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Stereoselective Biocatalytic Synthesis of (*S*)-2-Hydroxy-2-Methylbutyric Acid via Substrate Engineering by Using "Thio-Disguised" Precursors and Oxynitrilase Catalysis

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Abstract: 3-Tetrahydrothiophenone (**4**) and 4-phenylthiobutan-2-one (**7**) were used as masked 2-butanone equivalents to give the corresponding cyanohydrins **5** (79% yield, 91% *ee*) and **8** (95% yield, 96% *ee*) in an enzymatic cyanohydrin reaction applying the hydroxynitrile lyase (HNL) from *Hevea brasiliensis*. After hydrolysis and desulphurisation the desired intermediate (*S*)-2-hydroxy-2-methylbutyric acid (**10**) was obtained with 99% *ee*. Interestingly, when applying (*R*)-selective HNL from *Prunus amygdalus* again the (*S*)-cyanohydrin **5** was formed (62% *ee*). The absolute configuration of **5** was verified by crystal structure determination of the corresponding hydrolysis derived carboxylate. The fact that both enzymes yield the same enantiomer was analysed and interpreted by molecular modelling calculations.

Keywords: cyanohydrins · enzyme catalysis · heterocycles · hydroxy acids · lyases

Introduction

Although the enzyme-catalysed cyanohydrin reaction is one of the oldest examples of biocatalysis[1] a more comprehensive consideration of the synthetic potential began not before the 80s of the 20th century. Oxynitrilases or hydroxynitrile lyases (HNLs) were brought up as (*R*)- or (*S*)-selective biocatalysts for the addition of hydrogen cyanide to carbonyl functions of aldehydes and ketones in an enormous substrate variety reviewed frequently and recently.[2–8] Enantiomerically pure α-hydroxynitriles can be obtained from aliphatic and aromatic aldehydes. An extraordinary example is the double transformation of 1,1′-diformylferrocene into the (*R*,*R*)-biscyanohydrin in high yield and stereochemical purity.[9] With respect to the industrial application of HNLs, for example, (*S*)-3-phenoxybenzaldehyde cyanohydrin, an intermediate for synthetic pyrethroids, was produced on large industrial scale.[10]

Regarding ketones as educts for the biocatalysed cyanide attack, besides methyl and ethyl ketones[11–15] fewer results on cyclic ketones were published.[16–18] Surprisingly, although acalyphin, a cyanogenic plant glycoside, emerges from a pyridone derivative,[19] except our primary publication[20] and the results presented here, no HNL-catalysed HCN addition to heterocyclic ketones has been reported up to date. It is known from rational structure–activity relationship studies in biocatalytic cyanohydrin reactions that it is difficult for the enzyme to differentiate between similar small sized groups such as methyl and ethyl as in 2-butanone (**1**). However, as shown in Scheme 1, the cyanohydrin of 2-butanone would be a perfect target molecule to be hydrolysed to the desired intermediate 2-hydroxy-2-methylbutyric acid. In particular, the *S*-enantiomer **10** is used for the synthesis of a COX inhibitor.[21,22] Acid **10** can be obtained by resolution with chiral amines,[21] or by cyclisation with chiral hemiamidals giving diastereoisomeric mixtures which are chromato-

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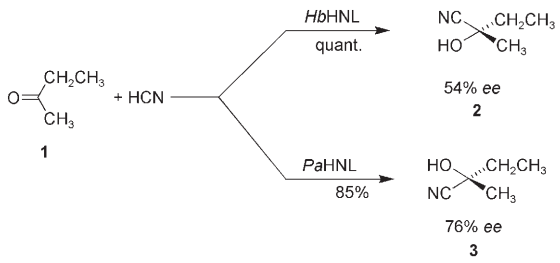
graphically separated and hydrolysed to give the desired α-hydroxy acid.[23] Recently it has been reported that lipase OF (*Candida cylindracea*) catalysed kinetic resolution of the benzyloxyalkanoate gave *ee* values from 60 to 81% for the desired product.[24] In the work described here another approach is shown by application of substrate engineering in biocatalysis, the docking/protecting group strategy recently published.[25]

Results and Discussion

In Scheme 1 the synthesis of (*R*)- and (*S*)-cyanohydrin **2** and **3** starting from 2-butanone (**1**) is illustrated. The biocatalytic cyanide addition employing the (*S*)-selective HNL from *Hevea brasiliensis* (*Hb*HNL) was performed in a numerous series of experiments with diverse reaction conditions. Nevertheless, no solvent change or alteration of the ratio of the biphasic solvent mixture, pH of buffer, equivalents of HCN, units of the biocatalyst used, could raise the stereochemical purity of 2-hydroxy-2-methylbutyronitrile (**2**) to more than 54% *ee* best.



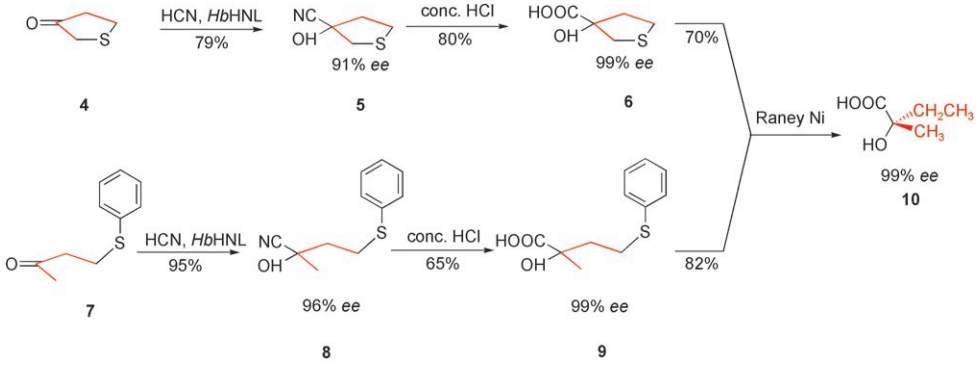
Scheme 1. Enzyme-catalysed cyanohydrin reaction of 2-butanone.

