Fungal Biosynthesis of 3-Nitropropanoic Acid

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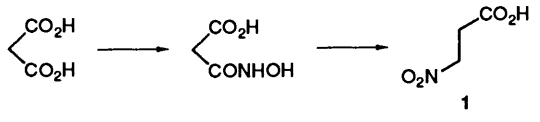
The origin of 3-nitropropanoic acid **1** in the fungus *Penicillium atrovenetum* has been examined using a combination of stable isotope methods. The incorporation of ^{13}C and ^{15}N from DL-[2-^{13}C, ^{15}N]aspartic acid **2a**, ^{18}O from $^{18}O_2$, ^{15}N from DL-diethyl [^{15}N] nitrosuccinate **6** and ^{2}H from L-[2,3,3-$^{2}H_3$]aspartate, DL-[4-^{13}C, 2,3,3-$^{2}H_3$]aspartate and from (2*S*,3*R*)-[3-^{2}H]- and (2*S*,3*S*)-[2,3-$^{2}H_2$]-aspartates indicate a biosynthetic pathway L-aspartate **2** → (*S*)-nitrosuccinate **5** → **1**. Mature cells of *P. atrovenetum* which produce 3-nitropropanoate dehydrogenase catalyse an apparent futile cycle between **1** and 3-nitroacrylate **3** with loss of the stereospecific integrity of the hydrogen at the 2-position of **1**.

3-Nitropropanoic acid **1** is a toxic metabolite produced by plants of the family *Fabeaceae*, in which it occurs both in the free form and as a component of the glycoside hiptagin,[1] and by fungi of the *Penicillium* and *Aspergillus* genera.[2] The compound has been shown to be a suicide inhibitor of mammalian succinate dehydrogenases, being converted into 3-nitroacrylate which subsequently inactivates the enzyme by alkylation of an essential cysteine sulfydryl.[3] Biosynthesis of the metabolite appears, however, to occur by quite different routes in fungi and higher plants. Radiochemical studies with whole plants and cuttings of *Indigofera spincata* indicate a precursorial relationship involving malonate and malonylhydroxamate[4] (Scheme 1). In contrast, studies on the biosynthesis of **1** in *P. atrovenetum* have shown that the carbon skeleton of the fungal metabolite is derived from L-aspartate **2**.[5–9] In view of the fact that the producing fungi and several other microorganisms produce dioxygenases capable of oxidising nitropropionate to nitrite and malonyl hemialdehyde it has been suggested that the metabolite may play a key role in a fungal nitrification pathway whereby the nitrogen of L-aspartate, derived originally from ammonia, is ultimately metabolised to nitrite and nitrate.[10,11]



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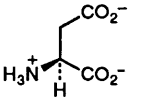
Scheme 1 Biosynthesis of 3-nitropropanoic acid in plants[4]

Early investigations of the fungal pathway[5–8] using ^{14}C labelled precursors showed that the 2, 3 and 4 carbon atoms of L-aspartate, but not those of the D-isomer, were incorporated into the 3-, 2- and 1-positions of the propionate skeleton respectively. Birch and his co-workers[5] suggested that the nitro group of **1** arises by oxidation of the amino group of L-aspartate, a hypothesis supported by subsequent results of Gatenbeck and Forsgren[6] who found that both ^{14}C and ^{15}N from [U-^{14}C, ^{15}N]aspartate were incorporated in the skeleton, albeit to different extents. The *in situ* oxidation of amino groups to nitro groups has been shown to occur in other biosynthetic pathways, for example in the formation of chloroampheticol[12] and pyrrolnitrin.[13] However, this proposal has been criticised by Shaw *et al.* who found that the composition of nitrogen salts in *P. atrovenetum* growth medium strongly influences production of the metabolite[8,14] and suggested that an inorganic nitrogen donor could be involved. Biological nitration has recently been shown to occur in the biosynthesis of dioxapyrrolomycin by *Streptomyces fumanus*.[15] This rationalisation implies that the earlier results could be explained by liberation of the nitrogen of aspartate through transamination prior to transformation of the carbon skeleton and subsequent reincorporation of nitrogen at a higher oxidation level. However, although both hydroxylamine and nitrite have been detected in *P. atrovenetum* culture media[6] exogenous hydroxylamine does not appear to depress the incorporation of ^{15}N from [^{15}N]aspartate into the metabolite[6] and ^{18}O from [^{18}O]nitrite was not found to be incorporated into **1** by *P. atrovenetum*.[8] This latter result, in itself, does not preclude nitrite as an intermediate since nitrite oxygen exchange has been shown to occur under metabolic conditions in *Nitromonas* bacteria.[16] Shaw has reported[17] that a partially purified protein fraction from *P. atrovenetum* can catalyse the *in vitro* reduction of 3-nitroacrylic acid **3** to **1** in the presence of NADPH (but not the reverse reaction), and the presence of this enzymic activity (3-nitropropanoate dehydrogenase) suggests that **3** could be an intermediate in the biosynthesis of **1**. Indeed, although 3-nitroacrylate is extremely toxic to the organism, radioactivity from [1-^{14}C]-**3** has been shown to be incorporated into **1** in low levels.[8]



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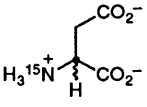
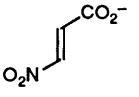


2a (• = ^{13}C)



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In this paper we summarise our studies on the biosynthesis of **1** in *P. atrovenetum*, some of which have been described, in part, in preliminary communications,[9,18,19] which have resulted in the delineation of the steps involved in the biosynthetic pathway in this organism and a clarification of the role played by 3-nitropropanoate dehydrogenase in the producing organism.

Origin of the Nitrogen of 3-Nitropropanoate.—To determine whether the nitrogen atom of L-aspartate is incorporated directly into **1** without cleavage of the carbon–nitrogen bond of the precursor or whether it is incorporated indirectly by a route involving amino acid degradation, DL-[2-^{13}C, ^{15}N]aspartic acid **2a** was synthesised[20] and its incorporation into **1** by *P. atrovenetum* examined. To avoid complications arising from catabolism of the enriched aspartic acid and reassimilation of the ^{15}N in another form the incorporation experiments were carried out using surface cultures of *P. atrovenetum* grown on an ammonium-rich medium. A mixture of the sodium salts of

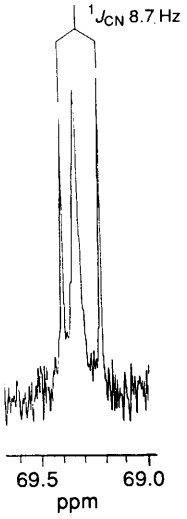


Fig. 1 The C-3 methylene resonance of the 50 MHz {^{1}H} ^{13}C NMR spectrum of **1** biosynthetically derived from DL-[2^{13}C, ^{15}N]aspartate **2a**. Spectral width 1000 Hz, acquisition time 8.2 s, 668 transients, solvent deuterioacetone.

