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Surface plasmon resonance-based detection of ladder-shaped polyethers by inhibition detection method

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ABSTRACT

Ladder-shaped polyether (LSP) compounds represented by brevetoxins and ciguatoxins were largely discovered in association with seafood poisoning. Thus, a quick quantification method for LSPs is potentially important. We examined a surface plasmon resonance method using desulfated-yessotoxin (dsYTX) immobilized on a sensor chip and phosphodiesterase PDEII in a inhibition detection mode. Yessotoxin, brevetoxin B and synthetic LSP derivatives showed clear inhibition against PDEII binding to the immobilized dsYTX, by which their half inhibitory concentrations were successfully estimated. This inhibition method appeared to be superior in specificity to direct binding assays where binding proteins to LSP was immobilized on a sensor chip.

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Ladder-shaped polyether (LSP) compounds with unique structures and very potent toxicity, such as ciguatoxin,[1] brevetoxin B (PbTx2)[2] and yessotoxin (YTX),[3] have been discovered in association with seafood poisoning in the last three decades (Fig. 1). Their structures are best characterized by a molecular stem comprising continuously trans-fused ether rings. Among those, YTX was first obtained from the scallop *Patinopecten yessoensis* that were contaminated with the toxins of diarrhetic shellfish poisoning. Afterward, YTX turned out to have weak acute toxicity to human. YTX and its homologues are produced by the dinoflagellates *Protoceratium reticulatum*[4] and *Ligulodinium polyedrum*[5] and exhibits multiple biological activities such as the intraperitoneal toxicity against mice,[6a] modulation of cellular calcium levels of human lymphocytes,[6b] and activation of caspases.[6c] In addition, it was recently reported by Botana et al. that YTX interacts with phosphodiesterase (PDE) to enhance its enzymatic activity, which results in higher AMP levels in human lymphocytes.[7] Very recently, we reported that YTX and other LSP compounds[8a,b] interact with the α-helix domain of glycophorin A on the basis of surface plasmon resonance (SPR),[8c,d] SDS–PAGE,[8] and NMR.[8d] Despite their remarkable biological activities, the mechanism of action of LSPs is still unclear at the

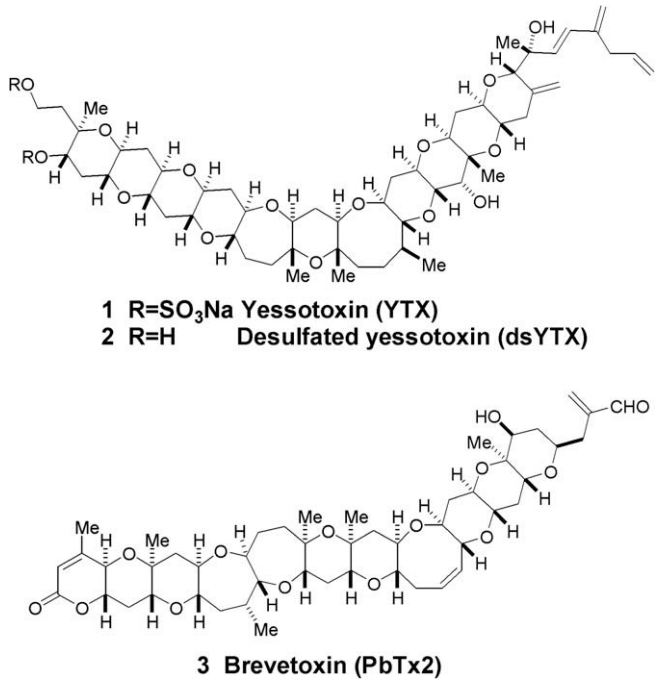

Figure 1. Structures of yessotoxin (YTX), desulfated yessotoxin (dsYTX), and brevetoxin B (PbTx2).

