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Microbial biotransformation of water-insoluble herbimycin A to 11-hydroxy-(11-demethoxy)-herbimycin C by *Eupenicillium* sp. SD017

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ABSTRACT

Herbimycin A, a typically water-insoluble anti-tumor drug and an analog of geldanamycin, was transformed by fungus *Eupenicillium* sp. SD017. The water solubility and anti-tumor activity of converted products were evaluated after separation, purification, and structure elucidation. The results showed that herbimycin A can be converted into herbimycin C **1** and 11-hydroxy-(11-demethoxy)-herbimycin C **2** with water solubility increased. The latter is a novel compound, and its IC_{50} to A549, MCF-7, Ehrlich and HeLa cells is 152.9, 0.46, 10.7 and 109.3 μg/ml, respectively, which are nearly in the range of those between herbimycin A and herbimycin C. It is suggested that an *O*-demethylase or/and methyltransferase may play an important role on biotransformation in this case. This study will firstly serve as a fundamental methodology for further structural alteration and functional improvement of the water-insoluble anti-tumor drugs using microbial resources.

1. Introduction

The benzoquinonoid ansamycin, herbimycins including herbimycin A, B and C, as geldanamycin analogs, had been firstly isolated from the fermentation broth of *Streptomyces hygroscopicus* AM-3672 by Omura and his colleagues from 1978 to 1986 [1–3]. Herbimycin possess a wide range of promising bioactivities, including herbicidal effect and the inhibition of radial tumor, bacterial and fungal cell growth [4–6]. The reported IC_{50} values of herbimycin and other geldanamycin derivates against several tumor cell lines range from 1 ng/ml to 30 μg/ml, such as IC_{50} values of herbimycin A, B and C are 3.5 μg/ml, 3.2 μg/ml and 7.3 μg/ml to HeLa cells, and 0.4 μg/ml, 0.8 μg/ml and 1.2 μg/ml to Ehrlich cells, respectively [2]. Herbimycin A (Fig. 1), like geldanamycin and macbecin, has been observed to reverse the morphology of cancer cells by reducing kinase activity and cellular phophotyrosine content of various tyrosine kinases and receptors coding oncogenes [7]. And now 17-allylamino-17-demethoxygeldanamycin (17-AAG), one of geldanamycin derivates, is currently undergoing phaseland II clinical trials [8,9]. However, experience with the behavior of geldanamycin, 17-AAG in animals and humans has pointed to the need for more water-soluble and less hapatotoxic form of these drugs [10]. The apparent hepatoxicity of water-insoluble geldanamycin deviates could be attributed to the reactivity of the quinine ring with cellular nucleophiles, low target binding affinity, and degradation of the molecular into a toxic metabolite [11]. So, it is crucially necessary to modify the chemical structure of water-insoluble drug into more water-soluble and less toxic derivate with high performance. Biotransformation is an important tool in the structural modification of organic compounds, such as natural products, due to its significant regio- and stereo-selectivities [12,13] which is usually difficult to achieve by chemical means. In this paper, we report the successfully microbial biotransformation of herbimycin A into products with enhanced bioactivity and increased polarity by the fungus cultures of *Eupenicillium* sp. SD017, which can produce several bioactive products like curvularin derivates we described before [14].

2. Materials and methods

2.1. General experimental procedures

Melting points were determined on a XRS-1 digital-melting point apparatus, and the values were uncorrected. The ^1H and ^{13}C NMR data were collected on a Bruker AVANCE-500 (Bruker, Switzerland) spectrometer at 500 MHz, and the chemical shifts were recorded in δ (ppm) relative to $Si(Me)_4$ with coupling constants J in Hz. Electron ionization-mass spectrometry (EI-MS)

