Bripiodionen, a New Inhibitor of Human Cytomegalovirus Protease from *Streptomyces* sp. WC76599

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Received January 17, 1997[⊗]

Bripiodionen (**1**), a new natural product, was isolated from *Streptomyces* sp. WC76599 during the screening of microbial fermentation extracts for their ability to inhibit human cytomegalovirus protease. The structure of **1** was elucidated by spectroscopic methods. Compound **1** displayed inhibitory activity against human cytomegalovirus protease with an IC_{50} value of 30 μM.

Human cytomegalovirus (HCMV), a β-herpes DNA virus, is a major cause of a variety of diseases referred to as cytomegalic inclusion diseases (CID). It is an ubiquitous opportunistic pathogen that causes debilitating disease in congenitally infected infants and in immunocompromised and immunosuppressed individuals such as AIDS patients and recipients of bone marrow or organ transplantation.[1] For example, HCMV infection of the central nervous system is a probable cause of major learning disabilities. HCMV-induced pneumonia and hepatitis can be acquired from infections as a result of transplant surgery. HCMV also causes retinitis, especially in AIDS patients.[2] The drugs currently approved for the treatment of HCMV are the nucleoside analogs ganciclovir (DHPG) and cidofovir (HPMPC) and the pyrophosphate derivative foscarnet (PFA). However, the clinical use of these compounds is limited due to their host toxicity and emerging resistant strains of HCMV.[3] Thus, the search for a novel and more effective class of compounds for antiviral chemotherapy of HCMV infections has to be broadened by investigating new targets that are essential for HCMV replication.

The HCMV virus produces a virally-encoded protease whose structure and function is similar to that of herpes simplex virus 1 (HSV-1). Like HSV-1 protease, it is thought that HCMV protease is a crucial capsid assembly protein,[4] and its autoproteolytic cleavage is thought to be a requisite for successful DNA packaging and completion of the viral replicative cycle.[5]

The HCMV open reading frame UL80 encodes both protease and assembly protein precursor (pAP) from its N-terminal and C-terminal regions, respectively. Sequence and expression analysis reveals a nested gene family of in-frame reading sequence from UL80, resulting in five 3′ coterminal overlapping proteins.[6,7] Four autoproteolytic cleavage sites within the 85 kDa protein are cleaved in a time-ordered fashion and allow multifunctionality.[7,8] Selective inhibition of this viral-encoded obligate proteolytic processing would provide an effective approach for anti-HCMV therapy. Research results of HCMV protease inhibitors have begun to emerge; Sch 65676, a fungal metabolite,[9] and a number of compounds causing irreversible inactivation *via* enzyme disulfide bond formation[10,11] were reported as low micromolar inhibitors.

A screen for identifying novel inhibitors of HCMV protease from synthetic and natural sources was developed and implemented in a high throughput mode (unpublished results). The screen is based on the measurement of radioisotopically-labeled cleavage product. As a result, an extract of *Streptomyces* sp. WC76599 was found to exhibit the enzyme inhibitory activity. The activity was confirmed in both primary and secondary fermentations. When subjected to bioassay-guided fractionation, the extract yielded several active compounds. This paper describes the isolation, structural elucidation, and inhibitory effect against HCMV protease of one of the active compounds, bripiodionen (**1**).

Early steps of bioassay guided fractionation were conducted by solvent extraction and sequential solvent partitions by the modified Kupchan's procedure.[12] Enrichment of activity and final purification of the active **1** was achieved by repeated column chromatography (CC) on Sephadex LH-20 (Figure 1). Compound **1** displayed inhibitory activity against HCMV protease with an IC_{50} value of 30 μM. The compound also demonstrated moderate cytotoxicity with an IC_{50} value of 34 μM on the murine tumor cell M109, a Madison lung carcinoma-derived cell line.

