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# Pteridic Acids A and B, Novel Plant Growth Promoters with Auxin-like Activity from *Streptomyces hygroscopicus* TP-A0451

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In the course of screening for plant growth regulators from microbial secondary metabolites, we isolated pteridic acids A and B from the fermentation broth of *Streptomyces hygroscopicus* TP-A0451 as plant growth promoters with auxin-like activity[1,2]. Pteridic acids induce the formation of adventitious roots in hypocotyl of kidney beans at 1 nM as effectively as auxin (indoleacetic acid), a natural plant growth hormone. We herein describe the fermentation, isolation and structure determination of pteridic acids.

The producing organism, strain TP-A0451 was isolated from a stem of bracken, *Pteridium aquilinum*, collected in Toyama, Japan. The seed culture was incubated in a medium consisting of 1% soluble starch, 0.5% glucose, 0.3% NZ-case, 0.2% yeast extract, 0.5% tryptone, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into thirty 500-ml K-1 flasks each containing 100 ml of the production medium consisting of 0.5% glucose, 2% glycerol, 2% soluble starch, 1.5% Pharmamedia, 0.3% yeast extract, 1% Diaion HP-20 (adjusted to pH 7.0 before sterilization). Fermentation was carried out for 6 days at 30°C on a rotary shaker (200 rpm). The fermented whole broth (3 liters) was centrifuged to separate the mycelium and the supernatant. The mycelium was extracted with 80% aqueous acetone (1 liter×3). After evaporation, the resultant aqueous solution was combined with the supernatant, extracted with ethyl acetate (2 liters×3) at pH 8.0, and concentrated *in vacuo*. The residual solid was washed with acetonitrile to give 2.3 g of azalomycin B (~95% purity on HPLC) as a white powder. The aqueous layer was then extracted with ethyl acetate (1 liter×3) at pH 3.5 and the organic layer was evaporated to dryness. The residue was chromatographed on silica gel with the eluent of $CHCl_3$-MeOH (100:1~50:1). The active fractions were combined, dried and rechromatographed on ODS RP-18 with $CH_3CN$/0.15% $KH_2PO_4$ buffer (pH 3.5) (2:8~3:7). The acetone of the combined active fractions was evaporated and then extracted with ethyl acetate and concentrated *in vacuo* to give pteridic acids A (**1**, 11.2 mg) and B (**2**, 3.5 mg) as a pale yellow oil.

The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formula of **1** and **2** was determined as $C_{21}H_{32}O_5$ based on the HRFAB-MS (**1**: $[M+H]^+$, *m/z* 365.2327, $\Delta$ −0.1 mmu; **2**: $[M+H]^+$, *m/z* 365.2326, $\Delta$ −0.2 mmu) and NMR data. The presence of $\alpha$, $\beta$, $\gamma$, $\delta$-unsaturated carboxylic acid was indicated by the IR (1690, 1640 $cm^{-1}$) and UV ($\lambda_{max}$ 256 nm) spectra. The $^1H$ and $^{13}C$ NMR spectral data of **1** and **2** are shown in Table 2. Both of the $^{13}C$ NMR spectra of **1** and **2** displayed 21 signals which consisted of five methyls, one methylene, thirteen methines and two quaternary carbons by DEPT and HMQC experiments. The planar structure of **1** was determined by the analysis of DQF-COSY and HMBC spectra (Fig. 2). The presence of spiroketal was revealed by the $^1H$-$^{13}C$ long-range couplings from H-7, H-12, H-13, H-15 and H-19 to C-11 (96.86 ppm). In addition, the long-range couplings from H-2 and H-3 to the carbonyl carbon C-1 (171.97 ppm) confirmed the $\alpha$, $\beta$, $\gamma$, $\delta$-unsaturated carboxylic acid residue. The geometries of C-2/C-3 and C-4/C-5 were proved to be *E* by virtue of the coupling constants of $J_{2,3}$ (15.4 Hz) and $J_{4,5}$ (15.4 Hz).

