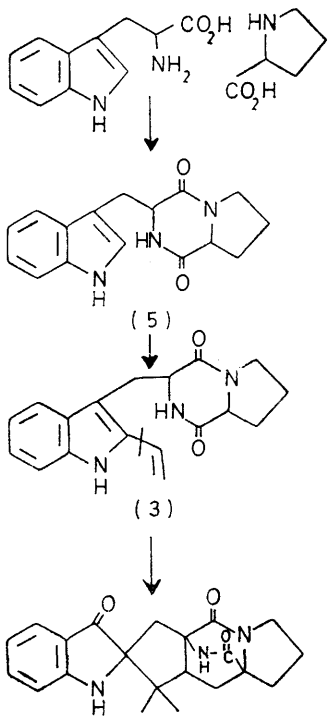
Studies in Relation to Biosynthesis. Part XLVI.[1] Incorporation of *cyclo*-L-Tryptophyl-L-proline into Brevianamide A

By **John Baldas, Arthur J. Birch,* and Richard A. Russell,** Research School of Chemistry, Australian National University, P.O. Box 4, Canberra ACT 2600, Australia

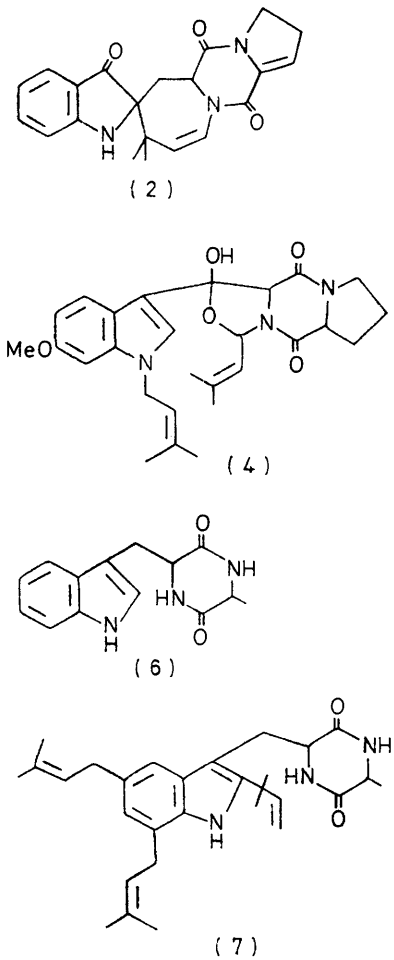
The culture conditions and method of isolation of brevianamide A (1) from *Penicillium brevicompactum* have been improved. Use of ^{3}H and ^{14}C labelling has shown that *cyclo*-L-tryptophyl-L-proline (5) is incorporated efficiently into brevianamide A (1) and a biosynthetic sequence is proposed.

BREVIANAMIDE A (1), isolated as the major component of a related group of pigments from *Penicillium brevicompactum*,[2] was the first characterised [3] of a growing number of fungal dioxopiperazines which may be derived from tryptophan, proline, and one or more units of mevalonic acid. Steyn [4] has isolated the related austamide (2) and congeners from *Aspergillus ustus*, including the predicted biosynthetic precursor of (1) [2] and (2), *cyclo*-L-2-(1,1-dimethylallyl)tryptophyl-L-proline (3). Lanosulin

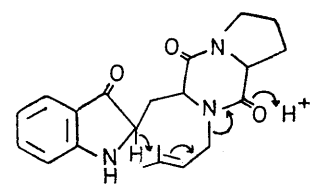


SCHEME

(4), recently isolated [5] from *Penicillium lanosum*, differs from the brevianamides and echinulin (7) in lacking an isoprene unit attached in the reversed manner to the 2-position of the indole; both structures (2) and (4) have a C_5 unit (or units) attached in the normal manner to nitrogen. The reversed unit could be attached directly through a cation or might be biogenetically transferred in the brevianamides from the dioxopiperazine nitrogen atom, either in an indole or in an indoxyl precursor. A possible process for the latter is shown (A).



An alternative possibility is ring formation leading to structure (2) by simultaneous addition to carbon and



(A)

[1] Part XLV, A. J. Birch and D. J. Thompson, *Austral. J. Chem.*, 1972, **25**, 2731.
[2] A. J. Birch and J. J. Wright, *Tetrahedron*, 1970, **26**, 2329.
[3] A. J. Birch and J. J. Wright, *Chem. Comm.*, 1969, 644.
[4] P. S. Steyn, *Tetrahedron*, 1973, **29**, 107.
[5] D. T. Dix, J. Martin, and C. E. Moppett, *J.C.S. Chem. Comm.*, 1972, 1168.

nitrogen in a carbene-type reaction, such as that postulated [6] for the introduction of a terpene unit into a non-phenolic benzene ring.

