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Amino Acid Sequence and Chemical Modification of a Novel α-Neurotoxin (Oh-5) from King Cobra (*Ophiophagus hannah*) Venom[1]

Shinne-Ren Lin,* Lei-Fen Leu,† Long-Sen Chang,† and Chun-Chang Chang†,[2]

**School of Chemistry and* †*Department of Biochemistry, Kaohsiung Medical College, Kaohsiung, Taiwan 807*

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A novel α-neurotoxin, Oh-5, was isolated from king cobra (*Ophiophagus hannah*) venom and purified by successive SP-Sephadex C-25 column chromatography and reversed-phase HPLC. The complete sequence of Oh-5 was determined by Edman degradation of peptide fragments generated by endopeptidases, *i.e.*, trypsin, *Saccharomyces aureus* V_8 protease and lysyl endopeptidase. This novel toxin comprises 72 amino acid residues with 10 cysteines. The sequence shows 89% sequence homology with Oh-4, and 60% with Toxins a and b from the same venom. The tyrosine, tryptophan, lysine and arginine residues in Oh-5 were modified with tetranitromethane (TNM), 2-nitrophenylsulfenyl (NPS) chloride, trinitrobenzene sulfonate (TNBS), and *p*-hydroxyphenylglyoxal (HPG), respectively. Modification of Tyr-4 or Trp-27 did not affect the lethal toxicity at all, while the Tyr-4 and 23 nitrated derivative retained about 50% of the lethality of native toxin. Selective trinitrophenylation of Lys-51 or 69 resulted in a decrease in lethality by 29%, and 50% lethality was retained after modification of Lys-2, 51, and 69. A drastic decrease in lethality to 26% was observed when both Arg-35 and 37 were modified. The neurotoxicity was further decreased when Arg-9 was additionally modified. These results suggest that the aromatic residues, Tyr-4 and Trp-27, are not crucial for the neurotoxicity, whereas the cationic residues are involved in multipoint contact between the toxin molecule and the nicotinic acetylcholine receptor (nAChR). The residues Tyr-23 and Arg-35 and 37 in the central loop of Oh-5 seem to contribute greatly to the neurotoxicity.

Key words: amino acid sequence, aromatic residue, cationic residue, king cobra α-neurotoxin.

α-Neurotoxins such as curare bind specifically to nicotinic acetylcholine receptors (nAChR) on the postsynaptic membranes of skeletal muscles to block the neuromuscular transmission. About 100 α-neurotoxins from over 30 species of *Elapid* and *Hydrophid* venoms have been fully sequenced, and they can be structurally classified into two groups, short-chain neurotoxins containing 60–62 amino acid residues with four disulfide bonds, and long-chain neurotoxins of 70–74 residues with five disulfide bonds (*1-3*). X-ray crystallographic analyses of erabutoxin *b* (*4, 5*), α-cobratoxin (*6*), and α-bungarotoxin (α-BuTX) (*7, 8*) have provided a model of the three-dimensional structure of α-neurotoxins. The sequence homology and common models of toxic action of α-neurotoxins imply that they all possess a similar overall folding, but differ in details such as the extent of secondary structure and positioning of a conserved side chain.

Our previous experiments on chemical modification of the conserved amino acid residues in cobrotoxin and α-BuTX have suggested that the status and functional importance of these homologous residues for the neurotoxicity probably differ somewhat between short and long α-neurotoxins (*9-14*). Recently, six α-neurotoxins were isolated from the king cobra (*Ophiophagus hannah*) venom in our laboratory, and one of them, Oh-4 (a long toxin), has been sequenced (*15*). The roles of its Trp and cationic residues in neurotoxicity have been investigated (*16-18*). However, the contribution of these residues to the neurotoxicity of Oh-4 was different from that noted with other long α-neurotoxins. In the present study, another α-neurotoxin (Oh-5) was examined to determine its amino acid sequence, and the contributions of aromatic and cationic residues to its neurotoxicity were explored by chemical modification. The results may throw more light on the roles of these residues in long α-neurotoxins.

