STUDIES IN RELATION TO BIOSYNTHESIS

XLIII.* THE INCORPORATION OF L-LYSINE INTO MYCOBACTIN-P

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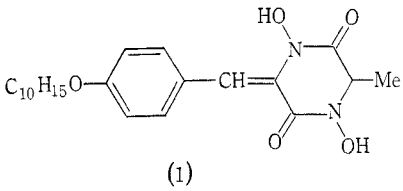
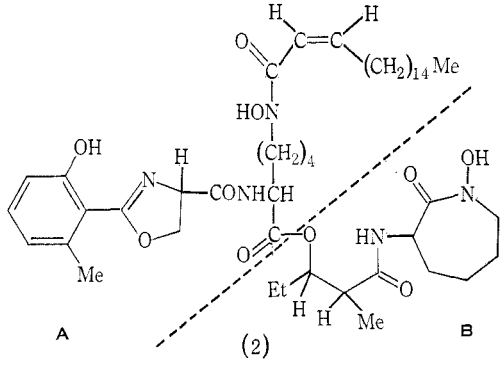
We suggested[1] that a number of biosynthetic reactions may be initiated by oxidation of a nitrogen in a molecule, leading further to products containing N–O structures, or to cyclized or cleaved products. The largest class contains oxidized peptides and amino acids including penicillin (ring closures), 3-nitropropionic acid (amino into nitro), acylhydroxylamines (COHN into CONOH), and the related N-hydroxydioxopiperazines such as mycelianamide (1). The incorporation of [^{14}C]-tyrosine and [^{14}C]alanine into mycelianamide has been demonstrated,[1,2] confirming the origins of the carbon skeleton, but in neither this nor any other case of an oxidized peptide of which we are aware has nitrogen retention been demonstrated by the use of [^{15}N]. To assess whether it is technically possible to do this we have examined a number of cases where [^{15}N] precursors might be sufficiently incorporated, including mycobactin-P (2) from *Mycobacterium phlei*.[3]



(1)



(2)

Mycobactin-P is one of a number of growth factors, and related antibiotics, which contains several acylhydroxylamine units, which can be assumed by inspection of the structures to be derived from ornithine or lysine, in this case lysine. The hydroxylamine unit of (2) is not α to a carboxyl and is therefore less likely to have

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[1] Birch, A. J., and Smith, H., in "CIBA Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity." p. 247. (J. & A. Churchill: London 1958.)

[2] Birch, A. J., and Massy-Westropp, R. A., unpublished data.

[3] Snow, G. A., *J. chem. Soc.*, 1954, 4080; *Biochem. J.*, 1965, **94**, 160.

been introduced through deamination and reaction of an α-keto acid with hydroxylamine than is the case with the *N*-hydroxydioxopiperazines. Failing the technical feasibility of the [^{15}N] experiments, an alternative approach would be to feed the organism with fragments corresponding to the A or B portions of (2) with the *N*-hydroxyls removed, in the hope of complete incorporation.

From inspection the origins of the skeleton of (2) can be defined with a fair degree of certainty. There is a fatty-acid chain (acetate–malonate origin), an aromatic ring (polyketide in origin in this case, although probably not in mycobactin-T which has an unsubstituted salicylic acid unit), a branched chain acid (from propionate–methyl malonate), two oxidized lysine units and a serine unit in the oxazoline ring.

The addition of U-[^{14}C]-L-lysine to *M. phlei* resulted in incorporation of radioactivity to the extent of 2%. This is a satisfactory indication of direct incorporation in view of the complex beef medium used and the difficult growth of the organism.

Alkaline hydrolysis[3] of mycobactin-P caused fission of the molecule into part A (mycobactic acid) and part B (cobactin) (see (2)). The r.m.a.[4] of these two products were in the ratio 1·45 : 1. Hydrogenation of the mycobactic acid to remove the *N*-hydroxyl, hydrolysis with acid, and examination of the resulting lysine and serine showed that these together contained all of the radioactivity, 17% of it in the serine. Cobactin, similarly treated, gave lysine containing all of the initial radioactivity. The octadecenoic acid and methylpentanoic acid units contained no radioactivity, indicating little if any degradation of the added lysine to small fragments.

