L-696,474, A NOVEL CYTOCHALASIN AS AN INHIBITOR OF HIV-1 PROTEASE

II. ISOLATION AND STRUCTURE

J. ONDEYKA, O. D. HENSENS, D. ZINK, R. BALL[a], R. B. LINGHAM[b], G. BILLS[c], A. DOMBROWSKI[b] and M. GOETZ

Department of Natural Products Chemistry,
[a] Department of Biophysical Chemistry,
[b] Department of Microbial Chemotherapeutics and Molecular Genetics,
[c] Department of Basic Microbiology,
Merck Sharp and Dohme Research Laboratories,
Rahway, New Jersey 07065-0900, U.S.A.

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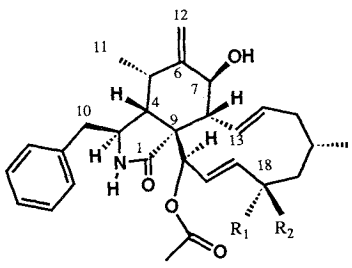
A novel HIV-1 protease inhibitor, L-696,474 ($C_{30}H_{39}NO_4$, 477), was isolated from the fermentations of the fungus *Hypoxylon fragiforme* (ATCC 20995, MF5511) and purified by silica gel chromatography followed by crystallization. Spectroscopic studies have shown the competitive inhibitor L-696,474 to be a novel cytochalasin. Two related novel cytochalasins were also isolated and had no effect on the enzyme.

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus type 1 (HIV), a retro-virus. HIV-1 protease is one of several key therapeutic targets due to its critical role in the life cycle of HIV. HIV protease activity is essential for viral replication and it can be assumed that identification of a compound capable of blocking this aspartyl protease could be an important step leading to a therapeutic agent for AIDS[1]. While screening for HIV protease inhibitors a novel cytochalasin L-696,474 (**1**) (Fig. 1) of MW of 477 and molecular formula of $C_{30}H_{39}NO_4$, was purified from *Hypoxylon fragiforme* (ATCC 20995), a fungus isolated from the bark of the American beech tree[2]. Two structurally related novel compounds L-696,475 (**2**) and L-697,318 (**3**) were also identified during the course of isolation but exhibit little if any activity against HIV-1 protease (Fig. 2).

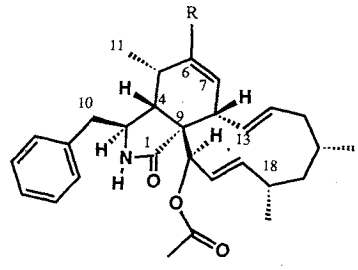
Cytochalasins are fungal secondary metabolites[3~7] that have been isolated from *Phomopsis*, *Chaetomium*, *Zygosporium* spp. and most recently from *Hypoxylon* sp.[8]. The structures of the novel cytochalasins (**1**~**3**) wre established on the basis of MS, NMR and X-ray crystallography. The relative

Fig. 1. Structure of L-696,474 (**1**) and cytochalasin H (**4**).



1 $R_1=CH_3$ $R_2=H$
4 $R_1=CH_3$ $R_2=OH$

Fig. 2. Structure of L-696,475 (**2**) and L-697,318 (**3**).



2 $R=CH_3$
3 $R=CH_2OH$

stereochemistry of **1** was determined to be the same as in cytochalasin H (**4**) (Fig. 1). This paper presents the isolation and spectroscopic and physical data used for determination of the structures; the following paper[9] will discuss the biological properties.

Materials and Methods

General Procedures

UV spectra, taken in methanol, were recorded on a Beckman DU-8 spectrophotometer. Mass spectra were recorded on a Finnigan MAT 90 in the electron impact (EI) mode at 70 eV. Exact mass measurements were performed at high resolution (HREI) using perfluorokerosene (PKF) as internal standard. Trimethylsilyl derivatives were prepared with a 1:1 mixture of bistrimethylsilyltrifluoroacetamide and pyridine (BSTFA-pyridine) at room temperature.

^{13}C NMR spectra were recorded in $CDCl_3$ at 100 MHz on a Varian XL400 spectrometer at 22°C. Chemical shifts are given in ppm relative to tetramethylsilane (TMS) at 0 ppm using the solvent peak at 77.0 ppm as internal standard. ^1H NMR spectra were recorded in $CDCl_3$ at 400 MHz on a Varian XL400 NMR spectrometer at 22°C. Chemical shifts are in ppm relative to TMS at 0 ppm using the solvent peak at 7.24 ppm as internal standard.

