Structure Determination, Pharmacological Evaluation, and Structure-Activity Studies of a New Cyclic Peptide Substance P Antagonist Containing the New Amino Acid 3-Prenyl-β-hydroxytyrosine, Isolated from *Aspergillus flavipes*

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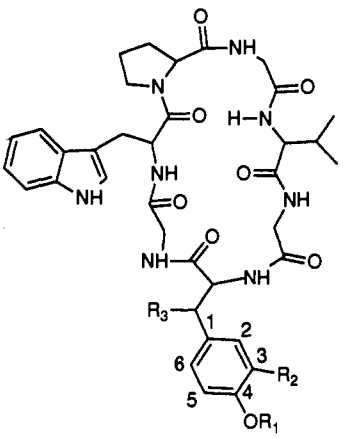
Two novel cyclic heptapeptides, peptides **1a** and **1c**, were isolated from an *Aspergillus flavipes* culture, originally isolated from soil, and their structures established by chemical and spectroscopic evidence. Peptide **1a** contains a new amino acid, 3-prenyl-β-hydroxytyrosine, and is a competitive antagonist to substance P at the human NK1 receptor, with an inhibitor affinity constant (K_i) of $8 \pm 4\ \mu M$. Methylation of **1a** gave the monomethyl derivative **1b**, which is a more potent competitive antagonist, with a K_i of $0.12 \pm 0.03\ \mu M$ at the human NK1 receptor. Herein we report the structure determinations of **1a** and **1c**, and some structure-activity results. Several analogs of **1a** were prepared by derivatization and synthesis. Structure-activity results for these analogs confirmed that the 3-prenyl-β-hydroxytyrosine moiety is critical for the biological potency of **1a** and **1b**.

Substance P (SP) is the most well studied member of the neurokinin family, a family of peptide neurotransmitters that are characterized by a common C-terminal sequence. SP and its receptor, the neurokinin-1 receptor (NK1), are widely distributed in both the central and peripheral nervous systems. Although the exact physiological role of SP is not well defined, this peptide is involved in numerous physiological activities, such as vasodilation,[1] smooth muscle contraction,[2] and stimulation of salivary secretion.[3] One area in which the importance of SP has been clearly demonstrated is in pain and inflammation.[4]

Some disease states in which evidence for the involvement of SP has been explored most extensively are arthritis[4] and inflammatory bowel disease.[5] It is believed that a SP antagonist is potentially useful as a nonnarcotic analgesic or as an antiinflammatory agent. We have used a natural products screening approach in an attempt to find small molecules with high affinity for the NK1 receptor.[6] This approach has now led to the discovery of the SP antagonist **1a** (WIN 66306) and the related compound **1c** (WIN 68577). Herein we report the structure determinations of **1a** and **1c**, the formation of the more potent derivative **1b**, the pharmacological evaluation of these compounds, and structure-activity studies.

Structure Determination of Cyclic Peptides 1a and 1c

From an ethyl acetate extract of whole culture fermentation broths of *Aspergillus flavipes*, SC230,[7] **1a** was isolated as a white solid. The UV spectrum of **1a** indicated the presence of an indole moiety and amide bonds. Quantitative amino acid analysis and Marfey's derivatization showed the presence of three glycines, one L-valine, one L-tryptophan, and one L-proline per molecule of **1a**. Sequence determination by Edman degradation for **1a** gave no sequence, suggesting an N-terminal blocked or cyclic peptide. The molecular formula of **1a** was determined to be $C_{41}H_{52}N_8O_9$ by high-resolution FAB mass spectrometry. Low-resolution positive-ion FAB spectra gave an intense ion at 611 Da, shown by MSMS to be a daughter ion from the MH^+ parent at 801 Da.



	R1	R2	R3
1a	H	prenyl	OH
1b	Me	prenyl	OH
1c	H	H	OH
1d	Me	H	OH
1e	Me	2-me-butyl	OH
1f	Me	2-me-butyl	H
2a	t-butyl	H	H
2b	Me	H	H
2c	H	H	H
3[a]	t-butyl	H	H
4[b]	*S*-tryptophan		

[a] α-C of tyrosine analog residue has *R* stereochemistry
[b] tryptophan replaces tyrosine related residue

Reductive cleavage of **1a** in base with sodium borohydride, in an attempt to selectively cleave at the N-terminal of proline, gave four major cleavage products. The most abundant product was confirmed by amino acid analysis and MSMS sequencing to be cyclic-Pro-Gly-Val-Gly-Gly-Gly-Trp-, which contains one more glycine than observed for **1a**, and has a molecular ion at 611 Da. The remaining three degradation products were amenable to Edman sequencing. The first gave the sequence Pro-Gly-Val-Gly, the second the sequence Pro-Gly-Val-Gly-Gly-Gly, and the third the sequence X-Gly-Trp-Pro-Gly-Val. For the latter peptide no standard PTH-amino acid was detected in the first degradation cycle, but the second cycle clearly revealed PTH-glycine. These results confirm that **1a** is a cyclic peptide of the sequence -X-Gly-Trp-Pro-Gly-Val-Gly-, where X is a nonstandard amino acid with the formula $C_{14}H_{17}NO_3$. NMR data from a HMBC experiment (Figure

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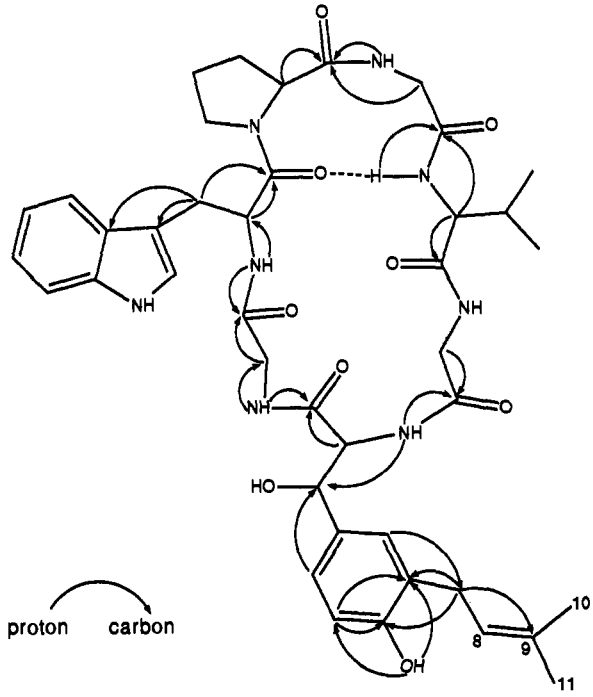
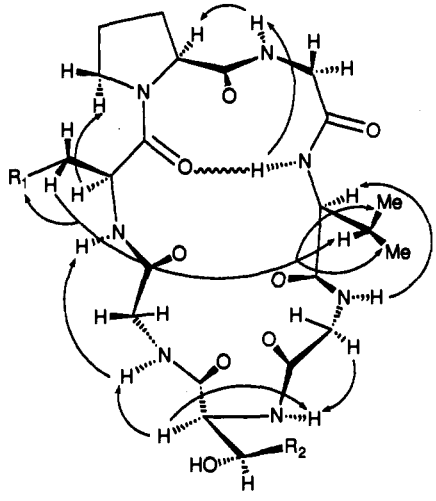


Figure 1. Selected long-range ^1H–^{13}C coupling observed from an HMBC experiment for WIN 66306 (**1a**).



R_1= indole; R_2=3-prenyl-phenol

Figure 2. Selected NOE data from ROESY and HMQC-NOESY experiments for WIN 66306 (**1a**). This NOE data gives structural conformational information for **1a**, including establishing the presence of a Trp-Pro-Gly-Val type II β-turn.

1) and from ROESY experiments (Figure 2) were consistent with the above sequence.

