The isolation and identification of 6-hydroxycyclohepta-1,4-dione as a novel intermediate in the bacterial degradation of atropine

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Growth of *Pseudomonas* AT3 on the alkaloid atropine as its sole source of carbon and nitrogen is nitrogen-limited and proceeds by degradation of the tropic acid part of the molecule, with the metabolism of the tropine being limited to the point of release of its nitrogen. A nitrogen-free compound accumulated in the growth medium and was isolated and identified as 6-hydroxy-cyclohepta-1,4-dione. This novel compound is proposed as an intermediate in tropine metabolism. It served as a growth substrate for the organism and was also the substrate for an NAD^+-linked dehydrogenase present in cell extracts. The enzyme was induced during the tropine phase of diauxic growth on atropine or during growth on tropine alone.

INTRODUCTION

Atropine is one of the tropane alkaloids that occurs in plants of the family Solanaceae, including *Atropa belladonna* (deadly nightshade) (Dalton, 1979) and was first isolated by Brandes (1832). The molecule contains two cyclic structures, the alicyclic nitrogen-containing alcohol tropine and the aromatic tropic acid, joined together by an ester linkage (Figure 1). Its degradation by micro-organisms has been reported by several groups (Kackowski, 1959; Niemer et al., 1959; Kackowski and Mazejko-Toczko, 1960; Berends et al., 1967; Rorsch et al., 1971), and in those cases where the mechanism has been studied, the initial step is the hydrolysis of the ester bond to give the two separate cyclic components. The tropic acid moiety has been reported to be metabolized via phenylacetic acid (Stevens and Rorsch, 1971), but much less is known about the fate of the tropine molecule, or indeed about the metabolism of complex N-heterocycles in general. This is partly because suitable compounds for testing as putative intermediates or for use as analytical standards are not readily available and this has hindered studies of their degradation. However, oxidation of the alcohol group of tropine by an NAD^+-linked dehydrogenase in *Corynebacterium belladonna* to give tropinone has been reported by Niemer and Bucherer (1961). They also reported the isolation of methylamine and small amounts of tropinic acid (Figure 1) from culture medium and suggested metabolism of the tropinone through tropinic acid to 2,6-dioxopimelic acid. This implies cleavage of the alicyclic ring of tropine at the carbon bearing the hydroxy group before release of nitrogen and plausible biochemical mechanisms to accomplish this can be postulated. However, the evidence was not conclusive. There has been little progress in the elucidation of the mechanism of tropine degradation since that time, reflecting in part some of the difficulties mentioned above.

In this study an organism capable of growth on atropine as its sole carbon or carbon and nitrogen source has been isolated from soil and identified as a species of *Pseudomonas*. It, too, initiates degradation by an esterase-catalysed cleavage of the substrate and then preferentially utilizes the aromatic fragment (Long et al., 1993). This fragment (tropic acid) is also preferentially utilized when the organism is grown on atropine as its sole source of both carbon and nitrogen but, under these conditions, there also has to be partial metabolism of the tropine to provide nitrogen for growth. This results in the accumulation of a nitrogen-free product from tropine, and the present paper deals with the isolation and identification of this compound with some evidence that it is an intermediate in tropine metabolism.

MATERIALS AND METHODS

Maintenance and growth of organism

The organism was maintained and grown as described by Long et al. (1993). Medium contained 1 g of growth substrate/l. Growth was monitored by measuring attenuance (D) at 580 nm.

Preparation of cell extracts

Cell paste was resuspended in an equal volume of 42 mM potassium/sodium phosphate buffer, pH 7.1, and cells were disrupted by ultrasonic disintegration using a Soniprep 150 (MSE, Crawley, Sussex, U.K.) sonifier at an amplitude of 14 μm with four 30 s bursts interspersed with cooling on ice. The disrupted suspension was then centrifuged at 28000 *g* for 30 min at 4 °C and the supernatant taken as crude extract.

Partial purification of 6-hydroxycyclohepta-1,4-dione dehydrogenase

A portion of crude extract containing 5 mg of protein was loaded on to a column of 0.5 ml of Reactive Red 120–agarose (Sigma, Poole, Dorset, U.K.) in 21 mM potassium/sodium phosphate buffer, pH 7.1, contained in a Pasteur pipette. The column was washed with 5 ml of buffer and then enzyme was eluted with 2 ml of buffer containing 5 mM NAD^+. This gave a 10.7-fold purification with 36 % recovery of activity.

