CALBISTRINS, NOVEL ANTIFUNGAL AGENTS PRODUCED BY *Penicillium restrictum*

II. ISOLATION AND ELUCIDATION OF STRUCTURE

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The novel antifungal agents, calbistrins A, B, C and D have been isolated from a strain of *Penicillium restrictum* (AB 1875C-28). The four congeners were separated by bioactivity directed fractionation using countercurrent chromatography and preparative-HPLC. NMR studies revealed that the calbistrins each contain a carboxylic acid conjugated tetraene attached through an aliphatic ester linkage to a hexahydronaphthalene system.

In the course of screening microorganisms for the production of bioactive metabolites, a penicillium was discovered which produced potent activity against *Candida albicans*. Bioactivity directed fractionation of a fermentation extract provided four new related antifungal agents. The majority of the bioactivity was found in the mycelia, which was filtered off and extracted with acetone. The concentrated extract was partitioned between ethyl acetate and water, $CHCl_3$-MeOH-H_2O and methanol-*n*-heptane. The four calbistrins were recovered as a mixture from the methanol solubles by droplet countercurrent chromatography. They were then separated and further purified by preparative-HPLC using a silica-bonded phenyl column.

High resolution FAB mass spectrometry in the positive ion mode gave an $(M+Na)^+$ parent mass of 563.2624 for calbistrins A and B, and 565.2774 for C and D; indicating molecular formulae for A and B of $C_{31}H_{40}O_8$ and for C and D of $C_{31}H_{42}O_8$. NMR studies revealed that the calbistrins each contain a carboxylic acid conjugated tetraene which differentiates the isomers A from B, and C form D. In each compound this conjugated olefin is attached through an aliphatic ester linkage to a hexahydronaphthalene system. In calbistrins A and B, the hexahydronaphthalene system is fused with a cyclic hemiacetal. In calbistrins C and D this group has been reduced to the open ring diol. Calbistrins A and B exhibit potent activity against *Candida albicans* and some related species. Calbistrins C and D are less active. The structural characterization of the calbistrins is outlined in this paper. Microbiological and fermentation data are presented in a comparison paper.[1)]

Isolation

The isolation of the calbistrins was guided by the use of disc diffusion bioassays on agar plates seeded with *Candida albicans*. Ninety liters of whole beer was filtered through glass wool impregnated paper and the filter cake was dispersed and soaked in sixteen liters of acetone for 16 hours (at 4°C). After additional stirring, the mixture was filtered through glass wool impregnated paper and the filter cake was washed with an additional eight liters of acetone. The acetone extract was concentrated on a circulating flash evaporator until approximately four liters of residual aqueous acetone remained. This was extracted with

three equal volumes of ethyl acetate. The ethyl acetate extracts were combined and concentrated to a residue which was then partitioned in the solvent system MeOH - H_2O - $CHCl_3$ (5 : 2 : 5, total volume 800 ml), the upper phase being washed with four portions of the lower. The lower layers were combined and concentrated to an oily residue. The material was then partitioned between methanol and *n*-heptane (a total of 800 ml), the methanol layer being washed with four 300~400 ml portions of the *n*-heptane.

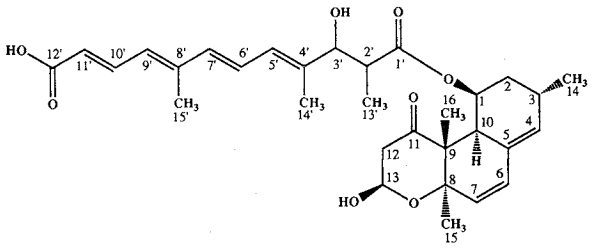
The active methanolic concentrate was chromatographed in two equal portions, with the solvent system MeOH - 50 mM NH_4OAc (pH 5.0) - $CHCl_3$ - CCl_4 (10 : 4 : 5 : 5), using a droplet countercurrent with the lower phase as the stationary phase. This custom-made device consists of 100 Teflon loops with an approximate total volume of 450 ml. 90~95% of this volume is retained during use as stationary phase when a flow rate of 1~1.5 ml/minute is maintained. The active material from the two chromatographies, fractions 70~170 and 66~124 (10~12 ml in each fraction), was combined and rechromatographed in two portions, with the solvent system $CHCl_3$ - CCl_4 - MeOH - 50 mM NH_4OAc (pH 5.0) (3 : 7 : 10 : 4), on the same instrument with the upper phase as the stationary phase. Using chromatographic parameters similar to those of the previous chromatography, active material was eluted in fractions 30~120 of the first run and 52~104 of the second.

