Patulosides A and B, Novel Xanthone Glycosides from Cell Suspension Cultures of *Hypericum patulum*

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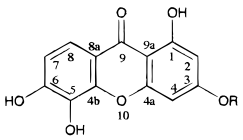
Two new xanthone glycosides, patuloside A (3-*β*-D-glucopyranosyloxy-1,5,6-trihydroxy-9H-xanthene-9-one, **1**) and patuloside B [3-(2-*O*-α-L-rhamnopyranosyl-*β*-D-glucopyranosyl)oxy-1,5,6-trihydroxy-9H-xanthene-9-one, **2**], have been isolated from cell suspension cultures of *Hypericum patulum*. Their structures were elucidated by spectral techniques.

Plants of the genus *Hypericum* have been used as traditional medicinal plants in various parts of the world.[1] Recently, antifungal,[2] antibiotic,[3] antiviral,[4] and anticancer[5] compounds were isolated from these species. *Hypericum patulum* Thunb. (Guttiferae) has been used in traditional Chinese herbal medicine for the treatment of hepatitis, bacterial diseases, and nasal hemorrhage.[6] Previously, as part of a continuing study of *Hypericum*,[3] we reported the isolation and structure determination of 14 prenylated xanthones and epicatechins from chloroform or methanol extracts of cell suspension cultures induced from flowers of *H. patulum*[7−11] and discussed the possible biosynthetic relationships among these xanthones.[9,12] Recently, Takamiya et al. isolated anthocyanins and procyanidins from tissue, cell suspension, and adventitious root cultures of *H. patulum*.[13]

Further investigation of the *n*-BuOH extract from cell suspension cultures of *H. patulum* has led to the isolation of two new xanthone glycosides named patulosides A (**1**) and B (**2**). We describe herein the isolation, characterization, and structure elucidation of these compounds. This is the first report on the isolation of 1,3,5,6-tetrahydroxyxanthone glycosides from cell suspension cultures of *H. patulum*.

Suspension cells of *H. patulum* maintained in Linsmaier−Skoog (LS) medium[14] containing 2,4-D and kinetin at 27 °C in the dark were harvested at 2−3-week intervals. The harvested cells were dried and extracted successively with $CHCl_3$ and MeOH. The MeOH extract was suspended in water and extracted with EtOAc and *n*-BuOH, in turn. The *n*-BuOH extract was subjected to polyamide and Si gel column chromatography followed by further purification using gel filtration over Sephadex LH-20, and recrystallization from MeOH led to the isolation of two new xanthone glycosides, patulosides A (**1**) and B (**2**).

Patuloside A (**1**) crystallized as pale yellow needles, mp 282 °C. FABMS showed a molecular ion at *m*/*z* 423 $[M + H]^+$, and a HRMS measurement established a molecular formula of $C_{19}H_{18}O_{11}$. The IR spectrum suggested the presence of phenolic hydroxyl groups and a conjugated carbonyl group from bands at 3320 cm^{-1} and 1643 cm^{-1}. The UV spectrum exhibited characteristic absorption bands[15] of a 1,3,5,6-tetraoxygenated xanthone at λ_{max} 256, 281, and 325 nm. Acid hydrolysis of **1** yielded an aglycon and a sugar. The aglycon was identified as 1,3,5,6-tetrahydroxyxanthone (**3**),[16] which has been isolated previously from the same culture, by direct comparison with an authentic sample.[11] The sugar unit was determined to be glucose by TLC.



1 R = Glc
2 R = Glc-(2←1)-Rha
3 R = H

The ^1H NMR spectrum of **1** showed the presence of two characteristic pairs of *meta*-coupled aromatic protons (*δ* 6.40, 6.63) and *ortho*-coupled aromatic protons (*δ* 6.87, 7.50), a hydrogen-bonded hydroxyl proton (*δ* 13.25), an anomeric proton (*δ* 5.07), and other sugar protons in the range of *δ* 3.16−3.72 (Table 1). The lowfield shift of H-8 (*δ* 7.50), *ortho*-coupled with H-7, indicated that H-8 is located in an area deshielded by a carbonyl group. Thus, the two hydroxyl groups should be located at C-5 and C-6. The ^{13}C NMR spectrum of **1** showed signals for 19 carbons in the molecule, with there being six saccharide carbons, including an anomeric carbon at *δ* 99.8 and 13 carbons corresponding to a tetrahydroxyxanthone moiety, with a carbonyl carbon at *δ* 179.8 (C-9). The spectroscopic data of **1** indicated it to be a monoglucoside of 1,3,5,6-tetrahydroxyxanthone (**3**). All signal assignments in the ^1H and ^{13}C NMR spectra were confirmed with $^1H-^1H$ COSY and $^1H-^{13}C$ COSY 2D NMR techniques (Table 1).

