68. *Studies in Relation to Biosynthesis. Part XV.* Origin of Terpenoid Structures in Mycelianamide and Mycophenolic Acid.*

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

Incorporations of [*carboxy*-^{14}C]acetic acid, 3-methyl[1-^{14}C]but-2-enoic acid, and βδ-dihydroxy-β-methyl[α-^{14}C]valeric (mevalonic) lactone into the terpenoid chains of mycelianamide (II) and mycophenolic acid (XVI) have been examined and compared in the first case with the incorporation into griseofulvin (IX) and in the second case with the incorporation into the acetic acid-derived portion of the molecule. The biogenesis of *iso*pentane units is discussed.

The way in which the methyl- and carboxyl-carbon atoms of acetic acid contribute to the *iso*pentane unit in natural compounds was first elucidated by work on steroid biosynthesis.[1] In 1952 one of us independently advanced [2] the same pattern (I). The active intermediate in mind at the time was 3-methylglutaconic acid. Work was begun in conjunction with A. R. Penfold and J. L. Willis (Museum of Applied Arts and Sciences, Sydney) to examine the mode of incorporation of [*carboxy*-^{14}C]acetic acid into the citronellal of the essential oil of *Eucalyptus citriodora* Hook but failed owing to insufficient incorporation of the tracer. Our joint views were set out in a lecture.[3] Much experimental evidence has accumulated during the last five years in the fields of carotenoids,[4] steroids,[1] and triterpenes [5] that the pattern (I) is correct. However, intermediate stages between C_2 and C_5 compounds still require investigation.

We sought mould metabolic products containing simple terpenoid chains for study with isotopically labelled compounds. Two suitable substances appeared to be mycelianamide [6,7] (II) containing a C_{10} terpenoid chain readily isolated as methylgeraniolene (III) by reduction with sodium and liquid ammonia,[7] and mycophenolic acid [8] (XIV), the C_7 side-chain of which we regard as the remnant of an oxidised geranyl group.

Mycelianamide.—Mycelianamide is produced by *Penicillium griseofulvum* Dierckx (L.S.H. Cat. No. P. 38) simultaneously with griseofulvin (VIII) which previous studies [9] have shown to be directly derived from acetic acid. This mould therefore provides an opportunity to study the relative effectiveness of various sources for the production of an isoprenoid chain and of a substance directly formed from acetic acid. The two compounds are produced by the mould in approximately constant ratio throughout its growth and so the extent of incorporation into griseofulvin is a measure of the biochemical conversion of the added labelled compound into acetic acid.

Addition of Me·$^{14}CO_2H$ gave rise to labelled mycelianamide and griseofulvin. The degradation of the former was carried out as below, the results being set out as in our previous work.[9] It can be seen that they agree quantitatively with the expected distribution (II). The efficiency of incorporation of acetic acid is of the same order in both cases.

Addition to the mould of $Me_2C{:}CH{\cdot}^{14}CO_2H$, another possible source of *iso*pentane units, also gave rise to mycelianamide with the distribution of ^{14}C found in (II), as shown by the annexed degradations. The griseofulvin simultaneously produced has the activity to be

* Part XIV, preceding paper.

[1] For reviews see Cornforth, *Rev. Pure Appl. Chem.* (*Australia*), 1954, **4**, 275; Friedman, Byers, and St. George, *Ann. Rev. Biochem.*, 1956, **25**, 613; cf. Cornforth, Gore, and Popjak, *Biochem. J.*, 1956, **25**, 613, and earlier papers.

[2] Birch, *Perfumery Essent. Oil Record*, 1952, **43**, 110.

[3] Penfold, *Perfumery Essent. Oil Record*, 1954, **45**, 213.

[4] *Inter al.*, Reichel and Wallis, *Naturwiss.*, 1957, **44**, 234; Grob and Bütler, *Experientia*, 1955, **7**, 259.

[5] Dauben and Richards, *J. Amer. Chem. Soc.*, 1956, **78**, 5329.