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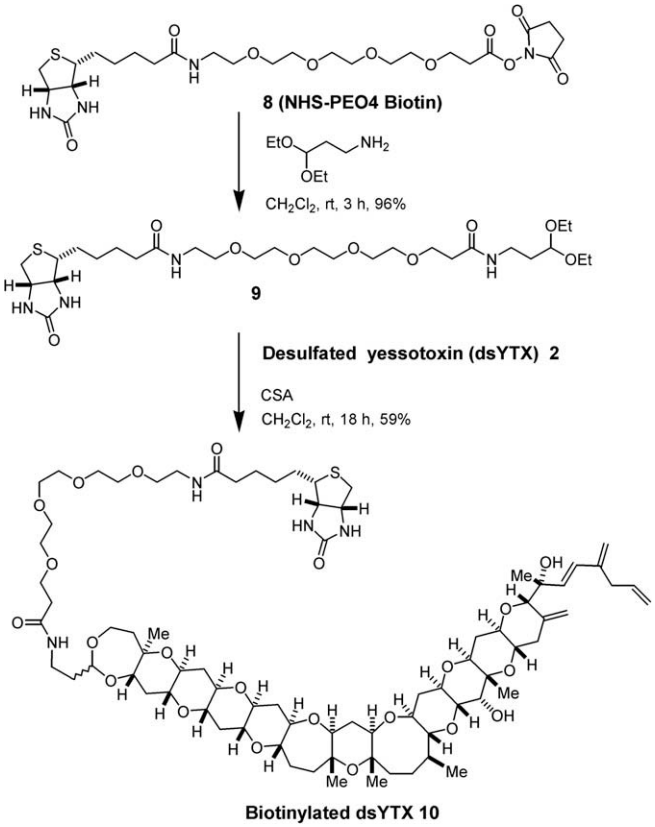
molecular level. Evaluation of their affinity to proteins is, therefore, particularly important for facilitating mechanism-of-action studies and also for estimating their potential risk to human health.

Surface plasmon resonance (SPR) is utilized not only to examine biological interactions but also to quantify the relevant molecules. The methodology is generally suitable for large molecules since its sensitivity chiefly depends on the mass change on the SPR chip. To improve the specificity and sensitivity of SPR assays, inhibition detection[9a–c] and sandwich protocols[9d] have been invented, the former of which was successfully applied to small compounds such as trinitrotoluene.[9a–c] Moreover, in the inhibition assays, receptor proteins are not necessary to be constrained on the sensor chip surface, which should greatly facilitate the screening of small molecules for affinity to variety of proteins.

Among LSPs of natural origins, we focused on YTX since the toxin, which can be produced by laboratory cultures of the dinoflagellate *P. reticulatum*, is known to interact with soluble proteins such as PDEs. In this study, therefore, we adopted YTX as a competitive ligand and cGMP-stimulated phosphodiesterase (PDEII)[7] as a binding protein to LSPs, and aimed to establish a practical SPR method to evaluate the potential biological activities of LSPs by measuring affinity to the physiologically relevant protein.

By the SPR inhibition assays, we examined YTX, desulfo-YTX, PbTx2 (**1**, **2**, and **3** in Fig. 1), and artificial ladder-shaped polyethers (ALPs **4**–**6** in Fig. 2) which were previously synthesized in our laboratory.[8a–c] Biotinylated dsYTX (Scheme 1, see Supplementary data for details) was prepared from YTX by desulfation and conjugation with biotinylated PEG aldehyde, and then immobilized on the surface of a sensor chip via streptavidin. Detection of LSPs was carried out in HBS-EP+ buffer (BiacoreAB) containing 1% DMSO as running and injection media, and PDEII (5 μg/mL) dissolved in HBS-EP+ buffer containing 1% DMSO was passed on the surface of the sensor chip modified with biotinylated dsYTX at a flow rate of 10 μL/min for 2 min. The SPR response increased immediately after injection due to interaction between free PDEII in a sample solution and biotinylated dsYTX immobilized on the surface of sensor chip as shown in Figure 3. Dose-dependent SPR responses were recorded for a series of concentrations of PDEII. After each measurement, the surface of a sensor chip was regenerated by injecting 0.5% SDS, which brought the SPR response back to the original level. In order to obtain high responses in a stable sensorgram, we implemented the inhibition assays at the PDEII concentration of 5 μg/mL. We then incubated various LSPs as analyte in the range of 0–50 μM (or 100 μM in the case of a sample with low affinity). Figure 4 shows the sensorgrams in the presence of various concentrations of YTX, dsYTX and PbTx2. In these assays, the SPR response decreased with increasing concentrations of LSPs in a sample solution. We adopted the increment of the response during 20–40 s after the sample loading for evaluating the inhibition by LSP (Fig. 5) as often used in the inhibition protocol.[10] Curve fitting to the experimental data was carried out by using BIAevaluation software (BiacoreAB) with four-parameter logistic equation which was widely adopted to produce dose–response curves (see Supplementary data).[11] Figure 5 demonstrates the dose–response curves of the inhibition assays for YTX (A), dsYTX (B), and PbTx2 (C), which



