8-*O*-Methylsclerotiorinamine, Antagonist of the Grb2−SH2 Domain, Isolated from *Penicillium multicolor*

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A new secondary metabolite, 8-*O*-methylsclerotiorinamine (**1**), was isolated from a strain of *Penicillium multicolor*, and its structure was established using NMR spectroscopy and chemical evidence. The metabolite inhibited significantly the binding between the Grb2−SH2 domain and the phosphopeptide derived from the Shc protein and also blocked the protein−protein interactions of Grb2−Shc in cell-based experiments, with IC_{50} values of 5.3 and 50 μM, respectively.

Signal transduction is critical for biological processes including cell growth and differentiation, and aberrations in some signal pathways cause adverse effects such as cancers.[1] The growth factor receptor-bound protein-2 (Grb2) is one adaptor protein involved in the Ras signaling pathway. It binds several tyrosine-phosphorylated receptors via Src homology 2 (SH2) domain, including erbB2 (a subfamily of EGFR), EGFR (epidermal growth factor receptor), and PDGFR (platelet-derived growth factor receptor), as well as other phosphotyrosine-containing proteins such as Shc (Src homology and collagen protein) and IRS-1 (insulin receptor substrate 1).[2] Therefore, development of Grb2 SH2 domain binding blockers has important implications for treatment of a variety of diseases, including several cancers.[3] Recently, we found that actinomycins, well-known DNA intercalators, inhibited the Grb2−Shc binding in *in vitro* and cell-based assays.[4,5]

During a search for SH2 domain antagonists from natural sources, 8-*O*-methylsclerotiorinamine (**1**) was isolated from the fermentation broth of *Penicillium multicolor*. Many sclerotiorin analogues have been reported to exhibit a variety of biological activities.[6−9] In this paper, we describe the isolation, structure determination, and biological activity (Grb2−Shc interaction) of **1**.

The structure of **1** was determined by spectroscopic analysis and comparison with spectral data of sclerotiorin analogues.[8,9] A molecular formula of $C_{22}H_{26}NO_4Cl$ was determined by HREIMS ($[M]^+$, *m*/*z* 403.1576, calcd 403.1551 for $C_{22}H_{26}NO_4Cl$). The number of carbon atoms was consistent with 22 individual ^{13}C NMR signals. The HMQC experiment and DEPT spectra revealed six methyl, one methylene, six methine, and nine quaternary carbons. $^1H-^1H$ correlations in COSY indicated the presence of a 3,5-dimethyl-1,3-heptadiene residue, a typical group in sclerotiorin analogues. The azaphilone skeleton was confirmed by the strong two- and three-bond HMBC correlations from C-3 (δ 162.6) to H-1 (δ 8.97) and H-4 (δ 7.49) and by the correlations of the quaternary C-8 (δ 160.6) with the protons on C-18 and C-19 together with the correlation of C-4 (δ 116.3) with H-9 as shown in Figure 1.

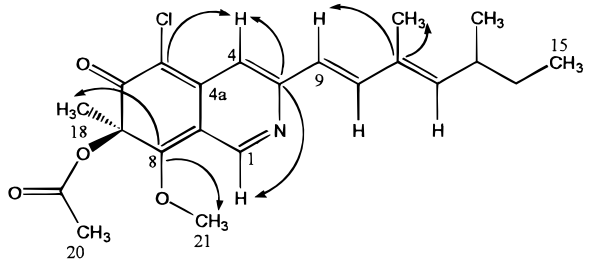
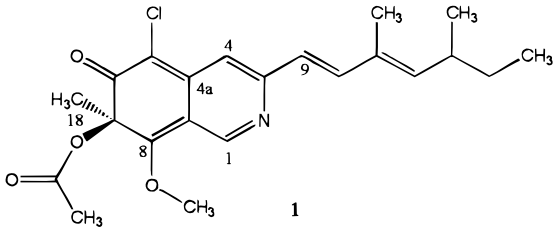
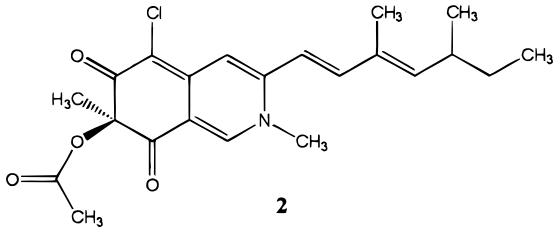


Figure 1. Some important HMBC correlations for compound **1**.





To confirm the proposed structure, **1** was treated with CH_3I in CH_3CN under reflux to obtain **2**, where 8-*O*-CH_3 had been converted to 2-*N*-CH_3 to give *N*-methylsclerotiorinamine (**2**). On the basis of HREIMS ($[M]^+$, *m*/*z* 403.1552, calcd 403.1551 for $C_{22}H_{26}NO_4Cl$), ^{13}C NMR, and DEPT, **2** had the same molecular formula as **1**. This chemical conversion was confirmed by the chemical shift of the 8-*O*-methyl from δ 3.96 to δ 3.61 (2-*N*-methyl) in ^1H NMR and from δ 62.6 to δ 42.3 in ^{13}C NMR. In addition, a large chemical shift change was seen for H-1 (from δ 8.97 for **1** to δ 7.76 for **2**).

