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Promiscuity and Selectivity of Hydroxysteroid Dehydrogenases in the Biocatalyzed Reduction of 1,2-Diketones

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Hydroxysteroid dehydrogenases (HSDHs) are NAD(P)H-dependent alcohol dehydrogenases (ADHs) known for their exceptional stereo- and regioselectivity when acting on natural substrates, including neutral steroids, bile acids, and various steroid derivatives. Notably, in recent studies this specific subfamily of oxidoreductases has displayed intriguing substrate promiscuity, exhibiting the capacity to accommodate a diverse array of substrates, such as sterically hindered ketones and even α-keto esters. Herein, the promiscuous nature of HSDHs was further explored by investigating their catalytic activity with representative 1,2-diketones. This set encompasses symmetric aliphatic/aromatic diketones – namely, 3,4-hexandione and benzil – as well as the asymmetric synthon 1-phenyl-1,2-propanedione. In the case of 3,4-hexandione, substrate conversion and selectivity closely resembled that previously observed with aliphatic α-keto esters. On the contrary, a more heterogeneous behavior was observed in the case of aromatic substrates, with diverse performances in terms of conversions and stereo- or regioselectivity. Additionally, docking studies were carried out to get a deeper insight in the stereochemistry of 1,2-diketones reduction catalyzed by the broad substrate scope and steroid-active ketoreductase Is2-SDR.

Introduction

Hydroxysteroid dehydrogenases (HSDHs) belong to the short-chain dehydrogenases/reductases (SDR) superfamily and are NAD(P)(H)-dependent oxidoreductases capable to catalyze the oxidation or reduction of hydroxyl or keto groups of steroids in an exquisitely regio- and stereoselective manner.[1]

During the last decades, HSDHs have been widely studied for either their physiological function[2] or their biocatalytic applications in steroid chemistry, for example for the development of sustainable approaches in the synthesis of bile acids of pharmacological interest, such as the drug ursodeoxycholic acid.[1,3–6]

Conversely, the substrate scope of HSDHs towards non-steroidal substrates, i.e., their substrate promiscuity, has been scarcely investigated so far.[7–10]

Following the discovery of a set of novel HSDHs by (meta)genomes mining, a first systematic look into this issue has been recently carried out by us.[11] In this work, a library of microbial HSDHs, showing either 7α-, 7β-, or 12α-HSDH activity, and originated from already reported sources, as well as from newly identified sequences, were tested as biocatalysts for the stereoselective reduction of different non-steroid molecules.

α-Keto esters (Figure 1A) of interest as synthons of pharmaceutical compounds were well accepted by the majority of the tested HSDHs, in most cases with the formation of the corresponding reduced products with high conversion and enantiomeric excess (ee) values. On the contrary, moving to bicyclic compounds, tetralones (Figure 1B) were not accepted as substrates and only two 7β-HSDHs could reduce (±)-*trans*-1-decalone (Figure 1C), although with remarkable stereoselectivity.[11]

Interesting results were obtained with our HSDHs library when it was applied to the reduction of Wieland-Miescher ketone (Figure 1D)[12] and of other complex bioactive natural products, like 6-gingerol (Figure 1E),[13] as well as towards "bulky-bulky" ketones such as aryl decorated γ-keto esters, synthetic precursors of valuable bioactive metabolites (Figure 1F).[14,15]

In this work, to get further insights into HSDHs promiscuity, we considered the biocatalyzed reduction of 1,2-diketones to optically active α-hydroxy ketones, a topic previously investigated by various groups who used generic alcohol dehydro-

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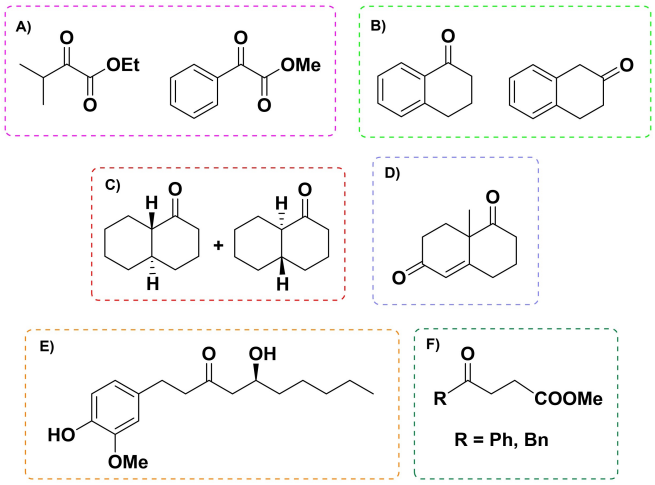


Figure 1. Examples of substrates tested in our previous studies on HSDHs promiscuity. A) α-keto esters; B) tetralones; C) (±)-*trans*-1-decalone; D) Wieland-Miescher ketone; E) 6-gingerol; F) aryl decorated γ-keto esters.

genases (ADHs) to achieve the enzyme-mediated preparation of these useful chiral building blocks.[16]

Concerning aliphatic vicinal diketones, the selective asymmetric reduction of these substrates has been mainly studied using 2,3-butanediol dehydrogenases (BDHs). Specifically, the study of the (*S*)-selective enzyme BudC originating from *Serratia marcescens* revealed a relatively wide substrate scope, although with restrictions involving sterically hindered substrates. Remarkably, this enzyme displayed exceptional stereoselectivity, especially when acting on small aliphatic diketones.[17] Additionally, the (*R*)-selective BDH from *Bacillus clausii* has been recently subjected to a comprehensive characterization, underscoring its ability to stereoselectively reduce not only aliphatic α-diketones, but also various bulky substrates, for example diketones and α-hydroxy ketones carrying a phenyl moiety.[18] However, in agreement with their physiological role, both BDHs formed diols along with α-hydroxy ketones.[17,18]

