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NOTES

Sulphostin, a Potent Inhibitor for Dipeptidyl Peptidase IV from *Streptomyces* sp. MK251-43F3

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In the course of screening for new microbial bioactive metabolites, the culture broth of *Streptomyces* sp. MK251-43F3 showed inhibitory activity in the dipeptidyl peptidase IV (EC 3.4.14.5, DPP-IV, CD26) assays[1]. DPP-IV, one of exopeptidases, is featured by the preferential cleavage of X-L-Pro and X-L-Ala from the NH_2 terminus of oligopeptides[2] and cleaves several biologically important peptides including chemokines[3~5] and peptide hormones[6,7]. Therefore, DPP-IV inhibitors are currently tested as therapeutic agents for immune-related disorders[8,9] and type 2 diabetes[10,11]. We isolated sulphostin (**1**) from the culture broth of *Streptomyces* sp. MK251-43F3 together with its epimer (**2**), which was found to be formed during the isolation process (Fig.1). In this paper, we briefly report on the production, isolation and structure elucidation of **1** and **2**.

The producing strain *Streptomyces* sp. MK251-43F3 was isolated from a soil sample collected in Sado-gun, Niigata Prefecture, Japan, and identified as a species of *Streptomyces* on the basis of taxonomic characterizations.

The producing organism was cultivated at 27°C for 6 days on a rotary shaker (180 rpm) in 500-ml Erlenmeyer flasks containing 110 ml of a medium consisting of potato starch 1.0%, glucose 4.0%, soybean meal 1.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.3%, $CuSO_4 \cdot 5H_2O$ 0.07%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MnCl_2 \cdot 4H_2O$ 0.08%, $ZnSO_4 \cdot 7H_2O$ 0.02%, $CaCO_3$ 0.2% and 1 drop of antifoaming agent (pH adjusted to 7.4 with 2 N NaOH before sterilization). The antifoaming agent consisted of silicone oil KM-70 (Shin-etsu Chemical Industry) and soybean oil (1 : 1).

The decolorized filtrate (105 liters), obtained by activated charcoal treatment of the broth filtrate, was sequentially passed through a column of Dowex 50 [H^+] and Dowex 1 [OH^-] three times. The active fraction was finally adsorbed onto the third circle of Dowex 1 column. The active material was eluted with 0.2 M aq. AcOH. The eluate was adjusted to pH 6.5 with Dowex WGR [OH^-], and lyophilized to yield a crude powder (41 g). The crude powder was subjected to a column chromatography using microcrystalline cellulose (*n*-BuOH - AcOH - H_2O (3 : 1 : 1)) and then to centrifugal partition chromatography (*n*-BuOH - AcOH - H_2O (5 : 1 : 5)) for seven times to give 3.7 mg of colorless powder (**A**), which was found to be a mixture of **1** and **2** by following studies.

By negative ion HRFAB-MS, the molecular formula of **A** was found to be $C_5H_{13}N_4O_5PS$ (*m/z* 271.0276, calcd for $C_5H_{12}N_4O_5PS$ $[M-H]^-$ 271.0266), implying 5 degrees of unsaturation. In addition, the presence of a sulfonic acid group was suggested by a fragment ion at *m/z* 191.0712 (requires $[M-SO_3H]^-$ 191.0698).

The ^1H and ^{13}C NMR data ($D_2O+CD_3CO_2D$, pD 6.0) for **A** are summarized in Table 1. A pair of 3-H signals in **1** and **2** (δ_H 4.17 and 4.15) in pD 6.0 shifted to δ_H 3.38 in pD 8.0

Fig. 1. Structures of sulphostin (**1**) and its epimer (**2**).

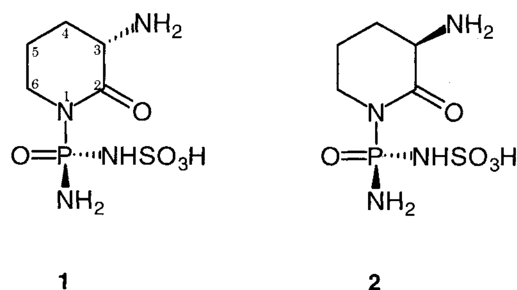

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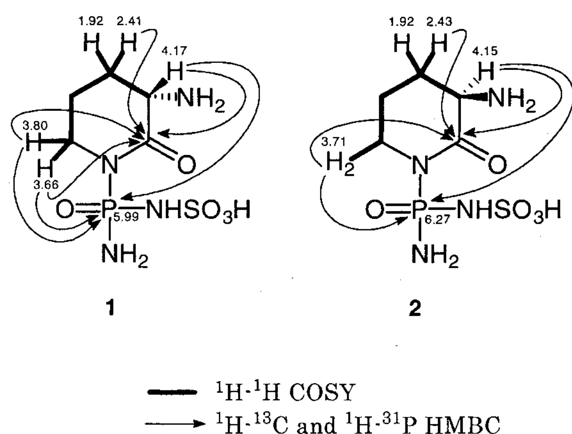
Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **2**.

