Hydroxylation of the Triterpenoid Nigranoic Acid by the Fungus *Gliocladium roseum* YMF1.00133

by **Jin-Yan Dong**[a]), **Ye-Gao Chen**[b]), **Hong-Chuan Song**[c]), **Yan-Hui Zhu**[a]), **Yong-Ping Zhou**[a]), **Lei Li**[a]), **Yan-Ping He**[a]), **Jie Cao**[a]), and **Ke-Qin Zhang***[a])

[a]) Key Laboratory for Conservation and Utilization of Bioresources, Yunnan University, Kunming, Yunnan 650091, People's Republic of China (phone: 86-871-5034878; fax: 86-871-5034838; e-mail: Kqzhang111@yahoo.com.cn)

[b]) Department of Chemistry, Yunnan Normal University, Kunming 650092, People's Republic of China

[c]) Provincial Key Lab of Rural Energy Engineering, Yunnan Normal University, Kunming 650092, People's Republic of China

The ability of the fungus *Gliocladium roseum* YMF1.00133 to transform the bioactive nigranoic acid (=(24*Z*)-9,19-cyclo-3,4-secolanosta-4(28),24-diene-3,26-dioic acid) was investigated. Three new products from the co-cultures of nigranoic acid and *G. roseum* YMF1.00133 were obtained by employing a combination of *Sephadex LH-20* and silica-gel column chromatography. The major metabolite was identified as 15β-hydroxynigranoic acid, and the minor metabolites as 6α,15β-dihydroxynigranoic acid and 7β,15β-dihydroxynigranoic acid by mass spectrometry and NMR spectroscopy. This is the first report of the biotransformation of the A-ring-secocycloartene triterpenoid, nigranoic acid.

Introduction. – Biotransformation is an attractive approach to generate structural diversity in a chemical library [1–3]. Fungi are often used as whole cell biocatalysts by organic chemists searching for new compounds as a consequence of hydrolysis, reduction, oxidation, C–C bond formation, addition and elimination, glycosidic transfer reaction, and halogenation and dehalogenation reactions of natural and unnatural substrates with complicated structures [4]. They can be used to synthesize chemical structures that are difficult to obtain by other means [5].

Nigranoic acid (=(24*Z*)-9,19-cyclo-3,4-secolanosta-4(28),24-diene-3,26-dioic acid; **1**) is an A-ring-secocycloartene triterpenoid produced by plants belonging to the genera *Schisandra* and has been reported to possess a variety of biological activities, including cytotoxic activity toward leukemia and Hela cells, and inhibition of expression of HIV reverse transcriptase and polymerase [6–9]. Our recent isolation of much nigranoic acid from *Schisandra propinqua* prompted us to obtain further nigranoic acid analogues by microbial biotransformation in order to assess the structure–activity relationships of this class of compounds. Thus, in this study, the utility of *Gliocladium roseum* in nigranoic acid biotransformation was investigated. The characterization of the new compounds was performed by various spectroscopic methods.

Results and Discussion. – Incubation of nigranoic acid (**1**) with the culture of *G. roseum* YMF1.00133 for 10 days at 28° led to the formation of compounds **2**–**4**

(*Fig.*), all three more polar than the substrate itself. All the microbial products were isolated and purified from an AcOEt extract by a combination of *Sephadex LH-20* and silica-gel column chromatography.

