67. *Studies in Relation to Biosynthesis. Part XIV.* The Origin of the Nuclear Methyl Groups in Mycophenolic Acid.*

By A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor, and Herchel Smith.

Feeding experiments with [$^{14}CH_3$]methionine in the culture of *Penicillium brevi-compactum* have shown that the methoxyl and methyl groups attached to the benzene ring of the resulting mycophenolic acid (II; $R^1 = R^2 = H$, $R^3 = Me$) are derived from methionine.

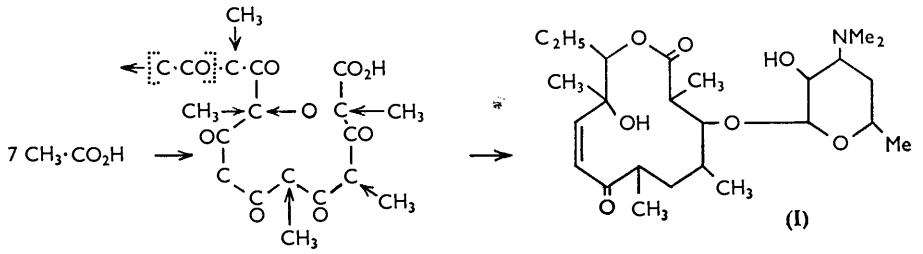
Structural comparisons led to the conclusion [1] that many natural products otherwise derived from acetic acid contain extra C_1 units (often methyl groups) introduced in a biosynthetic stage distinct from the formation of the main skeleton. The C_1-donors involved could be methionine, choline, or known carriers of formyl groups such as tetrahydrofolic acid, thus relating the process to the attachment of C_1 units to oxygen, nitrogen, or sulphur.[2] The nuclei to which attachment occurs are usually capable of high anionoid reactivity, notably in acylphloroglucinol or orcinol rings, and laboratory analogies for the postulated *C*-alkylations are available.[3] In some cases alkylation may occur in non-aromatic β-polyketone precursors.

A different hypothesis has been put forward [4] to explain the biosynthesis of the macrolides, which in our view are merely particular examples of the general type above. This postulates the intervention of propionic acid or an equivalent. To have any meaning in biochemical terms this amounts to postulating the introduction of C_3 units while the main skeleton is being formed. We recognise that this assumption explains very satisfactorily the structural relations in the macrolide field alone, particularly the presence in

* Part XIII, preceding paper.

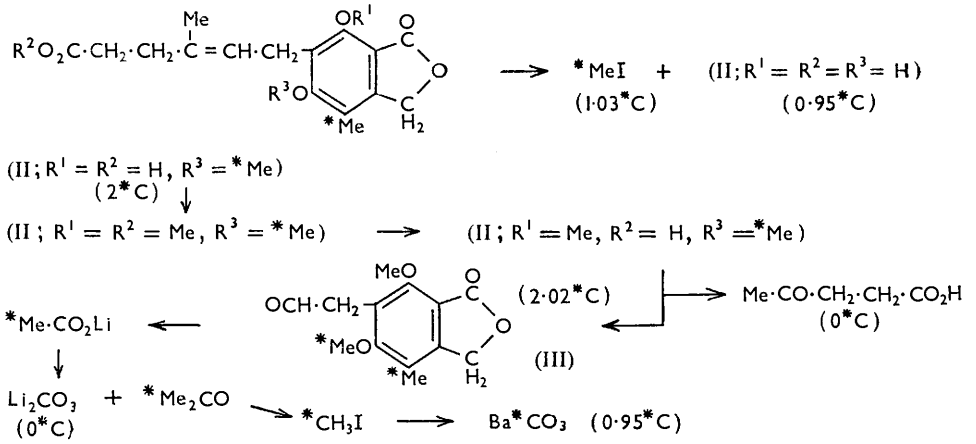
[1] Birch, Elliott, and Penfold, *Austral. J. Chem.*, 1954, **7**, 169.
[2] Challenger, *Quart. Rev.*, 1955, **9**, 255.
[3] Riedl, *Annalen*, 1954, **585**, 38; Riedl and Risse, *ibid.*, p. 209.
[4] Flynn, Gerzon, Monahan, Quarck, Sigal, Weaver, and Wiley, *Chem. Eng. News*, 1956, **34**, 5138; Woodward, *Angew. Chem.*, 1957, **69**, 50.

some cases of terminal ethyl groups. As a general hypothesis it has several unsatisfactory features, particularly the difficulty of including compounds containing *gem*-dimethyl or *iso*pentenyl or derived groups. Methymycin [5] (I) could arise as illustrated here.



