Tetrahedron Vol. 49, No. 48, pp. 11155-11168, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00

Characterization and Synthesis of a New Calcium Antagonist from the Venom of a Fishing Spider

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(*Received in USA* 26 *August* 1993; *accepted* 4 *October* 1993)

Abstract: A new calcium antagonist, CNS 2103, is isolated from the venom of a fishing spider, *Dolomedes okefinokensis*. The structure of this compound is derived from spectroscopic data, including tandem mass spectrometry. A flexible, convergent synthesis of CNS 2103 is described.

INTRODUCTION

Fishing spiders (family Pisauridae, genus *Dolomedes*) are among the few spiders that routinely prey upon vertebrates. These spiders regularly catch and kill small fish, frogs, and tadpoles.[1] They might therefore be expected to have neurotoxins specific to vertebrate nervous systems in their venom. We anticipated that one mechanism of spider venom action would involve blocking of neurochemically important ion channels. Since such agents might have significant therapeutic value, we have now screened the venom from one of the larger fishing spiders (*Dolomedes okefinokensis*) for such activity, and report the isolation, characterization, and synthesis of a new polyamine, "CNS 2103", of particular neurochemical interest because of its reversible block of voltage sensitive calcium channels.[2,3]

ISOLATION AND CHARACTERIZATION

Preparative HPLC fractionation of this venom (Figure 1) was monitored by assaying each fraction for various types of neurochemical activity.[3] One fraction was found to be of particular interest because of its reversible blocking of voltage sensitive calcium channels. Further HPLC purification of this fraction yielded a homogenous sample of CNS 2103, along with another related component (CNS 2104) of lesser activity.

Characterization of CNS 2103 began with amino acid analysis and ^{1}H-NMR spectroscopic study of a small sample. No amino acids were found in the hydrochloric acid hydrolyzate. The initial sample's ^{1}H-NMR spectrum, although of poor quality because of the small sample size, did yield some useful information. Several broad peaks between 0.9 and 3.1 ppm hinted at a polyamine structure, and a few peaks in the aromatic region

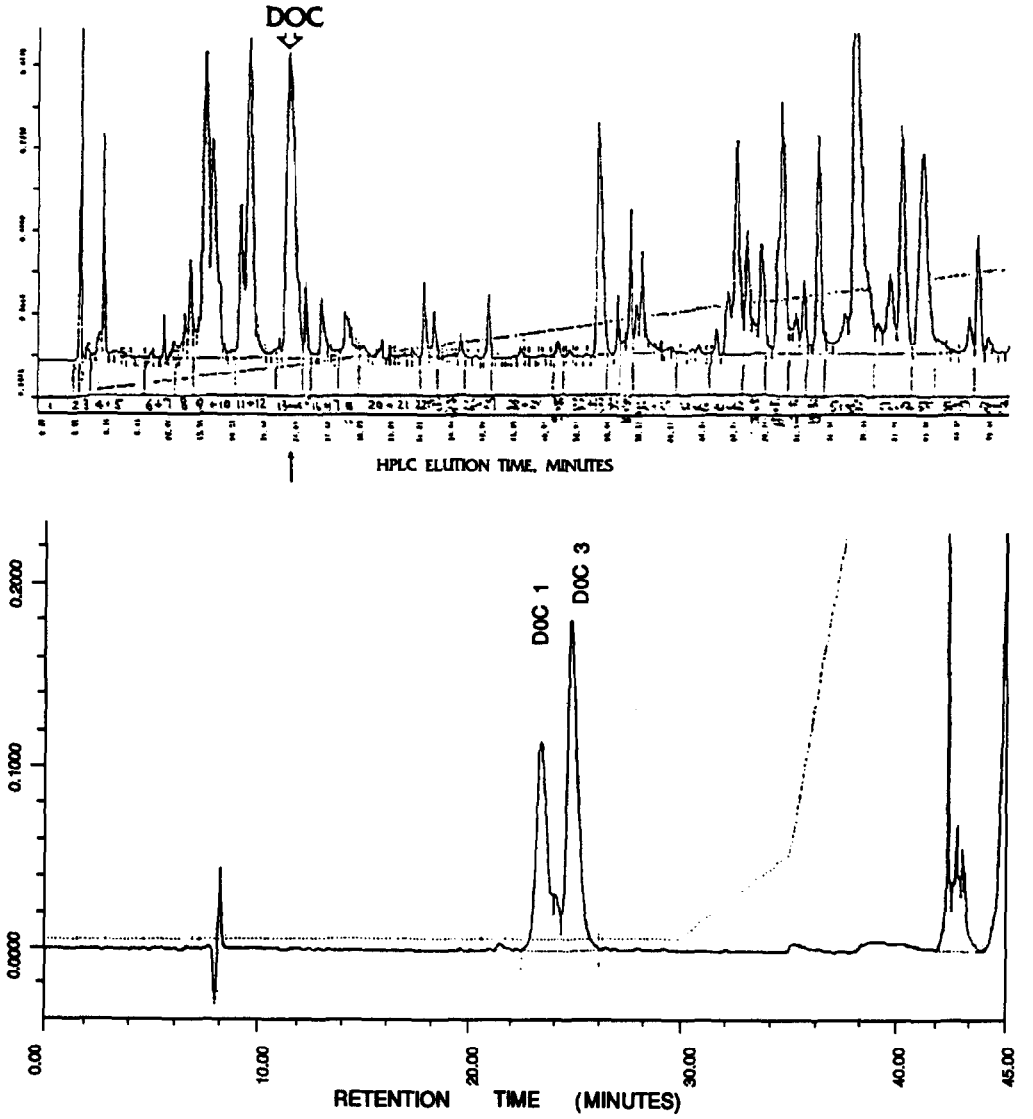


Fig. 1. HPLC purification of CNS 2103 from the venom of the spider *Dolomedes okefinokensis*. (a) A chromatogram of the crude venom showing the location of the DOC fraction. (b) A chromatogram showing the separation of the DOC fraction into its main components: CNS 2103 and CNS 2104.

