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STUDIES IN RELATION TO BIOSYNTHESIS—XLII[1]

THE STRUCTURAL ELUCIDATION AND SOME ASPECTS OF THE BIOSYNTHESIS OF THE BREVIANAMIDES-A AND -E

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Abstract—Five related metabolites have been isolated from the mould, *Penicillium brevi-compactum* Dierckx. The structural elucidation and biosynthesis of two of these, brevianamides-A and -E, is discussed. They are shown to be biogenetically derived from tryptophan, proline and mevalonic acid. The UV chromophores of four of these compounds are related to the 2,2-disubstituted ψ-indoxyl system. Brevianamide-A probably has the structure **8**, and brevianamide-E is probably **25**.

THE culture medium of *Penicillium brevi-compactum* was observed to be yellow and extraction gave a number of neutral metabolites. Those so far characterized have been named brevianamides-A to -E. Substance E is the only one of the series which is not yellow, orange or red. We report here structural investigations on brevianamides-A, and -E. Because of the small yields (0·1 to 10 mg/l), structural elucidations relied heavily on biogenetic theory and prediction together with the use of physical measurements.

Brevianamide-A

This yellow neutral compound crystallized from chloroform with one molecule of solvent of crystallization and was appreciably soluble only in strongly polar solvents. Although insoluble in water it dissolved in dilute alkali on heating. The mass spectrum revealed a molecular ion at *m/e* 365 of composition $C_{21}H_{23}N_3O_3$.* The primary fragmentation was loss of mass 69 of composition C_5H_9 from the molecular ion.

The UV absorption spectrum had maxima at 235, 256 and 404 mμ and the spectrum did not alter with the addition of acid or base to the solution. The spectrum is typical of a ψ-indoxyl chromophore,[2] occurring naturally in several indole alkaloids.[3] Hydroxyindolines (e.g. 2) obtained from 2,2-disubstituted ψ-indoxyls (e.g. 1) by reduction with sodium borohydride have been shown to undergo a well-characterized rearrangement[4] to 2,3-disubstituted indoles (e.g. 3) on treatment with acid. Brevianamide-A was similarly found to undergo conversion into the indole deoxybrevianamide-A, the UV spectra of the intermediate indolines and the indole being almost identical with those of the models. In the case of brevianamide-A two diastereoisomeric hydroxyindolines formed on reduction were observed on TLC but were not isolated. Quantitative conversion of both products into deoxybrevianamide-A was

* The assignment of composition to mass spectral ions in this discussion is supported, unless stated otherwise, by accurate mass measurement.

immediate on acidification. The formation of the same product from both alcohols indicates that the rearrangement reaction in this case is not concerted, since it must be independent of the hydroxyl configuration.

The IR absorption spectrum of brevianamide-A contains a sharp band at 3420 cm^{-1}, a broad band at 3300 cm^{-1} and multiple absorption in the 1660–1710 cm^{-1} region, with a sharp band at 1625 cm^{-1}. The model ψ-indoxyl (1) has a sharp absorption band at 3420 cm^{-1}, assigned to the NH hydrogen, carbonyl absorption at 1670 cm^{-1}, and bands at 1620 cm^{-1} and 750 cm^{-1}. The band at 1620 cm^{-1} has been assigned to the structural unit C_6H_5—N—C,[4b] where C is saturated. The UV spectrum of deoxybrevianamide-A shows no indication of unsaturation conjugated with the aromatic system. The partial structure **4** can therefore at this stage be assigned tentatively to brevianamide-A. The ψ-indoxyl nitrogen is secondary since the N-nitroso derivative of brevianamide-A could be prepared and its UV spectrum is almost identical with that of the same derivative of the model compound **1**. The NMR spectrum of brevianamide-A confirms the presence of four aromatic protons in the molecule, their distinctive splitting pattern being identical with that of the aromatic protons of the model **1**. The partial structure **4** is therefore confirmed.

The empirical formula of brevianamide-A indicates that the substance must contain in addition to the ψ-indoxyl system, six double bonds or rings. Evidence for the presence or absence of olefinic bonds was therefore sought. No resonances in the NMR spectrum could be assigned to olefinic protons, so that only tetrasubstituted double bonds, if any, could be present. Catalytic hydrogenation of brevianamide-A with platinum oxide resulted in the uptake of two molar proportions of hydrogen but with reduction of the aromatic ring. This result is analogous to that obtained[4a] in the reduction of 2,2-tetramethylene-ψ-indoxyl **1** to the tetrahydroderivative under the same conditions. Hydrogenation of deoxybrevianamide-A with platinum oxide and acetic acid gave a product homogeneous by TLC and mass spectrometry, which had resulted from the absorption of four molar proportions of hydrogen. The lack of UV absorption in the substance is in accord with the conclusion that reduction of the indole system to an octahydroindole had taken place.

In a further attempt to degrade the molecule, ozonolysis of deoxybrevianamide-A was examined under conditions which have been used for the ozonolysis of ibogaine lactam.[5] After methylation of the acidic product with diazomethane, a derivative of composition $C_{17}H_{24}N_2O_6$ was obtained. This product was therefore formed by complete fission of the indole ring as in the ibogaine model and its formation indicates the absence of any other C=C double bonds in the molecule.

Brevianamide-A gave a crystalline dibromoderivative with bromine in chloroform. This is however a substitution product, presumably with bromine atoms in the 5,7-positions of the aromatic ring. The model compound behaved in a similar manner. This dibromoderivative was particularly useful in assisting in the interpretation of mass spectra.

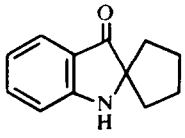
The combined evidence from the preceding work is that no C=C unsaturation occurs in the molecule other than that in the ψ-indoxyl system.

There is evidence of the presence of CO groups. The IR spectrum of deoxybrevianamide-A contains strong absorption bands at 1670 cm^{-1} and 1690 cm^{-1}, possibly indicative of the presence of two CO groups in the molecule. In view of the positions of the peaks and the absence of conjugated unsaturation, the CO's are likely to be

present in amide groupings. This expectation was confirmed by reduction with diborane[6] to a diamine, $C_{21}H_{27}N_3$, corresponding to the reduction of two CO groups in deoxybrevianamide-A to two methylene groups. Deuterium exchange experiments to be discussed below indicate that these two amide groupings contain only one NH between them.

To sum up: brevianamide-A has been unambiguously demonstrated to have a 2,2-disubstituted ψ-indoxyl portion, two other CO groups in amide functions, and no olefinic bonds. The composition $C_{21}H_{23}N_3O_3$ of brevianamide-A together with this evidence leads to the conclusion that it is hexacyclic.

