Multi-enzymatic routes for the targeted synthesis of enantiopure vicinal amino alcohols

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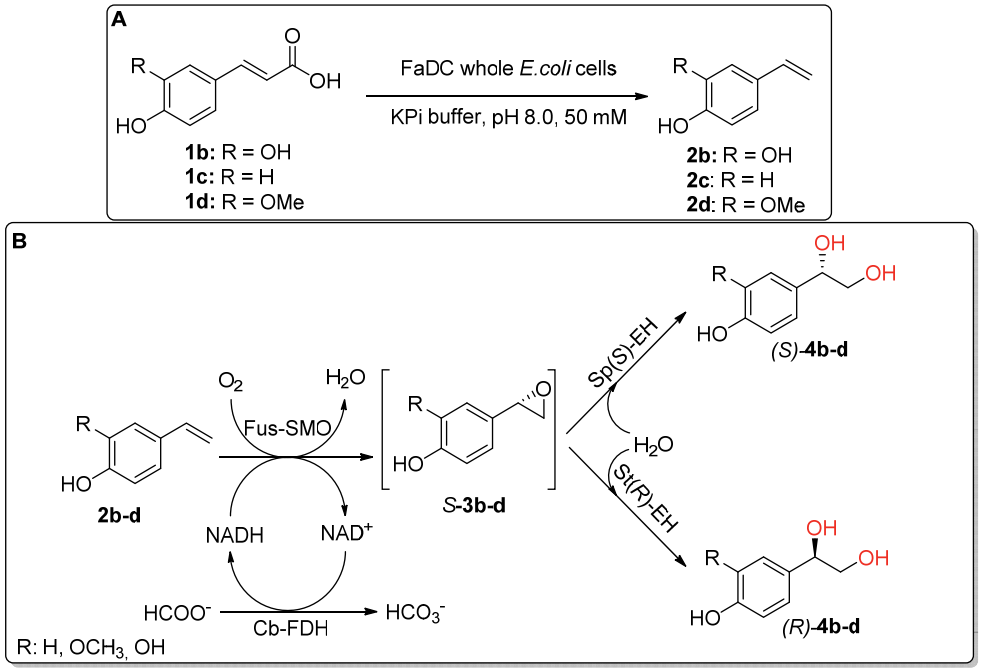
Chapter 6

Towards the synthesis of valuable chiral vicinal amino alcohols from potential renewable raw materials

6.1 Introduction

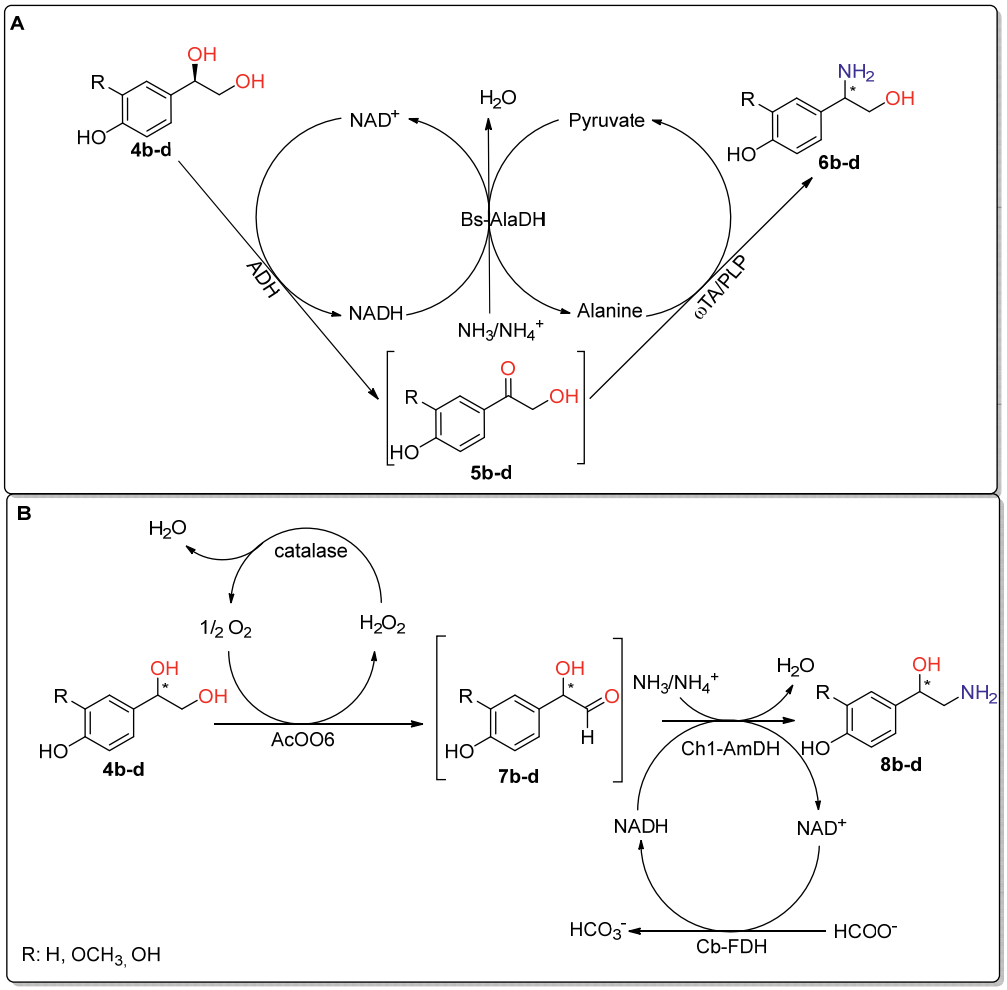
Alkenes are highly reactive substrates due to their versatile C-C double bond. However, the origin of these compounds still relies on fossil-based feedstock. Alternative greener raw materials are needed to meet the requirements for a sustainable development towards a circular economy. For instance, a certain class of aromatic olefins can be derived from the decarboxylation of potential renewable substrates, such as caffeic, *p*-coumaric and ferulic acids. These hydroxycinnamic acids are very common in fruits and vegetables.[1] Moreover, they constitute the main units in the various forms of lignin found within different plant species.[2] Decarboxylation of such compounds is usually performed by heating up to reflux a solution of the carboxylic acid in a high boiling organic solvent, e.g., dimethylformamide (DMF).[3, 4] On the other hand, enzymatic systems are known to perform such reaction under milder conditions. Among these methods, ferulic acid decarboxylase from *Enterobacter* sp. (FaDC) was recently applied for the decarboxylation of these type of substrates.[5] In this study, we investigated the potential of the multi-enzymatic cascades developed in this thesis for accessing high valuable vicinal amino alcohols (e.g., adrenaline, *p*-synephrine, normetanephrine)[6, 7] from potential renewable raw materials. Indeed, the aim of this project is to prepare the olefin substrates through the enzymatic decarboxylation of the related carboxylic acids **1b-d** (caffeic, coumaric and ferulic acids) catalyzed by FaDC. In the subsequent steps, the one-pot cascade for the synthesis of the corresponding diols **4b-d** catalyzed by the chimeric styrene monooxygenase (Fus-SMO)[8] and two stereocomplementary epoxide hydrolases (EHs)[9] is implemented (**Scheme 6.1**).[10] Notably, there are no publications about the bio-epoxidation of these aromatic mono- and di-hydroxylated olefin substrates catalyzed by flavin-dependent styrene monooxygenase systems.



