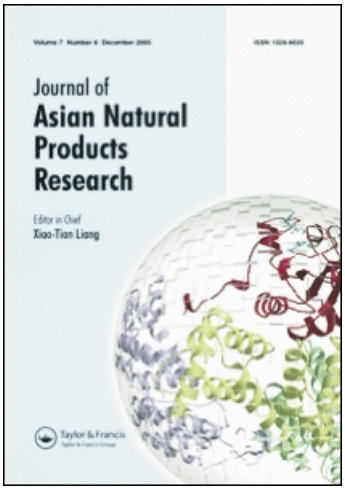

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L. Yang[a]; J. Dai[a]; J. -I. Sakai[a]; M. Ando[a]
[a] Department of Chemistry and Chemical Engineering, Niigata University, Niigata, Japan

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Biotransformation of α- and 6β-santonin by fungus and plant cell cultures

L. YANG†§, J. DAI‡§*, J.-I. SAKAI§ and M. ANDO§

†College of Life and Environmental Sciences, The Central University for Nationalities, 27 South Zhongguancun Street, Beijing 100081, China
‡Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, China
§Department of Chemistry and Chemical Engineering, Niigata University, Ikarashi 2-8050, Niigata 950-2181, Japan

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One fungus, *Abisidia coerulea* IFO 4011, and suspended cell cultures of one plant, *Asparagus officinalis*, were employed to bioconvert α- and 6β-santonin. Incubation of α-santonin with the cell cultures of the fungus afforded two products, 11β-hydroxy-α-santonin (**1**, in 76.5% yield) and 8α-hydroxy-α-santonin (**2**, in 2.0% yield). And from 6β-santonin, four major products (**3**, **4**, **5** and **6**) and four minor products (**7**, **8**, **9** and **10**) were obtained, including 8α-hydroxylated products in trace yields. Very interestingly, a skeletal rearrangement occurred and a guaiane product (**13**) formed in a very low yield when α-santonin incubating with *A. officinalis* cell cultures, while not in the case of 6β-santonin as substrate. Among the obtained 15 products, **2**, **7**, **8**, **9**, **10** and **12** are new compounds. The fact of 8α hydroxylation of santonin enables the formation of 8,12-eudesmanolide instead of 6,12-eudesmanolide and some useful modification at C-8 position. In addition, these reactions would provide evidence for the biogenesis between different types of eudesmane and/or guaiane compounds in the plants in nature.

Keywords: α-Santonin; 6β-Santonin; Biotransformation; *Abisidia coerulea* IFO 4011; Cell suspension cultures; *Asparagus officinalis*

1. Introduction

Owing to the presence of many diverse functional groups, the most cheap and abundant, α- and 6β-santonin, have been extensively used as the starting materials to obtain several naturally bioactive terpenoid compounds bearing A eudesmane or guaiane skeleton [1–5]. These compounds have attracted a great deal of interest from chemists on account of their wide range of biological activities, such as cytotoxic, antitumour, immunosuppressive, insecticidal and anti-HIV activities [6]. There have been a number of reports on the biotransformation of these compounds, and already many interesting results have been achieved [2,5,7–14]. In order to obtain more convenient and useful intermediates for the

*Corresponding author. E-mail: jgdai@imm.ac.cn

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synthesis of these types of bioactive compounds, the study on the bioconversion of α- and 6β-santonin by a variety of fungi and plant cell cultures have been carried out in our laboratory. In this paper, we report their biotransformation by a fungus, *Abisidia coerulea* IFO 4011, and suspended cell cultures of *Asparagus officinalis*.

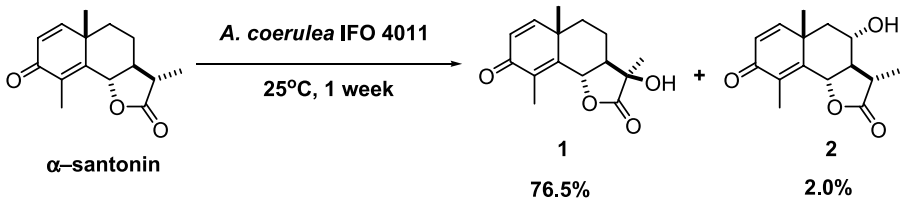
2. Results and discussion

2.1 Biotransformation of α- and 6β-santonin by Abisidia coerulea

α-Santonin was added to 2-day-old cell cultures of fungus *A. coerulea* and incubated for additional 7 days to give two products, **1** (76.5%) and **2** (2.0%) (scheme 1). Their structures were determined as 11β-hydroxy-α-santonin (**1**) and 8α-hydroxy-α-santonin (**2**) on the basis of ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, DEPT, HMQC, HMBC, NOE, IR and HRMS spectral data. 11β Hydroxylation of α-santonin by microbial transformation has been reported, the highest yield being 83% [14]; this reaction is the key step for the synthesis of natural compound decipienin [5]. However, there is no report on the 8α hydroxylation of α-santonin by enzymatic approach. The fact of 8α hydroxylation of α-santonin enables the formation of 8,12-eudesmanolide instead of 6, 12-eudesmanolide and some other useful modification at C-8 position.

