1010. *Studies in Relation to Biosynthesis. Part XXXV.*[1] *Macrolide Antibiotics. Part XII.*[2] *Methymycin.*

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The biosynthesis of the antibiotic methymycin (II) has been investigated by growing *Streptomyces venezuelae* in media containing [1-^{14}C]- and [2-^{14}C]-propionic acid, [*Me*-^{14}C]methionine, [1-^{14}C]acetic acid, diethyl [2-^{14}C]malonate, [2-^{14}C]pyruvic acid, and [1-^{14}C]formic acid. Degradations of the labelled macrolide show that the aglycone arises by the polyketide route from five " propionic acid " units and one " acetic acid " unit.* A preliminary report of this work has been published.[11]

Branched aliphatic chains are now known to arise biogenetically by several distinct routes.[3] One of these involves construction of a chain from an acyl-coenzyme-A initiating unit (which is frequently acetyl-coenzyme-A) with the addition of malonyl-coenzyme-A units and the introduction of methyl groups from cation donors such as methionine or choline. Each *C*-methylation occurs at a position derived from the methylene-carbon atom of a malonate unit. Since labelled acetic acid is commonly used experimentally as the precursor, its coenzyme-A ester being reversibly carboxylated to the malonyl derivative, the introduced methyl groups appear on carbon atoms that were originally acetate-methyl groups. This route, which was the first for which experimental evidence was provided, very clearly operates in many organisms varying from trees to moulds,[3,4] and it was originally suggested[5] also as a possibility for the macrolide antibiotics[6] produced by *Streptomyces* species.

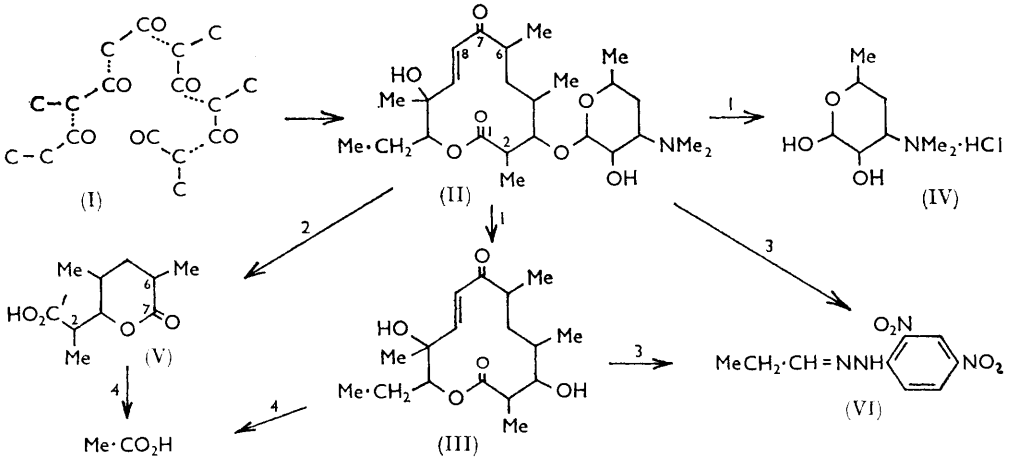
An alternative biogenetic route to the macrolides, which was postulated by Gerzon[7] and Woodward,[6,8] involved direct incorporation of propionic acid units instead of acetic acid units into the chain. From later work demonstrating the role of malonyl-coenzyme-A in the formation of " acetate "-derived compounds, the biochemical unit active in this case would be expected to be methylmalonyl-coenzyme-A,[9] and evidence relating to this has

* The terms " propionic acid " and " acetic acid " units are used here with the understanding that their actual incorporation involves the coenzyme-A esters, which probably undergo reversible carboxylation to the corresponding malonyl derivatives.

