Quanolirones I and II, Two New Human Cytomegalovirus Protease Inhibitors Produced by *Streptomyces* sp. WC76535

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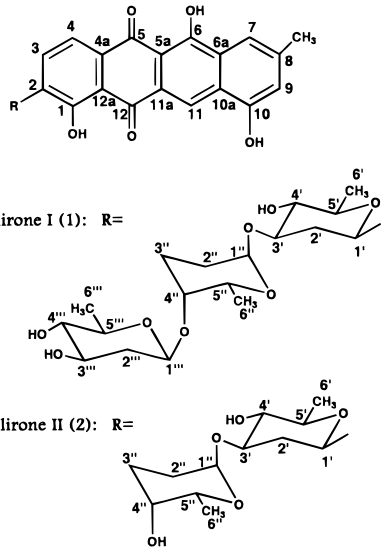
Two new naphthacenequinone glycosides, quanolirones I (**1**) and II (**2**) were isolated, together with the known compound galtamycin from the fermentation broth of *Streptomyces* sp. WC76535. The structures **1** and **2** were established by analysis of their spectroscopic data and by comparison of their data to those of galtamycin. Compounds **1**, **2**, and galtamycin showed inhibitory activity against HCMV protease with IC_{50} values of 14, 35, and 52 μM, respectively.

Human cytomegalovirus (HCMV) is a β-herpes virus, a ubiquitous opportunistic pathogen that causes a broad range of disease states from mild to severe. The virus causes debilitating disease in infected infants and immunocompromised or immunosuppressed adults, including HCMV-induced retinitis, colitis, and pneumonia.[1] This virus produces a protease whose structure and function is similar to that of herpes simplex virus 1 (HSV-1) protease. The auto-processed HCMV protease, termed *assemblin*, is an essential capsid-assembly protein.[2,3] By analogy to HSV-1, the self-processing step is requisite for successful DNA packaging and completion of the viral replicative cycle.[4] A drug that could effectively inhibit HCMV replication and infection by inhibiting this essential protease activity would have great utility.

The HCMV open-reading frame UL80 encodes both protease and assembly protein precursor (pAP) from its N- and C-terminal regions, respectively. Sequence and expression analyses reveal a nested gene family from UL80, resulting in four in-frame, overlapping proteins, 3′ coterminal.[5,6] The full-length form of the protein is found primarily in the infected cell cytoplasm.[7] The autoproteolytic cleavage sites within the 85 kDa protein are cleaved in a time-ordered fashion and allow multifunctionality. The first to be cleaved is the M site (ala643/ser644), and this is the maturational cleavage site.[8] M site cleavage occurs in the nuclei of infected cells and is the step required for viral capsid maturation and DNA packaging.[7] The R site (ala256/ser257) allows the release of the 256-residue catalytic protein, called assemblin. The third site, called the I site (ala143/ala144), was originally thought to be involved in inactivation and is now referred to as the "internal" site. Its cleavage converts single-chain assemblin to the equally active two-chain assemblin.[9,10] Typical mammalian serine protease inhibitors do not inhibit the HCMV protease to any great extent.[9,11] Recently, benzimidazolylmethyl sulfoxides were reported as potent inhibitors with in vivo activity; these compounds are thought to interact with the unusual number of cysteine sulfhydryl groups present in the HCMV protease sequence.[7]

A high-throughput screen to identify inhibitors of HCMV protease activity was developed, which was able to accommodate reliably a variety of sample types, including natural products. During the screening of microbial fermentation extracts for their ability to inhibit HCMV protease, the ethyl acetate extract of *Streptmyces* sp. WC76535 was found to be active. When subjected to bioassay-guided fractionation, three naphthacenequinone glycosides, quanolirones I (**1**) and II (**2**) and galtamycin, were isolated. Compounds **1** and **2** are new representatives in the class of C-glycosyl naphthacenequinone antibiotics, while galtamycin was isolated for the first time in 1986.[12] Structures of the galtamycin aglycon, galtamycinone, and galtamycin were subsequently characterized.[13,14] However, the initial structures of galtamycinone and galtamycin in terms of the positions of C-glycosidation and substitution of a hydroxy group, were later revised.[15] Because all the literature on structure elucidation of galtamycinone and galtamycin were written in Russian and were not readily accessible, a detailed structure elucidation of **1** and **2** became necessary and beneficial despite their structural similarity to galtamycin. This report describes the fermentative production, isolation, structure elucidation, and biological activities of these compounds.



