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THE INCORPORATION OF SHIKIMIC ACID INTO MYCOBACTIN S AND SALICYLIC ACID BY MYCOBACTERIUM SMEGMATIS

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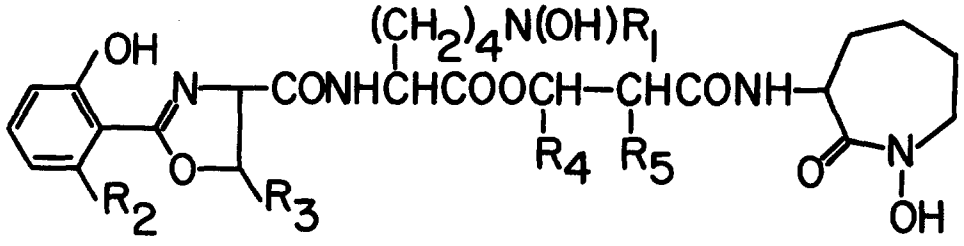
Although production of members of the mycobactin series by various Mycobacteria has been known for some time[1], the biosynthetic precursors of the aromatic components of these interesting metabolites have not been defined. We have, therefore, investigated the biosynthesis of both the salicylic acid unit of the intracellular product, mycobactin S (I), and the extracellular salicylic acid produced by Mycobacterium smegmatis (NCIB 8548).



I. $R_1 = CH_3(CH_2)_nCH = CHCO$; n = 10, 12, 14, 16

$R_2 = R_3 = R_5 = H$, $R_4 = CH_3$

II. $R_1 = CH_3(CH_2)_nCH = CHCO$; n = 10, 12, 14, 16

$R_2 = CH_3$, $R_3 = H$, $R_4 = C_2H_5$, $R_5 = CH_3$

In preliminary studies, the growth of this organism in shake culture was investigated. The medium was that used previously[2]; since zinc has been reported[3] to stimulate production of mycobactin S by an unidentified strain of Mycobacterium smegmatis this metal was routinely added (0.46 μg/ml medium). Salicylic acid production reached a maximum after 1 days growth (3.6 mg/g dry weight of cells) and then declined rapidly, only trace quantities of this compound being isolated after 4 days and none after 5 days. In contrast, only small amounts of mycobactin S were formed after 1 days growth (4.4 mg/g dry weight), the maximum production occurring after a 2 day growth period (29.8 mg/g dry weight). The mycobactin S concentration then declined slowly, 15.8 mg/g dry weight being present after 5 days. As a result of these observations, a 48 hour growth period was used in tracer experiments to allow isolation of adequate amounts of both mycobactin S and salicylic acid.

The results obtained for the incorporation of radioactivity from 20 μc of generally labeled shikimic acid (G-^{14}C-shikimate) into both metabolites are shown in Table I. The total volume of culture medium was 1400 ml distributed in two flasks. From the dried cells (4.0 g) was obtained 128 mg of mycobactin S using a modification of the method of White and Snow[2]; degradation of this metabolite to cobactin S and mycobactic acid S was also carried out by their procedure. Mycobactic acid S was further hydrolyzed to salicylic acid and a fatty acid mixture. The mycobactin S contained 0.112% of the total radioactivity fed as shikimic acid; virtually all (97.8%) of the radioactivity was present in the salicylic acid moiety, the remaining activity being located in cobactin S.

TABLE I

Tracer fed-G-^{14}C-shikimate (20 μc). Specific Activity (S.A.) 4.1269 x 10^6 dpm/μmole - added at time of inoculation

Compound isolated	% incorporation	S.A. dpm/μmole	Dilution value
salicylic acid	0.138	709	5,824
mycobactin S	0.112	332	12,423

All radioactive measurements were made with a scintillation counter

Isolation of the extracellular salicylic acid was achieved by continuous ether extraction of the acidified medium (pH 1), followed by chromatography on silicic acid with benzene/1% acetic acid. The salicylic acid (12 mg) contained 0.138% of the radioactivity fed and was purified to constant activity by recrystallization from benzene/petroleum ether.

In order to establish accurately that both the salicylic acid formed in the culture medium and that derived by degradation of mycobactin S were derived directly from shikimic acid, the two samples were diluted with inactive material and decarboxylated with copper chromite catalyst in quinoline. The evolved carbon dioxide was collected as barium carbonate; phenol was purified on silicic acid with benzene/1% acetic acid, and then converted to the benzoate derivative for counting. The results shown in Table II establish conclusively that shikimic acid was incorporated intact into both salicylic acid units.

TABLE II

Degradation of salicylic acid

Compound counted	S. A. dpm/μ mole	% Activity
salicylic acid (extracellular)	50	100
phenol benzoate	42	84
barium carbonate	7	14
salicylic acid (from mycobactin S)	75	100
phenol benzoate	64	85
barium carbonate	11	15

In conclusion, the level of activity incorporated from shikimate into salicylic acid and mycobactin S is good in view of the fact that the medium contains an endogenous source of shikimic acid - glucose. The dilution values are also reasonable and the differences between them are probably a reflection upon the different times of synthesis of the two metabolites.

The findings represent the first evidence for participation of shikimic acid in mycobactin biosynthesis, and conclusively establish shikimic acid as the precursor of extracellular salicylic acid in M. smegmatis. Ratledge[4] had recently drawn the latter conclusion from work in which washed cell suspensions of an unidentified strain of M. smegmatis were incubated with various ^{14}C labeled compounds. In this latter work only very small quantities of salicylic acid were obtained, and no attempt was made to degrade the molecule. This procedure is of considerable importance since some mycobacteria degrade shikimic acid via a pathway which allows incorporation of radioactivity into other metabolic pools[5].

It is of interest that other mycobactins (e. g. mycobactin P (II)) contain 6-methylsalicylic acid, rather than salicylic acid. Since we have obtained evidence[6] that 6-methylsalicylic acid biosynthesis by Mycobacterium phlei does not involve a role for shikimic acid but proceeds via polyketides, it appears likely that a polyketide route is also involved for the 6-methylsalicylic acid moiety of mycobactin P.

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