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

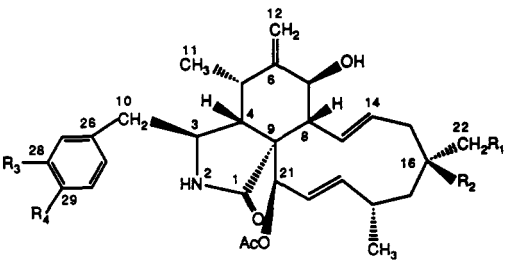
TOM S. CHEN,* GEORGE A. DOSS, ANNJIA HSU, AMY HSU, RUSSELL B. LINGHAM, RAYMOND F. WHITE, and RICHARD L. MONAGHAN

Merck Research Laboratories, Rahway, New Jersey 07065

ABSTRACT.—The microbiological transformation of L-696,474 [**1**], a novel cytochalasin that is an inhibitor of HIV-1 protease, was investigated using *Actinoplanes* sp. ATCC 53771. Six hydroxylated metabolites **2–7** of **1** were isolated and purified using reversed-phase hplc. All six metabolites were found to have undergone hydroxylation at the C-16 methyl group (C-22) of **1**. Three of the compounds, **3**, **4**, and **5**, were further hydroxylated at the para (C-29), the meta (C-28), and both the para and the meta, positions of the phenyl ring, respectively. Metabolites **6** and **7** were shown to result from vicinal dihydroxylation on both C-16 and its attached Me (C-22). The metabolite **7** was further hydroxylated on the meta position of the phenyl ring. The structures of the metabolites were established using spectroscopic techniques including ms, ^{1}H nmr, ^{13}C nmr, and various 2D nmr spectroscopy experiments.

A retrovirus designated human immunodeficiency virus (HIV) is the etiological agent of the complex disease that includes progressive destruction of the immune system (acquired immune deficiency syndrome; AIDS) a degeneration of the central and peripheral nervous system. A common feature of retrovirus replication is the extensive post-translational processing of precursor polyproteins by a virally encoded protease to generate mature viral proteins required for virus assembly and function. Interruption of this processing prevents the production of normally infectious virus and is therefore an attractive therapeutic target (1).

L-696,474 [**1**], a new cytochalasin (2) isolated from a fermentation broth of *Hypoxylon fragiforme* ATCC 20995 (3), was found to inhibit HIV protease activity with an IC_{50} of 3 μM, and the mode of inhibition was competitive with respect to substrate (4). In view of the complexity of the structure, we initiated studies on microbial modification of the compound in the hope of finding derivatives with increased potency and that would further extend structure-activity relationships. In this communication, we report the microbial hydroxylation of **1** by *Actinoplanes* sp. ATCC 53771.



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	OH	H	H	H
3	OH	H	H	OH
4	OH	H	OH	H
5	OH	H	OH	OH
6	OH	OH	H	H
7	OH	OH	OH	H

EXPERIMENTAL

CHEMICALS.—All organic solvents (EM Science; Gibbstown, NJ) were hplc grade. H_2O was purified in a Millipore Milli-Q system (Bedford, MA). Solvent for nmr analysis ($CDCl_3$) was purchased from MSD Isotopes. L-696,474 [**1**] was prepared at Merck Research Laboratories. The peptide substrate [^{3}H]-acetyl-Val-Ser-Gln-Asn-β-naphthyl-Ala-Pro-Ile-Val-Gln-Gly-Arg-Arg was synthesized according to the procedure of Merrifield (5) and labeled with [^{3}H]-Ac_2O (4).

NMR AND MS SPECTROSCOPY.—Nmr experiments were performed on either a Varian Unity 400 or VXR-500 NMR spectrometer, and chemical shifts were referenced to the $CDCl_3$ solvent peak ($\delta_H = 7.26$, $\delta_C = 77.0$ ppm). Fabms measurements were obtained on a Finnigan Mat TSQ 70 instrument.

