Regioselective Hydrolysis of Aromatic Dinitriles Using a Whole Cell Catalyst

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A series of aromatic dinitriles have been examined as substrates for an immobilised whole cell *Rhodococcus* sp. that catalyses the hydrolysis of nitriles to amides and/or carboxylic acids. The fluorinated aromatic dinitriles **48** and **49** were regioselectively hydrolysed to the corresponding cyano amides **48a** and **49a** whereas the non-fluorinated analogues **41–44** were converted to cyano acids but with poorer regioselectivity.

Introduction

During the past 10–15 years there has been an enormous increase in the use of enzymes as catalysts for organic synthesis.[1] In particular hydrolytic enzymes[2] (*e.g.* lipases, esterases and acylases) have proved to be particularly useful catalysts for the preparation of homochiral molecules. There are two major reasons for the widespread use of these catalysts by a variety of laboratories: (i) the enzymes operate on a wide range of substrates with very high stereoselectivity and (ii) they are robust and extremely easy to use requiring no co-factors. More recently, the replacement of water by organic solvents[3] has expanded the range of reactions that can be effected (*e.g.* esterification and peptide bond synthesis).

By comparison, a related group of enzymes, those that catalyse the hydrolysis of nitriles to amides and/or carboxylic acids, have remained largely unexplored.[4] This is a somewhat surprising situation considering the importance of nitriles as intermediates in organic synthesis. Thus, organonitriles can be readily prepared by a number of methods including the addition of cyanide ion (or its equivalent) to alkyl halides,[5] the Strecker reaction,[6] the Sandmeyer reaction,[7] the reaction of aryl halides with copper cyanide,[8] the reaction of ketones with tosylmethyl isocyanide,[9] and the dehydration of amides.[10] Perhaps, more importantly, the enzymic hydrolysis of nitriles can be carried out under mild conditions (pH 6–8, 25–35 °C, aqueous buffer). These conditions should be compared with the more severe conditions required for the non-enzymic hydrolysis (*e.g.* for nitrile to amide: H_2O_2–NaOH and for nitrile to carboxylic acid: 6 mol dm^{-3} HCl-reflux or 2 mol dm^{-3} NaOH-reflux).[11] In addition to operating under milder conditions, enzyme-catalysed hydrolysis offers the potential for carrying out selective (chemo-, regio-, stereo-) transformations that are difficult to achieve non-enzymically.[12] However, despite the success of certain well-known large-scale processes that utilise biocatalytic nitile hydrolysis [*e.g.* the conversion of acrylonitrile **1** into acrylamide **2** using an immobilised bacterial organism carried out by the Nitto Chemical Company in Japan (*ca.* 20 000 tons per annum) (Scheme 1)][13] there has not, until very

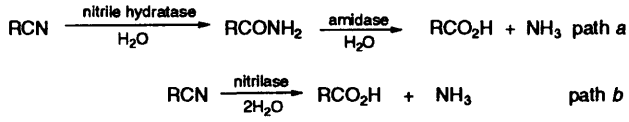
1 **2**

Scheme 1 *Reagents:* i, immobilised bacteria

recently, been any systematic examination of either the substrate specificity, or stereoselectivity, of the enzymes involved. The aim of this review therefore is to assess the current position regarding the use of nitrile hydrolysing enzymes in organic synthesis.

Enzymes Involved in Nitrile and Amide Hydrolysis

It has been known for many years that a diverse range of microorganisms are able to metabolise organonitriles.[14] Investigation of the metabolic pathways involved has revealed two distinct routes from a nitrile to a carboxylic acid (Scheme 2). In path *a*, the nitrile is first hydrated to an amide, catalysed



Scheme 2

by a *hydratase* and subsequently the amide is hydrolysed to a carboxylic acid in the presence of an *amidase*. In path *b*, the nitrile is converted directly into the acid *via* a *nitrilase*. As a general guideline, aliphatic nitriles are hydrolysed *via* path *a* whereas aromatic and heterocyclic nitriles are metabolised *via* path *b*. By growth of the organism on an appropriate substrate, it is often possible to induce any or all three of the enzymes involved.

Both pathways *a* and *b* have been demonstrated to occur in a range of microorganisms including *Rhodococcus*, *Brevibacterium*, and *Nocardia*. From the point of view of biotransformations, the organisms can be conveniently used as whole-cell preparations to carry out preparative conversions. With respect to the individual enzymes, only the nitrile hydratases have been studied in any detail. Nitrile hydratases have been purified from two different *Rhodococcus* species,[15,16] a *Corynebacterium* sp.[17] and from *Brevibacterium* R312[18] and *Pseudomonas chlororaphis* B23.[19] By the use of ESR studies[20] Yamada has shown that these nitrile hydratases are non-haem ferric iron-containing enzymes. Moreover, on the basis of UV/visible spectroscopic studies he has suggested that the enzyme also binds the cofactor pyrroloquinoline quinone (PQQ)[19] leading to a proposed mechanism of action shown in Fig. 1.

Application of Nitrile Hydrolases in Organic Synthesis

(i) *Substrate Specificity*.—Early work using a range of different micro-organisms established that all three enzymes, *i.e.* hydratase, amidase and nitrilase were able to catalyse the hydrolysis of a wide range of structurally diverse substrates. For example, using *Rhodococcus rhodochrous* J1 that contained both a nitrilase[21] and nitrile hydratase[22,23] Yamada *et al.*, prepared the compounds **3–11** from the corresponding nitriles. The synthesis of *p*-aminobenzoic acid **3** is representative of the procedure used. Thus, using resting cells of *R. rhodochrous* J1, *p*-aminobenzonitrile was added batchwise such that the concentration of substrate did not exceed 200 mol dm^{-3} (NB

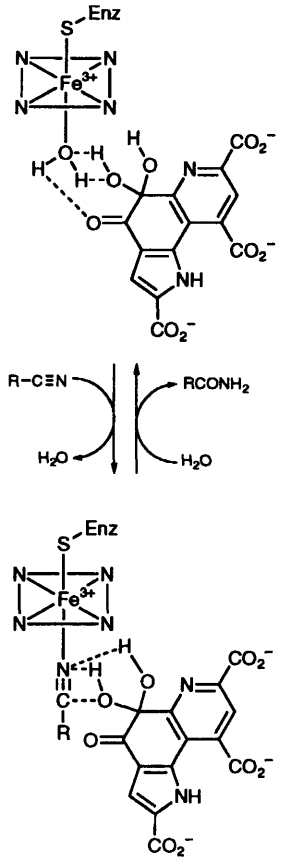
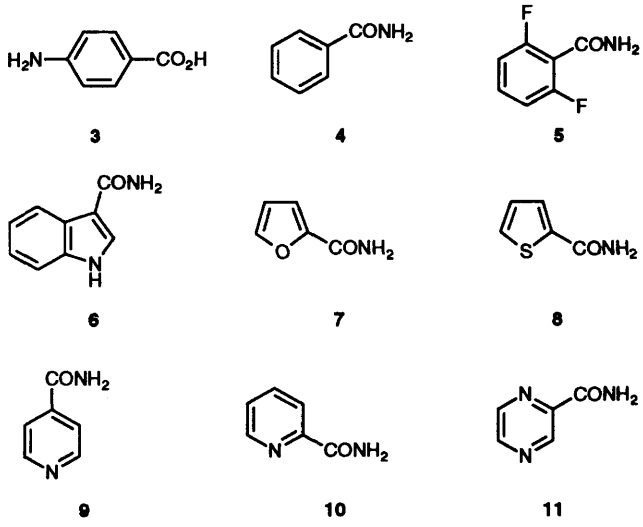
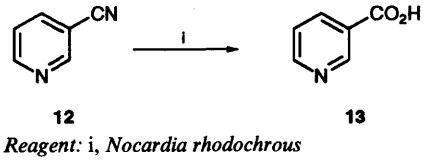


