Biologically Active Compounds

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Synthesis and Biological Activity of Argiotoxin 636 and Analogues: Selective Antagonists for Ionotropic Glutamate Receptors**

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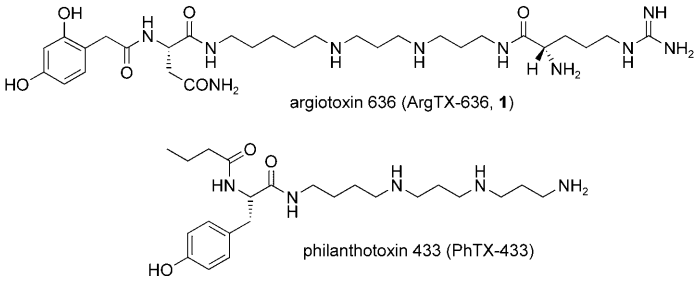
The family of ionotropic glutamate (iGlu) receptors (iGluRs) are ligand-gated ion channels that mediate the majority of excitatory synaptic transmission in the vertebrate brain and are crucial for normal brain function. Dysfunction of iGlu receptors is involved in a range of neurological and psychiatric diseases, and iGlu receptors are considered important drug targets for brain diseases.[1]

In particular, the inhibition of iGlu receptors is a promising strategy for the treatment of neurodegenerative diseases, such as stroke and Alzheimer's disease.[1a,2] However, the development of selective and clinically effective inhibitors of iGlu receptors has proven difficult. Only a few drugs directed against iGlu receptors have been approved, one of which is memantine, an open-channel blocker of the *N*-methyl-D-aspartate (NMDA) subtype of iGlu receptors. Memantine is used in the treatment of Alzheimer's disease.[3]

Polyamine toxins, a group of small molecules found in spiders and wasps, are also open-channel blockers of iGlu receptors.[4] They have found valuable use as pharmacological tools on the basis of their high affinity and selectivity for iGlu receptors,[4] but have not yet been explored as templates for the development of iGlu receptor drugs. A main concern is that native polyamine toxins generally distinguish poorly among individual iGluR subtypes, such as NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.

Argiotoxin 636 (ArgTX-636, **1**; Scheme 1) is a polyamine toxin isolated from the venom of the orb weaver spider *Argiope lobata*.[4,5] It is one of the most potent inhibitors of iGlu receptors, which it blocks in a use- and voltage-dependent manner, presumably by binding to the ion-channel region of these receptors. ArgTX-636 has demonstrated neuroprotective properties;[6] however, it does not distinguish iGluR subtypes. We have now investigated the rational design of analogues of **1** in an attempt to produce iGluR inhibitors with marked improvement in subtype selectivity.



Scheme 1. Structures of key polyamine toxins.

ArgTX-636 was synthesized previously by using conventional solution-phase chemistry. Cumbersome purification of highly polar polyamine intermediates was required, with extensive use of protection groups, and ArgTX-636 was obtained in very low overall yield.[7] Inspired by the solid-phase synthesis of the related polyamine toxin philanthotoxin 433 (PhTX-433, Scheme 1) and analogues,[8] we initially attempted the solid-phase synthesis of ArgTX-636 (**1**) from preloaded PS-Trt-Arg-OH (PS = polystyrene). This strategy required an inverse (C→N) coupling as the initial step of the synthesis. However, only small quantities of the desired product were obtained, and the use of a variety of different conditions did not improve conversion.

