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Novel Stachyflin Derivatives from *Stachybotrys* sp. RF-7260

Fermentation, Isolation, Structure Elucidation and Biological Activities

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Stachybotrys sp. RF-7260 was found to produce stachyflins, novel anti-influenza virus agents, under solid-state fermentation conditions. Feeding DL-lysine to a culture of *Stachybotrys* sp. RF-7260 induced the formation of the novel compounds, SQ-02-S-L2 and -L1, and feeding DL-valine the formation of SQ-02-S-V1 and -V2. The structures of these metabolites were determined by detailed 2D NMR analyses in comparison with acetylstachyflin. SQ-02-S-L2 and -L1 have the lysine moiety and SQ-02-S-V1 has the valine moiety. SQ-02-S-V2 has an amidine moiety instead of the lactam moiety in acetylstachyflin. SQ-02-S-L2, -L1 and -V1, substituted on the lactam amide hydrogen, displayed only a low level of the antiviral activity. However, deacetyl SQ-02-S-V2 showed potent antiviral activity similar to stachyflin.

Acetylstachyflin (**1**) and stachyflin (**2**), which have a novel pentacyclic moiety including *cis*-fused decalin, can be isolated by solid-state fermentation of *Stachybotrys* sp. RF-7260[1]. Stachyflin (**2**) displays potent anti-influenza A virus (H1N1) *in vitro*. The mechanism of the antiviral action had been elucidated as inhibition of the fusion process between the viral envelope and the host cell membrane, which is an early step in the entry of virus into host cell[2,3]. Therefore **2** was considered to be an attractive lead compound for the development of antiviral agents. Preliminary investigation of structure-activity relationship by chemical modification was reported in our previous paper[4]. We here report the modification of the fermentation conditions by addition of amino acids to the fermentation of *Stachybotrys* sp. RF-7260 to produce new stachyflin derivatives. Two new metabolites, SQ-02-S-L2 (**3**) and SQ-02-S-L1 (**4**), were isolated from the fermentation with DL-lysine, and two new metabolites, SQ-02-S-V1 (**5**) and SQ-02-S-V2 (**6**), from the fermentation with DL-valine. In this paper, we describe the fermentation, isolation, structure elucidation and biological activities of these new compounds.

Results

Fermentation

We fermented *Stachybotrys* sp. RF-7260 in brown rice medium with several kinds of amino acids. Poor growth resulted with the feeding of some amino acids (glycine, D-glutamic acid, L-alanine, DL-aspartic acid, DL-methionine and L-asparagine). However, several new metabolites resulted on the feeding of DL-lysine and DL-valine.

Isolation

The isolation and purification procedures for SQ-02-S-L2 (**3**), -L1 (**4**) and SQ-02-S-V1 (**5**), -V2 (**6**) are summarized in Fig. 2 and 3, respectively. The metabolites **3**, **4** and **5**, **6** were extracted with acetone from the fermented rice media of *Stachybotrys* sp. RF-7260 supplemented with DL-lysine and DL-valine, respectively. The extract was purified by a combination of solvent partition, column chromatography and preparative HPLC. All compounds were finally obtained as white powders.

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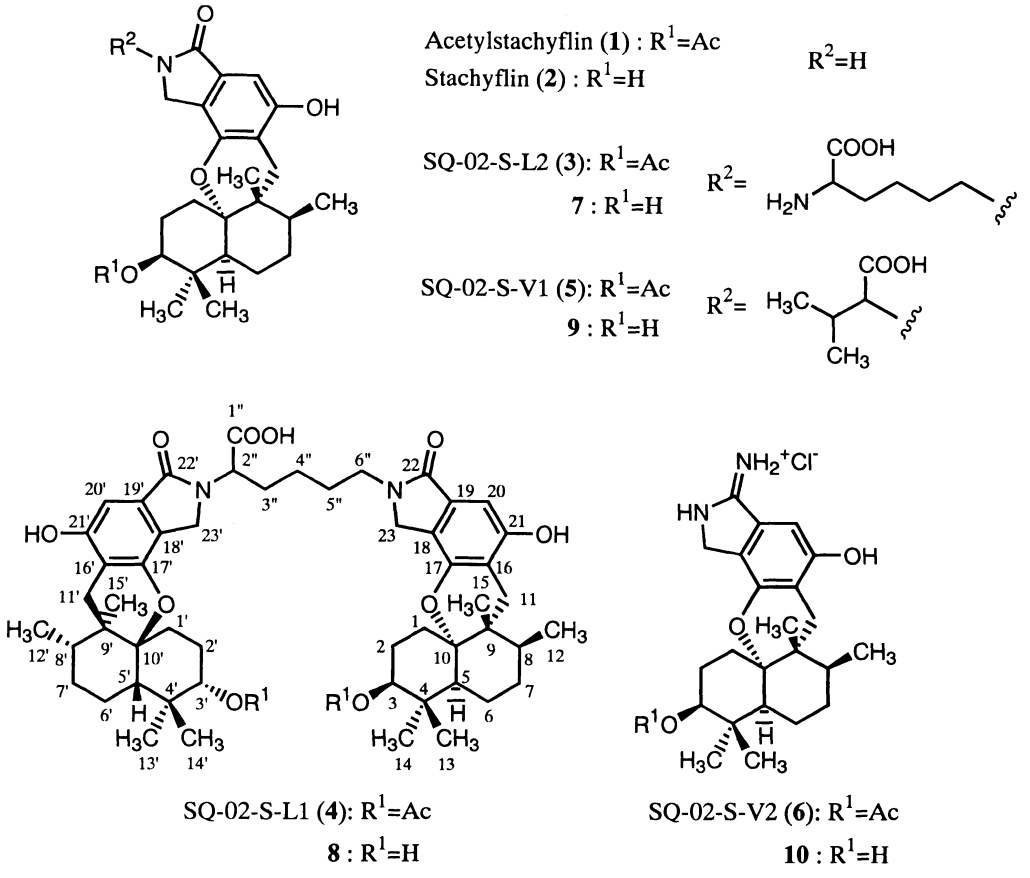
Fig. 1. Structures of compounds **1**~**10**.



Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of SQ-02-S-L2 (**3**), -L1 (**4**), -V1 (**5**) and -V2 (**6**) are summarized in Table 1. These four compounds were soluble in MeOH, acetone and DMSO but insoluble in *n*-hexane. The UV and IR spectra of **3**, **4** and **5** were similar to those of acetylstachyflin (**1**)[1]. The ^{1}H and ^{13}C NMR data of **3** and **4** and those of **5** and **6** are summarized in Tables 2 and 3, respectively.

SQ-02-S-L2 (**3**): The molecular formula of **3** was determined to be $C_{31}H_{44}N_2O_7$ on the basis of HR-FABMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **1** but also showed one carbonyl, four methylene, and one methine signals (Table 2). The long-range ^{1}H-^{13}C correlations as shown in Fig. 4 revealed that those additional signals constituted a lysine moiety. This lysine moiety was thought to be connected to an acetylstachyflin moiety, the stereochemistry of which was confirmed by detailed 2D NMR analyses. The linkage of the two moieties was determined by the long-range ^{1}H-^{13}C correlations of H-6″ with C-22 and C-23 (Fig. 4). These results revealed the structure of **3** in which the 6-amino group of lysine was connected to the lactam amide of **1**.

SQ-02-S-L1 (**4**): The molecular formula of **4** was determined to be $C_{56}H_{74}N_2O_{12}$ on the basis of HR-FABMS and ^{13}C NMR data. In the ^{1}H and ^{13}C NMR spectra of **4**, one additional set of signals ascribable to an acetystachyflin moiety was observed compared to those of **3**. This observation suggested that **4** had a dimeric structure like stachybocins[5] and SMTP-8[6]. The linkage of two acetylstachyflin units to a lysine moiety was determined by the long-range ^{1}H-^{13}C correlations of H-6″ with C-23 and of H-2″ with C-23′ as shown in Fig. 4.

SQ-02-S-V1 (**5**): The molecular formula of **5** was determined to be $C_{30}H_{41}NO_7$ on the basis of HR-FABMS and ^{13}C NMR data. Comparison of ^{1}H and ^{13}C NMR spectra of **5** with those of **1** revealed that **5** had a valine moiety in addition to an acetylstachyflin moiety (Table 3). That was confirmed by detailed 2D NMR analyses, and the linkage of the acetylstachyflin and the valine moieties was

Fig. 2. Isolation procedure of SQ-02-S-L1 and -L2.

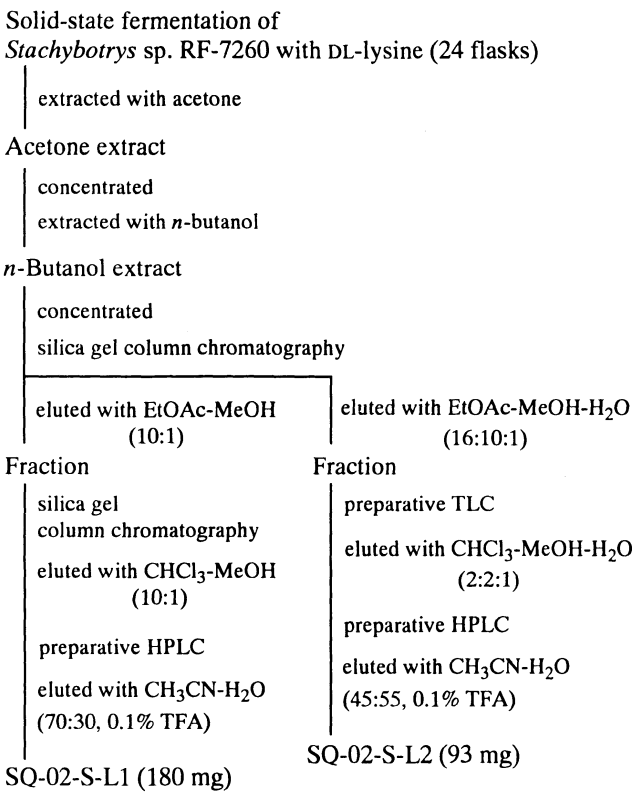


Fig. 3. Isolation procedure of SQ-02-S-V1 and -V2.

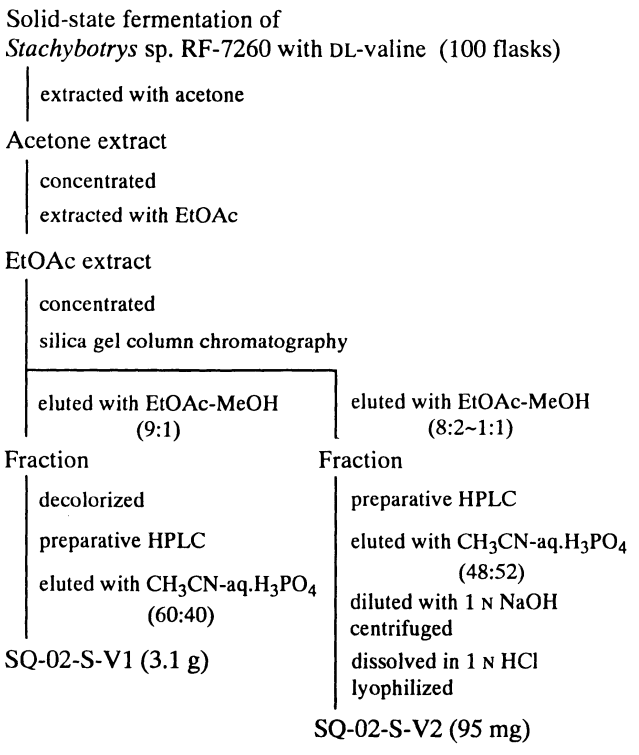


Table 1. Physico-chemical properties of SQ-02-S-L2 (**3**), -L1 (**4**), -V1 (**5**) and -V2 (**6**).

