Biosynthesis of 6-Methylsalicylic Acid by *Mycobacterium phlei**

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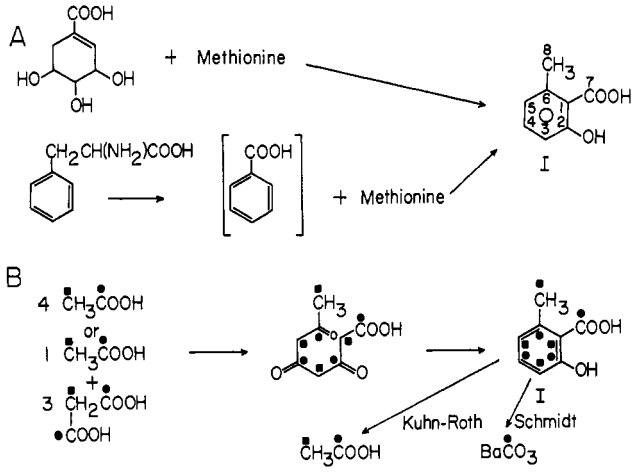
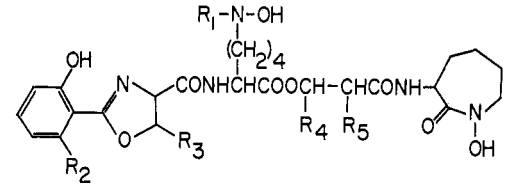
ABSTRACT: Two strains of *Mycobacterium phlei*, found to accumulate 6-methylsalicylic acid extracellularly, were used to study the biosynthesis of this aromatic acid by the tracer technique. Methionine-$^{14}CH_3$ did not provide the methyl group, and the efficiency and dilution observed using shikimate-*G*-^{14}C and phenylalanine-*U*-^{14}C were not compatible with either of these materials being obligatory intermediates. In experiments with labeled acetates, the distribution patterns obtained by chemical degradation of the 6-methylsalicylic acid samples were in keeping with a polyketide biosynthetic route.

6-Methylsalicylic acid (I, Scheme I) is a well-known fungal secondary metabolite whose carbon skeleton is formed from acetate and malonate units through the agency of a polyketide intermediate (for review, see Bentley and Campbell, 1968). There are, however, very few reports of the occurrence of this material in bacteria. In 1966 an acid produced by *Mycobacterium phlei*, and previously identified as salicylic acid, was reidentified as 6-methylsalicylic acid (Ratledge and Winder, 1962, 1966). The only other reported incidence of this acid in bacteria is as part of the mycobactin complexes P (II), H (III), and A (IV) (Snow, 1954, 1965; White and Snow, 1969; Snow and White, 1969). In view of our work on the biosynthesis of salicylic acid and mycobactin S in *M. smegmatis* (Hudson and Bentley, 1970), we sought, initially, to prove conclusively that 6-methylsalicylic acid was a metabolic product of *M. phlei* and secondly to determine how it is biosynthesized. While the polyketide biosynthetic pathway may operate in the bacterial system, alternative routes such as methylation of salicylic acid or related compounds had to be given due consideration.

SCHEME I





P(II) $R_1 = CH_3(CH_2)_nCH=CHCO$, n = 10, 12, 14, 16; $R_2 = CH_3$, $R_3 = H$, $R_4 = C_2H_5$, $R_5 = CH_3$

H(III) $R_1 = CH_3(CH_2)_nCH=CHCO$, n = 14, 16; $R_2 = CH_3$, $R_3 = CH_3$, $R_4 = CH_3$, $R_5 = H$

A(IV) $R_1 = CH_3(CH_2)_{10}CH=CHCO$; $R_2 = CH_3$, $R_3 = H$, $R_4 = CH_3$, $R_5 = H$

This paper reports that at least two strains of *M. phlei* produce 6-methylsalicylic acid and that the polyketide pathway is involved in its biosynthesis.

Experimental Section

General. All solvents were redistilled. Silicic acid for column chromatography was Clarkson Chemical Co.'s Unisil, mesh 200–325. Sephadex refers to LH-20 obtained from Pharmacia, Inc. Alumina for column chromatography was Woelm alumina, neutral, activity grade 1. Silica gel for thin-layer chromatography was Stahl silica gel G.

