Pergamon

Tetrahedron Letters 42 (2001) 41–44

TETRAHEDRON LETTERS

Spiruchostatins A and B, novel gene expression-enhancing substances produced by *Pseudomonas* sp.

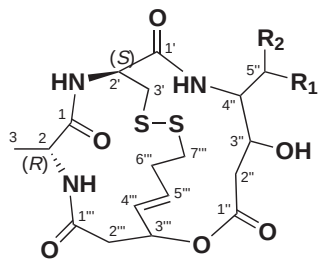
Yuhta Masuoka,[a] Atsuko Nagai,[a] Kazuo Shin-ya,[a,*] Kazuo Furihata,[b] Koji Nagai,[c] Ken-ichi Suzuki,[c] Yoichi Hayakawa[a] and Haruo Seto[a]

[a]*Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan*
[b]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan*
[c]*Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co. Ltd., 1-1-8, Azusawa Itabashi-ku, Tokyo 174-8511, Japan*

Received 11 September 2000; accepted 20 October 2000

Abstract—Spiruchostatins A and B were isolated from *Pseudomonas* sp. as gene expression-enhancing substances. They possess novel bicyclic depsipeptides involving 4-amino-3-hydroxy-5-methylhexanoic acid and 4-amino-3-hydroxy-5-methylheptanoic acid residues, respectively, as shown in Fig. 1.

A variety of growth factors and cytokines control proliferation, differentiation and cell survival in various types of cells by regulating gene expression. Transforming growth factor-β (TGF-β) was first discovered as a trophic factor to some cell lines,[1] but is now widely known as a growth inhibitor to various kinds of mammalian cells.[2] The loss of this negative regulation is thought to contribute to tumor development. Studies have shown that TGF-β suppresses the growth of certain cancer cell lines,[3] and restoration of TGF-β signaling in LNCaP human prostate cancer cells suppresses tumorigenicity by inducing apoptosis.[4] Furthermore, it was reported that the reduction of TGF-β type I receptor expression contributes to the malignancy of human colon carcinoma cells.[5] The mode of action of TGF-β is not fully understood, but it has been revealed to express a variety of genes such as a tumor suppression gene *p21*,[6] plasminogen activator inhibitor-1 (PAI-1)[7] to suppress tumor progression. Thus, substances which mimic the gene expression-enhancing activity of TGF-β are expected to be useful chemotherapeutic agents for cancer treatment.



spiruchostatin A (**1**): $R_1 = R_2 = {}^{6'',7''}CH_3$

spiruchostatin B (**2**): $R_1 = {}^{6''}CH_2{}^{7''}CH_3$, $R_2 = {}^{8''}CH_3$

Figure 1. Structures of spiruchostatins A (**1**) and B (**2**).

For detecting TGF-β induced gene expression, we constructed a screening system utilizing reporter gene expression. Mink lung epithelial (Mv1Lu) cells, which express excess TGF-β receptors and respond to TGF-β resulting in the expression of plasminogen activator inhibitor-1 (PAI-1), were transfected with the firefly luciferase reporter gene at the downstream of the PAI-1 promoter gene.[8] During the course of our screening using this system, we isolated histone deacetylase inhibitors such as trichostatin A (TSA)[9] and diheteropeptin.[10] Further investigation resulted in the isolation of novel metabolites with gene expression-enhancing activities, spiruchostatins A and B (**1**[11] and **2**,[12] respectively Fig. 1). We report herein the fermentation, isolation and structure determination of **1** and **2**.