Compound **1**, a white powder, has a molecular weight of 292 and a molecular formula of $C_{15}H_{20}N_2O_4$ from electrospray MS and HRFABMS measurements, indicating an unsaturation degree of seven. The presence of a major UV maximal absorption at 328 nm and absence of benzenoid UV bands (230−270 nm) indicated an extensively conjugated but not an aromatic system; the presence of a γ-lactam (1707 cm^{-1}), a conjugated cyclic ketone (1660 cm^{-1}), and a primary amide (1660 cm^{-1} overlapped, 1600 cm^{-1}) was suggested by the IR spectrum. When ^{1}H- and ^{13}C-NMR (Table 1, *E*-form) data were taken of a fresh sample of **1** immediately after its isolation, the compound proved to be predominantly a single isomer. Fifteen carbons were accounted for by the following functional groups; three methyls (δ_C 10.5, 17.6, 19.3), one methylene (δ_C 37.9), two methines (δ_C 28.9, 29.6), one aminomethine (δ_C 57.4), one oxymethine (δ_C 84.4), one highly polarized ring double bond with a proton (δ_C 118.6, 150.5) attached to each carbon, two fully substituted olefinic carbons including one enol carbon (δ_C 102.5, 166.9), two amido (δ_C 167.5, 171.1), and one ketone (δ_C 199.3) carbons. There are also three exchangeable protons assignable to one primary (δ_H 6.88, 7.36) and one secondary (lactam, δ_H 7.7) amido

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

S0163-3864(97)00054-2 CCC: $14.00 © 1997 American Chemical Society and American Society of Pharmacognosy

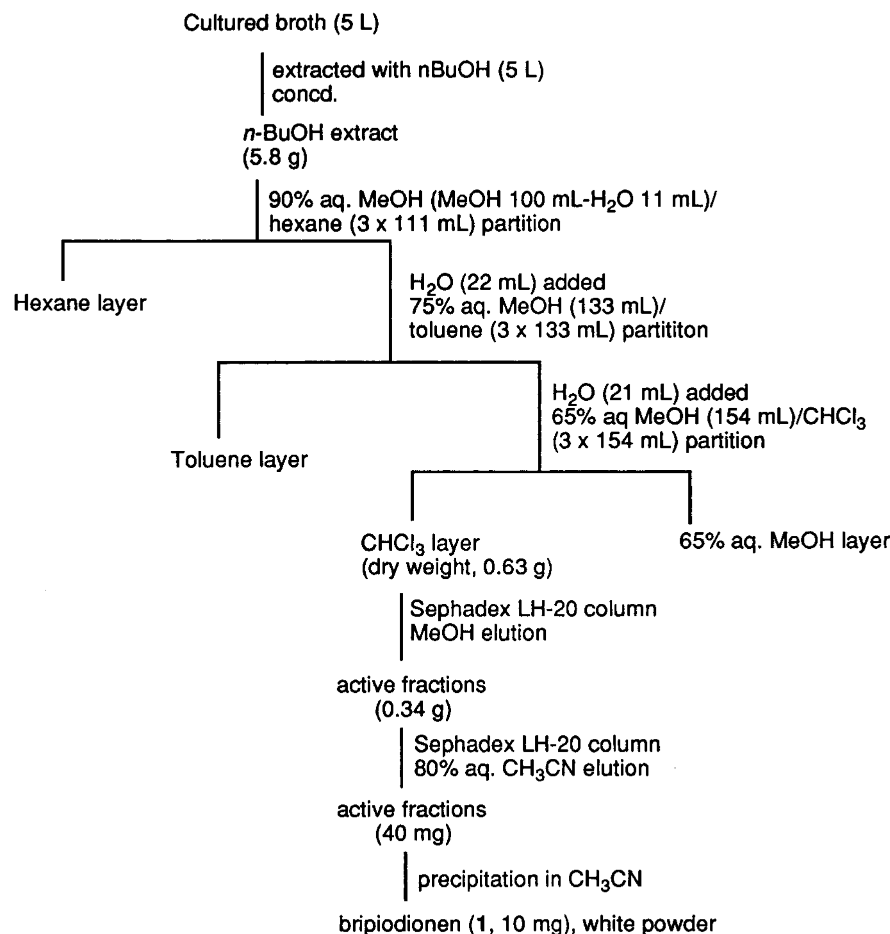

Figure 1. Extraction and isolation of bripiodionen (**1**).

