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Studies of Platelet Activating Factor (PAF) Antagonists from Microbial Products. I. Bisdethiobis(methylthio)gliotoxin and Its Derivatives

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A platelet activating factor (PAF) antagonist, designated as FR-49175, was isolated from fermentation products of *Penicillium terlikowskii* and identified as bisdethiobis(methylthio)-gliotoxin (**1**). The IC_{50} value of this compound for PAF-induced rabbit platelet aggregation was 8.4 μM.

Some derivatives of bisdethiobis(methylthio)gliotoxin (**1**) were synthesized and their inhibitory activities on PAF-induced platelet aggregation were examined. Among these compounds, 5a,6-anhydrobisdethio-3,10a-bis(methylthio)gliotoxin (**8**) showed the most potent PAF inhibitory activity (IC_{50}; 4.4 μM).

Keywords——platelet activating factor (PAF); PAF inhibitor; FR-49175; bisdethiobis(methylthio)gliotoxin; structure–activity relationship

Platelet activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine) is released from rabbit basophils through an immunoglobulin E (IgE)-dependent mechanism.[1] This compound is an extremely potent inducer of platelet aggregation[2] as well as hypotension[3] and bronchoconstriction.[4] An important role of PAF in various allergic and inflammatory reactions has recently been suggested.[5]

In our continuing search for potential PAF inhibitors, we have tested a wide range of fermented broths for inhibitory effects on PAF-induced rabbit platelet aggregation. As a result, bisdethiobis(methylthio)gliotoxin (**1**) was isolated from the fermented broth of strain No. 5348. This strain was identified as *Penicillium terlikowskii* No. 5348.

Compound **1** was first isolated from cultures of the wood fungus *Gliocladium delquescens* as a minor metabolite by Kirby *et al.*, in 1979.[6] They also obtained this natural product (**1**) by chemical conversion from gliotoxin (**2**), the major metabolite from the same cultured filtrate. However, its PAF inhibitory activity has not been reported.

This report decribes the isolation and structure identification of **1**, the synthesis of its derivatives, and the determination of their anti-PAF activities.

Results and Discussion

Ninety-two milligrams of **1** was obtained from 15 l of the culture filtrate of *Penicillium terlikowskii* No. 5348 by means of ethyl acetate extraction under acidic conditions, silica gel column chromatography and preparative thin layer chromatography (pTLC). The substance (**1**) inhibited PAF-induced platelet aggregation with an IC_{50} of 8.4 μM, and slightly (84.2 μM) inhibited collagen-induced aggregation, but had no effect (400 μM) on the aggregation induced

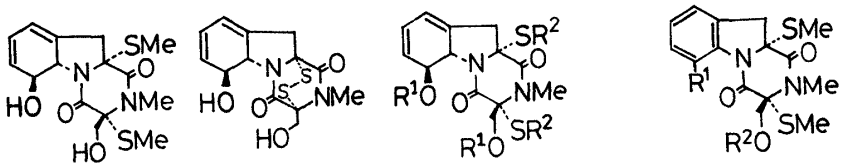
TABLE I. Inhibition of Platelet Aggregation by Bisdethiobis(methylthio)-gliotoxin (**1**), Tiaramide and Indomethacin

Inducer	IC_{50} value (μM)		
	1	Tiaramide	Indomethacin
PAF	8.4	15.3	>400
Collagen	84.2	7.7	5.8
Arachidonic acid	>400	37.0	1.3
ADP	>400	33.0	>400

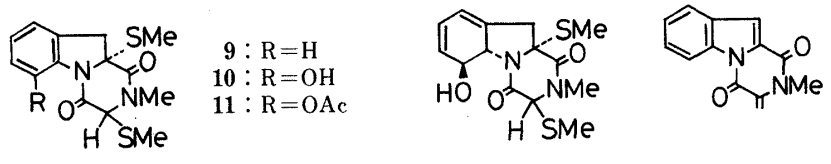
Each drug was added 2 min before an aggregating agent, PAF (0.1 μM), collagen (2.5 μg/ml), arachidonic acid (100 μM) or ADP (2.5 μM). The results are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