PRODUCTION OF METABOLITES.—A frozen vial (2.0 ml) of *Actinoplanes* sp. ATCC 53771 was used to inoculate a 250-ml 3-baffled shake flask containing 50 ml of an autoclaved seed medium consisting of (in g/liter) dextrin 10.0, glucose 1.0, beef extract 3.0, Ardamine PH (Yeast Products, Inc.) 5.0, N-Z Amine type E 5.0, $MgSO_4 \cdot 7H_2O$ 0.05, K_2HPO_4 0.37, and $CaCO_3$ 0.5. The pH of the seed medium was adjusted to 7.1 before autoclaving. The seed flask was incubated on a rotary shaker (220 rpm) at 27° for 24 h. A 2.5-ml aliquot of the developed seed was used to inoculate a 250-ml non-baffled Erlenmeyer shake flask containing 50 ml of production medium. Production medium consisted of (in g/liter) glucose 10.0, Hycase SF 2.0, beef extract 1.0, corn steep liquor 3.0; pH was adjusted to 7.0 before autoclaving. Substrate **1** was added as a solution in MeOH to achieve a final concentration of 0.05 mg/ml. The shake flasks were subsequently incubated at 27° on a rotary shaker for 30 h.

ISOLATION AND PURIFICATION.—The whole broth (200 ml) was extracted with CH_2Cl_2 (3 × 200 ml). CH_2Cl_2 extracts were combined and concentrated under vacuum to an oily residue. The residue was dissolved in MeOH and subjected to hplc. Hplc was carried out on a Whatman Partisil 10 ODS-3 9.4 mm × 25 cm column at 45° and monitored at 215 nm. The column was developed at 3 ml/min with a linear gradient from 20% aqueous MeCN to 80% aqueous MeCN in 40 min. The compounds were collected during repeated injections of the above-described extract. The fractions at retention times 26.1, 27.6, 29.2, 30.0, 31.2, and 35.4 min, were pooled and evaporated to yield **5** (1.0 mg), **7** (0.9 mg), **3** (0.9 mg), **4** (1.3 mg), **6** (0.8 mg), and **2** (1.0 mg), respectively.

ASSAY OF HIV-1 PROTEASE ACTIVITY.—Compounds dissolved in MeOH were mixed with 1.05 μM [^{3}H]-acetyl-Val-Ser-Gln-Asn-β-naphthyl-Ala-Pro-Ile-Val-Gln-Gly-Arg-Arg and 2 nM HIV-1 protease in a final volume of 100 μl containing 100 mM NaOAc buffer, pH 5.5, and 0.05% bovine serum albumin. After 60 min at 37° the reaction was stopped with 100 μl of 5% H_3PO_4, and 150 μl of the reaction mix was loaded onto a 1.0 ml column of Dowex Ag50W-X8 resin (H^+ form). The column was washed with 1.85 ml of H_2O, and the ^{3}H-acetyl product was collected into scintillation vials for counting in a liquid scintillation counter. The IC_{50} for inhibition of HIV-1 protease activity by each compound was calculated using a 4-parameter algorithm.

RESULTS AND DISCUSSIONS

The antiviral compound L-696,474 [**1**], whose structural complexity makes chemical modification by organic synthesis difficult, is an ideal substrate for microbiological modification. Our goal was to provide metabolites of **1** in sufficient quantity for biological evaluation to better understand the structure-activity relationships of **1**. The microorganism *Actinoplanes* sp. ATCC 53771 was cultured on a transformation medium, and substrate **1** was added at the start. After a 30 h incubation at 27° on a rotary shaker, the hplc examination of whole broth showed the presence of six metabolites in addition to some unconverted substrate **1**. The products were separated by hplc on a reversed-phase C-18 column.

Positive ion fabms gave $[M + H]^+$ signals at *m/z* 494, 510, 510, 526, 510, and 510 for the microbial transformation metabolites **2**, **3**, **4**, **5**, **6**, and **7**, indicating that they are mono-, di-, di-, tri-, di-, and dihydroxylated derivatives, respectively. The final structure identifications were based on unambiguous ^{1}H and ^{13}C signal assignments of the metabolites **2–7** and comparison with the parent compound **1** (2). The ^{1}H assignments were supported by ^{1}H-^{1}H COSY and TOCSY experiments. All six metabolites were found to have undergone hydroxylation on the methyl group corresponding to C-22 of **1**. Three of the compounds, **3**, **4**, and **5**, have been further hydroxylated at the

TABLE 1. ^{1}H-nmr Spectral Data for Compounds **1–7**.[a]