Fermentation

Fermentation and characterization of the producing culture (ATCC 20994) was described by BILLS and DOMBROWSKI[2].

Isolation

Isolation was accomplished by use of silica gel chromatography followed by crystallization techniques to produce L-696,474 (**1**). Silica gel 60 TLC (E. Merck) was employed for detection of compound **1** using a hexane-acetone (4:1) solvent system. Reverse phase HPLC was used to monitor fermentation titers as well as to assess the purity of L-696,474 (**1**). Chromatography was carried out on a C-18 bonded phase column (4.6 mm × 25 cm, Whatman Partisil-5 ODS-3) eluted with 60% aqueous acetonitrile at 1 ml/minute. Detection was by UV absorption at 213 nm (Micromerectics-788).

Results

Isolation

Four roller jars (4-liter) containing solid substrate fermentation were extracted with 500 ml each of methyl ethyl ketone for 2 hours at 100 rpm, and the extracts were filtered and combined. After removal

Table 1. Physical and spectroscopic data for compounds **1**~**3**.

		1	**2**	**3**
Appearance		White crystalline solid	White amorphous solid	White amorphous solid
MP		201°C		
Molecular formula		$C_{30}H_{39}NO_4$	$C_{30}H_{39}NO_3$	$C_{30}H_{39}NO_4$
Elemental analysis				
Calcd for $C_{30}H_{39}NO_4$:		C 75.5, H 8.2, N 2.9		
Found:		C 75.3, H 8.2, N 2.9		
EI-MS	Calcd:	477.2877	461.2929	477.2877
	Found:	477.2854	461.2911	477.2889
UV, λ_{max}		End absorption	End absorption	End absorption
TLC[a] (Rf)		0.25	0.30	0.20
HPLC[b] (K')		3.0	3.6	10.0

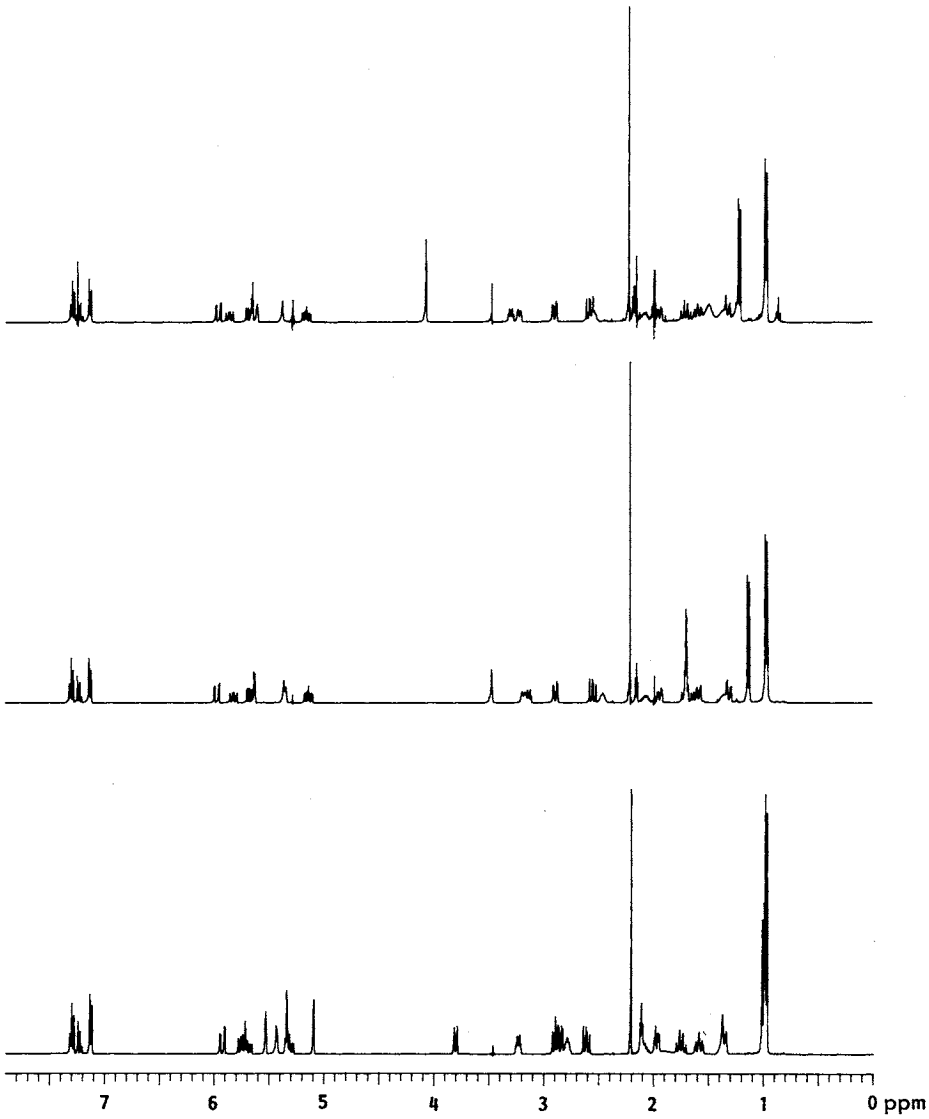
[a] E. Merck silica gel TLC: Hexane-acetone (4:1).
[b] Whatman Partisil-5 ODS-3, 70% aqueous acetonitrile at 1 ml/minute at 40°C, 213 nm.