Moving to aromatic vicinal diketones, enzymes capable to reduce typical diaryl substrates, such as benzil, have been found in different microorganisms,[19] producing alternatively the (*R*) or (*S*)-benzoin with excellent optical purity.[20,21]

Among other biocatalysts studied in the reduction of vicinal diketones, worth of mentioning are also the recently discovered NAD(P)H-dependent pseudoephedrine or ephedrine dehydrogenases (PseDH or EDH) from *Arthrobacter* sp. TS-15. They converted 1,2-diarylketones into chiral α-hydroxy ketones in an enantiocomplementary way and with exceptional ee values of up to 99%, thus showing an interesting applicative potential.[22,23]

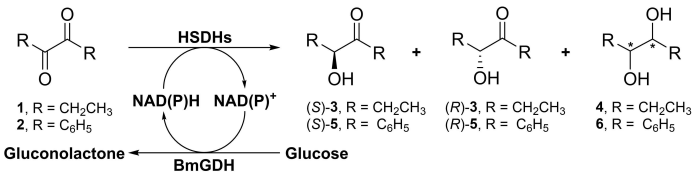
Results and Discussion

To study the enzymatic reduction of the target vicinal diketones, a library of 14 steroid-active biocatalysts was selected from our in-house collection. It comprised 13 HSDHs from various sources and showing different stereo- and regioselectivity, i.e., 7α-, 7β- and 12α-HSDHs, along with the broad scope reductase Is2-SDR recently discovered by us in a hot spring metagenome. This enzyme was included in the study because it shows some phylogenetic relationship with known HSDHs and is active as a 3β-reductase with different bile acids and steroidal substrates.[15] All these enzymes were obtained by heterologous expression in *Escherichia coli* and purified to homogeneity by affinity chromatography. It is worth noting that none of them show any relevant sequence similarity to those previously mentioned in the Introduction, such as 2,3-butanediol dehydrogenases[17,18] and benzil reductases.[19] The reduction reactions were coupled to the *B. megaterium* glucose dehydrogenase (BmGDH)-glucose system for NAD(P)H cofactor regeneration.

HSDHs screening for the stereoselective reduction of symmetric 1,2-diketones

The results of the HSDH-mediated reduction of the symmetric aliphatic diketone **1** (Scheme 1) are reported in Table 1. In general, all the enzymes demonstrated the ability to transform this substrate into the corresponding α-hydroxy ketone **3** without the formation of diols (**4**). Only in the case of the Is2-SDR catalyzed reduction (Table 1, entry 14), diols (**4**) were predominantly formed in the respect of the corresponding mono-reduced 1,2-hydroxy ketone (**3**).

Different degrees of enantioselectivity were obtained; Ls12α-HSDH, Hh7α-HSDH, Hh7β-HSDH, Sc7β-HSDH and Ca7β-HSDH were identified as the most promising in terms of conversion and/or enantiomeric excess (ee >90%). Interestingly, regardless their stereospecificity towards steroids, all the different HDSHs and the promiscuous reductase Is2-SDR catalyzed the stereoselective reduction of **1** into the (*R*)-enantiomer of **3**, the *anti*-Prelog reduction product obtained by



Scheme 1. Biocatalyzed reduction of the vicinal ketones **1** and **2** using different HSDHs and the *B. megaterium* glucose dehydrogenase (BmGDH)-glucose system for NAD(P)H cofactor regeneration.

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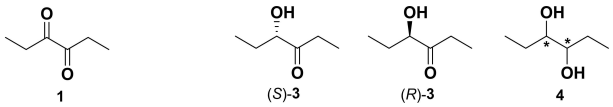
Table 1. HSDHs screening for the bioreduction of **1** to the α-hydroxy ketone **3** and/or diol **4**.



Entry	Enzyme [a]	Conversion (%)[b]	3, ee (%)[b]	% diols
1	Csp12α-HSDH	21.3	86.4 (*R*)	n.d.[c]
2	Ls12α-HSDH	70.0	97.7 (*R*)	n.d.[c]
3	Dm7α-HSDH	26.0	86.0 (*R*)	n.d.[c]
4	Ec7α-HSDH	31.1	70.7 (*R*)	n.d.[c]
5	Hh7α-HSDH	88.9	94.7 (*R*)	n.d.[c]
6	Ngi1_7α-HSDH	29.0	80.9 (*R*)	n.d.[c]
7	Ca7α-HSDH	73.3	86.8 (*R*)	n.d.[c]
8	Bsp7β-HSDH	25.3	87.0 (*R*)	n.d.[c]
9	Hh7β-HSDH	84.1	96.7 (*R*)	n.d.[c]
10	Rs7β-HSDH	16.8	91.8 (*R*)	n.d.[c]
11	Sc7β-HSDH	25.4	96.2 (*R*)	n.d.[c]
12	Ca7β-HSDH	68.6	95.6 (*R*)	n.d.[c]
13	Cae7β-HSDH	75.2	82.6 (*R*)	n.d.[c]
14	Is2-SDR	73.5	75.6 (*R*)	87.7

[a] For details about enzyme sources, see Table S1, Supporting Information. [b] Reaction conditions: 10 mM of **1** dissolved in a 5% (*v/v*) solution of DMSO in 50 mM potassium phosphate buffer (PB), pH 7.0, 1 U mL^{-1} BmGDH, 2 U mL^{-1} HSDH and 0.4 mM NAD(P)$^+$, final volume 1 mL, reaction time: 24 h. Conversions and ee values were calculated *via* GC analyses on a chiral column (see Experimental Section for details). [c] n.d.: not detected.

