An in-depth study of the biotransformation of nitriles into amides and/or acids using *Rhodococcus rhodochrous* AJ270[1]

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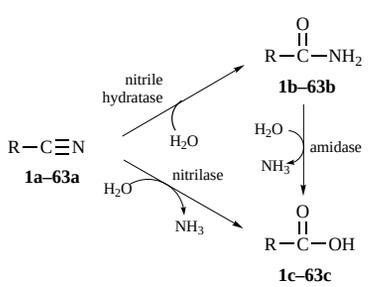
A variety of aliphatic, aromatic and heterocyclic nitriles have been readily hydrolysed into the corresponding amides and/or acids under very mild conditions using *Rhodococcus sp.* AJ270. The nitrile hydratase involved in this novel nitrile-hydrolysing microorganism efficiently hydrates most nitriles tested, irrespective of the electronic and steric effects of the substituents, to form the amides. Conversion of amides into acids catalysed by the associated amidase is rapid and efficient in most cases. Substrates bearing an adjacent substituent (which may be an *ortho* substituent on an aromatic nitrile, an adjacent heteroatom in a heterocyclic ring or a geminal substituent in an α,β-unsaturated nitrile) undergo slow hydrolysis of the amides allowing efficient amide isolation. The scope, limitations and reaction mechanism of this enzymatic process have been systematically studied. A molecular size of >7 Å diameter and the presence of functions capable of metal complexation near to the nitrile inhibit hydrolysis.

Introduction

The organisms and their enzymes

There has been increasing interest in enzyme-catalysed organic reactions due to their high chemo-, regio- and stereo-selectivity and very mild conditions.[2] Applications of hydrolytic enzymes such as lipases and esterases have been well documented while, surprisingly, the use of nitrile-hydrolysing enzymes in synthesis has received scant attention until recently despite the fact that the biotransformation of nitriles into the corresponding carboxylic acids has been known for decades.[2,3] Enzyme-catalysed hydrolysis of nitriles has been shown to proceed by two distinct routes; a nitrilase[4] transforms the nitriles directly into acids, or a nitrile hydratase forms the amide which is hydrolysed to the acid by an amidase.[5] (Scheme 1). So far a range of microorganisms, including *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Nocardia*, *Pseudomonas* and *Rhodococcus*, has been shown to contain a nitrilase or nitrile hydratase–amidase, or both.[3] For examples, *Nocardia rhodochrous* NCIB 11216 is a long-used nitrilase system,[4,6] while *Brevibacterium* B312 is a well-studied nitrile hydratase-containing organism.[3b,3c,7] *Rhodococcus sp.* N-774[8] and *Pseudomonas chlororaphis* B23[9] were selected as the first- and second-generation strains in the 1980s for the industrial production of acrylamide from acrylonitrile. It has been demonstrated more recently that both nitrilase and nitrile hydratase–amidase pathways can occur in the same strains, such as *Nocardia rhodochrous* LL100-21[10] and *Rhodococcus rhodochrous* J1.[11] More importantly the three different enzymes have been induced selectively by the growth of the organism under optimum culture conditions. *Rhodococcus rhodochrous* J1 is currently being used in Japan to produce tens of thousands of tonnes of acrylamide per annum.[12] The mechanisms of enzymatic hydrolyses of nitriles using nitrilase and nitrile hydratase have also been proposed[4b,13] and for those transition metal-containing nitrile hydratases it is believed that the hydration stage involves the 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] It should be noted here that for a long time it was mistakenly assumed that aliphatic nitrile hydrolyses were mediated by the hydratase enzyme, while aromatic and heterocyclic systems utilised a nitrilase enzyme.[15]