	3	**4**	**5**	**6**
Appearance	white powder	white powder	white powder	white powder
$[\alpha]_D^{24}$	+96.6° (*c* 0.56, MeOH)	+131.0° (*c* 0.63, MeOH)	+113.5° (*c* 0.34, MeOH)	+113.6° (*c* 0.47, MeOH)
Molecular weight	556	966	527	463
Molecular formula	$C_{31}H_{44}N_2O_7$	$C_{56}H_{74}N_2O_{12}$	$C_{30}H_{41}NO_7$	$C_{25}H_{35}N_2O_4Cl$
HR-FABMS (m/z)				
calcd :	557.3227 (as $C_{31}H_{45}N_2O_7$)	967.5320 (as $C_{56}H_{75}N_2O_{12}$)	528.2961 (as $C_{30}H_{42}NO_7$)	461.2207 (as $C_{25}H_{34}N_2O_4{}^{35}Cl$)
found :	557.3226 $(M+H)^+$	967.5317 $(M+H)^+$	528.2969 $(M+H)^+$	461.2213 $(M-H)^-$
UV λ_{max} nm (ε) in MeOH	218 (36,000) 261 (7,600) 301 (2,500)	218 (64,100) 261 (14,000) 301 (4,300)	218 (38,500) 262 (9,200) 300 (2,900)	223 (27,700) 273 (6,200) 314 (2,600)
IR ν_{max} cm^{-1} (KBr)	3423, 2876, 1734, 1671, 1626, 1466	3422, 2875, 1735, 1705, 1670, 1626, 1466	3424, 2876, 1737, 1708, 1668, 1626, 1467	3380, 3271, 1735, 1698, 1624, 1510, 1464, 1438

determined by the long-range ^1H-^{13}C correlations of H-2′ with C-22 and C-23 (Fig. 4).

SQ-02-S-V2 (**6**): The molecular formula of **6** was determined to be $C_{25}H_{35}N_2O_4Cl$ on the basis of HR-FABMS and ^{13}C NMR data, which indicated the presence of one more nitrogen and two more hydrogen atoms but one

Table 2. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectral data of SQ-02-S-L2 and -L1 in DMSO-d_6.

	SQ-02-S-L2		SQ-02-S-L1			
Position	δ_C (ppm)	δ_H (ppm, J in Hz)	δ_C (ppm)		δ_H (ppm, J in Hz)	
1, 1'	23.63 (t)	1.67 (m), 2.06 (m)	23.43 (t)[c]	23.48 (t)[c]	1.70 (m), 2.09 (m)	1.70 (m), 2.09 (m)
2, 2'	22.28 (t)[a]	1.60 (m), 2.41 (m)	22.10 (t)	22.10 (t)	1.60 (m), 2.40 (m)	1.60 (m), 2.40 (m)
3, 3'	74.92 (d)	4.65 (br.s)	74.92 (d)	74.92 (d)	4.65 (m)	4.65 (m)
4, 4'	36.33 (s)		36.15 (s)[d]	36.17 (s)[d]		
5, 5'	43.56 (d)	1.58 (m)	43.53 (d)	43.53 (d)	1.58 (m)	1.58 (m)
6, 6'	22.60 (t)	1.76 (m), 1.92 (m)	22.38 (t)	22.38 (t)	1.72 (m), 1.90 (m)	1.72 (m), 1.90 (m)
7, 7'	26.94 (t)	1.34 (m), 2.02 (m)	26.83 (t)	26.83 (t)	1.32 (m), 2.01 (m)	1.32 (m), 2.01 (m)
8, 8'	38.5 (d)	1.75 (m)	38.3 (d)	38.3 (d)	1.78 (m)	1.78 (m)
9, 9'	36.73 (s)		36.62 (s)	36.62 (s)		
10, 10'	82.14 (s)		82.11 (s)[e]	82.24 (s)[e]		
11, 11'	31.43 (t)	2.13 (d, 17.9) 3.08 (d, 17.9)	31.28 (t)	31.28 (t)	2.15 (d, 17.9) 3.11 (d, 17.9)	2.13 (d, 17.9) 3.09 (d, 17.9)
12, 12'	16.60 (q)	1.11 (d, 7.3)	16.31 (q)	16.31 (q)	1.11 (d, 7.3)[j]	1.12 (d, 7.3)[j]
13, 13'	29.36 (q)	1.04 (s)	29.10 (q)[f]	28.95 (q)[f]	1.02 (s)	1.02 (s)
14, 14'	25.71 (q)	0.81 (s)	25.44 (q)	25.44 (q)	0.80 (s)	0.81 (s)
15, 15'	19.44 (q)	0.84 (s)	19.19 (q)[g]	19.26 (q)[g]	0.83 (s)	0.84 (s)
16, 16'	111.30 (s)		111.86 (s)	111.31 (s)		
17, 17'	146.07 (s)		146.27 (s)	146.15 (s)		
18, 18'	117.71 (s)		117.82 (s)	118.48 (s)		
19, 19'	131.02 (s)		131.00 (s)	131.09 (s)		
20, 20'	98.92 (d)	6.65 (s)	98.90 (d)[h]	98.95 (d)[h]	6.66 (s)	6.65 (s)
21, 21'	155.50 (s)		155.38 (s)[i]	154.44 (s)[i]		
22, 22'	166.95 (s)		167.02 (s)	167.88 (s)		
23, 23'	46.78 (t)	4.16 (d, 17.2) 4.31 (d, 17.2)	46.47 (t)	46.47 (t)	4.13 (d, 17.2) 4.26 (d, 17.2)	4.21 (d, 16.3) 4.31 (d, 16.3)
24, 24'	169.06 (s)[b]		169.03 (s)	169.03 (s)		
25, 25'	20.74 (q)	2.05 (s)	20.47 (q)	20.47 (q)	2.04 (s)	2.04 (s)
1"	169.25 (s)[b]		171.94 (s)			
2"	53.59 (d)	3.13 (m)	52.80 (d)		4.68 (m)	
3"	30.40 (t)	1.72 (m)	27.86 (t)		1.95 (m)	
4"	22.22 (t)[a]	1.33 (m)	22.73 (t)		1.20 (m)	
5"	27.34 (t)	1.54 (m)	26.46 (t)		1.61 (m)	
6"	41.18 (t)	3.30 (dt, 13.8, 7.3) 3.54 (dt, 13.8, 7.3)	40.73 (t)		3.33 (dt, 13.9, 7.0) 3.51 (dt, 13.9, 7.0)	

