# BEQUINOSTATINS A AND B, NEW INHIBITORS OF GLUTATHIONE *S*-TRANSFERASE, PRODUCED BY *Streptomyces sp.* MI384-DF12

## PRODUCTION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITIES

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New benzo[*a*]naphthacenequinone metabolites, designated bequinostatins A and B, have been isolated from the culture broth of the benastatin-producing strain *Streptomyces* sp. MI384-DF12. The structures of bequinostatins A and B were determined by spectral analyses to be 5,6,8,13-tetrahydro-1,6,7,9,11-pentahydroxy-8,13-dioxo-3-pentylbenzo[*a*]naphthacene-2-carboxylic acid and 2-decarboxybequinostatin A, respectively. Bequinostatin A showed considerable inhibitory activity against human pi class glutathione *S*-transferase (GST $\pi$).

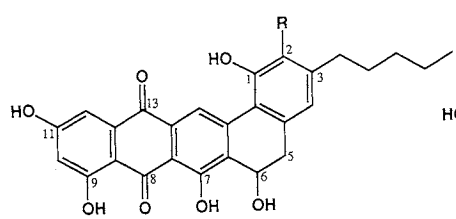
Isolation, physico-chemical, biological properties, and structures of benastatins A (**1**), B, C and D were reported previously[1~3]. In our continuing study of the benastatin-producing strain, we have found that this strain produces other inhibitors of glutathione *S*-transferase (GST, EC2.5.1.18). Bequinostatins A (**2**) and B (**3**) possess the benzo[*a*]naphthacenequinone skeleton (Fig. 1) and are classified as a new family of metabolites along with G-2N, G-2A[4] and KS-619-1[5,6]. In this report, we describe the production, isolation, structure determination and biological activities of the inhibitors.

## Materials and Methods

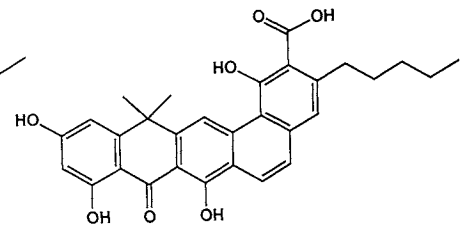
### Chemicals

Chemicals employed were as follows: Silanised silica gel 60, Silica gel 60 and TLC-plate Silica gel $F_{254}$ (0.25 mm thickness) from E. Merck, Darmstadt, FRG; YMC GEL (ODS-A60-200/60) from

Fig. 1. Structures of bequinostatins and benastatin A.




Bequinostatin A (**2**) R=COOH
Bequinostatin B (**3**) R=H




Benastatin A (**1**)

Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; packed column of Capcell Pak $C_{18}$ from Shiseido Co., Tokyo, Japan; glutathione, reduced form (GSH) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; 1-chloro-2,4-dinitrobenzene (CDNB) from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were of analytical grade.

### Analytical Instruments

HPLC was performed by a GILSON's system equipped with a Waters 991J photodiode array detector. Melting points were taken using a Yanaco MP-500D apparatus and were uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using micro-cell (light path 10 cm). UV spectra were recorded on a Hitachi U-3210 spectrophotometer, and IR spectra on a Hitachi I-5020 FT-IR spectrophotometer. Mass spectra were obtained on a JEOL JMS-SX 102 mass spectrometer. NMR spectra were recorded on a JEOL JNM-GX400 NMR spectrometer with $^1H$ NMR at 400 MHz and $^{13}C$ NMR at 100 MHz.

### Production of Bequinostatins

A loopful of slant culture of *Streptomyces* sp. MI384-DF12 (FERM P-11270) was inoculated into 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto Soytone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 30°C for 3 days on a rotary shaker (180 rpm). Two ml of this seed culture were inoculated into 110 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, $K_2HPO_4$ 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 6.2 adjusted with 1 M $KH_2PO_4$ before sterilization) in a 500-ml Erlenmeyer flask and cultured at 27°C for 4 days on a rotary shaker (180 rpm).

