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Bioorganic & Medicinal Chemistry 13 (2005) 5104–5112
Bioorganic & Medicinal Chemistry

Design and synthesis of labeled analogs of PhTX-56, a potent and selective AMPA receptor antagonist

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Received 29 March 2005; revised 19 May 2005; accepted 20 May 2005
Available online 28 June 2005

Dedicated to Professor Koji Nakanishi on receipt of the 2004 Tetrahedron Prize.

Abstract—Polyamines and polyamine toxins are biologically important molecules, having modulatory effects on nucleotides and proteins. The wasp toxin, philanthotoxin-433 (PhTX-433), is a non-selective and uncompetitive antagonist of ionotropic receptors, such as ionotropic glutamate receptors and nicotinic acetylcholine receptors. Polyamine toxins are used for the characterization of subtypes of ionotropic glutamate receptors, the Ca^{2+}-permeable AMPA and kainate receptors. A derivative of the native polyamine toxin, philanthotoxin-56 (PhTX-56), has recently been shown to be an exceptionally potent and selective antagonist of Ca^{2+}-permeable AMPA receptors. PhTX-56 and its labeled derivatives are promising tools for structure–function studies of the ion channel of the AMPA receptor. We now describe the design and synthesis of ^{3}H-, ^{13}C-, and ^{15}N-labeled derivatives of PhTX-56 for molecular level studies of AMPA receptors. [^{3}H]PhTX-56 was prepared from a diiodo-precursor with high specific radioactivity, providing the first radiolabeled ligand binding to the pore-forming part of AMPA receptors. For advanced biological NMR studies, ^{13}C and ^{15}N-labeled PhTX-56 were synthesized using solid-phase synthesis. These analogs can provide detailed information on the ligand–receptor interaction. In conclusion, synthesis of labeled derivatives of PhTX-56 provides important tools for future studies of the pore-forming region of AMPA receptors.

1. Introduction

Endogenous polyamines are present in a vast majority of cells. They play important roles in the synthesis of proteins, cell division, and differentiation, and bind to nucleic acids resulting in their condensation, thereby affecting gene expression.[1] Endogenous polyamines and polyamine derivatives also bind and affect several important ion channel proteins, such as potassium channels and ionotropic glutamate (iGlu) receptors.[2]

The iGlu receptors are important mediators of fast synaptic transmission in the mammalian central nervous system (CNS) and are classified into three subtypes according to selective activation by the agonists, namely *N*-methyl-D-aspartate (NMDA), (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA), and kainic acid. The iGlu receptors are particularly interesting, as there is strong evidence to suggest that glutamate-induced excessive Ca^{2+} entry under pathophysiological conditions leads to a wide range of neurological insults including ischemia-induced brain damage, epileptic seizures, and chronic neurodegenerative disorders, such as Alzheimer's disease, as well as other neurological and psychiatric disorders.[3,4] Moreover, iGlu receptors are believed to be important for the basic cellular and molecular mechanism by which memories are formed and stored, as characterized by long-term potentiation (LTP)[5] and long-term depression (LTD).[6]

AMPA receptors are distributed ubiquitously throughout the CNS and mediate fast excitatory neurotransmission. The involvement of AMPA receptors in LTP and LTD, and the physical transport of AMPA receptors into and out of the synaptic membrane have only recently received widespread attention.[7] AMPA receptors are composed of four subunits designated GluR1–4, and the receptor is formed by a tetrameric assembly of these subunits.[3] They can be classified according to their Ca^{2+}

Keywords: Natural products; Polyamine toxins; Glutamate receptors; AMPA receptors.
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doi:10.1016/j.bmc.2005.05.023

permeability; in GluR1, GluR3, and GluR4 subunits, a glutamine (Q) residue is present at a site known as the Q/R site that is located in the pore-forming region of AMPA receptors (Fig. 2). This residue has been edited in virtually all GluR2 subunits to an arginine (R), which determines the Ca^{2+} permeability of AMPA receptors. Hence, Ca^{2+} permeability is inversely correlated to the relative abundance of the GluR2 subunit.[3] It has been suggested that Ca^{2+}-permeable AMPA receptors are important for the pathogenesis of neurological disorders.[8] Thus, the use of AMPA receptor antagonists is believed to be helpful in treating neurodegenerative disorders and/or reducing the amount of neurological damage associated with these disorders.[8,9]

A thorough understanding of the structure and function of iGlu receptors in general and AMPA receptors, in particular, is of major scientific interest and might have pathbreaking consequences for the development of therapeutics to treat neurological disorders. Gouaux and coworkers have done a pioneering work in this field by solving the X-ray crystallographic structure of a construct of the extracellular ligand-binding domain.[10] This structure has been used to explain several important functions of an AMPA receptor, such as a mechanism for desensitization[11] and a structural basis for partial agonism.[12] On the other hand, the membrane-spanning (or pore) region of AMPA receptors, and iGlu receptors in general, is much less well-defined.[13] It is believed that iGlu receptors are structurally and evolutionarily related to K^+-channels, and the discovery of the GluR0 channel/receptor, a hybrid between K^+-channels and iGlu receptors, has substantiated this belief.[14] The current hypothesis suggests that the pore-regions of iGlu receptors are similar to inverted K^+-channels,[13b] but still properties, such as ion selectivity, are strikingly different. Currently, very little is known about this region and no concrete structural information is available. Therefore, pharmacological tools that can allude to some of these issues are of major interest.