The HREI mass spectrum of **2** showed a molecular ion peak $[M]^+$ at *m/z* 262.1202, consistent with the molecular formula of $C_{15}H_{18}O_4$, suggesting that a hydroxyl group may be introduced. The presence of OH group in **2** was supported by the IR absorption at 3628 cm^{-1}. The ^{1}H NMR, ^{13}C NMR, DEPT, HMQC spectra of **2** also showed the existence of a new oxymethine proton signal at δ 4.15 (*ddd*, $J = 4.6$, 11.0, 11.0 Hz) and the connected carbon signal at δ 68.74 (*d*). In addition, the ^{1}H NMR spectrum of **2** was similar to that of α-santonin except that the signals of H-8 [Ha: δ 2.16 (*dddd*, $J = 3.4$, 4.9, 12.4, 13.0 Hz); Hb: δ 1.76 (m)] had disappeared; HMBC correlations of the signals of the above oxymethine proton signal with C-7 and C-9 and ^{1}H-^{1}H COSY correlations with H-7 and H-9 strongly indicated that the OH group was introduced at C-8 position. The stereochemistry of 8-OH was determined to be α-configuration by the NOE spectrum experiment; in the NOE spectrum, the integration values of H-6, H-11 and H-14 were enhanced by 6.06%, 3.88% and 3.77%, respectively, when H-8 was irradiated; the integration values of H-8, H-11 and H-14 were enhanced by 4.48%, 3.72% and 6.40%, respectively, when H-6 was irradiated. Accordingly, the structure of **2** was determined as 8α-hydroxyl α-santonin.

In an effort to investigate the biotransformation capacity of the fungus and the effects of different configurational substituents on the biotransformation, the epimer of α-santonin, 6β-santonin, was employed as substrate and bioconverted by the same fungus under the same



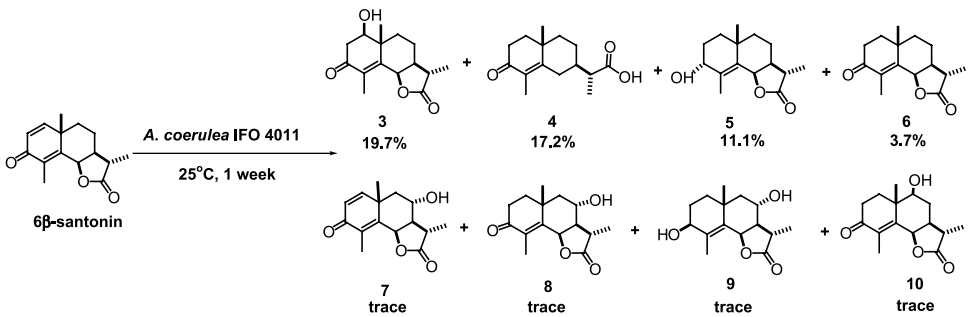
Scheme 1. The biotransformation of α-santonin by *A. coerulea*.

conditions. However, four major products (**3**, **4**, **5** and **6**) [13], four minor and new products (**7**, **8**, **9** and **10**) (scheme 2) were obtained, their structures were determined based on ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, DEPT, HMQC, HMBC, NOE, IR and HRMS spectral data (see section 3). In this bioprocess, the occurred reactions involved dehydrogenation [C1(2)], reduction (C-3), hydroxylation (C-1, C-8 and C-9). C-11 hydroxylation did not occur, but selective C-8α hydroxylation still occurred as well as C-9 hydroxylation although in very low yields. These results suggested that substrates only with a different configurational substituent might result in different biotransformation modes by the same biocatalyst under the same reaction conditions, that is to say that the corresponding enzyme(s) possess strict stereochemical selectivity.

The HREI mass spectrum of **7** showed a molecular ion peak $[M]^{+}$ at *m/z* 262.1195, consistent with the molecular formula of $C_{15}H_{18}O_4$, suggesting that a hydroxyl group may be introduced. The presence of OH group in **7** was supported by the IR absorption at 3620 cm^{-1}. The ^{1}H NMR, ^{13}C NMR, DEPT, HMQC spectra of **7** also showed the existence of a new oxymethine proton signal at δ 4.00 (*ddd*, $J = 3.9$, 10.0, 13.0 Hz) and the connected carbon signal at δ 66.89 (*d*). In addition, the ^{1}H-NMR spectrum of **7** was similar to that of 6β-santonin except that the signals of H-8 had disappeared; HMBC correlations of the signals of the above oxymethine proton signal with C-7 and C-9 and ^{1}H-^{1}H COSY correlations with H-7 and H-9 clearly indicated that the OH group was introduced at C-8 position. The stereochemistry of 8-OH was determined to be α-configuration by the NOE spectrum experiment; in the NOE spectrum, the integration values of H-11 and H-14 were enhanced by 8.25% and 7.56%, respectively, when H-8 was irradiated. Accordingly, the structure of **7** was determined as 8α-hydroxyl 6β-santonin.

