Journal of Molecular Catalysis B: Enzymatic 61 (2009) 268–273

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

α- and β-oxygenated aldehydes derived from Diels–Alder reactions as substrates for hydroxynitrile lyases

Manuela Avi[a], Georg Steinkellner[b,c], Juliane Keilitz[d], Chakib Hajji[d], Rainer Haag[d], Karl Gruber[b,c], Herfried Griengl[a,c,*]

[a] Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 16, 8010 Graz, Austria
[b] Institute of Molecular Biosciences, University of Graz, Humboldtstraße 50/3, 8010 Graz, Austria
[c] Research Centre Applied Biocatalysis, Petersgasse 14, 8010 Graz, Austria
[d] Institute of Chemistry and Biochemistry, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 2 June 2009
Received in revised form 10 August 2009
Accepted 12 August 2009
Available online 19 August 2009

Keywords:
Cyanohydrins
Enzyme catalysis
Hydroxynitrile lyases
Molecular modelling
Biocatalysis

ABSTRACT

3,4-Dihydro-2H-pyran-2-carbaldehyde (**1**) and 2-methoxycyclohex-3-encarbaldehyde (**2**) obtained by thermal or chemocatalytic Diels–Alder reactions were converted into the corresponding cyanohydrins by hydroxynitrile lyase catalysis. Modelling investigations give a clear interpretation for the steric course of the biocatalytic cyanohydrin reaction of these α- and β-oxygenated aldehydes.

1. Introduction

Biocatalysts are of increasing importance for the pharmaceutical and agrochemical industry for the synthesis of enantiopure complex molecules [1]. Especially C–C bond forming enzymes are gaining momentum in the last years [2,3]. Hydroxynitrile lyases (HNLs), which catalyse the enantioselective synthesis of cyanohydrins, for example find application in the production of valuable precursors for many pharmaceuticals and agrochemicals, as ACE-inhibitors and pyrethroids [3,4].

Recently, α- and β-oxygenated aldehydes were investigated as substrates for the HNLs from *Prunus amygdalus* (*Pa*HNL) and *Hevea brasiliensis* (*Hb*HNL) [5–7]. α-Oxygenation influences strongly the HNL catalysed transformation. The selectivity decreases in comparison to the corresponding alkylated aldehydes. Most of these substrates also had an additional stereocentre adjacent to the carbonyl group, but the HNLs employed showed no chiral discrimination [5–7].

Aldehydes **1** and **2** (see Fig. 1) were available by Diels–Alder reactions from a cooperation project within the CERC-3 framework. The joint structural element is an oxygen functionality α and β to the carbonyl group. This opened the opportunity to extend the substrate spectrum of the biocatalytic cyanohydrin reaction and to finalize earlier work [5–7] on the influence of α- or β-oxygenation on this transformation both by experimental work and by molecular modelling.

2. Experimental

All solvents and materials not described in this chapter are commercially available and were appropriately purified, if necessary. The hydroxynitrile lyases were kindly provided by DSM Fine Chemicals Austria. Reactions were monitored by TLC (Merck silica gel 60 F_{254} or aluminium oxide 60 F_{254} neutral) and the compounds were visualised by spraying with Mo-reagent (10% H_2SO_4, 10% $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ and 0.8% $Ce(SO_4)_2{\cdot}4H_2O$ in water) or vanillin/H_2SO_4 solution (1 g vanillin in 1000 mL H_2SO_4 conc.). Flash chromatography was performed on Silica gel 60 (70–230 mesh, Merck). ^1H and ^{13}C NMR spectra were recorded on a VARIAN INOVA 500 MHz or a Bruker AMX 500 MHz spectrometer (^1H 500 MHz, ^{13}C 125 MHz) with TMS as an internal reference. NOE spectra were recorded on a Bruker AC 250 MHz spectrometer. Enantiomeric purities were analysed using a Hewlett Packard 6890 instrument equipped with a FID and a Chirasil-DEX CB column (25 m × 0.32 mm, 0.25 μm film). GC/MS measurements were per-

* Corresponding author at: Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 16, 8010 Graz, Austria.
E-mail address: herfried.griengl@a-b.at (H. Griengl).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.08.005

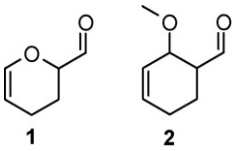

Fig. 1. Substrates.

formed employing a Hewlett Packard HP6890 Series-II GC system equipped with a HP 5973N mass selective detector with methane as reactant, a HP5-MS column (30 m × 0.25 mm, 0.25 μm film) and He as carrier gas. For analytical data *vide infra*.

2.1. Synthesis and safe-handling of anhydrous HCN—caution

All reaction equipment, in which HCN or cyanides were involved, was placed in a well ventilated hood. For continuous warning, an electrochemical sensor for HCN detection was used. The required amount of HCN was freshly prepared by adding dropwise a saturated NaCN solution to aqueous sulphuric acid (60%) at 80 °C. HCN was transferred in a nitrogen stream through a drying column and collected in a cooling trap at −12 °C. Waste solutions containing cyanides were treated with aqueous sodium hypochlorite (10%). Subsequently the pH was adjusted to 7.0 with aqueous sulphuric acid.

2.2. 3,4-Dihydro-2H-pyran-2-carbaldehyde (**1**)

The synthesis was performed according to literature and the spectroscopic data are identical with those reported [8]. The ratio aldehyde **1**/acrolein after purification is 5/1.