In the active site of the enzyme the similarity of the ethyl and methyl group directs the substrate with only minor selectivity of spatial orientation. Therefore, the cyanide can attack from the *Re* or the *Si* side.

These considerations correspond to experimental results for **2**, which was obtained in 18% *ee* with the *Hb*HNL-similar enzyme from *Manihot esculenta*.[26] This unsuitability of **1** as a substrate is in accord with the reported synthesis of (*R*)-cyanohydrin **3** using HNL from *Prunus amygdalus* (*Pa*HNL) with 76% *ee*[12] and from *Linum usitatissimum* HNL with 77% *ee*.[27] Very recently 2-butanone (**1**) has again been transformed into the (*R*)-cyanohydrin with a new HNL from *Prunus mume* to give 72% *ee*.[28]

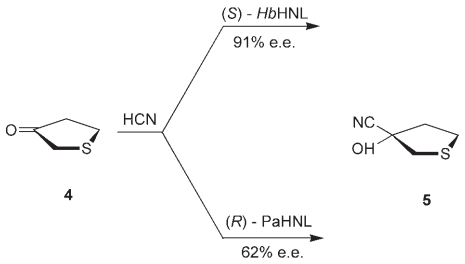
Consideration of the "docking/protecting group" technique[29–31] used in regio- and stereoselective biohydroxylations prompted us to choose 3-tetrahydrothiophenone (**4**) and 4-phenylthiobutan-2-one (**7**) which might be considered "thio-disguised" substrates as precursors for the enzymatic cyanohydrin reaction, as shown in Scheme 2. By this masking the spatial similarity of the vicinity of the carbonyl group is abolished and a clear preference of the cyanide attack might be the result. After the cyanohydrin reaction, the sulphur-containing groups can be removed to release the desired target molecule. Therefore, the commercially available heterocyclic ketone **4** was reacted in a biphasic mixture using *Hb*HNL catalysis with HCN to give 3-hydroxytetrahydrothiophene-3-carbonitrile (**5**) in 79% yield with 91% *ee*. Alternatively, the open chain ketone **7** was synthesised in situ by Michael addition of benzenethiol to 3-buten-2-one and subsequently transformed in a one-pot procedure to the corresponding cyanohydrin **8**. The yield was optimised over both steps to be 95% (96% *ee*). Cyanohydrins **5** and **8** were hydrolysed in excellent yields using concentrated HCl to give 3-hydroxytetrahydrothiophene-3-carboxylic acid (**6**) and 2-hydroxy-2-methyl-4-(phenylthio)butanoic acid (**9**). α-Hydroxy acid **9** was obtained with 99% *ee* after recrystallisation. Subsequent desulphurisation with Raney nickel transformed **6** and **9** into the required product **10** without loss of enantiopurity.

For advanced investigations concerning substrate acceptance we tried to synthesise the corresponding (*R*)-cyanohydrins by converting ketones **4** and **7** with HCN using the (*R*)-specific *Pa*HNL. However, we could not find any cyanohydrin formation with the open chain ketone **7** and *Pa*HNL. The reason is probably that the active site of *Pa*HNL is quite tight. Therefore, bulky substrates are not easily accepted. Regarding the heterocyclic derivative **4** we measured 62% *ee* for cyanohydrin **5** under *Pa*HNL catalysis as can be seen in Scheme 3. However, much to our surprise the same



Scheme 2. Cyanohydrin reaction of masked synthons for 2-butanone.

enantiomer was formed as obtained by *Hb*HNL catalysis. To the best of our knowledge this is the first substrate which shows the same stereochemistry for cyanide addition both by catalysis of the (*S*)-selective *Hb*HNL and the (*R*)-selective *Pa*HNL. For experimental elucidation of the absolute configuration of cyanohydrin **5** it was hydrolysed and recrystallised to give hydroxy acid **6** as the (*S*)-phenylethylamine salt. The X-ray structure determination shows clearly the (*S*)-configuration of **6** (see Figure 3), which is also proof for the (*S*)-configuration of 3-hydroxytetrahydrothiophene-3-carbonitrile (**5**).



Scheme 3. HNL-catalysed synthesis of (*S*)-cyanohydrin **5**.

These findings correspond to molecular dynamic calculations of the substrate binding in the active site of the two different enzymes. The structures of complexes of both *Hb*HNL and *Pa*HNL5 with (*S*)- and (*R*)-**5** were modelled using molecular docking calculations followed by molecular mechanics optimizations. The low-energy binding modes thus obtained (see Figures 1 and 2) are very similar to the binding modes of the natural substrates of these enzymes which were determined experimentally in the case of acetone cyanohydrin and *Hb*HNL[32] and by molecular modelling in the case of mandelonitrile and *Pa*HNL.[33] In particular, the polar interactions of the hydroxy and the cyano groups of the substrates are conserved in the complexes with the non natural substrate **5**.

