WIN 64821, A NOVEL NEUROKININ ANTAGONIST PRODUCED BY AN *Aspergillus* sp.

III. BIOSYNTHETIC ANALOGS

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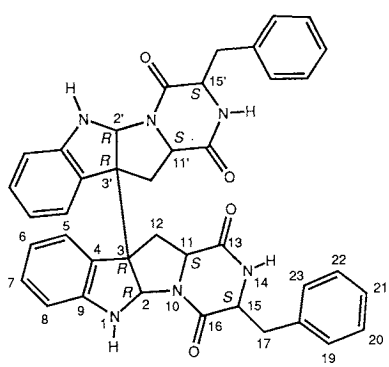
WIN 64821 (**1**) is a substance P (SP) antagonist isolated from a fungal culture (*Aspergillus* sp., SC319). It is a symmetrical dimer biosynthesized from four aromatic amino acid molecules: each equivalent half of the dimer is constructed from one molecule of phenylalanine (Phe) and one molecule of tryptophan (Trp). Feeding analogs of Phe, Trp, and other amino acids to intact cells of SC319 has yielded 36 biosynthetic analogs of WIN 64821. The analogs fall into three categories: substitutions on the indoline ring, substitutions on the Phe-derived phenyl ring, and replacement of the phenyl ring by an aliphatic group. In addition, these directed biosynthesis experiments generated asymmetrical dimers (derived from three amino acids) and, often, symmetrical dimers (derived from two amino acids). The relative SP binding affinities of several analogs suggest involvement of both the indoline and phenyl moieties in SP receptor binding.

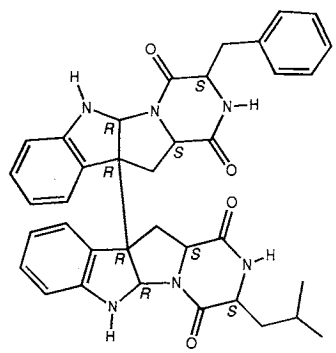
Substance P (SP), an undecapeptide, is a member of the neurokinin family and is believed to be the endogenous ligand for the neurokinin-1 receptor. Selective antagonists of SP may have utility as analgesics or anti-inflammatory agents. Our search for novel SP antagonists from microbial sources led to the discovery of WIN 64821 (**1**)[1]. This molecule was isolated from an *Aspergillus* sp. (SC319, ATCC 74177) and fully characterized[1]. It is a competitive antagonist of SP binding at the human NK1 receptor and displaces [^{125}I]SP with a *Ki* of 230 ± 30 nM. It also has significant NK2 receptor antagonist activity[2]. Information regarding biological activity, fermentation, and isolation of **1** is provided in the accompanying reports of this issue[2,3]

Inspection of the structure (**1**, Fig. 1), suggests that L-phenylalanine (Phe) and L-tryptophan (Trp)

Fig. 1. Structures of the natural products originally isolated from SC 319[1]: WIN 64821 (**1**) and WIN 64745 (**27**).



1 (WIN 64821)



27 (WIN 64745)

are the biosynthetic precursors of WIN 64821. The identification of **27** (Fig. 1) in the original culture extracts[1] supports the hypothesis that amino acids are the natural precursors; biosynthesis of **27** would presumably begin with Phe, Trp and leucine. This indicates the potential for directed biosynthesis of additional analogs by supplementing culture media with amino acids. This approach has been successful for the preparation of analogs of other microbial products, such as cyclosporin A[4] and asperlicin[5]. In this study, 36 biosynthetic analogs of **1** were prepared by feeding amino acids to cultures of SC319. The production, isolation, and biological activity of these biosynthetic compounds is the subject of this report.

Experimental

Microorganism

Fungal culture SC319 (ATCC 74177) was isolated in Taiwan by Panlabs, Inc. from a soil sample and identified as an *Aspergillus* sp. Taxonomy is described in the accompanying paper[3].

