Bioorganic & Medicinal Chemistry Letters 22 (2012) 3332–3335

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Penicacids A–C, three new mycophenolic acid derivatives and immunosuppressive activities from the marine-derived fungus *Penicillium* sp. SOF07

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ARTICLE INFO

Article history:
Received 27 November 2011
Revised 13 February 2012
Accepted 28 February 2012
Available online 11 March 2012

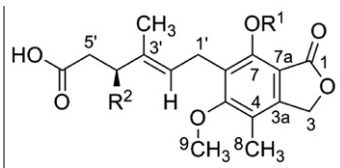
Keywords:
Marine fungi
Penicillium sp.
Penicacid A–C
Inosine-monophosphate dehydrogenase
Structure–activity relationship

ABSTRACT

Three new mycophenolic acid derivatives, penicacids A–C (**1–3**), together with two known analogues, mycophenolic acid (MPA, **4**) and 4′-hydroxy-MPA (**5**), were isolated from a fungus *Penicillium* sp. SOF07 derived from a South China Sea marine sediment. The structures of compounds **1–3** were elucidated on the basis of MS and NMR (^{1}H, ^{13}C, HSQC and HMBC) data analyses and comparisons with the known compounds. Structure–activity relationship studies of compounds **1–5** focused on inosine-monophosphate dehydrogenase inhibition revealed that hydroxylation at C-4′, methylation at C-7-OH, dual hydroxylation at C-2′/C-3′ double bond of MPA diminished bioactivity whereas glucosyl hydroxylation at C-4′ correlated to bioactivity comparable to that observed for MPA.

Marine-derived fungi are prolific producers of new, structurally intriguing and bioactive compounds as reflected by the fact that, at present, more than 1000 new compounds have been isolated and identified from marine fungi.[1] In our efforts to discover new bioactive natural products from marine fungi originating from the South China Sea, we have isolated new cytotoxic cytochalasins from the marine-derived fungus *Xylaria* sp. SCSIO 156.[2] Another strain, *Penicillium* sp. SOF07, attracted our attention because a crude extract of this fungal culture was lethal to brine shrimp (*Artemia salina*) and displayed cytotoxicity against human cancer cell lines A549, HCT15 and HEP3B. HPLC-(DAD)-UV analysis of this extract unveiled an array of structurally related secondary metabolites (**1–5**, Fig. 1) with similar characteristic UV absorptions; subsequent structure elucidation efforts revealed a conserved mycophenolic acid (MPA, **4**)-based structural scaffold.[3]

The fungal metabolite **4** displays a wide range of biological activities including antiviral, antitumor, and RNA capping inhibitory activity.[4–7] MPA is also a potent inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH), an essential rate-limiting enzyme in the purine metabolic pathway.[8] IMPDH controls the size of the guanine nucleotide pool which, in turn, controls many physiological processes including replication, transcription, signaling and glycosylation.[9] Consequently, IMPDH is an important drug target for immunosuppressive, antiviral and cancer chemotherapy.[10] Mycophenolate mofetil (MMF) is the 2-morpholinoethyl ester prodrug of MPA and has been widely used as an immunosuppressant in kidney, heart, and liver transplantation procedures.



1 $R^1 = CH_3$, $R^2 = OH$
2 $R^1 = H$, $R^2 = OGlu$
4 $R^1 = R^2 = H$
5 $R^1 = H$, $R^2 = OH$

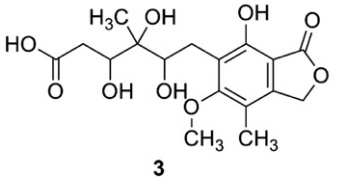


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Figure 1. Structures of compounds **1–5**.

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http://dx.doi.org/10.1016/j.bmcl.2012.02.106

Strain SOF07 was isolated from a sediment collected in the South China Sea at a depth of 675 m. The strain was identified as *Penicillium* sp. SOF07 on the basis of morphology and analysis of the ITS region sequence. The voucher specimen was deposited in RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences (Guangzhou, China).

