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(*E*)-Selective hydrolysis of (*E*,*Z*)-α,β-unsaturated nitriles by the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana*†

Franz Effenberger* and Steffen Oßwald‡

Institut für Organische Chemie der Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany

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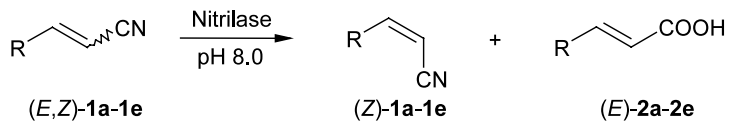
Abstract—From stereoisomeric α,β-unsaturated nitriles (*E*,*Z*)-**1**, the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana* hydrolyses the (*E*)-isomers exclusively to the corresponding (*E*)-carboxylic acids (*E*)-**2** with high specificity. The (*E*)-selectivity can also be utilised for the preparation of the isomerically pure nitriles (*Z*)-**1**. From (*E*,*Z*)-2-hydroxycinnamonitrile (*E*,*Z*)-**3**, the otherwise difficult obtainable (*Z*)-**3** was prepared in 66% isolated yield. With β,γ-unsaturated (*E*,*Z*)-3-heptenenitrile (*E*,*Z*)-**4**, however, (*E*)-selectivity was not observed. AtNIT1 exhibits not only diastereoselectivity but also regioselectivity. From a mixture of the four isomers **A**–**D** of 3-(2-cyanocyclohex-3-enyl)propenenitrile **6**, exclusively isomer **D** ((*E*)-*cis*-**6**) was hydrolysed to 3-(2-cyanocyclohex-3-enyl)propenoic acid (*E*)-*cis*-**7**, as stated by X-ray crystal structure. Only after complete conversion of **D** and high enzyme concentrations, isomer **C** ((*E*)-*trans*-**6**) was hydrolysed to a small extent.

1. Introduction

In general, α,β-unsaturated nitriles are prepared via Wittig-type reactions or via *Knoevenagel* reaction from cyanoacetates and aldehydes or ketones. Apart from a few exceptions, complete control of the (*E*,*Z*)-selectivity has not been possible. Due to the possibly different biological activities of stereoisomers, methods for the preparation of pure (*E*)- and (*Z*)-isomers are of particular interest, especially for the synthesis of pharmaceuticals. The (*Z*)-isomer of the β-lactamase inhibitor 2β-acrylonitrile penam sulfone Ro 48-1220, for example, is known to be 20 times as active as the corresponding (*E*)-isomer.[2] By addition of a lithium salt the isomeric ratio in the Wittig reaction was improved to 4:1 in favor of the (*Z*)-isomer, but isolation of the pure (*Z*)-isomer requires chromatographic purification steps and crystallization.[2]

Enzymes are applied mainly for the preparation of pure enantiomers by kinetic resolution and by enantioselective synthesis, respectively. On the contrary, only very few examples of enzymatic (*E*,*Z*)-selectivity of α,β-unsaturated carboxylic acid derivatives are published.



(*E*,*Z*)-**1a-1e** (*Z*)-**1a-1e** (*E*)-**2a-2e**

1,2	a	b	c	d	e
R	Me	Ph			OMe

Scheme 1.

* Corresponding author. E-mail: franz.effenberger@po.uni-stuttgart.de

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The only known nitrile-hydrolysing enzyme with (*E*)-selectivity is the nitrilase from *Rhodococcus* ATCC 39484, which is capable of hydrolysing selectively (*E*)-3-furylacrylic nitrile but not the corresponding (*Z*)-isomer.[3] The extremely small substrate range of this enzyme, however, is disadvantageous for synthetic applications. Even closely structurally related compounds such as cinnamonitrile are not accepted as substrates. Further examples of (*E*,*Z*)-selective enzymes include a lipase from *Candida antarctica*, which hydrolyses exclusively diethyl fumarate but not diethyl maleate,[4] and a porcine liver carboxylesterase converting different methyl 3-arylacrylates with poor (*E*,*Z*)-selectivity.[5]

In our investigations on the synthetic applications of the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana* we also used α,β-unsaturated nitriles as substrates for the enzyme-catalyzed hydrolysis. In the present publication we report on the results obtained with these compounds.

