Biosynthetic ^{15}N-Enrichment and ^{15}N N.M.R. Spectra of Neosaxitoxin and Gonyautoxin-II: Application to Structure Determination

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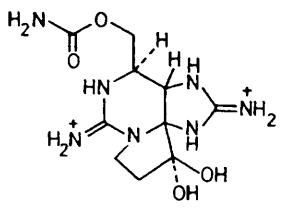
Nitrogen-15 enriched samples of two important neurotoxins, neosaxitoxin and gonyautoxin-II, from the red tide-causing organism, *Gonyaulax tamarensis*, were prepared by feeding of $Na^{15}NO_3$; ^{15}N n.m.r. spectra of the enriched toxins support the structural assignments previously made for these toxins.

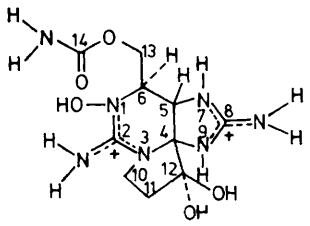
Neosaxitoxin is one of the major toxic components found in toxic shellfish,[1,2] crabs,[3,4] and the toxic dinoflagellates, *Gonyaulax tamarensis*,[1,2] *Pyrodinium bahamense* var. *compressa*.[5] The related toxins include saxitoxin (**1**),[6] and gonyautoxin-I—VIII.[7,8] Most of them are recognized as important tools in the study of excitable membranes.[9]

The structure of neosaxitoxin is tentatively proposed as N^1-hydroxysaxitoxin (**2a**) on the basis of spectroscopic studies and correlation with saxitoxin,[10] whose structure (**1**) has been unequivocally established by *X*-ray crystallography[11] and total synthesis.[12] However, considerable uncertainty still remains regarding the exact nature and location of the additional functional group. Knowledge of the correct structure of neosaxitoxin is particularly important because the structures of several other toxins were based on correlation with this toxin.[8,13–15] The structure (**2a**) is supported by ^1H and ^{13}C n.m.r. observations. In comparison with the spectra of saxitoxin (**1**), significant differences were seen in the chemical shifts of C-6 and the attached proton of neosaxitoxin. A pH dependency study of the ^1H shifts showed that deprotonation associated with one of the toxin's pK_a's (pK_a 6.75) induces a significant change in the chemical shift of 6-H.† However, this evidence does not rule out alternative structures such as

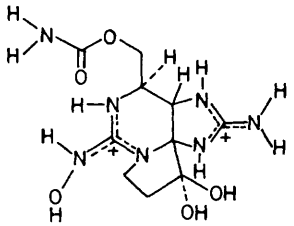
† Initially this pK_a was assigned to dissociation of the hydroxy-proton. However, a recent pK_a study of δ-*N*-hydroxyarginine suggests that it may be associated with the substituted guanidinium itself (Y. Shimizu, A. Hori, and M. Ghazala, unpublished result).



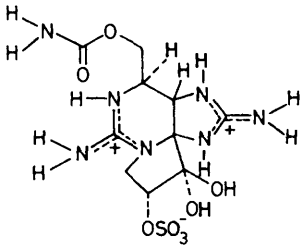
(1)



(2a)



(2b)



(3)

the C(2)-*N*-hydroxy derivative (**2b**). The gummy toxin is unsuitable for *X*-ray crystallography and does not afford reliable analysis nor mass spectroscopic data because of its highly hygroscopic, non-volatile nature. Further structural work has been severely hindered by the paucity of available samples and the lack of an effective method to differentiate the seven nitrogen atoms present in the toxin molecule. As an experimental approach to resolve the problem, we considered the use of ^{15}N n.m.r. spectra of biosynthetically enriched samples. The correct assignment of ^{15}N n.m.r. spectra of these neurotoxins was also considered necessary in order to carry out future biosynthetic studies.

