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(*R*)-Oxynitrilase Catalyzed Synthesis of (*R*)-Ketone Cyanohydrins#,1

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Abstract: (*R*)-Oxynitrilase from almonds (*Prunus amygdalus*) catalyzes the enantioselective addition of HCN to ethyl alkyl ketones **1** in diisopropyl ether yielding (*R*)-ethyl alkyl ketone cyanohydrins (*R*)-**2**, which are hydrolyzed under acid catalysis to give the α-hydroxy acids (*R*)-**3**. This (*R*)-oxynitrilase also catalyzes the enantioselective addition in aqueous citrate buffer (50 mM, pH 4.0), as demonstrated for the preparation of (*R*)-methyl alkyl ketone cyanohydrins (*R*)-**5** which are obtained in high enantiomeric excesses comparable to those in diisopropyl ether as solvent.

Chiral α-hydroxy carboxylic acids with further substituents in the α-position are difficult to prepare.[3] Since compounds of this type, however, are of interest as structural elements in natural products, numerous routes for their synthesis have been described.[4-8] Acid catalyzed hydrolysis of cyanohydrins represents an important general access to α-hydroxy carboxylic acids. Optically active aldehyde cyanohydrins, for example, easily accessible by enzyme catalyzed syntheses,[9] can be hydrolyzed without any racemization to give chiral α-hydroxy carboxylic acids.[10,11]

In contrast to chiral aldehyde cyanohydrins, very few optically active ketone cyanohydrins are described in the literature.[12] We therefore became interested in the preparation of optically active ketone cyanohydrins via oxynitrilase catalyzed addition of hydrocyanic acid to ketones. Recently we have described the enantioselective addition of hydrocyanic acid to methyl alkyl ketones, catalyzed by (*R*)-oxynitrilase [EC 4.1.2.10] from bitter almonds (*Prunus amygdalus*).[13a] In organic solvents (*R*)-ketone cyanohydrins are obtained in high enantiomeric excess.[13a] By hydrolyzing these (*R*)-ketone cyanohydrins with concentrated hydrochloric acid, (*R*)-α-hydroxy-α-methyl carboxylic acids are obtained in good chemical yields and enantiomeric excesses.[13a]

Conn et al. isolated from *Linum usitatissimum* (flax) an (*R*)-oxynitrilase, which catalyzes the addition of HCN to ketones.[14] M.-R. Kula et al.[15a] have investigated the substrate range of this enzyme with various aldehydes and ketones. Most of the reactions were performed, however, solely in a qualitative manner. Only in case of ethyl methyl ketone (butan-2-one) the reaction was carried out on a preparative scale.[15a] In a very recent paper[15b] the synthesis of (*S*)-ketone cyanohydrins via (*R*)-oxynitrilase catalyzed transcyanation from racemic ketone cyanohydrins was reported.

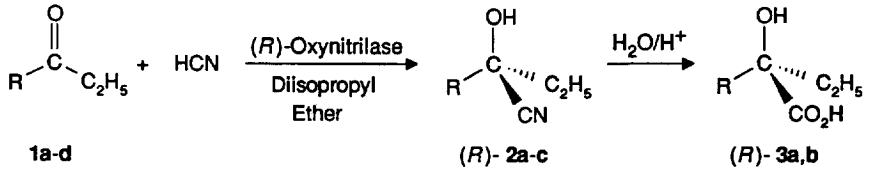
Because of the importance of chiral tertiary α-hydroxy acids for the synthesis of biologically active compounds, we have reinvestigated the enantioselective addition of HCN to ketones catalyzed by (*R*)-oxynitrilase from almond particularly under the aspects of large scale preparations of tertiary α-hydroxy acids.

In the present paper we report, on the one hand, the (*R*)-oxynitrilase catalyzed addition of HCN to ethyl alkyl ketones (alkan-3-ones) **1** in organic solvents under the reaction conditions described before.[13a] On the other hand, we report on the reactions of methyl alkyl ketones (alkan-2-ones) **4** with HCN in an aqueous medium using (*R*)-oxynitrilase from bitter almonds as catalyst, especially in comparison with the additions catalyzed by the (*R*)-oxynitrilase from flax.[15a]

Preparation of (*R*)-Ethyl Alkyl Ketone Cyanohydrins (*R*)-2 and the Corresponding Acids (*R*)-3

For the preparation of optically active ethyl alkyl ketone cyanohydrins neither a chemical nor an enzymatic process has been described in the literature. By analogy to the preparation of (*R*)-methyl alkyl ketone cyanohydrins,[13a] the addition of HCN to the ethyl alkyl ketones **1**, catalyzed by the (*R*)-oxynitrilase from almonds, was performed in anhydrous diisopropyl ether at pH 4.5 to give the corresponding (*R*)-ethyl ketone cyanohydrins (*R*)-**2** (Scheme 1, Table 1).

