Production of the Staurosporine Aglycon K-252c with a Blocked Mutant of the Staurosporine Producer Strain *Streptomyces longisporoflavus* and by Biotransformation of Staurosporine with *Streptomyces mediocidicus* ATCC 13279

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Staurosporine (**1**) was first isolated in 1977 by OMURA *et al.*[1] from *Streptomyces staurosporeus*. About 10 years later in 1986, NAKANISHI *et al.* reported the first isolation of K-252c, the staurosporine aglycon moiety, from the culture broth of *Nocardiopsis* sp. K-290[2,3].

K-252c (**2**) acts like staurosporine as an inhibitor of protein kinase C. While studying the mechanism of action of this indolocarbazole compound, it appeared, that the interactions between the protein kinase and this kind of alkaloids were due to the rigid aromatic ring[4]. The fact, that the aromatic system is important for the inhibition of the kinase activity, suggests that other indolcarbazoles may develop interesting biological activity.

In the course of our screening program for staurosporine metabolites two original ways for the production of the staurosporine aglycon moiety K-252c (**2**) could be demonstrated. Compound (**2**) could be achieved with a blocked mutant of the staurosporine producer strain *Streptomyces longisporoflavus* R19/col15 (TÜ 2399) and *via* biotransformation of staurosporine with *Streptomyces mediocidicus* ATCC 13279.

Mutation and Selection, Metabolic Analysis

The staurosporine overproducing strain used as parental strain in our mutation program was isolated by the group of Professor ZÄHNER (University of Tübingen, Germany) from a soil sample from Ellora (India) in 1980.

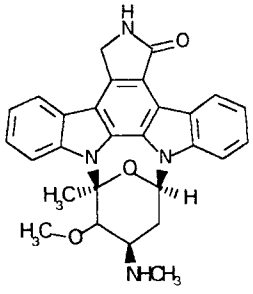
Spore suspensions with spore titers of about 10^7 spores per ml containing 0.1% TWEEN 80 were irradiated with a UV-lamp under suitable conditions (500 μW/cm^2, 50 seconds) to obtain 0.01 to 0.1% of surviving spores. Spore suspensions were diluted with phosphate buffer (pH 7.0), plated on sporulation agar (SPA) and incubated at 28°C for 5 to 7 days. SPA is composed of maize starch (Fluka, 20 g/liter), yeast extract (Fluka, 4 g/liter) and agar (Difco, 20 g/liter). After incubation, mutant colonies were selected for a random screening. Selection aspects were based on morphological differences during the growth on sporulation agar and on minimal agars. Selected colonies were transfered to liquid medium NL 148 (mannitol) in microtiter plates and incubated for 3 days at 28°C and an agitation rate of 180 rpm in a rotary shaker (first preculture). Medium NL 148 (mannitol) consisted of mannitol (22 g/liter), Lab Lemco (Oxoid, 4 g/liter), Peptone C (American Laboratories Inc., 5 g/liter), yeast extract (Difco, 0.5 g/liter), Casitone (Difco, 3 g/liter) and NaCl (1.5 g/liter). Before sterilization the pH was adjusted to 7.6 with 2 N NaOH and 2 N HCl. Starting from the first preculture with 1 ml of seed culture, a second one was carried out and incubated for 2 days at 28°C and 220 rpm in a rotary shaker. Bacteria from selected colonies were then incubated in 100-ml Erlenmeyer flasks with 20 ml production medium NL 3 for five days. NL 3 consisted of mannitol (40 g/liter), Sunpro (Sundatta Food & Fibres Division, India, 20 g/liter), KH_2PO_4 (0.5 g/liter) and SAG 471 (0.5 g/liter). The pH was adjusted to 6.5 before sterilization.

The resulting fermentation broth of each studied colony was first analyzed by TLC. Subsequently metabolites of the most interesting samples were then analyzed by HPLC.

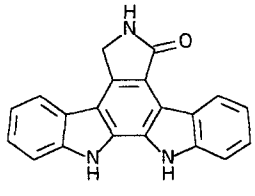
Several mutants of *S. longisporoflavus* did not produce staurosporine (**1**). Some of these mutants, such as M 13 accumulated the staurosporine aglycon K-252c (**2**).

M 13 is a stable mutant. Even after 10 or more successive fermentations, M 13 still produced K-252c as a single compound. In cofermentations with an other blocked mutant M 14†, producing a late biosynthetic intermediate in the biosynthetic pathway between K-252c and staurosporine, the staurosporine production was restored. This suggests, that only early steps after K-252c formation were blocked.

Fig. 1. Structures of staurosporine (**1**) and K-252c (**2**).



Staurosporine (**1**)



K-252c (**2**)

Screening for Biotransformation with Microbial Strains

Various strains from culture collections (*Streptomyces, Chromobacterium, Mycobacterium, Alcaligenes, Lactobacillus, Bacillus, Pseudomonas, Aspergillus, Trichoderma, Cunninghamella, Absidia, Cylindrocarpon, Chaetomium, Mortiella, Saccharomyces, Candida, Fusarium, Wojnowi-*

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† Manuscript in preparation.

