UNIWERSYTET PRZYRODNICZY WE WROCŁAWIU

WYDZIAŁ BIOTECHNOLOGII I NAUK O ŻYWNOŚCI

KATEDRA CHEMII ŻYWNOŚCI I BIOKATALIZY

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MIKROBIOLOGICZNA SYNTEZA ZWIĄZKÓW ZAPACHOWYCH I ICH POCHODNYCH O AKTYWNOŚCIACH BIOLOGICZNYCH

MICROBIOLOGICAL SYNTHESIS OF FRAGRANCE COMPOUNDS AND THEIR DERIVATIVES WITH BIOLOGICAL ACTIVITIES

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Wrocław 2023

Badania prowadzone w ramach projektu pn. „UPWR 2.0: międzynarodowy i interdyscyplinarny program rozwoju Uniwersytetu Przyrodniczego we Wrocławiu", współfinansowanego ze środków Europejskiego Funduszu Społecznego w ramach Działania 3.5. Kompleksowe programy szkół wyższych Osi III Szkolnictwo wyższe dla gospodarki i rozwoju Programu Operacyjnego Wiedza Edukacja Rozwój oraz budżetu państwa na podstawie umowy o dofinansowanie nr POWR.03.05.00-00-Z062/18 z dnia 4 czerwca 2019 r.

Składam serdeczne podziękowania:

Promotorowi Pracy
Panu dr. hab. Filipowi Boratyńskiemu, prof. UPWr
Za cztery lata doktoratu, w czasie których był moim mentorem, a także za cierpliwość, poświęcony czas i wszystkie udzielone mi rady.

For my second Supervisor
Professor Maria Elisabetta Brenna
For all the advice given during my PhD studies, a good welcome to her research team at Politecnico di Milano and help in finding an apartment in Italy.

Pani prof. Teresie Olejniczak
Za to, że zawsze miała czas, żeby pomóc mi w rozwiązywaniu różnych problemów, które napotkałem w czasie realizacji doktoratu.

Pracownikom i Doktorantom
Katedry Chemii Żywności i Biokatalizy
Uniwersytetu Przyrodniczego we Wrocławiu
Za chętne dzielenie się posiadaną wiedzą i doświadczeniem.

Moim Rodzicom
Za wsparcie, na które zawsze mogłem liczyć i zachęcanie mnie do rozwijania moich pasji oraz wiarę w moje możliwości.

Mojej żonie Darii
Za jej miłość, pomoc i życzliwe słowo każdego dnia oraz za to, że bez wahania pojechała ze mną na roczny staż naukowy do Mediolanu i wspierała mnie w trudach doktoratu.

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PUBLIKACJE STANOWIĄCE ROZPRAWĘ DOKTORSKĄ

PUBLIKACJA 1 (P1)

Hernik, D.*; Pannek, J.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.*
Bacterial whole cells synthesis of whisky lactones in a solid-state fermentation bioreactor prototype. *Catalysts*, 2021, 11, 320.

(IF = 4,501; 100 pkt MEiN)

PUBLIKACJA 2 (P2)

Hernik, D.*; Gatti, F.; Brenna, E.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.*
Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*. *Frontiers in Microbiology*, 2023, 14, 1117835.

(IF = 6,064; 140 pkt MEiN)

PUBLIKACJA 3 (P3)

Hernik, D.*; Szczepańska, E.; Brenna, E.; Patejuk, K.; Olejniczak, T.; Strzała, T.; Boratyński, F.*
Trametes hirsuta as an attractive biocatalyst for the preparative scale biotransformation of isosafrole into piperonal. *Molecules*, 2023, 28, 3643.

(IF = 4,927; 140 pkt MEiN)

PUBLIKACJA 4 (P4)

Hernik, D.*; Szczepańska, E.; Ghezzi, M. C.; Brenna, E.; Włoch, A.; Pruchnik, H.; Mularczyk, M.; Marycz, K.; Olejniczak, T.; Boratyński, F.*
Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives. *Frontiers in Microbiology*, 2023, 14, 1223123.

(IF = 6,064; 140 pkt MEiN)

Całkowity współczynnik IF dla publikacji wchodzących w skład rozprawy doktorskiej wynosi: **21,328**

Suma punktów według czasopism punktowanych MEiN dla publikacji wchodzących w skład rozprawy doktorskiej wynosi: **520**

* Autor korespondencyjny

Wartość współczynnika Impact Factor zostały podane zgodnie z rokiem opublikowania, a punktacja Ministerstwa Edukacji i Nauki w oparciu o dane dostępne w dniu: 17.07.2023 r.

STRESZCZENIE W JĘZYKU POLSKIM

Związki zapachowe stanowią liczną grupę, do której należą między innymi laktony oraz związki arylopropenowe i ich pochodne. Laktony przede wszystkim nadają smak i zapach wielu produktów spożywczych, a także posiadają bardzo szerokie spektrum aktywności biologicznej potwierdzone licznymi doniesieniami literaturowymi. Z kolei związki z pierścieniem aromatycznym są powszechnie spotykane w olejkach eterycznych wielu gatunków roślin. Z uwagi na ich szeroką aktywność biologiczną, związki z tej grupy są często stosowane w przemyśle spożywczym, perfumeryjnym, farmaceutycznym i kosmetycznym.

W ostatnich latach biotechnologiczne metody otrzymywania związków chemicznych, do których zaliczają się biotransformacje, rozwijają się bardzo intensywnie, stając się coraz bardziej konkurencyjną alternatywą dla metod chemicznych. Zastosowanie biokatalizatorów w hodowlach na podłożach stałych (SSF) i wgłębnych (SmF) pozwala na równoczesne prowadzenie wielu reakcji enzymatycznych i jest ekonomicznie uzasadnionym rozwiązaniem charakteryzującym procesy wysokimi wydajnościami. Z uwagi na ogromną różnorodność mikroorganizmów oraz złożoność ich aparatu enzymatycznego użycie ich w formie całych komórek umożliwia otrzymanie produktów nieosiągalnych innymi metodami.

Celem pracy była mikrobiologiczna synteza związków z ugrupowaniem laktonowym oraz arylopropenów i ich pochodnych, określenie zdolności katalitycznych wyselekcjonowanych szczepów bakterii i grzybów przy zastosowaniu różnych technik biotechnologicznych, a także ocena określonych aktywności biologicznych wybranych związków.

W pracy doktorskiej wykorzystywałem bakterie do otrzymania poszczególnych enancjomerów whisky laktonu. Badania te prowadziłem zarówno w hodowlach na podłożu stałym, jak i w hodowlach wgłębnych w celu porównania i doboru optymalnej techniki umożliwiającej otrzymanie chiralnych laktonów. Jako podłoże do SSF wykorzystałem różne makuchy: lniany, rzepakowy oraz z wiesiołka. Produkty uboczne przemysłu olejarskiego stanowią bowiem tanią alternatywę dla podłoży dedykowanych mikroorganizmom. W wyniku utlenienia dioli **1a** i **1b** prowadzonych w zwiększonej skali na makuchu lnianym otrzymałem nieznacznie enancjomerycznie wzbogacone izomery *trans*-(+)-(4*S*,5*R* (**2a**), *cis*-(−)-(4*S*,5*S*) (**2c**) i *cis*-(+)-(4*R*,5*R*) (**2d**) whisky laktonu z udziałem bakterii *R. erythropolis* DSM44534, *R. erythropolis* PCM2150 i *G. rubripertincta* PCM2144. Natomiast w bioutlenieniu prowadzonym techniką SmF z *R. erythropolis* DSM44534 i *R. erythropolis* PCM2150 otrzymałem poszczególne enancjomery *trans*-(−)-(4*R*,5*S*) (**2b**) i *cis*-(+)-(4*R*,5*R*) (**2d**) oraz wysoce wzbogacony izomer *trans*-(+)-(4*S*,5*R*) (**2a**) whisky laktonu. Dodatkowo, w wyniku zastosowania biokatalizatora w postaci proszków acetonowych z *R. erythropolis* DSM44534 powstawał enancjomerycznie wzbogacony *cis*-(−)-(4*S*,5*S*) (**2c**) whisky lakton. Przeprowadziłem również utlenienie z udziałem *R. erythropolis* DSM44534

dioli **3a** i **3b**, w wyniku czego powstawał wysoce wzbogacony izomer *trans*-(+)-(5*S*,6*R*) (**4a**) oraz enancjomer *cis*-(–)-(5*S*,6*S*) (**4c**) aerangis laktonu. Potwierdziło to możliwość wykorzystania tej bakterii w stereoselektywnej syntezie γ- oraz δ-laktonów.

Dalsza część badań dotyczyła zastosowania wybranych bakterii i grzybów do otrzymywania szeregu związków z pierścieniem aromatycznym, w tym piperonalu (**5**) oraz tlenowych pochodnych arylopropenu – hydroksyketonów **6c-9c**. W wyniku transformacji izosafrolu (**6a**) z wyselekcjonowanymi szczepami grzybów *Trametes hirsuta* d28 i *T. hirsuta* Th2_2 otrzymałem piperonal (**5**) z wysoką wydajnością. Opracowałem również dwuetapową biokatalityczną metodę otrzymywania tlenowych pochodnych wybranych arylopropenów. Stosując diole **6b-10b**, otrzymane na drodze chemo-enzymatycznej syntezy, uzyskałem z udziałem bakterii *Dietzia* sp. DSM44016, *Rhodococcus erythropolis*, *R. erythropolis* PCM2150 i *R. ruber* PCM2166 odpowiednie hydroksyketony **6c-9c** z wysokimi wydajnościami. Ostatnim etapem badań była ocena aktywności biologicznej arylopropenów **6a-10a** oraz ich pochodnych, dioli **6b-10b** i hydroksyketonów **6c-9c**. Analizowałem właściwości fungistatyczne, przeciwutleniające, hemolityczne, przeciwproliferacyjne oraz wpływ na sztywność błony biologicznej otrzymanych związków. Wyniki tych badań sugerują, że otrzymane przeze mnie związki **6a-9c** wpływają w zróżnicowany sposób na badane aktywności, m. in. w zależności od stężenia, obecności dodatkowych grup funkcyjnych i typu wiązań występujących w danym związku.

STRESZCZENIE W JĘZYKU ANGIELSKIM

Fragrance compounds are a large group, which includes, among others, lactones and arylpropenes, and their derivatives. Lactones primarily are responsible for taste and smell of many food products, and also have a wide spectrum of biological activities confirmed by numerous literature reports. Compounds with the aromatic ring are commonly found in the essential oils of many plant species. Due to their wide biological activity, compounds from this group are often used in the food, perfumery, pharmaceutical and cosmetic industries.

In recent years, biotechnological methods of obtaining chemical compounds, which include biotransformations, have been developed very intensively, becoming an increasingly competitive alternative to chemical methods. The use of biocatalysts in Solid-State Fermentation (SSF) and Submerged Fermentation (SmF) allows for simultaneous conducting of many enzymatic reactions. It is also an economically viable solution characterized processes by high efficiency. Due to the large variety of microorganisms and the complexity of their enzymatic systems, using them in the form of whole cells allows to obtain products unattainable by other methods.

The aim of the study was the microbiological synthesis of compounds with lactone moiety and arylpropenes, and their derivatives, determination of catalytic properties of selected strains of bacteria and fungi using various biotechnological techniques, as well as assessment of specific biological activities of selected compounds.

In my doctoral dissertation, to obtain individual enantiomers of whisky lactone selected bacteria were used. The studies were conducted both in solid-state fermentation and submerged fermentation, in order to compare and select the optimal technique to obtain chiral lactones. As a substrate for SSF I used various oilcakes: linseed, rapeseed and evening primrose. By-products of the oil industry are a cheap alternative comparing to medium components dedicated to microorganisms. As a result of oxidation of diols **1a** and **1b** carried out on preparative scale on linseed oilcake slightly enantiomericaly enriched *trans*-(+)-(4*S*,5*R* (**2a**), *cis*-(−)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones with bacteria *R. erythropolis* DSM44534, *R. erythropolis* PCM2150 and *G. rubripertincta* PCM2144 were obtained. However, in the SmF bio-oxidation with *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150 individual enantiomers of *trans*-(−)-(4*R*,5*S*) (**2b**) and *cis*-(+)-(4*R*,5*R*) (**2d**), and a highly enriched *trans*-(+)-(4*S*,5*R*) (**2a**) isomer of whisky lactones were obtained. In addition, as a result of the use of a biocatalyst *R. erythropolis* DSM44534 in the form of acetone powders, enantiomeric enriched *cis*-(−)-(4*S*,5*S*) (**2c**) whisky lactone was formed. Moreover, oxidation of diols **3a** and **3b** with *R. erythropolis* DSM44534, resulting in the formation of a highly enriched *trans*-(+)-(5*S*,6*R*) (**4a**) isomer and a *cis*-(–)-(5*S*,6*S*) enantiomer (**4c**) of aerangis lactone was carried out. This confirmed the possibility of using this bacterium in stereoselective synthesis of γ- and δ-lactones.

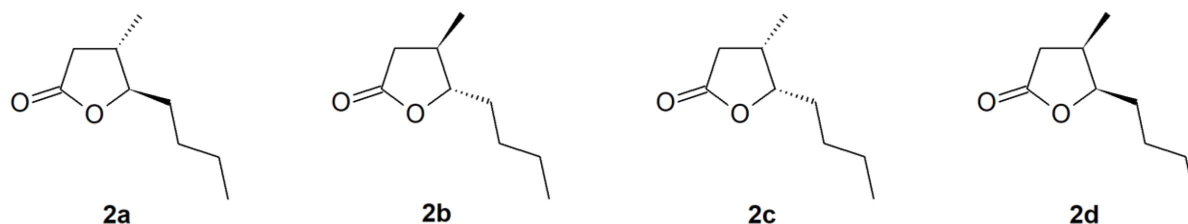
The next part of the research concerned the use of selected bacteria and fungi to obtain a number of compounds with the aromatic ring, including piperonal (**5**) and derivatives of arylpropene – hydroxyketones **6c-9c**. As a result of the transformation of isosafrole (**6a**) with selected fungal strains, *Trametes hirsuta* d28 and *T. hirsuta* Th2_2, piperonal (**5**) with high yield was obtained. Moreover, a two-step biocatalytic method for obtaining oxygenated derivatives of selected arylpropenes was developed. Diols **6b-10b**, obtained by chemo-enzymatic synthesis, were applied for transformations with *Dietzia* sp. DSM44016, *Rhodococcus erythropolis*, *R. erythropolis* PCM2150 and *R. ruber* PCM2166 affording corresponding hydroxyketones **6c-9c** with high yield. The final stage of the study assumes the assessment of the biological activity of arylpropenes **6a-10a** and their derivatives, diols **6b-10b** and hydroxyketones **6c-9c**. I analyzed fungistatic, antioxidant, hemolytic, antiproliferative properties, and the effect on the stiffness of the biological membrane of the obtained compounds. The results of these studies suggest that the compounds **6a-9c** affect the examined activities in a different way, depending on the concentration, the presence of additional functional groups and the type of bonds occurring in a given compound.

1. WSTĘP

Związki zapachowe stanowią liczną grupę związków chemicznych, występujących naturalnie w wielu produktach spożywczych takich jak owoce, warzywa, przyprawy, napoje, a także w kwiatach, olejkach eterycznych czy też perfumach. Związki te są również syntezowane przez zwierzęta, które wytwarzają je w celach komunikacji z innymi osobnikami, odstraszania drapieżników lub też jako feromony. Związki zapachowe charakteryzują się wysoką lotnością, co pozwala im na szybkie przenoszenie się do układu węchowego zwierząt, a następnie w wyniku interakcji z receptorami węchowymi, odczuć bodźce zapachowe w mózgu. Opierając się na strukturze chemicznej, wśród związków zapachowych występujących w żywności wyróżniamy takie grupy jak: alkohole, aldehydy, ketony, estry, laktony, kwasy tłuszczowe, pirazyny i związki aromatyczne [1]. W moich badaniach postanowiłem skupić się na dwóch grupach związków – laktonach i związkach z pierścieniem aromatycznym, które zostaną przedstawione poniżej.

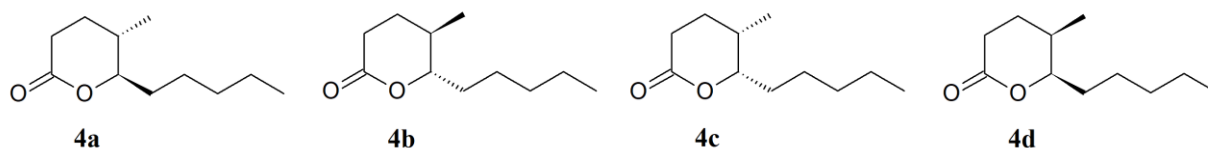
Do pierwszej grupy związków należą laktony będące cyklicznymi estrami powstającymi w wyniku wewnątrzcząsteczkowej estryfikacji γ-, δ-, ε-hydroksykwasów [2,3]. Laktony są wszechobecne w żywności, przyczyniając się do odczuwania bodźców smakowych i zapachowych, takich jak owocowy, kokosowy, maślany, kremowy, słodki lub orzechowy [4,5]. Należą do dobrze poznanych związków organicznych wykazujących szerokie działanie biologiczne, między innymi: antybakteryjne przeciwbakteryjne, przeciwwirusowe, przeciwzapalne czy przeciwnowotworowe [6,7].

Whisky lakton jest kluczowym składnikiem wielu długo dojrzewających napojów alkoholowych, takich jak whisky, koniak i brandy, oraz składnikiem zapachowym różnych produktów spożywczych (np. słodyczy, pieczonych potraw) oraz napojów bezalkoholowych [8]. Jest również stosowany jako środek odstraszający komary i muchy [9]. Whisky lakton został po raz pierwszy zidentyfikowany w 1970 roku przez Suomalainena i Nykanena jako pojedynczy związek obecny w alkoholach dojrzewających w dębowych beczkach, dlatego w języku angielskim jest powszechnie nazywany jako „oak lactone” [10]. Masuda i Nishimura odkryli później, że z drzewa dębowego można wyizolować dwa diastereoizomery tego laktonu[11]. Następnie, w 1989 roku, Gunther i Mosandl rozdzielili cztery stereoizomery whisky laktonu (**Rysunek 1**). Zapach mieszaniny izomerów został opisany, jako przypominający kokos, podczas gdy izomery *cis* zostały scharakteryzowane jako drzewne i ziemiste, a izomery *trans* jako przypominające zapach selera. Warto zwrócić uwagę, że w naturze drewno dębu zawiera tylko *trans*-(+)-(4*S*,5*R*) i *cis*-(-)-(4*S*,5*S*) izomery whisky laktonu [12]. Natomiast whisky lakton wykorzystywany komercyjnie w przemyśle spożywczym jest chemicznie otrzymywaną mieszaniną racemiczną *trans* i *cis* izomerów. Dlatego obecnie ważne jest opracowanie metod pozwalających na otrzymanie w skali przemysłowej poszczególnych stereoizomerów oraz analiza zależności pomiędzy ich strukturą a aktywnością biologiczną.



Rysunek 1. Wzory strukturalne *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**), *cis*-(−)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky laktonów.

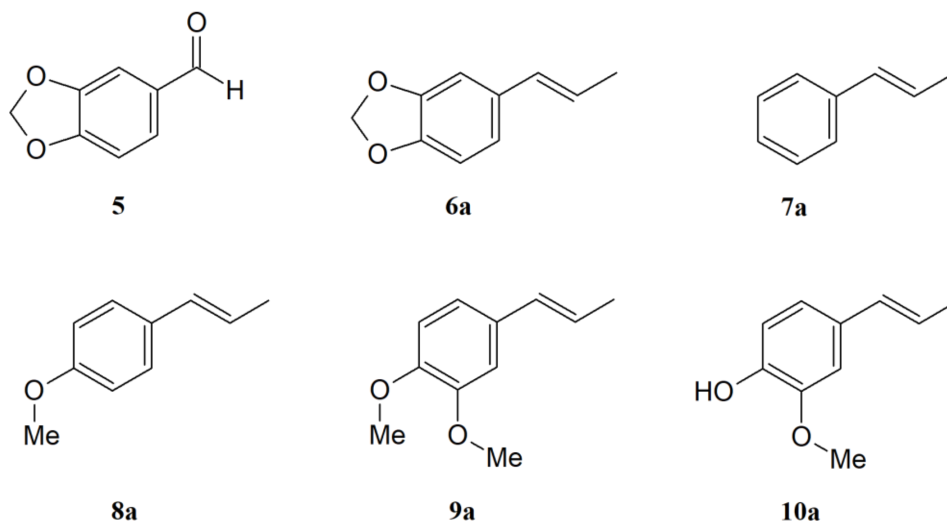
Aerangis lakton, należący do grupy δ-laktonów, został odkryty przez Kaisera w 1993 roku w postaci izomeru *cis*-(–)-(5*S*,6*S*) jako główny związek zapachowy afrykańskich storczyków *Aerangis confusa* i *Aerangis kirkii* (**Rysunek 2**) [13]. Stwierdzono, że izomery *cis* mają bardzo przyjemny zapach określany jako „wypełniający pomieszczenie", który z jednej strony przypomina zapach kwiatu tuberozy (*Polianthes tuberosa*) i odmian gardenii, a z drugiej karmel, mleko skondensowane i kokos, zwłaszcza mleko kokosowe [14]. Natomiast zapach izomerów *trans* aerangis laktonu kojarzony jest z zapachem brzoskwini i kokosu, jednak ma zdecydowanie niższy próg wyczuwalności zapachu niż izomery *cis*. Atrakcyjny zapach izomerów aerangis laktonu spowodował, że jest on najczęściej stosowany w przemyśle kosmetycznym i perfumeryjnym.



Rysunek 2. Wzory strukturalne *trans*-(+)-(5*S*,6*R*) (**4a**), *trans*-(−)-(5*R*,6*S*) (**4b**), *cis*-(−)-(5*S*,6*S*) (**4c**) and *cis*-(+)-(5*R*,6*R*) (**4d**) aerangis laktonów.

Chiralność jest jedną z najszerzej badanych właściwości związków od czasu jej odkrycia na początku XIX wieku i odgrywa kluczową rolę w procesach biologicznych [15-17]. W przypadku produkcji chiralnych leków enancjomery mogą w różny sposób oddziaływać z poszczególnymi układami metabolicznymi [18], z tego powodu optycznie czyste związki są zazwyczaj przynajmniej kilkukrotnie droższe niż ich forma racemiczna. W 1992 roku organizacja Food & Drug Administration (FDA) przedstawiła szereg wytycznych dotyczących farmaceutycznej produkcji enancjomerów i racematów [19]. W ostatnich latach zaobserwowano tendencję wzrostową w stosowaniu leków zawierających optycznie czyste związki chemiczne w celu zmniejszenia toksyczności lub skutków ubocznych związanych ze stosowaniem mieszanin racemicznych. Dobrze znanym przykładem wpływu chiralności na działanie leku jest Talidomid - lek stosowany w łagodzeniu mdłości u kobiet w ciąży, który w formie racemicznej powodował nieprawidłowy rozwój płodu [20]. Wykazano, że toksyczność talidomidu jest związana z enancjomerem *S*, natomiast enancjomer *R* pozytywnie wpływa na zmniejszenie nudności [21].

Drugą grupą związków związanych z prowadzonymi badaniami w ramach mojej pracy doktorskiej były arylopropeny i ich pochodne, między innymi piperonal (**5**), izosafrol (**6a**), anetol (**8a**), izoeugenol (**10a**) (**Rysunek 3**) [22]. Związki te są szeroko spotykane w olejkach eterycznych z roślin takich jak anyż, lukrecja i jagodlin wonny [23-26]. Związki z tej grupy są ważne gospodarczo i szeroko stosowane w przemyśle spożywczym, zapachowym, farmaceutycznym i kosmetycznym, a także jako półprodukty w syntezie bardziej złożonych produktów [27-29]. Arylopropeny mają szerokie spektrum aktywności biologicznej, między innymi wykazują działanie przeciwutleniające, przeciwdrobnoustrojowe, przeciwzapalne i antyproliferacyjne. Przykładowo, olejki eteryczne zawierające izosafrol wykazują działanie przeciwutleniające i przeciwdrobnoustrojowe względem bakterii z rodzaju *Helicobacter*, *Staphylococcus* i *Escherichia* [30]. Olej safrolowy charakteryzuje się aktywnością antyproliferacyjną na wybranych liniach komórkowych raka wątroby [31]. Natomiast anetol wywiera działanie hamujące na zapalenie przyzębia [32-33], a bogaty w anetol olej z liści *Clausena heptaphylla* ma działanie przeciwcukrzycowe, hamujące tyrozynazę i antycholinoesterazy [33]. Z kolei izoeugenol wykazuje aktywność przeciwdrobnoustrojową względem takich patogenów żywności jak *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas fluorescens* i *Leuconostoc mesenteroides*, a także odznacza się właściwościami przeciwutleniającymi i przeciwzapalnymi [34].



Rysunek 3. Wzory arylopropenów i ich pochodnych wykorzystanych w badaniach: piperonal (**5**), izosafrol (**6a**), prop-1-en-1-yl benzen (**7a**), anetol (**8a**), 1,2-dimetoksy-4-prop-1-en-1-ylobenzen (**9a**), izoeugenol (**10a**).

Piperonal (**5**), jest metabolitem wtórnym wytwarzany przez rośliny, takie jak heliotrop, wanilia, kamfora, fiołek i pieprz czarny [35]. Pomimo rzadkiego występowania w przyrodzie, piperonal odgrywa istotną rolę w przemyśle spożywczym i zapachowym. Zapach jego przypomina wanilię, z przyjemną nutą kwiatowo-pudrową, dlatego jest stosowany, jako aromat w perfumach, świecach zapachowych, detergentach i odświeżaczach powietrza. Dodatkowo stanowi użyteczny półprodukt do syntezy

substancji chemicznych, takich jak Tropional® (zapach handlowy), piperyna (suplement diety zwiększający biodostępność innych związków w żywności), α-metylodopa (lek przeciwnadciśnieniowy), piribedil (środek chorobie Parkinsona). Ponadto piperonal może być stosowany w syntezie leku psychoaktywnego MDMA (3,4-metylenodioksy-N-metyloamfetamina), w związku z tym produkcja piperonalu i jego prekursorów oraz handel nimi podlegają ścisłej kontroli i regulacjom w wielu krajach.

Izosafrol (**6a**) jest związkiem zapachowym często stosowanym w przemyśle perfumeryjnym [25]. Występuje w niewielkich ilościach w różnych olejkach eterycznych, a jego zapach przypomina anyż lub lukrecję i ma charakterystyczny słodki aromat. Głównymi źródłami izosafrolu oraz jego izomerycznej formy – safrolu są rośliny z gatunku *Ocotea pretiosa* oraz *Sassafras albidum*, w których działa jako naturalny antyfidant [36]. Najczęściej otrzymuje się go poprzez izomeryzację safrolu z oleju roślinnego [37-39]. Izosafrol jest również prekursorem piperonalu i może być przekształcany za pośrednictwem związku pośredniego MDP2P w psychoaktywny MDMA. W związku z tym wymaga zezwoleń na zakup lub sprzedaż w znaczącej ilości w USA [40].

Anetol (**8a**) jest związkiem organicznym, popularnie stosowanym jako substancja aromatyzująca [25]. Występuje szeroko w przyrodzie, w olejkach eterycznych roślin takich jak koper włoski, anyż, lukrecja, i znacząco wpływa na smak i zapach tych roślin. Anetol jest bardzo słodki (13 razy słodszy od cukru) i jest postrzegany przez konsumentów, jako przyjemny w smaku nawet przy wysokich stężeniach. Znalazł zastosowanie w takich napojach alkoholowych jak ouzo, raki, anisette i absyncie. Jest również stosowany w przyprawach i wyrobach cukierniczych oraz produktach do higieny jamy ustnej [41]. Anetol jest również niedrogim prekursorem chemicznym parametoksyamfetaminy (PMA), co sprawia, że jest stosowany w jej nielegalnej produkcji [42, 43].

Izoeugenol (**10a**) występuje w olejkach eterycznych roślin takich jak *Cananga odorata* i jest składnikiem dymu drzewnego i dymu płynnego. Ma przyjemny, pikantny, goździkowy, korzenny zapach [44]. Jest jednym z kilku związków fenolowych odpowiedzialnych za hamujący wpływ dymu na rozwój pleśni w mięsie i serach. Izoeugenol może być syntetyzowany z eugenolu i jest stosowany w produkcji waniliny [45].

W literaturze opisano wiele chemicznych metod otrzymywania wyżej wymienionych związków oraz ich pochodnych. Dostępne chemiczne metody syntezy *trans* i *cis* izomerów whisky laktonu opierają się na wieloetapowej syntezie chemicznej z wykorzystaniem katalizatorów metalicznych i rozpuszczalników organicznych [46-54]. Pomimo szerokiego zastosowania, katalizatory na bazie metali są często szkodliwe dla środowiska. Opisano również metodę otrzymywania stereoizomerów whisky laktonu z katalizatorami niemetalicznymi [54], jednak wieloetapowe podejście zastosowane w tym procesie charakteryzowało się stosunkowo niską konwersją. Z kolei komercyjna droga otrzymywania piperonalu (**5**) opiera się na izomeryzacji safrolu

do izosafrolu (**6a**) i jego późniejszym utlenieniu kwasem chromowym lub ozonem, w połączeniu z siarką lub redukcją cynku [55]. Inna metoda jest związana z elektrochemicznym utlenianiem alkoholu piperonylowego za pomocą katalizatora Au / CeO_2 [56]. Szereg aromatycznych aldehydów można także otrzymać w wyniku utleniania arylopropenów pod wpływem promieniowania mikrofalowego i użyciu komercyjnych utleniaczy ($PhI(OAc)_2$ na NaY i $PhI(OAc)_2$ na Al_2O_3) [57]. Została również zaproponowana metoda utlenienia alkoholu piperonylowego różnymi komercyjnymi heterogenicznymi katalizatorami, przy użyciu paraformaldehydu, jako reagenta [58]. Ze względu na coraz większą uwagę poświęcaną tematyce zielonej chemii i zaletom metod biotechnologicznych, ważne jest sukcesywne zastępowanie metod chemicznych metodami wykorzystującymi biokatalizatory w postaci całych komórek lub izolowanych enzymów.

Biotransformacje to przemiany ksenobiotycznego substratu, zachodzące z udziałem biokatalizatorów. Jako biokatalizatory stosuje się drobnoustroje, rośliny, tkanki zwierzęce lub całe organizmy, a także enzymy wyizolowane z wymienionych źródeł [59]. Enzymy katalizujące procesy kataboliczne i anaboliczne w organizmach żywych, w prosty sposób mogą być wykorzystane do prowadzenia wielu reakcji chemicznych, których produkty zazwyczaj nie posiadają żadnej funkcji metabolicznej i gromadzą się w środowisku reakcji, z którego mogą zostać wyizolowane. W obecności enzymu reakcja przebiega od 10^8 do nawet 10^{12} razy szybciej. Biotransformacje posiadają wiele zalet. Biokatalizatory cechują się wyższą aktywnością jak również chemo-, regio-, stereo- i enancjoselektywnością, co pozwala na przeprowadzanie reakcji, które są nieosiągalne lub nieopłacalne w przypadku syntez chemicznych [60]. Biotransformacje są zazwyczaj prowadzone w bardzo łagodnych warunkach w porównaniu z metodami chemicznymi. Istnieje również możliwość prowadzenia wieloetapowych transformacji w jednym bioreaktorze bez izolowania produktów pośrednich. Natomiast do wad biotransformacji można zaliczyć podatność na wpływ wielu czynników takich jak: temperatura, ciśnienie, stężenie jonów wodorowych, i powstającego produktu, jak również obecność niektórych jonów [61]. Jest to powodem, dla którego większość procesów biotechnologicznych wymaga ścisłej kontroli parametrów reakcji. Ponadto, reakcje prowadzone z wykorzystaniem izolowanych enzymów, potrzebują do prawidłowego działania odpowiednich, często drogich, kofaktorów takich jak donory równoważników redoks (NADP) lub energii (ATP). Zbyt duże stężenie substratów lub powstających produktów może również wpłynąć na zahamowanie procesu lub może dezaktywować biokatalizatory [62].

W procesach biotechnologicznych z zastosowaniem całych komórek mikroorganizmów zastosowanie znalazły dwie metody prowadzenia hodowli – hodowle wgłębne (SmF) (ang. Submerged Fermentation) oraz hodowle na podłożu stałym (SSF) (ang. Solid State Fermentation). Hodowla wgłębna to proces, w którym wzrost mikroorganizmów odbywa się w płynnym podłożu, które jest zoptymalizowane pod kątem wymaganych składników odżywczych [63]. Mikroorganizmy są namnażane w zamkniętym bioreaktorze zawierającym podłoże fermentacyjne i wysokie stężenie

tlenu. Z kolei hodowla na podłożu stałym jest procesem, gdzie mikroorganizmy wzrastają na stałych podłożach. Surowce wykorzystywane do SSF zwykle powinny charakteryzować się niską zawartością wody wolnej. Dodatkowo zazwyczaj w procesach SSF podłoże stałe jest jedynym źródłem składników odżywczych w czasie prowadzenia reakcji. W SSF mogą być prowadzone zróżnicowane procesy biotransformacji, od prostych przekształceń konkretnego fragmentu substratu z udziałem jednego enzymu, po wieloetapowe metody biokonwersji przy udziale wielu enzymów. Obecnie coraz większą uwagę zwraca się na odpady przemysłu rolno-spożywczego i metody ich zagospodarowania. Dodatkowo coraz częściej poszukuje się również procesów odpowiadających zasadom zielonej chemii, aby tworzyć metody bardziej przyjazne dla środowiska i konkurencyjne dla klasycznych metod chemicznych [64].

Hodowle na podłożu stałym posiadają wiele zalet. Podłoża wykorzystywane w tych procesach są tanie, między innymi stosuje się odpady po obróbce zbóż, drzew, wysłodków buraczanych, makuchów jak również inne części roślin, które nie zostały przetworzone na żywność. Oczyszczanie produktów jest zazwyczaj mniej skomplikowane niż w procesach prowadzonych w hodowlach wgłębnych, a w czasie prowadzenia tych procesów nie powstają ścieki [65]. W procesach SSF można produkować żywność o zwiększonej wartości spożywczej i walorach smakowych. Przykładem może być tempach, który powstaje w czasie fermentacji ziaren soi i jest wykorzystywany jako zamiennik mięsa. Hodowla na podłożu stałym posiada też pewne wady. Nie wszystkie mikroorganizmy mogą przyswajać związki organiczne znajdujące się w danym surowcu, a niektóre substancje w nich zawarte mogą działać na nie inhibicyjne, a nawet silnie toksycznie. Niska zawartość wody wolnej może być również przeszkodą dla rozwoju niektórych bakterii i grzybów. Z uwagi na różnorodność odpadów przemysłu rolno-spożywczego, procesy SSF często wymagają zastosowania innych konstrukcji bioreaktora.

W literaturze zaproponowano kilka metod z wykorzystaniem szlaków biokatalitycznych, które prowadzą do biosyntezy opisanych wcześniej związków zapachowych [51, 52, 66-68]. Dobrym przykładem jest dehydrogenaza alkoholowa wyizolowana z końskiej wątroby (HLADH) enancjoselektywnie utleniająca racemiczne *syn-* i *anty*-3-metylooktano-1,4-diole do *trans-* i *cis*-whisky laktonów [51]. Inna metoda wykorzystuje całe komórki *Beauveria bassiana* AM278 i *Pycnidiella resinae* KCH50 do laktonizacji γ-ketokwasów [52]. Alternatywnie, enancjomer *trans*-(+)-(4*S*,5*R*) whisky laktonu otrzymuje się przez redukcję odpowiednich γ-ketokwasów katalizowanych przez drożdże piekarskie [47]. Dotychczas opisano również kilka metod syntezy piperonalu (**5**) przy użyciu biokatalizatorów. Głównym substratem stosowanym w biotechnologicznych metodach otrzymywania piperonalu (**5**) był izosafrol (**6a**) [55, 69–71]. Trzyetapowa chemo-enzymatyczna procedura konwersji izosafrolu w piperonal została w ostatnim czasie zaproponowana przez Tentori i in. [72]. Dodatkowo w literaturze przedstawiono procesy biotechnologicznego otrzymywania piperonalu (**5**), w których

jako substraty wyjściowe zastosowano kwas piperonylowy i alkohol piperonylowy [73, 74].

W XXI wieku przykłada się coraz większą uwagę do poszukiwania procesów odpowiadających zasadom zielonej chemii stanowiących alternatywę względem klasycznych metod chemicznych. Konsumenci coraz częściej wybierają produkty pochodzenia naturalnego zwracając uwagę na to, w jaki sposób powstał dany produkt. Aktywność biologiczna związków obecnych w produkcie ma kluczowe znaczenie. Mając na uwadze dalszą potrzebę opracowywania biotechnologicznych metod syntezy związków biologicznie aktywnych, w badaniach opisanych w pracy doktorskiej stosowałem biotransformacje z użyciem całych komórek bakterii i grzybów. Pierwszą grupę związków otrzymanych przeze mnie stanowią znane ze swoich właściwości zapachowych whisky i aerangis laktony oraz piperonal. Do drugiej grupy należą hydroksyketony, będące pochodnymi komercyjnie znanych arylopropenów tj. anetol, izoeugenol czy izosafrol, których aktywność biologiczna, w tym fungistatyczna, antyoksydacyjna, hemolityczna, antyproliferacyjna, a także wpływ na płynność błon komórkowych, została oceniona.

2. CEL

Głównym celem pracy była mikrobiologiczne synteza związków zapachowych oraz ich pochodnych, wraz z oceną aktywności biologicznych wybranych związków.

Dodatkowo należy wyszczególnić następujące cele szczegółowe:

- Określenie zdolności biokatalitycznych badanych szczepów bakterii oraz grzybów.
- Stereoselektywna synteza poszczególnych enancjomerów whisky laktonu w wyniku bioutlenienia prowadzonego w hodowlach z udziałem bakterii.
- Otrzymanie piperonalu z izosafrolu w wyniku transformacji z udziałem grzybów.
- Opracowanie metody otrzymywania tlenowych pochodnych arylopropenowych – dioli i hydroksyketonów.
- Zwiększenie skali prowadzenia procesów transformacji z użyciem całych komórek mikroorganizmów.
- Ocena potencjału zastosowania biokatalizatorów w postaci proszków acetonowych.
- Porównanie biotransformacji dioli w hodowlach wgłębnych (SmF) oraz na podłożu stałym (SSF).
- Ocena możliwości wykorzystania produktów ubocznych przemysłu olejarskiego (makuchów) jako medium hodowlanego i podłoża do biotransformacji.
- Dobór optymalnej metody ekstrakcji produktów biotransformacji z hodowli na podłożu stałym (SSF).
- Ocena wybranych aktywności biologicznych arylopropenów i ich pochodnych.

3. MATERIAŁY I METODY

3.1 Mikroorganizmy

W badaniach opisanych w publikacjach **P1**, **P2** oraz **P4** jako biokatalizatorów używałem następujące szczepy bakterii: *Bacillus subtilis* PCM2238, *B. subtilis* PCM2850, *Dietzia maris* PCM2292, *Gordonia bronchialis* PCM2167, *G. rubripertincta* PCM2144, *Micrococcus luteus* PCM525, *Pseudomonas aeruginosa* PCM2720, *P. aeruginosa* PCM3035, *Rhodococcus coprophilus* PCM2174, *R. erythropolis* PCM2150, *R. rhodnii* PCM2157, *R. rhodochrous* PCM909, *R. ruber* PCM2166, *R. ruber* PCM2171, *R. ruber* PCM2216, *Serratia liquefaciens* PCM2830, *S. marcescens* PCM549, *S. plumuthica* PCM550, *Serratia* sp. PCM1324, *Streptomyces griseus* subsp. *griseus* PCM2331 pochodzące z Polskiej Akademii Nauk (Wrocław, Poland) oraz *Dietzia* sp. DSM44016 i *Rhodococcus erythropolis* DSM44534, które zostały zakupione w German Collection of Microorganisms and Cell Cultures (Braunschweig, Niemcy).

W biotransformacjach prowadzących do otrzymywania piperonalu opisanych w publikacji **P3** biokatalizatorami były wymienione szczepy grzybów: *Absidia cylindrospora* AM336, *Armillaria mellea* AM296, *A. mellea* AM461, *Aspergillus ochraceus* AM456, *Chaetomium* sp. AM432, *Fusarium culmorum* AM282, *F. equiseti* AM22, *F. oxysporum* AM21, *Inonotus radiates* AM70, *Laetiporus sulphurens* AM498, *L. sulphurens* AM515, *Mortierella isabelina* AM212, *Papularia rosea* AM17, *Pholiota aurivella* AM522, *Piptoporus betulinus* AM40, *P. betulinus* AM57, *Pleurotus ostreatus* AM482, *Poria placenta* AM36, *P. placenta* AM38, *Trametes versicolor* AM536 obecne w kolekcji mikroorganizmów Katedry Chemii Żywności i Biokatalizy, Uniwersytetu Przyrodniczego we Wrocławiu. Szczepy *Agrocybe aegerita* DSM 22459 i *Pleurotus sapidus* DSM 8266 zostały zakupione w German Collection of Microorganisms and Cell Cultures (Braunschweig, Niemcy), a *Pycnoporus cinnabarinus* CBS 353.63 został zakupiony w Westerdijk Fungal Biodiversity Institute (Utrecht, Holandia). Dodatkowo wiele szczepów pozyskanych przez dr Katarzynę Patejuk bezpośrednio z różnych środowisk naturalnych (rozkładające się drewno, owocniki grzybów i zdrowe tkanki roślinne) i zdeponowanych w kolekcji mikrobiologicznej Katedry Ochrony Roślin Uniwersytetu Przyrodniczego we Wrocławiu zostało użytych do badań i opisanych w publikacji **P3**.

3.2 Substraty, enzymy i koenzymy

Jako substratów do syntez chemo-enyzmatycznych używałem: diasteroizomeryczną mieszaninę whisky laktonu (**2**), aerangis lakton (**4**), mieszaninę (8:2) diastereoizomerów (*E*)/(*Z*) izosafrolu (**6a**) oraz prop-1-en-1-ylobenzen (**7a**), anetol

(**8a**), 1,2-dimetoksy-4-prop-1-en-1-ylobenzen (**9a**) i izoeugenol (**10a**). W reakcjach z proszkami acetonowymi stosowałem dehydrogenazę glutaminianową (GDH) oraz następujące koenzymy: dinukleotyd nikotynoamidoadeninowy (NAD^+), fosforan dinukleotydu nikotynamidoadeninowego ($NADP^+$), mononukleotyd flawinowy (FMN).

3.3 Biotransformacje na podłożu stałym

Biotransformacje na podłożu stałym (Solid-State Fermentation, SSF) prowadziłem w sterylnym podłożu, składającym się z makuchu lnianego, rzepakowego lub z wiesiołka oraz sterylnej wody destylowanej, w ilości pozwalającej na osiągnięcie 60% wilgotności podłoża. Wilgotność każdego z makuchów określiłem za pomocą pomiaru masy próbek przed i po liofilizacji. Badania przesiewowe w SSF prowadziłem w kolbach Elenmayera o pojemności 100 mL, zawierających 5 g odpowiedniego makucha oraz sterylną wodę destylowaną. Biotransformacje w zwiększonej skali prowadziłem w prototypowym bioreaktorze SSF, do którego dodawałem 50 g makucha lnianego oraz 75 mL wody destylowanej. Substraty do biotransformacji SSF rozpuszczone w acetonie dodawałem w warunkach sterylnych w końcowej fazie wzrostu mikroorganizmów. Produkty biotransformacji SmF ekstrahowałem octanem etylu, a następnie analizowałem za pomocą technik chromatograficznych TLC oraz GC.

3.4 Prototypowy bioreaktor do hodowli na podłożu stałym

System kontrolno-pomiarowy prototypowego bioreaktora do hodowli na podłożu stałym składał się z Raspberry Pi Zero W podłączonego do przetwornika analogowo-cyfrowego i do optoizolowanego przekaźnika dwukanałowego. Dla obu przekaźników, jako sygnał sterujący wykorzystałem sygnał 3,3 V z pinów GPIO, a same przekaźniki stosowałem do sterowania dwiema pompami. W komorze bioreaktora umieściłem dwie elektrody grafitowe, czujnik z sondą do pomiaru wilgotności gleby Waveshare 9527, rurkę stalową do czujnika temperatury, szklaną kapilarę odpowiedzialną za dodawanie wody oraz dwie szklane nasadki z filtrami mikrobiologicznymi do wymiany gazowej. Aby zminimalizować wpływ prądu elektrycznego, elektrody czujnika wilgotności zostały podłączone do tranzystora. Do podłączenia przewodów powietrznych użyto węży teflonowych. Komora bioreaktora została wykonana z polipropylenu przeznaczonego do sterylizacji.

3.5 Ekstrakcja produktów biotransformacji z podłoża stałego

Dobór optymalnej metody ekstrakcji produktów biotransformacji z podłoża stałego zakładał przeprowadzenie szeregu testów z zastosowaniem: ekstrakcji prostej, destylacji z parą wodną (z modyfikacjami) oraz ekstrakcji z wykorzystaniem aparatu Derynga.

Destylację z parą wodną prowadziłem w następujący sposób: hodowle SSF po biotransformacji razem z biomasą przenosiłem do odpowiedniej kolby okrągłodennej a następnie dodawałem wodę destylowaną. Tak przygotowaną próbkę destylowałem następnie w aparacie do destylacji, a następnie zbierałem powstający destylat. Po zebraniu odpowiedniej ilości destylatu ekstrahowałem go trzykrotnie rozpuszczalnikiem organicznym (octanem etylu). Po ekstrakcji osuszyłem otrzymaną próbkę i odparowałem nadmiar rozpuszczalnika na wyparce rotacyjnej pod zmniejszonym ciśnieniem. Dodatkowo zastosowałem również modyfikację tej metody, w której destylacji poddałem ekstrakt hodowli SSF z biomasą po biotransformacji uzyskany w wyniku ekstrakcji prostej. Ekstrakcję w aparacie Derynga prowadziłem umieszczając w nim podłoże po biotransformacji w kolbie okrągłodennej z dodatkiem wody destylowanej. Kolba była ogrzewana, a powstające opary zostały skroplone za pomocą zimnego czynnika chłodniczego. Tak powstający ekstrakt zebrałem w formie ekstraktu z cykloheksanem.

3.6 Biotransformacje wgłębne

Biotransformacje wgłębne (Submerged Fermentation, SmF) prowadziłem w sterylnym podłożu płynnym dedykowanym dla bakterii, zawierającym 2% glukozy, 1% peptonu bakteriologicznego, 0,2% hydrolizatu kazeinowego, 0,2% ekstraktu drożdżowego oraz 0,6% NaCl lub w podłożu przeznaczonym dla grzybów zawierającym 3% glukozy oraz 1% peptonu bakteriologicznego rozpuszczonych w wodzie destylowanej. Biotransformacje przesiewowe prowadziłem w 48-dołkowych płytkach MTP (4 mL podłoża na dołek), oraz w kolbach Elenmayera o pojemności 100 mL zawierających 40 mL podłoża). Natomiast biotransformacje w zwiększonej skali prowadziłem w kolbach Elenmayera 300 mL (100 mL podłoża) oraz 2 L (zawierających 500 mL). Substraty rozpuszczone w acetonie lub DMSO (dimetylosulfotlenek) dodawałem w warunkach sterylnych w końcowej fazie wzrostu mikroorganizmów. Produkty biotransformacji ekstrahowałem octanem etylu, a następnie analizowałem za pomocą technik chromatograficznych TLC oraz GC.

3.7 Techniki analiz chemicznych

Przebieg biotransformacji sprawdzałem za pomocą chromatografii cienkowarstwowej (TLC) przy użyciu płytek z folii aluminiowej pokrytych żelem krzemionkowym oraz chromatografii gazowej (GC; detektor płomieniowo-jonizacyjny, gaz nośny H_2) w aparacie Agilent Technologies 7890N wyposażonym w różne kolumny kapilarne, w tym z wypełnieniem chiralnym w zależności od analizowanego związku. Dla wybranych związków przeprowadziłem również analizy GC-MS. Struktury związków potwierdziłem za pomocą magnetycznego rezonansu jądrowego (NMR) widm ^{1}H i ^{13}C NMR próbek rozpuszczonych w $CDCl_3$ zarejestrowanych na spektrometrach Bruker Avance DRX 600 (600 MHz) i Avance II (400 MHz). Widma w podczerwieni (IR) wyznaczono za pomocą spektrometru FTIR Thermo-Mattson IR 300. Skręcalność optyczną mierzyłem na polarymetrze Jasco P-2000.

3.8 Testy aktywności biologicznej

Test aktywności fungistatycznej wykonałem względem czterech szczepów z gatunku *C. albicans* (*C. albicans* ATTC 90028, *C. albicans* 636/20, *C. albicans* 595/20, i *C. albicans* 38) przy pomocy metody mikrorozcieńczeń z wykorzystaniem płytek 96-dołkowych. Mikropłytki inkubowałem w termostacie Biosan PST-60 HL Plate Shaker-Thermostat. Aktywność fungistatyczną badanych związków oceniałem poprzez pomiar absorbancji przy długości fali 595 nm (Epoch, BioTek) w celu określenia wartości MIC_{50} (tj. stężenia związku wymaganego do zahamowania 50% wzrostu mikroorganizmów).

Aktywność antyoksydacyjną sprawdzałem za pomocą metody z wykorzystaniem DPPH (2,2-difenylo-1-pikrylohydrazyl) z wykorzystaniem mikropłytek. Mikropłytki inkubowałem na termowytrząsarce Biosan PST-60 HL, a aktywność przeciwutleniającą związków oceniłem poprzez pomiar absorbancji przy długości fali 517 nm w celu określenia wartości EC_{50} (tj. stężenia związku, który daje 50% maksymalnej odpowiedzi). Wartości EC_{50} zostały obliczone przy użyciu kalkulatora Quest Graph™ EC50 (AAT Bioquest, Inc.).

Aktywność hemolityczna została określona na ludzkich erytrocytach otrzymanych ze stacji krwiodawstwa. Absorbancję mierzono przy 540 nm za pomocą spektrofotometru UV–Vis (Specord 40, Analytik Jena). Aktywność hemolityczną związków określono jako stosunek absorbancji hemoglobiny w badanych próbkach do absorbancji w komórkach poddanych całkowitej hemolizie pomnożonej przez 100%.

Wpływ na sztywność błony erytrocytów zbadano za pomocą sondy DPH, dzięki której zmierzono wartości anizotropii błon komórkowych krwinek czerwonych zmodyfikowanych badanymi związkami. Pomiary wykonano w kuwetach kwarcowych przy użyciu fluorymetru CARY Eclipse (Varian). Długość fali wzbudzenia dla sondy

DPH wynosiła λ_{exc} = 360 nm, a długość fali emisji λ_{em} = 426 nm. Na podstawie zmian w natężeniu DPH w świetle spolaryzowanym określono wartość anizotropii.

Właściwości przeciwproliferacyjne zostały ocenione na komórkach HepG2 (linia komórkowa ludzkiego raka wątroby), Caco-2 (linia komórkowa ludzkiego raka jelita) i MG63 (linia komórkowa ludzkiego kostniakomięsaka) za pomocą testu bazującego na resazurynie. Poziomy absorbancji dla resazuryny mierzono spektrofotometrycznie (Epoch) przy długości fali 600 nm.

Szczegółowe opisy warunków prowadzonych biotransformacji, syntez chemo-enzymatycznych, testów biologicznych, analizę spektroskopową struktur chemicznych uzyskanych produktów biotransformacji oraz stosowana aparatura zostały przedstawione w publikacjach **P1**-**P4** wchodzących w skład dysertacji.

4. WYNIKI I DYSKUSJA

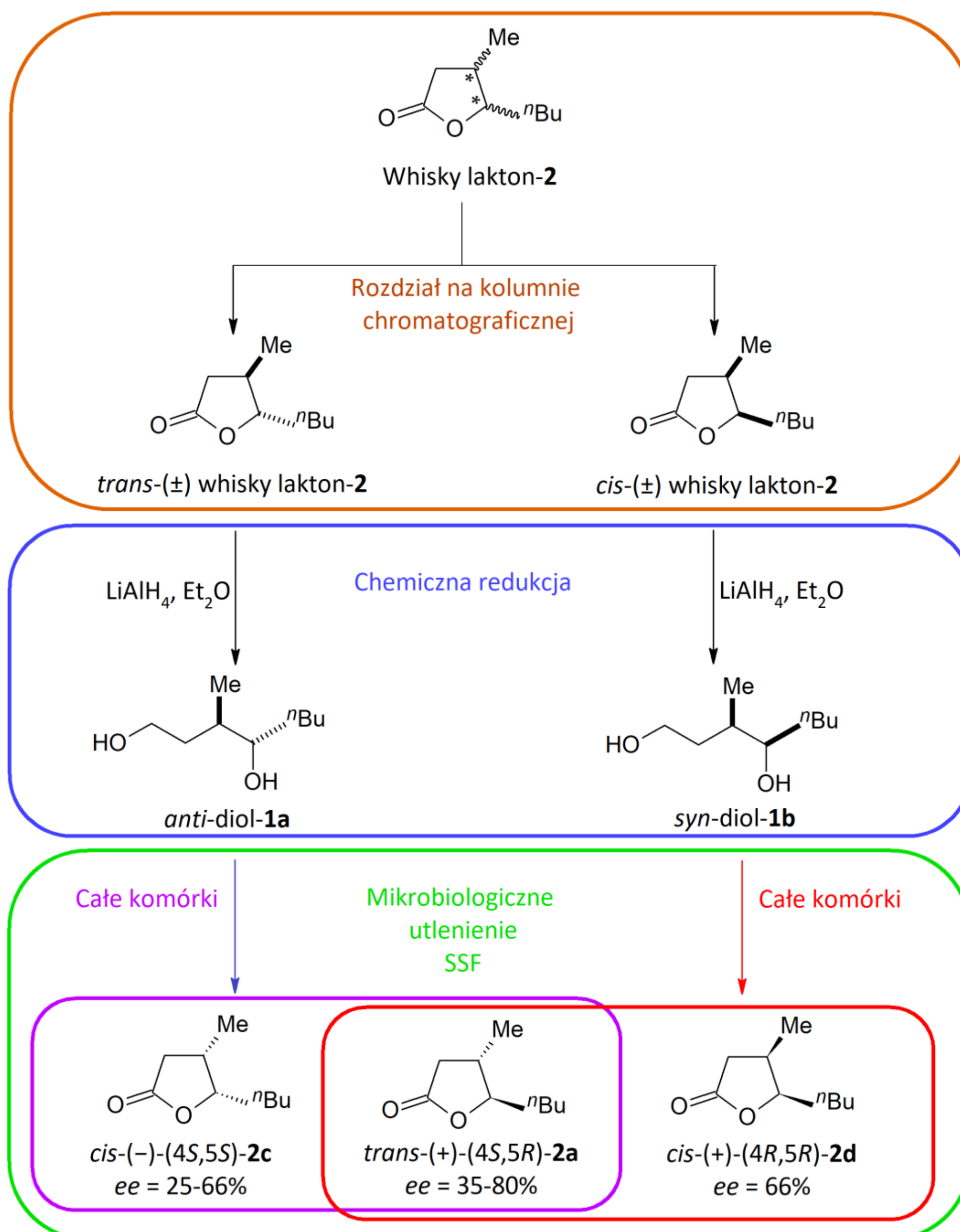
Dysertacja została napisana w oparciu o cztery publikacje (**P1-P4**), w których opisałem mikrobiologiczne utlenianie dioli z wykorzystaniem hodowli prowadzonych na produktach ubocznych przemysłu rolno-spożywczego (**P1**) oraz podłóż dedykowanych dla poszczególnych typów mikroorganizmów (**P2**) otrzymując chiralne whisky laktony. Mikrobiologiczną transformacje kontynuowałem w dwóch kolejnych publikacjach (**P3** i **P4**) otrzymując z isosafrolu, piperonal (**P3**) oraz tlenowe pochodne arylopropenowe w postaci dioli i hydroksyketonów, które były następnie analizowane pod kątem szeregu aktywności biologicznych (**P4**).

W publikacjach **P1** i **P2** biokatalizatorami było 14 szczepów bakterii z rodzaju: *Dietzia*, *Gordonia*, *Micrococcus*, *Rhodococcus* i *Streptomyces* pochodzących z kolekcji Polskiej Akademii Nauk (PCM) oraz z German Collection of Microorganisms and Cell Cultures (DSMZ). W publikacji **P4**, oprócz wyżej wymienionych mikroorganizmów, korzystałem również z bakterii z rodzaju: *Bacillus*, *Pseudomonas* oraz *Serratia* pochodzących z kolekcji Polskiej Akademii Nauk (PCM). Natomiast biokatalizatorami w publikacji **P3** były grzyby z rodzaju *Absidia*, *Agrocybe*, *Armillaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Inonotus*, *Laetiporus*, *Mortierella*, *Papularia*, *Pholiota*, *Piptoporus*, *Pleurotus*, *Poria*, *Pycnoporus* i *Trametes* pochodzące z kolekcji Katedry Chemii Żywności i Biokatalizy Uniwersytetu Przyrodniczego we Wrocławiu (AM), German Collection of Microorganisms and Cell Cultures (DSMZ) i Westerdijk Fungal Biodiversity Institute (CBS) w Utrecht. Dodatkowo wiele szczepów użytych do badań opisanych w **P3** pozyskano bezpośrednio z ich środowisk naturalnych, na podstawie ich cech makro- i mikroskopowych zostały one oznaczone, jako szczepy należące do rodzaju: *Trametes*, *Marasmius*, *Peniophora*, *Trichoderma*, *Aureobasidium*, *Chaetopsis*, *Mucor*, *Penicillium*, *Daedalea*.

4.1 Mikrobiologiczne otrzymywanie zapachowych laktonów

Badania rozpocząłem od utlenienia dioli do whisky laktonów katalizowane przez bakterie wzrastające na produktach ubocznych przemysłu olejarskiego. Warto wspomnieć, że procesy hodowli na podłożu stałym (SSF) wykorzystujące aktywność utleniającą mikroorganizmów nie są powszechnie znane w literaturze. Do tej pory opisano mikrobiologiczne utlenianie ksenobiotyków na produktach ubocznych przemysłu naftowego przy użyciu grzybów strzępkowych, które katalizują stereoselektywną hydrolizę mieszaniny racemicznej octanu fenyloetylu, a następnie utlenienie 1-fenyloetanolu do acetofenonu [53]. Utylizacja opadów i zagospodarowanie produktów ubocznych przemysłu mogą prowadzić wręcz do nieopłacalności produkcji. Poszukując rozwiązań w tym zakresie w publikacji **P1** opisałem biotransformacje w hodowli na podłożu stałym prowadzone w nisko-kosztowym prototypowym bioreaktorze SSF umożliwiającym prowadzenie mikrobiologicznej transformacji w zwiększonej skali z możliwością kontroli przebiegu procesu.

Przedstawiona w publikacji **P1** trójetapowa metoda polega kolejno na separacji diastereoizomerycznej mieszaniny whisky laktonów za pomocą chromatografii kolumnowej; chemicznej redukcji racemicznych *trans*- i *cis*-whisky laktonów (**2a-d**) do odpowiednich *syn*- i *anti*-dioli (**1a-b**); mikrobiologicznym utlenieniu w hodowli na podłożu stałym racemicznych dioli do poszczególnych optycznie czynnych izomerów whisky laktonu (**Rysunek 4**).



Rysunek 4. Trójetapowa metoda otrzymywania optycznie czynnych izomerów whisky laktonu w hodowli na podłożu stałym (SSF).

Na podstawie wcześniejszych doświadczeń grupy badawczej dr hab. Filipa Boratyńskiego w tematyce bioutlenienia [51, 75, 76], bakterie z rodzaju *Gordonia* i *Rhodococcus* zostały wyselekcjonowane, jako potencjalne katalizatory mikrobiologicznego utleniania dioli (**1a-b**). Bakterie te charakteryzowały się wysoką aktywnością dehydrogenaz alkoholowych (ADH) odpowiedzialnych za jednoetapowe utlenianie dioli do laktonów.

Wzrost biokatalizatorów, a także proces biotransformacji prowadziłem w hodowli na podłożu stałym (SSF) przy użyciu trzech różnych produktów ubocznych przemysłu olejarskiego makuchów: lnianego, rzepakowego i z wiesiołka. Są one bogate

w składniki odżywcze, takie jak węglowodany, białka i tłuszcze, a zatem mogą stanowić idealną pożywkę dla wzrostu mikroorganizmów [77, 78]. Średnia wilgotność badanych przeze nie makuchów wynosiła 7,9% dla makucha lnianego, 6,5% dla rzepakowego i 4,2% z wiesiołka. Transformacje mikrobiologiczne prowadziłem przy wilgotności podłoża 60%.

W pierwszym etapie badań przeprowadziłem biotransformacje osobno dla poszczególnych dioli **1a** i **1b** (10 mg substratu/5g makuchu) na trzech makuchach z 14 szczepami bakterii. W wyniku biotransformacji przesiewowych okazało się, że transformacje o najwyższej enancjoselektywności obserwowałem na makuchu lnianym, zarówno dla diolu **1a** jak i **1b**. W transformacjach katalizowanych z udziałem bakterii *G. bronchialis* PCM2167, *G. rubripertincta* PCM2144, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150, *R. rhodochrous* PCM909 i *R. ruber* PCM2166 na makuchu lnianym z dodatkiem substratu **1a** po 3 dniach powstawały enancjomerycznie czyste *cis*-(−)-(4*S*,5*S*)-**2c** i *cis*-(+)-(4*R*,5*R*)-**2d** whisky laktony w ilościach 3-20%. Izomery *trans* dominowały w badanych próbkach (80-97%), jednak ich nadmiary enancjomeryczne były niskie. Natomiast w wyniku bioutlenienia diolu **1b** przez te same szczepy na podłożu lnianym po 3 dniach powstawał enancjomer *trans*-(+)-(4*S*,5*R*)-**2a** w ilościach 19-37% oraz enancjomerycznie wzbogacone izomery *cis*, które stanowiły dominujący produkt w badanych próbkach. Biorąc pod uwagę powyższe wyniki, do biotransformacji w zwiększonej skali (gdzie użyłem 100 mg diolu zwiększając ilość makuchu do 50 g) wybrałem trzy mikroorganizmy: *G. rubripertincta* PCM2144, *R. erythropolis* DSM44534 oraz *R. erythropolis* PCM2150. Na makuchu rzepakowym obserwowałem powstawanie mniejszych ilości whisky laktonów, dodatkowo z niższymi nadmiarami enancjomerycznymi. Z kolei w próbkach z makuchu z wiesiołka identyfikowałem jedynie substrat. Z tego powodu do dalszych badań wybrałem jedynie makuch lniany.

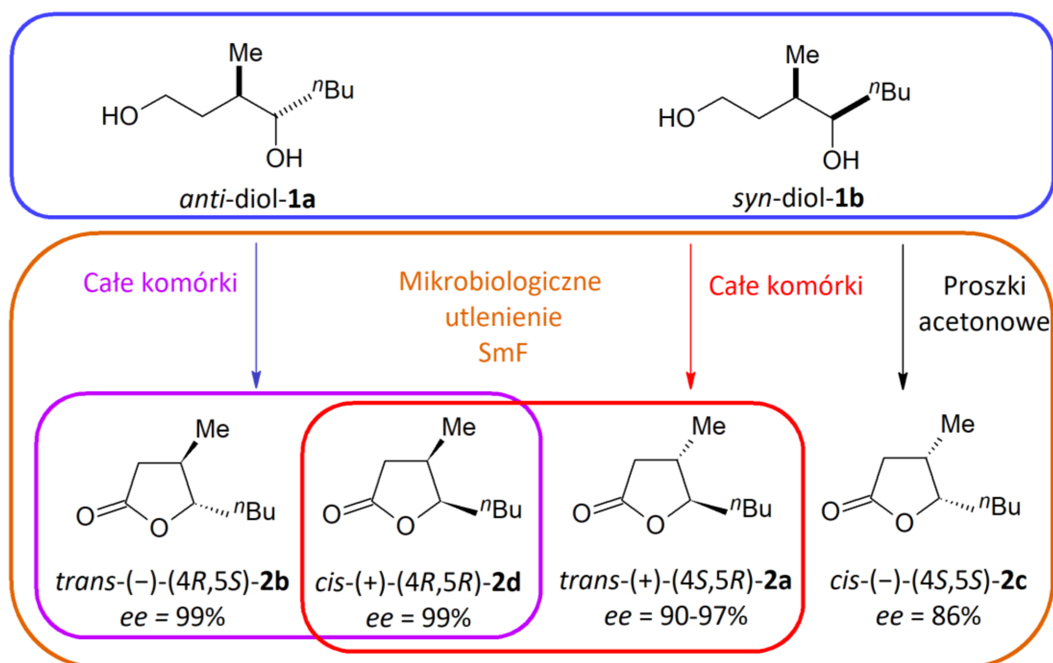
W celu przeprowadzenia biotransformacji w zwiększonej skali na podłożu stałym z właściwą kontrolą parametrów hodowli, został zaprojektowany i skonstruowany prototypowy bioreaktor SSF pozwalający na utrzymywanie stałych warunkach wilgotności i natlenienia. Podczas wielokrotnych testów bioreaktora SSF potwierdziłem, że materiały użyte do jego opracowania, zostały dobrane w odpowiedni sposób. Pokrywa bioreaktora z polipropylenu, szklana kapilara, elektrody grafitowe, sonda Waveshare 9527 i czujnik temperatury TMP36GT9Z okazały się odporne na wielokrotną sterylizację, a także kontakt z mikroorganizmami i rozpuszczalnikami organicznymi. Pompa powietrza, którą zastosowałem w bioreaktorze, zapewniała właściwą wymianę gazową. Aby kontrolować wilgotność podczas biotransformacji w bioreaktorze, przetestowano dwa rodzaje elektrod. Pomiary przeprowadzono najpierw elektrodami grafitowymi, a następnie sondą do pomiaru wilgotności gleby Waveshare 9527 na makuchach lnianym i rzepakowym. Porównując wartości uzyskane podczas testów z dwoma różnymi elektrodami, zdecydowałem się na zastosowanie czujnika z sondą Waveshare 9527 do pomiaru wilgotności. Umożliwiło to uzyskanie

bardziej liniowych wartości w szerokim zakresie wilgotności, co pozwoliło na dokładniejsze pomiary.

Biotransformacje diolu **1a** prowadzone w zwiększonej skali, katalizowane przez *R. erythropolis* PCM2150 i *G. rubripertincta* PCM2144 pozwoliły na otrzymanie enancjomerycznie wzbogaconych izomerów *trans*-(+)-(4*S*,5*R*) (**2a**) (*ee* = 35-64%, wydajność izolowana = 15-45%) i *cis*-(−)-(4*S*,5*S*) (**2c**) (*ee* = 25-66%, wydajność izolowana = 14-42%) whisky laktonu. Natomiast w wyniku bioutlenienia diolu **1b** przez *R. erythropolis* DSM44534 i *G. rubripertincta* PCM2144 otrzymałem enancjomerycznie wzbogacone izomery *trans*-(+)-(4*S*,5*R*) (**2a**) (*ee* = 66-80%, wydajność izolowana = 17-68%) i *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 66%, wydajność izolowana = 62%) (**Rysunek 4**).

Podczas wykonywania ekstrakcji po biotransformacji prowadzonej w bioreaktorze SSF izolowałem duże ilości tłuszczu zawartego w makuchach. Ich obecność uniemożliwiała dokładne oddzielenie frakcji lipidowej od interesujących mnie laktonów. Dlatego kolejne badania poświęcone były doborowi metody izolacji, która pozwoliłaby na rozwiązanie tego problemu. Do tego celu wykorzystałem trzy metody ekstrakcji: w aparacie Derynga, destylację z parą wodną oraz jej modyfikację. W ekstrakcji w aparacie Derynga i destylacji z parą wodną, produkty były destylowane bezpośrednio z hodowli po biotransformacji. Natomiast osobno przeprowadziłem destylację z parą wodną z ekstraktu organicznego, który otrzymałem w wyniku ekstrakcji prostej podłoża i biomasy po biotransformacji. Okazało się, że spośród tych metod, ostatnia z nich pozwoliła na osiągnięcie wysokiej wydajności ekstrakcji (81%) oraz nie powodowała dodatkowego pienienia oraz częściowej degradacji próbek tak jak w wypadku pozostałych badanych metod.

Ze względu na niską enancjoselektywność i wydajność biotransformacji w hodowlach na podłożu stałym (SSF) opisanych w publikacji **P1**, kolejnym problemem badawczym, przed którym stanąłem było opracowanie wydajnego i wysoce stereoselektywnego procesu syntezy poszczególnych enancjomerycznych form whisky laktonów. Opisana w publikacji **P2** trójetapowa metoda była modyfikacją procesu przedstawionego wcześniej dla biotransformacji w hodowli na podłożu stałym (SSF) (**Rysunek 4**). Zdecydowałem się na utlenienie racemicznych dioli **1a** i **1b** do poszczególnych enancjomerów whisky laktonu (**2a-d**) z udziałem bakterii w hodowli wgłębnej (SmF) z wykorzystaniem podłóż dedykowanych dla tych mikroorganizmów, jak również stosując proszki acetonowe (**Rysunek 5**).



Rysunek 5. Mikrobiologiczne otrzymywanie poszczególnych enancjomerów whisky laktonu w hodowli wgłębnej (SmF).

Do pierwszego etapu badań przesiewowych wyselekcjonowałem szczepy bakterii opisane we wcześniejszych badaniach (**P1**), sądząc, że ich aktywność biokatalityczna będzie wyższa w hodowlach wgłębnych (SmF) niż na podłożu stałym (SSF). Badania przesiewowe prowadziłem w 24-dołkowych mikropłytkach w podłożu dedykowanym dla bakterii (PCM) z dodatkiem 1 mg substratu **1a** lub **1b** w 4 mL hodowli. Użycie płytek pozwoliło na szybką identyfikację, które z testowanych mikroorganizmów wykazują najwyższą aktywność dehydrogenaz alkoholowych (ADH). Podczas tych badań zauważyłem, że enancjomerycznie czyste lub wysoce wzbogacone *trans*- i *cis*-whisky laktony powstawały głównie w biotransformacjach katalizowanych przez następujące mikroorganizmy: *Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150 i *R. ruber* PCM2166.

Następnym krokiem było 10-krotne zwiększenie skali eksperymentu (10 mg substratu **1a** lub **1b**), ocena wpływu na przebieg bioutlenienia i wyselekcjonowanie na tej podstawie mikroorganizmów do kolejnych procesów. Podczas prowadzenia tego eksperymentu bardzo szybko zauważyłem, że dwie z badanych bakterii *R. erythropolis* DSM44534 i *R. erythropolis* PCM2150 wyróżniają się szczególnie wysoką enancjoselektywnością i szybką konwersją. Podczas biotransformacji diolu **1a** z tymi dwoma szczepami już po 48 godz. powstawało od 25 do 82% enancjomeru *trans*-(−)-(4*R*,5*S*) (**2b**) (*ee* = 99%), oraz od 18 do 75% enancjomeru *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 99%). Natomiast po dodaniu diolu **1b** do hodowli *R. erythropolis* DSM44534 powstawał jedynie enancjomer *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 99%). W wyniku biotransformacji tego samego diolu przez *R. erythropolis* PCM2150 po 24 godz. powstawały enancjomery *trans*-(−)-(4*R*,5*S*) (**2b**) (31%) oraz *cis*-(+)-(4*R*,5*R*) (**2d**) (69%) whisky laktonu.

Podczas biotransformacji *anty*-3-metylooktano-1,4-diolu (**1a**) w ponownie zwiększonej skali (100 mg substratu) czas potrzebny na konwersję był znacznie dłuższy niż w przypadku reakcji w mniejszej skali. Ponadto zaobserwowano powstawanie produktów ubocznych. W bioutlenieniu z *R. erythropolis* DSM44534 po 144 godz. otrzymałem enancjomery *trans*-(−)-(4*R*,5*S*) (**2b**) (*ee* = 99%, wydajność = 22%) oraz *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 99%, wydajność = 8%) whisky laktonu. W biotransformacjach *syn*-3-metylooktano-1,4-diolu (**1b**) konwersja zachodziła dużo szybciej. W wyniku bioutlenienia tego diolu z *R. erythropolis* PCM2150 po 42 godz. otrzymałem enancjomerycznie wzbogacony *trans*-(+)-(4*S*,5*R*) (**2a**) (*ee* = 97%, wydajność 14%) oraz optycznie czysty *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 99%, wydajność = 60%) whisky lakton. Natomiast biotransformacje z z *R. erythropolis* DSM44534 po 42 godz. pozwoliły na otrzymanie enancjomerycznie wzbogaconego *trans*-(+)-(4*S*,5*R*) (**2a**) (*ee* = 90%, wydajność 28%) oraz enancjomeru *cis*-(+)-(4*R*,5*R*) (**2d**) (*ee* = 99%, wydajność = 40%) whisky laktonu.

