# WIN 64821, a New Competitive Antagonist to Substance P, Isolated from an *Aspergillus* Species: Structure Determination and Solution Conformation

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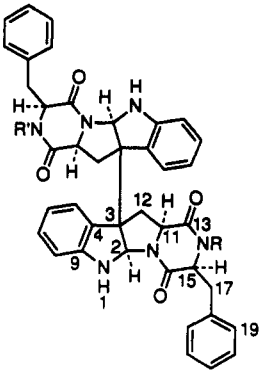
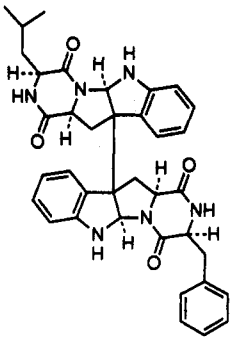
Two new diketopiperazine dimers, WIN 64821 (**1a**) and WIN 64745 (**2**), were isolated from an *Aspergillus* culture originally isolated from soil and their structures established on the basis of chemical and spectroscopic evidence. The dimer **1a** has *C*1 symmetry with each of two equivalent monomeric subunits biosynthetically constructed from one phenylalanine and one tryptophan residue. Dimer **1a** is a competitive antagonist to substance P (SP) at the human NK1 receptor with an inhibitor affinity constant ($K_i$) of 230 ± 30 nM against [$^{125}$I]SP in human astrocytoma cells. The solution structures of **1a** and the nonsymmetrical methylation derivative **1b** were determined by analysis of NMR data and molecular modeling. The solution structures, together with some structure–activity data, suggest a probable binding conformation for these molecules at the NK1 receptor.

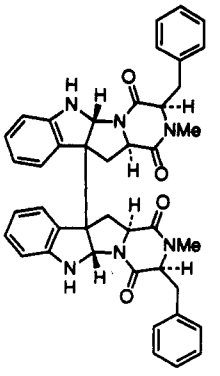
Substance P (SP), an undecapeptide and a member of the neurokinin family, has been implicated in a number of physiological activities, including vasodilation,[1] stimulation of salivary secretion,[2] and airway smooth muscle contraction.[3] SP is a potent agonist and believed to be the endogenous ligand for the neurokinin-1 (NK1) receptor, which is the most well-studied subtype of the tachykinin family of cell surface receptors. Selective antagonists of SP at the NK1 receptor might prove to be novel analgesics or antiinflammatory agents and have potential in the treatment of arthritis, asthma, and inflammatory bowel disease.[4] Our continuing search[5] for new SP antagonists from microbial culture has led to the discovery of WIN 64821 (**1a**), a new competitive antagonist of SP at the NK1 receptor. Compound **1a** and the less-potent analog WIN 64745 (**2**) were produced in culture by an *Aspergillus* sp., isolated originally from soil. To determine which structural features of these complex molecules are important for substance P antagonism we used NOE analysis, molecular modeling, and structure–bioactivity data. This analysis allowed us to determine the three-dimensional solution structure of **1a** and suggested a probable binding conformation for **1a** at the NK1 receptor. Examination of the two-dimensional structure of **1a** indicated a close relationship to ditryptophenaline (**3**).[6] However **3** was much less potent than **1a** in our assays, and we believe this is due to their dissimilar three-dimensional structure. Herein we report the structure determination of **1a** and **2**, their three-dimensional structure in solution, and suggest a probable binding conformation for **1a** at the NK1 receptor.



**1a** R=R'=H
**1b** R=H, R'=Me
**1c** R=R'=Me



**2**



**3**

## Results and Discussion

**Structure Determination of WIN 64821 (1a) and WIN 64745 (2).** From an ethyl acetate extract of whole culture fermentation broths of *Aspergillus* sp., SC319 (ATCC 74177), **1a** was isolated as a white solid. The UV

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Table I. $^1H$, $^{13}C$, and HMBC NMR Data ($\delta$, $J$ in Hz) for WIN 64821 (1a) in Acetonitrile-$d_3$ (360 MHz)

| position | $^1H$ (Hz) | $^{13}C$ | HMBC coupling to carbon |
|---|---|---|---|
| 1 | 5.84 s | | |
| 2 | 4.85 bs | 80.82 d | H12a |
| 3 | | 61.14 s | H1, H2, H12a,b, H5 |
| 4 | | 131.90 s | H1, H2, H12a,b, H6 |
| 5 | 7.34 d (8.0) | 126.53 d | H7 |
| 6 | 6.73 td (8.0, 1.2) | 120.86 d | H8 |
| 7 | 7.10 td (8.0, 1.2) | 130.60 d | H5 |
| 8 | 6.67 d (8.0) | 110.82 d | H6 |
| 9 | | 150.56 s | H1, H2, H5, H7 |
| 11 | 4.05 t (8.5) | 57.93 s | H1, H2, H12a,b |
| 12a | 2.98 dd (14.0, 8.0) | 36.88 t | H11, H14 |
| 12b | 2.50 dd (14.0, 7.5) | | |
| 13 | | 170.36 s | H11, H12b |
| 14 | 5.88 bs | | |
| 15 | 4.15 bt (5.5) | 57.17 d | H14, H17a,b |
| 16 | | 169.55 s | H14, H15, H17a |
| 17a | 3.09 dd (14.5, 5.0) | 36.17 t | H15 |
| 17b | 2.98 dd (14.5, 6.0) | | |
| 18 | | 137.88 s | H17a,b |
| 19(23) | 7.13 s | 130.82 d | H17a,b, H19–23 |
| 20(22) | 7.13 s | 129.84 d | |
| 21 | 7.13 s | 128.11 d | H19–23 |