2. Results and discussion

2.1. (*E*,*Z*)-Selectivity in the hydrolysis of α,β-unsaturated nitriles 1

The (*E*,*Z*)-selectivity of recombinant AtNIT1 was first detected and investigated in detail on crotononitrile **1a**, which is commercially available only as an (*E*,*Z*)-isomeric mixture (Scheme 1, Table 1). The nitrilase hydrolyses exclusively the minor isomer to crotonic acid **2a**. The formed acid **2a** (as methyl ester) was unambiguously identified as (*E*)-crotonic acid by gas chromatography and coinjection of pure (*E*)-**2a**. Additionally the configuration of the double bond was determined by ^{1}H NMR spectroscopy. (*E*)-**1a** was hydrolysed completely even at high concentrations of 0.25 M, giving (*E*)-**2a** in 27% isolated yield (referred to starting (*E*,*Z*)-**1a** 4:6) and >99% GC purity. The corresponding (*Z*)-**2a** (detection limit <0.5% referred to (*Z*)-**1a**) could not be found neither using higher enzyme concentrations nor by doubling the reaction time. The remaining (*Z*)-**1a** was isolated in isomerically pure form in 57% yield after distillation (determined by GC and NMR).

Since the nitrilase shows complete (*E*)-selectivity already in the case of the small methyl substituent in crotononitrile, cinnamonitrile **1b**, 4-phenyl-2-pentenenitrile **1c**,[6] and 3-methoxypropenenitrile **1e** were applied as substrates (Scheme 1, Table 1).

As can be seen in Table 1, the (*E*)-isomers of nitriles (*E*,*Z*)-**1b**–**1e** were exclusively hydrolysed to the corresponding (*E*)-acids with high specificity. After workup, in all cases only the (*E*)-acids **2b**–**2e** were detected by GC, which were identified unambiguously by coinjection (for **2b**) or by ^{1}H NMR spectroscopy (for **2c**–**2e**). 1-Cyano-1,3-butadiene (*E*,*Z*)-**1d** was prepared by reaction of acetonitrile with acrolein following a literature method.[7] After elimination of the mesyloxy group from intermediate 1-(cyanomethyl)prop-2-enyl methylsulfonate, (*E*,*Z*)-**1d** was obtained in a ratio of *E*:*Z* = 68:32 (see Section 4).

Besides the important (*E*)-selectivity of the AtNIT1-catalyzed hydrolysis of isomeric mixtures of α,β-unsaturated nitriles, in many cases chemical hydrolysis of α,β-unsaturated nitriles is not possible at all. For example, the enol ether (*E*,*Z*)-**1e** and the diene (*E*,*Z*)-**1d**, which decompose under acid or base catalysis, were hydrolysed by the nitrilase to (*E*)-**2d** and (*E*)-**2e** in good yields. (*E*)-3-Methoxyacrylic acid (*E*)-**2e**, for example, is used as the starting material for the preparation of the antibiotic chlorothricin.[8]

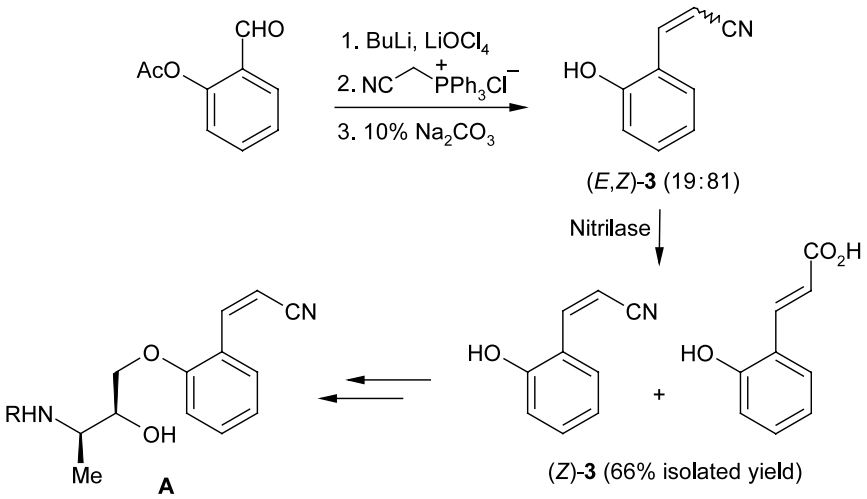
The (*E*)-selectivity in the hydrolysis of structurally very different α,β-unsaturated nitriles with *A. thaliana* nitrilase AtNIT1 can be used advantageously for the preparation of isomerically pure (*Z*)-nitriles, as demonstrated for the preparation of nitriles (*Z*)-**1a** and (*Z*)-**1e**. A further interesting example in connection with this is the preparation of (*Z*)-2-hydroxycinnamonitrile (*Z*)-**3**, a precursor for the β-antagonists 1-(cyanovinylphenoxy)-3-(alkylamino)butan-2-ols **A**[9] (Scheme 2).