The ^1H and ^{13}C NMR spectra of **1a** contained resonances that were assigned to three glycines, one valine, one proline, and one tryptophan residue (Tables 1 and 2). The remaining resonances were assigned to a substituted tyrosine, with the aid of two-dimensional NMR experiments. A ^1H COSY experiment showed a connected spin system from the substituted tyrosine NH resonance through the αH and βH resonances to an exchangeable doublet at 5.58 ppm (βOH), consistent with the presence of a β-hydroxy amino acid. A relayed COSY showed a correlation from the βH resonance to an aromatic doublet at 6.96 ppm (H6), which was further coupled to another aromatic doublet at 6.70 ppm (H5) and an aromatic singlet at 6.96 ppm (H2). The presence of H2 as a singlet, and a long-range C-H connectivity from H5 to a quaternary

Table 1. ^1H NMR Data for **1a**, **1b**, and **1c**

position		^1H (J, Hz)		
		1a	**1b**	**1c**
Pro[1]	αH	4.10m	4.11m	4.07m
	βHh	1.79m	1.78m	1.78m
	βH1	2.10m	1.09m	2.05m
	γHh	1.80m	1.79m	1.80m
	γH1	1.95m	1.95m	1.95m
	δHh	3.24m	3.22m	3.26m
	δH1	3.49m	3.49m	3.50m
Gly[2]	NH	8.90dd (8.0, 4.6)	8.91dd (7.8, 4.6)	8.97dd (7.2, 4.5)
	αHh	3.38dd (17.0, 4.6)	3.37m	3.36m
	αH1	4.10m	4.08dd (17.1, 7.7)	4.08m
Val[3]	NH	8.02d (10.2)	8.03d (10.4)	8.08d (10.5)
	αH	4.00t (10.2)	3.97t (10.5)	3.93t (10.4)
	βH	1.62m	1.61m	1.53m
	γHa	0.40d (6.4)	0.37d (6.5)	0.30d (6.5)
	γHb	0.55d (6.4)	0.53d (6.5)	0.47d (6.5)
Gly[4]	NH	8.08dd (4.0, 3.0)	8.09bs	8.11bw
	αHh	3.70dd (18.0, 3.0)	3.69dd (17.5, 3.0)	3.70m
	αH1	4.11dd (18.0, 4.0)	4.12dd (17.5, 4.2)	4.10m
βRTyr[5]	NH	8.62d (4.6)	8.74bs	9.12bs
	αH	3.98dd (7.5, 4.6)	4.00m	3.96m
	βH	4.70dd (7.5, 4.6)	4.75d (7.5)	4.68d (8.2)
	βOH	5.58d (4.6)		
	H2	6.97s	7.05s	7.09d (8.6)
	H3			6.68d (8.6)
	H4	9.17s (OH)	3.75s (Me)	
	H5	6.70d (9.0)	6.86d (8.5)	6.66d (8.6)
	H6	6.96d (9.0)	7.12d (8.4)	7.09d (8.6)
	H7	3.18t (6.5)	3.20t (6.7)	
	H8	5.25tt (6.5, 1.4)	5.21bt (6.7)	
	H10	1.68s		
	H11	1.70s		
Gly[6]	NH	8.45t (6.5)	8.49t (6.4)	8.54t (6.0)
	αHh	3.34dd (17.0, 6.5)	3.34m	3.35m
	αH1	3.67dd (17.0, 6.5)	3.68dd (17.0, 6.4)	3.64m
Trp[7]	NH	7.85d (9.0)	7.85d (8.8)	7.94d (8.8)
	αH	4.88dt (4.3, 9.3)	4.87dt (4.5, 9.0)	4.87dt (4.2, 9.2)
	βHh	2.77dd (14.5, 4.3)	2.78dd (14.5, 4.0)	2.71dd (14.7, 4.7)
	βH1	2.91dd (14.5, 9.3)	2.91dd (14.3, 9.0)	2.89dd (14.7, 9.2)
	NH	10.88d (1.2)	10.89s	10.92s
	H2	7.10d (1.2)	7.08s	7.07s
	H4	7.50d (8.0)	7.49d (7.9)	7.46d (7.8)
	H5	6.98t (8.0)	6.96t (7.4)	6.95t (7.2)
	H6	7.06t (8.0)	7.05t (7.1)	7.04t (7.4)
	H7	7.30d (8.0)	7.28d (8.2)	7.28d (8.0)

carbon signal at 126.64 (C3), established that the β-hydroxytyrosine has a substituent of C3. One- and two-dimensional NMR experiments clearly established the presence of a prenyl group, while a long-range COSY correlation from H2 to H7, and HMBC correlations from C2 to H7, and from H7 to C3, C4 and C2, established that the prenyl group is the meta substituent of the β-hydroxytyrosine (Figure 1).

The UV spectrum of **1c** was almost identical to that of **1a**, indicating that these compounds are structurally related. Amino acid analysis showed the presence of three glycines, one valine, one proline, and one tryptophan residue per molecule of **1c**. Resonances for these amino acids were readily identified in the ^1H and ^{13}C NMR spectra of **1c**. The molecular formula of **1c** was determined to be $C_{36}H_{44}N_8O_9$ by high-resolution FAB mass spectrometry. Low-resolution positive-ion FABMS and FABMSMS spectra gave similar fragments to those observed in spectra of **1a**, including an intense signal at 611 Da, indicating the same amino acid sequence for **1a** and **1c**. NMR spectral data also supported the same amino acid sequence (Tables 1 and 2, Figure 1 and 2), and circular dichroism spectra indicated the same relative and absolute stereochemistry for **1a** and **1c**. ^1H and ^{13}C NMR and COSY spectra for **1c** indicated the presence of an unsubstituted β-hydroxytyrosine moiety (Tables 2 and 3). Therefore **1c** differs

Table 2. ^{13}C NMR Data for 1a, 1b, and 1c

position		^{13}C 1a	1b	1c
Pro[1]	αC	61.03	61.08	61.12
	βC	28.82	28.88	28.92
	γC	24.54	24.59	24.61
	δC	47.24	47.29	47.31
	CO	171.16	171.27	171.25
Gly[2]	αC	42.40	42.46	42.50
	CO	168.40	168.49	168.50
Val[3]	αC	60.34	60.40	60.50
	βC	31.61	31.65	31.66
	δCa	18.63	18.66	18.68
	δCb	19.02	19.06	19.06
	CO	170.49	170.58	170.56
Gly[4]	αC	42.75	42.77	42.77
	CO	169.20	169.35	169.32
βR-Tyr[5]	αC	61.89	61.87	62.20
	βC	71.50	71.37	71.44
	C1	131.64	133.17	131.67
	C2	127.74	127.64	127.83
	C3	126.64	128.36	114.60
	C4	154.04	156.13	156.64
	C5	114.22	109.92	114.60
	C6	124.82	125.23	127.83
	C7	28.01	28.15	
	C8	122.97	122.67	
	C9	130.74	131.23	
	C10	17.58	17.60	
	C11	25.48	25.54	
	CO	170.38	170.44	170.56
	OMe	53.30		
Gly[6]	αC	42.47	42.50	42.40
	CO	168.77	168.85	168.89
Trp[7]	αC	50.55	50.58	50.52
	βC	27.62	27.66	27.64
	C2	122.94	123.01	122.96
	C3	109.00	109.05	109.11
	C4	117.62	117.69	117.69
	C5	118.13	118.21	118.20
	C6	120.78	120.86	120.86
	C7	111.04	111.11	111.11
	C8	127.01	127.06	127.07
	C9	135.74	135.81	135.81
	CO	169.09	169.18	169.14

Table 3. Ability of 1a–4 To Displace [^{125}I]SP Binding to Human Astrocytoma Cells (NK1 Binding) and To Displace [^{125}I]NKA Binding to Human Urinary Bladder Membrane Protein (NK2 Binding)[a]

compd	K_i (μM) NK1	NK2
1a	8 ± 4	3.8 ± 2
1b	0.12 ± 0.03	1.6 ± 0.4
1c	>100	>100
1d	18	33
1e	0.12 ± 0.02	2.4
1f	2.5	ND[b]
2a	93	31
2b	72	73
2c	>100	>100
3	48	27
4	>100	>100

[a] K_i values are averages of from two to eight determinations. Assays were performed as previously described.[22] Unlabeled SP as a NK1 reference standard gave a K_i of 0.16 nM. [Nle[10]]NKA(4–10) as a NK2 reference standard gave a K_i of 13 nM. [b] Not determined.

from 1a by the absence of the prenyl substituent on the β-hydroxytyrosine moiety.