spectrum of **1a** agreed with the presence of an indoline moiety. Quantitative amino acid analysis, followed by Marfey's derivatization,[7] showed the presence of two L-phenylalanine residues per molecule of **1a**. The molecular formula of **1a** was established as $C_{40}H_{36}N_6O_4$ by HRFABMS. An intense daughter ion in the FABMSMS spectrum was shown by HRFABMS to correspond to the formula $C_{20}H_{18}N_3O_2$, indicating that **1a** contained two equivalent monomeric subunits. The $^1H$ NMR spectrum of **1a** showed a four-proton aromatic ABCD spin system, a five-proton aromatic singlet, two exchangeable protons, and three methine and two methylene systems. The $^{13}C$ NMR spectrum and a DEPT experiment showed the presence of 12 methine, 2 methylene, and 6 unprotonated carbons. Since the $^1H$ and $^{13}C$ NMR spectra revealed the presence of only 20 carbons and 18 protons, while the mass spectral data required 40 carbons and 36 protons, **1a** was constructed as a symmetrical dimer where both halves were equivalent by NMR spectroscopy.

A COSY spectrum showed that a methine proton at 4.15 ppm (H15) was coupled to an exchangeable proton at 5.88 ppm (NH14), and NH14 was long-range coupled to a methine at 4.05 ppm (H11). Furthermore, in an HMBC experiment the H11 proton was coupled to a carbonyl carbon at 170.36 ppm (C13), and H15 was coupled to a second carbonyl carbon at 169.55 ppm (C16), indicating the presence of a diketopiperazine moiety within each monomeric subunit of **1a**. Additional COSY correlations, from H15 to protons at 3.09 and 2.98 ppm (H17a, H17b), and from H17a and H17b to a 5-proton aromatic singlet at 7.13 ppm (H19–H23), established the presence of phenylalanine in the diketopiperazine ring of each monomeric subunit.

In the HMBC spectrum long-range correlations from the ABCD spin system to carbons at 131.90 ppm (C4) and 150.56 ppm (C9) (Table I), and correlations from both C4 and C9 to an exchangeable proton at 5.84 ppm (NH1) and a methine proton at 4.85 ppm (H2), were consistent with the presence of an indoline moiety. In the COSY spectrum H11 was coupled to protons at 2.50 and 2.98 ppm (H12a, H12b), and both H12a and H12b were long-range coupled to the H2 methine, indicating that the indoline is attached to the diketopiperazine ring through C12. Furthermore, the presence of a methine (H2) and not a methylene suggested that C2 and N10 are covalently bonded. Long-range HMBC correlations from the quaternary carbon at 61.14 ppm (C3) to NH1, H12a, H12b, H2 and H5, indicated that C3 is the site where the two spectroscopically equivalent monomeric subunits are linked by a carbon–carbon bond (C3–C3′). Complete $^1H$ and $^{13}C$ NMR spectral assignments are given in Table I.

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Table II. $^1H$ and $^{13}C$ NMR Data ($\delta$, $J$ in Hz) for WIN 64745 (2) in Acetonitrile-$d_3$ (360 MHz)

| position | $^1H$ (Hz) | $^{13}C$ | position | $^1H$ (Hz) | $^{13}C$ |
|---|---|---|---|---|---|
| 1 | 5.84 s | | 21 | 7.14 s | 127.85 |
| 2 | 4.87 s | 80.83 | 1′ | 5.76 s | |
| 3 | | 61.20 | 2′ | 4.87 s | 80.79 |
| 4 | | 132.13 | 3′ | 3.00 m | 61.27 |
| 5 | 7.36 d (7.7) | 126.53 | 4′ | | 132.26 |
| 6 | 6.73 td (7.7, 1.2) | 120.24 | 5′ | 7.39 d (7.7) | 126.55 |
| 7 | 7.10 td (7.7, 1.2) | 130.36 | 6′ | 6.74 dd (7.7, 6.6) | 120.20 |
| 8 | 6.67 d (7.7) | 110.69 | 7′ | 7.10 dd (7.7, 6.6) | 130.36 |
| 9 | | 150.93 | 8′ | 6.61 d (7.7) | 110.69 |
| 11 | 4.08 m | 58.08 | 9′ | | 150.85 |
| 12a | 3.01 m | 37.25 | 11′ | 4.12 m | 58.23 |
| 12b | 2.51 dd (14.1, 7.7) | | 12a′ | 3.00 m | 61.63 |
| 13 | | 170.74 | 12b′ | 2.75 dd (14.1, 7.0) | |
| 14 | 5.93 s | | 13′ | | 170.74 |
| 15 | 4.16 dd (5.5, 4.9) | 57.36 | 14′ | 6.10 s | |
| 16 | | 170.83 | 15′ | 3.83 dd (8.5, 4.4) | 54.52 |
| 17a | 3.10 dd (14.7, 4.9) | 36.44 | 16′ | | 170.79 |
| 17b | 3.02 dd (14.7, 5.5) | | 17a′ | 1.40 m | 39.17 |
| 18 | | 138.24 | 17b′ | 1.31 m | |
| 19(23) | 7.14 s | 130.70 | 18′ | 1.75 m | 25.26 |
| 20(22) | 7.14 s | 129.66 | 19′ | 0.83 d (1.9) | 22.08 |
| 20′ | 0.81 d (1.8) | 23.27 | | | |

