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Study of Structure–Activity Correlation in Destruxins, a Class of Cyclodepsipeptides Possessing Suppressive Effect on the Generation of Hepatitis B Virus Surface Antigen in Human Hepatoma Cells

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A new destruxin [destruxin E2 chlorohydrin] was isolated from the culture medium of *Metarrhizium anisopliae* and its structure was determined by NMR spectroscopy and mass spectrometry. As compared with other destruxins, the new destruxin showed a lower suppressive activity on the production of hepatitis B virus surface antigen in human hepatoma Hep3B cells. NMR study coupled with molecular modeling by computer graphics has revealed that the hydrophobicity nature of the convex surface characteristic of all destruxin molecules plays an important role in their biological activity.

Destruxins are cyclic hexadepsipeptides first isolated from the culture filtrate of *Metarrhizium anisopliae,* an insect-pathogenic fungus [1-5]. Recently, these conformation-constrained peptide compounds were reported to possess inhibitory effects on the proliferation of leukemic cells [6] and the expression of hepatitis B surface antigen (HBsAg) in human hepatoma Hep3B cells [7]. Hepatitis B virus (HBV) is recognized as an infectious agent of worldwide health concern. Infection by HBV often results in acute and chronic hepatitis, which is sometimes associated with high risks of developing primary hepatocellular carcinoma and liver cirrhosis in those affected patients [8-11]. Although vaccination against HBV has been shown to be effective in preventing both vertical and horizontal viral infection [12], effective anti-HBV drugs are still not available and in urgent need for further development.

Cultured human hepatoma Hep3B cells contain one or two copies of integrated HBV DNA and were shown to actively secrete the hepatitis B surface antigen (HBsAg) into the culture medium [13]. We demonstrated previously that the HBsAg gene expression in Hep3B cells can be regulated by various agents, including costunolide and insulin [14-15]. This cell line has thus provided a rapid and effective assay system for screening potential anti-HBV drugs from natural products.

In our continued effort of searching for therapeutically effective anti-HBV agents from the fungus *Metarhizium anisopliae,* we have recently shown that cyclodepsipeptide destruxins strongly suppress *in vitro* the secretion of HBsAg from human hepatoma Hep3B cells [16]. In the present communication, we have identified a new cyclic depsipeptide, destruxin E2 chlorohydrin, in the culture medium of *M. anisopliae,* which can also suppress the secretion of HBsAg in human hepatoma Hep3B cells. We apply NMR analysis and computer graphics to derive three-dimensional structures for several destruxins, synthetic destruxinlactam and

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linear destruxins in order to make a defined structure/activity comparison of these structurally related destruxin analogues with different HBsAg suppressive activities.

MATERIALS AND METHODS

General Experimental Procedures

^{1}H-NMR spectra were obtained on a Bruker AM-400 NMR spectrometer and were reported as ppm downfield from DMSO-d_6(d=2.48). Mass spectral data were obtained on a Jeol JMS-HX 110 mass spectrometer operating in the positive FAB mode. HBsAg enzyme immunoassay (EIA) kits were purchased from Ever New Corp. (Taipei, Taiwan). Fetal calf serum was obtained from Hyclone (Logan, UT, USA). Linear peptides and lactams were synthesized according to the previous report [17].

Computer Modeling

The peptide conformation of destruxin B was determined based on data from the X-ray crystallography [18] and NMR study of desmethyldestruxin B (unpublished results). The conformation of destruxin B_2 was modified from the X-ray crystalline structure of destruxin B by computer modeling on a Silicon Graphics workstation, Iris 4D/35 computer with molecular modeling software. Structures were analyzed, examined and plotted by the computer-modeling system ''Insight II'' graphics program from Biosym Technologies Inc. (version 2.2). Firstly, the initial structure for molecular dynamic calculation was carried out by conjugate gradient minimization for 600 steps. The structure was heated gradually from 0 to 300 K with 1.0-fs integral and equilibrated at 300 K in 200 steps and then computer-simulated for 1000 steps at 300 K. Finally, we extract the average structure from ten structures obtained from computer simulation.