It has been established[7] that trans secondary amides exhibit an amide-I band near 1650 cm^{-1}, and an amide-II band near 1550 cm^{-1}, while *cis* secondary amides show only the amide-I band. Alkyl 2,5-diketopiperazines are examples in the latter category and exhibit the amide-I band at positions from 1670 to 1690 cm^{-1}.[8] The IR spectrum of brevianamide-A reveals nothing that can be attributed to an amide-II band while the amide-I bands at 1670 and 1690 cm^{-1} are in the range expected for a 2,5-diketopiperazine. Alkaline hydrolysis gave rise to a water-soluble product which, on acidification and attempted methylation, regenerated to a large extent the starting brevianamide-A. This result can be accommodated by the hydrolytic opening, but not the complete fission, of a diketopiperazine ring. Acid hydrolysis under standard peptide fission conditions gave rise to no recognisable amino-acids. The hydrolysis results indicate the absence of any other reactions such as retro-aldol or other cleavage processes.



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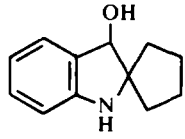

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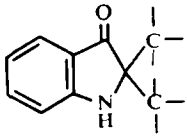

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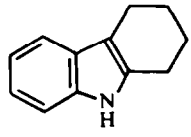

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Biosynthetic study

The presence of an indole-derived unit suggests the possibility of tryptophan as a biological precursor, and the possible presence of a diketopiperazine ring indicates that this may be linked with some other amino-acid. Additionally, the loss of C_5H_9 in the mass spectrum indicates the possible presence of a C_5-terpene unit. On the basis of these ideas, an examination was made of possible biogenetic precursors of the substance.

A number of diketopiperazine derivatives are known as mould metabolites. These include echinulin **5**[9] and mycelianamide **6**.[10] In the case of echinulin the incorporation of tryptophan and alanine,[11] and in the case of mycelianamide the incorporations of tyrosine and alanine[12] have been observed. The structure of echinulin was also independently derived[11] partly as a result of these tracer incorporations.

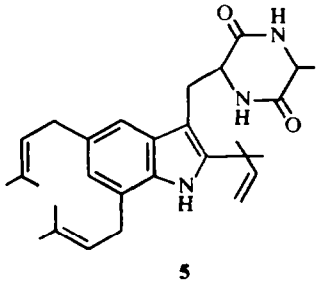
The incorporation of [methylene-^{14}C]tryptophan into brevianamide-A was examined and radioactive brevianamide-A was isolated, indicating that the indole ring and the methylene carbon are probably incorporated. More desirably, [carboxyl-^{14}C]tryptophan could be used but this was not available.

The presence, as revealed by the NMR spectrum of brevianamide-A, of two methyl groups, each attached to a quaternary carbon atom suggested that the isoprene unit, if present, may be attached in the reverse position, as in echinulin **5**. Feeding experiments with [1-^{14}C]acetate and [2-^{14}C]mevalonolactone were conducted and the results are shown in Table 1. Both of these precursors were incorporated, and although degradation experiments could not be carried out on the products in view of the low yields of the pigments, the incorporations are considered to be significant. Feeding experiments with [$^{14}CH_3$]methionine gave no incorporation whatsoever. Mevalonic acid has been shown to be a virtually irreversible intermediate in terpenoid biosynthesis in moulds[13] and its incorporation is therefore significant of the presence of the terpene unit.

From the working hypothesis that a tryptophan unit and an isoprene unit are involved, there remain five carbons to be accounted for. If a diketopiperazine unit is present in the molecule these should be part of an amino acid not containing Me groups; the most attractive possibility is proline. The absorption in the NMR spectrum at τ 6·7 integrating for 2 protons could then be correlated with the CH_2N group in the proline ring. However, hydrolysis under either acid or alkaline conditions produced no recognisable amino acid, and proline, if present, must be joined by some further linkage than those of peptide types.

TABLE 1. RADIOACTIVE TRACER EXPERIMENTS

Substrate	Activity of Brevianamide-A cts/100 secs	Incorporation (% activity fed)
DL[3-^{14}C]tryptophan	1430	6×10^{-1}
[2-^{14}C]mevalonic lactone	28	3×10^{-3}
Na[2-^{14}C]acetate	425	25×10^{-2}
[$^{14}CH_3$]methionine	0	0
L[U-^{14}C]proline	4880	9×10^{-2}



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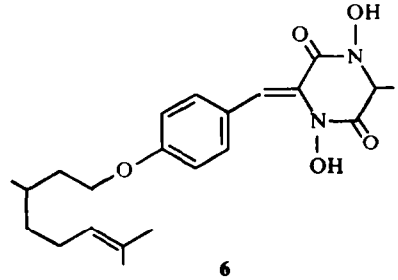

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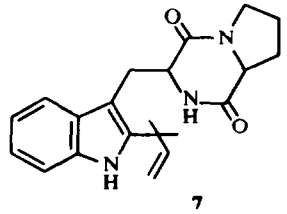

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When L-[U-^{14}C]proline was added to cultures of the organism, the incorporation was comparable to that of tryptophan. Very tentatively, a hypothetical biogenetic precursor could now be written as **7**.

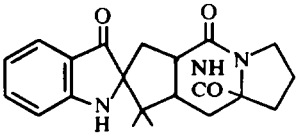
Spectral examinations of possible structures

Protons at the 3,6-positions of 2,5-diketopiperazines have distinctive chemical shifts in NMR spectra with resonances between τ 5·5 and τ 6·3.[14] There are no signals in the NMR spectrum of brevianamide-A between τ 4·0 and τ 6·7 in d^5-pyridine. The inference that protons in this environment are not present is critical, and an attempt was made further to confirm the deduction.

Early work on the alkaline hydrolysis of optically active 2,5-diketopiperazines to dipeptides showed that extensive racemisation had occurred. L-Leucine anhydride is completely and rapidly racemised under conditions which lead to no cleavage of the 2,5-diketopiperazine ring.[15] Hydrogens adjacent to the amide carbonyl should therefore be exchangeable with deuterium under basic conditions. This was confirmed with proline anhydride using sodium methoxide in deuteromethanol, the dideutero proline anhydride being formed in 92% yield. Treatment of brevianamide-A under identical conditions resulted in no incorporation of deuterium other than that into NH groups. Hydrogens in the equivalent positions to the 3,6-hydrogens of the diketopiperazine are therefore not present in brevianamide, unless steric effects prevent enolisation.

The absence of the unsaturation to be expected in a terpene unit, the probable absence of the diketopiperazine 3,6-hydrogens and the necessity for constructing two further rings to explain the composition of the substance, led to the hypothesis that these rings may have been formed between these two positions and the termini of the double bond. On this basis a number of possible formulae can be written, most of which, however, are ruled out on the basis of steric or NMR considerations. The two most likely formulae are **8** and **9**, depending on whether the isoprene unit is attached in the "normal" or "reversed" position to the indole system. On the basis of these alternative tentative structures, several structures can be written for deoxybrevianamide-A, **10**, **11**, **12** and **13**. The fact that only one product results from the non-concerted rearrangement is an argument in favour of the structure **8**, where a consideration of migratory aptitudes would predict **11** to be at least the major product of rearrangement. In the case of **9** there seems little to choose between the two possible rearrangements. The distinction between **8** and **9** in regard to the situation of the *gem* dimethyl groups was arrived at by considerations of the NMR spectra. The two C-Me groups present in brevianamide-A are both situated on a quaternary position from their chemical shifts and the absence of spin-spin splitting. A Dreiding model of structure **8** reveals that the two Me groups lie near the ψ-indoxyl ring system, one above the CO and the other above the nitrogen. The chemical shift difference between them can be explained in this case by their quite different environments and particularly by the shielding of one relative to the other because of its proximity to the CO group. The Me groups in the alternative structure **9** should be magnetically non-equivalent but from molecular models the different environment should not be sufficient to cause the chemical shift difference observed.