The structure of **2** was determined in a similar manner to that of **1a**. The UV spectrum of **2** implied the presence of an indoline moiety. Quantitative amino acid analysis, followed by Marfey's derivatization, showed the presence of one L-phenylalanine and one L-leucine residue per molecule of **2**. HRFABMS established the molecular formula $C_{37}H_{38}N_6O_4$. Daughter ions in the FABMSMS spectrum of **2** were similar to those observed for **1a**, with an intense ion corresponding to $C_{20}H_{18}N_3O_2$. This suggested that one-half of **2** was equivalent to the monomeric subunit of **1a**. Several fragment ions observed in the FABMSMS spectrum of **2**, including one corresponding to $C_{17}H_{20}N_3O_2$, were 34 mass units lower than those observed for **1a**. This mass difference corresponds to the difference in mass between a phenylalanine and a leucine residue, suggesting that **2** is a nonsymmetrical dimer equivalent to **1a** with leucine replacing phenylalanine in one of the monomeric subunits. $^1H$, COSY, and $^{13}C$ NMR data were consistent with the proposed structure and reflect the nonsymmetrical dimeric structure of **2**. $^1H$ and $^{13}C$ NMR data for **2** were assigned with the aid of COSY spectra and by comparison with data for **1a** (Table II).

**Determination of the Stereochemistry of WIN 64821 (1a) and WIN 64745 (2).** Reaction of **1a** with hot acetic acid for 8 h gave cyclo(*S*-Trp-*S*-Phe) (**4**) as the major degradation product. The structure and stereochemistry of the degradation product **4** were confirmed by comparison with an authentic sample of synthetic material. Compound **4** was also isolated as a major component of the extract and is most likely the biosynthetic precursor to **1a**. The isolation of **4** after mild acid hydrolysis of **1a**, together with the isolation of (*S*)-phenylalanine after strong acid hydrolysis, established that both C11 and C15 of **1a** have the *S* configuration. For **1a**, the $^5J$(H,H)

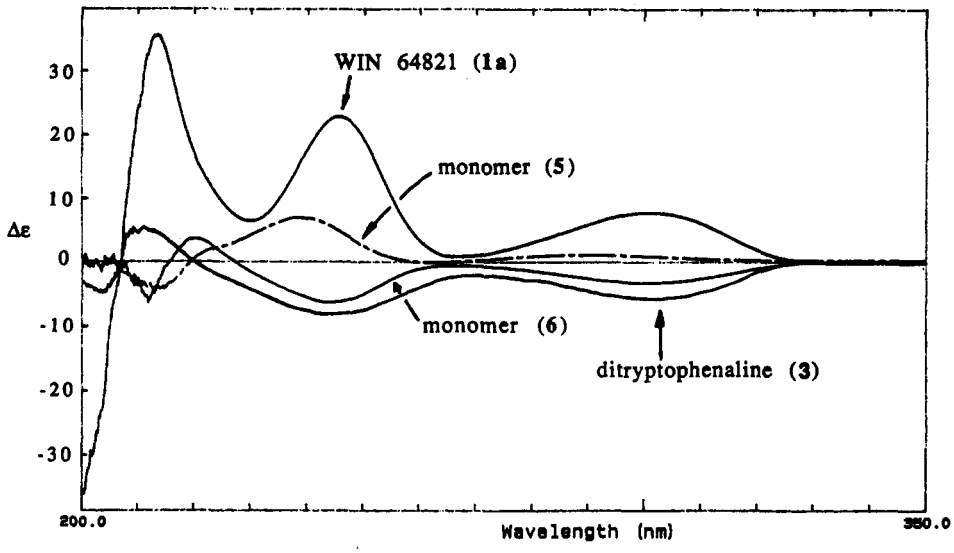
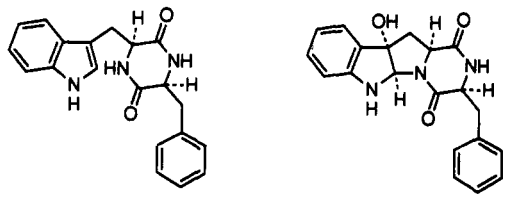



**Figure 1.** Circular dichroism (CD) spectra for WIN 64821 (1a), ditryptophenaline (3), and the oxygenated monomeric diastereoisomers 5 and 6 recorded in methanol at 21 °C. Results differ from those previously published.[8]

coupling observed between H11 and H15 was consistent with the cis relationship between these protons.[8]



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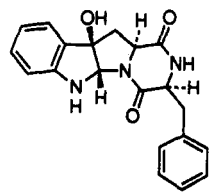

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The stereochemistry at C2 and C3 for **1a** was assigned, as described below, from NOE data for **1a** and its monomethyl WIN 64821 (**1b**) and by CD comparisons with the model compounds, ditryptophenaline (**3**),[6,8,9] and monomers **5** and **6**, prepared synthetically by reaction of **4** with singlet oxygen.[8] Monomethyl WIN 64821 (**1b**) was prepared for use in the conformational analysis of **1a** and for structure–bioactivity information, but **1b** was also useful for the assignment of the C2 and C3 stereochemistries of **1a**.

