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Derivatives of Saxitoxin

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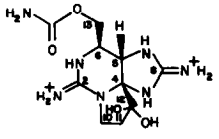
The preparation, spectral characterization, chromatographic and biological properties of several derivatives of saxitoxin, a highly potent neurotoxin, are described. Derivatives include decarbamoylsaxitoxin, both epimers of dihydrosaxitoxin (α- and β-saxitoxinol) and both epimers of decarbamoylsaxitoxinol, as well as several isotopically labeled variants. Natural toxin is readily regenerated from these C13 and C12 alcohols by carbamoylation or oxidation procedures. Evaluation of the biological properties of these compounds provides information on the structural features required for toxin binding to the sodium channel. Hemisuccinate esters of decarbamoylsaxitoxin and saxitoxinol have also been prepared, and the latter derivative was coupled to bovine serum albumin to provide a conjugate for possible antibody production.

INTRODUCTION

Toxic dinoflagellate blooms are fairly common environmental problems in many coastal and/or estuarine waters around the world (*1, 2*). Organisms of the genus *Gonyaulax,* notorious for their periodic blooms, the "red tides," along both Atlantic and Pacific coasts of the United States and Canada, are known to elaborate several potent neurotoxins, among which saxitoxin (STX) (**1**) has received most attention (*3, 4*). Human ingestion of shellfish which feed on



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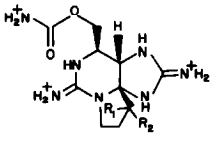
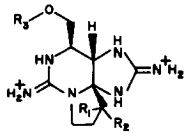


2 R_1=H, R_2=OH

3 R_1=OH, R_2=H

4 R_1/R_2=H/OCOCH$_2$CH$_2$COOH (epimeric mixture)



5 R_1=R_2=OH R_3=H

6 R_1=H, R_2=OH, R_3=H

7 R_1=OH, R_2=R_3=H

8 R_1=R_2=OH, R_3=COCH$_2$CH$_2$COOH

microflora and concentrate the toxin is the cause of "paralytic shellfish poisoning" a frequently fatal syndrome. Neurophysiologists have long been interested in these dinoflagellate toxins because of their potency and specificity of action. Saxitoxin, like the puffer fish poison tetrodotoxin, binds to the sodium channel in nerve and muscle cells with high affinity and specificity, thereby blocking the

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inward sodium current underlying the action potential of excitable tissues. Saxitoxin has, therefore, been exploited as a biochemical tool in many neurophysiological studies and its use has contributed important information on the Na channel and ion transport (*5–7*).

In connection with our work on the naturally occurring structural relatives of saxitoxin, we have explored the chemical modification of this natural product to obtain derivatives which, together with the natural analogs, could give insight into the structural requirements for toxin binding to its receptor (the Na channel) and the nature of toxin/channel interaction. In addition, such saxitoxin derivatives might be exploited for the elaboration of toxin/protein conjugates for the generation of antibodies and radioimmunoassay development, or the preparation of specific receptor (photo)-affinity labels or perhaps affinity column ligands. Early studies on saxitoxin led to a number of degradation products (8, 9) and one close structural analog, dihydrosaxitoxin, prepared originally by catalytic hydrogenation (*10*), then by borohydride reduction (*4, 9*) of STX, and described (*10*) as nontoxic.

We report here preparative details and spectroscopic characterization of several saxitoxin analogs, including decarbamoyl-STX (**5**) (*11*), a surprisingly active analog both *in vivo* and *in vitro*; two epimers of dihydrosaxitoxin (here named α- and β-saxitoxinol, **2, 3**) which do retain some toxicity and receptor binding affinity; α and β epimers of decarbamoylsaxitoxinol (**6, 7**); the hemisuccinate esters of decarbamoyl-STX (**8**) and of saxitoxinol (**4**); and the BSA conjugate of the latter. In addition, procedures for the conversion of **2** and **5** to the natural product (**1**) are presented. The biological evaluation of these saxitoxin derivatives offers insight into certain aspects of toxin/receptor binding.

MATERIALS AND METHODS

Paper electrophoresis (Whatman 1, 30 V/cm) was performed on a Savant flat plate electrophoresis system at 4°C with pH 4.5 acetate buffer. Compounds were visualized by spraying with Weber reagent (*12*).

Thin-layer chromatography was carried out on silica gel G (EM Reagents) and on precoated carboxymethyl cellulose plates (MN 300 CM 0.1 mm, Brinkman Instruments). Charring and Weber reagents (*12*) were used for visualization on silica gel and cellulose, respectively. Sephadex G-25 and LH-20 were obtained from Pharmacia Fine Chemicals. Bio-Gel P-2 was obtained from Bio-Rad Laboratories.

High-pressure liquid chromatography was carried out with a Waters Model M-6000A high-pressure system on either Waters μPorasil (10 μ, 3.9 mm × 30 cm) or Corasil (37–50 μ, 2 mm × 60 cm) columns in aqueous ammonium acetate solvents. Monitoring was carried out at 220 nm by means of a dual beam variable wavelength uv detector.

^{1}H nmr spectra were obtained on a 270-MHz Bruker instrument equipped with a Nicolet 1180 data system with 100% D_2O as solvent and chloroform at δ 7.27 (relative to TMS=O) as internal reference signal. Proton noise decoupled ^{13}C nmr

spectra were recorded either on a Bruker HX90-E or JOEL FX-200 spectrometer in 99.9% D_2O with tetramethylsilane (internal capillary) or dioxane as internal standard.

Radiochemically labeled compounds were counted in Aquasol (New England Nuclear) with a Beckman LS-100C liquid scintillation counting system.

Toxicity in mouse units (MU) was determined by interperitoneal injection of 0.5-ml solutions of STX or the appropriate derivative into white mice (19–22 g ARS Sprague–Dawley) and potency determined as previously described (*13*).

