3′-Demethoxy-3′-hydroxystaurosporine, a Novel Staurosporine Analogue Produced by a Blocked Mutant

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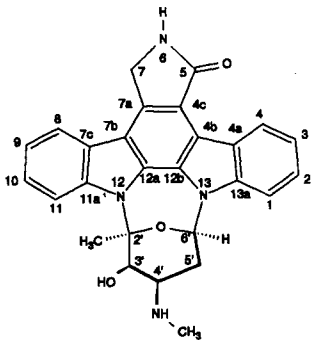
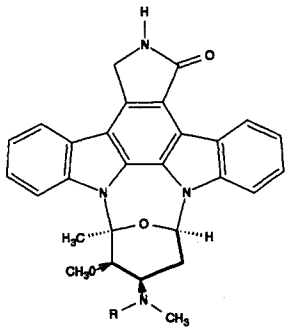
3′-Demethoxy-3′-hydroxystaurosporine, **1** (CGP 58 546), a novel staurosporine analogue, was isolated from a mutant of *Streptomyces longisporoflavus* R19 blocked in the last step of the biosynthetic pathway. CGP 58 546 was less potent than staurosporine, but it showed a more selective inhibition pattern against various subtypes of protein kinase C.

Staurosporine, **2**, an indolo (2,3-a) carbazole alkaloid, was first isolated in 1977 by S. ŌMURA and co-workers from *Streptomyces* sp. AM-2282[1], later reclassified as *Saccharothrix* sp. Its structure and relative stereochemistry were determined by X-ray crystallographic analysis[2,3] and by ^{1}H and ^{13}C NMR studies[4]. The absolute stereochemistry could only recently be established[5]. The latter publication also mentions its many biological activities which were subsequently detected over a period of 10 years, such as antimicrobial, hypotensive, cell cytotoxic activities and inhibition of platelet aggregation and protein kinase C.

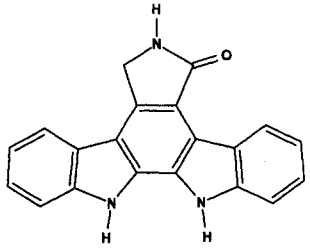
Fig. 1. Structures of staurosporine and selected derivatives and analogues.



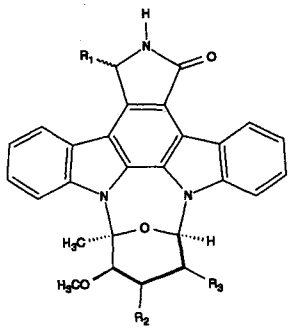
1
3′-demethoxy-3′-hydroxy staurosporine, CGP 58 546



2 R=H staurosporine
3 R=COC_6H_5 CGP 41 251



4
staurosporine aglycone (K-252c)



	R_1	R_2	R_3	
5	H	OH	OH	MLR-52 (14)
6	H	OH	H	RK-286c (15, 16)
7	H	OH	H	RK-1409 B (*3′-epi*) (17)

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In our own studies we were particularly interested in its potent activity against the latter enzyme which plays a key role in cellular signal transduction[6] and is therefore a promising target for antitumor compounds. Since staurosporine itself does not show the desired subtype specificity a number of semisynthetic derivatives were synthesized and tested in an appropriate test panel. Among these derivatives the 4′ *N*-benzoyl derivative CGP 41 251, **3**, exhibited the desired specificity and was selected for further development as an antitumor drug[7~9].

In order to improve the preparative access to the starting material staurosporine **2** we initiated a program of strain and process development. The producing strain was subjected to a mutagenic treatment. The isolated mutants were tested for improved productivity and for the production of novel intermediates. Such intermediates from blocked mutants could provide valuable information on the biosynthetic pathway of staurosporine only the first steps of which have been investigated to date[4,10].

This paper deals with the isolation and characterization of 3′-demethoxy-3′-hydroxystaurosporine **1** (CGP 58 546), which was shown to be an intermediate in the biosynthetic pathway. In addition, the interesting *in vitro* activity of the new metabolite and the isolation of the producing mutant strain M14 (DSM 8325) will also be described.

