11 Chader, G.J., Fletcher, R.T., O'Brien, P.J., and Krishna, G., Biochemistry *15* (1976) 1615.
12 Chader, G.J., Fletcher, R.T., Russel, P., and Krishna, G., Biochemistry *19*, (1980) 2634.
13 Smith, H.G., Stubbs, G.W., and Litman, B.J., Exp. Eye Res. *20* (1975) 211.
14 Fesenko, E.E., and Orlov, N.Ya., Molec. Biol.-Engl. Tr. *14* (1980) 617.
15 Miller, J.A., and Paulsen, R., J. biol. Chem. *250* (1975) 4427.
16 Fesenko, E.E., Orlov, N.Ya., and Gasparian, A.G., Studia biophys. *82* (1981) 75
17 Livrea, M.A., Nicotra, C., Bongiorno, A., Ciaramitaro, G., and Romano, M., Experientia *36* (1980) 894.

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Isolation and amino acid compositions of geographutoxin I and II from the marine snail *Conus geographus* Linné[1]

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Summary. The peptide toxins having inhibitory effects on the contractile response of the mouse diaphragm to direct stimulation, geographutoxin I and II, have been isolated from *Conus geographus*, and their amino acid compositions have been determined.

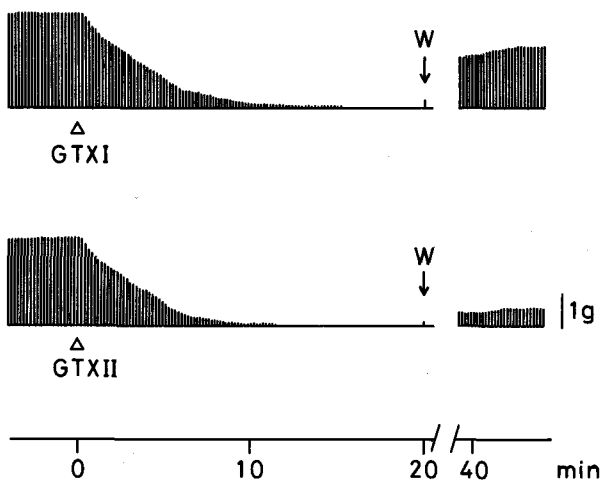
The species of the family Conidae use their specialized venom apparatus in the capture of prey organisms[2]. Among them, the venom of *Conus geographus* is most toxic to vertebrates and has been responsible for human fatilities[3]. It has been shown that the venom caused an inhibitory effect on the contractile response of directly stimulated isolated skeletal muscle[4-7]. Furthermore, preliminary purification of the substances having such a direct action on skeletal muscles has been reported[6]. Recently, 3 peptides having inhibitory actions on the post synaptic membrane of a vertebrate neuromuscular junction have been isolated from *C. geographus* and their amino acid sequences have been determined[8-10]. The present paper deals with the isolation and the amino acid compositions of 2 peptide toxins, geographutoxin (GTX) I and II from the venom of *C. geographus*.

Specimens of *C. geographus* were obtained from reefs in Okinawa waters. The gastropods were immediately frozen, shipped via air to Tokyo and stored at −20 °C until the specimens were used. The venom ducts (8.2 g) dissected from 30 animals (shell length 6.5–10.5 cm) were cut into small segments and the contents were squeezed out. The contents were extracted 3 times with 0.2 M acetic acid (80 ml each) and lyophilized to give a yellowish crude venom (1.2 g). The mouse isolated diaphragm preparation was set up and the contractile response to direct stimulation (0.1 Hz, 5 msec, supramaximal voltage) was measured as previously described[11]. Purification of the crude venom was carried out as the following procedure by monitoring the inhibitory effect on the diaphragm. The crude venom was chromatographed on a column of Sephadex G-50 (55 cm × 2.2 cm ID, Vo = 80 ml) using 1/30 M sodium phosphate buffer, pH 5.6, containing 0.15 M NaCl as eluant. The active portion (relative elution volume, Ve/Vo 1.7-2.9) was applied on a column of CM-Sephadex C-25 (50 cm × 2.6 cm ID) previously equilibrated with 1/30 M sodium phosphate buffer (pH 5.6) containing 0.15 M NaCl. The column was eluted with the same buffer (300 ml) and then with a linear gradient consisting of 1000 ml of 0.15 M and 1000 ml of 1.0 M NaCl in 1/30 M sodium phosphate

