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Hydroxynitrile Lyases, Interesting Biocatalysts in Stereoselective Organic Syntheses

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Abstract: Hydroxynitrile lyases (HNLs) from *Prunus amygdalus* (PaHNL), *Sorghum bicolor* (SbHNL), *Manihot esculenta* (MeHNL) and *Hevea brasiliensis* (HbHNL) are excellent biocatalysts for the preparation of optically active cyanohydrins. (*R*)- as well as (*S*)-cyanohydrins are obtained in high optical and chemical yields. The synthetic potential of the now easily available optically pure cyanohydrins is demonstrated by stereoselective transformations of the cyanohydrins to biologically active compounds with one or two stereogenic centers.

The release of HCN (cyanogenesis) as a defence mechanism against herbivores, is not only widely distributed in higher plants, including important food products like cassava and sorghum, but is in addition found in several species of ferns, bacteria, fungi and insects (ref. 1).

During cyanogenesis cyanohydrin-O-glycosides are decomposed to a sugar, HCN and a carbonyl compound by a two step mechanism. In the first step β-glycosidase catalyzed hydrolysis of the glycosides into carbohydrates and the corresponding cyanohydrins occur. In the second step the cyanohydrins are cleaved to give HCN and aldehydes or ketones, respectively (ref. 2). The latter step occurs (base catalyzed) spontaneously or enzymatically by action of an α-hydroxynitrile lyase (HNL) (ref. 1). Hydroxynitrile lyases not only catalyze the cleavage of cyanohydrins, but also their formation from carbonyl compounds and HCN.

CHARACTERIZATION, MOLECULAR CLONING AND OVEREXPRESSION OF HYDROXYNITRILE LYASES

HNLs have been purified and characterized from almost a dozen cyanogenic plants. In all cases where the cyanohydrins have a chirality center, only one stereoisomer (enantiomer) is found in naturally occuring cyanohydrin glycosides (ref. 1). Traditionally, HNLs have been divided in two groups, flavoprotein HNLs and nonflavoprotein HNLs. Flavoprotein HNLs are major seed proteins of the rosaceous stone fruits and have (*R*)-mandelonitrile as natural substrate, they are monomeric glycoproteins. Nonflavoprotein HNLs have been isolated from three families of dicotyledons (*Linaceae*, *Euphorbiaceae*, *Olacaceae*), one family of monocotyledons (*Gramineae*) and one family of ferns (*Polypodiaceae*) (ref. 1). In contrast to the flavoprotein group of HNLs, the nonflavoprotein HNLs comprise enzymes forming a rather heterogeneous group regarding their biochemical properties.

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In recent years HNLs from *Prunus serotina* (PsHNL) (ref. 3), *Sorghum bicolor* (SbHNL) (ref. 4), *Manihot esculenta* (MeHNL) (ref. 5) and *Hevea brasiliensis* (HbHNL) (ref. 6) have been cloned and their primary sequence analyzed with regard to sequence homologies to other proteins.

While HbHNL and MeHNL have 74% identity, there is no sequence homology between them and any other cloned HNL. Moreover, there is no obvious homology among other HNLs. Analysis of the SbHNL revealed extensive homologies to serine carboxypeptidases which belong to the structurally well investigated group of α/β hydrolase fold enzymes (ref. 4). The active site of carboxypeptidases and other α/β hydrolases is a catalytic triad.

Crystallographic studies, the mutational analysis of MeHNL (ref. 7) and HbHNL (ref. 6,8) and inhibitor studies suggest that these HNLs also utilize a catalytic triad. The order of the catalytic triad residues in both HNLs is in favour of an α/β hydrolase fold structure of these enzymes.

The HNL encoding cDNAs for HbHNL (ref. 6) and MeHNL (ref. 9), respectively, were used to construct expression vectors for overexpression of the HNLs in *Escherichia coli* (ref. 9), *Saccharomyces cerevisiae* (ref. 6) and *Pichia pastoris* (ref. 6).