Scheme 1

The transformations observed

Despite the fact that commercial production of acrylamide through microbial hydrolysis of acrylonitrile has been operating successfully for some years, preparative biotransformations of nitriles into amides and/or acids have remained largely unexplored.[15] No systematic study of substrate specificity or stereoselectivity of the enzymes involved has appeared. The literature data is rather confused in terms of predicting effective hydrolysis and the associated selectivity. This is partly due to the variety of systems used and their mode of generation. Nagasawa, Yamada and their co-workers[16] have reported that a few aromatic and heterocyclic nitriles give amides as the sole product when reacted with *R. rhodochrous* J1 organism (a system lacking significant amidase action) grown in a medium containing cobalt ions and crotonamide or urea. Several groups[17] showed that an immobilised whole cell *Rhodococcus sp.* (a nitrile hydratase system) developed by Novo Industri of Denmark slowly hydrolysed *para*- and *meta*-substituted benzonitriles though with poor conversions. Using the same Novo preparation, several heterocyclic nitriles were slowly converted into amides and/or acids.[18] When aliphatic nitriles were subject to nitrile hydratase systems, apart from the formation of acrylamide from acrylonitrile, acids were produced in most cases.[17b,19] A Hammett-type linear free energy correlation was observed between initial rates and the nature of *para*-substituents in the Novo enzyme-catalysed hydrolysis of *para*-substituted benzyl cyanides.[20]

Synthetic applications of *Rhodococcus sp.* AJ270

At Sunderland over a number of years a large number of soil-derived nitrile hydrolysing organisms have been screened[21] and we have currently focused on the most robust and versatile, *Rhodococcus sp.* AJ270, a nitrile hydratase system. We herein

Table 1 Hydrolysis of mono-substituted benzonitriles RC_6H_4CN[a]

Substrate **a**	R	Reaction time/h	Product yield (%) Amide **b**	Acid **c**	Nitrile **a**
1	H	24	—	97.5	—
2	*p*-Me	24	—	82.2	—
3	*p*-MeO	28	—	86.6	—
4	*p*-PhO	72	—	59.3	40.5
5	*p*-Cl	48	—	89.4	—
6	*p*-F	24	—	93.3	—
7	*p*-NO_2	44	—	71.5	—
8	*p*-Ac	24	—	99.3	—
9	*p*-MeO_2C	22	—	96.5	—
10	*p*-HO	20	—	28.5	—
11	*p*-H_2N	9	63.7	9.7	—
		48	30.1	37.5	—
12	*m*-NO_2	28	—	91.7	—
13	*m*-MeO	96	—	91.3	—
14	*m*-HO	7	—	56.0	—
15	*m*-H_2N	7	—	78.5	—
16	*o*-Me	5.5	92.4	6.6	—
		120	—	98.9	—
17	*o*-MeO	4	93.1	4.9	—
		18	57.5	27.7	—
		96	—	94.8	—
18	*o*-PhO	24	—	—	100
		72	—	—	85.0
19	*o*-Cl	6.5	94.1	4.4	—
		168	—	79.9	—
20	*o*-NO_2	4	89.5	10.0	—
		18	60.2	29.5	—
		90	—	85.7	—
21	*o*-HO	3	80.1	5.0	—
		28	—	69.0	—
22	*o*-H_2N	1	97.7	—	—
		24	—	62.3	—

[a] 3 mmol of the nitriles were used and reaction conditions were not optimised.

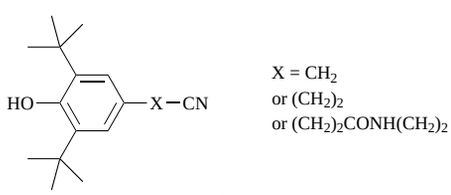
report the first stage of a systematic study of the applications of this novel organism in organic synthesis.[1] In order to examine the scope and limitations of these biotransformations, the substrate specificity of *Rhodococcus* AJ270 was tested against both saturated and unsaturated aliphatic nitriles, aromatic nitriles with different substituents and substitution patterns and heterocyclic nitriles. We present for the first time a rationalisation of the enzyme-catalysed hydrolysis of nitriles.

It is generally more convenient and economical in preparative biotransformation to use whole cell systems rather than the purified or semi-purified enzymes. The biomass of *Rhodococcus* AJ270 was prepared in bulk in a 20 l fermentor using acetamide as both the carbon and nitrogen source. The cells, harvested by continuous centrifuge at 4 °C, were kept at −20 °C without losing activity for several months. The biotransformations were simply effected by resuspending the cells in potassium phosphate buffer (pH 7.0) and incubating with the nitriles at 30 °C. The reactions were monitored by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) and the products obtained either through continuous extraction of the whole reaction mass or, in sensitive cases, by extraction with organic solvent after removal of the cells by filtration with the aid of Celite.