Materials and Methods

Microorganism

The origin of our staurosporine-producing strain *Streptomyces longisporoflavus* R19 was reported before[11]. The selected colony R19/col 15 was picked from a series of cultures on agar plates and used as the parental strain for the mutation program. The mutant strain producing 3′-demethoxy-3′-hydroxystaurosporine was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) in Braunschweig, FRG under accession number DSM 8325 on May 27, 1993.

Media Used (ingredients in g/liter)

SPA (sporulation agar): maize starch (Fluka), 20; yeast extract (Fluka), 4, and agar (Difco), 20. MA (minimal agar): glucose, 10; L-asparagine, 0.5; $K_2HPO_4 \cdot 3H_2O$, 0.65; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$ 0.01. pH 7.0~7.2. NL 148: mannitol, 22; Lab Lemco (Oxoid), 4; peptone C (American Laboratories Inc.), 5; yeast extract (Difco), 0.5; casitone (Difco), 3; and NaCl, 1.5. The pH was adjusted to 7.6 with 2 N HCl before sterilization. NL 3 (production medium): mannitol, 40; Sunpro (Sundatta Food and Fibres Division, India), 20; KH_2PO_4, 0.5. The pH was adjusted to 6.5 before sterilization.

All fermentations were carried out at 28°C.

Mutation and Selection Methods

Three cultures of *Streptomyces longisporoflavus* R19/col 15 on SPA plates were incubated for 5~7 days until they exhibited a white to grey color on the surface of the mycelium. For the mutation experiments using UV-irradiation a spore suspension was prepared as follows: A sterile 0.1% (w/v) solution of Tween 80 was added to each plate. Spores were removed by slightly scraping the surface of the mycelium with gentle pressure. The suspension was transferred to a Petri dish and the concentration of the spores in the suspension adjusted to about 10^7/ml after control under a microscope. The mutagenic treatment was performed by irradiating with a UV-lamp (model UVG-254, 254 nm; 500 $\mu W/cm^2$) under constant agitation. Based on an experimental survival curve, irradiation in the dark was carried out during 50 seconds to obtain 0.01 to 0.1% of surviving spores[12]. The irradiated spore suspension was diluted 100-fold with phosphate buffer of pH 7.0, stored in the dark for one day at room temperature, plated on SPA and incubated for 5~7 days. The incubated agar cultures were replica-plated on MA and SPA plates. After 4 days of incubation the replicas were compared to the master plates. All colonies differing in shape, color and/or size on MA were isolated, cultivated in shake flasks and examined with respect to metabolite production by TLC and HPLC. Selected colonies were transferred to 50-ml Erlenmeyer flasks containing 10 ml of medium NL 148 and incubated for 3 days on a rotary shaker with an agitation of 220 rpm. One ml of this first preculture was used to seed a second one which was cultivated for 2 days under the same conditions. One ml of this seed was used to inoculate 20 ml of production medium NL 3 in 100-ml shake flasks. Incubation was carried out at 28° during 5 days.

Analytical Methods

For TLC analysis culture broth samples were extracted with methylene chloride or ethyl acetate. Aliquots of the organic phase (25 μl) were deposited on silica gel plates. After development with the solvent mixture CH_2Cl_2-2-propanol (10:1) the separated spots were visualized by UV light at 254 and 366 nm. The metabolite pattern of the most interesting mutants was further characterized by HPLC coupled with a diode-array UV detector. For HPLC analysis samples of the whole culture broth were extracted with the same volume of methanol and centrifuged. 20 μl of supernatant were then injected in a reversed phase column (Merck, LiChrospher 100 RP-8, 5 μm, 4×125 mm). Mobile phase A consisted of 2.5 mmol potassium phosphate buffer pH 3.0. Mobile phase B was prepared by mixing acetonitrile with mobile phase A (80:20, v/v). With a constant flow rate of 1.25 ml/minute a linear gradient was run from 40 to 100% of mobile phase B within 16 minutes.