Amino acid compositions of GTX I and II

Amino acid	GTX I Nano-moles analyzed	Amino acid ratio	GTX II Nano-moles analyzed	Amino acid ratio
Lysine	7.10	4.10 (4)	6.61	4.01 (4)
Histidine	–	–	–	–
Arginine	5.09	2.94 (3)	4.33	2.62 (3)
Aspartic acid	3.57	2.06 (2)	3.39	2.05 (2)
Threonine	1.73	1.00 (1)	1.65	1.00 (1)
Serine	–	–	–	–
Glutamic acid	3.44	1.99 (2)	0.15	0.09 (0)
Proline	–	–	–	–
Hydroxyproline	5.20	3.01 (3)	4.46	2.70 (3)
Cysteine[a]	10.6	6.13 (6)	9.08	5.50 (6)
Glycine	–	–	–	–
Alanine	1.68	0.97 (1)	1.64	1.00 (1)
Valine	–	–	–	–
Methionine	–	–	1.01	0.61 (1)
Isoleucine	–	–	0.25	0.15 (0)
Tyrosine	–	–	–	–
Phenylalanine	–	–	–	–
Tryptophan[b]		–		–

[a] Determined as cysteic acid; [b] determined by UV absorption.



Effects of geographutoxin (GTX) I (2×10^{-7} M) and II (2×10^{-7} M) on the contractile response of the isolated mouse diaphragm to direct stimulation (0.1 Hz, 5 msec, supramaximal voltage). At △ and ↓, toxins were added and washed twice with fresh medium, respectively.

buffer, pH 5.6. Two active fractions A and B were eluted at the NaCl concentrations of 0.8 M and 0.95 M, respectively. After diafiltering both the fractions using Amicon UM 05 filter, the active retentates were lyophilized to yield C (188 mg) from A and D (190 mg) from B. Each lyophilizate was purified by HPLC using a column of MCI gel CQP-10 (60 cm × 7.6 mm ID, Mitsubishi Chemical Industries, Ltd) under the condition of 0.15 M ammonium formate as mobile phase and a flow rate of 2 ml/min. The active fractions E ($t_R = 6.4$ min) from C and F ($t_R = 8.6$ min) from D were chromatographed on a column of Sephadex G-25 (0.2 M acetic acid) and lyophilized to afford GTX I (13 mg) and GTX II (12 mg) as colorless powders, respectively.

GTX I, II and their performic acid-oxidized peptides were subjected to cellulose plate electrophoresis in 0.2 M pyridine acetate buffer, pH 6.5 (45 min at 40 V/cm). Only one cathodally moving band (ninhydrin positive) was observed in all cases and a clear separation between them could be achieved under these conditions. The amino acid compositions of the 2 peptide toxins were determined by amino acid analysis of hydrolysates with constant boiling hydrochloric acid for 20 h at 105 °C (table). The compositions of GTX I and II are similar to each other. GTX II possesses methionine residues instead of glutamine (or glutamic acid) residues in GTX I. Furthermore, hydrolysis of dansylated GTX I and II gave only dansylated arginine as the α-labeled amino acid. These results suggest that each toxin is almost homogeneous. It has been reported[9] that all 3 conotoxins isolated from *C. geographus* have glutamic acid in the N-terminal and histidine, serine and glycine as the internal residues. On the other hand, both of GTX I and II have arginine in the N-terminal and threonine and hydroxyproline residues but not histidine, serine and glycine residues.