Results and Discussion

Compounds **1**, **2**, and galtamycin were isolated from an ethyl acetate extract of *Streptmyces* sp. WC76535 by bioassay-guided fractionation. HRFABMS measurement revealed compound **1** to have a molecular formula of $C_{37}H_{42}O_{13}$, which indicated 17 degrees of unsaturation.

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Table 1. ^{1}H NMR Data of Quanolirones I (**1**), II (**2**), and Galtamycin (**3**)[a]

proton position	**1** (δ in ppm, mult, J in Hz)	**2** (δ in ppm, mult, J in Hz)	galtamycin (δ in ppm, mult, J in Hz)
3-H	7.78 (d, $J_{3,4} = 7.6$)	7.85 (d, $J_{3,4} = 7.4$)	7. 84 (d, $J_{3,4} = 7.8$)
4-H	7.68 (d, $J_{4,3} = 7.7$)	7.83 (d, $J_{4,3} = 7.7$)	7.80 (d, $J_{4,3} = 7.7$)
7-H	7.44 (s)	7.61 (s)	7.58(s)
9-H	6.90 (s)	6.96 (s)	6.93 (s)
11-H	8.32 (s)	8.46 (s)	8.44 (s)
13-Hs	2.36 (s)	2.44 (s)	2.43 (s)
1′-H	4.73 (d, $J_{1',2'} = 11.3$)	4.82 (d, $J_{1',2'} = 11.0$)	4.80 (d, $J_{1',2'} = 11.1$)
2′-Ha	1.21 (t, $J_{2',1'} = J_{2',3'} = 11.2$)	1.23 (m)	1.22 (m)
2′-He	2.46 (m)	2.47 (m)	2.43 (m)
3′-H	3.68 (m, $J_{3',2'} = 11.3$; $J_{3',4'} = 8.9$)	3.70 (m)	3.67 (m)
4′-H	3.05 (m, $J_{4',3'} = J_{4',5'} = 8.9$; $J_{4',4'-OH} = 4.8$)	3.05 (m)	3.05 (m)
5′-H	3.44 (m, $J_{5',4'} = 8.9$; $J_{5',6'} = 6.0$)	3.44 (m)	3.44 (m)
6′-Hs	1.30 (d, $J_{6',5'} = 5.9$)	1.29 (d, $J_{6',5'} = 6.0$)	1.29 (d, $J_{6',5'} = 6.0$)
1″-H	4.90 (br s)	4.88 (br s)	4.89 (br s)
2″-Ha	1.98 (m)	1.98 (m)	1.98 (m)
2″-He	1.83 (m)	1.90 (m)	1.82 (m)
3″-Ha	1.30 (m)	1.43 (m)	1.27 (m)
3″-He	1.75 (m)	1.89 (m)	1.76 (m)
4″-H	3.44 (m, $J_{4'',5''} = 1.6$)	3.49 (m)	3.45 (m)
5″-H	4.15 (m, $J_{5'',4''} = 1.6$, $J_{5'',6''} = 6.5$)	4.12 (m, $J_{5'',4''} = 1.6$, $J_{5'',6''} = 6.5$)	4.15 (m, $J_{5'',4''} = 1.6$, $J_{5'',6''} = 6.5$)
6″-H	1.04 ($J_{6'',5''} = 6.4$)	1.03 ($J_{6'',5''} = 6.4$)	1.04 ($J_{6'',5''} = 6.4$)
1‴-H	4.47 (d, $J_{1''',2'''} = 9.7$)		4.49 (d, $J_{1''',2'''} = 9.6$)
2‴-Ha	1.36 (m, $J_{Ha,He} = 11.8$, $J_{2''',1'''} = J_{2''',3'''} = 9.8$)		1.23 (m)
2‴-He	2.02 (m, $J_{He,Ha} = 11.8$)		2.23 (m)
3‴-H	3.31 (m, $J_{3''',3'''-OH} = 4.0$)		3.49 (m)
4‴-H	2.71 (m, $J_{4''',3'''} = J_{4''',5'''} = 9.0$, $J_{4''',4'''-OH} = 4.8$)		2.88 (m)
5‴-H	3.11 (m, $J_{5''',4'''} = 9.0$; $J_{5''',6'''} = 6.2$)		3.19 (m)
6‴-H	1.13 (d, $J_{6''',5'''} = 6.2$)		1.16 (d, $J_{6''',5'''} = 6.0$)
1⁗-H			4.86 (br s)
2⁗-H			1.92 (m)
			1.28 (m)
3⁗-H			1.95 (m)
			1.49 (m)
4⁗-H			3.35 (m)
5⁗-H			4.06 (m)
6⁗-H			0.99 (d, $J_{6'''',5''''} = 6.5$)
4′-OH	5.09 (d, $J_{4'-OH,4'} = 4.8$)		5.06 (d, $J_{4'-OH,4'} = 4.7$)
3‴-OH	4.80 (d, $J_{3'''-OH,3'''} = 4.0$)		
4‴-OH	4.87 (d, $J_{4'''-OH,4'} = 4.8$)		4.93 (d, $J_{4'''-OH,4'''} = 4.4$)
4⁗-OH			4.35 (br s)

