Synthesis of the Spider Toxins Nephilatoxin-9 and -11 by a Novel Solid-Phase Strategy

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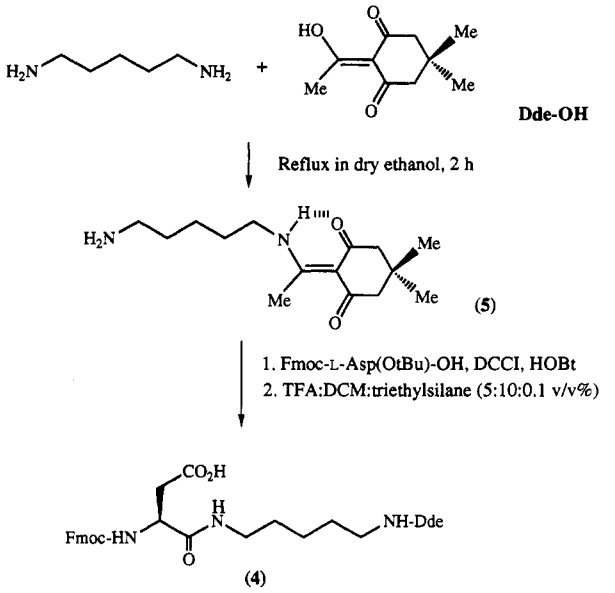
Spider venoms have been the source of several families of important neurotoxins, most of which have been characterized as low molecular weight polyamine peptides, and they are known to act as antagonists at ionotropic glutamate receptor (iGluR) channels.[1] Since glutamate receptors are associated with neuromuscular transmission in many invertebrates and probably mediate the major excitatory neural pathways in the vertebrate central nervous system, the specific blockade of these processes is of considerable interest.

The nephilatoxins (NPTX), isolated from the venom of the Joro spider *Nephila clavata*,[2] are polyamine peptides[3,4] comprising an aryl L-asparaginyl residue and a polyamine chain attached to a number of cationic L-amino acids. Common features of this family of neurotoxin are the presence of an L-asparaginylcadaverine moiety and either an indole-3-acetyl or 6-hydroxy-indole-3-acetyl residue.

To date, the only reported total synthesis of members of the nephilatoxins, e.g., NPTX-9 (**1**) and NPTX-11 (**2**), are based on difficult solution chemistry and are somewhat inflexible.[3] We now report an efficient and facile solid-phase route to the nephilatoxins and other polyamine peptides based on the orthogonally *N*-protected key component N^{α}-(Fmoc-L-Asp)-N^{ϵ}-Dde-cadaverine (**4**). This intermediate can be coupled *via* the aspartyl β-carboxyl group to a solid support,[5] and the complete assembly of a wide range of polyamine peptides was achieved using continuous-flow solid-phase methodology. This conceptually novel strategy relies on the ability to remove selectively the amino protecting groups from the resin-bound N^{α}-[Fmoc-L-Asp-("amide linker")]-N^{ϵ}-Dde-cadaverine, thus enabling the peptide synthesis to proceed in a stepwise manner from either end.

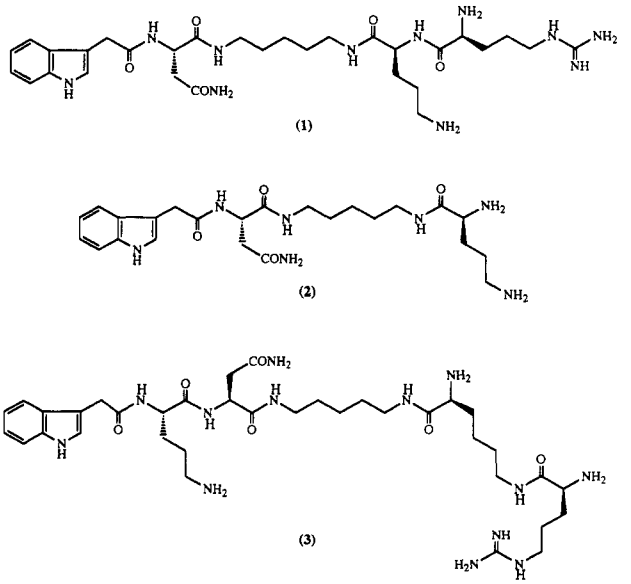
This selectivity is achieved utilizing our recently reported amino protecting group, *N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl] (Dde).[6] Dde is stable to 20% piperidine in DMF, the reagent frequently used for the removal of the Fmoc group, but is readily removed by 2% hydrazine in DMF within minutes. The driving force of the Dde deprotection step is the formation of 3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazole, and this can be monitored by the UV absorption at either 270 or 290 nm.