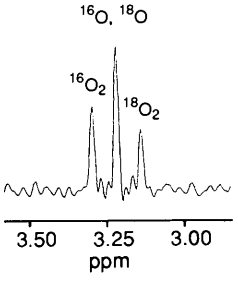
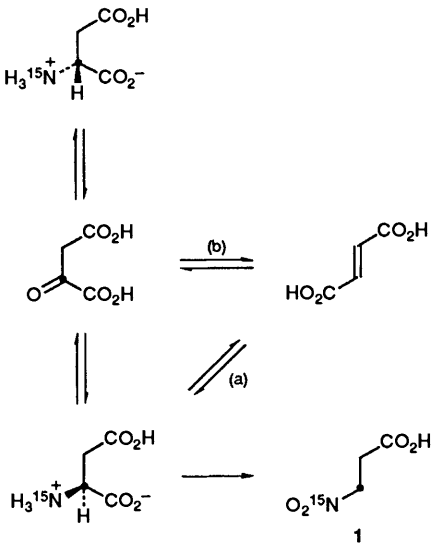


Fig. 2 36.5 MHz ^{15}N NMR spectrum of 3-nitropropanoic acid enriched biosynthetically with [^{15}N]ammonium and $^{18}O_2/^{16}O_2$. The spectrum was obtained using a DEPT pulse sequence.[23] Spectral width 400 Hz, acquisition time 1.28 s, 200 transients, solvent deuterioacetone, chemical shifts were referenced to nitromethane. The observed ^{18}O isotope shift was 0.08 ppm and assignment of the higher frequency signal to [$^{16}O_2$ ^{15}N]-**1** is based on the enhancement of this resonance upon addition of unenriched **1**.

DL-[2-^{13}C, ^{15}N]aspartic acid and L-[U-^{14}C]aspartic acid were fed over a period of 24 h starting 48 h after inoculation of the culture and the enriched metabolite isolated after 96 h of growth.

In the {^{1}H} ^{13}C NMR spectrum of isolated **1** the signal corresponding to the C-3 methylene carbon was observed as a composite of a broad singlet ($\omega_{\frac{1}{2}}$ 1.8 Hz, ^{13}C–^{14}N) and a sharp doublet ($^1J_{CN}$ 8.7 Hz, ^{13}C–^{15}N). The presence of the doublet, offset to lower frequency by the ^{15}N isotope shift, is consistent with the intact incorporation of the ^{13}C–^{15}N moiety of the labelled precursor (Fig. 1). An upper limit of *ca.* 20% for exchange of ^{15}N by ^{14}N by transamination prior to incorporation can be calculated from the integral ratios of the C-3 signals and the 58-fold dilution of ^{14}C from administered L-[U-^{14}C]aspartate. This figure must also include any incorporation of ^{13}C from D-[2–^{13}C, ^{15}N]aspartate *via* [2–^{13}C]oxaloacetate (Scheme 2) in addition, the lack of significant enhancement of the C-2 signal in the spectrum eliminates the possibility of equilibration of enrichment of the C-2 and C-3 either through aspartase activity or *via* the Kreb's cycle, shown as routes (a) and (b) in Scheme 2. The biosynthesis of **1** from L-aspartate must, therefore, involve *in situ* oxidation of the amino group of the amino acid.



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Scheme 2 Potential routes for incorporation of ^{13}C and ^{15}N from D- and L-[2–^{13}C, ^{15}N]aspartates into **1**

Origin of the Oxygen Atoms of the Nitro Group.—Analogy with the bacterial oxidation of ammonia to nitrite by *Nitromonas spp.*, in which at least one of the oxygen atoms of the product has been shown to come from molecular oxygen,[15,21] suggests that oxidation of alkyl or aryl amino groups to the corresponding nitro groups may involve a similar mechanism. To find out whether dioxygen is the direct source of one or both the oxygens of the nitro group of **1** we exploited the fact that ^{18}O substitution on nitrogen results in a relatively large isotope shift in the ^{15}N NMR spectrum.[21,22]

Cultures of *P. atrovenetum* were grown under an atmosphere of $^{16}O_2$–$^{18}O_2$ (1:1) in a defined medium in which $^{15}NH_4Cl$ (98 atom % ^{15}N) was the sole nitrogen source. Not surprisingly, the ^{1}H spectrum of the isolated 3-nitropropanoic acid showed two- and three-bond ^{15}N–^{1}H coupling [$^2J_{NH}$ 2.2 Hz, $^3J_{NH}$ 3.7 Hz] and integration of the proton signals indicated a ^{15}N abundance of >95%. The {^{1}H} ^{15}N DEPT NMR spectrum[23] showed three signals corresponding to $^{15}N^{16}O_2$, $^{15}N^{16}O^{18}O$ and $^{15}N^{18}O_2$ species (Fig. 2). This distribution of oxygen isotopes provides convincing evidence that both oxygen atoms of the nitro group are derived from molecular oxygen.

Intermediacy of (S)-*Nitrosuccinate.*—The intact incorporation of L-aspartate into **1** effectively limits the number of possible metabolic routes to those shown in Scheme 3. While route (a), *via* β-alanine, can be immediately discounted by the earlier failure to incoporate radiolabel from [^{14}C]β-alanine into **1**,[5] the alternative routes, involving decarboxylation of *N*-hydroxyaspartate **4** or of nitrosuccinate **5** have proven difficult to test because of the reactivity of these compounds.[24] To circumvent the problem of instability we elected to prepare and feed labelled diethyl nitrosuccinate, reasoning that *in vivo* hydrolysis of the diester would be slow enough to liberate small quantities of the putative intermediate within the cells.

DL-Diethy [^{15}N]nitrosuccinate **6** (94 atom% ^{15}N) was prepared by treatment of diethyl bromosuccinate with $Na^{15}NO_2$ in the presence of phloroglucinol and the labelled diester was administered to a surface culture of *P. atrovenetum* over a period of 24 h beginning 48 h after innoculation. The {^{1}H} ^{15}N DEPT NMR spectrum of **1** isolated from the organism showed an intense signal at 3.4 ppm corresponding to a 41-fold increase of the ^{15}N signal over natural abundance (Fig. 3).

