A New Inhibitor of Synovial Phospholipase A2 from Fermentations of Penicillium Sp. 62–92

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Z. Naturforsch. 53c, 60–64 (1998); received September 29, 1997

Penidiamide, a new tripeptide containing dehydrotryptamine, glycine and anthranilic acid linked together by two amide bonds, and oxindole were isolated from submerged cultures of Penicillium sp. 62–92. Both compounds preferentially inhibited human synovial phospholipase A2, penidiamide with an IC50 of 30 μM and oxindole of 380 μM. With the exception of U937 cells (leukemia, human), no cytotoxic activities were detected against HL-60 (leukemia, human), HeLa S3 (epitheloid carcinoma, human), BHK 21 (kidney fibroblasts, hamster), and L1210-cells (leukemia, mouse). No antimicrobial activity was detected for oxindole, and only weak antibacterial activity for penidiamide. The structure of penidiamide was elucidated by spectroscopic methods.

Introduction

Phospholipases A2 (PLA2’s) catalyze the hydrolysis of the ester linkage in membrane glycerophospholipids at the sn-2 position, liberating a fatty acid (e.g. arachidonic acid) and lysophospholipid. The enzymes are found ubiquitously in nature, taking part in food digestion, membrane turnover, host defense and signal transduction (Glaser et al., 1993, 1995). The best characterized PLA2’s are the extracellular secretory enzymes (sPLA2’s) with a molecular mass of about 14 kDa, a high disulfide bond content and the requirement of Ca2+ for catalysis. They are subdivided into three main groups, I, II, and III, based on their primary structures (Dennis, 1994). In many inflammatory diseases, high levels of type II sPLA2 enzymes are detected and thought responsible for part of the inflammatory reactions. Inhibition of type II sPLA2 enzymes is therefore considered of therapeutic relevance. Several synthetic and natural products, many of these from marine sources (Potts et al., 1992), have been shown to inhibit PLA2 enzymes and to have antiinflammatory activity (Glaser, 1995). Inhibitors derived from fungi are the thielocins (Yoshida et al., 1991; Matsumoto et al., 1995), the cinatrans (Tanaka et al., 1992), folipastatin (Hamano et al., 1992), and 5-hydroxy-3-vinyl-2(5H)-furanone (Lorenzen et al., 1995). In the following we describe the fermentation, isolation, structure elucidation, and biological properties of penidiamide, a new inhibitor of human recombinant sPLA2, from Penicillium sp. 62–92.

Experimental

Enzymes

Recombinant human synovial phospholipase A2 (type II), phospholipase A2 from porcine pancreas (type I) and cytosolic phospholipase A2 from human THP-1 cells (type IV) were generous gifts of Boehringer Mannheim GmbH (Mannheim, FRG). Phospholipase A2 from Apis mellifera (type III) was purchased from Sigma Chemical Co. (St. Louis, USA).

Fungal strain

Penicillium sp. 62–92 was obtained from the culture collection of H. Anke, LB Biotechnology, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG medium (g/l): glucose (10), maltose (10), yeast extract (4), pH 5.5.

Fermentation

Fermentations were carried out in a Biostat U fermenter (B. Braun-Diessel Biotech, Melsungen)
containing 201 of YMG medium. Prior to sterilization the pH was adjusted to 5.5 with hydrochloric acid and 2 ml silicone antifoam were added to the medium. For inoculation a well grown seed culture (250 ml) was used. The culture was grown at 24 °C, stirred with 130 rpm, and aerated with 31 air per minute. During fermentation the mycelial dry weight, the pH value and the glucose concentration (hexokinase method, Boehringer Mannheim) were measured daily. The production of PLA₂-inhibitors (penidiamide and oxindole) was measured using the assay for the determination of synovial PLA₂-activity.

Isolation

After 250–270 hours the mycelia were separated from the culture broth by filtration and discarded. The inhibitors were removed from the culture fluid (18 l) by adsorption to HP-21 resin (Mitsubishi) and eluted with methanol. The crude extract obtained after concentration was applied to a column containing silica gel (0.063–0.2 mesh, Merck 60, 15 x 5 cm) and eluted with cyclohexane-ethylacetate (3:7). Further purification was achieved by chromatography on Sephadex LH 20 in methanol (71.5 x 2.5 cm) and preparative HPLC (LiChrospher 100 RP-18, 10 µm; column 250 x 25 mm, elution with water – acetonitrile, 0–100% in 8 min.; flow rate 45 ml/min. Oxindole eluted at 30% and penidamide at 40% acetonitrile. Penidiamide was purified in a final step using a preparative HPLC on Merck LiChrogel PS1, 10 µm (column 250 x 25 mm, flow rate 5 ml/min.) elution with 2-propanol. The purification of oxindol and penidiamide was monitored by the enzymatic colorimetric assay, TLC and analytical HPLC.