MATERIALS AND METHODS

A novel α-neurotoxin, Oh-5, was isolated from king cobra (*O. hannah*) venom as previously described (*15*). 2-Nitrophenylsulfenyl chloride (NPS-Cl), trinitrobenzene sulfonate (TNBS), trypsin, and *Saccharomyces aureus* V_8 protease were purchased from Sigma Chemical. Acetonitrile and trifluoroacetic acid (TFA) were obtained from E. Merck, lysyl endopeptidase and tetranitromethane (TNM) were from Wako, Japan, and *p*-hydroxyphenylglyoxal

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[2] To whom correspondence should be addressed. Fax: +886-7-3218309.

Abbreviations: α-BuTX, α-bungarotoxin; HPG, *p*-hydroxyphenylglyoxal; nAChR, nicotinic acetylcholine receptor; NPS-Cl, 2-nitrophenylsulfenyl chloride; TNBS, trinitrobenzene sulfonate; TNM, tetranitromethane.

(HPG) was from Pierce (Rockford, IL). A SynChropak RP-P column (0.46×25 cm) was purchased from SynChrom, and a TSK gel ODS-120T (0.46×25 cm) column from Toyo Soda, Japan. All other reagents were of analytical grade.

Determination of Amino Acid Sequence—The toxins were reduced and *S*-carboxymethylated (RCM) according to the procedures described by Crestfield *et al.* (*19*), followed by proteolytic digestion. RCM-protein (1 mg) was digested with trypsin or V_8 protease in 1 ml of 0.1 M ammonium bicarbonate (pH 8.0), or with lysyl endopeptidase in 0.01 M Tris buffer (pH 9.0) for 6 h at 37°C, at a substrate/enzyme ratio of 50 : 1 (w/w). The hydrolysates were separated by RP-HPLC on a Toyo Soda ODS-120T column (0.46×25 cm) equilibrated with 0.1% TFA as shown in the figure legends. The peptides were lyophilized for amino acid analysis with a Beckman 6300 amino acid analyzer and sequence determination with an Applied Biosystems 477 A protein sequencer.

Modification of Trp Residue with NPS-Cl—Oh-5 was modified with NPS-Cl according to the procedure described by Chang *et al.* (*16*). Oh-5 (2 μmol) in 3 ml of 30% acetic acid was incubated with a 6-fold molar excess of NPS-Cl. The reaction was allowed to proceed at 30°C for 2 h and the mixture was desalted by passage through a Sephadex G-25 column (2.5×50 cm) equilibrated with 0.1 M acetic acid. The modified proteins were separated by reversed-phase HPLC on a SynChropak RP-P column equilibrated with 0.1% TFA and eluted with a linear gradient of 20-60% acetonitrile for 100 min. The flow rate was 1.0 ml/min and the eluate was monitored at 280 nm.

Modification of Tyr Residues with TNM—The Tyr residues were nitrated with TNM essentially according to the procedure described by Chang *et al.* (*9*). To a solution of Oh-5 (2 μmol) in 2 ml of 0.05 M Tris-HCl buffer (pH 8.0), a 10-fold molar excess of TNM was added, and the reaction was allowed to proceed at 30°C for 2 h. The reaction products were purified as described for the preparation of the NPS-derivative.

Modification of Arg Residues with HPG—Arginine-modified derivatives were prepared according to the procedure described previously (*14*). Oh-5 (1.5 μmol) in 2 ml of 0.2 M $NaHCO_3$ buffer (pH 9.0) was incubated with a 30-fold molar excess of HPG, and the reaction was allowed to proceed at room temperature for 1 h. The reaction products were separated on a CM-52 column (1.5×28 cm) and eluted with a linear gradient from 0.03 M (pH 5.0)-0.3 M ammonium acetate buffer. The two major fractions were further purified by RP-HPLC.