Hydrolysis of aromatic nitriles

When reacted with *Rhodococcus* AJ270, *para*- or *meta*-substituted benzonitriles were hydrolysed efficiently to give the corresponding acids irrespective of the nature of the substituents. Only in one case, 4-aminobenzonitrile, were both amide and acid isolated, indicating that the amide-to-acid transformation was slower than the nitrile-to-amide step (Table 1). Traces of the amide are occasionally visible by TLC after a few hours action. However, shortening the reaction time only led to low conversions of nitriles to acids without isolation of amides. In sharp contrast, *ortho*-substituted benzonitriles were rapidly and efficiently transformed into amides while conversion of amides to acids proceeded slowly. Amides or acids are thus both separately available from the same starting nitrile simply by controlling the reaction time (Table 1). This *ortho*-substituent effect, which operates irrespective of the nature of the substituent, suggests that while the first step, hydration of the linear nitrile group, is not significantly hindered by steric and electronic factors, the second amidase-mediated step is very sensitive to steric factors. In other words, in reactions of benzonitriles without an *ortho*-substituent the amidase reaction is significantly faster than the hydratase step, while for with *ortho*-substituted compounds the reverse is true. To examine the generality of this steric effect a number of other aromatic nitriles were studied. The results (Table 2) further illustrate convincingly the generality of the steric effect.

More sterically-hindered compounds, such as 2,6-difluoro- and 2,6-dichloro-benzonitriles, gave amides as the major or the sole product, while cyanonaphthalenes yielded acids. The lower conversion (or slower rate of conversion) of 4-phenoxybenzonitrile **4a**, 2,6-dichlorobenzonitrile **24a** and 1-naphthonitrile **28a**, and the total lack of reaction of 2-phenoxybenzonitrile **18a**, 2,6-dimethylbenzonitrile **25a**, 2,6-dimethoxybenzonitrile **26a**, 9-cyanoanthracene **30a** and 9-cyanophenanthrene **31a** also reflect the size limitation of the nitrile hydratase in *Rhodococcus* AJ270. It would seem that the metal of the nitrile hydratase enzyme complexes the nitrile nitrogen and performs the hydration of this complex in a pocket of limited size, or that there is a 'bottle-neck' *en route* to the active site. This restriction is of a diameter no greater than *ca.* 7 Å. Therefore 2,6-disubstituted benzonitriles (9-cyanoanthracene being viewed as a special example of such a molecule) are excluded, with the exception of 2,6-difluoro- and 2,6-dichloro-benzonitrile. This size restriction explains why, for example, other bulky nitriles reported elsewhere are unaffected by nitrile hydratases, even when the nitrile function is quite remote from the steric problem. For example, the interesting 4-hydroxy-2,6-di-*tert*-butylphenyl-substituted nitriles **32** are unaffected by nitrile hydratases even when a long



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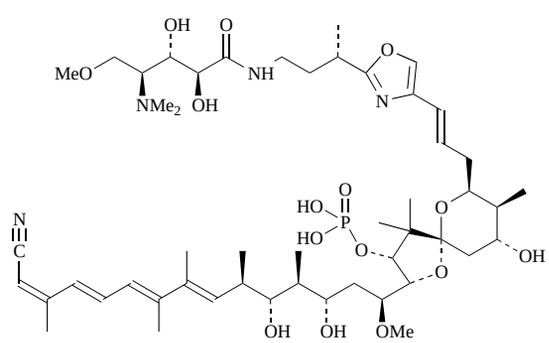


33 (–)-Calyculin A

chain interposes between the aryl unit and the nitrile.[22] Also we find that remote nitrile functions attached to large molecules suffer similar non-reactivity as exemplified by calyculin A **33**, though the AJ270 remains viable after several days attempted hydrolysis.[23]

