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CAF-603: A NEW ANTIFUNGAL CAROTANE SESQUITERPENE. ISOLATION AND STRUCTURE ELUCIDATION

NAOHARU WATANABE*[1], MICHIO YAMAGISHI, TAKU MIZUTANI, HIDEAKI KONDOH, SADAFUMI OMURA, KAZUNORI HANADA, and KATSUHIKO KUSHIDA[2]

Research Institute, Taisho Pharmaceutical, 1-403, Yoshino-cho, Omiya-shi, Saitama-ken, 330, Japan

ABSTRACT.—A new antifungal compound, CAF-603, was isolated from the culture broth of *Gliocladium virens* IFO 9166. The structure of compound **1** has been elucidated by spectroscopic methods that also allowed the relative stereochemistry to be assigned. CAF-603 [**1**] was active against yeasts and dermatophytes.

During the course of our screening program for antifungal compounds, a fungus, *Gliocladium virens* IFO 9166, was found to produce a novel antifungal carotane-type sesquiterpene, CAF-603 [**1**]. Here we describe the isolation and structure elucidation of **1**, based on spectral data including C-H COSY, 2D-INADEQUATE, and differential nOe studies. The antifungal activity of **1** is also discussed.

RESULTS AND DISCUSSION

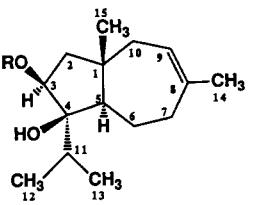
The *n*-hexane fraction obtained from the filtrate of culture broth was fractionated on a Si gel column (C_6H_6/Me_2CO). The eluate showing antifungal activity was further purified by preparative hplc (ODS, 63% MeCN); after evaporation of the solvent, it yielded an antifungal compound CAF-603.

CAF-603 [**1**] had a molecular formula $C_{15}H_{26}O_2$ from the hrms data *m/z* 238.1907 $[M]^+$ (calcd 238.1909).

The ^{13}C-nmr spectrum (Table 1), the insensitive nuclei enhanced by polarization transfer (INEPT) experiment, and the ^1H-^{13}C shift correlation 2D nmr spectrum (C-H COSY) of **1** indicated a carbon count of 15 carbons and a hydrogen count of 24 carbon-bound hydrogens. This was consistent with the ms empirical formula and also showed the presence of 2 exchangeable hydrogens (-OH) and 3 degrees of unsaturation/rings. The carbon types included 4 × Me, 4 × CH_2, 2 × CH, 1 × O-CH, 1 × C, 1 × O-C, 1 × CH=, and 1 × C=, accounting for one degree of unsaturation and two rings.

Acetylation of **1** in pyridine/Ac_2O afforded its monoacetate **2**, eims *m/z* $[M]^+$ 280; ^1H-nmr δ ($CDCl_3$) 2.09 (3H, s, CO*Me*), 2.22 (1H, broad s, 4-OH), 4.04 (1H, dd, 5.5, 1.0, H-3), indicating that one of the hydroxyl groups was tertiary.

Analyses of ^1H, ^{13}C, and ^1H-^1H correlated 2D-nmr spectra of **1** in $CDCl_3$ defined four partial structures **A**, **B**, **C**, and **D** as shown in Figure 1. The remaining carbons



1 R=H
2 R=Ac

[1]Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836, Ohya, Shizuoka-shi, Shizuoka-ken, 422, Japan.

[2]Varian Instruments Ltd.

TABLE 1. ^{1}H- and ^{13}C-nmr Chemical Shifts of CAF-603 [**1**] in $CDCl_3$.

Position	δ H [multiplicity, *J* (Hz)]	δ C	Multiplicity
1	—	42.3	s
2	H_a 1.39 (dd, 14.0, 7.1) H_b 1.57 (dd, 14.0, 1.8)	50.3	t
3	3.77 (dd, 7.1, 1.8)	72.4	d
4	—	84.8	s
5	1.32 (dd, 7.9, 5.0)	58.4	d
6	1.54 (m)	20.8	t
7	1.99 (m)	34.5	t
8	—	139.5	s
9	5.42 (m)	122.5	d
10	H_a 1.78 (dd, 8.8, 14.0) H_b 1.93 (dd, 8.8, 14.0)	42.8	t
11	1.66 (sep. 6.9)	35.2	d
12	0.93 (d, 6.9)	17.1	q
13	0.74 (d, 6.9)	17.8	q
14	1.73 (broad s)	27.4	q
15	1.19 (s)	21.1	q
3-OH	2.08 (broad s)	—	
4-OH	2.42 (broad s)	—	

were as shown in fragments **E–G**. The ^{1}H- and ^{13}C-nmr spectral data of **1** are given in Table 1.

