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# Stachyflin and Acetylstachyflin, Novel Anti-influenza A Virus Substances, Produced by *Stachybotrys* sp. RF-7260

## I. Isolation, Structure Elucidation and Biological Activities

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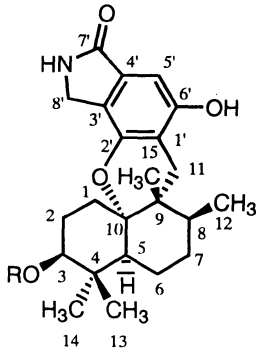
Two novel compounds, stachyflin and acetylstachyflin, have been isolated by solid-state fermentation of *Stachybotrys* sp. RF-7260. The structures of both metabolites, determined by detailed NMR analyses and X-ray crystallographic analysis, are novel with a pentacyclic moiety including *cis*-fused decalin. The absolute stereochemistry of stachyflins was determined by circular dichroism analysis. Stachyflin showed antiviral activity against influenza A virus (H1N1) *in vitro* with an $IC_{50}$ value of 0.003 $\mu$M. Acetylstachyflin was about 77-fold less active than stachyflin.

Human influenza viruses, which cause infectious disease of the respiratory tract, are classified into types A, B and C, depending on their internal antigens. Types A and B are known to cause severe symptoms. One chemotherapeutic agent known for more than 20 years is amantadine, although its efficacy has not yet been established[1]. Zanamivir is an inhibitor of neuraminidases from both influenza A and B viruses and is currently used for treatment of influenza viral infection although it does not show satisfactory activity when administered orally or intraperitoneally[2]. Thus, there is a strong demand for the development of more effective anti-influenza agents.

In our continuing search for anti-influenza substances from microorganisms, we have isolated stachyflin and acetylstachyflin, novel substances with potent antiviral activity against influenza A virus from *Stachybotrys* sp. RF-7260. In this paper, we describe the isolation, structure elucidation and biological activities of these two new compounds.

Fig. 1. Structures of acetylstachyflin (**1**) and stachyflin (**2**).




Acetylstachyflin (**1**) R=Ac
Stachyflin (**2**) R=H

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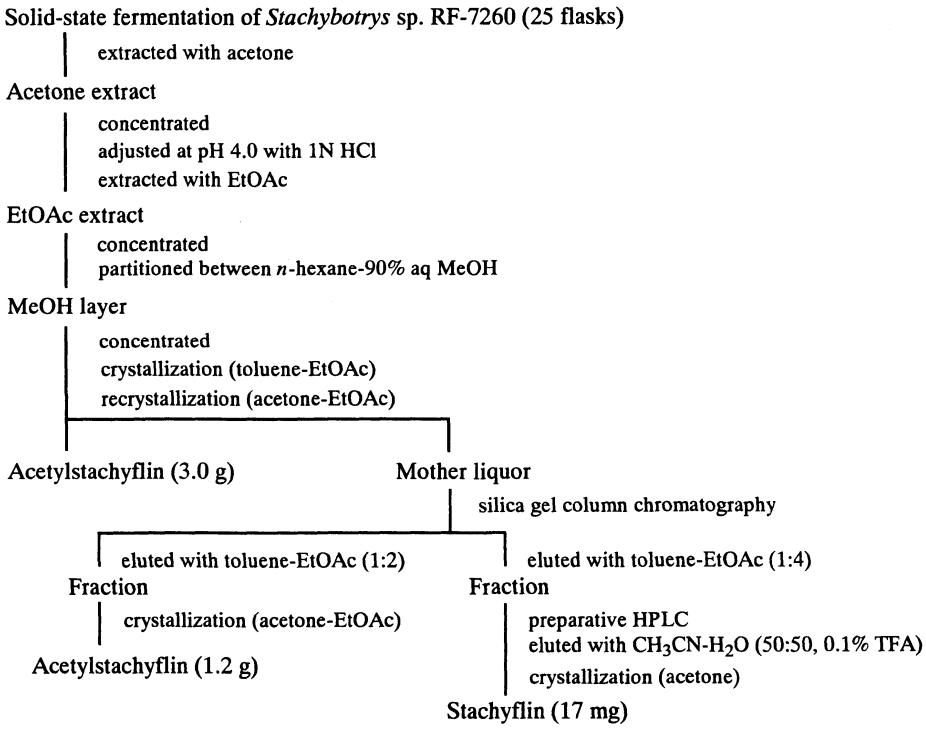
Fig. 2. Isolation procedure of acetylstachyflin and stachyflin.




