Origin of Nitrogen in the Indolocarbazole Unit of Staurosporine†

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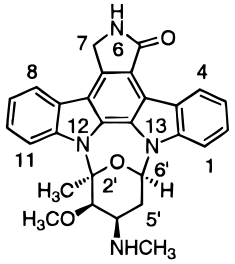
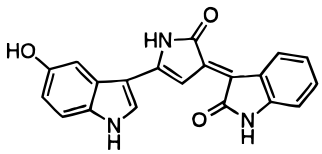
The fate of the nitrogen atoms in tryptophan during biosynthesis of the antitumor antibiotic staurosporine (**1**), a potent PKC inhibitor, has been studied using [$^{15}N_2$]-L-tryptophan and $^{15}NH_4NO_3$. The results indicate that the nitrogen atom on the side chain of tryptophan is cleaved during staurosporine synthesis.

Staurosporine (**1**), an indolo[2,3-*a*]carbazole alkaloid, was first isolated from *Streptomyces staurosporeus* (AM-2282) in 1977.[2] It has been found that **1** has very interesting biological properties, including antifungal,[2] hypotensive,[3] and platelet aggregation activities.[4] Its cytotoxic activity against tumor cells, based on the inhibition of protein kinases, is the most important aspect of its biological profile.[5,6] In recent studies, its absolute configuration was determined by X-ray crystallographic analysis,[7] and the total synthesis of **1** has also been successfully completed.[8] Some derivatives of **1**, including NA-382, UCN-01, and CGP41-251, have demonstrated the ability to reverse multidrug resistance, especially when they are combined with conventional chemotherapy during administration.[9−12] From this recent progress, it has become apparent that a PKC inhibitor may well become a candidate for a new class of clinically useful antineoplastic drugs or as an adjunct against clinically resistant tumor cells.

Previously, we described the ^{13}C- and ^{2}H-labeling of staurosporine (**1**) in studies of its biogenetic origin using electron impact mass spectrometry (EIMS) and NMR techniques.[1,13] Hence, the biosynthetic building blocks of **1** were determined as two tryptophan moieties,[14] one glucose unit,[1] and two methyl groups from methionine.[15] In our continuous work, [α-^{14}C]tryptamine was successfully incorporated into staurosporine; therefore, interest focused on the fate of the nitrogen atoms on tryptophan and of the α-protons on tryptamine during incorporation.



1 Staurosporine



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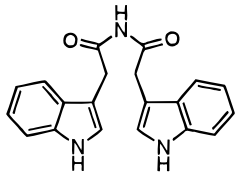

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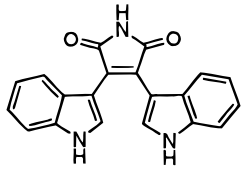

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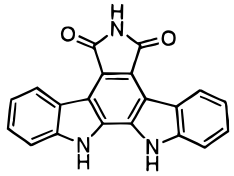

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Results and Discussion

Tryptamine was determined to be a precursor of staurosporine through the detection of radioactivity in staurosporine using radiolabeled [β-^{14}C]tryptamine (Experimental Section). Whether the hydrogens at C-7 are derived directly from tryptamine is a critical point with respect to determining the intermediates in the pathway. Therefore, [α-$^{2}H_2$]tryptamine was used to observe the fate of the α-protons in tryptamine. Staurosporine obtained from the feeding of [α-$^{2}H_2$]tryptamine was examined by ^{1}H-NMR and EIMS. In the ^{1}H-NMR, the integration of H_2-7 remained at two protons compared to H-3′, H-4′, and H-6′. In the EIMS, no significant shift of the molecular fragment ion *m*/*z* 466 was observed, and the calculation of relative enrichment of the ions at *m*/*z* 467 and 468 did not show significant enhancement due to deuterium labeling (5% and 2% enhancement in M + 1 and M + 2 peaks, respectively). By contrast, the tryptamine metabolites, *N*-acetyltryptamine and (3a*R*,8a*S*)-1-acetyl-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-*b*]indol-3a-ol, were isolated with high deuterium incorporation (>90%) which could be directly observed in the ^{1}H NMR.[16] From the fact that no significant retention of either of the α-deuterium atoms of tryptamine occurred during the biosynthesis of **1**, the proposed pathway in which tryptamine directly conjugates with an indole-3-acetate moiety to form an intermediate bisindole moiety should not be the major biosynthetic pathway of **1**. Tryptamine probably undergoes side

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chain α-oxidation leading to the loss of both α-protons followed by conjugation with another unit.