[a ~ j] Assignments may be interchanged.

less oxygen atom compared with that of **1**. The ^{1}H NMR spectrum of **6** was similar to that of **1**, but there were two more deuterium exchangeable protons (δ 9.06 and 9.48) than that of **1**. In addition, the aromatic proton H-20, the hydroxy proton 21-OH, the methylene protons H-23 and the NH proton **c** appeared further downfield than that of **1**. In the ^{13}C NMR spectrum of **6**, the upfield shift of C-22 signal (from δ_C 170.27 in **1** to δ_C 163.70) was observed.

Table 3. ^{1}H and ^{13}C NMR spectral data of SQ-02-S-V1 and -V2.

	SQ-02-S-V1			SQ-02-S-V2	
Position	δ_C (ppm)[a]	δ_H (ppm, J in Hz)[b]	Position	δ_C (ppm)[c]	δ_H (ppm, J in Hz)[d]
1	25.67 (t)	1.80 (m), 2.24 (m)	1	23.87 (t)	1.69 (m), 2.08 (m)
2	24.12 (t)	1.66 (m), 2.50 (tt, 14.0, 3.1)	2	22.52 (t)	1.61 (m), 2.24 (m)
3	77.79 (d)	4.77 (t, 3.1)	3	75.18 (d)	4.66 (t-like)
4	38.27 (s)		4	36.64 (s)	
5	45.98 (d)	1.66 (m)	5	44.03 (d)	1.59 (m)
6	24.72 (t)	2.04 (m), 2.10 (m)	6	22.92 (t)	1.72 (m), 1.93 (m)
7	28.99 (t)	1.41 (m), 1.84 (m)	7	27.16 (t)	1.32 (m), 2.03 (m)
8	40.93 (d)	1.84 (m)	8	38.68 (d)	1.79 (m)
9	38.80 (s)		9	37.07 (s)	
10	84.67 (s)		10	83.41 (s)	
11	33.35 (t)	2.24 (d, 18.2) 3.23 (d, 18.2)	11	31.79 (t)	2.19 (d, 17.8) 3.13 (d, 17.8)
12	17.49 (q)	1.19 (d, 7.6)	12	16.77 (q)	1.11 (d, 7.2)
13	30.67 (q)	1.11 (s)	13	29.68 (q)	1.01 (s)
14	26.82 (q)	0.88 (s)	14	25.96 (q)	0.82 (s)
15	20.50 (q)	0.94 (s)	15	19.65 (q)	0.84 (s)
16	114.81 (s)		16	115.11 (s)	
17	148.29 (s)		17	147.05 (s)	
18	120.84 (s)		18	121.53 (s)	
19	131.33 (s)		19	126.73 (s)	
20	100.55 (d)	6.74 (s)	20	99.70 (d)	7.10 (s)
21	157.58 (s)		21	156.66 (s)	
22	171.52 (s)		22	163.70 (s)	
23	46.72 (t)	4.36 (d, 17.2) 4.60 (d, 17.2)	23	48.71 (t)	4.47 (d, 18.9) 4.65 (d, 18.9)
24	171.98 (s)		24	169.76 (s)	
25	21.30 (q)	2.08 (s)	25	21.03 (q)	2.05 (s)
1'	173.32 (s)		21-OH		10.24 (s)
2'	61.78 (d)	4.58 (d, 10.3)	NHa		9.48 (s)
3'	29.97 (t)	2.34 (m)	NHb		9.06 (s)
4'	19.64 (t)[e]	0.89 (d, 6.6)[f]	NHc		10.20 (s)
5'	19.95 (t)[e]	1.08 (d, 6.6)[f]			

[a] Recorded at 75 MHz in CD_3OD.

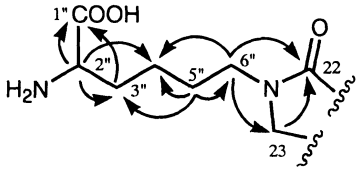
[b] Recorded at 300 MHz in CD_3OD.

[c] Recorded at 150 MHz in DMSO-d_6.

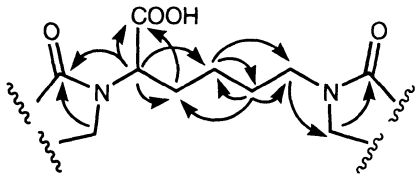
[d] Recorded at 600 MHz in DMSO-d_6.

[e, f] Assignments may be interchanged.

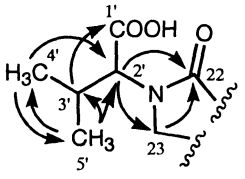
Fig. 4. Key long-range ^{1}H-^{13}C correlations of **3**~**5**.



SQ-02-S-L2 (**3**)

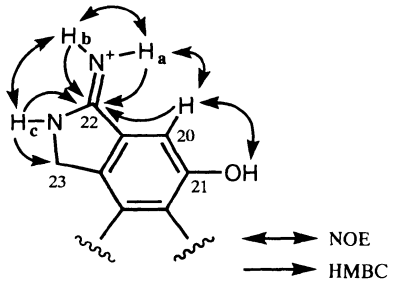


SQ-02-S-L1 (**4**)



SQ-02-S-V1 (**5**)

Fig. 5. Key NOEs and long-range ^{1}H-^{13}C correlations of **6**.



