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Transformation of Isosteviol Lactam by Fungi and the Suppressive Effects of Its Transformed Products on LPS-Induced iNOS Expression in Macrophages

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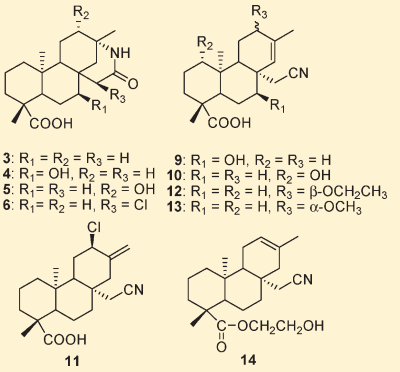
Supporting Information

ABSTRACT: From the screening of 21 microbial strains, *Absidia pseudocylindrospora* ATCC 24169 and *Aspergillus niger* BCRC 32720 were found to reproducibly transform isosteviol lactam (4α-carboxy-13α-amino-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactam) (**3**) into various compounds. Preparative-scale transformation of **3** with *Abs. pseudocylindrospora* yielded two new hydroxylated compounds (**4** and **5**), with conservation of the lactam ring. Preparative-scale transformation of **3** with *Asp. niger* afforded seven new compounds, **6** and **9**−**14**, together with the known compounds **7** and **8**. A single-crystal X-ray diffraction experiment confirmed the structure of **14**. The suppressive effects of compounds **1**−**14** on the lipopolysaccharide-induced expression of the inducible nitric oxide synthase gene in RAW 264.7 macrophages were examined by a reverse-transcription real-time PCR analysis. With the exception of **7**, all other compounds significantly reduced levels of iNOS mRNA relative to control cells, which were induced by LPS alone. Compounds **2**, **3**, and **5** were similar in activity to dexamethasone, while **9** was more potent.



Nitric oxide (NO), an important small signaling molecule involved in the regulation of diverse physiological and pathophysiological processes, is produced by NO synthase (NOS).[1] NOS exists in three isoforms, namely, endothelial (e)NOS, neuronal (n)NOS, and inducible (i)NOS. eNOS and nNOS are called constitutive (c)NOSs and constitutively produce low levels of NO, which help maintain cellular functions.[2] iNOS, which is expressed in various cell types including macrophages, is induced in response to proinflammatory cytokines and bacterial lipopolysaccharide (LPS) and plays a pivotal role in host defense and inflammatory processes.[3] Overexpression of iNOS can result in high levels of NO, leading to tissue damage and immunosuppression, and it was implicated in a number of inflammatory disorders.[2,4,5] Thus, effective suppression of NO production, via inhibition of iNOS expression, has become an alternative strategy to develop new compounds for treating inflammatory diseases.[2,6] Clinically, glucocorticoids (GCs) are used to treat a wide variety of autoimmune and chronic inflammatory conditions by interfering with many inflammatory mediators and influencing the expression of several genes.[7] One putative target for glucocorticoids' action is iNOS, which is produced by host immune cells.[8] Thus, GCs, such as dexamethasone, were reported to reduce iNOS expression in many cell types, suggesting that inhibition of iNOS induction may underlie the pharmacological effects of anti-inflammatory glucocorticoids.[3,6,8−10] Prolonged treatment of chronic conditions with GCs is hampered by insensitivity/resistance or serious adverse effects, thus limiting the use of these valuable drugs.[7] Since iNOS is responsible for the overproduction of NO in inflammation and a wide range of undesirable effects accompanying GC therapy, the inhibition of NO production by blocking iNOS expression may provide a useful strategy for alleviating or treating inflammatory disorders.[2,4,11]

A variety of enzymes that exist in several microorganisms are effective in biotransforming a wide range of natural and synthetic compounds by maintaining their exquisite catalytic properties with respect to regio- and stereoselectivity.[12] Over the past few decades, the utilization of microorganisms to accomplish difficult stereospecific modifications of organic compounds was reported, and numerous biocatalysts and substrates were revealed.[13] Thus, microbial transformation offers a useful alterative to chemical methods, enabling the specific access to remote positions on a molecule under mild reaction conditions. Natural products always play a central role in discovering molecular targets for developing drugs.[14] They are also ideal training compounds for

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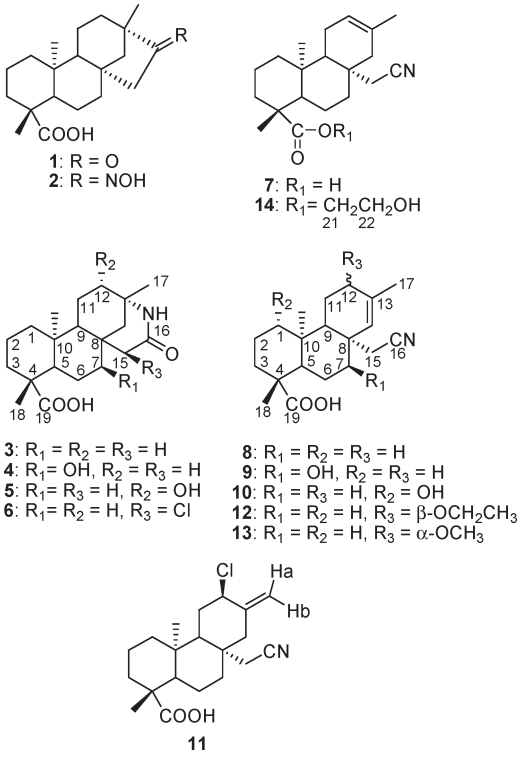
biocatalysis, especially terpenoids.[15,16] Isosteviol (**1**), a tetracyclic diterpenoid with an *ent*-beyerane skeleton that possesses several biological activities,[17] can readily be obtained from the acid hydrolysis of stevioside.[18] Small modifications in the structure of a compound can modify its biological activities or lead to new activities.[19,20] Incorporation of biocatalytic steps using microorganisms and/or enzymes has increasingly been exploited in both industrial and academic synthesis laboratories.[21] Thus, the D-ring of **1** was modified to lactone and oxime moieties and subjected to microbial transformation to yield diverse transformed compounds for biological evaluation.[22−24] In order to obtain more structurally interesting and biologically active compounds, the D-ring of **1** was further modified to the lactam ring instead of the lactone ring, based on the concept of isosterism. By reacting isosteviol oxime (**2**) with thionyl chloride in dioxane, isosteviol lactam (4α-carboxy-13α-amino-13,16-*seco*-*ent*-19-norbeyeran-16-oic acid 13,16-lactam) (**3**)[25] was obtained. In continuing our studies on bioconversion to generate various derivatives with a search for potential lead compounds, the biocatalytic modification of **3** was carried out. Since it was reported that some lactam-based compounds possess anti-inflammatory properties via inhibition of LPS-induced iNOS expression[26] and tetracyclic diterpenoids possess steroid-like skeletons,[27] the suppressive effects of compounds **1**−**14** on LPS-induced iNOS gene expression in RAW 264.7 macrophages were also examined by a reverse-transcription real-time polymerase chain reaction (RT-PCR) analysis, with dexamethasone used as a positive control. Herein, we describe the production, isolation, and structural elucidation as well as the biological activities of these compounds.



