600. *Studies in Relation to Biosynthesis. Part XXIV.* Some Remarks on the Structure of Echinulin.*

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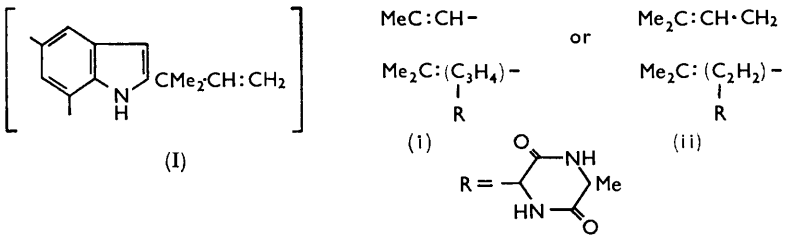
Biological incorporations of [2-^{14}C]mevalonic lactone, [1-^{14}C]acetate, [1-^{14}C]alanine, and [1-^{14}C]glycine into echinulin have independently indicated the presence of three isoprene units and support other aspects of structure and biosynthesis postulated by Quilico's school.[1]

The traditional method of examining structure–biosynthetic relations has been to determine the structure of a natural product and then to try to rationalise it in terms of possible biosynthetic precursors and reactions.[2] We have now attempted to reverse this process in certain cases by examining with tracer methods the units involved in biosynthesis in order to apply the results to elucidation of structure. This approach becomes possible with an increasing knowledge of biosynthetic routes [3] in cases where incorporation of radioactive precursors can readily be achieved, as with micro-organisms. We now report such an examination of echinulin, produced by *A. echinulatus* [4] or *A. amstelodami*.[5]

* Part XXIII, Birch, Cameron, and Rickards, *J.*, 1960, 4395.

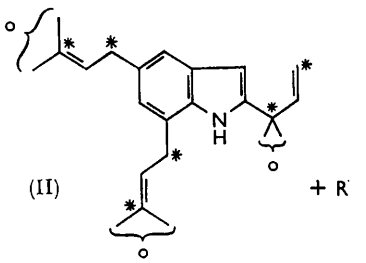
[1] Cardani, Casnati, Piozzi, and Quilico, *Tetrahedron Letters*, 1959, No. 16, 1.
[2] Robinson, " The Structural Relations of Natural Products," The Clarendon Press, Oxford, 1955.
[3] Birch, *Fortschr. Chem. org. Naturstoffe*, 1957, **16**, 186.
[4] Quilico and Panizzi, *Ber.*, 1943, **76**, 348.
[5] Quilico and Cardani, *Rend. Accad. Lincei*, 1950, **9**, 220.

When this work was begun knowledge of the structure of echinulin, due to the chemical work of Quilico and his colleagues [6] could be summarised in the expression (I).



(I)

One isoprene unit is clearly distinguishable, attached in the reverse manner to the 2-position of the indole nucleus, and the presence of the isopropylidene group suggests the possibility of the presence of other isoprene units. Accordingly we fed to *A. amstelodami* the specific terpene precursor [2-^{14}C]mevalonic acid which should place one labelled carbon



(II)

atom at one Me position in each unit. Incorporation was of the expected order (4·25%). Acetone was produced by ozonolysis, the derived 2,4-dinitrophenylhydrazone having 35·2% of the activity of the whole molecule, indicating the presence of *three* isoprene units. The most probable skeleton on this basis corresponds to (I, ii) as above, *i.e.*, to (II) with the labelled atoms denoted °.

Kuhn–Roth oxidation of the acetone derivative to acetic acid, and degradation of the latter showed that all of the label was, as expected, on the methyl group. As a further check the organism was fed with Me·$^{14}CO_2Na$, and in this case the acetone derivative contained 16·1%, *i.e.*, about one sixth, of the total activity, corresponding to the expected labelling pattern (II; * = ^{14}C). As required by this pattern all of the activity in the acetone was shown to be on the carbonyl group. To confirm that acetic acid was incorporated only to a notable extent into the isoprene residues one of these was isolated in the known fashion.[6] Hydrogenation, oxidation, and hydrolysis of echinulin gave αα-dimethylvaleric acid which had, as expected, one-third of the activity of the echinulin.

These results all agree with the structure (II) although they do not define the point of attachment of the dioxopiperazine ring. At this stage, in a personal discussion with Professor Quilico (mentioned also by him [1]), we learned that his further chemical work had led to the same conclusion for the skeleton of the molecule.