The active concentrate, containing 3.24 g of a mixture of the calbistrins, was purified by preparative HPLC in 500 mg aliquots dissolved in 1.8 ml of CH_3CN. The chromatography hardware consisted of a SepTech ST/LAB 800C preparative chromatograph and a Prochrom LC 50 column, containing 250 g of YMC phenyl packing (15 μm), packed under an axial pressure of 50 bar. The mobile phase was pumped as a binary system at a rate of 50 ml/minute and consisted of a 45 minute linear gradient, starting with CH_3CN - 2% HOAc/H_2O (20 : 80) and ending with CH_3CN - H_2O (80 : 20). The final solvent proportions were delivered at 50 ml/minute for an additional 15 minutes before reequilibrating the column. The effluent was monitored by UV detection at 275 nm. One major peak and three minor peaks were observed and collected as tight cuts to maximize purity. Each was reinjected on the column and recollected to obtain compounds suitable for structure elucidation and biological testing. Each 500 mg run afforded 33.7 mg of the major (t_R 36.3 minutes) which was characterized as calbistrin A. The minors from each run (t_R 35.1 minutes, 37.0 minutes and 38.0 minutes) were characterized as calbistrin C (9.3 mg), calbistrin D (1.7 mg) and calbistrin B (12.5 mg), respectively.

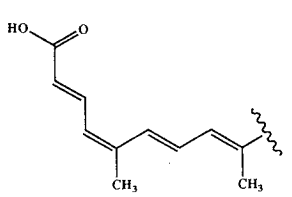
Characterization

The calbistrins are a novel family of antifungal agents (Fig. 1). They are soluble in common organic solvents such as dimethyl sulfoxide, methanol, ethanol, acetonitrile, acetone, ethyl acetate, chloroform and benzene. High resolution fast atom bombardment (FAB) mass spectrometry in the positive ion mode gave an $(M+Na)^+$ parent mass of 563.2624 (calc. 563.2621) for calbistrin A, indicating a molecular formula of $C_{31}H_{40}O_8$. Calbistrin B gave an identical $(M+Na)^+$ parent mass of 563.2624 (calc. 563.2621) also indicating a molecular formula of $C_{31}H_{40}O_8$. The $(M+Na)^+$ parent mass of calbistrin C and calbistrin D were identical to each other; 565.2774 (calc. 565.2777) indicating $C_{31}H_{42}O_8$. Each of the compounds contains an acid conjugated substituted tetraene chromophore and a cyclic diene chromophore. The characteristic ultraviolet absorption spectra of calbistrins A~D in methanol reveal both chromophores, with the heteroannular diene giving rise to an absorption maximum at approximately 237 nm, having extinction coefficients in the range of 19,800~22,800 (Table 1). The all *trans* tetraene of calbistrins A and C give rise to an absorption at approximately 330 nm in methanol, having extinction coefficients of 42,500 and

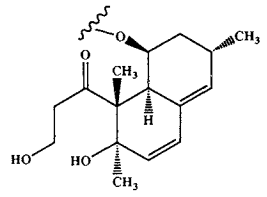
Fig. 1. The structures of calbistrins A~D.



Calbistrin A



Calbistrin B and D



Calbistrin C and D

Table 1. Ultraviolet (UV) absorption spectra maxima of the calbistrins in methanol.