Preparation of Saxitoxinol (*2, 3*)

(*a*) *Sodium borohydride reduction in methanol.* To a rapidly stirred solution of 20 mg of saxitoxin (**1**) dihydrochloride in 10 ml of methanol at 55°C was added 71 mg sodium borohydride and the mixture was stirred for 75 min. The mixture was allowed to cool to room temperature and was diluted with 10 ml of water, after which the pH was adjusted to 3 by dropwise addition of 3 *N* aqueous HCl. The mixture was evaporated to dryness *in vacuo* and the resultant residue was taken up in 4 ml of methanol with the insoluble remainder removed by centrifugation. The supernatant was chromatographed on a 2.0 × 60-cm column of Sephadex LH-20 with methanol as eluting solvent. Fractions (4.0 ml) were collected at a flow rate of 0.8 ml/min and a spot test with Weber reagent showed the product to be present in fractions 23–30. These were pooled to yield 15.5 mg (83%) of a 2.8 : 1 mixture of α-saxitoxinol (**2**) and β-saxitoxinol (**3**) by HPLC and nmr analysis. Spectral and chromatographic data are given in Tables 1, 2, and 3.

(*b*) *Sodium borohydride reduction in water at 90°C.* To a hot solution of 300 μg saxitoxin (**1**) dihydrochloride in 0.4 ml H_2O was added 3.6 mg sodium borohydride. The reaction was allowed to proceed under positive nitrogen pressure for 20 min after which an additional 2 mg sodium borohydride was added. After 20 additional min the mixture was allowed to cool, the pH adjusted to 1 with aqueous HCl, and the mixture evaporated to dryness *in vacuo*. The resulting residue was taken up in a minimal amount of methanol and chromatographed on a 1.0 × 30-cm Sephadex LH-20 column with methanol as eluting solvent. Collection of 0.75-ml fractions at a flow rate of 0.8 ml/min eluted the product in fractions 32–45 which HPLC analysis showed to be a mixture of α- and β-saxitoxinol in a greater than 8 : 1 ratio.

Sodium borohydride reduction in water at 0°C. The experimental procedure carried out was identical to that described for the elevated temperature case except that the entire reaction was performed with the vessel cooled in an ice bath with the temperature kept below 4°C during the reaction. Product isolation and chromatography on Sephadex LH-20 were identical to that described in the previous case. LH-20 fractions 30–46 contained the product which was shown by HPLC analysis to be pure α-saxitoxinol (**2**) with no detectable β epimer present.

(*c*) *Catalytic reduction.* A flask was charged with 1.85 mg of PtO_2 and 0.5 ml of 0.1 *N* aqueous HCl was added. The system was flushed with nitrogen and then sealed with a septum and stirred under a positive pressure of hydrogen. After 2–5 min the catalyst was observed to turn black and a solution of 5.4 mg saxitoxin

TABLE 1

^{1}H nmr DATA FOR SAXITOXIN AND DERIVATIVES

Compound	H5	H6	H10	H11	H13	H12
STX (1)	4.33(d)	3.47(ddd)	3.40(dd)J = 3, 10 Hz		3.88(dd)J = 9, 12 Hz	
	J = 1, 2 Hz	J = 1.2, 6, 9 Hz	3.18(ddd)J = 8, 10, 11 Hz	2.00(m)	3.65(dd)J = 6, 12 Hz	—
α-STXOL (2)	4.13(d)	3.40(t)	3.09	1.99(m)	3.77(m)	3.90(dd)
	J = 1.2 Hz		3.26	1.48(m)		J = 8.7, 11.4 Hz
β-STXOL (3)	4.33(s)	3.57(m)	3.35(m)	1.78(m)	3.68(dd)J = 12.0, 5.6 Hz	3.79(d)
	J = 1.2 Hz			2.02(m)	3.86(dd)J = 12.0, 9.6 Hz	J = 4.6 Hz
dcSTX (5)	4.33(s)	3.25(m)	3.35(m)	2.00(m)	3.25(m)	
			3.25(m)			—
α-dcSTXOL (6)	4.03(s)	3.25(m)	3.05	1.95(m)	3.22(m)	3.83(dd)
			3.18	1.58(m)		J = 8.6, 11.0 Hz
β-dcSTXOL (7)	4.26(s)	3.26(m)	3.16(m)	2.01(m)	3.22(m)	3.85(d)
				1.75(m)		J = 4.5 Hz

TABLE 2

^{13}C nmr DATA FOR SAXITOXIN AND DERIVATIVES

Compound	C14	C8	C2	C12	C4	C13	C5	C6	C10	C11
STX (1)	159.7	158.5	156.8	99.4	83.2	64.0	57.8	53.8	43.8	33.8
α-STXOL (2)	159.3	158.9	156.4	74.5	80.7	64.6	60.1	53.6	44.0	27.7
β-STXOL (3)	159.3	158.9	156.4	75.2	80.7	63.8	58.4	53.6	28.2	27.7
dcSTX (5)	—	158.5	156.7	99.4	83.1	62.0	57.3	56.3	43.6	33.6

dihydrochloride (1) in 0.5 ml of 0.1 *N* aqueous HCl was injected by syringe. An additional 0.4 ml of solvent was used to rinse the sample vial and this was also added to the reaction mixture. The reaction mixture was stirred under a hydrogen pressure of 10–11 psi, and aliquots were taken at time points and assayed to determine the progress of the reaction. After 13 hr the mixture was filtered, the catalyst washed with a small amount of H_2O, and the combined filtrate and washings lyophilized to a clear film. The crude product was subjected to HPLC analysis. The crude product was dissolved in 1.0 ml of 0.1 *N* acetic acid and chromatographed on a 2.0 × 100-cm column of Bio-Gel P-2 with 0.1 *N* acetic acid as eluting solvent. Weber-positive fractions were pooled to give an epimeric mixture of α- and β-saxitoxinol (**2, 3**) in an approximate 10: 1 ratio.

Oxidation of α-Saxitoxinol (2) to Saxitoxin (1)

To a solution of 0.92 mg of α-saxitoxinol (**2**) dihydrochloride in 0.3 ml dimethylsulfoxide (dried over molecular sieves) was added 6.5 mg dicyclohexylcarbodiimide followed by 3.9 mg pyridinium trifluoroacetate (*14*). The reaction mixture was stirred at room temperature under positive nitrogen pressure for 7.5 hr, was then frozen, and the solvent removed under reduced pressure. The residue was taken up in 0.5 ml methanol, centrifuged, and the supernatant chromatographed on a 2.0 × 100-cm column of Sephadex LH-20 with methanol as eluting solvent.

