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Microbial transformation of (−)-Huperzine A

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ABSTRACT

Five products were yielded from the transformation of (−)-Huperzine A (**1**) by *Streptomyces griseus* CACC 200300. Their structures were determined as 16-hydroxyl huperzine A (**2**), 14α-hydroxyl huperzine A (**3**), huperzine A 8α,15α-epoxide (**4**), 13*N*-formyl huperzine A (**5**), and 13*N*-acetyl huperzine A (**6**) on the basis of their chemical and physical data. It is the first report on the microbial transformation of (−)-Huperzine A and would facilitate further structural modification by chemo-enzymatic method.

(−)-Huperzine A (**1**, Fig. 1) is an enantiomeric lycodine alkaloid isolated from the club moss, *Huperzia serrata* (Thunb.) (Huperziaceae family).[1,2] This plant is known as Qian Ceng Ta in China and used as Chinese folk medicine for the treatment of various maladies. (−)-Huperzine A has been identified as a potent, specific, and reversible inhibitor of the enzyme acetylcholinesterase (EC 3.1.1.7, AChE), relative to butyryl cholinesterase (EC 3.1.1.8, BuChE). (−)-Huperzine A has received extensive studies in many aspects, such as chemical synthesis, structural modification, structure–activity relationship, various biological effects, and mechanism of action. Due to its rigid molecular configuration, the structural modification of natural (−)-Huperzine A is very difficult by traditional chemical method, and only focused on the pyridone ring and the primary amino group. However, biotransformation has proven powerful tool to structural modification of natural/non-natural compounds. Therefore, the transformations of (−)-Huperzine A by microbes/plant cells have been systematically investigated in our laboratory. Herein, we first report the microbial transformation of (−)-Huperzine A and the structural elucidation of its products.

A variety of microbial strains which might possess various enzymes were collected and employed for the biotransformation. These strains were distributed to prokaryotic organisms (including bacteria, actinomycete) and eukaryotic organisms (including yeast, filamentous fungi, etc.). Totally, 85 microbial strains were used through routinely two-step procedure. Among them only four actinomycetic strains (*Streptomyces griseus* CACC200300, *S. roseochromogenes* CACC200427, *S.* sp. CACC201936, and *Chainia* sp. CACC200049, from China Center for Antibiotics Culture Collection, Beijing), have been observed to possess the capacity of converting (−)-Huperzine A on the basis of HPLC and LC–UV analyses. According to the analyses, the biotransformation patterns of (−)-Huperzine A with the four strains were very similar (see Supplementary data), there existed difference only in the yields of metabolites. The yields of metabolites (especially for **2**) were the highest in the case of *S. griseus* as a biocatalyst. Thus, this strain was selected for the further preparative biotransformation. After a two-step procedure and various chromatographic techniques, five products were obtained.[3] On the basis of the physical and chemical data (HRMS, 1D NMR, 2D NMR, IR, etc.), their structures were identified as 16-hydroxyl huperzine A (**2**, 63.2%), 14α-hydroxyl huperzine A (**3**, 8.8%), huperzine A 8α,15α-epoxide (**4**, 1.0%), 13*N*-formyl huperzine A (**5**, 1.0%), and 13*N*-acetyl huperzine A (**6**, 0.5%), respectively. The occurred reactions included specific hydroxylation, epoxidation, and acylation. Among these products, **2**–**4** were three new compounds, **5** and **6** were reported in a patent.[4]

HRESI-positive mass spectrum of **2** exhibited two quasi molecular ion peaks at $[M+H]^+$ 259.1384 and $[M+Na]^+$ 281.1199, respectively, consistent with the molecular formula of $C_{15}H_{18}N_2O_2$, suggesting the substitution of an additional OH group as compared with **1**. The ^{1}H NMR spectrum of **2** was similar to that of **1** except that the signal corresponding to 15-Me (δ 1.46, s, H-16) in **1** disappeared, while the oxygen-bearing methylene signals [δ 3.78 (d, J = 13.2 Hz), δ 3.74 (d, J = 13.2 Hz)] were observed. These indicated the introduction of OH group at C-16 position, which was further supported by the signal of C-16 in **2** which significantly shifted downfield to δ 65.9 (t) when compared with δ 22.5 (q) in **1**. Additionally, the HMBC correlations of the signals of δ_H 3.78 and δ_H 3.74 with δ_C 125.5 (d, C-8) and δ_C 45.4 (t, C-14) in **2** strongly revealed