The HREI mass spectrum of **8** displayed a molecular ion peak $[M]^{+}$ at *m/z* 264.1350, consistent with the molecular formula of $C_{15}H_{20}O_4$. Its IR absorption at 3628 cm^{-1} suggested the presence of OH group in **8**. The ^{1}H NMR and ^{13}C NMR spectra (see section 3) were very similar to those of compound **7** except that there was no olefinic proton signal and one olefinic carbon signals at δ 137.38 (*s*, C-4) and 149.05 (*s*, C-5), hence it could be deduced that the C-1 (2) double bond was reduced. Accordingly, **8** was the 1,2-hydrogenated and 8α-hydroxylated derivative of 6β-santonin, and produced from **7** via reduction.

The HREI mass spectrum of **9** showed a molecular ion peak $[M]^{+}$ at *m/z* 266.1503, consistent with the molecular formula of $C_{15}H_{22}O_4$. The ^{1}H NMR and ^{13}C NMR spectra of **9** were similar to those of **8** except that the signals of C-3 [δ 198.48 (*s*)] had disappeared, while the signal of another oxymethine [H-3: δ 4.08 (*dd*, $J = 6.9$, 9.5 Hz), C-3: δ 71.32 (*d*)] appeared, so product **9** could be formed from **8** through C-3 carbonyl



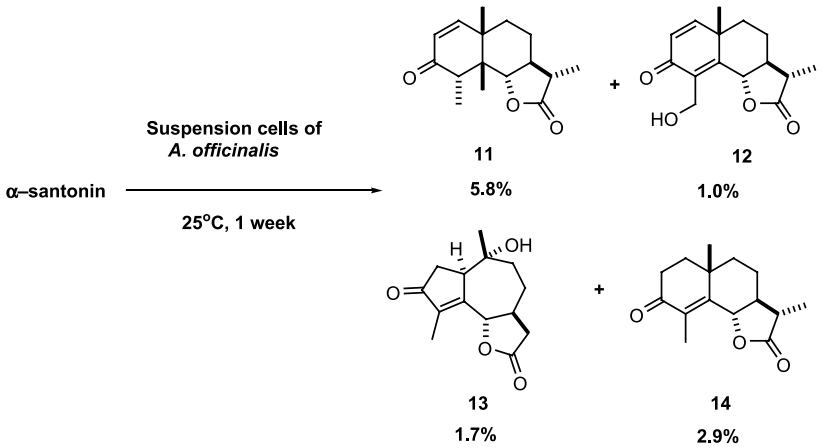
Scheme 2. The biotransformation of 6β-santonin by *A. coerulea*.

reduction. The stereochemistry of 3-OH was determined by NOE spectrum experiment; in the NOE spectrum, the integration values of H-6 were enhanced by 1.26%, when H-3 was irradiated.

The HREI mass spectrum of **10** revealed a molecular ion peak $[M]^+$ at *m/z* 264.1374, consistent with the molecular formula of $C_{15}H_{20}O_4$. Its IR absorption at 3628 cm^{-1} suggested the presence of OH group in **10**. The ^1H NMR and ^{13}C NMR spectra (see section 3) showed that there was no olefinic proton signal and one olefinic carbon signals at δ 138.43 (*s*, C-4) and 149.85 (*s*, C-5), hence it could be deduced that the C-1 (2) double bond was reduced. The ^1H NMR, ^{13}C NMR, DEPT, HMQC spectra of **10** also showed the existence of a new oxymethine proton signal at δ 3.46 (*dd*, $J = 3.7$, 12.0 Hz) and the connected carbon signal at δ 74.90 (*d*). In addition, the ^1H NMR spectrum of **10** was similar to that of 6β-santonin except that the signals of H-9 had disappeared; HMBC correlations of the signals of the above oxymethine proton signal with C-8 and C-10 and ^1H-^1H COSY correlations with H-8 clearly indicated that the OH group was introduced at C-9 position. The stereochemistry of 9-OH was determined to be β-configuration by the NOE spectrum experiment; in the NOE difference spectrum, the integration values of H-6 and H-7 were enhanced by 3.35% and 2.56%, respectively, when H-9 was irradiated. Accordingly, **10** was determined as a 1,2-hydrogenated and 9β-hydroxylated derivative of 6β-santonin.

2.2 *Biotransformation of α- and 6β-Santonin by* Asparagus officinalis

α-Santonin was incubated with suspended cells of *A. officinalis* for 7 days to give four products, **11** (5.8%) [4], **12** (1.0%) and **13** (1.7%) and **14** (2.9%) [5] (scheme 3). All the structures were determined on the basis of their NMR, IR and HRMS spectral data, among them, compounds **12** and **13** were two new compounds from enzymatic bioconversion. Very interestingly, a skeletal rearranged guaiane product (**13**) formed in this bioprocess although in a very low yield, which provides a very strong evidence for the biogenetic relationships between eudesmane and guaiane compounds in the natural plants.

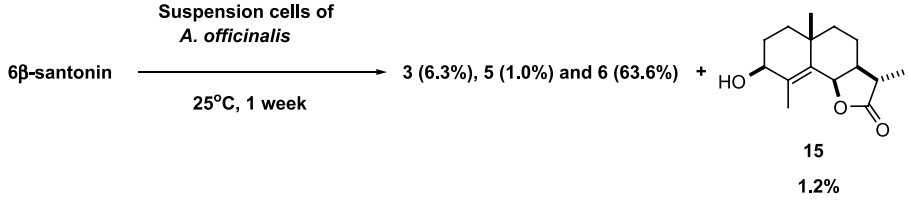


Scheme 3. The biotransformation of α-santonin by A. officinalis.

