

# Photolabile $\mu$ -conotoxins with a chromogenic phenyldiazirine. A novel probe for muscle-type sodium channels

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Three photoreactive derivatives of  $\mu$ -conotoxin G IIIA have been prepared as photoaffinity labeling reagents for muscle-type sodium channels. The reagents competitively inhibited the binding of saxitoxin to the eel sodium channel with  $K_i$  values of 11–18 nM. The introduced chromogenic phenyldiazirine group on the toxin was photolyzed efficiently, and spectroscopic properties of the reagents demonstrated that irradiation and detection can be performed in a spectral region where the absorptions due to most of biological macromolecules are negligible.

Diazirine;  $\mu$ -Conotoxin; Photoaffinity labeling; Sodium channel

## 1. INTRODUCTION

The voltage-sensitive sodium channel is responsible for the increase in sodium ion permeability during the initial rapidly rising phase of the action potential in the most excitable cells such as nerve, skeletal muscle, and heart [1]. The primary structure of the channel protein from eel electroplax was first deduced from cDNA clone [2], and similar complete sequence information is also available for the principal  $\alpha$ -subunit of the channels from rat brain [3] and *Drosophila* [4]. These proteins are highly homologous with each other, and each contains four internal homology units with six proposed transmembrane helices. Although several models of their transmembrane topology have been proposed, the relations between molecular structure and the mechanism of ion permeation and channel gating are still to be solved. Photoaffinity labeling may have a crucial role in the identification of ligand binding regions within a polypeptide [5]. Five receptor sites for distinct families of neurotoxins have been shown to be present on voltage-sensitive sodium channels by ligand binding studies [1]. Photoreactive derivatives of those toxins are potentially useful tools for structural analyses of the binding sites at the molecular level. We have reported a specific photolabeling of the sodium channel protein from eel electroplax with tetrodotoxin derivatives carrying azido [6] and other photoactivatable groups [7]. Recently, the region in the rat brain sodium channel photolabeled with a  $\alpha$ -scorpion toxin derivative was analysed using a battery of antipeptide antibodies [8].

$\mu$ -Conotoxins from *Conus geographus*, polypeptide neurotoxins which differ from  $\alpha$ -scorpion toxin in the binding site, share a common binding site with tetrodotoxin and saxitoxin [9,10]. Since  $\mu$ -conotoxins specifically block ion flow through muscle channels [9], determination of the location of their receptor region within the primary structure of the sodium channel  $\alpha$ -subunit will help to define the molecular mechanisms of ion flux. The chemical synthesis of  $\mu$ -conotoxin G IIIA in our lab has been reported very recently [11], and we also developed an efficient synthetic route for supplying substantial amounts of this toxin as a molecular probe of the channel structure [12]. The present paper deals with preparation and characterization of photoreactive derivatives of  $\mu$ -conotoxin G IIIA carrying a chromogenic diazirine, [[2-Nitro-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]]phenoxy]acetic acid (NDPA) [13], as a photoreactive part, which we have very recently described as a new potential photoreactive chromogenic group in the photolabeling work.

## 2. MATERIALS AND METHODS

### 2.1. Materials

$\mu$ -Conotoxin G IIIA was isolated from the venom of *Conus geographus* as described before [14]. The *N*-hydroxy succinimide ester of NDPA (NDP-OSu) was prepared from *p*-bromophenol in nine steps and the synthetic part of this new diazirine has been reported separately [13]. Medium-size specimens of *Electrophorus electricus* were obtained from World Wide Scientific Animals. All the other reagents were of analytical grade unless otherwise stated.

### 2.2. Modification of $\mu$ -conotoxin G IIIA with NDP-OSu

To a vigorously stirred solution of  $\mu$ -conotoxin G IIIA (2.6 mg, 1  $\mu$ mol) in 50 mM borate buffer, pH 8.5, a 10 mM solution of NDP-OSu in acetonitrile (100  $\mu$ l, 1  $\mu$ mol) was added at room temperature.

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After the addition, the reaction was allowed to proceed for 1 h. The reaction mixture was directly applied to HPLC. The yields of products (I, II, and III in fig.2) were determined from the results of amino acid analyses with a Hitachi 835 analyzer after the hydrolysis of samples with 6 N HCl for 24 h at 110°C.

### 2.3. High-performance liquid chromatography (HPLC) procedure

Reversed-phase HPLC was performed with a Waters chromatography unit, a 250 × 4.6 mm Chemcosorb C<sub>18</sub> Column (Chemco Scientific Co., Japan; 7 μm particles, 8 nm pores), and various gradients between 0.1% trifluoroacetic acid in water and in acetonitrile at a flow rate of 1 ml/min at room temperature.