Culture Techniques and Feeding Experiments

The common amino acids and the following compounds were purchased from Sigma: D-Phe, D-Trp, DL-4-fluorotryptophan, DL-5-fluorotryptophan, DL-6-fluorotryptophan, DL-*o*-fluorophenylalanine, DL-*m*-fluorophenylalanine, DL-*p*-fluorophenylalanine, DL-4-methyltryptophan, DL-5-methyltryptophan, DL-6-methyltryptophan, DL-norvaline, DL-*β*-2-thienylalanine, DL-allylglycine, DL-*o*-chlorophenylalanine, DL-*p*-chlorophenylalanine. DL-5-Bromotryptophan was purchased from Aldrich, DL-crotylglycine from U.S. Biochemicals, and DL-*p*-methylphenylalanine from Schweizerhall (S. Plainfield, NJ). DL-*o*-Methylphenylalanine was obtained from JPS Chimie (Beaviax, Switzerland) and DL-5,5,5-trifluoroleucine from Columbia Organic Chemical Co. (Camden, SC). DL-Cyclohexylalanine was obtained from the in-house chemical collection of Sterling Winthrop, Inc. Ditryptophenaline was isolated from fermentations of *Aspergillus flavus* (SC1661, MIT M25-27)[1,6].

Fermentations in 250-ml shake flasks and 5-liter fermentors were accomplished with glycerol-beef extract medium according to procedures given in the preceding paper[3]. Most feeding experiments were conducted with resting cells. These were prepared after 48 hours of growth (late log phase); cells were harvested and washed twice with two volumes of 50 mM MOPS at pH 6.7. The cells were resuspended in the same buffer to yield the original culture volume. Without exogenous carbon or nitrogen sources, resting cells produced **1** and the analogs in buffer using standard incubation conditions: 27°C, 210 rpm, for up to 72 hours. Amino acids were added as dry powders directly to the incubation vessel (final concentration 5 mM). Screening for analog production was accomplished in pilot studies using small culture volumes, typically 2 ml or 4 ml in 17 × 100-mm (polypropylene) or 25 × 75-mm (glass) tubes, respectively. Feeding experiments were also successful when amino acids were added directly to cultures grown in glycerol-beef extract medium. In these cases, the cells were not washed before the incubations. Preparative scale incubations were performed in 250-ml shake-flasks or 5-liter stirred fermentors. At the end of the incubation period, the entire mixture (cells + buffer/medium) was extracted with an equal volume of ethyl acetate and the extract evaporated to dryness.

NK1 Binding Assay

Assay of crude extract and isolated analogs was conducted as previously described[3]. Reported *Ki* values are the average of at least two determinations. Differences of less than two fold are not considered significant.

Isolations

Culture extracts were analyzed by reverse phase HPLC. The system included a C_{18} reverse phase column, Waters model 600 gradient controller, model 510 pumps, and model 990 photodiode array detector. Various methanol - water gradient programs were employed and are described in the footnote to Table 1. For preparative scale purification of analogs, semipreparative columns were used: Microsorb C_{18}, 5 μm, 1 × 30 cm with guard cartridge, or YMC ODS A-323. Methanol - water gradients were optimized for each separation. All compounds were determined to be pure by HPLC coupled with photodiode array detection.

Spectroscopy

One dimensional proton and carbon-13 spectroscopy, and two dimensional proton homonuclear correlation (COSY) experiments were carried out using a Varian Gemini 300 spectrometer with tetramethylsilane as an internal standard in CD_3CN. Circular dichroism (CD) curves were measured in methanol using a JASCO J-600 spectropolarimeter. The molar circular dichroism ($\Delta\varepsilon$) was calculated at three different wavelengths. Mass spectral analysis was performed with a Nermag R10-10C mass spectrometer (CI using CH_4), a Finnigan MAT TSQ 70 mass spectrometer and a VG analytical ZAB 2-SE high field mass spectrometer. The FAB spectra were recorded using a Finnigan MAT TSQ 70 triple quadrupole mass spectrometer equipped with a Finnigan MAT Cesium Ion Gun. Approximately 500 ng of each analyte was dissolved in methanol and 0.5 μl of this solution was mixed with glycerol on the tip of the FAB probe. The energy of the ionizing cesium ion beam was 10 keV. The thermospray spectra (Fig. 2) were also recorded on a Finnigan MAT TSQ 700 with a Finnigan MAT TSP 2 ion source. For LCMS, a Microsorb C_{18} column (5 μm, 0.41 × 15 cm) was developed isocratically with water-methanol (35:65) at 1.2 ml/minute; a UV detector in series with the mass spectrometer was used to monitor 242 nm. The thermospray ion source conditions were: vaporizer 90°C, source block 200°C and repeller 40 volts. UV spectra were recorded on either a Varian Cary-1, or a Shimadzu 160 U UV-visible spectrophotometer.

