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BIOSYNTHESIS OF STAUROSPORINE, 2. INCORPORATION OF TRYPTOPHAN[1,2]

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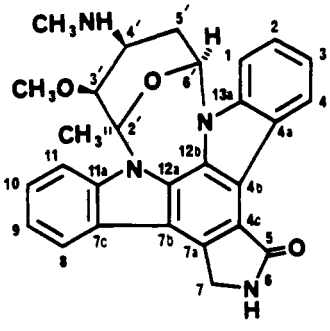
ABSTRACT.—Following studies to define the time course, media, and nutrient parameters for the production of the potent cytotoxic antibiotic staurosporine [**1**] in *Streptomyces staurosporeus*, biosynthetic studies of staurosporine with singly- and doubly-labeled radioactive precursors established that either one or two units of tryptophan was incorporated efficiently. From the ^{13}C-nmr spectrum of staurosporine subsequent to stable isotope incorporation experiments, it was established that the aglycone moiety was derived from two units of tryptophan with the carbon skeleton incorporated intact.

Staurosporine [**1**], an indolo[2,3-*a*]carbazole alkaloid, was first isolated from *Streptomyces staurosporeus* Awaya (1) and subsequently from several other actinomycetes (2,3), and the structure and stereochemistry were deduced by X-ray crystallography (4,5). Staurosporine possesses inhibitory activity against fungi and yeasts, strong antihypertensive activity (6,7), and pronounced in vitro activity against a number of experimental tumors (2,8,9). It is a potent inhibitor of protein kinase C (8) and platelet aggregation (3) but has no significant effects on bacteria (1). The aglycone moiety **2** is important for biological activity (10), strongly suggesting that other indolo[2,3-*a*]carbazole derivatives might also possess important biological activity.

Several naturally occurring indolo[2,3-*a*]carbazoles have recently been reported (10–15), and three biogenetic syntheses of staurosporine aglycone **2** and related derivatives have been completed (16–18). However, to date there have been no reports on the biosynthesis of these interesting metabolites, knowledge which might ultimately be applicable for the production of systematically modified staurosporine derivatives. As a working hypothesis, staurosporine is most likely constructed from a tryptophan-derived aromatic unit and an amino sugar moiety that is connected stereospecifically by an unusual double *N*-glycosidic linkage. As the initial steps in a program to examine the biosynthesis of staurosporine, we reported (19) on the unambiguous determination of the ^{1}H- and ^{13}C-nmr spectral parameters of staurosporine [**1**]. Here, we report on the



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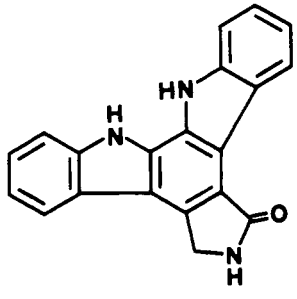

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production of **1** and the nature of the aromatic building blocks involved in its biosynthesis.

For the production of staurosporine, the seed inoculum is usually initiated directly from spores of *Streptomyces*, and in the past, **1** has been extracted and isolated from the fermentation broth. Several compositions of fermentation medium have been used for the production of staurosporine (2,3,10,14), but after evaluation of these, the fermentation medium selected was that described previously by Omura *et al.* (1). More growth occurred in agitated cultures than in stationary cultures, and it was also noted that the growth of *S. staurosporeus* was very sensitive to changes in pH, temperature, presence of trace surfactants, aeration, and agitation.

Staurosporine [**1**] was purified by overpressured layer chromatography (20,21), where reproducible separation was achieved within 1.5 h. Careful plate-edge impregnation was found to be necessary to prevent solvent leakage at overpressure (22). Additionally, pre-equilibration with a suitable solvent prior to any separation was important in order to eliminate any air and/or gas within the adsorbent (22) and to avoid the solvent demixing effect, causing "zig-zag fronts," which often results in a loss of resolution (23).