Most of the activity is therefore found in the two lysine units, in the ratio 1·24 : 1 in parts A and B respectively. This result suggests separate elaboration of the larger structural units corresponding to A and B, possibly with a pool of precursors. The process by which serine becomes labelled is not clear, but it cannot involve complete degradation, otherwise the molecule would show label elsewhere.

Due to the high dilution of label, the use of [^{15}N] precursors does not seem to be feasible using the present method of growing *M. phlei*.

Experimental

For radioactivity assay, compounds were crystallized to constant activity and were assayed as "infinitely thick" (30 mg/cm^2) samples on a 0·3 cm^2 planchette using an end-window counter as previously described.[4] In each case 10^4 counts were taken. Estimates of incorporation were made by comparison with a polymethylmethacrylate specimen of identical geometry and known activity. The r.m.a. are counts/100 sec times the molecular weight.

Incorporation of U-[^{14}C]-L-Lysine

U-[^{14}C]-L-lysine (100 μCi) was incorporated by addition to a standard fermentation[3] of *Mycobacterium phlei* and fermentation continued for 3 days. The product was extracted and purified[3] as the aluminium salt (2·0 μCi). The salt (150 mg) in chloroform (30 ml) was washed with hydrochloric acid (5N, 8 × 40 ml) and water (4 × 40 ml), and evaporated to give mycobactin-P (122 mg), m.p. 170–171°. It was diluted with inactive mycobactin-P (500 mg), kindly provided by Dr G. A. Snow, I.C.I. Pharmaceuticals Division, and the product purified further by solution in chloroform and precipitation with ether. The [^{14}C]mycobactin-P (530 mg), $C_{47}H_{75}N_5O_{10}$, mol. wt. 869, had 1055 c.p.c., r.m.a. 9·17 × 10^5.

[4] Birch, A. J., Massy-Westropp, R. A., Rickards, R. W., and Smith, H., *J. chem. Soc.*, 1958, 360.

The hydrolysis was carried out according to the literature[3] giving mycobactic acid, m.p. 131° (lit.[3] 131°) (412 mg), $C_{35}H_{55}N_3O_7$, mol. wt. 629, c.p.c. 858·5, r.m.a. $5{\cdot}40\times10^5$, and in the neutral fraction, cobactin, crystallized from butan-2-one, m.p. 150° (lit.[3] 152·5°) (48 mg), $C_{12}H_{22}N_2O_4$, mol. wt. 268, c.p.c. 144·5, r.m.a. $3{\cdot}73\times10^5$. The sum of the r.m.a. $\times10^{-5}$ (9·13) should be identical with that of the mycobactin-P (9·17).

Mycobactic acid was reduced as described[3] by means of zinc dust to hydromycobactic acid, and the cobactin similarly[3] to hydrocobactin.

The hydromycobactic acid was hydrolysed[3] with hydrochloric acid (5N). Separation of the products gave octadec-2-enoic acid as a waxy solid which was not detectably radioactive. The aqueous phase was evaporated to dryness and the residue separated on Whatman's MM paper[3] into lysine and serine hydrochlorides. The amino acids were assayed as their picrates: lysine picrate (28 mg), $C_{12}H_{17}N_5O_9$, mol. wt. 375, c.p.c. 1187, r.m.a. $4{\cdot}45\times10^5$; serine picrate (24 mg), $C_9H_{10}N_4O_{10}$, mol. wt. 334, c.p.c. 27, r.m.a. $0{\cdot}9\times10^5$.

The hydrocobactin (40 mg) was hydrolysed as above[3] and the 3-hydroxy-2-methylpentanoic acid obtained assayed as the *p*-bromophenacyl ester, m.p. 88°, c.p.c. 0·0. The lysine picrate obtained as above (20 mg) had c.p.c. 960, r.m.a. $3{\cdot}60\times10^5$.

Acknowledgments

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