	1		**2**	
Position	δC ppm (multiplicity)	δH ppm (multiplicity, *J* in Hz)	δC ppm (multiplicity)	δH ppm (multiplicity, *J* in Hz)
2	172.45 (s)		172.42 (s)	
3	51.38 (d)	4.17 (1H, dd, 7.0, 11.6)	51.40 (d)	4.15 (1H, dd, 6.9, 11.9)
4	24.27 (t)	2.41 (1H, m) 1.92 (1H, m)	24.68 (t)	2.43 (1H, m) 1.92 (1H, m)
5	20.55 (t)	2.13 (1H, m) 1.96 (1H, m)	21.01 (t)	2.06 (2H, m)
6	45.49 (t)	3.80 (1H, m) 3.66 (1H, m)	45.75 (t)	3.71 (2H, m)

Fig. 2. ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{31}P HMBC experiments of a mixture of **1** and **2**.



(D_2O+ND_4OD) indicating that each C-3 in **1** and **2** had an amino group. Analyses of the ^{1}H-^{1}H COSY, ^{13}C NMR (125 MHz), DEPT and HMQC spectra suggested that **A** was a mixture of stereoisomers and the both contained ornithine skeleton (Fig. 2). The ^{1}H-^{13}C HMBC experiment clearly indicated long-range cross peaks from each two pairs of 6-H_2 and 3-H to a pair of carbonyl carbon (C-2), respectively. This information indicated the presence of ornithine lactam, that is: 3-amino -2-piperidone moieties in **1** and **2**.

The ^{31}P NMR spectrum (200 MHz) exhibited a pair of signal in almost the same intensity at δ_P 5.99 and 6.27 (85% H_3PO_4 as an external ref. =0 ppm). The long-range spin couplings between ^{31}P and ^{13}C were observed in the ^{13}C NMR spectrum (C-3: δ_C 51.38 ($^{3}J_{P\text{-}N\text{-}C}$=6.28 Hz), δ_C 51.40 ($^{3}J_{P\text{-}N\text{-}C}$=5.03 Hz), C-6: δ_C 20.55 ($^{3}J_{P\text{-}N\text{-}C}$=5.03 Hz), δ_C 21.01 ($^{3}J_{P\text{-}N\text{-}C}$=5.03 Hz)). The ^{1}H-^{31}P HMBC spectrum showed long-range couplings from 6-H_2 and 3-H in the piperidone moiety to a phosphorus. By the interpretation of HRFAB-MS spectra, the remaining partial structure containing a phosphorous was determined to be an amino(sulfoamino)phosphinyl group which was already found in phaseolotoxin and octicidin isolated from a *Pseudomonas* and its infected plant[12]. Thus, planar structure of **A** was elucidated to be a mixture of the stereoisomers of 3-amino-1-[amino(sulfoamino)phosphinyl]-2-piperidone.

Acid hydrolysis (6 N HCl) of **A** yielded DL-ornithine, which was proved by ORD spectrum of the di-2,4-dinitrophenyl derivative. Furthermore, HPLC analysis of **A** on CHIROBIOTIC T for chiral separation showed only two peaks (RT; 76.5 and 70 minutes at 215 nm) with the same integrated area (MeOH - AcOH - triethylamine (100 : 0.015 : 0.01)). Therefore, it was concluded that **A** was a mixture of two diastereomers at C-3 of 3-amino-1-[amino(sulfoamino)phosphinyl]-2-piperidone.

To minimize the epimerization during the isolation process, the decolorized filtrate (1.7 liters) was purified under milder conditions than the previous procedure. The decolorized filtrate was lyophilized and the lyophilized material (44 g) was sequentially washed with EtOH and MeOH. The insoluble residue (6.6 g) was subjected to a column of microcrystalline cellulose (*n*-BuOH - AcOH - H_2O (3 : 1 : 1)) three times. The active fraction (376 mg) was developed onto DEAE Sephadex A-25 column with 0.2 M aq. NH_4HCO_3 two times. The active fraction (8.3 mg) was applied to HPLC on Shodex IEC DEAE-825 (0.2 M aq.

NH_4HCO_3) to give a colorless powder (less than 0.1 mg). The ^{31}P NMR spectrum showed two signals at δ_P 6.03 and 6.31, but the integration ratio of these signals was approximately 4 : 1. On HPLC analysis by CHIROBIOTIC T, two peaks (RT; 76.5 and 70 minutes) were detected, and the integration ratio of these peaks was 3.8 : 1. These results indicated that the major product is natural sulphostin and the minor one is the epimerized product during the isolation.

As described above, production of sulphostin by fermentation was extremely hard due to low productivity, tedious isolation procedure and unavoidable epimerization during the isolation process. Recently, we are successful in chemical syntheses of sulphostin (**1**) and its three diastereomers. The four stereoisomers were identified by separation by HPLC using CHIROBIOTIC T. The X-ray crystal analysis of synthesized **1** revealed that the absolute configurations of the C-3 and the phosphorous atoms of **1** are *S* and *R*, respectively. Compound **1** was found to be readily epimerized under basic conditions to yield **2**. Thus, the structure of sulphostin was determined to be 3(*S*)-amino-1-[(*R*)-amino(sulfoamino)phosphinyl]-2-piperidone. The synthetic studies will be reported separately.

Sulphostin exhibited inhibitory activities to DPP-IV with dose-dependent manner, and the IC_{50} value was 6.0 ng/ml. This activity was 100-fold stronger than that of diprotin A, a known DPP-IV inhibitor discovered in our Institute[13].

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