Paeciloquinones A, B, C, D, E and F: New Potent Inhibitors of Protein Tyrosine Kinases Produced by *Paecilomyces carneus*

I. Taxonomy, Fermentation, Isolation and Biological Activity

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Paeciloquinones A to F as well as versiconol have been isolated as inhibitors of protein tyrosine kinase from the culture broth of the fungus *Paecilomyces carneus* P-177. The novel anthraquinones inhibit epidermal growth factor receptor protein tyrosine kinase in the micromolar range. Two compounds, paeciloquinones A and C, are potent and selective inhibitors of the v-abl protein tyrosine kinase with an IC_{50} of 0.4 μM. Dependent on the fermentation conditions, partially different sets of paeciloquinones may be produced. An HPLC method allows separation of all major active components.

Protein tyrosine kinases (PTK's) are a family of enzymes catalyzing phosphorylation of tyrosine residues of protein substrates. These enzymes play a key role in the response of cells to various signals involved in cell proliferation and transformation. One of the best understood and most intensely investigated PTK's is the epidermal growth factor receptor (EGF-R) protein tyrosine kinase[1] which belongs to a class of ligand-activated, membrane-spanning PTK's. The binding of EGF to the extracellular domain of the receptor activates the intracellular EGF-R tyrosine kinase domain leading to tyrosine phosphorylation of target proteins and finally to cell division. The protooncogene c-erbB-2 codes for the closely related $erbB_2$ PTK which is frequently overexpressed in breast cancer[2,3]. Thus the deregulation of PTK activity within normal cells as observed in oncogenic transformation, is thought to be a primary cause of some forms of cancer[4]. Potent and selective inhibitors of individual EGF-R PTK's are currently in preclinical evaluation as antitumor agents[5]. We turned our attention to the v-abl PTK†††, for which, unlike for the EGF-R PTK, no highly potent and specific inhibitors are known to date[6,7].

The abl PTK belongs to a group of cytoplasmic PTK's which can not be activated by direct interaction with an extracellular signal. Deregulation of the c-abl PTK††† due to gene translocation is believed to be the cause of two forms of cancer, chronic myelogenous leukemia and acute lymphoblastic leukemia[8]. Inhibition of the enzymatic activity of c-abl PTK or as a model system of v-abl PKT which is largely homologous to the former enzyme[9] may therefore be of therapeutic value and offer a new mechanism-based approach for treating these types of leukemia. In addition to a high potency, selectivity with respect to other PTK's is a desirable property of a clinically useful inhibitor of PTK's††††.

In our search for naturally occurring inhibitors of the v-abl PTK among secondary metabolites of microorganisms, new inhibitors from the fungus *Paecilomyces carneus* P-177 were identified. In the present communication, the taxonomy of this strain and the production, isolation, and enzyme-inhibitory properties of the new compounds are described. The structure elucidation and the physico-chemical data of these novel anthraquinones will be described in the subsequent paper[11].

Dedicated to Prof. Dr. HANS ZÄHNER on the occasion of his 65th birthday.

††† v-abl PTK: viral Abelson PKT, originally obtained from the Abelson murine leucemia virus c-abl PTK: cellular Abelson PTK.

†††† In this context it is noteworthy that many investigations analyzing the role of protein kinase C using the kinase inhibitor staurosporine are of rather limited value, since a broad spectrum of different kinases present in a cell are inhibited by this microbial metabolite[10].

Materials and Methods

Microorganism: Isolation and Preservation

2-(*N*-Morpholino)-ethanesulphonic acid (MES) buffer, 2-[(2-amino-2-oxoethyl)-amino]ethanesulphonic acid (ACES), 3-(*N*-morpholino)-propanesulphonic acid (MOPS), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), and *N*-tris-(hydroxymethyl)-methylglycine (Tricine) were obtained from Serva Feinbiochemica, Heidelberg, Germany.