The spiruchostatins A and B producing organism, identified as *Pseudomonas* sp., was cultivated in a seed medium containing glucose 1.0%, potato starch 2.0%, polypepton 0.5%, yeast extract 0.5%, $CaCO_3$ 0.4% (pH

Keywords: spiruchostatin; bicyclic depsipeptide; *Pseudomonas*.
* Corresponding author. Tel.: +81-3-5841-7840; fax: +81-3-5841-8485; e-mail: kshin@iam.u-tokyo.ac.jp

0040-4039/01/$ - see front matter
PII: S0040-4039(00)01874-8

Table 1. ^{13}C and ^{1}H NMR data of **1** and **2** in $CDCl_3$

	1				**2**		
	No.	δ_C	δ_H (multiplicity, J = Hz)		No.	δ_C	δ_H (multiplicity, J = Hz)
Ala	1	171.3		Ala	1	171.2	
	2	52.2	4.21 (dq, J = 4.0, 7.5)		2	52.2	4.22 (dq, J = 4.0, 7.0)
	3	16.5	1.48 (d, J = 7.5, 3H)		3	16.6	1.48 (d, J = 7.0, 3H)
	NH		6.28 (d, J = 4.0)		NH		6.18 (d, J = 4.0)
Cys	1′	169.1		Cys	1′	169.2	
	2′	54.9	4.84 (dt, J = 9.0, 3.5)		2′	54.5	4.87 (dt, J = 9.0, 3.0)
	3′	40.9	3.13 (m)		3′	41.3	3.10 (m)
			3.28 (m)				3.33 (m)
	NH		6.79 (d, J = 9.0)		NH		6.75 (d, J = 9.0)
Ahhx[a]	1″	171.7		Ahhp[b]	1″	171.8	
	2″	39.5	2.68 (d, J = 4.0, 2H)		2″	39.5	2.70 (d, J = 4.0, 2H)
	3″	69.1	4.52 (m)		3″	68.2	4.60 (m)
	4″	63.4	2.77 (ddd, J = 4.0, 7.0, 9.0)		4″	61.7	2.94 (ddd, J = 4.0, 7.0, 9.0)
	5″	29.7	2.34 (m)		5″	36.3	2.05 (m)
	6″	19.7	0.90 (d, J = 7.0, 3H)		6″	27.1	1.21 (m)
	7″	20.6	1.00 (d, J = 7.0, 3H)				1.53 (m)
	NH		7.38 (d, J = 7.0)		7″	11.5	0.89 (t, J = 7.5, 3H)
	OH		3.09 (d, J = 10.0)		8″	15.4	0.90 (d, J = 7.0, 3H)
					NH		7.25 (d, J = 7.0)
					OH		2.93 (m)
Acyl	1‴	170.8		Acyl	1‴	170.6	
	2‴	40.3	2.59 (d, J = 13.0)		2‴	40.7	2.58 (d, J = 13.0)
			3.31 (dd, J = 7.0, 13.0)				3.31 (dd, J = 7.0, 13.0)
	3‴	70.7	5.48 (m)		3‴	70.6	5.48 (m)
	4‴	128.9	5.68 (d, J = 15.0)		4‴	128.6	5.67 (d, J = 15.0)
	5‴	133.3	6.31 (m)		5‴	133.4	6.36 (m)
	6‴	33.1	2.43 (m)		6‴	33.3	2.44 (m)
			2.68 (m)				2.71 (m)
	7‴	40.9	2.73 (m)		7‴	40.5	2.72 (m)
			3.24 (m)				3.20 (m)

^{13}C and ^{1}H NMR spectra were recorded at 125 MHz and 500 MHz, respectively.
[a] Ahhx: 4-amino-3-hydroxy-5-methylhexanoic acid.
[b] Ahhp: 4-amino-3-hydroxy-5-methylheptanoic acid.

