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Biotransformation of chinensiolide B and the cytotoxic activities of the transformed products

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Abstract

Biotransformation of chinensiolide B, 10α-hydroxy-1α,5α,15-H-3-oxoguaia-11(13)-en-6α,12-olide (**1**), yielded three selectively reduced products, 3β,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide (**2**), 3α,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide (**3**), and 3β,10α-dihydroxy-1α,5α,11β,15α-H-guaia-6α,12-olide (**4**) by the cell suspension cultures of *Catharanthus roseus*. **2** and **3** were also obtained from **1** incubated with cell cultures of a fungus *Abisidia coerulea* IFO 4011 and *Platycodon grandiflorum*, respectively. Among them, **2**, **3** are two new compounds. The three products, **2**–**4**, along with **1** were preliminarily evaluated for their in vitro cytotoxic activity against 3 cell lines (HepG2, WI-38 and VA-13) and all showed potent inhibitory effects on the cell proliferation. Of the four compounds, **3** was the most toxic to the three cell lines tested with IC_{50} values of 22.7, 0.33 and 3.30 μM, respectively.
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Keywords: *Catharanthus roseus* (Apocynaceae); *Platycodon grandiflorum* (Campanulaceae); *Abisidia coerulea* IFO 4011; Chinensiolide B; Biotransformation; Cytotoxic activity

1. Introduction

Living organism systems, such as microbes (fungi, bacteria, etc.), plant cells and organs, insects and animals (including cells in vitro), are the multi-enzymes systems, therefore, it is possible that a great many products could be yielded from one natural product bioconverted by these systems as an exogenous substrate. The type of biotransformation reactions involve hydroxylation, glycosylation, methylation, acylation, prenylation, sulfation and many others, and this approach possesses more advantages over chemical reactions by regio- and stereo-selectivity, mild conditions and so on, and there have been many reviews and reports on it [1–8]. Chinensiolide B [10α-hydroxy-1α,5α,15α-H-3-oxoguaia-11(13)-en-6α,12-olide, **1**], a guaianolide with α-methylene-γ-lactone isolated from *Ixeris chinensis* Nakai (Compositae) recently [9], exhibited high cytotoxic activities against several tumor cells. In an effort to obtain more derivatives with potential for lead compound searching, we have tried biocatalytic modification of **1**. Herein, we report the biotransformation of **1** by cell cultures of two plants and one fungus, and the cytotoxic activities of the products as well.

2. Materials and methods

2.1. General

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 $CDCl_3$ as solvent and internal standard. ^{1}H NMR and ^{13}C NMR assignments were determined by ^{1}H–^{1}H COSY, DEPT,

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HMQC and HMBC experiments. HRFABMS were carried out on a JEOL-HX 110 FAB-mate instrument and HREIMS 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 (GL Science, 25 cm × 10 mm i.d.) stainless steel column and an YRU-883 RI/UV bi-detector, the flow rate was 4.0 ml/min. Analytic HPLC was recorded on the same instruments with an Inertsil Sil (GL Science, 25 cm × 4.6 mm i.d.) stainless steel column eluting with ethyl acetate at the flow rate of 1.0 ml/min, and detected at 254 nm. Silica gel (230–300 mesh) was employed for flash column chromatography, analytical TLC plates (silica gel 60 F_{254}, Merck) were visualized at UV_{254} and by spraying H_2SO_4 followed by heating.