The fungal organism was isolated from a soil sample collected in a jungle region of Bolivia. The strain was taxonomically determined by the DSM (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) as *Paecilomyces carneus* and has been deposited there under the accession number DSM 7243. The strain was grown on agar with the following composition: Lactose 1.5%, cornsteep liquor 0.5%, peptone 0.5%, NaCl 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.06%, $FeCl_3 \cdot 6H_2O$ 0.0005%, $CuSO_4 \cdot 5H_2O$ 0.0002%, agar 3.0%. The pH was adjusted to 4.8 with 5 N H_2SO_4 or 5 N NaOH. The fungus was incubated at 28°C for ten days until complete sporulation had occurred. Working stocks are stored at 4°C, whereas long-term storage is at −80°C and −169°C, respectively.

Fermentation

DSM 7243 was inoculated from a slant culture into a 500-ml Erlenmeyer flask with one baffle containing 100 ml of medium A with the following composition: Glucose 2%, Pharmamedia (Southern Cotton Oil Co., Memphis, USA) 1.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. The pH was adjusted to 7.0 as above. The preculture was shaken under aerobic conditions for 72 hours at 28°C and 250 rpm. 5% of the seed culture was transferred into 500 ml of the above medium in a 2,000-ml Erlenmeyer flask with four baffles and incubated under the same conditions. 5% of the second seed culture was transferred into a 50 liter fermenter containing 30 liters of the following medium B driven by a blade stirrer: Saccharose 2.5%, mannitol 0.85%, meat extract (Difco) 2.5%, ammonia nitrate 0.04%. Prior to sterilization, the pH was adjusted to 7.0 with 98% H_2SO_4 and 5 N NaOH. The fermentation was carried out at 33°C for 117 hours with an aeration rate of 30 liters/minute and an agitation rate of 700 rpm.

To cultivate the fungal strain on shake flask scale, the preculture was performed as described above. After 72 hours an inoculum of 5% of the culture broth was transferred to 500-ml Erlenmeyer flasks filled with 100 ml of medium B or medium C (sucrose 2.5%, mannitol 0.85%, peanut meal 2.0%, meat extract (Difco) 2.5%, ammonia nitrate 0.04%, pH adjusted to 7.0 with 5 N H_2SO_4 or 5 N NaOH). The flasks were incubated at 30°C on a rotary shaker at 250 rpm for 96 hours.

Optimization of the Fermentation Temperature

This investigation was carried out using a temperature gradient incubator TN-3 (Advantec Toyo Co., Ltd., Tokyo, Japan). The shake vessels were filled with 5 ml of medium B and inoculated with 10% of the preculture prepared as described above. A continuous temperature gradient was established from 20°C to 40°C. After 120 hours the paeciloquinone A titers were determined by HPLC.

Isolation

General remarks: Melting points are uncorrected. Large scale liquid chromatography was done using a medium pressure system equipped with a Büchi pump B-681, a Büchi glass column B-685 (volume 920 ml) filled with LiChroprep Si60 (15~25 μm), a Kontron Uvikon 725 detector (1 mm pathlength) and a Büchi B-684 fraction collector. Preparative reversed phase chromatography was performed using a Labomat VS200 gradient mixer, a Besta-HD-200 pump, a conically shaped Büchi column (300 ml) filled with LiChroprep RP-18 (15~25 μm), a Shimadzu UV-120-02 UV detector (1 mm path) and a Büchi B-684 fraction collector. For semipreparative HPLC a Beckman 100B pump and a Kratos SF769 UV detector were used. A Nucleosil 100-5 silica gel column (100 Å, 5 μm, 16×250 mm) was employed for semipreparative separations.