RESULTS AND DISCUSSION

Isosteviol lactam (**3**) was obtained by reacting isosteviol oxime (**2**) with thionyl chloride in dioxane, and their identities were verified by comparison of experimental and literature spectroscopic data.[25] Small-scale screening experiments found that *Absidia pseudocylindrospora* ATCC 24169 and *Aspergillus niger* BCRC 32720 were capable of reproducibly converting **3** into diverse products. Thus, preparative-scale biotransformation of **3** was performed to produce sufficient quantities of compounds for structural characterization and biological evaluation. Incubation of **3** with *Abs. pseudocylindrospora* gave two new compounds, **4** and **5**. However, incubation of **3** with *Asp. niger* led to the isolation of two known compounds, **7** and **8**,[24] along with seven unreported compounds, **6** and **9**−**14**. Compounds **4** and **5**, respectively, showed quasi-molecular ions $[M + H]^+$ at *m*/*z* 350.2339 and 350.2341 in the HRESIMS analysis, suggesting the molecular formula of $C_{20}H_{31}NO_4$. The ^{13}C NMR and DEPT spectra of **4** and **5** revealed 20 resonances, due to three CH_3, eight CH_2, three CH, and six quaternary carbons. The DEPT and HSQC spectra of **4** and **5** showed the disappearance of one CH_2 resonance and the presence of a proton geminal to a carbinol moiety at δ_H 3.77 (δ_C 75.8) and δ_H 3.72 (δ_C 77.4), respectively, suggesting the introduction of an OH group in both molecules. In the HMBC spectrum of **4**, the resonance at δ_H 3.77 exhibited connectivities with the resonances at δ_C 48.3 (C-5), 50.8 (C-9), and 40.6 (C-15). In **5**, the resonance at δ_H 3.72 showed connectivities with the resonances at δ_C 29.3 (C-11), 56.1 (C-9), and 25.7 (CH_3-17). Thus, the additional OH group present in both **4** and **5**, respectively, was located at C-7 and C-12. In **4**, the 7β-OH orientation was established due to the multiplicity of H-7, which was displayed as a broad singlet.[28] The NOESY spectrum also showed cross-peaks of H-7 (δ 3.77) with H-6 (δ 2.39 and 2.59), H-14 (δ 1.43−1.52), and H-15 (δ 2.14). In **5**, the α-OH orientation at C-12 was suggested from the cross-peaks of the resonance at δ 3.72 (dd, J = 10.5, 4.5 Hz) with H-9β (δ 1.02−1.11), H-11 (δ 1.56−1.67 and 2.08), and H-14 (δ 1.31 and 1.44−1.54) in the NOESY spectrum. On the basis of the above evidence, **4** and **5** were characterized as 4α-carboxy-13α-amino-13,16-*seco*-*ent*-7α-hydroxy-19-norbeyeran-16-oic acid 13,16-lactam and 4α-carboxy-13α-amino-13,16-*seco*-*ent*-12β-hydroxy-19-norbeyeran-16-oic acid 13,16-lactam, respectively.

Compound **6** was obtained as a white powder. Its HRESIMS spectrum revealed the presence of one chlorine atom, due to the observed characteristic isotope patterns in a ratio of 3:1 (*m*/*z* 368.2012 $[M+H]^+$ and 370.1990 $[M+H+2]^+$, respectively). Thus, the molecular formula of **6** was established as $C_{20}H_{30}ClNO_3$. The ^{13}C NMR, DEPT, and HSQC spectra, compared to those of **3**, showed the disappearance of one CH_2 resonance and the presence of new CH resonances at δ_H 5.26 and δ_C 61.8, suggesting **6** to be a chlorinated product of **3**. The DEPT and HSQC spectra showed that the resonances of C-7 and C-14 had shifted upfield from δ 44.6 to 40.4 ($\Delta\delta$ −4.2 ppm) and 49.8 to 43.3 ($\Delta\delta$ −6.5 ppm), respectively, by comparison with **3**. In the HMBC spectrum, the resonance at δ_H 5.26 exhibited correlations with the resonances at δ_C 40.4 (C-7), 40.6 (C-8), 57.8 (C-9), 43.3 (C-14), and 171.3 (C-16). Thus, the chlorine atom was attached at C-15. The β-orientation of 15-Cl was suggested from the cross-peaks of δ 5.26 with CH_3-20 (δ 1.21), H-11 (δ 1.37−1.45), and H-6 (δ 2.33) in the NOESY spectrum. Accordingly, the structure of **6** was elucidated to be 4α-carboxy-13α-amino-13,16-*seco*-*ent*-15α-chloro-19-norbeyeran-16-oic acid 13,16-lactam.

Compounds **9** and **10** displayed their respective quasi-molecular ions $[M - H]^-$ at *m*/*z* 330.2066 and 330.2074 (calcd 330.2069) (HRESIMS), corresponding to the molecular formula of $C_{20}H_{29}NO_3$, consistent with their ^{13}C NMR and DEPT spectra. Their ^1H and ^{13}C NMR spectra showed close