HYDROXYNITRILE LYASE CATALYZED PREPARATIONS OF (*R*)- AND (*S*)-CYANOHYDRINS

For enantioselective syntheses of optically active cyanohydrins the following four hydroxynitrile lyases are applied mainly: the (*R*)-HNL from bitter almond (EC 4.1.2.10) and the (*S*)-HNLs from *Sorghum bicolor* (EC 4.1.2.11), from *Hevea brasiliensis* (EC 4.1.2.39) as well as from *Manihot esculenta* (EC 4.1.2.37).

The first asymmetric synthesis effected by enzymes was the preparation of optically active mandelonitrile from benzaldehyde and HCN, with emulsin as source of the enzyme (ref. 10). More than fifty years later E. Pfeil et al. used isolated PaHNL from bitter almonds for a more general study of the PaHNL catalyzed snythesis of (*R*)-cyanohydrins (ref. 11). (*R*)-Mandelonitrile was prepared with 86% *ee*, but enzymatically less reactive aldehydes gave only poor optical yields. Thus, this simple procedure for the preparation of (*R*)-cyanohydrins found virtually no practical application. All efforts failed to improve the optical yields under the conditions common for enzyme catalyzed reactions, namely water or water/ethanol as solvents and working at pH 5-6, which is the optimum for PaHNL. Under these conditions the chemical addition of HCN to aldehydes leading to racemic products cannot be suppressed and prevails higher optical yields especially when the enzyme catalyzed reaction becomes slow.

The decisive breakthrough of the application of HNLs in the synthesis of optically active cyanohydrins came with the discovery that the undesirable chemical addition is suppressed in organic solvents that are not miscible with water (ref. 12). It was found that there is no loss of enzyme activity in solvents like diisopropyl ether (ref. 13). Thus, even for aldehydes that are poor substrates for the enzyme, the enzymatic process predominates the chemical addition in these media. In aqueous medium the chemical addition of HCN to aldehydes can also be suppressed by lowering the pH below 4.5 (ref. 14). Under these conditions optically active cyanohydrins of high optical purity can be obtained (ref. 14).

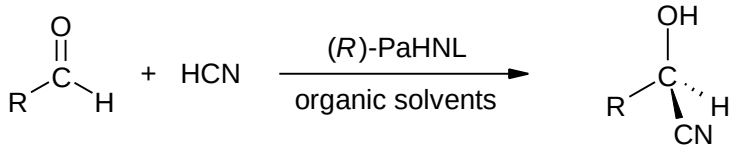
For industrial biocatalytic processes a two-phase system in many cases is advantageous. The principle is to combine optimum enzyme performance in the buffered aqueous phase with a high productivity by employing the organic phase as a reservoir of both substrate and product. The use of this procedure for the preparation of optically active cyanohydrins was published in the early nineties (ref. 15,16). It is likely that two-phase processes will become highly efficient, cost-effective production methods for a wide variety of optically pure cyanohydrins on a multi-kilogram scale (ref. 17).

PaHNL catalyzed preparation of (*R*)-cyanohydrins

For synthetic purposes the (*R*)-hydroxynitrile lyase can easily be isolated in sufficient quantities from almond meal (ref. 18). Its high stability made it possible to investigate a wide range of substrates. It could be shown that aliphatic, aromatic and heterocyclic aldehydes (ref. 13) as well as ketones (ref. 19) are good substrates for the enzyme. In organic solvents the corresponding (*R*)-cyanohydrins are obtained in high optical purity (Table 1).

For the reactions carried out in organic solvents it is particularly advantageous to employ enzymes bound to a suitable support, for example to cellulose. Thus the "support-bound" enzyme may be filtered off after the reaction is complete and the catalyst can be reused. PaHNL in organic solvents accepts high substrate concentrations (up to 2 mol L^{-1}) without decreasing *ee*-values (ref. 20).

