The Fungal Biosynthesis of 3-Nitropropionic Acid: Is the Decarboxylation of L-Nitrosuccinate an Enzymatic Reaction?

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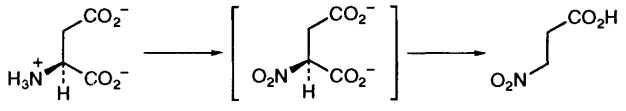
In vitro enzymatic hydrolysis of diethyl (*RS*)-nitrosuccinate **2c** by pig liver esterase at pH 7.8 affords 3-nitropropionic acid **1** indicating that the decarboxylation of (*RS*)-nitrosuccinate **2b** is spontaneous under physiological conditions. In the *in vivo* synthesis of **1** from L-aspartate by cells of *Penicillium atrovenetum*, which involves oxidation of L-aspartate to nitrosuccinic acid, the decarboxylation of the nitrosuccinate may not require a specific biological catalyst.

We have recently shown[1] that the biosynthesis of the fungal toxin 3-nitropropionic acid **1** by *Penicillium atrovenetum* involves the oxidation of the amino group of L-aspartic acid and loss of the C-1 carboxylate with retention of all of the hydrogen atoms of the precursor (see Scheme 1).



L-aspartate 1

Scheme 1

In the course of this study we examined the diethyl ester of racemic nitrosuccinate as a precursor of **1** and found that diethyl (±)-[^{15}N]nitrosuccinate **2d** is efficiently converted into [^{15}N]-**1** by cells of *P. atrovenetum*. The logic of this experiment was that the diester should be slowly hydrolysed *in vivo* to afford nitrosuccinic acid, which would be subsequently converted into **1**. While this experiment, in conjunction with other data,[1] suggested that (*S*)-nitrosuccinate **2a** is a likely intermediate in the biosynthetic pathway, it immediately poses the question whether the nitrosuccinate produced in the cell, derived either biosynthetically by direct oxidation of the amino group of L-aspartate or artificially by enzymatic hydrolysis of the artificially introduced racemic diester, requires an enzymatic decarboxylation to give 3-nitropropionate or whether the decarboxylation of **2a** is spontaneous under physiological conditions.

In an effort to resolve this question we carried out a large number of hydrolyses of the diester **2c** under a wide variety of alkaline and acidic conditions, but were unable to detect either the free acid **2b** or the decarboxylation product **1** in the mixture of products formed. This is perhaps predictable in that deprotonation at the C-2 of the α-nitro ester **2c** is facile and the resultant *aci* nitro anion would be expected to be extremely reactive.

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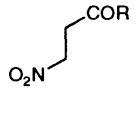
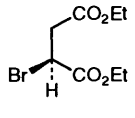
a (*S*) $R^1 = R^2 = H$
b (*RS*) $R^1 = R^2 = H$
c (*RS*) $R^1 = R^2 = Et$
d (*RS*) [^{15}N] $R^1 = R^2 = Et$
e (*RS*) $R^1 = H$, $R^2 = Et$



1 R = OH
4 R = (*R*) OCH(Ph)CO_2Me
5 R = (*S*) NHCH(CO_2Me)CHMe$_2$



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However, the rationale of the *in vivo* diester experiment requires that esterase action by the fungal cells releases the free diacid at a controlled rate at cytoplasmic pH, a condition which cannot be easily fulfilled in an acid or base hydrolysis. An alternative *in vitro* experiment is to effect hydrolysis of the diester by an esterase with catholic substrate requirements, which would produce the free α-nitro acid under essentially neutral conditions. Accordingly, we treated the racemic diethyl ester **2c** with pig liver esterase (PLE, EC 3.1.1.1) at pH 7.8 and monitored the reaction by ^{1}H NMR spectroscopy of ether extracts of aliquots of the incubation mixture. Under these conditions two products were observed. While after 3 h no signals for unchanged **2c** were detectable, signals attributable to the monoester **2e** and 3-nitropropionic acid **1** were evident. Integration indicated a **2e**:**1** ratio of 1:2. After 8 h 3-nitropropionic acid **1** was the sole detectable product.

