Stachybocins, Novel Endothelin Receptor Antagonists, Produced by *Stachybotrys* sp. M6222

II. Structure Determination of Stachybocins A, B and C

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The structures of stachybocins A, B and C, new endothelin receptor antagonists, were determined by NMR spectral analysis using pulse-field-gradient techniques. Stachybocin A consists of two spirobenzofuran units each fused to a substituted decalin, which were connected by a lysine residue. Stachybocins B and C are derivatives of stachybocin A with an additional hydroxy group at the same position in the different decalin unit.

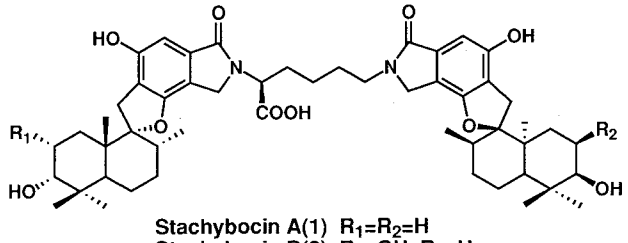
In a preceding paper[1], we described the fermentation, isolation, physico-chemical properties and biological activities of stachybocins A, B and C. In this paper, we describe the structure determination of these compounds (Fig. 1).

Results and Discussion

Stachybocins are produced by the fungus, *Stachybotrys* sp. M6222 and are isolated as white powders by solvent extraction followed by alumina and silica gel column chromatography. The molecular formula of stachybocin A (**1**) by HR-FAB spectral data and elemental analysis[1] was determined to be $C_{52}H_{70}N_2O_{10}$.

The characteristic feature of the ^{13}C NMR spectrum of **1** is the presence of 23 pairs of identical or almost equivalent carbon signals, which are assigned to 18 quaternary carbons, 8 methines, 12 methylenes and 8 methyls by DEPT experiments (Table 1). This ^{13}C NMR feature implies that **1** consists of two identical units connected by a fragment (hereafter referred to as the dimeric unit and a connecting unit, respectively) comprised of one carbonyl carbon (172.8 ppm), one methine (53.5 ppm) and four methylenes (28.5, 23.4, 27.2 and 41.4 ppm) which accounts for the total of the 52 carbons of **1**. The presence of an amide carbonyl function was confirmed by the IR spectral absorption at 1670 cm^{-1}.

Fig. 1. Structures of stachybocin A (**1**), B (**2**) and C (**3**).



Stachybocin A(1) $R_1=R_2=H$
Stachybocin B(2) $R_1=OH$, $R_2=H$
Stachybocin C(3) $R_1=H$, $R_2=OH$

All one-bond ^1H-^{13}C connectivities of **1** were established by a heteronuclear multiple quantum coherence (HMQC) spectrum using pulse field gradient (PFG)[2~4] techniques as summarized in Table 1.

A double-quantum-filtered COSY (DQF-COSY)[5] experiment indicated the presence of a proton spin network of the connecting unit, $-C(25)H-C(26)H_2-C(27)H_2-C(28)H_2-$. The chemical shifts of 25-H (4.71 ppm) and C-25 (53.5 ppm) unequivocally showed that an acylated amino group was attached to C-25. Since the 25-H showed ^{13}C-^1H long range couplings to two carbonyl carbons (168.3 and 172.8 ppm) in one of the aromatic units of the dimeric structure and connecting unit in the heteronuclear mulitple bond coherence (HMBC) spectrum (Fig. 2), the connecting unit was extended to give $-(O=)C-C(25)H(N-C=O)-C(26)H_2-C(27)H_2-C(28)H_2-$; the terminal methylene must be linked to the only remaining residue, the $-C(29)H_2-N$, of the connecting unit. Although the overlapping signal of the methylene proton region (1.2 to 2.0 ppm) prevented us

Table 1. ^{1}H and ^{13}C NMR data for stachybocin A (**1**), B (**2**) and C (**3**) in DMSO-d_6.