Isolation of Paeciloquinones A, B, C, D and of Versiconol

Extraction and isolation of paeciloquinones are shown in Scheme 1. The solid obtained from fermentation in medium B (7.95 g) was dissolved in ethyl acetate (70 ml) and MeOH (20 ml) and adsorbed on silica gel (16 g). After removal of the polar solvents *in vacuo* the material was separated into five fractions by silica gel column chromatography (CH_2Cl_2 - MeOH (98 : 2, 700 ml), (95 : 5, 4 liters), (90 : 10, 2 liters), (85 : 15, 2 liters), (75 : 25, 1 liter), all solvents water saturated) which were combined according to UV trace (290 nm) and HPLC (see below):

Fraction 1 (3.1~4.5 liters; 1.22 g of a red solid) was crystallized twice from MeOH to give paeciloquinone A (85 mg) as yellow crystals with MP >350°C (sublimes).

Fraction 2 (4.5~5.7 liters; 0.32 g) a part of which (77 mg) was rechromatographed on reversed phase (solvent A: water - TFA (100 : 0.1); solvent B: acetonitrile - water - TFA (80 : 20 : 0.07); gradient from 40% B to 75% B in 35 minutes; 30 ml/minute; sample dissolved in DMSO (200 μl) and MeOH (4 ml)) to give paeciloquinone C (20~22.5 minutes; 5.4 mg) as an orange solid with MP >300°C and paeciloquinone A (27~32.4 minutes; 10.2 mg) as yellow crystals after recrystallization from MeOH.

Fraction 3 (6.5~6.9 liters; 435 mg) was crystallized from a mixture of MeOH and ethyl acetate to give 140 mg of paeciloquinone B as orange crystals (MP 240~260°C).

Fraction 4 (6.9~7.9 liters; 1.2 g) contained paecilo-

quinone B (700 mg) and versiconol (149 mg). Pure versiconol was obtained from similar material by semi-preparative silica gel HPLC chromatography (CH_2Cl_2-EtOH (92 : 8), solvent saturated with water; 8 ml/minutes; detected at 290 nm; Rt 12 minutes; 16 mg in 250 μl ethyl acetate injected) to give orange crystals after crystallization from MeOH (MP 245~60°C).

Fraction 5 (8.3~9.5 liters; 507 mg): A part of it (288 mg) was crystallized from MeOH (1.5 ml) to give paeciloquinone D (74 mg) as orange crystals with MP >300°C.

Isolation of Paeciloquinones B, E and F

The shake flasks containing medium C were extracted are shown in Scheme 1. The solid (7.61 g) was dissolved in ethyl acetate (100 ml) and MeOH (30 ml) and adsorbed on silica gel (10 g). After removal of the polar solvents *in vacuo* the material was separated into two fractions by column chromatography on silica gel (CH_2Cl_2-MeOH (99 : 1, 500 ml), (98 : 2, 2 liters), (95 : 5, 2 liters), (90 : 10, 4 liters), (85 : 15, 2 liters), all solvents water saturated) which were combined according to UV trace (290 nm) and HPLC results (see below):

Fraction 1 (1.1~2.4 liters; 260 mg) was crystallized from MeOH to give 114 mg of paeciloquinone E as orange crystals (MP 212~214°C).

Fraction 2 (4.7~10.5 liters; 3.48 g) a part of which (1.76 g) was rechromatographed on reversed phase (solvent A: water - TFA (100 : 0.1); solvent B: acetonitrile - water - TFA (80 : 20 : 0.07); gradient from 40%B to 75%B in 35 minutes; 30 ml/minute; sample dissolved in MeOH (2.5 ml) and water (5 ml); 7 runs) to give paeciloquinone B (17.7~24 minutes; 875 mg) as an orange solid after removal of the acetonitrile *in vacuo* and crystallization from the remaining water and paeciloquinone F (30.3~33.6 minutes; 164 mg; yellow crystals, MP 201~5°C from CH_2Cl_2/EtOH) after extraction with CH_2Cl_2 and removal of the organic solvent *in vacuo*.

