Synthesis, Conformational Properties, and Antibody Recognition of Peptides Containing β-Turn Mimetics Based on α-Alkylproline Derivatives†

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Peptide recognition by monoclonal antibodies may provide a useful model for drug development, in particular to test the effects of conformational restriction on ligand binding. We have tested the influence of novel peptide mimetics upon conformation and binding affinity for the case of monoclonal antibodies raised to a peptide antigen which displays a preference for a β-turn conformation in aqueous solution. Two monoclonals were isolated that recognized the peptide Ac-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala specifically at the β-turn formed by Tyr-Pro-Tyr-Asp. Peptide analogues were then synthesized containing mimetics designed to stabilize this conformation. One, analogue (3), contained a spirocyclic γ-lactam bridge between the α-position of proline-2 and the N atom of tyrosine-3, while another (2) contained (*S*)-α-methylproline at position 2. NMR spectroscopy and molecular modeling suggest that both analogues adopt reverse-turn conformations stabilized relative to that in the native sequence. For the (*S*)-α-methylproline analogue binding to both monoclonal antibodies was substantially improved, compared with the native antigen, whereas the γ-lactam analogue (3) was not recognized by either antibody. Quantitative equilibrium ultrafiltration binding assays showed that the affinities of the (*S*)-α-methylproline analogue (2) for the two antibodies were improved over those measured with the native antigen by −2.3 and −0.65 kcal/mol. The origins of these free energy differences cannot be explained wholly on the basis of presumed extra hydrophobic contacts between the new methyl substituent and the antigen binding sites. We propose that the increased conformational stability of the analogue plays a decisive role, implying that the reverse turn detected in the native antigen, possibly a type-I turn, is important for recognition by the two antibodies.

Although linear peptides of less than about 20 residues frequently occupy a random coil conformation, recent evidence has indicated the adoption of stable secondary structure, including β-turns[1-4] and regions of α-helix,[5-13] by various short peptide and protein fragments dissolved in water at, or near, room temperature. Even in these cases, the molecules probably interconvert rapidly between many low-energy conformations, but populate to a greater extent those possessing the respective turn or helix motifs.[15] However, the factors which govern the adoption of stable conformations in peptides and proteins dissolved in water remain ill-defined.[1-18]

A related issue concerns the mechanism of peptide recognition by a complementary protein receptor in aqueous solution. The forces governing peptide–receptor recognition are likely to be the same as those controlling protein folding,[19] although entropic factors may be of special importance when the ligand is a short conformationally mobile peptide. The relationships between peptide conformation and receptor recognition and the influence of novel conformational constraints upon both are therefore of interest. Anti-peptide monoclonal antibodies are attractive surrogates for studies of such molecular recognition processes, since with the application of modern biological and biophysical techniques it should be possible to elucidate and manipulate the three-dimensional structures of antigen–antibody complexes more easily than might be the case with other membrane-bound receptor–ligand protein complexes.

We have investigated how a nonapeptide with a known preference for a β-turn conformation is recognized by monoclonal antibodies complementary to the β-turn structure. The nonapeptide Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala (termed here peptide-P9) had been studied extensively[1,15,20] by use of ^{1}H NMR spectroscopy, and this revealed a highly populated type-II reverse turn conformation at the N-terminus[21] in water at 5 °C (as depicted

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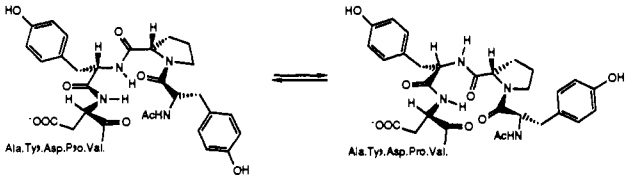
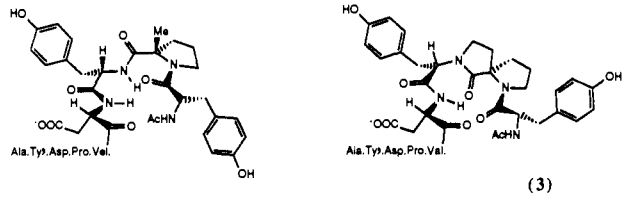
in 1). This sequence was also identified[22] as the immunodominant epitope of a 36-residue peptide immunogen comprising residues 75–110 of the influenza virus hemagglutinin HA1 chain,[23] thereby supporting the hypothesis that short peptides which are immunogenic also exhibit conformational preferences for secondary structural elements in aqueous solution.[24] We raised two monoclonal antibodies that recognize the N-acetylated form of P9 (i.e. NAcP9 1) at the N-terminal residues Ac-Tyr-Pro-Tyr-Asp, where the molecule populates the β-turn conformation.[25] With these monoclonals we then addressed the question of how the reverse-turn conformation is related to the mechanism(s) of binding to the two antibodies.

We describe here chemical studies which involved the synthesis of NAcP9 analogues containing nonproteinogenic amino acid replacements chosen for their potential to stabilize β-turn conformations. Of most interest were two analogues in which proline-2 had been methylated at the α-position to give 2αMePro-NAcP9 (2) or incorporated into a spirocyclic γ-lactam to afford 3. We report the synthesis of these molecules and the results of conformational studies using NMR spectroscopy and molecular modeling, together with the effects of these and other structural changes on the binding affinity to the two monoclonal antibodies specific for NAcP9.[26,27]



NAcP9 (1)



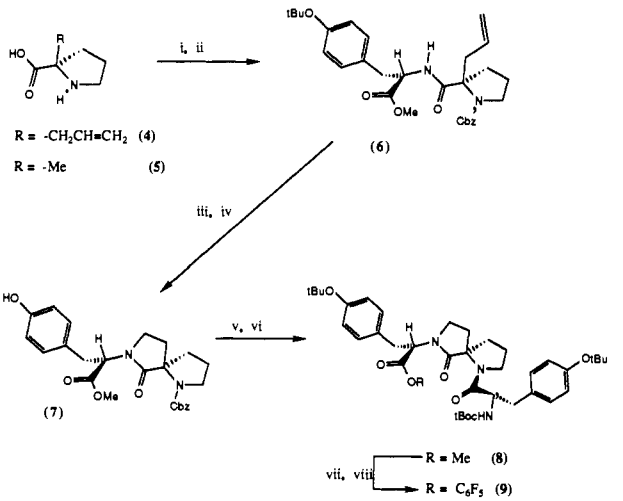
(3)

2αMePro-NAcP9 (2)

Peptide Design and Synthesis

Our first aim was to modify the backbone of NAcP9 in order to strongly favor the type-II β-turn conformation deduced from earlier[1,15,20,21] NMR studies, while perturbing minimally the other steric and electrostatic properties of the peptide. The γ-spirolactam in 3 appeared well suited to this purpose, since the value of γ-lactams in stabilizing

Scheme I[a]



[a] Reagents: i, $PhCH_2OCO\text{-}Cl$, Et_3N; ii, H_2N-Tyr(OtBu)-COOMe, $(Pr^iN{=})_2C$, HOBt, DMF; iii, OsO_4, $NaIO_4$, MeOH, then $NaBH_4$; iv, EtOOCN=NCOOEt, PPh_3, THF; v, H_2 Pd/C; vi, Boc-NH-Tyr-(OtBu)-COOH, $(Pr^iN{=})_2C$, HOBt, DMF; vii, aq NaOH, THF; viii, C_6F_5OH, $(PrN{=})_2C$, HOBT, DMF.

turn conformations in linear polypeptides was well precedented,[28–30] and molecular modeling studies suggested that the spirolactam unit functioned as a conformational mimic of a type-II β-turn (vide infra). The synthetic route to 3 (Scheme I) represents an extension of the chemistry developed by Freidinger[31] for the synthesis of γ-lactams, in that new conditions for the closure of the lactam were developed, which importantly, allowed the incorporation of the proline α-carbon within the lactam unit.[32–37] It was envisaged that the spirocyclic unit could be incorporated into the target peptide by using standard Fmoc solid phase peptide synthesis methods[38] (Fmoc = (fluoren-9-ylmethoxy)carbonyl).

An important component of the synthesis was the approach developed by Seebach and co-workers[39] for the α-alkylation of proline with self-reproduction of chirality. Thus (*R*)-2-allylproline (4) ($[\alpha]_D$ −53° (*c* = 2.0, MeOH)) and (*S*)-2-methylproline (5) ($[\alpha]_D$ −75° (*c* = 2.0, MeOH)) were prepared in optically pure form, starting from (*S*)-proline, the configuration of the quaternary centers being assigned by analogy to previous work.[39] Upon hydrogenation, (*R*)-2-allylproline afforded (*S*)-2-propylproline ($[\alpha]_D$

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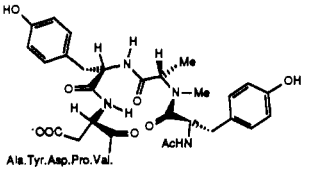
−76° (c = 2.0, MeOH)). The conversion of **4** into the protected dipeptide **6** was accomplished in two steps, and no difficulties were experienced with coupling protected **4** to H_2N-Tyr(OtBu)-OMe by standard procedures. Oxidative cleavage of the double bond in **6** by ozonolysis afforded only poor yields of aldehyde (dimethyl sulfide workup) or alcohol ($NaBH_4$ workup). However, upon reaction with catalytic OsO_4 and periodate at room temperature, followed by immediate reduction ($NaBH_4$ at −78 °C) of the resulting aldehyde, **6** was transformed into the required alcohol in 69% yield. This alcohol then reacted smoothly under Mitsunobu conditions[40] to afford the γ-lactam **7**, under conditions which had been described earlier in a β-lactam synthesis.[41] After cleavage of the N-protecting group by hydrogenation, coupling of **7** to *N*-*t*-Boc(OtBu)tyrosine proceeded very slowly in DMF, with activation by diisopropylcarbodiimide and hydroxybenzotriazole (HOBt), presumably due to the steric hindrance at the carbonyl group caused by the adjacent quaternary center. After several days at room temperature, **8** was isolated in 95% yield without contamination by epimers, as judged by high-field NMR analysis. Hydrolysis of the ester using aqueous NaOH yielded the corresponding acid, which without purification, was coupled to pentafluorophenol to afford the activated tripeptide ester **9**, again without epimerization. This derivative is a convenient form for use in solid-phase peptide synthesis, and the general utility of the spirocyclic lactam unit in constructing peptides of defined secondary structure should become evident (see also ref 42).