The HREI-MS spectrum of **12** showed a molecular ion peak at *m/z* 262.1202 $[M]^+$, consistent with the molecular formula of $C_{15}H_{19}O_4$, suggesting that a hydroxyl group may be introduced. The existence of OH group in **12** was supported by its IR absorption at 3620 cm^{-1}. The ^1H NMR spectrum of **12** was similar to that of α-santonin except that the signal of H-15 [δ 2.07 (*d*, $J = 1.5$ Hz)] had disappeared, however, one new oxy-methylene [δ 4.80 (*s*)] and its connected carbon signal at δ 56.06 (*t*) appeared. HMBC correlations of the signals of the above oxy-methylene proton signal with C-4 clearly indicated that the hydroxylation occurred at C-15. Therefore, the structure of **12** was identified to be 15-hydroxyl α-santonin.

The HREI-MS, and ^1H NMR and ^{13}C NMR spectra of **13** displayed an elemental composition of $C_{15}H_{20}O_4$. The IR spectrum of **13** showed the absorption of hydroxyl group at 3620 cm^{-1} and it was confirmed by ^{13}C NMR and DEPT spectra in which a new signal for quarternary carbon bearing oxygen were observed at δ 74.50 (*s*). These showed that one hydroxyl group might exist in the molecule of **13**. According to ^1H NMR, ^{13}C NMR, DEPT and HMQC spectra, there was no olefinic proton signal and one olefinic carbon signals at δ 143.18 (*s*, C-4) and 161.09 (*s*, C-5), hence it could be deduced that the C-1 (2) double bond was reduced. In HMBC spectrum, the correlations between H-2 [Ha: δ 2.61 (*dd*, $J = 3.4$, 20.0 Hz); Hb: δ 2.61(*dd*, $J = 5.9$, 20.0 Hz)] with C-5 and H-6 [δ 4.82 (*brs*)] with C-1 (δ 50.44, *d*) suggested that **13** was a rearranged product bearing guaiane skeleton from eudesmane skeleton by enzyme(s). HMBC correlations of the quarternary carbon with H-1, H-2, H-8, H-9 and H-14 clearly indicated that the introduced OH group was at C-10 position, and the stereochemistry was determined as α-configuration based upon the NOE difference (see section 3). Also, the stereochemistry of H-1 was determined to be α-configuration on the basis of NOE difference spectrum (see section 3). Thus, the structure of **13** was determined as 10**β**-hydroxy-1α-H-3-oxoguai-4-en-6α,12-olide. This compound has already obtained by chemical approach [15], but this is the first time to obtain via enzymatic approach and report its complete NMR data (see section 3).

Both 6α-and 6β-eudesmane or guaiane compounds exist in nature, thus, in order to gain further insight into the effects of different configuration of the substrate on the biotransformation, 6β-santonin was used as substrate and converted by the suspended cells of *A. officinalis* under the same incubation conditions. As a result, four products (**3**, **5** and **6**, **15** [1]) were obtained in 6.3%, 1.0%, 63.6% and 1.2% yields, respectively (scheme 4). The reactions that occurred involved dehydrogenation [C-1 (2)], reduction (C-3) and hydroxylation (C-1). However, the above rearrangement did not occur in the case of 6β-santonin as substrate. These results also demonstrated that substrates with different configuration led to different reactions in biotransformation process, and gave evidence for the substrate-specificity of the enzyme(s).



Scheme 4. The biotransformation of 6β-santonin by *A. officinalis*.

2.3 Conclusions

There are a variety of reactions (reduction, hydroxylation, skeletal rearrangement, etc.) involving in the biotransformation of α- and 6β-santonin by the fungus *A. coerulea* and the cell suspension cultures of *A. officinalis*. To our best knowledge, here is the first report on C-8 and C-15 hydroxylation and skeletal rearrangement of α-santonin through biotransformation. Among the reported reactions, some would facilitate their modification to bioactive compounds, for example, C-1, C-8 and C-15 hydroxylation and the skeletal rearrangement. The fact of different biotransformation modes caused by different configurational substituents indicates the strict configuration-specificity of the enzyme(s). Furthermore, C-8 hydroxylation would provide the evidence for the biogenetic relationships between 6,12-eudesmanolide and 8,12-eudesmanolide; and the skeletal rearrangement provide the evidence for the biogenetic relationships between eudesmane and guaiane sesquiterpenoids in nature.

3. Experimental

3.1 General experimental procedures

Melting points were measured with a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were obtained using a Horiba SEPA-200 polarimeter. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded with a Varian Unity-PS instrument using $CDCl_3$ or pyridine-d_5 as solvents. ^{1}H NMR and ^{13}C NMR assignments were determined by H-H COSY, DEPT, HMQC and HMBC experiments. HREI-MS were carried out on a JEOL-HX 110 instrument. IR spectra were taken on a Hitachi 270-30 spectrometer in $CHCl_3$. Semi-preparative HPLC was performed on a Hitachi L-6200 HPLC instrument with an Inertsil Prep-sil or Pre-ODS (GL Science, 25 cm × 10 mm i.d.) stainless steel column and a YRU-883 RI/UV bi-detector; the flow rate was 5 mL/min unless stated elsewhere. Silica gel (230–300 mesh) was employed for flash column chromatography; analytical TLC plates (silica gel 60 F_{254}, Merck) were visualised at UV_{254}.