(*E*,*Z*)-**3** was prepared by Wittig reaction as outlined in Scheme 2. The *E*:*Z* ratio could be improved in favor of the (*Z*)-isomer to a ratio *E*:*Z* = 19:81 by addition of lithium perchlorate. After enzymatic hydrolysis, the (*E*)-2-hydroxycinnamic acid was separated by extraction with sodium bicarbonate solution, and the nitrile (*Z*)-**3** was isolated in 66% total yield referred to starting (*E*,*Z*)-**3**.

Table 1. (*E*)-Selective hydrolysis of α,β-unsaturated nitriles (*E*,*Z*)-**1** catalyzed by *Arabidopsis thaliana* nitrilase

Substrate		Reaction time (h)	Rel. activity (%)[a]	Products			
1	Ratio *E*:*Z*			(*Z*)-**1**	Yield (%)[b]	(*E*)-**2**	Yield (%)[b]
1a	4:6	3 (24)[c]	37	**1a**	51	**2a**	27
1b	6:4	4	48	**1b**	–	**2b**	–
1c	3:1	3 (21)[c]	22	**1c**	–	**2c**	61
1d	68:32	2 (24)[c]	44	**1d**	–	**2d**	41
1e	37:63	3 (48)[c]	27	**1e**	47	**2e**	30

[a] Rel. activity of the (*E*)-isomer.
[b] Isolated yield referred to total amount of starting (*E*,*Z*)-**1**.
[c] Reaction time of the preparative enzymatic hydrolysis at pH 8.5 and room temperature.



Scheme 2.

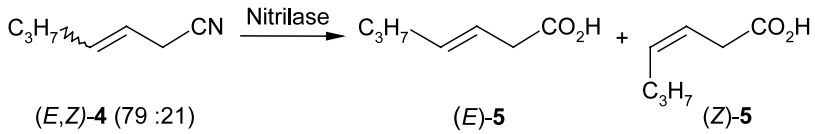
2.2. Investigation of *E*:*Z* selectivity in the hydrolysis of the β,γ-unsaturated nitrile (*E*,*Z*)-4

On the basis of the results described for α,β-unsaturated nitriles, the scope of (*E*)-selectivity of recombinant AtNIT1 was investigated with the β,γ-unsaturated nitrile (*E*,*Z*)-3-heptenenitrile **4**[10] (Scheme 3, Fig. 1).

As can be seen from Fig. 1, the conversion rate of both stereoisomers is very similar. Thus, the enrichment of one isomer was not observed neither for the nitrile nor the acid. Nevertheless, the enzymatic hydrolysis is of synthetic interest, because β,γ-unsaturated nitriles as well as β,γ-unsaturated acids tend to isomerize to the thermodynamically more stable α,β-unsaturated compounds at higher temperature in acidic or basic medium.[11] Chemical hydrolysis of these compounds is therefore accompanied by the formation of by-products. The *A. thaliana* nitrilase hydrolyses (*E*,*Z*)-**4** with high specific activity exclusively to (*E*)- and (*Z*)-3-heptenoic acid **5** in 91% yield with an isomeric ratio of *E*:*Z* = 79:21, identical to that of the starting material (*E*,*Z*)-**4**.

2.3. Regio- and stereoselective hydrolysis of 3-(2-cyanocyclohex-3-enyl)propenenitriles 6

3-(2-Cyanocyclohex-3-enyl)propenenitrile **6**, a cyclic dinitrile with non-equivalent nitrile groups, was prepared by Diels–Alder reaction of 1-cyano-1,3-butadiene



Scheme 3.