Gas–liquid chromatography was carried out on an F & M 402 gas chromatograph, operating at 180° and employing a 6-ft glass column packed with 3% OV-1 on silanized Gas-Chrom Q, 60–80 mesh. All radioactive materials were obtained from commercial sources. Radioactivity determinations were carried out as described in the preceding paper (Hudson and Bentley, 1970).

Growth of Mycobacterium phlei (ATCC 10142). This organism, obtained from the American Type Culture Collection, Rockville, Md., was maintained on nutrient agar slopes at 37° and grown in either of the two following media: (A) L-asparagine, 14.0 g; K_2HPO_4, 1.4 g; sodium citrate, 7.0×10^{-1} g; $MgSO_4 \cdot 7H_2O$, 1.5 g; glycerol, 100 ml; and deionized water to make a final volume of 1 l. and a pH of 6.9; and (B) L-asparagine, 7.66 g; K_2HPO_4, 1.4 g; sodium citrate, 7.0×10^{-1} g; $MgSO_4 \cdot 7H_2O$, 1.5 g; glycerol, 50 ml; and deionized water to make a final volume of 1 l. and a pH of 6.9.

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Liquid inocula were prepared by growth for 24 hr at 37° as shake cultures in 250-ml erlenmeyer flasks containing 25 ml of either medium A or B. The liquid inocula so produced were used to seed 2.8-l. fernbach flasks containing 700 ml of the corresponding medium plus a sterile solution of glucose (7.5 g) in water (25 ml). Growth as shake cultures was continued at 37° for a further 5-day period. The cells were then filtered and washed with distilled water; yield 1 g of dry cells/flask. Growth of the organism in either medium resulted in virtually identical yields of the metabolites.

Growth of M. phlei (ATCC 354). This organism, the gift of Professor M. Weber, was maintained on nutrient agar slopes and liquid inocula were prepared with medium of the following composition: (C) L-glutamic acid, 5.25 g; Tween 80, 5.25 g; K_2HPO_4, 5.25 g; NH_4Cl, 1.05 g; $MgSO_4 \cdot 7H_2O$, 5.25×10^{-2} g; $FeSO_4 \cdot 7H_2O$, 5.25×10^{-3} g; $ZnSO_4 \cdot 7H_2O$, 1.5×10^{-3} g; $CuSO_4 \cdot 5H_2O$, 1.05×10^{-3} g; $MnCl_2 \cdot 4H_2O$, 3.15×10^{-4} g; and deionized water to make a final volume of 1 l. Prior to sterilization the medium was adjusted to pH 7.2 with 100% KOH solution. Portions (25 ml) of the liquid inocula were used to seed 2.8-l. fernbach flasks containing 700 ml of the above medium plus a sterile solution of glucose (12.5 g) in water (25 ml). Growth as shake cultures was continued at 37° for a further 24-hr period. The cells were then freed from culture fluid by continuous centrifugation and washed twice with distilled water; yield 2 g of wet cells/flask.

Isolation of Metabolites from M. phlei (ATCC 10142). For the isolation of genistein and prunetin, the filtered medium (7 l.) was adjusted to pH 9 and continuously ether extracted overnight. Genistein (19 mg) and prunetin (16 mg) were isolated from the resulting ether extract as previously described (Hudson and Bentley, 1969).

The medium extracted at pH 9 was next acidified to pH 1 with 8 N HCl and continuously ether extracted overnight to remove the aromatic acids. The crude extract (900 mg) obtained by removal of the ether *in vacuo* was dissolved in benzene–ethyl acetate (1:1, v/v) and chromatographed on a column of silicic acid (160 g, 61 × 2 cm) in the system benzene–ethyl acetate–acetic acid (80:20:1, v/v), 7-ml fractions being collected. Fractions 5–6 contained 6-methylsalicylic acid (20 mg), mp 168–169°. Recrystallization from benzene–petroleum ether (bp 30–60°) raised the melting point to 170–171° (lit. mp 170–171°);[1] *O*-acetate, mp 130–131° (lit. mp 131°).[1] Continued elution of the above column gave, in fractions 14–17, phenyllactic acid as a crystalline solid (350 mg), mp 121–123°. Recrystallization from benzene raised the melting point to 124–126° (lit. mp 124–126°).[1]