Scheme 1



1a-d (*R*)- **2a-c** (*R*)- **3a,b**

1-3	a	b	c	d
R	*n*-C_3H_7	*n*-C_4H_9	*n*-C_5H_{11}	$(CH_3)_2CHCH_2$

Table 1. Ketone Cyanohydrins (*R*)-2 by (*R*)-Oxynitrilase Catalyzed Addition of HCN to Alkan-3-ones **1** and Subsequent Hydrolysis to the Corresponding α-Hydroxy Acids (*R*)-3

Ketones	Reaction Conditions		(*R*)-Cyanohydrins			(*R*)-α-Hydroxy Acids		
1	Time [d]	Temp. [°C]	(*R*)-**2**	Yield [%][a]	*ee* [%][b]	(*R*)-**3**	Yield [%]	*ee* [%][b]
a	3	20	**a**	33	85[c]	**a**	82[d]	85 (94)[e]
a	7	0	**a**	7	83			
b	3	20	**b**	21	90	**b**	89[d]	90 (95)[e]
b	7	0	**b**	5	90			
c	5	20	**c**	7	66	**c**	-	-
d	14	20	**d**	-	-	**d**	-	-

[a] Determined by ^1H NMR. [b] Determined by gas chromatography on β-cyclodextrin phases after hydrolysis to the corresponding acid and subsequent esterification with diazomethane. [c] Configuration by comparison of optical rotation values, see Ref.[13b] [d] Preparative batch size. [e] ee-Values after recrystallization in parentheses.

The results listed in Table 1 show that, as expected, ethyl alkyl ketones **1** are poorer substrates for the (*R*)-oxynitrilase in comparison to methyl alkyl ketones.[13a] The ethyl cyanohydrin (*R*)-**2a**, for example, was ob-

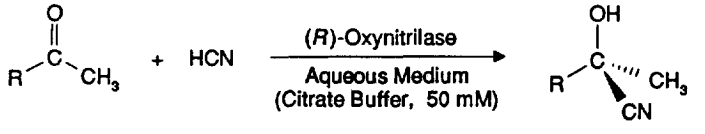
tained from hexan-3-one **1a** in 33% yield and 85%*ee* in 3 days at 20°C, whereas the cyanohydrin from hexan-2-one was obtained after 2 days at 0°C in 90% yield and 98%*ee*.[13a] While the enantiomeric excesses of the addition of HCN to methyl ketones significantly depend on the temperature[13a] this is not the case with the ethyl ketones **1**. Since ethyl ketones react very slow and the enantiomeric excesses are not temperature dependent, a reaction temperature of 20°C is recommendable. The enantiomeric purity of the ketone cyanohydrins (*R*)-**2** could not be determined via derivatization as MTPA esters, acetic or trifluoroacetic acid esters. Therefore the enantiomeric excesses of (*R*)-**2a-c** were determined after hydrolysis to the corresponding α-hydroxy acids (*R*)-**3a-c**.

(*R*)-Methyl ketone cyanohydrins are hydrolyzed to (*R*)-α-hydroxy-α-methyl carboxylic acids without any racemization.[13a] It was to assume that the acid catalyzed hydrolysis of (*R*)-ethyl ketone cyanohydrins occurs also without racemization. We have therefore hydrolyzed the cyanohydrins (*R*)-**2a-c** to the hydroxy acids (*R*)-**3a-c**. The enantiomeric excesses of (*R*)-**3a-c** were determined by gas chromatography on β-cyclodextrin phases after reaction of the acids with diazomethane to the corresponding methyl esters.

