3′-Demethoxy-3′-hydroxystaurosporine-*O*-methyltransferase from *Streptomyces longisporoflavus* Catalyzing the Last Step in the Biosynthesis of Staurosporine

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Staurosporine (STP), an alkaloidal antibiotic, first isolated in 1977 by S. ŌMURA *et al.* from *Streptomyces* sp. AM 2282, is a potent inhibitor of protein kinases, especially of protein kinase C ($IC_{50} \approx 2.7$ nM). This enzyme plays a key role in signal transduction and cellular regulation so that inhibitors of protein kinase C are interesting drug canditates for cancer chemotherapy[1,2].

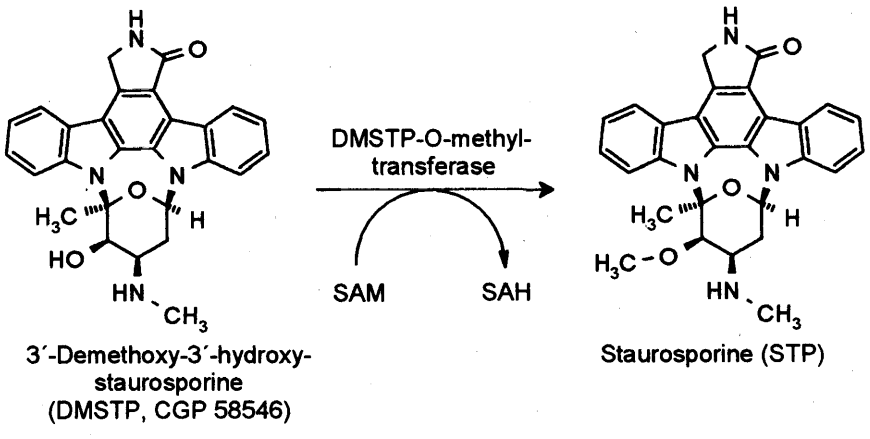
Until recently the knowledge of the STP biosynthesis has been limited to the early steps, particularly the incorporation of tryptophan by the formation of the aglycone moiety[3]. As part of a strain development program HOEHN *et al.* (1995) isolated the UV-mutant M14 from an STP overproducing strain of *Streptomyces longisporoflavus*, which excretes a novel staurosporine analogue, 3′-demethoxy-3′-hydroxy-staurosporine (DMSTP, CGP 58546). Cofermentation experiments demonstrated that this compound is the direct precursor of STP, indicating that *O*-methylation is the final step in the biosynthetic pathway of STP[4]. In this reaction (Fig. 1) *S*-adenosyl-methionine (SAM) can be supposed to be the methyl donor, corresponding to other *O*-methyltransferases, which are involved in the biogenesis of other antibiotics[5~9].

In this note we describe the detection of a DMSTP-*O*-methyltransferase, which catalyses the final step in the biosynthesis of STP.

A first evidence for the existence of a DMSTP-*O*-methyltransferase in *Streptomyces longisporoflavus* was the methylation of purified DMSTP via biotransformation by growing cells of the *S. longisporoflavus* mutant strain M13. This stable mutant produces the aglycon of STP as a single compound[10].

The fermentation was preceded by two inoculum stages. For the first preculture 1 ml of a cryo ampoule of the mutant strain M13 was transfered to 500 ml Erlenmeyer flasks containing 100 ml of medium SCR/12 mod + HEPES containing soy bean flour, full fat (20 g/liter); sucrose (20 g/liter); HEPES (12 g/liter), and antifoam agent SAG 471 (Union Carbide, New York, NY, USA, 0.1 g/liter); pH adjusted to 7.7 before sterilization. The flasks were incubated for 48 hours at 28°C and 180 rpm on a rotary shaker. 5 ml of this culture was used to inoculate the second preculture of medium SCR/12 mod. All ingredients are identical with the composi-

Fig. 1. Methylation of 3′-demethoxy-3′-hydroxystaurosporine (DMSTP) by DMSTP-*O*-methyltransferase with *S*-(5′-adenosyl)-L-methionine as the methyl donor.



SAM: *S*-(5′-adenosyl)-L-methionine; SAH: *S*-(5′-adenosyl)-L-homocysteine.

tion of the medium described above, except that HEPES was omitted. After 24 hours 1 ml of this second preculture were used to inoculate the main culture. The biotransformation of DMSTP with strain M13 was carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium NL3 consisting 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); pH adjusted to 6.8 before sterilization. The fermentation was run for a period of 180 hours at 250 rpm on a rotary shaker. After 42 hours of incubation DMSTP was added to each flask at a final concentration of 100 mg/liter in form of a concentrated solution (50 mg/ml) dissolved in DMSO. During the fermentation the progress of the biotransformation was periodically analyzed by HPLC. Samples of the culture broth (1 ml) were extracted with 5 ml of MeOH by shaking with 220 rpm at room temperature for 20 minutes. After centrifugation at 18000 × *g* for 15 minutes the supernatant was analyzed by HPLC: injection volume 10 µl; reversed phase column LiChrospher Nucleosil 100 RP-18, 5 µm, 4 × 125 mm (Merck, Darmstadt, Germany); mobile phase A consisted of 2.5 mM potassium phosphate buffer, pH 3.0; mobile phase B was prepared by mixing acetonitrile with mobile phase A (80 : 20, v/v); a linear gradient was run from 40% to 77% of mobile phase B within 10 minutes with a constant flow rate of 1.25 ml per minute; detection at 290 nm. The retention times are 6.14 minutes for the aglycone, 6.58 minutes for DMSTP, and 7.99 minutes for STP. DMSTP could not be detected in the culture broth any more 48 hours after addition, and after 78 hours it was quantitatively converted to STP.

