Substrate Specificity of Mutants of the Hydroxynitrile Lyase from *Manihot esculenta***

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Dedicated to Professor Volker Jäger on the occasion of his 60th birthday

Several tryptophan128-substituted mutants of the hydroxynitrile lyase from Manihot esculenta *(MeHNL) are constructed and applied in the MeHNL-catalyzed addition of HCN to various aromatic and aliphatic aldehydes as well as to methyl and ethyl ketones to yield the corresponding cyanohydrins. The mutants (especially MeHNL-W128A) are in most cases superior to the wild-type (wt) enzyme when diisopropyl ether is used as the solvent. Substitution of tryptophan128 by an alanine residue enlarges the entrance channel to the active site of MeHNL and thus facilitates access of sterically demanding substrates to the active site, as clearly demonstrated for aromatic aldehydes, especially 3-phenoxybenzaldehyde. These experimental results are in accordance with the X-ray crystal structure of MeHNL-W128A. Aliphatic aldehydes, surprisingly, do not demonstrate this reactivity dependence of mutants on substrate bulkiness. Comparative reactions of 3-phenoxybenzaldehyde with wtMeHNL and MeHNL-W128A in both aqueous citrate buffer and a two-phase system of water/methyl* tert*-butyl ether again reveal the superiority of the mutant enzyme: 3-phenoxybenzaldehyde was converted quantitatively into a cyanohydrin nearly independently of the amount of enzyme present, with a space-time yield of 57 g L^{-1} h^{-1}.*

KEYWORDS:

aldehydes · bioorganic chemistry · cyanohydrins · enzymes · ketones

Introduction

Hydroxynitrile lyases (HNLs) are of great importance as biocatalysts for the stereoselective synthesis of cyanohydrins.[1] The HNLs of *Hevea brasiliensis* (HbHNL)[2] and *Manihot esculenta* (MeHNL)[3] have recently become the subject of intensive structural studies. Although MeHNL accepts a wide range of carbonyl compounds as substrate,[4] the enzyme-catalyzed preparation of optically active cyanohydrins is limited by bulky substituents with respect to conversion and enantiomeric excesses, as shown, for example, for the reaction of 3-phenoxybenzaldehyde, an important starting material for pyrethroids.[4b]

Based on the X-ray crystal structure of MeHNL,[3a] first attempts have been undertaken to correlate substrate specificity with structural features of the enzyme. The X-ray crystal structure of wild-type MeHNL (wtMeHNL) revealed that the active site of the enzyme is only accessible by a narrow channel through the surface of the protein.[3b] The channel entrance is capped by a tryptophan residue (Trp128), a relatively bulky amino acid, which might, therefore, be a primary determinant for substrate transport into the active site of the enzyme.

In order to investigate the influence and importance of Trp128 for the substrate specificity and activity of the enzyme, this tryptophan residue had to be exchanged with other amino acids with diminished steric demand.[5] Herein, we report the activity and specificity of the wtMeHNL mutants with alanine (W128A), cysteine (W128C), leucine (W128 L), and tyrosine (W128Y) mutations as catalysts of cyanohydrin formation from a variety of substrates.

Results and Discussion

Production and purification of the MeHNL mutants

In order to produce the recombinant MeHNL mutants, the respective transformed *E. coli* strains were grown in a 30-L fermentor at 20 °C for 48 h and were induced by addition of a low concentration of isopropyl-D-thiogalactopyranoside (IPTG; 100 mM) after 24 h (Table 1).

Wild-type MeHNL and the mutant proteins showed similar expression levels (~2 g MeHNL/fermentation), have the same native molecular weights, and behave identically in the purification procedure; this suggests that the amino acid substitutions have no impact on the overall global structure of the enzyme. The purified MeHNL mutants were initially characterized with respect to their specific activity towards the natural

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[**] *Enzyme-Catalyzed Reactions, Part 47. Part 46: J. Roos, F. Effenberger,* Tetrahedron: Asymmetry ***2002***, 13, *1855 – 1862*.