Porównując wyniki biotransformacji prowadzonych w zwiększonej skali w hodowlach wgłębnych (SmF) z wynikami uzyskanymi w procesie fermentacji na podłożu stałym (SSF), procesy prowadzone w SmF charakteryzowały się znacznie szybszą konwersją dioli **1a** i **1b** (**Tabela 1**). Ponadto, jedynie w wyniku bioutlenienia w hodowlach wgłębnych możliwe było otrzymanie enancjomerycznie czystych laktonów. Spośród testowanych mikroorganizmów bakterie z gatunku *Rhodococcus erythropolis* były najefektywniejszymi katalizatorami.

Tabela 1. Porównanie biotransformacji w zwiększonej skali dioli **1a** i **1b** w hodowlach wgłębnych (SmF) i na podłożu stałym (SSF) z wybranymi szczepami bakterii.

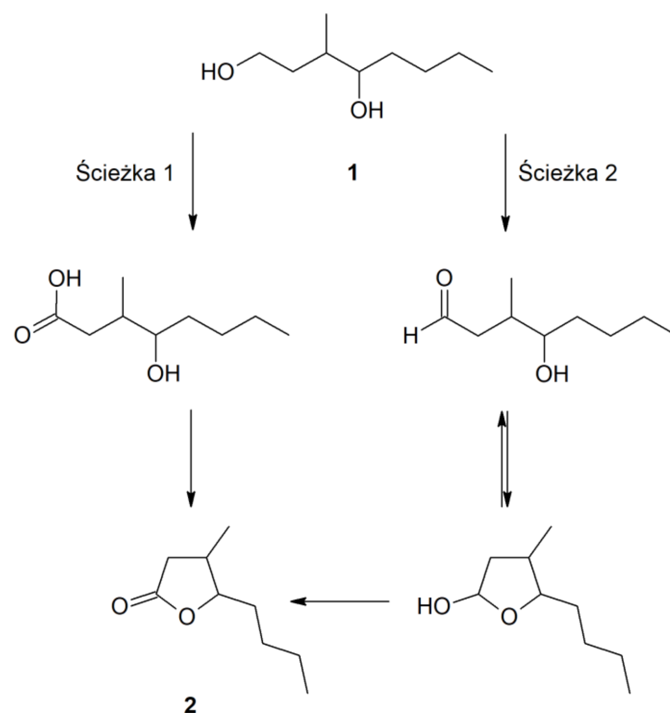
Typ hodowli	Szczepy bakterii	Czas [godz.]	Substrat	Izomer *trans*	Wydajność [%]	Izomer *cis*	Wydajność [%]
SSF	*G. rubripertincta* PCM2144	168	**1a**	**2a** (*ee* = 64%)	15	**2c** (*ee* = 25%)	42
		168	**1b**	**2a** (*ee* = 66%)	68		
	R. erythropolis DSM44534	168	**1b**	**2a** (*ee* = 80%)	17	**2d** (*ee* = 66%)	62
	R. erythropolis PCM2150	168	**1a**	**2a** (*ee* = 35%)	45	**2c** (*ee* = 66%)	14
SmF	*R. erythropolis* DSM44534	144	**1a**	**2b** (*ee* = 99%)	22	**2d** (*ee* = 99%)	8
		42	**1b**	**2a** (*ee* = 90%)	28	**2d** (*ee* = 99%)	40
	R. erythropolis PCM2150	42	**1b**	**2a** (*ee* = 97%)	14	**2d** (*ee* = 99%)	60

Zgodnie z literaturą enancjomery whisky laktonu otrzymuje się poprzez mikrobiologiczną redukcję *γ*-oksokwasów całymi komórkami grzybów [52]. Enancjomer *trans*-(+)-(4*S*,5*R*) (**2a**) identyfikowano (*ee* = 99%) jako jedyny produkt biotransformacji katalizowanej przez *Didymosphaeria igniaria* KCH6651, *Laetiporus sulphurens* AM525, *Chaetomium* sp. KCH6670 i *Saccharomyces cerevisiae* AM464. Natomiast

w wyniku transformacji z udziałem *Beauveria bassiana* AM278 i *Pycnidiella resinae* KCH50 otrzymano mieszaninę izomerów *trans*-(+)-(4*S*, 5*R*) (**2a**) (*ee* = 99%) i *cis*-(−)-(4*S*,5*S*) (**2c**) (*ee*= 45–77%). Z kolei podczas reakcji enzymatycznych katalizowanych przez komercyjnie dostępne dehydrogenazy alkoholowe HLADH i PADH I otrzymano enancjomerycznie wzbogacone izomery *trans*-(−)-(4*R*, 5*S*) (**2b**) i *cis*-(+)- (4*R*,5*R*) (**2d**) (*ee* = 27–82%). W opisanej wcześniej metodzie otrzymywania enancjomeru *trans*-(+)-(4*S*,5*R*) (**2a**) (*ee* = 99%) wydajność procesu wynosiła 38% [47]. W porównaniu z tymi wynikami, w biotransformacjach dioli **1a** i **1b** w hodowlach wgłębnych otrzymałem czyste lub wysoce enancjomerycznie wzbogacone izomery *trans*-(+)-(4S,5R) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**) i *cis*-(+)-(4*R*,5*R*) (**2d**) whisky laktonu (*ee* = 97–99%) sięgającymi wydajności 68%.

W związku z faktem, iż w zwiększonej skali prowadzenia transformacji z całymi komórkami nie otrzymałem enancjomerycznie czystego *cis*-(−)-(4*S*,5*S*) (**2c**) whisky laktonu postanowiłem zastosować biokatalizator w innej formie. Proszki acetonowe są często stosowane w biotransformacjach, aby zwiększyć stabilności enzymów i poprawić ich enancjoselektywność. Ponadto, biokatalizatory te często zwiększają wydajność i są proste w przechowywaniu oraz użytkowaniu. Przykładowo, zastosowanie proszków acetonowych z *Geotrichum candidum* zwiększyło enancjoselektywność i stopień redukcji ketonów do alkoholi i umożliwiło otrzymanie produktu o odwrotnej konfiguracji absolutnej [75]. Biorąc pod uwagę powyższe, przeprowadziłem utlenianie za pomocą proszków acetonowych przygotowanych z wybranych gatunków bakterii wyselekcjonowanych na podstawie wcześniejszych eksperymentów. Do transformacji użyłem NAD^+ i $NADP^+$ jako koenzymów oraz FMN i GDH do ich regeneracji. Spośród testowanych szczepów bakterii tylko *R. erythropolis* DSM44534 katalizował reakcję w wyniku, której z diolu **1b** powstawał enancjomerycznie wzbogacony izomer *cis*-(−)-(4*S*,5*S*) (**2c**) whisky laktonu (*ee* = 86%).

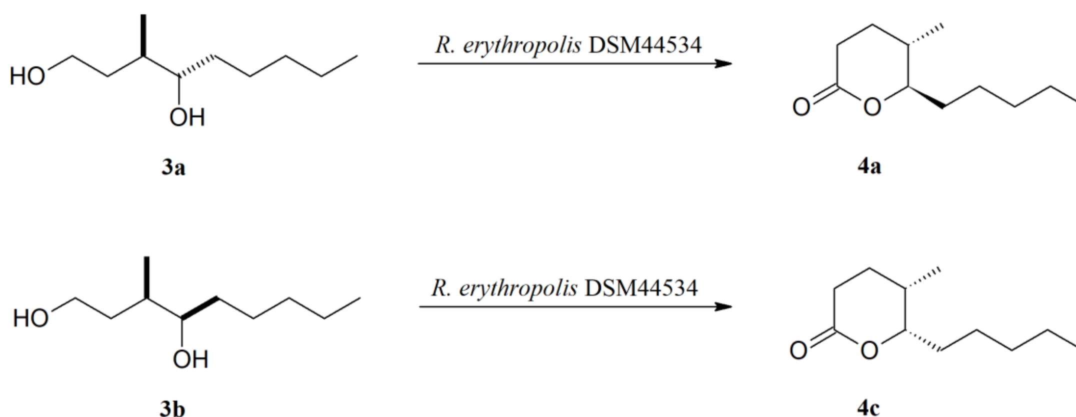
Na podstawie doświadczeń, które przeprowadziłem zainteresowałem się dlaczego izomer *cis*-(−)-(4*S*,5*S*) (**2c**) whisky laktonu nie powstaje w optycznie czystej formie. Z tego powodu postanowiłem przeanalizować możliwe ścieżki bioutlenienia [76]. W pierwszej z nich (**Rysunek 6**), pierwszorzędowa grupa hydroksylowa diolu jest chemoselektywnie utleniana do grupy karboksylowej, a następnie powstający hydroksykwas ulega cyklizacji do odpowiedniego laktonu. Jednak w drugiej ścieżce zachodzi dwuetapowy proces utleniania. Najpierw, diol jest utleniany do odpowiedniego hydroksyaldehydu, z którego następnie powstaje spontanicznie półacetal, a ten w dalszym etapie utleniany jest do laktonu.



Rysunek 6. Możliwe ścieżki biotransformacji diolu **1** do whisky laktonu (**2**).

Na podstawie badań, które przeprowadziłem w hodowlach SSF i SmF nie można jednoznacznie potwierdzić, który z wyżej wymienionych mechanizmów zachodzi w czasie utleniania katalizowanego przez całe komórki bakteryjne oraz z nich otrzymane proszki acetonowe. W wyniku tych biotransformacji otrzymałem jedynie enancjomerycznie czyste lub wzbogacone izomery whisky laktonu oraz dodatkowe produkty niebędące związkami pośrednimi w dwóch przedstawionych ścieżkach bioutlenienia.

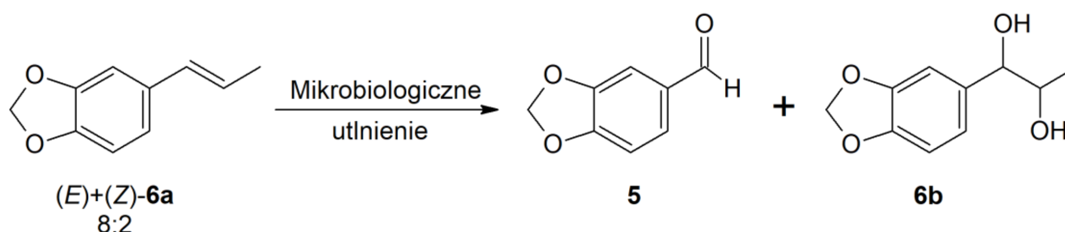
Przeprowadziłem również badania na innym substracie – diolu **3** w celu otrzymania aerangis laktonu. Chciałem bowiem sprawdzić, czy bakterie z gatunku *R. erythropolis* mogą także katalizować reakcję prowadzącą do otrzymania strukturalnie większych, chiralnych δ-laktonów. Przy okazji miałem nadzieję na potwierdzenie jednej z opisanych wyżej ścieżek bioutlenienia. Badanie te prowadziłem we współpracy z zespołem dr Francesco Gilberto Gattiego z Politechniki Mediolańskiej, dzięki któremu otrzymałem *anty*- i *syn*-4-metylodekan-1,5-diole (**3a** i **3b**). W wyniku biotransformacji substratu **3a** z udziałem *R. erythropolis* DSM44534 otrzymałem enancjomerycznie wzbogacony izomer *trans*-(+)-(5*S*,6*R*) aerangis laktonu (**4a**) (*ee* = 94%) z wydajnością izolowania 64%. Z kolei biotransformacja diolu **3b** dostarczyła czystego optycznie *cis*-(–)-(5*S*,6*S*) aerangis laktonu (**4c**) (*ee* = 99%, wydajność 54%) (**Rysunek 7**). Warto zauważyć, że biotransformacje w tym przypadku prowadziły tylko do jednego izomeru aerangis laktonu **4a** lub **4c**, w przeciwieństwie do dwóch izomerów w przypadku wcześniej opisanego whisky laktonu. Ponadto, w czasie biotransformacji nie powstawały żadne produkty pośrednie wskazujące na mechanizm reakcji. Opisane wyżej wyniki dotyczące otrzymywania stereoizomerów aerangis laktonu wymagają dalszych badań i nie zostały jeszcze przez nas opublikowane.



Rysunek 7. Biotransformacje dioli **3a** i **3b** do aerangis laktonów **4a** i **4c** katalizowane prze *R. erythropolis* DSM44534.

4.2 Biotransformacje arylopropenów i ich tlenowych pochodnych

Drugą część badań, związanych ze związkami zawierającymi w swojej strukturze pierścień aromatyczny, arylopropenami i ich tlenowymi pochodnymi, zacząłem od biotransformacji związanych z otrzymywaniem piperonalu (**5**) z izosafrolu (**6a**) (**P3**). Początkowo testowałem 23 szczepy grzybów oraz 22 szczepy bakterii pod kątem transformacji izosafrolu (**6a**) występującego w formie mieszaniny diastereoizomerów (E/Z) w stosunku 8:2. Szczepy te zostały wyselekcjonowane na podstawie danych literaturowych. Zdecydowana większość testowanych przeze mnie mikroorganizmów nie wykazywała aktywności wobec substratu **6a**. Jest to prawdopodobnie związane z toksycznością izosafrolu (**6a**) i jego aktywnością hamującą wzrost mikroorganizmów [55]. Spośród badanej grupy mikroorganizmów jedynie kilka szczepów grzybów wykazywało zdolność do przekształcania izosafrolu (**6a**) do piperonalu (**5**) oraz diolu **6b** (**Rysunek 8**).



Rysunek 8. Produkty biotransformacji izosafrolu (**6a**): piperonal (**5**) i diol **6b**.

Analiza ekstraktów z hodowli *Trametes versicolor* AM536, *T. hirsuta* d28 i *Mortierella isabelina* AM212 po 7 i 14 dniach pokazała, że podczas biotransformacji powstawały niewielkie ilości (8-14%) piperonalu (**5**). Warto zauważyć, że w biotransformacjach z *Piptoporus betulinus* AM40 i *Laetiporus sulphurens* AM515 zaobserwowano powstawanie diolu **6b**, jako jedynego produktu. Biorąc to pod uwagę uznaliśmy, że w kolejnej części badań opisanych w dalszej części tej dysertacji, jako

substraty do biotransformacji użyte zostaną pochodne arylopropenowe w postaci szeregu dioli **6b-10b**.

Ze względu na niezadowalającą ilość piperonalu (**5**) (14%) otrzymanego w kulturze *T. versicolor* AM536, postanowiłem określić, czy zmiana źródeł węgla i azotu w medium hodowlanym wpłynie na poprawę wydajności utleniania izosafrolu (**6a**) do piperonalu (**5**). Wstępne testy przeprowadziłem przy użyciu *T. versicolor* AM536, zastępując glukozę w pożywce Saboraud innymi cukrami, takimi jak: fruktoza, mannoza, ryboza, skrobia i galaktoza. Stosowałem również alternatywne źródła azotu organicznego zastępując pepton, hydrolizatami: kazeiny, albuminy mleka, mięsnym, ziemniaczanym. Otrzymane wyniki okazały się mało satysfakcjonujące, więc postanowiłem rozszerzyć badania przesiewowe o inne szczepy grzybów.

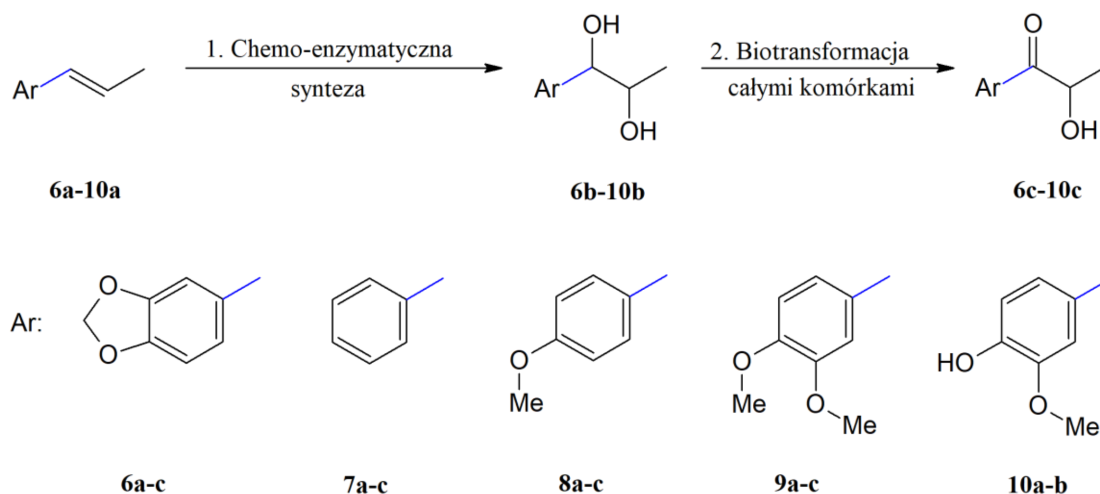
Dzięki uprzejmości dr Katarzyny Patejuk z Katedry Ochrony Roślin Uniwersytetu Przyrodniczego we Wrocławiu do dalszych badań otrzymałem różne szczepy grzybów wyizolowane bezpośrednio z ich naturalnych środowisk. Biotransformacje z tymi szczepami potwierdziły, że zdecydowana większość tych mikroorganizmów nie wykazywała zdolności do przekształcania izosafrolu (**6a**). Wyjątkiem były szczepy z gatunku *Trametes hirsuta*, które okazały się jedynymi biokatalizatorami wykazującymi aktywność wobec substratu **6a**. Dane literaturowe wskazują, że szczepy z rodzaju *Trametes* są znane z rakcji utlenienia alkenów w szerokim zakresie związków chemicznych [78-86]. Obiecujące wyniki otrzymałem podczas procesów z wykorzystaniem *T. hirsuta* Th2_2 i *T. hirsuta* d28, gdzie wyizolowałem odpowiednio 38% i 43% piperonalu (**5**) po 14 dniach biotransformacji.

Następnie przeprowadziłem biotransformacje w zwiększonej skali z dodatkiem 200 mg substratu **6a**. Proces kontrolowałem w czasie zatrzymując biotransformację w 11 dniu i izolując produkty. Po oczyszczeniu mieszaniny reakcyjnej, otrzymałem piperonal (**5**) z wydajnością 62% (0,124 g) i 50,5% (0,101 g) odpowiednio w kulturach szczepów *T. hirsuta* Th2_2 i *T. hirsuta* d28.

Obecnie znane biotechnologiczne metody syntezy piperonalu (**5**) polegają na wykorzystaniu modyfikowanych genetycznie bakterii *Escherichia coli*. Zaproponowane przez nas rozwiązanie obejmuje zastosowanie biokatalizatora w postaci całych komórek, co pozwala pominąć czasochłonne i kosztowne procedury, takie jak oczyszczanie enzymów lub stosowanie genetycznie zmodyfikowanych systemów ekspresji bakterii. Z tego powodu na podstawie badań opisanych w **P3** powstały dwa zgłoszenia patentowe o numerach P.444274 i P.444273.

Ze względu na fakt, iż w opisanych przez mnie wcześniej badaniach dotyczących arylopropenów wykazywały one działanie inhibicyjne na większość testowanych mikroorganizmów postanowiłem, we współpracy z zespołem mojej drugiej promotor, profesor Marii Elisabetty Brenny (pod której naukową opieką odbyłem roczny staż w ramach projektu POWER 3.5), otrzymać mniej toksyczne pochodne arylopropenowe w postaci dioli **6b–10b**. Zastosowałem je jako substraty do biotransformacji mając

nadzieję na otrzymanie aldehydów o właściwościach aromatycznych. Okazało się jednak, że głównymi produktami reakcji były hydroksyketony **6c–9c**. Opracowaliśmy więc dwuetapową biokatalityczną syntezę tlenowych pochodnych arylopropenowych, która obejmowała (1) chemo-enzymatyczną epoksydację, a następnie hydrolizę związków wyjściowych **6a–10a** do odpowiednich dioli **6b–10b** oraz (2) mikrobiologiczne utlenianie dioli **6b–10b** do hydroksyketonów **6c–9c** (**Rysunek 9**). Badania te opisałem w publikacji **P4**.



Rysunek 9. Dwuetapowa synteza pochodnych arylopropenowych: dioli **6b-10b** i hydroksyketonów **6c-10c**.

W pierwszym etapie związki **6a–10a** przekształcono zgodnie z metodą opisaną przez Tentori i in. dla izosafrolu (**6a**) [72], otrzymując diole **6b–10b** z wysoką wydajnością w zakresie 65–80%. Następnie wykorzystałem je jako substraty w biotransformacjach przesiewowych prowadzonych z 22 szczepami bakterii z rodzajów: *Bacillus*, *Dietzia*, *Gordonia*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, *Serratia* and *Streptomyces* oraz ponad 30 grzybami z rodzajów *Absidia*, *Aspergillus*, *Beauveria*, *Chaetomium*, *Cladosporium*, *Fusarium oxysporum*, *Fusicoccum*, *Neurospora*, *Paecilomyces*, *Penicilium*, *Phanerochaete*, *Rhizopus*, *Spicaria* i *Trichoderma.* W wyniku biotransformacji jako główne produkty otrzymałem hydroksyketony **6c-10c**. Dodatkowo w mieszaninie reakcyjnej można było wykryć niewielkie ilości diketonów i izomerycznych form hydroksyketonów. Najbardziej wydajnymi szczepami w biotransformacjach selekcyjnych były bakterie z rodzaju *Dietzia* sp. DSM44016, *R. erythropolis* PCM2150, *R. erythropolis* DSM44534 i *R. ruber* PCM2166. We wszystkich biotransformacjach zawartość hydroksyketonów **6c-10c** rosła wraz z czasem aż do 11 dnia. Najwięcej produktu **6c** (85%) powstawało w biotransformacji z *Dietzia* sp. DSM44016. Natomiast hydroksyketony **7c** i **8c** były najwydajniej otrzymywane w biotransformacji z *R. ruber* PCM2166, a dużą ilość produktu **9c** (77–79%) uzyskano w biotransformacjach przeprowadzonych przez *R. erythropolis* DSM44534 i *R. ruber* PCM2166. Wszystkie wymienione szczepy skutecznie utleniały diole **6b-10b** do hydroksyketonów **6c-10c**, z wyjątkiem biotransformacji diolu **8b** ze szczepem *R. erythropolis* PCM2150, gdzie otrzymano 38% hydroksyketonu **8c**.

W wyniku biotransformacji diolu **10b** nie powstawały hydroksyketony ani inne produkty, dlatego substrat ten nie był testowany w zwiększonej skali. Z kolei równocześnie testowane grzyby wykazywały zdecydowanie mniejszą zdolność do biotransformacji dioli **6b-10b**, w wyniku czego powstawały niewielkie ilości hydroksyketonów **6c-9c**, dlatego nie były wykorzystywane w kolejnych badaniach.

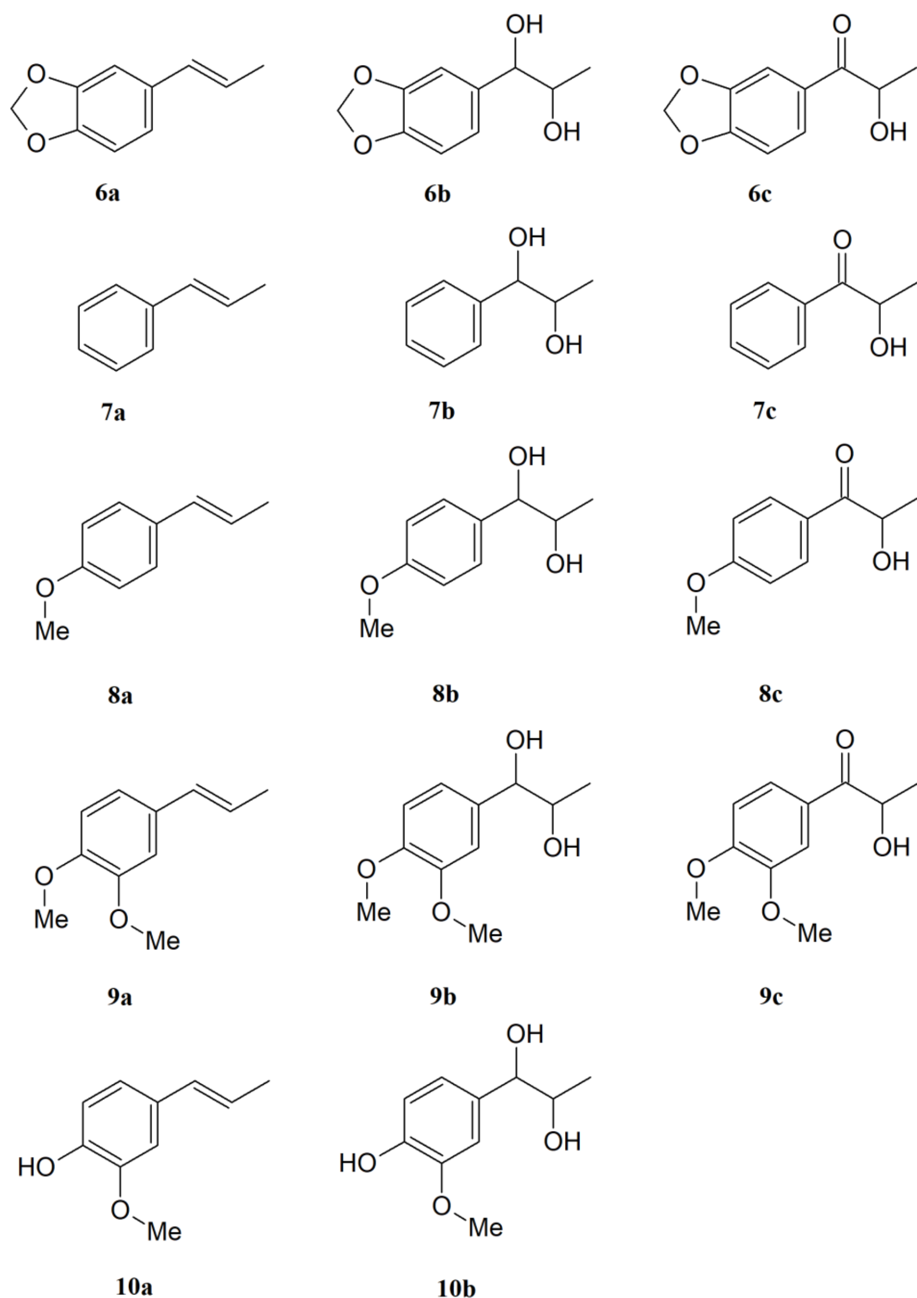
Biotransformacje w zwiększonej skali prowadziłem z wybranymi szczepami bakterii przez 11 dni z dodatkiem 0,2 g poszczególnych dioli **6a-9a**. Najwięcej hydroksyketonu **6c** otrzymałem w wyniku biotransformacji z *Dietzia* sp. DSM44016 (0,125 g, wydajność = 62,5%). W przypadku bioutlenienia diolu **7b**, tylko *R. ruber* PCM2166 katalizował reakcję, w wyniku której powstało 0,081 g produktu **7c** (wydajność = 40,5%). Natomiast w biotransformacji z *R. erythropolis* DSM44534 otrzymałem 0,115 g hydroksyketonu **8c** (wydajność = 57,5%), a reakcja z innym szczepem z tego samego gatunku - *R. erythropolis* PCM2150 pozwoliła na otrzymanie produktu **9c** (0,119 g, wydajność = 59,5%).

W literaturze opisano kilka metod syntezy hydroksyketonów **6c–9c** [87-91, 92-94]. W metodzie wykorzystującej 2,3-dichloro-5,6-dicyjano-1,4-benzochinon i fale ultradźwiękowe otrzymano związek **6c** z 72% wydajnością [88]. Reakcje enzymatyczne z zastosowaniem liazy benzaldehydowej lub dekarboksylazy benzoilomrówczanu dostarczyły hydroksyketony **7c** i **8c** z wydajnością 81-95% [91, 92]. Utlenianie dioli do α-hydroksyketonów z wykorzystaniem nadtlenku wodoru i katalizatora manganowego [89], dimetylodioksyranu, jego analogu trifluorometylowego [87] lub kwasu 2-jodoksybenzoesowego (IBX) zostało również opisane w literaturze [88]. Zaproponowana w publikacji **P4** metoda biokatalityczna stanowi atrakcyjną alternatywę dla metod opisanych w literaturze. Nie wymaga ona drogich odczynników i enzymów, a substratami są arylopropeny wszechobecne w olejkach eterycznych oraz ekstraktów roślinnych.

Z uwagi na fakt, że arylopropeny **6a-10a** charakteryzują się szerokim spektrum aktywności biologicznej [30, 32, 34], interesujące było zbadanie i analiza wpływu struktury związków na aktywność otrzymanych pochodnych tlenowych: dioli **6b–10b** i hydroksyketonów **6c–9c**.

4.3 Ocena wybranych aktywności biologicznych arylopropenów i ich tlenowych pochodnych

W następnym etapie badań substraty arylopropeny **6a-10a**, oraz zsyntezowane diole **6b-10b** i hydroksyketony **6c-9c** zostały zbadane pod kątem wybranych aktywności biologicznych (**Rysunek 10**).



Rysunek 10. Związki arylopropenowe oraz ich pochodne wybrane do badań aktywności biologicznych.

Aktywność fungistatyczną sprawdzałem względem 4 szczepów drożdży z rodzaju *Candida albicans*: 636/20, 595/20, 38, i ATTC90028. Została ona wyrażona w postaci wartości MIC_{50} (tj. stężenia związku wymaganego do zahamowania wzrostu 50% mikroorganizmów). Porównując różne związki i ich pochodne, zauważyliśmy, że obecność dodatkowych podstawników w pierścieniu aromatycznym arylopropenów **6a** i **8a-10a** wpływała pozytywnie na ich aktywność fungistatyczną w porównaniu ze związkiem **7a**. Obecność grup hydroksylowych w diolach **7b–9b** zwiększała ich aktywność inhibicyjną wobec większości badanych szczepów w porównaniu ze związkami wyjściowymi **7a–9a**. Wszystkie hydroksyketony, z wyjątkiem **8c**, były mniej aktywne niż związki wyjściowe i diole (**Tabela 2**). W literaturze opisana jest

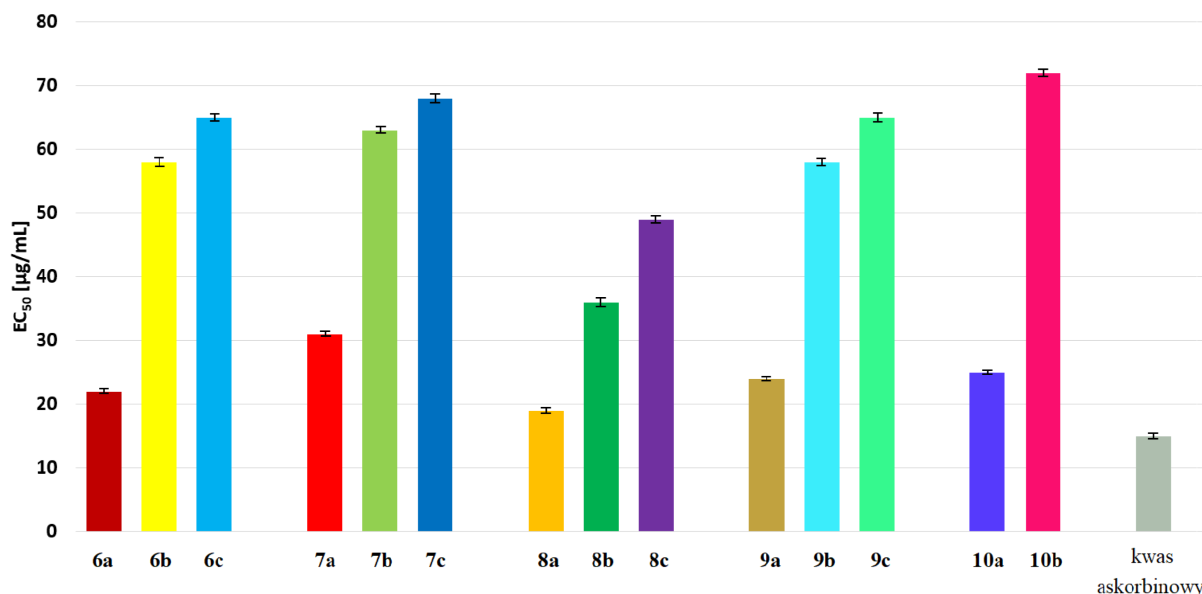
aktywność przeciwdrobnoustrojowa izosafrolu (**6a**), anetolu (**8a**) i izoeugenolu (**10a**), jednak nie porównano aktywności przeciwdrobnoustrojowej ich pochodnych, takich jak diole i hydroksyketony [30, 32-34, 78].

Tabela 2. Minimalne stężenie hamujące (MIC_{50}) [µg/mL] dla związków (**6a-10b**).

Badany związek	***C. albicans* 636/20**	***C. albicans* 595/20**	***C. albicans* 38**	***C. albicans* ATTC 90028**
6a	99	65	76	85
6b	177	107	113	135
6c	>250[1]	200	178	199
7a	195	168	>250[1]	>250[1]
7b	121	147	183	125
7c	220	230	>250[1]	>250[1]
8a	75	62	93	100
8b	61	47	76	124
8c	77	64	61	103
9a	43	59	39	67
9b	37	46	63	52
9c	111	135	97	154
10a	69	75	52	88
10b	95	103	89	130

[1]Związki nieaktywne przy najwyższym badanym stężeniu (250 µg/mL).

Kolejną aktywnością biologiczną, którą oceniłem była aktywność przeciwutleniająca z wykorzystaniem metody DPPH. Aktywność przeciwutleniającą wszystkich związków porównałem z kontrolą pozytywną, którą był kwas askorbinowy (EC_{50} = 15,21 µg/mL). Wśród badanych związków, te z grupą propenylową miały najniższe wartości EC_{50}. Wśród nich anetol (**8a**) wykazał najwyższą aktywność przeciwutleniającą (EC_{50} = 19,13 µg/ml). Istotnie niższą aktywność antyoksydacyjną zaobserwowałem dla dioli **6b-10b** i hydroksyketonów **6c-9c**, przy wartościach EC_{50} w zakresie od 36,34 do 72,08 µg/ml. Wśród dioli, **8b** wykazywał najwyższą aktywność przeciwutleniającą, natomiast wszystkie hydroksyketony wykazywały niższą aktywność niż związki wyjściowe i diole (**Rysunek 11**). Warto podkreślić, że w dostępnej literaturze znajdują się jedynie dane na temat aktywności przeciwutleniającej wybranych związków komercyjnie dostępny (**6a, 8a, 10a**) [92-93].



Rysunek 11. Wartości EC_{50} [µg/mL] dla wszystkich badanych związków w porównaniu z kwasem askorbinowym.

Aktywność hemolityczna została wykonana na ludzkich erytrocytach zgodnie z klasyfikacją toksyczności, według której, związki są wysoce toksyczne, jeśli szybkość hemolizy wynosi 90–100% i nietoksyczne, jeśli szybkość hemolizy wynosi 0–9% [94]. Szybkość hemolizy wszystkich badanych związków była bardzo podobna do tej w grupie kontrolnej i nie przekraczał 3%, co świadczy o tym, że arylopropeny **6a-10a** i ich pochodne **6b-10b** i **6c-10c** nie mają toksycznego wpływu na ludzkie krwinki czerwone (**Tabela 3**). Wyniki otrzymane dla związków wyjściowych **6a-10a** pokrywają się z tymi dostępnymi w literaturze, natomiast dla ich pochodnych otrzymanych przeze mnie nie zostały do tej pory opisane.

Tabela 3. Procent hemolizy ludzkich krwinek czerwonych po 1 godzinie inkubacji ze związkami w różnych stężeniach.

Związek	**Stężenie [µM]**						
	0	**10**	**20**	**40**	**60**	**80**	**100**
Kontrola	1.340	1.583	1.741	1.820	2.017	1.756	2.146
6a	1.340	2.412	1.544	2.180	2.397	2.585	2.550
6b	1.340	2.279	1.864	1.978	1.884	2.387	2.575
6c	1.340	1.460	1.171	1.806	1.848	1.412	1.507
7a	1.340	1.273	1.665	1.475	1.767	1.250	2.042
7b	1.340	1.038	1.081	1.005	1.317	1.787	1.706
7c	1.340	1.156	1.858	1.422	1.744	1.938	2.464
8a	1.340	1.362	1.143	2.235	2.536	2.199	2.500
8b	1.340	2.451	2.979	1.904	2.294	2.397	2.604
8c	1.340	1.834	1.649	1.725	2.327	2.152	1.526
9a	1.340	1.760	1.480	1.669	1.577	2.475	1.786
9b	1.340	1.635	1.517	1.417	1.199	1.185	2.057
9c	1.340	1.483	1.934	2.099	1.768	2.024	2.445
10a	1.340	1.379	1.161	1.753	1.881	2.047	2.185
10b	1.340	1.270	1.550	1.521	1.621	2.583	1.981

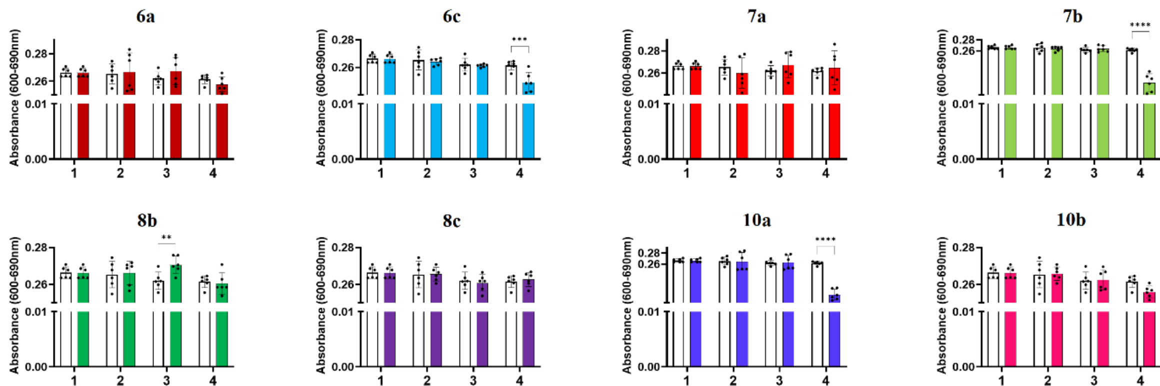
Następnie zbadano wpływ wszystkich badanych związków arylopropenowych na płynność ludzkich krwinek czerwonych, którą badano za pomocą markera fluorescencyjnego DPH [95]. Na podstawie otrzymanych wyników stwierdziliśmy, że diole, szczególnie **7b** i **9b**, powodują wzrost sztywności błony (**Tabela 4**). Co ciekawe, związek **8b** powodował wzrost sztywności błony przy niższych stężeniach i spadek przy wyższych stężeniach. Natomiast hydroksyketony **7c**, **8c** i, w mniejszym stopniu, **9c** spowodowały spadek anizotropii, co wskazuje na wzrost płynności błony. Aktywność biologiczna badanych związków jest prawdopodobnie również związana z ich różnym wpływem na błonę komórkową a określenie ich dokładnej lokalizacji w błonie komórkowej wymaga dalszych badań.

Tabela 4 Wartości anizotropii fluorescencyjnej sondy DPH w błonach komórkowych ludzkich erytrocytów poddanych działaniu badanych związków.

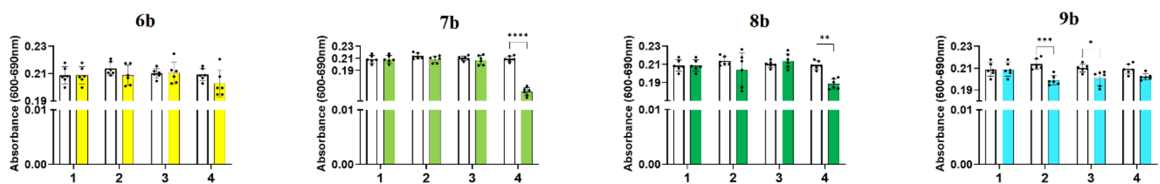
Związek	Stężenie (µM)			
	0	20	60	100
Kontrola	0.266	0.267	0.269	0.266
6a	0.266	0.262	0.264	0.237
6b	0.266	0.247	0.274	0.252
6c	0.266	0.260	0.267	0.277
7a	0.266	0.262	0.268	0.267
7b	0.266	0.286	0.277	0.278
7c	0.266	0.236	0.240	0.238
8a	0.266	0.263	0.261	0.259
8b	0.266	0.272	0.249	0.262
8c	0.266	0.239	0.249	0.244
9a	0.266	0.264	0.261	0.269
9b	0.266	0.289	0.228	0.290
9c	0.266	0.257	0.243	0.263
10a	0.266	0.259	0.272	0.256
10b	0.266	0.268	0.277	0.272

Ostatnia z ocenianych przez nas aktywności biologicznych - antyproliferacyjna była sprawdzana na liniach komórkowych HepG2, Caco-2 i MG63. Badane przez nas arylopropeny **6a-10a**, diole **6b-10b** i hydroksyketony **6c-10c** wpływały w różny sposób na wybrane linie komórkowe w zależności od dawki. Związek **8b** znacząco zwiększył potencjał proliferacyjny komórek HepG-2, podczas gdy związek **7b** wykazał najwyższy efekt hamujący żywotność komórek HepG2. Odmienną odpowiedź komórkową na związki zaobserwowano w linii komórkowej Caco-2. Najsilniejsze hamowanie wzrostu komórek Caco-2 obserwowaliśmy, gdy do ich komórek dodawano związki **6b-9b**. Arylopropeny **6a** i **7a** silnie zmniejszały żywotność komórek HepG-2. Proliferacja komórek MG63 była różnie modulowana przez związki, w zależności od dawki. Związki **6c**, **8b** i **10b** hamowały proliferację tej linii komórkowej jednak przy stężeniach 50 µg/ml związek **10b** znacząco zwiększał żywotność komórek. Wyniki uzyskane przez nas dla arylopropenów **6a-10a** są zgodne z literaturą [96-100], natomiast wpływ związków **6b-10b** i **6c-9c** na badane przez nas linie komórkowe nie został dotychcząs opisany w dostępnej literaturze. Opisane wyżej dane zostały zebrane poniżej w formie wykresów (**Rysunek 12**).

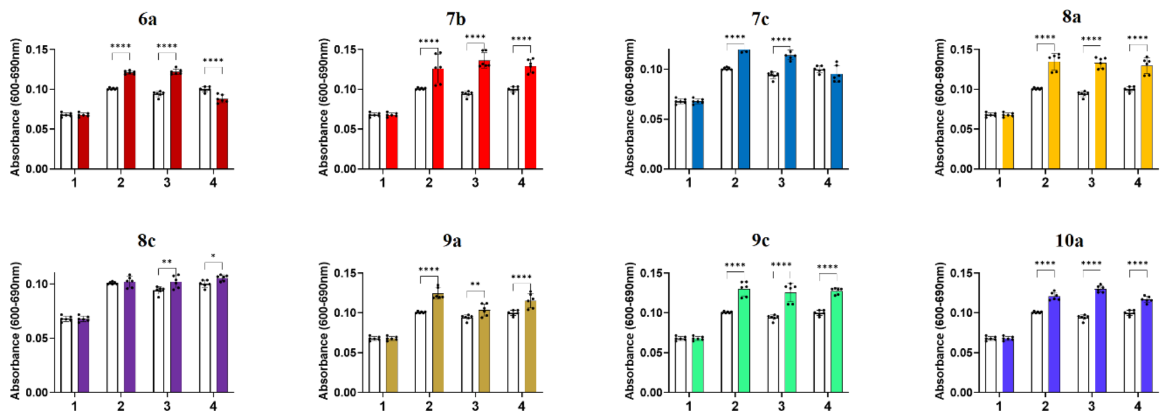
HEPG2



Caco-2



MG63



Rysunek 12. Wartości absorbancji dla linii komórkowych HepG2, Caco-2 i MG63 traktowanych związkami w stężeniach od 0 do 200 mg/ml, gdzie 1. 0 mg/ml (0% EtOH); 2. 1 mg/ml (0,01% EtOH); 3. 50 mg/ml (0,5% EtOH); 4. 200 mg/ml (2% EtOH).

5. PODSUMOWANIE

1. Oceniono potencjał biokatalityczny 22 szczepów bakterii z rodzajów *Bacillus*, *Gordonia*, *Micrococcus*, *Pseudomaons*, *Rhodococcus*, *Serratia* i *Streptomyces* oraz ponad 50 szczepów grzybów z rodzaju *Absidia*, *Agrocybe*, *Armillaria*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Chaetopsis*, *Daedalea*, *Fusarium*, *Inonotus*, *Laetiporus*, *Marasmius*, *Mortierella*, *Mucor*, *Papularia*, *Peniophora*, *Penicillium*, *Pholiota*, *Piptoporus*, *Pleurotus*, *Poria*, *Pycnoporus*, *Trametes* i *Trichoderma* względem substratów, w tym szeregu dioli **1a-b**, **3a-b**, **6b-10b** oraz izosafrolu (**6a**).

2. Przeprowadzono biotransformacje dioli **1a** i **1b** z udziałem *Gordonia rubripertincta* PCM2144, *Rhodococcus erythropolis* DSM44534, *R. erythropolis* PCM2150 w prototypowym bioreaktorze SSF, w wyniku czego otrzymano whisky laktony charakteryzujące się niską czystością optyczną.

3. Zastosowanie hodowli wgłębnych (SmF) z *R. erythropolis* DSM44534, *R. erythropolis* PCM2150 pozwoliło na otrzymanie czystych optycznie *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(–)-(4*R*,5*S*) (**2b**) i *cis*-(+)-(4*R*,5*R*) (**2d**) whisky laktonów bezpośrednio z racemicznych dioli **1a** i **1b**.

4. Transformacje izosafrolu (**6a**) prowadzone w zwiększonej skali z udziałem grzybów z rodzaju *Trametes* dostarczyły piperonalu (**5**) z wysoką wydajnością izolowania.

5. Opracowano dwuetapową biokatalityczną metodę otrzymywania tlenowych pochodnych wybranych arylopropenów.

6. Spośród testowanych bakterii, wyselekcjonowano cztery (*Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150 i *R. ruber* PCM2166) efektywnie utleniające diole **6b-9b** do hydroksyketonów **6c-9c**. Zwiększenie skali procesu skutkowało wysokimi wydajnościami izolowania.

7. Zastosowanie *R. erythropolis* DSM44534 w formie proszków acetonowych dostarczyło enancjomerycznie wzbogaconego *cis*-(–)-(4*S*,5*S*) izomeru whisky laktonu (**2c**).

8. Potwierdzono możliwość wykorzystania *R. erythropolis* DSM44534 w stereoselektywnej syntezie γ- oraz δ-laktonów. W wyniku utlenienie dioli **3a** i **3b** powstawał wysoce wzbogacony izomer *trans*-(+)-(5*S*,6*R*) (**4a**) oraz enancjomer *cis*-(–)-(5*S*,6*S*) (**4c**) aerangis laktonu.

9. Produkty uboczne przemysłu olejarskiego mogą być wykorzystane jako podłoże mikrobiologiczne w procesie utlenienia dioli katalizowanego przez bakterie.

10. Ekstrakcja prosta, a następnie destylacja z parą wodną była najbardziej wydajną metodą izolowania laktonów z podłoża stałego.

11. Związki arylopropenowe (**6a-10a**) oraz ich pochodne (**6b-10b**, **6c-9c**) zostały przeanalizowane pod kątem aktywności fungistatycznej, przeciwutleniającej, hemolitycznej oraz przeciwproliferacyjnej. Większość z testowanych związków wykazywała aktywność fungistatyczną. Związki arylopropenowe wykazywały wyższą aktywność przeciwutleniającą względem ich tlenowych pochodnych. Diole zwiększały sztywność błony, natomiast hydroksyketony wpływały na zwiększenie jej płynności. Szeroka grupa testowanych związków wykazywała inhibicję lub promocję proliferacji komórek HepG2, Caco-2, MG63 w zależności od zastosowanego stężenia.

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7. OŚWIADCZENIA O WKŁADZIE W PUBLIKACJE

Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

Dawid Hernik
imię i nazwisko

Wrocław 23.08.2023
miejscowość i data

Katedra Chemii Żywności i Biokatalizy,
Uniwersytet Przyrodniczy we Wrocławiu,
C. K. Norwida 25, 50-375 Wrocław
afiliacja

OŚWIADCZENIE

Oświadczam, że w pracy Hernik, D.; Pannek, J.; Szczepańska, E.; Olejniczak, T.; Boratyński, F. Bacterial whole cells synthesis of whisky lactones in a solid-state fermentation bioreactor prototype. *Catalysts*, 2021, 11, 320; mój udział polegał na wykonaniu biotransformacji *anty* i *syn*-3-metylo-oktan-1,4-dioli w hodowli na podłożu stałym (SSF) w skali przesiewowej i preparatywnej katalizowanych przez bakterie, liofilizacji makuchów i ich analizie pod kątem przydatności do prowadzonych biotransformacji, sprawdzeniu różnych metod ekstrakcji produktów z podłoży stałych (destylacja z parą wodną i modyfikacje tej metody, ekstrakcja w aparacie Derynga), przygotowaniu prób i ich analizie za pomocą chromatografii gazowej z detektorem FID, oczyszczaniu chromatograficznym produktów, przygotowaniu prób i wykonywaniu pomiarów skręcalności optycznej, przygotowaniu prób i analizie widm magnetycznego rezonansu jądrowego, konstrukcji prototypowego bioreaktora SSF, kalibracji czujników temperatury i wilgotności podłoża, przetworzeniu uzyskanych danych, wizualizacji danych i napisaniu manuskryptu.

23.08.2023 Dawid Hernik
data i podpis

Potwierdzam treść oświadczenia.

23.08.2023 Filip Boratyński
data i podpis promotora

Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

Dawid Hernik
imię i nazwisko

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afiliacja

OŚWIADCZENIE

Oświadczam, że w pracy Hernik, D.; Gatti, F.; Brenna, E.; Szczepańska, E.; Olejniczak, T.; Boratyński, F. Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*. *Frontiers in Microbiology*, 2023, 14, 1117835; mój udział polegał na wykonaniu biotransformacji *anty* i *syn*-3-metylo-oktan-1,4-dioli w hodowlach wgłębnych (SmF) w mikropłytkach, skali przesiewowej i preparatywnej katalizowanych przez bakterie, analizie wpływu gęstości optycznej biomasy na przebieg biotransformacji, przygotowaniu biokatalizatorów w postaci proszków acetonowych i prowadzeniu biotransformacji z ich udziałem, przygotowaniu prób i ich analizie za pomocą chromatografii gazowej z detektorem FID, oczyszczaniu chromatograficznym produktów, przygotowaniu prób i wykonywaniu pomiarów skręcalności optycznej, przygotowaniu prób i analizie widm magnetycznego rezonansu jądrowego, przetworzeniu uzyskanych danych, wizualizacji danych i napisaniu manuskryptu.

23.08.2023
data i podpis

Potwierdzam treść oświadczenia.

23.08.2023
data i podpis promotora

Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora

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OŚWIADCZENIE

Oświadczam, że w pracy Hernik, D.; Szczepańska, E.; Brenna, E.; Patejuk, K.; Olejniczak, T.; Strzała, T.; Boratyński, F. *Trametes hirsuta* as an attractive biocatalyst for the preparative scale biotransformation of isosafrole into piperonal. *Molecules*, 2023, 28, 3643; mój udział polegał na wykonaniu biotransformacji izosafrolu w hodowlach wgłębnych (SmF) w skali przesiewowej i preparatywnej katalizowanych przez grzyby, przygotowaniu prób i ich analizie za pomocą chromatografii gazowej z detektorem FID, oczyszczaniu chromatograficznym produktów, przygotowaniu prób i analizie widm magnetycznego rezonansu jądrowego, przetworzeniu uzyskanych danych i napisaniu manuskryptu.

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Potwierdzam treść oświadczenia.

23.08.2023 Filip Boratyński
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OŚWIADCZENIE

Oświadczam, że w pracy Hernik, D.; Szczepańska, E.; Ghezzi, M. C.; Brenna, E.; Włoch, A.; Pruchnik, H.; Mularczyk, M.; Marycz, K.; Olejniczak, T.; Boratyński, F. Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives. *Frontiers in Microbiology*, 2023, 14, 1223123.; mój udział polegał na wykonaniu biotransformacji dioli 1b-5b w hodowli wgłębnej (SmF) w skali przesiewowej i preparatywnej katalizowanych przez bakterie, przygotowaniu prób i ich analizie za pomocą chromatografii gazowej z detektorem FID, oczyszczaniu chromatograficznym produktów, przygotowaniu prób i analizie widm magnetycznego rezonansu jądrowego, wykonywaniu oznaczenia aktywności fungistatycznej i przeciwutleniającej, przetworzeniu uzyskanych danych, wizualizacji danych i napisaniu manuskryptu.

23.08.2023……Dawid Hernik……
data i podpis

Potwierdzam treść oświadczenia.

23.08.2023…Filip Boratyński…
data i podpis promotora

8. PUBLIKACJE

7.1 PUBLIKACJA 1 (P1)

Hernik, D.*; Pannek, J.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.*

Bacterial whole cells synthesis of whisky lactones in a solid-state fermentation bioreactor prototype. *Catalysts*, 2021, 11, 320.

catalysts

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Article

Bacterial Whole Cells Synthesis of Whisky Lactones in a Solid-State Fermentation Bioreactor Prototype

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Citation: Hernik, D.; Pannek, J.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.; Bacterial Whole Cell Synthesis of Whisky Lactones in a Solid-State Fermentation Bioreactor Prototype. *Catalysts* **2021**, *11*, 320. https://doi.org/10.3390/catal11030320

Academic Editor: Evangelos Topakas

Received: 31 December 2020
Accepted: 25 February 2021
Published: 1 March 2021

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Abstract: Agro-industrial side streams such as oilseed cakes were used as a medium in solid-state fermentation (SSF) for microbial oxidation of *anti*- and *syn*-3-methyl-octane-1,4-diols to obtain corresponding *trans*- and *cis*-whisky lactones. In preliminary screening transformations, a wide range of whole bacterial cells were tested on the basis of oxidation activity, which is rarely described in the literature, in contrast to the widely studied lipolytic activity on SSF. Among the different oil cakes tested, biotransformations carried out on linseed cake were characterized by the highest conversion and stereoselectivity. Several preparative-scale oxidations performed in a self-constructed SSF bioreactor catalyzed by *Rhodococcus erythropolis* DSM44534, *Rhodococcus erythropolis* PCM2150 and *Gordonia rubripertincta* PCM2144 afforded optically active *trans*-(+)-(4*S*,5*R*), *cis*-(+)-(4*R*,5*R*) and *cis*-(-)-(4*S*,5*S*) isomers of whisky lactones, respectively. Bacteria of the *Rhodococcu*s, *Gordonia*, *Dietzia* and *Streptomyces* genera carried out transformations with complete conversion after three days. Various extraction methods were applied for the isolation of the products, and among them, the combination of steam distillation with simple extraction were the most efficient. Biotransformations were conducted under precise control of conditions in a bioreactor based on a Raspberry Pi Zero W. The proposed low-cost (ca. USD 100) bioreactor is a standalone system that is fully autoclavable and easy to use.

Keywords: biotransformation; microbial oxidation; whisky lactones; diols; solid-state fermentation (SSF); bioreactor; Raspberry Pi

1. Introduction

Solid-state fermentation (SSF) is defined as a microbial cultivation process conducted on a solid substrate with a low content of water [1]. Several bioprocesses based on agro-industrial residues, such as the production of enzymes, single-cell proteins, aromas, organic acids, ethanol, biopolymers, and other secondary metabolites, have been developed [2–11]. In both laboratory- and industrial-scale microbial processes, it is essential to control culture conditions such as temperature, moisture content, aeration, and pH [12]. In comparison to submerged fermentation (SmF), monitoring the aforementioned process parameters during SSF is a challenge because of the solid nature of the substrate. However, due to numerous advantages (low cost of growth media, low energy requirement, low contamination risk, low pollutant production, and high isolation yields), it is worth applying SSF in industry. Of note, SSF use low-cost raw materials such as agro-industrial which significantly lowers the capital investment required for particular bioprocesses in comparison to submerged fermentation, in which liquid media are used [13].

In recent years, attention has been given to significantly reducing the generation of food waste. Agro-industrial residues have special biotechnological potential and can be

used to solve this problem. They meet the criteria enabling their use as raw materials for the SSF process, i.e., low water content and optimal particle size allowing penetration of the substrate by microorganisms (especially by filamentous fungi) [14]. Oilseed cakes are solid residues obtained as a result of the pressing of oilseeds during vegetable oil production. They can constitute up to 65–70% of seeds' weight. The oleo industry generates millions of tons of these organic byproducts every year; therefore, focusing on the various processes related to the value addition of oil cakes is needed.

Biotransformation is a process in which microorganisms or isolated enzymes are applied to obtain fine chemicals, such as enantiopure forms of chiral compounds [6,15–17]. Obtaining the desired products by the use of environmentally safe biocatalysts and raw materials makes this approach possible to meet the requirements of sustainable development and green chemistry [18]. Compounds obtained by this method are regarded as natural [19].

The increase in the application of SSF as an alternate production method to SmF by industry has been recently noted [20,21]. Despite the progress made in research on the use of SSF, the main obstacle to scaling up the process is the lack of simple, economical and easily scalable bioreactors that eliminate the problem of controlling the process conditions. The basic SSF bioreactors are tray, horizontal drum, packed-bed, and fluidized bed bioreactors, where tray bioreactors have the simplest construction and process performance. The approach proposed in this study constitutes the application of whole-cell biotransformation on oilseed cake as a medium for microorganisms using an upscaled tray bioreactor with sensors controlling the basic culture conditions (temperature and moisture). Due to a number of SSF applications, a new bioprocess involving bacterial oxidoreductases to produce industrially demanded whisky lactone was developed.

Whisky lactone is an essential component of aged alcoholic beverages such as whisky, cognac, and brandy beverages [22]. Presently, it is used as an odor ingredient in various food products (beverages and sweet and baked foods). This commercial food additive contains a racemic mixture of *trans*- and *cis*-isomers. However, in nature, individual *trans*-(+)-(4*S*,5*R*) and *cis*-(+)-(4*S*,5*S*) isomers of whisky lactone, which exhibit individual odoriferous properties, occur [23,24]. *Cis*-isomers are described as earthy and woody fragrances, while *trans*-isomers are reminiscent of celery. Therefore, the manufacture of individual stereoisomers of whisky lactone to study the relationship between its structure and biological activity is necessary. Whisky lactone is not only an aroma; the mixture of *cis*-and *trans*-isomers is used as a repellent against mosquitoes and flies [25].

Several stereoselective pathways for the synthesis of individual *trans*- and *cis*-isomers of whisky lactone have been presented in the literature [14,26–29]. However, they rely on a multistep chemical synthesis using metal catalysts and organic solvents. Catalysis involving metal compounds is widely engaged in the laboratory and industrial practice. Nevertheless, metal-based catalysts as well as organic solvents also can be dangerous for environment, and significant amounts of them are released to environment annually. The employment of biocatalysts in organic synthesis is seen as the key to green chemistry. It is worth mentioning that the method of obtaining whisky lactone isomers using a non-metallic catalyst has also been described [30]. This process is promising because it is an alternative to metal catalysts, however, it is a multi-step process with a relatively low conversion. Therefore, there is a need for the development of safe procedures that fulfills green chemistry requirements to obtain stereoisomers. To date, two pathways of biotransformation have been proposed: *via* alcohol dehydrogenase isolated from horse liver (HLADH) enantioselectivity oxidizing racemic *syn*- and *anti*-3-methyloctane-1,4-diols as well as with the use of *Beauveria bassiana* AM278 and *Pycnidiella resinae* KCH50 microorganisms catalyzing lactonization of γ-oxo acids. The application of the second strategy allowed us to obtain enantiomerically pure *trans*-(+)-(4*S*,5*R*)-whisky lactone [31].

The study aimed to perform the oxidation of *anti*-3-methyl-octane-1,4-diol (1a) and *syn*-3-methyl-octane-1,4-diol (1b) catalyzed by bacteria growing on oilseed cakes. It is

worth mentioning that the SSF processes involving oxidation on a solid substrate are not commonly known in the literature. To date, microbial oxidation of xenobiotics performed on oil industry byproducts has been reported with the use of filamentous fungi, which catalyze stereoselective hydrolysis of a racemic mixture of phenylethyl acetate and subsequent oxidation of 1-phenylethanol to acetophenone [32]. Research on reprocessing waste often requires very large financial contributions, including research equipment. In this article, we described a low-cost bioreactor with a potential environmental impact. To increase the efficiency of whisky lactone production, monitoring of cultivation parameters in the prototype bioreactor was performed.

2. Results and Discussion

Substrates for bioxidation, *anti*- (1a) and *syn*-3-methyl-octane-1,4-diols (1b), were obtained by chemical reduction of corresponding *trans*- (2a–b) and *cis*-whisky lactones (2c-d), which were previously separated by column chromatography (Figure 1). On the basis of our previous experience in the biooxidation reactions [33–35], four bacterial strains (*Gordonia bronchialis* PCM2167, *Rhodococcus ruber* PCM2166, *Rhodococcus erythropolis* DSM44534 and *Rhodococcus rhodochrous* PCM909) were selected as potential candidates for microbial oxidation of *anti*- (1a) and *syn*-3-methyl-octane-1,4-diols (1b) to obtain corresponding *trans*-(+)-(4*S*,5*R*) (2a) and *trans*-(−)-(4*R*,5*S*) (2b) or *cis*-(−)-(4*S*,5*S*) (2c) and *cis*-(+)-(4*R*,5*R*) (2d) whisky lactones (Figure 2). The same microorganisms were also used in our recent studies (pending patent protection), in which 3-n-butylphthalide was obtained as a result of oxidation of the corresponding diol (1-hydroxymethyl-2-(1-hydroxypentyl)benzene. Bacteria from selected species were characterized by high activity of alcohol dehydrogenases (ADH) responsible for one-pot oxidation of diols to lactones which was confirmed in our previous studies [33–35].

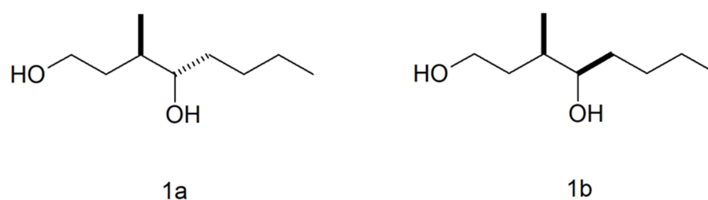

Figure 1. Structures of *anti*- (1a) and *syn*-3-methyl-octane-1,4-diols (1b).

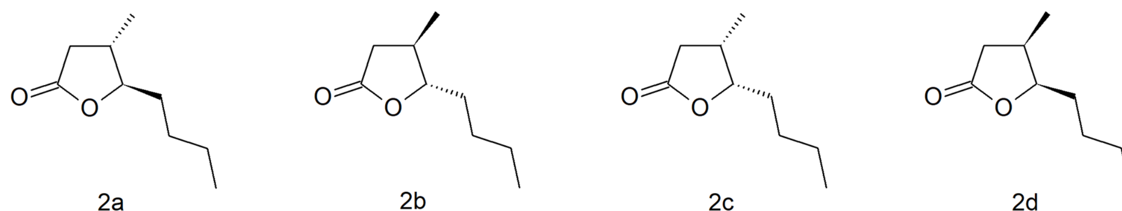

Figure 2. Structures of *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**), *cis*-(−)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones.

The growth of biocatalysts as well as the biotransformation process was performed *via* solid-state fermentation using three different oleoindustry byproducts: linseed, rapeseed and evening primrose cakes. These residues are abundant in nutritional compounds such as carbohydrates, proteins, fats, and cellulose and therefore constitute excellent media for the growth of microorganisms [36,37]. The average moisture of the studied oil cakes (7.9% for linseed cake, 6.5% for rapeseed cake and 4.2% for evening primrose cake) was calculated by the lyophilization of individual oil cake samples. This confirmed our observation, during which the linseed cake had the highest hygroscopic properties, while the evening primrose cake absorbed water slightly.

2.1. Preliminary Screening Scale Biotransformations with Anti-3-Methyl-Octane-1,4-Diol (1a) on Different Oil Cakes.

In the oxidation of *anti*-3-methyl-octane-1,4-diol (1a) with all tested strains growing on the linseed cake, the complete conversion (*conv.* = 100%) after three days was observed (Table 1). Even though c*is*-whisky lactone was obtained in enantiopure form (*ee* > 99%) in all biotransformations, enantiomerically enriched *trans*-whisky lactone (*ee* = 20–70%) was a dominant product.

Table 1. Comparison of microbial oxidation of *anti*-3-methyl-octane-1,4-diol (1a) on linseed and rapeseed cake (in % according to GC).

Strain	Oil cake	Time [days]	Conv. 1a [%]	Products			
				Trans 2a–b [%]	ee [%]	*Cis* 2c–d [%]	ee [%]
Gordonia bronchialis PCM2167	linseed	3	100	83 (±0.7)	33 (+)-(4*S*,5*R*)-2a	17 (±1.5)	>99 (−)-(4*S*,5*S*)-2c
		7	100	81 (±1.2)	33 (+)-(4*S*,5*R*)-2a	19 (±1.3)	>99 (−)-(4*S*,5*S*)-2c
	rapeseed	3	9 (±0.9)	7 (±0.4)	32 (+)-(4*S*,5*R*)-2a	2 (±0.1)	0
		7	12 (±1.1)	10 (±0.8)	18 (+)-(4*S*,5*R*)-2a	2 (±0.1)	0
Rhodococcus erythropolis DSM44534	linseed	3	100	97 (±1.2)	20 (−)-(4*R*,5*S*)-2b	3 (±0.2)	>99 (+)-(4*R*,5*R*)-2d
		7	100	90 (±0.9)	70 (−)-(4*R*,5*S*)-2b	10 (±0.5)	>99 (+)-(4*R*,5*R*)-2d
	rapeseed	3	15 (±0.7)	8 (±0.7)	nd*	7 (±0.2)	nd
		7	43 (±2.1)	9 (±0.8)	nd	34 (±1.2)	nd
Rhodococcus rhodochrous PCM909	linseed	3	100	92 (±0.7)	20 (+)-(4*S*,5*R*)-2a	8 (±0.3)	>99 (+)-(4*R*,5*R*)-2d
		7	100	90 (±0.9)	42 (+)-(4*S*,5*R*)-2a	10 (±0.4)	>99 (+)-(4*R*,5*R*)-2d
	rapeseed	3	0	0	0	0	0
		7	0	0	0	0	0
Rhodococcus ruber PCM2166	linseed	3	100	92 (±1.3)	37 (+)-(4*S*,5*R*)-2a	8 (±0.1)	>99 (−)-(4*S*,5*S*)-2c
		7	100	86 (±0.6)	33 (+)-(4*S*,5*R*)-2a	14 (±0.8)	65 (−)-(4*S*,5*S*)-2c
	rapeseed	3	0	0	0	0	0
		7	84 (±0.6)	67 (±1.2)	5 (+)-(4*S*,5*R*)-2a	17 (±1.2)	0

nd*—not determined.

Oxidation with *Gordonia bronchialis* PCM2167 on linseed cake afforded optically active *trans*-(+)-(4*S*,5*R*)-isomer (2a) (81-83%, *ee* = 33%) and enantiomerically pure *cis*-(−)-(4*S*,5*S*) whisky lactone (2c) (17–19%, *ee* >99%). In biotransformations performed on linseed cake with *Rhodococcus erythropolis* DSM44534, *R. rhodochrous* PCM909 and *R. ruber* PCM2166, optically active *trans*-whisky lactone isomers were obtained after three days in amounts of 97%, 92% and 92%, respectively. It is significant that after 7 days, *R. erythropolis* DSM44534 produced *trans*-(−)-(4*R*,5*S*)-isomer (2b) (*ee* = 70%), while *R. ruber* PCM2166 and *R. rhodochrous* PCM909 gave the opposite *trans*-(+)-(4*S*,5*R*)-isomer (2a) (*ee* = 33–42%). The enantioselectivity of biotransformation increases over time; thus, the enantiomeric excess of lactone was higher after 7 days. On the other hand, transformation catalyzed by *G. bronchialis* PCM2167 afforded higher amounts (*ca.* 20%) of enantiomerically pure *cis*-(−)-(4*S*,5*S*) whisky lactone (2c) compared with *Rhodococcus* strains.

A very low conversion (9 to 43%) for *G. bronchialis* PCM2167 and *R. erythropolis* DSM44534, except oxidation with *R. ruber* PCM2166 (84% after 7 days), was observed in biotransformations performed on rapeseed cake. No conversion was observed during the biotransformation with *R. ruber* PCM2166 after three days which indicates that alcohol dehydrogenases are produced by this strain in the later stages of biotransformation. In the bioxidation with *R. rhodochrous* PCM909, no conversion on the rapeseed cake occurred. This indicates that rapeseed cake is an inadequate medium for ADH production by aforementioned biocatalyst. No whisky lactones were detected by using primrose cake for solid-state fermentation; thus, this byproduct is not applicable for diol oxidation.

2.2. Preliminary Screening Scale biotransformations with Syn-3-Methyl-Octane-1,4-Diol (1b) on Different Oil Cakes.

During the oxidation of *syn*-3-methyl-octane-1,4-diol (1b) on the linseed cake with all bacterial strains, 100% conversion was observed after the 3rd day (Table 2). Although the transformations catalyzed by *Gordonia bronchialis* PCM2167, *Rhodococcus rhodochrous* PCM909 and *R. ruber* PCM2166 afforded enantiomerically pure *trans*-(+)-(4*S*,5*R*) whisky lactone (*ee* >99%), enantiomerically enriched *cis*-(+)-(4*R*,5*R*) isomer (2d) constituted the majority of the reaction mixture (63–81%). Biotransformation with *G. bronchialis* PCM2167 on linseed cake after three days gave *trans*-(+)-(4*S*,5*R*) enantiomer (2a) (19%, *ee* >99%) and enantiomerically enriched *cis*-(+)-(4*R*,5*R*) whisky lactone (2d) (83%, *ee* = 93%). In the biotransformations with *G. bronchialis* PCM2167, it was observed that from days 3 to 7, the enantiomeric excess of *cis*-(+)-(4*R*,5*R*) (2d) isomer decreased from *ee* = 93% to 85%.

Table 2. Comparison of microbial oxidation of *syn*-3-methyl-octane-1,4-diol (1b) on linseed and rapeseed cake (in % according to GC).

Strain	Oil cake	Time [days]	Conv. 1b [%]	Products			
				Trans 2a–b [%]	*ee* [%]	*Cis* 2c–d [%]	*ee* [%]
Gordonia bronchialis PCM2167	linseed	3	100	19 (±0.7)	>99 (+)-(4*S*,5*R*)-2a	81 (±0.7)	93 (+)-(4*R*,5*R*)-2d
		7	100	21 (±1.3)	>99 (+)-(4*S*,5*R*)-2a	79 (±1.7)	85 (+)-(4*R*,5*R*)-2d
	rapeseed	3	0	0	0	0	0
		7	12 (±0.9)	10	0	2 (±0.4)	82 (+)-(4*R*,5*R*)-2d
Rhodococcus erythropolis DSM44534	linseed	3	100	27 (±0.9)	37 (+)-(4*S*,5*R*)-2a	73 (±1.0)	83 (+)-(4*R*,5*R*)-2d
		7	100	23 (±0.6)	78 (+)-(4*S*,5*R*)-2a	77 (±1.1)	86 (+)-(4*R*,5*R*)-2d
	rapeseed	3	18 (±0.7)	4 (±0.1)	0	14 (±0.3)	0
		7	57 (±1.5)	9 (±0.3)	0	48 (±1.6)	4 (+)-(4*R*,5*R*)-2d
Rhodococcus rhodochrous PCM909	linseed	3	100	25 (±0.9)	>99 (+)-(4*S*,5*R*)-2a	75 (±1.2)	65 (+)-(4*R*,5*R*)-2d
		7	100	31 (±0.4)	>99 (+)-(4*S*,5*R*)-2a	69 (±0.7)	67 (+)-(4*R*,5*R*)-2d
	rapeseed	3	0	0	0	0	0
		7	0	0	0	0	0
Rhodococcus ruber PCM2166	linseed	3	100	37 (±0.9)	>99 (+)-(4*S*,5*R*)-2a	63 (±1.6)	79 (+)-(4*R*,5*R*)-2d
		7	100	37 (±1.2)	>99 (+)-(4*S*,5*R*)-2a	63 (±0.8)	83 (+)-(4*R*,5*R*)-2d
	rapeseed	3	0	0	0	0	0
		7	22 (±0.7)	6 (±0.3)	0	16 (±0.9)	0

On the rapeseed cake, the low conversion of diol and only small amounts of both *trans*- and *cis*-whisky lactone isomers were observed. The highest conversion of *syn*-3-methyl-octane-1,4-diol (1b) was observed on rapeseed cake with *R. erythropolis* DSM44534. There was no conversion in transformation with *R. rhodochrous* PCM909 on rapeseed cake, likewise in oxidation with *anti*-3-methyl-octane-1,4-diol (1a). In biotransformations on primrose oilcake, no conversion of *syn*-3-methyl-octane-1,4-diol (1b), similar to that of *anti*-3-methyl-octane-1,4-diol (1a), was detected.