Instead, the synthesis of ArgTX-636 was attempted by using a backbone amide linker (BAL) as a handle (Scheme 2).[9,10] We reasoned that this approach would provide an opportunity for late-stage diversification of the parent molecule for the generation of a focused library. A monoprotected diamine was loaded onto the resin by reductive amination, and two amino acid coupling reactions then provided **11** on the resin (Scheme 2). Extended reaction times were required for complete conversion in the first coupling reaction, and it was necessary to protect the amide side chain of asparagine for efficient coupling, to avoid a competing cyclization.[11]

The polyamine chain was prepared was prepared by a Fukuyama–Mitsunobu alkylation strategy[12] with amino alcohols as building blocks. Intermediate **11** was transformed efficiently into the Teoc-protected amine **12** by treatment with *N*-Teoc-protected 3-hydroxypropylamine in the presence of 1,1′-azodicarbonyldipiperidine (ADDP) and tributylphosphane as redox reagents (Scheme 2). The Teoc group was removed selectively with tetrabutylammonium fluoride

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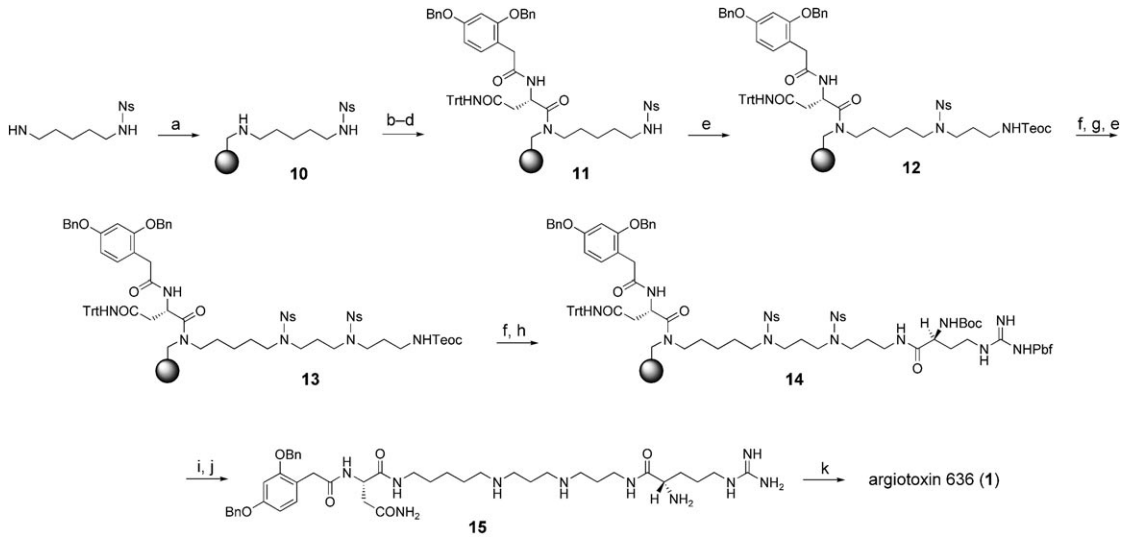
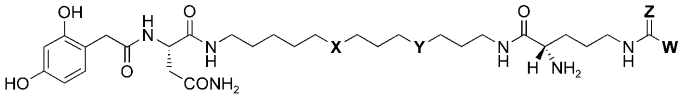


Scheme 2. Solid-phase synthesis of ArgTX-636 (**1**): a) BAL PS resin (loading: 0.92 mmol g^{-1}), $NaCNBH_3$, DMF/AcOH (99:1), 1 h; b) Fmoc-L-Asn(Trt)-OH, HATU, collidine, DMF, 16 h followed by purification/washing and a further 16 h; c) 20% piperidine in DMF, 2 min followed by purification/washing and a further 20 min; d) 2-(2,4-bis(benzyloxy)phenyl)acetic acid, HATU, collidine, DMF, 2 h; e) $HO(CH_2)_3NHTeoc$, Bu_3P, ADDP, CH_2Cl_2/THF (1:1), 3×3 h; f) TBAF, THF, 50 °C, 30 min; g) Ns-Cl, THF/CH_2Cl_2 (2:1), 3 h; h) Boc-L-Arg(Pbf)-OH, HATU, collidine, DMF, 2 h; i) DBU, β-mercaptoethanol, DMF, 3×20 min; j) TFA/CH_2Cl_2/TIS/H_2O (47.5:47.5:2.5:2.5), 2 h; k) H_2, Pd/C, MeOH, 16 h. ADDP = 1,1′-azodicarbonyldipiperidine, Bn = benzyl, Boc = *tert*-butoxycarbonyl, DBU = 1,8-diazabicyclo[5.4.0]undecane, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, HATU = *N*-((dimethylamino)-1*H*-1,2,3-triazolo[4,5,*b*]pyridin-1-ylmethylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, Ns = *o*-nitrobenzenesulfonyl, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, TBAF = tetrabutylammonium fluoride, Teoc = 2-trimethylsilylethoxycarbonyl, TIS = triisopropylsilane, TFA = trifluoroacetic acid, Trt = trityl.