Reaction of 1c with triethylsilane in TFA gave a compound with the same HPLC retention time, CD, and mass spectrum as that obtained for the synthetic peptide 2a. This defined the α-carbon chirality of the β-hydroxytyrosine in 1c as *S*. Similarity of the NMR and CD spectra

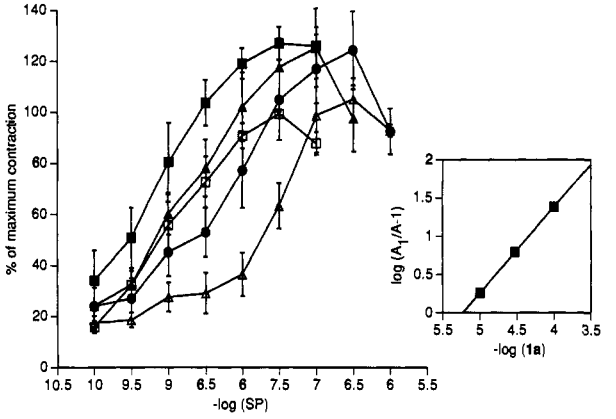


Figure 3. Concentration–response curves for SP-induced contractions of GP ileum in the absence (□) and the presence of 1a at 3 μM (■), 10 μM (▲), 30 μM (●), and 100 μM (△). Each point indicates the mean ± SEM from four experiments. Data obtained from concentration–response curves were plotted as $\log(A_1/A - 1)$ vs −log[1a] (inset), where A and A_1 represent 50% effective concentration of SP on GP ileum contraction in the absence and in the presence of 1a, respectively.

for 1a to that obtained for 1c indicates that the α-carbon of the *m*-prenyl-β-hydroxytyrosine in 1a also has the *S* chirality, although this chirality was not confirmed. The chirality at the β-carbon remains undefined for both 1a and 1c.

NOESY and ROESY spectra obtained in d_6-DMSO solutions gave information about the solution conformation of 1a. Only one set of peaks was observed in the ^1H NMR spectrum, and reasonably intense interresidue NOEs were observed for 1a, indicating that a single conformation dominates in solution. A D_2O exchange experiment showed that the valine amide proton undergoes significantly slower exchange than do the other amide protons, indicating involvement of the valine amide proton in a hydrogen bond. NOEs from the β-protons of tryptophan to the β-protons of valine, and from the NH proton of glycine-2 to the slowly exchanging valine amide proton, indicated that a type I or type II β-turn exists with proline at position 2 of this turn. A type II turn is indicated by the NOE data (Figure 3), although overlap of the αH proline signal with one of the glycine methylene proton signals made this assignment ambiguous. However, $^3J_{NH\alpha}$ coupling constants for glycine-2 of 4.6 and 8.0 Hz were consistent with φ angles of 90° and −150°, as expected for a type II turn.[8] The coupling constant and NOE data were not consistent with a type I turn. To confirm the presence of the type II turn an HMQC-NOESY[9] experiment was performed. This clearly showed an intense NOE between the glycine-2 amide proton and the alpha proton of the proline, consistent only with the type II turn.[8]

Other interresidue NOEs giving conformational information for 1a included an NOE from the αH of tryptophan to the δH2 of proline, which defined the proline amide bond as being in the standard trans conformation. Further αH_i to NH_{i+1} NOEs defined trans conformations for the amide bonds between valine and glycine-4 and between the substituted tyrosine (βR-Try) and glycine-6. NOEs observed between the tryptophan and valine side chains indicated that these side chains were close in space (Figure 3). The absence of NOEs from the peptide backbone to the βR-Tyr side chain and coupling constants of 7.5 Hz between the αH and βH of 1a suggests that this side chain has some rotational mobility in solution. Similar NOE

results were obtained from NOESY spectra of **1b**, indicating that methylation of the βR-Tyr phenolic oxygen has little influence on the solution conformation of this peptide.

Structure–Bioactivity Studies

The structure of other known substance P antagonists indicates that hydrophobic aromatic groups are important for binding at the NK1 receptor.[10] Therefore, in an attempt to increase binding potency, **1a** was methylated with diazomethane to give **1b**, which has increased hydrophobicity at the new amino acid (βR-Tyr). Bioassay data showed that **1b** was approximately 60 times more potent than **1a**, confirming that βR-Tyr is important for biological potency.

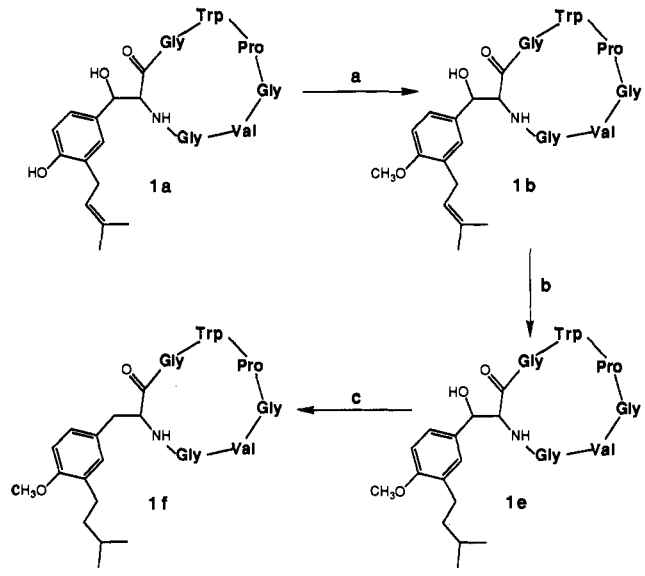
The presence of a β-hydroxy group and a *m*-prenyl group in **1b** makes this a difficult molecule to synthesize by traditional methods. In an attempt to obtain a simplified analog which still retained the potency of **1a**, synthetic peptides were prepared which contained *S*-tyrosine (**2c**) *S*-methyltyrosine (**2b**), *S-tert*-butyltyrosine (**2a**), *R-tert*-butyltyrosine (**3**), or *S*-tryptophan (**4**) in place of βR-Tyr. These amino acids were chosen because of their similarity to βR-Tyr. The alkylated tyrosine analogs were prepared to resemble the hydrophobicity of the methylated and prenylated βR-Tyr of **1b**.

The general synthetic procedure used to prepare the cyclic peptides **2a** to **4** firstly required manual solid-phase synthesis of the linear peptide Trp-Pro-Gly-Val-Gly-X-Gly, where X was a Tyr analog or Trp. This sequence was chosen to eliminate the possibility of C-terminal racemization during cyclization. Also, this sequence avoided low yields due to diketopiperazine formation, which occurred if Val and Gly or Pro and Gly were coupled in the first synthetic cycle. Peptides were synthesized containing *tert*-butyl-protected tyrosine and free tryptophan and cyclized at low dilution using BOP active esters.[11] Peptides were extracted with ethyl acetate and purified using reversed-phase HPLC. A portion of **2a** was deprotected with TFA in dichloromethane to give **2c**, and a portion of **2c** was subsequently methylated with diazomethane to give **2b**. The NK1 and NK2 receptor binding data for these synthetic peptides are listed in Table 3. None showed activity better than 25 μM, indicating that the prenyl and/or the β-hydroxy group are necessary for the potency observed for **1b**.

To determine the importance of the *m*-prenyl substituent for the potency of **1b**, **1c**, which lacks this group, was methylated with diazomethane to give **1d**. Because **1d** differs from **1b** only by the absence of a *m*-prenyl substituent, the difference in biological activity for these compounds should give the contribution of the *m*-prenyl group to binding potency. The NK1 binding activity of **1d** was 18 μM, while that of **1b** was 0.12 μM, indicating that the prenyl group contributes to binding potency by greater than a factor of 100. The NK2 binding for **1d** was 33 μM, compared to 1.6 μM for **1b**, indicating that the prenyl group also contributes significantly to the binding potency at the NK2 receptor.

The importance of the β-hydroxy group to the potency of **1b** was determined by first hydrogenating the prenyl double bond, using hydrogen over 10% palladium-on-carbon, and subsequently removing the β-hydroxy group with triethylsilane in trifluoroacetic acid and dichloromethane (Scheme 1). The hydrogenation step gave **1e**, which showed substance P binding inhibition similar to that of **1b**. Removal of the β-hydroxy group gave **1f**, which was approximately 25 times less potent than **1e** and **1b**. These results indicate that both the *m*-prenyl group and the β-hydroxy group are necessary for the potent substance P antagonism of **1b**.

Scheme 1[a]



[a] Reagents: (a) $TMSCHN_2$; (b) H_2, Pd/C; (c) $(Et)_3SiH$/TFA, 0 °C.

Pharmacological Activity

Both **1a** and **1b** appear to be competitive antagonists of SP at both the guinea pig (GP) and human NK1 receptors. Peptide **1a** displaced [^{125}I]SP binding to human astrocytoma cells with a median inhibition concentration (K_i) of 8 ± 4 μM. The corresponding K_i values for **1b** and **1e** were 0.12 ± 0.03 and 0.12 ± 0.02 μM, respectively. The K_i values for binding to the human NK2 receptor were 3.8 μM and 1.6 μM for **1a** and **1b**, respectively. The NK1 and NK2 binding potency of other analogs and derivatives of **1a** are given in Table 3.