2.3. Procedure for the synthesis of 2-methoxycyclohex-3-enecarbaldehyde (**2**) by thermal Diels–Alder reaction

To a solution of acrolein (1.5 equiv.) in toluene a small amount of *p*-hydroquinone and 1-methoxy-1,3-butadiene (1 equiv.) is added. The mixture is heated to reflux until quantitative conversion. After cooling to rt the solution is filtered and the solvent and excessive acrolein are evaporated under reduced pressure yielding the crude Diels–Alder adduct **2** (81%, *cis*/*trans* = 6/1) as a light yellow liquid. Separation of the *cis*- and *trans*-product is achieved by HPLC (eluent: hexane/ethyl acetate = 85/15). Spectroscopic data are slightly different from literature [9]. ^{1}H NMR ($CDCl_3$) *cis*: δ 9.75 (d, J = 1.10 Hz, 1H, CHO), 5.96–5.88 (m, 2H, H3, H4), 4.05 (m, 1H, H2), 3.32 (s, 3H, OMe), 2.49–2.44 (m, 1H, H1), 2.20–2.10 (m, 1H, H5), 2.00–1.90 (m, 1H, H5′), 1.90–1.75 (m, 2H, H6); *trans*: δ 9.74 (d, J = 1.51 Hz, 1H, CHO), 5.84–5.76 (m, 2H, H3, H4), 4.05 (m, 1H, H2), 3.34 (s, 3H, OMe), 2.57–2.51 (m, 1H, H1), 2.07–2.01 (m, 1H, H5), 2.00–1.75 (m, 3H, H5′, H6). ^{13}C ($CDCl_3$) *cis*: δ 203.7 (CO), 132.2, 125.0 (olefinic), 72.6 (C2), 56.4 (OCH_3), 50.2 (C1), 23.9 (C6), 18.2 (C5); *trans*: δ 203.6 (CO), 132.6, 124.4 (olefinic), 73.2 (C2), 56.9 (OCH_3), 51.3 (C1), 24.4 (C6), 17.7 (C5). MS: 141 (M^+), 109 (M-MeO), 81 (M^+-(MeO + CO)), 79 (C_6H_7).

2.4. Procedure for the synthesis of 2-methoxycyclohex-3-enecarbaldehyde (**2**) catalysed by salen–chromium complexes

The reaction was performed under anhydrous conditions. To a solution of Jacobsen's salen–chromium complex [10] (63 mg, 5 mol%) or polyglycerol-supported salen–chromium complex [11] (1.41 mmol g^{-1}, 78 mg, 2.2 mol%) in anhydrous CH_2Cl_2 (1 mL/mmol) 4 Å molecular sieves is added and the mixture is cooled to 0 °C. Subsequently 1-methoxy-1,3-butadiene (1 equiv.) and acrolein (1 equiv.) are added and the mixture is stirred for 24 h (Jacobsen's salen) or 48 h (polyglycerol-supported salen) at 0 °C. The solvent was removed in vacuo and the residue was purified by column chromatography (hexane/ethyl acetate = 9/1) to yield the *cis*/*trans*-mixture of **2** in a ratio of 9/1.

2.5. General procedure for the synthesis of racemic cyanohydrins (blank reaction)

To a solution of aldehyde in *tert*-butyl methyl ether (*t*BME) an aqueous buffer (30 mM, citrate, 1/1, v/v) is added. The resulting mixture is stirred at 0 °C until an emulsion is formed. After addition of freshly prepared prussic acid (3.6 equiv.), the mixture is stirred at 0 °C until quantitative conversion. The emulsion is broken with Celite 545, filtered and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yields the crude cyanohydrins as light yellow liquids. For determination of the product distribution a small amount is acetylated with acetic anhydride and pyridine in dichloromethane using standard methods.

2.6. General procedure for the enzymatic synthesis of cyanohydrins

Method A: HbHNL. To a solution of aldehyde in *t*BME an aqueous solution (1/1, v/v), containing particular amounts of *Hb*HNL (approx. 4000–5000 U/mmol aldehyde) in buffer (30 mM, citrate, pH 5.0), is added and the resulting mixture is stirred at 0 °C until an emulsion is formed. After addition of freshly prepared prussic acid (1.8–3.6 equiv.), the mixture is stirred at 0 °C until quantitative conversion. The emulsion is broken with Celite 545, filtered and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yields the crude cyanohydrins as a light yellow liquid. For the determination of the distribution of the stereoisomers, a small amount is acetylated.

Method B: PaHNL. To a solution of aldehyde in *t*BME an aqueous solution (1/1, v/v), containing particular amounts of *Pa*HNL (approx. 2000–2500 U/mmol aldehyde) in buffer (30 mM, citrate, pH 5.0), is added and the resulting mixture is stirred at 0 °C until an emulsion is formed. Freshly prepared prussic acid (2.0–3.6 equiv.) is added and the mixture is stirred at 0 °C until quantitative conversion. The emulsion is broken with Celite 545, filtered and dried over Na_2SO_4. Solvent removal in vacuo gives the crude cyanohydrins as light yellow liquid. For the determination of the distribution of the stereoisomers, a small amount is acetylated using standard procedures.

Table 1
HNL catalysed conversion of **1** to give cyanohydrins **3**.

