The Structure of Clavicipitic Acid, an Azepinoindole Derivative from *Claviceps fusiformis*

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Clavicipitic acid, an amphoteric indole derivative from *Claviceps fusiformis*, is identified by mass spectral and ^{1}H n.m.r. studies as 3,4,5,6-tetrahydro-6-(2-methylprop-1-enyl)-1*H*-azepino[5,4,3-*cd*]indole-4-carboxylic acid (4) and shown to exist in two diastereoisomeric forms.

An amphoteric indole derivative has been isolated from submerged culture of *Claviceps fusiformis* strain 139/2/1G.[1] While this compound was being investigated, Robbers and Floss [2] reported the isolation of an amino-acid, which they named clavicipitic acid, from submerged culture of *Claviceps* strain SD-58 to which DL-ethionine had been added to inhibit *N*-methylation in clavine alkaloid biosynthesis. In spite of a statement to the contrary,[3] *Claviceps* strain SD-58 is probably identical with *C. fusiformis*.[4-7] The chromatographic behaviour of the compound from *C. fusiformis* is identical with that of clavicipitic acid and their mass spectral fragmentations are very similar; † there is no doubt that the two compounds are identical.

† We are indebted to Dr. H. G. Floss for a sample of clavicipitic acid and a copy of its mass spectral element map.

[1] C. A. Szczybrbak, Ph.D. Thesis, University of London, 1972.
[2] J. E. Robbers and H. G. Floss, *Tetrahedron Letters*, 1969, 1857.

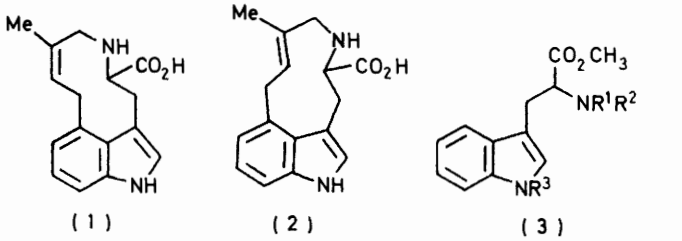
Robbers and Floss [2] attributed the structure (1) to clavicipitic acid on the basis of biosynthetic and spectroscopic evidence. Models indicate large steric interactions in the ten-membered heterocycle, but these are considerably reduced in the double bond isomer (2). The mass spectral fragmentation is difficult to accommodate on the basis of structure (1) or (2) (see below) and, owing to the poor solubility of the compound in any suitable solvent, no satisfactory ^{1}H n.m.r. spectrum has been obtained. Further definitive structural evidence could only be obtained by the preparation and examination of derivatives.

[3] P. A. Stadler and P. Stutz, 'The Alkaloids,' ed. R. H. F. Manske, Academic Press, London, vol. 15, 1975, p. 5.
[4] A. R. Loveless, *Trans. Brit. Mycol. Soc.*, 1967, **50**, 15.
[5] A. Jindra, E. Ramstad, and H. G. Floss, *Lloydia*, 1968, **31**, 190.
[6] E. H. Taylor and E. Ramstad, *J. Pharm. Sci.*, 1961, **50**, 681.
[7] K. Waiblinger and D. Gröger, *Biochem. Physiol. Pflanzen*, 1972, **163**, 468.

Robbers and Floss [2] reported n.m.r. data for a trimethyl derivative obtained by the action of diazomethane on clavicipitic acid. In our hands the reaction of the acid with an excess of ethereal diazomethane gave a complex mixture which could be separated by two-dimensional t.l.c. Mass spectrometry revealed the main components to be unchanged clavicipitic acid and mono- and di-methylated derivatives; no trimethylated compound was found. This result agrees with our investigation of the reaction of tryptophan with diazomethane, which gives a mixture of methyl esters (3a—c) none of which has the indolic nitrogen atom methylated.