Hydrolysis of heterocyclic nitriles

Rhodococcus AJ270 also shows high nitrile hydratase activity against heterocyclic nitriles (Table 3). However, six-membered heterocycles bearing a C=O or C=N group adjacent to the nitrile are poor substrates.[18] Thus, after six days' interaction with the biomass cyanopyridones **43a** and **44a** and pyridazinone **45a** were recovered intact, while the hydrolyses of 2-cyanopyridine **38a** and 1-cyanoisoquinoline **42a** proceeded sluggishly with low conversion. This is in contrast with the hydrolysis of 2-amino- and 2-hydroxy-benzonitriles. It would appear that coordination of the adjacent group with the metal could interfere with nitrile hydrolysis. To eliminate inefficient diffusion of the substrates through the cell wall, the reactions were repeated using a crude extract of the enzymes instead of whole cells; no hydrolysis was observed. Interestingly, the adjacent substituent effect discovered in the hydrolysis of aromatic nitriles was also encountered in the cases of heterocyclic nitriles. The adjacent ring heteroatoms, including oxygen, sulfur and nitrogen, behave like an *ortho*-substituent allowing selective heterocyclic amide or acid formation. Isolation of isonicotinamide **40b**, as with that of 4-aminobenzamide **11b**, shows that the presence of an amino group or a nitrogen in the heterocyclic ring at the *para*-position also lowers the rate of amide hydrolysis. This effect, which totally differs from that of adjacent substituent, may result from competitive interaction of the nucleophilic nitrogen atom with the active site of the amidase.

Table 2 Hydrolysis of other aromatic nitriles RCN[a]

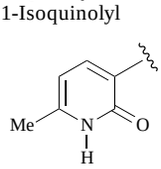
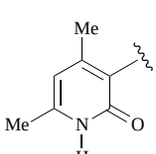
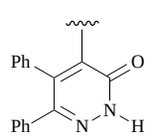
Substrate **a**	R	Reaction time/h	Product yield (%) Amide **b**	Acid **c**	Nitrile **c**
23	2,6-$F_2C_6H_3$	24	80.0	14.9	—
24	2,6-$Cl_2C_6H_3$	192	23.1	—	69.6
25	2,6-$Me_2C_6H_3$	120	—	—	87.5
26	2,6-$(MeO)_2C_6H_3$	216	—	—	80.1
27	3,4-$(OCH_2O)C_6H_3$	24	—	88.9	—
28	1-Naphthyl	168	—	80.3	16.3
29	2-Naphthyl	120	—	69.3	—
30	9-Anthracenyl	168	—	—	96.1
31	9-Phenanthrenyl	168	—	—	92.7

[a] 3 Mmol of the nitriles were used and reaction conditions were not optimised.

Hydrolysis of aliphatic nitriles

Almost all of the aliphatic nitriles tested, whether saturated or unsaturated, were efficiently hydrolysed to the corresponding acids (Table 4). It is worth noting that acrylonitrile and cinnamonitrile were transformed into the acids without significant formation of the amide while methacrylamide was isolated after short action with *Rhodococcus* AJ270. This shows again that an adjacent substituent slows down the second hydrolytic step. However, when the pivalo- **51a** and 1-adamantylcarbonitriles **52a** having a very bulky group attached to the CN were hydrolysed, no amide was found and only acid was isolated with high conversion. The rate of the amide hydrolysis by amidase here is greater than that of nitrile hydration. Apparently the amidase activity is not only affected by the size but also by the orientation of the substituents. The difference between aromatic or α,β-unsaturated amides bearing an adjacent group and 1-adamantylcarboxamide or pivalamide is that only in the non-aliphatic molecules are adjacent substituents locked in the same plane as the carbonyl group. This suggests that a coplanar adjacent substituent hinders the attack of an amino acid residue of the amidase on the amide carbonyl. Surprisingly, little[2] has been reported on the mechanism of amidase action.

Table 3 Hydrolysis of heterocyclic nitriles RCN[a]

Substrate **a**	R	Amount/ mmol	Reaction time/h	Product yield (%) Amide **b**	Acid **c**	Nitrile **a**
34	2-Furyl	3	1	82.2	12.5	—
		5	7	—	83.1	—
35	2-Thienyl	3	1.5	72.8	28.1	—
		5	48	—	85.8	—
36	1,5-Dimethylpyrrolyl	3	2	81.3	—	16.1
		3	72	87.1	—	11.1
37	3-Indolyl	2	144	—	50.4	29.3
38	2-Pyridyl	3	144	—	46.3	24.6
39	3-Pyridyl	5	24	—	61.8	—
40	4-Pyridyl	3	2	58.9	—	—
		3	72	—	50.1	—
41	3-Quinolyl	3	55	—	57.7	—
42	1-Isoquinolyl	3	120	12.0	—	62.5
43		3	144	—	—	93.2
44		3	144	—	—	96.4
45		2	144	—	—	98.9

[a] Reaction conditions were not optimised.

