Deuterium Labeling and Mass Fragmentation Studies of Staurosporine†

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Received July 26, 1996⊗

The mass fragmentation pattern of the antitumor antibiotic staurosporine (**1**), a potent PKC inhibitor, has been studied by HREIMS and by using deuterium-labeled samples of **1**, derived from incorporation experiments with [6-$^2H_2$]-D-glucose, [2-^{2}H]-D-glucose, [U-$^2H_7$]-D-glucose, and deuterium oxide.

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 kinase C, 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** have demonstrated the ability to reverse multidrug resistance to sensitive cell lines. NA-382 (**2**), a 7-oxostaurosporine (**3**) derivative, exhibits higher selectivity against protein kinase C (PKC) and also shows the effect of intracellular accumulation of vinblastine in adriamycin-resistant P-388 cells that have the multiple-drug-resistant phenotype.[9,10] The combination of UCN-01 (**4**, a 7α-hydroxystaurosporine) and mitomycin C was active against mitomycin-insensitive tumors, and this has resulted in a clinical trial.[11] 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 deuterium labeling of staurosporine (**1**) in studies of its biogenetic origin using electron-impact mass spectrometry (EIMS) and ^{1}H-NMR.[1] Mass fragmentation studies have aided in the identification of the labeled positions of the stable isotopes in secondary metabolites. During the course of this work, it became clear that no studies had been described examining the mass spectra of this important series of compounds. In this paper, we describe how the two principal mass fragmentation pathways of **1** under electron impact were elucidated by high-resolution electron-impact mass spectrometry (HREIMS) and the EIMS of labeled and unlabeled **1**.

The individually ^{2}H-labeled staurosporines were obtained from [6-$^2H_2$]-D-glucose, [2-^{2}H]-D-glucose, [U-$^2H_7$]-D-glucose, and D_2O feeding experiments with *Streptomyces staurosporeus*.[1] From the EIMS of these metabolites, combined with the HREIMS of unlabeled **1**, the major fragmentation pathway of **1** in EIMS was proposed. This evidence could further assist the identification of the locations of deuterium incorporation during staurosporine biosynthesis, especially in deuterium-labeled **1** obtained from a deuterium oxide incorporation experiment.

Results and Discussion

In the EIMS of staurosporine (**1**), a molecular ion peak was observed at *m/z* 466 together with principal fragment ions at *m/z* 379 (10), 337 (59), 308 (8), 156 (100), and 87 (40) (Table 1). The aminosugar fragment ion B (*m/z* 156, $C_8H_{14}NO_2$, 100) was identified by HREIMS (obsd 156.1020; calcd 156.1024). Both UCN-01 (**4**) and UCN-02 (**5**), which have the same glycon moiety as that in staurosporine, also display a glycon fragment ion at *m/z* 156.[12] Fragment ion B, *m/z* 156, was also corroborated by deuterium-labeling experiments. From the differently deuterium-labeled samples of **1**, the glycon peaks (*m/z* 156) primarily shift 1, 2, and 5 units, compared to the EIMS of unlabeled **1**, for the [2-^{2}H]-D-glucose, [6-$^2H_2$]-D-glucose, and [U-$^2H_7$]-D-glucose feeding experiments, respectively. The specific labeling has been determined by ^{1}H NMR and described in a previous paper.[1] Other evidence comes from the [C^2H_3]-L-methionine feeding experiment; fragment ion B was shifted from *m/z* 156 (natural abundance) to *m/z* 159 and 162 due to one or two deuterium-labeled methyl(s) incorporation (data will be published elsewhere). The mechanism considered for this fragmentation is shown in Figure 1, where it is proposed that charge localization occurred on the nitrogen atom at the 4′-*N*HCH$_3$ group, followed by electron migration causing double *N*-glycosidic linkage cleavage, and then the loss of a hydrogen radical from 3′-H resulting in the generation of the glycon fragment ion B (*m/z* 156, pathway I in Figure 1). The fragment ion cluster with a relative weak intensity, which appeared at *m/z* 308, was proposed as the aglycon fragment ions, aglycon A (*m/z* 309) and A′ (*m/z* 308) (pathway II in Figure 1).

