Selective Hydrolysis of Aliphatic Dinitriles to Monocarboxylic Acids by a Nitrilase from *Arabidopsis thaliana*[1]

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Abstract: The hydrolysis of a variety of dinitriles including α,ω-dicyanoalkanes **1**, β-substituted glutaronitriles **5**, and γ-cyanopimelonitrile **7** with a recombinant plant nitrilase from *Arabidopsis thaliana*, expressed in *E. coli*, is described. Conversion rate and selectivity of the hydrolysis of dinitriles **1a–f** to ω-cyanocarboxylic acids **2a–f** depend on the chain length. The enzyme activity markedly increases from malononitrile (**1a**) to octanedinitrile (**1f**). The selectivity, however, does not correlate with the rates. Up to a chain length of 6 C-atoms, the cyanocarboxylic acid is the only product, even at complete conversion of the starting material. Pimelonitrile (**1e**) is hydrolyzed to the cyanocarboxylic acid **2e** without formation of diacid (<1%) up to 73% conversion. Glutaronitriles **5a–c** were also hydrolyzed to the corresponding cyanobutanoic acids **6a–c** with perfect selectivity. The nitrilase hydrolyzes exclusively the primary cyano group of **7** to give 3,5-dicyanoheptanoic acid (**8a**), whereby the selectivity is slightly reduced compared to the unsubstituted pimelonitrile (**1e**). If the hydrolysis is terminated at conversions ≤90%, pure **8a** can be isolated in 72% yield (92% referred to conversion). After esterification of **8a** to the methyl ester **8b**, only the 5-cyano group but not the ester function was hydrolyzed enzymatically to give cyanoheptanedioic acid monoester (**10**).

Key words: catalysis, enzymes, hydrolyses, nitriles, regioselectivity

Introduction

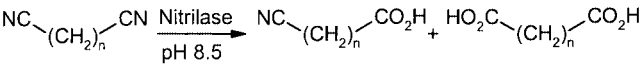
The nitrilase-mediated selective hydrolysis of dinitriles to cyanocarboxylic acids is of particular synthetic interest, since a selective chemical hydrolysis of dinitriles is virtually impossible. While dinitriles are readily available not only on bench scale but also in technical quantities, the preparation of compounds bearing both a cyano group and a carboxyl function is much more difficult. Nevertheless, cyanocarboxylic acids are of interest for a variety of applications. For example, ω-cyanocarboxylic acids, derived from aliphatic α,ω-dinitriles by nitrilase-catalyzed selective hydrolysis, could be converted directly to the corresponding five- and six-membered ring lactams after hydrogenation of the cyano group to the primary amine.[3]

All nitrilases used so far in the selective hydrolysis of dinitriles to monocarboxylic acids were obtained from bacterial sources. Depending on the growth substrates, resting cells of *Rhodococcus rhodochrous* N.C.I.B 11216, for example, were capable of hydrolyzing selectively isophthalonitrile and fumaronitrile,[4] whereas aliphatic α,ω-dinitriles such as glutaronitrile were hydro-lyzed effectively but not selectively to the monoacids.[4] The nitrilase from *Rhodococcus rhodochrous* J1 exhibits specificity for dicyanobenzenes, yielding selectively the corresponding cyanobenzoic acids.[5,6] A nitrilase from *Acidovorax facilis* 72 W having aliphatic nitrilase activity converted α,ω-dinitriles from malononitrile to glutaronitrile selectively to the corresponding ω-cyanocarboxylic acids in 92–99% yield, depending on the chain length.[3] Pétré et al. reported a selective enzymatic hydrolysis particularly of adiponitrile to 5-cyanovaleric acid, an intermediate for the preparation of ε-caprolactam, using the nitrilase from *Comamonas* NI1.[7]

Cloning and overexpression of a nitrilase from *Arabidopsis thaliana*[8] (EC 3.5.5.1) offered for the first time access to a plant nitrilase in amounts sufficient for synthetic applications. Investigations of the substrate range of *A. thaliana* demonstrated the acceptance of aliphatic nitriles with longer chain length.[2,9] Because, as mentioned above, the monohydrolysis of aliphatic dinitriles is of great industrial interest, we have examined the possibility of hydrolyzing selectively aliphatic dinitriles to monocarboxylic acids using an *Arabidopsis thaliana* nitrilase.

Selective Hydrolysis of Unsubstituted α,ω-Dicyanoalkanes

α,ω-Dicyanoalkanes **1** with varying chain lengths were hydrolyzed with a nitrilase from *Arabidopsis thaliana* as catalyst as outlined in Scheme 1.



