Regioselective biotransformations of dinitriles using *Rhodococcus* sp. AJ270[1]

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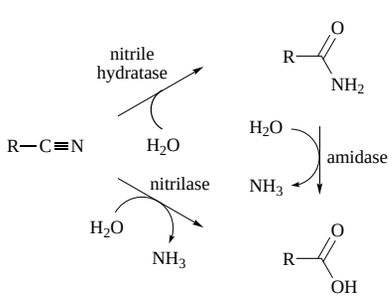
A variety of dinitriles have been hydrolysed selectively under very mild conditions using *Rhodococcus* sp. AJ270. Aliphatic dinitriles $NC[CH_2]_nCN$ 1 undergo regioselective hydrolysis to give the mono acids 2 with up to 4 methylenes between the nitrile functions while those with $n > 4$ give the diacids 3 in good yield. Dinitriles $NC[CH_2]_nX[CH_2]_nCN$ 4 bearing an ether or sulfide linkage are efficiently transformed into the mono acids 5 when an oxygen is placed β, γ or δ to the cyano group or a β- or γ-sulfur is present. Hydrolysis of *N*,*N*-bis(2-cyanoethyl)anilines 4h–j takes place slowly affording exclusively the monoacids 5h–j while the monocyano amides 5o–p are obtained as the sole isolable product from rapid hydrolysis of the corresponding *N*,*N*-bis(2-cyanomethyl)butylamine 4o and *N*,*N*-bis(3-cyanopropyl)butylamine 4p. Higher homologues of arylimino- and butylimino-dinitriles are inert to enzymatic hydrolysis. A variety of other aliphatic dinitriles have been converted readily into mono acids in good to excellent yields except for *o*-phenylenediacetonitrile which gives *o*-phenylenediacetamide as the major product. The title organism also effects the hydrolysis of aromatic dinitriles with regiocontrol such as *m*- and *p*-dicyanobenzenes, but not the *ortho*-substituted analogue. The scope and limitations of this enzymatic process have been systematically studied and the mechanism of regioselective hydrolysis has been discussed in terms of a chelation–deactivation effect.

Introduction

Biotransformations in organic synthesis using micro-organisms and isolated enzymes have received tremendous attention during last two decades.[2] Since they are performed under very mild conditions such as at ambient temperature and in aqueous media at pH *ca*. 7, biotransformations are regarded as environmentally benign processes.[3] More importantly, most enzyme-catalysed reactions proceed in a chemo-, regio- and stereo-selective manner. Therefore, they provide powerful and efficient synthetic routes to compounds which are sometimes not easily accessible by 'conventional' chemical means. For example, lipases, esterases and acylases have been widely used for the kinetic resolution of racemic esters and for the preparation of homochiral molecules from prochiral and *meso* diesters.[2,4] In contrast to these extensively studied hydrolytic enzymes, nitrile-hydrolysing enzymes in synthesis have remained largely unexplored until recently.[5] In fact, bioconversion of nitriles into the corresponding carboxylic acids has been known for decades.[6] Enzyme-catalysed hydrolyses of nitriles have been shown to proceed by two distinct routes: a nitrilase[7] transforms the nitriles directly into acids while a nitrile hydratase produces amides which are subsequently hydrolysed to the acids by an amidase.[8] (Scheme 1). So far a range of



Scheme 1

nitrile-hydrolysing micro-organisms has been isolated[9] and some nitrilases,[7a,10] nitrile hydratases[11] and amidases[12] have been purified. The mechanisms of enzymatic hydrolysis of nitriles using nitrilase[10a,13] and nitrile hydratase[11a,g] systems have been proposed and for the latter the hydration stage is believed to involve complexion of the nitrile nitrogen to a transition metal (iron or cobalt) followed by hydration mediated by a possible prosthetic group such as a pyrroloquinoline quinone.[14]

In our previous studies[9] it has been demonstrated that *Rhodococcus* sp. AJ270 is a powerful and robust nitrile hydratase/amidase-containing organism. It has very broad activity against all types of nitriles including aromatic, heterocyclic and aliphatic ones. To further explore its potential in organic synthesis, and also as a prelude to investigating the hydrolysis of prochiral dinitriles we have made a systematic study of the effect of structure on the regioselectivity of hydrolysis of dinitriles.

In 1980 Yamada *et al*. observed that biodegradation of glutaronitrile by *Pseudomonas* sp. K-9 proceeded through 4-cyanobutyramide, 4-cyanobutyric acid and glutaric acid,[15] but stopped at the 4-cyanobutyric acid stage using the fungus *Fusarium merismoides* TG-1.[16] Recently it has been reported that a series of dinitriles, $NC(CH_2)_nCN$ [n = 3-5], were hydrolysed to the diacids with no selectivity using *Rhodococcus rhodochrous* NCIB 11216[17] while *Rhodococcus rhodochrous* K-22 catalysed selective monohydrolysis of glutaronitrile in quantitative yield,[18] though both organisms are nitrilase-containing strains. Using an immobilised whole cell *Rhodococcus* SP361, a nitrile hydratase system developed by Novo Industri of Denmark, Hönicke-Schmidt and Schneider[19] observed exclusive formation of 4-cyanobutyric acid from glutaronitrile. Turner *et al*. showed that succinonitrile gave solely 3-cyanopropionic acid while glutaronitrile gave mono- and di-acids, the ratios of which depended upon the reaction.[20] Biotransformation of adiponitrile into adipic acid has been closely studied by a French group utilising the wild type and mutants of *Brevibacterium* sp. R312[21] while efficient monohydrolysis of cyclohexane-1,4-dicarbonitrile has been effected with organisms such as *Acremonium* sp. D9K[22] and *Corynebacterium* sp. CH.[23] Two groups studied hydrolysis of prochiral dinitriles. Ohta[24] and Turner[25] and their co-workers have shown that some 3-hydroxyglutaronitrile derivatives, when treated with *Rhodococcus rhodochrous* ATCC 21197 and *Rhodococcus* SP361, respectively, were converted into the monocyano acids

Table 1 Conversion of $NC[CH_2]_nCN$ **1** into $NC[CH_2]_nCO_2H$ **2** and/or $HO_2C[CH_2]_nCO_2H$ **3**

Substrate		Reaction conditions[a]		Product yield[b] (%)	
1	*n*	Amount (mmol)	Time (h)	**2**	**3**
1a	2	5	3	30	—
	2	5	24	17	—
1b	3	5	3	41	—
	3	5	16	35	—
1c	4	5	3	41	4
	4	5	24	28	23
	4	5	48	—	26
1d	5	3	3	—	46
	5	3	48	—	74
1e	6	3	48	—	78
1f	7	3	48	—	88
1g	8	3	48	—	89

[a] Reaction conditions were not optimised. [b] Isolated yield.