by arachidonic acid and adenosine diphosphate (ADP) (Table I). Antiinflammatory drugs, tiaramide and indomethacin were also examined for comparison. The inhibitory effect of tiaramide on rabbit platelet aggregation has already been reported.[7] This drug inhibited not only PAF-induced aggregation (IC_{50}; 15.3 μM) but also collagen-, arachidonic acid- and ADP-induced aggregations (IC_{50}; 7.7, 37.0 and 33.0 μM, respectively). The mode of action of this drug on platelet aggregation has not been elucidated yet. Indomethacin is known to block cyclooxygenase; this drug inhibited collagen-induced (IC_{50}; 5.8 μM) and arachidonic acid-induced (IC_{50}; 1.3 μM) aggregation but it did not inhibit PAF- or ADP-induced aggregation up to 400 μM. The experimental results presented here indicate that **1** is a specific inhibitor of PAF-induced aggregation. Although Nunn[8] found that endogenous PAF did not mediate collagen-induced platelet aggregation by using desensitization techniques, we still presume that PAF might play some role in collagen-induced aggregation.

Compound **2** was also isolated from the same culture filtrate but its anti-PAF activity was very weak (IC_{50}; 93.0 μM). Even though **2** exhibits remarkable antifungal and antiviral activities,[9] the toxicity observed in animal experiments precluded its therapeutic use.[10] The LD_{50} value of gliotoxin (**2**) when given intraperitoneally to male ddY mice was 17.9 mg/kg. In contrast, **1** is less toxic (LD_{50}; >500 mg/kg. male ddY mice, *i.p.*) and has neither antifungal nor antiviral activities (data not shown). These observations suggest that **1** could be useful in investigations of the role of PAF in anaphylaxis and inflammation.



1 **2** **3** : R^1=Ac, R^2=CH_3; **4** : R^1=H, R^2=CH_2CH_3; **5** : R^1=H, R^2=$(CH_2)_3CH_3$; **6** : R^1=OH, R^2=H; **7** : R^1=OAc, R^2=Ac; **8** : R^1=R^2=H



9 : R=H; **10** : R=OH; **11** : R=OAc

12 **13**

Chart 1

TABLE II. Inhibitory Activity of Bisdethiobis(methylthio)gliotoxin Derivatives on PAF-Induced Platelet Aggregation

Compound No.	IC_{50} (μM)	Compound No.	IC_{50} (μM)
1	8.4	**8**	4.4
2	93.0	**9**	38.8
3	30.2	**10**	95.2
4	7.8	**11**	16.3
5	136.4	**12**	48.9
6	16.9	**13**	>200
7	68.5		

IC_{50} is the concentration of a compound required for 50% inhibition of rabbit platelet aggregation induced by PAF (0.1 μM).

Moreover, the foregoing new finding of the biological activity of **1** led us to synthesize some derivatives, starting from **1** or **2**, to test their inhibitory activities on PAF-induced platelet aggregation. All the compounds **3** through **12**, but not **13**, possess a dialkylthiopiperazinedione skeleton in the molecule. The PAF-induced rabbit platelet aggregation inhibitory activities of these derivatives are summarized in Table II. Although it is difficult to elucidate the structure–activity relationships from the test results, the following speculations are presented. Long-chain alkyl sulfide in the dialkylthio-piperazinedione system decreases the inhibitory activity (see compounds **1**, **4** and **5**). The dihydrobenzene ring system seems not to be essential for the activity since the phenolic dihydro derivative (**6**) still showed significant activity and the anhydro compound (**8**) showed higher inhibitory activity than the natural product (**1**). The hydroxymethyl group at position 3 appears to play an important role, since the dehydroxymethylated compounds (**9**), (**10**) and (**12**) were less active than the corresponding compounds (**8**), (**6**) and (**1**), respectively, although an exception was found in the case of compound **11**. As anticipated, the desulfurized compound **13** had lost the activity completely. Based on these apparent structure–activity relationships, chemists in our laboratories have started to synthesize analogous compounds in search of potential PAF antagonists, and the results will be published in due course.