3.2 Organism, media and culture conditions

Abisidea coerulea IFO4011 was purchased from Institute for Fermentation, Osaka, Japan (IFO) and was kept in solid medium containing potato (200 g/L), sucrose (20 g/L) and agar (2%) at 4°C. The seed cultures was prepared in a 500-mL Erlenmeyer flask with 150 mL of liquid medium and incubated for 2 days. To 150 mL of medium in a 500-mL flask was added 5 mL of the seed cultures and cultured on a rotary shaker at 110 rpm at (25 ± 2)°C in the dark for the use of biotransformation.

The seedlings of *Asparagus officinalis* (identified by Professor M. Suzuki, Faculty of Agriculture, Niigata University) were collected from the campus of Niigata University, Japan, in May 2002. Young stems were used to initiate calli. The explants were disinfected by immersing in 70% ethanol for 30 s, followed in saturated calcium hypochlorite solution for 15 min, washed five times with sterilised water, then cut into small pieces (about 1.0 cm in length) and aseptically transferred to Murashige and Skoog's medium (MS) supplemented with 0.5 mg/L of naphthalene acetic acid (NAA), 0.5 mg/L of 6-benzylaminopurine (6-BA)

and 0.2 mg/L of 2,4-dichlorophenoxy acetic acid (2,4-D). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. The calli initiated from all the explants within 4 weeks of cultivation in the dark at (25 ± 2)°C. The callus cultures were maintained under the same culture conditions by sub-culturing every four weeks. After five generations, 3-week-old friable calli were used for initiation of suspension cultures. Cell suspension cultures were sub-cultured every three weeks at the inoculum size of 5.0 g/L dry weight in a 500-mL Erlenmeyer flask with 150 mL of fresh medium and incubated on a rotary shaker at 110 rpm in the dark at (25 ± 2)°C for the biotransformation use.

3.3 Biotransformation of α-santonin with A. coerulea

α-Santonin (100 mg, purchased from Sigma Company) was dissolved in acetone (1.8 mL), distributed among three Erlenmeyer flasks of 2-day-old cultures with a micropipette and subsequently incubated for additional 7 days, after which time the cultures were filtered and washed with EtOAc (3 × 100 mL), and the combined filtrate was extracted with EtOAc (5 × 500 mL). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40°C to give 260 mg of residue. The residue was dissolved in acetone. Micro-crystals formed in acetone solution and were filtered to afford 62.3 mg of **1** (62.3%). The remaining solution was chromatographed on a silica gel flash column eluting gradiently with the mixtures of Hexane and EtOAc (70% Hexane and 30% EtOA/100% EtOAc) to give six fractions monitored by TLC. Further purification was performed by semi-preparative HPLC. 21.0 mg of substrate (21.0%) was obtained from fractions 2, 3 and 4 (t_R = 14.74 min; mobile phase: Hexane/EtOAc = 6:4). 14.2 mg of **1** (14.2%) was obtained from fractions 4, 5 (t_R = 15.83 min; mobile phase: Hexane/EtOAc = 6:4). 2 mg of **2** (2.0%) (t_R = 8.47 min; mobile phase: 100% EtOAc) was obtained from fraction 6.

Compound 2: colourless plates: 191–193°C; $[\alpha]_D^{20}$ −41.1 (*c* 0.513, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3628, 2988, 2944, 1788, 1734, 1668, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.67 (1H, *d*, *J* = 10.0 Hz, H-1), 6.25 (1H, *d*, *J* = 9.8 Hz, H-2), 4.81 (1H, *dd*, *J* = 1.2, 12.0 Hz, H-6), 4.15 (1H, *ddd*, *J* = 4.6, 11.0, 11.0 Hz, H-8), 2.64 (1H, *dq*, *J* = 6.9, 6.9 Hz, H-11), 2.25 (3H, *d*, *J* = 1.2 Hz, H-15), 2.14 (1H, *dd*, *J* = 4.5, 12.7 Hz, H-9β), 1.91 (1H, *ddd*, *J* = 11.0, 11.0, 12.0 Hz, H-7), 1.50 (1H, *dd*, *J* = 11.0, 12.7 Hz, H-9α), 1.43 (3H, *d*, *J* = 6.9 Hz, H-13), 1.34 (3H, *s*, H-14); NOE: (1) H-6 (−100%), H-8 (+4.48%), H-11 (+3.72%), H-14 (+6.40%); (2) H-8 (−100%), H-6 (+6.06%), H-9β (+4.41%), H-11 (+3.88%), H-14 (+3.71%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 186.00 (*s*, C-3), 177.18 (*s*, C-12), 153.50 (*d*, C-1), 149.26 (*s*, C-5), 129.59 (*s*, C-4), 125.93 (*d*, C-2), 78.12 (*d*, C-6), 68.74 (*d*, C-8), 58.67 (*d*, C-7), 46.92 (*t*, C-9), 40.84 (*d*, C-11), 40.15 (*s*, C-10), 26.54 (*q*, C-14), 14.24 (*q*, C-13), 10.88 (*q*, C-15); HREI-MS *m/z* 262.1202 (calcd for $C_{15}H_{18}O_4$, 262.1205).

