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Ohioensins F and G: Protein tyrosine phosphatase 1B inhibitory benzonaphthoxanthenones from the Antarctic moss *Polytrichastrum alpinum*

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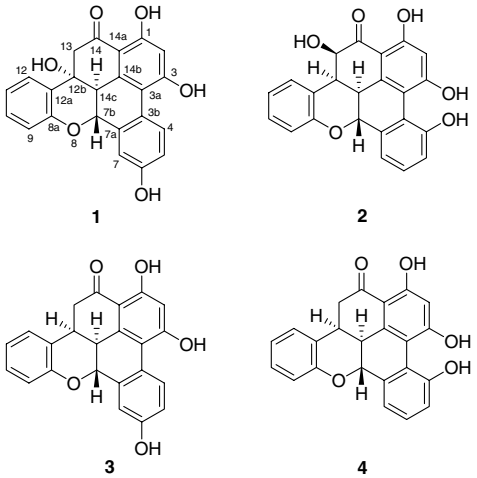
Abstract—Ohioensins F and G (**1** and **2**), two new benzonaphthoxanthenones, have been isolated from the MeOH extract of Antarctic moss *Polytrichastrum alpinum* by various chromatographic methods. The structures of these compounds were determined mainly by analysis of NMR spectroscopic data. The known compounds ohioensins A and C (**3** and **4**) were also obtained. Compounds **1**–**4** showed potent inhibitory activity against therapeutically targeted protein tyrosine phosphatase 1B (PTP1B). Kinetic analysis of PTP1B inhibition by ohioensin F (**1**) suggested that benzonaphthoxanthenones inhibited PTP1B activity in a non-competitive manner.

The bryophytes are classified into liverworts, hornworts, and mosses depending on their morphology.[1] Although the chemistry of liverworts has been intensively investigated over the last several decades, much less effort has been devoted to the mosses which are currently represented by approximately 10,000 species that colonize diverse habitats.[2] It is also conceivable that the chemistry of mosses is different from that of liverworts since mosses do not contain cellular oil bodies which are common in liverworts.[3,4] Thus, mosses represent a relatively untapped natural source to be explored for new bioactive metabolites.

During the course of chemical studies of mosses from Antarctic region as potential sources of new bioactive secondary metabolites, we investigated a MeOH extract of a sample of *Polytrichastrum alpinum* (Hedw.) G.L. Sm.[5] Chemical studies of this extract led to the isolation of two new metabolites named ohioensin F (**1**) and ohioensin G (**2**), along with two known metabolites, ohioensins **A** and **C** (**3** and **4**). This report describes the isolation, structure elucidation, and biological activities of compounds **1**–**4**.



A dried sample of *P. alpinum* (50 g) was extracted with MeOH (1 L × 2) for 24 h. The resulting crude MeOH extract (4.7 g) was subjected to C_{18} functionalized silica gel

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flash column chromatography (3 × 15 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 70%, 80%, 90%, and 100% (v/v) MeOH in H_2O (400 mL each). The fraction eluted at 80% MeOH (41 mg) was then subjected to semi-preparative reversed-phase HPLC using a gradient from 40 to 60% CH_3CN in H_2O (0.1% formic acid) over 27 min to yield **1** (3.9 mg; t_R = 22.4 min). The fraction eluted with 70% MeOH in water (32 mg) was subjected to semi-preparative reversed-phase HPLC using a gradient from 40 to 82% CH_3CN in H_2O (0.1% formic acid) over 42 min to yield **2** (2.6 mg; t_R = 29.5 min) and **4** (4.0 mg; t_R = 40.5 min). Compound **3** (4.5 mg, t_R = 16.0 min) was isolated by semi-preparative reversed-phase HPLC [eluting with gradient from 40 to 80% CH_3CN in H_2O (0.1% formic acid) over 30 min] using the fraction eluted with 90% MeOH from the C_{18} functionalized silica gel flash column.