For **1b**, an NOE between H11′ and H2′ was observed, establishing that these protons were on the same side of the five-membered ring and that the cis-fused ring system must have the *R* stereochemistry at both C2′ and C3′. A further NOE from H12b′ to H5′ also supported the stereochemical assignment of C3′ as *R*. For **1a** no NOE was observed between H11 and H2, probably due to a difference in the conformation of the diketopiperazine ring in the absence of an *N*-methyl group. An NOE from H12b to H5 for **1a**, long-range H11′ to H15′ coupling for **1b**, and almost identical CD spectra for **1a** and **1b** confirm that no change in chirality occurred upon *N*-methylation. Both **1a** and **1b** must therefore have the 2*R*, 2′*R*, 3*R*, 3′*R*, 11*S*, 11′*S*, 15*S*, 15′*S* configuration. The relative stereochemistry of **2** was defined by NMR spectroscopy and CD comparison with **1a** and the absolute configuration defined by isolation and identification of (*S*)-phenylalanine and (*S*)-leucine after acid hydrolysis. Therefore **2** also has the 2*R*, 2′*R*, 3*R*, 3′*R*, 11*S*, 11′*S*, 15*S*, 15′*S* configuration.

The relative stereochemistry of ditryptophenaline (**3**) was previously determined by crystal structure[6] and its absolute configuration by synthesis.[9] However, we noted an inconsistency in the reported data, where the CD spectrum for **3** was quoted as negative for the synthetic material[9] and positive in the case of reisolated natural product.[8] If the chiralities at C2 and C3 were opposite for **1a** and **3** we would expect these compounds to exhibit essentially opposite CD spectra. To further support the stereochemical assignments of **1a** and to resolve doubts about the sign of the CD curve of **3** we reisolated **3** from a fermentation broth of *Apergillus flavus* SC1661 (originally *A. flavus* MIT-M25).[6] We found that the CD spectrum of **3** isolated from SC1661 had a negative sign and was opposite in sign to that obtained for **1a** and **2**, consistent with the opposite stereochemistry close to the dominant indoline chromophores in these molecules. Therefore CD comparisons among authentic **3**, the synthetically prepared monomers **5** and **6**, and **1a** supported our stereochemical assignments for **1a** Figure 1.

**Three-Dimensional Solution Structures of WIN 64821 (1a), Monomethyl WIN 64821 (1b) and Dimethyl WIN 64821 (1c).** Because the inherent symmetry of **1a** caused ambiguity for the assignment of NOEs we initially determined the solution structure of the closely related and biological active derivative **1b**. Using the results from this analysis of **1b**, together with NMR and modeling data obtained for **1a** and **1c**, we also determined the solution conformations of **1a** and **1c**. These solution conformations, together with structure–bioactivity data, gave a possible binding conformation for **1a** at the NK1 receptor.

The conformational uncertainties within **1b** were (1) the conformation of the two diketopiperazine rings, (2)

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the spatial orientation of the benzyl rings, and (3) the spatial orientation of the two monomeric subunits relative to one another. The conformations of the diketopiperazine rings were established by a combination of coupling constant and NOE data. For the nonmethylated monomeric subunit a 1.0-Hz coupling was observed between H11 and H15 in the COSY spectrum and confirmed by selective decoupling experiments. This coupling is unusual and is consistent with an axial arrangement for these protons,[10,11] implying that the diketopiperazine ring exists in a boat conformation, with H11 and H15 in a coaxial arrangement. For the methylated monomeric subunit we observed a 1.2-Hz coupling between H11′ and H15′. Also, irradiation of the *N*-methyl group gave an NOE enhancement to both H11′ and H15′, implying that all three groups were on the same side of the diketopiperazine ring. A further NOE from the *N*-methyl group to H19′(23′) suggested a pseudoequatorial orientation for the *N*-methyl group, with a half-boat conformation for the diketopiperazine ring.

The aromatic protons in the $^1$H NMR spectrum of the nonmethylated monomeric subunit of **1b** were degenerate, indicating that this benzyl group was not confined in a single orientation in solution. Nearly equivalent $^3J$(a,b) coupling constants of 5.0 Hz, from H15 to H17a, and 5.5 Hz, from H15 to H17b, also supported some rotation about the C15–C17 bond. However, the phenyl group of the methylated monomeric subunit was much more constrained in space. An NOE from H17b′, but not from H17a′, to the *N*-methyl group indicated that a preferred conformation existed where H17b′ was close in space to the *N*-methyl group. The nondegeneracy of the phenyl protons indicated that the phenyl group interacted with the diketopiperazine nucleus. The large upfield shift of H12b′ on methylation of **1a** also supported a conformation for **1b** in which the phenyl group near the *N*-methyl group lay over the diketopiperazine ring, close to H12b′. Analysis of $^3J$(a,b) coupling constants of 5.4 Hz (H17b′ to H15′) and 2.7 Hz (H17a′ to H15′), using a modified Karplus relationship,[12] gave H17′–H15′ dihedral angles of approximately 40° and −75°, respectively.