	Calbistrin A λ_{max} nm (ε)	Calbistrin B λ_{max} nm (ε)	Calbistrin C λ_{max} nm (ε)	Calbistrin D λ_{max} nm (ε)
MeOH	237 (22,300), 331 (42,500)	237 (22,800), 330 (29,400)	237 (19,800), 329 (52,400)	238 (21,800), 331 (32,600)
0.1 N NaOH - MeOH	237 (24,300), 324 (52,900)	237 (23,200), 320 (38,300)	237 (20,000), 326 (53,400)	237 (22,000), 322 (38,400)
0.1 N HCl - MeOH	237 (25,700), 339 (42,900)	237 (22,600), 338 (28,700)	237 (18,600), 342 (47,200)	238 (21,200), 338 (32,300)

52,400, respectively. In the isomerized compounds B and D, this band is at very much the same wavelength, but of considerably reduced intensity (ε=29,400 and 32,600, respectively). These changes are similar to those found in carotenoid-like compound *cis-trans* isomerizations[2]. The IR spectra for the calbistrins are presented in Fig. 5a~d.

Structure Determination

Each of the calbistrins has a carboxylic acid conjugated tetraene which differentiates the isomers A from B, and C from D. In each compound this conjugated olefin is attached through an aliphatic ester linkage to a hexahydronaphthalene system. In calbistrins A and B, the hexahydronaphthalene system is fused with a cyclic hemiacetal. In calbistrins C and D, this group has been reduced to the open ring diol.

^{1}H NMR experiments delineate three olefinic spin systems and also a single olefinic proton coupled into an aliphatic system. In the ^{1}H NMR of calbistrin A, two doublet signals, one at δ 5.89 (11′-H) and 6.30 (9′-H) are coupled into a signal at δ 7.70 (10′-H) (Table 2). A similar spin system is seen with doublets at δ 6.13 (5′-H) and 6.41 (7′-H) coupled into a signal at δ 6.74 (6′-H). ^{1}H NMR correlation spectroscopy (COSY) experiments show a coupling of a singlet methyl at δ 1.77 (14′-H_3) to 5′-H and a similar long range coupling of another singlet methyl at δ 2.04 (15′-H_3) to 9′-H. Proton chemical shifts indicate that the 9′-H through 11′-H spin system is polarized by an electron withdrawing group attached to C-11′. A

^{13}C-^{1}H heteronuclear multiple bond correlation spectroscopy (HMBC) experiment shows couplings between a carbonyl signal at δ 171.0 (C-12′) and the 10′-H and 11′-H protons (Fig. 2). In addition, a quaternary carbon signal seen in the ^{13}C NMR at δ 145.5 (C-8′) shows HMBC couplings into this spin system as well as to the singlet methyl 15′-H_3 (Table 3). Similarly, the quaternary signal at δ 141.2 (C-4′) shows HMBC couplings into the 5′-H through 7′-H spin system and the singlet methyl 14′-H_3. With the quaternary carbon C-8′ also showing HMBC couplings into the 5′-H through 7′-H spin system, the conjugated dimethyl substituted tetraene system is defined.

Two of the three remaining olefinic protons appear as a pair of coupled doublets at δ 6.00 and δ 5.64 (6-H and 7-H, $J_{6,7}=9.8$ Hz), and the third is a broad singlet at δ 5.74 (4-H). In the HMBC experiment,

Fig. 2. HMBC couplings of the quaternary carbons in calbistrin A.

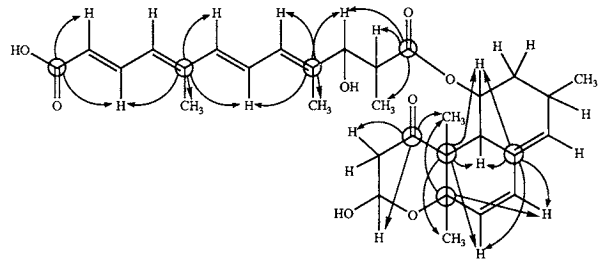


Table 2. ^{1}H NMR chemical shift data for the calbistrins[a].