Results

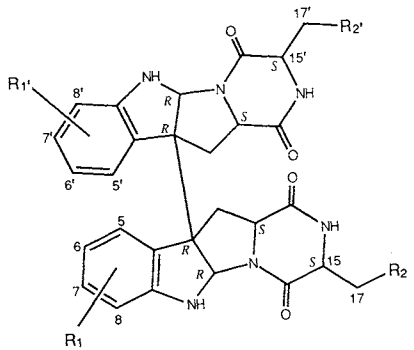
Biosynthetic Analogs

Approximately 70 different amino acids were individually incubated with SC319 cultures. The precursors included the 20 common amino acids, the D-isomers of Phe and Trp, and substituted analogs of Phe and Trp. Of these, 23 amino acids were incorporated to give 36 novel analogs of **1** (Table 1). Either heterodimers (asymmetrical dimers derived from three amino acids) or, often, both hetero- and homodimers (symmetrical dimers derived from two amino acids) were produced. Without exception, homodimers were not observed in the absence of the analogous heterodimers, and **1** was always produced in addition to any analogs. As demonstrated in fermentation studies[3], addition of L-Trp and/or L-Phe increased the production **1**, and L-Leu increased the production of **27**. However, other exogenous amino acids added to the culture medium did suppress the production of **1** to varying degrees. Isolated yields of the directed analogs were typically 1 to 15 mg/liter.

Detection of potential biosynthetic analogs of **1** in crude extracts was accomplished by analytical HPLC and UV spectroscopy (photodiode array detector). Compounds having a UV spectrum similar to **1** were isolated and characterized (see reference[3] for the UV spectrum of **1** and **27**). As an example of a typical HPLC profile, the UV-trace of an extract of a culture that was fed *m*-F-Phe is shown in Fig. 2A. Using authentic material as a standard, WIN 64821 (**1**) was identified by its retention time and UV spectrum. The two following peaks in the chromatogram have UV spectra very similar to that of **1**. These compounds were isolated by HPLC and subsequently identified as the *m*-F-phenyl heterodimer and homodimer analogs, **10** and **11**, respectively. It should be noted that the elution order of the compounds was typically **1**, followed by the heterodimer and homodimer analogs, respectively. When the culture was simultaneously fed fluorinated analogs of both precursor amino acids (*m*-F-Phe and 6-F-Trp), five HPLC peaks with UV-spectra similar to WIN 64821 were observed (Fig. 2B). Thermospray LC-mass spectroscopy demonstrated that those peaks were the parent compound (MH^+, *m/z* 665), the mono- (683), di- (701), tri- (719), and tetra- (737) F- analogs. Positional isomers were not determined for the mono-F- (two potential isomers), di-F- (four), or tri-F- (two) analogs.

Structural assignments were made using NMR, MS, and CD spectroscopy and by comparison of

Table 1. Structures, HPLC retention times, and mass spectra of WIN 64821 and biosynthetic analogs.