Many attempts have been reported to avoid the use of free HCN in the synthesis of cyanohydrins (ref. 15a). Transcyanation with the stable acetone cyanohydrin as HCN donor in diisopropyl ether and with almond meal as enzyme source gives (*R*)-cyanohydrins with high conversion and in many cases satisfactory optical purity (ref. 21).



1 (*R*)-2

TABLE 1. Synthesis of (*R*)-Cyanohydrins 2 by (*R*)-Hydroxynitrile Lyase Catalyzed Addition of HCN to Aldehydes 1

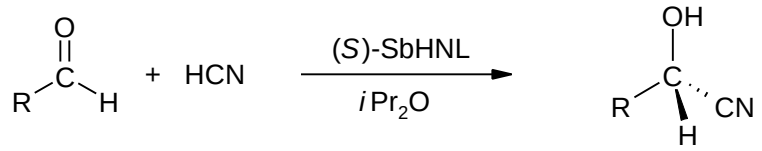
R =	in ethyl acetate/Avicel[a]			in diisopropyl ether/Avicel[a]		
	t, h	yield, %	*ee*, %[b]	*t*, h	yield, %	*ee*, %
C_6H_5	2.5	95	99	3	96	>99
3-C_6H_5O-C_6H_4	192	99	98	-	-	-
2-furyl	4	88	99	-	-	-
3-thienyl	-	-	-	6	95	>99
3-pyridyl	4.5	89	14	3	97	82
$H_3CCH=CH$	3	68	97	-	-	-
$H_3CS(CH_2)_2$	6.5	97	80	16	98	96
C_3H_7	-	-	-	16	99	98
$C_6H_5(CH_2)_3$	-	-	-	45	94	90

[a] The enzyme was bound on crystalline cellulose (Avicel). [b] Determined by gas chromatography (Ref. (12)).

SbHNL catalyzed preparation of (*S*)-cyanohydrins

The enzyme was first isolated from *Sorghum bicolor* and characterized in 1961 (ref. 22). It is substantially more time-consuming to isolate larger amounts of the SbHNL from *Sorghum* than it is to isolate PaHNL from bitter almonds.

SbHNL from *Sorghum* catalyzes exclusively the addition of HCN to aromatic and heteroaromatic aldehydes to yield the respective (*S*)-cyanohydrins (Table 2), but it does not accept aliphatic aldehydes or ketones as substrates (ref. 23,24).



1 (*S*)-2

TABLE 2. Synthesis of (*S*)-Cyanohydrins 2 by (*S*)-Hydroxynitrile Lyase Catalyzed Addition of HCN to Aldehydes 1

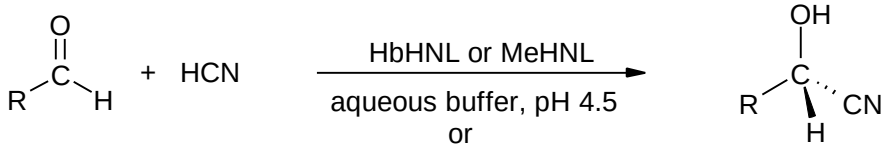
R =	*t*, h	yield, %	*ee*, %[a]	R =	*t*, h	yield, %	*ee*, %[a]
C_6H_5	3	91	97	3-$F_3CC_6H_4$	20	87	52
4-ClC_6H_4	48	87	54	3-$H_3COC_6H_4$	20	93	89
4-$H_3CC_6H_4$	32	78	87	3-$C_6H_5OC_6H_4$	144	93	96
3-HOC_6H_4	24	97	91	2-furyl	9	80	80
3-BrC_6H_4	18	94	92	3-thienyl	20	85	97
3-ClC_6H_4	48	95	91				

[a] Determined by gas chromatography.

Despite all improvements in the isolation of SbHNL from *Sorghum bicolor*, industrial applications of this enzyme requires the respective gene to be cloned in pro- or eucaryotic organisms and overexpressed.

