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Modification of Tetrodotoxin with Succinic Anhydride

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With an aim toward the synthesis of physiologically active derivatives of the powerful neurotoxin tetrodotoxin and the use of such derivatives in biochemical studies of the molecular makeup of the tetrodotoxin binding site in nerve, the modification of tetrodotoxin with succinic anhydride has been examined. A new toxin derivative, 11-succinylanhydrotetrodotoxin, is described which contains a remote carboxylic acid grouping suitable for subsequent modification or attachment to an affinity column. High voltage electrophoresis and radiochemical techniques served to establish the purity of the derivative and its relative toxicity, namely, 0.4% that of tetrodotoxin (frog nerve assay). Structure assignment was made on the basis of the FT 100 MHz nmr spectrum.

INTRODUCTION

Owing to its ability at nanomolar concentrations to block selectively the early transient inward Na current associated with the passage of an action potential in nerve, tetrodotoxin (TTX)[2] (1) has enjoyed wide application as a pharmacological tool in neurophysiology (*1*). Concurrently, aided by the recent availability of [G-^{3}H]TTX, several laboratories have been working toward the isolation and characterization of the biomolecules which specifically bind TTX (*2–6*). Affinity chromatography has proven invaluable in similar situations; however, none of the known derivatives (*7–9*) of TTX appears to be structurally suitable for convenient construction of an affinity column.[3,4] We now describe the chemical modification of TTX with a bifunctional reagent, succinic anhydride, in aqueous solution. The utility of our new, active TTX derivative in biochemical studies with nerve stems from the presence of the remote carboxyl group which may serve not only as a point of attachment of the molecule to an insoluble polymer support but also as a potential site for the incorporation of a photoaffinity label.

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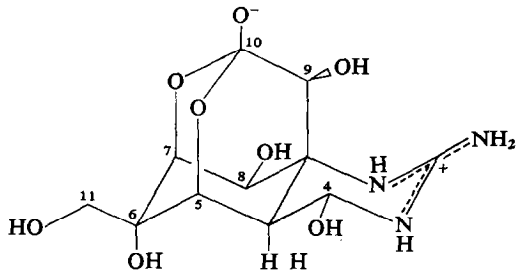
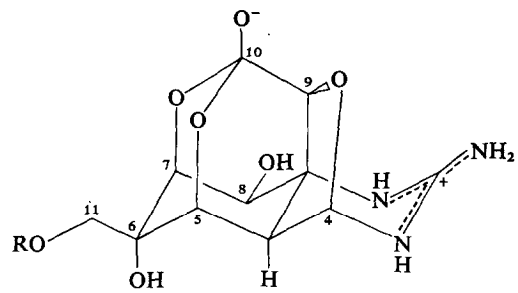
[2] Abbreviations used are: TTX, tetrodotoxin; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazoyl)]benzene; ε-DNP-Lys, ε-dinitrophenyl lysine.

[3] Recently, R. Y. Tsien *et al.* (*16*) have reported an *in situ* synthesis of a methoxyamine derivative of 11-nor TTX and have suggested the possibility of covalent attachment of other functional groups to 11-nor TTX.

[4] V. E. Ghazarossian *et al.* (*17*) have prepared decarbamylsaxitoxin and have pointed out its potential for the construction of an affinity column.



TTX (1) (1)



[5]

AnhydroTTX (2), $R = H$

11-SuccinylanhydroTTX (3), $R = HO_2CCH_2CH_2C(=O)$

RESULTS AND DISCUSSION

Succinic anhydride modification of TTX. Through use of a large molar excess of succinic anhydride and maintaining pH 5–6 with the aid of a pH-Stat, both succinylation of [G-^{3}H]TTX and the reaction of [1,4-^{14}C]succinic anhydride with unlabeled TTX produced succinylTTX[5] clearly distinct and separable from unmodified TTX by high voltage electrophoresis at pH 6.5 (Fig. 1). SuccinylTTX comigrated with ε-DNP-Lys and sucrose at pH 6.5, indicating the derivative to be electrically neutral at this pH. Since TTX itself has a net single positive charge below its pK_a (=8.5) (*8*) and since each succinyl residue attached to TTX brings with it a single negative charge at pH 6.5, there must be one succinyl group in succinylTTX. This was confirmed by electrophoresing succinylTTX at pH 1.9 where the monosuccinate anion is protonated and the derivative migrates as a singly positively charged species (with respect to sucrose), approximately migrating with ε-DNP-Lys.