Table 1. *MeHNL mutants generated by site-directed mutagenesis.*

Amino acid substitution	Sequence of mutagenic oligonucleotide 5′ – 3′[a]
W128A	AAG CTT TTG GAG TCG TTT CCT GAC **GCG** AGA GAC ACA GAG TAT TTT ACG TTC AC
W128C	GGA AAG CTT TTG GAG TCG TTT CCT GAC **TGC** AGA GAC ACA GAG TAT TTT ACG TTC ACT
W128L	GGA AAG CTT TTG GAG TCG TTT CCT GAC **TTA** AGA GAC ACA GAG TAT TTT ACG TTC ACT
W128Y	GGA AAG CTT TTG GAG TCG TTT CCT GAC **TAT** AGA GAC ACA GAG TAT TTT ACG TTC ACT

[a] Triplets encoding the mutated amino acid at position 128 of MeHNL are underlined and shown in bold.

substrate acetone cyanohydrin and the bulky unnatural substrate 4-hydroxymandelonitrile (**2 f**).[5] While all MeHNL proteins have roughly similar specific activities towards acetone cyanohydrin (between 20 and 40 U mg^{-1}), there were huge differences in their specific activities towards 4-hydroxymandelonitrile (Table 2).

Table 2. *Specific activities of wtMeHNL and MeHNL mutants.*

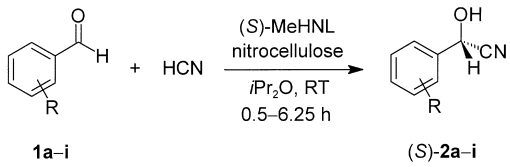
Enzyme	Specific activity [U mg^{-1}]	
	Acetone cyanohydrin	4-Hydroxymandelonitrile (**2 f**)
wtMeHNL	44	0.002
MeHNL-W128A	20	0.89
MeHNL-W128C	28	1.87
MeHNL-W128L	31	0.36
MeHNL-W128Y	40	0.17

Substitution of tryptophan128 by alanine, cysteine, leucine, or tyrosine resulted in an 85- (W128Y) to 935-fold (W128C) increase in the specific activity towards 4-hydroxymandelonitrile of the various MeHNL mutants compared to the wild-type enzyme. This result again underscores the importance of the accessibility of the active site of the wtMeHNL through the tryptophan-covered channel, as discussed based on the crystal structure of the enzyme.[5]

Substituted benzaldehydes 1 as substrates

In general, substituted benzaldehyde cyanohydrins have great synthetic potential for the preparation of the corresponding mandelic acids and 1,2-amino alcohols,[1] while 3-phenoxy- and 4-fluoro-3-phenoxybenzaldehyde cyanohydrins are important intermediates for the synthesis of pyrethroids.[6]

The addition of HCN to a variety of substituted benzaldehydes **1** catalyzed by wtMeHNL and the channel mutants MeHNL-W128A, MeHNL-W128C, MeHNL-W128 L, and MeHNL-W128Y, respectively, was investigated (Scheme 1). In order to compare the activity of the different mutants, identically purified enzyme solutions (4.6 mg protein), substrate (1 mmol), and anhydrous HCN (2 mmol) were applied. The reaction time was varied depending on the reactivity of the different aldehydes. The results are summarized in Table 3.



1a–i (*S*)-**2a–i**

1,**2**	R=	**1**,**2**	R=
a	H	**f**	4-HO
b	2-Cl	**g**	4-MeO
c	2-Br	**h**	4-Me
d	2-HO	**i**	3-PhO
e	3-HO		

Scheme 1. *MeHNL-catalyzed addition of HCN to substituted benzaldehydes **1**.*

As can be seen from the results shown in Table 3, the mutant enzymes are superior to the wild type in the cases of the sterically demanding aldehydes **1 h**, **1 i**; excellent enantiomeric excesses at high conversion rates were achieved with most of the applied aldehydes (Table 3), especially with the mutant MeHNL-W128A. Only moderate conversion rates were obtained with the hydroxybenzaldehydes: 44 – 57 % for 2-hydroxybenzaldehyde (**1 d**) and 51 – 63 % for 4-hydroxybenzaldehyde (**1 f**). The excellent results obtained for 3-phenoxybenzaldehyde (**1 i**) with several mutants led us to investigate the reaction with the wild-type enzyme and the mutant MeHNL-W128A at two different concentrations (Figure 1). As can be seen from the figure, after 75 min reaction time, the mutant MeHNL-W128A has ten times higher activity than wtMeHNL towards **1 i**. At substrate concen-