similarities to those of **8**, implying that **9** and **10** possessed the same basic skeleton as **8**.[24] The DEPT spectra of **9** and **10** showed the disappearance of one CH_2 resonance and the presence of a new CH resonance at δ 70.4 and 80.7, respectively, suggesting that each metabolite contained one more oxygen atom than does **8**. Examination of the HSQC and HMBC spectra of **9** and **10** and a comparison with **8** showed that the new CH resonance at δ_H 4.46 showed connectivities with the resonances at δ_C 21.5 (C-15), 46.6 (C-9), and 48.2 (C-5) in **9**, suggesting the presence of an OH group at C-7. The C-7 hydrogen resonated as a broad singlet at δ 4.46, indicating that the proton was in the α-orientation.[28] The 7β-OH orientation was also suggested from the cross-peaks of H-7 with H-6 (δ 2.46−2.52 and 2.72) and H-15 (δ 2.84) in the NOESY experiment. For **10**, the CH resonance at δ_H 3.68 showed connectivities with the resonances at δ_C 11.5 (CH_3-20), 44.4 (C-10), and 56.3 (C-9), suggesting the presence of an OH group at C-1. The orientation of the C-1 OH group was assigned as α from the H-1 resonating as a doublet of doublets (J = 11.0, 5.0 Hz) due to coupling with the protons of the neighboring C-2 in the COSY spectrum.[28] The 1α-OH orientation was also established from the cross-peaks of H-1 with H-2 (δ 1.97−2.09), H-5β (δ 1.17), and H-9β (δ 1.51) in the NOESY experiment. On the basis of the above evidence, **9** and **10** were characterized as *ent*-8β-cyanomethyl-7α-hydroxy-13-methyl-13-podocarpen-19-oic acid and *ent*-8β-cyanomethyl-1β-hydroxy-13-methyl-13-podocarpen-19-oic acid, respectively.

Compound **11** was obtained as a white powder, and two quasi-molecular ions $[M - H]^-$ were observed at m/z 348.1743 ($C_{20}H_{27}{}^{35}ClNO_2$) and m/z 350.1733 ($C_{20}H_{27}{}^{37}ClNO_2$) in the ratio of 3:1 in the HRESIMS, which proved that **11** contains one chlorine atom. The DEPT and HSQC data showed the disappearance of one CH resonance (δ_H 5.35, δ_C 121.3) and one CH_3 resonance relative to **7**[24] and the presence of one new CH resonating at δ_H 5.03 (δ_C 64.6) and an exocyclic methylene resonating at δ_H 5.06 and 5.16 (each br s) (δ_C 115.0). In the DEPT spectrum, the resonances of C-11 and C-13 shifted downfield from δ 23.0 to 31.6 ($\Delta\delta$ +8.6 ppm) and 131.4 to 144.3 ($\Delta\delta$ +12.9 ppm), respectively; the resonances of C-9 and C-14, respectively, shifted upfield from δ 52.4 to 50.3 ($\Delta\delta$ −2.1 ppm) and 46.6 to 44.3 ($\Delta\delta$ −2.3 ppm) compared to **7**.[24] In the HMBC spectrum, one-proton resonances at δ_H 5.06 and 5.16 showed correlations with the resonances at δ_C 44.3 (C-14), 64.6, and 144.3 (C-13), thus suggesting an exocyclic double bond at C-13. Additionally, the resonance at δ_C 64.6 exhibited correlations with the resonances at δ_H 1.86 (H-9), 2.01−2.12 (H-11), 2.39−2.49 (H-14), 5.06 (H-17b), and 5.16 (H-17a), indicating chlorination at C-12. A β-orientation of the C-12 chlorine atom was suggested from the cross-peaks of the resonance at δ 5.03 with the resonances at δ 1.74 (H-11), 2.01−2.12 (H-11), and 5.16 (H-17a) in the NOESY experiment. Thus, **11** was characterized as *ent*-12α-chloro-8β-cyanomethyl-podocarp-13(17)-en-19-oic acid.

Compound **12** was obtained as a white powder. Its HRESIMS spectrum displayed a quasi-molecular ion at m/z 358.2397 $[M - H]^-$ (calcd for $C_{22}H_{32}NO_3$, 358.2382), corresponding to the molecular formula $C_{22}H_{33}NO_3$. Examination of the ^{13}C NMR and DEPT data for **12** revealed resonances closely related to those for **8**,[24] except for replacement of a methylene in **8** by an oxymethine and an *O*-ethyl moiety in **12**. This suggested that **12** was an ethoxy-substituted analogue of **8**. Examination of the HSQC and HMBC spectra of **12** showed that the new CH resonance at δ_H 3.60 had connectivities with the resonances at

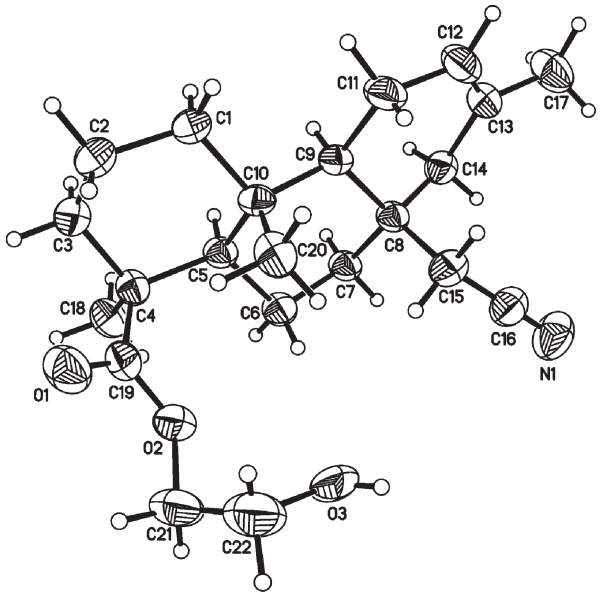


Figure 1. ORTEP drawing of the X-ray structure of **14**.

δ_C 21.2 (CH_3-17), 49.9 (C-9), 65.2 ($-OCH_2$), and 135.1 (C-13). Accordingly, the substituent was at C-12. The presence of an ethoxy group at C-12 was supported by the new CH_3 signal resonating at δ_H 1.24 as a triplet and showing an HMBC correlation with the resonance at δ_C 65.2 ($-OCH_2$). The β-orientation of 12-OCH_2CH_3 was deduced from the cross-peaks of H-12 (δ 3.60) with H-11 (δ 1.37 and 1.93), CH_3-17 (δ 1.82), and $-OCH_2$ (δ 3.40) in the NOESY experiment. The multiplicity of H-12 in the ^1H NMR spectrum, as a broad singlet, also indicated that it was α-oriented.[29] Thus, the structure of **12** was determined to be *ent*-8β-cyanomethyl-12α-ethoxy-13-methyl-13-podocarpen-19-oic acid.