### 2.4. Binding experiment

A partially purified sodium channel protein, obtained by DEAE ion-exchange chromatography [15,16], was used for binding experiments. The DEAE fraction (10 μl, 0.5 mg protein/ml) was mixed with 1.1 μM of [<sup>3</sup>H]saxitoxin (Amersham, sp. act. 21 Ci/mmol) and various concentrations of native or modified μ-conotoxins. After dilution to 200 μl with 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5, the assay mixture was incubated at 0°C for 30 min. Toxin binding was measured according to a gel filtration procedure described in the literature [17].

### 2.5. Determination of modified amino acid residue

Isolated II (10 nmol) was incubated with 75 μg of endoproteinase Lys-C (from *Achromobacter lyticus*, Wako Chemicals Co., Japan; 4.5 U/mg) in 60 μl of 50 mM Tris chloride, pH 9.0, for 72 h at 37°C. The digest was directly injected to HPLC applying a linear gradient of acetonitrile from 10% to 40% over 20 min. The main product eluting at 17 min was reduced with 100 μl of 0.1% β-mercaptoethanol in 0.25 M Tris chloride, 6 M guanidine-HCl, 1 mM EDTA, pH 8.5, for 18 h, subsequently pyridylethylated with 0.9 μl of 4-vinylpyridine (Aldrich) for 4 h at room temperature. The main peak eluting at 15 min with HPLC using same conditions as above was collected and subjected to sequence analysis.

### 2.6. Sequence analysis

This was performed with an Applied Biosystems 477A sequencer equipped with an on-line PTH 120A analyzer. A standard programme was used and the reagents were provided by the manufacturer.

### 2.7. Photolysis

The sample solution was placed on ice and irradiated with a 20 W black light (National FL 20S-BL-B) at 5 cm distance.

## 3. RESULTS AND DISCUSSION

The photoreactive trifluoromethyldiazirine group of NDP yields a highly reactive carbene which inserts the C-H bond of cyclohexane intermolecularly. NDP derivatives and the insertion products have preferable spectroscopic properties which enable to perform the photoaffinity labeling experiments at the spectral region (320–350 nm) where the absorptions of most of proteins and nucleic acids have little effect on the irradiation of samples as well as the spectrophotometric detection of products [13]. As a new photoaffinity labeling reagent for muscle type sodium channels, photoactivable derivatives of μ-conotoxin G IIIA carrying with it this new diazine have been synthesized (fig.1). μ-Conotoxin G IIIA is a basic 22-peptide amide having four lysyl ε-amino residues and a N-terminal amino group for the potential sites of acylation. Reaction of the μ-conotoxin with NDP-OSu gave a complex mixture supposedly composed with NDP derivatives different in

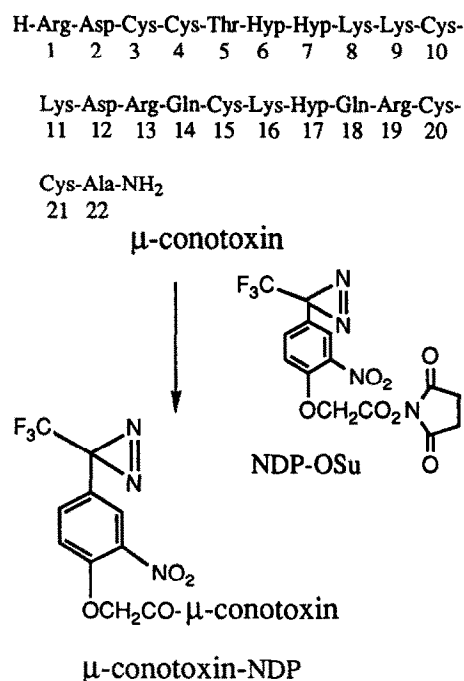


Fig.1. Modification of μ-conotoxin G IIIA with NDP-OSu.

the sites and degrees of the acylation. HPLC of the product was performed by the simultaneous monitoring of absorptions at 215 and 320 nm. HPLC profile monitored the absorption of introduced NDP at 320 nm revealed the existence of a number of NDP derivatives which presumably differ in degree of acylation and introduced sites on the peptide (fig.2). The first three peaks, I–III in fig.2, were similar in the ratio of the absorption at 320 nm to that of 215 nm. After the purification of peaks I–III with HPLC, concentration of peptide and amount of NDP group introduced on the toxin was determined from the amino acid analyses and UV absorption at 325 nm, respectively. The products I–III all correspond to mono-NDP derivatives which are