The two Me groups of deoxybrevianamide-A have nearly identical chemical shifts (τ 8·51, τ 8·54), and the resultant deshielding of these Me groups on conversion from



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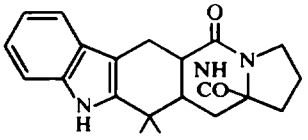

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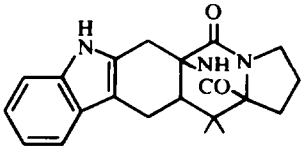

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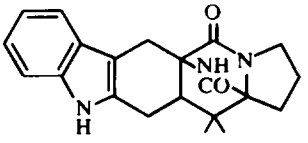

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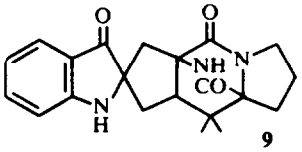

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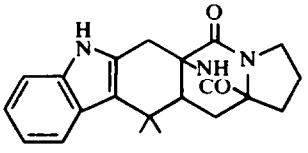

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a ψ-indoxyl into an indole is consistent only with a conversion of **8** into either **10** or **11**. Hence, structure **9** may be discarded in favour of structure **8** for brevianamide-A. Further analysis of the NMR spectra provides evidence in support of structure **8**.

A 2-proton quartet in the spectrum of brevianamide-A can be attributed to a geminally coupled, isolated methylene group (τ 6·82, τ 7·06; J = 15 Hz). The spectrum of deoxybrevianamide-A also contains this system (τ 5·76, τ 6·80; J = 18 Hz). These signals may be respectively assigned to the methylene group attached to the ψ-indoxyl system in **8** (H_A, H'_A) and the indole system in **10** or **11**. The large coupling constant of 18 Hz is consistent with either of the structures **10** and **11** as, from models, the H—H axis is almost at right angles to the plane of the ring.[16]

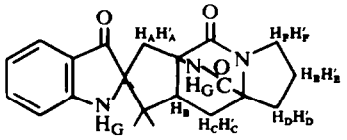
The absorption centred at τ 6·7 may be correlated with the methylene group adjacent to the nitrogen of the proline ring system. This correlation is assisted by comparison with the spectrum of proline anhydride. The absorption occurs as a triplet in brevianamide-A when deuterochloroform and deuteroacetone are employed as solvents but when deuteropyridine is employed a multiplet resembling two overlapping triplets is observed. With the addition of D_2O the multiplet collapses into a triplet. A possible explanation is that in chloroform and acetone inversion of the proline nitrogen results in an averaging of the methylene group environment giving rise to a triplet, whereas in dry prydine this inversion is hindered, leading to different chemical shifts for the methylene protons. The corresponding methylene group in deoxybrevianamide-A gives rise to a triplet in dry pyridine. Irradiation in the region near τ 8·2 leads to collapse of the multiplet centred at τ 6·7 into an AB quartet (J = 11 Hz). With D_2O added, irradiation in the same region leads to collapse of the triplet near τ 6·7 and the multiplet near τ 7·2 to singlets. If the low field multiplet can

be assigned to H_F and H'_F and the high field multiplet to H_D and H'_D then the result is that to be expected from irradiation of H_E and H'_E. Complete assignment of the NMR spectrum of brevianamide-A as structure **8** is given below.

TABLE 2. 100 MHz NMR SPECTRUM OF BREVIANAMIDE-A (d^5 PURIDINE)

Assignment	No. of Protons	Chemical Shift (τ)	Pattern*
Aromatic	4	2·3–3·5	AA'XX'
C-methyl	3	8·7	s
C-methyl	3	9·1	s
H_A, H'_A	2	6·82, 7·06	q($J_{AA'}$ = 15 Hz)
H_D, H'_D	2	7·2	m
H_F, H'_F	2	6·7	m[c]
H_G[b]	1	1·6	s
H'_G[b]	1	5·2 or	s
H_B, H_C, H'_C, H_E, H'_E	5	8·2–8·5	m

[a] s = singlet; q = quartet; m = multiplet
[b] disappears on addition of D_2O
[c] collapses to triplet on addition of D_2O



The mass spectrum of brevianamide-A also supports the assigned structure. The primary fragmentation of brevianamide-A is the loss of a C_5H_9 radical to give a stable even-electron species, *m/e* 296. The loss of C_5H_9 proceeds through the fission of three bonds; all these are very favourable for cleavage being β to both a nitrogen and a CO group. It has been shown that in the fragmentation of 3,6-dialkyl-2,5-diketopiperazines the alkyl groups may be lost as radicals or olefins.[17] Some fragmentations of brevianamide-A for which there are metastable ions are outlined in Figs 1 and 2.

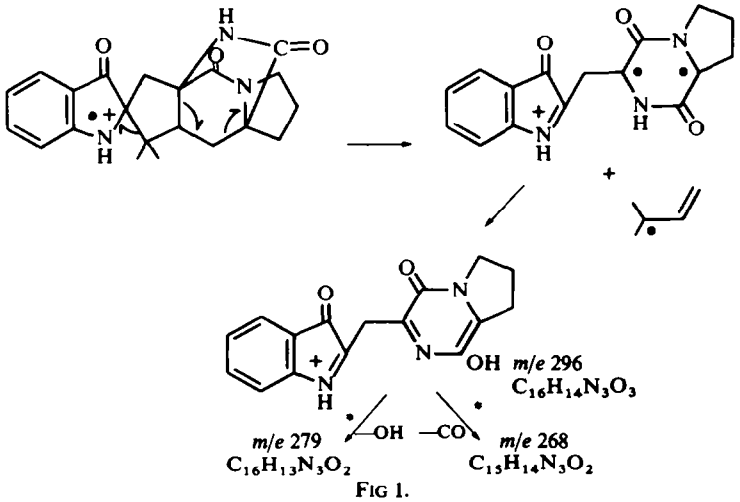


FIG 1.

