COMMUNICATIONS TO THE EDITOR

CRM646-A and -B, Novel Fungal Metabolites that Inhibit Heparinase

Sir,

Heparin-like glycosaminoglycans (HLGs), such as heparin and heparan sulfate (HS), polysulfated copolymers of alternating 1→4 linked glucosamine and hexuronic acid found both at the cell surface and in the extracellular matrix (ECM), constitute the principal structural unit of tissues and play a role in diverse biological processes such as cell proliferation and signaling[1,2]. To degrade extracellular HLGs, cells express endoglycosidases that cleave HLG chains at a limited number of sites along the polysaccharide chains[3]; the enzymes may be involved in the remodeling of the ECM and aid migration of cells such as macrophages[4] and tumor cells[5]. The endoglycosidases are also reported to be related to metastasis[6], inflammation[7] and angiogenesis[8]. However, more detailed investigation of the endoglycosidases has been limited due to the lack of cloned genes of the enzymes; it was only very recently that the relevant genes for the enzymes have been cloned from placenta, platelet and hepatoma cells[9~11].

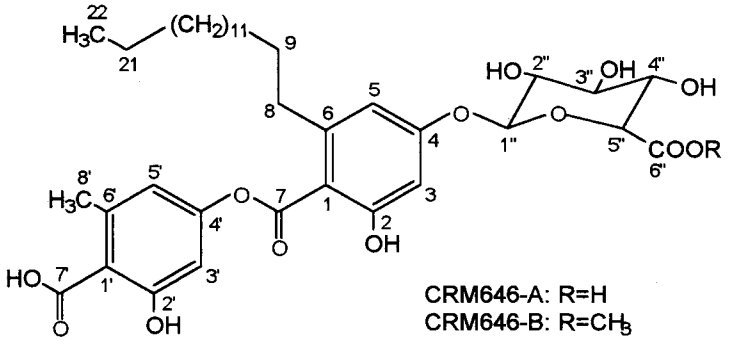
Heparinase I, II and III cloned from *Flavobacterium heparinum*[12], are the other enzymes that degrade such HLG molecules that have been actively studied. These three enzymes are unique in their action since they cleave along the HLGs backbone with high degree of specificity[13]. In addition, heparinases were implicated in many growth factor-mediated cellular signaling[14]. There also were reports about the inhibition of the metastatic melanoma cells and other tumor cells by heparin and its derivatives[15,16]. Hence, heparinase inhibitors might provide useful tools for the elucidation of biological functions of heparin and HS and can be used as antimetastatic agents.

In the course of screening for heparinase inhibitors from microbial metabolite through a modified assay system for heparinase, we isolated novel inhibitors, CRM646-A (**1**) and -B (**2**) from *Acremonium* sp. MT70646 (Fig. 1). In this communication, we report the fermentation, isolation, structural determination and biological activity of **1** and **2**.

For the assay of heparinase activity, we measured the residual amount of heparin after the enzyme reaction by a slight modification of conventional method. Briefly, 0.2 unit of heparinase (Sigma) was preincubated with test samples for 5 minutes and then enzyme reaction was started by adding 10 ng of porcine heparin (Sigma) in 25 μl of a buffer (14 mM sodium acetate and 1.4 mM $CaCl_2$, pH 7.0) for further 15 minutes at room temperature. For the measurement of the amount of heparin, the degree of complex formation between antithrombin and factor Xa was determined using the Sigma Diagnostics kit (Sigma Catalog No. CRS106-A), and then, the absorbance was measured on spectrophotometer at 405 nm.

