Monamidocin, a Novel Fibrinogen Receptor Antagonist

I. Production, Isolation, Characterization and Structural Elucidation

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(Received for publication May, 29, 1995)

Monamidocin, a novel fibrinogen receptor antagonist, has been isolated from the culture broth of *Streptomyces* sp. NR 0637 by carbon adsorption, n-BuOH extraction, SP-Toyopearl, silica gel 60 silanised and Sephadex LH-20 column chromatographies and preparative HPLC. The molecular formula of monamidocin has been determined to be $C_{15}H_{22}N_4O_4$ from HRFAB-MS and ^{13}C NMR spectral data. The structure of monamidocin has been determined to be *N*-[(*S*)-5-guanidino-2-hydroxypentanoyl]-L-phenylalanine by 2D NMR experiments.

To keep the blood flowing in our bodies, we have several mechanisms to keep the balance between the thromboplastic and thrombolytic states. When, for some reason, this balance tips towards the thromboplastic state, thrombosis occurs. The formation of thrombi causes many of the cerebrovascular or ischemic heart diseases such as cerebral thrombosis or angina pectoris. Platelet aggregation plays a key role in the formation of thrombi. Platelets first adhere and spread onto the thrombogenic components of the vascular subendothelium at the sites of vascular lesions. When the fibrinogen receptors (GPIIb/IIIa) existing as Ca^{2+}-dependent heterodimer complexes are stimulated by an agonist such as ADP, collagen or thrombin, they acquire the ability to bind fibrinogen by changing the conformation of the receptors. Fibrinogen binding to platelets is prerequisite for platelet aggregation. Therefore, fibrinogen receptor antagonism is a good target for a platelet aggregation inhibitor.

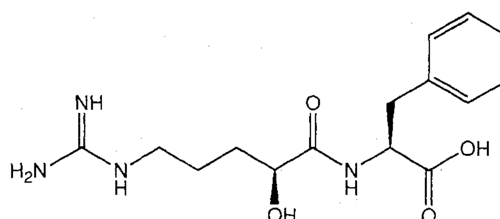
In the course of our screening program for fibrinogen receptor antagonists, we isolated a low molecular weight antagonist, monamidocin (**1**), from the culture broth of *Streptomyces* sp. NR 0637 (Fig. 1)[1]. It inhibits the binding of fibrinogen to its receptors with an IC_{50} of 0.21 μM and also inhibits the aggregation of human platelets induced by ADP, collagen and thrombin. In this paper, we report on the production, isolation, physico-chemical properties and structural elucidation of **1**. We describe the biological activity and derivatization of **1** in the accompanying paper[2].

Production

The organism producing **1**, strain NR 0637, was isolated from a soil sample collected in Iriomote Island, Okinawa Prefecture, Japan, and was identified as *Streptomyces* sp. based on its morphological and physiological properties and cell wall type.

The frozen mycelial suspension of strain NR 0637 (200 μl) was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a medium consisting of glucose 2.0%, potato starch 2.0%, yeast extract 0.5%, Toast soya 2.0%, NaCl 0.25%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $CaCO_3$ 0.32%, and Nissan Disfoam CA-115 0.05% (adjusted to pH 7.0 before sterilization). The inoculated flask was shaken at 220 rpm on a rotary shaker at 27°C for 3 days. Then, 2 ml of the resultant vegetative inoculum was transferred into one hundred 500-ml baffled Erlenmeyer flasks containing 100 ml of the same medium as above mentioned medium. The flasks were shaken at 220 rpm on a rotary shaker at 27°C. The production of monamidocin reached maximum on the 4th day of incubation.

Fig. 1. Structure of monamidocin (**1**).



Isolation

The isolation scheme is shown in Fig. 2. Each fraction was monitored by the solid phase assay (fibrinogen binding to immobilized GP IIb/IIIa) described in the accompanying paper[2]. Activated charcoal (74 g) was added to the broth filtrate (7.4 liters). The mixture was stirred at room temperature for 30 minutes and filtered to collect the carbon. The collected carbon cake was suspended in 10% aq acetone (3.7 liters). The suspension was adjusted to pH 2 with 6 N HCl and stirred for 30 minutes. The carbon was again collected by filtration and suspended in 50% aq acetone (5 liters). The suspension was adjusted to pH 2 with 6 N HCl, stirred for 30 minutes and filtered. The filtrate was adjusted to pH 7 and concentrated under reduced pressure. The concentrate (19 g) was partitioned between water (660 ml) and EtOAc (600 ml) at pH 2, and the organic layer was discarded. The water layer (600 ml) was shaken with *n*-BuOH (600 ml) at pH 2, then the separated organic layer was concentrated under reduced pressure. The concentrate (2 g) was dissolved in water (2 liters), adjusting to pH 2.4 with 1 N HCl, and subjected to column chromatography on SP-Toyopearl (550 ml) developed with water (4 liters) and 0.1 M NaCl (5 liters). The active eluate (0.1 M NaCl) was concentrated under reduced pressure. The concentrate was dissolved in water (100 ml) and subjected to a column chromatography on silica gel 60 silanised (Merck) developed stepwise with water, 10% MeOH, and MeOH. The active eluate (MeOH) was concentrated under reduced pressure. The concentrate (98 mg) was dissolved in MeOH (5 ml) and subjected to column chromatography on Sephadex LH-20 (1 liter) developed with MeOH. The active fractions were combined and concentrated under reduced pressure. The concentrate (38 mg) was purified by preparative HPLC (column: YMC D-ODS-10-A (20 mm i.d. × 250 mm); solvent: 20% MeOH; flow rate: 25 ml/minute; detection: UV 210 nm). The fraction containing monamidocin was concentrated under reduced pressure to give 19.7 mg of monamidocin hydrochloride as a white powder.

