# Sporidesmins. Part 16.[1] The Structure of Chetomin, a Toxic Metabolite of *Chaetomium cochliodes*, by Nitrogen-15 and Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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Analysis of the $^{13}C$ and $^{15}N$ n.m.r. spectra of [$^{15}N_6$]chetomin biosynthesized by *Chaetomium cochliodes* established that the sporidesmin-like and 3-(ω-skatyl)-3,6-epidithiopiperazine-2,5-dione fragments are linked by a bond between the indole nitrogen and the quaternary β-indoline carbon.

CHETOMIN,[2] a toxic metabolite of *Chaetomium* spp,[3,4] is thought to be associated with poor growth in young ruminants. Initial chemical and spectroscopic studies on the metabolite and its derivatives indicated the presence of a sporidesmin-like residue [5] bonded to a 3,6-epidithiopiperazine-2,5-dione system as shown in (1). Structure (2) was selected because the $^1H$ n.m.r. evidence showed that one *N*-methyl group ($\delta_H$ 3.00) was chemically shifted from the other two which were, fortuitously, chemically equivalent ($\delta_H$ 3.16).[2] In a recent preliminary account [6] of our $^{13}C$ and $^{15}N$ n.m.r. studies of [$^{15}N$]chetomin biosynthesised by *Chaetomium cochliodes* it was established that the metabolite had structure (3), and here the detailed evidence in support of this structure is presented.

To provide optimum conditions for producing and isolating chetomin it was necessary to modify the original isolation procedure [2] and the chemically defined medium used by Waksman to produce chetomin from *C. cochliodes*.[7] The [$^{15}N_6$]chetomin was obtained by growing the fungus (HLX 833) on the modified medium with $Na^{15}NO_3$ as the sole nitrogen source. Analyses by n.m.r. suggested that this material had a purity of >95%, whereas its optical rotation and biological activity indicated *ca.* 80% purity.

The $^1H$ resonance assignments for chetomin in $C^2HCl_3$ were based on previous parameters obtained with $(C^2H_3)_2CO$ as the solvent, and the close correspondence of parts of the spectrum with that of chaetocin [8] and

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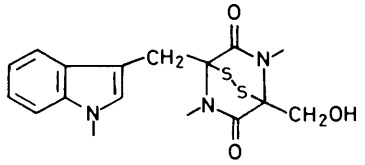
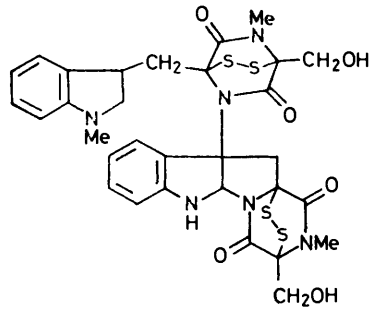
[2] S. Safe and A. Taylor, *J.C.S. Perkin I*, 1972, 472.
[3] S. A. Waksman and E. Bugie, *J. Bacteriol.*, 1944, **48**, 527.
[4] D. Brewer, J. M. Duncan, W. A. Jerram, C. K. Leech, S. Safe, A. Taylor, L. C. Vining, R. McG. Archibald, R. G. Stevenson, C. J. Mirocha, and C. J. Christensen, *Canad. J. Microbiol.*, 1972, **18**, 1129.
[5] R. Hodges, J. W. Ronaldson, A. Taylor, and E. P. White, *Chem. and Ind.*, 1963, 42.
[6] A. G. McInnes, A. Taylor, and J. A. Walter, *J. Amer. Chem. Soc.*, 1976, **98**, 6741.
[7] W. B. Geiger, J. E. Conn, and S. A. Waksman, *J. Bacteriol.*, 1944, **48**, 531.
[8] D. Hauser, H. W. Weber, and H. P. Sigg, *Helv. Chim. Acta*, 1970, **53**, 1061.

substituted indoles.[9] Chemical shifts and spin–spin couplings for the benzene-type protons were obtained by spectral simulation techniques.[10]

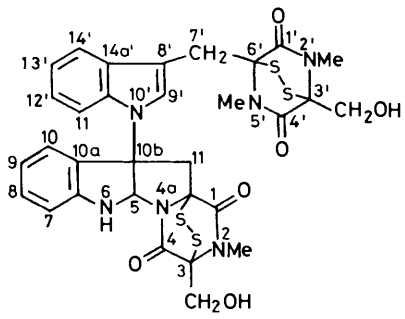
Although chetomin possesses 31 carbons the broadband $^1$H-decoupled $^{13}$C spectrum contained only 30 distinct resonances (see Table) because of peak overlap (C-1, C-1′). Signals could be assigned using known chemical-shift trends, and published chemical-shift data for indoline,[11] chaetocin,[12] and substituted indoles.[13-15] The high-resolution (h.r.) and off-resonance decoupled spectra, which permitted the proton and carbon resonances to be correlated,[16] provided confirmation of many of the assignments for carbons bonded to hydrogen.