Scheme 6.1. **A)** Decarboxylation of potential renewable carboxylic acid catalyzed by a ferulic acid decarboxylase (FaDC); **B)** one-pot cascade for the dihydroxylation of aromatic olefins catalyzed by a Fus-SMO paired with two EHs.

The next step would be the conversion of these highly functionalized diols **4b-d** to the corresponding vicinal amino alcohols, as depicted in **Scheme 6.2**. This can be accomplished by pairing in a one-pot selected alcohol dehydrogenases (in this case, either Aa-ADH from *Aromatoleum aromaticum*[11] or Bs-BDHA from *Bacillus subtilis* BGSC1A1[12, 13]) with stereocomplementary ω-transaminases (in this case, Cv(*S*)-ωTA from *Chromobacterium violaceum* DSM 30191[14] and At(*R*)- ωTA from *Aspergillus terreus*[15, 16]), as described previously in chapters 4 and 5. On the other hand, another cascade was developed previously for accessing amino alcohols bearing a terminal amino group. This involves the coupling of an alcohol oxidase (AcCO6)[17] with an amine dehydrogenase (Ch1-AmDH).[18]

Overall, this chapter provides a proof-of-concept for the potential application of these routes for the synthesis of this class of amino alcohols. Nevertheless, conclusions cannot be drawn based on these results, yet, because significantly more investigation is required to fairly assess the actual relevance of this approach.



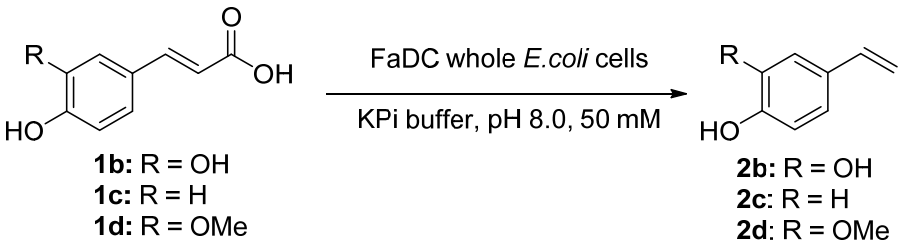
Scheme 6.2. **A)** one-pot cascade approach for the conversion of diols **4b-d** catalyzed by an alcohol dehydrogenase (ADH) paired with stereocomplementary ω-transaminases (ω-TAs) for accessing amino alcohols **6b-d**; **B)** one-pot cascade route for the conversion of diol **4b-d** catalyzed by an alcohol oxidase (AcCO6) paired with an amine dehydrogenase (Ch1-AmDH) for the synthesis of amino alcohols **8b-d**.

6.2 Results and discussion

6.2.1 Testing various conditions for the synthesis of chiral diols from caffeic acid and derivatives

6.2.1.1 Synthesis of olefin substrates by enzymatic decarboxylation of carboxylic acids

Initially, we investigated the decarboxylation step by screening the activity of lyophilized *E. coli* whole cells (20 mg mL^{-1}) carrying overexpressed ferulic acid decarboxylase from *Enterobacter* sp. (FaDC) on three substrates, namely: caffeic acid (**1b**), cumaric acid (**1c**) and ferulic acid (**1d**) at 50 mM concentration (**Scheme 6.3**). The biotransformations were carried out in KPi buffer (pH 8.0, 50 mM) and glass vials were used to prevent (or alleviate) volatility of the olefin products. The decarboxylation of all three substrates proceeded quantitatively into the desired aromatic olefin products in 24 h at 30 °C. We did not perform any further optimization at this stage.

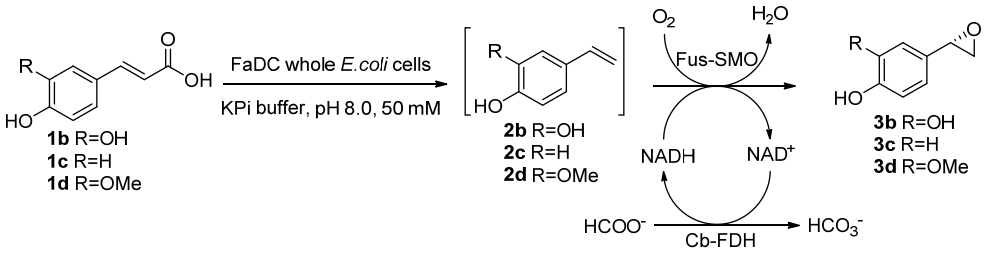


Scheme 6.3. Reaction scheme for the decarboxylation step catalyzed by ferulic acid decarboxylase as lyophilized *E. coli* whole cells

6.2.1.2 Towards the bio-catalytic synthesis of chiral epoxides from caffeic acid substrates

The next step was to investigate the possibility of pairing, in a one-pot fashion, the decarboxylation and the epoxidation steps by adding the fused-styrene monooxygenase (Fus-SMO),[8] thereby obtaining enantiopure epoxides from the correspondent carboxylic acids (50 mM), as depicted in **Scheme 6.4**. The first attempt was to perform the two reactions in a stepwise fashion. Therefore, the decarboxylation step was performed first for 24 h followed by the addition of the reagents required for the bio-epoxidation of the generated olefins. The latter step was performed by using lyophilized *E. coli* whole cells carrying the co-expressed Fus-SMO/Cb-FDH system (10 mg mL^{-1}) for 6 h reaction time. Both steps were carried out in KPi buffer (pH 8.0, 50 mM). However, while the FaDC is a coenzyme-independent enzyme, the Fus-SMO/Cb-FDH system requires the addition of some

