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ON THE STRUCTURE AND BIOLOGICAL PROPERTIES OF DECARBAMOYLSAXITOXIN

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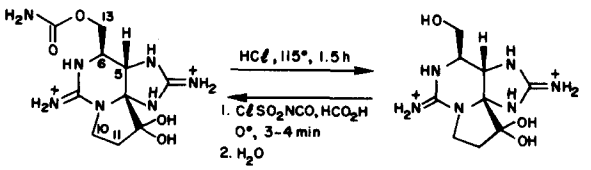
The acid hydrolysis product of saxitoxin is shown to be decarbamoylsaxitoxin by spectral characterization and its reconversion to saxitoxin by carbamoylation. Natural and resynthesized saxitoxin are identical in chromatographic and spectral properties and in their potencies in blocking the sodium channel in squid giant axon. The hydrolysis product, decarbamoylsaxitoxin, exhibits 20% of the potency of saxitoxin in the squid axon system. These results confirm the structure of the hydrolysis product and its biological activity relative to saxitoxin.

Introduction

Saxitoxin ((1), Fig. 1) is the best-known member of a family of neurotoxins elaborated by marine dinoflagellates [1–6]. Its specific and high-affinity binding to the voltage-dependent sodium channel of nerve and muscle cells has been exploited in a broad range of electrophysiological and neurobiochemical studies [7–9]. Current efforts to define toxin-channel interactions and the structural requirements for toxin binding [10–12] have led to the prepration and assay of selected saxitoxin derivatives. One of these derivatives, decarbamoylsaxitoxin ((2), Fig. 1) [13,14] is of particular interest, in part because (2) exhibits high potency, and in part because the primary hydroxy function in (2) offers a site for potential further modification to experimentally useful toxin analogs. Hydrolysis product (2) retains about 60% of the potency of (1) in the standard mouse-toxicity assay [13,15], and 20% of the activity of (1) in blocking the sodium channels in frog sartorius muscle [11]. Furthermore, Koehn et al. [15] have reported that carbamoylation of (2) with chlorosulfonyl isocyanate regenerates saxitoxin, (1), in agreement with the earlier results of Kishi and co-workers [16] who carbamoylated racemic (2), prepared by total synthesis, to obtain racemic saxitoxin. However, the above chemical and biochemical results have been questioned recently by Rubinson [17], who reported obtaining a hydrolysis product at least 100-times less effective than saxitoxin in blocking the propagated action potential in crab leg nerves. This finding, and his failure to regenerate saxitoxin from the hydrolysis product after attempted carbamoylation, were interpreted to "show that the acid hydrolysis product may



(1) (2)

Fig. 1. Structures and interconversion of saxitoxin (1) and decarbamoylsaxitoxin (2).

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

not be, in fact, decarbamoylsaxitoxin, or the chlorosulfonylisocyanate reaction does not proceed as believed" [17]. We now present further experimental work, both chemical and electrophysiological, which shows unambiguously that hydrolysis of saxitoxin to (2) and reconversion of the latter to the former proceeds as depicted in Fig. 1 and that the respective compounds exhibit biological potencies as previously described.

Materials and Methods

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, and Bio-Gel P-2 and Bio-Rex 70 from Bio-Rad Laboratories. Chlorosulfonyl isocyanate was obtained from Aldrich. Formic acid was purified by refluxing over phthalic anhydride for 24 h followed by distillation.

Thin-layer chromatography (TLC) was performed on precoated aluminum-backed silica gel G (EM reagents) in pyridine/ethyl acetate/acetic acid/water (75 : 25 : 15 : 30, v/v), or butanol/acetic acid/water (12 : 5 : 9, v/v) as solvent. Compounds were visualized by heating at 120°C for 10 min and observing the resultant fluorescent spots under long-wavelength ultraviolet illumination, or by spraying with Weber reagent [18]. ^{1}H-NMR spectra of the compounds in 100% ^2H_2O solution were obtained on a Bruker instrument at 270 MHz, equipped with a Nicolet 1180 data system. The 600 MHz NMR spectrum of (2) was obtained at the Eastern Regional NMR Center at Carnegie-Mellon University. Chloroform ($\delta = 7.27$) served as internal reference.

Hydrolysis of saxitoxin (1) to decarbamoylsaxitoxin (2)

This reaction was performed as described previously [15]. Specifically, 13.7 mg of saxitoxin dihydrochloride (1) in 4.0 ml of 7.5 M aqueous HCl was heated at 115°C under nitrogen for 90 min. The resulting light-brown solution was diluted with water, frozen and lyophilized. The residue was loaded on a 2 × 100 cm column of Bio-Gel P-2 and eluted with 0.02 M aqueous acetic acid. Fractions yielding fluorescent material upon heating were pooled and lyophilized. The resulting residue was taken up in H_2O and loaded on a 1 × 100 cm column of Bio-Rex 70 (H^+ form). The column was washed with 70 ml H_2O, followed by elution with 20 mM HCl, to give 7.1 mg (58% yield) of decarbamoylsaxitoxin (2). Chromatographic and spectral data for the product are shown in Table I and Fig. 2, respectively.

