Enzyme Catalysis

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Tetrahydroxynaphthalene Reductase: Catalytic Properties of an Enzyme Involved in Reductive Asymmetric Naphthol Dearomatization**

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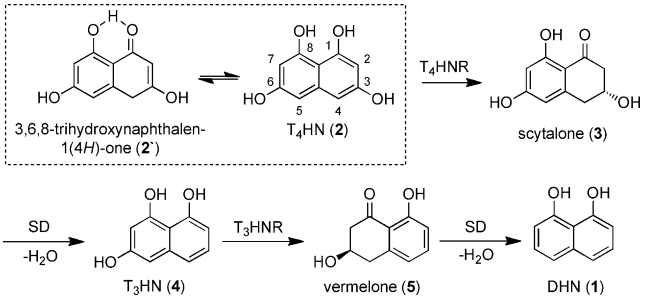
Alcohol dehydrogenases are mainly applied for the reduction of aliphatic ketones. By contrast, a biosynthetic point of view hints at broader catalytic activities. For example, the metabolism of aromatic compounds in fungi and bacteria proceeds through an aerobic or anaerobic route. While mono- or dioxygenases are involved in the aerobic pathways, oxidoreductases are required for the anaerobic metabolism, thus leading to the formation of acetyl coenzyme A. Additionally, polyphenolic polyketide synthase (PKS) products degrade by a reduction–dehydration sequence during secondary metabolite synthesis.[1] This deoxygenation strategy was found as a key step in 1,8-dihydroxynaphthalene (DHN, **1**)-melanin biosynthesis of various fungi[2–6] and is also proposed for the formation of aflatoxin,[7,8] actinorhodin,[9] and chrysophanol.[10,11] Furthermore, polyhydroxynaphthalenes represent branching points in several secondary metabolite syntheses.[12–14] A high degree of metabolic diversity can be found, especially in the group of spirodioxynaphthalenes.[12]

DHN (**1**), as the monomeric unit of DHN-melanin, is produced by means of a double deoxygenation (Scheme 1).[2–6] The PKS product 1,3,6,8-tetrahydroxynaphthalene (T_4HN, **2**) is reduced by tetrahydroxynaphthalene reductase (T_4HNR) to scytalone (**3**),[15] which is readily dehydrated by scytalone dehydratase (SD) to 1,3,8-trihydroxynaphthalene (T_3HN, **4**).[16–18] In the same manner, **4** is reduced by trihydroxynaphthalene reductase (T_3HNR) to vermelone (**5**) and aromatized by SD to **1**.[19] The reduction



Scheme 1. Fungal DHN-melanin biosynthesis; dashed box: prevalent keto–enol tautomerism of **2** in solution.[2–6,15–21]

steps are believed to take place via the 3-keto tautomers of **2** and **4**.[20,21] T_4- and T_3HNR of *Magnaporthe grisea* involved in this route show 46% sequence identity and exhibit a preference for T_4HN (**2**) and T_3HN (**4**), respectively.[15] The two reductases are members of the short-chain dehydrogenase/reductase (SDR) family. This family is known for sharing the same structural motif, though the members exhibit few sequence similarities.[15,19,22–25] The common conserved motif is the core structure for divergent reductases, but also represents the backbone for a catalytic-promiscuous family.[26]

Dearomatization strategies represent a powerful and direct approach to cyclic building blocks in natural product synthesis.[27,28] However, dearomatization reactions concurrent with asymmetric catalysis in one step are very challenging.[27] Thus, T_4HNR and T_3HNR may represent valuable tools for catalytic, asymmetric dearomatization.

Herein, we focus on the structure–activity relationship of T_4HNR to gain insights into the dynamics of its catalytic cycle. A broad substrate range allowed for the identification of an essential structural motif of naphtholic substrates and of major active-site interactions. The mutational effect of C-terminal truncation agrees with the concept of stabilization by the C-terminal carboxylate as an explanation for the substrate preference of T_4HNR. This structural feature may help to find further naphthol reductases.

