Sulfobacins A and B, Novel von Willebrand Factor Receptor Antagonists

I. Production, Isolation, Characterization and Biological Activities

TSUTOMU KAMIYAMA, TAKAYUKI UMINO, TOMOKO SATOH, SAYOKO SAWAIRI, MICHIKO SHIRANE, SHOICHI OHSHIMA and KAZUTERU YOKOSE

Nippon Roche Research Center,
200 Kajiwara, Kamakura 247, Japan

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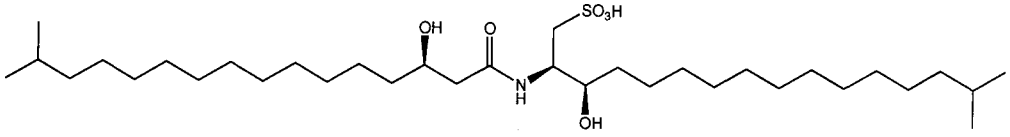
Sulfobacins A and B, novel von Willebrand factor (vWF) receptor antagonists, have been isolated from the culture broth of *Chryseobacterium* sp. (*Flavobacterium* sp.)† NR 2993 by ethyl acetate extraction, and by Sephadex LH-20 and silica gel column chromatographies. The physico-chemical properties of the sulfobacins indicate that their structures are completely different from that of aurintricarboxylic acid, the one known vWF receptor antagonist. Sulfobacins A and B inhibit the binding of vWF to its receptor with IC_{50}s of 0.47 and 2.2 μM, respectively. Sulfobacin A also inhibits ristocetin-induced agglutination in human platelets fixed with paraformaldehyde with an IC_{50} of 0.58 μM.

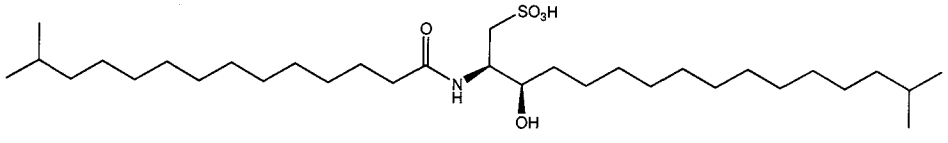
Thrombosis is most often the primary manifestation of diffuse intravascular coagulation for a wide variety of diseases. Formation of an intraluminal thrombus is initiated by the adherence of platelets to a blood vessel and is followed by the formation of platelet aggregates on adhered platelets. When a blood vessel is damaged, the platelets adhere to von Willebrand factor (vWF) bound to a disrupted subendothelial matrix, such as collagen. The vWF absorbed on the vascular surface interacts with glycoprotein (GP) Ib/IX on the platelet membrane, hence initiating platelet aggregation, wherein fibrinogen binds to another receptor, GP IIb/IIIa, crosslinking the platelets. If the binding of GP Ib/IX to vWF is inhibited, thrombosis will be blocked.

This would lead us to expect that a vWF receptor antagonist could be an antithrombosis agent. Therefore, we screened microbial metabolites to obtain the receptor antagonists. In this screening program, we discovered the vWF receptor antagonists, sulfobacins A (**1**) and B (**2**), in the culture broth of strain NR 2993 (Fig. 1). The strain was isolated from a soil sample collected in Iriomote Island, Okinawa Prefecture, Japan and was identified as a *Chryseobacterium* sp. based on its morphological and physiological properties[1,2]. In this paper, we report on the production, isolation, physico-chemical properties, and biological activity of the sulfobacins. We describe the structural elucidation of the sulfobacins in the accompanying paper[3].

Fig. 1. Structures of sulfobacins A and B.



Sulfobacin A (**1**)



Sulfobacin B (**2**)

† *Flavobacterium* was transferred to a new genus, *Chryseobacterium*, based on the recent taxonomical revision[1].