SQ-02-S-V2 (**6**)

Table 4. *In vitro* anti-influenza A virus activity of stachyflin and its analogues.

	anti-influenza A virus activity	cytotoxicity
compound	IC_{50} (μM)	CC_{50} (μM)
1	0.23	44
2	0.003	65
3	>190	>190
4	>100	>100
5	>200	>200
6	0.2	27
7	1.2	>190
8	3.6	>100
9	3.3	>200
10	0.002	45

virus: A/WSN/33(H1N1), cells: Madin-Darby bovine kidney cells

Moreover, the aromatic proton H-20 and the amidino protons **a**, **b** and **c** showed long-range ^{1}H-^{13}C correlations with C-22 (Fig. 5). The NOEs were observed between H-20 and both amidino proton **a** and 21-OH, and between amidino proton **b** and amidino proton **a** and **c** as shown in Fig. 5. These observations confirmed that **6** had an amidine moiety instead of the lactam moiety of **1**.

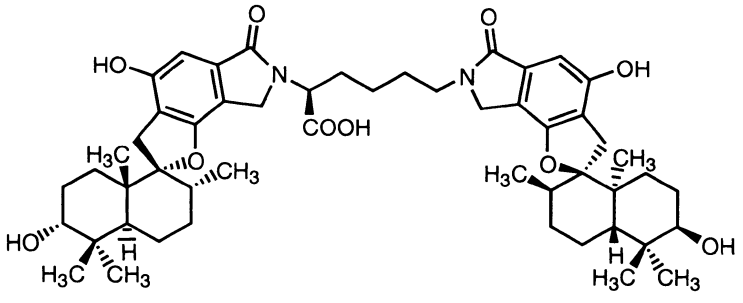
Biological Activity

The *in vitro* antiviral activity against influenza A virus and the cytotoxic activity against Madin-Darby bovine kidney (MDBK) cells were measured according to a method described previously[2]. All four compounds **3**~**6** were deacetylated with 1 M NaOMe/MeOH to give **7**~**10**, respectively. The anti-influenza A virus activity and cytotoxicity of stachyflin analogues are shown in Table 4. Compounds **3**~**5** did not show antiviral activity at concentrations of >100 μM. The antiviral activity of compound **7**, which had a lysine moiety, was 400 times weaker than that of **2**. Interestingly, dimeric compound **8** was about 3-fold less active than the compound **7**. Compound **9**, which had a valine moiety also displayed reduced antiviral activity. On the other hand, compound **6**, which had an amidine moiety, had antiviral activity with an IC_{50} value of 0.2 μM. Moreover, the antiviral activity of compound **10** (IC_{50} of 0.002 μM) was 100 times more potent than that of **6**, which was comparable to that of **2**.

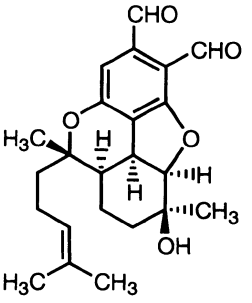
Discussion

In the present study, four new stachyflin analogues, SQ-02-S-L2 (**3**), SQ-02-S-L1 (**4**), SQ-02-S-V1 (**5**) and SQ-02-S-V2 (**6**) were isolated from *Stachybotrys* sp.

Fig. 6. Structures of stachybocin A (**11**) and bisabosqual A (**12**).



Stachybocin A (**11**)



Bisabosqual A (**12**)

RF-7260 fermented under modified conditions. Compounds **3** and **4** and compounds **5** and **6** were obtained by feeding *Stachybotrys* sp. RF-7260 with DL-lysine and DL-valine, respectively. The amino acids were incorporated into **3**, **4** and **5**. A lysine and a valine moiety were connected to the lactam amide moiety of **1** in **3** and **5**, respectively. Two acetylstachyflin units were bridged at the 2- and 6-amino group of lysine in **4**. Interestingly, compound **6** has a unique amidine moiety instead of the lactam moiety in **1**.

The substitution at the lactam amide hydrogen decreased the antiviral activity as judged by the weak activities of compounds **7**, **8** and **9**. It is interesting that the unique amidine analogue **10** exhibited antiviral activity similar to **2**. Moreover, compound **10** was far more soluble in water than **2**. Thus, compound **10** is an attractive analogue as a potential anti-influenza virus agent.

Various biologically active metabolites such as stachybocin A (**11**)[5] and spirodihydrobenzofuranlactam VI[7] with endothelin receptor antagonistic activity and bisabosqual A (**12**) with squalene synthase inhibitory activity[8,9] have been isolated from *Stachybotrys* organisms. Stachybocin A (**11**) and spirodihydrobenzofuranlactam VI are different from stachyflins in that the formers have phenylspirodrimane moieties. However, they have a lysine moiety that bridges two sesquiterpenic benzolactam moieties similar to SQ-02-S-L1. On the other hand, SETO *et al.* reported that the biogenetic precursor of stachybocin A would be related to K-76[10] which has a phthaldialdehyde moiety[5]. Bisabosqual A (**12**) also has a phthaldialdehyde moiety. In addition, GRIGG *et al.*[11] reported that the reaction of *o*-phthaldialdehyde with α-amino acids gave *N*-substituted isoindolin-1-ones. Thus, stachyflin and *N*-substituted analogues were considered to be formed by the non-enzymatic reaction of a phthaldialdehyde-type biogenetic precursor corresponding to stachyflin precursor with amino acid/amine, although we have not detected any of such precursors on solid-state fermentation of stachyflin producing organisms. The above hypothesis about stachyflin biogenesis was supported by the formation of an amino acid-containing compound, similar to SQ-02-S-V1, in the treatment of bisabosqual A with a α-amino acid in acetone-water (data not shown). Further investigation is needed to clarify the mode of the reaction between biogenetic precursors with phthaldialdehyde and amino acid/amine.