Table 4 Hydrolysis of aliphatic nitriles RCN[a]

Substrate **a**	R	Amount/ mmol	Reaction time/h	Product yield (%) Amide **b**	Acid **c**	Nitrile **a**
46	Vinyl	10	0.5	—	98.1	—
		10	16	—	68.6	—
47	*trans*-Styryl	3	16	—	91.5	—
48	Propen-2-yl	10	0.5	43.4	44.5	—
		10	16	—	98.0	—
49	Butyl	10	17	—	85.8	—
50	Isobutyl	5	19	—	90.2	—
51	*tert*-Butyl	5	2.5	—	75.4	trace
		5	19	—	83.9	—
52	1-Adamantyl	1.5	26	—	60.3	30.6
		1	72	—	94.4	—
53	Allyl	10	16	—	71.9	—
54	*trans*-But-2-enyl	10	12	—	99.5	—
55	3-Phenylpropyl	5	24	—	89.2	—
56	2-Phenylethyl	5	17	—	98.3	—
57	Benzyl	5	17	—	91.3	—
58	*p*-Chlorobenzyl	3.6	19	—	89.8	—
59	*p*-Nitrobenzyl	5	24	—	99.4	—
60	*p*-Methylbenzyl	5.1	19	—	82.6	—
61	*p*-Methoxybenzyl	5	19	—	85.5	—
62	1-Naphthylmethyl	3	96	—	91.3	trace
63	2-Naphthylmethyl	3	72	—	93.9	—

[a] Reaction conditions were not optimised.

In conclusion, it is clear that *Rhodococcus* AJ270 tolerates a broad spectrum of nitrile substrates, in contradistinction to some earlier reports of related organisms.[3,7–10] Both the nitrile hydratase and amidase activities involved in *Rhodococcus* AJ270 are mainly influenced by steric and coordinating factors, in particular the presence of α- or *ortho*-substituents. For effective nitrile hydrolysis, a molecular diameter of the substrate of no more than *ca.* 7 Å appears the limit. The amidase is more sensitive than the nitrile hydratase to the geometry of the molecule. Without adjacent substituents such as an *ortho*-substituent in benzonitriles, an adjacent heteroatom in heterocycles or a geminal substituent in an α,β-unsaturated nitrile, the amidase catalyses the conversion of amides to acids rapidly and efficiently. When such adjacent substituents are present in the molecule, on the contrary, biotransformation of an amide into an acid proceeds sluggishly. The presence of an amino group at the *para*-position but not the *meta*-position of a benzamide (or an amino group in a 6-membered heterocycle γ to the amide group) also has a deleterious effect on amidase efficiency.

It is worth noting that the adjacent-substituent effect is also generally observed in most of the published examples using the nitrile hydratase–amidase systems. Thus, the combination of electronic and steric insensitivity of the nitrile hydratase and the steric sensitivity of the amidase accounts for the formation of amides and the poor regioselectivity observed in the hydrolysis of perfluoro and unsymmetrical aromatic dinitriles.[15] The outcomes of the published hydrolyses of heterocyclic nitriles also fit this generalisation.[18]

There is one last factor that causes low yields in the biotransformation of nitriles using whole cell systems. Small aliphatic nitriles and those bearing amino and hydroxy groups appear to be metabolised in a different manner by the organism (presumably by other enzymes), leading to lowered yields of acids and amides. Thus aminobenzoic acids **10c**, **14c** and **21c** and hydroxybenzoic acids **11c**, **15c** and **21c** as well as aceto- and propio-nitriles give lower yields. Also, acrylonitrile **46a** and allyl cyanide **53a** gave about a 70% yield of the acid in 16 h while cinnamonitrile **47a** and methacrylonitrile **48a** gave the corresponding acid almost quantitatively in the same reaction period. Longer reaction times with the smaller nitriles lead consistently to lower yields. Similar problems were also encountered in the hydrolysis of dinitriles[24] which will be the subject of a further full study. It is strongly recommended, therefore, that variation of the concentration of the substrates and careful monitoring of the reaction process is undertaken to achieve optimal conversion and chemical yield.