Table 3. *Conversion rate (%) and enantiomeric excess (%, in parentheses) for the MeHNL-catalyzed preparation of benzaldehyde cyanohydrins (S)-**2** by HCN addition to benzaldehydes **1** in diisopropyl ether.*

Substrate	**1 a**	**1 b**	**1 c**	**1 d**	**1 e**	**1 f**	**1 g**	**1 h**	**1 i**
t [h]	0.5	1.0	4.0	1.0	1.0	1.5	0.75	0.75	6.25
Wild-type	97 (99)	96 (98)	96 (96)	47 (91)	88 (97)	51 (94)	79 (99)	50 (99)	47 (96)
W128Y	98 (98)	99 (96)	91 (96)	43 (85)	95 (98)	62 (92)	81 (94)	80 (95)	85 (96)
W128L	97 (99)	99 (98)	95 (97)	57 (87)	93 (97)	61 (97)	84 (99)	86 (99)	95 (98)
W128C	98 (99)	99 (99)	98 (98)	43 (85)	95 (98)	61 (95)	84 (98)	56 (96)	97 (96)
W128A	97 (97)	99 (93)	98 (93)	44 (82)	97 (94)	63 (92)	84 (95)	93 (97)	98 (90)

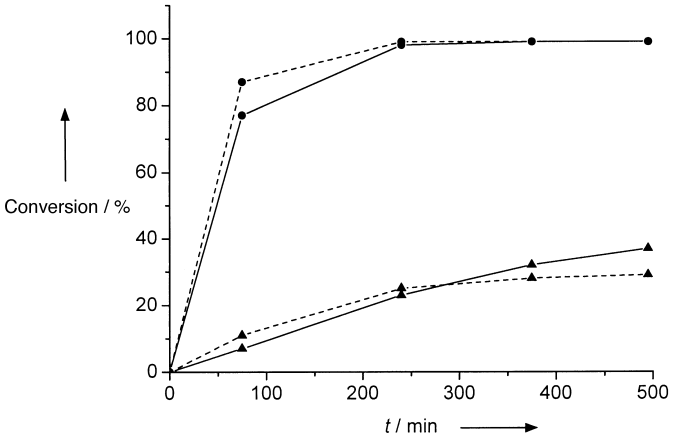
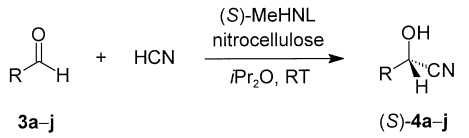


Figure 1. *Data from the conversion of 3-phenoxybenzaldehyde (**1 i**) to 3-phenoxybenzaldehyde cyanohydrin (S)-**2 i** catalyzed by wtMeHNL (▲) and mutant MeHNL-W128A (●) at concentrations of 600 mM (——) and 1 M (- - - -).*



3a–j (*S*)-**4a–j**

3,4	R=	3,4	R=
a	$PhCH_2$	**f**	$CH_3(CH_2)_2CH{=}CH$
b	$4\text{-}Me_3SiOC_6H_4CH_2$	**g**	$CH_3(CH_2)_{10}$
c	$4\text{-}HOC_6H_4CH_2$	**h**	$CH_3(CH_2)_8$
d	PhCH=CH	**i**	$CH_3(CH_2)_7$
e	$PhCH_2CH_2$	**j**	$CH_3(CH_2)_6$

Scheme 2. *MeHNL-catalyzed addition of HCN to aliphatic saturated and unsaturated aldehydes **3 a–j**.*

trations of 1 M, **1 i** was converted quantitatively by MeHNL-W128A, to give a space-time yield of 56 g L^{-1} h^{-1}. After the reaction was complete, 1.13 g cyanohydrin **2 i** was obtained in 5 mL diisopropyl ether. These experimental data are in accordance with the crystal structure of the MeHNL-W128A substrate-free form of the enzyme and indicate that W128A substitution significantly enlarged the active-site channel entrance.[5] Hence, access to the active site is facilitated even for bulky substrates such as **1 i**. The enzyme-catalyzed addition of HCN to 4-fluoro-3-phenoxybenzaldehyde, a further important pyrethroid precursor, afforded results comparable with those found for **1 i**.[4c]