Enrichment of the nitro nitrogen was also evident from the appearance of ^{15}N satellites of both methylene signals in the

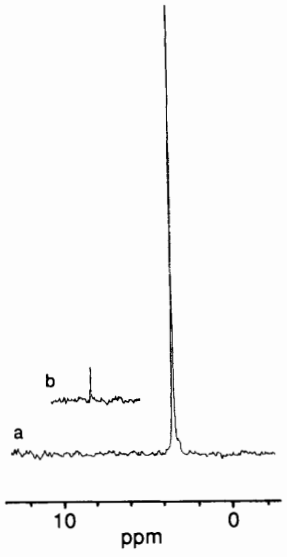


Fig. 3 36.5 MHz ^{15}N NMR spectrum of **1** derived from diethyl [^{15}N]nitrosuccinic acid **6**; the spectrum (a) was recorded on a 1 mol dm^{-3} solution of enriched **1** and the control spectrum (b) on a 2.85 mmol dm^{-3} solution of unenriched material. Spectral width 2000 Hz, acquisition time 1.02 s, 20 640 transients, solvent deuterioacetone, chemical shifts are relative to nitromethane.

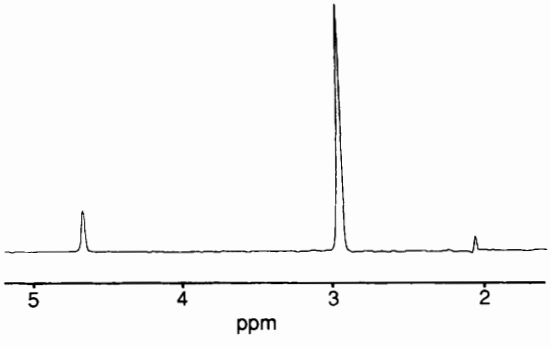
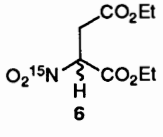


Fig. 4 55.3 MHz ^{2}H NMR spectrum of **1** derived from L-[2,3,3-$^{2}H_3$]aspartic acid. The spectrum was obtained on a 1 mol dm^{-3} solution of **1** in acetone. Spectral width 600 Hz, acquisition time 0.83 s, 2000 transients. The reasonance at 2.04 ppm is due to natural abundance deuterioacetone.

^{1}H spectrum of the metabolite which corresponded in intensity to a 20-fold dilution of ^{15}N enrichment relative to that of the racemic diester.



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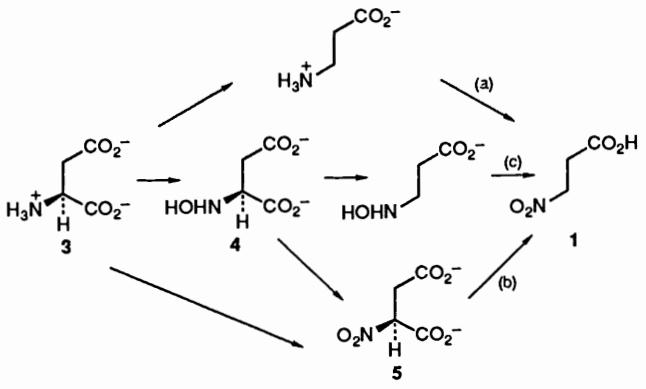
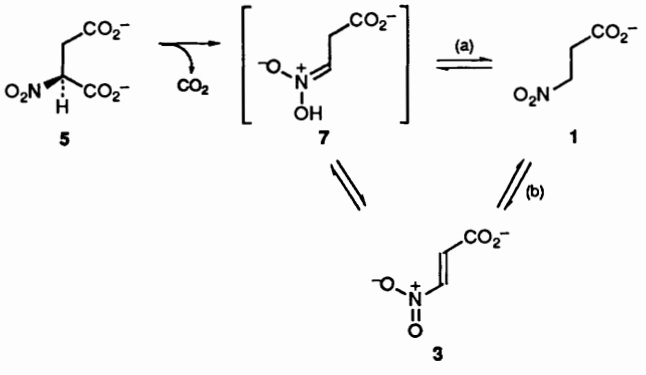
Assignment of the stereochemistry of the intermediate nitrosuccinic acid was ascertained indirectly by examining the incorporation of deuterium from L-[2,3,3-$^{2}H_3$]aspartate into **1**. The ^{2}H NMR spectrum of the enriched 3-nitropropanoic acid shows that deuterium is retained at both the 2- and 3-positions



Scheme 3 Oxidation of the amino group of L-aspartate



Scheme 4 Decarboxylation of **5**

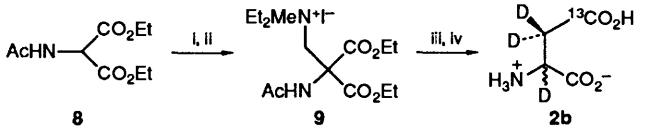
(Fig. 4).* It follows that the stereochemistry of the L-aspartate is most probably retained in the oxidation of the amino acid to nitrosuccinate since if inversion were to occur we should expect no deuterium enrichment at the 3-position of **1**. These results support biosynthesis *via* route (b) shown in Scheme 3.

Mechanism of Decarboxylation of (S)-*Nitrosuccinate.*—The intermediacy of (*S*)-nitrosuccinic acid and the retention of 2-H of L-aspartate in the final product limit the possible mechanisms for the decarboxylation step, eliminating, for example, a pyridoxal phosphate-dependent mechanism or the involvement of nitrofumaric or nitromaleic acids. Two feasible routes, shown in Scheme 4, involve either decarboxylation and, subsequently, protonation to give 3-nitropropionate directly [route (a)] or decarboxylation followed by hydride abstraction from the β carbon to afford 3-nitroacrylate [route (b)]. While we would advance the proposition, on mechanistic grounds, that the *aci* nitro compound **7** is a possible intermediate in each case, the final results can be considered to be *formally* similar to situations where no α nitro substituent is involved. Thus, in the case of route (a) there are analogies for decarboxylation and protonation occurring with retention[25] or inversion of the stereochemistry at the α carbon, while in the case of route (b) analogies exist for the loss of either of the prochiral β hydrogens.[26,27]

Since these two alternatives could apparently be distinguished by the loss or retention of the hydrogens derived from the C-3 methylene of L-aspartate we examined the incorporation of deuterium from [4-^{13}C, 2,3,3-$^{2}H_3$]aspartic acid into **1**. DL-[4-^{13}C, 2,3,3-$^{2}H_3$]Aspartic acid **2b** was prepared from diethyl acetylaminomalonate **8** by the route shown in Scheme 5.