(TBAF), and the resulting primary amine was subsequently protected/activated with *o*-nitrobenzenesulfonyl chloride (Ns-Cl). The Fukuyama–Mitsunobu alkylation was repeated to complete the installation of the polyamine chain. Removal of the Teoc group was then followed by peptide coupling to generate fully protected ArgTX-636 (**1**) on the resin. The Ns-protected amines were deprotected, and the sample was cleaved from the resin with subsequent removal of the Pbf, Boc, and Trt protecting groups to give bisbenzyl ArgTX-636 **15** in 3.4% overall yield for the 13 steps on the solid phase: an average yield per reaction of 77%. ArgTX-636 (**1**) was obtained in 58% yield from **15** by treatment with H_2.

Having completed the total synthesis of ArgTX-636 (**1**), we were interested in applying the methodology to the preparation of analogues that could be used to probe biological activity at the NMDA and AMPA receptors. We envisioned that the structure of the polyamine moiety would confer differences in potency and selectivity, so we designed a number of analogues by systematic modification of the polyamine and the terminal arginine moieties. The resulting focused library consisted of nine compounds with variations in the polyamine moiety and the guanidinyl side chain of arginine (Scheme 3).

We chose to synthesize three different polyamine units by replacing the secondary amino groups with methylene groups systematically. Moreover, we modified the guanidine group of the arginine moiety by introducing either citrulline or N^{ε}-acetylornithine in place of arginine. These analogues should provide subtle information on the biological importance of internal nitrogen atoms as well as the terminal guanidine group. The synthetic route developed for ArgTX-636 (**1**) was



2 (X=Y=NH, Z=O, W=NH_2)
3 (X=Y=NH, Z=O, W=CH_3)
4 (X=NH, Y=CH_2, Z=NH, W=NH_2)
5 (X=NH, Y=CH_2, Z=O, W=NH_2)
6 (X=NH, Y=CH_2, Z=O, W=CH_3)
7 (X=CH_2, Y=NH, Z=NH, W=NH_2)
8 (X=CH_2, Y=NH, Z=O, W=NH_2)
9 (X=CH_2, Y=NH, Z=O, W=CH_3)

Scheme 3. ArgTX-636 analogues selected for synthesis and biological evaluation.

then applied to the synthesis of the eight analogues (Scheme 3). The analogues were prepared readily by using appropriate amino alcohol building blocks and replacing the arginine building block with citrulline or N^{ε}-acetylornithine. Following HPLC purification and characterization, the analogues were subjected to biological testing.

The potency of ArgTX-636 and analogues in blocking the NMDA and AMPA receptors was evaluated by two-electrode voltage-clamp electrophysiology on *Xenopus oocytes* expressing the AMPA receptor GluR1 or the NMDA receptor subunits NR1 and NR2A. Initially, the inhibitory activity of a compound was compared to that of the parent ArgTX-636 (**1**) by measuring the inhibition of agonist-induced currents at oocytes expressing NMDA and AMPA receptors (Figure 1).

