PHENOXAN, A NEW OXAZOLE-PYRONE FROM MYXOBACTERIA: PRODUCTION, ANTIMICROBIAL ACTIVITY AND ITS INHIBITION OF THE ELECTRON TRANSPORT IN COMPLEX I OF THE RESPIRATORY CHAIN†

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During our continuing search for new biologically active metabolites from myxobacteria[1], a new screening system using an MT-4 cell culture assay with HIV-1[2,3] led to the discovery of the novel secondary metabolite, phenoxan (Fig. 1). It was isolated from an acetone-extract of the cell mass of *Polyangium* spec., strain PI VO19 and purified as described elsewhere[4]. The chemical and spectroscopic structure elucidation revealed phenoxan to be a new substituted oxazole-γ-pyrone[4] showing a structural similarity to the microbial metabolites aureothin[5,6] and spectinabilin[7]. The cytotoxicity of phenoxan for different human lymphoblastoid cell lines *in vitro* and its suppression of HIV-1 infection in the MT-4 cell culture assay has been described[2]. In this paper we wish to report on the production of phenoxan, its antimicrobial activity, and on experiments with beef heart submitochondrial particles showing that phenoxan is a powerful inhibitor of the eukaryotic respiratory chain at the site of complex I, *i.e.*, NADH: ubiquinone oxidoreductase.

The producing organism was *Polyangium* spec., strain PI VO19, isolated at the GBF in March 1988 from a soil sample collected in Ephesos, Turkey. The bacterium was initially cultivated in a lean standard peptone liquid medium (MD1 l.m.: peptone from casein, tryptically digested, from Merck, Darmstadt, 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.0; supplemented with 1 ml/liter of each of a standard vitamin and a trace element solution). Batch cultures of 100 or 400 ml in 250 or 1,000 ml Erlenmeyer flasks, respectively, were incubated at 30°C on a rotary shaker at 160 rpm for 3 ~ 5 days. PI VO19 grew in small lumps. It could readily be cultivated on technical media based on Probion (single cell protein prepared from *Methylomonas clarae*; Hoechst AG, Frankfurt), soy flour, skim milk powder (Humana Milchwerke, Herford, FRG), or peanut meal. In shake cultures with 100 ml

Fig. 1. The chemical structure of phenoxan[4].

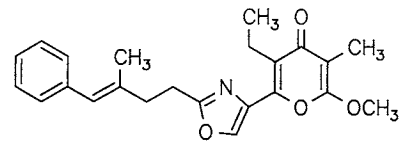

Table 1. Influence of various technical substrates on the production of phenoxan.

Substrate[a]	Concentration (%)	Antibiotic titer[b] (mg/liter)
Probion	0.4	2.5
Probion	0.9	5.2
Skim milk powder	0.4	1.2
Skim milk powder	0.9	9.6
Soy meal	0.4	2.8
Soy meal	0.9	4.8
Peanut meal	0.5	2.4
Peanut meal	0.9	No growth
Cornsteep	0.9	0.8
Zein (maize gluten)	0.9	0.8
Wheat gluten	0.9	No growth

[a] The basal medium was: soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%, HEPES buffer 50 mM (pH 7.2), supplemented with standard vitamin and trace element solutions, 1 ml/liter each. Harvest was at the end of the growth phase after about 4 days.

[b] The concentration of phenoxan was determined by TLC-analysis. Aliquots of acetone extracts of the cell mass were chromatographed on thin-layer plates (silica-gel F_{254}, Merck 5553) with toluene - methanol 85 : 15 as the solvent. The plates were scanned at 250 nm using a Shimadzu CS 920 high speed scanner. The concentrations of phenoxan were calculated from the peak areas of the phenoxan spots by comparison with those of the pure substance.

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of MD1 l.m. in 250 ml Erlenmeyer flasks, the average yield of phenoxan was below 1 mg/liter. Among the technical substrates tested for phenoxan production, skim milk powder and Probion gave the best results (Table 1). Phenoxan production on a larger scale was therefore performed in media based on Probion or skim milk powder. For example, 5 liters of shake culture was grown for three days in Probion liquid medium (Probion 0.9%, soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05%, 1 ml/liter of each of a standard vitamin and of a trace element solution, pH 7.0). The bacteria were then inoculated into a type b 50 bioreactor from Giovanola Frères (Monthey, Switzerland) containing 65 liters of Probion liquid medium. The fermenter was kept at 30°C and agitated with a turbine plate stirrer at 200 rpm. The aeration rate was 200 liters of air per hour. The pH was not regulated and rose during the fermentation from 7.0 to 7.5. The pO_2 in the culture was recorded continuously with a polarographic oxygen electrode. At the beginning of the fermentation the pO_2 was about 90% saturation and fell to about 50% at the end of the fermentation at 100 hours. A typical 60 liters fermentation yielded 253 mg of crystalline phenoxan[4].