[a] 500 Hz, DMSO-d_6.

Absorptions at 250, 265, 295, and 490 nm in the UV spectrum were in agreement with a substituted 1,6-dihydroxy-naphthacenequinone nucleus. The IR spectrum showed absorption bands at 3390, 1616, and 1578 cm^{-1}, implying that **1** should possess at least one hydroxyl group and quinone carbonyl groups. The ^{1}H and ^{13}C NMR spectra of **1** closely resemble those of galtamycin and contained signals characteristic of substituted naphthacenequinone chromophore and sugar moieties. For the naphthacenequinone chromophore portion, the ^{13}C NMR spectrum showed the signals of two quinone carbonyl groups (δ 186.3 and 187.2) and 16 olefinic carbons between δ 110 and 165. Among them, 11 are fully substituted. The ^{1}H NMR spectrum showed the signals of five aromatic protons. The protons at δ 7.78 (3-H, d, $J = 7.7$ Hz) showed coupling to the proton at δ 7.68 (4-H, d, $J = 7.7$ Hz), indicating the ortho-relation of these two protons. The other three aromatic protons (7-H, 9-H, and 11-H) appeared as singlets at δ 7.44, 6.90, and 8.32. One methyl group (δ 22.0) should be attached to the aromatic chromophore at C-8 due to the long-range couplings of the methyl protons at δ 2.63 to C-7 at δ 114.3 and C-8 at δ 141.8. The structure of the naphthacenequinone chromophore was established by detailed NMR studies including HETCOR and HMBC (Tables 1and 2, Figure 1), and by comparing the NMR data of compound **1** to those of galtamycin. The ^{13}C NMR spectrum further revealed 18 carbon signals arising from the deoxygenated trisaccharide moiety. Among them, there were only two anomeric carbon signals at δ 92.1 and 101.1. This phenomenon was consistent with the C-glycosyl bond between the chromophore and the trisaccharide moiety. Moreover, there were signals of nine oxymethine, four methylene, and three methyl groups. The ^{1}H NMR spectrum of sugar fragments in DMSO-d_6 also showed the signals of three hydroxyl groups (δ 5.09, 4.80, and 4.87), and the positions of hydroxyl groups at C-4′, C-3‴, and C-4‴ were indicated by ^{1}H−^{1}H couplings of these hydroxyl groups to 4′-H, 3‴-H and 4‴-H, respectively. The linkages between C-3′−*O*−C-1″ and C-4″−*O*−C-1‴ were confirmed by the strong NOEs (Figure 2) between 3′-H (δ 3.68) and 1″-H (δ 4.90), and between 4″-H (δ 3.44) and 1‴-H (δ 4.47), respectively. The relative stereochemistry of trisaccharide moiety was suggested by ^{1}H−^{1}H coupling constants (Table 1) and NOEs (Figure 2). To measure some important ^{1}H−^{1}H coupling constants, a trace of D_2O was added to DMSO-d_6 solution of the sample. Thus, the structure of the trisaccharide moiety was established by analyzing its NMR data including COSY, HETCOR, HMBC, and NOE (Tables 1, 2, Figures 1, 2), as well as by the comparison of these data to those of galtamycin. Moreover, the position of C-glycosidation between the chromophore and the sugar moiety was proven by the HMBC correlation (Figure 1) between 3-H (δ 7.78) and C-1′ (δ 76.2) and NOE (not shown) between 3-H and 1′-H (δ 4.73). Thus, compound **1** was characterized as a glycoside of galtamycinone, and the disaccharide contained one fragment each of rodinose and

