Prunus Armeniaca Hydroxynitrile Lyase (ParHNL) Catalyzed Asymmetric Synthesis of δ,ε-Unsaturated Cyanohydrins

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Received 8 December 2008

Abstract: Hydroxynitrile lyases (HNL) are one of the key enzymes in cyanogenic plants, catalyzing the final step in the biodegradation pathway of cyanogenic glycosides releasing HCN and the corresponding carbonyl components. We have been able to find some new plant HNL from drupe available in the northern part of the Indian subcontinent. Asymmetric cyanohydrin synthesis from γ,δ-unsaturated aldehydes by applying those new HNL is reported in this communication.

Key Words: hydroxynitrile lyase, cyanohydrins, asymmetric synthesis

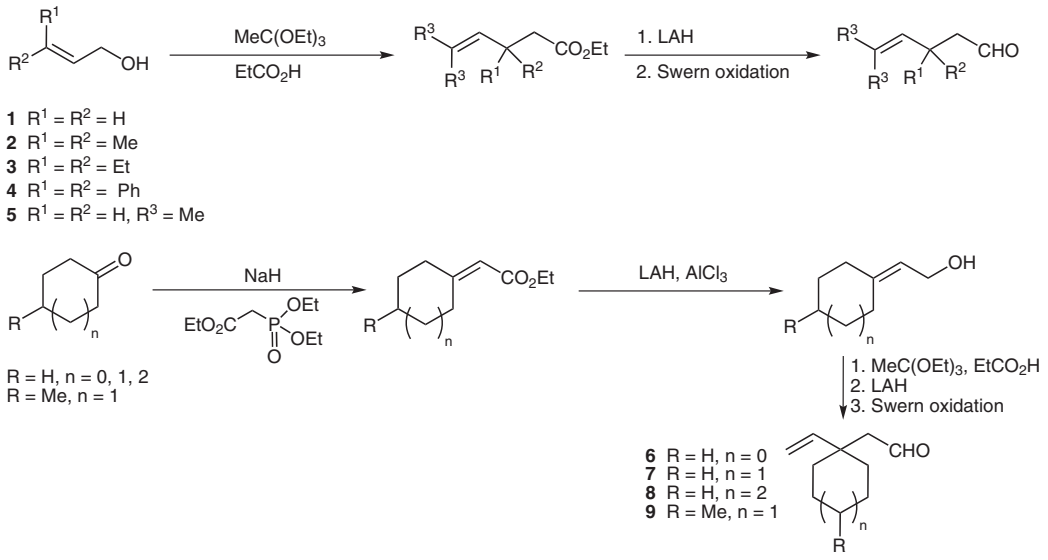
Enantiomerically pure cyanohydrins have attracted the attention of organic chemists, as well as enzymologists, due to their immense potential as chiral building blocks and interesting biological properties.[1] Enantiopure cyanohydrins serve as intermediates for several industrially useful chemicals, and the use of chiral cyanohydrins as building blocks for the production of important chemicals is likely to continue growing, as it avoids problems associated with the optical resolution of or asymmetric synthesis of certain products.[2] One of the most promising and interesting ways to obtain enantiomerically pure cyanohydrins is the HNL-catalyzed addition of cyanide to the requisite carbonyl compound.[3] The chemistry and biology of HNL have been extensively explored over the past two decades,[4] and active research to find new HNL with interesting catalytic properties is still ongoing. Hydroxynitrile lyase has been reckoned as an efficient industrial biocatalyst for the production of active pharmaceutical ingredients (APIs), chiral building blocks, and agrochemicals. Both the alcohol and the nitrile parts of the cyanohydrin functionality can undergo transformation to a range of groups. These are general methods that proceed via racemization-free process so that the optical purity is retained. Pioneering work by Effenberger, Griengl, and others opened a new era in the area of asymmetric cyanohydrin synthesis by HNL,[4] readily available from the kernels of almond, apple, cherry, apricots, and plums.