4o, *n* = 2; **4p,** *n* = 3
4q, *n* = 4; **4r,** *n* = 5

5o, *n* = 2, 17%
5p, *n* = 3, 31.5%

Figure 1

in an enantioselective manner. Interestingly, *R. rhodochrous* ATCC 21197 hydrolysed a prochiral malononitrile to afford its monoacid monoamide derivative with high enantiomeric excess.[26] Some aromatic and unsaturated dinitriles were hydrolysed regioselectively with both nitrilase[17,27] and nitrile hydratase[5] systems except for *o*-phthalonitrile which gave different products with different organisms.[20,28]

Results and discussion

The purpose of our research was to define the extent of regioselective control in the hydrolysis of a wide variety of dinitriles and to try to understand the mechanism of this control. It is probable that all nitrile hydratase/amidase systems differ more in a quantitative than a qualitative manner, and that our findings should be of wider generality. We first systematically examined the regioselectivity of hydrolysis of a series of aliphatic α, ω-dinitriles **1**, monitoring the reaction against time (Table 1). Unless otherwise specified, all hydrolyses gave acids as products, the amides being occasionally seen by TLC but not isolated since they were rapidly transformed into acids. Both succinonitrile **1a** and glutaronitrile **1b** were hydrolysed efficiently to give solely 3-cyanopropionic acid **2a** and 4-cyanobutyric acid **2b**, respectively. Only the diacids **3d**–**g** were obtained from pimelo-, subero-, azelao- and sebaco-nitriles **1d**–**g** while adiponitrile **1c** was selectively converted into 5-cyanovaleric acid **2c** or adipic acid **3c** depending upon time of reaction. It was quite clear that dinitriles with more than four methylenes separating the functions gave the diacid as the sole product irrespective of reaction time. With less than four methylenes, however, regioselective monohydrolysis occurred. It should be pointed out that, as we noted in earlier work,[9] low molecular weight nitriles tend to be metabolised by other enzymes in the organism and therefore overall chemical yields were limited and reduced with increasing time of interaction.

Next examined were a range of α, ω-dinitriles $NC[CH_2]_nX[CH_2]_nCN$ **4** containing a heteroatom X in the chain (Table 2). Significant regioselectivity was observed. With the ether series **4a**–**d**, hydrolysis was rapid and generally efficient. Regiocontrol was optimally efficient when an oxygen atom was placed δ to the cyano group in the chain. Hydrolysis was also effected selectively with dinitriles **4a**–**b** bearing a β- or γ-oxygen, while regioselectively was lost entirely when bis(5-cyanopentyl) ether **4d** was used. The sulfide substrates **4e**–**g** underwent similar rapid hydrolysis; however, a γ-sulfur **4f** allowed optimal regiocontrol, while the β-analogue **4e** showed selectivity but not the δ-analogue **4g**. 3,3′-Imino- and 3,3′-methylimino-dipropionitriles proved to be unstable under the conditions used, even in the absence of the organism. We therefore included the *N*-aryl **4h**–**n** and *N*-butyl **4o**–**r** analogues in the study. Probably because of steric factors, all reactions utilising the *N*-aryl derivatives proceeded slowly to give the monoacids **5h**–**j** and with $n > 2$, not at all, even with added co-solvents such as acetone and methanol. Because of their limited reactivity, small steric changes remote from the nitrile group resulted in a dramatic difference in hydrolysis rate; the *para*-substituted substrate **4i** was hydrolysed after one week compared to 64 h of complete conversion for the parent aniline **4h**. Steric limitations of the nitrile hydratase were further exemplified with the substrate *N*,*N*-bis(cyanoalkyl)butylamines **4o**–**r** (Fig. 1). The longer homologues bearing four and five methylenes such as **4q** and **4r** remained intact and were recovered after a week of interaction with *Rhodococcus* sp. AJ270. In contrast, the butylamine analogue with two methylenes **4o** was hydrolysed rapidly, being converted within 1 h, while that with three methylenes, **4p**, completely reacted within 8 h, low yields of monocyano amides **5o** and **5p** being the sole product; this suggested that the amidase as well as the nitrile hydratase enzyme suffers similar steric limitations in the amine series. Longer incubation of **4o** and **4p** led to the total loss of products, indicating that the organism rapidly metabolises the derived amino amides or, most likely, amino acids utilising other enzymes. At this stage, we cannot yet define the limits of regiocontrol with chain length in the nitrogen series but good selectivity was shown with a β- or a γ-nitrogen. Nevertheless, regioselectivity in the biotransformation of simple aliphatic dinitriles with *Rhodococcus* sp. AJ270 is principally dependent upon the placement of the heteroatom rather than the overall chain length.

Scheme 2

The regioselectivity of the hydrolysis of aliphatic dinitriles was interpreted on the basis of chelation–deactivation of the enzyme (Scheme 2). It is believed that hydrolysis occurs by complexation of the nitrile nitrogen to a metal (iron or cobalt) followed by hydration of the nitrile function by the presumed co-factor present in such nitrile hydratase, pyrroloquinoline quinone, as documented elsewhere.[14] When a suitably placed ligand atom is also present in the nitrile, bidentate complexation to the metal occurs, which interferes with this hydration process. This ligand may be a suitably positioned CO_2^- group generated from a CN function, or a heteroatom. 4-Cyanobutyric acid **2b**, the hydrolysis product of glutaronitrile **1b** ($n = 3$), bears a δ-oxygen ligand as does the ether **5c** obtained from **4c** ($n = 4$, X = O). The C–S bond is considerably longer than the C–O bond (1.81 and 1.43 Å respectively) and, not surprisingly, therefore a γ placement of sulfur lends optimal regioselectively. Clearly, if this mechanism is correct, constraints in the chain between the nitrile functions such as multiple bonds or trigonal groups will change the preferred regioselective effect of heteroatoms.