## Results

### Fermentation and Isolation

The origin and taxonomy of the producing strains were described in our previous paper[3]. *Stachybotrys* sp. RF-7260 was fermented in a brown rice medium under static condition. The fermented rice medium was extracted with acetone and the extract was separated by solvent partition, silica gel chromatography, crystallization and preparative HPLC. The isolation procedure of acetylstachyflin (**1**) and stachyflin (**2**) is shown in Fig. 2.

### Physico-chemical Properties

The physico-chemical properties of acetylstachyflin (**1**) and stachyflin (**2**) are summarized in Table 1. Both compounds were soluble in MeOH, acetone, EtOAc and DMSO, slightly soluble in chloroform, but insoluble in *n*-hexane and water. Compound **1** showed UV absorption maxima at 215, 256 and 301 nm characteristic of some triprenyl phenol compounds[4]. The carbonyl groups were observed in the IR spectrum as strong bands at 1735 (ester) and 1627 (amide) $cm^{-1}$, while the strong absorption at 1613 $cm^{-1}$ was attributed to an aromatic ring. The UV spectra of **1** and **2** indicated the existence of the same chromophore in both compounds. The IR spectrum of **2** was similar to that of **1** except for the absorption at 1735 $cm^{-1}$.

### Structure Elucidation of Acetylstachyflin

The molecular formula of **1** was determined to be $C_{25}H_{33}NO_5$ on the basis of HR-SIMS and NMR spectral data. The $^{13}C$ NMR and DEPT spectra (DMSO-$d_6$) showed 25 resolved signals, consisting of five methyl, six methylene, four methine and ten quaternary carbons (Table 2). The $^1H$ NMR spectrum (DMSO-$d_6$) displayed 33 protons. The two proton signals due to deuterium exchangeable protons were observed at $\delta$ 8.31 and 9.73. The five unsaturated quaternary carbons ($\delta_C$ 111.81, 120.59, 131.62, 146.78 and 155.81) and an unsaturated methine carbon ($\delta_C$ 99.13) indicated the presence of a penta-substituted aromatic ring moiety. The geminal-coupled methylene signals at H-8′ ($\delta$ 4.09 and 4.17) form a typical AB system, which was similar to that of isoindolinone compounds[5]. The long-range $^1H$-$^{13}C$ correlations of the amide proton ($\delta$ 8.31) with C-8′ ($\delta_C$

Table 1. Physico-chemical properties of acetylstachyflin (**1**) and stachyflin (**2**).

| | **1** | **2** |
|---|---|---|
| Appearance | colorless needles | colorless needles |
| MP | >300°C | >300°C |
| $[\alpha]_D^{24.5}$ | +136.4° (*c* 1.0, MeOH) | +138.7° (*c* 1.0, MeOH) |
| Molecular weight | 427 | 385 |
| Molecular formula | $C_{25}H_{33}NO_5$ | $C_{23}H_{31}NO_4$ |
| HR-LSIMS (m/z) | | |
| calcd : | 428.2435 (as $C_{25}H_{34}NO_5$) | 386.2329 (as $C_{23}H_{32}NO_4$) |
| found : | 428.2445 | 386.2328 |
| UV $\lambda_{max}$ nm ($\varepsilon$) in MeOH | 215(45,700)<br>256(7,000)<br>301(3,200) | 216(42,700)<br>257(6,600)<br>302(3,000) |
| IR $\nu_{max}$ $cm^{-1}$ (KBr) | 3405,1735,1689,1627,1613,1466 | 3410,1684,1625,1465 |