TABLE 3

TLC R_f AND TOXICITY VALUES FOR STX AND DERIVATIVES

Compound	TLC R_f		Specific toxicity (mouse units/mg)
	Solvent 1[a]	Solvent 2	
STX (1)	0.51	0.42	5500 ± 500
decSTX (5)	0.45	0.42	3700 ± 400
α-STXOL (2)	0.46	0.37	187 ± 28
β-STXOL (3)	0.52	0.37	<18
α-dcSTXOL (6)	0.44	0.35	25 ± 3
β-dcSTXOL (7)	0.46	0.35	<0.5

[a] Solvent 1, Pyr: EtoAC: HoAc: H_2O, 75: 25: 15: 30; Solvent 2, EtOH: HoAc: H_2O, 100: 40: 25.

Collection of 4.0-ml fractions at a flow of 1.0 ml/min yielded a Weber-positive product in fractions 42–47, chromatographically and spectrally identical with saxitoxin (**1**). The preparation at this stage contains a small amount of dicyclohexylurea, which, if necessary, can be removed by ion-exchange chromatography.

Preparation of [12-^{3}H]Saxitoxinol Hemisuccinate (**4**)

The procedure used was an adaptation of that used to prepare the tetrodotoxin hemisuccinate (*15*). [12-^{3}H]saxitoxinol (**2, 3**) (25 mg, 1.4 mC/mmol) was dissolved in pH 6.8 phosphate buffer and 500 mg of succinic anhydride was added. The reaction mixture was maintained at 10 ± 1°C under vigorous stirring. The pH was maintained at 5.8 with 1–4 *M* NaOH. The reaction was allowed to proceed for 2 hr after which an additional 500 mg succinic anhydride was added. Temperature and pH were again maintained at 10°C and 5.8, respectively. After 2 hr, 500 mg additional succinic anhydride was added and the conditions again maintained as above for 2 hr. Subsequently, the reaction mixture was cooled to 0°C and the pH adjusted to approximately 2 with 7.5 *N* HCl. The resulting precipitate was removed by centrifugation and washed twice with volumes of ice-cold 5% HCl in water. The supernatant and washings were combined and lyophilized. The resulting material was dried under vacuum over P_2O_5 for approximately 40 hr at 60°C and was washed with small volumes of acetone (total volume, 100 ml). The acetone insoluble material was then dissolved in distilled water and chromatographed on a 2.2 × 120-cm Bio-Gel P-2 column with distilled water as eluting solvent. Two radioactive peaks were eluted, pooled separately, and lyophilized. Each product was chromatographed on Sephadex LH-20 with methanol as eluting solvent and fractions containing radioactivity were pooled. The starting material could be eluted from the original P-2 column with 0.1 *M* acetic acid. The second peak contained the desired product, saxitoxinol hemisuccinate (7.2 mg). Nuclear magnetic resonance δ 4.96 (m, H12), 4.33 (s, H5), 3.42 (m, H6), 3.35 (m, H10), 3.25 (m, H10), 2.30–2.00 (m, succinate, 4H); (TLC R_f = 0.43, EtOH, HoAc, H_2O); (electrophoresis R_m = 0.62, relative to saxitoxinol (**2, 3**) R_m = 1.00, pH 4.5 acetate buffer). The material in the first peak has not yet been identified.

Preparation of Decarbamolysaxitoxin (**5**)

A solution of 80 mg saxitoxin (**1**) dihydrochloride in 25 ml of 7.5 *N* HCl was heated at 100°C for 7.5 hr after which the resulting brown solution was evaporated to dryness *in vacuo*. The residue was taken up in a small amount of ethanol and chromatographed on a 2.3 × 30-cm column of acidic alumina with ethanol as eluting solvent. The toxic fractions were collected, concentrated, and further purified by chromatography on a 2.3 × 30-cm column of Sephadex LH-20 using methanol as eluting solvent to yield 47.5 mg (67%) of decarbamoyl saxitoxin (**5**). Spectral and chromatographic data are given in Tables 1, 2, and 3. Subsequent experiments have shown the reaction to be complete in 3 hr at 110°C, with some improvement in the product yield (75%).

Preparation of Decarbamoylsaxitoxinol (6, 7)

Decarbamoylsaxitoxin (**5**) dihydrochloride (32 mg) was treated with sodium borohydride in methanol at 55°C as described for the corresponding preparation of saxitoxinol. Product isolation and chromatography were identical to that described for saxitoxinol. Final yield following chromatography on Sephadex LH-20 was 29.5 mg (95%) of an approximately 3:1 mixture of α-decarbamoylsaxitoxinol (**6**) and β-decarbamoylsaxitoxinol (**7**). Spectral and chromatographic data are given in Tables 1, 2, and 3.

Nitrous Acid Decarbamoylation of Saxitoxin

To a solution of 10.4 mg saxitoxin (**1**) dihydrochloride in 2.0 ml of H_2O at 45°C was added 0.05 ml concentrated HCl and 30.5 mg $NaNO_2$. The addition of 30-mg portions of $NaNO_2$ and 0.05 ml HCl was repeated at 2.5, 6, and 23 hr. After 27 hr the solution was allowed to cool to room temperature and lyophilized. The resulting solid was dissolved in a small amount of 12 m*M* aqueous HCl and chromatographed on a 2.0 × 100-cm column of Bio-Gel P-2 with 12 m*M* HCl as eluting solvent. Collection of 5.0-ml fractions at a flow of 0.5 ml/min yielded in fractions 43–46 a small amount of saxitoxin along with a minor product (<10%) of lower polarity on TLC. Fractions 48–62 contained decarbamoylsaxitoxin (**5**, 4.1 mg, 45%) by nmr and TLC analysis.

Carbamoylation of (5) to (1)

A solution of 1.1 mg of decarbamoylsaxitoxin (**5**) dihydrochloride in 0.25 ml of methanol was evaporated to dryness *in vacuo*. To the residue, dissolved in 0.2 ml dry formic acid (*16*) (distilled from phthalic anhydride) under positive N_2 pressure and cooled to 0°C by immersion in an ice bath for 10 min, 0.075 ml chlorosulfonylisocyanate (*17*) was added dropwise and the mixture was stirred for 2 min. The bulk of the solvent and reagent was removed under vacuum, and the sample was subsequently dried at low pressure (50–100 μm) for 1.5 hr to remove remaining volatiles. After addition of H_2O the mixture was stirred for 5 min before it was lyophilized. The resulting gray powder was taken up in a small amount of methanol, centrifuged, and the supernatant was chromatographed on a 2.0 × 100-cm Sephadex LH-20 column. Elution with methanol and collection of 4.0-ml fractions gave a 50–60% yield of product in fractions 43–47, which was pooled and shown to be saxitoxin (**1**) by TLC and nmr analysis.