Proton	Multiplicity	Calbistrin A	Calbistrin B	Calbistrin C	Calbistrin D
1	m	6.02	6.03	5.37	5.37
2a	m	2.19	2.21	2.06	2.07
2b	br dd	1.34	1.34	1.24	1.25
3	m	2.50	2.50	2.42	2.43
4	br s	5.74	5.73	5.65	5.65
6	d	6.00	5.99	5.97	5.97
7	d	5.64	5.64	5.39	5.39
10	m	2.89	2.89	3.13	3.13
12a	—	2.80 dd	2.81 dd	3.02 dt	3.02 dt
12b	—	2.39 dd	2.39 dd	2.78 dt	2.78 dt
13	dd	5.21	5.21	—	—
13a	m	—	—	3.81	3.81
13b	m	—	—	3.72	3.72
14	d	1.04	1.04	1.02	1.02
15	s	1.24	1.24	1.12	1.12
16	s	1.33	1.34	1.40	1.40
2′	m	2.52	2.53	2.54	2.54
3′	d	4.07	4.11	4.09	4.12
5′	d	6.13	6.19	6.14	6.22
6′	dd	6.74	6.70	6.74	6.74
7′	d	6.41	6.95	6.41	6.97
9′	d	6.30	6.15	6.30	6.18
10′	dd	7.70	7.80	7.70	7.84
11′	d	5.89	5.82	5.89	5.83
13′	d	0.89	0.92	0.89	0.91
14′	s	1.77	1.77	1.78	1.79
15′	s	2.04	2.02	2.04	2.03

[a] 500 MHz in CD_3OD at -5.0°C.

Table 3. ^{13}C NMR chemical shift and DEPT data for the calbistrins[a].

Carbon number	Carbon type[b]	Calbistrin A	Calbistrin B	Calbistrin C	Calbistrin D
1	CH	71.1	71.1	70.9	70.9
2	CH_2	36.2	36.2	36.6	36.6
3	CH	28.2	28.2	27.5	27.5
4	=CH	136.7	136.7	134.8	134.9
5	=C quat.	131.6	131.4	132.4	132.4
6	=CH	129.7	129.9	129.0	129.1
7	=CH	131.6	131.6	134.4	134.4
8	Q	78.7	78.7	75.3	75.3
9	Q	54.8	54.8	58.2	58.2
10	CH	41.1	41.1	42.0	42.1
11	C=O	210.9	210.9	215.3	215.3
12	CH_2	48.0	48.0	45.1	45.1
13	CH	92.5	92.5	—	—
13	CH_2	—	—	58.4	58.4
14	CH_3	21.3	21.4	21.5	21.5
15	CH_3	23.5	23.5	26.5	26.4
16	CH_3	18.4	18.4	14.3	14.3
1′	OC=O	176.6	176.6	176.9	177.0
2′	CH	45.7	45.7	45.6	45.6
3′	CH	81.1	81.0	81.2	81.2
4′	=C quat.	141.2	141.8	141.2	141.9
5′	=CH	129.8	129.8	129.7	129.8
6′	=CH	128.5	129.5	128.6	129.5
7′	=CH	137.9	129.7	137.9	129.6
8′	=C quat.	145.5	144.4	145.5	144.4
9′	=CH	129.8	128.2	129.8	128.2
10′	=CH	141.9	140.9	142.0	140.9
11′	=CH	122.0	121.2	121.8	121.2
12′	HOC=O	171.0	171.0	170.8	171.0
13′	CH_3	14.9	14.9	14.9	14.9
14′	CH_3	11.4	11.5	11.4	11.4
15′	CH_3	13.1	21.1	13.1	21.1

[a] 500 MHz in CD_3OD at −5.0°C.
[b] Based on ^{13}C distortionless enhancement by polarization transfer (DEPT) NMR experiments. Q: Saturated quaternary carbon.

a quaternary carbon at δ 131.6 (C-5) shows coupling to 6-H as does C-4 (δ 136.7). The diene thus constructed shows a COSY coupling from the 4-H proton to a broad multiplet at δ 2.50 (3-H) and a COSY coupling to a methine signal at δ 2.89 (10-H). Vicinal couplings can be followed from this broad doublet signal through an upfield methine at δ 6.02 (1-H) and then a methylene (δ 1.34, 2.19; 2b-H, 2a-H) back to the 3-H multiplet, which is also coupled to a methyl doublet at δ 1.04 (14-H_3). The remainder of the hexahydronaphthalene system is established by chemical shift considerations and observing the HMBC couplings of the quaternary carbon C-8 (δ 78.7) to protons 6-H and 16-H_3, in addition to the couplings of the quaternary carbon C-9 (δ 54.8) to 1-H, 7-H, 10-H and 15-H_3.