		δH	δC					δH	δC		
No.		**1**	**1**	**2**	**3**	No.		**1**	**1**	**2**	**3**
1			168.3	168.1	168.2	1'			167.4	167.5	167.4
2			133.2	134.3	133.8	2'			134.1	134.1	134.1
3	CH=	6.59	100.9	101.0	101.0	3'	CH=	6.53	100.8	101.0	100.9
4			153.8	153.7	153.8	4'			153.7	153.9	153.7
5			116.9	116.2	116.5	5'			116.4	116.4	116.3
6			155.9	155.8	155.9	6'			155.8	155.8	155.8
7			112.3	112.4	112.4	7'			111.9	111.7	111.9
8	CH_2	4.28	43.9	43.8	43.8	8'	CH_2	4.18	46.6	46.7	46.6
9	CH_2	3.11, 2.75	31.7	31.9	31.8	9'	CH_2	3.11, 2.75	31.7	31.7	31.8
10			98.3	97.5	97.9	10'			97.8	97.8	97.6
11	CH	1.77	36.6	36.4	36.1	11'	CH	1.77	36.5	36.4	36.6
12	CH_2	1.51, 1.36	30.7	30.8	30.8	12'	CH_2	1.51, 1.36	30.8	30.6	30.8
13	CH_2	1.46, 1.38	20.4	20.3	20.5	13'	CH_2	1.46, 1.38	20.4	20.5	20.3
14	CH	2.01	39.4	38.9	39.4	14'	CH	2.01	39.4	39.4	38.8
15			41.8	42.9	41.8	15'			41.8	41.8	42.9
16	CH_2	1.75, 0.92	23.8	32.9	23.9	16'	CH_2	1.75, 0.92	23.8	22.9	32.8
17	CH	1.75, 1.39	24.9	65.0	24.9	17'	CH	1.75, 1.39	24.8	24.9	64.9
18	CH	3.18	73.5	77.7	73.5	18'	CH	3.18	73.5	73.5	77.7
19			37.3	38.1	37.3	19'			37.3	37.3	38.1
20	CH_3	0.64	15.5	15.5	15.4	20'	CH_3	0.64	15.5	15.5	15.6
21	CH_3	0.94	15.8	16.9	15.9	21'	CH_3	0.94	15.8	15.8	16.7
22	CH_3	0.79	22.4	22.1	22.4	22'	CH_3	0.79	22.4	22.4	22.0
23	CH_3	0.88	28.6	29.0	28.7	23'	CH_3	0.88	28.6	28.7	29.0
24			172.8	172.8	172.7						
25	CH	4.71	53.5	55.5	54.6						
26	CH_2	2.03, 1.98	28.5	29.6	29.1						
27	CH_2	1.23	23.4	23.9	23.7						
28	CH_2	1.64	27.2	27.5	27.3						
29	CH_2	3.43, 3.38	41.4	41.6	41.5						

from determining the complete proton spin system of the connecting unit just explained, the ^{13}C-^{1}H long range couplings shown in partial structure **6** in Fig. 3, established the connecting unit to be a substituted lysine residue. The ε-methylene protons (29-H) of this unit were long range coupled to a carbonyl carbon (C-1′, 167.4 ppm) and a methylene carbon (C-8′, 46.6 ppm) as was the methine proton 25-H (*vide supra*). Therefore, it was clear that the two identical structural units were linked through the lysine residue. The structure of this connecting unit was confirmed by isolation of L-lysine by Jones oxidation followed by acid hydrolysis of a trimethyl derivative of **1** (*vide infra*).

An aromatic singlet proton at 6.59 ppm (3-H) is strongly long range coupled to two *meta*-carbons (116.9 and 112.3 ppm), and weakly coupled to oxygenated *ortho* or *para* carbons (153.8 and 155.9 ppm) as well as to the carbonyl carbon (C-1, 168.3 ppm) which is linked to the α-methine (25-H) of the lysine unit *via* an amide bond (partial structure **4** in Fig. 3).