In the *Hb*HNL complexes the hydroxy group of cyanohydrin **5** is hydrogen bonded to the side chains of Ser80 and Thr11, while the cyano group interacts with the ammonium group of Lys236 (Figure 1). Ser80 is part of the catalytic triad of *Hb*HNL (Ser80, His235, Asp207) which supposedly acts as a general base in the enzyme catalysed reaction. The positive charge of Lys236 is thought to contribute to substrate binding and orientation as well as to stabilizing the negative charge at the cyano group.[32] These mechanistically important polar interactions with the enzyme are the same for (*S*)- and (*R*)-**5**. The two enzyme bound enantiomers differ slightly in tetrahydrothiophene ring puckering. The main differences, however, are—not surprisingly—the position of the sulfur atom and the 5-methylene group. In the complex with (*S*)-**5**, the sulfur atom interacts with Ile12 and Leu148 (Figure 1a), whereas in (*R*)-**5** the sulfur atom is oriented towards a hydrophobic pocket formed by Ile209 and Trp128 (Figure 1b).

In the *Pa*HNL5 complexes the substrates are bound close to the flavin cofactor with the hydroxy groups being hydrogen bonded to His498 and Tyr458 (Figure 2). The cyano groups are pointing towards His459. In the complex with the more favoured *S*-enantiomer of **5** the sulfur atom points towards a hydrophobic pocket (Figure 2a) which otherwise binds the phenyl of the natural substrate (*R*)-mandelonitrile.[33] In (*R*)-**5** the sulfur interacts with the methyl group of Ala-111 (Figure 2b). Val361 on one side of the substrate

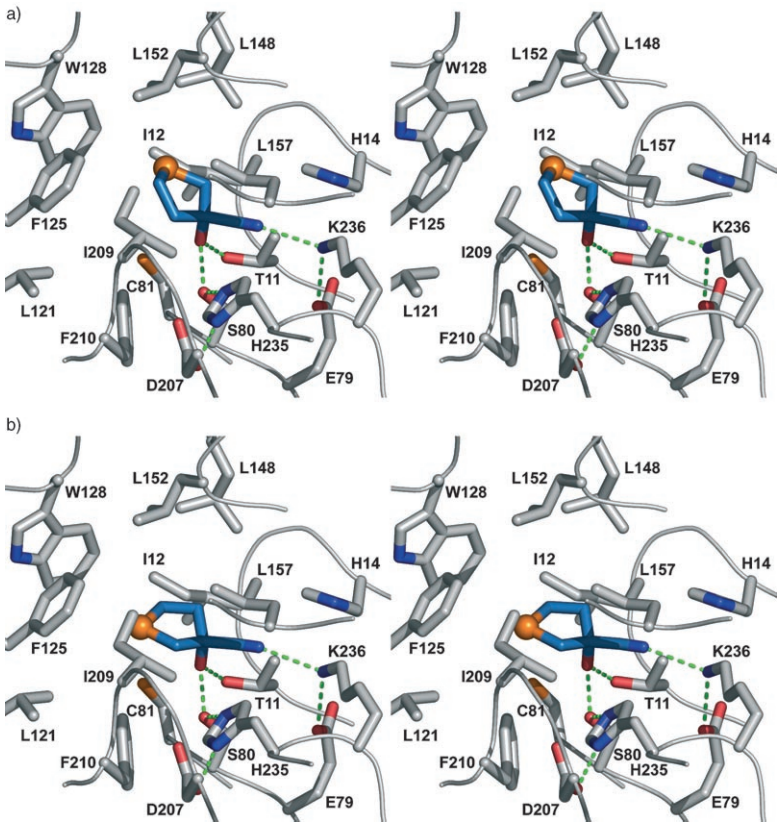


Figure 1. Stereoview of the active site of the hydroxynitrile lyase from *Hevea brasiliensis* in the modeled complexes of the enzyme with a) (*S*)-**5** and b) (*R*)-**5**. Amino acid residues are shown in grey, the substrate in light-blue. Nitrogen and oxygen atoms are coloured blue and red, respectively sulfur atoms are shown in orange. The sulfur atom in the thiophene ring of the substrate is shown as a small sphere. Hydrogen bonding interactions are depicted as green dashed lines. Figures 1 and 2 were obtained using the program PyMol (www.pymol.org).