1 **2** **3**

1-3	a	b	c	d	e	f
n =	1	2	3	4	5	6

Scheme 1

Both hydrolysis selectivity and hydrolysis rate were determined by gas chromatography. Thereby, as stated by coinjection, diacids **3**, which are commercially available, could be detected at concentrations of 1% referred to the

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initial concentration of **1**. The results of enzymatic hydrolysis are summarized in Table 1.

The hydrolysis of dinitriles **1a–d** catalyzed by *A. thaliana* afforded selectively the corresponding cyanocarboxylic acids **2a–d**. In all cases, the amount of the corresponding diacid **3a–d** is <1% at 100% conversion. Table 1 clearly shows the dependence of the nitrilase activity on the chain length. While the dinitriles **1a–c** (n = 1–3) were found to be moderate substrates for the nitrilase, adiponitrile (**1d**), pimelonitrile (**1e**) and octanedinitrile (**1f**) (n = 4–6) were converted very quickly to the monoacids **2d–f**. The selectivity of *A. thaliana*, however, is diminished in the hydrolysis of **1e** and **1f** to the cyanocarboxylic acids **2e** and **2f**. The percentage of diacid **3e** and **3f** amounts to 1.1% at 83% conversion of **1e** and 12.5% at 66% conversion of **1f**, respectively (Table 1). It was found that the initial ratio **2f**:**3f** of approximately 5 remained constant in the course of hydrolysis. The crystalline 7-cyanoheptanoic acid (**2f**) could be separated easily from the diacid **3f** and was isolated in 61% yield. In order to obtain pure 6-cyanohexanoic acid (**2e**), the hydrolysis of **1e** was terminated at 73% conversion (percentage of diacid **3e** <1%). After distillation, **2e** was obtained in 66% yield (91% referred to conversion).

Table 1 Selective Hydrolysis of α,ω-Dinitriles **1** to ω-Cyanocarboxylic Acids **2** and Diacids **3** Catalyzed by a Nitrilase from *Arabidopsis thaliana*

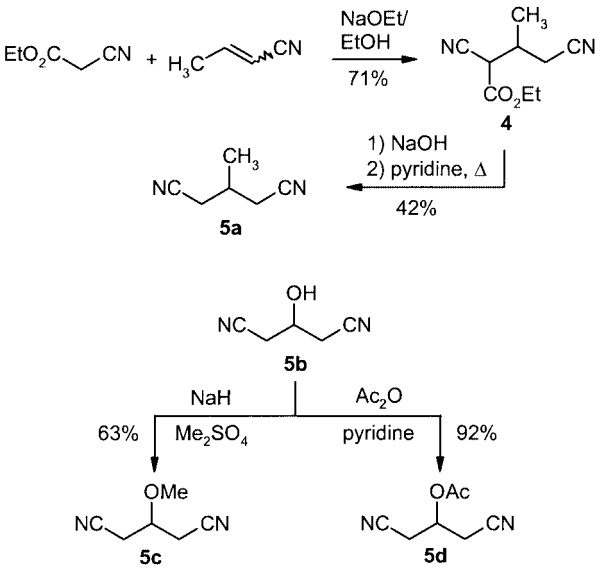
Substrate	Rel. Activity (%)[a]	Conversion (%)[b]	Selectivity **2**:**3** (%)	Monoacids	Yield (%)
1a	3.2	100	>99:<1	**2a**	–
1b	14	100	>99:<1	**2b**	–
1c	38	100	>99:<1	**2c**	–
1d	123	100	>99:<1	**2d**	88
1e	271	83	98.9:1.1	**2e**	66
1f	326	66	87.5:12.5	**2f**	61

[a] Activity referred to butyronitrile.
[b] Conversion is defined as: conversion = [**2**] + [**3**]/[**1**]$_0$.

The ω-cyanocarboxylic acids **2**, required as reference compounds, were prepared as follows: **2b**[10] and **2c**[11] were obtained by reacting β-propiolactone and γ-butyrolactone, respectively, with sodium cyanide. The monoacids **2d–f** were prepared by enzymatic hydrolysis as described on a preparative scale.

