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Identification of L-Nitrosuccinate as an Intermediate in the Fungal Biosynthesis of 3-Nitropropanoic Acid

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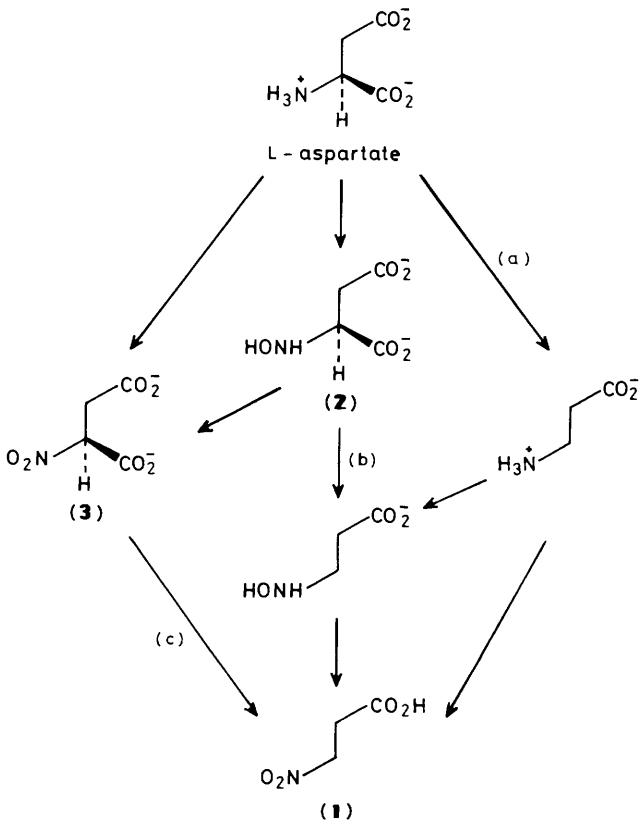
D,L-Diethyl [^{15}N]nitrosuccinate is efficiently incorporated into 3-nitropropanoic acid (**1**) by cells of *Penicillium atrovenetum*; incorporation of L-[2,3,3-$^{2}H_3$]aspartate into (**1**) with retention of the C-2 deuterium of the amino acid allows assignment of the chirality of the intermediate.

3-Nitropropanoic acid (**1**) is a toxin produced by a number of fungi[1] and several plants of the family *Fabaceae*.[2] The biosynthetic routes to this metabolite, although not characterised in detail, appear to be markedly different in the two types of organism.[3] The fungal pathway is of especial interest since 3-nitropropanoic acid is implicated as a key intermediate in the nitrification pathway of *Aspergillus* and *Penicillium* strains.[4] Previous investigations with *P. atrovenetum* have shown that the amino nitrogen and carbons-2, -3 and -4 of the L-aspartate skeleton are incorporated as an intact unit[5—7] and that both oxygens of the nitro group are derived from dioxygen.[8] On this basis, three distinct routes from L-aspartate to (**1**) are possible in theory (Scheme 1). While route (a), *via* β-alanine, can be discounted by the failure to incorporate label from β-alanine into (**1**),[5] the alternative routes, involving decarboxylation either of *N*-hydroxyaspartate (**2**), or of nitrosuccinate (**3**), have proven difficult to test because of the instability of these compounds.[9] We have examined the incorporation of ^{15}N from D,L-diethyl [^{15}N]nitrosuccinate into (**1**), reasoning that *in vivo* hydrolysis of the diester would be slow enough to liberate small quantities of the free acid within the cells and that subsequent incorporation of (**3**), while it would not preclude *N*-hydroxyaspartate as an earlier intermediate, would show that complete oxidation of the amino group of aspartate to a nitro group precedes decarboxylation.

Accordingly, D,L-diethyl [^{15}N]nitrosuccinate was prepared by treatment of diethyl bromosuccinate with $Na^{15}NO_2$ (94 atom % ^{15}N) in the presence of phloroglucinol[10] and the diester was pulse fed to surface cultures of *P. atrovenetum* over a period of 36 h beginning 48 h after inoculation. The ^{1}H-decoupled ^{15}N DEPT n.m.r. spectrum of the isolated 3-nitropropanoic acid in deuteriomethanol showed an intense signal at 3.4 p.p.m. (relative to $MeNO_2$) corresponding to a 14-fold increase of the ^{15}N signal over natural abundance. The enrichment of the nitro nitrogen was also evident from the appearance of ^{15}N satellites of both methylene signals in the ^{1}H n.m.r. spectrum of the metabolite ($^{2}J_{NH}$ 2.2 Hz, $^{3}J_{NH}$ 3.7 Hz) which corresponded in intensity to a 20 fold dilution of ^{15}N enrichment from the racemic diester.

The stereochemistry of the intermediate nitrosuccinic acid was determined indirectly by examining the incorporation of deuterium from L-[2,3,3-$^{2}H_3$]aspartic acid (98 atom % ^{2}H) into (**1**). The ^{2}H n.m.r. spectrum of the enriched 3-nitropropanoic acid shows that deuterium is retained at both the 2- and



Scheme 1

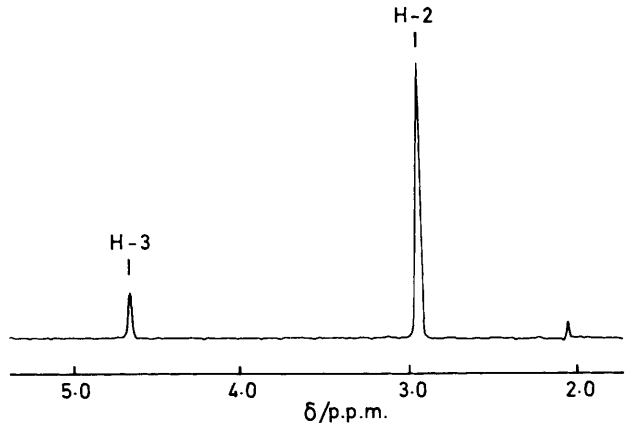


Figure 1. 55.3 MHz ^2H N.m.r. spectrum of 3-nitropropanoic acid obtained from material biosynthetically enriched with ^2H from L-[2,3,3-$^2H_3$]aspartic acid. The spectrum was measured on a 1 M solution in acetone, spectral width 600 Hz, acquisition time 0.85 s, 2000 transients.

3-positions of the metabolite (Figure 1).† If inversion of the stereochemistry at the carbon derived from the C-2 of L-asparatate had occurred in the formation of nitrosuccinic acid then no retention of deuterium of the 3-position of (**1**) would be expected. It follows that the stereochemistry at the C-2 of L-aspartate is probably retained in the metabolic oxidation of the amino acid to nitrosuccinic acid.

On the basis of these results it appears evident that the biosynthetic pathway involves oxidation of L-aspartate to L-nitrosuccinate prior to decarboxylation [route (c) in Scheme 1] and that the subsequent steps do not involve loss of the H-2 of this intermediate. The mechanism and stereochemistry of the decarboxylation step are however unknown.

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† Partial loss of ^2H derived from the 2-position of L-[2,3,3-^2H]asapartate may arise through aspartate aminotransferase activity[7] or by chemical exchange at a later stage in the pathway. In a control experiment (data not shown) the intensity ratio of the ^2H signals for 3-nitropropanoic acid isolated from a culture grown in medium containing 20% ^2H_2O was *ca.* 1:1, indicating that no significant exchange of ^2H at C-3 occurred during isolation of the metabolite.

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