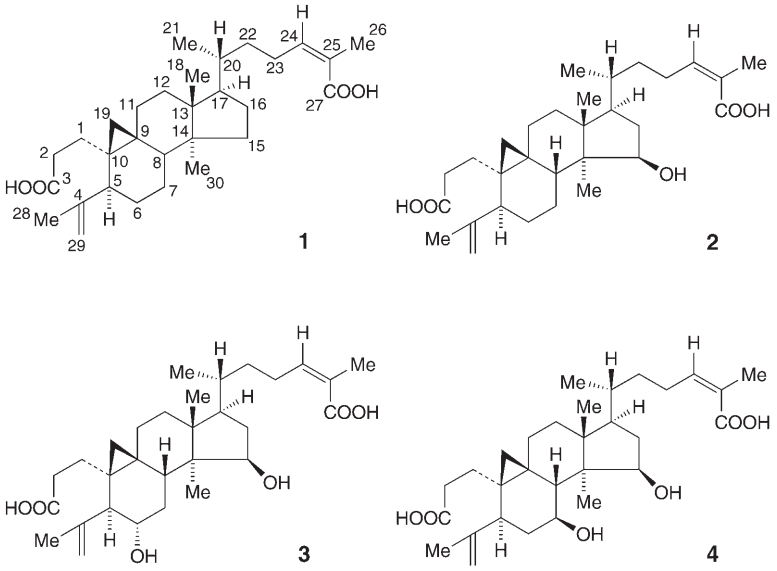

Figure. *The structures of nigranoic acid* (**1**), *and the biotransformed products 15β-hydroxynigranoic acid* (**2**), *6β,15β-dihydroxynigranoic acid* (**3**), *and 7β,15β-dihydroxynigranoic acid* (**4**)

The high-resolution mass spectrum of compound **2** displayed the $[M+Na]^+$ ion peak at *m/z* 509.3247 (calc. 509.3242), suggesting a molecular formula of $C_{30}H_{46}NaO_5$. The ^{13}C-NMR spectrum (*cf. Table 2* below) showed an additional oxygenated CH signal at δ 78.4. Compared to the corresponding data for nigranoic acid (**1**), the C(14) (δ 54.0) and C(16) (δ 42.3) signals shifted downfield by 3.4 and 12.8 ppm, respectively, whereas C(8) (δ 42.5) shifted upfield by 7.2 ppm, suggesting the hydroxylation at C(15). This conclusion was confirmed by the correlations of Me(30) (δ 0.93, *s*)/C(15) (δ 78.4), H–C(15) (δ 3.67, br. *d*, $J=8.0$ Hz)/C(13) (δ 46.3), and H–C(15)/C(17) (δ 54.1) in the HMBC spectrum. The enhancement between H–C(15) and Me(30) in the NOESY spectrum suggested that OH group at C(15) was β-oriented. When D_2O was added, the H–C(15) signal appeared as a typical double *doublet*, resulting from the couplings of H–C(15) with H_β–C(16)/(^3J(ax,ax) = 8.0 Hz) and H_α–(16)/(^3J(ax,eq) = 4.5 Hz). This split pattern is consistent with the β-configuration of H–C(15) [10]. Thus compound **2** was identified as 15β-hydroxynigranoic acid.

The high-resolution mass spectrum (HR-MS) of compound **3** showed the $[M+Na]^+$ ion peak at *m/z* 525.3207, suggesting a molecular formula of $C_{30}H_{46}NaO_6$. The ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) indicated that compound **3** has two secondary OH groups. The first one (δ(H) 3.67, δ(C) 78.4) at C(15) must be pseudo-equatorial as could be established by observing the same O-bearing tertiary C-atom signal at δ 78.4, and the same NOESY correlation between H–C(15) and Me(30) as compound **2**. On the basis of the secondary OH group signal (δ(H) 4.22–4.27, δ(C) 69.6), there was

evidence that it is attached to C(5) (δ(H) 2.58, $J(5\alpha,6\beta)=8.2$ Hz), which was confirmed by the HMBC correlations from H–C(6) to C(4) (δ 151.0) and C(1) (δ 31.2), and the COSY correlation between H–C(5) and H–C(6). The relative configuration of the OH group at C(6) was assigned as axial by the $J(H_\alpha-C(5), H_\beta-C(6)$ value of 8.2 Hz, which was reconfirmed by the absence of NOESY correlation between these H-atoms. Thus, compound **3** was identified as 6α,15β-dihydroxynigranoic acid.