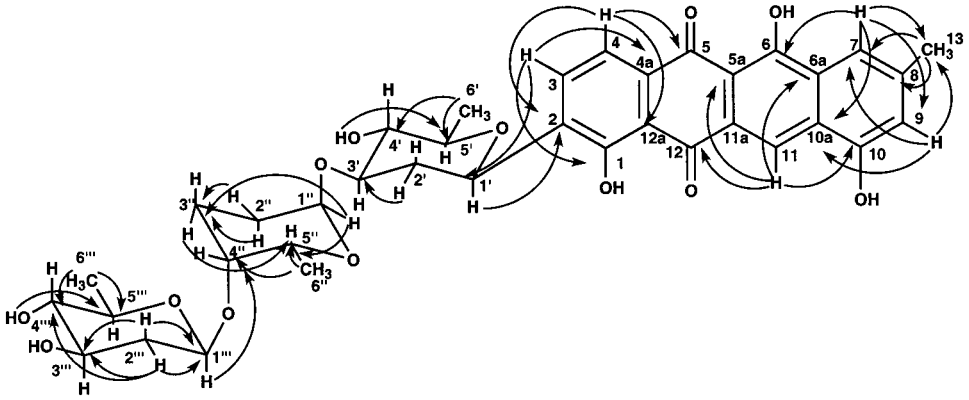

Figure 1. HMBC correlations of quanolirone I (**1**).

Table 2. ^{13}C NMR Data of Quanolirone I (**1**), Quanomycin II (**2**), and Galtamycin[a]

carbon position	**1** (δ in ppm)	**2** (δ in ppm)	galtamycin (δ in ppm)
C-1	158.4	158.4	158.4
C-2	137.3	137.2	137.2
C-3	133.0	131.9	132.9
C-4	118.5	118.5	118.5
C-4a	132.2	131.7	132.2
C-5	186.3	186.1	186.0
C-5a	108.7	108.7	108.7
C-6	162.2	162.2	162.5
C-6a	128.2	128.8	128.3
C-7	114.3	114.3	114.3
C-8	141.8	141.7	141.7
C-9	116.3	116.3	116.3
C-10	156.0	156.1	156.1
C-10a	124.2	124.1	124.2
C-11	116.8	116.8	116.8
C-11a	125.1	124.9	125.0
C-12	187.2	187.2	187.2
C-12a	116.1	116.0	116.0
C-13	22.0	22.5	22.0
C-1′	70.5	69.5	70.6
C-2′	36.0	36.2	36.1
C-3′	74.8	75.0	74.8
C-4′	74.6	74.7	74.5
C-5′	76.2	75.5	76.3
C-6′	18.6	18.4	18.6
C-1″	92.1	98.1	92.4
C-2″	24.2	24.7	24.2
C-3″	24.2	24.6	24.3
C-4″	75.5	70.0	75.0
C-5″	65.4	67.6	65.5
C-6″	17.1	17.2	17.4
C-1‴	101.1		101.0
C-2‴	39.8		38.2
C-3‴	70.5		75.8
C-4‴	76.9		73.8
C-5‴	71.6		71.8
C-6‴	18.6		18.6
C-1⁗			92.3
C-2⁗			23.6
C-3⁗			25.4
C-4⁗			65.6
C-5⁗			66.0
C-6⁗			17.3

[a] 125 Hz, DMSO-d_6.

oliviose as shown. Although the structures in Figure 1 are presented as D-sugars, no determination of absolute stereochemistry has been made due to insufficient quantity of the material.

Compound **2** showed a molecular formula of $C_{31}H_{32}O_{10}$ by HRFABMS. It showed almost identical UV and similar IR spectra to those of **1**. Both ^{13}C and ^{1}H NMR spectra of **2** closely resemble those of **1** and galtamycin. The NMR

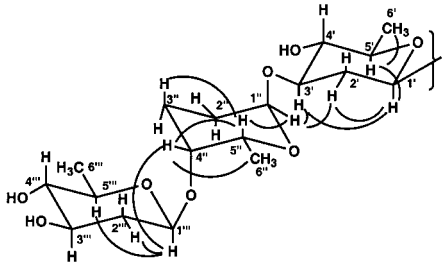

Figure 2. Important NOEs of trisaccharide moiety of quanolirone I (**1**).

spectra of **2** differed only in the sugar moieties from that of **1**. The ^{13}C NMR spectrum showed only 12 carbons instead of 18 arising from the sugar moieties, which indicated the presence of a disaccharide moiety. The structure of this disaccharide moiety was further characterized based on its NMR data (Tables 1, 2). Thus, compound **2** is characterized as a glycoside of galtamycinone and one fragment of rodinose, as shown in Figure 1. Again, no determination of absolute stereochemistry has been made for compound **2** due to insufficient quantity of the material.