Polyamine toxins form a class of low molecular weight compounds, isolated from the venom of spiders and wasps, which are non-selective antagonists of ionotropic receptors, such as iGlu and nicotinic acetylcholine (nACh) receptors.[15] Polyamine toxins are uncompetitive antagonists, that is, they require the activation of the receptor before antagonism can take place, probably by binding to the pore-forming region of the receptor (Fig. 2). This mode of action is interesting from a drug discovery perspective, as recently an uncompetitive antagonist of NMDA receptors, memantine (Ebixa®), was introduced for treating patients with moderate-to-severe Alzheimer's disease.[16]

The polyamine wasp toxin philanthotoxin-433 [(*S*)-PhTX-433 (**1**)] (Fig. 1), isolated from the venom of a female digger wasp *Philanthus triangulum*, is a prototypical example.[17] Polyamine toxin **1** and its synthetic analog (*S*)-PhTX-343 (**2**) are antagonists of Ca^{2+}-permeable AMPA and kainate receptors, which make them useful pharmacological tools for determination of the subunit composition of these receptors.[18] The potential of polyamine toxins, as drug candidates, has so far been considered to be limited due to the non-specific action on other ionotropic receptors. But, recently, it has been demonstrated that modification of the polyamine portion of PhTX-343 (**2**) led to selective antagonism on various classes of ionotropic receptors.[19] Removal of one of the inner basic sites in PhTX-343 (**2**) led to an enhanced potency at AMPA receptors, whereas the activity at other ionotropic receptors remained low.[19c,d] The most striking example was that of philanthotoxin-56 [(*S*)-PhTX-56 (**3**)] (Fig. 1), which inhibited AMPA receptors that were composed of GluR1 subunits with a K_i value of 3.3 ± 0.78 nM, thus being the most potent polyamine toxin analog ever described.[19d] PhTX-56 (**3**) showed selectivity for Ca^{2+}-permeable AMPA receptors, as it was more than 1000-fold less potent at GluR2-containing AMPA receptors. Quite remarkably, PhTX-56 (**3**) was also highly selective for AMPA, relative to kainate

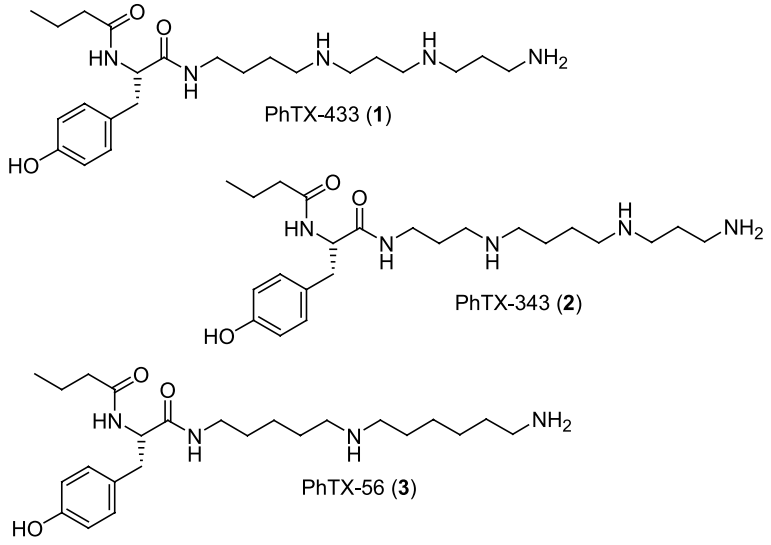


Figure 1. Structures of polyamine toxins. PhTX-433 (**1**) is the native philanthotoxin isolated from the wasp *P. triangulum*, while PhTX-343 (**2**) and PhTX-56 (**3**) are derivatives, the latter being a very potent and highly selective AMPA receptor antagonist.

receptors, as it was more than 500-fold less potent at kainate receptors.[19d]

PhTX-56 is therefore an excellent tool to study ligand–receptor interactions at the pore-forming part of AMPA receptors. To the best of our knowledge, no radiolabeled ligand exists that would allow binding studies with this region; hence, we wanted to provide the first radioligand that would allow such binding studies on the AMPA receptor. Furthermore, we are interested in employing advanced biological NMR spectroscopy to study ligand–receptor interactions at the molecular level, which require isotope labeling of PhTX-56 with either ^{13}C, ^{15}N, or both. Recently, one example of ^{15}N-labeling of polyamine toxin derivatives has appeared; Bienz and co-workers prepared ^{15}N-labeled acylpentamines, to be used as reference compounds to explain some unexpected observations during their mass spectrometric investigations of novel polyamine toxins.[20]

Previously, photolabeling studies using analogs of philanthotoxins have been carried out at the nicotinic acetylcholine (nACh) receptor, showing that the aromatic head-group of philanthotoxins binds to the lumen just above the pore, but specific interactions of the polyamine portion remain elusive (Fig. 2).[21] Recent studies have shown that the polyamine moiety is crucial for interaction with receptors, and this part of the molecule determines selectivity, and in many cases the potency.[19] Therefore, studying the interaction between the polyamine moiety of PhTX-56 (**3**) and the AMPA receptor is of major interest, but has so far not been possible.

Biological interest in polyamines and polyamine toxins has paved the way for an efficient synthesis of these compounds, including native polyamines and polyamine toxins, as well as their derivatives for the investigation of structure–activity relationship (SAR) studies.[15] The use of solid-phase synthesis has greatly facilitated the synthesis of these compounds, the major advantage being that purification of highly polar intermediates is avoided and the use of protecting groups is reduced.[15a,b] Generally, two approaches have been used for the synthesis of polyamines on solid phase, either by reductive amination or by alkylation. The latter strategy has extensively adopted the Fukuyama amination, where an amine is protected and activated as a nitrobenzene sulfonamide, which is subsequently alkylated either by conventional alkylation or by a Mitsunobu reaction.[15a,b]

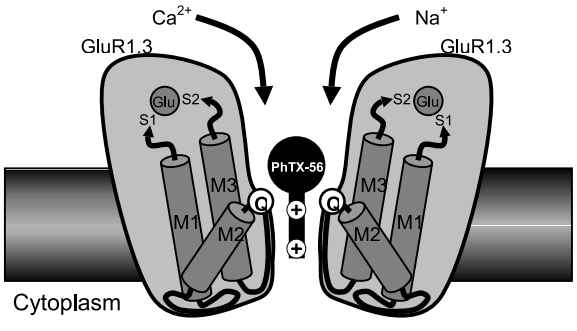


Figure 2. Suggested mode of binding of PhTX-56 (**3**) to the pore-forming region of AMPA receptors. Polyamine toxins, such as PhTX-56 (**3**), are believed to antagonize ionotropic receptors by binding to the pore-forming region, inside the ion channel. Antagonism of AMPA receptors only takes place when receptors are composed of GluR1, 3, and 4 subunits, whereas block is prevented if the GluR2 subunit is present. This selectivity is explained by editing of the Q/R site; in GluR1, 3, and 4 subunits glutamine (Q) resides, whereas virtually all GluR2 subunits are edited and contain an arginine (R). Modified from Ref. 10b.