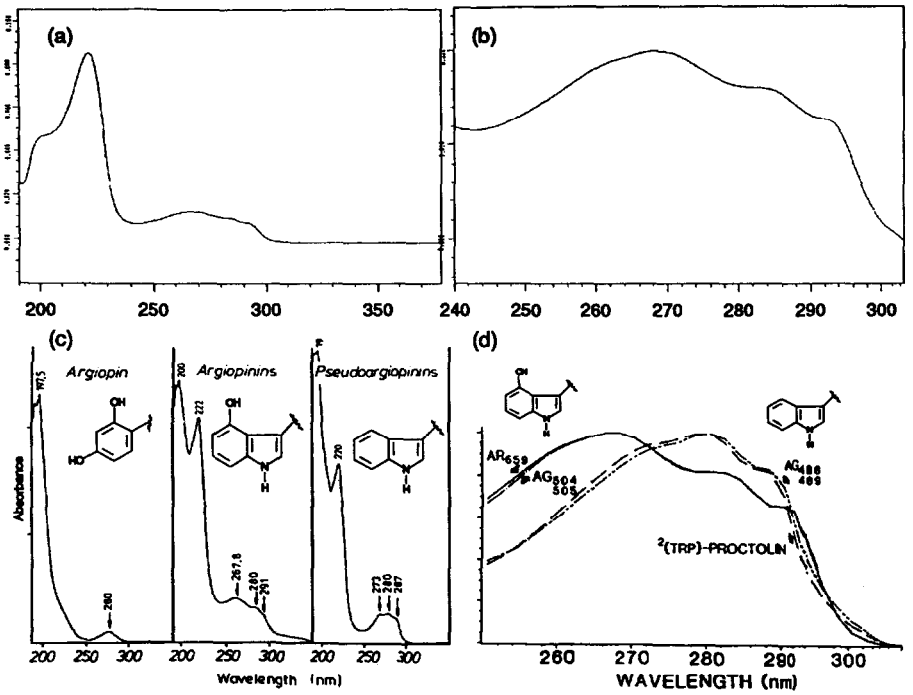
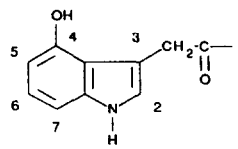


Fig. 2. A comparison of UV spectra of CNS 2103 and other spider venom acylpolyamines: (a) and (b) CNS 2103; (c) argiopin, argiopinins, and pseudoargiopinins;[4] (d) argiotoxins-504, -505, -488, -489, and argiopinin III.[5]

between 6.3 and 7.1 ppm suggested the presence of an aromatic acyl group. On the basis of these early observations, this toxin appeared to be a non-amino acid containing acylpolyamine. At the time of this assignment, only a few non-amino acid containing acylpolyamines were known, and their structures had not yet been fully determined. While sixteen non-amino acid containing acylpolyamines have now been characterized, none of these compounds correspond to CNS 2103,[5-11] nor do they show any voltage-activated calcium channel blocking activity.

The UV absorption spectrum of CNS 2103 has a strong 223 nm absorption maximum and three weaker maxima at 269, 284, and 292 nm. This pattern is characteristic of a 4-hydroxyindole (Figure 2). The presence of a 4-hydroxyindole moiety was confirmed by ^{1}H-NMR spectroscopy once sufficient material had been purified. The 400 MHz ^{1}H-NMR spectrum of the toxin in D_2O (Figure 3) shows the four expected aromatic 4-hydroxyindole resonances: a singlet at 7.05 ppm for H-2, a triplet at 6.40 ppm (J = 4.1 Hz) for H-6, and two overlapping doublets at 6.94 ppm (J=4.5 and J = 3.4 Hz) for H-5 and H-7. The spectrum also includes a two proton singlet at 3.65 ppm which indicates that the indole ring is bound to an acetic acid unit, as shown below.



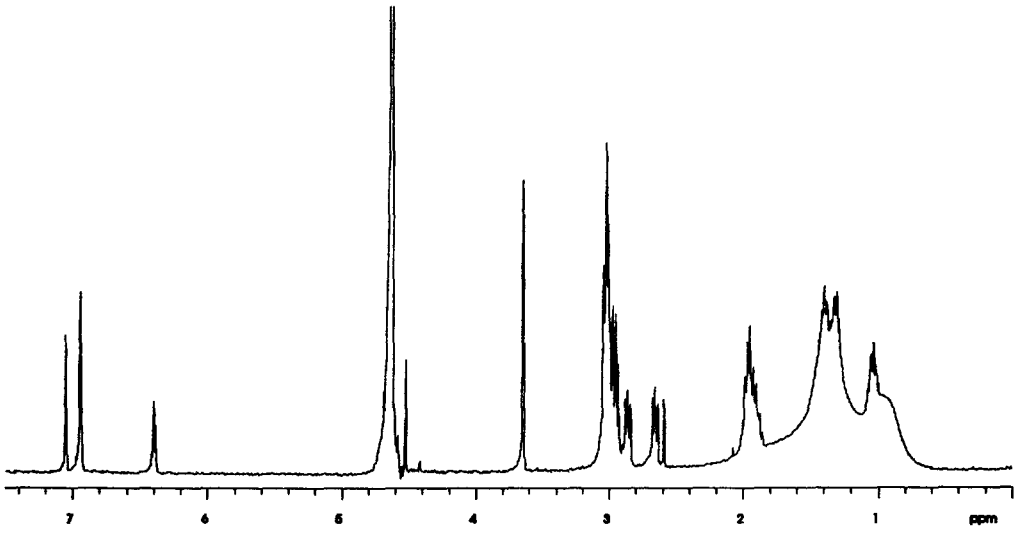


Fig. 3. 400 MHz ^{1}H-NMR spectrum of CNS 2103 in D_2O.

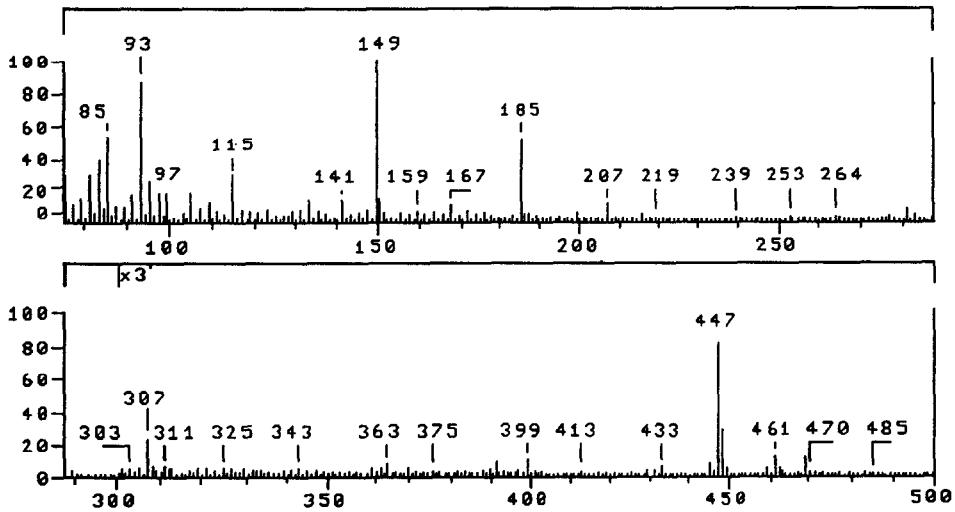


Fig. 4. FAB mass spectrum of CNS 2103.