Compound **13** displayed an $[M - H]^-$ ion at m/z 344.2238 (HRESIMS), corresponding to the molecular formula $C_{21}H_{31}NO_3$. Analysis of the ^{13}C NMR, DEPT, and HSQC spectra indicated that **13** exhibited features similar to **8**,[24] except for the resonances of C-11, C-12, and a methoxy group. Examination of the HSQC and HMBC spectra revealed that the carbon resonating at δ 81.0 (δ_H 3.82) correlated with the resonances at δ_H 1.20−1.27 (H-9), 2.16−2.20 (H-11), 5.65 (H-14), 1.82 (CH_3-17), and 3.39 ($-OCH_3$). It could be inferred from these observations that the substituent was at C-12. The proton resonating at δ 3.39 showed only one cross-peak with the resonance at δ_C 81.0, thus indicating the presence of a methoxy group at C-12. The α-orientation of 12-OCH_3 was established from the cross-peaks of H-12 (δ 3.82) with $-OCH_3$ (δ 3.39), H-9β (δ 1.20−1.27), CH_3-17 (δ 1.82), and H-11 (δ 2.16−2.20) in the NOESY spectrum. Thus, the structure of **13** was characterized as *ent*-8β-cyanomethyl-12β-methoxy-13-methyl-13-podocarpen-19-oic acid.

Compound **14** displayed an $[M + H]^+$ ion at m/z 360.2554 (calcd 360.2539) (HRESIMS), corresponding to the molecular formula of $C_{22}H_{33}NO_3$. The NMR data of **14** were similar to those of **7** except for the C-19 resonance, which resonated at δ_C 177.9 ($\Delta\delta$ −2.5 ppm vs **7**).[24] The DEPT and HSQC spectra showed two new CH_2 resonances at δ_H 4.07 (δ_C 60.5) and δ_H 4.42 (δ_C 67.2) compared to those of **7**. In the HMBC spectrum, the resonance at δ_C 177.9 exhibited one cross-peak with the

Table 1. ^{1}H NMR Chemical Shifts of Compounds 3−6 (pyridine-d_5, δ values in ppm)[a]

position	3	4	5	6
1	1.67, d (12.5)	1.78, d (12.5)	1.70, br d (13.0)	1.66−1.71, m[b]
	0.78−0.84, m[b]	1.08, m	0.85, td (13.0, 3.0)	0.86, td (13.0, 3.5)
2	2.15−2.22, m[b]	2.31−2.33, m[b]	2.21−2.26, m[b]	2.22, m
	1.44−1.50, m[b]	1.54, dd (12.5. 3.0)	1.44−1.54, m[b]	1.51, br d (14.0)
3	2.41, d (13.5)	2.49, d (13.0)	2.43, br d (12.5)	2.44, d (13.5)
	1.02−1.08, m[b]	1.19, m	1.02−1.11, m[b]	1.06−1.13, m[b]
5	1.02−1.08, m[b]	2.31−2.33, m[b]	1.02−1.11, m[b]	1.19−1.24, m[b]
6	2.15−2.22, m[b]	2.59, t (13.0)	2.21−2.26, m[b]	2.33, m
	1.93, d (12.0)	2.39, d (13.0)	1.98, m	2.04, dd (14.5, 2.5)
7	1.44−1.50, m[b]	α 3.77, br s	1.56−1.67, m[b]	2.54, d (13.5)
	1.16−1.19, m[b]		1.19, m	1.06−1.13, m[b]
9	0.78−0.84, m[b]	1.72, dd (12.5, 2.5)	1.02−1.11, m[b]	1.19−1.24, m[b]
11	1.44−1.50, m[b]	1.61−1.67, 2H, m[b]	2.08, m	1.59, m
	1.34−1.39, m[b]		1.56−1.67, m[b]	1.37−1.45, m[b]
12	1.61, d (9.5)	1.61−1.67, m[b]	β 3.72, dd (10.5, 4.5)	1.66−1.71, m[b]
	1.34−1.39, m[b]	1.43−1.52, m[b]		1.37−1.45, m[b]
14	1.34−1.39, m[b]	2.24, d (12.5)	1.44−1.54, m[b]	2.12, dd (13.0, 2.5)
	1.16−1.19, m[b]	1.43−1.52, m[b]	1.31, m	1.06−1.13, m[b]
15	3.19, dd (18.0, 2.5)	3.25, d (18.0)	3.25, d (18.0)	α 5.26, s
	2.03, d (18.0)	2.14, d (18.0)	2.14, d (18.0)	
17	1.20, s	1.28, s	1.48, s	1.29, s
18-CH_3	1.32, s	1.43, s	1.36, s	1.37, s
20-CH_3	1.12, s	1.27, s	1.14, s	1.21, s
N*H*	8.05, s	8.06, s	8.11, s	8.53, s

[a] Assignments based on DEPT, HSQC, and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses. [b] Overlapping signals.

resonance at δ_H 4.42 (2H). However, the resonance at δ_C 60.5 displayed only one cross-peak with the resonance at δ_H 4.42, thus suggesting the presence of an ester linkage of a 2-hydroxyethyl group at C-19. The structure of **14** was confirmed by an X-ray crystallographic analysis (Figure 1). Consequently, the structure of **14** was established to be *ent*-8β-cyanomethyl-13-methyl-12-podocarpene-19-hydroxyethyl ester.

Results of the current study demonstrate that *Abs. pseudocylindrospora* has the ability to only regio- and stereoselectively hydroxylate **3**. However, *Asp. niger* can perform diverse characteristic reactions including chlorination at the lactam ring and opening the lactam ring to afford two cyanomethyl products, **7** and **8**. The two cyanomethyl analogues were further modified by regio- and stereoselective hydroxylation, chlorination, *O*-methylation, *O*-ethylation, and 19-esterification. The mechanism for the production of both chlorinated compounds (**6** and **11**) is not clear. It may involve the action of chloroperoxidases, and the chlorine atom is probably derived from the addition of NaCl to the fermentation medium.[30] To evaluate the suppressive effects of chemical compounds on iNOS expression for potential anti-inflammatory activity, LPS-activated RAW 264.7 mouse macrophage cells can be used as an in vitro model.[31] In this study, the effects of all compounds on iNOS gene expression were examined by a real-time PCR analysis, with dexamethasone as a positive control. Data presented herein demonstrate that compounds **1**−**14** significantly reduced the levels of iNOS mRNA relative to control cells induced with LPS alone except for **7**. Among these, compounds **2**, **3**, and **5** were similar in activity to dexamethasone, while **9** was more potent (Table 4). The results of the biological activity assay suggested that these potentially active compounds may be ideal targets for development of anti-inflammatory compounds due to the decrease in iNOS gene expression. Detailed investigations of the underlying mechanisms of these potentially active compounds should be further addressed. The current study also demonstrates that the incorporation of biocatalytic steps using microorganisms may offer an alternative approach to generate diverse interesting derivatives of diterpenoids for pharmacological evaluation.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were obtained with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-1020 digital polarimeter. The NMR spectra were taken on a Varian Unity Inova-500 spectrometer and a Bruker Avance-500 spectrometer with pyridine-d_5 as the solvent. Chemical shifts are expressed in parts per million (ppm) with respect to the corresponding solvent as the internal standard, and coupling constants (*J*) are in hertz (Hz). Low- and high-resolution ESIMS measurements were recorded on a VG platform electrospray mass spectrometer. Column chromatography (CC) was performed with Kieselgel silica (70−230 and 230−400 mesh, Merck, Darmstadt, Germany). High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-2130 (Tokyo, Japan) apparatus equipped with a refractometer detector (Hitachi L-2490). Purification by means of HPLC was conducted using a silica gel column (250 × 10 mm, 5 μm, at a flow rate of 2 mL/min) (Thermo Scientific, Waltham, MA) for semipreparative and analytical HPLC. X-ray single-crystal diffraction was measured on a Siemens SMART CCD XRD. TLC was performed on F_{254} precoated silica gel (0.25 mm) (Merck), and spots were detected by 10% H_2SO_4 spray reagent followed by heating.