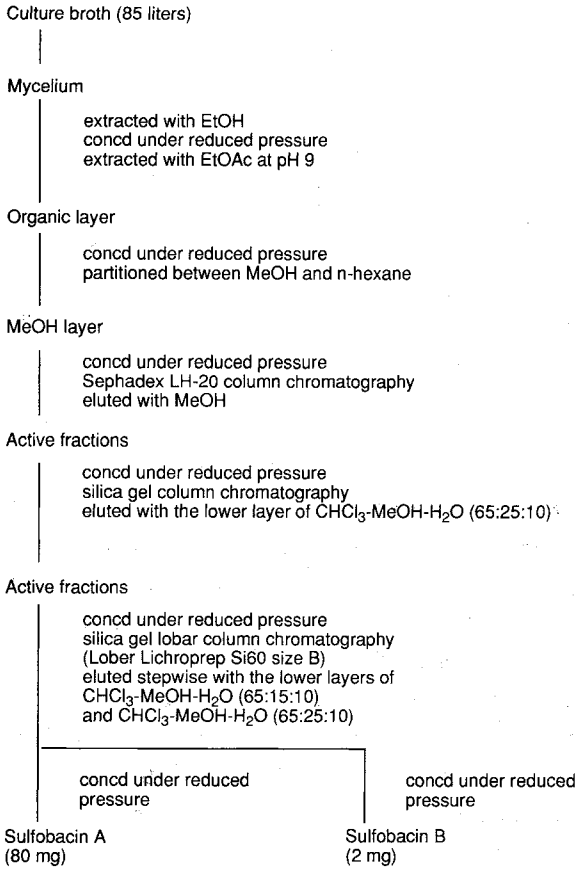
Production

The stock culture of *Chryseobacterium* sp. NR 2993 stored at −80°C was thawed, and 100 μl of the culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, dextrin 1.0%, S-III meat (Ajinomoto Co. Inc.) 1.0%, Pharmamedia (Traders Protein) 1.0%, K_2HPO_4 0.06%, KH_2PO_4 0.025% and Nissan disfoam 0.03%. The flask was shaken at 220 rpm on a rotary shaker at 27°C for one day. Then 90 ml of the culture broth was transferred to a 50-liter jar fermentor containing 30 liters of production medium having the same composition as the seed medium except for the addition of 0.6% Nissan disfoam. The fermentation was carried out at 27°C for 70 hours with an aeration of 30 liters/minute and an agitation speed of 300 rpm.

Isolation

The isolation procedure is shown in Fig. 2. The culture broth (85 liters) was separated into the filtrate and the mycelium by centrifugation. The mycelium was extracted with EtOH (30 liters). After the mycelium was removed, the EtOH extract was concentrated to dryness under reduced pressure and then suspended in water (30 liters). The suspension was extracted with EtOAc (30 liters) at pH 9, and then the organic layer was concentrated under reduced pressure. The concentrate (32 g) was partitioned between MeOH (500 ml) and *n*-hexane (1 liter). The MeOH layer was then concentrated to dryness under reduced pressure, and the concentrate (14 g) was subjected to column chromatography on Sephadex LH-20 (Pharmacia) developed with MeOH. The active fractions were combined and concentrated under reduced pressure. The concentrate (2.5 g) was subjected to column chromatography on silica gel (250 ml) developed with the lower layer of $CHCl_3$-MeOH-H_2O (65:25:10). The active fractions were combined and concentrated under reduced pressure. The concentrate (800 mg) was applied to a silica gel column, Lobar Lichroprep Si60 size B (Merck). The column was developed stepwise with the lower layers of $CHCl_3$-MeOH-H_2O (65:15:10) and $CHCl_3$-MeOH-H_2O (65:25:10). Active fractions from this column were combined based on their TLC behavior to yield **1** (80 mg) and **2** (2 mg) as white powders.

Fig. 2. Isolation procedure of sulfobacins A and B.



Physico-chemical Properties

The physico-chemical properties of the sulfobacins are summarized in Table 1. Sulfobacins A and B were both acidic and lipophilic. They are soluble in DMSO and pyridine, only slightly soluble in MeOH, but insoluble in water and *n*-hexane. The IR spectra of **1** (Fig. 3) and **2** were almost identical and suggested the presence of sulfonic acid (1200 and 1070 cm^{-1}) and amide (1660 and 1560 cm^{-1}) functional groups. The positive color ob-

Table 1. Physico-chemical properties of sulfobacins A and B.