The remainder of the ^{1}H-NMR spectrum shows a group of highly coupled methylene resonances between 1.0 and 3.1 ppm. The FAB mass spectrum revealed an $[M+1]^+$ ion at $m/z = 447$ (Figure 4). Of this, 370 amus are accounted for by the 3-(4-hydroxyindole)-acetyl moiety ($C_{10}H_8NO_2$) and fourteen methylenes ($C_{14}H_{28}$). This leaves 76 amus, which can best be assigned to a residual N_5H_6. It follows that CNS 2103 contains a four segmented polyamine chain with no further functionalization.

Determining how the fourteen methylenes are distributed between the nitrogen links was accomplished from analysis of the ^{1}H-NMR coupling patterns observed in a double quantum filtered (DQ) COSY spectrum (Figure 5). The sixteen proton signals between 2.6 and 3.1 ppm belong to eight methylene groups which are

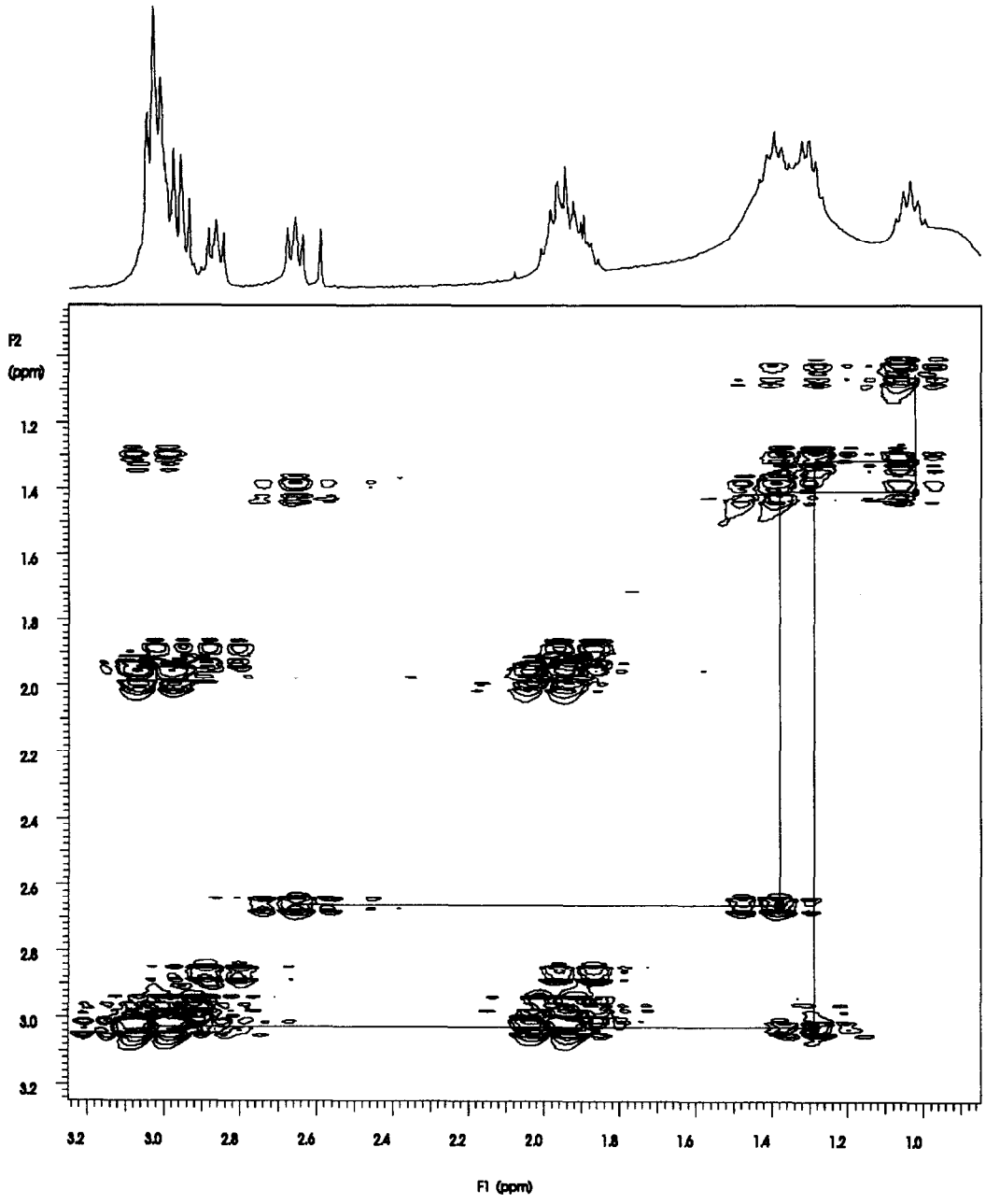


Fig. 5. Double quantum filtered COSY spectrum of the polyamine region of CNS 2103.

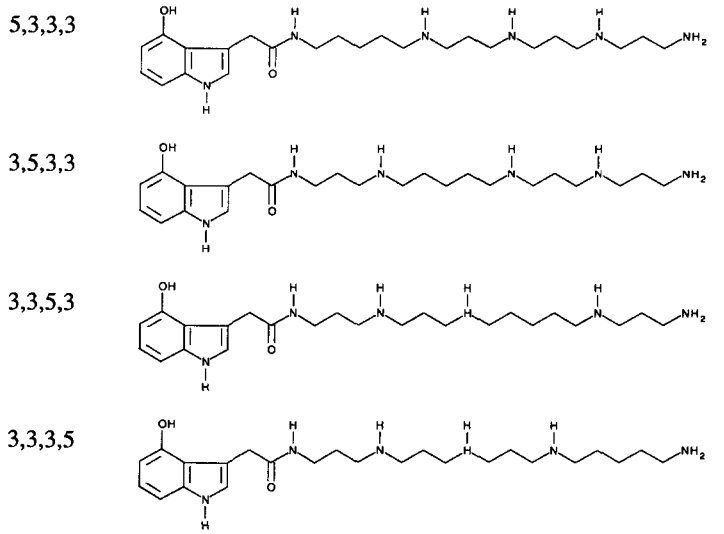
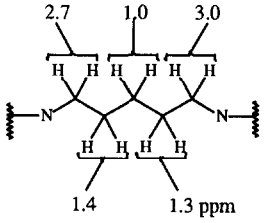


Fig. 6. The four possible structures of CNS 2103.

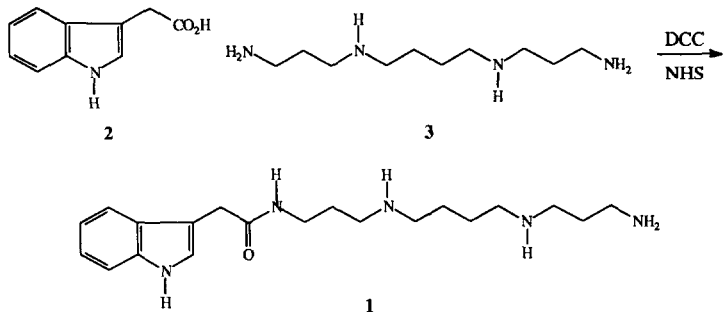
adjacent to nitrogens. Six of these methylenes are coupled to three methylenes whose signals lie between 1.9 and 2.0 ppm. This pattern is indicative of three trimethylene units. The remaining five methylenes are coupled in the form of a linear pentamethylene unit as shown below.