While PLE has never been reported to have any decarboxylase activity it was possible that the decarboxylation of nitrosuccinic acid observed in the *in vitro* experiment was the result of a PLE-catalysed reaction. To test whether PLE could effect a decarboxylation reaction we examined the enzyme catalysed hydrolysis of ethyl 3-oxo-3-phenylpropanoate **3** under the same *in vitro* conditions. PLE hydrolysis of this ester afforded a quantitative yield of 3-oxo-3-phenylpropanoic acid and no acetophenone was detected even on prolonged incubations (>24 h) of **3** with large amounts of the enzyme, indicating that PLE does not catalyse decarboxylation of 3-oxo acids. It therefore seems reasonable to assume that decarboxylation of (*R*)- and (*S*)-nitrosuccinate, produced *in vitro* by the hydrolysis of **2c** by the esterase, is not an enzymatically facilitated reaction, but rather a chemical reaction which occurs spontaneously under the mild conditions of the incubation.

Extension of this result to the *in vivo* biosynthetic situation is tenuous however, since it can be argued that many chemical reactions, which occur spontaneously *in vitro*, can involve specific enzymic catalysis *in vivo*. In this case we might predict that if an enzyme-mediated decarboxylation is involved *in vivo*, then the protonation step following decarboxylation is likely to be stereospecific, whereas a non-enzymatic decarboxylation would be followed by a non-stereospecific protonation. Thus, if an enzymatic decarboxylation is involved, biosynthesis of **1** from [2-^{2}H]-L-aspartate should afford either (3*S*)- or (3*R*)-

[3-^{2}H]-**1**, depending on whether the reaction proceeds with retention or inversion of stereochemistry. Conversely, if decarboxylation proceeds by a non-enzyme catalysed route then we should expect formation of a racemic product.

To test this we prepared (2*S*,3*S*)-[2,3-^{2}H]aspartate[2] and fed this to *P. atrovenetum* cultures during the log phase of growth. The ^{2}H NMR spectrum of the isolated **1** showed that deuterium was retained at both the C-2 and C-3 positions of the metabolite. However, the deuterium enrichment at C-3 of the metabolite was approximately 30% of that at C-2 indicating that some loss of ^{2}H at C-3 occurs during the biosynthetic process.* In previous work we had noted that the ^{1}H NMR spectra of the methyl (*R*)-mandelate ester and of the methyl (*S*)-valine amide of 3-nitropropanoate, **4** and **5** respectively, both showed significant shift dispersion for the C-3 methylene protons and we prepared both of these derivatives of the ^{2}H enriched material. However, the ^{2}H NMR spectra of **4** and **5** prepared from [2,3-$^2H_2$]-**1** showed that although ^{2}H was retained in the 2-position >95% of the ^{2}H was lost from the 3-position of the derivatised metabolite during the preparation of the derivatives. While tuning of the conditions of the derivatisation reactions enabled a reduction of the ^{2}H-loss from the C-3 position no enantiomeric excess at this position could be detected in the ^{2}H spectra. Since the absence of chirality at this position could result either from a non-stereospecific *in vivo* decarboxylation or by epimerisation at C-3 in the course of the derivatisation reactions no conclusion can be drawn from this experiment.

As an alternative approach to this problem we elected to prepare both diethyl (2*R*)- and (2*S*)-nitrosuccinates and evaluate these as competitive substrates for both the *P. atrovenetum* system *in vivo* and for pig liver esterase *in vitro*. Chiral diethyl (*S*)- and (*R*)-bromosuccinates (**6** and its enantiomer, respectively) were prepared by straightforward methods from L- and D-aspartic acids.[3] However, conversion of these into the corresponding nitro compounds was accompanied by complete racemisation of the chiral centre giving diethyl (*RS*)-nitrosuccinate **2c** in each case. The ease of racemisation of the 2-position of diethyl nitrosuccinate is exemplified by the fact that complete exchange of the C-2 proton by ^{2}H within 20 min in 10% ^2H_2O–$C^2H_3O^2H$ at 18 °C. It is therefore not surprising that chiral esters of nitrosuccinic acid could not be prepared.