Fermentation

a) Shake flasks: Inocula grown on SPA slants were transferred into 100-ml Erlenmeyer flasks containing 20 ml of medium NL 148 and incubated for 3 days on a rotatory shaker (220 rpm). 0.5 ~ 1 ml of this seed culture was then used to inoculate 20 ml of the same medium in 100-ml Erlenmeyer flasks and cultured for 2 days at 220 rpm. 1 ~ 2 ml of the second seed culture were then transferred to 500-ml shake flasks containing 100 ml of fermentation medium NL 3 and incubated under the same conditions for 5 days.

b) Cofermentation: precultures of mutant strain DSM 8325 and mutant strain M13 producing the aglycone **4** as the only metabolite were prepared separately as described above. One ml of each seed culture was transferred to 500-ml shake flasks containing 100 ml of medium NL 3. The pattern of metabolites was analyzed after 5 days of incubation at 220 rpm.

c) 30-liter fermenters: the fermentation was preceded by 2 inoculum stages (initial pH of about 7.6), whereas the production fermentation was performed at pH values ranging from 6.0 to 7.0. For the first preculture the contents of a cryo ampoule or a loopful of the mutant strain grown on agar were transferred to 50-ml Erlenmeyer flasks containing 10 ml of medium NL 148. After incubation at 220 rpm for 48 hours 1 ml of this culture was used to inoculate 100 ml of the same medium in 500-ml flasks with 1 baffle. The flasks were incubated for 48 hours at 250 rpm. A second preculture was prepared by using 25 ml of the first preculture for the inoculation of 500 ml of NL 148 in 2-liter Erlenmeyer flasks with 4 baffles. After 48 hours at 120 rpm 600 ml of this second preculture were used to inoculate 30 liters of medium NL 3 in a MBR fermenter. The production fermentation was run for a period of 117 hours (aeration, 1 liter/1 liter/minute; agitation, 600 rpm; head space pressure 0.5 bar). The stirring velocity was controlled in order to maintain dissolved oxygen above 30%. No pH adjustment was made during the fermentation. The production of secondary metabolites was periodically analyzed by HPLC.

Strain Preservation

Cultures of the mutant strains were stored at −80°C. A 50-ml Erlenmeyer flask containing 10 ml of medium NL 148 was inoculated with 1 ml of the first preculture obtained as described above. After growth for 2 days at 28°C and 220 rpm aliquots of 1.5 ml were stored in cryo-ampoules.

Spectroscopy

The following instruments were used in this study: NMR Varian VXR-400S and Varian Unity 500, UV/VIS spectrophotometer Perkin Elmer Lamba 5, FT-IR spectrophotometer Bruker IFS-48 and mass spectrometer V6 70-4SE. Thioglycine was used as matrix for the HR-FAB-MS.

Results

Isolation and Characterization of Mutant Strain M14 (DSM 8325)

Comparison of a series of mutant strains on the replica plates yielded one colony showing poor and thin growth on MA plates, but normal development on SPA plates. This interesting mutant designated M14 produced a yellow to brown mycelium on SPA, whereas the parental strain R19/col 15 formed a grey colored mature spore mass. Chromatographic analysis by TLC revealed the presence of only one major metabolite, **1**, which was more polar than staurosporine. In the HPLC/diode array detection system it showed one major peak with a UV-spectrum almost superimposable over that of staurosporine. Therefore, this mutant was further investigated with first priority.

In liquid medium NL 148, mutant M14 formed small pellets with a diameter of up to 3 mm. The titer of the new metabolite in medium NL 3 amounted to 55 mg/liter after 4 days of culture in fermenters and could be significantly increased by stepwise optimization of the composition of the medium. The metabolite pattern was unchanged after 10 or more successive fermentations confirming the genetic stability of this mutant.

Isolation and Purification of Metabolite **1**

a) From shake flask cultures:

1.5 liter of the whole fermentation broth was vigorously stirred with 2 liters of methylene chloride. The extracted cell material was removed by filtration using Hyflo (diatomaceous earth) as filter aid. The aqueous layer of the clear filtrate was separated and subjected to further extraction with 2 liters of ethyl acetate. The combined organic extracts were washed with a small volume of water, dried over anhydrous sodium sulfate and evaporated to dryness. Crystallization of the residue from a small volume of ethyl acetate yielded pure **1** (30 mg) in the form of pale yellowish crystals.

b) From fermenter culture:

Culture broths from 3 different fermenter runs were combined (77 liters). The whole broth was vigorously stirred with twice its volume of ethyl acetate. After addition of Hyflo (10 kg) the suspension was filtered and the filter cake washed with additional ethyl acetate (30 liters). The organic phase was washed with saturated brine and the solvent evaporated *in vacuo*. The crude extract (55.5 g) was triturated with *n*-heptane (1 liter) to remove 44.84 g of a thin oil. The insoluble residue (10.57 g) was dissolved in a minimum amount of methylene chloride. A first crop of crystals which

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) assignments of CGP 58 546 in DMSO-d_6 at 80°C.