The conformation of the six-membered ring from C-7 to C-11 was deduced from the coupling constants between H-6 and H-7 ($J$=10.0 Hz), H-7 and H-8 ($J$=2.2 Hz), H-8 and H-9 ($J$=4.9 Hz), and H-9 and H-10 ($J$=11.2 Hz). The stereochemistry at C-11 was determined by the NOEs observed between H-10 and H-12, and H-7 and H-16. Furthermore, the NOEs between H-16 and H-5, and H-16 and H-14, established the stereochemistry at C-6 and C-14, respectively, as shown in Fig. 3. **2** was determined to be the stereoisomer of **1** regarding to the spiro carbon at C-11 by the differential NOE experiments in which NOEs were observed among H-7, H-9 and H-12 but not between H-10 and H-12.

The absolute configuration of pteridic acids was determined by applying the modified Mosher's method[3]. After protecting the carboxyl residue of **1** by methylation, the hydroxyl group at C-9 was esterified with (*R*)- or (*S*)-MTPA. In the $^1H$ NMR spectra of MTPA esters, the positive

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Table 1. Physico-chemical properties of pteridic acids A (**1**) and B (**2**).

| | **1** | **2** |
|---|---|---|
| Appearance | Pale yellow oil | Pale yellow oil |
| $[\alpha]_D^{24}$ | -22.3 (*c* 1.0, $CHCl_3$) | -20.8 (*c* 0.68, $CHCl_3$) |
| HRFAB-MS | | |
| Found: | 365.2327 $[M+H]^+$ | 365.2326 $[M+H]^+$ |
| Calcd: | 365.2328 (for $C_{21}H_{33}O_5$) | 365.2328 (for $C_{21}H_{33}O_5$) |
| Molecular formula | $C_{21}H_{32}O_5$ | $C_{21}H_{32}O_5$ |
| UV $\lambda_{max}^{MeOH}$ nm (log ε) | 256 (4.13) | 256 (4.13) |
| IR $\nu_{max}$ ($cm^{-1}$) | 3420, 1690, 1640 | 3425, 1695, 1640 |
| TLC (Rf)[a] | 0.52 | 0.50 |
| HPLC (Rt)[b] | 7.9 min | 5.2 min |

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)
[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm, i.d.), Mobile phase: $CH_3CN$-0.15% $KH_2PO_4$ (pH 3.5) (50:50), Flow rate: 1.0 ml/min, Detection: UV-254 nm.

Table 2. $^1H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data for pteridic acids A and B.

| | Pteridic acid A | | Pteridic acid B | |
|---|---|---|---|---|
| Position | $^{13}C$ | $^1H$ | $^{13}C$ | $^1H$ |
| 1 | 171.97 | | 171.85 | |
| 2 | 118.37 | 5.77 (1H, d, 15.4) | 118.28 | 5.76 (1H, d, 15.4) |
| 3 | 147.47 | 7.25 (1H, dd, 10.0, 15.4) | 147.57 | 7.26 (1H, dd, 10.5, 15.4) |
| 4 | 126.84 | 6.18 (1H, dd, 9.8, 15.4) | 127.44 | 6.24 (1H, dd, 10.5, 15.4) |
| 5 | 150.11 | 6.25 (1H, dd, 6.8, 15.4) | 149.33 | 6.14 (1H, dd, 7.5, 15.4) |
| 6 | 38.49 | 2.48 (1H, ddq, 9.8, 6.8, 6.8) | 38.37 | 2.53 (1H, ddq, 10.0, 6.8, 6.8) |
| 7 | 74.48 | 3.75 (1H, dd, 2.2, 10.0) | 75.57 | 3.26 (1H, dd, 2.0, 9.8) |
| 8 | 36.24 | 2.06 (1H, ddq, 2.2, 4.6, 6.8) | 36.24 | 2.07 (1H, ddq, 1.7, 4.9, 6.8) |
| 9 | 72.48 | 3.85 (1H, dd, 2.2, 10.0) | 74.25 | 3.70 (1H, dd, 4.6, 11.2) |
| 10 | 40.86 | 1.62 (1H, quint, 6.9) | 40.74 | 1.78 (1H, dq, 11.4, 6.8) |
| 11 | 96.86 | | 97.87 | |
| 12 | 127.52 | 5.51 (1H, dd, 1.2, 10.2) | 134.00 | 5.92 (1H, dd, 1.9, 10.7) |
| 13 | 130.25 | 5.96 (1H, ddd, 1.0, 5.8, 10.2) | 123.43 | 5.89 (1H, d, 10.7) |
| 14 | 40.35 | 1.61 (1H, dq, 11.0, 6.8) | 42.26 | 1.86 (1H, m) |
| 15 | 71.60 | 3.91 (1H, q, 6.8) | 68.13 | 3.89 (1H, dq, 9.8, 6.1) |
| 16 | 22.87 | 1.24 (3H, d, 6.8) | 19.51 | 1.22 (3H, d, 6.1) |
| 17 | 15.17 | 1.00 (3H, d, 6.8) | 15.29 | 0.968 (3H, d, 6.8) |
| 18 | 4.51 | 0.91 (3H, d, 7.0) | 4.85 | 0.974 (3H, d, 6.8) |
| 19 | 12.48 | 0.90 (3H, d, 6.8) | 11.48 | 0.91 (3H, d, 6.8) |
| 20 | 26.20 | 1.45 (2H, quint, 7.3) | 23.34 | 1.20 (1H, m), 1.49 (1H, m) |
| 21 | 11.88 | 0.93 (3H, t, 7.3) | 9.92 | 0.87 (3H, t, 7.6) |