If this mechanism is correct the above-mentioned ligands, *i.e.* monocyano acids derived from dinitriles should behave as competitive inhibitors in the hydrolysis of other easily hydrolysed nitriles. To clarify this issue, hydrolysis of benzonitrile was studied in the presence and absence of 4-(3-cyanopropylthio)butyric acid **5f**. Using HPLC we were able to record the

Table 2 Conversion of $NC[CH_2]_nX[CH_2]_nCN$ **4** into $NC[CH_2]_nX[CH_2]_nCO_2H$ **5** and/or $HO_2C[CH_2]_nX[CH_2]_nCO_2H$ **6**

Substrate			Reaction conditions[a]		Product yield[b] (%)		
4	*n*	X	Amount (mmol)	Time (h)	**5**	**6**	**4**
4a	2	O	3	48	61	—	—
4b	3	O	5	1	35	Trace	35
	3	O	3	72	—	70	—
4c	4	O	3	2	73	—	—
	4	O	3	120	75	5	—
4d	5	O	3	2	—	25	60
	5	O	3	48	—	97.5	—
4e	2	S	5	1	45	—	10
	2	S	3	48	—	60	—
4f	3	S	3	2	52	—	29
	3	S	3	96	83	—	—
4g	4	S	3	2	—	83	Trace
	4	S	3	96	—	86	—
4h	2	NPh	2	64	93	—	—
4i	2	NC_6H_4Cl-*p*	2	168	71	—	26
4j	2	NC_6H_4OMe-*p*	2	72	91	—	—
4k	3	NPh	1.5	168	—	—	95
4l	3	NC_6H_4OMe-*p*	1.5	168	—	—	92
4m	4	NPh	1.5	168	—	—	94
4n	5	NPh	1.5	168	—	—	99

[a] Reaction conditions were not optimised. [b] Isolated yield.

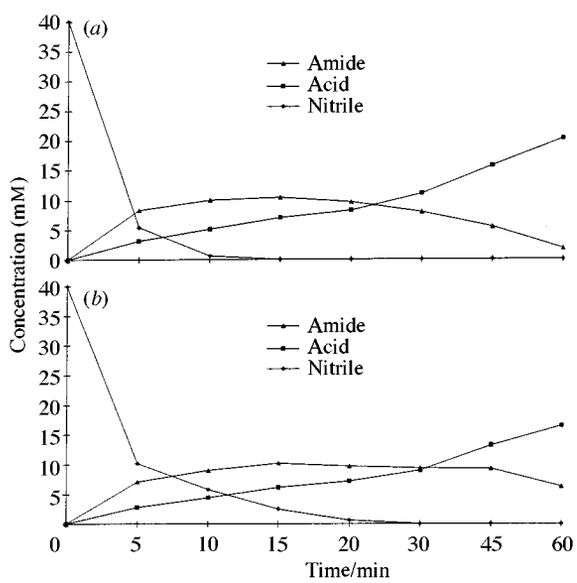
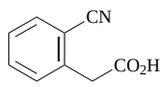
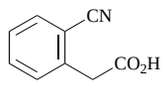
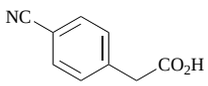
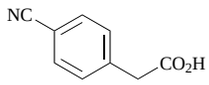
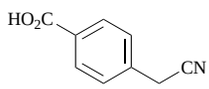
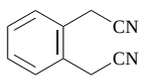
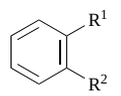
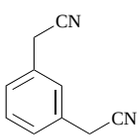
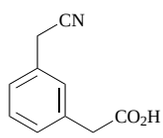
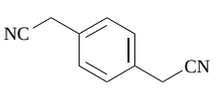
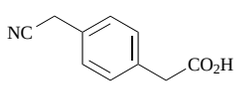
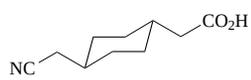
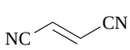
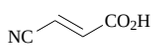
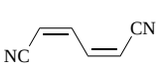
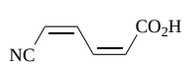


Fig. 2 Hydrolysis of benzonitrile in the absence (*a*) and presence (*b*) of inhibitor 4-(3-cyanopropylthio)butyric acid **5f**

course of the hydrolysis over time. As illustrated in Fig. 2, **5f** indeed significantly slowed the hydrolysis of benzonitrile; thus after reaction for 5 min, about 12% of unchanged benzonitrile remained with no added cyano acid, while in the presence of the inhibitor almost 25% remained. After 10 min the figures were *ca*. 1 and 15%. Furthermore, the rate of decrease of benzamide and the rate of formation of benzoic acid were both significantly slower in the presence of **5f**. *Rhodococcus* sp. AJ270 also effectively catalysed the hydrolysis of a variety of miscellaneous aliphatic dinitriles (Table 3). Excellent regioselectivity was evident in almost all cases that we have examined. *o*-Cyanophenylacetonitrile **7a** was transformed into *o*-cyanophenylacetic acid in good yield while *p*-cyanophenylacetonitrile gave a mixture of primarily *p*-cyanophenylacetic acid **8b** and *p*-cyanomethylbenzoic acid **8c** (4 : 1 ratio) in a combined yield of 81%. These results suggest a preference of *Rhodococcus* sp. AJ270 for the hydrolysis of aliphatic nitriles over aromatic ones. The preferential attack on the aliphatic cyano group is most probably attributable to the lower steric demand of the aliphatic substrates. For example, the aliphatic cyano group in *p*-cyanophenylacetonitrile **8a** is more accessible than the aromatic one. This accessibility is even more true for the *o*-cyanophenylacetonitrile **7a** where the aliphatic nitrile moiety is hydrolysed while aromatic nitrile remains intact, since an *ortho* substituent poses further steric hindrance. *m*-Phenylenediacetonitrile **10a** was hydrolysed to give the monocyano acid **10b** as sole product, as was the *para*-analogue **11**. Interestingly, however, hydrolysis of *o*-phenylenediacetonitrile **9a** afforded *o*-phenylenediacetamide **9c** as the major product, with the monocyano acid **9b** being formed only in 11% yield. This is the first example of the diamide formation from enzymatic dinitrile hydrolysis. The reason for the accumulation of diamide **9c** is not clear at this stage though one may suggest that either hydration of the second group is faster and non-selective or the amidase is less effective towards the monocyano amide intermediate and *o*-phenylenediacetamide **9c**, or both. It is worth noting that *trans*-cyclohexane-1,4-diyldiacetonitrile **12a** was also converted into the corresponding monocyano acid **12b** in almost quantitative yield although the two cyano groups are separated by six carbons. Compared with the outcome of the hydrolysis of suberonitrile **1e**, it is apparent that the conformation of the cyclohexane moiety plays an important role in determining regiospecificity. *Rhodococcus* sp. AJ270 was able to hydrolyse unsaturated dinitriles such as fumaronitrile **13a** and *cis*,*cis*-mocononitrile **14a** regioselectively in a manner similar to those hydrolyses utilising other organisms.[17b,19]