Proton	Compound						
	1[b]	**2**	**3**	**4**	**5**	**6**	**7**
H-2	5.48	5.53 br s	5.43 br s	5.58 br s	5.45 br s	5.42 br s	5.49
H-3	3.25 m	3.27 m	3.21 m	3.36 m	3.23 m	3.28 m	3.37 m
H-4	2.13 t	2.14 m	2.12 m	2.13 m	2.12 m	2.15 t (4.4)	2.15 t (4.4)
H-5	2.8 m	2.81 br m	2.80 m	2.80 m	2.80 m	2.84 br m	2.84 br m
H-7	3.82 d (12.0)	3.85 d (11.0)	3.85 d (10.6)	3.84 d (10.5)	3.85 d (11.0)	3.86 d (11.0)	3.86 d (11.0)
H-8	2.91 t	2.95 t (10.3)	2.95 t (10.3)	2.95 t (9, 10.3)	2.94 t (9.9)	3.00 t (10.3)	3.00 t (10.3)
H_a-10	2.85 dd (4.2, 13.3)	2.88 dd (4.4, 13.5)	2.80 m	2.80 m	2.76 dd (2.5, 13.5)	2.90 dd (4.2, 13.6)	2.85 dd (4.2, 13.6)
H_b-10	2.62 dd (9.8, 13.5)	2.65 dd (9.3, 13.2)	2.57 dd (9.7, 13.3)	2.61 dd (9.7, 13.3)	2.61 dd (8.8, 13.5)	2.62 m	2.58 dd (8.8, 13.5)
H-11	1.03 d (6.8)	1.03[c] d (6.9)	1.02[c] d (6.6)	1.02 d (ca. 7.0)	1.02 d (ca. 7.0)	1.07[c] d (6.4)	1.07[c] d (6.6)
H_a-12	5.36 s	5.37 br s	5.37 br s	5.37 br s	5.37 br s	5.39 br s	5.40 br s
H_b-12	5.12 s	5.13 br s	5.12 br s	5.12 br s	5.12 br s	5.14 br s	5.14 br s
H-13	5.75 ddd (1.2, 9.6, 15.4)	5.84 ddd (15.7, 10.0, 1.0)	5.84 dd (10.7, 15.7)	5.83 dd (10.7, 15.7)	5.83 dd (10.0, 15.7)	5.90 ddd (15.6, 9.7, 1.3)	5.89 m
H-14	5.30 ddd (4.6, 10.6, 15.4)	5.23 ddd (5.2, 10.8, 15.6)	5.35 ddd (4.8, 10.8, 15.6)	5.35 ddd (4.8, 10.8, 15.6)	5.33 ddd (4.7, 10.5, 15.0)	5.59 ddd (4.2, 10.8, 15.2)	5.60 ddd (4.2, 10.8, 15.1)
H_a-15	2.0 m	2.29 dd (5.3, 12.8)	2.29 m	2.29 m	2.28 m	2.48 dd (4.3, 13.2)	2.48 dd (4.3, 13.2)
H_b-15	1.76 m	1.69 m	1.69 m	1.70 m	1.70 m	1.88 dd (11.0, 13.4)	1.85 dd (11.0, 13.4)
H-16	1.4 m	1.41 m	1.48 m	1.44 m	ca 1.46 m	—	—
H_a-17	1.35 m	1.69 m	1.69 m	1.70 m	1.70 m	1.83 dd (11.4, 14.1)	1.82 dd (11.4, 14.1)
H_b-17	1.6 m	1.48 m	1.48 m	1.47 m	ca 1.46 m	1.69 dd (2.9, 14.3)	1.69 dd (2.9, 14.3)
H-18	2.05 m	2.14 m	2.12 m	2.13 m	2.12 m	2.40 m	2.41 m
H-19	5.69 ddd (2.2, 7.1, 16.4)	5.72 ddd (7.1, 16.5, 2.6)	5.71 ddd (6.9, 16.5, 2.2)	5.71 ddd (6.9, 16.5, 2.2)	5.70 ddd (7.0, 16.5, 2.2)	5.80 ddd (7.1, 16.6, 2.0)	5.80 ddd (7.1, 16.6, 2.1)
H-20	5.93 dd (2.3, 16.3)	5.99 dd (2.6, 16.5)	5.99 dd (2.6, 16.5)	5.97 dd (2.6, 16.5)	5.95 dd (2.6, 16.5)	5.93 dd (3.3, 16.6)	5.91 dd (2.6, 16.5)
H-21	5.55 dd (2.4, 2.4)	5.57 br t (2.6)	5.55 br t (2.4)	5.53 br t (2.4)	5.47 br t (2.5)	5.55 m	5.52 br t (2.6)
H_a-22	1.02 d (6.8)	3.54 dd (6.2, 10.3)	3.53 m	3.54 m	3.53 m	3.37 tight AB	3.37 A of ABX
H_b-22	—	3.42 dd (7.2, 10.3)	3.43 m	3.42 m	3.42 m	3.37 tight AB	3.37 B of ABX
H-23	1.0 d (6.8)	1.04[c] d (7.0)	1.03[c] d (7.0)	1.02 d (ca. 7.0)	1.02 d (ca. 7.0)	1.06[c] d (6.9)	1.05[c] d (7.0)