Ohioensin F (**1**)[6] has the molecular formula of $C_{23}H_{16}O_6$, as deduced from ^{13}C NMR and HRESIMS [*m*/*z* 389.1000 $(M+H)^+$; Δ + 2.5 mmu] data. This formula indicated 16 degrees of unsaturation. The ^1H NMR and DEPT data revealed the presence of eight aromatic methines, two sp^3 methines (one oxygenated), and a methylene unit. In addition to signals corresponding to the above carbons, analysis of the ^{13}C NMR and DEPT data revealed the presence of one carbonyl carbon, ten non-protonated aromatic carbons, and a quaternary sp^3 carbon, indicating the highly aromatic nature of **1**. Analysis of the COSY NMR data led to the identification of the 1,2-disubstituted, 1,2,4-trisubstituted, and pentasubstituted benzene rings. These structural features accounted for 13 unsaturation equivalents. The remaining unsaturation equivalents must be accounted for by the presence of three additional rings. The presence of isolated diastereotopic methylene unit and mutually coupled sp^3 methines was also evident in the COSY spectrum. ^1H and ^{13}C NMR assignments and connections of the aforementioned structural units were determined on the basis of HMQC and HMBC data (Table 1). HMBC correlation of H-4 with C-3a required attachment of the 1,2,4-trisubstituted ring to the pentasubstituted ring at C-3a. A partial skeleton composed of aliphatic carbons C13–C12b–C14c–C7b was established by analysis of HMBC data, including key correlations of H-7b/H-14c/H-13 with oxygenated sp^3 quaternary carbon C-12b, and of H-14c with C-13. HMBC correlations of H-14c with C-14b/C-3a, and of H-7b with C-7a/C-3b/C-7, enabled connection of the biphenyl unit and the aliphatic fragment via C-14b/C-14c and C-7a/C-7b connections. Linkages of the ketone carbonyl C-14 to C-13 and the aromatic carbon C-14a were made on the basis of HMBC correlations of H-13 with C-14 and C-14a. The presence of an intramolecular hydrogen-bonded phenolic OH proton (δ 11.95) and HMBC correlations of this signal to C-1/C-2/C-14a supported this assignment. HMBC correlation of H-12 with oxygenated quaternary carbon C-12b enabled connection of C-12a to C-12b. At this point, assignments of the three remaining hydroxy groups and the construction of an additional ring to fulfill the unsaturation requirement were necessary to complete the gross structure of **1**. However, no positive spectral information for these assignments was available except for chemical shift data (C3/δ 162.7; C6/δ 156.1; C7b/δ 72.4; C8a/δ 151.8; C12b/δ 65.5). Thus, an acetylated derivative of **1** was prepared in the presence of pyridine/DMAP.[7] Formation of the tetraacetate for **1** was confirmed by observation of ^1H NMR signals for four acetate methyl groups. In addition, the chemical shifts of H-7b and H-9 were almost unaffected upon acetylation while downfield shifts of the signals for H-2 (Δδ 0.5), H-5 (Δδ 0.33), and H-7 (Δδ 0.23) were evident. Thus, the four hydroxy groups were located at C-1, C-3, C-6, and C-12b, and the remaining two oxygenated carbons (i.e., C-8a and C-7b) were connected via the final oxygen atom to complete the gross structure of **1**.

Table 1. NMR Spectroscopic data for Ohioensin F (**1**) in DMSO-d_6

Position	δ_H (int, mult., *J* in Hz)[a]	δ_C[b]	HMBC (H → C#)
1		160.4	
2	6.39 (1H, s)	101.8	1, 3, 3a, 14a
3		162.7	
3a		114.4	
3b		121.3	
4	8.25 (1H, d, 8.4)	129.0	3a, 6, 7a
5	6.77 (1H, dd, 8.7, 2.6)	113.7	3b, 7
6		156.1	
7	7.26 (1H, br s)	110.8	3b, 5, 6, 7b
7a		139.1	
7b	5.25 (1H, d, 14.3)	72.4	3b, 6,[c] 7, 7a, 12b, 14b, 14c
8a		151.8	
9	7.02 (1H, br d, 8.0)	116.5	8a, 10, 11, 12b[c]
10	7.28 (1H, br t, 7.7)	128.6	8a, 12
11	7.05 (1H, br t, 7.7)	121.4	9, 10
12	7.62 (1H, dd, 7.7, 1.4)	127.8	8a, 12a, 12b
12a		129.3	
12b		65.5	
13	2.85 (1H, d, 15.2) 3.24 (1H, d, 15.2)	49.0	12b, 14, 14a, 14c 7b,[c] 12b, 14
14		199.9	
14a		109.4	
14b		137.2	
14c	3.28 (1H, d, 14.3)	46.6	3a, 7a, 13, 14a, 14b
1-OH	11.95 (1H, s)		1, 2, 14a

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.
[c] Weak four-bond correlations.

