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# F-11334s, New Inhibitors of Membrane-bound Neutral Sphingomyelinase

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Ceramide, the product of sphingomyelin hydrolysis by neutral pH-optimum and magnesium-dependent sphingomyelinase (N-SMase), has been reported to increase in response to several inflammatory stimuli, including TNF$\alpha$ and IL-1$\beta$ and to play an important role in such stimuli-mediated physiological and pathological processes[1~9]. Intervention of the ceramide production, therefore, might have clinical potential, and thus inhibitors of N-SMase were sought in fermentation products. As a result, novel hydroquinones, F-11334s, were discovered in the mycerial extract of a fungal strain. The producing organism was identified as *Acremonium murorum* (Corda) W. Gams SANK 20793 by the characteristics of the slime drop of dark amerospores arising from hyaline phialides. Under neutral condition, they showed variable inhibitory activity to N-SMase derived from rat brain microsome fraction[10]. Herein we report fermentation, isolation and structural studies on F-11334s.

The producing organism was inoculated to a 500 ml Erlenmeyer flask containing 100 ml GPMY medium (composed of: 5% (v/v) glycerol, 5% potato, 0.5% yeast extract, 0.5% malt extract, 0.005% CB-422) and incubated on a rotatory shaker (200 rpm) at 23°C for 4 days. Two milliliters of the seed culture was inoculated into seven 500 ml Erlenmeyer flasks, each containing 100 ml of GPMY medium, and cultivation was carried out for 7 days under the same condition. The mycelial cake was separated by centrifugation and extracted with 80% aqueous acetone (700 ml). The extract was concentrated *in vacuo* to remove acetone, and the resulting aqueous solution was extracted three times with ethyl acetate. The ethyl acetate extract (1.6 g) was applied to a silica gel column equilibrated with dichloromethane - methanol (15 : 1). The column was eluted with the same solvent, and the fractions showing N-SMase inhibitory activity were combined. Further purification was accomplished by HPLC (column: Nacalai tesque, COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 9 ml/minute, mobile phase: 50% aqueous methanol, detection: 210 nm) to give F-11334 $A_1$ (19 mg), a mixture of $A_2$ and $A_3$ (34 mg), $B_1$ (4.5 mg) and $B_2$ (10 mg). The retention times were 7.0 minutes for $A_1$, 10.0 minutes for $A_2$ and $A_3$, 8.0 minutes for $B_1$, and 12.0 minutes for $B_2$. Separation of $A_2$ (7 mg) and $A_3$ (23 mg) was achieved by normal phase HPLC (column: Senshu Pak-Silica-4251-N, 10 i.d.×250 mm, flow rate: 5 ml/minute mobile phase: hexane - EtOAc (2 : 1), detection: differential refractive index). The retention times of $A_2$ and $A_3$ were 14.0 minutes and 15.0 minutes, respectively.

The physico-chemical properties of F-11334s are summarized in Table 1. UV absorption bands due to the hydroquinone chromophore were observed at about 216, 226 and 293 nm for compounds of the A group and at about 248 and 322 nm for those of the B group. Only the compounds of the A group were optically active, and $A_1$, $B_1$ and $B_2$ spectra showed IR absorption bands at 1660 and 1610 $cm^{-1}$; the bands were not detected in spectra for $A_2$ and $A_3$. Since these results suggested that all F-11334s are closely related compounds, structural studies were first carried out on the major active component, $A_1$, by interpreting the NMR spectra taken in $CD_3OD$.

$^1H$- and $^{13}C$-NMR spectral data of F-11334 $A_1$ are summarized in Table 2. Spin-spin coupling between 7-H and 8-H and long-range correlations of methyl proton (1.23 ppm, 6H, s) with C-8 and C-9 in the COLOC experiment showed the existence of a 2,3-dihydroxy-3-methylbutyl group, which was in turn attached to the C-2 aryl quaternary carbon based on long-range correlation of the 7-H methylene proton with C-1, C-2 and C-3. The 2-alkylhydroquinone chromophore was revealed by the coupling constants between 3-H and 5-H (3 Hz, *meta*) and between 5-H and 6-H (8.5 Hz, *ortho*). These results gave the full structure of F-11334 $A_1$ as shown in Fig. 1.