In summary, the structure of CNS 2103 consists of a 3-(4-hydroxyindole)-acetyl moiety joined to a polyamine chain made up of one linear C_5 unit and three C_3 units. There are four ways that these segments can be arranged, as shown in Figure 6.

The most commonly used techniques for determining the sequence of atoms within a polyamine are FAB and CI mass spectrometry.[7,9,11] However, these mass spectra are difficult to interpret because polyamines give only very low intensity fragment ions, which are consequently difficult to differentiate from extraneous peaks due to impurities, sample matrix, and instrumental noise. With only a limited amount of CNS 2103 available, it was important to find a more definitive technique. Tandem mass spectroscopy seemed promising, since this method shows only the fragmentation pattern derived from a single, selected parent ion which is isolated by the spectrometer's first stage. Since at that time, tandem mass spectrometry had not been applied to any related compounds, it was not known whether polyamines would fragment in a predictable and useful way. A model study was therefore carried out. Indole-3-acetamide-3,4,3-polyamine, **1**, (with the numerals preceding the polyamine denoting the number of methylene groups between the amino groups) was chosen as the model compound because of its structural similarity to CNS 2103 and its ease of synthesis. This compound was

Scheme I



2 3 1

prepared in one step by coupling indole-3-acetic acid (**2**) to spermine (**3**) as shown in Scheme I. The FAB-MS-MS of **1** turned out to be very useful, and clearly showed the location of all of the nitrogen atoms in the polyamine chain (Figure 7). Almost all of the observed fragment ions could be attributed to charge-site induced cleavage[12] adjacent to the nitrogen atoms. Interestingly, the fragmentations of polyamines observed by conventional FAB-MS usually occur by α-cleavage[7] or two proton rearrangement[7,13] rather than charge-site induced cleavage.

Since tandem mass spectroscopy performed very well with the model compound, it was applied to CNS 2103. The results identified the correct structure (Figure 8) unambiguously. The fragment ion at $m/z = 172$ requires that the three trimethylene units be connected sequentially, and the ion at $m/z = 316$ shows that the 4-hydroxyindole unit, the pentamethylene unit, and one trimethylene unit are joined. These fragmentations are possible only if the C_5 segment is located between the 4-hydroxyindole-3-acetic acid and the three C_3 segments. The CNS 2103 structure is thus defined.

SYNTHESIS

Developing a synthetic route for CNS 2103 was important not only to confirm the structure, but also to provide sufficient material with which to complete the evaluation of its biological activity. A convergent and flexible scheme was sought. Our synthesis of CNS 2103 begins with the preparation of the aromatic acyl moiety and assembly of the polyamine chain. These units are then coupled to form the desired product. This approach makes possible the convenient study of structure-activity relationships, since it is easy to vary the aromatic acyl and the polyamine units independently at a late stage in the synthesis.

The route to an activated 4-hydroxyindole-3-acetic acid ester is shown in Scheme II. Benzylation of the commercially available 4-hydroxyindole (**4**) gives 4-benzyloxyindole (**5**), which is then converted to 4-benzyloxyindole-3-acetic acid (**8**) by a convenient three step sequence which had already been developed.[14,15] This method begins with the conversion of indole **5** to the gramine **6** by a Mannich reaction,[15] followed by cyanide ion displacement of trimethylamine from the gramine methiodide to give 4-benzyloxyindole-3-acetonitrile (**7**).[14] Finally, the nitrile is hydrolyzed under basic conditions to give the desired 4-benzyloxyindole-3-acetic acid (**8**).[14] To couple the indole with a polyamine, the acetic acid group is converted into its activated *p*-nitrophenol ester **9** using DCC coupling conditions. This product is purified by crystallization, and stored in the freezer until needed.

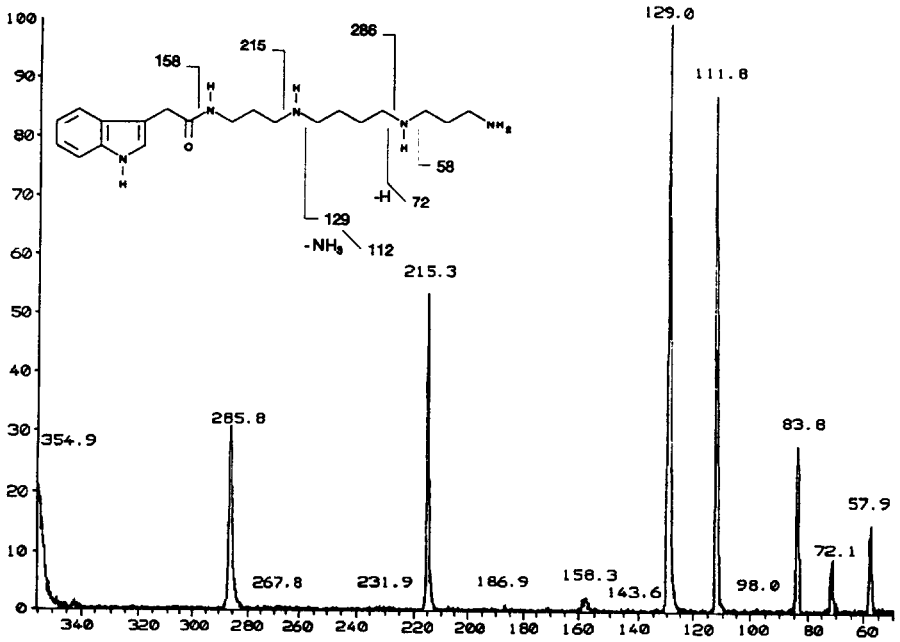
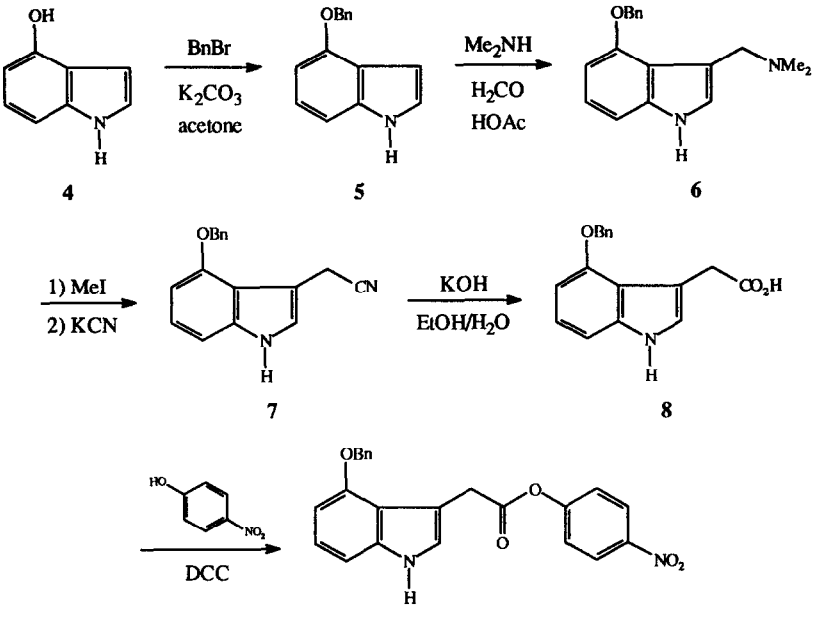


Fig. 7. Tandem mass spectrum of the molecular ion for indole-3-acetamide-3,4,3-polyamine **1**.