Compounds **1**, **2**, and galtamycin showed inhibitory activity against HCMV protease with IC_{50} values of 14, 35, and 52 μM, respectively. In addition, they demonstrated moderate cytotoxicity against the murine tumor cell line M-109 IC_{50} values of 14, 89, and 12 μM, respectively.

Experimental Section

General Experimental Procedure. HPLC/UV analysis was carried out on a Hewlett−Packard HP-1090 liquid chromatograph by using a Rainin analytical C18 column; preparative TLC was performed on Si gel 60 F_{254}s plates (2 mm thick); preparative HPLC was carried out on Rainin Microsorb semipreparative column, 10 × 250 mm, 5 μm. Electrospray MS were taken on a Finnigan TSQ7000 triple quadruple mass spectrometer, the HRFABMS analysis was performed with a Kratos MS50 mass spectrometer, and all ^{1}H and ^{13}C NMR spectra, including COSY, HETCOR, and HMBC, were taken on a Bruker AM-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz).

Media and Culture Conditions. *Streptomyces* sp. was grown in test tubes on agar slants, which 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 gyrotary 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 were frozen in a dry ice−Me_2CO 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. The extraction and fractionation were monitored by HCMV protease assay. The fermentation broth (5 L) was stirred vigorously with EtOAc (3 L) for 1 h. The EtOAc extract was evaporated in vacuo to dryness (2.2 g). The dark-red residue (200 mg) was then separated by preparative TLC, eluted with CH_2Cl_2−MeOH (9:1). Two active fractions were obtained. Compound **1** (40 mg) was purified from fraction A by preparative HPLC on a C18 column, eluted with a gradient solvent system of MeCN and H_2O (from 80% MeCN to 100% MeCN over 40 min). Preparative HPLC separation of fraction B on C18 with the same solvent system, afforded compounds **2** (6 mg) and **3** (10 mg).

Compound 1: dark-red needle; UV (MeOH) λ_{max} 200, 265, 495 nm; IR (KBr) ν_{max} 3390, 2930, 1722, 1578, 1616, 1437, 1385, 1263, 1067, 1011, 850 cm^{-1}; HREIMS *m/z* 693.2530 [M − H]$^+$ (calcd for $C_{37}H_{41}O_{13}$, 693.2547); ^1H and ^{13}C NMR data, see Table 1.

Compound 2: dark-red needle; UV (MeOH) λ_{max} 200, 265, 495 nm; IR (KBr) ν_{max} 3401, 2933, 1707, 1629, 1613, 1385, 1259, 1060, 1010, 850 cm^{-1}; HREIMS *m/z* 563.1906 [M − H]$^+$ (calcd for $C_{31}H_{31}O_{10}$, 563.1917); ^1H and ^{13}C NMR data, see Table 1.

Human Cytomegalovirus Protease Assay. Biological activity of the HCMV protease was determined in an in vitro assay in which generation of cleavage product from the substrate results in an increase in radioactive counts, subsequently quantitated by scintillation counting (TopCount).[16] The substrate is a GST-fusion protein expressed in *Escherichia coli* and contains the authentic natural HCMV protease cleavage site.[17] The protease enzyme is also a GST-fusion protein expressed in *E. coli* and contains the wild-type HCMV protease catalytic domain.[7] The HCMV protease is intrinsically more active than the related HSV-1 protease, and cleavage activity can be measured under physiological salt conditions. The enzyme is more stable, however, and the assay more suited to high-throughput screening constraints under antichaeotropic conditions similar to those described for HSV-1 protease.[17]

Cytotoxicity Assay. Cytotoxicity was assessed by the XTT assay using a murine cell line M-109 (Madison lung carcinoma 109).[16,18,19]

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