For the isolation of fatty acids, a portion of the filtered, washed, and dried cells (10 g) was refluxed with 10% methanolic potassium hydroxide solution (25 ml) for 1 hr. The mixture was then filtered and the filtrate evaporated and acidified (8 N HCl, 20 ml) prior to ether extraction. Removal of the ether afforded a pale yellow oil (1 g), a portion of which (5 mg) was treated with ethereal diazomethane. After several minutes the ether was removed; the resulting methyl esters were purified by thin-layer chromatography on silica gel in the system benzene–petroleum ether (1:1, v/v). Analysis of the methyl esters by gas–liquid chromatography showed the principal component to be methyl palmitate (identified by reference to an authentic sample).

[1] From Heilbron and Bunbury (1953).

TABLE I: Utilization of L-Methionine-$^{14}CH_3$ by *M. phlei* ATCC 10142.[a]

Metabolite Isolated	Sp Act. (dpm/μmole)	Incorp (%)	Dilution Value
6-Methylsalicylic acid[b]	184	0.01	6.5×10^5
Prunetin	21,192	0.57	5.6×10^3
Genistein	0	0.0	

[a] The L-methionine-$^{14}CH_3$ (100 μCi, specific activity 53.6 μCi/μmole) was added to 10 flasks of the organism which had grown for 2 days on medium A; growth was then continued for a further 3 days. [b] The acetic acid derived from this metabolite by Kuhn–Roth oxidation had a specific activity of 20 dpm/μmole. Hence, only 11% of the total activity in 6-methylsalicyclic acid was present in C-6 + C-8.

Isolation of Metabolites from M. phlei (ATCC 354). The centrifuged culture fluid and washings (*ca.* 5 l.) were combined and reduced in volume by approximately 90%. The resulting syrup (500 ml) was strongly acidified (concentrated HCl) and continuously ether extracted for 24 hr. The ether solubles (266 mg) were then chromatographed on a column of silicic acid (50 g, 30 × 2 cm) established in benzene. The column was developed with an approximately linear gradient of benzene–acetic acid 95:5 (v/v, 150 ml) to benzene–acetic acid 75:25 (v/v, 300 ml), 4.5-ml fractions being taken. 6-Methylsalicylic acid (73 mg) was located in fractions 33–38 and was purified by sublimation (95°, 0.1 mm), preparative thin-layer chromatography (silica gel, solvent system of benzene–dioxane–acetic acid (90:45:4, v/v), and crystallization from ethyl acetate–petroleum ether;[2] yield 21 mg, mp 166–167°.

For isolation of tyrosine from cell protein, a portion of the paste (2.7 g) was hydrolyzed with aqueous HCl (5 N, 100 ml) for 24 hr under nitrogen. Charcoal (1 g) was then added to the hot solution and the mixture was filtered. Evaporation of the filtrate yielded a yellow oil (3.0 g), from a portion (718 mg) of which tyrosine (6 mg) was isolated by chromatography on Dowex 1-X8 and crystallization from water (Hirs *et al.*, 1954).

Fatty acids were isolated in the same way as those for *M. phlei* (ATCC 10142). Analysis of the fatty acid esters by gas–liquid chromatography–mass spectrometry (Campbell and Naworal, 1969) showed the principal components to be methyl palmitate and methyl 10-methyloctadecanoate.

Degradation of 6-Methylsalicylic Acid (Scheme I). Atoms C-6 and C-8 were obtained as acetic acid by Kuhn–Roth oxidation, essentially as described by Eisenbraun *et al.* (1954). The sodium acetate obtained by neutralization of the acetic acid with 0.05 N NaOH was then converted into *p*-bromo-

[2] In several cases the crude 6-methylsalicylic acid fraction obtained from chromatography on silicic acid was purified on Sephadex prior to sublimation as follows: the crude material was dissolved in ethyl acetate–acetic acid (32:1, v/v) and chromatographed on a column of Sephadex LH-20 (40 g, 34 × 1.8 cm, swelling time 24 hr) established in the same solvent system. The column was then eluted with ethyl acetate–acetic acid (32:1, v/v). Fractions (25 ml) were collected; 6-methylsalicyclic acid was present in fractions 20–46.