HbHNL and MeHNL catalyzed preparation of (*S*)-cyanohydrins

As already mentioned, both HbHNL and MeHNL have 74% sequence homologies and both have been cloned and overexpressed. Both enzymes therefore are available in large amounts also for technical applications. In contrast to the (*S*)-HNL from *Sorghum bicolor*, which only accepts aromatic and heteroaromatic aldehydes as substrates, HbHNL as well as MeHNL catalyze the enantioselective formation of (*S*)-cyanohydrins from aliphatic, aromatic and heterocyclic aldehydes (ref. 8,25,26). Even ketones are accepted as substrates by HbHNL and MeHNL, respectively (ref. 8,26).



1 (*S*)-2

TABLE 3. Synthesis of (*S*)-Cyanohydrins 2 Catalyzed by HbHNL and MeHNL, Respectively

R =	HbHNL in aqueous buffer, pH 4.5[a]		MeHNL in diisopropyl ether[b]	
	yield, %	*ee*, %	yield, %	*ee*, %
C_6H_5	67	99	100	98
4-H_3CO-C_6H_4	49	95	82	98
H_2C=CH	38	94	100	47
3-furyl	61	99	98	92
2-thienyl	52	99	85	96
3-thienyl	49	99	98	98
E-$H_3C(CH_2)_2CH$=CH	-	-	82	97

cC$_6$H$_{11}$	94	99	100	92
nC$_4$H$_9$	-	80[c]	100	91
(H$_3$C)$_2$CH	-	81[c]	91	95

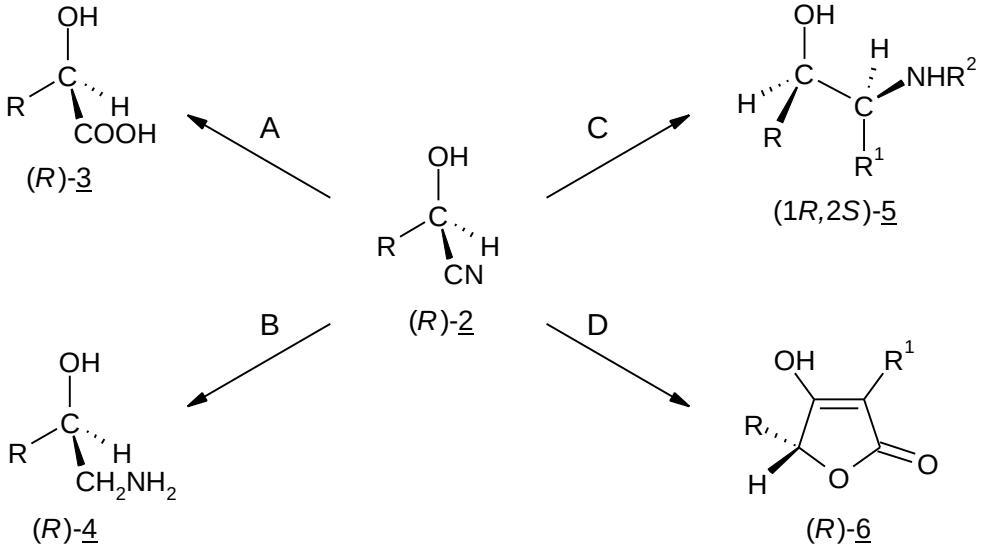
[a] Ref. (8a). [b] Ref. (26). [c] Ref. (8b)

Stereoselective reactions of optically active cyanohydrins (ref. 8,13,17)

Chiral cyanohydrins have a considerable synthetic potential, and subsequent stereoselective reactions lead to other important classes of compounds with stereogenic centers. Two types of reactions of cyanohydrins can be differentiated: follow-up reactions of the cyano group alone which do not involve the hydroxyl function and those in which the hydroxy group is transformed into a good leaving group for further reactions.

Most of the stereoselective follow-up reactions were performed starting from (*R*)-cyanohydrins only. But it could be demonstrated - as expected - that (*S*)-cyanohydrins react in the same manner.