Entry	Method	pH	Enzyme amount (U/mmol)	HCN (equiv.)	(2*S*,2′*R*)- [a] **3** (%)	(2*R*,2′*R*)- **3** (%)	*de*- **3** (%)	(2*R*,2′*S*)- **3** (%)	(2*S*,2′*S*)- **3** (%)	*de* **3** (%)	Yield (%)
1	*Hb*HNL	4.8	4000	3.6	5.7	43.7	76.9	7.1	43.5	71.9	73
2	*Pa*HNL	3.7	2500	3.6	46.7	4.2	83.5	46.3	2.8	88.6	68
3	Blank	3.2	–	3.6	22.7	27.1	8.8	28.3	21.9	12.7	86

[a] Configuration at position 2′might be exchanged.

2.6.1. 2-(3′,4′-Dihydro-2′H-pyran-2′-yl)-2-hydroxy acetonitrile (**3**)

Blank: 86% yield. Enzymatic. The yields are shown in Table 1. ^{1}H ($CDCl_3$): δ 6.35 (m, 1H, H6′), 4.78 (m, 1H, H2), 4.50 (d, J = 4.88, 1H, H5′), 4.00 (m, 1H, H2′), 2.40–1.90 (m, 3H, H3′, H3″, H4′), 1.78–1.70 (m, 1H, H4″); δ 6.35 (m, 1H, H6′), 4.78 (m, 1H, H2), 4.57 (d, J = 4.88, 1H, H5′), 4.0 (m, 1H, H2′), 2.40–1.90 (m, 3H, H3′, H3″, H4′), 1.78–1.70 (m, 1H, H4″). ^{13}C ($CDCl_3$) δ 142.9, 118.3, 101.6, 75.0, 63.8, 23.4, 19.0; δ 142.9, 118.3, 101.7, 75.0, 64.2, 23.2, 19.1.

2.6.2. 2-Hydroxy-2-(2′-methoxycyclohex-3′-enyl) acetonitrile (**4**)

Blank: 84% yield. Enzymatic. Yields are shown in Table 2. The *cis*- and *trans*-products can be separated by flash chromatography (cyclohexane/EtOAc = 2/1 + acetic acid). ^{1}H ($CDCl_3$) *cis*: δ 6.02–5.90 (m, 3H, H3′((2*R*, 1′*R*, 2′*R*) + *ent*), H4′ (both diastereomers)), 4.64 (d, J = 4.88, 1H, H3′ ((2*R*, 1′*S*, 2′*S*) + *ent*)), 4.46 (m, 1H, H2 ((2*R*, 1′*R*, 2′*R*) + *ent*)), 4.34 (m, 1H, H2 ((2*R*, 1′*S*, 2′*S*) + *ent*)), 4.13 (m, 1H, H2′ ((2*R*, 1′*R*, 2′*R*) + *ent*)), 3.81 (m, 1H, H2′ ((2*R*, 1′*S*, 2′*S*) + *ent*)), 3.42 (s, 3H, OCH_3 ((2*R*, 1′*R*, 2′*R*) + *ent*)), 3.34 (s, 3H, OCH_3 ((2*R*, 1′*S*, 2′*S*) + *ent*)), 2.26–1.52 (m, 10H, H1′, 2×H5′, 2×H6′ (both diastereomers)). ^{13}C ($CDCl_3$) *cis*: (2*R*, 1′*R*, 2′*R*) + *ent*: δ 133.5, 123.9 (olefinic), 119.4 (CN), 74.1 (C2), 64.5 (C2′), 56.5 (OCH_3), 43.4 (C1′), 25.7 (C6′), 20.3 (C5′); (2*R*, 1′*S*, 2′*S*) + *ent*: δ 133.8, 123.7 (olefinic), 119.8 (CN), 74.4 (C2), 65.2 (C2′), 56.6 (OCH_3), 41.9 (C1), 25.7 (C6′), 18.6 (C5′).

2.7. General procedure for the acetylation of cyanohydrins **3** and **4**

Cyanohydrins **2** are acetylated according to standard procedures with 1.5 equiv. acetic anhydride and 1.5 equiv. pyridine in CH_2Cl_2 over night.

2.7.1. Cyano-(3′,4′-dihydro-2′H-pyran-2′-yl)-methylacetate (**5**)

The diastereomers are separated by flash chromatography (cyclohexane/EtOAc = 3/1). ^{1}H ($CDCl_3$) δ 6.28 (m, 1H, H6′), 5.48 (d, J = 5.86, 1H, H2), 4.74 (m, 1H, H5′), 4.08–4.03 (m, 1H, H2′), 2.12 (s, 3H, CH_3(Ac)), 2.12–1.97 (m, 3H, H3′, H3″, H4′), 1.86–1.77 (m, 1H, H4″); δ 6.38 (m, 1H, H6′), 5.54 (d, J = 5.80, 1H, H2), 4.80 (d, 1H, H5′), 4.18–408 (m, 1H, H2′), 2.18 (s, 3H, CH_3(Ac)), 2.16–2.01 (m, 3H, H3′, H3″, H4′), 2.00–1.94 (m, 1H, H4″). ^{13}C ($CDCl_3$) δ 169.1, 142.8, 115.0, 101.6, 72.9, 62.8, 23.4, 20.6, 18.7; δ 169.2, 143.1, 114.9, 101.4, 73.2, 63.3, 23.3, 20.5, 18.9. MS: 182 (M^+), 155 (M-CN), 140 (M-(CN + CH_3)). Chiral GC: 120 °C isotherm, 20 min, 0.45 bar H_2, 10.1 min, 11.7 min (2*R*, 2′*RS*), 12.4 min, 14.0 min (2*S*, 2′*RS*).