TABLE 1. Continued

Proton	Compound						
	1[b]	2	3	4	5	6	7
H-25	2.23 s	2.24 s	2.23 s	2.23 s	2.23 s	2.23 s	2.23 s
H-27	7.14 d	7.15 m	6.78 AA'XX' (8.4)	6.72 d (ca. 8.0)	6.58 dd (8.1, 2.2)	7.15 m	6.71 d (8.0)
H-28	7.3 dd	7.23 m	7.02 AA'XX' (8.4)	7.18 t (ca. 8.0)	6.81 d (8.1)	7.33 m	7.19 t (7.8)
H-29	7.25 t	7.26 m	—	6.72 d (ca. 8.0)	—	7.26 m	6.72 d (7.8)
H-30	same as H-28	same as H-28	same as H-28	—	—	same as H-28	—
H-31	same as H-27	same as H-27	same as H-27	6.64 br s	6.67 d (2.2)	same as H-27	6.63 br s
OH's	—	—	—	—	5.44, 5.16 s	—	5.10, 1.94 s; 1.91 d

[a]Values in parentheses are *J* (in Hz).
[b]Values for this compound are from Ondeyka *et al.* (2).
[c]Assignment interchangeable.

para (C-29), the meta (C-28), and both the para (C-29) and the meta (C-28) positions of the phenyl ring, respectively. Metabolites **6** and **7** were shown to result from vicinal dihydroxylation on both C-16 and its attached Me (C-22). The metabolite **7** was also hydroxylated on the meta (C-28) position of the phenyl ring. The stereochemistry of hydroxylation at C-16 in **6** and **7** was shown to proceed with retention rather than inversion of the configuration of C-16. The nmr features that led to the structure determination of these metabolites are discussed below.

The ^{1}H nmr of **2** (Table 1) revealed the absence of one methyl signal and the appearance of two novel protons around 3.44 ppm, consistent with a CH_2OH. Analysis of the ^{1}H-^{1}H COSY coupling network in **2** unequivocally indicated that the affected methyl was located in position C-22 rather than in positions C-11 or C-23. For example, the two remaining methyl doublets in **2** were correlated with two protons at 2.2 and 2.8 ppm, each of which was further correlated to an olefinic signal, thus showing that these methyls must be those in positions in C-23 and C-11 and not C-22. All other COSY correlations supported the proposed structure. The structure of **2** was further confirmed by the ^{13}C-nmr data (Table 2) which, compared with that of the parent compound **1**, indicated the loss of one of the methyl signals at 25.1 ppm and the appearance of a new methylene signal at 68.5 ppm consistent with a CH_2OH group. The ^{13}C signals of C-

TABLE 2. ^{13}C-nmr Chemical Shifts of Compounds **1**, **2**, and **6**.

Carbon	Compound		
	1[a]	**2**	**6**
C-1	174.2	174.3	174.3
C-3	53.7	53.8	54.0
C-4	50.6	50.6	50.5
C-5	33.0	33.0	33.0
C-6	148.1	147.9	148.2
C-7	69.5	69.7	69.5
C-8	47.3	47.3	47.3
C-9	51.8	51.9	51.3
C-10	45.7	45.7	45.7
C-11	14.3	14.3	14.6
C-12	113.9	114.4	113.8
C-13	127.4	128.3[b]	131.0[b]
C-14	138.4	137.3	137.1[b]
C-15	42.5	36.7	42.0
C-16	33.3	40.9	75.1
C-17	48.4	42.9	45.7
C-18	34.2	34.4	29.5
C-19	135.8	135.0	134.4[b]
C-20	125.4	125.8[b]	124.9[b]
C-21	78.6	78.5	78.5
C-22	22.1	68.5	72.1
C-23	25.3	21.9	22.5
C-24	170.1	170.1	170.1
C-25	20.9	21.0	21.0
C-26	137.5	137.4	137.5
C-27/31	129.0	128.9	129.0
C-28/30	129.0	128.9	129.0
C-29	127.1	127.1	127.0[b]

[a]Values for this compound are from Ondeyka *et al.* (2).
[b]Assignments are interchangeable.