Experimental

Melting points, which are uncorrected, were determined using a Reichert Kofler hot stage apparatus. Infrared spectra were obtained on a Unicam Research Series 1 FTIR instrument as liquid films or KBr discs. NMR Spectra were recorded in $CDCl_3$ or [$^2H_6$]DMSO solution with $SiMe_4$ as internal standard on a JEOL 270 spectrometer. Mass spectra were measured on a Kratos MS8ORF mass spectrometer and microanalyses were carried out at Newcastle University on a Carlo Erba 1106 Elemental Analyser. Thin layer chromatography (TLC) was performed with Merk silica 60 F_{254} plates, and for flash chromatography Janssen silica (35–70 mm) was used.

Preparation of biocatalyst

The medium used was the chelate mineral medium (CMM) plus vitamins.[25] *Rhodococcus sp.* AJ270, previously isolated from a soil sample, was subcultured at 30 °C in a conical flask (1 dm^3) containing 300 cm^3 of the medium omitting ammonium sulfate. Acetonitrile (3 cm^3) was used as the carbon and nitrogen source. After 24 h the culture was transferred into a 20 dm^3 fermentor containing the same medium (15 dm^3) and an antifoam agent [antifoam 204 (Sigma)] (1 cm^3). Acetamide (44.3 g, 50 mM) was used instead of acetonitrile as the carbon and nitrogen source. After inoculation at 30 °C for about 14 h (late log phase), cells were collected by continuous centrifugation at 4 °C. Cells were washed twice with potassium phosphate buffer (0.1 M, pH 7.0) and were stored at −20 °C in a freezer for further use.

Biotransformation of nitriles

To an Erlenmeyer flask (250 cm^3) with a screw cap was added *Rhodococcus sp.* AJ270 cells (2 g wet weight) and potassium phosphate buffer (0.1 M, pH 7.0, 50 cm^3) and the resting cells were activated at 30 °C for 0.5 h with orbital shaking. Nitrile (1–10 mmol, see Tables 1–4 for each substrate) was added in one portion 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) and worked up following one of two methods.

Method A, mainly used for the aliphatic nitriles, involved filtration through a Celite pad to remove the biomass followed by basifying the aqueous solution to pH 11 with aqueous NaOH (2 M). Extraction with diethyl ether or ethyl acetate (2×30 cm^3) gave, after drying over magnesium sulfate and concentration, the amide and unreacted nitrile. Separation of nitrile and amide was effected by flash chromatography. The aqueous solution was then acidified using concentrated HCl to pH 2 and extracted with diethyl ether or ethyl acetate (2×30 cm^3). The acid was obtained after the solvent had been removed *in vacuo*.

Method B was used mainly for aromatic and heterocyclic nitriles. The reaction mixture was adjusted to pH 11 with aqueous NaOH (2 M) and was extracted continuously with dichloromethane for 12–24 h. The aqueous phase was acidified with concentrated HCl to pH 2 and was extracted again with dichloromethane for another 12–24 h. The amide and/or nitrile and acid were obtained respectively from the two extractions. For pyridinecarboxylic acids, the pH was adjusted to their isoelectric point (isonicotinic acid 3.2; nicotinic acid 3.4; picolinic acid 3.6) and extracted continuously with dichloromethane.

Method A was used for the isolation of aminobenzoic acids and after addition of glacial acetic acid and concentration of the solution *in vacuo*, anthranilic acid was obtained as a crystalline product. 3- And 4-aminobenzoic acids were obtained when the aqueous solution was concentrated to dryness and the residue was extracted with ethanol.

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.

1,5-Dimethyl-1*H*-pyrrole-2-carboxamide 36b

Colourless crystalline solid, mp 152.5 °C; ν_{max}/cm^{-1} 3370, 3185, 1637 and 1612; δ_H 6.54 (1 H, d, *J* 3.8), 5.89 (1 H, dd, *J* 3.8 and 0.8), 5.44 (2 H, br s, NH_2), 3.85 (3 H, Me) and 2.24 (3 H, Me); *m*/*z* (El) 138 (M^+, 100%), 122 (76) and 94 (26) (Found: C, 60.75; H, 7.24; N, 20.28. $C_6H_{10}N_2O$ requires C, 60.83; H, 7.30; N, 20.28%).