TABLE II: Utilization of Phenylalanine and Shikimic Acid by *M. phlei* ATCC 10142.

Compound Isolated	L-Phenylalanine-*U*-^{14}C[a] Sp Act. (dpm/μmole)	Incorp (%)	Dilution	(−)-Shikimic-*G*-^{14}C Acid[b] Sp Act. (dpm/μmole)	Incorp (%)	Dilution
6-Methylsalicylic acid	39	0.003	5.8×10^5	41[c]	0.003	1.8×10^5
Phenyllactic acid	15,874	8.8	1.4×10^3	1,364	1.85	5.5×10^3
Fatty acids				1	0.001	1.3×10^7

[a] L-Phenylalanine-*U*-^{14}C (50 μCi, specific activity 10 μCi/μmole) was added to five flasks of the organism after a 2-day growth period on medium B; growth was then continued for a further 3 days. [b] (−)-Shikimic-*G*-^{14}C acid (33 μCi, specific activity 3.35 μCi/μmole) was added to three flasks of the organism after a 2-day growth period on medium B; growth was then continued for a further 3 days.

phenacyl acetate according to the method of Vogel (1956). The derivative was purified by chromatography on silicic acid with benzene–chloroform (9:1, v/v) to give colorless needles, mp 83–84°. Further purification, to mp 85–86°, was achieved by recrystallization from aqueous ethanol. In a typical experiment 4 mg of *p*-bromophenacyl acetate was obtained from 15 mg of 6-methylsalicylic acid.

C-7 of 6-methylsalicylic acid was obtained by a Schmidt decarboxylation according to the method of Phares (1951), using a final temperature of 180°. The resulting barium carbonate was washed successively with water, ethanol, and ether, and then dried. Typically, 8 mg of barium carbonate was obtained from 14 mg of 6-methylsalicylic acid.

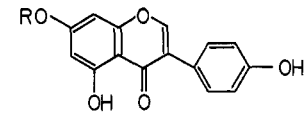
Degradation of Tyrosine. Using the method of Baddiley *et al.* (1950), suitably diluted tyrosine (32.2 mg) was converted to *p*-hydroxybenzoic acid (10 mg, mp 208–209°). This latter acid was decarboxylated by the Phares (1951) technique to give barium carbonate (8.8 mg).

Results

To determine whether 6-methylsalicylic acid formation involved C methylation of salicylic acid, a material derivable directly from shikimic acid or indirectly *via* phenylalanine and a C_6–C_1 unit (Scheme I), tracer experiments with labeled precursors were undertaken (Tables I and II). The results indicate first of all that although methionine-$^{14}CH_3$ was appreciably incorporated (0.01%) into 6-methylsalicylic acid by *M. phlei*, it suffered significant dilution[3] (6.5×10^5). Moreover, the chemical degradation proved the incorporated activity was not specifically located in the methyl group, C-8, an absolute requirement for methionine involvement in 6-methylsalicylic acid biosynthesis by the route under discussion.

That the organism was well able to conduct methylations with added methionine was clearly demonstrated by the effective methylation (0.57%) of genistein (V) to its *O*-methyl ether prunetin (VI). Genistein is liberated by the bacterium from a contaminant, likely the glucoside genistin, present in small amounts in the L-asparagine component of the culture medium (Hudson and Bentley, manuscript in preparation, 1970).

[3] Dilution value is defined as specific activity of precursor/specific activity of product.



V, R = H
VI, R = CH_3

On feeding shikimate-*G*-^{14}C and phenylalanine-*U*-^{14}C, a small measure of radioactivity from both precursors was incorporated into 6-methylsalicylic acid (specific activities 41 and 39 dpm per μmole, respectively), but the efficiency and dilution factors recorded were not compatible with either material being an obligatory intermediate in 6-methylsalicylic acid biosynthesis (Table II). The incorporation data for phenyllactic acid produced by the organism as a co-metabolite of 6-methylsalicylic acid demonstrates impressively, however, that under the prevailing conditions *M. phlei* was capable of utilizing added shikimic acid for aromatic biosynthesis. Samples of fatty acids isolated during the feeding of shikimate-*G*-^{14}C were not significantly labeled (Table II).