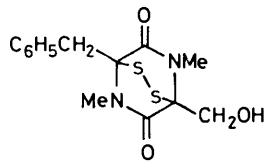
(1)



(2)



(3)



(4)

The six nitrogens in $^{15}$N-enriched chetomin each gave rise to a separate signal in the broadband $^1$H-decoupled $^{15}$N spectrum, and one-bond N–H spin–spin coupling[17] in the corresponding h.r. spectrum identified the indoline nitrogen of the eserine moiety, N-6 ($\delta_N$ 51.0; $^1J_{N-H}$ 87.8 Hz). It was also clear from the broadband $^1$H-decoupled $^{13}$C spectrum of the $^{15}$N-labelled metabolite that 20 carbons were spin–spin coupled to nitrogen.[17] Broadband $^1$H-decoupling with concomitant single frequency $^{15}$N-irradiation at the resonance frequency of each nitrogen in turn established all $^{13}$C–$^{15}$N spin–spin couplings, and consequently the structure of chetomin. Coupling constants were corrected for off-resonance decoupled effects and spectral simulation techniques were used to confirm their magnitudes and derive values for $^3J_{6,11a}$ and $^2J_{10',5'}$.

Thus, N-6 was coupled to five carbons, one of which was clearly identifiable as C-5 because of its distinctive chemical shift ($\delta_C$ 80.2) and one-bond coupling to hydrogen ($^1J_{CH}$ 174 Hz). This carbon was also one-bond coupled ($^1J_{NC}$ 5 ± 1 Hz) to another nitrogen ($\delta_N$ 123.0) which could only be N-4a since it was coupled to five additional carbons including one bonded to two hydrogens (C-11, $\delta_C$ 42.7; $^1J_{CH}$ 138.1 Hz). Another nitrogen resonating at $\delta_N$ 91.1 was coupled to two carbons in common with N-4a, thereby identifying it as N-2.

The identity of the nitrogen bonded to the β-indoline quaternary carbon C-10b ($\delta_C$ 73.8) followed immediately from the observation that this carbon was coupled to a nitrogen ($\delta_N$ 117.4; $^1J_{NC}$ 11.5 ± 0.4 Hz) which was also coupled to five other carbons. One was C-5 ($^2J_{NC}$ 1.5 Hz), and the remainder clearly belonged to the indole heterocyclic ring. In particular one carbon belonged to a methine group, with a high $^1J_{CH}$ value characteristic of indole 2-CH groups,[18] and had a chemical shift close to that expected for C-2 in a 1,3-disubstituted indole.[14]

[9] T. J. Batterham, 'NMR Spectra of Simple Heterocycles,' John Wiley and Sons, New York, 1973, 250.
[10] P. Diehl, H. Kellerhals, and E. Lustig, 'NMR. Basic Principles and Progress,' eds. P. Diehl, E. Fluck, and R. Kosfeld, Springer–Verlag, New York, 1972, 6.
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[17] T. Axenrod, 'Nitrogen NMR,' eds. M. Witanowski and G. A. Webb, Plenum Press, New York, 1973, p. 261.
[18] W. A. Jerram, A. G. McInnes, W. S. G. Maass, D. G. Smith, A. Taylor, and J. A. Walter, *Canad. J. Chem.*, 1975, **53**, 727.

This carbon therefore was C-9′ ($\delta_C$ 127.3; $^1J_{CH}$ 186 ± 2 Hz). Also H-9′ was allylic-coupled ($J_{7',9'}$ <0.5 Hz) to the methylene hydrogens H-7′ in the $^1$H n.m.r. spectrum. The two remaining nitrogens ($\delta_N$ 90.3, 95.3), like N-2, were one-bond coupled to methyl, carbonyl, and quaternary carbons, and therefore part of the other epidithiodioxopiperazine ring. Because its chemical shift and ing $^{15}$N–$^{13}$C coupled carbons was trivial, the complete n.m.r. analysis leaving only the signals for the two hydroxymethyl carbons to be distinguished. Thus the combined evidence unequivocally establishes that C-10b and the indole nitrogen N-10′ are directly bonded, and, consequently, that chetomin has structure (3). The configurations of the chiral centres are, therefore, the

