PURIFICATION AND CHARACTERIZATION OF ASEANOSTATINS: ACTINOMYCETE-DERIVED FATTY ACID INHIBITORS TO MYELOPEROXIDASE RELEASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

AKIKO ISHIDA-OKAWARA[a], YUKIO KIMOTO[a], KAZUO WATANABE[b], KOICHI TOKUDA[a], MICHIO SHIBATA[a,c], KAORI MASUDA[a], YUKIE TAKANO[a], KAZUE KAWAGUCHI[a], HISAYOSHI AKAGAWA[a], NALINE NILUBOL[d], KUNIMOTO HOTTA[a], KAZUNAGA YAZAWA[b], SATOSHI MIZUNO[a] and KAZUO SUZUKI[a,*]

[a]Department of Antibiotics, National Institute of Health, 2-10-35, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
[b]Sagami Chemical Research Center, 4-4-1 Nishi-ohnuma, Sagamihara, Kanagawa 229, Japan
[c]Department of Biology, Faculty of Science, Tokyo Metropolitan University, 2-1-1 Fukazawa, Setagaya-ku, Tokyo 158, Japan
[d]The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Phayathai Road, Bangkok 10500, Thailand

(Received for publication December 25, 1990)

We found inhibitors, designated aseanostatins P1 and P5, against myeloperoxidase (MPO) release from human polymorphonuclear leukocytes (PMN). Aseanostatins were extracted from an actinomycete isolated in Thailand and purified by a series of column chromatography of charcoal and silica gel, and HPLC. Physico-chemical characterization by gas liquid chromatography and GC-MS indicated that aseanostatins were fatty acids. The active forms of aseanostatins were recovered by hydrolyzing their methyl esters after HPLC. Two components P1 and P5 with the IC_{50} of 0.96 and 0.54 μg/ml to the MPO release were obtained as pure forms, indicating aseanostatin P5 was higher activity than aseanostatin P1. The component P1 was identical with 12-methyltridecanoic acid and P5 was indistinguishable to 12-methyltetradecanoic acid (ante-i-15 : 0). Aseanostatin P5 (1 μg/ml) did not inhibit β-glucuronidase release, but O_2^- production a little. It has no effect on chemotaxis of PMN to fMet-Leu-Phe (10^{-8} M), PMN adhesion or phosphorylation of a 64-kD protein in the PMN cell-lysate system.

Polymorphonuclear leukocytes (PMN) take part in the defense mechanism against the primary infection; they reach infectious sites and eliminate invaders by O_2^- production and release of lysosomal enzymes. The function of PMN *in vivo* is believed to be regulated by chemotactic factors/activators such as fMet-Leu-Phe (a bacterial chemotactic peptide[1]), C5a[2], LTB_4[3] and LUCT/IL-8 (lung giant cell-derived chemotactic protein/interleukin-8[4]). *In vitro* these chemotaxin/activators[5~8], cytochalasin B[9~11], colchicine[12], some local anesthetics[13,14] and nicotine[15] are known as modulators for PMN functions such as chemotaxis, release of lysosomal enzymes and O_2^- production. These modulators have been employed to elucidate mechanism of PMN activation. However, mechanism of exocytosis in PMN including enzyme release has not been clarified.

In order to obtain a new microbial modulator of PMN function we screened actinomycete strains isolated in Thailand, and obtained a strain showing inhibition to the myeloperoxidase (MPO) release from PMN. In this paper, purification, structure and some characteristics of the active principles were described.

Materials and Methods

Materials

Cytochalasin B, 3,3',5,5'-tetramethylbenzidine and cytochrome *c* were purchased from Sigma Chemical Company, St. Louis. fMet-Leu-Phe was purchased from Peptide Institute Inc., Osaka. Charcoal and Wakogel C-200 for chromatography and 4-methylumbelliferyl-β-D-glucuronide were obtained from Wako Pure Chemical Industries, Osaka. TLC plates (Silica gel 60 F_{254}) was purchased from Merck Inc., Darmstadt.

Production and Purification of Inhibitor of PMN Activity

Actinomycete strains isolated in various areas in Thailand were cultivated in two kinds of media. Medium A consisted of soybean powder 1.5%, glycerol 2.0%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005%. Medium B consisted of Polypeptone 1.0%, yeast extract 0.2%, corn starch 2.5% and $CaCO_3$ 0.6%. After cultivation in a 4-ml tube for 4 days at 27°C on a reciprocal shaker at 120 rpm using medium A or B, the cultured broths were centrifuged at 18,000 × *g* for 5 minutes and filtered through a Millipore filter (0.22 μm) and the resulting supernatants were used for screening of PMN modulator.