Carbon No.	^{13}C NMR shift, multiplicity	^{1}H NMR shift, multiplicity	Carbon No.	^{13}C NMR shift, multiplicity	^{1}H NMR shift, multiplicity
1	108.2 (d)	7.61 (m)	11	113.8 (d)	7.98 (d)
2	125.1 (d)	7.50 (dd)	11a	138.8 (s)	
3	119.0 (d)	7.35 (t)	12a	129.3 (s)	
4	125.4 (d)	9.30 (d)	12b	125.1 (s)	
4a	122.5 (s)		13a	136.0 (s)	
4b	114.9 (s)		2′	94.3 (s)	
4c	119.0 (s)		3′	68.4 (d)	4.84 (dd)
5	171.5 (s)		4′	53.7 (d)	3.77 (ddd)
7	45.1 (t)	4.99 (d), 4.94 (d)	5′	25.8 (t)	2.95~3.02 (m, H-5′a), 2.26 (ddd, H-5′b)
7a	132.3 (s)		6′	80.5 (d)	6.92 (dd)
7b	113.9 (s)		2′-CH_3	28.5 (q)	2.31 (s, 3H)
7c	123.9 (s)		4′-CH_3	30.1 (q)	2.62 (s, 3H)
8	120.7 (d)	8.02 (d)	NH		8.27 (d)
9	119.8 (d)	7.29 (t)	OH		6.12 (br)
10	124.3 (d)	7.46 (dd)			

Coupling constants (Hz): $J_{1,2}=J_{3,4}=8.0$; $J_{8,9}=J_{10,11}=8.0$; $J_{3',4'}=3.2$; $J_{3',5}=1.2$; $J_{4',5'a}=4.0$; $J_{4',5'b}=12.4$; $J_{5'a,5'b}=12.5$; $J_{5'a,6'}=8.2$; $J_{5'b,6'}=6.2$.

precipitated spontaneously was separated, dissolved in methanol, treated with active charcoal and filtered. The clear filtrate was concentrated and recrystallized from ethyl acetate. The mother liquor was combined with the previously obtained mother liquor from the spontaneously formed crystals in methylene chloride and the pooled material further purified by column chromatography on silica gel (Si 60 Merck, particle size, 15~25 μ; 1 liter). The desired product was eluted with methylene chloride - methanol (95 : 5). Crystallization and repeated chromatography of mother liquors and impure fractions eventually yielded a second and a third crops of crystals from ethyl acetate. The combined crystallizates were recrystallized from ethyl acetate, giving a total yield of 1.13 g of pale yellow crystals of pure compound (from 77 liters of culture broth).

Structure Elucidation of Compound 1

The structure elucidation of the new metabolite from mutant strain M14 was based on results of MS- and NMR spectroscopy. The signals of the ^{1}H and ^{13}C NMR spectra (listed in Table 1) were very similar to those of staurosporine[4,11]. The major difference was the lack of the OCH_3 group documented by a missing singlet signal at 3.32 ppm in the ^{1}H NMR and of the primary carbon signal at 57.2 ppm in the ^{13}C NMR. In addition, the signal for the C-atom in position 3′ was significantly shifted downfield from 82.7 to 68.9 ppm. The assumed elemental composition $C_{27}H_{24}N_4O_3$ of **1** was confirmed by a positive mode high resolution FAB-MS which gave a molecular weight of 452.517. Final proof of the structure of **1** was provided by the bioconversion experiments described below.

Results of Bioconversion Experiments

The first bioconversions were carried out with another blocked mutant, M13, which produced the aglycone K-252c **4** as the only detectable metabolite[13]. Cultures of this strain converted **1** quantitatively into staurosporine **2**. The same results were observed when the parental strain R19/col 15 was used in bioconversion experiments.

Physico-chemical and Biological Properties

mp: >220°C (dec.)

