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THIANGAZOLE, A NEW THIAZOLINE ANTIBIOTIC FROM *Polyangium* sp. (MYXOBACTERIA): PRODUCTION, ANTIMICROBIAL ACTIVITY AND MECHANISM OF ACTION†

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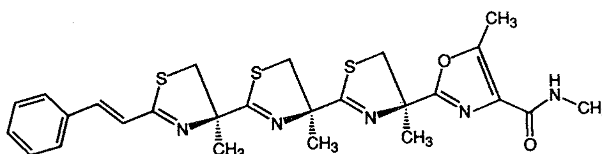
Thiangazole (Fig. 1) is the third compound from myxobacteria after phenoxan[1,2] and the phenalamids[3] which has been discovered by screening crude extracts in an MT-4 cell culture assay with HIV-1[4]. It was isolated from an acetone-extract of the cell mass of *Polyangium* sp., strain Pl 3007 and purified as described elsewhere[5]. The structure of the new compound was determined by spectroscopic methods to be a new tris-thiazoline derivative[5] showing a structural similarity to the cytotoxic alkaloid tantazole B[6], isolated from the blue-green alga *Scytonema mirabile*. The cytotoxicity of thiangazole for different human lymphoblastoid cell lines *in vitro*, and its suppression of HIV-1 infection of cultivated MT-4 cells has recently been described[4]. In this article we report on the production of thiangazole, its antimicrobial activity, and on experiments on its mechanism of action showing that thiangazole strongly inhibits the electron transport in beef heart submitochondrial particles (SMP) at the site of complex I, *i.e.*, NADH: ubiquinone oxidoreductase.

The producing organism was *Polyangium* sp., strain Pl 3007, isolated at the GBF in October 1986 from a soil sample collected in the gardens of the Alhambra in Granada, Spain. Stock cultures were stored in a deep freezer at $-80°C$ or in liquid nitrogen. 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%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.0; supplemented with 1 ml/liter each of a standard vitamin and a trace element solution). Batch cultures of 100 ml or 400 ml in 250 ml or 1,000 ml Erlenmeyer flasks, respectively, were incubated at 30°C on a rotary shaker at 160 rpm for 3∼5 days. Pl 3007 grew in small lumps. It could also be cultivated in technical media as described for other *Polyangium* strains[2]. In shake cultures with a medium containing 0.9% Probion (single cell protein prepared from *Methylomonas clarae*: Hoechst AG., Frankfurt) thiangazole was produced in quantities of up to 5.5 mg/liter, and in soy flour media in yields of up to 4.2 mg/liter. The thiangazole concentrations were determined from acetone extracts of the cell mass by HPLC analysis (column 25×4 mm, HD-Sil 18-5-100 (Kronwald); solvent: methanol-water (80:20); flow rate 1.5 ml/minute; detection 290 nm).

For thiangazole production on a larger scale, a medium based on 0.4% Probion appeared to be best. For example, 5 liters of shake culture grown for three days in Probion liquid medium (Probion 0.4%, soluble starch 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, vitamin B_{12} 0.25 mg/liter, and 1 ml/liter of a standard trace element solution, pH 7.0) were inoculated into a type b 50 bioreactor from

Fig. 1. The chemical structure of thiangazole[5].

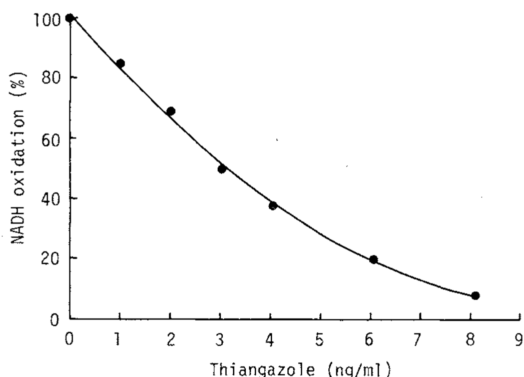


† Article No. 56 on antibiotics from gliding bacteria. Article No. 55: TROWITZSCH-KIENAST, W.; K. GERTH, V. WRAY, H. REICHENBACH and G. HÖFLE: Liebigs Ann. Chem. 1993: in print.