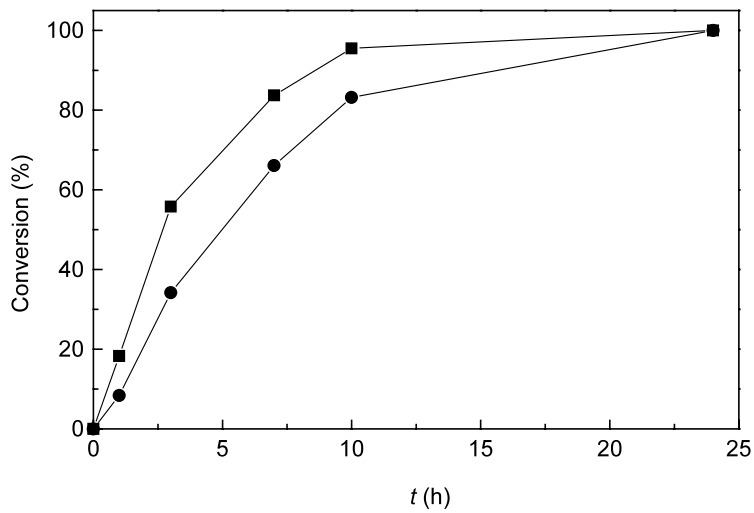
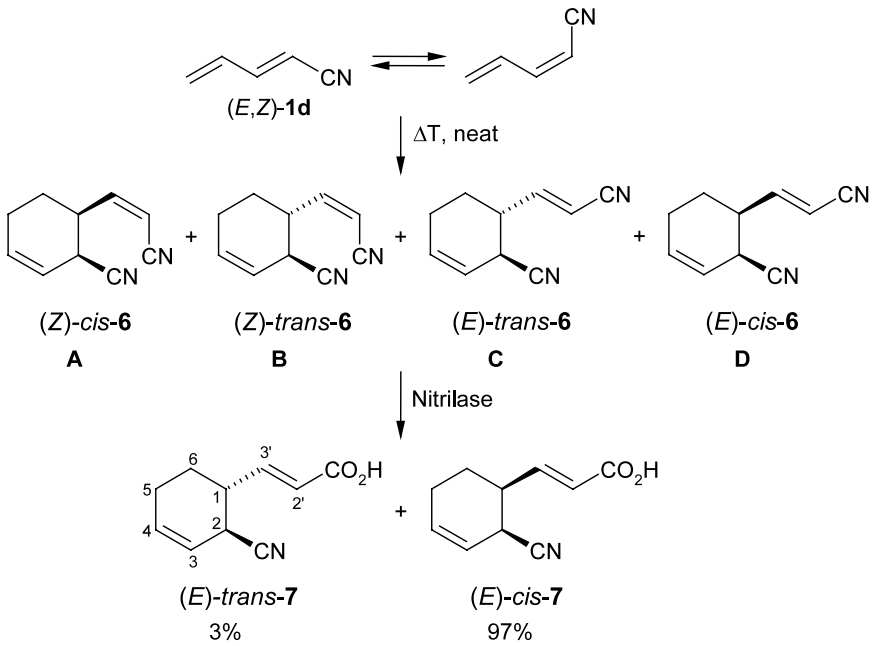


Figure 1. Course of conversion of (*E*,*Z*)-3-heptenenitrile, (*E*,*Z*)-**4** (10 mM) at pH 8.0; (*E*)-isomer (●) and (*Z*)-isomer (■).



Scheme 4.

1d (Scheme 4).[12] After distillative and gas chromatographic separation and enrichment, respectively, four main products could be isolated and identified by ^{1}H NMR as *E*/*Z* (with respect to the exocyclic double bond) and *cis*/*trans* (with respect to the ring substituents) isomers **A**–**D**[12b] (Scheme 4).

We have prepared the analytically pure compounds **A**–**D** starting from (*E*,*Z*)-**1d** (*E*:*Z* = 68:32) by heating at 160°C for 0.5 h in 69% yield with a purity of 95% for the four isomers **A**–**D**, determined by gas chromatography. The ratios **A**:**B**:**C**:**D** were 11:5:15:69. From this isomeric mixture of dinitriles exclusively isomer **D** was hydrolysed by the *A. thaliana* nitrilase. Only after complete conversion of **D** and at higher enzyme concentrations was isomer **C** also hydrolysed to a small extent. The isomers **A** and **B**, however, remained totally unchanged even at high enzyme concentrations and long reaction times. The hydrolysis of both **C** and **D** occurs exclusively on the alkylidene nitrile group yielding the corresponding (*E*)-3-(2-cyanocyclohex-3-enyl)-propenoic acids **7**, as confirmed by NMR spectroscopy. For the (*E*)-cyanocyclohexenylpropenoic acid (*E*)-*cis*-**7** derived from isomer **D** the *cis*-configuration of the ring substituents could unambiguously be assigned by X-ray crystal structure (Fig. 2).[13] Thus, isomer **D** has (*E*)-*cis*-configuration ((*E*)-*cis*-**6**), while isomer **C** is assumed to be (*E*)-*trans*-configurated [(*E*)-*trans*-**6**].