An ir absorption at 3620 cm^{-1} and 3520 cm^{-1} ($\Delta\nu = 100$) in a diluted solution of dried CCl_4 indicated the presence of hydrogen-bonded vicinal hydroxy groups in **1** (1); thus the partial structures **B** and **F** should be connected at C-3 and C-4.

The complexity of the signals due to methines and methylenes in ^{1}H nmr of **1** precluded a direct assignment of its structure. Consequently the analysis of ^{13}C-^{13}C-correlated 2D-nmr spectrometry (INADEQUATE) of **1** was embarked upon, as shown in Figure 2.

The carbon skeleton of **1** disclosed by INADEQUATE, except the connection between C-8 and C-9, is depicted in Figure 3. Thus, **1** was shown to be a novel carotane sesquiterpene diol.

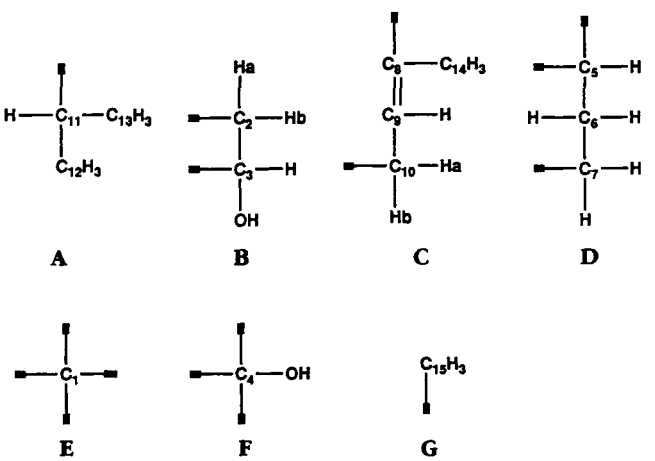


FIGURE 1. Substructures and fragments of CAF-603 [**1**] inferred from the 2D ^{1}H-^{1}H COSY, 2D ^{1}H-^{13}C COSY, and ^{13}C, ^{1}H-nmr spectra.