2.7.2. Cyano-(2′-methoxycyclohex-3′-enyl)-methylacetate (**6**)

The *cis*-stereoisomers can be separated by flash chromatography (cyclohexane/EtOAc = 5/1). *cis*-(2*S*, 1′*S*, 2′*S*): $[\alpha]_D^{24}$ +71.6 (c 1.37, $CHCl_3$), *de* = 90%, *ee* = 57%; *cis*-((2*S*, 1′*R*, 2′*R*) + *ent*): $[\alpha]_D^{24}$ −103.6 (c 1.14, $CHCl_3$), *de* = 70%, *ee* = 45%. ^{1}H ($CDCl_3$) *cis*: (2*R*, 1′*R*, 2′*R*) + *ent*: δ 5.98 (m, 2H, H3′, H4′), 5.31 (d, J = 10.26, 1H, H2), 3.80 (m, 1H, H2′), 3.34 (s, 3H, OCH_3), 2.20–1.92 (m, 2H, H1′, H5′), 2.09 (s, 3H, CH_3(Ac)), 1.65–1.55 (m, 1H, H5′), 1.55–1.44 (m, 2H, H6′, H6″); (2*R*, 1′*S*, 2′*S*) + *ent*: δ 5.94 (m, 2H, H3′, H4′), 5.35 (d, J = 10.26, 1H, H2), 3.57 (m, 1H, H2′), 3.27 (s, 3H, OCH_3), 2.24–2.16 (m, 1H, H1′), 2.09 (s, 3H, CH_3(Ac)), 2.16–2.00 (m, 1H, H5′), 1.82–1.74 (m, 1H, H5′), 1.66–1.52 (m, 2H, H6′, H6″). ^{13}C ($CDCl_3$) *cis*: (2*R*, 1′*R*, 2′*R*) + *ent*: δ 169.4 (CO), 132.5, 124.0 (olefinic), 116.5 (CN), 71.1 (C2), 63.3 (C2′), 56.6 (OCH_3), 41.5 (C1′), 25.2 (C6′), 20.4 (C5′), 19.6 (CH_3); (2*R*, 1′*S*, 2′*S*) + *ent*: δ 168.9 (CO), 132.6, 123.9 (olefinic), 117.0 (CN), 69.7 (C2), 62.6 (C2′), 56.5 (OCH_3), 41.2 (C1′), 25.2 (C6′), 20.3 (C5′), 19.1 (CH_3). MS: 210 (M^+), 178 (M-OCH_3), 150 (M-OAc), 118 (M-(OAc + OCH_3). Chiral GC: 120 °C isotherm, 25 min, 0.45 bar H_2, 15.9, 17.0 min (*trans* (2*S*)), 16.1, 17.5 min (*trans* (2*R*)), 18.0 min (*cis*-(2*R*, 1′*R*, 2′*R*)), 18.3 min (*cis*-(2*S*, 1′*S*, 2′*S*)), 19.5 min (*cis*-(2*S*, 1′*R*, 2′*R*)), 19.9 min (*cis*-(2*R*, 1′*S*, 2′*S*)).

2.8. Computational methods

Models of the enantiomers of cyanohydrins **3** and **4** were build and optimised using the program Sybyl v.7.3 (Tripos inc) [12]. In order to probe the different conformations of the six-membered rings a conformational search was applied using an MM3 stochastic search algorithm integrated within the Sybyl program. The conformations with the lowest energies were selected for docking and partial atomic charges were calculated for those conformations with antechamber [13] using the AM1-BCC charge model. The protein coordinates for *Hb*HNL were taken from the high resolution X-ray crystal structure of the complex with (*S*)-mandelonitrile (PDB Code 1yb6) [14]. Alternate conformations were discarded. Asp, Glu, Arg and Lys residues were treated as charged. Protonation and tautomerisation states of His-residues were chosen according to sensible hydrogen bonding networks using the program reduce [15] and by additional visual inspection. Hydrogen atoms were added using the program protonate which is part of the amber package [16]. Only polar hydrogen atoms of the protein and the ligands were retained for the docking simulations. The anionic cyanohydrins were docked to the active site using the program AutoDock v.4.0 [17,18] restricting the search to a 22.5 Å box around the active site. The protein was kept rigid in all calculations while the position and orientation of ligands as well as torsion angles around single bonds were allowed to vary. A genetic algorithm optimisation was employed using fifty independent simulations with populations consisting of 450 random structures evolving in about 130 generations. The probability for performing a local search which consists up to 300 iterations of a pseudo Solis and Wets optimisation [19] was set to 26%. For clustering the lowest energy structures of each independent run the rms-tolerance was set to 0.5 Å.

Table 2
HNL catalysed conversion of **2** to give cyanohydrins **4**. The columns *trans* and *cis* refer to the relative configuration of the starting material **2** which is not changed during the reaction. 2*R* and 2*S* denotes the configuration of the newly formed centre of chirality at C-2. Since the amounts of the products with *trans*-stereochemistry are small and due to separation difficulties during GC-analysis only the sum of the products with 2*R*- and 2*S*-configuration on the newly formed centre of chirality at C-2 is given.