The producing organism, *Acremonium* sp. MT70646 was isolated from a soil sample collected in Mt. Geryong, Kongju, in Korea. The seed culture was incubated in a medium consisting of 2.0% glucose, 0.5% Bactopeptone, 0.3% yeast extract, 0.3% malt extract, 0.05% $MgSO_4$ and 0.1% KH_2PO_4 at 26°C for 4 days on a rotary shaker (150 rpm), and then transferred into a 15 liters jar fermentor containing 10 liters of the same medium. The

Fig. 1. Chemical structures of CRM646-A (**1**) and -B (**2**).



production of **1** and **2** reached the maximum at 6 days culture. After the fermentation, an equivalent volume of acetone was added to 10 liters of culture broth and then the mixture was filtered. The filtrate was concentrated *in vacuo* and suspended in 1 liter of deionized water. After the suspension was extracted with *n*-BuOH, the organic phase was concentrated. The residue (25.5 g) was chromatographed on a silica gel (Kiesegel 60, 0.063~0.2 mm, Merck) using the solvent system of $CHCl_3$/MeOH/H_2O (5/1/0.05). The active fractions were combined and concentrated *in vacuo*. The residue (3.7 g) was rechromatographed on RP-18 (70~230 mesh, YMC Co.) with CH_3CN/H_2O (1/1) as eluant. The active fraction (340 mg) was concentrated *in vacuo* and was loaded onto Sephadex LH-20 column (Sigma Co.) using CH_3CN/H_2O (6/4). The active fractions were concentrated *in vacuo* to give a yellowish crude powder (150 mg) and dissolved in a small amount of CH_3CN. Finally, **1** and **2** were purified with preparative HPLC (J'sphere ODS-H80, i.d. 20× 150 mm, eluant; 80% CH_3CN containing 0.05% TFA, flow rate; 5 ml/minute, 210 nm). Compound **1** (85 mg) and **2** (23 mg) were collected and concentrated *in vacuo* to give white powders.

The IR absorption bands of **1** and **2** at 1730, 1666, and 1614 cm^{-1} suggested the presence of ester, carbonyl and carboxyl group, and also the presence of –OH and C=C group were showed at 3397 cm^{-1}, 2900 cm^{-1}, respectively. The FAB-MS spectrum (in negative mode) of **1** gave a

Fig. 2. ^{1}H-NMR spectrum of CRM646-A (**1**) (300 MHz, in DMSO-d_6).

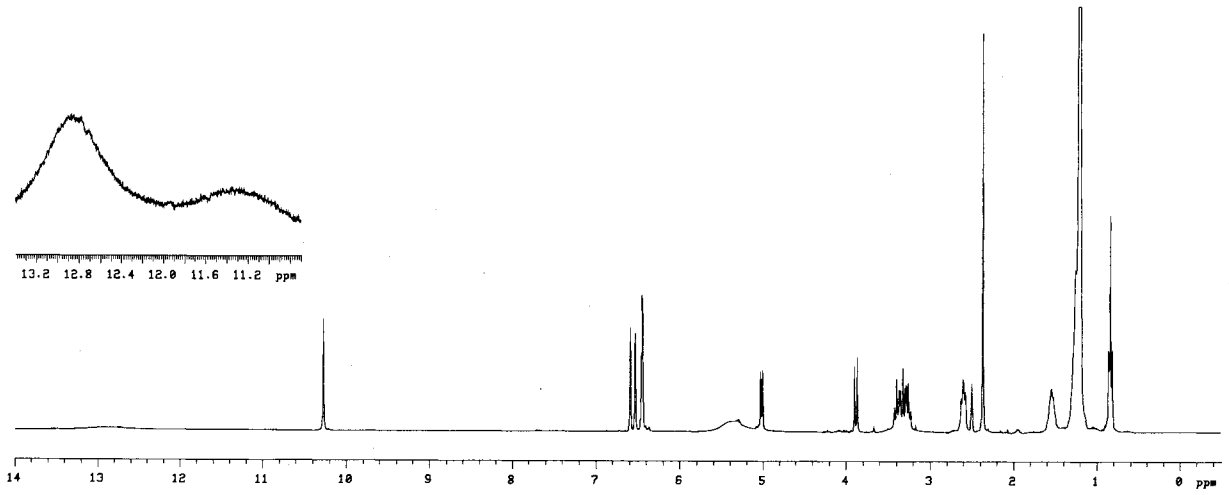


Fig. 3. ^{13}C-NMR spectrum of CRM646-A (**1**) (75 MHz, in DMSO-d_6).