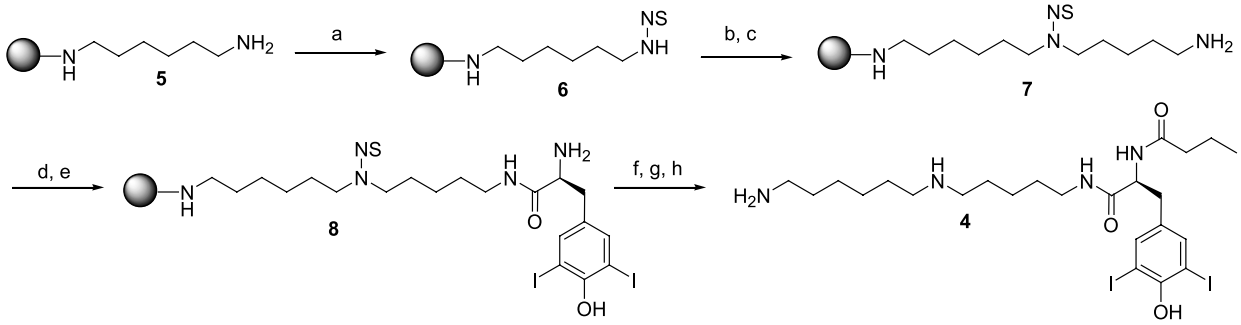
We have previously shown how a small library of polyamine toxins could be synthesized by a sequential strategy using the Fukuyama amination under Mitsunobu conditions,[22] and the methodology has since been used for the synthesis of several philanthotoxin analogs.[19d] Recently, a slightly modified version was presented using N-protected aminoalkyl halides, rather than amino alcohols, as a more convenient reaction sequence.[23] Here, we describe the synthesis of a number of labeled PhTX-56 derivatives, which, in general, were synthesized by a solid-phase synthesis strategy.

2. Results

Two principally different labeling strategies were carried out, which in both cases left the native structure of PhTX-56 unchanged; only the isotope labeling was changed. In one case, a ^{3}H-labeled version of PhTX-56 was synthesized by preparing a precursor on solid phase, which was subsequently hydrogenated using tritium gas. The other strategy involved labeling the polyamine moiety of PhTX-56 with ^{13}C and ^{15}N, which could be carried out by attaching labeled precursors to a solid phase, and carrying out solid phase synthesis, as previously described, providing ^{13}C- and ^{15}N-labeled PhTX-56.

2.1. [^{3}H]PhTX-56

For the design and synthesis of a tritiated version of PhTX-56, it was quite clear where to incorporate the tritium label; one of the most frequently adopted methods for the labeling of peptides is to convert a diiodo-tyrosine precursor into a tritium-labeled tyrosine by simple hydrogenation. Therefore, we decided to prepare diiodo-PhTX-56 (**4**), as shown in Scheme 1. The polyamine portion was synthesized, as outlined in Scheme 1, and as previously described;[19d,22] a resin-bound diamine (**5**) was reacted with *o*-nitrobenzenesulfonyl chloride to give the corresponding *o*-nitrobenzene sulfonamide **6**. This was reacted with a protected amino alcohol in a modified Mitsunobu reaction, and the protecting group was removed by treatment with tetrabutyl ammonium fluoride (TBAF) to give the resin-bound polyamine precursor (**7**). The crucial step was the coupling with *N*-Fmoc 3,5-diiodo-tyrosine, as the tyrosine derivatives are generally protected at the phenol group with a *tert*-butyl group. However, it was believed that steric hindrance induced by the two iodo-atoms, as well as the ability of *N*-[(dimethylamino)-1*H*-1,2,3-triazol[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) to be less prone to reaction with phenolic groups, would be



Scheme 1. Reagents: (a) *o*-nitrobenzenesulfonyl chloride, DIEA; (b) 3-[*N*-[2-(trimethylsilyl)ethoxycarbonyl]amino]pentanol, Bu_3P, ADDP; (c) TBAF; (d) (*S*)-*N*-Fmoc-*O*-(*tert*-butyl)tyrosine, HATU, collidine; (e) 20% piperidine in DMF; (f) butyric acid, HATU, collidine; (g) $HSCH_2CH_2OH$, DBU; (h) TFA, triisopropylsilane, CH_2Cl_2, H_2O.

sufficient for a hasslefree reaction, and this turned out to be true. Subsequently, the Fmoc group was removed to give **8**, which was reacted with butyric acid, followed by the removal of the 2-nitrobenzene sulfonamide (NS) group. Finally, the compound was cleaved from the resin to give the desired diiodo-PhTX-56 (**4**). The crude product was purified by preparative HPLC-MS, to give diiodo-PhTX-56 (**4**) in 35% yield.

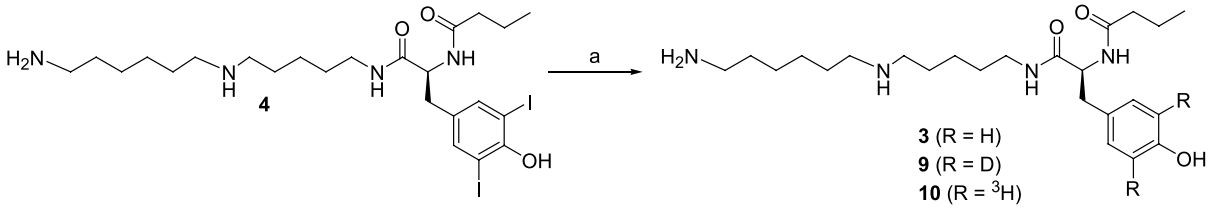
With **4** in hand, conditions for reduction were investigated; compound **4** was dissolved in DMF, Pd/C was added, and the reaction mixture was stirred for 20 h under an atmosphere of hydrogen providing PhTX-56 (**3**). The same synthetic procedure was repeated, but using deuterium, instead of hydrogen, gas, providing deuterated PhTX-56 (**9**) (Scheme 2). The synthesis of compound **9** confirmed the feasibility of the procedure and showed that the iodine–deuterium exchange was quantitative. Both PhTX-56 (**3**) and **9** were purified by preparative HPLC-MS and were obtained in good yield and high purity (>99% by UV).