The relative orientation of the methylated and nonmethylated monomeric subunits is defined by the C4–C3–C3′–C4′ dihedral angle. The value for this angle was determined using difference NOE spectroscopy and refined using molecular modeling. The absence of symmetry and the presence of sufficient dispersion for signals in the $^1$H NMR spectrum were optimally achieved for **1b** in $CD_3CN$ in order to observe NOEs between the monomeric subunits.

Using difference NOE spectroscopy, irradiation of either H12a′ or H11′ in **1b** gave an enhancement of H5. Conversely, irradiation of H5 gave an enhancement of both H11′ and H12a′. Irradiation of H12b′ gave an enhancement of H5′ and a negative enhancement of H5, consistent with a homonuclear dipolar polarization transfer via H12a′. Irradiation of H11 gave an enhancement of H5′ and irradiation of H12b gave an enhancement of H5, with a negative enhancement of H5′. These results are consistent only with a highly populated conformation in which the two indoline moieties are twisted away from each other and the C4–C3–C3′–C4′ angle is close to 180°, as shown in Figure 3. To obtain a refined structure molecular modelling was performed, without NMR constraints, using the MULTIC method on MACROMODEL.[13] Starting structures were generated with C4–C3–C3′–C4′, C16–C15–C17–C18 and C16′–C15′–C17′–C18′ angles each varying by 30°, throughout the possible 360° range. The structures were then energy minimized (MM2 forcefield[14] and BDNR optimization,[15] 250 iterations per structure). The lowest energy structures had a C4–C3–C3′–C4′ dihedral angle of 165 ± 5° and were the only structures consistent with the NMR data. In all the low-energy structures the phenyl of the monomethylated monomer lay over the diketopiperazine ring (Figure 3). The distance from H5′ to H11 and also from H5′ to H12b, in the low-energy structures obtained from modeling, was 2.4 ± 0.1 Å, which was consistent with the observation of NOEs between these protons in solution. Higher energy structures were inconsistent with the observed NOE data.

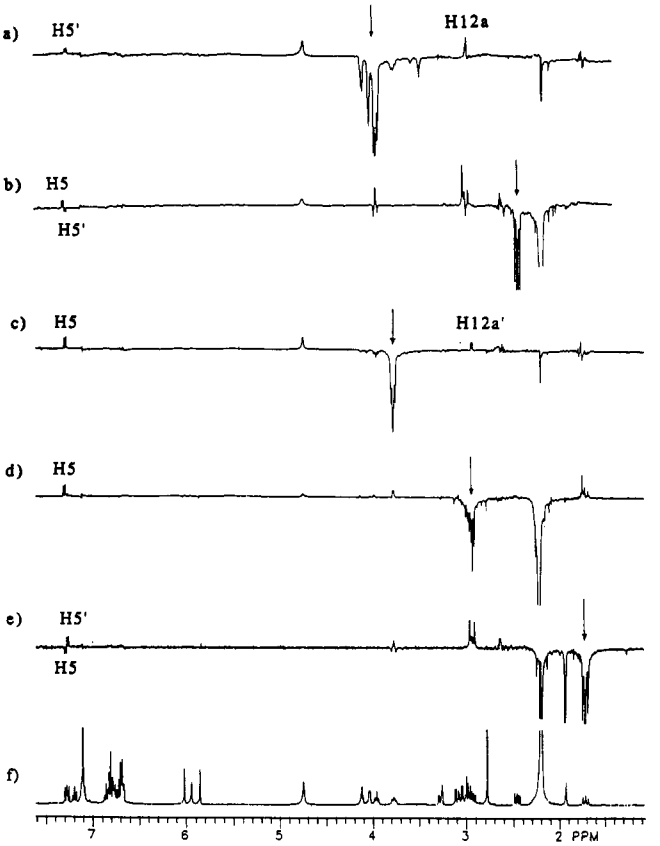


**Figure 2.** Selected difference NOE spectra showing NOEs between monomeric subunits for monomethyl WIN 64821 (**1b**): spectrum a, irradiation of H11; spectrum b, irradiation of H12b; spectrum c, irradiation of H11′; spectrum d, irradiation of H12a′; spectrum e, irradiation of H12b′; spectrum f, $^1$H NMR spectrum of **1b** in acetonitrile-$d_3$ at 300 MHz.

Once the solution structure of **1b** was determined, it was possible to determine the solution structure of **1a**. $^1$H and $^{13}$C NMR data were identical for each monomeric subunit in **1a** due to symmetry (Table I). In the NOESY spectrum of **1a** NOEs were observed between H11 and H5, and between H12a and H5. These NOEs were assigned