The enriched aspartate was fed as the sodium salt to *P. atrovenetum* surface cultures at the beginning of 3-nitropropanoate production (48 h) and metabolite was isolated after 96 h growth. To avoid complications in the ^{13}C NMR spectrum due to γ-shifts of the carbonyl resonance the ^{2}H incorporated at

* The relative intensities of the ^{2}H resonances of **1** derived from L-[2,3,3-$^{2}H_2$]aspartate was 2:0.5 deviating significantly from the expected 2:1 ratio predicted on the basis of incorporation of two deuterium atoms at C-2 and a single deuterium atom at C-3. The most reasonable rationalisation of this result is that some exchange of deuterium from the C-3 position occurs during growth or in the isolation process. In a control experiment the intensity ratio of the ^{2}H signals from **1** derived from a culture grown in 20% $^{2}H_2O$ was 1:1 indicating that no significant exchange of deuterium from the C-3 position occurred during isolation and purification of the metabolite.



Scheme 5 Synthesis of DL-[4-^{13}C, 2,3,3-$^{2}H_3$]aspartic acid **2b**. *Reagents:* i, HCHO, Et_2NH; ii, MeI; iii, $Na^{13}CN$; iv, 20% ^{2}HCl–$^{2}H_2O$

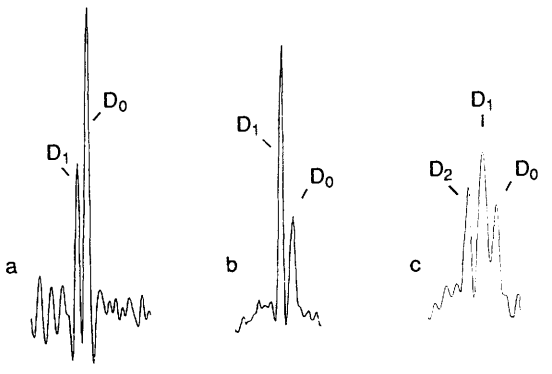


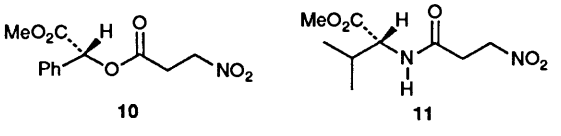
Fig. 5 The carboxy resonance in the 50 MHz {^{1}H} ^{13}C NMR spectra of: (a) enriched **1** derived from DL-[1-^{13}C, 2,3,3-$^{2}H_3$]aspartate **2b** [spectral width 100 Hz, acquisition time 3.2 s, 4040 transients]; (b) enriched **1** derived from [1-^{13}C, 2,2-$^{2}H_2$]nitropropanoic acid **1a** [spectral width 300 Hz, acquisition time 5.2 s, 10 690 transients]; (c) mixture of enriched **1** derived from [1-^{13}C, 2,2-$^{2}H_2$]nitropropanoic acid **1a** and authentic **1a** (showing two β deuterium shifts) [spectral width 300 Hz, acquisition time 5.2 s, 9250 transients]. Spectra were recorded in deuterioacetone and in each case the FID was weighed using a Lorentzian to Gaussian function (GB 0.2, LB-1) prior to transformation. The chemical shift of the unshifted carboxy carbon (D_0) was 170.0 ppm and the observed β deuterium shift was +0.012 ppm/deuterium.

the 3-position of **1** (*i.e.* that derived from the 2-position of L-aspartate) was removed by exchange in 0.5 mol dm^{-3} aqueous K_2CO_3 prior to spectroscopy. Under these conditions no exchange at the 2-position of **1** occurs. The carboxy resonance in the {^{1}H} ^{13}C NMR spectrum of enriched **1** derived from this experiment is shown in Fig. 5(*a*). Only a single β ^{2}H isotope shift of the carboxy resonance was observed indicating that only one of the deuterium atoms at the 3 position of the aspartate was retained in the product. It should be noted that while this result is consistent with biosynthesis *via* route (b) in Scheme 4, it is also consistent with biosynthesis *via* route (a) if the product is in equilibrium with 3-nitroacrylic acid through a reversible reaction catalysed by 3-nitropropanoate dehydrogenase.

To determine whether 3-nitropropanoate dehydrogenase was present in cells of *P. atrovenetum* grown under surface culture conditions cell-free extracts of mycelium were assayed for their ability to reduce 3-nitroacrylate to **1** in the presence of NADPH. While **1** is produced by the cultures from *ca.* 48 h of growth onwards 3-nitropropanoate dehydrogenase activity could not be detected during the first 82 h but rapidly increased thereafter reaching a maximum between 90 and 110 h. This suggests that 3-nitropropanoate dehydrogenase is not necessarily involved in the synthesis of **1** and that hydrogen abstraction from the 2-position could be a post-biosynthetic event. Confirmation of this was obtained by comparison of the mass spectra of samples of **1** derived from cultures fed with L-[2,3,3-$^{2}H_3$]aspartate and isolated after 72 and 96 h of growth. While the negative ion FAB mass spectrum of material isolated at 72 h showed (in addition to the molecular ion of undeuteriated **1** at *m/z* 119) an ion at *m/z* 122, corresponding to the (M^-) ion of [$^{2}H_3$]-**1**, this was absent in the spectrum obtained on material isolated after 96 h of growth which showed an ion at *m/z* 121, corresponding to the (M^-) ion of [$^{2}H_2$]-**1**.

To investigate the stereochemistry of hydrogen loss from the 2-position of the product the incorporation of deuterium from (2*S*,3*R*)-[3-^{2}H]aspartate **2c** and (2*S*,3*S*)-[2,3-$^{2}H_2$]aspartate **2d** into **1** both prior to and after induction of 3-nitropropanoate dehydrogenase was examined. 3-Nitropropanoic acid derived from each of the stereospecifically deuteriated L-aspartates was isolated from cultures after 72 h and 96 h of growth. The ^{2}H NMR spectra of enriched **1** derived from both the 72 and 96 h feeding experiments indicated that both the deuterium atoms at the 3-position of the labelled L-aspartates were retained to an apparently equal extent irrespective of the stereochemistry of the precursor and of the presence or absence of 3-nitropropanoate dehydrogenase (Table 1).