Selective Hydrolysis of β-Substituted Glutaronitriles

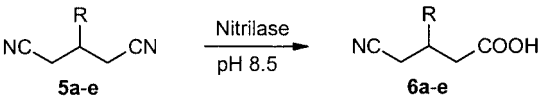
In order to clarify if the hydrolytic selectivity of the nitrilase is restricted to α,ω-dicyanoalkanes, substituted glutaronitriles were investigated. Substituents in α-position to the cyano group inhibit the *A. thaliana* catalyzed hydrolysis of a cyano group.[2,9] The only exception are α-fluoroni-triles, which could be hydrolyzed with *A. thaliana*.[1] Therefore the selective hydrolysis of β-substituted glutaronitriles **5** has been examined. Whereas β-hydroxyglutaronitrile (**5b**)[12] and β-phenylglutaronitrile (**5e**)[13] are known, the glutaronitriles **5a**,**c** and **d** had to be prepared (Scheme 2).



Scheme 2

In a Michael reaction comparable to a known method,[13] **5a** was obtained by reacting ethyl cyanoacetate with crotononitrile. The resulting Michael adduct, ethyl 2,4-dicyano-3-methylbutanoate (**4**), was hydrolyzed in NaOH and subsequently decarboxylated in boiling pyridine to give **5a** in 42% yield. For the preparation of **5c** and **5d**, β-hydroxyglutaronitrile (**5b**) was either methylated with dimethyl sulfate to give **5c** in 63% yield or acetylated with acetic anhydride to afford **5d** in 92% yield. The corresponding cyanobutanoic acids **6a–e** required as reference compounds were accessible by selective enzymatic hydrolysis of **5a–e**. For characterization, the monoacids **6b–e** were transformed into the corresponding methyl esters.

The *A. thaliana* nitrilase-mediated hydrolysis of glutaronitriles **5a–e** is shown in Scheme 3 and summarized in Table 2.



5,6	a	b	c	d	e
R	CH_3	OH	OCH_3	OAc	Ph

Scheme 3

Table 2 Selective Hydrolysis of Glutaronitriles **5** to 4-Cyanobutanoic Acid Derivatives **6** Catalyzed by a Nitrilase from *Arabidopsis thaliana* with 100% Conversion

Substrate	Rel. Activity	4-Cyanobutanoicacids **6**		
5	(%)[a]		Selectivity (%)	Yield (%)
5a	2.3	**6a**	>99	53
5b	9.7	**6b**	>99	41[b]
5c	0.5	**6c**	>99	29[b]
5d	<0.1	**6d**	–	–
5e	<0.1	**6e**	–	–

[a] Activity referred to butyronitrile.
[b] Isolated as methyl ester (see experimental part).

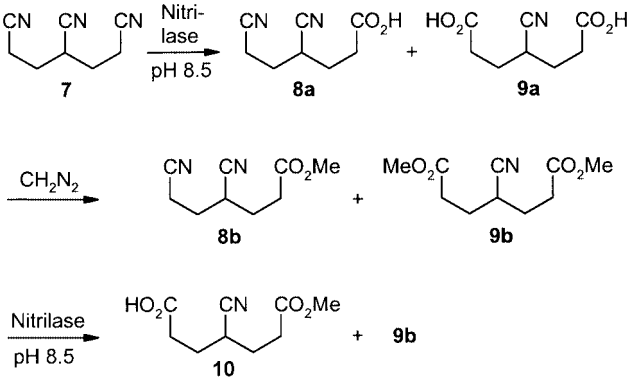
As can be seen from Table 2, the β-substituted glutaronitriles **5** were found to be poor substrates for *A. thaliana*. The nitrilase activity and thus acceptance of compounds **5** as substrates was strongly influenced by the steric demand of the substituents in β-position. The hydrolysis rates of **5a** and **5b** are comparable, **5c**, however, is converted 19 times slower than **5b**, whereas **5d** and **5e** are not accepted as substrates any more. In all cases, the enzymatic hydrolysis of the dinitriles **5** proceeded selectively to give only the corresponding 4-cyanobutanoic acids **6**.

Taking into account the results from the hydrolysis of dinitriles **1a**–**d** and **5a**–**c**, *A. thaliana* nitrilase hydrolyzes dinitriles but not the corresponding cyanocarboxylic acids. Due to almost complete deprotonation of the acid function at pH 8.5, we assume that this chemoselectivity arises from a repulsion of the carboxylate group and the hydrophobic binding site of the enzyme.

The selective hydrolysis of prochiral 3-substituted glutarate diesters using hydrolytic enzymes is a common method to prepare the corresponding chiral monoesters with high enantiomeric excesses.[14] Recently, prochiral 3-substituted glutaronitrile derivatives were subjected to hydrolysis with a nitrile hydratase/amidase system to afford enantioselectively the optically active cyanobutanoic acids.[15] The enantioselectivity of *A. thaliana* nitrilase, however, is relatively low. The cyanobutanoic acids **6a**–**c** were obtained from **5a**–**c** with enantiomeric excesses of only 13–60%, neither sufficient for practical purposes nor for an assignment of the enantiomers.