Finally, the aromatic dinitriles **15–17** were subjected to the hydrolysis (Table 4). The same reaction pattern as those reported in the literature[20,28] was found for *meta*- and *para*-phthalonitriles, *i.e.*, high chemical conversion with total regiocontrol to give monocyano acids **16b** and **17b**, respectively. However, hydrolysis of *o*-phthalonitrile **15a** proceeded sluggishly, with more than half the starting material being recovered after 70 h interaction with *Rhodococcus* sp. AJ270, the products being *o*-cyanobenzoic acid **15b** (12%) and phthalimide **15c** (12%). It has been shown previously that the same substrate gave diacid or *o*-cyanobenzamide and phthalimide with *Rhodococcus* SP361[20] or *Rhodococcus equi* A4,[28] respectively. Reaction probably involves the hydration of one cyano group with a nitrile hydratase forming *o*-cyanobenzamide followed either by hydrolysis with amidase yielding *o*-cyanobenzoic acid **15b** or a non-enzymatic ring-closure affording phthalimide **15c** during the time of reaction and/or work-up. As we have noted earlier all *ortho* or adjacently substituted aromatic and alkenic nitriles undergo a slow amidase mediated hydrolysis, allowing isolation of the amide.[9] It is tempting to

Table 3 Conversion of miscellaneous dinitriles into acids

Substrate		Conditions[a] mmol	Time (h)	Product(s) and yields[b] (%)		
7a		2	24	**7b**		80
8a		2	24	**8b**		81[c] **8b/8c** = 4/1[d]
				8c		
9a		3	24			
				9a	$R^1 = R^2 = CH_2CN$	13
				9b	$R^1 = CH_2CN$, $R^2 = CH_2CONH_2$	11
				9c	$R^1 = R^2 = CH_2CONH_2$	65
10a		3	30	**10b**		67
11a		3	30	**11b**		69
12a		1.5	39	**12b**		99
13a		5	14	**13b**		67
14a		5	14	**14b**		86

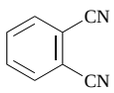
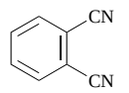
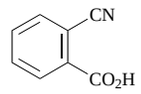
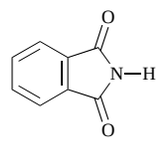
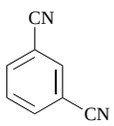
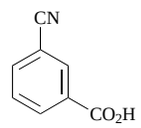
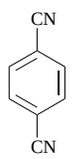
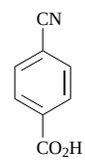
[a] Reaction conditions were not optimised. [b] Isolated yield. [c] Total isolated yield. [d] Ratio of **8b/8c** (4:1) was determined on the basis of ^{1}H NMR measurements.

suggest that π-systems embedded in the molecule can also act as effective ligands for the metal (iron or cobalt) when chelation allows. Also included is *trans*-cyclohexane-1,4-diyldiacetonitrile **12a** which shows total regioselective hydrolysis, presumably by way of chelation of its axial or twist-boat conformer.

Conclusion

Chemical hydrolysis of nitriles usually requires forceful conditions such as strong acids or bases and proceeds without regioselectivity when a dinitrile is employed. Using a whole cell *Rhodococcus* sp. AJ270 system we were able to hydrolyse various types of dinitriles efficiently under very mild conditions. Most significantly this enzymatic process showed excellent regioselectivity against almost all the dinitriles we examined, and it provided a powerful synthetic route to monocyano acids which would be difficult to obtain utilising other methods. For simple aliphatic dinitriles and those bearing heteroatoms including oxygen, sulfur and nitrogen, efficiency of regiocontrol is dependent upon the distance between cyano function and heteroatom; an oxygen placed β, γ or δ (optimally δ) to the cyano group allows regiocontrol as does sulfur positioned β or δ (optimally δ). Nitrogen substituents appear to behave as for oxygen but suffer a steric limitation due to the size of the nitrogen substituent when it is trisubstituted. We explain the regioselectivity in the hydrolysis of these dinitriles by a chelation–deactivation effect, *i.e.* a suitably placed heteroatom (which can be derived from the first-formed CO_2^- group) also participates in binding to the metalloenzyme and, therefore, interferes with the nitrile hydration. This mechanism is consistent with the fact that monocyano acids act as competitive inhibitors in the hydrolysis of benzonitrile. The reason for the regioselective

Table 4 Conversion of aromatic dinitriles into acids

Substrate	Reaction conditions[a] Amount (mmol)	Time (h)	Product(s) and yields[b] (%)	
15a 	3	70	**15a** 	52
			15b 	12
			15c 	12
16a 	3	24	**16b** 	71
17a 	3	20	**17b** 	81

[a] Reaction conditions were not optimised. [b] Isolated yield.

action on other dinitriles including aromatic examples is not known at this moment. However, a possible chelation interaction of π-system with the enzyme has been considered. The extent of regiocontrol in dinitrile hydrolysis would be useful in designing and conducting syntheses utilising prochiral and chiral dinitriles, which we intend now to study.

Experimental

Both melting points, which were determined using a Reichert Kofler hot-stage apparatus, and boiling points are uncorrected. IR spectra were obtained on a Unicam Research Series 1 FTIR instrument as liquid films of KBr discs. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or [$^2H_6$]-DMSO solution with $SiMe_4$ as internal standard on a JEOL 270 spectrometer. Chemical shifts are reported in ppm and coupling constants are given in Hz. Mass spectra were measured on a Kratos MS8ORF mass spectrometer using EI or CI. Exact mass was measured on a Kratos MSED mass spectrometer and microanalyses were carried out on a Carlo Erba 1106 Elemental Analyser, both at Newcastle University. High performance liquid chromatography (HPLC) was performed using a Lichrosorb RP-18 10 micron column (250 × 4.6 mm) (Alltech UK) with a Gilson Gradient System and a LDC/Milton Roy spectroMonitor^R 3000 UV detector at 204 nm. Potassium phosphate buffer (pH 2.8, 0.01 M) and acetonitrile were used as mobile phase and furan-2-carboxamide was used as an internal standard throughout the analyses. Thin layer chromatography (TLC) was performed with Merck silica $60F_{254}$ plates, and for flash chromatography Janssen silica (35–70 mm) was used.