42.34), C-3′ ($\delta_C$ 120.59), C-4′ ($\delta_C$ 131.62) and C-7′ ($\delta_C$ 170.27) revealed a lactam ring moiety. In addition to these results, the correlations of the methylene proton H-8′ with C-3′, C-4′ and C-2′ ($\delta_C$ 146.78), and of the aromatic proton H-5′ ($\delta$ 6.63) with C-1′ ($\delta_C$ 111.81), C-3′, C-6′ ($\delta_C$ 155.81) and C-7′, and of the phenolic proton 6′-OH ($\delta$ 9.73) with C-1′ confirmed the presence of an isoindolinone subunit. The $^1H$-$^1H$ COSY experiment revealed the partial structures of H-1 to H-3 and H-5 to $H_3$-12, as shown in Fig. 3. The geminal dimethyl system was hypothesized to be connected to C-3 and C-5 by the long-range $^1H$-$^{13}C$ correlations of $H_3$-13 ($\delta$ 1.03) with C-4 ($\delta_C$ 36.53) and C-5 ($\delta_C$ 43.76), and of $H_3$-14 ($\delta$ 0.81) with C-3 ($\delta_C$ 75.24) and C-4. The decalin ring moiety was established on the basis of the long-range $^1H$-$^{13}C$ correlations of H-2 ($\delta$ 1.59 and 2.38), H-5 ($\delta$ 1.58) and H-8 ($\delta$ 1.77) with C-10 ($\delta_C$ 82.43), and of $H_3$-12 ($\delta$ 1.11) with C-9 ($\delta_C$ 36.95). Moreover, the long-range $^1H$-$^{13}C$ correlations of $H_3$-15 ($\delta$ 0.84) with C-8 ($\delta_C$ 38.66), C-9 and C-10 revealed the presence of the methyl group at C-9. The long-range $^1H$-$^{13}C$ correlations of H-3 ($\delta$ 4.65) with the acetyl carbonyl C-16 ($\delta_C$ 169.62), and of the acetyl methyl $H_3$-17 ($\delta$ 2.05) with C-16 indicated the C-3 acetoxyl functionality. Connection of the decalin moiety and the isoindolinone moiety through C-11 was confirmed by the correlations of H-11 ($\delta$ 2.13 and 3.09) with C-1′, C-2′, C-9, C-10 and C-15 ($\delta_C$ 19.62), and of $H_3$-15 with C-11 as shown in Fig. 3. Unfortunately, no long-range $^1H$-$^{13}C$ correlation across the pyran oxygen was observed. However, C-10 and C-2′ were hypothesized to be linked to an oxygen atom based on its chemical shift ($\delta_C$ 82.43 and $\delta_C$ 146.78, respectively), and the molecular formula of **1** confirmed the presence of the pyran ring and the connectivity of C-10 to C-2′ through the oxygen atom. Thus, the planar structure of **1** was established as shown in Fig. 1.

The relative stereochemistry of **1** was determined on the basis of the NOE experiments and X-ray diffraction analysis. The NOEs were observed between $H_3$-15 and both H-1 and $H_3$-12, between H-8 and H-11. Thus, $H_3$-12 and $H_3$-15 were located on the top face of the molecule. In addition, the NOEs between $H_3$-13 and H-6 and between H-5 and H-11 established that the two cyclohexane rings of the sesquiterpene fragment are *cis*-fused as shown in Fig. 4. The acetoxyl group at C-3 was assigned an axial orientation on the basis of the small coupling constants of H-3 ($\delta$ 4.65, $J$=~3 Hz, t-like) and the NOEs between H-3 and both $H_3$-13 and $H_3$-14. The relative stereostructure of **1** was confirmed by X-ray diffraction analysis as shown in Fig. 5.

## Structure Elucidation of Stachyflin

The molecular formula of stachyflin (**2**) was determined to be $C_{23}H_{31}NO_4$ on the basis of HR-SIMS and NMR data. In the $^1H$ and $^{13}C$ NMR spectra of **2**, the signals due to $H_3$-17 ($\delta$ 2.05, $\delta_C$ 20.92) and C-16 ($\delta_C$ 169.62) of the acetyl group seen in **1** were absent and the upfield shift of H-3

Table 2. $^{1}H$ (600 MHz) and $^{13}C$ (150 MHz) NMR spectral data of acetylstachyflin and stachyflin in DMSO-$d_6$.

