O- and *N*-Methylation in the Biosynthesis of Staurosporine[1]

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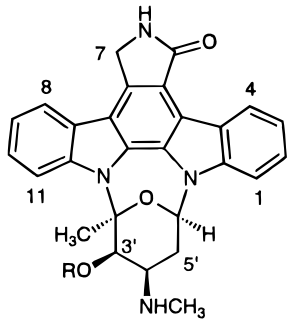
The feeding of ^{13}C- and ^{2}H-enriched methionine to *Streptomyces staurosporeus* established that the methyl carbon and proton source of both the 3′-*O*- and 4′-*N*-methyl groups of staurosporine (**1**) was methionine and that all three methyl protons from methionine were retained on **1**. In the presence of the methyltransferase inhibitor, sinefungin, the biosynthesis of staurosporine was blocked at the last step, *O*-methylation. An intermediate, 3′-demethoxy-3′-hydroxystaurosporine (**2**), was efficiently accumulated in the medium. Other general methyltransferase inhibitors failed to produce any other staurosporine intermediates or analogues.

Staurosporine (**1**), an indolo[2,3-a]carbazole alkaloid, was first isolated from *Streptomyces staurosporeus* (AM-2282) in 1977.[2] Its absolute configuration was determined by X-ray crystallographic analysis[3] and the total synthesis described.[4] Staurosporine (**1**) has various biological effects, including antifungal,[2] hypotensive,[5] and platelet aggregation activities.[6] However, its cytotoxic activity against tumor cells, based on the inhibition of protein kinase C (PKC), is one of the most important aspects of its biological profile.[7,8] Staurosporine derivatives are also capable of reversing the MDR phenotype to sensitive cell lines,[9−11] therefore, indolocarbazole-type alkaloids could potentially serve as anticancer drugs or as therapeutic adjuncts. Two staurosporine derivatives, NB-506 and BMY-27557, are currently in clinical trials for their antitumor activity.[12]

Previous biosynthetic studies disclosed that staurosporine was derived from two tryptophan units.[13,14] Staurosporine aglycon (K-252c), also found in a blocked mutant *Streptomyces longisporoflavus*,[15,16] was probably an intermediate, which conjugated with a glycone moiety derived from one glucose unit with selective proton incorporation and retention.[17,18] Precursor-directed biosynthesis was not successful.[19,20] The 3′-*O*-demethyl derivative of **1**, 3′-demethoxy-3′-hydroxystaurosporine (**2**), was found in a blocked mutant of *S. longisporoflavus* R19[16] and more recently was isolated from the ascidian *Eudistoma toealensis*.[21] Alkaloid **2** was described as a less potent PKC inhibitor than staurosporine,[16] although it showed a selective inhibition pattern to PKC isotypes.[22] The methyl transferase enzyme responsible for the *O*-methylation was partially isolated and used to transform **2** to **1**.[23] These results strongly suggested that *O*-methylation was the final step in staurosporine biosynthesis and prompted us to report our results in this area.[24]

Addition of a methyltransferase inhibitor has been used to accumulate intermediates in the medium. General methyltransferase inhibitors include sinefungin, ethionine, and (*S*)-adenosylhomocysteine,[25] and several successful examples were established from studies on well-known



1 Staurosporine R= CH_3
2 3'-Demethoxy-3'-hydroxy-staurosporine R= H

antibiotics.[26−28] Sinefungin, an antiprotozoal antibiotic isolated from *Streptomyces griseolus*,[29] showed broad inhibition against methyltransferases (Figure 1) and was used to block the *O*-methylation step to accumulate the *O*-demethyl product, *O*-demethyl-citreamicine from *Micromonospora citrea* NRRL 189351.[25] In this paper, several methyltransferase inhibitors, including sinefungin, D,L-ethionine, (*S*)-adenosylhomocysteine, aminopterin, amethopterin, and D-methionine, were evaluated for their ability to inhibit the methyltransferases in staurosporine biosynthesis. We also identify the origin of the *O*- and *N*-methyl groups of staurosporine.