The initial screening revealed a number of important points (Figure 1; see Table S1 in the Supporting Information). First, ArgTX-636 was confirmed to be a very potent blocker of NMDA and AMPA receptors. Second, the terminal guanidine group is important for biological activity, as the three compounds containing this group, **1**, **4**, and **7**, were the

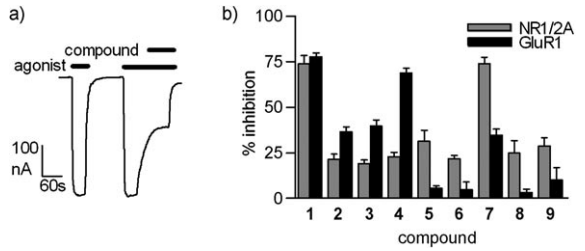


Figure 1. Screening of ArgTX-636 (**1**) and analogues at NMDA and AMPA receptors. a) Representative trace showing the electrophysiological protocol used to determine the inhibitory effect of a test compound (1 μM) on agonist-evoked (100 μM) iGlu receptor currents. b) Graphical summary of the percent inhibition (mean ± standard deviation; $N > 3$) by compounds at NMDA (gray bars) and AMPA (black bars) receptors in oocytes held at a membrane potential of −60 mV.

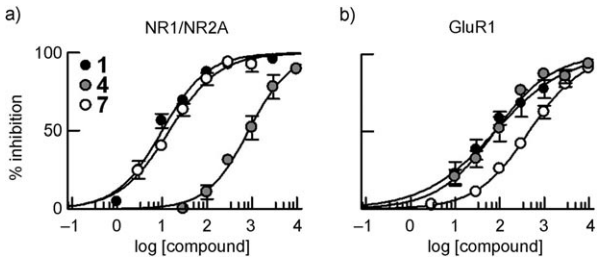


Figure 2. Determination of IC_{50} values (concentration of the compound required for 50% inhibition) for ArgTX-636 (**1**) and analogues **4** and **7** at a) NMDA and b) AMPA receptors. Data points represent the mean ± SEM (standard error of the mean) from at least three different experiments with different oocytes.

most potent at NMDA or AMPA receptors (Figure 1). In contrast, compounds **2**, **3**, **5**, **6**, **8**, and **9**, with either citrulline or N^{ε}-acetylornithine in place of arginine, showed less than 50% inhibition of both receptor subtypes. Finally, and most importantly, the screen indicated that compounds **4** and **7** were selective for the AMPA and NMDA receptor subtype, respectively, in contrast to the parent compound.

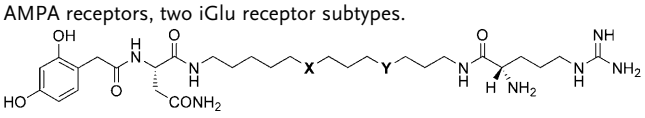
These results prompted further characterization of ArgTX-636 (**1**) and analogues **4** and **7**. Full dose-response curves were generated at both AMPA and NMDA receptors at three different membrane potentials. At a membrane potential of −80 mV, which is close to the typical neuronal resting potential, ArgTX-636 (**1**) was a very potent NMDA receptor antagonist with an IC_{50} value of 10 ± 1 nM, but also quite potent at AMPA receptors (IC_{50} = 77 ± 14 nM; Table 1). Subsequent testing of compounds **4** and **7** confirmed the initial observation regarding selectivity: Compound **4** was equipotent to ArgTX-636 at AMPA receptors (IC_{50} = 78 ± 12 nM), but showed a remarkable 84-fold decrease in activity at NMDA receptors (IC_{50} = 842 ± 117 nM; Figure 2 and Table 1). Thus, the replacement of the secondary amino group in position Y of the polyamine chain of nonselective ArgTX-636 (Scheme 3 and Table 1) with a methylene group leads to the potent and selective AMPA receptor antagonist **4**.

Intriguingly, the reverse selectivity was observed for compound **7**. In this case, substitution of the secondary amino group in position X with a methylene group (Scheme 3 and Table 1) led to 32-fold selectivity for the NMDA receptor subtype. Thus, compound **7** is equipotent to ArgTX-636 at NMDA receptors (IC_{50} = 14 ± 1 nM) but several times less potent at AMPA receptors (IC_{50} = 454 ± 27 nM).

