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PTP1B Inhibitory Secondary Metabolites from Marine-Derived Fungal Strains *Penicillium* spp. and *Eurotium* sp.

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The selective inhibition of PTP1B has been widely recognized as a potential drug target for the treatment of type 2 diabetes and obesity. In the course of screening for PTP1B inhibitory fungal metabolites, the organic extracts of several fungal species isolated from marine environments were found to exhibit significant inhibitory effects, and the bioassay-guided investigation of these extracts resulted in the isolation of fructigenine A (1), cyclopenol (2), echinulin (3), flavoglaucin (4), and viridicatol (5). The structures of these compounds were determined mainly by analysis of NMR and MS data. These compounds inhibited PTP1B activity with 50% inhibitory concentration values of 10.7, 30.0, 29.4, 13.4, and 64.0 μM, respectively. Furthermore, the kinetic analysis of PTP1B inhibition by compounds 1 and 5 suggested that compound 1 inhibited PTP1B activity in a noncompetitive manner, whereas compound 5 inhibited PTP1B activity in a competitive manner.

Keywords: *Penicillium*, *Eurotium*, fungal metabolites, protein tyrosine phosphatase 1B (PTP1B) inhibition, enzyme kinetic analysis

Protein tyrosine phosphatase 1B (PTP1B) plays a key role in the negative regulation of insulin signaling by dephosphorylating the insulin receptors (IR), insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) [6, 15, 25]. It has been shown that PTP1B levels are increased in insulin-resistant diabetes patients, and the deletion of PTP1B in mice has been shown to increase insulin sensitivity [2, 8, 16]. In addition, small molecule inhibitors of PTP1B work synergistically with insulin to increase signaling and augment insulin-stimulated glucose uptake [30, 34]. Several genetic and biochemical studies indicate that PTP1B is also implicated in the inhibition of leptin signaling, which inhibits food intake and promotes energy expenditure [14, 22, 33], and there are several recent studies showing that body weight, adiposity, and leptin action can be regulated by neuronal PTP1B [21]. Taken all together, PTP1B has emerged as one of the well-validated drug targets for the treatment of both diabetes and obesity. Recognition of PTP1B as a novel drug target has led to a number of studies on the design and development of synthetic PTP1B inhibitors [13, 17]. Several classes of plant-derived secondary metabolites have also been described as PTP1B inhibitors [17].

Marine microorganisms have been recognized as a rich source of structurally novel and pharmacologically active secondary metabolites [10, 20]. In particular, fungi from the marine environment have been shown to produce diverse secondary metabolites that are more or less similar to those produced by terrestrial fungi [5, 24]. As part of our ongoing studies on PTP1B inhibitors from marine microorganisms collected from Korea, fungal isolates *Penicillium* sp. SF-5203, *Eurotium* sp. SF-5130, and *Penicillium* sp. SF-5295 were selected for further investigation on the basis of their potent inhibitory effects against PTP1B.

Penicillium sp. SF-5203 (deposited at College of Medical and Life Sciences fungal strain repository, Silla University) was isolated from an intertidal sediment sample collected from Wan Island, Korea, in January 2008. The sediment sample was stored in a sterile plastic bag and transported to the laboratory where it was kept frozen until processed. The sample was diluted 10-fold using sterile seawater. One