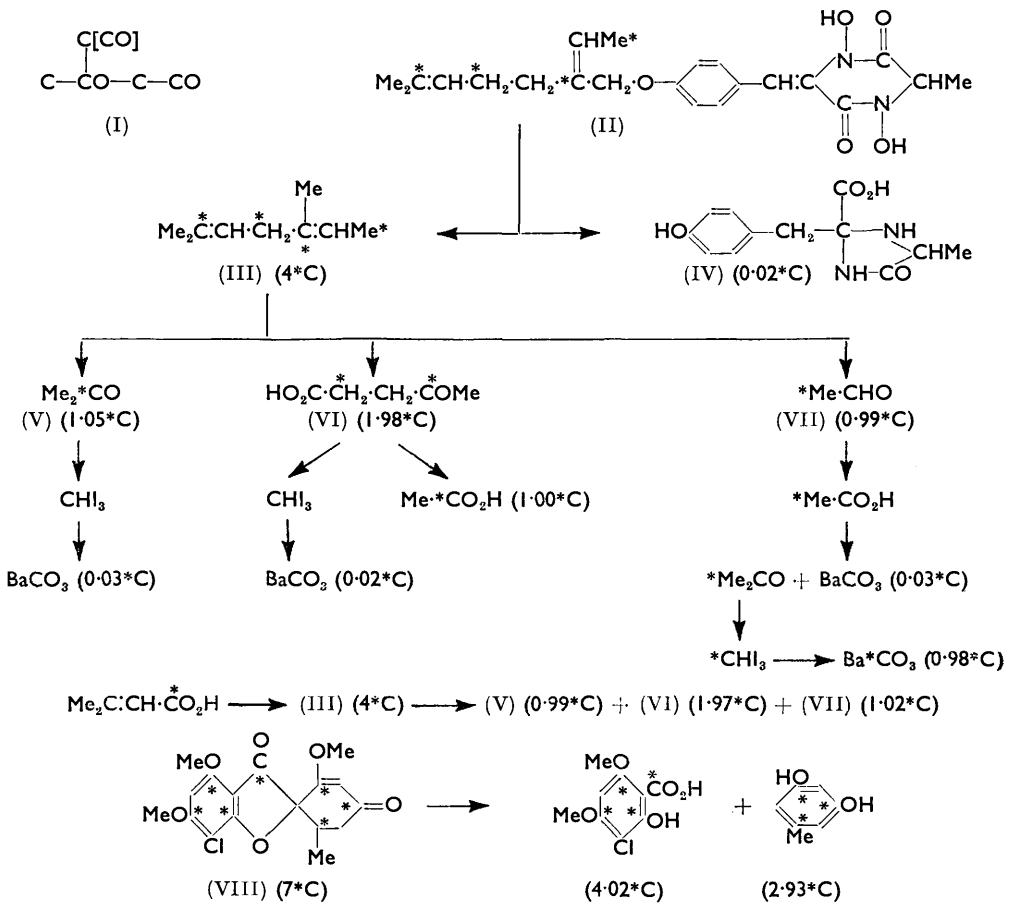
[6] Oxford and Raistrick, *Biochem. J.*, 1948, **42**, 323.

[7] Birch, Massy-Westropp, and Rickards, *J.*, 1956, 3717.

[8] Birkinshaw, Raistrick, and Ross, *Biochem. J.*, 1952, **50**, 630.

[9] Birch, Massy-Westropp, Rickards, and Smith, *Proc. Chem. Soc.*, 1957, 98; *J.*, 1957, 360.