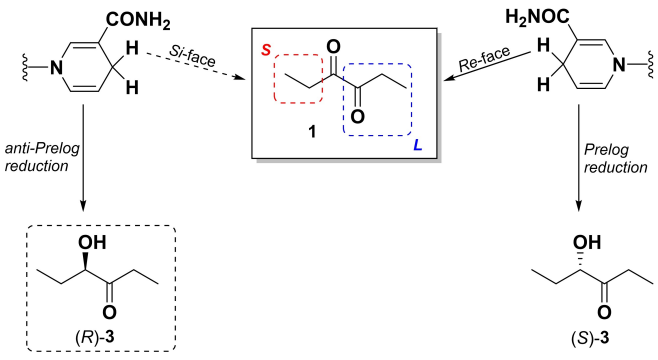
a hydride nucleophilic attack to the *Si*-face of the C=O double bond (Scheme 2).

As a second representative substrate to assess the performances of our library of hydroxysteroid dehydrogenases, we investigated the stereoselectivity (if any could be achieved) of the bioreduction of the "bulky-bulky", aromatic diketone **2**. The challenging aspect of asymmetric reduction for ketones with large, similar-sized groups lies in the need for the catalyst to differentiate between two groups that have subtle size differences.[19,24]

Table 2 summarizes the obtained results, again both in terms of substrates conversion and ee of the obtained product.

At variance to **1**, the reduction of this bulkier diketone under the same experimental conditions was generally characterized by lower conversions. The best performing enzyme in this context was Ca7β-HSDH (Table 2, entry 12) with a conversion around 65% to the α-hydroxy ketone benzoin (**5**) after 48 h. Interestingly, only in the case of Dm7α-HSDH (Table 2, entry 3) a moderate formation (around 10%) of a mixture of diols (**6**) was observed.

As far as stereoselectivity concerns, the HSDH-catalyzed reduction of **2** was characterized by poor to modest enantioselectivity. Moreover, even if with lower enantioselectivity when compared to the reduction of **1**, the screened enzymes showed different stereoselectivity forming both the Prelog and *anti*-Prelog products, i.e. (*S*)-**5** and (*R*)-**5**. Dm7α-HSDH and Is2-SDR were the most selective enzyme producing the Prelog-product (*S*)-**5** (ee 73.2% and 77.3%, respectively), while the (*R*)-**3** α-hydroxy ketone was predominantly formed from **1** by these two enzymes.



Scheme 2. Representation of the Prelog and *anti*-Prelog reduction of **1** to **3**. S = sterically-small substituent, L = sterically-large substituent.

HSDHs-mediated regio- and enantioselective reduction of the asymmetric vicinal diketone 7

1-Phenyl-1,2-propanedione (**7**), a commercially available asymmetric 1,2-diketone, represents a valuable synthon in biocatalyzed processes since it can be subjected to, for instance, enzymatic stereo/regioselective reductions or C=O reductive aminations.[25,26] The reduction of **7** (Scheme 3) can lead to three different products: the two regioisomeric α-hydroxy ketones, 1-hydroxy-1-phenylpropan-2-one (**8**) and 2-hydroxy-1-phenylpropan-1-one (**9**), and the fully reduced diol (**10**). While efficient biocatalytic entries to **8** and families of structurally related α-hydroxy ketones have been built around the use of oxidor-

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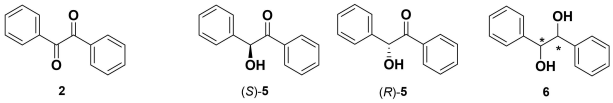
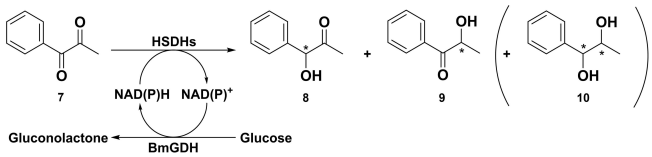
Table 2. HSDHs-catalyzed reduction of benzil (**2**) to benzoin (**5**) and/or diol (**6**).



2 (*S*)-**5** (*R*)-**5** **6**

Entry	Enzyme[a]	Conversion (%)[b]	**5**, ee (%)[b]	% diols
1	Csp12α-HSDH	< 5	–	n.d.[c]
2	Ls12α-HSDH	< 5	–	n.d.[c]
3	Dm7α-HSDH[d]	32.2	73.2 (*S*)	10.6
4	Ec7α-HSDH	9.0	18.3 (*R*)	n.d.[c]
5	Hh7α-HSDH	< 5	–	n.d.[c]
6	Ngi1_7α-HSDH	8.7	37 (*R*)	n.d.[c]
7	Ca7α-HSDH	17.4	rac	n.d.[c]
8	Bsp7β-HSDH	6.1	28.6 (*R*)	n.d.[c]
9	Hh7β-HSDH	< 5	–	n.d.[c]
10	Rs7β-HSDH	5.0	12.4 (*R*)	n.d.[c]
11	Sc7β-HSDH	< 5	–	n.d.[c]
12	Ca7β-HSDH[d]	64.8	63.4 (*R*)	n.d.[c]
13	Cae7β-HSDH[d]	38.0	37.8 (*R*)	n.d.[c]
14	Is2-SDR	47.2	77.3 (*S*)	n.d.[c]

[a] For details about enzyme sources, see Table S1, Supporting Information. [b] Reaction conditions: 10 mM of **2** dissolved in a 5% (*v/v*) solution of DMSO in 50 mM PB, pH 7.0, 1 U mL^{-1} BmGDH, 2 U mL^{-1} HSDH and 0.4 mM NAD(P)$^+$, final volume 1 mL. Conversions were calculated *via* GC analyses, while ee values and absolute configurations were assigned by chiral phase HPLC (see Experimental Section for details). All reactions were run for 24 h unless otherwise stated. [c] n.d.: not detected. [d] Reaction was run for 48 h.



