Thus, the Amaryllidaceae alkaloid haemanthidine (**1**) is available in 2.3% overall yield via a linear synthetic sequence that involves only 12 chemical operations from commercially available piperonal. Further extensions of this and related methodologies in the alkaloid field are in progress and will be reported in due course.

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(19) We thank Professor J. B. Hendrickson (Brandeis University), Dr. P. Jeffs (Smith-Kline-French), and Dr. H. Fales (National Institutes of Health) for providing authentic samples of haemanthidine, tazettine, and related alkaloids and Professor E. Furusawa (University of Hawaii) for an authentic sample of pretazettine hydrochloride.

Biosynthesis of Saxitoxin Analogues: The Unexpected Pathway

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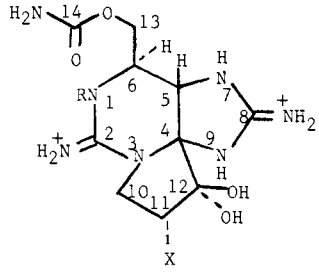
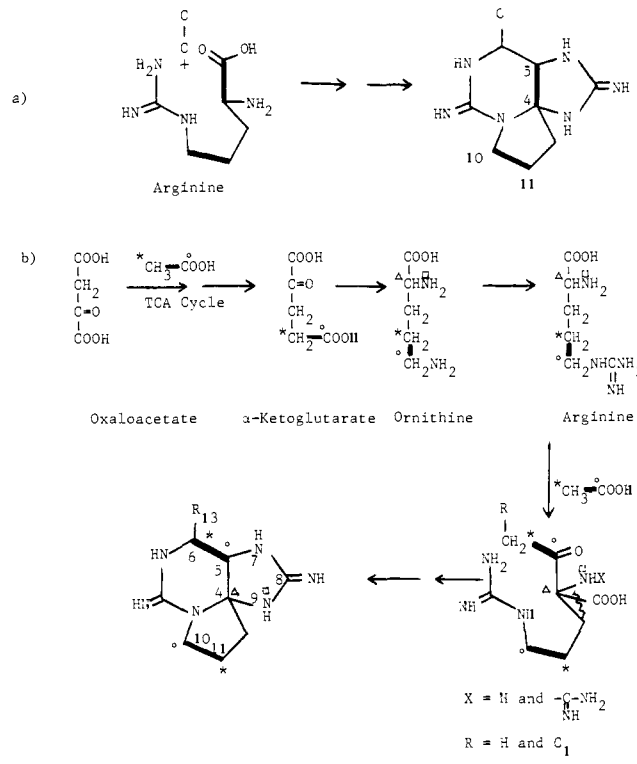
A group of dinoflagellate toxins represented by saxitoxin (**1**) have been extensively investigated because of their occurrences in edible shellfish and their importance as pharmacological tools.[1-3]



1: R = H, X = H
2: R = H, X = OSO_3^-
3: R = OH, X = H

However, only very limited knowledge is available regarding the biosynthetic origin of the unique tricyclic systems having perhydropurine rings.[4] In fact, such compounds as purine nucleotides,[5] C_7 sugars,[6] or arginine[3,7] were implicated as possible precursors, but actual feeding studies have been severely impeded by the nonheterotrophic nature of the photosynthetic toxin-producing dinoflagellates, which resist the utilization of exogeneous organic compounds.[6] After many unsuccessful feeding experiments with various amino acids and other plausible precursors, we decided to try feeding small simple molecules, which might penetrate more easily into the system. In an earlier experiment[3] feeding [2-^{13}C]glycine to a culture of *Gonyaulax tamarensis* resulted in the enrichment of all carbons in isolated gonyautoxin II (**2**)[8] but extra enrichment was observed with C-11 and C-12. This rather unusual enrichment of the two neighboring carbons from the single-labeled precursor was explained by assuming that glycine was incorporated into α-ketoglutarate via glyoxalate–TCA cycle pathway.[3] Since α-ketoglutarate is a precursor of arginine and related compounds, the result was considered to support the arginine precursor theory of the toxins (Scheme Ia),[3,7]

