A New Inhibitor of Synovial Phospholipase A_2 from Fermentations of *Penicillium* Sp. 62–92

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Penidiamide, a new tripetide 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 A_2, penidiamide with an IC_{50} of 30 μM and oxindole of 380 μM. With the exception of 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 A_2 (PLA_2'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 PLA_2's are the extracellular secretory enzymes ($sPLA_2$'s) with a molecular mass of about 14 kDa, a high disulfide bond content and the requirement of Ca^{2+} 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 $sPLA_2$ enzymes are detected and thought responsible for part of the inflammatory reactions. Inhibition of type II $sPLA_2$ 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 PLA_2 enzymes and to have antiinflammatory activity (Glaser, 1995). Inhibitors derived from fungi are the thielocins (Yoshida *et al.*, 1991; Matsumoto *et al.*, 1995), the cinatrins (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 $sPLA_2$, from *Penicillium* sp. 62–92.

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Experimental

Enzymes

Recombinant human synovial phospholipase A_2 (type II), phospholipase A_2 from porcine pancreas (type I) and cytosolic phospholipase A_2 from human THP-1 cells (type IV) were generous gifts of Boehringer Mannheim GmbH (Mannheim, FRG). Phospholipase A_2 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)

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containing 20 l 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 3 l 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 PLA_2-inhibitors (penidiamide and oxindole) was measured using the assay for the determination of synovial PLA_2-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 penidiamide 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 CD_3OD) with a Bruker ARX500 spectrometer. The chemical shifts are reported in ppm with the solvent signals (δ_H=3.31 and δ_C=49.15) as reference, and the coupling constants (*J*) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$=145 Hz and $^nJ_{CH}$=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

Penidiamide (**2**) was obtained as a colourless oil. UV (methanol) λ_{max} (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 (CD_3OD, 500 MHz), δ, mult. *J* (Hz): 7.74, ddd, J_{5-6}=7.8, J_{5-7}=1.1, J_{5-8}=0.7, 5-H; 7.56, dd, $J_{5''-7''}$=1.4, $J_{6''-7''}$=7.9, 7''-H; 7.42, d, J_{1-2}= 14.7, 1-H; 7.35, ddd, J_{5-8}=0.7, J_{6-8}=1.1, J_{7-8}=8.0, 8-H; 7.24, s, 10-H; 7.21, ddd, $J_{4''-5''}$=7.2, $J_{5''-6''}$=7.2, $J_{5''-7''}$=1.4, 5''-H; 7.12, ddd, J_{5-7}=1.1, J_{6-7}=7.1, J_{7-8}= 8.0, 7-H; 7.07, ddd, J_{5-6}=7.8, J_{6-7}=7.1, J_{6-8}=1.1, 6-H; 6.77, dd, $J_{4''-5''}$=8.2, $J_{4''-6''}$=1.0, 4''-H; 6.66, ddd, $J_{4''-6''}$=1.0, $J_{5''-6''}$=7.2, $J_{6''-7''}$=7.9, 6''-H; 6.52, d, J_{1-2}=14.7, 2-H, 4.10, s, 2'-H_2. ^{13}C NMR (CD_3OD, 125 MHz), d: 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%, $C_{19}H_{18}O_2N_4$ requires 334.1430), 158.0842 (100%, $C_{10}H_{10}N_2$ requires 158.0844), 120. 0452 (37%, C_7H_6ON requires 120.0449). CIMS (NH_3): 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 A_2 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 $CaCl_2 x 2H_2O$, 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 PLA_2-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 PLA_2 as determined by the enzymatic colorimetric