Fig. 1 Proposed mechanism of nitrile hydration catalysed by a nitrile hydratase

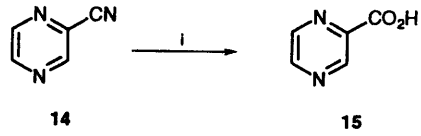


higher concentrations of substrate were found to be inhibitory) resulting in a quantitative yield of **3** at a concentration of 110 g dm^{-3}. *p*-Aminobenzoic acid is an important intermediate in the synthesis of anaesthetics, dyes and developers. Knowles reported the conversion of 3-cyanopyridine **12** into nicotinic acid **13** using *Nocardia rhodochrous*[24] (Scheme 3) this reaction



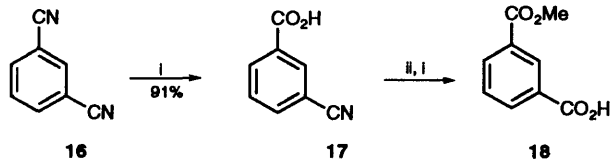
Scheme 3 *Reagent:* i, *Nocardia rhodochrous*

being catalysed by a nitrilase. The nitrilase activity was induced in *N. rhodochrous* by addition of benzonitrile to stationary phase cultures of the organism. A related transformation is the conversion of cyanopyrazine **14** into pyrazinoic acid **15** using a nitrilase from *R. rhodochrous* J1 (Scheme 4).[25]



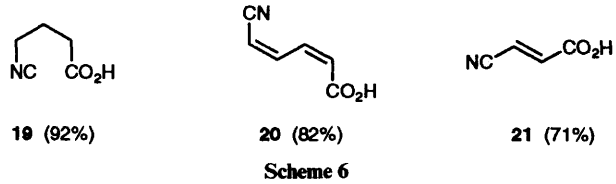
Scheme 4 *Reagent:* i, *Rhodococcus rhodochrous*

Three groups[26–30] have independently reported a more detailed study of an immobilised whole cell *Rhodococcus* sp. developed by Novo Industri of Denmark. This organism contains only a nitrile hydratase and a amidase with no nitrilase activity being present. The immobilised catalyst hydrolyses a wide range of substrates inluding both aromatic and aliphatic nitriles. Moreover, the catalyst is very simple to use and can be recovered at the end of the reaction. In addition to the hydrolysis of simple organonitriles, the catalyst can be used for the selective hydrolysis of isophthalonitrile **16** to the cyano carboxylic acid **17** (Scheme 5).[26] The initial hydrolysis results



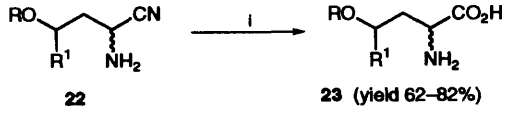
Scheme 5 *Reagents:* i, immobilised *Rhodococcus* sp.; ii, CH_2N_2

in a 91% yield of the cyano acid with none of the diacid being obtained. Esterification of the carboxylic acid group of **17** followed by re-exposure to the catalyst resulted in hydrolysis of the second nitrile group to give **18**. The conversion of isophthalonitrile **16** into **17** has also been reported using *R. rhodochrous*[31,32] *via* a single-step nitrilase. Similar selective hydrolysis of aliphatic dinitriles can also be achieved. Thus the cyano acids **19–21** were obtained from the corresponding dinitriles (Scheme 6).[33,34]



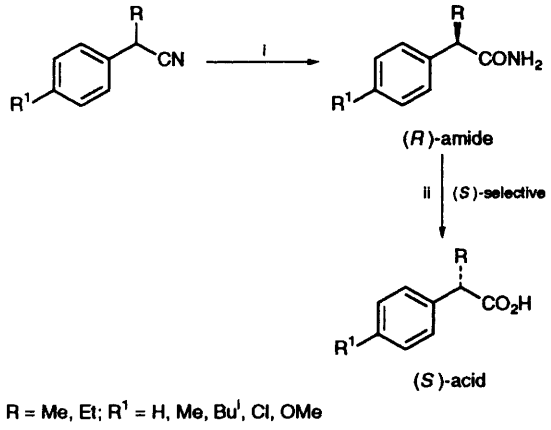
Scheme 6

(ii) *Stereoselective Transformations.*—Much of the recent work using nitrile hydrolases has focussed on the stereoselective hydrolysis of nitriles. One of the earliest reported examples involved the conversion of racemic α-hydroxy nitriles to optically active L-α-hydroxy acids using *Torulopsis candida*.[35] Similarly, α-aminopropionitrile was hydrolysed to L-alanine using a bacterial isolate.[36] A *Brevibacterium* strain has also been used to prepare optically active L or D-amino acids from racemic α-amino nitriles.[37] Interestingly, the hydrolysis of γ-alkoxy-α-amino nitriles **22** to γ-alkoxy-α-amino acids **23** was found to proceed smoothly but without any attendant stereoselectivity (Scheme 7).[38]



Scheme 7 *Reagent:* i, *Brevibacterium* sp. R312

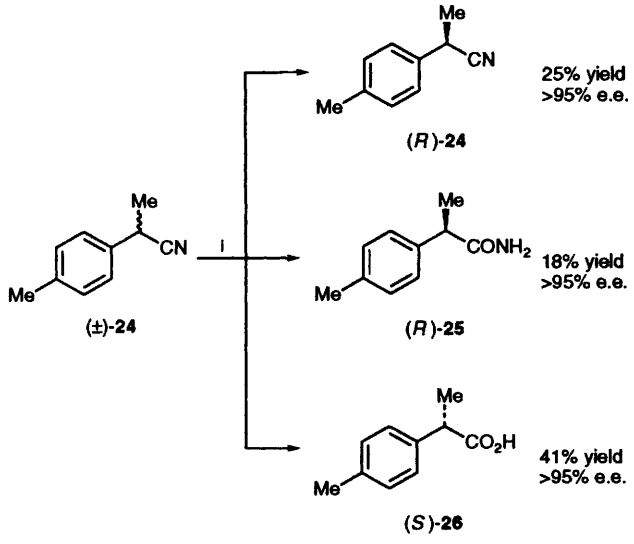
More recently, two groups have made a more detailed investigation of the stereoselectivity of nitrile hydrolysis. Using either the Novo immobilised *Rhodococcus* catalyst[39,40] or *Rhodococcus butanica* ATCC 21197[41,42] a range of substrates have been investigated. Racemic aryl substituted alkanenitriles (Scheme 8) have been hydrolysed to give the corresponding



R = Me, Et; R^1 = H, Me, Bu^t, Cl, OMe

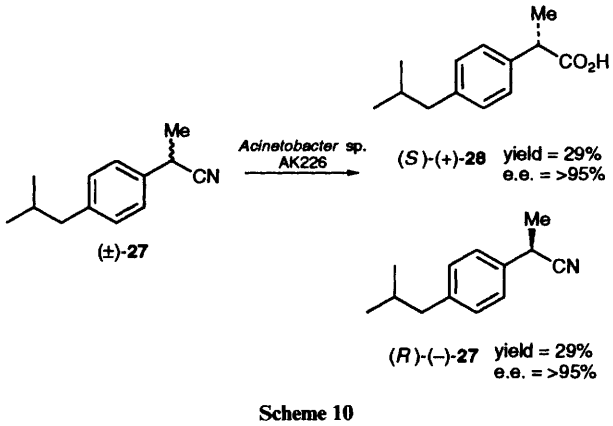
Scheme 8 *Reagents:* i, nitrile hydratase; ii, amidase

optically active amides and carboxylic acids.[39,40] In certain instances the recovered nitrile was also found to be optically active (Scheme 9).[43] Overall, the absolute configuration of the