For the preparation of tritium-labeled PhTX-56 and [^{3}H]PhTX-56 (**10**), similar reaction conditions were applied; a mixture of diiodo-precursor **4**, diisopropylamine (DIPA), and Pd/C in DMF was frozen at −80 °C and evacuated three times. Tritium gas was then introduced at atmospheric pressure, and the reaction mixture was stirred overnight, which provided the crude product. Following purification by preparative HPLC, [^{3}H]PhTX-56 (**10**) was obtained in high radioactive purity (>99.5%) and with high specific activity (>50 Ci/mmol).[24]

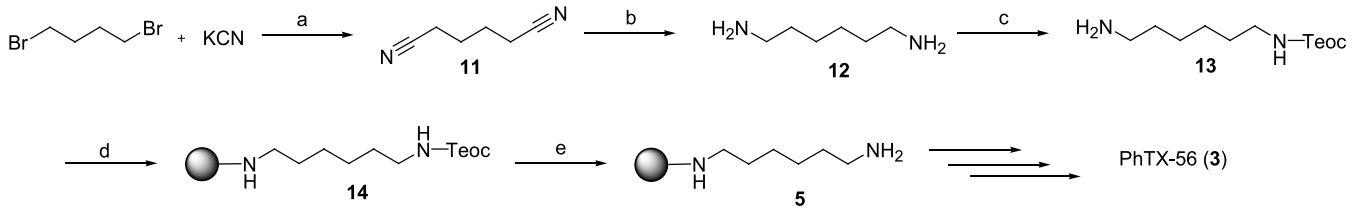
2.2. $^{13}C_2$, $^{15}N_2$, and $^{13}C_2$,$^{15}N_2$-labeled PhTX-56

In the design of ^{13}C- and ^{15}N-labeled derivatives of PhTX-56 for advanced biological NMR studies, it was important that the labels be placed in the polyamine portion of the molecule since recent studies have shown that the polyamine portion determines the selectivity and, to a certain extent, the potency of philanthotoxins.[19] Moreover, studies with photolabile PhTX-343 derivatives of nACh receptors have indicated that the aromatic head-group was located at the outer mouth of the ion channel.[21] Hence, the head-group probably interacts with the receptor in a non-specific manner, but plays an important role in anchoring the molecule to the receptor.

We wanted to incorporate the labels into the polyamine moiety and envisaged that a synthetic approach similar to that described above would be feasible. Since 1,6-diaminohexane is commercially available with both $^{13}C_2$-



Scheme 2. Reagents: (a) Pd/C, hydrogen, deuterium or tritium gas.



Scheme 3. Reagents: (a) 18-crown-6; (b) $LiAlH_4$; (c) 2-(trimethylsilyl)-ethyl 4-nitrophenyl carbonate, Et_3N; (d) trityl chloride resin, DIEA; (e) TBAF.

and $^{15}N_2$-labels, we have used them. However, as an alternative approach we employed [^{13}C], [^{15}N], and [^{13}C,^{15}N]potassium cyanide, which is much cheaper, as outlined in Scheme 3. Starting from 1,4-dibromobutane and potassium cyanide, 1,4-dicyanobutane (**11**) was prepared in high yield by refluxing in acetonitrile in the presence of 18-crown-6, and **11** was reduced to 1,6-diaminohexane (**12**) by treatment with $LiAlH_4$.

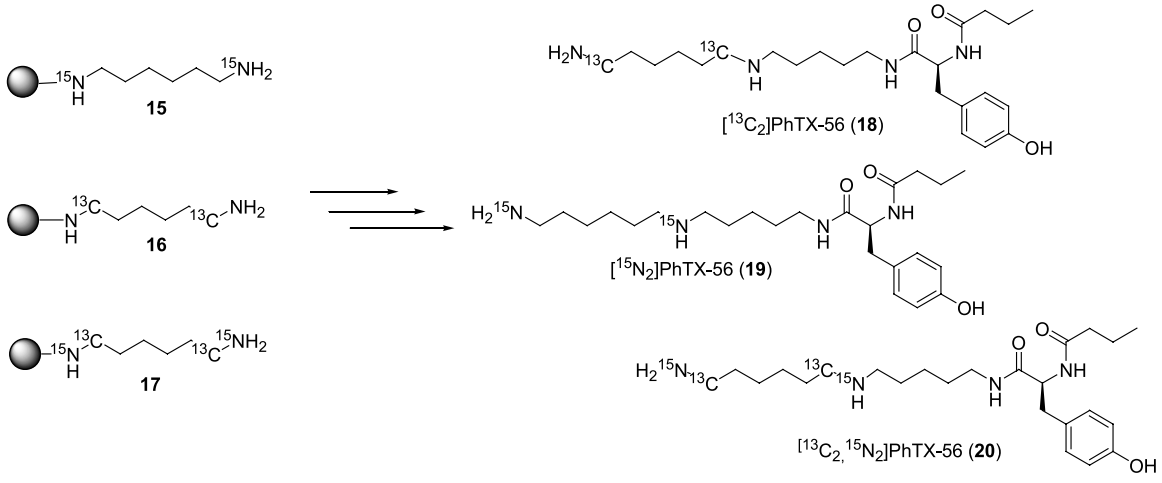
Unprotected diamines are normally reacted with trityl chloride resin, using a 10-fold excess of the diamine however; in this case we were interested in minimizing the use of diamines. We investigated the efficiency of loading 1,6-diaminohexane to the resin, by comparing the reaction with the trityl chloride resin with either 5 or 10 equivalents of 1,6-diaminohexane or 1 equivalent of 1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane. The latter was treated with TBAF, and all three resins were reacted with butyric acid in the presence of HATU and collidine. The products were cleaved from the resins and were subsequently analyzed by HPLC-MS, which showed that the yields using either 5 or 10 equivalents of 1,6-diaminohexane or 1 equivalent of 1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane were 40%, 68%, and 87%, respectively. This underlines the increased efficiency by using mono-protected diaminohexane; hence, the diamines were mono-protected by the reaction with 2-(trimethylsilyl)-ethyl 4-nitrophenyl carbonate to give 1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane (**13**). The latter compound was reacted with trityl chloride resin to give **14** and subsequently treated with TBAF to provide the resin-bound 1,6-diaminohexane (**5**).