milliliter of the diluted sample was processed by using the spread plate method, in potato dextrose agar (PDA) medium containing 3% NaCl. The plate was incubated at 25°C for 14 days. After purifying the isolates several times, the final pure cultures were selected and preserved at -70°C. A GenBank search with the 28S rRNA gene of SF-5203 indicated *Penicillium expansum* (JN938952) and *P. solitum* (JN642222) as the closest matches, showing sequence identities of 98.85%. Therefore, the marine-derived fungal strain SF-5203 was identified as a *Penicillium* sp. The fungal strain SF-5203 was cultured on 30 petri plates (90 mm), each containing 20 ml of PDA with 3% NaCl. The plates were individually inoculated with 2 ml of seed culture of the fungal strain and incubated at 25°C for a period of 10 days. Extraction of the combined agar media with EtOAc (2 L) provided an organic phase, which was then concentrated *in vacuo* to yield 400 mg of extract. The EtOAc extract was subjected to C_{18} flash column chromatography (5 × 25 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 70%, 80%, 90%, and 100% (v/v) MeOH in H_2O (300 ml each). The fraction eluted at 70% MeOH was identified as fructigenine A (**1**, 2.5 mg). The fraction eluted at 60% MeOH (39.7 mg) was further purified by semi-preparative reversed-phase HPLC, eluting with a gradient from 40% to 60% MeOH in H_2O (0.1% formic acid) over 80 min to yield **2** (1.6 mg, t_R = 18.0 min). Solvents for extractions and flash column chromatography were of reagent grade and used without further purification. Solvents used for HPLC were of analytical grade. Flash column chromatography was carried out using YMC octadecyl-functionalized silica gel (ODS-A, 75 μm particle size). HPLC separations were performed on a Shiseido Capcell Pak C_{18} column (10 × 250 mm; 5 μm particle size) with a flow rate of 2 ml/min. Compounds were detected by UV absorption at 254 nm.

The structures of compounds **1** and **2** were determined as fructigenin A and cyclopenol, respectively, by analysis of NMR and MS data, along with comparisons with those in the literature [1,11]. ESIMS data were obtained using an API-2000 ESI-MS instrument (Applied Bio System, USA). NMR spectra (1D and 2D) were recorded in DMSO-d_6, CD_3OD, and acetone-d_6 using a JEOL JNM ECP-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to the respective residual solvents (δ_H/δ_C = 2.49/39.5 for DMSO-d_6; δ_H/δ_C = 3.30/49.0 for CD_3OD; δ_H/δ_C = 7.26/77.0 for $CDCl_3$). HMQC and HMBC experiments were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively.

Fructigenine A (1): $C_{27}H_{29}N_3O_3$, ^1H NMR (400 MHz, DMSO-d_6): δ 8.22 (1H, s, H-2), 7.84 (1H, d, *J* = 8.0 Hz, H-7), 7.38 (1H, *J* = 7.7 Hz, H-10), 7.29 (2H, m, H-15/17), 7, 26 (2H, m, H-8, H-16), 7.24 (2H, m, H-14/18), 7.13 (1H, dd, *J* = 7 Hz, H-9), 5.92 (1H, s, H-5a), 5.64 (1H, dd, *J* = 17.6, 10.6, H-4′), 5.04 (1H, d, *J* = 17.6 Hz), 5.035 (1H, d, *J* = 10.6 Hz), 4.44 (1H, m, H-3), 3.58 (1H, m, H-11a), 3.12 (1H, dd, *J* = 14.3, 4.4 Hz, H-12), 3.00 (1H, dd, *J* = 14.3, 5.1, H-12), 2.55 (3H, s, H-2′), 2.32 (1H, dd, *J* = 11.2, 6.2 Hz, H-11), 1.61 (1H, dd, *J* = 11.2 Hz, H-11), 0.94 (3H, s, H-6′), 0.79 (3H, s, H-7′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.5 (C-1′), 166.6 (C-1), 165.2 (C-4), 143.4 (C-4′), 143.2 (C-6a), 136.6 (C-13), 132.4 (C-10a), 130.0 (C-14/18), 128.4 (C-8), 128.0 (C-15/17), 126.5 (C-16), 124.9 (C-10), 124.1 (C-9), 117.7 (C-7), 114.1 (C-5′), 78.6 (C-5a), 60.5 (C-10b), 58.5 (C-11a), 55.5 (C-3), 40.3 (C-3′), 36.3 (C-11), 36.0 (C-12), 23.7 (C-2′), 22.9 (C-7′), 22.1 (C-6′); LRESI-MS: 442.2 $[M - H]^-$, 466.1 $[M + Na]^+$.