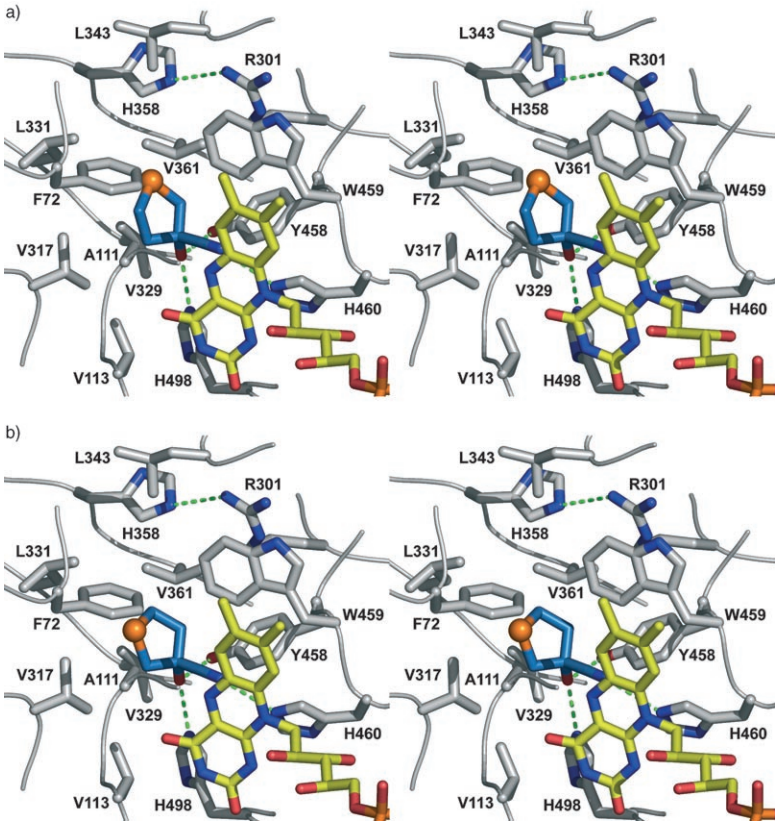


Figure 2. Stereoview of the active site of the hydroxynitrile lyase from *Prunus amygdalus* (isoenzyme 5) in the modeled complexes of this enzyme with a) (*S*)-**5** and b) (*R*)-**5**. Amino acid residues are shown in grey, the substrate in light-blue and the flavin cofactor in yellow. Nitrogen and oxygen atoms are coloured blue and red, respectively sulfur atoms are shown in orange. The sulfur atom in the thiophene ring of the substrate is shown as a small sphere. Hydrogen bonding interactions are depicted as green dashed lines.

binding side and Ala111 on the opposite side have recently been the prime targets for structure guided design efforts to improve the substrate acceptance and the enantioselectivity of *Pa*HNL5 for different non-natural substrates.[34,35]

Relative binding energies for (*S*)- and (*R*)-**5** were estimated from the force field energies after geometry optimisation (see Computational Methods). This approach—analysing relative substrate binding energies instead of energies of transition states—has been successful in the past to understand enantioselectivities of hydroxynitrile lyases.[34–36] In the *Hb*HNL case the complex with (*S*)-**5** is lower in energy by about 2 kcal mol^{-1} in close agreement with the observed *ee* of 91 % (see Scheme 3). For the complexes with *Pa*HNL5, a lower preference for the (*S*)-enantiomer is predicted (ΔE = 1.5 kcal mol^{-1}), which slightly overestimates the actual experimental values for the selectivity of this enzyme (62 % *ee*, Scheme 3). In the case of *Hb*HNL we also conducted thermodynamic integration calculations in order to estimate relative free energies of binding of (*S*)- and (*R*)-**5**. In these calculations we obtained a $\Delta\Delta G$ of 2.0(0.7) kcal mol^{-1} which is again in good agreement with the experimental data.

The computational results consequently reproduce the observed *S* selectivity of both enzymes with tetrahydrothiophene-3-one (**4**) as substrate. In the *Hb*HNL case, differences in electrostatic terms contribute most to the lower energy of the complex of (*S*)-**5**, and outweigh the counteracting van der Waals terms. In contrast to that, both electrostatic and van der Waals energy terms contribute almost equally to the lower energy of the complex with the (*S*)-enantiomer in the *Pa*HNL case. A similar result was obtained in the thermodynamic integration calculations with the estimated $\Delta\Delta G$ being dominated by contributions from electrostatic terms. Breaking up $\Delta\Delta G$ into contributions from interactions of the substrate with different amino acids in the active site of *Hb*HNL, however, revealed no single residue to be solely responsible for the observed selectivity. The largest effects were calculated to originate from interactions with Ile12 and Trp128 (see Figure 1).

Conclusions

For the first time the concept of docking/protecting groups was applied to enzyme-catalysed cyanohydrin reactions. 3-Tetrahydrothiophen-3-one (**4**) and 4-phenylthiobutan-2-one (**7**) are considered as masked 2-butanone. Thereby both substituents flanking the carbonyl group were sufficiently distinct to ensure unambiguous orientation within the active site and therefore a clear enantiopreference for cyanide attack catalysed by *Hb*HNL. After removal of the sulphur containing parts of the molecule the target molecule **10** could be obtained with high *ee*. Interestingly, the same (*S*)-enantiomer **5** was obtained both by (*R*)-selective *Pa*HNL as well as (*S*)-selective *Hb*HNL catalysed cyanide addition to **4**. In silico modelling using molecular docking calculations followed by molecular mechanics optimizations estimating binding energies for (*S*)- and (*R*)-**5** from the force field energies after geometry optimisation are in good agreement with the experimental data. The computational methods therefore reproduce the observed *S* selectivity of both enzymes with the heterocyclic ketone **4** as substrate. The absolute stereochemistry of 3-hydroxytetrahydrothiophene-3-carbonitrile (**5**) was proved by crystallographic data of the (*S*)-phenylethylamine salt of hydroxy acid **6**.

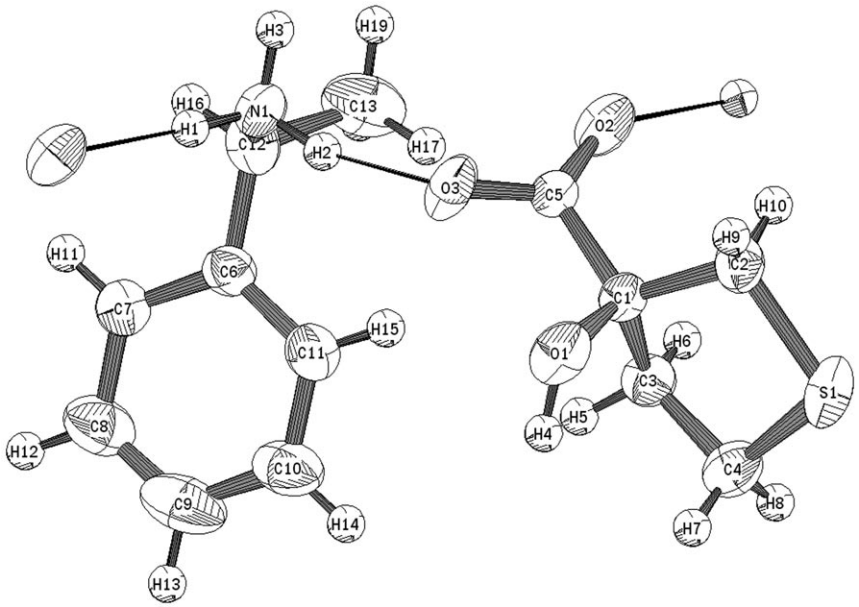


Figure 3. Detail of the crystal structure of (*S*)-phenylethylammonium (*S*)-3-hydroxytetrahydrothiophene-carboxylate ((*S*)-phenylethylammonium salt of **6**). Hydrogen bonds are indicated as thin sticks. In order to improve the visualisation an arbitrary displacement parameter is used for the representation of the hydrogen atoms.