TABLE I

CHROMATOGRAPHIC (TLC) COMPARISON OF TOXINS

Solvent 1: pyridine/ethylacetate/acetic acid/water (75 : 25 : 15 : 30, v/v); solvent 2: butanol/acetic acid/water (12 : 5 : 9, v/v).

Compound	R_F (TLC)	
	Solvent 1	Solvent 2
Saxitoxin ((1), natural)	0.51	0.40
Decarbamoylsaxitoxin (2)	0.45	0.32
Saxitoxin ((1), synthetic)	0.51	0.40

Carbamoylation of (2) to (1)

A methanolic solution of 2.8 mg decarbomoylsaxitoxin dihydrochloride (2) was evaporated at reduced pressure and further dried under high vacuum for 6 h. The residue, dissolved in 0.2 ml formic acid was stirred under N_2 for 15 min in an ice-bath, and then treated with 75 µl chlorosulfonyl isocyanate [15].

After 3.5 min, 1.5 ml cold H_2O was added and the resulting mixture was frozen and lyophilized. The residue was loaded on a 2 × 100 cm Bio-Gel

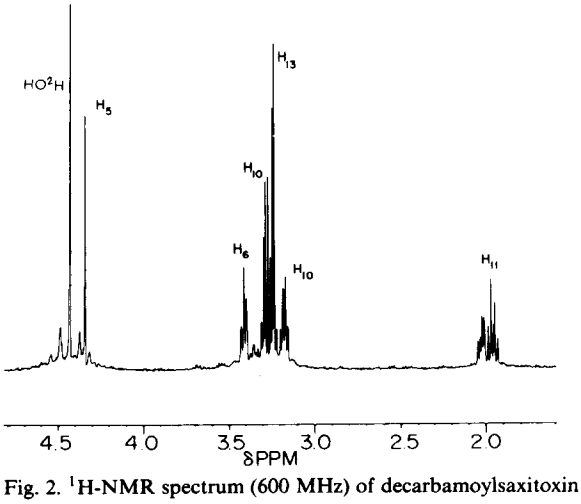


Fig. 2. ^{1}H-NMR spectrum (600 MHz) of decarbamoylsaxitoxin (2).

P-2 column and washed with 160 ml H_2O. Subsequent elution with 0.02 M HOAc gave a peak of Weber-positive material of which the lead portion was observed to contain contaminating inorganic salts. This fraction was lyophilized, taken up in methanol and chromatographed on a 2 × 100 cm column of Sephadex LH-20 with methanol as eluting solvent. Fractions containing Weber-positive material were pooled to give 0.42 mg saxitoxin (1). The remaining major portion of the peak from the P-2 column was lyophilized, taken up in H_2O and loaded on a 1 × 6 cm column of Bio-Rex 70 (H^+ form). The column was washed with 70 ml of H_2O and then eluted with 36 mM HCl to give one peak of Weber-positive material. Peaks from LH-20 and ion exchange were pooled to give 0.84 mg saxitoxin (34% yield), spectrally and chromatographically identical to authentic material (Fig. 2, Table I).

Electrophysiological experiments

Because the actions of (1) and (2) are known to be limited to a blockade of the sodium channel [11], the relative potencies of these compounds on the squid giant axon were assayed by their depression of the maximum rate of rise of the propagated action potential ($\dot{V}_{max}$). Periodic checks were made until a steady-state of toxin action was reached. The $\dot{V}_{max}$ values in the presence of toxin were normalized to those in the absence of toxin ($\dot{V}'_{max}/\dot{V}_{max}$), and plotted against toxin concentration. The data were then compared with theoretical relations which were predicted on the assumption of a bimolecular reaction between the toxin and the membrane [11]. For (1), both natural and synthetic, the predicted curve was fitted to the data by eye. For (2), it was shifted towards higher concentrations on the assumption that (2) was 20% as active as (1) [11].

Results and Discussion

Acid hydrolysis of saxitoxin (1) under the conditions previously reported [15] results in the removal of the carbamate group and yields decarbamoylsaxitoxin (2) (Fig. 1). This conclusion is based on the ^{1}H-NMR spectrum of product (2) which contains all the resonances for the carbon-bonded protons exhibited by the starting material