Entry	Method	HCN (equiv.)	Enzyme amount (U/mmol)	pH	*trans*		cis (1′*R*, 2′*R*)[a]			(1′*S*, 2′*S*)[a]		
					2*R* (%)	2*S* (%)	2*R* (%)	2*S* (%)	*de* (%)	2*R* (%)	2*S* (%)	*de* (%)
1	Blank[b]	3.5	–	3.8	8.3	5.4	19.0	14.9	12.1	18.5	21.9	8.4
2	*Hb*HNL[b]	3.5	5400	4.6	2.4	11.8	4.0	36.4	80.2	12.5	32.8	44.8
3	*Hb*HNL[c]	2.1	4000	5.5	n.d	n.d	10.3	39.2	58.4	14.0	36.5	44.6
4	*Hb*HNL[d]	3.5	4000	4.2	n.d	n.d	12.2	33.5	46.6	18.3	29.6	23.6
5	*Pa*HNL[b]	3.5	2175	3.6	12.4	1.1	41.9	0.7	96.7	42.4	1.5	93.2

[a] Configuration might be exchanged.
[b] Educt from thermal DA reaction, *cis*/*trans* 6/1.
[c] Educt from thermal DA reaction followed by separation of *cis*-, *trans*-**2**.
[d] DA reaction catalysed by PG-salen, *cis*/*trans* 9/1.

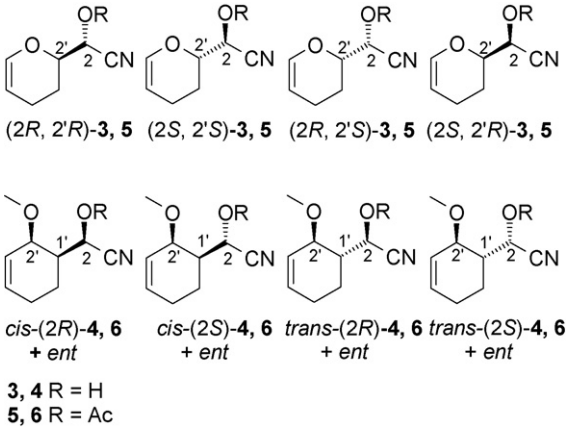

Fig. 2. Stereoisomers of the products from the cyanohydrin conversion of **1** and **2**.

3. Results and discussion

The two selected substrates (Fig. 1) were obtained from a thermal Diels–Alder reaction of acrolein with itself or with 1-methoxybuta-1,3-diene and are racemic. The reaction of acrolein to its dimer proceeds under harsh conditions. This solvent free reaction is carried out in a stainless steel bomb at 180 °C [8]. The purification of the hetero-DA adduct **1** as described in literature [8] turned out to be inefficient, due to the low stability of the product. Therefore, the substrate contained always some acrolein, which is also a substrate for HNLs [20]. However, the acrolein did not influence the enzymatic conversion of **1**.

The thermal Diels–Alder reaction of acrolein with 1-methoxybuta-1,3-diene afforded **2** in a *cis*/*trans* ratio of 6/1. Additionally, this reaction was carried out applying Jacobsen's salen–chromium catalyst [10] and a polyglycerol-supported salen–chromium complex [11], whereby the *cis*/*trans* ratio was enhanced to 9/1.

The cyanohydrin formation catalysed by HNLs was performed in an emulsion of *tert*-butyl methyl ether (*t*BME)/aqueous buffer (citrate buffer, pH 3.5–4.5, 1/1, v/v) at 0 °C. The conversion of **1** gave a mixture of four stereoisomers in 65–90% chemical yield after 2–3 h, whereas **2** was converted into a mixture of eight stereoisomers quantitatively after 4–5 h (Fig. 2). In both cases the non-enzymatic reaction shows no selectivity induced by the already existing stereocentres.

As Riva et al. showed for *Pa*HNL and *Hb*HNL [5–7] and other groups for the hydroxynitrile lyases from *Manihot esculenta* [21] and *Prunus mume* [22], HNLs make no chiral discrimination with racemic aldehydes. Substrates **1** and **2** are no exception. Therefore, major attention was given to the selectivities regarding the new centre formed. The absolute configurations were not assigned.

Complementary selectivities with respect to the new stereocentre were obtained by employing *Hb*HNL and *Pa*HNL as catalysts. By *Hb*HNL catalysis in product **3** at C-2 mainly the formation of the (2*R*)[1]-diastereomer is observed and in product **4** mainly (2*S*)[1]-stereochemistry. Employing *Pa*HNL in product **3** (2*S*)[1]-formation and in product **4** (2*R*)-stereochemistry was seen. This is in accordance with the known stereoselectivities of these two enzymes [2].

The results of the cyanohydrin reactions of substrate **2**, given in Table 2, need some comments. In entry 3 diastereomerically pure *cis*-**2** was used as substrate. Since the starting material was racemic the amounts of the (1′*R*,2′*R*)- and (1′*S*,2′*S*)-stereoisomers are the same within the experimental error of the GC-integration. As expected for *Hb*HNL catalysis the transformation of the carbonyl group into the 2*S*-alcohol is slightly preferred leading to a *de* of 58.4% and 44.6%, respectively, depending on the configuration at c-1′ and c-2′. The relative configuration of the centres c-1′/c-2′ and c-2 was assigned arbitrarily, what is, however, not of relevance with respect to the discussion of the steric course of the reaction.