	Sulfobacin A	Sulfobacin B
Appearance	White powder	White powder
$[\alpha]_D^{24}$	-35° (*c* 0.14, MeOH)	-19° (*c* 0.14, MeOH)
Molecular formula	$C_{34}H_{69}NO_6S$	$C_{32}H_{65}NO_5S$
UV λ_{max}^{MeOH}	End	End
IR ν_{max} (KBr) cm^{-1}	3350, 2945, 2860, 1660, 1560, 1480, 1200, 1070	3300, 2925, 2860, 1655, 1550, 1470, 1220, 1060
HRFAB-MS (*m/z*) $(M-H)^-$		
Calcd:	618.4768	574.4505
Found:	618.4766	574.4496
TLC (R_f value)*	0.26	0.22
Color reaction	Rydon-Smith (+), Pinacryptol yellow (+)	Rydon-Smith (+), Pinacryptol yellow (+)

* TLC conditions: plate: HPTLC plate Kieselgel 60 F_{254} Art. 15696 (Merck); solvent: the lower layer of $CHCl_3$-MeOH-H_2O (65:25:10).

Fig. 3. IR spectrum of sulfobacin A.

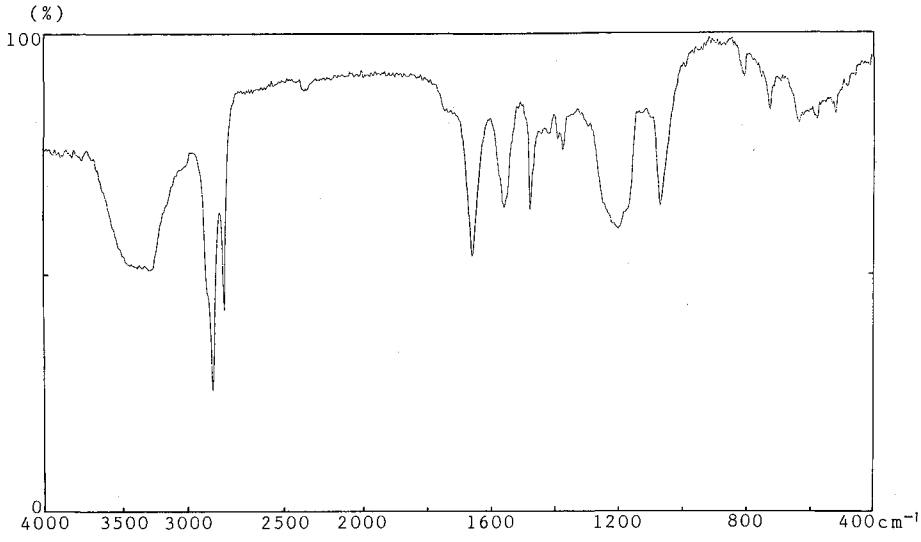
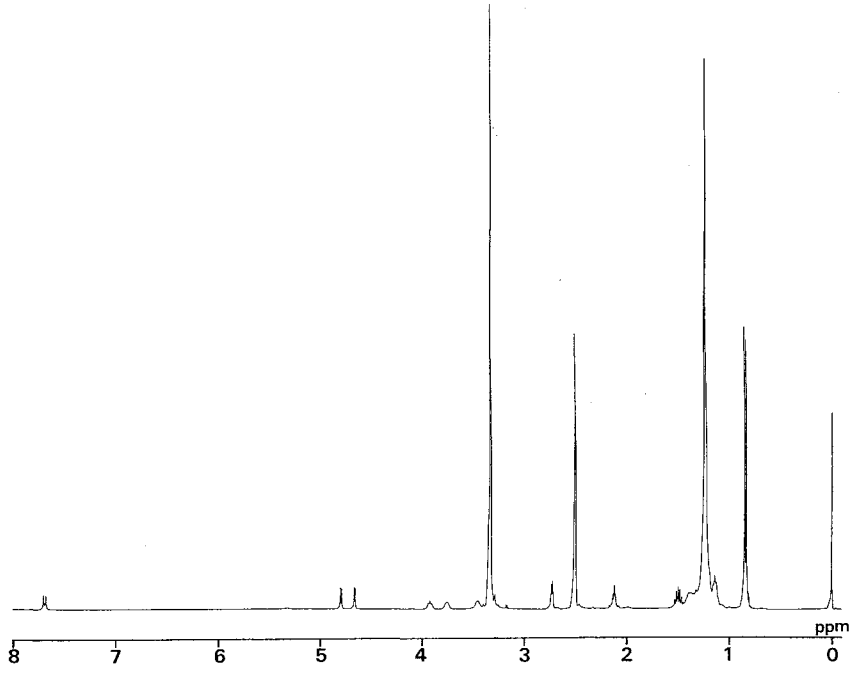