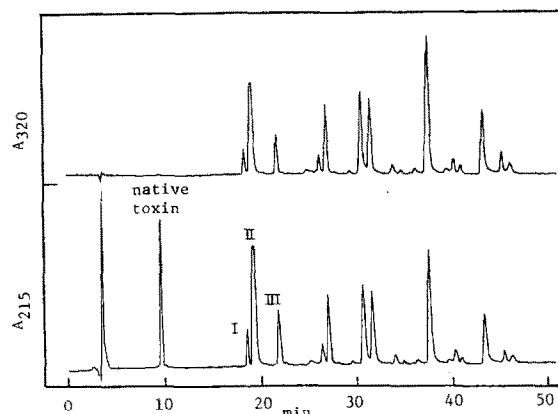


Fig.2. HPLC profile of reaction mixture after modification of μ-conotoxin G IIIA with NDP-OSu. HPLC analyses were performed applying a linear gradient of acetonitrile (0–50%) in 60 min.

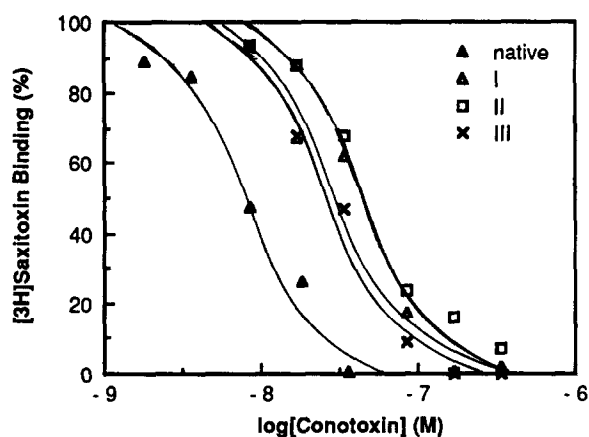


Fig. 3. Inhibition of [ $^3$ H]saxitoxin binding to the sodium channel by  $\mu$ -conotoxin GIIIA or  $\mu$ -conotoxin NDP I-III.

probably different in the acylation site on the toxin. Isolation yields of the products were: I, 1%, II, 10%, and III, 3%.

Binding of I-III to the solubilized sodium channel (DEAE fraction [I]) from eel electroplax membrane is competitive with respect to [ $^3$ H]saxitoxin (fig. 3). Using the  $IC_{50}$  values obtained from fig. 3 ( $\mu$ -conotoxin GIIIA, 9 nM; I, 35 nM; II, 50 nM; III, 30 nM), the inhibition constants ( $K_i$ ) of saxitoxin binding can be estimated [18] from the  $K_d$  value of competitive radioligand, [ $^3$ H]saxitoxin whose  $K_d$  was determined as 30 nM from a separate saturation experiment. The  $K_i$  values obtained were 3 nM (native toxin), 12 nM (I),

Table 1

Yields of PTH amino acids upon sequence analysis of endoproteinase Lys-C digest of  $\mu$ -conotoxin-NDP-II

Cycle	Residue	Yield (pmol)
1	Arg	21
2	Asp	84
3	Cys	171
4	Cys	170
5	Thr	61
6	Hyp	112
7	Hyp	112
8	(Lys)	-
9	Lys	48

18 nM (II), 11 nM (III). Although the modification decreased the affinity of the toxin,  $K_i$  values of I-III are still smaller than that of saxitoxin. Thus the NDP derivatives obtained here have desirable binding activity as the photoaffinity labeling reagent.

Sequence analyses of I-III showed significant decrease of the yields of PTH-lysine at cycles 9 (I), 8 (II), and 11 (III) compared to that of native toxin (fig. 4). Although the yields are not completely depressed probably due to a partial hydrolysis of the NDP group under the conditions for sequencing, these results indicate the possibility that the modification occurs at the corresponding lysines. To identify the modification site, major product II was further examined as described in section 2.5. After the enzyme digestion, a single peptide which has the absorption of the NDP group at 320 nm