Scheme 3. HSDHs-catalyzed regio- and enantioselective reduction of the vicinal, asymmetric diketone **7**.

eductases or carboligases,[18,22,27–29] the enzymatic preparation of **9** from **7** is far from being trivial, as deductible by the limited number of literature reports.[21,30] Moreover, preparative scale chromatographic separation of **8** and **9** *via* TLC, flash column chromatography and/or HPLC, to the best of our knowledge, still needs to be developed. Usually, NMR spectroscopy is used to quantify **8** and **9** in mixtures obtained from the reduction of **7** or from the oxidation of the corresponding diols **10**.[31–33] Also, no commercial suppliers for **9** can be found at variance to **7** and **8**.

In the light of these considerations, it became of interest the investigation of the behavior of our library of HSDHs in the reduction of **7** with a particular focus on the possibility to obtain the hydroxyl ketone **9** as the main or even the only product.

Screening results are summarized in Table 3 in which the conversions and the relative amounts of **8**, **9** and **10** (as percentage) have been calculated by ^{1}H NMR of the crude mixtures, following diagnostics signals for the three mentioned compounds and for the starting material **7** (see Supporting Information for details). At variance to Tables 1 and 2, reactions were run for 4 h instead of 24 h, since our aim was to depict a regioselectivity profile of our library of enzymes in kinetically controlled biotransformations.

With the exception of Ls12α-HSDH, Ngi1_7α-HSDH and Hh7β-HSDH (Table 3, entries 2, 6 and 9, respectively), all the tested enzymes reduced **7** with modest to very good conversions.

As far as regioselectivity concerns, all the active HSDHs (entries 1, 3–5, 7, 8, 10–13) showed a modest to strong preference for the reduction of the C_1 carbonyl group leading to the formation of **8**.

The best result was obtained with Dm7α-HSDH (entry 3), which gave a quantitative conversion of **7** with a regioselectivity higher that 99%, however, unfortunately, producing **8** and not the desired regioisomer **9**. The reaction was scaled up for (a) the evaluation of the enantiomeric excess of the process, using a method based on direct phase HPLC on a chiral column (see Experimental Section for details), and (b) the determination of the absolute configuration of the product **8**, in case it was enantiomerically pure or enriched.

In this way, the Prelog product (*R*)-**8**, (Figure S1, Supporting Information), was identified and its ee evaluated (30% ee, $[a]_D^{25} = -84.4°$, c: 1.00 EtOH).[34] Therefore, the stereoselectivity of Dm7α-HSDH was consistent with that observed with benzil (**2**, Table 2, entry 3).

Similarly to the reductions of compounds **1** and **2**, the formation of the mixture of diols **10** was not observed with the vast majority of the enzymes tested (Table 3, entries 1–13). This

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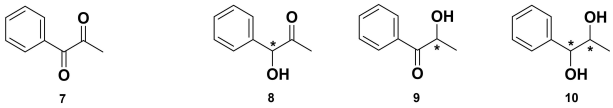
Table 3. HSDHs screening for the regioselective reduction of (**7**).



Entry	Enzyme[a]	Conversion (%)[b]	% of **8**[b]	% of **9**[b]	% of **10**[b]
1	Csp12α-HSDH	78.5	53.7	46.3	n.d.[c]
2	Ls12α-HSDH	n.d.[c]	–	–	–
3	Dm7α-HSDH	>99	>99	n.d.[c]	n.d.[c]
4	Ec7α-HSDH	89.7	86.6	13.4	n.d.[c]
5	Hh7α-HSDH	55.6	54.9	45.1	n.d.[c]
6	Ngi1_7α-HSDH	n.d.[c]	–	–	–
7	Ca7α-HSDH	77.5	82.0	18.0	n.d.[c]
8	Bsp7β-HSDH	70.1	66.5	33.5	n.d.[c]
9	Hh7β-HSDH	n.d.[c]	–	–	–
10	Rs7β-HSDH	62.5	81.3	18.7	n.d.[c]
11	Sc7β-HSDH	62.5	71.3	28.7	n.d.[c]
12	Ca7β-HSDH	52.0	64.6	35.4	n.d.[c]
13	Cae7β-HSDH	48.0	71.4	28.6	n.d.[c]
14	Is2-SDR	>99 >99[d]	78.1 33.2[d]	7.2 n.d.[c]	14.7 66.8[d]

[a] For details about enzyme sources, see Table S1, Supporting Information. [b] Reaction conditions: 10 mM of **7** dissolved in a 5% (*v/v*) solution of DMSO in 50 mM PB, pH 7.0, 1 U mL^{-1} BmGDH, 1 U mL^{-1} HSDH and 0.4 mM NAD(P)$^+$, final volume 10 mL. Conversions of **7** and the ratios of products **8**, **9** and **10** were calculated *via* ^{1}H NMR of the crude screening mixtures following specific signals characterizing the different species. All reactions were run for 4 h unless otherwise stated. [c] n.d.: not detected. [d] Reaction was run for 24 h.