A ketonic carbonyl signal at δ 210.9 (C-11) shows HMBC coupling to exchangeable methylene protons at δ 2.39 and δ 2.80 (12b-H, 12a-H), and also to a methine at δ 5.21 (13-H) which is vicinally coupled to the methylene. In 2D ^1H nuclear Overhauser effect experiments (ROESY) on calbistrins A and B, 12a-H demonstrates an NOE to 16-H_3 and 13-H shows an NOE to 15-H_3. C-11 also shows a HMBC coupling to 16-H_3, and C-13 is found at δ 92.5 in the ^{13}C NMR. These observations are accommodated by

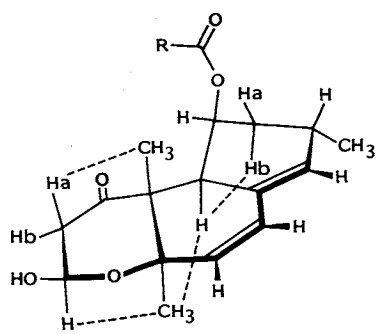
formulating a third ring, containing a cyclic hemiacetal fused to the hexahydronaphthalene system. In the ^{13}C NMR spectra of C and D, a new hydroxy-methylene signal appears at δ 58.4 with attached protons at δ 3.72 and δ 3.81, and there is no signal corresponding to the hemiacetal methine (C-13) in the vicinity of δ 92.5.

^{13}C NMR H→D carbon shift experiments were performed. In this technique, ^{13}C NMR spectra of a compound are electronically overlaid after acquisitions in a protic non-deuterated and then deuterated solvent are captured. Carbons which bear free exchangeable hydroxyl groups will show a recordable directional difference in chemical shift. Data from these experiments indicate that in all of the calbistrins there is a free hydroxyl on C-3′ ($\sim\delta$ 81), which bears a proton vicinally coupled to a methine at δ ~2.53 (2′-H). The only other coupling to this methine is from a methyl doublet at δ ~0.9 (13′-H_3). An NOE interaction is seen between 3′-H and 14′-H_3 as well as HMBC couplings between C-4′ and 3′-H, and an ester carbonyl at $\sim\delta$ 177 (C-1′) and H-2′/3′. Since the only carbon on the tricyclic ring portion to show a free hydroxyl (in calbistrins A and B) is C-13, and NOE interactions are between H-2′ and 16-H_3, the point of attachment of this aliphatic ester is determined as C-1. The carbonyl (C-12′) conjugated to the tetraene portion of the molecule, is seen as a free acid in the ^{13}C NMR H→D carbon shift experiment for all of the calbistrins. Using this technique on calbistrins C and D, a free hydroxyl is shown to be on C-8 of the hexahydronaphthalene system. These data, combined with ^1H NMR and mass spectral studies, lead to the identification of calbistrins C and D as the open ring reduction analogs of A and B, respectively.

Stereochemical Considerations

Although a crystal structure is not available at this time, tentative relative stereochemical assignments can be made on the basis of ROESY data and coupling constant considerations for the calbistrins. A *trans* to *cis* isomerization of the C-8′/C-9′ double bond on the tetraene moiety is the single difference between calbistrins A (C) and B (D). In calbistrin A, $J_{6',7'}$ and $J_{10',11'}$ = 14.9 Hz, $J_{5',6'}$ = 11.9 Hz, and $J_{9'10'}$ = 12.4 Hz. In the ROESY, strong interaction is seen between 6′-H and both 14′-H_3 and 15′-H_3, as well as a strong signal for the 10′-H/15′-H_3 and 7′-H/9′-H interaction. These data indicate that calbistrin A contains the all *trans* tetraene. In the data for calbistrin B, proton coupling constants differ little from that of A, since C-8′ is a quaternary carbon. However, a strong NOE interaction is seen between 7′-H and 10′-H, and the interaction between 7′-H and 9′-H disappears. An additional cross peak appears between 9′-H and 15′-H_3. This indicates that the C-8′/C-9′ double bond is in the *cis* conformation in calbistrin B, as is corroborated by the reduced extinction coefficient of the 330 nm absorption maxima in the ultraviolet absorption spectra of B as opposed to A.