3.4 Biotransformation of 6β-santonin with A. coerulea

6β-Santonin (500 mg, purchased from Sigma) was dissolved in acetone (5.0 mL), distributed among fifteen Erlenmeyer flasks of 2-day-old cultures with a micropipette and incubated for additional 7 days, after which time the cultures were filtered and washed with EtOAc (3 × 500 mL), and the combined filtrate was extracted with EtOAc (5 × 2000 mL). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40°C to give 1560 mg of residue. The residue was subjected onto a silica gel flash

chromatographic column eluting gradiently with the mixture of Hexane and EtOAc (80% Hexane and 20% EtOA/100% EtOAc) to give seven fractions monitored by TLC analysis. Further purification was performed by semi-preparative HPLC. From fraction 3 (53.4 mg) was obtained 9.0 mg of **6** ($t_R = 8.22$ min; mobile phase: Hexane/EtOAc = 6:4) and 29.1 mg of **4** ($t_R = 6.32$ min; mobile phase: Hexane/EtOAc = 6:4). From fraction 4 (202.2 mg) was obtained 9.5 mg of **6** ($t_R = 8.22$ min; mobile phase: Hexane/EtOAc = 6:4), 56.8 mg of **4** ($t_R = 6.32$ min; mobile phase: Hexane/EtOAc = 6:4), 55.7 mg of **5** ($t_R = 9.76$ min; mobile phase: Hexane/EtOAc = 6:4) and 43.2 mg of substrate ($t_R = 11.04$ min; mobile phase: Hexane/EtOAc = 6:4). 100.5 Mg of substrate ($t_R = 11.04$ min; mobile phase: Hexane/EtOAc = 6:4) was obtained from fraction 5 (287.4 mg). From faction 6 (290.9 mg) was obtained 95.0 mg of **3** ($t_R = 7.33$ min; mobile phase: Hexane/EtOAc = 1:4) and 1.4 mg of **8** ($t_R = 11.30$ min; mobile phase: Hexane/EtOAc = 1:4). First, fraction 7 were separated by HPLC with Hexane/EtOAc = 1:4 as the mobile phase, and gave 3.6 mg of **3**, 2.7 mg of **8**, 2.7 mg of **9** ($t_R = 9.76$ min) and a mixture (5.0 mg). The mixture was further separated with Hexane/EtOAc = 1:2 as the mobile phase to afford 1.4 mg of **10** ($t_R = 16.22$ min), 2.1 mg of **7** ($t_R = 17.84$ min).

Compound 7: oil-like; $[\alpha]_D^{20}$ −91.3 (*c* 0.11, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 3032, 2936, 1788, 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.76 (1H, *d*, *J* = 9.8 Hz, H-1), 6.27 (1H, *d*, *J* = 10.0 Hz, H-2), 5.61 (1H, *d*, *J* = 5.4 Hz, H-6), 4.00 (1H, *ddd*, *J* = 3.9, 10.0, 13.0 Hz, H-8), 2.99 (1H, *q*, *J* = 7.8, H-11), 2.08 (3H, *s*, H-15), 2.03 (1H, *dd*, *J* = 5.4, 10.0 Hz, H-7), 2.02 (1H, *m*, H-9a), 1.52 (1H, *m*, H-9b), 1.46 (3H, *d*, *J* = 7.6 Hz, H-13), 1.33 (3H, *s*, H-14); NOE: H-8 (−100%), H-11 (+8.25%), H-14 (+7.56%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 196.00 (*s*, C-3), 179.00 (*s*, C-12), 155.96 (*d*, C-1), 146.80 (*s*, C-5), 138.19 (*s*, C-4), 125.87 (*d*, C-2), 76.74 (*d*, C-6), 66.89 (*d*, C-8), 51.35 (*d*, C-7), 42.83 (*t*, C-9), 41.27 (*d*, C-11), 40.07 (*s*, C-10), 25.86 (*q*, C-14), 15.02 (*q*, C-13), 11.21 (*q*, C-15); HREI-MS *m/z* 262.1195 (calcd for $C_{15}H_{18}O_4$, 262.1205).

Compound 8: oil-like; $[\alpha]_D^{20}$ −29.5 (*c* 0.10, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3624, 3032, 2936, 1780, 1716, 1672 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.53 (1H, *d*, *J* = 5.6 Hz, H-6), 3.91 (1H, *ddd*, *J* = 3.9, 10.0, 13.0 Hz, H-8), 2.97 (1H, *q*, *J* = 7.8 Hz, H-11), 2.71 (1H, *ddd*, *J* = 5.6, 13.9, 17.8 Hz, H-2a), 2.52 (1H, *ddd*, *J* = 2.2, 4.9, 18.0 Hz, H-2b), 2.03 (1H, *dd*, *J* = 5.8, 10.0 Hz, H-7), 1.94 (1H, *m*, H-1a), 1.93 (3H, *s*, H-15), 1.85 (1H, *dd*, *J* = 3.7, 12.7 Hz, H-9a), 1.80 (1H, *ddd*, *J* = 2.2, 5.6, 13.4 Hz, H-1b), 1.43 (3H, *d*, *J* = 7.8 Hz, H-13), 1.41 (1H, *m*, H-9b), 1.29 (3H, *s*, H-14); NOE: H-8 (−100%), H-11 (+7.78%), H-14 (+7.43%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.48 (*s*, C-3), 179.01 (*s*, C-12), 149.05 (*s*, C-5), 137.38 (*s*, C-4), 76.94 (*d*, C-6), 66.30 (*d*, C-8), 51.25 (*d*, C-7), 47.33 (*t*, C-9), 41.00 (*d*, C-11), 37.80 (*t*, C-1), 35.74 (*s*, C-10), 33.45 (*t*, C-2), 24.11 (*q*, C-14), 15.16 (*q*, C-13), 11.15 (*q*, C-15); HREI-MS *m/z* 264.1350 (calcd for $C_{15}H_{20}O_4$, 264.1361).