### Isolation of Bequinostatins

The culture broth was filtered and separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with methanol; the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the culture filtrate and extracted with an equal volume of ethyl acetate. The extract was concentrated to dryness under reduced pressure. Purification was followed by UV detection, which suggested that **2** and **3** were similar. The dried material was chromatographed on a column of silanised silica gel ( × 50 w/w powder) with a linear gradient 40 to 100% aq. MeOH and then on a column of YMC GEL ( × 100 w/w powder) with the same linear gradient. The fractions containing **2** and **3** were collected and evaporated to give a reddish powder. The crude powder was further purified by a reversed phase HPLC using a Capcell Pak $C_{18}$ column (2.0 × 25 cm, flow rate 8 ml/minute) with a solvent mixture of $CH_3CN$-$H_2O$-AcOH (58 : 42 : 1). Early fractions contain **2** followed next by **3**. Each eluate was evaporated to dryness.

### Enzymes

Partially purified GST was prepared from rat liver as described by WU and MATHEWS[7]. Human pi class GST (GST $\pi$) was kindly provided by Dr. K. TAKAHASHI, University of Tokyo.

### Assay for Glutathione *S*-Transferase (GST) and Inhibitory Activity

GST activity was measured by a modification of the method of HABIG *et al.*[8]. The reaction mixture (total 2.0 ml) consisted of 100 mM potassium phosphate buffer (pH 7.4 for partially purified GST and pH 6.5 for GST $\pi$), 1.0 mM GSH, 1.0 mM CDNB, partially purified GST or GST $\pi$ and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, following incubation at 30°C for 5 minutes. The conjugate was determined spectrophotometrically at 340 nm, corrections were made for chemical reactivity.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the enzyme conjugate without an inhibitor and B is that with an inhibitor. $IC_{50}$ value shows the concentration of inhibitor at 50% inhibition of enzyme activity.

### Assay of Mitogen Induced Murine Lymphocyte Blastogenesis *In Vitro*

The tissue culture medium employed was a complete medium designated Roswell Park Memorial

Institute(RPMI)-1640 supplemented with 20% fetal calf serum, 25 mM Hepes buffer, 100 $\mu$g/ml streptomycin sulfate and 100 units/ml of benzyl penicillin. Lipopolysaccharide (LPS) was utilized to stimulate unseparated spleen cells at final concentration of 100 $\mu$g/ml.

After spleens were aseptically removed from BALB/c mice, single cell suspension of the spleen cells was prepared and subjected to a hyper shock treatment to remove erythrocytes therefrom. Two $\times 10^5$ spleen cells were mixed with the prescribed concentration of the test compound (total 0.2 ml) in each well of a microplate (COSTAR) with a flat bottom, and were incubated at 37°C in a humidified atmosphere (95% air, 5% $CO_2$) for 72 hours, Eight hours from the completion of cultivation, 37 KBq of [$^3$H]thymidine was added to each well. The resultant cells were harvested and [$^3$H]thymidine incorporation into DNA measured.

## Results and Discussion

### Production and Isolation of Bequinostatins

The strain of *Streptomyces* sp. MI384-DF12 was cultured in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. As shown in Fig. 2, **2** and **3** were isolated from the culture broth (30 liters) by chromatography on reversed phase silica gel and Capcell Pak $C_{18}$ (HPLC) followed by solvent extraction. The total yields were 129.2 mg and 16.0 mg, respectively. The purity of each preparation was confirmed by TLC and HPLC.

### Structure of **2**

The physico-chemical properties of **2** are summarized in Table 1. **2** is soluble in dimethyl sulfoxide, sparingly soluble in methanol, acetone and ethyl acetate, but insoluble in water. The molecular weight and formula of **2** were elucidated as $C_{28}H_{24}O_9$ (MW 504) from the FAB-MS peaks at $m/z$ 505 $(M+H)^+$ and $m/z$ 503 $(M-H)^-$ and $^1$H and $^{13}$C NMR spectra of **2** (Table 2). The UV spectrum of **2** was similar

Fig. 2. Isolation procedure of bequinostatins.