Fig. 3. 1~3 diaxial NOE interactions of the calbistrin tricyclic moiety.



The stereochemical configuration of the hexahydronaphthalene ring system does not differ among the calbistrins. In ROESY experiments in MeOD and benzene titrated MeOD, the absence of

Fig. 4. The absolute stereochemistry of versiol.

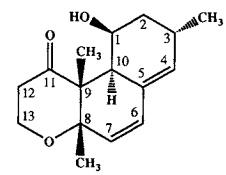


Fig. 5a. The FTIR spectrum of calbistrin A in $CDCl_3$.

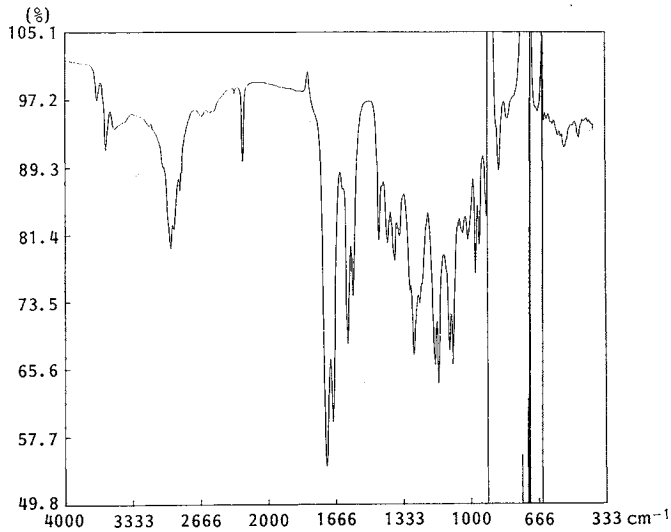
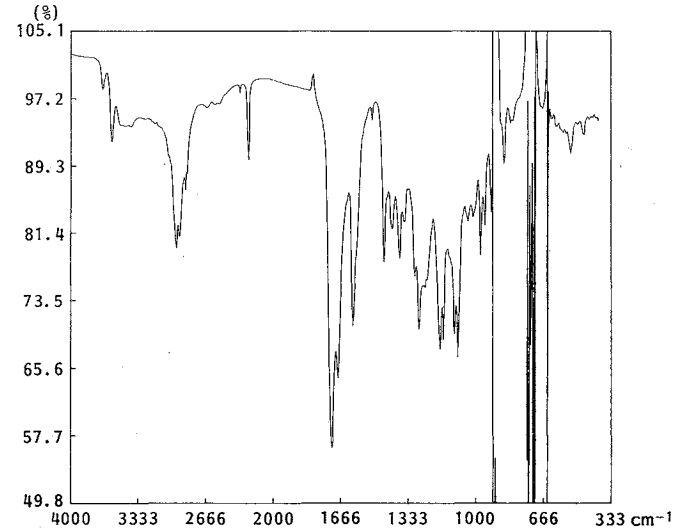


Fig. 5b. The FTIR spectrum of calbistrin B in $CDCl_3$.



a cross peak between the protons of the methyl groups 15-H_3 and 16-H_3, strongly suggest that these methyl groups are in the *anti*-orientation (Fig. 3). In addition, 12a-H shows a 1~3 diaxial NOE with 16-H_3, and 13-H exhibits a 1~3 diaxial NOE with 15-H_3 (calbistrins A and B). Since 12a-H shows a diaxial vicinal coupling of 8.7 Hz to 13-H, it is further evidenced that the bridgehead methyls are *anti*. The data show a vicinal coupling constant of less than 4 Hz between the 10-H multiplet and 1-H. 1~3 diaxial NOEs are seen between 10-H and both 2b-H and 15-H_3. 2b-H shows one large axial-axial vicinal coupling to 3-H ($J_{2b,3}=12.4$ Hz) and only couplings of less than 2 Hz are seen between the 2-H_2 protons and the 1-H multiplet. Therefore, 10-H, 1-H, 2b-H and 14-H_3 were determined to all be on the same side of the hexahydronaphthalene ring system as 15-H_3, with 10-H and 2b-H axial and 1-H equatorial.