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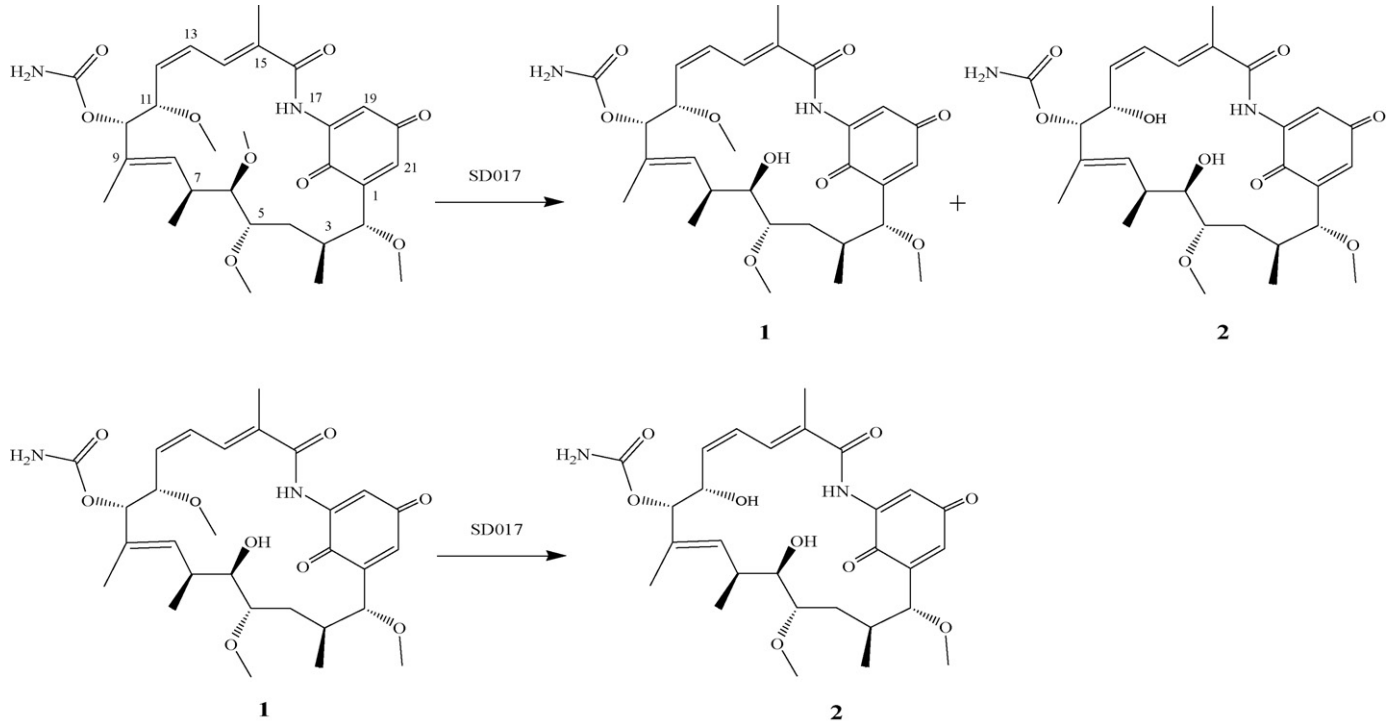

Fig. 1. The reaction formula for microbial biotransformation of herbimycin A by *Eupenicillium* sp. SD017.

was performed using a MAT95XP mass spectrometer (Thermo, USA).

2.2. Chemicals

Herbimycin A was provided by New Drug Research & Development Center of North China Pharmaceutical Group Corporation. Silica gel (100–200 mesh) for open column chromatography was produced by the Qingdao Marine Chemical Factory (Qingdao, China). Sephadex^{TM} LH-20 was from GE Healthcare (GE, USA, made in Sweden). All other chemicals used in this study were of analytical grade.

2.3. Tumor cell lines

The anti-tumor screening for purified biotransformation products involved 4 types of tumor cell including human lung adenocarcinoma epithelial cell line (A549), human Henrietta Lacks cervical cancer cell line (HeLa), mice Ehrlich ascites carcinoma cell line (Ehrlich) and human breast adenocarcinoma cell line (MCF-7).