TLC: Rf 0.69, staurosporine 0.14 (for conditions see Analytical Methods)

UV-spectrum: (95% EtOH, 10^{-4} M): absorption maxima in nm (ε-values): 207 (23,300), 244 (22,300), 293 (50,700), 335 (12,400), 355 (8,170) and 372 (8,780).

Optical rotation: $[\alpha]^{20}$ (*c* 1% in DMSO): at 546 nm: +96.2°; 578 nm: +84.6°; 589 nm: +82°±1.1°.

IR spectrum (KBr): 3420, **3252**, 3049, **2930**, **1660**, **1587**, **1458**, 1420, **1398**, **1348**, **1317**, 1283.

FAB-MS (positive ion mode, high resolution): *m/z* 452.517; additional signals at 453 ($M-H^+$), 338, 311, 295 and 142; calc. for $C_{27}H_{24}N_4O_3$: 452.513).

^{1}H and ^{13}C NMR spectra: see Table 1.

In Vitro Biological Properties

The activity of **1** against different subtypes of protein kinase C was determined by comparison with staurosporine **2** and its *N*-benzoyl derivative CGP 41 251. The results are compiled in Table 2.

Table 2. *In vitro* inhibition of protein kinase C (PKC) subtypes.

	Staurosporine (2)	CGP 41 251 (3)	CGP 58 546 (1)
PKC alpha IC_{50} (mM)	0.003	0.024	0.039
PKC beta-1 IC_{50} (mM)	0.009	0.030	0.15
PKC beta-2 IC_{50} (mM)	0.003	0.031	0.048
PKC gamma IC_{50} (mM)	0.004	0.024	0.044
PKC delta IC_{50} (mM)	0.027	0.33	0.95
PKC epsilon IC_{50} (mM)	0.049	2.0	2.1
PKC zeta IC_{50} (mM)	1.29	>500	58
PKC eta IC_{50} (mM)	0.010	0.160	0.36
T24 Bla.Ca. IC_{50} (mM)	0.007	0.2	0.007

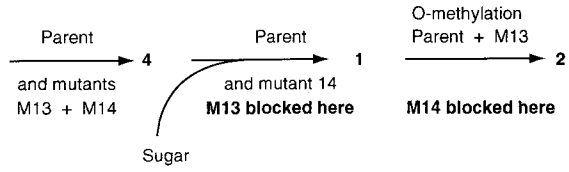
Discussion

In our efforts to improve the productivity of the staurosporine producing strain *Streptomyces longisporoflavus* R19/col 15, we isolated a mutant, M14, which produced a novel intermediate of the biosynthesis of staurosporine. Up to now, knowledge on the biosynthesis of this pharmacologically interesting molecule has been limited to the early steps, specifically the incorporation of tryptophan[10]. Our own studies of blocked mutants of a staurosporine producer strain has led to the isolation of the 3′-demethoxy-3′-hydroxy analogue **1**. This new metabolite has been shown to be a direct precursor of staurosporine. By cofermentation and bioconversion experiments we have excluded the possibility that **1** is produced by demethylation of the end product by an enzyme which could be repressed in the parental strain. Subsequent experiments have corroborated our conclusion that *O*-methylation is the final step in the biosynthetic pathway of staurosporine (Fig. 2) and that mutant M14 (DSM 8325) lacks the *O*-methylase enzyme required for this step. These data and further information on the isolation and characterization of this enzyme will be published in a separate paper.

To date only a few staurosporine analogues carrying a free hydroxy group in the sugar moiety have been isolated from microbial sources, *e.g.* MLR-52, **5**[14], RK-286c, **6**[15,16], and RK-1409B, **7**[17]. In contrast to the new metabolite **1** none of these compounds contains a hydroxy group in position 3′ while retaining the pharmacologically important aminomethyl group in position 4′.

As an inhibitor of protein kinase C, **1** turned out to be less potent than staurosporine itself. However, it showed a higher selectivity against the various subtypes of this enzyme. These *in vitro* results demonstrated once more that comparatively minor modifications of the staurosporine molecule can have a profound effect on its biological properties. Results of our efforts to modify the activity of **1** by chemical transformation will be reported elsewhere.

Fig. 2. Steps in the staurosporine biosynthetic pathway.



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