Results and Discussion

Methionine, considered as a general methyl source of *O*- and *N*-methyl groups in secondary metabolism, was evaluated as a precursor of staurosporine. [*methyl*-^{13}C]-L-Methionine (50 mg/100 mL) was fed at 24 h after cell inoculation. Enriched **1** was isolated and its ^{13}C NMR spectrum examined. In the ^{13}C NMR spectrum of enriched **1**, the resonances of the *O*-Me (57.3) and *N*-Me (33.3) groups were enhanced 49- and 44-fold, respectively, compared to natural abundance staurosporine, thereby indicating a high incorporation rate of methionine into both methyl groups in staurosporine (**1**). The further fate of the methyl protons from methionine was examined by [*methyl*-$^{2}H_3$]-L-methionine. When the concentration of deuterium-labeled methionine was lower than 20 mg/100 mL (5, 10, and 20 mg/mL), incorporation was observed in EIMS with a low incorporation rate, and in the ^{1}H NMR the *O*- and *N*-methyl resonances were observed as two broadened

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singlets without a significant diminution of integration. When the concentration of [*methyl*-$^2H_3$]-L-methionine was increased to 30, 40, and 50 mg/100 mL, the methyl proton signals were both strongly suppressed to about half the proton integration compared to unlabeled 2′-CH_3 (3H) and 3′-H (1H) in **1**. The EIMS of labeled **1** also supported that all of the deuterium atoms on labeled-methionine were retained. The *O*- and *N*-methyl protons are directly derived from methionine during the biosynthesis of **1**, especially from the observation of molecular ion peaks of *m*/*z* 472, 469, and 466, and the glycone fragment ion peaks, *m*/*z* 162, 159, and 156 in the EIMS spectrum. Low concentrations of labeled methionine did not show a good incorporation rate, probably because they were utilized for cell growth before staurosporine biosynthesis and were diluted by the internal biosynthesis of methionine. The incorporation of deuterium oxide into the *O*- and *N*-methyl groups[18] was observed, possibly caused by deuterium incorporation during methionine biosynthesis in the microorganism instead of during the biosynthesis of **1**. After the methyl group source of **1** was determined, interest was focused on the inhibition of these processes to generate the 3′-demethoxy-3′-hydroxy-, 4′-*N*-demethyl- or 3′-demethoxy-4′-*N*-demethyl-3′-hydroxy intermediates.

Various methyltransferase inhibitors (5 mg/100 mL) were preliminarily examined for their ability to inhibit the methyltransferases involved in staurosporine biosynthesis. *S. staurosporeus* culture was supplemented individually with sinefungin, D,L-ethionine, (*S*)-adenosylhomocysteine, aminopterin, amethopterin, and D-methionine, and the broth was analyzed for the production of demethyl intermediates. From the analysis of the EtOAc extract of the broth by TLC, only sinefungin was able to produce a derivative with a lower R_f value and with the same UV absorption as **1**. All of the other methyltransferase inhibitors failed to produce any new staurosporine intermediates or analogues, and the biosynthesis of staurosporine was not blocked. A derivative of **1** accumulated when sinefungin was added at 2 mg/100 mL. The product was isolated, purified, and evaluated by ^1H NMR and EIMS. The ^1H NMR spectrum did not show the *O*-Me signal in the region of δ 3.3, and the molecular ion in EIMS was observed at *m*/*z* 452, which was 14 daltons less than that of **1**. The remaining NMR spectral characteristics in $CDCl_3$ at 25 °C corresponded to those of **1** and were close to the data published by Hoehn's group (DMSO, 80 °C).[16] The assignments of the ^1H NMR resonances in $CDCl_3$ are based on the $^1H-^1H$ COSY spectrum and the comparison to those of **1**.

Sinefungin was added in different final concentrations of 0.1, 0.5, 1.0, 1.5, 2.8, and 5 mg/100 mL to each 100 mL broth of *S. staurosporeus* at 24 h after inoculation of seed medium, respectively. Sinefungin did inhibit the growth of *S. staurosporeus*. Cell growth was inhibited at the concentrations of 1.5 to 2.8 mg/100 mL to the extent of 1/2 to 2/3 volume of full growth, and at 5.0 mg/100 mL supported only 1/10 cell volume of full growth. The harvested cells were extracted and analyzed by TLC. Metabolite **2** was observed in the presence of sinefungin at concentrations of 0.5 to 5.0 mg/100 mL, and the yield of **2** was proportional to the applied concentration of sinefungin. At higher applied concentrations of sinefungin (>1.5 mg/100 mL) inhibition of cell growth was observed, further lowering the yield of **2**.

The quantitative analysis of the production of **1** and **2** vs different concentrations of sinefungin was monitored by HPLC with an internal standard, reserpine (Figure 1).