$$\underset{\text{(pH 1.9)}}{HO_2C(CH_2)_2CO_2\text{-TTX}^+} \qquad \underset{\text{(pH 6.5)}}{\bar{O}_2C(CH_2)_2CO_2\text{-TTX}^+}.$$

Two preparative high voltage electrophoreses (pH 6.5) afforded a sample of [1,4-^{14}C]succinylTTX that was 52% radiochemically pure. At this point the sample was

[5] "SuccinylTTX" refers to the electrophoretically pure mixture of succinylated derivatives in which the major component is 11-succinylanhydroTTX (see text).

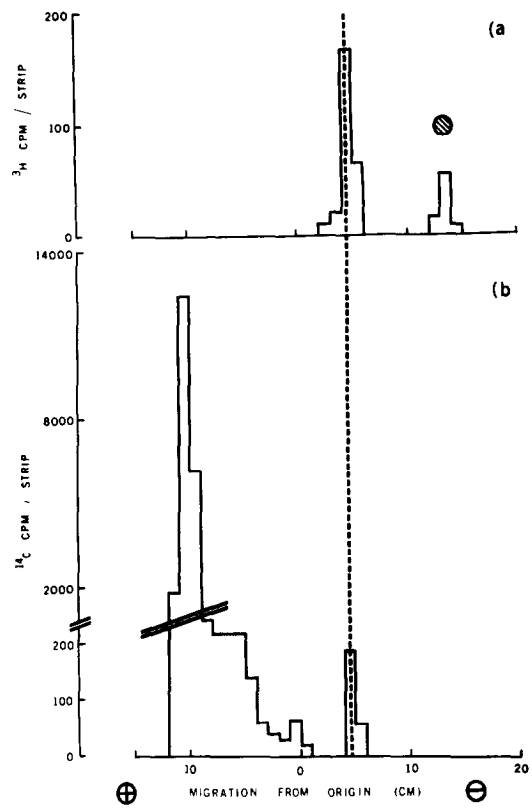


FIG. 1. High voltage electrophoresis (pH 6.5) of (a) the crude reaction product from [G-^{3}H]TTX and succinic anhydride and (b) the crude reaction product from TTX and [1,4-^{14}C]succinic anhydride. The large peak of radioactivity migrating to the anode in (b) is [1,4-^{14}C]succinate. The broken line indicates the migration of an uncharged marker (sucrose). Background activity (ca. 25 cpm) is subtracted in both (a) and (b).

doped with 20 μl (1.5×10^{3} dmp, ca. 0.02 μg) of [G-^{3}H]TTX in order to facilitate detection of any natural TTX in subsequent purifications of the succinylTTX. A further electrophoresis enhanced the radiochemical purity of [1,4-^{14}C]succinylTTX to 93%. This material showed a toxicity equivalent to 1.28 μg of TTX and contained <1% (as shown by an analytical electropherogram) of the [G-^{3}H]TTX added in the previous step.

Since it was conceivable that the observed toxicity resulted from some hydrolysis (see below) of the succinylTTX (regenerating starting TTX), a fourth preparative electrophoresis was performed in which the [1,4-^{14}C]succinylTTX was eluted, lyophilized, analyzed for impurities, and bioassayed within the shortest possible time period (ca. 5 hr). All stages of the final purification were carried out at <4°C. Thus, the [1,4-^{14}C]succinylTTX so obtained was 99.4% radiochemically pure (Fig. 2) and showed a toxicity equivalent to 0.19 μg of TTX. Knowing the weight of succinylTTX present in the sample (from the specific activity of [1,4-^{14}C]succinic anhydride and the fact that one succinyl group is present in succinylTTX) and after a small correction for the TTX

produced by hydrolysis (see below) during the 10-min bioassay equilibration time, it can be estimated that succinylTTX is 0.4% as toxic as TTX.