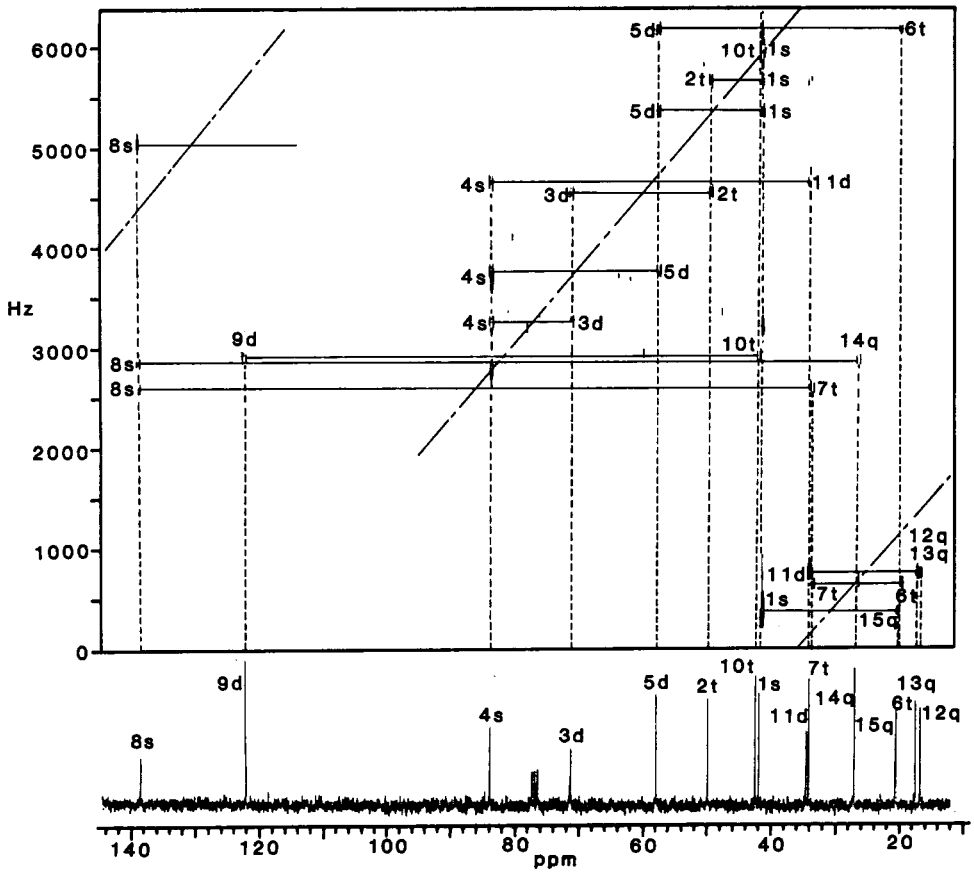


FIGURE 2. 2D INADEQUATE spectrum of CAF-603 [1].

The relative stereochemical features of **1** were resolved by nOe measurements as shown in Figure 4. Irradiation of the gem dimethyl group at δ 0.74 (H_3-12) and 0.93 (H_3-13) resulted in the enhancement of both the H-5 (δ 1.32) and H-3 (δ 3.77) resonances. A clear nOe enhancement was observed between H-3 and H_a-2 (δ 1.39) and not with H_b-2 (δ 1.57). These results indicated that H_a-2, H-3, and H-5 were on the same side of the molecule as the isopropyl group at C-4 and that H_b-2 was located on the opposite face.

Irradiation of H_3-15 (δ 1.19) gave nOe's with H_b-2 and H_b-10 (δ 1.93), which were thus on the same face of the molecule. No nOe was observed with H-5 which was lo-

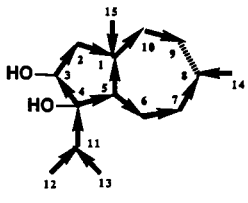


FIGURE 3. C–C Connectivities of CAF-603 [1] confirmed by INADEQUATE. ←: observed correlation; IIIIIII: incomplete result.

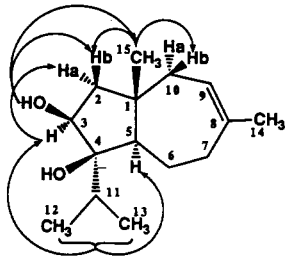


FIGURE 4. Observed nOe enhancements of CAF-603 [1].

cated on the opposite face. This result indicated that 5- and 7-membered rings were trans-fused.

The relative stereochemistries of C-1, C-2, and C-3 were further confirmed by the nOe's between the 3-OH frequency at δ 2.08, H_b-2, and H_3-15.

These experiments were fortuitous in that they successfully related the relative stereochemistries of six carbon atoms (C-1, -2, -3, -4, -5, and -10). Thus the stereochemistry of **1** was deduced.

To the best of our knowledge this is the first instance of the natural occurrence of a carotane derivative carrying an oxygen atom at C-3 rather than the more usual 2, 5, 6, or 10 positions (2–9). The carotane sesquiterpenes previously isolated were derived from the plant kingdom. This is the first isolation of a carotane sesquiterpene, to our knowledge, from a fungus species.

The antifungal activities of **1** were determined by agar-dilution methods as shown in Table 2. Compound **1** shows remarkable antifungal activity against *Candida albicans* strains, while the antifungal activities against the other *Candida* species, yeasts, and dermatophytes are weaker than those of miconazole, which was used as the positive control. Chemical modification of **1** presently under way will elucidate the essential component necessary for its antifungal activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp was determined on a Yanagimoto micropoint apparatus and is uncorrected. The following instruments were used: optical rotations, Jasco DIP360 polarimeter; uv, Hitachi 220 spectrophotometer; hplc, Waters 6000A with a uv detector; eims and hreims, Hitachi M-80-M-003 system; nmr, JEOL GX-400 or Varian XL-300 with TMS as an internal reference.

TABLE 2. Antifungal Activities of CAF-603 [**1**] and Miconazole Nitrate (MCZ).