In order to demonstrate the presence of the methyltransferase *in vitro*, crude cell extracts of different *Streptomyces longisporoflavus* strains were prepared. For this purpose, cells were grown in shake flask cultures (see above) using the chemically defined culture medium SYNT/33 for the main cultures. The medium contained sucrose (100 g/liter); $(NH_4)_2SO_4$ (7.5 g/liter); $CaCO_3$ (10 g/liter); K_2HPO_4 (1.5 g/liter); mineral solution 10 ml; SAG 471 (5% suspension) 1 ml; pH 7.5. The mineral solution consisted of $MgSO_4 \cdot 7H_2O$ (100 g/liter); $FeSO_4 \cdot 7H_2O$ (2 g/liter); $ZnSO_4 \cdot 7H_2O$ (1 g/liter); $CuSO_4 \cdot 5H_2O$ (0.4 g/liter); $CoCl_2 \cdot 6H_2O$ (0.1 g/liter), HCl (37%) 1 ml/liter. After 48 hours of growth the mycelium was collected by centrifugation at 9000 × *g* and 4°C for 20 minutes. The supernatant was discarded and the pellet was washed twice by resuspending in disruption buffer (100 mM potassium phosphate, pH 7.5; 0.1 mM EDTA; 1 mM 1,4-dithio-D,L-threitol, DTT). For the ultrasonic treatment the cell material was resupended in 1.5 volumes of disruption buffer and subsequently disintegrated with a W-385 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, New York, USA). After removal of the cell debris by centrifugation at 28000 × *g* and 4°C for 30 minutes the supernatant (crude cell extract) was used as enzyme source.

The enzyme assay for the DMSTP-*O*-methyltransferase activity, which was derived from an enzyme test used by CORCORAN (1975), contained the following components in a total volume of 100 µl: crude cell extract 80 µl; DMSTP solution (5.525 mM in DMSO) 4 µl; SAM-chloride solution (5.68 mM) 2 µl; *S*-adenosyl-L-[methyl-^{14}C]-methionine-solution (^{14}C-SAM, Amersham International plc, Buckinghamshire, U.K., 0.489 mM, 550000 cpm/ml, in H_2SO_4) 1 µl; deionized water 13 µl. Since the crude extracts frequently contained some DMSTP, it was not possible to carry out control assays without DMSTP in order to determine the background activity of the enzymatic decomposition of SAM. For control complete enzyme assays were stopped immediately by mixing with 200 µl ethyl acetate and blank enzyme tests without crude cell extract were prepared simultaneously.

After 4 hours of incubation at 30°C under gentle shaking the assay was mixed rigorously with 200 µl of ethyl acetate, centrifuged at 18000 × *g* for 15 minutes, and 25 µl of the supernatant containing the extracted STP and DMSTP and standard solutions of DMSTP and STP were analyzed by TLC: silica gel 60 F_{254}, 0.25 mm (Merck, Darmstadt, Germany), solvent system 1% triethylamine in methylene dichloride CH_2Cl_2-methanol (9 : 1), UV detection at 254 and 366 nm. The spots of STP and DMSTP from the standards were marked radioactively with 1 µl of ^{14}C-SAM-solution, and the radioactivity of the different spots on the plate were counted with a TLC-plate radioscanner (Digital Autoradiograph, Laboratorium Prof. Dr. BERTHOLD GmbH & Co., Wildbad, Germany).

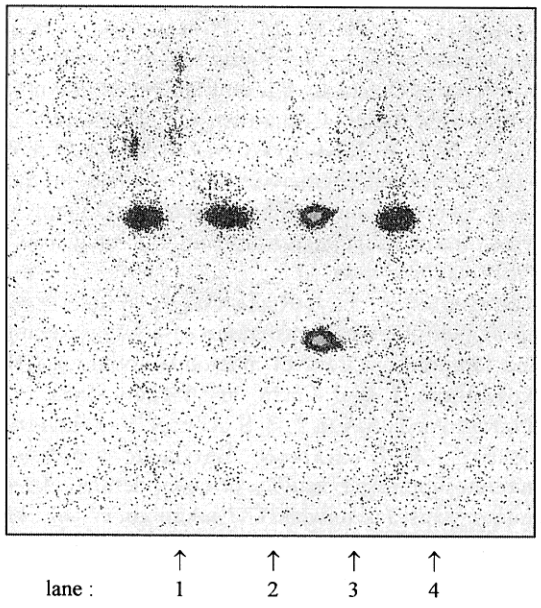
Fig. 2 shows clearly, that the majority of the radioactivity extracted from the assays contributed to ^{14}C-STP. In control assays without crude extract no radioactivity could be extracted. Using this *in vitro* test the existence of a DMSTP-*O*-methyltransferase was demonstrated in the crude extracts of *Streptomyces longisporoflavus* strains PHT3, R19/col15, SG71, all three producers of STP, and M13, the aglycon producer. As expected in the cell extract of the DMSTP-producing block mutant M14 the enzyme was not found.