Two fragment ions, D (C_4H_9NO, *m/z* 87) and E ($C_{22}H_{11}N_3O$, *m/z* 337), were identified by HREIMS (obsd 87.0695 and 337.1210; calcd 87.0684 and 337.1215). The EIMS spectra of both UCN-01 (**4**) and UCN-02 (**5**) show a corresponding fragment ion at *m/z* 353,[12] which resembles fragment ion E plus an oxygen atom, and indicates that fragment E contains C-7 in the aglycon part. The proposed mechanism considered for this fragmentation pathway is shown in Figure 2. Decyclization of the glycon ring of **1** leads to two fragment ions C (*m/z* 379) and D (*m/z* 87). Fragment C is then

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

S0163-3864(96)00567-8 CCC: $14.00 © 1997 American Chemical Society and American Society of Pharmacognosy

Table 1. Major Fragment Ion Peaks and Relative Intensities or Incorporation Intensities of Naturally Occurring and Deuterium-Labeled Staurosporine[a]

staurosporine ion fragments	precursor fed: glucose	[2-^{2}H]-D-glucose	[6-$^2H_2$]-D-glucose	[U-$^2H_7$]-D-glucose	D_2O (40%) (with tryptophan)	D_2O (40%) (without tryptophan)
molecular ion[b]	466 (78) 467 (26) 468 (4)	466 (100) d (53)	467 (38) d (62), d_2 (60), d_3 (35)	466 (5), d (13), d_2 (28), d_3 (45), d_4 (56), d_5 (64), d_6 (62), d_7 (49), d_8 (37), d_9 (15), d_{10} (15), d_{11} (5)	466 (56), d (44), d_2 (40), d_3 (44), d_4 (40), d_5 (31), d_6 (18), d_7 (9)	466 (19), d (31), d_2 (55), d_3 (67), d_4 (61), d_5 (45), d_6 (26), d_7 (13)
fragment ion B[b] (aminosugar)	156 (100) 157 (9)	156 (69) d (35)	156 (37) d (24), d_2 (75), d_3 (29)	156 (14), d (26), d_2 (43), d_3 (47), d_4 (55), d_5 (44), d_6 (32)	156 (30), d (30), d_2 (27), d_3 (29), d_4 (24), d_5 (16)	156 (16), d (31), d_2 (44), d_3 (42), d_4 (28), d_5 (15)
fragment ion E[b]	337 (59) 338 (18) 339 (3)	337 (92) d (37)	337 (95) d (71), d_2 (39)	337 (46), d (72), d_2 (75), d_3 (66), d_4 (52), d_5 (30), d_6 (16)	337 (98), d (70), d_2 (38), d_3 (16), d_4 (4)	337 (79), d (76), d_2 (58), d_3 (32), d_4 (14)
fragment ion A[c] (aglycon)	308 (8) 309 (6)	308 (17) 309 (16)	309 (25) 310 (28) 311 (36) 312 (24)	310 (26), 311 (32), 312 (28), 313 (22)	308 (18), 309 (22), 310 (21), 311 (19)	308 (14), 309 (19), 310 (22), 311 (11), 312 (12)
fragment ion C[c]	379 (10)	379 (13) 380 (10) 381 (12)	380 (12) 381 (14) 382 (12) 383 (11)	381 (12) 382 (14), 383 (15), 384 (15), 385 (14)	380 (11), 381 (15), 382 (12), 383 (7)	380 (7), 381 (13), 382 (14), 383 (11)
fragment ion D[c]	87 (40) 88 (41)	87 (44) 88 (47)	87 (82) 88 (66) 89 (51)	87 (60), 88 (72), 89 (73), 90 (47), 91 (22)	87 (26), 88 (40), 89 (31), 90 (25), 91 (16)	87 (16), 88 (32), 89 (38), 90 (27), 91 (15)
fragment ion F[c]	281 (7) 282 (6)	281 (14) 282 (11)	281 (20) 282 (30) 283 (22)	281 (17), 282 (23) 283 (23), 284 (19)	281 (19) 282 (18)	281 (16) 282 (17) 283 (13)
fragment ion G[c]	255 (3) 254 (2)	255 (6) 254 (4)	255 (15) 254 (10)	256 (10) 255 (6)	255 (10) 254 (6)	255 (8) 254 (5)