Fig. 8. Tandem mass spectrum of the molecular ion for CNS 2103.

Scheme II

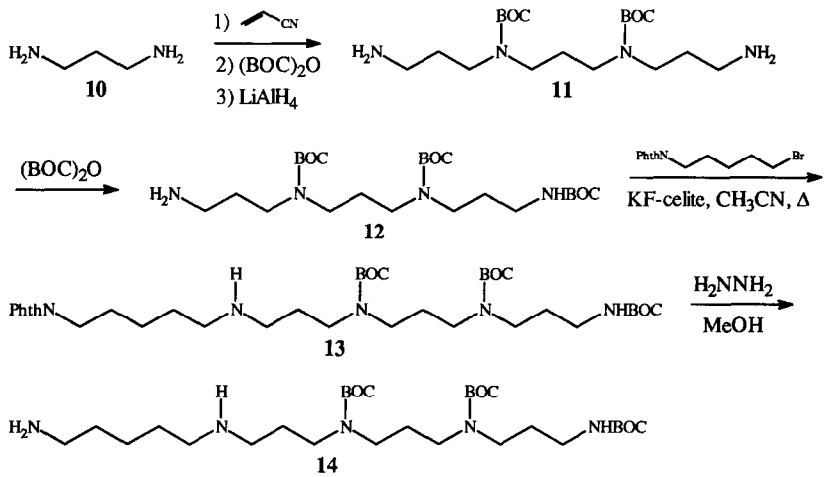


The synthetic route to the 5,3,3,3 polyamine is simple, efficient, and versatile. To ensure proper coupling, all of the basic nitrogen atoms except for the terminal amino group are protected. CNS 2103 itself is a highly polar compound that is difficult to purify by conventional methods, particularly because it decomposes readily in air, especially under basic conditions. It was therefore necessary to carry out a careful purification at the penultimate step, before removal of the protecting groups. Subsequent deprotections must then be clean and quantitative. The BOC group was chosen because it meets these deprotection requirements; it is easily introduced, easily removed with trifluoroacetic acid or HCl, and stable under most non-acidic reaction conditions.

Our route to the 5,3,3,3 polyamine is shown in Scheme III. It begins with dicyanoethylation of 1,3-diaminopropane (**10**) to give a quantitative yield of the expected dinitrile. Without further purification of this intermediate, its two newly formed secondary amino groups are protected by treatment with $(BOC)_2O$. The two cyano groups are then reduced with $LiAlH_4$ to give the symmetrical 3,3,3 polyamine, **11**, in 50% overall yield from **10**. The cyanoethylation procedure is particularly attractive, since at room temperature only primary amines are alkylated. Other polyamine forming reactions (reductive aminations, alkyl halide alkylations, polyamide formation and subsequent reduction[16]) give poorer yields, produce more side products, or are not compatible with BOC protection. The one disadvantage of this route is the disappointing yield (40 to 55 %) in the nitrile reduction. The reduction was also carried out *via* catalytic hydrogenation (Adam's catalyst PtO_2/ EtOH/ $CHCl_3$,[17] Raney Ni/ NaOH/ EtOH,[18] and Pearlman's catalyst $Pd(OH)_2$-C/ HOAc[7]), but none of these reactions represented an improvement.

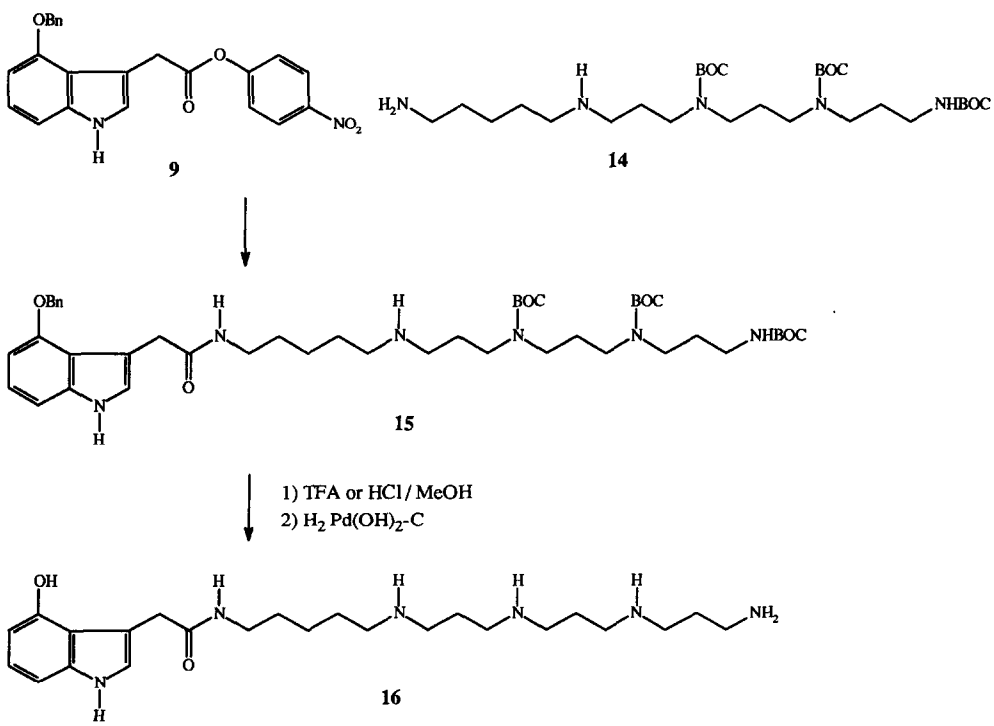
The symmetrical 3,3,3 amine, **11**, is treated with one equivalent of $(BOC)_2O$ in CH_2Cl_2 to form the triply protected amine, **12**, alkylation of which with a N-(5-bromopentyl)-phthalimide in the presence of KF-celite,[19]

Scheme III



yielded the 5,3,3,3 polyamine, **13**, in 50% yield. The new secondary amino group between the C_5 and C_3 segments can be protected with $(BOC)_2O$, but this was found to be unnecessary; leaving this amino group unprotected did not interfere with subsequent end group coupling. Finally, the phthalimide protecting group is removed by treatment with hydrazine to give the desired 5,3,3,3 polyamine, **14**.