In order to establish the time-course production of **1**, the culture fermentation broth was harvested after different time periods, basified, and extracted with EtOAc containing reserpine as an internal standard. Quantitative hplc analysis was conducted using reversed-phase silica (Novapak column) with MeCN-0.01 M NaOAc solution (55:45) as the solvent and uv detection (294 nm). Induction of the biosynthesis of staurosporine was observed to occur in the lag phase before production of **1** began at 10 h after inoculation. Maximum production was reached at about 55–60 h and then declined throughout the idiophase. Data from the time-course study also permitted determination of the time of exhaustion of the nitrogen source. This was followed by the production of staurosporine and was, therefore, a suitable time for the addition of labeled precursor prior to the active production of a metabolite (24). Hplc analysis of the culture fermentation broth following centrifugation showed that **1** was detected in the mycelium precipitate (8.69 mg/liter medium) at a level greater than in the supernatant portion (1.18 mg/liter medium).

Utilization of exogenous amino acids for antibiotic production in different media does not usually change the time course of biosynthesis (25); the optimal level of exogenous tryptophan that would not interfere with the growth and fermentation pattern was determined. Varying the levels of tryptophan in tryptophan-free medium demonstrated that the level of **1** progressively increased as the level of exogenous L-tryptophan was increased from 0.05 mM to 0.5 mM. Levels of tryptophan beyond 0.5 mM resulted in a decrease in staurosporine production. But it should be noted that the organism can also synthesize staurosporine in the absence of exogenous tryptophan, because **1** was present in tryptophan-free cultures. These results suggested that staurosporine biosynthesis in *S. staurosporeus* was at least partly under the control of endogenous tryptophan (26), and that the observed effect of exogenous tryptophan may represent an induction of enzyme(s) in the biosynthetic pathway such as is seen in ergot alkaloid biosynthesis (27). Experiments varying the levels of tryptophan in the fermentation medium resulted in no significant change of staurosporine production, because the soybean medium contained 0.79 mM tryptophan, which exceeds the optimum concentration of either D- or L- exogenous tryptophan. *S. staurosporeus* can utilize both isomers for staurosporine biosynthesis with the slight preference being for L-tryptophan.

As discussed previously, inspection of the structure of staurosporine suggested a biosynthetic origin from tryptophan. Furthermore, based on the time-course study, the optimal time for precursor addition was found to be at the onset of significant antibiotic

production, which should result in high incorporation (28,29). At this time, after 24 h of fermentation, staurosporine biosynthetic activity was indicated by hplc to be approximately 0.16–2.0 mg/liter of staurosporine in the culture medium. After L-[β-^{14}C] tryptophan was fed to 24-hour-old cultures of *S. staurosporeus*, staurosporine was isolated from the cultures 41 h later and purified to constant specific radioactivity. The result (1.34% incorporation) indicated an effective precursor role for L-tryptophan in staurosporine [**1**] biosynthesis.

In order to establish whether tryptophan was incorporated intact, a mixture of L-[5-^{3}H] tryptophan and L-[β-^{14}C] tryptophan (^{3}H/^{14}C = 11.93) was fed. If the ratio of the two radionuclides is doubled, it would show that only one unit of tryptophan is incorporated intact while the other indole unit is incorporated after loss of the side chain. The results revealed that the ^{3}H/^{14}C ratio in the isolated staurosporine remained the same (^{3}H/^{14}C = 11.17) as that in the precursor, indicating that essentially no loss of tritium or ^{14}C had occurred in the formation of **1**. Therefore, either one or two units of tryptophan were incorporated intact.