Aliphatic saturated and unsaturated aldehydes 3 as substrates

Aliphatic saturated and unsaturated aldehydes **3 a – j** were also investigated comparatively by using wtMeHNL and the channel mutants described above (Scheme 2, Table 4). The results in Table 4 reveal that 2-phenylacetaldehyde (**3 a**) was converted quantitatively by all mutants and also by wtMeHNL. The enantiomeric excesses, however, become lower with decreasing size of the amino acid at the channel entrance, that is, in the sequence: wild type > W128Y > W128L > W128C > W128A. In contrast to the aromatic aldehydes **1**, the saturated aliphatic aldehydes do not show the expected dependence of substrate specificity on the bulkiness of the channel mutants. A plausible explanation of these results cannot currently be deduced from the X-ray structure of the enzyme.

The unsaturated cinnamaldehyde (**3 d**) and 2-hexenal (**3 f**) are excellent substrates for both wtMeHNL and all mutants. In the case of 3-phenylpropanal (**3 e**), the enantiomeric excesses (*ee*) vary; the best *ee* value (82%) was achieved with the mutant MeHNL-W128A. In the series of longer chain aldehydes **3 g – 3 j**, dodecanal **3 g** was still accepted as substrate, although double the amount of protein generally used and 72 h reaction time were necessary to obtain satisfying conversion. Quantitative conversion and 90% *ee* was obtained with **3 g** and mutant MeHNL-W128Y (Table 4). Substrates **3 h – j** show similar results to the other compounds concerning conversion and optical purity, but the reaction times for these substrates had to be varied considerably.

Methyl and ethyl ketones 5 as substrates

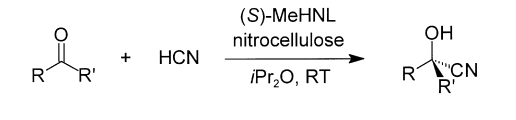
The reactions of several methyl ketones with HCN catalyzed by the wild-type enzyme wtMeHNL have already been reported.[4a] In the study described herein, some more methyl (**5 a – d**) and ethyl ketones (**5 e**, **f**) have been investigated, not only catalyzed by wtMeHNL but also by the mutants described above (Scheme 3, Table 5).

Table 5 shows that the reaction of acetophenone (**5 a**) proceeds with a very low degree of conversion with all mutants, while the enantiomeric excesses of 81 – 90% *ee* are satisfactory. Phenylacetone (**5 b**), however, was found to be an optimal substrate for the wild-type enzyme and for all mutants, and gives the corresponding cyanohydrin (*S*)-**6 b** with *ee* values of 89 –

Table 4. *Conversion rate (%) and enantiomeric excess (%, in parentheses) for the MeHNL-catalyzed preparation of (S)-cyanohydrins (S)-**4** by HCN addition to aldehydes **3** in diisopropyl ether.*

Substrate	3 a	3 b	3 c	3 d	3 e	3 f	3 g[a]	3 h	3 i	3 j
t [h]	1.0	24	0.5	4.0	1.0	5.0	72	17	26	3.0
Wild type	99 (98)	89 (82)	88 (96)	80 (95)	90 (67)	58 (97)	80 (71)	65 (78)	99 (80)	96 (79)
W128Y	99 (95)	99 (64)	91 (93)	87 (95)	99 (73)	92 (99)	99 (90)	93 (88)	94 (89)	99 (82)
W128L	99 (93)	99 (79)	96 (56)	80 (98)	89 (32)	92 (99)	91 (87)	64 (76)	96 (73)	99 (72)
W128C	99 (87)	98 (49)	62 (76)	71 (96)	91 (50)	26 (93)	99 (86)	28 (66)	98 (72)	99 (80)
W128A	99 (85)	99 (50)	94 (64)	87 (97)	97 (82)	87 (99)	95 (87)	99 (81)	98 (80)	99 (78)

[a] 9.2 mg protein used.