Enzyme Catalyzed Preparation of (*R*)-Methyl Alkyl Ketone Cyanohydrins (*R*)-5 in Aqueous Medium

In the (*R*)-oxynitrilase catalyzed preparation of (*R*)-aldehyde cyanohydrins high enantiomeric excesses were obtained only in organic solvents or in aqueous medium at low pH values (pH ≤ 4).[9] Under these conditions the normal chemical addition of HCN to aldehydes, leading to racemic products and thus to a decrease of enantiomeric excesses, can be suppressed. Addition reactions in aqueous medium at pH values in the range of 3.25 to 4 are described in a patent.[16] In this patent, however, only 2-methylcyclohexanone is listed as an example for a ketone reaction without any specification of the enantiomeric excess of the cyanohydrin obtained.[16] We have now investigated the enantioselective addition of HCN to methyl alkyl ketones **4** in aqueous medium in detail (Scheme 2).

Scheme 2



4a-d (*R*)-**5a-d**

4, 5	a	b	c	d
R	*n*-C_3H_7	*n*-C_4H_9	*n*-C_5H_{11}	$(CH_3)_2CHCH_2$

For all conversions in citrate buffer we have applied 50 Units enzyme per mmol substrate deviating from the patent mentioned,[16] in which no enzyme activity is given. One has to assume, however, that probably considerable larger amounts of enzyme were used.[17]

Since the enzyme catalyzed reaction of methyl alkyl ketones in organic solvents is markedly influenced by temperature,[13a] we have performed the conversion of **4a** to (*R*)-**5a** at different temperatures (Table 2).

Table 2. Variation of Temperature and Reaction Time in the (*R*)-Oxynitrilase Catalyzed Preparation of (*R*)-**5a** from **4a** in Citrate Buffer (50 mM, pH 4.0)

Reaction Conditions		Cyanohydrin (*R*)-**5a**	
Temp. [°C]	Time [h]	Yield [%][a]	*ee* [%][b]
2	4	34	95
2	8	59	94
2	24	78	95[c]
20	2.5	41	93
20	5	52	88
20	24	70	81
30	1.75	50	83

[a] Determined by ^{1}H NMR. [b] As (*R*)-MTPA ester. [c] As (*S*)-MTPA ester.

As shown in Table 2, a temperature of 2°C is particularly advantageous with regard to chemical yields and enantiomeric excesses, whereby a longer reaction time is required. At 20°C and a short reaction time especially the chemical yield is diminished and at 30°C and short reaction times the chemical yield is still satisfying but the enantiomeric excess is decreased considerably compared to the conversion at 2°C or 20°C. The influence of reaction times on the enantiomeric excess and chemical yields at the different temperatures are listed also in Table 2. At 20°C the best enantiomeric excess of (*R*)-**5a** was obtained after 2.5 h. Longer reaction times lead to better chemical yields but the enantiomeric excess decreases (Table 2). Lowering the temperature to 2°C, in contrast to reactions at 20°C, the enantiomeric excess remains almost constant even after long reaction times (see Table 2).

At pH values below 4.0 the chemical addition of HCN to aldehydes is largely suppressed. But also (*R*)-oxynitrilase from almonds is deactivated with decreasing pH values by splitting off the prosthetic group FAD.[18] We have therefore investigated the pH dependence of the reaction at 20°C in the range of pH 3.5 to pH 5.4, the pH optimum of the enzyme[19] (Table 3).

Table 3. pH Variation in (*R*)-Oxynitrilase Catalyzed Reactions of Ketones **4a-d** to Cyanohydrins (*R*)-**5a-d** in Citrate Buffer (2.5 h Reaction Time at 20°C)

	(*R*)-**5a**		(*R*)-**5b**		(*R*)-**5c**		(*R*)-**5d**	
pH	Yield [%][a]	*ee* [%][b]	Yield [%][a]	*ee* [%][b]	Yield [%][a]	*ee* [%][b]	Yield [%][a]	*ee* [%][b]
3.5	33	94	36	97	21	93	9	96
4.0	41	93	62	95	27	95	23	95
4.5	61	89	68	91	37	88	24	90
5.0	67	68	75	80	49	74	29	75
5.4	77	56	83	56	65	59	29	55

[a] Determined by ^{1}H NMR. [b] As (*S*)-MTPA esters.

Table 3 shows a pH optimum of 4.0 for the conversion of methyl alkyl ketones **4a-d** to methyl ketone cyanohydrins (*R*)-**5a-d**. Due to the diminished enzyme activity at pH 3.5[20] the yields in all cases are low. At pH 4.0, however, a significant increase of yields combined with high enantiomeric excess is observed. Increasing the pH further causes better yields but optical purities are decreased indicating an increase of the non-catalyzed chemical addition of HCN.