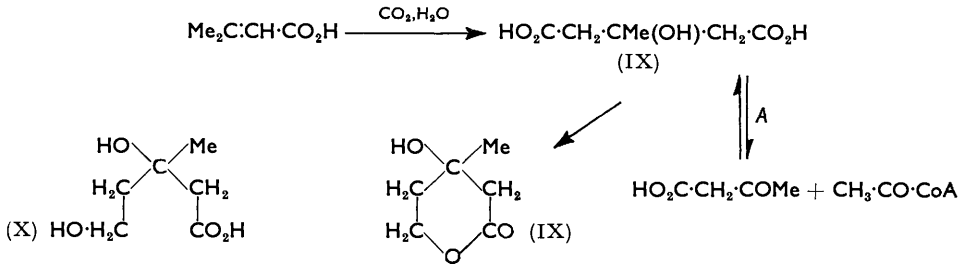
predicted on the basis of its formation from $Me\cdot{}^{14}CO_2H$ of the same degree of labelling as that giving rise to the mycelianamide. To confirm that the distribution of labelling in the griseofulvin is the same as that already observed,[9] hydrolytic fission was carried out by Mr. R. W. Rickards and the resulting orcinol and 3-chloro-2-hydroxy-4 : 6-dimethoxybenzoic acid shown to have the calculated activities. These results are conclusive in showing that the $Me_2C{:}CH\cdot{}^{14}CO_2H$ is not incorporated as a unit, but undergoes degradation to acetic acid before incorporation. The equilibrium *A* must therefore be set up, in this organism at least, far more rapidly than the β-hydroxy-β-methylglutaric acid



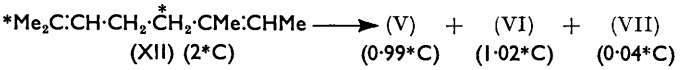
(IX) is converted into the direct *iso*pentane precursor, probably mevalonic acid [10] (X). In contrast to these results Bloch, Clarke, and Harary [11] reported the incorporation of $Me_2C{:}CH\cdot{}^{14}CO_2H$ *en bloc* into cholesterol in rats, and Sanderman and Stockman [12] made similar observations on the biosynthesis of pulegone in *Mentha pulegium* L.

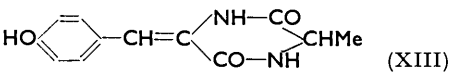
A study of the incorporation of [2-^{14}C]mevalonic lactone (XI) reveals that this substance is indeed an irreversible intermediate in terpene biosynthesis, no longer giving rise to labelled acetic acid. The degradations of the resulting methylgeraniolene shown on p. 371 support the distribution (XII). It is particularly notable that the labelled carbon atom at position 4 is not interchanged with that in the 3-methyl group. This should be important when tracing biosynthetic routes in terpenoid compounds with complex ring

[10] Wolf, Hoffman, Aldrich, Skeggs, Wright, and Folkers, *J. Amer. Chem. Soc.*, 1956, **78**, 4497; 1957, **79**, 1486.
[11] Bloch, Clarke, and Harary, *ibid.*, 1954, **76**, 3859.
[12] Sanderman and Stockman, *Naturwiss.*, 1956, **43**, 580.



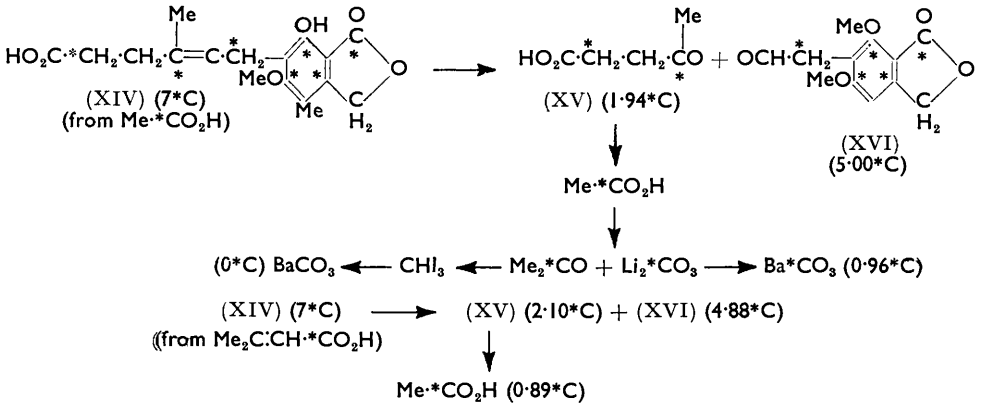
systems. The griseofulvin produced simultaneously is completely inactive, indicating the complete absence of $^{14}CH_3 \cdot CO_2H$.