Selective Hydrolysis of γ-Cyanopimelonitrile

The hydrolysis of γ-cyanopimelonitrile (**7**),[16] containing cyano groups on primary and secondary C-atoms, offers further possibilities to investigate the regiospecificity and the selectivity of *A. thaliana* (Scheme 4, Table 3).



Scheme 4

Table 3 Selective Hydrolysis of 3-Cyanopimelonitrile **7** and its Derivatives **8a**,**b** Catalyzed by a Nitrilase from *Arabidopsis thaliana*

Substrate	Rel. Activity (%)[a]	Conversion (%)	Product	Selectivity (%)
1e	271	83	**2e**	98.9
7	167	77	**8a**	96.7
8a	1.3	96	**9a**	100
8b[b]	131	100	**10**	100

[a] Activity referred to butyronitrile.
[b] Hydrolysis of a mixture **8b**/**9b**.

Like the unsubstituted pimelonitrile (**1e**), compound **7** is also an excellent substrate for *A. thaliana*. Compound **7** was converted significantly faster than the reference compound butyronitrile (Table 3). The specific enzyme activity was diminished by about 40% compared to **1e**, indicating less influence of the secondary cyano group on the reaction rate. The selectivity of hydrolysis of **7** is less than that of **1e** with 96.7% of 3,5-dicyanoheptanoic acid (**8a**) and 3.3% of diacid **9a** formed at 77% conversion. Compound **8a** crystallized when the purity was at least 90%. Terminating the hydrolysis of **7** at conversions below 90% therefore yielded pure **8a** after recrystallization from petroleum ether/ethyl acetate.

At higher enzyme concentrations and with longer reaction times, **7** was hydrolyzed completely to 3-cyanopimelic acid (**9a**). Although monoacid **8a** thereby formed as intermediate was a markedly poorer substrate, as can be seen from the enzyme activity (Table 3), it was converted exclusively to **9a**. Since, as mentioned above, the carboxylate form of **8a** dominating at pH 9 was assumed to be responsible for the selectivity, a mixture of **8a**/**9a** (95:5), yielded by the enzymatic hydrolysis of **7** at 95% conversion, was esterified with diazomethane[17] to give the methyl esters **8b**/**9b**. From this mixture monoester **8b** was hydrolyzed about 100 times faster than **8a** (Table 3) exclusively to methyl hydrogen 3-cyanoheptanedioate (**10**), while diester **9b** remained unchanged under these reaction conditions (Scheme 4). This result was confirmed using

isolated **9b** for hydrolysis. Compound **9b** could be separated from **10** by extraction with base. In the reaction sequence shown (Scheme 4), cyanoheptanedioic acid monoester **10** was isolated in 74% total yield without necessity to isolate and purify the intermediates. In no case, hydrolysis of the secondary cyano group was observed. The enantioselectivity of *A. thaliana* nitrilase in the hydrolysis of **7** is even lower than in the hydrolysis of **5a**–**c**, yielding **8a** and **10** with <10% ee.

The experimental results clearly demonstrate that the *A. thaliana* nitrilase exhibited not only regiospecificity in the case of dinitrile but also selectivity for cyano groups in presence of ester functions. Comparable selectivity has been described for the nitrilase from *Rhodococcus* ATCC 39484, which converts benzonitrile but not methyl benzoate.[18]

Conclusion

In summary, among the nitrile-hydrolyzing enzymes investigated so far the nitrilase from *Arabidopsis thaliana* described shows the highest selectivity in the hydrolysis of aliphatic α,ω-dini-triles to cyanocarboxylic acids known today. Thereby the enzyme activity raises with increasing chain length of the dinitrile. Moreover, this enzyme differentiates chemically non-equivalent cyano groups, hydrolyzing exclusively the cyano groups on primary C-atoms. In contrast to many other nitrilases this enzyme is stable under the applied reaction conditions.

The *A. thaliana* nitrilase-catalyzed hydrolysis, starting from readily available di- and trinitriles, therefore opens access to a wide range of cyanocarboxylic and dicyanocarboxylic acids, which cannot be obtained by chemical hydrolysis. Since cyanocarboxylic acids are of interest for the preparation of other important compounds such as amino carboxylic acids and the corresponding lactams, this new method might have interesting synthetic potential.