2.4. Isolation and identification of strain Eupenicillium sp.

The fungal isolate was obtained by serial dilution method [15] from sponge samples collected from South China Sea (18° 13′ N; 109° 29′ E) near Sanya, Hainan, China in July, 2005. The sponge sample was later identified as *Axinella* sp. by Dr. K.J. Lee (Department of Biology, Hannam University, 133 Ojungdong, Daedukgu, Daejeon, Korea) through personal communication. The bioactive fungal strain was identified to be *Eupenicillium* sp. by comparing its morphological features with the reference description [16] and by online BLAST analysis of its 18S rDNA sequence with those submitted sequences on GenBank database.

2.5. Culture media

The strain isolation was performed on the starch casein KNO_3 Agar (SCKA) medium composed of 20 g starch, 2 g KNO_3, 2 g K_2HPO_4, 2 g NaCl, 0.3 g casein, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4{\cdot}7H_2O$, and 18 g agar in 500 ml sterile distilled water premixed with 500 ml filtered sea water.

The germination and growth of the marine fungal isolate was undertaken on the Gauze's No. 1 sea water medium (GSW), which was composed of 20 g soluble starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4{\cdot}7H_2O$, and 30 g agar in 500 ml sterile distilled water premixed with 500 ml filtered sea water. All above inorganic chemicals were from Guangzhou Chemical Reagent Factory, and the organic reagents from Amresco Inc.

2.6. Preparative HPLC conditions

The purification of converted products was mainly performed on a HITCHI L-2000 preparative high performance liquid chromatography (HITCHI, Japan) with one YMC semi-preparative ODS column (5 mm, Φ 10 mm × 250 mm) and MeOH–H_2O solution as the mobile phase. The flow rate was 1.5 ml/min, and the detection wavelength was 271 nm.

2.7. Biotransformation procedure

The fresh mycelium grew on GSW agar at 28 °C for 5 days was inoculated into 500 ml Erlenmeyer flasks containing 150 ml GSW medium. After 2 days of incubation at 28 °C on rotary shaker at 200 rpm, a 10 ml seed culture liquid was transferred into each 500 ml Erlenmeyer flask containing 150 ml GSW medium. After 5 days of incubation, a total amount of 200 mg of herbimycin A dissolved in 20 ml of DMSO was distributed equally into 40 flasks. The incubation was allowed to continue for additional 5 days on the shaker. The cultures were then pooled and filtered in vacuo. The filtrate was extracted with 6 L of ethyl acetate for three times. The organic extract was evaporated to dryness in a rotary evaporator under reduced pressure at 50 °C to yield 1.3 g of a brownish residue.

Table 1
NMR spectral data for biotransformation compound **1** and **2**.