In biotransformations performed on the linseed cake with *G. bronchialis* PCM2167, *R. ruber* PCM2166, *R. erythropolis* DSM44534 and *R. rhodochrous* PCM909 (2.1 and 2.2), significantly more *trans* whisky lactone (81–97%) was formed when *anti*-3-methyl-octane-1,4-diol (1a) was used as a substrate (Table 1). On the other hand, when *syn*-3-methyl-octane-1,4-diol (1b) was applied, *cis*-isomer (63–81%) was formed predominantly (Table 2). Among tested microorganisms it was noticed that in transformations with *R. ruber* PCM2166 on rapeseed cake, higher conversion (84%) was obtained with 1a compared to 1b (22%)."

According to our previous results [31], *trans*-(+)-(4*S*,5*R*) (2a) and *cis*-(−)-(4*S*,5*S*) (2c) enantiomers of whisky lactones were produced as a result of microbial whole-cell lactonization of the corresponding γ-oxoacids. The opposite enantiomerically enriched *trans*-(−)-(4*R*,5*S*) (2b) and *cis*-(+)-(4*R*,5*R*) (2d) whisky lactones were obtained in enzymatic oxidation catalyzed by commercially available alcohol dehydrogenases. We developed a SSF oxidation process as an interesting alternative against redox reactions catalyzed by expensive enzymes and required coenzymes.

There are two possible approaches to obtain chiral lactones from racemic diols [33]. In the first one (Figure 3), the primary hydroxy group of diol is chemoselectively oxidized to a carboxylic group, and then, the corresponding hydroxy carboxylic acid is cyclized to a lactone product. However, in the second pathway, there is a two-step oxidation process. First, diol is oxidized to the corresponding hydroxyaldehyde; then, hemiacetal is formed spontaneously, and it is further oxidized to lactone. Our previous studies, due to isolated hemiacetals, confirmed the second pathway of enzymatic oxidation of diols to whisky lactones. However, in the second pathway, there is a two-step oxidation process. First, diol is oxidized to the corresponding hydroxyaldehyde; then, hemiacetal is formed spontaneously, and it is further oxidized to lactone. Our previous studies, due to isolated hemiacetals, confirmed the second pathway of enzymatic oxidation of diols to whisky lactones [33].

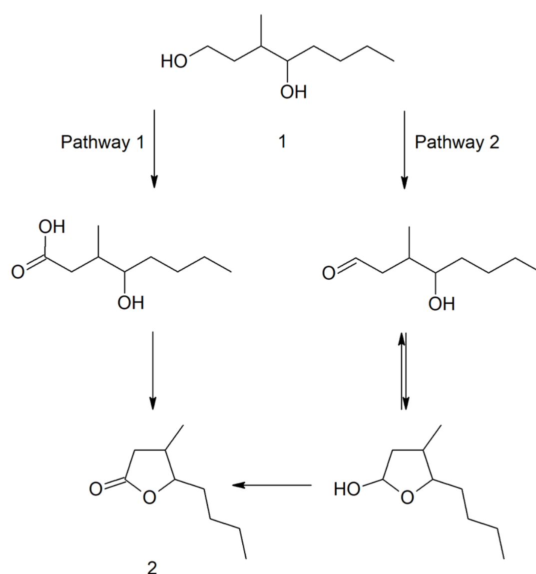

Figure 3. Two possible pathways of biotransformation of 3-methyl-octane-1,4-diol (**1**).

On the basis of our research, we cannot clearly confirm which of the abovementioned mechanisms takes place during the oxidation catalyzed by bacterial cells. As a result of bacterial oxidation of the diols (1a and 1b) in the preliminary screening, we obtained enantiomerically pure or enriched isomers opposite to those isolated in our previous research [31,33]. Therefore, further research is required to confirm which path is actually involved in this process because no intermediate products were isolated.

2.3. Preliminary Screening Scale Biotransformations with a Diastereoisomeric Mixture of Anti- and Syn-3-Methyl-Octane-1,4-Diols (1a–b)

On the basis of previous screening, only linseed cake was selected for these studies. It is supposed to be related to facts that the linseed cake is characterized by the highest ability to absorb water [37]. In the case of bacteria, it is of great importance because they grow in the entire volume of the substrate, and the even distribution of water throughout the growth medium also causes even bacterial growth. In the case of evening primrose

cake, only a part of the water is absorbed, and the rest is on the surface of the substrate, which can reduce bacterial growth. In addition, evening primrose oilcake contains much more fiber, which can slow down the growth of bacteria because it makes them less accessible to nutrients [38]. Rapeseed cake contains sinapic acid, which has antimicrobial activity therefore probably causes worse bacterial growth on this byproduct [39].

Biotransformations with all tested strains with a diastereoisomeric mixture of *anti*- and *syn*-3-methyl-octane-1,4-diols (1a—b) were performed to determine how the mixture of substrate stereoisomers affects the obtained products concerning the conversion and enantiomeric purity of lactones (Table 3). The results obtained from the oxidation of a mixture of *anti*- and *syn*-diols were the resultant of those received in individual biotransformations presented in paragraphs 2.1 and 2.2. In most biotransformations the *trans*/*cis* ratio of whisky lactone isomers was in the range of 39–60% for *trans*-2a 40–61% for *cis*-2d isomer. Among tested strains the ratio of formed *trans*/*cis*-whisky lactones differs significantly only in the transformation with *Gordonia bronchialis* PCM2167 after three days affording 23% of the *trans*-isomer 2a and 77% of the *cis*-isomer 2d. However, after seven days it was 45/55%, similar ratio to the rest of the biotransformations. The highest enantiomeric excess (*ee* = 90%) of *cis*-(+)-(4*R*,5*R*) lactone (2d) was obtained in the *G. bronchialis* PCM2167 culture after three days. In all biotransformations the conversion was 100%. Since a diastereoisomeric mixture of diols was used as a substrate, a significant decrease in the biotransformation enantioselectivity was observed; thus, separate biotransformations with the individual isomers in subsequent studies were subsequently performed.

Table 3. Microbial oxidation of a mixture of *anti*- and *syn*-3-methyl-octane-1,4-diols (1a–b) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a–1b [%]	Products			
			***Trans* 2a–b [%]**	***ee* (+)-(4*S*,5*R*)-2a[%]**	***Cis* 2c–d [%]**	***ee* (+)-(4*R*,5*R*)-2d[%]**
Gordonia bronchialis PCM2167	3	100	23 (±0.9)	55	77 (±1.2)	90
	7	100	45 (±0.7)	52	55 (±0.6)	70
Rhodococcus erythropolis DSM44534	3	100	47 (±1.1)	32	53 (±1.4)	77
	7	100	47 (±0.4)	35	53 (±0.7)	79
Rhodococcus rhodochrous PCM909	3	100	39 (±0.8)	70	61 (±1.1)	60
	7	100	47 (±1.7)	76	53 (±1.9)	74
Rhodococcus ruber PCM2166	3	100	58 (±1.4)	30	42 (±1.1)	32
	7	100	60 (±1.7)	37	40 (±0.3)	35

2.4. Screening Scale Biotransformations with Anti-3-Methyl-Octane-1,4-Diol (1a) on Linseed Cake

During the first round of biotransformations performed on three different oil cakes, only decent amounts of optically pure isomers of whisky lactones were obtained. Therefore, further screening studies were conducted to select bacteria with significant dehydrogenase activity that effectively catalyze the oxidation of diols to lactones. Based on previous experiments, linseed cake was selected as the growth medium for biotransformation. Next, several strains of bacteria from the *Gordonia*, *Rhodococcus*, *Micrococcus*, *Dietzia* and *Streptomyces* species were selected to test their oxidation activity (Table 4).

Table 4. Microbial oxidation of *anti*-3-methyl-octane-1,4-diol (1a) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a [%]	Products			
			Trans 2a–b [%]	*ee* [%]	*Cis* 2c–d [%]	*ee* [%]
Dietzia sp.	3	100	80 (±1.1)	31 (−)-(4*R*,5*S*)-2b	20 (±0.6)	84 (+)-(4*R*,5*R*)-2d
DSM44016	7	100	82 (±0.8)	44 (−)-(4*R*,5*S*)-2b	18 (±0.7)	85 (+)-(4*R*,5*R*)-2d
Gordonia rubripertincta	3	100	100	78 (+)-(4*S*,5*R*)-2a	0	0
PCM2144	7	100	100	62 (+)-(4*S*,5*R*)-2a	0	0
Micrococcus luteus	3	20 (±0.5)	20 (±0.7)	12 (+)-(4*S*,5*R*)-2a	0	0
PCM525	7	25 (±0.9)	25 (±0.2)	15 (+)-(4*S*,5*R*)-2a	0	0
Rhodococcus coprophilus	3	100	95 (±0.6)	7 (+)-(4*S*,5*R*)-2a	5 (±0.1)	20 (+)-(4*R*,5*R*)-2d
PCM2174	7	100	93 (±0.5)	3 (+)-(4*S*,5*R*)-2a	7 (±0.7)	30 (+)-(4*R*,5*R*)-2d
Rhodococcus erythropolis	3	100	80 (±1.1)	0	20 (±0.9)	99 (−)-(4*S*,5*S*)-2c
PCM2150	7	100	82 (±0.8)	27 (+)-(4*S*,5*R*)-2a	18 (±0.7)	50 (−)-(4*S*,5*S*)-2c
Rhodococcus ruber	3	30 (±0.2)	30 (±0.6)	30 (+)-(4*S*,5*R*)-2a	0	0
PCM2171	7	80 (±1.1)	80 (±1.5)	9 (+)-(4*S*,5*R*)-2a	0	0
Rhodococcus ruber	3	0	0	0	0	0
PCM2216	7	35 (±0.9)	35 (±0.3)	10 (+)-(4*S*,5*R*)-2a	0	0
Streptomyces griseus subsp. griseus	3	100	78 (±1.5)	50 (+)-(4*S*,5*R*)-2a	22 (±0.7)	8 (+)-(4*R*,5*R*)-2d
PCM2331	7	100	85 (±1.2)	50 (+)-(4*S*,5*R*)-2a	15 (±0.4)	25 (+)-(4*R*,5*R*)-2d

In transformations carried out with *Gordonia rubripertincta* PCM2144, *Rhodococcus erythropolis* PCM2150, *Rhodococcus coprophilus* PCM2174, *Dietzia* sp. DSM44016 and *Streptomyces griseus subsp. griseus* PCM2331, after three days, *trans*-whisky lactones in amounts ranging from 78% to 100% were produced. The highest enantiomeric excess of *trans*-(+)-(4*S*,5*R*) isomer 2a (*ee* = 78%) was detected on the 3rd day of transformations with *G. rubripertincta* PCM2144. As a result of oxidation, the *cis*-isomer was obtained with the strains *R. erythropolis* PCM2150, *R. coprophilus* PCM2174, *Dietzia* sp. DSM44016 and *S. griseus subsp. griseus* PCM2331. The enantiomerically pure *cis*-(−)-(4*S*,5*S*) whisky lactone (2c) (*ee* > 99%) was obtained in the culture of *R. erythropolis* PCM2150.

By comparing the same genus of bacteria (*G. bronchialis* PCM2167 *vs G. rubripertincta* PCM2144, *R. erythropolis* DSM44534 *vs R. erythropolis* PCM2150, *R. ruber* PCM2171 *vs R. ruber* PCM2216) used in both screening experiments (Table 2 and Table 4), there is a similarity in the biotransformations carried out by the strains of the same type.

2.5. Screening Scale Biotransformations with Syn-3-Methyl-Octane-1,4-Diol (1b) on Linseed Cake

Analysis of the oxidation performed with *G. rubripertincta* PCM2144, *R. erythropolis* PCM2150, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016 showed 100% conversion of substrate after three days. During oxidation with most of the strains, enantiomerically enriched *cis*-(+)-(4*R*,5*R*) whisky lactone was obtained (Table 5). The highest amounts from 77% to 100% of this isomer were obtained when *G. rubripertincta* PCM2144, *R. erythropolis* PCM2150, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016 were used as the biocatalyst. In the oxidation with *Dietzia* sp. DSM44016 *cis*-(+)-(4*R*,5*R*) isomer was obtained on the 7th day (77%, *ee* = 79%). As a result of transformation, an optically active *trans*-isomer (*ee* = 50-77%) was obtained with *G. rubripertincta* PCM2144, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016. Biotransformations with *S. griseus subsp. griseus* PCM2331 showed low conversion with a solely small amounts of *trans*- and *cis*-isomers (5–16%).

Table 5. Microbial oxidation of *syn*-3-methyl-octane-1,4-diol (1b) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1b [%]	Products			
			Trans 2a–b [%]	*ee* [%]	*Cis* 2c–d [%]	*ee* [%]
Dietzia sp.	3	100	27 (±0.7)	77 (+)-(4*S*,5*R*)-2a	73 (±1.2)	75 (+)-(4*R*,5*R*)-2d
DSM44016	7	100	23 (±0.9)	50 (+)-(4*S*,5*R*)-2a	77 (±0.4)	79 (+)-(4*R*,5*R*)-2d
Gordonia rubripertincta	3	100	40 (±0.3)	77 (+)-(4*S*,5*R*)-2a	60 (±1.2)	32 (−)-(4*S*,5*S*)-2c
PCM2144	7	100	16 (±0.5)	62 (+)-(4*S*,5*R*)-2a	84 (±2.1)	30 (−)-(4*S*,5*S*)-2c
Micrococcus luteus	3	30 (±0.4)	0	0	30 (±0.9)	20 (+)-(4*R*,5*R*)-2d
PCM525	7	35 (±1.3)	0	0	35 (±1.1)	29 (+)-(4*R*,5*R*)-2d
Rhodococcus coprophilus	3	100	15 (±0.4)	60 (+)-(4*S*,5*R*)-2a	85 (±1.2)	33 (+)-(4*R*,5*R*)-2d
PCM2174	7	100	30 (±0.9)	75 (+)-(4*S*,5*R*)-2a	70 (±0.7)	33 (+)-(4*R*,5*R*)-2d
Rhodococcus erythropolis	3	100	0	0	100	5 (+)-(4*R*,5*R*)-2d
PCM2150	7	100	0	0	100	2 (+)-(4*R*,5*R*)-2d
Rhodococcus ruber	3	30 (±0.2)	0	0	30 (±1.7)	10 (−)-(4*S*,5*S*)-2c
PCM2171	7	60 (±1.3)	0	0	60 (±1.8)	0
Rhodococcus ruber	3	35 (±0.7)	0	0	35 (±1.1)	30 (+)-(4*R*,5*R*)-2c
PCM2216	7	35 (±0.4)	0	0	35 (±1.3)	0
Streptomyces griseus subsp. griseus	3	10 (±0.2)	5	0	5 (±0.3)	60 (+)-(4*R*,5*R*)-2d
PCM2331	7	27 (±1.2)	16	0	11 (±0.3)	60 (+)-(4*R*,5*R*)-2d

Comparing the same bacteria from *Gordonia* genus only with *G. bronchialis* PCM2167, enantiomerically pure *trans*-whisky lactone was formed (Table 2). *R. erythropolis* PCM2150 (Table 5) transformed diol (1b) to *cis*-whisky lactone with lower enantiomeric excess than *R. erythropolis* DSM44534 (Table 2), and no *trans*-isomer was formed. *R. ruber* PCM2166 (Table 2) oxidized diol (1b) with higher conversion and enantioselectivity than *R. ruber* PCM2171 and *R. ruber* PCM2216 (Table 5).

During biotransformation of *anti*- and *syn*-diols with strains *Dietzia maris* PCM2292 and *Rhodococcus rhodnii* PCM2157 (not mentioned in Tables 4 and 5), there was no conversion, which proves that these bacteria do not have the ability to oxidize 3-methyl-octane-1,4-diol on linseed cake. Biotransformations with *Micrococcus luteus* PCM525 and *Rhodococcus ruber* PCM2216 showed low conversion (20–35%). With *anti*-diol (1a), only a small amount of *trans*-isomer (20–35%) was formed, and with *syn*-diol (1b) only *cis*-isomer of whisky lactone was produced (30–35%).

In bioxidation carried out with the following strains *Dietzia* sp. DSM44016, *G. rubripertincta* PCM2144, *R. coprophilus* PCM2174, *R. erythropolis* PCM2150, *R. ruber* PCM2171, and *S. griseus subsp. griseus* PCM2331 (Table 4 and 5), it was also noticed that more *trans*-whisky lactone was produced after seven days (80–100%) when the *anti*-diol (1a) was used as the substrate. However, when *syn*-diol (1b) was added, more *cis*-whisky lactone isomer was formed predominantly after seven days (60–100%) within the same strains, except for *S. griseus subsp. griseus* PCM2331 (5–11%). We identified three biocatalysts *M. luteus* PCM525, *R. ruber* PCM2171, *R. ruber* PCM2216, which catalyzed highly diastereoselective oxidation independently of the substrate used, affording only *trans*-whisky isomers from *anti*-diol and *cis*-whisky isomers from *syn*-diol. Application of two stereochemically different substrates in biotransformations catalyzed by *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 was significant concerning diastereoselectivity of the process. In oxidation catalyzed by *G. rubripertincta* PCM2144 using *anti*-diol only *trans*-isomer was obtained, however with *syn*-diol a mixture of *cis*/*trans*-isomers was observed. In contrary, *R. erythropolis* PCM2150 catalyzed oxidation of *syn*-diol to only *cis*-lactone, while from *anti*-diol a mixture of *cis*/*trans*-whisky isomers was detected.

2.6.Selection of the Extraction Method

On the basis of screening studies, a simple extraction method using organic solvents was applied to isolate biotransformation products on a preparative scale. While conducting simple extraction in preparative biotransformations, a problem arose related to the extraction of excessive amounts of fat from linseed cake. Attempts to separate the lipid fraction *via* column chromatography were unsuccessful; the columns clogged, and only 10-15% of the product was obtained. Therefore, it was decided to test other extraction methods. Extraction with the Deryng apparatus was then tested, but the cake foamed and burned easily when the flask was heated (even at low heating temperatures). In next method steam distillation was tested. The efficiency during steam distillation was 85%; however, due to the high content of protein in the cake, it foamed considerably. Therefore, the extraction process had to be performed several times because the resulting foam filled the entire distillation flask, which significantly extended the time of this method. Taking into consideration this fact, it was decided to conduct steam distillation from organic extract first to obtain a simple extraction of the preparative biotransformation. The yield of this method was 81%, and distillation could be performed in one step due to the volume of the obtained extract was small and did not foam. This method turned out to be the most effective among all tested and was used in all preparative biotransformations.

2.7. Calibration of Moisture Sensor

To control humidity during biotransformation in the bioreactor, two types of electrodes were tested. First, measurements were carried out with graphite electrodes and then with a probe for measuring soil moisture (Waveshare 9527) on linseed and rapeseed cake. Based on measurements conducted with graphite electrodes, a correlation graph of moisture and voltage in rapeseed cakes was created (Figure 4a). The lowest value of moisture was 15% for rapeseed cake. With moisture lower than 15%, the sensor could not detect voltage. The voltage increased for a moisture from 15 to 90%. Figure 4b shows that for linseed cake, the lowest value of moisture conducted with graphite electrodes, which could be marked, was 20%. Voltage increased for the moisture from 20 to 90%. A steady increase in voltage was observed for moisture in the range from 40 to 50%. The smallest increase in voltage was observed from 60 to 70% moisture, and the largest increase was from 30 to 60% moisture.

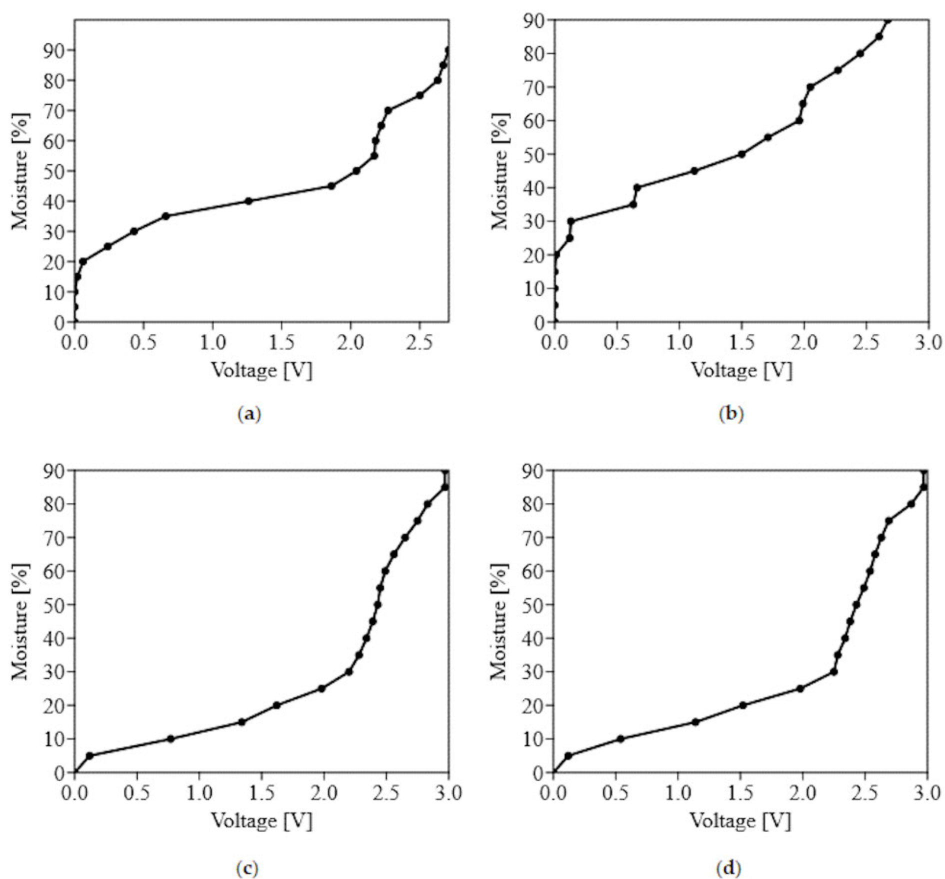

Figure 4. Correlation curves of moisture and voltage: (**a**) measured with graphite electrodes for rapeseed cake; (**b**) measured with graphite electrodes for linseed cake; (**c**) measured with probe (Waveshare 9527) for rapeseed cake; (**d**) measured with probe (Waveshare 9527) for linseed cake.

When measuring the moisture in the rapeseed cake with a probe (Waveshare 9527), the first measurable voltage reading was 5% medium moisture (Figure 4c). The maximum reading range ended at 85% medium moisture. In the ranges of 5% to 30%, 30% to 60% and 60% to 80%, the values were similar to linear, allowing humidity control between these values. The first measurement at 5% humidity was also obtained in the measurement of moisture on the linseed cake using a probe with a humidity sensor (Waveshare 9527). The maximum range also ended at 85% humidity because the voltage was constant above this value (Figure 4d). Linear values were obtained in the range from 5 to 30% and from 30 to 75% humidity, allowing for the precise control of humidity in the medium.

Comparing the values obtained when controlling the medium with different electrodes, we decided to use a sensor with a probe to measure the humidity. This made it possible to obtain more linear values in wide ranges of humidity, which allowed for more accurate measurements. Additionally, this sensor was easier to work with and to keep clean.

2.8. Preparative Biotransformations with Anti- and Syn-3-Methyl-Octane-1,4-Diols (1a-b)

A prototype SSF bioreactor was designed and constructed to conduct preparative biotransformations under constant conditions of humidity and oxygenation. During multiple tests of the SSF bioreactor, we confirmed that materials that were used for its design were chosen in an appropriate manner. The cover of the bioreactor (polypropyl-

ene), glass capillary, graphite electrodes, probe (Waveshare 9527) and TMP36GT9Z temperature sensor survived multiple sterilization as well as contact with microorganisms and organic solvents. All the elements of the bioreactor were easy to clean and maintain. In the case of damage, electrodes or capillaries can be quickly and simply replaced with new electrodes or capillaries. The air pump that we used in bioreactor ensures good gas exchange.

In preparative biotransformations, bacterial cultures with OD_{600} = 0.3 were added to the oilcakes. It was observed that the growth of bacteria was associated with the production of metabolites causing sludge formation and a slight discoloration of the medium. During preliminary studies on preparative scale, it was noticed that the addition of substrate before 4th day after bacterial inoculation led to lower conversion and enantioselectivity of the biotransformation.

On the basis of previous screening experiments for preparative biotransformations of diols (1a–b), the following strains were selected: *Rhodococcus erythropolis* DSM44534, *R. erythropolis* PCM2150 and *Gordonia rubripertincta* PCM2144. Biotransformations of *syn*-3-methyl-octane-1,4-diol (1b) catalyzed by *R. erythropolis* DSM44534 and *G. rubripertincta* PCM2144 showed 80–100% conversion of the substrate after seven days (Table 6). The isolation yield of a mixture of optically active *trans*-(+)-(4*S*,5*R*) (2a) and *cis*-(+)-(4*R*,5*R*) (2d) whisky lactone isomers in the transformation with *R. erythropolis* DSM44534 was 79.4%. Specific rotations of the enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (2a) ($[\alpha]_D^{20}$ = +79.7 (c = 0.25, CH_3OH, *ee* = 80%, *yield* = 17.4%); ref. $[\alpha]_D^{20}$ = +97.0 (c = 0.34, CH_3OH, *ee* = 99%) [40]) and *cis*-(+)-(4*R*,5*R*) (2d) ($[\alpha]_D^{20}$ = +52.1 (c = 0.2, CH_3OH, *ee* = 66%, *yield* = 62%); ref. $[\alpha]_D^{20}$ = +79.0 (c = 0.5, CH_3OH, *ee* = 99%) [40]) whisky lactones were measured and compared with data from the literature. The biotransformation with *G. rubripertincta* PCM2144 was highly selective and afforded only enantiomerically enriched *trans*-(+)-(4*S*,5*R*)-isomer (2a) ($[\alpha]_D^{20}$ = +61.2 (c = 0.15, CH_3OH, *ee* = 66%) with an isolation *yield* = 68%.

Table 6. Comparison of microbial oxidation of *syn*-3-methyl-octane-1,4-diol (1b) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1b [%]	Products			
			***Trans* 2a** [%]	***ee*** [%]	***Cis* 2d** [%]	***ee*** [%]
Gordonia rubripertincta PCM2144	4	25 (±0.6)	25 (±0.9)	55 (+)-(4*S*,5*R*)-2a	0	0
	7	100	100	66 (+)-(4*S*,5*R*)-2a	0	0
Rhodococcus erythropolis DSM44534	4	15 (±0.3)	3 (±0.2)	65 (+)-(4*S*,5*R*)-2a	12 (±0.5)	54 (+)-(4*R*,5*R*)-2d
	7	80 (±1.2)	22 (±0.6)	80(+)-(4*S*,5*R*)-2a	58 (±0.5)	66 (+)-(4*R*,5*R*)-2d

Analysis of the oxidation of *anti*-3-methyl-octane-1,4-diol (1a) with *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 showed 90–100% conversion of the substrate after seven days (Table 7). In both biotransformations, a mixture of enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (2a) and *cis*-(−)-(4*S*,5*S*) (2c) whisky lactones was obtained. It is worth emphasizing that in the biotransformation of substrate 1a, opposite to the oxidation of 1b, the optically active *cis*-(−)-(4*S*,5*S*) isomer (2c) of whisky lactone was produced. The overall isolation yields of the biotransformations catalyzed by *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 were 65% and 61%, respectively. In the transformation with *R. erythropolis* PCM2150, enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (2a) ($[\alpha]_D^{20}$ = +32.8 (c = 0.1, CH_3OH, *ee* = 35%, *yield* = 45%) and *cis*-(−)-(4*S*,5*S*) (2c) ($[\alpha]_D^{20}$ = −54.5 (c = 0.1, CH_3OH, *ee* = 66%, *yield* = 14%); ref. $[\alpha]_D^{20}$ = −79.0 (c = 0.5, CH_3OH, *ee* = 99%) [40]), whisky lactones were isolated. Preparative oxidation catalyzed by *G. rubripertincta* PCM2144 delivered enantiomerically enriched *trans*-(+)-(4*S*,5*R*)-isomer (2a) ($[\alpha]_D^{20}$ = +60.7 (c = 0.086, CH_3OH, *ee* = 64%, *yield* = 15.1%) and *cis*-(−)-(4*S*,5*S*)-isomer (2c) ($[\alpha]_D^{20}$ = −24.3 (c = 0.1, CH_3OH, *ee* = 25%, *yield* = 42%) of whisky lactones.

Table 7. Comparison of microbial oxidation of *anti*-3-methyl-octane-1,4-diol (1a) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a [%]	Products			
			Trans 2a [%]	*ee* [%]	*Cis* 2c [%]	*ee* [%]
Gordonia rubripertincta PCM2144	4	54 (±1.1)	18 (±0.4)	51 (+)-(4*S*,5*R*)-2a	36 (±0.9)	27 (−)-(4*S*,5*S*)-2c
	7	90 (±1.5)	21 (±0.7)	64 (+)-(4*S*,5*R*)-2a	69 (±1.1)	25 (−)-(4*S*,5*S*)-2c
Rhodococcus erythropolis PCM2150	4	56 (±0.3)	45 (±0.2)	0	11 (±0.4)	68 (−)-(4*S*,5*S*)-2c
	7	100	77 (±1.3)	35 (+)-(4*S*,5*R*)-2a	23 (±0.8)	66 (−)-(4*S*,5*S*)-2c

3. Materials and Methods

3.1. Microorganisms

Micrococcus luteus PCM525, *Streptomyces griseus* subsp. *griseus* PCM2331, *Dietzia maris* PCM2292, *Rhodococcus coprophilus* PCM2174, *Rhodococcus erythropolis* PCM2150, *Rhodococcus rhodnii* PCM2157, *Rhodococcus rhodochrous* PCM909, *Rhodococcus ruber* PCM2166, *Rhodococcus ruber* PCM2171, *Rhodococcus ruber* PCM2216, *Gordonia bronchialis* PCM2167, *Gordonia rubripertincta* PCM2144 came from the Polish Academy of Sciences. *Dietzia* sp. DSM44016 and *Rhodococcus erythropolis* DSM44534 came from the Department of Chemistry at Wroclaw University of Environmental and Life Sciences. Biocatalysts were maintained at 4°C on PCM agar slants then transferred into conical flasks with PCM medium containing sodium chlorine (6 g), glucose (20 g), casein (2 g), bacteriological peptone (10 g) and yeast extract (2 g) dissolved in distilled water (1 L) at 25 °C pH 5.5.

3.2. Materials

A diastereoisomeric mixture of whisky lactones, $LiAlH_4$ and PCM medium ingredients were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Oilseed cakes were purchased from Oleofarm, Wroclaw, Poland.

3.3. Measurement of Oilseed Cake Moisture

Five grams of linseed, rapeseed and primrose cakes were weighed into a round-bottom flask. The outlet of the flasks was clogged with cotton wool, and each sample was weighed on an analytical balance and placed in a freeze dryer. The prepared samples were freeze-dried for 24 h and then weighed on an analytical balance. For each oilseed cakes, five repetitions were performed.

3.4. Separation of the cis/trans-Whisky Lactones

A diastereoisomeric mixture of *cis*/*trans*-whisky lactones was separated using column chromatography. For this purpose, a column filled with silica gel dissolved in hexane was prepared, and then, 1.0 g of the *cis*/*trans*-whisky lactone mixture was applied. The column was eluted with a mixture of hexane:ethylacetate:diethylether:methylene chloride in a ratio of 20:1:1:1. Fractions were collected and controlled using gas chromatography (GC) [8]. Finally, *trans*- (0.430 g) and *cis*- (0.500 g) whisky lactones were collected separately. Spectral data were attached in supporting information (SI).

Trans-whisky lactone ^1H NMR (600 MHz, $CDCl_3$) δ: 0.91 (t, J = 7.2 Hz, 3H, CH_3-4′); 1.13 (d, J = 6.5 Hz, 3H, CH_3-4); 1.32–1.42 (m, 3H, CH_2-3′, one of CH_2-2′); 1.50 (m, 1H, one of CH_2-2′); 1.60 (m, 1H, one of CH_2-1′); 1.68 (m, 1H, one of CH_2-1′); 2.15–2.25 (m, 2H, one of CH_2-3, H-4); 2.66 (m, 1H, one of CH_2-3); 4.00 (td, J = 7.9, 4.0 Hz, 1H, H-5); ^{13}C NMR (150 MHz, $CDCl_3$): δ 13.89 (C-4′), 17.49 (CH_3-4), 22.49 (C-3′), 27.85 (C-2′), 33.70 (C-1′), 36.08 (C-4), 37.13 (C-3), 87.46 (C-5), 176.61 (C-2); IR (film, cm^{-1}): 1787 (s), 1222 (s), 1187 (s) (Figures S2–S4 SI).

Cis-whisky lactone ^1H NMR (600 MHz,$CDCl_3$) δ: 0.91 (t, J = 7.3 Hz, 3H, CH_3-4′); 1.00 (d, J = 7.0 Hz, 3H, CH_3-4); 1.29-1.40 (m, 3H, CH_2-3′, one of CH_2-2′); 1.45-1.54 (m, 2H, one of CH_2-2′, one of CH_2-1′); 1.65 (m, 1H, one of CH_2-1′); 2.18 (dd, J = 17.0, 4.0 Hz, 1H, one of CH_2-3); 2.57 (m, 1H, H-4); 2.67 (dd, J = 17.0, 7.8 Hz, 1H, one of CH_2-3); 4.42 (ddd, J = 10.1, 5.6, 4.1 Hz, 1H, H-5); ^{13}C NMR (150 MHz, $CDCl_3$): δ 13.82 (CH_3-4), 13.90 (C-4′), 22.51 (C-3′), 28.03 (C-2′), 29.57 (C-4), 33.01 (C-1′), 37.56 (C-3), 83.70 (C-5), 176.94 (C-2); IR (film, cm^{-1}): 1787 (s), 1219 (m), 1180 (s) (Figures S5–S7 SI).

3.5. Chemical Reduction of Whisky Lactones

In the round-bottom flask, 0.420 g of racemic *trans*-whisky lactone (2a–b) dissolved in 50 mL of diethyl ether was placed, followed by the addition of 0.128 g $LiAlH_4$. Similarly, 0.480 g of racemic *cis*-whisky lactone (2c–d) was dissolved in diethyl ether, followed by the addition of 0.146 g $LiAlH_4$. The flask with the attached condenser was placed on a magnetic stirrer. The reduction was carried out for 24 h at 20 °C. The reaction was controlled by thin-layer chromatography (TLC) and gas chromatography (GC). After completion of the reaction, 10% HCl was added to a mixture to spread the excess $LiAlH_4$. The content of the flask was then transferred to the splitter and extracted three times with diethyl ether. The collected organic layer was then extracted with saturated NaCl solution and dried with $MgSO_4$, passed through a paper filter and evaporated under reduced pressure. As a result of the reduction, 0.380 g of *anti*-3-methyl-octane-1,4-diol (1a) (yield = 90.4%) and 0.456 g of *syn*-3-methyl-octane-1,4-diol (1b) (yield = 95%) were obtained. Spectral data were attached in supporting information (SI).

Anti-3-methyl-octane-1,4-diol (1a) ^1H NMR (600 MHz,$CDCl_3$) δ: 0.87 (d, J = 6.8 Hz, 3H, CH_3-3); 0.89 (t, J = 7.1 Hz, 3H, CH_3-8); 1.22–1.36 (m, 3H, one of CH_2-6, CH_2-7); 1.38–1.46 (m, 3H, CH_2-5, one of CH_2-6); 1.50 (m, 1H, one of CH_2-2); 1.67-1.77 (m, 2H, one of CH_2-2, H-3); 2.81 i 3.00 (two s, 2H, 2xOH); 3.55 (m, 1H, H-4); 3.62 (ddd, J = 10.9, 7.1, 5.0 Hz, 1H, one of CH_2-1); 3.73 (ddd, J = 10.9, 6.4, 5.0 Hz, 1H, one of CH_2-1); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.89 (CH_3-3), 14.12 (C-8), 22.79 (C-7), 28.70 (C-6), 33.35 (C-5), 35.99 (C-3), 36.20 (C-2), 60.65 (C-1), 74.97 (C-4); IR (film, cm^{-1}): 3342 (s), 1475 (m), 1395 (m), 1065 (m), 1018 (m) (Figures S8–S10 SI).

Syn-3-methyl-octane-1,4-diol (1b) ^1H NMR (600 MHz, $CDCl_3$) δ: 0,89 (t, J = 7.1 Hz, 3H, CH_3-8); 0.92 (d, J = 6.8 Hz, 3H, CH_3-3); 1.23–1.36 (m, 3H, one of CH_2-6, CH_2-7); 1.37–1.51 (m, 3H, CH_2-5, one of CH_2-6); 1.56 (m, 1H, one of CH_2-2); 1.62–1.70 (m, 2H, one of CH_2-2, H-3);3.13 (s, 2H, 2xOH); 3.38 (ddd, J = 8.4, 5.5, 3.3 Hz, 1H, H-4); 3.59 (ddd, J = 11.4, 6.9, 5.1 Hz, 1H, one of CH_2-1); 3.72 (ddd, J = 11.4, 6.7, 5.0 Hz, 1H, one of CH_2-1); ^{13}C NMR (150MHz, $CDCl_3$) δ: 14.12 (C-8), 16.60 (CH3-3), 22.81 (C-7), 28.06 (C-6), 34.14 (C-5), 35.26 (C-2), 36.43 (C-3), 60.31 (C-1), 75.82 (C-4); IR (film, cm^{-1}): 3333 (s), 1480 (s), 1386 (s),1069 (s), 1018 (s) (Figures S11–S13 SI).

3.6. Screening Scale Biotransformations

Five grams of oilseed cake (linseed, rapeseed or primrose) was weighed into 100 mL tapered flasks and then sterilized at 121 °C at a pressure of 1 atm. The medium was inoculated with 0.5 mL of preprepared cultures of bacteria at OD_{600} = 0.3. The prepared bacterial cultures were placed for 4 days at 30 °C. After this time, 0.01 g of the substrate (*anti*-3-methyl-octane-1,4-diol (1a) or *syn*-3-methyl-octane-1,4-diol (1b)) dissolved in 0.5 mL of acetone and 0.5 mL of water was sprayed onto each of the flasks. For simple extraction, ethyl acetate (25 mL) was added to the samples and shaken for 5 min at 200 rpm in Falcon tubes. The organic phase was transferred to a vial and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a paper filter to a GC vial. Biotransformation was controlled after three and seven days on the GC. Control experiments were also performed in which microorganisms were cultured on the medium without the addition

of substrate to check their metabolites. The stability of the substrate was also checked by the addition of the substrate to the medium without microorganisms.

3.7. Statystical Analysis

All the described experiments were performed in triplicate, and the values presented in the tables are the mean of the obtained results. Student's t-test showed that all the data did not differ significantly. Additionally, the values of the standard deviation were calculated for the conversion and a percentage of whisky lactone isomers are shown in the tables. Statistical analyses were performed by Past 4.02.

3.8. Preparative Biotransformations

Linseed cake (50 g) was placed in the bioreactor vessel. The bioreactor was sterilized at 121 °C for 15 min. Subsequently, the medium was inoculated with 5 mL of preprepared cultures of bacteria at OD_{600} = 0.3. Then, 75 mL of sterile water was added to obtain 60% medium moisture. The bioreactor was connected to the rest of the apparatus and placed in a thermostatic cabinet at 30 °C. The culture was incubated for 4 days with continuous gas exchange under 60% humidity. The air pump, guaranteed gas exchange and sterile flow of air in the chamber, was turned on for 5 min with 30 min intervals. Then, 0.1 g of the substrate (*anti*-3-methyl-octane-1,4-diol (1a) or *syn*-3-methyl-octane-1,4-diol (1b)) dissolved in 2.5 mL of acetone and 2.5 mL of water was sprayed onto the culture. Samples were extracted after 2, 3, 4, 5 and seven days and checked by GC to estimate the progress of the biotransformation.

3.9. Design of the Bioreactor

The SSF bioreactor is based on calculating the voltage measurement. A Raspberry Pi Zero W was connected to a 10-bit analog-to-digital converter MCP3008 and to an opto-isolated two-channel relay. For both relays, as a control signal, 3.3 V signal from a GPIO pins was used. Relays were used to control two 4.8 W air pumps with maximal efficiency of 2 × 270 L/h and continuous regulation. Two graphite electrodes, sensor with a probe for measuring soil moisture Waveshare 9527, steel tube for temperature sensor, one glass capillary with tapered tip responsible for water additions and two glass capillary with microbiological filters with 0.2 μm pore size for gas exchange were placed in the bioreactor chamber. To minimize the impact of electric current, the humidity sensor electrodes were connected to the MOSFET transistor as a switch. Microbiological filters with 0.2 μm input and output as well as microbiological filters with input of air in bottles with sterile water guaranteed sterile air conditions. To connected air wires Teflon hoses were used. The chamber was made of polypropylene to be sterilized. The dimensions of the vessel were 120 mm × 85 mm × 65 mm and were selected to be able to work in the chamber under sterile conditions. The graphite electrodes connected to MCP3008 were 22 mm in diameter and 160 mm in height (Figures S14–S17 SI).

3.10. Extraction Methods

3.10.1. Simple Extraction

When the biotransformation was completed, growth medium with microorganisms was transferred into a 1000 mL flask and extracted three times with ethyl acetate (100 mL). During the first extraction, the reaction mixture was shaken with solvent for 4 h, and then the organic phase was filtered under reduced pressure. Subsequent extractions were carried out by analogy lasting 2 h and 1 h, respectively. The collected organic phase was transferred into a flask and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a paper filter, and the solvent was evaporated under reduced pressure.

3.10.2. Steam Distillation

After biotransformation, growth medium with biomass was transferred into a 500 mL round-bottom flask, and then 200 mL of distilled water was added. The flask was connected to the distillation apparatus. Distillation was carried out for 2 h, and the distillate was collected into a 250 mL round-bottom flask. The distillates were transferred to a 500 mL splitter and then extracted three times with 80 mL of ethyl acetate. Then, the extract was dried with anhydrous $MgSO_4$, and the organic solvent was evaporated under reduced pressure.

3.10.3. Steam Distillation from the Extract

In this modification of steam distillation, the content of the bioreactor vessel was transferred into a 1000 mL flask and extracted three times with ethyl acetate (100 mL). During the first extraction, the reaction mixture was shaken with solvent for 4 h, and then the organic phase was filtered under reduced pressure. Subsequent extractions were carried out by analogy lasting 2 h and 1 h. The organic phase was transferred to a flask and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a paper filter, and the solvent was evaporated under reduced pressure. The extract obtained from three extractions was placed in a 250 mL round-bottom flask, and 100 mL of distilled water was added and connected to the distillation apparatus. Distillation was carried out for 2 h, and 250 mL of distillate was collected. The distillates were transferred to a 500 mL splitter and then extracted three times with 80 mL of ethyl acetate. Then, the extract was dried with anhydrous $MgSO_4$, and the organic solvent was evaporated under reduced pressure.

3.10.4. Extraction with a Dryng Apparatus

After biotransformation, reaction mixture was placed in a 500 mL round-bottom flask, and then 200 mL of distilled water was added. The sample flask was heated for 2 h. The vapors were condensed by means of a cold refrigerant. After extraction, 1 mL of cyclohexane containing biotransformation products was collected.

3.11. Analysis Procedure

Separation of the diastereoisomeric mixture of *cis*/*trans*-whisky lactones and chemical reduction of whisky lactones to corresponding diols were controlled by thin layer chromatography (TLC), using aluminum fail plates coated with silica gel. Compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$ and 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4. Gas chromatography analysis (GC, FID, carrier gas H_2) was carried out on Agilent Technologies 7890N (GC System, Santa Clara, CA, USA). Enantiomeric excesses of the products were determined on chiral column Cyclosil-B (30 m × 0.25 mm × 0.25 μm, Santa Clara, CA, USA) according to the next temperature program: 80 °C, 160 °C (3 °C/min), 250 °C (20 °C/min) (3 min). Samples (2 μL) were injected with split 9:1; the flow of carrying gas was 1 mL/min. The total run time was 34.0 min. Retention times were established as follow: t_R = 20.74 min for *trans*-(+)-(4*S*,5*R*) (2a), t_R = 21.05 min for *trans*-(−)-(4*R*,5*S*) (2b), t_R = 22.42 min for *cis*-(−)-(4*S*,5*S*) (2c), t_R = 22.54 min for *cis*-(+)-(4*R*,5*R*) (2d) (Figure S1 SI). The substrates were determined on the chiral column CP-Chirasil L-Val (25 m × 0.25 mm × 0.12 μm, Santa Clara, CA, USA) according to the next temperature program: 80 °C, 165 °C (3 °C/min), 200 °C (20 °C/min) (1 min). Samples (2 μL) were injected with split 9:1; the flow of carrying gas was 1 mL/min. The total run time was 31.0 min. Retention times were established as follow: t_R = 18.553 for *anti*-3-methyl-octane-1,4-diol (1a), t_R = 18.630 min for *syn*-3-methyl-octane-1,4-diol (1b). The structures of the compounds were confirmed on the basis of ^1H NMR and ^{13}C NMR, which were recorded for $CDCl_3$ solutions on a Bruker Avance DRX 600 (600 MHz) spectrometer (Billerica, MA, USA). IR spectra were determined using FTIR Thermo-Mattson IR 300 Spectrometer. Optical rotations were measured on a Jasco P-2000 Polarimeter.

3.12. Moisiture Analysis

To ensure maximum chemical protection and to minimalize the impact of metals on microorganisms, we decided to use graphite electrodes to measure the moisture content. A sensor with a probe for measuring soil moisture Waveshare 9527 was also checked to compare them with graphite electrodes and determine which electrodes would be better for controlling the humidity. The moisture sensor was calibrated using an oil cake with known water content. The bioreactor chamber was placed in a temperature controller at an air temperature of 30 °C. The preliminary study showed that an equilibrium state was established in 15 min, so the measurements were carried out after this time. Water was added to the bioreactor vessel in a liquid state by spraying through a septum with small holes (d = 1 mm). The moisture sensor monitored the oil cake moisture and water was added automatically when the humidity level in the bioreactor vessel decreased by 3% from the set point. Sensor measurements were fluctuating in range ±1%.

The relation between the water additive and voltage was determined using an analog digital converter. A separate curve depending on the moisture from the analog to digital converter was designed for each of the oilcakes. We also examine the effect of temperature and electrode distance on the response of the sensor. When we increased the moisture, the resistance between electrodes decreased, which resulted in more voltage. Electrodes were placed at a distance of 3 cm from each other and submerged in the culture to a depth of 1 cm. Measurement of the water content depending on the conductivity was carried out by adding water successively, which corresponds to 5% to 90% by increasing the oilcake content by 5 to 50 g. Research was carried out at a temperature of 30 °C. Moisture was calculated by the following equation (1) which allows to calculate the amount of water required to achieve the desired humidity value taking into consideration the water contained presently in the cake.

$$Ww = Ow * \frac{W}{100\% - W} - Ow * Mw \quad (1)$$

Ww—Water weight [g]

Ow—Oilcake weight [g]

W—Moisture content that we need to obtain [%]

Mw—Moisture contet in oilcakes [%]

The amount of water added ranged from 2.6 g for 5% moisture to 450 g for 90% moisture. Each addition of water required 15 min of waiting for water absorption. Then, measurements were carried out every 10 sec until 10 matching results were obtained.

3.13. Software

In the presented bioreactor, we used a Raspbian Stretch Linux distribution specifically designed for Raspberry Pi. To control the relay and MCP3008, Python programming was used. When the moisture value was too low, the resistance between the graphite electrodes increased, and one of the GPIO pins that controlled the relay changed the LOW/HIGH state. The air pumps, enabled by the relay, pumped sterile air to the bottle with water, the pressure pushed water to capillary, and consequently, the medium was moistened. The second pump was used to exchange the gas by removal of carbon dioxide and other gases and was controlled by the GPIO pins switched on at certain intervals. The temperature sensor TMP36GT9Z was used only for remote temperature reading and was not connected to the heating cabinet. All data from sensors were collected and sent to a server.

4. Conclusions

The byproducts from the oil industry can be used as a valuable microbial medium for the bacterial oxidation process, leading from diols to whisky lactones. Biotransformations carried out on a preparative scale delivered corresponding enantiomerically en-

riched isomers *trans*-(+)-(4*S*,5*R*) (2a), *cis*-(−)-(4*S*,5*S*) (2c) and *cis*-(+)-(4*R*,5*R*) (2d). Among the different oil cakes tested, biotransformations carried out on linseed cake were characterized by a high conversion and stereoselectivity. During the research, it was confirmed that biotransformations should be carried out separately for *anti*- (1a) and *syn*-3-methyl-octane-1,4-diol (1b) because the oxidation of the diastereoisomeric mixture of diols was characterized by low stereoselectivity. Steam distillation followed by simple extraction with the use of organic solvents was proven to be the most efficient method of extracting products after biotransformation. This method allowed for the extraction of whisky lactones without the fats in the oilcake. In our opinion, our bioreactor prototype is definitively a better alternative to classic biotransformations performed in Erlenmeyer flasks. The measurements of moisture in the solid medium using graphite electrodes were satisfactory, and the irrigation and gas exchange system worked properly. Our results showed that by using simple solutions, it is possible to create a fully functional bioreactor prototype.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/11/3/320/s1, Figure S1: Chromatogram of the mixture of trans and cis whisky lactone, Figure S2: ^{1}H NMR spectrum of trans-whisky lactone, Figure S3: ^{13}C NMR spectrum of trans-whisky lactone, Figure S4: IR spectrum of trans-whisky lactone, Figure S5: ^{1}H NMR spectrum of cis-whisky lactone, Figure S6: ^{13}C NMR spectrum of cis-whisky lactone, Figure S7: IR spectrum of cis-whisky lactone, Figure S8: 1H NMR spectrum of anti-3-methyl-octane-1,4-diol, Figure S9: ^{13}C NMR spectrum of anti-3-methyl-octane-1,4-diol, Figure S10: IR spectrum of anti-3-methyl-octane-1,4-diol, Figure S11: ^{1}H NMR spectrum syn-3-methyl-octane-1,4-diol, Figure S12: ^{13}C NMR spectrum syn-3-methyl-octane-1,4-diol, Figure S13: IR spectrum syn-3-methyl-octane-1,4-diol, Figure S14: A bioreactor vessel with graphite electrodes, a vessel with water, air and water pumps, Figure S15: Control system in the plastic cover, Figure S16: Moisture sensor, Figure S17: Bacteria in the linseed cake.

Author Contributions: Conceptualization, D.H., J.P., F.B.; methodology, D.H., J.P., E.S., T.O., F.B.; formal analysis, D.H., F.B.; investigation, D.H., J.P.; resources, D.H., F.B.; writing—original draft preparation, D.H., J.P., E.S.; writing—review and editing, F.B.; visualization, D.H., E.S.; supervision, F.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research and APC was funded by the project "UPWR 2.0:international and interdisciplinary program of development of Wrocław University of Environmental and Life Sciences" ,co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062 / 18 of June 4, 2019. The work was supported by Wroclaw Centre of Biotechnology, program The Leading National Research Centre (KNOW) for years 2014–2018.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank American Journal Experts (AJE) for providing the editing service.

Conflicts of Interest: The authors declare no conflict of interest.

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catalysts

MDPI

Supporting Information

Bacterial Whole Cells Synthesis of Whisky Lactones in a Solid-State Fermentation Bioreactor Prototype

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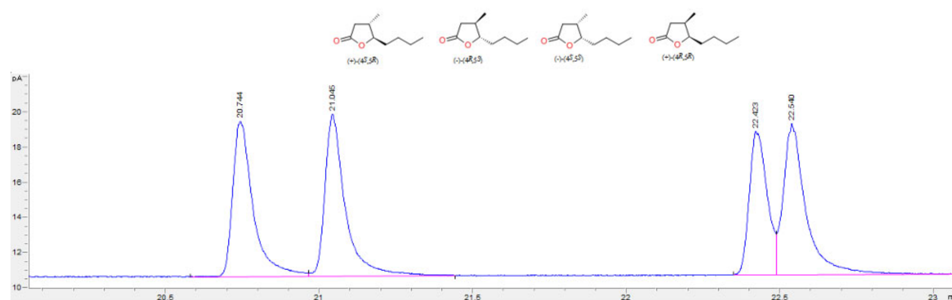

Figure 1. Chromatogram of the mixture of *trans* and *cis* whisky lactone.

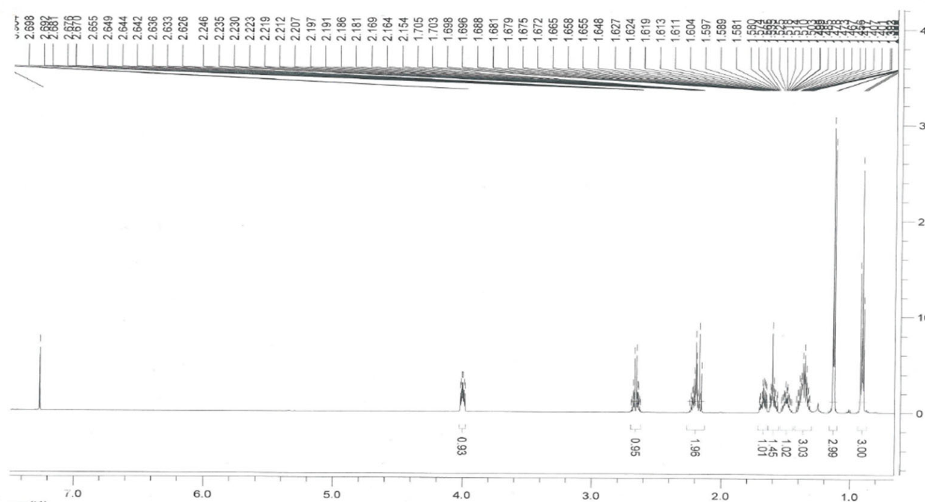

Figure S2. ^{1}H-NMR spectrum of *trans*-whisky lactone.

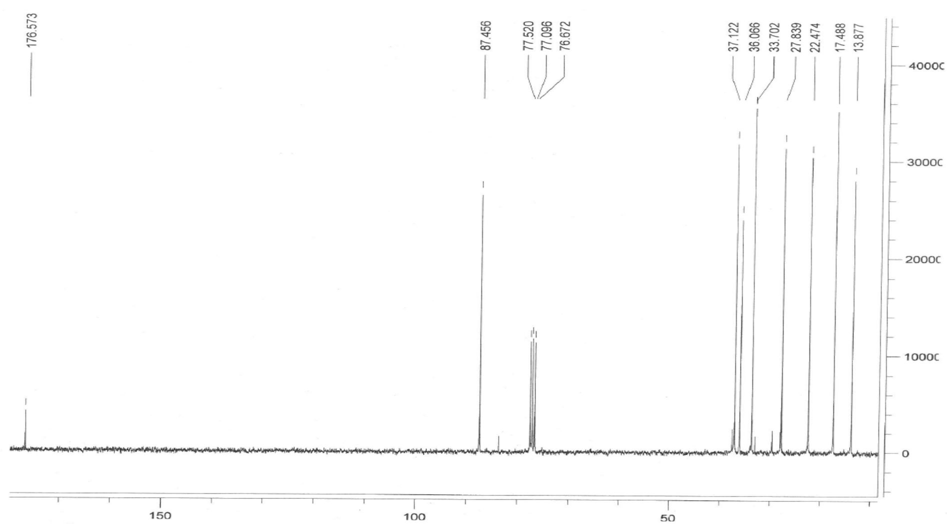

Figure S3. ^{13}C NMR spectrum of *trans*-whisky lactone.

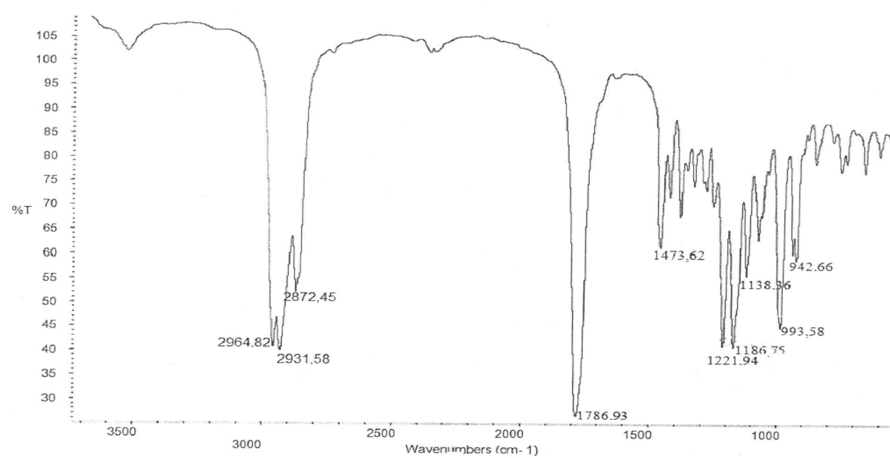

Figure 4S. IR spectrum of *trans*-whisky lactone.

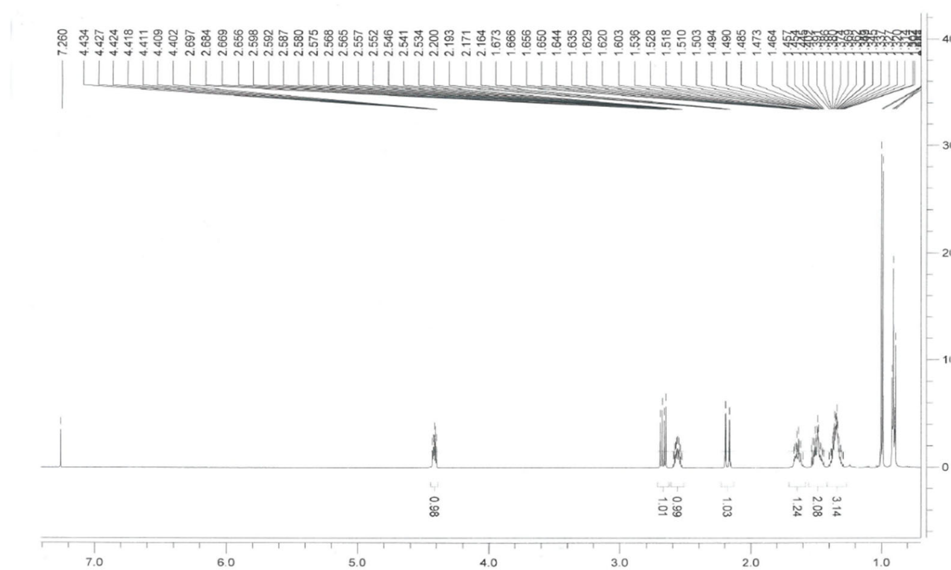

Figure S5. ^{1}H NMR spectrum of *cis*-whisky lactone.

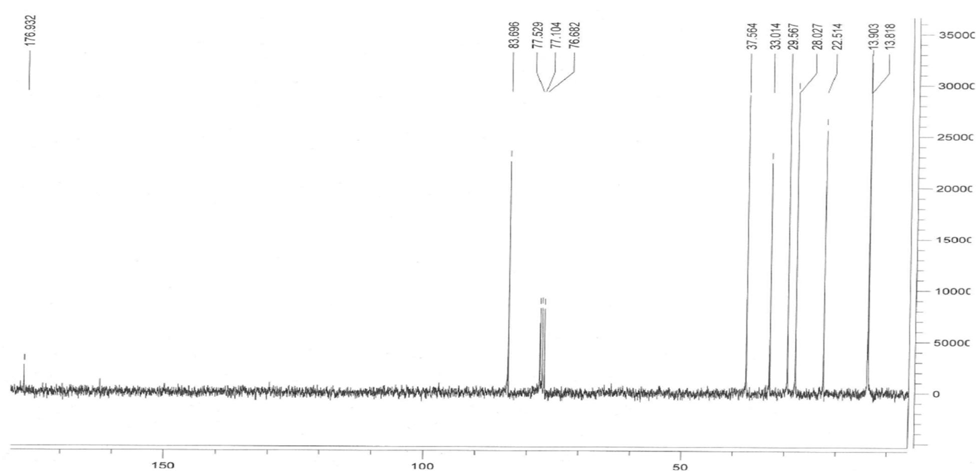

Figure S6. ^{13}C NMR spectrum of *cis*-whisky lactone.

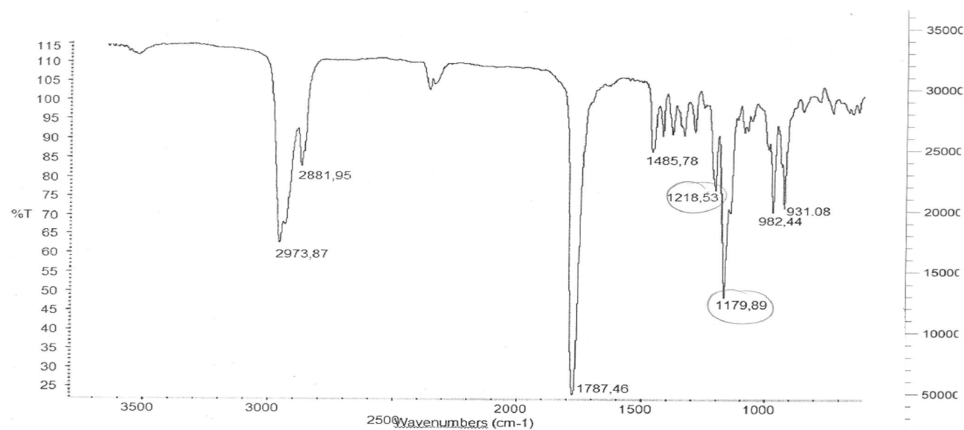

Figure S7. IR spectrum of *cis*-whisky lactone.

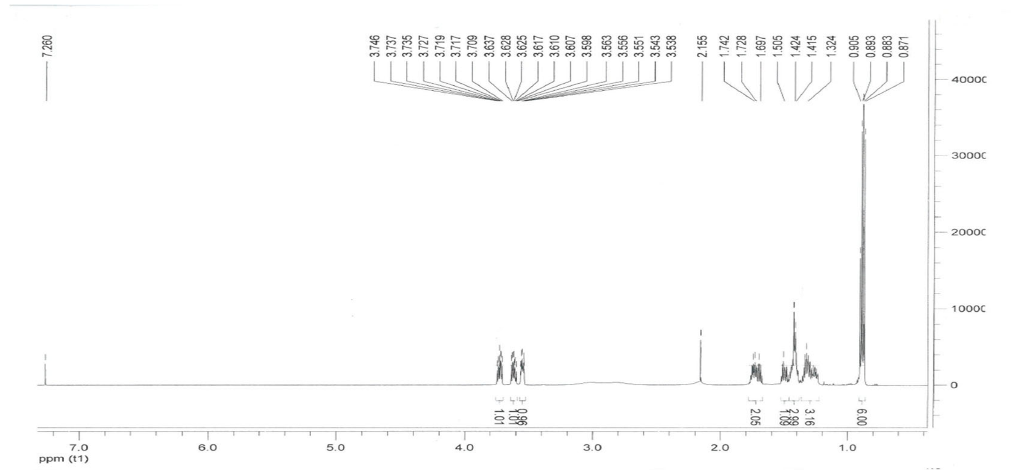

Figure 8. H^1 NMR spectrum of *anti*-3-methyl-octane-1,4-diol.

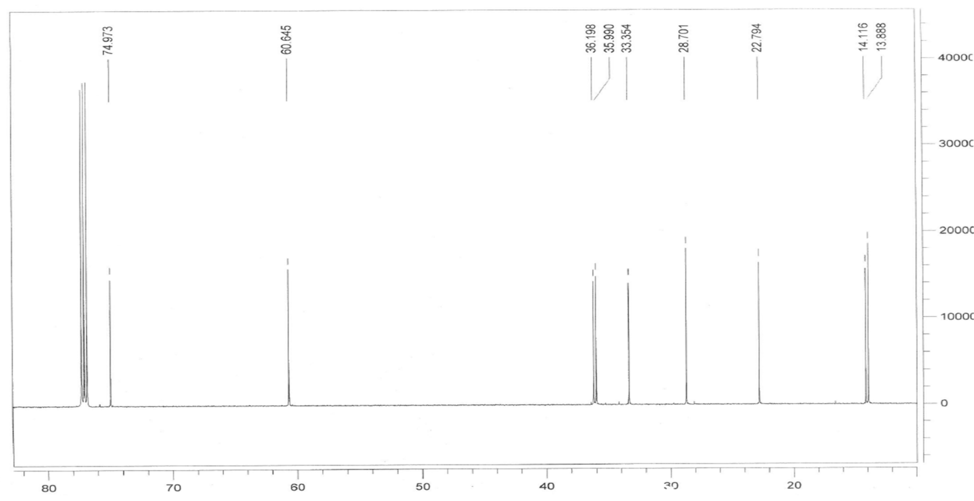

Figure S9. ^{13}C NMR spectrum of *anti*-3-methyl-octane-1,4-diol.

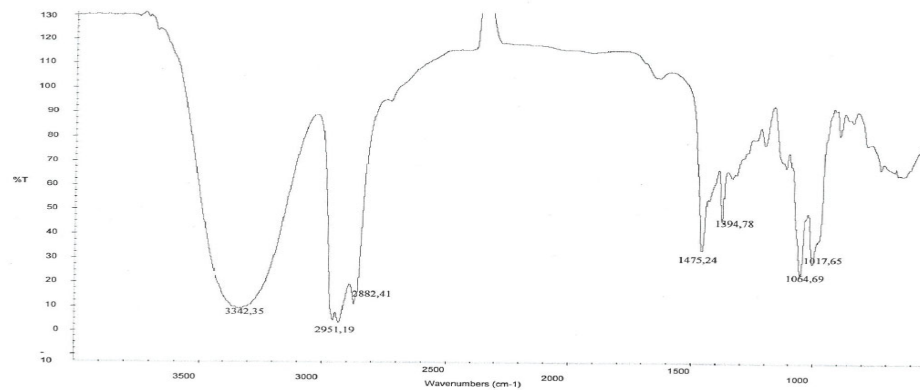

Figure S10. IR spectrum of *anti*-3-methyl-octane-1,4-diol.

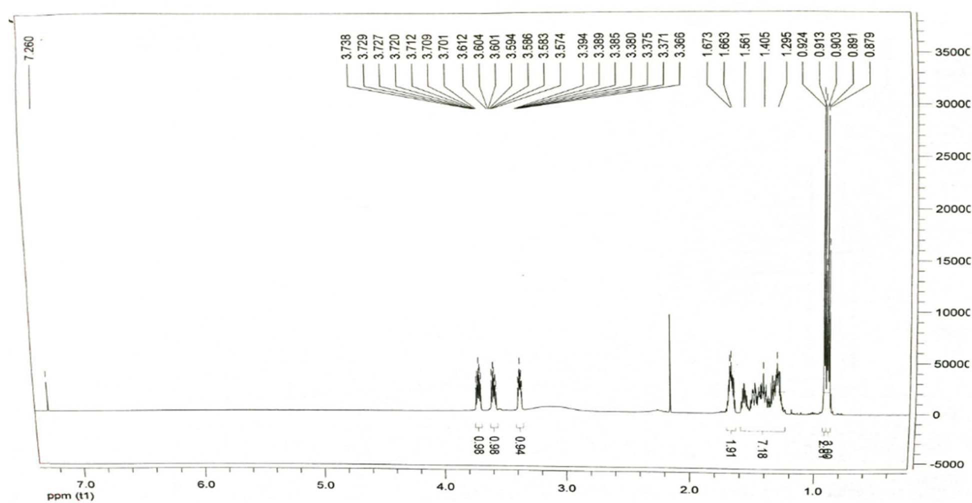

Figure S11. ^{1}H NMR spectrum *syn*-3-methyl-octane-1,4-diol.

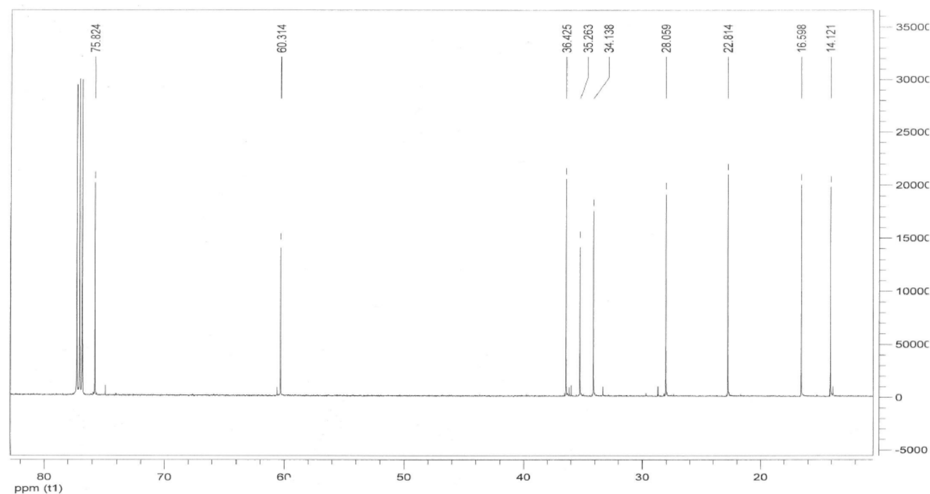

Figure S12. ^{13}C NMR spectrum *syn*-3-methyl-octane-1,4-diol.

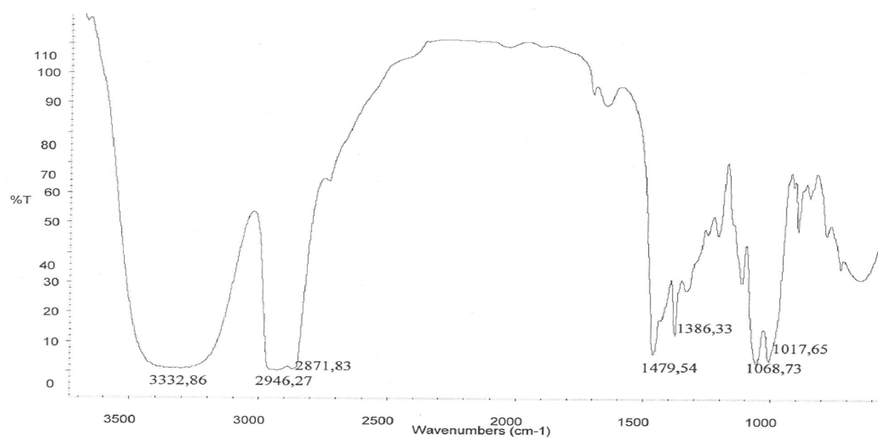

Figure S13. IR spectrum *syn*-3-methyl-octane-1,4-diol.

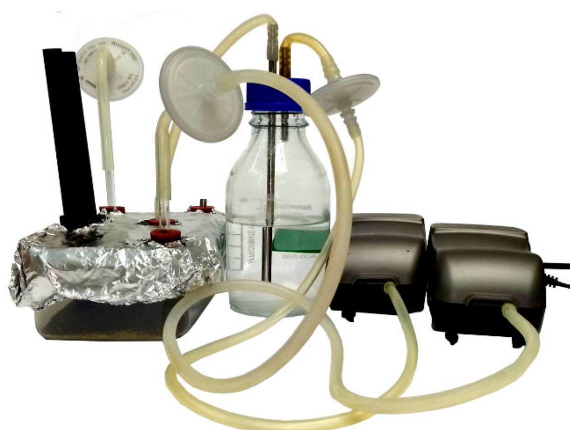

Figure S14. A bioreactor vessel with graphite electrodes, a vessel with water, air and water pumps.

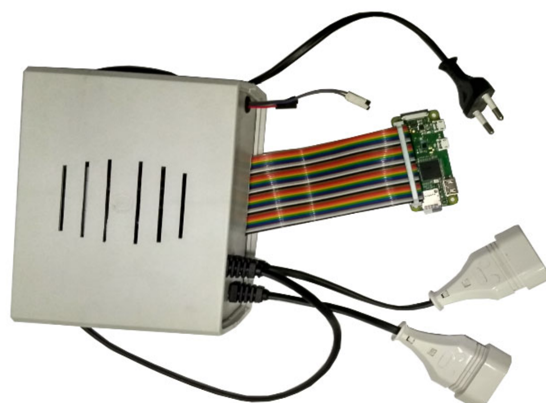

Figure S15. Control system in the plastic cover.