The dinoflagellate, *G. tamarensis* (Ipswich strain)[16] was cultured in Guillard F sea water-based media[17] (7 × 11 l) containing $Na^{15}NO_3$ (99% enrichment, 0.1 g/l) at 13 °C under fluorescent illumination. After 33 days, the organisms (2×10^9 cells) were harvested and extracted according to the previously reported procedure.[1] The toxic components were then fractionated by Bio-Gel P-2 and Bio-Rex 70 chromatography. The major toxins, gonyautoxin-II and neosaxitoxin, were pooled (*ca.* 15 and 6 mg respectively as estimated by mouse assay),[18,19] and further purified for ^{15}N n.m.r. study by Bio-Gel P-2 and Chelex-100 chromatography.‡ A high degree of ^{15}N-enrichment in the toxins (*ca.* 90%) was expected from the low content of intrinsic nitrogen in the sea water which was used. This expectation was corroborated by the ^{15}N–^{13}C and ^{15}N–^{1}H coupling patterns in the ^{13}C and ^{1}H n.m.r. spectra (Bruker 500 MHz instrument).

^{15}N N.m.r. studies of neosaxitoxin were conducted using a Bruker WH360 (36.5 MHz) instrument for 1.8 mM solutions in D_2O–H_2O (5:95; D_2O and doubly distilled H_2O were passed through a Chelex 100 column prior to use) using a 20 mm sample tube equipped with co-axial tubing containing NH_4NO_3 as an external standard. The gonyautoxin-II spectra were measured likewise for a 3.3 mM solution. In both instances the parameters were set in two modes: (i) gated proton decoupled with nuclear Overhauser enhancement (n.O.e.) (45 degree pulse, acquisition time 0.328 s, time delay 3 s, and decoupler power 7H); (ii) gated proton coupled with n.O.e. (45 degree pulse, acquisition time 0.819 s, decoupler power 18H). Chemical shifts are expressed in δ p.p.m. downfield from liquid NH_3 using a conversion factor of 21.60 p.p.m. for ammonium nitrogen of the external reference.

All seven nitrogen atoms in both toxin molecules give signals within the expected range[20] (Figure 1). In the proton-coupled spectrum, gonyautoxin-II gave three triplets [87.0 ($^1J_{NH}$ 92.8 Hz), 70.4 ($^1J_{NH}$ 93.2 Hz), and 76.6 p.p.m. ($^1J_{NH}$ 90.9 Hz)], three doublets [80.4 ($^1J_{NH}$ 95.5 Hz), 101.0 ($^1J_{NH}$ 99.6 Hz), and 86.2 p.p.m. ($^1J_{NH}$ 99.1 Hz)], and one singlet (96.4 p.p.m.).§ Since the two guanidinium groups in the molecule are expected to be fully protonated at pH 4,[21] the observed coupling patterns are in good accordance with the structure (**3**).[14,16,22] Significantly, in the neosaxitoxin spectrum, the doublet signal, δ 80.4 p.p.m., seen in the gonyautoxin-II spectrum is replaced by a downfield singlet signal, δ 140.1 p.p.m. The other signals paralleled closely those of gonyautoxin-II, except for small downfield shifts observed with the singlet for N-3 (−2.6 p.p.m.) and one of the triplet nitrogens [probably C(2)–N, −1.7 p.p.m.]. The result clearly excludes such structures as (**2b**) having a substituent on an imino nitrogen which would appear as a doublet, leaving the structure (**2a**) as the most reasonable choice. The remarkable downfield shift induced by the substitution (59.7 p.p.m.) also supports the earlier assumption that the substituent is a hydroxy group or a similar electron-withdrawing function.

Owing to limited sensitivity, ^{15}N n.m.r. analysis has not yet been fully utilized in the structure elucidation of natural products which are available only in small quantities. In this case, however, the highly enriched toxins have demonstrated excellent responses despite their small sample sizes.¶ Thus the approach to use biosynthetically enriched samples for ^{15}N n.m.r. studies appears to have more general applications in the structure determination of complex nitrogenous metabolites.

‡ This step was found to be absolutely necessary for ^{15}N n.m.r. measurements. The untreated samples, which gave excellent ^{13}C and ^{1}H n.m.r. spectra failed to give any ^{15}N n.m.r. signals. The minute sample sizes used in this study seem to make the signals much more susceptible to quenching by metal ions.