Reduction of mycelianamide with sodium and methanol in liquid ammonia gives, in addition to methylgeraniolene, a substance $C_{12}H_{14}O_4N_2$ which is soluble in aqueous sodium hydrogen carbonate, insoluble in mineral acid, and fails to react with nitrous acid. It is a carboxylic acid (infrared spectrum) and on acid hydrolysis gives *p*-hydroxyphenylpyruvic acid. Its light absorption is similar to that of *p*-ethylphenol [13] and it is therefore formulated as the tetrahydroglyoxaline derivative (IV), presumably formed by hydrolysis and recyclisation of the initially formed piperazine (XIII).

Mycophenolic Acid.—With this substance (XIV) we are able, as will be shown, to study the incorporation of precursors into an isoprenoid chain and an acetic acid-derived nucleus

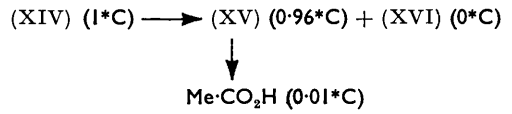


in the same molecule. The degradations below establish that $Me \cdot {}^{14}CO_2H$ produces the expected labelling pattern (XIV) and it is notable that the extent of incorporation into the nucleus and into the side chain is exactly the same. The results obtained with mycelianamide from $Me_2C{:}CH \cdot {}^{14}CO_2H$ are substantially confirmed, in that the labelling pattern is closely similar to that produced directly from $Me \cdot {}^{14}CO_2H$. It will be noted that the activities of the lævulic acid (XV) and the aldehyde (XVI) are respectively higher (by 5%) and lower (by 2·5%) than the values predicted for complete derivation from $Me \cdot {}^{14}CO_2H$. We regard these variations as indicating some incorporation of $Me_2C{:}CH \cdot {}^{14}CO_2H$ as a unit into (XIV). If it is assumed that $Me_2C{:}CH \cdot {}^{14}CO_2H$ gives rise as above to $Me \cdot {}^{14}CO_2H$ with one-third of the original specific molar activity, and that the

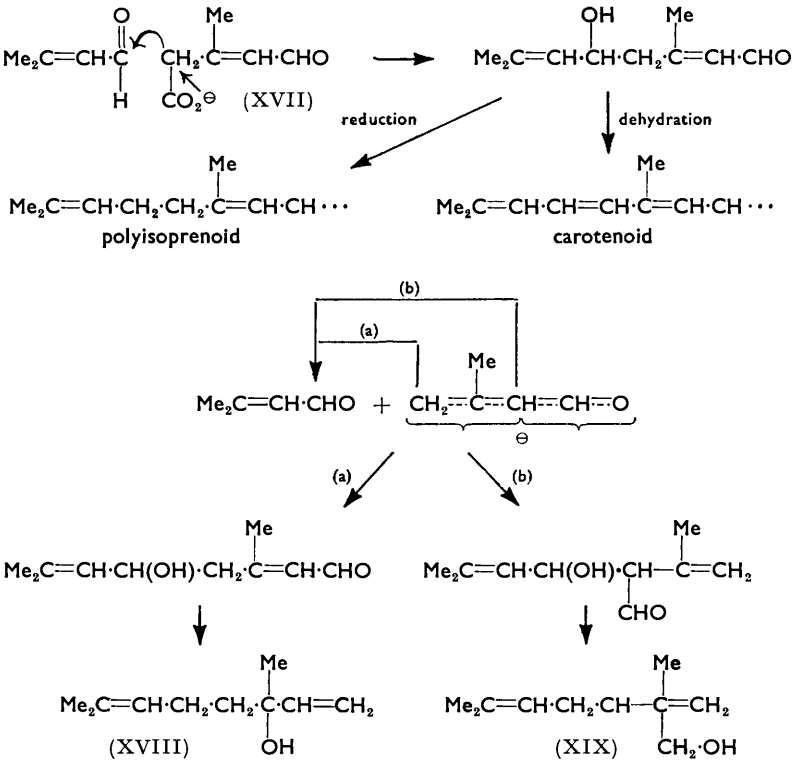
[13] Friedel and Orchin, " Ultraviolet Spectra of Aromatic Compounds," Wiley and Sons Inc., New York, 1951, p. 37.