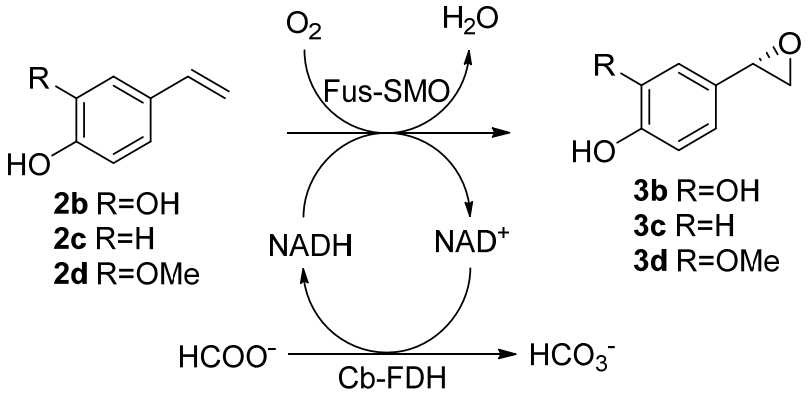
reagents, namely: FAD (50 µM), NAD^+ (1 mM), HCOONa (250 mM, 5 eq.) and a catalase (0.1 mg mL^{-1}). Moreover, the epoxidation step is usually performed in a biphasic system by using *n*-heptane as the organic solvent, which was added together with the reagents mentioned above.[8] After a careful analysis of the outcome of this biotransformation, we observed the decarboxylated products. However, no traces of the epoxides were detected. We then proceeded by performing the two steps in a concurrent mode under the same reaction conditions described above. Hence, all the reagents required for both reactions were added in the same pot from the beginning. Surprisingly, in this case, even the decarboxylation of carboxylic acids **1b-d** did not proceed, and the substrates were the only compounds detected after 24 h. Hence, based on these results, we excluded the possibility to perform the two steps in a one-pot cascade because of deactivation and incompatibility of the enzymatic systems.



Scheme 6.4. One-pot decarboxylation/epoxidation of carboxylic acid substrates **1b-d** catalyzed by lyophilized *E. coli* whole cells.

The next step was to investigate the bio-epoxidation of aromatic olefins **2b-d** (20 mM), since no epoxides were detected when the biotransformation was carried out in the same pot with the decarboxylase. Thus, we aimed at elucidating whether the lack of epoxidation activity stemmed from the decarboxylation step due to incompatibility with either FaDC or the carboxylic acid substrates. Furthermore, we could examine the activity of the Fus-SMO/Cb-FDH system on these hydroxyl functionalized aromatic olefins, since no data were available in literature for the bio-epoxidation of these types of substrates catalyzed by flavin-dependent styrene monooxygenases. While substrates **2c-d** are commercially available (**2c** as 10% solution in propylene glycol), substrate **2b** was chemically synthesized. Nevertheless, we found out that olefins **2b-d** are not soluble in *n*-heptane, hence, we carried out the bio-epoxidation in KPi buffer only (pH 8.0, 50 mM). Additionally, the reaction depicted in **Scheme 6.5** was tested with whole *E. coli* cells carrying

either the co-expressed Fus-SMO/Cb-FDH system or only the Fus-SMO enzyme. In this last case, purified Cb-FDH (10 µM) was then added separately.



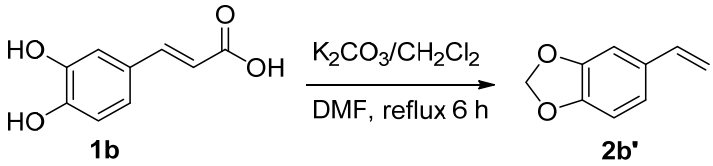
Scheme 6.5. Bio-epoxidation of aromatic olefins **2b-d** catalyzed by a fused-styrene monooxygenase.

The outcome of these biotransformations was examined first by direct analysis of the aqueous phase by RP-HPLC. Unfortunately, the reactions did not proceed as expected and none of the epoxides were detected. Notably, **2b-c** were not observed either, whereas **2d** was the sole substrate that could be recovered back; therefore, it seems that **2d** is not accepted by our enzymatic system at all. Two main hypotheses were raised at this stage: *i)* the targeted epoxides are highly unstable and, once formed, they might undergo spontaneous ring-opening to the corresponding diols; *ii)* substrates **2b-c** are the most polar of the tested series, therefore either the substrates or the corresponding epoxide/diol products might be difficult to extract. In contrast, substrate **2d**, the least polar, is surely not accepted by the Fus-SMO because it is recovered at the end of the reaction. We analyzed the aqueous phase to ascertain the possible presence of diols (e.g., TLC analysis of aqueous and organic layers; extraction of the aqueous layer with a mixture of EtOAc:MeOH (95:5) and analysis by GC-MS; protection of the hydroxyl moieties as acetates for both the organic and aqueous phase). However, none of these analyses and methods could reveal any presence of either substrates **2b-c**, or epoxide, or diol products. Therefore, it might be that either the olefin substrates (**2b-c**), or the epoxides (**3b-c**), or the corresponding diol products cannot be easily extracted from the aqueous buffer containing the lyophilized cells.

6.2.1.3 (Chemo)-enzymatic synthesis of protected 4-vinylbenzene-1,2-diol from caffeic acid

6.2.1.3.1 Chemical synthesis of protected vinyl catechol (2b') from caffeic acid

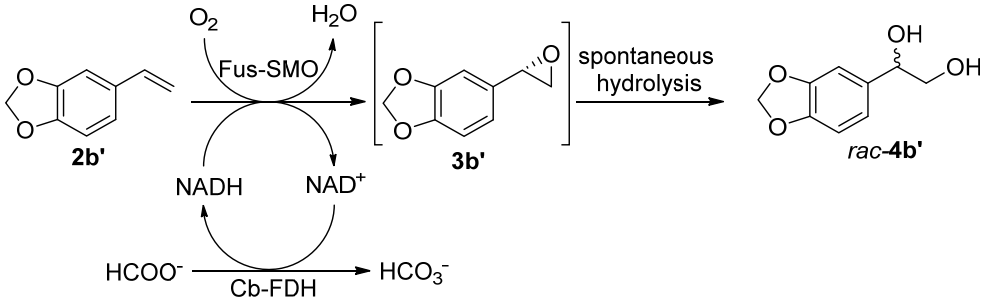
To evaluate the possibility that the highly polar substrates can interact with our bio-epoxidation system, we focused on testing the protected olefin substrates and caffeic acid **1b** was initially used as the model substrate. The decarboxylation and protection steps were carried out in the same pot by following a literature procedure.[4] Caffeic acid in dimethylformamide (DMF) was refluxed for 6 hours in the presence of potassium carbonate (K_2CO_3) and CH_2Cl_2 yielding the desired product **2b'** (30%).