Table 2. ^{1}H NMR Chemical Shifts of Compounds 9−14 (pyridine-d_5, δ values in ppm)[a]

position	9	10	11	12	13	14
1	1.68, m	β 3.68, dd (11.0, 5.0)	1.59, d (12.5)	1.75, d (12.0)	1.71, br d (13.0)	1.55, br d (12.5)
	1.04, m		0.95, m	0.95, m	0.84, m	0.81, m
2	2.24−2.32, m[b]	2.63, dd (14.0, 4.0)	2.16−2.29, m[b]	2.24−2.34, m[b]	2.23−2.36, m[b]	2.07−2.17, m[b]
	1.53, m	1.97−2.09, m[b]	1.51, m	1.52, d (13.5)	1.54, br d (14.0)	1.40, br d (14.0)
3	2.46−2.52, m[b]	2.40−2.48, m[b]	2.39−2.49, m[b]	2.43, d (13.0)	2.46, br d (13.0)	2.32, d (13.5)
	1.15, td (13.5, 4.0)	1.24−1.33, m[b]	1.07, m	1.04, m	1.08−1.14, m[b]	0.99, td (13.5, 4.0)
5	2.24−2.32, m[b]	1.17, dd (12.5, 2.5)	1.15, br d (11.0)	1.09, d (12.0)	1.08−1.14, m[b]	1.07−1.14, m[b]
6	2.72, m	2.40−2.48, m[b]	2.16−2.29, m[b]	2.24−2.34, m[b]	2.23−2.36, m[b]	2.07−2.17, m[b]
	2.46−2.52, m[b]	1.97−2.09, m[b]	2.01−2.12, m[b]	2.01, d (14.0)	2.03, br d (14.0)	1.90−1.93, m[b]
7	α 4.46, br s	2.17, m	2.01−2.12, m[b]	2.11, d (13.5)	2.16−2.20, m[b]	2.07−2.17, m[b]
		1.24−1.33, m[b]	1.24, m	1.21, m	1.20−1.27, m[b]	1.07−1.14, m[b]
9	2.00−2.08, m[b]	1.51, d (11.5)	1.86, m	1.61, d (13.0)	1.20−1.27, m[b]	1.21, m
11	2.00−2.08, m[b]	3.50, dd (14.0, 7.0)	2.01−2.12, m[b]	1.93, d (14.0)	2.16−2.20, m[b]	1.90−1.93, m[b]
	1.82, m	1.59, m	1.74, m	1.37, m	1.43, m	1.66, m
12	3.17, d (16.5)	1.97−2.09, 2H, m[b]	α 5.03, s	α 3.60, br s	β 3.82, t (8.0)	5.33, s
	2.16, d (16.5)					
12-OCH_3					3.39, s	
12-OCH_2CH$_3$				3.67, quint (7.0)		
				3.40, quint (7.0)		
12-OCH$_2$CH_3				1.24, t (7.0)		
14	5.36, s	5.54, s	2.39−2.49, 2H, m[b]	5.67, s	5.65, s	2.07−2.17, m[b]
						1.78, br d (16.0)
15	2.84, d (17.0)	2.99, d (16.5)	2.84, d (16.5)	2.93, d (16.0)	3.00, br d (16.0)	2.76, d (16.5)
	2.66, d (17.0)	2.68, d (16.5)	2.39−2.49, m[b]	2.57, d (16.0)	2.64, br d (16.0)	2.41, d (16.5)
17	1.71, s	1.62, s	5.16, s	1.82, s	1.82, s	1.62, s
			5.06, s			
18-CH_3	1.42, s	1.35, s	1.36, s	1.31, s	1.35, s	1.24, s
20-CH_3	1.07, s	1.29, s	0.91, s	1.00, s	0.99, s	0.83, s
21						4.42, 2H, dt (15.0, 5.0)
22						4.07, 2H, d (5.0)

[a] Assignments based on DEPT, HSQC, and HMBC. Signal multiplicity and coupling constants (Hz) are in parentheses. [b] Overlapping signals.

Substrate. Isosteviol oxime (**2**) was prepared as previously reported.[32] The preparation of isosteviol lactam (**3**) was obtained via a modified literature procedure.[33] Briefly, a solution of oxime **2** (1.0 g, 3.0 mmol) in dioxane (40 mL) was cooled to 0 °C, and $SOCl_2$ (6 mL, 82.0 mmol) in dioxane (10 mL) was added. The mixture was heated at 70 °C for 10 h. The reaction solution was treated with cold 1 N aqueous NaOH (200 mL). The white precipitate was filtered to give a solid, which was purified by CC over silica gel eluted with CH_2Cl_2−CH_3OH (8:1) to give the crude product **3**. Recrystallization from EtOAc yielded pure **3** (0.7 g, 70%) as cubic crystals [mp >300 °C; $[\alpha]^{25}_D$ −47.5 (*c* 0.6, CH_3OH); HRESIMS *m*/*z* 334.2413 $[M + H]^+$ (calcd for $C_{20}H_{32}NO_3$, 334.2382)]. The identity of **3** was verified by comparison of experimental and literature spectroscopic data.[25]

Microorganisms and Culture Conditions. Twenty-one microorganisms, obtained from the Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa (Iowa City, IA), and Bioresources Collection and Research Center (Hsinchu, Taiwan), were used for the preliminary screening of **3**. Organisms belonging to the following genera (with the number of species screened) were screened for their abilities to metabolize **3**: *Aspergillus* (four), *Absidia* (one), *Bacillus* (one), *Beauveria* (two), *Cunninghamella* (four), *Mortierella* (one), *Mucor* (two), *Nocardia* (two), *Pseudomonas* (two), and *Streptomyces* (two). Stock cultures were maintained at 4 °C on Sabouraud-maltose agar slants, or those recommended by the ATCC and BCRC, and subcultured periodically. The standard two-stage fermentation protocol for screening was identical to that previously described.[34] Culture and substrate controls were both run.