To test whether the postulated *C*-methylation can occur we have examined the production of mycophenolic acid in *Penicillium brevi-compactum*. This substance is particularly suitable, being readily available and containing methoxyl, almost certainly derived from methionine, and an " extra " nuclear methyl group which can be compared with it: the rest of the molecule is entirely derivable from acetic acid.[6] The mould was grown in the presence of methionine containing $^{14}CH_3$. Labelled mycophenolic acid resulted with an astonishingly high incorporation (77%) of the added ^{14}C, and was degraded as shown in the chart. The results are expressed as in Part XIII; they leave no doubt that the hypothesis is correct in this case. The extent of labelling of the methoxyl is significantly higher than that of the nuclear methyl group. We attribute this to the presence of some unmethylated phenolic precursor at the stage of growth when methionine was introduced. The high utilisation of the latter indicates it is probably a limiting factor in synthesis. Mycophenolic acid grown on Me·$^{14}CO_2H$ gave rise from the nucleus to Me·CO_2H which was, as expected, completely unlabelled.[6]

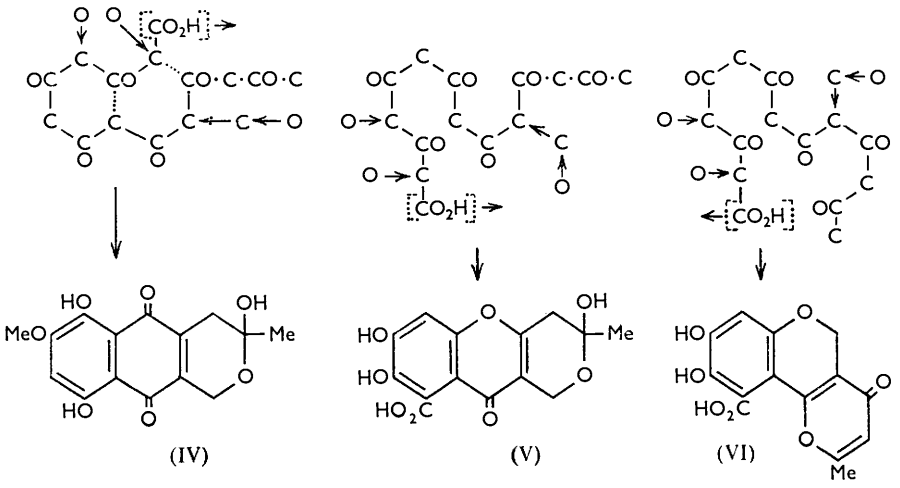
Many examples of the application of the theory could be cited. A most interesting series of mould metabolic products which can be structurally correlated are fusarubin (IV), fulvic acid (V), and citromycetin (VI). Despite superficial dissimilarities in the formulæ



all of these can be derived (on paper) from the same skeleton composed of seven acetic acid units with an introduced C_1 unit and two introduced oxygen atoms. It is significant that by changing the growth medium,[7] griseofulvin results instead of fulvic acid. This compound has been shown [8] to be derived from seven acetic acid units. For some reason the change of medium has apparently led to methylation on oxygen rather than on carbon.

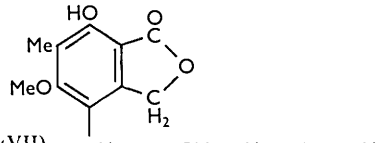
[5] Djerassi and Zderic, *J. Amer. Chem. Soc.*, 1956, **78**, 2907.
[6] Birch, English, Massy-Westropp, and Smith, following paper.
[7] Dean, Eade, Moubasher, and Robertson, *Nature*, 1957, **179**, 366.
[8] Birch, Massy-Westropp, Rickards, and Smith, *Proc. Chem. Soc.*, 1957, 98; preceding paper.

The Structure of Mycophenolic Acid.—When this work was begun one observation in the literature appeared to indicate preferentially the structure (VII) rather than (II; $R^1 = R^2 = H$, $R^3 = Me$) despite the very strong evidence presented for the latter.[9,10,11]

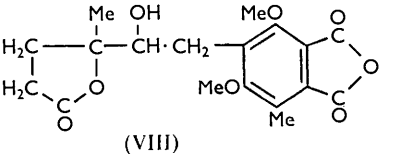


(IV) (V) (VI)

Oxidation of the methyl ether (II; $R^1 = R^3 = Me$, $R^2 = H$) by permanganate gave a substance formulated by Raistrick and his colleagues [9,10] as (VIII). After dissolution in hot ethanol it titrated as a monobasic acid and, after being heated in alkaline solution, as a tribasic acid. This behaviour was attributed to initial formation of an acid ethyl ester, which seemed to us unlikely in view of lack of reactivity with ethanol under similar conditions of other 2-alkyl-5-methoxyphthalic anhydrides which we have examined. Repetition of the oxidation and examination of the infrared spectrum shows, however, that (VIII) is correct. In chloroform solution no band is found attributable to a carboxyl group, but a hydroxyl is indicated by a broad band at 3550 cm.$^{-1}$. Strong bands at 1830, 1820, and 1760 cm.$^{-1}$ (broad) must be due to the anhydride ring (phthalic anhydride has bands at 1845 and 1775 cm.$^{-1}$). The expected absorption of the γ-lactone ring is probably included in the 1760 cm.$^{-1}$ band. Logan and Newbold [12] have proved structure (IX) by synthesis of a degradation product.



(VII) $CH_2 \cdot CH = CMe \cdot CH_2 \cdot CH_2 \cdot CO_2H$



(VIII)

Experimental

Experimental directions are as for Part XIII. Where possible, substances were crystallised to constant specific activity.