A voltage dependency of the IC_{50} value was observed for all three compounds in the form of a decrease in affinity as the membrane potential becomes less negative (see Table S2 and Figure S1 in the Supporting Information). This relationship is a hallmark of polyamine channel blockers[1a] and suggests that the mode of blockade by **4** and **7** is similar to that of **1**.

To better understand the structural basis for the differences in AMPA and NMDA receptor selectivity of **1**, **4**, and **7**, these compounds were docked into models of the AMPA and NMDA receptor ion channels (Figure 3).[13] Generally, this class of ligands is assumed to bind to both AMPA and NMDA receptors in a similar fashion: The aromatic head group is positioned in the ion-channel vestibule, and the polyamine tail penetrates the narrowest part of the ion channel, the selectivity filter, also known as the N/Q/R site (Figure 3).[14,15] The latter site is highly critical for the binding of polyamine blockers, as binding is abolished when there is an arginine residue (R) in the N/Q/R site.[1a] The N/Q/R site is structurally

Table 1: Potency of ArgTX-636 (**1**) and analogues **4** and **7** at NMDA and AMPA receptors, two iGlu receptor subtypes.



Compound	X	Y	IC_{50} [nM] NMDA[a]	AMPA[b]	Selectivity[c]
1	NH	NH	10 ± 1(4)	77 ± 14(3)	8
4	NH	CH_2	842 ± 117(3)	78 ± 12(3)	0.09
7	CH_2	NH	14 ± 1(3)	454 ± 27(3)	32

[a] Inhibition of response to an agonist (glutamate (100 μM) and glycine (100 μM)) in oocytes expressing heteromeric NR1/NR2A receptors at a holding potential of −80 mV. [b] Inhibition of response to an agonist (glutamate (100 μM)) in oocytes expressing homomeric GluR1 flip receptors at a holding potential −80 mV. Values are the mean inhibition ± SEM. The number of replicates is indicated in parentheses. [c] IC_{50}-(AMPA)/IC_{50}(NMDA).

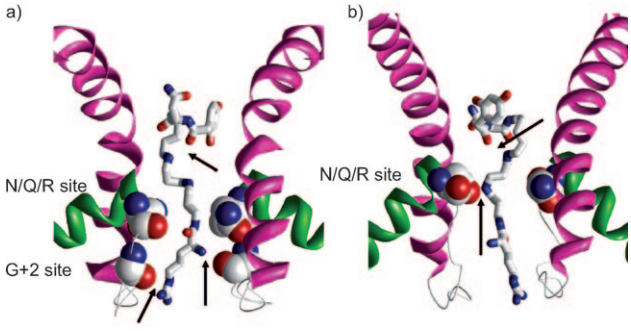


Figure 3. Docking of ArgTX-636 (**1**) into the models of a) AMPA and b) NMDA receptor ion channels. The M2 and M3 helices are shown as green and magenta ribbons, respectively, and the N/Q/R site and G+2 site (in the model of the AMPA receptor ion channel) are space-filling models. Specific hydrogen bonds are indicated by arrows.

different in the AMPA and NMDA receptor ion channels: The glutamine side chain of the AMPA receptor is inaccessible to channel blockers, whereas the corresponding asparagine (N) side chain in the NMDA receptor ion channel faces the pore and provides opportunity for interaction (Figure 3B).[14,15] In addition to penetrating the N/Q/R site, the terminal amino group probably binds to a backbone carbonyl group of the so-called G + 2 site, a glycine residue located two residues downstream from the N/Q/R site. The G + 2 site is crucial for AMPA receptor blockade (Figure 3).[13,15,16]