[1] Part XXXIV, Birch, Hussain, and Rickards, *J.*, 1964, 3494.
[2] Part XI, Djerassi, Ishikawa, Budzikiewicz, Shoolery, and Johnson, *Tetrahedron Letters*, 1961, 383.
[3] Birch, Simonsen Lecture, *Proc. Chem. Soc.*, 1962, 3.
[4] Rickards, in " Recent Developments in the Chemistry of Natural Phenolic Compounds," ed. Ollis, Pergamon Press, London, 1961, p. 1.
[5] Birch, *Fortschr. Chem. Org. Naturstoffe*, 1957, **14**, 186; Birch, English, Massy-Westropp, Slaytor, and Smith, *J.*, 1958, 365.
[6] Woodward, *Angew. Chem.*, 1957, **69**, 50.
[7] Gerzon, XIVth Internat. Congr. Pure Applied Chem., Zurich, 1955; Gerzon, Flynn, Sigal, Wiley, Monahan, and Quarck, *J. Amer. Chem. Soc.*, 1956, **78**, 6396.
[8] Woodward, *Angew. Chem.*, 1956, **68**, 13.
[9] Lynen, *J. Cell. Comp. Physiol.*, 1959, **54**, 33.

recently been obtained.[10] The two routes are not biochemically equivalent, *i.e.*, malonic acid is not methylated by C_1-donors before incorporation as methylmalonic acid. The routes are, however, structurally equivalent, and which route is involved in a particular case cannot be distinguished by mere inspection of formulæ. Our original suggestion of the involvement of acetate and methionine was dropped at an early stage [11] when it was shown that propionic acid was, in fact, involved in the formation of the aglycones of the macrolides erythromycin [10,12] and methymycin.[11] Further work has shown that propionate is utilised in the biosynthesis of the macrolides magnamycin [13] and nystatin,[14] and in the production of branched-chain lipids by biological systems as diverse as *Mycobacteria*,[15] the intestinal helminth *Ascaris lumbricoides*,[16] and the preen gland of the goose.[17] It can also, like many other acids, initiate acetate chain formation, as in the rutilantins.[18] The carbon skeleton of erythronolide [7] arises [12] entirely from propionic acid units. Evidence has been presented [11] that methynolide [19] (III), the "aglycone" of methymycin (II), is formed from five propionic acid units and one acetic acid unit (as in I); in more precise biochemical terms, this would mean initiation of the aglycone chain by propionyl-coenzyme-A, to which are added one methylmalonyl-, one malonyl-, and three further methylmalonyl-coenzyme-A units, in that order. The present work amplifies and confirms this conclusion,[11] and provides some further evidence as to the origin of propionic acid units in *Streptomyces* species.

The principal degradations employed for labelled methymycin samples, shown below, are from the original literature,[19,20] modified where necessary to improve yields for isotope work, together with Kuhn–Roth oxidations where appropriate.



[10] Vanek, Puza, Majer, and Dolezilova, *Folia Microbiologica*, 1961, **6**, 408; Grisebach, Achenbach, and Hofheinz, *Z. Naturforsch.*, 1962, **17**b, 64; Corcoran, 142nd Amer. Chem. Soc. Meeting, Atlantic City, 1962.

[11] Birch, Pride, Rickards, Thomson, Dutcher, Perlman, and Djerassi, *Chem. and Ind.*, 1960, 1245.

[12] Vanek, Majer, Liebster, Veres, and Dolezilova, Proc. Synposium Antibiotics, Prague, 1960, p. 143; Corcoran, Kaneda, and Butte, *J. Biol. Chem.*, 1960, **235**, PC29; Kaneda, Butte, Taubman, and Corcoran, *ibid.*, 1962, **237**, 322; Grisebach, Achenbach, and Hofheinz, *Z. Naturforsch.*, 1960, **15**b, 560.

[13] Grisebach and Achenbach, *Z. Naturforsch.*, 1962, **17**b, 6; *Tetrahedron Letters*, 1962, 569; Gilner and Srinivasan, *Biochem. Biophys. Res. Comm.*, 1962, **8**, 299.

[14] Birch, Holzapfel, Rickards, Djerassi, Seidel, Westley, Dutcher, and Thomas, 142nd Amer. Chem. Soc. Meeting, Atlantic City, 1962.

[15] Gastambide-Odier, Delaumery, and Lederer, *Chem. and Ind.*, 1963, 1285.

[16] Saz and Weil, *J. Biol. Chem.*, 1960, **235**, 914; 1962, **237**, 2053.

[17] Noble, Stjernholm, Mercier, and Lederer, *Nature*, 1963, **199**, 600.

[18] Ollis and Sutherland, *Proc. Chem. Soc.*, 1960, 347.

[19] Djerassi and Zderic, *J. Amer. Chem. Soc.*, 1956, **78**, 6390.