of the solvent by evaporation, the residue was dissolved in 20 ml of acetone - methylene chloride (1 : 1). Fractionation proceeded by column chromatography on silica gel (500 ml) with 1 liter of hexane - acetone (4 : 1) followed by 500 ml each of the following solvents: hexane - acetone (3 : 1), (1 : 1), acetone and methanol. The chromatography was monitored by silica gel TLC using short-wavelength UV light and 50% sulfuric acid spray reagent for visualization. L-696,474 (**1**) was observed in the 3 : 1 and 1 : 1 fractions. These were pooled and taken to dryness to yield 600 mg of a white powder. This material was dissolved in methanol and allowed to crystallize. 300 mg of crystals were recovered and the purity determined by HPLC, TLC and ^{1}H NMR.

Compound **2** was detected in the 4 : 1 fractions from the above fractionation scheme, based on its UV and TLC color response, and 3 mg was isolated from a semipreparative C-18 HPLC system using

Fig. 3. ^{1}H NMR spectra of compounds **1**~**3** (400 MHz, $CDCl_3$).

Under: **1**, middle: **2**, upper: **3**.



65% aqueous acetonitrile delivered at 10 ml/minute with detection at 205 nm at ambient temperature. Compound **3** was detected in the 1 : 1 fractions from the above scheme and 10 mg was isolated by HPLC as above except using 60% aqueous acetonitrile as the solvent system.

Physico-chemical Properties

L-696,474 (**1**) was isolated as a white, crystalline compound. It was soluble in methanol, methylene chloride, DMSO, ethyl acetate, chloroform and acetone, but not soluble in water or hexane. The molecular formula of the compound was determined to be $C_{30}H_{39}NO_4$ by HREI-MS and elemental analysis (Table 1). Compounds **2** and **3** behaved similarly and the physico-chemical properties and chromatographic properties of compounds **1**~**3** are given in Table 1.

The UV spectrum of L-696,474 (**1**) exhibits only end absorption. The IR spectrum of **1** showed ester carbonyl at 1740 cm^{-1} and amide carbonyl at 1680 cm^{-1}. The ^{13}C NMR of **1** displayed 30 carbon resonances and the ^{1}H NMR (Fig. 3) had 25 distinct resonances integrating for 39 protons. The ^{1}H and ^{13}C NMR assignments of **1**~**3** are listed in Tables 2 and 3, along with those of cytochalasin H for comparison.

A structure of the novel HIV-1 protease inhibitor (**1**) was proposed based on its NMR and MS evidence and verified by X-ray crystallography (Fig. 1). The novel cytochalasin has a MW of 477 which corresponds to a molecular formula of $C_{30}H_{39}NO_4$. It formed a di-trimethylsilyl derivative and the

Table 2. ^{1}H NMR assignments for compounds **1**~**4**.