Cyclopenol (2): $C_{17}H_{14}N_2O_4$, ^1H NMR (400 MHz, CD_3OD): δ 7.56 (1H, m, H-8), 7.17 (1H, m, H-9), 7.16 (1H, m, H-7), 7.15 (1H, m, H-6), 7, 00 (1H, dd, *J* = 7.7 Hz, H-17), 6.70 (1H, d, *J* = 7.7 Hz, H-16), 6.14 (1H, s, H-14), 6.10 (1H, d, *J* = 7.7 Hz, H-18), 4.08 (1H, s, H-10), 3.19 (3H, s, CH_3-4); ^{13}C NMR (100 MHz, CD_3OD): δ 168.7 (C-2), 168.4 (C-5), 158.5 (C-15), 136.5 (C-11), 134.0 (C-8), 133.5 (C-13), 132.2 (C-6), 130.3 (C-17), 128.0 (C-12), 126.1 (C-7), 122.3 (C-9), 118.5 (C-18), 117.0 (C-16), 113.9 (C-14), 71.7 (C-3), 65.9 (C-10), 31.7 (4-CH_3); LRESI-MS: 309.1 $[M - H]^-$, 311.0 $[M + H]^+$.

The strain SF-5130 was isolated from an intertidal sediment sample collected from Wan Island, Korea in January 2008. A GenBank search with the 28S rRNA gene of SF-5130 indicated *Eurotium rubrum* (AY004346) as the closest match, showing a sequence identity of 98.86%. Therefore, the marine-derived fungal strain SF-5130 was identified as an *Eurotium* sp. The fungal strain SF-5130 was cultured on 30 petri plates (90 mm), each containing 20 ml of PDA with 3% NaCl. Plates were individually inoculated with 2 ml of seed culture of the fungal strain and incubated at 25°C for a period of 10 days. Extraction of the combined agar media with EtOAc (2 L) provided an organic phase, which was then concentrated *in vacuo* to yield 4.9 g of extract. The EtOAc extract was subjected to C_{18} flash column chromatography (5 × 40 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (500 ml each). A portion (40.6 mg) of the fraction eluted at 100% MeOH (116.4 mg) was dissolved in MeOH (4.6 ml), and the precipitants was separated to yield compound **3** (9.4 mg). The remaining MeOH-soluble portion was further purified by semi-preparative reversed-phase HPLC, eluting with a gradient from 83% to 90% MeOH in H_2O (0.1% formic acid) over 50 min to yield **4** (4.0 mg, t_R = 41.1 min). The structures of compounds **3** and **4** were determined as echinulin and flavoglaucin A, respectively, by analysis of NMR and MS data, along with comparisons with those in the literature [27, 31].

Echinulin (3): $C_{29}H_{39}N_3O_2$, ^1H NMR (400 MHz, $CDCl_3$):

δ 8.06 (1H, s, H-1), 7.14 (1H, s, H-4), 6.81 (1H, s, H-6), 6.48 (1H, s, H-10), 6.10 (1H, dd, *J* = 17.2, 10.6 Hz, H-2′), 5.69 (1H, s, H-14), 5.42 (1H, m, H-2‴), 5.34 (1H, m, H-2″), 5.16 (1H, d, *J* = 18.3 Hz, H-3′), 5.15 (1H, d, *J* = 10.3 Hz, H-3′), 4.41 (1H, m, H-9), 4.10 (1H, q, *J* = 6.6 Hz, H-12), 3.65 (1H, dd, *J* = 14.6, 3.7 Hz, H-8), 3.53 (1H, d, *J* = 7.3 Hz, H-1‴), 3.39 (1H, d, *J* = 7.3 Hz, H-1″), 3.19 (1H, dd, *J* = 14.6, 11.7 Hz, H-8), 1.87 (3H, s, H-5‴), 1.81 (3H, s, H-4‴), 1.74 (3H, s, H-5″), 1.735 (3H, s, H-4″), 1.54 (3H, d, 7.6 Hz, H-15), 1.51 (6H, s, 2 x CH_3-1′); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.5 (C-10), 167.8 (C-13), 145.7 (C-2′), 141.3 (C-2), 133.8 (C-5), 132.9 (C-3‴), 132.2 (C-7a), 131.6 (C-3″), 128.9 (C-3a), 124.5 (C-2″), 123.4 (C-7), 122.8 (C-6), 115.0 (C-4), 112.3 (C-3′), 104.0 (C-3), 54.5 (C-9), 50.8 (C-12), 38.9 (C-1′), 34.6 (C-1″), 31.4 (C-1‴), 29.4 (C-8), 27.9 (CH_3-1′), 27.8 (CH_3-1′), 25.8 (C-4‴), 25.7 (C-4″), 19.8 (C-15), 17.90 (C-5‴), 17.88 (C-5″); LRESI-MS: 484.6 $[M + Na]^+$.