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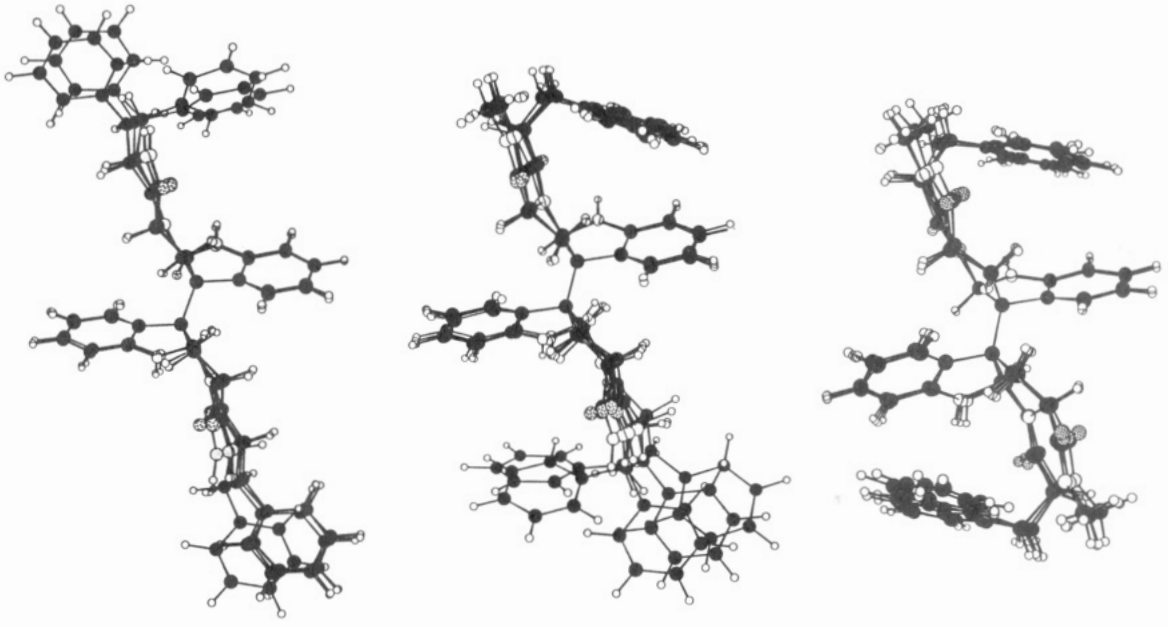

**Figure 3.** The five lowest energy structures overlaid for WIN 64821 (**1a**), monomethyl WIN 64821 (**1b**), and dimethyl WIN 64821 (**1c**), respectively. Structures were obtained using a MULTIC procedure about the three rotatable bonds defined by C2–C3–C3′–C4′, C16–C15–C17–C18, and C16′–C15′–C17′–C18′. Each structure was minimized using an MM2 force field and 250 iterations per structure with BDNR optimization. These structures agree well with the solution structures obtained from NMR spectroscopy.

as being between monomeric subunits by comparison with **1b** and because these protons within the same monomeric subunit are probably too far apart to expect an NOE enhancement. These results suggested a monomer–monomer spatial relationship similar to that of **1b**. Molecular modeling, performed as described for **1b**, again gave results consistent with the NMR data. The five lowest energy structures were overlaid for each compound and are shown in Figure 3. In the case that no methyl group is present on the amide nitrogen there are several low-energy positions for the phenyl group. This is consistent with the observation of degeneracy for the benzyl aromatic protons in the $^1$H NMR spectrum. The solution structure of **1c** was determined in a similar manner. For **1c** both phenylalanine phenyl rings are constrained and lie over their respective diketopipirazine rings (Figure 3).

**Biological Activity and Binding Pharmacophore Model for WIN 64821 (1a).** WIN 64821(**1a**) competes with [$^{125}$I]SP in NK1 binding experiments using human astrocytoma cells and has a mean inhibitor affinity constant ($K_i$) of 0.23 ± 0.03 μM. The corresponding $K_i$ values for **1b**, **1c**, **2**, and **3** were 0.55, 4.0, 3.9, and 12.0 μM, respectively, while those for the monomeric compounds **4**, **5**, and **6** were all greater than 50 μM. The corresponding $K_i$ value for **1a** in normal human fetal tissue was 0.74 ± 0.05 μM and in guinea pig (GP) submaxillary gland membrane was 0.6 ± 0.04 μM. Functional data for **1a** are discussed elsewhere.[16]

The low potency of the monomeric compounds **4**, **5**,and **6** suggested that more than one-half of **1a** is necessary for NK1 binding activity. The low activity of **2** suggested that both phenylalanyl phenyl groups are necessary for potency. Further studies indicated that one but not both indoline aromatic groups are necessary for the binding of **1a** to the NK1 receptor.[17]

Many of the low-energy structures for both **1a** and **1b** have one indoline and both phenylalanyl phenyl groups on one side of the molecule and a single indoline on the other side. These low-energy structures, together with structure–activity data, suggest a binding conformation in which both phenylalanyl phenyl groups and the indoline on the same side of the molecule bind to the NK1 receptor. This suggested binding conformation is observed in solution for both **1a** and **1b**, but not **1c**. This model is consistent with the low potency observed for **1c**, which would have to bind in a high energy conformation with one phenylalanyl phenyl group close in space to a methyl group.

## Experimental Section

**General.** One- and two-dimensional NMR spectra were recorded at 300, 400, or 500 MHz. Chemical shifts are given in δ (ppm) and were recorded in acetonitrile-$d_3$ or dimethyl sulfoxide-$d_6$ using the corresponding solvent signals as references. CD spectra were obtained at room temperature in methanol. Molecular modeling was performed using MACROMODEL and BatchMin V2.6. Energy minimizations were performed using an MM2 forcefield and BDNR optimization. Conformational searching was performed using the MULTIC method with 250 iterations of minimization with BDNR optimization for each structure.