5a–**d** (R'=Me)
5e,**f** (R'=Et)

(*S*)-**6a**–**d** (R'=Me)
(*S*)-**6e**,**f** (R'=Et)

5,**6**	R=	**5**,**6**	R=
a	Ph	**d**	$(CH_3)_2C=CH(CH_2)_2$
b	$PhCH_2$	**e**	$CH_3(CH_2)_3$
c	$PhCH_2CH_2$	**f**	$CH_3(CH_2)_4$

Scheme 3. *MeHNL-catalyzed addition of HCN to methyl (**5a**–**d**) and ethyl (**5e**,**f**) ketones.*

Table 5. *Conversion rate (%) and enantiomeric excess (%, in parentheses) for the MeHNL-catalyzed preparation of (S)-cyanohydrins (S)-**6** by HCN addition to ketones **5** in diisopropyl ether.*

Substrate	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**
t [h]	3	1	1	1	2	6
Wild type	13 (87)	82 (97)	36 (49)	78 (61)	14 (46)	24 (61)
W128Y	13 (86)	86 (91)	86 (19)	84 (49)	61 (91)	62 (90)
W128L	13 (90)	85 (92)	85 (18)	64 (43)	87 (96)	77 (97)
W128C	14 (90)	86 (89)	70 (22)	67 (30)	47 (90)	66 (86)
W128A	12 (81)	85 (92)	85 (13)	85 (28)	40 (80)	54 (93)

97% at 82–86% conversion. In contrast, the conversion rates as well as the enantiomeric excesses in the reaction of benzylacetone (**5c**) are in all cases low. Similar experimental results to those observed for **5c** were obtained for the reaction of the unsaturated ketone **5d**.

Both ethyl ketones **5e** and **5f** are poor substrates for wtMeHNL. By using the mutant MeHNL-W128L, however, the corresponding cyanohydrins **6e** and **6f** were surprisingly obtained with 96 and 97% *ee* at 87 and 77% conversion, respectively.

MeHNL-catalyzed reactions in water and in a two-phase system

The MeHNL-catalyzed reactions of aldehydes and ketones with HCN described so far were performed in diisopropyl ether with the enzymes adsorbed on nitrocellulose and gave high chemical and optical yields of (*S*)-cyanohydrins. Nevertheless it was of interest to see if HCN applied in a free form could be substituted by HCN prepared in situ. Two procedures that use HCN prepared in situ are described in the literature: reaction in an aqueous medium[7] and reaction in a two-phase system.[8] In both cases, the required HCN is released from an alkali cyanide by acids. Both routes were investigated with respect to their application to catalysis by MeHNL mutants.

Two factors are decisive in obtaining high conversion rates and satisfying optical yields in aqueous solution for HNL-catalyzed enantioselective HCN addition to carbonyl compounds: first, the solubility of the carbonyl compounds, and second, avoidance of chemical addition of HCN. Several aldehydes that are interesting as potent starting compounds are not soluble in water and thus gave low space-time yields. To suppress the base-catalyzed chemical addition, relatively low pH values (pH < 4) are required for reactions in aqueous solution.[7] To achieve efficient space-time yields despite the low pH conditions, much greater amounts of enzyme are necessary to compensate for the markedly reduced enzyme activity at the lower pH value. The conditions developed for reactions with HbHNL in aqueous citrate buffer[7c] were also applied to the reactions with MeHNL mutants. The results for the reactions of the aldehydes **1d**, **1i**, and **3e** in aqueous citrate buffer are summarized in Table 6.

Table 6. *Conversion rate (%) and enantiomeric excess (%, in parentheses) for the MeHNL-catalyzed preparation of (S)-cyanohydrins (S)-**2d**,**i**, **4e** by HCN addition to aldehydes **1d**, **i**, **3e** in aqueous citrate buffer.*[a]

Substrate	**1d**	**1i**	**3e**
t [h]	1	6.25	3
Wild type	77 (98)	46 (98)	90 (67)
W128Y	32 (94)	50 (98)	98 (81)
W128L	87 (94)	54 (97)	89 (39)
W128C	25 (92)	35 (98)	83 (40)
W128A	81 (91)	72 (95)	99 (85)

[a] MeHNL: 4.6 mg; aldehydes **1d**, **i**, **3e**: 1 mmol; citrate buffer: pH 3.8, 0.5 M. Experiments were carried out at 0 °C.