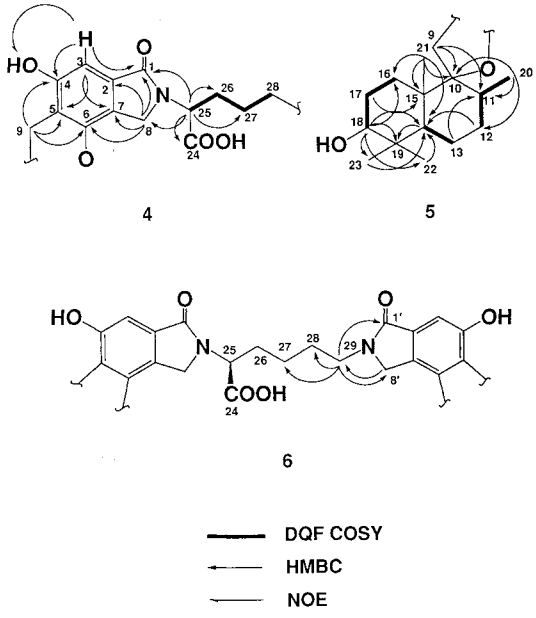
The isolated methylene protons at 4.28 ppm (8-H) are long range coupled to an oxygenated aromatic carbon (C-6, 155.9 ppm), a non-oxygenated aromatic carbon (C-7, 112.3 ppm) and a quaternary aromatic carbon at 133.2 ppm (C-2); the former two carbons are long-range coupled to 3-H. In addition, C-8 is long range coupled to the α-methine proton of the lysine unit. These results establish the presence of a phthalimidine system in **1**

(partial structure **4** in Fig. 3). The presence of a benzylic methylene unit at C-5 was confirmed by long-range couplings of its methylene protons (H-9, 3.11 and 2.75 ppm) to two oxygenated aromatic carbons (C-4 and C-6) and a non protonated aromatic carbon (C-5). The C-4 position of a phenolic hydroxy group was proven by the NOE observed between an exchangeable proton at 9.66 ppm and the aromatic proton 3-H. These results establish the partial structure **4** in Fig. 3.

The sesquiterpene residue consisting of C-9 to C-23 (partial structure **5** in Fig. 3) was established by analyzing ^{1}H-^{13}C long-range couplings observed with partial structures of the 16-H to 18-H, the 20-H to 12-H and the 13-H to 14-H, these relations were not previously revealed by the DQF-COSY spectral data.

The oxymethine proton at 3.18 ppm (18-H), which was directly linked to a carbon at 73.5 ppm (C-18), showed ^{1}H-^{13}C long range correlations to C-14 (39.4 ppm), C-16 (23.8 ppm), C-19 (37.3 ppm), C-22 (22.4 ppm) and C-23 (28.6 ppm). Among these, the correlations of the quaternary carbon (C-19) and two methyl carbons (C-22 and C-23) indicated the presence of a gem-dimethyl group. These dimethyl carbons showed cross peaks to the methine proton (2.01 ppm, 14-H) which had been proven to be connected to 13-H based on the DQF-COSY spectral data. The proton 14-H was coupled to signals at 15.8 ppm (C-21), 30.7 ppm (C-12), and 41.8 ppm (C-15). Since the relationship between 20-H to 12-H has been established (*vide supra*), the connectivities were extended to include 11-H to 14-H, 16-H to 19-H and three methyls (20-H, 22-H and 23-H) based on the ^{1}H-^{13}C long-range correlations. The remaining singlet methyl proton signal at 0.94 ppm (21-H) displayed long-range correlations to two quaternary carbons (98.3 and 41.8 ppm); one at lower field (C-10) was also coupled to a methyl proton signal (20-H) and a methylene proton signal (9-H) which had been assigned to a benzylic position. The proton 9-H showed cross peaks to the quaternary carbon (C-15) and methine carbon (C-11). These correlations revealed the presence of the sesqui-

Fig. 2. Partial structures of stachybocin A (**1**).



4 5

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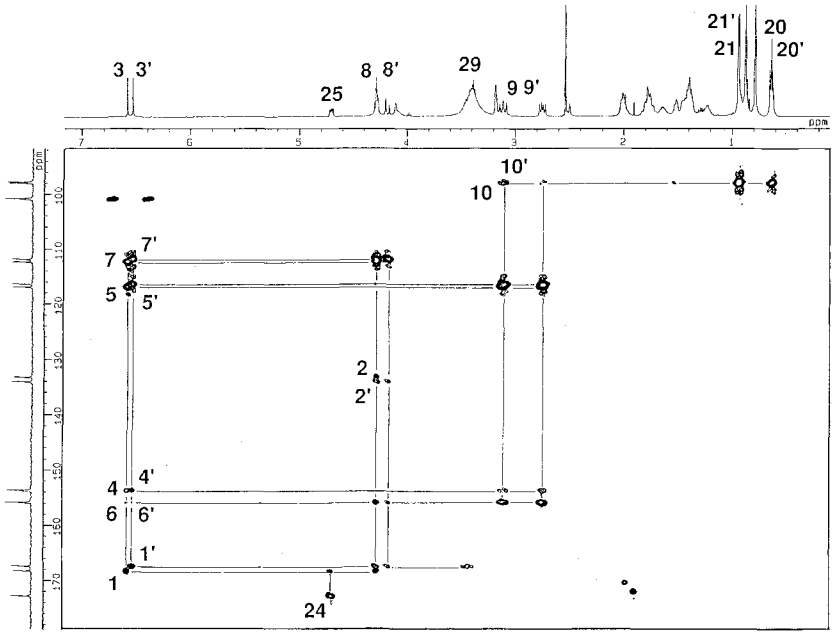
Fig. 3. PFG-HMBC spectrum of stachybocin A (**1**).