We have since carried out some further examinations of echinulin biosynthesis. Incorporation of radioactivity from H·$^{14}CO_2H$, was, as expected, very small, and appeared to be random, but incorporation of (±)-[1-^{14}C]alanine and [1-^{14}C]glycine both took place. Hydrolysis of the alanine-derived material gave alanine with 65·8% of the total radioactivity. There is little doubt therefore that, as previously suggested,[1] this is a biosynthetic precursor of the dioxopiperazine ring. Kuhn–Roth oxidation of the alanine-derived echinulin showed that some radioactivity had been randomly incorporated into

[6] Quilico, Piozzi, and Cardani, *Gazzetta*, 1958, **88**, 125, and earlier papers.

the isoprene unit (probably *via* pyruvate and acetylcoenzyme A). If the usual distribution of the label is assumed, the isoprene units could account for about 19% of the remaining activity. This leaves 14% unaccounted for, in either the glycine or the indole portion. It may be noted that if the latter is derived from shikimic acid, some incorporation of pyruvate (from deamination of alanine) would be expected. Further degradations are necessary to test this possibility.

The glycine-derived echinulin gave rise to alanine with 4·25% of the total labelling (possibly incorporated *via* serine) and the acetic acid from Kuhn–Roth oxidation contained only 1·06% of the total activity. The site of incorporation of the glycine could therefore be either in the dioxopiperazine ring as previously suggested, or in the indole nucleus. Insufficient material was available to decide this point.

By a reaction sequence starting from the potassium salt of echinulin and involving pyrolysis, hydrogenation, and oxidation, Quilico *et al.*[6] obtained a substance $C_{22}H_{35}NO_3$ which was considered to have the nuclear skeleton of (II) lacking the R group. In confirmation we have found that the compound formed from [2-^{14}C]mevalonate-derived echinulin has the same molar activity as the parent, and so must have retained all the labelled carbon atoms.

Clearly, tracer incorporation in this case could have provided valuable structural information. It did, in fact, lead from the partial structure (I) to the more complete (II) independently of the chemical work.

[ADDED, May 29th 1961.] Professor Quilico informs us [6a] that echinulin is $C_{29}H_{39}N_3O_2$, instead of $C_{28}H_{37}N_3O_2$, and carries the dioxopiperazine ring attached to the 3-position by CH_2. The substance is therefore satisfactorily related biogenetically to tryptophan. Our general conclusions are unaltered; the r.m.a. figures for echinulin and its derivatives should be multiplied by 1·031 and the figures are then a better fit. We had in fact mentioned to Professor Quilico in a personal communication that the tracer results fitted better for a C_{29} than a C_{28} formula.

EXPERIMENTAL

General instructions are as for Part XIII.[7]

Echinulin.—A. amstelodami, from the collection of the Musée Nationale d'Histoire Naturelle, Paris, was sub-cultured for 6 days on Czapek–Dox agar slopes, and then grown on a medium made by adding potassium dihydrogen phosphate to sugar-beet molasses and making up the solution to 1 l. The cultures from 7 l. of medium were harvested after 11 days and the mycelium was dried at room temperature. It was then finely powdered, extracted continuously (Soxhlet) with light petroleum (b. p. 30—40°), and exhaustively extracted with ether (Soxhlet) for 3 days. The precipitate from the ether reservoir recrystallised from ethanol, to give echinulin (4·5 g.) as needles, m. p. 230—242°, spectroscopically identical [6] with a sample from Professor Quilico (Milan).

[^{14}C]*Echinulin.*—The mould was grown as above, the [^{14}C]-precursors being introduced into the culture medium 4 days after inoculation when a mycelial mat had grown. Growth was allowed to continue for a further 6 days and the product isolated. The precursors, amount of culture solution, yields, and incorporations were as follows: Me·$^{14}CO_2Na$(0·3 mc), 900 c.c., 0·78 g., 0·74%; [2-^{14}C]mevalonic lactone (0·05 mc), 600 c.c., 0·55 g., 4·25%; H·$^{14}CO_2Na$ (0·2 mc), 600 c.c., 0·35 g., 0·04%; (±)-[1-^{14}C]alanine (0·25 mc), 600 c.c., 0·51 g., 0·12%; [1-^{14}C]glycine (0·2 mc), 600 c.c., 0·78 g., 0·84%.

Ozonolysis of Echinulin.—Echinulin (0·6 g.) was ozonised [8] in acetic acid (40 c.c.), the ozonide was worked up as usual with zinc powder, and the volatile carbonyl compounds were

[6a] Quilico, personal communication; Casnati, Piozzi, Quilico, and Ricca, *Chimica e Industria*, 1961, 412.

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

[8] Cardani, Casnati, Cavelleri, and Quilico, *Rend. Accad. Lincei*, 1958, **24**, 488.

swept by nitrogen into Brady's reagent. The 2,4-dinitrophenylhydrazones were separated by chromatography on bentonite—kieselguhr with ether containing 6% of ethanol as eluent.[9] The band corresponding to acetone 2,4-dinitrophenylhydrazone gave a product, m. p. 126—127° undepressed by the authentic material.