Preparation of Decarbamoylsaxitoxin [^{14}C]Hemisuccinate (8)

Decarbamoylsaxitoxin[^{14}C]hemisuccinate (**8**) was prepared from decarbamoylsaxitoxin (**5**) by the procedure described for the preparation of [12-^{3}H]saxitoxinol hemisuccinate (**4**) except that [1,4-^{14}C]succinic anhydride (0.1 mCi, 1 mg) was added with the first portion of succinic anhydride. This label was subsequently used as a tag for the product.

The toxicity of the reaction mixture was observed to decrease substantially during the course of the reaction. After chromatography on Bio-Gel P-2 and

Sephadex LH-20, 13 mg of impure product with a total toxicity of 17,170 mouse units and 7.3×10^5 dpm of radioactivity was obtained. The product was further purified by electrophoresis (pH 4.5 acetate buffer) to remove a small amount of decarbamoylsaxitoxin starting material. The desired product was extracted from the paper with water and chromatographed on Sephadex LH-20 to remove material leached from the paper. The final product (5.7 mg, 16%) displayed a specific toxicity of 34.4 mu/mg and 218×10^5 dpm of radioactivity. Nuclear magnetic resonance (D_2O) δ 4.00 (dd, H13, J = 12.5, 10 Hz), 3.70 (dd, H13, J = 12.5, 5 Hz), 3.48 (m, H6), 3.42 (m, H10), 3.18 (m, H10), 2.00 (m, H11), 2.33–2.13 (bm, succinate, 4H); (TLC R_f = 0.48, EtOH; HoAc, H_2O); (electrophoresis R_m = 0.50, relative to decarbomoylsaxitoxin (**5**) R_m = 1.00, pH 4.5 acetate buffer).

In a control experiment a small amount of saxitoxin (**1**) was mixed with succinic anhydride under the same conditions as described above. In this case the toxin was recovered unchanged as evidenced by toxicity and electrophoresis, indicating that no reaction had occurred with the guanidinium groups.

Isotopically Labeled Derivatives

Complete exchange of the C-11 protons for deuterium was affected by dissolving the sample, STX, in 99.8% D_2O and heating for 15–20 min at 100°C. The exchange, monitored by ^1H nmr spectroscopy was observed to be complete after 20 min as evidenced by a disappearance of the H11 multiplet at δ 2.00 ppm. Nuclear magnetic resonance (D_2O) δ 4.33 (d, H5, J = 1.3 Hz), 3.88 (dd, H13, J = 9, 12 Hz), 3.65 (dd, H13, J = 6, 12 Hz), 3.47 (dq, H6, J = 1.3, 6, 9 Hz), 3.40 (d, H10, J = 10 Hz), 3.18 (d, H10, J = 10 Hz).

[12-^{2}H]saxitoxinol and [12-^{3}H]saxitoxinol were prepared by reduction of saxitoxin dihydrochloride in methanol with sodium borodeuteride and sodium [^{3}H]borohydride, respectively. Reaction conditions and product isolation were identical to those outlined for the nonlabeled case.

[11,11,12-^{2}H]saxitoxinol was prepared by sodium borodeuteride reduction of [11,11-$^2H_2$]saxitoxin obtained by exchange in D_2O. Reaction conditions and product isolation were identical to those described above.

*Separation of Epimeric Mixture of α- and β-Saxitoxinol (**2**, **3**)*

An epimeric mixture of α- and β-saxitoxinol (**2**, **3**) obtained by sodium borohydride reduction of saxitoxin in water at 75°C was separated by semipreparative high-pressure liquid chromatography on silica gel (10 μ, 3.9 mm × 30-cm Waters μPorasil) in 0.05 M aqueous ammonium acetate at a flow rate of 2.0 ml/min. Column eluant was monitored at 220 nm and the absorbing peaks after one recycle were collected, pooled, and lyophilized. Further epimeric purity as well as desalting was achieved by repeated chromatography on a 2.0 × 100-cm column of Bio-Gel P-2 with 0.1 N acetic acid as eluting solvent. Collection of 5.0-ml fractions at a flow rate of 0.5 ml/min showed α-saxitoxinol to be eluted in fractions 39 through 41 followed by β-saxitoxinol. Fractions from P-2 were monitored for epimeric purity by rechromatography on HPLC as well as TLC on silica gel G (Pyr; EtOAc; HoAc; H_2O).

Separation of Epimeric Mixture of α- and β-Decarbamoylsaxitoxinol Epimers

An epimeric mixture of α- and β-decarbamoylsaxitoxinol (**6, 7**) was chromatographed on a 2.0 × 100-cm column of Bio-Gel P-2 under identical conditions as those described for saxitoxinol except that the original fraction size of 5.0 ml was reduced to 2.0 ml after fraction 25. Weber-positive fractions 45–70 were monitored for epimeric purity by HPLC as previously described for saxitoxinol and those fractions containing pure α or β epimer were pooled. The remaining tubes containing a mixture were pooled and rechromatographed on P-2. Final epimeric purity was assured by HPLC and ^{1}H nmr.

Coupling of [12-^{3}H]STXOL Hemisuccinate (4) to Bovine Serum Albumin

Bovine serum albumin (10 mg) and [12-^{3}H]STXOL hemisuccinate (**4**) (7.2 mg) were dissolved in 1.0 ml of distilled water and the pH of the solution adjusted to 6.8 with 10 m*M* NaOH. 1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide–HCl (10 mg) was then added and the mixture stirred for 20 hr at 28°C. Subsequently the reaction mixture was loaded onto a 50 × 1.0-cm Sephadex G-25 column and eluted with 0.5 m*N* NaCl in pH 6.8 phosphate buffer. The column fractions were assayed for both radioactivity and protein (*18*) and fractions containing both were pooled, lyophilized, and stored at −20°C.