Since these results, at face value, appeared inconsistent with the incorporation of only one deuterium atom at C-2 of 3-nitropropanoate in the [4-^{13}C, 2,3,3-$^{2}H_3$]aspartate feeding experiment, the chirality of deuterium incorporation was further examined by conversion of 3-nitropropanoic acid samples derived from (2*S*,3*R*)-[3-^{2}H]- and (2*S*,3*S*)-[2,3-$^{2}H_2$]-aspartates into a chiral derivative in the spectra of which the 2-*pro-R* and 2-*pro-S* proton resonances could be distinguished. Earlier workers[28] have shown that the diastereotopic β-methylene ^{1}H resonances of chiral methyl mandelate esters of simple carboxylic acids show significant shift dispersion. However the methyl (2*R*) mandelate ester of 3-nitropropionate **10** perversely exhibits a negligible difference between the chemical shifts of its C-2 methylene protons. In contrast the ^{1}H NMR spectrum of the 3-nitropropanoyl amide of (2*S*) valine methyl ester **11** shows distinction between both the C-2 methylene (AA'BB' centred at 2.86 ppm, $\Delta\sigma$ = 0.08 ppm) and C-3 methylene (AA'BB' centred at 4.67 ppm, $\Delta\sigma$ = 0.11 ppm) proton resonances.



The ^{2}H NMR spectrum of the amide **11** prepared from enriched **1**, which was isolated after 72 h of growth from a culture to which (2*S*,3*S*)-[2,3-$^{2}H_2$]aspartate had been administered, exhibited a single resonance at 3.04 ppm while that from the (2*S*,3*R*)-[3-^{2}H]aspartate experiment showed a resonance at 2.94 ppm. This implies that in the absence of dehydrogenase activity there is either no racemisation or else complete inversion* at the 2-position of **1**. In contrast, in both of the 96 h incubation experiments the ^{2}H NMR spectra of the derivative prepared from enriched **1** were identical, both exhibiting two C-2 methylene deuterium resonances, at 2.94 and 3.04 ppm, with an intensity ratio of 1:2 (Table 1). Thus, in both cases the same mixture of [2-^{2}H] stereoisomers were formed and the ratio of these isomers was independent of the original stereochemistry of the methylene group of the precursor. The deuterium labelling results are consistent with a biosynthetic pathway, operative in late log phase cells, in which decarboxylation of (*S*)-nitrosuccinate affords 3-nitropropanoate by route (a) in Scheme 4. In mature cells, which produce 3-nitropropanoate dehydrogenase, some racemisation of the 2-position of **1** occurs.

* While inversion of the C-2 position at some point in the biosynthesis cannot be ruled out the authors have been unable to find an analogy for this process.

Table 1 Chemical shifts of **1** and the derivative **11** derived from ^2H enriched L-aspartates

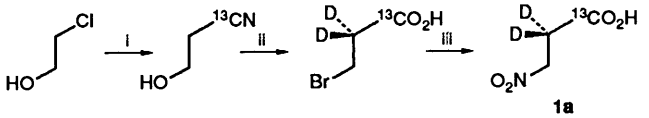
L-Aspartate substrate	Age of culture at harvesting (h)	^2H Chemical shifts (ppm) in **1** (rel. intensity)	^2H Chemical shifts (ppm)[a] in **11** (rel. intensity)
[2-^2H, 3-$^2H_2$]	96	2.97, 4.69 (3:1)	2.94, 3.04 (1:2)
(2*S*, 3*S*)-[2-^2H, 3-^2H]	72	2.98, 4.70 (3:1)	3.04
(2*S*, 3*S*)-[2-^2H, 3-^2H]	96	2.97, 4.69 (3:1)	2.94, 3.04 (1:2)
(2*S*, 3*R*)-[3-^2H]	72	2.96	2.94
(2*S*, 3*R*)-[3-^2H]	96	2.96	2.94, 3.04 (1:2)

[a] Exchange of ^2H at C-3 occurs during preparation of **11** from **1**.

3-Nitropropanoate Dehydrogenase Activity and the Post-biosynthetic Cycling of 3-Nitropropanoate in vivo.—An implication of the above results is that the action of 3-nitropropanoate dehydrogenase on biosynthetically produced (2*S*)-[2-^2H]-**1** or on (2*R*)-[2-^2H]-**1** affords the same mixture of products. Assuming that the action of the dehydrogenase is reversible—a contention at variance with earlier *in vitro* studies,[17] this suggests that either: (i) the enzyme does not have a rigid regiochemical requirement for its substrate; being able to accommodate it in two different orientations allowing abstraction of a hydride from *either* the 2- or 3-methylene positions of the molecule; or (ii) that the enzyme is regiospecific but can accommodate the substrate in two different conformations permitting abstraction of *either* the *pro-S* or *pro-R* hydrogen of one of the methylene groups.

The regiospecificity of the enzyme *in vitro* was examined by carrying out the reduction of 3-nitroacrylate with NADPH in ^2H_2O using the partially purified enzyme from *P. atrovenetum*. The ^1H NMR spectrum of the 3-nitropropanoic acid produced showed a significant reduction of the intensity of the 3-H triplet and the appearance of a doublet superimposed on the 2-H methylene triplet indicating that the ^2H was incorporated at the 3-position of **1**. Confirmation of the regioselectivity of ^2H incorporation was also evident from the ^2H NMR spectrum of the product which showed a single resonance at 4.45 ppm indicating incorporation of deuterium only at the 3-position. This implies that hydride addition occurs exclusively at the 2-position. It is noteworthy that, in agreement with earlier findings,[17] we were unable to demonstrate any reversibility of the reaction under *in vitro* conditions. Incubation of **1** and an excess of NADP with the dehydrogenase preparation showed no evidence of NADPH or 3-nitroacrylate formation nor was deuterium incorporated in **1** when the incubation was carried out in ^2H_2O.

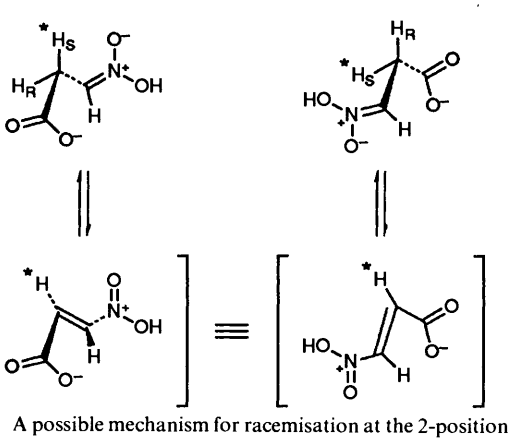
To examine the reversibility of the *in vivo* reaction [1-^{13}C, 2,2-$^2H_2$]nitropropanoic acid **1a** was synthesised by the route shown in Scheme 6. The enriched material was administered to cells after 84 h of growth and reisolated at 96 h—over a period when 3-nitropropanoate dehydrogenase activity could be demonstrated in cell extracts.



