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An *R*-Selective Hydroxynitrile Lyase from *Arabidopsis thaliana* with an α/β-Hydrolase Fold**

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Hydroxynitrile lyases (HNLs) catalyze the stereoselective formation of C–C bonds between HCN and aldehydes or ketones yielding chiral cyanohydrins, which are versatile building blocks for the pharmaceutical and agrochemical industries.[1] Among the most important cyanohdrins are chiral α-hydroxy acids such as substituted mandelic acids,[1e,f,2a] *m*-phenoxybenzaldehyde derivatives,[2c] and structures with additional aliphatic linkers between the aldehyde moiety and aromatic ring which are useful for the synthesis of "prils".[2d] In nature HNLs catalyze the cleavage of cyanohydrins, known as cyanogenesis. The currently known HNLs can be divided into two groups: *R*-selective enzymes evolved from oxidoreductase ancestors, such as HNLs from various *Rosaceae*[2] and from *Linum usitatissimum*,[3a] and *S*-selective enzymes derived from hydrolases with an α/β-hydrolase fold; these encompassing the enzymes from *Hevea brasiliensis* (*Hb*HNL),[3b] *Manihot esculenta* (*Me*HNL),[3c] and *Sorghum bicolor* (*Sb*HNL).[3d] Here we present the first exception to this accepted rule with the first *R*-selective HNL containing an α/β-hydrolase fold from the noncyanogenic plant *Arabidopsis thaliana* (mouse-ear cress).

Owing to the growing demand for chiral compounds like cyanohydrins there is a strong motivation to identify new stereoselective HNLs with a broad substrate range which can be easily and economically produced. These demands are fulfilled by the currently available *S*-selective enzymes *Hb*HNL and *Me*HNL: they can be expressed in bacterial hosts like *Escherichia coli* and accept a broad range of aromatic and aliphatic aldehydes as well as ketones.[4] A similar broad substrate range has been reported for the *R*-selective HNLs isolated from some *Prunus* species (*P. amygdalus* (*Pa*HNL) and *P. mume* (*Pm*HNL)). These biocatalysts are either used as defatted seed meals or, in the case of *Pa*HNL (isoenzyme 5), are expressed in the yeast *Pichia pastoris*.[2a,e]

Recently, several approaches were reported to identify new HNLs for biocatalytic processes by screening different cyanogenic plant extracts for HNL activity, yielding some new enzyme sources.[5] Attempts to identify new enzymes based on sequence similarities to known HNLs have not yet been successful.[6,7]

Several sequences similar to *Me*HNL and *Hb*HNL are found in the genome of the noncyanogenic model plant *Arabidopsis thaliana*.[7] In the course of our studies on structure–function relationships of α/β-hydrolases we cloned several genes encoding *Arabidopsis* proteins with high sequence similarity to *Me*HNL and *Hb*HNL and expressed them in *E. coli*. Unexpectedly, one of them (gene bank entry: AAN13041) shows high activity towards mandelonitrile and catalyzes also the cleavage of some other cyanohydrins derived from cyclohexanone and *m*-phenoxybenzaldehyde, while acetaldehyde, propionaldehyde, and acetone cyanohydrin are poor substrates.[8]

A subsequent investigation of the cyanohydrin-forming activity revealed that the new enzyme is highly *R*-selective with a broad substrate range including various aromatic and aliphatic aldehydes as well as ketones, which are converted to *R*-cyanohydrins with good to excellent yields and mainly excellent enantioselectivities (Table 1).[9] As can be seen, a whole range of substituted benzaldehydes are converted with excellent activity and enantioselectivity. There was no optimization of the reaction time, but substrates such as **3**, **4**, and **6**, which react even in the absence of the enzyme, gave products with 99% *ee* indicating a high enzymatic activity towards these substrates. To obtain complete conversion of substrates with the more bulky substituents the reaction time had to be increased slightly. It should be also noted that the reaction was performed at pH 5. Lowering the pH could of course suppress the nonenzymatic reaction even further. But even at pH 5 the *ee* obtained is higher for *o*-chlorobenzaldehyde cyanohydrin than that in earlier studies with optimized *Pa*HNL[2a] or with the wild-type enzyme.[10] Subsequent hydrolysis yields (*R*)-*o*-chloromandelic acid, which is a key