T_4HNR was cloned and expressed using a slightly modified method of Thompson et al.[15] and the cell extract was used without further purification. Despite its susceptibility to oxidation by air, the physiological substrate **2** gave a conversion of 47% with 33% yield of scytalone (**3**) being obtained. The absolute configuration was determined by CD spectroscopy to be *R*, and the enantiomeric excess (*ee*) was found to be > 95% (Table 1 and the Supporting Information).

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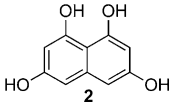
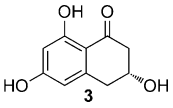
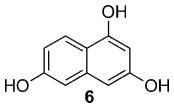
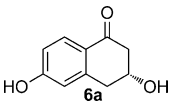
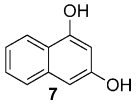
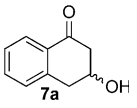
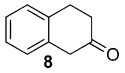
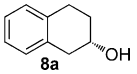
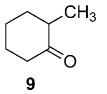
Table 1: Substrate promiscuity of T_4HNR.[a]

Substrate	Product	Conv. [%][b]	Yield [%]	*ee* [%]	Abs. config.[c]
 **2**	 **3**	47	33	>95	*R*
 **6**	 **6a**	49	39	>99	*R*
 **7**	 **7a**	3	n.d.	n.d.	n.d.
 **8**	 **8a**	99	77	61	*S*
 **9**	 **9a**	50	24[d]	93	*S*,*S*

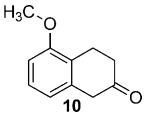
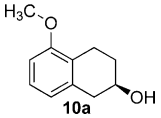
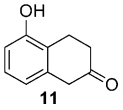
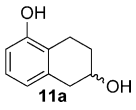
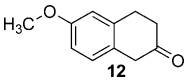
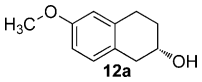
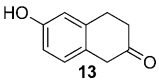
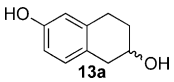
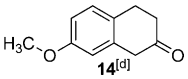
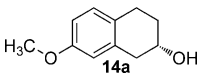
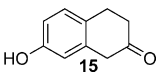
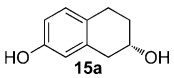
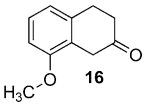
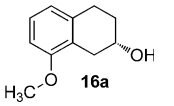
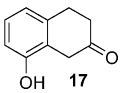
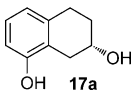
[a] Reactions were performed with substrate concentrations of 9.5 or 10 mM. Glucose dehydrogenase (GDH)/D-glucose or malic enzyme (MAE)/L-malic acid were used as a cofactor regeneration system (5–10 mol% of $NADP^+$). [b] Conversions were determined by ^{1}H NMR spectroscopy. [c] The absolute configuration was determined by using CD (**3**, **6a**) and vibrational circular dichroism (VCD, **9a**) spectroscopy. The absolute configuration of **8a** was determined according to the method of Mosher and Dale.[29] [d] The low yield is possibly due to the formation of an azeotrope. n.d.: not determined.

The 3-hydroxynaphthalen-1(4*H*)-one tautomer **2′** (Scheme 1) and the respective keto tautomer of T_3HN (**4**) are stabilized by hydrogen bonding through the 8-hydroxy proton, in solution.[20,21] 1,3,6-Trihydroxynaphthalene (**6**) was converted to (*R*)-8-deoxyscytalone (**6a**) with a yield and *ee* that suggest that the intramolecular hydrogen bond to the 1-ketone does not facilitate the reduction step and that the 8-hydroxyl group is not essential for substrate recognition. However, **6** will also exhibit keto–enol tautomerism, even if to a smaller extent, and it remains unknown if the 3-hydroxynaphthalen-1(4*H*)-one tautomer is the actual substrate, either for Michael addition of hydride or for an enzyme-catalyzed rearrangement to the 3-keto tautomer before keto reduction. In comparison to **6**, the conversion of 1,3-dihydroxynaphthalene (**7**) dropped dramatically, because of the loss of the 6-hydroxyl group.[21] Nevertheless, the 1,3-dihydroxyl substitution pattern apparently represents the essential structural motif for phenolic substrates, since we have not measured any conversion of 2-naphthols (as exemplified by the strictly regioselective reduction of **6**).