terpene unit fused to the spirobenzofuran residue in **1**. In addition to the signals explained above, another set of almost identical signals are observed that are assignable to the other structural unit (C-1′ to C-23′).

The relative stereochemistry of the sesquiterpene portion was established by the NOESY data. The absolute configulation at the stereogenic center of the connecting unit was determined by isolation of lysine by Jones oxidation followed by acid hydrolysis. The trimethyl derivative of **1** prepared by treatment with trimethylsilyldiazomethane was converted into the phthalimide form by Jones reagent. The hydrolysate of the oxidation product was purified to give lysine which was established to have the L-configuration by the chiral HPLC system. Therefore, the absolute configuration of C-25 was determined to be S.

The molecular formula $C_{52}H_{70}N_2O_{11}$ of stachybocin B (**2**) showed that **2** was a monohydroxy derivative of **1**. In the ^1H NMR spectrum of **2**, one of the oxymethine protons (18-H or 18′-H) located next to the methylene protons in **1**, was coupled to a new oxymethine proton at 3.78 ppm (J=2.5, 11.0 Hz) indicating the introduction of a hydroxyl group into the C-17 or C-17′ position. In agreement with this spectral change, downfield shifts were observed with signals due to the relevant carbons, C-16, C-17 and C-18 (or C-16′, C-17′ and C-18′) and all other carbon signals remained unchanged (Table 1). The position of this new hydroxyl group was established by tracing the ^{13}C-^1H long range correlations from the asymmetric center at C-25 through those interactions; 25-H–C-8, 8-H–C-6–9-H–C-15–16-H–C-17.

These results revealed that methylene carbon (C-17) of **1** was replaced by an oxymethine carbon (65.0 ppm). The stereochemistries of 17-H and 18-H were established to be in a diaxial relationship by the large vicinal coupling constants (J_{17-18}=11.0 Hz). Thus, the structure of **2** was determined as shown in Fig. 1.

Stachybocin C (**3**) possessing the same molecular formula with **2** ($C_{52}H_{70}N_2O_{11}$) showed very similar ^1H and ^{13}C NMR spectra to **2**. These results suggested that the additional hydroxyl group was located at the same position of the different terpene unit (C-17′) in **2**. In the HMBC spectrum, continuous two or three bond correlations were observed from the oxymethine carbon (C-17′) to the benzylic carbon (C-8′), which was coupled to the terminal methylene (29-H) of the connecting unit. Thus, the structure of **3** was established as shown in Fig. 1.

The structure of the sesquiterpene-benzofuran unit of stachybocins is very closely related to the fungal metabolites, stachybotramide[6] and K-76[7]. Presumably the stachybocins are biosynthesized by reaction between lysine and the biosynthetic intermediates related to K-76 (or its monodeoxy derivative) *via* Schiff-base formation, reduction, and lactam ring formation. In our studies conducted to date, the expected biosynthetic intermediates with a sesquiterpene-benzofuran structure have not be detected as metabolites of the stachybocin producing organism. Stachybotramide and K-76 are reported to be a phytotoxin and act as a complement inhibitor, respectively, but their endothelin binding inhibitory activity is not reported.

Stachybocins A, B and C are new endothelin antagonists isolated from the culture broth of *Stachybotrys* sp. M6222, and are structurally unrelated to WS009[8], asterric acid[9] and myriceron caffeoyl ester[10] which are the known non-peptide endothelin antagonists. The unique structural features of the stachybocins combined with their endothelin binding inhibitory activity makes them interesting lead compounds for further possible development into novel cardiovascular agents.