Starting materials

The dinitriles **1a–g**, **4a**, **7a**, **9a–11a**, **13a** and **14a**, and the aromatic dinitriles **15a–17a** were purchased from commercial sources. The sulfides **4e**[29] and **4g**,[30] the anilines **4h–j**[31] and **4k–l**[32] and *trans*-cyclohexane-1,4-dicarbonitrile **12a**[33] were prepared according to the literature. The bis(cyanoalkyl) ethers **4b–d** and the sulfide **4f** were synthesized by allowing sodium cyanide to react with bis(chloroalkyl) ethers and sulfide, respectively, according to a literature method.[34] Compound **4o** was obtained through cyanoethylation of aqueous butylamine[35] while other amine derivatives **4m–n** or **4p–r** were prepared by alkylation of aniline or butylamine with ω-iodoalkyl cyanides following the methods reported in the literature.[32,36]

General procedure for the preparation of the bis(cyanoalkyl) ethers 4b–d and the sulfide 4f[34]

To a three-necked flask equipped with a mechanical stirrer was added sodium cyanide (0.22 mol) and dry dimethyl sulfoxide (90 cm^3). The mixture was stirred and heated to 90 °C after which bis(chloroalkyl) ether or sulfide (0.1 mol) was added dropwise to it at such a rate that the temperature of the reaction mixture was <110 °C. The reaction mixture was kept at *ca.* 100 °C for a further 30 min after the addition was complete, and then allowed to cool to room temperature. After dilution with dichloromethane (150 dm^3), the mixture was poured into an excess of saturated brine in a separatory funnel to which water was then added to dissolve precipitated salts. After this, the organic layer was separated and the aqueous layer was extracted once with dichloromethane. The combined organic phase and extract were washed with brine, dried ($MgSO_4$) and evaporated. The residue was then distilled *in vacuo* to give the desired product.

Bis(3-cyanopropyl) ether 4c. Bis(3-chloropropyl) ether[37] gave the ether **4c** as a colourless oil (12.6 g, 83%), bp 103 °C/0.02 mmHg (Found: M^+ 152.0942. $C_8H_{12}N_2O$ requires *M*, 152.0949); ν_{max}/cm^{-1} 2247 (CN); δ_H 3.56 (4H, t, *J* 5.6), 2.48 (4H, t, *J* 6.9) and 1.88–1.97 (4H, m); *m*/*z* (CI) 153 (M^+ + 1, 100%), 86 (92), 82 (26) and 68 (79).

Bis(4-cyanobutyl) ether 4d. Bis(4-chlorobutyl) ether[38] gave the ether **4d** as a colourless oil (16.2 g, 90%), bp 128 °C/0.03 mmHg (lit.,[38] bp 75–80 °C/0.0001 mmHg);ν_{max}/cm^{-1} 2244 (CN); δ_H 3.47 (4H, t, *J* 5.6), 2.39 (4H, t, *J* 6.9) and 1.69–1.80 (8H, m); *m*/*z* (CI) 181 (M^+ + 1, 100%), 100 (55) and 82 (99).

Bis(5-cyanopentyl) ether 4e. Bis(5-chloropentyl) ether[39] gave the ether **4e** as a pale yellow oil (17.9 g, 86%), bp 146–150 °C/0.015 mmHg (Found: M^+ 208.1579. $C_{12}H_{20}N_2O$ requires *M*, 208.1576); ν_{max}/cm^{-1} 2244 (CN); δ_H 3.41 (4H, t, *J* 5.9), 2.36 (4H, t, *J* 6.9), 1.70 (4H, quin, *J* 6.9) and 1.47–1.67 (8H, m); *m*/*z* (CI) 209 (M^+ + 1, 92%) and 96 (100).

Bis(3-cyanopropyl) sulfide 4f. Bis(3-chloropropyl) sulfide[40] gave the sulfide **4f** as a colourless oil (13.2 g, 79%), bp 128–133 °C/0.018 mmHg (Found: M^+ 168.0719. $C_8H_{12}N_2S$ requires *M*, 168.0721); ν_{max}/cm^{-1} 2246 (CN); δ_H 2.67 (4H, t, *J* 7.0), 2.53 (4H, t, *J* 7.0) and 1.96 (4H, quin, *J* 7.0); *m*/*z* (CI) 169 (M^+ + 1, 100%) and 102 (69).

Preparation of *N*,*N*-bis(4-cyanobutyl)aniline 4m[32]

A mixture of aniline (4.6 g, 50 mmol), 5-iodovaleronitrile (20.9 g, 100 mmol) and potassium carbonate (6.9 g, 50 mmol) in water (70 dm^{-3}) was refluxed for 24 h. After cooling, further potassium carbonate (1 g) was added to the mixture which was then extracted with dichloromethane. The extract was dried ($MgSO_4$) and evaporated *in vacuo*, to give the title compound as a yellow solid, recrystallisation of which from light petroleum–ethyl acetate gave pale yellow prisms (7.1 g, 56%), mp 48.5–49 °C (Found: C, 75.70; H, 8.64; N, 16.75. $C_{16}H_{21}N_3$ requires C, 75.26; H, 8.29; N, 16.46%); ν_{max}(KBr)/cm^{-1} 2246 (CN); δ_H 6.68–7.26 (5H, m), 3.32 (4H, t, *J* 7.0), 2.40 (4H, t, *J* 6.5) and 1.71–1.74 (8H, m); δ_C 147.6, 129.4, 119.4, 116.7, 112.6, 50.3, 26.5, 23.1 and 17.2; *m*/*z* (CI) 256 (M^+ + 1, 100%), 213 (33) and 187 (44).