Compound **1** strongly inhibited the binding between the GST−Grb2 SH2 domain and [^{3}H]-phosphopeptide-derived from Shc with an IC_{50} value of 7.2 μM. We also found that compound **1** inhibited the Grb2−Shc interactions in cell-based experiments using B104-1-1 cells.[5] First, we exam-

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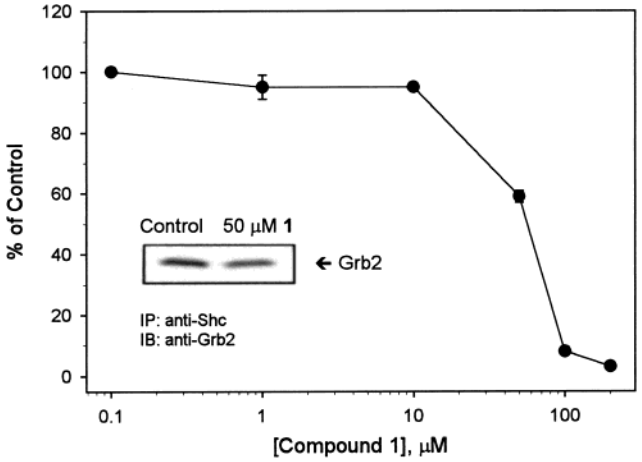

Figure 2. Growth-inhibition assay using the colorimetric method with WST-1. B104-1-1 cells were seeded at a density of 5000 cells/well in a 96-well microtiter plate. One day after seeding, cells were replenished with fresh complete medium containing **1** or 0.1% DMSO. After incubation for 48 h, cell proliferation reagent WST-1 (Roche Biochemicals) was added to each well. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad). In inset, B104-1-1 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EGTA, 1 mM sodium vanadate, 50 mM NaF, 30 mM $Na_4P_2O_7$, 10% glycerol, 1 mM PMSF, 5 μg/mL aprotinin, 10 μg/mL leupeptin), and then lysates were treated with DMSO or **1**. The Shc−Grb2 complexes were immunoprecipitated with rabbit antiserum against Shc. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using anti-Grb2 antibody.

ined whether **1** inhibited proliferation of B104-1-1 cells with the colorimetric method with WST-1 (Roche Biochemicals) and found that **1** inhibited proliferation of B104-1-1 cells with a GI_{50} value of 50 μM (see Figure 2). Therefore, we measured the inhibitory activity of **1** for the Grb2-Shc interaction in B104-1-1 cells at the concentration of 50 μM by the following method. Associated protein complexes containing Shc were immunoprecipitated from DMSO (control) or from 50 μM solution (in DMSO) **1**-treated cell lysates with polyclonal anti-Shc antibody. Then the association with Grb2 was analyzed by immunoblotting with monoclonal anti-Grb2 antibody, which revealed that **1** at a concentration of 50 μM inhibited Shc/Grb2 interaction about 50% (inset box in Figure 2).[5]

8-*O*-Methylsclerotioriamine (**1**) has a unique structure in comparison with other reported SH2 domain antagonists.[10] Therefore, **1** may be a useful lead compound for the development and design the Grb2−SH2 domain antagonists.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a digital spectropolarimeter. High-resolution mass spectra were obtained on an Autospec-UltimaE, VG. NMR spectra were recorded on a Bruker AMX 400 spectrometer.

Organism and Fermentation. The *Penicillium multicolor* fungal strain was isolated from a soil sample collected in Mt. Chilgap, Chungnam Province, as reported earlier.[11] *P. multicolor* was maintained on a potato-dextrose agar plate. Seed cultures were inoculated with two agar plugs added to a 500 mL Erlenmeyer flask containing 50 mL of seed medium containing 2% glucose, 0.2% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in distilled H_2O. The flasks of inoculated seed medium were incubated on an orbital shaker rotating at 150 rpm at 26 °C for 36−48 h. The seed culture was inoculated into the 1 L Erlenmeyer flask (32 flasks) containing 150 mL of production medium of the following composition: 2% soluble starch, 0.4% Bacto-soytone, 0.5% pharma media, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, and 0.3% $CaCO_3$ in distilled H_2O. The mixture was incubated for 5 days.

Isolation. After incubation, 150 mL of acetone was added to each flask, and the substrate was homogenized. The suspension was filtered, and the filtrate was concentrated *in vacuo*. The residue was resuspended in 500 mL of CH_2Cl_2 and filtered, and the filtrate was concentrated to give a dark residue (7.4 g). The residue was loaded onto a silica gel column and eluted with increasing concentrations of EtOAc in *n*-hexane. The active constituent was separated and further purified by preparative TLC with 30% EtOAc−hexane to obtain **1** (50 mg) as an orange gum.