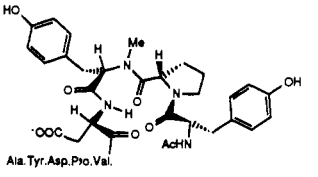
With the synthesis of the protected tripeptide **9** complete, the analogue **3** could be assembled by using Fmoc solid-phase methodology.[43] The first Fmoc-protected amino acid residue was coupled to commercial Pepsyn-KA resin (Milligen) as its preformed symmetrical anhydride in the presence of 4-(dimethylamino)pyridine as catalyst. This resin contains a *p*-(hydroxymethyl)phenoxyacetic ester linked to a poly(dimethylacrylamide) gel, supported on macroporous kieselguhr particles. Thereafter, the amino acid residues were added in the standard manner[43] until the N-terminus of the resin bound hexapeptide (H_2N-Asp-Val-Pro-Asp-Tyr-Ala-polymer) was released with use of piperidine. Acylation of this with 1.4 equiv of the pentafluorophenyl ester **9** generated the fully protected form of the desired resin-bound nonapeptide. Cleavage of the peptide from the resin using 95% TFA/H_2O (TFA = trifluoroacetic acid) yielded a crude product that was fractionated by chromatography on LH-20 Sephadex:1% AcOH–H_2O into two components (ca. 1:1 ratio), identified as the desired nonapeptide, and a hexapeptide resulting from incomplete coupling of **9** onto the free N-terminus in the final stage of the assembly. Further purification by preparative reverse-phase HPLC afforded homogeneous material, which was then acetylated at the N-terminus to afford **3**.

The synthesis of analogue 2αMePro-NAcP9 (**2**), containing (*S*)-α-methylproline in place of proline-2, was also accomplished by the Fmoc method, but using instead commercially available Sasrin resin[44] on an ABI 430A automated peptide synthesizer. Although the coupling of Fmoc-(*S*)-α-methylproline to resin-bound heptapeptide proceeded smoothly, the final coupling step with Fmoc-Tyr(OtBu)-NH_2 was sluggish. An unoptimized triple-coupling protocol was employed, using 2 equiv of Fmoc-Tyr(OtBu)-OH in each cycle. Upon completion of the synthesis, the nonapeptide was deprotected (piperidine) and removed from the resin with use of 90% TFA–H_2O. Homogeneous material was obtained following chromatography on LH-20 Sephadex and preparative reverse-phase HPLC before being acetylated to afford **2**.

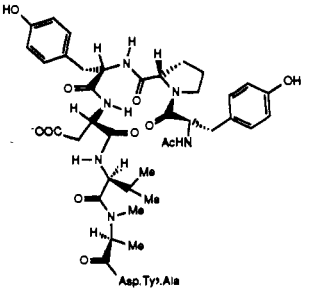
Two further analogues of NAcP9 (**1**) were synthesized in which the prolines at positions 2 or 6 were replaced with (*S*)-*N*-methylalanine. We envisaged that this substitution would relax any conformational constraint imposed by the proline rings, without seriously affecting the electrostatic properties of the peptides. The effects upon solvation energy, of course, would be unknown. These analogues, 2NMeAla-NAcP9 (**10**) and 6NMeAla-NAcP9 (**11**), were again assembled by normal sequential Fmoc-coupling methodology, employing Fmoc-(*S*)-*N*-methylalanine in the eighth or fourth round of assembly, respectively. Finally, the analogue 3NMeTyr-NAcP9 (**12**), containing (*S*)-*N*-methyltyrosine at position 3, was synthesized to assess the role of the Tyr3 amide NH in the recognition of the antigen by monoclonal antibodies. The necessary (*S*)-*N*-methyltyrosine was synthesized in a protected form using the NaH–MeI method of Benoiton[45,46] starting with *N*-Cbz-(OtBu)Tyr. After methylation, the Cbz-protecting group was replaced by Fmoc by using standard chemistry, and 3NMeTyr-NAcP9 (**12**) was assembled as before using the Fmoc solid-phase methodology.



2NMeAla-NAcP9 (**10**)



3NMeTyr-NAcP9 (**12**)



6NMeAla-NAcP9 (**11**)

For quantitative binding measurements to the monoclonal antibodies, labeled forms of the peptides **1**, **2**, **10**, and **11** were readily prepared with use of [^{3}H]acetic anhydride for the N-terminal acetylation step.

Conformational Studies by NMR Spectroscopy

The conformational behavior of NAcP9 (**1**) and the analogues 2αMePro-NAcP9 (**2**) and spirocycle **3** were studied in aqueous solution by ^{1}H NMR spectroscopy. Proton spectra were assigned by making spin-system as-

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Table I. Temperature Coefficients ($\Delta\delta$ ppb per °C) and Chemical Shifts at 25 °C (δ ppm), for the NH Resonances in the Peptides NAcP9 (1) (Cis and Trans Forms), 2NMeAla-NAcP9 (10) (Trans Form), 2αMePro-NAcP9 (2), 2αMePro-P9, and Spirocycle 3[a]

	trans-NAcP9 (1)		*cis*-NAcP9 (1)		*trans*-2NMeAla-NAcP9 (10)		2αMePro-NAcP9 (2)		2αMePro-P9		spirocycle 3	
residue	Δδ	δ	Δδ	δ	Δδ	δ	Δδ	δ	Δδ	δ	Δδ	δ
Tyr1	-8.0	8.20	-7.9	8.12	-8.9	8.14	-7.6	8.36			-7.3	8.05
Tyr3	-7.1	7.53	-6.7	8.10	-6.9	7.43	-3.3	6.67	-2.6	6.55		
Asp4	-4.6	8.19	-5.7	8.29	-5.0	8.38	0.0	7.92	0.0	7.86	-1.5	8.18
Val5	-7.4	7.95	-7.6	7.93	-8.0	7.85	-7.1	7.92	-11.6	7.96	-9.4	7.88
Asp7	-8.3	8.24	-8.0	8.22	-8.2	8.18	-8.7	8.26	-9.4	8.18	-8.0	8.25
Tyr8	-7.2	7.86	-7.3	7.88	-7.3	7.81	-8.0	7.89	-11.0	7.82	-8.5	7.81
Ala9	-6.2	7.74	-6.2	7.69	-6.3	7.68	-6.6	7.74	-7.9	7.68	-6.8	7.70

[a] ^{1}H NMR spectra were recorded at 500 MHz in H_2O/D_2O (90:10) (peptide concentration 10 mM), pH 6.0 (uncorrected meter reading). Coefficients ($\Delta\delta$ in ppb per °C) were determined by least-squares linear regression analysis from measurements over the range 3–30 °C.

signments from phase-sensitive COSY[47] or TOCSY[48,49] spectra, and then the connectivity was established from $d_{\alpha N}(i, i+1)$ NOEs detected in phase-sensitive ROESY[50] spectra. The ^{1}H NMR spectrum of NAcP9 (1) showed two sets of overlapping NH resonances, whose integrated intensities were in 3:2 ratio, due to slow trans–cis isomerism about the Tyr1 to Pro2 peptide bond. Earlier 2D NMR experiments on peptide-P9 by Wright and co-workers[1,20,21] had indicated that a β-turn conformation around residues Tyr-Pro-Tyr-Asp is significantly populated by the trans rotomer in water at 5 °C. On the other hand, the ^{1}H NMR spectra of 2αMePro-NAcP9 (2) and spirocycle 3 in 90% H_2O–D_2O each showed only one major set of NH resonances, and a 2D-ROESY spectrum of 2 showed a NOE between Tyr1-αCH and Pro2-δ(CH_2), as expected for a trans peptide bond. In a series of 1D NMR spectra of 2αMePro-NAcP9 (2), which were obtained at pH 6 over a peptide concentration range of 0.1–10 mM, there was no significant change in either the chemical shifts or the line widths of the amide protons. We conclude, therefore, that this peptide shows no tendency to aggregate in the concentration ranges used in this study.

A convenient indicator of stable secondary structure, frequently applied in conformational analyses of short oligopeptides dissolved in either water or organic solvents, is the temperature dependence of the peptide amide NH ^{1}H chemical shifts.[51] An amide proton involved in a stable intramolecular hydrogen bond, or one inaccessible to solvent for steric reasons, typically shows a reduced temperature coefficient of $> -6.0 \times 10^{-3}$ ppm/°C. The temperature coefficients for the amide protons in NAcP9 (1), 2αMePro-NAcP9 (2), and spirocycle 3 were measured in 90% H_2O–10% D_2O at pH 6, over the temperature range 3–30 °C (Table I). All plots of chemical shift against temperature were linear over this range, and there was no significant change in $^3J_{NH-C\alpha H}$ values, suggesting that no major changes in peptide conformation occurred. In the peptide NAcP9 (1) a reduced coefficient was apparent for the Asp4 NH in the trans rotomer, suggesting its participation in an intramolecular hydrogen bonded β-turn structure[1,20] (structure 1). The cis rotomer provides an ideal internal control, since a similar β-turn cannot form, and its Asp4 NH resonance showed a slightly higher temperature coefficient. More dramatic effects were seen for the Asp4 NH in 2αMePro-NAcP9 (2) and spirocycle 3.

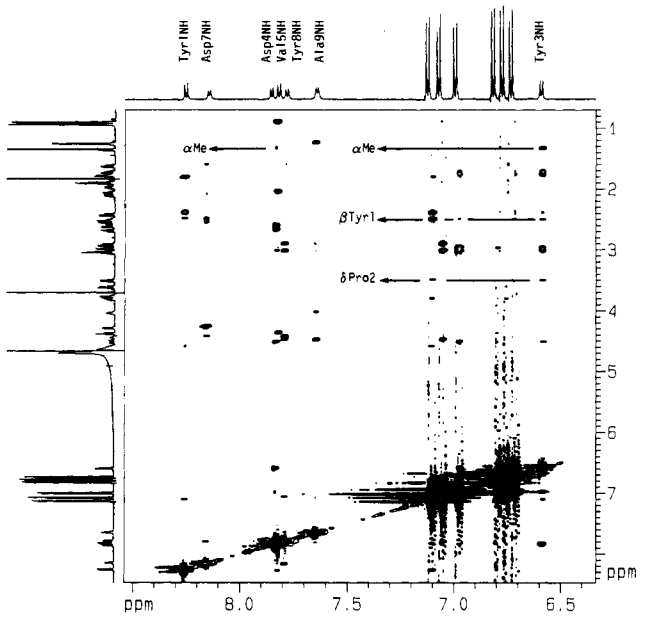

Figure 1. Section from a 2D ROESY spectrum measured at 600 MHz in 90% H_2O–10% D_2O, pH 6, at 30 °C, for the peptide 2αMePro-NAcP9, using a 100 ms mixing time (see Experimental Section for further details). The ^{1}H NMR assignments are given in Table II.

