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Identification of Thioether Intermediates in the Reductive Transformation of Gonyautoxins into Saxitoxins by Thiols

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Abstract—*O*-Sulfate group of gonyautoxin I and IV is transformed into methylene to form neosaxitoxin by thiols such as glutathione, a common cellular scavenger, in mild conditions. We isolated the intermediate of this conversion and propose that this reaction proceeds through formation of thiohemiketal, 1,2 shift to form stable thioether intermediate, and then redox exchange at sulfur atom to form the final product. © 2000 Elsevier Science Ltd. All rights reserved.

Saxitoxin (STX) and its analogues such as gonyautoxins (GTXs) are causative toxins responsible for paralytic shellfish poisoning, one of the most problematic seafood poisonings (Chart 1). These compounds are thought to be produced primarily by toxic dinoflagellates and are introduced to the higher filter feeders through the food chains.[1]

Some of the GTXs have been shown to be transformed reductively into STX or neoSTX by homogenate of toxin-accumulating bivalve *Placopecten magellanicus*[2a] or by some bacteria,[2b] and thus the conversion was thought to be catalyzed by enzymes. However, mild chemical treatment of GTXs with biologically available thiol reagents such as glutathione (GSH) results in formation of STX.[3] Our recent studies suggested the presence of a stable intermediate in the course of reductive transformation of GTXs with 2-mercaptoethanol (ME) as well as GSH.[4] Herein, we describe the isolation and characterization of the intermediate and propose the mechanism of this conversion (Scheme 1).

An equilibrated mixture of GTX I, IV (**1** and **2**)[5] was treated with GSH at pH 7.4 in phosphate buffer at 70 °C for 20 min, the reaction was quenched by adding acetic acid before emergence of the corresponding final product, neoSTX (**3**), and the product **4** was isolated.[6] The mass spectral analysis of **4** suggested a formula of $C_{20}H_{32}N_{10}O_{11}S$, which corresponds to a formula for GSH adduct of **3**. The HMBC spectrum of **4** indicated a covalent bond between the sulfur atom of the Cys residue of GSH and C-11 by crosspeaks between the Cys β protons and C-11. The β configuration at C-11 of **4** was deduced by comparison to $^3J_{H10\alpha-H11}$ and $^3J_{H10\beta-H11}$ of GTX II (0 and 5 Hz, respectively) and GTX III (**5**, 8.8 and 7.0 Hz, respectively) where those of **4**, both 8.7 Hz, are analogous to GTX III, the 11β-hydroxySTX-*O*-sulfate.[7] Treatment of **4** with a large excess of GSH gave **3**, proving that **4** is an intermediate of this transformation reaction (Scheme 1).[8]

We further investigated the mechanism of this transformation by using **4** as well as a simple conjugate: a ME adduct of neoSTX (**6**). A formula of **6**, $C_{12}H_{19}N_7O_5S$ (FABHRMS), was 18 da. less than the anticipated product (**7**), an 11-ME adduct of neoSTX. The cyclic structure of **6** was confirmed by a HMBC cross peak between H1′a and C-11 showing the sulfide linkage between C-1′ and C-11,[9] and that between H2′a and C-12 showing a linkage between C-2′ and C-12 via an oxygen atom. Treatment of **6** with ME gave **3** as in the case of **4**.[10]

These results showed that the chemical transformation of GTX I (**1**) and GTX IV (**2**) to neoSTX (**3**) is at least a two step conversion consisting of: (1) replacement of *O*-sulfate by a thiol reagent to give a thioether intermediate, and (2) an attack of another thiol at the sulfur atom of the intermediate to give the products, **3** and a disulfide. Treatment of **4** with ME in fact gave the disulfide **8** (Scheme 1). It is, however, less likely that GSH can directly replace *O*-sulfate in the rather mild reaction conditions employed.[6] We thus suspected the contribution of the electrophilic keto equivalent C-12 (exists mainly as hydrated form). When the reaction was carried out by using 12-reduced derivatives of GTXI, IV,