8-*O*-Methylsclerotiorinamine (1): $[\alpha]^{25}_D$ +102° (*c* 0.15, MeOH); UV (MeOH) λ_{max} 204 (5600), 271 (16500), 286 (17800), 364 (22500) nm; IR (film) ν_{max} 2962, 1743, 1697, 1611, 1579, 1278 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.97 (1H, s, H-1), 7.49 (1H, s, H-4), 7.46 (1H, d, J = 15.9 Hz, H-10), 6.54 (1H, d, J = 16 Hz, H-9), 5.66 (1H, d, J = 9.6 Hz, H-12), 3.96 (3H, s, H-21), 2.45 (1H, m, H-13), 2.09 (3H, s, H-20), 1.83 (3H, s, H-17), 1.52 (3H, s, H-18), 1.42 (1H, m, H-14a), 1.32 (1H, m, H-14b), 0.93 (3H, d, J = 6.9 Hz, H-16), 0.85 (3H, t, J = 7.5 Hz, H-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.1 (s, C-6), 170.7 (s, $COOCH_3$), 162.6 (s, C-3), 160.6 (s, C-8), 149.6 (d, C-1), 147.7 (d, C-12), 143.7 (d, C-10), 133.2 (s, C-11), 130.5 (s, C-4a), 124.4 (d, C-9), 119.7 (s, C-8a), 116.3 (d, C-4), 111.9 (s, C-5), 81.3 (s, C-7), 62.6 (q, OCH_3), 35.6 (d, C-13), 30.8 (t, C-14), 23.7 (q, C-18), 20.9 (q, C-16), 20.9 (q, $COOCH_3$), 13.2 (q, C-17), 12.5 (q, C-15); EI ms *m/z* 403 (7.9), 381 (5.9), 346 (27.5), 318 (11.2), 288 (21.5), 219 (21.7), 69 (100); HREIMS *m/z* 403.1576 (calcd for $C_{22}H_{26}NO_4Cl$, 403.1551).

3-*N*-Methylsclerotiorinamine: gum, $[\alpha]^{25}_D$ +112° (*c* 0.12, MeOH); UV (MeOH) λ_{max} 207 (6600), 236 (16500), 343 (21800), 368 (23500) nm; $[\alpha]^{25}_D$ +112° (*c* 0.12, MeOH); IR (film) ν_{max} 2962, 1740, 1698, 1621, 1579, 1278 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.76 (1H, s, H-1), 7.01 (1H, s, H-4), 6.95 (1H, d, J = 15.6 Hz, H-10), 6.13 (1H, d, J = 15.6 Hz, H-9), 5.71 (1H, d, J = 9.8 Hz, H-12), 3.61 (3H, s, H-21), 2.46 (1H, m, H-13), 2.16 (3H, s, H-20), 1.85 (3H, s, H-17), 1.53 (3H, s, H-18), 1.41 (1H, m, H-14a), 1.31 (1H, m, H-14b), 1.0 (3H, d, J = 6.9 Hz, H-16), 0.87 (3H, t, J = 7.5 Hz, H-15); ^{13}C NMR ($CDCl_3$, 100 MHz) 193.8 (s, C-8), 184.1 (s, C-6),) δ 170.4 (s, $COOCH_3$), 148.8 (d, C-12), 146.2 (s, C-5), 145.2 (d, C-10), 145.0 (s, C-3), 142.4 (d, C-1), 132.1 (s, C-11), 115.0 (s, C-8a), 114.9 (d, C-9), 111.5 (d, C-4), 102.1 (s, C-4a), 85.0 (s, C-7), 42.3 (q, NCH_3), 35.4 (d, C-13), 30.4 (t, C-14), 23.5 (q, C-18), 20.6 (q, $COOCH_3$), 20.5 (q, C-16), 12.9 (q, C-17), 12.3 (q, C-15); EI ms *m/z* 403 (41.0), 361 (100), 345 (12.5), 318 (70.7), 75 (87.8); HREIMS *m/z* 403.1552 (calcd for $C_{22}H_{26}NO_4Cl$, 403.1551).

Grb2−SH2 Domain Binding Assay. The binding affinity against the GST−Grb2 SH2 domain was measured using Amersham protein A-coated SPA beads and a rabbit IgG anti-GST antibody (Molecular Probes) to bind the fusion protein to the bead. The reaction mixture contained 0.25 μg of GST−Grb2 SH2 domain fusion protein, 6 μg of anti-GST antibody, 0.24 mg of protein A SPA beads (Amersham), and 0.2 μCi of [^3H]propionyl-labeled AcSpYVNVK−NH_2, which was derived from the SH2 domain binding sequence of Shc pY317. The final volume of 200 μL was obtained by adding a buffer of 20 mM Tris-HCl, pH 7.4, containing 250 mM NaCl and 0.1% bovine serum albumin. Compounds dissolved in DMSO were added to give a final DMSO concentration of 2 vol %. Plates were typically incubated with shaking at room temperature for 3 h. The inhibitory activity was measured by the released energy from SPA bead-bound [^3H]-peptide by counting with a 1450 Microbeta counter (Wallac).

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