The large *J*-value (14.3 Hz) between the two bridgehead protons H-7b and H-14c indicated a *trans*-diaxial relationship for these protons. In addition, NOESY correlation of H-7b with one of the diastereotopic (H_2-13) protons at δ 2.85 suggested the proximity of these two protons, indicating the presence of a *cis* fusion between the cyclohexenone and dihydropyran rings. Thus, the relative configuration of **1** was assigned as shown.

Ohioensin G (**2**)[8] was assigned the same molecular formula of $C_{23}H_{16}O_6$ as ohioensin F (**1**) on the basis of HRESIMS and NMR data. Analysis of ^1H and ^{13}C NMR data for **2** (Table 2) revealed the presence of the same highly aromatic polycyclic skeleton as found in ohioensin F (**1**). The presence of a signal for non-oxygenated sp^3 methine and the absence of the isolated dia-

Table 2. NMR Spectroscopic data for Ohioensin G (**2**) in DMSO-d_6

Position	δ_H (int, mult., J in Hz)[a]	δ_C[b]	HMBC (H → C#)
1		163.5	
2	5.81 (1H, s)	103.9	1, 3a, 14a
3		175.7	
3a		118.3	
3b		121.23	
4		158.2	
5	6.60 (1H, d, 8.3)	118.6	3b, 7
6	7.04 (1H, t, 8.3)	126.4	4, 7a
7	7.11 (1H, d, 8.3)	111.7	3b, 5
7a		140.1	
7b	5.02 (1H, d, 13.2)	73.2	7a, 14c
8a		154.1	
9	7.00 (1H, br d, 8.4)	116.4	8a, 12a
10	7.18 (1H, br t, 8.4)	127.5	8a, 12
11	6.95 (1H, br t, 8.4)	121.15	9, 12a
12	7.38 (1H, br d, 8.4)	129.2	8a, 10, 12b
12a		120.4	
12b	3.42 (1H, m)	33.7	7b, 12a
13	4.17 (br s)	73.5	
14		198.1	
14a		103.3	
14b		140.2	
14c	2.95 (1H, dd, 13.2, 7.7)	36.2	3a, 12b, 13, 14b
1-OH	12.12 (1H, s)		1, 2, 14a

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.

stereotopic methylene signals in the ^{1}H NMR spectrum of **2** suggested translocation of the hydroxy group from C-12b of **1** to C-13 in **2**. The ^{1}H and ^{13}C NMR chemical shifts and ^{1}H NMR coupling patterns in the aromatic region were also different, suggesting some modification of the substitution patterns in the benzene rings as well. Detailed analysis of COSY data for **2** confirmed the presence of the C7b–C14c–C12b–C13 spin system, and the 1,2-disubstitured, 1,2,3-trisubstituted, and pentasubstituted benzene rings. ^{1}H and ^{13}C NMR chemical shifts assignments for **2** were established by analysis of HMQC data. Analysis of HMBC data confirmed the presence of the benzonaphthoxanthenone skeleton. Based on above considerations, the structure of ohioensin G was assigned as shown in **2**.

Literature search found similar benzonaphthoxanthenones called ohioensins that possess the same ring system as appeared in compounds **1** and **2**.[9–11] Thus, the relative configuration of **2** was determined by comparison of its ^{1}H–^{1}H coupling constants with those reported for ohioensin **C** (**4**).[10] The similarities of the coupling constants between H-7b and H-14c (J = 13.2 Hz), and H-14c and H-12b (J = 7.7 Hz), in both **2** and **4** led to assignment of the analogous, *tans* H-7b/H-14c and *cis* H-14c/H-12b relative configuration in **2**. The small J-value observed between H-12b and H-13 indicated that H-13 adopted a pseudoequatorial orientation with respect to the cyclohexenone ring. Thus, the relative configuration between H-12b and H-13 was assigned as *cis*.