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. Monamidocin (**1**) was soluble in water, MeOH, and DMSO, but insoluble in *n*-hexane. The positive color obtained from reactions to Sakaguchi and Rydon-Smith reagents suggested the presence of guanidino and amide groups, respectively. The IR spectrum of **1** (Fig. 3) also suggested the presence of an amide (1660 and 1535 cm^{-1}) functional group. The molecular formula of **1** was determined to be $C_{15}H_{22}N_4O_4$ from the positive ion HRFAB-MS and ^{13}C NMR spectral data. The ^1H NMR spectrum of **1** in DMSO-d_6 is shown in Fig. 4. The ^1H and ^{13}C NMR spectral data of **1** are summarized in Table 2.

Fig. 2. Isolation procedure of monamidocin.

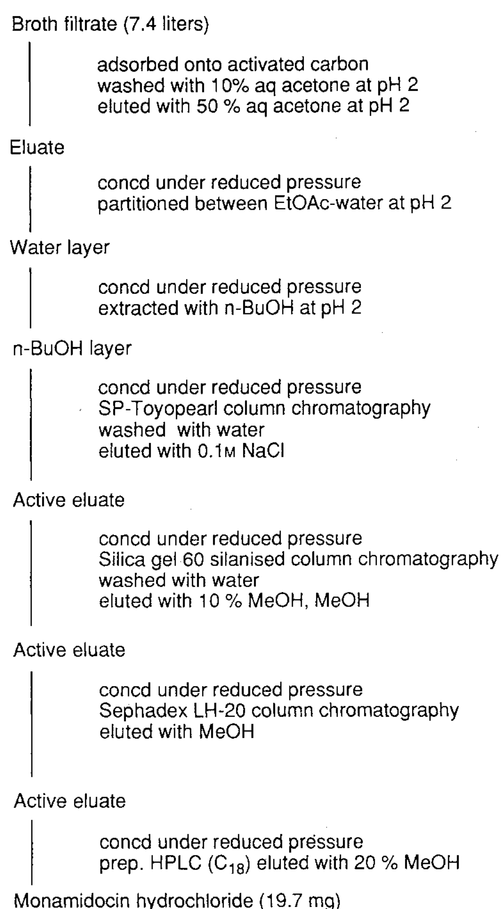


Table 1. Physico-chemical properties of monamidocin (**1**).

	1
Appearance	White powder
$[\alpha]_D^{24}$	+21.5° (*c* 0.5, MeOH)
Molecular formula	$C_{15}H_{22}N_4O_4$
HRFAB-MS (*m/z*)	
Calcd:	323.1719
Found:	323.1720 $(M+H)^+$
UV λ_{max}^{MeOH}	End
IR ν_{max} (KBr) cm^{-1}	3400, 3200, 1660, 1600, 1535, 1400, 1125, 700
Color reaction	Rydon-Smith (+), Sakaguchi (+)

Fig. 3. IR spectrum of monamidocin.

