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THE STRUCTURES OF FIVE DIKETOPIPERAZINES FROM *ASPERGILLUS USTUS*

P. S. STEYN
National Chemical Research Laboratory, C.S.I.R., Pretoria, Republic of South Africa.

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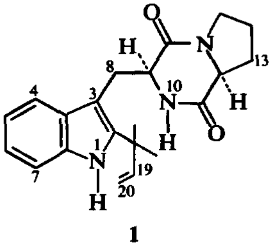
Abstract—Evidence is presented which confirms the structures of the five metabolites as shown in 1–5. The absolute configuration of compounds 1 and 2 was established; for compound 5 the absolute configuration was established at position 12 only.

Strains of *Aspergillus ustus* (Bainier) Thom and Church were isolated from stored foodstuffs in the course of a continuing search for toxigenic fungi. Maize meal cultures of *A. ustus* C.S.I.R. 1128 were found to cause acute toxicosis in day-old ducklings. The toxic principles were quantitatively extracted from the mouldy maize meal and systematic fractionation, guided by bio-assay led to the isolation of austamide as one of the active components. The structural elucidation of austamide (4) and of prolyl-2-(1',1'-dimethylallyl)tryptophyldiketopiperacine (1)* has been briefly reported.[1] The detailed structural and stereochemical studies as well as the structures of three new metabolites, *viz* 12,13-dihydroaustamide (5), 12,13-dehydroprolyl-2-(1',1'-dimethylallyl)-tryptophyldiketopiperazine (2) and of 10,20-dehydro[12,13-dehydroprolyl-2-1',1'-dimethylallyl)tryptophyldiketopiperazine] (3) are described herein.

A. THE STRUCTURES OF THE THREE INDOLE ALKALOIDS

1. *Prolyl*-2-(1',1'-*dimethylallyl*)*tryptophyldiketopiperazine* (1).

The indoles (1–3) gave a negative Ehrlich colour reaction indicating substitution in the positions 2 and 3 relative to the indole N—H[3]. The diketopeperazine (1) $C_{21}H_{25}N_3O_2$ was the major constituent and showed a typical indole UV absorption λ_{max}^{EtOH} 225, 275 (sh), 283 and 291 nm (log ε 4·51, 3·85, 3·91 and 3·85, respectively). Its IR spectrum exhibited characteristic N—H absorption at 3480, 3460 and 3365 cm^{-1}, while CO absorption at 1685



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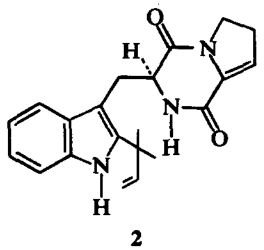

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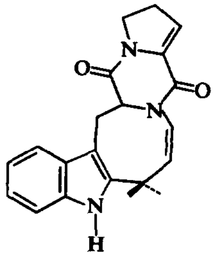

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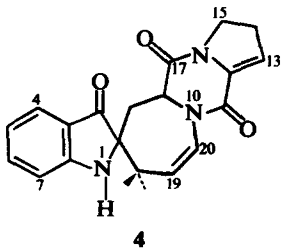

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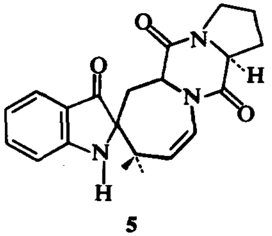

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*The trivial name desoxybrevianamide E proposed by Birch[2] for compound (1) has been discarded in favour of a systematic name, as 1 does not involve the mere loss of one O atom from brevianamide E. The numbering system of the indole and ψ-indoxyl alkaloids described in this paper are shown in formulae 1 and 4.

(weak sh) and 1670 m^{-1} together with the absence of the amide 2 band clearly supported the presence of the diketopiperazine system.

The NMR spectrum possessed signals which were interpreted as follows. Two exchangeable singlets at τ 1·25 and τ 4·28 were assigned to the NH protons. A multiplet at τ 2·48–3·05 was attributed to the four neighbouring aromatic protons. A 6-proton singlet at τ 8·50 was due to the *gem*-dimethyl group while the three exocyclic olefinic protons appeared as an AA'X system at τ 3·90 (1H, X part, J_{AX} 18·2, $J_{A'X}$ 9 Hz, C_{19}—H) and τ 4·92 (2H, AA' part of AA'X system, J_{AX} 18·2, $J_{A'X}$ 9 Hz, $\overset{H}{\diagdown}C{=}C\underline{H}_2$). The protons at C_8 and C_9 resonated as an ABX system at τ 5·56 [1H, X part of ABX system, J_{AX} 4, J_{BX} 11 Hz, C_9—$\underline{H}$), 6·25 and 6·83 [2H, AB part of an ABX system, H_A (τ 6·25) J_{AB} 15·5, J_{AX} 4 Hz and H_B (τ 6·83) J_{AB} 15·5, J_{BX} 11 Hz, 8 $C\underline{H}_2$]. The triplet at τ 5·95 (J 7 Hz), broadened by the nitrogen quadrupole moment was assigned to the methine proton at position 12. The protons adjacent to the proline nitrogen at position 15 resonated as an ill-defined triplet at τ 6·34, while the other four protons comprising the proline ring

$$[-\overset{|}{C}H-C\underline{H}_2-C\underline{H}_2-CH_2-\overset{|}{N}-]$$

gave rise to an unstructured multiplet between τ 7·6 and 8·2.

In the mass spectrum of 1 only one prominent fragment appeared, namely the base peak at *m/e* 198, $C_{14}H_{16}N$ which originated via cleavage of the 8,9-bond.

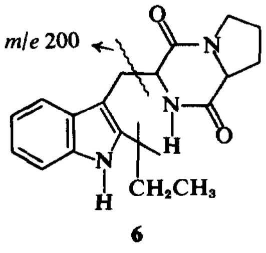
Collateral support for the structure of **1** was provided by hydrogenation which gave prolyl-2-(1',1'-dimethylpropyl)tryptophyldiketopiperazine (**6**). Its mass spectrum showed a molecular ion at *m/e* 353, $C_{21}H_{27}N_3O_2$ and the base peak at *m/e* 200, $C_{14}H_{18}N$ confirming that the indole fragment contained the Et group. The NMR spectrum showed the absence of olefinic absorption, but instead, resonance due to the methylene protons at τ 8·29 (2H, q, J 7Hz) and the Me group at τ 9·27 (3H, t, J 7Hz).

A Kuhn–Roth oxidation of **6** with concomitant distillation of the volatile acids gave 2,2-dimethylbutyric acid in 50% yield, unambiguously establishing the reverse linkage of the isoprenoid unit to an sp^2 hybridized carbon atom as in echinulin (**7**)[4] and neo-echinulin (**8**)[5]. Birch postulated[6] that this unusual orientation could arise by preliminary normal alkylation of the indole nitrogen and a subsequent cyclic rearrangement. Casnati and Pochini[7] recently supported this proposal by an acid-catalyzed rearrangement of 1-γγ-dimethylallyl-3-methylindole.

Acid hydrolysis of **6** gave proline, identified by an automatic amino acid analyzer and had M^+ 115.

Prolyl-2-(1',1'-dimethylallyl)tryptophyldiketopiperazine (**1**) is clearly identical to the compound obtained by Birch[2] upon treatment of brevianamide E (**9**) with Zn and AcOH.

The absolute configuration and conformation of prolyl-2-(1',1'-dimethylallyl)tryptophyldiketopiperazine (**1**). Our chemical studies established that compound (**1**) was a dipeptide incorporating proline and 2-(1',1'-dimethylallyl)tryptophan. Several



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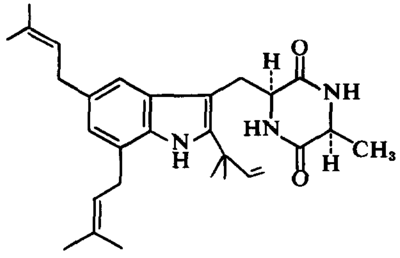

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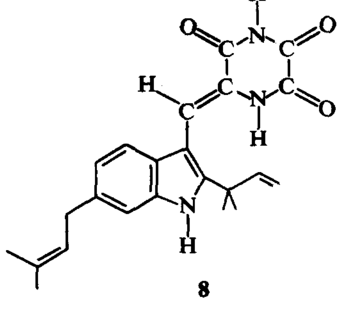

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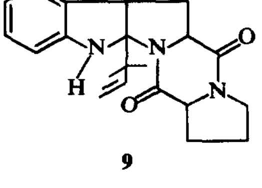

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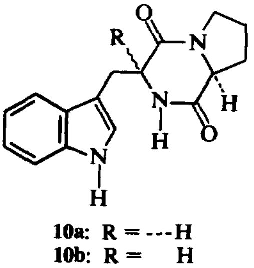
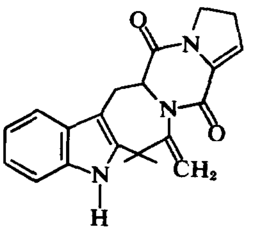
studies[8] led to the conclusion that the related fungal diketopiperazine echinulin (7) had the LL-configuration.