For isolation of active principles, strain T261 that showed the highest inhibitory activity was cultured for 2 days in medium A, and transferred into fresh medium A at a ratio of 2% and cultured for 5 days. The T261 cultured broth (7.6 liters) with pH 6.2~7.6 were filtrated through a Whatman No. 2 filter paper. Mycelial mass thus separated was extracted with methanol, and the extract was evaporated to dryness and stored at −20°C until use. Active principles were purified by charcoal chromatography followed by silica gel chromatography and HPLC.

Charcoal Chromatography: The methanol extract of the T261 mycelium (1 g) was dissolved in 60 ml of water and applied to a charcoal column (1.5 × 40 cm) equilibrated with water. After the column was washed with 80 ml of water at 2 ml/minute of flow rate, active principles were eluted with butyl alcohol - acetic acid - water (8 : 1 : 1) at 1 ml/minute of flow rate. Active fractions (Nos. 2~8; 20 ml each) were combined and evaporated. This procedure was repeated once using 2.7 g of separate mycelium extracts with methanol.

Silica Gel Chromatography: The active fraction in the charcoal chromatography was dissolved with 70% methanol, mixed with 2 g of silica gel powder and dried in a desiccator overnight. The mixture was dissolved with approximately 2 ml of butyl alcohol - acetic acid - water (22 : 2 : 1) and applied to a silica gel column (1.8 × 55 cm) equilibrated with the same solvent. The column was eluted with 140 ml (fractions 1~35) of the same solvent followed by 84 ml (fractions 36~56) of butyl alcohol - acetic acid - water (13 : 8 : 4) at a flow rate of 0.5~1.0 ml/minute. The first active peak fractions (Nos. 6~9; 4 ml each) that gave rise to one spot upon TLC monitoring were evaporated. This procedure was repeated once using 1.2 g of the active fraction in a separate charcoal chromatography. Active fractions thus obtained were combined, dissolved in approximately 1.5 ml of methanol, filtrated through a glass filter No. 4, and evaporated. The evaporated fraction was mixed with *n*-hexane which was then passed through a glass filter and evaporated. The *n*-hexane soluble fraction was dissolved in approximately 1 ml of chloroform and applied to a silica gel column (1.5 × 40 cm) equilibrated with chloroform. The column was eluted with chloroform (fractions 1~20) followed by chloroform - methanol (9 : 1; fractions 21~29) at a flow rate of 0.5 ml/minute. Active fractions (Nos. 7~15; 4 ml each) were evaporated and dissolved in approximately 1 ml of methanol which was then filtrated with glass filter No. 4 and air dried at 4°C.

HPLC: The active fractions in the second silica gel chromatography were methyl esterified. The esterification was performed at room temperature for 1 hour using diazomethane diethyl ether solution. The ester solution was applied to HPLC using an ODS column (YMC-Pack A312, 6 × 150 mm) and eluted with methanol - water (9 : 1) at a flow rate of 1 ml/minute. The eluates were extracted with *n*-hexane, and evaporated. Each fraction was assayed for the inhibitory activity to the MPO release and analyzed by gas liquid chromatography (GLC).

Hydrolysis of Methyl Esters in HPLC Fractions: After *n*-hexane was removed, fractions of HPLC were added with 1.2 ml of 90% ethanol containing 3 N NaOH, and then incubated for 1 hour at 85°C to hydrolyze. After neutralization with 6 N HCl, the fractions were extracted with *n*-hexane and filled with N_2 gas. For assay of MPO release inhibition, the extracts were completely evaporated, dissolved with DMSO and then diluted with PBS(−) to the final concentration of DMSO to 0.1%.

TLC: Samples in each purification step were applied to a TLC plate and developed with the same solvent employed in each purification step. Substances in fractions were detected by exposing to UV or spraying with sulfuric acid followed by heating.

GLC: The methyl esterified samples were applied to a 0.25 mm × 25 m capillary column coated with PEG 20M. The column temperature was initially kept at 150°C for 3 minutes and raised up to 196°C at a rate of 2°C/minute. Analysis was performed using a detector equipped with FID.

GC-MS: The samples were applied to a 0.25 mm × 25 m capillary column coated with OV-1. The column temperature was initially kept at 50°C for 1 minute and increased to 200°C at a rate of 25°C/minute and then to 230°C at a rate of 4°C/minute. Mass spectra were determined by a combined GC-MS, model 70SE (VG Analytical, UK), with an ionizing current of 200 μA, an electron-accelerating voltage of 35 eV and an ion source temperature 230°C.

Release and Assay of Lysosomal Enzymes from PMN

Human PMN were prepared as described previously[16]. Briefly, the fraction containing PMN and red blood cells were obtained from heparinized peripheral blood (20 IU/ml blood) of healthy adult volunteers. PMN were suspended in Hanks' balanced salt solution (HBSS) at a concentration of 2×10^6/ml.