Culture Conditions and Activity Assay

M. anisopliae (ATCC 26474) was cultured in 2-liter flasks containing 1 liter of 3.9% Czapek-Dox broth (Difco) supplemented with 0.5% Bacto-peptone (Difco).

Extraction and isolation. After incubation for 15 days at 26°C on a gyro-rotary incubator run at 120 rpm, the liquid culture was acidified with acetic acid, vacuum-filtered to get rid of the mycelia, and extracted three times with EtOAc. The crude extract was neutralized by the addition of Na_2CO_3 and fractionated repeatedly by silica-gel column chromatography employing a stepwise n-hexane-to-EtOAc solvent gradient to give three fractions. Further purification was carried out by reversed-phase HPLC on a C-18-AR semi-preparative column (5 mm Cosmosil, 6 mm $\times$ 25 cm) using H_2O-MeCN-MeOH (55.0 : 35.5 : 7.5 (v/v), 1.0 ml/min) as the eluent. The following compounds were separated and identified in HPLC: destruxin A (Rt, 16.18 min), A_2 (Rt, 12.62 min), B (Rt, 30.28 min), B_2 (Rt, 22.20 min), E chlorohydrin (Rt, 10.40 min), E_2 chlorohydrin (Rt, 9.73 min); desmethyl-destruxin A (Rt, 12.03 min), B (Rt, 20.93 min); Rt. retention time.

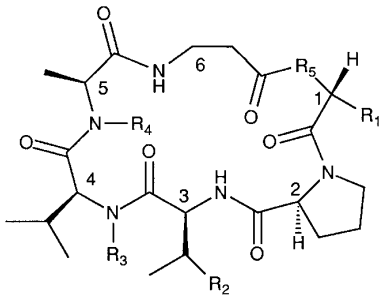
Cell culture. Stock cultures of human hepatoma cells Hep3B were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 units/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The cultures were passaged by trypsinization every fourth day. For bioassay, cells were plated in 24-well plates at a density of 1.0×10^5 cells/well in DMEM containing 10% fetal calf serum.

Preparation of test compounds. For bioassay, the compound was first dissolved in EtOH, filtered through a 0.25 μm fluoropore filter (Millipore) and added to cell cultures.

Determination of HBsAg. Hep3B cells were seeded in 24-well plates at a density of 1.0×10^5 cells/well in DMEM containing 10% fetal calf serum. After 24 h incubation, cells were washed three times with phosphate-buffered saline (pH 7.0) and incubated in serum-free DMEM containing various concentrations of test compounds for 48 h. The HBsAg in the culture medium were routinely measured by enzyme immunoassay (EIA kits). The viability of cells was determined by trypan blue exclusion assay and counted in a hemocytometer.

RESULTS AND DISCUSSION

In the present study, one novel and unique analogue of destruxins was isolated and purified from the broth medium of fungal culture. We have applied NMR and mass spectral analysis to identify and determine the structure. Further structure-activity comparison of this new destruxin with various existing destruxin analogues should provide some insight into the structural basis underlying the distinct activity differences among these closely related HBsAg suppressive agents, which may further assist in the rational design of these compounds.