Scheme 1. Preparation of biotinylated dsYTX **10**.

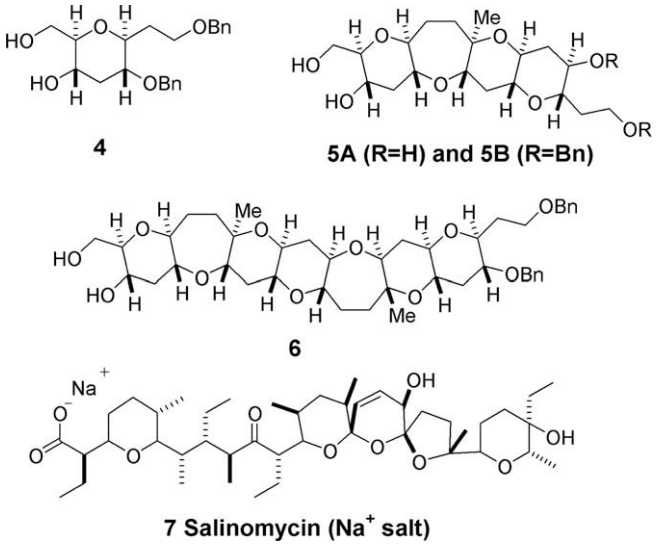


Figure 2. Structures of monocyclic ether **4**, tetracyclic **5A**, **5B**, heptacyclic artificial ladder-shaped polyethers **6** (ALPs) and salinomycin (Na^+ salt) **7**.

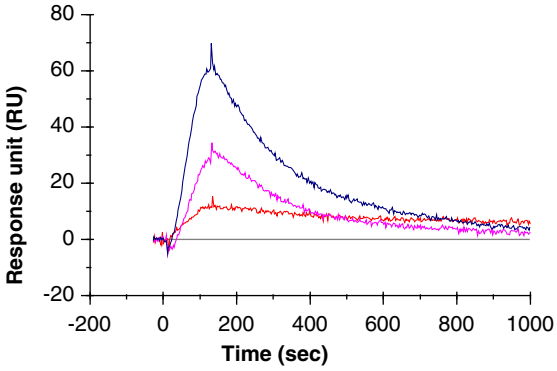


Figure 3. SPR sensorgrams of PDEII for biotinylated dsYTX immobilized on the surface of sensor chip via streptavidin. PDEII solutions were injected at 0 (gray), 1 (red), 2 (magenta), and 5 (blue) μg/mL in HBS-EP+ containing 1% DMSO. After 120 s, the PDEII solutions was changed to wash buffer.