Applying the optimized reaction conditions (2°C, 24 h reaction time, pH 4.0), (*R*)-oxynitrilase catalyzed addition of HCN to methyl alkyl ketones **4** yielding methyl ketone cyanohydrins (*R*)-**5** was performed on a preparative scale (Table 4). In comparison to the reactions in aqueous medium the corresponding reactions in diisopropyl ether[13a] are listed also in Table 4.

Table 4. Methyl Ketone Cyanohydrins (*R*)-**5a-d** from Methyl Ketones **4a-d** in Aqueous Medium at 2°C and 24 h Reaction Time in Comparison with Reactions in Diisopropyl Ether

	Citrate Buffer, pH 4.0[a]		Diisopropyl Ether/Avicel[b]			
Ketones	Products (*R*)-**5**		Products (*R*)-**5**			
4	Yield [%][c]	*ee* [%][d]	Temp. [°C]	R.time [h]	Yield [%]	*ee* [%][e]
a	78	95	0	43	70	97
b	94	98	0	42	90	98
c	56	96	20	72	88	98
d	40	98	0	40	57	98

[a] 50 U enzyme/mmol substrate. [b] Ref.[13a], 20 U enzyme/mmol substrate. [c] Determined by ^{1}H NMR. [d] As (*S*)-MTPA esters. [e] As (*R*)-MTPA esters.

The yields and the enantiomeric excesses of the cyanohydrins (*R*)-**5a,b** obtained are comparable in both solvent systems. In aqueous medium a larger amount of enzyme (50 U/mmol substrate) compared with the reactions in diisopropyl ether (20 U/mmol substrate) is necessary. Whereas the enantiomeric excesses of (*R*)-**5a-d** are almost identically in both systems, the yields differ. **4c,d** are poorer soluble in water than **4a,b** that may explain the lower yields.

The optical purity of (*R*)-ketone cyanohydrins (*R*)-**5** was determined by gas chromatography after derivatization with Mosher's reagent (*R*)- or (*S*)-MTPA-Cl[21] to diastereomeric esters as described in Ref.[13a]

Comparison of the (*R*)-Oxynitrilases from Almond and from Flax in the Synthesis of (*R*)-Ketone Cyanohydrins

The natural substrate for the addition of HCN to carbonyl compounds catalyzed by the (*R*)-oxynitrilase from bitter almonds is benzaldehyde. It could be shown that numerous other aldehydes[9,19] are accepted as substrates by the (*R*)-oxynitrilase from almonds, and surprisingly, in organic solvents not miscible with water, also (*R*)-ketone cyanohydrins are easily obtained with this enzyme in good enantiomeric excesses.[13a]

In the present paper we could show that the (*R*)-oxynitrilase catalyzed addition of HCN to ketones is also possible in aqueous medium with enantiomeric excesses comparable to those in the reaction in diisopropyl

ether (see Table 4). This result was not to expect since in the addition of HCN to aldehydes in an aqueous medium far lower enantiomeric excesses were obtained,[9] although benzaldehyde is the natural substrate for (*R*)-oxynitrilase from almond.

The investigations by Kula et al.[15a] show that the (*R*)-oxynitrilase from *Linum usitatissimum* affords (*R*)-ketone cyanohydrins in good optical yields *only* in an organic solvent and citrate/phosphate buffer system but not in an aqueous medium alone, although acetone and butan-2-one are the natural substrates for this enzyme.

With regard to the application, the (*R*)-oxynitrilase from bitter almonds is unambiguously superior to the (*R*)-oxynitrilase from flax for the synthesis of (*R*)-ketone cyanohydrins. This concerns the availability of the enzyme as well as the broad substrate range and the great independence of the enantioselectivity of the addition from the medium applied.

Experimental

Materials and Methods: 5-Methyl-3-hexanone (**1d**) was prepared according to Ref.,[22] the methyl ketones **4a-c** according to Ref.[23] All other ketones **1a-c** and **4d** were purchased from Fluka, Avicel cellulose from Merck. The organic solvents were purified and dried. ^{1}H NMR spectra were recorded on a Varian T 60, Bruker WP 80 and CXP 250 with TMS as internal standard. GC for the determination of enantiomeric excess: a) Carlo Erba Fractovap 4160 with FID, Spectra Physics Minigrator, 0.5 bar hydrogen, column 20 m, phase PS086; b) Carlo Erba HRGC 5360 Mega Series with FID, C.E. DP Data Processor, 0.5 bar hydrogen, column 20 m, phase OV 1701; c) Carlo Erba HRGC 5300 Mega Series with FID, Carlo Erba Mega Series Integrator, 0.5 bar hydrogen, column 20 m, phases OV 1701 and 225 with 10% permethylated β-cyclodextrin.