Scheme IV



The completion of the CNS 2103 synthesis is outlined in Scheme IV. First, the indole and polyamine units are coupled by adding the *p*-nitrophenol ester **9** to the 5,3,3,3 BOC protected polyamine **14** in DMF, to give an 81% yield of the desired product, **15,** after careful purification by flash chromatography. The benzyl and BOC groups are then removed by acid treatment followed by catalytic hydrogenation. Interestingly, if the order of these deprotections is reversed, extensive product decomposition occurs during the BOC removal. Extreme care is required in the benzyl group hydrogenolysis step and the subsequent handling of the CNS 2103, since the free 4-hydroxyindole moiety is very susceptible to oxidation. All solvents used in the final steps were degassed prior to use.

Synthetic and natural CNS 2103 were indistinguishable both chromatographically and spectroscopically. With the completion of this work, CNS 2103 now becomes available for more extensive biological evaluation.

EXPERIMENTAL

Mass spectra were recorded on either a Kratos MS 50 or a Finnigan MAT 70 mass spectrometer. Tandem mass spectroscopy was carried out using argon gas in the collision chamber. FAB mass spectra samples were prepared in a glycerol matrix. UV spectra were measured using a Hewlett Packard 8451A diode array spectrophotometer.

NMR spectra were measured using either Varian Unity-500, XL-400 or Bruker WM-300 spectrometer. All NMR spectra of natural samples were taken in high purity "100 % Deutero" solvents from MSD Isotopes. Synthetic samples were dissolved in normal grade deutero solvents from MSD Isotopes or Cambridge Isotopes. The phase sensitive DQ-COSY of CNS 2103 was acquired with a 64x2048 matrix using 256 scans per increment. A presaturation of the HOD resonance was used during the 1.7 s relaxation delay between scans. A narrow 1279.3 Hz sweep width was used to maximize resolution and sensitivity. The peak folding of the indole and HOD resonances were carefully controlled to prevent any interferences with the polyamine signals.

High pressure liquid chromatography was accomplished with a Beckman Gold HPLC system. Thin layer chromatography was performed using Bakerflex silica gel IB2-F plates. Polyamine visualizations were achieved with ninhydrin staining, and all other visualizations were made by phosphomolybdic acid staining. Reaction products were purified by flash chromatography using EM Science silica gel 60 (230-400 mesh).

All reactions were carried out under an atmosphere of argon using reagent grade solvents. When necessary, solvents and reagents were distilled prior to use. Acetone was distilled from anhydrous potassium carbonate. Dichloromethane was distilled from calcium hydride. Ether was distilled from sodium. Tetrahydrofuran was distilled from potassium benzophenone ketyl. Solvents were degassed just prior to use by, bubbling either Ar or He through the solvent for at least 1 h.

Dolomedes okefinokensis spiders were collected in northern Florida and milked by The Spider Pharm (Pennsylvania) using electro-stimulation. The crude venom was then kept frozen at -80°C until just prior to purification. Aliquots of the thawed venom (80 µL) were diluted with 160 µL of 0.1% trifluoroacetic acid (TFA) in H_2O and centrifuged at 13,000 rpm for 5 min. The supernatant was chromatographed by reverse-phase HPLC using a C18 column (10.0 x 250 mm, 5 µm, 100 Å, YMC material from The NEST Group). Preliminary fractionation was performed using an acetonitrile gradient of 0 to 30% in 0.1% TFA over 105 min at 4.0 mL/min. The DOC fraction was collected after 40 min, concentrated with a Speed Vac, and resuspended in 250 µL of 0.1% TFA. The DOC fraction was further purified by reverse-phase HPLC in 50 µL portions using an isocratic elution of 9% methanol in 0.1% TFA. This gave >95% pure CNS 2103 and CNS 2104.

N-(12-Amino-4,9-diazadodec-1-yl)-1H-indole-3-acetamide (1): To a solution of indole-3-acetic acid (**2**) (68 mg, 0.39 mmol) and N-hydroxysuccinimide (45 mg, 0.39 mmol) in THF (6.0 mL) was slowly added a 1.4 M THF solution of 1,3-dicyclohexylcarbodiimide (275 µL, 0.39 mmol). The reaction mixture was stirred at ambient temperature for 3h during which dicyclohexylurea precipitated. The supernatant was then drawn off, and added to a solution of spermine (**3**) (115 mg, 0.57 mmol) in THF (10 mL). After stirring the reaction mixture at room temperature for 20 h, it was added to 0.1 M NaOH and extracted with ether. The organic layer was dried with K_2CO_3 and concentrated to give the crude product as a white solid. A portion of the

crude product was purified by HPLC (RP-18 column, eluting with 9:91 CH_3CN-H_2O with 0.1 % TFA) to give pure acylpolyamine **1**: **^{1}H-NMR:** δ (300 MHz, D_2O) 7.53 (d, 1H, J=8.0 Hz), 7.35 (d, 1H, J=8.0), 7.17 (s, 1H), 7.11 (t, 1H, J=8.0), 7.01 (t, 1H, J=8.0), 3.63 (s, 2H), 3.15 (t, 2H, J=6.6), 2.78 (t, 2H, J=6.8), 2.53 (t, 2H, J=6.4), 2.46 (t, 2H, J=7.6), 1.42 (m, 4H), 1.23 ppm (m, 2H); **FAB-MS:** *m/z* 360 (m+1).

4-Benzyloxyindole (5): A mixture of 4-hydroxyindole (**4**) (845 mg, 6.34 mmol), K_2CO_3 (2.64 g, 19.0 mmol), benzyl bromide (1.1 mL, 9.5 mmol), and dry acetone (17 mL) was stirred at room temperature for 20 h and then concentrated in vacuo to give a dark thick tar. The crude product was purified by flash chromatography (25:1:0.1 $CHCl_3$-MeOH-$NH_{3\ aq}$) to give 1.34 g of benzyl ether **5** (6.02 mmol, 95 %) as a white solid: **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 7.51 (br d, 2H, J=7.5 Hz), 7.44-7.30 (m. 3H), 7.23-7.06 (m, 4H), 7.02 (dt, 1H, J=8.2, 0.8), 6.72 (ddd, 1H, J=3.2, 2.2, 1.0), 6.59 (dd, 1H, J=7.5, 0.5), 5.23 ppm (s, 2H).