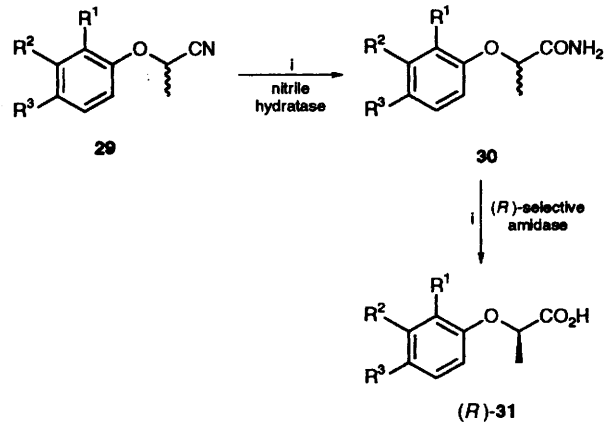


Scheme 9 *Reagent: Rhodococcus* sp. SP361

products can be rationalised according to the model shown in Scheme 8. The preparation of (*S*)-(+)-ibuprofen **28** by hydrolysis of the nitrile **27** has also been achieved using an *Acinetobacter* sp. that contained a nitrilase (Scheme 10).[44] In this reaction both the recovered nitrile (*R*)-(+)-**27** and the product acid (*S*)-(+)-**28** were found to have e.e.s >95%. Evidence for a single-step nitrilase-catalysed reaction derived from the observations that (i) none of the amide could be detected and (ii) the racemic amide was not hydrolysed to the carboxylic acid under the reaction conditions. 2-Aryloxypropionic acids **31**, compounds with high herbicidal activity, have been prepared in almost optically pure form by hydrolysis of the corresponding racemic nitriles **29** (Scheme 11).[45] By using the racemic nitrile **29** as substrate, a fast non-selective hydrolysis to the amides **30** was observed followed by a slow stereoselective conversion into the acids (*R*)-**31** catalysed by an (*R*)-selective amidase. The (*S*)-amides **30** were also recovered (e.e.s 76–95%).

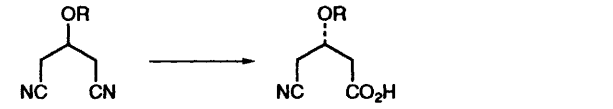


Scheme 10



Scheme 11 *Reagent: Brevibacterium imperiale*

Prochiral dinitrile substrates can be asymmetrically hydrolysed to optically active cyano acids.[40,42] A series of 3-substituted glutaronitriles were transformed to the corresponding cyano acids having the 3*S* configuration (Scheme 12). No evidence was obtained for the presumed cyano amide

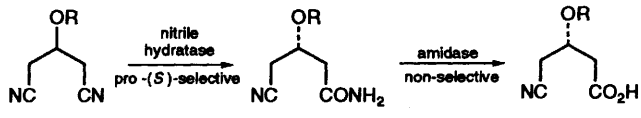


32 R = Bn
34 R = Bz

33 R = Bn yield = 73%; e.e. = 83%
35 R = Bz yield = 25%; e.e. = 84%

Scheme 12

intermediate. The enantiomeric excess of the product depended upon the protecting group, the best results being obtained when R = Bn (e.e. = 83%) or Bz (e.e. = 84%). The observed stereoselectivity can be rationalised according to the mechanism shown in Scheme 13. The prochiral dinitrile is hydrolysed

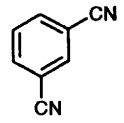
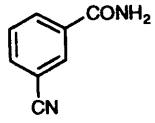
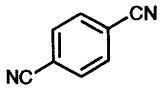
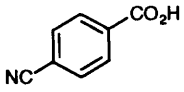
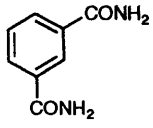
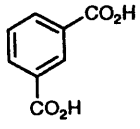


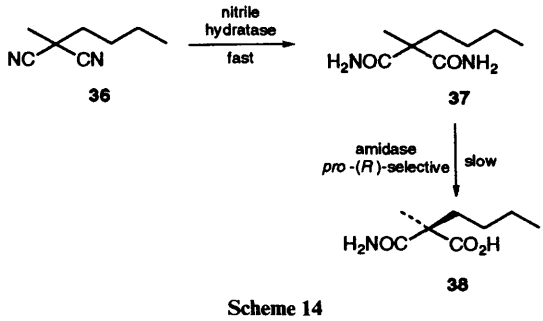
Scheme 13

initially by an *S*-selective hydratase followed by a fast, non-selective amidase.

By employing disubstituted malononitrile substrates with *Rhodococcus rhodochrous* ATCC 21197, the reaction has been shown to take a slightly different course (Scheme 14).[46] Thus butylmethylmalononitrile **36** was hydrolysed to the amido carboxylic acid **38** in 94% yield and 96% e.e. The reaction was shown to proceed *via* a fast non-selective hydrolysis to the diamide **37** followed by a slow *pro-R*-selective amidase reaction.

Table 1

Substrate	Product	c/mmol dm^{-3}	t/h	Yield (%)
 **16**	 **16a**	48	49	11
 **39**	 **39a**	25	15	62
 **40**	 **40a**	10	23	62



Scheme 14

In summary, it is clear that the enzyme-catalysed hydrolysis of nitriles and amides represents a potentially useful methodology for organic synthesis. The mildness of the reaction conditions leads to a selective hydrolysis of nitrile groups in the presence of acid and base sensitive functionality (*e.g.* acetals and esters). The recent reports of stereoselective transformations indicate that in certain cases the nitrile hydrolases may be as useful as the lipases and esterases for the synthesis of chiral building blocks in homochiral form.

Regioselective Hydrolysis of Dinitriles

The preceding sections have outlined clearly that nitrile hydrolases are able to catalyse the hydrolysis of organonitriles with various types of selectivity. Thus, examples have been given of chemoselectivity (Scheme 5), enantioselectivity, including kinetic resolutions (Schemes 8–11) and enantiotopic group discrimination (Schemes 12–14) and finally the selective hydrolysis of one nitrile group in the presence of another (Schemes 5, 6). The latter type of selectivity encouraged us to investigate whether these enzymes might be able to carry out regioselective transformations, particularly on aromatic dinitriles. By taking a non-symmetrical dinitrile we would attempt selective hydrolysis of one of the two nitrile groups thus generating a single regioisomer. We now report the realisation of this objective and demonstrate for the first time a novel type of selectivity of nitrile hydrolases.