Experimental Section

General: All solvents and bulk materials not described in below are commercially available and were appropriately purified, if necessary. Melting points where determined on a Büchi 530 and are uncorrected. Optical rotations were measured on a Perkin–Elmer Polarimeter 341. ^{1}H and ^{13}C NMR spectra were recorded either on a Varian INOVA 500 (^{1}H 499.82 MHz, ^{13}C 125.69 MHz) or a Varian GEMINI 200 (^{1}H 199.92 MHz, ^{13}C 50.25 MHz). GC analyses were performed using a Hewlett Packard 6890 instrument and a Chirasil-DEX CB column.

HCN formation—CAUTION: All reaction equipment in which cyanides were used or produced was placed in a well ventilated hood. The required amount of HCN was freshly formed by dropping a saturated NaCN solution into aqueous sulfuric acid (60%) at 80°C and trapping HCN at −12°C in a cooling trap. For continued warning, an electrochemical sensor for HCN detection was used. Waste solutions containing cyanides were treated with aqueous sodium hypochlorite (10%). Subsequently the pH was adjusted to 7.0 with aqueous sulfuric acid.

General procedure A (GP A) for the synthesis of the cyanohydrins with *Hb*HNL: An aqueous solution of *Hb*HNL (700–1500 IU mmol^{-1} ketone) was added to a solution of ketone in *tert*-butyl methyl ether (*t*BME), and the resulting mixture was stirred until an emulsion was formed. The aqueous enzyme preparation (approx 5 kIU mL^{-1}) was previously diluted with distilled water (1:2 v/v) and the pH was adjusted with 10% citric acid. After addition of freshly generated HCN (2 equiv), the mixture was stirred at room temperature until quantitative conversion. The emulsion was broken with Celite 545, filtered and dried over Na_2SO_4. Evaporation of the solvent yielded the crude cyanohydrins.

General procedure B (GP B) for the synthesis of the cyanohydrins with *Pa*HNL: A solution of *Pa*HNL (200–450 IU mmol^{-1} ketone, *t*BME/enzyme 5:7 v/v) was added to a solution of ketone in *t*BME, and the resulting mixture was stirred until an emulsion was formed. The pH of the aqueous enzyme solution (approx. 3 kIU mL^{-1}) was previously adjusted with 10% citric acid. After addition of freshly generated HCN (2 equiv), the mixture was stirred at room temperature until quantitative conversion. The emulsion was broken with Celite 545, filtered and dried over Na_2SO_4. Evaporation of the solvent yielded the crude cyanohydrins.

(*S*)-2-Hydroxy-2-methylbutyronitrile (2): As described above 2-butanone (**1**) (10.8 g,150 mmol) diluted with *t*BME (35 mL) was reacted at 0°C according GP A. *Hb*HNL (35 mL, 6000 IU mL^{-1}) as catalyst was adjusted to pH 5.0 and upon HCN addition (14.6 mL, 375 mmol) afforded hydroxynitrile **2** (13.3 g, 90%) as a colourless oil with 58% *ee* measured by chiral GC after TMS protection (120°C isotherm). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 2.91$ (br s, 1H, OH), 2.81 (m, 2H, $J = 7.3$, 2.0 Hz, H3, H3′), 1.60 (s, 3H, CH_3), 1.11 ppm (dt, 3H, $J = 7.3$, 2.0 Hz, CH_3-Et); ^{13}C NMR (125 MHz, $CDCl_3$): corresponded well to the published data:[27] $\delta = 122.10$ (CN), 69.54 (C-OH), 35.05 (C3), 27.35 (C1), 8.75 ppm (C4); elemental analysis calcd (%) for C_5H_9NO: C 60.58, H 9.15, N 14.13; found: C 59.67, H 9.08, N 14.30.

(*S*)-3-Hydroxytetrahydrothiophene-3-carbonitrile (5): Following GP A a solution of **4** (10.2 g, 100 mmol) in *t*BME (35 mL) was emulsified with *Hb*HNL (31 mL, 6000 IU mL^{-1} pH 4.5). Successive addition of HCN (19.6 mL, 500 mol) at 0°C afforded after workup crude **5** (10.26 g, 80%) as a slightly yellow oil with 91% *ee* measured by chiral GC after TMS protection (120°C isotherm).