In conclusion, we have shown that *in vitro* enzymic hydrolysis of diethyl (*RS*)-nitrosuccinate **2c** by pig liver esterase proceeds by hydrolysis of the C-4 ester as the first step to afford the mono ester **2e** and that hydrolysis of **2e** results in decarboxylation to afford 3-nitropropionic acid **1**. Since PLE appears devoid of decarboxylase activity this suggests that decarboxylation of **2b** *in vitro* is a spontaneous chemical reaction. However, the status of the decarboxylation step affording **1** *in vivo* must remain in some doubt since it has not been possible to ascertain whether a separate enzymatic step is involved in the decarboxylation of **2a**. From a chemical point of view, however, it is evident that in the biosynthesis of 3-nitropropionate **1**, oxidation of the amino group of L-aspartate to a nitro group must predispose the product (which may epimerise at this stage) to a rapid decarboxylation, which may not require specific enzymatic catalysis. It is noteworthy that a specific decarboxylase activity need not be implicated. The essential economy of a one-enzyme process for the conversion of L-aspartate to **1** is ecologically appealing, in view of the role of the nitropropionate pathway in fungal nitrification,[4] since it reduces the number of enzymatic steps between L-aspartate and nitrite anion to two.

* As a control experiment 3-nitropropionic acid **1** was isolated from a culture grown in medium containing 20% ^2H_2O. The ^{2}H NMR spectrum of the deuterium-enriched metabolite {recorded under identical conditions to that of **1** enriched from (2*S*,3*S*)-[2,3-^{2}H]-aspartate} showed peaks of equal intensity for both positions indicating that no loss of ^{2}H from C-3 was involved in the isolation procedure. The fact that D-aspartate is not a precursor of **1**[1] suggests that exchange occurs after oxidation to **2a**.

Experimental

NMR spectra were recorded on a Bruker WH200 spectrometer and mass spectra using a Kratos MS50 TC instrument. Diethyl (*RS*)-nitrosuccinate was prepared as described previously[1] and pig liver esterase was purchased from Sigma Chemical Co., UK. The growth of *P. atrovenetum* cultures, feeding of enriched precursors and the isolation and purification of 3-nitropropionic acid were carried out essentially as previously described.[1]

Enzymatic Hydrolyses. Typically diethyl (*RS*)-nitrosuccinate **2b** (10 mg, 0.46 mmol) was dissolved in DMSO (100 mm^3) and added to a solution of pig liver esterase (1 mg, 200 units) in sodium phosphate buffer (pH 7.8, 200 mmol dm^{-3}; 10 cm^3) at 25 °C. Aliquots (2 cm^3) were removed after 3, 6 and 8 h, acidified with HCl (to pH 3) and extracted with ether (5 cm^3 × 2). The ethereal extracts were dried (Na_2SO_4), evaporated and redissolved in $CDCl_3$ for NMR analysis. Product ratios were determinated from the ^{1}H NMR spectra of the extracts.

Product identification. To verify the identity of the hydrolysis products a larger scale incubation of **2b** with the esterase (2 mg esterase, 57 mg **2b**, in 20 cm^3 buffer, 2 h) was carried out, extracted as above and the products separated by TLC affording 3-nitropropionic acid **1** (10 mg, TLC, MS and ^{1}H NMR identical with an authentic sample) and monoethyl nitrosuccinate **2c** (30 mg) [Found: M − (HNO_2 + OH), 127.0390. Calc. for $C_6H_7O_3$: 127.0395]; δ_H 1.30 (3 H, t, *J* 7.1), 3.29 (2 H, AB of ABX, *J* 22.9, 9.2 and 4.8), 4.30 (2 H, q, *J* 7.1), 5.52 (1 H, X of ABX, *J* 9.2 and 4.8); *m*/*z* (EI) 127 (7.5%), 99 (48), 55 (100).

Methyl (R)-O-(3-*nitropropanoyl*)*mandelate* **4**.—3-Nitropropanoic acid (10 mg, 0.08 mmol) in dry THF (1 cm^3) was treated with trifluoroacetic anhydride (18 mg, 0.08 mmol) and the mixture shaken vigorously for 2 min and periodically over a further 1 h. Methyl (*R*)-mandelate (20 mg, 0.085 mmol) was added and the mixture stored at room temp. for 12 h 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 **4** (8 mg, 37%) m.p. 120–122 °C; δ_H([$^2H_6$]benzene) 2.45 (2 H, t, *J* 10, CH_2CO_2R), 3.40 (3 H, s, OCH_3), 3.70 and 3.90 (2 H, 2 m, CH_2NO_2), 5.50 (1 H, s, $CHCO_2CH_3$), 7.00 (5 H, ArH); *m*/*z* (EI) 267 (M^+, 10%).