Hydroxynitrile lyase biocatalysis with α,β-unsaturated aliphatic aldehydes has been well reported in the literature.[5] However, HNL biocatalysis with structurally similar γ,δ-unsaturated aldehydes has not been studied. The δ,ε-unsaturated cyanohydrins can serve as very good chiral intermediates for accessing various small organic molecules in asymmetric fashion as described by Marcus et al.[6] ω-Unsaturated chiral nitrones have been synthesized from δ,ε-unsaturated cyanohydrins and dipolar cycloaddition reactions afford highly substituted carbocyclic analogues in a stereoselective manner. The synthesized cyanohydrins from γ,δ-unsaturated aldehydes can also lead to various interesting small carbocyclic molecules by functional group modification.

The required γ,δ-unsaturated aldehydes can be synthesized by a three-step method starting from allylic alcohols (Scheme 1). The allylic alcohols for accessing **1**, **2**, and **5** are commercially available; whereas the others were synthesized from the respective ketones by a two step method (Wittig–Horner–Emmons olefination with triethyl phosphonoacetate followed by reduction with $LiAlH_4$ and $AlCl_3$). The allylic alcohols are subjected to Johnson orthoester Claisen rearrangement to yield the corresponding γ,δ-unsaturated esters in good yield.[7] Reduction of the ester functionality followed by oxidation under Swern conditions afforded the γ,δ-unsaturated aldehydes in good yield.[8]

As part of our ongoing HNL screening program from several cyanogenic plant species, we have been successful in finding new HNL from various stone fruits collected from the state of Himachalpradesh (Northern territory of India). We mainly focused our attention on fruits from members of the *Rosaceae* family (genus: *Prunus*). The fruits collected comprised peach (*Prunus persica*), wild cherry (*Prunus avium*), Red Indian plum (*Prunus domestica*), Himalayan plum (*Prunus americana*), apricot (*Prunus armeniaca*) and shakarpara (white apricot, a hybrid cultivar). The enzymes isolated from the seed portion of all the above collected fruit exhibit HNL activity as measured by chiral HPLC based HNL assay method.[9] All of the plant materials produced (*R*)-mandelonitrile by *Si*-facial attack as detected by chiral HPLC measurements when benzaldehyde was used as the standard assay substrate. Among the plant HNL we screened, HNL from shakarpara (white apricot, *Prunus armeniaca*) is superior in terms of enantioselection (Table 1); whereas HNL from wild cherry (*Prunus avium*) and apricot (*Prunus armeniaca*) also exhibit good enantioselection under the standard assay conditions. The partially purified enzyme solution can be stored at 4 °C for 2–3 months without significant loss of activity.

SYNLETT 2009, No. 8, pp 1237–1240
Advanced online publication: 17.04.2009
DOI: 10.1055/s-0029-1216732; Art ID: D40508ST



Scheme 1 Orthoester Claisen rearrangement for the synthesis of γ,δ-unsaturated aldehydes

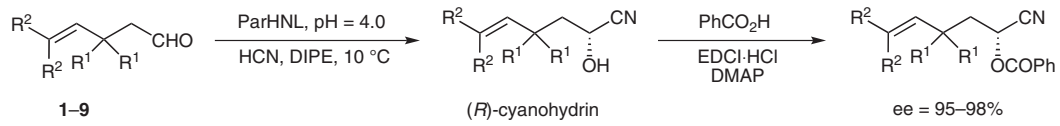
Table 1 Comparision of HNL Activity in Various Plants

Plant material	Material assayed	HNL activity (U/mL)	ee (%)[a]
Prunus avium (wild cherry)	Seed	80 (*R*)	93
Prunus domestica (Indian plum)	Seed	90 (*R*)	88
Prunus persica (Peach)	Seed	95 (*R*)	72
Prunus armeniaca (Apricot)	Seed	206 (*R*)	93
Prunus americana (Himalayan plum)	Seed	102 (*R*)	89
Prunus armeniaca Shakarpara cultivar (Apricot)	Seed	280 (*R*)	96

[a] Enantiomeric excess (%) was measured by chiral HPLC (OJ-H column; hexane–*i*-PrOH; 9:1; flow rate 1 mL/min. Benzaldehyde was taken as a standard substrate. Retention times for (*R*)- and (*S*)-mandelonitrile are 14.4 and 19.1 min, respectively.