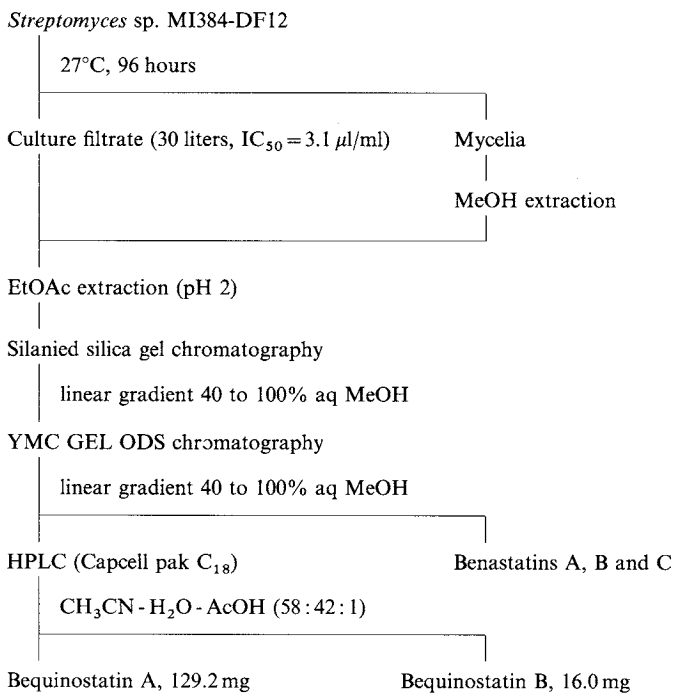


Table 1. Physico-chemical properties of bequinostatins.

| | Bequinostatin A | Bequinostatin B |
|---|---|---|
| Appearance | Red powder | Red powder |
| MP | 312~314°C (dec) | 274~276°C (dec) |
| $[\alpha]_D$ ($c$ 0.01) | −320.0° (25°C, $CHCl_3$-MeOH, 1:1) | −250° (23°C, MeOH) |
| Molecular formula | $C_{28}H_{24}O_9$ | $C_{27}H_{24}O_7$ |
| FAB-MS ($m/z$, Pos.) | 505 $(M+H)^+$ | 461 $(M+H)^+$ |
| FAB-MS ($m/z$, Neg.) | 503 $(M-H)^-$ | 459 $(M-H)^-$ |
| UV ($\log\varepsilon$) nm $\lambda_{max}^{MeOH}$: | 232 (4.50), 267 (sh, 4.28), 304 (4.30), 317 (4.30), 483 (4.08) | 203 (4.64), 228 (4.56), 267 (4.34), 294 (sh, 4.43), 313 (4.54), 479 (4.24) |
| $\lambda_{max}^{MeOH-HCl}$: | 241 (4.55), 301 (4.41), 466 (4.13) | 204 (4.62), 228 (4.57), 267 (4.35), 312 (4.57), 472 (4.27) |
| $\lambda_{max}^{MeOH-NaOH}$: | 242 (4.54), 296 (sh, 4.29), 310 (sh, 4.34), 330 (4.37), 392 (sh, 3.88), 529 (4.04) | 239 (4.54), 255 (4.49), 318 (4.36), 347 (4.38), 432 (sh, 3.86), 544 (4.14) |
| IR $\nu_{max}^{KBr}$ $cm^{-1}$ | 3439, 2946, 1614, 1473, 1400, 1365, 1288, 1249, 1195, 1181, 1042, 775 | 3429, 2922, 1616, 1649, 1400, 1253, 1167, 1104, 1035, 1015, 869, 765 |
| Rf value on TLC | 0.17 ($CHCl_3$-MeOH, 4:1, silica gel) | 0.81 ($CHCl_3$-MeOH, 4:1, silica gel) |
| Color reaction | Phosphomolybdate-$H_2SO_4$, $FeCl_3$ | Phosphomolybdate-$H_2SO_4$, $FeCl_3$ |
| Solubility Soluble: | DMSO, MeOH, $Me_2CO$, EtOAc | DMSO, MeOH, $Me_2CO$, EtOAc |
| Insoluble: | $H_2O$ | $H_2O$ |