Preparation of Paeciloquinone B Methyl Ester (8)

A solution of paeciloquinone B (76 mg) in methanol/BF_3 reagent (1.3 M; 4 ml) was heated for 2 hours at 65 °C. The reaction mixture was poured on ice, almost neutralized with a saturated Na_2CO_3 solution (pH 6) and extracted with ethyl acetate (50 ml). The organic phase was filtered to remove water and the solvent removed *in vacuo*. The almost pure product (90%) was dissolved in 2-PrOH (100 μl), EtOAc (400 μl), CH_2Cl_2 (500 μl) and chromatographed on the semipreparative silica gel HPLC column (CH_2Cl_2-EtOH (95 : 5) saturated with water; 8 ml/minutes; 290 nm; 10 runs; Rt 14.3 minutes) to give paeciloquinone B methyl ester (64 mg; 81% yield; MP >210°C).

HPLC Analysis

The following equipment was used: low pressure gradient mixing system (Merck-Hitachi gradient pump L 6200 A); autosampler (AS-4000); UV detector (L-4200); data processing with integration software NELSON model 2600 (Nelson/Perkin-Elmer, USA) with data interface NELSON 900 and an IBM-AT03 personal computer.

Results and Discussion

The investigations of incubation time and inoculum volume showed that a cultivation time of 72 hours and an inoculum volume of 5% yielded the highest titers of paeciloquinone A. To determine the most suitable pH for productivity, several pH values were adjusted with 50 mM Good-buffers[12]. MES-buffer was used for the pH-adjustment at 5.5 and 6.0, ACES for pH 6.5, MOPS for pH 7.0, TES for pH 7.5, and Tricine for the maintenance of the pH at 8.0. The optimal value for production was found to be equal to or higher than pH 8. This pH range was reached during normal cultivation of the fungus. Therefore a pH regulation during fermentation was not required.

The production of the paeciloquinones in fermenters in relation to the temperature and aeration conditions was intensively investigated. Both parameters were found to be critical for optimization of the production on bioreactor scale. A higher impeller speed to improve the insufficient oxygen supply destroyed the fungal hyphae and had a negative impact on the production of the paeciloquinones. The reduction of cell mass by lower concentrations of C- or N-sources in order to decrease the oxygen requirement similarly resulted in lower amounts of the paeciloquinones.

When the fermentation was carried out in shake flasks in medium B at 30°C, the titer of paeciloquinone A reached about 50 mg/liter, whereas only traces of paeciloquinones could be detected on bioreactor scale at the same temperature, due to an insufficient oxygen supply.

The physiological requirement for oxygen can be modified by running the fermentation at different temperatures. Using the temperature gradient incubator we could determine the maximum of cell mass production to be at 32°C. At a temperature of 33°C, the mycelial mass was slightly lower and a further increase of the paeciloquinones titers were observed (Fig. 1). In a 50 liter-bioreactor, the paeciloquinones could only be produced in appreciable amounts when the temperature was increased to 33°C. At this temperature the oxygen input was satisfactory and the partial pressure was kept under control (Fig. 2.).

Some of the components turned out to be rather

Fig. 1. Temperature dependence of the paeciloquinones A, B, F and versiconol production in a temperature gradient incubator using medium B.

● Paeciloquinone A, ⊟ paeciloquinone B, ▲ paeciloquinone F, ⊖ versiconol.