The cognate $\Delta\delta$ value in the spirocyclic analogue 3 is significantly lower relative to (1-trans) (Table I), supporting the notion that the type-II β-turn conformation is stabilized by the γ-lactam bridge. More surprisingly, this coefficient is still lower in 2αMePro-NAcP9 (2), and the Tyr3 NH now also shows a reduced-temperature coefficient, consistent with both Tyr3 and Asp4 NH groups being shielded from bulk solvent. The Tyr3 NH resonance in 2 also appears unusually far upfield, at 6.6 ppm, well removed from the other NH resonances. This large upfield shift is unlikely to arise solely due to H-bonding, but is possibly caused also by shielding from either the Tyr3 or Tyr1 aromatic rings. Evidence that the *N*-acetyl group was not responsible for these effects came from NMR data on the peptide 2αMePro-P9, possessing a free N-terminus, where again the Asp4 and Tyr3 NH resonances have low-temperature coefficients, and the Tyr3 NH resonates unusually far upfield (Table I).

Complementary NOE data were also obtained. 2D ROESY spectra for NAcP9 (1), recorded at 30 °C, revealed all four of the possible $d_{NN}(i, i+1)$ NOEs, and data derived from a series of sequential ROESY experiments, with various mixing times, showed that within experimental error all these weak NOEs had similar build-up rates. This suggests that average NH–NH distances are similar because the peptide populates many rapidly in-

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Table II. ^{1}H NMR Assignments for 2αMePro-NAcP9 in 90% H_2O–D_2O at 30 °C, pH 6

residue[a]	chemical shift assignments (δ) and C_αH–NH coupling constant					
	NH (*J*, Hz)	αH(Me)	βH	γH	δH	aromatic
Tyr1	8.29 (7.8)	4.62	2.51			7.12
			2.41			6.81
αMePro2		(1.35)	1.97	1.79	3.81 (*pro-R*)	
			1.74		3.52 (*pro-S*)	
Tyr3	6.61 (7.5)	4.53	3.04			7.00
			2.98			6.73
Asp4	7.87 (6.6)	4.67	2.69			
			2.61			
Val5	7.84 (6.6)	4.38	2.06	0.94		
				0.92		
Pro6		4.28	2.10	1.90	3.77	
			1.61		3.61	
Asp7	8.19 (6.5)	4.44	2.56			
			2.50			
Tyr8	7.81 (7.4)	4.49	3.03			7.07
			2.92			6.77
Ala9	7.67 (6.7)	4.03	1.26			

[a] MeCO = 1.83.

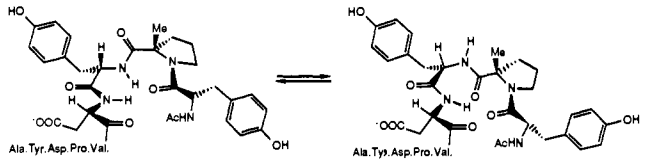

Figure 2. Representations of type-I and type-II reverse turns occupied by 2αMePro-NAcP9 (**2**). The type-I and type-II β-turns shown are principally related by rotation of the peptide bond between 2αMePro and Tyr3 by 180° (see text also and Figure 1).

terconverting conformational states. In contrast, 2αMePro-NAcP9 (**2**) showed a d_{NN} (i, i + 1) NOE between Tyr3NH and Asp4NH that was substantially larger than the other observable d_{NN} (i, i + 1) NOEs in this peptide. In addition, the 2D ROESY spectrum of **2** (Figure 1 and Table II), revealed other interesting NOEs, including Tyr3NH to the *pro-S* proton at the δ-position of Pro2, Tyr3NH to a proton at C_β in Tryl, Asp4NH to the α-methyl of 2αMePro, and Tyr3NH to the α-methyl of 2αMePro (Figure 2). These NMR data, in particular the observed NOE between Asp4NH and the α-methyl of 2αMePro, are consistent with a stabilized turn structure about residues Tyr-αMePro-Tyr-Asp, relative to that detected in P9 and NAcP9 (**1**). Surprisingly, by these criteria the α-methyl group of Pro2 appeared more effective in stabilizing the turn conformation than did the spirocyclic unit in **3**. There was little, if any, evidence for conformational order in the C-terminal halves of these peptides.

Two backbone conformations of 2αMePro-NAcP9 (**2**) in the tetrapeptide segment that would account for much of the NMR data are shown in Figure 2, where the residues occupy type-I or type-II turns. The α-methyl substituent seems to restrict the conformational space available to the tetrapeptide unit so that it occupies for a longer time reverse-turn conformations, which include the relatively strain-free (see molecular modeling results below) type-I turn, and only the trans-amide link. *We emphasize, however, that we do not view the peptide as being rigid; it must still populate other conformations, but on average spends more time in the reverse-turn geometry.*

Finally, the replacement of Pro2 in NAcP9 by *N*-methylalanine was expected to result in a higher temperature coefficient for the Asp4 NH resonance due to increased conformational mobility. In fact, this coefficient was increased in analogue 2NMeAla-NAcP9 (**10**), but by a smaller amount than anticipated (Table I). Furthermore, d_{NN} (i, i + 1) NOEs in the ROESY spectra of this peptide were small or not detected, consistent with a randon-coil conformation. The analogous replacement of Pro6 by *N*-methylalanine, however, had little effect on the turn conformation at the N-terminus, as detected by NMR.

Computer Modeling Studies

In order to examine whether any adverse steric or electrostatic effects might arise from the introduction of the non-proteinogenic amino acids, computer modeling studies were undertaken. First, we wished to determine whether the spirocyclic γ-lactam in peptide **3** would indeed be effective in constraining the peptide into a type-II reverse turn. It also became of interest to determine the low-energy conformations accessible to the critical tetrapeptide segment in the nonapeptide analogues 2αMePro-NAcP9 (**2**) and 2NMeAla-NAcP9 (**10**), primarily to assess whether steric buttressing between the adjacent methyl groups in the *N*-methylalanine residue would prevent the N-terminus of the 2NMeAla-NAcP9 analogue from adopting a reverse-turn geometry.

The determination of all possible conformations for the nonapeptides represented too difficult a problem[52] for the available computer resources, especially if explicit water molecules were to be included in the calculations. Although this problem could have been addressed by using Monte-Carlo search algorithms, it is often difficult to assess whether important minima have been missed by the random-search procedure. Therefore, our studies were carried out upon tetrapeptides AcTyr-2MePro-Tyr-Asp-NHMe (**13**) and AcTyr-NMeAla-Tyr-Asp-NHMe (**14**) (Table III) as models of the critical segments of **2** and **10**, respectively, and we sought to determine whether the conformational minima of **13** were a subset of those adopted by **14**. A systematic grid search[53,54] was carried out using the MULTICONFORMER option of the MacroModel V2.5 molecular modeling package[55] which generated sets of initial molecular geometries for **13** and **14** (2904 and 3661, respectively). Conformations in which nonbonded atoms were

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Table III. Conformational Properties of Low-Energy Structures Computed for Tetrapeptide Model Peptides **13** and **14**

rel energy, kJ mol^{-1}	φ_{i+1},[a] deg	ψ_{i+1},[a] deg	φ_{i+2},[a] deg	ψ_{i+2},[a] deg	β-turn type[b]
(a) AcNH-Tyr-αMePro-Tyr-Asp-NHMe (**13**)					
0.0	−54.7	−23.5	−86.3	−8.7	I
0.6	−55.3	−27.1	−91.5	−39.5	VIII
4.3	−71.9	45.9	−161.7	−51.2	
7.1	−71.0	57.2	−78.5	64.7	
(b) AcNH-Tyr-NMeAla-Tyr-Asp-NHMe (**14**)					
0.0	57.7	−119.9	−82.4	−44.7	II′
0.2	−57.5	−39.1	−60.0	−47.6	I
4.3	59.1	−126.3	−66.9	−48.5	II′
6.2	−125.3	62.3	59.6	38.5	
8.8	59.0	−122.3	−81.9	−43.8	II′

[a] The torsion angles in the table are defined such that residues $i+1$ and $i+2$ correspond to the amino acids in positions 2 and 3 relative to the N-terminus of the oligopeptide, i.e. in **13** residue $i+1$ is (*S*)-α-methylproline, while in **14** residue $i+1$ is (*S*)-*N*-methylalanine. [b] Although there have been various classifications of β-turn types, the proposed types correspond to those described by Wilmot and Thornton (ref 59).

closer than 1.5 Å were eliminated, and the remaining structures were energy minimized using the all-atom AMBER potential energy functions and parameters[56,57] as implemented in BATCHMIN V2.5.[55] We note that BATCHMIN is a computational package which merely *reproduces* AMBER energies by using the appropriate potential energy function. It should not be confused with the widely used AMBER software. The tetrapeptides were modeled as the N-acetylated, *N*′-methylamide derivatives in order to minimize the introduction of conformational artifacts due to strong charge–charge interactions. Partial charge distributions for the nonproteinogenic amino acids, *N*-methylalanine and (*S*)-α-methylproline, were obtained by using MOPAC[58] V4.0, and nonbonded cutoffs were employed in all calculations (electrostatic 12.0 Å, van der Waals 6.0 Å, hydrogen bonding 4.0 Å).

After energy minimization and elimination of duplicate conformations, all structures which possessed strain energies greater than 20 kJ/mol relative to the global energy minimum were excluded from further analysis. Given the low barriers to side-chain rotation, the backbone torsion angles (ϕ,ψ) of the peptides were selected as measures of the molecular conformation (Table III). For the tetramer **13** containing (*S*)-α-methylproline a single, well-defined minimum-energy structure was obtained in which the (ϕ,ψ) angles of the α-methylproline–tyrosine segment corresponded to those present in a type I β-turn.[59] The next structure, determined to be 0.6 kJ/mol higher in energy, was merely a distorted form of the lowest energy conformation and was almost identical about the critical α-methylproline residue. However, in the case of **14** containing (*S*)-*N*-methylalanine, the minimum-energy structure **14a** was located on the potential energy surface only 0.2 kJ/mol below a conformationally unrelated minimum **14b**. Significantly, the ϕ backbone torsion angle of the *N*-methylalanine residue in **14a** had a value of +57.7°, and therefore its peptide backbone could not be identical with that of oligopeptide **1** since the corresponding angle of (*S*)-proline must be negative. On the other hand, the backbone of **14b** superimposed almost identically onto the

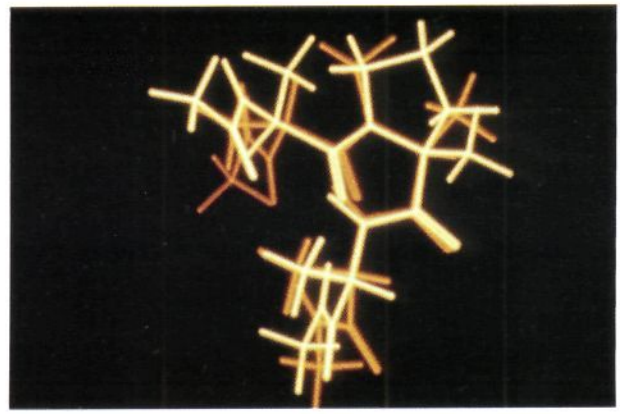

Figure 3. Graphical representation of the global minimum-energy structure deduced for the tetramer AcTyr-2-MePro-Tyr-Asp-NHMe (**13**) (in yellow) and the minimum-energy conformer (**14b**) (in orange) (see text and Table III) found for AcTyr-NMeAla-Tyr-Asp-NHMe. The backbones of these two conformers superimpose almost identically.

global minimum found for **13** in the β-turn region, as illustrated in Figure 3.