Table 1. *^{1}H-NMR Spectral Data (*CD_3OD, 500 MHz*) of Compounds* **1**–**4**

Position	**1**	**2**	**3**	**4**
1	2.11–2.05 (*m*), 1.46–1.38 (*m*)	2.12–2.08 (*m*), 1.44–1.37 (*m*)	2.12–2.08 (*m*), 1.48–1.38 (*m*)	2.12–2.08 (*m*), 1.48–1.40 (*m*)
2	2.56–2.46 (*m*), 2.32–2.26 (*m*)	2.53–2.48 (*m*), 2.32–2.24 (*m*)	2.56–2.45 (*m*), 2.31–2.23 (*m*)	2.50–2.45 (*m*), 2.11–2.05 (*m*)
5	2.60–2.51 (*m*)	2.57–2.51 (*m*)	2.58 (*d*, *J*=8.2)	2.72 (*dd*, *J*=4.1, 13.0)
6	1.60–1.51 (*m*), 1.22–1.15 (*m*)	1.57–1.51 (*m*), 1.21–1.13 (*m*)	4.27–4.22 (*m*)	1.67–1.58 (*m*), 1.40–1.32 (*m*)
7	1.43–1.35 (*m*), 1.23–1.15 (*m*)	1.51–1.44 (*m*), 1.20–1.12 (*m*)	1.78–1.70 (*m*)	3.75–3.68 (*m*)
8	1.68–1.60 (*m*)	2.09–2.04 (*m*)	1.77–1.69 (*m*)	2.18 (*d*, *J*=9.0)
11	1.62–1.53 (*m*), 1.36–1.31 (*m*)	1.62–1.52 (*m*), 1.22–1.17 (*m*)	1.64–1.50 (*m*), 1.28–1.18 (*m*)	1.60–1.51 (*m*), 1.28–1.18 (*m*)
12	1.62–1.53 (*m*), 1.24–1.18 (*m*)	1.75–1.65 (*m*), 1.61–1.57 (*m*)	1.74–1.68 (*m*)	1.70–1.64 (*m*), 1.66–1.60 (*m*)
15	1.78–1.70 (*m*)	3.67 (br. *d*, *J*=8.0)	3.67 (*d*, *J*=6.2)	3.84–3.79 (*m*)
16	2.02–1.94 (*m*), 1.41–1.33 (*m*)	2.58–2.52 (*m*), 1.38–1.31 (*m*)	2.58–2.53 (*m*), 1.37–1.32 (*m*)	2.53–2.44 (*m*), 1.38–1.32 (*m*)
17	1.70–1.66 (*m*)	1.64–1.58 (*t*, *J*=9.7)	1.65–1.56 (*m*)	1.70–1.59 (*m*)
18	1.02 (*s*)	1.23 (*s*)	1.23 (*s*)	1.23 (*s*)
19	0.78 (*d*, *J*=3.4, H_β), 0.46 (*d*, *J*=3.4, H_α)	0.86 (*d*, *J*=4.2, H_β), 0.46 (*d*, *J*=4.2, H_α)	0.80 (*d*, *J*=4.2, H_β), 0.58 (*d*, *J*=4.2, H_α)	0.38 (*d*, *J*=4.2, H_β), 0.95 (*d*, *J*=4.2, H_α)
20	1.48–1.43 (*m*)	1.75–1.65 (*m*)	1.60–1.48 (*m*)	1.70–1.58 (*m*)
21	0.96 (*d*, *J*=6.1)	0.96 (*d*, *J*=7.8)	0.98 (*d*, *J*=7.8)	0.97 (*d*, *J*=7.8)
22	2.22–2.15 (*m*), 1.37–1.30 (*m*)	2.24–2.18 (*m*), 1.38–1.31 (*m*)	2.23–2.18 (*m*), 1.35–1.28 (*m*)	2.24–2.16 (*m*), 1.36–1.30 (*m*)
23	2.62–2.50 (*m*), 2.48–2.34 (*m*)	2.57–2.51 (*m*), 2.45–2.35 (*m*)	2.60–2.50 (*m*), 2.47–2.38 (*m*)	2.52–2.42 (*m*), 2.37–2.31 (*m*)
24	5.89 (*t*, *J*=6.7)	5.93 (*t*, *J*=7.2)	5.95 (*t*, *J*=7.2)	5.67 (*t*, *J*=7.0)
26	1.90 (*s*)	1.90 (*s*)	1.90 (*s*)	1.90 (*s*)
28	4.88 (*s*), 4.77 (*s*)	4.88 (*s*), 4.76 (*s*)	4.90 (*s*), 4.78 (*s*)	4.87 (*s*), 4.77 (*s*)
29	1.73 (*s*)	1.74 (*s*)	1.74 (*s*)	1.74 (*s*)
30	1.04 (*s*)	0.93 (*s*)	1.08 (*s*)	0.99 (*s*)