was not the case with Is2-SDR (entry 14) and 11% of the diols were detected after 4 h. At longer reaction times (24 h), the percentage of **10** reached the 66.8%, along with the complete disappearance of the monohydroxylated product **9** which accumulated during the first reaction hours. The reaction was analyzed in more details, evaluating the formation of **9** and **10** in the first 5 h. Our hypothesis, based on the data reported in Table 3, was that diols (**10**) are rapidly formed from **9** that, at variance to **8**, is not present anymore after 24 h. Accordingly, the progress of the reaction was monitored by ^{1}H NMR analysis, which provided the degree of substrate conversion and product(s) production at scheduled times (15, 30, 60, 90, 120, 180, 240 and 300 min). The results are reported in Figure 2.

Substrate **7** was rapidly reduced by the enzyme: complete conversion was achieved after 60 min (red line) and the main product **8** was produced (80%) after the same time. According to our hypothesis, **9** was initially accumulated (up to the 15% in the first 30 min of biotransformation), but, after 1 h, it started to be converted into diols (**10**). At longer time the main product **8** was also partially transformed into the diols **10**.

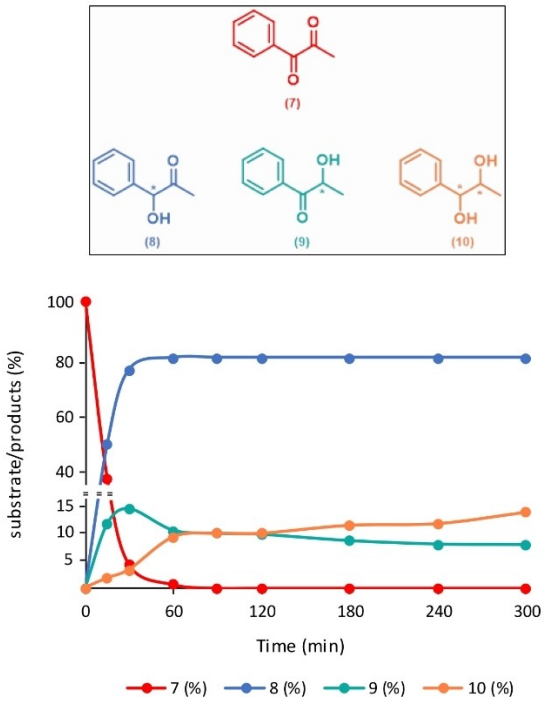


Figure 2. Reaction progress for the bioreduction of **7** catalyzed by Is2-SDR.

Docking studies: the case of Is2-SDR

Intrigued by the performances of Is2-SDR, a biocatalyst whose broad substrate scope was shown in previous works,[11,12,15] we decided to further analyze its distinctive reductase activity and substrate promiscuity by conducting a computational investigation of its interaction with the diketones **1**, **2** and **7**.

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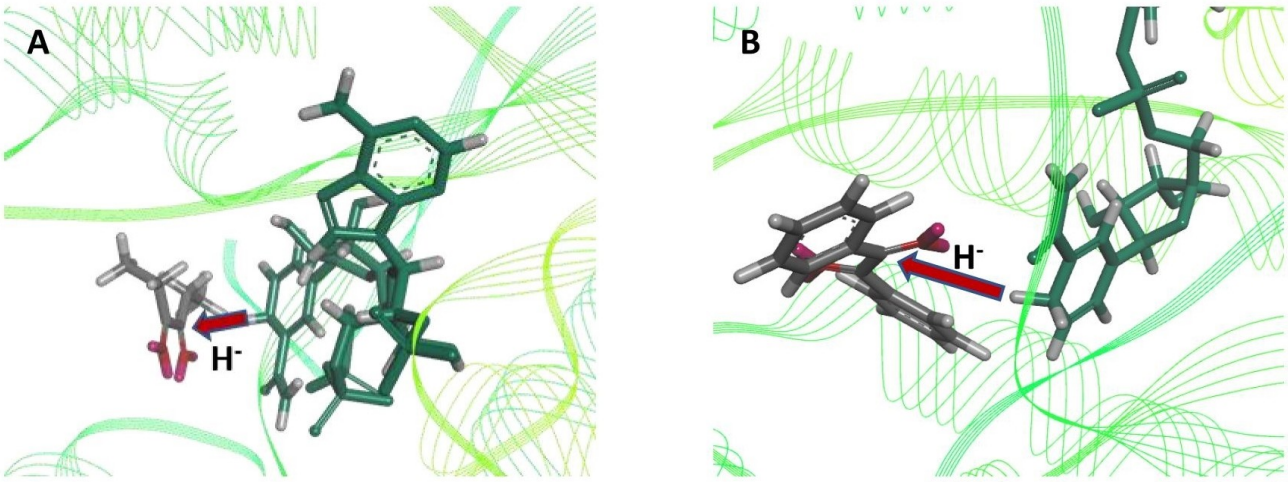

Figure 3. Docking of substrates **1** (A) and **2** (B) in Is2-SDR active site. The arrows indicate the direction of the cofactor hydride attack, NADPH cofactor is shown in green, the substrates (**1** in A, **2** in B) are in gray (see text for details).