Scheme 1. Synthesis of N^{α}-(Fmoc-L-Asp)-N^{ϵ}-Dde-cadaverine



The versatility of the overall methodology is illustrated by the synthesis of two natural nephilatoxins NPTX-9 (**1**) and NPTX-11 (**2**) and an analogue (**3**).

An efficient and simple synthesis of N^{α}-(Fmoc-L-Asp)-N^{ϵ}-Dde-cadaverine (**4**) was achieved in three steps as outlined in Scheme 1. Cadaverine (1,5-diaminopentane) readily reacts with 2-acetyldimedone in refluxing ethanol, within 2 h to afford the monoprotected *N*-Dde-Cad (**5**) in 49% yield.[7] The active ester of Fmoc-L-Asp(OtBu)-OH (obtained by carboxyl activation using an equimolar mixture of DCCI and HOBt) was coupled with (**5**) at room temperature overnight to yield the orthogonally protected N^{α}-[Fmoc-L-Asp(OtBu)]-N^{ϵ}-Dde-cadaverine.[8] Selective removal of the *tert*-butyl protecting group by exposure to TFA:DCM (1:2 v/v) in the presence of triethylsilane (0.5%) afforded, after recrystallization, **4** as a white solid in 90% yield.[9]



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(4) Miyashita, M.; Matsushita, M.; Sato, H.; Toki, T.; Nakajima, T.; Irie, H. *Chem. Lett.* **1993**, 929–932.

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(7) The monoprotected Dde-Cad is unstable and is therefore reacted with activated Fmoc-Asp(OtBu)-OH immediately following its isolation by silica flash column chromatography. The undesired bis-Dde-protected compound occurred in ~20% yield which is readily removed by flash chromatography.

(8) Fast-atom-bombardment (FAB-MS) *m/z* 660 (M+ + H): The ^{1}H NMR (250 MHz, [^{2}H]CCl_3) spectrum is in accord with the proposed structure. The assigned spectrum is similar to **4**,[9] except for the presence of a singlet at δ 1.44 (9H, OtBu $C(CH_3)_3$).

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The assemblies of the desired nephilatoxins were then readily accomplished by application of established solid-phase peptide synthetic procedures. Attachment of N^{α}-(Fmoc-L-Asp)-N^{ϵ}-Dde-cadaverine (**4**) *via* the aspartyl side chain to an amide resin[10] followed by selective Fmoc deprotection releases the aspartyl amino group. In the cases of NPTX-9 and -11 (**1** and **2**) this was acylated with indole-3-acetic acid, while for the analogue (**3**) Fmoc-L-Orn(Boc)-OH was first attached, deprotected, and then acylated with indole-3-acetic acid. Following Dde deprotection with 2% hydrazine,[6] further peptide construction was in each case carried out from the cadaverine N^{ϵ}-terminus. The acylating species were activated Fmoc-L-Orn(Boc)-OH for (**1**), Fmoc-L-Orn(Boc)-OH followed by Fmoc-L-Arg(Pmc)-OH for (**2**), and Dde-L-Lys(Fmoc)-OH followed by Fmoc-L-Arg(Pmc)-OH for (**3**). Deprotection and cleavage[11] from the resin afforded crude products which were purified by reverse-phase HPLC[12] to give the synthetic nephilatoxins (**1–3**) as white solids in 70–80% yields.[13]

In conclusion, we have outlined a rapid and facile route for the total chemical synthesis of the nephilatoxin polyamine peptides, in significant quantities, by application of orthogonal amino protecting groups and solid-phase methods. The implication of this novel solid-phase strategy for the construction of chemical combinatorial libraries and labeled spider toxins as well as affinity chromatography columns for isolation of iGluR proteins are presently being explored.