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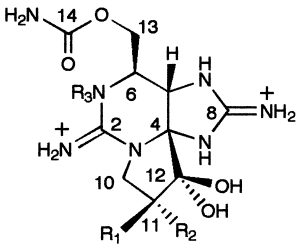
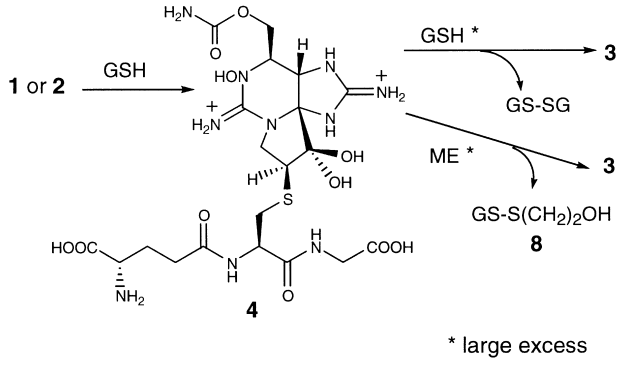


	R_1	R_2	R_3
GTX I (**1**)	H	OSO_3^-	OH
GTX IV (**2**)	OSO_3^-	H	OH
neoSTX (**3**)	H	H	OH
GTX III (**5**)	OSO_3^-	H	H
GTX II	H	OSO_3^-	H

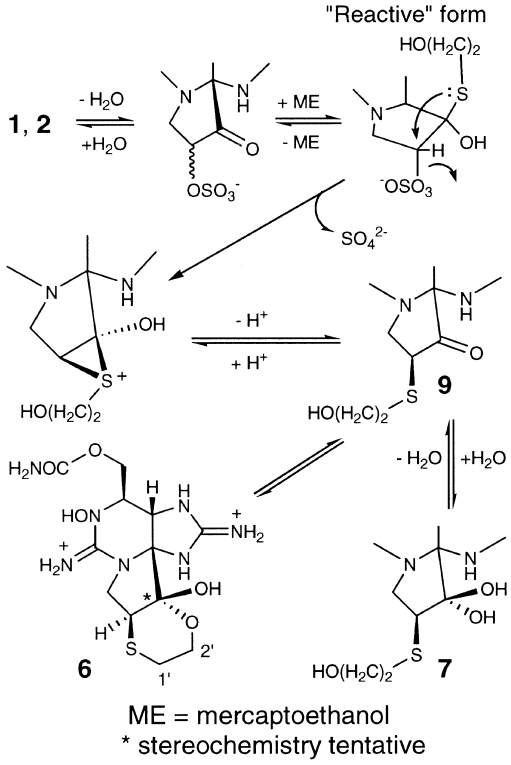
Chart 1.



Scheme 1.

no corresponding product was obtained over three h, indicating an importance of C-12 functionality. Since the keto character of C-12 is known to be pH dependent,[11] the reaction rate was monitored between pH 4.2 and 8.4, where the keto character at C-12 would change significantly.[5] As shown in Fig. 1, formation rate of **6** corresponded with the increase of pH between 4.2 and 7.4. This trend was concurrent with increase in the keto character at C-12 reported previously for STX.[11] It should be noted, however, that the reaction rate declined at higher pH (>8.0). This could be due to deterioration of electrophilicity at C-12, since deprotonated guadinium groups are less electron withdrawing than the protonated counterpart.[12]

These results allowed us to propose the following mechanism for the formation of the intermediates. First, the sulfur atom of the thiol reagent attacks the electrophilic C-12 to form a thiohemiketal which can be transformed to the thioether through an episulfonium ion intermediate,[13,14] when the leaving *O*-sulfate group is oriented *anti* to the sulfur atom (Scheme 2). As shown in Fig. 2, **1** was consumed in preference to **2** indicating **1** to be a "reactive" isomer (Scheme 2). This observation was consistent with the NMR data of **1** which indicated the *O*-sulfate group to be *pseudo* axail.[15] When we plotted ln[GTXI or IV]



Scheme 2.

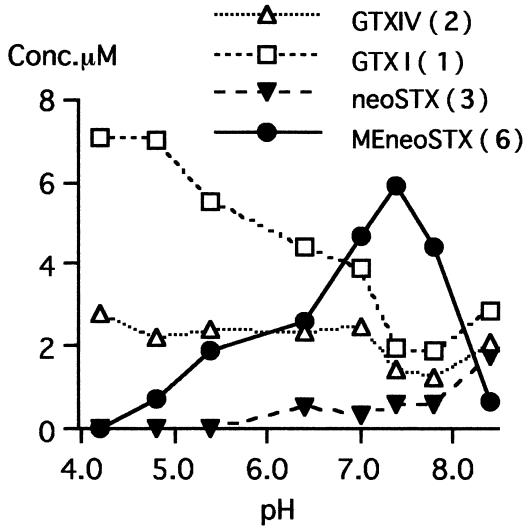


Figure 1.