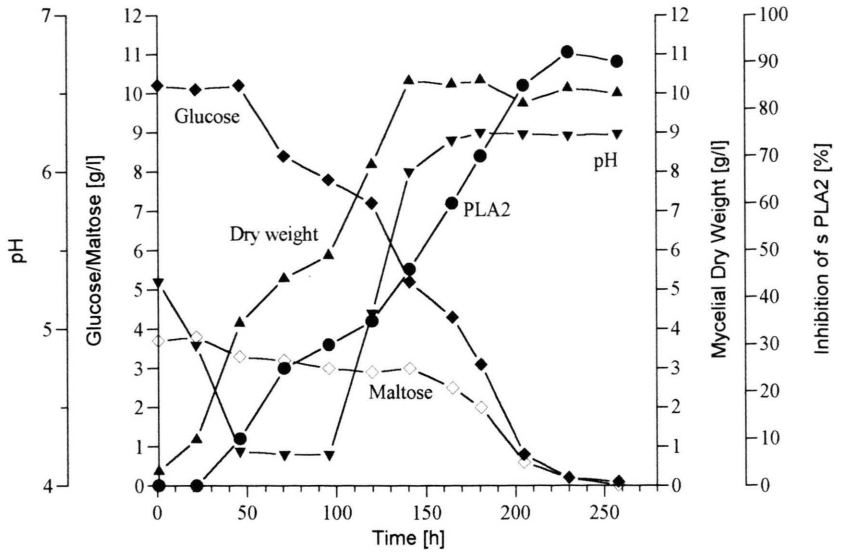
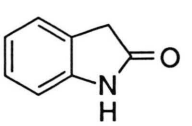


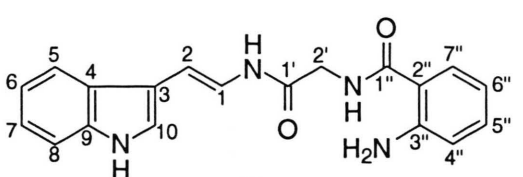
Fig. 1. Time course of a fermentation of *Penicillium* sp. 62–92 in 20 l of YMG medium. To determine the presence of $sPLA_2$ inhibitors, 100 ml of culture filtrate were applied to a column (30 x 5 cm) with HP21 resin and oxindole and ascodiamide eluted with 200 ml of methanol. The crude extract was dissolved in methanol (10 mg/ml) and 10 µl assayed for PLA_2 inhibition as described in the experimental section.

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.



1



2

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 ^{13}C 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

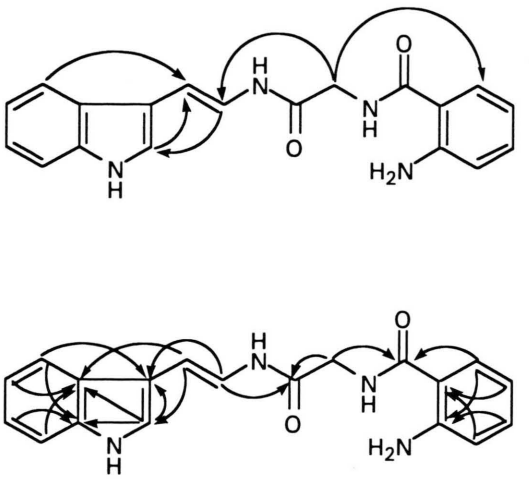


Fig. 2. Pertinent NOESY (top) and HMBC (bottom) correlations observed with penidiamide (**2**) in CD_3OD.

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_7H_6ON. 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 insidiosum, Streptomyces spec., Micrococcus luteus, Nadsonia 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 oxindole (**1**) and penidiamide (**2**) towards various cell-lines.

	LD_{50} [μg/ml]				
Compound	HL60	L1210	U937	BHK	HeLa S3
1	>100	>100	80	>100	>100
2	>100	>100	40	>100	>100

Table I. Inhibition of different PLA_2 enzymes by oxindole (**1**) and penidiamide (**2**).

	IC_{50} [μg/ml]; (μM]			
Compound	**Type I-PLA$_2$** porcine pancreas	**Type II-PLA$_2$** human synovial fluid	**Type III-PLA$_2$** bee venom	**Type IV-PLA$_2$** human cytosolic
1	>100	51 (383)	>100	>100
2	39 (117)	10 (30)	>100	>100

(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.

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