Scheme I



Feeding of [1,2-^{13}C]acetate to *G. tamarensis* also resulted in the modest enrichment of all carbons, but in this case extra enrichment was observed with C-5 and C-6 gonyautoxin II (**2**) and neosaxitoxin (**3**).[9,10] This enrichment of the two adjacent carbons, C-5 and C-6, by one acetate unit as indicated by the coupling pattern was in clear contradiction to the arginine precursor theory in which C-5 must come from C-1 of arginine (Scheme Ia). The experiment was further repeated using a toxic strain of *Aphanizomenon flos-aquae*, a blue-green alga, which had been reported to produce neosaxitoxin and other saxitoxin analogues.[11] We confirmed again the incorporation of [1,2-^{13}C]acetate into C-5

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(2) Shimizu, Y. "Marine Natural Products, Chemical and Biological Perspectives"; Scheuer, P. J., Ed.; Academic Press: New York, 1979; Vol. I, p 1.
(3) Shimizu, Y. *Pure Appl. Chem.* **1982**, *54*, 1973.
(4) Schantz, E. J.; Ghazarossian, V. E.; Schnoes, H. K.; Strong, F. M.; Springer, J. P.; Pezzanite, J. O.; Clardy, J. *J. Am. Chem. Soc.* **1975**, *97*, 1238. Bordner, J.; Thiessen, W. E.; Bates, H. A.; Rapoport, H., *Ibid.* **1975**, *97*, 6008.
(5) Mickelson, C. "Toxic Dinoflagellate Blooms"; Taylor, D. L., Seliger, H. H., Eds.; Elsevier North Holland: New York, 1979; p 494.
(6) Shimizu, Y.; Hori, A.; Genenah, A.; Kobayashi, M.; Ichihara, N. "Abstract of Papers", 186th National Meeting of the American Chemical Society, Washington, DC, Aug 1983; American Chemical Society: Washington, DC, 1983; AGFD 116.
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(8) Shimizu, Y.; Buckley, L. J.; Alam, M.; Oshima, Y.; Fallon, W. E.; Kasai, H.; Miura, I.; Gullo, V. P.; Nakanishi, K. *J. Am. Chem. Soc.* **1976**, *98*, 5414. Boyer, G. L.; Schantz, E. J.; Schnoes, H. K. *J. Chem. Soc., Chem. Commun.* **1978**, 889. Shimizu, Y.; Hsu, C. P. *Ibid.* **1981**, 314.
(9) Shimizu, Y.; Hsu, C. P.; Fallon, W. E.; Oshima, Y.; Miura, I.; Nakanishi, K. *J. Am. Chem. Soc.* **1978**, *100*, 6791.
(10) *G. tamarensis* was cultured in the enriched seawater, Guillard F medium (80 L) under fluorescent illumination at 12 °C. Labeled sodium acetate (800 mg) was fed 20 days after inoculation, and the culture was left to grow for additional 8 days. The separation of the toxins was accomplished by a combination of Bio-Gel P-2 gel filtration chromatography and Bio-Rex 70 ion-exchange chromatography as previously reported (Oshima, Y.; Buckley, L. J.; Alam, M.; Shimizu, Y. *Comp. Biochem. Physiol. C* **1977**, *57C*, 31). Gonyautoxin II (**2**) (5.3 mg) and neosaxitoxin (**3**) (8 mg) were the major toxins in this culture, and the other toxins were insufficient for ^{13}C NMR measurement.
(11) Ikawa, M.; Wagner, K.; Faxall, T. L.; Sasner, J. J., Jr. *Toxicon* **1982**, *20*, 747.