[20] Donin, Pagano, Dutcher, and McKee, "Antibiotics Annual 1953—54," Medical Encyclopedia, Inc., New York, p. 179; Djerassi, Bowers, Hodges, and Riniker, *J. Amer. Chem. Soc.*, 1956, **78**, 1733; Djerassi and Zderick, p. 2907; Djerassi and Halpern, *ibid.*, 1957, **79**, 3926.

S. venezuelae fermentations of media supplemented with [*Me*-^{14}C]methionine gave methymycin (II) (with 7% isotope incorporation) labelled almost exclusively in the desosamine moiety (IV), indicating the occurrence of *N*-methylation and the absence of *C*-methylation during the biosynthesis. The incorporation (2—3%) of either [1-^{14}C]- or [2-^{14}C]-propionic acid occurred exclusively into the " aglycone " with little redistribution of isotope, and gave unequivocal evidence of the presence of five such units in the aglycone (III) (cf. the Table; radioactivity of methynolide itself is obtained by difference). The radioactivity associated with each propionate unit is effectively the same: the lactonic acid (V) [19,21] (containing three units) has three-fifths of the molar activity of the parent antibiotic (II); the propionaldehyde dinitrophenylhydrazone (VI) (one unit) has one-fifth. Acetic acid samples resulting from Kuhn–Roth oxidation of [1-^{14}C]- and [2-^{14}C]-propionate-derived methynolide preparations carried, respectively, insignificant radioactivity and one-fifth of the methymycin activity (localised, in this latter case, in the carboxyl group as expected). Little, if any, propionate incorporation occurred by enzymic degradation [22] into the supposed acetic acid unit (C-8 and C-7).

The incorporation of [1-^{14}C] acetic acid into the antibiotic was lower (*ca.* 1%) than that of propionate and, in view of the extensive randomisation of isotope, probably occurs largely by prior conversion through succinate into methylmalonate (see below). It is somewhat surprising that its incorporation into the acetate unit of the chain is not more marked. However, since such utilisation would in fact occur biochemically through malonyl-coenzyme-A, the organism was fed with diethyl [2-^{14}C]malonate. Although incorporation was extremely low (0·02%), consequently severely limiting the material available for detailed degradations, both the derived desosamine (IV) and the lactonic acid (V) were inactive. This provides strong evidence for the expected specific labelling of C-8, which was qualitatively confirmed by the presence of significant radioactivity in the carbon dioxide evolved during oxidation with hydrogen peroxide of the total ozonolysis product from methymycin. This product would contain C-8 as the carboxyl of the α-keto-acid which, after oxidative decarboxylation and hydrolysis, yields the lactone (V).

On the basis of published data,[11] Bentley [23] suggested that C-7 and C-8 of methynolide do not represent an isolated acetate unit, but rather the carboxyl and methylene carbon, respectively, of a sixth propionate unit that has suffered oxidative loss of its methyl carbon. While admissible on the [1-^{14}C]propionate data,[11] if the chain-initiating propionate unit [contained in the propionaldehyde (VI)] is more highly labelled than the chain-extending units (cf. erythromycin [10]), this possibility is not compatible with the [2-^{14}C]malonate or [2-^{14}C]propionate results described here. For example, considering the labelling pattern that would result from [2-^{14}C]propionate precursor if the macrolide were entirely propionate-derived, we can see that the lactonic acid (V) would still contain only three labelled atoms, whose average activity (19·6%) is the same as that in the propionaldehyde (VI; 19·9%), leaving only 21·4% of the total macrolide activity for distribution between C-8 and C-10.

With regard to the ultimate origin of propionate units themselves in *Streptomycetes*, the incorporations of [2-^{14}C]pyruvate and [^{14}C]formate, although both low (0·3 and 0·2%, respectively), are of interest. While both tracers were utilised exclusively for aglycone synthesis, the former gave nearly equal labelling in the methylene- and methyl-carbon atoms of the five propionate units involved, the latter yielded apparently carboxyl-labelled propionate as shown by the virtual inactivity of the Kuhn–Roth acetic acid (cf. Table). This clearly indicates that a symmetrical intermediate is involved in the conversion of pyruvic acid into propionyl- or methylmalonyl-coenzyme-A by *S. venezuelae*. In view of the observed labelling pattern from these two tracers, and by analogy with known paths of