For the further investigations, which were performed

with the STP producing strain PHT3, ^{14}C labeled STP was quantified by mixing 100 μl of the ethyl acetate extract of the activity assays with 2 ml of scintillation liquid (Irgascint A 300, Ciba-Geigy AG, Switzerland) and counting the radioactivity in a Tri-Carb Liquid Scintillation Analyser 2000CA (Canberra Packard, Zürich, Switzerland). The protein concentration was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Glattbrugg, Switzerland). Using this method it was shown, that the product formation in the standard activity assay with a protein concentration of 4.7 mg/ml was linear with the incubation time up to approximately 120 minutes. When incubating the assays for 240 minutes with different amounts of crude extract, the assays proved to be linear up to a final protein concentration of about 4 mg/liter.

Fig. 2. Distribution of radioactivity on a TLC-plate detected with the Digital Autoradiograph-radioscanner.

Lanes 1, 2 and 4: standard activity assays with crude extract of strain M13; lane 3: control = standard solution containing STP (higher Rf-value) and DMSTP (lower Rf-value) (spots were marked radioactively with *S*-adenosyl-L-[methyl-^{14}C]-methionine-solution after visualisation under UV light).



The DMSTP-*O*-methyltransferase was partially purified from 30 ml of crude cell extract by anion exchange chromatography on a 1.6 × 30 cm DEAE-Sephadex A-25 column (Pharmacia, Uppsala, Sveden), equilibrated with standard buffer containing 1 mM DDT and 0.1 mM EDTA. The elution was performed by a linear gradient from 0 ~ 1 M NaCl with a total volume of 420 ml at a flow rate of 25 ml/hour and 4.7 ml fraction size. The enzyme eluted between 0.2 and 0.4 M NaCl with 5.8-fold increased specific activity and a recovery rate of 76%.

The partially purified enzyme lost 80% of its activity during storage at 4°C for 6 days. This material was concentrated 12-fold by ultrafiltration with a CEC-column concentrator equipped with a YM30 membrane (Amicon Corp., Boston, Mass., USA) at 4°C, whereby another 85% of the activity were lost. However, no

Table 1. Influence of potentially inhibiting or activating substances on the acytivity of the DMSTP-*O*-methyltransferase (avarage of three parallel assays in each case).

	Residual activity (%)			
Concentration (mM)	0.111	0.221	0.442	1.105
STP and STP derivatives:				
DMSTP	94	100	83	69
STP-aglycon	93	90	n.d.	n.d.
STP	79	108	103	99
Biosynthetic precursors of the aglycon and of tryptophan:				
Tryptophan	n.d.	236	n.d.	64
Indol	n.d.	63	n.d.	62
Anthranilate	n.d.	76	n.d.	75
Indolpyruvate	n.d.	47	n.d.	44
Possible activated biosynthetic precursors of STP:				
TDP-glucose	n.d.	82	n.d.	n.d.
Concentration (mM)	0.057	0.114	0.227	0.568
S-(5'-Adenosyl)-L-homocysteine	46	32	21	8

deactivation occured during storage at −20°C for at least two weeks.

In order to examine the influence of derivatives of STP and of potential metabolites from the STP metabolism on the activity of the DMSTP-*O*-methyltransferase standard activity assays were performed in the presence of the various substances in the concentrations given in Table 1. DMSTP exhibits a certain substrate inhibition, but neither the aglycon of STP nor the endproduct of the biosynthetic pathway, STP itself, inhibit the enzyme. In the presence of only 0.2 mM tryptophan the activity is increased more than two-fold, whereas this amino acid at higher concentrations, like the other biosynthetic precursors of the aglycon, exhibits a moderate inhibition. The enzyme is strongly inhibited by *S*-(5'-adenosyl)-L-homocysteine, propably in a competitive manner to SAM, providing further evidence for SAM being the methyl donor for the methyltransfer to DMSTP.

The existence of a DMSTP-*O*-methyl transferase could clearly be demonstrated by biotransformation of DMSTP to STP by the STP-aglycon producing mutant M13 and by measuring the activity in crude cell extracts of *Streptomyces longisporoflavus* PHT3, SG71, R19/col15 and M13. In the crude cell extract of the DMSTP producing block mutant M14 the activity could not be detected. These results strongly support the assumption by HOEHN *et al.* (1995) that methylation of DMSTP represents the last step in the biosynthesis of STP. SAM serves as the methyl donor for the reaction like *e.g.* in the erythromycin biosynthesis. The demethylated cosubstrate SAH strongly inhibits the methyltransferase reaction, which is also known for many SAM dependent methyltransferases[11~13].

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