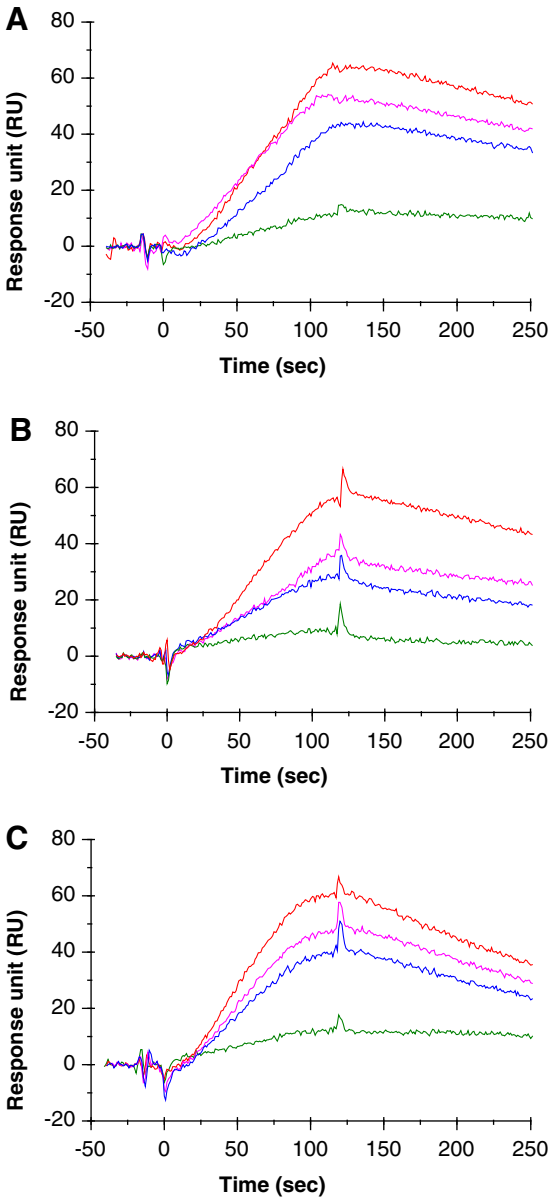


Figure 4. SPR sensorgrams of 5 μg/mL PDEII in HBS-EP+ containing 1% DMSO solution with 0 (red), 0.5 (magenta), 2 (blue), and 20 (green) μM of inhibiting LSPs, **A**, **YTX**, **B, dsYTX**, and **C**, **PbTx2,** which were injected to biotinylated dsYTX immobilized on the surface of sensor chip via streptavidin.