Melting points were determined on a Büchi SMP-20 and are uncorrected. Unless otherwise stated, ^{1}H NMR spectra were recorded on a Bruker AC 250 F (250 MHz) and ARX 500 (500 MHz) in $CDCl_3$ with TMS as internal standard. Chromatography was performed using silica gel S (Riedel-de Haën), grain size 0.032–0.063 mm. GC separations were conducted using capillary glass columns (20 m × 0.32 mm) with OV 1701, carrier gas hydrogen. GC determination of enantiomeric excess was performed with a Chiraldex G-TA (ICT) column (30 m × 0.32 mm), carrier gas hydrogen. All solvents were dried and distilled. The recombinant nitrilase from *Arabidopsis thaliana* was obtained after overexpression in *Escherichia coli* as described elsewhere.[8,9] Petroleum ether used refers to the fraction boiling at 30−75°C.

Ethyl 2,4-Dicyano-3-methylbutanoate (4)

To sodium (1.03 g, 44.8 mmol) in anhyd EtOH (150 mL) was added portionwise ethyl cyanoacetate (32.88 g, 290.7 mmol). Crotononitrile (15.0 g, 223.6 mmol) was slowly added dropwise (ice-cooling), and the reaction mixture was stirred at r.t. for 2 h. After heating at 60 °C for 5 h, the mixture was poured onto the same volume of ice/concd HCl (10 mL). Approximately 100 mL of EtOH were distilled off, and the aqueous layer was extracted with Et_2O (3 × 100 mL). The combined organic layers were dried (Na_2SO_4), and concentrated. Distillation through a Vigreux column under high vacuum gave 28.53 g (71%) of **4** as a colorless oil; bp 101–103 °C/0.001 torr.

^{1}H NMR (250 MHz): δ = 1.26–1.38 (m, 6 H, 2 CH_3), 2.50–2.73 (m, 3 H, CH, CH_2), 3.61 and 3.73 (d, 1 H, *J* = 4.9 Hz, CH, diastereomeric mixture).

^{13}C NMR (63 MHz): δ = 13.99 (OCH_2CH_3), 16.53, 17.71, 21.72, 22.72 (C-2,3), 31.73, 31.86, 42.50, 42.63 (CH_3, C-4), 63.50 (OCH_2CH_3), 113.97, 114.30 (C-1), 116.92, 117.00 (C-5), 164.45 (CO_2Et).

Anal. Calcd for $C_9H_{12}N_2O_2$: C, 59.99; H, 6.71; N, 15.55. Found: C, 60.15; H, 6.78; N, 15.51.

β-Methylglutaronitrile (5a)

Butanoate **4** (17.16 g, 95.2 mmol) was slowly added dropwise to ice-cold aq NaOH (5%, 100 mL). After stirring for 75 min at r.t., the mixture was acidified with concd HCl (ice-cooling) and extracted with EtOAc (3 × 100 mL). The combined extracts were dried ($MgSO_4$), and concentrated. The crude product was dissolved in pyridine (100 mL) and heated at reflux for 4 h. The solution was concentrated, and the residue was taken up in Et_2O and washed with a 0.1 M solution of HCl. The organic layer was dried (Na_2SO_4), and concentrated. Distillation through a Vigreux column under vacuum gave 4.33 g (42%) of **5a** as a colorless oil; bp 133–134 °C/15 torr.

^{1}H NMR (250 MHz): δ = 1.26 (d, 3 H, *J* = 6.6 Hz, CH_3), 2.21–2.41 (m, 1 H, CH), 2.49 (d, 4 H, *J* = 6.1 Hz, 2 CH_2).

^{13}C NMR (63 MHz): δ = 19.02 (CH_3), 23.58 (C-3), 28.25 (C-2,4), 117.13 (CN).

Anal. Calcd for $C_6H_8N_2$: C, 66.64; H, 7.46; N, 25.90. Found: C, 66.62; H, 7.70; N, 25.84.

β-Methoxyglutaronitrile (5c)

To a suspension of NaH (0.7 g, 29.2 mmol) in anhyd THF (40 mL) at 0 °C under inert gas atmosphere was slowly added dropwise a solution of **5b**[12] (3.0 g, 27.2 mmol) in anhyd THF (10 mL) followed by addition of neat dimethyl sulfate (3.61 g, 28.6 mmol), and the mixture was stirred for 15 min at 0 °C. After stirring for 6 h at r.t., the mixture was poured onto ice (100 mL)/1 M solution of HCl (50 mL), and extracted with Et_2O (3 × 100 mL). The combined extracts were dried (Na_2SO_4), and concentrated. The residue was chromatographed on silica gel with petroleum ether–EtOAc (80:20) to give 2.13 g (63%) of **5c**.