2.2. Organisms, media and cultivation conditions

The *Catharanthus roseus* [10] and *Platycodon grandiflorum* [11] cell suspension cultures were cultivated in 500 ml Erlenmeyer flask with 150 ml of liquid MS medium 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) on the rotary shaker at 110 rpm at 25 ± 2 °C in the dark at the inoculum size of 5 g/l of cell cultures (dry weight). The pH value was adjusted to 5.8 before autoclaving for 20 min at 121 °C. Both the cell cultures were maintained in the above conditions before use for the biotransformation. Fungi, *Abisidia coerulea* IFO4011, *Aspergillus niger* JCM 5546, *Mucor genevensis* JCM 10585, *Cunninghamella elegans* CGMCC 3.3402, *C. echinulata* CGMCC 3.400, *Curvularia sp*. CGMCC 3.1471, *Fusarium solani* CGMCC 3.1791 were purchased from Institute for Fermentation, Osaka, Japan (IFO), Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, Japan (JCM), and China General Microbiological Culture Collection Center, China (CGMCC), respectively. All the fungi were kept on solid medium (PDA) containing potato (200 g/l), sucrose (20 g/l) and agar (2%) at 4 °C. The seed was prepared in a 500 ml flask with 150 ml of liquid medium (PDA medium without agar) and incubated for 2 days. A volume of 5 ml of the seed was added to one flask and shaken at 110 rpm at 25 ± 2 °C in the dark for the use of biotransformation.

2.3. Screening test

Substrate, **1** (isolated from *Ixeris chinensis* Nakai by our laboratory [9], the purity was >95% by HPLC analysis) was dissolved in acetone to make 10 mg/ml. For screening test, the plant and fungi cell cultures was incubated as described above, 15-day-old plant cell cultures and 2-day-old fungi cell cultures were employed for biotransformation. A volume of 0.5 ml of substrate solution was fed to the systems separately. The incubation was allowed to proceed for 7 days, after which the cultures were filtered and washed with ethyl acetate (3 × 30 ml), each filtrate was saturated with NaCl and extracted with ethyl acetate (4 × 50 ml). The extract was dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40 °C to give residue. At the meantime, each system was performed a control into which only 0.5 ml of acetone was added. All the residues were analyzed by TLC (each treatment, its control and substrate as a group).

2.4. Biotransformation of ***1*** by cell suspension cultures of C. roseus

2.4.1. Preparative biotransformation of ***1***

An amount of 50 mg of **1** was dissolved in acetone (5.0 ml), distributed among 10 flasks of 15-day-old cultures, the cultures were filtered and washed with ethyl acetate (3 × 100 ml) after 7 days of incubation, the combined filtrates were saturated with NaCl and extracted with ethyl acetate (4 × 1000 ml). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40 °C to give 100 mg of residue. The residue was then applied to a silica gel (10 g) flash chromatographic column and gradiently eluted with the mixtures of hexane and ethyl acetate (70% hexane and 30% ethyl acetate-100% ethyl acetate) to afford six fractions. Semi-preparative normal-phase HPLC (mobile phase: 100% ethyl acetate) of fraction 6 yielded **1** (10.8 mg, 21.6%, t_R = 14.74 min, determined by HPLC and ^{1}H NMR analyses), **2** (21.2 mg, ca. 42.4%, t_R = 16.98 min), **3** (5.2 mg, ca. 14.0%, t_R = 21.31 min), and **4** (2.5 mg, ca. 5.0%, t_R = 24.52 min).

3β,10α-Dihydroxy-1α,5α,15α-H-guaia-11(13)*-en-6α,12-olide* (**2**): colorless plates; $[\alpha]_D^{20}$ −26.0° (*c* 0.50, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 2940, 1764, 1646 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.18 (1H, d, *J* = 3.7 Hz, H-13a), 5.46 (1H, d, *J* = 3.7 Hz, H-13b), 4.23 (1H, dd, *J* = 9.8, 9.8 Hz, H-6), 3.74 (1H, ddd, *J* = 7.1, 7.2, 15.1 Hz, H-3), 2.88 (1H, m, H-7), 2.32 (1H, ddd, *J* = 6.9, 8.3, 9.6 Hz, H-1), 2.26 (1H, ddd, *J* = 7.1, 13.1, 15.1 Hz, H-2α), 2.18 (1H, m, H-9a), 1.98 (1H, m, H-9b), 1.96 (1H, m, H-4), 1.93 (1H, m, H-5), 1.71 (1H, ddd, *J* = 5.6, 8.5, 13.4 Hz, H-8a), 1.62 (1H, *J* = 8.3, 13.1, 15.1 Hz, H-2α), 1.47 (1H, m, H-8b), 1.27 (3H, s, H-14), 1.16 (3H, d, *J* = 6.5 Hz, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.15 (*s*, C-12), 140.28 (*s*, C-11), 119.63 (*t*, C-13), 84.87 (*d*, C-6), 78.26 (*d*, C-3), 74.76 (*s*, C-10), 51.15 (*d*, C-5), 48.31 (*d*, C-1), 46.77 (*d*, C-4), 45.54 (*d*, C-7), 38.88 (*t*, C-9), 36.46 (*t*, C-2), 27.97 (*q*, C-14), 24.82 (*t*, C-8), 17.90 (*q*, C-15); HRFABMS (positive) *m/z* $[M+H]^+$ 267.1597 (calcd for $C_{15}H_{23}O_4$, 267.1596).