Tryptophan is readily esterified with dimethyl sulphite and hydrogen chloride in methanol at 50 °C.[8] Treatment of clavicipitic acid under the same conditions resulted in extensive decomposition and only a poor yield of the methyl ester. The methylation–acetylation procedure of Morris *et al.*,[9] which gives a nearly quantitative yield of the *N*-acetyl-*NN'*-dimethyl methyl ester (3d) when applied to tryptophan (*cf.* methylation of indole [10]), was next investigated. Dissolution of clavicipitic acid in acetic anhydride and methanol gave a nearly quantitative yield of acetylclavicipitic acid methyl ester, treatment of which with sodium hydride in dimethyl sulphoxide followed by methyl iodide gave a monomethyl derivative. Both these clavicipitic esters were soluble in deuteriochloroform.

Mass Spectra.—The 70 eV electron-impact spectrum of clavicipitic acid shows an abundant molecular ion peak at *m/e* 270 (100%) corresponding to $C_{16}H_{18}N_2O_2$



a; $R^1 = R^2 = R^3 = H$
b; $R^1 = R^3 = H, R^2 = Me$
c; $R^1 = R^2 = Me, R^3 = H$
d; $R^1 = Ac, R^2 = R^3 = Me$

(precise mass measurement). A peak corresponding to loss of a hydrogen atom from $M^{+\cdot}$ is typical of clavine alkaloids with a benzylic hydrogen atom at C-10; further intense peaks at *m/e* 154 (98%) 167, 168, 181, 182, 183, 196, and 197 are also typical of clavine alkaloids.[11] Two particularly significant peaks are those with *m/e* 225 and 215, $C_{15}H_{17}N_2$ and $C_{12}H_{11}N_2O_2$, respectively, by mass measurement. The former is thus formed by loss of CO_2H, confirming that the compound is a carboxylic acid. The latter results from loss of C_4H_7 from the molecular ion; bearing in mind the biosynthetic evidence,[2] this suggests the presence of the unit $Me_2C{:}CH$. Structures (1) and (2) do not contain such a unit and it is difficult to devise a rational mechanism for the elimination of C_4H_7 from their molecular ions.

The mass spectrum of clavicipitic acid methyl ester is identical with that of one of the products of the diazomethane reaction. It shows an intense peak at *m/e* 284 (95%), due to the molecular ion, and strong peaks at *m/e* 154 (100%), 167, 169, 181, 182, 183, 196, and 197 typical of the clavine alkaloids. The peaks at 215 and 269 in the spectrum of the acid are shifted to 229 and 283 in that of the ester, whereas that at 225 is not shifted, indicative of the methylation of the carboxylic acid function. The mass spectrum of the other diazomethane product shows a molecular ion peak at *m/e* 298 ($C_{18}H_{22}N_2O_2$) as expected for a dimethylated clavicipitic acid. Peaks at *m/e* 297, 243 ($C_{14}H_{15}N_2O_2$), and 239 ($C_{16}H_{19}N_2$) again indicate losses of H, C_4H_7, and CO_2CH_3 from $M^{+\cdot}$. The characteristic clavine alkaloid peak at *m/e* 154 is also present, thus indicating that the indole nitrogen atom has not been methylated. The mass spectrum of this product, together with the observation that no trimethylated product is formed, supports the formulation of clavicipitic acid as a secondary amino-acid. The characteristic clavine alkaloid peaks starting at *m/e* 154 are also present in the mass spectrum of acetylclavicipitic acid methyl ester; this spectrum is, however, dominated by the peak (*m/e* 283) due to loss of CH_3CO from the molecular ion (*m/e* 326, $C_{19}H_{22}N_2O_3$). The same process dominates the fragmentation of acetylmethylclavicipitic acid methyl ester, the peak resulting being at *m/e* 297. In the case of this compound, however, the characteristic *m/e* 154 peak is not present, being replaced by a peak at 168 due to methylation of the indole nitrogen atom. The *m/e* 154 peak seems typical of tryptophans alkylated at C-4; tri- or tetracyclic structures are not necessary for its appearance. Thus 4-(dimethylallyl)tryptophan shows a fairly intense peak (33%) at *m/e* 154 with the base peak (*m/e* 198) resulting from scission in the amino-acid side chain; this compound does not show loss of H or C_4H_7 from the molecular ion. The mass spectra of clavicipitic acid and its derivatives are consistent with the structure (4) proposed in our preliminary publication.[12]