The HR-MS of metabolite **4** displayed the $[M+Na]^+$ ion peak at *m*/*z* 525.3206, suggesting a molecular formula of $C_{30}H_{46}NaO_6$. The ^{1}H- and ^{13}C-NMR indicated that compound **4** has also two secondary OH groups. As compound **3**, one OH group at C(15) must be pseudo-equatorial as could be established by observing the almost same O-bearing tertiary C-atom signal at δ 80.3, and the same NOESY correlation between H–C(15) and Me(30). As we can see from the secondary OH group signal (δ(H) 3.68–3.75, δ(C) 70.1), there was evidence that it is attached to C(8) (δ(H) 2.18, $J(7\alpha,8\beta)=$

Table 2. *^{13}C-NMR Spectral Data for Compounds* **1**–**4**[a])

Position	**1**	**2**	**3**	**4**
1	30.8 (*t*)	31.1 (*t*)	31.2 (*t*)	31.1 (*t*)
2	33.0 (*t*)	33.2 (*t*)	33.1 (*t*)	33.4 (*t*)
3	178.2 (*s*)	178.7 (*s*)	178.6 (*s*)	178.6 (*s*)
4	151.2 (*s*)	151.4 (*s*)	151.0 (*s*)	150.2 (*s*)
5	47.6 (*d*)	47.7 (*d*)	47.7 (*d*)	46.1 (*d*)
6	29.6 (*t*)	29.5 (*t*)	69.6 (*d*)	38.0 (*t*)
7	26.7 (*t*)	26.0 (*t*)	33.8 (*t*)	70.1 (*d*)
8	49.7 (*d*)	42.5 (*d*)	45.1 (*d*)	48.7 (*d*)
9	23.1 (*s*)	23.2 (*s*)	23.6 (*s*)	22.4 (*s*)
10	28.9 (*s*)	29.0 (*s*)	29.3 (*s*)	29.3 (*s*)
11	37.5 (*t*)	37.4 (*t*)	37.3 (*t*)	37.5 (*t*)
12	37.3 (*t*)	36.2 (*t*)	36.2 (*t*)	35.7 (*t*)
13	46.8 (*s*)	46.3 (*s*)	46.3 (*s*)	46.4 (*s*)
14	50.6 (*s*)	54.0 (*s*)	58.2 (*s*)	53.8 (*s*)
15	34.8 (*t*)	78.4 (*d*)	78.4 (*d*)	80.3 (*d*)
16	29.5 (*t*)	42.3 (*t*)	42.5 (*t*)	39.8 (*t*)
17	53.9 (*d*)	54.1 (*d*)	54.1 (*d*)	53.5 (*d*)
18	20.4 (*q*)	19.8 (*q*)	19.9 (*q*)	19.7 (*q*)
19	31.6 (*t*)	31.8 (*t*)	31.8 (*t*)	29.9 (*t*)
20	37.8 (*d*)	37.5 (*d*)	37.5 (*d*)	37.4 (*d*)
21	19.3 (*q*)	19.2 (*q*)	19.2 (*q*)	19.2 (*q*)
22	28.6 (*t*)	28.6 (*t*)	27.7 (*t*)	27.8 (*t*)
23	28.1 (*t*)	27.8 (*t*)	26.2 (*t*)	26.8 (*t*)
24	144.8 (*d*)	143.2 (*d*)	143.3 (*d*)	143.1 (*d*)
25	128.9 (*s*)	129.7 (*s*)	129.4 (*s*)	128.3 (*s*)
26	21.6 (*q*)	21.5 (*q*)	20.8 (*q*)	21.4 (*q*)
27	172.1 (*s*)	172.9 (*s*)	172.6 (*s*)	172.1 (*s*)
28	112.7 (*t*)	112.5 (*t*)	112.5 (*t*)	112.9 (*t*)
29	20.7 (*q*)	20.6 (*q*)	20.6 (*q*)	20.5 (*q*)
30	19.3 (*q*)	19.7 (*q*)	19.8 (*q*)	19.3 (*q*)

[a]) Signal multiplicities were determined from DEPT experiments.

9.0 Hz), which was confirmed by the COSY correlation between H−C(7) and H−C(8), and the HMBC correlations from H−C(8) to C(7) (δ 70.1), C(14) (δ 53.8), C(1) (δ 31.1), C(19) (δ 29.9), C(9) (δ 22.4), and C(30) (δ 19.3). The relative configuration of the OH group at C(7) was assigned as equatorial by the $J(H_\alpha-C(7), H_\beta-C(8))$ value of 9.0 Hz, which was reconfirmed by the presence of NOESY correlation between $H_\alpha-C(5)$ and $H_\alpha-C(7)$. Thus, compound **4** was identified as 7β,15β-dihydroxynigranoic acid.