Acid hydrolysis of **6** destroyed the tryptophan moiety and gave proline. The proline was purified by high voltage electrophoresis at pH 1·9 and its ORD spectrum showed a peak at 224 nm, $[\theta]^{0\cdot 1\mathrm{N\,HCl}}$ +225°. L-proline is known to have a positive Cotton effect at the first extremum.[9] Compound **1** therefore has the 12*S*-configuration.

In order to establish the absolute configuration at position 9, two model diastereoisomers were prepared, *viz* L-tryptophyl-L-prolyldiketopiperazine (LTLPDKP)(**10a**) and D-tryptophyl-L-prolyldiketopiperazine (DTLPDKP) (**10b**). Treatment of L-tryptophan methylester hydrochloride with carbobenzoxy-L-prolyl-*p*-nitrophenylester in aqueous dioxane and triethylamine gave the dipeptide L-tryptophan-N-(1-carbobenzoxy-L-prolyl)methylester in excellent yield. Removal of the protecting group was achieved by hydrogenation over Pd/C in ethanol yielding the free amine which was converted into the formate salt and cyclized by azeotropic distillation in a solution of sec-butylalcohol: toluene (4:1)[10] to furnish LTLPDKP (**10a**), m.p. 174°. DTLPDKP (**10b**) was similarly prepared by employing D-tryptophan methylester hydrochloride as a starting material.



10a: R = ---H
10b: R = H



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From a comparison of the CD spectra (Fig 1) of LTLPDKP (**10a**) and DTLPDKP (**10b**) with that of **6** it was evident that **6** and therefore **I** was stereochemically related to L-tryptophan and L-proline. Compounds **10a**, **10b** and **6** showed similar CD characteristics above 250 nm, but the main difference being at lower wave-length where DTLPDKP (**10b**) showed a prominent negative Cotton effect $\Delta\epsilon$ 237 nm = −3·7. The latter absorption was absent in the spectra of **10a** and **6**.

The configuration of **1** at position 9 was also studied by Westley's method[11] which involves epiermization of diketopiperazines from several amino acids and an investigation of the products by TLC. Westley *et al.*[11] observed from fourteen pairs of diketopiperazine diastereoisomers studied that the compounds which contained an LL-isomer (*cis*) had a lower R_f value than the LD-isomer (*trans*) except for the diketopiperazine of proline and leucine. The diketopiperazines containing a proline unit are exceptional in being able to assume a stable boat conformation[12] for the diketopiperazine ring compared to the planar ring[13] of other diketopiperazines. Epimerization of LTLPDKP (**10a**) in boiling ethanol and triethylamine gave an equilibrium mixture of LTLPDKP (**10a**) and DTLPDKP (**10b**) as established by TLC; DTLPDKP (**10b**) had a lower R_f value than (**10a**). Epimerization therefore occurred at position 9. A similar epimerization of compound (**1**) also gave an epimer of lower R_f value. The results are confirmatory for an LL-configuration for prolyl-2-(1′,1′-dimethylallyl)tryptophyl-diketopiperazine (**1**) and for the finding of Westley[11] on the exceptional behaviour of cycloprolyl-leucine.

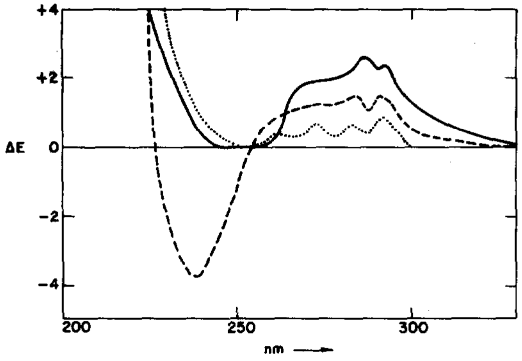


Fig 1. The CD spectra of compound **6** (———), LTLPDKP **10a** (·········) and DTLPDKP **10b** (--------).

In conclusion, inspection of Dreiding models of **1**, **10a** and **10b** indicated a boat conformation for the diketopiperazine ring and a half-chair conformation for the proline ring, these findings are in agreement with the NMR data obtained by Siemion.[12] The molecular models furthermore indicated a conformation for **1** and **10a** in which the indole ring is swung away from the diketopiperazine. However, compound **10b** can either be in the folded conformation in which the indole ring faces the diketopiperazine ring or in a conformation where the indole ring lies away from the diketopiperazine. From a NMR study of the conformation of cyclic peptides which contain an aromatic side chain, with the diketopiperazine ring constrained to be planar, Kopple and Marr[14] concluded that the folded conformation is preferred.

Table 1 reports the positions of the protons comprising the diketopiperazine derivatives excluding those of aromatic protons. Any differences observed in the chemical shift of protons, *e.g.* at position 12 can not be attributed to the anisotropy of the carbonyl groups, but to the contribution of the anisotropy of the carbonyl groups, but to the contribution of the anisotropy of the aromatic part of the diketopiperazine. Therefore, chemical shift differences can be used as a probe to evaluate these predicted conformations.

Table 1. NMR data of some diketopiperazine derivatives.

Compound	Chemical shift (τ) and multiplicity (Hz)					
	H_8eq.	H_8ax	H_9	H_{12}	H_{13}—H_{14}(4H)	H_{15}
1	6·25(q) (J = 4, 15)	6·83(q) (J = 11, 15)	5·56(q) (J = 4, 11)	5·95(t) J = 7	7·60–8·20(m)	6·34(m)
10a	6·38(q) (J = 4, 15)	7·06(q) (J = 10, 15)	5·76(q) (J = 4, 10)	6·11(q) (J = 6, 6)	7·6–8·6	*ca* 6·55
10b	6·93(q) (J = 4, 14)	6·74(q) (J = 6, 14)	5·85(q) (J = 4, 6)	7·34(q) (J = 7, 10)	7·9–8·9	*ca* 6·8

From Table 1 it was clear that the protons of the diketopiperazine ring in **1** and **10a** have similar chemical shifts. Small differences could be due to the bulky isoprenoid unit in **1** which could interfere with free rotation around the C_3—C_8 and C_8—C_9 bonds. The protons at C_8 and C_9 for **1** and **10a** have the same semi-eclipsed conformation (J = 4, 10 Hz, requiring dihedral angles of close to 60° and 180°).[15] A comparison of data obtained for **10a** and **10b** revealed that the proton at position 12 was shifted upfield by 1·23 ppm in **10b**. In the predicted folded conformation of **10b** H_{12} will be close to the mid-point of the bond common to the 6- and 5-membered rings and therefore experience a strong upfield shift. The other proline protons experienced a weaker upfield shift (*ca* 0·3 ppm). A folded conformation for **10b** would require the protons at C_8 and C_9 to assume a staggered conformation which is in agreement with the coupling constants of 4 and 6 Hz, representing dihedral angles of close to 60°, as observed for the constituent protons. The degree of shielding by the indole ring will depend on the contribution of folded conformation in **10b**, which clearly makes an important contribution to the total rotamer population. Similar shielding effects were recently observed by Houghton and Saxton[8a] and Kishi *et al.*[16]

The proposed conformations are illustrated in Fig 2.

2. 12,13-*Dehydroprolyl*-2-(1′,1′-*dimethylallyltryptophyl*)*diketopiperazine* (**2**)

Compound **2** had a molecular constitution of $C_{21}H_{23}N_3O_2$, showed a typical indole UV absorption and its IR spectrum was similar to that of **1** except for an additional sharp band at 1648 cm^{-1}, attributable to the enamide moiety. Its mass spectrum exhibited a base peak at *m/e* 198, $C_{14}H_{16}N$ indicating unsaturation in the proline part of the molecule.

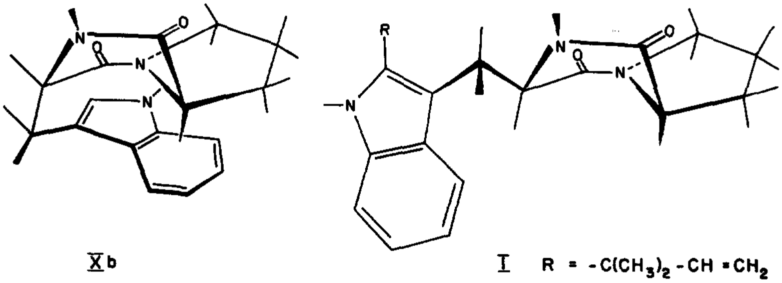
The NMR spectra of **1** and **2** have many features in common, the most striking difference being due to protons of the proline ring. These protons appeared as an A_2M_2X pattern, represented by a triplet at τ 6·0 (2H, J 9Hz, 15 $C\underline{H}_2$), a sextet at τ 7·31 (2H, J 3, 9, 9 Hz, 14 $C\underline{H}_2$) and a triplet at τ 3·94 (1H, J 3Hz, 13 $C\underline{H}$).

Hydrogenation of **2** gave only one stereoisomer, identical in every respect (NMR, CD, IR, mass spectra and TLC) to **6**. Compound **2** therefore had the 9*S*-configuration.