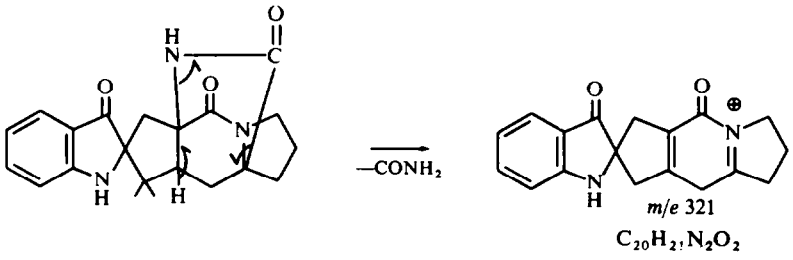


FIG 2

An initially puzzling feature was the loss of $CONH_2$ from the molecular ion which becomes one of CONHD when both of the NH hydrogens have been exchanged with deuterium. The tendency to lose $CONH_2$ rather than CONH as normally observed in the mass spectra of 3,6-dialkyl-1,5-diketopiperazines is presumably characteristic of the bridged ring present and may involve hydrogen migration as shown (Fig 2).

There are a number of fragments of lower mass which are most significant and their formation can be rationalised on the basis of the structure **8** for brevianamide-A. By observing which of these fragments are not present in the spectrum of dibromobrevianamide-A, but are replaced by ones at higher *m/e* values corresponding to the substitution of two Br atoms, differentiation of fragments which contain the aromatic system from fragments arising from other parts of the molecule can be made. The fragments containing the aromatic system are not found at 14 mass units higher in the spectrum of monomethylbrevianamide-A and so methylation must have occurred on the secondary amide nitrogen and not on the ψ-indoxyl nitrogen. The fragments that have increased by 14 units in the spectrum of this compound compared with that of the unmethylated material must contain CONMe unless the Me group undergoes an unlikely migration during the fragmentation. The loss of $CONH_2$ in brevianamide-A becomes one of $CONHCH_3$ in monomethylbrevianamide-A, further supporting the previous argument that monomethylbrevianamide-A carries the methyl on the amide nitrogen. Two fragments, *m/e* 138 and *m/e* 149 were found to be unaltered in mass by either bromination or methylation. The results are shown in Table 3, the values being calculated from exact mass measurements. With this information, the postulated structure **8** can accommodate plausible representations of the structures of these ions and these are shown below (Fig. 3).

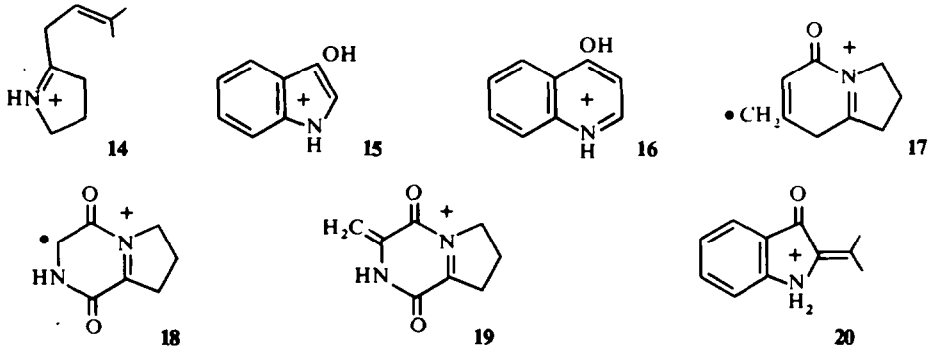
The spectrum of dideuterobrevianamide-A (NH protons exchanged with deuteromethanol) reveals that five of the fragments, (**14**, **15**, **18**, **19** and **20**) increase by one mass unit. For the last four of these, this increase is to be expected from the structure **8** and the first one has gained a deuterium by migration. Fragment **16** gains two deuteriums inferring that the extra one obtained by migration comes from the amide nitrogen. Finally fragment **17** does not alter in the deuterated product, a result entirely in accord with its assigned structure, as it does not retain a hydrogen from an exchangeable position in the original structure, nor does it gain a hydrogen by migration.

The most probable structure of deoxybrevianamide-A, on the basis of migratory aptitudes, is **11**.

TABLE 3

Fragment	m/e	Composition[a]	Category[b]
14	138	$C_9H_{16}N$	C
15	133	C_8H_7NO	A
16	146	C_9H_8NO	A
17	149	$C_9H_{11}NO$	C
18	152	$C_7H_8N_2O_2$	B
19	165	$C_8H_9N_2O_2$	B
20	174	$C_{11}H_{12}NO$	A

[a] From accurate mass measurement
[b] A—contains aromatic ring
B—contains lactam secondary nitrogen
C—in neither category A nor B



14 15 16 17 18 19 20

FIG 3.

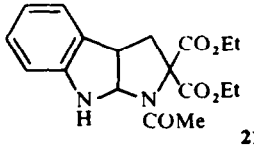
Brevianamide-E

Brevianamide-E, a colourless glass, was isolated by TLC of the neutral extract containing the four pigments. The mass spectrum had a molecular ion at *m/e* 367 of composition $C_{21}H_{15}N_3O_3$ and the primary fragmentation was a loss of mass 69 to give the base peak, *m/e* 298. The close relationship between brevianamide-E and brevianamide-A is immediately apparent. The UV spectrum contained maxima at 239 mμ and 296 mμ similar to that of an indoline, except that no change was observed in acid solution. This behaviour can be explained by the presence of an electronegative group in the 2-position of the indoline system. The presence of a basic nitrogen in that position would have lessened the basicity of the indoline nitrogen but also would have led to a hypsochromic shift in acid solution. An amide nitrogen in that position, as in the synthetic compound **21** which has a superimposable UV spectrum, would have however led to the observed behaviour. Absorption in the IR spectrum of brevianamide-E at 3600 cm^{-1} and 3370 cm^{-1} was assigned to hydrogens on oxygen and nitrogen respectively. Carbonyl absorption of 1680 cm^{-1} and 1690 cm^{-1} and the absence of absorption near 1550 cm^{-1} (amide-II) indicated that a diketopiperazine system is probably present. The NMR spectrum of brevianamide-E contains a singlet τ 8·73 integrating for 6 protons and 3 olefinic protons whose splitting pattern resembled that of an ABX system characteristic of vinyl groups. Calculation of the

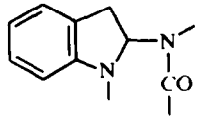
parameters with a digital computer gave chemical shifts τ_A 4·9, τ_B 4·96, τ_X 3·65; J_{AX} = 18·5 Hz, J_{BX} = 10·5 Hz, J_{AB} = 1·5 Hz. Catalytic hydrogenation gave dihydrobrevianamide-E, the mass spectrum of which contained a molecular ion at *m/e* 369, from which a loss of mass 71 led to the base peak *m/e* 298. The NMR spectrum of dihydrobrevianamide-E revealed that no olefinic protons remained in the molecule but that an ethyl group had been produced by the reduction. The unit C—H═CH_2 is therefore present in brevianamide-E with the allylic carbon tetrasubstituted. The fragment of mass 69 lost from brevianamide-E contained the unsaturation as the loss became that of 71 in dihydrobrevianamide-E. Combined evidence strongly suggested that this fragmentation represents the loss of a dimethylallyl group (C_5H_9) from the molecular ion.