The starting point for this work was the previously reported conversion of isophthalonitrile **16** into the cyano acid **17** (Scheme 5).[43] Using the immobilised *Rhodococcus* sp. (SP361) under standard conditions the cyano acid **17** was obtained in 91% yield. As outlined above (Scheme 5) the SP361 catalyst will only hydrolyse the second nitrile group after esterification of the carboxylic acid to the methyl ester. The immobilised catalyst SP361 is known to contain both a nitrile hydratase and an amidase but no nitrilase. Although the amide **16a** is an intermediate in the hydrolysis of **16**, its rate of hydrolysis greatly exceeds its rate of production. However, by lowering the reaction temperature to 4 °C it was possible to isolate the intermediate amido acid **16a** in low yield (11%) (Table 1). Selective hydrolysis of a dinitrile was also observed in the conversion of terephthalonitrile **39** into 4-cyanobenzoic acid **39a** (62%) (Table 1). Interestingly, if the isophthalamide **40** is used as a substrate then no selectivity is observed and isophthalic acid **40a** is obtained (62%). We turned next to the hydrolysis of a series of simple non-symmetrical dinitriles **41**–**44** (Table 2) in an attempt to obtain regioselective discrimination between two non-equivalent nitrile groups. In all cases, the products obtained were the result of hydrolysis of only one of the nitrile groups to the carboxylic acid. However, only with the substrates **41** and **43** was any selectivity observed and even then it was poor (3.6:1 and 2:1, respectively).

At this point we felt that it would be more productive to investigate regioselective transformations by choosing a system in which only the first hydrolysis (nitrile to amide) could occur without any secondary hydrolysis of amide to carboxylic acid. It had previously been shown by Yamada *et al.*, that, 2,6-difluorobenzonitrile can be hydrolysed to 2,6-difluorobenzamide **5** without subsequent hydrolysis to the carboxylic acid.[22,23] Presumably, the amide **5** was not a substrate for the amidase that would normally convert the amides directly through into carboxylic acids. Using the fluoro dinitriles **45** and **46** we were able to obtain analogous results, namely conversion into the corresponding cyano amides **45a** and **46a** in 40 and 73% yield, respectively (Table 3). Furthermore 5-chloroisophthalonitrile **47** was mono-hydrolysed to the cyano amide **47a** (20% yield). From the results obtained it appears that the effect of the fluorine groups was to depress the rate of the second enzyme (hydratase) catalysed hydrolysis and thus allow cyano amides to be isolated.

A second series of fluorinated substrates **48**–**50** (Table 4) was then tested in an attempt to obtain regioselective conversions. The dinitriles **48** and **49** were selected since it was felt that the amino group might enhance the selectivity of hydrolysis by exerting both steric and electronic effects at the enzyme active

Table 2

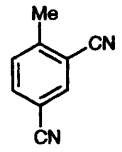
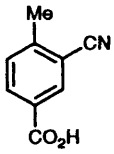
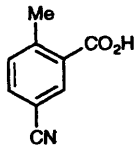
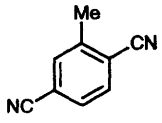
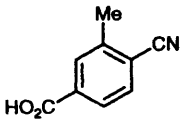
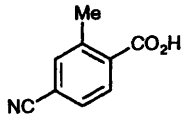
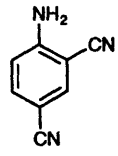
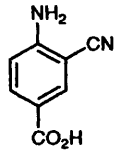
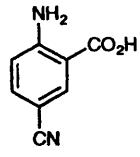
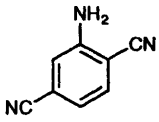
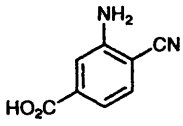
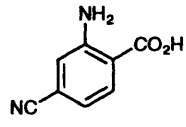
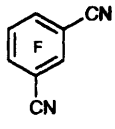
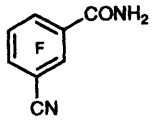
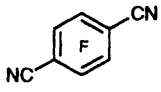
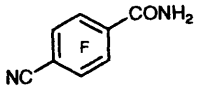
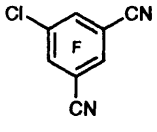
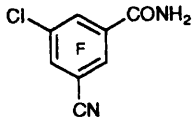
Substrate	Products		c/mmol dm^{-3}	t/h	Ratio [yield (%)]
 **41**	 **41a**	 **41b**	30	183	**41a:41b** = 3.6:1 [86%]
 **42**	 **42a**	 **42b**	30	48	**42a:42b** = 1:1 [53%]
 **43**	 **43a**	 **43b**	5	49	**43a:43b** = 2:1 [81%]
 **44**	 **44a**	 **44b**	5	95	**44a:44b** = 1:1 [80%]

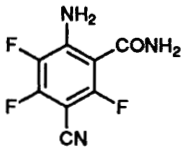
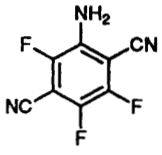
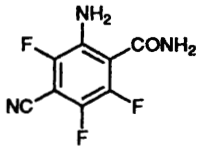
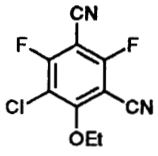
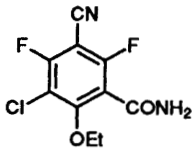
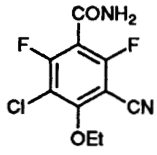
Table 3

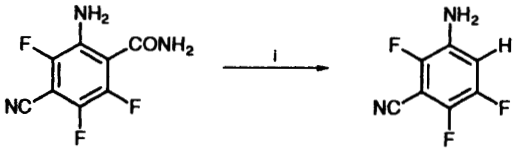
Substrate	Product	c/mmol dm^{-3}	t/h	Yield (%)
 **45**	 **45a**	5	21	40
 **46**	 **46a**	5	120	73
 **47**	 **47a**	5	44	20

site. It is notable that both **48** and **49** were hydrolysed to a single regioisomer and, significantly, both products contained an amide group. The nitrile group undergoing hydrolysis was clearly established as the one *ortho* to the amino group by analysis of the ^{13}C NMR spectra and comparison of the chemical shifts and coupling constants [$J(^{13}C$-$^{19}F)$] with theoretically calculated values.[47] In the case of **48a** further evidence for the structure was provided by the synthetic sequence shown in Scheme 15. Hydrolysis of the amido group in **46a** to the acid followed by decarboxylation gave the known amino nitrile **51**.

The reason for the selective hydrolysis of the nitrile group *ortho* to the amine is unclear at this stage. When the results from substrates **48** and **49** are compared with the result from the hydrolysis of **41** (Table 2), in which the nitrile remote from the methyl group is attacked, then it is clear that both steric and electronic factors influence the regioselectivity. One possibility is that the *ortho*-amino group may assist in the protonation step

Table 4

Substrate	Products	c/mmol dm^{-3}	t/h	Ratio [yield (%)]
 **48**	 **48a**	5	115	Single isomer [15%]
 **49**	 **49a**	5	70	Single isomer [52%]
 **50**	 **50a**  **50b**	5	310	**50a:50b** = 1:1 [47%]



48a **51**

Scheme 15 *Reagents:* i, AcOH, 6 mol dm^{-3} H_2SO_4, reflux

that is required for the conversion of the nitrile into the amide. Clearly, the various factors controlling the regioselectivity of the hydrolysis will only be understood by a systematic investigation using a wider range of substrates.

Experimental

General.—Unless otherwise stated, all reagents were obtained from commercial suppliers and used without further purification. Light petroleum (b.p. 40–60 °C) and ethyl acetate were distilled prior to use. DMF was distilled from calcium hydride and pyridine was distilled from potassium hydroxide. Dried solvents were stored over 4 Å molecular sieves under an inert atmosphere or argon.

Reactions were monitored by TLC on Merck Kieselgel 60 F_{254}, 0.25 mm plates. Plates were visualised by alkaline potassium permanganate dip or UV (254 nm) fluorescence. Preparative column chromatography was performed using silica gel 60H (0.04–0.063 mm/230–400 mesh) (Merck 9385). Solvent mixtures are expressed in volume:volume ratios.