Modification of Amino Groups with TNBS—Oh-5 was modified with TNBS according to the procedure described by Lin and Chang (*13*). Oh-5 (1.5 μmol) in 2 ml of 0.2 M $NaHCO_3$ buffer (pH 8.3) was incubated with a 2-fold molar excess of TNBS. The reaction was allowed to proceed at 30°C for 1 h and the reaction product was purified under the same conditions used for separation of HPG-derivatives. The extent of modification was determined spectrophotometrically based on the molar absorbance coefficient of 11,500 $M^{-1} \cdot cm^{-1}$ at 345 nm in 0.1 M HCl (*20*).

Circular Dichroism—CD spectra of Oh-5 and its derivatives were recorded with a Jasco J-700 spectropolarimeter from 250 to 190 nm at a concentration of 0.8 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) with a cell path length of 0.1 mm. The CD spectra were obtained by averaging five scans.

Fluorescence Quenching Measurement—Quenching of Trp fluorescence was studied essentially according to the procedure described previously (*21*).

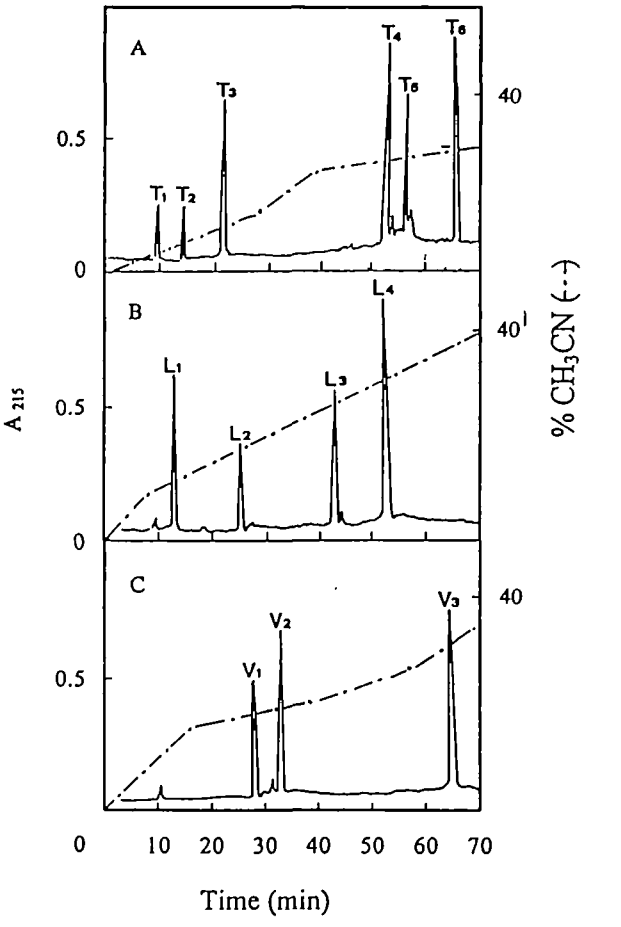

Fig. 1. **Separation of protease-digested RCM-Oh-5 by RP-HPLC.** A, trypsin; B, lysyl endopeptidase; C, *S. aureus* V_8 protease. The hydrolysates were chromatographed on a TSK-gel ODS-120T column (0.46×25 cm, Toyo Soda) at a rate of 1.0 ml/min with a linear gradient of 0-50% acetonitrile in 0.1% TFA as shown by a dotted line.

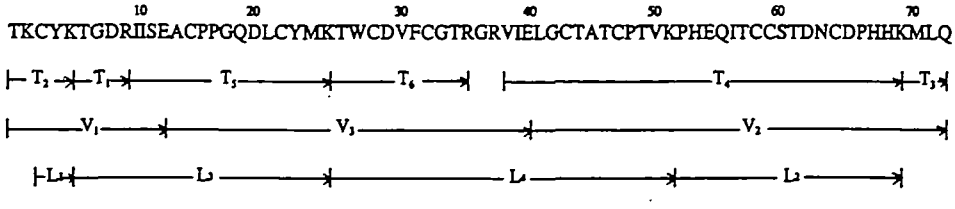

Fig. 2. **The complete amino acid sequence of Oh-5.** The peptides derived from the various digestions with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease correspond to those in Fig. 1.

Assay for Lethal Toxicity—The lethal toxicity, LD_{50} (in μg toxin/g mouse), was determined by the procedure described previously (*22*).