Hydrolysis of succinylTTX. SuccinylTTX undergoes slow hydrolysis in Ringer's solution (pH 7.2) at 25°C with an apparent pseudo-first-order rate constant $k = 0.11$ hr^{-1} (six points taken over four half-lives, $r = 0.97$) ($t_{1/2} = 6.3$ hr). The rate constant was determined by monitoring the progressive increase in toxicity of the [1,4-^{14}C]succinylTTX

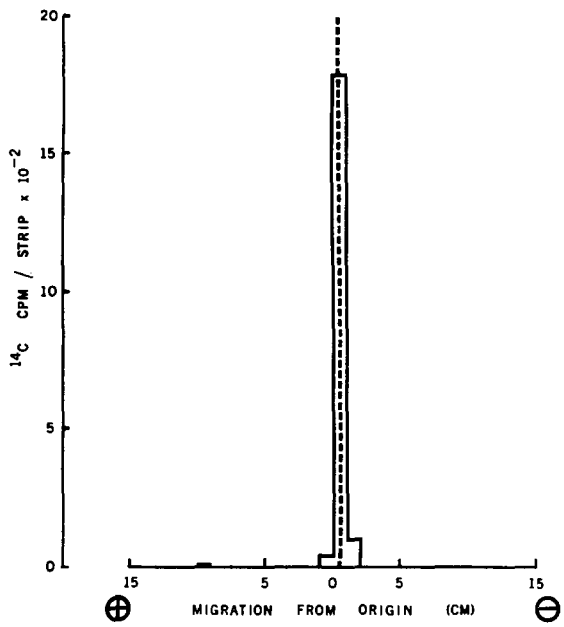


FIG. 2. High voltage electrophoresis (pH 6.5) of 99.4% radiochemically pure [1,4-^{14}C]succinylTTX. The broken line indicates the migration of an uncharged marker (sucrose). Background activity (ca. 25 cpm) is subtracted.

Ringer's solution with the frog bioassay. Assuming that the increase in toxicity was due to formation of TTX, then the yield of TTX at t_∞ based on starting [1,4-^{14}C]succinylTTX was ca. 5%.[6] A second hydrolysis of [1,4-^{14}C]succinylTTX was carried out in pH 9.1 buffer at 25°C. Analysis by high voltage electrophoresis after 12 hr revealed that the major hydrolysis product was [1,4-^{14}C]succinate with 36% of the radioactivity remaining in the original peak ($t_{1/2} \approx 8$ hr.)

Structure of succinylTTX. An inspection of the structure of TTX (**1**) reveals several possible sites for succinylation. Since acylguanidines, however, are highly labile toward hydrolysis in aqueous solution ($t_{1/2}$ ~8 min (*10*)), it is unlikely that the succinyl

[6] We do observe that succinylTTX is significantly more stable at 4°C in both pH ~5 distilled water and in the pH 6.5 electrophoresis buffer (as qualitatively shown by the lack of liberation of [G-^{3}H]TTX or [^{14}C]succinate by electrophoresis) than in Ringer's solution at 25°C (as measured by the progressive increase in toxicity in the bioassay). Thus, rather than hydrolysis of both the ester linkage and the anhydro linkage to produce TTX, the "hydrolysis" may actually involve only reversion of the anhydro linkage back to that present in TTX, with the ester function remaining attached. This latter molecule might be more toxic than 11-succinylanhydroTTX but less so than TTX, explaining the fact that we observe an increase in toxicity corresponding to only a 5% yield of TTX.

group in succinylTTX is attached to the guanidinium group. The relatively unhindered primary hydroxyl group attached to C-11 constitutes the most reasonable possibility and the most attractive one from the point of view of eventual construction of an affinity column. Monoacylation of TTX at C-11 with hot 99% formic acid has already been reported (*7*), though the resulting "nontoxic" derivative possessed the undesired C_4–C_9 anhydro framework.