Specifically, we used the *in silico* generated 3D structural model of Is2-SDR already described and validated by us[15] to carry out docking studies using the GOLD module of the Hermes CCDC-suite.[35] The different docking poses of **1**, **2** and **7** were thus visualized into the active site of the protein, with a special focus on stereoselectivity in the case of **1** and **2** and on regioselectivity in the analyses conducted on **7**.

We started with the simplest diketone **1** (Figure 3A). As expected, in the best binding pose the hydride transfer from the nicotinamide ring of the cofactor occurred from the *Si*-face of the C–O double bond plain of **1** leading preferentially to the formation of an (*R*) configured secondary alcohol, the *anti*-Prelog reduction product.

At variance to **1**, in the case of the more sterically hindered substrate **2** the NADPH nicotinamide ring was positioned in a way that hydride transfer was allowed preferentially from the *Re*-face of the C–O double bond plain promoting the formation of the Prelog product, i.e., the (*S*)-configured alcohol derivative (Figure 3B). Moreover, we observed that one of the aromatic rings of **2** docked into a hydrophobic binding pocket where it interacted by π–π staking and π–alkyl interactions with Ile190, Ala199, Ala209, and Phe218. The second aromatic ring was also interacting with the protein *via* π–alkyl interactions and π–π staking as well, but with Ala147 and the pyridine ring of NADPH. These interactions keep **2** in distinct and rigid binding position, thus hypothetically limiting the formation of diols, when compared to the same analyses conducted on the fully aliphatic substrate **1** (see Figure S2A, Supporting Information, for detailed contact map).

In the case of 1-phenyl-1,2-propanedione (**7**), we focused our attention on regioselectivity. As discussed before (Table 3 and Figure 2), Is2-SDR resulted able to reduce **7** forming both the hydroxy ketones **8** and **9** and the corresponding diol (**10**).

Substrate **7** finds its place in the same binding pocket as **2** (Figure S2B, Supporting Information), with an additional π-cation interaction with the residue Lys205. However, due to the lack of the second aromatic ring, the alkyl chain seems to remain more conformationally free when compared to **2**, thus allowing the NADPH hydride to attack both the carbonyl moieties in agreement with the experimental data. In particular, as shown in Figure 4, the docking analysis highlighted two binding poses for **7**, one, with higher docking scores, leading to the formation of **8**, while the other, less favorable, to the regioisomer **9**. This result is in agreement with the previously described outcome of Is2-SDR reaction progress analyses (Figure 2), which showed how the enzyme preferentially reduces **7** to **8**, while accumulating small amount of **9** that are rapidly converted into **10**.

Conclusions

In this work we have further analyzed the substrate scope and stereo- and regioselectivity of a library of microbial HSDHs, showing either 7α-, 7β-, or 12α-HSDH activity and including some novel enzymes discovered by (meta)genomes mining.

α-Keto esters, synthons of pharmaceutical compounds, were previously investigated as possible HSDHs substrates. Specifically, the α-keto esters reported in Figure 1A inspired the

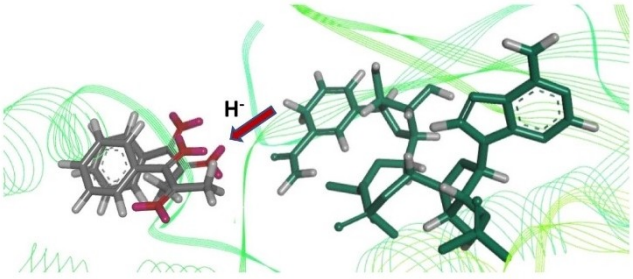

Figure 4. Docking of substrate **7** in Is2-SDR active site, two binding posed overlapped. The arrow indicates the direction of the cofactor hydride attack, NADPH cofactor is shown in green, the substrate is in gray (see text for details).

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current investigation since they were generally reduced to the corresponding (*R*)-alcohols with good to excellent conversions and ee up > 99%.[11]

Substrate **1**, a symmetric aliphatic 1,2 diketone, was processed by the HSDH library as expected based on the data previously collected on ethyl 3-methyl-2-oxobutanoate (Figure 1A, left),[11] forming the corresponding (*R*)-alcohol with modest to good conversions and good to excellent ee. (Table 1).

By changing the stereo-properties of the substrate, *i.e.* using the bulky-bulky 1,2 diketone **2** characterized by two terminal phenyl moieties, we evaluated the ability of the HSDHs library to reduce sterically hindered, symmetric 1,2-diketones. Interestingly, both the conversion and the enantioselectivity of the bioreduction were drastically decreased when compared to substrate **1**, with Dm7α-HSDH and Is2-SDR being the best performing enzymes for the productions of the (*S*)-enantiomer of the corresponding alcohol (Table 2).

Finally, intrigued by the good performances we reported for the reduction of the α-keto ester methyl 2-oxo-2-phenylacetate (Figure 1A, right),[11] we tested the performance of the HSDHs library in the reduction of the asymmetric, aryl-alkyl 1,2-diketone **7**, obtaining quite interesting results. The substitution of the terminal methyl ester group with a second reducible α-methyl ketone, in fact, introduced a second layer to the selectivity analysis: a problem of regioselectivity. This resulted in the formation of mixtures of the corresponding alcohols as mixtures of regioisomers. Only Dm7α-HSDH catalyzed the formation of only one regioisomer, (*R*)-2-hydroxy-1-phenylpropan-1-one, but with a poor ee.