7.0) for 3 days at 28°C on a rotary shaker. The seed culture (2 ml) was inoculated to a production medium containing glycerol 3.0%, glucose 1.0%, polypepton 0.5%, meat extract 0.5%, NaCl 0.5%, antifoam NKL5430 0.05% (pH 7.0) and cultivated for 3 days at 28°C. The active principle was extracted from the broth filtrate with EtOAc. The solvent layer was dried over Na_2SO_4, and concentrated to give an oily residue. The residue was applied to a silica gel column and washed with $CHCl_3$–MeOH (20:1) followed by eluting with $CHCl_3$–MeOH (5:1). The active fraction was collected and further purified by Sephadex LH-20 column chromatography ($CHCl_3$–MeOH = 1:1). The active fraction was subjected to centrifugal partition chromatography (CPC) using a solvent system $CHCl_3$–MeOH–H_2O (5:6:4) of lower stationary phase and upper mobile phase in an ascending mode. Pure samples of **1** and **2** were finally obtained by HPLC using a PEGASIL ODS column (Senshu-Pak, 20 i. d.×250 mm) developed with 35% CH_3CN.

The molecular formula of **1** was established as $C_{20}H_{31}N_3O_6S_2$ by a high-resolution FAB-MS spectrum. The nature of the two sulfur atoms was respectively assigned as a disulfide functionality from the FAB-MS. FAB-MS spectra of **1** using thioglycerol as a matrix showed a *quasi* molecular ion at m/z 476, compared to a protonated molecular ion at m/z 474 measured in 3-nitrobenzylalcohol. The difference of 2 mass units is ascribable to reduction of the disulfide to a dithiol by the reducing action of thioglycerol. IR absorptions at 3350 ~ 3400, and 1660 and 1720 cm^{-1} implied the presence of –OH, –NH, amide and ester functions. Together with the IR absorptions, amide or ester carbons observed in the ^{13}C NMR (169.1, 170.8, 171.3 and 171.7 ppm) suggested **1** to be a peptide derivative. The ^{13}C and ^{1}H NMR spectral data are shown in Table 1.

Analysis of the COSY spectrum of **1** revealed the four spin systems. The sequence from an amide proton at 6.79 ppm to methylene protons 3′-H through a α-methine proton 2′-H was detected. An amide carbonyl carbon C-1′ was long-range coupled to 2′-H and 3′-H in the HMBC[13] spectrum of **1**. Eventually, the presence of sulfur atoms in this partial structure was substantiated by the chemical shift C-3′ (40.9 ppm) and the detection of cysteic acid in the acid hydrolysate of the performic acid oxidation product of **1**. These results established a cysteinic moiety in Fig. 2.

The proton spin system from 2″-H to 6″-H through 3″-H, 4″-H and 5″-H was detected in the COSY spectrum, which was also coupled to 3″-OH, 4″-NH and a methyl proton 7″-H, respectively. The 2″-H and 3″-H were long-range coupled to an amide carbonyl carbon C-1″ to prove the presence of a 4-amino-3-hydroxy-5-methylhexanoic acid moiety (Fig. 2).

Another analysis of the COSY spectrum of **1** revealed the sequence from an amide proton at 6.28 ppm to methyl proton 3-H through an α-methine proton 2-H. The methyl proton 3-H was long-range coupled to C-2, and an amide carbonyl carbon C-1 in the HMBC spectrum of **1** revealed the presence of an alanyl moiety (Fig. 2).

The residual moieties involving ester or amide functions were revealed by proton spin systems from 2‴-H to 7‴-H. The long-range couplings from 2‴-H and 3‴-H to an amide carbon C-1‴ as well as the chemical shift of C-3‴ (70.7 ppm) revealed the presence of 3-oxy-4-heptenoyl-7-yl. The (*E*) geometry of the double bond in this acyl component was evident from the vicinal coupling constant of 15 Hz.

The connectivities among these amide and ester functional units were established by long-range couplings from the amide protons to their neighboring carbonyl carbons, i.e. the amide protons at 6.79, 7.38 and 6.28 ppm to C-1, C-1′ and C-1‴, respectively (Fig. 2). Although the long-range coupling from the oxymethine proton 3‴-H to carbonyl carbon C-1″ could not be detected in the HMBC experiment, the connectivity between C-3‴ and C-1″ was established from the low-field chemical shift of 3‴-H (5.48 ppm) caused by an acylated shift. The remaining two methylenes, C-3′ and C-7‴, were connected through a disulfide, vide supra, and the structure of **1** was determined to be a bicyclic depsipeptide (Fig. 2).