According to GP B, a solution of **4** (1.02 g, 10 mmol) in *t*BME (3.5 mL) was emulsified with *Pa*HNL (3.1 mL, 2500 IU mL^{-1} pH 4.5). Successive addition of HCN (2 mL, 50 mol) at 0°C afforded after workup crude **5** (1.05 g, 81%) as a slightly yellow oil with 62% *ee* measured by chiral GC after TMS protection (120°C isotherm: 11.9 min (*S*)-**5**, 12.1 min (*R*)-**5**). $[\alpha]_D^{20} = -11.0°$ ($c = 1.0$ in $CHCl_3$ for 50% *ee*); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.94$ (s, 1H, OH), 3.28 (d, 1H, $J = 12.2$ Hz, H2), 3.12 (dd, 1H, $J = 11.7$, 1.0 Hz, H2′), 3.08–2.98 (m, 2H, H5, H5′), 2.48 (ddd, 1H, $J = 3.9$, 0.5 Hz, H4), 2.28 ppm (ddd, 1H, $J = 7.8$ Hz, 1.5 Hz, H4′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 120.11$ (CN), 74.3191 (C3-OH), 42.85 (C2), 42.71 (C4), 28.30 ppm (C5); elemental analysis calcd (%) for C_5H_7NOS: C 46.49, H 5.46. N 10.84, S 24.82; found: C 46.41, H 5.50, N 10.90, S 24.83.

(*S*)-3-Hydroxytetrahydrothiophene-3-carboxylic acid (6): A solution of (*S*)-**5** (6.0 g, 46 mmol) was stirred with conc. HCl (15 mL) at 70°C for 15 h. After cooling NaOH (40 mL, 20%) was added and the mixture (pH 10) was extracted with *t*BME twice; the aqueous phase was acidified again with conc. HCl (10 mL) and after extraction the *t*BME phase was dried over Na_2SO_4. The filtrate was concentrated in vacuo to give crude (*S*)-hydroxy acid **6** (5.47 g, 80%) as a beige solid. M.p. 74–76°C; $[\alpha]_D^{20} = -50.3°$ ($c = 2.0$ in $CHCl_3$) 99% *ee* (after recrystallisation with (*S*)-phenylethylamine and hydrolysis of the salt); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.33$ (d, 1H, $J = 11.2$ Hz, H2), 3.09–2.98 (m, 2H, $J = 10.3$ Hz, H5, H5′), 2.90 (dd, 1H, $J = 11.7$, 1.5 Hz, H2′), 2.33–2.21 ppm (m, 2H, $J = 10.7$ Hz, H4, H4′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 178.45$ (COOH), 82.91 (C3-OH), 42.52 (C2), 42.10 (C4), 30.15 ppm (C5); elemental analysis calcd (%) for $C_5H_8O_3S$: C 40.53, H 5.44, S 21.64; found: C 41.13, H 5.38, S 21.64.

(*S*)-2-Hydroxy-2-methyl-4-phenylthiobutyronitrile (8): Methyl vinyl ketone (3.2 mL, 40 mmol) was slowly added to a solution of thiophenol (4.2 mL, 41 mmol) and trimethylamine (55 μL, 0.4 mmol) *t*BME (55 mL) under nitrogen atmosphere cooled to 0°C. After completion of the reaction the organic phase is washed with 1 M NaOH and twice with water to give a *t*BME solution of freshly prepared 4-phenylthiobutan-2-one (**7**) according to literature.[37] The latter was mixed with K_2HPO_4 buffer (10 mL, 30 mmol L^{-1}, pH 6.0) and at 0°C reacted according to GP A.

*Hb*HNL (2.5 mL, 3000 IU mL^{-1}) as catalyst was adjusted to pH 6.0 and upon HCN addition (3.2 mL, 82 mmol) afforded cyanohydrin **8** (7.84 g, 95%) as a colourless oil with 96% *ee*. $[\alpha]_D^{20} = -6.0°$ (c = 5.5 in $CHCl_3$) for 87% *ee*; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.13$ (d, 2H, $J = 7.8$ Hz, H2″, H6″), 7.08 (t, 2H, $J = 7.8$ Hz, H3″, H5″), 6.99 (t, 1H, $J = 7.8$ Hz, H4″), 3.38 (br s, 1H, OH), 2.92 (m, 2H, $J = 8.3$, 2.4 Hz, H4, H4′), 1.81 (m, 2H, $J = 8.3$, 2.4 Hz, H3, H3′), 1.36 ppm (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 130.14$, 129.48, 129.36, 127.08 (aryl), 121.61 (CN), 68.79 (C-OH), 40.45 (C-S), 29.07 (C3), 28.38 ppm (CH_3); elemental analysis calcd (%) for $C_{11}H_{13}NOS$: C 63.74, H 6.32, N 6.76, S 15.47; found: C 63.93, H 6.38, N 6.69, S 15.51.

(*S*)-2-Methyl-4-phenylthio-2-trimethylsilyloxybutyronitrile (8a): Standard TMS protection for chiral GC analysis afforded **8a** as a colourless oil. $[\alpha]_D^{20} = -12.8°$ (c = 2.0 in $CHCl_3$) for 87% *ee;* GC (140 °C isotherm): 28.0 min (*R*)-**8a**, 28.5 min (*S*)-**8a**; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.10$ (d, 2H, $J = 7.3$ Hz, H2″, H6″), 7.06 (t, 2H, $J = 7.3$ Hz, H3″, H5″), 6.95 (t, 1H, $J = 7.3$ Hz, H4″), 2.83 (m, 2H, $J = 7.8$ Hz, H4, H4′), 1.79 (t, 2H, $J =$ 7.8 Hz, H3, H3′), 1.38 (s, 3H, CH_3), 0.01 ppm (s, 9H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 135.70$, 129.28, 129.27, 126.43 (arom), 121.60 (CN), 69.10 (C-OTMS), 43.25 (C-S), 29.10 (C3), 28.04 ppm (CH_3); GC-MS: m/z: 308 $[M+C_2H_5]^+$, 281 $[M]^+$, 253 $[M-CN]^+$, 123 (Ph-S-CH_2); elemental analysis calcd (%) for $C_{14}H_{21}NOSSi$: C 60.17, H 7.57, N 5.01, S, 11.47; found: C 60.18, H 7.52, N 5.12, S 11.63.