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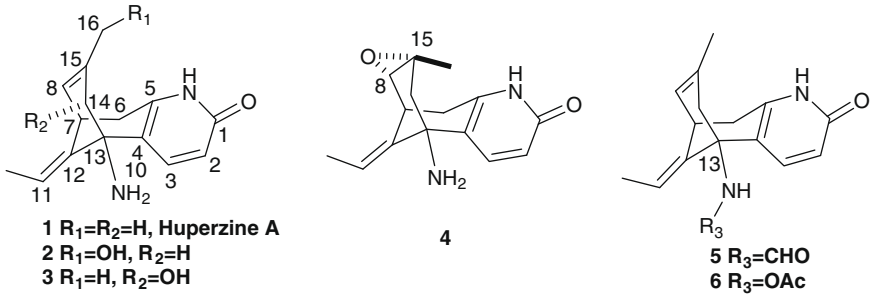

Figure 1. The structures of huperzine A and its metabolites by *Streptomyces griseus* CPCC 200300.

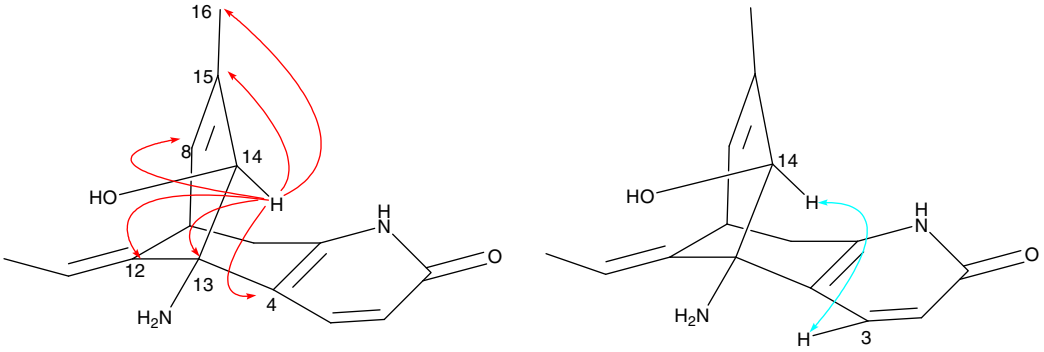

Figure 2. Key HMBC (→) and NOE (↔) correlations of compound **3**.

that the OH group was attached at C-16 position. Thus, the structure of **2** was determined as 16-hydroxyl huperzine A.[5]

The molecular formula of **3** was established to be $C_{15}H_{18}N_2O_2$ by combined analyses of the HRESIMS, ^{1}H NMR, and ^{13}C NMR spectroscopic data, with 16 amu more than the corresponding value of **1**. The ^{1}H NMR spectrum displayed the disappearance of the signals responsible for H-14 [δ 2.25 (d, J = 16.8 Hz) and δ 2.21 (d, J = 16.8 Hz)] and the appearance of one new oxygen-bearing methine proton signal at δ 3.57 (s). Its connected carbon was designated to δ 77.7 (d) by HSQC experiment. These indicated that compound **3** was a 14-hydroxylated product of **1** by *S. griseus*. The observation of the correlations of δ_H 3.57 (s) with C-8 and C-16 confirmed this deduction further (Fig. 2). The stereochemistry of 14-OH group was determined to be α (*syn*-) oriented with respect to the C-10/11 unsaturated side chain by NOE difference spectrum,[6] in which the integration value of the olefinic proton H-3 was enhanced when H-14 was irradiated (Fig. 2). That is to say, therefore, the structure of **3** was deduced to be 14α-hydroxyl huperzine A.[7] It is the first derivative with OH group and other substituents at C-14 in this type of compounds.