3. 10,20-*Dehydro*[12,13-*dehydroprolyl*]-2-(1′,1′-*dimethylallyltryptophyl*)*diketopiperazine* (**3**)

This biogenetically important metabolite $C_{21}H_{21}N_3O_2$ (**3**) was elaborated by *A. ustus* irregularly and in poor yield. It showed UV and IR characteristics virtually identical to those of **2**, differing in the NH region where only two peaks occurred [3485 cm^{-1} (sharp peak) and 3350 cm^{-1} (weak broad band)] assigned to the indole NH-group. Mass spectroscopy established the presence of only one exchangeable proton compared to the two exchangeable protons in **1** and **2**.

The NMR spectrum showed the presence of an indole NH-proton τ 1·70(s) and four contiguous aromatic protons τ 2·4–3·1(m). The two 3-proton singlets at τ 8·39 and τ 8·66 were assigned to the



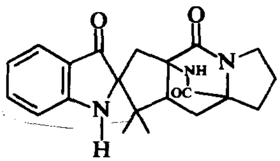
$\underline{X}$b

$\underline{I}$ R = $-C(CH_3)_2-CH=CH_2$

$\underline{X}$a R = H

Fig 2. Proposed conformations of the aromatic side chain in compounds **1**, **10a** and **10b**.

gem-dimethyl group and the 2-proton singlet at τ 4·26 to the olefinic protons at positions 19 and 20. The three protons at C_8 and C_9 appeared as an ABX pattern as quartets *viz* H_X at τ 5·75 (J 1, 6 Hz, C_9—H); H_A at τ 6·40 (J 1, 15 Hz, C_8—$H_{eq.}$) and H_B at τ 6·54 (J 6, 15 Hz, C_8—$H_{ax.}$). The small coupling constant (1 Hz) between C_9—H and C_8—$H_{eq.}$ is consonant with a dihedral angle of close to 90°, while J_{BX} 6 Hz is consistent with a dihedral angle of close to 135°; the dihedral values are in agreement with those obtained from an inspection of a Dreiding model of **3**. A two-proton triplet at τ 6·42 was assigned to the protons at C_{15}; a 2-proton sextet at τ 8·17 (J 3, 10, 10 Hz) was assigned to the methylene protons at C_{14} while the olefinic proton at C_{13} resonated as a triplet at τ 4·58 (J 3 Hz). Irradiation at the centre of this latter triplet led to the collapse of the sextet to a triplet at τ 8·17 (J 10 Hz).



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The data can be formulated as **3** or **11**. Formula **3** is regarded as correct on the following grounds. Addition of 0·6 mole equivalent of europium-111-tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octadione[17] to a solution of **3** in $CDCl_3$ led to a downfield shift of the two olefinic protons from τ 4·26 to form an AB system at τ 2·62 and τ 3·12 (J_{AB} 10 Hz). The observed J-value is identical to that observed for these protons in **4** and **5** (see below) and larger than *J-gem* across as sp^2 hybridized carbonatom for ethylene derivatives. Hydrogenation of **3** gave a tetrahydroderivative $C_{21}H_{25}N_3O_2$, whose NMR spectrum exhibited no olefinic absorption or a secondary Me group. Formula **3** is in agreement with biosynthetic considerations and can be regarded as the probable precursor of **4**. Therefore closure of the diketopiperazine on to the terpene unit occurs prior to the indole oxidation which furnishes the ψ-indoxyl moiety as in the austamides and in brevianamide A (**12**).

B. STRUCTURES OF THE TWO ψ-INDOXYL ALKALOIDS

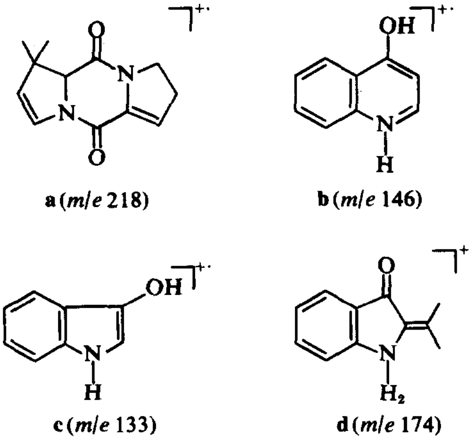
1. *Austamide* 4

The molecular composition $C_{21}H_{21}N_3O_3$ of **4** was established by high-resolution mass spectroscopy. The UV spectrum, *viz* λ_{max}^{EtOH} 234, 256, 268 (sh), 282 and 392 nm (log ϵ 4·42, 3·07, 3·04, 3·94 and 3·43, respectively) was unchanged upon the addition of acid or base. The absorption at 234, 256 and 392 nm is typical of the ψ-indoxyl chromophore which occurs in brevianamide A (**12**) and in other ψ-indoxyl alkaloids.[18] The long wave-length electron transfer band of **4** is shifted hypsochromically by 12 nm compared to that of **12** and most naturally occurring ψ-indoxyls. This shift is apparently due to the linking of the ψ-indoxyl moiety of **4** to a less strained 7-membered ring, in contrast to the strained 5-membered C-ring in **12** and other ψ-indoxyls. The absorption at 268 and 282 nm is assigned to the cyclic enamide chromophore since it is absent in the tetrahydroaustamide (**14a** and **b**).

The IR spectrum of **4** contained bands assignable to an NH group (3420 cm^{-1}), a ψ-indoxyl CO group (1700 cm^{-1}), a diketopiperazine unit (strong absorption at 1680 cm^{-1} and the absence of the amide 2 band) an enamide group (1650 cm^{-1}) and the C_6H_5N—C≡ moiety (1622 cm^{-1}).[19] NMR and mass spectroscopy established the presence of only one exchangeable proton. Preparation of the N-nitrosoderivative of the tetrahydroaustamide (**14a**) established the secondary nature of the ψ-indoxyl nitrogen as well as the location of the exchangeable proton in the molecule.

An exceptionally revealing NMR spectrum of austamide had the following characteristic features. Two three-proton singlets at τ 8·50 and τ 9·20 were assigned to the geminal Me groups. An AB pattern at τ 5·20 and τ 3·34 (J_{AB} 10 Hz) was attributable to the *cis*-olefinic protons at positions 19 and 20, respectively. The protons at C_8 and C_9 appeared as an ABX system, H_A being $H_{8eq.}$, τ 7·04 (q, J_{AB} 14, J_{AX} 5 Hz), H_B being H_{8ax}, τ 8·19 (q, J_{AB} 14, J_{BX} 12 Hz) and the X-proton, H_9 represented by pair of doublets at τ 5·20 (J_{AX} 5, J_{BX} 12 Hz). The protons comprising the unsaturated proline part of the molecule resonated as an A_2M_2X system. The two double triplets centred at τ 6·04 (J 9 Hz) were assigned to the methylene protons adjacent to the nitrogen of the proline ring. The difference in chemical shift (3 Hz) between the two triplets is apparently due to the N atom acting as a chiral centre in this strained system leading to anisochronism of the geminal protons.[20] A distinct sextet at τ 7·24 (J 3·0, 9·0, 9·0 Hz) was assigned to the allylic methylene protons, while the protons at position 13 gave rise to a triplet at τ 3·76 (J 3·0 Hz). These assignments were confirmed by decoupling experiments. A broad signal at τ 4·74 was attributable to the exchangeable NH-group. The resonance between τ 2·4 to 3·4 was assigned to the four aromatic protons which were arranged in a 1,2,3,4-pattern. The NMR spectra of austamide (**4**) and of brevianamide A (**12**) both recorded in deuteropyridine revealed essentially identical splitting patterns in the aromatic region.

The most important mass spectral fragmentation of **4** originated from cleavage of the spiran ring[21] to lead to the alicyclic fragment *m/e* 218 $C_{12}H_{14}N_2O_2$ (60%) (a) which lost a Me group to give the base peak at *m/e* 203 $C_{11}H_{11}N_2O_2$. Minor fragments representing the aromatic part occurred at *m/e*


a (m/e 218) b (m/e 146) c (m/e 133) d (m/e 174)

Mass spectral fragments.

146 C_9H_8NO (8%) (b) and m/e 133 C_8H_7NO (5%) (c).

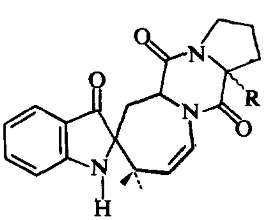
Catalytic hydrogenation of austamide led to the uptake of 1·2 mole equivalents of hydrogen within 12 min. Preparative TLC gave two pairs of crystalline diastereoisomers each pair in a ratio of 3 : 1. The dihydroderivatives $C_{21}H_{23}N_3O_3$ (**13a** and **b**) being the major products and the tetrahydroderivatives $C_{21}H_{25}N_3O_3$ (**14a** and **b**) the minor pair, prolonged hydrogenation (2 hr) gave the tetrahydroderivatives only. The impeded hydrogenation of the 19,20-double bond is due to steric interference of the neighbouring geminal Me groups, an observation substantiated by the resistance of dihydroaustamide (**13a**) to an oxidation with osmium tetroxide.