2-Tetralone (**8**), as a non-naphtholic substrate, was converted quantitatively. The smaller size of **8** allows for a second orientation with *si-face* attack of the hydride, which is reflected in the decreased *ee* of 61%. Monocyclic 2-methylcyclohexanone (**9**) was exclusively transformed to *trans*-2-methylcyclohexanol (**9a**, >99% *de*). The latter two substrates (**8**, **9**) represent an overlap with the medium-chain dehydrogenase/reductase (MDR) family, for example horse liver ADH,[30] and with the SDR family, namely tropinone reductases I and II (also see below).[31,32]

The decreased enantiomeric excess observed in the reduction of 2-tetralone (**8**) suggested that sterically demanding substitution patterns on the aromatic ring will have an impact on the stereochemical outcome. Indeed, reduction of 5-methoxy-2-tetralone (**10**) showed reversed facial selectivity, giving **10a** of *R* configuration in high enantiomeric excess (Table 2). Most other tetralones tested gave the *S* enantiomer

Table 2: Reduction of 2-tetralone derivatives by T_4HNR.[a]

Substrate	Product	Conv. [%][b]	Yield [%]	*ee* [%]	Abs. config.[c]
 **10**	 **10a**	99	75	99	*R*
 **11**	 **11a**	80	59	<5	n.d.
 **12**	 **12a**	98	66	49	*S*
 **13**	 **13a**	17	n.d.	n.d.	n.d.
 **14**[d]	 **14a**	99	71	>99	*S*
 **15**	 **15a**	99	72	99	*S*
 **16**	 **16a**	99	75	99	*S*
 **17**	 **17a**	99	81	96	*S*

[a,b] According to Table 1. [c] The absolute configuration was determined according to the method of Mosher and Dale[29] (**10a**, **12a**, **14a**, **16a**) and according to the method of Harada and Nakanishi (**13a**, **15a**, **17a**).[34] [d] For this substrate the scalability of the reduction was shown representatively (see the Supporting Information).

corresponding to the natural product **3**, but 5-hydroxy-2-tetralol (**11a**) was obtained with an *ee* value of <5%, which represents the limiting case between a possible *re-face* and the preference of *si-face* attack of the hydride.

To understand these results we performed docking experiments with the 2-tetralones. The 3D structure of the catalytic center of T_4HNR has been described elsewhere based on the enzyme co-crystallized with the inhibitor pyroquilon.[22] According to this model, the *R* or *S* configuration of the product depends on the relatively fixed position of the carbonyl oxygen atom and two possible side orientations of the stacked aromatic ring. Both orientations of the substrates (Figure 1) were modeled using the software program "Glide",[33] and the respective docking scores of the *re*- and *si-face* forms were compared.

The models of the complexes indicate the following relationships: 1) a methoxy group of the substrate preferentially interacts with a hydrophobic region consisting of