Biogenetic considerations and physical methods were combined to elucidate the structure of (1).[2,3] In the initial work the incorporation of ^{14}C-labelled DL-tryptophan, DL-mevalonate, and L-proline into (1) was low (0·6, 0·003, and 0·09%, respectively) [3] probably owing largely to the instability and the very low yield of (1) produced. In the present work it has been found that improved culture conditions, including the partial replacement of the culture medium by sterile water prior to precursor feeding, give more efficient incorporations. Also, owing to a reduced growing period, brevianamide A (1) can be isolated from the mycelium instead of from the medium.

The isolation of *cyclo*-L-tryptophyl-L-proline (5) from the culture medium of *Penicillium brevicompactum* [7] together with the formation of (3) by *Aspergillus ustus* suggests that the sequence leading to brevianamide A (1) may be as shown in the Scheme. The postulated precursor (3) has, however, not so far been detected in cultures of *P. brevicompactum*.

It has been shown [8] that ^{14}C-labelled *cyclo*-L-tryptophyl-L-alanine (6) is incorporated efficiently into echinulin (7) by *Aspergillus amstelodami* and that a cell-free extract will convert a mixture of 3,3-dimethylallyl pyrophosphate and (6) into a derivative with the single isoprene substituent [9,10] on the pyrrole ring.

Table 1 shows the incorporation of DL-[*methylene*-^{14}C]-tryptophan, L-[5-^{3}H]proline, DL-[2-^{14}C]mevalonic acid lactone, and *cyclo*-L-[*methylene*-^{14}C]tryptophyl-L-proline (5) into brevianamide A (1). The specific molar incorporations were determined by feeding precursors of known specific molar activity and recrystallising the brevianamide A produced to constant activity without the addition of inactive material. The results show that the total activity recovered as brevianamide A is considerably less than the actual total incorporation. This is undoubtedly due to the instability of brevianamide A during the fermentation and isolation procedures.

TABLE 1

Incorporations into brevianamide A (1)

Precursor	Activity fed (μCi)	% Total incorporation	% Specific incorporation
DL-[*methylene*-^{14}C]-Tryptophan [a]	100	5·1	
	100		17·6
L-[5-^{3}H]Proline	100	0·40	
DL-[2-^{14}C]-Mevalonic acid lactone [a]	75	0·16	
cyclo-L-[*methylene*-^{14}C] Tryptophyl-L-proline	12·9	1·8	
	12·5		14·0

[a] Incorporations are based on the expected utilisation of the L-isomer only.

To establish that (5) is incorporated as a unit into brevianamide A without any prior hydrolysis into the amino-acids, followed by recombination, *cyclo*-L-[*methylene*-^{14}C]tryptophyl-L-[5-^{3}H]proline was prepared. Feeding of the doubly labelled material (^{3}H : ^{14}C ratio 3·67 : 1; specific activity 306 × 10^{3} and 83·5 × 10^{3} disint. min^{-1} mg^{-1} for ^{3}H and ^{14}C) gave (Table 2) brevianamide A with ^{3}H : ^{14}C ratios of 3·82 : 1 and 3·74 : 1 and total incorporations of 3·2 and 3·6%, respectively. The results show that *cyclo*-L-tryptophyl-L-proline (5) is incorporated intact into brevianamide A (1). Furthermore, the high specific incorporation of (5) (14·0%) confirms the route shown in the Scheme. The introduction of an isoprene unit into the indole ring of (5) followed by further elaboration of (3) to brevianamide A (1) must represent a major (if not the only) biosynthetic sequence. The details of the final stages are still unknown.

TABLE 2

Incorporation of *cyclo*-L-[*methylene*-^{14}C]tryptophyl-L-[5-^{3}H]proline into brevianamide A

Precursor	Brevianamide A	
^{3}H : ^{14}C Ratio	^{3}H : ^{14}C Ratio	% Total incorporation
3·67 : 1 (105 mg)	3·82 : 1	3·2
3·67 : 1 (106 mg)	3·74 : 1	3·6

EXPERIMENTAL

M.p.s were determined on a Kofler hot-stage apparatus. Analytical and preparative t.l.c. was carried out with Merck Kieselgel GF_{254}. Radioactive samples were counted on a Beckman LS-150 liquid scintillation counter (internal standardisation with ^{3}H and ^{14}C standards).