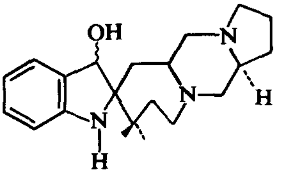
The two dihydroderivatives had virtually identical UV and IR spectra. The NMR spectra of **13a** and **13b** (Table 2) showed the absence of the olefinic triplet at τ 3·74 and the newly formed proton at C_{12} at τ 5·82 and concurrently the peaks comprising the proline part became more complex. The remaining olefinic protons were still clearly displayed, *e.g.* at τ 4·96 and τ 3·43 (J_{AB} 10 Hz) for **13a**. The NMR spectra of the two tetrahydroderivatives (**14a** and **14b**) showed the absence of the olefinic absorption but additional resonance due to the methylene protons at C_{19} and C_{20} (Table 2).

The mass spectra of these hydrogenated compounds showed a conspicuous peak at m/e 70 C_4H_8N from the proline ring and major fragments representing the alicyclic part of the molecule at m/e 220 $C_{12}H_{16}N_2O_2$ (corresponding to fragment a) for **13a** and **13b** and at m/e 222 $C_{12}H_{18}N_2O_2$ (corresponding to fragment a) for **14a** and **14b**. The fragment at m/e 174 $C_{11}H_{12}NO$ (d) from **14a** and **14b** is important in confirming the reverse linking of the isoprene side chain to the ψ-indoxyl moiety.

Reduction of the major tetrahydroderivative **14a** with LAH yielded a pair of diastereoisomeric hydroxyindolines (**15**). The major crystalline product $C_{21}H_{31}N_3O$ exhibited typical indoline UV absorption and lacked carbonyl absorption in its IR spectrum. The proton on the hydroxylbearing C atom gave rise to a singlet at τ 4·97. Compound (**15**) was converted with difficulty to the indole derivative upon treatment with hydrochloric acid, probably due to preferential protonation of the tertiary N atoms. The major indole was isolated by TLC on Al_2O_3. The formation of two products is possible. The C atom (C_{18}) bearing the geminal Me groups is known[19] to have a much greater migratory aptitude than the methylene C atom (C_8); the formation of compound (**16**) would therefore be favoured.

From NMR data it was not possible to assign the stereochemistry of the newly created chiral centre at position 12. The major tetrahydroderiva-


13a: R = ---H
13b: R = H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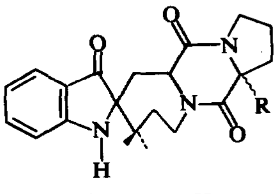
14a: R = ---H
14b: R = ◂H

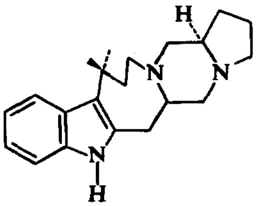
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Table 2. NMR data of austamide and of its hydrogenation products

	Chemical shift (τ) and multiplicity (Hz)										
Compound	$H_{8ax.}$	$H_{8eq.}$	H_9	H_{12}	H_{13}	H_{14}	H_{15}	H_{19}	H_{20}	Me	Me
4	8·19(q) $J = 12, 14$	7·04(q) $J = 5, 14$	5·20(q) $J = 5, 12$		5·76(t) $J = 3$	7·24(sextet) $J = 3, 9, 9$	6·04(t) $J = 9$	4·99(d) $J = 10$	3·34(d) $J = 10$	8·50(s)	9·20(s)
13a	7·87(q) $J = 9, 15$	7·22(q) $J = 5, 15$	5·38(q) $J = 5, 9$	5·82(q) $J = 8, 16$	7·60 TO 8·20		6·44(m)	4·96(d) $J = 10$	3·43(d) $J = 10$	8·85(s)	9·02(s)
13b	8·17(q) $J = 13, 14$	7·15(q) $J = 6, 14$	5·28(q) $J = 6, 13$	5·82(q) $J = 10, 7$	7·60 TO 8·20		6·44(m)	5·04(d) $J = 10$	3·34(d) $J = 10$	8·48(s)	9·22(s)
14a	7·36(q) $J = 9, 16$	7·70(q) $J = 2, 16$	5·33(q) $J = 2, 9$	5·88(t) $J = 8$	7·60 TO 8·05		6·54(q) $J = 6, 8$	8·50(q) $J = <1, 8, 14$ 7·6	5·65(q) $J = <1, 8, 14$ 6·80(q) $J = <1, 8, 14$	8·96(s)	9·16(s)
14b	8·02(q) $J = 10, 16$	7·66(q) $J = 4·2, 16$	5·27(q) $J = 4·2, 10$	5·86(t)	7·60 TO 8·05		6·50(m)	obscure	5·70 $J = 5, 6, 15$ 6·94 $J = 5, 6, 15$	8·93(s)	9·12(s)

tive (**14a**) was submitted to an acid hydrolysis which yielded approximately one mole equivalent of proline in the hydrolysate as determined by an automatic amino acid analyzer. The proline was separated preparatively by high voltage electrophoresis at pH 1·9. The ORD spectrum of the proline showed a peak ($[\theta]^{0\cdot 1N\,HCl}_{224\,nm}+480°$) establishing its *S*-configuration.[9] At position 12 compounds **13a** and **14a** have the *S*-configuration while **13b** and **14b** have the *R*-configuration.

2. 12,13-*Dihydroaustamide* (**5**)

A minor fluorescent metabolite was always associated with austamide in cultures of *A. ustus*. The compound (**5**) had identical physical properties (m.p.; UV, IR, NMR, CD and mass spectra) to compound **13a**, the major dihydroderivative obtained upon hydrogenation of austamide. Subsequent hydrogenation of each of these compounds gave the major tetrahydroderivative (**14a**) m.p. 246–247°.

12,13-Dihydroaustamide (**5**) therefore has the 12*S*-configuration. The chirality of its apparent precursor prolyl-1-(1′,1′-dimethylallyltryptophyl)diketopiperazine **1** was thus retained at position 12.

Proposed stereochemistry of austamide (**4**), *12,13-dehydroaustamide* (**5**) *and the hydrogenation products* (**13a**, **13b**, **14a** *and* **14b**). Our chemical studies established the structural features of austamide (**4**) and of 12,13-dihydroaustamide (**5**). Both compounds contain chiral centres at positions 2 and 9; in addition 12,13-dihydroaustamide (**5**) contains a chiral centre at position 12. The *S*-chirality at position 12 in **5** was established by correlation with 12*S*-tetrahydroaustamide (**14a**) see above. Compound **5** therefore retained at position 12 the same absolute configuration as its apparent precursor 9*S*, 12*S*-prolyl-2-(1′,1′-dimethylallyl)-tryptophyldiketopiperazine (**1**). The diketopiperazines **1** and **2** are most likely the biogenetic precursors of the ψ-indoxyls **5** and **4**, respectively; this hypothesis is strongly supported by their co-occurrence in cultures of *A. ustus*. From general biosynthetic considerations it is mechanistically unlikely that the 9*S*-chiral centre of **1** and **2** would be involved in the cyclization leading to an 8-membered biogenetic intermediate e.g. (**3**), thus clearly supporting the 9*S*-configuration in both **4** and **5**. Inspection of molecular models of the rigid brevianamide A (**12**) molecule shows that it will similarly require that both chiral centres on the diketopiperazine ring must have the same configuration. This *cis*-orientation proposed for 12,13-dihydroaustamide (**13a**) is the same as depicted in Fig 5 for 12*S*-tetrahydroaustamide (**14a**); **14a** is obtained upon hydrogenation of **13a**.

The proposal of the stereochemistry at position 2, the spiro atom, relative to position 9 is based exclusively on NMR data. The values of the different NMR parameters which could be obtained from the NMR spectra of austamide and its hydrogenation products are given in Table 2, excluding data on the ψ-indoxyl moiety. From Table 2 it was evident that the proton on the diketopiperazine ring at position 9 resonated between τ 5·20–5·38. In **1** and the model compounds **10a** and **10b** the proton at position 9 appeared between τ 5·56–5·85; these values are in accordance with the data (τ 5·5–6·3) given by Romanet *et al.*[22] for protons at the 3,6-positions of 2,5-diketopiperazines. The lower field absorption of the protons at position 9 in **4**, **13a**, **13b**, **14a** and **14b** is therefore thought to be due to the anisotropy of the ψ-indoxyl carbonyl group as shown in Fig 3. With an opposite configuration at