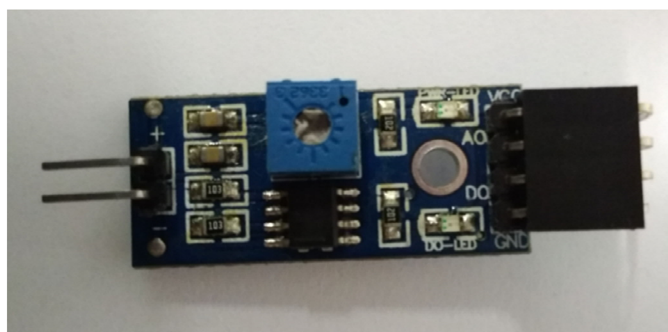

Figure S16. Moisture sensor**.**

Figure S17. Bacteria in the linseed cake**.**

7.2 PUBLIKACJA 2 (P2)

Hernik, D.*; Gatti, F.; Brenna, E.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.*
Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*. *Frontiers in Microbiology*, 2023, 14, 1117835.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 19 January 2023
DOI 10.3389/fmicb.2023.1117835

Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 06 December 2022
ACCEPTED 03 January 2023
PUBLISHED 19 January 2023

CITATION
Hernik D, Gatti F, Brenna E, Szczepańska E, Olejniczak T and Boratyński F (2023) Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*. *Front. Microbiol.* 14:1117835. doi: 10.3389/fmicb.2023.1117835

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Whisky lactone is a naturally occurring fragrance compound in oak wood and is widely used as a sensory additive in food products. However, safe and efficient methods for the production of its individual enantiomers for applications in the food industry are lacking. The aim of this study was to develop an efficient and highly stereoselective process for the synthesis of individual enantiomeric forms of whisky lactones. The proposed three-step method involves (1) column chromatography separation of a diastereoisomeric mixture of whisky lactone, (2) chemical reduction of *cis-and trans*-whisky lactones to corresponding *syn-and anti*-diols, and (3) microbial oxidation of racemic diols to individual enantiomers of whisky lactone. Among various bacteria in the genera *Dietzia*, *Gordonia*, *Micrococcus*, *Rhodococcus,* and *Streptomyces*, *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150 effectively oxidized *anti-and syn*-3-methyl-octane-1,4-diols (**1a-b**) to corresponding enantiomerically pure *cis-and trans*-whisky lactones, indicating high alcohol dehydrogenase activity. Bio-oxidation catalyzed by whole cells of these strains yielded enantiomerically pure isomers of *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**), and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones. The optical density of bacterial cultures and the impact of the use of acetone powders as catalysts on the course of the reaction were also evaluated. Finally, the application of *R. erythropolis* DSM44534 in the form of an acetone powder generated the enantiomerically enriched *cis*-(−)-(4*S*,5*S*)-isomer (**2c**) from the corresponding *syn*-diol (**1b**). The newly developed method provides an improved approach for the synthesis of chiral whisky lactones.

KEYWORDS

biotransformation, fragrances, *Rhodococcus erythropolis*, lactones, oxidation, enantioselectivity

1. Introduction

Whisky lactone is a crucial ingredient in aged alcoholic beverages, such as whisky, cognac, and brandy (Maga, 1996) and a fragrance ingredient in various foods (e.g., sweet and baked foods) and beverages. It is also used as a repellent against mosquitoes and flies (Suzukt et al., 1992). Whisky lactone was first identified in 1970 by Suomalainen and Nykanen (Suomalainen and Nykanen, 1970) as a single compound in many alcohols in oak barrels; therefore, it is commonly named oak lactone. Masuda and Nishimura (Masuda and Nishimura, 1971) later discovered that two diastereoisomers can be isolated from oak wood species. Then, in 1989, Gunther and Mosandl separated four whisky lactone stereoisomers. Mixtures of isomers have been described as reminiscent of coconut, while *cis*-isomers have been characterized as woody and earthy and *trans*-isomers as celery-like. It should

be noted that in nature, oak wood contains only *trans*-(+)-(4*S*,5*R*) and *cis*-(−)-(4*S*,5*S*) whisky lactone isomers (Abbott et al., 1995).

Several processes for *trans-and cis*-isomers of whisky lactone synthesis have been described (Ito et al., 1996; Brenna et al., 2001; Armstrong et al., 2009; Jiang et al., 2010; Pisani et al., 2012; Boratyński et al., 2013, 2014, 2018; Xie et al., 2017). However, these processes are based on multi-stage chemical synthesis using metal catalysts and organic solvents. Despite their wide use, metal-based catalysts are often harmful to the environment. A method for obtaining lactone stereoisomers with non-metallic catalysts (Xie et al., 2017) has also been described; however, the multistep approach was characterized by a relatively low conversion. Therefore, safe methods for the production of each stereoisomers of whisky lactone that fulfill green chemistry requirements are needed. Several biocatalytic pathways lead to optically active stereoisomers of whisky lactone. For example, alcohol dehydrogenase isolated from horse liver (HLADH) enantioselectively oxidizes racemic *syn*- and *anti*-3-methyloctane-1,4-diols (Boratyński et al., 2014). Another method uses whole cells of *Beauveria bassiana* AM278 and *Pycnidiella resinae* KCH50 for the lactonization of γ-oxo acids (Boratyński et al., 2013). Alternatively, the *trans*-(+)-(4*S*,5*R*) enantiomer of whisky lactone is obtained by reduction of the corresponding γ-oxo acids catalyzed by baker's yeast (Brenna et al., 2001).

The chirality of chemical compounds is very important in biological processes. In the case of the production of chiral drugs, enantiomers may interact differently with individual metabolic systems (Beck, 2002). Enantiomers can also evoke different aroma sensations or have a different odor intensity (expressed as the odor threshold). Since the properties of individual enantiomers can vary substantially, it is important to develop methods for obtaining enantiomerically pure compounds. One such method is biotransformation involving whole microbial cells (bacteria, yeast, or fungi) or isolated enzymes (Nagy et al., 2006; Marie et al., 2011; Chreptowicz et al., 2016). This method can be used to generate optically active compounds that occur naturally and are difficult to obtain by chemical methods (Braga and Belo, 2016).

The genus *Rhodococcus* (phylum *Actinobacteria*) includes Gram-positive, non-motile aerobic bacteria (Alvarez, 2019). Bacteria in this genus have been isolated from soil, groundwater, marine sediments, and diseased and healthy animals and plants (Larkin et al., 2006). Only a few species are pathogenic, e.g., *R. equi* (a cause of foal pneumonia) and *R. fascians* (a cause of leafy gall disease). Various *Rhodococcus* strains have been used as biocatalysts for the degradation of natural organic compounds as well as xenobiotics (Kim et al., 2018). These bacteria show, *inter alia*, the ability to biodegrade short-and long-chain alkanes and aromatic, heterocyclic, and polycyclic compounds (Larkin et al., 2005). They are characterized by high metabolic diversity, indicating high tolerance against a wide range of substrates and solvents (Liang et al., 2019). For this reason, the use of *Rhodococcus* strains in the bioremediation of organic pollutants from petroleum, like *o*-xylene, has been investigated (Kim et al., 2002, 2010). The degradation of lignins *via R. jostii* RHA1 can lead to the production of vanillin, a valuable flavor compound (Ahmad et al., 2011). *Rhodococcus* members are also able to carry out the desulfurization reaction and therefore can degrade sulfur-containing compounds found in fossil fuels, like benzothiophene or dibenzothiophene (Khairy et al., 2015). *Rhodococcus* has a wide range of enzymatic activities and is therefore a biocatalyst of choice in various biotransformation processes involving alcohol dehydrogenases (ADHs), oxidases, monooxygenases, dioxygenases, reductases, etc. (Stampfer et al., 2002; Nikodinovic et al., 2006; Kim et al., 2013; Ewing et al., 2015; Nolte and Urlacher, 2015; Biermann et al., 2016; Müller et al., 2016; Wu and Li, 2018; Sheldon and Brady, 2019).

The aim of this study was to develop a biocatalytic method of obtaining industrially valuable optically active whisky lactones. Established methods are limited and usually characterized by low stereoselectivity. Herein, selected bacterial strains were tested for the bio-oxidation of *anti*- and *syn*-3-methyloctane-1,4-diols (1a-b). Two strains of *R. erythropolis* with high ADH activity stereoselectively catalyzed biotransformation, yielding highly enantioenriched whisky lactone isomers. The newly described method is a cost-efficient strategy for the asymmetric synthesis of each stereoisomer of whisky lactones.

2. Materials and methods

2.1. Microorganisms

Dietzia maris PCM2292, *Gordonia bronchialis* PCM2167, *Gordonia rubripertincta* PCM2144, *Micrococcus luteus* PCM525, *Rhodococcus coprophilus* PCM2174, *Rhodococcus erythropolis* PCM2150, *Rhodococcus rhodnii* PCM2157, *Rhodococcus rhodochrous* PCM909, *Rhodococcus ruber* PCM2166, *Rhodococcus ruber* PCM2171, *Rhodococcus ruber* PCM2216, and *Streptomyces griseus* subsp. *griseus* PCM2331 were purchased from the Polish Academy of Sciences. *Dietzia* sp. DSM44016 and *Rhodococcus erythropolis* DSM44534 were purchased from the German Collection of Microorganisms and Cell Cultures. Biocatalysts were maintained at 4°C on PCM medium agar slants and were then transferred into conical flasks with PCM medium containing sodium chlorine (6 g), glucose (20 g), casein (2 g), bacteriological peptone (10 g), and yeast extract (2 g) dissolved in distilled water (1 L) at 25°C and pH 5.5.

2.2. Materials

A diastereoisomeric mixture of whisky lactones, nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), flavin mononucleotide (FMN), glutamate dehydrogenase (GDH), $LiAlH_4$, and PCM medium ingredients was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Preparation of substrates for biotransformation

A commercially available diastereoisomeric mixture of *cis*/*trans*-whisky lactones was separated by column chromatography to obtain individual *trans* (0.430 g) and *cis* (0.500 g)-whisky lactones, which were subsequently chemically reduced to corresponding *anti*-(0.380 g) and *syn*-3-methyl-octane-1,4-diol (0.456 g) diols (**1a**-**b**). This procedure has been described by us in detail (Hernik et al., 2021). The NMR and IR spectra of lactones and diols are as follows:

Trans-whisky lactone (**2a**-**b**) ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.91 (t, J = 7.2 Hz, 3H, CH_3-4′); 1.13 (d, J = 6.5 Hz, 3H, CH_3-4); 1.32–1.42 (m, 3H, CH_2-3′, one of CH_2-2′); 1.50 (m, 1H, one of CH_2-2′); 1.60 (m, 1H, one of CH_2-1′); 1.68 (m, 1H, one of CH_2-1′); 2.15–2.25 (m, 2H, one of CH_2-3, H-4); 2.66 (m, 1H, one of CH_2-3); 4.00 (td, J = 7.9, 4.0 Hz, 1H, H-5); ^{13}C NMR (150 MHz, $CDCl_3$): δ 13.89 (C-4′), 17.49 (CH_3-4), 22.49 (C-3′),

27.85 (C-2′), 33.70 (C-1′), 36.08 (C-4), 37.13 (C-3), 87.46 (C-5), 176.61 (C-2); IR (film, cm^{-1}): 1787 (s), 1222 (s), 1187 (s).

Cis-whisky lactone (**2c-d**) ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.91 (t, J = 7.3 Hz, 3H, CH_3-4′); 1.00 (d, J = 7.0 Hz, 3H, CH_3-4); 1.29–1.40 (m, 3H, CH_2-3′, one of CH_2-2′); 1.45–1.54 (m, 2H, one of CH_2-2′, one of CH_2-1′); 1.65 (m, 1H, one of CH_2-1′); 2.18 (dd, J = 17.0, 4.0 Hz, 1H, one of CH_2-3); 2.57 (m, 1H, H-4); 2.67 (dd, J = 17.0, 7.8 Hz, 1H, one of CH_2-3); 4.42 (ddd, J = 10.1, 5.6, 4.1 Hz, 1H, H-5); ^{13}C NMR (150 MHz, $CDCl_3$): δ 13.82 (CH_3-4), 13.90 (C-4′), 22.51 (C-3′), 28.03 (C-2′), 29.57 (C-4), 33.01 (C-1′), 37.56 (C-3), 83.70 (C-5), 176.94 (C-2); IR (film, cm^{-1}): 1787 (s), 1,219 (m), 1,180 (s).

Anti-3-methyloctane-1,4-diol (**1a**) ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.87 (d, J = 6.8 Hz, 3H, CH_3-3); 0.89 (t, J = 7.1 Hz, 3H, CH_3-8); 1.22–1.36 (m, 3H, one of CH_2-6, CH_2-7); 1.38–1.46 (m, 3H, CH_2-5, one of CH_2-6); 1.50 (m, 1H, one of CH_2-2); 1.67–1.77 (m, 2H, one of CH_2-2, H-3); 2.81 i 3.00 (two s, 2H, 2xOH); 3.55 (m, 1H, H-4); 3.62 (ddd, J = 10.9, 7.1, 5.0 Hz, 1H, one of CH_2-1); 3.73 (ddd, J = 10.9, 6.4, 5.0 Hz, 1H, one of CH_2-1); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 13.89 (CH_3-3), 14.12 (C-8), 22.79 (C-7), 28.70 (C-6), 33.35 (C-5), 35.99 (C-3), 36.20 (C-2), 60.65 (C-1), 74.97 (C-4); IR (film, cm^{-1}): 3342 (s), 1475 (m), 1,395 (m), 1065 (m), 1,018 (m).

Syn-3-methyloctane-1,4-diol (**1b**) ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0,89 (t, J = 7.1 Hz, 3H, CH_3-8); 0.92 (d, J = 6.8 Hz, 3H, CH_3-3); 1.23–1.36 (m, 3H, one of CH_2-6, CH_2-7); 1.37–1.51 (m, 3H, CH_2-5, one of CH_2-6); 1.56 (m, 1H, one of CH_2-2); 1.62–1.70 (m, 2H, one of CH_2-2, H-3);3.13 (s, 2H, 2xOH); 3.38 (ddd, J = 8.4, 5.5, 3.3 Hz, 1H, H-4); 3.59 (ddd, J = 11.4, 6.9, 5.1 Hz, 1H, one of CH_2-1); 3.72 (ddd, J = 11.4, 6.7, 5.0 Hz, 1H, one of CH_2-1); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.12 (C-8), 16.60 (CH3-3), 22.81 (C-7), 28.06 (C-6), 34.14 (C-5), 35.26 (C-2), 36.43 (C-3), 60.31 (C-1), 75.82 (C-4); IR (film, cm^{-1}): 3333 (s), 1,480 (s), 1386 (s), 1069 (s), 1018 (s).

2.4. Preliminary screening-scale biotransformations in microtiter plates

Twenty-four well MTPs were sterilized at 121°C and 1 atm. Then, 4 mL of sterile PCM medium was added to each well of the MTP. Holes were inoculated with 0.2 mL of pre-prepared cultures of bacteria at OD_{600} = 0.3 and shaken (200 rpm) for 24 h at 22°C. Then, 0.002 g of the substrate (*anti*-3-methyloctane-1,4-diol (**1a**) or *syn*-3-methyloctane-1,4-diol (**1b**)) dissolved in 0.1 mL of acetone was added to every well. For simple extraction, ethyl acetate (0.7 mL) was added to the samples (1 mL) and shaken for 5 min at 200 rpm in 2 mL Eppendorf tubes. The organic phase was transferred to a vial and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a paper filter to a GC vial. Biotransformation was controlled after 6, 24, and 48 h on the GC. Control experiments were also performed in which microorganisms were cultured on the medium without the addition of substrate to check metabolites.

2.5. Screening-scale biotransformations

Forty milliliters of PCM medium were added to 100 mL tapered flasks and then sterilized at 121°C at a pressure of 1 atm. The medium was inoculated with 0.5 mL of pre-culture of bacteria at OD_{600} = 0.3. The prepared bacterial cultures were placed for 3 days at 22°C and shaken at 150 rpm. Then, 0.01 g of the substrate (*anti*-3-methyloctane-1,4-diol (**1a**) or *syn*-3-methyloctane-1,4-diol (**1b**)) dissolved in 0.5 mL of acetone water was added to each of the flasks. For simple extraction, ethyl acetate (5 mL) was added to the samples (10 mL) and shaken for 5 min at 200 rpm in Falcon tubes. The organic phase was transferred to a vial and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a filter paper to a GC vial. Biotransformation was controlled after 3 and 7 days on the GC.

2.6. Preparative biotransformations

Eighty milliliters of PCM medium were placed in 250 mL Erlenmeyer flask and sterilized at 121°C for 15 min. The medium was inoculated with 5 mL of preprepared cultures of bacteria at OD_{600} = 0.3. Erlenmeyer flasks with bacterial cultures were placed for 3 days at 22°C and shaken at 150 rpm. After incubation for 3 days, 0.05 g of the substrate (*anti*-3-methyloctane-1,4-diol (**1a**) or *syn*-3-methyloctane-1,4-diol (**1b**)) dissolved in 2 mL of acetone was added to the culture. Samples were extracted after 24, 48, and 72 h and evaluated by GC.

2.7. Preparation of acetone powders

Five hundred milliliters of a 3-day bacterial culture were centrifuged at 12,000× *g* for 10 min at 4°C and the medium was separated from the bacterial cells. Cells were suspended in acetone (−20°C), centrifuged at 12,000× *g* for 10 min at 4°C, and the acetone was removed. This process was repeated three times and finally the cells were dried for 1 h to obtain dry and non-sticky acetone powders.

2.8. Biotransformations with acetone powders

Twenty-four well MTPs were sterilized at 121°C at a pressure of 1 atm. Then, 0.1 g of acetone powder was added with 3 mL of phosphate buffer (pH = 8.0) to each well of the MTP. Into each well, 0.002 g of NAD^+ or $NADP^+$ as coenzymes and 0.004 g of FMN or 0.001 g GDH as coenzyme regeneration agents were added in 0.1 ml of phosphate buffer (pH = 8.0). Subsequently, 0,010 g of the substrate (*anti*-3-methyloctane-1,4-diol (**1a**) or *syn*-3-methyloctane-1,4-diol (**1b**)) dissolved in 0.1 mL of acetone was added to every well and the MTP was shaken (200 rpm) at 22°C. For simple extraction, ethyl acetate (0.7 mL) was added to the samples (1 mL) and shaken for 5 min at 200 rpm in 2 mL Eppendorf tubes. The organic phase was transferred to a vial and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a filter paper to a GC vial. Biotransformation was controlled after 6, 18, 42, and 66 h on the GC.

2.9. Analytical procedure

The separation of the diastereoisomeric mixture of *cis/trans*-whisky lactones and chemical reduction of whisky lactones to corresponding diols were controlled by thin layer chromatography, using aluminum foil plates coated with silica gel. Compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$ and 2%

$H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4. Gas chromatography (GC, FID, carrier gas H_2) was carried out using the Agilent Technologies 7,890 N (GC System, Santa Clara, CA, USA). Enantiomeric excesses of the products were determined on the chiral column Cyclosil-B (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) according to the following temperature program: 80°C, 160°C (3°C/min), 250°C (20°C/min) (3 min). Samples (2 μL) were injected at a split ratio of 9:1; the carrier gas flow rate was 1 ml/min. The total run time was 34.0 min. Retention times (t) were established as follows: t_R = 20.74 min for *trans*-(+)-(4*S*,5*R*) (**2a**), t_R = 21.05 min for *trans*-(−)-(4*R*,5*S*) (**2b**), t_R = 22.42 min for *cis*-(−)-(4*S*,5*S*) (**2c**), t_R = 22.54 min for *cis*-(+)-(4*R*,5*R*) (**2d**) (Supplementary Figure S1). The substrates were determined on the chiral column CP-Chirasil L-Val (25 m × 0.25 mm × 0.12 μm; Agilent Technologies) according to the following temperature program: 80°C, 165°C (3°C/min), 200°C (20°C/min) (1 min). Samples (2 μL) were injected with a split ratio of 9:1; the flow of carrier gas was 1 mL/min. The total run time was 31.0 min. Retention times (t_R) were established as follows: t_R = 18.553 for *anti*-3-methyloctane-1,4-diol (**1a**), t_R = 18.630 min for *syn*-3-methyloctane-1,4-diol (**1b**). The structures of the compounds were confirmed on the basis of ^{1}H NMR and ^{13}C NMR, which were recorded for $CDCl_3$ solutions using a Bruker Advance DRX 600 (600 MHz) spectrometer (Billerica, MA, USA). IR spectra were determined using the FTIR Thermo-Mattson IR 300 Spectrometer. Optical rotations were measured on a Jasco P-2000 Polarimeter.

2.10. Statistical analysis

All experiments were performed in triplicate, and mean values are presented. For all comparisons, differences were not significant, as determined by Student's *t*-tests. Additionally, the standard deviations were calculated for the conversion rate and percentages of whisky lactone isomers are shown in the tables. Statistical analyses were performed using Past 4.02.

3. Results and discussion

Fourteen bacterial strains in the genera *Dietzia*, *Gordonia*, *Micrococcus*, *Rhodococcus,* and *Streptomyces* were selected for screening-scale transformation in microtiter plates (MTPs) to obtain enantiomerically pure *trans*- and *cis*-whisky lactones (**2a–d**) from corresponding diols (**1a–b**). These microorganisms were chosen on the basis of our previous research focused on the sustainable management of oleo industry by-products in the microbial synthesis of whisky lactones (Hernik et al., 2021). Owing to the modest enantioselectivity and low yield in these previous semi-preparative-scale processes, our goal was to develop an efficient, and highly stereoselective process for the synthesis of individual enantiomeric forms of whisky lactones. Proposed by us three-step method involves (1) column chromatography separation of a diastereoisomeric mixture of whisky lactones, (2) chemical reduction of *trans*- and *cis*-whisky lactones (**2a-d**) (*de* > 99% determined by GC) with $LiAlH_4$ to give the corresponding *syn-and anti*-diol, respectively (**1a-b**) (*de* > 99% determined by GC), and (3) microbial oxidation of racemic diols to individual enantiomers of whisky lactone (Scheme 1).

3.1. Preliminary screening-scale biotransformation in microtiter plates with *anti*-diol 1a

Initial biotransformations were carried out in MTPs. This allowed us a rapid selection of microorganisms with high ADH activity, responsible for the stereoselective oxidation of racemic diols to corresponding chiral lactones (Boratyński et al., 2013, 2016, 2020).

In oxidation of *anti*-3-methyloctane-1,4-diol (**1a**) the highest enantiomeric excess of *trans*-(+)-(4*S*,5*R*) isomer (**2a**) (*ee* = 96%) was detected after 24 h of transformation with *Dietzia* sp. DSM44016. In bio-oxidation with *R. erythropolis* DSM44534, *R. rhodnii* PCM2157, and *R. ruber* PCM 2166 for 24 h, enantiomeric excesses of *trans*-(+)-(4*S*,5*R*) isomer (**2a**) were lower (*ee* = 70–80%). It is worth mentioning that the opposite enantiomerically enriched *trans*-(−)-(4*R*, 5*S*) isomer (**2b**) (*ee* = 73%) was detected after 48 h of transformation with *R. erythropolis* PCM2150. The enantiomerically pure *cis*-(+)-(4*R*,5*R*) isomer (**2d**) (*ee* > 99%) was obtained by biotransformation with four strains: *Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150, and *R. ruber* PCM2166. In bio-oxidation with the remaining strains, enantiomeric excesses of *trans*- and *cis*-whisky lactones were substantially lower than those for these four strains; using *S. griseus* subsp. *griseus* PCM2331, *D. maris* PCM2292, and *R. ruber* PCM2216, no conversion was observed (Table 1).

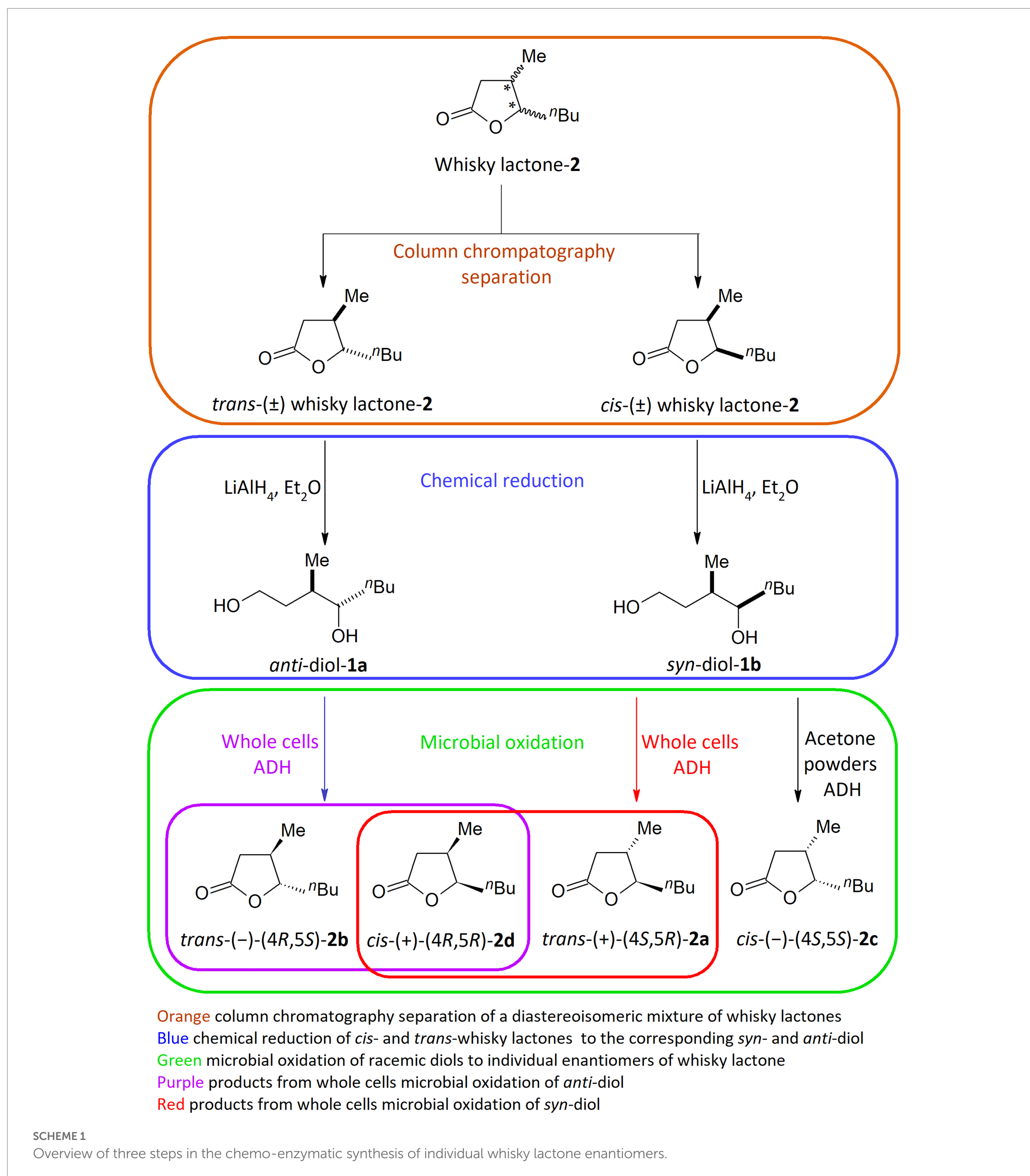
3.2. Preliminary screening-scale biotransformations in microtiter plates with *syn*-diol 1b

In biotransformations with *syn*-3-methyloctane-1,4-diol (**1b**) enantiomerically pure *trans*-(+)-(4*S*,5*R*) lactone (**2a**) was produced by *Dietzia* sp. DSM44016 and *R. erythropolis* PCM2150 after 24 h. On the other hand, enantiomerically pure *cis*-(+)-(4*R*,5*R*) whisky lactone (**2d**) was obtained with *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150. For oxidation with *M. luteus* PCM525, *R. erythropolis* DSM44534, and *R. rhodnii* PCM2157, only *cis*-isomers of whisky lactone were formed. *M. luteus* PCM525 delivered *cis*-(−)-(4*S*,5*S*) (**2c**) with the highest enantiomeric excess (*ee* = 70%). In biotransformations with *S. griseus* subsp. *griseus* PCM2331, *R. coprophilus* PCM2174, *R. rhodochrous* PCM909, *R. ruber* PCM2171, and *G. rubripertincta* PCM2144, substantially lower enantiomeric excesses were observed than those achieved with other strains. No substrate conversion was detected using *D. maris* PCM2292 and *R. ruber* PCM2216 (Table 2).

Surprisingly, the enantiomerically pure or highly enriched *trans-and cis*-whisky lactones dominantly formed by selected microorganisms: *Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150, and *R. ruber* PCM2166, indicate on dynamic kinetic resolution processes in performed biotransformations. Based on this preliminary results carried out in MTPs with *anti-and syn*-diols, aforementioned five microorganisms were selected for subsequent analyses.

3.3. Screening-scale biotransformations with *anti*-diol 1a

In the oxidation of *anti*-3-methyloctane-1,4-diol (**1a**) with *R. erythropolis* PCM2150, *R. erythropolis* DSM44534, and *Dietzia* sp.



SCHEME 1
Overview of three steps in the chemo-enzymatic synthesis of individual whisky lactone enantiomers.

DSM44016, complete conversion (*conv.* = 100%) was observed after 6, 24, and 48 h, respectively. Oxidation with *R. erythropolis* PCM2150 afforded enantiomerically pure *trans*-(−)-(4*R*, 5*S*) (**2b**) (25%, *ee* = 99%) and *cis*-(+)-(4*R*,5*R*) (**2d**) (75%, *ee* = 99%) whisky lactone isomers after 48 h. In biotransformations conducted with *R. erythropolis* DSM44534, enantiomerically pure *trans*-(−)-(4*R*, 5*S*) (**2b**) (82%, *ee* = 99%) and *cis*-(+)-(4*R*,5*R*) (**2d**) (18%, *ee* = 99%) enantiomers were obtained after 48 h. After 72 h with the same strain, only the *trans*-(−)-(4*R*, 5*S*) (**2b**) isomer (100%, *ee* = 99%) was obtained. During transformation with *Dietzia* sp. DSM44016 after 72 h, enantiomerically enriched *trans*-(−)-(4*R*, 5*S*) (**2b**) (87%, *ee* = 54%) and enantiomerically pure *cis*-(+)-(4*R*,5*R*) (13%, *ee* = 99%) whisky lactone isomer (**2d**) were detected. In the course of biotransformation with *R. erythropolis* DSM44534 and *Dietzia* sp. DSM44016, *trans*-(+)-(4*S*,5*R*) (**2a**) enantiomer formation was relatively high, while *trans*-(−)-(4*R*, 5*S*) (**2b**) enantiomer became dominant over time. This trend was consistent with those in biotransformations in MTP, in which the formation of the *trans*-(+)-(4*S*, 5*R*) isomer (**2a**) in the initial stage was also observed. In addition, during biotransformation with *R. erythropolis*

TABLE 1 Results of the microbial oxidation of *anti*-3-methyloctane-1,4-diol (1a) in MTPs.

Strain	Time [hours]	Conv. 1a [%]	Products			
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]
Dietzia sp. DSM44016	6	8 (±0.3)	–	–	8 (±0.8)	50 (+)
	24	100	72 (±1.2)	96 (+)	28 (±1.6)	99 (+)
	48	100	82 (±1.9)	67 (+)	18 (±0.7)	99 (+)
Gordonia bronchialis PCM2167	6	0	–	–	–	–
	24	45 (±1.9)	31 (±1.1)	50 (+)	14 (±0.9)	43 (+)
	48	100	64 (±2.1)	85 (+)	36 (±1.1)	71 (+)
Gordonia rubripertincta PCM2144	6	0	–	–	–	–
	24	43 (±1.3)	37 (±1.3)	–	6 (±0.4)	–
	48	89 (±2.0)	71 (±2.3)	67 (+)	18 (±0.9)	62 (+)
Micrococcus luteus PCM525	6	0	–	–	–	–
	24	0	–	–	–	–
	48	100	75 (±1.6)	65 (+)	25 (±1.2)	98 (+)
Rhodococcus coprophilus PCM2174	6	0	–	–	–	–
	24	60 (±0.9)	37 (±1.3)	5 (+)	23 (±1.0)	–
	48	100	62 (±0.9)	8 (+)	38 (±1.3)	3 (+)
Rhodococcus erythropolis DSM44534	6	27 (±1.0)	21 (±1.3)	50 (+)	6 (±0.2)	99 (+)
	24	89 (±1.2)	76 (±0.9)	71 (+)	13 (±0.4)	99 (+)
	48	100	72 (±1.7)	63 (+)	28 (±1.1)	99 (+)
Rhodococcus erythropolis PCM2150	6	0	–	–	–	–
	24	75 (±1.7)	65 (±2.1)	20 (−)	10 (±0.5)	99 (+)
	48	100	84 (±1.9)	73 (−)	16 (±0.7)	99 (+)
Rhodococcus ruber PCM2166	6	0	–	–	–	–
	24	13 (±0.5)	11 (±0.2)	70 (+)	2 (±0.1)	40 (+)
	48	100	80 (±2.3)	89 (+)	15 (±0.4)	99 (+)
Rhodococcus ruber PCM2171	6	0	–	–	–	–
	24	0	–	–	–	–
	48	72 (±1.1)	47 (±1.5)	17 (+)	25 (±0.5)	26 (+)
Rhodococcus rhodnii PCM2157	6	0	–	–	–	–
	24	52 (±1.3)	29 (±0.7)	80 (+)	23 (±0.3)	72 (+)
	48	93 (±2.5)	52 (±1.4)	82 (+)	41 (±1.9)	70 (+)
Rhodococcus rhodochrous PCM909	6	0	–	–	–	–
	24	80 (±1.4)	45 (±1.1)	50 (+)	35 (±0.9)	67 (+)
	48	81 (±1.6)	61 (±2.1)	61 (+)	20 (±0.2)	62 (+)

%, determined by CGC.

DSM44534 and *R. erythropolis* PCM2150, the same enantiomers of whisky lactone formed but in different amounts. Biotransformation with *R. ruber* PCM2166 showed a lower conversion rate (69%), and enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (**2a**) (58%, *ee* = 64%) and *cis*-(+)-(4*R*,5*R*) (**2d**) (11%, *ee* = 72%) whisky lactones were acquired. The transformation with *R. ruber* PCM2157 showed very low conversion (5%) after 72 h (Table 3).

3.4. Screening-scale biotransformation with *syn*-diol 1b

During the biotransformation of *syn*-3-methyloctane-1,4-diol (**1b**) with *R. erythropolis* DSM2150, *R. erythropolis* DSM44534, and *Dietzia* sp. DSM44016, the conversion rate was analogous to those for the transformation of *anti*-diol (**1a**). With *R. erythropolis* DSM2150, after 24 h, enantiomerically pure *trans*-(+)-(4*S*, 5*R*) (**2a**) (31%, *ee* = 99%) and *cis*-(+)-(4*R*,5*R*) (**2d**) (69%, *ee* = 99%) enantiomers of whisky lactone were obtained. After 48 h with the same strain, only the *cis*-(+)-(4*R*,5*R*) (**2d**) (100%, *ee* = 99%) isomer was acquired. In bio-oxidation with *R. erythropolis* DSM44534, after 24 h, only *cis*-(+)-(4*R*,5*R*) (**2d**) (100%, *ee* = 99%) whisky lactone was obtained. With *Dietzia* sp. DSM44016, after 48 h, enantiomerically enriched *trans*-(+)-(4*S*, 5*R*) (**2a**) (34%, *ee* = 94%) and *cis*-(+)-(4*R*,5*R*) (**2d**) (66%, *ee* = 97%) isomers formed. In biotransformation with *R. ruber* PCM2166, after 72 h, enantiomerically pure *trans*-(+)-(4*S*, 5*R*) (**2a**) enantiomer (11%, *ee* = 99%) and enantiomerically enriched

TABLE 2 Results of the microbial oxidation of *syn*-3-methyloctane-1,4-diol (1b) in MTPs.

Strain	Time [hours]	Conv. 1b [%]	Products			
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]
Dietzia sp. DSM44016	6	0	–	–	–	–
	24	78 (±2.2)	15 (±1.0)	99 (+)	63 (±1.3)	64 (+)
	48	100	23 (±0.4)	98 (+)	77 (±1.5)	97 (+)
Gordonia bronchialis PCM2167	6	0	–	–	–	–
	24	79 (±1.5)	35 (±0.8)	62 (+)	44 (±0.5)	73 (+)
	48	100	45 (±1.5)	87 (+)	55 (±1.2)	92 (+)
Gordonia rubripertincta PCM2144	6	0	–	–	–	–
	24	50 (±0.9)	31 (±1.1)	5 (+)	19 (±0.4)	13 (+)
	48	74 (±1.5)	63 (±0.7)	58 (+)	11 (±1.1)	41 (+)
Micrococcus luteus PCM525	6	0	–	–	–	–
	24	85 (±2.1)	–	–	85 (±1.2)	70 (−)
	48	100	–	–	100 (±0.0)	20 (−)
Rhodococcus coprophilus PCM2174	6	0	–	–	–	–
	24	43 (±0.9)	24 (±1.1)	33 (+)	19 (±0.8)	17 (+)
	48	90 (±1.1)	47 (±2.1)	23 (+)	43 (±1.7)	7 (+)
Rhodococcus erythropolis DSM44534	6	40 (±1.1)	–	–	40 (±0.4)	90 (+)
	24	100	–	–	100	99 (+)
	48	100	–	–	100	99 (+)
Rhodococcus erythropolis PCM2150	6	11 (±0.2)	4 (±0.2)	–	7	–
	24	100	26 (±1.2)	99 (+)	74 (±1.5)	99 (+)
	48	100	11 (±0.9)	74 (+)	89 (±0.7)	99 (+)
Rhodococcus ruber PCM2166	6	0	–	–	–	–
	24	40 (±0.8)	–	–	40 (±0.9)	73 (+)
	48	89 (±1.3)	14 (±0.8)	98 (+)	75 (±1.0)	82 (+)
Rhodococcus ruber PCM2171	6	0	–	–	–	–
	24	32 (±0.4)	17 (±0.6)	–	15 (±0.8)	–
	48	80 (±1.5)	51 (±1.4)	19 (+)	29 (±1.1)	22 (+)
Rhodococcus rhodnii PCM2157	6	0	–	–	–	–
	24	52 (±2.0)	–	–	52 (±2.3)	67 (+)
	48	87 (±0.9)	–	–	87 (±1.9)	77 (+)
Rhodococcus rhodochrous PCM909	6	0	–	–	–	–
	24	60 (±1.7)	41 (±0.8)	35 (+)	19 (±0.3)	59 (+)
	48	100	63 (±1.2)	44 (+)	37 (±0.7)	63 (+)
Streptomyces griseus subsp. *griseus* PCM2331	6	0	–	–	–	–
	24	20 (±0.4)	8 (±0.2)	–	12 (±0.7)	10 (+)
	48	60 (±1.3)	23 (±0.8)	–	37 (±1.2)	9 (+)

%, determined by CGC.

cis-(+)-(4*R*,5*R*) (**2d**) (66%, *ee* = 97%) whisky lactone were obtained. Biotransformation with *M. luteus* PCM525 and *R. ruber* PCM2157 showed low conversion rates, while no conversion was observed with *G. bronchialis* PCM2167 (Table 4).

Based on preliminary screening experiments for the preparative biotransformation of *anti*- and *syn*-3-methyl-octane-1,4-diols (**1a–b**) *R. erythropolis* DSM2150 and *R. erythropolis* DSM44534 were selected.

3.5. Preparative biotransformation with anti-diol 1a

The effect of the biomass concentration based on optical density (OD_{600} = 0.3, 0.5, and 1.0) during the course of biotransformation was evaluated. In a comparison of results obtained after 24, 48, and 72 h, no significant differences were detected in the enantiomeric

TABLE 3 Results of the biotransformation of *anti*-3-methyloctane-1,4-diol (1a).

Strain	Time [hours]	Conv. 1a [%]	Products			
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]
Dietzia sp. DSM44016	6	2 (±0.3)	-	-	2 (±0.1)	50 (+)
	24	25 (±1.3)	24 (±0.9)	92 (+)	1 (±0.1)	99 (+)
	48	100	95 (±1.0)	40 (+)	5 (±0.7)	99 (+)
	72	100	87 (±1.2)	54 (−)	13 (±0.9)	99 (+)
Rhodococcus erythropolis DSM44534	6	33 (±1.0)	29 (±0.8)	62 (+)	4 (±0.4)	99 (+)
	24	100	87 (±1.1)	30 (−)	13 (±0.7)	99 (+)
	48	100	82 (±1.3)	99 (−)	18 (±0.5)	99 (+)
	72	100	100	99 (−)	-	-
Rhodococcus erythropolis PCM2150	6	100	96 (±1.0)	0	4 (±0.7)	99 (+)
	24	100	47 (±0.3)	88 (−)	53 (±1.2)	99 (+)
	48	100	25 (±1.1)	99 (−)	75 (±1.8)	99 (+)
	72	100	22 (±0.6)	99 (−)	78 (±1.1)	99 (+)
Rhodococcus ruber PCM2166	6	0	-	-	-	-
	24	13 (±0.3)	11 (±0.9)	70 (+)	2 (±0.2)	40 (+)
	48	38 (±0.7)	34 (±0.5)	64 (+)	4 (±0.7)	75 (+)
	72	69 (±1.4)	58 (±1.6)	64 (+)	11 (±0.5)	72 (+)

%, determined by CGC.

TABLE 4 Results of the biotransformation of *syn*-3-methyloctane-1,4-diol (1b).

Strain	Time [hours]	Conv. 1b [%]	Products			
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]
Dietzia sp. DSM44016	6	12 (±0.6)	–	–	12 (±0.3)	92 (+)
	24	67 (±1.6)	9 (±0.5)	99 (+)	58 (±1.7)	56 (+)
	48	100	34 (±1.4)	94 (+)	66 (±2.3)	97 (+)
	72	100	18 (±0.9)	94 (+)	82 (±2.5)	99 (+)
Rhodococcus erythropolis DSM44534	6	37 (±0.9)	–	–	37 (±1.0)	84 (+)
	24	100	–	–	100	99 (+)
	48	100	–	–	–	–
	72	100	–	–	–	–
Rhodococcus erythropolis PCM2150	6	100	7 (±0.2)	99 (+)	93 (±1.1)	34 (+)
	24	100	31 (±0.5)	99 (+)	69 (±1.4)	99 (+)
	48	100	0	–	100	99 (+)
	72	100	0	–	100	99 (+)
Rhodococcus ruber PCM2166	6	0	–	–	–	–
	24	9 (±0.8)	–	–	9 (±0.6)	68 (+)
	48	26 (±1.2)	2 (±0.1)	99 (+)	24 (±0.9)	70 (+)
	72	66 (±1.5)	11 (±0.7)	99 (+)	55 (±1.3)	68 (+)

%, determined by CGC.

excess of lactones and the conversion of diols. For this reason, preparative biotransformations were performed at $OD_{600} = 1.0$.

During biotransformation with *anti*-3-methyloctane-1,4-diol (**1a**), the time needed for conversion was substantially longer than that for biotransformation at a smaller scale. Moreover, the formation of byproducts was observed. In bio-oxidation with *R. erythropolis* DSM44534, after 144 h, enantiomerically pure *trans*-(−)-(4*R*,5*S*) (**2b**) ($[\alpha]_D^{20}$ = −96.3 (c = 0.25, CH_3OH, *ee* = 99%, *yield* = 22%); ref. $[\alpha]_D^{20}$ = −97.0 (c = 0.34, CH_3OH, *ee* = 99%) (Wilkinson et al., 2004)) and *cis*-(+)-(4*R*,5*R*) (**2d**) ($[\alpha]_D^{20}$ = +79.4 (c = 0.2, CH_3OH, *ee* = 99%, *yield* = 8%); ref. $[\alpha]_D^{20}$ = +79.0 (c = 0.5, CH_3OH, *ee* = 99%) (Wilkinson et al., 2004)) whisky lactones were detected (Table 5).

TABLE 5 Results of microbial oxidation of *anti*-3-methyloctane-1,4-diol (1a).

Strain	Time [hours]	Conv. 1a [%]	Products				Byproducts
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]	
Rhodococcus erythropolis DSM44534	96	100	92 (±1.2)	0	5 (±0.4)	99 (+)	2
	120	100	87 (±0.9)	0	5 (±0.4)	99 (+)	8
	144	100	37 (±0.5)	99 (−)	13 (±0.8)	99 (+)	55
Rhodococcus erythropolis PCM2150	96	100	100	5 (+)	–	–	0
	120	100	94 (±1.0)	5 (+)	6 (±0.7)	99 (+)	0
	144	100	0	0	0	0	100

%, determined by CGC.

TABLE 6 Results of the microbial oxidation of *syn*-3-methyloctane-1,4-diol (1b).

Strain	Time [hours]	Conv. 1b [%]	Products				Byproducts
			Trans [%]	*ee* [%]	*Cis* [%]	*ee* [%]	
Rhodococcus erythropolis DSM44534	18	77 (±1.0)	27 (±0.4)	18 (+)	50 (±1.1)	99 (+)	0
	42	100	37 (±1.1)	90 (+)	47 (±0.6)	99 (+)	16
Rhodococcus erythropolis PCM2150	18	100	23 (±0.7)	99 (+)	77 (±1.2)	58 (+)	0
	42	100	53 (±1.3)	97 (+)	47 (±0.9)	99 (+)	0

%, determined by CGC.

3.6. Preparative biotransformation with *syn*-diol 1b

The conversion of *syn*-3-methyloctane-1,4-diol (**1b**) was fastest during bio-oxidation with *R. erythropolis* PCM2150 (after 18 h). With *R. erythropolis* DSM44534, complete conversion required 42 h. As a result of biotransformation catalyzed by *R. erythropolis* PCM2150, after 42 h, enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (**2a**) ($[\alpha]_D^{20}$ = +98.1 (c = 0.2, CH_3OH, *ee* = 97%, *yield* = 14%); ref. $[\alpha]_D^{20}$ = +97.0 (c = 0.34, CH_3OH, *ee* = 99%) (Wilkinson et al., 2004)) and optically pure *cis*-(+)-(4*R*,5*R*) (**2d**) ($[\alpha]_D^{20}$ = +78.1 (c = 0.15, CH_3OH, *ee* = 99%, *yield* = 60%); ref. $[\alpha]_D^{20}$ = +79.0 (c = 0.5, CH_3OH, *ee* = 99%) (Wilkinson et al., 2004)) whisky lactones were obtained. During bio-oxidation with *R. erythropolis* PCM44534, after 42 h, enantiomerically enriched *trans*-(+)-(4*S*,5*R*) (**2a**) ($[\alpha]_D^{20}$ = +94.8 (c = 0.1, CH_3OH, *ee* = 90%, *yield* = 28%); ref. $[\alpha]_D^{20}$ = +97.0 (c = 0.34, CH_3OH, *ee* = 99%) and pure *cis*-(+)-(4*R*,5*R*) (**2d**) ($[\alpha]_D^{20}$ = +79.9 (c = 0.2, CH_3OH, *ee* = 99%, *yield* = 40%); ref. $[\alpha]_D^{20}$ = +79.0 (c = 0.5, CH_3OH, *ee* = 99%) (Wilkinson et al., 2004)) whisky lactones were obtained (Table 6).

By comparing the results from the submerged preparative biotransformations described herein with our previous results obtained by solid-state fermentation (Boratyński et al., 2020), processes conducted in the SmF were characterized by the substantially greater and faster conversion of diols to corresponding whisky lactones. Moreover, the bio-oxidation carried out in SmF afforded enantiomerically pure lactones on a preparative scale, which could not be obtained in preparative SSF biotransformations. Among fourteen bacteria tested, bio-oxidation by *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150 showed the highest efficiency and stereoselectivity, yielding *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones.

In our previous study, whisky lactone enantiomers were obtained by the microbial whole-cell reduction of γ-oxoacids (Boratyński et al., 2013). The *trans*-(+)-(4*S*,5*R*) (**2a**) enantiomer was obtained (*ee* = 99%) as the only product of the biotransformation catalyzed by *Didymosphaeria igniaria* KCH6651, *Laetiporus sulphurens* AM525, *Chaetomium* sp. KCH6670, and *Saccharomyces cerevisiae* AM464. However, during the biotransformation of the same γ-oxoacid by *Beauveria bassiana* AM278 and *Pycnidiella resinae* KCH50, a mixture of *trans*-(+)-(4*S*, 5*R*) (**2a**) (*ee* = 99%) and *cis*-(−)-(4*S*,5*S*) (**2c**) (*ee* = 45–77%) isomers was obtained. During enzymatic reactions catalyzed by the alcohol dehydrogenases HLADH and PADH I, enantiomerically enriched *trans*-(−)-(4*R*, 5*S*) (**2b**) and *cis*-(+)-(4*R*,5*R*) (**2d**) isomers were obtained (*ee* = 27–82%). In a previously described method of obtaining the *trans*-(+)-(4*S*, 5*R*) (**2a**) enantiomer (*ee* = 99%) by lactonization biocatalyzed by baker's yeast, the efficiency on a preparative scale was 38% (Brenna et al., 2001). Compared to these results, *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**), and *cis*-(+)-(4*R*,5*R*) (**2d**) enantiomers of whisky lactone (*ee* = 97–99%) were obtained by the newly developed approach.

3.7. Screening-scale biotransformation with acetone powders in microtiter plates with anti and *syn*-diols 1a-b

Since the enantiomerically pure *cis*-(−)-(4*S*,5*S*) (**2c**) whisky lactone was not obtained, oxidation was carried out with acetone powders prepared from selected bacteria (*Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, and *R. erythropolis* PCM2150). For enzymatic transformation, NAD^+ and $NADP^+$ as coenzymes and FMN and GDH for coenzyme regeneration were selected. Only $NADP^+$ and GDH were suitable for biotransformations (Schenkels and Duine, 2000).

In all biotransformations of *anti*-3-methyloctane-1,4-diol (**1a**), only *trans*-whisky lactone isomers formed. In bio-oxidation with acetone powders from *Dietzia* sp. DSM44016, after 66 h, *trans*-(+)-(4*S*,5*R*) (**2a**)

whisky lactone was detected (*ee* = 74%) with 100% conversion. Transformation with acetone powder from *R. erythropolis* DSM44534 after 42 h led to the *trans*-whisky lactone isomer *trans*-(−)-(4*R*,5*S*) (**2b**) (*ee* = 87%) with 85% conversion. It is worth noting that when using acetone powders from *Dietzia* sp. DSM44016 and *R. erythropolis* PCM2150, during the biotransformation of *anti*-diol (1a), an opposite *trans*-(+)-(4*S*,5*R*) (**2a**) isomer formed in the screening-scale whole-cell biotransformations. In transformations of *syn*-3-methyloctane-1,4-diol (**1a**) with acetone powders from *Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, and *R. erythropolis* PCM2150, conversion was complete after 66 h. Oxidation with *R. erythropolis* DSM44534 acetone powders after 42 h afforded the highest enantiomeric excess (*ee* = 86%) of *cis*-(−)-(4*S*,5*S*) (**2c**) isomer, instead of *cis*-(+)-(4*R*,5*R*) (**2d**), which was obtained in whole-cell biotransformations with these bacteria. Biotransformations with acetone powders were characterized by lower and slower conversion than those in screening-scale bio-oxidation involving whole cells from the same strains. Additionally, in transformations with acetone powders, only one isomer always formed; when *anti*-diol (**1a**) was added as substrate, *trans*-whisky lactone formed, while *syn*-diol (**1b**) produced *cis*-whisky lactone. The application of acetone powders in biotransformations is often used to increase the stability of enzymes and improve enantioselectivity. Additionally, these biocatalysts frequently increase the yield and benefit from simple storage and use. For instance, the use of acetone powders from *Geotrichum candidum* by Nakamura and Madsuda (Nakamura and Matsuda, 1998) increased the enantioselectivity and efficiency of the reduction of ketones to alcohols and generated products with the opposite configuration. However, we did not observe a benefit of this biocatalyst over whole cell oxidation.

4. Conclusion

A chemo-enzymatic three-step method for obtaining whisky lactone isomers was developed. This method combined the separation of a diastereoisomeric mixture of whisky lactone isomers by column chromatography followed by chemical reduction to corresponding racemic diols. The latter are submitted to microbial oxidation to obtain each stereoisomer of whisky lactone. Among bacteria from different genera, *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150 effectively oxidized *anti*- and *syn*-3-methyloctane-1,4-diols (**1a-b**) to corresponding whisky lactones, indicating high ADH activity. Bio-oxidation carried out on a preparative scale yielded enantiomerically pure isomers of *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(−)-(4*R*,5*S*) (**2b**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones. In addition, it was developed that acetone powders prepared from selected bacteria could be used to generate enantiomerically enriched *cis*-(−)-(4*S*,5*S*) (**2c**) whisky lactone isomers, although the reactions were characterized by lower conversion. Based on the obtained results, it was noticed that the dynamic kinetic resolution processes are probably involved in the described whole cells transformations. Therefore, further studies are currently ongoing on wider portfolio of substrates to confirm the mechanism that occurs during this process.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

DH and FB: conceptualization, formal analysis, methodology, resources, and writing – original draft. DH: funding acquisition and investigation. FB and EB: supervision. DH and ES: visualization. TO, EB, and FG: writing – review and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research and APC were funded by the project "UPWR 2.0: International and Interdisciplinary Program of Development of Wrocław University of Environmental and Life Sciences," co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of 4 June 2019.

Acknowledgments

We would like to thank Editage for providing the editing service.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1117835/full#supplementary-material

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Supplementary Material

Stereoselective synthesis of whisky lactone isomers catalyzed by bacteria in the genus *Rhodococcus*

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1 Supplementary Data

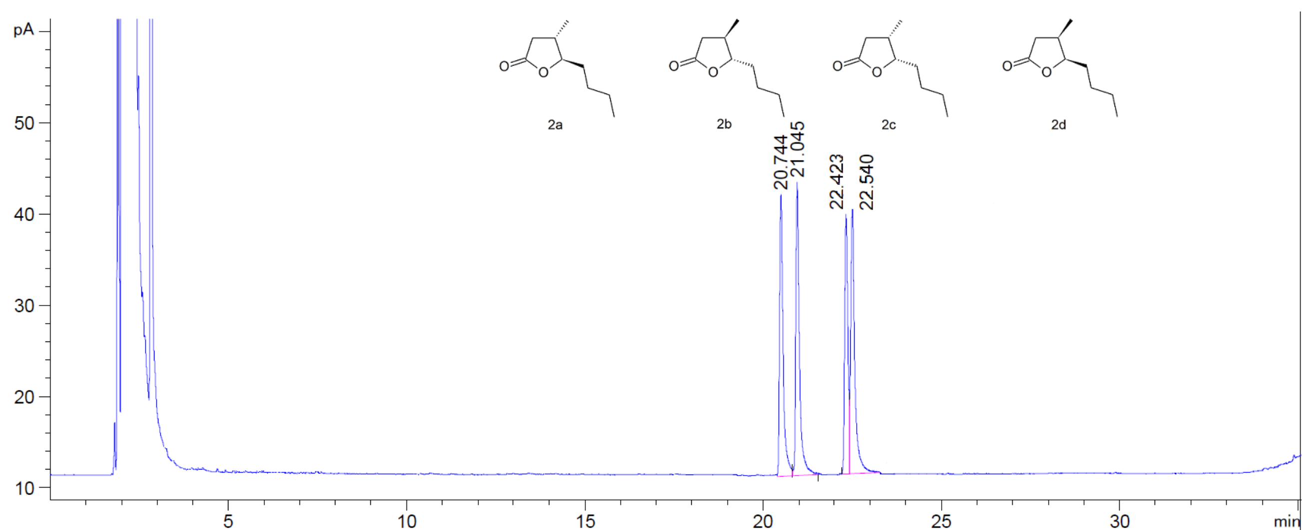

Figure S1. Chromatogram of the mixture of *trans* and *cis* whisky lactone

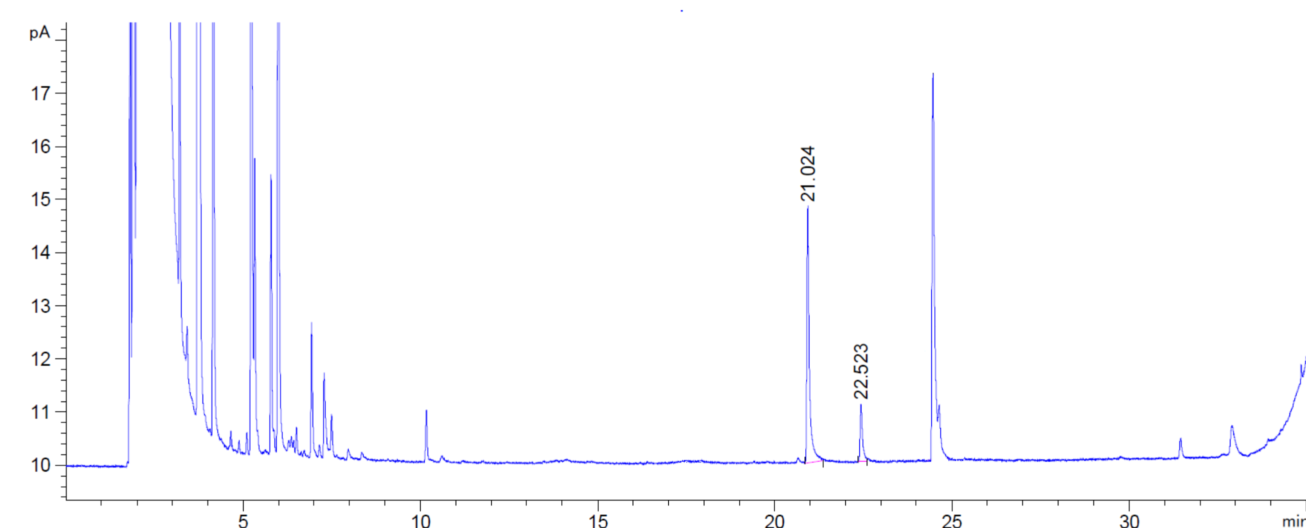

Figure S2. Chromatogram of the preparative scale oxidation of *anti*-3-methyloctane-1,4-diol with *R. erythropolis* DSM44534 after 144 hours.

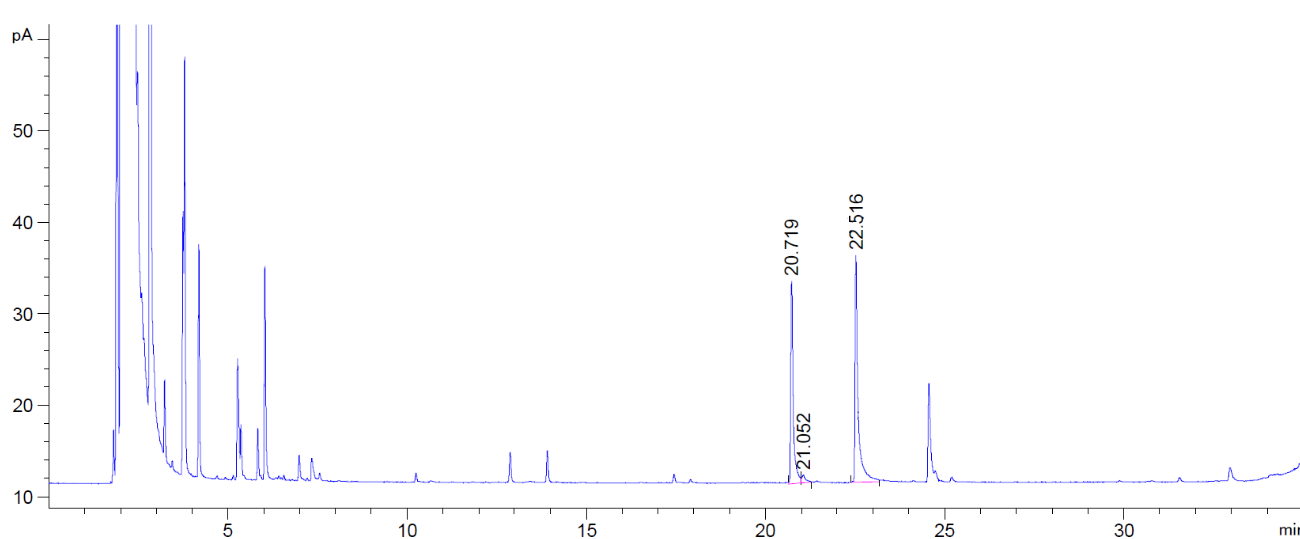

Figure S3. Chromatogram of the preparative scale oxidation of *syn*-3-methyloctane-1,4-diol with *R. erythropolis* DSM44534 after 42 hours.

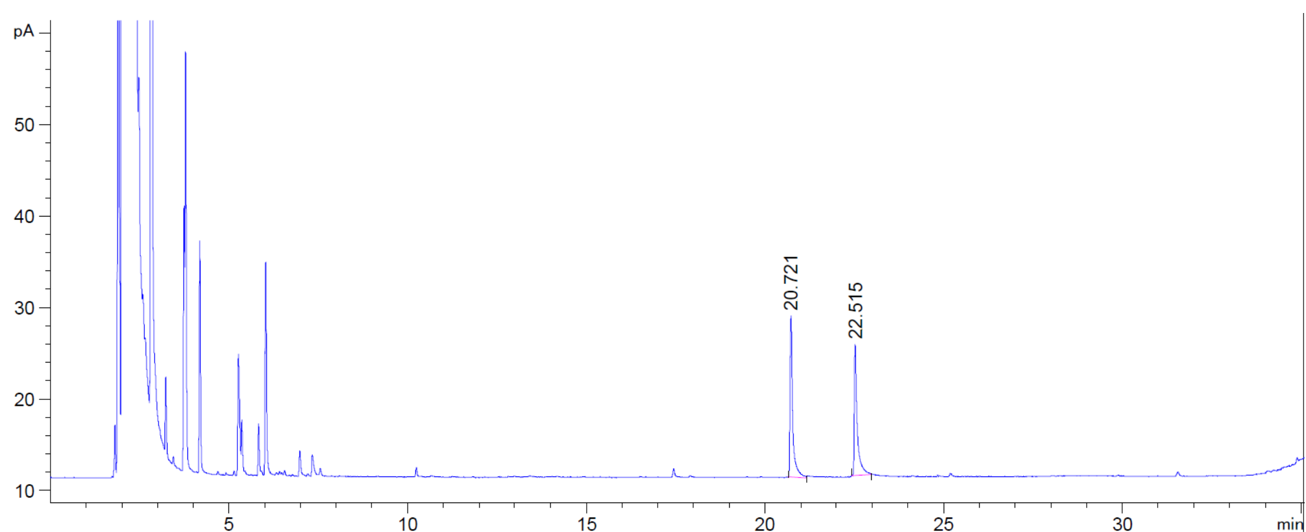

Figure S4. Chromatogram of the preparative scale oxidation of *syn*-3-methyloctane-1,4-diol with *R. erythropolis* PCM2150 after 42 hours.