efficiencies of incorporation of both acids into the product (XIV) are the same, it may be calculated that the ratio of the specific molar activities of (XV) and (XVI) represents direct incorporation of $Me_2C{:}CH{\cdot}{}^{14}CO_2H$ into (XIV) to the extent of 7·5%. This view is reinforced by the observation that the acetic acid obtained by Kuhn–Roth oxidation of the acid (XV) has a lower activity than that required for biosynthesis from acetate. The ratio of the specific molar activities of this acetic acid and of (XV) gives a value of 11% for the direct incorporation of $Me_2C{:}CH{\cdot}{}^{14}CO_2H$ into (XIV), in fair agreement with the first value in view of the probable experimental errors.

The incorporation of [2-^{14}C]mevalonic acid confirms our view on the origin of the C_7 chain, because the nucleus is then completely unlabelled, and all of the labelling is present in the lævulic acid produced by ozonolysis. Proof is again provided that mevalonic acid is a specific intermediate in terpene biosynthesis.



The mode of incorporation of the C_6 compound mevalonic acid into substances containing the C_5 *iso*pentane unit involves decarboxylation.[14] We believe that the process may involve initial oxidation to the aldehyde-acid (XVII), decarboxylation of which, as its



anion, will be facilitated by its structure as a " vinylogue " of a β-aldehydo-acid. The resulting anion could then react with the carbonyl group of another similar unit, with later further decarboxylation, or with β-methylcrotonaldehyde produced by proton-addition to the anion. An advantage of this view is that the process can continue indefinitely, since the necessary activation is supplied by the unit added to the end of the chain. The

[14] Tavormina, Gibbs, and Huff, *J. Amer. Chem. Soc.*, 1956, **78**, 4498.

intermediate alcohol could be dehydrated to give conjugated polyenes of the carotenoid type, or hydrogenolysed to polyisoprenes, possibly by the kind of process postulated [15] for hydrogenolysis of some natural allyl alcohol derivatives.

The head-to-head or tail-to-tail linkage of *iso*pentane units which appears to occur in some compounds is explicable on the basis of migrations, usually of methyl groups. An interesting case whose significance has already been noted [3] is the co-occurrence of linaloöl (XVIII) and lavendulol (XIX) where the relationship could be as illustrated.

Experimental

General directions are as in the two preceding papers.

[^{14}C]*Mycelianamide and* [^{14}C]*Griseofulvin.*—(*a*) *Penicillium griseofulvum* Dierckx (L.S.H. Catalogue No. P. 38) was cultured under the conditions described previously.[6] After 18 days a solution of sodium [*carboxy*-^{14}C]acetate (1·0 mc; 7·6 mg.) was distributed among 8 flasks, each containing 350 ml. of medium. After a further 9 days at 30°, [^{14}C]mycelianamide (1·2 g.), m. p. 168—170° (decomp.), and griseofulvin (0·2 g.), m. p. 219—220°, were isolated. An aliquot part (900 mg.) of the impure mycelianamide was diluted with pure, inactive mycelianamide (17·5 g.) and on recrystallisation from ethanol had m. p. 171—172° (decomp.). The incorporation of tracer into mycelianamide and griseofulvin was about 2% and 1% respectively.

(*b*) The mould was grown as above, except that 3-methyl[1-^{14}C]but-2-enoic acid (136 μc) was added in place of sodium [*carboxy*-^{14}C]acetate. Mycelianamide (800 mg., 0·71%; 0·96 μc) and griseofulvin (210 mg., 0·41%; 0·56 μc) were obtained. The ratio of the molar specific activities is 7·2 : 4; biosynthesis from Me·$^{14}CO_2Na$ of the same activity requires a theoretical ratio 7 : 4. Both metabolites were diluted before degradation.