1a

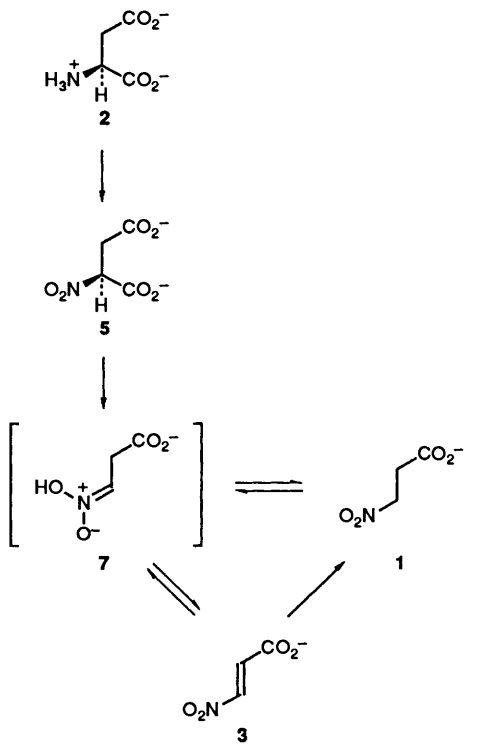
Scheme 6 Synthesis of [1-^{13}C, 2,2-$^2H_2$]nitropropanoic acid **1a**; i, Na^{13}CN, 18-crown-6; ii, 40% ^2HBr–^2H_2O; iii, $NaNO_2$, DMF

The ^{13}C NMR spectrum of the reisolated metabolite showed two carboxy resonances, separated by 0.012 ppm, corresponding to [1-^{13}C, 2-^2H]-**1** and a smaller amount of [1-^{13}C]-**1** (principally due to natural abundance material) showing that loss of one of the deuterons from the 2-position had occurred [Fig. 5(*b*)]. This result indicated that cycling of the metabolite *via* the dehydrogenase-catalysed reaction can occur *in vivo*. The contradiction implicit in the reversibility of the *in vivo* reaction and the apparent irreversibility of the *in vitro* reaction may be resolved if we consider that the *aci* nitro tautomer **7**, rather than **1**, is a substrate of the dehydrogenase. If **7** is the kinetic product of the (*S*)-nitrosuccinate decarboxylase then equilibration between **1** and **7** might be possible *in vivo* but not necessarily under *in vitro* conditions. The observed loss of one of the deuterium atoms from the 2-position of [1-^{13}C, 2-$^2H_2$]-**1** catalysed by cells producing 3-nitropropanoate dehydrogenase is in agreement with the results obtained with L-aspartates enriched with a single deuteron at the 3-position if either of the hydrogens at this carbon can be removed by the enzyme. In a dehydrogenase catalysed redox cycle the loss of one deuterium atom from a 2-$^2H_2$ labelled substrate is obviously obligatory. If *stereospecific* hydride abstraction was involved either the 2-*pro-S* or the 2-*pro-R* hydrogen would be lost. The retention of the deuterium atoms at the 2-position of **1** derived from both (2*S*,3*R*)-[3-^2H]- and (2*S*,3*S*)-[2,3-$^2H_2$]-aspartates (**2c** and **2d**) suggests that the substrate can be bound to the dehydrogenase in either of two different conformations. This is shown in Scheme 7 in which we envisage that the chirality of the hydrogen abstracted depends on the conformation of the bound state of the substrate. Indeed, there are several well authenticated examples of lack of stereospecificity in dehydrogenase-catalysed reactions.[29] A consequence of this stereochemical flexibility would be the preferential loss of hydride, rather than of deuteride, from this position since the cleavage of carbon–deuterium bonds is slowed by a primary isotope effect. The inbalance of the stereospecificity of deuterium labelling at C-2 of **1** observed at equilibrium suggests, however, that there could be a favoured enzyme-bound conformation of the substrate. If one allows the assumption that inversion at the methylene carbon does not occur* during, or subsequent to, decarboxylation of (*S*)-nitrosuccinate then abstraction of the 2-*pro-R* hydrogen of **1** (or **7**) and addition of the NADPH derived hydride on the same face (*i.e. re* face attack) at the 2 position of 3-nitroacrylate appears to be preferred [route (b) in Scheme 7].



Scheme 7 A possible mechanism for racemisation at the 2-position of **7**

* See footnote on preceding page.



Scheme 8 Nitropropanoate biosynthesis

Conclusions.—The pathway shown in Scheme 8 rationalises the available evidence from this and earlier studies. Oxidation of L-aspartate affords (*S*)-nitrosuccinate **5**. Decarboxylation of **5** presumably gives rise to an *aci* nitro intermediate **7** as the kinetic product which tautomerises to afford **1**. When nitropropanoate dehydrogenase is present **7** (or **1**) may be intercepted and cycled *via* 3-nitroacrylate **3**. The role of the futile cycle between 3-nitropropanoate and 3-nitroacrylate in *P. atrovenetum* is unclear especially since the latter compound is toxic to the organism. One possible explanation, which has analogy with other metabolic futile cycles,[30] is that it may serve as an additional mechanism for regulating NADP/NADPH levels in the cell.

Experimental

NMR spectra were determined on Bruker WH200 and WM360 and Varian VXR600 spectrometers and mass spectra using a Kratos MS50 TC spectrometer. Extracts made with organic solvents were typically dried over $MgSO_4$, filtered and evaporated under reduced pressure using a rotary evaporator. Melting points were determined on a Reichart hot-stage apparatus and are uncorrected. DL-[2-^{13}C, ^{15}N]Aspartic acid **2a**,[20] 3-nitroacrylic acid **3**[16] and (2*S*,3*R*)-[3-^{2}H]-**2c** and (2*S*,3*S*)-[2,3-$^{2}H_2$]-aspartates **2d**[31] were prepared by methods previously described. L-[2,3,3-$^{2}H_3$]Aspartatic acid was purchased from Merck & Co. Inc., Rahway, NJ, USA.