1	R_1= $CH_2CH(OH)CH_2Cl$	R_2= H	R_3= CH_3	R_4= CH_3	R_5= O
2	R_1= $CH_2CH=CH_2$	R_2= CH_3	R_3= CH_3	R_4= CH_3	R_5= O
3	R_1= $CH_2CH=CH_2$	R_2= H	R_3= CH_3	R_4= CH_3	R_5= O
4	R_1= $CH_2CH(CH_3)CH_3$	R_2= CH_3	R_3= CH_3	R_4= CH_3	R_5= O
5	R_1= $CH_2CH(CH_3)CH_3$	R_2= H	R_3= CH_3	R_4= CH_3	R_5= O
6	R_1= $CH_2CH(OH)CH_2Cl$	R_2= CH_3	R_3= CH_3	R_4= CH_3	R_5= O
7	R_1= $CH_2CH(OH)CH_2OH$	R_2= CH_3	R_3= CH_3	R_4= CH_3	R_5= O
8	R_1= $CH_2CH=CH_2$	R_2= CH_3	R_3= H	R_4= CH_3	R_5= O
9	R_1= $CH_2CH(CH_3)CH_3$	R_2= CH_3	R_3= H	R_4= CH_3	R_5= O
11	R_1= $CH_2CH(CH_3)CH_3$	R_2= CH_3	R_3= CH_3	R_4= CH_3	R_5= N
12	R_1= $CH_2CH(CH_3)CH_3$	R_2= CH_3	R_3= CH_3	R_4= H	R_5= N
10	(DL-Leucic acid)-Pro-Ile-Val-(N-methyl-Ala)-(β-Ala)				
13	(D-Leu)-Pro-Ile-Val-(N-methyl-Ala)-(β-Ala)				
14	(D-Leu)-Pro-Ile-Val-Ala-(β-Ala)				

FIG. 1. Chemical structures of destruxins. The listed numbers refer to various destruxin analogues in Table 2, *e.g.* compound (**1**) is the newly characterized destruxin E_2 chlorohydrin.

Isolation and Structural Determination of a New Destruxin

Culture broth of *M. anisopliae* was extracted with ethyl acetate and the crude extract was fractionated into acidic and neutral fractions. These two fractions were composed mainly of cyclodepsipeptides, which based on their characteristic ^{1}H-NMR signals could be identified as destruxins. A combination of silica-gel column chromatography and extensive reversed-phase HPLC gave nine compounds, including the new destruxin E_2 chlorohydrin (**1**), and previously described destruxins A (**2**), A_2 (**3**), B (**4**), B_2 (**5**), E chlorohydrin (**6**), E diol (**7**), desmethyldestruxin A (**8**), and B (**9**), all these destruxin analogues (**1-9**) were easily identified and structurally assigned as such by comparison of their ^{1}H-NMR spectral data with those reported previously [2,19-22], as summarized in Fig. 1.

The new destruxin (**1**) has a molecular formula of C_{28} $H_{46}ClN_5O_8$ ($[MH]^+$, m/z 616). The mass spectrum of **1** exhibited a peak ratio for m/z 616/618 of about 2:1. This is indicative of the presence of one chlorine atom in **1.** ^{1}H-NMR spectrum of **1** contained characteristic signals for two N-Me group (2.59 and 3.13 ppm), two amide protons (6.96 and 8.02 ppm), and a methyl doublet (1.18 ppm) that belongs to the alanine side chain. The ^{1}H-NMR spectrum of compound **1** was compared with those of destruxin E chlorohydrin (**6**) and destruxin E diol (**7**) (Table 1). The spectrum of **1** was similar to that of destruxin E chlorohydrin (**6**) except for the absence of the γ-methylene group at Ile_3. The mass and NMR spectroscopic data therefore confirmed that **1** was destruxin E_2 chlorohydrin. The ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY spectra provided additional support and corroborated the proposed structure (Fig. 2).

Activity Assays for HBsAg Suppressive Effect

The cells were plated in 24-well plates and allowed to attach overnight. The medium was subsequently replaced by serum-free Dulbecco's Modified Eagle's Medium (DMEM), and

TABLE 1
^{1}H-NMR Chemical Shifts for Destruxin E2 Chlorohydrin (1), Destruxin E Chlorohydrin (6), and Destruxin E Diol (7)