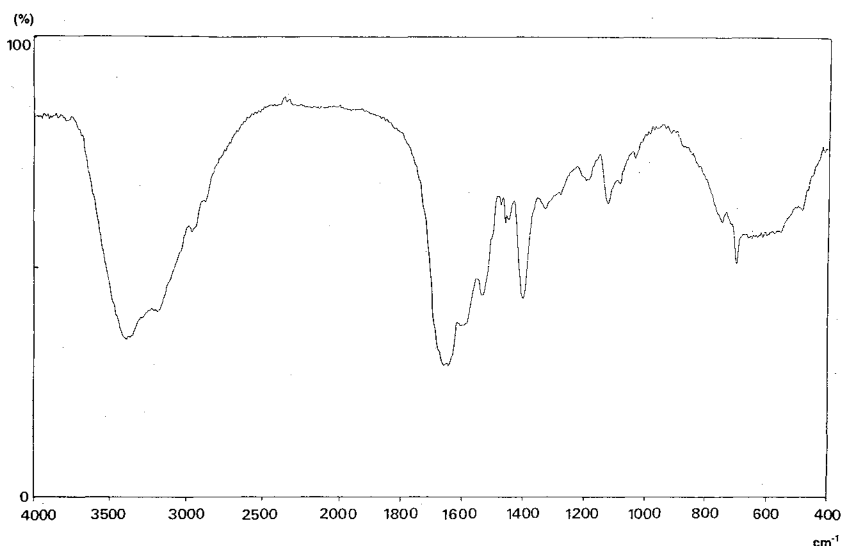
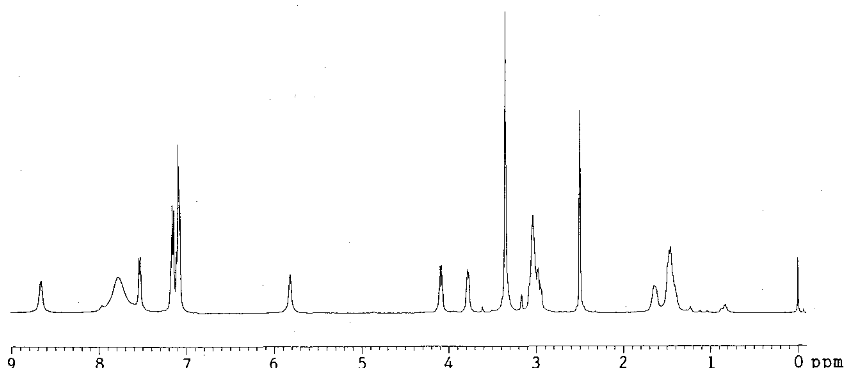


Fig. 4. ^{1}H NMR spectrum of monamidocin (400 MHz, in DMSO-d_6).



Structural Elucidation

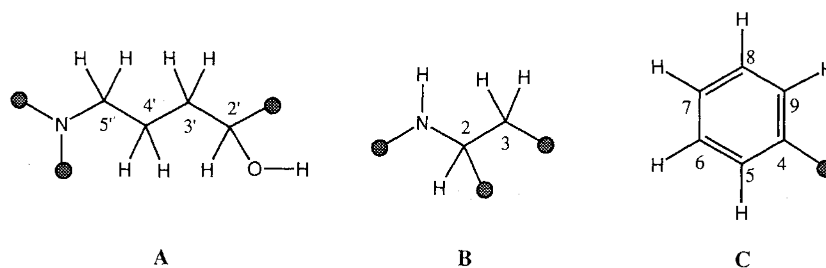
Partial structures A, B and C (Fig. 5) were elucidated by homo decoupling experiments and by interpretation of ^{1}H and ^{13}C NMR spectra. Homo decoupling experiments on **1** established proton connectivities in partial structures A and B. The methine at position 2′ was assigned to be adjacent to an oxygen atom because of its ^{13}C (δ 70.6) and ^{1}H (δ 3.79) chemical shifts. An exchangable proton signal of 2′-OH (δ 5.81) was coupled with the methine at position 2′. Judging from the chemical shifts of the methine at position 2 (δ_H 4.07, δ_C 54.5) and the methylene at position 5′ (δ_H 3.03, δ_C 40.5), the methine and the methylene were assigned to be adjacent to nitrogen atoms. A mono-substituted phenyl group in partial structure C was deduced from the ^{13}C and ^{1}H NMR spectral data of positions from 4 to 9. Further connectivities were disclosed by interpretation of ^{1}H-^{13}C long range couplings obtained by the HMBC experiments on **1**. Partial structures B and C and a quaternary carbon C-1 (δ 174.5) were connected to form a phenylalanine residue by the observation of the following ^{1}H-^{13}C long range couplings: (2-H)-(C-1), (3-H)-(C-1), (2-NH)-(C-1), (2-H)-(C-4) and (3-H)-(C-4). Judging from the chemical shift of C-6′ (δ 157.4), C-6′ was assigned to be the carbon atom of the guanidino group suggested by the color reaction. The guanidino group and carbonyl carbon C-1′ were concluded to connect with partial structure A to form a 5-guanidino-2-hydroxypentanoic acid residue because of the presence of ^{1}H-^{13}C long range couplings between H-5′ and C-6′ and between H-3′ and C-1′. The connectivity between a phenylalanine and a 5-guanidino-2-hydroxypentanoic acid residue was also clarified by the ^{1}H-^{13}C long range couplings between C-1′ and protons, 2-H and 2-NH. The structure of **1** was thus determined

Table 2. NMR spectral data for **1** in DMSO-d_6.