We subsequently used HNL from shakarpara (ParHNL) for the asymmetric synthesis of cyanohydrins. In the general procedure, a vigorously stirred biphasic reaction medium was employed,[10] with HCN in diisopropylether (DIPE) as the cyanating source (Scheme 2). As HCN is a strong cyanating agent the reaction is complete within 2–6 hours at 10 °C. We also performed a biocatalytic transcyanation approach with acetonecyanohydrin as the cyanide source for accessing the δ,ε-unsaturated cyanohydrins,[11] but the slow decomposition of acetonecyanohydrin under the reaction conditions made the process time consuming, and a large excess of acetone cyanohydrin is required (6 equiv with respect to aldehydes) to obtain appreciable amount of conversion. After completion of the reaction (as indicated by TLC) the product cyanohydrins were isolated by extractive workup and purified. To determine the enantioselectivity of the reactions, synthesized cyanohydrins are derivatized as their benzoate esters and chiral HPLC measurements were performed (compared with the HPLC trace of racemic benzoate esters prepared from racemic δ,ε-unsaturated cyanohydrins).

The chemical yield and optical purity of the cyanohydrins thus synthesized from γ,δ-unsaturated aldehydes by HNL biocatalysis are listed in Table 2. As can be seen from Table 2 it is evident that all the γ,δ-unsaturated aldehydes (**1–9**) are very good substrates for ParHNL as they yield the respective cyanohydrins with good chemical yield (70–85%) and excellent optical purity (ca. 96–98% ee). The absolute configuration of all the newly synthesized cyanohydrins was assigned as *R* as ParHNL is *R*-selective. The γ,δ-unsaturated aldehydes **1** and **5** yield the respective cyanohydrins in comparatively lesser time than the other

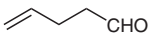
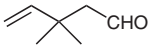
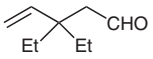
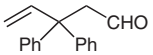
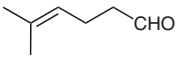
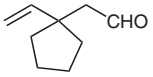
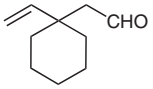
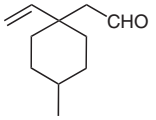
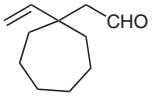


Scheme 2 Biocatalyzed synthesis of δ,ε-unsaturated cyanohydrins

compounds, possibly due to the fact that the β-carbon is unsubstituted, whereas for other aldehydes (**2**–**4** and **6**–**9**)[12] the β-carbon is fully substituted and that might hinder binding in the HNL active site.

In conclusion we have described an efficient biocatalytic asymmetric synthesis of δ,ε-unsaturated cyanohydrins from the corresponding aldehydes. The synthesized cyanohydrins exhibit excellent enantioselection and are obtained in good chemical yield. Further functionalization of those optically pure δ,ε-unsaturated cyanohydrins to afford enantiomerically pure small organic molecules are currently in progress in our laboratory.

Table 2 ParHNL-Catalyzed Asymmetric Synthesis of δ,ε-Unsaturated Cyanohydrins

Substrate	Reaction time (h)	Yield (%)	ee (%)[a]
	2	80	98
	4.5	82	98
	5	78	96
	5	70	97
	3	85	98
	5	82	98
	5	76	96
	6	80	98
	6	72	95

[a] Enantiomeric excess (%) was measured by chiral HPLC measurement of the benzoate derivatives of the cyanohydrins (CHIRALPAK AS-H; hexane–*i*-PrOH, 49:1; flow rate 0.8 mL/min).

Acknowledgment

Financial support from IFS (Sweden, Grant agreement No: F-4192/1) is gratefully acknowledged. Three of the authors (RB, NJ & TD) are greateful to CSIR (New Delhi, India) for research fellowships.