Table 1. ^{1}H- and ^{13}C-NMR Data of Bripiodionen (**1**) (DMSO-d_6)

C no.	^{13}C (5*E*-form)	^{1}H (5*E*-form)	HMBC correlns (C no.)	^{13}C (5*Z*-form)	^{1}H (5*Z*-form)
1	171.1 (s)			171.1 (s)	
2	37.9 (t)	2.27 (dd, 7.3, 15.5) 2.46 (dd, 4.2, 15.5)	1, 4	37.8 (t)	2.24 (dd, 7.4, 15.5) 2.45 (dd, 4.3, 15.5)
3	57.4 (d)	3.91 (dd, 4.2, 7.3)	1, 4	56.8 (d)	3.89 (dd, 4.3, 7.4)
4	199.3 (s)			196.2 (s)	
5	102.5 (s)			102.5 (s)	
6	167.5 (s)			169.1 (s)	
7	166.9 (s)			165.4 (s)	
8	118.6 (d)	7.45 (d, 9.8)	7	118.3 (d)	7.61 (d, 9.8)
9	150.5 (d)	7.04 (dd, 9.8, 6.7)	7, 10, 11	149.9 (d)	7.03 (dd, 9.8, 6.7)
10	29.6 (d)	2.64 (m)	8, 9	29.6 (d)	2.64 (m)
11	84.4 (d)	3.74 (dd, 3.1, 10.4)	15	84.4 (d)	3.74 (dd, 3.1, 10.4)
12	28.9 (d)	1.89 (m)		28.9 (d)	1.89 (m)
13	19.3 (q)	1.15 (d, 6.4)	11	19.3 (q)	1.15 (d, 6.4)
14	17.6 (q)	0.87 (d, 4.7)	11	17.6 (q)	0.87 (d, 4.7)
15	10.5 (q)	0.88 (d, 4.9)		10.5 (q)	0.88 (d, 4.9)
H_2N		6.88 (s), 7.36 (s)	1, 2		6.88 (s), 7.36 (s)
HN		7.7 0 (s)	3, 4, 5		7.94 (s)

groups. The COSY spectrum of **1** enabled the assembly of a major branched segment (I) consisting of eight carbons, C(13,14)−C12−C11−C10(C15)−C9−C8 (Scheme 1). The segment was confirmed and further extended from C8 to C7 (δ_C 102.5) by an HMBC experiment (Scheme 1, Table 1). HMBC and COSY data also revealed the presence of another branched segment (II) formed by the connectivity of NH_2−C1−C2−C3(NH)−C4 and NH−C6−C5 (Scheme 1). To meet the total unsaturation degree (USD) of seven for **1**, the branched form of segments I (2 USD) and II (3 USD) must be cyclized respectively; cyclic segment I was established by linking C7−O(enol oxygen)−C11 and cyclic segment II by linking C5 and C6 to form a γ-lactam ring (Scheme 1), despite no notable C−H long-range couplings across these linkages observed in HMBC. The connection of segments I and II can only be made *via* the C5−C7 double bond to give rise to the gross structure of bripiodionen (**1**). The proposed structure was strongly supported by tandem mass spectrometry (MS/MS) data of the *quasi*molecular ion (MH^+, m/z 293) of **1** as shown in Figure 2. The relative stereochemistry of segments I and II was independently examined by NOE study. As a result, H-10 and H-11 were found to be *cis*-oriented to each other; so were NH and H-3. However, when segments I and II were linked to form **1**, the stereochemistry of the entire molecule no longer became easily accessible by NMR methods and is at present unknown.