250 MHz ^{1}H, 62.9 MHz ^{13}C and 235 MHz ^{19}F NMR spectra were recorded on a Bruker AM250 spectrometer with 300 MHz ^{1}H and 75.5 MHz ^{13}C NMR spectra being recorded on a Bruker AM300 spectrometer. A Bruker AMX400 spectrometer was employed to record 100.6 MHz ^{13}C NMR spectra. Chemical shift is expressed in ppm downfield from tetramethylsilane and reported as: δ_H, δ_C, or δ_F, number of equivalent nuclei (by integration), multiplicity (s = singlet, d = doublet, q = quartet, m = multiplet, br = broad), coupling constants (J in Hz) and assignment, as appropriate. IR spectra (ν_{max}) were recorded on a Perkin-Elmer 881 IR grating spectrometer and are recorded as wavenumbers (cm^{-1}) relative to a polystyrene standard (1601 cm^{-1}). High resolution mass spectra (m/z) were recorded at the SERC Mass Spectrometry Centre, Swansea using a VG ZAB-E mass spectrometer or at Exeter University using a Kratos Profile HV3 instrument. Melting points were measured using an Electrothermal melting point instrument and are uncorrected.

GC-MS experiments were carried out at Zeneca Specialities, Blackley, Manchester, using a Trio-1 mass spectrometer (EI) and a 30-m DB624 capillary column with an injection temperature of 250 °C and an oven temperature of 80–250 °C (20 °C min^{-1}). Elemental analysis was also performed at Zeneca.

All HPLC were run using a Gilson 303 dual pump machine with a UV detector set at 254 nm. Preparative HPLC was achieved using an ODS Hypersil 5 μm column (28 cm × 2.5 cm i.d.) with a flow rate of 12 cm^3 min^{-1} and water–acetonitrile (3:1) as the eluent.

The pH 7.0 phosphate buffer solution used for the biotransformations was prepared using 100 mmol dm^{-3} solutions of K_2HPO_4 and KH_2PO_4. A CSI AGB 2000 pH meter and combination electrode was used to determine the pH of the buffer solutions during preparation.

General Procedure for the Enzymic Hydrolysis of Nitriles and Amides.—The substrate was suspended in potassium phosphate buffer (100 mmol dm^{-3}, pH 7.0). The immobilised enzyme system (Nitrilase SP 361, 1 g 100 cm^{-3} of buffer) was added and the reaction shaken, in an orbital incubator, at 220 rev min^{-1}, 30 °C. The reaction was terminated by filtration of the enzyme (through a Celite pad). The aqueous filtrate was basified (pH 10, 2 mol dm^{-3} NaOH) and extracted with ethyl acetate, ether or chloroform. The combined extracts were washed with brine (1 × 50 cm^3), dried ($MgSO_4$), filtrered and the filtrate subjected to rotary evaporation to afford any unchanged nitrile and/or amide.

The aqueous portion was then acidified (pH 2, 2 mol dm^{-3} HCl) and again extracted with ethyl acetate, ether or chloroform. The combined extracts were washed with brine (1 × 50 cm^3), dried ($MgSO_4$) and subjected to rotary evaporation to afford the acid product. Quantities of substrate and buffer used, as well as reaction times, purification techniques and yields for each individual reaction are given below.

Enzymic Hydrolysis of Isophthalonitrile **16** *at Low Temperature.*—Isophthalonitrile **16** (400 mg, 3.13 mmol) was suspended in buffer solution (65 cm^3), to obtain a substrate concentration of 48.0 mmol dm^{-3}, and stirred at 4 °C for 49 h. This yielded, after work-up, 3-cyanobenzoic acid (375 mg, 2.55 mmol, 82%) and 3-amidobenzonitrile **16a** (50 mg, 0.34 mmol, 11%), as a white solids; R_F 0.10 [ethyl acetate–light petroleum (1:1)]; m.p. 205–209 °C (decomp.) [Found (EI): M^+, 146.0481 $C_8H_6N_2O$ requires 146.0480]; ν_{max}(Nujol)/cm^{-1} 3423, 3156 (NH str), 2230 (CN str) and 1707, 1626 (amide I and II); δ_H[250 MHz, $(CD_3)_2SO$] 7.62 (1 H, br s, NH), 7.69 (1 H, t, *J* 7.8, 5-H), 7.95–8.03 (1 H, m, 6-H), 8.13–8.22 (2 H, m, 4-H and NH) and 8.30 (1 H, s, 2-H); δ_C[62.9 MHz, $(CD_3)_2SO$] 111.4 (CN), 118.2 (C-1), 129.6, 130.9, 132.1, 134.5 (CH, Ph × 4), 135.2 (C-3) and 165.9 (CO); *m*/*z* (EI) 149 (100%), 146 (M^+, 45), 130 [$(M - NH_2)^+$, 75], 102 [$(M - CONH_2)^+$, 45], 83 (50), 71 (42) and 57 (70).

Enzymic Hydrolysis of Toluene-2,4-dicarbonitrile **41**.—Toluene-2,4-dicarbonitrile **41** (150 mg, 1.05 mmol) was suspended in buffer solution (35 cm^3), to afford a substrate concentration of 30.0 mmol dm^{-3}, and incubated for 183 h. A white solid was isolated and shown by NMR spectroscopy to be a mixture of 3-cyano-4-methylbenzoic acid **41a** and 3-cyano-6-methylbenzoic acid **41b** (3.6:1) (146 mg, 0.91 mmol, 86%). The two components were inseparable by TLC.

3-*Cyano*-4-*methylbenzoic acid* **41a** R_F 0.80 (ethyl acetate + 2% acetic acid); δ_H[300 MHz, $(CD_3)_2CO$] 2.60 (3 H, s, Me), 7.59 (1 H, d, *J* 8.0, 5-H), 8.16 (1 H, dd, *J* 8.0, *J* 2.0, 6-H) and 8.24 (1 H, d, *J* 2.0, 2-H); δ_C[75.5 MHz, $(CD_3)_2CO$] 20.6 (Me), 113.9 (C-3), 117.6 (CN), 130.1 (C-1), 131.6, 134.2, 134.3 (*C*H, Ph × 3), 147.5 (C-4) and 166.0 (CO).

3-*Cyano*-6-*methylbenzoic acid* **41b**. R_F 0.80 (ethyl acetate + 2% acetic acid); δ_H[300 MHz, $(CD_3)_2CO$] 2.67 (3 H, s, Me), 7.53 (1 H, d, *J* 8.0, 3-H), 7.82 (1 H, dd, *J* 8.0, *J* 2.0, 4-H) and 8.23 (1 H, d, *J* 2.0, 6-H); δ_C[75.5 MHz, $(CD_3)_2CO$] 21.9 (Me), 110.7 (C-5), 118.6 (CN), 132.0 (C-1), 133.7, 134.9, 135.5 (*C*H, Ph × 3), 146.4 (C-2) and 167.1 (CO).

The mixture was treated with diazomethane to afford the corresponding methyl esters, in quantitative yield, which were inseparable by TLC.

Methyl 3-*cyano*-4-*methylbenzoate*. R_F 0.90 (ethyl acetate); δ_H[300 MHz, $(CD_3)_2CO$] 2.61 (3 H, s, Me), 3.93 (3 H, s, OMe), 7.61 (1 H, d, *J* 8.0, 5-H), 8.14 (1 H, dd, *J* 8.0, 2.0, 6-H) and 8.22 (1 H, d, *J* 2.0, 2-H).

Methyl 3-*cyano*-6-*methylbenzoate*. R_F 0.90 (ethyl acetate); δ_H[300 MHz, $(CD_3)_2CO$] 2.65 (3 H, s, Me), 3.93 (3 H, s, OMe), 7.55 (1 H, d, *J* 8.0, 3-H), 7.84 (1 H, dd, *J* 8.0, 2.0, 4-H) and 8.18 (1 H, d, *J* 2.0, 6-H).