Culture and Isolation of Labelled Brevianamide A (1).—A strain was selected of *Penicillium brevicompactum* Dierckx (University of Manchester Acc. 382) which when cultured on corn-steep liquor–glucose slopes produced dark green spores. A medium consisting of glucose (20 g), corn-steep liquor (11 g), and distilled water (500 ml) in 2 l penicillin flasks was inoculated with a heavy spore suspension of the mould and the culture was grown in the dark at 25°. Growth was rapid in comparison with cultures grown on Czapec-Dox medium. After 2 days the surface of the medium was covered with thick white mycelium and sporulation had just begun. At this stage *ca.* 400 ml of the medium was removed and replaced with an equal volume of sterile distilled water containing the labelled precursor. Alternatively, the labelled precursor dissolved in the minimum amount of ethanol was added to the reflooded culture. Concentrations of ethanol greater than 10 ml per l of medium inhibited spore development and subsequently pigment production. By the third day of growth the mycelial mat was completely covered with dark green spores and the cultures were harvested after 7 days. In a typical feeding experiment the mycelium from five cultures was filtered off and the medium discarded. Care was taken to exclude light in this and subsequent operations in order to minimise the decomposition of brevianamide A. The mycelium was dried under reduced pressure (P_2O_5) and powdered before being stirred with light petroleum (b.p. 60—80°; 600 ml) for 4 h to remove lipids. The light petroleum was removed

[6] A. J. Birch, *J. Agric. and Food Chem.*, 1971, **19**, 1088.
[7] A. J. Birch and R. A. Russell, *Tetrahedron*, 1972, **28**, 2999.
[8] G. P. Slater, J. C. McDonald, and R. Nakashima, *Biochemistry*, 1970, **9**, 2886.
[9] C. M. Allen, jun., *Biochemistry*, 1972, **11**, 2154.
[10] C. M. Allen, jun., *J. Amer. Chem. Soc.*, 1973, **95**, 2386.

by filtration and the dark green powder was stirred overnight with methanol (400 ml). The deep yellow solution was filtered and evaporated to dryness under reduced pressure. The residue was extracted with ethyl acetate (600 ml) and the resulting yellow solution was washed with saturated aqueous sodium carbonate (3 × 100 ml) and brine (1 × 100 ml), dried ($MgSO_4$), and evaporated to a small volume. Brevianamide A was isolated by preparative t.l.c. on silica (10% ethanol–ether as solvent) and recrystallised to constant activity from chloroform; m.p. 220—250° (sublimation) [lit.,[2] 220—250° (sublimation)]. In experiments where total recovered activity was required inactive brevianamide A (10—20 mg) was added before crystallisation.

Preparation of Labelled cyclo-L-*Tryptophyl*-L-*prolines* (5).—*cyclo*-L-[*methylene*-^{14}C]Tryptophyl-L-[5-^{3}H]proline was prepared from benzyloxycarbonyl-L-[*methylene*-^{14}C]tryptophan [11] and L-[5-^{3}H]proline benzyl ester hydrochloride [12] by the method described for the unlabelled compound.[7] The doubly labelled compound was recrystallised to constant ^{3}H : ^{14}C ratio (3·67 : 1; specific activities 306 × 10^3 and 83·5 × 10^3 disint. min^{-1} mg^{-1}, respectively) from ethanol; m.p. 173—175° (lit.,[7] 173—175°). *cyclo*-L-[*methylene*-^{14}C]Tryptophyl-L-proline was similarly prepared from benzyloxycarbonyl-L-[*methylene*-^{14}C]tryptophan and inactive L-proline (specific activity 278 × 10^3 disint. min^{-1} mg^{-1}).

Calculation of ^{3}H : ^{14}C *Ratios*.—The ^{3}H : ^{14}C ratios for doubly labelled brevianamide A and doubly labelled compound (5) were determined by making corrections for the non-linear relationship between the counting efficiency for tritium, and to a lesser extent for ^{14}C, with the quenching factor. A correction was also made for the varying spill-over of ^{14}C counts into the ^{3}H channel with quenching factor. A direct comparison between the doubly labelled (5) fed and the brevianamide A produced can be made. When a sample of [^{3}H,^{14}C]brevianamide A was compared directly with a sample of [^{3}H,^{14}C]-(5) made up to the same quenching factor with inactive brevianamide A, the ^{3}H : ^{14}C ratios were found to agree to within 3%.

We thank Mrs. M. Anderson for the culture work.

[3/1213 *Received*, 11*th June*, 1973]

[11] E. I. Smith, *J. Biol. Chem.*, 1948, **175**, 39.
[12] R. E. Neuman and E. L. Smith, *J. Biol. Chem.*, 1951, **193**, 97.