SNA-60-367 Components, New Peptide Enzyme Inhibitors of Aromatase: Structure of the Fatty Acid Side Chain and Amino Acid Sequence by Mass Spectrometry

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SNA-60-367 components, new peptide enzyme inhibitors of aromatase, were isolated from the culture broth of soil bacterium, Bacillus sp. SNA-60-367. These inhibitors are a family of acylated decapetides that differ from each other in terms of amino acid composition and the nature of the fatty acid side chain. The structures of the fatty acid moieties were shown to be (3-hydroxy)heptadecanoic acid and (3-hydroxy)hexadecanoic acid that possess normal-, iso- or anteiso-type alkyl groups. The amino acid sequence of the open form of the lactone ring of the acylpeptides is RCO-L-Glu-D-Orn-L(or D)-Tyr3-D-allo-Thr-L-Glu-D-X1 (Ala, Aba or Val)-L-Pro-L-Gln-D(or L)-Tyr-L-X102 (Ile or Val)-OH. The lactone ring of SNA-60-367 components is formed between Tyr3 and X102.

In the course of our screening program for new aromatase (estrogen synthase) inhibitors, we have isolated new plipastatin1-3) analogues from the broth of soil bacterium, Bacillus sp. SNA-60-367. Such compounds having aromatase inhibition activity are of potential clinical value for controlling estrogen-mediated events, such as ovulation and the growth of estrogen-dependent tumors. The isolation, characterization and biological properties of SNA-60-367 components have been described in a previous paper4). As described in that paper, over 23 peaks were detected in the HPLC profile. Finally, 17 components were isolated in pure form as white amorphous powder and used for structure determination. UV, IR, 1H and 13C NMR, amino acid analysis, HR-FAB/MS and elemental composition data suggested that SNA-60-367 components are new analogues of plipastatins that were reported to be phospholipase A2 inhibitors. In this paper, we report the structures of the fatty acid side chain and the amino acid sequences, including the results of DL-amino acid analyses of SNA-60-367 components.

Structures of Fatty Acid Side Chain

Each SNA-60-367 component was hydrolyzed with 6N hydrochloric acid and the hydrolyzate was extracted with ethyl acetate and subjected to negative mode fast atom bombardment (FAB) collision-induced dissociation (CID)/linked-scan measurement. High-energy CID of compounds that have a long hydrocarbon chain and localized charge causes charge-remote fragmentation, and
is quite useful for determining the locations of double bonds, branch points, and functional groups in a long hydrocarbon chain\(^5\). For instance, a saturated long-chain fatty acid gives a pattern of peaks that are evenly spaced by 14 amu with losses of \(\text{C}_n\text{H}_{2n+2}\) that arise from the alkyl terminus via a 1,4-elimination of \(\text{H}_2\), and the highest mass fragment ion results from the loss of \(\text{CH}_4\)\(^5\). Fig. 1 shows the CID spectra of the fatty acids derived from SNA-60-367-8, -13 and -12. No. 8 has a typical pattern of peaks evenly spaced by 14 amu but Nos. 13 and 12 clearly lack the peaks at \(\text{m/z} 255\) and \(241\), respectively, among the peaks evenly spaced by 14 amu. Thus, the normal-, iso- and anteiso-type alkyl chains were assigned to Nos. 8, 13 and 12, respectively. Also, the peaks at \(\text{m/z} 59\) and \(87\) indicated the presence of a hydroxyl group on the 3-carbon. Therefore, the structures of the fatty acids in SNA-60-367-8, -13 and -12 were found to be 3-hydroxyheptadecanoic acid, 15-methyl-3-hydroxyhexadecanoic acid and 14-methyl-hexadecanoic acid, respectively. The stereochemistry of the hydroxyl group and the anteiso-type alkyl chain was not determined. The structures of the fatty acid moieties of the other SNA-60-367 components were also determined in a similar manner.

**Amino Acid Sequence**

The FAB-CID/linked-scan spectrum of SNA-60-367-2 is shown in Fig. 3-A (precursor ion: \(\text{m/z} 1463\), \(\text{MH}^+\)). There are many fragment ions in the higher mass region, but almost no ions useful for amino acid sequencing in the mass region lower than near \(\text{m/z} 966\). This suggests that the ion of \(\text{m/z} 966\) corresponds to a cyclic structure originating from a lactone ring. The mass differences of the three \(C\)-terminal (\(Y^*\)-type) ions at \(\text{m/z} 966\), 1080 and 1209 indicated that Orn and Glu are connected to the lactone ring in this order.
The FAB/MS spectrum of SNA-60-367-2 is shown in Fig. 3-B. Two types of the fragment ion series were detected. The C-terminal (Y"-type) ion series confirmed that Orn and Glu are connected to the lactone ring. The N-terminal (C"-type) ion series that were derived from the opened form of the lactone ring generated on a FAB target, showed the amino acid sequence of RCO-Glu-Orn-Tyr-Thr-Glu(OH). Thus, it was deduced that the hydroxyl group of Tyr forms the lactone ring.