^{1}H NMR (250 MHz): δ = 2.73 (d, 4 H, *J* = 5.6 Hz, CH_2), 3.50 (s, 3 H, OCH_3), 3.82 (quin, 1 H, CH).

^{13}C NMR (63 MHz): δ = 22.50 (C-2,4), 58.13 (OCH_3), 72.65 (C-3), 115.79 (CN).

Anal. Calcd for $C_6H_8N_2O$: C, 58.05; H, 6.50; N, 22.57. Found: C, 57.91; H, 6.60; N, 22.57.

β-Acetoxyglutaronitrile (5d)

A solution of **5b**[12] (2.0 g, 18.2 mmol), Ac_2O (3.71 g, 36.3 mmol) and pyridine (2.15 g, 27.2 mmol) in CH_2Cl_2 (25 mL) was heated at 60 °C for 19 h. The reaction mixture was then taken up in Et_2O (100 mL), washed with a 1 M solution of HCl (2 × 25 mL) followed by aq sat. solution of $NaHCO_3$ (2 × 25 mL), dried (Na_2SO_4), and concentrated. The residue was chromatographed on silica gel with petroleum ether–EtOAc (50:50) to give 2.53 g (92%) of **5d**.

^{1}H NMR (250 MHz): δ = 2.73 (d, 4 H, *J* = 5.6 Hz, CH_2), 3.50 (s, 3 H, CH_3CO), 3.82 (quin, 1 H, CH).

^{13}C NMR (63 MHz): δ = 20.63 (CH_3CO), 22.27 (C-2,4), 64.24 (C-3), 114.87 (CN), 169.50 (CH_3CO).

Anal. Calcd for $C_7H_8N_2O_2$: C, 55.26; H, 5.30; N, 18.41. Found: C, 55.27; H, 5.48; N, 18.33.

Crude Extract Preparation and Enzymatic Hydrolysis of Dinitriles 1, 5, and 7 with a Nitrilase from *Arabidopsis thaliana*; General Procedure

Cells of *E. coli*, grown in LB medium supplemented with ampillicin and kanamycin at 30 °C and induced with isopropyl-β-D-thiogalactopyranoside,[9] were separated from the nutrient medium by centrifugation for 30 min at 4 °C (5700×g) and washed with Tris/HCl buffer (70 mM, pH 8.5). The pellet was resuspended in Tris/HCl buffer (100 mL/10 g wet weight), sonicated with ice-cooling (3 × 5 min), and centrifuged for 40 min (186000×g). The supernatant containing the crude enzyme was diluted with Tris/HCl buffer, pH 8.5 (to reach 15–40% conversion after 2–4 h), and a solution of the respective dinitrile **1**, **5**, **7** in MeOH was added (10 mM end concentration; the concentration of the stock solution must be as high as the added volume and should not exceed 100 µL/5 mL reaction solution). After stirring for 2–4 h, samples of 1 mL volume were taken and analyzed as described below.

Determination of Conversion by Gas Chromatography

The sample taken was acidified with a 5 M HCl solution (50 µL) and extracted with Et_2O (5 mL). After centrifugation (2000×g) and cooling at −30 °C for 30 min to freeze the aqueous layer, the organic layer was decanted and a 0.2 M solution of diazomethane in Et_2O[17] was added. The excess of diazomethane and Et_2O was distilled off under vacuum. The residue was taken up in Et_2O (1 mL), and the conversion was determined by GC from the ratio area of methyl ester to area of nitrile.

Determination of the Enzyme Activity

To 5 mL of a solution of the enzyme in Tris/HCl buffer (70 mM, pH 8.5), warmed at 35 °C for 10 min, was added a 0.25 M solution of 3-phenylpropionitrile in MeOH (50 µL), and the mixture was stirred at 35 °C for 1 h. A sample of 1 mL was worked up and analyzed by GC as described for the determination of conversion. For the calibration of peak areas 3-phenylpropionitrile (181.5 mg) and 3-phenylpropionic acid (205.3 mg) were dissolved in MeOH/buffer (10 mL/990 mL). Amounts of 5 mL of each solution were mixed, and 5 mL from this mixture added to 5 mL of a solution of 3-phenylpropionitrile. Samples of 1 mL from the respective dilution were treated and analyzed by GC as described. The conversion was calculated from the ratio of peak areas of nitrile and acid considering the calibration factor. One unit is defined as 1 µmol conversion per minute.