| | Acetylstachyflin | | Stachyflin | |
|---|---|---|---|---|
| Position | $\delta_C$ (ppm) | $\delta_H$ (ppm, $J$ in Hz) | $\delta_C$ (ppm) | $\delta_H$ (ppm, $J$ in Hz) |
| 1 | 23.82 (t) | 1.68 (m), 2.07 (m) | 23.39 (t) | 1.57 (m), 1.21 (m) |
| 2 | 22.46 (t) | 1.59 (m), 2.38 (m) | 25.63 (t) | 1.54 (m), 2.34 (m) |
| 3 | 75.24 (d) | 4.65 (t-like) | 72.08 (d) | 3.34 (m) |
| 3-OH | | | | 4.46 (d, 3.0) |
| 4 | 36.53 (s) | | 37.34 (s) | |
| 5 | 43.76 (d) | 1.58 (m) | 44.23 (d) | 1.46 (dd, 13.0 & 3.0) |
| 6 | 22.77 (t) | 1.71 (m), 1.93 (m) | 23.34 (t) | 1.64 (m), 2.12 (m) |
| 7 | 27.14 (t) | 1.32 (m), 2.04 (m) | 27.50 (t) | 1.25 (m), 1.97 (m) |
| 8 | 38.66 (d) | 1.77 (m) | 38.95 (d) | 1.73 (m) |
| 9 | 36.95 (s) | | 37.02 (s) | |
| 10 | 82.43 (s) | | 83.16 (s) | |
| 11 | 31.63 (t) | 2.13 (d, 17.8), 3.09 (d, 17.8) | 31.83 (t) | 2.09 (d, 17.9), 3.07 (d, 17.9) |
| 12 | 16.73 (q) | 1.11 (d, 7.3) | 16.88 (q) | 1.09 (d, 7.2) |
| 13 | 29.41 (q) | 1.03 (s) | 29.99 (q) | 0.92 (s) |
| 14 | 25.87 (q) | 0.81 (s) | 26.94 (q) | 0.89 (s) |
| 15 | 19.62 (q) | 0.84 (s) | 19.84 (q) | 0.83 (s) |
| 16 | 169.62 (s) | | | |
| 17 | 20.92 (q) | 2.05 (s) | | |
| 1' | 111.81 (s) | | 111.94 (s) | |
| 2' | 146.78 (s) | | 147.10 (s) | |
| 3' | 120.59 (s) | | 120.67 (s) | |
| 4' | 131.62 (s) | | 131.46 (s) | |
| 5' | 99.13 (d) | 6.63 (s) | 98.95 (d) | 6.61 (s) |
| 6' | 155.81 (s) | | 155.80 (s) | |
| 6'-OH | | 9.73 (s) | | 9.70 (s) |
| 7' | 170.27 (s) | | 170.36 (s) | |
| 8' | 42.34 (t) | 4.09 (d, 16.9), 4.17 (d, 16.9) | 42.44 (t) | 4.06 (d, 16.8), 4.16 (d, 16.8) |
| NH | | 8.31 (s) | | 8.29 (s) |

signal (from $\delta$ 4.65 in **1** to $\delta$ 3.34) was observed. These data suggested that **2** was the deacetyl analogue of **1**, which was confirmed by detailed 2D NMR analysis. In addition, treatment of **1** with 1 M NaOMe/MeOH gave deacetyl compound (**2**). The spectroscopic data and optical rotation of synthetic **2** were in complete agreement with those of natural **2**. This also revealed that **1** and **2** have the same relative stereochemistry.

## Absolute Stereochemistry

The absolute configuration of stachyflins was determined by circular dichroism (CD) using 3,8′-dioxo analogue (**4**) as follows. Acetylstachyflin (**1**) was first protected as a benzyl ether at the C-6′ position and then deacetylated with NaOMe/MeOH to give **3** in good yield. Treatment of **3** with Jones reagent (8.0 equivalents), followed by catalytic hydrogenation gave **4** (Scheme 1).

Applying the octant rule for *cis*-decalone[6,7], the alpha-

Fig. 3. $^{1}H$-$^{1}H$ COSY and key HMBC correlations of acetylstachyflin.

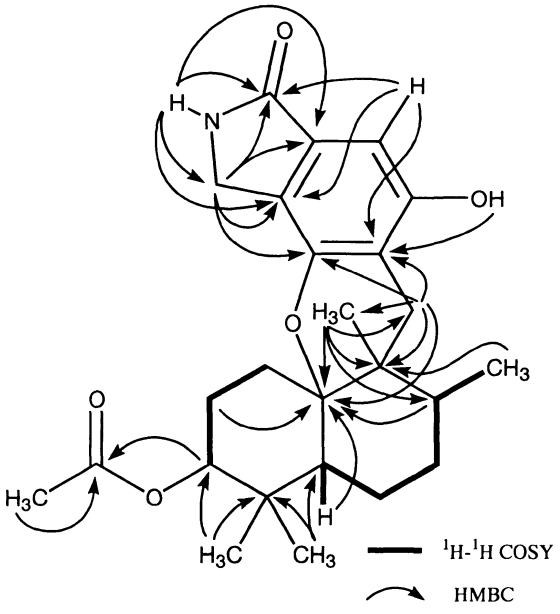
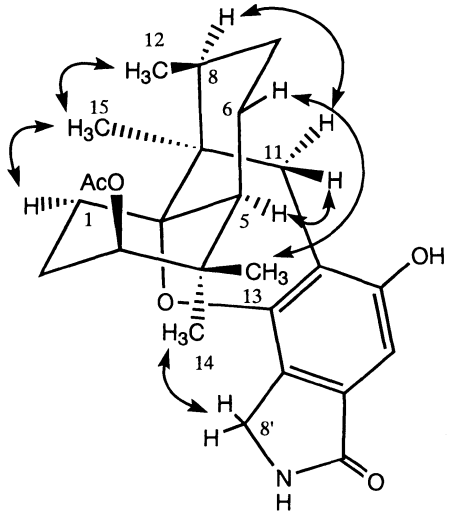
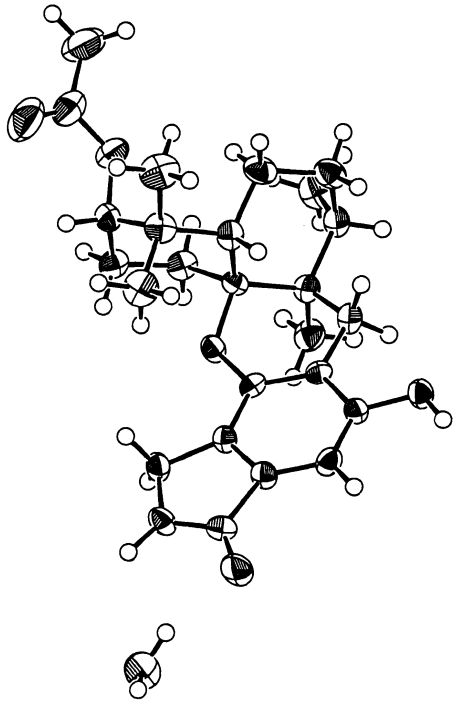