Conversion and enantiomeric excesses are enhanced for 2-hydroxybenzaldehyde (**1d**), compared with the reaction in diisopropyl ether. 3-Phenoxybenzaldehyde (**1i**), however, has a low conversion rate due to its insolubility in citrate buffer. As observed for the organic solvent, in citrate buffer the mutant MeHNL-W128A was the most efficient catalyst for the bulky substrate **1i** with respect to conversion. The aldehyde **3e** is also less soluble in water than in organic solvents, therefore longer reaction times are required.

Since the conversion rate of **1i** in aqueous medium is fairly low, the preparation of 3-phenoxybenzaldehyde cyanohydrin (*S*)-**2i** was also investigated in the two-phase system (water/methyl *tert*-butyl ether (MTBE); Figure 2) previously applied in the HbHNL-catalyzed reaction of **1i**.[8b] Figure 2 shows the results obtained with the wild-type enzyme and the mutant MeHNL-W128A. The reaction times are clearly diminished compared to the reaction in diisopropyl ether as a result of the formation of an emulsion in the two-phase system, which increases the specific surface of the catalyst. Since the quantity of enzyme used for all substrate concentrations investigated was identical, it can be deduced from Figure 2 that the reaction rate is nearly independent of the amount of enzyme.

The mutant MeHNL-W128A is superior to the wild-type enzyme for bulky substrates in the two-phase system. Aldehyde **1i** (10 mmol) was converted by MeHNL-W128A quantitatively in roughly one hour, while the reaction catalyzed by wtMeHNL reached only approximately 42% conversion after two hours. Moreover, the enantiomeric excess decreased with increasing substrate concentration when the wild-type enzyme was used.

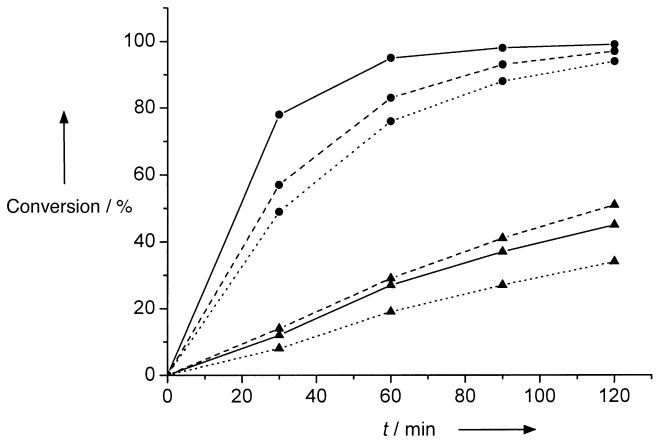


Figure 2. *Data from the conversion of 3-phenoxybenzaldehyde (**1i**) into 3-phenoxybenzaldehyde cyanohydrin (S)-**2i** catalyzed by wtMeHNL (▲) and mutant MeHNL-W128A (●) with 1 mmol (——), 5 mmol (- - - -), and 10 mmol (•••••) substrate in a two-phase system.*

The space-time yield of 57 g L^{-1} h^{-1} for 10 mmol **1i** under MeHNL-W128A catalysis corresponds with that obtained in organic solvent (56 g L^{-1} h^{-1}).

Conclusions

The tryptophan128 residue of the wild-type hydroxynitrile lyase of *Manihot esculenta*, situated at the channel entrance to the active site of MeHNL, was substituted by amino acids with decreasing size to give the MeHNL mutants MeHNL-W128A, MeHNL-W128C, MeHNL-W128L, and MeHNL-W128Y investigated in this study. It can be seen from the X-ray crystal structure of the mutant MeHNL-W128A that the entrance to the active site of the enzyme is significantly enlarged by alanine substitution, which facilitates access to the active site. The role of Trp128 as a structure determinant of wtMeHNL was unambiguously supported by the addition of HCN to the bulky 3-phenoxybenzaldehyde (**1i**) to yield the pyrethroid precursor (*S*)-3-phenoxybenzaldehyde cyanohydrin ((*S*)-**2i**). The Trp128 channel mutants were generally found to have higher activity than the wild-type enzyme for aromatic aldehydes. Surprisingly, aliphatic aldehydes do not show this effect. The reactions of aliphatic aldehydes with bulky structures with MeHNL mutants are comparable to those with the wild-type enzyme. Besides steric effects, solubility could therefore also have an influence on substrate specificity. Catalysis by the mutant MeHNL-W128A enabled **1i** to be converted to the corresponding cyanohydrin (*S*)-**2i** quantitatively and with high enantiomeric excess, with a space-time yield of 56 g L^{-1} h^{-1} at a substrate concentration of 1 M. This result could also be achieved in a two-phase system of water/methyl *tert*-butyl ether. However, simple workup conditions make the organic solvent preferable to the aqueous or the two-phase system.