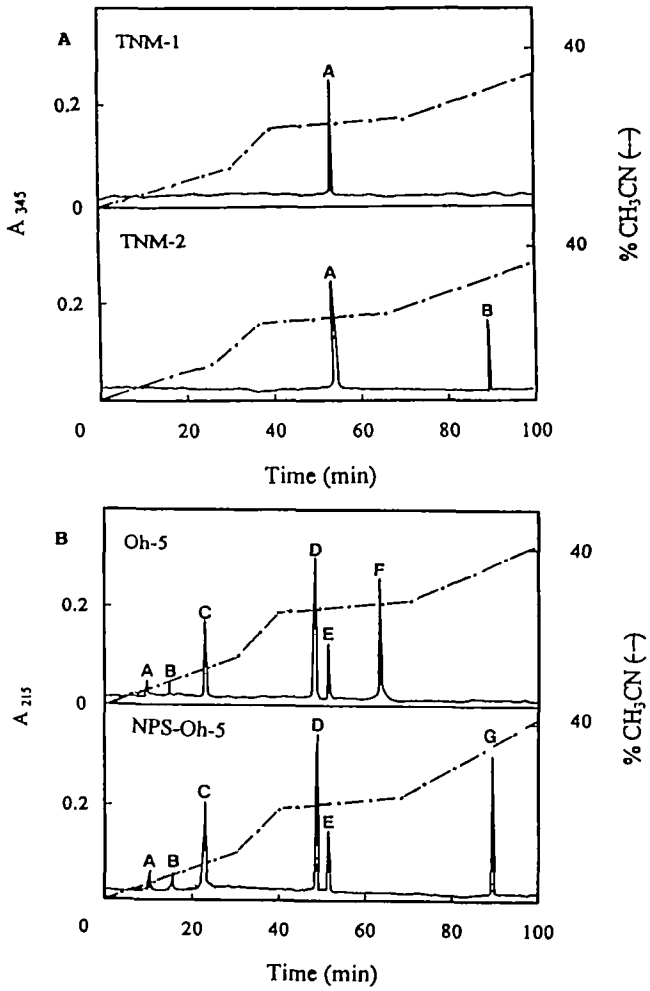

Fig. 4. **RP-HPLC profiles of the tryptic digests of RCM-derivatives of Oh-5 modified at Tyr (A) and Trp (B).** Chromatography was performed under the same condition as in Fig. 1 except for a linear gradient shown by a dotted line. Peaks eluted at the same retention time were assigned the same letters.