Strain SOF07 was fermented in rice solid medium supplemented with 3% sea salt and the harvested whole culture broth extracted with methylethylketone to afford a residue after solvent evaporation. The residue was subjected to chromatography over normal phase silica gel and reverse phase C18 silica gel chromatography using MPLC and semi-preparative HPLC, respectively, to yield compounds **1**–**5** (Fig. 1).[11] Compounds **4** and **5** were identified as MPA and 4′-hydroxy-MPA, respectively, by comparing their MS, ^{1}H and ^{13}C NMR spectroscopic data with those previously reported.[12–14] Compounds **1**–**3** were elucidated as new compounds, designated penicacids A–C, on the basis of MS, 1D and 2D NMR data analyses and comparisons with known compounds.

Penicacid A (**1**) was isolated as an amorphous solid. Its molecular formula was established as $C_{18}H_{22}O_7$ by HRESIMS which displayed a quasimolecular peak at *m/z* 349.1521 $[M-H]^-$.[15a] The ^{1}H NMR spectrum of **1** was characterized by signals corresponding to a methyl group at δ_H 1.81 (3H, s, 3′-Me), an aromatic methyl group at δ_H 2.14 (3H, s, C-8), two methoxy moieties at δ_H 3.75 (3H, s, 9-OMe) and δ_H 3.66 (3H, s, 7-OMe), and an oxygen-bearing methylene singlet at δ_H 5.19 ppm. Detailed comparisons of the ^{1}H and ^{13}C NMR data for **1** with those of the known compound **5** showed a high degree of similarity although the hydroxy group of **5** was replaced in **1** with a methoxy group (Table 1). HMBC correlations involving C-7 and this OMe moiety clearly established OMe connectivity to C-7 of the MPA-based scaffold of **1**. Other HMBC correlations in **1** confirmed the highly conserved nature of **1** with **5**; numerous structural features of **1** and **5** were found to be identical. Thus, **1** was identified as 7-*O*-methyl-4′-hydroxy-MPA, a new compound.

Penicacid B (**2**) was obtained as an amorphous solid and its molecular formula determined to be $C_{23}H_{30}O_{12}$ on the basis of the pseudomolecular ion in the HRESIMS at *m/z* 497.1624 $[M-H]^-$.[15b] The molecular formula of **2** suggested the presence of nine degrees of unsaturation. The UV spectrum of **2** displayed characteristic absorption maxima for the MPA chromophore at 216, 250 and 304 nm. The ^{1}H, ^{13}C and DEPT NMR data for **2** strongly resembled those of **5** with the exception of an additional set of signals consistent with the presence of a sugar unit. The ^{1}H NMR data for **2** showed two methyl groups at δ_H 1.86 (3H, s, 3′-Me) and δ_H 2.18 (3H, s, C-8), and one methoxy group at δ_H 3.80 (3H, s, 9-OMe). A set of 2D NMR spectra (^{1}H–^{1}H COSY, HSQC and HMBC) of **2** was acquired allowing full assignment of all its ^{1}H and ^{13}C signals (Table 1). Examination of the COSY and HMBC correlations observed in **2** confirmed the presence of 4′-hydroxy-MPA (**5**) as the aglycone. Further HMBC correlation of the anomeric H-1″ to C-4′ confirmed the sugar connectivity to C-4′ of aglycone core **5** (Fig. 2). The sugar moiety was identified as glucopyranose on the basis of ^{1}H and ^{13}C NMR chemical shifts. The coupling constant of the anomeric proton obtained in DMSO-d_6 (J = 4.0 Hz) suggested the α linkage in the molecule. The optical rotation ($[\alpha]_D^{25}$ +48 (*c* 0.41, H_2O)) of the sugar obtained through acid hydrolysis of **2**, and the ^{1}H NMR data of the acetylated sugar confirmed that the sugar moiety was D-glucose.[16] Thus, compound **2** was elucidated to be α-D-glucopyranosyl-4′-*O*-MPA.