Unfortunately, since only very small quantities of 15β-hydroxynigranoic acid (**2**), 6α,15β-dihydroxynigranoic acid (**3**), and 7β,15β-dihydroxynigranoic acid (**4**) were obtained from the biotransformation products, it was not possible to screen their bioactivity against tumor cell lines and HIV. However, the present results have suggested that the selected microorganisms can be useful for biotransformation of nigranoic acid (**1**) leading to hydroxy derivatives, which are difficult to obtain by chemical methods. Thus, further studies on how the fungus transforms these

compounds, in order to achieve higher yields, more diverse structures, and optimized functions are in progress.

This study was financially supported by the *National Natural Science Foundation of China* (NSFC30570059, NSF 30560179 and NSFC20562015), *Yunnan Provincial Natural Science Foundation* (2005C0005Q, 2005NG03 and 2005NG05), and *State Key Laboratory of Phytochemistry and Plant Resources* in west China, *Kunming Institute of Botany*, China. We are grateful to Dr. *L. Cai* for providing cultures of *Gliocladium roseum* YMF1.00133.

Experimental Part

General. TLC: plates precoated with silica gel F_{254} (*Qingdao Marine Chemical Corp.*, Qingdao, China), visualization by spraying with 5% (*v*/*v*) H_2SO_4 in EtOH. Column chromatography (CC): silica gel (200–300 mesh; *Qingdao Marine Chemical Corp.*, Qingdao, China) and *Sephadex LH-20* gel (*Pharmacia*, Uppsala, Sweden). Optical rotations: *Horiba SEPA-300* polarimeter. IR Spectra: in KBr pellets, with a *Bio-Rad FTS-135* spectrophotometer (*Bio-Rad*, Richmond, CA, USA). ^{1}H- and ^{13}C-NMR spectra: *DRX-500* spectrometer (*Bruker*, D-Karlsruhe) in CD_3OD at r.t. with TMS as the internal standard. The chemical shifts (δ) are reported in ppm. Standard pulse sequences were used for distortionless enhancement by polarization transfer (DEPT), nuclear *Overhauser* effect correlation spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) experiments. HR-MS: *VG Auto Spec-3000* mass spectrometer (*VG*, GB-Manchester).

Chemicals. Nigranoic acid (=(24*Z*)-9,19-cyclo-3,4-secolanosta-4(28),24-diene-3,26-dioic acid; **1**) was isolated from the Chinese medicine *Schisandra propinqua*, and identified unambiguously by NMR spectroscopy and MS, and by comparison with authentic samples from the China Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The purity was >99% based on high-performance liquid chromatography (HPLC) and TLC analyses. All solvents used for product isolation were of anal. grade or higher.

Strain and Culture Conditions. G. roseum YMF1.00133 was maintained on potato dextrose agar slants. Cultures on agar slants were incubated for 10–14 days at 28° for abundant spore production. The spores were washed from the surface of the agar with 5 ml of sterilized water and used to inoculate 500-ml *Erlenmeyer* flasks containing 100 ml of a synthetic medium containing (l^{-1}): peptone, 0.7143 g; $CaCl_2$, 0.7143 g; $NaNO_3$, 1.4285 g; glycerol, 7.1428 g; $ZnSO_4 \cdot 7\,H_2O$, 0.0714 g; K_2HPO_4, 0.1429 g; yeast extracts, 4 g; dextrose, 15 g; the pH was adjusted to 7.0. The fungus was grown for 72 h on a rotary shaker at 28° with shaking at 180 rpm. *Nigranoic acid* (**1**; 600 mg) was dissolved in 20 ml of abs. EtOH; 2 ml of the EtOH soln. was added to each 500-ml *Erlenmeyer* flask. Incubation was continued for 10 days under the same conditions.