Preparation of *N*,*N*-bis(5-cyanopentyl)aniline 4n

Following the same synthetic procedure as for **4m**, the title compound **4n** was obtained from distillation (Kugelrohr) at 225 °C/0.07 mmHg as pale yellow oil (8.6 g, 61%) (Found: M^+ 283.2050. $C_{18}H_{25}N_3$ requires *M*, 283.2048); ν_{max}/cm^{-1} 2246

(CN); δ_H 6.62–7.25 (5H, m), 3.28 (4H, t, *J* 7.0), 2.34 (4H, t, *J* 7.0) and 1.42–1.75 (12H, m); δ_C 147.6, 129.2, 119.5, 115.7, 111.9, 50.7, 26.4, 26.1, 25.2 and 17.1; *m*/*z* (CI) 284 ($M^+ + 1$, 100%), 201 (36) and 96 (64).

General procedure for the preparation of *N*,*N*-bis(2-cyanoalkyl)-butylamines 4p–r [36]

A mixture of butylamine (2.2 g, 30 mmol), ω-iodoalkyl cyanide (60 mmol) and potassium carbonate (8.3 g, 60 mmol) in ethanol (50 dm^{-1}) was refluxed for 48 h after which it was concentrated by removal of ethanol *in vacuo* and then diluted with water (*ca.* 50 cm^3); the aqueous phase was separated and extracted with ether. The extract was dried ($MgSO_4$) and concentrated, and the residue was distilled under reduced pressure to give the product.

***N*,*N*-Bis(3-cyanopropyl)butylamine 4p.** The butylamine **4p** was obtained as a colourless oil (4.8 g, 77%), bp 136–139 °C/0.015 mmHg (Found: M^+ 207.1727. $C_{12}H_{21}N_3$ requires *M*, 207.1735); ν_{max}/cm^{-1} 2246 (CN); δ_H 2.53 (4H, t, *J* 6.6), 2.42 (4H, t, *J* 6.9), 2.40 (2H, t, *J* 5.9), 1.78 (4H, quin, *J* 6.6), 1.18–1.47 (4H, m) and 0.95 (3H, t, *J* 7.2); δ_C 119.7, 53.3, 52.1, 28.9, 23.3, 20.6, 14.7 and 14.0; *m*/*z* (CI) 208 ($M^+ + 1$, 100%), 164 (82) and 153 (39).

***N*,*N*-Bis(4-cyanobutyl)butylamine 4q.** The butylamine **4q** was obtained as a colourless oil (5.6 g, 79%), bp 164 °C/0.02 mmHg (Found: M^+ 235.2054. $C_{14}H_{25}N_3$ requires *M*, 235. 2048); ν_{max}/cm^{-1} 2245 (CN); δ_H 2.34–2.44 (10H, m), 1.51–1.75 (8H, m), 1.20–1.44 (4H, m) and 0.94 (3H, t, *J* 7.3); δ_C 119.7, 53.6, 52.9, 29.2, 26.2, 23.2, 20.6, 17.1 and 14.0; *m*/*z* (CI) 236 ($M^+ + 1$, 100%), 193 (65) and 167 (23).

***N*,*N*-Bis(5-cyanopentyl)butylamine 4r.** The butylamine **4r** was obtained as a colourless oil (6.9 g, 87%), bp 155–158 °C/0.01 mmHg (Found: M^+ 263.2353. $C_{16}H_{29}N_3$ requires *M*, 263.2361); ν_{max}/cm^{-1} 2245 (CN); δ_H 2.32–2.41 (10H, m), 1.63–1.74 (4H, m), 1.25–1.50 (12H, m) and 0.91 (3H, t, *J* 6.9); δ_C 119.64, 53.75, 53.64, 29.16, 26.55, 26.45, 25.29, 20.61, 17.08 and 14.00; *m*/*z* (CI) 264 ($M^+ + 1$, 100), 220 (58), 181 (80) and 169 (34).

Preparation of *Rhodococcus* sp. AJ270 resting cells

Rhodococcus sp. AJ270 was cultured in a 20-dm^3 fermentor as we described in our previous paper.[9] The biomass was stored at −20 °C in a freezer.

Biotransformation of dinitriles

An Erlenmeyer flask (250 cm^3) with a screw cap was charged with *Rhodococcus* sp. AJ270 cells (2 g wet weight) and the potassium phosphate buffer (0.1M, pH 7.0; 50 cm^3) and the resting cells were activated at 30 °C for 0.5 h with orbital shaking. The dinitrile (1.5–5 mmol, see Tables 1–4 for each substrate) was added in one portion to the flask and the mixture was incubated at 30 °C using an orbital shaker (200 rpm). The reaction, monitored by TLC, was stopped after a period of time (see Tables 1–4 for each individual substrate). Biomass was removed by filtration through a Celite pad and the aqueous solution was basified to pH 12 with aqueous NaOH (2 M). Extraction with diethyl ether gave, after drying ($MgSO_4$) and concentration, the amide and unchanged nitrile. Separation of nitrile and amide was effected by flash chromatography. The aqueous solution was then acidified using aqueous HCl (2 M) to pH 2 and extracted with diethyl ether. Mono- and/or di-acid were obtained after evaporation of the extracts *in vacuo*. Solid and oily products were purified by recrystallisation and flash chromatography, respectively. All products were characterised by their spectral data and comparison of the melting points with the known compounds, which are listed below, or by full characterisation.

3-(2-Cyanoethoxy)propionic acid 5a. Pale yellow oil (Found: $M^+ - OH$, 126.0558. $C_6H_9NO_3 - OH$ requires 126.0555); ν_{max}/cm^{-1} 2657–3442 (CO_2H), 2252 (CN), 1720 (C=O) and 1116 (C–O–C); δ_H 6.39 (1H, s), 3.79 (2H, t, *J* 6.0), 3.70 (2H, t, *J* 6.8), 2.65 (2H, t, *J* 6.2) and 2.60 (2H, t, *J* 6.5); δ_C 176.1, 117.7, 66.2, 65.5, 34.6 and 18.6; *m*/*z* (CI) 144 ($M^+ + 1$, 15%), 126 (100) and 84 (33).