RESULTS

The catalytic reduction of saxitoxin (**1**) to a product called dihydrosaxitoxin was originally reported many years ago (*10, 19*), but the nature of the transformation as a ketone to alcohol conversion became apparent only with the initiation of structural work (*9*) and the eventual definition of the STX structure (*3, 4*). We report here that, depending on reagents and conditions, reduction of STX yields one, or a mixture of both, C12 alcohol epimers (**2, 3**), a result in accord with two very recent reports (*20, 21*), published while this paper was under review.

Formation of two epimers is evident from nmr, TLC, and HPLC analysis of the reaction products resulting from sodium borohydride reduction in methanol. At 55°C, reduction with sodium borohydride in methanol yields an epimer ratio of approximately 2.8:1 on the basis of ^{1}H nmr and HPLC analysis. As the reaction temperature is lowered the ratio of α epimer **2** to β epimer **3** increases. The same is true when water is used as the solvent. Thus in water at 0°C sodium borohydride reduction yields no detectable β epimer. Catalytic reduction in 0.1 *N* aqueous HCl yields an α to β ratio of approximately 10:1. The major material from catalytic reduction is identical to the α epimer from sodium borohydride reduction by ^{1}H nmr, HPLC, and TLC. It is interesting to note that on silica gel HPLC the β epimer is the more polar of the two isomers, while on TLC (silica gel G) this situation is reversed (Fig. 3 and Table 3). This reversal is probably the result of the differing solvent systems.

The α-hydroxy configuration has been assigned to the major epimer **2**, and the β stereochemistry to the minor product **3**, on the basis of the following ^{1}H nmr

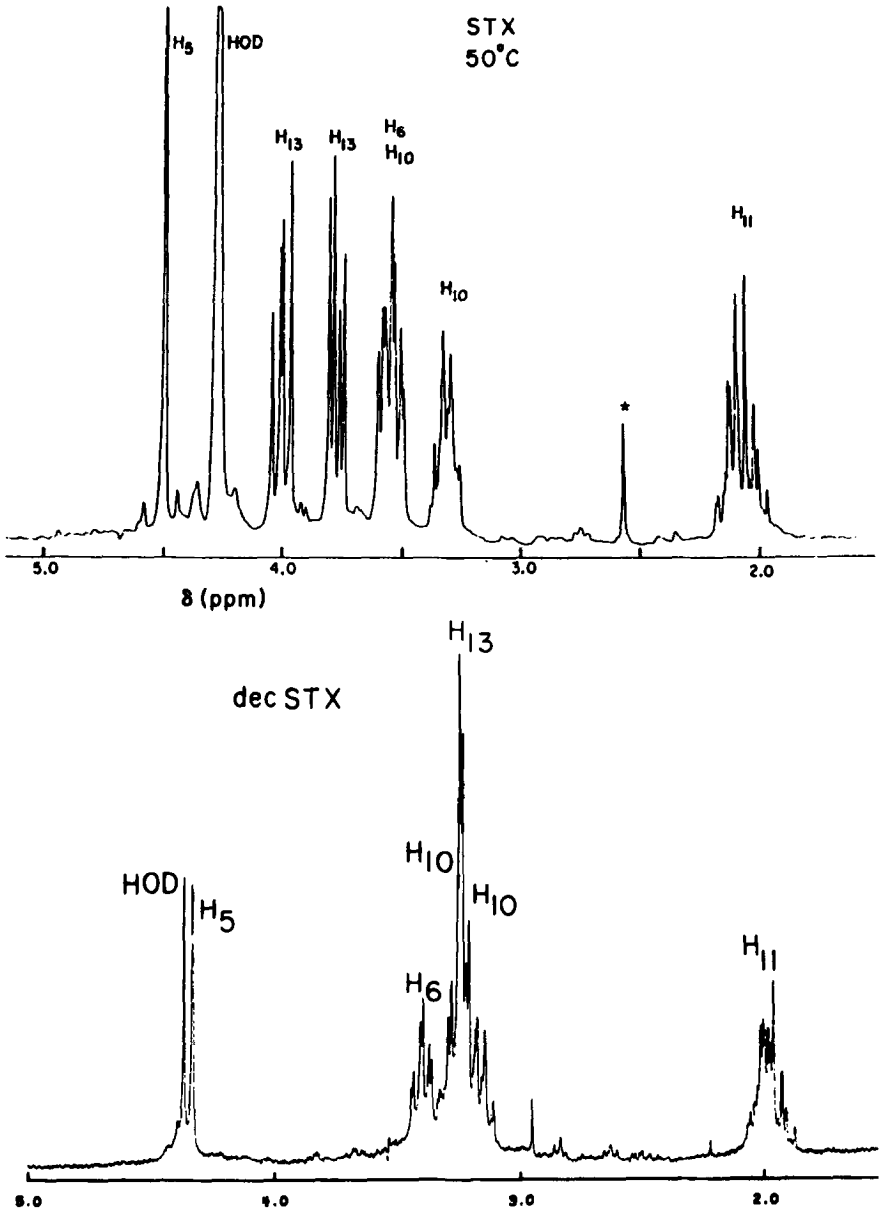

FIG. 1. Proton nmr spectra (270 MHz) of saxitoxin (STX, 1) and decarbamoylsaxitoxin (decSTX, 5).

criteria (Fig. 2): (i) The signal for H13 is a two-proton multiplet in the major epimer **2**, while in the minor epimer **3** the H13 protons give rise to two distinct doublets of doublets. This behavior obviously implies nonequivalency of the two C13 protons in the β-hydroxy epimer **3**, presumably resulting from restricted side chain rotation, because of the presence of the β-hydroxy group. A similar phenomenon is observed in the ^{1}H nmr spectrum of saxitoxin (*21, 22*), but no steric interaction is possible when the C12 hydroxyl is in the α configuration as in epimer