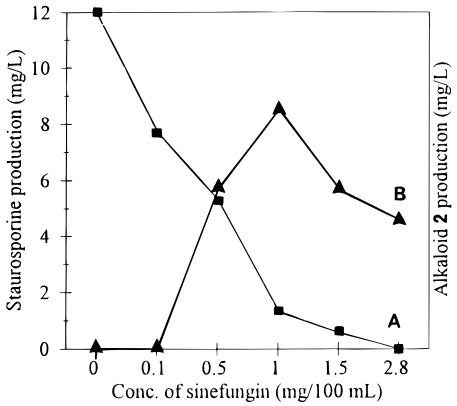

Figure 1. Effect of sinefungin on staurosporine biosynthesis: A (■), staurosporine (**1**); B (▲), 3′-demethoxy-3′-hydroxy-staurosporine (**2**).

Under the conditions described in the Experimental Section, the retention times of compounds **1**, **2**, and reserpine were 5.2, 3.2, and 7.8 min, respectively. It was found that the production of staurosporine was inversely proportional to the concentration of added sinefungin. The yield of **2** was 0.5 mg/100 mL after growth for an additional 7−10 days at a concentration of 2.8 mg/100 mL of sinefungin. The results showed that inhibition is efficient when the concentration of sinefungin is higher than 1 mg/100 mL, and that it was the 3′-*O*-methylation step which was almost totally inhibited. This result supported 3′-*O*-methylation as the final step in the pathway of staurosporine biosynthesis.

In conclusion, the carbon and proton source of the *O*- and *N*-methyl groups of staurosporine (**1**) from methionine was determined. The 3′-*O*-methyltransferase could be inhibited selectively by sinefungin, and, as a result, intermediate **2** accumulated. The 4′-*N*-demethyl product was not found during the sinefungin feeding experiment, indicating that sinefungin is not an inhibitor of an *N*-methyltransferase in this case.

Experimental Section

General Experimental Conditions. ^1H NMR and $^1H-^1H$ COSY spectra were recorded on either a Nicolet NT-360 instrument operating at 360 MHz or a Varian XL-300 instrument operating at 300 MHz. EIMS were obtained using a Finnigan MAT 90 instrument. ^{13}C NMR spectra were obtained using a Nicolet NT-360 (360 MHz) spectrometer operating at 90.8 MHz. The samples were dissolved in $CDCl_3$. [*methyl*-^{13}C]-L-Methionine (98%) and [*methyl*-$^2H_3$]-L-methionine (98%) were purchased from Cambridge Isotopes Laboratory (Andover, MA). Reserpine was purchased from Sigma, and sinefungin was obtained as a gift from Rhone−Poulenc−Rorer (Vitry-sur-Seine, France).

Maintenance and Fermentation of *S. staurosporeus*. *S. staurosporeus* strain NRRL 11184 was generously supplied in the lyophilized form by Dr. J. L. Swezey of the Midwest Area National Center for Agricultural Utilization Research, ARS, USDA (Peoria, IL). The activation, maintenance, and fermentation of *S. staurosporeus* are the same as previously described.[13,17,30]

Feeding Experiments with Stable Isotopes. Aliquots (10 mL) of [*methyl*-^{13}C]-L-methionine (50 mg/mL) were added in a sterile manner through disposable Millipore filters to five 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 5 days. Incubation was conducted on a rotary shaker at 250 rpm at room temperature. After harvesting, the resulting alkaloid mixture was purified as usual.[17,18] The labeling pattern was

deduced using ^{13}C NMR spectral analysis, and the significantly enriched carbons were determined.

[*methyl*-$^2H_3$]-L-Methionine was added to the 250-mL flask containing 100 mL of the fermentation medium at the final concentrations of 5, 10, 20, 30, 40, and 50 mg/100 mL individually at 12 h after inoculation of seed medium. The cultures were allowed to grow for 4 days more. Further extraction and isolation procedures were the same as previously described.[16] The yield of labeled **1** at the feeding concentration of [*methyl*-$^2H_3$]-L-methionine 50 mg/mL was 0.4 mg/100 mL: EIMS *m/z* 472 $[M + d_6]^+$ (48), 469 $[M + d_3]^+$ (32), 466 $[M]^+$ (17), 349 (36), 348 (42), 337 (93), 308 (37), 282 (24), 254 (15), 162 [glycone + d_6]$^+$ (78), 159 [glycone + d_3]$^+$ (55), 156 [glycone]$^+$ (28), 94 (92), 91 (100). The labeling pattern was deduced using ^1H NMR and EIMS spectral analysis to determine the significant deuterium labeling.