Compounds **1**, **4**, and **7** differ only in the presence or absence of secondary amino groups in positions X and Y (Scheme 3 and Table 1). Therefore, we focused on the interactions of these groups in the ion-channel models. Notable differences were observed that may contribute to changes in selectivity. First, the three-dimensional (3D) structure of the aryl head group is dependent on the functional group in position X. When a secondary amine is present, as in **1** and **4**, intramolecular hydrogen bonds form between the amine and the carbonyl oxygen atoms on the head group. In contrast, substitution with a methylene group, as in **7**, shifts hydrogen bonding to the amine in position Y and leads to enlargement of the head group. This effect reduces the ability of the polyamine tail to reach past the selectivity filter into the channel pore. Thus, interactions between the terminal amino groups of the tail and the G + 2 site—important interactions for AMPA receptor affinity—are compromised (Figure 3A). In contrast, the decrease in pore penetration of the tail does not affect the key interaction between the tail and the NMDA receptor channel: hydrogen-bond formation between the asparagine side chain at the N/Q/R site and the amino group at position Y. Hence, **7** and **1** have a similar affinity for the NMDA receptor subtype.

The models also show that substitution of the secondary amine at position Y with methylene, as in **4**, leads to loss of the important hydrogen-bonding interaction between this position and the asparagine residue at the N/Q/R site of the NMDA receptor channel. This hydrogen bond is not present in the AMPA receptor model and appears to be the primary determinant for the 8-fold decrease in the affinity of NMDA receptors for **4** relative to their affinity for **1** and **7**.

Thus, high-affinity binding of ArgTX-636 and analogues to NMDA receptors is mainly determined by the presence of the secondary amino group in position Y, which is probably involved in a direct interaction with an asparagine residue in the N/Q/R site. On the other hand, AMPA receptor affinity is primarily determined by binding of the terminal amine group to the G + 2 site. This interaction is only possible when the secondary amino group in position X is also present.

In conclusion, we have developed an efficient solid-phase synthesis of the polyamine toxin ArgTX-636 (**1**) by using a BAL linker. The versatility of the procedure was demonstrated by the synthesis of analogues of ArgTX-636. Variations were introduced in the polyamine chain and the guanidine group; however, the synthetic procedure enables the modification of other parts of the molecule. Characterization of the affinity of AMPA and NMDA receptor subtypes of the iGluR family for compounds **1**–**9** demonstrated the general importance of the guanidine group for iGlu receptor affinity. Three analogues with subtle variations in the polyamine moiety were further characterized. A remarkable shift in selectivity was revealed that depended on the modification of the secondary amino groups. Whereas the native toxin, ArgTX-636 (**1**), was almost equipotent at the two receptor subtypes, changes in the amino groups led to significant differences in the selectivity profile of the resulting analogues, **4** and **7**, which are potent and selective inhibitors of AMPA and NMDA receptors, respectively. The structural principles underlying the effects of these subtle changes in toxin structure were investigated by the docking of compounds **1**, **4**, and **7** into models of the NMDA and AMPA receptor ion channels, and a rationale was established as to how the composition of the polyamine tail of ArgTX derivatives can determine selectivity for a particular iGluR subtype. The results should be useful for tailoring future compounds with a fine-tuned preference for certain iGluR species.

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[1] a) R. Dingledine, K. Borges, D. Bowie, S. F. Traynelis, *Pharmacol. Rev.* **1999**, *51*, 7; b) M. L. Mayer, N. Armstrong, *Annu. Rev. Physiol.* **2004**, *66*, 161; c) P. S. Pinheiro, C. Mulle, *Nat. Rev. Neurosci.* **2008**, *9*, 423.

[2] a) H. Bräuner-Osborne, J. Egebjerg, E. Ø. Nielsen, U. Madsen, P. Krogsgaard-Larsen, *J. Med. Chem.* **2000**, *43*, 2609; b) R. Planells-Cases, J. Lerma, A. Ferrer-Montiel, *Curr. Pharm. Des.* **2006**, *12*, 3583.

[3] a) S. A. Lipton, *Nat. Rev. Neurosci.* **2007**, *8*, 803; b) K. M. Cosman, L. L. Boyle, A. P. Porsteinsson, *Expert Opin. Pharmacother.* **2007**, *8*, 203.