The different reaction rates for the enzyme-catalyzed hydrolysis of (*E*)-*trans*-**6** and (*E*)-*cis*-**6** can be used to separate these isomers. As shown in Scheme 4, the enzymatic hydrolysis gave 97% (*E*)-*cis*-**7** and 3% (*E*)-*trans*-**7** at 95% conversion referred to isomer **D**. After recrystallization from petroleum ether/chloroform, (*E*)-*cis*-**7** was isolated in 84% yield (referred to **D** in the starting isomeric mixture).

3. Conclusion

In summary, the extremely high selectivity in hydrolysing only the dinitrile **D** from the mixture of the four dinitriles **A**–**D** is due firstly to the high regioselectivity of the *A. thaliana* nitrilase, preferring nitrile groups unbranched in the α-position,[1] secondly, due to complete (*E*)/(*Z*) selectivity, hydrolysing exclusively the isomer with the (*E*)-configurated exocyclic double bond and thirdly, due to the very high *cis*/*trans* selectivity (diastereoselectivity), favoring the *cis*-1,2-disubstituted cyclohex-3-ene.

4. Experimental

4.1. Materials and methods

Melting points were determined on a Büchi SMP-20 and are uncorrected. Unless otherwise stated, ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 F

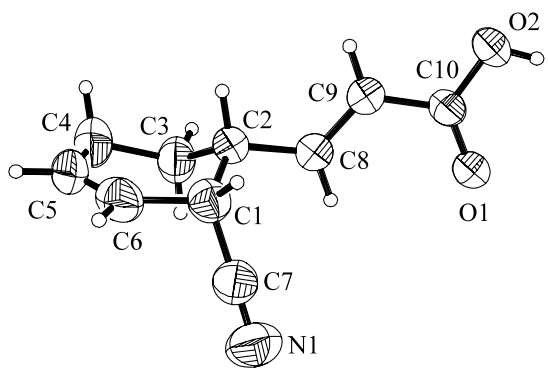


Figure 2. ORTEP view of (*E*)-*cis*-3-(2-cyanohex-3-enyl)-propenoic acid **7** derived from isomer **D**.

(250 MHz) and ARX 500 (500 MHz) in $CDCl_3$ with TMS as internal standard. Chromatography was performed using silica gel S (Riedel-de Haen), grain size 0.032–0.063 mm. Gas chromatography separations were conducted using capillary glass columns (20 m× 0.32 mm) with OV 1701, carrier gas hydrogen. All solvents were dried and distilled. The recombinant AtNIT1 was obtained by overexpression in *E. coli* as described elsewhere.[1]

4.2. 1-Cyano-1,3-butadiene (*E*,*Z*)-1d

To a solution of BuLi in hexane (1.6 M, 131 mL, 210 mmol) and dry THF (160 mL) at −78°C under an inert gas atmosphere was slowly added a solution of acetonitrile (8.21 g, 200 mmol) in THF (40 mL). After stirring for 15 min, acrolein (13.45 g, 240 mmol) was added over 5 min. The reaction mixture was warmed to −20°C and methanesulfonyl chloride (27.49 g, 240 mmol) was added over 5 min. After stirring for a further 1 h at −20°C and 6 h at room temperature, the mixture was poured into ice (250 mL)/satd NH_4Cl solution (50 mL). The layers were separated and the aqueous layer was extracted with diethyl ether (2×100 mL). The combined organic layers were washed with water until neutral, dried (Na_2SO_4) and concentrated to give crude 1-(cyanomethyl)prop-2-enyl methylsulfonate (39.13 g). To a solution of crude intermediate (10 g, 57.1 mmol) in THF (100 mL) was added dry sodium acetate (6.28 g, 76.5 mmol). After stirring at 40°C for 6 h, the mixture was poured on ice (50 mL) and the aqueous layer was extracted with diethyl ether (2×50 mL). The combined organic layers were washed with water until neutral, dried (Na_2SO_4) and concentrated. Distillation through a Vigreux column under vacuum gave 2.40 g (53%) of (*E*,*Z*)-**1d**, *E*:*Z* = 68:32, bp 43°C/20 mbar. (*E*)-Isomer: ^{13}C NMR (63 MHz): δ 98.24 (CH), 116.01 (CN), 127.00, 132.74, 149.53 (CH, CH_2=). (*Z*)-Isomer: ^{13}C NMR (63 MHz): δ 99.90 (CH), 117.75 (CN), 126.77, 134.14, 150.62 (CH, CH_2=). Anal. calcd for C_5H_5N (79.1): C, 75.92; H, 6.37; N, 17.71. Found: C, 75.95; H, 6.43; N, 17.59%.