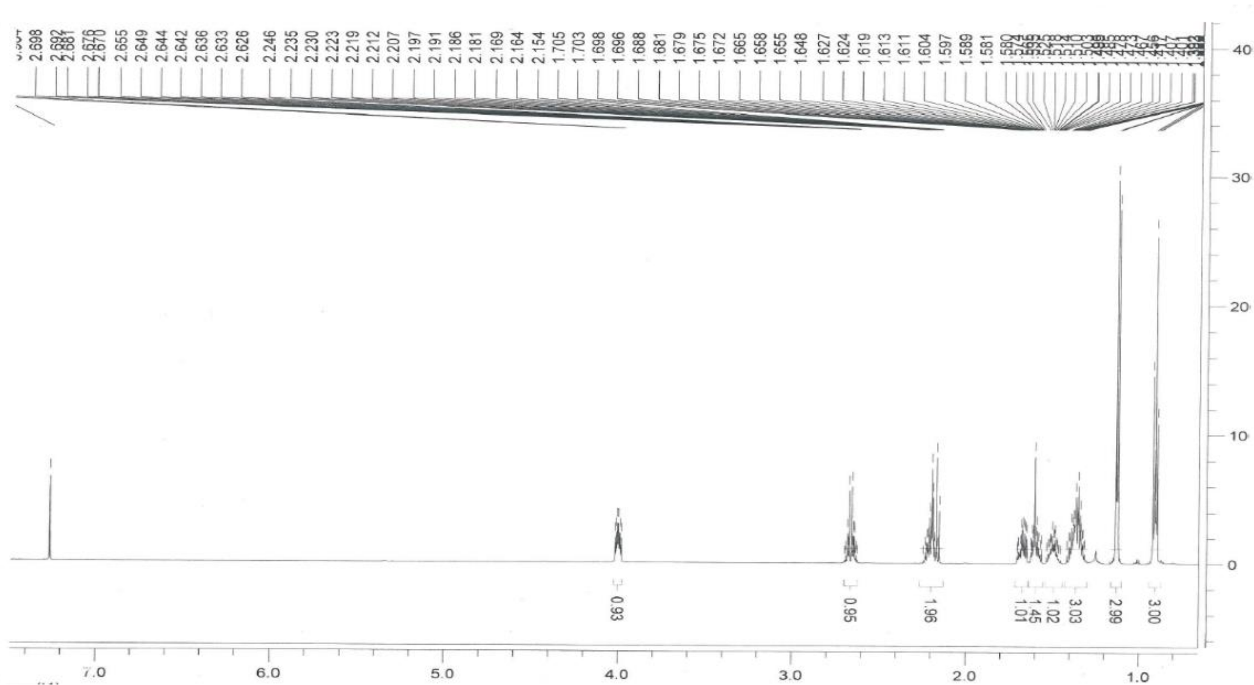

Figure S5. H[1] NMR spectrum of *trans*-whisky lactone

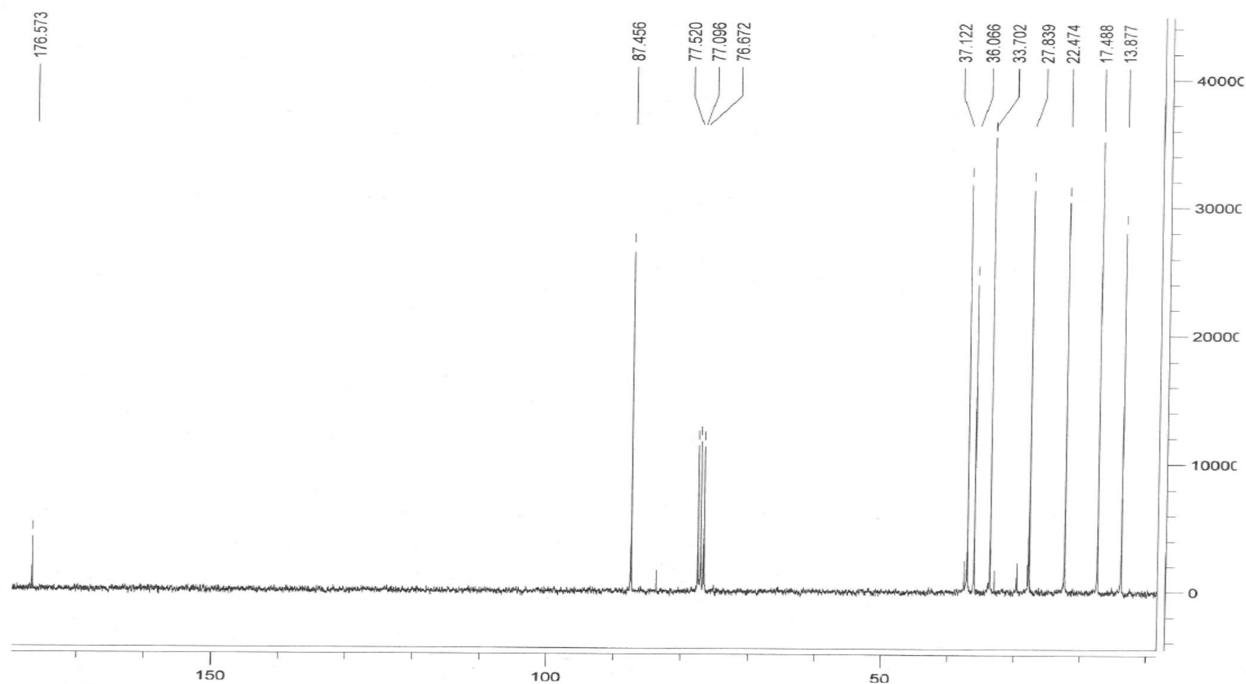

Figure S6. C[13] NMR spectrum of *trans*-whisky lactone

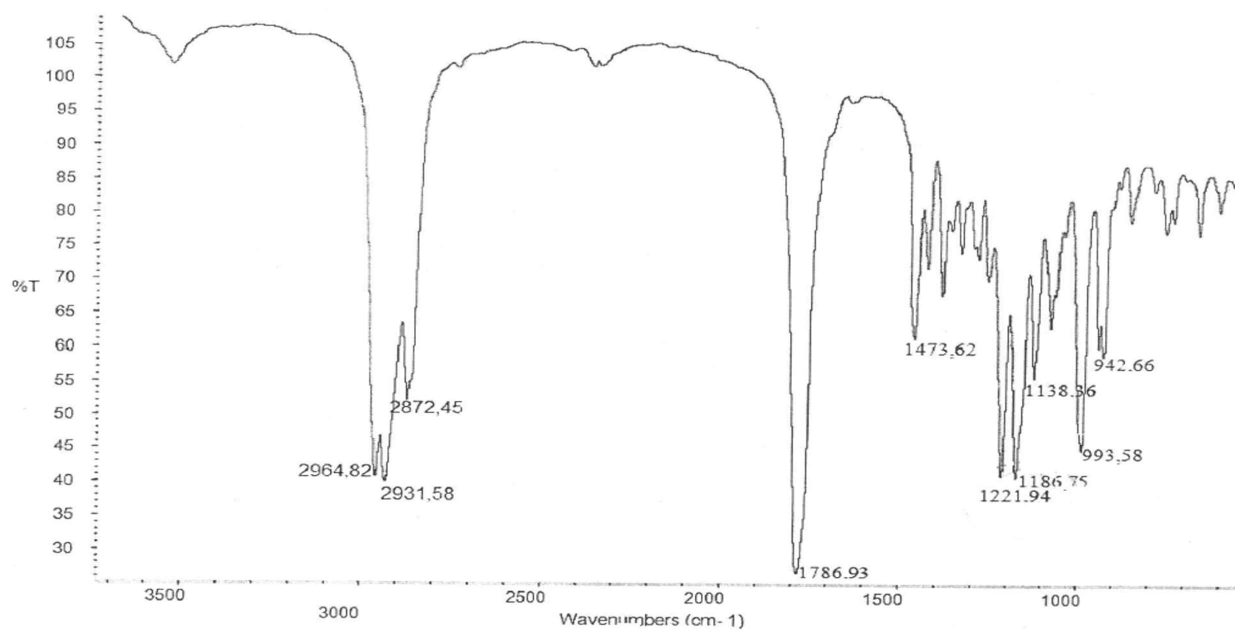

Figure S7. IR spectrum of *trans*-whisky lactone

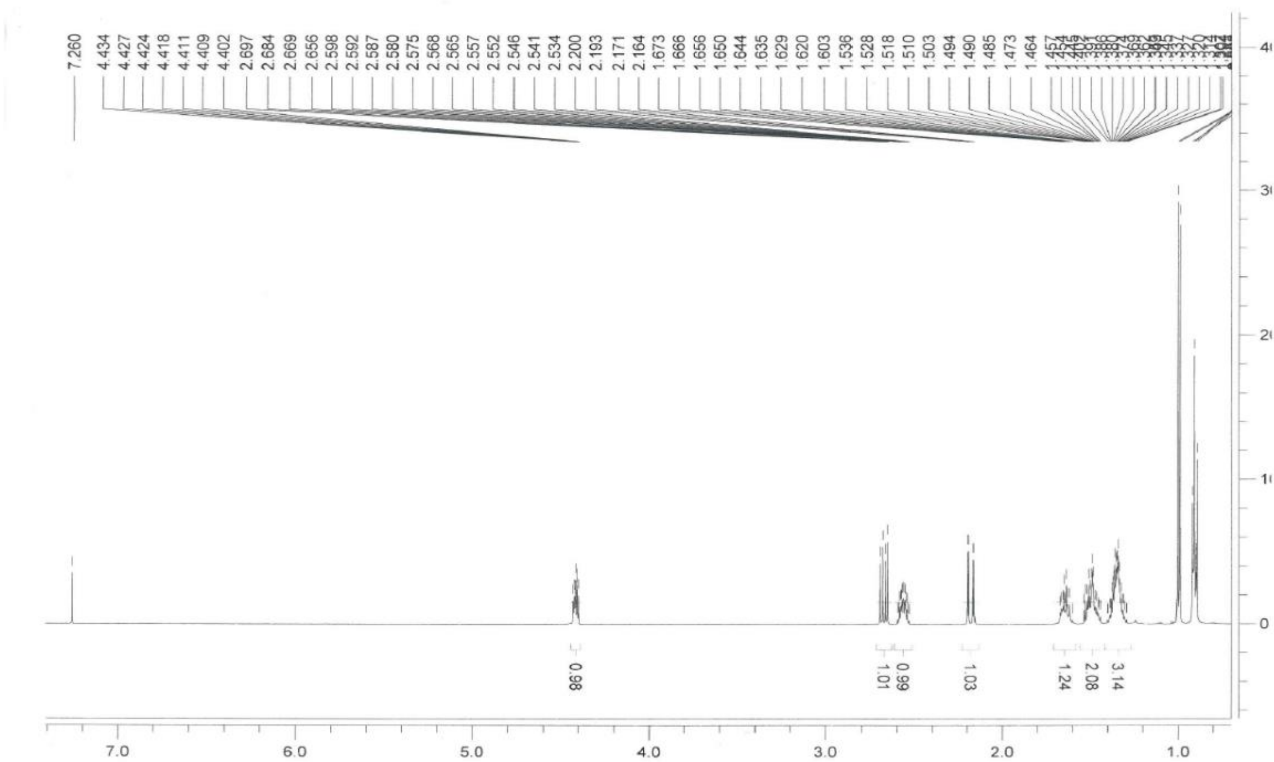

Figure S8. H[1] NMR spectrum of *cis*-whisky lactone

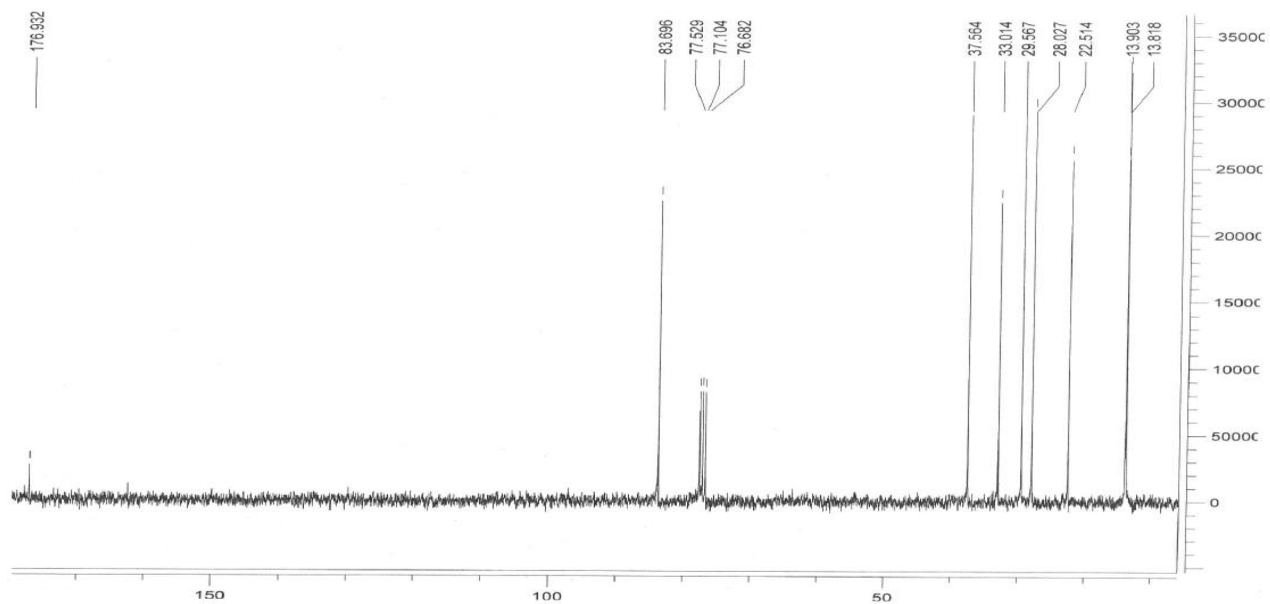

Figure S9. C[13] NMR spectrum of *cis*-whisky lactone

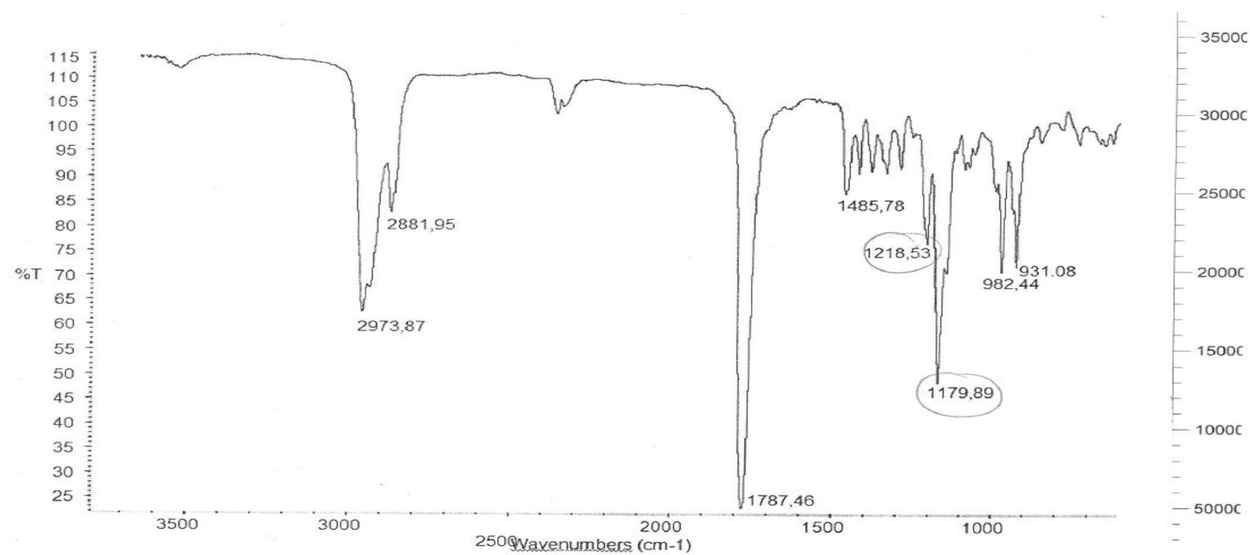

Figure S10. IR spectrum of *cis*-whisky lactone

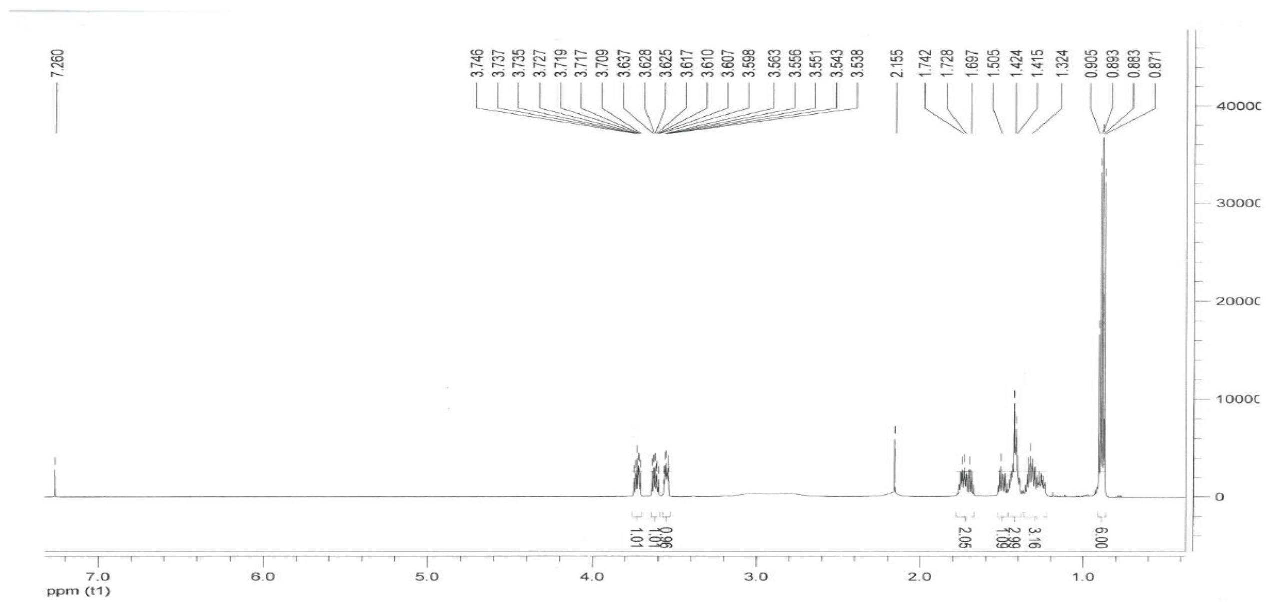

Figure S11. H^1 NMR spectrum of *anti*-3-methyl-octane-1,4-diol

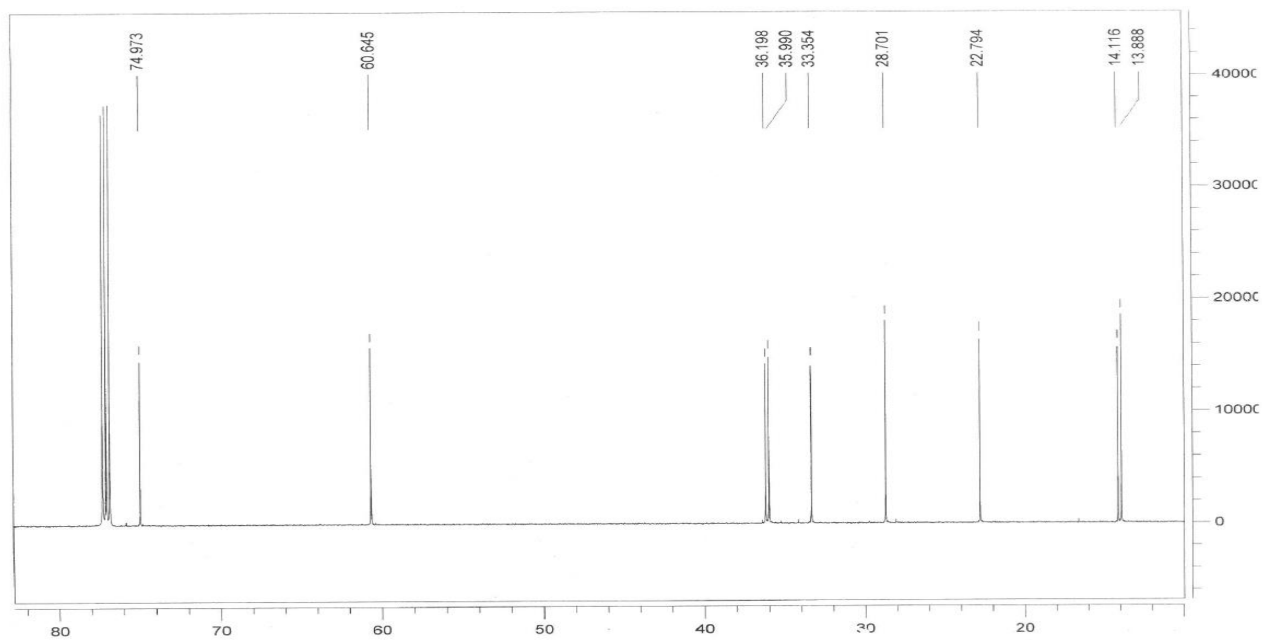

Figure S12. C^{13} NMR spectrum of *anti*-3-methyl-octane-1,4-diol

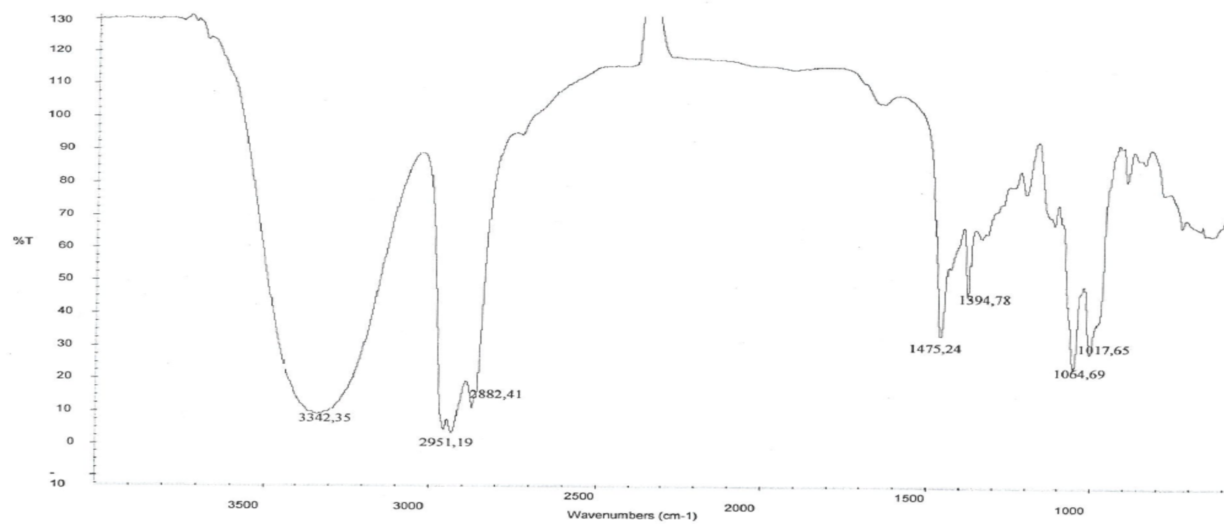

Figure S13. IR spectrum of *anti*-3-methyl-octane-1,4-diol

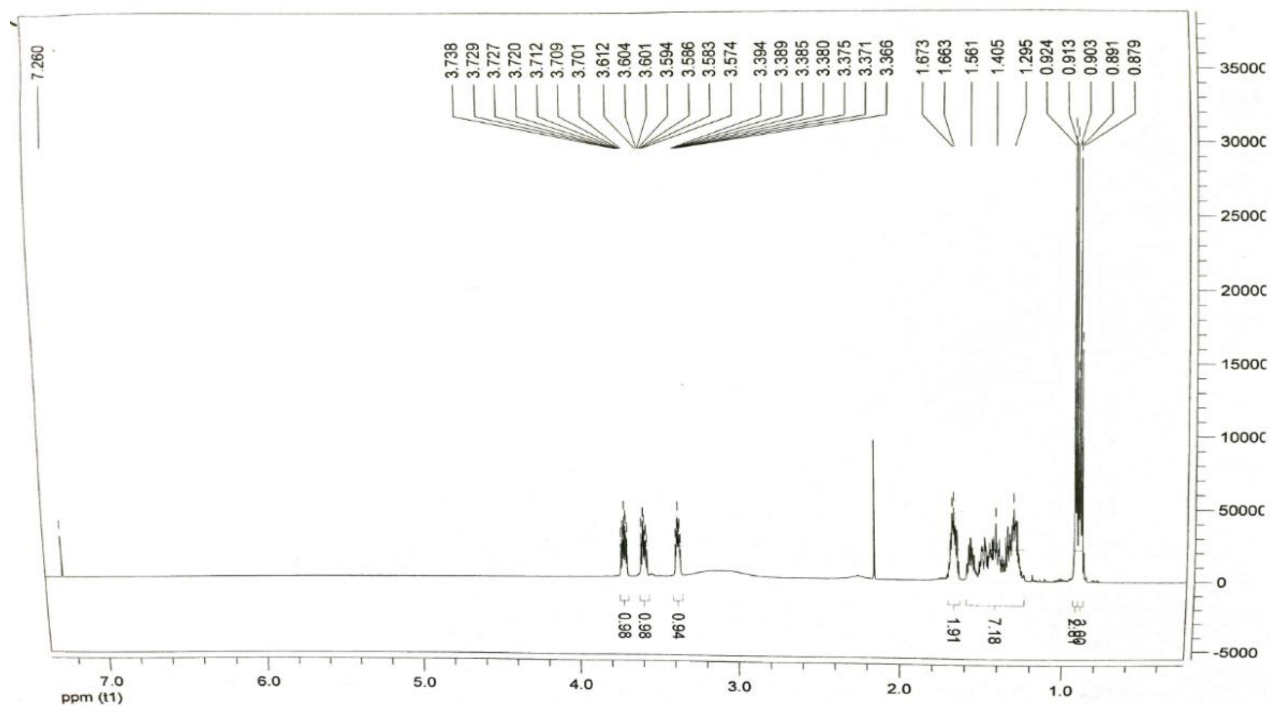

Figure S14. H^1 NMR spectrum *syn*-3-methyl-octane-1,4-diol

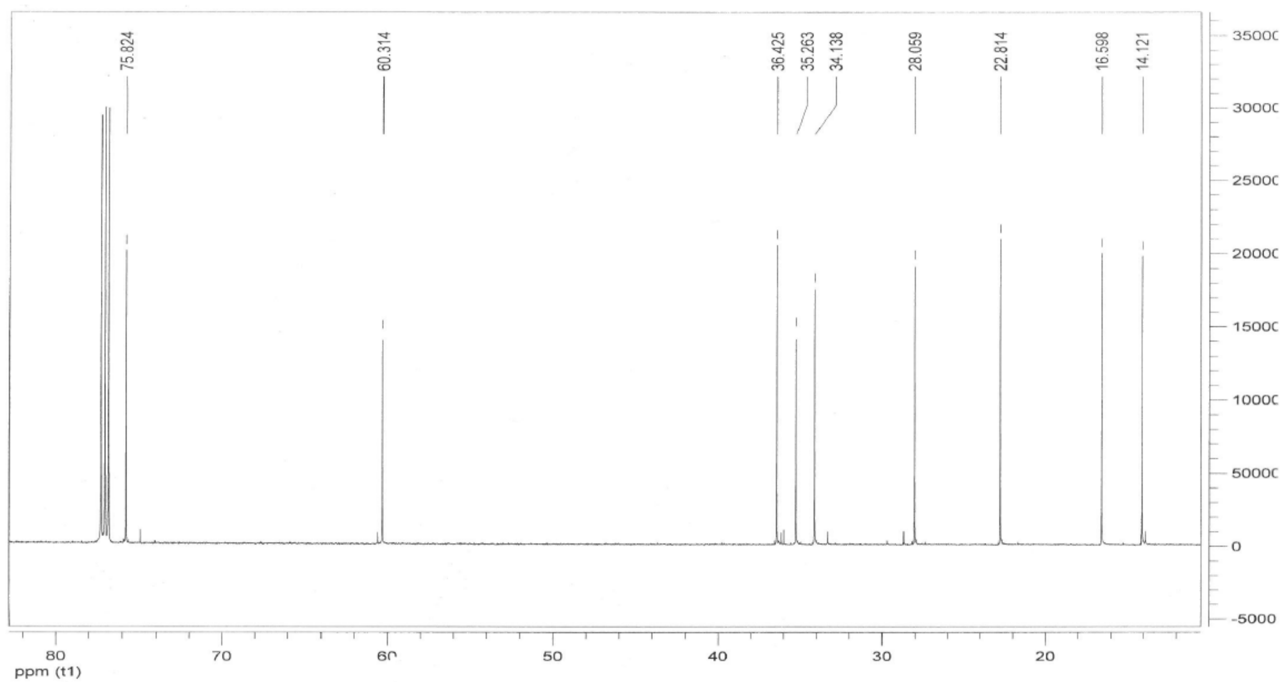

Figure S15. C^{13} NMR spectrum *syn*-3-methyl-octane-1,4-diol

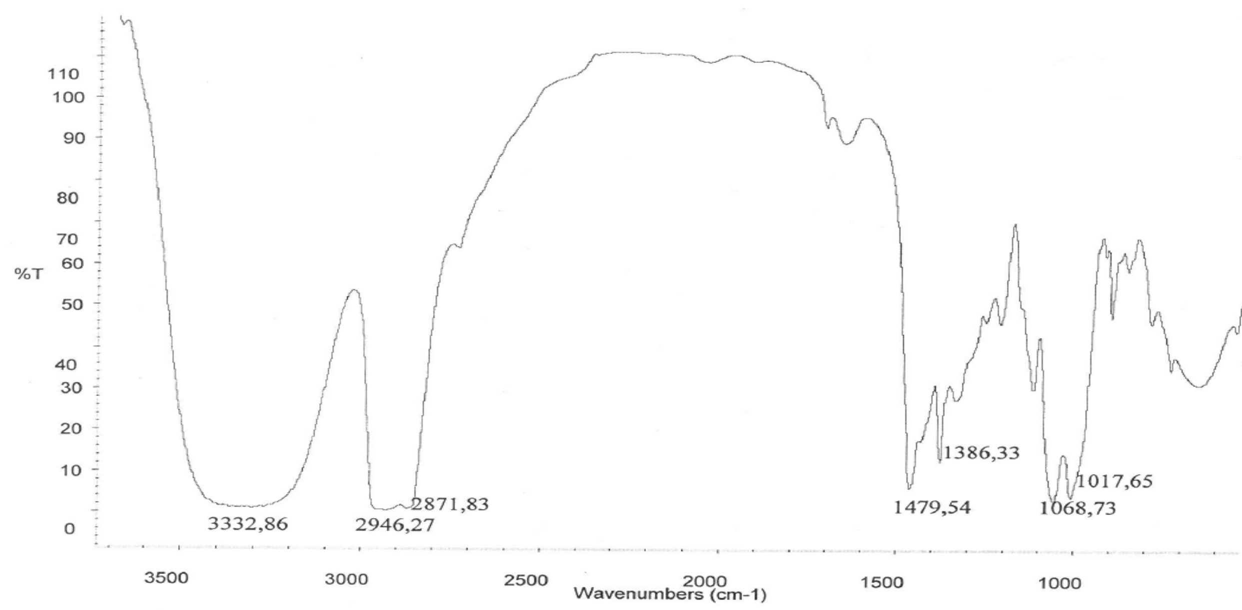

Figure S16. IR spectrum *syn*-3-methyl-octane-1,4-diol

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Hernik, D.*; Szczepańska, E.; Brenna, E.; Patejuk, K.; Olejniczak, T.; Strzała, T.; Boratyński, F.*

***Trametes hirsuta* as an attractive biocatalyst for the preparative scale biotransformation of isosafrole into piperonal.** *Molecules*, 2023, 28, 3643.

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Trametes hirsuta as an Attractive Biocatalyst for the Preparative Scale Biotransformation of Isosafrole into Piperonal

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Abstract: Piperonal is a compound of key industrial importance due to its attractive olfactory and biological properties. It has been shown that among the fifty-six various fungal strains tested, the ability to cleave the toxic isosafrole into piperonal through alkene cleavage is mainly found in strains of the genus *Trametes*. Further studies involving strains isolated directly from different environments (decaying wood, fungal fruiting bodies, and healthy plant tissues) allowed the selection of two *Trametes* strains, *T. hirsuta* Th2_2 and *T. hirsuta* d28, as the most effective biocatalysts for the oxidation of isosafrole. The preparative scale of biotransformation with these strains provided 124 mg (conv. 82%, isolated yield 62%) and 101 mg (conv. 69%, isolated yield 50.5%) of piperonal, respectively. Due to the toxic impact of isosafrole on cells, preparative scale processes with *Trametes* strains have not yet been successfully performed and described in the literature.

Keywords: piperonal; isosafrole; alkene cleavage; biotransformation; whole cells; microbial oxidation

Citation: Hernik, D.; Szczepańska, E.; Brenna, E.; Patejuk, K.; Olejniczak, T.; Strzała, T.; Boratyński, F. *Trametes hirsuta* as an Attractive Biocatalyst for the Preparative Scale Biotransformation of Isosafrole into Piperonal. *Molecules* **2023**, *28*, 3643. https://doi.org/10.3390/molecules28083643

Academic Editor: Renata Riva

Received: 13 March 2023
Revised: 5 April 2023
Accepted: 19 April 2023
Published: 21 April 2023

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1. Introduction

Piperonal (**1b**), also known as heliotropin (3,4-methylenedioxybenzaldehyde), is an organic compound belonging to aromatic aldehydes. It is a secondary metabolite produced by plants such as heliotrope, vanilla, camphor, violet, and black pepper [1]. Despite its rare occurrence in nature, piperonal has a relevant role in the flavor and fragrance industries. It is reminiscent of vanilla, with a pleasant floral-powder note, and therefore, it is used as an aroma in perfumes, scented candles, detergents, and air fresheners. Additionally, it constitutes a useful intermediate for the synthesis of fine chemicals, such as Tropional® (a commercial fragrance), piperine (a dietary supplement that increases the bioavailability of other compounds in food), α-methyldopa (an antihypertensive), and piribedil (an antiparkinson agent). Furthermore, piperonal (**1b**) can be used for conversion into the psychoactive drug MDMA (3,4-methylenedioxy-*N*-methylamphetamine); therefore, the production and trading of piperonal (**1b**) and its precursors are subject to strict control and regulation in many countries. It is noteworthy that piperonal (**1b**) has been approved for use as a food additive by the U.S. Food and Drug Administration (FDA) and the European Union.

The commercial route of obtaining the piperonal (**1b**) is based on the isomerization of safrole into isosafrole (**1a**) and its subsequent oxidation with chromic acid or ozone, combined with sulfur or a zinc reduction. These processes have a strong environmental

impact in terms of the toxicity of the employed chemicals and their high energy consumption for ozone production [2]. Besides the abovementioned method, other synthetic routes have been studied. Lucarelli et al. applied the electrochemical oxidation of piperonyl alcohol using an Au/CeO_2 catalyst [3]. Alvarez et al. [4] described a process of oxidation of isopropenylbenzenes to the corresponding aldehydes under the influence of microwave radiation with the use of commercial oxidants ($PhI(OAc)_2$ on NaY and $PhI(OAc)_2$ on Al_2O_3). Oppenauer's oxidation of piperonylic alcohol with different heterogeneous commercial catalysts using paraformaldehyde as a reactant was proposed by Borzatta et al. as an alternative route to obtain piperonal [5].

Due to the growing awareness of consumers regarding the origin of food additives, as well as the growing demand in the industry for piperonal (**1b**), the development of an alternative method for its synthesis is currently being researched. Biotechnological methods constitute the most attractive solution as they are more sustainable and environmentally friendly processes with an emphasis on the prevention of waste generation and avoidance of hazardous compounds. Several methods for piperonal synthesis using biocatalysts have been proposed in the literature. The main substrate used in these methods has been isosafrole (**1a**) [2,6–8]. Recently, a chemo-enzymatic three-step procedure for the conversion of isosafrole into piperonal was proposed by Tentori et al. [9]. Additionally, processes for the biotechnological preparation of piperonal, where piperonylic acid and piperonyl alcohol were used as the starting substrates, have been presented in the literature [10,11].

This paper reports a biotransformation process of isosafrole (**1a**) that leads to the production of piperonal (**1b**), a compound of outstanding importance in the fragrance and pharmaceutical industries. A selection of microbial strains exhibiting the desired biocatalytic properties was performed, which resulted in determining strains from the genus *Trametes* as the best biocatalyst agents able to provide piperonal with high conversion and isolation yield. In addition, during the screening process, vicinal diol **1c**, a dihydroxy derivative of isosafrole (**1a**), was obtained as one of the biotransformation products, and it may constitute a novel alternative substrate to produce piperonal (**1b**). This is the first report on the successful application of preparative scale biotransformation using whole cells of Basidiomycetes strains, which allows for obtaining piperonal **1b** from isosafrole **1a** in a considerable amount despite its known inhibitory effect on microbial cells [2].

2. Results and Discussion

2.1. Screening Scale Biotransformations

Initially, twenty-three different fungal strains were tested for the biotransformation of commercial isosafrole (**1a**), which was used as an 8:2 mixture of (*E*)/(*Z*) diastereoisomers for the desired product (piperonal (**1b**)). These strains were selected from various sources on the basis of literature data, as well as our research group's experience with the bio-oxidation process on numerous chemical compounds. The vast majority of the tested strains showed no activity towards substrate **1a**, nor did they metabolize it. This is likely related to the toxicity of isosafrole (**1a**) and its growth inhibitory activity towards microorganisms [2]. However, several fungal strains were selected to deliver products **1b** and **1c**, which are shown in Figure 1.

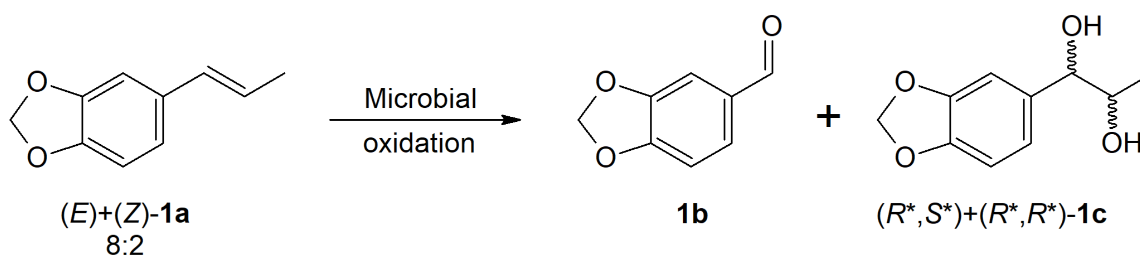

Figure 1. Products of isosafrole (**1a**) biotransformation: piperonal (**1b**) and vicinal diol (**1c**).

The Table 1 shows the strains that exhibited peroxidase activity towards isosafrole (**1a**) in the preliminary screening studies. The presence of piperonal (**1b**) was detected in extracts from the cultures of *Trametes versicolor* AM536, *T. hirsuta* d28, and *Mortierella isabelina* AM212. It was noteworthy that in the extracts collected from *Piptoporus betulinus* AM40 and *Laetiporus sulphurens* AM515, vicinal diol **1c** was observed as a sole product.

Table 1. Results of the preliminary screening of strains with peroxidase activity in relation to isosafrole (**1a**) (percentage values according to GC).

Strain	Time (Days)	Substrate (%)	Products (%)	
		1a	1b	1c
Laetiporus sulphurens AM515	7	0	0	100
	14	0	0	100
Mortierella isabelina AM212	7	100	0	0
	14	87	13	0
Piptoporus betulinus AM40	7	23	0	77
	14	17	0	83
Trametes versicolor AM536	7	63	10	27
	14	36	14	50
Trametes hirsuta d28	7	79	8	13
	14	42	10	48

Vicinal diol **1c** could be considered an intermediate product for obtaining piperonal (**1b**) in a more effective way. Given the toxicity of isosafrole (**1a**) in relation to microorganisms, the use of diol **1c** as a starting material might be a superior approach for obtaining piperonal (**1b**). Our research team is currently investigating this alternative route by using bacteria known for their oxidizing activity after producing diol **1c** from isosafrole by chemical synthesis. The microbiological transformation of diol **1c** into piperonal could then be considered as an alternative to the MnO_2 oxidative cleavage of **1c** described by Tentori et al. [9].

Santos et al. tested several strains of fungi (*Aspergillus flavus*, *A. niger*, and *Cladosporium sphaerospermum*) and bacteria (*Pseudomonas aeruginosa* and *P. putida*) for the conversion of isosafrole (**1a**) to piperonal (**1b**) [6]. The highest conversion (only 9.8%) was achieved with *C. sphaerospermum* after 7 days of biotransformation. Similar research was conducted on a broader screening of several dozen strains of fungi, yeasts, and bacteria which primarily belonged to the genus *Saccharomyces*, *SAspergillus*, *SFusarium*, and *Trichoderma*. *SPeacilomyces variotii* was selected as the most promising biocatalyst as the filamentous fungi showed better oxidative activity towards compound **1a** than the other microorganisms. As a result, only 20% conversion was obtained after 68 h of using a *P. variotii* post-culture medium containing extracellular peroxidases [2]. However, no isolation yields were reported in the aforementioned studies. Zhao et al. screened bacteria isolated from the soil for the bioconversion of isosafrole (**1a**) to piperonal (**1b**). For this purpose, a color screening method was used. Aromatic aldehydes were detected by a reaction with 2,4-dinitrophenylhydrazine (DNPH) to produce the corresponding colored hydrazone derivative (in the form of a red precipitate), with positive correlation between the amount of precipitate and the concentration of piperonal (**1b**). One of the tested strains, identified as *Serratia liquefaciens*, provided the highest piperonal (**1b**) concentration at the level of 282.32 mg/L with a molar efficiency of 35.8% after the optimization of culture conditions [7]. A fungus of the genus *Trametes* proved to be a very interesting biocatalyst for obtaining piperonal (**1b**) as in its culture the product showed higher stability and was not metabolized by cells. The research conducted by Lara et al. confirmed the ability of these fungi to produce the peroxidases responsible for biocatalytic alkene cleavage in the *trans*-anethol, leading to the production of the corresponding aldehyde [8]. They also attempted to convert isosafrole (**1a**); however, only 4% of piperonal (**1b**) was obtained.

2.2. The Influence of Carbon and Nitrogen Source on Biotransformation

Due to the unsatisfying amount of piperonal (**1b**) (14%) obtained in the culture of *T. versicolor* AM536, it was decided to determine whether changing the carbon and nitrogen sources in the biotransformation media would improve the oxidation efficiency of the isosafrole (**1a**) to piperonal (**1b**). Preliminary tests were carried out using *T. versicolor* AM536 where the glucose in Saboraud medium was replaced with various sugars such as fructose, mannose, ribose, starch, and galactose. Several attempts were also made to replace the peptone by using alternative sources of organic nitrogen, such as casein hydrolysate, meat, potatoes and soy peptones, and lactalbumin hydrolysate. These studies showed that when in a modified Sabouraud medium with ribose and peptone as sources of carbon and nitrogen, respectively, the amount of piperonal (**1b**) obtained increased to 18% (Table 2). When the carbon source was glucose and the nitrogen source was casein peptone, it reached 33%.

Table 2. Biotransformation of isosafrole (**1a**) by the *Trametes versicolor* AM536 strain in various carbon and nitrogen sources.

Source		Time (Days)	Substrate (%)	Products (%)	
Carbon	Nitrogen		1a	1b	1c
Fructose	Peptone	7	44	3	53
		14	31	3	66
Mannose		7	38	3	59
		14	29	3	68
Ribose		7	33	14	53
		14	18	18	64
Starch		7	52	2	46
		14	34	4	62
Galactose		7	62	0	38
		14	14	0	86
Glucose	Casein hydrolysate	7	19	33	48
		14	7	28	65
	Lactalbumin hydrolysate	7	17	10	73
		14	17	10	73
	Meat peptone	7	51	15	34
		14	61	14	25
	Potatoes peptone	7	83	3	14
		14	65	4	31
	Soy peptone	7	51	3	46
		14	32	4	64

Subsequently, the biotransformations were performed with *T. versicolor* AM536 and *T. hirsuta* d28 by increasing the amount of added substrate (50 mg) to the growing biomass and using various selected combinations of the optimal carbon and nitrogen sources (Table 3).

Unexpectedly, it appeared that with both strains, better results were obtained in the standard Sabouraud medium. Glucose as a carbon source had a positive effect on the conversion of isosafrole (**1a**) to piperonal (**1b**) while the nitrogen source did not have a significant impact on the biotransformation. The highest conversion of **1a** was reported for the *T. hirsuta* d28 culture, where 43% of aldehyde **1b** was obtained.

Due to the unsatisfactory amount of biocatalysts selected during the screening study (out of 23 tested strains, only 5 showed activity against isosafrole **1a**) (Table 1), it was decided to extend the search and examine the abilities of other fungi for the biooxidation of an unsaturated bond in substrate **1a**. Due to the kindness of researchers from the Department of Plant Protection, various strains of fungi isolated directly from different environments (decaying wood, fungal fruiting bodies, and healthy plant tissues) were provided to us for further tests (see Materials and Methods, Section 3.2). The study confirmed that the vast majority of the tested strains did not bring the expected results

and did not exhibit the ability to transform isosafrole (**1a**). The exceptions were the strains of the species *Trametes hirsuta*, which turned out to be the only biocatalysts that showed activity towards substrate **1a** among all the tested strains (Table 4). The literature data has indicated that strains of the *Trametes* genus are well-known for having alkene cleaving properties in a wide range of chemical compounds [12–21]. In particular, very promising results were obtained during the process using *T. hirsuta* Th2_2 as a biocatalyst, where 38% of piperonal (**1b**) was obtained after 14 days of biotransformation. The other tested strains of *T. hirsuta* delivered only diol **1c**.

Table 3. Biotransformation of isosafrole (**1a**) by *Trametes* strains in selected carbon and nitrogen sources (percentage values according to the GC as the mean value of two replicates).

Strain	Source		Time (Days)	Substrate (%) 1a	Products (%)	
	Carbon	Nitrogen			1b	1c
Trametes versicolor AM536	Glucose	Peptone	7	40	20	40
			14	34	24	42
	Ribose	Peptone	7	72	0	28
			14	53	16	31
	Ribose	Casein hydrolysate	7	57	5	38
			14	45	16	39
	Glucose	Casein hydrolysate	7	47	13	40
			14	32	20	48
Trametes hirsuta d28	Glucose	Peptone	7	33	35	32
			14	11	43	46
	Ribose	Peptone	7	100	0	0
			14	100	0	0
	Ribose	Casein hydrolysate	7	100	0	0
			14	100	0	0
	Glucose	Casein hydrolysate	7	17	32	51
			14	10	33	57

Table 4. Biotransformation of isosafrole (**1a**) by *Trametes hirsuta* strains (percentage values according to the GC).

Strain	Time (Days)	Substrate (%) 1a	Products (%)	
			1b	1c
T. hirsuta Th1_1	7	68	0	32
	14	66	0	34
T. hirsuta Th2_2	7	24	23	53
	14	8	38	54
T. hirsuta Th5_2	7	36	0	64
	14	22	0	78
T. hirsuta Th5_3	7	33	0	67
	14	34	0	66

2.3. *Preparative Biotransformations of Isosafrole (**1a**) to Piperonal (**1b**)*

Considering the most promising biocatalysts for producing piperonal (**1b**) (*T. hirsuta* Th2_2 and *T. hirsuta* d28), it was decided to perform preparative scale biotransformations. In the transformation conducted with *T. hirsuta* Th2_2 using 100 mg of substrate **1a**, after 11 days, the conversion of isosafrole (**1a**) reached 95%, where 38% of piperonal (**1b**) and 34% of vicinal diol (**1c**) were recorded. When *T. hirsuta* d28 was employed, 41% of product **1b** and 54% of product **1c** were obtained. The latter was subsequently isolated with a yield of 49%. The isolated compound was a 3:1 mixture of (*R**,*S**)- and (*R**,*R**)-diols **1c**, which were assigned based on the spectroscopic data. Our results are in accordance with the fact that isosafrole (**1a**), which was submitted for biotransformation, is a commercial mixture of (*E*)/(*Z*) diastereoisomers. The relative configuration of the two diastereoisomers

of diol (**1c**) was established on the basis of the fact that the starting isosafrole was enriched in the (*E*)-stereoisomer and that the hydrolytic epoxide cleavage occurred with an anti-mechanism [9].

Despite the use of a fairly high amount of isosafrole (**1a**), no negative effect of this compound on the cells of the tested fungi was observed, and as a result, it was decided to conduct the process by adding twice as much substrate **1a** (200 mg) to the *T. hirsuta* d28 and *T. hirsuta* Th2_2 cultures. The process was controlled at the appropriate intervals and stopped on the 11th day when a satisfactory conversion of isosafrole (**1a**) was obtained and no increase in product **1b** over that time period had been observed. After product purification, piperonal (**1b**) was obtained from the *T. hirsuta* Th2_2 and *T. hirsuta* d28 cultures with isolation yields of 62% (124 mg) and 50.5% (101 mg), respectively (Table 5).

Table 5. Summary of the biotransformation process on a preparative scale with 200 mg of isosafrole (**1a**) after 11 days (percentage values according to the GC).

Strain	Substrate (%) 1a	Products (%)		Isolation Yield of 1b (%)	Amount of 1b Obtained (mg)
		1b	1c		
T. hirsuta Th2_2	11	82	7	62	124
T. hirsuta d28	25	69	6	50.5	101

It is worth mentioning that the use of whole microbial cells in the piperonal (**1b**) synthesis from isosafrole (**1a**) has not yet been successfully accomplished in the preparative process. Currently known methods involve the use of purified enzymes or *Escherichia coli* bacteria which, as a result of genetic modifications, express the enzymes leading to the production of the aldehyde **1b**. Schwendenwein et al. proposed an alternative method of obtaining piperonal (**1b**) by the enzymatic reduction of piperonylic acid using carboxylate reductase (CAR) from *Neurospora crassa* expressed in *E. coli*. The preparative scale was conducted in two 2 L flasks (200 mL of medium), adding 0.99 g of piperonylic acid to each flask. They obtained 100% piperonal with a 92% isolation yield (1.66 g) [10]. The purified aryl-alcohol oxidase PeAAO2 from *Pleurotus eryngii* P34 was used to obtain piperonal (**1b**) in the preparative scale from piperonyl alcohol as a substrate [11]. The scale-up biotransformation was conducted in two 100 mL flasks containing 10 mL of potassium phosphate buffer with 0.5 μM of purified enzyme and catalase. This approach allowed for achieving a 95% of conversion within 3 h, and the final product was extracted with an 85% yield (244.6 mg of piperonal). The biosynthesis of piperonal (**1b**) by engineered *E. coli* co-expressing two enzymes (*trans*-anethole oxygenase and formate dehydrogenase) was proposed as a novel method by Wen et al. [22]. The final concentration of product **1b** was 19.45 g/L, and the maximum yield and space-time yield of the isosafrole (**1a**) bioconversion reached 96.02% and 3.89 g/L/h, respectively. The table below (Table 6) summarizes the approaches to piperonal (**1b**) preparation published so far using various types of biocatalysts. Both preparative and screening scale processes are included.

The approaches discussed above are undoubtedly attractive solutions that allow obtaining piperonal (**1b**) in considerable amounts. The use of biocatalysts in the form of genetically modified microorganisms, as well as purified enzymes, allowed the researchers to solve the problem of the toxic effect of isosafrole (**1a**) on microbial cells, which was the main limiting factor described in studies conducted by other scientists. As Table 6 indicates, the approaches constituting the application of fungal whole cells for the biotransformation of isosafrole (**1a**) have not been successfully performed in a preparative scale. The method we have proposed includes the use of a biocatalyst in the form of whole cells; therefore, time-consuming and expensive procedures such as an enzyme purification or the use of genetically modified bacterial expression systems can be omitted.

Table 6. Juxtaposition of the biotechnological approaches for obtaining piperonal (**1b**).

Biotransformation Approach	Biocatalysts	Substrate	Conversion (%)	Obtained Amount	Ref.
Whole cells	*Trametes hirsuta*	Isosafrole	4	np *	[8]
Whole cells	*Cladosporium sphaerospermum*	Isosafrole	9.8	np *	[6]
Post-culture medium	*Paecilomyces variotii*	Isosafrole	20	np *	[2]
Whole cells	*Serratia liquefaciens*	Isosafrole	38.5	282.32 mg/L **	[7]
GMO co-expressing system	*Escherichia coli (FDH* and TAO_{3G2})	Isosafrole	96.02	19.45 g/L	[22]
Purified enzyme	POAA2 from *Pleurotus eryngii* P34	Piperonylic alcohol	95	244.6 mg	[11]
GMO co-expressing system	*E. coli* (CAR from *Neurospora crassa*)	Piperonylic acid	100	1.66 g ***	[10]

* np—information not provided; ** results obtained after applying DOE technique (RSM); *** reaction performed in two 2 L flasks (200 mL of medium with 0.99 g of substrate each).

2.4. Species Identification

The Th2_2 and d28 strains sequencing showed two different DNA haplotypes (NCBI accession numbers OQ608086 and OQ608087, respectively). Blast analyses showed more than 99% identity to more than one *Trametes* species, and thus, exact species identification with that method was not possible. Phylogenetic trees (see Supplementary Materials, Figure S10) showed the same topology, though with minor differences for BA (*Bayesian analysis*) and ML (maximum likelihood), and we grouped both analyzed sequences with *T. hirsuta* representatives. The node connecting the Th2_2 and d28 sequences with other *T. hirsuta* samples had a high Bayesian probability (100%) and a high a-LRT bootstrap value (94%), suggesting that both analyzed samples belonged to the *T. hirsuta* species.

3. Materials and Methods

3.1. Materials

The isosafrole as an 8:2 mixture of (*E*)/(*Z*) diastereoisomers was purchased from Zentek s.r.l. (Milan, Italy). All chemicals used for the media preparation were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. The solvents used for the extractions and the GC analysis were purchased from Stanlab (Lublin, Poland).

3.2. Microorganisms

The strains *Absidia cylindrospora* AM336, *Armillaria mellea* AM296, *A. mellea* AM461, *Aspergillus ochraceus* AM456, *Chaetomium* sp. AM432, *Fusarium culmorum* AM282, *F. equiseti* AM22, *F. oxysporum* AM21, *Inonotus radiates* AM70, *Laetiporus sulphurens* AM498, *Laetiporus sulphurens* AM515, *Mortierella isabelina* AM212, *Papularia rosea* AM17, *Pholiota aurivella* AM522, *Piptoporus betulinus* AM40, *P. betulinus* AM57, *Pleurotus ostreatus* AM482, *Poria placenta* AM36, *P. placenta* AM38, and *Trametes versicolor* AM536 were obtained from the microbial collection of the Department of Food Chemistry and Biocatalysis at the Wroclaw University of Environmental and Life Sciences (AM). *Agrocybe aegerita* DSM 22459 and *Pleurotus sapidus* DSM 8266 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) in Braunschweig. *Pycnoporus cinnabarinus* CBS 353.63 was purchased from the Westerdijk Fungal Biodiversity Institute (CBS) in Utrecht (The Netherlands). The fungal strains were maintained at 4 °C on Sabouraud (SB) agar slants containing peptone (10 g), glucose (30 g), and agar (15 g) dissolved in water (1 L) at a pH of 5.5, and then they were transferred into conical flasks with SB medium.

Many strains used for the screening were obtained directly for this study from different environments (decaying wood, fungal fruiting bodies, and healthy plant tissues) and

deposited in the microbial collection of the Department of Plant Protection at the Wroclaw University of Environmental and Life Sciences. Based on their macro- and microscopic features, they were labeled as: *Trametes hirsuta*: d28, TH_ID007, TH1_1, TH2_2, TH5_2, TH5_3, TH3_2, TH5_1, TH1/2, TH3_4, and TH3_1; *Marasmius cohaerens* ID636_Mar_cohaerens; *Corpinus* sp.: 41_Corp and BS_Corp1/1; *Peniophora quercina*: Perenochaeta_3, Perenochaeta_1, and Perenochaeta_2; *Trichoderma* sp. WR1_1 and *Aureobasidium pullulans* Aureo; *Chaetopsis* sp. *Chaetopsis*; *Mucor* sp. BS_skórnik2 and *Penicillium* sp. BSwr6_4; *Daedalea quercina* BS_Gmatwek2/3; and unidentified: WR4_1, WR4_3, WR3_4, WR3_3, WR3_1, WR3_2, WR4_2, WR2_6, TH3_1, Th4, and wr2_6.

3.3. Fungi Isolation Methods

During the fieldwork (2021–2022), samples were collected in sterile envelopes and transported to the laboratory where the isolates underwent mycological analysis. To obtain pure fungal strains from the environmental samples for the screening, different methods of isolation were used.

Most strains were isolated from decaying wood tissues (white-rot) in accordance with the methodology proposed by Arhipova et al. [23] using PDA medium (potato dextrose agar, Biocorp). They were isolated from fir's wood, Gorce National Park, Poland (d28); beech wood, Złoty Stok mine region, Poland (TH2_2, TH3_2, Perenochaeta_1, Perenochaeta_2, TH5_1, TH1/2, TH3_4, TH3_1, WR1_1, WR4_1, WR4_3, WR3_4, WR3_3, WR3_1, WR3_2, WR4_2, WR2_6, TH5_2, TH3_1, Th4, and wr2_6); spruce wood, Jetřichovice village, region of Bohemian Switzerland, Czech Republic (BS_skórnik2, BSwr6_4, and BS_Corp1/1); and sycamore wood, Jetřichovice village, region of Bohemian Switzerland, Czech Republic (BS_Gmatwek2/3).

Some strains were isolated from fungal fruiting bodies. In this method, small pieces (3 mm × 3 mm) of the mycelium obtained from the inner part of fungal cup (to avoid environmental cross-contamination) were placed on PDA medium. They were isolated from *T. hirsuta* cup, Racibórz forest, Poland (TH_ID007); *T. hirsuta* cup, Złoty Stok mine region, Poland (TH1_1 and TH5_3); *P. quercina* fruiting body, Złoty Stok mine region, Poland (Perenochaeta_3); and *M. cohaerens* cup, Wigry National Park, Poland (ID636_Mar_cohaerens).

Three strains were obtained from living plant tissues with no visible symptoms of pathogenesis or decay. Two fungal strains (41_Corp and *Chaetopsis*) were isolated from the flowers of *Impatiens glandulifera*, which were growing in the Kraków municipality. One strain (Aureo) was isolated from a plum's leaf from the Wrocław municipality. A rinsing method using Martin medium (BTL Ltd., Potomac, MD, USA) was used to obtain all these strains.

In every method, growing colonies of fungi were passaged, and then clean mycelium was obtained by the method of monospore cultures.

3.4. Molecular Identification of Fungal Strains

To confirm the species identity of the two most promising biocatalysts that produced piperonal (the *T. hirsuta* Th2_2 and *T. hirsuta* d28 strains), molecular identification was performed. DNA was isolated from the culture growths on slants with TSA medium. Mycelial fragments were placed in 1.5 mL tubes, together with 500 µL of TE buffer, and heated in a microwave at 600 W for two minutes. Next, the tubes were centrifuged at ~6000 g for two minutes and the supernatant from each tube was transferred onto an isolation column from a Sherlock AX isolation kit (A&A Biotechnology, Gdańsk, Poland). Finally, DNA was extracted according to the producer's manual and suspended in TE buffer.

The isolated DNA was quantified with a Qubit 4 Fluorometer and diluted to unify the concentration among the samples. An internal transcribed fragment was amplified with the primers ITS4 and ITS5 with the following conditions: initial denaturation at 95 °C—2 min; 35 cycles at 95 °C—60 s; 55 °C—60 s; 72 °C—90 s; and final elongation at 72 °C—10 min. The PCR products were verified with agarose gel (1%) electrophoresis, cleaned with an Eppic

enzymatic cleanup kit (A&A Biotechnology, Poland), and, finally, sequenced in both directions using an Applied Biosystems 3730 XL DNA analyzer in Genomed S.A.

After sequencing, both strands of each sample were aligned using Bioedit 7.2.5 software [24] in order to obtain consensus sequences. Both sequences obtained in this way were used in Blast [25] and implemented in the Genbank database (http://www.ncbi.nlm.nih.gov, accessed on 9 March 2023) to search for homologues. Next, phylogenetic analysis was performed using the PolyPeet ITS database used previously in the phylogenetic study of *Trametes* sp. [26]. The trees were created with both the Bayesian and maximum likelihood approaches. MrBayes 3.2.7a [27] was used to estimate the Bayesian trees using the SYM + G + I substitution model, which was chosen as the best-fit model with jModelTest 2.1.10 [28]. The MrBayes analysis consisted of two independent runs (each with four chains) starting from random trees. The trees were sampled every 100th generation for 10,000,000 generations (with 25% burn-in) until the average standard deviation of split frequencies was stabilized at below 0.01 for all trees used to construct the consensus tree. IQTree [29] was used to estimate the maximum likelihood tree with the SYM + G + I model and a Shimodara–Hasegawa-like approximate likelihood ratio test (SH-aLRT) [30] as the tree branch support.

3.5. Screening Scale Biotransformations

The biotransformations with the fungal strains were carried out in 250 mL Erlenmayer flasks with 75 mL of liquid culture medium. The flasks were sterilized at 121 °C at a pressure of 1 atm (15 min) and the medium was inoculated with 0.5 mL of pre-prepared fungal cultures under a laminar chamber under sterile conditions. Then, the cultures were shaken at 150 rpm for 5–7 days at room temperature. After the growth of the biomasses, 15 mg (460 mM) of substrate (**1a**) dissolved in 0.2 mL of DMSO was added to the cultures.

3.6. Modification of Culture Media Composition

During the study, the impact of various carbon and nitrogen sources on the process was examined with a strain T. versicolor AM536. The biotransformations were carried out in 250 mL Erlenmayer flasks with 75 mL of modified Sabouraud medium where the glucose was replaced by sugars such as fructose, mannose, ribose, starch, and galactose. Instead of peptone, casein hydrolysate, meat peptone, potatoes peptone, soy peptone, and lactalbumin hydrolysate were used. After the growth of the biomasses, 15 mg of substrate (**1a**) dissolved in 0.2 mL of DMSO was added to the cultures. In further studies, the *T. hirsuta* d28 and *T. hirsuta* Th2_2 strains were used, where Sabouraud medium and its modifications (glucose/casein hydrolysate, ribose/peptone, and ribose/casein hydrolysate) were used. During this experiment, 50 mg of substrate (**1a**) dissolved in 0.2 mL of DMSO was added to the culture. The experiments were conducted in duplicates.

3.7. Extraction Procedure

To observe the progress of the biotransformation, samples (3 mL) of the reaction mixtures were taken after 7 and 14 days. The aqueous phase was acidified with 0.1 M HCl to pH = 3, salted out with NaCl, and extracted with ethyl acetate (1.5 mL), followed by centrifugation (4000 rpm/3100× *g*, 10 min, 4 °C). The extract was dried over anhydrous magnesium sulfate, filtered, concentrated by rotary evaporator, and analyzed by the GC method.

3.8. Preparative Biotransformations

Five-hundred milliliters of Sabouraud medium were placed in 2000 mL flasks and sterilized at 121 °C for 15 min. The medium was inoculated with 50 mL of preprepared cultures of the microorganisms. The flasks with fungal cultures were stored for 7 days at 25 °C and shaken at 150 rpm. After this time, 100 mg or 200 mg of isosafrole dissolved in 5 mL of DMSO was added into the culture and the process was continued at 25 °C and 150 rpm. Samples were extracted after 11 days and observed by GC to estimate the

progress of the biotransformation. Subsequently, the products were then extracted three times with 50 mL of ethyl acetate, collected, dehydrated by anhydrous $MgSO_4$, and the organic solvent was evaporated under reduced pressure. The samples prepared in this way were then purified using a puriFlash apparatus with hexane:ethyl acetate 4:1 (v/v). The fractions collected in this way were verified using the GC method, and finally, only those containing the pure product were combined.

3.9. Analysis Procedure

Gas chromatography analysis (GC, FID, with the carrier gas H_2) was carried out on an Agilent Technologies 6890 N (GC System, Santa Clara, CA, USA) with use of a column HP-5 (30 m × 0.32 mm × 0.25 µm, Santa Clara, CA, USA) according the following temperature program: 70 °C and 300 °C (30 °C/min) (1 min). Samples (1 µL) were injected with split 20:1, and the flow of the carrying gas was 1 mL/min. The total run time was 9.8 min. Retention times were established as follow: t_R = 4.19 min for piperonal (**1b**), t_R = 4.37 min for isosafrole (**1a**), and t_R = 5.58 min for the (1*R**,2*S**)-**1c** and 5.62 min for the (1*R**,2*R**)-**1c** vicinal diols. GC/MS analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 µm) from Agilent Technologies Italia S.p.A. (Cernusco sul Naviglio, Italy). The following temperature program was employed: 60 °C (1 min), 150 °C (6 °C/min) (1 min), and 280 °C (12 °C/min) (5 min). The total run time was 32 min. The structures of the compounds were confirmed on the basis of ^1H NMR and ^{13}C NMR, which were recorded for the $CDCl_3$ solutions on a Bruker Avance DRX 600 (600 MHz) spectrometer (Billerica, MA, USA).

Separation of the biotranformation products during flash chromatography were controlled by thin layer chromatography (TLC) using aluminum foil plates coated with silica gel. The compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$ and 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4.

The NMR spectra and GC-MS chromatograms (see Supplementary Materials, Figures S1–S9) of the obtained products were as follows:

Piperonal (**1b**) ^1H NMR ($CDCl_3$, 600 MHz): δ = 9.79 (s, 1H, *CHO*), 7.40 (dd, 2H, *J* = 7.9 Hz and 1.5 Hz, *Ar–H*), 7.32 (d, 1H, *J* = 1.5 Hz, *Ar–H*), 6.91 (d, 1H, *J* = 7.9 Hz, *Ar–H*), 6.06 (s, 2H; CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 190.4, 153.2, 148.8, 131.9, 128.8, 108.4, 106.9, 102.2; and GC/MS (EI) tr = 15.26 min: m/z (%) = 149 (M+ −1, 100), 121 (40), 91 (12), 63 (25).

(1*R**,2*S**)-1-(Benzo[1,3]dioxol-5-yl)propane-1,2-diol ((1*R**,2*S**)-**1c**) ^1H NMR ($CDCl_3$, 600 MHz): δ = 6.86–6.84 (m, 1H, *Ar–H*), 6.81–6.76 (m, 2H, *Ar–H*), 5.96 (s, 2H, CH_2), 4.28 (d, 1H, *J* = 7.5 Hz, *CHOH*), 3.80 (m, 1H, *CHOH*), 2.22 (s, 2H; 2x*OH*), 1.03 (d, 3H, *J* = 6.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 147.91, 147.5, 135.1, 120.5, 108.3, 107.2, 101.1, 79.4, 72.3, 18.9; and GC/MS (EI) tr = 21.23 min: m/z (%) = 196 (M+, 16), 178 (8), 162 (8), 151 (100), 135 (25), 123 (25).

(1*R**,2*R**)-1-(Benzo[1,3]dioxol-5-yl)propane-1,2-diol ((1*R**,2*R**)-**1c**) ^1H NMR ($CDCl_3$, 600 MHz): δ = 6.90–6.89 (m, 1H, *Ar–H*), 6.80–6.78 (m, 2H, *Ar–H*), 5.95 (s, 2H, CH_2), 4.56 (d, 1H, *J* = 4.6 Hz, *CHOH*), 3.95 (m, 1H, *CHOH*), 2.22 (s, 2H; 2x*OH*), 1.10 (d, 3H, *J* = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 147.87, 147.3, 134.4, 120.2, 108.2, 107.1, 101.2, 77.5, 71.3, 17.6; and GC/MS (EI) tr = 21.31 min: m/z (%) = 196 (M+, 16), 178 (8), 162 (8), 151 (100), 135 (25), 123 (25).

4. Conclusions

In this study, a preliminary screening was performed on fifty-six fungal strains. Only a few of them showed the ability to convert isosafrole (**1a**). Of these, the *Trametes* strains were the most promising biocatalysts. Due to literature reports on the ability of *Trametes* to perform biooxidation on a wide range of compounds, it was decided to extend the screening to include strains from different *Trametes* species. The most efficient biocatalyst appeared to be *T. hirsuta* Th2_2, which provided piperonal (**1b**) with a 62% isolation yield under the preparative biotransformation scale. It is worth mentioning that there are no literature reports on the production of piperonal (**1b**) from isosafrole (**1a**) on a preparative

scale using whole cells of fungi. Known methods involve the use of purified enzymes or genetically modified bacteria *E. coli*. Therefore, due to the simplicity of the process, it is important to continue research on this strain to improve its capacity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28083643/s1, Figure S1: Chromatogram of the preparative scale biotransformation of isosafrole (**1a**) to piperonal (**1b**) with *T. hirsuta* d28 after 11 days; Figure S2: Chromatogram of the preparative scale biotransformation of isosafrole (**1a**) to piperonal (**1b**) with *T. hirsuta* TH2_2 after 11 days; Figure S3: H^1 NMR spectrum of piperonal (**1b**); Figure S4: C^{13} NMR spectrum of piperonal (**1b**); Figure S5: GC/MS chromatogram of piperonal (**1b**); Figure S6: H^1NMR spectra of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**); Figure S7: C^{13} NMR spectra of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**); Figure S8: GC/MS chromatogram of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**); Figure S9: GC/MS chromatogram of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**); Figure S10: Bayesian phylogenetic tree of the PolyPeet ITS database sequences of *Trametes* (and other species), along with two sequences from the strains d28 and Th2_2 described in this manuscript. Numbers along the nodes are the posterior probabilities of the nodes (BA) and maximum likelihood bootstrap values (ML) (values below 0.7 are not shown (-)). The *Trametes hirsuta* clade is marked with a red line and both analyzed samples are marked with red stars.

Author Contributions: Conceptualization, E.B. and F.B.; methodology, D.H. and F.B.; formal analysis, D.H., E.S. and F.B.; provided fungal strains, K.P.; genetic determination of fungal strains, T.S.; investigation, D.H. and F.B.; writing—original draft preparation, D.H. and E.S.; writing—review and editing, F.B., E.B., K.P. and T.O.; visualization, E.S.; supervision, F.B. and E.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project "UPWR 2.0: international and interdisciplinary program of development of Wrocław University of Environmental and Life Sciences" and co-financed by the European Social Fund under the Operational Program Knowledge Education Development under contract No. POWR.03.05.00-00-Z062/18 of 4 June 2019. The APC is co-financed by Wrocław University of Environmental and Life Sciences.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to express our gratitude to the students Kinga Bobyk and Anna Sudomirska for their contributions to the research. This publication is the result of the activity of the research group "Biocatalysis and biological activity—BioActiv".

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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Supplementary Materials

Trametes hirsuta as an attractive biocatalyst for the preparative scale biotransformation of isosafrole into piperonal

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Number of pages: 9
Number of figures: 10

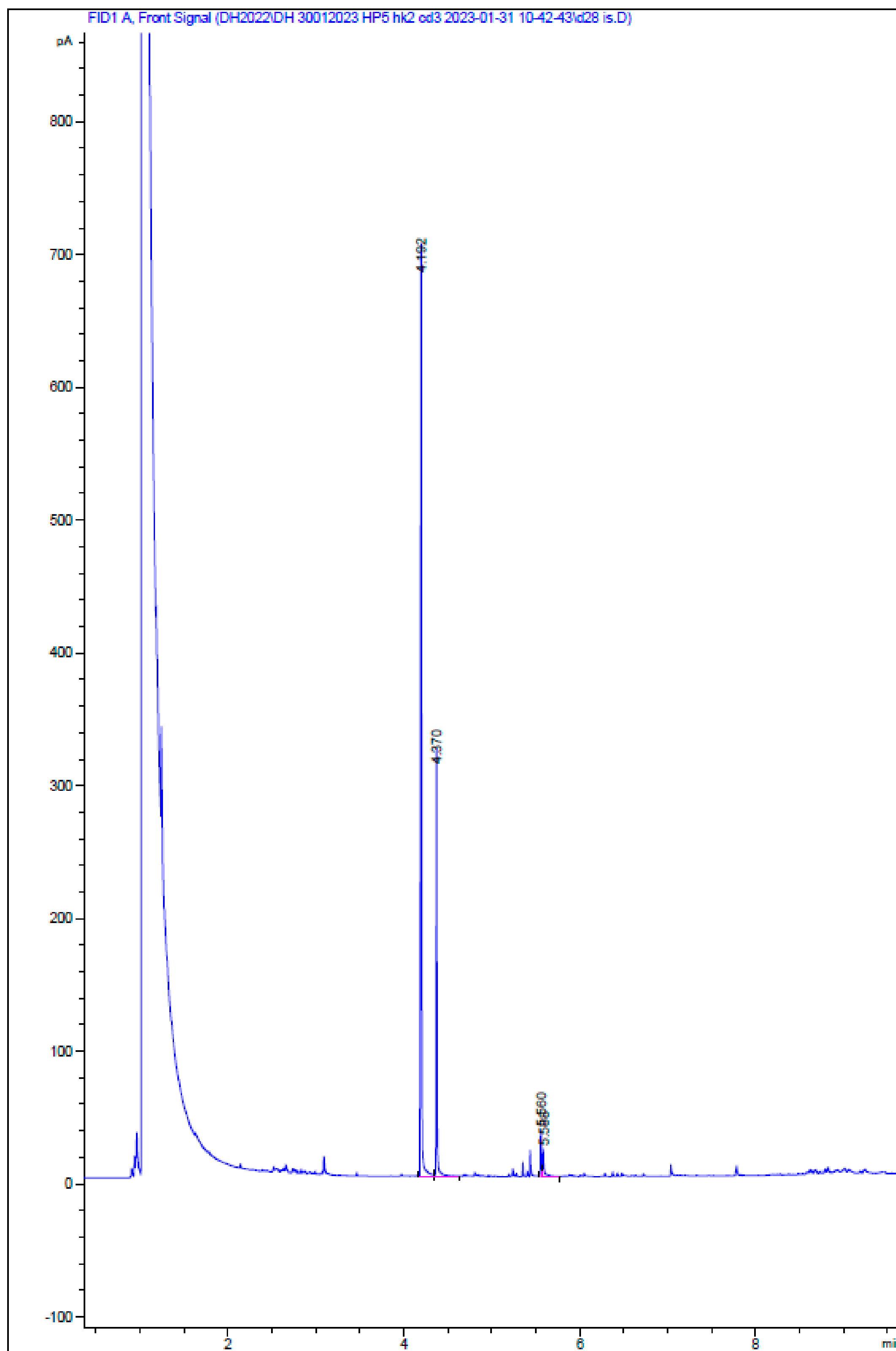


Figure S1. Chromatogram of the preparative scale biotransformation of isosafrole (**1a**) to piperonal (**1b**) with *T. hirsuta* d28 after 11 days.

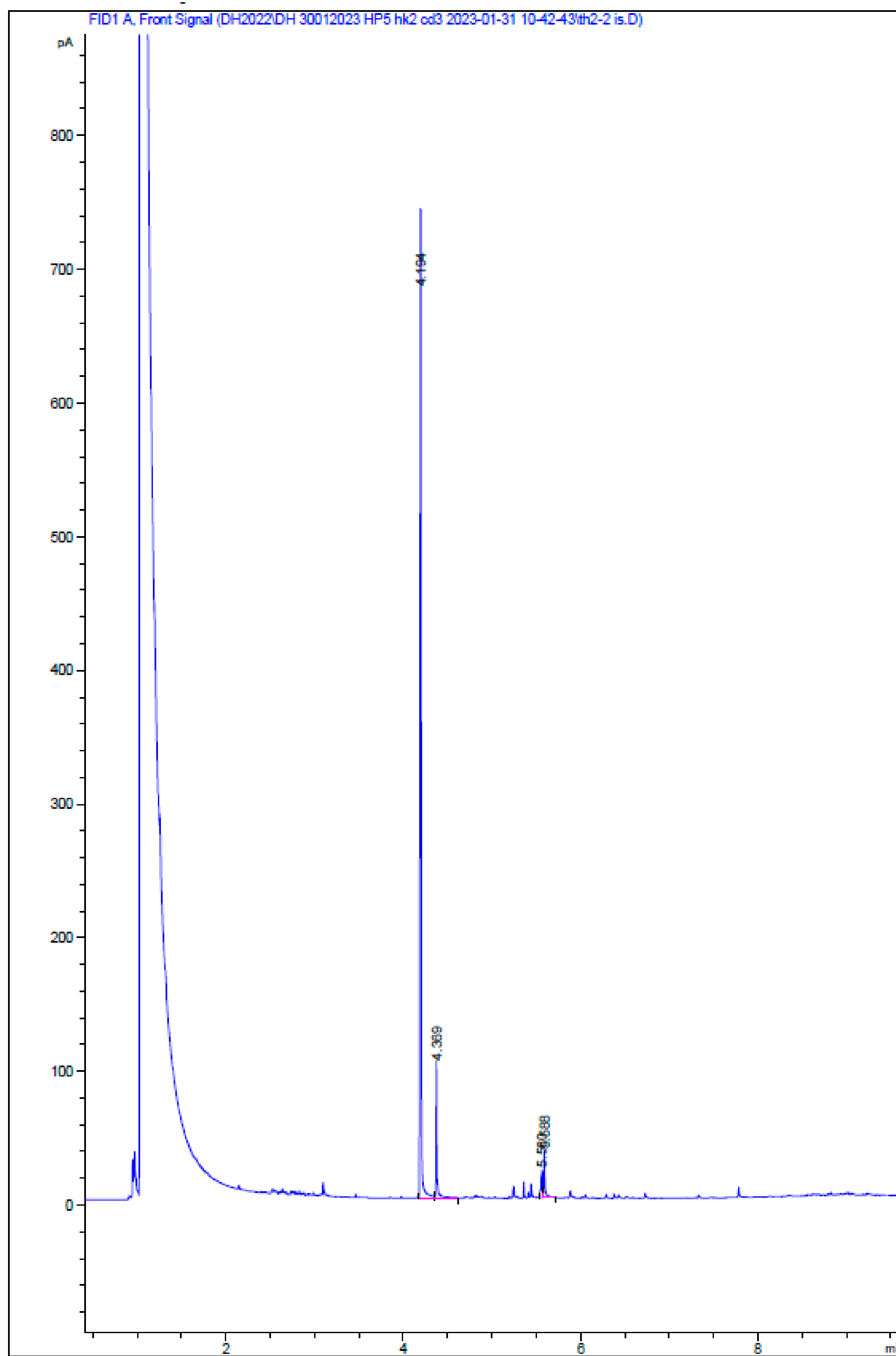


Figure S2. Chromatogram of the preparative scale biotransformation of isosafrole (**1a**) to piperonal (**1b**) with *T. hirsuta* TH2_2 after 11 days.

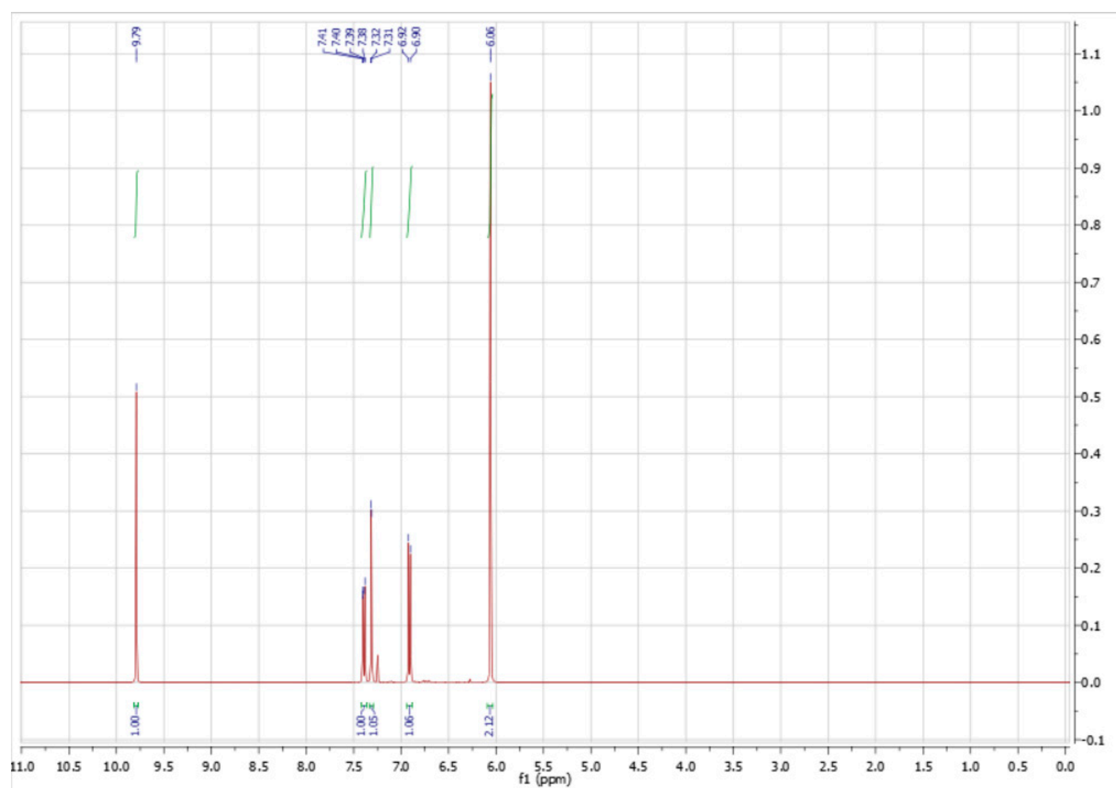


Figure S3. H[1] NMR spectrum of piperonal (**1b**).

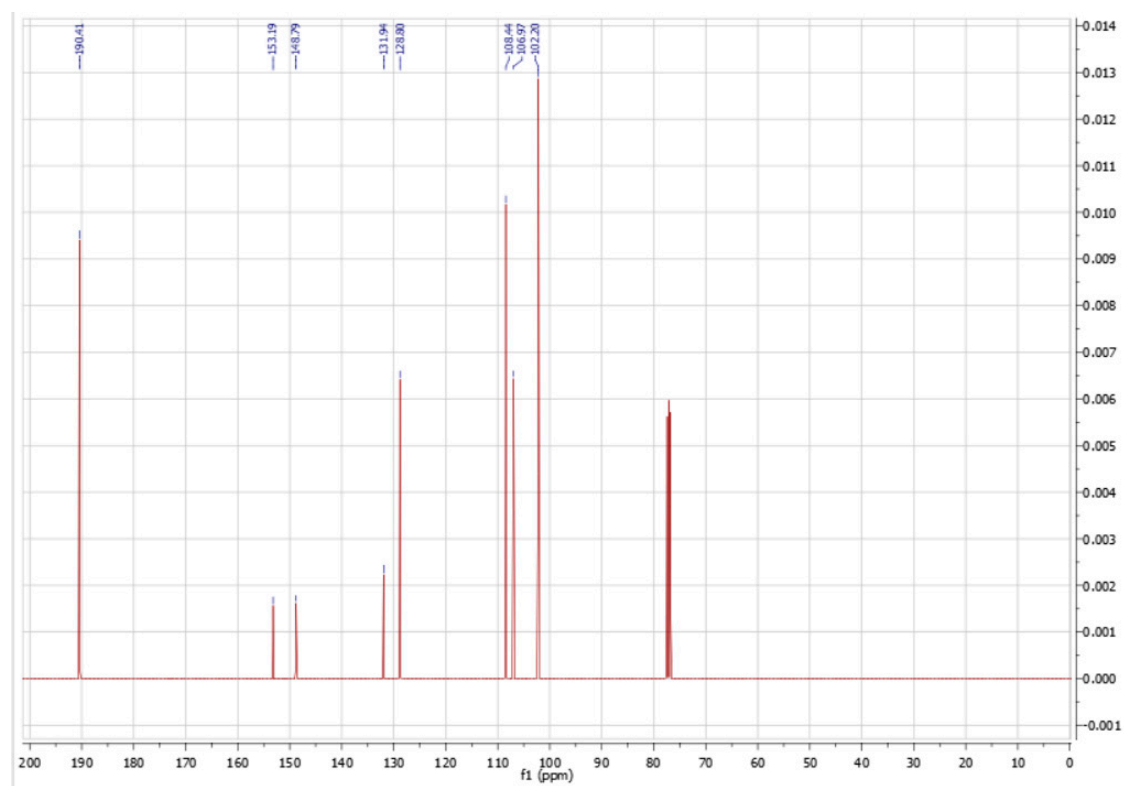


Figure S4. C[13] NMR spectrum of piperonal (**1b**).