Compound 9: oil-like; $[\alpha]_D^{20}$ −123.5 (*c* 0.10, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3624, 3432, 2936, 1762 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.46 (1H, *d*, *J* = 5.6 Hz, H-6), 4.08 (1H, *dd*, *J* = 6.9, 9.5 Hz, H-3), 3.85 (1H, *ddd*, *J* = 3.9, 10.0, 13.0 Hz, H-8), 2.92 (1H, *q*, *J* = 7.6 Hz, H-11), 2.07 (1H, *m*, H-2a), 1.91 (3H, *s*, H-15), 1.88 (1H, *dd*, *J* = 5.4, 9.8 Hz, H-7), 1.80 (1H, *m*, H-2b), 1.73 (1H, *dd*, *J* = 4.1, 12.4 Hz, H-9a), 1.52 (2H, *m*, H-1a and H-1b), 1.40 (3H, *d*, *J* = 7.6 Hz, H-13), 1.27 (1H, *m*, H-9b), 1.16 (3H, *s*, H-14); NOE: H-8 (−100%), H-11 (+8.35%), H-14 (+7.32%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.79 (*s*, C-12), 141.17 (*s*, C-4), 130.16 (*s*, C-5), 77.47 (*d*, C-6), 71.32 (*d*, C-3), 66.95 (*d*, C-8), 51.95 (*d*, C-7), 48.45 (*t*, C-9), 41.61 (*d*, C-11), 37.47 (*t*, C-1), 35.19 (*s*, C-10), 28.61 (*t*, C-2), 26.05 (*q*, C-14), 15.12 (*q*, C-13), 14.55 (*q*, C-15); HREI-MS *m/z* 266.1503 (calcd for $C_{15}H_{22}O_4$, 266.1518).

Compound 10: oil-like; $[\alpha]_D^{20}$ −101.3 (*c* 0.12, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 3034, 2936, 1778, 1720, 1668 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.39 (1H, *d*, *J* = 5.1 Hz, H-6), 3.46 (1H, *dd*, *J* = 3.7, 12.0 Hz, H-9), 2.66 (1H, *ddd*, *J* = 5.1, 14.6, 17.8 Hz, H-2a), 2.56 (1H, *ddd*, *J* = 2.5, 5.1, 17.8 Hz, H-2b), 2.55 (1H, *q*, *J* = 7.6 Hz, H-11), 2.32 (1H, *ddd*, *J* = 6.1, 7.0, 13.0 Hz, H-7), 2.22 (1H, *ddd*, *J* = 2.7, 5.4, 13.4 Hz, H-1a), 1.95 (3H, *s*, H-15), 1.91 (1H, *ddd*, *J* = 2.4, 6.4, 11.0 Hz, H-8a), 1.79 (1H, *ddd*, *J* = 5.1, 5.4, 13.9 Hz, H-1b), 1.71 (1H, *ddd*, *J* = 12.7, 12.7, 12.0 Hz, H-8b), 1.39 (3H, *d*, *J* = 7.6 Hz, H-13), 1.22 (3H, *s*, H-14); NOE: H-9 (−100%), H-6 (+3.35%), H-7 (+2.56%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.52 (*s*, C-3), 179.00 (*s*, C-12), 149.85 (*s*, C-5), 138.43 (*s*, C-4), 75.19 (*d*, C-6), 74.90 (*d*, C-9), 51.35 (*d*, C-7), 43.74 (*d*, C-11), 41.45 (*t*, C-8), 39.74 (*s*, C-10), 33.95 (*t*, C-1), 33.53 (*t*, C-2), 15.95 (*q*, C-14), 14.94 (*q*, C-13), 11.37 (*q*, C-15); HREI-MS *m/z* 264.1374 (calcd for $C_{15}H_{20}O_4$, 264.1361).