Fig. 4. Key NOE correlations of acetylstachyflin.




axial methyl group at C-4 was considered to show a significant effect on the CD spectrum. The CD spectrum of **4** showed a positive Cotton effect ($\Delta\varepsilon=+1.36$ at 292 nm in MeOH), from which the absolute structure of **4** was deduced by referring to the octant projection shown in Fig. 6. Thus the absolute configuration of stachyflins was established as shown in Fig. 1.

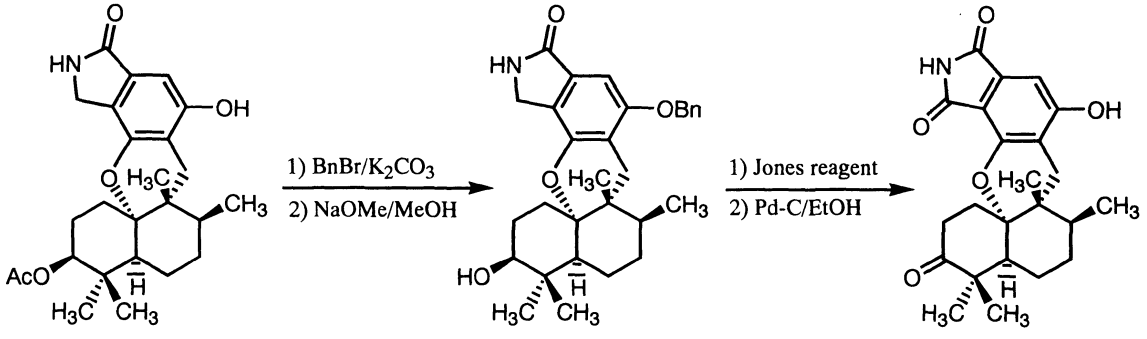
Fig. 5. ORTEP drawing of **1**.



## Biological Activities

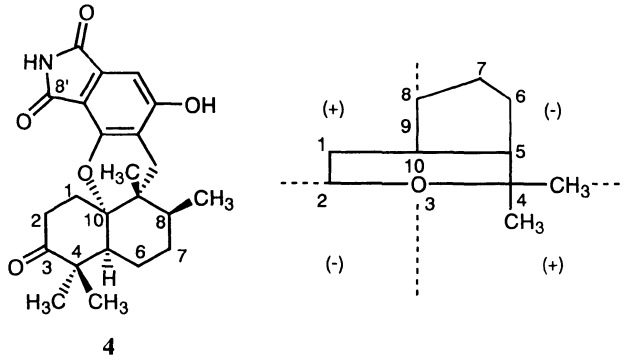
The *in vitro* antiviral activities against influenza A virus and cytotoxicities of stachyflins, amantadine and zanamivir, as measured using Madin-Darby bovine kidney (MDBK) cells according to a method described previously[8], are shown in Table 3. Acetylstachyflin (**1**) and stachyflin (**2**) had antiviral activity against influenza A/WSN/33 (H1N1) virus with an $IC_{50}$ value of 0.23 $\mu$M for **1** and 0.003 $\mu$M for **2**. The anti-influenza A virus activity of **2** was about 77 times more potent than that of **1**. This result suggested that the hydroxyl group at C-3 position might be important for the antiviral activity. Stachyflin (**2**) was about 1760 times more active than amantadine ($IC_{50}$ of 5.3 $\mu$M) and was about 250 times more active than zanamivir ($IC_{50}$ of 0.75 $\mu$M). The cytotoxicities of **1** and **2** against MDBK cells were 44 $\mu$M and 65 $\mu$M, respectively. Detailed studies on biological activities of **2** have been described by YOSHIMOTO *et al.*[8,9]. Stachyflin inhibits fusion between the viral envelope and the endosome constituting the cell membrane, which is the initial step in the entry of influenza virus into cells. On the other hand, zanamivir is a neuraminidase inhibitor[2], which blocks influenza virus replication. Amantadine has been known to inhibit