Carbon no.	Herbimycin A		Compound **1**		Compound **2**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	144.6	–	144.8	–	144.8	–
2	78.7	4.50 (1H, br s)	79.2	4.50 (1H, br s)	79.2	4.49 (1H, br s)
3	36.7	1.64 (1H, m)	32.3	1.65 (1H, m)	32.0	1.65 (1H, m)
4	34.0	1.75 (2H, m)	34.0	1.76 (2H, m)	34.0	1.75 (2H, br s)
5	83.4	3.48 (1H, m)	83.5	3.53 (1H, m)	83.5	3.53 (1H, m)
6	82.3	5.50 1H, br s)	72.9	3.50 (1H, m)	72.7	3.48 (1H, m)
7	34.1	2.64 (1H, br s)	29.7	2.62 (1H, br s)	34.1	2.64 (1H, br s)
8	130.1	5.65 (1H, br s)	132.8	5.67 (1H,br s)	131.5	5.65 (1H, br s)
9	131.6	–	131.8	–	131.6	–
10	79.2	5.34 (1H, br s)	79.4	5.33 (1H, s)	82.4	5.34 (1H, s)
11	78.3	4.48 (1H, s)	78.6	4.51 (1H, s)	66.6	4.50 (1H, s)
12	136.7	5.86 (1H, t)	136.8	5.87 (1H, t)	136.8	5.86 (1H, t)
13	125.6	6.50 (1H, dd)	125.7	6.51 (1H, dd)	125.7	6.50 (1H, dd)
14	128.3	6.97 (1H, br d)	128.3	6.98 (1H, br d)	128.3	6.98 (1H, br d)
15	134.5	–	134.7	–	134.7	–
16	168.7	8.60 (1H, s)	168.2	8.81 (1H, s)	168.7	8.80 (1H, s)
18	138.2	–	138.3	–	138.3	–
19	112.9	7.34 (1H, d)	113.1	7.35 (1H, d)	113.1	7.34 (1H, d)
20	187.7	–	187.9	–	187.9	–
21	132.6	6.63 (1H, s)	132.8	6.64 (1H, s)	132.7	6.63 (1H, s)
22	183.9	–	184.2	–	184.1	–
2-OCH_3	59.8	3.36 (3H, s)	58.5	3.35 (3H, s)	58.4	3.35 (3H, s)
3-CH_3	13.6	0.81 (3H, d)	13.7	0.82 (3H, d)	13.6	0.82 (3H, d)
5-OCH_3	57.6	3.31 (3H, s)	57.7	3.32 (3H, s)	57.7	3.31 (3H, s)
6-OCH_3	58.4	3.53 (3H, s)	–	–	–	–
6-OH		–		2.63 (1H, s)		2.64 (1H, br s)
7-CH_3	16.3	1.08 (3H, d)	14.2	1.09 (3H, d)	16.6	1.09(3H, d)
9-CH_3	14.1	1.65 (3H, br s)	12.4	1.63 (3H, br s)	14.2	1.65 (3H, br s)
10-$OCONH_2$	155.9	–	155.8	–	155.8	–
11-OCH_3	56.0	3.34 (3H, s)	56.0	3.35 (3H, s)	–	–
11-OH	–	–	–	–	–	2.64 (1H br s)
15-CH_3	12.4	2.03 (3H, s)	12.6	2.02 (3H, br s)	12.4	2.01 (3H, s)

2.8. Separation and purification of biotransformation products

As we described elsewhere [17], the obtained residue was first fractionated by silica gel open column chromatography and eluted with $CHCl_3$–MeOH (95:5–75:25, v/v) to afford six fractions. Fraction II was subjected to preparative HPLC eluted with MeOH–H_2O (65:35, v/v) and recrystallized from MeOH to yield 17.2 mg of compound **1** as a yellowish amorphous powder. Fraction IV was separated by preparative HPLC eluted with MeOH–H_2O (55:45, v/v) and further purified on a Sephadex LH-20 column ($CHCl_3$–MeOH, 1:1) and then recrystallized from MeOH to yield 15.4 mg of compound **2** as a yellowish amorphous powder. Purities of two products were over 95%, determined by HPLC with UV detector.

2.9. Bioassay

Using above anti-tumor screening cells, IC_{50} values of compound **1** and **2** (in DMSO) against the 4 types of tumor cell lines was tested in triplicate according to the MTT method described in the literature [18] with DMSO as a negative control and taxol™ as a positive control. And the bioassay was carried out at the Institute of Nutrisciences and Health, National Research Council of Canada.

3. Results and discussion

In screening of the most active strain for biotransformation of herbimycin A, it was found that herbimycin A was completely metabolized in the cultures of one isolate SD017 from marine sponge *Axinella* sp. collected in South China Sea near Sanya, later identified as fungus *Eupenicillium* sp. Its main morphological characteristics are its tough, dense penicilli bearing long, broad columns of conidia, and its smooth-walled, unflanged ascospores which are produced within 14 days of inoculation onto GSW medium, the optimal temperature for growth is 28 °C. *Eupenicillium* sp. SD017 (CGMCC 3.5706) was later preserved in the China General Microbiological Culture Collection Center.