Flavoglaucin (4): $C_{19}H_{28}O_3$, ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.92 (1H, s, 2-OH), 10.25 (1H, s, H-7), 6.89 (1H, s, H-4), 5.28 (1H, t, *J* = 8.0 Hz, H-2″), 3.26 (2H, d, *J* = 8.0 Hz, H-1″), 2.90 (2H, t, *J* = 6.2 Hz, H-1′), 1.76 (3H, s, H-4″), 1.70 (3H, s, H-5″), 1.70 ~ 1.10 (10 H, m, H-2′ ~ H6′), 0.86 (3H, t, *J* = 6.2 Hz, H-7′); LRESI-MS: 303.3 $[M - H]^-$, 327.1 $[M + Na]^+$.

The strain SF-5295 was isolated from an unidentified sponge that was manually collected using scuba diving equipment off the shores of Jeju Island in February 2009. The sample was stored in a sterile plastic bag and transported to the laboratory, where it was kept frozen until further processing. This fungus was identified based on the analysis of the ribosomal RNA (rRNA) sequence. A GenBank search with the 28S rRNA gene of SF-5295 indicated *Penicillium polonicum* (JN938933), *Penicillium aurantiogriseum* (JN938945), *Penicillium expansum* (JN938952), *Penicillium solitum* (JN642222), and *Penicillium fuscoglaucum* (JQ434691) as the closest matches, showing sequence identities of 99.88%, 99.88%, 99.76%, 99.76%, and 99.76%%, respectively. Therefore, the marine-derived fungal strain SF-5295 was characterized as *Penicillium* sp. The fungal strain SF-5295 was cultured on 50 petri plates (90 mm), each containing 20 ml of PDA with 3% NaCl. Plates were individually inoculated with 2 ml of seed culture of the fungal strain and incubated at 25°C for a period of 10 days. Extraction of the combined agar media with EtOAc (2 L) provided an organic phase, which was then concentrated *in vacuo* to yield 513.6 mg of extract. The EtOAc extract was subjected to C_{18} flash column chromatography (5 × 40 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (300 ml each). The fraction eluted at 60% MeOH (63.3 mg) was further purified by semi-preparative reversed-phase HPLC, eluting with a gradient from 40% to 80% MeOH in H_2O (0.1% formic acid) over 80 min, and then to yield **5** (2.8 mg, t_R = 48.0 min). The structure of compound **5** was determined as viridicatol by analysis of NMR and MS data, along with comparisons with those in the literature [11].

Viridicatol (5): $C_{15}H_{11}NO_3$, ^{1}H NMR (400 MHz, CD_3OD): δ 12.22 (1H, s, 1-NH), 9.55 (1H, s, 3′-OH), 9.15 (1H, s, 3-OH), 7.33 (1H, s, H-9), 7, 32 (1H, m, H-6), 7.29 (1H, dd, *J* = 8.3 Hz, H-5′), 7.09 (1H, s, H-8), 7.08 (1H, m, H-7), 6.82 (1H, d, *J* = 8.3 Hz, H-4′), 6.72 (1H, m, H-6′), 6.71 (1H, s, H-2′); ^{13}C NMR (100 MHz, CD_3OD): δ 158.2 (C-2), 157.3 (C-3′), 142.2 (C-10), 134.8 (C-1′), 133.1 (C-4), 129.3 (C-5′), 126.3 (C-6), 124.4 (C-8), 124.0 (C-3), 122.0 (C-7), 120.9 (C-5), 120.3 (C-6′), 116.6 (C-2′), 115.2 (C-9), 114.6 (C-4′); HRESI-MS: 254.0812 $[M + H]^+$.

