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Sclerotiorin and Isochromophilone IV: Inhibitors of Grb2-Shc Interaction, Isolated from *Penicillium multicolor* F1753

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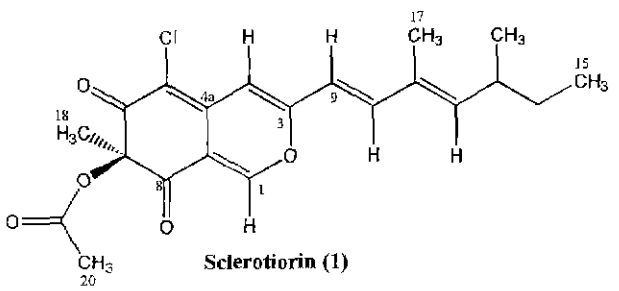
Abstract Grb2 is an important adaptor protein in the mitogenic Ras signaling pathway of receptor tyrosine kinases, and contains one SH2 domain and two SH3 domains. The SH2 domain binds to specific phosphotyrosine motifs on receptors or adaptor proteins such as Shc. The SH2 domain antagonists may lead to blocking of the oncogenic Ras signals and to developing new antitumor agents. In the course of screening SH2 antagonists from natural sources, sclerotiorin (**1**) and isochromophilone IV (**2**) were isolated from a strain, *Penicillium multicolor* F1753, and their structures were established by NMR spectral data. The metabolites significantly inhibited the binding between the Grb2-SH2 domain and phosphopeptide derived from the Shc protein, with IC_{50} values of 22 μM and 48 μM for (**1**) and (**2**), respectively. The compounds are the first non-peptidic inhibitors of the SH2 domain from a natural source.

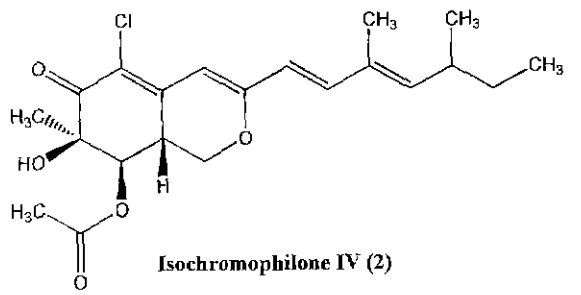
Key words: Grb2, Shc, signal transduction, antitumor

The adaptor protein Grb2 plays an important role in signaling-via-receptor tyrosine kinases [12]. Grb2 is an ubiquitously expressed 25 kDa protein, composed of a single SH2 domain flanked by an amino-terminal along with carboxy-terminal SH3 domains [2]. One of the functions of Grb2 is to link receptor tyrosine kinases to the Ras signaling pathway. The Grb2-SH2 domain binds several tyrosine-phosphorylated receptor type molecules, including erbB2, EGFR, and PDGFR, as well as other phosphotyrosine-containing proteins like Shc and insulin receptor substrate 1(IRS-1) [13]. Sequences of the Grb2-SH3 domain-binding have been localized to a proline-rich region in the C-terminus of Sos. Upon growth factor stimulation, the Grb2-Sos complex binds to activated receptors or to an adaptor protein, Shc [15]. It is proposed that connection of the Grb2-Sos complex to phosphorylated receptors activates the Ras pathway by bringing Sos to the proximity of the membrane-bound Ras. This process of activation can lead to runaway cell proliferation, differentiation, and apoptosis in many different kinds of cell types, resulting in diseases, including cancer. Thus, blocking of the Grb2-Shc binding may lead to intervention of the oncogenic signal transduction pathways and to developing a new antitumor drug.

We recently reported actinomycins as blockers of Grb2-Shc binding [6, 9]. In the course of screening for Grb2-SH2 domain antagonists of microbial origin, we have isolated sclerotiorin (**1**) [4] and isochromophilone IV (**2**) [1] as antagonists of the SH2 domain, from the culture broth of *Penicillium multicolor* F1753. In this paper, we describe the fermentation and isolation of sclerotiorin (**1**)



Sclerotiorin (1)



Isochromophilone IV (2)

Fig. 1. Structures of sclerotiorin (**1**) and isochromophilone IV (**2**).

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and isochromophilone IV (**2**) and their inhibition of Grb2-Shc interaction.

During the course of a screening program to identify fermentation-derived compounds, inhibitory activity for Grb2-Shc interaction was detected in the culture broth of F1753, an organism subsequently classified as a strain of *P. multicolor*. The fungal strain was isolated from a soil sample collected in Mt. Chilgap, Chungnam, Korea, as reported earlier [10]. Seed cultures were inoculated with two agar plugs added to a 500-ml Erlenmeyer flask containing 50 ml of the seed medium in the following composition: glucose 2%, yeast extract 0.2%, peptone 0.5%, KH_2PO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.05%, in distilled water. The flasks of inoculated seed medium were incubated on an orbital shaker rotating at 150 rpm for 36-48 h. Growth was at 25.5°C throughout. The seed culture was inoculated into a 1-l Erlenmeyer flask containing 150 ml of production medium of the following composition: soluble starch 2%, Bacto-soytone 0.4%, Pharma media 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.2%, and $CaCO_3$ 0.3%, in distilled water. The pH of the medium was adjusted to 6.0 by adding 1 N NaOH before being autoclaved. The culture was harvested after 5 days.