Unfortunately, L-[β-^{13}C] tryptophan which might label C-4c and C-7a was unavailable; consequently it was decided to feed commercially available DL-[α-^{13}C] tryptophan (0.46 mM). It was anticipated that the addition of a mixture of two enantiomers would have no effect on staurosporine production, based on the results from the study of regulation by amino acid discussed previously. Subsequent to the feeding and isolation, the proton-decoupled ^{13}C-nmr spectrum of enriched staurosporine was compared with the reference spectrum under identical conditions. At 173.62 and 45.95 ppm, enhanced signals were present which, according to the resonance assignments for staurosporine [**1**] (19), were C-5 and C-7 of the aglycone moiety, the atoms corresponding to the α-carbons of the tryptophan precursor units. Enrichment factors of C-5 and C-7 over natural abundance were 17.7 and 9.2, respectively, thereby establishing that staurosporine [**1**] is indeed produced from two intact tryptophan units. It remains to be seen if the differential incorporation of tryptophan into the two units of staurosporine represents the involvement of an unsymmetrical intermediate or is due to some other factors.

To obtain more conclusive evidence for the potential participation of acetate in the biosynthetic route, feeding experiments with [1,2-$^{13}C_2$] NaOAc and [1-^{13}C] NaOAc were investigated in a similar manner. The ^{13}C-nmr spectral results indicated that no specific incorporation had occurred.

Based on the results of the feeding experiments, the proposed biosynthetic building blocks which comprise the staurosporine aglycone moiety are two units of tryptophan with the two carbon side-chains intact. In the metabolic pathway from tryptophan to staurosporine, at least two different pathways (that is, unsymmetrical or symmetrical modification of tryptophan) are possible. Experiments that would serve to distinguish these alternative routes of formation, as well as to investigate the mechanism of formation of the indolo[2,3-*a*]carbazole moiety, and determine the precursor units of the amino sugar in staurosporine [**1**] are in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler-type hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Uv spectra were recorded with a Beckman model DU-7 spectrophotometer and ir spectra were obtained with a Nicolet MX-1 interferometer. Mass spectra were determined with a Varian MAT 112S double focusing mass spectrometer at 80 eV. The ^{1}H-nmr spectra were obtained with either a Nicolet NMC 360 instrument operating at 360 MHz or a Varian XL-300 instrument operating at 300 MHz. TMS was used as the internal standard, and chemical shifts are reported in δ ppm downfield from TMS.

L-[β-^{14}C] Tryptophan (specific activity 57.1 mCi/mmol; concentration 0.036 mg/0.5 ml for 0.01

mCi) was purchased from New England Nuclear, Boston, MA. L-[5-^{3}H] Tryptophan (specific activity 28 Ci/mmol; concentration 1 mCi/ml) was purchased from Amersham, Arlington Heights, IL. DL-[α-^{13}C] Tryptophan, [1,2-$^{13}C_2$] NaOAc, and [1-^{13}C] NaOAc were purchased from MSD Isotopes.

Adsorption chromatography was performed on columns of Si gel-60 (70–230 mesh, E. Merck, Darmstadt, West Germany) for gravity cc and Si gel-60 (230–400 mesh, E. Merck) for low pressure cc. Tlc was conducted using Si gel GHLF precoated plates (250 μm, 20 × 20 cm, Analtech, Newark, DE) for analytical tlc and precoated PSC-Si gel 60 F_{254} plates (20 × 20 cm, 1 mm layer thickness, E. Merck) for preparative scale. Analytical plates were visualized under uv light, and sprayed with 50% H_2SO_4 or with Dragendorff's reagent. For preparative tlc, the plates were washed by development in Me_2CO prior to application of material. Overpressured layer chromatography (oplc) was carried out with a Chrompres 25 OPLC system (Labor MIM, Budapest, Hungary). Hplc was conducted on an instrument consisting of a system controller, Beckman microprocessor model 421 (Beckman Instruments, Fullerton, CA), Beckman model 110A and 100A pumps, an Altex model 210 injector (Altex Scientific, Berkeley, CA), a model LC-85 variable wavelength uv spectrophotometric detector with a model LC-75 autocontrol (Perkin-Elmer, Norwalk, CT), and an Altex C-R1A integrator recorder (Shimadsu Seisakusho, Kyoto, Japan).