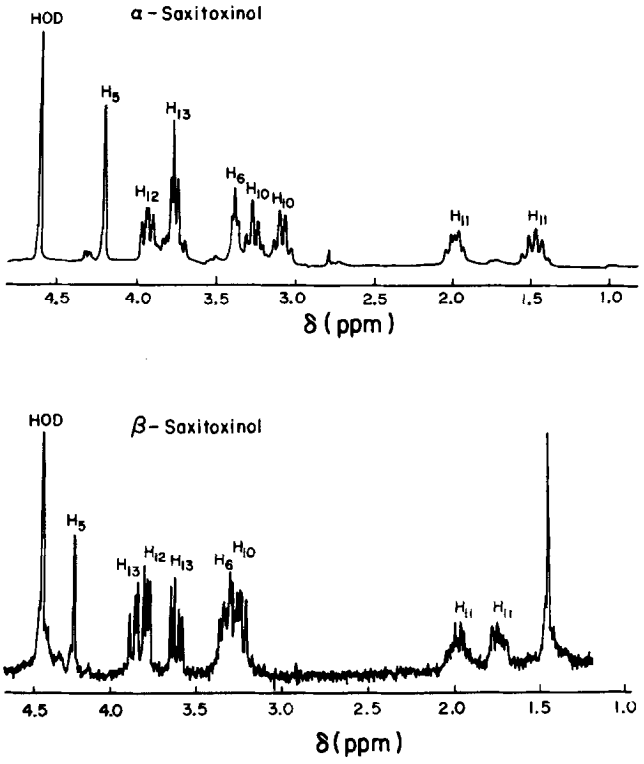

FIG. 2. Proton nmr spectra (270 MHz) of α-saxitoxinol (2) and β-saxitoxinol (3).

2; (ii) a small nuclear Overhauser effect observed on the H5 signal upon irradiation of the H12 signal in the major epimer in accord with the *cis* arrangement of these two protons in the α-hydroxy compound; (iii) the H12 signal in the α epimer is a doublet of doublets with values of 11.4 and 8.7 Hz, indicating coupling with both H11 protons. However, in the minor epimer, the H12 signal is a simple doublet with a coupling constant of 4.6 Hz, thus indicating a dihedral angle of 90° with one of the H11 protons. Models show that a β-hydroxy configuration would result in an angle of ca. 90° between H12α and H11β. This combination of NOE experiments and coupling analysis defines the C12 stereochemistry of the alcohol epimers explicitly; our results are in agreement also with the recent configurational assignments based on interpretation of ^{13}C nmr data (*20*) and proton coupling patterns (*21*).

The assignments in the ^{1}H nmr spectrum of saxitoxinol are confirmed by the spectral data on [11,11,12-$^{2}H_3$]saxitoxinol and [12-^{2}H]saxitoxinol. The former derivative showed the absence of the H11 signals at 2.05 and 1.58 ppm as well as of the resonance at δ 3.96 ppm due to H12, and the H10 multiplets at δ 3.35 and δ 3.15 now appeared as a simple AB quartet pattern. The spectrum of [12-^{2}H]saxitoxinol, as expected, lacked the H12 proton signal at δ 3.96.

The assignments for the ^{13}C nmr spectra of saxitoxin, decarbamoylsaxitoxin, and α- and β-saxitoxinol are given in Table 2, and the spectra are shown in Fig. 4.

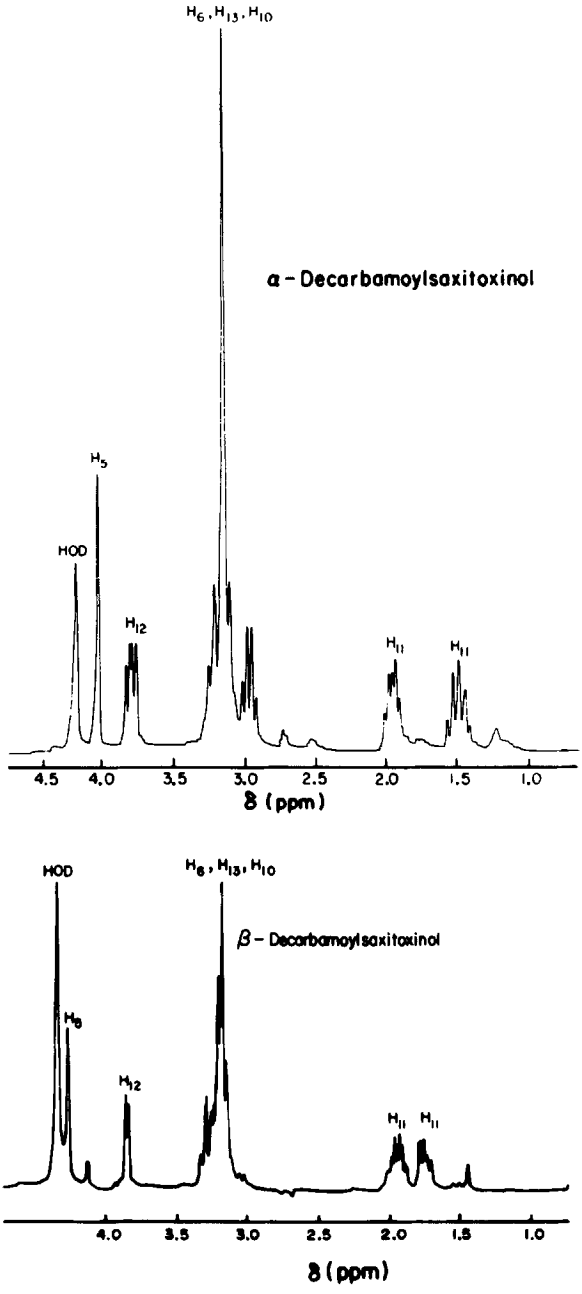

FIG. 3. Proton nmr spectra of α-decarbamoylsaxitoxinol (6) and β-decarbamoylsaxitoxinol (7).