To confirm functional activity for **1a** in SP-mediated biological events, **1a** was tested in a guinea pig ileum contractility model.[12] Peptide **1a** dose-dependently inhibited SP-induced contraction of GP ileum (NK1), with the contractility curves being shifted to the right in parallel (Figure 3). A Schild analysis of the data[13] gave a pA_2 of 5.23 ± 0.3 μM and a slope not significantly different from unity (Figure 3, inset). This indicates that the mechanism of **1a** antagonism may be competitive. Peptide **1b** also behaves as a functional SP antagonist, decreasing the SP induced Ca^{2+} efflux in human astrocytoma cells with an IC_{50} of 430 ± 40 nM (Figure 4).

Discussion

Peptide **1a**, a novel and competitive antagonist to substance P at the NK1 and NK2 receptor, was isolated from *Aspergillus flavipes*. The structure of **1a**, and the related natural product **1c**, was determined by spectroscopic and chemical means and shown to contain a new tyrosine-related amino acid. Conversion of **1a** to **1b** by methylation of the novel tyrosine-related amino acid resulted in a 60-fold increase in biological activity. Several related synthetic analogs and derivatives of **1b** were prepared for structure–activity information, confirming the importance of both the *m*-prenyl and β-hydroxy groups

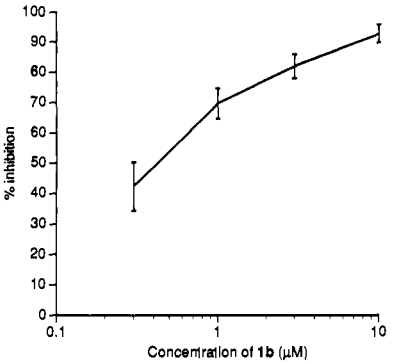


Figure 4. Ability of **1b** to inhibit SP (3 nM) induced $^{45}Ca^{2+}$ efflux from human astrocytoma cells.[22] Each data point represents the mean ±SEM from at least three experiments, after subtracting the efflux observed in the absence of SP and antagonist.

to the biological potency of **1b**. NOE data for **1a** and **1b** confirmed the presence of a type II Trp-Pro-Gly-Val β-turn in the solution structure of these peptides.

Recently, several substance P antagonists have been discovered. Most have at least two large aromatic groups which appear to be important for biological potency.[6,14-17] In some cases biological potency tends to increase with the presence of an additional hydrophobic substituent on one of these aromatic groups,[14,15] and it has been suggested that hydrophobicity and not aromaticity is necessary for binding potency.[6] For peptide **1b** both the tyrosine methyl and prenyl groups contribute significantly to biological potency, consistent with a strong hydrophobic interaction at this amino acid with the NK1 receptor. Hydrophobicity appears to be less important at the NK2 receptor, where peptides **1a**, **1b**, **1d**, and **1e** all have similar biological potency (Table 1). From comparison with the structures of other SP antagonists, where two or more aromatic moieties contribute to NK1 activity, it is likely that the tryptophan side chain and perhaps the valine side chain also contribute to the biological activity of **1b**. Further work is necessary to determine the actual contribution that these side chains make to the biological potency of **1b**.

The recent synthesis of a nonpeptidal peptidomimetic SP antagonist, using a glucose scaffold with an indole and two benzyl containing side chains,[16] indicates that the peptide backbone of **1b** may not be important for biological activity. The known solution structure of **1b** and the presence of three glycines and only seven amino acids makes a peptidomimetic approach particularly appealing for the production of further analogs of **1b**.

Experimental Section

General. All chemicals were reagent grade and used as received unless otherwise specified. One- and two-dimensional NMR spectra were recorded on Bruker AMX360 or AMX500 spectrometers. Chemical shifts are given in δ (ppm) and were recorded in dimethyl-d_6 sulfoxide using the solvent signals as reference. CD curves were measured on a JASCO J-600 spectropolarimeter. UV traces were recorded on a Shimadzu UV160U spectrometer. IR spectra were recorded on a Nicolet IBM IR/3X spectrometer. MS and MSMS were performed on a Finnigan MAT TSQ 70 mass spectrometer, and HRMS was obtained using a VG analytical ZAB 2-SE high-field mass spectrometer. Photodiode array HPLC was performed on a Waters system.

Isolation of Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl)-Gly-Trp (1a) and Cyclic Pro-Gly-Val-Gly-Tyr(β-OH)-Gly-Trp (1c). Whole culture fermentation broth of SC230 (1 L) was extracted with ethyl acetate (1 L, 2×). The dried ethyl acetate extract (1.2 g) was then solvent partitioned using hexane/ethyl acetate/methanol/water (5:6:5:3). Solvent was removed from the aqueous lower layer to give an oil (405 mg) which was further separated using centrifugal countercurrent chromatography with a chloroform/methanol/water (4:4:3) solvent system. Two biologically active fractions were obtained. Further purification using HPLC on an ODS C-18 reverse-phase column (YMC) with an isocratic (acetonitrile/water, 35:65) solvent system, gave two pure compounds. The least polar compound **1a** was obtained as a white solid (23 mg): UV μ_{max} (MeOH) 208.5 (ε 44 500), 224.0 (48 400), 281.0 (8400), 289.5 nm (6200); IR 3305, 2965, 2880, 1650, 1570, 1450, 1270, 1030, 740 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables I and II; HRFABMS MH$^+$ 801.3903 ($C_{41}H_{53}N_8O_9$ requires 801.3870); FABMSMS (801) 782 ($-H_2O$), 610 ($-C_{12}H_{15}O_2$), 425 (-Trp), 368 (-TrpGly), 311 (-TrpGlyGly), 254 (-TrpGlyGlyGly), 155 (base peak, -TrpGlyGlyGlyVal); CD max^m 200 nm ([ϑ] 72 000), 225 (28 600), 251.0 (-400), 283.0 (-1350), 290.2 (-650), min^m 215.5 (-1200), 240.0 (-1700), 275.0 (-2000), 285.8 (-1900), 294.2 (-1500) (MeOH); $[\alpha]_D$ = +30.1 (*c* 0.98, MeOH); mp 160-164 °C; amino acid analysis (AAA) 1.1 *S*-Val, 3.0 Gly, 1.0 *S*-Pro, 0.8 *S*-Trp. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 98% (t_R 21.4 min).

The more polar compound **1c** was also obtained as a white solid (2.0 mg): UV λ_{max} (MeOH) 207.5 (ε 42 400), 223.5 (46 500), 280.5 (8050), 289.0 (5800); IR 3300, 2970, 1650, 1570, 1440, 1270, 1020, 740 cm^{-1}; ^{1}H and ^{13}C NMR spectra, Tables I and II; HRFABMS MH$^+$ 733.3337 ($C_{36}H_{45}N_8O_9$ requires 733.3252); FABMS 733 (MH$^+$), 714 ($-H_2O$), 610 ($-C_7H_7O_2$), 425 (-Trp), 368 (-TrpGly), 311, 254, 155 (base peak); CD max^m 226.2 nm ([ϑ] 25 500), 288.5 (-150), 250.2 (-100), min^m 216.8 (-7500), 294.2 (-850), 271.0 (-1300), 238.0 (-700) (MeOH); $[\alpha]_D$ = +35.5 (*c* 0.05, MeOH); mp 185-190 °C; AAA 1.0 Val, 2.9 Gly, 1.1 Pro, Trp (not quantitated). Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 95% (t_R 16.1 min).

Sequence Determinations for Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl)-Gly-Trp (1a). A solution of **1a** in 1:1 methanol/sodium hydroxide (0.1 N) with excess sodium borohydride was stirred for 4 h at 35 °C. Four products were separated and purified by C18 reversed-phase HPLC and then analyzed by amino acid analysis, Edman degradation, and MSMS sequencing. The four products were confirmed to be (1) cyclic Pro-Gly-Val-Gly-Gly-Gly-Trp, (2) Pro-Gly-Val-Gly, (3) X-Gly-Trp-Pro-Gly-Val, and (4) X-Gly-Trp-Pro-Gly-Val-Gly, where X is a nonstandard amino acid.

Sequence Determination for Cyclic Pro-Gly-Val-Gly-Tyr-(β-OH)-Gly-Trp (1c). Compound **1c** was methylated as described below and the β-hydroxy group removed with TFA/triethylsilane, as described below for **1e**, which afforded a white powder with CD, mass spectral data, and HPLC retention time identical to that of synthetic **2c**.