Interestingly, the corresponding diols were formed exclusively by Is2-SDR as a complex mixture of stereoisomers. This reductase confirmed thus its pronounced promiscuity, and the docking results obtained in this work pave the way to perform structure-guided rational mutagenesis aimed to manipulate Is2-SDR stereo-preference as well as to improve its regioselectivity.

Experimental Section

General information

All reagents and solvents were purchased from Merck (Darmstadt, Germany), TCI (Zwijndrecht, Belgium), or Fluorochem (Hadfield, UK) and used without further purification, unless otherwise stated. Expression and purification of HSDHs, Is2-SDR and of the glucose dehydrogenase from *Bacillus megaterium* (BmGDH) were carried out as previously described.[11,12]

Analytical methods

The NMR spectra were acquired in $CDCl_3$ at room temperature (rt) on a Bruker AV 400 MHz spectrometer with a *z* gradient at 400 MHz for ^{1}H NMR analysis. Reactions were monitored via TLC (thin-layer chromatography) on pre-coated glass plates silica gel 60 with fluorescent indicator UV_{254} and treated with an oxidizing solution (phosphomolybdic reagent: $(NH_4)_6MoO_4$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4, 62 mL; H_2O, 1 L).

At scheduled times, reaction samples were extracted with EtOAc and dried over Na_2SO_4, then submitted to chiral HPLC, GC, or NMR as specified in the following.

GC analyses for compounds **1** and **2** were performed on an AGILENT 6850 (Network GC System) gas chromatograph equipped with a capillary column (MEGA DEX DAC-BETA, Legnano, Italy; 25 m×0.25 mm×0.25 μm, for **1**; MEGA DEX DMEPE-BETA, Legnano, Italy; 25 m×0.25 mm×0.25 μm, for **2**) and a flame ionization detector.

HPLC analyses of substrate **2** and product **5** were conducted using a Shimadzu LC-20AD high-performance liquid chromatography system equipped with a Shimadzu SPD-20 A UV detector. The samples, as EtOAc solutions, were analyzed on a Lux 3u Cellulose-3 (250×4.6 mm) through an isocratic elution (petroleum ether: isopropanol 80 : 20), flow rate: 1 mL min^{-1}, λ: 254 nm.

HPLC analyses for the determination of the enantiomeric excess of **8** were conducted using the same apparatus described above equipped with a Lux 3u Cellulose-1 (250×4.6 mm) through an isocratic elution (petroleum ether: isopropanol 97 : 03), flow rate: 0.7 mL min^{-1}, λ: 220 nm.

Activity assays

Dehydrogenase activities of HSDHs and BmGDH were determined spectrophotometrically by measuring the reduction of NAD(P)$^+$ at 340 nm (ε: 6.22 mM^{-1} cm^{-1}), while the activity of Is2-SDR was measured by following the oxidation of NADPH at the same wavelength. Assays were carried out in polyethylene cuvettes at room temperature by adding the opportune purified dehydrogenase (1—20 μL) to the following assay mixtures (1 mL final volume):

HSDH assay: 2.5 mM substrate (cholic acid for 7α- and 12α-HSDHs, ursodeoxycholic acid for 7β-HSDHs); 50 mM potassium phosphate buffer, pH 9.0; 0.20 mM NAD(P)$^+$.

Is2-SDR assay: 10 mM methyl benzoylformate; 50 mM potassium phosphate buffer, pH 8.0; 0.20 mM NADPH.

BmGDH assay: 50 mM glucose; 50 mM potassium phosphate buffer, pH 7.0; 0.20 mM NAD(P)$^+$.

One unit (U) is defined as the enzyme activity that reduces/oxidizes 1 μmol of NAD(P)(H) per min under the assay conditions described above.

Preparation of standard racemates

Chemical reductions of the substrates **1**, **2** and **7** were performed following a standard protocol using $NaBH_4$ as reducing agent. To a stirred solution of 0.15 M substrate (1 eq) in EtOH at 0 °C, $NaBH_4$ (0.25 eq) was added. The reaction was maintained at 0 °C until the complete dissolution of $NaBH_4$, then at rt for 30 min. After this time, the reaction was quenched with a saturated solution of NH_4Cl, then extracted with EtOAc (3×). Combined organic layers were dried over Na_2SO_4, concentrated *in vacuo*, affording the desired racemic alcohols that were used without any further purification. The racemic products were characterized by GC, HPLC, or NMR (see Supporting Information for details).

General procedure for enzymatic reduction

Enzymatic reductions of 3,4-hexanedione (**1**), benzil (**2**) and 1-phenyl-1,2-propanedione (**7**) were set up as follows: 10 mM substrate dissolved in a 5% (*v/v*) solution of DMSO in 50 mM potassium phosphate buffer (PB), pH 7.0, 50 mM glucose, 0.4 mM

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$NAD(P)^+$, 1 U mL^{-1} BmGDH and variable amounts of HSDHs (see details below). Reaction mixtures were incubated at rt under gently shaking for 4, 24 or 48 h. Conversions and enantiomeric excesses were determined by GC, HPLC and/or NMR analysis after extraction with EtOAc.