Scheme 6.6. One-pot decarboxylation and protection of caffeic acid.

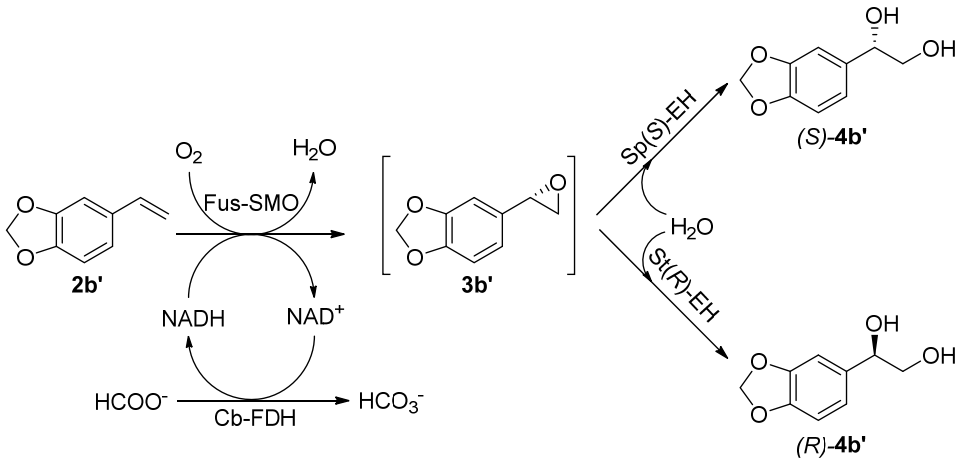
6.2.1.3.2 One-pot enzymatic cascade for the synthesis of chiral diols 4b'

The next step was to perform the bio-epoxidation by using the Fus-SMO/Cb-FDH (10 mg mL^{-1}) system on the protected vinyl catechol substrate **2b'** (22 mM). The biotransformation was performed under optimized conditions: KPi/heptane buffer (pH 8.0, 50 mM; 1:1 v v^{-1}; 5 mL final reaction volume), NAD^+ (1 mM), HCOONa (110 mM, 5 eq.), FAD (50 µM), catalase (0.1 mg mL^{-1}) as previously reported.[8] The qualitative analysis by GC-MS showed the quantitative conversion of substrate **2b'**. It should be noted that no compounds were detected in the *n*-heptane phase. The EtOAc extract analysis showed the presence of traces of epoxide **3b'** and the main product observed was the hydrolyzed diol product **4b'**, as depicted in **Scheme 6.7**. In an attempt to prevent the spontaneous hydrolysis of the *in situ* generated epoxide, we performed the bio-epoxidation of substrate **2b'** (20 mM) at different pH values of KPi buffer (pH = 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0) under the above-mentioned reaction conditions. From GC-MS analysis, we did not observe any significant influence of the pH on the outcome of the reaction. In fact, under all tested pH values, the substrate **2b'** was completely converted and the diol product **4b'** was the only compound observed after extraction. Nevertheless, slightly acidic and neutral pH values (pH 6.6 and 7.0) afforded slightly better results. At this stage, we also determined the enantiomeric excess of the spontaneously formed diol product by chiral HPLC analysis, which, unfortunately, turned out to be a racemic mixture.



Scheme 6.7. Bio-epoxidation of substrate **2b'** catalyzed by Fus-SMO/Cb-FDH system followed by spontaneous hydrolysis of the *in situ* generated epoxide.

Based on these results, we hypothesized that the presence of the epoxide hydrolase in the same pot of the bio-epoxidation would enable the stereoselective opening of the epoxide intermediate. Thus, we performed the one-pot enzymatic epoxidation/hydrolysis on substrate **2b'** (20 mM) by pairing the co-expressed Fus-SMO/Cb-FDH system (20 mg mL^{-1}) with two stereocomplementary epoxide hydrolases,[9] either Sp(*S*)-EH from *Sphingomonas sp.* HXN200 or St(*R*)-EH from *Solanum tuberosum*, as depicted in **Scheme 6.8**. The biotransformations were carried out under the same reaction conditions described above with the exception that lyophilized *E. coli* whole cells carrying the desired EH (20 mg mL^{-1}) were added in the same pot. Moreover, different pH values (6.6, 7.0 and 8.0; KPi buffer) were tested since slightly better results were observed in the bio-epoxidation step, as described above.

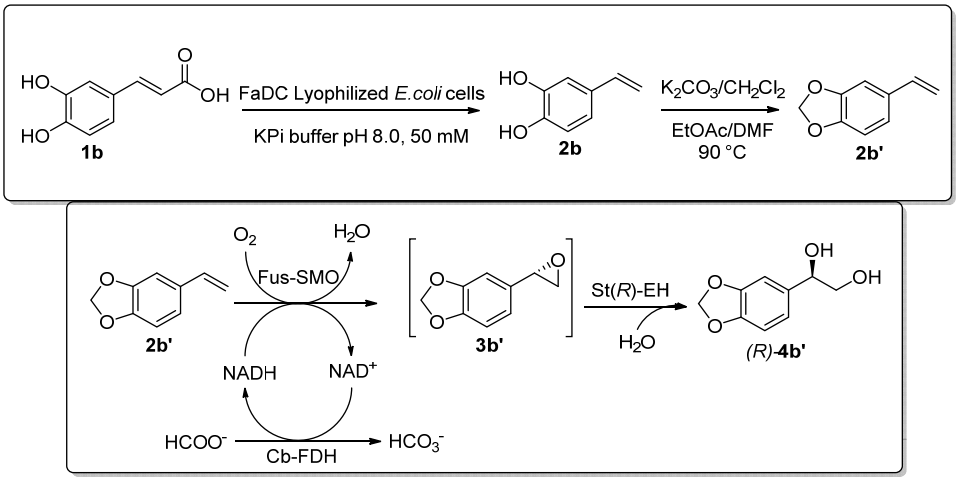


Scheme 6.8. One-pot enzymatic cascade for the epoxidation/hydrolysis of substrate **2b'**.