Initial tests showed approximately an equal inhibitory activity for Grb2-Shc interaction in both the cell pellet and supernatant of the fermentation broth (1.5 l). Therefore, the cultured broth (1.5 l) was centrifuged, and the pellet was extracted with acetone while the supernatant was extracted with chloroform. The organic extract of the supernatant and the pellet were combined and concentrated under reduced pressure. The remaining dark-brown oil (ca. 28.5 g) was suspended in CH_2Cl_2 and loaded onto a silica gel column. The compounds were eluted with increasing concentrations of ethyl acetate in n-hexane up to 80%. The active constituents were separated on silica gel chromatography according to their polarities. The active fractions were pooled and concentrated to dryness to give an orange powder of compound **1** (50 mg). The inhibitor **2** was obtained from the further purification with preparative TLC (Merck 5715 plates, 20 cm×20 cm, 0.25 mm thickness of Kieselgel60F_{254}). Development of the material with *n*-hexane/ethyl acetate (16:4) produced a yellow band with an R_f value of 0.4, yielding 25 mg of **2**. Active compounds were isolated based on the monitoring of their binding affinity against the GST-Grb2-SH2 domain using the reported method [7, 9]. The structures of **1** and **2** were determined by the extensive studies of NMR, IR, and mass spectral data. Analyses of HREIMS and the ^{13}C NMR spectra of **1** and **2** led to a molecular formula of $C_{21}H_{23}ClO_5$ and $C_{21}H_{27}ClO_5$, respectively. The compounds were identified as the known metabolites sclerotiorin (**1**) [4] and isochromophilone IV (**2**) [1] according to spectral data that included UV, IR, MS, ^1H, and ^{13}C NMR. They were originally isolated from *P. sclertiorum* and *P. multicolor*, respectively, and reported to have an

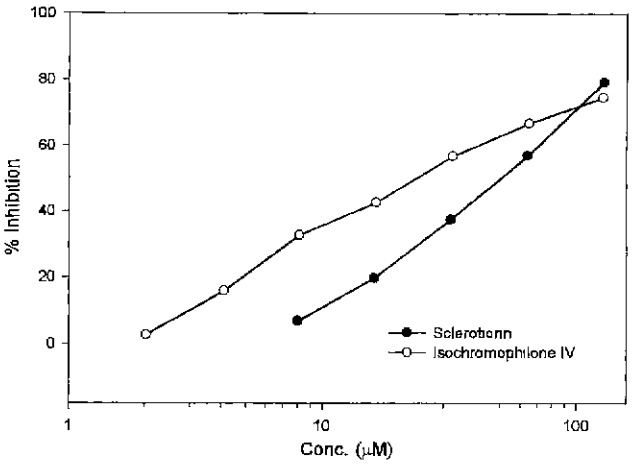

Fig. 2. The inhibitory effects of sclerotiorin (**1**) and isochromophilone IV (**2**) on Grb2-Shc interaction.

endothelin receptor binding activity [12] and acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor activity [1].

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, Eugene, U.S.A.) to bind the fusion protein to the beads. Assay 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, Piscataway, U.S.A.), and 0.2 μCi of [^{3}H]propionyl-labeled AcSpYVNVK-NH_2, which was derived from the SH2 domain binding sequence of Shc pY317. The final reaction volume of 200 μl contained 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, and 0.1% bovine serum albumin. Compounds dissolved in DMSO were added to give a final DMSO concentration of 2% by volume. Plates were routinely incubated by shaking at room temperature for 3 h. The inhibitory activity was measured by counting the released energy from SPA bead-bound [^{3}H]-peptide by a 1450 Microbeta counter (Wallac, Piscataway, U.S.A.). As shown in Fig. 2, the SH2 domain binding inhibitors **1** and **2** inhibited the interaction between the Grb2-SH2 domain and [^{3}H]propionyl-labeled AcSpYVNVK-NH_2 in a dose-dependent manner, with IC_{50} values of 22 and 48 μM, respectively.

In summary, we have described two azaphilones sclerotiorin (**1**) and isochromophilone IV (**2**), produced by *P. multicolor* F1753, which inhibit the Grb2-Shc interaction. The biological activities of the azaphilones include the inhibition of phospholipase A_2 [8] and endothelin receptor binding activity by **1**, and inhibition of the gp120-CD4 interaction by the recently reported isochromophilones [11]. Inhibitor **1** has also been reported to induce morphological changes in fungi [16]. Moreover, azaphilones have been reported as inhibitors of acylcholesterol acyl-transferase [1]. At present, there are few non-peptidic inhibitors for the Grb2-Shc interaction [5]. In the present study, we isolated, for the

first time, the non-peptidic inhibitors of the Grb2-Shc interaction from natural products, and identified their unique structure in comparison with other reported SH2 domain antagonists [3]. Therefore, the isolated antagonists should be useful for the development and designing of a SH2 domain antagonist. The results from this study also drew interest in understanding the biological activities of azaphilones.

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