[a] The EIMS of each labeled or unlabeled **1** was run at least twice under the same conditions. Single spectral data were recorded; however, the error of relative intensity in each cluster (e.g., M, M + 1, and M + 2) obtained from two different experiments was less than 5%. The relative intensity in different fragment ions (e.g., ions B and E) may vary in different labeled **1** or different EIMS, but this difference did not affect the calculation of the deuterium incorporation intensities (d, d_2, d_n). [b] d_n Indicates that the deuterium number incorporated and the intensity were justified by subtraction of the factor of natural abundance ^{13}C isotope. [c] Deuterium incorporation rate was not justified by subtraction of the factor of natural abundant ^{13}C isotope due to weak intensities of these peaks, which may result in high error.

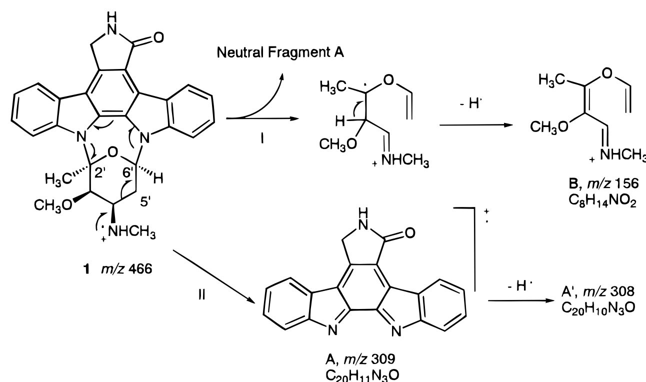


Figure 1. Proposed double *N*-glycosidic linkage cleavage in the fragmentation of staurosporine (**1**) in EIMS. In pathway I, charge localization occurred on the 4′-*N*HCH$_3$ group followed by electron migration and the loss of a hydrogen radical resulting in the generation of the fragment ion B. In pathway II, charge localization occurred on the bisindole resulting in the production of fragments, A and A′.

further cleaved on the amide bond with the loss of an acetyl group and an accompanying hydrogen transfer, resulting in fragment ion E (*m/z* 337). The aglycon fragment A (*m/z* 308) also could be generated from the cleavage of the *N*-vinyl group in fragment E. Proposed fragment ion C ($C_{24}H_{17}N_3O_2$, *m/z* 379) was also observed in the EIMS, but a higher error value was observed in the HREIMS due to the weak intensity (obsd 379.1445; calcd 379.1321). The identification of fragment ion E (*m/z* 337) further indirectly identifies the specific [2-^{2}H]-D-glucose labeling position (shift of one mass unit to *m/z* 338). In this way, it was established that the labeled position is at C-5′ from [2-^{2}H]-D-glucose, since in fragment ion E only the 5′-H_2 and 6′-H in the glycon unit remain attached. From the knowledge that the 6′-H was identified as being specifically derived from 1-H in glucose,[1] 5′-H could only be derived from 2-H in glucose, although low incorporation occurred due to glycolysis of glucose.[1] The calculated incorporation rate of 34% (obtained from fragment B, *m/z* 156 and 157) is close to the rate of glycolysis (38%) calculated from the [U-$^{13}C_6$]-glucose feeding experiment.[1]

The EIMS of 3′-demethoxy-3′-hydroxystaurosporine (**8**, *m/z* 452), a derivative of **1** obtained from a mutant of *Streptomyces longisporoflavus* R19,[14] showed the glycon fragment ion at *m/z* 142 (fragment B minus a CH_2 unit, *m/z* 156−14) and *m/z* 73 (fragment D minus a CH_2 unit, *m/z* 87−14). The other principal fragment ions, at *m/z* 337 and 308, were not influenced by the 3′-*O*-demethylation in the glycon moiety.