Scanning of thin-layer chromatograms was performed with a Packard model 7201 radiochromatogram scanner (Packard Instrument, Downers Grove, IL). All radioactive measurements were carried out on a Packard Tri-Carb 4640 liquid scintillation counter (Packard Instrument) in aquasol-2 scintillation solution (New England Nuclear). All measurements were done in triplicate. Counting efficiencies at each level of quenching were determined by using a series of quenched standards of [^{14}C]- and [^{3}H]-toluene. Activity of each isotope for dual-label study was calculated in the standard manner (30).

MAINTENANCE OF *S. STAUROSPOREUS*.—*S. staurosporeus* strains AM-2282 and NRRL 11184 were generously supplied in the lyophilized form by Professor S. Omura of the Kitasato Institute, Tokyo, Japan and Dr. A.J. Lyons at the Northern Regional Research Laboratories, ARS, USDA, Peoria, Illinois, respectively. *S. staurosporeus* strains AM-2282 and NRRL 11184 were brought to an active state of growth by transfer to glucose-asparagine agar slants (22) and allowed to grow at 27° for 14 days. The agar slants were stored at 5° and were transferred periodically to a fresh medium every 3 to 4 months. For longer storage, suspensions of spores or mycelia in glucose-asparagine broth were frozen at a controlled rate in a liquid nitrogen cooled container.

FERMENTATION.—*Small scale fermentation*.—Organisms were transferred from glucose-asparagine agar slants to seed medium in cotton-plugged 500-ml Erlenmeyer flasks containing medium (100 ml) on a rotary shaker (250 rpm) and cultured for 2 days at 27°. The seed culture (2 ml) was transferred into a series of cotton-plugged 500-ml Erlenmeyer flasks containing fermentation medium (100 ml) on a rotary shaker and cultivated for 65 h at 27°.

Large scale fermentation.—The seed culture (200 ml) was transferred into a 14-liter jar fermentor (Modular Magnaferm Fermentor Model MA-100, New Brunswick Scientific) containing fermentation medium (7.5 liters) and maintained for 65 h at 27° under the following conditions: aeration, 8 liters/min; agitation, 250 rpm; and foam breaker speed, 2400 rpm.

ISOLATION AND PURIFICATION OF STAUROSPORINE [**1**].—Culture fermentation broth (2 liters) containing mycelia was adjusted to pH 10 with NH_4OH and extracted with EtOAc (3 × 1 liter). The pooled organic phase was further extracted with 0.1 N HCl (3 × 1 liter), and the pooled aqueous fraction was subsequently adjusted to pH 10 and extracted with EtOAc (4 × 1 liter). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness in vacuo at 40°, and the staurosporine was isolated by cc (31), low pressure chromatography (31), or oplc as described below.

Precoated PSC-Si gel 60 F_{254} plates, 20 × 20 cm, 1 mm layer thickness, were obliquely scraped at the plate edges to a width of 3 mm as described by Nyiredy *et al.* (22). The tlc plate edge was polished and then impregnated with Impress II polymer suspension (Labor MIM) to a width of 7–8 mm and allowed to dry at room temperature for several hours. Two thin channels (about 0.5 mm width) were scratched out of the layer at the position that coincided with solvent inlet and drainage on the Teflon sheet. Crude extract was applied to this prepared tlc plate above the solvent inlet channel and the plate placed in the chamber. The water cushion pressure during separation was kept at 20 bar. The plate was pre-equilibrated with hexane within 25 min at a flow rate of 4 ml/min and subsequently run with CH_2Cl_2-Me_2CO (5:1) within 1.5 h at a flow rate of 1.5 ml/min. The eluent was collected with an automatic fraction collector (4.5 ml per fraction), and fractions containing staurosporine were combined and evaporated to dryness in vacuo and further recrystallized from MeOH to afford yellow crystals of staurosporine [**1**].