Scheme 1

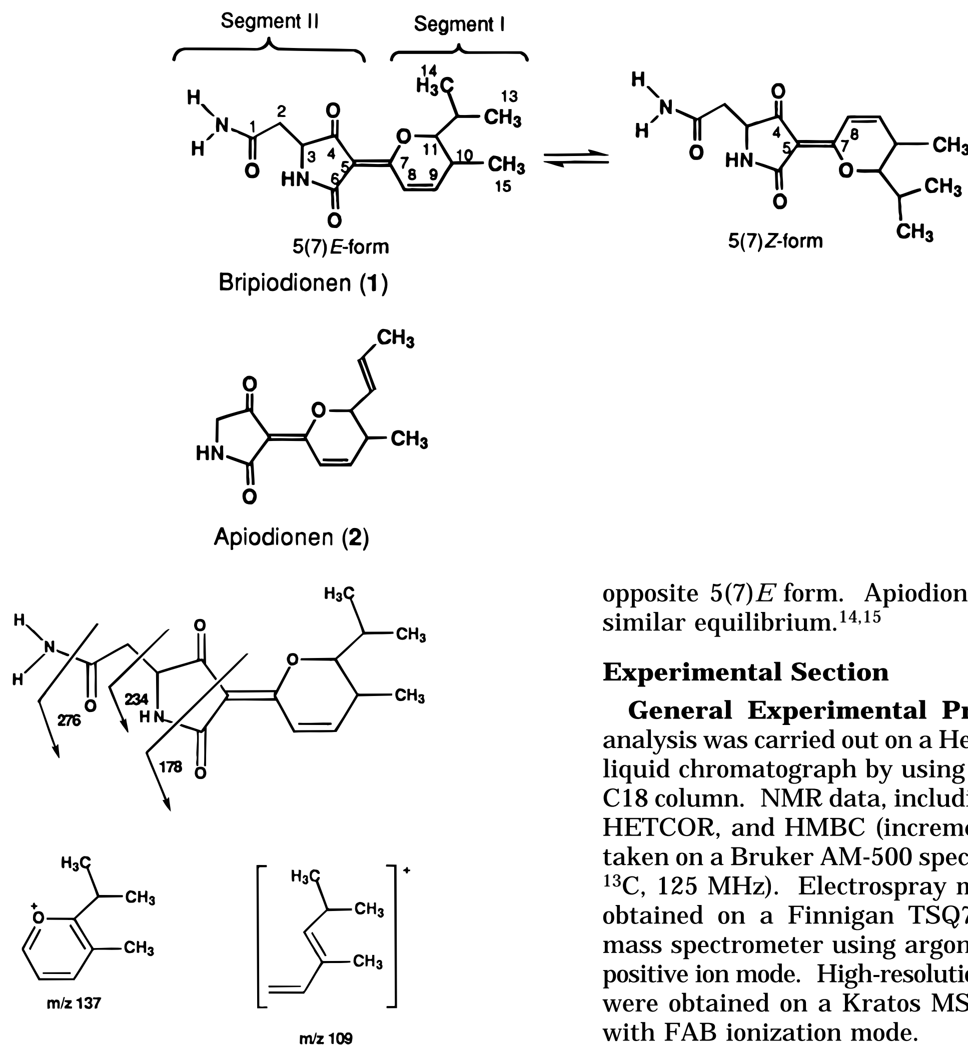

Figure 2. MS/MS product ions of the *m/z* 293 (MH^+) ion of **1** and their substructures.

The structure of **1** represents a new natural product possessing a tetramic acid moiety; however, it is substantially different from the tirandamycin–streptolydigin families of tetramic acid antibiotics.[13] The most closely related natural product to **1** seems to be apiodionen (**2**), an inhibitor of topoisomerase I and II, and a suppressor of chemiluminescence, isolated from the fermentation culture of *Apiosordaria effusa*.[14,15] During the spectral and chromatographic studies of **1**, we recognized that **1**, when dissolved in an organic solvent such as MeOH or DMSO for prolonged periods of time, had undergone geometric isomerization. The presence of a newly formed geometric isomer became evident after overnight NMR study in DMSO-d_6, and the isomerization appeared to reach equilibrium (1:1 of original and second isomers) in 3–4 days at room temperature as suggested by NMR and HPLC analyses (data not shown). The most critical difference between the two isomers proved to be the chemical shift of H-8, *i.e.*, δ 7.45 (d, $J = 9.8$ Hz) of the original isomer *versus* a more downfield shifted H-8 (δ 7.61) of the second (later) isomer. In view of the strong deshielding effect caused by the C4 ketone, the H-8 of the second isomer should be in a closer position to C4 than that of the original isomer; the second isomer was therefore assigned as the 5(7)*Z* form, and the original isomer as the opposite 5(7)*E* form. Apiodionen (**2**) also displayed a similar equilibrium.[14,15]

Experimental Section

General Experimental Procedures. HPLC/UV analysis was carried out on a Hewlett-Packard HP-1090 liquid chromatograph by using a Microsorb Short One C18 column. NMR data, including COSY, NOE, DEPT, HETCOR, and HMBC (increment delay, 0.06 s), were taken on a Bruker AM-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz). Electrospray mass spectra (MS) were obtained on a Finnigan TSQ7000 triple quadrupole mass spectrometer using argon as collision gas and in positive ion mode. High-resolution mass spectra (HRMS) were obtained on a Kratos MS 50 mass spectrometer with FAB ionization mode.