These results indicate that a relatively strain free type-I reverse-turn conformation is accessible to the 2αMePro analogue, and that there is no significant *enthalpic* penalty preventing the *N*-methylalanine residue from adopting a conformation identical with that of (*S*)-proline, i.e. the accessible conformation space of **13** is a subset of that for peptide **14**. Consequently, steric effects should not be a significant factor in reducing the binding constant, with respect to NAcP9 (**1**), of 2NMeAla-NAcP9 (**10**) for both monoclonal antibodies (vide infra). In addition, visualization of the electrostatic fields[60] about the energy-minimized structures **13** and **14b** suggested that the molecular electrostatics were essentially unaffected by the amino acid replacements. We also note that while these modeling studies were carried out with gas-phase potentials and AM1 charge distributions,[61] unmodified to account for the effects of solvent dielectric,[62,63] recent work aimed at modeling the peptide recognition site from the primary sequence of DB19/1 has revealed the highly hydrophobic nature of the pocket.[64] Attempts to include the effects of water upon the peptide conformation may not, therefore, be entirely relevant. However, calculations which include the effects of an aqueous environment are currently in progress.

Computational studies were also undertaken to evaluate the ability of the spirocyclic lactam unit in **3** to hold this peptide in the desired type-II β-turn. A detailed conformational analysis of **3** in which all of its low-energy minima were determined, including the effects of solvation, was not required for the resolution of this modeling problem, given that we only wished to ascertain the similarity of the spirocyclic unit to the dipeptide sequence, Pro-Tyr, when the latter was in a minimum-energy structure corresponding to a type-II β-turn. Hence, a full systematic search was performed upon the protected peptide Ac-

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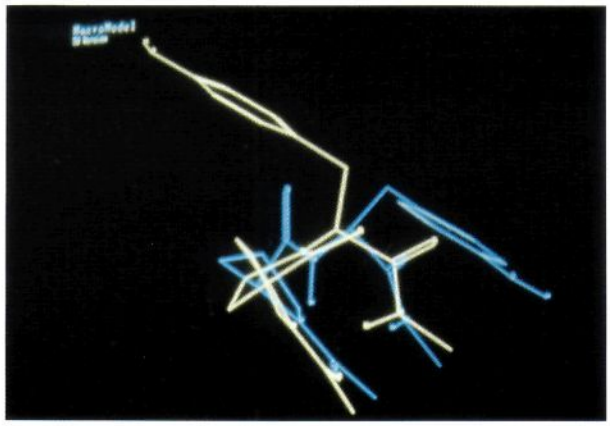
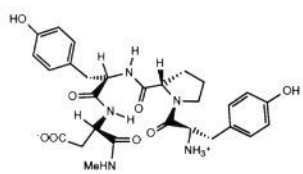


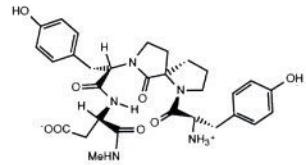
Figure 4. Graphical representation of the minimum-energy structures found for the tetramers **16** (in blue) and **17** (in green), showing the high degree of similarity in the backbone conformations around the type-II β-turn. Note, however, that the carbonyl group of Pro2 in **16** and the carbonyl group within the proline portion of the spirocycle in **17** do not superimpose well; their positions are related by about a 60° rotation.

Pro-Tyr-NMe (**15**) by examining all combinations of rotatable torsion angles, as the latter were varied through 60° increments.[52-54] Then all of these initial structures were energy minimized using the "united atom" AMBER potential functions and parameters,[56] as implemented in the program BATCHMIN,[55] to yield low-energy conformations. This set of structures was analyzed to obtain the lowest energy minimum possessing backbone torsions best resembling a type-II β-turn.[59] Flanking residues, tyrosine and aspartic acid, were then built onto this structure to generate the model tetrapeptide Tyr-Pro-Tyr-Asp-NHMe (**16**). Systematic variation of all rotatable bonds was then



(16)

carried out for **16** with the exception of the backbone torsion angles of the central Pro-Tyr segment. Subsequent energy optimization using a block-diagonal Newton-Raphson algorithm,[65,66] coupled with removal of conformations lying higher than 20 kJ/mol above the global minimum then yielded 32 low-energy structures which incorporated the β-turn. In all of these search procedures, duplicate conformational minima are removed by a standard superimposition procedure.[67] Such "build-up" algorithms have been demonstrated to allow effective conformational searches to be carried out for complex peptides.[68] Although our initial calculations were carried out using gas-phase minimization, making the interaction between the protonated N-terminal amino group and the charged aspartate side chain dominant, repetition of these calculations using a recent continuum solvation model[69] for introducing the effects of the aqueous environment into the conformational search has not yielded significantly different type-II β-turn structures. The low-energy structures of the γ-spirolactam peptide **17** which incor-



(17)

porated the novel spirocyclic amino acid unit were mapped in a similar fashion. All energy minimizations were carried out using a point charge model for the electrostatic interactions.[70] In the case of the novel spirocyclic unit, partial charges were obtained from ab initio computations using an STO-31G basis set. Superimposition of the global minima of **16** and **17** using standard superimposition methods[67] revealed an rms deviation of the main chain atoms of only 0.510 Å, indicating that a high degree of similarity existed in the crucial turn region of these tetrapeptide units, as shown in Figure 4. In both compounds the appropriate distances and torsion angles were in good agreement with those expected for type-II β-turns. Solvent accessible surface area[71] and volume calculations[72] revealed that the ethano bridge in the spirocyclic unit of **17** contributed an extra 24 Å^3 of volume, relative to **16**, an increase of approximately 4%.

Although these calculations indicated that the α-methylproline residue induces a type-I β-turn into the nonapeptide 2αMePro-NAcP9 (**2**), this may have been due to the parameterization procedures used to derive the AMBER potentials used in the simulation. However, AMBER has been shown to reproduce observed peptide conformations in a number of other studies.[73]

Antibody Binding Studies

The two monoclonal antibodies of interest, designated DB19/1 and DB19/25, were raised[25] in mice against a synthetic 19-residue peptide immunogen Asn-Ser-Lys-Ala-Phe-Ser-Asn-Ala-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser-Leu-Cys which was conjugated via cysteine to keyhole limpet hemocyanin. The monoclonals were selected on the basis of their ability, using a solution-phase competitive ELISA, to bind to the nine residue peptide NAcP9 (**1**) in the region of the nascent β-turn conformation.[25] The four residues Tyr-Pro-Tyr-Asp at the N-terminus of NAcP9 (**1**) comprise the principal epitope for recognition by both monoclonals. The following observations support this conclusion: when the N-terminal tyrosine is deleted from NAcP9 (**1**), binding to both monoclonal antibodies is abolished; the replacement of proline-2 in **1** by (*S*)-*N*-methylalanine again results in loss of binding, whereas when proline-6 is replaced by (*S*)-*N*-methylalanine binding is not significantly perturbed (see below); when Asp4 in **1** is replaced by Asn, binding to both antibodies is again abolished, whereas when Asp7 in **1** is replaced by Asn, binding is not significantly affected; a shifted epitope encompassed by the peptide Phe-Ser-Asn-Ala-Tyr-Pro-Tyr-Asp-Val was bound tightly by monoclonal DB19/1, although binding to DB19/25 was sub-

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Table IV. Dissociation Constants for the Equilibrium Binding of [Ac-^{3}H]Peptides to the Antibodies DB19/1 and DB19/25[a]

peptide	dissociation constant (K_d, M) DB19/1	DB19/25
NAcP9	$(1.8 \pm 0.2) \times 10^{-7}$	$(1.8 \pm 0.2) \times 10^{-8}$
2NMeAla-NAcP9	$(4.2 \pm 0.3) \times 10^{-6}$	$(6.0 \pm 0.5) \times 10^{-7}$
6NMeAla-NAcP9	$(8.8 \pm 0.8) \times 10^{-8}$	$(3.8 \pm 0.2) \times 10^{-8}$
2αMePro-NAcP9	$(3.6 \pm 0.5) \times 10^{-9}$	$(5.7 \pm 0.4) \times 10^{-9}$

[a] The dissociation constants were determined by equilibrium ultrafiltration. General procedure: The antibody and each [Ac-^{3}H]-peptide were mixed in PBS (5 mL) and incubated at 20 °C for 16 h. Two 1-mL aliquots were each filtered separately through an Amicon Centrifree micropartition membrane by centrifugation at 1900g (Heraeus Bench top centrifuge) for 5 min. The concentration of unbound peptide in the filtrate was measured by liquid scintillation counting, and the dissociation constant was determined by Scatchard analysis.[82] Nonspecific binding of the antigen was estimated using an antibody (RJD 2A10) with different specificity. For [Ac-^{3}H]2αMePro-NAcP9 the assay was carried out in PBS containing casein (0.1 μM), in order to reduce nonspecific binding of antigen to the filter. The error limits represent $\pm 2\sigma$ from the Scatchard plot. The changes in free energy of binding are related to the change in dissociation constant by the relation: $\Delta\Delta G = -RT \ln \Delta K_d$.

stantially diminished. The pentapeptide Ac-Tyr-Pro-Tyr-Asp-Val-NHMe (C-terminal methylamide) seems to comprise the minimum sequence necessary for recognition by both antibodies, and further evidence for the importance of the β-turn region around Tyr-Pro-Tyr-Asp came from our studies with the β-turn mimetic containing α-methylproline, described below.

When a competition ELISA was used to investigate binding of the analogues to these monoclonals, the spirocyclic analogue **3** failed to bind to either antibody. In order to test the importance of the Tyr3 NH group for recognition (lost in the spirocycle), the analogue of NAcP9 containing 3-*N*-methyltyrosine (**12**) was synthesized. This also failed to bind to either antibody. We can conclude, therefore, that the peptide backbone Tyr3 NH places a key role in both recognition processes, possibly by direct hydrogen bonding with residues in the antibody binding sites. The extra bridging methylene group in the spirocyclic turn mimetic would then introduce unacceptable steric buttressing, hence precluding its recognition by either antibody.