To identify the PTP1B inhibitory active principle(s) from the organic extract of the cultures of the three marine-derived fungi, we performed bioassays on each of the 5 compounds isolated. PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc. The enzyme activity was measured in a reaction mixture containing 2 mM *p*-nitrophenyl phosphate (*p*NPP) in 50 mM citrate, pH 6.0, 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol. The reaction mixture was placed in a 37°C incubator for 30 min, and the reaction was terminated by addition of 10 N NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the increase in absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

Among the tested compounds, fructigenine A (**1**) exhibited the most potent PTP1B inhibitory activity in a dose-dependent manner with an IC_{50} value of 10.7 μM. The fungal alkaloids, **2, 3,** and **4**, inhibited PTP1B activity in a dose-dependent manner with IC_{50} values of 30.0, 29.4, and 64.0 μM, respectively. The IC_{50} value of flavoglaucin was determined as 13.4 μM. A known phosphatase inhibitor, ursolic acid (IC_{50} = 3.08 μM), was used as a positive control

Table 1. PTP1B inhibitory activity of compounds **1**–**5**.

Compounds	PTP1B inhibitory activity (IC_{50} = μM)
1	10.7
2	30.0
3	29.4
4	13.4
5	64.0
Ursolic acid[a]	3.08

[a]Positive control.

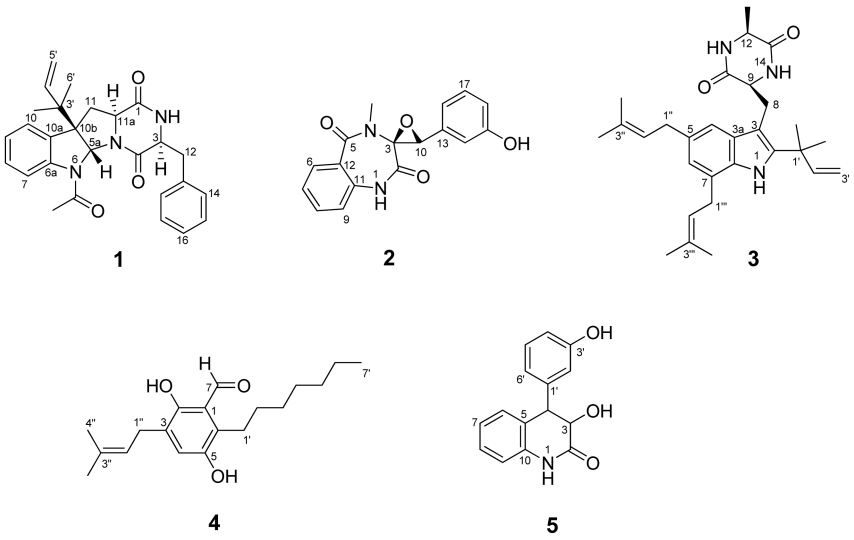

Fig. 1. The chemical structures of **1**–**5**.

in the assay [26]. The PTP1B inhibitory activity of the metabolites is summarized in Table 1.

To investigate the inhibition mode of the most potent inhibitor (*i.e.*, compound **1**) on the activity of PTP1B, substrate titration studies using the small substrate *p*NPP were conducted. The reaction mixture consisted of different concentrations of *p*NPP as a PTP1B substrate in the absence or presence of compound **1**, and was assayed as described above. Data were fitted by nonlinear regression analysis according to a Michaelis–Menten kinetic model (Graphpad Prism version 4.02). As shown in Fig. 2, compound **1** decreased the V_{max} value, but did not alter the K_m value of PTP1B, suggesting that the inhibition mode was noncompetitive toward *p*NPP. This observation implied that this compound may bind to the enzyme–substrate complex or to an allosteric site within PTP1B [19]. It is noteworthy that the identification of noncompetitive inhibitors targeting the allosteric site in PTP1B or the active site in the inactive conformation of PTP1B would have potential to be developed into selective and bioavailable PTP1B inhibitors, which are much required characteristics for PTP1B inhibition-based drugs [19, 29, 34]. On the other hand, the characteristics of the inhibition of PTP1B by compound **5** were determined to be in a competitive manner. When PTP1B was incubated with increasing concentrations of compound **5** and full velocity curves were determined (Fig. 3), nonlinear regression analysis showed that the data best fit a competitive model of inhibition, and re-plotting of the data as Lineweaver–Burk transformations confirmed this result. Therefore, it was