3α,10α-Dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide (**3**): colorless plates; $[\alpha]_D^{20}$ +7.3° (*c* 0.133, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3624, 2940, 1770, 1646 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.18 (1H, d, *J* = 3.6 Hz, H-13a), 5.46 (1H, d, *J* = 3.6 Hz, H-13b), 4.31 (1H, dd, *J* = 10.0, 10.0 Hz, H-6), 4.24 (1H, ddd, *J* = 6.1, 6.3, 6.5 Hz, H-3), 2.82 (1H, m, H-7), 2.37 (1H, m, H-5), 2.33 (1H, m, H-4), 2.31 (1H, m, H-1), 2.19 (1H, m, H-2α), 2.17 (1H, m, H-9a), 2.01 (1H, m, H-8a), 1.69 (1H, m, H-2β), 1.65 (1H, m, H-9b), 1.47

(1H, m, H-8b), 1.27 (3H, s, H-14), 1.07 (3H, d, $J = 7.3$ Hz, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.00 (*s*, C-12), 140.01 (*s*, C-11), 119.53 (*t*, C-13), 82.20 (*d*, C-6), 74.76 (*d*, C-3), 73.55 (*s*, C-10), 50.05 (*d*, C-5), 49.00 (*d*, C-1), 47.36 (*d*, C-4), 46.54 (*d*, C-7), 41.11 (*t*, C-9), 34.78 (*t*, C-2), 25.00 (*q*, C-14), 24.82 (*t*, C-8), 9.30 (*q*, C-15); HRFABMS (positive) *m/z* $[M+H]^+$ 267.1595 (calcd for $C_{15}H_{23}O_4$, 267.1596).

3β,10α-Dihydroxy-1α,5α,11β,15α-H-guaia-6α,12-olide (**4**): colorless plates; $[\alpha]_D^{20}$ +3.5° {*c* 0.067, $CHCl_3$; lit. [12], $[\alpha]_D^{24}$ +3° (*c* 1.4, $CHCl_3$)}; IR ($CHCl_3$) ν_{max} 3620, 2948, 1764 cm^{-1}; HREIMS *m/z* $[M]^+$ 268.1672 (calcd for $C_{15}H_{24}O_4$, 268.1674); ^{1}H NMR and ^{13}C NMR data were in good agreement with those reported [12].

2.4.2. Time course of the biotransformation of **1** to **2, 3** and **4**

On the day 15, 5 mg/flask (33.3 mg/l, in triplicates) of **1** was added, the broth was collected every 1 day and performed as described as in Section 2.4.1. The organic extract was evaporated to dryness, and the residue was dissolved in 1 ml of ethyl acetate. Samples were filtered through 0.45 μm-pore-size membranes just prior to HPLC analysis. An aliquot of 10 μl was used for each injection.