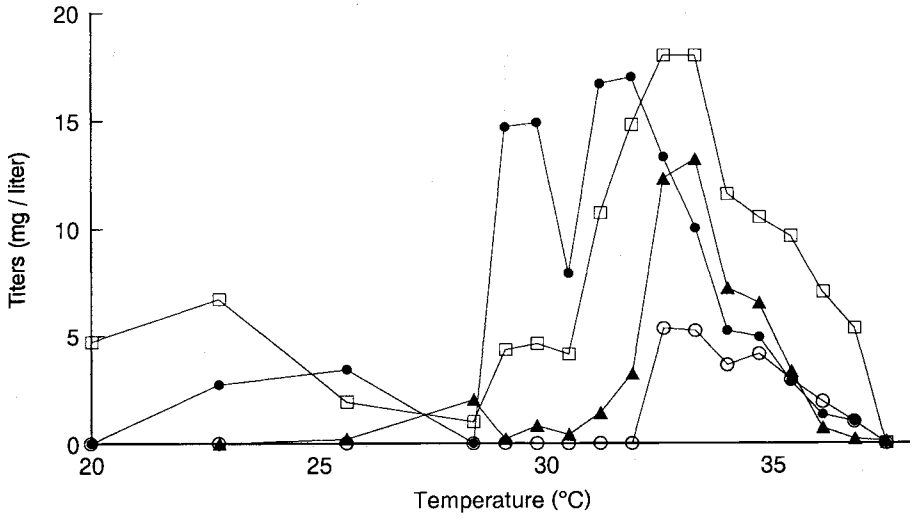
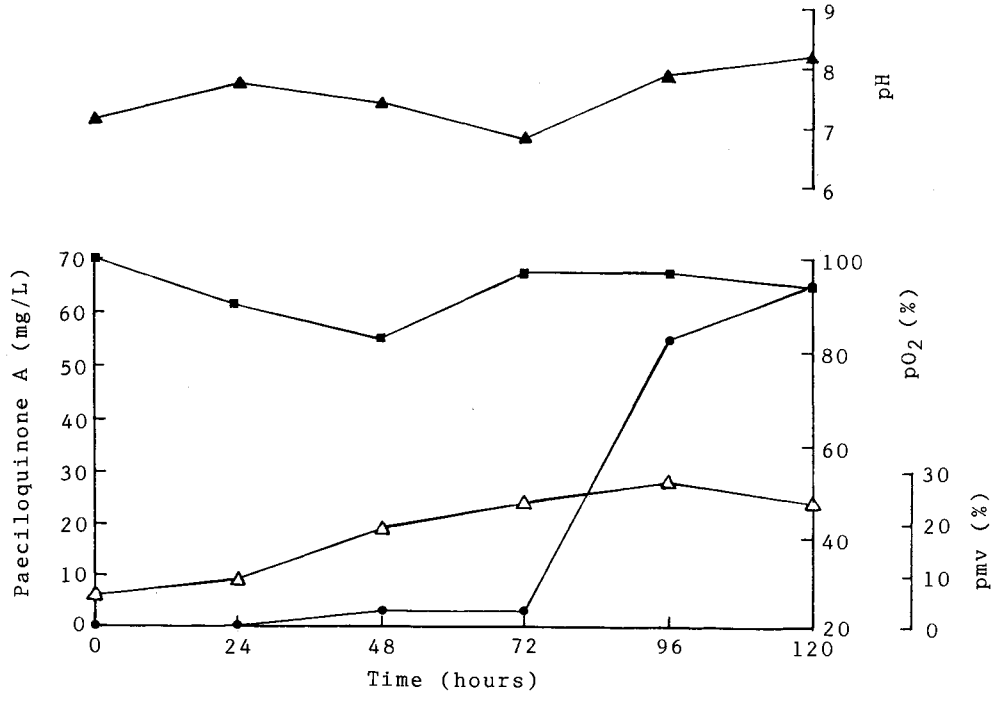