Product from 6-hydroxycyclohepta-1,4-dione dehydrogenase reaction

A reaction mixture containing, in 30 ml of 0.1 M sodium pyrophosphate buffer, pH 8.8, NAD^+ (90 μmol), 6-hydroxy-cyclohepta-1,4-dione (4.9 mg) and partially purified enzyme (0.23 mg) was incubated at 30 °C for 4 h. The mixture was then
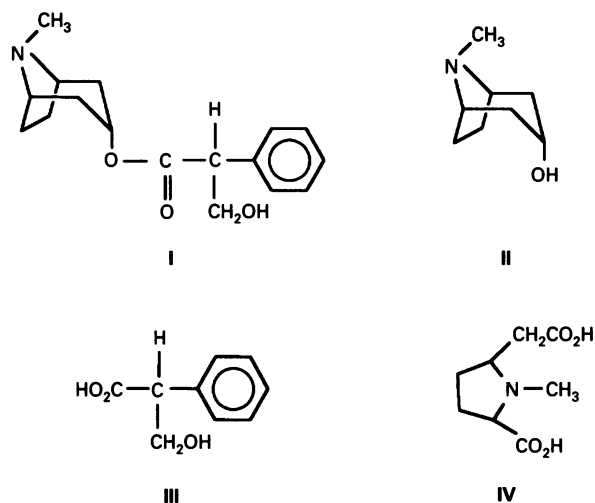

Figure 1 Formulae of (I) atropine, (II) tropine, (III) tropic acid and (IV) tropinic acid

acidified to pH 1–2 with 5 M HCl and extracted nine times with 30 ml of diethyl ether. The pooled extracts were dried over anhydrous Na_2SO_4 and the diethyl ether removed on a rotary evaporator to leave a small amount of an oil as product.

6-Hydroxycyclohepta-1,4-dione dehydrogenase assay

The hydroxycyclohepta-1,4-dione dehydrogenase was assayed spectrophotometrically by monitoring the reduction of NAD^+ at 340 nm at 30 °C in a 1-cm-pathlength cuvette containing in 3.0 ml of 0.1 M sodium pyrophosphate buffer, pH 8.8, 2 μmol of NAD^+, 0.2 μmol of substrate and enzyme. A unit of activity was that causing the reduction of 1 μmol of NAD^+/min.

Protein assays

Protein was assayed by the method of Lowry et al. (1951), with BSA as standard.

Assay for ketones

Ketones were detected by their reaction with 2,4-dinitrophenyl-hydrazine (2,4-DNPH). The reagent contained 0.1 % (w/v) 2,4-DNPH in 2 M HCl. A semiquantitative assay for ketones in culture medium was performed, after removal of cells from a sample of culture by centrifuging, by incubation of a portion of the supernatant, diluted to 0.5 ml, with 0.5 ml of 2,4-DNPH reagent at 30 °C for 30 min. After addition of 2 ml of 1 M NaOH and incubation for 5 min at room temperature, the absorbance of the mixture was measured at 427 nm.

Chromatography

Flash chromatography was performed on a 15 cm × 2 cm column of silica gel 60 (230–400 mesh; Merck 9385) with ethanol as solvent. The product was located by testing a small portion of each fraction with 2,4-DNPH reagent.

T.l.c. was performed using precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) with ethanol/ethyl acetate (1:1, v/v) as solvent. Ketonic compounds were located as yellow spots after spraying with 2,4-DNPH reagent.

G.c.–m.s.

G.c.–m.s. was performed on a Hewlett–Packard 5890 instrument with a 5971 mass-selective detector. An HP-5 (cross-linked 5 % phenylmethylsilicone) column (25 m × 0.2 mm × 0.33 μm film) with helium as the carrier gas, and a temperature programme of 4 min at 70 °C rising at 10 °C/min to 275 °C, were used.

N.m.r. spectra

N.m.r. spectra were performed by Dr. O. W. Howart at the SERC n.m.r. Service, The University of Warwick, using a Bruker WH 400 spectrometer with the sample dissolved in [^{2}H]-chloroform.

I.r. spectra

The i.r. spectrum of a 1 % KBr mull of the product was obtained using a Perkin–Elmer 1310 instrument.

Microanalysis

Microanalysis for C and H was performed by M. Hart, Micro-analytical Laboratory, Department of Chemistry, University of Manchester, Manchester, U.K.