Scheme 1 summarizes some of the important stereoselective transformations of the nitrile group of (*R*)-cyanohydrins. Route A: the hydrolysis of chiral cyanohydrins with aqueous HCl to α-hydroxy acids (*R*)-3 occurs without any racemization and is probably the easiest and most general route for the preparation of these compounds (ref. 27). An interesting example of economical importance is the synthesis of (*R*)-pantolactone by this procedure (ref. 28). Route B: hydrogenation of (*R*)-2 with LiAlH$_4$ leads - without protection of the hydroxyl group - directly to the corresponding 2-amino alcohols (*R*)-4 without racemization in excellent yields (ref. 29). The adrenergic bronchodilators (*R*)-terbutaline and (*R*)-salbutamol were synthesized recently starting from (*R*)-cyanohydrins (ref. 29). Route C: 2-amino alcohols of the ephedrine type (1*R*,2*S*)-5 can easily be obtained from (*R*)-cyanohydrins. The O-protected (*R*)-2 are reacted with Grignard reagents to give the corresponding imino intermediates which by hydrogenation with NaBH$_4$ form the (1*R*,2*S*)-amino alcohols with high diastereoselectivity (ref. 30). This synthetic route could also be applied for the preparation of thienyl and furyl analogues of ephedrine (ref. 31). Route D: optically active tetronic acids (*R*)-6 are obtained from O-protected (*R*)-cyanohydrins by addition of Reformatsky reagents (Blaise reaction) and aqueous workup (ref. 32).