Proton Magnetic Resonance Studies.—The 100 MHz ^1H n.m.r. spectrum of acetylclavicipitic acid methyl ester in deuteriochloroform is shown in Figure 1 and can be analysed in support of structure (5). The signals at τ 1.8, 2.8—3.2, and 3.3 are due to the indole aromatic protons (a)—(c). The one-proton doublet at τ 4.26 was shown by spin decoupling to be coupled to another one-proton doublet at τ 4.86 (J 7 Hz). By analogy with the results of Mrtek *et al.*[13] and of Plieninger *et al.*,[14] the

[8] D. R. Clarke, Ph.D. Thesis, University of London, 1970.
[9] H. Morris, D. H. Williams, and R. P. Ambler, *Biochem. J.*, 1971, **125**, 189.
[10] H. Heaney and S. V. Ley, *J.C.S. Perkin I*, 1973, 499.
[11] M. Barber, J. A. Weisbach, B. Douglas, and G. O. Dudek, *Chem. and Ind.*, 1965, 1072.
[12] G. S. King, P. G. Mantle, C. A. Szczyrbak, and E. S. Waight, *Tetrahedron Letters*, 1973, 215.
[13] R. G. Mrtek, H. L. Crespi, G. Norman, M. Blake, and J. J. Katz, *Phytochemistry*, 1968, **7**, 1535.
[14] H. Plieninger, C. Wagner, and H. Immel, *Annalen*, 1971, **743**, 95.

former is due to a vinylic proton (d) and the latter to a benzylic proton (e), the downfield shift from its position in the spectrum of agroclavine resulting from the effect of the amido-substituent. Spin-decoupling experiments similarly prove that the one-proton multiplet at τ 5.70 is coupled to the two-proton multiplet at τ 6.3—6.8 and that these signals can be identified with protons (f) and (g) is to be expected. The three-proton singlets at τ 6.36 and 7.94 are due, respectively, to the ester (h) and acetyl methyl (j) groups, and the three-proton singlets at τ 8.18 and 8.32 are due to two olefinic methyl groups (k) and (l) in different environments. Position 6 is a chiral centre (see below) but the configuration of the proton (e) is unlikely to have much effect on its