(*c*) The mould was grown with βδ-dihydroxy-β-methyl[α-^{14}C]valeric lactone [16] (42·8 μc). Radioactive mycelianamide (0·34 μc; 0·79%) was obtained; the incorporation of tracer into the griseofulvin was insignificant.

Reduction of [^{14}C]*Mycelianamide.*—Mycelianamide (14·8 g.) was reduced with sodium and methanol in liquid ammonia as previously described,[7] to give methylgeraniolene (3·22 g., 62%). The solution, after removal of the latter, was kept at room temperature for 48 hr., filtered, and acidified with concentrated hydrochloric acid. The precipitate was dissolved in aqueous potassium hydrogen carbonate solution and recrystallised from ethanol, to give *tetrahydro*-2-(4-*hydroxybenzyl*)-5-*methyl*-4-*oxoglyoxaline*-2-*carboxylic acid* (XIV), m. p. 285—288° (Found: C, 57·4; H, 5·7; N, 11·8. $C_{12}H_{14}O_4N_2$ requires C, 57·6; H, 5·6; N, 11·2%). The infrared absorption spectrum (Nujol mull) showed broad absorption between 3400 and 2500 cm.$^{-1}$ (hydrogen-bonded carboxylic-hydroxyl stretching) and bands at 1697 (carboxylic-carbonyl), 1657 (amide-carbonyl), 1610 and 1525 (aromatic C=C stretching) and 830 cm.$^{-1}$ (out-of-plane C–H deformation in 1 : 4-disubstituted benzene ring). In EtOH $\lambda_{max.}$ 226 and 277 mμ (ε 10,500 and 2000); $\lambda_{min.}$ 252 mμ (ε 910). The acid (XIV) with 2N-hydrochloric acid gave, in 13 hr., *p*-hydroxyphenylpyruvic acid identified as the 2 : 4-dinitrophenylhydrazone, m. p. 169—171°, undepressed by admixture with an authentic specimen of m. p. 173°.

Ozonolysis of [^{14}C]*Methylgeraniolene.*—(i) Ozonised oxygen was passed through a solution of methylgeraniolene (600 mg.) in ethyl chloride (15 c.c.) at −30° until the effluent gas liberated iodine from aqueous potassium iodide. Water (10 c.c.) was added and the solution refluxed for 30 min. in a stream of nitrogen, the emerging vapours being collected in Brady's reagent, to give a mixture of acetone and acetaldehyde 2 : 4-dinitrophenylhydrazones. A solution of potassium dichromate (2·85 g.) in water (30 ml.) and concentrated sulphuric acid (3·75 g.) was added to the residual mixture, the whole being kept for 3 hr. at room temperature, whereafter water (300 ml.) was added. Continuous ether-extraction gave lævulic acid obtained as the 2 : 4-dinitrophenylhydrazone, m. p. 206°. Chromatography of the mixture of acetone and acetaldehyde 2 : 4-dinitrophenylhydrazones on bentonite (21 g.) and kieselguhr (7 g.) in ether–ethanol gave acetone and acetaldehyde 2 : 4-dinitrophenylhydrazones, m. p. 127° and 169° respectively.

(ii) Methylgeraniolene (324 mg.) was ozonised in sulphur-free light petroleum (10 c.c.; b. p. 60—80°) at 0°. Acetic acid (2 c.c.) was added, the light petroleum distilled off, and the residue shaken for 1 hr. at room temperature with 30% hydrogen peroxide (0·5 c.c.). Water (3 c.c.) and more 30% hydrogen peroxide (2 c.c.) were added and the mixture was heated for 3 hr. at

[15] Birch and Slaytor, *Chem. and Ind.*, 1956, 1524.
[16] Cornforth, Cornforth, and Youhotsky-Gore, *Biochem. J.*, 1957, **66**, 10P.