Based on qualitative GC-MS analysis, the conversion of substrate **2b'** did not proceed to completion. The unreacted substrate was detected only in the *n*-heptane phase, while the diol products were the sole detected compounds after extraction with EtOAc. Next, we determined by chiral HPLC the enantiomeric excess of the diol **4b'** coming from each combination of enzymes. As expected, the presence of the EH in the same pot of the epoxidation reaction enabled the formation of both enantiomers of diol **4b'** with high enantioselectivity, hence showing that the presence of the epoxide hydrolase prevented non-stereoselective ring-opening of the epoxide intermediate. The best results in terms of enantioselectivity were observed at slightly acidic pH (6.6), thereby giving the (*S*)-**4b'** with *ee* 86% and (*R*)-**4b'** with *ee* >98%. In contrast, the enantioselectivity dropped significantly for (*S*)-**4b'** at both pH 7.0 and 8.0 (*ee* 73% and 75%, respectively), while the *R*-enantiomer was still obtained with high enantiomeric excess (92% and 97%, respectively). The low enantioselectivity observed in the case of (*S*)-**4b'** may stem from a combination of two factors: *i*) the intrinsic lower selectivity of Sp(*S*)-EH compared with St(*R*)-EH, as previously observed[10]; *ii*) the higher pH values (7.0 and 8.0) favor the spontaneous non-stereoselective ring-opening of the epoxide intermediate.

6.2.1.3.3 Chemo-enzymatic route to give access to protected (*R*)-4-vinylbenzene-1,2-diol ((*R*)-4b') from caffeic acid (1b)

At this point, we could access both enantiomers of diol **4b'**, albeit we could not perform all of the reactions in one-pot starting from caffeic acid **1b**. Thus, we decided to perform the whole transformation of **1b** to optically active diol **4b'** through two distinct cascade reactions. The first route was the chemo-enzymatic decarboxylation/protection of caffeic acid, followed by a one-pot enzymatic cascade for the conversion of the isolated **2b'** to the chiral *R*-**4b'** (**Scheme 6.9**).

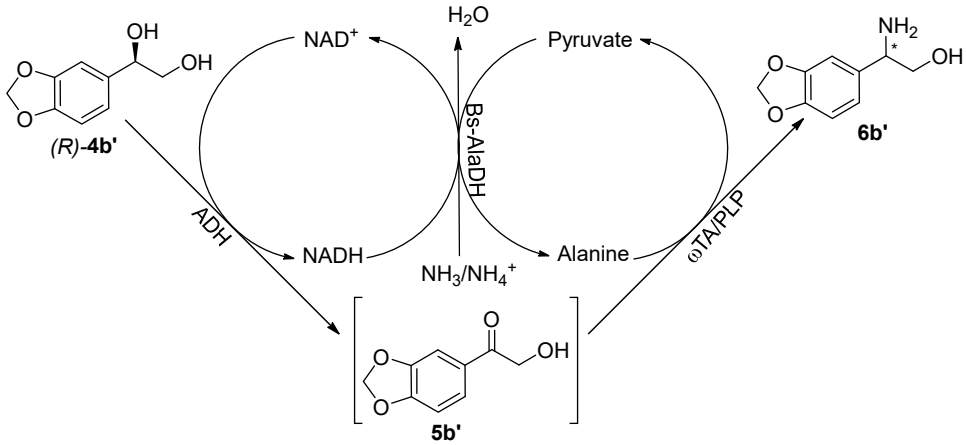


Scheme 6.9. Chemo-enzymatic route for the asymmetric synthesis of diol *R*-**4b'** from caffeic acid **1b**.

The first cascade was performed in a one-pot sequential mode: first, decarboxylation of caffeic acid **1b** (50 mM, 11.1 mmol) was carried out catalyzed by lyophilized *E. coli* whole cells carrying overexpressed FaDC (20 mg mL^{-1}) in KPi buffer (pH 8.0, 50 mM; 222 mL total reaction volume) for 24 h at 30 °C. After removal of the cells debris and extraction of olefin **2b** in EtOAc, the reagents for the protection step were added by assuming quantitative conversion of **1b** into **2b**. The protected vinyl catechol **2b'** was then used as substrate for the follow-up cascade for the synthesis in 100 mg scale of chiral diol (*R*)-**4b'** under the same conditions reported in paragraph 6.2.1.3.2.

6.2.2 Multi-enzymatic cascade for the synthesis of vicinal amino alcohols

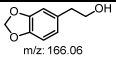
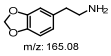
With the chiral diol in hands, the next step was to test the one-pot multi enzymatic cascade depicted in **Scheme 6.10** for the synthesis of the targeted vicinal amino alcohol **6b'**. The cascade comprises an alcohol dehydrogenase (50 µM) (either Aa-ADH from *Aromatoleum aromaticum*[11] or Bs-BDHA from *Bacillus subtilis* BGSC1A1)[12, 13] and one between the two stereocomplementary ω-transaminases (50 µM) namely At(*R*)-ωTA from *Aspergillus terreus*[15, 16] and Cv(*S*)-ωTA from *Chromobacterium violaceum* DSM 3019.[14] As described in chapter 5, the cascade was performed in $HCOONH_4$ buffer (pH 8.5, 1 M) supplemented with NAD^+ (1 mM), PLP (1 mM), D- or L-alanine (5 eq.) and Bs-AlaDH[19] (20 µM).



Scheme 6.10. One-pot multi-enzymatic cascade for the conversion of chiral diol **4b'** into the targeted vicinal amino alcohol **6b'.**