Since these findings appeared to exclude an obligatory role for shikimic acid in 6-methylsalicylic acid biosynthesis, results in keeping with the polyketide route were sought through feedings with acetate-1-^{14}C and acetate-2-^{14}C (Table III). Under a variety of experimental conditions, the incorporation of acetate-2-^{14}C activity into 6-methylsalicylic acid ranged from 0.01 to 0.06% while dilution values varied from 4000 to 50,000. The highest incorporation was obtained when the tracer was added on the third day (Table III, expt 1). The addition of four times as much total activity, spaced over the 5-day growth period, gave a lower incorporation level but comparable dilution value and product specific activity. Since, therefore, product specific activity did not seem to be dependent critically on the time of tracer addition, the labeled acetates were added to the culture medium on day zero to obtain samples of 6-methylsalicylic acid for chemical degradation.

The results of these degradations are shown in Table IV. If the polyketide route operates, in accordance with Scheme I, 25% of the total radioactivity of the sample of 6-methylsalicylic acid derived from acetate-2-^{14}C should be located in the acetic acid representing C-6 + C-8; 0% in the barium carbonate representing C-7. The corresponding figures for the

sample biosynthesized in the presence of acetate-1-^{14}C would be 25% in both acetic acid and barium carbonate. The experimental values are 23 and 6% from acetate-2-^{14}C and 24 and 27% from acetate-1-^{14}C, respectively.

Co-metabolites were isolated from the various acetate feeding experiments. The cellular fatty acids were massively labeled from acetate-1-^{14}C and acetate-2-^{14}C, their high incorporation level relative to 6-methylsalicylic acid reflecting the fact that they were produced in a much larger quantity than the aromatic acid. The dilution values of 6-methylsalicylic acid and fatty acids are, however, comparable.

Phenyllactic acid and tyrosine were also isolated and were found to contain appreciable acetate-derived activity. Degradative removal of the side chain of tyrosine revealed that this activity was distributed between ring and side chain (footnote *e*, Table III).

Discussion

From the results we conclude that 6-methylsalicylic acid is a metabolite of *M. phlei* and is biosynthesized by the polyketide route. Whether the biosynthesis involves four acetate units or one acetate and three malonate units remains to be seen. Three lines of evidence support this conclusion. (1) Methionine does not function as a specific methyl donor in 6-methylsalicylic acid formation. This evidence rules out any process which requires a C methylation, either at the aromatic or prearomatic level. (2) Neither shikimic acid nor phenylalanine is effectively incorporated into 6-methylsalicylic acid. This finding complements the methionine result. (3) Acetate is incorporated into 6-methylsalicylic acid with a dilution value comparable to the fatty acids. Furthermore, the labeling pattern in 6-methylsalicylic acid following feeding with acetate-1-^{14}C and acetate-2-^{14}C is that expected of a polyketide-derived substance.

While the issue seems fairly clear cut, some experimental observations appear worthy of further discussion. Activity from acetate was incorporated into phenyllactate and tyrosine at levels comparable to those observed with 6-methylsalicylate. On chemical degradation of the labeled tyrosine 67.4% of the total activity was found in the shikimate-derived ring. This finding is in keeping with the previously observed "spillover" of acetate activity into the shikimate-derived portion of the menaquinones of this organism (Kelsey *et al.*, manuscript in preparation, 1970) and in no way prejudices our experimental conclusions.

It was also disturbing that incorporations of acetate into 6-methylsalicylic acid were not higher, and that activity from shikimate-*G*-^{14}C suffered a greater dilution (1.3×10^7) in going to fatty acids than it did in going to 6-methylsalicylic acid (1.8×10^5). Since the incorporation presumably proceeds by degradation of shikimate to acetate and synthesis of the aromatic and aliphatic acids therefrom, comparable dilutions might have been expected. While such anomalies may be attributed to simple differences in pool sizes, rates, and loci of synthesis, and the nature of intermediates, etc., more complex factors may be responsible.

As yet, we have not resolved conclusively whether in *M. phlei* 6-methylsalicylic acid is synthesized *de novo*, or is a degradation product of mycobactin P (II). It is conceivable that the phenyloxazolone unit of this mycobactin is formed without the involvement of *free* 6-methylsalicylic acid. If this is the case, the 6-methylsalicylic acid we eventually isolate from the

TABLE III: Utilization of Labeled Acetates by *M. phlei* Strains.