RESULTS AND DISCUSSION

Accumulation of intermediate

Growth of *Pseudomonas* AT3 on atropine as its sole source of carbon is diauxic, with hydrolysis of the molecule and utilization of the tropic acid portion in the first phase of growth followed, after a lag, by growth on the tropine (Long et al., 1993). Assay of the growth medium showed the transient appearance of 2,4-DNPH-reactive material, indicative of an aldehyde or ketone, during the tropine growth phase. Similar transient formation of small amounts of such material was seen when tropine alone was used as the sole carbon, or carbon and nitrogen, source(s). Under conditions where atropine was the sole carbon and nitrogen source, the culture was nitrogen-limited and the extent of growth was reduced to the equivalent of that for the tropic acid phase. Under these conditions there was accumulation of much larger amounts of 2,4-DNPH-positive material which declined slowly on prolonged incubation of the culture (Figure 2), suggesting only limited metabolism of the tropine, mainly to the point of removal of the nitrogen required for growth. To accumulate sufficient of this material for identification a 500 ml culture was monitored for 2,4-DNPH-positive material during growth on 500 mg of atropine as the sole carbon and nitrogen source. At maximum metabolite accumulation the cells were removed and the medium was extracted continuously with diethyl ether. The diethyl ether extract was dried over anhydrous Na_2SO_4 and evaporated to dryness to yield a pale yellow oil. This was purified by flash chromatography on silica gel with ethanol as solvent. The solvent was removed by evaporation and the product crystallized from diethyl ether, typically giving 100 mg of white crystals, m.p. 57–58 °C. The reaction of a known amount of product with the 2,4-DNPH reagent in the assay for ketones gave an $A^{1\%}_{1cm,427}$ of 350. From this it can be calculated that at its maximum the A_{427} in Figure 1 represents a total of about 180 mg of product in the culture, assuming that this is the only ketone present. Clearly this is a major product and represents a high proportion of the tropine segment (244 mg) of the atropine growth substrate.
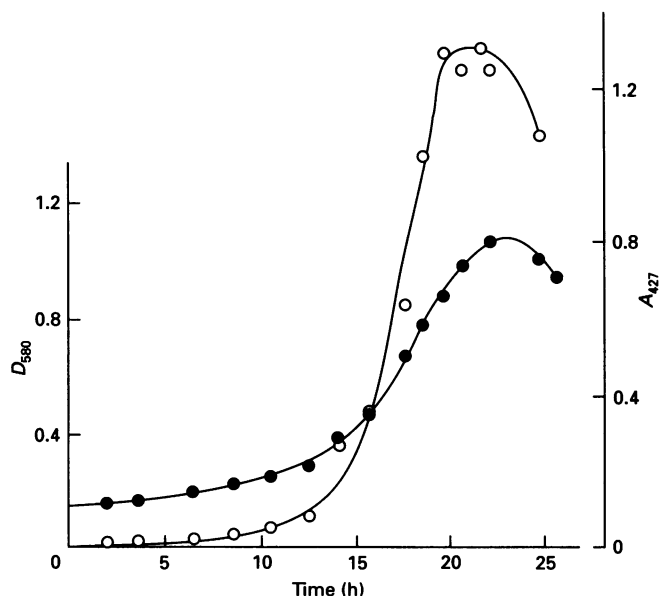
Figure 2 Growth of *Pseudomonas* AT3 on atropine and accumulation of intermediate

The growth the organism in liquid medium with atropine (1 g/l) as the sole carbon and nitrogen sources was monitored by the attenuance *(D)* at 580 nm (●). Accumulation of 2,4-DNPH-reactive material was monitored by allowing samples of culture medium to react with 2,4-DNPH reagent and reading the absorbance in alkali at 427 nm. The absorbance (○) is that equivalent to 0.05 ml of culture medium.
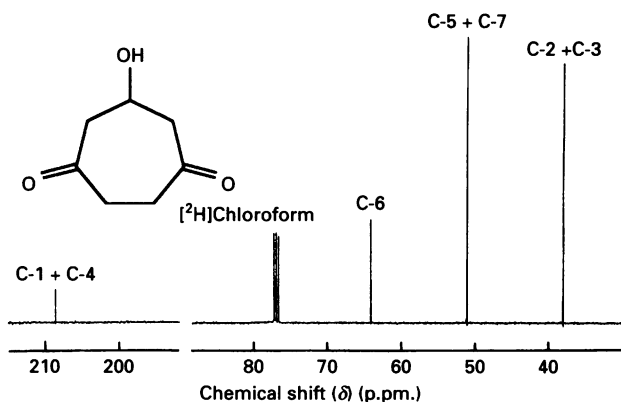

Figure 3 ¹³C-n.m.r. spectrum and proposed structure of isolated compound

The spectrum of 10 mg of the isolated compound dissolved in [²H]chloroform was recorded with tetramethylsilane included as the reference compound for calculation of the chemical shifts. The peak designations refer to the carbon atoms of 6-hydroxy-cyclohepta-1,4-dione, the chemical structure shown.