Table 2. Antimicrobial spectrum of phenoxan.

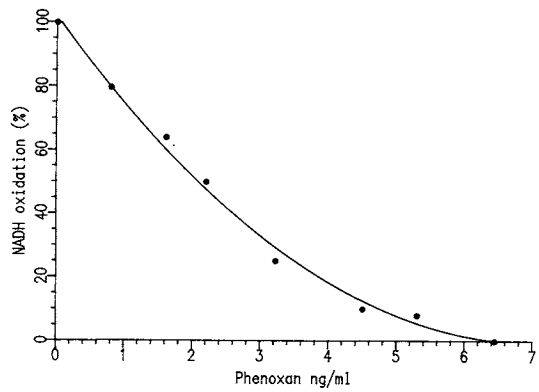
Test organism[a]	Diameter of inhibition zone[b] (mm)
Bacillus cereus DSM 621	0
B. megaterium DSM 32	0
B. subtilis DSM 10	0
B. thuringiensis DSM 2046	0
Micrococcus luteus GBF 26	0
Staphylococcus aureus GBF 16	0
Brevibacterium ammoniagenes	0
Corynebacterium fascians DSM 20131	0
Escherichia coli DSM 423	0
Salmonella typhimurium DSM 50912	0
Pseudomonas aeruginosa	0
Candida albicans GBF 129	0
Saccharomyces cerevisiae GBF 36	0
Mucor hiemalis	(20)
Botrytis cinerea	(17)
Trichoderma koningii	(10)
Gibberella fujikuori	(13)
Pythium debaryanum	(12)
Rhizopus arrhizus	(16)
Ustilago maydis	(35)

[a] The organisms were tested on standard complex media.

[b] Determined by the agar diffusion assay using paper discs of 6 mm diameter with 40 μg of phenoxan. Figures in parentheses indicate an incomplete inhibition.

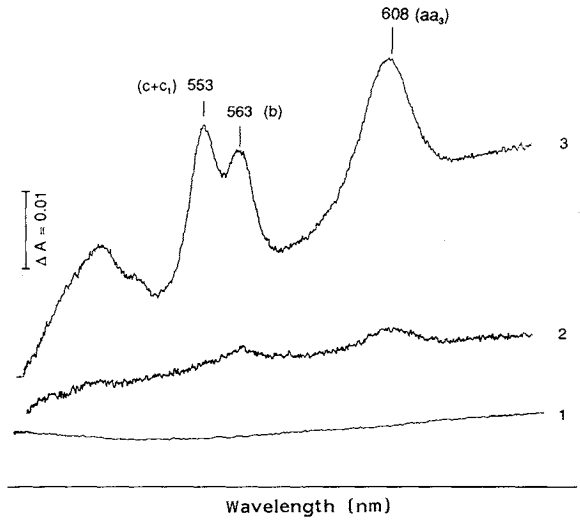
The antibiotic activity of phenoxan was determined by the agar diffusion test using paper discs. Phenoxan was inactive against bacteria and yeasts but inhibited the growth of some fungi (Table 2). The MIC determined by the serial dilution assay with *Ustilago maydis* was 19 ng/ml. To determine the mechanism of action of phenoxan we tested its influence on NADH oxidation of beef heart submitochondrial particles (SMP). The isolation and characterization of SMP as well as the performance of the experiments has been described previously[8]. As Fig. 2 shows, phenoxan strongly inhibited NADH oxidation in SMP. At a concentration of 5.8 nM (2.2 ng/ml), an inhibition of 50% was achieved. The site of inhibition within the electron transport chain was investigated by difference spectroscopy[8]. Starting with fully oxidized cytochromes the cytochromes in front of the block become reduced in the presence of a physiological substrate, *e.g.*, NADH, while those behind it remain oxidized. Treatment of SMP with 25 μg/ml phenoxan completely inhibited reduction of cytochrome aa_3 (α band at 605 nm), cytochrome b (α band at 563 nm) and the cytochromes $c+c_1$ (α band at 554 nm) by NADH (Fig. 3). This indicated that the site of action of phenoxan is on the substrate site of cytochrome b. Cytochrome b of complex III can be reduced by NADH *via* complex I (NADH: ubiquinone

Fig. 2. The effect of phenoxan on NADH oxidation by beef heart submitochondrial particles (SMP).