Expt No.	Organism	Nature and Vol of Culture Medium (l.)	Tracer Added (μCi)	Time of Precursor Addn (hr)	Time of Harvest (hr)	6-Methylsalicylic Acid			Fatty Acids		
						Sp Act. (dpm/μmole)	Incorp (%)	Dilution[a]	Sp Act. (dpm/μmole)	Incorp (%)	Dilution[a]
1	ATCC 10142	A, 7	Ac-2,[b] 100	48	120	375	0.057	5.0×10^4			
2	ATCC 10142	A, 7	Ac-2, 400	*c*	120	700	0.009	2.7×10^4	15,865	9.8	1.2×10^3
3[d]	ATCC 10142	B, 7	Ac-2, 500	0	120	4,546	0.029	4.2×10^3	65,408	8.8	2.9×10^2
4	ATCC 354	C, 4.2	Ac-2, 500	0	24	1,621	0.01	3.7×10^4			
5[e]	ATCC 354	C, 4.2	Ac-1, 150	0	24	896	0.017	8.0×10^4	4,723		1.5×10^4

[a] Specific activity of tracers added: expt 1–3, 8.56 mCi/mmole; expt 4, 27.4 mCi/mmole; expt 5, 32.5 mCi/mmole. [b] Ac-2 = acetate-2-^{14}C, Ac-1 = acetate-1-^{14}C. [c] Additions were made as follows: after 24 hr, 40 μCi; after 48 hr, 40 μCi; after 72 hr, 40 μCi; after 96 hr, 150 μCi, and after 120 hr, 130 μCi. [d] Phenyllactic acid was also isolated, with specific activity 634 dpm/μmole (incorporation = 0.06%, dilution = 3.0×10^4). [e] Tyrosine was also isolated, with specific activity 273 dpm/μmole (dilution = 2.6×10^4). Degradation revealed that 67.4% of the total activity was located in the aromatic ring, 1.2% in C-3, and 31.4% in C-2 + C-1.

TABLE IV: Degradations of Labeled 6-Methylsalicylic Acid.

		6-Methylsalicylic Acid		C-6 + C-8		C-7	
Sample Degraded[a]	Precursor[b]	dpm/μmole	% Total	dpm/μmole	% Total	dpm/μmole	% Total
Expt 3	Ac-2	1,053	100	240	23	62	6
Expt 4	Ac-2	27	100	5	19	1	4
Expt 5	Ac-1	121	100	29	24	33	27

[a] The experiment numbers given in this column refer to the experiments described in Table III. [b] The abbreviations are the same as those in Table III.

culture medium will have been the subject of much metabolic action and comparison of incorporation and dilution values with other metabolites might not be too meaningful.

An even more serious difficulty may beset attempts to record higher incorporations of acetate activity into 6-methylsalicylic acid in *M. phlei*. Plants and fungi represent the major stronghold of the polyketide pathway and it must be assumed that it is in these organisms that the most effective polyketide synthases have developed. For example, in the classical work of Birch *et al.* (1955) on 6-methylsalicylic acid biosynthesis in the fungus *Penicillium griseofulvum* P68, it can be calculated that 1.2% of the activity added as acetate was converted into the aromatic acid (compare bacterial incorporations in Table III). Apart from the observations that the fatty acid synthase of *E. coli* can under some conditions form the polyketide products, triacetic and tetraacetic lactones (M. Soucek and K. Bloch, 1970, personal communication), and that a soil *Pseudomonas* can metabolize the "preformed" polyketide, dehydroacetic acid, to triacetic acid and its lactone (Kotani *et al.*, 1964), polyketide products are apparently restricted to the order *Actinomycetales*. Within this order, the family *Mycobacteriaceae* occupies the unique position of producing little or no mycelium (Couch, 1957). This intermediate position which the *Mycobacteriaceae* hold between the polyketide-producing, mycelium-forming members of the *Actinomycetales* and the polyketide-lacking, nonmycelium-forming true bacteria may also be reflected in the intermediate development of their polyketide-synthesizing enzyme systems, and in their consequent intermediate effectiveness in utilizing acetate for aromatic polyketide biosynthesis.

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