In entries 2 and 4 diastereomerically enriched *cis*-starting material was used, entry 2 *cis*/*trans* 6/1, entry 4 *cis*/*trans* 9/1. Again by employing *Hb*HNL the formation of the (*S*)-configured centre at c-2 is preferred.

The slightly higher selectivities achieved by *Pa*HNL are probably due to the lower reaction pH, where the competitive chemical reaction is suppressed more strictly (Tables 1 and 2).

For the conversion of **1** the results are comparable with those obtained by the group of Riva with the saturated pyran-2-carbaldehyde [6]. Applying approximately the same amount of enzyme (U/mmol aldehyde) similar selectivities were achieved. With *Hb*HNL catalysis *de*-values of 77% (2′*R*) and 72% (2′*S*) were obtained. *Pa*HNL catalysis showed slightly higher selectivities and *de*-values of 84% and 89% for (2′*S*) and (2′*R*), respectively, were obtained (Table 1). The modelling calculations for the *Hb*HNL show

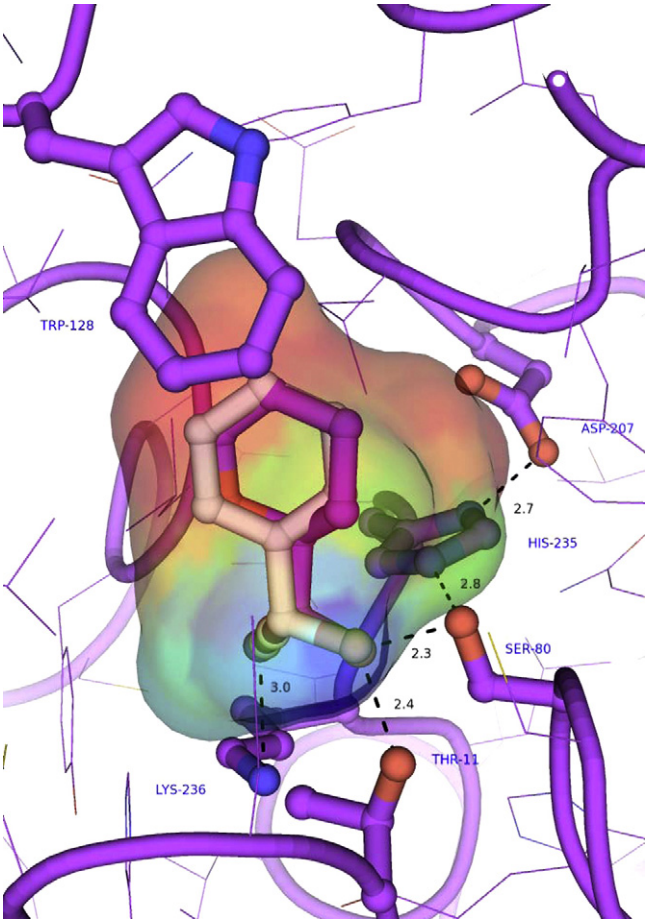

Fig. 3. View to the active site of *Hb*HNL as obtained by X-ray crystallography (1YB6) with docked cyanohydrin (2*R*,2′*S*)-**3** shown in the centre of the figure in purple as ball and stick representation. In a similar manner the active site residues responsible for catalysis of the cyanohydrin reaction are given: His-235, Asp-207, Ser-80, Thr-11 and Lys-236. On the top Trp-128 located at the tunnel for the entrance of the substrate to the active site is shown. Oxygen atoms are coloured in red, nitrogen atoms in blue. For comparison and discussion (*S*)-mandelonitrile (in white) is also laid into the active site. The active site pocket is shown as a transparent surface and coloured according to hydrophobic values where red indicated a hydrophobic and blue a hydrophilic environment.

[1] Priority replacement according to CIP rules.

that the docked cyanohydrin **3** binds within the active site very similarly to the (*S*)-mandelonitrile from the X-ray crystal structure (PDB Code: 1yb6, Fig. 3). There are no significant differences between (2*R*,2′*R*)-**3** and (2*R*,2′*S*)-**3** regarding to docking energies and binding modes, therefore no directing effect of the configuration at C-2′ to the formation of the newly formed chiral centre at C-2 is expected.

In contrast (2*S*,2′*R*)- and (2*S*,2′*S*)-**3** have higher docking energies and they do not bind in a productive binding mode (preserving the mechanistically important polar interactions with Ser-80, Thr-11 and Lys-236). This is in accordance with the already known properties of *Hb*HNL and verified by the data of Table 1. As the compounds are rather small and they all fit into the active site pocket without *in silico* mutation of the flexible Trp-128 residue, which closes the entry of the active site, the Trp-128 was kept rigid as well for the docking studies.

By transformation of **2** with *Hb*HNL catalysis the corresponding cyanohydrins **4** were obtained in slightly higher selectivities compared to β-oxygenated aldehydes investigated by Riva et al. The *cis*-enantiomers were converted with *de*-values of 24–45% and 47–80%, respectively, with approximately the same amount of enzyme. The lower selectivity for one enantiomer of a racemic aldehyde was also already observed earlier [7]. On the other hand, with *Pa*HNL catalysis *de*-values of 97% and 93% were obtained. In contrast to Riva's findings for β-oxygenated racemic aldehydes [5] approximately the same selectivities with both *cis*-enantiomers were achieved (Table 2).