Giovanola Frères (Monthey, Switzerland) containing 65 liters of the same medium. Because of foam problems, 0.05% silicone antifoam agent (Tegosipon, Goldschmidt AG, Essen) had to be added. The fermentor was kept at 30°C and agitated at 200 rpm with a turbine plate stirrer. The aeration rate was 200 liters air per hour. The pH was not regulated and rose during the fermentation from 7.0 to 7.2. The pO_2 in the culture was recorded continuously with a polarographic oxygen electrode. At the beginning of the fermentation, oxygen was at 100% saturation and fell till the end of the fermentation after 95 hours to about 85%. The cells were harvested by centrifugation and extracted with acetone. The concentration of thiangazole, analyzed by HPLC as described above, was 3.3 mg/liter.

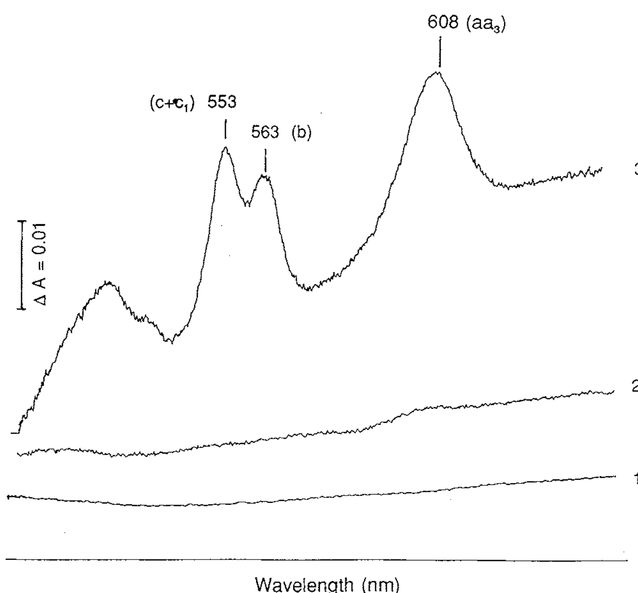
The antibiotic activity of thiangazole was determined by the agar diffusion test using paper discs. Thiangazole incompletely inhibited growth of the following fungi: *Mucor hiemalis*, *Botrytis cinerea*, *Trichoderma koningii*, *Gibberrella fujikuroi*, *Pythium debaryanum*, *Rhizopus arrhizus* and *Ustilago maydis*, but was inactive against yeasts and bacteria. The MIC determined by the serial dilution assay with *Ustilago maydis* was 3.12 μg/ml. To investigate the mechanism of action of thiangazole its influence on the electron flow in the respiratory chain of beef heart submitochondrial particles (SMP) was tested. The isolation and characterization of SMP as well as the execution of the experiments has been described previously[7]. In all assays, the submitochondrial particles were suspended in 75 mM sodium phosphate buffer, pH 7.4, supplemented with 1 mM each EDTA and $MgCl_2$. Thiangazole was

Fig. 2. The effect of thiangazole on NADH oxidation in beef heat submitochondrial particles (SMP).



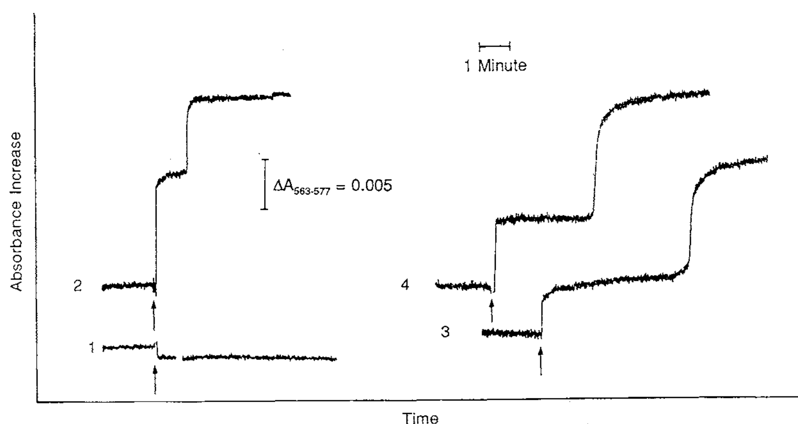
The SMP were dissolved in air saturated buffer at a concentration of 0.070 mg/ml. NADH was added to a final concentration of 0.16 mM. The rate of NADH oxidation in the control without thiangazole was 1.6 nmol/minute × mg protein.