	^{13}C 1)	^{1}H 2)
Phenylalanine		
1	174.5	-
2	54.5	4.07 (1H, dt, J = 7.3 Hz)
3	37.6	a 2.93 (1H, dd, J = 13.2, 5.9 Hz)
		b 3.05 (1H, dd, J = 13.2, 5.9 Hz)
4	138.6	-
5, 9	129.5	7.09 (2H, d, J = 6.6 Hz)
6, 8	127.7	7.15 (2H, dd, J = 6.6, 6.6 Hz)
7	125.7	7.11 (1H, dd, J = 6.6, 6.6 Hz)
2-NH	-	7.58 (1H, d, J = 7.3 Hz)
5-Guanidino-2-hydroxy pentanoic acid		
1'	172.5	-
2'	70.6	3.79 (1H, m)
3'	31.4	1.46 (2H, m)
4'	24.4	a 1.46 (1H, m)
		b 1.64 (1H, m)
5'	40.5	3.03 (2H, m)
6'	157.4	-
2'-OH	-	5.81 (1H, br)
Guanidino-NH		7.60 (3H, br)
		8.70 (1H, br s)

1) Assigned by DEPT, HSQC and HMBC experiments; 100 MHz, δ in ppm.
2) 400 MHz, δ in ppm.

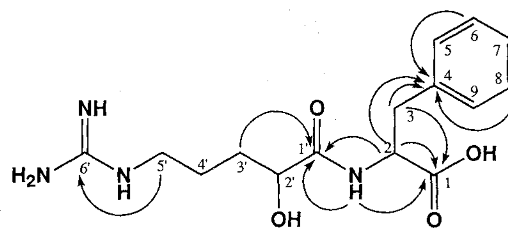
Fig. 5. Partial structures clarified by homo decoupling experiments and by the interpretation of ^{1}H and ^{13}C NMR spectra.



to be N-(5-guanidino-2-hydroxypentanoyl)-phenylalanine (Fig. 6).

The absolute configuration of the phenylalanine residue in **1** was determined to be L by the chiral HPLC analysis† of the hydrolysate (6 N HCl, 110°C, 16 hours) of **1**. To determine the absolute configuration of C-2′, we synthesized N-[(S)-5-guanidino-2-hydroxypentanoyl]-L-phenylalanine and N-[(R)-5-guanidino-2-hydroxypentanoyl]-L-phenylalanine and compared their

Fig. 6. ^{1}H-^{13}C long range couplings (arrows) obtained by the HMBC experiments on **1**.



† The following conditions were used. Column: TSK-GEL Enantio L-1 (4.6 mm i.d. × 250 mm); solvent: 1 mM copper sulfate; flow rate: 1.0 ml/minute; detection: UV 210 nm.

physico-chemical properties with those of **1**. The syntheses of these authentic samples are described in the accompanying paper[2]. Since the physico-chemical properties (IR, ^{1}H NMR, $[\alpha]_D$ and HPLC retention time) of **1** were identical with those ($[\alpha]_D^{24}$: 24° (*c* 0.5, MeOH) of *N*-[(*S*)-5-guanidino-2-hydroxypentanoyl]-L-phenylalanine but different from those ($[\alpha]_D^{24}$: 31° (*c* 0.5, MeOH) of its epimer, the absolute configuration of C-2′ was determined to be *S*. Thus, the total structure of monamidocin was determined as shown in Fig. 1.

Discussion

Most of the fibrinogen receptor antagonists of natural origin are peptides having molecular masses greater than 5,000 daltons. Among them, decorsin[3] isolated from the North American leech, *Macrobdella decora*., and disintegrins such as echistatin[4], kistrin[5], trigramin[5] and bitan[5] isolated from the venom of various snakes are peptides containing the RGD (Arg-Gly-Asp) sequence. RGD sequence is the minimal sequence in fibrinogen required for the binding of fibrinogen to its receptor during aggregation[6]. Recently, we reported novel fibrinogen receptor antagonist, tetrafibricin[7,8] whose structure is completely different from that of **1**. Tetrafibricin is a linear compound containing primary amine, conjugated tetraenoic acid and polyhydroxy functional groups, whereas **1** is a dipeptide containing the guanidine and carboxylic acid functional groups, which seem to be essential part for the binding in RGD peptides. It will be interesting to investigate the binding mechanism of the fibrinogen receptor using a small and simple molecule like **1**.

Since the structure of monamidocin is much simpler than those of tetrafibricin, decorcin and disintegrins, monamidocin seems to be a good lead of synthetic study. We report the syntheses of the analogs of **1** and their structure-activity relationships in the accompanying paper[2].

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