4-Benzyloxygramine (6): Indole **5** (1.34 g, 6.0 mmol) was treated with a mixture of 37% aqueous formaldehyde (520 μL, 6.9 mol), 40% aqueous dimethylamine (940 μL, 7.5 mmol), acetic acid (600 μL), and water (280 μL) as described by Poon *et al.*,[15] and purified by flash chromatography (9:1 hexane-ethyl acetate) to give 1.06 g of gramine **6** (5.4 mmol, 90%): **^{1}H-NMR:** δ (400 MHz, d_6-acetone) 8.013(s, 1H), 7.610(br d, 2H, J=7.5), 7.40 (br t, 2H, J=7.4), 7.32 (br t, 1H, J=7.5), 7.07 (s, 1H), 6.94-7.00 (m, 2H), 6.57 (dd, 1H, J=6.4, 2.2), 5.19 (s, 2H), 3.71 (s, 2H), 2.14 ppm (s, 6H).

4-Benzyloxyindole-3-acetonitrile (7): Gramine **6** (0.76 g, 2.7 mmol) was treated with methyl iodide (11 mL, 0.18 mol) followed by a 1.1 M aqueous sodium cyanide solution (28 mL, 31 mmol) as described by Stoll *et al.*[14] The product was purified by flash chromatography ($CHCl_3$) to give 0.52 g of nitrile **7** (2.0 mmol, 74%) a white powder: **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 8.08 (br s, 1H), 7.47 (br d, 2H, J=7.3), 7.40 (br t, 2H, J=7.2), 7.36 (br t, 1H J=7.3), 7.11 (t, 1H, J=1.0), 7.10 (t, 1H, J=8.0), 6.98 (d, 1H, J=8.2), 6.57 (d, 1H, J=7.8), 5.16 (s, 2H), 4.01 ppm (d, 2H, J=1.0); **CI-MS:** (CH_4) *m/z* 264 (47), 263 (m+1, bp, 100), 262 (17), 237 (12), 236 (55), 171 (15), 146 (87), 119 (11), 91 (77%).

4-Benzyloxyindole-3-acetic acid (8): Nitrile **7** (215 mg, 0.82 mmol) was added to a solution of KOH (1.5 g), ethyl alcohol (2.0 mL), and water (2.0 mL) and heated as described by Stoll et al.,[14] The crude product was purified by flash chromatography (2:1:0.1 hexane-ethyl acetate-acetic acid) to give 182 mg of acid **8** (0.65 mmol, 80%) as an amorphous solid: **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 8.04 (br s, 1H), 7.43 (br d, 2H, J=6.8 Hz), 7.28-7.38 (m, 3H), 6.96-7.12 (m, 3H), 6.56 (d, 1H, J=7.2), 5.18 (s, 2H), 3.91 ppm (s, 2H); **FAB-MS:** *m/z* 282 (m+1).

***p*-Nitrophenyl 4-benzyloxyindole-3-acetate (9):** To a mixture of indole **8** (17.4 mg, 62 μmol) and *p*-nitrophenol (13.1 mg, 94 μmol) in CH_2Cl_2 (400 μL) was added dicyclohexylcarbodiimide (19.2 mg, 93 μmol) and stirred at room temperature for 1 h. Initially indole **8** was insoluble, but after 5 min it had completely dissolved. During the course of the reaction, the byproduct dicyclohexylurea slowly precipitated. The reaction mixture was filtered and chromatographed on silica gel (3:1 hexane-ethyl acetate) to give 25 mg of pure *p*-nitrophenol ester **9** (62 μmol, 100 %) as yellow crystals: **m.p.** 129.5-130.0°C; **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 8.1 (d, 2H, J=9.2 Hz), 7.45 (br d, 2H, J=7.8), 7.34-7.26 (m, 3H), 7.14-7.01 (m, 3H), 7.00 (dt, 1H, J=8.2, 0.6), 6.94 (d, 2H, J=9.2), 6.59 (d, 1H, J=7.8), 5.17 (s, 2H), 4.20 ppm (d, 2H, J=0.6); **EIMS** *m/z*: 402 (26%), 311 (11), 236 (30), 208 (18), 173 (29), 172 (19), 145 (61), 117 (33), 92 (22), 91 (100, bp), 90 (12), 89 (15), 65 (26), 63 (11), 43 (22), 40 (14); **HREI-MS**: $C_{23}H_{18}N_2O_5$ Calcd: 402.1216. Found: 402.1214.

1,11-Diamino-4,8-*bis*[(1,1-dimethylethoxy)carbonyl]-4,8-diazaundecane (11): Freshly distilled acrylonitrile (7.9 mL, 120 mmol) was added dropwise to a solution of 1,3-diaminopropane **10** (5.0 mL, 60 mmol) in methanol (1.0 mL) over 30 min. After stirring at room temperature for 14 h, the reaction mixture was concentrated in vacuo to give a quantitative yield of desired dicyanoethylated product as a colorless oil: **^{1}H-NMR:** δ (400 MHz, DMSO) 2.71 (t, 4H, J=6.5 Hz), 2.54 (t, 4H, J=6.3), 2.54 (t, 4H, J=6.8), 1.90 (br s, 2H), 1.50 ppm (qn, 2H, J=6.8).

To a solution of dicyanoethylated product (7.78 g, 43.2 mmol) in CH_2Cl_2 (40 mL) was slowly added di-*tert*-butyl dicarbonate (20.8 mL, 90.5 mmol) over 30 min. After stirring for 4 h at room temperature, the reaction mixture was concentrated in vacuo to yield pure di-BOC protected product as a colorless oil: **^{1}H-NMR:** δ

(400 MHz, DMSO) 3.41 (t, 4H, J=6.6 Hz), 3.16 (t, 4H, J=7.0), 2.69 (t, 4H, J=6.6), 1.67 (qn, 2H, J=6.9), 1.40 ppm (s, 18H).

A solution of the BOC protected dinitrile (3.58 g, 9.4 mmol) in THF (50 mL) was added dropwise to a suspension of $LiAlH_4$ (2.60 g, 69 mmol) in ether (200 mL). The reaction mixture was stirred 2 h at 0°C, then the excess hydride was quenched with the careful addition of 1 M NaOH. The organic layer was decanted from the aluminum salts, and the salts were washed with Et_2O (3 x 50 mL). The combined organic layers were concentrated and chromatographed (2:1:0.1 CH_2Cl_2-MeOH-$NH_{3\ aq}$) to give 1.81 g of pure 3,3,3 polyamine **11** (4.7 mmol, 50 %) as a colorless oil: **^{1}H-NMR:** δ (400 MHz, DMSO) 3.15 (t, 4H, J=6.9 Hz), 3.07 (m, 12H), 2.50 (t, 4H, J=6.8), 1.64 (qn, 2H, J=7.1), 1.53 (qn, 4H, J=6.9), 1.38 ppm (s, 18H); **HRFAB-MS:** (m+1) $C_{19}H_{41}N_4O_4$ Calcd: 389.3128. Found: 389.3130.