		Chemical shifts		
		1a	6a	7a
Hia_1	α	5.09 (6.8)	5.07 (6.9)	5.09 t (6.6)
	β	1.96 m	1.95 m	1.91 m
	γ	3.72 d (5.93)	3.72 d (5.93)	3.73 d (5.1)
	δ	3.61 m	3.62 m	4.46 dd (6.5, 11.0), 3.6, 11.2)
	γOH	5.33 d (5.2)	5.33 (5.3)	—
Pro_2	α	4.40 d (7.5)	4.39 d (7.1)	4.38 d (7.7)
	β	2.00 m, 2.09 m	2.00 m, 2.10 m	2.00 m, 2.10 m
	γ	1.76 m	1.75 m	1.75 m
	δ	3.72 m, 3.82 m	3.70 m, 3.80 m	3.68, m, 3.82 m
Val_3/Ile_3	NH	6.96 d (9.5)	6.94 d (9.2)	6.93 d (9.5)
	α	4.82 d (10.7)	4.78 dd (6.2, 9.3)	4.82 dd (8.9, 6.5)
	β	2.06 m	1.81 m	1.81 m
	γ	0.81 d (7.2), 0.84 d (6.8)	1.23 m, 1.39 m	1.20 m, 1.48 m
	δ	—	0.77 d (7.4)	0.77 d (7.6)
	γCH_3	—	0.78 d (6.9)	0.78 d (7.0)
Me-Val_4	NCH_3	3.13 s	3.13 s	3.12 s
	α	4.97 d (10.7)	5.00 d (10.9)	4.98 d (10.8)
	β	2.19 m	2.19 m	2.19 m
	γ	0.85 d (6.8), 0.87 d (6.5)	0.84 d (6.5), 0.87 d (6.5)	0.83 d (6.5), 0.86 d (6.4)
Ala_5	α	5.14 q (6.6)	5.14 q (6.7)	5.15 q (6.8)
	β	1.18 d (6.7)	1.17 d (6.6)	1.15 d (6.7)
	NCH_3	2.59 s	2.54 s	2.50 s
β-Ala_6	NH	8.02 d (8.3)	8.05 d (8.0)	8.04 d (9.6)
	α	2.35 dd (12.0, 1.9)	2.36 dd (11.5, 2.9)	2.38 dd (12.1, 1.4)
		2.75 dd (17.0, 5.2)	2.77 dd (18.5, 4.8)	2.77 dd (19.6, 4,8)
	β	2.94 t (11.9), 3,82 m	2.93 t (11.8), 3.82 m	2.94 t (11.70), 3.83 m

[a] Recorded at 4000 MHz in DMSO-d_6 and assigned on the basis of a ^{1}H-^{1}H and ^{1}H-^{13}C COSY NMR experiment. Chemical shifts are expressed in δ values, with coupling constants (J in Hz) in parentheses.

various concentrations of test compounds were added. After 48-h incubation, secreted HBsAg in the medium was determined. The results showed that destruxin E_2 chlorohydrin suppressed HBsAg production of Hep3B cells with an IC_{50} of about 0.10 μM. The drug itself did not interfere with the enzyme immunoassay used for HBsAg determination. The suppressive effect of destruxin E_2 chlorohydrin was not due to the cytotoxicity of the compound since treated cells were still viable and continued to proliferate during the 48-h period of incubation. The isolated compounds (**1-9**) and the linear-chain molecule (**10**), resulting from the ring-opening of destruxin B, and the synthesized desmethyldestruxin B lactam (**11**), protodestruxin lactam (**12**) plus linear molecules of uncyclized destruxin lactam analogues (**13,14**) were assayed for their activities. The inhibitory concentrations (IC_{50}, in μM) for these 14 destruxins were listed in Table 2.

Structure/Activity Correlation of Various Destruxin Analogues

From the comparison of IC_{50} values (Table 2), the following structure/function relationships among these related destruxins can be established:

(*a*) Destruxin A (**2**) and destruxin B (**4**) possess higher anti-HBsAg activity than destruxin A_2 (**3**) and destruxin B_2 (**5**) respectively, which points to the fact that the side chain of the third residue has some effect on the biological activity. The more hydrophobic is the side chain of the third residue, the more potent is the biological activity of the destruxin molecule.