The $^1$H ($\delta_H$), $^{13}$C ($\delta_C$), and $^{15}$N ($\delta_N$) chemical shifts, together with the $^1$H–$^1$H ($^nJ_{HH}$), $^{13}$C–$^1$H ($^1J_{CH}$), and $^{15}$N–$^{13}$C ($^nJ_{CN}$) spin–spin coupling constants for chetomin in $C^2HCl_3$ *

| Locant | $\delta_H$ | $J_{HH}$ † (Hz) | $\delta_C$ | $^1J_{CH}$ (Hz) | $\delta_N$ | $^1J_{NH}$ and $J_{NC}$ (Hz) |
|---|---|---|---|---|---|---|
| 1 | | | 165.6 | | | |
| 2 | | | | | 91.1 | $J_{2,1}$ 13.7; $J_{2,3}$ 7.3; $J_{2,11a}$ 7.3; $J_{2,13}$ 9.3 |
| 3 | | | 76.3 | | | |
| 4 | | | 163.1 | | | |
| 4a | | | | | 123.0 | $J_{4a,3}$ 6.2; $J_{4a,4}$ 14.6; $J_{4a,5}$ 5 ± 1; $J_{4a,10b}$ 4.2 ± 0.6; $J_{4a,11}$ <1.0; $J_{4a,11a}$ 5.0 |
| 5 | 6.17 | | 80.2 | 174 ± 1 | | |
| 6 | 5.44 | | | | 51.0 | $^1J_{NH}$ 87.8; $J_{6,5}$ 8.1; $J_{6,6a}$ 11.8; $J_{6,10a}$ 3.7; $J_{6,10b}$ 1.1; $J_{6,11a}$ 1.3 |
| 6a | | | 148.4 | | | |
| 7 | 6.75 | $J_{7,8}$ 7.5 | 111.1 | 161.7 | | |
| 8 | 7.23 | $J_{8,9}$ 7.3 | 125.0 | 160.0 | | |
| 9 | 6.90 | $J_{9,10}$ 8.0 | 120.3 | 163 ± 1 | | |
| 10 | 7.22 | | 131.4 | 159.9 | | |
| 10a | | | 126.7 | | | |
| 10b | | | 73.8 | | | |
| 11 | 4.49; 3.09 | $J_g$ 15.4 | 42.7 | 138.1 | | |
| 11a | | | 73.6 | | | |
| 12 | 4.72 ‡ ($\Delta\nu_{AB}$ 8.9 Hz) | $J_g$ 12.7 | 60.4 or 61.1 | 149.3 or 148.5 | | |
| 13 | 2.93 | | 27.5 | 141.4 | | |
| 1′ | | | 165.6 | | | |
| 2′ | | | | | 90.3 | $J_{2',1'}$ 13.7; $J_{2',3'}$ 7.3; $J_{2',6'}$ 7.3; $J_{2'16'}$ 9.3 |
| 3′ | | | 76.6 | | | |
| 4′ | | | 166.8 | | | |
| 5′ | | | | | 95.3 | $J_{5',3'}$ 6.7; $J_{5',4'}$ 13.7; $J_{5',6'}$ 6.3; $J_{5',17'}$ 9.3 |
| 6′ | | | 74.9 | | | |
| 7′ | 2.62 ‡ ($\Delta\nu_{AB}$ 16.6 Hz) | $J_g$ 15.5; $J_{7',9'}$ <0.5 | 27.1 | 131.7 | | |
| 8′ | | | 107.8 | | | |
| 9′ | 7.34 | | 127.3 | 186 ± 2 | | |
| 10′ | | | | | 117.4 | $J_{10',8'}$ 4.3; $J_{10',9'}$ 13.8; $J_{10',10a'}$ 14.5; $J_{10',14a'}$ 4.7; $J_{10'10b}$ 11.5 ± 0.4; $J_{10',5}$ 1.5 |
| 10a′ | | | 134.1 | | | |
| 11′ | 7.29 | $J_{11',12'}$ 8.0; $J_{11',13'}$ 1.2; $J_{11',14'}$ 0.5 | 111.5 | 162 ± 1 | | |
| 12′ | 7.19 | $J_{12',13'}$ 7.0; $J_{12',14'}$ 1.2 | 122.8 | 160.4 | | |
| 13′ | 7.19 | $J_{13',14'}$ 7.8 | 120.6 | 161 ± 2 | | |
| 14′ | 7.64 | | 119.2 | 159 ± 2 | | |
| 14a′ | | | 130.4 | | | |
| 15′ | 4.72 ‡ ($\Delta\nu_{AB}$ 8.9 Hz) | $J_g$ 12.7 | 61.1 or 60.4 | 148.5 or 149.3 | | |
| 16′ | 3.14 or 3.15 | | 27.5 | 141.4 | | |
| 17′ | 3.15 or 3.14 | | 28.3 | 141.4 | | |