Fig. 1. Structures of pteridic acids A and B.

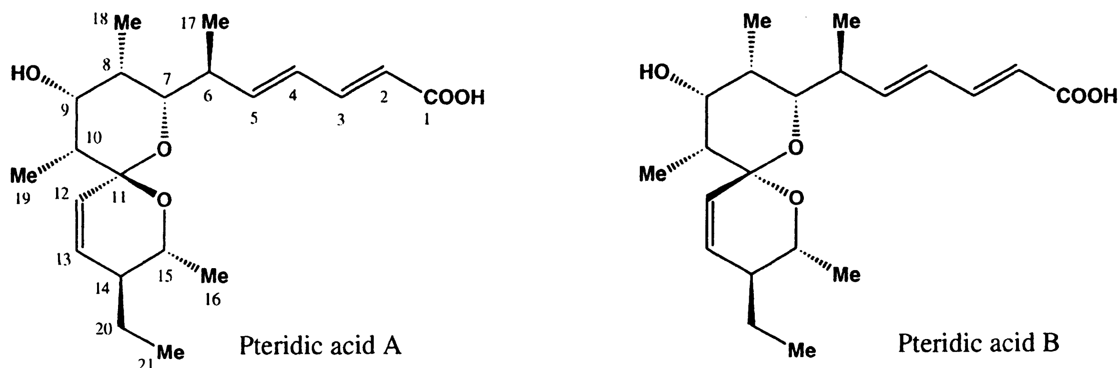
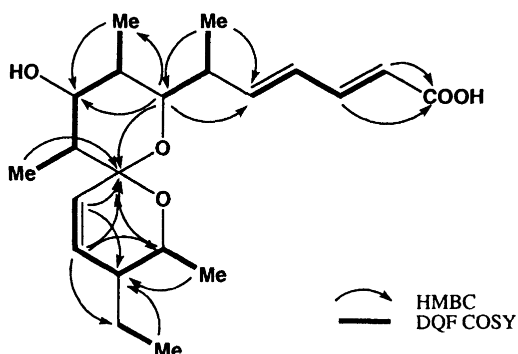



Fig. 2. DQF-COSY and HMBC correlations observed with **1**.




and negative $\Delta\delta$ ($\delta_S - \delta_R$) values were well arranged on both side of the carbinyl carbon at C-9 as shown in Fig. 4. The general tendency of $\Delta\delta$ values, which were negative on the left side and positive on the right side of the MTPA plane, indicated the 9*R* configuration.