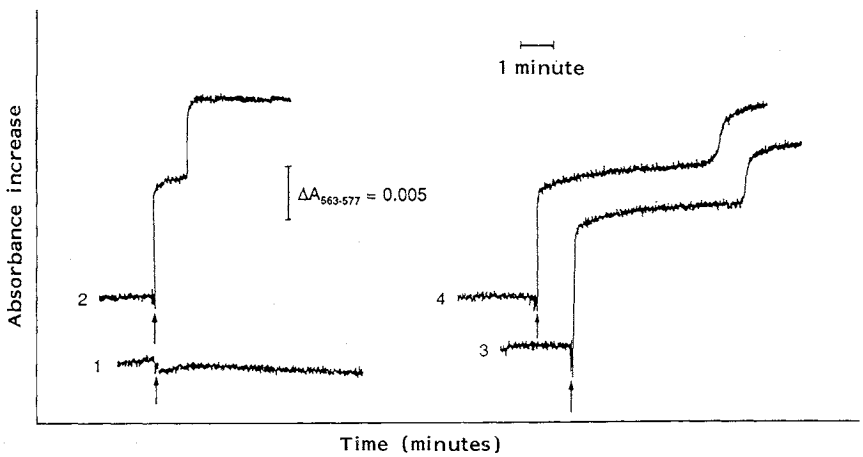
Phenoxan was dissolved in MeOH. The methanol concentration in the tests did not exceed 2%. The test suspension (70 μg protein/ml) was preincubated with the substance for 4 minutes before the reaction was started by the addition of NADH. The rate of NADH oxidation in the control without phenoxan was 1.6 nmol/minute × mg protein. Each value gives the average of 2~3 experiments.

Fig. 3. The effect of phenoxan on the reduction of cytochromes by NADH.



A suspension of air-oxidized beef heart submitochondrial particles (SMP) was diluted to give a density of 3.2 mg protein/ml. Cuvettes with an optical pathway of 1 cm were filled with 1.1 ml of the suspension and placed in the reference and sample position of a DW 2A UV/VIS double-beam spectrophotometer (American Instruments, Silver Springs, MD, USA). After the baseline (trace 1) had been recorded, phenoxan was added to the sample cuvette to a final concentration of 25 μg/ml. After preincubation for two minutes, NADH (final concentration 2 mM) was added to the sample cuvette. Volume corrections were performed in the reference cuvette. After two minutes the resulting difference spectrum was recorded (trace 2). As a control, the difference spectrum (reduced minus oxidized) of SMP reduced with NADH in the absence of phenoxan is also given (trace 3).

Fig. 4. The effect of phenoxan on the kinetics of cytochrome b reduction by NADH or succinate.



A suspension of air-oxidized submitochondrial particles was diluted as described in Fig. 3. The time course of cytochrome b reduction was measured by dual wavelength spectroscopy at the wavelength pair 563 minus 577 nm. The suspension was preincubated as described in Fig. 3. At the time indicated by the arrow either NADH (traces 1 and 2) or succinate (traces 3 and 4) were added to the sample and reference cuvettes, giving a final concentration of 2 mM and 5 mM, respectively. The sample cuvettes contained in addition phenoxan (traces 1 and 3) or methanol (traces 2 and 4).

oxidoreductase), or by succinate *via* complex II (succinate: ubiquinone oxidoreductase). We therefore investigated the effect of the antibiotic on the reduction kinetics of cytochrome b using either NADH or succinate as the substrate. As Fig. 4 shows, phenoxan inhibited the reduction of cytochrome b only when NADH was the electron donor. This suggests that phenoxan, like myxalamids[9], piericidin[10] and aurachins A and B[11], specifically blocked the electron flow in complex I, *i.e.* NADH: ubiquinone oxidoreductase.

It may be mentioned that meanwhile we detected phenoxan also in cultures of further *Polyangium* strains, *viz.* PI 3890, PI 6245, PI 6353 and PI 6370.

Acknowledgments

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