Proton	1	2	3	4
2	5.48 s	5.36 s	5.38 s	5.6
3	3.25 m	3.12 m	3.33 m	3.23 m
4	2.13 t	2.15 t	2.15 t	2.12 t
5	2.8 m	2.45 m	2.55 m	2.77 m
7	3.82 d (12)	5.35 m	5.6 m	3.82 d (10.8)
8	2.91 t	3.2 m	3.21 m	2.93 t
10a	2.85 dd (4.2, 13.3)	2.9 dd (3.8, 13.5)	2.9 dd (4.1, 13.5)	2.85 dd (4.4, 13.5)
10b	2.62 dd (9.8, 13.5)	2.55 dd (10.1, 13.5)	2.58 dd (10.0, 13.4)	2.64 dd (9.5, 13.3)
11	1.03 d (6.8)	1.13 d (9.4)	1.22 d (7.4)	1.0 d (6.7)
12a	5.12 s	1.72 s	4.1 s	5.15 s
12b	5.36 s			5.35 s
13	5.75 ddd (1.2, 9.6, 15.4)	5.82 ddd (1.5, 10.1, 15.2)	5.86 ddd (1.5, 10.0, 15.3)	5.75 ddd (1.2, 9.2, 15.6)
14	5.30 ddd (4.6, 10.6, 15.4)	5.14 ddd (4.5, 11.1, 15.4)	5.16 ddd (4.5, 11.1, 15.4)	5.40 ddd (5.4, 10.5, 15.6)
15a	2.0 m	1.95 m	1.95 m	2.0 m
15b	1.76 m	1.70 m	1.70 m	1.77 m
16	1.4 m	1.35 m	1.32 m	1.8 m
17a	1.35 m	1.60 m	1.60 m	1.86 dd (2.4, 14.2)
17b	1.6 m	1.31 m	1.32 m	1.56 dd (2.0, 14.0)
18	2.05 m	2.07 m	2.07 m	—
19	5.69 ddd (2.2, 7.1, 16.4)	5.66 ddd (2.3, 7.1, 16.4)	5.68 ddd (2.2, 7.0, 16.2)	5.56 dd (2.1, 13.2)
20	5.93 dd (2.3, 16.3)	5.97 dd (3.0, 16.5)	5.96 dd (3.3, 16.6)	5.86 dd (3.3, 13.6)
21	5.55 dd (2.4, 2.4)	5.63 d (3.0)	5.65 d	5.55 dd (2.4, 2.4)
22	1.02 d (6.8)	0.98 d (2.2)	0.98 d (2.1)	1.05 d (6.3)
23	1.0 d (6.8)	0.96 d (2.2)	0.98 d (2.1)	1.32 s
25	2.23 s	2.23 s	2.23 s	2.23 s
27, 31	7.14 d	7.14 d	7.14 d	7.14 d
28, 30	7.3 dd	7.3 dd	7.3 dd	7.3 dd
29	7.25 t	7.25 t	7.25 t	7.25 t

400 MHz chemical shifts (ppm) in $CDCl_3$.

fragmentation pattern suggested the presence of an acetate group, indicated by the base peak at m/z 418 (M^+-59), and a benzyl group with a major ion at m/z 386 (M^+-91) as well as an ion at m/z 91.

In the ^1H NMR spectrum there were 3 methyl doublets between 0.8 ppm and 1.1 ppm and an acetate methyl at 2.23 ppm (Fig. 3). Also present were olefinic protons at 5.69, 5.93, 5.75, and 5.30 ppm and exocyclic double bond protons at 5.17 and 5.36 ppm. ^1H-^1H connectivities were determined from COSY and decoupling experiments where as ^{13}C NMR assignments were obtained from HETCOR data. Compound **1** differs from cytochalasin H by one less hydroxyl group at C-18.