The molecular formula of **2** was established as $C_{21}H_{33}N_3O_6S_2$ by high-resolution FAB-MS spectrum. From the physico-chemical properties of **2**,[12] it was assumed to be a derivative of **1**. The ^1H and ^{13}C NMR spectra as well as FAB-MS revealed the presence of an additional methylene unit in **2**. In the COSY experiment, a proton spin system from 2″-H to 7″-H through 3″-H, 4″-H, 5″-H and 6″-H was detected. An additional spin coupling between a methyl proton 8″-H and 5″-H

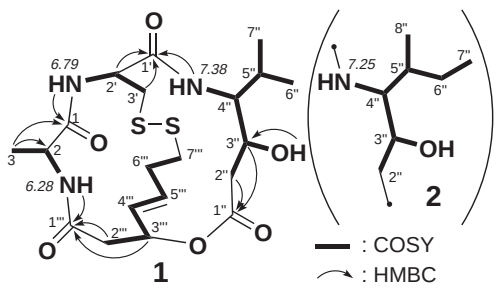


Figure 2. NMR analyses of **1** and **2**.

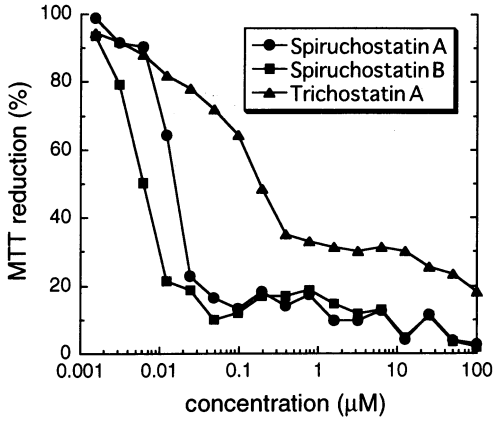


Figure 3. Cytostatic activities of spiruchostatins A (**1**), B (**2**) and trichostatin A.

together with spin couplings involving two exchangeable protons 3″-OH and 4″-NH confirmed a 4-amino-3-hydroxy-5-methylheptanoic acid substructure (Fig. 2). Since the residual subunits and their connectivities were the same as those of **1**, the structure of **2** was determined as shown in Fig. 1.

Stereochemistries of normal amino acid residues Ala and Cys in **2** were revealed using the reagents that can separate stereoisomers of amino acids. **2** was treated with performic acid followed by acid hydrolysis to give an alanine and a cysteic acid. The acid-hydrolyzed products were subjected to ODS column (PEGASIL ODS) and developed with 1 mM $CuAc_2$ and 2 mM *N*,*N*-dimethyl-l-Phe (pH 4.5) as a solvent system. As a result, the stereochemistries of both the Ala and the cysteic acid were determined to be D configuration. The establishment of the remaining stereochemistries of **1** and **2** is now under way. **1** and **2** are structurally similar to FR901228[14] that has the same acyl component containing the disulfide function; however, they have a new 15-membered macrocyclic lactone ring.

In the evaluation system we employed, the treatment of Mv1Lu cells with 40 ng/ml of TGF-β increased the expression of luciferase three times. **1** and **2** induced reporter gene expression under the control of the PAI-1 promoter more than three times at concentrations from 7 nM to 100 μM and 3 nM to 65 μM, respectively. Trichostatin A (TSA) also induced transcriptional activation, but its activity was one-tenth of that of **1** and **2**. TSA was reported to show strong cytostatic activity to a variety of cell lines. In order to compare the cytostatic activities of **1**, **2** and TSA, we evaluated the effects of **1** and **2** on cell growth using the MTT reduction (Fig. 3) and LDH release method.[15] **1** and **2** exhibited the cytostatic effects to Mv1Lu cells with IC_{50} values at 15.2 nM and 6.1 nM, respectively, whereas that of TSA was 180 nM. No LDH release was observed at the growth inhibitory concentration, suggesting that **1** and **2** exhibited cytostatic activity without cytotoxicity in Mv1Lu cells. Detailed investigations on other biological activities of **1** and **2** are now under way.