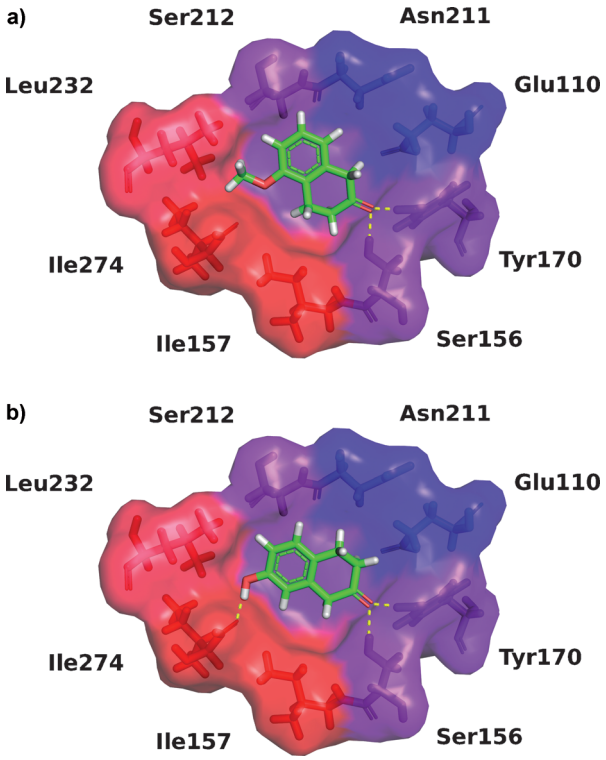


Figure 1. The two orientations of tetralones in the active site of T_4HNR revealed by docking studies: a) *si-face* orientation of **10** representing the preferential hydrophobic interaction of the methoxy group and b) hydrogen-bonded *re-face* orientation of **15** within the active site. Coloring based on hydrophobicity scale (red: hydrophobic; blue: hydrophilic). The substrate is viewed from the face occupied by NADPH in the crystal structure.

Leu232, Ile157, and the side chain of Ile274 (**10**, **12**, **14**, Figure 1a); and 2) if a hydrogen bond can be formed between a hydroxyl group of the substrate and the carboxyl group of Ile274, this diastereomeric complex is stabilized and favored over the other non-hydrogen-bonding orientation (**15** and **17**, Figure 1b). In the case of **10**, a large difference in the docking scores predicted that the inverted *si-face* orientation would be favored. The small enantiomeric excess of **11a** was predicted by small differences in the docking scores of **11** and is probably caused by hydrogen bonding in the *si-face* form. In substrate **13** neither the *re-* nor the *si-face* orientation can form a hydrogen bond between the hydroxyl group and the carboxylate of Ile274, which may be a reason for the low conversion of this derivative, along with its rapid oxidative decomposition.

Only in the case of substrate **16** did the docking fail to predict the observed configuration of the product. The *si-face* orientation of **16** scores higher than the *re-face*, with the methoxy group directed towards Met207 (which is just above Glu110 and Asn211 in Figure 1). However, in actual fact the *re-face* orientation is strongly favored, presumably because the methoxy group preferentially interacts with the hydrophobic region described above. These observations indicate that the presence of one of the interactions (1) or (2) helps to stabilize the substrate within the binding pocket for an enantioselective reaction.

As mentioned above, T_4HNR and T_3HNR prefer **2** and **4**, respectively, as a substrate.[15] Based on the X-ray structure with modeled substrates, Liao et al. have postulated that the C-terminal carboxylate of T_4HNR binds to the 6-hydroxyl group of **2** and is the origin of the substrate discrimination.[22] Our docking experiments agree with this concept and suggest that the carboxylate of Ile274 might also have an impact on substrate orientation. To test this idea kinetically, we generated the C-terminal, Ile274-truncated variant T_4HNR(1-273). Due to the high instability of the native substrates **2** and **4**, tetralones **8**, **14**, and **15** were used as model substrates (Table 3).

Table 3: Kinetic parameters for T_4HNR and T_4HNR(1-273).[a]

Substrate	T_4HNR		T_4HNR(1-273)	
	K_m(app.) [μM] (±SEM)	v_{max}(app.) [mM min^{-1}] (±SEM)	K_m(app.) [μM] (±SEM)	v_{max}(app.) [mM min^{-1}] (±SEM)
8	1347 ± 51	0.120 ± 0.002	1023 ± 37	0.136 ± 0.002
14	256 ± 13	0.078 ± 0.001	377 ± 15	0.198 ± 0.002
15	150 ± 36	0.180 ± 0.003	707 ± 31	0.081 ± 0.001

[a] For details see the Supporting Information. SEM: standard error of the mean.