15 and C-17 were both shifted upfield by around 3 ppm due to the γ effect of the new hydroxy group. In contrast, the C-16 signal was shifted downfield by about 8 ppm as expected for the β effect of the OH group. All other signals were virtually unaffected.

In addition to hydroxylation on the methyl group located in position C-22 hydroxylation of the phenyl rings in **3**, **4**, and **5** has also been established as follows. The ^{1}H-nmr data of the aromatic region of these compounds (Table 1) showed splitting patterns characteristic of 1,4-, 1,3-, and 1,3,4-substituted benzenes indicating hydroxylation at the para (C-29), the meta (C-28), and both the para and the meta carbons of the phenyl ring, respectively. The upfield shift of the protons ortho (C-27) and/or para to the hydroxy groups reinforces these assignments. An alternative structure for **5**, where hydroxylation occurred at both the meta (C-28) and ortho (C-31) positions, could be ruled out by comparison with the nmr spectra of the model compounds methylhydroquinone and 4-methylcatechol.

Compounds **6** and **7** have been shown to result from a vicinal dihydroxylation on C-16 and the methyl carbon corresponding to C-22. The latter compound was further hydroxylated at the meta position of the phenyl ring. Comparing the ^{1}H nmr data of **2** with those of **6** and **7** (Table 1) showed a simpler splitting pattern for the CH_2OH protons (a tight AB system at 3.39 ppm) suggesting the absence of H-16. The COSY spectra showed no coupling to any other signal except to an exchangeable OH proton at 1.9 ppm, and the cross peaks to H-16 at 1.21 ppm were absent, supporting the proposed structures as the CH_2OH methylene protons. Except for the aromatic region, the ^{1}H-nmr spectra of **6** and **7** (Table 1) were virtually identical. The structure of **6** was further confirmed by ^{13}C nmr (Table 2). The C-16 (methine) signal was conspicuously absent at around 40 ppm, and a new quaternary signal at 75.1 ppm was observed instead, strongly indicating hydroxylation at this carbon. Compared with those of **2**, the signals of C-15, C-17, and the CH_2OH (C-22) were shifted downfield in **6** and **7** whereas C-18 was shifted upfield, in agreement with the β and γ effects of the new OH group at C-16.

The stereochemistry of C-16 in **6** and **7** was determined to have been retained during hydroxylation as follows. Irradiation of the CH_2OH methylene protons in **7** resulted in nOe enhancement of only the C-15 and C-17 protons. A molecular model of **7** based on the X-ray structure of **2** (2) showed clearly that such nOe results are consistent with the 16*S* (retention) and not the 16*R* (inverson) configuration. The latter would have exhibited strong nOe enhancements of H-14, H-18, and, to a lesser extent, H-19 which showed close proximity to the irradiated protons in the 16*R* model. Furthermore, the axial protons at C-15 and C-17 were shifted downfield in **6** and **7** compared with **2** due to the deshielding effect of the axial OH at C-16 in the 16*S* configuration.

The HIV-1 protease inhibition data of the hydroxylated metabolites of **1** are summarized in Table 3. None of the metabolites were more active than the parent com-

TABLE 3. HIV Protease Activity of L-696,474 [**1**] and its Biotransformation derivatives **2**–**7**.

Compound	IC_{50} (μM)
1	3
2	22
3	35
4	38
5	>50
6	45
7	>50

pound **1**, suggesting that the further hydroxylation reactions are microbial inactivation processes.

The potential value of microbiological transformations as a powerful tool in drug development work is illustrated in this paper. Obtaining six microbial oxidation products of **1** with one culture, coupled with the specificity and mild conditions associated with biologically catalyzed reactions, is a good indication that microbial transformation reactions can be a useful adjunct to chemical modification for the production of novel derivatives for evaluation as therapeutic agents.

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