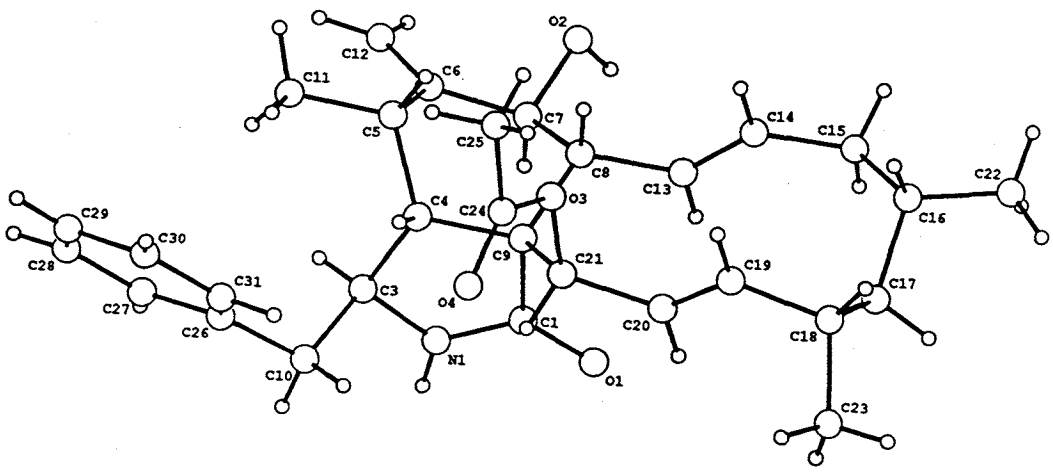
The proposed structure was shown to have the same relative stereochemistry as in cytochalasin H by X-ray crystallography[10,11]. The data were collected on an Enraf-Nonius CAD4 differctometer to a 2θ limit of 140° with 2,632 observed, $I>3\sigma(I)$, reflections out of 3,061 measured. Crystal structure details: $C_{30}H_{39}NO_4$, $M_r=477.65$, monoclinic, $P2_1$, $a=13.192$ (2), $b=7.360$ (3), $c=14.852$ (2) Å, $\beta=98.14$ (1°), $V=1427.5$ Å^3, $Z=2$, $D_X=1.111$ g/cm^3, monochromatized radiation λ(CuKα)$=1.54184$ Å, $\mu=0.54$ mm^{-1}, F(000)$=516$, T$=296$ K. The structure was solved by direct methods and refined using full-matrix least-squares on F. All non-hydrogen atoms were refined with anisotropic thermal displacements. Hydrogen atom contributions were included in calculations. Final agreement

Table 3. ^{13}C NMR assignments for compounds **1**~**4**.

Carbon	1	2	3	4
1	174.2	175.2	175.2	174.3
2	—	—	—	—
3	53.7	55.6	55.8	53.8
4	50.6	54.3	53.7	50.3
5	33.0	33.8	33.6	32.8
6	148.1	137.8	140.8	148.0
7	69.5	129.6	131.5	69.7
8	47.3	43.0	43.0	47.2
9	51.8	55.9	56.0	51.8
10	45.7	46.1	46.0	45.6
11	14.3	14.0	13.0	14.1
12	113.9	19.9	63.6	114.1
13	127.4	128.2	127.1	125.9
14	138.4	134.9	135.3	138.1
15	42.5	42.5	42.5	42.8
16	33.3	34.5	34.5	31.1
17	48.4	47.9	48.0	53.8
18	34.2	35.2	34.6	74.3
19	135.8	134.4	134.7	127.0
20	125.4	125.9	125.9	138.6
21	78.6	78.2	78.1	77.5
22	22.1	22.5	22.4	26.5
23	25.3	25.3	25.3	28.4
24	170.1	170.1	170.1	170.1
25	20.9	20.9	21.0	20.9
26	137.5	137.7	137.6	137.4
27, 31	129.0	128.9	129.0	128.9
28, 30	129.0	128.9	128.9	128.9
29	127.1	127.1	127.1	127.0

100 MHz chemical shifts (ppm) $CDCl_3$.

Fig. 4. X-Ray molecular structure of L-696,474 (**1**).



statistics are: R=0.041, wR=0.045, S=3.21, $(\Delta/\sigma)_{max}=0.02$. Weighting scheme is $1/\sigma 2(F)$. Maximum peak height in final difference Fourier map 0.17(3) eA-3 with no chemical significance. The atomic coordinates have been deposited with the Cambridge Crystallographic Data Centre.

The results of the crystallographic investigation are depicted in Fig. 4. The molecule displays no abnormal bond distances or angles and has a conformation very similar to that of cytochalasin H. There are two intermolecular hydrogen bonds: One between the carbonyl and hydroxyl of the isoindole structure and the second between the hydroxyl and the nitrogen of this same ring system.

Compound **2**, $C_{30}H_{39}NO_3$ *m/z* 461, has one less oxygen and forms a mono trimethylsilyl derivative. Compound **2** was determined to be a deshydroxy analog of **1** as indicated by an additional olefinic proton at 5.3 ppm (multiplet), one extra methyl signal at 1.72 ppm and the absence of the exocyclic methylene protons in the ^1H NMR (Fig. 3). This was supported by the ^{13}C NMR where C-7 moved upfield to 129.6 ppm and C-12 moved downfield to 19.9 ppm.