Fig. 4. ^{1}H NMR spectrum of sulfobacin A (400 MHz, in DMSO-d_6).



tained from reactions with pinacryptol yellow[4] and Rydon-Smith reagents also supported the presence of the sulfonic acid and amide functional groups, respectively. The molecular formulae of **1** and **2** were determined to be $C_{34}H_{69}NO_6S$ and $C_{32}H_{65}NO_5S$ from our negative ion HRFAB-MS data, respectively. The presence of an S atom was confirmed by ion chromatography. The ^{1}H NMR spectra of **1** and **2** in DMSO-d_6 are shown in Figs. 4 and 5, respectively. The ^{13}C NMR spectrum of **1** is also shown in Fig. 6. The UV spectra of sulfobacins showed no characteristic absorption. The physico-chemical properties indicated that the structures of **1** and **2** were completely different from that of the one known low-molecular-weight vWF receptor antagonist, aurintricarboxylic acid[5,6], which is a triphenyl dye and the peptide fragment derived from vWF-GP Ib/IX binding domain[7]. The physico-chemical properties also indicated that the structures can be related to sulfonolipids, major components of the cell envelope of some gliding bacteria[8].

Fig. 5. ^{1}H NMR spectrum of sulfobacin B (400 MHz, in DMSO-d_6).

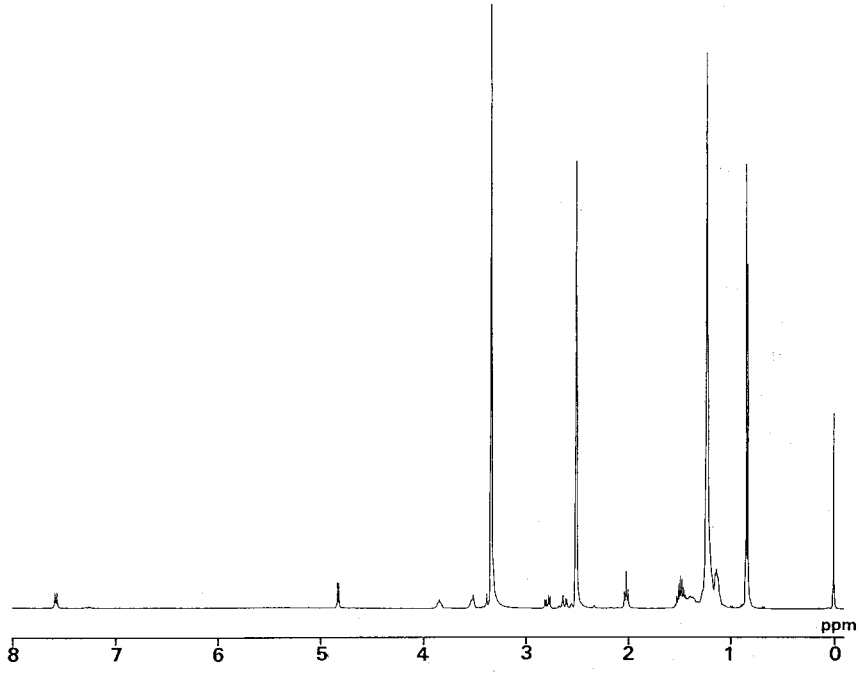
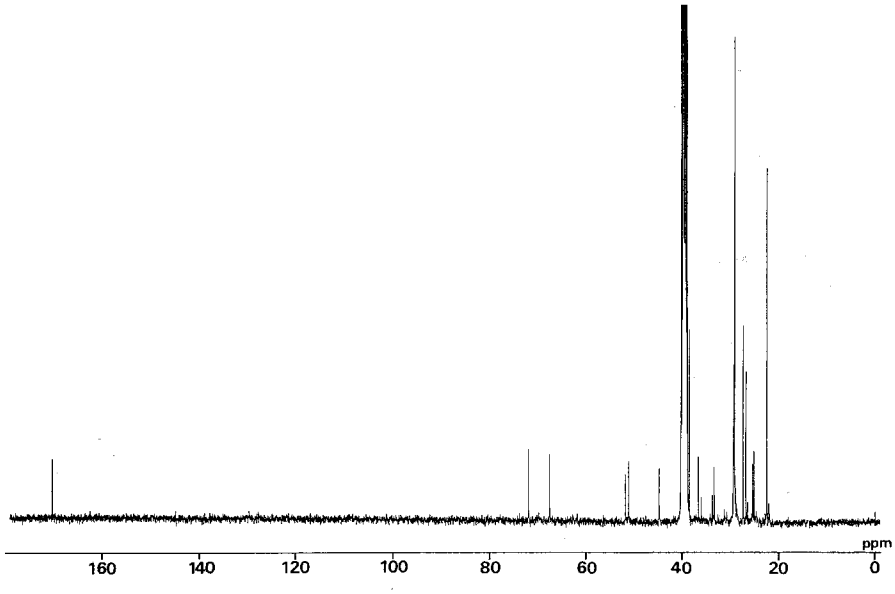