70°. The solution was brought to pH 5 with N-sodium hydroxide and steam-distilled. The distillate contained acetone (obtained as the 2 : 4-dinitrophenylhydrazone); a portion was oxidised with aqueous sodium hypoiodite to iodoform (70 mg.) which was recrystallised from methanol and oxidised by the Van Slyke–Folch procedure to barium carbonate. The residual solution from the steam-distillation was continuously extracted with ether, to give lævulic acid, oxidation of which with alkaline hypoiodite solution gave iodoform converted, as before, into barium carbonate. Kuhn–Roth oxidation of the above acetaldehyde 2 : 4-dinitrophenylhydrazone gave acetic acid, isolated as lithium acetate, pyrolysis of which *in vacuo* at 380° for 15 min. gave acetone and a residue of lithium carbonate. This salt was converted into barium carbonate. Alkaline hypoiodite treatment of the acetone gave iodoform, converted, as above, into barium carbonate.

[^{14}C]*Mycophenolic Acid.*—(*a*) Sodium [*carboxy*-^{14}C]*acetate* was incorporated to the extent of 0·4% into mycophenolic acid produced by *Penicillium brevi-compactum* Dierckx as described in Part XIV.

(*b*) Addition of 3-methyl[1-^{14}C]but-2-enoic acid (136 μc) to the culture medium gave mycophenolic acid (0·66 μc; 0·49%).

(*c*) Addition of βδ-dihydroxy-β-methyl[α-^{14}C]valeric lactone (20·7 μc) to the culture medium gave mycophenolic acid (0·31 μc; 1·5%). The degradations of labelled mycophenolic acid were carried out as described in Part XIV.

Radioactive Assay.—The results of the radioactive assay of the degradation products of various samples of mycelianamide, griseofulvin, and mycophenolic acid are given in the following Tables. Acetone, lævulic acid, acetaldehyde, and the aldehyde (XVI) were assayed as the 2 : 4-dinitrophenylhydrazones, acetic acid as the *p*-bromophenacyl ester.

Substance	Relative molar activities ($\times 10^{-3}$) Found	Required
Mycelianamide (II) from Me·$^{14}CO_2H$	457	—
2-(4′-Hydroxybenzyl)-5-methyl-4-oxoglyoxaline-2-carboxylic acid (IV)	3	—
Methylgeraniolene (III)	454 (by diff.)	—
Acetone (V) from (III)	119	113
Barium carbonate *ex* iodoform from (V)	3	0
Lævulic acid (VI) from (III)	225	227
Barium carbonate *ex* iodoform from (VI)	2	0
Acetic acid from (VI)	113	113
Acetaldehyde (VII)	112	113
Ba carbonate *ex* acetic acid from (VII)	3	0
Ba carbonate *ex* iodoform from acetone from (VII)	111	113
Mycelianamide from $Me_2C{:}CH{\cdot}^{14}CO_2H$	69·2	—
Methylgeraniolene	67·8	—
Acetone	16·9	17·0
Lævulic acid	33·3	33·9
Acetaldehyde	17·3	17·0
Griseofulvin (VIII) from $Me_2C{:}CH{\cdot}^{14}CO_2H$	164	—
3-Chloro-2-hydroxy-4 : 6-dimethoxybenzoic acid	94·2	93·6
Orcinol	68·6	70·2
Methylgeraniolene (XII) from lactone of [2-^{14}C]mevalonic acid	33·4	—
Acetone from (XII)	16·5	16·7
Lævulic acid from (XII)	17·1	16·7
Acetaldehyde from (XII)	0·7	0
Mycophenolic acid (XIV) from Me·$^{14}CO_2H$	212	—
Aldehyde (XVI)	152	152
Lævulic acid (XV) from (XIV)	58·9	60·6
Ba carbonate *ex* acetic acid from (XV)	29·1	30·3
Ba carbonate *ex* iodoform from acetone from above acetic acid	0	0
Mycophenolic acid from $Me_2C{:}CH{\cdot}^{14}CO_2H$	86·4	—
Aldehyde (XVI)	60·2	61·7
Lævulic acid (XV)	25·9	24·7
Acetic acid	11·0	12·4
Mycophenolic acid (XIV) from mevalonic lactone	65·1	—
Aldehyde (XVI)	0	0
Lævulic acid (XV)	62·5	65·1
Acetic acid from lævulic acid	0·9	0

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