Enzymatic Hydrolysis of Dinitriles 1 and 5 on a Preparative Scale; General Procedure

To a solution of crude enzyme in Tris/HCl buffer (70 mM, pH 8.5) at r.t. was added the respective dinitrile **1** or **5** (Table 4), and the mixture was stirred for the time given in Table 4. Then the mixture was acidified to pH 4 with a 5 M solution of HCl and extracted with Et_2O. The combined organic layers were extracted twice with 10% (v/v) sat. aq $NaHCO_3$ solution. The aqueous layer was washed with the double volume of Et_2O, acidified to pH 4 and extracted with Et_2O. The combined extracts were dried (Na_2SO_4), and concentrated. Crude cyanocarboxylic acids **2d**, **2e**, and **6a** were purified by distillation under vacuum, **2f** by recrystallization from petroleum ether/EtOAc. Compounds **6b** and **6c** were reacted with diazomethane[17] to give the corresponding methyl esters and chromatographed on silica gel with petroleum ether–EtOAc (60:40 and 70:30, respectively). For physical data see Table 5.

3,5-Dicyanohexanoic Acid (8a)

Compound **7** (4.0 g, 27.2 mmol) was hydrolyzed as described above with nitrilase (39.2 U) in Tris/HCl buffer (200 mL, 70 mM, pH 8.5). After 9.5 h at 78% conversion of **7**, the reaction was terminated. Recrystallization from petroleum ether–EtOAc (95:5) gave 3.25 g (72%) of **8a** as white needles; mp 45–46 °C.

^{1}H NMR (250 MHz): δ = 1.86–2.09 (m, 4 H, 2 CH_2), 2.51–2.70 (m, 4 H, 2 CH_2), 2.72–2.98 (m, 1 H, CH), 8.60 (br s, 1 H, CO_2H).

^{13}C NMR (63 MHz): δ = 15.34, 26.64, 28.09, 30.12, 30.96 (C-2,3,4,5,6), 117.89, 119.59 (CN), 177.25 (CO_2H).

Anal. Calcd for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.66; H, 6.15; N, 16.81.

Methyl 3-Cyanoheptane-1,7-dioate (9b)

Compound **7** (0.22 g, 1.5 mmol) was hydrolyzed as described above with nitrilase (78.4 U) in Tris/HCl buffer (25 mL, 70 mM, pH 8.5). After 72 h at 100% conversion of **7** and 74% conversion of **8a**, the reaction was terminated. The mixture of **8a**/**9a** was separated by chromatography on silica gel with petroleum ether–EtOAc (85:15), and reaction of **9a** with diazomethane gave 0.18 g (57%) of **9b**.

^{1}H NMR (250 MHz): δ = 1.89–2.06 (m, 4 H, 2 CH_2), 2.44–2.66 (m, 4 H, 2 CH_2), 2.76–2.88 (m, 1 H, CH), 3.71 (s, 6 H, 2 CO_2CH_3).

^{13}C NMR (63 MHz): δ = 27.32, 30.35, 31.19 (C-2,3,4), 51.95 (CO_2CH_3), 120.80 (CN), 172.52 (CO_2CH_3).

Anal. Calcd for $C_{10}H_{15}NO_4$: C, 56.33; H, 7.09; N, 6.57. Found: C, 56.29; H, 7.13; N, 6.49.

Table 4 Preparative Enzymatic Hydrolysis of Dinitriles **1** and **5** to Cyanocarboxylic Acids **2** and **6**

Substrates		Nitrilase	Buffer	Time	Conversion	Products	
1, **5**	g (mmol)	(U)	(mL)	(h)	(%)	**2**, **6**	Yield (g)
1d	3.78 (35.0)	27.7	250	19	100	**2d**	3.92
1e	6.11 (50.0)	55.4	500	6.75	73	**2e**	4.67
1f	2.72 (20.0)	27.7	250	8.5	94	**2f**	1.91
5a	1.00 (9.2)	68.0	50	48	92	**6a**	0.62
5b	1.00 (9.1)	13.1	50	50	97	**6b**	0.53[a]
5c	0.25 (2.0)	68.0	50	48	83	**6c**	0.09[a]

[a] Isolated as methyl ester.