[^{14}C]Echinulin (r.m.a. $1{\cdot}0 \times 10^5$) from Me·$^{14}CO_2Na$ gave acetone 2,4-dinitrophenylhydrazone (r.m.a. $1{\cdot}61 \times 10^4$). Kuhn–Roth oxidation of the latter to acetic acid and degradation of this in the usual manner [10] gave barium carbonate from Me with r.m.a. 0·0 and from CO_2H with r.m.a. $1{\cdot}62 \times 10^4$.

[^{14}C]Echinulin (r.m.a. $2{\cdot}66 \times 10^5$) from [2-^{14}C]mevalonic lactone gave acetone 2,4-dinitrophenylhydrazone (r.m.a. $9{\cdot}36 \times 10^4$). Kuhn–Roth oxidation of the latter and degradation of the resulting acetic acid as above gave barium carbonate from Me with r.m.a. $4{\cdot}56 \times 10^4$ (Calc. for three equally labelled methyl groups, $4{\cdot}685 \times 10^4$) and from CO_2H with r.m.a. 0·0.

Oxidation of Hexahydroechinulin by Peracetic Acid.—[^{14}C]Hexahydroechinulin (r.m.a. $1{\cdot}00 \times 10^3$) from Me·$^{14}CO_2Na$ was oxidised, and the product hydrolysed as previously described [6] to αα-dimethylvaleric acid which was converted in the usual manner into the 4-phenylphenacyl ester m. p. 85—86° (r.m.a. $3{\cdot}42 \times 10^4$. Calc. for three equally labelled isoprenoid units, r.m.a. $3{\cdot}33 \times 10^4$).

Pyrolysis of the Potassium Derivative of Echinulin.—[^{14}C]Echinulin (5 g.; r.m.a. $2{\cdot}66 \times 10^5$), derived from [2-^{14}C]mevalonic lactone, was converted into the potassium derivative and pyrolysed as previously described.[11] Oxidation of the product in the known manner [6] with hydrogen peroxide gave the substance $C_{23}H_{37}NO_3$, m. p. 119—121° (r.m.a. $2{\cdot}69 \times 10^5$).

Kuhn–Roth Oxidations.—Oxidation of echinulin gave acetic acid in 55% yield (for 4*C*-Me groups). The acid was degraded as before to barium carbonate from the Me and CO_2H groups. [^{14}C]Echinulin (r.m.a. $4{\cdot}80 \times 10^4$) from H·$^{14}CO_2Na$ gave barium carbonate from Me with r.m.a. $1{\cdot}46 \times 10^3$ and from CO_2H with r.m.a. $9{\cdot}34 \times 10^2$. [^{14}C]Echinulin (r.m.a. $7{\cdot}15 \times 10^4$) from (±)-[1-^{14}C]alanine gave barium carbonate from Me with r.m.a. $2{\cdot}66 \times 10^2$ and from CO_2H with r.m.a. $1{\cdot}16 \times 10^3$. [^{14}C]Echinulin (r.m.a. $1{\cdot}34 \times 10^5$) from [1-^{14}C]glycine gave barium carbonate from Me with r.m.a. $3{\cdot}66 \times 10^2$ and from CO_2H with r.m.a. $1{\cdot}06 \times 10^3$.

Hydrolysis of Hexahydroechinulin.—Hydrolysis as previously described [12] gave alanine, which was converted in the usual manner into the picrolonate. [^{14}C]Hexahydroechinulin (r.m.a. $7{\cdot}07 \times 10^4$) from (±)-[1-^{14}C]alanine gave alanine (r.m.a. $4{\cdot}65 \times 10^4$) [picrolonate, m. p. 214—215° (r.m.a. $4{\cdot}72 \times 10^4$)]. [^{14}C]Hexahydroechinulin (r.m.a. $7{\cdot}07 \times 10^4$) from [1-^{14}C]glycine gave alanine (r.m.a. $5{\cdot}69 \times 10^3$) [picrolonate, m. p. 214—215° (r.m.a. $5{\cdot}54 \times 10^3$); lit.,[13] 215°].

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[10] Cornforth, Hunter, and Popjak, *Biochem. J.*, 1953, **54**, 597.
[11] Quilico, Cardani, and Piozzi, *Gazzetta*, 1955, **85**, 3.
[12] Quilico, Cardani, and Piozzi, *Gazzetta*, 1956, **86**, 211.
[13] Levene and van Slyke, *J. Biochem.*, 1912, **12**, 127.