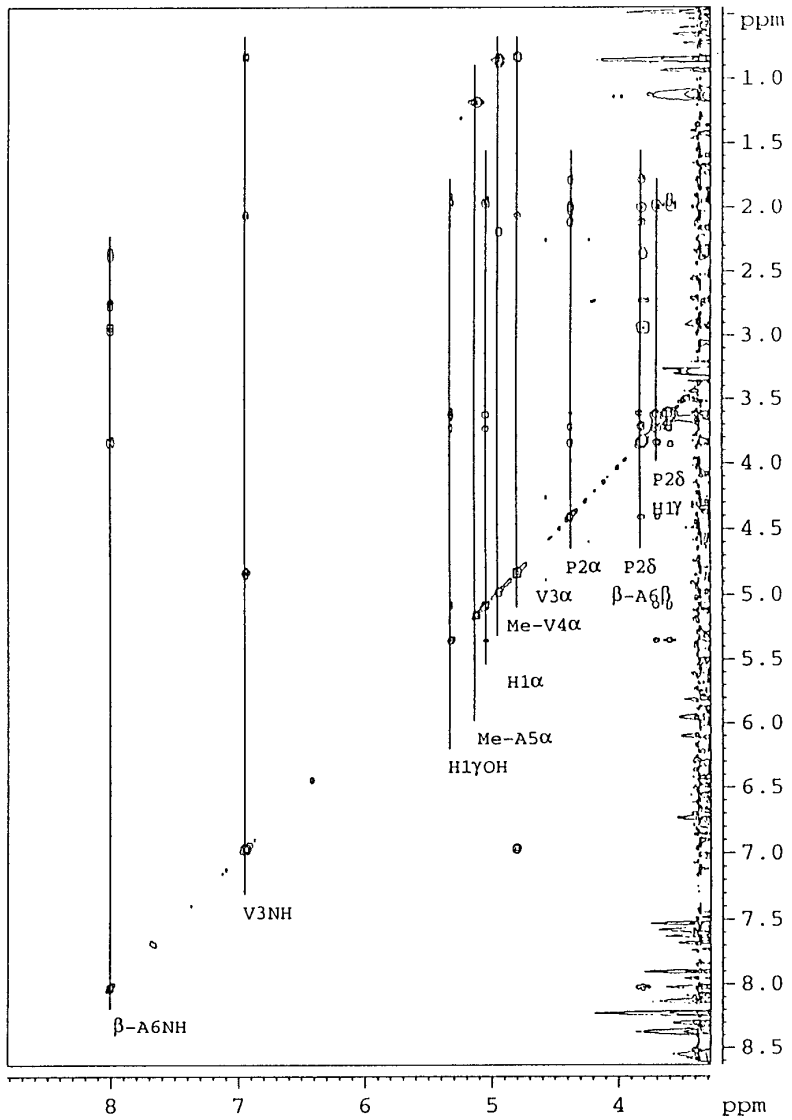


FIG. 2. ^{1}H-^{1}H TOCSY spectrum of destruxin E_2 chlorohydrin (**1**).

(*b*) Destruxin A (**2**) and destruxin B (**4**) are also more active than desmethyldestruxin A (**8**) and desmethyldestruxin B (**9**). It revealed that the N-methyl group in the fourth residue also plays a role in the biological activity.

(*c*) Destruxin A (**2**) and destruxin B (**4**) show higher activities than destruxin E chlorohydrin (**6**) and destruxin E diol (**7**), which in turn possess higher biological activities than **1.** This would indicate that the hydrophobicity of side chain in the first residue of these destruxins also influences strongly the biological activity.

(*d*) Desmethyldestruxin B (**9**) shows a strong biological activity whereas desmethyldestruxin B lactam (**11**) has no activity. The lactone part seems to be essential for the biological activity.

The structure of destruxin B solved by X-ray crystallographic analysis showed a bent rectangle fixed by two hydrogen bonds and possesses a hydrophobic convex surface and

TABLE 2
Effects of Various Destruxins on HBsAg Secretion in Hep3B Cells[a]

Compound	IC_{50} (μM)[b]
1	6.50
2	0.20
3	1.00
4	0.20
5	1.30
6	0.50
7	0.60
8	0.50
9	0.60
10	—[c]
11	—[c]
12	—[c]
13	—[c]
14	—[c]

[a] All compounds in different concentrations were tested in triplicate for their effects on the secretion of HBsAg by Hep3B cells in 48 h [7, 13].