Preparation of 2,4-*Dicyanoaniline* **43**.—The title compound was prepared by the same method as was used to prepare 2,5-dicyanoaniline **44**, using 2,4-dibromoaniline (5.00 g, 20.0 mmol), copper(I) cyanide (3.94 g, 44.0 mmol) and dimethylformamide (DMF) (40 cm^3). Purification by column chromatography [ethyl acetate–light petroleum (1:2) as the eluent] afforded the required compound as a pale yellow solid (880 mg, 6.15 mmol, 31%); R_F 0.22 [ethyl acetate–light petroleum (1:2)]; m.p. 206–209 °C (lit.,[48] 217–218 °C) [Found (EI): M^+, 143.0471. $C_8H_5N_3$ requires 143.0483]; ν_{max}(Nujol)/cm^{-1} 3470, 3375, 3240 (NH str), 2222 (CN str) and 1641 (CC str); δ_H[250 MHz, $(CD_3)_2CO$] 6.45 (2 H, br s, NH × 2), 7.02 (1 H, d, *J* 9.0, 6-H), 7.63 (1 H, dd, *J* 9.0, *J* 2.0, 5-H) and 7.88 (1 H, d, *J* 2.0, 3-H); δ_C[62.9 MHz, $(CD_3)_2CO$] 96.1 (C-2), 99.9 (C-4), 116.3 (CN), 116.6 (C-6), 118.8 (CN), 137.4 (C-3 or C-5), 138.3 (C-5 or C-3) and 154.8 (C-1); *m*/*z* (EI) 143 (M^+, 10%), 142 [$(M - H)^+$, 100] and 116 (22).

Enzymic Hydrolysis of 2,4-*Dicyanoaniline* **43**.—2,4-Dicyanoaniline **43** (250 mg, 1.75 mmol) was suspended in buffer solution (350 cm^3), to afford a substrate concentration of 5.0 mmol dm^{-3}, and incubated for 49 h. A yellow solid was isolated and shown by ^1H NMR spectroscopy to be a mixture of 4-amino-3-cyanobenzoic acid **43a** and 6-amino-3-cyanobenzoic acid **43b** (2:1) (231 mg, 1.43 mmol, 81%). The isolated mixture was dissolved in methanol (15 cm^3) and concentrated hydrochloric acid (0.1 cm^3) was added to the solution. The reaction mixture was then refluxed for 65 h and extracted with ethyl acetate (3 × 20 cm^3). The combined extracts were washed with saturated aqueous sodium hydrogen carbonate (2 × 20 cm^3) and brine (1 × 20 cm^3), dried ($MgSO_4$), filtered and subjected to rotary evaporation to afford a mixture of the corresponding methyl esters (206 mg, 1.17 mmol, 67%). Purification by preparative HPLC allowed the two regioisomers to be separated.

Methyl 4-*amino*-3-*cyanobenzoate* (70 mg, 0.40 mmol, 23%); R_t 35.5 min; m.p. 154–155 °C [Found (EI): M^+, 176.0595. $C_9H_8N_2O_2$ requires 176.0585]; ν_{max}(Nujol)/cm^{-1} 3465, 3375 (NH str), 2222 (CN str) and 1729 (CO str); δ_H[300 MHz, $(CD_3)_2CO$] 3.84 (3 H, s, OMe), 6.30 (2 H, br s, NH × 2), 6.98 (1 H, d, *J* 8.5, 5-H), 7.92 (1 H, dd, *J* 8.5, 2.0, 6-H) and 8.04 (1 H, d, *J* 2.0, 2-H); δ_C[75.5 MHz, $(CD_3)_2CO$] 52.0 (OMe), 95.2 (C-3), 115.5 (C-5), 117.2 (CN), 119.2 (C-1), 135.5 (C-2 or C-6), 135.6 (C-6 or C-2), 155.3 (C-4) and 165.8 (CO); *m*/*z* (EI) 176 (M^+, 40%), 145 (100) and 117 [$(M - CO_2Me)^+$, 21].

Methyl 2-*amino*-5-*cyanobenzoate* (35 mg, 0.20 mmol, 11%); R_t 55.5 min; m.p. 126–128 °C [Found (EI) M^+: 176.0593. $C_9H_8N_2O_2$ requires 176.0585]; ν_{max}(Nujol)/cm^{-1} 3473, 3366 (NH str), 2215 (CN str) and 1712 (CO str); δ_H[300 MHz, $(CD_3)_2CO$] 3.90 (3 H, s, OMe), 6.97 (1 H, d, *J* 8.5, 3-H), 7.20 (2 H, br s, NH × 2), 7.55 (1 H, dd, *J* 8.5, 2.0, 4-H) and 8.13 (1 H, d, *J* 2.0, 6-H); δ_C[75.5 MHz, $(CD_3)_2CO$] 52.2 (OMe), 98.2 (C-5), 110.5 (CN), 118.2 (C-3), 119.7 (C-1), 136.8 (C-4 or C-6), 137.1 (C-6 or C-4), 155.1 (C-2) and 167.6 (CO); *m*/*z* (EI) 176 (M^+, 60%), 144 (100) and 117 [$(M - CO_2Me)^+$, 40].

Preparation of 2,5-*Dicyanoaniline* **44**.—Copper(I) cyanide (7.00 g, 78.0 mmol) was added to a solution of 2,5-dibromoaniline (5.00 g, 20.0 mmol) in DMF (40 cm^3). The reaction mixture was stirred at 140 °C for 21 h and then poured, whilst hot, into 10% aqueous sodium cyanide (100 cm^3). Upon cooling to room temperature the mixture was extracted with ethyl acetate (3 × 150 cm^3). The combined extracts were washed with water (2 × 100 cm^3) and brine (1 × 100 cm^3), dried ($MgSO_4$), filtered and subjected to rotary evaporation to afford an orange solid (1.89 g). Purification of this by column chromatography [ethyl acetate–light petroleum (1:2) as the eluent] afforded the title compound as a pale yellow solid (830 mg, 5.80 mmol, 29%); R_f 0.45 [ethyl acetate–light petroleum (1:2)]; m.p. 202–204 °C (lit.,[49] 188–192 °C) [Found (EI): M^+, 143.0480. $C_8H_5N_3$ requires 143.0483]; ν_{max}(Nujol)/cm^{-1} 3456, 3364 (NH str) and 2221 (CN str); δ_H[300 MHz, $(CD_3)_2CO$] 6.05 (2 H, br s, NH × 2), 7.02 (1 H, dd, *J* 8.0, 2.0, 4-H), 7.27 (1 H, d, *J* 2.0, 6-H) and 7.61 (1 H, d, *J* 8.0, 3-H); δ_C[75.5 MHz, $(CD_3)_2CO$] 99.56 (C-2), 116.8 (CN), 117.7 (C-5), 118.4 (CN), 119.2 (C-4 or C-6), 119.7 (C-6 or C-4), 134.5 (C-3) and 152.1 (C-1); *m*/*z* (EI) 143 (M^+, 100%), 116 (48), 89 (10) and 58 (15).