[21] Anliker, Dvornik, Gubler, Heusser, and Prelog, *Helv. Chim. Acta*, 1956, **39**, 1785.
[22] Cf. Stumpf, *Ann. Rev. Biochem.*, 1960, **29**, 267.
[23] Bentley, *Ann. Rev. Biochem.*, 1962, **31**, 606.

propionate metabolism in *Propionibacteria*[24] and mammalian tissue,[25] this conversion probably involves carboxylation (for which formate would provide a carbon source) to oxaloacetate, reduction to succinate, and isomerisation to methylmalonate. Intermediates of a di- or tri-carboxylic acid cycle are also involved in the conversion of acetate into the propionate units of erythronolide by *S. erythreus*.[26]

The results above confirm not only the predicted [6-8] propionate origin of the macrolide antibiotics but also further illustrate the biochemical non-equivalence of the propionate and the acetate-plus-methylation routes. The structural equivalence of the two routes as regards the resulting metabolite, however, emphasises the necessity for actual tracer studies when considering the origins of such compounds.

Percentage distribution of radioactivity in methymycin preparations.

Compound assayed	Percentage r.m.a.[27] present according to tracer used: *MeS·CH_2·CH_2·CH(NH_2)·CO_2H	Me·CH_2·*CO_2H
Methymycin (II)	100	100
Desosamine hydrochloride (IV)	87·6	—
Lactonic acid (V)	1·66	62·0
Propionaldehyde 2,4-dinitrophenylhydrazone (VI)	0·15	21·7
Kuhn–Roth acetic acid:		†
methyl-carbon	—	0·48
carboxyl-carbon	—	0·18

Compound assayed	Me·*CH_2·CO_2H	Me·*CO·CO_2H	H·*CO_2H
Methymycin (II)	100	100	100
Desosamine hydrochloride (IV)	0·15	0	1·94
Lactonic acid (V)	58·7	—	—
Propionaldehyde 2,4-dinitrophenylhydrazone (VI)	19·9	17·9	—
Kuhn–Roth acetic acid:	‡	‡	‡
methyl-carbon	2·17	7·72	0·8
carboxyl-carbon	20·5	8·25	1·4

† Acetic acid obtained by oxidation of lactonic acid (V). ‡ Acetic acid obtained by oxidation of methynolide (III).

Experimental

Degradations are based on the original literature [19,20] and only modifications are described in detail. Compounds were purified to constant radioactivity and assayed as infinitely thick solid samples of 0·3 or 1·0 cm.^{2} cross-sectional area as described by Birch *et al.*[27,28] Relative molar activities [27] (r.m.a.'s) (a), (b), (c), (d), (e), and (f) refer to labelled methymycin and corresponding degradation products derived from [*Me*-^{14}C]methionine, [1-^{14}C]propionic acid, [2-^{14}C]propionic acid, [2-^{14}C]pyruvic acid, [^{14}C]formic acid, and diethyl [2-^{14}C]malonate, respectively.

[^{14}C]*Methymycin.*—*Streptomyces venezuelae* (Institute of Microbiology, Rutgers University strain 3629) was grown in submerged culture on a rotary shaker at 25 ± 1° in a medium containing (per l.) soyabean meal (30 g.), glucose (50 g.), and powdered calcium carbonate (1 g.). Equal amounts of tracer (total 50—100 μc.) were added to each of two flasks (250-ml. Erlenmeyer containing 50 ml. of medium) 24 and 48 hr. after inoculation. After 72 hours' incubation, cultures were harvested by adjustment of the broth to pH 9·5 with potassium hydroxide and extraction of the suspension with pentyl acetate. Inactive methymycin was added to the concentrated extract; the resulting [^{14}C]methymycin, crystallised from ethyl acetate, had m. p. 204—205° ([Found: r.m.a. × 10^{-3}, (a) 200; (b) 166; (c) 65·5; (d) 7·50; (e) 16·4; (f) 22·7].

Degradation of [^{14}C]*Methymycin.*—Hydrolysis of methymycin with 5N-sulphuric or -hydrochloric acid as described by Djerassi, Bowers, Hodges, and Riniker [20] afforded, respectively,

[24] Swick and Wood, *Proc. Nat. Acad. Sci. U.S.A.*, 1960, **46**, 28; Stjernholm and Wood, *ibid.*, 1961, **47**, 289, 303; Swick, *ibid.*, 1962, **48**, 288; Eggerer, Stadtmann, Overath, and Lynen, *Biochem. Z.*, 1960, **333**, 1.