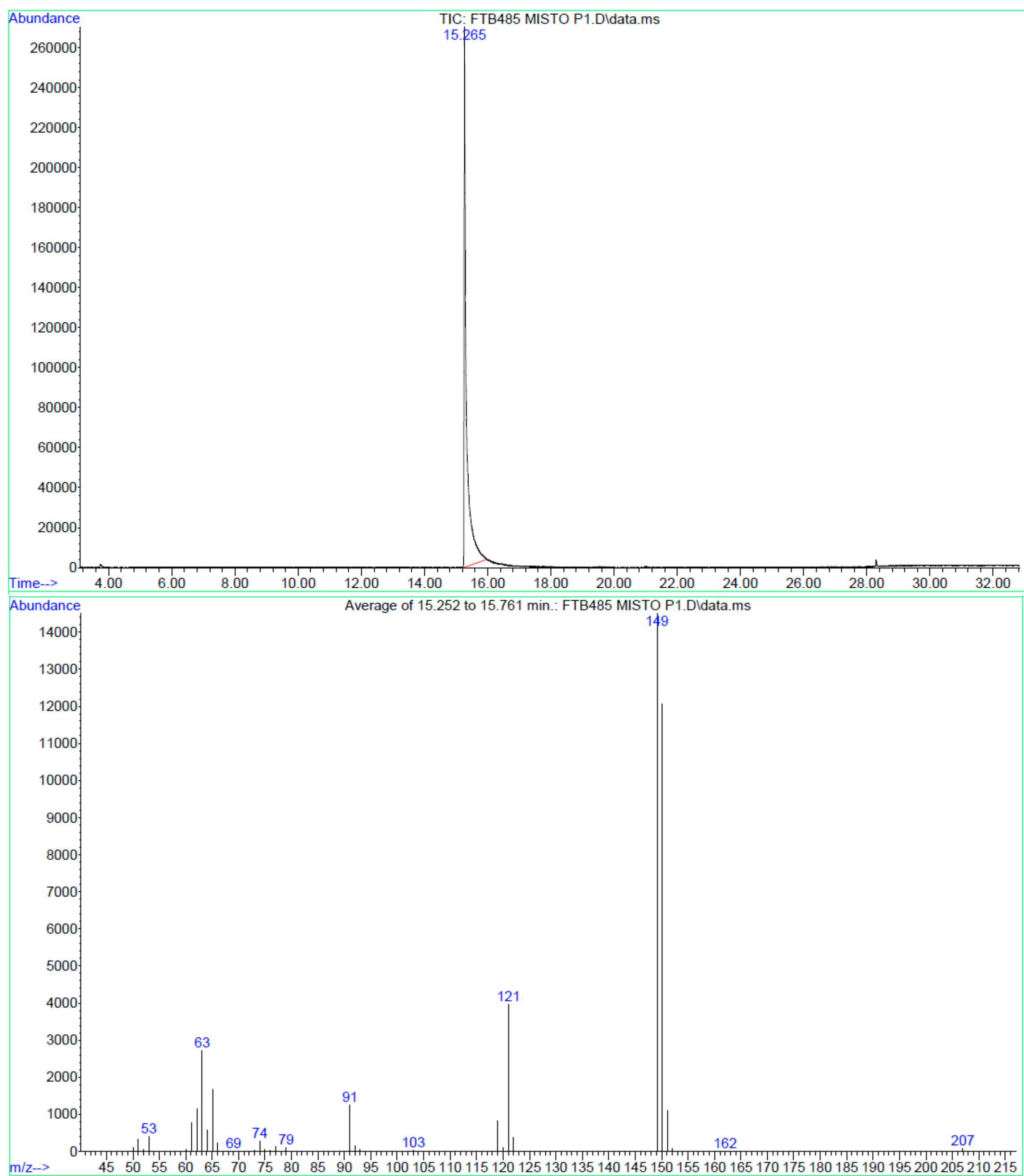


Figure S5. GC/MS chromatogram of piperonal (**1b**).

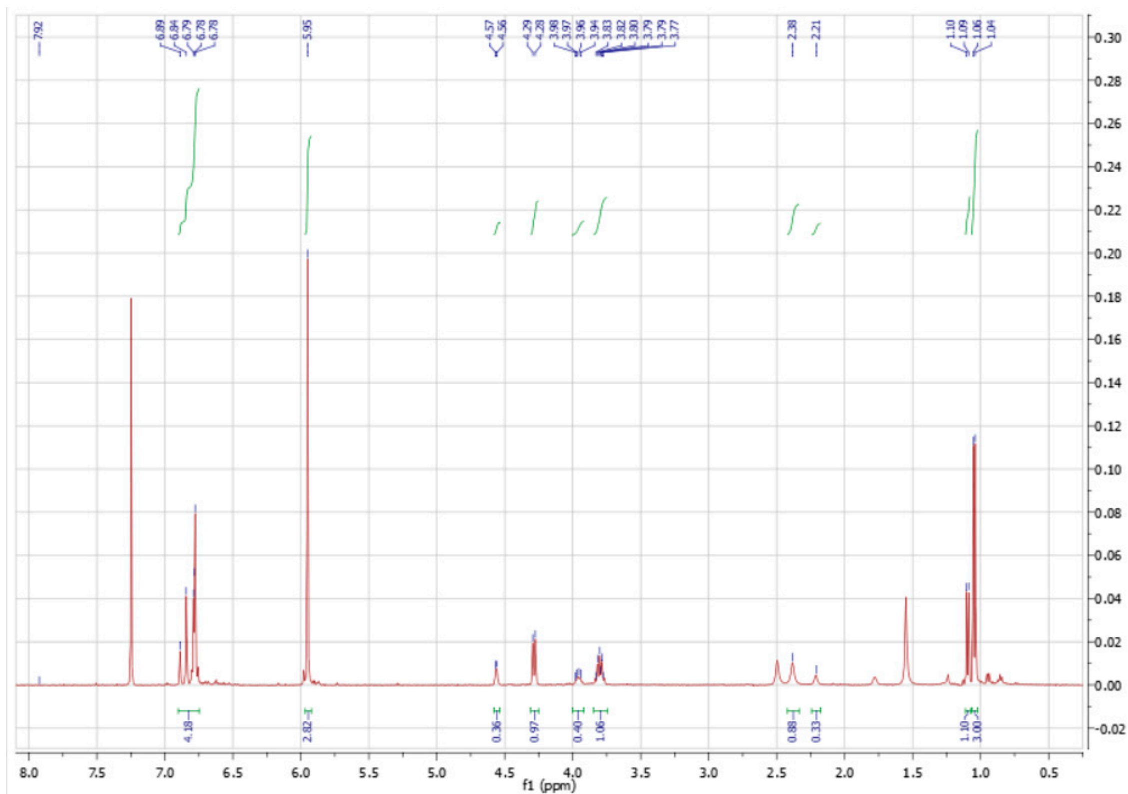


Figure S6. H[1] NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**).

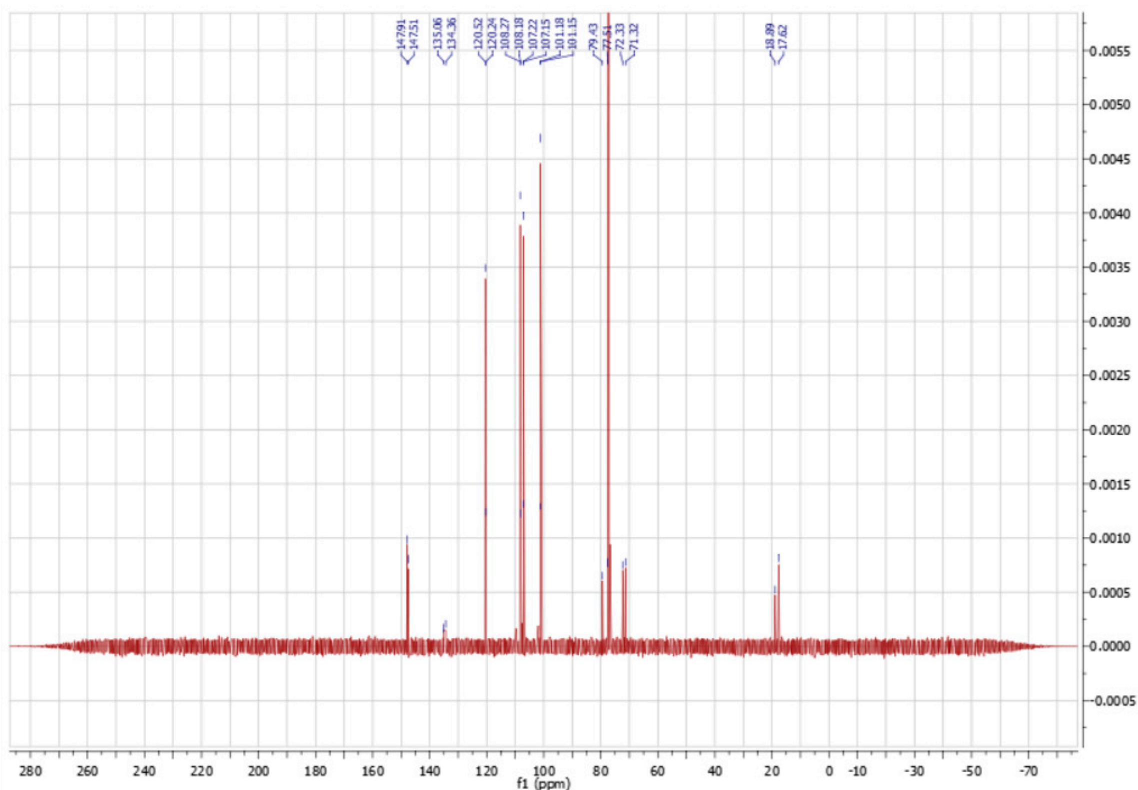
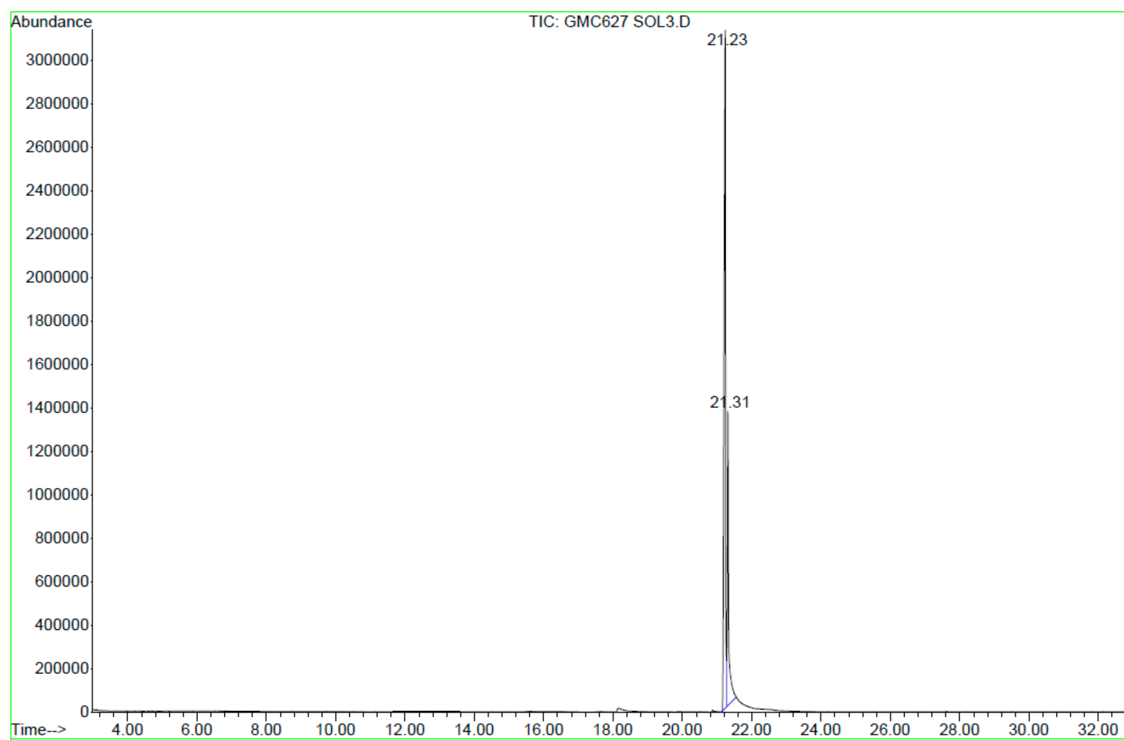


Figure S7. C[13] NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**).



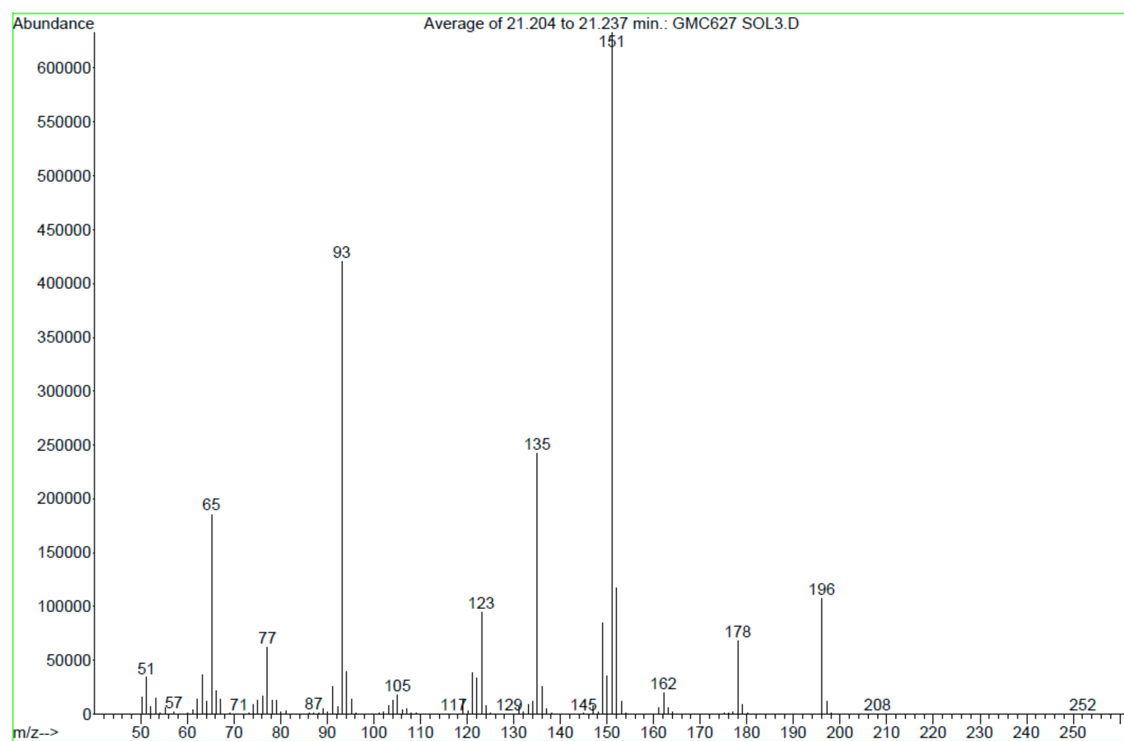
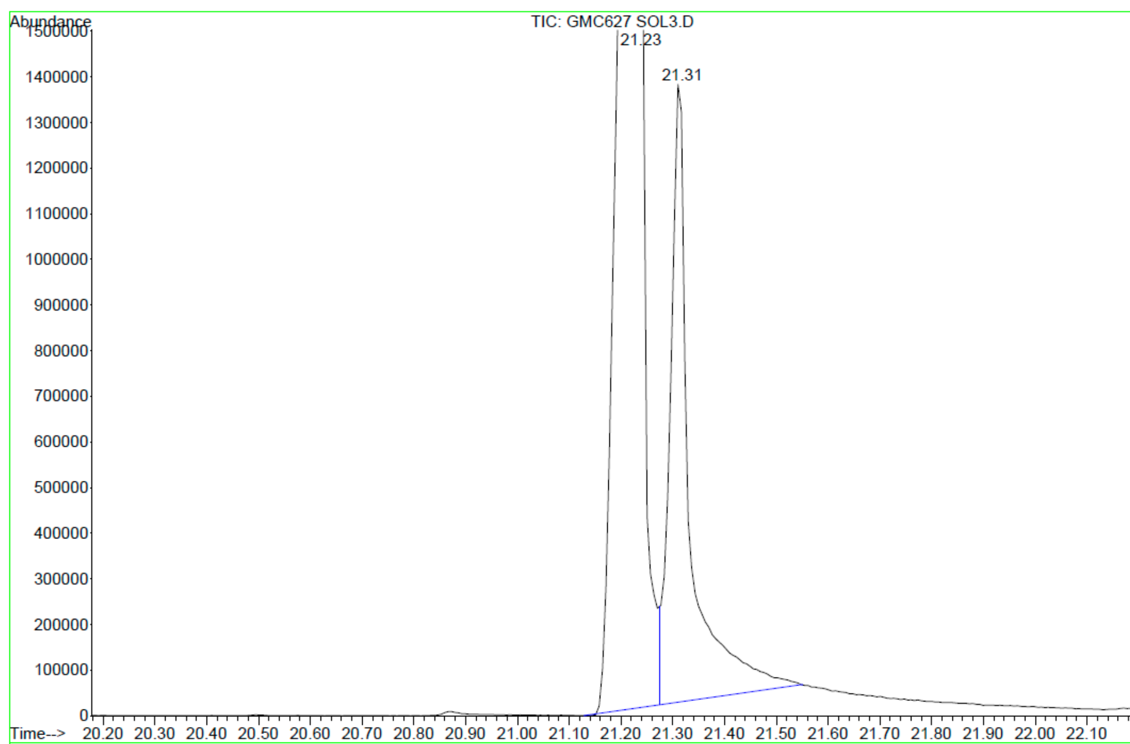


Figure S8. GC/MS chromatogram of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**).



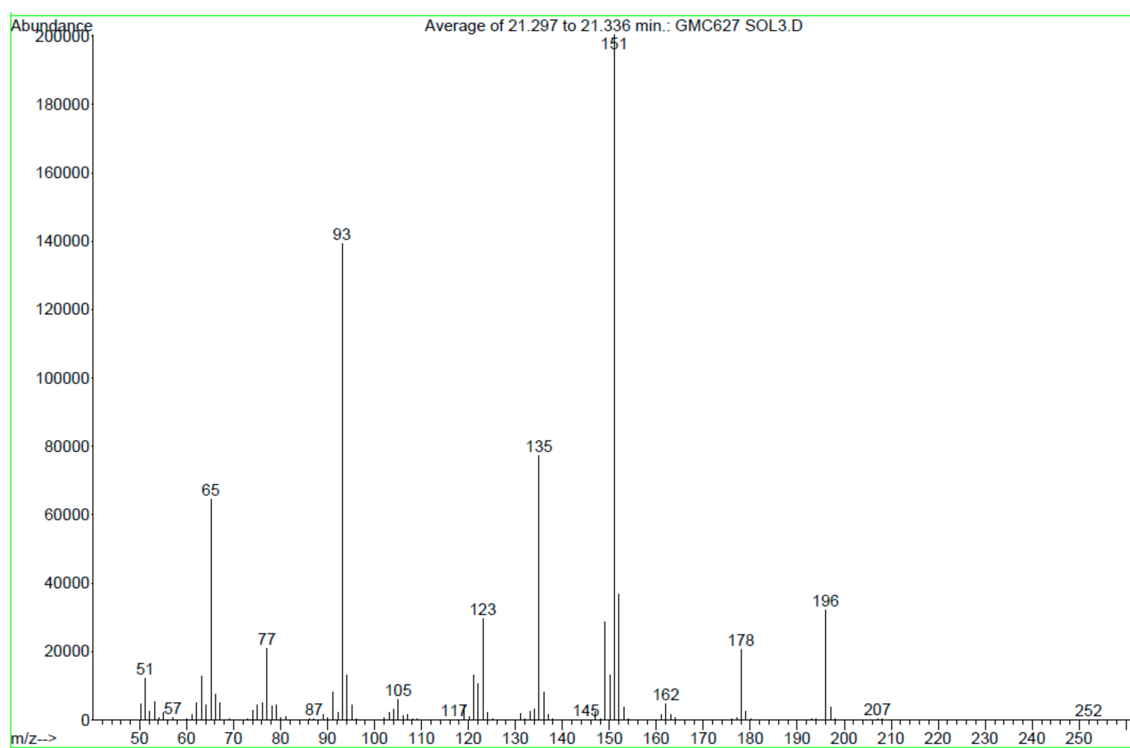


Figure S9. GC/MS chromatogram of (1*R**,2*S**) and (1*R**,2*R**)-1-(benzo[1,3]dioxol-5-yl)propane-1,2-diol (**1c**).

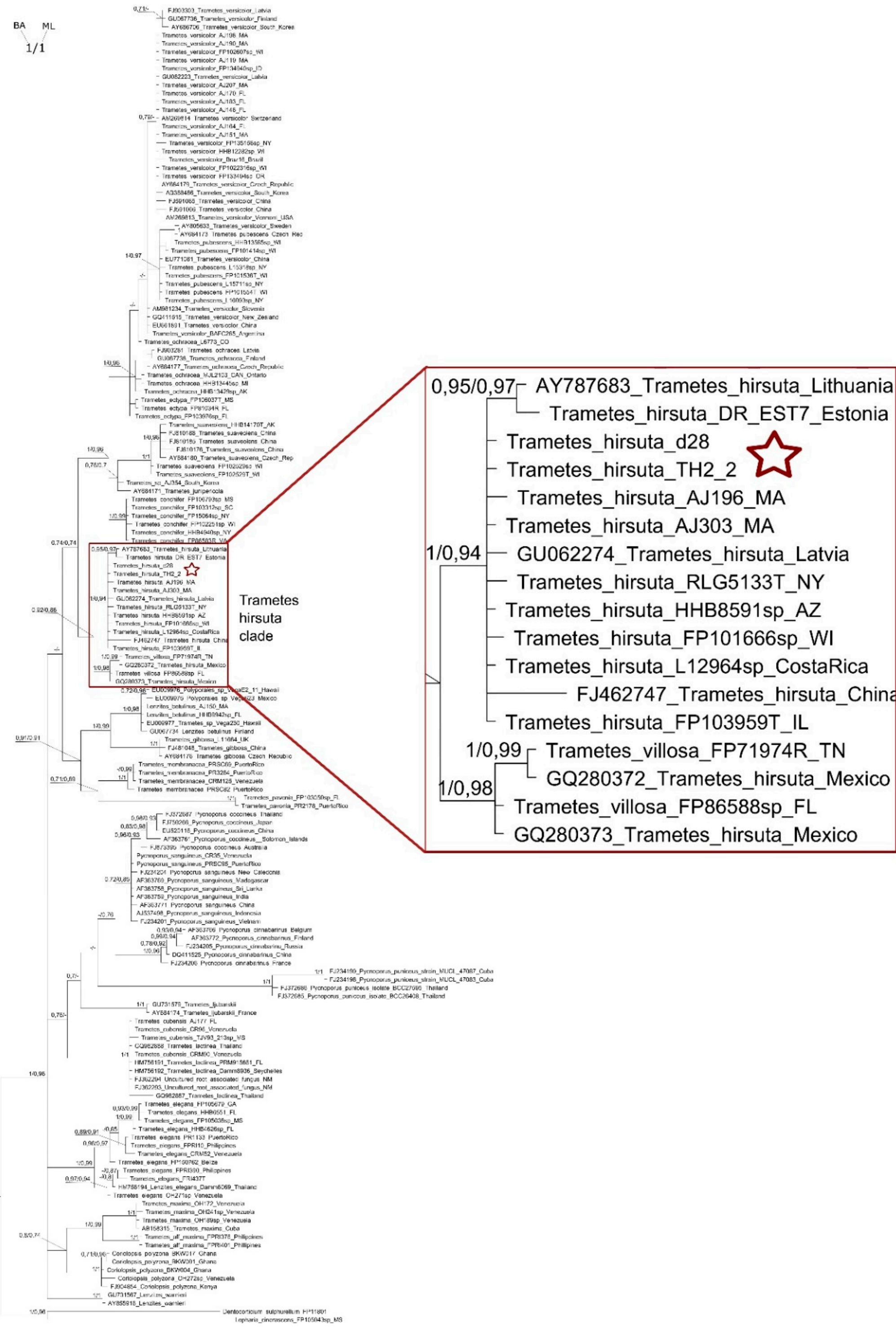


Figure S10. Bayesian phylogenetic tree of PolyPeet ITS database sequences of *Trametes* (and other species) along with two sequences from strains d28 and Th2_2 described in this manuscript. Numbers along the nodes are posterior probability of the node (BA) and maximum likelihood bootstrap values (ML) (values below 0,7 were not shown (-)). *Trametes hirsuta* clade was marked with red line and both analysed samples were marked with red star.

7.4 PUBLIKACJA 4 (P4)

Hernik, D.*; Szczepańska, E.; Ghezzi, M. C.; Brenna, E.; Włoch, A.; Pruchnik, H.; Mularczyk, M.; Marycz, K.; Olejniczak, T.; Boratyński, F.*

Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives. *Frontiers in Microbiology*, 2023, 14, 1223123.

| Frontiers in Microbiology

TYPE Original Research
PUBLISHED 26 June 2023
DOI 10.3389/fmicb.2023.1223123

Check for updates

OPEN ACCESS

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RECEIVED 15 May 2023
ACCEPTED 12 June 2023
PUBLISHED 26 June 2023

CITATION
Hernik D, Szczepańska E, Ghezzi MC, Brenna E, Włoch A, Pruchnik H, Mularczyk M, Marycz K, Olejniczak T and Boratyński F (2023) Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives. *Front. Microbiol.* 14:1223123.
doi: 10.3389/fmicb.2023.1223123

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Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives

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Propenylbenzenes, including isosafrole, anethole, isoeugenol, and their derivatives, are natural compounds found in essential oils from various plants. Compounds of this group are important and valuable, and are used in the flavour and fragrance industries as well as the pharmaceutical and cosmetic industries. The aim of this study was to develop an efficient process for synthesising oxygenated derivatives of these compounds and evaluate their potential biological activities. In this paper, we propose a two-step chemo-enzymatic method. The first step involves the synthesis of corresponding diols **1b–5b** from propenylbenzenes **1a–5a** *via* lipase catalysed epoxidation followed by epoxide hydrolysis. The second step involves the microbial oxidation of a diasteroisomeric mixture of diols **1b–5b** to yield the corresponding hydroxy ketones **1c–4c**, which in this study was performed on a preparative scale using *Dietzia* sp. DSM44016, *Rhodococcus erythropolis* DSM44534, *R. erythropolis* PCM2150, and *Rhodococcus ruber* PCM2166. Application of scaled-up processes allowed to obtain hydroxy ketones **1-4c** with the following yield range 36–62.5%. The propenylbenzene derivatives thus obtained and the starting compounds were tested for various biological activities, including antimicrobial, antioxidant, haemolytic, and anticancer activities, and their impact on membrane fluidity. Fungistatic activity assay against selected strains of *Candida albicans* results in MIC_{50} value varied from 37 to 124µg/mL for compounds **1a**, **3a–c**, **4a,b**, and **5a,b**. The highest antiradical activity was shown by propenylbenzenes **1-5a** with a double bond in their structure with EC_{50} value ranged from 19 to 31µg/mL. Haemolytic activity assay showed no cytotoxicity of the tested compounds on human RBCs whereas, compounds **2b–4b** and **2c–4c** affected the fluidity of the RBCs membrane. The tested compounds depending on their concentration showed different antiproliferative activity against HepG2, Caco-2, and MG63. The results indicate the potential utility of these compounds as fungistatics, antioxidants, and proliferation inhibitors of selected cell lines.

KEYWORDS

biotransformation, fragrances, propenylbenzenes, oxidation, fungistatic activity, antioxidant activity, haemolytic activity, proliferative activity

1. Introduction

Propenylbenzenes, such as isosafrole, anethol, isoeugenol, and their derivatives, are widely found in essential oils from plants such as the aniseed tree, liquorice, and the cananga tree (Newberne et al., 1999; Thi Luu et al., 2009; Fahlbusch et al., 2003; Lummiss et al., 2012). Compounds of this group are economically important and widely used in the flavour and fragrance industries as well as the pharmaceutical and cosmetic industries, and as intermediates in the synthesis of more complex products (Atsumi et al., 2005; Cabral et al., 2014; Aprotosoaie et al., 2016). In recent years, various commercial processes have been developed to obtain these compounds through isomerization of safrole, estragole, and eugenol (Petersen et al., 2010; Rajagopalan et al., 2013; Hassam et al., 2015). Propenylbenzenes have broad biological activities, such as antioxidant, antimicrobial, anti-inflammatory, and antiproliferative actions. For example, essential oils containing isosafrole have shown antioxidant activity and antimicrobial effects on clinical isolates of *Helicobacter pylori*, *Staphylococcus aureus*, and *Escherichia coli* (Bruna et al., 2022). Additionally, safrole oil and its nanoemulgel had an antiproliferative effect on hepatocellular carcinoma cells Hep3B (Eid and Hawash, 2021). Anethole exerts an inhibitory effect on periodontitis by suppressing pro-inflammatory molecules (Moradi et al., 2014; Lal et al., 2022). Anethole-rich oil from *Clausena heptaphylla* leaf has anti-diabetic, tyrosinase-inhibiting, and anti-cholinesterase activities (Lal et al., 2022). Emulsion-encapsulated isoeugenol has antimicrobial effects against food pathogens and spoilage bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, and *Leuconostoc mesenteroides* as well as antioxidant and anti-inflammatory properties (Siva et al., 2019).

Given the different biological activities of propenylbenzenes and their widespread use in industry, it is worth looking for derivatives of these compounds. As the interest in compounds obtained *via* environmentally friendly methods is currently growing, the necessity for the development of new methods for producing propenylbenzene derivatives such as diols and hydroxy ketones has increased in recent years. Various methods for obtaining propenylbenzenes and their derivatives have been described in the literature (D'Accolti et al., 1993; Peng et al., 2005; Mecozzi et al., 2017; Nair, 2020; Tentori et al., 2021). In one chemo-enzymatic method, isosafrole is epoxidated and then hydrolysed to yield a stereoisomeric mixture of corresponding diols (Tentori et al., 2021). Peng et al. (2005) presented a method involving selective oxidation of *sec*-1,2-diols, using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and ultrasound waves to produce α-hydroxy ketones. The oxidation of vicinal diols to α-hydroxy ketones can also be accomplished using hydrogen peroxide (H_2O_2) and a manganese catalyst (Mecozzi et al., 2017), dimethyldioxirane or its trifluoromethyl analogue (D'Accolti et al., 1993), or 2-iodoxybenzoic acid (IBX) (Nair, 2020).

Due to the increasing attention paid to green chemistry and the advantages of biotechnological methods, it is important to replace chemical methods with those using biocatalysts in the form of whole cells or isolated enzymes. For instance, Kihumbu et al. (2002) described a method that allowed synthesising all four stereoisomers of 1-phenylpropane-1,2-diol as well as the corresponding hydroxy ketones with high yields starting from benzaldehyde and acetaldehyde using different lyase–alcohol dehydrogenase combinations. Oeggl et al. (2018) reported a method to synthesise hydroxy ketone isomers using lyase or decarboxylase and then 4-methoxyphenyl-1,2-propanediol using alcohol dehydrogenase, with good yields. Finally, the use of benzoylformate decarboxylase or benzaldehyde lyase from *P. putida* and *P. fluorescens* allows the synthesis of hydroxy ketone derivatives of propenylbenzenes (Kurlemann et al., 2009; Kulig et al., 2013; Pérez-Sánchez et al., 2013). However, methods that use whole cells of microorganisms for cost reduction are lacking.

In the presented work, we aimed to obtain oxygenated propenylbenzene derivatives, including diols **1b–5b** and hydroxy ketones **1c–4c**, starting from propenylbenzenes **1a–5a**, using chemo-enzymatic synthesis followed by whole-cell transformation. In addition, biological activity tests were performed on the obtained compounds. This approach allowed us to assess the influence of various chemical groups attached to the propenyl chain and aromatic ring on the investigated biological activities. Based on our previous experience, we decided to test the following biological activities: antimicrobial, antioxidant, haemolytic, and anticancer actions, and the impact on membrane fluidity.

2. Materials and methods

2.1. Microorganisms

Bacillus subtilis PCM2238, *B. subtilis* PCM2850, *Dietzia maris* PCM2292, *Gordonia bronchialis* PCM2167, *Gordonia rubripertincta* PCM2144, *Micrococcus luteus* PCM525, *Pseudomonas aeruginosa* PCM2720, *P. aeruginosa* PCM3035, *Rhodococcus coprophilus* PCM2174, *Rhodococcus erythropolis* PCM2150, *Rhodococcus rhodnii* PCM2157, *Rhodococcus rhodochrous* PCM909, *Rhodococcus ruber* PCM2166, *R. ruber* PCM2171, *R. ruber* PCM2216, *Serratia liquefaciens* PCM2830, *Serratia marcescens* PCM549, *Serratia plumuthica* PCM550, *Serratia* sp. PCM1324, *Streptomyces griseus* subsp. *griseus* PCM2331 were obtained from the Polish Academy of Sciences (Wrocław, Poland). *Dietzia* sp. DSM44016 and *Rhodococcus erythropolis* DSM44534 were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The biocatalysts were maintained at 4°C on PCM medium agar slants. For use in experiments, they were transferred into conical flasks containing PCM medium composed of sodium chlorine (6 g) (Chempur, Piekary Śląskie, Poland), glucose (20 g) (Chempur), casein (2 g) (Biocorp, Warszawa, Poland), bacteriological peptone (10 g) (Biocorp), and yeast extract (2 g) (Chempur) dissolved in distilled water (1 L) at 25°C, pH 5.5.

Fungistatic activity was determined using *Candida albicans* ATCC 90028 from the American Type Culture Collection (ATCC, Manassas, VA, United States), and clinical isolates, *C. albicans* 636/20, *C. albicans* 595/20, and *C. albicans* 38 obtained from Wrocław Medical University, Wrocław, Poland.

2.2. Materials

Propenylbenzenes: isosafrole (**1a**), prop-1-en-1-yl benzene (**2a**), anethole (**3a**), 1,2-dimethoxy-4-prop-1-en-1-yl benzene (**4a**), and isoeugenol (**5a**) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). All chemicals and solvents were

purchased from Zentek s.r.l. (Milan, Italy) and used without further purification.

2.3. Chemo-enzymatic synthesis

2.3.1. Chemo-enzymatic synthesis of diols as substrates for biotransformations

Vicinal diols 1-(1,3-benzodioxol-5-yl)propane-1,2-diol (**1b**), 1-phenylpropane-1,2-diol (**2b**), 1-(4-methoxyphenyl)propane-1,2-diol (**3b**), 1-(3,4-dimethoxyphenyl)propane-1,2-diol (**4b**), and 1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (**5b**) were obtained as mixtures of two racemic diastereoisomers by chemo-enzymatic synthesis according to the procedure herein exemplified (Tentori et al., 2021).

Propenylbenzenes **1a–5a** (2.96 mmol) were dissolved in EtOAc (15 mL). A 35% w/w aqueous solution of H_2O_2 (382 µL, 4.44 mmol) and Novozym 435 (10 mg) was added to the solution, which was incubated in a thermoshaker at 30°C for 18 h. Then, the enzyme was filtered out and the reaction was quenched first with Na_2SO_3, then with a saturated $NaHCO_3$ solution. Extraction with EtOAc afforded an organic phase that was dried (Na_2SO_4) and concentrated under reduced pressure. The resulting residue was dissolved in MeOH (15 mL), and an excess of KOH (250 mg, 1.5 equiv.) was added to the solution. The reaction was left under magnetic stirring at room temperature for 24 h. The solution volume was reduced to one-third under vacuum, poured into diluted H_2SO_4 solution, and extracted with EtOAc. The organic phase was dried (Na_2SO_4) and concentrated under vacuum. Trituration of the solid residue with hexane/EtOAc (8,2) afforded mixtures of (*R**,*S**)- and (*R**,*R**)-diols **1b–5b**. The structure of the final compounds and the diastereoisomeric ratio, in which they were obtained, were established by GC and NMR analyses.

2.3.2. Tempo-mediated oxidation of diols to corresponding diketones and hydroxy ketones

In order to obtain reference compounds for the identification of products after biotransformation, the following oxidation reaction was implemented. A mixture of corresponding diol **1-4b** (4.9 mmol), TEMPO (8.0 mg, 0.049 mmol) and NaCl (3 mg, 0.049 mmol) in toluene (45 mL) was stirred at 100°C for 4–5 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic phase was dried and concentrated under reduced pressure to give the corresponding diketone and hydroxy ketone derivatives (approximately 1.8 g from a mixture of each diol). The extract obtained in this way was then purified and allowed to obtain GC/MS chromatograms, ^{1}H and ^{13}C NMR spectra of corresponding diketones and hydroxy ketones.

2.4. Whole-cell biotransformations

2.4.1. Screening-scale biotransformations

Forty millilitres of PCM medium was added into 100-mL tapered flasks and sterilized at 121°C under a pressure of 1 atm. The medium was inoculated with 0.5 mL of pre-cultured bacteria at $OD_{600} = 0.3–0.5$. The bacterial cultures were incubated at 22°C under shaking at 150 rpm for 3 days. Then, 0.001 g of diols **1b–5b** dissolved in 0.5 mL of dimethyl sulfoxide (DMSO) was added into the flasks. For simple extraction, ethyl acetate (3 mL) was added to the samples (5 mL) in Falcon tubes and shaken at 200 rpm for 5 min. The organic phase was transferred to a vial and dehydrated with anhydrous $MgSO_4$. Then, it was filtered through a filter paper into a GC vial. Biotransformation was controlled after 3, 7, and 11 days by GC.

2.4.2. Preparative biotransformations

Five hundred millilitres of PCM medium was added into a 2,000-mL Erlenmeyer flask and sterilized at 121°C for 15 min. The medium was inoculated with 5 mL of pre-prepared bacterial cultures at $OD_{600} = 0.3–0.5$. The cultures were incubated at 22°C under shaking at 150 rpm for 3 days. Then, 0.2 g of diols **1b–5b** dissolved in 5 mL of DMSO was added into the cultures. The samples were extracted after 3, 7, 11 days and evaluated by GC to estimate the progress of the biotransformation.

2.5. Evaluation of biological activity

2.5.1. Fungistatic activity assay

All compounds were tested for biological activity against *C. albicans* ATTC 90028, *C. albicans* 636/20, *C. albicans* 595/20, and *C. albicans* 38 using the broth microdilution method. YPD medium (20 g glucose, 20 g Bacto Peptone, 10 g yeast extract, and 1 L of distilled water, pH 6.5) was used for the tests. The compound solutions were prepared in DMSO and diluted in YPD to obtain final concentrations in the range of 10–250 µg/mL. One hundred microlitres of each solution was pipetted into the wells of a 96-well microtiter plate. The inoculum was standardized to 0.5 McFarland standard and then diluted to obtain a final suspension with a cell density of $0.5–2.5 \times 10^3$ CFU/mL. The inoculum size was 100 µL. The positive control comprised DMSO added in the same concentration as the tested compounds in the inoculum, whereas the negative control consisted of DMSO diluted in the broth without the addition of inoculum. The microtiter plates were incubated in a Biosan PST-60 HL Plate Shaker-Thermostat (Riga, Latvia) at 35°C under shaking at 1,000 rpm for 24 h. The fungistatic activity of the compounds was assessed by measuring the absorbance at a wavelength of 595 nm (Epoch; BioTek, Winooski, VT, United States) to determine the MIC_{50} value (i.e., the concentration of a compound required to inhibit the growth of 50% of the microorganisms).

2.5.2. Free-radical scavenging assay

The antiradical activity of all compounds was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test according to a previously described method (Herald et al., 2012; Dudek et al., 2022). One litre of a fresh solution of 190 µM DPPH in pure methanol was prepared in a dark glass bottle. The solution was kept protected from light and used within a week. The tested compounds and ascorbic acid as a positive control were prepared at concentrations in the range of 5–100 µg/mL. Two hundred microlitres of the test compound dilutions was added to each well of a 96-well microtiter plate. Then, 800 µL of a 190 µM solution of DPPH in methanol was added. DPPH was also added to the negative control, in which 200 µL of methanol was added instead of the test compounds. All samples were tested at least in triplicate. The microtiter plates were incubated in the Biosan PST-60 HL thermoshaker at 25°C under shaking at 1,000 rpm for 1 h. The antiradical activity of the compounds was assessed by measuring the

absorbance at 517 nm (Epoch) to determine the EC_{50} value (i.e., the concentration of a compound that gives 50% maximal response). EC_{50} values were calculated using Quest Graph™ EC50 Calculator (AAT Bioquest, Inc.).

2.5.3. Haemolytic activity assay

Haemolytic activity was tested using the method described by Włoch et al. (2020) with minor modifications. This test is based on comparison of the cytotoxicity of all tested compounds in human red blood cells (RBCs) with that in a control group. RBCs for this method were obtained from a blood center from examined and healthy donors. The compounds were tested at 10, 20, 40, 60, 80, and 100 µM concentrations. The control sample contained only ethanol in the same volume as the test samples. The final haematocrit in the test samples was 1.2%. The samples were incubated at 37°C for 1 h. Then, the absorbance at 540 nm was measured using a UV–Vis spectrophotometer (Specord 40, Analytik Jena, Jena, Germany). The haemolytic activity of the compounds was determined as the ratio of the absorbance of haemoglobin in the test samples to that in completely haemolyzed cells multiplied by 100%.

2.5.4. Fluorescence spectroscopy of the RBCs membrane

Using the DPH probe, the anisotropy values of cell membranes of RBCs modified with the test compounds were measured. RBCs for this analysis were prepared according to the method described in subsection 2.5.3. Then, the DPH probe was added to RBCs with a haematocrit of 0.2%. The final concentration of the probe in the sample was 1.3 µM. The mixture was incubated protected from light at 37°C for 30 min. Then, the compounds dissolved in ethanol were added. The compounds were tested at 20, 60, and 100 µM concentrations. Samples with compounds and control samples with alcohol of the appropriate concentration were incubated at 37°C for 1 h. Measurements in triplicate were made in quartz cuvettes using a CARY Eclipse fluorimeter (Varian, San Diego, CA, United States) at 37°C. The excitation wavelength for the DPH probe is $\lambda_{exc} = 360$ nm and the emission wavelength is $\lambda_{em} = 426$ nm. Based on the changes in DPH on the intensity under polarized light, the anisotropy value was determined according to the formula used in our previous publication (Pruchnik et al., 2018).

2.5.5. Cell culture

The human HepG2 and Caco-2 cell lines were obtained from the American Type Culture Collection (HB-8065™ and HTB-37™) and the MG-63 cell line was obtained from the European Collection of Authenticated Cell Cultures (Merck, Poznań, Poland). HepG2 cells were cultured in Dulbecco's modified Eagle's Medium - low glucose (Merck) supplemented with 10% foetal bovine serum (FBS) (Merck). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium - high glucose (Merck) supplemented with 10% FBS, 2% HEPES buffer (Thermo Fisher Scientific, Warszawa, Poland), 1% Penicillin–Streptomycin-Amphotericin B Solution (100×, Merck), 1% MEM solution (100×, Thermo Fisher Scientific), and 1% Gentamycin Solution (10 mg/mL, Merck). MG-63 cells were cultured in Minimum Essential Medium (Merck) supplemented with 10% FBS. All cells were cultured in an atmosphere of 5% CO_2 at 37°C.

2.5.6. Resazurin-based viability assay

HepG2, Caco-2, and MG63 cells were seeded in 96-well plates at 20,000 cells per well, and MG-63 cells at 10,000 cells per well, as described previously (Marycz et al., 2012; Kornicka et al., 2017). The tested compounds were dissolved in ethanol at 10 mg/mL. The concentrations were then adjusted to 1, 50, and 200 µg/mL in culture medium. Four controls were prepared: no ethanol added, and 0.01, 0.5, and 2% EtOH. The cells were treated with the compounds for 24 h. Then, cell viability was evaluated using the resazurin-based assay kit (TOX8), as described previously (Grzesiak et al., 2011; Marycz et al., 2018). The culture medium was replaced with medium containing 10% resazurin dye. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 2 h. Absorbance levels were measured spectrophotometrically (Epoch) at a wavelengths of 600 nm for resazurin and 690 nm as a reference.

2.6. Chemical analysis procedure

Thin-layer chromatography was conducted using aluminium foil plates coated with silica gel. Compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$ and 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4. Gas chromatography (GC; flame ionisation detection, carrier gas H_2) was carried out on a 7,890 N GC system (Agilent Technologies, Santa Clara, CA, United States) equipped with an HP-5 column (30 m × 0.32 mm × 0.25 µm, Agilent Technologies) according to the following temperature program: 70°C, 300°C (30°C/min) (1 min). Samples (2 µL) were injected with split 9:1; the carrier gas flow was 1 mL/min. The total run time was 9.8 min. Retention times (t_R) were established as follow: $t_R = 5.62$ min for (1*R**,2*S**)-**1b** and 5.65 min for (1*R**,2*R**)-**1b**, $t_R = 5.5$ min for **1c**; $t_R = 4.16$ min for (1*R**,2*S**)-**2b** and 4.20 min for (1*R**,2*R**)-**2b**, $t_R = 3.92$ min for **2c**; $t_R = 5.22$ min for (1*R**,2*S**)-**3b** and 5.26 min for (1*R**,2*R**)-**3b**, $t_R = 5.17$ min for **3c**; $t_R = 5.91$ min for (1*R**,2*S**)-**4b** and 5.94 min for (1*R**,2*R**)-**4b**, $t_R = 5.89$ min for **4c**, and $t_R = 5.05$ min for (1*R**,2*S**)-**5b** and (1*R**,2*R**)-**5b**. The structures of the compounds were confirmed using ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra of $CDCl_3$ solutions recorded on Avance DRX 600 (600 MHz) and Avance II (400 MHz) spectrometers (Bruker, Billerica, MA, United States). GC–MS analyses were conducted using a HP-5MS column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Italy) with the following temperature program: 60°C (1 min), 150°C (6°C/min) (1 min), 280°C (12°C/min) (5 min).

The NMR spectra of obtained products are as follows:

1-(1,3-benzodioxol-5-yl)propane-1,2-diol as a 3:1 diastereoisomeric mixture of (1R*,2S*) and (1R*,2R*)-**1b**.

(1R*,2S*)-**1b** ^1H NMR ($CDCl_3$, 400 MHz): δ = 6.86–6.84 (m, 1H, Ar–H), 6.81–6.76 (m, 2H, Ar–H), 5.96 (s, 2H, CH_2), 4.29 (d, 1H, J = 7.4 Hz, CHOH), 3.81 (m, 1H, CHOH), 2.56 (s, 1H, OH), 2.42 (s, 1H; OH), 1.06 (d, 3H, J = 6.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 147.91, 147.5, 135.1, 120.5, 108.3, 107.2, 101.1, 79.4, 72.3, 18.9; GC/MS (EI) tr = 21.23 min: m/z (%) = 196 (M^+, 16), 178 (8), 162 (8), 151 (100), 135 (25), 123 (25).

(1R*,2R*)-**1b** ^1H NMR ($CDCl_3$, 400 MHz): δ = 6.90–6.89 (m, 1H, Ar–H), 6.80–6.78 (m, 2H, Ar–H), 5.95 (s, 2H, CH_2), 4.57 (d, 1H, J = 4.6 Hz, CHOH), 3.96 (m,1H, CHOH), 2.28 (s, 1H, OH), 1.83 (s, 1H; OH), 1.11 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 147.87, 147.3, 134.4, 120.2, 108.2, 107.1, 101.2, 77.5, 71.3, 17.6; GC/

MS (EI) tr = 21.31 min: m/z (%) = 196 (M^+, 16), 178 (8), 162 (8), 151 (100), 135 (25), 123 (25).

1-(1,3-benzodioxol-5-yl)-2-hydroxypropan-1-one **1c** ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.52–7.50 (m, 1H, Ar–H), 7.40–7.42 (m, 1H, Ar–H), 6.86–6.90 (m, 1H, Ar–H), 6.06 (s, 2H, CH_2), 5.05 (q, 1H, J = 7.0 Hz, CHOH), 1.43 (d, 3H, J = 7.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 200.4, 152.6, 148.5, 127.9, 125.2, 108.4, 108.3, 102.2, 69.1, 22.8.; GC/MS (EI) tr = 20.82 min: m/z (%) = 194 (M^+, 11), 149 (100), 121 (21), 91 (4), 65 (15).

1-(1,3-benzodioxol-5-yl)-1-hydroxypropan-2-one GC/MS (EI) tr = 19.95 min: m/z (%) = 194 (M^+, 13), 178 (3), 151 (100), 135 (5), 123 (13), 93 (95), 65 (52).

1-(1,3-benzodioxol-5-yl)propane-1,2-dione ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.64–7.62 (m, 1H, Ar–H), 7.49–7.49 (m, 1H, Ar–H), 6.89–6.87 (m, 1H, Ar–H), 6.07 (s, 2H, CH_2), 2.49 (s, 3H, CH_3);

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 200.6, 189.4, 153.0, 148.2, 127.8, 126.2, 108.8, 108.1, 102.0, 26.3.; GC/MS (EI) tr = 19.21 min: m/z (%) = 192 (M^+, 6), 149 (100), 121 (31), 91 (6), 65 (19).

1-phenylpropane-1,2-diol as a 1.25:1 diastereoisomeric mixture of (1R*,2S*) and (1R*,2R*)-**2b**.

(1R*,2S*)-**2b** ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.39–7.27 (m, 5H, Ar–H), 4.38 (dd, 1H, J^1 = 7.3 Hz, J^2 = 2.7 Hz, CHOH), 3.91–3.82 (m, 1H, CHOH), 2.63–2.62 (m, 1H, OH), 2.47–2.46 (m, 1H, OH), 1.07 (d, 3H, J = 6.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 128.4, 128.0, 126.6, 125.6, 59.5, 59.0, 17.9.; GC/MS (EI) tr = 14.40 min: m/z (%) = 134 ($M^+ - 18$, 3), 134 (3), 117 (7), 108 (100), 103 (2), 91 (17), 79 (93).

(1R*,2R*)-**2b** ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.38–7.33 (m, 5H, Ar–H), 4.69–4.67 (m, 1H, CHOH), 4.06–3.97 (m, 1H, CHOH), 2.38–2.37 (m, 1H,OH), 1.88–1.87 (m, 1H, OH), 1.09 (d, 3H, J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 128.4, 128.0, 126.6, 125.6, 59.5, 59.0, 17.9.; GC/MS (EI) tr = 14.60 min: m/z (%) = 134 ($M^+ - 18$, 3), 134 (3), 117 (13), 108 (100), 103 (3), 91 (18), 79 (93).

2-hydroxy-1-phenylpropan-1-one **2c** ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.93–7.91 (m, 2H, Ar–H), 7.93–7.91 (m, 1H, Ar–H), 7.50–7.47 (m, 2H, Ar–H), 5.19–5.14 (q, 1H, J = 7.0 Hz, CHOH), 1.24 (s, 1H, OH), 1.21 (d, 3H, J = 9.5 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 202.5, 134.1, 133.7, 130.3, 128.9, 128.8, 128.6, 69.4, 13.3. GC/MS (EI) tr = 13.07 min: m/z (%) = 135 ($M^+ - 15$, 1)*, 135 (1), 105 (100), 77 (43), 51 (12).*

1-hydroxy-1-phenylpropan-2-one GC/MS (EI) tr = 12.34 min: m/z (%) = 150 (M^+, 2), 107 (100), 89 (2), 79 (85).

1-phenylpropane-1,2-dione ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.02–8.00 (m, 2H, Ar–H), 7.66–7.60 (m, 1H, Ar–H), 7.52–7.48 (m, 2H, Ar–H)*, 2.53 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz):* δ = 200.5, 191.4, 134.6, 131.8, 130.3, 128.9, 26.4.; GC/MS (EI) tr = 11.23 min: m/z (%) = 148 (M^+, 4), 105 *(100), 77 (71), 51 (20).*

1-(4-methoxyphenyl)propane-1,2-diol as a 3:1 diastereoisomeric mixture of (1R*,2S*) and (1R*,2R*)-**3b**.

(1R*,2S*)-**3b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 7.30–7.25 (m, 2H, Ar-H), 6.91–6.87 (m, 2H, Ar-H), 4.33 (dd, 1H, J^1 = 7.7 Hz, J^2 = 2.9 Hz, CHOH), 3.89–3.82 (m, 1H, CHOH), 3.81 (s, 3H, OCH_3), 2.47–2.42 (m, 2H, OH), 1.05 (d, 3H, J = 7.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 159.5, 146.6, 133.2, 128.0, 114.0, 79.2, 72.3, 18.8.; GC/MS (EI) tr = 19.75 min: m/z (%): 182 (M^+, 4), 164 (6), 137 (100), 121 (35), 109 (20), 94 (17).

(1R*,2R*)-**3b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 7.30–7.25 (m, 2H, Ar-H), 6.91–6.87 (m, 2H, Ar-H), 4.61–4.59 (m, 1H, CHOH), 4.01–3.97 (m, 1H, CHOH), 3.81 (s, 3H, OCH_3), 2.21–2.20 (m, 1H, OH), 1.79–1.78 (m, 1H, OH), 1.11 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 159.5, 146.6, 133.2, 128.0, 114.0, 79.2, 72.3, 18.8.; GC/MS (EI) tr = 19.83: m/z (%): 182 (M^+, 3), 164 (6), 137 (100), 121 (37), 109 (19), 94 (17).

2-hydroxy-1-(4-methoxyphenyl)propan-1-one **3c** ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.51–7.49 (m, 2H, Ar–H), 6.92–6.88 (m, 2H, Ar–H), 5.16–5.06 (m, 1H, CHOH), 3.93 (s, 3H, OCH_3), 1.43 (d, 3H, J = 7.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 201.2, 164.1, 132.9, 132.4, 131.1, 114.2, 113.8, 69.0, 55.6, 22.8; GC/MS (EI) tr = 19.96: m/z (%): 180 (M^+, 3), 135 (100), 107 (9), 92 (10), 77 (14).

1-hydroxy-1-(4-methoxyphenyl)propan-2-one GC/MS (EI) tr = 18.79: m/z (%): 180 (M^+, 3), 137 (100), 109 (25), 94 (25), 77 (23).

1-(4-methoxyphenyl)propane-1,2-dione ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.03–8.00 (m, 2H, Ar–H), 6.98–6.94 (m, 2H, Ar-H), 3.89 (s, 3H, OCH_3), 2.50 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 201.1, 190.0, 164.8, 132.8, 124.7, 114.2, 55.6, 26.5.; GC/MS (EI) tr = 17.63 min: m/z (%) = 178 (M^+, 2), 135 (100), 107 (11), 92 (16), 77 (22).

1-(3,4-dimethoxyphenyl)propane-1,2-diol as a 3:1 diastereoisomeric mixture of (1R*,2S*) and (1R*,2R*)-**4b**.

(1R*,2S*)-**4b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 6.94–6.84 (m, 3H, Ar-H), 4.32–4.30 (d, 1H, CHOH), 3.89 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.85–3.81 (m, 1H, CHOH), 2.69 (s, 1H, OH), 2.54 (s, 1H, OH), 1.06 (d, 3H, J = 6.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 149.1, 148.9, 133.7, 119.3, 119.1, 111.1, 109.8, 79.3, 72.2, 55.9, 18.8.; GC/MS (EI) tr = 22.22 min: m/z (%) = 212 (M^+, 2), 194 (24), 178 (9), 167 (12), 151 (100), 139 (8).

(1R*,2R*)-**4b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 6.84–6.82 (m, 3H, Ar-H), 4.59–4.58 (m, 1H, CHOH), 3.89 (s, 3H, OCH_3), 4.01–3.95 (m, 1H, CHOH), 3.87 (s, 3H, OCH_3), 2.41 (s, 1H, OH), 1.91 (s, 1H, OH), 1.12 (d, 3H, J = 6.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 149.0, 148.9, 133.1, 119.1, 111.0, 109.8, 77.5, 71.4, 55.9, 17.5; GC/MS (EI) tr = 22.22 min: m/z (%) = 212 (M^+, 2), 194 (24), 178 (9), 167 (12), 151 (100), 139 (8).

1-(3,4-dimethoxyphenyl)-2-hydroxypropan-1-one **4c** ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.52–7.49 (m, 2H, Ar–H), 6.91–6.89 (m, 1H, Ar–H), 5.14–5.08 (m, 1H, CHOH), 3.94 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 1.43 (d, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 200.9, 154.1, 149.4, 126.2, 123.5, 110.8, 110.2, 68.9, 56.2, 56.1, 23.0.; GC/MS (EI) tr = 22.20 min: m/z (%) = 210 (M^+, 9), 192 (2), 165 (100), 151 (5), 137 (9), 122 (6).

1-(3,4-dimethoxyphenyl)-1-hydroxypropan-2-one GC/MS (EI) tr = 21.20 min: m/z (%) = 210 (M^+, 6), 192 (7), 167 (100), 151 (22), 139 (61), 124 (23).

1-(3,4-dimethoxyphenyl)propane-1,2-dione ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.67–7.65 (m, 1H, Ar–H), δ = 7.58–7.76 (m, 1H, Ar–H), 6.92–6.90 (m, 1H, Ar–H), 3.97 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 2.51 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 200.9, 190.0, 154.7, 149.3, 126.6, 124.6, 111.0, 110.2, 56.0, 55.9, 26.5.; GC/MS (EI) tr = 21.00 min: m/z (%) = 208 (M^+, 3), 165 (100), 137 (9), 122 (8).

1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol as a 3:1 diastereoisomeric mixture of (1R*,2S*) and (1R*,2R*)-**5b**.

(1R*,2S*)-**5b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 6.94–6.87 (m, 3H, Ar-H), 5.62 (s, 1H, Ar-OH), 4.31 (d, 1H, J = 7.5 Hz, CHOH), 3.90 (s, 3H, OCH_3), 3.87–3.81 (m, 1H, CHOH), 1.06 (d, 3H, J = 6.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 146.7, 145.6, 133.0, 120.0, 114.3,

109.0, 79.4, 72.3, 56.0, 18.9.; GC/MS (EI) tr = 21.71 min: m/z (%) = 198 (M^+, 12), 180 (9), 153 (100), 137 (40), 125 (19).

(1*R**,2*R**)-**5b** ^1H NMR ($CDCl_3$, 400 MHz) δ = 6.84–6.80 (m, 3H, Ar-H), 5.62 (s, 1H, Ar-OH), 4.57 (d, 1H, *J* = 4.8 Hz, CHOH), 3.99–3.96 (m, 1H, CHOH), 3.90 (s, 3H, OCH_3), 1.12 (d, 3H, *J* = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 146.0, 145.4, 132.4, 119.9, 114.2, 109.2, 77.6, 71.4, 56.0, 17.6.; GC/MS (EI) tr = 21.71 min: m/z (%) = 198 (M^+,12), 180 (9), 153 (100), 137 (40), 125 (19).

2.7. Statistical analysis

The biotransformation and biological activity experiments were performed in triplicate, and the data are presented in tables and figures as means and standard deviations. Student's t-test was used to compare the means. Differences with $p < 0.05$ were considered significant. Statistical analyses were performed using Past 4.02 (Oyvind Hammer).

3. Results and discussion

In this research, we focused on propenylbenzenes **1a–5a** (Figure 1), as these compounds have been reported to possess various biological activities, such as antimicrobial, antioxidant, and antiproliferative activities (Moradi et al., 2014; Siva et al., 2019; Bruna et al., 2022; Lal et al., 2022). Taking this into consideration, several derivatives of these compounds were obtained in order to study the influence of their structure on biological activity.

The two-step biocatalytic synthesis (Figure 2) of oxygenated propenylbenzene derivatives involved (1) chemo-enzymatic epoxidation followed by hydrolysis of the starting compounds **1a–5a** to the corresponding diols **1b–5b** and (2) microbial oxidation of the diols **1b–5b** into hydroxy ketones **1c–4c**. The thus-obtained propenylbenzenes derivatives were tested for their antimicrobial, antioxidant, haemolytic, and anticancer activities and their impact on membrane fluidity.

3.1. Chemo-enzymatic synthesis of diols

In the first step, compounds **1a–5a** were converted into the corresponding diols accordingly to the method recently described by Tentori et al. (2021) for isosafrole (**1a**). This method is based on the Prilezhaev reaction of isosafrole with a peroxycarboxylic acid obtained *in situ* by lipase-catalysed perhydrolysis of the corresponding carboxylic acid in the presence of H_2O_2. We used aqueous H_2O_2 and commercial immobilized lipase B from *Candida antarctica* (Novozym 435) based on a report by Björkling et al. (1990). We modified this method, using ethyl acetate, which undergoes lipase-mediated perhydrolysis, resulting in the formation of ethanol and peroxyacetic acid. This avoided the addition of octanoic acid and other carboxylic acids to promote the reaction. However, after applying this method to compound **1a**, we noticed that diol **1b** was formed only in small amounts, whereas the dominant products were epoxide and monoacetate derivatives. Therefore, methanolic KOH was added to the compound mixture, which allowed for complete oxirane ring cleavage and promoted monoacetate hydrolysis. As a result of this reaction, epoxide and the monoacetate derivatives were readily converted into vicinal diol **1b**, with a high yield (69%). Considering these results, we decided to apply this method also to the other structurally similar propenylbenzenes **2a–5a**, which allowed to obtain corresponding diols **2b–5b** for use as substrates in biotransformation processes. As a result of implementing the aforementioned method, a diasteroisomeric mixture of (*R**,*S**)- and (*R**,*R**)-diols **1b–5b** was obtained, with high isolation yields in the range of 65–80%. Compared to other methods described in the literature, the method we proposeis highly efficient, using an immobilized biocatalyst that can be easily recovered from the reaction by filtration, and employing cheap H_2O_2 as an oxidant. Other enzymatic methods described so far (Kihumbu et al., 2002; Oeggl et al., 2018) were aimed at obtaining pure enantiomers of selected propenylbenzenes, whereas in our method we focused on obtaining diols as racemic diastereoisomers to be submitted to further microbial transformations.

3.2. Biotransformations of diols

Screening-scale whole-cell transformations of diols **1b–5b** were conducted using 22 bacterial strains belonging to the genera *Bacillus*, *Dietzia*, *Gordonia*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, and *Streptomyces*. In biotransformations with diols **1b–4b**, corresponding hydroxy ketones **1c–4c** were formed as the main products. In addition, small amounts of diketones and isomeric forms of hydroxy ketones were detected in the reaction mixture (data are shown in the Supplementary Tables S1–S4). The most efficient biocatalysts (*Dietzia* sp. DSM44016, *R. erythropolis* PCM2150, *R. erythropolis* DSM44534, and *R. ruber* PCM2166) in the screening-scale experiments are listed in Table 1. In all biotransformations, the amount of hydroxy ketones **1c–4c** (determined by GC) clearly increased over time up to day 11. The highest percentage of product **1c** (85%) was observed in the biotransformation with *Dietzia* sp. DSM44016. In contrast, hydroxy ketones **2c** and **3c** were the most efficiently produced in the biotransformation with *R. ruber* PCM2166, and a high amount of product **4c** (77–79%) was obtained in the biotransformations conducted by *R. erythropolis* DSM44534 and *R. ruber* PCM2166. All strains mentioned effectively produced

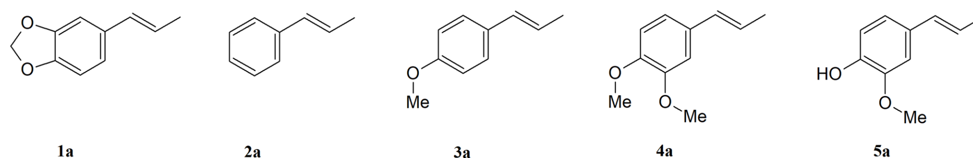

FIGURE 1
Structures of the studied propenylbenzenes **1a–5a**.

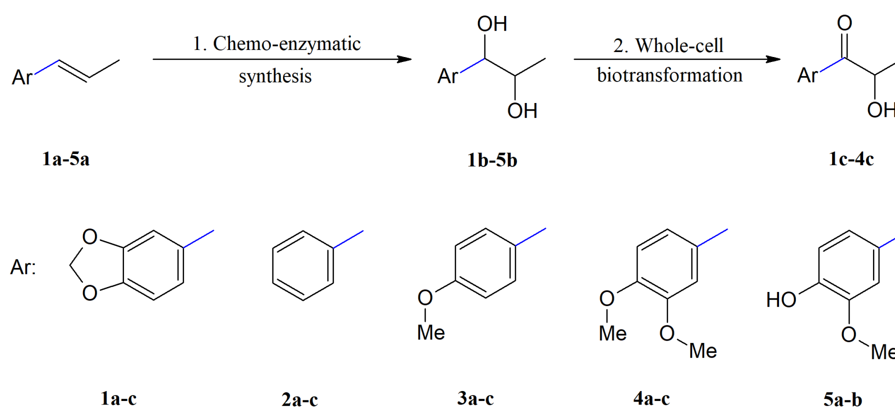

FIGURE 2
Two-step synthesis of propenylbenzene derivatives: diols **1b–5b** and hydroxy ketones **1c–4c**. 1. aq. H_2O_2, Novozym 435, EtOAc, 30°C, 18h, KOH, MeOH; 2. PCM medium, 23°C, 3–11days.

TABLE 1 Selected results of the screening-scale biotransformations of diols 1b–4b.

Strain	Time (days)	1c (%)	2c (%)	3c (%)	4c (%)
Dietzia sp. DSM44016	3	48 (±2.1)	45 (±4.1)	12 (±1.5)	39 (±3.6)
	7	66 (±3.3)	70 (±2.9)	41 (±1.9)	58 (±4.7)
	11	85 (±3.9)	74 (±3.4)	74 (±4.3)	69 (±1.9)
Rhodococcus erythropolis PCM2150	3	30 (±3.5)	19 (±1.0)	18 (±2.6)	25 (±3.3)
	7	46 (±4.2)	46 (±2.4)	27 (±3.1)	51 (±4.2)
	11	63 (±2.9)	67 (±4.1)	38 (±3.0)	71 (±2.1)
Rhodococcus erythropolis DSM44534	3	53 (±3.3)	24 (±1.9)	27 (±3.2)	27 (±1.7)
	7	72 (±1.3)	52 (±3.4)	61 (±3.0)	52 (±2.4)
	11	73 (±3.6)	70 (±2.1)	82 (±2.0)	79 (±2.9)
Rhodococcus ruber PCM2166	3	40 (±3.1)	24 (±2.1)	38 (±4.2)	34 (±1.8)
	7	45 (±4.2)	48 (±2.8)	64 (±3.1)	51 (±2.5)
	11	62 (±2.6)	87 (±3.3)	88 (±2.1)	77 (±2.0)

%, determined by GC.

hydroxy ketones, except for the biotransformation of compound **3b** with strain *R. erythropolis* PCM2150, after which only 38% hydroxy ketone **3c** was detected. Due to the small differences in biotransformations with these bacteria, all strains were selected for preparative scale biotransformations, which allowed to isolate products and determine their structures by NMR analysis. Biotransformation of diol **5b** did not yield hydroxy ketones or other products; therefore, this substrate was not studied on a preparative scale.

When planning the scale-up process, we decided to add substrates **1b–4b** in an amount of 0.2 g each and to conduct the biotransformation process for 11 days (Table 2). During preparative-scale biotransformation of substrate **1b**, the highest amount of product **1c** (0.125 g, yield = 62.5%) was obtained using *Dietzia* sp. DSM44016. In the course of biotransformation of diol **2b**, only *R. ruber* PCM2166 catalysed the reaction, affording 0.081 g of **2c** (yield = 40.5%). The high concentration of substrate **2b** had an inhibitory effect on all other strains, and only 10–24% of hydroxy ketone **2c** and unreacted substrate were detected in the samples. The highest amount of hydroxy ketone **3c** (0.115 g, yield = 57.5%) was obtained with *R. erythropolis* DSM44534. Finally, *R. erythropolis* PCM2150 yielded 0.119 g (59.5%) of product **4c**.

During the preparative-scale biotransformations, we noticed that an increase in the substrate concentration significantly affected the course of the process with selected bacterial strains. In the case of *Dietzia* sp. DSM44016, the highest yields were achieved in the biotransformation of substrate **1b**, which has a dioxolane group in its structure, whereas hydroxy ketones **3c** and **4c** (with one and two methoxy groups, respectively) were obtained with lower yields. However, diol **2b** (without additional substituent) was toxic to this strain and only small amount of product **2c** was detected by GC. These findings indicated that the dioxolane, methoxy, and dimethoxy groups reduce the toxicity of diols derived from propenylbenzenes on *Dietzia* sp. DSM44016. Biotransformation with *R. ruber* PCM2166 yielded a significantly smaller amount of compound **2c**, and with substrates **1b** and **3b–4b**, no products were formed. This indicates that *R. ruber* PCM2166 accepts only propenylbenzene diol derivatives without aromatic ring substituents. Biotransformations with *R. erythropolis* DSM44534 and *R. erythropolis* PCM2150 were characterized by a similar course of bio-oxidation, which is not surprising considering

TABLE 2 Summary of the biotransformations of propenylbenzenes 1a–4a performed on a preparative scale after 11days.

Strain	Substrate	Product	Isolation yield (%)	Amount of product (g)
Dietzia sp. DSM44016	**1b**	**1c**	62.5	0.125
R. erythropolis PCM2150			45.5	0.091
R. erythropolis DSM44534			36	0.072
R. ruber PCM2166	**2b**	**2c**	40.5	0.081
Dietzia sp. DSM44016	**3b**	**3c**	44.5	0.089
R. erythropolis PCM2150			36.5	0.073
R. erythropolis DSM44534			57.5	0.115
Dietzia sp. DSM44016	**4b**	**4c**	48.5	0.097
R. erythropolis PCM2150			59.5	0.119
R. erythropolis DSM44534			42.5	0.085

that they belong to the same species. These microorganisms effectively catalysed the oxidation of diols **1b** and **3b–4b** to corresponding hydroxy ketones **1c** and **3c–4c**. The high concentration of substrate **2b** had an inhibitory effect on these strains, as observed with *Dietzia* sp. DSM44016.

Several methods for the synthesis of hydroxy ketones **1c–4c** have been reported (Kihumbu et al., 2002; Peng et al., 2005; Kurlemann et al., 2009). In one method using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and ultrasound waves, compound **1c** was obtained with a 72% yield (Peng et al., 2005). Enzymatic reaction with benzaldehyde lyase or benzoylformate decarboxylase afforded hydroxy ketone **2c** with a yield of 95% (Kihumbu et al., 2002). Hydroxy ketone **3c** (yield = 81–84%) was obtained in a two-step process involving oxidation of *trans*-anethol using *Trametes hirsuta* lyophilisate followed by ligation of *para*-anisaldehyde with acetaldehyde using benzaldehyde lyase or benzoylformate decarboxylase (Kurlemann et al., 2009). The biocatalytic method presented in this paper is an attractive alternative to the methods described in the literature. It does not require expensive reagents and enzymes and allows to work starting from propenylbenzenes, most of which can be recovered from renewable feedstocks (natural essential oils and plants extracts).

3.3. Fungistatic activity

The fungistatic activities of propenylbenzenes **1a–5a** have been described in the literature (Moradi et al., 2014; Siva et al., 2019; Bruna et al., 2022; Lal et al., 2022). The MIC values of these compounds range from 100 to 400 μg/mL, with the lowest value reported for isosafrole (**1a**) and the highest for isoeugenol (**5a**) and its derivative **4a** (Kubo et al., 1993). These studies were conducted using *C. albicans* ATTC18804, and it should be noted that MIC_{50} values were not calculated. Additionally, studies using the disc diffusion method for *C. albicans* ATCC 66027 and the microdilution method (with a different cell density) for *C. albicans* ATCC 22019 showed that anethole (**3a**) and essential oil containing isosafrole (**1a**) have antifungal activity (Moradi et al., 2014; Bruna et al., 2022; Lal et al., 2022). The antimicrobial activities of isosafrole (**1a**), anethol (**3a**), and isoeugenol (**5a**) have been reported; however, antimicrobial activities of their derivatives, such as diols and hydroxy ketones, have not been reported. Therefore, we assessed how the structures of these compounds affect their biological activity (Moradi et al., 2014; Siva et al., 2019; Bruna et al., 2022; Lal et al., 2022). In our previous studies on fungistatic activity, we used yeasts of the genus *Candida* (Gach et al., 2021; Krężel et al., 2022) therefore, we used *C. albicans* strains as the test model in the current study.