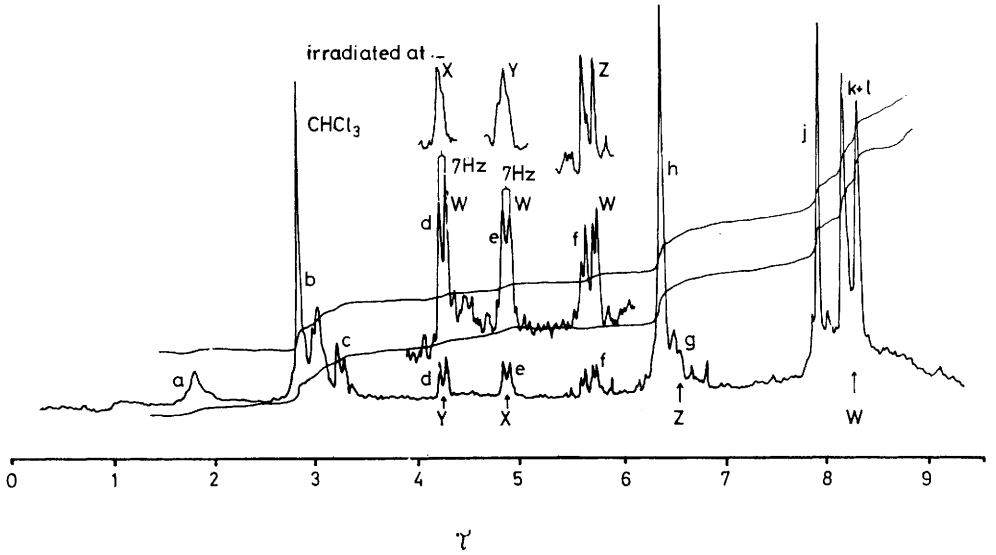


FIGURE 1 The 100 MHz ^{1}H n.m.r. spectrum of acetylclaviciptic acid methyl ester

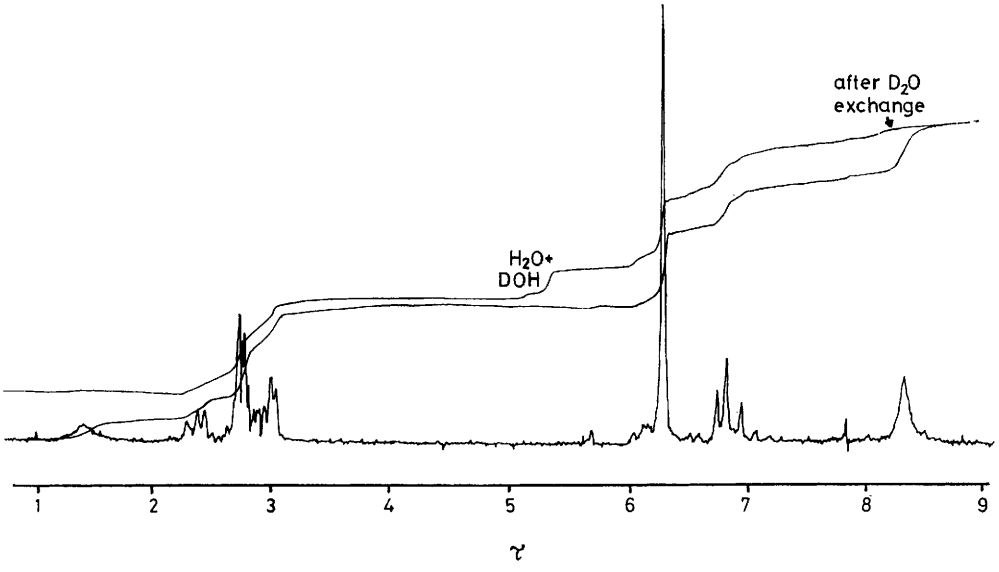
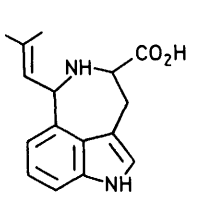


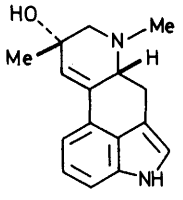
FIGURE 2 The 60 MHz ^{1}H n.m.r. spectrum of tryptophan methyl ester

and (g), respectively, the chemical shifts being similar to those of analogous protons in tryptophan methyl ester (Figure 2). The results of Mrtek *et al.*[13] indicate that a chemical shift of τ 5.70 is reasonable for proton (f) in comparison with the value of τ 6.47 for setoclavine (6), allowance being made for the effect of the carboxylic acid and acetyl functions. The value for the protons (g) is comparable with those (6.90 and 7.33) for the two protons at position 4 in setoclavine; extensive coupling between chemical shift: models indicate that the ester carbonyl group is far removed and other major anisotropic influences, the carbon–carbon double bond, the aromatic ring, and the acetyl carbonyl group, are similarly placed for both isomers. On the other hand a change in configuration at C-6 would cause a change in the anisotropic effect of the double bond on proton (f); the observed signal (τ 5.70) is probably a combination of two multiplet resonances due to the two stereoisomers.