CHARACTERIZATION OF STAUROSPORINE [**1**].—Staurosporine [**1**], identical with an authentic sample by means of mmp, uv, ir, ms, and ^{1}H-nmr (1), exhibited the following physical and spectroscopic data: mp 288–291° (dec.); $[\alpha]^{22}D$ +56.1° (c = 0.14, MeOH); uv λ max (MeOH) 241.0 (log ε 4.25),

266.0 (4.26), 292.5 (4.53), 321.5 (3.88), 335.0 (3.96), 355.0 (3.81), 372.5 nm (3.85); ir ν max (KBr) 3414, 3054, 2933, 1686, 1673, 1466, 1460, 1453, 1353, 1317, 1117, 744 cm^{-1}; ^{1}H-nmr (360 MHz, $CDCl_3$) see Meksuriyen and Cordell (19); ^{13}C-nmr (90.8 MHz, $CDCl_3$) see Meksuriyen and Cordell (19); ms *m/z* $[M]^+$ 466 (23%), 379 (7), 337 (39), 311 (47), 282 (25), 255 (16), 156 (55).

HPLC ANALYSIS OF STAUROSPORINE [1].—The following operating conditions were used for the hplc analysis of staurosporine: guard column (Waters Associates) packed with μ-Bondapak C_{18}; column, Novapak C_{18} (4 μm particle size), 10 cm × 5 mm i.d. (Waters); eluent, MeCN-0.01 M NaOAc solution (55:45), flow rate 1.8 ml/min, uv detection at 294 nm and sensitivity setting of 0.08 aufs. For quantitative analysis, reserpine (Sigma, 36.67 mg in MeOH, 50 ml) was used as an internal standard. Aliquots were taken to dissolve staurosporine (0.43, 1.05, and 1.44 mg) in a volumetric flask and the volume adjusted to 10 ml. Samples of different concentrations of staurosporine were filtered through a disposable filter unit, and two 5-μl volumes were injected for hplc analysis. Under these hplc conditions, staurosporine and reserpine possessed k′ values of 4.53 and 7.18, respectively. Peak-area ratio and peak-height ratio calibrations with internal standard were performed, and a peak-area ratio calibration was selected to use for the entire study.

TIME-COURSE STUDY OF STAUROSPORINE PRODUCTION.—The titer of staurosporine accumulated in culture broth was analyzed by hplc as follows. The culture broth (100 ml) was harvested at desired time intervals and was basified to pH 10 with NH_4OH. To the basic culture broth was added reserpine (1 mg) as an internal standard for quantitative hplc analysis, and the aqueous phase was extracted with EtOAc (2 × 50 ml). The pooled organic phase was dried (Na_2SO_4), evaporated in vacuo at 40°, and the residue dissolved in MeOH (10 ml), filtered using a disposable filter unit, and analyzed on a reversed-phase Novapak C_{18} column with a guard column attached. Double 20-μl injections were performed for each extract. All samples were extracted in duplicate, and the results indicated that the maximum concentration occurred after about 55 h (31).

ESTABLISHMENT OF THE OPTIMAL UTILIZATION OF EXOGENOUS TRYPTOPHAN FOR STAUROSPORINE BIOSYNTHESIS.—*In fermentation medium.*—A stock solution of L-tryptophan (Sigma) in deionized H_2O (100 mg/100 ml) was prepared and sterilized through a disposable Millipore filter (Sterivex-GS, 0.22 μm, Millipore Co.). Aliquots were transferred in a sterile manner into 500-ml Erlenmeyer flasks containing sterile fermentation medium (100 ml), and the final amino acid concentration was adjusted to 0.005–0.5 mM. Seed cultures (2 ml) were then inoculated and incubated at 27° on a rotary shaker at 250 rpm for 65 h. The cultures were adjusted to pH 10 with NH_4OH, reserpine (1 mg) was added, and the mixture was extracted with EtOAc (2 × 50 ml). The pooled organic layers were dried (Na_2SO_4) and evaporated in vacuo at 40°, and the residue was dissolved in MeOH (10 ml), filtered using a disposable filter unit, and analyzed by hplc using the established conditions. Double 6-μl injections were made at each selected concentration, and experiments at each concentration were performed in duplicate. Staurosporine production varied in a non-systematic manner between 3.12–4.49 mg/liter medium with no trends being discernable.