Growth of the Organism.—Freeze-dried spores of *Penicillium atrovenetum* (ATCC 13352; CMI 61837) obtained from the Commonwealth Mycological Institute, London, were applied to Czapex-Dox slants which were grown in the light at 25 °C for 5 d and stored at 4 °C. Subculturing of the organism resulted in loss of the ability to produce 3-nitropropanoate. Seed cultures were prepared by scraping a single slant into 50 cm^3 of modified Raulin Thom medium[14] in a 250 cm^3 shake flask which was shaken at 200 rev min^{-1} for 24 h. Surface cultures were initiated by inoculating 50 cm^3 of medium in 250 cm^3 Erlenmyer flasks with a 5 cm^3 aliquot of the seed culture and were grown, at 25 °C, under illumination by fluorescent tubes. The production of metabolite was monitored using the procedure described by Shaw and Wang;[14] typically the concentrations of **1** in the culture filtrate were 0.6, 0.8, 1.2 and 1.5 mg cm^{-3} at 48, 72, 96 and 120 h after inoculation. $^{18}O_2/^{16}O_2$ Experiments were carried out in single flasks fitted with sterile inlet and exhaust tubes and a stream of the gas mixture (*ca.* 2 cm^3/h) was passed through the flasks for the duration of the experiment. The medium used for these experiments contained; glucose (4.6 g), Na_2SO_4 (18 mg), K_2HPO_4 (36 mg), K_2CO_3 (40 mg), $MgCO_3$ (27 mg), $FeSO_4 \cdot 6H_2O$ (5 mg), $ZnSO_4 \cdot 7H_2O$ (5 mg), tartaric acid (320 mg), sodium tartrate (220 mg) and $^{15}NH_4Cl$ (530 mg) in water (100 cm^3).

Feeding Experiments.—Isotopically enriched aspartates and 3-[1-^{13}C, 2-$^{2}H_2$]nitropropanoic acid **1a** were administered to cultures as solutions of their sodium salts in sterile water which were prepared *in situ* by adding the appropriate amount of 5 mol dm^{-3} NaOH to an aqueous suspension (2 cm^3) containing *ca.* 400 μmol substrate. Except where stated otherwise, substrates (0.5 mg/cm^3 of culture) were administered to two flasks of surface culture (50 cm^3/flask) in equal aliquots after 36, 42 and 48 h of growth. DL-[2-^{13}C, ^{15}N]Aspartic acid **2a** (16 mg) and L-[U-^{14}C]aspartic acid (21.5 KBq, 7.5 GBq $mmol^{-1}$) were administered as equal aliquots of an aqueous solution (1 cm^3) to a single flask at 48, 60 and 72 h growth and diethyl DL-[^{15}N]nitrosuccinate **6** (50 mg) was fed as a solution in EtOH (1 cm^3) in equal aliquots to two flasks at 48, 60 and 72 h of growth.

Isolation of 3-Nitropropanoic Acid.—After removal of the mycelial mat, culture filtrates were acidified with 6 mol dm^{-3} HCl to pH 2 and extracted with an equal volume of ether. The process was repeated and the combined organic extracts were dried and evaporated. The residue was sublimed (80 °C at 5 mmHg) and the sublimate recrystallised from $CHCl_3$ to give **1**, m.p. 64–65 °C. Material isolated from control experiments was identical (mixed m.p., TLC, MS and ^{1}H NMR) with an authentic sample.

3-Nitropropanoate Dehydrogenase.—The preparation of cell-free extracts, protein fractionation and assay procedures were carried out essentially as described earlier.[16] Mycelial mats from two 50 cm^3 light-grown static cultures were frozen at −20 °C, ground in a mortar with an equal volume of phosphate buffer (50 mmol dm^{-3}, pH 7.2) and cleared by centrifugation (3000 g, 20 min). Ammonium sulfate fractionation of the extract concentrated the activity in the 40–65% precipitate, which was dialysed with phosphate buffer (50 mmol, pH 7.2), diluted to a standard concentration of 4 cm^3 g^{-1} of mycelium used and stored at −20 °C. Typical preparations from a 96 h culture had activities *ca.* 0.5 U g^{-1} of mycelium extracted. (1 U = 1 μmol of 3-nitropropanoate reduced/min). Assays were carried out in phosphate buffer (67 mmol dm^{-3}, pH 5.0) containing NADPH (0.2 mmol), 3-nitroacrylic acid (2 mmol dm^{-3}) and extract (typically 0.2 cm^3/cm^3). The rate of oxidation of NADPH was monitored spectrophotometrically at 340 nm.

DL-*Diethyl*[^{15}N]-*Nitrosuccinate* **6**.—Bromosuccinic acid (2 g) was dissolved in 3% HCl–EtOH (20 cm^3). After 12 h the solution was evaporated to afford diethyl bromosuccinate (2.5 g). $Na^{15}NO_2$ (234 mg, 0.33 mmol; 99 atom % ^{15}N) and phloroglucinol dihydrate (288 mg) were added to a solution of the diester (0.5 g, 2 mmol) in DMF (3 cm^3). The solution was stirred at room temperature for 2.5 h, diluted with ice–water (10 cm^3) and extracted with ether (5 cm^3 × 2). The dried ether extract was evaporated and the resultant oil subjected to

column chromatography on SiO_2 (80–100 mesh, 10 g) using 25% ether–light petroleum (b.p. 30–40 °C) as eluent. Fractions containing **6** (0.4 g) were evaporated and distilled (80–100 °C at 1 mmHg) using a Kugelrohr apparatus to afford **8** (200 mg); $\delta_H(CHCl_3)$ 1.26, 1.30 (6 H, 2t, *J* 7.0), 3.22 (2 H, AB of ABMX, $\Delta\sigma_A$ 0.03, $\Delta\sigma_B$ −0.05 ppm, $^2J_{AB}$ 17.4, $^3J_{AX}$ 5.4, $^3J_{BX}$ 8.4, $^3J_{AM}$ 4.2, $^3J_{BM}$ 2.4), 4.19, 4.28 (4 H, 2q, *J* 7.0) and 5.53 (1 H, X of ABMX, $^3J_{AX}$ 5.4, $^3J_{BX}$ 8.4, $^2J_{XM}$ 2.0); MS (EI) *m/z* (rel int) 175 (M − OEt, 23), 147 (M − CO_2Et, 32) and 127 (174 − $H^{15}NO_2$, 100).