2.5. Biotransformation of **1** by cell suspension cultures of P. grandiflorum

An amount of 9.0 mg of **1** was dissolved in acetone (1.0 ml), and added into two flasks of 15-day-old cultures and incubated for additional 7 days, after which the cultures were filtered and washed with ethyl acetate (3 × 30 ml), the combined filtrates were saturated with NaCl and extracted with ethyl acetate (4 × 200 ml). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40 °C to give 15.0 mg of residue. The residue was filtered by through 0.45 μm-pore-size membrane and directly separated by semi-preparative normal-phase HPLC (mobile phase: 100% ethyl acetate) to give **1** (1.8 mg, 20%), **3** (6.0 mg, ca. 66.7%).

2.6. Preparative biotransformation of **1** by A. coerulea

30.0 Mg of **1** was dissolved in acetone (3.0 ml), distributed among six flasks of 2-day-old cultures, the cultures were filtered and washed with ethyl acetate (3 × 100 ml) after additional 7 days of incubation, the combined filtrates were saturated with NaCl and extracted with ethyl acetate (4 × 500 ml). All the extracts were pooled and dried with anhydrous Na_2SO_4 and concentrated under vacuum at 40 °C to give 100 mg of residue. The residue was then applied to a silica gel flash chromatographic column and gradiently eluted with the mixtures of hexane and ethyl acetate (90% hexane and 10%ethyl acetate–100%ethyl acetate) to give five fractions. Semi-preparatve normal-phase HPLC (mobile phase: 100% ethyl acetate) of fraction 5 yielded **1** (23.2 mg, ca. 77.3%), **2** (3.6 mg, ca. 12.0%).

2.7. Cytotoxicity bioassays

Cytotoxic assays were performed as described in Rubinstein et al. [13]. The cell lines used were a human primary liver cancer (HepG2), and two human lung fibroblasts [WI-38, VA-13 (transformed by SV40 virus)]. Cytotoxicity, IC_{50} for each cell line, is the concentration of compound that causes a 50% inhibition of cell proliferation. Paclitaxel was used as the positive agent.

3. Results and discussion

3.1. Screening test

Cell suspension cultures of two plants, *C. roseus* and *P. grandiflorum*, and seven fungi (Table 1) were screened for the ability to transform **1**. Among them, the cell cultures of the two plants and a fungus, *A. coerulea* IFO 4011 transformed **1** into more polar metabolite(s) by TLC analysis, therefore, they were further used for the preparative biotransformation.

3.2. Biotransformation of **1** by cell suspension cultures of C. roseus and the structural elucidation of the products

In the case of cell suspension cultures of *C. roseus* as the biocatalyst, three more polar metabolites were obtained. On the basis of their ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, DEPT, HMQC, HMBC, NOE, IR and HRMS spectral data, their structures were determined to be 3β,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide (**2**), 3α,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide (**3**) and 3α,10α-dihydroxy-1α,5α,11β,15α-H-guaia-6α,12-olide (**4**) (Fig. 1), all of them were the reduced products. The yields of the products **2**, **3** and **4** were about 42.4, 14.0, and 5.0%, respectively. Among them, **2** and **3** were new compounds, **4** has already been obtained by chemical synthesis from α-santonin [12].

Table 1
Results of screenings for the biotransformation of **1**

Biocatalysts	Products (yield)
C. roseus (cell suspension cultures)	**2** (ca. 42.4%), **3** (ca. 14.0%), **4** (ca. 5.0%)
P. grandiflorum (cell suspension cultures)	**3** (ca. 66.7%)
A. coerulea IFO4011	**2** (ca. 12.0%)
A. niger JCM 5546	ND
M. genevensis JCM 10585	ND
C. elegans CGMCC 3.3402	ND
C. echinulata CGMCC 3.400	ND
Curvularia sp. CGMCC 3.1471	ND
F. solani CGMCC 3.1791	ND

ND: not detected.