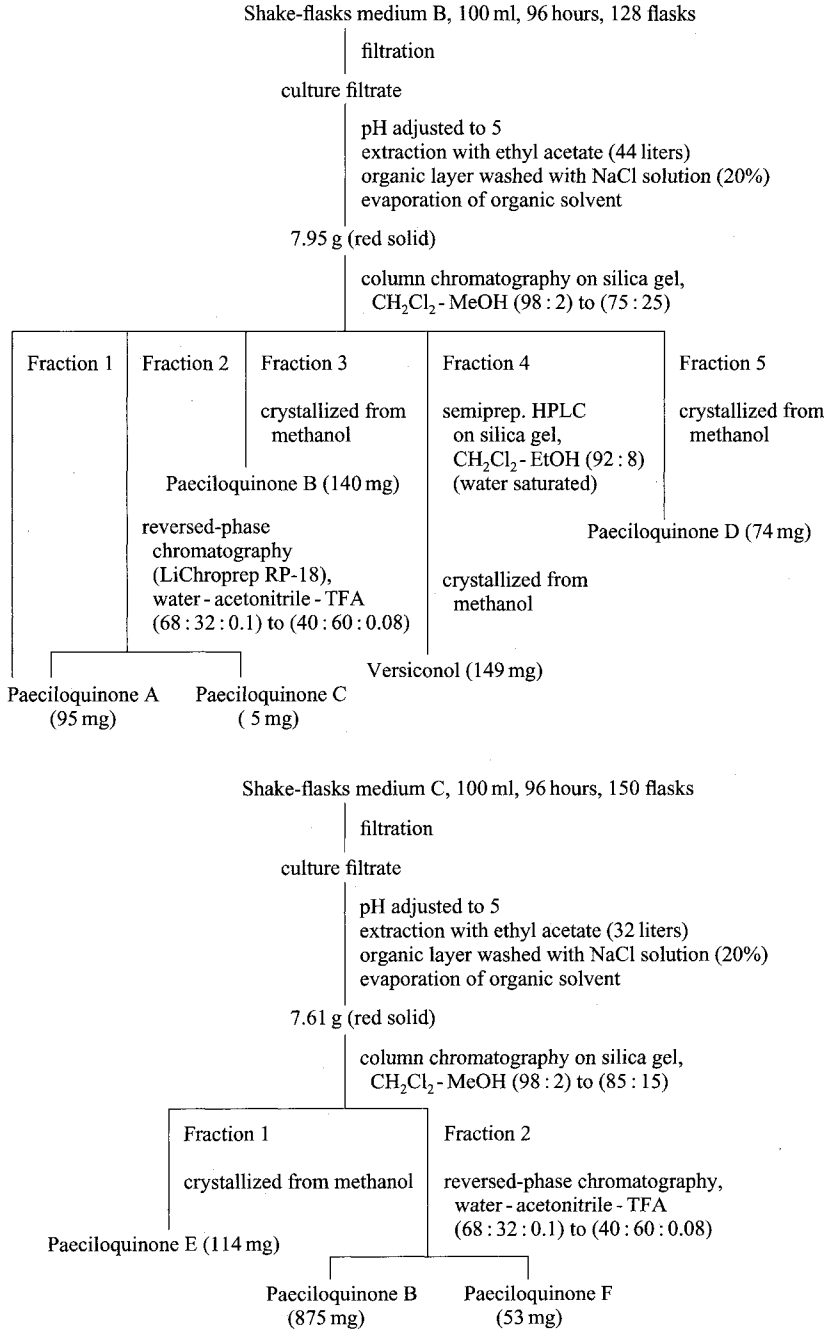
Fig. 2. Paeciloquinone A production time course on 50 liters bioreactor scale at 33°C with fermentation medium B.

● Paeciloquinone A, △ pmv, ■ pO_2, ▲ pH.



difficult to separate. This problem could be overcome by growing this strain in two fermentation media whereby a significantly different pattern of metabolites was produced and by isolating the metabolites from both series as shown in Scheme 1. In the fermentation broth of *Paecilomyces carneus* the paeciloquinones are found exclusively in the culture centrifugate. The solubility of the paeciloquinones is rather limited in organic solvents and pH dependent due to the carboxylic acid and phenolic functions. Therefore the pH of culture filtrate was adjusted to 5.0 prior to extraction with ethyl acetate. The crude product was chromatographed on preparative silica gel columns using a stepwise elution with water-saturated CH_2Cl_2-methanol mixtures. For some of the compounds a further purification step with reversed-phase chromatography was necessary. Isolation and analysis of shake flasks and fermentation experiments were assisted by an analytical HPLC method (see Fig. 3). The structures are shown in Fig. 4. Versiconol has been described earlier as a metabolite of *Aspergillus*

Scheme 1. Extraction and isolation of paeciloquinones A to F from *Paecilomyces carneus*.