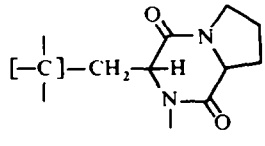
The NMR spectrum also indicated the presence of 4 protons in the molecule with resonances between τ 6·0 and τ6·6. This is the region in which the CH_2N group of brevianamide-A absorbs and is also the region in which the protons in the 3- and 6-positions of 2,5-diketopiperazines are known to absorb. It was therefore thought that brevianamide-E may contain a diketopiperazine unit which is unsubstituted in the 3- and 6-positions and which would therefore be hydrolysable to yield a free amino acid. Indeed, the acid hydrolysate was found to contain one molar proportion of proline.

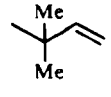
Another feature of the NMR spectrum of brevianamide-E is a doublet of a doublet of doublets (AB part of an ABX system) which could have arisen from two non-equivalent protons of a methylene group (A and B) coupling with each other and with a vicinal hydrogen (X) (τ_A 7·14, τ_B 7·39; J_{AX} = 11 Hz, J_{BX} = 8 Hz, J_{AB} = 13 Hz). Absorption by X in the region τ 6·0 to 6·6 is consistent with this being a proton at either the 3- or 6-position of a diketopiperazine ring. These three protons are not part of the proline ring as the methylene group couples with only one vicinal hydrogen. The combined evidence leads to the partial structure **22** for brevianamide-E. A negative ferric chloride test established that the hydroxyl group is not present as part of an acylhydroxylamine function.



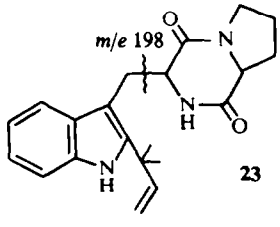
21



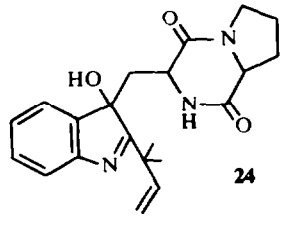




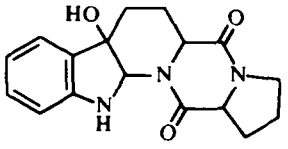
22



23



24



25

Deoxybrevianamide-E

Reaction of brevianamide-E with zinc dust in hot acetic acid gave an indole, deoxybrevianamide-E. The UV spectrum indicated the presence of an indole chromophore not conjugated with other unsaturation. The molecular ion occurred at *m/e* 351, corresponding to the formal loss of one oxygen atom from brevianamide-E. The spectrum revealed one major cleavage of the molecular ion to give the fragment *m/e* 198 which was the base peak. The second most intense peak in the spectrum has only 12% of the intensity of the base peak. NMR spectroscopy showed that two exchangeable protons were also present in deoxybrevianamide-E.

The combined evidence together with the partial structure **22** for brevianamide-E shows that deoxybrevianamide-E has the structure **23**. This structure is that previously considered, as a working hypothesis, to be the biosynthetic precursor of brevianamide-A. The fragment *m/e* 198 in the mass spectrum would therefore have arisen from cleavage in the expected position, β to the aromatic system.

The NMR spectrum revealed the presence of a vinyl group, and of two methyl groups (s, τ 8·46) which are equivalent to those in echinulin **5** and have a very similar chemical shift (τ 8·49). Evidence is also available for the presence of a CH_2 coupled with CH. The CH in this system appears to be coupled to another proton (J = 1·5–2·0 Hz) which may be on the amide group or another position of the diketopiperazine ring. Other absorption peaks are readily assigned to the proline residue.

Structure of brevianamide-E

The formula and biogenetic considerations could lead to the hydroxyindoline structure **24** for brevianamide-E, but this structure does not fit the UV evidence. This compound could however well be an intermediate in the reduction of brevianamide-E to deoxybrevianamide-E. The cyclized analogue **25** of this structure in fact explains all of the presently known evidence and is assumed to be the correct structure for brevianamide-E. This structure agrees with the NMR evidence including the fact that the Me groups in brevianamide-E are shifted downfield on conversion to the indole deoxybrevianamide-E. The hydroxyindoline **24** is likely to be the biogenetic intermediate from the postulated indole precurosr **23**.

Possible mechanisms for the ring-closures on to the terpene unit will be discussed later. It is sufficient to note that oxidations of diketopiperazine rings by fungi are well known[18] (cf. 6) and a reaction of this type could be the source of a carbonium ion to initiate the reaction.

We wish to re-emphasise the part which biogenetic considerations and experiments played in determining the structures. The physical evidence alone is too complex to lead directly to complete structures; the biogenetic basis permits possible structures to be drawn which can then be tested out against the available physical evidence. An X-ray structure determination of brevianamide-A is in progress.

EXPERIMENTAL

M.ps were determined on a Kofler block and are uncorrected. Optical rotations were measured on an ETL–NPL automatic polarimeter. UV spectra were measured on a Unicam SP800 spectrometer, in EtOH unless otherwise stated. IR spectra were measured on a Perkin–Elmer 237 or 257 spectrometer. NMR spectra were measured on a Varian HA-100 spectrometer using TMS as an internal reference. Mass spectra were determined using an A.E.I. MS9 or MS902 spectrometer. GLC was carried out on a Perkin–Elmer 800 chromatograph. Radioactive assay was carried out as described by Birch *et al.*[19] and counting figures are accurate within a statistical error of not more than 3%.

Isolation. The mould, *Penicillium brevi-compactum* Dierckx (Univ. of Manchester Acc. 382), was grown on Czapek-Dox medium for four to five weeks at 25°. The medium was filtered from the mycelium, reduced to half its original volume in a cyclone evaporator, acidified to pH3 with 2N HCl, and extracted with EtOAc. Following saturation with NaCl, it was extracted again. The combined extracts were reduced in volume, extracted with 5% Na_2CO_3aq, washed with saturated brine, and dried. The Na_2CO_3 soln was neutralized with 2N HCl and extracted with EtOAc. This extract contained mainly mycophenolic acid and its methyl ester. Mycophenolic acid could be crystallized from the extract, m.p. 141° (EtOH) after two recrystallizations.

The average yield of material obtained from one litre of medium was 70 mg of carbonate-insolubles and 110 mg of carbonate-solubles.

Separation of brevianamides-A, -B, -C, -D and -E. Initial isolation of brevianamide-A was by preparative TLC. Subsequently, about one third to one half of the material present in the extract could be crystallized directly by seeding its solution in chloroform. Column chromatography on florex, $MgSO_4$ and Kieselgel G achieved partial separation of the pigments, but TLC on Kieselgel G254 proved to be more efficient. A separation into four main pigments could be achieved by repetitive chromatography, the most useful solvent systems being 5% MeOH in $CHCl_3$ and 1% EtOH in ether. The separated pigments in order of decreasing R_f are brevianamide-C (orange), -D (red), -A (yellow), and -B (yellow). Brevianamide-E, a colourless compound, could be separated from the pigments by using ether as a developing solvent. The yields of isolated metabolites varied from 0·1 to 10 mg/l of culture fluid.