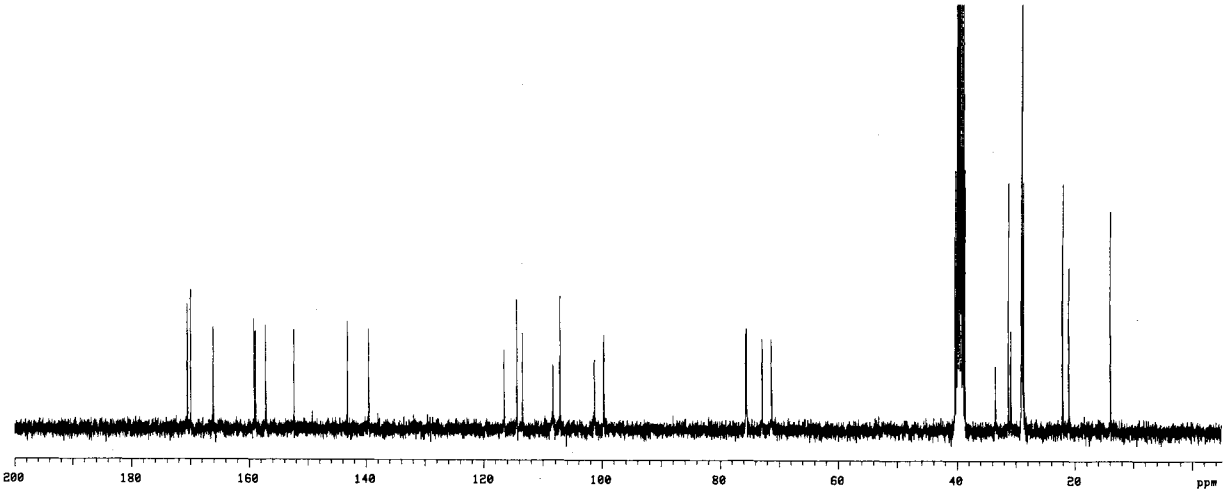
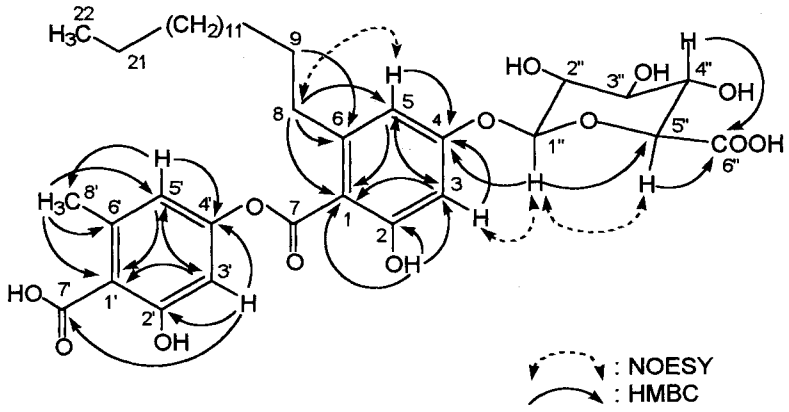


Fig. 4. Significant correlations observed in the HMBC and NOESY spectra of CRM646-A (**1**).