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(9) As confirmed by ^{1}H NMR (250 MHz, [$^2H_6$]-DMSO, 303 K): δ 0.94 (6H, s, Dde C(CH_3)$_2$), 1.42 (6H, m, Cad (CH_2)$_3$), 2.26 (4H, s, Dde 2 × CH_2), 2.45 (3H, s, Dde C=C-CH_3), 2.45, 2.50 (2H, 2 × dd, J = 16.5, 5 Hz, Asp CβH_2), 3.06 (2H, m, Cad CH_2), 3.37 (2H, m, Cad CH_2NH-Dde), 4.25 (3H, m, Fmoc CH-CH_2), 4.31 (1H, m Asp CαH), 7.32, 7.42, 7.71, 7.88 (8H, Fmoc ArH), 7.58 (1H, d, J = 8 Hz, Asp NH), 7.80 (1H, t, J = 5 Hz, Cad NH), 13.24 (1H, br t, Cad NH-Dde); FAB-MS m/z 604 (M^+ + H); mp 95–97 °C.

(10) Solid-phase assembly of polyamine peptides were accomplished on either Fmoc-UltraSyn C (*Pharmacia*) or Fmoc-PAL-PEG/PS (*Millipore*) resin. Carboxyl activation was achieved by the mixture HBTU:HOBt:DIPEA (1:1:2 molar ratios), and N^{α}-Fmoc deprotection with 20 v/v% piperidine in DMF.

(11) NPTX-11 was cleaved from the resin and the side chains were deprotected by treatment with TFA:H_2O:EDT (93:2:5 v/v%) for 2 h. NPTX-9 and the synthetic NPTX analogue (**3**) were obtained by treatment with TFA: triisopropylsilane:H_2O:EDT (88:5:2:5 v/v%) for 2–3 h. The yields of the crude polyamine peptides, as trifluoroacetate salts, were typically 90–95%.

(12) All compounds were a single peak by RP-HPLC. NPTX-11 was purified on Hypersil Pep-100 C18 column (4.6 × 150 mm, flow rate 1.20 mL min^{-1}) with a retention time of 8.6 min. NPTX-9 and synthetic NPTX analogue were purified on a semi-prep column Kromasil KR100-5C8 (8 × 250 mm, flow rate 2.50 mL min^{-1}) with retention times of 12.0 and 12.9 min, respectively. The elution gradient was 10–40 %B in 20 min at either 1.20 or 2.50 mL min^{-1} (A = 0.06% aqueous TFA, B = 0.06% TFA in 90% aqueous acetonitrile).

(13) All synthesized nephilatoxins gave expected TOF-MS data: NPTX-11 requires 487.6, found 486.8; NPTX-9 requires 655.7, found 655.4; synthetic NPTX analogue requires 772.0, found 774.0. The ^{1}H NMR (400 MHz, [$^2H_6$]-DMSO, 303 K) spectra were in accord with the assigned structures. The assignment for NPTX-9 is as follows: indole-3-acetyl, δ 3.55 (CH_2), 9.60 (NH), 7.18 (C^2H), 7.51 (C^5H), 6.95 (C^6H), 7.05 (C^7H), 7.32 (C^8H); Asn, 8.07 (NαH), 4.50 (CαH), 2.38, 2.46 (CβH_2), 6.82, 7.30 (NβH_2); cadaverine, 7.66 (NαH), 3.10 (CαH_2), 1.50 (CβH_2), 1.18 (CγH_2), 1.32 (CδH_2), 2.99 (CϵH_2), 8.07 (NϵH); Orn, 8.61 (NαH), 4.27 (CαH), 1.56, 1.66 (CβH_2), 2.77 (CγH_2), 7.15 (NH_3^+); Arg, 5.50 (CαH), 3.92 (CβH_2), 2.83 (CγH_2), 4.62 (CδH_2), 7.15 (NH_3^+).

(14) Abbrevations: Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; *N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl], Dde; DIPEA, *N,N*-diisopropylethylamine; DCCI, 1,3-dicyclohexyl-carbodiimide; DMF, *N,N*-dimethylformamide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethyloxy-carbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMR, nuclear magnetic resonance spectroscopy; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TOF-MS, time-of-flight mass spectrometry.