The HRESIMS, ^{1}H NMR, and ^{13}C NMR spectroscopic data of **4** were in accordance with the molecular formula $C_{15}H_{18}N_2O_2$, indicating that a new oxygen was introduced in the molecule of **4**. The ^{1}H NMR spectrum of **4** showed the absence of olefinic signal corresponding to H-8 (δ 5.55, d, J = 6.6 Hz) in **1**, whereas the presence of one oxymethine proton at δ 2.97 (s). Furthermore, two olefinic carbon signals in **1** disappeared responsible for C-8 (δ_C 124.3, d) and C-15 (δ_C 134.0, s), while a pair of oxygenated carbon signals at δ_C 63.8 (d) and δ_C 56.8 (s) were observed. These suggested the existence of an epoxide moiety at C-8(15) instead of a double bond. The HMBC correlations of H-8 with C-6, C-7, and C-12 along with H-16 and H-14 with C-8 further supported this deduction (Fig. 3). The stereochemistry of epoxide in compound **4** was characterized by 1D and 2D NOE difference spectra experiments. The enhancement of integration values of H-8, H-14β (*syn*-oriented with the lactam)[8] was observed when H-16 was irradiated, which indicated that H-16 was *syn*-oriented with H-8, whereas *anti*-oriented with epoxide moiety (Fig. 3). In the NOESY spectrum, the cross peak of H-16 with H-3 (one of the lactam protons) was observed (Fig. 3), which suggested that H-16 and H-14β were *syn*-ori-

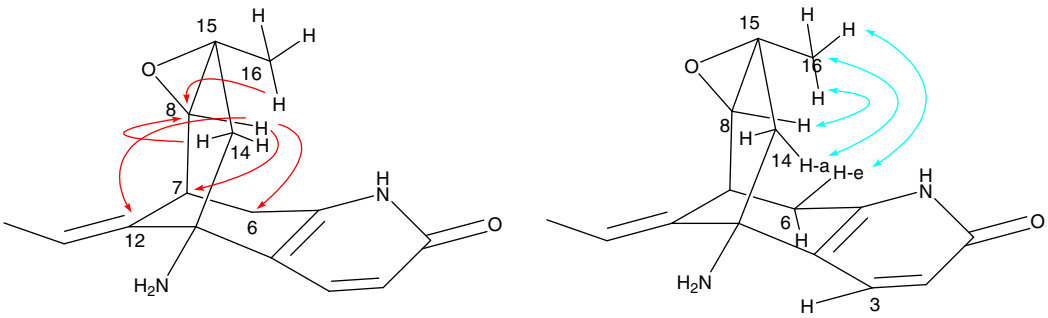

Figure 3. Key HMBC (→) and NOE (↔) correlations of compound **4**.

ented with respect to the lactam ring. Thus, the epoxide moiety was deduced to orient on the *anti* face with respect to the lactam ring and *syn* face to the C-10/11 unsaturated side chain,[6] and oriented 8α,15α. Therefore, the structure of **4** was identified as Huperzine A 8α,15α-epoxide.[8]

Products **5** and **6** were characterized as two 13-N acylated derivatives, 13*N*-formyl huperzine A (**5**) and 13*N*-acetyl huperzine A (**6**), which were reported in a patent.[4] In the NMR spectra of **5**, there existed a pair of proton and carbon signals designated to the two corresponding rotamers, which might be resulted from the rotation of the 13-*N* lactam bond. The HRESIMS, ^{1}H NMR, and ^{13}C NMR spectroscopic data are shown in Supplementary data.

In summary, five products of (−)-Huperzine A were obtained by *S. griseus* CACC200300, the involved reactions were specific hydroxylation, epoxidation, and acylation. To the best of our knowledge, this is the first report on the microbial transformation of (−)-Huperzine A. Some of these reactions are very difficult to conduct by chemical transformation because of its rigid molecule, especially for hydroxylation and epoxidation. It would facilitate further structural modification by chemo-enzymatic method for the discovery of new more potent AChE inhibitors from (−)-Huperzine A and better study of the structure–activity relationships of this type of compounds.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.05.073.