3.5 Biotransformation of α-santonin with cell cultures of A. officinalis

α-Santonin (300 mg) was dissolved in acetone (5.0 mL), distributed among 10 Erlenmeyer flasks of 14-day-old cultures with micropipette and incubated for additional 7 days, after which the cultures were filtered and washed with EtOAc (3 × 200 mL). The combined filtrate was extracted with EtOAc (5 × 1500 mL). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40°C to afford 544.7 mg of residue. Then the extract was chromatographed on a silica gel flash column eluting gradiently with the mixture of Hexane and EtOAc (80% Hexane and 20% EtOAc/100%EtOAc) to give three fractions; among them, fraction 2 (230.6 mg) was the recovered substrate monitored by TLC and ^1H NMR analyses. Metabolites **14** (8.7 mg, t_R = 8.30 min), **11** (17.5 mg t_R = 7.62 min) and substrate (8.0 mg) were obtained from fraction 1 (34.2 mg) by semi-preparative HPLC with Hexane/EtOAc = 6:4 as the mobile phase. And, metabolites **13** (5.2 mg, t_R = 8.90 min) and **12** (3.0 mg, t_R = 9.73 min) were obtained from fraction 3 (21.4 mg) by semi-preparative HPLC with 100% EtOAc as the mobile phase.

Compound 12: colourless needles: 167–169°C; $[\alpha]_D^{20}$ −92.0 (*c* 0.113, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 2944, 1792, 1666, 1634 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.74 (1H, *d*, J = 10.0 Hz, H-1), 6.28 (1H, *d*, J = 9.7 Hz, H-2), 4.81 (1H, *d*, J = 11.0 Hz, H-6), 4.80 (2H, *s*, H-15), 2.43 (1H, *dq*, J = 6.0, 7.0 Hz, H-11), 2.05 (1H, *m*, H-8a), 1.95 (1H, *m*, H-9a), 1.88 (1H, *m*, H-7), 1.70 (1H, *m*, H-8b), 1.54 (1H, *m*, H-9b), 1.37 (3H, *s*, H-14), 1.28 (3H, *d*, *J* = 6.9 Hz, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.24 (*s*, C-3), 176.65 (*s*, C-13), 155.51 (*d*, C-1), 153.70 (*s*, C-5), 131.62 (*s*, C-4), 126.20 (*s*, C-2), 80.90 (*d*, C-6), 56.06 (*t*, C-15), 53.55 (*d*, C-7), 41.63 (*s*, C-10), 41.04 (*d*, C-11), 37.82 (*t*, C-9), 25.30 (*q*, C-14), 22.98 (*t*, C-8), 12.49 (*q*, C-13); HREI-MS *m/z* 262.1208 (calcd for $C_{15}H_{18}O_4$, 262.1205); HRFAB-MS (positive) *m/z* $[M + H]^+$263.1307 (calcd for $C_{15}H_{19}O_4$, 263.1309).

Compound 13: white powder: 67–69°C; $[\alpha]_D^{20}$ + 27.3 (*c* 0.133, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 2984, 2940, 1786, 1708, 1646 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.82 (1H, *brd*, *J* = 11.0 Hz, H-6), 3.23 (1H, *brs*, H-1), 2.61 (1H, *dd*, *J* = 3.4, 20.0 Hz, H-2a), 2.55 (1H, *dd*, *J* = 5.9, 20.0 Hz, H-2b), 2.33 (1H, *dq*, *J* = 7.1, 6.8 Hz, H-11), 2.15 (1H, *ddd*, *J* = 7.1, 11.0, 12.4 Hz, H-7), 2.10 (1H, *m*, H-9a), 2.07 (1H, *m*, H-8a), 1.90 (3H, *t*, *J* = 2.0 Hz, H-15), 1.80 (1H, *ddd*, *J* = 5.0, 5.0, 14.7 Hz, H-9b), 1.45 (1H, *m*, H-8b), 1.29 (3H, *d*, *J* = 6.8 Hz, H-13), 0.97 (3H, *s*, H-14); NOE: H-1 (−100%), H-7 (+4.24%); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 207.57 (*s*, C-3), 177.08 (*s*, C-12), 161.09 (*s*, C-5), 143.18 (*s*, C-4), 81.44 (*d*, C-6), 74.50 (*s*, C-10), 50.44 (*d*, C-1), 48.47 (*d*, C-11), 45.40 (*t*, C-9), 41.41 (*d*, C-7), 37.11 (*t*, C-2), 25.87

(*t*, C-8), 21.35 (*q*, C-14), 12.50 (*q*, C-13), 9.47 (*q*, C-15); HREI-MS *m/z* 264.1362 (calcd for $C_{15}H_{20}O_4$, 264.1361).

3.6 Biotransformation of 6β-santonin with cell cultures of A. officinalis

The procedures were performed as described as the above, except that the amount of added substrate 6β-santonin was 210 mg, the final concentration was 30 mg/flask. 231 Mg of the resulting residue was subjected onto a silica gel flash chromatographic column eluting gradiently with the mixture of Hexane and EtOAc (80%Hexane and 20%EtOAc ∼ 100% EtOAc) to give 4 fractions. 133.6 Mg of **6** was given from fraction 1 after recrystallisation in the solution of Hexane and EtOAc. 2.0 mg of **5** ($t_R = 6.60$ min) and 2.6 mg of **15** ($t_R = 10.79$ min) were obtained from fraction 3 (13.6 mg) by semi-preparative reversed phase HPLC with methanol/acetonitrile/H_2O = 25/25/50 (v/v/v) as the mobile phase. 13.2 Mg of **3** ($t_R = 10.83$ min) was obtained from fraction 4 (24.7 mg) by semi-preparative HPLC with Hexane/EtOAc = 1/2 as the mobile phase.

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