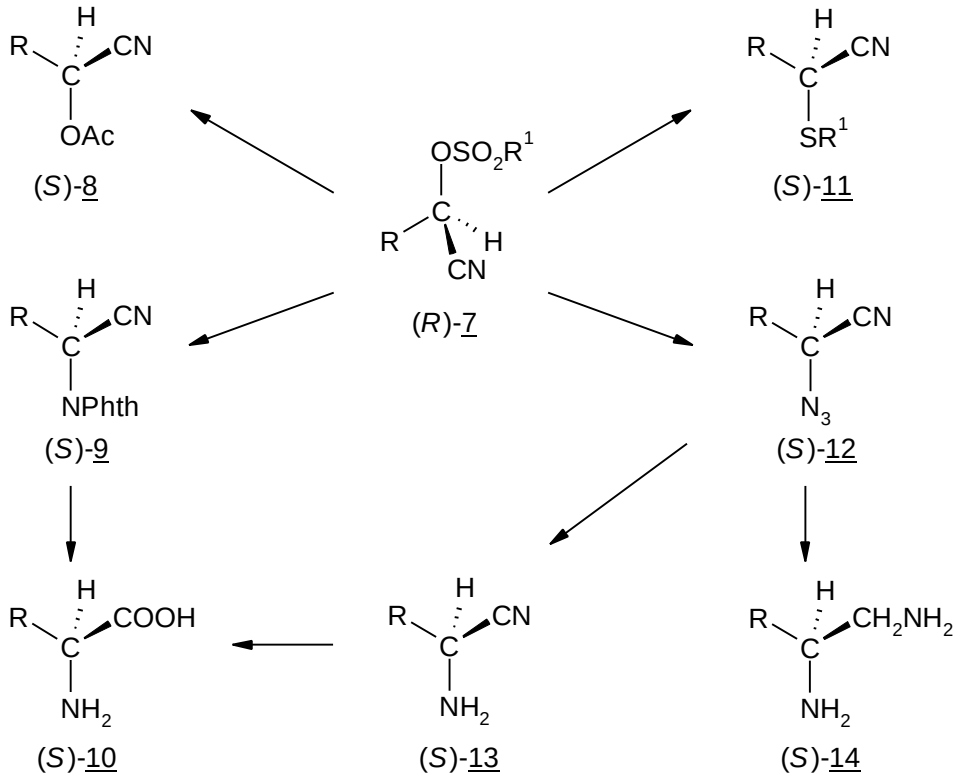


Scheme 1. Follow-up reactions of (*R*)-cyanohydrins (2) at the nitrile group

Activation and reactions of the hydroxyl group of (*R*)-cyanohydrins

The synthetic potential of optically active cyanohydrins can be extended considerably by transforming the OH function into a good leaving group which may be exchanged stereoselectively with a wide range of nucleophiles. Activation and nucleophilic exchange under inversion of configuration was applied for the inversion of the configuration of (*R*)-cyanohydrins (ref. 33). Sulfonylation of (*R*)-cyanohydrins gives the corresponding α-sulfonyloxynitriles (*R*)-7 without racemization (ref. 34).

Scheme 2 summarizes the most important reactions of sulfonyl activated cyanohydrins (*R*)-7 with various nucleophiles. The substitution occurs under complete inversion of configuration.



Scheme 2. Reactions of (*R*)-sulfonyl activated cyanohydrins (7)

Normally the reactions are carried out under mild conditions (room temperature) in an appropriate solvent. With potassium acetate the esters of (*S*)-cyanohydrins 8 are obtained (ref. 34) which can be hydrolyzed to (*S*)-cyanohydrins. With phthalimide potassium the N-protected α-aminonitriles (*S*)-9 are formed which can be transformed to the amino acids (*S*)-10 (ref. 34). α-Azidonitriles (*S*)-12 result in the reaction with alkali azides. Selective hydrogenation of (*S*)-12 to the aminonitriles (*S*)-13 or to the 1,2-diamino compounds (*S*)-14 is possible (ref. 35). With sulfur nucleophiles like thioacetate, benzyl mercaptide etc. α-mercaptonitriles (*S*)-11 result (ref. 36). The thio compounds (*S*)-11 can be hydrogenated or hydrolyzed to give optically active 1,2 amino thiols or α-mercapto carboxylic acids (ref. 36).

In addition to the reactions of sulfonyl activated cyanohydrins, direct stereoselective reactions of the OH group are feasible with special reagents. (*S*)-2-fluoronitriles for instance, may be prepared directly from (*R*)-cyanohydrins with diethylaminosulfur trifluoride (DAST) (ref. 37). Many of the follow-up products of (*R*)-cyanohydrins presented in Schemes 1 and 2 are of interest for further chemical transformations. Starting from 1,2-amino alcohols, 1,2-diamines or 1,2-amino thiols optically active heterocycles or metal complexes can be prepared which are of interest as optically active catalysts or as chiral auxiliaries in asymmetric synthesis. In ephedrine type compounds (1*R*,2*S*)-5

(Scheme 1) the OH function can be eliminated by catalytic hydrogenation yielding (*S*)-amphetamines. By this procedure (*S*)-3,4-methylenedioxyamphetamines, known as "designer drugs" (Ecstasy, Eve etc.), were obtained in optically pure form (ref. 38).

SUMMARY AND OUTLOOK

It was one intention of this article to demonstrate the considerable synthetic potential of optically active cyanohydrins. In most cases the reactions can be conducted with retention of chirality and further stereogenic centers can be introduced stereoselectively. Many of the compounds derived from cyanohydrins are biologically active and are used as pharmaceuticals (L-ephedrine, (*R*)-terbutaline, (*R*)-salbutamol) or agrochemicals (pyrethroids). Stereoselective syntheses of these compounds in industrial scales will therefore become very important. Through the stormy development in the last three years in getting recombinant (*S*)-hydroxynitrile lyases HbHNL and MeHNL, respectively, both types of enzymes (*R*)- and (*S*)-HNLs are available practically unrestricted and cheap. Since both enzymes accept all types of aldehydes and many ketones, the synthetic potential of optically active cyanohydrins is almost unlimited. Modifications of the active center by mutation will enable further improvements in reactivity and selectivity in special cases.

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