Prewarmed PMN (10^6 cells/ml) were exposed to samples (final concentration 1% and 0.3%), cytochlasin B (final concentration 5 μg/ml) and fMet-Leu-Phe (final concentration 10^{-6} M) in a 96 well-V-plate (Nunc, No. 2-45128) for 10 minutes at 37°C in a total volume 75 μl. After incubation, the plate was immersed on the ice and centrifuged at 350 × g for 5 minutes at 4°C to separate the supernatant from the cell pellet. The cell pellet was mixed with 150 μl of 0.1% Triton X-100 in HBSS in order to measure lysosomal enzyme activity remained.

MPO Activity: MPO activities in the supernatant and cell homogenate were assayed as described previously[17]. The reaction mixture consisted of PMN supernatant or homogenate, 1.7 mM 3,3',5,5'-tetramethylbenzidine, 0.39 mM H_2O_2, 84.2 mM sodium citrate buffer (pH 5.4), 7.2% DMF, PBS(−) and HBSS in a total volume of 200 μl in 96 well-F-plate (Nunc, No. 2-69620). Increase in absorbance at 650 nm in the reaction mixture at 37°C was measured with an automatic analyzer LFA-096 (Japan Spectroscopic Co., Tokyo, Japan) at 30 seconds interval. One unit was defined as the activity producing an increase of 1.0 in absorbance at 655 nm/minute/ml of original MPO preparation. MPO release activity was expressed as relative release (percent release) which was calculated using the formula of 100 × released activity/(released activity + remained activity in cells). Samples were examined at the final concentration of 1% and 0.3%. The sample was selected when 50% inhibition at the concentration of 1% with dose-dependent manner was confirmed.

β-Glucuronidase (BGL) Activity: BGL activities in the supernatant and cell homogenate were assayed by the method modifying as described previously[18]. Briefly, reaction mixture (40 μl) consisted of PMN supernatant or the cell homogenate, 1 mM 4-methylumbelliferyl-β-D-glucuronide, 0.05% TX-100, 0.1 M sodium acetate buffer (pH 3.5) in 96 well-F-plate. After incubation for 30 minutes at 37°C, 210 μl of a termination buffer (50 mM sodium glycine buffer pH 10.4 containing 5 mM EDTA-disodium salt) was added. Fluorescence intensity was measured by an automatic fluorescence analyzer LFA-96F (Japan Spectroscopic Co., Tokyo, Japan) with wavelength of excitation 365 nm and emission 405 nm. One unit of BGL activity was defined as the activity liberating 1 pmol of 4-methylumbelliferone/minute/ml of the original enzyme preparation.

Assay of Other PMN Functions

O_2^- Production of PMN: PMN suspension (2×10^6 cells/ml, 100 μl) and 66 μM of ferri cytochrome c were mixed in 96 well-F-Plate and kept for approximately 2 minutes. Then, sample and a mixture of cytochlasin B (final concentration 5 μg/ml) and fMet-Leu-Phe (final concentration 10^{-6} M), were subsequently added to the suspension and stood still for approximately 30 seconds at 37°C. O_2^- production was determined by measuring the increase in absorbance at 546 nm at 0.269 minute interval using the automatic analyzer LFA-096.

Chemotaxis Assay: Chemotactic activity was determined by a modified Boyden chamber method[19] using Millipore filter (3.0 μm pore size).

PMN Adhesion to Glass: PMN (2×10^6 cells/ml, 150 μl) were placed to the well of 6-well-silicon rubber containing 1.5 μl of samples on the slide-glass. After incubation for 15 minutes at 37°C, cells were

immediately transferred to the micro-test tube immersed in ice. Ratio of adherent PMN were calculated as percent adhesion by counting non-adherent cells.

Phosphorylation of 64-kD Protein in Cell-lysate System of PMN: The phosphorylation was assayed as described previously[20].

