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Alutenusin, a Specific Neutral Sphingomyelinase Inhibitor, Produced by *Penicillium* sp. FO-7436

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Sphingomyelin is an abundant constituent of the plasma membranes of mammalian cells[1]. Recently the catabolic intermediates of sphingomyelin *e.g.*, ceramides[2], sphingosine[3], sphingosin 1-phosphate[4], have been recognized to function as a potential second messenger. Among them, ceramide is the primary catabolic intermediate of sphingomyelin, and is released by either acid sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase). Activation of the sphingomyelin pathway by aSMase or nSMase in several normal and myeloid cell lines has been described to increase the production of ceramides, which subsequently trigger signaling pathways leading to either cell proliferation and differentiation or to apoptosis[5,6]. Two kinds of microbial nSMase inhibitors, F-10463a (scyphostatin)[7] and F-11334A1[8], were discovered as potential pharmaceutical agents by Sankyo group.

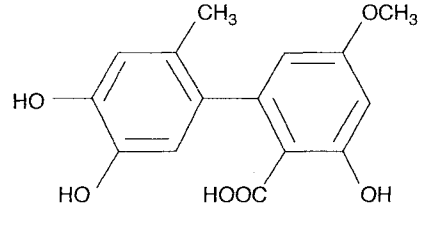
In the course of our screening program for SMase inhibitors of microbial origin, we have isolated a known compound, alutenusin (Fig. 1)[9], as a specific nSMase inhibitor from the culture broth of *Penicillium* sp. FO-7436. In this paper, we describe the fermentation, isolation, and SMase inhibitory activity of alutenusin.

A slant culture of the strain FO-7436 was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, yeast extract (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals) 0.5%, KH_2PO_4 0.1%, and agar 0.1%, pH 6.5), which was shaken on a rotary shaker (210 rpm) for 2 days at 27°C. Two ml of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (sucrose 2.0%, glucose 1.0%, corn steep powder 0.5%, Meat extract 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, $FeSO_4 \cdot 7H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg, $CuSO_4 \cdot 7H_2O$ 10 mg, and $CoCl_2 \cdot 6H_2O$ 10 mg, pH 6.5). The fermentation was carried out at 27°C for 6 days on a rotary shaker (210 rpm).

The cultured broth (900 ml) was centrifuged, and the supernatant was adjusted to pH 3 with 2N HCl and extracted with EtOAc. After dried over Na_2SO_4, the organic layer was concentrated to give a yellow oil (168 mg). It was applied on a silica gel column (7.5 g, Silica gel 60, 63 ~ 200 μm, Merck) prepared with $CHCl_3$, and the materials were eluted stepwise $CHCl_3$-CH_3OH solutions. The fractions eluted with $CHCl_3$-CH_3OH (10 : 1 and 5 : 1) were concentrated to give a yellow powder (70 mg). Then the powder was purified by preparative silica gel TLC (Merck) using $CHCl_3$-CH_3OH (1 : 1) as a developing solvent. The band with an Rf value of 0.55 was scraped off and extracted with MeOH to give a yellow powder (32 mg). It was finally purified by preparative HPLC (Senshu pak Pegasil C8, 20 × 250 mm; 48% CH_3CN in 50 mM sodium phosphate buffer, pH7.0; flow rate, 8 ml/minute; UV at 210 nm). The peak eluded with a retention time of 35 minutes was collected and concentrated to give a white powdery material (16 mg). By the spectral data including ^1H NMR, ^{13}C NMR, UV, and MS, the structure was found to be identified with alutenusin, which was originally isolated from *Alternaria* sp.[9], and subsequently reported as a myosin light chain kinase inhibitor.[10]