Enzymic Hydrolysis of 2,5-*Dicyanoaniline* **44**.—2,5-Dicyanoaniline **44** (200 mg, 1.40 mmol) suspended in buffer solution

(275 cm^3), to afford a substrate concentration of 5.1 mmol dm^{-3}, was incubated for 95 h. A yellow solid was isolated and shown by ^1H NMR spectroscopy to be a mixture of 3-amino-4-cyanobenzoic acid **44a** and 2-amino-4-cyanobenzoic acid **44b** (1:1) (181 mg, 1.12 mmol, 80%). The corresponding methyl esters were prepared using methanol and concentrated hydrochloric acid, as described for **43a** and **43b**. Purification by column chromatography [ethyl acetate–light petroleum (1:4) +2% Et_3N as the eluent] allowed separation of the two regio-isomers.

Methyl 3-amino-4-cyanobenzoate (50 mg, 0.28 mmol, 20%); R_F 0.18 [ethyl acetate–light petroleum (1:4)]; m.p. 137–138 °C [Found (EI): M^+, 176.0589. $C_9H_8N_2O_2$ requires 176.0585]; ν_{max}(Nujol)/cm^{-1} 3466, 3395 (NH str), 2212 (CN str) and 1705 (CO str); δ_H[300 MHz, $(CD_3)_2SO$] 3.84 (3 H, s, OMe), 6.35 (2 H, br s, NH × 2), 7.09 (1 H, dd, *J* 8.5, 2.0, 6-H), 7.43 (1 H, d, *J* 2.0, 2-H) and 7.51 (1 H, d, *J* 8.5, 5-H); δ_C[75.5 MHz, $(CD_3)_2SO$] 52.3 (OMe), 96.9 (C-4), 115.4 (C-2 or C-6), 115.8 (C-6 or C-2), 117.3 (CN), 133.0 (C-5), 134.2 (C-1), 151.6 (C-3) and 165.5 (CO); *m/z* (EI) 176 (M^+, 70%), 145 (100), 117 [$(M - CO_2Me)^+$, 75], 90 (48), 63 (35) and 56 (53).

Methyl 2-amino-4-cyanobenzoate (55 mg, 0.31 mmol, 22%); R_F 0.30 [ethyl acetate–light petroleum (1:4)] m.p. 115–117 °C [Found (EI): M^+, 176.0579. $C_9H_8N_2O_2$ requires 176.0585]; ν_{max}(Nujol)/cm^{-1} 3463, 3360 (NH str), 2231 (CN str) and 1708 (CO); δ_H[300 MHz, $(CD_3)_2SO$] 3.82 (3 H, s, OMe), 6.85 (1 H, dd, *J* 8.5, 2.0, 5-H), 6.95 (2 H, br s, NH × 2), 7.17 (1 H, d, *J* 2.0, 3-H) and 7.81 (1 H, d, *J* 8.5, 6-H); δ_C[75.5 MHz, $(CD_3)_2SO$] 51.8 (OMe), 112.1 (CN), 116.5 (C-5), 118.3 (C-4), 120.1 (C-3), 128.1 (C-1), 131.9 (C-6), 150.8 (C-2) and 166.7 (CO); *m/z* (EI) 176 (M^+, 50%), 144 (100), 117 [$(M - CO_2Me)^+$, 60] and 90 (30).

Enzymic Hydrolysis of 2,4,5,6-Tetrafluoroisophthalonitrile **45**.—2,4,5,6-Tetrafluoroisophthalonitrile **45** (300 mg, 1.50 mmol) suspended in buffer solution (300 cm^3) to afford a substrate concentration of 5.0 mmol dm^{-3} was incubated for 21 h. After work-up, purification of the product by column chromatography [ethyl acetate–light petroleum (1:3) as the eluent] yielded 3-carbamoyl-2,4,5,6-tetrafluorobenzonitrile **45a** (130 mg, 0.60 mmol, 40%) as a white crystalline solid; R_F 0.65 (ethyl acetate); m.p. 142–143 °C (Found: C, 44.2; H, 1.1; F, 34.3; N, 12.9. $C_8H_2F_4N_2O$ requires C, 44.05; H, 0.92; F, 34.84; N, 12.84%) [Found (EI): M^+, 218.0103. $C_8H_2F_4N_2O$ requires 218.0103]; ν_{max}(Nujol)/cm^{-1} 3379, 3201 (NH str), 2248 (CN str) and 1674, 1641 (amide I and II); δ_F[235 MHz, $(CD_3)_2CO$] 1.73 (1 F, ddd, J_{FF} 22.0, J_{FF} 20.0, J_{FF} 10.5, 5-F), 37.75 (1 F, ddd, J_{FF} 20.0, J_{FF} 11.5, J_{FF} 3.5.4-F or 6-F), 39.42 (1 F, ddd, J_{FF} 22.0, J_{FF} 11.5, J_{FF} 3.5, 6-F or 4-F) and 52.58 (1 F, dt, J_{FF} 10.5, J_{FF} 3.5, 2-F); *m/z* (EI) 218 (M^+, 60%), 202 [$(M - NH_2)^+$, 100], 174 [$(M - CONH_2)^+$, 28], 124 (47) and 44 [$(CONH_2)^+$, 45].

Enzymic Hydrolysis of 2,3,5,6-Tetrafluoroterephthalonitrile **46**.—2,3,5,6-Tetrafluoroterephthalonitrile **46** (300 mg, 1.50 mmol) suspended in buffer solution (300 cm^3) to afford a substrate concentration of 5.0 mmol dm^{-3} was incubated for 120 h. Purification by column chromatography [ethyl acetate–light petroleum (1:1) as the eluent] yielded 4-carbamoyl-2,3,5,6-tetrafluorobenzonitrile **46a** (240 mg, 1.10 mmol, 73%) as a crystalline solid; R_F 0.48 [ethyl acetate–light petroleum (1:1)]; m.p. 146–148 °C [Found (EI): M^+, 218.0103. $C_8H_2F_4N_2O$ requires 218.0103]; ν_{max}(Nujol)/cm^{-1} 3446, 3330 (NH str), 2255 (CN str) and 1677, 1623 (amide I and II); δ_F[235 MHz, $(CD_3)_2CO$] 23.68–23.88 (2 F, m) and 30.40–30.60 (2 F, m); *m/z* (EI) 218 (M^+, 90%), 202 [$(M - NH_2)^+$, 100], 174 [$(M - CONH_2)^+$, 28], 124 (60) and 44 [$(CONH_2)^+$, 65].

Enzymic Hydrolysis of 5-Chloro-2,4,6-trifluoroisophthalonitrile **47**.—5-Chloro-2,4,6-trifluoroisophthalonitrile **47** (300 mg, 1.39 mmol) suspended in buffer solution (275 cm^3) to afford a substrate concentration of 5.1 mmol dm^{-3} was incubated for 43.75 h. After work-up, purification of the product by column chromatography (ethyl acetate as the eluent) afforded 3-carbamoyl-5-chloro-2,4,6-trifluorobenzonitrile **47a** (66 mg, 0.28 mmol, 20%), as a white solid; R_F 0.58 [ethyl acetate–light petroleum (1:1)], m.p. 161–162 °C [Found (CI): M^+, 233.9808. $C_8H_2ClF_3N_2O$ requires 233.9808]; ν_{max}(Nujol)/cm^{-1} 3365, 3195 (NH str), 2249 (CN str) and 1665, 1626 (amide I and II); δ_F[235 MHz, $(CD_3)_2CO$] 56.79 (1 F, d, J_{FF} 7.4), 59.71 (1 F, d, J_{FF} 7.4) and 61.35 (1 F, t, J_{FF} 7.4); *m/z* (CI) 233 (M^+, 10%), 231 (80), 214 (100), 172 (40), 140 (55), 75 (44), 58 (40) and 44 [$(CONH_2)^+$, 98].