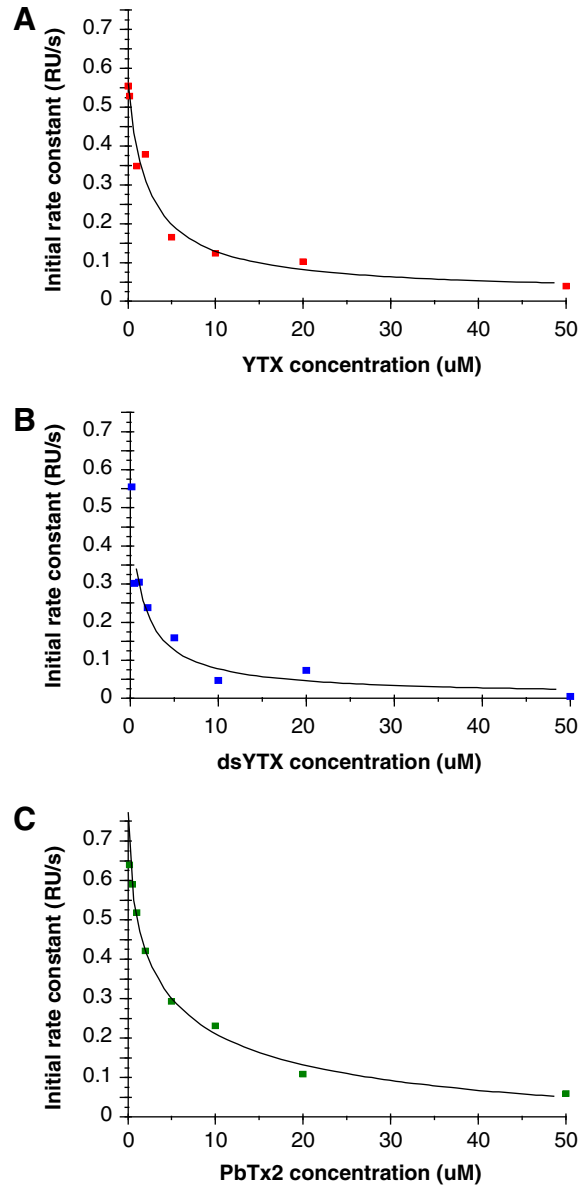


Figure 5. Dose–response inhibition curves for 5 μg/mL PDEII by 0.2, 0.5, 1, 2, 5, 10, 20, and 50 μM LSPs against biotinylated dsYTX immobilized on the surface of sensor chip via streptavidin. These curves demonstrate on **A**, **YTX**; **B**, **dsYTX** and **C**, **PbTx2**.

led to estimation of 50% inhibitory concentrations (IC_{50}) as shown in Table 1. In particular, PbTx2 showed comparable affinity for PDEII with the parent dsYTX, which suggested that PDEII widely interacts with LSPs. These observations prompted us to examine another series of LSP compounds, ALPS, and non-ladder-shape polyether, salimomycin, to gain a better understanding of structural requirements for the affinity to PDEs. The sensorgrams for ALPs (**4**, **5A**, **5B**, and **6**) and salinomycin are provided as Supplementary data. ALPs **4**, **5A,** or salinomycin **7** did not show significant inhibition. ALPs **5B** and **6** possessing a dibenzyl ether moiety, however, revealed high affinity to PDEII. The affinity increased with increasing number of rings when compared with their IC_{50} values of ALPs **4**, **5B** and **6** (Table 1). In comparison with cyclic polyether salinomycin without a ladder-shaped skeleton, ALP **6** with a similar molecular weight has much higher affinity. These results clearly indicate that PDEII interacts with LSP skeletal structures with certain molecular length although its recognition is not particularly influenced by the sequence of ether rings or by substituents on a polyether stem. This moderate specificity implies

Table 1
Half inhibitory concentrations (IC_{50}, μM) of LSPs as analyte against PDEII in inhibition detection assays

Assay methods	IC_{50} ± SEM (μM)
YTX **1**	3.0 ± 1.0
DsYTX **2**	1.7 ± 0.2
PbTx2 **3**	1.8 ± 1.0
4	—*
5A	—*
5B	13.0 ± 2.7
6	9.2 ± 0.9
Salinomycin **7**	—*

SEM was determined from three independent experiments.
* IC_{50} was estimated to be over 100 μM. Experimental details are provided in Supplementary data.