Pteridic acid is structurally related to azalomycin B (elaiophilin)[4], the major secondary metabolite of strain TP-A0451. Azalomycin B is a symmetric bislactone macrolide composed of two molecules of polyketide-derived hydroxy acids and deoxysugars. The functionalities on the carbon skelton of the hydroxy acid and pteridic acid are identical regarding to their positions and chirality except for the presence of a *cis*-olefin at C-12 and C-13 and a spiroacetal structure between C-11 carbonyl and 7- and 15-hydroxy groups in pteridic acid. Therefore, pteridic acid and azalomycin B is considered to be biosynthesized *via* a common pathway.

## Experimental

Methyl Ester of Pteridic Acid A

Pteridic acid A (3.6 mg, 10 $\mu$mol) was warmed with methyl iodide (25 $\mu$l, 0.4 mmol) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (8 $\mu$l, 0.05 mmol) in acetonitrile (100 $\mu$l) and acetone (100 $\mu$l) at 50°C for 2 hours. The solution was poured onto ice-water and extracted with ethyl acetate. After evaporation, the residue was purified on a silica gel column (hexane - ethyl acetate=10 : 1~2 : 1) to give a methyl ester of pteridic acid A (3.2 mg, 85%).

FAB-MS: *m/z* 379 $[M+H]^+$; $^1$H-NMR ($CDCl_3$): 0.90 (3H, d, 7.3 Hz, H19), 0.92 (3H, d, 7.3 Hz, H18), 0.92 (3H, t, 7.4 Hz, H21), 0.99 (3H, d, 6.8 Hz, H17), 1.25 (3H, d, 6.6 Hz, H16), 1.45 (2H, quint, 7.3 Hz, H20), 1.5~1.65 (2H, m, H10 and H14), 2.06 (1H, m, H8), 2.48 (1H, ddq, 6.8, 9.8 and 9.8 Hz, H6), 3.72 (3H, s, $COOCH_3$), 3.74 (1H, dd, 2.2 and 10.0 Hz, H7), 3.84 (1H, m, H9), 3.91 (1H, q, 6.8 Hz, H15), 5.50 (1H, dd, 1.2 and 9.9 Hz, H12), 5.78 (1H, d, 15.4 Hz, H2), 5.95 (1H, dd, 5.8 and 9.9 Hz, H13), 6.16 (1H, dd, 10.0 and 15.4 Hz, H4), 6.19 (1H, dd, 6.8 and 15.4 Hz, H5), 7.18 (1H, dd, 10.0 and 15.4 Hz, H3).

MTPA Derivatization of the Methyl Ester of Pteridic Acid A

*N,N*-Dimethylaminopyridine (2.4 mg, 19 $\mu$mol), dicyclo-hexylcarbodiimide (5.3 mg, 25 $\mu$mol) and (*R*)- or (*S*)-MTPA acid (5 mg, 21 $\mu$mol) were added to a solution of the methyl

Fig. 3. NOEs observed with pteridic acids A (**1**) and B (**2**).

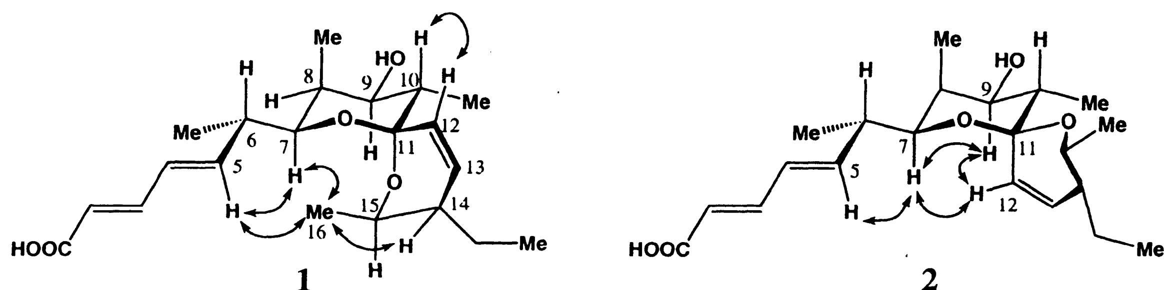
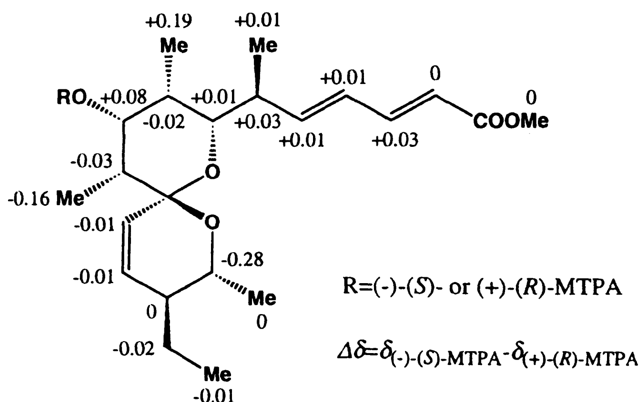