Specific assignments are based on the relative chemical shifts of signals in the spectra of STX and the derived alcohols, and are fairly straightforward for all carbons, except the guanidino carbons at C2 and C8, for which our data do not allow explicit assignments. However, the recent definitive pH titration studies of

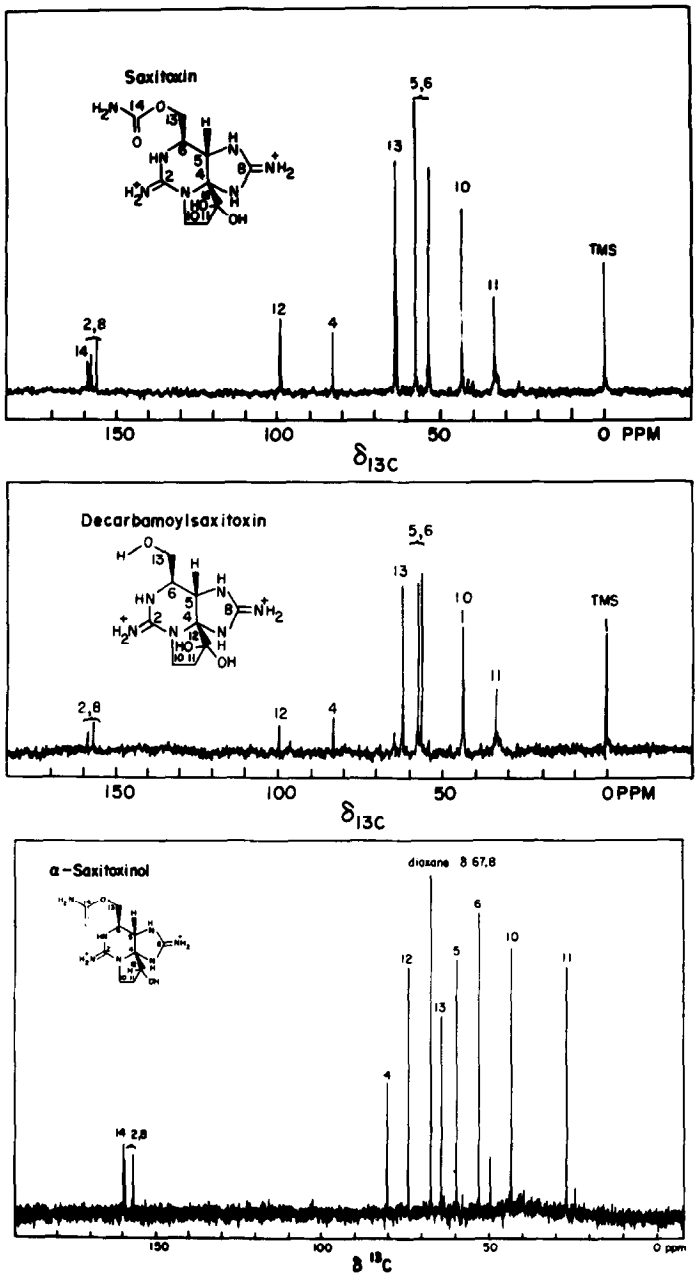

FIG. 4. ^{13}C nmr spectra of saxitoxin and derivatives.

Rogers and Rapoport (*20*) distinguish between the guanidinium carbons, and the specific shifts for C2 and C8 listed in Table 2 conform to their assignments.

In the ^{13}C spectrum of α-saxitoxinol (2), the absence of a C12 signal at 99.4 is noted, and a new peak is observed at 74.5 ppm. This is consistent with the conversion of the hydrated ketone to the corresponding alcohol. Although we

were unable to isolate a sufficient quantity of the β-saxitoxinol epimer (**3**) to permit ^{13}C nmr analysis, it was possible with spectra of pure α epimer and of an epimeric mixture, to deduce the spectrum of the β epimer. The assignments in Table 2 are the results of this procedure.

Hydrolysis of STX in strong acid removed the carbamate group and gave decarbamoylsaxitoxin (**5**). Decarbamoylation can also be accomplished under less strongly acidic conditions, i.e., by reaction in nitrous acid, although strong acid hydrolysis gives better yields. Proton and ^{13}C data for this compound are presented in Figs. 1 and 4 and Tables 1 and 2. The C13 protons of **5** are shifted to higher field, as expected, but noteworthy is the loss of signal multiplicity. The protons now appear as a single complex multiplet, instead of the double quartet pattern observed for STX (**1**), implying that the rotational restrictions on the side chain have been removed. Treatment of alcohol **5** with chlorosulfonylisocyanate (*17*) under conditions similar to those of Kishi and co-workers (*16*) affords the natural product, STX (**1**), in good yield. Interestingly, attempts to oxidize alcohol **5** to the corresponding aldehyde led to recovery of starting material only, whereas under the same conditions the C12 alcohol **2** was smoothly reoxidized to saxitoxin (**1**).

Sodium borohydride reduction of decarbamoylsaxitoxin (**5**) in methanol at 55°C gives the alcohols **6** and **7** in an epimer ratio similar to that obtained in the reduction of saxitoxin under similar conditions. The epimers could be separated on HPLC (see Fig. 5). Proton nmr data are presented in Fig. 3 and Table 1. Again one sees a doublet of doublets at δ 3.83, J = 8.6 and 11.6 Hz for the H12 signal of the α epimer (**6**), while the β isomer (**7**) shows a single doublet at δ 3.85, J = 4.5 Hz; the H13 signal for both epimers is a two-proton multiplet at 3.22 ppm. Apparently the carbamate side chain plays little role in the conformation of the lower ring in either saxitoxinol epimer since the corresponding decarbamoyl compounds show no change in the multiplicity of the H12 signals. The polarity reversal between HPLC and TLC is observed for these compounds as well.

Reaction of either saxitoxinol (as an epimeric mixture of **2** and **3**) or decarbamoylsaxitoxin (**5**) with succinic anhydride furnished the corresponding hemisuccinate derivatives (**4**) and (**8**), which were characterized on the basis of their proton nmr spectra (see Experimental). One might note that the spectrum of (**8**) now shows two clear quartets (doublets of doublets) for the C13 protons, implying restricted rotation of the ester side chain as in the case of the carbamoyl side chain of STX (**1**). The nmr of **4** (see Experimental) shows the expected downfield shift of H12 to 5 ppm. Hemisuccinate derivative **4** (as the 12-^{3}H derivative) was also coupled to bovine serum albumin (BSA) via a carbodiimide reaction, to yield a conjugate of 3 mol of (**4**)/mole BSA. Attempts to generate antibodies to this conjugate were not successful, probably because of the low degree of saxitoxinol substitution on the protein carrier.