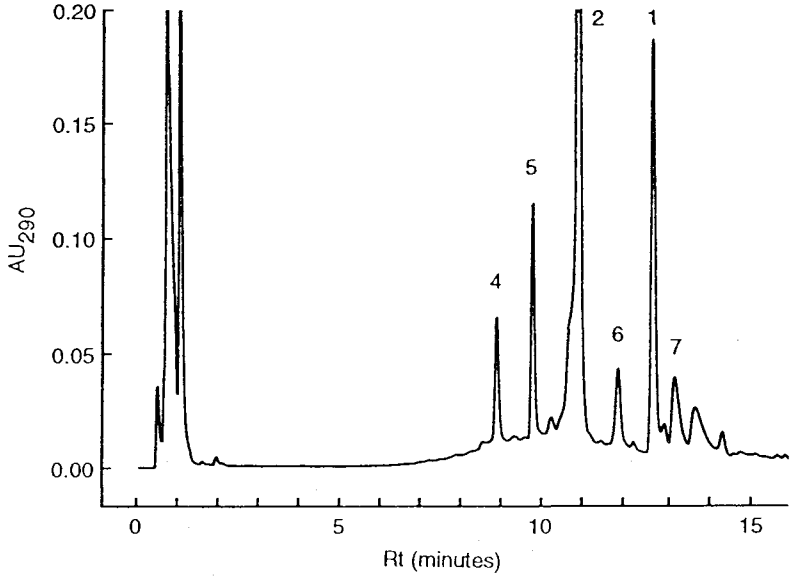
versicolor[13]. Paeciloquinone C has been synthesized by a Japanese group previously[14] as in intermediate in an attempt to synthesize rhodocladonic acid.

Biological Properties

The compounds were tested for inhibition of v-abl, c-src and EGF-R PTK as described previously[1,9,15,16]. As shown in Table 1 the paeciloquinones inhibited the enzymes with IC_{50} values in the micromolar range. The intracelluar domain of EGF-R PTK was inhibited by all compounds in a similar range suggesting that the binding site of that enzyme could accommodate all structural modifications of anthraquinones examined. The intracellular inhibitory activity of the paeciloquinones against EGF-R PTK was tested in an antiproliferative assay using BALB/MK mouse epidermal keratinocytes, which are strongly dependent on EGF for proliferation[17]. As shown in Table 1, the observed antiproliferative potency of these compounds was in a similar range as in the enzyme inhibitory activity against EGF-R PTK, suggesting that the compounds penetrated into the MK cells. It remains to be shown, whether the antiproliferative action is exclusively mediated by inhibition of EGF-R PTK.

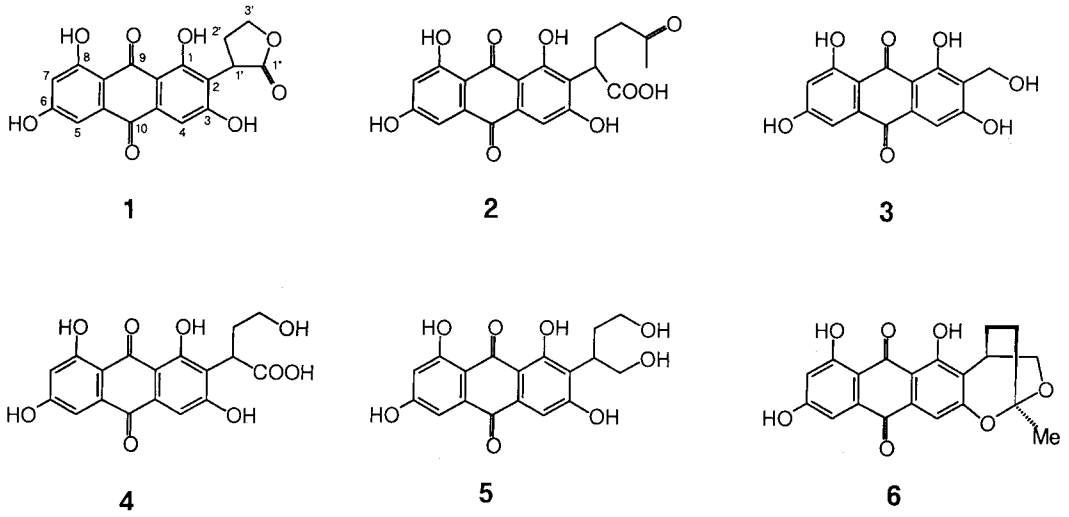
The compounds differed largely with respect to inhibition of v-abl PTK. Paeciloquinones A (**1**) and C (**3**)