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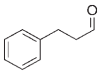
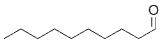
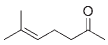
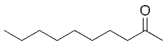
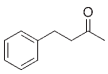
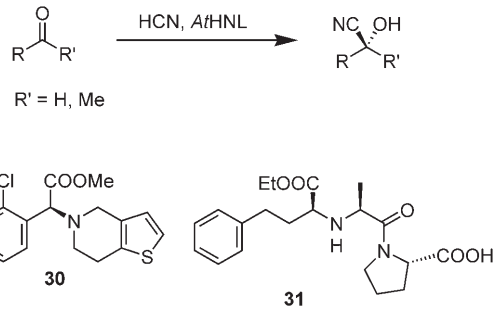
Table 1: Substrate range of *At*HNL.[a]

Substrate		*t* [h]	X_{enz}[b] [%]	*ee* (*R*) [%]	X_{nenz}[b] [%]
					
R = H	**1**	2	> 99	> 99	14
R = *o*-F	**2**	2	> 99	99	17
R = *o*-Cl	**3**	2	> 99	99	26
R = *o*-Br	**4**	6	99	98	42
R = *o*-I	**5**	3	> 99	> 95	26
R = *m*-F	**6**	2	> 99	> 99	22
R = *m*-Cl	**7**	3	99	> 99	7
R = *m*-Br	**8**	6	99	95	9
R = *m*-I	**9**	6	98	93	5
R = *m*-PhO	**10**	22	83	> 95	0
R = *p*-F	**11**	2	> 99	> 99	7
R = *p*-Cl	**12**	2	> 99	> 99	4
R = *p*-Br	**13**	3	99	> 99	4
R = *p*-I-	**14**	6	99	92	7
R = *p*-OH-	**15**	3	96	97	3
R = *p*-OMe-	**16**	22	87	68	14
	**17**	22	97	96	97
	**18**	22	99	68	97
	**19**	6	68	n.d.[c]	6
	**20**	6	99	98	78
	**21**	22	56	> 95	0
	**22**	3	53	n.d.[c]	0
	**23**	22	0	–	0
	**24**	6	48	95	2
	**25**	22	2	–	0
	**26**	3	94	–[d]	76
	**27**	22	7	n.d.	0
	**28**	22	8	95	0
	**29**	3	1	–	0

[a] All conversions were performed in a two-phase system; conversion (*X*) and enantiomeric excess (*ee*) were determined by gas chromatography.[9] n.d. = not determined. [b] enz: enzymatic; nenz: nonenzymatic. [c] Separation of enantiomers by the Chiraldex capillary GC column (G-PN-γ-cyclodextrin, propionyl) was not possible. [d] Achiral product.



Scheme 1. *At*HNL-catalyzed synthesis of chiral cyanohydrins and examples of compounds obtained after subsequent reactions.

intermediate for the antithrombotic agent clopidogrel (**30**). The cyanohydrin of **18** can be transferred to the corresponding α-hydroxyester, which is a building block of ACE inhibitors such as enalapril (**31**; Scheme 1).

The reaction of substrate **18** is somewhat less selective than that of **17**, indicating that the enzymatic reaction is slower and therefore the reaction conditions, primarily the pH, must be fine-tuned. Also an increasing chain length of the aliphatic aldehydes reduces the activity but not the stereoselectivity. In comparison, the enzyme is less active towards aliphatic and aromatic ketones.

In order to rationalize similarities and differences concerning the reaction mechanism and stereoselectivity of *At*HNL relative to the structurally similar, but *S*-selective *Hb*HNL and *Me*HNL, a homology model was created, based on the crystal structures of *Hb*HNL.[9,11] A comparison of both structures suggests a typical catalytic triad consisting of Ser81, Asp208, and His236 also in *At*HNL (Figure 1).