The EIMS of labeled **1** from the feeding of [6-$^2H_2$]-D-glucose showed mass shifts of fragments C (*m/z* 379 to 382 and 383) corresponding to labeling on the 2′-Me and two deuteriums in the aglycon. Because the sites of deuterium labeling have been determined by ^{1}H NMR,[1] fragment ion C (shifted from *m/z* 379 to 384 and 385) from the feeding of [U-$^2H_7$]-D-glucose could be explained by noting that three deuteriums are on the 2′-Me, one deuterium is on 6′-H, and the other label is on 5′-H (low incorporation rate) or on the aglycon. Fragment ion E (shifted from *m/z* 337 to 341 and 342) could be ex-

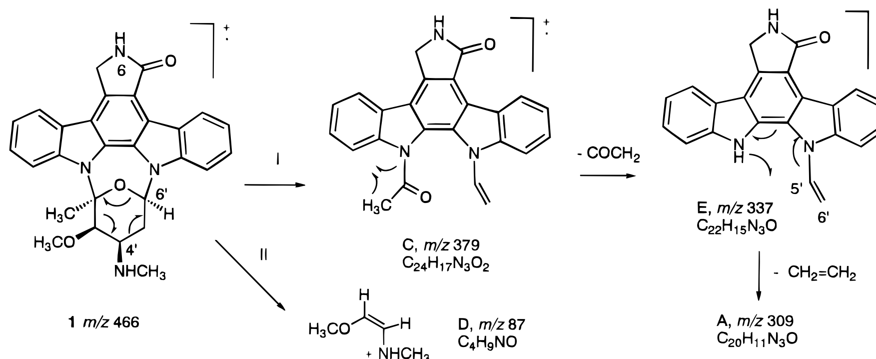


Figure 2. Proposed glycon decyclization of **1**. In pathway I, charge localization occurred on the bisindole, followed by the ring decyclization and loss of $-COCH_2$ and $-CH_2{=}CH_2$ functional groups which generated the fragment ions C, E, and A, respectively. In pathway II, charge localization was initiated on the 4′-*N*HCH$_3$ group; the decyclization fragment ion D was detected.

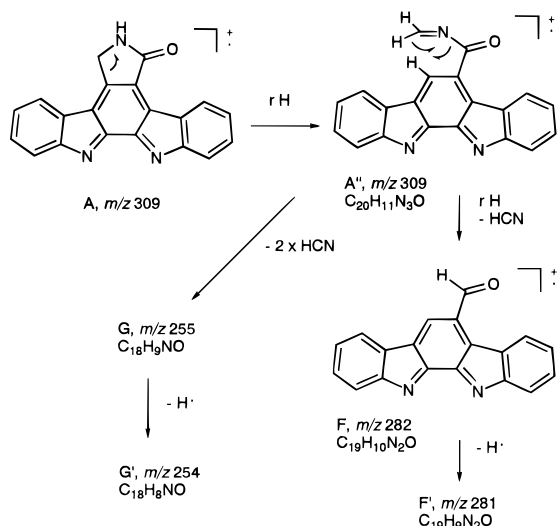


Figure 3. Proposed fragmentation pathway of the indolocarbazole unit in **1**. rH presents proton rearrangement.

plained by deuterium labeling on 6′-H, 5′-H, and the bisindole unit. The shift of fragment ion D [from *m/z* 87 (60) and 88 (72) to 88 (72) and 89 (73)] in uniformly labeled **1** is due to the incorporation of deuterium into the methyl of the *O*-Me and *N*-Me groups during methionine biosynthesis. From ^{1}H NMR, the incorporation of deuterium was also observed in the *O*-Me and *N*-Me groups based on the observation of the smaller integration and upfield shift of the resonance ($\Delta\delta$ -0.02 and -0.01 ppm, respectively). From the EIMS of deuterium-labeled **1** obtained from the [C^2H_3]-L-methionine feeding experiment, fragment ion D appeared as pairs at *m/z* 90 (91) and 93 (94) (original at *m/z* 87 and 88) due to the incorporation of one or two $-C^2H_3$ into the *O*- and *N*-Me groups (data will be published elsewhere).