Media and Culture Conditions. *Streptomyces* sp. WC76599 from culture collections of Bristol-Myers Squibb Pharmaceutical Research Institute was grown in test tubes on agar slants that consisted of the following per liter of distilled H_2O: Japanese soluble starch, 5 g; glucose, 5 g; fish meat extract, 1 g; yeast extract, 1 g; N-Z case, 2 g; NaCl, 2 g; $CaCO_3$, 1 g; agar, 15 g. The culture was incubated at 32 °C for 14 days, and then the surface was swabbed into 50 mL of vegetative medium in a 250 mL flask, which contained the following per liter of distilled H_2O: Japanese potato starch, 20 g; dextrose, 5 g; N-Z case, 3 g; yeast extract, 2 g; fish meat extract, 5 g; $CaCO_3$, 3 g. The flasks were incubated at 32 °C at 250 rpm on a rotary shaker. Frozen vegetative preparations were prepared by mixing a culture grown for 3 days in the vegetative medium with an equal volume of 20% (w/v) glycerol/10% (w/v) sucrose and aliquots frozen in a dry ice–acetone bath, and stored at −80 °C. From the frozen stock, 4 mL was used as an inoculum into 100 mL of the vegetative medium described above. The culture was grown for 3 days at 32 °C, and then 4 mL was used to inoculate 100 mL of the production medium in a 500 mL flask, which contained the following per liter of distilled H_2O: lactose, 20 g; dextrin, 10 g; Pharmamedia, 10 g; allophane, 10 g; glucose, 5 g. The culture was incubated for 6 days at 32 °C at 250 rpm.

Extraction and Isolation. Each step of fractionation was monitored by the *in vitro* HCMV protease

assay; in the case of CC, an aliquot (100 μL) of column fractions (8 mL/fraction) was transferred by a Hamilton liquid handler to a corresponding well in a 96 well mictrotiter plate, and the plate was dried on Solvant and submitted to the enzyme assay. The extraction and isolation of bripiodionen (**1**) is described in Figure 1.

Bripiodionen (1): white amorphous powder; mp 195−198 °C; $[\alpha]_D$ −260° (*c* 0.22, MeOH); UV (MeOH) λ max (log ϵ) 228 (3.56), 328 (4.12) nm; IR (KBr) ν max 3346, 2961, 2542, 1707, 1659, 1599, 1557, 1434, 1264 cm^{-1}; HRFABMS found 293.1508, calcd for MH^+, $C_{15}H_{21}N_2O_4$ 293.1501; electrospray MS *m/z* $[MH]^+$ 293 (100); ^{1}H- and ^{13}C-NMR Table 1.

Human Cytomegalovirus Protease Assay. Activity of the HCMV protease enzyme was determined in an *in vitro* assay that measures the cleavage of a GST-fusion protein containing the authentic HCMV protease cleavage site. The substrate was radiolabeled and activity measured by the release of cleaved substrate and quantitated subsequently by scintillation counting.

Cytotoxicity Assay. Cytotoxcity was assessed by the XTT-assay[16] using murine tumor cell line M-109. Cells were seeded at 4000 cells/well in 96-well microtiter plates, and 24 h later, drugs at serially diluted concentrations were added. The cells were incubated at 37 °C for 72 h, at which time the tetrazolium dye XTT was added. The results are expressed as an IC_{50}, which is the drug concentration required to inhibit cell proliferation (absorbance at 450 nm) to 50% of that of untreated control cells.

Acknowledgment. We thank Mr. Y. Hoshino for isolating the producing organism WC76599, Ms. B. Sarba for technical support in the completion of some of the enzyme assays, Dr. I. Bursuker and Ms. K. Neddermann for cytotoxicity testing, and Drs. K. Volk, S. Klohr, and R. Delterio for part of the spectral data measurements.

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NP970054V