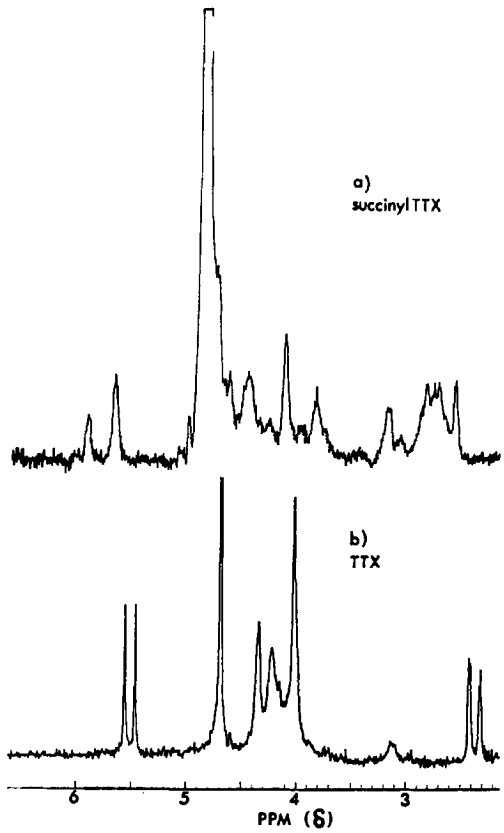


FIG. 3. Fourier transform nmr spectra of (a) succinyl-[G-^{3}H]TTX (79% radiochemically pure), 500 transients; (b) TTX, 200 transients. Spectra run in D_2O. Chemical shift scale (δ) measured with respect to DSS.

Sufficient succinyl-[G-^{3}H]TTX (900 μg) was synthesized and purified (79% radiochemically pure) for spectral analysis by FT nmr spectroscopy. The nmr spectrum (D_2O) of succinylTTX is shown in Fig. 3 together with the spectrum (D_2O) of our starting TTX (cf (*7*, *9*, *11*, *12*)). Spectrum (a) suggests that succinylTTX, although pure by electrophoresis,[7] is probably a mixture. The absence of the pair of doublets ($J = 9$ Hz) at δ 2.35 and 5.50 (axial protons on C_{4a} and C_4 in TTX) and the presence of a new

[7] An nmr spectrum of a further purified sample (~400 μg) of succinyl-[G-^{3}H]TTX (94% radiochemically pure) was complicated by the presence of impurities that were subsequently shown to have been leached from the electrophoresis paper. Apart from these impurities, the two spectra were very similar.

broad singlet at δ 5.63 (equatorial proton at C_4 (*7*, *9*) in succinylTTX) indicates that even under aqueous reaction conditions succinylation has been accompanied by formation of a C_4–C_9 anhydro ether bridge. The shift of the characteristic C_{11} methylene singlet from δ 4.01 in TTX to $\delta \sim 4.70$ (just under the HOD peak) in succinylTTX requires that the succinyl group is attached to the oxygen at C-11. The methylene protons of the succinyl group appear as an AA'BB' multiplet at δ 2.6–3.0, similar to the pattern observed for the anion of methyl hydrogen succinate.

The major component in succinylTTX is therefore assigned the structure 11-succinylanhydrotetrodotoxin (**3**).[8] The physiological activity of succinylTTX together with the presence of a remote carboxyl group for subsequent coupling reactions make succinylTTX a promising tool for the isolation of TTX binding sites in nerve using affinity chromatographic (and possibly affinity labeling) techniques.