During analysis of the incubation mixture added with herbimycin A, two new peaks with bigger area were observed in HPLC, which were not found in control tests. One peak showed UV absorption maxima at 269 nm and the other at 271 nm, which is characteristic adsorption wavelength for the benzoquinone-ringed ansamycin. Thus, they should be the biotransformed products of herbimycin A. In the preparative biotransformation, a total amount of 200 mg of herbimycin A was fed to the microbial cultures. After 5 days incubation, the products were obtained by extracting with ethyl acetate for three times. The obtained extract was subjected to silica gel open column chromatography, Sephadex LH-20 column chromatography and preparative HPLC to afford two pure products. The products were structurally characterized by comparing their IR, EI-MS data and NMR spectra including ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC, ^{1}H–^{1}H COSY and NOESY with the substrate and related compounds. Compound **1** is yellowish amorphous powder, $[\alpha]_D^{25} + 210.4$ (c 0.5, $CHCl_3$); IR λ_{max} (KBr): 3530, 3410, 3350, 1730, 1695, 1660, 1645, 1600, 1075 cm^{-1}; EI-MS (*m*/*z*): 583 (M+Na, 56), 562 (M+2, 9), 560 (M^+, 5), 517 (100). Compound **2** is yellowish amorphous powder, $[\alpha]_D^{25} + 231.6$ (c 0.5, $CHCl_3$); IR λ_{max} (KBr): 3534, 3410, 3350, 1725, 1690, 1655, 1645, 1605, 1082 cm^{-1}; EI-BMS (*m*/*z*): 569 (M+Na, 34), 548 (M+2, 17), 546 (M^+, 9), 503 (100). The NMR data of compound **1** and **2** with substrate are given in Table 1. The results showed that the molecular backbone of products was not destroyed but only demethylation occurred on C-6 and/or C-11. Thereby, these two products were structurally identified as herbimycin C **1** and 11-hydroxy-(11-demethoxy)-herbimycin C **2** (Fig. 1), and the latter is a novel compound.

In optimizing the conditions for biotransformation, the growth curve for *Eupenicillium* sp. SD017 cultures was recorded (data not

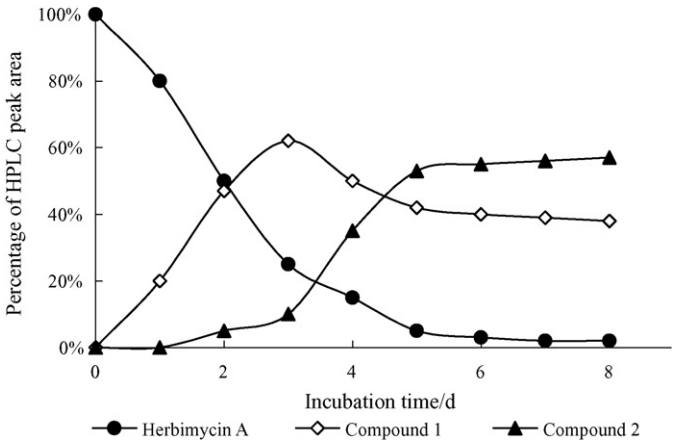

Fig. 2. The biotransformation curves for herbimycin A.

shown). The results showed that the optimal time for substrate addition was at the late logarithmic-phase to the early stationary-phase (5th day) during the strain growth period. Substrate addition at this period of time resulted in an efficient bioconversion of substrate and yield of products; for example, the yields for **1** and **2** were 2-fold more than those of substrate addition at the beginning of the strain growth period.

In general, the concentration of substrate in fermentation broth affects the yield of converted products in the biotransformation process. Thus, the effects of six different concentrations of substrates (10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, 50 mg/l and 60 mg/l) on the substrate bioconversion were investigated and the results indicated that the optimal amount of substrate addition was 30 mg/l. At this concentration, the substrate was efficiently converted (almost 100%), and simultaneously the products (**1** and **2**) reached the highest yields (data not shown).