Chirality Determinations for Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl)-Gly-Trp (1a) and Cyclic Pro-Gly-Val-Gly-Tyr(β-OH)-Gly-Trp (1c). Mild amino acid hydrolysis was performed on **1a** and the mixture derivatized with Marfey's reagent.[18] By comparison with *S* and *R* derivatized standards, it was confirmed that **1a** contained *S*-Pro, *S*-Val, and *S*-Trp. In a similar way it was confirmed that **1c** also contained *S*-Pro, *S*-Val, and *S*-Trp. Conversion of **1c** to **2c** confirmed the α-carbon chirality of the β-hydroxytyrosine as *S*.

Methylation of Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl)-Gly-Trp (1a) To Give Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl, 4-methyl)-Gly-Trp (1b). Compound **1a** (20 mg) was dissolved in methanol (4 mL), and (trimethylsilyl)diazomethane (10 wt % in methylene chloride) (1 mL) was added. The solution was left overnight and the solvent removed under nitrogen. Purification using HPLC as above, with an isocratic (acetonitrile/water, 42:58) solvent system, gave **1b** as a white solid (yield 85%): UV λ_{max} (MeOH) 210.2 (ε 55 540), 221.4 (58 080), 281.4 (9580), 289.8 (6420), λ_{min} 212.8 (55 180), 248.4 (2870) 288.2 (6290); IR 3270, 2960, 2930, 1740, 1550, 1450, 1250, 1100, 1050, 1010, 760, 740 cm^{-1}; ^{1}H and ^{13}C NMR spectra, Tables 1 and 2; HRFABMS MH$^+$ 815.4092 ($C_{42}H_{55}N_8O_9$ requires 815.4092); CD max^m 197.0 nm ([ϑ] 10 400), 225.5 (30 800), 249.4 (-335), 283.0 (-1260), 290.0 (+122); min^m 214.4 (2400), 242.2 (-930), 270.0

(−2490), 284.4 (−1555), 295.0 (−1240) (MeOH); $[\alpha]_D$ = +45.2 (*c* 0.35, MeOH); mp 124–128 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 99% (t_R 24.5 min).

Methylation of Cyclic Pro-Gly-Val-Gly-Tyr(β-OH)-Gly-Trp (1c) To Give Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 4-methyl)-Gly-Trp (1d). Compound 1c (2 mg) was dissolved in methanol (2 mL), and (trimethylsilyl)diazomethane (10 wt % in methylene chloride) (0.5 mL) was added. The solution was left overnight and the solvent removed under nitrogen. Purification using HPLC as above, with an isocratic (acetonitrile/water, 30:70) solvent system, gave 1d as a white solid (yield 75%). UV λ_{max} (MeOH) 209.0 (ε 52 600), 222.8 (56 100), 283.5 (8850), 289.4 (6200); IR 3300, 2980, 1645, 1580, 1440, 1260, 1020, 735 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.05 (Pα, m), 1.78 (Pβ, m), 2.08 (Pβ, m), 1.78 (Pγ, m), 1.95 (Pγ, m), 3.25 (Pδ, m), 3.49 (Pδ, m), 8.88 (GNH, dd, *J* = 7.2, 4.4 Hz), 3.36 (Gα, m), 4.05 (Gα, m), 8.00 (VNH, d, *J* = 10.4 Hz), 4.00 (Vα, t, *J* = 10.3 Hz), 1.63 (Vβ, m), 0.41 (Vγ, d, 6.3), 0.55 (Vγ, d, 6.5), 8 .05 (GNH, d, *J* = 4.8 Hz), 3.68 (Gα, m), 4.08 (Gα, m), 8.64 (YNH, bs), 4.03 (Yα, m), 4.79 (Yβ, t, *J* = 4.8 Hz), 5.67 (YβOH, d, *J* = 4.6 Hz), 7.24 (2H, Y2,6, d, *J* = 8.6 Hz), 6.86 (2H, Y3,5, d, *J* = 8.6 Hz), 3.73 (3H, Y4, s), 8.46 (GNH, t, *J* = 5.8 Hz), 3.35 (Gα, m), 3.62 (Gα, m), 7.82 (WNH, d, *J* = 8.7 Hz), 4.87 (Wα, dt, *J* = 4.7, 9.0 Hz), 2.78 (Wβ, dd, *J* = 4.3, 14.5 Hz), 2.91 (Wβ, dd, *J* = 14.5, 9.0), 10.87 (W1, s), 7.10 (W2, s), 7.49 (W4, d, *J* = 7.8 Hz), 6.97 (W5, t, *J* = 7.4 Hz), 7.05 (W6, t, *J* = 7.5 Hz), 7.28 (W7, d, *J* = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 61.12 (Pα), 28.92 (pβ), 24.55 (Pγ), 47.26 (Pδ), 171.25 (PCO), 42.45 (Gα), 168.50 (PCO), 60.32 (Vα), 31.59 (Pβ), 18.64 (Pγ), 19.04 (Pγ), 170.56 (PCO), 42.45 (Gα), 169.38 (GCO), 61.90 (Yα), 71.32 (Yβ), 133.45 (Y1), 127.82 (Y2,6), 113.19 (Y3,5), 158.53 (Y4), 55.34 (OMe), 170.30 (YCO), 42.45 (Gα), 168.89 (GCO), 50.57 (Wα), 27.60 (Wβ), 123.03 (W2), 109.00 (W3), 117.63 (W4), 118.17 (W5), 120.80 (W6), 111.08 (W7), 127.04 (W8), 135.78 (W9), 169.20 (WCO); HRFABMS MH$^+$ 746.3380 ($C_{37}H_{46}N_8O_9$ requires 746.3388); CD max^m 227.0 nm ([ϑ] 26 660), 289.5 (−200), 250.0 (−95), min^m 217.0 (−8400), 294.0 (−920), 272.0 (−1340), 239.0 (−750); $[\alpha]_D$ = +36.7 (*c* 0.09, MeOH); mp 154–158 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 98% (t_R 18.3 min).

Hydrogenation of Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 3-prenyl, 4-methyl)-Gly-Trp (1b) To Give Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 4-methyl, 3-(2-methylbutyl))-Gly-Trp (1e). Compound 1b (10 mg) was dissolved in methanol (5 mL), and Pd on C (10%) (50 mg) was added. The solution was stirred at 30 psi of hydrogen pressure for 3 h. After the reaction was complete the solution was centrifuged and decanted. Removal of solvent under vacuum followed by HPLC purification as above gave 1e as a white solid (yield 80%); UV λ_{max} (MeOH) 205.0 (ε 39 550), 221.8 (34 800), 281.8 (4856), 290.0 (2950), λ_{max} 212.8 (30 100), 251.0 (1830), 288.0 (2810); IR 3250, 2950, 1740, 1560, 1460, 1250, 1100, 1040, 1000, 765, 746 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.08 (Pα, m), 1.78 (Pβ, m), 2.11 (Pβ, m), 1.79 (Pγ, m), 1.95 (Pγ, m), 3.23 (Pδ, m), 3.49 (P δ, m), 8.93 (GNH, dd, *J* = 7.4, 4.5 Hz), 3.35 (Gα, m), 4.07 (Gα, m), 8.04 (VNH, d, *J* = 10.4 Hz), 3.97 (Vα, t, *J* = 10.2 Hz), 1.60 (Vβ, m), 0.37 (Vγ, d, 6.4), 0.53 (Vγ, d, 6.6), 8.09 (GNH, bs), 3.70 (Gα, m), 4.10 (Gα, m), 8.81 (YNH, bs), 4.03 (Yα, dd, *J* = 7.7, 4.4 Hz), 4.75 (Yβ, d, *J* = 7.4 Hz), 5.83 (YβOH, bs), 7.06 (Y2, s), 3.75 (3H, Y4, s), 6.85 (Y5, d, *J* = 8.5 Hz), 7.13 (Y6, d, *J* = 7.5 Hz), 2.48 (Y7, m), 1.37 (Y8, q, *J* = 7.0 Hz), 1.51 (Y9, m, *J* = 6.6 Hz), 0.90 (3H, Y10, s), 0.91 (3H, Y11, s), 8.51 (GNH, *t*, *J* = 6.4 Hz), 3.34 (Gα, m), 3.67 (Gα, dd, *J* = 17.1, 6.0 Hz), 7.87 (WNH, d, *J* = 8.8 Hz), 4.87 (Wα, dt, *J* = 4.75, 9.0 Hz), 2.75 (Wβ, dd, *J* = 4.1, 14.5 Hz), 2.91 (Wβ, dd, *J* = 14.8, 10.5 Hz), 10.88 (W1, s), 7.09 (W2, s), 7.49 (W4, d, *J* = 7.8 Hz), 6.95 (W5, t, *J* = 7.5 Hz), 7.05 (W6, t, *J* = 7.4 Hz), 7.28 (W7, d, *J* = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 61.10 (Pα), 28.88 (pβ), 24.59 (Pγ), 47.30 (Pδ), 171.25 (PCO), 42.44 (Gα), 168.49 (PCO), 60.43 (Vα), 31.66 (Pβ), 18.67 (Pγ), 19.06 (Pγ), 170.58 (PCO), 42.78 (Gα), 169.35 (GCO), 61.95 (Yα), 71.38 (Yβ), 133.06 (Y1), 127.88 (Y2,6), 129.63 (Y3), 156.28 (Y4), 109.93 (Y5), 125.19 (Y6), 29.56 (Y7), 27.32 (Y8), 27.36 (Y9), 22.45 (Y10), 22.48 (Y11), 170.49 (YCO), 55.28 (OMe), 42.50 (Gα), 168.85 (GCO), 50.57 (Wα), 27.67 (Wβ), 122.98 (W2), 109.05 (W3), 117.69 (W4), 118.20 (W5), 120.86 (W6), 111.11 (W7), 127.06 (W8), 135.80 (W9), 169.13 (WCO); HRFABMS MH$^+$ 817.4260 ($C_{42}H_{57}N_8O_9$ requires 817.4249); CD max^m 197.5 nm ([ϑ] 18 790), min^m 215.8 (−2190); $[\alpha]_D$ = −7.5 (*c* 0.20, MeOH); mp 135–140 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 99% (t_R 25.8 min).