0002-7863/84/1506-6433$01.50/0

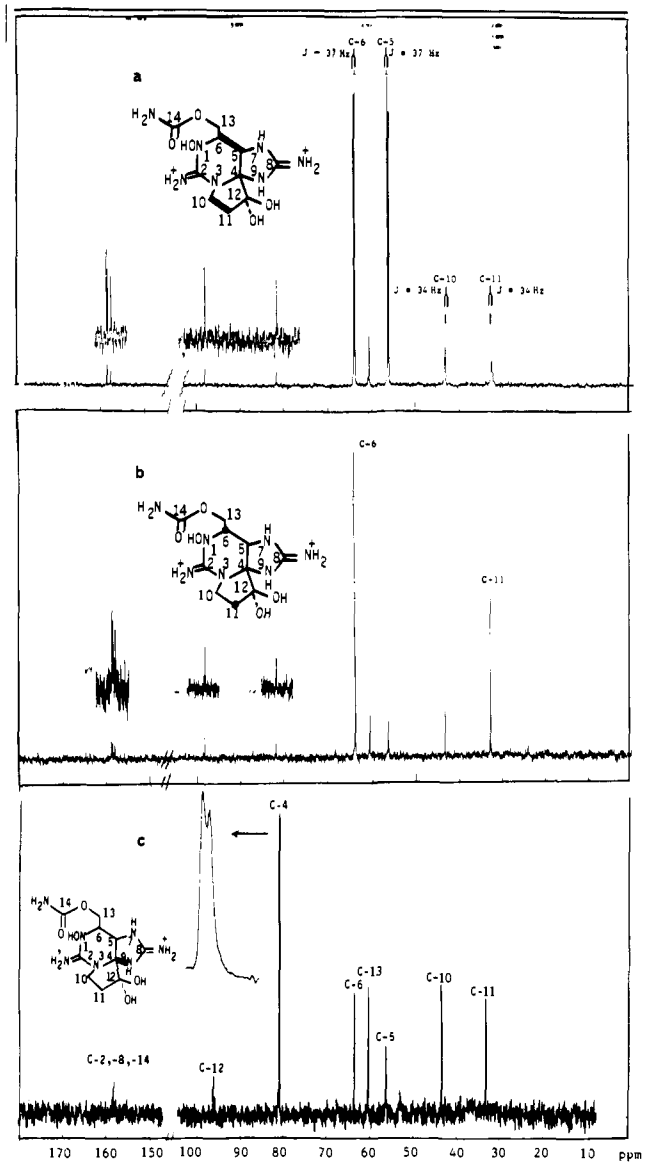

Figure 1. ^{13}C NMR spectra of neosaxitoxin (**3**) isolated from *Aphanizomenon flos-aquae* fed with [1,2-^{13}C]acetate (a), [2-^{13}C]acetate (b), and [2-^{13}C,2-^{15}N]ornithine (c).

and C-6 in isolated neosaxitoxin, but additionally we could observe the lesser incorporation of another acetate unit into C-10 and C-11 (Figure 1a).[12] The orientations of the incorporated acetate units were determined by feeding 2-^{13}C single-labeled acetate, which resulted in the enrichment of C-6 and C-11 (Figure 1b). The results were totally unexpected and do not conform to any biosynthetic pathways previously postulated for these toxins. To accommodate the new findings with our previous experimental results, we now propose a new pathway in which the key step is the Claisen-type condensation of an acetate unit or its derivative to the amino group bearing α-carbon of arginine or an equivalent and a subsequent loss of the carboxyl carbon and imidazole ring formation on the adjacent carbonyl carbon (Scheme Ib).[14] In such a scheme, the C-2 and α-amino group of arginine or its precursors should be still incorporated into the toxin molecule in intact form. Thus we prepared 2-^{13}C,2-^{15}N double-labeled ornithine, the direct biosynthetic precursor of arginine, from diethyl [2-^{13}C]malonate (99% enrichment) and potassium [^{15}N]phthalimide (99% enrichment) according to the procedure described by Martinkus et al.[15] and fed it to a culture of toxin-producing *Aphanizomenon flos-aquae*.[16] Neosaxitoxin (**3**) was isolated and subjected to ^{13}C NMR measurement.[17] A distinct enrichment was observed with C-4, whose signal appeared as a clear doublet (J = 9.1 Hz)[18] due to a spin–spin coupling with the neighboring nitrogen (Figure 1c). The result, concurrently with the previous observation that [1-^{13}C]-arginine or [1-^{13}C]ornithine feedings did not result in specific enrichment,[6] provides strong support for the newly proposed pathway.

Condensations of acyl groups with α-amino acids have some precedents. A well-known example is the condensation of succinate with glycine to form δ-aminolevulinate in porphyrin biosynthesis. The most recent and more direct analogy is found in the biosynthesis of antibiotics arphamenine A and B, which proceeds through the condensation of acetate to the α-position of arginine and subsequent decarboxylation.[19] The new pathway also involves the differential uptake of acetate, which explains the observed disparity of the two incorporated acetate units.

This work establishes the origins of all the carbons in the toxin ring system. Regarding the origin of an extra carbon, C-13, feeding experiments with precursors considered to be a general source of C_1 units have so far failed to effect the special enrichment, and it is conceivable that C-13 was derived from CO_2 at an earlier stage via malonate.