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(11) **Enzyme Extraction from *Prunus armeniaca* (Shakarpara Spricot)**
Ripened fruits were taken and the fleshy cover was removed to obtain the seeds. The upper layers of the seeds were cracked with a hammer to obtain the soft kernels. The kernels were homogenized at 4 °C, with aq K_3PO_4 buffer (10

mM, pH = 6.0), to give a milky suspension. The suspension was filtered through four layers of cheese cloth to remove the insoluble part. After that it was centrifuged (18800 g, 30 min), and removal of the residue gave a crude preparation of HNL. The crude preparation was fractionated with $(NH_4)_2SO_4$. Proteins precipitating with 30% saturation were collected by centrifugation (18800 g, 20 min), dissolved in the minimum volume of phosphate buffer and dialyzed against the same buffer with three changes. The dialyzed soln was then centrifuged and the supernatant was stored at 4 °C and assayed for HNL activity.

ParHNL Assay

In a typical assay reaction 1.0 M of benzaldehyde soln (in DMSO, 40 μL) was dissolved in 400 mM citrate buffer (760 μL, pH = 4.0), followed by addition of enzyme soln (100 μL) and 1.0 M NaCN soln (100 μL, total reaction volume 1 mL), and the reaction mixture was incubated in a rotary shaker. After 5 min, the 100 μL of the reaction mixture was removed and extracted with 900 μL hexane–2-PrOH (9:1), the organic layer was analyzed with chiral HPLC for the formation of (*R*)-mandelonitrile. A blank reaction was also performed without enzyme, and the amount of mandelonitrile obtained was deducted from the biocatalyzed reaction product. One unit of the enzyme is defined as the amount of the enzyme that produces 1 mmol of (*R*)-mandelonitrile under the above reaction conditions in 1 min. The protein content in all the HNL was measured by the Bradford method using a Bio-Rad protein assay kit with BSA as the standard.

General Procedure for the Synthesis of δ,ε-Unsaturated Cyanohydrins by ParHNL

To a soln of γ,δ-unsaturated aldehyde **1**–**9** in DIPE, a soln of ParHNL (300 IU/mmol of aldehyde, DIPE/enzyme; 1:1 v/v) was added, and the resulting mixture was stirred vigorously until an emulsion was formed (the pH of the enzyme soln having been previously adjusted to 4.0 with 10% citric acid soln). Freshly prepared HCN in DIPE (2 equiv) was added to the mixture, and the temperature was kept at 10 °C. After completion of the reaction it was extracted thoroughly with Et_2O several times, and the organic layer was dried (Na_2SO_4). Evaporation of the solvent yielded the crude cyanohydrins, which were purified by chromatography.

Preparation of HCN in DIPE

Sodium cyanide (10 g) and citric acid (0.1 g) were dissolved in H_2O (100 mL). The soln was cooled in an ice–water bath and extracted with DIPE (50 mL), while acidifying with 33% HCl until pH 5.5. The H_2O layer, which contained a suspension of NaCl, was extracted twice with DIPE (25 mL). The combined DIPE layers were stored in a dark bottle. The above procedure must be performed in a well-ventilated fume hood and impermeable gloves must be worn.

(12) **Cyanohydrin from 2**

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.8 (dd, *J* = 17.7, 10.6 Hz, 1 H), 5.1 (dd, *J* = 10.6, 1.4 Hz, 1 H), 5.00 (dd, *J* = 17.7, 1.4 Hz, 1 H), 4.48 (t, *J* = 7.6 Hz, 1 H), 1.88 (m, 2 H), 1.1 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 121.8, 119.4, 113.4, 59.8, 48.7, 36.5, 25.8, 24.6. $[\alpha]_D^{27}$ +8.8 (*c* 1.0, $CHCl_3$).