References and notes

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2. Ayer, W. A.; Browne, L. M.; Orszanska, H.; Valenta, Z.; Liu, J.-S. *Can. J. Chem.* **1989**, *67*, 1538.
3. A two-stage fermentation procedure was used. The cultural medium contained 20 g dextrose, 5 g yeast extract, 5 g soy peptone, 5 g NaCl, and 1.0 g $K_2HPO_4{\cdot}3H_2O$ in 1 L distilled water. A 1 mL sample of two-day-old seed culture was added to one flask (200 mL of medium per 1000 mL flask), and 300 mg substrate in 15 mL DMF was evenly distributed among 15 flasks after cultivation 2 days. After incubating for additional 7 days, the cultures were pooled and centrifuged. The supernatant was subjected to a macroporous resin (Amberlite XAD16) column and eluted with gradient ethanol/H_2O (0%, 25%, 50%, 100%). 25% and 50% fractions were further isolated by repeated reverse-phase semi-preparative HPLC followed by Sephadex LH-20 (desalinization). From 25% fraction were obtained compounds **2** [190 mg, ∼60%; t_R = 18.50 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 95/5 (v/v), flow rate: 2 mL/min)], **3** [24.5 mg, ∼8%; t_R = 16.63 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 93/7 (v/v), flow rate: 2 mL/min)], and **4** [4.0 mg, ∼1%; t_R = 18.11 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 93/7 (v/v), flow rate: 2 mL/min)]. From 50% fraction were obtained compounds **5** [4.9 mg, ∼1%; t_R = 47.71 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 85/15 (v/v), flow rate: 2 mL/min)], **6** [2.3 mg, ∼0.5%; t_R = 53.50 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 85/15 (v/v), flow rate: 2 mL/min)], and substrate (**1**) [60 mg, ∼20%; t_R = 42.15 min, mobile phase: 20 mM ammonium acetate (pH 5.5)/MeCN = 85/15 (v/v), flow rate: 2 mL/min)].
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5. *16-Hydroxyl huperzine A* (**2**): White powder (mp >250 °C); $[\alpha]_D^{20}$ − 100 (*c* 0.115, $CHCl_3$); UV–vis λ_{max}: 230, 310 nm (HPLC/UV); IR ν_{max} ($CHCl_3$): 3278, 2924, 1650, 1606, 1552, 1449, 1422, 1303, 1173, 1118, 1051, 1081, 933, 834, 772, 730, 660, 627 cm^{-1}; ^{1}H NMR (600 MHz, CD_3OD) δ 6.33 (1H, d, *J* = 9.6 Hz, H-2), 7.86 (1H, d, *J* = 9.6 Hz, H-3), 2.79 (1H, dd, *J* = 5.4, 16.2 Hz, H-6ax), 2.59 (1H, dd, *J* = 1.2, 16.2 Hz, H-6eq), 3.70 (1H, br s, H-7), 5.66 (1H, d, *J* = 4.8 Hz, H-8), 1.67 (3H, d, *J* = 6.6 Hz, H-10), 5.53 (1H, q, *J* = 6.6 Hz, H-11), 2.25 (1H, d, *J* = 16.8 Hz, H-14*anti*, with respect to the C-10/11 unsaturated side chain[6]), 2.21 (1H, d, *J* = 16.8 Hz, H-14*syn*, with respect to the C-10/11 unsaturated side chain[6]), 3.78 (1H, d, *J* = 13.2 Hz, H-16a), 3.74 (1H, d, *J* = 13.2 Hz, H-16b); ^{13}C NMR (150 MHz, CD_3OD) δ 165.7 (s, C-1), 118.0 (d, C-2), 141.5 (d, C-3), 124.2 (s, C-4), 144.3 (s, C-5), 36.0 (t, C-6), 33.7 (d, C-7), 125.5 (d, C-8), 12.5 (q, C-10), 113.5 (d, C-11), 141.4 (s, C-12), 55.3 (s, C-13), 45.4 (t, C-14), 139.2 (s, C-15), 65.9 (t, C-16); positive-HRESIMS *m*/*z* $[M+H]^+$ calcd for $C_{15}H_{19}N_2O_2$ 259.1446, found 259.1384; $[M+Na]^+$ calcd for $C_{15}H_{18}N_2O_2Na$ 281.1266, found 281.1199.