The wild-type enzyme showed an apparent Michaelis–Menten constant for **8** of 1347 ± 51 (± SEM, [μM]). For **14** the K_m(app.) value decreased to 256 ± 13 and in the case of **15** further down to 150 ± 36.[35] In terms of v_{max}/K_m **15** was preferred over **8** by a factor of 13.5 ± 3.6 (± SEM) in the noninhibited region (v_{max}(app.)) and by a factor of 5.9 ± 1.8 in the inhibited region (v_2(app.)). In accordance with the affinities observed, **8** possesses no possibility of any substituent interaction, while the methoxy group could interact with the hydrophobic region described above, thereby leading to a more rigid substrate orientation (> 99 % *ee*) and also higher affinity. The low K_m(app.) value for **15** is presumably caused by hydrogen bonding to the terminal carboxylate of Ile274 and reflects the preferential binding mode.

The K_m(app.) values for **8** (1.3-fold decrease) and **14** (1.5-fold increase), as well as the preference between **8** and **14** (1.2-fold higher preference for **14**), were only slightly affected by truncating T_4HNR by one amino acid. However, the variant T_4HNR(1-273) showed a 4.7-fold increased K_m(app.) value for **15** and a preference for **8** over **15** of 1.2 ± 0.1 (reversing the 13.5-fold preference of the wild-type enzyme for **15**).

The significant alteration of the affinity and the inversion of the preference between **15** and **8** result from a missing, hydroxyl-group-dependent stabilization effect. Therefore, T_4HNR discriminates between its substrates through C-terminal hydrogen bonding via the carboxylate of Ile274.[36]

Sequence alignments with particular reference to the C-terminal residues brought our attention to the 17β-hydroxysteroid dehydrogenase of *Cochliobolus lunatus* (17β-HSDcl, melanin producer).[37] In both cases, the C-terminal residue comes 13 amino acids after a highly conserved Trp (261 in T_4HNR, 257 in 17β-HSDcl), which suggests a similar location for the C-terminal residue and its possible involve-

ment in substrate stabilization for this enzyme. We tested 17β-HSDcl, for which the physiological substrate is still unknown,[32] for its ability to reduce naphthol **2** and obtained **3** with identical configuration and enantiomeric excess.[38]

In summary, a broad substrate range allowed for detailed studies on the catalytic features of T_4HNR. The use of 2-tetralone derivatives as model substrates revealed major enzyme–substrate interactions, as well as an involvement of the C-terminal residue in substrate discrimination and probably orientation. On the basis of findings made on 17β-HSDcl one may ask, as it is unknown what creates the difference between a native naphthol and a "classical" oxidoreductase, whether "classical" oxidoreductases have been treated too restrictively concerning alternate activities.

Experimental Section

Nitrogen was bubbled through the buffer solution and 2-propanol for 30 min, followed by degassing under reduced pressure before use.

General procedure for the reduction of phenolic compounds (**2**, **6**, **7**): The substrate (710 μmol) was dissolved in 2-propanol (3.5 mL) and added to a solution of D-glucose (3.55 mmol, 639 mg) and $NADP^+$-Na (71 μmol, 54 mg) in KPi buffer (67.5 mL, 50 mM, pH 7.0; 1 mM EDTA, 1 mM dithiothreitol). T_4HNR (8 U; U = μmol min^{-1}) and GDH (215 U) were added slowly and the reaction mixture was stirred under nitrogen for 24 h. The solution was acidified to pH 6, extracted three times with ethyl acetate, dried over $MgSO_4$, and the solvent was removed under reduced pressure. For product-specific purification procedures and the reduction of cyclic ketones (**8**–**17**), see the Supporting Information.

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