Enzymic Hydrolysis of 2,4-Dicyano-3,5,6-trifluoroaniline **48**.—2,4-Dicyano-3,5,6-trifluoroaniline **48** (250 mg, 1.27 mmol) suspended in buffer solution (250 cm^3) to afford a substrate concentration of 5.1 mmol dm^{-3} was incubated for 114.5 h. After work-up, purification of the product by column chromatography [chloroform–acetone (23:2) +2% Et_3N as the eluent] yielded 2-carbamoyl-4-cyano-3,5,6-trifluoroaniline **48a** (40 mg, 0.19 mmol, 15%), as a white solid, and recovered substrate **48** (115 mg, 0.53 mmol, 46%); R_F 0.65 (ethyl acetate); m.p. 213–215 °C [Found (CI): M^+, 215.0306. $C_8H_4F_3N_3O$ requires 215.0306]; ν_{max}(Nujol)/cm^{-1} 3509, 3434 (NH str), 2233 (CN str) and 1659 (CO str); δ_F[235 MHz, $(CD_3)_2CO$] 0.00–0.20 (1 F, m), 32.16–32.28 (1 F, m) and 55.87–56.02 (1 F, m); δ_C[100.6 MHz, $(CD_3)_2SO$] 75.6 (dd, J_{CF} 24.0, 16.0, C-4), 104.3 (d, J_{CF} 19.0, C-2), 110.2 (s, CN), 134.4 (dd, J_{CF} 239.0, 10.0, C-6), 143.7–143.9 (m, C-1), 149.8 (ddd, J_{CF} 254.0, 13.0, 10.0, C-5), 157.3 (dd, J_{CF} 253.0, 7.0, C-3) and 163.1 (s, CO); *m/z* (CI) 215 (M^+, 5%) and 102 (100).

Enzymic Hydrolysis of 2,5-Dicyano-3,4,6-trifluoroaniline **49**.—2,5-Dicyano-3,4,6-trifluoroaniline **49** (300 mg, 1.52 mmol) suspended in buffer solution (300 cm^3) to afford a substrate concentration of 5.1 mmol dm^{-3} was incubated for 69.5 h. After work-up purification of the product by column chromatography [ethyl acetate–light petroleum (1:1) as the eluent] afforded 2-carbamoyl-5-cyano-3,4,6-trifluoroaniline **49a** (170 mg, 0.79 mmol, 52%) as a white solid; R_F 0.66 (ethyl acetate); m.p. 193–195 °C [Found (CI): M^+, 215.0306. $C_8H_4F_3N_3O$ requires 215.0306]; ν_{max}($CHCl_3$)/cm^{-1} 3496, 3447, 3375, 3328 (NH str), 2252 (CN str) and 1662, 1607 (amide I and II); δ_F[235 MHz, $(CD_3)_2CO$] 12.70 (1 F, dd, J_{FF} 23.0, J_{FF} 9.5, 3-F), 23.27 (1 F, ddd, J_{FF} 23.0, 13.0, J_{HF} 3.5, 4-F) and 32.04 (1 F, dd, J_{FF} 13.0, 9.5, 6-F); δ_H[250 MHz, $(CD_3)_2CO$] 6.20 (2 H, br s, NH × 2) and 7.50 (2 H, br s, NH × 2); δ_C[100.6 MHz, $(CD_3)_2SO$] 92.2 (ddd, J_{CF} 18.0, 18.0, 3.0, C-5), 109.1 (d, J_{CF} 3.0, CN), 114.9 (dd, J_{CF} 18.0, 6.0, C-2), 133.1 (ddd, J_{CF} 13.0, 5.0, 3.0, C-1), 139.0 (ddd, J_{CF} 247.0, 12.0, 3.0, C-3 or C-4), 143.4 (ddd, J_{CF} 243.0, 12.0, 3.0, C-4 or C-3), 145.6 (d, J_{CF} 252.0, C-6) and 162.7 (s, CO); *m/z* (CI) 215 (M^+, 100%), 198 (10) and 171 [$(M - CONH_2)^+$, 6].

Chemical Hydrolysis of 2-Carbamoyl-5-cyano-3,4,6-trifluoroaniline **49a**.—2-Carbamoyl-5-cyano-3,4,6-trifluoroaniline was heated at 110 °C for 6 h in a solution of sulfuric acid (6 mmol dm^{-3}; 2.5 cm^3) and glacial acetic acid (5 cm^3). Upon cooling of the reaction mixture to room temperature; sodium hydroxide (2 mmol dm^{-3}) was added to it until it reached pH 10. The basic solution was extracted with ethyl acetate (3 × 10 cm^3) and the combined extracts were washed with brine (1 × 25 cm^3), dried (Na_2SO_4), filtered and subjected to rotary evaporation to afford an off-white solid (10 mg). Analysis of this by GC indicated that it was a multi-component mixture. GC-MS studies revealed that one of the components was 5-cyano-3,4,6-trifluoroaniline **51**; GC-MS: R_t 9.46 min; *m/z* (EI) 172 (M^+, 100%).

Enzymic Hydrolysis of 5-Chloro-4-ethoxy-2,6-difluoroisophthalonitrile **50**.—5-Chloro-4-ethoxy-2,6-difluoroisophthalonitrile **50** (300 mg, 1.24 mmol) suspended in buffer solution (250 cm^3) to afford a substrate concentration of 5.0 mmol dm^{-3} was incubated for 310 h. A pale brown solid (151 mg, 0.58 mmol, 47%) was isolated which consisted of a (1:1) mixture of regioisomers **50a** and **50b**, as evidenced by ^1H and ^{19}F NMR spectroscopy. Separation of the two isomers by column chromatography [ethyl acetate–light petroleum (3:2) as eluent] afforded two off-white solids.

Compound **50a** (27 mg, 0.10 mmol, 8%), R_F 0.54 [ethyl acetate–light petroleum (1:1)] [Found (EI): M^+, 260.0165. $C_{10}H_7ClF_2N_2O_2$ requires 260.0164]; δ_F[235 MHz, $(CD_3)_2CO$] 55.57 (1 F, d, J_{FF} 7.0), 60.16 (1 F, d, J_{FF} 7.0); δ_H[250 MHz, $(CD_3)_2CO$] 1.50 (3 H,t, J 6.7, Me), 4.48 (2 H, q, J 6.7, CH_2), 7.45 (1 H, br s, NH) and 7.60 (1 H, br s, NH); *m/z* (EI) 260 (M^+, 15%), 232 (35), 216 [$(M - CONH_2)^+$, 100] and 44 [$(CONH_2)^+$, 70].

Compound **50b** (28 mg, 0.10 mmol, 8%), R_F 0.36 [ethyl acetate–light petroleum (1:1)] [Found (CI): $(M + H)^+$, 261.0242. $C_{10}H_8ClF_2N_2O_2$ requires 261.0242]; δ_F[235 MHz, $(CD_3)_2CO$] 54.21 (1 F, s) and 58.60 (1 F, s); δ_H[250 MHz, $(CD_3)_2CO$] 1.42 (3 H, t, J 6.7, Me), 4.38 (2 H, q, J 6.7, CH_2), 7.30 (1 H, br s, NH) and 7.55 (1 H, br s, NH); *m/z* (CI) 261 [$(M + H)^+$, 100w], 215 [$(M - OEt)^+$, 30] and 44 [$(CONH_2)^+$, 10].

Acknowledgements

We are grateful to the SERC and Zeneca for the provision of a CASE Award (J. S. P.) and Novo Nordisk, Denmark for a gift of immobilised *Rhodococcus* sp. SP361.

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Paper 4/00864B
Received 14*th February* 1994
Accepted 16*th February* 1994