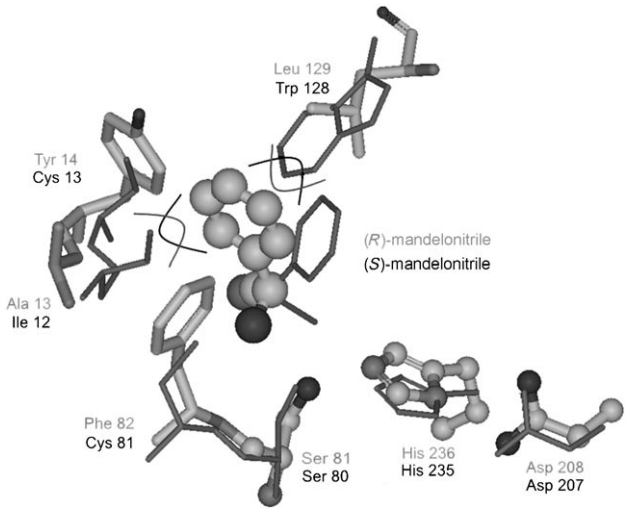


Figure 1. Overlay of the crystal structure of *Hb*HNL (dark gray, thin lines)[11] and the structural model of *At*HNL (light gray, thick rods). The catalytic triad (Ser/His/Asp) and the residues in contact with bound mandelonitrile are shown: (*R*)-mandelonitrile in the *At*HNL model and (*S*)-mandelonitrile in the crystal structure of *Hb*HNL (1YB8). *At*HNL reveals a specific binding pocket for (*R*)-mandelonitrile between Leu129 and Ala13 which is blocked by Trp128 and Ile12 in *Hb*HNL.

These residues were exchanged by nonfunctional, but sterically similar residues using site-directed mutagenesis, and the resulting variants (Ser81Ala, Asp208Asn, His236Phe) showed drastically impaired catalytic activity (< 2%), supporting their catalytically important function.[9] A further catalytically important residue (Lys236), which has been identified in *Hb*HNL,[11a] is replaced by Met237 in *At*HNL.

To analyze the differences in stereoselectivity a structural model of *At*HNL with (*R*)-mandelonitrile bound to the active

site was created based on the structure of *Hb*HNL containing (*S*)-mandelonitrile.[9,11] In comparison to *Hb*HNL, two side chains of the potential substrate-binding pocket in *At*HNL are exchanged. These are Trp128 and Cys13 in *Hb*HNL, which are replaced by Leu129 and Tyr14, respectively, in *At*HNL (Figure 1). The strict *S* selectivity of *Hb*HNL can be understood from the constructed model, since Trp128 and Ile12 sterically hinder the binding of (*R*)-mandelonitrile. On the other hand, it can be expected that the aromatic side chains of Tyr14 and Phe82 might stabilize (*R*)-mandelonitrile in the binding pocket of *At*HNL.

In first experiments with an *At*HNL variant (Tyr14Cys) still exclusively (*R*)-mandelonitrile was produced, suggesting that a single exchange is not sufficient to alter the stereoselectivity of *At*HNL. Studies on a double mutant (Tyr14Cys/Leu129Trp) and the crystal structure of the enzyme are in progress. The homology model is not yet accurate enough to explain the differences in activity or substrate selectivity as discussed before.

We have described a novel *R*-specific HNL (E.C. 4.2.1.–) from *Arabidopsis thaliana* and its application in biocatalytic processes. The enzyme is a good alternative to currently known *R*-selective HNLs, such as *Pa*HNL,[2e] for the production of *R*-cyanohydrins as it is readily available in technically relevant amounts by overexpression in *E. coli*. Its broad substrate range includes aliphatic and aromatic aldehydes as well as ketones.[14] As the first *R*-specific HNL based on an α/β-hydrolase fold, its structure will provide valuable information concerning the enzyme mechanism of α/β-hydrolase fold based HNLs.

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[9] For experimental details see the Supporting Information.

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[14] *At*HNL and its application have been filed for patent (J. Andexer, T. Eggert, evocatal GmbH, German patent application DE 102006058373.6, **2006**).