molecular weight of 690 (*m/z*; 689 $(M-H)^-$), whereas *m/z* 691 ($(M-H)^+$) was found in the positive FAB-MS spectrum. The molecular formula of **1** and **2** were determined to be $C_{36}H_{50}O_{13}$ and $C_{37}H_{52}O_{13}$ on the basis of HRFAB-MS and NMR data, respectively. The ^{1}H- and ^{13}C-NMR spectra of **1** were shown in Fig. 2 and Fig. 3 (in DMSO-d_6). In the ^{13}C-NMR spectrum of **1**, 31 resolved signals consisting of two $-CH_3-$, nine $-CH_2-$, nine $-CH-$ and eleven sp^2 quaternary carbons were showed. Of the nine $-CH-$ carbons, four sp^2 carbons (δ_C; 114.42, 108.31, 107.15 and 101.27) suggested the existence of two substituted benzene ring moieties by the chemical shifts and correlations in HMBC spectrum. Also, the remaining five carbons including an anomeric carbon (δ_C 99.67) indicated the presence of a sugar moiety. It was suggested that all of nine $-CH_2-$ carbons were originated from a saturated aliphatic chain. Therefore, it was confirmed that the aliphatic chain in **1** was consisted of fourteen $-CH_2$ carbons and a terminal $-CH_3$ (δ_C 13.95). In the HMBC spectrum of **1**, the long-range coupling from H-8 (2H, δ_H 1.26) to C-6 (δ_C 143.18), C-5 (δ_C 108.31) and C-1 (δ_C 113.53), from H-9 (2H, δ_H 1.54) to C-6 suggested that the aliphatic chain was connected to C-6 carbon in ring A moiety. On the other hand, the structural elucidation of two substituted ring moieties was achieved through the HMBC and FAB-MS experiments. In fact, there are no correlations to connect two ring moieties as shown in Fig. 4. But, because the molecular weight (*m/z* 167) of ring B moiety was showed in the FAB-MS spectrum, it could be concluded that the two ring moieties were connected with an ester bond between C-4′ and C-7. Both H-3 (δ_H 6.45) on ring A and H-3′ (δ_H 6.58) on ring B were showed correlations with the respective terminal carbonyl carbons at δ_C 166.15 and δ_C 170.55. The sugar moiety was determined as glucuronic acid by analyzing the coupling constants. In addition, the anomeric proton of H-1″ was long-range coupled to C-4 (δ_C 159.12) and C-5″ (δ_C 75.53). From these data, it was confirmed that the C-1″ carbon of glucuronic acid was linked to C-4 on ring A moiety. The stereochemistry at C-1″ was elucidated to β by the ^{13}C-^{1}H coupling constant of C-1″ ($^1J_{C-H}$=161.2 Hz). Thus, the structure of **1** was determined as shown in Fig. 1.

The ^{1}H- and ^{13}C-NMR data of **2** were similar to those of **1** except the peak of one methoxy proton (δ_H 3.66). In the HMBC spectrum, the methoxy proton showed a long-range coupling with C-6″ carbon (δ_C 169.14). Therefore, it was concluded that **2** was substituted with methoxy group at the position of C-6″ of **1**.

The structure of **1** was similar to those of TPI-3 and -4, known as the inhibitors of cAMP phosphodiesterase[17]. However, there are clearly differences in the structures such as the sugar moiety (β-D-glucose instead of glucuronic acid in **1**), the length of aliphatic chain and the presence of carboxyl residue on the ring in the TPIs. Therefore, **1** and **2** are novel inhibitors for heparinase and this paper is a first report for heparinase inhibitor.

Compounds **1** and **2** inhibited heparinase in a dose-dependent manner with the IC_{50} values of 3 μM and 10 μM, respectively. Suramin, known as a potent inhibitor of melanoma heparanase[15] showed an IC_{50} value of 5 μM in our assay system. Since there can be a possibility that the inhibitors may affect any other components employed in the assay system (especially factor Xa, a serine protease), we examined the effect of the inhibitors on each components.

We found that **1** and **2** had no effects on the assay system, and only showed the inhibitory activity against heparinase itself.

To examine the antimetastatic capability of the compounds **1** and **2**, an invasion assay of B16-F10 melanoma cells through laminin-coated matrigel was performed. Very hopefully, **1** (IC_{50}=15 μM) and **2** (IC_{50}= 30 μM) showed a strong inhibtion of B16-F10 cell migration. However, both the compounds showed negligible cytotoxicity against various cells up to 100 μM (data not shown). Therefore, **1** and **2** would be new agents for the study of metastasis and angiogenesis, or possible candidates for anticancer therapeutics. Thus, the detailed biological activities and *in vivo* experiments are now under investigation.

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