Extraction and Purification of Nigranoic Acid-Transformation Metabolites. After harvest, all the fermentation media were put together and then extracted three times with AcOEt. The org. phase was separated and filtered to make it more clear. The extract was washed with H_2O and evaporated under reduced pressure. The residue was dissolved in 1 ml of MeOH. The sample was applied to a *Sephadex LH-20* column and eluted with MeOH to give 198 mg of a yellowish solid that was subjected to silica-gel CC (200–300 mesh; 1.5 × 30 cm, with 20 g of silica gel), and eluted with gradient mixtures of petroleum ether (60–90°), acetone, and AcOH to yield *nigranoic acid* (**1**; 30 mg), *15β-hydroxynigranoic acid* (**2**; 3.4 mg), *6α,15β-dihydroxynigranoic acid* (**3**; 1.2 mg), and *7β,15β-dihydroxynigranoic acid* (**4**; 1.5 mg). Since the molar extinction coefficients of the metabolites are not known, the relative concentrations of metabolites were not determined.

15β-Hydroxynigranoic Acid (**2**). White amorphous powder (MeOH). $[\alpha]_D^{28.7} = +33.0$ ($c = 0.60$, in CH_3OH). IR (film): 3435, 2930, 2871, 1701, 1639, 1456, 1409, 1381, 1258, 1117, 1078, 1034, 891. ^{1}H- and ^{13}C-NMR (500 MHz, CD_3OD): see *Tables 1* and *2*, resp. TOF-MS: 509 ($[M+Na]^+$). HR-TOF-MS: 509.3247 ($[M+Na]^+$, $C_{30}H_{46}NaO_5$; calc. 509.3242).

6α,15β-Dihydroxynigranoic Acid (**3**). White amorphous powder (MeOH). $[\alpha]_D^{28.5}$ +25.1 ($c = 0.64$, MeOH). IR (film): 3437, 2950, 2870, 1700, 1643, 1456, 1381, 1268, 1075, 1044, 890. ^{1}H- and ^{13}C-NMR

(500 MHz, CD_3OD): see *Tables 1* and *2*, resp. TOF-MS: 525 ($[M+Na]^+$). HR-TOF-MS: 525.3207 ($[M+Na]^+$, $C_{30}H_{46}NaO_6$; calc. 525.3192).

7β,15β-Dihydroxynigranoic Acid (**4**). White amorphous powder (MeOH). $[\alpha]_D^{28.1} = +22.2$ ($c = 0.69$, in CH_3OH). IR (film): 3439, 2955, 2926, 2856, 1645, 1457, 1378, 1271, 1073, 1049, 893. ^1H- and ^{13}C-NMR (500 MHz, CD_3OD): see *Tables 1* and *2*, resp. TOF-MS: 509 ($[M+Na]^+$). HR-TOF-MS: 525.3206 ($[M+Na]^+$, $C_{30}H_{46}NaO_6$; calc. 525.3192).

REFERENCES

[1] W. A. Loughlin, *Bioresour. Technol.* **2000**, *74*, 49.
[2] S. Riva, *Curr. Opin. Chem. Biol.* **2001**, *5*, 106.
[3] A. Garcia-Granados, M. C. Gutierrez, F. Rivas, J. M. Arias, *Phytochemistry* **2001**, *58*, 891.
[4] L. R. Lehman, J. D. Stewart, *Curr. Org. Chem.* **2001**, *5*, 439.
[5] V. Urlacher, R. D. Schmid, *Curr. Opin. Biotechnol.* **2002**, *13*, 557.
[6] H. D. Sun, S. X. Qiu, L. Z. Lin, Z. Y. Wang, Z. W. Lin, T. Pengsuparp, J. M. Pezzuto, H. H. S. Fong, G.. A. Cordell, N. R. Farnsworth, *J. Nat. Prod.* **1996**, *59*, 525.
[7] Y. G. Chen, G. W. Qin, L. Cao, Y. Leng, Y. Y. Xie, *Fitoterapia* **2001**, *72*, 435.
[8] L. Huang, C. H. Chen, *Curr. Drug Targets-Infect. Disorders* **2002**, *2*, 33.
[9] I. C. Sun, Y. Kashiwada, S. L. Morris-Natschke, K. H. Lee, *Curr. Top. Med. Chem.* **2003**, *3*, 155.
[10] M. Ye, G. Q. Qu, H. Z. Guo, D. Guo, *Appl. Environ. Microbiol.* **2004**, *70*, 3521.

Received October 18, 2006