Brevianamide-A. Brevianamide-A, the major component of the mixture, crystallized from $CHCl_3$ in needles containing one molecule of solvent of crystallization; m.p. 175–180° with loss of solvent, recrystallization (190–220°) and sublimation (220–250°). Recovery of the material followed by recrystallization from $CHCl_3$ gave a compound identical with the original. (Found: C, 54·8; H, 5·0; N, 8·6. $C_{21}H_{23}N_3O_3$. $CHCl_3$ requires: C, 54·5; H, 5·0; N, 8·7%); $[\alpha]_D^{25} = +413°$ (EtOH); λ_{max} 235 mμ (28,600), 256 mμ (7,100), 404 mμ (3,260); ν_{max} ($CHCl_3$) 3420, 3300, 1680–1715, 1625 cm^{-1}; the NMR spectrum is given in Table 2.

Deoxybrevianamide-A. Brevianamide-A (20 mg) was dissolved in EtOH (5 ml) and cooled to 0°. Two drops of 2N NaOH were added followed by an excess of $NaBH_4$. After 1 hr the soln was left to return to room temp and stirring continued for 4 hr. The soln slowly became colourless. TLC (5% MeOH in $CHCl_3$) showed two new spots, close together and of equal intensity (R_f 0·5). The UV spectrum had maxima at 215 mμ, 245 mμ, and 300 mμ. Acidification immediately changed the spectrum to that of an indole. The soln, after acidification with 2N HCl, was evaporated to remove the EtOH, water added and the aqueous suspension extracted with $CHCl_3$. The $CHCl_3$ extract contained only one compound by TLC. The dried $CHCl_3$ soln was evaporated to leave a white solid (14 mg), which yielded crystalline product, m.p. 265–280°; λ_{max} 227 mμ (31,800), 283 mμ (6,630), 290 mμ (5,700); ν_{max} (KBr) 1690, 1670 cm^{-1}, τ(d^5 pyridine) 2·1–3·0 (m, 4H, aromatic protons), 0·02 (s, 1H, NH), −1·7 (s, 1H, NH), 5·76 (d, 1H, J = 18·0 Hz, Ind—CH_2—), 6·52 (t, 2H, J = 7·0 Hz, —CH_2—N—), 6·80 (d, 1H, J = 18 Hz, Ind—CH_2), 7·1 (m, 1H, —CH—CH_2—), 7·6–8·4 (m, 6H), 8·48 (s, 6H, —$C(CH_3)_2$); mass spectrum, M, 349·1790 ($C_{21}H_{23}N_3O_2$ requires 349·1790), *m/e* (rel. intensity) 349(60), 334(100), 306(11), 305(6), 289(21), 261(8), 208(12·5), 194(12), 180(9), 148(16).

Dideuterobrevianamide-A. Methanol-d was prepared by the method of Streitweiser *et al.*[20] with a D content of greater than 99%.

Brevianamide-A (1 mg) was dissolved in methanol-d, the solvent evaporated and the process repeated. The mass spectrum was run after 1 hr; *m/e* (rel intensity) 368(26), 367(100), 366(34), 299(25), 298(79), 297(45), 280(5), 279(5), 270(14),175(7), 167(7), 166(13), 153(8), 149(8), 148(8), 147(7), 139(8), 134(10), 133(6). Calculation shows that the spectrum obtained was that of a mixture of 73% dideutero- and 27% monodeuterobrevianamide-A.

Biological tracer experiments. After growth of 17 days, the radioactive precursor (10 μC), in aqueous soln, was distributed among two penicillin flasks (750 ml) containing Czapek-Dox soln. After 10 days, brevianamide-A was isolated as described above and crystallized to constant activity without dilution. Counting was carried out on the chloroform solvate as "infinitely thick" samples. For incorporations see Table 1.

2,2-Tetramethylene-ψ-indoxyl (1). This compound was synthesized by the method of Witkop and Patrick[4c] (catalytic oxidation) from tetrahydrocarbazole, and crystallized from light petroleum (b.p. 40–60°); m.p. 79°; τ($CDCl_3$) 2·3–3·4 (m, 4H, aromatic protons), 8·05–8·4 (m, 8H, methylene), 5·0 (s, 1H, NH); mass spectrum M, 187 ($C_{12}H_{13}NO$ requires: 187). Borohydride reduction as for deoxybrevianamide-A gave a quantitative yield of tetrahydrocarbazole.

Catalytic reduction of brevianamide-A. Brevianamide-A (3 mg) was dissolved in MeOH (2 ml) and shaken with PtO_2 (1–2 mg) in an atmosphere of H_2 at room temp. The solution rapidly decolourized. After 10 min the soln was filtered and the filtrate reduced to dryness to leave a non-crystalline residue; λ_{max} 229, 328 mμ; mass spectrum M, 369 ($C_{21}H_{27}N_3O_3$ requires: 369).

Brevianamide-A (4 mg) was dissolved in glacial AcOH and shaken with PtO_2 and H_2 as above, for 18 hr. The soln was filtered and reduced to dryness; mass spectrum *m/e* 373, 371, 369 in the intensity ratio 60:100:60, corresponding to the uptake of four, three and two molecules of H_2.

Catalytic reduction of deoxybrevianamide-A. Deoxybrevianamide-A (2 mg) was shaken in glacial AcOH (1·5 ml) with PtO_2 (1–2 mg) for 18 hr at room temp. The product had only end-absorption in its UV spectrum; its mass spectrum showed: M, 357 ($C_{21}H_{31}N_3O_2$ requires 357), *m/e* (rel. intensity) 357(30), 356(40), 355(25), 342(50), 314(80), 313(81), 166(98), 164(100).

N-Nitrosobrevianamide-A. Brevianamide-A (2 mg) was dissolved in glacial AcOH (0·2 ml) and $NaNO_2$ (5 mg) was added. The soln was left for 14 hr at room temp, the solvent removed *in vacuo*, water added, and the mixture extracted with $CHCl_3$. The dried $CHCl_3$ extracts were evaporated to leave a light yellow non-fluorescent residue; λ_{max} 206, 237, 252, 290, 330 mμ. The mass spectrum did not show a molecular ion. N-Nitroso-2,2-tetramethylene-ψ-indoxyl was prepared by the method of Witkop;[4a] λ_{max} 205, 235, 248, 290, 330 mμ; mass spectrum: M, 216 ($C_{12}H_{12}N_2O_2$ requires: 216).

Dibromobrevianamide-A. Brevianamide-A (5 mg) was dissolved in chloroform and a dilute solution of bromine added slowly until a permanent colour of bromine remained. The reaction was followed by TLC leading to the conclusion that one compound (presumably the monobromo derivative) was being formed initially and was then converted into another as the reaction neared completion. Evaporation of the solvent and the excess of Br_2 gave a yellow, non-fluorescent residue which crystallised from $CHCl_3$–light petroleum (b.p. 40–60°); m.p. 165–180° (loss of solvent of crystallization); λ_{max} 243 ($E_1^{0.01}$ = 7·5), 263 ($E_1^{0.01}$ = 1·65), 420 mμ ($E_1^{0.01}$ = 0·73); mass spectrum: M, (triplet characteristic of dibromo-compounds) 525, 523, 521 ($C_{21}H_{21}N_3O_3Br_2$ requires 525, 523, 521), *m/e* (rel intensity) M(100), 479(6), 454(82, t), 426(15, t), 220(26), 165(94), 152(43), 149(31), 138(25), 96(20).