In this manner, the three starting resins, **15**, **16**, and **17** labeled with $^{13}C_2$, $^{15}N_2$, and $^{13}C_2$,$^{15}N_2$, respectively, were prepared (Scheme 4). The synthesis of [$^{13}C_2$]PhTX-56 (**18**), [$^{15}N_2$]PhTX-56 (**19**), and [$^{13}C_2$,$^{15}N_2$]PhTX-56 (**20**) was then carried out using a methodology which is similar to that described for the synthesis of diiodo-PhTX-56 (**4**). The crude products were purified by preparative HPLC-MS, and the target molecules were achieved in yields of 32%, 29%, and 35%, respectively for the eight-step sequences.

3. Discussion

In the present work, we have prepared a range of labeled derivatives of PhTX-56 (**3**), which itself is an interesting pharmacological tool, as it is believed to bind to the pore-forming region of AMPA receptors (Fig. 2). It is highly sensitive to editing at the Q/R site and does not antagonize AMPA receptors with arginine in this position, that is, GluR2-containing receptors. PhTX-56 (**3**) binds with very high affinity ($K_i = 3.3 \pm 0.78$ nM) and has a unique selectivity, as it is 500-fold more potent at AMPA than at kainate, receptors. There are a number of possibilities when labeling PhTX-56; the classical approach is to introduce a radiolabel, such as ^{3}H; alternately, ^{13}C- and ^{15}N-labels can be introduced for biological NMR studies. In both cases, the labels can be introduced without changing the chemical structure of PhTX-56 (**3**). The introduction of fluorescent labels is also of major interest and has been performed (L. S. Jensen and K. Strømgaard, unpublished), but this obviously changes the parent structure and requires verification of the biological activity of the fluorescent analogs.

A tritiated version of PhTX-56 was obtained by treatment of a diiodo-precursor (**4**) with tritium gas, yielding [^{3}H]PhTX-56 (**10**) in high radiochemical purity (>99.5%) and, most importantly, with high specific radioactivity (>50 Ci/mmol). [^{3}H]PhTX-56 (**10**) will be an important tool in binding studies with AMPA receptors, as this is the first radioligand to bind to the pore-forming region of AMPA receptors. In addition, [^{3}H]PhTX-56 (**10**) can provide information on the pharmacokinetic properties of this class of compounds, and most importantly, whether these compounds penetrate the blood–brain barrier (BBB) or not. This is of utmost importance to evaluate the therapeutic properties of these compounds, and previous studies do not seem to have clarified this point. Finally, [^{3}H]PhTX-56 (**10**) can be used in ex vivo autoradiographic studies, which will provide information on regional binding, as well as binding kinetics (K_d and B_{max}), and when compared with similar experiments using [^{3}H]AMPA, a measurement of the



Scheme 4. Synthesis of [$^{13}C_2$]PhTX-56 (**18**), [$^{15}N_2$]PhTX-56 (**19**), and [$^{13}C_2$,$^{15}N_2$]PhTX-56 (**20**). Resin-bound 1,6-diaminohexanes labeled with either [$^{13}C_2$], [$^{15}N_2$] or [$^{13}C_2$,$^{15}N_2$] were used as starting materials and the target molecules were synthesized according to the procedure shown in Scheme 1.

occurrences of Ca^{2+}-permeable AMPA receptors might be feasible.

Three different PhTX-56 analogs, which incorporated ^{13}C, ^{15}N, or both into the polyamine moiety, were designed. Increasing evidence shows that the polyamine moiety determines both selectivity and, to some extent, the potency, and previous studies show an almost non-selective interaction of the aromatic head-group. Therefore, it is of major interest to study the interaction of the polyamine portion of PhTX-56 with the pore-forming region of the AMPA receptor. Labeled diamine precursors, which were either commercially available or prepared in two steps, were bound to a solid phase and using previously developed solid phase synthetic methods, the three compounds [$^{13}C_2$]PhTX-56 (**18**), [$^{15}N_2$]PhTX-56 (**19**), and [$^{13}C_2$,$^{15}N_2$]PhTX-56 (**20**) were synthesized in good yield.

Recent advances in biological NMR spectroscopic techniques are allowing for molecular level studies of ligand–receptor interactions.[25] Although transmembrane proteins, such as AMPA receptors, are particularly difficult to study owing to their lipid-embedded domains, there are a few examples of how these obstacles could be overcome, for example by studying fragments of the receptor.[25c] The ^{13}C and ^{15}N ligands prepared in this study can be applied in a similar fashion, thereby providing detailed information of ligand–receptor interaction.

4. Conclusion

The polyamine toxin derivative, PhTX-56 (**3**), is an important tool for the study of the pore-forming region of AMPA receptors. Here, we have prepared a range of labeled analogs of PhTX-56 that allow for molecular level studies of ligand–receptor interactions, as well as a range of other studies pertaining to AMPA receptor structure and function.

[^{3}H]PhTX-56 (**10**) was prepared in high radiochemical purity and high specific activity, and results from studies employing **10** will be reported in due course. Three analogs of PhTX-56, where ^{13}C- and ^{15}N-labels were incorporated into the polyamine moiety, as in compounds **18**, **19**, and **20**, were also prepared; these compounds are useful probes to study ligand–receptor interactions by means of biological NMR spectroscopy.