The known compounds ohioensins A (**3**) and C (**4**) were also obtained in this investigation, and were identified by comparison of their NMR data with those reported in the literature.[9,10]

Ohioensins F (**1**) and G (**2**) were new members of the benzonaphthoxanthenones, which are suggested to be derived by condensation of *o*-hydroxycinnamate with hydroxylated phenanthrenes or 9,10-dihydrophenanthrene units.[10] Occurrence of benzonaphthoxanthenone-type metabolites among natural products is uncommon, with the mosses *Polytrichum ohioense*[9,10] and *P. pallidisetum*[11] the only previously reported sources of this class of compounds. Ohioensins A–E were encountered in the course of the NCI screening program aiming for discovery of novel bioactive metabolites from mosses, and they displayed cytotoxicity against various tumor cells in culture.[9–11]

Compounds **1**–**4** inhibited the activity of PTP1B in a dose-dependent manner (data not shown), and their IC_{50} values were determined as 3.5 ± 0.2, 5.6 ± 0.7, 4.3 ± 0.3, and 7.6 ± 0.7 μM, respectively.[12] A known phosphatase inhibitor, RK-682 ($IC_{50} = 4.5 \pm 0.5$ μM), was used as a positive control in the assay.[13,14] To elucidate the inhibition mode of benzonaphthoxanthenones discovered in this study, inhibition kinetics of **1** were performed with different concentrations of substrate.[12] When *p*-nitro-phenyl phosphate (*p*NPP) was used as substrate, **1** decreased the V_{max} value, but did not alter the K_m value of PTP1B (Fig. 1) under our experimental conditions. Thus, **1** was determined as a non-competitive inhibitor with a K_i value of 1.5 μM. The inhibition modes of **2**–**4** were presumed to be analogous to that of **1** based on their structural similarities. Several non-competitive inhibitors of PTP1B were shown to interact with an allosteric site of PTP1B,[15] and the allosteric modulation of PTP1B activity has been suggested to be a promising approach for developing selective inhibitors.[15,16]

Inhibitors of PTP1B, a major nontransmembrane phosphotyrosine phosphatase in human tissues and a negative regulator of the insulin-stimulated signal transduction pathway,[17] are considered as potential agents in efforts to develop new treatments for type 2 diabetes and related metabolic syndromes.[18–20] Although several classes of natural products have been identified as PTP1B inhibitors,[21–23] PTP1B inhibitory ef-

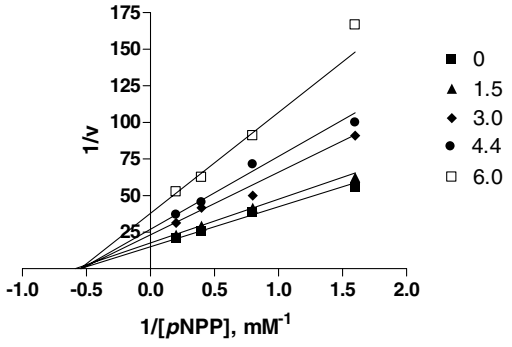

Figure 1. Kinetic analysis of PTP1B inhibition by ohioensin F (**1**). A Lineweaver-Burk plot for ohioensin F (**1**) inhibition of PTP1B. Data are expressed as mean initial velocity for n = 3 replicates at each substrate concentration. Concentrations (μM) of **1** are indicated in figure.

fects of the benzonaphthoxanthenones are now being reported for the first time, thus further investigation for therapeutic potential of these compounds is warranted.