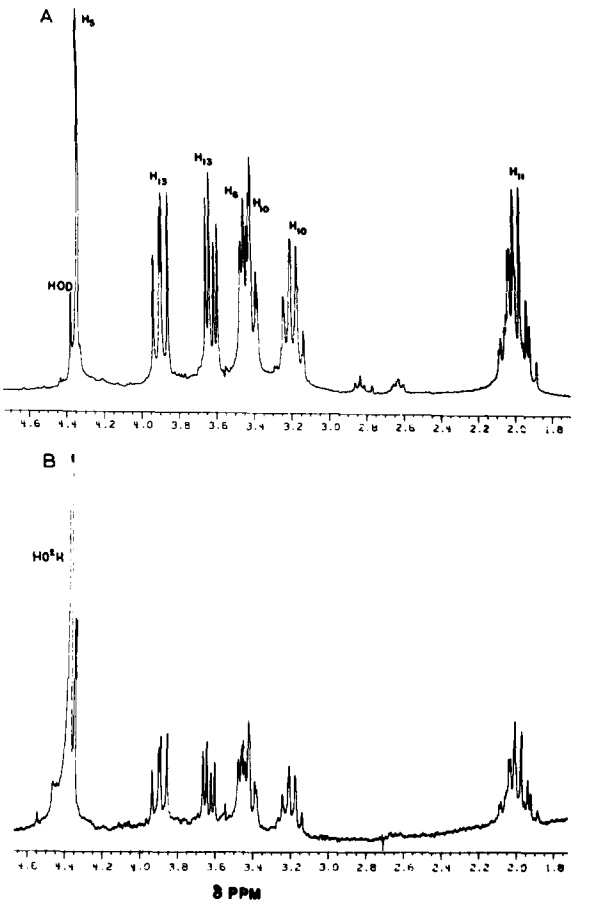


Fig. 3. ^{1}H-NMR spectra (270 MHz) of (A) natural saxitoxin, and (B) saxitoxin obtained by carbamoylation of (2).

(1), except that the C(13)-protons are shifted upfield as expected (see Fig. 2). The distinctive double four-line pattern (δ 3.88 and 3.65) of the C(13)-protons in the spectrum of (1) (see Fig. 3) coalesces into a sharp multiplet in the case of (2) (δ ~ 3.25, Fig. 2). At 600 MHz, the closely spaced signals for the C(6)-, C(10)- and C(13)-protons are, however, sufficiently well resolved to allow (together with decoupling and C(11)-deuterium exchange experiments) the specific proton assignments indicated in Fig. 2.

Unambiguous confirmation of structure (2) for the hydrolysis product is provided by the recarbamoylation of (2) to saxitoxin (1). Treatment of (2) with chlorosulfonyl isocyanate followed by aqueous work-up yields a product identical with natural saxitoxin as shown by direct comparison

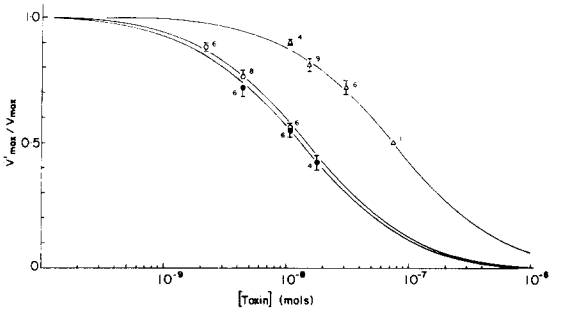


Fig. 4. Dose-response relations of natural saxitoxin, synthetic saxitoxin, and decarbamoylated saxitoxin. Series of ten propagated action potentials were elicited in single squid giant axons at intervals of 3–5 min. The action potentials were recorded with intracellular microelectrodes, differentiated and signal-averaged to obtain mean value of maximum rate of rise ($\dot{V}_{max}$). $\dot{V}_{max}$ values in toxin were normalized to those without toxin, and the ratio plotted against log of toxin concentration. Datum points represent mean ± S.E. with attached number showing number of axons used for the point. Solid lines are computed from the relation: $\dot{V}'_{max}/\dot{V}_{max} = 1/1 - (ED_{50}/[toxin])$. ○——○, natural saxitoxin; ●——●, synthetic saxitoxin; △——△, decarbamoylsaxitoxin. All data were collected at 6 ± 0.5°C. The artifical sea water contained (in mM): Na^+, 423; K^+, 9; Ca^{2+}, 9; Mg^{2+}, 50; Cl^-, 499; SO_4^{2-}, 26, and was buffered to pH of 7.25 with 10 mM Hepes.

of the respective ^{1}H-NMR spectra (Fig. 3) and chromatographic analysis (Table I). This result is in accord with earlier reports [15,16] and is in accord also with the more recent preparation of certain carbamoyl-*N*-sulfotoxins [6] using the same reagent under somewhat modified conditions.

We have also compared the relative potencies of hydrolysis product (2) with natural and synthetic saxitoxin (1). Fig. 4 shows the dose-response relations of (1) and (2) on the squid giant axon. Comparing the ED_{50} of the compounds, it is evident that whereas (2) is 20% as active as (1), as previously described for isolated frog muscle fibers [11], the recarbamoylated compound is equally active as the natural saxitoxin prior to acid hydrolysis.

The preceding results confirm the structure of the saxitoxin-hydrolysis product as (2) and its recarbamoylation to saxitoxin. We also show that (2), as reported previously [11], does retain substantial biological activity. An explanation of Rubinson's contrary findings [17] is not immediately apparent, and in the absence of physical data on his products, speculation as to their nature and purity is not profitable. We might note, however, that Rubinson's conditions for recarbamoylation of putative (2) to saxitoxin differ substantially from ours, and the prolonged reaction times chosen could well lead to undesired further modification of the initial carbamoylation product.

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