On the other hand, the 2αMePro-NAcP9 analogue (**2**) bound with *higher affinity* to the two monoclonals than did NAcP9 (**1**), an effect that was quantified by measuring the equilibrium dissociation constants using tritiated forms of NAcP9, 2αMePro-NAcP9, 2NMeAla-NAcP9, and 6NMeAla-NAcP9 and each of the antibodies. The 2- and 6-*N*-methylalanine analogues were investigated as controls. The 2αMePro substitution increased the affinity of the antigen to DB19/1 and DB19/25 by factors of 50 and 2, corresponding to changes in the free energy of binding of 2.3 and 0.65 kcal/mol, respectively (Table IV). On the other hand, the 2NMeAla-NAcP9 analogue (**10**) was bound more weakly to both antibodies than was NAcP9 (**1**), by a factor of *over 200-fold* in the case of DB19/1. These results emphasize the importance of the Pro2 residue in the epitope recognized by both antibodies. Likely causes for the reduced affinities are changes in the conformational flexibility of the peptide, or related solvation energy differences.

The origins of the enhanced affinities seen upon substituting proline by α-methylproline are of great interest and merit further consideration. Firstly, they may be due to extra hydrophobic binding energy arising from contacts made by the new methyl group in each of the antibody combining sites, or secondly, they may be due to the stabilized secondary structure in 2αMePro-NAcP9 that we detected by NMR, which would give rise to a reduced entropy loss upon binding (or a combination of these two factors). Recent estimates for the intrinsic (hydrophobic) binding energy of a methylene group in water, which fall in the range 1.0–1.5 kcal/mol, have been determined for the situation where a hydrophobic side chain is buried in a preformed protein cavity.[74] This value may not be entirely relevant here, since the presumed hydrophobic cavities into which the extra methyl group in 2αMePro-NAcP9 must fit would be performed for a hydrogen atom in NAcP9. Binding a methyl group in a cavity designed for a hydrogen atom would presumably lead to an unfavorable buttressing with groups in the protein. Nevertheless, the change in binding free energy observed with DB19/1 lies outside this range. At least in this case, therefore, the extra binding affinity presumably cannot be derived solely from such hydrophobic binding forces.

Our NMR data, on the other hand, show clear evidence for the stabilization of secondary structure in 2αMePro-NAcP9 (**2**), relative to NAcP9 (**1**), and this alone on entropic grounds would be expected to result in an improved binding affinity to the antibodies, as long as the new methyl group does not prevent binding per se. It seems unlikely that the electrostatic properties of the antigen have been altered significantly by introducing the methyl group (see computer modeling results). These arguments, therefore, point strongly to entropic or related solvation effects as being wholly responsible for the increased affinities of 2αMePro-NAcP9 for both monoclonals, especially DB19/1. Moreover, since this substitution stabilizes a reverse-turn conformation, which is also populated by the native antigen, it follows that this conformation is important for recognition of the native antigen by both monoclonal antibodies. These recognition events thus seem to be sensitive to the entropic penalty incurred when the peptide becomes constrained to a well-defined conformation upon binding, a conclusion that has clear implications for the design of synthetic vaccines based on flexible peptides.

The important question of whether a type-I or a type-II turn is recognized by these antibodies cannot be answered unambiguously at present, but our current prejudice lies in favor of the type-I turn, for the following reasons. Both type-I and type-II turns, as detected by NMR, are stabilized in 2αMePro-NAcP9 (**2**), relative to NAcP9 (**1**). However, the contrasting influences upon binding of the α-methyl group in **2** on the one hand and the spirocycle in **3** and 3NMeTyr in **12** on the other hand suggested to us that in both cases the Tyr3 NH is positioned into the binding cavity and makes direct contact with the antibodies, whereas the α-methyl group is directed out of the binding cavity into free solution. Divergently oriented Tyr3 NH and α-methyl groups are consistent with the type-I turn but not the type-II turn. This proposal provides a focus for further investigation aimed at determining for each antibody the bound antigen conformation, through transfer NOE NMR experiments and X-ray crystallographic studies of complexes between antibody fragments and the peptide antigen, and such work is currently underway.

It is not yet certain whether the effects of the (*S*)-α-methylproline substitution described here are unique to the Tyr-Pro-Tyr-Asp sequence and/or the two monoclonal antibodies. There are relatively few examples in which

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optically pure *chiral* α-methylamino acids have been used to define the relative importance of dynamic events in structure–activity relationships (SARs) with flexible peptide ligands. This aspect of molecular recognition is difficult to explore with proteinogenic amino acid replacements due to the difficulty of maintaining the essential steric and electrostatic features of the natural substrate for the receptor. We have found only one other case in which a similar use has been made of (*S*)-α-methylproline, namely its incorporation into a peptidic renin inhibitor, but its effects upon peptide conformation were not described.[75] Since optically pure α-methylamino acids are now available through synthesis, they should complement other methods (e.g. formation of cyclic peptides, or internal disulphide links) and become of general use for probing conformational factors in SAR studies.

Experimental Section

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. 1D NMR spectra were recorded on Bruker AM360 and JEOL GX270 spectrometers, and chemical shifts are expressed in ppm from Me_4Si. 2D NMR spectra on peptides dissolved in water were recorded on Varian VXR500 or Bruker AMX600 spectrometers, and chemical shifts are expressed in ppm from dioxane (δ 3.7) included as internal standard. Peptide samples for NMR were dissolved in 90% H_2O/10% D_2O, pH 6 (uncorrected meter reading). Probe temperatures were calibrated with use of ethylene glycol. COSY,[47] ROESY,[50] and TOCSY[48,49] experiments were performed in the phase-sensitive mode by using the TPPI method.[76] The water resonance was suppressed by presaturation over a 2-s period. 2048 Complex points were collected in 2D sets for each of 512 t_1 values with 16–64 scans per t_1 value. The ω_1 and ω_2 dimensions were processed with a $\pi/2$ phase shifted sine bell weighting function. The data were zero filled to ω_1 to yield a final matrix of 1024 × 1024 real points. A base-line correction was performed in 2D data sets before the ω_1 transform. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck 60F UV_{254}), and components were visualised either by UV light or by a vanillin–MeOH–H_2SO_4 spray reagent. Optical rotations were recorded on a Optical-Activity AA100 polarimeter. High-pressure liquid chromatography (HPLC) was performed with a Waters-600 instrument with a Model 481 UV detector. Analytical reverse-phase (RP-HPLC) separation used Waters C18 μ-Bondapak (4.6 mm × 25 cm), Brownlee RP300 (4.6 mm × 25 cm), or Vydac 214TP (4.6 mm × 25 cm) columns, whereas preparative separations used 19-mm- or 46-mm-diameter columns from the same manufacturers. Ion-exchange HPLC used Brownlee AX300 or CX300 (4.6 mm × 25 cm) columns. FAB mass spectra were recorded on a VG 70-250-Se double-focusing instrument, or a ZAB-2E double-focusing reverse-geometry mass spectrometer at the SERC Mass spectrometry centre, University College Swansea. Peptide syntheses were carried out either on a home-built continuous-flow instrument, as described by Dryland and Sheppard,[77] or on an ABI 430 automated instrument. All peptide syntheses were monitored at each step of assembly by the Kaiser[78] and isatin[79] tests. Amino acid analyses were carried out by the ninhydrin method on an LKB amino acid analyzer, and peptide sequencing was performed on an automated ABI 470A instrument. Pepsyn-KA resin was from Waters-Millipore, and SASRIN resin was from Bachem. Dimethylformamide (DMF) for peptide synthesis was distilled from CaH_2 under reduced pressure directly before use. Tetrahydrofuran (THF) was distilled just prior to use from sodium/benzophenone, and methylene chloride (CH_2Cl_2) was distilled from P_2O_5, acetonitrile (MeCN) from CaH_2, and ethyl acetate (EtOAc) from K_2CO_3. Solvents for HPLC were of HPLC-grade and filtered (0.25-mm filter) prior to use. Organic solutions were dried over Na_2SO_4 and evaporated on a Büchi rotary evaporator under reduced pressure. Column chromatography on silica gel was performed by the method of Still and co-workers.[80] [^{3}H]Ac_2O was from Amersham.

(*R*)-2-Allylproline (4). (2*R*,5*S*)-2-*tert*-Butyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one was prepared from (*S*)-proline and pivaldehyde and then alkylated with allyl bromide to give (2*R*,5*R*)-5-allyl-2-*tert*-butyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one, by the method of Seebach and co-workers.[39] This last product (11.83 g, 53 mmol) was stirred at room temperature in 6 M aqueous HCl (160 mL) for 2 days. The aqueous solution was washed with CH_2Cl_2 and evaporated, and the residue was chromatographed on DOWEX 50-W eluting with 1 N aqueous NH_3. Fractions containing product (4) were lyophilized to give 8.1 g (99%) of amorphous solid: $[\alpha]^{19.5}_D$ −53° (*c* 2.0, MeOH); IR (mull) 3080, 1630, 1475, 1383 cm^{-1}; ^{1}H NMR (D_2O) δ 1.80–2.10 (m, 3 H), 2.22–2.36 (m, 1 H), 2.40–2.51 (dd, 1 H), 2.73–2.85 (dd, 1 H), 3.20–3.40 (m, 2 H), 5.12–5.25 (dd, 2 H), 5.58–5.73 (m, 1 H); HR-MS *m/e* calcd for $C_8H_{14}NO_2$ (MH^+) 156.1024, found 156.0926.

(*R*)-*N*-Cbz-2-allylproline. Benzyl chloroformate (5.4 mL, 31 mmol) was added to a solution of 4 (4.04 g, 26 mmol) and triethylamine (7.2 mL) in MeCN–water (1:1) (30 mL) at 0 °C. After 48 h the solution was washed with EtOAc, and the aqueous phase was acidified and then extracted with EtOAc. The latter organic extracts were washed with H_2O and saturated NaCl and then dried and evaporated. The residue after chromatography on silica eluting with MeOH–CH_2Cl_2 (98:2) yielded 4.9 g (64%) of a colorless oil: $[\alpha]^{20}_D$ +20.1 (*c* 0.9, $CHCl_3$); IR ($CHCl_3$) 2995, 2720, 1710 cm^{-1}; NMR ($CDCl_3$) cis + trans rotomers δ 1.85, 2.05, 2.20, 2.35 (4 x m, 4 H), 2.6, 2.72 (2 x m, 1 H), 2.92, 3.02 (2 x m, 1 H), 3.43 (m, 1 H), 3.68, 3.78 (2 x m, 1 H), 5.12 (m, 4 H), 5.67 (m, 1 H), 7.32 (m, 5 H); MS (FAB) *m/e* 290 (M + H), 246. 200, 154,110, 91, 77, 65, 55.