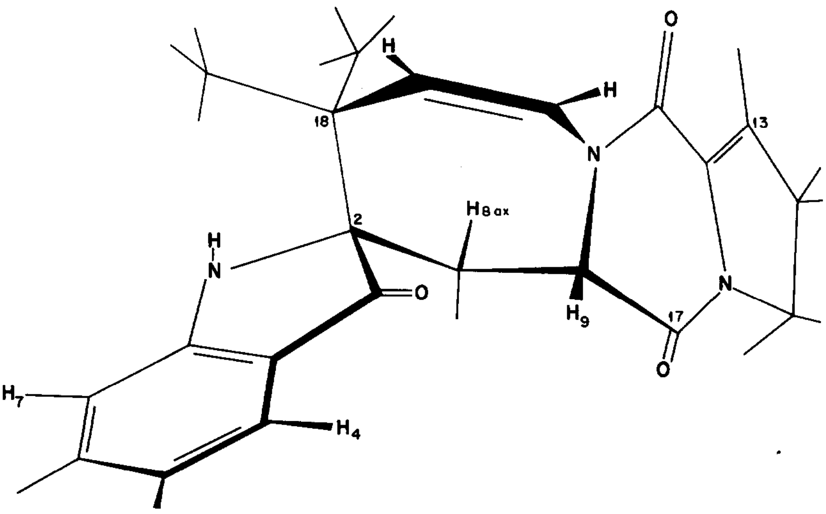
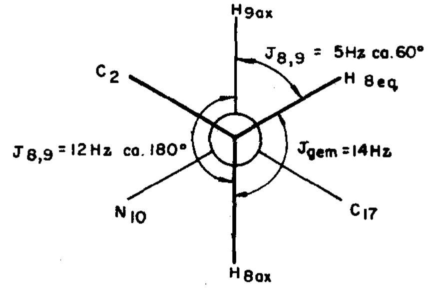


Fig 3. Proposed conformation of austamide.

position 2 it is unlikely that the proton at C_9 will suffer a down field shift.

In austamide **4** the diketopiperazine ring and the proline ring must be planar by the rigidity imposed in this system by the 12,13-double bond. The planarity of the proline ring was supported by the appearance of its constituent protons in the NMR spectrum as an A_2M_2X pattern. Information pertaining to the conformation of the 7-membered ring, dihedral angles based on *J*-values, are presented as Newman projections. For austamide (**4**) the coupling constants for the ABX system at C_8 and C_9 are compatible only as shown below. The unsaturated seven-membered ring is therefore in the preferred chair[23] conformation as shown in Fig 3.

The proposed conformation for austamide is in agreement with the isotropic shifts observed upon the addition of the paramagnetic shift reagent euorpium-111-tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octadione[17] to a solution of austamide in $CDCl_3$. Austamide **4** is a polifunctional compound, a variation in the proton shift ratios with concentration of the shift reagent is observed (Fig



Newman Projection I , austamide 4

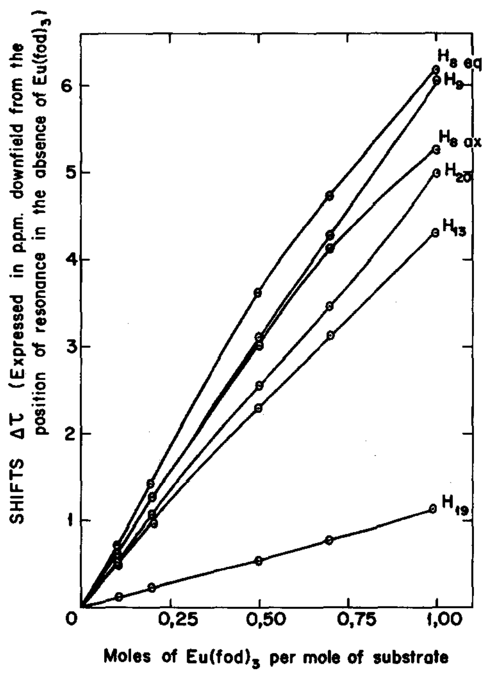


Fig 4. Shifts observed upon the addition of Eu $(fod)_3$ to a solution of austamide (**4**).

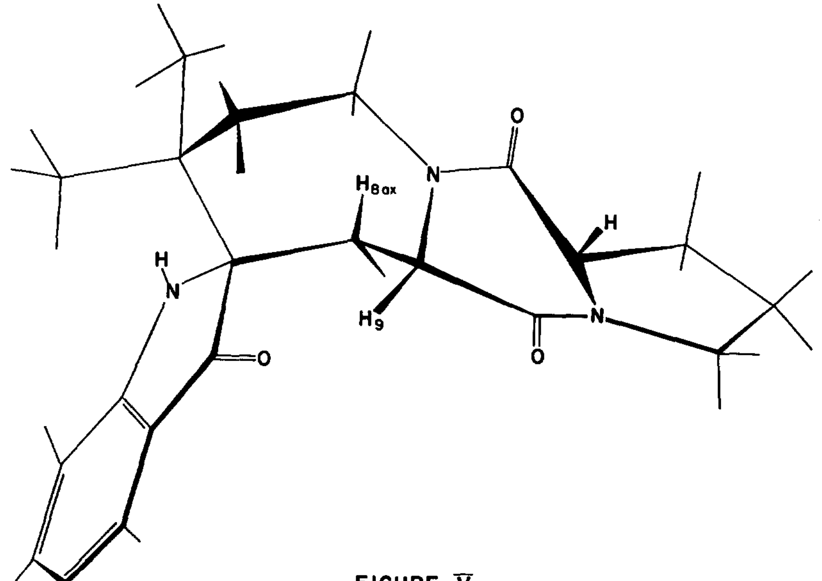
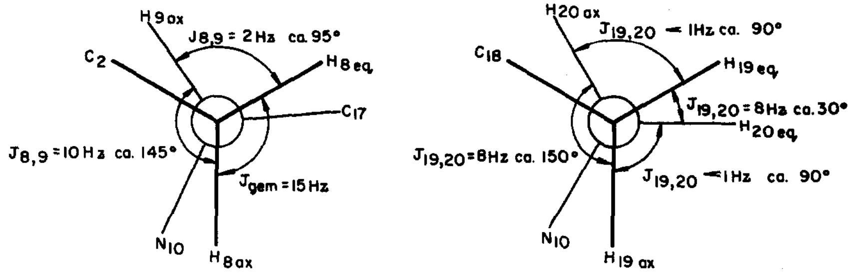


FIGURE V

Fig 5. Proposed conformation of 12*S*-tetrahydroaustamide (**14a**).



Newman projection 2, 12S-tetrahydroaustamide 14a

4), which is probably attributable to the influence of steric hindrance and differences in equilibrium constants for complex formations at the different co-ordination sites. To obtain some quantitative values, the shift ratios were calculated for the protons at C_8 and C_9, assuming the influence of complexation at C_{17} only and a distance of 3·0 A° between the Eu atom and the O atom of the C_{17}—CO group. Calculation by the McConnell and Robertson[24] formula gave values for $H_{8eq.}:H_9:H_{8ax}$ of 1:0·99:0·70; these ratios are in good agreement with the observed shifts (Fig 4). A similar calculation for the shift ratios of the protons at C_{13}, C_{19} and C_{20} gave values of $H_{20}:H_{13}:H_{19}$ of 1:0·76:0·41; these values have the same trend as the observed shifts. It is of importance to note that co-ordination occurred apparently preferentially at the diketopiperazine CO groups as very little isotropic shift was observed for the aromatic protons.

The tetrahydroderivatives **14a** and **14b** contained a saturated 7-membered ring; which according to Tochtermann[23] will have a preferred twist chair conformation. Inspection of a Dreiding model of these compounds indicated this twist chair conformation for the 7-membered ring, a boat conformation for the diketopiperazine ring and a buckled half-chair form for the proline ring. The information obtained on the conformation of the 7-membered ring is shown by Newman projections and the proposed stereoformula for **14a** is depicted in Fig 5. The NMR spectrum of the minor tetrahydroderivative (**14b**) taken at room temperature

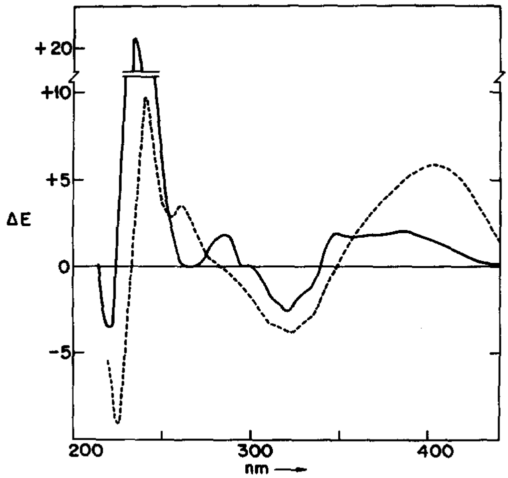


Fig 6. The CD spectra of austamide (4) (———) and of brevianamide A (12) (-----------).

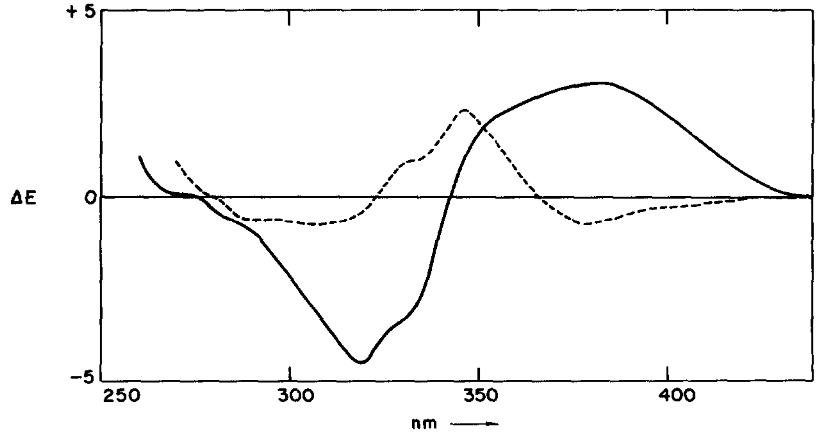


Fig 7. The CD spectra of compound 13a (---------) and of compound 13b (———).