Bioconversion of Isosteviol Lactam (3) by *Abs. pseudocylindrospora* ATCC 24169 and *Asp. niger* BCRC 32720. During initial screening experiments, *Abs. pseudocylindrospora* and *Asp. niger* were found to reproducibly metabolize **3** into various compounds. Thus, they were chosen for scale-up fermentation. Using 24-h-old stage II cultures, a solution of **3** (1.00 g dissolved in 10 mL of DMF) was evenly distributed among one hundred 125 mL stainless-steel-capped flasks containing stage II cultures. Substrate-containing cultures were incubated for 144 h. Extraction as previously described[34] produced 3.8 g of brown oil and 3.2 g of black oil after respective bioconversions with *Abs. pseudocylindrospora* and *Asp. niger*. The crude residue from *Abs. pseudocylindrospora* (3.8 g) was subjected to CC over silica gel (70−230 mesh, 4 × 60 cm). By eluting with mixtures of CH_2Cl_2−CH_3OH (500 mL each of 20:1, 15:1, 10:1, and 5:1), four fractions (1−4) were obtained on the basis of similar TLC profiles. With further chromatography of fraction 2 (98 mg) over silica gel (230−400 mesh, 3 × 55 cm) eluted with *n*-hexane−EtOAc (150 mL each of 3:1, 2:1, and 1:1), **4** (20 mg) was obtained. After recrystallization of fraction 3 (30 mg) from EtOAc−CH_3OH (2:1), **5** (17 mg) was obtained. Fraction 4 (800 mg) was subjected to CC over a silica gel column (230−400 mesh, 2 × 55 cm) eluted with *n*-hexane−EtOAc (2:1) to yield three fractions (4-1−4-3). Fraction 4-2 (690 mg) was recrystallized from EtOAc−CH_3OH (1:1), and 650 mg of **3** was recovered as white crystals.

Table 3. ^{13}C NMR Chemical Shifts of Compounds 3−6 and 9−14 (pyridine-d_5, δ values in ppm)[a]

no.	3	4	5	6	9	10	11	12	13	14
1	40.2	40.8	40.7	40.6	40.7	80.7	40.4	40.1	40.2	40.3
2	19.6	20.3	20.1	20.2	20.3	31.4	20.1	20.1	20.1	19.9
3	38.6	39.2	39.1	38.9	39.1	37.3	38.9	39.0	39.1	38.5
4	43.9	44.1	44.4	44.4	44.0	44.1	44.4	44.3	44.4	44.4
5	57.5	48.3	57.7	58.3	48.2	56.2	57.3	57.4	57.2	57.6
6	20.4	29.2	21.0	20.3	29.4	21.2	20.9	20.9	20.9	20.6
7	44.6	75.8	44.4	40.4	70.4	39.2	39.3	38.4	38.5	39.8
8	35.6	40.6	36.1	40.6	40.3	38.2	39.2	38.1	38.2	35.8
9	56.9	50.8	56.1	57.8	46.6	56.3	50.3	49.9	53.8	52.2
10	38.3	38.8	38.8	39.1	38.3	44.4	37.9	37.8	38.1	38.0
11	19.2	19.5	29.3	19.0	23.2	21.1	31.6	23.5	24.4	22.9
12	40.8	41.1	77.4	40.9	39.8	32.7	64.6	76.7	81.0	121.2
12-OCH_3									56.2	
12-OCH_2CH_3								65.2		
12-OCH_2CH_3								16.4		
13	51.4	52.3	56.0	52.5	132.2	134.9	144.3	135.1	135.7	131.3
14	49.8	45.2	49.1	43.3	120.3	131.8	44.3	134.8	135.2	46.4
15	40.7	40.6	41.3	61.8	21.5	25.4	21.0	23.6	24.9	20.6
16	173.1	173.3	173.8	171.3	120.2	120.4	119.5	120.1	120.0	120.1
17	28.9	29.7	25.7	28.8	24.3	23.7	115.0	21.2	20.2	23.9
18	29.3	29.7	29.8	29.8	29.7	29.6	29.6	29.5	29.6	29.2
19	179.9	180.8	180.4	180.3	181.0	180.2	180.3	180.3	180.3	177.9
20	14.2	14.6	14.7	15.2	13.9	11.5	14.9	14.8	14.8	13.9
21										67.2
22										60.5

[a] Assignments based on DEPT, HSQC, and HMBC.

The crude residue from *Asp. niger* (3.2 g) was subjected to silica gel CC (70−230 mesh, 5 × 90 cm) and eluted with mixtures of CH_2Cl_2−CH_3OH (600 mL each of 20:1, 15:1, 10:1, and 5:1). Six fractions (1−6) were obtained on the basis of similar TLC profiles. Further chromatography of fraction 4 (960 mg) over silica gel (230−400 mesh, 3 × 50 cm) eluted with *n*-hexane−EtOAc (1:1) yielded three fractions (4-1−4-3), and 690 mg of **3** was recovered from fraction 4-1 (810 mg). The mother liquid of fraction 4-1 was further purified by HPLC on a semipreparative column (*n*-hexane−EtOAc, 2:1) to give **7** (12 mg), **8** (17 mg), **9** (12 mg), and **10** (16 mg). Fraction 4-2 (35 mg) was purified by semipreparative HPLC (*n*-hexane−EtOAc, 1:1) to give **9** (16 mg) and **10** (5.5 mg). Fraction 5 (102 mg) was chromatographed over silica gel (230−400 mesh, 3 × 55 cm) eluted with *n*-hexane−EtOAc (1:1) to give two fractions (5-1 and 5-2). Fraction 5-1 (56 mg) was rechromatographed over silica gel (*n*-hexane−EtOAc with increasing polarity) to give a white solid. The white solid was further purified by HPLC on a semipreparative column to give **6** (10 mg), **11** (10 mg), and **12** (13 mg). Fraction 5-2 (26 mg) was subjected to repeated semipreparative HPLC separation (CH_2Cl_2−2-propanol, 14:1) to give **13** (5 mg) and **14** (3 mg).