$^1H$- and $^{13}C$-NMR spectral data of F-11334 $A_2$ and $A_3$ were similar to those of $A_1$ except for the chemical shifts of the oxymethine groups and the oxygenated quaternary

Table 1. Physico-chemical properties of F-11334s.

| | F-11334 $A_1$ | $A_2$ | $A_3$ |
|---|---|---|---|
| Appearance | Colorless powder | Colorless powder | Colorless powder |
| Molecular formula | $C_{11}H_{16}O_4$ | $C_{11}H_{14}O_3$ | $C_{11}H_{14}O_3$ |
| HREI-MS (*m/z*) Found | 212.1051 $(M)^{+\cdot}$ | 194.0955 $(M)^{+\cdot}$ | 194.0941 $(M)^{+\cdot}$ |
| Calcd. | 212.1049 | 194.0942 | 194.0942 |
| UV $\lambda_{max}$ nm (ε) in MeOH | 216 sh (4580), 226 sh (3690), 293 (3050) | 216 sh (4020), 229 (4310), 303 (3670) | 218 sh (5590), 227 (5240), 297 (3670) |
| IR $\nu_{max}$ $cm^{-1}$ (KBr) | 3340,1650, 1610, 1500, 1460 | 3230, 3160, 1490 | 3230, 3160, 1490 |
| $[\alpha]_D^{23}$ (MeOH) | +36.0 ° (*c* 0.2) | +49.8 ° (*c* 1.0) | -20.4 ° (*c* 1.0) |

| | $B_1$ | $B_2$ |
|---|---|---|
| Appearance | Colorless powder | Colorless powder |
| Molecular formula | $C_{11}H_{14}O_3$ | $C_{12}H_{16}O_3$ |
| HREI-MS (*m/z*) Found | 176.0844 $(M-H_2O)^{+\cdot}$ | 176.0835 $(M-MeOH)^{+\cdot}$ |
| Calcd. | 176.0837 | 176.0837 |
| UV $\lambda_{max}$ nm (ε) in MeOH | 248 sh (8500), 322 (3840) | 249 sh (9860), 325 (4490) |
| IR $\nu_{max}$ $cm^{-1}$ (KBr) | 3330,1660, 1610, 1500, 1460 | 3260, 1650, 1610 1510, 1460 |
| $[\alpha]_D^{23}$ (MeOH) | optically inactive | optically inactive |

Table 2. NMR Data of F-11334s ($CD_3OD$).

| | $A_1$ | | $A_2$ | | $A_3$ | |
|---|---|---|---|---|---|---|
| No. | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | 150.1 | | 155.0 | | 147.8 | |
| 2 | 129.6 | | 129.7 | | 122.4 | |
| 3 | 119.3 | 6.62 (1H, d, *J*=3 Hz) | 113.5 | 6.62 (1H, br s) | 116.9 | 6.62 (1H, br s) |
| 4 | 151.8 | | 152.6 | | 152.2 | |
| 5 | 115.2 | 6.50 (1H, dd, *J*=8.5, 3 Hz) | 115.3 | 6.48 (1H, dd, *J*=8.5, 2.5 Hz) | 116.1 | 6.52 (1H, dd, *J*=8.5, 2.5 Hz) |
| 6 | 117.7 | 6.63 (1H, d, *J*=8.5 Hz) | 110.2 | 6.52 (1H, br d, *J*=8.5 Hz) | 118.9 | 6.56 (1H, d, *J*=8.5 Hz) |
| 7 | 34.8 | 2.80 (1H, dd, *J*=13.5, 2 Hz) | 32.5 | 3.12 (1H, dd, *J*=15.5, 8.5 Hz) | 32.9 | 2.92 (1H, dd, *J*=16.5, 5.5 Hz) |
| | | 2.60 (1H, dd, *J*=13.5, 10 Hz) | | 3.06 (1H, dd, *J*=15.5, 9.5 Hz) | | 2.63 (1H, dd, *J*=16.5, 7.5 Hz) |
| 8 | 81.2 | 3.60 (1H, dd, *J*=10, 2 Hz) | 90.7 | 4.50 (1H, dd, *J*=9.5, 8.5 Hz) | 71.2 | 3.70 (1H, dd, *J*=7.5, 5.5 Hz) |
| 9 | 74.3 | | 73.0 | | 78.0 | |
| 10 | 26.2 | 1.23 (3H, s) | 25.9 | 1.20 (3H, s) | 26.3 | 1.29 (3H, s) |
| 11 | 25.4 | 1.23 (3H, s) | 25.6 | 1.22 (3H, s) | 21.4 | 1.20 (3H, s) |