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7. *14α-Hydroxyl huperzine A* (**3**): White powder (mp >250 °C); $[\alpha]_D^{20}$ − 138.5 (*c* 0.135, $CHCl_3$); UV–vis λ_{max}: 230, 310 nm (HPLC/UV); IR ν_{max} ($CHCl_3$): 3387, 3300, 3127, 2932, 2869, 1656, 1611, 1552, 1460, 1410, 1378, 1351, 1304, 1277, 1204, 1183, 1127, 1084, 1022, 923, 834, 794, 756, 735, 662, 637, 618, 586, 536, 516 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.42 (1H, d, *J* = 9.6 Hz, H-2), 7.94 (1H, d, *J* = 9.6 Hz, H-3), 2.92 (1H, dd, *J* = 5.4, 16.2 Hz, H-6ax), 2.68 (1H, dd, *J* = 1.2, 16.2 Hz, H-6eq), 3.62 (1H, br s, H-7), 5.55 (1H, d, *J* = 6.6 Hz, H-8), 1.72 (3H, d, *J* = 6.6 Hz, H-10), 5.56 (1H, q, *J* = 6.6 Hz, H-11), 3.57 (1H, s, H-14*anti*/β), 1.69 (3H, s, H-16); ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.4 (s, C-1), 117.0 (d, C-2), 140.9 (d, C-3), 120.8 (s, C-4), 143.1 (s, C-5), 33.8 (t, C-6), 32.8 (d, C-7), 127.0 (d, C-8), 12.6 (q, C-10), 115.3 (d, C-11), 138.1 (s, C-12), 58.1 (s, C-13), 77.7 (d, C-14), 136.0 (s, C-15), 20.8 (q, C-16); positive-HRESIMS *m*/*z* $[M+H]^+$calcd for $C_{15}H_{19}N_2O_2$ 259.1446, found 259.1389; $[M+Na]^+$ calcd for $C_{15}H_{18}N_2O_2Na$ 281.1266, found 281.1240.
8. *Huperzine A 8α,15α-epoxide* (**4**): White powder (mp >250 °C); $[\alpha]_D^{20}$ − 8 (*c* 0.05, $CHCl_3$); UV–vis λ_{max}: 230, 310 nm (HPLC/UV); IR ν_{max} ($CHCl_3$): 3275, 2941, 1656, 1576, 1413, 1353, 1180, 1117, 1049, 992, 855, 809, 789, 763, 717, 656, 621, 548, 520 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.48 (1H, d, *J* = 9.6 Hz, H-2), 7.94 (1H, d, *J* = 9.6 Hz, H-3), 3.01 (1H, dd, *J* = 5.4, 16.2 Hz, H-6ax), 2.87 (1H, dd, *J* = 1.2, 16.2 Hz, H-6eq), 3.65 (1H, br s, H-7), 2.97 (1H, s, H-8), 1.67 (3H, d, *J* = 6.6 Hz, H-10), 5.62 (1H, q, *J* = 6.6 Hz, H-11), 1.99 (1H, d, *J* = 14.4 Hz, H-14*anti*/β), 1.84 (1H, d, *J* = 14.4 Hz, H-14*syn*/α), 1.14 (3H, s, H-16); ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.1 (s, C-1), 117.9 (d, C-2), 140.8 (d, C-3), 121.9 (s, C-4), 141.7 (s, C-5), 31.2 (t, C-6), 32.7 (d, C-7), 63.8 (d, C-8), 12.4 (q, C-10), 117.1 (d, C-11), 139.0 (s, C-12), 52.6 (s, C-13), 47.5 (t, C-14), 56.8 (s, C-15), 24.2 (q, C-16); positive-HRESIMS *m*/*z* $[M+H]^+$calcd for $C_{15}H_{19}N_2O_2$ 259.1446, found 259.1410; $[M+Na]^+$ calcd for $C_{15}H_{18}N_2O_2Na$ 281.1266, found 281.1251.