From the result (1.34% incorporation) of the previous incorporation experiment using [β-^{14}C]-L-tryptophan and [α-^{13}C]-L-tryptophan, tryptophan was determined as a effective precursor in staurosporine biosynthesis.[14] [$^{15}N_2$]-L-Tryptophan was used to investigate the fate of the nitrogen atoms during the biosynthesis of **1**, especially the side-chain nitrogen atom. Since tryptophan has been determined to be a precursor of the bisindole moiety in **1**, it was anticipated that at least the nitrogen atoms of the indole ring would be incorporated into the indolocarbazole moiety of **1**.

Two sets of fermentation conditions with different concentrations of [$^{15}N_2$]-L-tryptophan, 10 and 30 mg/100 mL, were evaluated, and the yield of labeled **1** at the feeding concentrations was 1 and 0.5 mg/100 mL, respectively. The concentration of 10 mg/100 mL of labeled tryptophan was used to scale up the production of labeled **1** for ^{13}C NMR. The resulting labeled staurosporine (**1**) was investigated by both ^{1}H-NMR, ^{13}C-NMR, and EIMS. In the EIMS, when 10 mg/100 mL [$^{15}N_2$]-L-tryptophan was used, the molecular weight region of the labeled substrate was observed as *m*/*z* 466 (22), 467 (24), 468 (17), and 469 (4) which indicated that either none, one, or two units of labeled tryptophan were incorporated into **1** (incorporation ratio: 1:0.77:0.49:0; none:one:two:three ^{15}N incorporation). When 30 mg of [$^{15}N_2$]-L-tryptophan was fed, the molecular weight region of the labeled staurosporine was observed as *m*/*z* 466 (10), 467 (25), 468 (25), and 469 (7), which indicated that a higher incorporation rate had occurred (incorporation ratio: 1:2.2:1.7:0.03; none:one:two:three ^{15}N incorporation). The incorporation ratio for three ^{15}N atoms on the same molecule compared to all ^{15}N incorporation is close to zero percent (1%), which is in the error range, indicating that the nitrogen on the side chain was essentially lost at some point during staurosporine biosynthesis. This was not expected since when the side chain nitrogen is lost through transamination it commonly has a lower (20−40% retention), but not a zero incorporation rate for an amino acid precursor.[17] In the examination of the ^{1}H-NMR spectrum of labeled **1**, no ^{1}H−^{15}N coupling was detected at the 6-N*H* (δ 6.44) in 3000 scans, which also suggests that the side chain ^{15}N was not retained and that N-6 was not derived from tryptophan. By comparison, corresponding ^{15}N labeling experiments with violacein (**2**) showed that 29% of the nitrogen on the side chain from tryptophan was retained.[18] The specific site of ^{15}N incorporation into **1** was determined by ^{13}C-NMR through the observation of the couplings between ^{15}N−^{13}C, shown in Figure 1. The coupling constants were observed as 15.8 Hz for C-11a, 15.8 Hz for C-13a, 17 Hz for C-12a, 17 Hz for C-12b, 9 Hz for C-2′, and 11 Hz for C-6′. From the coupling constants and the intensity of the couplings, the symmetrical structure of the indolocarbazole and the symmetric incorporation of two tryptophan units were again demonstrated.

A high concentration of $^{15}NH_4NO_3$ fed with tryptophan was used to incorporate ^{15}N at the α position of the side chain of tryptophan through transamination,[18] and would be expected to further incorporate into N-6 in **1**, if the ^{15}N was not cleaved from the α-carbon in tryptophan during staurosporine biosynthesis. From

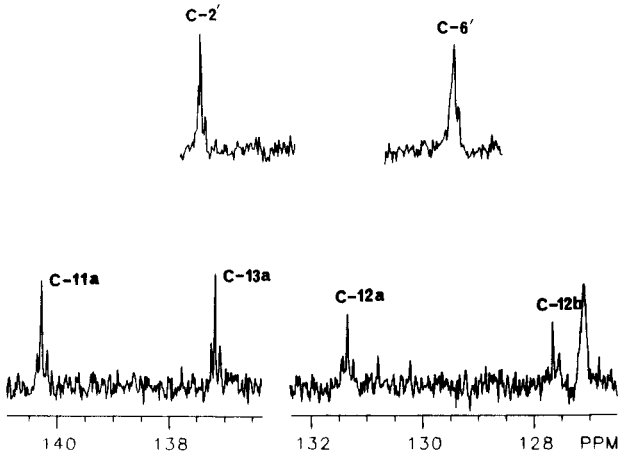

Figure 1. Partial ^{13}C-NMR of ^{15}N-labeled **1** from [$^{15}N_2$]-L-tryptophan feeding experiment.

the examination of the ^{1}H-NMR, ^{13}C-NMR, and EIMS spectra of the product, no significant incorporation of ^{15}N was observed into either indolocarbazole or glycon moieties. Compared to the 50% incorporation of ^{15}N using a similar method during violacein biosynthesis,[18] it appears that the side-chain nitrogen in tryptophan is cleaved during staurosporine biosynthesis.