Removal of the β-Hydroxy Group from Cyclic Pro-Gly-Val-Gly-Tyr(β-OH, 4-methyl, 3-(2-methylbutyl))-Gly-Trp (1e) To Give Cyclic Pro-Gly-Val-Gly-Tyr(4-methyl, 3-(2-methylbutyl))-Gly-Trp (1f). Compound 1e (5 mg) was dissolved in cold dichloromethane (1 mL), trifluoroacetic acid (1 mL), and triethylsilane (0.1 mL). The solution was stirred at 0 °C for 30 min and the solvent removed under nitrogen. HPLC purification as above (acetonitrile/water, 51:49) gave 1f as a white solid (yield 70%): UV λ_{max} (MeOH) 204.0 (ε 28 700), 222.0 (24 200), 282.0 (3330), 290.0 (2050), λ_{min} 212.4 (20 100), 251.0 (670), 282.2 (1980); IR 3320, 3000, 1670, 1550, 1250, 1180, 1050, 910, 760 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.13 (Pα, m), 1.81 (Pβ, m), 2.10 (Pβ, m), 1.81 (Pγ, m), 2.01 (Pγ, m), 3.21 (Pδ, m), 3.40 (Pδ, m), 8.88 (GNH, dd, *J* = 7 .4, 4.7 Hz), 3.35 (Gα, m), 4.08 (Gα, dd, *J* = 17.0, 7.6 Hz), 7.78 (VNH, d, *J* = 10.3 Hz), 4.02 (Vα, t, *J* = 10.1 Hz), 1.80 (Vβ, m), 0.59 (Vγ, d, 6.5), 0.68 (Vγ, d, 6.7), 7.04 (GNH, bd, *J* = 5.6 Hz), 3.62 (Gα, dd, *J* = 17.2, 1.6 Hz), 4.15 (Gα, dd, *J* = 17.0, 6.7 Hz), 8.76 (YNH, d, *J* = 5.7 Hz), 3.89 (Yα, m), 2.98 (Yβ, dd, *J* = 14.5, 5.7 Hz), 3.06 (Yβ, dd, *J* = 14.5, 4.8 Hz), 7.17 (Y2, d, *J* = 2.2 Hz), 3.74 (3H, Y4, s), 6.84 (Y5, d, *J* = 8.3 Hz), 6.97 (Y6, d, *J* = 7.4 Hz), 2.49 (Y7, m), 1.38 (Y8, q, *J* = 7.0 Hz), 1.53 (Y9, m, *J* = 6.7 Hz), 0.89 (3H, Y10, s), 0.91 (3H, Y11, s), 8.50 (GNH, dd, *J* = 7.3, 5.2 Hz), 3.35 (Gα, m), 3.97 (Gα, dd, *J* = 17.1, 7.6 Hz), 7.83 (WNH, d, *J* = 8.9 Hz), 4.89 (Wα, q, *J* = 8.3 Hz), 3.01 (Wβ, d, *J* = 7.8 Hz), 3.01 (Wβ, d, *J* = 7.8 Hz), 10.91 (W1, d, *J* = 1.8 Hz), 6.89 (W2, d, *J* = 1.9 Hz), 7.52 (W4, d, *J* = 7.8 Hz), 6.98 (W5, t, *J* = 7.2 Hz), 7.06 (W6, t, *J* = 7.2 Hz), 7.30 (W7, d, *J* = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.72 (Pα), 28.98 (Pβ), 24.64 (Pγ), 47.60 (Pδ), 171.77 (PCO), 42.33 (Gα), 168.54 (PCO), 60.32 (Vα), 31.60 (Pβ), 18.59 (Pγ), 18.97 (Pγ), 170.58 (PCO), 42.72 (Gα), 170.29 (GCO), 56.50 (Yα), 37.50 (Yβ), 130.14 (Y1), 129.59 (Y2,6), 130.14 (Y3), 155.60 (Y4), 110.47 (Y5), 127.30 (Y6), 34.10 (Y7), 22.37 (Y8), 27.27 (Y9), 22.36 (Y10), 22.38 (Y11), 171.06 (YCO), 55.27 (OMe), 41.69 (Gα), 168.77 (GCO), 50.45 (Wα), 26.97 (Wβ), 123.53 (W2), 109.03 (W3), 117.61 (W4), 118.28 (W5), 120.77 (W6), 111.17 (W7), 127.01 (W8), 135.83 (W9), 170.17 (WCO); HRFABMS MH$^+$ 801.4324 ($C_{42}H_{57}N_8O_8$ requires 801.4300); CD max^m 225.4 nm ([ϑ] 4810), 239.5 (5120), min^m 203.8 (−16 560), 215.0 (−7380), 284.2 (−2220) (MeOH); $[\alpha]_D$ = −12.2 (*c* 0.09, MeOH); mp 158–164 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 99% (t_R 26.7 min).

Synthesis of Cyclic Pro-Gly-Val-Gly-Tyr(4-*tert*-butyl)-Gly-Trp (2a). Linear Trp-Pro-Gly-Val-Gly-Tyr(tbu)-Gly was prepared manually using solid-phase Fmoc chemistry methodology[19] on a 2-methoxy-4-alkoxybenzyl alcohol resin support (SASRIN™),[20] with a *tert*-butyl side chain protection of tyrosine. *N*-α-Fmoc groups were removed before each coupling step with three treatments with 50% piperidine/DMF for 2 min each time. This procedure minimized diketopiperazine formation on deprotection of the N-terminal of tyrosine. Couplings were performed using the diisopropylcarbodiimide/1-hydroxybenzotriazole procedure.[21] The linear peptide was cleaved with 1% TFA in dichloromethane and neutralized with diisopropylethylamine. The peptide was prepared for cyclization by disolution in anhydrous tetrahydrofuran (800 mL). Diisopropylethylamine (0.75 mM) was added, and the solution was stirred vigorously while a solution of BOP in DMF (0.25 mM, 10 mL) was added over 7 h, after which the reaction was allowed to proceed for a further 9 h. Then water (100 mL) was added and the solution stirred for 1 h. The THF was then removed under reduced pressure and the solution extracted with ethyl acetate (3×). The ethyl acetate extractions were combined, and the solvent was removed under vacuo. Purification using C18 HPLC gave **2a** as a white solid (55% yield): UV λ_{max} (MeOH) 221.4 (ε 35 120), 280.8 (5500), 290.0 (4590), λ_{min} 248.0 (2160), 287.2 (4250); IR 3320, 1670, 1570, 1240, 1160, 750 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.13 (Pα, m), 1.82 (Pβ, m), 2.12 (Pβ, m), 1.82 (Pγ, m), 2.01 (Pγ, m), 3.34 (Pδ, m), 3.73 (Pδ, m), 8.90 (GNH, dd, *J* = 6.6, 5.1 Hz),