Experimental

General

Optical rotations were determined using the sodium D line on a Perkin-Elmer 241 polarimeter. UV spectra were measured on a Hitachi U-3410 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-700 spectrometer. FAB-MS and HR FAB-MS were obtained on a JEOL JMS-SX/SX 102A. ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity-600 and Varian Gemini-300 spectrometers. Column chromatography was carried out on silica gel (Silica gel 60, 70~230 mesh, E. Merck). For preparative TLC, silica gel plate (pre-coated TLC plates, Silica gel F-254, E. Merck) was used.

Fermentation with DL-Lysine

A slant culture of *Stachybotrys* sp. RF-7260 was inoculated into twenty-four 500-ml Erlenmeyer flasks containing brown rice lysine medium (25 g of brown rice,

0.5 g of glucose, 0.1 g of yeast extract (Difco), 0.5 g of DL-lysine hydrochloride and 50 ml of tap water), which had been sterilized at 121°C for 30 minutes. The fermentation was conducted at 28°C for 14 days under stationary condition.

Fermentation with DL-Valine

A slant culture of *Stachybotrys* sp. RF-7260 was inoculated into five 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of 2.0% glucose, 2.0% soluble starch, 1.0% polypeptone, 0.3% meat extract (Difco), 0.1% yeast extract (Difco) and 0.1% sodium chloride. The flasks were incubated at 28°C for 3 days on a rotary shaker at 180 rpm. Four ml of the seed culture was transferred into one hundred 500-ml Erlenmeyer flasks containing brown rice valine medium (25 g of brown rice, 0.5 g of glucose, 0.1 g of yeast extract (Difco), 0.5 g DL-valine and 50 ml of tap water), which had been sterilized at 121°C for 30 minutes. The fermentation was conducted at 28°C for 14 days under stationary condition.

Isolation of SQ-02-S-L1 and SQ-02-S-L2

The fermented rice medium was extracted with 2 liters of acetone and the extract was concentrated *in vacuo* to give an aqueous residue. This residue was adjusted to pH 2.0 with 1 N HCl, followed by extraction with *n*-butanol. The *n*-butanol layer was concentrated to dryness to give 4.5 g of a crude fraction, which was roughly separated by silica gel chromatography (Silica gel 60, 160 g) and eluted with EtOAc, EtOAc - MeOH (10 : 1) and EtOAc - MeOH - water (16 : 10 : 1). HPLC analysis revealed two new peaks when compared to fractions from the media without addition of amino acids. The fractions containing **4** were concentrated to dryness to give 2.7 g of a crude fraction. The fraction was subjected to silica gel chromatography (Silica gel 60, 100 g) and eluted with $CHCl_3$ - MeOH (10 : 1). The fractions containing **4** were concentrated and further purified by preparative HPLC (Ultron ODS-5, 20 i.d. ×250 mm, Shinwa Chemical Industries, Ltd., acetonitrile - water (70 : 30) containing 0.1% trifluoroacetic acid) to give 180 mg of **4** as a white powder. The fractions containing **3**, which were eluted with EtOAc - MeOH - water (16 : 10 : 1) were combined, and concentrated to dryness to give 640 mg of a crude fraction. The fraction was subjected to preparative TLC ($CHCl_3$ - MeOH - water (2 : 2 : 1)) to give 147 mg of crude powder, which was further purified by preparative HPLC (Ultron ODS-5, 20 i.d.×250 mm, Shinwa Chemical Industries, Ltd., acetonitrile - water (45 : 55) containing 0.1% trifluoroacetic acid) to give 93 mg of **3** as a white powder.

Isolation of SQ-02-S-V1 and SQ-02-S-V2

The fermented rice medium was extracted with 10 liters of acetone and the extract was concentrated *in vacuo* to give 3 liters of aqueous residue. Sodium chloride (100 g) was added to the aqueous residue and extracted with 5 liters of EtOAc. The EtOAc extract was concentrated to give 38 g of an oil. The oil was roughly separated by silica gel chromatography (Silica gel 60, 1.5 kg) and eluted with EtOAc - MeOH (9 : 1～8 : 2～1 : 1). HPLC analysis revealed two new peaks when compared to fractions from the media without addition of amino acids. The fractions containing **5** were concentrated to dryness to give 7.5 g of a crude fraction, which was then decolorized with 17.5 g of activated charcoal (Norit SX-3, Wako Pure Chemical Industries, Ltd.) and subjected to preparative HPLC (YMC ODS-AP S-15/30, 50 i.d.×500 mm, acetonitrile - 0.1% phosphoric acid (60 : 40)) to give 3.1 g of **5** as a white powder. The fractions containing **6**, which were eluted with EtOAc - MeOH (8 : 2～1 : 1) were concentrated to dryness to give 4.5 g of a crude fraction, which was subjected to preparative HPLC (YMC ODS-AP S-15/30, 50 i.d.×500 mm, acetonitrile - 0.1% phosphoric acid (48 : 52)). The pure fraction was concentrated and extracted with EtOAc. The EtOAc layer was washed with water and brine, and concentrated under reduced pressure. The residue was diluted in 1 N NaOH and centrifuged. The precipitate was dissolved in 1 N HCl and lyophilized to give compound **6** (95 mg) as a white powder.