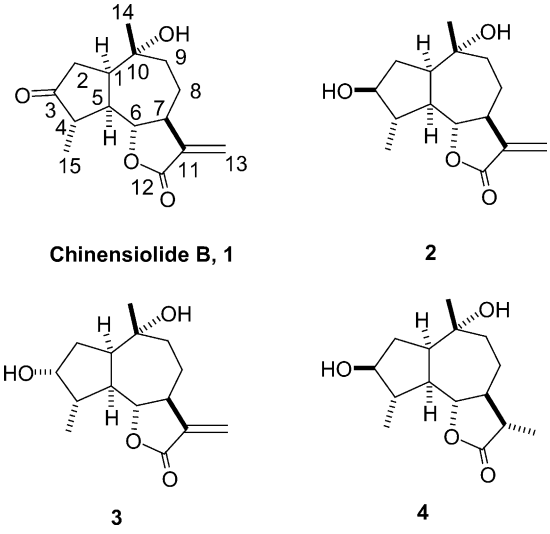

Fig. 1. The structures of **1** and its biotransformed products **2**, **3** and **4**.

The positive HRFAB mass spectrum of **2** showed a quasi molecular ion peak $[M+H]^+$ at *m/z* 267.1597 (calcd for $C_{15}H_{23}O_4$, 267.1596), suggesting that one C=O or C=C double bond was reduced. Its ^{13}C NMR spectrum was very similar to that of **1** except that C-3 keto carbonyl carbon signal at δ 219.3 (*s*) had disappeared, while one oxymethine carbon signal at δ 78.26 (*d*) appeared, which suggested the existence of one hydroxyl group at C-3 in **2** rather than one ketone group. Additionally, the fact that one new oxymethine proton signal at δ 3.74 (ddd, J = 7.1, 7.4, 15.1 Hz) was observed in the ^1H NMR spectrum, which was correlated with C-2, C-4, C-5 and C-15 in HMBC spectrum, strongly supported the above deduction. The OH stereochemistry was determined as β-configuration by the NOE difference spectral experiment, in which the integration values of H-2α, H-5 and H-15 were enhanced when H-3 was irradiated. Accordingly, the structure of **2** was identified to be 3β,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide, a 3β reduced derivative of **1**.

The positive HRFAB mass spectrum of **3** exhibited a quasi molecular ion peak $[M+H]^+$ at *m/z* 267.1595 (calcd for $C_{15}H_{23}O_4$, 267.1596), indicating that one C=O or C=C was reduced. Likewise, C-3 keto carbonyl carbon signal at δ 219.3 (*s*) had disappeared, while one oxymethine carbon signal at δ 74.76 (*d*) was observed, suggesting that the reduction occurred at C-3 position in different configuration compared with that of **2**. This deduction was confirmed by NOE difference spectral experiment, in which the integration values of H-2β, H-4, H-6 and H-14 were enhanced when H-3 was irradiated. Therefore, the structure of **3** was determined as 3α,10α-dihydroxy-1α,5α,15α-H-guaia-11(13)-en-6α,12-olide, a 3α reduced derivative of **1**. Thus, the cell cultures of *C. roseus* can transform ketone to alcohol(s) at C-3 regio-selectively, but not stereo-selectively, the reason might lie in different reductases in the cells responsible for the different reactions or non-critical stereo-selectivity of the same reductase.

*3.2.1. The time course of biotransformation of **1** to **2**, **3** and **4***

The time course of **1** to the metabolites **2**, **3** and **4** was investigated. The results (Fig. 2) displayed that: (1) after 5 days of incubation, the residual amount of the substrate, **1**, decreased slowly, however, the final amount in the broth was about 8.0 mg/l; (2) the yield of the major metabolite, **2**, reached the maximal amount, about 16.8 mg/l, after which it decreased; (3) the yields of the minor metabolites, **3** and **4**, increased very slowly, their yields were just 3.5 and 1.7 mg/l, respectively, after 7 days of incubation. The results of the decrease the yield of **2** and increase of the yield of **4** after the day 5 suggested that **4** might be biosynthesized from **2** *via* the reduction of C-11(13) double bond.