EXPERIMENTAL

TTX (free of citrate) was a generous gift from Sankyo Co., Ltd., Tokyo. D_2O (99.99 mole% D_2O) was obtained from Bio-Rad Laboratories. [1,4-^{14}C]succinic anhydride (9.6 mCi/mmole) was obtained from New England Nuclear Corp. and was diluted 50-fold with cold succinic anhydride before use. [G-^{3}H]TTX was purified by modification of published procedures (*2–6*). The sample showed a single peak of radioactivity when electrophoresed at pH 1.9, 3.5, and 6.5. Radiochemical purity was >94% with a specific activity of 1200 mCi/mmole (frog nerve assay). At pH 8.9 degradation occurred in accord with the known instability of TTX in alkaline solution (*13*, *14*). Samples were assayed for TTX-like activity using a frog nerve preparation (*15*). Analytical and preparative high voltage paper electrophoresis was performed either in a Gilson Model D High Voltage Electrophorator or on a Savant flat-plate instrument. Low voltage paper electrophoresis was performed on a Buchler apparatus. Whatman 3MM paper was used routinely and runs were carried out at 0–4°C. Electrophoresis buffers were of the following compositions: pH 1.9, acetic acid–88% formic acid–water, 87:25:888; pH 3.5, pyridine–acetic acid–water, 5:50:945; pH 6.5, pyridine–acetic acid–water, 100:4:896; pH 8.9, 0.03*M* NH_4HCO_3; pH 9.1, 0.1 *M* Na_2CO_3–0.1 *M* $NaHCO_3$, 1:9. SuccinylTTX and TTX were located on the electrophoresis paper using Mosher's technique (*14*). SuccinylTTX, like TTX, appears as a green fluorescent spot under these conditions. Sucrose (visualized using I_2) was employed as a neutral marker and ε-DNP-Lys was employed as a colored marker, having approximately the same migration characteristics as succinylated TTX throughout the range of pH's used.

Aqueous solutions (0.1-ml aliquots) were counted in a Triton scintillator (333 ml of Triton X-100, 4.0 g of PPO, 0.2 g of POPOP, and toluene to 1.0 liter) using a Packard Tri-Carb scintillation spectrometer; the counting efficiencies of ^{14}C and ^{3}H in this system were 0.90 and 0.34, respectively. After drying at 25°C overnight, the Whatman 3MM paper used in electrophoresis runs was cut into 1 × 3-cm strips and counted in 5 ml of toluene scintillator.

High resolution nmr spectra were obtained on a Varian XL-100-15 spectrometer

[8] Recently, a new highly toxic tetrodotoxin-like substance called chiriquitoxin has been isolated from a Costa Rican frog. The substance appears to be a C-6-modified tetrodotoxin (see (*18*)).

operating in the FT mode under control of a Varian 620/K 16K computer; free induction decay data were accumulated over ca. 500 transients using the block averaging technique and subsequently transformed into frequency nmr spectra. Chemical shifts were measured in D_2O, relative to DSS.

Succinylation of TTX. The succinylation of [G-^{3}H]TTX is illustrative. A mixture of 10 mg of TTX and 50 μl of [G-^{3}H]TTX (7.2×10^3 dpm/μl, ca. 0.05 μg) was stirred magnetically in a 100-ml cylindrical vessel with 10 ml of distilled water. During the first 40 min, 1.0 g of succinic anhydride (doubly sublimed, 120°C/4 mm) was added portionwise. The pH was kept in the range pH 5–6 with automatic addition of ca. 80 ml of saturated (ca. 0.15 *M*) $Ba(OH)_2$. The initial suspension of toxin dissolved within 2 min of adding the anhydride and, as the reaction proceeded, the mixture became milky in appearance as barium succinate precipitated. After 2.5 hr the reaction mixture was centrifuged and the supernatant was lyophilized to a white powder (ca. 1.2 g). Four times the lyophilized product was treated with 2–10 ml of water, centrifuged, and relyophilized. The final solution (2 ml) contained ca. 3.5×10^5 dpm. Lyophilization gave 29.7 mg which was dissolved in 0.3 ml of distilled water and purified by three successive high voltage electrophoresis runs at pH 6.5. At each stage the strip containing succinyl-TTX was cut out and the product was eluted from the paper with distilled water (six 10-ml, 30-min washes at 4°C) and lyophilized. Succinylation of TTX with [1,4-^{14}C]succinic anhydride was carried out in an analogous manner to that reported above with two exceptions: (i) NaOH (0.4 *M*) was used as the neutralizing base in the pH-Stat and (ii) ammonium formate (pH 6.2, 0.02 *M*) was used to elute product from the electropherograms.

Base hydrolysis of succinylTTX. To 0.1 ml of pH 9.1 buffer was added 0.1 ml of [1,4-^{14}C]succinylTTX (8.8×10^3 dpm, ca. 16 μg). The solution was shaken at 25°C, and the reaction products were analyzed by high voltage electrophoresis (pH 6.5) after 12 hr.

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