In tryptophan-free medium.—Tryptophan-free nutrient mixture,[3] used as the production medium, was suspended in deionized H_2O at different concentrations (2, 4, 6, 8, and 10%) and adjusted to pH 7 prior to sterilization. Seed cultures (2 ml) were inoculated into 500-ml cotton-plugged Erlenmeyer flasks containing tryptophan-free nutrient mixture (100 ml) and incubated at 27° on a rotary shaker at 250 rpm for 65 h. The cultures were rendered basic to pH 10 with NH_4OH, reserpine (1 mg) was added, and the mixture was extracted with EtOAc (2 × 50 ml). The organic layer was dried (Na_2SO_4) and evaporated in vacuo at 40°, and the residue was redissolved in MeOH (2 ml), filtered, and analyzed on a reversed-phase Novapak C_{18} column with a guard column attached. Double 10-μl volumes were injected, and all experiments were performed in duplicate. The results are shown in Table 1.

Addition of D- and L-tryptophan into tryptophan-free nutrient mixture.—Stock solutions of D- and L-tryptophan (Sigma) were prepared (0.25 g/50 ml deionized H_2O). Aliquots were transferred in a sterile manner through disposable Millipore filters into 500-ml cotton-plugged Erlenmeyer flasks containing 2% tryptophan-free nutrient mixture (100 ml) and adjusted to the final tryptophan concentration (0.05–2.5 mM). Seed cultures (2 ml) were inoculated and incubated at 27° on a rotary shaker at 250 rpm for 65 h. The cultures were basified to pH 10 with NH_4OH, reserpine (1 mg) was added, and the mixture was extracted with EtOAc (2 × 50 ml). The pooled organic layers were dried (Na_2SO_4) and evaporated in vacuo at 40°, and the residue was dissolved in MeOH (2 ml), filtered using a disposable filter unit, and analyzed by hplc.

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[3]Composition: vitamin free casein hydrolysate, 17.65%; sucrose, 68.63%; vegetable oil, 9.80%; salt mix No. 2 plus vitamin fortification mixture, 3.92%, purchased from United States Biochemical Corporation, Cleveland, Ohio.

TABLE 1. Effect of Tryptophan on Staurosporine Production

% Tryptophan-free nutrient mixture	Average peak-area ratio[a]	Staurosporine production (mg/liter TFD)
2	0.86	1.66
4	0.75	1.44
6	0.69	1.32
8	0.07	0.10
10	0	0
Control (fermentation medium)	8.62	16.94

[a]Average of two integrations.

Double 20-μl volumes were injected, and all experiments were performed in duplicate. The results showed that tryptophan levels beyond 0.5 mM resulted in a decrease in staurosporine production (31).

FEEDING EXPERIMENTS WITH RADIOLABELED SUBSTRATES.—*Incorporation of L-{β-^{14}C} tryptophan.*—Four 500-ml Erlenmeyer flasks containing fermentation medium (100 ml) were inoculated with seed cultures (2 ml). The fermentations were allowed to proceed for 65 h on a rotary shaker at 27°. Addition of L-[β-^{14}C] tryptophan (specific activity 1.49×10^{11} dpm/mmol) was carried out at 24 h. An aliquot (200 μl) of the isotopic medium was withdrawn to count the radioactivity after the immediate addition of the labeled precursor. The cultures were harvested after a total incubation of 65 h, the pH was adjusted to 10 with NH_4OH, and the mixture was extracted with EtOAc (2×50 ml). The combined EtOAc extracts were dried over Na_2SO_4, evaporated in vacuo, and dissolved in MeOH (2 ml). Aliquots of each extract (50 μl) were applied to tlc plates and developed with $CHCl_3$-MeOH (10:1) and C_6H_6-Me_2CO (1:2). These plates were scanned to determine the distribution of radioactivity. Radioactive **1** was isolated by preparative hplc. The conditions for semi-preparative separation were μ-Bondapak C_{18} column (30 cm × 7.8 mm i.d.) eluting with the mobile phase MeCN-0.01 M NaOAc solution (65:35), flow rate 3 ml/min, and an injection load of 2 mg/400 μl. Fractions corresponding to the staurosporine peak were collected and evaporated in vacuo at 40°. The residue was dissolved in a mixture of $CHCl_3$ and MeOH, passed through a small Si gel column to remove traces of inorganic salts, and then evaporated. The **1** isolated ws quantitated by analytical hplc using Novapak C_{18} reversed-phase column eluting with MeCN-0.01 M NaOAc solution (55:45), flow rate 1.7 ml/min, detection uv at 294 nm, with a sensitivity 0.08 aufs at ambient temperature. Aliquots were retained for radioactivity analysis. Cold carrier staurosporine (20 mg) was then added and the staurosporine recrystallized from MeOH to constant specific radioactivity (4.36×10^7 dpm/mmol); incorporation rate 1.34%.