*3.3. Biotransformation of **1** by cell cultures of A. coerulea and P. grandiflorum*

1 was bioconverted by cell cultures of *A. coerulea* and *P. grandiflorum* to yield **2** (ca. 12.0%) and **3** (ca. 66.7%),

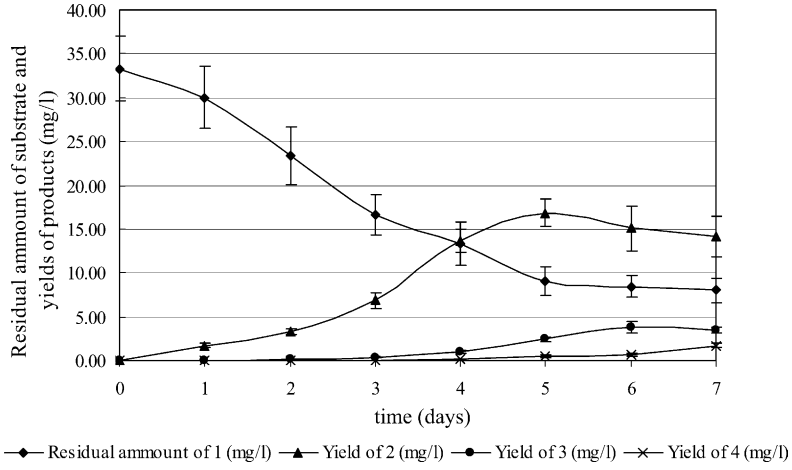

Fig. 2. Time course of the biotransformation of **1** to **2**, **3** and **4** by cell suspension cultures of *C. roseus*.

Table 2
The cytotoxicity of **1** and its metabolites **2**, **3** and **4** in vitro

Compound	IC_{50} in μM ($n = 3$)[a]		
	HepG2	WI-38	VA-13
1	26.5 ± 5.2	0.34 ± 0.05	26.8 ± 3.2
2	23.9 ± 4.8	1.69 ± 0.12	23.4 ± 5.8
3	22.7 ± 5.4	0.33 ± 0.04	3.30 ± 0.15
4	24.2 ± 3.6	1.94 ± 0.13	21.0 ± 4.8
Paclitaxel (control)	8.09 ± 1.2	0.005 ± 0.001	0.040 ± 0.008

[a] The IC_{50} values were the means of triplicates ± standard deviations.

respectively. From the results, **1** could highly regio- and stereo-selectively be reduced at C-3β by fungus *A. coerulea*, and at C-3α by the cell cultures of *P. grandiflorum*.

3.4. Cytotoxicities of the *1* and its products

1 and its bioconverted products **2**, **3** and **4** were tested for their in vitro cytotoxicity against one human primary liver cancer (HepG2) cell line and two human lung fibroblast (WI-38, VA-13) cell lines. The results of these bioassays are given in Table 2. For HepG2 and VA-13 cell lines, the products showed increased toxicities in comparison with **1**. For WI-38 cell line, **2** and **4** showed decreased toxicities, **3** showed almost the same toxicities as **1**. Furthermore, among these compounds, **3** exhibited the most toxic towards the three cell lines tested with the IC_{50} values of 22.7, 0.33 and 3.30 μM, respectively, suggesting that 3α-OH group could facilitate the cytotoxicity of this type of compounds comparing with 3-oxo and 3β-OH groups.

4. Conclusion

In conclusion, chinensiolide B can be specifically reduced [C-3 ketone to alcohol, and/or 11(13) methylene to methyl] by the cell suspension cultures of *C. roseus*, *P. grandiflorum* and a fungus *A. coerulea* IFO 4011. Bioassays showed that chinensiolide B and its three metabolites exhibited potent in vitro cytotoxicity against 3 cell lines. The results suggested that biotransformation is a useful approach to diversifying bioactive natural products and searching for more bioactive natural compounds and to provide various derivatives for biological evaluation, and for the structure-activity relationship (SAR) studies. In addition, with respect to the large-scale production of the most bioactive products, the best choose will be using cell suspension cultures of *P. grandiflorum* as the biocatalyst.

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