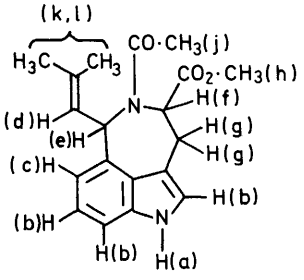
Stereochemistry.—Further evidence which supports the structure (4) but not (1) or (2) is found in the observation that t.l.c. on silica gel with chloroform–methanol–ammonia (75 : 25 : 1) as eluant separates clavicipitic acid



(4)



(6)



(5)

into two approximately equal fractions, R_F 0.25 and 0.29. Each component could be removed from the silica gel with weakly ammoniacal methanol and re-chromatographed with chloroform–benzene–methanol (2 : 1 : 1) as eluant, in which system the two components had the same R_F value. The two substances had almost identical u.v. spectra and mass spectral fragmentation patterns, which were also closely similar to those of the original clavicipitic acid sample.

These isomers could not be interconverted by heating to 200 °C and it seemed likely that they were diastereoisomers. That this is the case was shown by c.d. measurements, the component of R_F 0.29 having $\Delta\varepsilon_{max.}$ −2.95 at 289 nm and that of R_F 0.25 having $\Delta\varepsilon_{max.}$ −1.09 at 286 nm in methanol. Owing to the small amounts of material available, the concentrations of the solutions had to be estimated from their absorptivity at 285 nm and may be in error by as much as ±20%, but considerably less than the observed difference in $\Delta\varepsilon$ values. It seems unlikely that a centre of asymmetry resulting from slow interconversion of conformers of the ten-membered ring could survive heating to 200 °C; indeed compounds such as *trans*-cyclononene and *trans*-cyclodecene are either racemized easily or cannot be resolved at all.[15] On the other hand one would not expect the diastereoisomers of structure (4) to be interconverted at all easily.

Conclusion.—The mass spectra of clavicipitic acid, its methyl ester, and its *N*-methyl methyl ester, and the ^{1}H n.m.r. spectrum of *N*-acetylclavicipitic acid methyl ester are in agreement with the proposed structure (4) for the acid, and cannot easily be accommodated by structure (1) or (2). The definitive ^{1}H n.m.r. evidence and the biosynthetic work of Robbers and Floss [2] could be accommodated if a rearrangement occurred in the derivatization step as has been suggested by Joule.[16] However the identity of the mass spectral fragmentation pathways of clavicipitic acid and its methyl ester, whether this is prepared by treatment of the acid with diazomethane or with acetyl chloride and dimethyl sulphite, make it certain that rearrangement did not occur under the latter conditions. It is thus improbable that rearrangement would occur in the milder treatment, involving acetic anhydride and methanol, used to produced the *N*-acetyl ester. *

We have previously drawn attention to the possibility that clavicipitic acid is an artefact of the method of isolation.[12] However the recent studies of Bajwa *et al.*[17] make it clear that clavicipitic acid is a true metabolite of 4-(dimethylallyl)tryptophan. Bajwa and Anderson[18] have also converted 4-(dimethylallyl)tryptophan into clavicipitic acid in 4% yield using the same thioglycolate–iron(II) system as used for the 8-hydroxylation of agroclavine and elymoclavine. However, other extensive attempts to effect the conversion under a wide variety of oxidative conditions have been unsuccessful.[19]

EXPERIMENTAL

^{1}H N.m.r. spectra were measured with a Varian HA100 or A60 spectrometer with tetramethylsilane as internal standard and deuteriochloroform as solvent. Mass spectra were measured with an A.E.I. MS9 spectrometer operated at 70 eV ionizing energy, accelerating voltage 8 kV, and trap current 100 μA, samples being introduced directly into the ion source at 200 °C. Mass measurements were made at a resolving power of 15 000 (10% valley) with either perfluorokerosene or heptacosafluorotri-n-butylamine as standard. T.l.c. was carried out on silica gel GF_{254}.