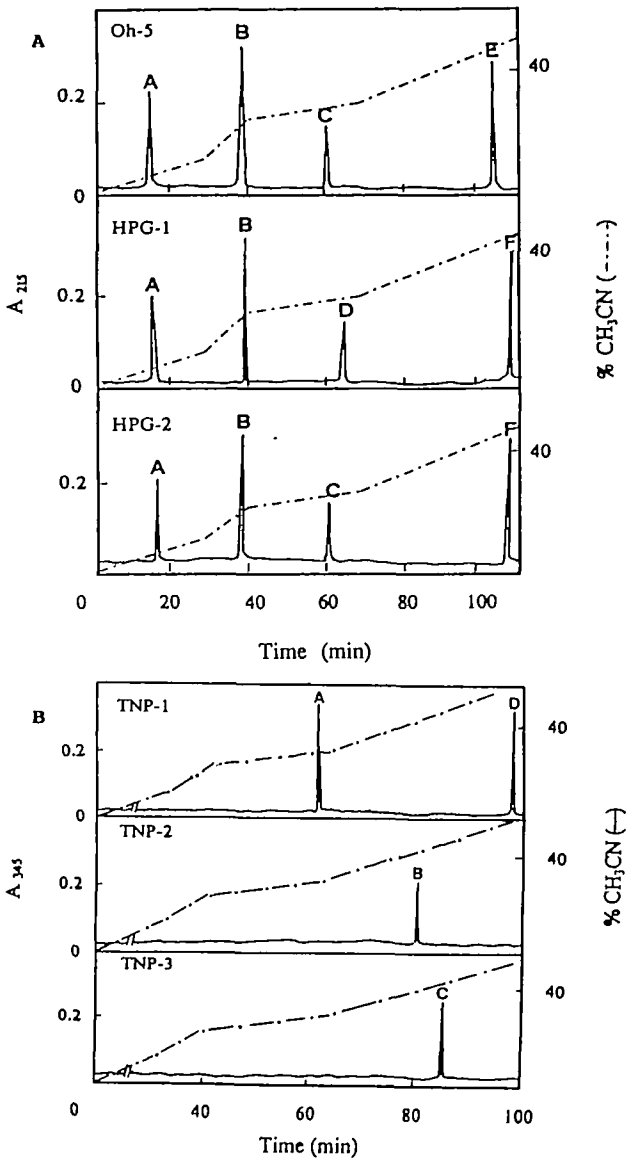

Fig. 5. **RP-HPLC profiles of digests of RCM-derivatives of Arg-modified Oh-5 (A) with lysyl endopeptidase, and amino modified Oh-5 (B) with trypsin.** Chromatography was performed under the same conditions as in Fig. 1, except for a linear gradient shown by a dotted line.

α-Neurotoxin	10 20 30 40 50 60 70	% identity
Oh-5	TKCY KTGDRIISEACPPGQDLCYMKTWCDVFCGTRGRVIELGCTATCPTVKPHEQITCCSTDNCDPHHKMLQ	100
Oh-4	.....--.....k.....k.....A.....N..PNKM.	89
Toxin a	.....--V.P.V-K.QT..A...I..TE....AW.TS..KRVN...A....I...GVE.K.....N.FPTWRKRP	56
Toxin b	.....--V.P.V---.T..D...I..T.....G..SS..KR.D...A....K...GVD.K.....N.FPTWKRKH	62
α-Cobratoxin	IR.F--I.P.I-T.KD..N.H-V..T.....A..SI..KRVD...A.....TGVD.Q.....N.FPTRKRP	52
α-BuTX	IV.HTTA.SPI-SAVT....EN...R.M...A..SS..K.V....A....SK..Y.EV.....K.N..P.QRPG	53
Toxin A	IR.F--I.P.I-T.KD..N.H-V..T.....G..SI..KRVD...A.....RTGVD.Q.....D...FPTRKRP	50

Fig. 3. **Comparison of amino acid sequence of Oh-5 with those of some long-chain α-neurotoxins from snake venoms.** Oh-4, Toxin *a* and Toxin *b* from *Ophiophagus hannah* venom (Refs. *15* and *23*); α-cobratoxin, *Naja naja siamensis* venom (Ref. *24*); α-BuTX, *Bungarus multicinctus* venom (Ref. *25*); Toxin A, *Naja naja* venom (Ref. *26*). Residues identical to that in the top line were designated with a dot, and gaps are marked with hyphens.

RESULTS AND DISCUSSION

Amino Acid Sequence of Oh-5—The Oh-5 was purified from the king cobra venom in a yield of about 1.5%. SDS-PAGE analysis showed that the toxin molecule had an apparent molecular weight of about 8 kDa (data not shown). Firstly, the N-terminal sequence of RCM-Oh-5 was determined up to 20 residues. Then, the RCM-toxin was digested with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease, respectively. The resulting hydrolysates were separated by RP-HPLC: six tryptic peptides (T_1 to T_6), four peptides derived from lysyl endopeptidase (L_1 to L_4), and three peptides (V_1 to V_3) from V_8 protease digestion were obtained as shown in Fig. 1. Based on the amino acid compositions and sequence determination of these peptides, the complete amino acid sequence of Oh-5 was constructed (Fig. 2). Apparently, Oh-5 is a long α-neurotoxin consisting of 72 amino acid residues with 10 cysteines. As shown in Fig. 3, Oh-5 shares about 89% sequence identity with Oh-4, and 50 to 61% identity with other long α-neurotoxins such as Toxins *a, b* from this venom (*23*), α-cobratoxin (*24*), α-BuTX (*25*), and Toxin A (*26*).