Spectroscopy

The EIMS spectrum (direct inlet, 70 eV) was recorded with a JEOL SX102 spectrometer, and the NMR spectra (in CD₃OD) with a Bruker ARX500 spectrometer. The chemical shifts are reported in ppm with the solvent signals (δH=3.31 and δC=49.15) as reference, and the coupling constants (J) in Hz. COSY, HMOC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the re-focusing delays were optimised for JCH=145 Hz and JCH=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). The IR spectrum was recorded with a Bruker IFS 48 spectrometer, and the UV spectrum with a Perkin Elmer λ 16 spectrometer.

Penidiamide (2) was obtained as a colourless oil. UV (methanol) λmax (ε): 303 nm (16,300) and 279 (13,900). IR (KBr): 3400, 2925, 2855, 1635, 1585, 1515, 1455, 1385, 1250, 1160, 945 and 745 cm⁻¹. 1H NMR (CD₃OD, 500 MHz), δ, mult. J (Hz): 7.74, ddd, J₆₋₅=7.8, J₆₋₇=1.1, J₅₋₆=0.7, 5-H; 7.56, dd, J₅₋₆=1.4, J₆₋₇=7.9, 7”-H; 7.42, d, J₁₋₂=14.7, 1-H; 7.35, ddd, J₅₋₆=0.7, J₆₋₇=1.1, J₇₋₈=8.0, 8-H; 7.24, s, 10-H; 7.21, ddd, J₄₋₅=7.2, J₅₋₆=7.2, J₅₋₇=1.4, 5”-H; 7.12, ddd, J₅₋₆=1.1, J₆₋₇=7.1, J₇₋₈=8.0, 7-H; 7.07, ddd, J₅₋₆=7.8, J₆₋₇=7.1, J₇₋₈=1.1, 6-H; 6.77, dd, J₄₋₅=8.2, J₄₋₆=1.0, 4”-H; 6.66, ddd, J₄₋₅=1.0, J₄₋₆=7.2, J₅₋₆=7.9, 6”-H; 6.52, d, J₁₋₂=14.7, 2-H; 4.10, s, 2’-H₂. ¹³C NMR (CD₃OD, 125 MHz), δ: 172.6 C-1’; 169.2 C-1’; 150.7 C-3’; 138.8 C-9; 133.6 C-5’; 129.4 C-7”; 126.7 C-4; 124.5 C-10; 123.0 C-7; 120.6 C-5; 120.6 C-6; 120.2 C-1; 118.4 C-4’; 117.5 C-6’; 117.0 C-2”; 113.5 C-3; 112.7 C-8; 109.8 C-2’; 43.8 C-2’. EIMS, m/z: 334.1424 (M⁺, 24%), C₁₀H₁₈O₃N₂ requires 334.1430, 158.0842 (100%), C₁₀H₁₈O₃N₂ requires 158.0844), 120.0452 (37%, C₆H₃ON requires 120.0449). CIMS (NH₃): 335 (M + H⁺, 13%), 244 (12%), 194 (100%), 162 (18%), 146 (14%), 118 (13%).

Biological tests

For the determination of type I and II phospholipase A₂ activity lecithin was used as substrate in a mixed micelle emulsion (100 mg/ml lecithin, 4 mm sodium-desoxycholate, 0.5% Triton X-100, 250 mm Tris-HCl, 8 mm CaCl₂x2H₂O, pH 8.0) and the free fatty acids liberated were measured using an enzymatic colorimetric assay (Boehringer Mannheim) according to Shimizu et al. [1980] and Scheuer [1989]. The cytotoxic type IV PLA₂-activity was measured with a scintillation proximity assay purchased from Amersham International (Buckinghamshire, England). The antimicrobial activities were tested in a serial dilution assay as described previously (Anke et al., 1989). The cytotoxicity against L1210 (ATCC CCL 219), HL-60 (ATCC CCL 240), BHK 21 (ATCC CCL 10), U 937 (ATCC CRL 1593), HeLa S3 (ATCC CCL 22) was measured as described by Erkel et al. [1991].