Identification of intermediate

The isolated material gave a single spot on t.l.c. (R_F 0.6) and was reactive with 2,4-DNPH. Examination of the compound by g.c.–m.s. gave a single peak whose mass spectrum by electron-impact ionization showed a molecular ion at m/z 142 and this was confirmed by chemical ionization. Other major peaks were at 124 ($M^+ - H_2O$), 114 ($M^+ - CO$), 98 ($M^+ - CH_2CO$), 71 and

56. The even number for the molecular mass indicates that the nitrogen of the original compound has been removed and, taking into account the C_7 ring of tropine, an empirical formula of $C_7H_{10}O_3$ can be proposed. Elemental analysis of the compound gave C, 58.7 and H, 6.7%; $C_7H_{10}O_3$ requires C, 59.14 and H, 7.09%.

The product was identified as an alcohol from its mass spectrum and as a keto-alcohol from its i.r. spectrum, which showed bands at 3420 cm⁻¹ (OH) and 1705 cm⁻¹ (C=O). From these data the most likely structure for such a compound derived from tropine is 6-hydroxy-1,4-cycloheptanedione (Figure 3). This was confirmed by the ¹³C n.m.r. spectrum, which showed only four lines (Figure 3), indicative of a highly symmetrical structure giving δ 208 (C-1 and C-4), 64 (C-6), 51 (C-5 and C-7) and 38 (C-2 and C-3). The ¹H n.m.r. spectrum gave δ 4.43 (1H, sextet, C*H*OH), 2.97 (1H, d, O*H*), 2.94 (4H, d, C-5 and C-7 methylenes), 2.66 (4H, m, C-2 and C-3 methylenes) which was in agreement with the proposed structure.

Further metabolism of intermediate

Pseudomonas AT3 gave good growth in liquid medium with the isolated intermediate as its sole carbon source. The generation time of 3–4 h was the same as that for growth on tropine under the same conditions and the final attenuance (2.03) was also similar to that for tropine-grown cells (2.25) with both substrates at an initial concentration of 1 g/l.

Extracts of cells grown on atropine, in the presence of NH_4^+ as nitrogen source, and harvested during the tropine phase of growth contained an NAD^+-linked dehydrogenase activity towards the 6-hydroxycycloheptane-1,4-dione with a specific activity of 0.083 μmol/min per mg of protein. This contrasts with values of 0.006 in the tropic acid phase of growth and < 0.0008 in succinate-grown cells and is comparable with the value of 0.21 in tropine-grown cells. Clearly the enzyme is induced by growth on tropine. The product expected from such a dehydrogenase reaction would be the triketone cyclohepta-1,3,5-trione and a peak with M^+ at m/z 140, which would correspond to this, was seen when 6-hydroxycyclohepta-1,4-dione was incubated with cell extract and NAD^+ and a diethyl ether extract of the reaction mixture examined by g.c.–m.s. However, even though the substrate had been completely degraded, this peak was only one among several products found in relatively small amounts in such an experiment. Thus to characterize more precisely this first step in the further metabolism of 6-hydroxycyclohepta-1,4-dione the dehydrogenase was first partially purified by affinity chromatography on Reactive Red–agarose 3000 CL.

With partially purified enzyme the stoicheiometry of NAD^+ reduced in the assay to substrate added approached a value of 1.0. The reaction was scaled up and products were isolated and examined by t.l.c. and g.c.–m.s. On t.l.c. there was a minor spot corresponding to residual substrate (R_F 0.6) and a single, major, additional spot (R_F 0.38) for the reaction product. On g.c.–m.s. the major peak (13.27 min retention time) represented 90% of the material detected and was well separated from the starting alcohol (15.03 min retention time). The m.s. gave an M^+ at m/z 140, also confirmed by the chemical-ionization spectrum. Other peaks were at m/z 112 ($M^+ - C\!=\!O$) and 98 ($M^+ - CH_2CO$).

Conclusions

The product formed in high yield from tropine during growth of *Pseudomonas* AT3 on atropine as the sole carbon and nitrogen

source has been identified as 6-hydroxycyclohepta-1,4-dione. This is a novel compound and its discovery points to attack not at the hydroxy group, as might have been predicted from the work of Niemer and Bucherer (1961), but at the nitrogen of tropine. Thus the intermediate role for tropinic acid, as suggested by Niemer and Bucherer (1961), is unlikely in this organism. There was the possibility that the new compound is a dead-end product formed by enzymes induced specifically under these growth conditions. However, evidence that it is a true intermediate of tropine metabolism, under other growth conditions where it does not accumulate to this extent, comes from the fact that it will itself serve as a good growth substrate and that there is an enzyme induced for its further metabolism. The enzyme is a dehydrogenase and its partial purification allowed accumulation of the reaction product. Although this has not been conclusively identified, it is probably the triketone cyclohepta-1,3,5-trione, and such a compound would be a ready substrate for cleavage of the alicyclic ring by a β-diketone hydrolase.

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