The biotransformation curves, namely the plot for the percentage of HPLC peak area of herbimycin A, compound **1** and **2** versus incubation time under 28 °C on rotary shaker at 200 rpm is given in Fig. 2. The results indicate the percentage of peak area (i.e. relative amount) of herbimycin A decreases along with the lapse of incubation time, and the relative amount of compound **1** increase until reaching a maximal value at 3th day before dropping down to an equilibrium value, while the concentration of compound **2** steadily increased until reaching an equilibrium value. It was suggested that the initiate biotransformed product was compound **1** whereas compound **2** was biosynthesized from compound **1** through a demethylation process catalyzed by an *O*-demethylase or/and methyltransferase which are occasionally found in biotransformation and biodegradation study. It was reported that dicamba *O*-demethylase from *Pseudomonas maltophilia* can catalyze the conversion of the herbicide dicamba to 3,6-dichlorosalicylic acid [19]. And Kaufmann et al. [20] isolated the *O*-demethylase from one homoacetogenic strain and proved the *O*-demethylase is an ether-cleaving enzyme system. Therefore, a proposed reaction formula for biotransformation of herbimycin A by *Eupenicillium* sp. SD017 is given as Fig. 1.

According to HPLC analysis on the incubation cultures, although the biotransformation yields for **1** and **2** were determined to be 42% and 53%, respectively, the recovery rate of isolated biotransformation products is relative less comparing with the amount of the substrate due to the loss in the separation and purification process.

To understand whether the extracellular enzyme or endocellular one, whether the constitutive enzyme or inducible one contributes the biotransformation, a series of experiments were designed to characterize the enzymes through the methods of cell-free culture and substrate/product concentration analysis.

Table 2
The IC_{50} values (in μg/ml) of biotransformed products against tumor cell lines.

Compounds	Tumor cell lines			
	A549[a]	MCF-7	Ehrlich	HeLa
Herbimycin A	90.4 ± 4.9[b]	0.03 ± 0.001	1.8 ± 0.06	83.6 ± 3.8
Compound **1**	219.3 ± 16.7	0.87 ± 0.06	5.4 ± 0.2	153.8 ± 7.3
Compound **2**	152.9 ± 11.9	0.46 ± 0.03	10.7 ± 0.6	109.3 ± 10.8
Taxol	4.5 ± 0.4	3.2 ± 0.2	8.6 ± 0.5	7.4 ± 0.4

[a] A549, human lung adenocarcinoma epithelial cell line; HeLa, human Henrietta Lacks cervical cancer cell line; Ehrlich, mice Ehrlich ascites carcinoma cell line; MCF-7, human breast adenocarcinoma cell line.
[b] Data with standard deviations under bioassay in triplicate according to the MTT method with taxol as a positive control.

It was found that the enzymes are extracellular and constitutive, since strain broth in vitro could convert the exogenous substrates to the two isolated products. It was suggested that an *O*-demethylase or/and methyltransferase may play an important role on biotransformation. Further work will focus on cDNA cloning and enzymatic expression to characterize the enzyme(s) involved. If the enzymes' properties and additional information were unearthed, the biotransformation rate and yield could be optimized through enhancing their expression and activities. Subsequently, the enzymes could be extracted, purified and immobilized for large-scale production of the desired products.

It is possible to obtain more water-soluble derivate(s) of herbimycin A because the demethylation reaction took place at the methoxyl moiety connected with C-6 or/and C-11. The water solubility of the compound **2**, the modified compound with new structure, has been increased about 10 times after microbial biotransformation through water resolve assay (data not shown). In addition, the water solubility of another product was much stronger than those of the substrate herbimycin A. Most importantly, the IC_{50} of 11-hydroxy-(11-demethoxy)-herbimycin C to the A549 cells is 152.9 μg/ml, to MCF-7 cells is 0.46 μg/ml, to Ehrlich cells is 10.7 μg/ml, and to HeLa cells is 109.3 μg/ml, which are nearly in the range of those between herbimycin A and herbimycin C (Table 2).