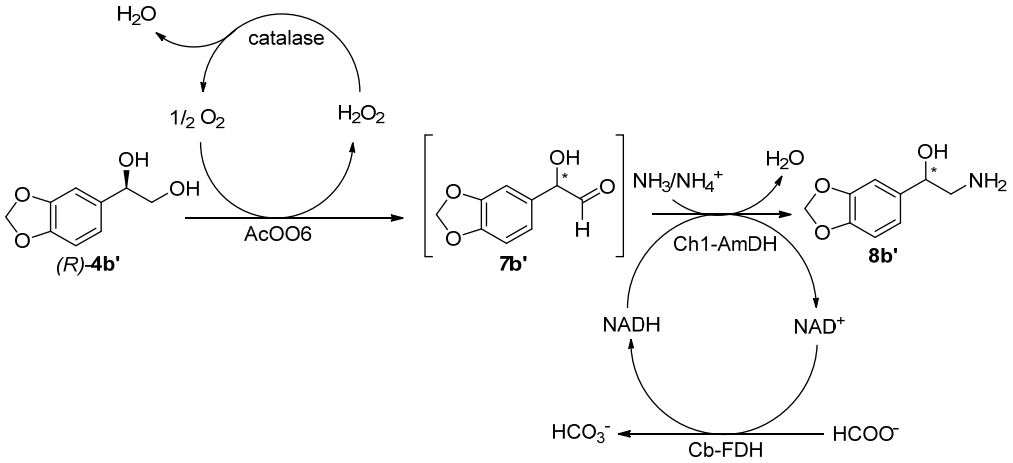
As show in **Table 6.1**, the formation of the targeted amino alcohol **6b'** was observed when either one of the two tested ADHs was combined with At(*R*)-ωTA (entry 2 and 4). However, Aa-ADH displayed higher activity compared with Bs-BDHA under the tested reaction conditions. Moreover, in both cases, intermediate **5b'** along with other compounds were detected. Two of them could be identified by GC-MS analysis, namely 2-(benzo[d][1,3]dioxol-5-yl)ethan-1-ol (**9b'**) and 2-(benzo[d][1,3]dioxol-5-yl)ethan-1-amine (**10 b'**), as depicted in **Table 6.1**. Conversely, the third observed compound could not be determined by mass analysis only, however, these reactions were performed in analytical scale, hence we could not analyze at this stage the reaction outcome by ^{1}H-NMR, for instance. On the other hand, conversion into the desired amino alcohol **6b'** was not detected when Cv(*S*)-ωTA was paired with the ADHs (entry 1 and 3). In contrast, in both cases, the main product formed was the intermediate **5b'**. These tests represented a preliminary investigation to assess the potentiality of this cascade; we acknowledge that an in-depth investigation is required to quantitatively determine the formation of the target amino alcohol. Moreover, the enantiomeric excess of the amino alcohol **6b'** was not determined at this stage. In fact, a suitable analytical method for *ee* analysis was still required and must be explained in the next step of this study along with the optimization of the cascade itself (e.g., testing other transaminases; balance enzymes loading; etc.).

Table 6.1. Preliminary results for the conversion of (*R*)-**4b'** (20 mM) catalyzed by ADH coupled with ωTA in a one-pot fashion.

Entry	1st step	2nd step	5b' [%][a]	6b' [%][a]	 m/z: 166.06 **9b'** [%][a]	 m/z: 165.08 **10b'** [%][a]	Unidentified [%][a]
1	Aa-ADH	Cv(*S*)-ωTA	43±1	n.d.	10±1	14±1	33±3
2	Aa-ADH	At(*R*)-ωTA	12±1	40±2	9±2	12±2	25±4
3	Bs-BDHA	Cv(*S*)-ωTA	33±2	n.d.	46±7	n.d.	21±4
4	Bs-BDHA	At(*R*)-ωTA	31±3	7±2	41±6	n.d.	20±4

[a]Qualitative conversions analyzed by GC-MS; the results reported are the average of two experiments; enantiomeric excess was not determined.

We have performed preliminary tests for synthesizing the regioisomer **8b'** through a one-pot multi enzymatic cascade as previously described in chapter 5. In this case, the alcohol oxidase AcCO6 variant from choline oxidase[17] was paired with the amine dehydrogenase Ch1-AmDH.[18] The cascade was carried out in $HCOONH_4$ (pH 8.5, 1 M) supplemented with NAD^+ (1 mM), catalase (0.1 mg mL^{-1})—for the disproportion of the *in situ* formed H_2O_2—and a formate dehydrogenase (Cb-FDH)[20]—for NADH recycling. Nevertheless, conversion of substrate (*R*)-**4b'** was not observed under the tested conditions and more investigation is needed in order to optimize the cascade and identify the potential issues.



Scheme 6.11. One-pot enzymatic cascade for the conversion of substrate *R*-**4b'** to chiral **8b'**.

6.3 Summary and future prospects

The present study was a proof of concept, which requires further investigation for possible application in the enzymatic synthesis of high valuable vicinal amino alcohols such as adrenaline and derivatives. In summary, we were able to obtain three aromatic olefins from the corresponding carboxylic acids, the latter of which could be sourced from renewable feedstocks. Moreover, we identified a suitable chemo-enzymatic route for the enzymatic synthesis of the high valuable optically active diol **4b'** by two sequential one-pot cascades. First, the olefin substrate was obtained by enzymatic decarboxylation of caffeic acid followed by chemical protection of the catechol moiety. In the second step, the free catechol was identified as the main issue in performing the epoxidation catalyzed by Fus-SMO due to the fact that catechol moieties are prone to further oxidation. This observation aligns with the fact that it is not possible to find in the literature any epoxidation of these types of compounds catalyzed by the styrene monooxygenase system. Thus, we performed the one-pot enzymatic epoxidation of olefin **2b'** by pairing the Fus-SMO with two stereocomplementary EHs in order to form both enantiomers of diol **4b'** ((*S*)-**4b'** 86% *ee*; (*R*)-**4b'** >98% *ee*). Nevertheless, this one-pot cascade requires some further optimization, such as the test of different pH values and types of buffers, and the exact quantification of the system's productivity. It is known that high pH values can lead to faster oxidation of catechols. Moreover, the use of a different buffers might also help to increase the enantiomeric excess of the (*S*)-enantiomer of diol **4b'**. It would also be interesting to investigate the possibility to perform the epoxidation/hydrolysis in an alternative biphasic system by using a "green" organic solvent which is more suitable for these highly polar compounds. Furthermore, the Fus-SMO/EH system could be investigated by using other substrates such as coumaric and ferulic acids. For this purpose, it will be necessary to evaluate different protecting groups for the hydroxyl substituent of the aromatic ring. Nevertheless, other enzymatic systems can be explored for the synthesis of the diol substrates. For example, naphthalene dioxygenase (NDO) is an enzyme that catalyzes the direct dihydroxylation of aromatic alkenes. Hence, the coupling of FaDC and NDO could be a viable alternative route to the one-pot epoxidation/hydrolysis of olefins. Regarding the one-pot cascade for the conversion of diols such as **4b'** to chiral amino alcohol such as **6b'**, more tests are necessary to assess the actual performance of the enzymatic system as well as determine the enantiomeric excess of the final product. Moreover, the combination of other ADHs and TAs could be investigated on both

enantiomers of **4b'** along with system's optimization. Finally, the one-pot cascade to synthesize the regioisomer amino alcohols such as **8b'** did not lead to any conversion of the diol substrate. Indeed, this system needs in-depth studies. An interesting approach would be the identification of other oxidases to perform the oxidation of the diol substrate as well as the use of other amine dehydrogenases or alternative aminating enzymes. Moreover, the use of computational biocatalysis might elucidate what is the actual issue, for instance, by identifying the interaction between the substrate and the enzymes involved. This work would provide the required knowledge to create new variants by protein engineering. This latter point is probably critical for the development of the herein described multi-enzymatic cascades for the synthesis high-value amino alcohols. In fact, the need to expand the substrate scope of these classes of enzymes and discover other enzymes will play a central role towards this aim.