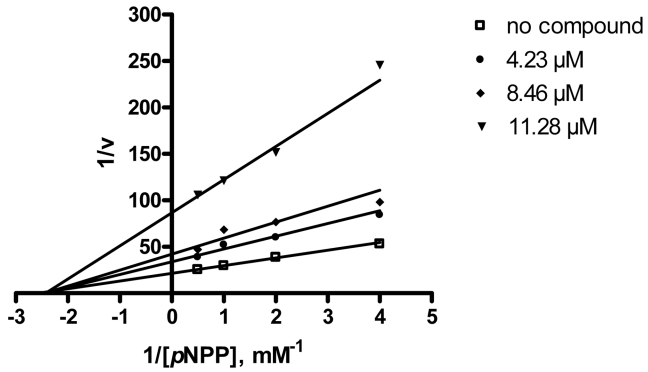

Fig. 2. A Lineweaver–Burk plot for fructigenine A (**1**) inhibition of PTP1B.

Data are expressed as the mean initial velocity for $n = 3$ replicates at each substrate concentration. Concentrations (μM) of **1** are indicated in the figure.

shown that compound **5** binds to the active site within PTP1B.

Fructigenine A (**1**) is a unique indole alkaloid bearing a reverse-prenyl group, and was originally isolated from *Penicillium fructigenum*. Growth-inhibitory activity against *Avena* coleoptile and leukemia L-5178 Y cells [1] and K562 cells [7] were reported. Cyclopenol (**2**) is a member of benzodiazepine-type mycotoxins, distributed in a number of *Penicillium* spp. [4]. Echinulin (**3**) is another mycotoxin that belongs to the family of diketopiperazine, which was isolated from several *Aspergillus* spp. [4]. Benzaldehyde fungal metabolite flavoglaucin (**4**) has been reported to have various biological effects such as binding affinity for

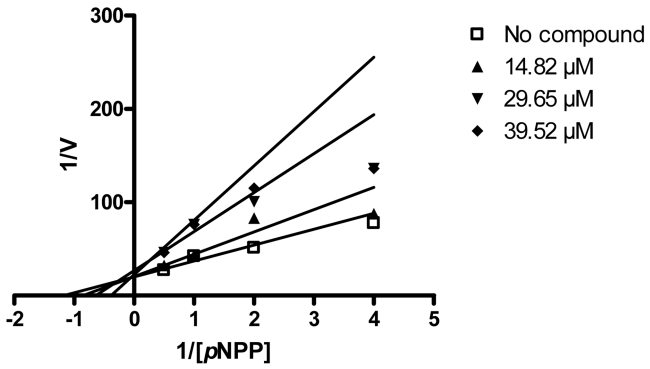

Fig. 3. A Lineweaver-Burk plot for viridicatol (**5**) inhibition of PTP1B.

Data are expressed as mean initial velocity for $n = 3$ replicates at each substrate concentration. Concentrations (μM) of **5** are indicated in the figure.

human opioid receptor [12], and antitumor-promoting [32] and radical-scavenging activities [18]. A quinoline alkaloid, viridicatol (**5**) has been isolated from *Penicillium* spp. [3], and displayed cytotoxicity against tumor cells [28]. The inhibitory effects of the isolated metabolites encountered in this study exhibited somewhat lower IC_{50} values in *in vitro* assays as compared with those of previously reported naturally occurring PTP1B inhibitors such as vanillic acid derivatives (IC_{50} = 1.7–3.7 μM) [9], ursolic acid (IC_{50} = 3.08 μM) [23], and depsidone-type lichen metabolites (IC_{50} = 0.87–2.48 μM) [26]. However, to the best of our knowledge, the PTP1B inhibitory effects by these fungal metabolites have not been previously reported. Further studies with this class of compounds with the aim of providing selectivity against other PTP panels as well as *in vivo* efficiency are necessary to evaluate their potential as lead compounds in the treatment of diabetes and obesity.

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