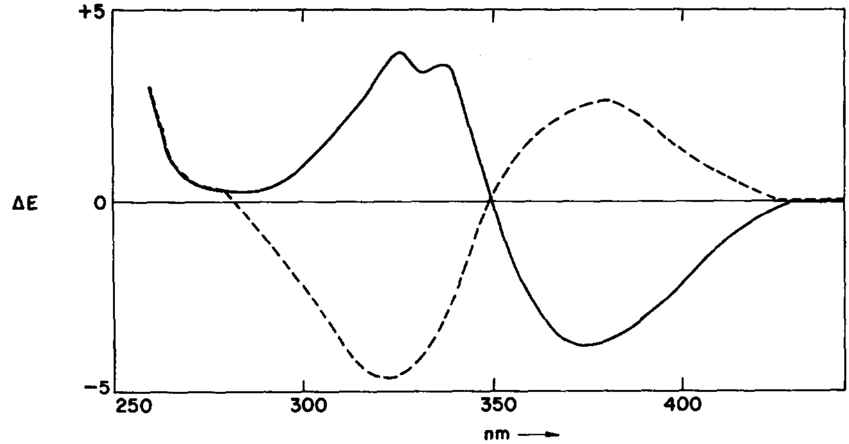


Fig 8. The CD spectra of compound **14a** (---------) and of compound **14b** (————).

in $CDCl_3$ revealed that the conformationally mobile 7-membered ring of this molecule existed in more than one conformation. These conformers interchange rapidly and the observed couplings of the protons at C_{20} (*J* 5, 6, 16 Hz) would then be the average values of the different conformers.

The CD data of compounds **4**, **5** (**13a**), **13b**, **14a** and **14b** are shown in Figs 6, 7, and 8. In austamide **4** the chromophore associated with the long wavelength electron transfer band of the ψ-indoxyl unit gave rise to a positive Cotton effect at 390 nm, $\Delta\epsilon +2$. The Cotton effect at 285 nm, $\Delta\epsilon + 1{\cdot}8$ is thought to be associated with the 12,13-enamide chromophore since it was absent in the spectrum of dihydroaustamide (**13a** and **13b**). The CD spectra of all of these compounds showed very strong Cotton effects at *ca* 234 nm, Δε 20–25 and are not reproduced in the figures. The absorption at 234 nm must be associated with the strong UV chromophore of the ψ-indoxyl moiety at this wave-length.

A most unusual feature of these CD data is the remarkable influence of conformational changes remote from the chiral spiro position on the sign of the Cotton effects above 300 nm. For example 12S-dihydroaustamide (**13a**) had Δε 380 nm − 0·8; 12*R*-dihydroaustamide (**13b**) had Δε 383 nm + 3·0; 12*S*-tetrahydroaustamide (**14a**) had Δε 379 nm + 2·6, while 21*R*-tetrahydroaustamide (**14b**) had Δε 375 nm − 3·6. From an empirical approach with consideration of conformational factors it appears that the sign of the Cotton effects above 300 nm, associated with the ψ-indoxyl moiety, must be governed by the orientation of the diketopiperazine part of the molecule relative to the ψ-indoxyl unit. The Cotton effect at 234 nm is therefore a better measure of the stereochemistry at position 2 in these compounds. The similarity in the CD spectra of austamide (**4**) and of brevianamide A (**12**) indicates that these compounds probably have the same configuration at position 2, (Fig 6).

EXPERIMENTAL

UV absorption refers to EtOH and IR absorption to $CHCl_3$. UV spectra (Unicam Model S.P. 800 Spectrometer) and IR spectra (Perkin-Elmer Model 237 Spectrometer). Mass spectra were taken on a MS-9 double focussing mass spectrometer. The CD spectra were recorded in MeOH at 21° with a JASCO ORD/UV-5 instrument with attachment for CD measurements. PMR spectra were recorded on a Varian HA-100 Spectrometer in $CDCl_3$.

Amino acid analysis was carried out on a Beckman model 120B automatic amino acid analyzer coupled to a Beckman 125 integrator. Electrophoresis was performed on a Gilson high voltage electrophorator, model D, with Varsol as cooling medium. The buffer system was AcOH : HCOOH : water (100 : 29·5 : 870) (v/v/v), pH 1·9. Whatman No. 3MM paper was used for preparative separation. Proline was located on the paper with the collidine-ninhydrin reagent.

Gas chromatography was carried out on a Packard gas chromatograph Model 846. TLC chromatography was carried out on Merck precoated Al_2O_3 and SiO_2 plates, layer thickness, 0·25 mm and 1·25 mm for analytical and preparative separations, respectively. Chromogenic reagent for TLC plates was a solution of 1% $Ce(SO_4)_2$ in $6NH_2SO_4$.

Isolation of compounds 1–5. *Aspergillus ustus* (Bainier) Thom and Church was grown in bulk on wet sterilized maize-meal for 20 days. The dried mouldy maize (6·3 kg) was extracted with $CHCl_3$–MeOH over a period of 2 days and the solvent removed under reduced pressure which yielded homogenous crystalline material (270 g)* which represented the main toxic component of the fungal culture and soluble material (470 g). The latter in $CHCl_3$ (4 l) was twice extracted with water (1·5 l). Evaporation of the $CHCl_3$ yielded 250 g of material which was partioned between 90% MeOH and hexane (3 l each), yielding 50 g of toxic material in the MeOH layer.

This toxic material (50 g) was separated by chromatog-

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*The structural elucidation of this compound will be described in a separate communication.

raphy on formamide-impregnated cellulose powder (1·5 kg).

The cellulose column was developed with mixture of hexane and benzene, 2500 fractions, each containing (20 ml), were collected.

Fraction A (4·9 g) derived from tubes 880–1400 contained compounds **4** and **5**.

Fraction B (2·6 g) derived from tubes 1401–1550 contained compound **1**.

Fraction C (2·0 g) derived from tubes 1550–1790 contained compounds **2** and **3**.

Purification of compound **1**. Fraction B (2·68 g) was separated on Al_2O_3, activity III (50 g) in benzene as solvent to give **1** as a homogenous powder (250 mg). It had $[\alpha]_D^{22°} -59°$ (*c*, 1·2 $CHCl_3$); λ_{max} 225, 275, 283 and 291 nm (log ϵ 4·51, 3·85, 3·91 and 3·85, respectively); ν_{max} 3480, 3458, 3365, 3000 and 1670 cm^{-1}. It had *m/e* 351·200 (M^+, $C_{21}H_{25}N_3O_2$ requires: 351·194), 198·127 ($C_{14}H_{16}N$, requires: 198·128).

Purification of compounds **2** *and* **3**. Fraction C (2·0 g) was separated on Al_2O_3 activity III (50 g). Elution with benzene gave a mixture of compounds (40 mg) containing **3**. The latter material was separated by TLC on SiO_2 in $CHCl_3$:MeOH (98:2) v/v yielding **3** (8 mg) it had λ_{max} 224, 272, 284 and 292 nm (log ϵ 4·44, 3·93, 3·84 and 3·71, respectively); ν_{max} 3482, 3350 (weak broad band), 3000, 1675 and 1650 cm^{-1}. It had *m/e* 347·161 (M^+, $C_{21}H_{21}N_3O_2$ requires: 347·162).

Elution of the foregoing Al_2O_3 column with $CHCl_3$: benzene (6:4) v/v gave **2** (120 mg) as a homogenous powder. It had $[\alpha]_D^{22°} -38°$ (*c*, 1·3 $CHCl_3$) λ_{max} 223, 268, 283 and 292 nm (log ϵ 4·54, 4·03, 4·00 and 3·89, respectively) ν_{max} 3482, 3460, 3380, 1670 and 1650 cm^{-1}. It had *m/e* 349·1720 (M^+, $C_{21}H_{23}N_3O_2$ requires: 349·1708).

Purification of compounds **4** *and* **5**. Fraction A (4·9 g) was separated on Al_2O_3, activity III (300 g) and elution with mixtures of benzene and $CHCl_3$ yielded **4** (2·7 g) and a mixture (150 mg) containing **4** and **5**. The latter mixture was separated by repeated chromatography on preparative SiO_2 chromatoplates, solvent $CHCl_3$:MeOH (97:3) v/v which gave pure **5** (40 mg).

Austamide **4** is a homogeneous powder and had $[\alpha]_D^{20°} +152°$ (*c*, 1 EtOH); λ_{max} 234, 256, 268 (sh), 282 and 392 nm (log ϵ 4·42, 3·07, 3·04, 3·94 and 3·43, respectively); ν_{max} 3420, 3000, 1700, 1680, 1650 and 1622 cm^{-1}.