Nitration of Oh-5—The reaction products of Oh-5 with TNM were separated by HPLC on a SynChropak RP-P column and two major nitrated derivatives (TNM-1 and TNM-2) were isolated. Spectral determination of the extent of modification based on the molar absorption coefficient and amino acid analysis of the tryptic hydrolysates of RCM-TNM derivatives indicated that TNM-1 and TNM-2 contained one and two nitrated tyrosines, respectively (Fig. 4A). As listed in Table I, the results of amino acid analysis and sequence determination showed that TNM-1 contained a nitrated Tyr residue at position 4, and both Tyr-4 and 23 were modified in TNM-2.

Sulfenylation of Oh-5—Oh-5 was incubated with a 6-fold molar excess of NPS-Cl in an acidic medium and the modified protein was separated by HPLC on a SynChropak RP-P column. Only one major NPS-derivative was isolated from the modified toxin (data not shown). Spectral determination of the extent of modification based on a molar absorption coefficient of 4,000 at 365 nm in 80% acetic acid indicated that only one NPS group had been incorporated into the NPS-Oh-5 derivative. Chromatographic profiles of the tryptic hydrolysates of the RCM-NPS derivative revealed that instead of the missing peak F, a new peak G

TABLE II. **Lethal toxicity of Oh-5 and its modified derivatives.**

Derivative	Modified position	Lethal toxicity[a]	
		LD_{50} (μg/g)	Relative (%)
Oh-5	–	0.25	100
TNM-1	Tyr-4	0.25	100
TNM-2	Tyr-4, 23	0.49	51
NPS	Trp-27	0.25	100
HPG-1	Arg-9, 35, 37	2.78	9
HPG-2	Arg-35, 37	0.98	26
TNP-1	Lys-2, 51, 69	0.49	51
TNP-2	Lys-51	0.35	71
TNP-3	Lys-69	0.35	71

TNM, NPS, HPG, and TNP derivatives denote toxins modified at tyrosine, tryptophan, arginine and lysine, respectively. [a]Measured i.p. with mice weighing 16–18 g.

TABLE I. **Amino acid sequences of proteolytic peptides from modified derivatives of Oh-5.**

Peak	Modified position	Sequence	Position
TNM (A)	Tyr-4	CYK | TNM	3-5
TNM(B)	Tyr-23	IISEACPPGQDLCYMK | TNM	10-25
NPS(G)	Trp-27	TWCDVFCGTR | NPS	26-35
HPG(D)	Arg-9	TGDRIISEACPPGQDLCYMK | HPG	6-25
HPG(F)	Arg-35,37	TWCDVFCGTRGRVIELGCTATCPTVK | | HPG HPG	26-51
TNP(A)	Lys-2	TKCYK | TNP	1-5
TNP(B)	Lys-51	VIELGCTATCPTVKPHEQITCCSTDNCDPHHK | TNP	38-69
TNP(C)	Lys-69	VIELGCTATCPTVKPHEQITCCSTDNCDPHHKMLQ | TNP	38-72
TNP(D)	Lys-51,69	VIELGCTATCPTVKPHEQITCCSTDNCDPHHKMLQ | | TNP TNP	38-72

TNM (A), (B), and NPS (G) as shown in Fig. 3 and TNP (A), (B), (C), and (D) in Fig. 4 were each digested with trypsin. HPG (D) and (F) in Fig. 4 were digested with lysyl endopeptidase.

had appeared (Fig. 4B). Peak G had the sequence TWCDV-
|
NPS
FCGTR, indicating that Trp-27 is modified in NPS-Oh-5.