Penicacid C (**3**) was isolated as an amorphous solid and its molecular formula determined to be $C_{17}H_{22}O_9$ by the HRESIMS $[(M-H_2O)-H]^-$ signal at *m/z* 351.1018, suggesting seven degrees of unsaturation.[15c] The ^{1}H NMR data of **3** revealed a methyl singlet at δ_H 1.47 (3H, s, 3′-Me), an aromatic methyl singlet at δ_H 2.19 (3H, s, C-8), and a OMe moiety at δ_H 3.84 (3H, s, 9-OMe). The ^{13}C NMR of **3** was characterized by the absence of two olefinic carbons (C-2′ and C-3′) readily found in **5**. In turn, two oxygen-bearing carbons appeared at δ_C 72.9 [methine, δ_H 4.33 (1H, br s, H-2′), C-2′] and δ_C 91.4 (quaternary carbon, C-3′), and one of the methyl groups

Table 1
Summay of ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for compounds **1**–**3**

Position	**1**[a]		**2**[b]		**3**[b]	
	δ_C	δ_H (mult.; *J* Hz)	δ_C	δ_H (mult.; *J* Hz)	δ_C	δ_H (mult.; *J* Hz)
1	172.9		173.8		173.5	
3	70.1	5.19 (2H, s)	70.9	5.27 (2H, s)	70.6	5.27 (2H, s)
3a	144.2		146.9		147.9	
4	116.8		117.9		117.9	
5	163.7		164.9		165.3	
6	124.1		123.1		121.9	
7	153.7		154.8		156.0	
7a	106.4		107.8		108.3	
7-OMe	51.8	3.66 (3H, s)				
8	11.6	2.14 (3H, s)	11.5	2.18 (3H, s)	11.5	2.19 (3H, s)
9-OMe	61.0	3.75 (3H, s)	61.8	3.80 (3H, s)	61.8	3.84 (3H, s)
1′	22.3	3.41 (2H, d, 6.5)	23.4	3.45 (2H, m)	27.4	2.98 (1H, dd, 12.5, 3.0) 3.11 (1H, d, 12.5)
2′	121.6	5.55 (1H, t, 7.0)	127.6	5.59 (1H, t, 6.5)	72.9	4.33 (1H, br s)
3′	136.0		135.8		91.4	
4′	73.2	4.42 (1H, dd, 9.0, 3.5)	83.5	4.35 (1H, dd, 11.5, 4.0)	74.0	4.35 (1H, d, 3.0)
5′	40.0	2.54 (2H, m)	40.6	2.45 (1H, dd, 16.0, 8.0) 2.74 (1H, dd, 16.0, 9.0)	39.4	2.48 (1H, d, 18.0) 3.17 (1H, dd, 18.0, 5.5)
6′	173.0		176.4		178.0	
3′-Me	12.1	1.81 (3H, s)	12.0	1.86 (3H, s)	18.1	1.47 (3H, s)
1″			100.2	4.66 (1H, d, 4.0)[c]		
2″			74.0	3.51 (1H, m)		
3″			75.4	3.59 (1H, m)		
4″			71.1	3.34 (1H, m)		
5″			74.1	3.31 (1H, m)		
6″			61.9	3.59 (1H, m); 3.51 (1H, m)		

[a] Recorded in $CDCl_3$.
[b] Recorded in MeOH-d_4.
[c] Recorded in DMSO-d_6.

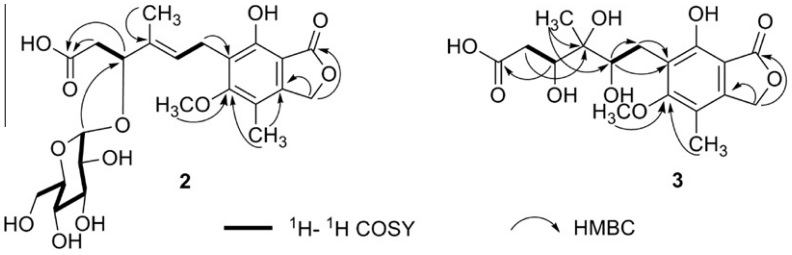

Figure 2. Key COSY and HMBC correlations of **2** and **3**.