Experimental

NMR spectra were obtained on JEOL JNM A500 and GSX 400 spectrometers with ^1H NMR at 500 MHz and 400 MHz, and with ^{13}C NMR at 125 MHz and 100 MHz. Chemical shifts are given in ppm using TMS as an internal standard. The various 2D NMR methods utilized were DQF-COSY, HMQC and HMBC using PFG techniques.

Determination of the Absolute Configuration at C-25 of Stachybocin A (1)

To a solution of **1** (3 mg) in MeOH (0.4 ml) and benzene (0.1 ml) was added a hexane solution of trimethylsilyldiazomethane (0.2 ml, Tokyo Kasei). After stirring at room temperature for 12 hours, the reaction mixture was evaporated to dryness. The residue was dissolved in acetone (1 ml) and then reacted with CrO_3(VI) and conc. H_2SO_4 (Jones reagent, 0.3 ml) at 20°C for 4 hours. The reaction mixture was treated with $NaHSO_3$, and then extracted with diethylether. The organic layer was washed successively with saturated aqueous NaCl and $NaHCO_3$, and evaporated to dryness. The oxidation product was hydrolyzed in 6 N HCl at 110°C for 48 hours. The released lysine was analyzed with a chiral HPLC column (MCI GEL CRS10W, 4.6 mm i.d. × 50 mm Mitsubishi Kasei Co., eluent 0.1 mM $CuSO_4$ aq., flow rate 0.2 ml/minute) for the separation of D,L-amino acids.

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References

1) NAKAMURA, M.; Y. ITO, K. OGAWA, Y. MICHISUJI, S. SATO, M. TAKADA, M. HAYASHI, S. YAGINUMA & S. YAMAMOTO: Stachybocins, novel endothelin receptor antagonists, produced by *Stachybotrys* sp. M6222. I. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 48: 1389~1395, 1995
2) HURD, R. E. & B. K. JOHN: Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy. J. Magn. Reson. 91: 648~653, 1991
3) TYBURN, J. M.; I. M. BRERETON & D. M. DODDRELL: Coherence selection in gradient-enhanced, heteronuclear correlation spectroscopy. J. Magn. Reson. 97: 305~312, 1992
4) VUISTER, W. G.; R. BOELENS, R. KAPTEIN, E. R. HURD, B. JOHN & M. C. P. VAN ZIJL: Gradient-Enhanced HMQC and HSQC spectroscopy. Applications to ^{15}N-Labeled Mnt Repressor. J. Am. Chem. Soc. 113: 9688~9689, 1991
5) PIANTINI, U.; O. W. SØRENSEN & R. R. ERNST: Multiple quantum filters for elucidating NMR coupling networks. J. Am. Chem. Soc. 104: 6800~6801, 1982
6) AYER, A. W. & S. MIAO: Secondary metabolites of the aspen fungus *Stachybotrys cylindrospora*. Can. J. Chem. 71: 487~493, 1993
7) KAISE, H.; M. SHINOHARA, W. MIYAZAKI, T. IZAWA, Y. NAKANO, M. SUGAWARA, K. SUGIURA & K. SASAKI: Structure of K-76, a complement inhibitor produced by *Stachybotrys complement* nov. sp. K-76. J. Chem. Soc. Chem. Commun. 726~727, 1979
8) MIYATA, S.; N. OHHATA, H. MURAI, Y. MASUI, M. EZAKI, S. TAKASE, M. NISHIKAWA, S. KIYOTO, M. OKUHARA & M. KOHSAKA: WS009 A and B, New endothelin receptor antagonists isolated from *Streptmyces* sp. No. 89009. J. Antibiotics 45: 1029~1040, 1992
9) OHASHI, H.; H. AKIYAMA, K. NISHIKIORI & J. MOCHIZUKI: Asterric acid, a new endothelin binding inhibitor. J. Antibiotics 45: 1684~1685, 1992
10) FUJIMOTO, M.; S. MIHARA, S. NAKAJIMA, M. UEDA, M. NAKAMURA & K. SAKURAI: A novel non-peptide endothelin antagonist isolated from bayberry, *Myrica cerifera*. FEBS Lett. 305: 41~44, 1992