6.4 Experimental section

General information. ADHs and ωTAs used in this study were expressed and purified as reported in chapter 5. FaDC was overexpressed in *E. coli* (50 μg mL^{-1} kanamycin; 0.5 mM IPTG, 25 °C overnight) according to the general enzymes expression protocol reported in chapter 3 and used as lyophilized whole cells.

General procedure for the biocatalytic decarboxylation of 1b-d. Lyophilized *E. coli* cells carrying FaDC (30 mg mL^{-1}) were rehydrated in 1.5 mL KPi buffer (50 mM, pH 8.0) in a 4 mL glass vial. After that, substrate **1b-d** (50 mM) was added. The mixture was shaken at 30 °C and 170 rpm on an orbital shaker for 24 h. Work-up was performed by acidification with HCl (1 M) followed by extraction with EtOAc (2 x 750 μL). Conversion was measured by GC-FID after derivatization or directly by RP-HPLC (100 μL aq. phase diluted with 1 mL H_2O:MeOH (1:1 v v^{-1} + 3% TFA), incubated at room temperature for 15 min, centrifuged and injected in RP-HPLC).

General derivatization procedure for GC-FID analysis. The extract (200 μL) was diluted with MeOH (200 μL) and EtOAc (600 μL) followed by addition of (trimethylsilyl)diazomethane (20 μL). The reaction was shaken at 30 °C, 170 rpm for 1 h. The excess of the derivatization reagent was neutralized by addition of acetic acid (4 μL). The mixture was shaken at 30 °C, 170 rpm for 30 minutes and analyzed by GC-FID.

Procedure for the one-pot reactions to epoxides 3b-d from carboxylic acids 1b-d. Lyophilized *E. coli* cells carrying FaDC (20 mg mL^{-1}) were rehydrated in 1.5 mL KPi buffer (50 mM, pH 8.0) in a 4 mL glass vial. After that, substrate **1b-d** (50 mM) was added. The mixture was shaken at 30 °C and 170 rpm on an orbital shaker for 24 h. The lyophilized *E. coli* whole cells co-expressing Fus-SMO and Cb-FDH (10 mg mL^{-1}) were added together with NAD^+ (1 mM), HCOONa (250 mM, 5 eq.), FAD (50 μM), catalase (0.1 mg mL^{-1}) and *n*-heptane (1.5 mL). The mixture was incubated at 30 °C and 180 rpm on an orbital shaker for 24 h. The organic phase was separated from the aqueous phase; *n*-heptane phase was analyzed by GC-MS, while the aqueous layer was injected in RP-HPLC (100 μL aq. phase diluted with 1 mL H_2O:MeOH (1:1 v v^{-1} + 3% TFA), incubated at room temperature for 15 min, centrifuged and injected in RP-HPLC).

Procedure for the bio-epoxidation of aromatic olefins 2b-d. Lyophilized *E. coli* cells carrying Fus-SMO were rehydrated in KPi buffer (1 mL; 50 mM, pH 8.0, otherwise stated) supplemented with NAD^+ (1 mM), HCOONa (100 mM, 5 eq.), FAD (50 μM), catalase (0.1 mg mL^{-1}) and Cb-FDH (10 μM) in a 4 mL glass vial. As last, substrate **2b-d** (20 mM) was added. The mixture was incubated at 30 °C and 180 rpm on an orbital shaker for 24 h. The aqueous phase was then directly analyzed by RP-HPLC (100 μL aq. phase diluted with 1 mL

H_2O:MeOH (1:1 v v^{-1} + 3% TFA), incubated at room temperature for 15 min, centrifuged and injected in RP-HPLC).

Chemical synthesis of protected vinyl catechol from caffeic acid.[4] To a 100 mL one-neck round bottom flask equipped with a cooling system, CH_2Cl_2 (1.4 mL, 1.8 eq.) and K_2CO_3 (4 g, 2.5 eq) were added to DMF (30 mL); then, a solution of caffeic acid **1b** (2 g, 0.011 mmol) in DMF (20 mL) was dropwise added to the flask. The mixture was refluxed and stirred under magnetic agitation for 18 h; after that, it was cooled down to room temperature and filtered. The filtrate was concentrated, diluted with H_2O, and extracted with EtOAc (3 x 100 mL). The organic layer was washed with 10% NaOH (30 mL), H_2O (25 mL), dried ($MgSO_4$), and evaporated to afford a bark brown oil, which was further filtered over silica (PE:EtOAc 1:1) thus yielding a light orange oil (390 mg, ca. 25%).

General procedure for the one-pot cascade for the synthesis of chiral 4b' from 2b'. Lyophilized *E. coli* cells co-expressing Fus-SMO/Cb-FDH (20 mg mL^{-1}) and lyophilized *E. coli* cells carrying either Sp(S)-EH or St(R)-EH (20 mg mL^{-1}) were rehydrated in KPi buffer (0.5 mL, 50 mM, pH 8.0, otherwise stated) in a 4 mL glass vial. After that, NAD^+ (1 mM), HCOONa (100 mM, 5 eq.), FAD (50 µM) and catalase (0.1 mg mL^{-1}) were added. *n*-Heptane (0.5 mL; 1:1 volumetric ratio with the buffer) was used as biphasic solvent. Finally, the biocatalytic reactions were initiated by the addition of substrate **2b'** (20 mM). The reactions were incubated at 30 °C and 170 rpm on an orbital shaker for 48 h. *n*-Heptane was removed, the aqueous phase was saturated with solid NaCl, and the organic compounds was extracted with EtOAc (1 x 500 µL). After drying over $MgSO_4$, the organic layer was analyzed by GC-MS and the enantiomeric excess determined by chiral HPLC.