4-(3-Cyanopropoxy)butyric acid 5b. Colourless oil (Found: $M^+ - OH$, 154.0867. $C_8H_{13}NO_3 - OH$ requires 154.0868); ν_{max}/cm^{-1} 2678–3444 (CO_2H), 2250 (CN), 1712 (C=O) and 1117 (C–O–C); δ_H 6.59 (1H, s), 3.52 (2H, t, *J* 5.6), 3.49 (2H, t, *J* 6.3), 2.46 (2H, t, *J* 7.3), 2.45 (2H, t, *J* 7.0), 1.92 (2H, quin, *J* 6.0) and 1.90 (2H, t, *J* 6.1); δ_C 179.2, 119.4, 69.7, 68.0, 30.7, 25.6, 24.6 and 14.0; *m*/*z* (CI) 172 ($M^+ + 1$, 18%), 154 (100) and 87 (97).

5-(4-Cyanobutoxy)valeric acid 5c. Colourless oil (Found: $M^+ - OH$, 182.1183. $C_{10}H_{17}NO_3 - OH$ requires 182.1181); ν_{max}/cm^{-1} 2678–3410 (CO_2H), 2246 (CN), 1709 (C=O) and 1116 (C–O–C); δ_H 6.87 (1H, s), 3.45 (2H, t, *J* 5.6), 3.43 (2H, t, *J* 5.9), 2.39 (2H, t, *J* 7.6), 2.38 (2H, t, *J* 7.2) and 1.57–1.82 8H, m); δ_C 179.4, 119.6, 69.5, 69.4, 33.6, 28.9, 28.5, 22.5, 21.4 and 16.9; *m*/*z* (CI) 200 ($M^+ + 1$, 38%), 182 (100) and 101 (91).

3-(2-Cyanoethylthio)propionic acid 5e. Pale yellow oil (Found: M^+, 158.9805. $C_6H_9NO_2S$ requires 159.0354); ν_{max}/cm^{-1} 2586–3446 (CO_2H), 2252 (CN) and 1712 (C=O); δ_H 8.50 (1H, s) and 2.62–2.89 (4H, m); δ_C 177.1, 118.2, 34.4, 27.7, 26.6 and 18.8; *m*/*z* (CI) 160 ($M^+ + 1$, 3%), 156 (11), 142 (80), 133 (13) and 114 (100).

4-(3-Cyanopropylthio)butyric acid 5f. Pale yellow oil (Found: M^+, 187.0431. $C_8H_{13}NO_2S$ requires 187.0667); ν_{max}/cm^{-1} 2668–3440 (CO_2H), 2248 (CN) and 1708 (C=O); δ_H 8.45 (1H, s), 2.65 (2H, t, *J* 6.9), 2.59 (2H, t, *J* 7.3), 2.51 (2H, t, *J* 6.9), 2.50 (2H, t, *J* 7.3), 1.94 (2H, quin, *J* 6.9) and 1.93 (2H, quin, *J* 6.9); δ_C 178.9, 119.0, 32.5, 31.0, 30.3, 25.0, 24.2 and 15.9; *m*/*z* (CI) 188 ($M^+ + 1$, 7%), 187 (28), 171 (27), 170 (100), 142 (14), 128 (12) and 87 (31).

3-(*N*-Cyanoethyl-*N*-phenylamino)propionic acid 5h. Colourless crystalline solid, mp 56.5–56.8 °C (Found: C, 66.25; H, 6.45; N, 12.72. $C_{12}H_{14}N_2O_2$ requires C, 66.04; H, 6.47; N, 12.84%); ν_{max}/cm^{-1} 2903–3549 (CO_2H), 2258 (CN) and 1732 (C=O); δ_H 9.50 (1H, s), 6.72–7.32 (5H, m), 3.72 (4H, t, *J* 6.9), 2.68 (2H, t, *J* 6.6) and 2.59 (2H, t, *J* 6.9); δ_C 177.9, 145.6, 129.7, 118.5, 118.2, 113.4, 47.9, 47.0, 32.4 and 15.9; *m*/*z* (EI) 218 (M^+, 14%), 178 (99), 159 (46), 136 (53), 106 (100), 104 (45) and 77 (55).

3-(*N*-*p*-Chlorophenyl-*N*-cyanoethylamino)propionic acid 5i. Colourless crystalline solid, mp 89.5–90 °C (Found: C, 57.26; H, 5.33; N, 11.23. $C_{12}H_{13}ClN_2O_2$ requires C, 57.04; H, 5.19; N, 11.09%); ν_{max}/cm^{-1} 3042–3550 (CO_2H), 2248 (CN) and 1720 (C=O); δ_H 9.86 (1H, s), 7.22 (2H, d, *J* 9.2), 6.66 (2H, d, *J* 9.2), 3.70 (4H, t, *J* 6.6), 3.69 (2H, t, *J* 6.6), 2.66 (2H, t, *J* 6.9) and 2.58 (2H, t, *J* 6.6); δ_C177.8, 144.3, 129.5, 123.5, 118.0, 114.6, 47.9, 47.0, 32.3 and 16.0; *m*/*z* (CI) 255 (7), 254 (9), 253 ($M^+ + 1$, 22%), 252 (20), 214 (32), 212 (100), 195 (18) and 193 (58).

3-(*N*-Cyanoethyl-*N*-*p*-methoxyphenylamino)propionic acid 5j. Colourless needles, mp 86–86.5 °C (Found: C, 63.01; H, 6.55; N, 11.20. $C_{13}H_{16}N_2O_3$ requires C, 62.87; H, 6.50; N, 11.28%); ν_{max}/cm^{-1} 2832–3548 (CO_2H), 2248 (CN), 1714, 1701 (C=O) and 1244 (C–O–C); δ_H 9.45 (1H, s), 6.88 (4H, s), 3.52 (2H, t, *J* 6.6), 3.51 (2H, t, *J* 6.9), 2.55 (2H, t, *J* 6.6) and 2.49 (2H, t, *J* 6.9); δ_C 177.3, 154.4,139.8, 119.0, 118.3, 115.1, 55.6, 49.7, 48.7, 32.5 and 16.2; *m*/*z* (EI) 248 (M^+, 16%), 208 (100), 189 (29), 166 (24), 136 (60), 135 (28), 134 (16) and 120 (40).

3-(*N*-Butyl-*N*-cyanoethylamino)propionamide 5o. Pale yellow oil (Found: M^+, 197.1535. $C_{10}H_{19}N_3O$ requires *M*, 197.1528); ν_{max}/cm^{-1} 3355, 3196 (NH_2), 2247 (CN) and 1666, 1621 (C=O); δ_H 7.13 (1H, s), 5.51 (1H, s), 2.81 (2H, t, *J* 6.6), 2.76 (2H, t, *J* 6.3), 2.48–2.54 (4H, m), 2.38 (2H, t, *J* 6.3), 1.79–1.53 (4H, m) and 0.93 (2H, t, *J* 7.2); δ_C 177.3, 118.9, 53.1, 50.1, 49.4, 33.5, 28.8, 20.3, 16.6 and 13.9; *m*/*z* (CI) 198 ($M^+ + 1$, 46%), 157 (100), 154 (63) and 139 (98).