5. Experimental

Resin-bound diamines (trityl chloride resin, 1% divinylbenzene, 200–400 mesh) and (*S*)-Fmoc-*O*-(*tert*-butyl)tyrosine were obtained from Novabiochem (Läufelingen, Switzerland). (*S*)-Fmoc-tyrosine(3,5-I_2)-OH was obtained from Advanced ChemTech (Louisville, KY, USA). All other starting materials were obtained commercially from Aldrich or Fluka. All reactions on solid phase were carried out using Bohdan MiniBlocks from Mettler-Toledo (Columbus, OH, USA). All starting materials and solvents were used without further purification, except for DMF, which was stored over 3 Å molecular sieves, and THF, which was distilled under N_2 from Na/benzophenone. TLC was carried out on Merck Kieselgel 60 F_{254} aluminum sheets. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury spectrometer at 300 MHz or on a Varian Gemini 200 BB at 300 MHz, using $CDCl_3$ or CD_3OD as solvents. Chemical shifts are reported in ppm (δ). Coupling constants (J) are given in Hz. Multiplicities of ^1H NMR signals are given as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; dt, doublet of triplets; dm, doublet of multiplets; q, quartet; p, pentet; m, multiplet. Analytical and preparative high-performance liquid chromatography (HPLC-MS) was performed on a Sciex API150ex instrument equipped with Atmospheric Pressure Chemical Ionization (APCI) ion source. The HPLC system consisted of two Shimadzu LC10ADvp pumps. UV trace was obtained with a Gilson UV/VIS 155 UV detector operating at 254 nm. Evaporative light scattering (ELS) trace was obtained with Sedere Sedex 55 Light Scattering Detector and was used for estimation of the purity of the final products. Analytical HPLC-MS was performed on a 50×4.6 mm YMC RP18 column, using 2 mL/min of a mixture of A: water with 0.05% TFA and B: acetonitrile with 5% water and 0.035% TFA. Preparative HPLC-MS (split-flow MS detection) was run with 500 μL injections (20 mg samples in 1.0 mL DMSO) to a Phenomenex Synergi Hydro 4 μm, 21.20×50 mm column eluted with the same solvent gradient at 22.7 mL/min. Accurate mass determination was performed on a JEOL JMS-HX110/100A HF mass spectrometer using a 3-nitrobenzyl alcohol (NBA) matrix and Xe ionizing gas, all being within ±5 ppm of theoretical values.

5.1. 1,4-Dicyanobutane (11)

To a solution of 1,4-dibromobutane (2.16 g, 10 mmol) and 18-crown-6 (0.22 g, 0.83 mmol) in dry acetonitrile (5 mL) was added dry KCN (2.61 g, 40 mmol). The mixture was refluxed for 15 h with vigorous magnetic stirring. After the addition of 15 mL water, the mixture was extracted three times with CH_2Cl_2 (5 mL). The combined extracts were dried ($MgSO_4$) and concentrated in vacuo to give a colorless oil, which was purified by silica gel column chromatography (EtOAC/Pet. ether 1:5 → 1:2) to give a colorless oil (1.06 g, 96%); ^1H and ^{13}C NMR as previously reported.[26]

5.1.1. [$^{13}C_2$]1,4-Dicyanobutane (11a). Yield: 392 mg, 95%; ^1H NMR ($CDCl_3$) δ 1.81–1.90 (m, 4H), 2.42–2.52 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 15.6, 16.3, 23.7 (2C), 118.6 (2C).

5.1.2. [$^{13}C_2$,$^{15}N_2$]1,4-Dicyanobutane (11b). Yield: 343 mg, 82%; ^1H NMR ($CDCl_3$) δ 1.75–1.98 (m, 4H), 2.41–2.62 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 16.6, 17.4, 24.8 (2C), 119.6 (2C).

5.2. 1,6-Diaminohexane (12)

To a suspension of lithium aluminum hydride (0.19 g, 5 mmol) in dry diethyl ether (10 mL) under nitrogen at

0 °C was added a solution of 1,4-dicyanobutane (**11**, 0.22 g, 2 mmol) in dry diethyl ether (7 mL). The mixture was heated under reflux for 1 h and then stirred at room temperature for 24 h. Water (1 mL) was then added to hydrolyze the remaining lithium aluminum hydride. The mixture was filtered and the granular solid was washed several times with diethyl ether and CH_2Cl_2. The organic layers were evaporated to give yellow crystals (163 mg, 70%), which were used without further purification. ^1H and ^{13}C NMR as previously reported.[27]

5.2.1. [$^{13}C_2$]1,6-Diaminohexane (12a). Yield: 67 mg, 67%; ^1H NMR ($CDCl_3$) δ 1.11–1.85 (m, 8H), 2.67 (dt, J = 132.9/6.8 Hz, 4H).

5.2.2. [$^{13}C_2$,$^{15}N_2$]1,6-Diaminohexane (12b). Yield: 133 mg, 36%; ^1H NMR ($CDCl_3$) δ 1.05–1.79 (m, 8H), 2.68 (dt, J = 133.0/6.6 Hz, 4H).

5.3. 1-*N*-(Trimethylsilyl)ethoxycarbonyl-6-aminohexane (13)

To a solution of 1,6-diaminohexane (**12**, 0.511 g, 5 mmol) in CH_2Cl_2 (8 mL) were added triethylamine (1.4 mL, 10 mmol) and a solution of 2-(trimethylsilyl)-ethyl 4-nitrophenyl carbonate (1.56 g, 5.5 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred overnight at room temperature. The solvent was evaporated in vacuo and the resulting yellow oil was redissolved in CH_2Cl_2 and washed several times with saturated NaCl, saturated $NaHCO_3$, and 2 M NaOH until the organic phase was only weakly yellow. The organic phase was dried ($MgSO_4$) and concentrated in vacuo to give yellow crystals (1.06 g, 86%), which were used without further purification. ^1H $CDCl_3$: δ 0.02 [s, $Si(CH_3)_3$], 0.91 (t, J = 8.5 Hz, $SiCH_2$), 1.24–1.32 (m, 4H), 1.49–1.62 (m, 4H), 2.60 (t, J = 6.9 Hz, 2H), 3.14 (t, J = 7.1 Hz, 2H), 4.11 (t, J = 7.9 Hz, OCO–CH_2), 4.31 (br s, NH).

5.3.1. [$^{13}C_2$]1-*N*-(Trimethylsilyl)ethoxycarbonyl-6-aminohexane (13a). Yield: 130 mg, 87%; ^1H NMR ($CDCl_3$) δ 0.04 [s, $Si(CH_3)_3$], 0.97 (t, J = 8.5 Hz, $SiCH_2$), 1.27–1.39 (m, 4H), 1.43–1.67 (m, 4H), 2.69 (qd, J = 5.8/132.1 Hz, 2H), 3.14 (qd, J = 6.1/136.7 Hz, 2H), 4.13 (t, J = 8.2 Hz, OCO–CH_2), 4.59 (br s, NH).