In case of cyanohydrin **4** the modelling calculations showed that the binding modes of (2*S*,1′*S*,2′*R*)-, (2*S*,1′*R*,2′*S*)-, (2*S*,1′*R*,2′*R*)- and (2*S*,1′*S*,2′*S*)-**4** are also very similar to the binding of (*S*)-mandelonitrile observed within the *Hb*HNL X-ray structure (Fig. 4, as example (2*S*,1′*S*,2′*R*)-**4** is drawn). The functional group of **4** is located close to the hydrophobic section of the pocket (Fig. 4) and therefore no hydrogen-bonds or electrostatic interactions with residues are formed. Hence the binding energies between these conformations do not differ significantly. Therefore, in this case no chiral discrimination is expected, underlining the experimental data. In contrast, the (2*R*)-stereoisomers do not have reasonable binding modes concerning interactions with the active site residues. However, visual inspection of the (2*R*,1′*R*,2′*S*)-**4** revealed that in principle a productive binding mode with a reasonable energy exists for the axial conformation which is arranged in the pocket in a way to be able to form polar interaction with the active site residues Ser-80, Thr-11 and Lys-236 (Fig. 5). It remains unclear why this conformation showed a reasonable binding mode whereas the experimental data show a drop of activity compared to the blank reaction (Table 2). All other docking predictions are supported by the experimental data.

Acetylation of **3** and **4** to give **5** and **6**, respectively, and separation of the diastereomers by flash chromatography yielded enantioenriched products with synthetic potential.

In conclusion it was demonstrated that the Diels–Alder adducts investigated are substrates for hydroxynitrile lyases. In accor-

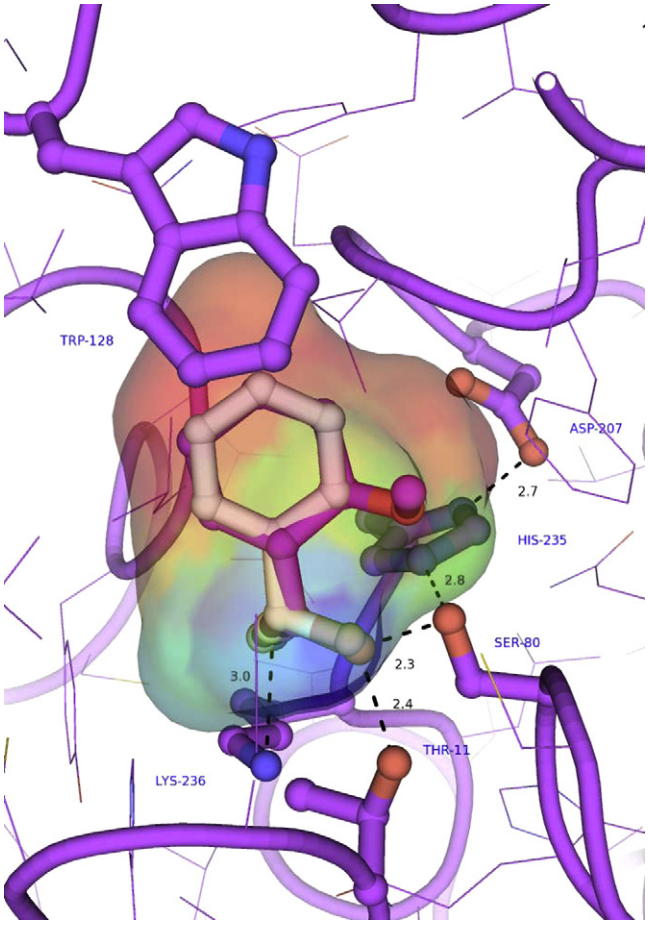

Fig. 4. View to the active site of X-ray crystal structure 1YB6 with docked cyanohydrin (2*S*,1′*S*,2′*R*)-**4**. The docked substrate and the active site residues His-235 Asp-207 Ser-80 Thr-11, Lys-236 and Trp-128 are shown in purple as ball and stick representation. Again for comparison (*S*)-mandelonitrile is also placed within the active centre. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