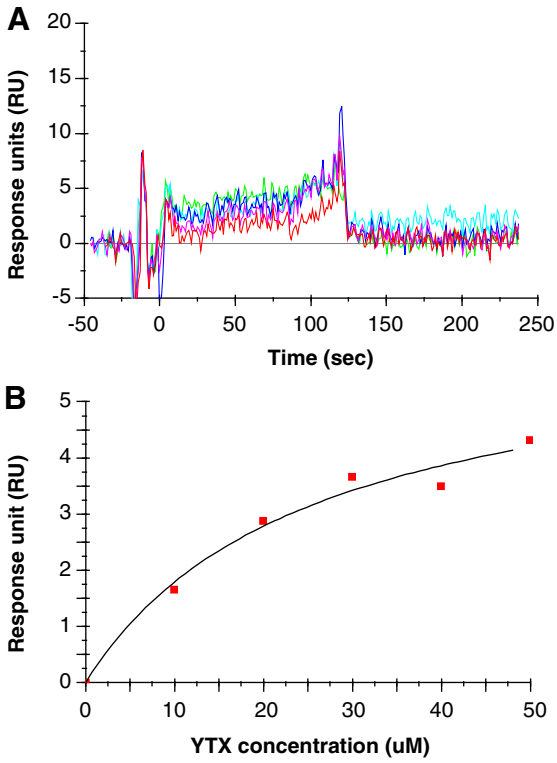


Figure 6. (**A**) SPR sensorgrams of direct method for YTX against PDEII. YTX was injected in the range from 0 to 50 μM. Color in trace corresponds to concentration of YTX; 0 (gray), 10 (red), 20 (magenta), 30 (blue), 40 (light blue), and 50 (light green) μM in HBS-EP+ containing 1% DMSO solution. (**B**) Concentration-dependent SPR responses and a theoretical curve for K_D of 2.2 μM.

the possibility that the SPR inhibition assay is applicable for estimating the total amount of diverse LSP toxins occurring marine organisms. The standard errors of the mean (SEM) in Table 1 were obtained from three independent experiments. To check the long-term reproducibility of the affinity index obtained from a single sensor chip, we carried out the inhibition assays over 6 months, which resulted in moderate dispersion of the IC_{50} values.

As the next step, we attempted to evaluate the specificity and sensitivity of the inhibition method in comparison with the direct method. The affinity (K_D) of YTX to immobilized PDEII was estimated to be 2.2 μM although the response of YTX binding was very small (Fig. 6). The value was comparable with the IC_{50} in the inhibition assays; under these concentrations of PDEII, IC_{50} is virtually same as K_D. These affinity indices are not much different from K_D values reported for YTX.[7] For example, Pazos et al. reported that the value was 8 ± 3 μM to the same enzyme in direct binding assays on a resonant mirror biosensor.[7c] Although we attempted to examine the other LSPs for the interaction of PDEII by the direct method, poor reproducibility and weak responses in sensorgrams prevented us from measuring their affinity. The smaller responses of LSPs for immobilized PDEII may be accounted for by the smaller amount (4600 RU) of PDEII immobilized on the chip as compared with that of the other phosphodiesterase PDE4D (9800 RU). In addition, the binding affinity of PDEII to LSPs might be impaired due to blocking the enzyme active site by the immobilization reaction. Instead, we run the direct assays using recombinant PDE4D. The dissociation constant of dsYTX for the enzyme immobilized on the sensor chip was 2.1 μM (see Supplementary data), which was comparable with those for PDEII by the inhibition method (Table 1). However, an SPR response in RU was markedly variable among LSPs tested (Table S1 in Supplementary data); ALPs with dibenzyl group such as **4**, **5B**, and **6** tend to bind abundantly to PDE4D as compared with YTX and dsYTX, which suggests that non-specific interactions predominantly occur in the direct binding experiments. In particular, **4** showed the binding of 49.7 RU in the direct method (Table S1), while the response for dsYTX was only 27.6 RU despite its larger molecular weight. A small ALP **4** should, therefore, substantially bind to the other sites than a specific binding pocket of dsYTX on PDE4D.

In the present study we demonstrated for the first time that the inhibition SPR method is useful for evaluating LSPs–protein interactions, particularly for screening receptor proteins, since it is not necessary to prepare a receptor-immobilized sensor chip. Some of LSPs are known to be responsible for seafood poisoning and need to be monitored carefully. Recently, enzyme-linked immunoassays have been developed for detecting ciguatoxin,[9d,12] for which high-affinity antibodies with nanomolar K_D values are used. These specific assays to the major causative toxin would partly address the serious problems of ciguatera. On the other hand, LSPs tested in this study showed relatively low affinity to binding proteins. Among those, ALPs **5B**, **6** and PbTx2 revealed higher affinity to PDEII but salinomycin or **4** did not. The results suggest that PDEII loosely recognizes the ladder-shaped skeletal structures, which sometimes works better than receptors with strict recognition since a great variety of LSP toxins are often involved in seafood poisoning, particularly in the case of shellfish poisoning. Among other SPR-based methods reported so far,[7,13] the present inhibition assays may provide a practical way for estimating LSP toxins in seafood and marine organisms.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.103.

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