Table 2
Summary of IMPDH and mouse splenocyte proliferation inhibition assays for compounds **1**–**5**

Compounds	IC_{50}[a] (μM) Inosine monophosphate dehydrogenase	IC_{50}[a] (μM) Mouse splenocyte proliferation
1	28.86 ± 2.50	2.46 ± 0.32
2	6.43 ± 1.10	>20
3	73.24 ± 6.40	>30
4	0.63 ± 0.09	0.32 ± 0.06
5	1.79 ± 0.13	1.10 ± 0.21

[a] Data represent the mean ± SD of three triplicate experiments.

(3′-Me) characteristic of **1**, **2**, **4** and **5** was down shifted to δ_C 18.1 ppm. ^{1}H–^{1}H COSY data revealed a H-1′/H-2′ spin system and HMBC experiments revealed correlations of H-2′/C-6, 3′-Me/C-3′, C-2′ (Fig. 2). Thus, the two olefinic side chain carbons characteristic of the other penicacids are replaced in **3** with the oxygen-bearing carbons C-2′ and C-3′. Limited amounts of **3**, coupled with our inability to obtain diffraction quality crystals have, thus far, abrogated stereochemical assignments for C-2′, C-3′ and C-4′ though efforts in this direction are ongoing. Consequently, **3** is assigned as 2′,3′-dihydroxy-4′-hydroxy-MPA.

To assess the biological activity of the penicacids we first evaluated **1**–**5** for their ability to inhibit IMPDH (type II) activity using the method reported by Magasanik et al.[17] Enzyme assays, in the presence or absence of varying concentrations of **1**–**5**, were carried out in 96-well microtiter plates and rates of reaction determined by monitoring absorbance at 340 nm resulting from enzymatic NADH production. Compounds **1**–**5** were dissolved in DMSO and serially diluted before being added to the initial assay mixture for pre-incubation with the enzyme. Concentrations of DMSO to dissolve compounds in assay mixtures were not allowed to exceed 2% (v/v). Data acquisition and processing revealed that **1**–**5** inhibited IMPDH with IC_{50} values of 28.86, 6.43, 73.24, 0.63, and 1.79 μM, respectively. These data suggest that: (i) hydroxylation at C-4′, methylation at C-7-OH, and dual hydroxylation at the C-2′/C-3′ olefin of **4** all decrease IMPDH inhibitory activity; (ii) C-4′ glycosylation of **4** (yielding **2**) minimally affects IMPDH inhibition, and (iii) the parent compound **4** remains the best inhibitor of IMPDH.

Inspired to further dissect the impact of the subtle structural differences among **1**–**5** compounds were then subjected to a splenocyte T lymphocyte proliferation assay,[18] the results of which are shown in Table 2. With the exception of glycosylated **2**, the immunosuppresive activities of **1**–**5** at the cellular level paralleled their IMPDH inhibitory activities. The collective SAR data from both bioassays reveal the importance of the C-7 OH, the C-2′/C-3′ olefin, and the absence of the C-4′ OH in the immunosuppresive activities displayed by the MPA scaffold at both the enzymatic and cellular levels.

MPA was discovered in 1893 by the Italian physician Bartolomeo Gosio.[3] MPA and related derivatives attracted a great deal of attention due to their vast array of biological activities. Among these activities, the MPA scaffold was associated with antiviral and immunosuppressive activities. However, there have been very few reports on the isolation of MPA and derivatives from natural sources[12,13,19] and even fewer investigations aimed at understanding the SAR of these compounds.[20] We aim in this report to reconcile, in part, this scarcity of information by reporting three new MPA analogues from a marine-derived fungus. Moreover, the **1**–**3** producer *Penicillium* sp. SOF07, is a previously unrecognized producer of known compounds **4** and **5**. On the basis of bioassays exploiting isolated enzyme and intact cells we provide further insight into MPA scaffold characteristics that may be applied to the design of new MPA derivatives as drug leads particularly in the context of immunosuppressives.

Acknowledgments

We thank the analytical facility center of South China Sea Institute of Oceanology for recording NMR data. This work is supported, in part, by grants from the Knowledge Innovation Programs of the Chinese Academy of Sciences (KZCX2-YW-JC202, KZCX2-EW-G-12 and KSCX2-YW-G-065), '863' Project (2012AA092104), Science and Technology Planning Project of Guangdong Province (2010B030600010), and Scientific Research Foundation for the Returned Overseas Chinese Scholars of the State Education Ministry. J.J. is a scholar of the '100 Talents Project' of Chinese Academy of Sciences (08SL111001).

Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra of compounds **1**–**3**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.02.106.

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11. The strain *Penicillium* sp. SOF07 was cultured on PDA medium supplemented with 3% sea salt at 25 °C for a week. The agar plugs containing strain SOF07 were inoculated into Erlenmeyer flasks containing 50 mL potato dextrose liquid broth supplemented with 3% sea salt. Flask cultures were incubated at 28 °C on a rotary shaker at 200 rpm for 2 d as seed culture. Ten milliliter of the seed culture was then inoculated to autoclaved solid medium (100 g rice,

100 mL H2O, 3 g sea salt). Incubation was carried out in twenty 500 mL Fernbach flasks each containing solid medium at 25 °C for 21 days. The fermented rice substrate was extracted with methylethylketone (3 × 4 L) to afford 40.45 g of crude residue. This extract was subjected to column chromatography (CC) on silica gel eluted with a gradient of $CHCl_3$–MeOH (100:0–0:100, v/v) to afford fractions 1–5. Fraction 2 was further purified on silica gel CC by gradient elution using petroleum–EtOAc (90:10–0:100, v/v) to get subfractions 1–7. Subfractions 2 and 4 were further purified by MPLC (ODS) with H_2O/MeOH gradient elution to yield compound **1** (11.0 mg), **4** (13.0 mg), and **5** (1.25 g), respectively. Fraction 3 was further refined by semi-preparative HPLC (YMC-pack ODS-A, 5 μm; 10 × 250 mm) to render **2** (5.8 mg) and **3** (4.2 mg).

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15. Physicochemical properties of **1**–**3**. (a) Compound **1**: amorphous solid; $[\alpha]_D^{25}$ −14 (*c*, 0.65, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 216 (4.71), 250 (4.18), 304 (3.92) nm; ^1H and ^{13}C NMR, see Table 1; ESI-MS (negative) *m/z*: 349.2 ($[M-H]^-$); HR-ESI-MS (negative) *m/z*: 349.1521, calcd for $C_{18}H_{22}O_7$. (b) Compound **2**: amorphous solid; $[\alpha]_D^{25}$ 74 (*c*, 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 216 (4.70), 250 (4.15), 304 (3.88) nm; ^1H and ^{13}C NMR, see Table 1; ESI-MS (negative) *m/z*: 497.6 ($[M-H]^-$); HR-ESI-MS (negative) *m/z*: 497.1624, calcd for $C_{23}H_{30}O_{12}$. (c) Compound **3**: amorphous solid; $[\alpha]_D^{25}$ 25 (*c*, 0.28, MeOH); UV (MeOH) λ_{max} (log ε): 216 (4.68), 249 (4.10), 304 (3.79) nm; ^1H and ^{13}C NMR, see Table 1; ESI-MS (negative) *m/z*: 369.3 ($[M-H]^-$); ESI-MS (positive) *m/z*: 375.2 ($[(M-H_2O)+Na]^+$); HR-ESI-MS (negative) *m/z*: 351.1018 $[(M-H_2O)-H]^-$, calcd for $C_{17}H_{22}O_9$.
16. Penicacid B (**2**, 2.0 mg) was treated with 1 N aqueous HCl (2 mL) at 95 °C for 2 h. The acidic aqueous mixture was extracted with EtOAc (3 × 2 mL), the aqueous fraction was evaporated to dryness. The optical rotation of the dried residue was determined ($[\alpha]_D^{25}$ +48 (*c* 0.41, H_2O)). The residue of sugar fraction was treated with pyridine (100 μL), Ac_2O (100 μL) at room temperature for 24 h. The reaction mixture was subjected to column chromatography on silica gel, eluted with a gradient of $CHCl_3$–MeOH (100:0–90:10) to yield compound **6**. The ^1H NMR of **6** was in agreement with that of an authentic D-glucose penta acetate (**7**) (see Figures S8 and S9).
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