Acknowledgements

We are grateful to Dr. T. Natsume of Mikoshiba Calciosignal Net Project, Exploratory Research for Advanced Technology (ERATO) for providing us with Mv1Lu cells transfected with the luciferase reporter gene. This work was supported in part by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists to Y.M., Research for the Future, Japan Society for the Promotion of Sciences to H.S., a Grant-in-Aid for Scientific Research on Priority Areas to Y.H.

References

1. Moses, H. L.; Branum, E. L.; Proper, J. A.; Robinson, R. A. *Cancer Res.* **1981**, *41*, 2842–2848.
2. Moses, H. L.; Yang, E. Y.; Pietenpol, J. A. *Cell* **1990**, *63*, 245–247.
3. Voss, M.; Wolff, B.; Savitskaia, N.; Ungefroren, H.; Deppert, W.; Schmiegel, W.; Kalthoff, H.; Naumann, M. *Int. J. Oncol.* **1999**, *14*, 93–101.
4. Guo, Y.; Kyprianou, N. *Cancer Res.* **1999**, *59*, 1366–1371.
5. Wang, J.; Han, W.; Zborowska, E.; Liang, J.; Wang, X.; Willson, J. K. V.; Sun, L.; Brattain, M. G. *J. Biol. Chem.* **1996**, *271*, 17366–17371.
6. Li, C. Y.; Suardet, L.; Little, J. B. *J. Biol. Chem.* **1995**, *270*, 4971–4974.
7. Lund, L. R.; Riccio, A.; Andreasen, P. A.; Nielsen, L. S.; Kristensen, P.; Laiho, M.; Saksela, O.; Blasi, F.; Dano, K. *EMBO J.* **1987**, *6*, 1281–1286.
8. Abe, M.; Harpel, J. G.; Metz, C. N.; Nunes, I.; Loskutoff, D. J.; Rifkin, D. B. *Anal. Biochem.* **1994**, *216*, 276–284.
9. Yoshida, M.; Kijima, M.; Akita, M.; Beppu, T. *J. Biol. Chem.* **1990**, *265*, 17174–17179.
10. Masuoka, Y.; Shin-ya, K.; Kim, Y.-B.; Yoshida, M.; Nagai, K.; Suzuki, K.; Hayakawa, Y.; Seto, H. *J. Antibiot.* **2000**, *53*, 788–792.
11. $[\alpha]_D = -63.6°$ (*c* 0.14, MeOH); UV (MeOH): end absorption; IR ν_{max} (KBr): 3400, 3350, 1720, 1660, 1520, 1260, 980 cm^{-1}; HRFAB-MS (*m*/*z*) 474.1735 $(M+H)^+$ (calcd for $C_{20}H_{32}N_3O_6S_2$: 474.1733).
12. $[\alpha]_D = -58.6°$ (*c* 0.11, MeOH); UV (MeOH): end absorption; IR ν_{max} (KBr): 3400, 3350, 1720, 1660, 1520, 1260, 980 cm^{-1}; HRFAB-MS (*m*/*z*) 488.1865 $(M+H)^+$ (calcd for $C_{21}H_{34}N_3O_6S_2$: 488.1889).
13. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
14. Ueda, H.; Nakajima, H.; Hori, Y.; Fujita, T.; Nishimura, M.; Goto, T.; Okuhara, M. *J. Antibiot.* **1994**, *47*, 301–310.
15. Decker, T.; Lohmann-Matthes, M.-L. *J. Immunol. Methods* **1988**, *115*, 61–69.