Compound ***4***: white powder; $[\alpha]^{25}_D$ +22.7 (*c* 0.55, C_5H_5N); for ^{1}H and ^{13}C NMR data, see Tables 1 and 3; HRESIMS *m*/*z* 350.2339 $[M + H]^+$ (calcd for $C_{20}H_{32}NO_4$, 350.2331).

Compound ***5***: white powder; $[\alpha]^{25}_D$ +29.1 (*c* 1.3, C_5H_5N); for ^{1}H and ^{13}C NMR data, see Tables 1 and 3; HRESIMS *m*/*z* 350.2341 $[M + H]^+$ (calcd for $C_{20}H_{32}NO_4$, 350.2331).

Compound ***6***: white powder; $[\alpha]^{25}_D$ +21.2 (*c* 0.85, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 1 and 3; HRESIMS *m*/*z* 368.2012 $[M + H]^+$ (calcd for $C_{20}H_{31}{}^{35}ClNO_3$, 368.1992).

Table 4. Real-Time Polymerase Chain Reaction Analysis of the Inhibitory Effects of Compounds on LPS-Induced iNOS mRNA Expression in RAW 264.7 Cells[a]

compd	mRNA level (fold)	compd	mRNA level (fold)
control	1.00 ± 0.01	**7**	0.63 ± 0.34
Dex	0.16 ± 0.02	**8**	0.48 ± 0.22
1	0.29 ± 0.12	**9**	0.12 ± 0.01
2	0.15 ± 0.05	**10**	0.43 ± 0.21
3	0.15 ± 0.06	**11**	0.30 ± 0.08
4	0.61 ± 0.11	**12**	0.21 ± 0.14
5	0.13 ± 0.03	**13**	0.20 ± 0.11
6	0.85 ± 0.09	**14**	0.50 ± 0.19

[a] The concentration of each test compound was 10 μM. The data are expressed as the relative fold expression compared to the LPS-treated (control) group. Dexamethasone (Dex) is the reference compound. Each value is presented as the mean ± SEM (n = 3). With the exception of **7**, all other compounds showed significance. Significantly different equals $p < 0.05$, using Student's *t*-test for paired samples.

Compound ***9***: white powder; $[\alpha]^{25}_D$ −36.7 (*c* 1.1, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 330.2066 $[M - H]^-$ (calcd for $C_{20}H_{28}NO_3$, 330.2069).

Compound ***10***: white powder; $[\alpha]^{25}_D$ +45.8 (*c* 0.65, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 330.2074 $[M - H]^-$ (calcd for $C_{20}H_{28}NO_3$, 330.2069).

Compound ***11***: white powder; $[\alpha]^{25}_D$ −32.4 (*c* 1.1, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 348.1743 $[M - H]^-$ (calcd for $C_{20}H_{27}{}^{35}ClNO_2$, 348.1730).

Compound ***12***: white powder; $[\alpha]^{25}_D$ +22.8 (*c* 1.2, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 358.2397 $[M - H]^-$ (calcd for $C_{22}H_{32}NO_3$, 358.2382).

Compound ***13***: white powder; $[\alpha]^{25}_D$ +23.3 (*c* 0.55, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 344.2238 $[M - H]^-$ (calcd for $C_{21}H_{30}NO_3$, 344.2226).

Compound ***14***: white powder; $[\alpha]^{25}_D$ −30.5 (*c* 0.55, CH_3OH); for ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m*/*z* 360.2554 $[M + H]^+$ (calcd for $C_{22}H_{34}NO_3$, 360.2539).

X-ray Crystallographic Data for 14. Crystals of **14** were grown by slow evaporation from a 4:1 (v/v) mixture of CH_3OH−H_2O. A well-shaped crystal of **14** with dimensions of 0.25 × 0.20 × 0.10 mm^3 was selected for X-ray analysis. Crystal data: $C_{22}H_{33}NO_3$, *M* 359.49, orthorhombic, $P2_12_12_1$, *a* 7.7118(12) Å, *b* 10.430(3) Å, *c* 25.351(7) Å, *V* 2039.2(8) Å^3; *Z* 4, D_{calcd} 1.171 g cm^{-3}, *F*(000) 784, λ(Mo Kα) 1.54178 Å, *T* 295(2) K, 15 060 reflection collected. Final GooF 0.976, final *R* indices R_1 0.0611, wR_2 0.1239, 245 parameters, μ 0.605 mm^{-1}, *R* indices based on 3716 reflections with $I > 2\sigma(I)$ absorption corrections applied. Complete crystallographic data of **14** were deposited in the Cambridge Crystallographic Data Centre (CCDC 801020). These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; e-mail: data_request@ccdc.cam.ac.uk).

RNA Isolation and RT-PCR Analysis. The impacts of these compounds on inflammatory gene expressions were evaluated in LPS-treated murine RAW 264.7 macrophages. After 4 h of pretreatment of cells with LPS at 100 ng/mL, the indicated compounds were applied to cells at a 10 μM concentration and cultured for 24 h in the presence of LPS. Total RNA was extracted from treated cells with the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and the RNA concentrations were estimated from the absorbance at 260 nm. First-strand complementary (c)DNA was synthesized from total RNA using the SuperScript II cDNA synthesis method (Invitrogen) with the oligo(dT) primer. A real-time PCR was performed using SYBR Green PCR

master mix in an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). The messenger (m)RNA level of iNOS was determined by its relative level to the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative quantification values were obtained from the threshold cycle number, at which the increase in the signal associated with an exponential growth of PCR products began to be detected using sequence detection system software version 3.0. Primers for iNOS (forward: 5′-GAAACGCTTCACTTCCAATGC-3′ and reverse: 5′-GGCAGCCTGTGAGACCTTTG-3′) and GAPDH (forward: 5′-CATG-GCCTTCCGTGTTCCTA-3′ and reverse: 5′-GCGGCACGTCAGATC-CA-3′) were used. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. At the end of the cycles, amplification specificity was confirmed by a dissociation curve analysis. The expression level is reported relative to the expression level of the LPS-treated group.

Statistical Analysis. Data are from at least three individual experiments. The average of the amount of expression relative to the expression level of the LPS-treated (control) group was analyzed by two-tailed Student's *t*-test for paired samples. A p value < 0.05 was considered to be statistically significant.