(*R*)-*N*-Cbz-2-allylprolyl(*O*-*tert*-butyl)tyrosine Methyl Ester (6). Diisopropylcarbodiimide (780 μL, 4.98 mmol), (*R*)-allylproline (1.2 g, 4.2 mmol), (*O*-*tert*-butyl)tyrosine methyl ester (1.35 g, 5.4 mmol), and hydroxybenzotriazole (1.5 g, 11 mmol) in DMF (10 mL) were stirred at room temperature for 2 days before being poured into H_2O and extracted with EtOAc. The combined organic extracts were washed with H_2O, saturated NaCl, dried, and evaporated to afford a yellow solid. Chromatography on silica eluting with hexane–ethyl acetate (4:1) gave 2.06 g (95%) of dipeptide **6** as an oil: $[\alpha]^{20}_D$ −14.1° (*c* 1.57, $CHCl_3$); IR ($CHCl_3$) 2980, 2875, 1745, 1685, 1615, 1510 cm^{-1}; NMR ($CDCl_3$) δ 1.29 (s, 9 H), 1.49 (m, 1 H), 1.68 (m, 1 H), 1.76 (m, 1 H), 2.48 (m, 1 H), 2.80 (m, 1 H), 2.90 (m, 1 H), 2.97 (m, 1 H), 3.15 (m, 1 H), 3.30 (m, 1 H), 3.45 (m, 1 H), 3.72 (s, 3 H), 4.77 (m, 1 H), 5.02–5.20 (m, 4 H), 5.58 (m, 1 H), 6.80–6.98 (m, 4 H), 7.24–7.42 (m, 5 H), 7.90 (d, 1 H); MS (FAB) *m/z* 523 (M + H), 507, 493, 479, 467, 433, 423, 403, 91 (100).

Spirocyclic Dipeptide 7. Sodium periodate (455 mg, 2.12 mmol) was added to a solution of OsO_4 in *tert*-butyl alcohol (2.5% w/w, 0.6 mL) and dipeptide **6** (370 mg, 0.709 mmol) in methanol (15 mL) and water (7.5 mL), and the mixture was stirred for 2 h at room temperature, poured onto water, and extracted with EtOAc. The organic extracts were washed with H_2O, dried, and evaporated to give a brown oil which was used directly in the next step. A solution of sodium borohydride in 2-propanol (4 mg/mL, 1.5 mL) was added to a solution of the aldehyde from the previous step dissolved in EtOAc (20 mL) at −78 °C. The solution was stirred at this temperature for 1 h and then allowed to warm to room temperature over 30 min. The mixture was poured onto H_2O and extracted with EtOAc, and the organic extracts were washed with H_2O and saturated NaCl and then dried. Evaporation of the solvent gave a brown oil (350 mg), which after chromatography on silica eluting with toluene–ethyl acetate (gradient 100% to 0% toluene) gave 223 mg (60%) of product as a colorless oil: $[\alpha]^{21}_D$ −34.5 (*c* 0.5, $CHCl_3$); IR ($CHCl_3$) 3620, 3430, 3300, 2985, 1750, 1686 cm^{-1}; NMR ($CDCl_3$) δ 1.31 (s, 9 H), 1.51 (m, 1 H), 1.72 (m, 1 H), 1.81 (br m, 1 H), 2.08 (br m, 1 H), 2.55 (br m, 1 H), 2.70 (br m, 1 H), 2.83 (br m, 1 H), 2.90 (dd, 1 H), 3.15 (dd, 1 H), 3.4 (br m, 2 H), 3.6–3.8 (br m, 5 H), 4.73 (br

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Table V. NMR Assignments for Various Peptides Described in the Experimental Section[a]

peptide and position	residue								
	Ac	1 Tyr	2 Pro	3 Tyr	4 Asp	5 Val	6 Pro	7 Asp	8 Tyr	9 Ala
YPYDVPDYA (60% trans Pro2)										
trans form										
NH		nd	–	7.67	8.19	7.99	–	8.23	7.89	7.77
α		4.41	4.43	4.61	4.61	4.42	4.32	4.49	4.57	4.10
β		2.84	1.85	3.04	2.59	2.07	1.53	2.54	2.96	1.32
β		3.02	2.19	3.09	2.64	–	2.16	2.61	3.09	
γ/ortho		7.17	1.93	7.12		0.94	1.96		7.13	
γ/meta		6.90	1.93	6.82		0.98	1.96		6.83	
δ			3.32				3.67			
δ			3.69				3.83			
cis form										
		Tyr	Pro	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		nd	–	nd	8.50	8.02	–	8.29	7.92	7.78
α		3.49	3.25	4.70	4.59	4.42	4.35	4.50	4.57	4.10
β		2.92	1.62	2.84	2.59	2.07	1.53	2.54	2.96	1.32
β		3.07	1.66	3.21	2.64	–	2.17	2.61	3.09	
γ/ortho		7.02	1.38	7.17		0.91	1.96		7.13	
γ/meta		6.88	1.66	6.90		0.98	1.96		6.83	
δ			3.30				3.67			
δ			3.42				3.83			
Ac YPYDVPDYA (60% trans)										
trans form										
	Ac	Tyr	Pro	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		8.30	–	7.62	8.26	8.09	–	8.31	7.96	7.83
α	1.98	4.78	4.44	4.65	4.68	4.46	4.42	4.55	4.62	4.15
β		2.77	1.86	3.01	2.69	2.12	1.72	2.61	3.01	1.37
β		2.89	2.13	3.15	2.65	–	2.20	2.66	3.15	
γ/ortho		7.18	1.94	7.16		0.99	1.94		7.18	
γ/meta		6.91	1.94	6.88		1.03	1.94		6.89	
δ			3.43			3.72				
δ			3.79			3.88				
cis form										
	Ac	Tyr	Pro	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		8.21	–	8.19	8.37	8.02	–	8.34	7.97	7.83
α	2.04	4.04	3.46	4.50	4.66	4.46	4.39	4.55	4.62	4.15
β		2.84	1.33	2.96	2.65	2.13	1.72	2.61	3.01	1.37
β		3.00	1.79	3.23	2.65	–	2.20	2.66	3.15	
γ/ortho		7.14	1.21	7.18		0.98	1.94		7.18	
γ/meta		6.91	1.63	6.91		1.04	1.94		6.89	
δ			3.27				3.72			
δ			3.32				3.88			
N-deacetylspirocycle 3 (90% trans; cis n.d.)										
	Ac	Tyr	(Pro)	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		nd		–	8.19	8.07	–	8.14	7.88	7.75
α		4.33	–	4.34	4.54	4.43	4.27	4.49	4.54	4.09
β		2.95	1.72*	3.30	2.65	2.05	1.67	2.53	2.96	1.32
β		3.23	1.84*	3.30	2.70	–	2.12	2.60	3.09	
γ/ortho		7.27	1.95*	7.12		0.97	1.95		7.12	
γ/meta		6.91	2.01*	6.85		1.00	1.95		6.81	
δ			3.14				3.67			
δ			3.75				3.75			
ϵ			1.86							
ϵ			2.34							
η			2.76							
η			3.48							
spirocycle 3 (100% trans)										
	Ac	Tyr	(Pro)	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		8.29	–	–	8.21	7.93	–	8.10	7.86	7.74
α	1.96	4.67	–	4.47	4.55	4.37	4.19	4.48	4.54	4.06
β		2.70	1.83*	3.12	2.57	2.04	1.60	2.51	2.92	1.30
β		3.00	1.88*	3.25	2.67	–	2.07	2.57	3.06	
γ/ortho		7.18	1.97*	7.12		0.91	1.90		7.10	
γ/meta		6.85	2.05*	6.84		0.95	1.90		6.81	
δ			3.33				3.62			
δ			3.87				3.80			
ϵ			1.90							
ϵ			2.25							
η			2.91							
η			3.49							

Table V (Continued)

peptide and position	residue 1 Ac	1 Tyr	2 Pro	3 Tyr	4 Asp	5 Val	6 Pro	7 Asp	8 Tyr	9 Ala
2αMePro-P9 (100% trans)										
		Tyr	MePro	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		nd	-	6.60	7.91	8.01	-	8.22	7.87	7.75
α		4.27	1.47 αMe	4.62	4.71	4.44	4.32	4.50	4.56	4.09
β		2.36	1.97	3.05	2.70	2.13	1.67	2.56	2.98	1.32
β		2.73	2.12	3.18	2.70	-	2.16	2.61	3.09	
γ/o		7.25	1.97	7.06		0.98	2.00		7.13	
γ/m		6.93	1.97	6.77		1.01	2.00		6.83	
δ			3.59				3.67			
δ			3.87				3.84			
2NMeAla-NAcP9 (10) (50% trans)										
trans form										
	Ac	Tyr	MeAla	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		8.25	-	7.51	8.46	7.94	-	8.27	7.90	7.75
α	1.92	nd	nd	4.63	4.62	4.41	4.35	4.49	4.55	4.08
β		2.73	1.20	2.82	2.62	2.07	1.67	2.54	2.95	1.31
β		2.74	2.59 NMe	3.15	2.66	-	2.18	2.60	3.08	
γ/ortho		7.07		7.10		0.92	1.96		7.13	
γ/meta		6.84		6.84		0.98	1.96		6.83	
δ							3.67			
δ							3.83			
cis form										
	Ac	Tyr	MeAla	Tyr	Asp	Val	Pro	Asp	Tyr	Ala
NH		8.42	-	8.22	8.46	7.99	-	8.26	7.90	7.75
α	2.03	nd	4.52	4.51	4.62	4.41	4.35	4.49	4.55	4.08
β		2.87	0.38	2.85	2.62	2.07	1.67	2.54	2.95	1.31
β		3.02	2.12 NMe	3.15	2.66	-	2.18	2.60	3.08	
γ/ortho		7.09		7.03		0.93	1.96		7.13	
γ/meta		6.78		6.81		0.98	1.96		6.83	
δ				3.67						
δ				3.83						

[a] Chemical shifts are relative to dioxane at δ 3.7 as secondary reference. Conditions typically 20 °C, pH 4–6, in 90% H_2O–10% D_2O. The spirocycle is labeled from the quaternary carbon β/γ/β toward the N-terminus and ε/η toward the C-terminus. Methylene protons at prochiral centres are not assigned stereospecifically; they are listed in order of increasing chemical shift. nd = not determined, in the free amines due to fast exchange with water. Some α-protons are coincident with the water line and are not observed. An asterisk indicates uncertainty in the assignment of β vs γ protons in proline.

m, 1 H), 5.12 (br s, 2 H), 6.80–7.00 (m, 4 H), 7.25–7.46 (br m, 5 H), 7.92 (br d, 1 H); MS (FAB) *m/e* 527 (M + H), 509, 495, 483, 468, 453, 437, 427, 91 (100).