The results of the loss of the side chain nitrogen atom on tryptophan and the loss of both α deuterium atoms in the tryptamine-labeling experiment suggests that in the major pathway either tryptophan or tryptamine undergo oxidation on the α carbon, leading to complete loss of the side chain nitrogen and the α protons, respectively, before the incorporation of the indole units into **1**. Some potential intermediates which could meet these criteria are **3**, **4**, and **5**. The involvement of such intermediates in the biosynthesis of **1** is presently under investigation. The origin(s) of the nitrogen atoms for N-6 and for the 4′-*N*HCH$_3$ in **1** remain to be investigated.

Experimental Section

General Experimental Procedures. ^{1}H NMR spectra were recorded on a Varian XL-300 instrument operating at 300 MHz. ^{13}C NMR spectra were obtained using a Nicolet NT-360 (360 MHz) spectrometer operating at 90.8 MHz. EIMS spectra were obtained using a Finnigan MAT 90 instrument with the parameters as follows: scan range *m*/*z* 50-898; scan speed 1 scan/s; probe temperature 30−300 °C/10 min; acceleration voltage 4675 V; source temperature 200 °C; electron energy 70 eV. [β-^{14}C]-L-Tryptamine bisuccinate was purchased from American Radiolabeled Chemical, St. Louis, MO. [$^{15}N_2$]-L-Tryptophan (96−99%) and $^{15}NH_4NO_3$ (99.9%) were purchased from Cambridge Isotope Laboratories, Andover, MA.

Organism and Culture Conditions. *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 and maintenance of *S. staurosporeus* are the same as previously described.[1,14] The fermentation methods were the same as those previously described.[1,14]

[β-^{14}C]Tryptamine Bisuccinate Feeding Experiment. Radiolabeled substrate (specific activity 55 μCi/mmol; concentration 50 μCi/mL) was added to the

fermentation medium immediately after cell inoculation. The cells were allowed to grow for 3 days and were harvested in the same manner as described previously.[1] The EtOAc extract was dried and evaporated, and the residue was applied to a silica gel TLC plate and developed with EtOAc−$CHCl_3$ (1:1) followed by a second development using $CHCl_3$−CH_3OH (10:1) as the eluting system. Staurosporine was detected under UV light (254 nm), and the observed fluorescence quench from staurosporine was marked. The marked plate was scanned using a TLC radioactive scanner. The area of radioactivity exactly matched the area observed under UV light.

Synthesis and Feeding of [α-$^2H_2$]Tryptamine. The synthesis and feeding of [α-$^2H_2$]tryptamine was described in a previous paper in the metabolism studies.[16] The yield of **1** was 0.8 mg/100 mL, and the product was examined by ^{1}H-NMR and EIMS for determination of the labeling pattern.

Incorporation of [$^{15}N_2$]-L-Tryptophan. [$^{15}N_2$]-L-Tryptophan (10 mg each) was added, separately, to the five individual fermentation media (100 mL each) 12 h after inoculation of seed medium, and the cultures were allowed to grow for an additional 4 days. Another experiment using a higher concentration of [$^{15}N_2$]-L-tryptophan (30 mg/100 mL medium) was conducted using the same method in three individual fermentation media (100 mL each).

[12,13-$^{15}N_2$]-Enriched Staurosporine (1). The extraction and isolation procedures were the same as previously described.[1] The yields of labeled **1** at the feeding concentrations were 1 and 0.5 mg/100 mL, i.e. 6 and 1.5 mg total, from the 10 and 30 mg [$^{15}N_2$]-L-tryptophan feeding experiments, respectively. EIMS of **1** from the addition of 10 mg of [$^{15}N_2$]-L-tryptophan: 466 (22), 467 (24), 468 (17), 469 (4), 337 (25), 338 (22), 339 (16), 309 (13), 310 (11), 311 (9), 282 (10), 283 (9), 284 (7), 254 (28), 255 (9), 156 (69), 88 (100); ^{13}C-NMR of **1** from the addition of 10 mg of [$^{15}N_2$]-L-tryptophan, see Figure 1 and text.

Incorporation of $^{15}NH_4NO_3$ and L-Tryptophan. $^{15}NH_4NO_3$ (10 mg/100 mL) and L-tryptophan (10 mg/100 mL) were added to two individual fermentation media (100 mL each) immediately after inoculation of the seed medium, and the cultures were allowed to grow for an additional 4 days. Further extraction and isolation procedures were the same as previously described.[1] The yield of **1** at the feeding concentration was around 1.8 mg/100 mL, i.e. 3.6 mg total for each experiment, and the staurosporine isolates were examined using EIMS analysis and ^{1}H- and ^{13}C-NMR techniques.

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