Fig. 5c. The FTIR spectrum of calbistrin C in $CDCl_3$.

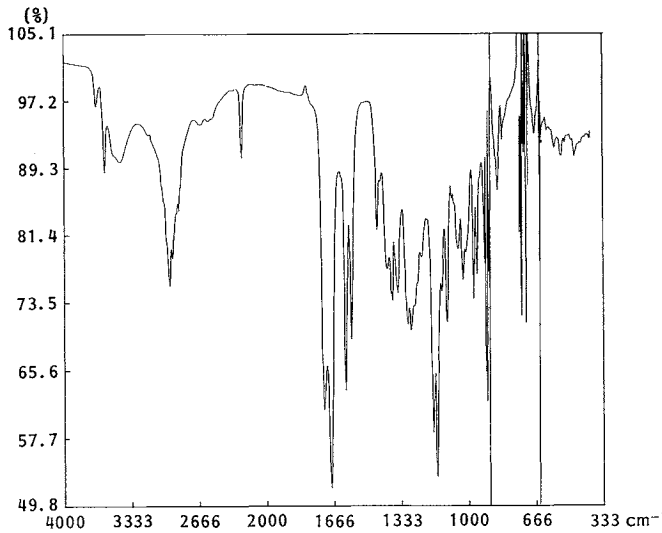
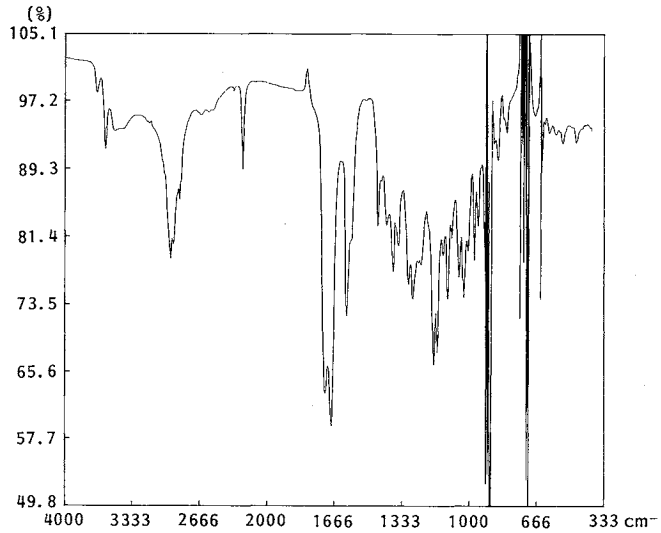


Fig. 5d. The FTIR spectrum of calbistrin D in $CDCl_3$.



The structure thus deduced for the calbistrins is defined except for the stereochemistry at C-2′ and C-3′, and for the absolute stereochemistry of the cyclic moiety. It presumably arises as two separate polyketide chains, although no biosynthetic studies have been done. The closest similar microbial metabolite is versiol (LL-N313ζ) isolated originally by McGAHREN *et al.* from *Sporormia affinis*[3], by FUKUYAMA *et al.* from *Aspergillus versicolor*[4] and more recently by KAWAHARA *et al.* from *Aspergillus silvaticus*[5]. Versiol has been the subject of a stereochemical revision[6], and X-ray structural determination defined the absolute stereochemistry as shown (Fig. 4). As such, the stereochemistry at the C-8 position is opposite of that for C-8 in the calbistrins (in a relative sense when compared with other substituents of the tricyclic system). Versiol also differs from the tricyclic moiety of the calbistrins in the oxidation state of C-13.

Experimental

NMR spectra were acquired employing a Varian Unity 500 spectrometer. Mass spectra were recorded on a Kratos MS50 spectrometer in the positive FAB mode (xenon/8kV) using a matrix of 3-nitrobenzyl alcohol-MeOH. UV spectra were recorded in methanol on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, and IR spectra on a Nicolet 5SXC FT-TR instrument in $CDCl_3$ solution.

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References

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