* $^1$H and $^{13}$C chemical shifts relative to $SiMe_4$, and $^{15}$N to 4M-$^{15}NH_4Cl$ in 2M-HCl. Error in $^1J_{CH}$ and $^nJ_{NC}$ *ca.* ±0.3 Hz except where shown. † $J_g$ = Geminal coupling constant. ‡ Average chemical shift of AB spin system.

couplings to neighbouring carbons were essentially the same as those for N-2 the nitrogen resonance at $\delta_N$ 90.3 was assigned to N-2′. Couplings to N-2′ were distinguished from those to N-2 by using a small $^{15}$N-decoupling field of $\gamma H_2/2\pi$ = 15 Hz. The resonance at $\delta_N$ 95.3 therefore belonged to N-5′. Assignment of the remaining features of chetomin that remain undefined.

It is thought [19,20] that sporidesmins are biosynthesised by oxidation at the β-position of a tryptophanyl dioxopiperazine and then subsequent cyclisation to the eserine. Since sporidesmins are 3-hydroxyindolines the oxidising

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[20] C. M. Atkinson, J. C. E. Simpson, and A. Taylor, *J. Chem. Soc.*, 1954, 165.

agent is presumably an oxidase; however the oxidising agent may be derived from a second tryptophanyl dioxopiperazine and oxidation may occur either at the 3 position of the indolenine tautomer, or at the nitrogen atom of the indole tautomer. In the first case oxidation and cyclisation leads to metabolites like chaetocin;[8] in the second only half the dimeric oxidation product can cyclise, and such cyclisation leads to metabolites like chetomin. Thus the indole moiety in chetomin is analogous to the hyalodendrin (4) type in the phenylalanine series.[21]

## EXPERIMENTAL

Inocula and production cultures were grown in Erlenmeyer flasks of capacity 125 and 500 ml respectively. Cultures were incubated at 25 °C and were shaken in a horizontal plane each flask describing a circle of 5.08 cm at 220 rev $min^{-1}$. I.r. spectra were determined on a Perkin-Elmer 237 spectrophotometer and u.v. spectra on a Cary 14 instrument.

A Varian HA-100 continuous wave n.m.r. spectrometer was used for the $^1H$ spectrum of chetomin dissolved in $C^2HCl_3$. Conditions were: sample concentration 180 mg $ml^{-1}$, frequency 100 MHz, probe temperature 30 °C, sweep width 1 000 Hz, sweep rate 2 Hz $s^{-1}$, frequency response 2 Hz, internal lock to tetramethylsilane, aromatic region expanded by factor of 10 to facilitate spectra simulation[10] of the proton multiplets. $^{13}C$ N.m.r spectra of chetomin in $C^2HCl_3$ were recorded at 30 °C with a Varian XL-100/15 pulse Fourier-transform spectrometer under the following conditions: frequency 25.16 MHz, spectral width 5 120 Hz, acquisition time 0.8 or 1.6 s, flip angle 40 or 55°, data resolution $\pm 0.6$ or $\pm 0.3$ Hz, $^1H$ decoupling field $\gamma H_2/2\pi \approx 3\,800$ Hz with 0 to 180° phase modulation at 150 Hz,[22] internal $^2H$ lock to $C^2HCl_3$ internal reference to $SiMe_4$, sample size 0.4 ml of 0.25 molar solutions of chetomin in 5 mm o.d. sample tubes, spin rate 25 Hz. Similar conditions were used for single frequency $^1H$ off-resonance decoupling except that the decoupling field was not phase modulated. $^{13}C$ Spectra were obtained with the decoupling field ($\gamma H_2/2\pi \approx 3\,800$ Hz) switched on for 1.6 s between data acquisition periods to retain the nuclear Overhauser enhancement.[23] Broadband $^1H$-decoupled $^{13}C$ spectra of $^{15}N$-enriched chetomin, with simultaneous single-frequency irradiation of each $^{15}N$ in turn, were obtained by feeding the $^{15}N$ decoupling frequency from a General Radio model 1 061 frequency synthesiser *via* a variable attenuator, broadband ENI 320L RF amplifier, and tuned filter to a double tuned network which matched both the $^{15}N$- and $^1H$-decoupling signals to the decoupling coils of the probe. Otherwise conditions were the same as for broadband $^1H$-decoupled $^{13}C$ spectra. The field strengths ($\gamma H_2/2\pi$) used to irradiate the six nitrogens were as follows: $^{15}N$-4a, $^{15}N$-6, $^{15}N$-5′, and $^{15}N$-10′, 65 Hz; $^{15}N$-2′, 32 Hz; $^{15}N$-2, 15 Hz. $^{15}N$ decoupling frequencies were obtained from a broadband $^1H$-decoupled pulse Fourier transform $^{15}N$ spectrum recorded with the Varian XL-100/15 spectrometer as follows: frequency 10.14 MHz, spectral width 2 000 Hz, acquisition time 2 s, flip angle 69°, pulse delay 2 s, data resolution $\pm 0.25$ Hz, internal $^2H$ lock to $C^2HCl_3$. A broadband $^1H$-decoupled $^{15}N$ spectrum was also recorded with an external $^{19}F$ lock to $C_6F_6$ in order to reference the $^{15}N$ chemical shifts to the signal from a separate sample of $4_M$-$^{15}NH_4Cl$ in $2_N$-HCl ($\delta_N$). A h.r. $^{15}N$ spectrum obtained with the external $^{19}F$ lock enabled N-6 to be identified by its spin–spin coupling to a directly bonded hydrogen. All $^{15}N$ chemical shifts are reported in p.p.m. downfield from the reference signal.