[25] Flavin and Ochoa, *J. Biol. Chem.*, 1957, **229**, 965; Kaziro and Ochoa, *ibid.*, 1961, **236**, 3131; Kaziro, Leone, and Ochoa, *Proc. Nat. Acad. Sci. U.S.A.*, 1960, **46**, 1319; Lengyel, Mazumder, and Ochoa, *ibid.*, p. 1312, and references cited therein.

[26] Vanek, Puza, Majer, and Dolezilova, *Folia Microbiologica*, 1961, **6**, 386; Grisebach, Hofheinz, and Achenbach, *Naturwiss.*, 1961, **48**, 101.

[27] Birch, Massy-Westropp, Rickards, and Smith, *J.*, 1958, 360.

[28] Birch, Snell, and Thomson, *J.*, 1962, 425.

methynolide (III) (cf. ref. 19), m. p. 161—165° (from ether–hexane), and desosamine hydrochloride (IV), m. p. 187—189° (from methanol–acetone) [Found for (IV): r.m.a. × 10^{-3}, (a) 175; (c) 0·098; (d) 0; (e) 0·318; (f) 0].

Reduction with lithium aluminium hydride followed by periodate oxidation of either methynolide [19] or methymycin [20] afforded propionaldehyde, isolated as the 2,4-dinitrophenylhydrazone (VI), m. p. 149—150° (from methanol; after purification by chromatography on alumina and on bentonite–kieselguhr [29]) [Found: r.m.a. × 10^{-3}, (a) 0·3; (b) 36·0; (c) 13·0; (d) 1·34]. The purity of this derivative was confirmed by paper chromatography.[30]

Kuhn–Roth oxidation of methynolide gave acetic acid, which was degraded by the Schmidt procedure [31] to carbon dioxide and methylamine. These fragments were assayed as barium carbonate [Found: r.m.a. × 10^{-3}, (c) 13·5; (d) 0·617; (e) 0·227] and *N*-methyl-2,4-dinitroaniline [Found: r.m.a. × 10^{-3}, (c) 1·44; (d) 0·579; (e) 0·137] respectively.

Kuhn–Roth oxidation of the lactonic acid (V) derived from the methymycin sample (b) afforded acetic acid, isolated as the lithium salt which, after pyrolysis,[27] gave barium carbonate [Found: r.m.a. × 10^{-3}, (b) 0·30] and acetone. Hypoiodite oxidation of the acetone and van Slyke–Folch oxidation of the resulting iodoform gave barium carbonate [Found: r.m.a. × 10^{-3}, (b) 0·80].

5-Hydroxy-2,4,6-trimethylpimelic Acid δ-Lactone (V).—Ozone-enriched oxygen was passed into methymycin (200 mg.) in ethyl acetate (20 ml.) at −80° until a permanent blue colour was obtained. The liquid was warmed to room temperature, and acetic acid (10 ml.), water (5 ml.), and hydrogen peroxide (0·5 ml.; 30-vol.) were added. After being kept overnight, the solution was concentrated, adjusted to pH 10 with 5% aqueous sodium hydroxide, and warmed on the water-bath for 4 hr. The acidified solution was continuously extracted with ether, to yield the lactonic acid (V) (43 mg., 53%), having m. p. 124—126° (refs. 19, 21, and 32 record m. p. 124—127°, 125—126°) when purified by sublimation at 110°/0·05 mm. and crystallisation from ether–hexane [Found: r.m.a. × 10^{-3}, (a) 3·30; (b) 103; (c) 38·5; (f) 0].

For the methymycin sample (f), the peroxide oxidation was performed under nitrogen and the evolved carbon dioxide (which is probably not derived exclusively from C-8) was collected as barium carbonate [Found: r.m.a. × 10^{-3}, (f) 9·6].

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[29] Elvidge and Whalley, *Chem. and Ind.*, 1955, 589.
[30] Horner and Kirmse, *Annalen*, 1955, **597**, 48.
[31] Cf. Phares, *Arch. Biochem. Biophys.*, 1951, **33**, 173.
[32] Djerassi and Halpern, *Tetrahedron*, 1958, **3**, 255.