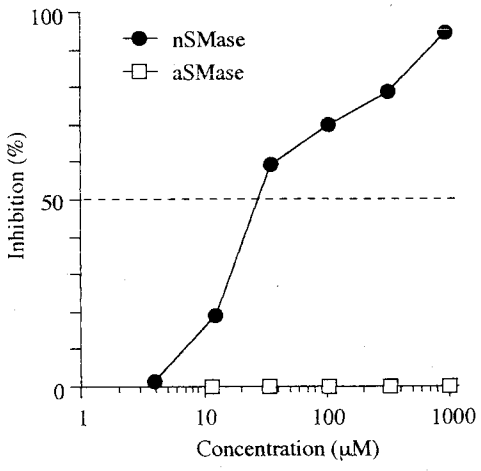
nSMase and aSMase activities were measured according to the method of QUINTERN *et al.*[11] with some

Fig. 1. Structure of alutenusin.



modifications. Briefly, the reaction mixture containing 100 μM [choline methyl-^{3}H]sphingomyelin (0.01 μCi, NEN), 20 mM HEPES-NaOH buffer (pH 7.4), 6.5 mM $MgCl_2$, 0.1% Triton X-100, a membrane fraction prepared (0.75 unit) from rat brains as nSMase source and a test sample (5 μl in 50% CH_3OH) in a total volume of 50 μl. As for aSMase activity, the reaction mixture contained 100 μM [choline methyl-^{3}H]sphingomyelin (0.01 μCi), 250 mM sodium acetate buffer (pH 5.0), 0.1% NP-40 (Sigma), aSMase (0.5 unit, Sigma) and a test sample in 50 μl. The reaction was started by adding the enzyme solution and the mixture were incubated at 37°C for 30 minutes. The reaction was stopped by addition of $CHCl_3$ - MeOH (1 : 1, 200 μl) to the mixture. After vortexing, the mixture was centrifuged and the aqueous layer (50 μl) containing the released [^{3}H]-phosphocholine was measured for radioactivity by a liquid scintillation counter (Beckman). As shown in Fig. 2, alutenusin inhibit nSMase activity in a dose-dependent fashion with an IC_{50} value of 28 μM. Whereas, aSMase activity was not inhibited at all even at 950 μM.

Fig. 2. Effect of alutenusin on nSMase and aSMase activities.



In order to elucidate the mechanism of nSMase inhibition by alutenusin, steady-state kinetics were obtained and the data are summarized in Fig. 3. The Lineweaver-Burk plots showed that alutenusin inhibited nSMase in a noncompetitive manner with respect to the substrate sphingomyelin. The *Ki* and *Km* values were calculated to be 20 and 35 μM, respectively.

Alutenusin was previously reported as an inhibitor of myosin light chain kinase[10], but the inhibitory activity was very weak (IC_{50}, 340 μM). Furthermore, the compound showed no antimicrobial activity against typical 16 fungal and bacterial strains[12] at 1 mg/ml (10 μg/disk, data not shown). Therefore, alutenusin is a specific nSMase inhibitor.

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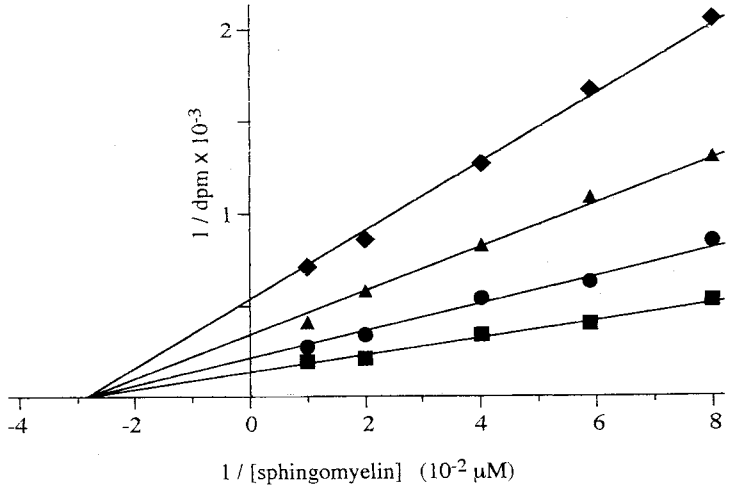
Fig. 3. Lineweaver-Burk plots of inhibition of nSMase by alutenusin.

Alutenusin; 0 (■), 20 (●), 40 (▲), and 60 μM (◆).



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