Experimental

The following instruments were used for obtaining physical data. The infrared (IR) spectra were recorded with a JASCO A-102 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a JEOL PMX-60 or a JEOL MH-100, and chemical shifts are given on the δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; br, broad; m, multiplet). Electron impact-mass spectra (EI-MS) were recorded on a Hitachi M-80 mass spectrometer. pTLC was carried out on Merck Silica gel F_{254} pre-coated plates, Art 5744.

Preparation of PAF——PAF (alkyl chain: C_{18}) was synthesized according to the method described by Heymans *et al.*[11] *Anal.* Calcd for $C_{28}H_{58}NO_7P \cdot H_2O$: C, 59.03; H, 10.61; N, 2.46; P, 5.43. Found: C, 59.28; H, 10.89; N, 2.46; P, 5.50. IR ν_{max}^{KBr} cm^{-1}: 3700—3100, 2900, 2830, 1730. ^{1}H-NMR (60 MHz, CD_3OD) δ: 0.71—1.01 (3H, m), 1.04—1.70 (32H, m), 2.08 (3H, s), 3.12—3.81 (15H, m), 3.93—4.08 (2H, m), 4.10—4.41 (2H, m,), 5.03—5.20 (1H, m). For use, PAF was dissolved in 0.15 M NaCl containing 2.5 mg/ml bovine serum albumin (Sigma).

Platelet Aggregation Method——Blood was collected from the central ear arteries of male Japanese white rabbits (2.5 to 3.0 kg body weight). Coagulation was prevented by adding 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 150 g for 10 min at 10 °C. The PRP was diluted with platelet-poor plasma obtained by further centrifugation of the blood at 1000 ***g*** for 20 min. The platelet counts in the PRP used for aggregation studies were about 4.0×10^5 platelet/mm^3. Aggregometry was performed using a SIENCO dual-sample aggregometer (DP-247 E) at 37 °C, with the following platelet aggregating agents: PAF, collagen (Tokyo Kasei), arachidonic acid (Sigma) and ADP (Boehringer). For drug studied, 0.3 ml of PRP was incubated with drugs or vehicle for 2 min before the addition of aggregating agent at a concentration which

gave maximum aggregation (PAF, 0.1 μM; collagen, 2.5 μg/ml; arachidonic acid, 100 μM; ADP, 2.5 μM). Indomethacin (Sigma), **1** and other derivatives were prepared in ethanol. The small amounts of ethanol (final concentration of 0.05—0.1%) employed as a vehicle had no effect on platelet aggregation. Tiaramide (Fujisawa) was dissolved in saline.

Acute Toxicity——Acute toxicity was studied in 5-week-old male ddY mice. Each drug was suspended in 0.5% methyl cellulose containing saline. After intraperitoneal administration of a single dose, the animals were observed for 10 d. LD_{50} was claculated by the Probit method.

Taxonomic Study of Strain No. 5348——Strain No. 5348 was originally isolated from a soil sample collected at Okayama City, Okayama Prefecture, Japan. Based on morphological and cultural characteristics, we identified this strain as *Penicillium terlikowskii* ZALESKI,[12] and named it *Penicillium terlikowskii* No. 5348.

Fermentation of *Penicillium terlikowski* No. 5348——Seed flasks (500 ml), containing 100 ml of sterile seed medium consisting of corn starch 1%, glucose 1%, corn steep liquor 0.5%, peanut meal 1%, molatein 0.5% and $CaCO_3$ 0.5% were inoculated from a well-grown slant culture of strain No. 5348. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) for 4 d at 30 °C. The contents of the flasks were inoculated into 20 l of fermentation medium in a 30-liter stainless steel fermenter. The composition of the medium was as follows: soluble starch 2%, glucose 2%, corn steep liquor 2%, molatein 1%, polypeptone 0.5% and $CaCO_3$ 0.2%. Fermentation was allowed to proceed for 3 d at 30 °C under aeration at 20 l/min and agitation at 200 rpm.