[4] a) A. L. Mueller, R. Roeloffs, H. Jackson in *The Alkaloids: Chemistry and Pharmacology*, *Vol. 46* (Ed.: G. A. Cordell), Academic Press, San Diego, **1995**, pp. 63–94; b) K. Strømgaard, I. R. Mellor, *Med. Res. Rev.* **2004**, *24*, 589; c) I. R. Mellor, P. N. R. Usherwood, *Toxicon* **2004**, *43*, 493.

[5] E. Grishin, T. Volkova, A. Arseniev, O. Reshetova, V. Onoprienko, L. Magazanic, S. Antonov, I. Fedorova, *Bioorg. Khim.* **1986**, *12*, 1121.

[6] a) A. Green, K. Nakanishi, P. Usherwood, *Brain Res.* **1996**, *717*, 135; b) B. C. Albensi, N. Alasti, A. L. Mueller, *J. Neurosci. Res.* **2000**, *62*, 177.

[7] a) T. L. Shih, J. Ruiz-Sanchez, H. Mrozik, *Tetrahedron Lett.* **1987**, *28*, 6015; b) J. V. Jasys, P. R. Kelbaugh, D. M. Nason, D. Phillips, N. A. Saccomano, R. A. Volkmann, *Tetrahedron Lett.* **1988**, *29*, 6223; c) I. Blagbrough, E. Moya, *Tetrahedron Lett.* **1995**, *36*, 9393.

[8] a) K. Strømgaard, K. Andersen, T. Ruhland, P. Krogsgaard-Larsen, J. W. Jaroszewski, *Synthesis* **2001**, 877; b) H. Kromann, S. Krikstolaityte, A. J. Andersen, K. Andersen, P. Krogsgaard-Larsen, J. W. Jaroszewski, J. Egebjerg, K. Strømgaard, *J. Med. Chem.* **2002**, *45*, 5745.

[9] K. J. Jensen, J. Alsina, M. F. Songster, J. Vagner, F. Albericio, G. Barany, *J. Am. Chem. Soc.* **1998**, *120*, 5441.

[10] During the course of this study, the BAL resin was used for the synthesis of other polyamine toxins: C. A. Olsen, M. Witt, H. Franzyk, J. W. Jaroszewski, *Tetrahedron Lett.* **2007**, *48*, 405.

[11] a) T. Fukuyama, C.-K. Jow, M. Cheung, *Tetrahedron Lett.* **1995**, *36*, 6373; b) C. A. Olsen, H. Franzyk, J. W. Jaroszewski, *Synthesis* **2005**, 2631; c) F. Hahn, U. Schepers in *Combinatorial Chemistry on Solid Supports*, *Vol. 278* (Ed. S. Bräse), Springer, Berlin **2007**, pp. 135 – 208.
[12] S. C. Vigil-Cruz, J. V. Aldrich, *Lett. Pept. Sci.* **1999**, *6*, 71.
[13] D. B. Tikhonov, *Mol. Membr. Biol.* **2007**, 24, 135.
[14] D. B. Tikhonov, L. G. Magazanik, I. R. Mellor, P. N. R. Usherwood, *Recept. Channels* **2000**, *7*, 227.
[15] a) L. Jin, H. Sugiyama, M. Takigawa, D. Katagiri, H. Tomitori, K. Nishimura, N. Kaur, O. Phanstiel IV, M. Kitajima, H. Takayama, T. Okawara, K. Williams, K. Kashiwagi, K. Igarashi, *J. Pharmacol. Exp. Ther.* **2007**, *320*, 47; b) K. Kashiwagi, K. Williams, K. Igarashi, *Amino Acids* **2007**, *33*, 299.
[16] a) D. B. Tikhonov, I. R. Mellor, P. N. R. Usherwood, L. G. Magazanik, *Biophys. J.* **2002**, *82*, 1884; b) T. F. Andersen, D. B. Tikhonov, U. Bølcho, K. Bolshakov, J. K. Nelson, F. Pluteanu, I. R. Mellor, J. Egebjerg, K. Strømgaard, *J. Med. Chem.* **2006**, *49*, 5414.