to those of G-2N, G-2A[4] and KS-619-1[5], and the absorption band in alkaline solution exhibited bathochromic shifts from 466 nm in HCl-MeOH to 529 nm in NaOH-MeOH, suggesting the presence of a polyhydroxyanthraquinone-like structure such as that in G-2N, G-2A and KS-619-1. In the IR spectrum of **2**, a strong absorption was obtained at 1614 $cm^{-1}$ due to the hydrogen-bonded quinone carbonyl group. Though the $^1H$ and $^{13}C$ NMR spectra of **2** were almost coincident with those of **1**, there were some differences. **1** has the *gem*-dimethyl group, but these corresponding signals were not observed in the $^1H$ and $^{13}C$ NMR spectra of **2**. Instead of this group, an additional aromatic ketone signal appeared at $\delta_C$ 181.3 (C-13) ppm and coupled with the aromatic proton signals at $\delta_H$ 7.14 (12-H) and 8.84 (14-H) ppm in the HMBC (Heteronuclear Multiple Bond Connectivity) spectrum of **2** (Fig. 3). Therefore, it was deduced that this aromatic ketone group was attributed to the anthraquinone moiety.

Furthermore, in the $^{13}C$ NMR spectrum, **2** revealed two additional $sp^3$ carbon signals at $\delta_C$ 37.2 (C-5) and 56.8 (C-6) ppm instead of the aromatic carbon signals at $\delta_C$ 126.5 (C-5) and 121.1 (C-6) ppm in the $^{13}C$ NMR spectrum of **1**, in which a signal (C-6) appeared in lower field ($\delta_C$ 56.8 ppm) indicating the oxygen-bearing carbon. Also in the $^1H$ NMR spectrum of **2**, a methylene proton signals at $\delta_H$ 2.82 and 2.99 (5-$H_2$) ppm, a methine proton signal at $\delta_H$ 5.19 (6-H) ppm and a hydroxyl proton signal at $\delta_H$ 5.03 (6-OH) ppm replaced the aromatic proton signals at $\delta_H$ 7.56 (5-H) and 8.12 (6-H) ppm in the $^1H$ NMR spectrum of **1**. These were seen coupling with each other in the $^1H$-$^1H$ COSY spectrum of **2**. Moreover, in the HMBC spectrum of **2**, the aromatic proton at $\delta_H$ 6.67 (4-H) ppm coupled to an additional $sp^3$ carbon at $\delta_C$ 37.2 (C-5) ppm, and an additional methine proton at $\delta_H$ 5.19 (6-H) ppm showed a cross peak with the aromatic carbon signal at $\delta_C$ 139.9 (C-14a) ppm. Consequently, the structure of **2** was determined to be 5,6,8,13-tetrahydro-1,6,7,9,11-pentahydroxy-8,13-dioxo-3-pentylbenzo[*a*]naphthacene-2-carboxylic acid (Fig. 1). The absolute configuration of C-6 remains to be determined.

## Structure of 3

The molecular weight and formula of **3** were elucidated as $C_{27}H_{24}O_7$ (MW 460) from the FAB-MS

Table 2. $^{13}C$ and $^{1}H$ NMR data of bequinostatins and benastatin A in DMSO-$d_6$.