The dipeptide alcohol (890 mg, 1.69 mmol), prepared as described above, was reacted with triphenylphosphine (930 mg, 3.5 mmol) and diethyl azodicarboxylate (318 mL, 2.0 mmol) in dry THF (10 mL) at room temperature for 1 h. The solvent was then evaporated and the residue chromatographed on flash silica eluting with hexane–EtOAc (gradient 0% to 50% EtOAc), to afford the spirocycle **7** (736 mg, 80%) as an oil: $[\alpha]^{21}_D$ −30.1° (*c* 0.59, $CHCl_3$); IR ($CHCl_3$) 2990, 2880, 1750, 1705, 1620, 1510 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (s, 9 H), 1.70 (m, 1 H), 1.78 (m, 2 H), 1.90 (m, 3 H), 2.87 (m, 1 H), 3.0 (dd, 1 H), 3.12 (m, 1 H), 3.26 (m, 2 H), 3.32 (dd, 1 H), 3.61 (s, 3 H), 4.90 (m, 1 H), 5.10 (m, 2 H), 7.4–6.8 (m, 9 H); MS (EI) *m/z* 508 (M^+), 452, 392, 345, 317, 275 (100); HR-MS *m/e* calcd for $C_{29}H_{36}H_2O_6$ (M^+) 508.2573, found 508.2574.

Deprotected Lactam. The spirocycle **7** (736 mg) was hydrogenolyzed in methanol (50 mL) in the presence of Pd/C (10%, 70 mg) under an atmosphere of H_2. The catalyst was then removed by filtration through Celite and the solvent evaporated to give an oil (528 mg), which after chromatography on silica eluting with EtOAc–MeOH (gradient 0% to 10% MeOH) gave the product as an oil (362 mg, 57%): $[\alpha]^{24}_D$ −18.9° (*c* 2.9, $CHCl_3$); IR ($CHCl_3$) 3340, 3000, 1740, 1690, 1605, 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.33 (s, 9 H), 1.70 (m, 1 H), 1.78 (m, 2 H), 1.90 (m, 3 H), 2.05 (br s, 1 H), 2.87 (m, 1 H), 3.01 (dd, 1 H), 3.12 (m, 1 H), 3.26 (m, 2 H), 3.32 (dd, 1 H), 3.72 (s, 3 H), 5.01 (dd, 1 H), 6.90 (d, 2 H), 7.09 (d, 2 H); ^{13}C NMR ($CDCl_3$) δ 25.9, 28.8, 33.9, 34.9, 35.3, 41.9, 47.3, 52.1, 55.1, 67.6, 78.3, 124.1, 129.0, 131.0, 154.3, 170.8, 178.1; MS (EI) *m/e* 374 (M^+), 359, 332, 317, 287, 276, 234, 216, 183, 178 (100%); HR-MS *m/e* calcd for $C_{21}H_{30}N_2O_4$ (M^+) 374.2205, found 374.2215.

Tripeptide 8. Diisopropylcarbodiimide (150 μL, 0.96 mmol) was added to a solution of the foregoing product (358 mg, 0.96 mmol), *N*-Boc-Tyr(OtBu)-OH (695 mg, 2.06 mmol), and hydroxybenzotriazole (550 mg, 4.07 mmol) in DMF (10 mL) and stirred at room temperature for 3 days, before being poured onto water and extracted with EtOAc. The EtOAc extracts were washed with H_2O and saturated NaCl and dried. Evaporation gave a yellow oil which was chromatographed on silica eluting with hexane–EtOAc to give **8** (624 mg, 94%) as an oil: $[\alpha]^{22}_D$ +2.5° (*c* 0.80, $CHCl_3$); IR ($CHCl_3$) 3420, 2990, 2880, 1745, 1710, 1650, 1615, 1510 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (s, 9 H), 1.33 (s, 9 H), 1.39 (s, 9 H), 1.80 (m, 3 H), 1.90 (m, 1 H), 2.02 (m, 1 H), 2.48 (m, 1 H), 2.86 (m, 2 H), 3.00 (dd, 1 H), 3.10 (dd, 1 H), 3.26 (dd, 1 H), 3.30 (m, 1 H), 3.48 (m, 1 H), 3.58 (m, 1 H), 3.62 (s, 3 H), 4.52 (m, 1 H), 4.97 (t, 1 H), 5.26 (d, 1 H), 6.91, 6.92 (2 x d, 4 H), 7.18 (d, 2 H), 7.24 (d, 2 H); ^{13}C NMR ($CDCl_3$) δ 23.9, 28.3, 28.8 (×2), 30.1, 34.2, 36.4, 38.9, 41.4, 47.8, 51.9, 53.8, 56.2, 68.0, 78.1, 78.3, 79.4, 124.1, 124.2, 129.4, 130.4, 131.3, 131.8, 154.1, 154.2, 169.8, 171.1, 173.7; MS (EI) *m/e* 693 (M^+), 678, 662, 634, 620, 579, 564, 534, 520, 481, 464 (100); HR-MS *m/e* calcd for $C_{39}H_{55}N_3O_8$ (M^+) 693.3989, found *m/z* 693.4045.

Tripeptide 9. Aqueous NaOH (500 μL, 2 M) and the tripeptide **8** (339 mg, 0.489 mmol) in THF (4 mL) and H_2O (2 mL) was stirred for 1 h at room temperature. The mixture was then acidified with 1 N HCl, poured onto H_2O, and extracted with EtOAc. The organic extracts were washed with H_2O and saturated NaCl, dried, and then evaporated and dried in vacuo to give an oil (340 mg). This oil was redissolved in EtOAc (5 mL) with diisopropylcarbodiimide (93 μL, 0.6 mmol) and pentafluorophenol (110 mg, 0.6 mmol), and the mixture was stirred overnight at room temperature. The solution was then evaporated and the residue was chromatographed on silica eluting with hexane–EtOAc, to afford **9** (234 mg, 55%) as an oil: $[\alpha]^{24}_D$ −21.1 (*c* 1.35, $CHCl_3$); IR ($CHCl_3$) 3440, 2990, 2890, 1790, 1710, 1650, 1615, 1525 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (s, 9 H), 1.32 (s, 9 H), 1.39 (s, 9 H), 1.85 (m, 3 H), 1.90 (m, 1 H), 2.01 (m, 1 H), 2.55 (m, 1 H), 2.85 (m, 2 H),

3.02 (dd, 1 H), 3.23 (dd, 1 H), 3.30 (dd, 1 H), 3.37 (m, 1 H), 3.60 (m, 2 H), 4.53 (m, 1 H), 5.26 (d, 1 H), 5.39 (dd, 1 H), 6.93 (d, 2 H), 6.97 (d, 2 H), 7.22 (d, 2 H), 7.23 (d, 2 H); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 23.9, 28.2, 28.8 (×2), 30.0, 33.9, 36.3, 38.7, 41.1, 47.7, 53.8, 55.9, 67.7, 78.1, 78.4, 79.5, 124.1, 124.4, 129.5, 130.3, 130.4, 131.1, 154.2, 154.4, 166.5, 170.1, 173.7, signals for C_6F_5 too weak for detection; MS (FAB) m/z 846 (M^+), 790, 746, 728, 702; HR-MS m/e calcd for $C_{44}H_{53}F_5N_3O_8$ (M + H) 846.37527, found m/z 846.37644.

Peptide 3. *N*-Fmoc-alanine (572 mg, 1.84 mmol) was converted to a symmetrical anhydride by reaction with diisopropylcarbodiimide (286 μL, 1 equiv) in DMF (1.5 mL) for 20 min. The solution was then filtered and circulated through a column of Pepsyn-KA resin (2.24 g, 0.198 mmol; 0.09 mequiv g^{-1}) with *N*,*N*-dimethyl-4-aminopyridine (30 mg) in DMF. The N-terminus was then deprotected with piperidine (20% v/v) in DMF and then acylated with the next symmetrical anhydride prepared from *N*-Fmoc-(OtBu)Tyr-OH (676 mg, 1.47 mmol) and diisopropylcarbodiimide (230 μL) in DMF. The subsequent additions were completed in a similar manner until a resin-bound hexapeptide (H_2N-Asp-Val-Pro-Asp-Tyr-Ala-pepsyn-KA) had been assembled. The tripeptide **9** (234 mg) was then circulated through the column with hydroxybenzotriazole (26 mg) until a negative Kaiser test was obtained. Thereafter, the resin was washed with DMF and diethyl ether and then air-dried. The dry resin (2.46 g) was shaken in 95% TFA–water (25 mL) for 4 h, the solution was filtered, and the solid residue was washed with TFA. The filtrate was then evaporated to give an oil (315 mg), which was chromatographed on LH20 Sephadex eluting with 1% acetic acid at a flow rate of 30 mL h^{-1}. Fractions (7.5 mL) were collected at 15-min intervals. Two UV-active bands eluted from the column, one in fractions 52–58 and the other in fractions 67–78. The respective fractions were pooled and freeze-dried. The second eluted band was fractionated twice by preparative HPLC on a μ-Bondapak C18 column eluting with a gradient of 10% MeCN + 0.2% TFA to 90% MeCN + 0.2% TFA over 30 min gave the product (66 mg, 30%, >99% purity by analytical reverse-phase HPLC) as a white powder after lyophilization: Amino acid analysis Asp 1.45, Ala 1.10, Pro 1.10, Tyr 1.70, Val 1.00; NMR (90% H_2O–10% D_2O, pH 6, 20 °C) see Table V for assignments; MS (FAB) m/e 1128 (M + H); HR-MS m/e calcd for $C_{55}H_{69}N_9O_{17}$ (M + H) 1128.4889, found 1128.48725. Acetic anhydride (38 μL) was added to a solution of the foregoing product (19 mg) in aqueous NaOH (380 μL, 100 mM) and water (1.5 mL). The mixture was shaken at room temperature and the disappearance of starting material was followed by reverse-phase HPLC. Material from several runs was combined and fractionated by RP-HPLC on a μ-Bondapak C18 column (19 ~ 150 mm) eluting with a gradient of 10% MeCN + 0.2% TFA to 90% MeCN + 0.2% TFA over 30 min: NMR (90% H_2O–10% D_2O) see Table V for assignments; MS (FAB) m/e 1192 (M + Na), 1170 (M + H).

General Procedure for the Synthesis of Peptides Containing Proteinogenic Amino Acids: Ac-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala (NAcP9) (1). Pepsyn-KA resin (2.0 g, 0.184 mmol equiv) was acylated with a symmetrical anhydride formed from alanine (572 mg, 1.84 mmol) by reaction with diisopropylcarbodiimide (280 μL) in the presence of DMAP (30 mg). The remaining amino acids (1.47 mmol) were attached sequentially to the growing peptide by using the standard method, and the N-terminus of the complete nonapeptide was then acetylated with acetylimidazole (202 mg, 1.84 mmol). The peptide was cleaved from the resin in 95% TFA–water, and after filtration and lyophilization the crude product was chromatographed on LH-20 Sephadex eluting with 1% ammonium acetate in water at pH 7.6. The product was further purified by RP-HPLC (same conditions as described above) to give NAcP9 (130 mg, 62%): Amino acid analysis Asp 2.01, Ala 1.19, Pro 2.02, Tyr 2.72, Val 1.07; NMR (90% H_2O–10% D_2O, pH 6, 27 °C) see Table V for assignments; MS (FAB) m/e 1166 (M + Na) 1144 (M + H). All other peptides prepared in this way also gave satisfactory analytical data by amino acid analysis, high field ^1H NMR, and FAB-MS.