Amino acid fed	Compound	$R_{1'}$	R_1	$R_{2'}$	R_2	HPLC method and retention time (minute)[a]	Mass spectrum		
							$(M+H)^+$	Fragment A	Fragment B
None	**1**	H	H	Phenyl	Phenyl	A: 11.5, B: 22, C: 17.1, D: 7.5, E: 16.5	665[b,c]	332	332
4-F-Trp	**2**	5′-F	H	Phenyl	Phenyl	C: 16.8	683[b]	332	350
4-F-Trp	**3**	5′-F	5-F	Phenyl	Phenyl	C: 17.6	701[b]	350	350
5-F-Trp	**4**	6′-F	H	Phenyl	Phenyl	C: 17.6	683[b]	332	350
5-F-Trp	**5**	6′-F	6-F	Phenyl	Phenyl	C: 18.3	701[b]	350	350
6-F-Trp	**6**	7′-F	H	Phenyl	Phenyl	C: 17.9	683[b]	332	350
6-F-Trp	**7**	7′-F	7-F	Phenyl	Phenyl	C: 18.9	701[b]	350	350
o-F-Phe	**8**	H	H	*o*-F-Phenyl	Phenyl	C: 17.5	683[b]	332	350
o-F-Phe	**9**	H	H	*o*-F-Phenyl	*o*-F-Phenyl	C: 18.0	701[b]	350	350
m-F-Phe	**10**	H	H	*m*-F-Phenyl	Phenyl	C: 18.0	683[b]	332	350
m-F-Phe	**11**	H	H	*m*-F-Phenyl	*m*-F-Phenyl	C: 18.9	701[b]	350	350
p-F-Phe	**12**	H	H	*p*-F-Phenyl	Phenyl	C: 17.7	683[b]	332	350
4-Methyl-Trp	**13**	5′-Methyl	H	Phenyl	Phenyl	D: 9.8	679[c]	332	346

4-Methyl-Trp	**14**	5′-Methyl	5-Methyl	Phenyl	Phenyl	D: 25.0	693[c]	346	346
5-Methyl-Trp	**15**	6′-Methyl	H	Phenyl	Phenyl	A: 16.1	679[c]	332	346
5-Methyl-Trp	**16**	6′-Methyl	6-Methyl	Phenyl	Phenyl	A: 22.8	693[c]	346	346
6-Methyl-Trp	**17**	7′-Methyl	H	Phenyl	Phenyl	D: 10.2	679[c]	332	346
5-Br-Trp	**18**	6′-Br	H	Phenyl	Phenyl	D: 13.8	743[c]	332	411
o-Cl-Phe	**19**	H	H	*o*-Cl-Phenyl	Phenyl	D: 12.8	699[c]	332	366
o-Cl-Phe	**20**	H	H	*o*-Cl-Phenyl	*o*-Cl-Phenyl	D: 20.8	734[c]	366	366
p-Cl-Phe	**21**	H	H	*p*-Cl-Phenyl	Phenyl	D: 13.5	699[c]	332	366
o-Methyl-Phe	**22**	H	H	*o*-Methyl-phenyl	Phenyl	E: 18.4	679[c]	332	346
o-Methyl-Phe	**23**	H	H	*o*-Methyl-phenyl	*o*-Methyl-phenyl	E: 19.7	693[c]	346	346
p-Methyl-Phe	**24**	H	H	*p*-Methyl-phenyl	Phenyl	D: 10.6	679[c]	332	346
p-Methyl-Phe	**25**	H	H	*p*-Methyl-phenyl	*p*-Methyl-phenyl	D: 15.1	693[c]	346	346
Methionine	**26**	H	H	CH_2-S-CH_3	Phenyl	B: 13.7	649[b]	332	316
Leucine	**27**	H	H	Isopropyl	Phenyl	C: 17.8	631[b]	332	298
5,5,5-tri-F-Leucine	**28**	H	H	tri-F-Isopropyl	Phenyl	B: 31.5	685[b]	332	352
Valine	**29**	H	H	Dimethyl	Phenyl	B: 12.5	617[b]	332	284[d]
Norvaline	**30**	H	H	Ethyl	Phenyl	B: 12.9	617[b]	332	284[d]
Allylglycine	**31**	H	H	Ethenyl	Phenyl	B: 12.9	615[b]	332	282[d]
Crotylglycine	**32**	H	H	1-Propenyl	Phenyl	B: 16.0	629[b]	332	296
Crotylglycine	**33**	H	H	1-Propenyl	1-Propenyl	B: 12.0	593[b]	296	296
β-2-Thienylalanine	**34**	H	H	2-Thienyl	Phenyl	E: 14.8	671[c]	332	338
β-2-Thienylalanine	**35**	H	H	2-Thienyl	2-Thienyl	E: 15.7	677[c]	338	338
Cyclohexylalanine	**36**	H	H	Cyclohexyl	Phenyl	E: 19.9	671[c]	332	338
Cyclohexylalanine	**37**	H	H	Cyclohexyl	Cyclohexyl	E: 22.1	677[c]	338	338