cia, *Beauveria*, *Mucor*, *Rhizopus*, *Comamonas*, *Stereum*, *Phanerochaete*, *Pleurotus*, *Curvularia*, *Psilocybe*, *Penicillium*, *Kloeckera* and *Irpex*) or isolated microorganism were inoculated from slants or from frozen cultures into Erlenmeyer flasks (50 ml) containing 20 ml of medium NL 148 (glucose). In NL 148 (glucose) all ingredients are identical with the composition of NL 148 (mannitol), except that glucose is used as carbon source instead of mannitol. The seed cultures were shaken at 28°C and an agitation rate of 220 rpm in a rotary shaker. 1 ml samples from two days grown seed cultures were transferred into 20 ml of biotransformation cultures with medium NL 148 (glucose) or medium MV-7 in Erlenmeyer flasks (50 ml). Medium MV-7 is chemically defined and consists of glucose (10 g/liter), NH_4NO_3 (2 g/liter), Na_2HPO_4 (1.75 g/liter), KH_2PO_4 (0.6 g/liter $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), $CaCl_2 \cdot 2H_2O$ (0.01 g/liter), $FeSO_4 \cdot 7H_2O$ (0.001 g/liter) and trace element solution (1.0 ml/liter). The trace element solution consisted of $Na_2MoO_4 \cdot 2H_2O$ (0.02 g/liter), $Na_2B_4O_7 \cdot 10H_2O$ (0.02 g/liter), $ZnSO_4 \cdot 7H_2O$ (0.02 g/liter), $MnSO_4 \cdot H_2O$ (0.02 g/liter) and $CuSO_4 \cdot 5H_2O$ (0.02 g/liter). After 48 hours of incubation 2 mg of staurosporine or of the staurosporine aglycon solved in 0.1 ml DMSO were added to each flask whereas to a parallel series of flasks 0.1 ml DMSO without educt were added. Every two to three days during the incubation the pH in the flasks was set to 7.0, glucose was added if no more glucose was detectable and samples of the cultures were checked for biotransformation of staurosporine or of the staurosporine aglycon by TLC and HPLC.

Out of 177 strains screened in a biotransformation program with the natural product staurosporine only *Streptomyces mediocidicus* ATCC 13279 was able to convert staurosporine to the free aglycon. *S. mediocidicus* ATCC 13279 is also known as biocatalyst for specific hydroxylations of steroids[5]. In our case, with this strain, no other biotransformation products (*i.e.* hydroxylated staurosporines) were observed.

To some of the biotransformation candidate strains staurosporine was quite toxic, this might be the reason for that no other biotransformation products were detected. The aglycon part of staurosporine did not seem to be a good educt for biotransformation processes. The low solubility in water might be an other reason for this observation. We also did not succeed to find any biotransformation product when the aglycon moiety itself was offered as educt (56 microorganisms reported with hydroxylating capacities for other substrates were tested).

Preparative-Scale Biotransformation and Production of the Metabolite K-252c (2)

The biotransformation of staurosporine was carried out in a 2.5 liter fermentor (MBR Reactor AG, Wetzikon) using two liters of the medium NL 148 (glucose) at 28°C, 600 rpm and an airation of 1 liter of air per minute. The fermentor was inoculated with a two days grown seed culture of *Streptomyces mediocidicus* ATCC 13279. After 30 hours, 200 mg of staurosporine in 10 ml of DMSO was added and the biotransformation to the aglycon moiety was followed by HPLC. The K-252c concentration reached 11.2 mg/liter (conversion yield: 11.2 %).

For the biosynthetic production of the metabolite K-252c with mutant M 13 the same fermentor was used under the conditions described above with 2 liters of the medium NL 3 using 100 ml inoculum. K-252c titers up to 37.5 mg/liter were obtained.

Analytical Methods

For the TLC analysis culture broths were extracted with CH_2Cl_2 or ethyl acetate, aliquots of the organic phase were deposited on silica gel TLC plates, developed in CH_2Cl_2-isopropanol (10:1) and products were localized with UV-light. Subsequently metabolites of the most interesting mutants or candidates for biotransformation with whole cells were analyzed by HPLC. For the HPLC analysis samples of culture broths were diluted with the same volume of methanol, extracted on a rotary shaker, centrifuged and analyzed. The instrumentation consisted of an HPLC pump (Spectra-Physics SP 8800), a UV/vis detector (Spectra-Physics SP 8450) and an integrator (Shimadzu C-R3A). A LiChrosper 100 RP-8, 5 μm, HPLC column (4 × 125 mm, Merck) was employed. Mobile phase A consisted of 2.5 mM potassium phosphate buffer with a pH of 3.0. Mobile phase B was prepared by mixing 80% CH_3CN with 20% of mobile phase A (v/v). The wavelength for detection was set to 290 nm and 20 μl of each sample were injected for an analysis. A linear gradient was run from 40 to 100% mobile phase B in 16 minutes with a constant flow rate of 1.25 ml per minute.

The metabolite of the mutant M 13 and the biotransformation product formed in the presence of *Streptomyces mediocidicus* were isolated and compared with authentical K-252c by ^1H NMR[6~9], by UV/vis spectroscopy and by the chromatographical behavior in various TLC and HPLC systems. It turned out that it was identical with K-252c.

Discussion

Two original ways to produce the agycon moiety of staurosporine have been demonstrated.

In the course of exploratory biosynthetic investigations on staurosporine and staurosporine related compounds, strain M 13 has further importance as tool for the investigation of the staurosporine biosynthesis and for information of the regulation of the production of staurosporine.

Precursor directed biosynthesis (*i.e.* with 5-Hydroxy-L-tryptophane) using the staurosporine producer strain might be a more promising way to get staurosporine derivatives than biotransformation of the quite toxic staurosporine. The degradation of staurosporine by *Streptomyces mediocidicus* ATCC 13279 to the less toxic

aglycon moiety could be a biological strategy for detoxification.

Chemically the cleavage of the *N*-glycosidic linkage and of the *N*/*O*-acetal is a quite interesting reaction.

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