Fig. 6. ^{13}C NMR spectrum of sulfobacin A (100 MHz, in DMSO-d_6).



Biological Activity

Bovine vWF was isolated from the cryoprecipitate of bovine plasma by gel-filtration of Sepharose 2B (Pharmacia). Glycoprotein Ib/IX was purified from human platelets as follows: the Triton X lysate of washed human platelets was subjected to wheat germ agglutinin (WGA) affinity chromatography; this eluate was put on a thrombin-Sepharose column and the retained protein was eluted by applying a linear salt gradient. The purified GP Ib/IX was coated onto a 96-well plate at a concentration of 0.05 μg/well. After the wells in the plate were washed, 35 μl of the Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4), 15 μl of a test sample and 100 μl of bovine vWF were added to each well and the plate was incubated at 37°C for 16 hours. Then the quantity of bound vWF was

assayed by the avidin-biotin method reported by us[9].

Agglutination in paraformaldehyde fixed platelets was measured by recording the velocity of light transmission changes with an aggregometer[5]. To a solution of paraformaldehyde fixed platelet (2×10^8 cells/ml) in the 84 mM imidazole-HCl buffer (pH 7.35) was added human vWF at a final concentration 4 μg/ml. To the solution, serial dilutions of the inhibitors were added, and the agglutination was induced by an agonist such as restocetin at a final concentration 1 mg/ml in the 84 mM imidazole-HCl buffer (pH 7.35).

Sulfobacins A and B inhibited the binding of vWF to the GPIb/IX receptors in a competitive manner with IC_{50}s of 0.47 and 2.2 μM, respectively (Table 2). The inhibitory activities of sulfobacins are almost same as those of aurintricarboxylic acid. Sulfobacin A also inhibited risticetin-induced agglutination in human platelets fixed with paraformaldehyde with an IC_{50} of 0.58 μM. However, the sulfobacins didn't inhibit the binding of fibrinogen to the GPIIb/IIIa receptors. These results meant that the sulfobacins were the first selective GP Ib/IX receptor antagonists of microbial origin and they would be very useful tools in the clarification of the binding of vWF to the GP Ib/IX receptors. Sulfobacins had no antibacterial and antifungal activity. The IC_{50} value of cytotoxicity against HeLa cells of **1** was greater than 80 μM. Its detailed biological activity will be reported elsewhere.

Table 2. Inhbitory activity against the binding of vWF to the GP Ib/IX receptors.

Compound	IC_{50} (μM)
Sulfobacin A	0.47
Sulfobacin B	2.2
Aurintiricarboxylic acid	0.45

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