Synthesis and Applications of Polyamine Amino Acid Residues: Improving the Bioactivity of an Analgesic Neuropeptide, Neurotensin

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Abstract: Conjugated polyamines are potential carriers for biotherapeutics targeting the central nervous system. We describe an efficient synthesis of a polyamine-based amino acid, lysine-trimethylene(diNosyl)-spermine(triBoc) with Dde or Fmoc orthogonal protecting groups. This nonnatural amino acid was incorporated into a neurotensin analogue using standard Fmoc-based protocols. The analogue maintained high affinity and agonist potency for neurotensin receptors and exhibited dramatically improved analgesia in mice. Our work provides a basis for use of polyamine amino acids in polypeptides.

Polyamines and polyamine-based compounds are ubiquitous in nature; these compounds are also broadly applicable for drug delivery, therapeutics, and engineering nanomaterials. Polyamine-modified polypeptides exhibit a significant increase in penetration across the blood−nerve and blood−brain barriers (BBB[a]).[1,2] Our recent study showed that a combination of cationization and lipidization applied to truncated galanin analogues improved their brain penetration, yielding very potent anticonvulsant compounds.[3] Furthermore, cell penetrating peptides and peptidic vectors that transfer conjugated cargos into the nervous system contain a large number of cationic amino acid residues. Although the mechanism by which cationized peptides can penetrate biological membranes is not fully understood, several potential pathways, including internalization and adsorptive-mediated endocytosis (AME), have been discussed.[4] Despite a potential of improving penetration of bioactive peptides into the nervous system by cationization, no reports exist on employing polyamine-based amino acids into neuropeptides.

To investigate how the structural addition of polyamines to the active moiety could improve pharmacological properties of neuroactive peptides, we selected neurotensin (NT) as the model peptide. NT is an endogenous neuropeptide that produces potent analgesic activity mediated spinally and/or supraspinally by NT receptors.[5,6] Two subtypes of NT receptors, NTS1 and NTS2, have been demonstrated to mediate the various antinociceptive effects, including stress-induced antinociception, or analgesia in acetic acid writhing and hot plate tests.[5] The spinal NTS1 was suggested to play an important role in reducing inflammatory response in the formalin test, a model of persistent pain.[6] The naturally occurring NT analogue, contulakin-G, containing site-specific *O*-glycosylation, exhibited picomolar potency in a rat model of inflammatory pain following intrathecal injection.[7,8] Therefore, NT is a convenient model peptide for improving its central nervous system (CNS) bioavailability because NT analogues with improved BBB penetration might be expected to produce enhanced antinociceptive effects in animals.[9−11] Numerous strategies have been attempted to improve the BBB permeability of NT analogues. These include backbone and/or side chain modifications to cyclic and mimetic analogues.[10,12−17] One of the modified NT(8−13) analogues that penetrates the BBB, NT69L ((*N*-methyl-Arg)-Lys-Pro-(L-*neo*-Trp)-(*tert*-Leu)-Leu), is a potent analgesic and it appeared a very useful pharmacological tool to study the role of neurotensin receptors in the CNS.[6,11,18,19] In the research presented here, we synthesized and characterized pharmacological properties of an NT analogue containing lysine-$CH_2CH_2CH_2$-spermine, with the main hypothesis that the polyamine-modified compound will exhibit improved analgesic properties.

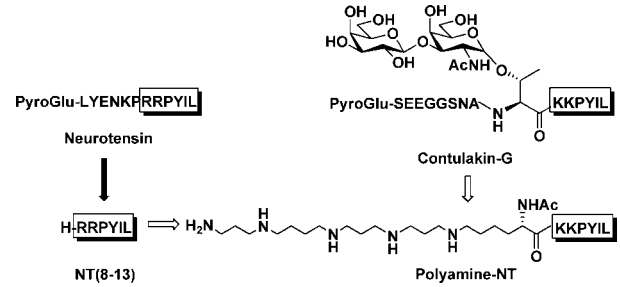

Figure 1. Design of polyamine-NT analogue. C-terminal fragments of NT and Contulakin-G (shown in boxes) are critical for binding to neurotensin receptors. In the truncated Contulakin-G analogue, a naturally occurring glycoamino acid, was replaced with a polyamine-based amino acid residue.