Fig. 3. Reversed phase HPLC curve of 25 μl of a centrifuged fermentation broth after 93 hours.



Experimental conditions: analytical cartridge (LiChrocart, Merck, Darmstadt, Germany; 4.0 × 125 mm) plus precolumn (4.0 × 30 mm), filled with spherical C-18 coated silica based reversed-phase material (LiChrospher RP-18, 5 μm); UV detection at 290 nm; mobile phase A: 2.5 mM aqueous phosphate buffer pH 3.0; mobile phase B: acetonitrile - mobile phase A (8 : 2); 1.5 ml/minute; gradient elution: 20% B for 2 minutes, then to 75% B in 13 minutes. The numbers indicate the assignment of individual peaks to the respective paeciloquinone component.

Fig. 4. Structure of paeciloquinones A (**1**), B (**2**), C (**3**), D (**4**), E (**6**) and of versiconol (**5**) isolated from *Paecilomyces carneus*.



Structure **6** represents the relative configuration. The structure of paeciloquinone F (**7**) is not shown, as the structure is not completely assured.

proved to be the most potent ones with IC_{50} values around 0.4 μM. Substituents in position 2 appear to play a crucial role for activity. It seems that small substituents like hydroxymethyl in **3** or a five-membered lactone ring like in **1** are well tolerated. Bulkier substituents like in versiconol (**5**) are not permitted. Above all, carboxylic acid groups lead to a dramatic decrease in activity: Paeciloquinone E (**4**) is three orders of magnitude less potent than paeciloquinone A (**1**). This was further corroborated by preparing the methyl ester of the main metabolite paeciloquinone B **8** which turned out to be 30 times more active than the parent compound. Paeciloquinones A (**1**) and C (**3**) are very selective inhibitors of c-src and EGF-R PTK. Further *in vitro* profiling of the most active compounds for v-abl PTK is ongoing and will be published elsewhere.

Recently emodin, another fungal metabolite, has been described as an inhibitor of $p56^{lck}$ PTK binding to the

Table 1. Inhibition of EGF-R, c-src and v-abl protein tyrosine kinases and of MK-cells by paeciloquinones A to F the methyl ester of paeciloquinone B and versiconol.

		IC_{50} (μM)			
		EGF-R[a]	v-Abl	c-Src	MK-cells
Paeciloquinone A	(1)	11 ± 4.3	0.59	2	35
Paeciloquinone B	(2)	21 ± 3.8	83	>100	>50
Methyl ester of 2	(8)	17 ±10	11	>100	28
Paeciloquinone C	(3)	6.7± 0.2	0.56	9	10
Paeciloquinone D	(4)	10.5± 2.3	11	35	16
Paeciloquinone E	(6)	38 ±24	220	51	18
Paeciloquinone F	(7)	>100	3.6	36	40
Versiconol	(5)	21 ± 2.8	23	>100	22

[a] Mean and standard derivation of 3 independent determinations.

ATP binding site with an IC_{50} of 19 mM[18]. The biological activity of some related anthraquinones will be described in the subsequent paper[19].

The activity of paecilochinone A (**1**) was tested against various microorganisms (10 μl of a solution 2 mg/ml per paper disc). No antimicrobial activity against yeasts, filamentous fungi, or bacteria could be observed (data not shown).

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