Modification of Arginine Residues—HPG is an arginine-specific reagent and the extent of modification can be determined by measuring the absorbance at 340 nm based on a molar extinction coefficient of $1.83\times10^4\ M^{-1}\cdot cm^{-1}$ (*14*). Oh-5 was reacted with HPG in 0.2 M $NaHCO_3$ buffer (pH 9.0) for 1 h, and two major protein fractions, HPG-1 and HPG-2 were separated from the reaction mixture on CM-52 column chromatography. Spectral determination of the extent of modification based on the molar absorption coefficient and amino acid analysis indicated that three and two HPG groups were incorporated into HPG-1 and HPG-2, respectively. Peptide mapping of the enzymatic hydrolysates of RCM-HPG derivatives with lysyl endopeptidase showed that peaks E and F, instead of peaks C and D appeared in the HPLC profile (Fig. 5A). The results of amino acid analysis and sequence determination indicated that HPG-1 contained modified Arg residues at positions 9, 35, and 37, while Arg-35 and 37 were modified in HPG-2 (Table I).

Modification of Amino Groups—After modification of Oh-5 with a 2-fold molar excess of TNBS, three trinitrophenylated derivatives (TNP-1 to TNP-3) could be separated on a CM-52 column. Spectral determination of the extent of modification based on a molar absorption coefficient of 11,500 at 345 nm in 0.1 M HCl and amino acid analysis indicated that three TNP groups had been incorporated into TNP-1 and only one into TNP-2 and TNP-3, respectively. Peptide mapping of the tryptic hydrolysates of RCM-TNP derivatives at 345 nm showed that peaks A, B, C, and D appeared in the hydrolysates of TNP-1 to TNP-3, respectively (Fig. 5B). The results of amino acid analysis and sequence determination revealed that TNP-1 contained modified Lys residues at positions 2, 51, and 69, while Lys-51 and Lys-69 were modified in TNP-2 and TNP-3, respectively (Table I).

Biological Activity of the Modified Toxins—Modification of Tyr-4 or Trp-27 in Oh-5 did not affect the lethal toxicity at all, whereas the derivative in which both Tyr-4 and 23 were nitrated showed a decrease in lethality by 50% (Table II). However, in contrast to the case of Oh-5, modification of the conserved Trp residue in Oh-4 and Oh-7 isolated from the same venom resulted in a decrease in toxicity (*16*). Previous studies showed that the neurotoxicity of cobrotoxin was greatly reduced, but that of α-BuTX remained almost intact after modification of the conserved Trp residue (*12*). These results indicate that the molecular interaction between nAChR and toxin molecules are not the