Melting points

1c, mp 122 °C (lit.,[26] 120–122 °C); **2c**, mp 181 °C (lit.,[27] 181 °C); **3c**, mp 184 °C (lit.,[28] 185 °C); **4c**, mp 159 °C (lit.,[29] 158–160 °C); **5c**, mp 243 °C (lit.,[30] 240 °C); **6c**, mp 185 °C (lit.,[31] 182 °C); **7c**, mp 241.5 °C (lit.,[27] 241 °C); **8c**, 208 °C (lit.,[32] 207.5–209.5 °C); **9c**, mp 230 °C (lit.,[33] 218–230 °C); **10c**, mp 213–214 °C (lit.,[29] 212–213 °C); **11b**, mp 183–183.5 °C (lit.,[34] 175–179 °C); **11c**, mp 186–187 °C (lit.,[35] 184 °C); **12c**, mp 140–141 °C (lit.,[36] 139–140 °C); **13c**, mp 110 °C (lit.,[27] 110.5 °C); **14c**, mp 200.8 °C (lit.,[29] 197–201 °C); **15c**, mp 174 °C (lit.,[35] 173 °C); **16b**, mp 143 °C (lit.,[37] 144–145 °C); **16c**, mp 105.5 °C (lit.,[38] 104.8–105.4 °C); **17b**, mp 130 °C (lit.,[39] 130–131 °C); **19b**, mp 142–143 °C (lit.,[40] 142–143.5 °C); **19c**, mp 141–143 °C (lit.,[31] 142 °C); **20b**, mp 177–178 °C (lit.,[41] 174–175 °C); **20c**, mp 146–147 °C (lit.,[42] 148 °C); **21b**, mp 142 °C (lit.,[40] 136.5–138.5 °C); **21c**, mp 160–162 °C (lit.,[29] 156–158 °C); **22b**, mp 111.5–112 °C (lit.,[43] 108.5–111 °C); **22c**, mp 147.5–148 °C (lit.,[35] 144–145 °C); **23b**, mp 146.5–147 °C (lit.,[44] 144.5–145.5 °C); **23c**, mp 156.5–157 °C (lit.,[44] 159–160 °C); **24b**, mp 200–201 °C (lit.,[45] 202 °C); **27c**, mp 230.5–232 °C (lit.,[46] 229–230 °C); **28c**, mp 162–163 °C (lit.,[47] 162 °C); **29c**, mp 187 °C (lit.,[47] 187.5 °C); **34b**, mp 143–144 °C (lit.,[48] 141–142 °C); **34c**, mp 134 °C (lit.,[49] 132–133 °C); **35b**, mp 183–184 °C (lit.,[50] 179–180 °C); **35c**, mp 132.5–133 °C (lit.,[51] 130 °C); **37c**, mp 221 °C (lit.,[52] 222 °C); **38c**, 135–137 °C (lit.,[53] 135–136 °C); **39c**, mp 235–236 °C (lit.,[54] 235 °C); **40b**, mp 156–156.5 °C (lit.,[55] 154–155 °C); **40c**, mp 315–316 °C (lit.,[56] 317–318 °C); **41c**, mp 276–277 °C (lit.,[57] 274–275 °C); **42b**, mp 171–172 °C (lit.,[58] 170–171 °C); **46c**, oil (lit.,[59] bp 141–142 °C); **47c**, mp 133–134 °C (lit.,[60] 132.4–132.7 °C); **48b**, mp 109 °C (lit.,[61] 108–109 °C); **48c**, oil (lit.,[62] bp 65 °C/11 mmHg); **49c**, oil (lit.,[63] bp 80–83 °C/10 mmHg); **50c**, oil (lit.,[64] bp 175–177 °C); **51**, mp 33–35 °C (lit.,[65] 34–35 °C); **52c**, mp 176 °C (lit.,[66] 175–176.5 °C); **53c**, oil (lit.,[67] bp 163–165 °C); **54c**, oil (lit.,[68] bp 97–98 °C/20 mmHg); **55c**, mp 51–52 °C (lit.,[69] 52 °C); **56c**, mp 46.5 °C (lit.,[60] 46.4–46.8 °C); **57c**, mp 77–78 °C (lit.,[60] 76.7–76.9 °C); **58c**, mp 103–105 °C (lit.,[70] 105 °C); **59c**, mp 151–153 °C (lit.,[70] 152 °C); **60c**, mp 93.5–95 °C (lit.,[71] 94 °C); **61c**, mp 84–86 °C (lit.,[72] 82 °C); **62c**, mp 132–132.5 °C (lit.,[47] 132 °C); **63c**, mp 143–144 °C (lit.,[47] 144 °C).

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