4.3. Enzymatic hydrolysis of nitriles (*E*,*Z*)-1 on an analytical scale; general procedure

A crude enzyme solution, prepared as previously described,[1,14] was diluted with Tris/HCl buffer, pH 8.5 (to reach 15–40% conversion after 2–4 h), and a solution of the respective nitrile in methanol (10 mM of the (*E*)-isomer for **1a**, **1d** and **1e**; 1.25 mM of the (*E*)-isomer for **1b** and **1c**) was added (the concentration of the stock solution must be as high as the added volume should not exceed 100 μL/5 mL reaction solution). After stirring for the time given in Table 1, samples of 1 mL volume were taken and analyzed by gas chromatography (see Section 4.4).

4.4. Determination of conversion by gas chromatography

The sample taken was acidified with a 5 M HCl solution (50 μL) and extracted with diethyl ether (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 diethyl ether[15] was added. The excess diazomethane and diethyl ether was distilled off under vacuum. The residue was taken up in diethyl ether (1 mL), and the conversion was determined from the ratio area of methyl ester to area of nitrile.

4.5. Enzymatic hydrolysis of nitriles (*E*,*Z*)-1 on a preparative scale; general procedure

To a crude enzyme solution was added the respective neat nitrile **1a**, **1c**–**1e** (Table 2), and the reaction mixture was stirred for the time given in Table 1. Then the mixture was acidified with 5 M HCl to pH 4.0 and extracted with diethyl ether. The combined extracts were washed twice with 10% (v/v) satd $NaHCO_3$ solution, dried (Na_2SO_4) and concentrated. The combined aqueous extracts were washed with a double volume of diethyl ether, acidified to pH 4.0 and again extracted with diethyl ether. The combined organic extracts were dried (Na_2SO_4) and concentrated. The crude products were purified as described.

4.5.1. (*E*)-4-Phenyl-2-pentenoic acid (*E*)-2c. Recrystallization from benzene/chloroform, mp 171–172°C. ^1H NMR (250 MHz): δ 1.44 (d, J = 7.0 Hz, 3H, CH_3), 3.64 (m, 1H, 4-CH), 5.81 (q, J_1 = 15.6, J_2 = 1.6 Hz, 1H, 2-CH), 7.18–7.42 (m, 6H, Ph, 3-CH). ^{13}C NMR (63 MHz): δ 20.09 (CH_3), 42.15 (C4), 119.39 (C2), 126.87, 127.35, 128.76, 142.92 (Ph), 155.46 (C3), 172.03 (CO_2H). Anal. calcd for $C_{11}H_{12}O_2$ (176.2.): C, 74.98; H, 6.86. Found: C, 75.20; H, 6.86%.

4.5.2. (*E*)-Penta-2,4-dienoic acid (*E*)-2d. Recrystallization from petroleum ether, mp 34–37°C. ^1H NMR (500 MHz): δ 5.56 (d, J = 11.2 Hz, 1H, CH_2=), 5.67 (d, J = 16.8 Hz, 1H, CH_2=), 5.91 (d, J = 15.4 Hz, 1H,

Table 2. Preparative enzymatic hydrolysis of nitriles (*E*,*Z*)-**1** at 100% conversion of the (*E*)-isomer

Substrate		Nitrilase (Units)	Buffer (mL)	Products			
1	g (mmol)			(*Z*)-**1**	(g)	(*E*)-**2**	(g)
1a	5.0 (74.5)	23.0	500	(*Z*)-**1a**	2.58	(*E*)-**2a**	1.76
1c	0.5 (3.18)	41.2	200	(*Z*)-**1c**	–	(*E*)-**2c**	0.34
1d	1.0 (12.6)	10.3	100	(*Z*)-**1d**	–	(*E*)-**2d**	0.51
1e	5.0 (60.2)	23.0	300	(*Z*)-**1e**	2.36	(*E*)-**2e**	1.86