against time in the presence of 8 mM GSH at 30 °C, the rate followed first order kinetic (Fig. 2). This result further supported that the replacement occurs through intramolecular 1,2-shift rather than bimolecular reaction.[14]

The second step (Scheme 3) is considered to be a nucleophilic substitution at the sulfur atom of the thioether **9** (keto form of **6**) with another SH reagent in a bimolecular mechanism. A formation of disulfide **8** in the reaction of **4** with ME supports the above mechanism. The resultant enolate could be readily hydrated to give **3**. The electrophilic carbonyl C-12 seems to assist the

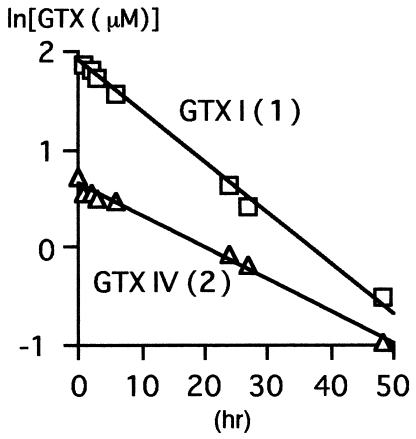
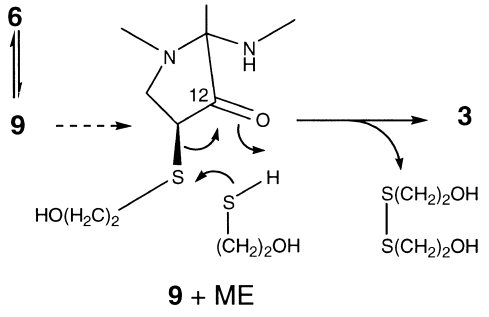


Figure 2.



Scheme 3.

reaction by stabilizing the leaving group, since C-12-reduced analogue of **6** was not reactive and since the reaction was faster in the higher pH when the reaction rate was monitored between pH 4 and 8.

The pH dependency of both first and second steps in the transformation is attributed to characteristic structure of GTX I (**1**), IV(**2**) in which the carbonyl-equivalent C-12 is adjacent to a carbon bearing two electron withdrawing guanidium groups. Degree of dissociation for each guanidium group modulates electrophilicity at C-12.[12] In the case of STX, each guanidium nitrogen has different pKa, 11.3 and 8.3 for C-2- and C-8-guanidium, respectively.[12] At the optimum pH for this reaction (near physiological pH), the keto character and electrophilicity at C-12 must be appropriate for the thiohemiketal formation.

Interestingly, several thiol mediated non-enzymatic reductions of flavin and its model compound undergo with the mechanism relevant, but not identical, to the present transformation.[16] Moreover, several enzymatic reduction processes are reminiscent of the above transformation: reductive cleavage of carbon–nitrogen bond catalyzed by glycine reductase,[17] proline reductase,[18] or reduction of tetrachlorohydroquinone (TCHQ) by TCHQ dehalogenase.[19]

Discovery of stable 11-thiol derivatives of STX is particularly important in developing biochemical probes, which would facilitate not only the study of the more detailed mechanisms of the transformation and dynamics of PSP toxins in nature, but also to confirm binding mechanisms of STX to the voltage-gated sodium channels.

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5. GTXs are known to exist as equilibrated mixture at C-11 due to the keto character at C-12. In this paper we denote 3:1 equilibrated mixture of **1** and **2** as GTX I, IV. It should be noted that no significant change in the ratio of **1** and **2** was observed between pH 4.2 and 8.4.
6. The intermediate forms at room temperature, but longer reaction time (more than 24 h) is required for completion.
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8. Production of **3** was confirmed by HPLC analysis of the product. For the HPLC analysis of PSP toxins see, Oshima, Y. *J. AOAC Int.* **1995**, *78*, 528.
9. The configuration at C-11 for **6** was deduced to be β based on $^{3}J_{H10\alpha-H11}$ and $^{3}J_{H10\beta-H11}$ values of both 9.3 and 9.3 Hz, respectively which are similar to **4**.
10. Production of **3** was unambiguously confirmed by FABMS and ^{1}H NMR of the product.
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