Isosafrole (**1a**) efficiently inhibited the growth of *C. albicans* strains 636/20, 595/20, 38, and ATTC90028, with MIC_{50} values below 100 μg/mL (Figure 3A). The highest inhibitory activity (MIC_{50} = 65 μg/mL) of this compound was noted for *C. albicans* 595/20. The fungistatic activity of dihydroxy derivative **1b** was decreased compared to that of the starting compound **1a**. The MIC_{50} values of diol **1b** for *C. albicans* 595/20 and 636/20 were 107 and 177 μg/mL, respectively. Compared to those of **1a** and **1b**, the inhibitory activity of hydroxy ketone **1c** against *C. albicans* strains 595/20, 38, and ATTC90028 was substantially decreased (MIC_{50} = 178–200 μg/mL). Compound **1c** showed no inhibitory activity against *C. albicans* 636/20, even at a concentration of 250 μg/mL. The presence of hydroxy and carbonyl groups in compounds **1b–c** suppressed their fungistatic activity against all tested strains, whereas the presence of two hydroxy groups in compounds **2a–c** increased their fungistatic activity against all tested strains (Figure 3B). Compared to **2a** and **2c**, diol **2b** showed the highest inhibitory activity against all tested strains, with the lowest noted for *C. albicans* 636/20 (MIC_{50} = 121 μg/mL) and ATTC90028 (MIC_{50} = 125 μg/mL). The growth of *C. albicans* 38 and ATTC90028 was not affected by compounds **2a** and **2c**, even at concentrations above 250 μg/mL. Anethole (**3a**) exhibited significant inhibitory activity against all tested strains, with MIC_{50} values of 62–100 μg/mL and the highest activity noted for *C. albicans* 595/20 (MIC_{50} = 62 μg/mL) (Figure 3C). Diol **3b** showed increased fungistatic activity against *C. albicans* 595/20, 636/20, and 38 (MIC_{50} = 47–76 μg/mL), with the lowest MIC_{50} of 47 μg/mL for *C. albicans* 595/20. Hydroxy ketone **3c** showed increased fungistatic activity against *C. albicans* 38 (MIC_{50} = 61 μg/mL). Compounds **3a–c** showed variable fungistatic activity against the tested strains; therefore, the impacts of their structures on fungistatic activity were difficult to determine. Among compounds **4a–c**, **4a** (MIC_{50} = 39–67 μg/mL) and **4b** (MIC_{50} = 37–63 μg/mL) showed the highest fungistatic activity against all strains, with diol **4b** showing higher activity against *C. albicans* 636/20, 595/20, and ATTC90028 (Figure 3D). The presence of a carboxyl group in compound **4c** substantial suppressed its fungistatic activity. Isoeugenol (**5a**) showed substantially higher fungistatic

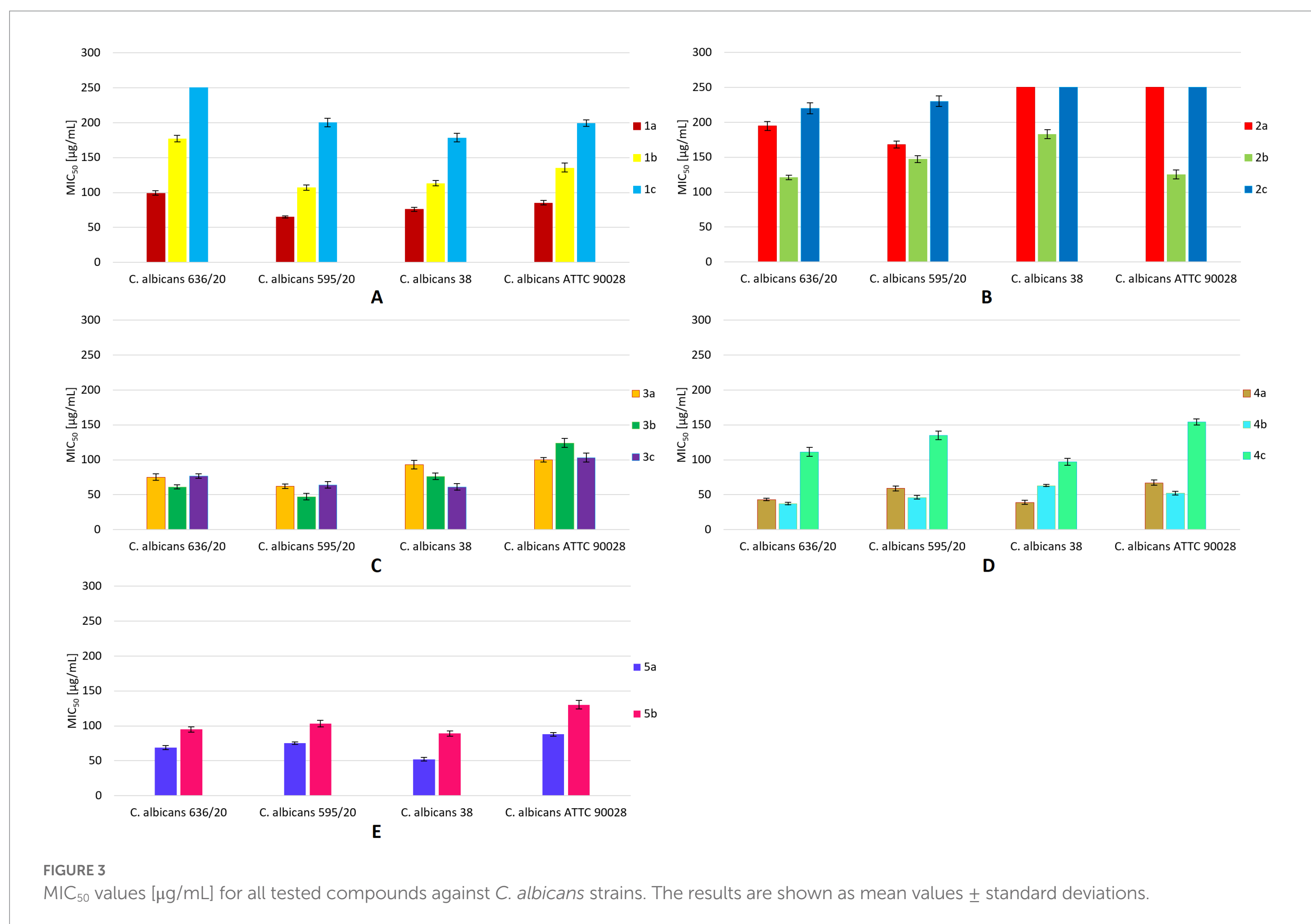

FIGURE 3
MIC_{50} values [µg/mL] for all tested compounds against *C. albicans* strains. The results are shown as mean values ± standard deviations.

activity than its diol derivative **5b** against all four strains (MIC_{50} = 52–88 µg/mL) (Figure 3E). By comparing the various compounds and their derivatives, we noticed that the presence of additional substituents in the aromatic ring of the starting propenylbenzenes **1a**, **3a**, **4a**, and **5a** had a positive effect on their fungistatic activity when compared to that of **2a**. The presence of hydroxy groups in diols **2b–4b** had a positive effect on their inhibitory activity against most strains tested when compared with that of the starting compounds **2a–4a**. All hydroxy diols, except **3c**, tended to be less active than the starting compounds and diols.

3.4. Antiradical activity

The antiradical activities of some propenylbenzenes or essential oils containing them have been reported in the literature. Eid and Hawash (2021) reported that safrole (the isomer of compound **1a**) exhibits antioxidant activity, with an IC_{50} value of 50.28 µg/mL. A study on the antiradical activity of isosafrole (**1a**) in essential oils containing a certain amount of this compound (19.5%) revealed that such mixtures of different compounds exhibit IC_{50} values >1,000 µg/mL (Bruna et al., 2022). For anethol (**3a**), an EC_{50} value of 8.69 has been reported (Lal et al., 2022). According to Siva et al. (2019), the antioxidant activity of isoeugenol (**5a**) was 86%.

The antioxidant activities of all compounds were compared with that of the standard, ascorbic acid, to estimate their free radical-scavenging power. The lowest EC_{50} value was noted for ascorbic acid (15.21 µg/mL), which was used as a positive control (Figure 4). Among the tested compounds, those with a double bond in the propenyl group had the lowest EC_{50} values. Among these, anethole (**3a**) showed the highest antiradical activity (EC_{50} = 19.13 µg/mL). Significantly lower antioxidant activity was observed for diols **1b–5b** and hydroxy ketones **1c–4c**, with EC_{50} values ranging from 36.34 to 72.08 µg/mL. Among the diols, **3b** showed the highest antiradical activity. All hydroxy ketones showed lower activity than the starting compounds and diols.

3.5. Haemolytic activity

The degree of cytotoxicity of the compounds was evaluated using a haemolytic activity assay, in which the haemolytic activity of 13 compounds at 10–100 µM on human RBCs was evaluated. According to toxicity classification, compounds are highly toxic if the haemolysis rate is 90–100% and nontoxic if the haemolysis rate is 0–9% (Pagano and Faggio, 2015). Data shown in the Supplementary Table S5 show the percentage of haemolysis after a 1 h incubation with the compounds at various concentrations at 37°C. The percentage of haemolysis was similar to that of the control for all compounds and did not exceed 3%; therefore, oxygenated derivatives of propenylbenzenes do not cause haemolysis of RBCs. Our results indicate that the compounds in the range of used concentrations do not have a toxic effect on human red blood cells.

The results obtained for compounds **1a** and **5a** are in agreement with the results of other authors, in which it was shown that

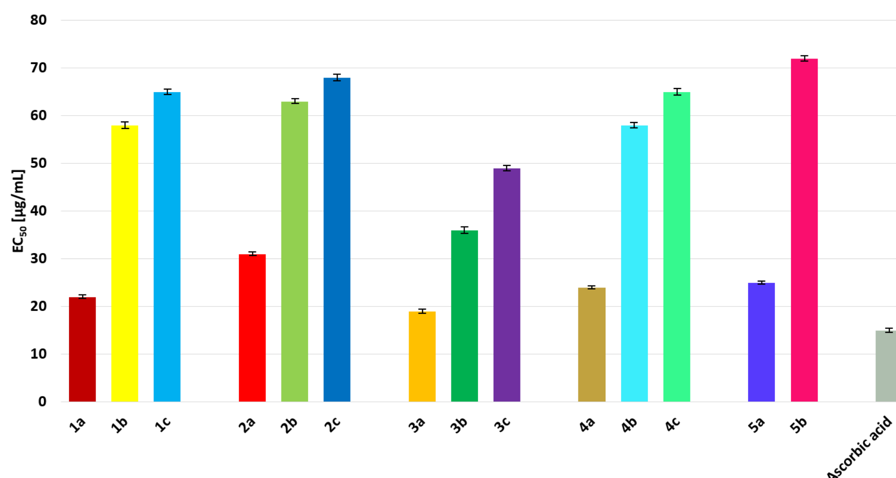

FIGURE 4
EC_{50} values [µg/mL] for all tested compounds in comparison to ascorbic acid. The results are shown as mean values ± standard deviations.

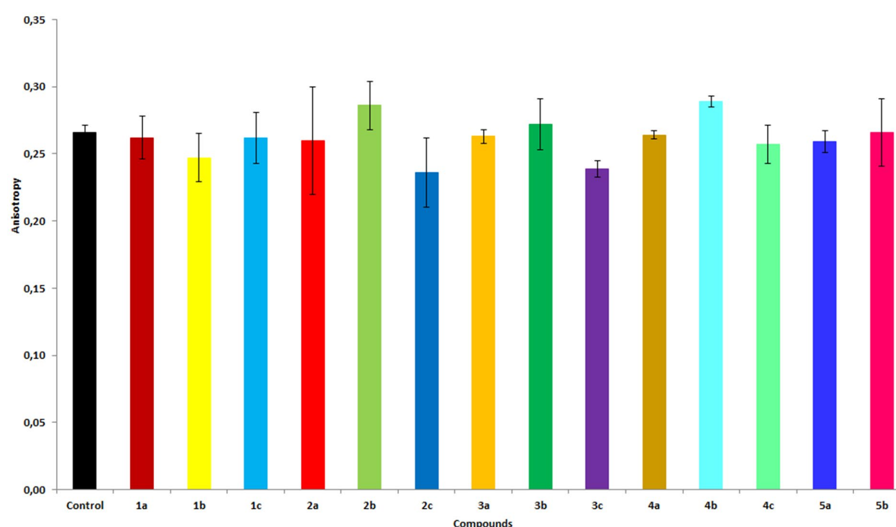

FIGURE 5
Fluorescence anisotropy values of the DPH probe in membranes of RBCs treated with the compounds at 20µM concentration. The results are shown as mean values ± standard deviations.

derivatives of safrole or eugenol present negligible hemolytic capacity (Hidalgo and De la Rosa, 2009; Madrid et al., 2014). However, all derivatives of safrole exhibited haemolytic activity (minor than 10%), and for the derivatives of eugenol activity lower than 1% was detected, which indicate their non-toxicity. Other authors have also shown that isoeugenol has low cytotoxic activity (Bhatia et al., 2011). Due to the fact that compounds **1-5b** and **1-4c** were described for the first time, the results obtained for these compounds are novel.

3.6. Effects of the compounds on RBCs membrane fluidity

The effect of each compound on the membrane fluidity of RBCs was examined using the fluorescent marker DPH. The DPH probe binds to the hydrophobic region of the membrane. Based on the change in anisotropy, one can infer the degree of change in the membrane fluidity of RBCs (Lakowicz, 2006). An increase in the anisotropy value indicates an increase in membrane stiffness, whereas a decrease suggests liquefaction of the membrane (complete data are shown in the Supplementary Table S6). In general, diols, particularly **2b** and **4b**, were found to cause an increase in anisotropy (Figure 5). Interestingly, compound **3b** caused an increase in anisotropy at lower concentrations and a decrease at higher concentrations. In contrast, hydroxy ketones **2c**, **3c**, and, to a lesser extent, **4c** caused a decrease in anisotropy, indicating an increase in membrane fluidity. The biological activity of the studied compounds likely is also related to their different effects on the cell membrane; depending on their structure, they cause an increase in membrane stiffness or membrane liquefaction. Determining the exact localization of the compounds in the membrane requires further research.

3.7. Proliferative activity

The proliferative activities of HepG2, Caco-2, and MG63 cells were differentially affected by compounds (**1a–5b**), in a dose-dependent manner (complete data are shown in the Supplementary Figures S27–S29). Compound **3b** significantly increased the proliferative potential of HepG-2 cells, whereas compound **2b** showed the highest inhibitory effect on HepG2 cell viability (Figure 6). A substantially different cellular response to the compounds was observed in the Caco-2 cell line. Caco-2 cells exhibited the lowest proliferative activity when treated with all tested compounds among all others tested cell lines. The strongest inhibition of Caco-2 cells was observed when the cells were treated with **1b**, **2b**, **3b**, and **4b**. Interestingly, **1a** and **2a** more strongly significantly suppressed the viability of HepG-2 cells. Compound **5b** at 1 μg/mL induced the proliferative activity of all cell lines to a similar level as compound **3c** did at 50 μg/mL. The proliferation of MG63 cells was differentially modulated by the compounds, depending on the dosage. Compounds **1c**, **3b**, and **5b** suppressed the proliferation of MG63 cells. However, at 50 μg/mL, **5b** significantly increased cell viability.

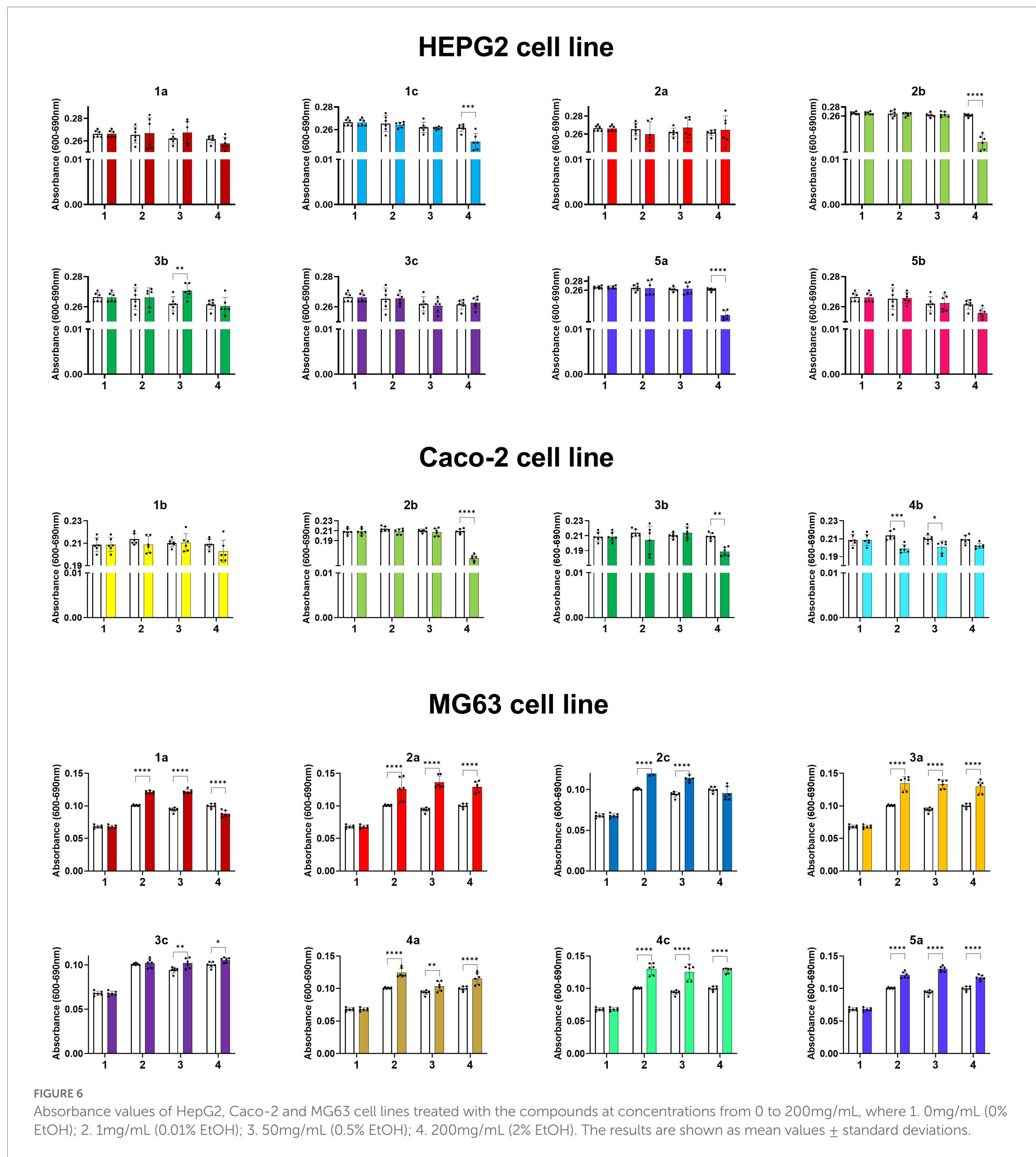

FIGURE 6
Absorbance values of HepG2, Caco-2 and MG63 cell lines treated with the compounds at concentrations from 0 to 200mg/mL, where 1. 0mg/mL (0% EtOH); 2. 1mg/mL (0.01% EtOH); 3. 50mg/mL (0.5% EtOH); 4. 200mg/mL (2% EtOH). The results are shown as mean values ± standard deviations.

Interestingly, compounds **1a**, **2a**, **2c**, **3a**, **3c**, **4a, 4c**, and **5a** promoted MGC63 proliferation.

The results obtained by us for starting propenylbenzenes **1-5a** are in agreement with the available literature. Anethol (**3a**), safrole and eugenol (isomers of compounds **1a** and **5a**) inhibited the growth of human hepatocellular carcinoma HepG2 cells (Yoo et al., 2005; Song et al., 2014, 2020). Whereas, isosafrole (**1a**) and eugenol induced apoptosis in human colon carcinoma Caco-2 cell line (Lea et al., 2016; Padhy et al., 2022). In human osteosarcoma MG63 cell line, *trans*-anethole (**3a**) caused abrogation in proliferation and induces apoptosis through the mitochondrial mediated pathway (Pandit et al., 2020). The effect of compounds **1-5b** and **1-4c** on the cell lines we studied has not been described in the available literature so far.

4. Conclusion

A two-step chemo-enzymatic method for obtaining oxygenated derivatives of commercially available propenylbenzenes was developed. The process involves chemo-enzymatic epoxidation followed by epoxide hydrolysis of starting compounds **1a–5a** to corresponding diols **1b–5b**, followed by microbial oxidation of thus obtained diols **1b–5b** into hydroxy ketones **1c–4c**. Among bacteria from different genera, *Dietzia* sp. DSM44016, *R. erythropolis* DSM44534, *R. erythropolis* PCM2150, and *R. ruber* PCM2166 effectively oxidized diols **1b–4b** to corresponding hydroxy ketones **1c–4c**, indicating high alcohol dehydrogenase activity. Bio-oxidation performed on preparative scale afforded hydroxy ketones **1c–4c** with good isolation yields. The obtained compounds and starting propenylbenzenes were tested for antimicrobial, antioxidant, haemolytic, and anticancer activities, and their impact on membrane fluidity. Compounds **1a**, **3a–c**, **4a,b**, and **5a,b** showed high fungistatic activity against selected strains of *C. albicans*. The type of substituent can significantly affect the MIC_{50} value. The starting propenylbenzenes **1a–5a** with a double bond in their structure showed the highest antiradical activity among the tested compounds. The compounds showed no cytotoxicity against human RBCs. However, compounds **2b–4b** and **2c–4c** affected the fluidity of the RBCs membrane; diols generally increased membrane stiffness, whereas hydroxy ketones increased membrane fluidity. Several compounds inhibited or promoted HepG2, Caco-2, and MG63 cell proliferation, depending on the concentration they were used at. Obtained data shed promising light on the application of tested compounds as hepatoprotective and promoting bone remodeling.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

DH, FB, and EB: conceptualization. DH, AW, HP, KM, and FB: formal analysis. DH: funding acquisition. DH, ES, MG, AW, HP, and MM: investigation. DH, AW, HP, KM, TO, EB, and FB: methodology. DH and FB: resources. FB and EB: supervision. DH, AW, HP, and KM: visualization. DH, AW, HP, KM, and FB: writing – original draft. ES, TO, and EB: writing – review and editing. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the project "UPWR 2.0: international and interdisciplinary program of development of Wrocław University of Environmental and Life Sciences," co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of 4 June 2019. The APC is financed by Wroclaw University of Environmental and Life Science.

Acknowledgments

The authors would like to thank Editage for providing the editing service.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1223123/full#supplementary-material

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Supplementary Material

Chemo-enzymatic Synthesis and Biological Activity Evaluation of Propenylbenzene Derivatives

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Number of pages: 27
Number of Figures: 29
Number of Tables: 6

1. Supplementary Data

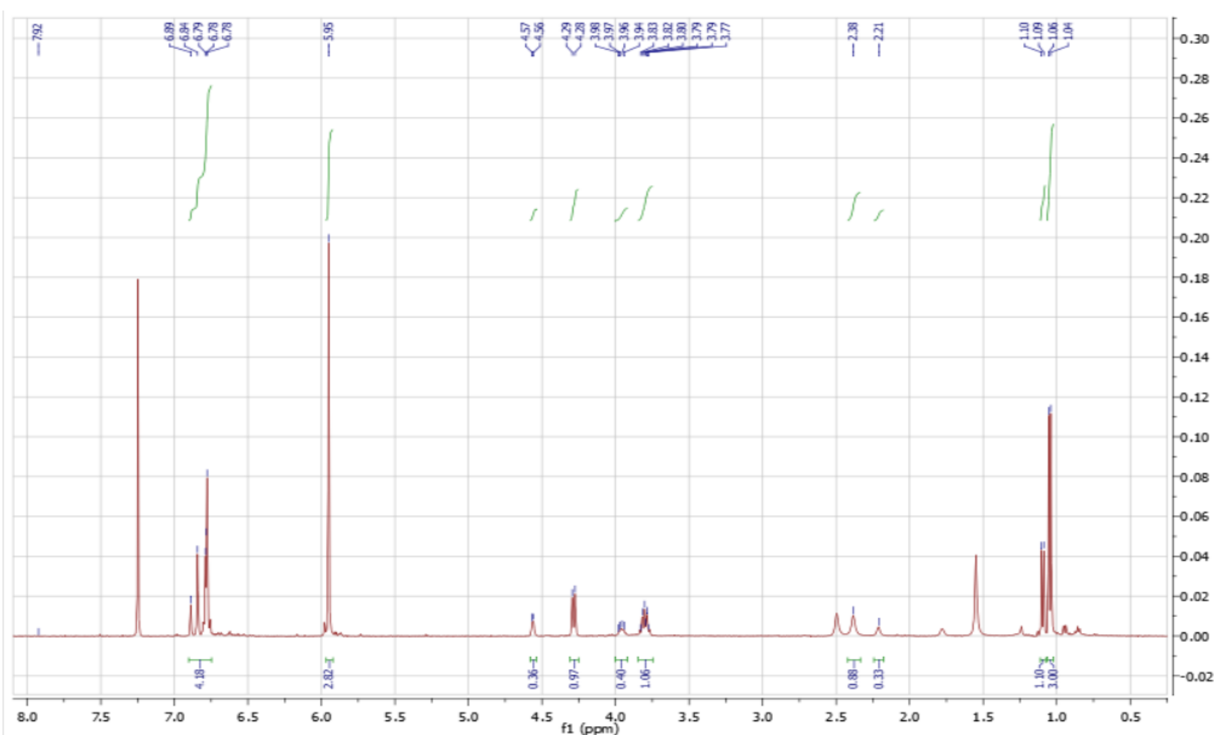

Figure S1. ^{1}H NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(1,3-benzodioxol-5-yl)propane-1,2-diol (**1b**).

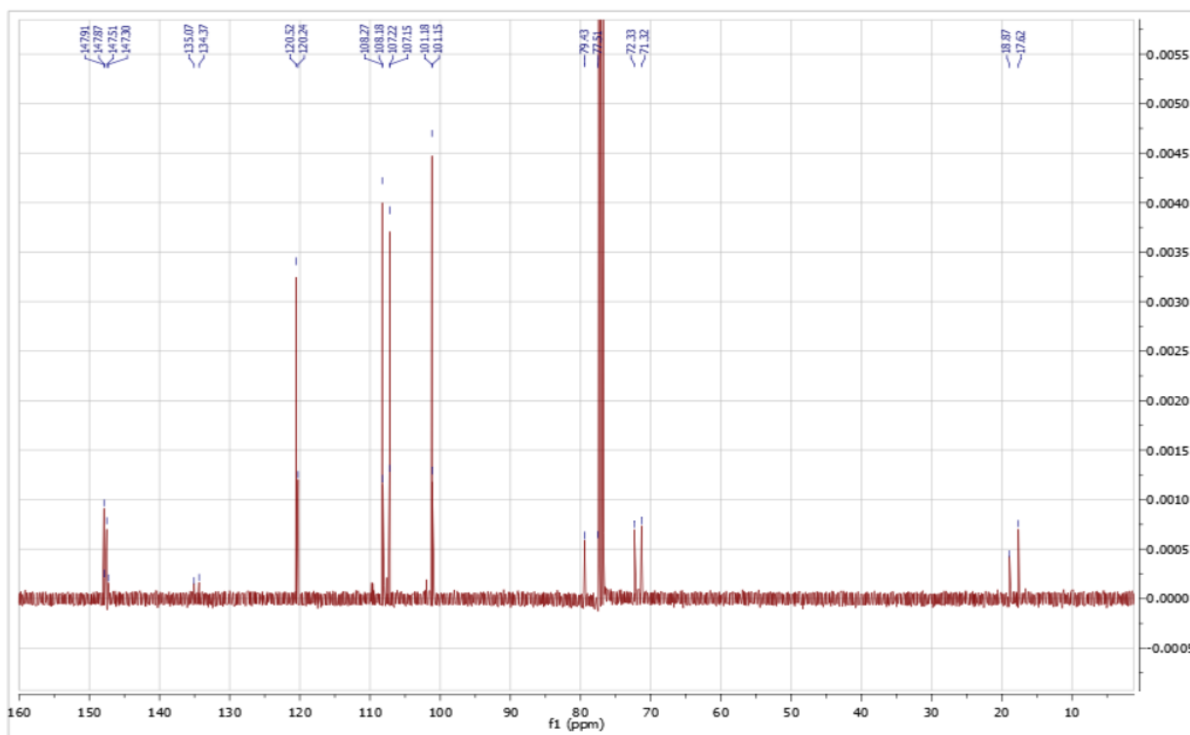

Figure S2. ^{13}C NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(1,3-benzodioxol-5-yl)propane-1,2-diol (**1b**).

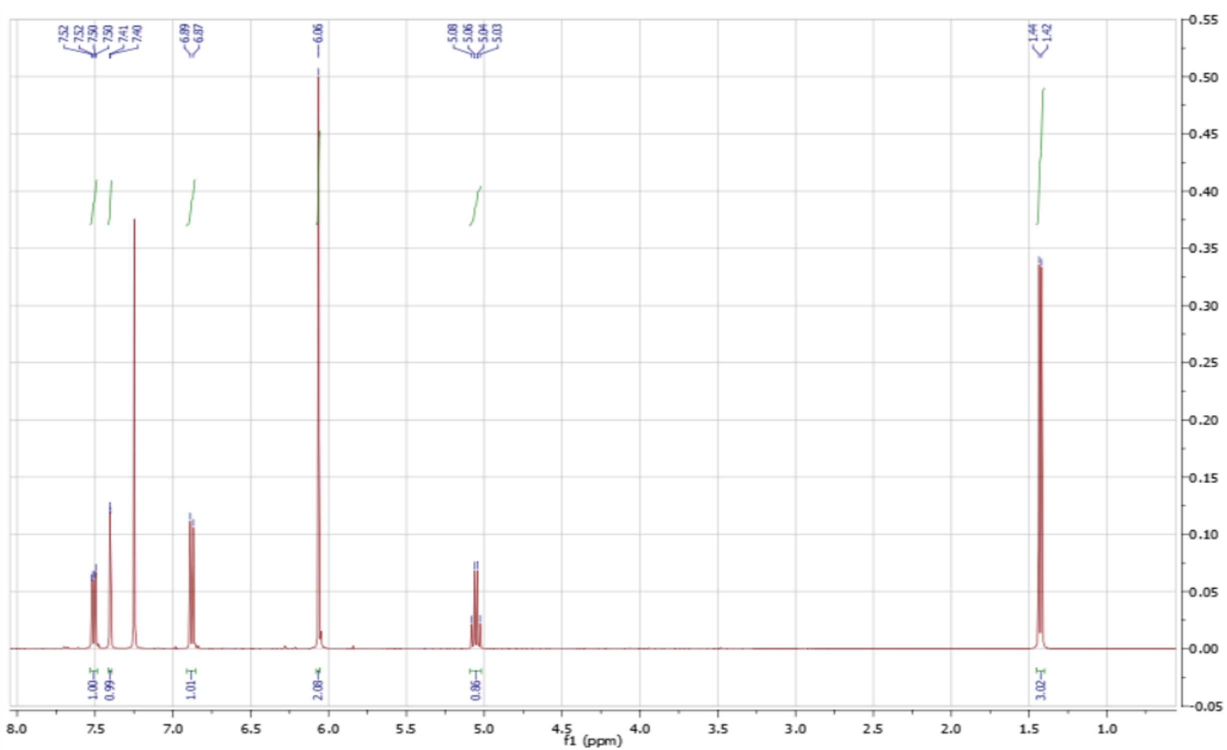

Figure S3. ^{1}H NMR spectrum of 1-(1,3-benzodioxol-5-yl)-2-hydroxypropan-1-one (**1c**).

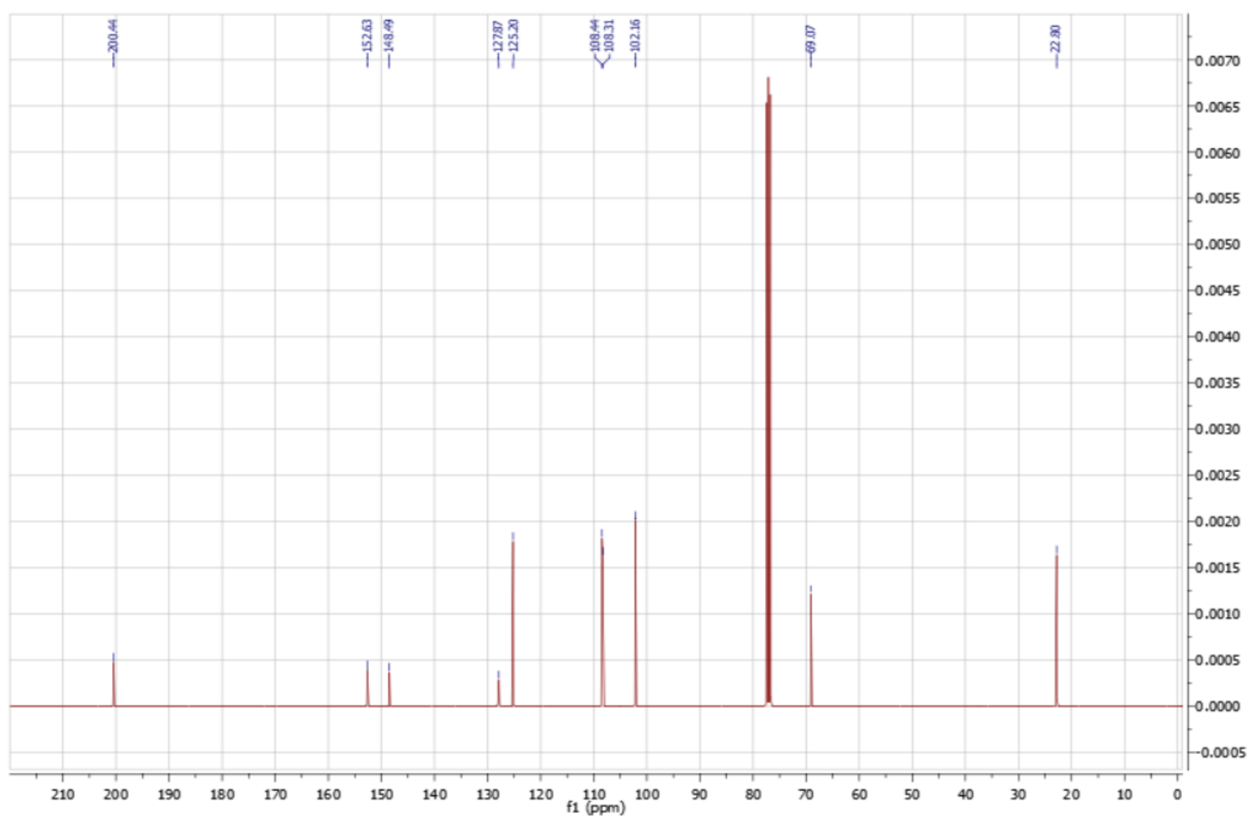

Figure S4. ^{13}C NMR spectrum of 1-(1,3-benzodioxol-5-yl)-2-hydroxypropan-1-one (**1c**).

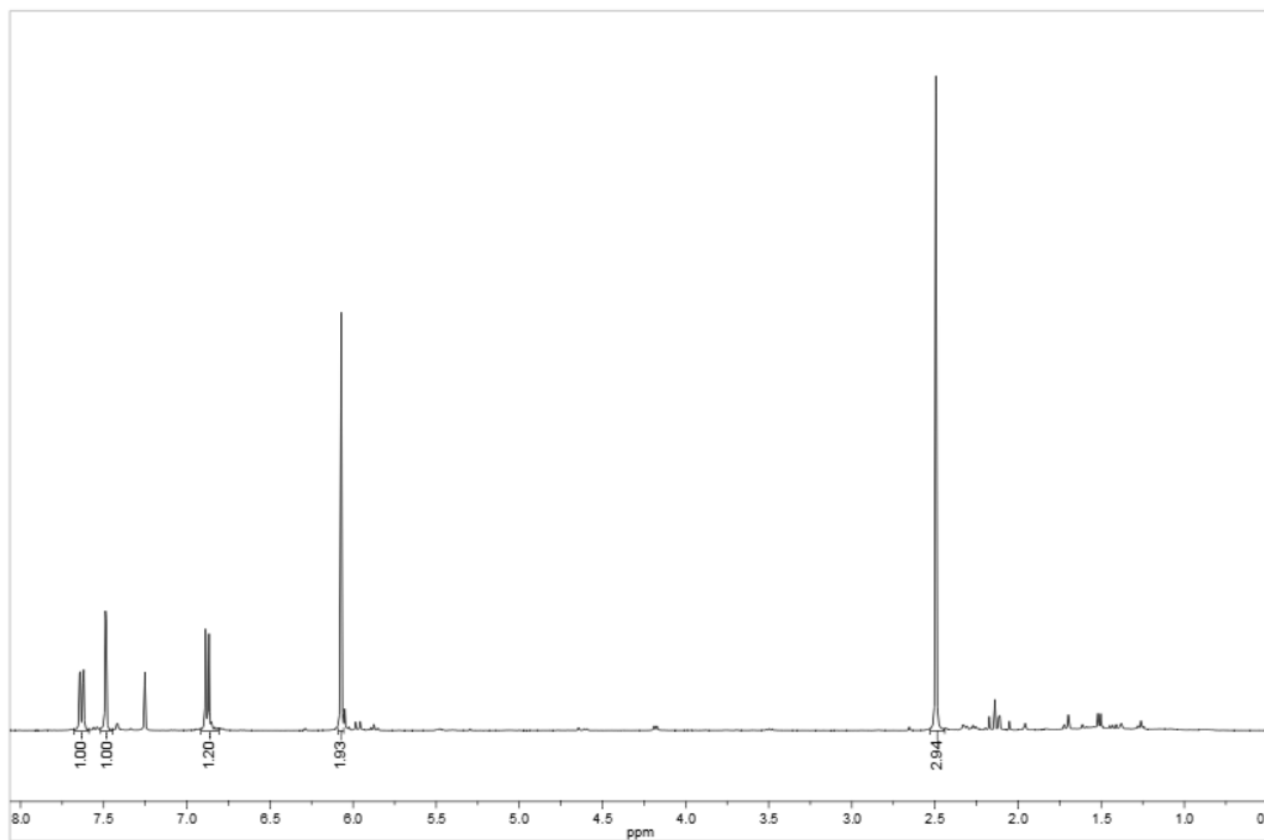

Figure S5. ^{1}H NMR spectrum of 1-(1,3-benzodioxol-5-yl)propane-1,2-dione.

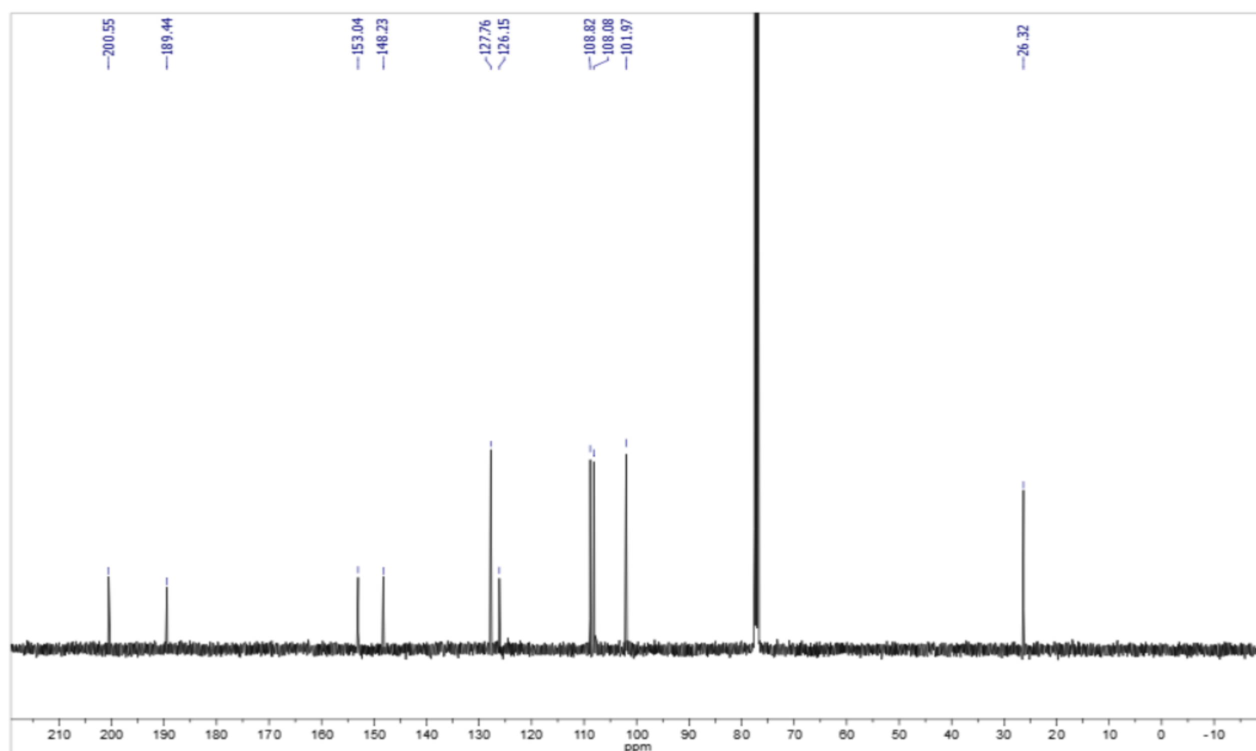

Figure S6. ^{13}C NMR spectrum of 1-(1,3-benzodioxol-5-yl)propane-1,2-dione.

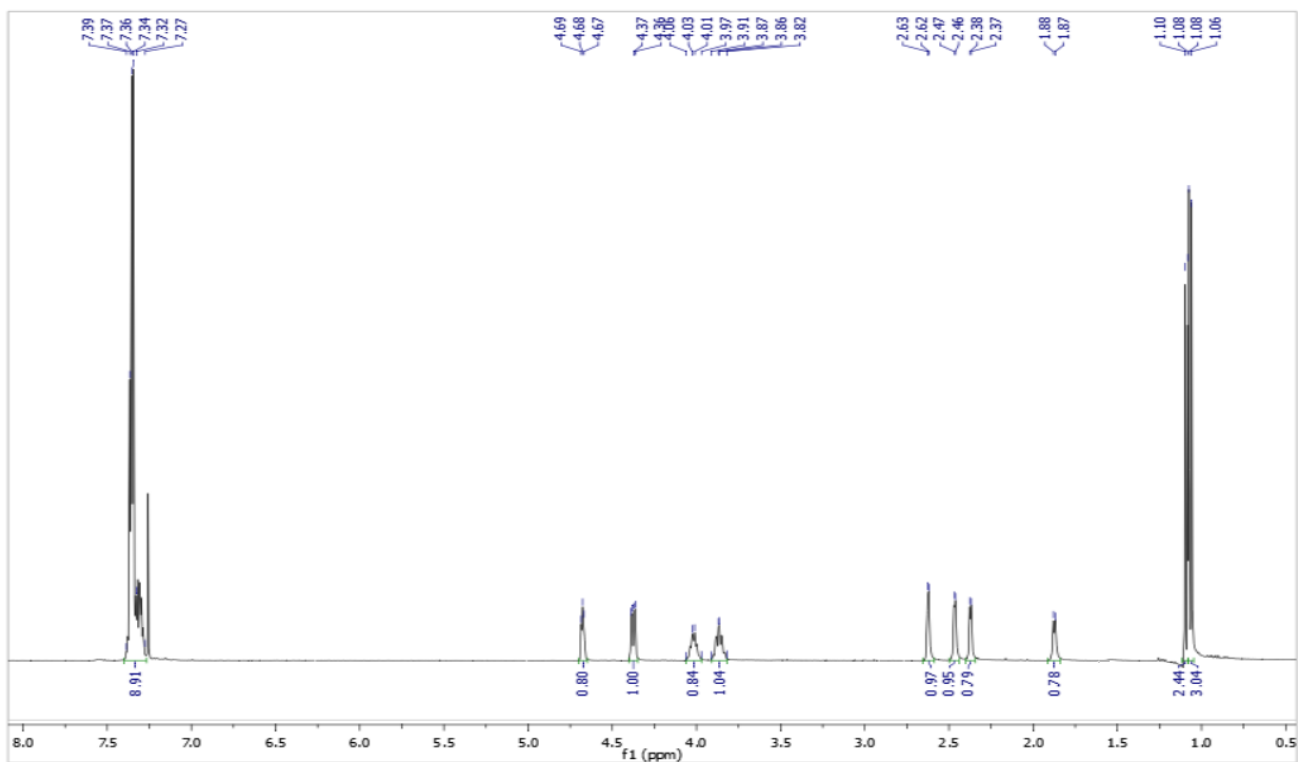

Figure S7. ^{1}H NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-phenylpropane-1,2-diol (**2b**).

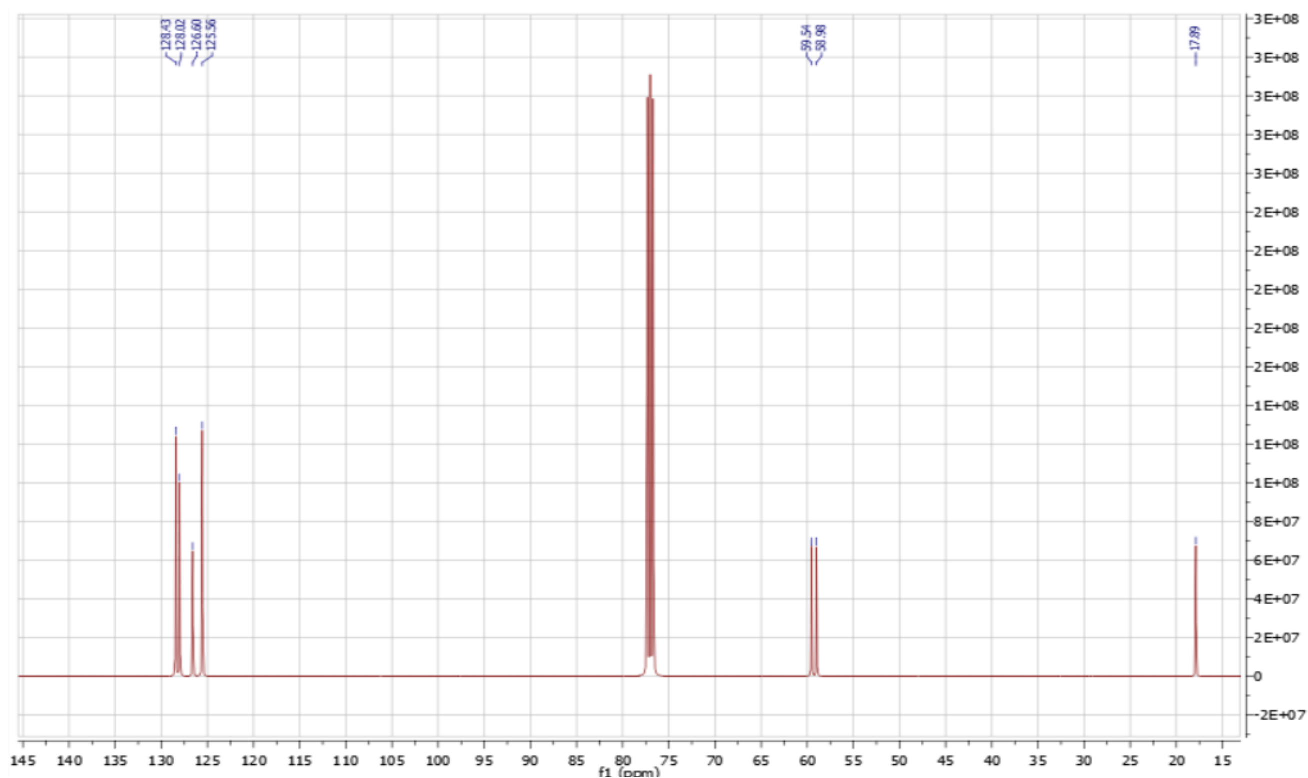

Figure S8. ^{13}C NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-phenylpropane-1,2-diol (**2b**).

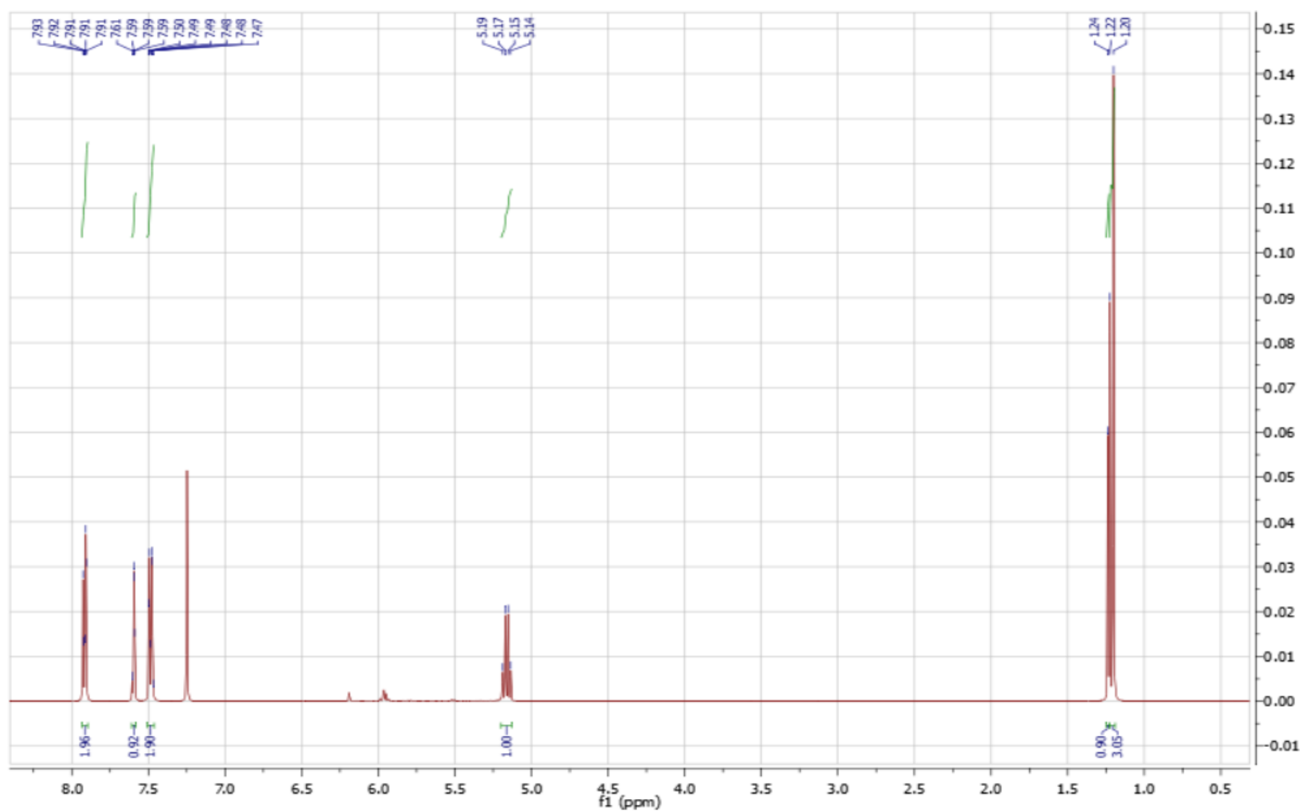

Figure S9. ^{1}H NMR spectrum of 2-hydroxy-1-phenylpropan-1-one (**2c**).

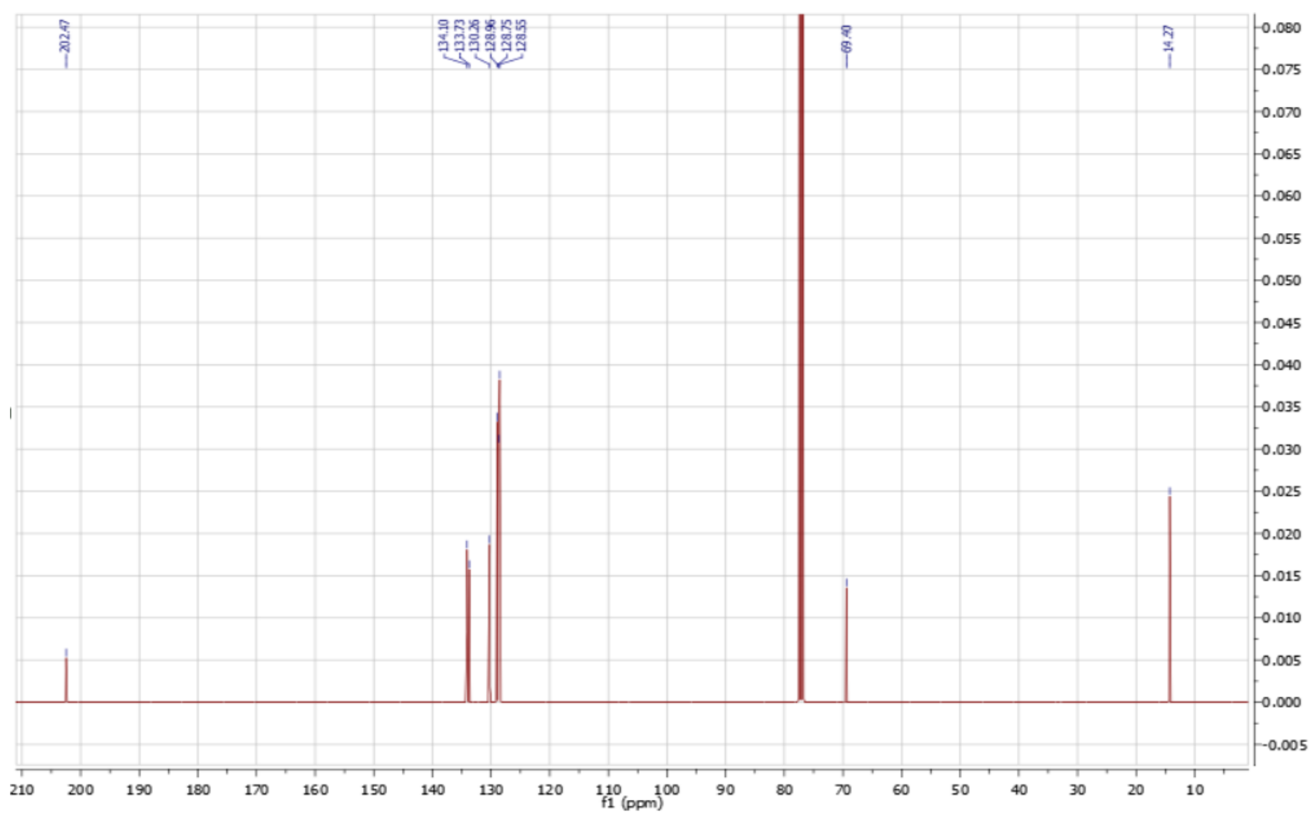

Figure S10. ^{13}C NMR spectrum of 2-hydroxy-1-phenylpropan-1-one (**2c**).

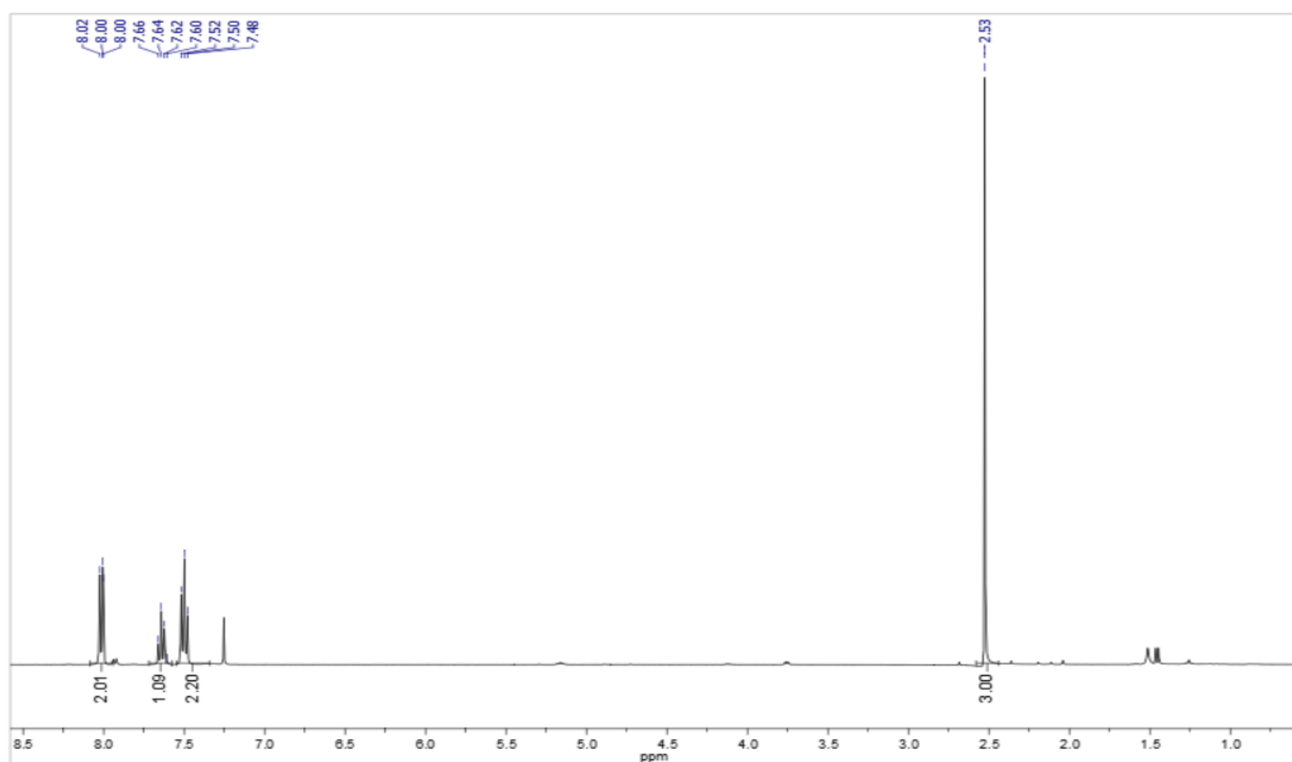

Figure S11. ^{1}H NMR spectrum of 1-phenylpropane-1,2-dione.

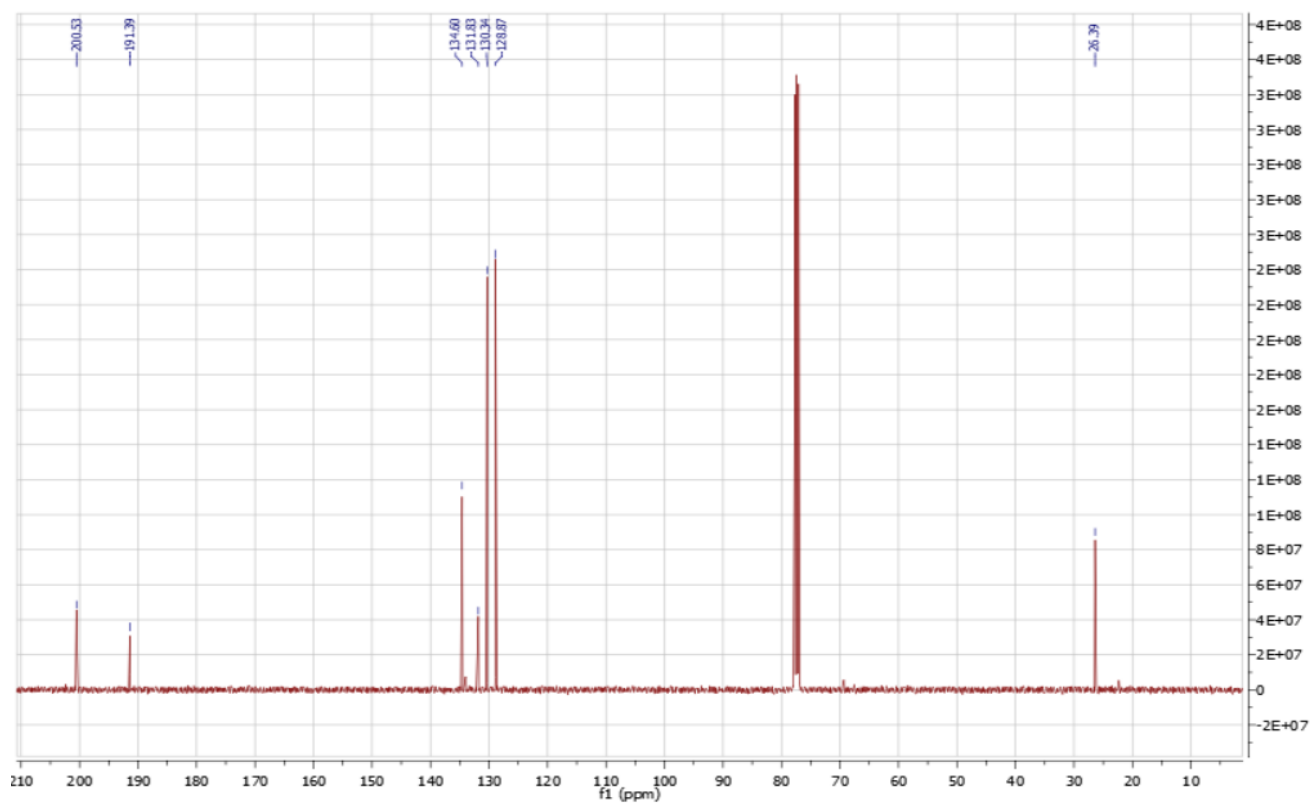

Figure S12. ^{13}C NMR spectrum of 1-phenylpropane-1,2-dione.

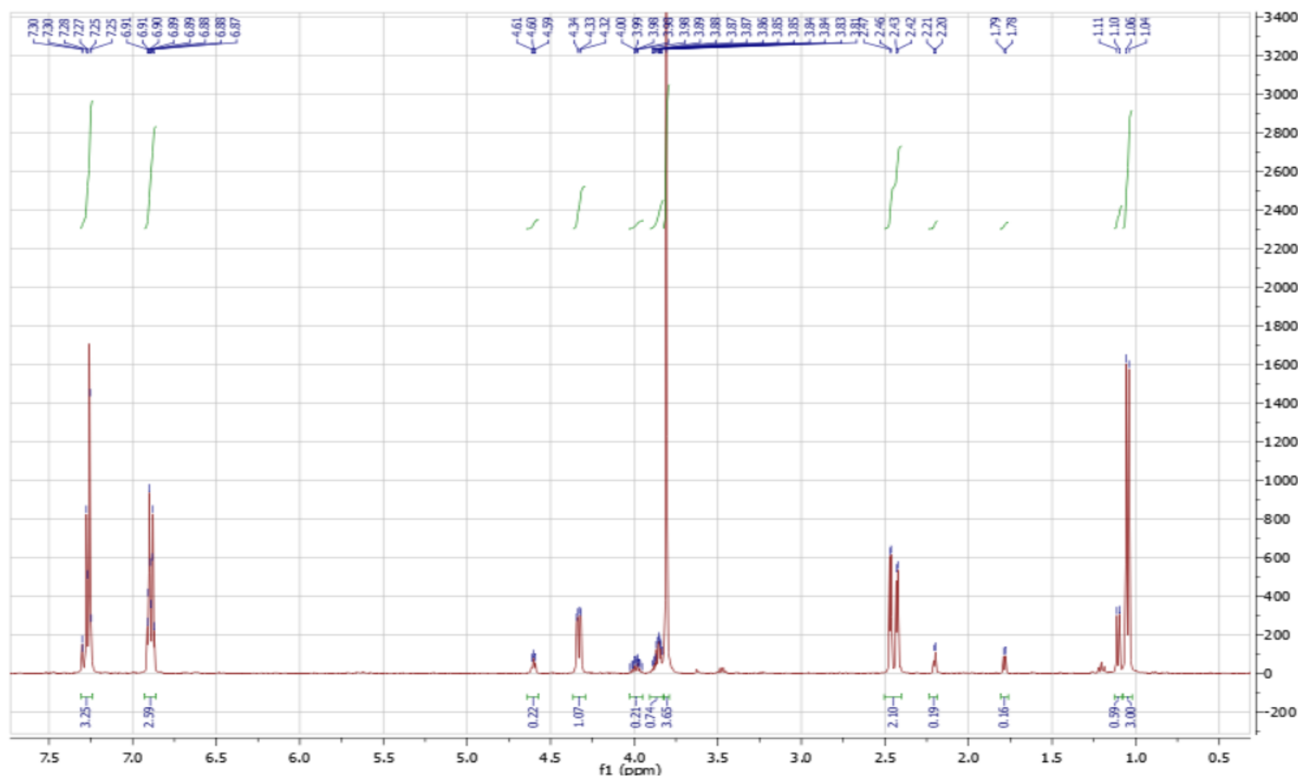

Figure S13. ^{1}H NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(4-methoxyphenyl)propane-1,2-diol (**3b**).

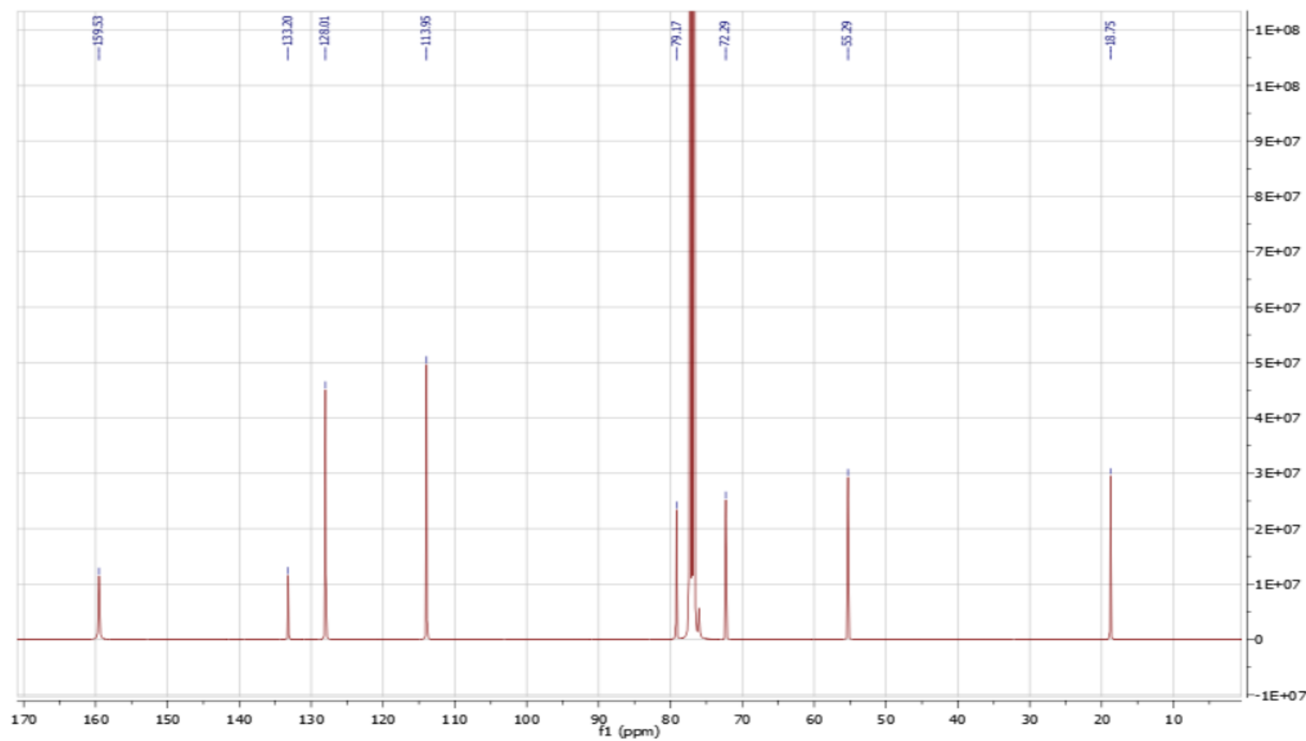

Figure S14. ^{13}C NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(4-methoxyphenyl)propane-1,2-diol (**3b**).

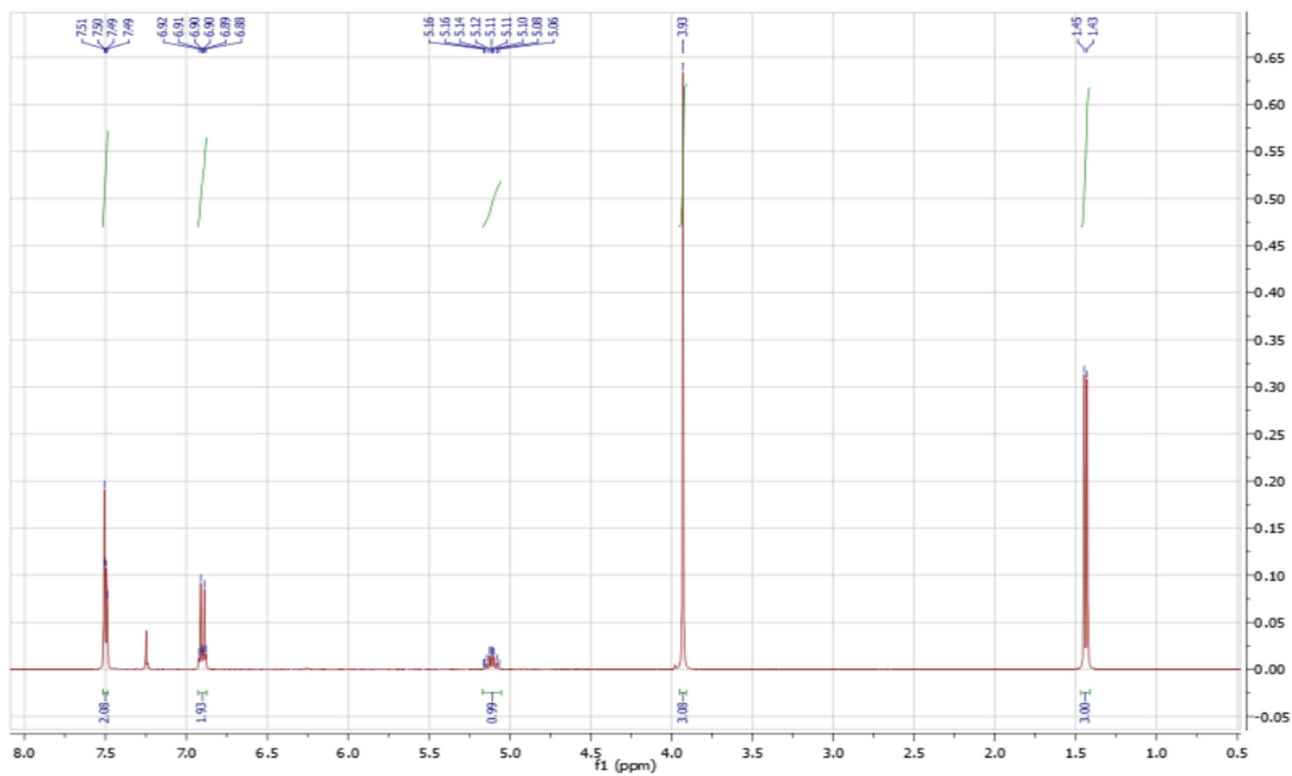

Figure S15. ^{1}H NMR spectrum of 2-hydroxy-1-(4-methoxyphenyl)propan-1-one (**3c**).

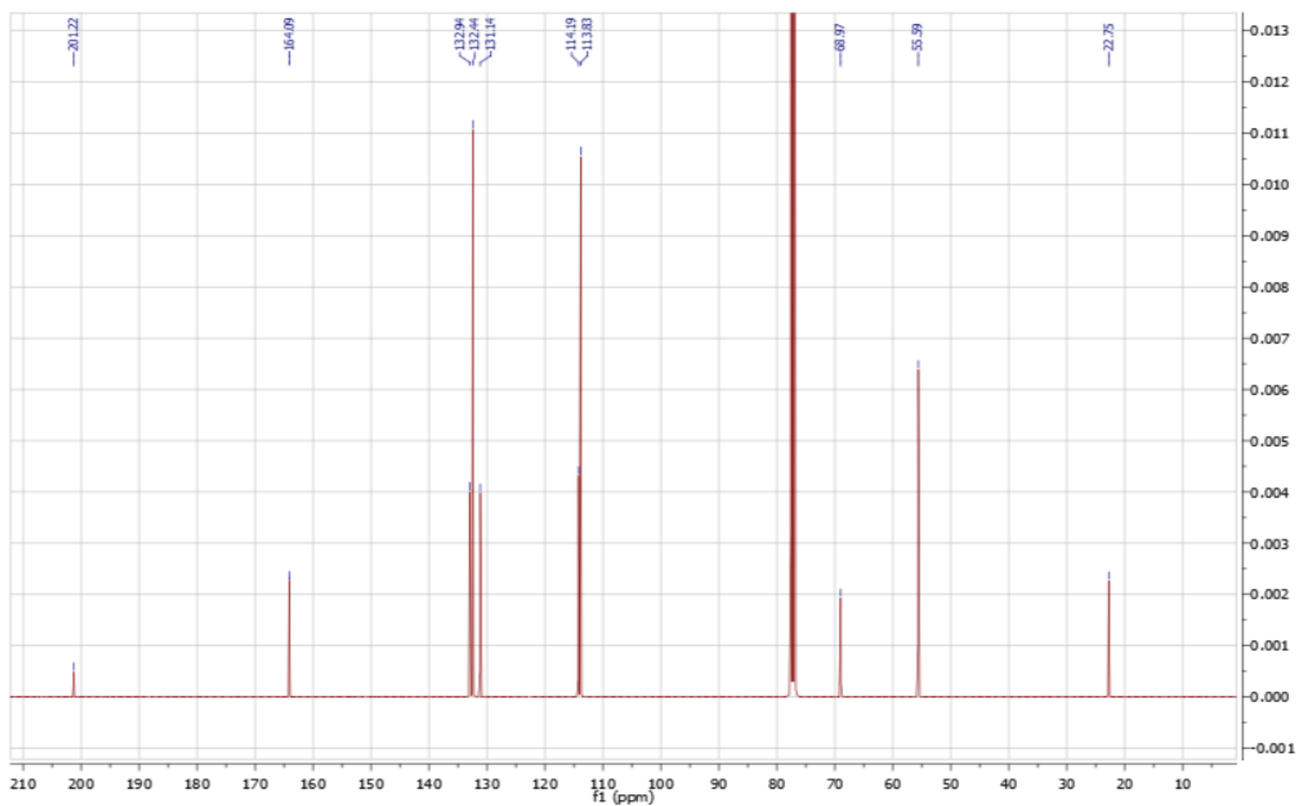

Figure S16. ^{13}C NMR spectrum of 2-hydroxy-1-(4-methoxyphenyl)propan-1-one (**3c**).