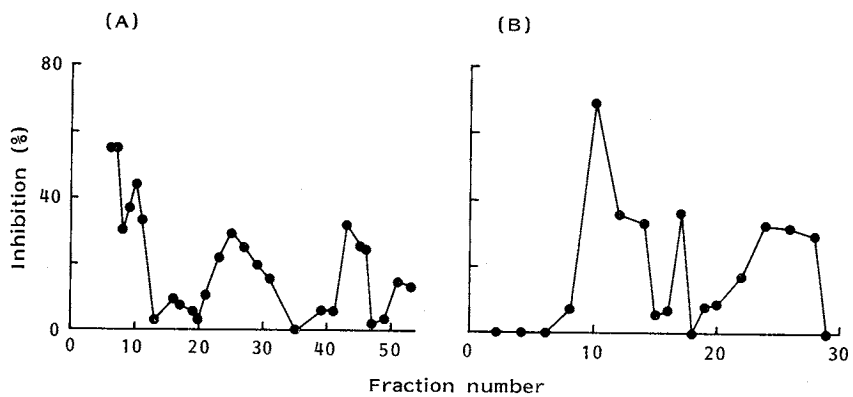
Results

Purification of Inhibitor of MPO Release from PMN

When the cultured broths from 506 actinomycete strains using media A and B were screened by the MPO release assay, 6 broths showed inhibitory activity to MPO release from PMN, while 11 broths enhanced the MPO release (data not shown). Inhibitors of the MPO release from PMN were purified from the T261 with the highest activity cultured in medium A. The T261 cultured broth showed no antibiotic activity to *Candida albicans*, *Bacillus subtilis* and *Escherichia coli* (data not shown).

Since T261 strain showed higher activity in its mycelium than in the culture supernatant, active principles were extracted from the mycelium with 99.6% methanol. The methanol extract was subjected to charcoal chromatography and then silica gel chromatography followed by HPLC. As shown in Fig. 1A, the active fractions obtained by charcoal chromatography gave three active peaks upon the 1st silica gel chromatography using butyl alcohol-acetic acid-water (22:2:1) and subsequently butyl alcohol-acetic acid-water (13:8:4) as developing solvents. When each active peak was monitored by TLC, the first peak (fractions 6~9) gave a single spot, whereas many spots were detected in the other peaks. Then, the first peak fraction (Nos. 6~9) was applied to the second silica gel chromatography (Fig. 1B). Elution with chloroform followed by chloroform-methanol (9:1) yielded fractions Nos. 7~15 that contained the high inhibitory activity and gave a single spot with Rf value 0.83 upon TLC developed with chloroform-methanol (9:1). Evaporation of the fractions gave rise to a pale yellow waxy substance which was changed to oily by warming. When this substance was methyl esterified and subjected to HPLC using an ODS column, 15 peaks were assigned (Fig. 2). However, no peak fraction showed inhibitory activity to the MPO release (data not shown). Since it seemed likely that esterification resulted in the loss of activity, the esters in the peak fractions were then hydrolyzed to produce the free acid form. As expected, free acid forms inhibited the fMet-Leu-Phe-induced MPO release from PMN. Fig. 3 shows the inhibition of the MPO release from PMN by peak fractions P1, P5 and P6. Their IC_{50} were estimated at 0.96, 0.54

Fig. 1. Silica gel chromatography.



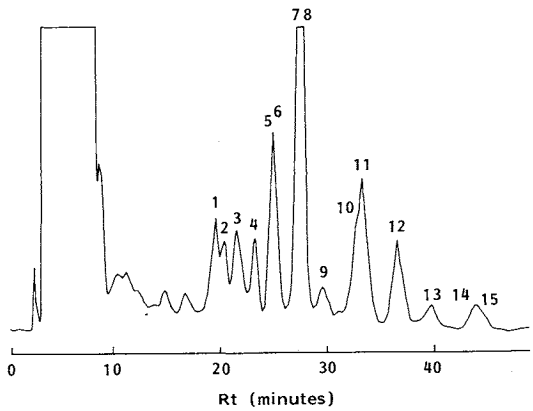
(A) The first silica gel chromatography. (B) The second silica gel chromatography.

and 0.49 μg/ml, respectively, according to Fig. 3. Two other major peaks (P10 and P14) slightly inhibited the MPO release at a concentration ranging from 0.05 to 1 μg/ml (Data not shown). Total MPO activity of PMN was 10.9 units/ml without fraction P5 and the value was constant in the presence of fraction P5 at the concentration ranging from 0.05 to 1 μg/ml (Table 1). P1 and P6 showed similar results (data not shown). Purification process of the inhibitor is summarized in Table 2. The specific activity of fraction P5 was increased by 145.7-folds. P5 showed higher specific activities than the activity of the active fraction in the second silica gel chromatography (Table 2).

Assignment of Aseanostatins P1 and P5

GC-MS analysis of the methyl esterified HPLC eluate indicated that the sample consisted of fatty acids with a branched methyl group. Fraction P1 gave the largest *m/z* 242 upon GC-MS and the Rt of 2.57 minutes upon GLC that were identical to 12-methyltridecanoic acid (i-14:0). Fraction P5 gave the largest *m/z* 256 and the predominant *m/z* 87 upon GC-MS (Fig. 4), and the Rt of 3.59 minutes upon GLC. These data indicated that P5 was indistinguishable with 12-methyltetradecanoic

Fig. 2. HPLC of the methyl esters of active components.