Addition of Methyltransferase Inhibitors. Preliminary evaluation of the application of the methyltransferase inhibitors sinefungin, D,L-ethionine, (*S*)-adenosyl-homocysteine, aminopterin, amethopterin, and D-methionine was performed at a concentration of 5 mg/100 mL. Inhibitor (5 mg) in sterilized H_2O (1 mL) was added through a disposable Millipore filter into the broth culture 12 h after inoculation of the seed medium. The cultures were allowed to grow for 5−10 days further, and were harvested, extracted, and evaluated by TLC.

Further sinefungin dose−response experiments were performed at the concentrations of 0, 0.1, 0.5, 1.0, 1.5, and 2.8 mg/100 mL. Solutions (1 mL) of different concentrations of sinefungin in H_2O were added in a sterilized manner to each 100 mL of broth of *S. staurosporeus* in Erlenmeyer flasks 24 h after inoculation of seed medium, respectively. After growth for 5 to 10 days, each broth sample was extracted with EtOAc (100 mL, 3 times), and the extract was dried and evaporated in vacuo. The dried extract was dissolved in MeCN (1 mL) for the TLC and HPLC analyses.

Isolation and Purification of 3′-Demethoxy-3′-hydroxystaurosporine (2). The isolation and separation methods were similar to those described for staurosporine.[14] Compound **2** (R_f 0.3, compared to R_f 0.5 of **1**) was isolated and purified twice by preparative TLC in the solvent system $CHCl_3$−MeOH (10:1).

3′-Demethoxy-3′-hydroxystaurosporine (2): ^1H NMR ($CDCl_3$, 300 MHz) 9.42 (1H, d, J = 7.9 Hz, H-4), 8.15 (1H, d, J = 8.9 Hz, H-11), 7.89 (1H, d, J = 7.3 Hz, H-8), 7.52 (1H, t, J = 7.9 Hz, H-2), 7.46 (1H, t, J = 7.9 Hz, H-10), 7.39 (1H, t, J = 7.5 Hz, H-3), 7.35 (1H, t, J = 7.9 Hz, H-9), 7.31 (1H, d, J = 7.9 Hz, H-1), 6.59 (1H, d, J = 4.9 Hz, H-6′), 6.27 (1H, br s, −NH), 5.06 (1H, d, J = 17.1 Hz, H-7a), 4.99 (1H, d, J = 17.1 Hz, H-7b), 4.17 (1H, d, J = 5.5 Hz, H-3′), 2.99 (1H, ddd, J = 4.3, 4.3, 4.3 Hz, H-4′), 2.78 (1H, dd, J = 15.7, 3.0 Hz, H-5′), 2.50 (1H, dt, J = 15.1, 5.2 Hz, H-5′), 2.29 (3H, s, 2′-CH_3), and 1.88 (3H, s, *N*-CH_3), EIMS *m/z* 452 $[M]^+$, 337, 311, and 142.

Analytical Methods. For TLC analysis, culture samples were extracted with an equal volume of EtOAc three times, and the EtOAc extract was dried in vacuo. Aliquots of the organic phase were deposited on Si gel plates. After development with the solvent system $CHCl_3$−MeOH (10:1) twice, the separated spots were visualized by UV at 254 nm or by spraying with Dragendorff's reagent.

A quantitative analysis of the yield of **2** under different concentrations of sinefungin was carried out by HPLC coupled with a photodiode-array UV detector. The calibration of the concentration of staurosporine by an internal standard, reserpine, was performed by calculation of the peak−area ratio of different known concentrations of staurosporine vs those of a fixed concentration of reserpine.[30] For quantitative HPLC analysis, an organic extract of the broth samples was dissolved in MeCN (1 mL) and mixed with reserpine (0.25 mg) in MeCN (1 mL). The mixed aliquot (10 L) was injected on a reversed-phase column (Waters, Nova-Pak C_{18}, 4 m, 3.9 × 150 mm), and the eluent consisted of 0.01 M NaOAc aqueous buffer and MeCN in a fixed ratio (45:55). With a constant flow rate of 1 mL/minute and UV detection at 290 nm, the retention times of alkaloids **1**, **2** and reserpine were 5.2, 3.2, and 7.8 min, respectively. Due to the similar chromophore of alkaloids **1** and **2**, and their similar molecular weight and ϵ value,[13,15] the concentration of compound **2** was calculated through the calibration of the peak-area ratio of staurosporine vs reserpine.

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References and Notes

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