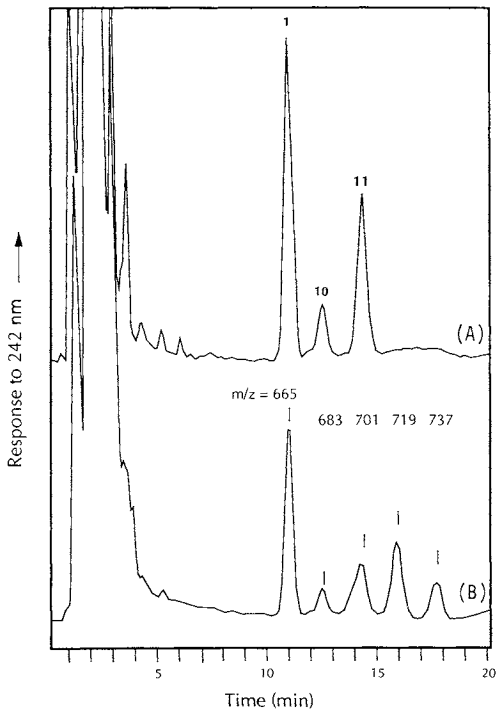
[a] Analytical HPLC methods: A: Column is Ranin Microsorb C_{18}, 5 μm particles, 4.1 × 150 mm with guard cartridge; solvent is 65% methanol in water, isocratic at 1.1 ml/minute. B: Column is Waters Nova-Pak C_{18}, 4 μm particles, 8 × 100 mm with guard cartridge; solvent is 54% methanol in water, isocratic at 2 ml/minute. C: Same as A except a solvent gradient of 55 ~ 70% methanol in water is run over 15 minutes followed by a 5 minutes hold at 70% methanol, flow is 1 ml/minute. D: Same as A except solvent is 68% methanol in water. E: Same as A except a gradient of 45 to 95% methanol (in water) in 20 minutes is run at 1.1 ml/minute.

[b] $(M+H)^+$ from positive ion FAB-MS.

[c] $(M+H)^+$ from CI-MS.

[d] Predicted fragment, MS not scanned below 300.

Fig. 2. HPLC traces of crude extracts from feeding experiments with (A) *m*-F-Phe, and (B) both *m*-F-Phe and 6-F-Trp.



In trace A, the peaks are WIN 64821 (**1**), its mono-fluoro analog (**10**) and di-fluoro analog (**11**). In trace B, thermospray LC/MS identified the mass-to-charge ratio (*m/z*) of each UV peak; the natural product (**1**) has *m/z* 665, and addition of one to four fluorine atoms increases *m/z* from 683 to 737.

spectral data with that of WIN 64821 (**1**) and the Leu-containing analog (**27**)[1,3]. The exact mass values obtained from high resolution MS agreed with the calculated mass values. Usually, FAB and chemical ionization MS lead to fragmentation at the C-3~C-3′ bond. Thus, homodimers would produce a single "monomer" fragment with a mass equal to one-half the molecular weight of the dimer; *e.g.*, **1** generated a fragment with *m/z* 332. Heterodimers split into two different "monomer" fragments, one of which was always *m/z* 332 (*e.g.* **27**). In every case, the molecular ion and the monomer ions were characteristic of the expected structures (Table 1). Proton, ^{13}C and COSY NMR spectroscopy further supported the assigned structures. Chemical shift, J_{HH}, and J_{CH} data clearly demonstrated that halo- and methyl-substitutions on the aromatic amino acids were incorporated into the analogous position of the hetero- and/or homodimers. For example, 5-fluoro-tryptophan was converted to analogs (**4** and **5**) in which the resonance for C-6 (and/or 6′) was a $^1J_{CF}$ doublet and the signals of H-5 and H-7 were $^3J_{HF}$ doublets. The NMR data of compounds **26**~**37** confirmed that, in each case, the R_2 (and $R_{2'}$ as in Table 1) was identical to the side chain of the amino acid added to the culture medium.