| | $B_1$ | | $B_2$ | |
|---|---|---|---|---|
| No. | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | 149.5 | | 149.6 | |
| 2 | 126.7 | | 126.2 | |
| 3 | 113.9 | 6.83 (1H, d, *J*=3 Hz) | 113.7 | 6.84 (1H, d, *J*=3 Hz) |
| 4 | 151.7 | | 151.7 | |
| 5 | 116.6 | 6.51 (1H, dd, *J*=8.5, 3 Hz) | 117.0 | 6.54 (1H, dd, *J*=8.5, 3 Hz) |
| 6 | 117.9 | 6.61 (1H, d, *J*=8.5 Hz) | 117.9 | 6.62 (1H, d, *J*=8.5 Hz) |
| 7 | 123.0 | 6.82 (1H, d, *J*=16 Hz) | 126.4 | 6.80 (1H, d, *J*=16.5 Hz) |
| 8 | 138.6 | 6.28 (1H, d, *J*=16 Hz) | 135.3 | 6.12 (1H, d, *J*=16.5 Hz) |
| 9 | 72.1 | | 77.5 | |
| 10 | 30.4 | 1.37 (3H, s) | 26.7 | 1.36 (3H, s) |
| 11 | 30.4 | 1.37 (3H, s) | 26.7 | 1.36 (3H, s) |
| 12 | | | 51.2 | 3.21 (3H, s) |

Fig. 1. Structure of F-11334 $A_1$.

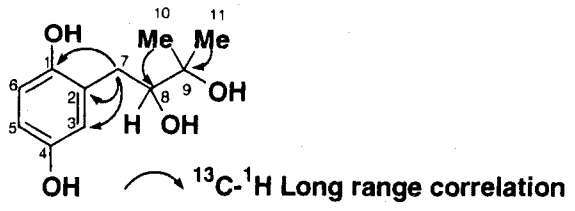
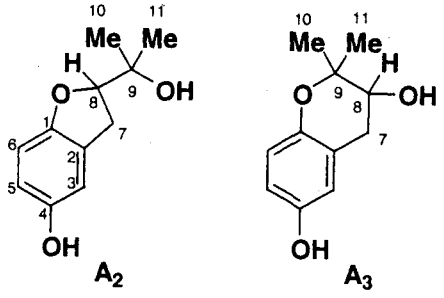



Fig. 2. Structures of F-11334 $A_2$ and $A_3$.

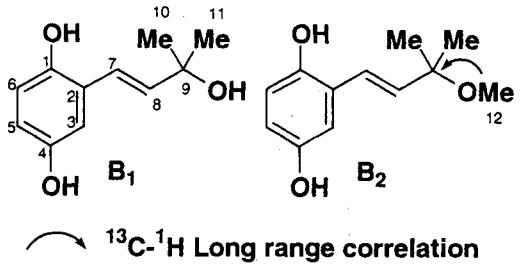



carbons (Table 2). The molecular formula for both $A_2$ and $A_3$ was $C_{11}H_{14}O_3$, suggesting that both were dehydrated derivatives of $A_1$. In the case of $A_3$, the cyclization between the C-9 and C-1 hydroxyl groups of $A_1$ was suggested since a large deuterium induced-$\beta$ shift[11,12] (0.12 ppm) was observed on the oxymethine carbon signal in the measurement of $^{13}$C-NMR in $CD_3OD/CD_3OH$. The oxymethine proton signal of $A_2$ was observed at 4.50 ppm in $CD_3OD$, suggesting the existence of a 2,3-dihydrobenzo[*b*]furan moiety in the structure. In the case of $A_2$, therefore, the cyclization between C-8 and C-1 hydroxyl groups of $A_1$ was relevant, and the structures of $A_2$ and $A_3$ were decided as shown in Fig. 2.

Signals due to both the oxymethine group and the methylene group were absent in $^1$H- and $^{13}$C-NMR spectra of F-11334 $B_1$ compared with those of $A_1$, and two olefinic methine groups were observed instead (Table 2). The geometry of the double bond was determined to be *E* based on the large coupling constants (16.0 Hz) of the olefinic proton signals. Although the 1D NMR spectrum of $B_2$ was very similar to that of $B_1$ (Table 2), the existence of a methoxy group on the C-9 quaternary carbon was verified by long-range coupling between 12-H and C-9 in the COLOC experiment. Based on those observations, the structures of $B_1$ and $B_2$ were decided as shown in Fig. 3.

F-11334s are hydroquinone derivatives that are substituted by alkyl groups derived from isoprene at the C-2 position. Nothing is known about the absolute configuration of the compounds. Although acidic conditions might account for the formation of F-11334 $A_2$, $A_3$, $B_1$ and $B_2$ from $A_1$, the fact that all of the compounds were detected in the acetone extract by HPLC analyses showed that F-11334s were actually natural products. $IC_{50}$ values of F-11334 $A_1$, $A_2$, $A_3$, $B_1$ and $B_2$ relative to N-SMase of rat brain microsome fraction under neutral conditions were 7.5, >200, >200, 3.6 and 3.2 $\mu$g/ml, respectively, suggesting that free hydroxyl groups in the hydroquinone moiety were required for F-11334s to exhibit enzyme inhibitory activity.

Fig. 3. Structures of F-11334 $B_1$ and $B_2$.

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