§ The triplet at δ 76.6 (δ 76.8 p.p.m. in neosaxitoxin spectrum) is the sharpest signal in both spectra, and evidently belongs to C(14)–N which is the sole nitrogen lacking long-range coupling. The singlet at δ 96.4 is unquestionably due to N-3, the doublet at δ 80.4 (which is missing from the neosaxitoxin spectrum) is due to N-1, and the triplet at δ 87.0 p.p.m. (which showed a downfield shift in the neosaxitoxin spectrum) was assumed to be due to C(2)–N. However, these assignments are still very tentative. In an attempt to establish unequivocal assignments from C–N couplings, toxins doubly enriched with ^{15}N and ^{13}C have also been harvested from a culture medium containing $Na^{15}NO_3$ and $^{13}CO_2$. We were able to detect one-bond C–N couplings in the ^{13}C n.m.r. spectra (9.4, 7.8, and 8.4 Hz for C-5, C-6, and C-10 of gonyautoxin-II and 6.4 Hz for C-10 of neosaxitoxin), but we have so far failed to obtain a reasonable nitrogen spectrum.

¶ In measurements on a Bruker WM250 instrument using a 15 mm sample tube, a 7.3 mM solution of enriched gonyautoxin-II gave distinctive signals on a single scan. Here again, the absolute absence of metal ions seems to be required for measurements of minute samples.

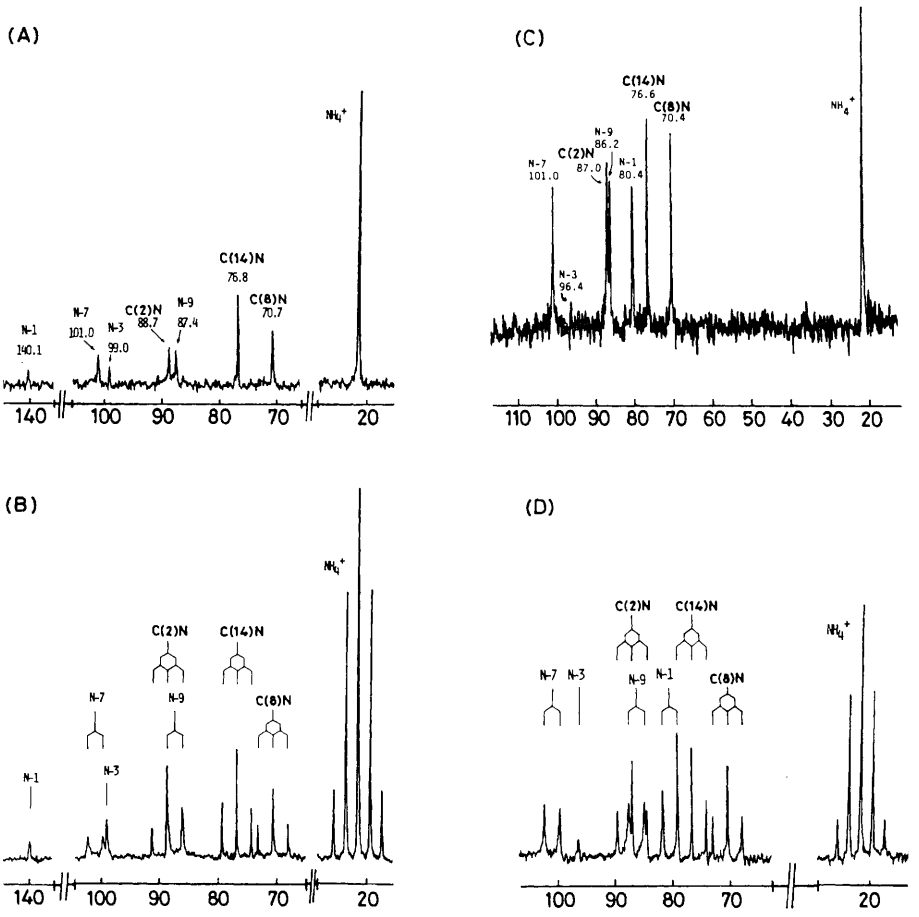


Figure 1. ^{15}N N.m.r. spectra of neosaxitoxin: (A) ^{1}H decoupled, (B) ^{1}H coupled, 10 370 scans; gonyautoxin II: (C) ^{1}H decoupled, 622 scans, (D) ^{1}H coupled, 4 395 scans.

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