(S)-N-(3-*nitropropanoyl*)*valine Methyl Ester* **5**.—L-Valine methyl ester (16.5 mg, 0.1 mmol) and 1,3-dicyclohexylcarbodiimide (10 mg, 0.1 mmol) were added to a stirred solution of 3-nitropropanoic acid (10 mg, 0.08 mmol) in dry THF (0.5 cm^3) at room temp. 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, 2 g) using 0–10% MeOH–$CHCl_3$ as eluent. Fractions containing the amide were pooled, evaporated and the residue crystallised from EtOAc–hexane to give **5** (9 mg, 50%) m.p. 42–44 °C (Found: M^+, 232.106. Calc. for $C_9H_{16}N_2O_5$: *M*, 232.1060); δ_H($CDCl_3$) 0.89 and 0.92 (6 H, 2 d, *J* 5.5, 2 × CH_3), 2.14 [1 H, m, $CH(CH_3)_2$], 2.86 (2 H, ABCD, $\Delta\delta$ 0.08, ^2J 8.5, ^3J 4.2 and 2.5, CH_2CONH), 3.75 (3 H, s, OCH_3), 4.60 (1 H, dd, *J* 5 and 10, *CH*NH), 4.67 (2 H, AA'BB' system, $\Delta\delta$ 0.11, ^2J 8.0, ^3J 2.5 and 4.2, CH_2NO_2), 6.25 (1 H, d, *J* 10, NH); *m*/*z* (EI) 232 (14%), 172 [(M − CO_2CH_3)$^+$, 5], 125 [(172 − NO_2)$^+$, 10].

Attempted Preparation of Diethyl (2R)-*Nitrosuccinate*.—(*S*)-Bromosuccinic acid was prepared from L-aspartic acid as previously described[3] and esterified with 3% HCl in EtOH to afford the diethyl ester **6** as a colourless oil, $[\alpha]_D^{20}$ −31.7 (*c* 2.3 in EtOH); $\delta_H(CDCl_3)$ 1.25, 1.29 (6 H, 2 d, *J* 7.1, 2 × CH_3), 3.22 (2 H, AB of ABX, $\Delta\delta$ 0.23, ^2J 17.7, ^3J 5.0 and 9.1, CH_2), 4.18 and 4.29 (4 H, 2 q, *J* 7.1, 2 × CH_2O), 5.53 (1 H, X of ABX, *J* 5.0 and 9.1, CH); *m*/*z* (EI) 208 (M − OEt, 20%). Sodium nitrite (234 mg, 0.33 mmol) and phloroglucinol dihydrate (288 mg) were added to a solution of **6** (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 (30–40 °C) as eluent. Fractions containing the nitro ester (0.4 g) were evaporated and distilled (80–100 °C at 1 mmHg) using a Kugelrohr apparatus to afford racemic diethyl nitrosuccinate **2c** (200 mg), $[\alpha]_D^{20}$ 0.0° (*c* 2.1 in EtOH), which was chromatographically and spectroscopically identical with authentic material.[1] Diethyl (*R*)-bromosuccinate, $[\alpha]_D^{20}$ + 31.0° (*c* 2.0 in EtOH), was prepared from D-aspartic acid as above and similarly gave racemic diethyl nitrosuccinate.

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References

1 R. L. Baxter, A. B. Hanley, H. W.-S. Chan, S. L. Greenwood, E. M. Abbot, I. J. McFarlane and K. Milne, *J. Chem. Soc., Perkin Trans 1*, 1992, 2495.
2 S. J. Field and D. W. Young, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2387.
3 J. A. Frick, J. B. Klassen, A. Bathe, J. M. Abramson and H. Rapoport, *Synthesis*, 1992, 621.
4 K. G. Dextader and M. Alexander, *Can. J. Microbiol.*, 1966, **12**, 807; J. A. E. Molina and M. Alexander, *J. Bacteriol.*, 1971, **105**, 489; B. J. Grunder, A. B. DeAngelo and P. D. Shaw, *Arch. Biochem. Biophys.*, 1972, **148**, 107; D. J. T. Porter and J. Bright, *J. Biol. Chem.*, 1987, **262**, 14 428; T. Kido, K. Soda and K. Asada, *J. Biol. Chem.*, 1978, **253**, 226; M. R. Dhawale and U. Hornemann, *J. Bacteriol.*, 1979, **137**, 916.

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