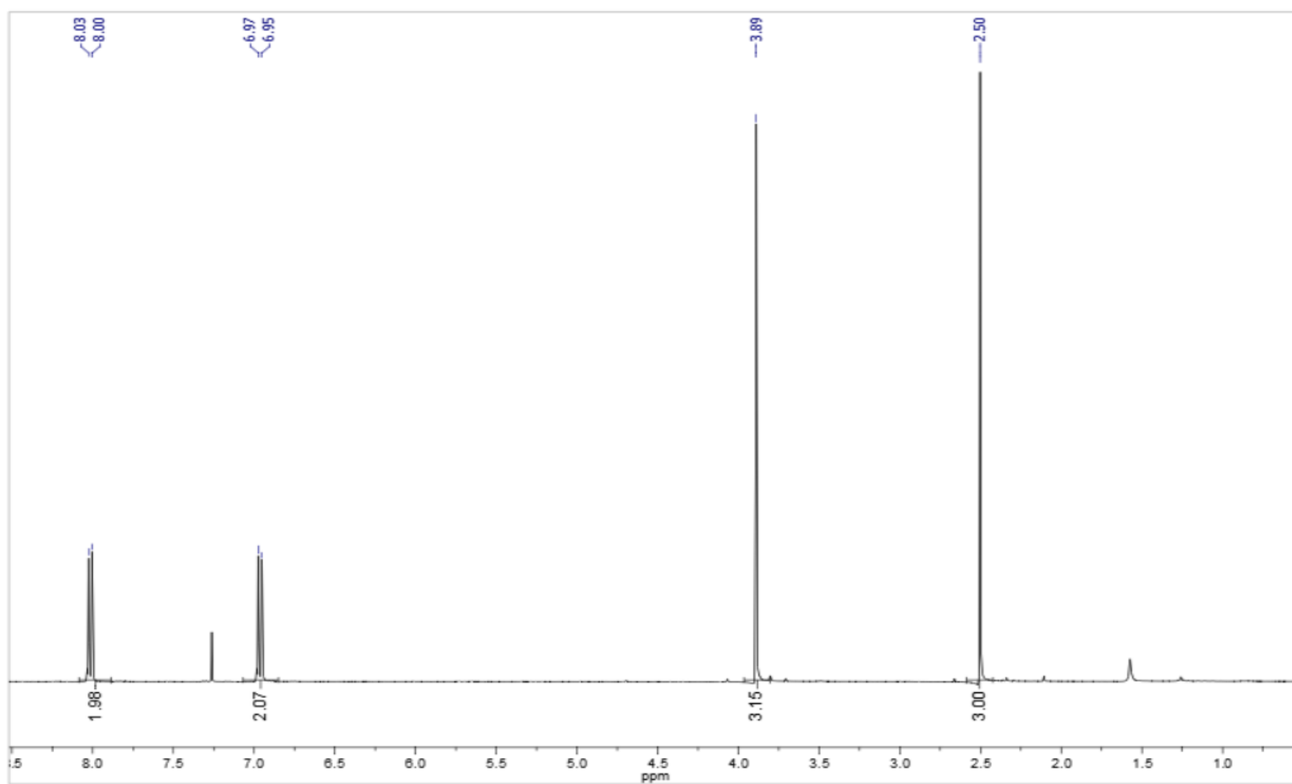

Figure S17. ^{1}H NMR spectrum of 1-(4-methoxyphenyl)propane-1,2-dione.

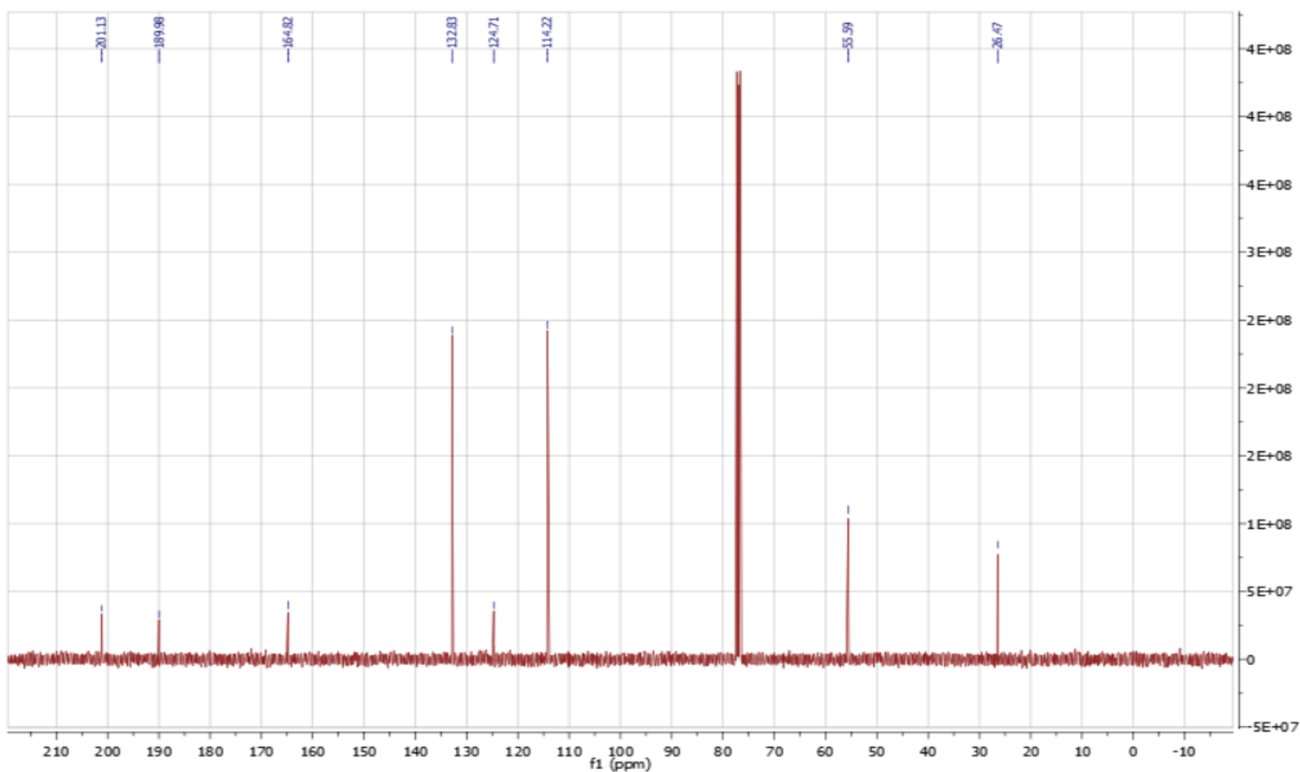

Figure S18. ^{13}C NMR spectrum of 1-(4-methoxyphenyl)propane-1,2-dione.

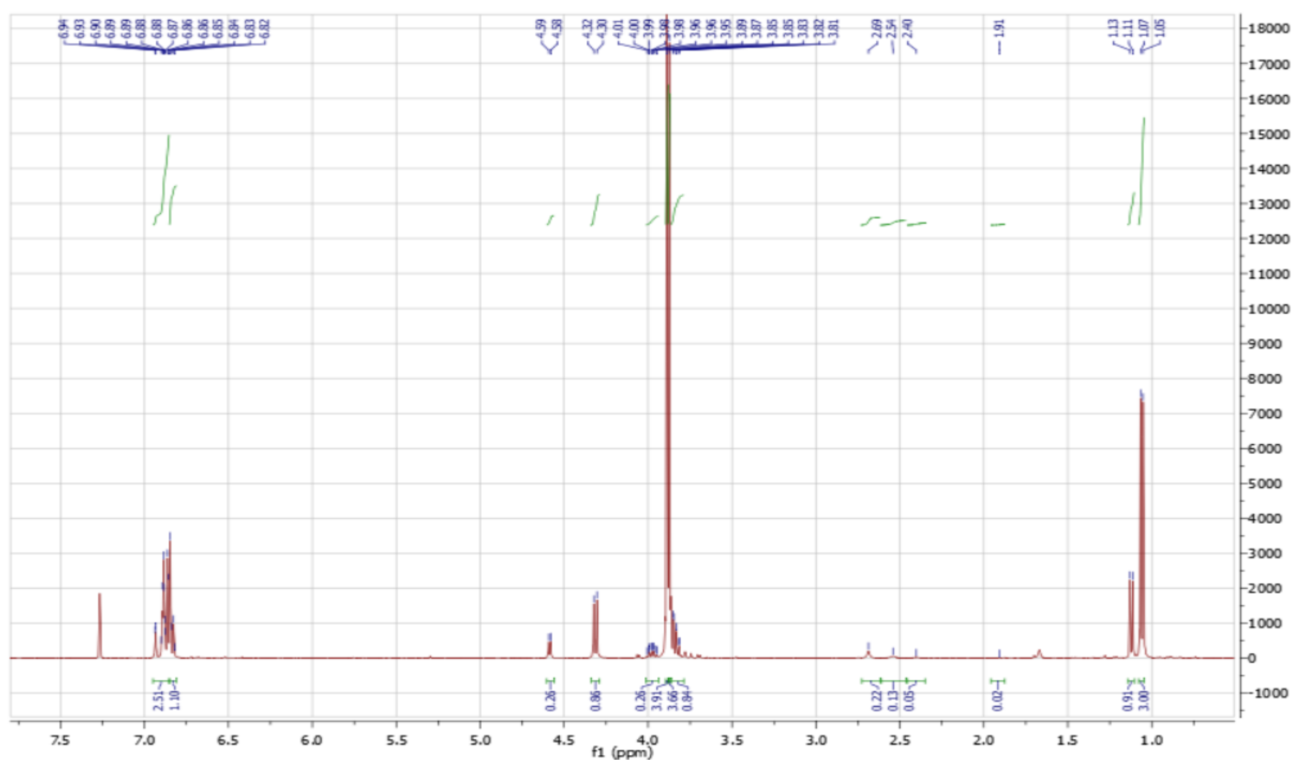

Figure S19. ^{1}H NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(3,4-dimethoxyphenyl)propane-1,2-diol (**4b**).

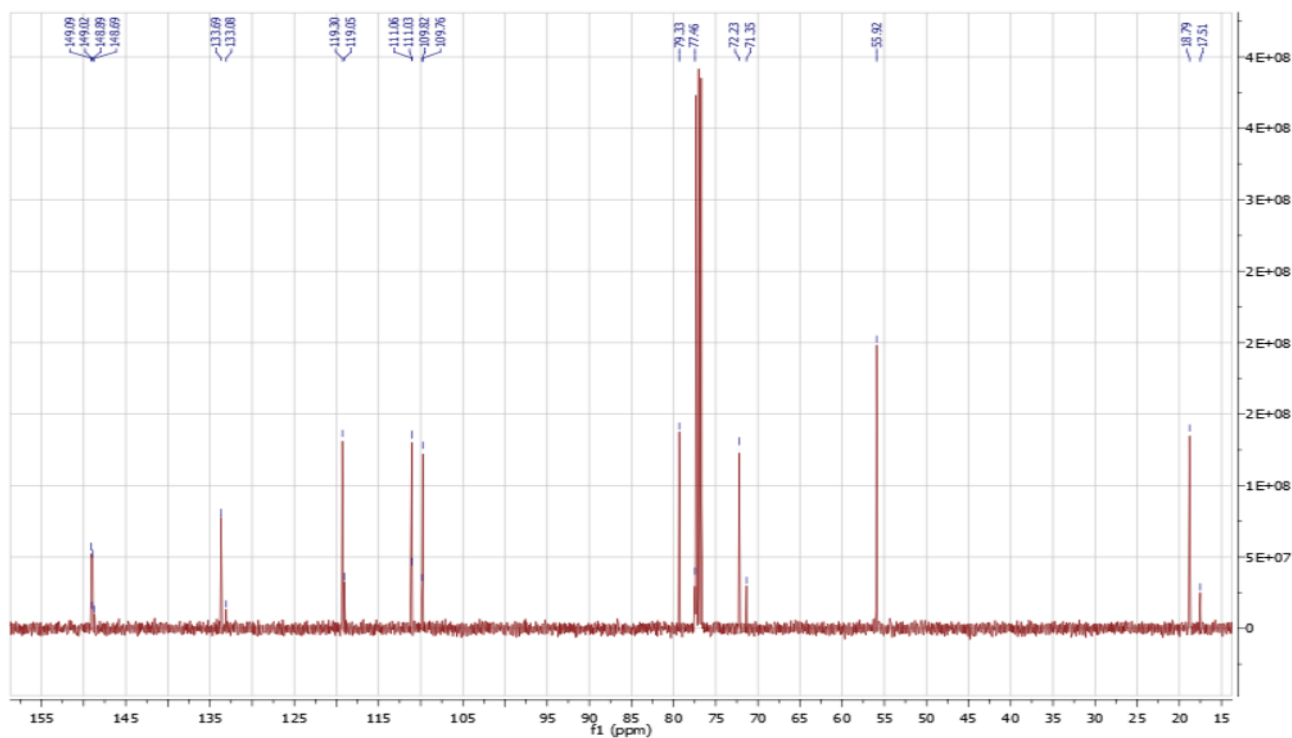

Figure S20. ^{13}C NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(3,4-dimethoxyphenyl)propane-1,2-diol (**4b**).

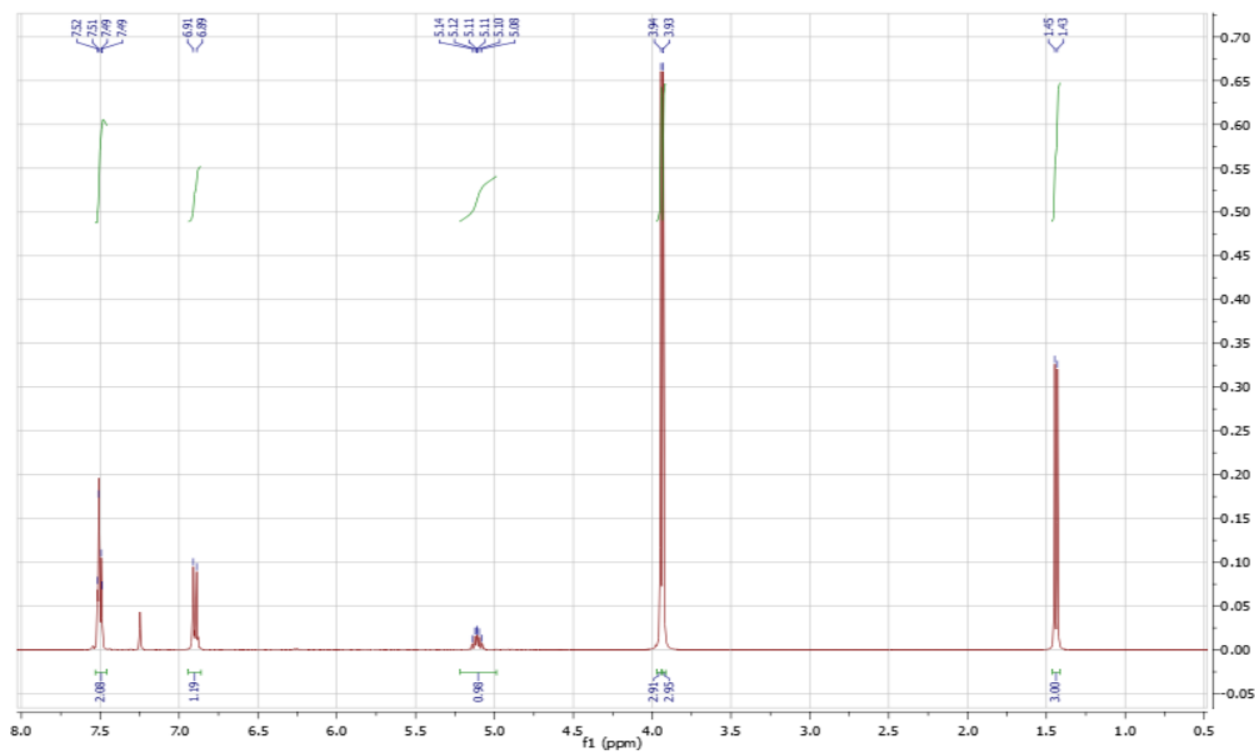

Figure S21. ^{1}H NMR spectrum of 1-(3,4-dimethoxyphenyl)-2-hydroxypropan-1-one (**4c**).

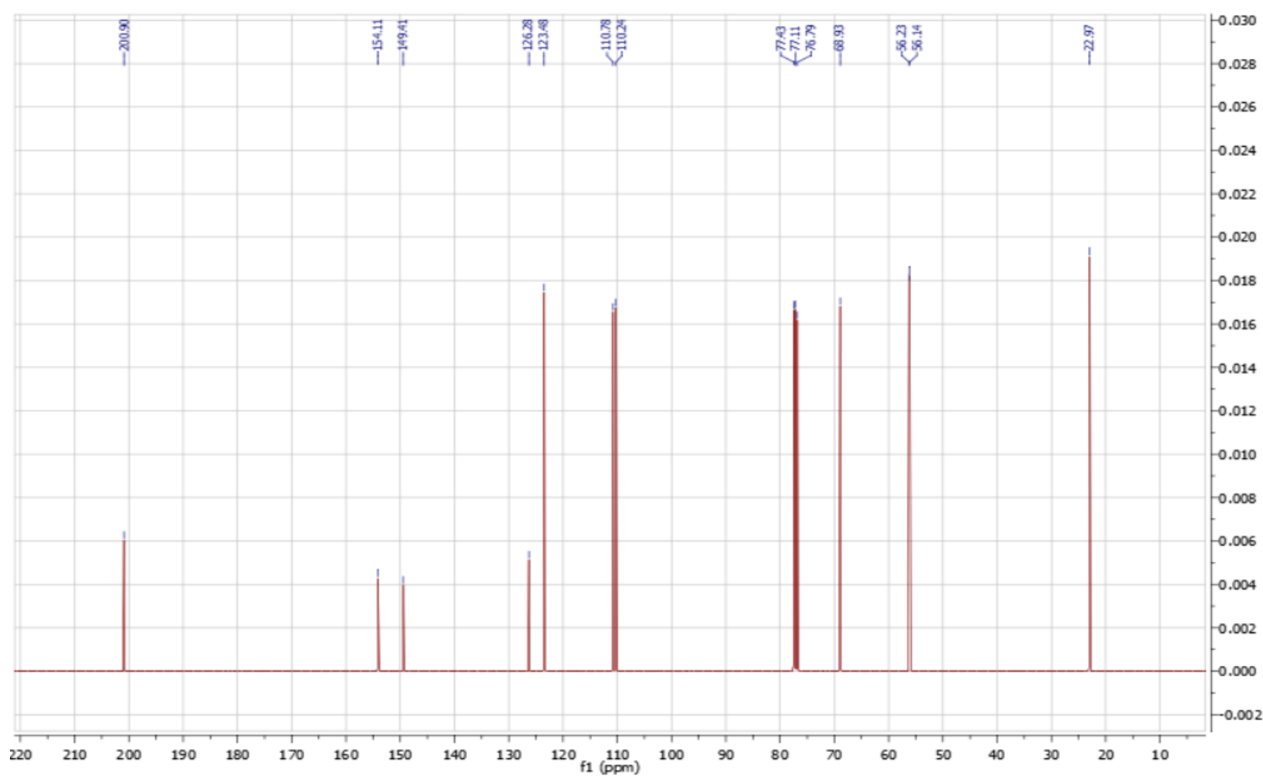

Figure S22. ^{13}C NMR spectrum of 1-(3,4-dimethoxyphenyl)-2-hydroxypropan-1-one (**4c**).

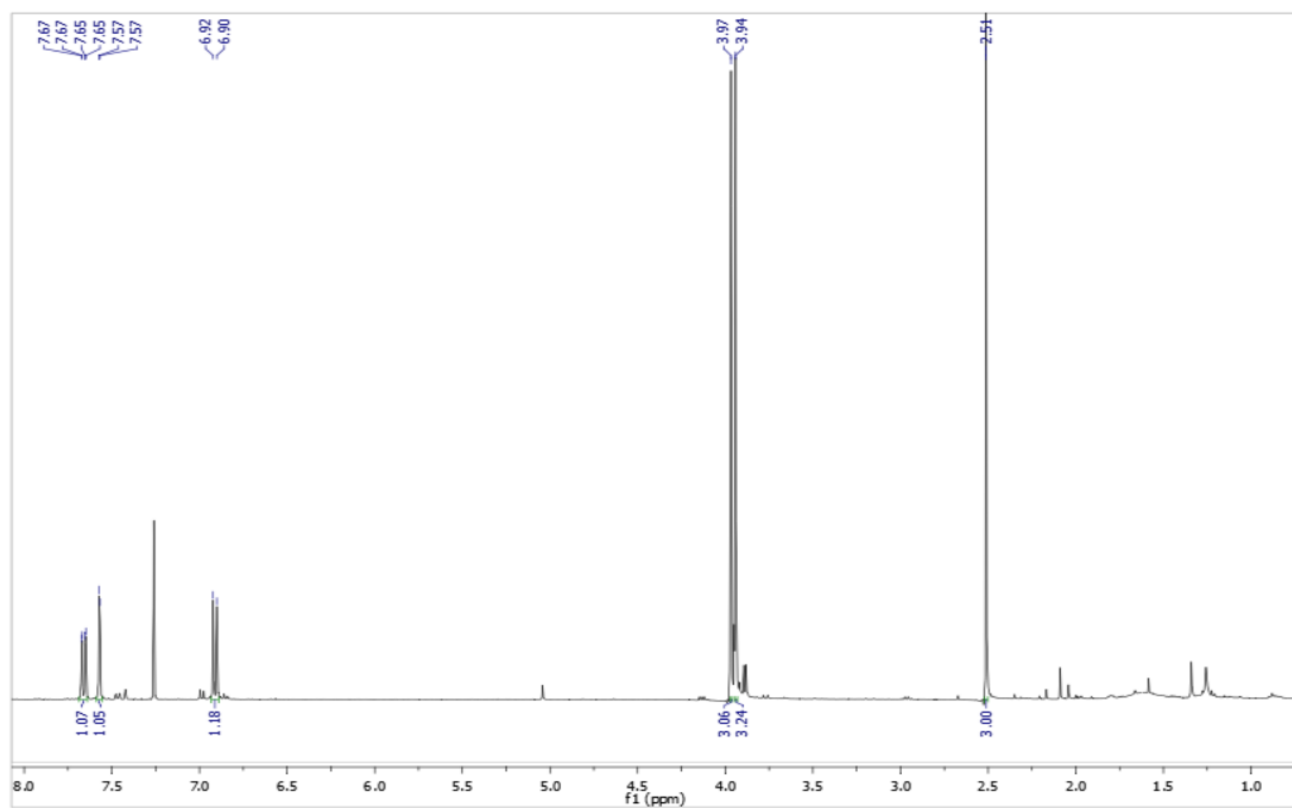

Figure S23. ^{1}H NMR spectrum of 1-(3,4-dimethoxyphenyl)propane-1,2-dione.

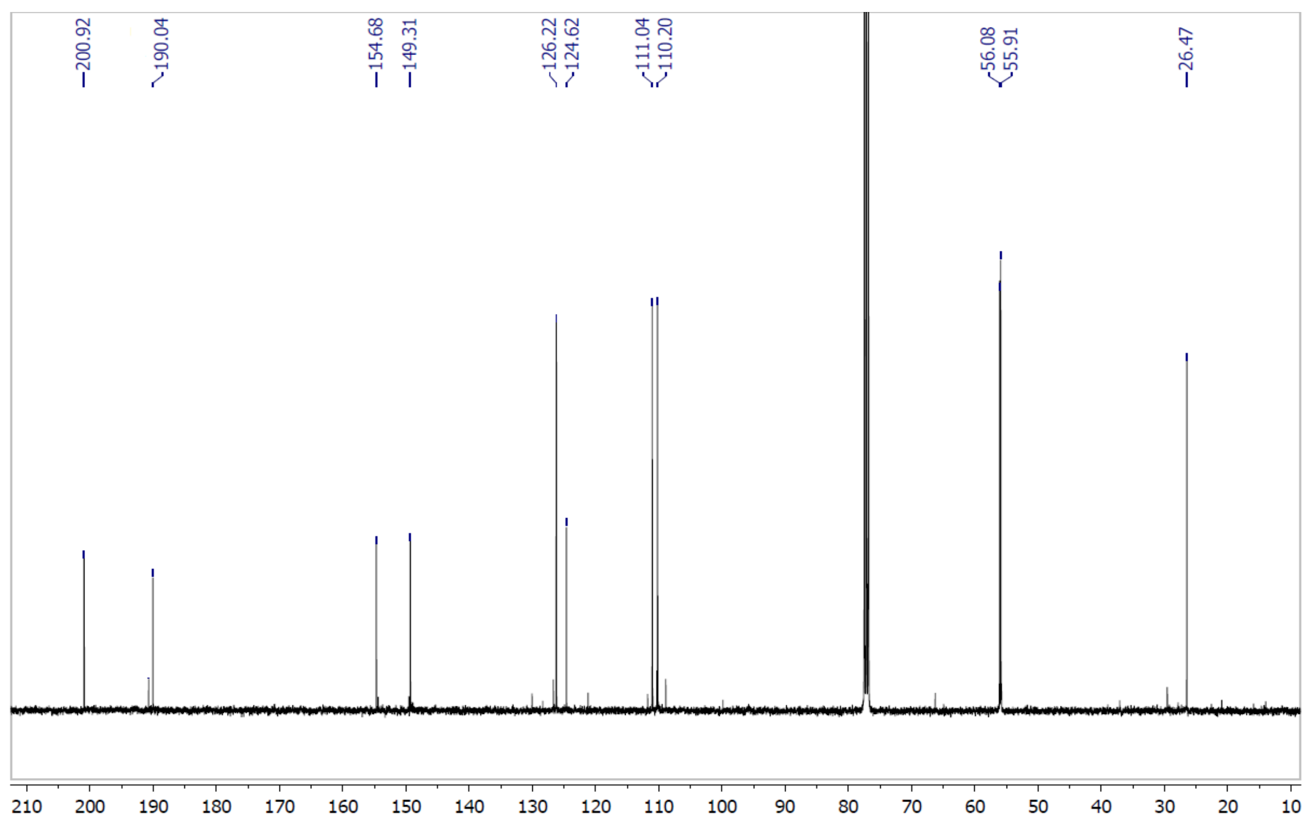

Figure S24. ^{13}C NMR spectrum of 1-(3,4-dimethoxyphenyl)propane-1,2-dione.

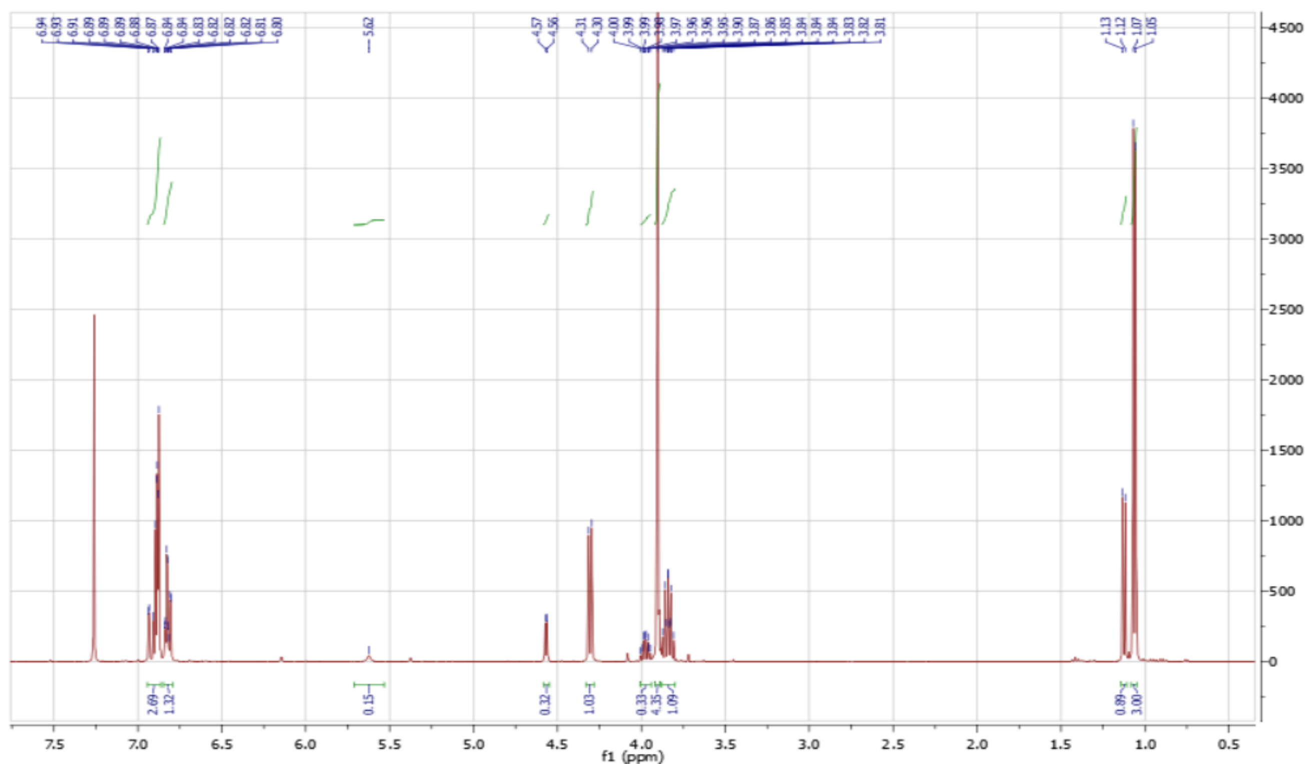

Figure S25. ^{1}H NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (**5b**).

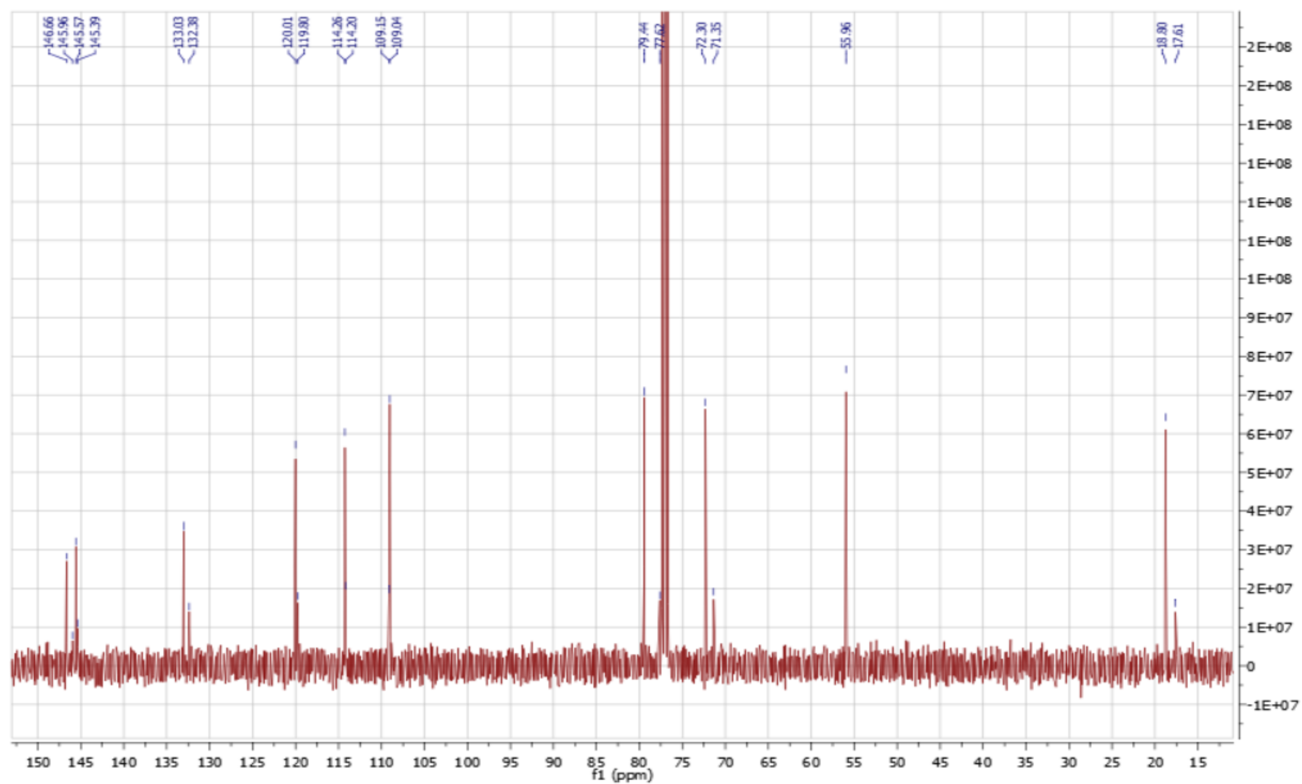

Figure S26. ^{13}C NMR spectrum of (1*R**,2*S**) and (1*R**,2*R**)-1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (**5b**).

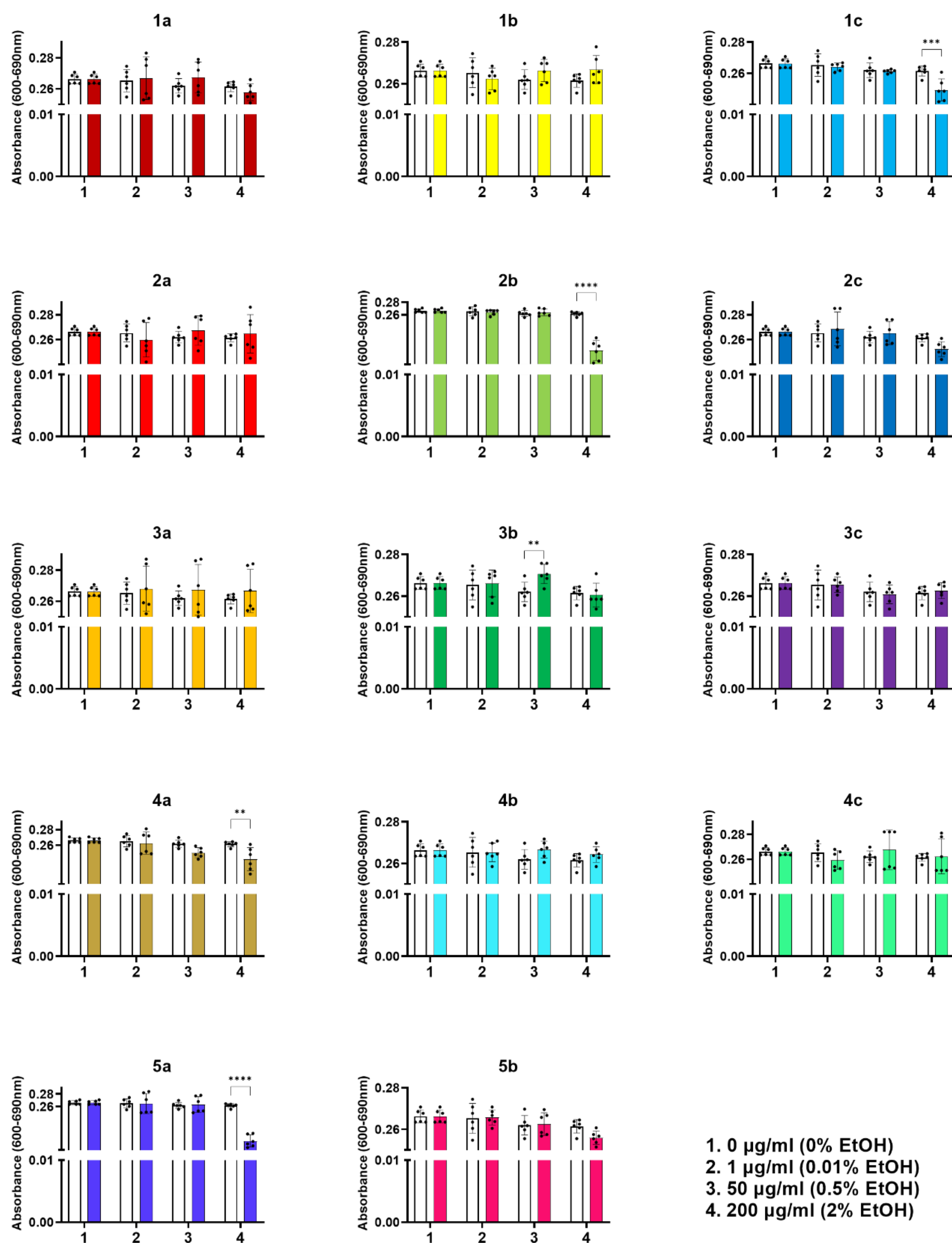

Figure S27. Absorbance values of HepG2 cell line treated with the compounds at concentrations from 0 to 200 µg/ml. The results are shown as mean values ± standard deviations.

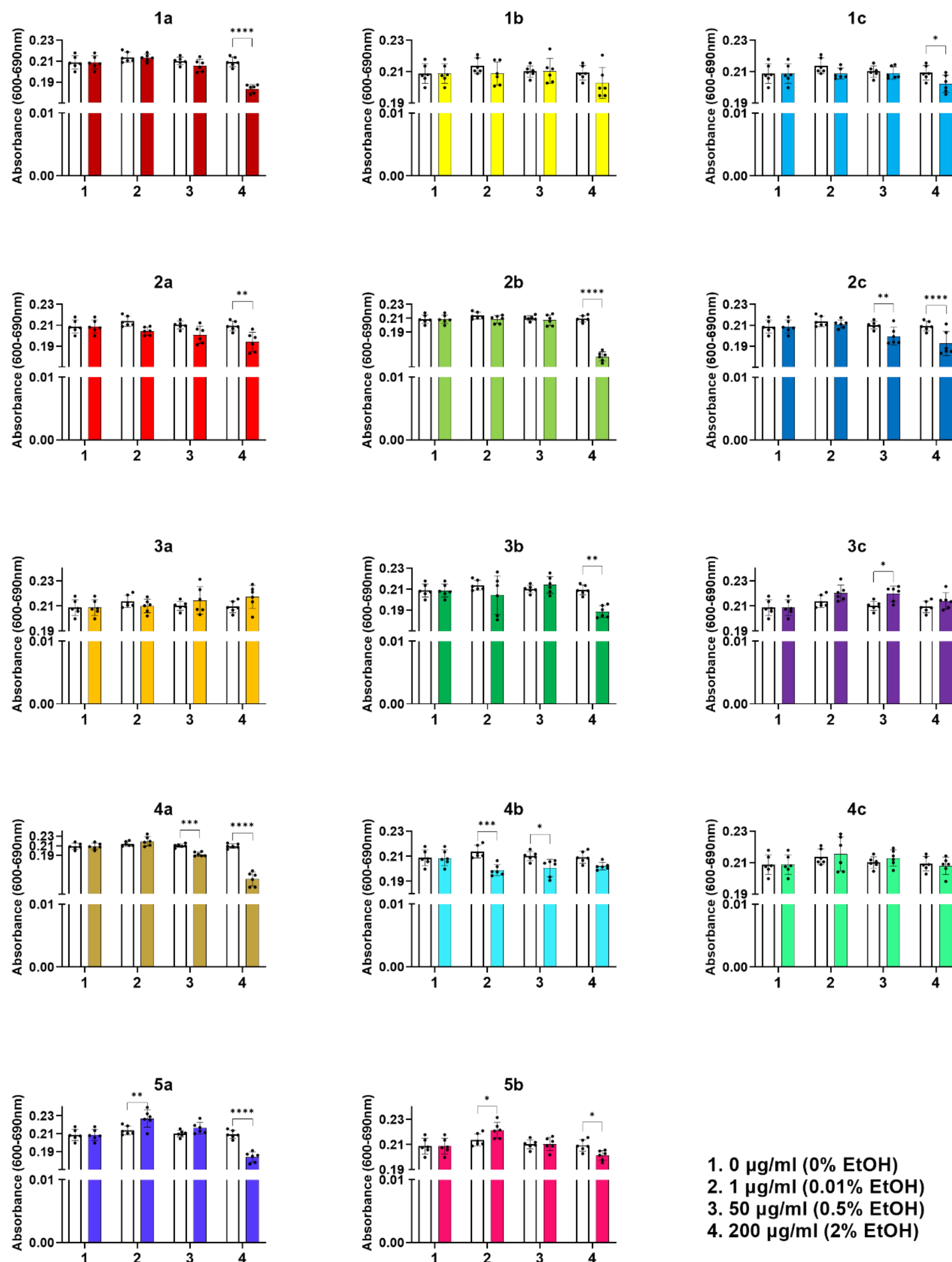

Figure S28. Absorbance values of Caco-2 cell line treated with the compounds at concentrations from 0 to 200 μg/ml. The results are shown as mean values ± standard deviations.

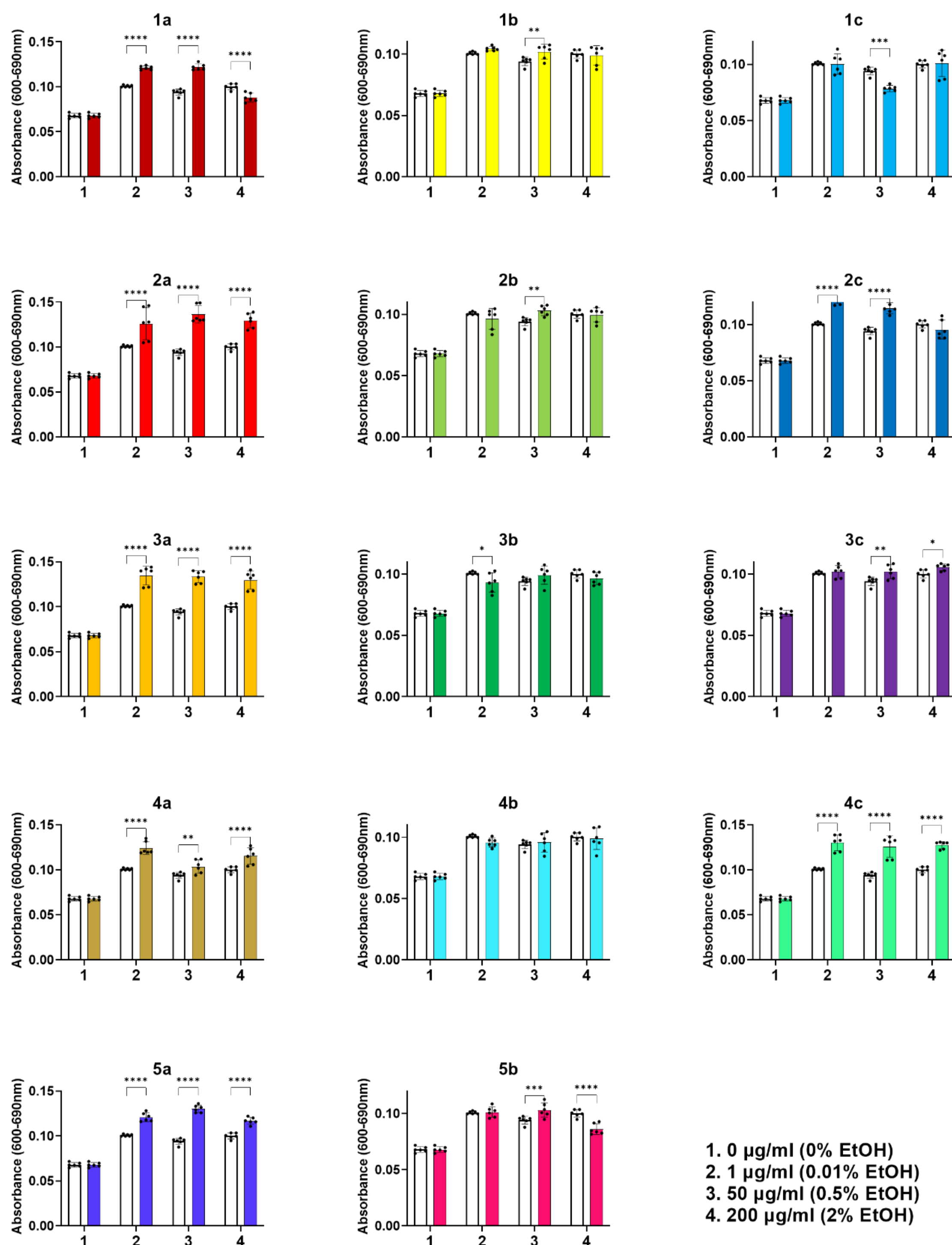

Figure S29. Absorbance values of MG63 cell line treated with the compounds at concentrations from 0 to 200 µg/ml. The results are shown as mean values ± standard deviations.

Table S1. Results of the screening-scale biotransformations of diol **1b**.

Strain	Time [days]	Substrate conversion 1-(1,3-benzodioxol-5-yl)propane-1,2-diol **1b [%]**	Chemical composition of biotransformation products 1-(1,3-benzodioxol-5-yl)propane-1,2-dione **[%]**	1-(1,3-benzodioxol-5-yl)-1-hydroxypropan-2-one **[%]**	1-(1,3-benzodioxol-5-yl)-2-hydroxypropan-1-one **1c [%]**
Bacillus subtilis PCM2238	3	44 (±2.1)	8 (±0.7)	10 (±1.7)	26 (±1.1)
	7	47 (±3.1)	4 (±0.5)	10 (±1.9)	33 (±3.2)
	11	58 (±3.4)	4 (±0.5)	10 (±0.9)	44 (±3.5)
Bacillus subtilis PCM2850	3	28 (±1.5)	7 (±0.7)	8 (±0.5)	13 (±0.9)
	7	26 (±2.3)	5 (±0.3)	10 (±0.9)	21 (±2.1)
	11	58 (±2.9)	5 (±0.3)	10 (±1.1)	43 (±2.3)
Dietzia maris PCM2292	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Dietzia sp. DSM44016	3	66 (±2.4)	6 (±0.2)	12 (±0.5)	48 (±2.1)
	7	84 (±3.1)	6 (±0.2)	12 (±1.0)	66 (±3.3)
	11	100	5 (±0.4)	10 (±0.9)	85 (±3.9)
Gordonia bronchialis PCM2167	3	21 (±1.0)	2 (±0.1)	5 (±0.1)	14 (±1.2)
	7	21 (±1.6)	4 (±0.8)	5 (±0.5)	12 (±1.1)
	11	63 (±2.0)	13 (±1.7)	17 (±0.9)	33 (±2.3)
Gordonia rubripertincta PCM2144	3	40 (±2.5)	2 (±0.2)	6 (±0.8)	32 (±2.1)
	7	53 (±3.1)	4 (±0.3)	11 (±1.4)	38 (±1.7)
	11	65 (±4.1)	7 (±0.6)	12 (±1.1)	46 (±3.4)
Micrococcus luteus PCM525	3	13 (±0.7)	-	-	13 (±0.7)
	7	10 (±1.3)	-	-	10 (±1.3)
	11	17 (±1.9)	-	-	17 (±1.9)
Pseudomonas aeruginosa PCM2720	3	0	-	-	-
	7	0	-	-	-
	1	0	-	-	-
Pseudomonas aeruginosa PCM3035	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Rhodococcus coprophilus PCM2174	3	0	-	-	-
	7	15 (±1.3)	3 (±0.1)	-	12 (±1.3)
	11	35 (±3.4)	2 (±0.1)	4 (±0.5)	29 (±1.9)
	3	45 (±4.2)	5 (±0.1)	10 (±1.0)	30 (±3.5)

Rhodococcus erythropolis PCM2150	7	61 (±3.1)	4 (±0.3)	11 (±0.7)	46 (±4.2)
	11	79 (±3.5)	4 (±0.6)	12 (±1.0)	63 (±2.9)
Rhodococcus erythropolis DSM44534	3	62 (±3.4)	4 (±0.3)	5 (±0.3)	53 (±3.3)
	7	83 (±2.3)	3 (±0.5)	8 (±0.6)	72 (±1.3)
	11	84 (±3.0)	3 (±0.1)	8 (±0.9)	73 (±3.6)
Rhodococcus rhodnii PCM2157	3	15 (±1.0)	-	5 (±0.6)	10 (±0.3)
	7	40 (±1.9)	2 (±0.1)	5 (±0.2)	33 (±1.6)
	11	49 (±2.8)	2 (±1.0)	4 (±0.1)	43 (±2.4)
Rhodococcus rhodochrous PCM909	3	30 (±1.5)	-	-	30 (±1.5)
	7	42 (±2.3)	-	-	42 (±2.3)
	11	47 (±2.7)	-	-	47 (±2.7)
Rhodococcus ruber PCM2166	3	61 (±3.7)	11 (±0.3)	10 (±1.2)	40 (±3.1)
	7	65 (±4.1)	10 (±1.2)	10 (±0.9)	45 (±4.2)
	11	95 (±3.7)	15 (±0.4)	18 (±1.1)	62 (±2.6)
Rhodococcus ruber PCM2171	3	40 (±2.1)	-	-	40 (±2.1)
	7	44 (±2.5)	2 (±0.1)	5 (±0.3)	37 (±1.9)
	11	49 (±3.5)	2 (±0.1)	5 (±0.5)	42 (±3.1
Rhodococcus ruber PCM2216	3	31 (±2.1)	-	-	31 (±2.1)
	7	40 (±2.1)	4 (±1.4)	2 (±0.1)	34 (±1.6)
	11	54 (±3.5)	5 (±1.0)	6 (±0.4)	43 (±3.3)
Serratia liquefaciens PCM2830	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia marcescens PCM549	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia plumuthica PCM550	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia sp. PCM1324	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Streptomyces griseus subsp. *Griseus* PCM2331	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-

%, determined by GC

Table S2. Results of the screening-scale biotransformations of diol **2b**.

Strain	Time [days]	Substrate Conversion	Chemical composition of biotransformation products		
		1-phenylpropane-1,2-diol **2b [%]**	1-phenylpropane-1,2-dione **[%]**	1-hydroxy-1-phenylpropan-2-one **[%]**	2-hydroxy-1-phenylpropan-1-one **2c [%]**
Bacillus subtilis PCM2238	3	10 (±0.9)	-	-	10 (±0.9)
	7	14 (±1.7)	-	-	14 (±1.7)
	11	29 (±3.1)	4 (±0.2)	5 (±0.6)	20 (±2.5)
Bacillus subtilis PCM2850	3	17 (±2.3)	-	-	17 (±2.3)
	7	20 (±1.8)	-	3 (±0.3)	17 (±1.5)
	11	26 (±3.3)	-	6 (±0.2)	20 (±3.1)
Dietzia maris PCM2292	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Dietzia sp. DSM44016	3	57 (±4.1)	2 (±0.1)	10 (±1.3)	45 (±4.1)
	7	84 (±3.7)	3 (±0.1)	11 (±1.7)	70 (±2.9)
	11	88 (±3.4)	3 (±0.1)	11 (±1.6)	74 (±3.4)
Gordonia bronchialis PCM2167	3	52 (±1.8)	-	7 (±0.5)	45 (±1.3)
	7	63 (±2.2)	-	8 (±0.7)	55 (±1.7)
	11	67 (±2.7)	-	8 (±0.4)	59 (±2.3)
Gordonia rubripertincta PCM2144	3	37 (±2.7)	3 (±0.1)	7 (±0.3)	27 (±2.3)
	7	35 (±3.3)	-	5 (±0.2)	30 (±3.1)
	11	41 (±2.6)	2 (±0.1)	7 (±0.4)	32 (±1.9)
Micrococcus luteus PCM525	3	0	-	-	-
	7	9 (±0.4)	-	2 (±0.1)	7 (±0.3)
	11	23 (±1.6)	-	6 (±0.2)	17 (±1.4)
Pseudomonas aeruginosa PCM2720	3	0	-	-	-
	7	22 (±2.0)	-	7 (±0.4)	15 (±1.6)
	1	58 (±3.1)	-	13 (±0.7)	45 (±2.4)
Pseudomonas aeruginosa PCM3035	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Rhodococcus coprophilus PCM2174	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
	3	19 (±1.0)	-	-	19 (±1.0)

Rhodococcus erythropolis PCM2150	7	52 (±2.7)	-	6 (±0.3)	46 (±2.4)
	11	84 (±5.0)	3	14 (±0.9)	67 (±4.1)
Rhodococcus erythropolis DSM44534	3	27 (±2.0)	-	3 (±0.1)	24 (±1.9)
	7	57 (±3.6)	-	5 (±0.2)	52 (±3.4)
	11	80 (±2.5)	5 (±0.2)	5 (±0.2)	70 (±2.1)
Rhodococcus rhodnii PCM2157	3	11 (±1.0)	-	5 (±0.3)	11 (±0.7)
	7	24 (±1.4)	-	5 (±0.2)	19 (±1.2)
	11	37 (±1.9)	-	9 (±0.5)	28 (±1.4)
Rhodococcus rhodochrous PCM909	3	7 (±0.5)	-	-	7 (±0.5)
	7	28 (±2.1)	-	7 (±0.6)	21 (±1.5)
	11	40 (±2.4)	-	6 (±0.3)	34 (±2.1)
Rhodococcus ruber PCM2166	3	38 (±2.9)	6 (±0.2)	8 (±0.6)	24 (±2.1)
	7	61 (±3.7)	3 (±0.1)	10 (±0.8)	48 (±2.8)
	11	96 (±3.8)	-	9 (±0.5)	87 (±3.3)
Rhodococcus ruber PCM2171	3	13 (±1.2)	-	5 (±0.7)	8 (±0.5)
	7	32 (±2.9)	-	14 (±1.8)	18 (±1.1)
	11	41 (±3.3)	-	14 (±1.3)	27 (±2.0)
Rhodococcus ruber PCM2216	3	17 (±0.9)	-	-	17 (±0.9)
	7	50 (±3.3)	5 (±0.1)	9 (±0.3)	36 (±2.9)
	11	52 (±4.1)	4 (±0.3)	13 (±0.7)	35 (±3.1)
Serratia liquefaciens PCM2830	3	25 (±1.7)	-	10 (±0.4)	15 (±1.3)
	7	28 (±2.5)	-	10 (±0.6)	18 (±1.9)
	11	46 (±3.8)	2 (±0.3)	11 (±1.2)	33 (±2.3)
Serratia marcescens PCM549	3	0	-	-	-
	7	20 (±2.0)	-	-	20 (±2.0)
	11	27 (±2.1)	-	4 (±0.2)	23 (±1.9)
Serratia plumuthica PCM550	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia sp. PCM1324	3	27 (±3.1)	-	8 (±0.9)	19 (±2.2)
	7	47 (±3.8)	-	11 (±0.4)	36 (±3.4)
	11	58 (±3.8)	4 (±0.2)	14 (±1.1)	40 (±2.5)
Streptomyces griseus subsp. *Griseus* PCM2331	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-

%, determined by GC

Table S3. Results of the screening-scale biotransformations of diol **3b**.

Strain	**Time [days]**	**Substrate Conversion**	**Chemical composition of biotransformation products**		
		1-(4-methoxyphenyl)propane-1,2-diol **3b [%]**	1-(4-methoxyphenyl)propane-1,2-dione **[%]**	1-hydroxy-1-(4-methoxyphenyl)propan-2-one **[%]**	2-hydroxy-1-(4-methoxyphenyl)propan-1-one **3c [%]**
Bacillus subtilis PCM2238	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Bacillus subtilis PCM2850	3	26 (±2.2)	-	-	26 (±2.2)
	7	36 (±2.4)	-	-	36 (±2.4)
	11	41 (±3.1)	-	-	41 (±3.1)
Dietzia maris PCM2292	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Dietzia sp. DSM44016	3	16 (±1.6)	-	4 (±0.1)	12 (±1.5)
	7	43 (±2.1)	1 (±0.1)	1 (±0.1)	41 (±1.9)
	11	88 (±5.1)	3 (±0.1)	11 (±0.7)	74 (±4.3)
Gordonia bronchialis PCM2167	3	0	-	-	-
	7	5 (±0.2)	-	1 (±0.1)	4 (±0.1)
	11	30 (±1.5)	2 (±0.1)	3 (±0.1)	25 (±1.3)
Gordonia rubripertincta PCM2144	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Micrococcus luteus PCM525	3	11 (±0.6)	-	4 (±0.1)	7 (±0.5)
	7	29 (±2.2)	1 (±0.1)	4 (±0.2)	24 (±1.9)
	11	43 (±2.0)	2 (±0.1)	5 (±0.2)	36 (±1.7)
Pseudomonas aeruginosa PCM2720	3	0	-	-	-
	7	0	-	-	-
	1	0	-	-	-
Pseudomonas aeruginosa PCM3035	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Rhodococcus coprophilus PCM2174	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
	3	21 (±2.8)	-	3 (±0.2)	18 (±2.6)

Rhodococcus erythropolis PCM2150	7	30 (±3.2)	-	3 (±0.1)	27 (±3.1)
	11	42 (±3.2)	2 (±0.1)	2 (±0.1)	38 (±3.0)
Rhodococcus erythropolis DSM44534	3	29 (±3.3)	-	2 (±0.1)	27 (±3.2)
	7	63 (±3.1)	-	2 (±0.1)	61 (±3.0)
	11	90 (±2.5)	1 (±0.1)	7 (±0.4)	82 (±2.0)
Rhodococcus rhodnii PCM2157	3	0	-	-	-
	7	9 (±0.7)	-	2 (±0.1)	7 (±0.6)
	11	21 (±1.8)	-	2 (±0.1)	19 (±1.7)
Rhodococcus rhodochrous PCM909	3	10 (±1.1)	-	-	10 (±1.1)
	7	17 (±1.4)	-	2 (±0.1)	15 (±1.3)
	11	28 (±2.0)	-	2 (±0.1)	26 (±1.9)
Rhodococcus ruber PCM2166	3	42 (±4.3)	-	4 (±0.1)	38 (±4.2)
	7	78 (±3.6)	-	14 (±0.5)	64 (±3.1)
	11	100 (±2.6)	-	12 (±0.5)	88 (±2.1)
Rhodococcus ruber PCM2171	3	21 (±1.3)	-	7 (±0.4)	14 (±0.9)
	7	37 (±2.0)	3 (±0.1)	10 (±0.8)	23 (±1.1)
	11	38 (±2.6)	4 (±0.2)	10 (±0.7)	24 (±1.7)
Rhodococcus ruber PCM2216	3	28 (±1.8)	-	-	28 (±1.8)
	7	59 (±3.5)	3 (±0.1)	13 (±1.0)	43 (±2.4)
	11	59 (±2.9)	2 (±0.1)	10 (±0.7)	47 (±2.1)
Serratia liquefaciens PCM2830	3	28 (±1.4)	-	12 (±0.3)	16 (±1.1)
	7	41 (±3.2)	-	10 (±0.9)	31 (±2.3)
	11	56 (±3.8)	4 (±0.1)	13 (±0.4)	39 (±3.3)
Serratia marcescens PCM549	3	13 (±0.5)	-	8 (±0.4)	5 (±0.1)
	7	50 (±2.6)	-	9 (±0.5)	41 (±2.1)
	11	63 (±3.6)	6 (±0.1)	14 (±1.1)	43 (±2.4)
Serratia plumuthica PCM550	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia sp. PCM1324	3	26 (±1.4)	-	5 (±0.1)	21 (±1.3)
	7	37 (±1.5)	-	10 (±0.3)	27 (±1.2)
	11	53 (±3.3)	-	11 (±0.9)	42 (±2.4)
Streptomyces griseus subsp. *Griseus* PCM2331	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-

%, determined by GC

Table S4. Results of the screening-scale biotransformations of diol **4b**.

Strain	Time [days]	Substrate Conversion	Chemical composition of biotransformation products		
		1-(3,4-dimethoxyphenyl)propane-1,2-diol **4b [%]**	1-(3,4-dimethoxyphenyl)propane-1,2-dione **[%]**	1-(3,4-dimethoxyphenyl)-1-hydroxypropan-2-one **[%]**	1-(3,4-dimethoxyphenyl)-2-hydroxypropan-1-one **4c [%]**
Bacillus subtilis PCM2238	3	7 (±0.6)	-	-	7 (±0.6)
	7	23 (±1.3)	3 (±0.1)	4 (±0.1)	16 (±1.1)
	11	34 (±2.0)	3 (±0.1)	7 (±0.3)	24 (±1.6)
Bacillus subtilis PCM2850	3	16 (±1.2)	-	2 (±0.1)	14 (±1.1)
	7	26 (±1.8)	-	3 (±0.1)	23 (±1.7)
	11	37 (±1.2)	-	6 (±0.2)	31 (±1.0)
Dietzia maris PCM2292	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Dietzia sp. DSM44016	3	47 (±3.9)	-	8 (±0.3)	39 (±3.6)
	7	70 (±5.1)	4 (±0.1)	8 (±0.3)	58 (±4.7)
	11	89 (±2.7)	7 (±0.1)	13 (±0.7)	69 (±1.9)
Gordonia bronchialis PCM2167	3	42 (±1.1)	-	5 (±0.1)	37 (±1.1)
	7	63 (±2.1)	-	5 (±0.1)	58 (±2.0)
	11	76 (±2.3)	-	6 (±0.5)	70 (±1.8)
Gordonia rubripertincta PCM2144	3	25 (±2.0)	-	8 (±0.3)	17 (±1.7)
	7	47 (±2.7)	4 (±0.1)	8 (±0.3)	35 (±2.3)
	11	54 (±2.5)	4 (±0.1)	7 (±0.6)	43 (±1.8)
Micrococcus luteus PCM525	3	7 (±0.9)	-	-	7 (±0.9)
	7	20 (±1.6)	-	7 (±0.1)	13 (±1.5)
	11	29 (±3.0)	-	7 (±1.1)	22 (±1.9)
Pseudomonas aeruginosa PCM2720	3	0	-	-	-
	7	0	-	-	-
	1	0	-	-	-
Pseudomonas aeruginosa PCM3035	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Rhodococcus coprophilus PCM2174	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
	3	25 (±3.3)	-	-	25 (±3.3)

Rhodococcus erythropolis PCM2150	7	67 (±5.3)	4 (±0.1)	12 (±1.0)	51 (±4.2)
	11	86 (±2.9)	2 (±0.1)	13 (±0.7)	71 (±2.1)
Rhodococcus erythropolis DSM44534	3	31 (±1.8)	-	4 (±0.1)	27 (±1.7)
	7	63 (±2.9)	5 (±0.1)	6 (±0.4)	52 (±2.4)
	11	94 (±4.6)	7 (±0.8)	8 (±0.9)	79 (±2.9)
Rhodococcus rhodnii PCM2157	3	21 (±2.6)	-	6 (±0.5)	15 (±2.1)
	7	23 (±2.5)	-	6 (±0.5)	17 (±2.0)
	11	31 (±3.6)	-	7 (±1.1)	24 (±2.5)
Rhodococcus rhodochrous PCM909	3	25 (±3.2)	-	-	25 (±3.2)
	7	41 (±4.7)	-	10 (±1.0)	31 (±3.7)
	11	50 (±3.1)	-	11 (±0.8)	39 (±2.3)
Rhodococcus ruber PCM2166	3	44 (±2.9)	-	10 (±1.1)	34 (±1.8)
	7	70 (±3.3)	6 (±0.1)	13 (±0.7)	51 (±2.5)
	11	98 (±2.0)	8 (±0.5)	13 (±1.0)	77 (±2.0)
Rhodococcus ruber PCM2171	3	15 (±0.9)	-	4 (±0.1)	11 (±0.8)
	7	35 (±2.4)	-	13 (±0.6)	22 (±1.8)
	11	41 (±3.6)	-	12 (±1.5)	29 (±2.1)
Rhodococcus ruber PCM2216	3	20 (±1.8)	-	-	20 (±1.8)
	7	24 (±1.8)	-	-	24 (±1.8)
	11	25 (±2.5)	-	-	25 (±2.5)
Serratia liquefaciens PCM2830	3	25 (±2.3)	-	10 (±0.8)	15 (±1.5)
	7	30 (±3.9)	-	13 (±1.5)	17 (±2.4)
	11	54 (±4.0)	5 (±0.3)	14 (±0.8)	35 (±2.9)
Serratia marcescens PCM549	3	7 (±0.9)	-	-	7 (±0.9)
	7	28 (±2.5)	-	5 (±0.6)	23 (±1.9)
	11	33 (±2.6)	-	6 (±0.3)	27 (±2.3)
Serratia plumuthica PCM550	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-
Serratia sp. PCM1324	3	21 (±1.7)	-	7 (±0.3)	14 (±1.4)
	7	45 (±3.2)	-	12 (±0.7)	33 (±2.5)
	11	59 (±3.5)	8 (±0.4)	12 (±0.9)	39 (±2.2)
Streptomyces griseus subsp. *Griseus* PCM2331	3	0	-	-	-
	7	0	-	-	-
	11	0	-	-	-

%, determined by GC

Table S5. Percentage of haemolysis of human RBCs after a 1h incubation with the compounds at various concentrations. The results are shown as mean values ± standard deviations.

Compound	Concentration [µM]						
	0	10	20	40	60	80	100
Control	1.340 ± 0.416	1.583 ± 0.202	1.741 ± 0.007	1.820 ± 0.188	2.017 ± 0.091	1.756 ± 0.363	2.146 ± 0.105
1a	1.340 ± 0.416	2.412 ± 0.481	1.544 ± 0.202	2.180 ± 0.153	2.397 ± 0.446	2.585 ± 0.014	2.550 ± 0.007
1b	1.340 ± 0.416	2.279 ± 0.251	1.864 ± 0.293	1.978 ± 0.272	1.884 ± 0.251	2.387 ± 0.349	2.575 ± 0.321
1c	1.340 ± 0.416	1.460 ± 0.080	1.171 ± 0.020	1.806 ± 0.302	1.848 ± 0.027	1.412 ± 0.295	1.507 ± 0.576
2a	1.340 ± 0.416	1.273 ± 0.319	1.665 ± 0.175	1.475 ± 0.034	1.767 ± 0.155	1.250 ± 0.263	2.042 ± 0.067
2b	1.340 ± 0.416	1.038 ± 0.275	1.081 ± 0.107	1.005 ± 0.295	1.317 ± 0.255	1.787 ± 0.382	1.706 ± 0.054
2c	1.340 ± 0.416	1.156 ± 0.027	1.858 ± 0.697	1.422 ± 0.550	1.744 ± 0.590	1.938 ± 0.154	2.464 ± 0.322
3a	1.340 ± 0.416	1.362 ± 0.296	1.143 ± 0.159	2.235 ± 0.130	2.536 ± 0.267	2.199 ± 0.108	2.500 ± 0.390
3b	1.340 ± 0.416	2.451 ± 0.509	2.979 ± 0.153	1.904 ± 0.209	2.294 ± 0.635	2.397 ± 0.209	2.604 ± 0.265
3c	1.340 ± 0.416	1.834 ± 0.355	1.649 ± 0.509	1.725 ± 0.174	2.327 ± 0.436	2.152 ± 0.161	1.526 ± 0.134
4a	1.340 ± 0.416	1.760 ± 0.152	1.480 ± 0.173	1.669 ± 0.195	1.577 ± 0.180	2.475 ± 0.152	1.786 ± 0.159
4b	1.340 ± 0.416	1.635 ± 0.127	1.517 ± 0.375	1.417 ± 0.074	1.199 ± 0.395	1.185 ± 0.040	2.057 ± 0.536
4c	1.340 ± 0.416	1.483 ± 0.007	1.934 ± 0.228	2.099 ± 0.395	1.768 ± 0.342	2.024 ± 0.047	2.445 ± 0.013
5a	1.340 ± 0.416	1.379 ± 0.047	1.161 ± 0.047	1.753 ±0.134	1.881 ± 0.020	2.047± 0.080	2.185± 0.261
5b	1.340 ± 0.416	1.270 ± 0.094	1.550 ± 0.101	1.521 ± 0.181	1.621± 0.174	2.583 ± 0.288	1.981± 0.308

Table S6. Fluorescence anisotropy values of the DPH probe in membranes of RBCs treated with the compounds at 37 °C . The results are shown as mean values ± standard deviations.

Compound	Concentration (µM)			
	0	20	60	100
Control	0.266 ± 0.003	0.267 ± 0.006	0.269 ± 0.006	0.266 ± 0.005
1a	0.266 ± 0.003	0.262 ± 0.016	0.264 ± 0.030	0.237 ± 0.041
1b	0.266 ± 0.003	0.247 ± 0.018	0.274 ± 0.036	0.252 ± 0.012
1c	0.266 ± 0.003	0.260 ± 0.011	0.267 ± 0.019	0.277 ± 0.078
2a	0.266 ± 0.003	0.262 ± 0.020	0.268 ± 0.023	0.267± 0.006
2b	0.266 ± 0.003	0.286 ± 0.015	0.277 ± 0.028	0.278 ± 0.011
2c	0.266 ± 0.003	0.236 ± 0.026	0.240 ± 0.019	0.238 ± 0.002
3a	0.266 ± 0.003	0.263 ± 0.005	0.261 ± 0.009	0.259 ± 0.008
3b	0.266 ± 0.003	0.272 ± 0.019	0.249 ± 0.083	0.262 ± 0.056
3c	0.266 ± 0.003	0.239 ± 0.006	0.249 ± 0.007	0.244 ± 0.003
4a	0.266 ± 0.003	0.264 ± 0.004	0.261 ± 0.008	0.269 ± 0.007
4b	0.266 ± 0.003	0.289 ± 0.004	0.228 ± 0.095	0.290 ± 0.003
4c	0.266 ± 0.003	0.257 ± 0.014	0.243 ± 0.019	0.263 ± 0.008
5a	0.266 ± 0.003	0.259 ± 0.009	0.272 ± 0.085	0.256 ± 0.023
5b	0.266 ± 0.003	0.268 ± 0.025	0.277 ± 0.003	0.272 ± 0.058

8. DOROBEK NAUKOWY

8.1 Publikacje

1. Hernik, D.; Pannek, J.; Szczepańska, E.; Olejniczak, T.; Boratyński, F.
   Bacterial whole cells synthesis of whisky lactones in a solid-state fermentation bioreactor prototype. *Catalysts*, 2021, 11, 320.
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   Chemo-enzymatic synthesis and biological activity evaluation of propenylbenzene derivatives. *Frontiers in Microbiology*, 2023, 14, 1223123.

8.2 Monografie

1. Hernik D., Boratyński F. 2018. Wykorzystanie zielonych rozpuszczalników w biotransformacjach, Nauka Badania i Doniesienia Naukowe 2018 Część II Nauki Przyrodnicze i Medyczne, ISBN 978-83-951445-1-6.

8.3 Patenty

1. Pannek J. Hernik D., Olejniczak T. Boratyński F., Szczepańska E., Gach J.
   Sposób wytwarzania (+)-izomeru-(3*R*)-3-butyloftalidu.
   P.430872_80_19 (14.08.2019)/22.07.2021

8.4 Zgłoszenia patentowe

1. Hernik D., Szczepańska E., Patejuk K., Olejniczak T. Boratyński F.
   Sposób wytwarzania piperonalu
   Zgłoszenie oznaczono numerem: P.444274 (31.03.2023)
2. Hernik D., Szczepańska E., Patejuk K., Olejniczak T. Boratyński F.
   Sposób wytwarzania piperonalu
   Zgłoszenie oznaczono numerem: P.444273 (31.03.2023)

8.5 Komunikaty konferencyjne

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8.6 Projekty badawcze

„UPWR 2.0: międzynarodowy i interdyscyplinarny program rozwoju Uniwersytetu Przyrodniczego we Wrocławiu, moduł V zadanie 4 – Interdyscyplinarna Międzynarodowa Szkoła Doktorska” nr POWR.03.05.00-00-Z062/18 z dnia 4 czerwca 2019 r., realizowanym przez Uniwersytet Przyrodniczy we Wrocławiu, współfinansowany ze środków Unii Europejskiej w ramach Europejskiego Funduszu Społecznego. Wysokość finansowania 261 360 zł. Czas realizacji 01.10.2019-30.09.2023 - udział w projekcie.