In conclusion, we have obtained a powerful method for preparation of C-6/C-11 demethylated herbimycin A derivatives by *Eupenicillium* sp. SD017 cultures. This might provide a useful tool to prepare bioactive geldanamycin derivates. This study will firstly serve as a fundamental methodology for further structural alteration and functional improvement of the water-insoluble antitumor drugs using microbial resources.

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References

[1] S. Omura, Y. Iwai, Y. Takahashi, N. Sadakane, A. Nakagawa, H. Oiwa, Y. Hasegawa, T. Ikai, J. Antibiot. 32 (1979) 255–261.
[2] Y. Iwai, A. Nakagawa, N. Sadakane, S. Omura, H. Oiwa, S. Matsumoto, M. Takahashi, T. Ikai, Y. Ochiai, J. Antibiot. 33 (1980) 1114–1119.
[3] K. Shibata, S. Satsumabayashi, A. Nakagawa, S. Omura, J. Antibiot. 39 (1986) 1630–1633.
[4] K.K. Svoboda, D.L. Orlow, C.L. Chu, W.R. Reenstra, Anat. Rec. 254 (1999) 348–359.
[5] R.D. Sheski, V. Natarajan, S.T. Porttratz, J. Lab. Clin. Med. 133 (1999) 265–273.
[6] T. Oikawa, H. Ogasawara, H. Sano, K. Shibata, S. Omura, Biol. Pharm. Bull. 17 (1994) 1430–1432.
[7] L. Whitesell, S.D. Shifrin, G. Schwab, L.M. Necker, Cancer Res. 52 (1992) 1721–1728.

[8] S.S. Ramalingam, M.J. Egorin, R.K. Ramanathan, S.C. Remick, R.P. Sikorski, T.F. Lagattuta, G.S. Chatta, D.M. Friedland, R.G. Stoller, D.M. Potter, S.P. Ivy, C.P. Belani, Clin. Cancer Res. 14 (2008) 3456–3461.
[9] D.B. Solit, I. Osman, D. Polsky, K.S. Panageas, A. Daud, J.S. Goydos, J. Teitcher, J.D. Wolchok, F.J. Germino, S.E. Krown, D. Coit, N. Rosen, P.B. Chapman, Clin. Cancer Res. 14 (2008) 8302–8307.
[10] M.J. Egorin, E.G. Zuhowski, D.M. Rosen, D.L. Sentz, J.M. Covey, J.L. Eiseman, Cancer Chemother. Pharmacol. 47 (2001) 291–302.
[11] Y. Uehara, H. Fukazawa, Y. Murakami, S. Mizuno, Biochem. Biophys. Res. Commun. 163 (1989) 803–809.
[12] S. Riva, Curr. Opin. Chem. Biol. 5 (2001) 106–111.
[13] A. Świzdor, A. Szpineter, T. Kołek, J. Mol. Catal. B: Enzym. 42 (2006) 10–13.
[14] L.W. Xie, O.Y. Ouyang, K. Zou, G.H. Wang, M.J. Chen, H.M. Sun, S.K. Dai, X. Li, Appl. Biochem. Biotechnol. 159 (2009) 284–293.
[15] L. Schoenborn, P.S. Yates, B.E. Grinton, P. Hugenholtz, P.H. Janssen, Appl. Environ. Microbiol. 70 (2004) 4363–4366.
[16] T.R. Nag Raj, Mycotaxon 53 (1995) 295–310.
[17] L.W. Xie, X. Li, Faming Zhuanli Shenqing Gongkai Shuomingshu, CN 101318930 A (2008) (in Chinese).
[18] F. Denizot, R. Lang, J. Immunol. Methods 89 (1986) 271–275.
[19] P.L. Herman, M. Behrens, S. Chakraborty, B.M. Chrastil, J. Barycki, D.P. Weeks, J. Biol. Chem. 280 (2005) 24759–24767.
[20] F. Kaufmann, G. Wohlfarth, G. Diekert, Arch. Microbiol. 168 (1997) 136–142.