Peak numbers 1~15 were named as P1 to P15, respectively.

Fig. 3. Effect of concentration of fractions P1, P5 and P6 on MPO release from PMN.

PMN cells were treated with P1 ▲, P5 ■ and P6 ● at the indicated concentrations.

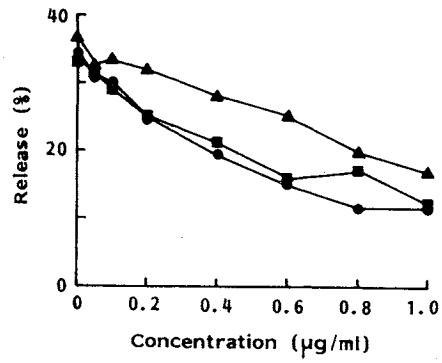


Table 1. The effect of P5 on total MPO activity.

P5 concentration (μg/ml)	Total MPO activity[a] (U/ml)	P5 concentration (μg/ml)	Total MPO activity (U/ml)
0	10.9	0.4	11.4
0.05	10.7	0.6	10.4
0.1	11.2	0.8	10.8
0.2	12.6	1.0	12.5

[a] Total MPO activity = extracellular MPO + remained MPO.

Table 2. Purification table.

Purification step	Total activity (U[a])	Total weight (mg)	Specific activity (U/mg)	Fold	Yield (%)
MeOH extract	6.27×10^5	3700.0	169.5	1.0	100.0
Charcoal chromatography	1.90×10^5	1574.5	120.5	0.7	30.3
1st silica gel chromatography	2.47×10^5	420.0	588.2	3.5	39.4
2nd silica gel chromatography	12.0×10^5	204.0	5882.4	34.7	191.4
HPLC (P5)	36.8×10^5	14.9	24691.4	145.7	58.7

[a] 1 U: IC_{50} in assay.

Fig. 4. Mass spectrum of P5.

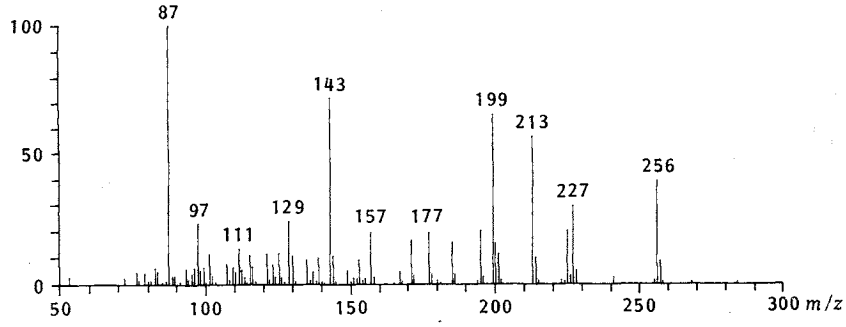


Fig. 5. The structure of aseanostatins.

(A) Aseanostatin P5, (B) aseanostatin P1, (C) i-15:0.

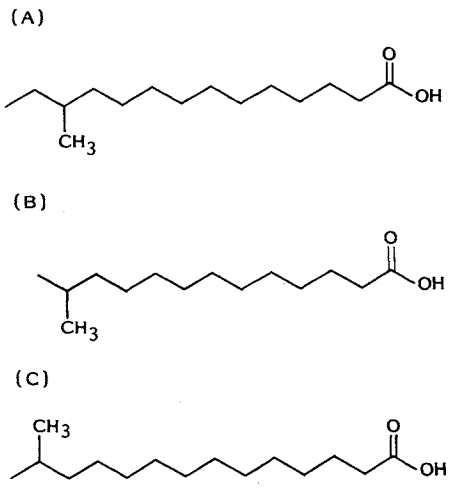
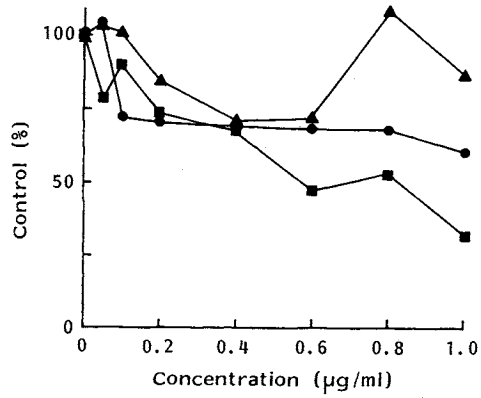


Fig. 6. Effect of concentration of aseanostatins P1 and P5 and fraction P6 on O_2^- production.