(*S*)-2-Methylproline (5). (2*R*,5*S*)-2-*tert*-Butyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one was prepared from (*S*)-proline and pivaldehyde and alkylated with methyl iodide to give (2*R*,5*S*)-2-*tert*-butyl-5-methyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one by the method of Seebach and co-workers.[39] The latter material (8.4 g, 43 mmol) in HCl (160 mL, 6 M) was stirred for 16 h and then evaporated. The resulting solid in water (10 mL) was chromatographed on Dowex 50Wx8 acid form (80 g) eluting with aqueous NH_3 (1 M). Fractions containing the product were collected and evaporated. **5** was obtained as a white crystals after recrystallization from MeOH–EtOAc (5.1 g, 92%): mp 262–264 °C; $[\alpha]^{20}_D$ = −75° (*c* 2.0, MeOH); IR 3420, 1618 cm^{-1}; NMR (D_2O) δ 1.60 (s, 3 H), 1.78–2.62 (m, 4 H), 3.25–3.48 (m, 2 H); MS (CI) m/e 130 (M^+), 84. Anal. Calcd for $C_5H_9O_2N$: C, 55.8; H, 8.6; N, 10.8. Found: C, 55.7; H, 8.5; N, 11.0.

AcN-Tyr-2MePro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala (2αMePro-NAcP9) (2). The peptide was synthesised on an Applied Biosystems 430A peptide synthesizer using the standard Fmoc programs provided by the manufacturer, and SASRIN[81] resin (0.51 g, 1.06 mequiv/g). Two equivalents of preformed HOBT ester were used in each elongation cycle. Three equivalents of activated N-Fmoc-(OtBu)tyrosine were used for the final coupling onto (*S*)-α-methylproline. The complete N-deprotected peptide was cleaved from the resin with 1% TFA in CH_2Cl_2. The remaining protecting groups were then cleaved with 20% TFA in CH_2Cl_2 (20 mL) for 2 h. The solvent was evaporated and the crude product was chromatographed on LH 20 Sephadex eluting with 1% acetic acid in water at a flow rate of 30 mL h^{-1}. The peptide was further purified by RP-HPLC on a μ-Bondapak C18 column (19 × 150 mm) eluting with a gradient of MeCN–water (0–30% MeCN + 0.2% TFA) over 30 min to afford after lyophilization 2αMePro-P9 (222 mg, 37%) as a white powder: NMR (90% H_2O–10% D_2O, pH 6, 20 °C) see Table V for assignments (2αMePro-P9); MS (FAB) m/z 1116 (M + H), 679, 652, 553, 465. Acetic anhydride (20 μL) was added to 2αMePro-P9 (10 mg) in aqueous sodium hydroxide (200 μL, 100 mM) and water (800 μL). The mixture was shaken and the disappearance of starting material was followed by HPLC. The product was purified by RP-HPLC on a μ-Bondapak C18 column (19 × 150 mm) eluting with a gradient of MeCN–water (0–30% MeCN + 0.2% TFA) over 30 min (9.5 mg, 91%): NMR see Table II; MS (FAB) m/e 1180 (M + Na), 1158 (M + H), 1069, 953, 204, 695, 652, 564.

Ac-Tyr-NMeAla-Tyr-Asp-Val-Pro-Asp-Tyr-Ala (2NMeAla-NAcP9) (10). This peptide was synthesized by using the procedure described above for NAcP9 with Pepsyn-KA resin (2.0 g, 0.18 mequiv), except (*S*)-*N*-Fmoc-*N*-methylalanine was used in place of proline-2. The crude peptide was chromatographed on Sephadex LH20 eluting with 1% ammonium acetate in water at pH 6.0, and then purified by RP-HPLC as above, to afford the product as a white powder after lyophilization (67 mg, 33%, >98% pure by anal. RP-HPLC): NMR (90% H_2O–10% D_2O, pH 6, 20 °C) see Table V for assignments; MS (FAB) m/z 1154 (M + Na), 1132 (M + H), 927, 765.

Ac-Tyr-Pro-Tyr-Asp-Val-NMeAla-Asp-Tyr-Ala (6NMeAla-NAcP9) (11). This peptide was synthesized by using the method described for **10** above. After cleavage from the solid support (95% TFA) and lyophilization, the product was chromatographed on LH-20 Sephadex eluting with 1% acetic acid and then purified further by RP-HPLC (yield 107 mg, 53%, >98% pure by anal. RP-HPLC): MS (FAB) 1154 (M + Na), 1132 (M + H), 927, 668, 465.

***N*-Fmoc-*N*-methyl-(*O*-*tert*-butyl)tyrosine.** *N*-Cbz-(*O*-*tert*-butyl)tyrosine methyl ester (2.34 g, 6.7 mmol), MeI (2.84 g, 20 mmol), and NaH (80% dispersion in oil, 400 mg, 13 mmol) were mixed in THF (50 mL) at 0 °C and then stirred at room temperature for 16 h. Water (5 mL) was added, and after stirring for 2 h the aqueous phase was acidified and extracted with diethyl ether. The ether extracts were washed with saturated NaCl, dried, and evaporated to afford *N*-Cbz-*N*-methyl-(*O*-*tert*-butyl)tyrosine (1.83 g, 78%). This product (1.63 g, 4.64 mmol) was stirred with Pd/C (5%, 500 mg) in CH_2Cl_2 (50 mL) under H_2 gas. After 13 h the solution was filtered through Celite and evaporated. The residue was redissolved in 10% Na_2CO_3 (50 mL) and cooled to 0 °C. Dioxane (50 mL) and Fmoc-Cl (1.15 g) in dioxane (50 mL) were added, and the mixture was stirred at 0 °C. After washing with diethyl ether, the aqueous layer was acidified and extracted

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with EtOAc. The extracts were washed with brine, dried, and evaporated to afford a white solid (1.35 g, 62%) which gave white needles after recrystallization from EtOAc: mp 165–166 °C; $[\alpha]^{21}_D$ −8.4 (*c* 1.0, CH_2Cl_2); IR 3440 br, 3060 br, 2980, 1750, 1650 cm^{-1}; NMR ($CDCl_3$) δ 1.27, 1.28 (2 s, 9 H, cis + trans rotomers), 2.69 (dd, 0.37 H), 2.76, 2.77 (2 s, 3 H), 3.09 (m, 1 H), 3.32 (dd, 0.63 H), 4.1–4.85 (m, 4 H), 6.85–7.80 (m, 12 H); MS (CI) 474.5 (M + H), 430, 418, 252. Anal. Calcd for $C_{29}H_{31}O_5N$: C, 73.6; H, 6.6; N, 3.0. Found: C, 73.4; H, 6.9; N, 2.8.

Ac-Tyr-Pro-MeTyr-Asp-Val-Pro-Asp-Tyr-Ala (3NMeTyr-NAcP9) (12). This peptide was synthesized by the method described above for **10**. (*S*)-*N*-Fmoc-*N*-methyl-(OtBu)-tyrosine was used in cycle 7 of the assembly. After cleavage from the resin with 95% TFA–H_2O and lyophilization, the product was chromatographed on LH-20 Sephadex eluting with 0.5% ammonium acetate and then purified further by RP-HPLC (yield 100 mg, 49%, >98% pure by RP-HPLC): MS (FAB) 1158 (M + H), 1061, 953.

Preparation of Tritium-Labeled Peptides for Scatchard Analysis. A general procedure follows: [^{3}H]Ac_2O (100 mCi, 52.5 mCi mg^{-1}) and peptide (10 mg) in dilute NaOH (250 μL, 25 mM) were incubated for 3 h at 20 °C. Exchangeable tritium was removed by repeated lyophilization from H_2O, and the product was purified by RP-HPLC using conditions described above for each peptide. The specific radioactivity was determined by liquid scintillation counting; [*Ac* – ^{3}H]NAcP9, 2.270 × 10^{11} dpm $mmol^{-1}$; [*Ac*-^{3}H]2NMeAla-NAcP9, 3.97 × 10^{10} dpm $mmol^{-1}$; [*Ac*-^{3}H]-6NMeAla-NAcP9, 1.51 × 10^{11} dpm $mmol^{-1}$; [*Ac*-^{3}H]2αMePro-NAcP9, 5.51 × 10^{12} dpm $mmol^{-1}$.

Registry No. 1, 129970-92-9; **2**, 133373-17-8; **3**, 133373-18-9; **4**, 121772-98-3; *N*-Cbz-**4**, 121773-05-5; **5**, 42856-71-3; **6**, 121772-99-4; **7**, 133373-19-0; **7** (N-deprotected), 121773-00-0; **8**, 121773-06-6; **9**, 121773-02-2; **10**, 133373-20-3; **11**, 133373-21-4; **12**, 133373-22-5; **13**, 130203-50-8; **14**, 130203-51-9; Cbz-Cl, 501-53-1; Fmoc-Cl, 28920-43-6; H-Tyr(Bu-*t*)-OMe, 52616-82-7; Cbz-α($HOCH_2CH_2$)-Pro-Tyr(Bu-*t*)-OMe, 133373-23-6; BOC-Tyr(Bu-*t*)-OH, 47375-34-8; Fmoc-Ala-OH, 35661-39-3; Fmoc-Tyr(Bu-*t*)-OH, 71989-38-3; Fmoc-MeAla-OH, 84000-07-7; Cbz-Tyr(Bu-*t*)-OMe, 5068-29-1; MeI, 74-88-4; *N*-Cbz-*N*-Me-Tyr(Bu-*t*)-OH, 67586-06-5; *N*-Fmoc-*N*-Me-Tyr(Bu-*t*)-OH, 133373-24-7; [Ac-^{3}*H*]NAcP9, 133373-25-8; [Ac-^{3}*H*]$_2$NMeAla-NAcP9, 133399-34-5; [Ac-^{3}*H*]-6NMeAla-NAcP9, 133399-35-6; [Ac-^{3}*H*]2αMePro-NAcP9, 133373-26-9; (2*R*,5*R*)-5-allyl-2-*tert*-butyl-1-aza-3-oxabicyclo-[3.3.0]octan-4-one, 81286-83-1; (2*R*,5*S*)-2-*tert*-butyl-5-methyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one, 86046-11-9.