Fungus strain	MIC (μg/ml)			
	Medium A		Medium B	
	CAF-603	MCZ	CAF-603	MCZ
Candida albicans IAM 4888[a]	12.5	6.25	1.56	3.13
C. albicans NHL 4019[b]	0.8	0.05	0.4	<0.05
Candida guiliermondii JTD[c]	>100	6.25	>100	3.13
Candida krusei IAM 4801[a]	25	0.8	25	0.8
Candida tropicalis IAM 4924[a]	>100	12.5	50	6.25
C. albicans MCNH-1[d]	6.25	12.5	3.13	12.5
C. albicans MCNH-2[d]	3.13	6.25	1.56	12.5
C. albicans MCNH-3[d]	12.5	3.13	3.13	12.5
C. albicans MCNH-4	6.25	6.25	1.56	6.25
Saccharomyces cerevisiae IAM 4500[a]	>100	3.13	>100	12.5
S. cerevisiae IAM 4203[a]	3.13	6.25	1.56	1.56
Torulopsis glabrata OUT 6189[e]	50	6.25	—[f]	—[f]
Aspergillus fumigatus IAM 2400[a]	25	0.8	12.5	1.56
Aspergillus niger NHL[b]	100	3.13	12.5	3.13
Rhizopus arrhizus IAM 6052[a]	>100	0.4	>100	0.2

[a]Deposited in the Institute of Applied Microbiology, the University of Tokyo.
[b]Deposited in the National Institute of Hygienic Science, Ministry of Health and Welfare, Japan.
[c]Deposited in the Medical Center of Juntendo University, Japan.
[d]Deposited in the Medical Center of National Hospital, Japan.
[e]Deposited in the Department of Fermentation Technology, Faculty of Engineering, Osaka University, Japan.
[f]Microorganism was not grown on this medium.

^{13}C-^{13}C-CORRELATED 2D NMR (INADEQUATE).—A Varian XL-300 nmr spectrometer system was used. The sequence 90°-Δ-180°-Δ-90°-t1-90°-Acquisition was applied to a solution (140 mg/0.5 ml of $CDCl_3$, 5 mm sample tube). To generate double quantum coherence, Δ was chosen appropriate to a mean carbon-carbon coupling constant of 37.0 Hz (Δ = 6.76 msec). The spectral width was 10,000 Hz in F2 and 6400 Hz in F1.

A total of 64 increments with 512 transients were collected along with t1 dimension. During acquisition time (1024 msec), 2048 data points were collected. The 4096 × 512 data table was transformed along with t2 and t1 dimensions, respectively.

NOe DIFFERENCE SPECTRA.—NOe difference spectra were acquired by JEOL GX-400 nmr spectrometer according to the method described by Ishida *et al.* (10).

FERMENTATION.—Cultured *G. virens* IFO 9166, deposited in the Institute for Fermentation, Osaka, Japan, was stored as a stock suspension at −20° in a growth medium containing 12% sucrose solution. A thawed suspension (3 ml) was used to inoculate 100 ml of growth medium consisting of polypeptone 1% and glucose 4% in a 500-ml Sakaguchi's flask. After 96 h incubation at 30° on a reciprocal shaker, 50 ml of the resulting cell suspension was used to inoculate 3 liters of the fermentation medium consisting of polypeptone 1% and glucose 4% in a 5-liter fermentor (Marubishi Bioengi, Japan). The fermentation was carried out at 30° with aeration of 3 liters/min and agitation of 500 rpm for 96 h. The amount of antibiotic produced was determined by a paper-disk agar diffusion method using *C. albicans* NHL 4019, deposited in the Institute of Applied Microbiology, the University of Tokyo, Japan, as the test organism.

ISOLATION OF CAF-603 [**1**].—Filtrate (5.5 liters) from the culture broth of *G. virens* IFO 9166 was subjected to an HP-20 (Mitsubishi Kasei) column (column volume: Vt = 50 ml, equilibrated with H_2O). The active antifungal fraction was recovered from the Me_2CO-MeOH (1:1) (200 ml) eluate after washing with H_2O (200 ml). Extraction by *n*-hexane from the aqueous residue after evaporation of the solvent afforded the crude materials. The residue was purified by cc on a Si gel column [Kieselgel, Merck, C_6H_6-Me_2CO (96:4)] and preparative hplc (Develosil ODS: 25↦250 mm, Senshu Scientific, 63% aqueous MeCN) to yield 220 mg of CAF-603 [**1**] as colorless needles.