2-CH), 6.49 (m, 1H, 4-CH), 7.36 (q, $J=11.3$ Hz, 1H, 3-CH), 11.30 (br s, 1H, CO_2H). ^{13}C NMR (126 MHz): δ 121.35, 126.84, 134.53, 147.05 (C2,3,4,5), 172.43 (CO_2H). MS (EI, 70 eV) m/z (%): 220.1 (32), 205.2 (80), 98.1 (100) $[M]^+$, 71.1 (70), 57.0 (99). HRMS (EI, 70 eV) calcd for $C_5H_6O_2$ (M^+) 98.03666. Found: 98.03678.

4.5.3. (*E*)-3-Methoxypropenoic acid (*E*)-2e. Recrystallization from petroleum ether/chloroform, mp 64°C. ^1H NMR (250 MHz): δ 3.73 (s, 3H, CH_3O), 5.19 (d, $J=12.6$ Hz, 1H, 2-CH), 7.72 (d, 1H, 3-CH), 11.50 (br s, 1H, CO_2H). ^{13}C NMR (63 MHz): δ 57.56 (CH_3O), 95.40 (C2), 165.17 (C3), 173.72 (CO_2H). Anal. calcd for $C_4H_6O_3$ (102.1): C, 47.06; H, 5.92. Found: C, 47.10; H, 5.94%.

4.5.4. (*Z*)-3-Methoxypropenenitrile (*Z*)-1e. Distillation through a Vigreux column under vacuum, bp 71°C/17 mbar. ^1H NMR (250 MHz): δ 3.92 (s, 3H, CH_3O), 4.40 (d, $J=6.4$ Hz, 1H, 2-CH), 6.73 (d, 1H, 3-CH). ^{13}C NMR (63 MHz): δ 61.79 (CH_3O), 74.35 (C2), 115.34 (CN), 164.31 (C3). Anal. calcd for C_4H_5NO (83.1): C, 57.82; H, 6.07; N, 16.86. Found: C, 57.64; H, 6.10; N, 16.62%.

4.6. (*Z*)-3-(2-Hydroxyphenyl)propenenitrile (*Z*)-3

(a) To a stirred suspension of cyanomethyltriphenylphosphonium chloride (4.52 g, 13.4 mmol) and lithium perchlorate (2.34 g, 22 mmol) in dry THF (50 mL) at −78°C under an inert gas atmosphere was added a 1.6 M solution of BuLi in hexane (8.37 mL, 13.4 mmol). After stirring for 1 h, a solution of 2-acetoxybenzaldehyde (2 g, 12.2 mmol) in dry THF (5 mL) was added. The mixture was stirred for a further 1 h at −78°C, and was then allowed to warm to room temperature within 6 h. The mixture was poured on ice (50 mL) and the layers were separated. The aqueous layer was extracted with diethyl ether (2×100 mL). The combined organic layers were washed with water until neutral, dried (Na_2SO_4) and concentrated. The residue was chromatographed on silica gel with petroleum ether/ethyl acetate (80:20) to separate triphenylphosphine oxide. To a solution of satd Na_2CO_3 (50 mL), water (350 mL) and methanol (50 mL) was added a solution of the remaining intermediate in methanol (50 mL). After stirring for 1.5 h at room temperature, the mixture was extracted with diethyl ether (3×150 mL). The combined extracts were washed with water (3×50 mL), dried (Na_2SO_4) and concentrated to give crude (*E*,*Z*)-**3** in an *E*:*Z* ratio of 19:81.

(b) To a solution of enzyme (123 U) in Tris/HCl buffer (300 mL, 70 mM, pH 8.5) was added crude (*E*,*Z*)-**3**. After complete conversion of (*E*)-**3** (28 h), the mixture was worked up as described (see Section 4.4) to give 1.17 g (66%) of (*Z*)-**3**, mp 63°C. ^1H NMR (250 MHz): δ 6.09 (d, $J=11.3$ Hz, 1H, 2-CH), 6.77–7.28 (m, 5H, Ph), 7.55 (d, 1H, 3-CH). ^{13}C NMR (63 MHz): δ 96.86 (C2), 116.89 (CN), 119.54, 121.27, 126.34, 129.86, 132.67 (Ph), 147.75 (C3), 155.89 (Ph). Anal. calcd for C_9H_7NO (145.2): C, 74.47; H, 4.86; N, 9.65. Found: C, 74.20; H, 4.88; N, 9.69%.