| Carbon | Benastatin A | | Bequinostatin A | | Bequinostatin B | |
|---|---|---|---|---|---|---|
| | $\delta_C$ ppm (100 MHz) | $\delta_H$ ppm ($J$ in Hz, 400 MHz) | $\delta_C$ ppm (100 MHz) | $\delta_H$ ppm ($J$ in Hz, 400 MHz) | $\delta_C$ ppm (100 MHz) | $\delta_H$ ppm ($J$ in Hz, 400 MHz) |
| 1 | 169.2 (s) | 10.96 (1-OH, br)[a] | 161.6 (s) | 11.40 (1-OH, br)[a] | 155.9 (s) | 10.16 (1-OH, br)[a] |
| 2 | 114.3 (s) | | 112.8 (s) | | 114.9 (d) | 6.69 (1H, br s) |
| 3 | 146.4 (s) | | 147.5 (s) | | 144.9 (s) | |
| 4 | 116.8 (d) | 6.89 (1H, s) | 122.7 (d) | 6.67 (1H, s) | 121.0 (d) | 6.63 (1H, br s) |
| 4a | 136.4 (s) | | 141.8 (s) | | 140.3 (s) | |
| 5 | 126.5 (d) | 7.56 (1H, d, 9.4) | 37.2 (t) | 2.82 (1H, br d, 16.6), 2.99 (1H, br d, 16.6) | 37.1 (t) | 2.79 (1H, dd, 16.4, 2.8), 2.97 (1H, dd, 16.4, 1.6) |
| 6 | 121.1 (d) | 8.12 (1H, d, 9.4) | 56.8 (d) | 5.19 (1H, br s), 5.03 (6-OH, br) | 57.1 (d) | 5.19 (1H, br s), 4.95 (6-OH, br) |
| 6a | 118.9 (s) | | 131.2 (s) | | 131.4 (s) | |
| 7 | 159.3 (s) | 13.78 (7-OH, s) | 158.5 (s) | 12.50 (7-OH, s) | 158.5 (s) | 12.70 (7-OH, br) |
| 7a | 107.7 (s) | | 113.1 (s) | | 113.2 (s) | |
| 8 | 189.2 (s) | | 189.3 (s) | | 188.2 (s) | |
| 8a | 106.9 (s) | | 109.1 (s) | | 108.0 (s) | |
| 9 | 165.0 (s) | 12.92 (9-OH, s) | 164.4 (s) | 12.06 (9-OH, s) | 164.7 (s) | 12.17 (9-OH, br) |
| 10 | 100.9 (d) | 6.28 (1H, d, 2.2) | 107.8 (d) | 6.54 (1H, d, 2.2) | 107.8 (d) | 6.46 (1H, br s) |
| 11 | 165.6 (s) | 10.96 (11-OH, br)[a] | 165.6 (s) | 11.40 (11-OH, br)[a] | 168.1 (s) | 10.16 (11-OH, br)[a] |
| 12 | 106.8 (d) | 6.78 (1H, d, 2.2) | 108.8 (d) | 7.14 (1H, d, 2.2) | 110.4 (d) | 7.07 (1H, d, 1.6) |
| 12a | 155.1 (s) | | 135.3 (s) | | 135.2 (s) | |
| 13 | 39.0 (s) | | 181.3 (s) | | 182.0 (s) | |
| 13a | 145.7 (s) | | 131.5 (s) | | 131.5 (s) | |
| 14 | 116.5 (d) | 10.12 (1H, s) | 119.9 (d) | 8.84 (1H, s) | 119.8 (d) | 8.90 (1H, s) |
| 14a | 137.6 (s) | | 139.9 (s) | | 137.4 (s) | |
| 14b | 117.5 (s) | | 117.4 (s) | | 116.1 (s) | |
| 15 | 171.6 (s) | | 173.3 (s) | | | |
| 16 | 35.8 (t) | 3.20 (2H, br t, 7.6) | 35.5 (t) | 2.90 (2H, m) | 34.9 (t) | 2.49 (2H, m) |
| 17 | 31.3 (t) | 1.60 (2H, m) | 31.1 (t) | 1.54 (2H, m) | 30.1 (t) | 1.57 (2H, m) |
| 18 | 31.7 (t) | 1.32 (2H, m) | 31.6 (t) | 1.32 (2H, m) | 31.0 (t) | 1.30 (2H, m) |
| 19 | 22.2 (t) | 1.32 (2H, m) | 22.0 (t) | 1.32 (2H, m) | 22.0 (t) | 1.33 (2H, m) |
| 20 | 14.0 (q) | 0.87 (3H, t, 7.0) | 13.9 (q) | 0.87 (3H, t, 6.6) | 13.9 (q) | 0.87 (3H, t, 6.8) |
| 21 | 34.4 (q) | 1.74 (3H, s) | | | | |
| 22 | 34.4 (q) | 1.74 (3H, s) | | | | |

[a] The intensity of this broad signal corresponded to two hydroxy groups.

Fig. 3. $^{1}H$-$^{13}C$ correlation for bequinostatin A by the HMBC experiment.