Brinkmann silica-gel thin-layer glass plates were used for analytical t.l.c. Disulphides were detected by spraying the plates with neutral aqueous silver nitrate.[24] Column chromatography using hydrophobic Hyflosupercel,[25] was carried out as previously described.[2]

*Production and Isolation of* $^{15}N_6$*-Chetomin.*—A small piece of a culture of *Chaetomium cochliodes* (HLX 833 *) growing on 2% (w/v) malt agar (Difco) was inoculated into each of 9 flasks containing malt extract medium [2% (w/v), 9 × 50 ml, Difco]. The flasks were incubated on the shaker for 4 days, the cultures combined, stirred at room temperature in a Waring blender for 1 min and the slurry (1 ml) used to inoculate production flasks (53 × 200 ml) containing the following medium: sodium nitrate [99 atom % $^{15}N$ (Merck, Sharpe and Dohme, Montreal), 2.44 g], dipotassium hydrogen phosphate (1 g), magnesium sulphate (0.5 g), potassium chloride (0.5 g), calcium carbonate (3 g), glucose (30 g), trace metal solution[26] (10 ml), and water (1 l). The flasks were incubated on the shaker for 14 days, combined, and the mycelium collected, mixed with ice (500 g), and mascerated in a Waring blender for 2 min. The lyophilised slurry (52.4 g) was extracted with benzene (4 × 200 ml), by stirring it in the Waring blender, and the combined benzenoid extracts were evaporated. The residue (0.9 g) was digested at room temperature with methanol (100 ml), light petroleum (b.p. 30—60 °C, 100 ml), and water (9 ml). The mixture was filtered and the methanol phase extracted with light petroleum (× 3). The methanol phase was evaporated, and the residue (0.1 g) chromatographed on hydrophobic Hyflosupercel as described[2] (column 18 × 2.5 cm, precision bore, solvent: benzene–chloroform–methanol–water 15 : 15 : 23 : 7, column dead volume 40 ml, flow rate 6 ml $min^{-1}$, head pressure 2 kg $cm^{-2}$). Fractions were collected after the appearance of Methyl Orange in the eluate as the solvent (upper phase) above the column descended each 6 cm. The following weight distribution was obtained (in mg): 1, 10.40; 2, 3.19; 3, 2.79; 4, 35.62; 5, 18.56; 6, 2.49; 7, 1.27; 8, 0.73; and 9, 0.61 (recovery 76%). Fractions 4 and 5 were combined, digested with ether (1 ml), and the residual $^{15}N_6$-chetomin had the following properties: minimum inhibitory concentration *vs. Bacillus subtilis*,[27] 0.1 μg/ml; $E_1^{1\%}{}_{cm}$ (MeOH) 133; $[\alpha]_D^{24}$ +258° (*c* 0.095, $CHCl_3$).

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* Accession number to the culture collection held at this laboratory.

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[22] J. B. Grutzner and R. E. Santini, *J. Magnetic Resonance*, 1975, **19**, 173.
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[27] D. Brewer, D. E. Hannah, and A. Taylor, *Canad. J. Microbiol.*, 1966, **12**, 1187.