Scheme 1.




Acetylstachyflin (**1**) **3** (82%) **4** (28%)

Fig. 6. Absolute stereostructure of **4**.



**4**

Table 3. *In vitro* anti-influenza A virus activity and cytotoxicity.

| | anti-influenza A virus activity | cytotoxicity |
|---|---|---|
| compound | $IC_{50}$ (μM) | $CC_{50}$ (μM) |
| Stachyflin | 0.003 | 65 |
| Acetylstachyflin | 0.23 | 44 |
| Amantadine | 5.3 | 533 |
| Zanamivir | 0.75 | 301 |

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

replication by interfering with the ion channel activity of the influenza Matrix 2 (M2) protein[1]. Thus, the mechanism of antiviral action of stachyflin is different from that of the known anti-influenza virus agents.

## Discussion

In the present study, we isolated stachyflins with a novel pentacyclic skeleton including a *cis*-fused decalin and a pyranoisoindolinone ring system. *Stachybotrys* organisms and *Memnoniella echinata* produced similar metabolites such as K-76[10], stachybotridial[11], memnobotrin A[12] and stachybotrins[13]. Stachyflins and other metabolites are structurally different in the connection between the sesquiterpene and aromatic moieties. A similar structure occures in aureol[14] and strongylin A[15] produced by the marine sponges *Smenospongia aurea* and *Strongylophora hartmani*, respectively. These compounds also possess a *cis*-fused decalin and phenol moieties. It is interesting that such similar metabolites were isolated both from the fungus *Stachybotrys* sp. and marine sponges.

We have previously reported the isolation of bisabosqual A which has a bisabolane-type sesquiterpene moiety as part of a *cis*-fused tetracyclic ring system, by liquid medium fermentation of *Stachybotrys* sp. RF-7260[3,16]. Here, we report that solid-state fermentation of the same strain produced different secondary metabolites, stachyflins. Alteration of the fermentation conditions from liquid-state to solid-state seems to change the gene expression involved in secondary metabolite production. Indeed, gene expression of glucoamylase in fungi was observed only under solid-state fermentation conditions, and not under liquid fermentation conditions[17]. Therefore, our results suggest that *Stachybotrys* sp. RF-7260 may have at least two types of sesquiterpene cyclase, which plays a critical role in controlling the structural and stereochemical course of a particular cyclization reaction[18], and their expression may be controlled by fermentation conditions. These findings indicate that combination of solid-state and liquid-state fermentation may be useful for the production of diverse metabolites.

Stachyflin is not structurally related to any known anti-influenza virus agent, including amantadine and zanamivir, and moreover, exhibits potent anti-influenza activity with a novel mechanism. It has potential as an interesting lead compound. (±)-Stachyflin has been successfully synthesized by TAISHI *et al.*[19]. The synthesis of several derivatives of **2** and the preliminary structure-activity relationships of stachyflin will be discussed in the accompanying paper[20].

## Experimental

### General

Melting points were obtained using a Yanagimoto micro melting point apparatus and are uncorrected. 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. CD spectrum was recorded on a JASCO J-720 spectropolarimeter. LSIMS and HR-LSIMS were obtained on a Hitachi M-90 instrument. FAB-MS and HRFAB-MS were obtained on a JEOL JMS-SX/SX 102A. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Varian Unity-600 and Varian Gemini-300 spectrometers.

Amantadine was purchased from E. Merck Co., Ltd. 4-Guanidino-Neu5Ac2en (zanamivir) was synthesized from Neu5Ac2en in the Shionogi Research Laboratories, Shionogi & Co., Ltd.

### Fermentation

Spores of *Stachybotrys* sp. RF-7260 slant-cultured in five test tubes were suspended into 50 ml of physiological saline. Two ml each of this suspension was inoculated into each of twenty-five 500-ml Erlenmeyer flasks containing brown rice mediums (25 g of brown rice, 0.5 g of glucose, 0.1 g of yeast extract (Difco) and 50 ml of tap water), which had been sterilized at 121°C for 30 minutes in an autoclave. The fermentation was conducted under static conditions at 28°C for 14 days.