Cyanohydrin from 3

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.7 (dd, *J* = 17.6, 10.6 Hz, 1 H), 5.22 (dd, *J* = 10.6, 1.4 Hz, 1 H), 5.00 (dd, *J* = 17.6, 1.4 Hz, 1 H), 4.49 (t, *J* = 7.6 Hz, 1 H), 1.99 (m, 2 H), 1.45 (m, 4 H), 0.86 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.1, 120.5, 114.7, 58.6, 41.9, 27.67, 27.0, 7.5. $[\alpha]_D^{27}$ +4.4 (*c* 1.0, $CHCl_3$).

Cyanohydrin from 4

^{1}H NMR (400 MHz, $CDCl_3$,): δ = 7.50–7.10 (m, 10 H), 6.6 (dd, *J* = 17.6, 10.6 Hz, 1 H), 5.32 (dd, *J* = 10.6, 1.4 Hz, 1 H), 4.98 (dd, *J* = 17.6, 1.4 Hz, 1 H), 4.42 (t, *J* = 7.6 Hz, 1 H), 2.80 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.0, 144.7, 143.2, 128.6, 128.5, 128.2, 127.7, 127.6, 119.9, 114.5, 59.3, 52.64, 44.8. $[\alpha]_D^{27}$ +10.5 (*c* 0.5, $CHCl_3$).

Cyanohydrin from 5

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.16 (t, *J* = 6.4 Hz, 1 H), 4.46 (t, *J* = 7.6 Hz, 1 H), 2.2 (m, 2 H), 1.85 (m, 2 H), 1.75 (s, 3 H), 1.7 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 134.6, 123.2, 119.4, 61.6, 36.7, 27.6, 23.4, 18.8. $[\alpha]_D^{27}$ +2.1 (*c* 1.0, $CHCl_3$).

Cyanohydrin from 6

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.75 (dd, *J* = 17.4, 10.6 Hz, 1 H), 5.20–5.08 (m, 2 H), 4.45 (t, *J* = 6.8 Hz, 1 H), 2.01 (d, *J* = 6.8 Hz, 2 H), 1.80–1.50 (m, 8 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 143.7, 120.7, 113.9, 59.3, 47.8, 45.4, 37.0, 36.7, 23.0, 22.9. $[\alpha]_D^{27}$ +6.4 (*c* 0.8, $CHCl_3$).

Cyanohydrin from 7

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.66 (dd, *J* = 17.4, 10.6 Hz, 1 H), 5.20 (d, *J* = 10.6 Hz, 1 H), 5.08 (d, *J* = 17.4 Hz, 1 H), 4.48 (t, *J* = 7.2 Hz, 1 H), 1.9 (d, *J* = 7.2 Hz, 2 H), 1.70–1.46 (m, 10 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 144.7, 120.8, 115.2, 58.5, 46.3, 39.2, 36.0, 35.5, 26.1, 21.9, 21.8. $[\alpha]_D^{27}$ +4.8 (*c* 1.2, $CHCl_3$).

Cyanohydrin from 8

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.75 (dd, *J* = 17.4, 10.6 Hz, 1 H), 5.2 (dd, *J* = 10.6, 1.4 Hz, 1 H), 5.05 (dd, *J* = 17.6, 1.4 Hz, 1 H), 4.48 (m, 1 H), 1.9 (m, 2 H), 1.70–1.40 (m, 12 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 146.1, 120.7, 113.4, 58.8, 46.9, 42.3, 38.0, 37.2, 30.0, 22.3. $[\alpha]_D^{27}$ +22.2 (*c* 0.6, $CHCl_3$).

Cyanohydrin from 9

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.75 (dd, *J* = 17.4, 10.6 Hz, 1 H), 5.15 (dd, *J* = 10.6, 1.4 Hz, 1 H), 5.00 (dd, *J* = 17.6, 1.4 Hz, 1 H), 4.48 (m, 1 H), 2.0 (m, 2 H), 1.60–1.20 (m, 9 H), 0.90 (d, *J* = 7.6 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 147.2, 120.5, 112.9, 58.8, 40.4, 38.1, 34.6, 33.9, 31.9, 29.9, 29.8, 21.9. $[\alpha]_D^{27}$ +11.5 (*c* 1.2, $CHCl_3$).