**Isolation of WIN 64821 (1a) and WIN 64745 (2).** Whole culture fermentation broths of *Aspergillus* sp. SC319 (100 mL) were extracted with ethyl acetate (100 mL × 2). C-18 Reverse-phase flash chromatography of the ethyl acetate extract (75 mg) was performed and a fraction eluting with 3:1 methanol–water contained **1a** and **2** as a mixture. Separation of **1a** and **2** was achieved using HPLC on an ODS C-18 reverse-phase column (YMC). **1a** was obtained as a white solid (23 mg). UV $\lambda_{max}$ (MeOH) 211 (ε 37800), 241 (12100), 301 nm (5300); IR 1670 cm$^{-1}$; $^1$H and $^{13}$C NMR data, Table I; HRFABMS MH$^+$ 665.2913

(16) Oleynek, J. J.; Sedlock, D. M.; Barrow, C. J.; Appell, K. C.; Casiano, F. M.; Haycock, D. A.; Ward, S. J.; Kaplita, P. V.; Gillum, A. M., manuscript in preparation.

(17) Popp, J. L.; Musza, L. L.; Rudewicz, P. J.; Barrow, C. J.; Houck, D. R., manuscript in preparation.

($C_{40}H_{37}N_8O_4$ requires 665.2876), [M - $C_{12}H_{12}N_2O_2$]$^+$ 448.1895 ($C_{28}H_{24}N_4O_2$ requires 448.1899), [M - $C_{20}H_{18}N_3O_2$]$^+$ 332.1400 ($C_{20}H_{18}N_3O_2$ requires 332.1399); CD (MeOH) 214 ([$\vartheta$] 121300), 246 (80700), 301 nm (27600), Figure 1; $[\alpha]_D$ = +200.0° (*c* 0.15, MeOH); mp 203–205 °C; AAA, 1.8 L-Phe. **2** was also obtained as a white solid (1 mg). UV $\lambda_{max}$ (MeOH) 213 ($\epsilon$ 35500), 242 (10000), 301 nm (4500); IR 1670 cm$^{-1}$; $^1$H and $^{13}$C NMR data, Table II; HRFABMS MH$^+$ 631.3033 ($C_{37}H_{39}N_6O_4$ requires 631.3033); FABMS *m/z* 448, 414, 332, 298, 130; CD (MeOH) 213 ([$\vartheta$] 122540), 246 (78600 ), 301 nm (28800); $[\alpha]_D$ = +280.0° (*c* 0.012, MeOH); mp 194–196 °C; AAA, 0.8 L-Leu, 1.3 L-Phe.

**Methylation of WIN 64821 (1a) To Give Monomethyl WIN 64821 (1b) and Dimethyl WIN 64821 (1c).** **1a** (50 mg) was dissolved in acetone (2.0 mL), and potassium carbonate (500 mg) and methyl iodide (1.5 mL) were added with stirring. The mixture was stirred at room temperature for 20 h after which the solution was filtered and the volatile reagent and solvent removed under vacuum. The two products were separated by HPLC affording **1b** (15 mg) and **1c** (12 mg). **1b** was obtained as a white solid. UV $\lambda_{max}$ (MeOH) 205 ($\epsilon$ 44000), 241 (9500), 304 nm (6400); IR 1680, cm$^{-1}$; $^1$H and $^{13}$C NMR data, Table III; HRFABMS MH$^+$ 679.3043 ($C_{41}H_{39}N_6O_4$ requires 679.3040); FABMS 462, 448, 346, 332, 130; CD (MeOH) 214 ([$\vartheta$] 112900), 246 (72600), 302 (24600); $[\alpha]_D$ = +170.3° (*c* 0.35, MeOH); mp 196–198 °C. **1c** was also obtained as a white solid. UV $\lambda_{max}$ (MeOH) 205 ($\epsilon$ 38000), 241 (8800), 304 nm (5400); IR 1670 cm$^{-1}$; $^1$H NMR (360 MHz, $CD_3CN$) $\delta$ 6.00 (H1, s), 4.63 (H2, bs), 7.22 (H5, d, *J* = 7.5 Hz), 6.85 (H6, m), 7.20 (H7, td, *J* = 7.7, 1.2 Hz), 6.82 (H8, m), 3.67 (H11, bt, *J* = 11.0 Hz), 2.95 (H12a, dd, *J* = 13.5, 7.4 Hz), 1.67 (H12b, dd, *J* = 13.5, 11.3 Hz), 4.02 (H15, m), 3.25 (H17a, dd, *J* = 14.8, 2.5 Hz), 3.08 (H17b, dd, *J* = 14.8, 5.7 Hz), 6.66 (H19, H23, m), 6.80 (H20, H21, H22, m), 2.77 ($NCH_3$, s); $^{13}$C NMR (75 MHz, $CD_3CN$) $\delta$ 81.07 (C2), 61.24 (C3), 132.72 (C4), 127.06 (C5), 121.42 (C6), 131.40 (C7), 111.56 (C8), 151.58 (C9), 58.19 (C11), 40.67 (C12), 167.68 (C13), 64.39 (C15), 167.54 (C16), 37.43 (C17), 137.13 (C18), 131.11 (C19, C22), 129.95 (C20, C23), 128.36 (C21), 32.71 ($NCH_3$); HRFABMS MH$^+$ 693.3177 ($C_{42}H_{41}N_6O_4$ requires 693.3165); FABMS 462, 448, 346, 332, 130; CD (MeOH) 215 ([$\vartheta$] 66800, 247 (45400), 305 (15200); $[\alpha]_D$ = +140.0° (*c* 0.30, MeOH); mp 198–201 °C.