The high space-time yields and the simple accessibility of recombinant enzymes, both the wtMeHNL and its mutants, now allow the preparation of (*S*)-cyanohydrins in preparative amounts. (*S*)-Cyanohydrins can be converted stereoselectively, for example, to the corresponding α-hydroxycarboxylic acids, 1,2-amino alcohols, or α-amino acids.

Material and Methods

Gas chromatography separations were conducted by using a) capillary glass columns (20 m), OV 1701, carrier gas: hydrogen; b) a Chiraldex B-TM (ICT) column (30 m × 0.32 mm), carrier gas: hydrogen. Starting aldehydes are either commercially available or were prepared according to known procedures.[9, 10] Racemic cyanohydrins were prepared according to a literature procedure.[11]

4-Trimethylsilyloxyphenylacetaldehyde (3b): a) Pyridine (4.7 mL, 57.8 mmol) was added dropwise to a solution of methyl 4-hydroxyphenylacetate (8 g, 48.2 mmol) and trimethylchlorosilane (7.3 mL, 57.8 mmol) in CH_2Cl_2 (70 mL). After stirring for 10 min (GC control), the reaction mixture was diluted with Et_2O (70 mL) and the precipitated pyridinium chloride was filtered off. The solvent was removed, the residue dissolved in Et_2O, and pyridinium chloride filtered off. This procedure was repeated until the pyridinium chloride had been completely removed. Crude methyl 4-trimethylsilyloxyphenylacetate was distilled under vacuum to give 8 g (70%) product as a colorless oil; b.p.: 118 °C/0.1 mm; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.25$ (s, 9H), 3.55 (s, 2H, $PhCH_2$), 3.67 (s, 3H, OCH_3), 6.79 (d, $J = 8.5$ Hz, 2H, Ph), 7.13 (d, $J = 8.5$ Hz, 2H, Ph); Elemental analysis: calcd for $C_{12}H_{18}O_3Si$ (238.4): C, 60.47; H, 7.61; found: C, 60.61; H, 7.52.

b) A 1 M solution of diisobutylaluminium (DIBAL) in hexane (40.5 mL, 40.5 mmol) was added dropwise to a solution of methyl 4-trimethylsilyloxyphenylacetate (7 g, 29.36 mmol) in absolute toluene at −78 °C under an N_2 atmosphere. When the reaction was complete (GC control), methanol (5 mL) was added to hydrolyze excess DIBAL. The reaction mixture was allowed to warm to room temperature and dilute HCl (5%) was slowly added until two layers formed. The organic layer was separated, dried (Na_2SO_4), and concentrated. Distillation under vacuum gave **3b** (3.5 g, 57%) as a colorless oil; b.p.: 110 °C/0.1 mm; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.26$ (s, 9H), 3.60 (d, $J = 2.5$ Hz, 2H, $PhCH_2$), 6.83 (d, $J = 8.5$ Hz, 2H, Ph), 7.08 (d, $J = 8.5$ Hz, 2H, Ph), 9.70 (t, 1H, CHO). Elemental analysis: calcd for $C_{11}H_{16}O_2Si$ (208.3): C, 63.41; H, 7.74; found: C, 63.24; H, 7.73.