Isolation of Clavicipitic Acid from C. fusiformis 139/2/1G. —A modification of the original method [1] was used. Culture filtrate at pH 10 was continuously extracted for several days first with ether and then with chloroform. The aqueous layer was then extracted at pH 5 with butan-1-ol under reduced pressure. The dried butanol extracts afforded, on evaporation under vacuum, a viscous tar which was applied to a Sephadex G10 column (12.5 × 20 cm) in 10% methanol–water. Elution with 10% methanol–water removed first brown high molecular weight material, followed by the amphoteric indole fraction. T.l.c. in chloroform–methanol–benzene (2 : 1 : 1) gave clavicipitic acid, m.p. 264° (decomp.) (from ethanol). T.l.c. in chloroform–methanol–ammonia (75 : 25 : 1) resolved this material

* Since this paper was submitted the synthesis of two isomeric lactams, formally oxidation products of structures (1) and (2), has been reported (N. G. Anderson and R. G. Lawton, *Tetrahedron Letters*, 1977, 1843). Their mass and ^{1}H n.m.r. spectra support the conclusion that clavicipitic acid cannot have structure (1) or (2).

[15] A. C. Cope and B. A. Pawson, *J. Amer. Chem. Soc.*, 1965, **87**, 3649.
[16] J. A. Joule, ' The Alkaloids,' Chem. Soc. Specialist Periodical Report, 1974, vol. 4, p. 287.
[17] R. S. Bajwa, R. D. Kohler, M. S. Saini, M. Cheng, and J. A. Anderson, *Phytochemistry*, 1975, **14**, 735.
[18] R. S. Bajwa and J. A. Anderson, *J. Pharm. Sci.*, 1975, **64**, 343.
[19] M. H. Rahman and E. S. Waight, unpublished observations.

into two components (R_F 0.25 and 0.29), which were isolated with weakly ammoniacal methanol and rechromatographed in chloroform–benzene–methanol (2 : 1 : 1). The two components then had identical R_F values, but rechromatography in the ammoniacal medium resulted in no interconversion and reproduced the original separation.

Reaction with Diazomethane.—A methanolic solution of clavicipitic acid was treated with an excess of ethereal diazomethane. The product was subjected to two-dimensional t.l.c. Six spots were obtained of which four gave a positive reaction in Ehrlich's test.[20] These components were isolated for mass spectrometry by an adaption of the technique of Rix *et al.*[21] No spectrum was obtained from the component of R_F 0.21 ($CHCl_3$) and 0.25 (EtOH–C_5H_6), but the others were identified as unchanged clavicipitic acid, $M^{+\cdot}$ 270, R_F 0.01 ($CHCl_3$) and 0.04 (EtOH–C_6H_6), monomethylclavicipitic acid, $M^{+\cdot}$ 284, R_F 0.25 ($CHCl_3$) and 0.33 (EtOH–C_6H_6), and dimethylclavicipitic acid, $M^{+\cdot}$ 298, R_F 0.47 ($CHCl_3$) and 0.53 (EtOH–C_6H_6).

Reaction of Tryptophan with Diazomethane.—DL-Tryptophan (0.2 g) dissolved in ether (7.5 ml) and methanol (2.5 ml) was added to diazomethane (0.17 g) in ether (50 ml). After 28 h the solution was taken to dryness and the residue examined by two-dimensional t.l.c. Five spots were observed of which three gave positive reactions in the Ehrlich test, and were identified by mass spectrometry as *NN*-dimethyltryptophan methyl ester ($M^{+\cdot}$ 246), *N*-methyltryptophan methyl ester ($M^{+\cdot}$ 232), and tryptophan methyl ester ($M^{+\cdot}$ 218).[22]

Tryptophan Methyl Ester.—(*A*) Tryptophan (5 g) was dissolved in anhydrous methanol (30 ml) and ether (10 ml) saturated with hydrogen chloride at 0 °C, and kept at room temperature (6 h). Removal of the solvent left a residue which was dissolved in aqueous sodium hydroxide (0.1M; 20 ml); the solution was extracted with chloroform (3 × 20 ml). The dried (Na_2SO_4) extracts afforded a pale yellow viscous oil (3.6 g), $\nu_{max.}$ ($CHCl_3$) 3 500, 3 400, 1 720, and 1 300 cm^{-1}; $\lambda_{max.}$ (EtOH) 285 nm (ε 6 030); ^1H n.m.r. spectrum in Figure 2.