At this point, there remained the question of stereochemistry. It has been established that both **1** and a related fungal product, ditryptophenaline[6], have the *S* configuration at the carbons that correspond to the α-carbons of the precursor amino acids, Trp and Phe (C-11, C-11′, C-15, and C-15′ of **1**)[1]. However, ditryptophenaline and **1** differ in the configurations at C-2, C-3, C-2′, and C-3′; **1** has *R,R,R,R* configuration, and ditryptophenaline has *S,S,S,S* configuration[1]. We used circular dichroism (CD) and NMR techniques to determine the stereochemistry of our biosynthetic analogs. The UV spectra of **1** and the analogs had absorbance maxima close to 210 nm, 240 nm and 290 nm. As shown in Table 2, the absolute values and signs of $\Delta\varepsilon$ for the analogs paralleled the values for **1**. On the other hand, the CD spectrum of ditryptophenaline was negative and almost opposite to **1** and the analogs produced in this strain of *Aspergillus*. (It should be noted that we could not detect a trace of ditryptophenaline in this culture). Thus CD demonstrates that the configurations at the carbons adjacent to the indoline chromophore are *R,R,R,R*, as in **1**. From ^1H NMR experiments on selected analogs, nuclear Overhauser enhancements (NOE) were observed from H-2 to H-11, to H-15; since C-2 is *R*, H-2 can be close in space to H-11 and H-15 only if C-11 and C-15 have the *S* configuration. Therefore the biosynthetic analogs produced in this *Aspergillus* species have the same stereochemistry as the natural product, **1**.

Table 2. Circular dichroism data for **1**, selected analogs produced by SC319, and ditryptophenaline.

Compound	210 nm ($\Delta\varepsilon$)	240 nm ($\Delta\varepsilon$)	290 nm ($\Delta\varepsilon$)	Compound	210 nm ($\Delta\varepsilon$)	240 nm ($\Delta\varepsilon$)	290 nm ($\Delta\varepsilon$)
1	+24.6	+17.6	+6.0	**10**	+20.8	+14.1	+5.4
4	+27.1	+16.3	+6.1	**11**	+23.3	+15.2	+6.0
5	+29.2	+15.4	+7.4	**27**	+22.5	+16.6	+7.2
6	+34.3	+26.4	+7.4	**32**	+22.0	+14.4	+5.8
7	+36.6	+32.0	+7.1	**33**	+22.4	+14.8	+5.8
8	+26.6	+17.4	+6.1	Ditryptophenaline	− 4.6	− 7.6	−4.7
9	+25.3	+17.0	+7.0				

Table 3. SP antagonist activity of WIN 64821 and directed biosynthesis analogs.

Compound	Analog structure[a]	*Ki* (μM)	Compound	Analog structure[a]	*Ki* (μM)
1	WIN 64821	0.2	Phenyl replacements		
Indoline substitutions			**27**	$R_{2'}$=Isopropyl	4.0
4	6′-F	1.9	**32**	$R_{2'}$=Propenyl	2.1
5	6′,6-diF	2.9	**33**	$R_{2'}$,R_2=Propenyl	11.0
13	5′-Methyl	0.4	**34**	$R_{2'}$=2-Thienyl	0.9
14	5′,5-Dimethyl	5.7	**35**	$R_{2'}$,R_2=2-Thienyl	3.0
15	6′-Methyl	0.6	**36**	$R_{2'}$=Cyclohexyl	0.4
16	6′,6-Dimethyl	2.9	**37**	$R_{2'}$,R_2=Cyclohexyl	0.8
18	5′-Bromo	1.5			
Phenyl substitutions					
22	18′-Methyl	0.2			
23	18′,18-Dimethyl	0.2			
24	20′-Methyl	0.4			
25	20′,20-Dimethyl	0.4			

[a] Refer to Fig. 1 and Table 1 for numbering and R_2 designation.