PMN were treated with P1 ▲, P5 ■ and P6 ● at the indicated concentrations.



acid (ante-i-15:0). On the other hand, P6 gave rise to two peaks with the Rt of 3.43 and 3.59 minutes upon GLC; the substances contained in the two peaks are identical to 13-methyltetradecanoic acid (i-15:0) and P5, respectively, in terms of the Rt and M^+ (256) upon GC-MS. Thus, P6 turned out to be a mixture of i-15:0 and P5 (1:2). Thus, P1 and P5 were obtained as pure forms and designated aseanostatins P1 and P5, respectively. Aseanostatin P5 showed the highest activity in these pure substances. The structures of aseanostatins P5 and P1 and i-15:0 were shown in Fig. 5.

Effect of the Inhibitor on Other PMN Functions

Aseanostatins P1, P5 and fraction P6 did not affect BGL release (data not shown), but O_2^- production slightly; aseanostatin P5 showed higher inhibition than that of P6 and P1 in a dose-dependent manner (Fig. 6). PMN migration toward fMet-Leu-Phe was not inhibited by 1 μg/ml of aseanostatin P5 and P1 (data not shown), but inhibited by fraction P6 approximately 20%. PMN adhesion to glass was not inhibited by aseanostatins (data not shown). No significant inhibition of phosphorylation of 64-kD protein in the PMN cell lysate system was observed with 1 μg/ml of aseanostatin P5 (data not shown).

Discussion

In the present study, we screened about 500 actinomycete strains for the productivity of inhibitors

against the MPO release from PMN. Since the assay employed is so sensitive, a number of strains showed the inhibitory activity. Out of these, the strain T261 with the highest inhibitory activity was chosen for extraction and purification of the active principles by a series of chromatography. Physico-chemical characterization revealed that the active principles were fatty acids with a branched methyl group. The present study showed that aseanostatins were inhibitors to MPO release from PMN, but not inhibitor of MPO itself, since total activity of MPO was not altered by the aseanostatins. Upon purification, the specific activity of P5 increased by 145.7-fold; the yield markedly increased by 2nd silica gel chromatography and the specific activity increased markedly by 2nd silica gel chromatography and HPLC. These marked increases were likely due to the elimination of substances that interfered with aseanostatins. It seems likely that the carboxyl group of ante-i-15:0 and i-14:0 are essential for the inhibitory activity of P5 and P1 because their methyl esterified forms were inactive to the MPO release.

Aseanostatin P5 had no effect on BGL release, indicating that MPO is contained in a different granule of PMN from that of BGL or that MPO exists in different manner from BGL[18]. Recently, it has been demonstrated that lipoprotein activates protein kinase C in P388D1[21] and lysozyme release but not BGL release from PMN[22]. To other PMN functions such as BGL release, O_2^- production, chemotaxis, adhesion and 64-kD protein phosphorylation, aseanostatin P5 showed weaker activity than that to the MPO release. We have previously reported that a 64-kD protein of PMN was phosphorylated in a dose-dependent manner when PMN was stimulated with fMet-Leu-Phe, LUCT/IL-8 or LU10[23]. Aseanostatin P5 did not influence the 64-kD protein phosphorylation of PMN.

Ante-i-15:0 and i-14:0 were saturated fatty acids with a branched methyl group and have been known as the bacterial membrane components[24,25]. It is known that exogenous fatty acids are incorporated into microbial phospholipids and glycolipids[24,25]. There have been several reports on the biological activity of various fatty acids in phagocytes; linoleic acid hydroperoxide inhibits O_2^- production by alveolar macrophages of rat[26] and LTB_4 increases adhesion of PMN to human endothelial cell *in vitro*[27]. LTB_4 and other fatty acids like 5-HETE have been known to induce chemotaxis[28,29], degranulation[30] and O_2^- production[31] of PMN. Moreover, arachidonic acid[32] and LTB_4[33] were reported to be related to the signal transduction of PMN. Thus, fatty acids stimulate various PMN functions. It is therefore of interest that aseanostatins function as specific inhibitors of the MPO release from PMN.

Another actinomycete metabolite inhibiting the substance P-induced MPO-release from PMN will be reported in a separate paper[34].

Acknowledgments

This study was supported in part by a Special Coordination Fund for Promoting Science and Technology from the Science and Technology Agency of Japan and a grant for cardiovascular diseases (64A-4) from the Ministry of Health and Welfare of Japan for KS.