Our design strategy was to introduce a polyamine amino acid into the truncated NT/contulakin-G analogue (Figure 1). In both NT and contulakin-G, the active fragment comprises the last six amino acid residues (RRPYIL and KKPYIL, respectively).[7] Replacements of Arg residues in NT(8−13) with many cationic amino acids did not change receptor binding properties.[10,16] Because contulakin-G contains a bulky moiety β-D-Gal-(1→3)-α-D-GalNAc-(1→) disaccharide attached to Thr10 that was apparently benign to the bioactivity of this NT analogue, we designed the truncated contulakin-G analogue containing the polyamine amino acid instead of glycoamino acid. Thus, our lead compound, named polyamine-NT, was based on the truncated contulakin-G analogue, cont-G(10−16), in which the glycoamino acid was replaced by a polyamine amino acid.

Because natural biogenic polyamines almost exclusively incorporate 1,3-diaminopropyl or 1,4-diaminobutyl units, the spacer $-CH_2CH_2CH_2-$ between lysine and spermine was selected to maintain the physicochemical properties of the side chain for our construct. Structure **1** was designed to be compatible with solid-phase peptide synthesis (SPPS, Scheme 1). Nosyl (*ortho*-nitrobenzenesulfonyl, Ns) strategy was used to conjugate lysine and spermine moieties because of synthetic accessibility, no undesired tertiary amine and/or quaternary ammonium salt formation, and highly efficient alkylation with

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[a] Abbreviations: AME, absorptive-mediated endocytosis; BBB, blood−brain barrier; CNS, central nervous system; Dde, 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl; DIPEA, *N,N*-diisopropylethylamine; ip, intraperitoneal; Ns, *ortho*-nitrobenzenesulfonyl; NT, neurotensin; NTS1, neurotensin receptor subtype 1; NTS2, neurotensin receptor subtype 2; PyBop, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis.

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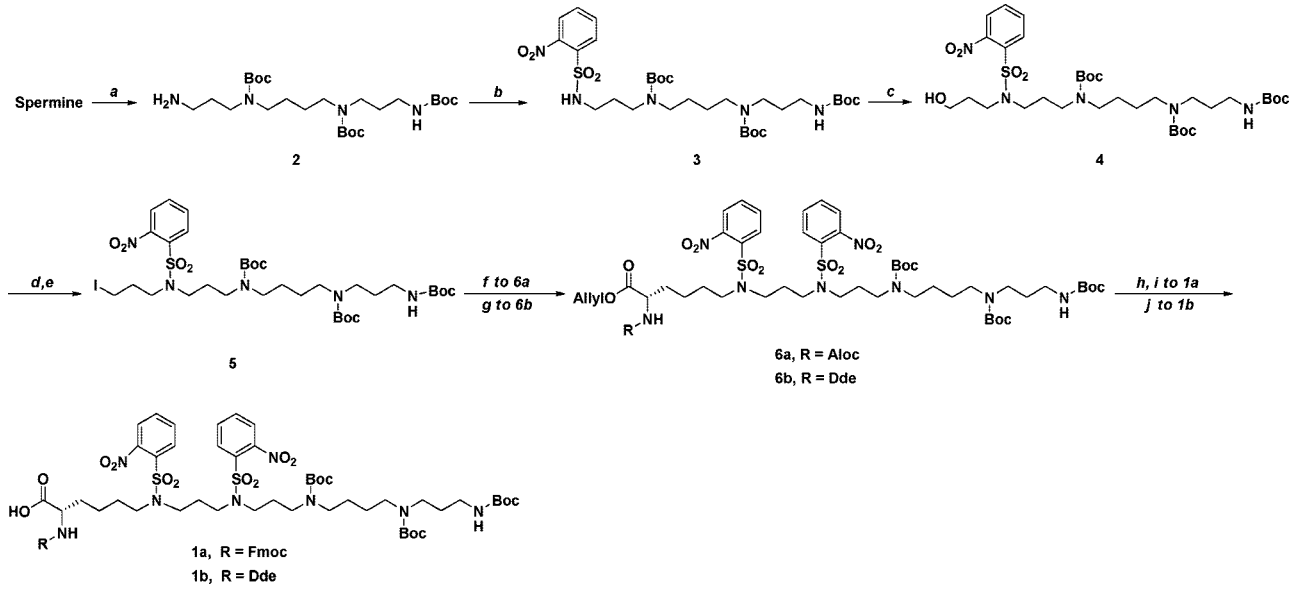
Scheme 1. Synthesis of Polyamine Amino Acids[a]