Toxicity

Mouse bioassay of the individual saxitoxinol epimers showed the α epimer (**2**) to have a specific toxicity of 187 ± 28 MU/mg, thus representing approximately

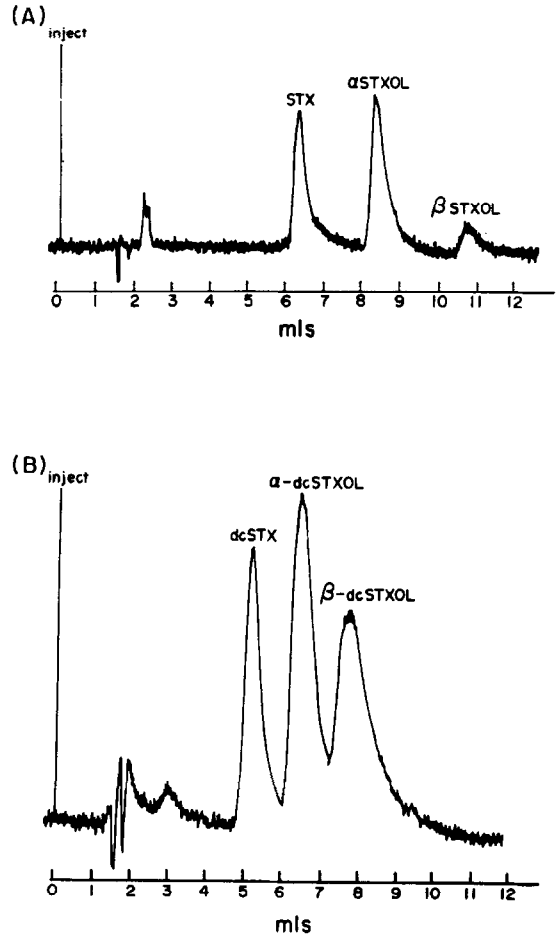

FIG. 5. High-pressure liquid chromatography of saxitoxinol (A) and decarbamoylsaxitoxinol (B) epimers.

3.7% of the biological activity of saxitoxin (5500 MU/mg). The β epimer of saxitoxinol (3) showed a specific toxicity of less than 1/10th that of the α epimer. There appears to be no synergistic effect between the two isomers, as a 2.8: 1 α:β mixture showed a specific toxicity of approximately 2.5% that of saxitoxin. The symptoms of toxicity and death in mice were the same as those observed for saxitoxin, although there appeared to be a prolonged duration of action for saxitoxinol as compared to an equivalent dose of saxitoxin.

Decarbamoylsaxitoxin (5) displayed a specific toxicity of 3700 ± 400 MU/mg, approximately 60% that of saxitoxin (1). The corresponding C12 alcohols, α- and β-decarbamoylsaxitoxinol, showed toxicities similar to that of α- and β-saxitoxinol, with α-decarbamoylsaxitoxinol having 0.5% of the activity of saxitoxin and the β epimer less than 1/10th of the activity of the α epimer. Succinylation of decarbamolysaxitoxin to hemisuccinate 8 significantly reduces toxicity (to 34 MU/mg).

DISCUSSION

The name "dihydrosaxitoxin" was originally given to the catalytic hydrogenation product of saxitoxin based on the experimental evidence that one mole of hydrogen was taken up per mole of toxin (*10, 19*). Since the reduction involves the conversion of the hydrated ketone function to two epimeric alcohols, we suggest "saxitoxinol" (or more specifically, α- and β-saxitoxinol) as a more descriptive and shorter designation for the product.

Previous reports have stated that the reduction product of saxitoxin was nontoxic (*10*). We have shown that this material does clearly possess a small but finite amount of biological activity, with the α epimer being the more toxic of the two by a factor of approximately 10.

The decrease of toxicity on going from the ketone hydrate to the alcohol at C12 raises a number of points concerning the binding of saxitoxin to its receptor (the sodium channel) in the nerve or muscle membrane. If hydrogen bonding plays a major role in binding, it appears that the gem diol configuration of saxitoxin at C12 is important, since both epimers of saxitoxinol (**2, 3**) show similarly decreased activity, which would not be expected if hydrogen bonding involving only one hydroxyl were a dominant factor in binding. Thus a hydrogen bonding model of binding is possible in which both hydroxyls participate synergistically in binding to the target site.

A second model features the dehydration of saxitoxin at C12 to the keto form, which in turn may react with a nucleophilic receptor component to form a labile covalent linkage (*23*). Neither epimer of saxitoxinol would be able to particpate in this type of interaction, and the formation of a covalent bond therefore appears highly unlikely.

Decarbamoylsaxitoxin (**5**) is a potent analog of saxitoxin, indicating that the carbamate side chain plays a secondary role in toxin/receptor interactions and suggesting also that other side chain derivatives (i.e., other esters or carbamates) might retain high binding affinity and thus serve as useful probes in neurophysiological work. The hemisuccinate ester (**8**) of decarbamoylsaxitoxin (**5**) is only weakly toxic (30–50 MU/mg), possibly because of charge interaction of the free carboxyl with a guanidino ring, one of which is certainly important for toxin/receptor binding; but the carboxy residue may of course also alter toxin distribution and transport in the organism, or promote nonspecific binding to other macromolecules. Reduction of the ketone in **5** gives the corresponding alcohol epimers **6** and **7**, which exhibit, as expected, much reduced toxicity.

Some of these derivatives have been used for *in vitro* studies on isolated frog muscle and squid giant axon (*24*). Preliminary results indicate that the relative binding affinity for saxitoxin, decarbamoylsaxitoxin, and saxitoxinol is 1 : 0.2 : 0.01, with α-saxitoxinol being about four times more active than the β epimer, a relative order that follows *in vivo* toxicity measurements. Furthermore, all toxins bind specifically to the sodium channel (*24*).

The saxitoxinol hemisuccinate–BSA conjugate is a potential antigen for the production of antibodies to saxitoxin. The low coupling efficiency of hapten to protein may be due to a number of factors. The hemisuccinate moiety may not be

of sufficient length to alleviate steric interactions with the protein during the coupling reaction. Alternatively, the residual negative charge of the carboxylic acid side chain may result in electrostatic interactions with the albumin molecule as well as possibly participating in the formation of an internal salt with one of the guanidino groups of the hapten molecule, resulting in a hapten of greatly reduced reactivity to coupling. Saxitoxinol adducts with dicarboxylic acids of increased chain length may circumvent a number of these complications. The present saxitoxinol hemisuccinate conjugate has been tested for antisera production in rabbits with no apparent success at this stage.

ACKNOWLEDGMENTS

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