Bromination of 1. Tetramethylene-ψ-indoxyl (25 mg) in $CHCl_3$ was treated with Br_2 as above. The product, *dibromotetramethylene-ψ-indoxyl* crystallised from $CHCl_3$–petroleum ether (b.p. 40–60°), m.p. 139–140°; (Found: C, 41·4; H, 3·6. $C_{12}H_{11}NOBr_2$ requires: C, 41·8; H, 3·2%); λ_{max} 243, 262, 418 mμ (ε 28,000, 7,700, 4,100).

Monomethylbrevianamide-A. Brevianamide-A (3 mg) was dissolved in 50% aqueous MeOH (1 ml); NaOH (8 mg) and Me_2SO_4 (25 mg) were added and the whole stirred at room temp for 3 hr. The reaction mixture was worked up in usual way and the products were submitted to preparative TLC (5% MeOH in $CHCl_3$). A yellow fluorescent product was isolated in very small yield which was homogeneous by TLC; mass spectrum: M, 379 ($C_{22}H_{25}N_3O_3$ requires: 379), *m/e* (rel intensity) 379(100), 321(6), 310(60), 282(6), 234(4), 181(11), 179(11), 174(5), 166(25), 138(6), 133(7).

Dimethylbrevianamide-A. Brevianamide-A (1·2 mg) was heated under reflux in dry ether with an excess of NaH and MeI for 3 hr and left at room temp for 10 hr. The mixture obtained by evaporation of the solvent was submitted to preparative TLC (5% MeOH in $CHCl_3$). A yellow product was isolated; mass spectrum: M, 393 ($C_{23}H_{27}N_3O_3$ requires: 393).

Attempted base-catalysed deuteration of brevianamide-A. Brevianamide-A (1·5 mg) was dissolved in methanol-d (1 ml) in which a piece of dry Na (2–3 mg) had been dissolved. The soln was left at room temp in a dry-box under N_2 for 20 hr. The solvent was removed *in vacuo* and D_2O (2 ml) added. This was extracted with ether and the ether layer washed twice with more D_2O and once with water, dried and evaporated to dryness. The mass spectrum of the residue (homogeneous by TLC) was identical with that of the natural brevianamide-A.

Proline anhydride. This compound was prepared by a standard method[21] and crystallized from $CHCl_3$–light petroleum (b.p. 40–60°); m.p. 178–180°; τ(d⁵pyridine) 5·88 (t, 2H, J = 8 Hz, —CH_2—C<u>H</u>—(CO)N—), 6·6 (m, 4H, —CH_2—N—), 7·9 (m, 4H, —C<u>H</u>$_2$—CH(CO)N—), 8·3 (m, 4H); mass spectrum: M, 194 ($C_{10}H_{14}N_2O_2$ requires: 194).

Deuteration of proline anhydride. The same procedure was used as for the attempted deuteration of brevianamide-A (base-catalysed), except that $CHCl_3$ was used in the extraction in place of ether; mass spectrum: M, 196 ($C_{10}H_{12}N_2O_2D_2$ requires: 196). Monodeuteroproline anhydride was also present (8%).

Alkaline hydrolysis of brevianamide-A. Brevianamide-A (15 mg) was heated on a steam-bath in 10% NaOHaq (1 ml) under N_2 for 1 hr. The soln was allowed to cool, neutralized with 2N HCl and the soln passed through a Dowex 50 ion exchange column (5 gm). After washing the column with water until the washings were neutral, the yellow material remaining on the column was eluted with dilute NH_4OH (10%). A trace of starting material was present by TLC, together with a yellow, highly polar compound.

Part of this product (6 mg) was treated with ethereal diazomethane. The products of this reaction were submitted to preparative TLC (5% MeOH in $CHCl_3$) and two bands were eluted. The major product of the reaction was starting material, brevianamide-A. The second was produced in poor yield and was not investigated further; mass spectrum *m/e* (rel intensity) 411(16), 382(70), 265(12), 355(8), 354(9), 282(17), 267(8), 151(2), 146(10).

Another portion of the hydrolysis product (0·5 mg) was treated with dry trifluoroacetic acid (0·1 ml) and trifluoroacetic anhydride (0·05 ml) at 0°. The soln was left to return to room temp after 15 min and the excess of trifluoroacetic anhydride and trifluoroacetic acid were removed *in vacuo*. The residue was submitted to preparative TLC as above to give two yellow products. The major product was again identical with brevianamide-A (by mass spectrometry). A more polar product was a trifluoroacetyl derivative of brevianamide-A, presumably formed by the dehydration (with cyclisation) of the acylated open chain dipeptide, mass spectrum: M, 461 ($C_{23}H_{22}N_3O_4F_3$ requires: 461).

Ozonolysis of tetrahydrocarbazole. Tetrahydrocarbazole (100 mg) was ozonised in MeOH at −78° for 10 min and allowed to return to room temp. H_2O_2 (1 ml; 100 vol) was added with formic acid (4 ml) and the soln stirred for 2 hr. Water was added and the whole evaporated to dryness. The residue was methylated with diazomethane and the products chromatographed by GLC (BDS; 120°). Adipic acid dimethyl ester, 85% of the volatile products, was identified by comparison with an authentic sample.

Ozonolysis of deoxybrevianamide-A. Deoxybrevianamide-A (8 mg) was ozonized by the same procedure as for tetrahydrocarbazole. The methylation products submitted to preparative TLC (5% MeOH in $CHCl_3$), and a band R_f 0·7) eluted to give a colourless residue (3·5 mg). This was shown to be a mixture by mass spectrometry, of which the major component had the composition expected from cleavage of the pyrrolic double bond; mass spectrum M, 352·1643 ($C_{17}H_{24}N_2O_6$ requires: 352·1634).

Diborane reduction of deoxybrevianamide-A. A soln of diborane in THF (approx molar) was prepared by the method of Brown and Zweifel.[22]

Deoxybrevianamide-A (8 mg) was dissolved in the diborane soln (4 ml) and heated under reflux for 24 hr in a dry-box under N_2. The solvent was removed *in vacuo* and the residue treated with N HCl (4 ml) and extracted with $CHCl_3$. The dried extract was evaporated to leave an oily residue (ca. 1 mg) which was a mixture and was discarded. The aqueous soln was basified with 2N NaOH and extracted with $CHCl_3$. The dried extract was evaporated to leave a residue (5·5 mg) which was submitted to preparative TLC (5% MeOH in $CHCl_3$). Several bands were present. The major one (R_f 0·75) was eluted to give a product homogeneous by TLC; λ_{max} 225, 275 (sh), 282, 290 mμ; mass spectrum: M, 321·2203 ($C_{21}H_{27}N_3$ requires: 321·2205), *m/e* (rel intensity) 321(32), 306(18), 291(17), 277(5), 275(5), 249(5), 194(13), 150(14), 138(12), 97(100).