[a] Reagents and conditions: (a) CF_3COOEt, −78 to 4 °C; Boc_2O, 4 °C; K_2CO_3, MeOH/H_2O (51%); (b) 2-Nitrobenzenesulfonyl chloride, Et_3N, 0 °C; (c) 3-Bromo-1-propanol, Cs_2CO_3, 50 °C (76% from **2** to **4**); (d) $MeSO_2Cl$; DIPEA, 0 °C; (e) NaI, acetone, reflux (74%); (f) Alloc-Lys(Ns)-OAll, Cs_2CO_3, 50 °C (72%); (g) Dde-Lys(Ns)-OAll, Cs_2CO_3, 50 °C (64%); (h) $Pd(PPh_3)_4$, pyrrolidine (48%); (i) FmocOSu, $NaHCO_3$ (76%); (j) $Pd(PPh_3)_4$, pyrrolidine (87%).

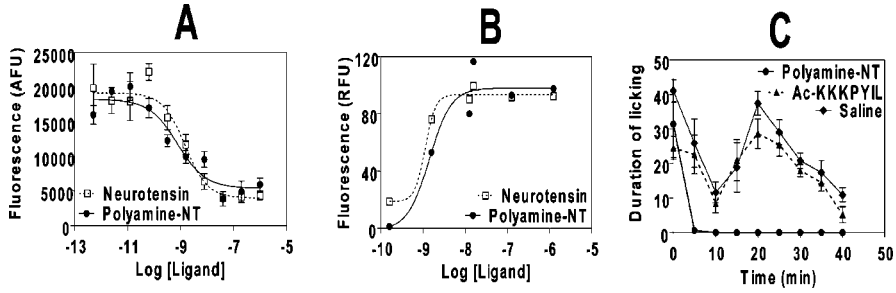


Figure 2. Pharmacological properties of polyamine-NT. (A) Competitive binding assay for the human NTS1 (membrane preparations containing the recombinant NTS1 used in the binding assays were derived from HEK-293T cells). (B) Agonist activity determined by stimulation of intracellular Ca^{2+} mobilization in HEK-293T cells expressing human NTS1. (C) Analgesic activity in the formalin pain assay in mice (ip, 4 mg/kg, injected 1 h prior to formalin injection in a paw). Paw-licking (expressed in seconds) was monitored continuously for 2 min every 5 min. Increased duration of licking between 0−5 min reflects the phase I response (acute pain), whereas that between 15−30 min is defined as the phase II response (inflammatory pain).

RX or ROH.[20] Ns strategy had been successfully applied to the synthesis of polyamine toxins such as spider venom HO-416b[21] and philanthotoxin-343.[20]

Compound **2** was prepared by selective trifluoroacetyl monoprotection of spermine, followed by Boc derivatization of the remaining amino groups. Subsequent hydrolysis of the trifluoroacetyl functionality was accomplished with methanolic K_2CO_3, which gave better results than other published methods (NaOH/H_2O/MeOH or MeOH/NH_4OH).[22,23] Nosylation of **2** gave **3**, which yielded **4** after alkylation with 3-bromo-1-propanol (overall 76% yield for the two steps). Mitsunobu coupling of **4** with Alloc-Lys(Ns)-OAll to form **6a** directly gave low yields and inseparable byproduct. The problem was circumvented via **5**, which was prepared conventionally in good yield by mesylation of **4** followed by displacement with iodide.