5.3.2. [$^{15}N_2$]1-*N*-(Trimethylsilyl)ethoxycarbonyl-6-aminohexane (13b). Yield: 215 mg, 64%; ^1H NMR ($CDCl_3$) δ 0.04 [s, $Si(CH_3)_3$], 0.97 (t, J = 8.5 Hz, $SiCH_2$), 1.28–1.55 (m, 8H), 2.67 (t, J = 6.8 Hz, 2H), 3.05–3.21 (m, 2H), 4.13 (t, J = 8.2 Hz, OCO–CH_2), 4.61 (dm, J = 90 Hz).

5.3.3. [$^{13}C_2$,$^{15}N_2$]1-*N*-(Trimethylsilyl)ethoxycarbonyl-6-aminohexane (13c). Yield: 144 mg, 27%; ^1H NMR ($CDCl_3$) δ 0.01 [s, $Si(CH_3)_3$], 0.94 (t, J = 8.4 Hz, $SiCH_2$), 1.17–1.56 (m, 8H), 2.64 (d, J 135.1 Hz, 2H), 3.20 (dm, J = 137.0 Hz, 2H), 4.10 (t, J = 8.3 Hz, OCO–CH_2), 4.71 (dm, J = 90 Hz).

5.4. Synthesis of resin-bound 1,6-diaminohexane (5)

To a suspension of trityl chloride resin (0.2 mmol, 125 mg loading 1.6 mmol/g) and Et_3N (139 μL, 0.8 mmol) in DMF/CH_2Cl_2 (1:1) (4 mL) was added a solution of 1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane (**13**, 104 mg, 0.4 mmol) in DMF/CH_2Cl_2 (1:1) (4 mL). The reaction mixture was stirred at room temperature for 2 h. The resin was quenched with MeOH (4 mL), drained, washed with DMF (3 × 5 mL), CH_2Cl_2 (3 × 5 mL), MeOH (3 × 5 mL), and CH_2Cl_2 (3 × 5 mL), and dried in vacuo to give resin-bound 1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane (**14**). The above resin was suspended in dry THF (5 mL) under nitrogen at 50 °C. A solution of TBAF (1.0 mL, 1 M in THF) was added slowly and the mixture was stirred at 50 °C for 30 min. The resin was drained, washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo to give **5**.

Similar reactions were carried out treating trityl chloride resin with [$^{13}C_2$]1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane (**13a**), [$^{15}N_2$]1-*N*-(trimethylsilyl)ethoxycarbonyl-6-aminohexane (**13b**), and [$^{13}C_2$,$^{15}N_2$]1-*N*-(trimethylsilyl) ethoxycarbonyl-6-aminohexane (**13c**), respectively, to give resin-bound [$^{13}C_2$]1,6-diaminohexane (**5a**), [$^{15}N_2$]1,6-diaminohexane (**5b**), and [$^{13}C_2$, $^{15}N_2$]1,6-diaminohexane (**5c**), respectively, which were reacted according to the procedure described below.

5.5. Synthesis of PhTX-56 (3) and derivatives. General procedure

Resin-bound 1,6-diaminohexane (0.16 mmol) was suspended in CH_2Cl_2 (4 mL). Diisopropylethylamine (DIEA) (0.96 mmol) and *o*-nitrobenzenesulfonyl chloride (0.64 mmol) were added successively and the reaction mixture was stirred under nitrogen at room temperature for 3 h. The resin was drained, washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo. The above resin was suspended in dry THF/CH_2Cl_2 (1:1) (2 mL) under nitrogen. A solution of 3-[*N*-[2-(trimethylsilyl)ethoxycarbonyl]amino]pentanol, prepared as previously described,[12d] (0.8 mmol) in dry THF/CH_2Cl_2 (1:1) (1 mL), tributylphosphine (0.8 mmol), and a solution of 1,1′-(azadicarbonyl)-dipiperidine (ADDP) in dry THF/CH_2Cl_2 (1:1) (1 mL) were added successively. The reaction mixture was stirred at room temperature under nitrogen for 3 h. The resin was drained, washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo. The procedure was repeated two more times, and the resulting resin was dried in vacuo. The above resin was suspended in dry THF (5 mL) under nitrogen at 50 °C. A solution of TBAF (1 M in THF, 0.64 mmol) was added slowly, and the reaction mixture was stirred at 50 °C for 30 min. The resin was drained, washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo. A solution of an *N*-Fmoc-protected (*S*)-tyrosine derivative (0.48 mmol) and HATU (0.48 mmol) in DMF (1 mL), followed by a solution of collidine (0.72 mmol) in DMF (0.5 mL), was added to the resin. The reaction mixture was stirred at room temperature for 2 h, and the resin was subsequently washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2

(3 × 2.5 mL). The product was treated with 20% piperidine in DMF (v/v, 2 mL) and the reaction mixture was agitated for 3 min at room temperature. The resulting resin was washed with DMF (3 × 2.5 mL), treated again with 20% piperidine in DMF (v/v, 2 mL) for 20 min, and then washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo. A solution of butyric acid (0.48 mmol) and HATU (0.48 mmol) in DMF (1 mL), followed by a solution of collidine (0.72 mmol) in DMF (0.5 mL), was added to the resin. The mixture was agitated at room temperature for 2 h. The resin was drained, washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and dried in vacuo. The resin was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.6 mmol) in DMF (1 mL) and mercaptoethanol (0.6 mmol) in DMF (1 mL) for 30 min. The resin was drained and washed with DMF (5 × 2.5 mL). The procedure was repeated 3 more times. The resin was washed with DMF (3 × 2.5 mL), CH_2Cl_2 (3 × 2.5 mL), MeOH (3 × 2.5 mL), and CH_2Cl_2 (3 × 2.5 mL), and then treated with a solution of CH_2Cl_2/TFA/triisopropylsilane/H_2O (47.5:47.5:2.5:2.5 v/v, 2 mL) for 2 h. The resin was drained and washed with MeOH (2 mL) and CH_2Cl_2 (2 mL). The solutions of the cleaved product and the washings were combined and evaporated in vacuo to give a sticky solid, which was purified by preparative HPLC to give the final product as a yellowish oil.