Isolation of Bisdethiobis(methylthio)gliotoxin (1) and Gliotoxin (2)——The culture filtrate (15 l) was adjusted to pH 2.0 and then extracted with 30 l of ethyl acetate. The extract was concentrated *in vacuo*. The resultant material was subjected to silica gel column chromatography (0.5 l) and the column was eluted with a mixture of *n*-hexane–acetone (1 : 1). The fractions containing active compound were concentrated and the product was recrystallized from methanol to give 3.1 g of yellow needles. The physical data for this product were identical with those of **2**.[13] IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 1680, 1660, 1415, 1370, 1060, 1015. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.90 (1H, d, J=18 Hz), 3.15 (3H, s), 3.70 (1H, d, J=18 Hz), 4.20 and 4.40 (2H, ABq, J=12 Hz), 4.75 (2H, s), 5.60—6.00 (3H, m). EI-MS *m/z*: 326 (M^+), 262. Needles, mp. 220 °C (dec), $[\alpha]_D^{25}$ −288° (c=0.1, EtOH). The mother liquor was concentrated and subjected to pTLC (Kieselgel 60); the plate was developed with a mixture of chloroform–methanol (20 : 1). The active zone was extracted with ethyl acetate and concentrated *in vacuo* to give 92 mg of pure powder, which was identified as **1** by comparison of the ^{1}H-NMR spectrum with the literature data.[6] IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 1660, 1640, 1420, 1385, 1190, 1080, 1055. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.28 (3H, s), 2.30 (3H, s), 3.00 (2H, br s), 3.10 (3H, s), 3.90 and 4.35 (2H, ABq, J=12 Hz), 4.90 (2H, s), 5.60—6.00 (3H, m). EI-MS *m/z*: 309 (M^+ −SMe), 261. **1** was synthesized from gliotoxin by Kirby's method.[6]

Synthesis of Analogous Compounds

Diacetylbisdethiobis(methylthio)gliotoxin (3)——**2** (15 mg) was treated with acetic anhydride (0.5 ml) in pyridine (1 ml). Usual work-up gave **3** (16 mg) as a gum. EI-MS *m/z*: 440 (M^+). ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.95 (3H, s), 2.12 (3H, s), 2.29 (3H, s), 2.31 (3H, s), 3.01 (3H, s), 3.03 (2H, br), 4.40 and 4.60 (2H, ABq, J=12 Hz), 5.00—5.40 (2H, m), 5.80—6.10 (3H, m).

Bisdethiobis(ethylthio)gliotoxin (4) and Bisdethiobis(*n*-butylthio)gliotoxin (5)——**4** and **5** were prepared from **2** according to Kirby's method.[6] **4**: EI-MS *m/z*: 384 (M^+). ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.22 (3H, t, J=7 Hz), 1.25 (3H, t, J=7 Hz), 2.79 (2H, q, J=7 Hz), 2.80 (2H, q, J=7 Hz), 3.01 (2H, br s), 3.11 (3H, s), 3.90 and 4.38 (2H, ABq, J=12 Hz), 4.90 (2H, br s), 5.60—5.95 (3H, m). **5**: EI-MS *m/z*: 440 (M^+). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 1650, 1425, 1390, 1195, 1060. ^{1}H-NMR (60 MHz, $CDCl_3$): δ: 0.90 (6H, t, J=7 Hz), 1.10—1.80 (8H, m), 2.50—2.95 (4H, m), 3.00 (2H, br s), 3.10 (3H, s), 3.90 and 4.32 (2H, ABq, J=12 Hz), 4.90 (2H, br s), 5.60—6.00 (3H, m).

5a,6-Dehydrobisdethio-3,10a-bis(methylthio)gliotoxin (6)——**6** was prepared from **1** by Kirby's method.[6] The ^{1}H-NMR spectral data (60 MHz, $CDCl_3$) were in accord with the literature values.[6]

Diacetyl-5a,6-dehydrobisdethio-3,10a-bis(methylthio)gliotoxin (7)——**6** (115 mg) was treated with acetic anhydride (2 ml) and pyridine (4 ml). Usual work-up gave **7** as an oil. EI-MS *m/z*: 438 (M^+). ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 1.95 (3H, s), 2.26 (3H, s), 2.31 (3H, s), 2.36 (3H, s), 3.10 (3H, s), 3.55 and 3.60 (2H, ABq, J=16 Hz), 4.50 and 4.80 (2H, ABq, J=12 Hz), 7.00—7.40 (3H, m).