Enzymatic reduction of 1

Reaction mixtures contained: 10 mM of **1**, 1 U mL^{-1} BmGDH, 2 U mL^{-1} HSDH and 0.4 mM $NAD(P)^+$, final volume 1 mL. After 24 h, the reactions were extracted with EtOAc and analyzed *via* chiral phase GC to determine conversions and enantiomeric excesses ($T_{injector}$: 200 °C; $T_{detector}$: 250 °C; flow rate: 1.4 mL min^{-1}. Elution conditions: 60 °C for 1 min, 8 °C min^{-1} until 200 °C, hold 5 min). R_t : **1**, 5.2 min; (*S*)-**3**, 8.5 min; (*R*)-**3**, 8.7 min. Absolute configurations were determined by assessing the optical rotatory power of the product obtained from the reaction catalyzed by Hh7α-HSDH (high conversion, ee >90%) and comparing it with reported data for both (*S*)-**3**[36] and (*R*)-**3**.[37]

Semipreparative scale reduction of 1 catalyzed by Hh7β-HSDH

Working on 100 mg of **1** (final volume 67 mL) and using Hh7β-HSDH as biocatalyst**,** **3** was isolated after a 24 h reaction *via* a sequence of (a) extraction with EtOAc; (b) drying of the combined organic layer over Na_2SO_4 and (c) flash column chromatography on silica gel (gradient of EtOAc in petroleum ether). Isolated yield: 80%, $[\alpha]_D^{24}$: −94.5° (c: 1.0 in EtOH), ee = 97.0%[37]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.39–4.02 (m, 1H), 3.48 (d, *J* = 4.8 Hz, 1H), 2.61–2.38 (m, 1H), 2.01–1.79 (m, 2H), 1.69–1.50 (m, 2H), 1.22–1.05 (m, 3H), 1.02–0.84 (m, 3H).

Enzymatic reduction of 2

Reaction mixtures contained: 10 mM of **2**, 1 U mL^{-1} BmGDH, 2 U mL^{-1} HSDH and 0.4 mM $NAD(P)^+$, final volume 1 mL. After 24 or 48 h (see Table 2 for details), the reactions were extracted with EtOAc and analyzed *via* GC to determine conversions ($T_{injector}$: 200 °C; $T_{detector}$: 250 °C; flow rate: 1.0 mL min^{-1}. Elution conditions: 180 °C for 1 min, 10 °C min^{-1} until 200 °C, hold 25 min). R_t : **2**, 14.8 min; rac-**5**, 15.4 min; **6**, 24.1 min. Enantiomeric excesses were calculated *via* chiral phase HPLC (petroleum ether: isopropanol 80:20; flow rate: 1.0 mL min^{-1}; λ: 254 nm) and comparing the obtained retention times (R_t : **2**, 6.9 min; (*R*)-**5**, 7.6 min; (*S*)-**5**, 8.2 min) with literature data.[38]

Enzymatic reduction of 7

Reaction mixtures contained: 10 mM of **7**, 1 U mL^{-1} BmGDH, 1 U mL^{-1} HSDH and 0.4 mM $NAD(P)^+$, final volume 10 mL. After 4 h (24 h for Cae7β-HSDH), the reactions were extracted with EtOAc and analyzed via ^{1}H NMR in $CDCl_3$ to determine conversions into **8**, **9** or **10**.

Semipreparative scale reduction of 7 catalyzed by Dm7α-HSDH

Working on 100 mg of **7** (final volume 67 mL) and using Dm7α-HSDH as biocatalyst, **8** was isolated after a 24 h reaction *via* a sequence of (a) extraction with EtOAc; (b) drying of the combined organic layer over Na_2SO_4 and (c) flash column chromatography on silica gel (gradient of EtOAc in petroleum ether). Isolated yield: 95%, $[\alpha]_D^{24}$: −84.2° (c: 1.1 in EtOH), ee = 30%[34]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4 7.64–7.13 (m, 1H), 5.07 (d, *J* = 9.6 Hz, 1H), 2.07 (s, 1H). R_t : **7**, 7.6 min; (*S*)-**8**, 21.6 min; (*R*)-**8**, 25.7 min.

Molecular docking analysis

The 3D structural model of Is2-SDR was previously generated by using the SWISS-MODEL automated server and the structure of the homo tetrameric 3-oxoacyl-[acyl-carrier protein] reductase from Listeria monocytogenes (Lm-FabG, PDB ID: 4JRO) as template as described in[15].

Protein visualization and ligand optimization were carried out using the Discovery Studio package 2021 (version 21.1.0 BIOVIA, San Diego, CA, USA). GOLD module of the Hermes CCDC-suite was used to dock in the Is2-SDR active site conformers of the optimized ligands generated using the Mercury module of the CCDC suite (http://www.CCDC.cam.ac.uk).

Supporting Information

GC chromatograms, HPLC chromatograms and ^{1}H NMR spectra are available in the Supporting Information, where the authors have cited additional references.[39–41]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biocatalyzed reduction · hydroxysteroid dehydrogenases · regioselectivity · stereoselectivity · vicinal diketones

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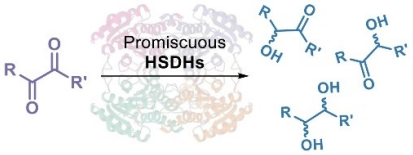
RESEARCH ARTICLE

Selected hydroxysteroid dehydrogenases (HSDHs) were screened against 1,2-diketones to investigate their promiscuous character. This study focused on the asymmetric synthon 1-phenyl-1,2-propanedione as well as the symmetric diketones 3,4-hexandione and benzil. The substrate conversion and selectivity for 3,4-hexandione nearly matched those previously noted for aliphatic α-keto esters, while HSDHs showed a more heterogeneous response with aromatic substrates, exhibiting different conversion rates and stereo- or regioselectivity.



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Promiscuity and Selectivity of Hydroxysteroid Dehydrogenases in the Biocatalyzed Reduction of 1,2-Diketones

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