Fig. 3-C shows the FAB-CID/linked-scan spectrum using the fragment ion at m/z 966 as precursor ion, which corresponds to the lactone-ring moiety of SNA-60-367-2. Many fragment ions that were presumed to be generated from the linear peptide with Pro as the N-terminal amino acid were observed. In general, under FAB conditions, Pro tends to protonate and be easily cleaved off at the N-terminal site of Pro. Based on the assignments of N-terminal (A- and B-type) ion series, the amino acid sequence of the lactone ring was determined to be (NH₂)Pro-Gln-Tyr-Ile-O·Tyr-Thr-Glu-Ala(OH). The amino group of O·Tyr connects to the carboxyl group of Orn in the chain moiety of SNA-60-367-2. Similarly, the amino acid sequences of the other SNA-60-367 components were also determined.

DL-Amino Acid Analysis

The SNA-60-367 components were subjected to acid hydrolysis and the hydrolysates were derivatized with (+)-1-(9-fluorenyl) ethyl chloroformate to give fluorescent amino acid enantiomers. The amino acid enantiomers were separated on a reversed-phase column. D-allo-Thr(1), L-Glu(3), L-Pro(1), D-Orn(1), D-Tyr(1) and L-Tyr(1) were detected as common constituents of all the SNA-60-367 components. Of the three molecules of L-Glu, one was identified to be L-Gln based on FAB/MS and FAB-CID/linked-scan spectra. The positions of D-Tyr and L-Tyr were not specified. The variable constituents of X₁ and X₂ were D-Ala (Aba or Val) and L-Ile (Val), respectively, as shown in Fig. 4. The expression “Thr” means allo-Thr in Figs. 3 and 4.

Thus, the structures of the SNA-60-367 components were determined, as listed in Fig. 4. Of these, SNA-60-367-3, -6 and -12 were identical with plipastatins A₁, A₂, and
B_2_, respectively. The retention times of SNA-60-367-3 and -6 on HPLC were in accord with those of authentic samples in both separate and coinjection experiments. The rest of the components were all new, differing in amino acid composition and fatty acid structure from the known plipastatins.

**Experimental**

**Fermentation and Isolation**

Fermentation of the bacterial strain *Bacillus* sp. SNA-60-367 and isolation of SNA-60-367 components were undertaken as described previously^{4).}

**Mass Spectrometry**

FAB/MS and FAB-CID/linked-scan measurements were performed using a JEOL JMS-HX110 mass spectrometer. FAB was carried out using xenon as the primary beam with 6 keV energy and the ion accelerating voltage was 10 kV. High-energy CID was performed by introducing helium as collision gas until the intensity of the precursor ion decreased to 1/3 of the initial value. Glycerol was used as the FAB ionization matrix.

**D/L-Amino Acid Analysis**

Analysis of D/L-amino acid isomers was carried out by precolumn derivatization with (+)-1-(9-fluorenyl)ethyl...
chloroformate (FLEC) and reversed-phase liquid chromatography\textsuperscript{6,7} with some modifications. The SNA-60-367 components were hydrolyzed with 6N hydrochloric acid at 110°C for 8 hours and the dried samples were dissolved in 0.1 M borate buffer (pH 9.0). Ten μl of FLEC reagent (15 mM in acetone/acetonitrile, 2/1 (v/v)) was added to 10 μl of sample in a vial tube. The reaction mixture was incubated for 25 minutes at 35°C, and then terminated by adding 10 μl of 100 mM cysteic acid (0.1 M borate buffer (pH 9.0)): excess FLEC was converted to a cysteic acid adduct. After 5 minutes, 70 μl of 0.1 M sodium acetate buffer (pH 4.0) was added to the reaction mixture. Derivatized fluorescent amino acid diastereomers were separated by a reversed-phase column (DOCOSIL-B 4.6 i.d. x 200 mm; Senshu Scientific). The column was eluted with a programmed gradient of 0.1 M sodium acetate buffer (pH 4.17)/acetonitrile/tetrahydrofuran (76:12:12, by vol.) to 0.1 M sodium acetate buffer (pH 4.46)/acetonitrile/tetrahydrofuran (4:3:3, by vol.). The excitation and emission wavelengths of fluorescence detector were 265 and 315 nm, respectively.

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References