The EIMS of staurosporine aglycon K252c (**6**) and staurosporine analog K252d (**7**) were observed to have *m/z* 311, 282, and 255 in common,[13] which resemble the fragment ion peaks at *m/z* 308, 281, and 255 in **1**. The identification of *m/z* 308, 281, and 255 from HREIMS failed due to the weak intensity of these ion peaks and the complexity of neighboring peaks (cluster like). However, the proposed mechanism of fragmentation is shown in Figure 3. Cleavage occurred at the bond β to the nitrogen atom (N-6) in the amide group and was followed by the loss of HCN to generate fragment ion F ($C_{19}H_{10}N_2O$, *m/z* 282), or alternatively, the pathway could go through the loss of two HCN units to produce fragment G ($C_{18}H_9NO$, *m/z* 255), followed by the loss of another hydrogen radical to generate fragment ion G′ ($C_{18}H_8NO$, *m/z* 254). These ion fragments could be observed in the EIMS of differently labeled samples of **1** and were used to support the deuterium incorporation into the aglycon moiety. From the EIMS of double ^{15}N-labeled **1** (labeling on both indole nitrogens), fragment ions A and F (or F′) shift one or two mass units due to one or two ^{15}N-labeling on indole nitrogen(s). On the other hand, fragment ion G′ did not show any mass shift from ^{15}N-labeling, indicating that two HCN groups were lost during formation of fragment ion G′ and that both nitrogen atoms are from indole nitrogen atoms (data for ^{15}N-labeled **1** will be published elsewhere).

Deuterium oxide incorporation into **1** was evaluated with 30%, 40%, 50%, and 100% D_2O in the medium. Cell growth was strongly inhibited at 100% D_2O. However, at 30%, 40%, and 50% of D_2O in the medium, cell growth continued, and staurosporine was produced. A limit of 40% of D_2O in the medium was chosen for a larger-scale fermentation. The yield of labeled staurosporine was inhibited when L-tryptophan (50 mg) was added to the medium (0.2−0.3 mg/100 mL) and when L-tryptophan (50 mg) with L-methionine (10 mg) were added. When L-tryptophan was not added to the 40% D_2O medium, the cell growth and yield of **1** (0.4−0.5 mg/100 mL) were enhanced compared with those when tryptophan was added.

The ^{1}H NMR of labeled **1** showed a more complicated pattern and shift of the resonances of 3′-H, 4′-H, 5′α-H, 5′β-H, 3′-*O*-Me, and 4′-*N*-Me (shown in Figure 4) due to partial deuterium substitution, indicating that deuterium was incorporated into these positions. The 3′-H (δ 3.860, d, in unlabeled **1**) resonance in the ^{1}H NMR of deuterium-labeled **1** appeared as a doublet mixed with a singlet (δ 3.855) due to partial loss of the coupling with 4′-H resulting from deuterium substitution on 4′-H. The original quartet-like 4′-H (δ 3.33, ddd) resonance was simplified to a triplet (or dd) by reason of the deuterium incorporation on 3′-H or 5′-H_2. Because the probability of deuterium incorporation into both 3′-H and 5′-H_2 in the same molecule is low, doublet or singlet signals of 4′-H in labeled **1** were not observed in the ^{1}H NMR. These two deuterium incorporations on 3′-H and 4′-H were confirmed by ^{13}C-NMR spectral analysis, which showed a resonance shift of 0.1 ppm for both C-3′ (δ 84.2) and C-4′ (δ 50.5) accompanying the non-