3.41 (Gα, m), 4.11 (Gα, dd, J = 16.5, 7.6 Hz), 7.78 (VNH, d, J = 10.3 Hz), 4.03 (Vα, t, J = 10.0 Hz), 1.78 (Vβ, m), 0.60 (Vγ, d, 6.5), 0.69 (Vγ, d, 6.7), 7.94 (GNH, bd, J = 4.7 Hz), 3.63 (Gα, bd, J = 15.9 Hz), 4.14 (Gα, dd, J = 16.5, 6.5 Hz), 8.83 (YNH, d, J = 5.4 Hz), 3.93 (Yα, m), 3.00 (Yβ, m), 3.14 (Yβ, m), 7.08 (Y2, d, J = 8.4 Hz), 6.88 (Y3, d, J = 8.3 Hz), 6.88 (Y5, d, J = 8.3 Hz), 7.08 (Y6, d, J = 8.4 Hz), 1.27 (9H, Y8, s), 8.51 (GNH, dd, J = 6.7, 5.3 Hz), 3.35 (Gα, m), 3.96 (Gα, m), 7.81 (WNH, d, J = 8.0 Hz), 4.89 (Wα, q, J = 7.6 Hz), 3.01 (Wβ, m), 3.01 (Wβ, m), 10.91 (W1, s), 7.18 (W2, d, J = 1.9 Hz), 7.52 (W4, d, J = 7.7 Hz), 6.99 (W5, t, J = 7.3 Hz), 7.06 (W6, t, J = 7.4 Hz), 7.31 (W7, d, J = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.82 (Pα), 28.08 (Pβ), 24.74 (Pγ), 47.69 (Pδ), 171.86 (PCO), 42.38 (Gα), 168.66 (PCO), 60.39 (Vα), 31.66 (Pβ), 18.64 (Pγ), 19.04 (Pγ), 170.74 (PCO), 41.80 (Gα), 170.26 (GCO), 56.34 (Yα), 34.38 (Yβ), 132.78 (Y1), 129.68 (Y2,6), 123.56 (Y3,5), 153.50 (Y4), 77.69 (Y7), 28.53 (Y8), 171.15 (YCO), 24.80 (Gα), 168.87 (GCO), 50.49 (Wα), 27.03 (Wβ), 123.56 (W2), 109.08 (W3), 117.70 (W4), 118.38 (W5), 120.88 (W6), 111.26 (W7), 127.07 (W8), 135.89 (W9), 170.26 (WCO); HRFABMS MH$^+$ 773.4017 ($C_{40}H_{53}N_8O_8$ requires 773.3986); [α]$_D$ = −3.8° (c 0.6, MeOH); mp 192–194 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 99% (t_R 24.0 minutes).

Deprotection of Cyclic Pro-Gly-Val-Gly-Tyr(4-*tert*-butyl)-Gly-Trp (2a) To Give Cyclic Pro-Gly-Val-Gly-Tyr-Gly-Trp (2c). Compound **2a** (20 mg) was dissolved in 50% TFA/dichloromethane and stirred for 2 h. The solvent was removed and the residue purified by C18 HPLC to give **2c** as a white solid (90% yield): UV λ$_{max}$ (MeOH) 222.2 (ε 31 150), 280.8 (5790), 289.0 (4430), λ$_{min}$ 247.0 (2120), 287.6 (4380); IR 3300, 1670, 1540, 1270, 740 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.13 (Pα, m), 1.82 (Pβ, m), 2.12 (Pβ, m), 1.82 (Pγ, m), 2.02 (Pγ, m), 3.36 (Pδ, m), 3.72 (Pδ, m), 8.95 (GNH, m), 3.40 (Gα, m), 4.11 (Gα, dd, J = 17.0, 7.7 Hz), 7.8 0 (VNH, d, J = 10.1 Hz), 4.02 (Vα, t, J = 9.8 Hz), 1.79 (Vβ, m), 0.58 (Vγ, d, 6.6), 0.68 (Vγ, d, 6.7), 7.95 (GNH, bd, J = 4.4 Hz), 3.63 (Gα, bd, J = 15.6 Hz), 4.13 (Gα, dd, J = 16.3, 6.8 Hz), 8.94 (YNH, d, J = 5.4 Hz), 3.86 (Yα, m), 2.92 (Yβ, dd, J = 13.9, 9.5 Hz), 3.08 (Yβ, m), 6.96 (Y2, d, J = 8.5 Hz), 6.67 (Y3, d, J = 8.4 Hz), 6.67 (Y5, d, J = 9.4 Hz), 6.96 (Y6, d, J = 8.5 Hz), 8.63 (GNH, m), 3.36 (Gα, m), 3.94 (Gα, dd, J = 17.2, 7.5 Hz), 7.81 (WNH, d, J = 8.5 Hz), 4.88 (Wα, q, J = 7.5 Hz), 3.01 (Wβ, m), 3.01 (Wβ, m), 10.92 (W1, s), 7.19 (W2, d, J = 1.9 Hz), 7.52 (W4, d, J = 7.8 Hz), 6.98 (W5, t, J = 7.3 Hz), 7.06 (W6, t, J = 7.3 Hz), 7.30 (W7, d, J = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.72 (Pα), 28.95 (Pβ), 24.62 (Pγ), 47.56 (Pδ), 171.76 (PCO), 42.32 (Gα), 168.54 (PCO), 60.27 (Vα), 31.54 (Pβ), 18.56 (Pγ), 18.96 (Pγ), 170.60 (PCO), 41.72 (Gα), 170.23 (GCO), 56.55 (Yα), 34.21 (Yβ), 128.02 (Y1), 129.90 (Y2,6), 115.03 (Y3,5), 155.85 (Y4), 171.21 (YCO), 42.71 (Gα), 168.77 (GCO), 50.44 (Wα), 26.98 (Wβ), 123.51 (W2), 109.01 (W3), 117.58 (W4), 118.25 (W5), 120.74 (W6), 111.14 (W7), 127.00 (W8), 135.82 (W9), 170.07 (WCO); HRFABMS MH$^+$ 717.3405 ($C_{36}H_{45}N_8O_8$ requires 717.3360); [α]$_D$ = +4.2° (c 0.8, MeOH); mp 222–225 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 99% (t_R 18.3 min).

Methylation of Cyclic Pro-Gly-Val-Gly-Tyr-Gly-Trp (2c) To Give Cyclic Pro-Gly-Val-Gly-Tyr(4-methyl)-Gly-Trp (2b). Compound **2c** (10 mg) was dissolved in methanol (1 mL), and excess (trimethylsilyl)diazomethane in dichloromethane was added. After 3 h the solvent was removed and the residue purified by C18 HPLC to give **2b** as a white solid (75% yield): UV λ$_{max}$ (MeOH) 208.4 (ε 23 170), 222.2 (28 060), 281.0 (4740), 289.8 (3620), λ$_{min}$ 250.0 (2630), 288.0 (3550); IR 3300, 1660, 1580, 1245, 760 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.11 (Pα, m), 1.82 (Pβ, m), 2.12 (Pβ, m), 1.82 (Pγ, m), 2.01 (Pγ, m), 3.35 (Pδ, m), 3.73 (Pδ, m), 8.91 (GNH, m), 3.40 (Gα, m), 4.08 (Gα, m), 7.81 (VNH, d, J = 10.1 Hz), 4.00 (Vα, t, J = 9.8 Hz), 1.80 (Vβ, m), 0.55 (Vγ, d, 6.6), 0.65 (Vγ, d, 6.5), 7.95 (GNH, bs), 3.62 (Gα, bd, J = 15.9 Hz), 4.15 (Gα, m), 8.78 (YNH, d, J = 5.4 Hz), 3.87 (Yα, m), 2.93 (Yβ, m), 3.10 (Yβ, m), 6.97 (Y2, d, J = 8.1 Hz), 6.67 (Y3, d, J = 8.3 Hz), 6.67 (Y5, d, J = 8.3 Hz), 6.97 (Y6, d, J = 8.1 Hz), 3.72 (3H, Y8, s), 8.53 (GNH, m), 3.36 (Gα, m), 3.93 (Gα, m), 7.82 (WNH, d, J = 8.3 Hz), 4.89 (Wα, q, J = 7.6 Hz), 3.00 (Wβ, m), 3.00 (Wβ, m), 10.92 (W1, s), 7.16 (W2, s), 7.51 (W4, d, J = 7.8 Hz), 6.99 (W5, m), 7.06 (W6, t, J = 7.5 Hz), 7.30 (W7, d, J = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.78 (Pα), 29.00 (Pβ), 24.67 (Pγ), 47.61 (Pδ), 171.77 (PCO), 42.36 (Gα), 168.58 (PCO), 60.34 (Vα), 31.60 (Pβ), 18.58 (Pγ), 18.99 (Pγ), 170.67 (PCO), 41.79 (Gα), 170.19 (GCO), 56.69 (Yα), 34.24 (Yβ), 130.10 (Y1), 129.99 (Y2,6), 113.69 (Y3,5), 157.83 (Y4), 54.95 (Y8), 171.21 (YCO), 42.73 (Gα), 168.82 (GCO), 50.44 (Wα), 27.02 (Wβ), 123.49 (W2), 109.04 (W3), 117.63 (W4), 118.30 (W5), 120.80 (W6), 111.19 (W7), 127.02 (W8), 135.85 (W9), 170.10 (WCO); HRFABMS MH$^+$ 731.3519 ($C_{37}H_{47}N_8O_8$ requires 731.3517); [α]$_D$ = −3.5° (c 0.8, MeOH); mp 170–174 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 92% (t_R 20.9 min).

