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

Ludwig Wittera, Timm Ankea and Olov Sternerb

a Lehrbereich Biotechnologie der Universität Kaiserslautern, Paul Ehrlich-Straße 23,
D-67663 Kaiserslautern, Germany
b Division of Organic Chemistry 2, Lund University, P. O. B. 124, S-221 00 Lund, Sweden

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

Penidiamide, Tripeptide, Oxindole, sPLA2, Inhibitor, Penicillium sp. 62–92

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 phospholi-
pase A2, penidiamide with an IC50 of 30 μM and oxindole of 380 μM. With the exception of the U
937 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 hy-
drolysis of the ester linkage in membrane glycerophospholipids at the sn-2 position, liberating a
fatty acid (e.g. arachidonic acid) and lysophospho-
lipid. The enzymes are found ubiquitously in na-
ture, 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 inflam-
matory diseases, high levels of type II sPLA2 en-
zymes 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 natu-
ral products, many of these from marine sources
(Potts et al., 1992), have been shown to inhibit
PLA2 enzymes and to have antiinflammatory ac-
tivity (Glaser, 1995). Inhibitors derived from fungi
are the thielocins (Yoshida et al., 1991; Matsumoto
et al., 1995), the cinatrin (Tanaka et al., 1992), foli-
pastatin (Hamano et al., 1992), and 5-hydroxy-3-
viny-2(5H)-furanone (Lorenzen et al., 1995). In
the following we describe the fermentation, iso-
lution, structure elucidation, and biological prop­
erties of penidiamide, a new inhibitor of human re-
combinant 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 hu-
man 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 cul-
ture collection of H. Anke, LB Biotechnology,
University of Kaiserslautern. For maintenance on
agar slants the fungus was grown on YMG me-
dium (g/l): glucose (10), maltose (10), yeast extract
(4), pH 5.5.

Fermentation

Fermentations were carried out in a Biostat U
fermenter (B. Braun-Diessl Biotech, Melsungen)

Reprint requests to Prof. Dr. T. Anke,
Telefax: +631–2052999;
or to Prof. Dr. O. Sterner, Telefax: +462228209.

0939–5075/98/0100–0060 $ 06.00 © 1998 Verlag der Zeitschrift für Naturforschung. All rights reserved.
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 monitored by the enzymatic colorimetric assay purchased from Amersham International (ATCC CRL 1593), HeLa S3 (ATCC CCL 240), BH K 21 (ATCC CCL 10), U 937 (ATCC CRL 1593), HeLa S3 (ATCC CCL 22) 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, 1100, 945 and 745 cm⁻¹. 1H NMR (CD₂OD, 500 MHz), δ, mult. J (Hz): 7.74, ddd, J = 7.8, J = 1.1, J = 0.7, 5-H; 7.56, dd, J = 7.9, 7-H; 7.42, d, J = 147, 1-H; 7.35, ddd, J = 0.7, J = 1.1, J = 8-H; 7.24, s, 10-H; 7.21, ddd, J = 7.2, J = 7.2, J = 1.4, 5-H; 7.12, ddd, J = 7.1, J = 7-H; 7.07, ddd, J = 7.8, J = 7.1, J = 1.1, 6-H; 6.77, dd, J = 8.2, J = 1.0, 4''-H; 6.66, ddd, J = 7.2, J = 7.9, 6''-H; 6.52, d, J = 14.7, 2-H; 4.10, s, 2''-H. 13C NMR (CD₂OD, 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; 119.8 C-2; 43.8 C-2''. EIMS, m/z: 334.1424 (M⁺, 24%), C₁₉H₁ₙO₂N₂ requires 334.1430), 158.0842 (100%, C₁₀H₁₀N₂ requires 158.0844), 120. 0452 (37%, C₇H₆ON requires 120.0449), CIMS (NH₃): 335 (M + H⁺, 13%), 244 (12%), 194 (100%), 162 (18%), 146 (11%), 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 cytosolic 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
assay started early and increased significantly after 60 h. The maximum was reached after 210 h. The fermentation was terminated after 260 h, after depletion of glucose and maltose in the culture broth.

Oxindole (1) and penidiamide (2) were isolated as described in the experimental section and characterized by spectroscopy.

![Structure of Oxindole (1)](image1)

The molecular ion of oxindole (1) appeared at $m/z$ 133, and high resolution measurements showed that this corresponds to the elemental composition C₈H₇ON. 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 NH₃ as ionizing gas, and high resolution EIMS together with $^{13}$C NMR established the elemental composition to C₁₉H₁₈O₂N₄. 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 NOEY 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 C₁₀H₁₀N₂. 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'-H₂ appears as a singlet.

![Structure of Penidiamide (2)](image2)
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, manoilide (Potts et al., 1992) inhibited the synovial PLA$_2$ with an IC$_{50}$ of 0.7 $\mu$m in the same assay. For 5-hydroxy-3-vinyl-2(5H)-furanone an IC$_{50}$ of 0.3 $\mu$m for human synovial and 32 $\mu$m 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 oxindol (1) showed no antibiotic activity against the following tested strains: Acinetobacter calcoaceticus, Entrobacter dissolvens, Escherichia coli K12, Salmonella typhimurium TA 98; Mycobacterium phlei, Arthrobacter citreus, Bacillus brevis, Bacillus subtilis, Corynebacterium inisidiosum, Streptomyces spec., Micrococcus luteus, Nodosia fulvescens, Nematospora coryli, Rhodotorula glutinis var. dairenensis, Saccharomyces cerevisiae S288c, Saccharomyces cerevisiae is1; Fusarium oxysporum, Mucor miehei, Paecilomyces variotii, Penicillium notatum, Ustilago nuda. Penidiamide (2) inhibited the growth of B. subtilis

Table II. Cytotoxicity (LD$_{50}$) of oxindol (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>
<th>HeLa S3</th>
</tr>
</thead>
<tbody>
<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>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table I. Inhibition of different PLA$_2$ enzymes by oxindol (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>
</thead>
<tbody>
<tr>
<td></td>
<td>porcine pancreas</td>
<td>human synovial fluid</td>
<td>bee venom</td>
<td>human cytosolic</td>
</tr>
<tr>
<td>1</td>
<td>&gt;100</td>
<td>51 (383)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2</td>
<td>39 (117)</td>
<td>10 (30)</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Penidiamide, a New Inhibitor of sPLA2 (MIC 80 μg/ml), C. insidiosum (MIC 50 μg/ml) and M. luteus (MIC 80 μg/ml) while the other bacteria and fungi were not affected.

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.