(*S*)-2-Hydroxy-2-methyl-4-phenylthiobutanoic acid (9): A solution of (*S*)-2-hydroxy-2-methyl-4-phenylthiobutyronitrile (**8**) (16.9 g, 82 mmol) was stirred with conc. HCl (135 mL) and distilled H_2O (35 mL) at 85 °C for 16 h. After extraction with toluene the organic phase was washed successively with NaOH (20%) and conc. HCl. Crystallisation directly from the toluene phase at −10 °C gave pure white crystals of the hydroxy acid **9** (11.9 g, 65%) with 99% *ee*, measured by chiral GC after derivatisation with bis(trimethylsilyl)trifluoroacetamide (150 °C isotherm: 6.6 min (*R*)-**9**, 6.8 min (*S*)-**9**). M.p. 33 °C; $[\alpha]_D^{20} = +16.0°$ (c = 2.0 in $CHCl_3$) for 99% *ee*; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.34$ (d, 2H, $J = 7.8$ Hz, H2″, H6″), 7.28 (t, 2H, $J = 7.3$ Hz, H3″, H5″), 7.19 (t, 1H, $J = 7.3$ Hz, H4″), 3.15–3.05 (m, 1H, $J = 10.7$, 5.4 Hz, H4), 2.95–2.87 (m, 1H, $J = 10.7$, 4.9 Hz, H4′), 2.22–2.14 (m, 1H, $J = 10.7$, 5.4 Hz, H3), 2.10–2.00 (m, 1H, $J = 10.7$, 4.9 Hz, H3′), 1.30 ppm (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 181.49 (COOH), 135.96, 129.25, 126.34, 120.61 (arom), 74.53 (C2-OH), 39.21 (C3), 28.09 (C4-S), 26.46 ppm (CH_3); elemental analysis calcd (%) for $C_{11}H_{14}OS$: C 58.38, H 6.24, S, 14.17; found: C 58.39, H 6.22, S, 14.14.

(*S*)-2-Hydroxy-2-methylbutanoic acid (10), from 6: A suspension of (*S*)-3-hydroxytetrahydrothiophene-3-carboxylic acid (**6**) (300 mg 2 mmol) and Raney nickel (5.5 g moist 50% Ni) in water (28 mL) and NaOH (1 mL 2 M) was stirred at 75 °C for 2 h. After acidification with H_2SO_4 (1 M) to pH 1, filtration and extraction with *t*BME the solvent is removed in vacuo to afford **10** (166 mg, 70%) as a white solid.

From 9: Raney nickel (135 g moist 50% Ni) was added in five portions to a solution of (*S*)-2-hydroxy-2-methyl-4-phenylthiobutanoic acid (**9**) (16 g 70,7 mmol) in aqueous NaOH (2 M, 35.4 mL, 70.8 mmol). The suspension was stirred at 75 °C for 2 h. After filtration, acidification with HCl (5 M) to pH 1, and extraction with *t*BME the solvent was dried over Na_2SO_4 and removed in vacuo to afford after recrystallisation from *n*-hexane at ambient temperature **10** (9.5 g, 82%) as a white solid in 99% *ee*, detected by chiral GC after derivatisation to the acetonide via acetone and conc. H_2SO_4 (GC: 100 °C, 3.5 min per 10 °C min^{-1}, 160 °C, 2.5 min). M.p. 74 °C; NMR data are identical to the literature;[38] elemental analysis calcd (%) for $C_5H_{10}O_3$: C 50.84, H 8.53; found: C 50.80, H 8.64.

X-ray diffraction data of 6: Due to severe twinning problems the structure of **6** could not be unambiguously determined. Thus in order to establish its absolute configuration a structure determination of its (*S*)-phenylethylammonium salt was carried out. Preliminary X-ray investigations showed that this compound is orthorhombic, that is, $P2_12_12_1$ (no. 19) with $a = 6.449(2)$, $b = 13.267(6)$, $c = 16.22(1)$ Å, $Z = 4$. For the determination of the crystal structure a coffin shaped colourless single crystal mounted on a glass fibre was transferred to a serial kappa-diffractometer (Nonius Turbo CAD4) operated with graphite monochromated $Mo_{K\alpha}$ radiation. Intensity data were collected at 294 K over one quadrant of r.s. up to $2\vartheta = 59.9°$. Data reduction led to a set of 4009 unique reflections out of which 3278 with $F^2 > 3\sigma(F^2)$ were considered significant. The crystal structure was solved with the SIR 92[39] program package, yielding the skeleton atoms, H atoms could be located from the difference Fourier map. They were treated as riding atoms in the final refinements. The absolute configuration was obtained from the Flack parameter[40] which refined to 0.028 for the correct enantiomorph and was proved by the fact that the refinement showed the correct configuration for the phenylethylammonium ion.

Details on the structure determination are given in Table 1. CCDC 615093 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

For the calculations programs of the Crystal Structure 2.0[41] crystallographic program package were used.