■ ASSOCIATED CONTENT

Supporting Information. NMR spectra of compounds **3**−**6** and **9**−**14** and X-ray crystallographic data (CIF file) of **14** are available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Chi, D. S.; Qui, M.; Krishnaswamy, G.; Li, C. F.; Stone, W. *Nitric Oxide* **2003**, *8*, 127–132.
(2) Hobbs, A. J.; Higgs, A.; Moncada, S. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 191–220.
(3) Korhonen, R.; Lahti, A.; Hämäläinen, M.; Kankaanranta, H.; Moilanen, E. *Mol. Pharmacol.* **2002**, *62*, 698–704.
(4) Giovine, M.; Gasparini, A.; Scarfi, S.; Damonte, G.; Sturla, L.; Millo, E.; Tonetti, M.; Benatti, U. *FEBS Lett.* **1998**, *426*, 33–36.
(5) Guslandi, M. *Eur. J. Clin. Invest.* **1998**, *28*, 904–907.
(6) Vallance, P.; Leiper, J. *Nat. Rev. Drug Discovery* **2002**, *1*, 939–950.
(7) Schäcke, H.; Döcke, W. D.; Asadullah, K. *Pharmacol. Ther.* **2002**, *96*, 23–43.
(8) Shinoda, J.; McLaughlin, K. E.; Bell, H. S.; Swaroop, G. R.; Yamaguchi, S. I.; Holmes, M. C.; Whittle, I. R. *Glia* **2003**, *42*, 68–76.
(9) Hämäläinen, M.; Lilja, R.; Kankaanranta, H.; Moilanen, E. *Pulm. Pharmacol. Ther.* **2008**, *21*, 331–339.
(10) Di Rosa, M.; Radomski, M.; Carnuccio, R.; Moncada, S. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1246–1252.
(11) Zhou, D.; Rothfuss, J. M.; Chen, D. L.; Ponde, D. E.; Welch, M. J.; Mach, R. H. *J. Med. Chem.* **2009**, *52*, 2443–2453.
(12) Patel, R. N. *Coord. Chem. Rev.* **2008**, *252*, 659–701.
(13) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258–268.
(14) Ganesan, A. *Curr. Opin. Chem. Biol.* **2008**, *12*, 306–317.
(15) Riva, S. *Curr. Opin. Chem. Biol.* **2001**, *5*, 106–111.
(16) Venisetty, R. K.; Ciddi, V. *Curr. Pharm. Biotechnol.* **2003**, *4*, 153–167.
(17) Chang, S. F.; Yang, L. M.; Lo, C. H.; Liaw, J. H.; Wang, L. H.; Lin, S. J. *J. Nat. Prod.* **2008**, *71*, 87–92.
(18) Avent, A. G.; Hanson, J. R.; de Oliveira, B. H. *Phytochemistry* **1990**, *29*, 2712–2715.
(19) Leuenberger, H. G. W. *Pure Appl. Chem.* **1990**, *62*, 753–768.
(20) Braguini, W. L.; Gomes, M. A. B.; de Oliveira, B. H.; Carnieri, E. G. S.; Rocha, M. E. M.; de Oliveira, M. B. M. *Toxicol. Lett.* **2003**, *143*, 83–92.
(21) Loughlin, W. A. *Bioresour. Technol.* **2000**, *74*, 49–62.
(22) Chou, B. H.; Yang, L. M.; Chang, S. F.; Hsu, F. L.; Lo, C. H.; Liaw, J. H.; Liu, P. C.; Lin, S. J. *J. Nat. Prod.* **2008**, *71*, 602–607.
(23) Chou, B. H.; Yang, L. M.; Chang, S. F.; Hsu, F. L.; Lo, C. H.; Lin, W. K.; Wang, L. H.; Liu, P. C.; Lin, S. J. *Phytochemistry* **2009**, *70*, 759–764.
(24) Chang, S. F.; Chou, B. H.; Yang, L. M.; Hsu, F. L.; Lin, W. K.; Ho, Y.; Lin, S. J. *Bioorg. Med. Chem.* **2009**, *17*, 6348–6353.
(25) Militsina, O. I.; Kovyljaeva, G. I.; Bakaleynik, G. A.; Strobykina, I. Y.; Kataev, V. E.; Alfonsov, V. A.; Musin, R. Z.; Beskrovny, D. V.; Litvinov, I. A. *Mendeleev Commun.* **2005**, *15*, 27–29.
(26) Kim, H. M.; Ryu, D. K.; Choi, Y.; Park, B. W.; Lee, K.; Han, S. B.; Lee, C. W.; Kang, M. R.; Kang, J. S.; Boovanahalli, S. K.; Park, S. K.; Han, J. W.; Chun, T. G.; Lee, H. Y.; Nam, K. Y.; Choi, E. H.; Han, G. *J. Med. Chem.* **2007**, *50*, 2737–2741.
(27) Hanson, J. R. *Nat. Prod. Rep.* **1992**, *9*, 139–151.
(28) De Oliveira, B. H.; dos Santos, M. C.; Leal, P. C. *Phytochemistry* **1999**, *51*, 737–741.
(29) De Oliveira, B. H.; Strapasson, R. A. *Phytochemistry* **1996**, *43*, 393–395.
(30) Clark, B. R.; Lacey, E.; Gill, J. H.; Capon, R. J. *J. Nat. Prod.* **2007**, *70*, 665–667.
(31) Harinantenaina, L.; Quang, D. N.; Takeshi, N.; Hashimoto, T.; Kohchi, C.; Soma, G. I.; Asakawa, Y. *J. Nat. Prod.* **2005**, *68*, 1779–1781.
(32) Al'fonsov, V. A.; Andreeva, O. V.; Bakaleinik, G. A.; Beskrovnyi, D. V.; Gubaidullin, A. T.; Kataev, V. E.; Kovylyaeva, G. I.; Konovalov, A. I.; Korochkina, M. G.; Litvinov, I. A.; Militsina, O. I.; Strobykina, I. Y. *Russ. J. Gen. Chem.* **2003**, *73*, 1255–1260.
(33) Tret'yakova, E. V.; Flekhter, O. B.; Galin, F. Z.; Baikova, I. P.; Tolstikov, G. A. *Chem. Nat. Compd.* **2003**, *39*, 16–18.
(34) Hsu, F. L.; Hou, C. C.; Yang, L. M.; Cheng, J. T.; Chi, T. C.; Liu, P. C.; Lin, S. J. *J. Nat. Prod.* **2002**, *65*, 273–277.