Preparation of (R)-α-ethyl-α-hydroxy alkanenitriles (R)-2: A solution of (*R*)-oxynitrilase (150 µl, 1000 Units/ml, 30 Units/mmol substrate) was dropped on Avicel cellulose (1 g, soaked in 0.02 M sodium acetate buffer, pH 4.5), then diisopropyl ether (20 ml), the ketone **1** (5 mmol) and hydrocyanic acid (600 µl, 15.4 mmol) were added. The reaction mixture and the Avicel cellulose loaded with the enzyme were stirred at the given temperature and time (Table 1). Avicel cellulose was filtered off and washed with diethyl ether. The combined filtrates were dried (Na_2SO_4) and concentrated. The residue was dried for 30 min *in vacuo*, and the yield was determined by ^{1}H NMR spectroscopy.

Determination of the enantiomeric excess of (R)-2: 10 µl of the crude product were hydrolyzed with 0.5 ml conc. HCl at 90°C for 5 h. HCl was removed, the residue was taken up in diethyl ether, filtered, dried (Na_2SO_4) and evaporated. Then 1 ml of a solution of diazomethane in diethyl ether was added. After 5 min the solvent was removed, 3 ml dichloromethane were added and the enantiomeric excess was determined on permethylated β-cyclodextrin phases by gas chromatography.

Preparation of (R)-α-ethyl-α-hydroxy acids (*R*)-3: To the crude cyanohydrin (*R*)-2 (2 mmol) 10 ml conc. HCl were added, the mixture was stirred for 7 h at room temperature, then heated to 60°C for 12 h and to 100°C for 5 h. After cooling HCl was removed, the residue mixed with 50 ml diethyl ether and stirred for

30 min. NH_4Cl was filtered off, the filtrate dried (Na_2SO_4), concentrated and dried for 1 h *in vacuo*. The residue was recrystallized from *n*-hexane. **3b**: $[\alpha]_D^{20}$ = -3.53 (*c* 1.84, $CHCl_3$); mp 75-77°C; ^1H NMR ($CDCl_3$): δ = 0.87-0.95 (2 t, 6 H, CH_3), 1.08-1.23 (m, 1 H, $CH_2CH_2CH_2$), 1.23-1.40 (m, 2 H, $CH_2CH_2CH_2$), 1.40-1.60 (m, 1 H, $CH_2CH_2CH_2$), 1.60-1.95 (m, 4 H, CH_2, $CH_2CH_2CH_2$). **3a** and **3b** gave correct elemental analyses.

Determination of the enantiomeric excess of (R)-3: A solution of diazomethane in diethyl ether (1 ml) was added to 5 mg of the carboxylic acid. After 5 min the solvent is removed, 3 ml dichloromethane were added and the enantiomeric excess was determined directly by gas chromatography.

Preparation of (R)-α-hydroxy-α-methyl alkanenitriles (R)-5: The ketone **4** (2 mmol) was added to citrate buffer (0.05 M, 40 ml) followed by the enzyme (100 µl, 1000 U/ml, 50 U/mmol substrate) and hydrocyanic acid (300 µl, 7.7 mmol), and the mixture was stirred (reaction conditions see Table 2-4). The reaction mixture was extracted four times with each 25 ml chloroform. The combined extracts were dried (Na_2SO_4) and concentrated. The residue was dried for 15 min *in vacuo*, and the yield was determined by ^1H NMR spectroscopy.

Determination of the enantiomeric excess of (R)-5: The crude product (10 µl) was dissolved in 200 µl dichloromethane, and 20 µl of (*R*)-(+)- or (*S*)-(-)-MTPA chloride[21] and 20 µl of pyridine were added. The mixture was heated to 60°C for 3 h and then allowed to stand at room temperature for 12 h. The solution was filtered through a silica gel column (3 x 0.5 cm) with dichloromethane (2 ml) as eluent. The enantiomeric excess was directly determined from the filtrate.

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