**Degradation of WIN 64821 (1a) with Acetic Acid To Give Cyclo(L-Trp-L-Phe) (4).** WIN 64821 (**1a**) (10 mg) was dissolved in 0.1 M acetic acid (20 mL) and refluxed for 3 days. Ethyl acetate extraction and subsequent HPLC purification afforded cyclo(L-Trp-L-Phe) (**4**) as a white solid (5 mg). UV, IR, HRMS, CD, $^1$H and $^{13}$C NMR spectral, and MP data were identical with that of a synthetic sample of **4**, which was synthesized according to established procedures.[18]

**Photooxidation of Cyclo(L-Trp-L-Phe) (4).** Cyclo(L-Trp-L-Phe)(**4**) was photooxidized as previously described to give oxidation products **5** and **6**.[8] Spectroscopic data, with the exception of CD spectra, were in agreement with that previously reported (see Figure 1).[3] For **5**: CD (MeOH) 238 ([$\vartheta$] 8480) (238), 263 (–220), 293 (1320); $[\alpha]_D$ = +4° (*c* 0.10, MeOH). For **6**: CD (MeOH) 220 ([$\vartheta$] 4350), 244 (–8040), 267 (–750), 299 (–4190); $[\alpha]_D$ = –310.7° (*c* 0.15, MeOH).

**Isolation of Ditryptophenaline (3).** *Aspergillus flavus* culture SC1661 (500 mL) (from strains MIT M25-27) was extracted with ethyl acetate (200 mL × 2). Fatty material was removed by trituration with hexane and the remaining material was subjected to reverse-phase HPLC. Ditryptophenaline (**3**) was obtained as a white solid (8 mg). Spectroscopic data including UV, MS, NMR, and optical rotation data were in agreement with literature data. CD data were in agreement with one set of previously reported data,[9] but not a second set (Figure 1).[8]

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**Table III. $^1$H $^{13}$C, Difference NOE, and Selective INEPT NMR Data ($\delta$, *J* in Hz) for Monomethyl WIN 64821 (1b) in Acetonitrile-$d_3$ (300 MHz)**

| position | $^1$H (Hz) | $^{13}$C | selective INEPT to carbon (difference NOE data) |
|---|---|---|---|
| 1 | 5.85 s | | |
| 2 | 4.75 bs | 80.25 d | |
| 3 | | 60.71 s | H1, H5, H12 |
| 4 | | 131.72 s | H1, H12 |
| 5 | 7.29 d (7.8) | 125.93 d | (H11′) |
| 6 | 6.71 d (7.8) | 120.21 d | |
| 7 | 7.09 m | 130.12 d | |
| 8 | 6.69 m | 110.48 d | |
| 9 | | 150.24 s | |
| 11 | 3.96 dd (8.6, 8.2) | 57.56 s | (H2, H12a) |
| 12a | 3.01 m | 37.03 t | (H11) |
| 12b | 2.46 dd (14.2, 8.2) | | (H5′) |
| 13 | | 169.52 | H11, H12, H15 |
| 14 | 5.90 s | | |
| 15 | 4.12 dd (6.0, 5.0) | 56.88 d | |
| 16 | | 168.79 s | |
| 17a | 3.03 m | 36.10 t | |
| 17b | 2.98 m | | |
| 18 | | 137.19 s | |
| 19(23) | 7.11 s | 130.23 d | |
| 20(22) | 7.11 s | 129.22 d | |
| 21 | 7.11 s | 127.52 d | |
| 1′ | 6.01 s | | |
| 2′ | 4.75 bs | 80.25 d | (H11′) |
| 3′ | | 60.39 s | |
| 4′ | | 131.20 s | |
| 5′ | 7.27 d (7.7) | 125.93 d | (H12b′) |
| 6′ | 6.78 d (7.7) | 120.21 d | |
| 7′ | 7.20 dd (7.7, 7.7) | 130.08 d | |
| 8′ | 6.76 m | 110.23 d | |
| 9′ | | 149.94 s | |
| 11′ | 2.77 bdd (10.8, 7.2) | 57.30 d | (H5, H2′, H12a′) |
| 12a′ | 2.93 dd (13.7, 7.2) | 39.51 t | (H5, H11′) |
| 12b′ | 1.72 dd (13.7, 10.8) | | (H5′) |
| 13′ | | 166.41 s | H11′, H12′, *N*-$CH_3$ |
| 15′ | 4.04 bdd (5.4, 2.7) | 63.37 d | |
| 16′ | | 166.40 s | H11′, H15′, H17′ |
| 17a′ | 3.26 dd (14.1, 2.7) | 36.50 t | |
| 17b′ | 3.10 dd (14.1, 5.4) | | (*N*-$CH_3$) |
| 18′ | | 135.59 s | |
| 19′(23′) | 6.82 d (7.5) | 129.90 d | |
| 20′(22′) | 6.67 m | 128.78 d | |
| 21′ | 6.86 dd (7.5, 7.4) | 127.16 d | |
| *N*-$CH_3$ | 2.78 s | 31.86 q | (H11′, H15′, H17b′, H19′(23′)) |

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**Supplementary Material Available:** Copies of $^1$H and $^{13}$C NMR spectra of compounds **1a–c** and **2** (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.