4-(*N*-Butyl-*N*-cyanopropylamino)butyramide 5p. Pale yellow oil (Found: M^+, 225.1846. $C_{12}H_{23}N_3O$ requires *M*, 225.1841); ν_{max}/cm^{-1} 3354, 3197 (NH_2), 2246 (CN) and 1667, 1622 (C=O); δ_H 5.82 (1H, s), 5.38 (1H, s), 2.52 (2H, t, *J* 6.6) 2.44 (2H, t, *J* 6.6), 2.42 (2H, t, *J* 6.9), 2.37 (2H, t, *J* 6.7), 2.27 (2H, t, *J* 7.6),

1.72–1.83 (4H, m), 1.17–1.45 (4H, m) and 0.91 (3H, t, *J* 7.3); δ_C 175.4, 120.1, 53.4, 53.2, 51.9, 33.6, 29.0, 23.3, 23.1, 20.6, 14.8 and 14.1; *m*/*z* (CI) 226 ($M^+ + 1$, 100%), 208 (86), 182 (93), 164 (79), 153 (93), 111 (67) and 86 (93).

***p*-Cyanophenylacetic acid 8b and *p*-cyanomethylbenzoic acid 8c.** A mixture of **8b** and **8c** was obtained as a colourless crystalline solid, mp 138–139.5 °C; ν_{max}/cm^{-1} 2562–3424 (CO_2H), 2252 and 2229 (CN) and 1695 (C=O); δ_H 8.07 (0.5H, d, *J* 8.4), 7.61 (2H, d, *J* 8.4), 7.42 (2.5H, d, *J* 8.4), 3.82 (0.5H, s) and 3.66 (2H, s).

***m*-Cyanomethylphenylacetic acid 10b.** Colourless crystalline solid,[20] mp 45–47 °C (Found: C, 68.72; H, 5.14; N, 7.65. $C_{10}H_9NO_2$ requires C, 68.56; H, 5.18; N, 8.00%); ν_{max}/cm^{-1} 2540–3432 (CO_2H), 2252 (CN) and 1702 (C=O); δ_H 7.25–7.36 (4H, m), 6.49 (1H, s), 3.75 (2H, s) and 3.67 (2H, s); δ_C 176.8, 134.4, 130.3, 129.4, 129.1, 128.9, 126.9, 117.6, 40.7 and 23.4; *m*/*z* (EI) 175 (M^+, 35%), 130 (100) and 104 (25).

***trans*-4-Cyanomethylcyclohexylacetic acid 12b.** Colourless needles, mp 89.5–90 °C (Found: C, 66.34; H, 8.20; N, 7.64. $C_{10}H_{15}NO_2$ requires C, 66.27; H, 8.34; N, 7.73%); ν_{max}/cm^{-1} 2632–3400 (CO_2H), 2250 (CN) and 1694 (C=O); δ_H 2.26 (4H, d, *J* 6.5), 1.89 (4H, d, *J* 9.7), 1.76 (1H, s), 1.65 (1H, s), 0.98–1.28 (4H, m); δ_C 178.8, 118.6, 41.3, 34.4, 33.9, 32.0, 31.9 and 24.5; *m*/*z* (CI) 182 ($M^+ + 1$, 1%), 164 (100) and 136 (12).

Melting points

2a, mp 50 °C (lit.,[41] 50–51 °C); **2b**, oil (lit.,[42] bp 110–115 °C/3 mmHg); **2c** (lit.,[42] bp 133–137 °C/3 mmHg); **3c**, mp 147–148 °C (lit.,[43] 146–147 °C); **3d**, mp 103–104 °C (lit.,[44] 103–105); **3e**, mp 139–141 °C (lit.,[45] 140 °C); **3f**, mp 105–106 °C (lit.,[46] 105–106 °C); **3g**, mp 134–135 °C (lit.,[47] 133.5–134 °C); **6b**, mp 81–82 °C (lit.,[48] 81 °C); **6c**, mp 87–89 °C (lit.,[38] 88.5–89.5 °C); **6d**, mp 90–91 °C (lit.,[48] 90–91 °C); **6e**, mp 127–130 °C (lit.,[49] 128–130 °C); **6g**, mp 97–97.5 °C (lit.,[50] 96.5–97.5 °C); **7b**, mp 123–124 °C (lit.,[51] 122–123 °C); **9b**, mp 99–100 °C (lit.,[52] 101 °C); **9c**, mp 197–198 °C (lit.,[53] 198 °C); **11b**, mp 122 °C (lit.,[54] 120–121.5 °C); **13b**, mp 75–77 °C (lit.,[17b] 73–77 °C); **14b**, 137.5–138.5 °C (lit.,[55] 136–138 °C); **15b**, 180–183 °C (lit.,[56] 178–183 °C); **15c**, mp 227–228 °C (lit.,[57] 227 °C); **16b**, mp 220 °C (lit.,[58] 220–221 °C); **17b**, mp 218–221 °C (lit.,[59] 219–220 °C).

Hydrolysis of benzonitrile

The same biotransformation procedure as described above was employed in the study of the hydrolysis of benzonitrile over time. Two experiments were conducted in parallel, one with added 4-(3-cyanopropylthio)butyric acid **5f** (99.5 mg, 0.53 mmol) and the other without. After incubation for 30 min at 30 °C with shaking, benzonitrile (206.2 mg, 2 mmol) was added in one portion to the mixture which was then incubated at 30 °C using an orbital shaker (200 rpm). A sample (0.1 cm^3) was taken from the mixture at different time intervals and the biomass was removed using a microcentrifuge (30 000 rpm). The supernatant (0.08 cm^3) was removed by pipette and mixed with furan-2-carboxamide solution (1 mM in methanol; 0.04 cm^3). HPLC analysis of the mixture (0.01 cm^3), after being calibrated with calibration curves, gave the concentrations of benzonitrile, benzamide and benzoic acid (Fig. 2).

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