Compound **7**

SQ-02-S-L2 (52 mg) was dissolved in 1 M NaOMe/MeOH solution (3.0 ml) and the mixture was heated to reflux for 5 hours. After cooling, water (1.5 ml) was added to the reaction mixture, and the pH was adjusted to 2.0 with diluted hydrochloric acid. MeOH was evaporated under reduced pressure, and the residue was extracted with *n*-butanol. The *n*-butanol layer was concentrated and separated by TLC ($CHCl_3$ - MeOH - water=2 : 2 : 1) to give **7** (29 mg, 60%); $[\alpha]_D^{24}$ =+116.3° (*c* 0.41, MeOH); HR FAB-MS (*m/z*) 515.3122 $(M+H)^+$ Calcd for $C_{29}H_{43}N_2O_6$: 515.3121; IR ν_{max} (KBr) cm^{-1}: 3427, 2932, 2870, 1734, 1655, 1464, 1381, 1363; ^1H NMR (DMSO-d_6, 300 MHz) δ 0.83 (3H, s), 0.89 (3H, s), 0.93 (3H, s), 1.09 (3H, d, *J*=7.2 Hz), 1.26 (1H, m), 1.3～1.5 (2H, m), 1.5 (4H, m), 1.6～1.8 (5H, m), 2.0～2.4 (4H, m), 2.10 (1H, d, *J*=17.7 Hz), 3.07 (1H, d, *J*=17.7 Hz), 3.35 (1H, m), 3.55 (1H, m), 3.90 (1H, t-like), 4.12 (1H, d, *J*=17.1 Hz), 4.32 (1H, d, *J*=17.1 Hz), 4.48 (1H, m), 6.61 (1H, s), 8.21 (2H, s), 9.74 (1H, s); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 17.37, 20.31, 22.10, 23.84, 23.91, 26.11, 27.42,

27.70, 27.98, 30.08, 30.64, 32.29, 37.51, 37.86, 41.63, 44.80, 47.55, 52.21, 72.58, 83.76, 99.56, 112.38, 118.82, 131.83, 147.41, 156.35, 168.08, 171.50.

Compound **8**

SQ-02-S-L1 (30 mg) was dissolved in 1 M NaOMe/MeOH solution (2.0 ml) and the mixture was heated to reflux for 5 hours. After cooling, water (0.5 ml) was added to the reaction mixture, and the pH was adjusted to 2.0 with diluted hydrochloric acid. MeOH was evaporated under reduced pressure, and the residue was extracted with *n*-butanol. The *n*-butanol layer was concentrated and separated by TLC ($CHCl_3$ - MeOH - water=2 : 2 : 1) to give **8** (16 mg, 58%); $[\alpha]_D^{24}$ = +157.4° (*c* 0.39, MeOH); HR FAB-MS (*m/z*) 883.5107 $(M+H)^+$ Calcd for $C_{52}H_{71}N_2O_{10}$: 883.5109; IR ν_{max} (KBr) cm^{-1}: 3430, 2958, 2871, 1668, 1625, 1465, 1384, 1365; ^1H NMR (DMSO-d_6, 300 MHz) δ 0.82 (3H, s), 0.84 (3H, s), 0.88 (6H, s), 0.91 (6H, s), 1.09 (3H, d, *J*=7.2 Hz), 1.10 (3H, d, *J*=7.2 Hz), 1.23 (2H, m), 1.27 (2H, m), 1.4~1.7 (10H, m), 1.8~2.4 (10H, m), 3.07 (1H, d, *J*=17.4 Hz), 3.08 (1H, d, *J*=17.4 Hz), 3.35 (3H, m), 3.50 (1H, m), 4.09 (1H, d, *J*=16.8 Hz), 4.21 (1H, d, *J*=17.4 Hz), 4.27 (1H, d, *J*=17.4 Hz), 4.29 (1H, d, *J*=16.8 Hz), 4.45 (1H, m), 4.68 (1H, dd, *J*=4.8, 10.5 Hz), 6.60 (1H, s), 6.64 (1H, s), 9.69 (1H, s), 9.75 (1H, s); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 16.86 (×2), 19.79, 19.86, 23.19, 23.32 (×6), 25.60 (×2), 26.86 (×2), 27.48, 29.91, 30.03, 30.54, 31.78 (×2), 36.97 (×2), 37.28 (×2), 41.09, 44.21 (×2), 46.91 (×2), 53.09, 71.97 (×2), 83.09, 83.23, 98.93 (×2), 111.67, 112.23, 118.10, 118.77, 130.31, 131.12, 146.67, 146.79, 155.59, 155.66, 167.25, 168.14, 172.28.

Compound **9**

SQ-02-S-V1 (16 mg) was dissolved in 1 M NaOMe/MeOH solution (1.5 ml) and the mixture was heated to reflux for 5 hours. After cooling, water (0.5 ml) was added to the reaction mixture, and the pH was adjusted to 2.0 with diluted hydrochloric acid. After evaporation of MeOH under reduced pressure, the residue was extracted with EtOAc. The EtOAc layer was concentrated and separated by TLC ($CHCl_3$ - MeOH - water=2 : 2 : 1) to give **9** (11 mg, 76%); $[\alpha]_D^{24}$ = +131.8° (*c* 0.28, MeOH); HR FAB-MS (*m/z*) 486.2855 $(M+H)^+$ Calcd for $C_{28}H_{40}NO_6$: 486.2856; IR ν_{max} (KBr) cm^{-1}: 3428, 2963, 2874, 1722, 1669, 1624, 1466, 1363; ^1H NMR (DMSO-d_6, 300 MHz) δ 0.78 (3H, d, *J*=6.3 Hz), 0.85 (3H, s), 0.89 (3H, s), 0.91(3H, s), 0.98 (3H, d, *J*=6.6 Hz), 1.09 (3H, d, *J*=7.5 Hz), 1.25 (1H, m), 1.4~1.8 (5H, m), 1.98 (1H, m), 2.1~2.4 (4H, m), 2.10 (1H, d, *J*=18.0 Hz), 3.08 (1H, d, *J*=18.0 Hz), 3.35 (1H, m), 4.25 (1H, d, *J*=16.8 Hz), 4.40 (1H, d, *J*=10.2 Hz), 4.42 (1H, d, *J*=16.8 Hz), 6.44 (1H, s), 9.76 (1H, s); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 16.89, 19.10, 19.33, 19.92, 23.36, 23.41, 25.68, 26.89, 27.52, 27.83, 29.95, 31.85, 37.05, 37.35, 44.31, 44.58, 59.80, 72.07, 83.43, 99.17, 112.49, 118.83, 130.20, 147.02, 155.96, 168.19, 171.83.