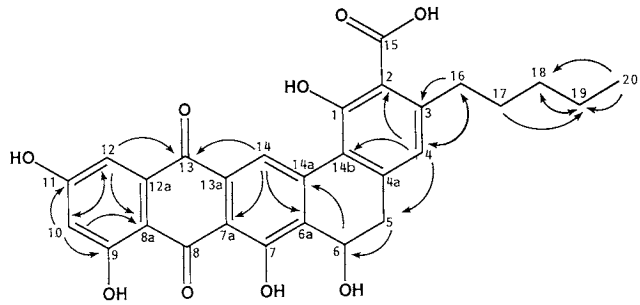
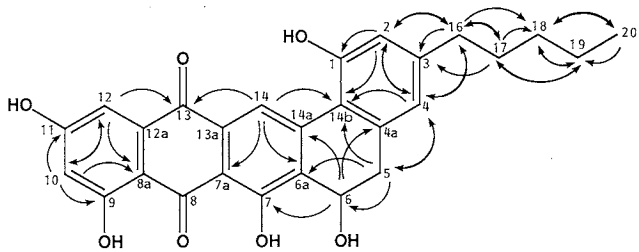


Fig. 4. $^{1}H$-$^{13}C$ correlation for bequinostatin B by the HMBC experiment.



peaks at $m/z$ 461 $(M+H)^+$ and $m/z$ 459 $(M-H)^-$ and $^{1}H$ and $^{13}C$ NMR spectra of **3** (Table 2). The UV and IR spectra, and the $^{1}H$ and $^{13}C$ NMR spectra of **3** were similar to those of **2**. **2** showed a carbonyl carbon signal at $\delta_C$ 173.3 (C-15) ppm, but this corresponding signal was not observed in the $^{13}C$ NMR spectrum of **3**. Instead of this carbon signal, an additional aromatic proton signal appeared at $\delta_H$ 6.69 (2-H) ppm and coupled with an aromatic proton at $\delta_H$ 6.63 (4-H) ppm in the $^{1}H$-$^{1}H$ COSY spectrum of **3**. Therefore, it was deduced that the aromatic proton located at *meta* position (C-2) to the 4-H. Furthermore, in the HMBC spectrum of **3** (Fig. 4), the aromatic proton at $\delta_H$ 6.69 (2-H) ppm coupled to four carbon at $\delta_C$ 155.9 (C-1), 121.0 (C-4), 116.1 (C-14b) and 34.9 (C-16) ppm. Consequently, it was determined that **3** was 2-decarboxybequinostatin A or 5,6,8,13-tetrahydro-1,6,7,9,11-pentahydroxy-8,13-dioxo-3-pentylbenzo[*a*]naphthacene (Fig. 1).

Table 3. Effect of bequinostatin B on LPS induced murine lymphocyte blastogenesis *in vitro*.

| Dose ($\mu$g/ml) | [$^{3}H$]Thymidine incorporation (mean dpm $\pm$ SD) | Stimulatory index (%)[a] |
|---|---|---|
| 0 | 14,762 $\pm$ 648 | 100.0 |
| 0.39 | 18,308 $\pm$ 642 | 124.0 |
| 0.78 | 22,420 $\pm$ 2,182 | 151.9 |
| 1.56 | 25,302 $\pm$ 1,564 | 171.4 |
| 3.13 | 30,416 $\pm$ 3,675 | 206.0 |
| 6.25 | 7,058 $\pm$ 434 | 47.8 |
| 12.5 | 118 $\pm$ 4 | 0.8 |

[a] (dpm of treated)/(dpm of non-treated) $\times$ 100.

Biological Activities of **2** and **3**

**2** has a significant inhibitory activity against partially purified GST which contains several isozymes. Considerably more activity was seen against GST $\pi$ which an isozyme of human pi class GST expressed in carcinogenesis[9]. Its $IC_{50}$ values were 4.6 and 0.6 $\mu$g/ml, respectively. **3** inhibited partially purified GST but only slightly the GST $\pi$ enzyme. Its $IC_{50}$ values were 15.0 and 100.0 $\mu$g/ml, respectively.

As reported previously benanostatins C (2-decarboxybenastatin A) and D (2-decarboxybenastatin B)

show excellent stimulatory activity on murine lymphocyte blastogenesis *in vitro*[3]. **3** (2-decarboxybequinostatin A) showed only a slightly stimulatory activity on LPS induced murine lymphocyte blastogenesis (Table 3). The maximum stimulation was observed at 3.13 $\mu$g/ml of **3** and the index was 206.0.

**2** and **3** had no significant antimicrobial activity at 100 $\mu$g/ml. They have no deaths or toxic indications after ip injection into mice at a dose of 100 mg/kg.

Acknowledgment

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