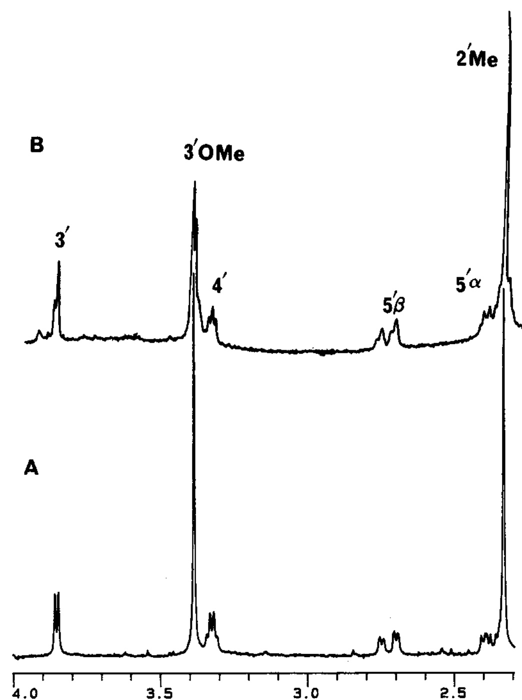


Figure 4. Expansion of the ^{1}H-NMR spectra of staurosporine (**1**) in the region of 2.3−4.0 ppm. (A) standard, without D_2O added; (B) obtained from 40% D_2O feeding experiment. 4′-*N*-Methyl resonance is not shown, but has similar pattern compared to that of 3′-*O*-Me.

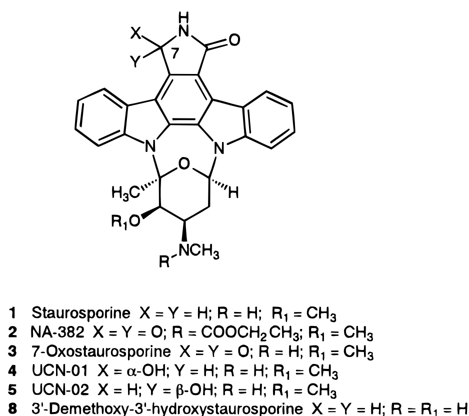
deuterium-labeled resonances (δ 84.1 and δ 50.4) with the same intensity, indicating around 50% deuterium incorporation. The proton signals of 5′β-H (δ 2.71, dd) and 5′α-H (δ 2.39, ddd) in natural abundance **1** were observed as a doublet (J = 14.7 Hz) and a doublet of doublets (J value not obtained due to overlapping), respectively, in labeled **1** from the D_2O feeding experiment and was intensified due to the loss of the neighboring proton coupling. In addition, in the ^{13}C-NMR spectrum, C-5′ was observed as a broadened signal (δ 30.1). The shifted signals of the singlets of the 3′-*O*-Me (δ 3.38, $\Delta\delta = -0.01$ ppm) and 4′-*N*-Me (δ 1.53, $\Delta\delta = -0.01$ ppm) due to partial deuterium incorporation were observed with the original signals (δ 3.39 and δ 1.54), indicating that deuterium substitution had occurred on the *O*- and *N*-methyl groups during biosynthesis from methionine.

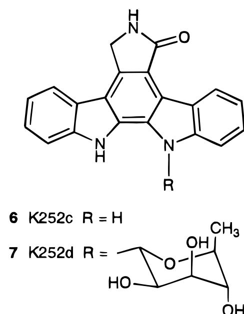
Determination of the specific deuterium incorporation was utilized for the evaluation of some of the mass fragmentation pathways. The mass unit shifts and the intensity pattern of the molecular ion related peaks [*m/z* 466 (19), d (31), d_2 (55), d_3 (67), d_4 (61), d_5 (45), and d_6 (26)] showed one or two units' difference compared to those of the aminosugar fragment ion B peaks [*m/z* 156 (16), d (31), d_2 (44), d_3 (42), d_4 (28), and d_5 (15)], indicating that incorporation of one or two deuterium atoms had occurred in the bisindole fragment. Principally, the retention of four deuterium atoms on fragment ion B from the aminosugar of **1** at a high incorporation rate was observed, and they were at 4′-H, 5′-H, and two on each of the *O*- and *N*-methyl groups. Deuterium labeling on 3′-H could not be observed from fragment ion B based on the loss of 3′-H in the proposed mechanism (Figure 1). Other minor incorporation might occur on another 5′-H due to the effect of glycolysis.