CAF-603 [**1**].—$C_{15}H_{26}O_2$; found C 75.54, H 10.84, requires C 75.58, H 10.99; mp 82–84°; hreims *m/z* 238.1908 for $C_{15}H_{26}O_2$ (calcd *m/z* 238.1909); eims *m/z* $[M]^+$ 238 (8.7), $[M-H_2O]^+$ 220 (8.3), $[M-Me-H_2O]^+$ 205 (5.0), $[M-2H_2O]^+$ 202 (10.1), $[M-Me_2CH]^+$ 195 (100.0), $[195-H_2O]^+$ 177 (79.5), $[177-H_2O]^+$ 159 (42.1); ir ν max (in dried CCl_4) cm^{-1} 3620, 3520; uv λ max (MeOH) end absorption; $[\alpha]^{25}D$ −26.2° (*c* = 0.5, MeOH); ^1H nmr ($CDCl_3$) and ^{13}C nmr ($CDCl_3$) see Table 1.

ANTIMICROBIAL ACTIVITY.—The MICs were determined by the agar dilution method. Sabouraud Agar (Medium A) (Eiken) and Yeast Morphology Agar (Medium B) (Difco Laboratories) were used for all assays. Overnight cultures of test organism, except dermatophytes, were diluted to final concentrations of approximately 10^6 CFU/ml with buffered saline gelatin containing (g/liter): NaCl, 8.5; NaH_2PO_4, 0.6; KH_2PO_4, 0.3; gelatin, 0.1. The dermatophytes, 10^6 CFU/ml of spores collected from the slant cultures, were used for assays.

One loopful of diluted culture containing ca. 10^4 CFU was spotted with an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan) onto agar plates containing serial twofold dilutions of CAF-603 [**1**] and miconazole nitrate (Kyowa Japan).

The MIC was defined as the lowest concentration of antibiotic which prevented visible growth on agar after incubation for 24 h at 30°.

ACKNOWLEDGMENTS

We thank Mr. J. Kokatsu and Mr. S. Jingu for eims and high resolution eims analyses.

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ERRATA

For the paper by Guerriero *et al.* entitled "Hydroxyicosatetraenoic, Hydroxyicosapentaenoic, Hydroxydocosapentaenoic, and Hydroxydocosahexaenoic Acids from the Sponge *Echinochalina mollis* of the Coral Sea," *J. Nat. Prod.*, **53**, 57 (1990), the authors request the following addition and deletions:

On pages 57 and 61, the 12*R** stereochemical designation for compounds (+)-**1**, (+)-**3**, and (+)-**4**, as well as reference 6 on page 57, line 6, should be deleted.

On page 57 add "(12*R*)-HETE has also been reported to have endogenous origins (1)."

The absolute configuration of compound (+)-**1**, isolated as a semisynthetic derivative from the red seaweed *Murrayella periclados*, has been extrapolated to be 12*S* from optical rotation data in comparison with (12*S*)-HETE methyl ester [M. Bernart and W.H. Gerwick, *Tetrahedron Lett.*, **29**, 2015 (1988)].

For the paper by Vasanth *et al.*, entitled "Isolation and Characterization of Vicodiol, a New Monoterpenediol from *Vicoa indica*," *J. Nat. Prod.*, **53**, 354 (1990), the following figure was omitted from the paper:

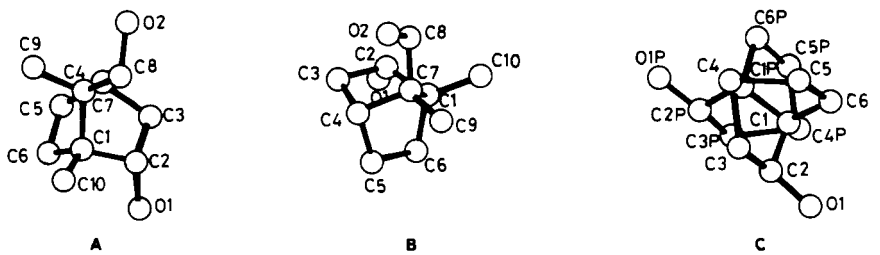

FIGURE 1. Stereoviews of molecules A, B, and C in the crystal structure of vicodiol.