[^{14}C]*Mycophenolic Acid.*—(*a*) *Penicillium brevi-compactum* was grown on standard Czapek-Dox medium at 25°.[13] After 27 days a solution of sodium [*carboxy*-^{14}C]acetate (0·5 mc) was

[9] Clutterbuck and Raistrick, *Biochem. J.*, 1933, **27**, 654.
[10] Birkinshaw, Bracken, Morgan, and Raistrick, *ibid.*, 1948, **43**, 216.
[11] Birkinshaw, Raistrick, and Ross, *ibid.*, 1952, **50**, 630.
[12] Logan and Newbold, *J.*, 1957, 1946.
[13] Clutterbuck, Oxford, Raistrick, and Smith, *Biochem. J.*, 1932, **26**, 1441.

added to three flasks, and growth continued for 14 days. The mould solution was filtered, acidified to pH 1 with hydrochloric acid, and extracted three times with ethyl acetate. The ethyl acetate extracts were dried and evaporated under reduced pressure at 35°. The residue was dissolved in absolute alcohol, and the potassium salt of mycophenolic acid isolated as described.[13] The residues were re-extracted three times, inactive acid being used as carrier, the total being diluted with pure acid (1·5 g.) and recrystallised to constant activity. The incorporation was 0·4%.

(*b*) [$^{14}CH_3$]Methionine [14] (165 mg.; 15·8 μc) was incorporated into mycophenolic acid as for sodium [1-^{14}C]acetate. Extraction gave mycophenolic acid (200 mg.; 12·2 μc).

Demethylation of [^{14}C]*Mycophenolic Acid.*—The acid (relative molar activity × 10^{-3}, 182) was demethylated by hydriodic acid.[9] The methyl iodide was collected at −20° and refluxed in ethanol (10 c.c.) with thiourea (1 g.) for 5 min. Picric acid (1 g.) was added and dissolved by further heating. *S*-Methylthiuronium picrate separated on cooling and after recrystallisation from alcohol had m. p. 223—224° (Brown and Campbell [15] give 224°) (Found: C, 30·2; H, 2·6; N, 21·2%; relative molar activity × 10^{-3}, 93·8. Calc. for $C_8H_9N_5O_7S$,1*C: C, 30·0; H, 2·8; N, 21·8%; relative molar activity × 10^{-3}, 91·0). The normycophenolic acid (II; $R^1 = R^2 = R^3 = H$) was sublimed at 158°/0·1 mm. and recrystallised from benzene–light petroleum. It had m. p. 183—184° (Found: C, 62·6; H, 5·8%; relative molar activity × 10^{-3}, 86·6. Calc. for $C_{16}H_{18}O_6$,1*C: C, 62·7; H, 5·9%; relative molar activity × 10^{-3}, 91·0).

Ozonolysis of [^{14}C]*Mycophenolic Acid Methyl Ether* (II; R^1 = Me, R^2 = H, $R^3 = {}^{14}CH_3$).—The acid (1 g.) (relative molar activity × 10^{-3}, 153) was ozonised in chloroform [10] (150 c.c.). Lævulic acid was isolated as the 2 : 4-dinitrophenylhydrazone which, after precipitation from aqueous sodium hydrogen carbonate by mineral acid and recrystallisation from methanol, had m. p. 204—205° (Found: C, 44·8; H, 3·9%; relative molar activity, 0. Calc. for $C_{11}H_{12}O_6N_4$: C, 44·6; H, 4·0%). The phenylacetaldehyde derivative also produced was obtained as the 2 : 4-dinitrophenylhydrazone which was purified by chromatography on bentonite (18 g.)–kieselguhr (6 g.) in 3 : 97 ethanol–chloroform and, after recrystallisation from methanol, had m. p. 213—214° (Found: C, 53·2; H, 4·3%; relative molar activity × 10^{-3}, 154. Calc. for $C_{19}H_{18}O_8N_4$,2*C: C, 53·0; H, 4·2%; relative molar activity × 10^{-3}, 153). The above 2 : 4-dinitrophenylhydrazone was oxidised by the Kuhn–Roth method. The resulting acetic acid was isolated as lithium acetate which was heated *in vacuo* at 500° for 10 min. to give lithium carbonate and acetone (cf. ref. 8). The former was converted into barium carbonate for radioactive assay (Found: relative molar activity, 0). The latter was treated with hypoiodite to give iodoform, Van Slyke–Folch oxidation of which gave further barium carbonate for counting (Found: relative molar activity × 10^{-3}, 71·6; 1*C requires 76·5).

Grants from the Nuffield Foundation (Australia) (to M. S.) and from the Cumberland Education Committee (to R. J. E.) are gratefully acknowledged. We also thank the University of Manchester for the award of a Science Research Scholarship (to R. M.-W.) and Imperial Chemical Industries Limited for the loan of counting equipment.

UNIVERSITY OF MANCHESTER, MANCHESTER, 13. [*Received, June 21st,* 1957.]

[14] Calvin, Heidelberger, Reid, Tolbert, and Yankwich, " Isotopic Carbon," Wiley, New York, 1949, p. 222.

[15] Brown and Campbell, *J.*, 1937, 1699.