DL-[4-^{13}C, 2,3,3-$^2H_3$]*Aspartic Acid* **2b**.—Diethyl acetamidomalonate (21.7 g, 0.1 mol) and aq formaldehyde (4%; 8.2 cm^3) were added to a stirred solution of diethylamine (15 cm^3, 0.01 mol) in glacial HOAc (15 cm^3) at 0 °C. After 0.5 h the solution was made alkaline with 20% NaOH and the precipitated Mannich base filtered off and dried *in vacuo*. Treatment of an ethanolic solution (60 cm^3) of the product (27.4 g) with an excess of MeI afforded the corresponding *methiodide* **9** which was filtered off and dried *in vacuo*. A solution of **9** (4.2 g, 10 mmol) and Na^{13}CN (90 atom % ^{13}C; 0.6 g, 12 mmol) in water (20 cm^3) was heated at reflux for 12 h; the reaction mixture was evaporated to dryness under reduced pressure at 40 °C, the residue dissolved in 20% ^{2}HCl-^2H_2O (99 atom % ^{2}H; 5 cm^3) and the solution heated at reflux for 18 h. The solution was evaporated to dryness under reduced pressure, the residue taken up in MeOH (20 cm^3) and precipitated NaI filtered off. Compound **2b** was precipitated with pyridine (3 cm^3 × 2), removed by centrifugation and dried *in vacuo* (0.98 g, 73%). The chromatographic (TLC, SiO_2, 33% NH_4OH–propan-2-ol) and electrophoretic (paper, pH, 2.1 and pH 6.9) behaviour of the product was identical with that of authentic DL-aspartic acid. Assessment of the ^{2}H enrichment of **2c** was carried out by conversion of a sample of **2b** into its *N*-acetyl derivative, with Ac_2O–NaOH under Schotten–Bauman conditions, and subsequent methylation with diazomethane, to afford enriched dimethyl *N*-acetylaspartate; $\delta_H(CDCl_3)$ 1.96 (3 H, s, CH_3-CO), 3.60 (3 H, d, $^2J_{CN}$ 3.9, 4-OCH_3), 3.67 (3 H, s, 1-OCH_3) and 6.74 (1 H, s, $^3J_{2,NH}$ 7.8). Integration of residual ^{1}H signals indicated an enrichment ($^2H_2$:$^2H_1$:$^2H_0$) of 9:1:0 at the 3-position.

Preparation of Chiral Derivatives of 3-*Nitropropanoic Acid*.—(a) *Methyl* (2R)-3-*Nitropropanoylmandelate* **10**.—3-Nitropropanoic acid (100 mg, 0.8 mmol) in dry THF (3 cm^3) was treated with trifluoroacetic anhydride (180 mg, 0.8 mmol) and the mixture shaken vigorously for 2 min and periodically over a further 1 h. Methyl (2*R*)-mandelate (200 mg, 0.85 mmol) was added and the mixture allowed to stand for 12 h at room temperature, after which it was cooled to 0 °C, taken to pH 7 with 3 mol dm^{-3} aq. NaOH and extracted with EtOAc (5 cm^3 × 3). Evaporation of the combined organic extracts, preparative TLC (SiO_2, 5% EtOH–$CHCl_3$) and crystallisation from $CHCl_3$–hexane afforded **10** (80 mg, 37%), m.p. 120–122 °C; $\delta_H([^2H_6]$benzene) 2.45 (2 H, t, *J* 10, CH_2CO_2R), 3.40 (3 H, s, OCH_3), 3.70, 3.90 (2 H, 2m, CH_2NO_2), 5.50 (1 H, s, $CHCO_2CH_3$) and 7.00 (5 H, ArH); *m/z* (EI) 267 (M$^+$).

(b) *Methyl* (2S)-3-*Nitropropanoylvaline* **11**. L-Valine methyl ester (165 mg, 1.0 mmol) and 1,3-dicyclohexylcarbodiimide (100 mg, 1.0 mmol) were added to a stirred solution of 3-nitropropanoic acid (100 mg, 0.8 mmol) in dry THF (2.5 cm^3) at room temperature. After 2 h the mixture was filtered, evaporated under reduced pressure and the resultant oil subjected to flash column chromatography (SiO_2, 80–100 mesh, 10 g) using 0–10% MeOH–$CHCl_3$ eluent. Fractions containing the amide were pooled, evaporated and the residue crystallised from EtOAc–hexane to give **11** (104 mg, 53%), m.p. 42–44 °C; $\delta_H(CDCl_3)$ 0.89, 0.92 (6 H, 2d, *J* 5.5, $2CH_3$), 2.14 [1 H, m, $CH(CH_3)_2$], 2.86 (2 H, AA'BB', dd = 0.08 ppm, ^2J 8.5, ^3J 4.2, 2.5, CH_2CONH), 3.75 (3 H, s, OCH_3), 4.60 (1 H, dd, *J* 5, 10, CHNH), 4.67 (2 H, AA'BB' system, $\Delta\delta$ = 0.11 ppm, ^2J 8.0, ^3J 2.5, 4.2, CH_2NO_2) and 6.25 (1 H, d, *J* 10, NH); *m/z* (EI) 232.1069 (M$^+$, calc. for $C_9H_{16}N_2O_5$: 232.1060), 172 (M − CO_2CH_3)$^+$ and 125 (172-NO_2)$^+$.

3-[1-^{13}C, 2,2-$^2H_2$]*Nitropropanoic Acid* **1a**.—A solution of Na^{13}CN (0.1 g, 2.0 mmol), 18-crown-6 (0.03 g, 0.11 μmol) and 2-chloroethanol (0.25 g, 0.3 mmol) in water (1 cm^3) was stirred at 80–90 °C for 3 h. The solution was cooled to room temperature, adsorbed on dry SiO_2 (2 g) and extracted with EtOAc (2 cm^3 × 5). The combined organic extracts were evaporated to afford 3-[1-^{13}C]*hydroxypropionitrile* **12** (160 mg), b.p. 74–76 °C at 1 mmHg. A solution of **12** (156 mg, 2.0 mmol) in 40% ^{2}HBr in ^2H_2O (3 cm^3) was heated at reflux for 2 h, cooled to room temperature, diluted with ^2H_2O (3 cm^3) and extracted with ether (10 cm^3 × 5). The combined extracts were evaporated and the residue crystallised from ethanol to give 3-[1-^{13}C, 2,2,3,3-$^2H_4$]*bromopropanoic acid* **13** (120 mg), m.p. 62–63 °C. Solid **13** (100 mg) was added to a stirred suspension of $NaNO_2$ (75 mg) in dry DMF and the solution stirred for 1.5 h at room temperature. The reaction mixture was diluted with water (10 cm^3), adjusted to pH 1 with 6 mol dm^{-3} HCl and extracted with ether (15 cm^3 × 5). Evaporation of the combined extracts and crystallisation of the residue from $CHCl_3$ afforded 3-[1-^{13}C, 2,2-$^2H_2$]*nitropropanoic acid* **1a** (25 mg), m.p. 62–63 °C; $\delta_H([^2H_6]$acetone) 4.45 (s, 3-CH_2NO_2); *m/z* (FAB, +ve) 122 (M$^-$) and 121 (M − 1)$^-$.

Acknowledgements

We thank the SERC and the AFRC for support. A. B. H. was supported by the AFRC Food Research Institute, K. M. acknowledges the support afforded by an SERC studentship and I. J. M. the award of a Royal Society Fellowship.

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Paper 2/02516G
Received 14*th May* 1992
Accepted 25*th June* 1992