References

1) SHOWELL, H. J.; R. J. FREER, S. H. ZIGMOND, E. SCHIFFMANN, S. ASWANIKUMAR, B. CORCORAN & E. L. BECKER: The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. J. Exp. Med. 143: 1154~1169, 1976
2) GERARD, C.; D. E. CHENOWETH & T. E. HUGLI: Response of human neutrophils to C5a: A role for the oligosaccharide moiety of human $C5a_{desArg-74}$ but not of C5a in biologic activity. J. Immunol. 127: 1978~1982, 1981
3) PRESCOTT, S. M.; G. A. ZIMMERMAN & A. R. SEEGER: Leukotriene B_4 is an incomplete agonist for the activation of human neutrophils. Biochem. Biophys. Res. Commun. 122: 535~541, 1984
4) SUZUKI, K.; H. MIYASAKA, H. OTA, Y. YAMAKAWA, M. TAGAWA, A. KURAMOTO & S. MIZUNO: Purification and partial primary sequence of a chemotactic protein for Polymorphonuclear leukocytes derived from human lung giant cell carcinoma LU65C cells. J. Exp. Med. 169: 1895~1901, 1989
5) SUZUKI, K.; M. HAZAMA, H. OTA, K. OGURO, H. UCHIYAMA & T. FUJIKURA: Phosphorylation of 64-kD protein, enzyme release, and increase in intracellular cAMP level of human polymorponuclear leukocytes induced by fMet-Leu-Phe stimulation. J. Clin. Biochem. Nutr. 8: 21~32, 1990
6) HUEY, R. & T. E. HUGLI: Characterization of a C5a receptor on polymorphonuclear leukocytes. J. Immunol. 135: 2063~2068, 1985
7) OMAN, G. M.; A. E. TRAYNOR, A. L. HARRIS & L. A. SKLAR: LTB_4-induced activation signals and responses in

neutrophils are short-lived compared to formylpeptide. J. Immunol. 138: 2626~2632, 1987

8) SUZUKI, K.; O. KOSHIO, A. ISHIDA-OKAWARA, M. SHIBATA, Y. YAMAKAWA, M. TAGAWA, H. OTA, A. KURAMOTO & S. MIZUNO: Localization of chemotactic activity and 64 kD protein phosphorylation for human polymorphonuclear leukocytes in *N*-terminus of the chemotactic protein LUCT/IL-8. Biochem. Biophys. Res. Commun. 163: 1298~1305, 1989

9) ZURIER, R. B.; S. HOFFSTEIN & G. WEISSMANN: Cytochalasin B: Effect on lysosomal enzyme release from human leukocytes. Proc. Natl. Acad. Sci. U.S.A. 70: 844~888, 1973

10) KITAGAWA, S. & F. TAKAKU: Effect of the chemotactic peptide on the subsequent superoxide releasing response in polymorphonuclear leukocytes. FEBS Lett. 128: 5~8, 1981

11) CHRISTOPHER, S. H.: The use of cytochalasins to probe the immunobiology of lymphocytes. *In* Cytochalasins-Biochemical and Cell Biological Aspects. *Ed.*, S. W. TANNENBAUM, pp. 191~215, North-Holland Biochemical Press, 1978

12) GOLDSTEIN, I.; S. HOFFSTEIN, J. GALLIN & G. WEISSMANN: Mechanisms of lysosomal enzyme release from human leukocytes: Microtubule assembly and membrane fusion induced by a component of complement. Proc. Natl. Acad. Sci. U.S.A. 70: 2916~2920, 1973

13) GOLDSTEIN, I. M.; S. LIND, S. HOFFSTEIN & S. WISSMAN: Influence of local anesthetics upon human polymorphonuclear leukocyte function *in vitro*. J. Exp. Med. 146: 483~494, 1977

14) SASAGAWA, S.; K. SUZUKI, T. SAKATANI & T. FUJIKURA: Effects of local anesthetics on O_2^- production in human polymorphonuclear leukocytes *in vitro*. Jpn. J. Pharmacol. 33: 127, 1983

15) SASAGAWA, S.; K. SUZUKI, T. SAKATANI & T. FUJIKURA: Effects of nicotine on the functions of human polymorphonuclear leukocytes *in vitro*. J. Leukocyte. Biol. 37: 493~502, 1985

16) SUZUKI, K.; T. UCHIDA, T. SAKATANI, S. SASAGAWA, S. HOSAKA & T. FUJIKURA: Measurement of active phagocytosis by polymorphonuclear leukocytes by fluorescence liberation from phagocytized microspheres. J. Leukocyte. Biol. 39: 475~488, 1986

17) SUZUKI, K.; M. YAMADA, K. AKASHI & T. FUJIKURA: Similarity of kinetics of three types of myeloperoxidase from human leukocytes and four types from HL-60 cells. Arch. Biochem. Biophys. 245: 167~173, 1986

18) SUZUKI, K. & M. YAMADA: Differences in release from differentiated HL-60 cells between myeloperoxidase and β-glucuronidase. J. Clin. Biochem. Nutr. 4: 103~109, 1988