### Isolation

The fermented rice medium was extracted with acetone (2 liters) and the extract was concentrated *in vacuo* to give an aqueous residue. The aqueous residue was adjusted to pH 4.0 with 1 N HCl, followed by extraction with EtOAc. The EtOAc layer was concentrated to dryness, and partitioned between *n*-hexane (500 ml) and 90% aqueous MeOH (500 ml). The MeOH layer was concentrated to dryness to give 24 g of a crude fraction, which was successively crystallized from toluene-EtOAc and acetone-EtOAc to give **1** (3.0 g) as colorless needles. The crystallization mother liquors were concentrated *in vacuo* and subjected to silica gel chromatography (pre-packed column size B, LiChroprep Si60, E. Merck) eluting with toluene - EtOAc (1 : 2~1 : 4). The fractions eluted with toluene - EtOAc (1 : 2) were concentrated to dryness and crystallized from acetone-EtOAc to give **1** (1.2 g). The fractions eluted with toluene - EtOAc (1 : 4) were

concentrated to dryness to give 19 g of a stachyflin containing fraction. The fraction was further purified by preparative HPLC (LiChroprep RP-18, 25~40 $\mu$m, 20 i.d.×500 mm, acetonitrile - 0.1% trifluoroacetic acid/water (50 : 50)) to give a stachyflin fraction, which was neutralized with 1 N NaOH, and evaporated under reduced pressure to remove acetonitrile. The residue was extracted with EtOAc. After evaporation of the solvent, **2** was crystallized from acetone as colorless needles (17 mg).

X-Ray Crystallographic Analysis of Acetylstachyflin (**1**)

Colorless prismatic crystals of **1**, $C_{25}H_{33}NO_5 \cdot H_2O$ were grown from EtOAc-acetone solution. A single crystal with dimensions of 0.10×0.15×0.30 mm was used for data collection. X-ray diffraction measurements were performed on a Rigaku AFC5R diffractometer using graphite monochromated Mo-K$\alpha$ radiation ($\lambda$=0.70926 Å) and a rotating anode generator. Cell constants were obtained by least-squares refinement using the setting angles of 25 carefully centered reflections in the range $17°<2\theta<19°$. Crystal data are as follows: orthorhombic; space group; $P2_12_12_1$; $a$=13.879(3) Å, $b$=19.381(4) Å, $c$=8.614(4) Å, $V$=2316(1) Å$^3$, $Z$=4, $D$cal=1.28 g/cm$^3$. The data were collected at a temperature of 295 K using the $\omega/2\theta$ scan technique to the maximum $2\theta$ value of 50.1°. Scans of $(1.1+0.3\tan\theta)°$ were done at the speed of 8°/minute (in $\omega$). A total of 2379 unique reflections were collected. The linear absorption coefficient, $\mu$, for Mo-K$\alpha$ radiation is 0.9 cm$^{-1}$. The data were corrected for Lorentz and polarization effects. The structure was solved by direct methods[21] and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. The positional parameters of H-atoms in the OH, NH and $H_2O$ were refined, while the rest were included in fixed positions. The final cycle of full-matrix least-squares refinement was based on 2356 observed reflections [$I>0$] and 302 variable parameters. Final $R$ and weighted $R$ values were 0.116 and 0.109, respectively, and $R1$ was 0.053. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.45 and −0.52 e/Å$^3$, respectively. All calculations were performed using the teXsan[22] crystallographic software package of the Molecular Structure Corporation.

Conversion of Acetylstachyflin (**1**) into Stachyflin (**2**)

Compound **1** (1.2 g) was dissolved in dry MeOH (50 ml) and 1 M NaOMe/MeOH solution (57 ml), and heated to reflux for 12 hours. To the cooled reaction mixture was added water (50 ml), and MeOH was evaporated under reduced pressure. To the residual aqueous suspension was added 1 N HCl to adjust the pH to 1.0, followed by extraction with EtOAc (500 ml). The EtOAc layer was washed with water and dried over anhydrous sodium sulfate. The solvent was then evaporated under reduced pressure. The residue was crystallized from MeOH to give **2** (720 mg, 67%). All spectral data of the synthetic product were identical to those of the natural **2**.