It had *m/e* 363·1579 (M^+, $C_{21}H_{21}N_3O_3$ requires: 363·1582), 218·1040 ($C_{12}H_{14}N_2O_2$ requires: 218·1055), 203·0769 ($C_{11}H_{11}N_2O_2$ requires: 203·082), 173·085 ($C_{11}H_{11}NO$ requires: 173·084).

12,13-Dihydroaustamide **5** was crystallised from acetone and had m.p. 235–238° and $[\alpha]_D^{22°} +55°$ (*c*, 1·1 $CHCl_3$); λ_{max} 238, 256 and 390 nm (log ϵ 4·49, 4·13 and 3·52, respectively); ν_{max} 3420, 3335, 3000, 1670 and 1620 cm^{-1}. It had *m/e* 365·1750 (M^+, $C_{21}H_{23}N_3O_3$ requires 365·1739), 192·1268 ($C_{11}H_{16}N_2O$ requires: 192·1262), 70·0656 (C_4H_8N requires: 70·0656).

Hydrogenation of compound **1**. Compound **1** (22 mg) was hydrogenated in EtOH (5 ml) over 10% Pd/C (15 mg). The absorption of H_2 ceased after 4 min upon the uptake of 1 mole of H_2. Standard work-up gave **6** (20 mg). It had λ_{max} 224, 275, 284 and 292 nm (log ϵ 4·42, 3·75, 3·80 and 3·75, respectively; ν_{max} 3480, 3360 and 1670 cm^{-1}; *m/e* 353 (M^+, $C_{21}H_{27}N_3O_2$ requires: 353).

Hydrogenation of compound **2**. Compound **2** (90 mg) was hydrogenated in EtOH (10 ml) over 10% Pd/C (50 mg) for 1 hr. Standard work-up gave **6** (95 mg). This compound had UV, IR, NMR, CD and mass spectra as well as chromatographic properties on Al_2O_3 and SiO_2 identical in detail to that obtained upon hydrogenation of **1**, see above.

Kuhn-Roth oxidation of compound **6**. Compound **6** (50 mg) in 4N chromic acid-conc H_2SO_4 (4:1) v/v (5 ml) was heated under reflux for 1 min. Water (5 ml) was added and the volatile acids were removed by steam distillation. This procedure was repeated 10 times. The acids were neutralised with 0·05 N NaOH and converted to the *p*-bromophenacyl esters.[25] The *p*-bromophenacyl-2,2-dimethylbutyrate (15 mg) was obtained by separation on SiO_2 TLC in $CHCl_3$:benzene (1:1) v/v on which it appeared at R_f 0·40.

This compound was compared with the *p*-bromophenacyl ester of 2,2-dimethylbutyric acid prepared by standard procedure. The two compounds had identical mobilities in several SiO_2 TLC systems; identical retention time (18 min) on GLC, [column packed with 5% OV 101 on 60/80 gas chrom. Q, inlet temp 140°, initial hold 5 min, program rate 10°/min, final temp 200°, carrier gas flow rate 35 cc/min] identical mass spectra and PMR characteristics, *viz* τ 2·28 and τ 2·46 (4 arom. $\underline{H}$, AB system J_{AB} 8 Hz); τ 4·80 (2H, s, $COC\underline{H}_2O$—); τ 8·37 (2H, q, *J* 7 Hz, $RC\underline{H}_2CH_3$); τ 8·90 (6H, s, *gem* dimethyl group) and τ 9·10 (3H, t, *J* 7 Hz, R—$CH_2\underline{CH}_3$).

Hydrolysis of prolyl-2-(1′,1′-dimethylallyl)tryptophyl-diketopiperazine **1**. Compound **1** (10 mg) suspended in 6N HCl (5 ml) was heated in a sealed tube at 110° for 20 hr. The soln was filtered, treated with charcoal and concentrated in a dessiccator over KOH and $CaCl_2$ to dryness. Automatic amino acid analysis revealed the presence of proline in the hydrolysate. The latter was separated on a Gilman electrophorator for 1·5 hr at 4500 volt. The proline was eluted from the paper with AcOH: *n*-propanol:water (1:1:8) v/v/v. It had $[\theta]_{224\,nm}^{0\cdot1\,N\,HCl} +225°$ and *m/e* 115 (calc. for $C_5H_9NO_2$: M^+, 115).

Preparation of LTLPDKP (**10a**). L-tryptophan methyl ester hydrochloride (1·2 g) in water (20 ml) was added to 1-carbobenzoxy-L-prolyl-*p*-nitrophenyl ester (1·4 g) in dioxane (40 ml) and kept at 80°. Triethylamine (1·2 ml) in dioxane (20 ml) was slowly added to the mixture. TLC indicated completion of the reaction after 4 hr. The solvent was removed at reduced pressure and the residue in $CHCl_3$ (150 ml) washed consecutively with water (2 × 50 ml), 0·5 N HCl (60 ml) and water (2 × 50 ml) and dried.

The solvent was removed under reduced pressure and the residue filtered through Al_2O_3 (50 g) by eluting with $CHCl_3$: MeOH (98:2) v/v to yield L-tryptophan-N-(1-carbobenzoxy-L-prolyl) methyl ester (1·2 g). After crystallization from MeOH, it had m.p. 60° (lit.,[26] 55–75°) and *m/e* 449 (calculated for $C_{25}H_{27}N_3O_3$: M^+, 449).

The above dipeptide (1·1 g) in EtOH (50 ml) was stirred over 10% Pd/C (500 mg) in a H_2 atmosphere overnight. The mixture was filtered and the filtrate concentrated. The residue in HCOOH (30 ml) was kept at room temp for 20 min. After removal of excess HCOOH below 10°, the residue containing the crude dipeptide ester formate was dissolved in *sec*-BuOH : toluene 4:1 (200 ml). The soln was boiled for 8 hr and the level maintained by the addition of fresh solvent. Excess solvent was removed in a stream of N_2. The product was separated on SiO_2 (150 g) in $CHCl_3$:MeOH (97:3) v/v yielding LTLPDKP (500 mg). After crystallization from acetone, it had m.p. 174°; $[\alpha]_D^{22°} -101°$ (*c* 1·1 AcOH); λ_{max} 220, 273, 280 and 290 nm (log ϵ 4·51, 3·72, 3·74 and 3·66, respectively); ν_{max} 3480, 3360, 3000 and 1660 cm^{-1}) and *m/e* 283 ($C_{16}H_{17}N_3O_2$ requires: 283).

Preparation of DTLPDKP (**10b**). DTLPDKP (**10b**) was prepared from D-tryptophan methyl ester hydrochloride under conditions similar to those described for **10a**. It had m.p. 204–206° (from acetone); $[\alpha]_{\mathrm{D}}^{22^\circ} - 101^\circ$ (c 1·78 AcOH) λ_{max} 220, 273, 280 and 290 nm (log ϵ 4·54, 3·75, 3·78 and 3·70, respectively); ν_{max} 3478, 3395, 3300 (broad), 3000, 1660 and 1448 cm^{-1}; and m/e 283 (M^+, $C_{16}H_{17}N_3O_2$ requires: 283). [Found: C, 67·70; H, 5·96; N, 14·48. $C_{16}H_{17}N_3O_2$ requires: C, 67·82; H, 6·05; N, 14·83%].

The epimerization of LTLPDKP (**10a**). Compound **10a** (10 mg) in ethanol : triethylamine 1 : 1 (2·0 ml) v/v was heated under reflux for 3 days under anhydrous conditions. The course of the reaction was followed by SiO_2 TLC using the solvent system $CHCl_3$: MeOH (94 : 6) v/v. In this system LTLPDKP appeared at R_f 0·30 while the epimerization product appeared at R_f 0·15. The newly formed product had chromatographic properties identical to those of DTLPDKP (**10b**) in several solvent systems.

The epimerization of compound **1**. Compound **1** (15 mg) in EtOH : triethylamine 1 : 1 (3·0 ml) v/v was heated under reflux for 3 days under anhydrous conditions. The course of the reaction was followed by SiO_2 TLC using the solvent system $CHCl_3$: MeOH (97 : 3) v/v. In this system compound **1** appeared at R_f 0·30 and its epimer at R_f 0·15.

Hydrogenation of compound **3**. Compound **3** (5 mg) was hydrogenated in EtOH (5 ml) over 10% Pd/C (3 mg) for 2 hr. Standard work-up gave the tetrahydro derivative, homogeneous by SiO_2 TLC. It had m/e 351·199 (M^+, $C_{21}H_{25}N_3O_2$ requires: 351·195).

Hydrogenation of austamide **4**. Austamide (300 mg) was hydrogenated in EtOH (50 ml) over 10% Pd/C (300 mg). The experiment was stopped after 12 min when 1·2 mole of H_2 was taken up. The mixture was filtered and the filtrate evaporated to give a mixture containing four compounds. The mixture was resolved by repeated chromatography on five preparative SiO_2 chromatoplates (40 × 20 cm) in $CHCl_3$: MeOH (97 : 3) v/v.