Construction and purification of MeHNL-mutants: Point mutations of tryptophan128 of MeHNL were introduced into the pQE4-MeHNL expression construct described elsewhere,[12] which encodes wild-type MeHNL. For this purpose, the Quick Change Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) was used according to the manufacturer's recommendations. In brief, for each amino acid substitution two complementary primers corresponding to nucleotides 380 – 436 (or 383 – 436 in the case of MeHNL-W128A) of MeHNL and containing the mutation of interest (Table 1) were extended during temperature cycling by means of Pfu DNA polymerase. This process produced mutated plasmids containing staggered nicks. Methylated (parental) and hemimethylated (semiparental) plasmids were selectively digested with DpnI and the remaining mutated plasmids were transformed into *E. coli* XL1-Blue. Several clones were analyzed for the presence of the desired mutation by restriction analysis and DNA sequencing with the modified chain termination method.[13] In order to express the clones, the pQE4-MeHNL-derived plasmids encoding the various MeHNL mutants were transformed in *E. coli* strain M15[pREP4] and IPTG-inducible production of the recombinant proteins was achieved as previously described for the wild-type MeHNL.[4a] Purification of the MeHNL mutants was performed by use of anion exchange chromatography.[4a]

Enzyme-catalyzed reactions in an organic solvent: Nitrocellulose (10 g) was soaked in sodium citrate buffer (100 mL, 0.02 M, pH 3.3) for 4 h. The nitrocellulose was then filtered off and dried under vacuum for 5 h. A solution of the respective MeHNL was added to the pretreated nitrocellulose (100 mg/4.6 mg enzyme). The mixture was left to stand for 15 min at room temperature, then diisopropyl ether (5 mL), the respective substrate **1**, **3**, or **5** (1 mmol), and anhydrous HCN[14] (2 equiv with respect to the substrate) were added, and the reaction mixture stirred for the times given in Tables 3 – 6. After the reaction was complete, the immobilized enzyme was filtered off and washed with diisopropyl ether. The filtrate was concentrated under vacuum and the crude products **2**, **4**, and **6** were analyzed by NMR spectroscopy.

Enzyme-catalyzed reactions in aqueous citrate buffer: The enzyme solution and the respective substrate **1 d**, **1 i**, or **3 e** (1 mmol) was added to aqueous sodium citrate buffer (7 mL, 0.5 M, pH 3.8). A solution of KCN (2 mmol) in sodium citrate buffer (5 mL, 0.5 M, pH 3.8) at 0 °C was then added dropwise over 30 min. To terminate the reaction, Na_2SO_4 (10 g) was added to the reaction mixture and extracted with diisopropyl ether. The combined extracts were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was concentrated under high vacuum and analyzed by NMR spectroscopy.

Enzyme-catalyzed conversion of 1 i in a two-phase system: An enzyme solution was made up to 10 mL with sodium citrate buffer (50 mM, pH 5.4), and a solution of substrate **1 i** in MTBE (1.5 mL) was added. The reaction was started by addition of HCN (2 equiv with respect to **1 i**) and the reaction mixture was vigorously stirred for the given time, whereby an emulsion was formed. At the given time intervals, aliquots of 300 μL were taken, mixed with MTBE (2 mL), and $CaCl_2$ was added to remove excess water. The clear organic supernatant was concentrated. The residual cyanohydrin **2 i** was acetylated in CH_2Cl_2 (800 μL) with acetic anhydride (100 μL) and pyridine (50 μL), as described below.

Determination of enantiomeric excess and conversion percentages: Acetic anhydride and pyridine (100 μL each) were added to a solution of crude product (10 μL) in CH_2Cl_2 (500 μL) and the reaction mixture was heated at 60 °C for 2 h. The mixture was then filtered through a silica gel column (3 × 0.5 cm) with CH_2Cl_2 (2 mL) as eluent. Enantiomeric excess and the extent of conversion achieved were directly determined from the filtrate by gas chromatography on chiral cyclodextrin phases.

Chemical reaction (blank experiment): The chemical HCN addition was performed analogously to the enzymatic reaction, with the enzyme solution replaced by the same volume of sodium acetate buffer (20 mM, pH 5.4).

This work was generously supported by the EU project "Hydroxynitrile Lyases for Industrial Enantioselective Synthesis" (Project no. ERB BIO4-CT96 – 0112), the Degussa AG, and the Fonds der Chemischen Industrie. We thank Dr. A. Baro for preparing the manuscript.

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Received: September 26, 2002 [F 497]