[b] IC_{50} is the concentration of destruxins which affords 50% reduction in HBsAg secretion by Hep3B cells.

[c] HBsAg secretion for these compounds does not change as compared with the control test.

a hydrophilic concave surface (Fig. 3) [18]. Neither the linear molecule, resulting from the opening of destruxin B, nor the synthetic linear hexapeptide and destruxin lactam analogues showed any suppressive effect on the secretion of HBsAg. Therefore the bent rectangular structure of destruxins may be a key feature for maintaining biological activity. Furthermore, from the study of structure-function relationship, the hydrophobic convex surface is considered to be important for the biological activity. The slight modification in the hydrophobic convex surface of the molecule, not only in the central part (**residues 3 and 4**) but also on the rim side (**residue 1**), would evoke drastic change in the biological activity. Judging from the results of molecular modeling (Fig. 3), the 19-member ring conformation of destruxins seemed to be common for these molecules. However, when comparing the IC_{50} value of (**4**) with those of (**5**) and (**9**), we found that the stronger is the hydrogen bond in the molecule, the more potent is the biological activity. In addition, the hydrophobicity of side chains and N-methyl group in the residues 1, 3 and 4 would also affect the rigid rectangular conformation of destruxin and thus influence their biological activity. In the case of AM-Toxin II, a cyclodepsitetrapeptide isolated from the culture filtrate of *Alternaria mali*, which causes venial necrosis on apple leaves, the hydrophobic side chain of the first residue (L-2-amino-5-phenylpentanoic acid, L-App) in this toxin is the most important part for its biological activity [23]. When L-App was replaced by a less hydrophobic L-Phe, [L-Phe^1]-AM-toxin showed a much weaker activity than the native toxin. This seems to strengthen our conclusion that the more hydrophobic lactone in D-leucic acid and N-methyl group in Ala of these destruxins are crucial in maintaining the basic ''bent rectangle'' structure which is essential for the biological activity. Furthermore,

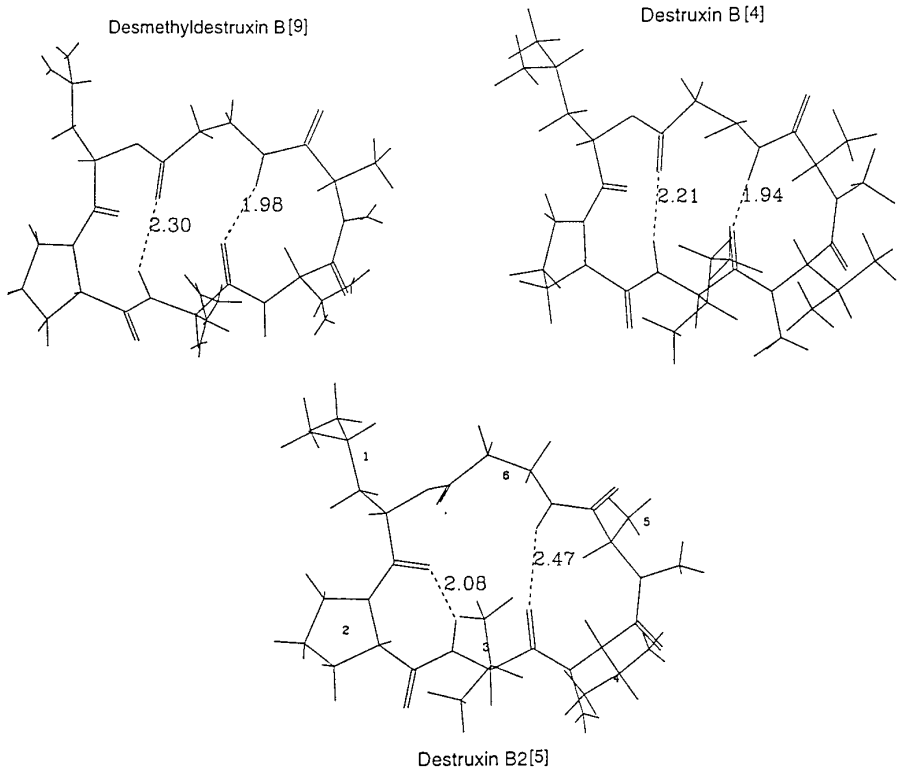


FIG. 3. The mean NMR-derived structures of destruxin B (**4**), destruxin B_2 (**5**), and desmethyldestruxin B (**9**). The dashed lines with numerical values indicate distances (in Å) for two hydrogen bonds proposed to be important for maintaining the bent rectangular conformation of destruxins.