12R-*Dihydroaustamide* (**13b**) (52 mg) m.p. 144° (from acetone). It had $[\alpha]_{\mathrm{D}}^{22^\circ} + 163^\circ$ (c, 0·5 $CHCl_3$); UV absorption identical to that of **13a**, see below; ν_{max} 3400, 1690 (sh) 1665 and 1620 cm^{-1} and m/e 365 (M^+, $C_{21}H_{23}N_3O_3$ requires: 365).

12S-*Dihydroaustamide* (**13a**) (160 mg) m.p. 236–238° (from acetone). It had $[\alpha]_{\mathrm{D}}^{22^\circ} + 47^\circ$ (c, 1·8 $CHCl_3$; λ_{max} 234, 234, 256 and 392 nm (log ϵ 4·40, 4·05 and 3·46, respectively); ν_{max} 3420, 3340, 3000, 1690 (sh), 1670 and 1620 cm^{-1}; and m/e 365·172 (M^+, $C_{21}H_{23}N_3O_3$ requires: 365·173).

12S-*Tetrahydroaustamide* (**14a**) (40 mg) m.p. 246–247° (from acetone). It had $[\alpha]_{\mathrm{D}}^{22^\circ} - 245^\circ$ (c, 1·1 $CHCl_3$); λ_{max} 225, 265 (sh) and 394 nm (log ϵ 4·32, 3·76 and 3·50, respectively); ν_{max} 3420, 3330, 3000, 1685 (sh), 1670 and 1620 cm^{-1}. It had m/e 367·1859 (M^+, $C_{21}H_{25}N_3O_3$ requires: 367·1895), 222·1368 ($C_{12}H_{18}N_2O_2$ requires: 222·1368) 201·1183 ($C_{13}H_{15}NO$ requires: 201·1153) 146·0585 (C_9H_8NO requires: 146·0605).

12R-*Tetrahydroaustamide* (**14b**) (12 mg) m.p. 250–252° (from acetone); $[\alpha]_{\mathrm{D}}^{22^\circ} + 135^\circ$ (c, 1·14 $CHCl_3$); UV, IR and mass spectral properties virtually identical to those of **14a**.

Prolonged hydrogenation (2·5 hr) of austamide (**4**) under these conditions furnished **14a** and **14b** only.

Hydrogenation of compounds **13a** *and* **5**. Compounds **13a** (10 mg) and **5** (10 mg) were separately hydrogenated in EtOH (10 ml) over 10% Pd/C for 2 hr. Standard work-up gave only 12S-tetrahydroaustamide **14a** (10 mg) m.p. 246–247° (from acetone) and IR spectra identical to that of **14a**.

Lithium aluminium hydride reduction of compound **14a**. Compound **14a** (170 mg) in dry THF (30 ml) was added dropwise to LAH (200 mg) in THF (30 ml), stirred at room temp for 30 min, and then heated under reflux for 1·5 hr. The mixture was poured onto ice and extraction into $CHCl_3$ yielded a crude mixture (150 mg). Al_2O_3 TLC indicated two main Dragendorff-positive products. The main product (**15**) was obtained by crystallisation from acetone which gave the hydroxyindoline (80 mg) m.p. 204–206°. It had $[\alpha]_{\mathrm{D}}^{21\cdot8^\circ} + 119^\circ$ (c, 1·1 $CHCl_3$), λ_{max} 212, 251 and 308 nm (log ϵ 4·22, 3·94 and 3·34, respectively), ν_{max} 3590, 3320 (broad), 2960 and 1600 cm^{-1}. It had m/e 341·244 ($C_{21}H_{31}N_3O$ requires: 341·246).

Acid catalysed rearrangement of compound **15**. The hydroxyindoline **15** (40 mg) in EtOH (10 ml) containing 5 drops of conc HCl was heated under reflux for 7 min. The solvent was evaporated under reduced pressure and the residue partitioned between $CHCl_3$ and 1N $NaHCO_3$ aq to yield a mixture (30 mg) of three products on Al_2O_3 TLC developed in $CHCl_3$: MeOH (92 : 8) v/v. This mixture was separated in the above system to give a major compound (**16**) (21 mg) noncrystalline and homogenous by TLC. It had λ_{max} 227, 276, 285 and 292 nm (log ϵ 4·44, 3·69, 3·73 and 3·71, respectively) and m/e 323·234 ($C_{21}H_{29}N_3$ requires: 323·236).

N-*nitroso*-12S-*tetrahydroaustamide*. Compound (**14a**) (4 mg) in glacial AcOH (0·4 ml) was treated with $NaNO_2$ (11 mg) and kept at room temp for 4 hr. The solvent was removed *in vacuo* and the residue separated on SiO_2 TLC in $CHCl_3$: MeOH (98 : 2) v/v giving the N-nitrosoderivative (3 mg) as a yellow residue. It had λ_{max} 206, 237, 252, 283 and 328 nm (log ϵ 4·20, 4·08, 4·10, 3·79 and 3·67, respectively) (similar value were reported[2] for N-nitrosobrevianamide A); and ν_{max} 1715 (ψ-indoxyl CO), 1660 (diketopiperazine CO groups), and 1455 cm^{-1} (N—NO). Its mass spectrum did not show a molecular ion.

Hydrolysis of tetrahydroaustamide (**14a**). Compound **14a** (6 mg) in 6N HCl (5 ml) was heated in a sealed tube at 110° for 20 hr. The solution became dark brown. The solvent was removed in a desiccator over KOH and $CaCl_2$. Automatic amino acid analysis revealed the presence of about one molar equivalent of proline in the hydrolysate. The latter was separated on a Gilman electrophorator for 1·5 hr at 4,500 volt. The proline was eluted from the paper with AcOH : n-propanol : water (1 : 1 : 8) v/v/v. It had $[\theta]_{224\,\mathrm{nm}}^{0\cdot1\mathrm{N\,HCl}} + 470^\circ$

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REFERENCES

[1] P. S. Steyn, *Tetrahedron Letters* 3331 (1971)
[2] A. J. Birch and J. J. Wright, *Tetrahedron* **26**, 2329 (1970).
[3] F. Feigl, *Spot tests in organic analysis* p. 279. Elsevier, Amsterdam (1956).
[4] G. Casnati, A. Quilico and A. Ricca, *Gazz. Chim. Ital.* **93**, 349 (1963).
[5] M. Barbetta, G. Casnati, A. Pochini and A. Selva, *Tetrahedron Letters* 4457 (1969).
[6] A. J. Birch and K. R. Farrar, *J. Chem. Soc.* 4277 (1963).
[7] G. Casnati and A. Pochini, *Ibid.* (D), 1328 (1970).

[8a] E. Houghton and J. E. Saxton, *Ibid.* (C), 1003 (1969).
[8b] R. Nakashima and G. P. Slater, *Tetrahedron Letters* 2649 (1971).
[9] J. P. Jennings, W. Klyne and P. M. Scopes, *J. Chem. Soc.* 294 (1965).
[10] D. E. Nitecki, B. Halpern and J. W. Westley, *J. Org. Chem.* **33**, 864 (1968).
[11] J. W. Westley, V. A. Close, D. N. Niteki and B. Halpern, *Analyt Chem.* **40**, 1888 (1968)
[12] I. Z. Siemion, *Liebigs Ann.* **748**, 88 (1971).
[13] R. Degeilh and R. E. Marsh, *Acta Cryst.* **12**, 1007 (1959).
[14] K. D. Kopple and D. H. Marr, *J. Am. Chem. Soc.* **89**, 6193 (1967).
[15] M. Karplus, *Ibid.* **85**, 2870 (1963).
[16] Y. Kishi, S. Nakatsuka, T. Fukuyama and T. Goto, *Tetrahedron Letters* 4657 (1971).
[17] R. E. Rondeau and R. E. Sievers, *J. Am. Chem. Soc.* **93**, 1522 (1971).
[18] A. W. Sangster and K. L. Stuart, *Chem. Revs.* 69 (1965).
[19] B. Witkop and J. B. Patrick, *J. Am. Chem. Soc.* **73**, 713 (1951).
[20] Z. Buczkowski, A. Gryff-Keller and P. Szczecinski, *Tetrahedron Letters* 607 (1971).
[21] N. Finch, I. Hsiu-Chu Hsu, W. I. Taylor, H. Budzikiewcz and C. Djerassi, *J. Am. Chem. Soc.* **86**, 2620 (1964).
[22] R. Romanet, A. Chemizard, S. Duhous, S. David, *Bull. Soc. Chim. Fr.* 1048 (1963).
[23] W. Tochtermann, *Fortschr. Chem. Forsch.* **15**(3), 378 (1970).
[24] H. M. McConnell and R. E. Robertson, *J. Chem. Phys.* **29**, 1361 (1958).
[25] A. I. Vogel, *Practical Organic Chemistry* p. 362. Longmans, London (1961).
[26] H. Zahn and D. Brandenburg, *Liebigs Ann.* **692**, 220 (1966).