In the 40% D_2O feeding experiment with concomitant tryptophan feeding, the incorporation rate of each deuterium was significantly decreased compared to that of the non-tryptophan-containing culture (40% D_2O). In this instance, the mass unit shifts and the intensity pattern of the molecular ion peaks [*m/z* 466 (56), d (44), d_2 (40), d_3 (44), d_4 (40), d_5 (31), and d_6 (18)] show one unit of difference from those of the aminosugar fragment ion B peaks [*m/z* 156 (30), d (30), d_2 (27), d_3 (29), d_4 (24), and d_5 (16)], supporting that one deuterium (3′-H) was lost during formation of fragment ion B. Hence, the incorporation of deuterium occurred primarily in the aminosugar or an aminosugar fragment and could be applied to identify the fragment ions D and E. Fragment ion E only contains two deuteriums from 5′-H_2 and is in accord with the Dalton shift from *m/z* 337 to *m/z* 338 (d, 70) and 339 (d_2, 38). Fragment D was shifted from a pair of ions at *m/z* 87 (40) and 88 (41%) to a pair of ions at *m/z* 89 (31) and 90 (25).

The fragment ions related to the bisindole unit, including the ions at *m/z* 381 (15), 338 (100), 281 (19), and 255 (10), could be satisfactorily explained according to the proposed fragmentation when tryptophan was added. In the D_2O feeding without tryptophan, there was a one-unit mass shift of the fragment ions at *m/z* 382, 350, 339, 310, and 282, compared with those obtained from tryptophan feeding, suggesting that one deuterium was incorporated into the aglycon fragment ions A and F during the biosynthesis of tryptophan. Unfortunately, the specific position of incorporation into the indolocarbazole unit was not observed in ^{1}H-NMR and ^{13}C-NMR spectra.

From a biosynthetic view, there are primarily six deuterium atoms incorporated from D_2O into staurosporine (**1**) in the D_2O with tryptophan feeding experiment. They are incorporated only into the glycon unit at the positions 3′-H, 4′-H, 5′α-H, 5′β-H, and into the *O*-Me and the *N*-Me. One of the 5′-H_2 was incorporated primarily during glycolysis (62−66%). The deuterium incorporation studies correspond to previously reported work on the biosynthesis of other 2,6-dideoxysugars or their glycosides.[15] The incorporation of deuterium into *O*- and *N*-Me groups might have occurred during methionine biosynthesis before the biosynthesis of **1**, based on the success of the high direct incorporation of [C-$^2H_3$]-methionine, in which all three deuterium atoms were retained on both of the *O*- and *N*-Me groups.[18] When tryptophan was added in the 40% D_2O medium, no significant deuterium incorporation occurred in the indolocarbazole moiety, compared to one- or two-deuterium incorporation into the aglycon that occurred without added tryptophan. K-252c (**6**) has been suggested as an intermediate of **1** in *S. longisporoflavus* R19,[14] indicating that UCN-01 (**4**) and 7-oxostaurosporine (**3**) may be the further metabolites or shunt products instead of intermediates in staurosporine biosynthesis. Hence, the origin of the 7-H_2 could be a key aspect in searching for the intermediates of **1**. Of further interest is whether the α-H of tryptophan is retained at the C-7 position in **1** and whether the 7-H_2 is derived from tryptamine for, although [α-^{14}C]-tryptamine shows good incorporation into staurosporine, low deuterium incorporation of the α-deuteriums from tryptamine into **1** was observed from an initial [α-$^2H_2$]-tryptamine labeling experiment.[18]





In conclusion, we have identified some of the principal fragment ions in the EIMS of staurosporine. This information is very useful for the assignment of the isotopic incorporation into the indolocarbazole and glycon moieties for biosynthetic study and, in addition, could be a useful tool for the identification of related derivatives for chemical study. Also from D_2O-labeling experiments we conclude that 3′-H, 4′-H, and one of the 5′-H_2 are derived from the aqueous medium (Figure 5) during staurosporine biosynthesis.