Downfield shifts[17] of 0.24 and 0.23 ppm for H-2 (*δ* 6.40) and H-4 (*δ* 6.63), respectively, in the ^1H NMR spectrum and downfield shifts of 0.50 and 0.7 ppm for C-2 (*δ* 98.3) and C-4 (*δ* 94.5), respectively, in the ^{13}C NMR spectrum of **1**, compared with analogous data for **3**,[16] suggested the location of a glucose moiety at the 3-hydroxyl group. The configuration of the anomeric proton was determined as α from the ^1H NMR (J = 7.3 Hz) and ^{13}C NMR (Glc-1 at *δ* 99.8) data.[18] Thus, the structure of patuloside A was established as 3-*O*-*β*-D-glucopyranosyl-1,5,6-trihydroxyxanthone (**1**).

Patuloside B (**2**) crystallized as pale yellow needles, mp 233 °C, and the molecular formula of $C_{25}H_{28}O_{15}$ was established by HRFABMS measurements. Positive-ion FABMS showed a $[M + H]^+$ ion at *m*/*z* 569. The IR spectrum suggested the presence of phenolic hydroxyl groups at 3200 cm^{-1} and a conjugated carbonyl group at 1650 cm^{-1}, while the UV spectrum exhibited maxima at 206, 251, 281, and 325 nm.

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Table 1. ^{1}H, ^{13}C NMR and HMBC Data for Patulosides A (**1**) and B (**2**)[a]

	patuloside (**1**)		patuloside (**2**)		
position	^{13}C	^{1}H (*J*)	^{13}C	^{1}H (*J*)	HMBC
1	162.4		162.5		
2	98.3	6.40 d (2.1)	98.1	6.38 d (2.5)	1, 4, 9a
3	163.6		163.3		
4	94.5	6.63 d (2.1)	94.3	6.62 d (2.5)	2, 3, 4a, 9a
4a	156.9		157.0		
4b	145.7		146.2		
5	132.6		132.6		
6	153.6		152.6		
7	113.5	6.87 d (8.5)	113.3	6.95 d (8.8)	5, 8a
8	116.1	7.50 d (8.5)	116.0	7.52 d (8.8)	4b, 6, 9
8a	112.2		112.9		
9	179.8		179.9		
9a	102.9		103.0		
1-OH		13.25 s		13.15 s	
glucose moiety					
1′	99.8	5.07 d (7.3)	97.6	5.26 d (7.3)	
2′	73.0	3.16−3.51 m	76.2	3.53 m	Glc-4
3′	76.2	3.16−3.51 m	76.9	3.49 m	Glc-2
4′	69.5	3.16−3.51 m	69.6	3.22 m	Glc-3
5′	77.0	3.16−3.51 m	77.1	3.49 m	
6′	60.6	3.72 dd (1.8, 11.6)	60.5	3.50 dd (5.4, 8.8) 3.73 d (5.4)	Glc-4
rhamnose moiety					
1″			100.4	5.15 d (1.5)	Glc-2, Rha-2, -3, -5
2″			70.4	3.71 dd (1.5, 3.4)	Rha-3, -4
3″			70.5	3.35 dd (3.4, 9.8)	Rha-4
4″			71.8	3.22 m	Rha-2, -3
5″			68.3	3.77 m	
6″			18.0	1.21 d (5.9)	Rha-4, -5

[a] Data were recorded in DMSO-d_6 at 500 Hz (^{1}H) or 125 MHz (^{13}C). Multiplicities and coupling constants are given in Hz.

The ^{1}H and ^{13}C NMR data of **2** (Table 1) were similar to those of patuloside A (**1**), but **2** had an additional rhamnose moiety. The ^{1}H NMR spectrum of **2** showed the presence of signals corresponding to a 1,3,5,6-tetrahydroxyxanthone skeleton, and the sugar moieties included two anomeric protons at δ 5.15 (J = 1.47 Hz, Rha) and δ 5.26 (J = 7.33 Hz, Glc). The ^{13}C NMR spectrum of **2** showed 25 signals, of which 12 were assigned to the saccharide protons and 13 to a tetrahydroxyxanthone moiety. The sugar proton resonances were assigned based on the ^{1}H−^{1}H COSY spectrum, and the ^{13}C NMR data were assigned from the ^{13}C−^{1}H COSY spectrum. The NMR data of **2** (Table 1) indicated that it was a monorhamnoside of patuloside A (**1**). Partial acid hydrolysis of **2** with 10% formic acid yielded L-rhamnose and **1**, which was identified by comparison with an authentic sample.

The position of the rhamnose linkage in **2** was established at the 2′-hydroxyl group of the glucose moiety by HMBC techniques (Table 1), which showed cross peaks between the signals at δ 5.15 (Rha-1) and δ 76.2 (Glc-2). The configuration of anomeric protons of the glucose and rhamnose were determined as α and β based on J values in the ^{1}H NMR spectrum and chemical shifts in the ^{13}C NMR spectrum.[18] Thus, the structure of patuloside B was established as 3-*O*-(2-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranosyl-1,5,6-trihydroxyxanthone (**2**).