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



The SPM were dissolved in air saturated buffer at a concentration of 3.2 mg/ml. Trace 1: Baseline, trace 2: Difference spectrum (reduced minus oxidized) of SMP reduced with NADH (2 mM) in the presence of 25 μg/ml thiangazole. 3: Difference spectrum of SMP reduced with NADH (2 mM) without inhibitor.

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



Air-oxidized SMP were diluted as described for Fig. 3. At the time indicated by the arrow, either NADH (trace 1 and 2) or succinate (trace 3 and 4) were added to the sample and reference cuvettes to a final concentration of 2 mM or 5 mM, respectively. The sample cuvettes contained in addition 10 μg/ml thiangazole (trace 1 and 3), the reference cuvettes the corresponding volume methanol (trace 2 and 4).

dissolved in methanol, and the methanol concentration in the tests did not exceed 2%. The effect of thiangazole on NADH oxidation in SMP was registrated with a 551 S UV/VIS spectrophotometer (Perkin-Elmer, Überlingen, FRG). As Fig. 2. shows, NADH oxidation was inhibited by 50% at a concentration of 3.2 ng/ml (5.9 nM) thiangazole. The site of inhibition within the electron transport chain was investigated by difference spectroscopy using a DW 2A UV/VIS double-beam spectrophotometer (American Instruments, Silver Springs, MD, USA)[7]. A suspension of air-oxidized beef heart submitochondrial particles (SMP) was filled into cuvettes with an optical pathway of 1 cm and placed in the reference and sample position of the spectrophotometer. After the baseline had been recorded, thiangazole was added to the sample cuvette. After preincubation for two minutes, NADH was added to the sample cuvette. Volume corrections were performed in the reference cuvette and after two minutes the resulting difference spectrum was recorded. Upon reduction with a physiological substrate, *e.g.*, NADH, fully oxidized cytochromes in front of the block become reduced, while those behind it remain oxidized. As Fig. 3 shows, treatment of SMP with thiangazole completely inhibited reduction by NADH of cytochrome aa_3 (α band at 608 nm), cytochrome *b* (α band at 563 nm) and the cytochromes $c+c_1$ (α band at 553 nm). This indicated that the site of action of thiangazole is on the substrate side of the cytochrome b, which can be reduced either by NADH *via* complex I (NADH: ubiquinone oxidoreductase), or by succinate *via* complex II (succinate: ubiquinone oxidoreductase). To decide whether thiangazole interferes with complex I, with complex II, or with both, we tested the effect of the compound on the reduction kinetics of cytochrome b using either NADH or succinate as the substrate. The time course of cytochrome *b* reduction was measured by dual wavelength spectroscopy at the wavelength pair 563 minus 577 nm. As can be seen in Fig. 4, thiangazole inhibited the reduction of cytochrome *b* only when NADH was the electron donor (the second reduction step in traces 2, 3 and 4 occurs after the oxygen in the sample cuvettes has been consumed). The result suggests that thiangazole blocks the electron flow in SMP specifically at the site of complex I, *i.e.*, NADH: ubiquinone-oxidoreductase, like the other two compounds found in the screening mentioned above, *viz.* phenoxan and the phenalamids. It is not known at the moment whether the inhibition of the respiratory chain is connected with the anti-HIV activity.

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