Experimental Section

General Experimental Procedures. 1D ^{1}H-NMR spectra were recorded on either a Nicolet NT-360 instrument operating at 360 MHz or 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 or a Varian XL-300 (75.4 MHz) instrument. A ^{13}C-tuned probe accommodating a 5-mm NMR tube with the samples dissolved in $CDCl_3$ was used. 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. The mass measurement method used for HREIMS was peak matching. [6-$^2H_2$]-D-Glucose (98%), [2-^{2}H]-D-glucose (98%), [U-$^2H_7$]-D-glucose (98%), and deuterium oxide (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.[1,16,17] The fermentation methods were the same as those previously described.[1,16,17]

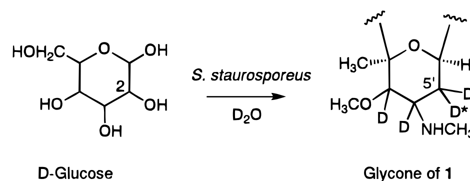


Figure 5. Deuterium atoms from deuterium oxide were incorporated into 3′-H, 4′-H, and 5′-H_2 of the aminosugar moiety of staurosporine (**1**). One deuterium incorporation on C-5′ position (marked with * sign) with a lower incorporation rate was caused by glucose glycolysis and gluconeogenesis and was retained from 2-H in glucose. The deuterium incorporation also occurred on *O*- and *N*-methyl groups due to biosynthesis of methionine and was not shown.

Isolation and Purification of Staurosporine (1). The isolation and separation methods were modified and summarized as below. Culture fermentation broth (100 mL) containing mycelia was adjusted to pH 10 with NH_4OH and extracted with EtOAc (3 × 100 mL). The pooled organic phase was dried (Na_2SO_4) and evaporated to dryness under vacuum at 40 °C, and the staurosporine was isolated by preparative TLC. The solvent system and detailed methods were described previously.[1] The yield of staurosporine produced from *S. staurosporeus* in the normal medium was 1.2−1.5 mg/100 mL.

Characterization of Staurosporine (1). The physical data, including melting point, UV, IR, $[\alpha]_D$, EIMS, and ^{1}H NMR, were described previously.[16,17] The EIMS of all deuterium-labeled staurosporines are summarized in Table 1.

Feeding Experiments with Stable Isotopes. For the feeding experiment with [6-$^2H_2$]-D-glucose, [2-^{2}H]-D-glucose, and [U-$^2H_7$]-D-glucose, labeled glucose was used at a concentration of 1 g/50 mL of fermentation medium in a plugged 250-mL Erlenmeyer flask, and no natural glucose was added. Cells were harvested after 72 h, and the subsequent isolation and separation procedures (preparative TLC) were the same as those described previously.[1,16,17] The yields of staurosporine production were 0.5−0.6 mg/50 mL in all experiments.

Deuterium Oxide Feeding Experiment with L-Tryptophan Added. Cells from the seed medium were transferred to a glucose-deficient fermentation medium (60 mL) with mannitol (1 g), $CaCO_3$ (0.1 g), and soy bean meal (2 g) and allowed to grow on a shaker for 24 h. D_2O (60 mL, 40 mL, and 27 mL), with D-glucose (4 g) and L-tryptophan (50 mg) was sterilized by filtration and added to the fermentation medium to reach the percentages of 50%, 40%, and 30% of D_2O (v/v), respectively. After cell growth for an additional four days, the mycelium was extracted and separated as previously described. Deuterium-labeled staurosporines from different cultures were isolated and examined by EIMS and ^{1}H NMR.

Deuterium Oxide Feeding Experiment without Tryptophan. Except that tryptophan (50 mg) was not added, the procedure was the same as that described previously. The cells were incubated in three sets of 40% D_2O medium (v/v, 100 mL), and processed as previously described.

Deuterium Oxide Feeding Experiment with L-Tryptophan and/or L-Methionine Added. The same method was applied except that L-tryptophan (50 mg/100 mL) and L-methionine (10 mg/100 mL) were added,

or L-methionine (4 mg/100 mL) alone was added. The experiment was run in triplicate.

Acknowledgment. We thank Dr. Richard van Breemen of this department for discussions related to the EI fragmentation pathways of **1**, the Research Resources Center of the University of Illinois at Chicago for the provision of NMR spectroscopic facilities, and Mr. Richard Dvorak of this department for obtaining the MS spectra.

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NP960567M