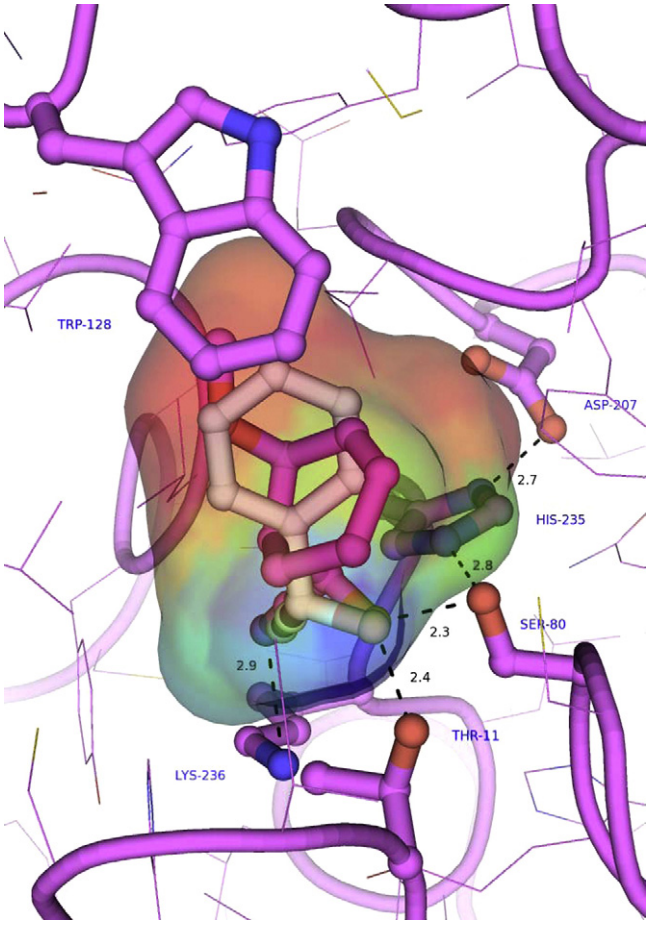

Fig. 5. View to the active site of X-ray crystal structure 1YB6 with docked cyanohydrin (2*R*,1′*R*,2′*S*)-**4** and for comparison (*S*) mandelonitrile (in white)

dance with previous investigations the enzymes are not able to catalyse the kinetic resolution of racemic aldehydes. *Pa*HNL gave products with higher *de*-values compared with those obtained by *Hb*HNL catalysis. Furthermore, *Pa*HNL catalysis did not show lower selectivities with one enantiomer of *cis*-**2**, as observed for the *Hb*HNL case with this substrate and for both enzymes with other racemic aldehydes [5,7]. The modelling calculations for *Hb*HNL indicate a (*R*)-selectivity in case of cyanohydrin **3** and (*S*)-selectivity in case of cyanohydrin **4** for the new stereocentre formed, which is coherent with the knowledge of *Hb*HNL being (*S*)-selective. No clear binding preference could be observed for the stereoisomers with productive binding modes in both cases, indicating that no chiral discrimination should be observed, which is consistent with the experimental data. The (2*S*)-**3** respectively (2*R*)-**4** enantiomers show higher docking energies and they do not have reasonable binding modes reflecting the experimental results.

Acknowledgments

The authors want to thank C. Illaszewicz for measurement of NMR spectra as well as Winfried Münch for HPLC-separation and NOE measurements. For providing the enzymes DSM Fine Chemicals Austria is gratefully acknowledged. Financial support was provided by the CERC3-Project "Sustainability by advanced chemoenzymatic technologies", FWF Nr. I20_B03.

References

[1] D.J. Pollard, J.M. Woodley, Trends Biotechnol. 25 (2006) 66–73.
[2] J. Sukumaran, U. Hanefeld, Chem. Soc. Rev. 34 (2005) 530–542.
[3] T. Purkarthofer, W. Skranc, C. Schuster, H. Griengl, Appl. Microbiol. Biotechnol. 76 (2007) 309–320.
[4] P. Poechlauer, Chim. Oggi 16 (1998) 15–19.
[5] G. Roda, S. Riva, B. Danieli, Tetrahedron: Asymm. 10 (1999) 3939–3949.
[6] P. Bianchi, G. Roda, S. Riva, B. Danieli, A. Zabelinskaja-Mackova, H. Griengl, Tetrahedron 57 (2001) 2213–2220.
[7] G. Roda, S. Riva, B. Danieli, H. Griengl, U. Rinner, M. Schmidt, A. Mackova Zabelinskaja, Tetrahedron 58 (2002) 2979–2983.
[8] T.M. Pedersen, E.L. Hansen, J. Kane, T. Rein, P. Helquist, P. Norrby, D. Tanner, J. Am. Chem. Soc. 123 (2001) 9738–9742.
[9] W.G. Dauben, H.O. Krabbenhoft, J. Org. Chem. 42 (1977) 282–287.
[10] S.E. Schaus, J. BranalT, E.N. Jacobsen, J. Org. Chem. 63 (1998) 403–405.
[11] C. Hajji, S. Roller, M. Beigi, A. Liese, R. Haag, Adv. Synth. Catal. 348 (2006) 1760–1771.
[12] SYBYL 7.3. Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.
[13] J. Wang, W. Wang, P.A. Kollman, D.A.J. Case, Mol. Graph. Model. 25 (2006) 247–260.
[14] G. Gartler, C. Kratky, G. Gruber, J. Biotechnol. 129 (2007) 87–97.
[15] J.M. Word, S.C. Lovell, J.S. Richardson, D.C. Richardson, J. Mol. Biol. 285 (1999) 1735–1747.
[16] D.A. Pearlman, D.A. Case, J.W. Caldwell, W.S. Ross, T.E. Cheatham III, S. DeBolt, D. Ferguson, G. Seibel, P. Kollman, Comput. Phys. Commun. 91 (1995)1–42.
[17] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, J. Comput. Chem. 19 (1998) 1639–1662.
[18] R. Huey, G.M. Morris, A.J. Olson, D.S. Goodsell, J. Comput. Chem. 28 (2007) 1145–1152.
[19] F.J. Solis, R.J.B. Wets, Math. Oper. Res. 6 (1981) 19–30.
[20] M.H. Fechter, R. Gaisberger, H.J. Griengl, Carbohydr. Chem. 20 (2001) 833–839.
[21] H. Buehler, B. Miehlich, F. Effenberger, ChemBioChem 6 (2005) 711–717.
[22] S. Nanda, Y. Kato, Y. Asano, Tetrahedron: Asymm. 17 (2006) 735–741.