Acknowledgments

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5. (a) *Polytrichastrum alpinum* (Hedw.) G.L. Sm. was collected and identified by one of us (J. H. Yim) from Barton Peninsular around King Sejong Station (S 62°13.3′, W 58°47.0′) on King George Island, Antarctica in January, 2003. *P. alpinum* is a common moss that occurred in most ice-free areas on King George Island; (b) Kim, J. H.; Ahn, I.-Y.; Lee, K. S.; Chung, H.; Choi, H.-G. *Polar Biol.* **2007**, *30*, 903.
6. Ohioensin F (**1**): yellow gum; $[\alpha]_D^{25} + 54$ (*c* 0.52, MeOH); UV (CH_3OH) λmax (log ε) 367 (3.4), 273 (4.4), 215 (4.6); ^1H, ^{13}C, and 2D NMR data, see Table 1; ^1H NMR (CD_3OD, 400 MHz) δ 8.29 (d, *J* = 8.4 Hz, H-4), 7.61 (d, *J* = 7.6 Hz, H-12), 7.35 (br s, H-7), 7.26 (t, *J* = 7.5 Hz, H-10), 7.05 (t, *J* = 7.5 Hz, H-11), 7.04 (d, *J* = 7.5 Hz, H-9), 6.77 (d, *J* = 8.4 Hz, H-5), 6.35 (br s, H-2), 5.10 (d, *J* = 13.4 Hz, H-7 b), 3.08 (d, *J* = 15.4 Hz, H-13), 3.03 (d, *J* = 15.4 Hz, H-13); the signal for H-14c was overlapped with residual solvent signals; ESIMS *m*/*z* 387 [$(M-H)^-$; rel int 100)]; HRESIMS *m*/*z* 389.1000 $(M+H)^+$ (calcd for $C_{23}H_{17}O_6$, 389.1025).
7. Approximately 1 mg of ohioensin A (**1**) was dissolved in acetone (1 mL) and combined with DMAP (2 mg) and acetic anhydride (0.5 mL). The reaction mixture was stirred at room temperature for 24 h. The resulting mixture was separated by reversed-phase HPLC (Capcell Pak C18 column; 10 × 250 mm; 5-μm particle size; 60-100% CH_3CN in H_2O over 16 min; 2 mL/min) to afford the acetylated product (0.4 mg, t_R 15.5 min): ^1H NMR (CD_3OD, 400 MHz) δ 8.48 (d, *J* = 7.5 Hz, H-4), 7.62 (d, *J* = 7.6 Hz, H-12), 7.58 (br s, H-7), 7.26 (t, *J* = 7.6 Hz, H-10), 7.10 (d, *J* = 7.5 Hz, H-5), 7.06 (t, *J* = 7.6 Hz, H-11), 7.05 (d, *J* = 7.6 Hz, H-9), 6.75 (br s, H-2), 5.13 (d, *J* = 16.4 Hz, H-7b), 2.89–2.97 (m, H-13), 2.32 (3H, s, CH_3), 2.02 (3H, s, CH_3), 2.01 (3H, s, CH_3), 1.95 (3H, s, CH_3); the signal for H-14c was overlapped with residual solvent signals; ESIMS *m*/*z* 557 [$(M+H)^+$; rel int 100)].
8. Ohioensin G (**2**): yellow gum; $[\alpha]_D^{25} - 12$ (*c* 0.43, MeOH); UV (CH_3OH) λmax (log ε) 357 (3.6), 269 (4.1), 215 (5.1); ^1H, ^{13}C, and 2D NMR data in DMSO-d_6, see Table 2; ESIMS *m*/*z* 387 [$(M-H)^-$; rel int 100)]; HRESIMS *m*/*z* 389.1011 $(M+H)^+$ (calcd for $C_{23}H_{17}O_6$, 389.1025).
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12. PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc., and the enzyme activity was measured using *p*-nitrophenyl phosphate (pNPP) as substrate. For inhibition assay, inhibitors were added to the reaction mixture (final volume 100 μL) containing PTP1B (0.05 – 0.1 μg) and 2 mM pNPP in a buffer solution [50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT)]. The reaction mixture was placed in a 30 °C incubator for 30 min, and the reaction was terminated by addition of 1 M NaOH solution (10 uL). The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM pNPP was corrected by measuring the absorbance at 405 nm in the absence of PTP1B enzyme. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of PTP1B were determined by the Lineweaver-Burk plot using a GraphPad Prism® 4 program (GraphPad Software Inc., USA).
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