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Registry No. **1**, 60508-89-6; **2**, 64296-20-4; **3**, 92096-46-3; diethyl [2-^{13}C]bromomalonate, 67035-94-3; diethyl [2-^{13}C, ^{15}N]phthalimidomalonate, 92096-47-4; *N*-(3-iodo-1-propyl)phthalimide, 5457-29-4.

(12) *A. flos-aquae* was cultured in ASM-1 medium without soil extract (total 40 L) and was fed on the eighth day, and the cells were harvested by centrifugation after 10 days. Toxins were separated as previously described,[10] and neosaxitoxin (ca. 4 mg) and a small amount of saxitoxin were obtained.

(13) The measurements were done in D_2O at 125.7 MHz, and the following data were recorded; ^{13}C NMR δ 32.4 (C-11, J = 34 Hz), 43.1 (C-10, J = 34 Hz), 56.1 (C-5, J = 37 Hz), 60.4 (C-13), 63.8 (C-6, J = 37 Hz), 81.4 (C-4), 98.0 (C-12), 157.5, 158.2, and 158.4 (C-2, C-8, and C-14). C-5 and C-6 are both methine carbons having very close chemical shifts. The assignment of these crucial signals was unequivocally established by heteronuclear decoupling[9] and also confirmed by the carbon connectivity study of uniformly enriched neosaxitoxin prepared by $^{13}CO_2$ feeding (unpublished results).

(14) The possibility that the toxin molecule was formed by the β-amination of a lysine molecule derived from the α-aminoadipate pathway was also considered. However, in such a scheme, the orientation of an acetate unit incorporated at C-5 and C-6 would be reversed.

(15) Martinkus, K. J.; Tann, C. H.; Gould, S. J. *Tetrahedron* **1983**, *39*, 3493. Diethyl [2-^{13}C]malonate was converted to diethyl [2-^{13}C]bromomalonate in 84.5% crude yield. Reaction of diethyl [2-^{13}C]bromomalonate with potassium [^{15}N]phthalimide using hexadecyltri-*n*-butylphosphonium bromide as catalyst gave diethyl [2-^{13}C,^{15}N]phthalimido malonate in 99% crude yield. This was condensed with *N*-(3-iodo-1-propyl)phthalimide in the presence of sodium hydride. The crude product was hydrolyzed with a mixture of HCl, AcOH, and H_2O (1:1:1) overnight at 100 °C. D,L-[2-^{13}C,2-^{15}N]-Ornithine was purified by chromatography on a Rexy 101 (Fisher Co.) column yielding 270 mg (59% yield). The purity of the obtained ornithine was confirmed by ^1H and ^{13}C NMR spectra.

(16) *Aphanizomenon flos-aquae* was cultured in three carboys (3 × 10 L), and the precursor (90 mg for each carboy) was added 10 days after inoculation. The culture was left to grow for another 10 days. Pure neosaxitoxin (2.0 mg) was obtained after the previously reported isolation procedure.[10]

(17) The measurement was done in a D_2O–H_2O mixture (1:3) at pH 2 on a Bruker instrument at 125.7 MHz. The following signals were observed in the proton noise-decoupled spectrum after 96 000 scans: ^{13}C NMR δ 32.7 (s, C-11), 43.2 (s, C-10), 56.2 (s, C-5), 60.5 (s, C-13), 63.8 (s, C-6), 81.6 (d, J = 9.1 Hz, C-4), 98.1 (s, C-10), 158 (C-2, C-8, C-14). The assignment of the signals was unequivocally established in previous works.[13]

(18) This one-bond C–N coupling constant is very close to what we expected from those observed with ^{13}C–^{15}N couplings of other C–N pairs in the molecule. The following values were previously obtained from the ^{13}C NMR spectrum of uniformly ^{15}N-enriched neosaxitoxin and gonyautoxin II (Hori, A.; Shimizu, Y. *J. Chem. Soc., Chem. Commun.* **1983**, 790): $^1J_{N\text{-}1,C\text{-}6}$ = 7.8 Hz, $^1J_{N\text{-}7,C\text{-}5}$ = 9.4 Hz, $^1J_{N\text{-}3,C\text{-}10}$ = 8.4 Hz.

(19) Ohuchi, S.; Okuyama, A.; Nakagawa, H.; Aoyagi, T.; Umezawa, H. *J. Antibiot.* **1984**, *37*, 518.