Prenylated or glycosylated 1,3,6,7-tetrahydroxyxanthone derivatives and prenylated 1,3,5,6-tetrahydroxyxanthone derivatives have been isolated to date from cell suspension cultures of *H. patulum*. However, glycosylated 1,3,5,6-tetrahydroxyxanthone derivatives (**1** and **2**) are reported for the first time in this study. The co-occurrence of 1,3,5,6- and 1,3,6,7-tetraoxygenated xanthones in species of the Guttiferae[19] and Moraceae[20] has been reported, and their coexistence in cell suspension cultures of *H. patulum* was shown for the first time by Ishiguro et al.[9]

In a previous study, we reported antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* of xanthone derivatives isolated from *H. patulum* cultures.[12] We considered it worth investigating the same biological effect for patulosides A (**1**) and B (**2**). However, these compounds did not suppress the growth of *S. aureus* and *E. coli* at 1 mg/mL using an agar-well method.[18] Therefore, this result supports our previous conclusion that the antibacterial activity of xanthone derivatives from *H. patulum* is dependent on the presence of either a prenyl group or a cyclopentane ring in the molecule.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micro melting-point apparatus. IR spectra were recorded on a Shimazu 435 spectrometer and the UV absorption spectra with Hitachi 323 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with JEOL JNM-GSX 500 spectrometers (TMS as internal reference). FABMS were performed on a JEOL JMS-DX 303 double-focusing spectrometer, with an ion-accelerating voltage of 3 kV. A fast-atom xenon beam was generated from Xe^+ ions that were accelerated to 6 kV. The mass maker was calibrated with poly(ethylene glycol)-400 for patuloside A (**1**) and poly(ethylene glycol)-600 for patuloside B (**2**) (resolution = 5000).

Plant Material. Seedlings of *H. patulum* were grown at Mukogawa Women's University and verified by Dr. G. Yoneda (Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan). A voucher sample (MWU P3144014) is kept at Mukogawa Women's University.

Suspension Culture. Callus tissues were induced from the flower of *H. patulum* on LS solid medium containing 3% sucrose; 10^{-5} M 2,4-D; 10^{-7} M kinetin; and 1.4% agar (pH 5.5) in the dark at 27 °C. Callus tissue cultures were transferred into liquid LS medium[14] containing 3% glucose; 10^{-5} M 2,4-D; and 10^{-7} M kinetin (pH 5.5) to initiate suspension cultures and were subcultured every 2−3 weeks for one year. Suspen-

sion cultures were maintained at 27 °C in the dark on a rotary shaker (100 rpm).

Extraction of Cultured Cells. Cells were collected by filtering the suspension cultures at 2−3-week intervals. Whole cells (1.2 kg), after being washed with H_2O and dried under a flow of hot air at 60 °C, were extracted successively with $CHCl_3$ and MeOH.

Isolation of 1 and 2. The MeOH extract (283 g) was dissolved in a minimal amount of H_2O and partitioned with EtOAc. The aqueous phase was then extracted with *n*-BuOH. The *n*-BuOH extract (30 g) was subjected to polyamide column chromatography using H_2O and MeOH as solvents. The MeOH-soluble eluent was subjected to Si gel column chromatography using $CHCl_3$−MeOH as eluent. A fraction eluted by $CHCl_3$−MeOH (5:1) was purified by gel filtration over Sephadex LH-20 with MeOH and was recrystallized from MeOH to obtain patuloside A (**1**, 3.8 mg). The $CHCl_3$−MeOH (3:1) fraction was recrystallized from MeOH to obtain patuloside B (**2**, 23 mg).

Patuloside A (1): pale yellow needles (MeOH), mp 282 °C; UV (MeOH) λ_{max} (log ϵ) 206 (4.20), 251 (4.52), 281 (3.89), 325 (4.14) nm; IR (KBr) ν_{max} 3320 (br, OH), 2870, 1643, 1600, 1495, 1460 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; positive FABMS *m*/*z* 423 $[M + 1]^+$ (99); HRFABMS *m*/*z* 423.0934 (calcd for $C_{19}H_{18}O_{11}$, 423.0927).

Patuloside B (2): pale yellow needles (MeOH), mp 233 °C; UV (MeOH) λ_{max} (log ϵ) 205 (4.25), 251 (4.02), 281 (4.63), 325 (4.35) nm; IR (KBr) ν_{max} 3400 (br, OH), 3200, 2850, 1650, 1590, 1610, 1490, 1460, 1400 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz,) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; positive FABMS *m*/*z* 569 $[M + 1]^+$ (99); HRFABMS *m*/*z* 569.1514, (calcd for $C_{25}H_{28}O_{15}$, 569.1507).

Antimicrobial Assay. The in vitro antimicrobial activity was determined by the agar-well method[21] using both *Staphylococcus aureus* 209P JC-1 and *Escherichia coli* NIHJ JC-2. Plates were prepared with 4-mm deep sensitivity test agar medium (Eiken Chemical Co., Ltd., Tokyo, Japan), which had previously been inoculated with 1% (v/v) of a test organism grown overnight at 37 °C in heart infusion broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Holes of 8-mm diameter were bored in the medium with a sterile borer and filled with a 40-μL solution (1 mg/mL) of each test sample. The plates were sealed and then incubated for 18 h at 37 °C. The activity was expressed as the diameter of the inhibition.

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