Brevianamide-E. Brevianamide-E (40 mg) was obtained as a glassy solid after TLC in ether (R_f 0·6). It gave a red colour reaction with 1% ceric sulphate in 2N H_2SO_4. It was unstable to light whilst on a TLC plate and was unstable to base, giving a mixture of compounds, some of which possessed the ψ-indoxyl chromophore. It had $[\alpha]_D^{25} = -30°$ (EtOH); λ_{max} 239, 296 mμ (ε 7,500, 2,050); ν_{max} ($CHCl_3$) 3600, 3370, 1690, 1680 cm^{-1}; τ($CDCl_3$) 2·7–3·32 (m, 4H, aromatic protons), 3·7 (s, 1H, NH), 3·6 (X part of ABX system, 1H, J_{AX} = 18·5 Hz, J_{BX} = 10·5 Hz, —CH=C<u>H</u>$_2$), 4·9 (A part of ABX system, 1H, J_{AX} = 18·5 Hz, J_{AB} = 1·5 Hz, CHC<u>H</u>$_2$), 4·96 (B part of ABX system), 1H, J_{BX} = 10·5 Hz, —CHC<u>H</u>$_2$), 6·06–6·4 (m, 2H, —CH(CO)N—), 6·48 (t, 2H, J = 7 Hz, —CH_2—N—), 7·14 (A part of ABX system, 1H, J_{AB} = 13·0 Hz, J_{AX} = 11·0 Hz, Indol—C<u>H</u>$_2$—), 7·39 (B part of ABX system, 1H, J_{BX} = 8·0 Hz, J_{AB} = 13·0 Hz, Indol—C<u>H</u>$_2$—), 7·5 (s, 1H, OH), 7·7–8·2 (m, 4H, —CH—C<u>H</u>$_2$—C<u>H</u>$_2$—C<u>H</u>$_2$—N—), 8·73 (s, 6H, —$C(CH_3)_2$); M, 367·1899 ($C_{21}H_{25}N_3O_3$ requires: 367·1896).

Hydrolysis of brevianamide-E. Brevianamide-E (0·54 mg) was heated in 6N HCl (0·5 ml) at 110° for 20 hr

in a sealed tube. The soln was reduced to dryness *in vacuo* and automatic amino-acid analysis revealed the presence in the hydrolysate of approximately one molar equiv of proline. There were no other natural amino acids present.

Dihydrobrevianamide-E. Brevianamide-E (10 mg) was shaken with PtO_2 (3 mg) in EtOH (2 ml) in an atmosphere of H_2 for 15 min at room temp. The soln was filtered and the solvent was evaporated to leave an oil, homogeneous by TLC (ether); λ_{max} 241, 297 mμ; ν_{max} (CCl_4) 3600, 3370, 1690, 1675 cm^{-1}; τ($CDCl_3$) 2·7–3·35 (m, 4H, aromatic protons), 3·7 (s, 1H, NH), 6·18–6·45 (m, 2H, —CH(CO)N—), 6·5 (ill-defined triplet, 2H, —CH_2—N—), 7·04 (A part of ABX system, 1H, J_{AB} = 12·5 Hz, J_{AX} = 10·5 Hz, Indol—C<u>H</u>$_2$—), 7·42 (B part of ABX system, 1H, J_{AB} = 12·5 Ha, J_{BX} = 8·0 Hz, Indol—C<u>H</u>$_2$—), 7·8 (s, 1H, OH), 7·8–8·2 (m, 4H, —CH—C<u>H</u>$_2$—C<u>H</u>$_2$—CH_2—N—), 8·3 (m, 2H, —C<u>H</u>$_2$—CH_3), 8·88 (s, 3H, C—CH_3), 8·92 (s, 3H, C—CH_3), 9·14 (t, 3H, J = 7·5 Hz, —CH_2—C<u>H</u>$_3$); mass spectrum: M, 369 ($C_{21}H_{27}N_3O_3$ requires: 369), *m/e* (rel. intensity) 369(18), 298(100), 281(4), 217(7), 216(32), 200(28), 174(31), 173(21), 154(54), 146(39).

Attempted acetylation of dihydrobrevianamide-E. Dihydrobrevianamide-E (3 mg) was treated with pyridine and Ac_2O at room temp for 12 hr. No reaction had occurred according to TLC examination. The solvent was removed *in vacuo* and the residue treated with Ac_2O at 85° for 1 hr. Starting material (2 mg) was recovered by preparative TLC.

Deoxydihydrobrevianamide-E. Dihydrobrevianamide-E (1·1 mg) was heated on a steam-bath in glacial AcOH (0·2 ml) and Zn dust (110 mg) for 5 min. The zinc was filtered from the soln and washed with EtOH. The filtrate and washings were reduced to dryness to leave an oil. This by TLC (ether), was a mixture of a small proportion of starting material with a single product; λ_{max} 228, 279, 292 mμ; mass spectrum: M, 353 ($C_{21}H_{27}N_3O_2$ requires: 353), *m/e* (rel intensity) 200(100).

Deoxybrevianamide-E. Brevianamide-E (10 mg) was heated on a steam-bath in glacial AcOH (0·3 ml) and Zn dust (20 mg) for 10 min. The Zn was filtered from the soln and washed with EtOH. The filtrate and washings were reduced to dryness to leave an oil which was submitted to preparative TLC (ether). Starting material (3 mg) was recovered. Deoxybrevianamide-E, a solid (5·5 mg), was also obtained (homogeneous by TLC); λ_{max} 227, 279, 291·5 mμ; τ($CDCl_3$) 1·88 (s, 1H, indole NH), 3·8 (X part of ABX system, 1H, C<u>H</u>CH_2), 4·84 (AB part of ABX system, 2H, —CHC<u>H</u>$_2$), 4·34 (s, 1H, amide NH), 5·58 (broad doublet, 1H, J = 11·6 Hz, Indol—C<u>H</u>—CH—), 6·26 (A part of ABX system, 1H, J_{AB} = 15·0 Hz, J_{AX} = 11·6 Hz, Indol—C<u>H</u>$_2$—CH—), 6·8 (B part of ABX system, 1H, J_{AB} = 15·0 Hz, J_{BX} = 4·0 Hz, Indol—C<u>H</u>$_2$—CH), 8·6–9·15 (m, 4H, —CH—C<u>H</u>$_2$—C<u>H</u>$_2$—CH—N—), 6·36 (ill-defined triplet, 2H, —CH_2—N—), 8·46 (s, 6H, C(CH_3)$_2$); mass spectrum: M, 351 ($C_{21}H_{25}N_3O_2$ requires 351), *m/e* (rel. intensity) 351(9), 198(100), 183(12), 182(11), 168(7), 167(6).

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