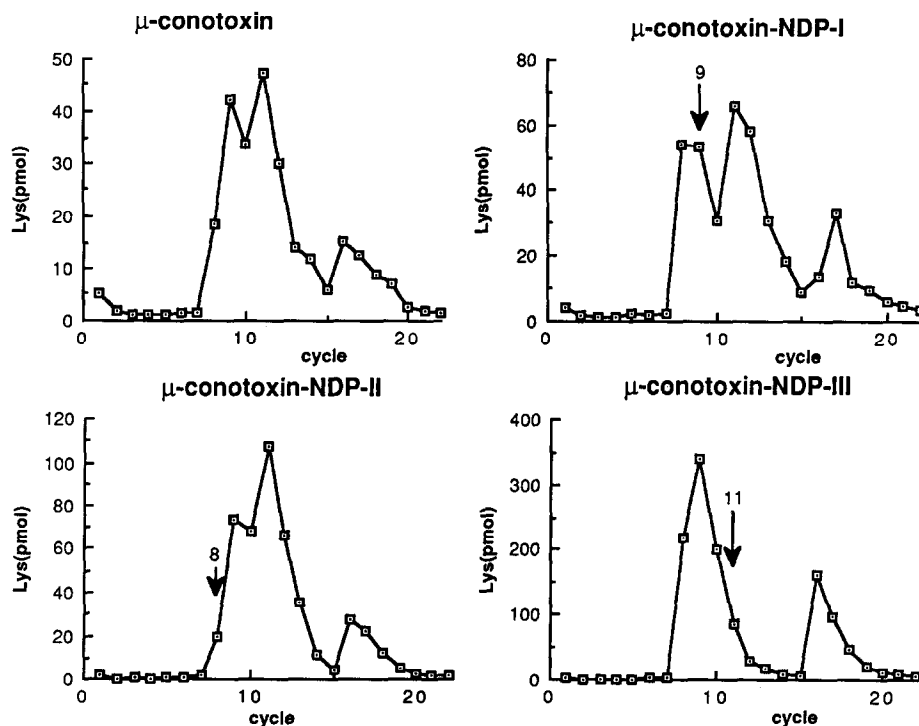


Fig. 4. Comparison of the yield of PTH-Lys upon sequence analysis of  $\mu$ -conotoxin GIIIA and  $\mu$ -conotoxin NDP I-III.

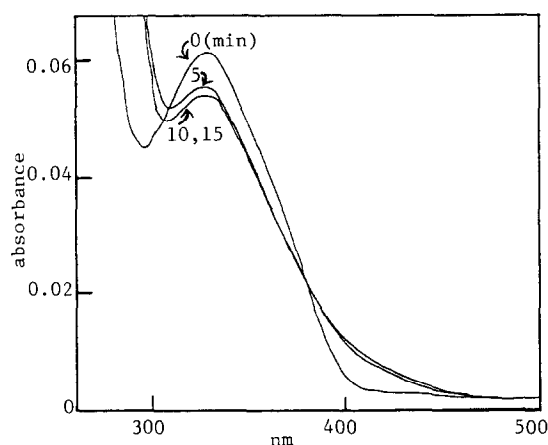


Fig.5. Spectral changes of  $\mu$ -conotoxin NDP II upon photolysis in water (28  $\mu$ M).

was isolated and its sequence was determined as Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-(Lys)-Lys. No PTH-Lys was detected at cycle 8 (table 1). Furthermore, the lysine at position 8 was enzymatically uncleavable by endoproteinase Lys-C which is known to selectively cleave normal lysine at its carboxyl end. Both results strongly suggest that Lys<sup>8</sup> is the site of modification on product II and this is in agreement with the results of sequence analysis of the product II itself.

The spectral changes of II upon photolysis are shown in fig.5. The absorption of the NDP group centered at 325 nm enables one to perform the photolysis at the spectral region where photochemical damages to the channel protein are minimized. The photolysis was completed within 10 min with a black light lamp which mainly emits light of wavelength 320–400 nm, and no detectable loss of binding activity of the solubilized sodium channel was observed under the same photolytic conditions. After the photolysis, photoproducts still have the UV absorption at the region where the absorptions due to protein itself are negligible.

The results demonstrate that modification of three of four lysines on the toxin does not abolish binding activity in terms of specific binding. A variety of biologically active derivatives of  $\mu$ -conotoxin GIIIA will be possibly made by the modification method for preparation of the NDP derivatives used here. The NDP group on the toxin was efficiently photolysed under mild conditions,

and the spectroscopic nature of the photoproducts will be effective in providing a practical approach for the real-time trace of photolabeled fragments on HPLC. The application of these new reagents for photoaffinity labeling of the sodium channels is currently under way.

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