Compound **10**

SQ-02-S-V2 (30 mg) was dissolved in 1 M NaOMe/MeOH solution (1.5 ml) and the mixture was heated to reflux for 4 hours. After cooling, water (1.5 ml) was added to the reaction mixture and the pH was adjusted to 1.0 with diluted hydrochloric acid. After evaporation of MeOH under reduced pressure, the residue was dissolved in water. The solution was then allowed to be adsorbed onto MCI GEL CHP20P (Mitsubishi Chemical Co.) column, washed with water, eluted with 50% aqueous acetone, and concentrated under reduced pressure. To the residue dissolved in MeOH (0.5 ml) was added diluted hydrochloric acid to adjust the pH to 2.0. Compound **10** (19.5 mg) was then precipitated with an excess of diethyl ether; $[\alpha]_D^{24}$ = +123.5° (*c* 0.26, MeOH); HR FAB-MS (*m/z*) 419.2109 $(M-H)^-$ Calcd for $C_{23}H_{32}N_2O_3Cl$: 419.2101; IR ν_{max} KBr cm^{-1}: 3426, 2960, 2872, 1685, 1622, 1463, 1382; ^1H NMR (DMSO-d_6, 300 MHz) δ 0.83 (3H, s), 0.89 (3H, s), 0.90 (3H, s), 1.09 (3H, d, *J*=7.2 Hz), 1.28 (1H, m), 1.4~1.8 (5H, m), 1.98 (1H, m), 2.1~2.4 (3H, m), 2.15 (1H, d, *J*=18.3 Hz), 3.11 (1H, d, *J*=18.3 Hz), 4.42 (1H, d, *J*=19.2 Hz), 4.51 (1H, m), 4.63 (1H, d, *J*=19.2 Hz), 7.14 (1H, s), 9.20 (1H, s), 9.60 (1H, s), 10.26 (2H, s); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 15.66, 18.61, 22.20 (×2), 24.42, 25.73, 26.27, 28.98, 30.75, 35.87, 36.18, 43.26, 47.51, 70.80, 82.87, 98.49, 113.97, 120.22, 125.42, 146.10, 155.41, 162.52.

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References

1) MINAGAWA, K.; S. KOUZUKI, J. YOSHIMOTO, Y. KAWAMURA, H. TANI, T. IWATA, Y. TERUI, H. NAKAI, S. YAGI, N. HATTORI, T. FUJIWARA & T. KAMIGAUCHI: Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by *Stachybotrys* sp. RF-7260. I. Isolation, structure elucidation and biological activities. J. Antibiotics 55: 155~164, 2002

2) YOSHIMOTO, J.; M. KAKUI, H. IWASAKI, T. FUJIWARA, H. SUGIMOTO & N. HATTORI: Identification of a novel HA conformational change inhibitor of human influenza virus. Arch. Virol. 144: 865~878, 1999
3) YOSHIMOTO, J.; M. KAKUI, H. IWASAKI, H. SUGIMOTO, T. FUJIWARA & N. HATTORI: Identification of amino acids of influenza virus HA responsible for resistance to a fusion inhibitor, stachyflin. Microbiol. Immunol. 44: 677~685, 2000
4) MINAGAWA, K.; S. KOUZUKI & T. KAMIGAUCHI: Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by *Stachybotrys* sp. RF-7260. II. Synthesis and preliminary structure-activity relationships of stachyflin derivatives. J. Antibiotics 55: 165~171, 2002
5) OGAWA, K.; M. NAKAMURA, M. HAYASHI, S. YAGINUMA, S. YAMAMOTO, K. FURIHATA, K. SHIN-YA & H. SETO: Stachybocins, novel endothelin receptor antagonists, produced by *Stachybotrys* sp. M6222. II. Structure determination of stachybocins A, B and C. J. Antibiotics 48: 1396~1400, 1995
6) HU, W.; S. OHYAMA & K. HASUMI: Activation of fibrinolysis by SMTP-7 and -8, novel staplabin analogs with a pseudosymmetric structure. J. Antibiotics 53: 241~247, 2000
7) ROGGO, B. E.; P. HUG, S. MOSS, A. STAMPFLI, H.-P. KRIEMLER & H. H. PETER: Novel spirodihydrobenzofuranlactams as antagonists of endothelin and as inhibitors of HIV-1 protease produced by *Stachybotrys* sp. II. Structure determination. J. Antibiotics 49: 374~379, 1996
8) MINAGAWA, K.; S. KOUZUKI, K. NOMURA, T. YAMAGUCHI, Y. KAWAMURA, K. MATSUSHIMA, H. TANI, K. ISHII, T. TANIMOTO & T. KAMIGAUCHI: Bisabosquals, novel squalene synthase inhibitors. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiotics 54: 890~895, 2001
9) MINAGAWA, K.; S. KOUZUKI, K. NOMURA, Y. KAWAMURA, H. TANI, Y. TERUI, H. NAKAI & T. KAMIGAUCHI: Bisabosquals, novel squalene synthase inhibitors. II. Physico-chemical properties and structure elucidation. J. Antibiotics 54: 896~903, 2001
10) KAISE, H.; M. SHINOHARA, W. MIYAZAKI, T. IZAWA, Y. NAKANO, M. SUGAWARA & K. SUGIURA: Structure of K-76, a complement inhibitor produced by *Stachybotrys complementi* nov. sp. K-76. J. Chem. Soc. Chem. Commun.: 726~727, 1979
11) GRIGG, R.; H. Q. N. GUNARATNE & V. SRIDHARAN: A simple one-step synthesis of *N*-substituted isoindolin-1-ones. Diastereofacially selective protonation of an intermediate isoindolinol. J. Chem. Soc. Chem. Commun.: 1183~1184, 1985