Results and Discussion

Fig. 1 shows a typical fermentation of the Penicillium sp. 62–92. The inhibition of the synovial PLA₂ as determined by the enzymatic colorimetric
The molecular ion of oxindole (1) appeared at m/z 133, and high resolution measurements showed that this corresponds to the elemental composition C_8H_7ON. 2D NMR experiments and comparison with published 1D NMR data (Pouchert and Behnke, 1993) confirmed the suggested structure. Oxindole (1) has previously been isolated from Chromobacterium violaceum (Haun et al., 1992) and Narcissus geranium (van Dort et al., 1993), but to our knowledge this is the first report of its isolation from a fungus. The second compound, which we have named penidiamide (2), is a new natural product. The EI mass spectrum suggested that its molecular weight is 334, which was confirmed by CIMS using NH_3 as ionizing gas, and high resolution EIMS together with ^13C NMR established the elemental composition to C_{19}H_{18}O_2N_4. The unsaturation index is thereby 13, and as NMR data indicate the presence of two 1,2-disubstituted benzene rings, two additional double bonds and two carbonyl functions, the molecule should contain one additional ring. The presence of an indole moiety in the form of a dehydrotryptamine residue is supported by both the 1D and 2D NMR data, and pertinent NOESY as well as HMBC correlations are shown in Fig. 2. 2-H gives HMBC correlations to C-3, C-4 and C-10, 5-H to C-3, and 10-H (which is a singlet) to C-3, C-4 and C-9. The base peak in the EI mass spectrum appears at m/z 158, corresponding to the expected fragment dehydrotryptamine with the composition C_{10}H_{10}N_2. The configuration of the C-1/C-2 double bond is E, as shown by the coupling constant between 1-H and 2-H (14.7 Hz). 1-H gives HMBC correlations to C-3 and, over the nitrogen, to C-1'. The signal for 2'-H_2 appears as a singlet.
Fig. 2. Pertinent NOESY (top) and HMBC (bottom) correlations observed with penidiamide (2) in CD$_3$OD.

at 4.10 ppm integrating for two protons, 2′-H$_2$ give HMBC correlations to both carbonyl carbons C-1′ and C-1″, the chemical shift for C-2′ is 43.8 ppm, which all is in agreement with the proposed glycine residue. A HMBC correlation to C-1″ is also observed from 7″-H, and that the remaining part of the molecule is anthranilic acid was suggested by the second important MS fragment (at m/z 120) which exact mass corresponds to the composition C$_7$H$_6$ON. The NOESY correlations observed are all in agreement with the suggested structure, which consists of dehydrotryptamine, glycine and anthranilic acid linked together by two amide bonds with the glycine in the center.

Table I summarizes the inhibitory effects (IC$_{50}$) of 1 and 2 on different PLA$_2$-enzymes using the enzymatic colorimetric assay. 2 preferentially inhibited the synovial PLA$_2$ (type II), while the enzyme from porcine pancreas was less affected. The PLA$_2$ of bee venom and the human cytosolic enzyme were not inhibited. For comparison, manoa­lide (Potts et al., 1992) inhibited the synovial PLA$_2$ with an IC$_{50}$ of 0.7 μM in the same assay. For 5-hydroxy-3-vinyl-2(5H)-furanone an IC$_{50}$ of 0.3 μM for human synovial and 32 μM for human pancreatic PLA$_2$ had been reported (Lorenzen et al., 1995). To determine the cytotoxic activities of the two isolated compounds, HL-60-, L1210-,U937-, BHK 21-, HeLa S3-cells were assayed as described previously (Erkel et al., 1991). Oxindol (1) and penidiamide (2) showed weak activity towards only towards U937-cells (Table II). In the serial dilution assay oxindole (1) showed no antibiotic activity against the following tested strains: Acinetobacter calcoaceticus, Enterobacter dissolvens, Escherichia coli K12, Salmonella typhimurium TA 98; Mycobacterium phlei, Arthrobacter citreus, Bacillus brevis, Bacillus subtilis, Corynebacterium in​sidiosum, Streptomyces spec., Micrococcus luteus, Nadjosina fulvescens, Nematospora coryli, Rhodotorula glutinis var. dairenensis, Saccharomyces cerevisea S288c, Saccharomyces cerevisea is1; Fusi​arum oxysporum, Mucor miehei, Paecilomyces variotii, Penicillium notatum, Ustilago nuda. Penidiamide (2) inhibited the growth of B. subtilis.

Table II. Cytotoxicity (LD$_{50}$) of oxindole (1) and penidiamide (2) towards various cell-lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HL60</th>
<th>L1210</th>
<th>U937</th>
<th>BHK</th>
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<tr>
<td>1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>80</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>40</td>
<td>&gt;100</td>
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</table>

Table I. Inhibition of different PLA$_2$ enzymes by oxindole (1) and penidiamide (2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type I-PLA$_2$</th>
<th>Type II-PLA$_2$</th>
<th>Type III-PLA$_2$</th>
<th>Type IV-PLA$_2$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>porcine pancreas</td>
<td>human synovial fluid</td>
<td>bee venom</td>
<td>human cytosolic</td>
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<tr>
<td>1</td>
<td>&gt;100</td>
<td>51 (383)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>2</td>
<td>39 (117)</td>
<td>10 (30)</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</tbody>
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edged. Boehringer Mannheim is thanked for the generous gifts of human recombinant synovial PLA2 and Dr. W. Scheuer, Boehringer Mannheim, for helpful discussions.

**Acknowledgments**

Financial support from BM BF and the Swedish Science Research Council is gratefully acknowledged. Boehringer Mannheim is thanked for the generous gifts of human recombinant synovial PLA2 and Dr. W. Scheuer, Boehringer Mannheim, for helpful discussions.