5a,6-Anhydrobisdethio-3,10a-bis(methylthio)gliotoxin (8), 5a,6-Anhydro-3-dehydroxymethylbisdethio-3,10a-bis(methylthio)gliotoxin (9) and 3-Dehydroxymethylbisdethio-3,10a-bis(methylthio)gliotoxin (12)——A solution of **1** (100 mg) and 1,8-diazabicyclo-5,4,0-undec-7-ene (46 μl) in dioxane (10 ml) was heated at 70 °C for 2 h. The cooled solution was diluted with ethyl acetate (50 ml), washed with water and dried over magnesium sulfate. Evaporation of the solvent gave a residue (70 mg), which was purified by pTLC [developed with ethyl acetate–hexane (2 : 3)] to afford **8** (30 mg), **9** (12 mg) and **12** (8 mg). **8**: EI-MS *m/z*: 338 (M^+). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3400, 1660, 1600, 1480, 1415, 1385. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.25 (3H, s), 2.33 (3H, s), 3.20 (3H, s), 3.62 (2H, br s), 4.02 and 4.50 (2H, ABq, J=12 Hz), 7.15—7.55 (3H, m), 8.10 (1H, m). **9**: EI-MS *m/z*: 309 (M^+ +1). ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 2.18 (3H, s), 2.48 (3H, s), 3.15 (3H, s), 3.50 and 3.67 (2H, ABq, J=16 Hz), 4.65 (1H, s), 7.00—7.43 (3H, m), 8.0 (1H, br d, J=8 Hz). **12**: EI-MS *m/z*: 327 (M^+ +1). ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 2.17 (3H, s), 2.42 (3H, s), 2.96 (2H, br s), 3.08 (3H, s), 4.58 (1H, s), 4.84 (2H, m), 5.62—5.90 (3H, m).

5a,6-Dehydro-3-dehydroxymethylbisdethio-3,10a-bis(methylthio)gliotoxin (10)——A mixture of **6** (50 mg), potas-

sium carbonate (20 mg) and acetone (10 ml) was refluxed for 8 h. The cooled solution was acidified with 1 N HCl (30 ml) and extracted with ethyl acetate. Usual work-up gave a residue (45 mg), which was purified by pTLC [developed with ethyl acetate–hexane (1 : 3)] to afford **10** (20 mg) as a powder. EI-MS *m/z*: 324 (M^+). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1665, 1640, 1605, 1480, 1435, 1400. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.20 (3H, s), 2.45 (3H, s), 3.15 (3H, s), 3.45 and 3.65 (2H, ABq, J=16 Hz), 4.70 (1H, s), 6.60—7.20 (3H, m), 10.30 (1H, s).

Acetyl-5a,6-dehydro-3-dehydroxymethylbisdethio-3,10a-bis(methylthio)gliotoxin (11)——**10** (5 mg) was treated with acetic anhydride (0.5 ml) and pyridine (1 ml). Usual work-up gave **11** (5 mg) as an oil. EI-MS *m/z*: 366 (M^+). ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.20 (3H, s), 2.30 (3H, s), 2.40 (3H, s), 3.12 (3H, s), 3.50 and 3.65 (2H, ABq, J=15 Hz), 4.61 (1H, s), 7.0—7.3 (3H, m).

2-Methyl-3-methylene-pyrazino[1,2-*a*]indole-1,4(2*H*,3*H*)-dione (13)——A solution of **2** (50 mg) and 1,8-diazabicyclo[5,4,0]undec-7-ene (40 μl) in dioxane (5 ml) was refluxed for 30 min. The cooled solution was diluted with ethyl acetate (20 ml), washed with water and dried over magnesium sulfate. Evaporation of the solvent gave a residue (40 mg), which was purified by pTLC [developed with ethyl acetate–chloroform (1 : 5)] to afford **13** (25 mg). EI-MS *m/z*: 226 (M^+). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1700, 1650, 1600, 1570, 1450, 1430, 1400, 1355, 1200, 890, 845. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 3.36 (3H, s), 5.20 (1H, d, J=1.5 Hz), 6.10 (1H, d, J=1.5 Hz), 7.45 (1H, s), 7.20—7.80 (3H, m), 8.45 (1H, br d, J=8 Hz).

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