5.5.1. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene-propaneamide bis(trifluoroacetate) (diiodo-PhTX-56, 4). Yield: 35%; ^{1}H NMR (CD_3OD) δ 0.86 (t, J = 7.4 Hz, 3H), 1.22–1.69 (m, 16H), 2.18 (t, J = 7.3 Hz, 2H), 2.78 (dd, J_{AB} = 13.6 Hz, J_{AX} = 8.0 Hz, 1H), 2.84–3.23 (m, 9H), 4.42 (t, J = 7.6 Hz, 1H), 7.61 (s, 2H); HRMS calcd for $C_{24}H_{41}I_2N_4O_3$ [M+1] 687.1268; found: 687.1249.

5.6. Synthesis of PhTX-56 (3) and D_2-PhTX-56 (9)

A dried, hydrogen- or deuterium-flushed 25 mL two-necked flask equipped with a balloon filled with either hydrogen or deuterium was charged with a suspension of Pd/C 10% (5 mg) in DMF (0.5 mL) and a solution of diiodo-PhTX-56 (**4**, 30 mg, 0.04 mmol) in DMF (1 mL). The mixture was stirred vigorously for 20 h. The reaction mixture was filtered over a patch of Celite and washed with EtOAc. The solvents were removed in vacuo and the resulting crude product was purified by preparative HPLC-MS.

5.6.1. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene-propaneamide bis(trifluoroacetate) (PhTX-56, 3). Yield: 35%; ^{1}H and ^{13}C NMR as previously reported.[19d]

5.6.2. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene(3,5-D_2)-propaneamide bis-(trifluoroacetate) (D_2-PhTX-56, 9). Yield: 33%; ^{1}H NMR (CD_3OD) δ 0.87 (t, J = 7 Hz, 3H), 1.35–1.60 (m, 16H), 2.17 (t, J = 7 Hz, 2H), 2.74–2.82 (m, 1H), 2.84–3.23 (m, 9H), 4.45 (t, J = 7 Hz, 1H), 7.04 (s, 2H).

5.7. Synthesis of [^{3}H]PhTX-56 (10)[24]

The reaction mixture containing **4** (10 mg, 14.6 μmol) in anhydrous DMF (0.8 mL), DIPA (10 μL, 57 μmol), and 10% palladium on charcoal (20 mg) was frozen at −80 °C and evacuated three times. Tritium gas was introduced at atmospheric pressure and the reaction mixture was stirred at room temperature overnight. After the removal of un-reacted tritium gas, the reaction mixture was filtered. The crude product was purified by preparative HPLC to give [^{3}H]PhTX-56. The HPLC conditions were as follows: Zorbax SB-C18 (4.5 × 250 mm) column, using a gradient, with A: water and 0.1% TFA, and B: acetonitrile, gradient: 5–50% in 20 min. Flow rate was 1 mL/min, with a radiodetector (^{3}Hβ-RAM) and UV detection at 235 nm. The radiochemical purity was 99.52% and the specific activity as determined by UV detection was found to be 50.2 Ci/mmol.

5.8. Synthesis of [$^{13}C_2$]PhTX-56 (18), [$^{15}N_2$]PhTX-56 (19), and [$^{13}C_2$,$^{15}N_2$]PhTX-56 (20)

The compounds were synthesized according to the general procedure using resin-bound 1,6-diaminohexane as starting materials. Resin-bound 1,6-diaminohexanes **15**, **16**, and **17** were used for the synthesis of **18**, **19**, and **20**, respectively (Scheme 4).

5.8.1. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene-propaneamide bis(trifluoroacetate) ([$^{13}C_2$]PhTX-56, 18). Yield: 32%; ^{1}H NMR (CD_3OD) δ 0.86 (t, J = 7.4 Hz, 3H), 1.18–1.31 (m, 2H), 1.37–1.77 (m, 14H), 2.17 (t, J = 7.1 Hz, 2H), 2.68–2.84 (m, 3H), 2.89–3.00 (m, 3H), 3.01–3.27 (m, 4H), 4.42 (t, J = 7.8 Hz, 1H), 6.69 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.5 Hz, 2H); HRMS calcd for $C_{22}{}^{13}C_2H_{43}N_4O_3$ [M+1] 437.6006; found: 437.6019.

5.8.2. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene-propaneamide bis(trifluoroacetate) ([$^{15}N_2$]PhTX-56, 19). Yield: 29%; ^{1}H NMR (CD_3OD) δ 0.86 (t, J = 7.3 Hz, 3H), 1.18–1.28 (m, 2H), 1.38–1.75 (m, 14H), 2.17 (t, J = 7.6 Hz, 2H), 2.79 (dd, J = 13.6/8.1, 1H), 2.80–3.22 (m, 9H), 4.43 (t, J = 7.9 Hz, 1H), 6.69 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.5 Hz, 2H); HRMS calcd for $C_{24}H_{43}N_2{}^{15}N_2O_3$ [M+1] 437.6021; found: 437.6029.

5.8.3. (*S*)-*N*-[5-[(6-Aminohexyl)amino]pentyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzene-propaneamide bis(trifluoroacetate) ([$^{13}C_2$,$^{15}N_2$]PhTX-56, 20). Yield: 35%; ^{1}H NMR δ 0.84 (t, J = 7.2 Hz, 3H), 1.14–1.28 (m, 2H), 1.31–1.80 (m, 14H), 2.21 (t, J = 6.9 Hz, 2H), 2.58–2.79 (m, 3H), 2.79–2.97 (m, 3H), 3.04–3.24 (m, 4H), 4.47 (t, J = 8.1 Hz, 1H), 6.63 (d, J = 8.1 Hz, 2H), 7.09 (d, J = 8.2 Hz, 2H); HRMS calcd for $C_{22}{}^{13}C_2H_{43}N_2{}^{15}N_2O_3$ [M+1] 439.3343; found: 439.3329.

Acknowledgments

Technical assistance of Ms. Dorthe Kroghave Toftdahl and Mr. Erik Damgård, Department of Spectroscopy,

Medicinal Chemistry Research, H. Lundbeck A/S (Valby, Denmark), is appreciated.

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