Alloc-Lys(Ns)-OAll was synthesized smoothly from Fmoc-Lys-OAll in three steps (see Supporting Information). However, attempts to synthesize N^{α}-Fmoc protected **1a** by way of **5** and Alloc-Lys(Ns)-OAll gave mixed results. Although alkylation of **5** with Alloc-Lys(Ns)-OAll proceeded well, simultaneous deprotection of both Alloc and allyl groups with catalytic $Pd(PPh_3)_4$ in the presence of pyrrolidine gave low yields (48%). Other scavengers such as morpholine[24] or barbituric acid[25] produced similar or lower yields. Carrasco's one-pot strategy for Alloc/allyl removal followed by Fmoc protection without purification gave only 10% yield.[26]

Because of relatively low yields of the above methods, **1b** with Dde protection at N^{α}-position of polyamine acid was designed. To this end, Dde-Lys(Ns)-OAll was synthesized in four steps from H-Lys(Boc)-OH: N^{α}-Dde protection, allyl ester formation, N^{ε}-Boc deprotection, and N^{ε}-nosylation (see Supporting Information). Dde-Lys(Ns)-OAll was then coupled with **5** to give **6b**. Finally allyl deprotection of **6b** with $Pd(PPh_3)_4$/pyrrolidine afforded **1b** in good yield (87%).

The polyamine-NT was synthesized on preloaded Wang resin using PyBop reagent. Amino acid coupling was performed on an automated peptide synthesizer. The final addition of **1b** was done manually. Common Dde deprotection with hydrazine could not be used due to the Ns nitro groups. An alternate deprotection method[27] ($NH_2OH \cdot HCl$/imidazole in NMP/CH_2Cl_2) was applied successfully, followed by *N*-terminal acetylation. Ns deprotection on the resin using (*p*-methoxythiophenol/K_2CO_3 in CH_3CN/

DMSO[28]) was accomplished in 2 h. The peptide was cleaved from the resin with reagent K and purified by HPLC (overall yield 12%).

Polyamine-NT was first tested for its ability to bind and activate the NT receptors, as described previously.[29] This analogue exhibited subnanomolar affinity (K_i 0.25 nM) as well as low nanomolar agonist potency (EC_{50} 1.4 nM) for the human NTS1 (Figure 2A,B). These values were similar to those for the full-length, unmodified NT (K_i 0.5 nM and EC_{50} 1.1 nM),[29] suggesting that the polyamine moiety did not affect the interactions between the polyamine-NT and the NTS1. This finding is in agreement with other reports (e.g. demotensin analogues[30]) and our recent study on glycosylated NT analogues, e.g., the presence of N-terminal extensions in NT(8−13), including addition of various glycoamino acids, did not change the affinity of the analogues toward NTS1.[29] The subnanomolar affinity of polyamine-NT is comparable with another BBB-permeable NT analogue, such as NT67L,[9] and with some of Arg8 substituted NT(8−13) analogues.[10,31] Two other previously described analgesic NT analogues, NT69L and JMV2012, exhibited K_d of 1.55 nM and 150 nM, respectively, for human NTS1.[11,12]

To test our main hypothesis that the polyamine-containing NT analogue may have improved CNS bioavailability, we compared the analgesic activity of polyamine-NT with the control analogue missing the spermine side chain, Ac-KKKPYIL (as referenced above,[30] the presence of the *N*-acetyl-Lys moiety preceding -KKPYIL is unlikely to affect the NT receptor binding properties). Polyamine-NT exhibited a pronounced analgesic effect in the mouse formalin pain model at a single bolus dose of 4 mg/kg following intraperitoneal (ip) administration. The inflammatory phase was completely eliminated at this dose, which did not induce any apparent motor impairment (Figure 2C). In contrast, the control analogue missing the spermine side chain was inactive as an analgesic and did not differentiate itself from the saline control.

Our work shows that incorporation of polyamine amino acid residues during SPPS is feasible, and the resulting neurotensin analogue displayed analgesic properties. Although the antinociception of polyamine-NT is likely mediated by NTS1 located in the spinal cord,[6] we cannot rule out an additional role of NTS2 in producing the apparent analgesia. More detailed studies are required, including measuring interactions between polyamine-NT analogue and NTS2, to address these pharmacological aspects. Nonetheless, an immediate outcome of our work is that polyamine amino acids should be explored more frequently when attempting to improve the CNS bioavailability of peptides. There are multiple mechanisms by which modified peptides can penetrate the BBB, and the polyamine-conjugated compounds are more likely to be transported by AME.[32] Other applications for polyamine amino acids are to explore them as substitutes for cell-penetrating peptides[33] or for engineering biosilica-based nanomaterials.[34] Taken together, our results support the use of polyamine amino acids to engineer novel properties of biopolymers assembled via SPPS.

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Supporting Information Available: Experimental details for synthetic procedures, full characterization of all compounds, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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