13-Amino-6,10-*bis*[(1,1-dimethylethoxy)carbonyl]-2,6,10-triazatridecanoic acid (1,1-dimethylethyl) ester (12): Di-*tert*-butyl dicarbonate (640 μL, 2.8 mmol) was slowly added to diamine **11** (1.18 g, 3.0 mmol) in CH_2Cl_2 (20 mL) over 20 min. After an additional 1 h, the reaction mixture was concentrated and purified by flash chromatography (10:1:0.1 CH_2Cl_2-MeOH-$NH_{3\ aq}$) to yield 872 mg of the triply protected polyamine **12** (1.79 mmol, 59 %) as a colorless oil: **^{1}H-NMR:** δ (400 MHz, DMSO) 6.74 (br s, 1H), 3.14 (t, 2H, J=7.0 Hz), 3.09 (t, 2H, J=7.3), 3.07 (m, 2H), 3.07 (t, 2H, J=7.2), 2.87 (q, 2H, J=6.5), 2.49 (t, 2H, J=6.6), 1.62 (qn, 2H, J=6.9), 1.54 (qn, 2H, J=7.3), 1.50 (qn, 2H, J=7.1), 1.37 ppm (s, 18H), 1.35 ppm (s, 9H); **HRFAB-MS:** (m+1) $C_{24}H_{49}N_4O_6$ Calcd: 489.3652. Found: 489.3664.

2,2-Dimethyl-4-oxo-3-oxa-22-phthalimido-5,9,13,17-tetraaza-docosane-5,9-dicarboxylic acid bis(1,1-dimethylethyl) ester (13): A mixture of amine **12** (439 mg, 0.90 mmol), N-(5-bromopentyl)-phthalimide[20] (277 mg, 0.94 mmol), KF-celite[19] (269 mg), and acetonitrile (6.0 mL) was heated at 60°C for 22 h. After which the mixture was filtered, concentrated, and purified by flash chromatography (15:1:0.1 CH_2Cl_2-methanol-$NH_{3\ aq}$) to produced 353 mg of the desired product **13** (0.50 mmol, 55 %) as a colorless oil: **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 7.80 (dd, 2H, J=5.4, 3.1 Hz), 7.68 (dd, 2H, J=5.4, 3.1), 3.65 (t, 2H, J=7.0), 3.0-3.4 (m, 8H), 2.55-2.75 (m, 4H), 1.6-1.8 (m, 8H), 1.43 (s, 9H), 1.41 (s, 9H), 1.40 (s, 9H), 1.00 ppm (m, 2H).

22-Amino-2,2-dimethyl-4-oxo-3-oxa-5,9,13,17-tetraazadocosane-5,9-dicarboxylic acid bis(1,1-dimethylethyl) ester (14): To a solution of phthalimide **13** (306 mg, 0.43 mmol) in methanol (4.5 mL) was added hydrazine hydrate (400 μL, 8.0 mmol) and stirred for 1 h at 60°C. The reaction mixture was then added to 4N NH_3 (20 mL) and extracted with CH_2Cl_2 (6 X 6 mL). The combined organic layers were dried with K_2CO_3, and concentrated to give 237 mg of pure 5,3,3,3 polyamine **14** (0.41 mmol, 96 %) as a colorless oil: **^{1}H-NMR:** δ (400 MHz, $CDCl_3$) 3.0-3.3 (m, 10H), 2.67 (t, 2H, J=6.9 Hz), 2.56 (t, 4H, J=7.0), 1.6 - 1.8 (m, 6H), 1.3 - 1.5 (m, 6H), 1.44 (s, 9H), 1.43 (s, 9H), 1.41 ppm (s, 9H); **FAB-MS:** *m/z* 574 (m+1).

25-[4-Benzyloxy-1H-indol-3-yl]-2,2-dimethyl-4,24-dioxo-3-oxa-5,9,13,17,23-pentaazapentacosane-9,13-dicarboxylic acid bis(1,1-dimethylethyl) ester (15). A solution of 4-benzyloxyindole *p*-nitrophenol ester (**9**) (164 mg, 0.42 mmol) in dry DMF (2.0 mL) was added to polyamine **14** (237 mg, 0.41 mmol) and stirred at ambient temperature for 1 h. The reaction mixture was then added to CH_2Cl_2 (50 mL), washed with 1N NaOH (4 x 20 mL), brine (20 mL), dried with K_2CO_3, concentrated in vacuo, and purified by flash chromatography (15:1:0.1 CH_2Cl_2-MeOH-NH_3 aq) to give 272 mg of pure product **15** (0.33 mmol, 80 %): **^{1}H-NMR:** δ (300 MHz, $CDCl_3$) 7.32-7.60 (m, 5H), 7.00-7.13 (m, 3H), 6.59 (d, 1H, J=8 Hz), 6.04 (br s, 1H), 5.19 (s, 2H), 3.77 (s, 2H), 3.04-3.27 (m, 12H), 2.99 (q, 2H, J=6.5), 2.50 (t, 1H, J=6.9), 2.37 (br t, 1H, J=6.3) 1.56-1.79 (m, 6H), 1.43 (s, 9H), 1.42 (s, 18H), 1.30 (m, 2H), 1.19 (m, 2H), 1.03 ppm (m, 2H); **FAB-MS:** *m/z* 837 (m+1).

CNS 2103 or N-(17-amino-6,10,14-triazaheptadec-1-yl)-4-hydroxy-1H-indole-3-acetamide (16): To a solution of compound **15** (55 mg, 66 μmol) in degassed CH_2Cl_2 (1.0 mL) was added degassed trifluoroacetic acid (1.0 mL). The reaction mixture was stirred at ambient temperature for 30 min and then concentrated under a stream of N_2. The crude product was then dissolved in degassed methanol (0.5 mL) and degassed water (0.5 mL). To this mixture was added $Pd(OH)_2$-carbon (11.8 mg, 20% Pd) and stirred under 1 atm of hydrogen for 1 h. The product was then filtered through a 0.45 mm filter while being kept under an atmosphere of N_2, and then concentrated with a Speed Vac to give a quantitative yield of pure product **16** as a fine white powder: **^{1}H-NMR:** δ (300 MHz, D_2O) 7.03 (s, 1H), 6.94 (d, 1H, J=4.3 Hz), 6.93 (d, 1H, J=3.9),

6.38 (dd, 1H, J=4.3, 3.9) 3.63 (s, 2H), 2.90-3.05 (m, 12H), 2.85 (t, 2H, J=8.3), 2.64 (t, 2H, J=8.0), 1.83-2.02 (m, 6H), 1.38 (qn, 2H, J=7.8), 1.29 (qn, 2H, J=7.2), 1.02 ppm (qn, 2H, J=7.7) ; **FAB-MS:** *m/z* 447 (m+1).

Acknowledgment. We wish to thank Dr. Braden Roach, Pei-Ying Gong, and Cheri Morgan for their help. The partial support of this project by SBIR grant GM 41547, NIH grant AI 12020 and NIH Traineeship GM 07273 is gratefully acknowledged.

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