Incorporation of L-{β-^{14}C} tryptophan and L-{5-^{3}H} tryptophan.—Seed culture (2 ml) was inoculated into the fermentation medium (100 ml) and incubated at 27° on a rotary shaker at a speed of 250 rpm. L-[5-^{3}H] Tryptophan and L-[β-^{14}C] tryptophan were added to the production cultures in a sterile manner 24 h after inoculation. An aliquot (200 μl) of the radiolabeled medium was immediately taken to count the radioactivity (^{3}H/^{14}C ratio 11.93). The fermentation was incubated for an additional 41 h following the addition of labeled precursors. Each fermentation was terminated by basification and extraction with EtOAc (2×50 ml); the extract was dried over anhydrous Na_2SO_4 and evaporated in vacuo. Radiolabeled **1** was purified by preparative hplc whose column effluent was quantitated and checked for chemical purity by analytical hplc. An aliquot was retained for radioactivity measurement as described previously. Cold carrier staurosporine (20 mg) was added and the sample recrystallized from MeOH to constant specific activity (^{3}H 3,367,065 dpm/mmol, ^{14}C 300,710 dpm/mmol). The experiment was evaluated in triplicate. The ^{3}H/^{14}C ratio in the isolated staurosporine was 11.17.

FEEDING EXPERIMENTS WITH STABLE ISOTOPES.—*DL-{α-^{13}C} tryptophan.*—Aliquots (10 ml) of DL-[α-^{13}C] tryptophan solution (0.94 mg/ml) were added in a sterile manner through disposable Millipore filters to ten 500-ml Erlenmeyer flasks each containing fermentation medium (100 ml) 24 h after inoculation with seed culture (2 ml). The fermentation was continued for an additional 41 h. Incubation was conducted on a rotary shaker at 250 rpm at 27°. The cultures were adjusted to pH 10 with NH_4OH and extracted twice with EtOAc. The pooled organic phase was dried over Na_2SO_4 and evaporated in vacuo, and the residue was subjected to preparative hplc. Semi-preparative separation of **1** from isotopically enriched incubations was accomplished using μ-Bondapak C_{18} column (30 cm × 7.8 mm i.d.) with an eluent of MeCN-0.01 M NaOAc solution (55:45), flow rate 2 ml/min and an injection load of 2 mg/400 μl. Removal of the solvent from labeled staurosporine-containing fractions was followed by passage through a Si

gel column eluting with mixtures of $CHCl_3$/MeOH for desalting to afford chromatographically pure **1**, which was subjected to mass spectral and ^{13}C-nmr spectral analysis.

{1,2-$^{13}C_2$} NaOAc and {1-^{13}C} NaOAc.—Each aliquot (10 ml) of each aqueous solution (5 mg/ml) of [1,2-$^{13}C_2$] NaOAc and [1-^{13}C] NaOAc was fed in a sterile manner through disposable Millipore filters to ten flasks of culture fermentation medium (100 ml) 24 h after inoculation with seed culture. The experiment was allowed to proceed and the same workup procedure used as in the feeding experiment with ^{13}C-tryptophan.

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