Table 1. Crystallographic data and details of the structure refinement for (*S*)-phenylethylammonium (*S*)-3-hydroxytetrahydrothiophenecarboxylate).

empirical formula	$C_{13}H_{19}NSO_3$
formula weight	269.35
lattice parameters	
a [Å]	6.449(2)
b [Å]	13.267(6)
c [Å]	16.22(1)
V [Å^3]	1387.8
space group	$P2_12_12_1$ (no. 19)
Z	4
ρ_{calcd} [g cm^{-3}]	1.285
$\lambda(Mo_{K\alpha})$ [cm^{-1}]	2.33
crystal colour, habit	colourless, prismatic
crystal dimensions [mm^3]	$0.4 \times 0.225 \times 0.125$
no. reflns	25
T [°C]	21(1)
no. reflns measured	4518
no. reflns above cutoff	3278
cutoff	$3.0\sigma(F^2)$
structure solution	Direct Methods (SIR92)
refinement	full-matrix least-squares on F
least squares weights	Chebychev polynomial with three parameters 102.207, 141.330, 44.169
no. observations	3278
no. variables	183
reflection/parameter ratio	17.91
residuals: R; Rw	0.041; 0.045
goodness of fit indicator	1.11
max shift/error in final cycle	0.00
maximum peak in final diff. map [e$^-$ Å^3]	0.57
minimum peak in final diff. map [e$^-$ Å^3]	−0.67

Description of the structure: The crystal structure of (*S*)-phenylethylammonium (*S*)-3-hydroxytetrahydrothiophenecarboxylate is characterised by an extended hydrogen bonding scheme which involves all three H atoms of the ammonium group and both oxygen atoms of the carboxylate group while the hydroxyl group remains isolated (see Figure 3). Thus, the cations and anions are alternatingly connected through the virtually linear hydrogen bonds N(1)-H(1)···O(2) and N(1)-H(2)···O(3) to yield infinite chains which are pairwise linked together through N(1)-H(3)···O(2) to form double chains which run along the [100] direction and are arranged in a distorted hexagonal rod packing.

Computational methods

Docking calculations and molecular mechanics: Models for both enantiomers of cyanohydrin **5** were built and optimised using the program Sybyl v6.5 (Tripos Inc.). Partial atomic charges for these compounds were calculated using the RESP protocol.[42] For the hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL), protein coordinates were taken from the respective atomic resolution X-ray crystal structure (PDB-entry: 1qj4).[43] For the enzyme from *Prunus amygdalus* (*Pa*HNL5), a homology model of isoenzyme no. 5 was used as in previous modelling and engineering studies.[34,35] This model is based on the crystal structure of isoenzyme no. 1 (*Pa*HNL1, PDB-entry: 1ju2)[44] which shares about 75% sequence identity. In both protein models, Asp, Glu, Arg and Lys residues were treated as charged. Protonation and tautomerisation states of His residues were chosen that resulted in sensible hydrogen bonding networks. Hydrogen atoms were added to the structure, followed by a geometry optimisation using AMBER,[45] applying harmonic restraints on the positions of all heavy atoms. Only polar hydrogen atoms of the protein and the ligands were retained for the docking simulations.

The two enantiomers were docked to the active sites of these enzymes using a Monte-Carlo simulated-annealing approach as implemented in the program AutoDock v3.0.[46] The structure of the protein was kept rigid, whereas the ligands possessed translational, rotational and three torsional degrees of freedom. In order to probe the conformational space of the five-membered rings, an open structure was created by breaking the bond between C4 and C5. During the simulations, a constraint was applied which kept the respective distance between 1.4 to 1.7 Å. For each ligand, 100 randomly chosen initial structures were subjected to 150 Monte Carlo steps starting at an RT value of 1000 and using a cooling factor of 0.95 per cycle. In each cycle, a maximum of 10000 accepted or rejected moves were allowed. The resulting structures were clustered with a root-mean-square tolerance of 1.5 Å. The structure of the lowest-energy representative of each cluster was further optimised using programs from the AMBER package.[45]

During minimization, only the bound ligand and amino acid residues within 12 Å of the ligand were allowed to move. Non-bonded interactions were truncated to a sphere of 10 Å radius. A distance dependent dielectric function was employed ($\varepsilon_r = r$). The protocol for the geometry optimisation consisted of three steps: a) an energy minimisation run employing weak harmonic restraints (1 kcal mol^{-1} Å^{-2}) on the ligand, b) a 100 ps molecular dynamics simulation ($T = 298$ K, $\Delta t = 2$ fs) with snapshots taken every 10 ps, and c) an unrestrained minimisation of the ten snapshot structures. The structure with the lowest energy was then used in the analysis.

Thermodynamic integration: In order to estimate the difference of binding free energy of the two enantiomers thermodynamic integration calculations were performed for the complexes with *Hb*HNL. The two states between which the free energy difference was calculated, were the *S*- and *R* enantiomer of **5** bound in the active site of the enzyme. Inversion of the absolute configuration was accomplished by perturbing the sulfur atom to methylene group and the C5-methylene group to a sulfur atom during a molecular dynamics simulation. The calculations were performed with the program GIBBS from the AMBER package.[45] Starting structures were the modeled complexes (see above) plus a 30 Å water cap around the active site. All atoms except those from residues in the vicinity of the ligand (within 12 Å), the water cap and the ligand itself were constrained. The time step for integration was set to 2 fs and all bonds involving hydrogen atoms were constrained using the SHAKE algorithm.[47] Each simulation was preceded by 3000 steps of geometry optimisation of the water cap, a 20 ps dynamics run during which the temperature was continuously raised from 10 to 298 K and a 80 ps equilibration run at 298 K. In the "forward" direction the coupling parameter λ was decremented in 20 steps from 1 (representing the complex with the *R* enantiomer) to 0 (representing in this case the complex with the *S* enantiomer) during a 210 ps molecular dynamics simulation (21 windows with 2 ps of equilibration and 8 ps of data collection) at 298 K. After that, the system was equilibrated for 20 ps at the final state ($\lambda = 0$), followed by a "backward" perturbation run ($\lambda = 0 \rightarrow 1$) using the same parameters. This protocol was repeated with both starting structures and the resulting free energy differences were averaged.

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