Biological Activity of WIN 64821 Analogs

Many of the analogs of **1** were assayed for SP antagonism activity at the human NK1 receptor (Table 3). In general, analogs containing substitutions on the phenyl groups were as active as the parent compound, while those with indoline substitutions were 2- to 28-fold less active. Analogs with the phenyl group replaced by an aliphatic moiety generally were less active, except the cyclohexyl heterodimer (**36**). Interestingly, the binding affinity of ditryptophenaline (Ki=12 μM) was 60-fold lower than the affinity of **1** for the NK1 receptor[1]. As noted above, ditryptophenaline and **1** have opposite stereochemistry at C-2(2′) and C-3(3′), and in addition ditryptophenaline has *N*-methyl groups at N-14 and N-14′.

Discussion

Herein is another example of how directed biosynthesis is useful for rapid preparation of analogs of complex natural products. Whereas we have succeeded in producing 36 novel analogs of WIN 64821, the chemical synthesis of these analogs would have been an arduous and long term project. One liability, however, is that directed biosyntheis is limited by the substrate flexibility of biosynthetic enzymes. In this case, the substrate specificity did limit the scope of potential substitutions at R_1 and R_2; for example, amino acids having polar or small alkyl (*e.g.*, Ala) side-chains were not substrates for this biosynthetic system, and an indole side chain was required for the dimerization. Nevertheless, because the enzymes were somewhat permissive, a variety of analogs were generated for biological evaluation.

The stringent regio- and stereospecificity of the biosynthetic system dictated the assembly of amino

acids into dimeric structures that were analogous to the natural products (Fig. 1 and Table 1); the biosynthetic sequence did not break C-C bonds, or alter the substitution patterns of the amino acid precursors. A variety of structural changes were effected and the chiral centers of the resulting analogs had the same stereochemistry as the natural product, **1**. In addition to the introduction of substituents on the aromatic rings, the benzyl group (derived from Phe) could be replaced by aliphatic moieties. One such replacement, isopropyl (**27**), was a natural product isolated from initial fermentations of this fungus[1]; *i.e.*, its biosynthesis did not require addition of exogenous leucine to the culture medium. However, the yield of **27** was greatly enhanced when SC319 was incubated with millimolar amounts of L-Leu[3].

To date, the most potent NK1 antagonists contain two or three aromatic groups available for receptor recognition and binding[7~12]. In fact, the sequence of the natural ligand, substance P, includes two neighboring phenylalanine residues[13]. Modeling and X-ray crystal studies with two potent antagonists, CP-96,345 and CP-99-994, suggest that a parallel stacking of two aromatic rings is an important determinant for receptor binding[7,10]. Compound **1** has two types of aromatic handles: indoline and benzyl moieties. Our equilibrium binding results indicate that both aromatic moieties are involved in binding to the NK1 receptor (Table 3). Substitutions on the indoline ring had a variable effect on activity, but potency was always decreased (except **13**). While halogen and methyl substitutions on the phenyl ring were essentially neutral with respect to potency (**22**~**25**), replacement of the aromatic ring with an aliphatic group did reduce activity. The fact that the cyclohexyl heterodimer (**36**) retains potency, relative to **1**, suggests that the receptor has a binding pocket for the phenyl group(s) of **1** that is hydrophobic, but not strictly specific for aromatic groups. Published data[1] has demonstrated that methylation of one or both N-14/N-14′ significantly reduces binding affinity. In addition, inverting the configurations at C-2, C-3, C-2′, and C-3′ (ditryptophenaline) lowers the potency even further, thus leading us to believe the *R,R,R,R* stereochemistry is required for tight binding.

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