Fig. 4. Determination of the absolute configuration of pteridic acid A.



ester of pteridic acid A (1.5 mg, 4 $\mu$mol) in dry $CH_2Cl_2$ (100 $\mu$l). After stirring for 5 hours at room temperature, the reaction mixture was applied on a silica gel column (hexane-ethyl acetate=10 : 1~6 : 1) to give 1.4 mg (60%) of (*R*)- or 1.5 mg (65%) of (*S*)-MTPA ester.

(*R*)-MTPA ester: FAB-MS: *m/z* 595 $[M+H]^+$; $^1$H-NMR ($CDCl_3$): 0.719 (3H, d, 6.8 Hz, H18), 0.792 (3H, d, 6.8 Hz, H19), 0.921 (3H, t, 7.3 Hz, H21), 0.985 (3H, d, 6.8 Hz, H17), 1.245 (3H, d, 6.8 Hz, H16), 1.439 (2H, m, H20), 1.56 (1H, overlapped with $H_2O$ signal), 1.854 (1H, m, H10), 2.304 (1H, m, H8), 2.410 (1H, m, H6), 3.550 (3H, s, $CH_3O$ of MTPA), 3.724 (3H, s, $COOCH_3$), 3.843 (1H, dd, 2.2 and 9.8 Hz, H7), 3.938 (1H, q, 6.8 Hz, H15), 5.224 (1H, dd, 4.6 and 11.7 Hz, H9), 5.482 (1H, dd, 1.0 and 10.2 Hz, H12), 5.784 (1H, d, 15.2 Hz, H2), 5.975 (1H, dd, 5.6 and 9.5 Hz, H13), 6.145 (1H, m, H4), 6.165 (1H, m, H5), 7.144 (1H, dd, 10.0 and 15.3 Hz, H3), 7.3~7.45 (5H, m, Ph of MTPA).

(*S*)-MTPA ester: FAB-MS: *m/z* 595 $[M+H]^+$; $^1$H-NMR ($CDCl_3$): 0.631 (3H, d, 6.8 Hz, H19), 0.910 (3H, t, 7.3 Hz, H21), 0.910 (3H, d, 6.8 Hz, H18), 0.998 (3H, d, 6.8 Hz, H17), 1.243 (3H, d, 6.8 Hz, H16), 1.421 (2H, m, H20), 1.56 (1H, overlapped with $H_2O$ signal), 1.827 (1H, m, H10), 2.285 (1H, m, H8), 2.438 (1H, m, H6), 3.560 (3H, s, $CH_3O$ of MTPA), 3.662 (1H, q, 6.6 Hz, H15), 3.725 (3H, s, $COOCH_3$), 3.854 (1H, dd, 2.4 and 10.0 Hz, H7), 5.300 (1H, dd, 4.6 and 11.7 Hz, H9), 5.471 (1H, dd, 1.0 and 10.2 Hz, H12), 5.788 (1H, d, 15.2 Hz, H2), 5.961 (1H, dd, 5.4 and 9.5 Hz, H13), 6.152 (1H, m, H4), 6.172 (1H, m, H5), 7.172 (1H, dd, 10.0 and 15.4 Hz, H3), 7.33~7.45 (5H, m, Ph of MTPA).

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