Table 5 Physical and Spectroscopic Data of Cyanocarboxylic Acids **2** and **6**

Product[a]	Appearance	Bp (°C/0.001 torr)	^{1}H NMR ($CDCl_3$/TMS, 500 MHz) δ, *J* (Hz)	^{13}C NMR ($CDCl_3$/TMS, 125.8 MHz) δ
2d	colorless oil	101–103	1.70–1.83 (m, 4 H, 2 CH_2), 2.36–2.47 (m, 4 H, 2 CH_2), 10.28 (br s, 1 H, CO_2H)	17.00 (C-5), 23.58, 24.69 (C-3,4), 33.05 (C-2), 119.30 (CN), 179.05 (CO_2H)
2e[b]	colorless oil	98–99	1.54–1.76 (m, 6 H, 3 CH_2), 2.34–2.42 (m, 4 H, 2 CH_2), 10.32 (br s, 1 H, CO_2H)	17.01 (C-6), 23.78, 25.07, 28.03 (C-3,4,5), 33.63 (C-2), 119.54 (CN), 179.54 (CO_2H)
2f	colorless crystals	38–39[c]	1.33–1.48 (m, 4 H, 2 CH_2), 1.60–1.67 (m, 4 H, 2 CH_2), 2.29–2.38 (m, 4 H, 2 CH_2), 11.50 (br s, 1 H, CO_2H)	17.46 (C-7), 24.64, 25.51, 28.53, 28.66 (C-3,4,5,6), 34.21 (C-2), 120.06 (CN), 180.32 (CO_2H)
6a	colorless crystals	85–87 41–43[c]	1.00 (d, 3 H, *J* = 6.3, CH_2), 2.09–2.63 (m, 5 H, CH, 2 CH_2), 12.39 (br s, 1 H, CO_2H)	18.77 (CH_3), 22.80 (C-4), 26.85 (C-2), 39.04 (C-3), 119.21 (CN), 172.76 (CO_2H)
6b[d]	-		2.63–2.72 (m, 4 H, 2 CH_2), 3.51 (br s, 1 H, OH), 3.75 (s, 3 H, CO_2CH_3), 4.33–4.39 (m, 1 H, CH)	25.05 (C-4), 39.76 (C-2), 52.21 (CO_2CH_3), 64.11 (C-3), 116.92 (CN), 172.01 (CO_2CH_3)
6c[b,d]	-		2.56–2.78 (m, 4 H, 2 CH_2), 3.43 (s, 3 H, OCH_3), 3.72 (s, 3 H, CO_2CH_3), 3.87–3.97 (m, 1 H, CH	22.49 (C-4), 38.35 (C-2), 52.07 (CO_2CH_3), 57.78 (OCH_3), 73.29 (C-3), 116.94 (CN), 170.66 (CO_2CH_3)

[a] Satisfactory microanlyses or HRMS obtained: C ±0.30, H ±0.10, N ±0.28.
[b] 250 MHz spectrum.
[c] Mp.
[d] Characterized as methyl ester.

Methyl Hydrogen 3-Cyanoheptane-1,7-dioate (10)
Compound **7** (1.77 g, 12.0 mmol) was hydrolyzed as described above with nitrilase (39.2 U) in Tris/HCl buffer (100 mL, 70 mM, pH 8.5). After 17 h at 95% conversion of **7**, the reaction was terminated. The mixture of **8a**/**9a** was reacted with diazomethane. The resulting mixture of methyl esters **8b**/**9b** was hydrolyzed with nitrilase (39.2 U) in Tris/HCl buffer (100 mL, 70 mM, pH 8.5) for 48 h. Workup as described above for the enzymatic hydrolysis gave 1.77 g (74%) of **10** as a colorless oil.

^{1}H NMR (500 MHz): δ = 1.87–2.02 (m, 4 H, 2 CH_2), 2.49–2.68 (m, 4 H, 2 CH_2), 2.81–2.87 (m, 1 H, CH), 3.75 (s, 3 H, CO_2CH_3), 8.40 (br s, 1 H, CO_2H).

^{13}C NMR (125.8 MHz): δ = 26.97, 27.24, 30.13, 30.24, 31.13 (C-2,3,4,5,6), 52.00 (CO_2CH_3), 120.64 (CN), 172.61 (CO_2CH_3), 177.58 (CO_2H).

HRMS (CI, 70 eV): *m*/*z* Calcd for $C_9H_{14}NO_4$ (MH^+): 200.09228. Found: 200.09247.

MS (CI): *m*/*z* (%) = 399.2 (3) [2 M + H]$^+$, 381.1 (4) [2 M - H_2O + H]$^+$, 200.1 (43) [MH]$^+$, 182.1 (100) [MH - H_2O]$^+$.

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