19) SUZUKI, K.; K. ASAOKA, K. TAKAHASI & T. FUJIKURA: Differences among primates in defence against infection: Sensitivity of polymorphonuclear leukocytes to fMet-Leu-Phe. Cell Biochem. Funct. 3: 297~303, 1985

20) KOSHIO, O.; S. MIZUNO & K. SUZUKI: Formyl-Met-Leu-Phe-dependent serine kinase for a 64,000 molecular weight protein of polymorphonuclear leukocytes in a cell-lysate system. Cellular Signalling 2: 471~477, 1990

21) HAUSCHILDT, S.; U. STEFFENS, L. WAGNER-ROOS & W. G. BESSLER: Role of proteinkinase C and phosphatidylinositol metabolism in lipopeptide-induced leukocyte activation as signal transducing mechanism. Mol. Immunol. 25: 1081~1086, 1988

22) SEIFERT, R.; G. SCHULTZ, M. R. FREUND, J. METZGER, K. H. WIESMÜLLER, G. JUNG, W. G. Bessler & S. HAUSCHILDT: Activation of superoxide formation and lysozyme release in human neutrophils by the synthetic lipopeptide Pam_3 Cys-Ser-$(Lys)_4$. Biochem. J. 267: 795~802, 1990

23) SHIBATA, M.; O. KOSHIO, T. OHOKA, S. MIZUNO & K. SUZUKI: Glucose-stimulated phosphorylation of the 64-kDa protein of human polymorphonuclear Leukocytes in a cell-free system. Immunol. Lett. 24: 159~164, 1990

24) CHARLES, O. R. & J. SUZANNE: Pathways for the incorporation of exogenous fatty acids into phosphatidylethanolamine in *Escherichia coli*. J. Biol. Chem. 260: 12720~12724, 1985

25) JOHN, P. W.; M. ELLEN, M. KIRK & U. K. MOBASHSHER: The manipulation of the fatty acid composition of glycerolipids in cyanobacteria using exogenous fatty acids. Plant Cell Physiol. 31: 493~503, 1990

26) FORMAN, H. J. & E. KIM: Inhibition by linoleic acid hydroperoxide of alveolar macrophage superoxide production: Effects upon mitochondrial and plasma membrane potentials. Arch. Biochem. Biophys. 274: 443~452, 1989

27) PALMBLAD, J.; P. LINDSTRÖM & R. LERNER: Leukotriene B_4 induced hyperadhesiveness of endothelial cells for neutrophils. Biochem. Biophys. Res. Commun. 166: 848~851, 1990

28) DAHINDEN, C. A.; R. M. CLANCY & T. E. HUGLI: Stereospecificity of leukotriene B_4 and structure-function relationships for chemotaxis of human neutrophils. Immunology 134: 1477~1482, 1984

29) GOETZL, E. J. & W. C. PICKETT: The human leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). J. Immunol. 125: 1789~1791, 1980

30) O'FLAHERTY, J. T.; R. L. WYKLE, C. J. LEES, T. SHEWMAKE, C. E. MCCALL & M. J. THOMAS: Neutrophil-degranulating action of 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid and 1-*O*-alkyl-2-*O*-acetyl-sn-glycero-3-phosphocholine. Am. J. Pathol. 105: 264~269, 1981

31) BADWEY, J. A.; J. M. ROBINSON, W. HORN, R. J. SOBERMAN, M. J. KARNOVSKY & M. L. KARNOVSKY: Synergistic stimulation of neutrophils. Possible involvement of 5-hydroxy-6,8,11,14-eicosatetraenoate in superoxide release. J.

Biol. Chem. 263: 2779~2786, 1988

32) HORIGUCHI, J.; D. SPRIGGS, K. IMAMURA, R. STONE, R. LUEBBERS & D. KUFE: Role of arachidonic acid metabolism in transcriptional induction of tumor necrosis factor gene expression by phorbol ester. Mol. Cell. Biol. 9: 252~258, 1989

33) PALMBLAD, J.; H. GYLLENHAMMER, BO. RINGERTZ, E. NILSSON & E. COTTELL: Leukotriene B_4 triggers highly characteristic and specific functional responses in neutrophils: Studies of stimulus specific mechanisms. Biochim. Biophys. Acta 970: 92~102, 1988

34) MASUDA, K.; K. SUZUKI, A. ISHIDA-OKAWARA, S. MIZUNO, K. HOTTA, S. MIYADOH, O. HARA & M. KOYAMA: Pyrrolomycin group antibiotics inhibit substance P-induced release of myeloperoxidase from human polymorphonuclear leukocytes. J. Antibiotics 44: 533~540, 1991