the hydrophobicity in the convex surface of destruxins may also affect the flexibility of these molecules in solution and thus influence their biological activity.

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REFERENCES

1. Kodaira, Y. (1961) *Agr. Biol. Chem.* **25,** 261–262.
2. Suzuki, A., and Tamura, S. (1972) *Agric. Biol. Chem.* **36,** 896–898.
3. Suzuki, A., Taguchi, H., and Taumura, S. (1970) *Agric. Biol. Chem.* **34,** 813–816.
4. Suzuki, A., Kawakami, K., and Taumura, S. (1971) *Agric. Biol. Chem.* **35,** 1641–1643.
5. Ayer, W. A., and Pena-Rodriguez, L. M. (1987) *J. Nat. Prod.* **50,** 408–417.
6. Odier, F., Vey, A., and Bureau, J. P. (1992) *Biol. Cell* **74,** 267–271.
7. Sun, C. M., Chen, H. C., and Yeh, S. F. (1994) *Planta Med.* **60,** 87–88.
8. Beasley, R. P., Hwang, L. Y., Lin, C. C., and Chien, C. S. (1981) *Lancet* **2,** 1129–1133.
9. Brechot, C., Pourecel, C., Louise, A., Rain, B., and Tiollais, P. (1980) *Nature* **286,** 533–535.
10. Lo, K. J., Tong, M. J., Chien, M. C., Tsai, Y. T., Liaw, Y. F., Yang, K. C., Chian, H., Liu, H. C., and Lee, S. D. (1982) *J. Infect. Dis.* **146,** 205–210.
11. Shafritz, D. R., Shouval, D., Sherman, H. I., Hadziyannis, S. J., and Kew, M. C. (1981) *New Engl. J. Med.* **305,** 1067–1073.
12. Lo, K. J., Lee, S. D., Tsai, Y. T., Wu, T. C., Chan, C. Y., Chen, G. H., and Yeh, C. L. (1988) *Hepatology* **6,** 1647–1650.
13. Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. B. (1979) *Nature* **282,** 615–616.

14. Chen, H. C., Chou, C. K., Lee, S. D., Wang, J. C., and Yeh, S. F. (1995) *Antiviral Res.* **27,** 99–109.
15. Lin, Y. L., Chen, H. C., Yeh, S. F., and Chou, C. K. (1995) *Endocrinology* **136,** 2922–2927.
16. Chen, H. C., Yeh, S. F., Ong, G. T., Wu, S. H., Sun, C. M., and Chou, C. K. (1995) *J. Nat. Prod.* **58,** 527–531.
17. Chiou, A. J., Ong, G. T., Wang, K. T., Chiou, S. H., and Wu, S. H. (1996) *Biochem. Biophys. Res. Comm.* **219,** 572–579.
18. Steiner, J. R., and Barnes, C. L. (1988) *Int. J. Peptide Protein Res.* **31,** 212–219.
19. Pais, M., Das, B. C., and Ferron, P. (1981) *Phytochemistry* **20,** 715–723.
20. Gupta, S., Roberts, D. W., and Renwick, R. (1989) *J. Chem. Soc., Perkin Trans.* **I,** 2347–2357.
21. Jegorov, A., Matha, V., Sedmera, P., and Roberts, D. W. (1992) *Phytochemistry* **31,** 2669–2670.
22. Wahlman, M., and Davidson, B. S. (1993) *J. Nat. Prod.* **56,** 643–647.
23. Shimohigashi, Y., and Izumiya, N. (1978) *Int. J. Peptide Protein Res.* **12,** 7–16.