Conversion of **1** into **3**

Benzyl bromide (1.52 g) and potassium carbonate (500 mg) were added to a solution of **1** (500 mg, 1.17 mmol) in acetone (30 ml), and the mixture was heated to reflux for 6 hours. The reaction mixture was filtered and the filtrate was concentrated. The resultant product was subjected to silica gel column chromatography (Silica gel 60, 70 g, E. Merck) using a mixed solvent of $n$-hexane - acetone (1 : 1) to give a benzyl derivative (600 mg, 99%). The benzyl derivative (102 mg, 0.24 mmol) was dissolved in dry MeOH (1 ml) and 1 M NaOMe/MeOH (4.8 ml), and heated to reflux for 5 hours. After cooling, water (3 ml) was added to the reaction mixture and MeOH was removed under reduced pressure. The pH of the residual aqueous layer was adjusted to 1.0 with 1 N HCl, followed by extraction with EtOAc (50 ml). The EtOAc layer was washed with water, dried over anhydrous sodium sulfate, and concentrated. The residue was crystallized from MeOH to give **3** (76 mg, 83%); HR FAB-MS ($m/z$) 476.2807 $(M+H)^+$ Calcd for $C_{30}H_{38}NO_4$: 476.2801; IR $\nu_{max}$ KBr cm$^{-1}$: 3423, 2957, 2871, 1682, 1625, 1601, 1465, 1452, 1366; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 0.92 (3H, s), 0.98 (3H, s), 1.02 (3H, s), 1.14 (3H, d, $J$=7.2 Hz), 1.34 (1H, m), 1.78 (6H, m), 2.05 (2H, m), 2.28 (1H, d, $J$=18.0 Hz), 2.30 (1H, m), 2.45 (1H, m), 3.19 (1H, d, $J$=18.0 Hz), 3.56 (1H, m), 4.33 (1H, d, $J$=16.8 Hz), 4.36 (1H, d, $J$=16.8 Hz), 5.11 (2H, s), 6.81 (1H, br.s), 6.98 (1H, s), 7.32~7.47 (5H, m); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 17.15, 20.27, 23.86, 24.15, 25.92, 26.53, 28.14, 30.36, 32.36, 37.66, 37.95, 39.48, 43.77, 44.78, 70.25, 74.39, 83.90, 96.68, 114.62, 123.57, 127.32 (×2), 127.81, 128.41 (×2), 130.98, 136.79, 147.33, 157.43, 172.54.

Conversion of **3** into **4**

Jones reagent (8 mol equiv) was added to a solution of **3** (24 mg) in acetone (2 ml), and the mixture was stirred at room temperature for 3 hours. Two drops of 2-PrOH were added to the reaction mixture, which was then neutralized with 10% aqueous sodium hydrogen carbonate. The solvent was evaporated under reduced pressure. The residue was then partitioned between EtOAc (20 ml) and water (20 ml). The EtOAc layer was concentrated and purified by

preparative TLC (pre-coated TLC plates, Silica gel F-254, E. Merck, $CH_2Cl_2$-MeOH (10:1)) to give an oxidation product (8.4 mg). A solution of the oxidation product (8.4 mg) in EtOH (2 ml) was treated with 10% palladium-charcoal (10 mg) under a hydrogen atmosphere for 3 hours. After the catalyst was filtered off, the filtrate was concentrated under reduced pressure to give **4** (5.6 mg, 28%); HR FAB-MS (*m/z*) 398.1970 $(M+H)^+$ Calcd for $C_{23}H_{28}NO_5$: 398.1967; CD $\lambda_{max}^{MeOH}$ nm ($\Delta\varepsilon$): 340 (+1.64), 292 (+1.36), 243 (+16.4), 221 (−1.99); IR $\nu_{max}$ KBr $cm^{-1}$: 3450, 3340, 2967, 2933, 2883, 1752, 1706, 1620, 1603, 1482, 1457, 1425, 1389; $^1H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.86 (3H, s), 0.89 (3H, s), 1.07 (3H, d, $J$=7.2 Hz), 1.22 (3H, s), 1.35 (1H, m), 1.80 (3H, m), 1.9~2.2 (6H, m), 2.19 (1H, d, $J$=18.0 Hz), 2.40 (1H, m), 3.05 (1H, d, $J$=18.0 Hz), 6.77 (1H, s), 10.73 (1H, s); $^{13}C$ NMR (75 MHz, DMSO-$d_6$) $\delta$ 16.83, 19.86, 23.68, 24.01, 27.03, 28.50, 30.21, 31.89, 33.39, 36.64, 47.45, 48.45, 82.74, 101.42, 107.72, 114.25, 133.49, 149.78, 161.77, 167.94, 168.91, 214.81.

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