Synthesis of Cyclic Pro-Gly-Val-Gly-*R*-Tyr(4-*tert*-butyl)-Gly-Trp (3). Linear Trp-Pro-Gly-Val-Gly-*R*-Tyr(*t*-Bu)-Gly was prepared by the general method described above, and cyclized as above. Purification by C18 HPLC gave **3** as a white powder (25% yield): UV λ$_{max}$ (MeOH) 220.4 (ε 33 960), 280.8 (5020), 289.8 (4200), λ$_{min}$ 249.0 (2440), 288.0 (3890); IR 3340, 1680, 1540, 1240, 1160, 750 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.19 (Pα, m), 1.82 (Pβ, m), 2.13 (Pβ, m), 1.84 (Pγ, m), 2.02 (Pγ, m), 3.47 (Pδ, m), 4.04 (Pδ, m), 8.94 (GNH, t, J = 6.0 Hz), 3.37 (Gα, dd, J = 16.7, 4.7 Hz), 4.07 (Gα, dd, J = 17.0, 7.7 Hz), 7.5 6 (VNH, d, J = 10.1 Hz), 4.01 (Vα, t, J = 9.9 Hz), 1.86 (Vβ, m), 0.61 (Vγ, d, 6.5), 0.71 (Vγ, d, 6.6), 7.79 (GNH, m), 3.62 (Gα, d, J = 17.3 Hz), 4.28 (Gα, dd, J = 17.6, 7.7 Hz), 8.80 (YNH, d, J = 4.3 Hz), 4.22 (Yα, m), 2.85 (Yβ, m), 2.85 (Yβ, m), 7.17 (Y2, d, J = 8.3 Hz), 6.89 (Y3, d, J = 8.3 Hz), 6.89 (Y5, d, J = 8.3 Hz), 7.17 (Y6, d, J = 8.3 Hz), 1.27 (9H, Y8, s), 8.62 (GNH, t, J = 6.2 Hz), 3.51 (Gα, dd, J = 17.3, 5.8 Hz), 3.69 (Gα, dd, J = 17.4, 6.7 Hz), 7.81 (WNH, m), 4.69 (Wα, q, J = 7.7 Hz), 3.09 (Wβ, dd, J = 14.9, 7.7 Hz), 3.18 (Wβ, Wβ, dd, J = 15.0, 6.6 Hz), 10.89 (W1, s), 7.18 (W2, s), 7.46 (W4, d, J = 7.7 Hz), 6.99 (W5, t, J = 7.4 Hz), 7.06 (W6, t, J = 7.4 Hz), 7.31 (W7, d, J = 8.0 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.45 (Pα), 29.08 (Pβ), 24.66 (Pγ), 47.94 (Pδ), 172.22 (PCO), 42.83 (Gα), 168.43 (PCO), 60.17 (Vα), 31.36 (Pβ), 18.45 (Pγ), 18.94 (Pγ), 170.60 (PCO), 41.23 (Gα), 170.53 (GCO), 56.78 (Yα), 35.11 (Yβ), 131.51 (Y1), 129.39 (Y2,6), 122.37 (Y3,5), 153.65 (Y4), 77.64 (Y7), 28.50 (Y8), 171.71 (YCO), 41.91 (Gα), 168.34 (GCO), 49.84 (Wα), 26.85 (Wβ), 123.21 (W2), 109.03 (W3), 117.39 (W4), 118.31 (W5), 120.82 (W6), 111.25 (W7), 126.99 (W8), 135.90 (W9), 170.82 (WCO); HRFABMS MH$^+$ 773.3998 ($C_{40}H_{53}N_8O_8$ requires 773.3986); [α]$_D$ = −44.4° (c 1.0, MeOH); mp 192–196 °C. Purity analysis by analytical HPLC, using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 95% (t_R 23.9 min).

Synthesis of Cyclic Pro-Gly-Val-Gly-Trp-Gly-Trp (4). Linear Trp-Pro-Gly-Val-Gly-Trp-Gly was prepared by the general method described above and cyclized as above. Purification by C18 HPLC gave **4** as a white powder (45% yield): UV λ$_{max}$ (MeOH) 221.4 (ε 35 970), 281.4 (9380), 290.2 (8090), λ$_{min}$ 247.4 (3630), 287.2 (7410); IR 3280, 1680, 1540, 1235, 1060, 1030, 750 cm^{-1}; ^{1}H NMR (360 MHz, d_6-DMSO) δ 4.11 (Pα, m), 1.83 (Pβ, m), 2.12 (Pβ, m), 1.83 (Pγ, m), 2.01 (Pγ, m), 3.33 (Pδ, m), 3.74 (Pδ, m), 8.91 (GNH, dd, J = 7.1, 4.9 Hz), 3.41 (Gα, m), 4.10 (Gα, m), 7.83 (VNH, d, J = 7.8 Hz), 4.0 2 (Vα, m), 1.79 (Vβ, m), 0.57 (Vγ, d, 6.5), 0.69 (Vγ, d, 6.7), 7.78 (GNH, bd, J = 4.7 Hz), 3.62 (Gα, bd, J = 16.5 Hz), 4.14 (Gα, dd, J = 17.0, 5.5 Hz), 8.82 (WNH, d, J = 5.4 Hz), 4.00 (Wα, m), 3.20 (Wβ, m), 3.30 (Wβ, m), 10.90 (W1, d, J = 2.2 Hz), 7.16 (W2, d, J = 2.2 Hz), 7.47 (W4, d, J = 7.8 Hz), 6.97 (W5, t, J = 7.4 Hz), 7.06 (W6, t, J = 7.5 Hz), 7.31 (W7, d, J = 8.8 Hz), 8.59 (GNH, dd, J = 7.3, 5.1 Hz), 3.35 (Gα, m), 3.96 (Gα, dd, J = 17.0, 7.5 Hz), 7.89 (WNH, d, J = 9.0 Hz), 4.91 (Wα, q, J = 7.7 Hz), 3.02 (Wβ, d, J = 7.7 Hz), 3.02 (Wβ, d, J = 7.0 Hz), 10.90 (W1, d, J = 2.1 Hz), 7.19 (W2, d, J = 2.2 Hz), 7.54 (W4, d, J = 7.5 Hz), 6.99 (W5, t, J = 7.8 Hz), 7.06 (W6, t, J = 7.5 Hz), 7.34 (W7, d, J = 8.08 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 60.77 (Pα), 28.96 (Pβ), 24.62 (Pγ), 47.59 (Pδ), 171.67 (PCO), 42.42 (Gα), 168.49 (PCO), 60.39 (Vα), 31.63 (Pβ), 18.62 (Pγ), 18.93 (Pγ), 170.53 (PCO), 41.83 (Gα), 170.18 (GCO), 55.83 (Wα), 25.06 (Wβ), 123.53 (W1), 110.23 (W3), 118.05 (W4), 118.26 (W5), 120.82 (W6), 111.29 (W7), 127.30 (W8), 136.08 (W9), 171.44 (WCO), 42.66 (Gα), 168.84 (GCO), 50.46 (Wα), 27.01 (Wβ), 123.53 (W2), 109.03 (W3), 117.59 (W4), 118.26 (W5), 120.76 (W6), 111.15 (W7), 127.02 (W8), 135.83 (W9), 170.06 (WCO); HRFABMS MH$^+$ 762.3353 ($C_{38}H_{45}N_9O_7$ requires 762.3340); [α]$_D$ = +66.6° (c 0.9, MeOH); mp 155–158 °C. Purity analysis by analytical HPLC,

using a linear gradient from 20/80 acetonitrile/water to 80/20 acetonitrile/water in 30 min, was 97% (t_R 20.8 min).

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