4.7. Enzymatic hydrolysis of 3-(2-cyanocyclohex-3-enyl)propenenitrile 6

4.7.1. (*E*)-*cis*-3-(2-Cyanocyclohex-3-enyl)propenoic acid (*E*)-*cis*-7. An isomeric mixture of (*E*,*Z*)-**6**[12] (1 g, 6.32 mmol) was hydrolysed as described above with nitrilase (34.3 U) in Tris/HCl buffer (250 mL, 70 mM, pH 8.5). After 24 h at 95% conversion, the reaction was terminated. Purification by recrystallization from petroleum ether/chloroform gave 0.65 g (84%, referred to **D** in starting (*E*,*Z*)-**6**) of (*E*)-*cis*-**7**, mp 81–82°C. ^1H NMR (500 MHz): δ 1.81–1.90 (m, 2H, 6-CH_2), 2.14–2.30 (m, 2H, 5-CH_2), 2.67–2.72 (m, 1H, 1-CH), 3.35–3.39 (m, 1H, 2-CH), 5.67–5.73 (m, 1H, 3-CH), 5.95–6.06 (m, 2H, 4-,2′-CH), 7.14 (dd, $J_1=15.7$, $J_2=7.8$ Hz, 1H, 3′-CH). ^{13}C NMR (126 MHz): δ 24.07, 24.14 (C5,6), 31.30 (C2), 38.10 (C1), 118.32 (CN), 119.94, 122.62 (C3,4), 131.95 (C2′), 149.56 (C3′), 170.63 (CO_2H). Anal. calcd for $C_{10}H_{11}NO_2$ (177.2): C, 67.78; H, 6.26; N, 7.90. Found: C, 67.82; H, 6.31; N, 8.06%.

4.7.2. (*E*)-*trans*-3-(2-Cyanocyclohex-3-enyl)propenoic acid (*E*)-*trans*-7. The reisolated isomeric mixture of (*E*,*Z*)-**6** (see above) was hydrolysed with nitrilase (3.4 U) in Tris/HCl buffer (70 mM, pH 8.5). After 6 h at complete conversion of (*E*)-*cis*-**6**, the reaction was terminated. Unreacted nitrile **6** was re-isolated and again hydrolysed with nitrilase (34.3 U) in Tris/HCl buffer (70 mM, pH 8.5). After 48 h at 63% conversion of (*E*)-*trans*-**6**, the reaction was terminated. Purification by recrystallization from petroleum ether/chloroform gave (*E*)-*trans*-**7**, mp 75–77°C. ^1H NMR (500 MHz): δ 1.54–2.03 (m, 2H, 6-CH_2), 2.16–2.21 (m, 2H, 5-CH_2), 2.72–2.78 (m, 1H, 1-CH), 1.36–3.20 (m, 1H, 2-CH), 5.64–5.67 (m, 1H, 3-CH), 5.97–6.07 (m, 2H, 4-,2′-CH), 7.01 (dd, $J_1=15.7$, $J_2=7.8$ Hz, 1H, 3′-CH).

4.8. Crystallography

The isomer (*E*)-*cis*-**7** was recrystallized from petroleum ether/chloroform to obtain single crystals for X-ray analysis. Intensity data were collected on a Nicolet P3 diffractometer with ω-scan technique (graphite-monochromated Mo Kα radiation, $\lambda=0.71073$ Å) at 293 K. The structure was solved by direct methods and refined[16] against F^2. Crystal data: formula, $C_{10}H_{11}NO_2$ (177.2); crystal size, 0.9×0.25×0.25 mm; $F(000)=376$; crystal system, monoclinic, space group, $P2_{(1)}/n$; $Z=4$; $a=5.9713(10)$, $b=15.143(2)$, $c=10.6172(14)$ Å; $\beta=93.388(12)°$; $V=958.4(3)$ Å^3; $D_{calcd}=1.228$ g/cm^3; number of reflections = 1849; number of independent reflections (with 2θ in the range of 2.4–25.0°) = 1682; number of reflections having $I>2\sigma(I)=1509$; GooF = 1.161; $R_1=0.0545$; w$R_2=0.116$.

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