Compound **3**, $C_{30}H_{39}NO_4$ *m/z* 477, is isomeric with **1** and forms a ditrimethylsilyl derivative. The exocyclic methylene protons were absent in the ^1H NMR (Fig. 3) spectrum and the appearance of an olefinic proton at 5.6 ppm (multiplet) and a methylene singlet at 4.1 ppm indicated the presence of a CH_2OH. This was verified by ^{13}C NMR where C-12 shifted downfield to 63.6 ppm and C-7 shifted upfield to 131.5 ppm.

Discussion

L-696,474 (**1**) is a specific and competitive HIV-1 protease inhibitor[9]. Since the closely related anlogs (**2** and **3**) and cytochalasin H have no effect on the protease, it is tempting to speculate that the C-7 hydroxyl group in **1** is a critical element for activity, possibly through hydrogen bonding.

Several commercially available cytochalasins were assayed and only one, cytochalasin A, had activity[9]. Cytochalasin A contains a 14-membered lactone ring while compound **1** contains an 11-membered carbocyclic ring. This novel non-peptide inhibitor (**1**) of HIV-1 protease may represent an important lead towards the development of potent therapeutic agents.

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References

1) KOHL, N. E.; E. A. EMINI, W. A. SCHLIEF, L. J. DAVIS, J. C. HEIMBACH, R. A. F. DIXON, E. M. SCOLNICK & I. S. SIGAL: Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. U.S.A. 85: 4686~4690, 1988
2) DOMBROWSKI, A. W.; G. F. BILLS, G. SABNIS, L. R. KOUPAL, R. MEYER, J. G. ONDEYKA, R. A. GIACOBBE, R. L. MONAGHAN & R. B. LINGHAM: L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease. I. The producing organism and its fermentation. J. Antibiotics 45: 671~678, 1992
3) COLE, R. & R. COX: Handbook of Toxic Matoblites. pp. 289~293, Academic Press, 1981
4) BERDY, J: CRC Handbook of Antibiotic Compounds. Vol II. pp. 427~443, CRC Press, Inc., 1980
5) PENDSE, G. S.: Recent Advances in Cytochalasins. pp. 1~23. Chapman and Hall, 1986
6) IZAWA, Y.; T. HIROSE, T. SHIMIZU, K. KOYAMA & S. NATORI: Six new 10-phenyl-[11]cytochalasans, cytochalasins N-S from *Phomopsis* sp.: Tetrahedron 45: 2323~2335, 1989
7) HIROSE, T.; Y. IZAWA, K. KOYAMA, S. NATORI, K. IIDA, I. YAHARA, S. SHIMAOKA & K. MARUYAMA: The effects of new cytochalasins from *Phomopsis* sp. and the derivatives on cellular structure and actin polymerization. Chem. Pharm. Bull. 38: 971~974, 1990
8) EDWARDS, R.; D. MAITLAND & A. WHALLEY: Metabolites of the higher fungi. Part 24. Cytochalasins NOPQ

and R. New cytochalasins from the fungus *Hypoxylon terricola* Mill. J. Chem. Soc. Perkin Trans. I 1989: 57～65, 1989

9) LINGHAM, R. B.; A. HSU, K. C. SILVERMAN, G. F. BILLS, A. DOMBROWSKI, M. E. GOLDMAN, P. L. DARKE, L. HUANG, G. KOCH, J. G. ONDEYKA & M. A. GOETZ: L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease. III. Biological activity. J. Antibiotics 45: 686～691, 1992

10) MCMILLAN, J. A.; C. C. HIANG, M. K. GREENSLEY, I. C. PAUL, S. H. PATWARACHAN, S. DEV, M. A. BENO & G. G CHRISTOPH: X-ray crystal and molecular structure of kodo-cytochalasin-1. J. Chem. Soc. Chem. Commun. 1977: 105～106, 1977

11) BENO, M. A.; R. H. COX, J. M. WELLS, R. J. COLE, J. W. KIRKSEY & G. G. CRISTOPH: Structure of a new [11]cytochalasin, cytochalasin H or kodo-cytochalasin-1. J. Am. Chem. Soc. 99: 4123～4130, 1977