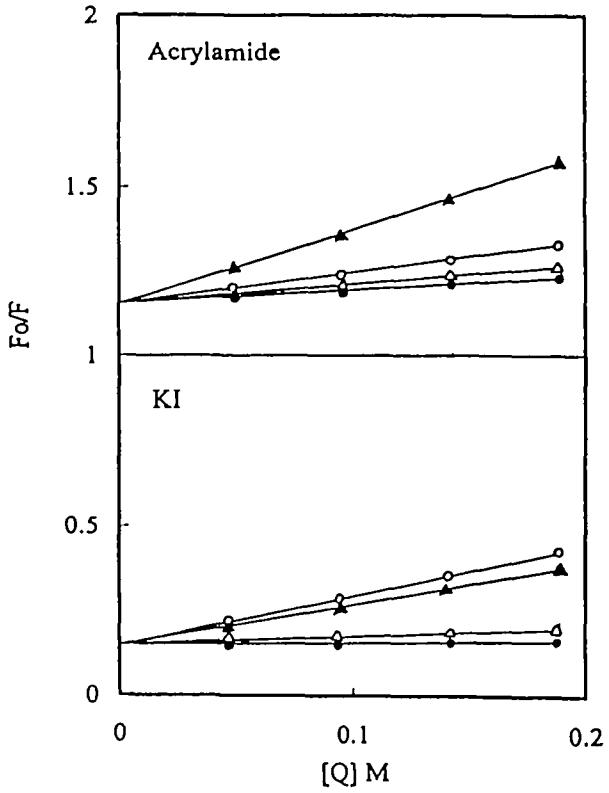

Fig. 6. **Stern-Volmer plots for quenching of the tryptophan fluorescence of cobrotoxin (○), α-BuTX (●), Oh-5 (△), and RCM-Oh-5 (▲) by acrylamide and iodide.** Toxins (0.1 μmol) in 2 mM Hepes-0.2 M NaCl (pH 7.5) were quenched with acrylamide and iodide. The fluorescence intensity was recorded at 345 nm upon excitation at 295 nm. Fluorescence quenching was analyzed according to the Stern-Volmer equation, $F_0/F=1+K_{sv}[Q]$, where F_0 is the fluorescence in the absence of quencher, F is the fluorescence at molar quencher concentration [Q], and K_{sv} is the Stern-Volmer quenching constant obtained from the slope of a plot of F_0/F *vs.* [Q]. In all experiments, the total salt concentration was kept constant at 0.2 M by the addition of an appropriate amount of NaCl. All values are the means of triplicate determinations.

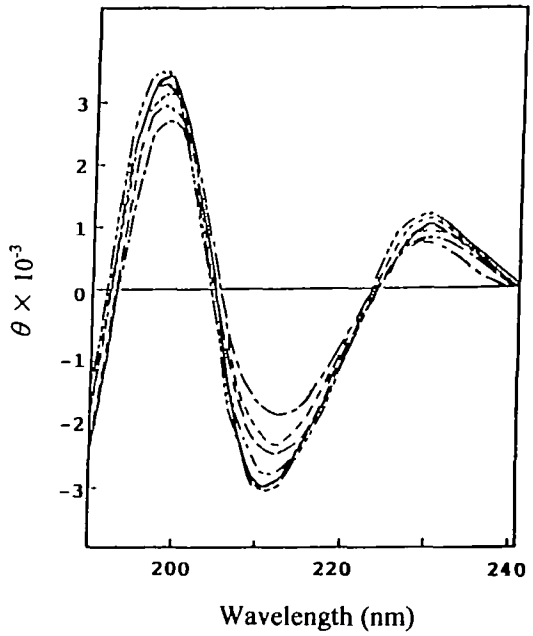

Fig. 7. **CD spectra of Oh-5 and its derivatives modified at cationic residues.** The spectra were recorded with a Jasco J-700 spectropolarimeter from 250 to 190 nm at a concentration of 0.8 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) with a cell path length of 0.1 mm. The CD spectra were obtained by averaging five scans. (—), Oh-5; (· · ·), TNP-1; (– –), TNP-2; (–·–), TNP-3; (–···–), HPG-1; (—···—), HPG-2. The spectra of the derivatives modified at aromatic groups are similar to those observed with Oh-5 and derivatives modified at cationic residues.

same for all α-neurotoxins. This is supported by the different roles of the conserved Trp in the lethal toxicity of cobrotoxin and α-BuTX (*21*). As shown in Fig. 6, quenching studies with acrylamide and iodide revealed that the microenvironment of Trp in Oh-5 is similar to that of α-BuTX. The inaccessibility of the Trp residue in Oh-5 to acrylamide and iodide indicates that Trp is located in a hydrophobic, buried environment, which is different from that in cobrotoxin (*21*). Thus, modification of Trp-27 in Oh-5 did have any appreciable influence on the lethal toxicity, as in the case of α-BuTX.

As shown in Table II, modifications of Arg-35 and 37 caused a drastic decrease in lethality to 26%, and there was a further decrease to 9% when Arg-9 was additionally modified. These results reflect the fact that all the arginine residues in Oh-5 are readily modified with HPG. Monotrinitrophenylation at Lys-51 or 69 slightly affected the lethality, while the derivative trinitrophenylated at Lys-2, 51, and 69 still retained about 50% of the neurotoxicity. The CD spectra of these modified derivatives exhibited almost the same profile as that of the native toxin (Fig. 7), revealing that the incorporated groups did not significantly perturb the native conformation of Oh-5. The present results indicate that Tyr-4 and Trp-27 of Oh-5 are not essential for neurotoxicity, whereas the cationic residues are involved in multipoint contact between the toxin molecule and the nicotinic acetylcholine receptor (nAChR). Tyr-23, Arg-35 and 37 in the central loop of Oh-5 seems to be closely related to the neurotoxicity. The results are in good agreement with previous studies showing that the loop 2 of α-neurotoxins is important for the interaction with nAChR (*27, 28*).

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