General procedure for the chemo-enzymatic synthesis of (*R*)-4b' from 1b. To a 500 mL Erlenmeyer flask, lyophilized *E. coli* whole cells carrying FaDC (20 mg mL^{-1}, 4.4 g) were rehydrated in KPi buffer (222 mL; pH 8.0, 50 mM) and substrate **1b** (50 mM, 2 g) was added. The mixture was incubated at 30 °C, 170 rpm for 24 h. After that, cell debris were removed by centrifugation and the supernatant was extracted with EtOAc (3 x 50 mL). The combined organic layers were concentrated under vacuum up to ca. 17 mL. The EtOAc extract (ca. 9 mL) was used for the follow-up step, considering a hypothetical quantitative conversion of **1b** to vinyl catechol **2b**.
In a 50 mL one-neck bottom flask, CH_2Cl_2 (6 mL) and K_2CO_3 (2.5 g) were added to DMF (15 mL); then, the EtOAc extract (9 mL) was added dropwise to the flask. The mixture was heated up at 90 °C and stirred under magnetic agitation for 18 h. After that, it was cooled down to room temperature and filtered. The filtrate was concentrated, diluted with H_2O, and extracted with EtOAc (3 x 10 mL). The organic layer was washed with 10% NaOH (30 mL), H_2O (30 mL), dried over $MgSO_4$ and evaporated to afford a dark orange oil, which was analyzed by GC-MS.

Lyophilized *E. coli* cells co-expressing Fus-SMO/Cb-FDH (20 mg mL^{-1}) and lyophilized *E. coli* cells carrying St(*R*)-EH (20 mg mL^{-1}) were rehydrated in KPi buffer (39 mL, 50 mM, pH 6.6) in a 250 mL Erlenmeyer flask. After that, NAD^+ (1 mM), HCOONa (100 mM, 5 eq.), FAD (50 µM) and catalase (0.1 mg mL^{-1}) were added. *n*-Heptane (39 mL; 1:1 volumetric ratio with the buffer) was used as biphasic solvent. Finally, the biocatalytic reactions were initiated by the addition of substrate **2b'** (20 mM, 0.675 mmol). The reactions were incubated at 30 °C and 170 rpm on an orbital shaker for 48 h. *n*-Heptane was removed, the aqueous phase was saturated with solid NaCl and the organic compounds extracted with EtOAc (2 x 20 mL). After drying over $MgSO_4$, the organic layer was analyzed by GC-MS and the enantiomeric excess determined by chiral HPLC.

General procedure for the conversion of (*R*)-4b' to chiral 6b'. $HCOONH_4$ buffer (pH 8.5, 1 M; 0.5 mL) was added to an Eppendorf tube (1.5 mL) and supplemented with NAD^+ (1 mM), PLP (1 mM), D-or L-Alanine (100 mM, 5 eq.) and Bs-AlaDH (20 µM). Then, ADH (50 µM) and ωTA (50 µM) were added followed by substrate (*R*)-**4b'** (20 mM). The mixture was incubated at 30 °C, 170 rpm for 48 h on an orbital shaker and, after that, quenched with 10 M KOH (100 µL). The aqueous layer was saturated with solid NaCl and the organic compounds extracted with EtOAc (1 x 500 µL). The organic layer was dried over $MgSO_4$ and analyzed by GC-MS.

General procedure for the conversion of (*R*)-4b' to chiral 8b'. In an Eppendorf tube (1.5 mL), $HCOONH_4$ buffer (0.5 mL, pH 8.5, 1 M), NAD^+ (1 mM), catalase (0.1 mg mL^{-1}) and purified Cb-FDH (10 µM) were added followed by purified AcCO6 (50 µM) and Ch1-AmDH (50 µM). (*R*)-**4b'** (20 mM) was added as last. The mixture was incubated at 30 °C, 170 rpm for 48 h on an orbital shaker and, after that, quenched with 10 M KOH (100 µL). The aqueous phase was saturated with solid NaCl and the organic compounds were extracted with EtOAc (1 x 500 µL). The organic layer was dried over $MgSO_4$ and analyzed by GC-MS.

Analytical methods

GC-FID method A: Column: Agilent DB1701 (30 m, 250 µm, 0.25 µm). Carrier gas: H_2**;** Parameter: T injector 250 °C; constant pressure 6.9 psi; temperature program: 80 °C, hold 6.5 min; gradient 5 °C min^{-1} up to 160 °C, hold 5 min; gradient 20 °C min^{-1} up to 200 °C, hold 2 min; gradient 20 °C min^{-1} up to 280 °C, hold 4 min.

GC-MS method B: Column Agilent DB-1701 (30 m, 250 µm, 0.25 µm); injector temperature 250 °C; constant pressure 71.8 kPa; temperature program: 80 °C/hold 6.5 min; 160 °C/rate 10 °C min^{-1}/hold 5 min; 200 °C/rate 20 °C min^{-1}/hold 2 min; 280 °C/rate 20 °C min^{-1}/hold 1 min.

RP-HPLC method C: Column Shimadzu Shim-pack GIST (4.6 mm x 150 mm); HPLC gradient program: 100% MilliQ (+ 0.1% TFA), down to 40% MilliQ (+ 0.1% TFA) in 20 min.; down to 35% MilliQ (+ 0.1% TFA) in 5 min.; up again to 100% MilliQ (+ 0.1% TFA) in 15 min; oven Temperature 30 °C, 1 mL min^{-1}

Chiral HPLC method D: Column Daicel IC-3 (0.46 cm x 25 cm); HPLC program: constant oven temperature 25 °C; constant pressure 70 bar; eluent composition: *n*-Hexane:Isopropanol 92:8, 1 mL/min

Table 6.2. Retention times of compounds analyzed in this study

Entry	Compound	Retention time [min]	Method
1	**1b**	14.5	C
2	**1c**	34.9	A[a]
		17.1	C
3	**1d**	17.5	C
		35.1	A[a]
4	**2b**	17.2	C
5	**2c**	21.3	A[a]
		20.9	C
6	**2d**	21.4	C
		21.5	A[a]
7	**2b'**	13.01	B
8	**3b'**	16.9	B
9	**4b'**	24.3	B
10	(*S*)-**4b'**	32.6	D
11	(*R*)-**4b'**	33.8	D
12	**5b'**	22.6	B
13	**6b'**	23.4	B

[a]After derivatization with (trimethylsilyl)diazomethane

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