(*B*) Acetyl chloride (3 ml) and dimethyl sulphite (3 ml) were added dropwise with stirring to anhydrous methanol (30 ml) at 0 °C. The mixture was added to tryptophan (1 g) and the solution kept at 50 °C for 1 h. Removal of the solvents *in vacuo* left a residue which was treated as above to yield a product (0.95 g) having almost identical spectroscopic properties.

Clavicipitic Acid Methyl Ester.—The acid (3 mg) was dissolved in dimethyl sulphite, methanol, methyl acetate, and hydrogen chloride (1 ml) as in method (*B*) above. The residue left after removal of the solvents was subjected to t.l.c. in 5% ethanol–benzene. The major product (R_F 0.32) gave a positive reaction in Ehrlich's test and had $M^{+\cdot}$ 284. On the basis of a molar absorptivity (ε) of 6 000 at $\lambda_{max.}$ 283 nm, *ca.* 0.8 mg of the title compound was obtained.

N^{α}-*Acetyl*-N(1)N^{α}-*dimethyltryptophan Methyl Ester.*—Tryptophan (10 mg), acetic anhydride (0.2 ml), and methanol (0.8 ml) were kept at room temperature for 3 h. After removal of the solvents *in vacuo* a solution (*ca.* 1 ml) of sodium hydride in dimethyl sulphoxide [9] was added, followed by methyl iodide. After 10 min at room temperature the solvents were removed *in vacuo* at 40 °C and the residue mixed with water (1 ml) and chloroform (3 ml). The dried (Na_2SO_4) chloroform layer yielded the title compound as an oil, $M^{+\cdot}$ 288; $\nu_{max.}$ ($CHCl_3$) 1 730 and 1 680 cm^{-1}; $\lambda_{max.}$ (EtOH) 285 nm.

Acetylation–Methylation of Clavicipitic Acid.—Clavicipitic acid (*ca.* 0.5 mg) was treated with methanol–acetic anhydride and sodium hydride–dimethyl sulphoxide–methyl iodide as above. T.l.c. of the product in 10% methanol–chloroform gave two spots showing a positive Ehrlich reaction (R_F *ca.* 0.3 and *ca.* 0.8). The compound having R_F 0.8 showed $M^{+\cdot}$ 340.179 5 (Calc. for $C_{20}H_{24}N_2O_3$: 340.178 3); $\lambda_{max.}$ (EtOH) 290 nm.

Acetylclavicipitic Acid Methyl Ester.—The acid (*ca.* 4 mg) dissolved in acetic anhydride (0.4 ml) and methanol (1.6 ml) was kept at room temperature for 3 h. The solvents were removed (nitrogen stream) and the residue was triturated with a little hexane, affording a solid, m.p. 107—109°, shown to be homogeneous by t.l.c. in 6% methanol–chloroform; $M^{+\cdot}$ 326.160 0 (Calc. for $C_{19}H_{22}N_2O_3$: 326.163 0); $\lambda_{max.}$ (MeOH) 285 nm. On the basis of a molar absorptivity (ε) of 6 000, the yield of the title compound was quantitative. The ^1H n.m.r. spectrum is shown in Figure 1.

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[20] S. Agurell and E. Ramstad, *Lloydia*, 1962, **26**, 67.
[21] M. J. Rix, B. R. Webster, and I. C. Wright, *Chem. and Ind.*, 1969, 452.
[22] J. S. Fitzgerald, *Austral. J. Chem.*, 1963, **16**, 246.