As shown in the figure the purified toxins GTX I and II at concentrations above 10^{-8} M markedly inhibited twitch responses of the mouse diaphragm to direct stimulation. After washout of both toxins with the fresh medium, the depressed contractile response to electrical stimulation was progressively restored. However, the conotoxins at 2×10^{-7} M have been shown to cause a marked decline of the twitch response of the mouse diaphragm to indirect nerve stimulation without effects on that to direct stimulation[10]. These observations indicate that nature of pharmacological actions of GTX I and II is quite different from that of conotoxins. The detailed chemical and pharmacological studies on GTX I and II are in progress.

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2 Kohn, A.J., J. Ecol. Monogr. *29* (1959) 47.
3 Kohn, A.J., Hawaii Med. J. *17* (1958) 528.
4 Whyte, J.M., and Endean, R., Toxicon *1* (1962) 25.
5 Endean, R. Parish, G., and Gyr, P., Toxicon *12* (1974) 131.
6 Spence, I., Gillessen, D., Gregson, R.P., and Quinn, R.J., Life Sci. *21* (1977) 1759.
7 Kobayashi, J., Nakamura, H., Hirata, Y., and Ohizumi, Y., Toxicon *20* (1982) 823.
8 Cruz, L.J., Gray, W.R., and Olivera, B.M., Archs Biochem. Biophys. *190* (1978) 539.
9 Gray, W.R., Luque, A., Olivera, B.M., Barrett, J., and Cruz, L.J., J. biol. Chem. *256* (1981) 4734.
10 McManus, O.B., Musick, J.R., and Gonzalez, C., Neurosci. Lett. *24* (1981) 57.
11 Kobayashi, J., Ohizumi, Y., Nakamura, H., and Hirata, Y., Toxicon *19* (1981) 757.

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Stereoselective binding of 4,5-dihydrodiazepam to human serum albumin

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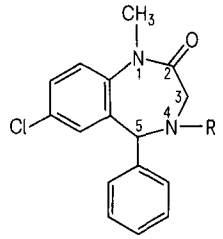
Summary. S(+)-4,5-dihydrodiazepam was found to have higher binding affinity for human serum albumin than its antipode. The binding of 4-carbamoyl-4,5-dihydrodiazepam is weak and not stereoselective.

Several chiral drugs have been found to exhibit stereoselective binding to serum proteins, indicating the specificity of binding sites[2]. The highest stereoselectivities were obtained for the binding of 3-substituted 1,4-benzodiazepines to human serum albumin (HSA)[2-5]. The phenomenon was interpreted in terms of the inversion of the diazepine ring giving preference to the conformation in which the C_3-substituent is found in the equatorial position. In this work, we studied the stereoselective binding of 4,5-dihydrodiazepam (DHD) as well as 4-carbamoyl-DHD (CDHD) to HSA. These compounds are 1,4-benzodiazepines with a chiral centre at the C_5 position (cf. formula). S(+)-DHD was found[6] to have about 2-3 times stronger anticonvulsant and antiaggressive effects in mice than its antipode.

Materials and methods. Synthesis and resolution of DHD and its 2-^{14}C-labelled form were carried out as previously described[7,8]. Samples of racemic and enantiomeric CDHD were obtained from Chemical Works of Gedeon Richter Ltd, Budapest. ^{3}H-diazepam and ^{14}C-warfarin were purchased from the Institute of Isotopes, Budapest, and The Radiochemical Centre Amersham, respectively. Binding studies with radioactive DHD enantiomers as well as competition experiments were performed by a method[9] applying HSA immobilized on polyacrylamide microparticles. After centrifugation, the concentration of the labelled free drug was measured by liquid scintillation counting of the supernatant. 0.005 M phosphate, 0.1 M KCl buffer



DHD: R = H
CDHD: R = $CONH_2$