

## Small Molecular Probes for G-Protein-Coupled C5a Receptors: Conformationally Constrained Antagonists Derived from the C Terminus of the Human Plasma Protein C5a

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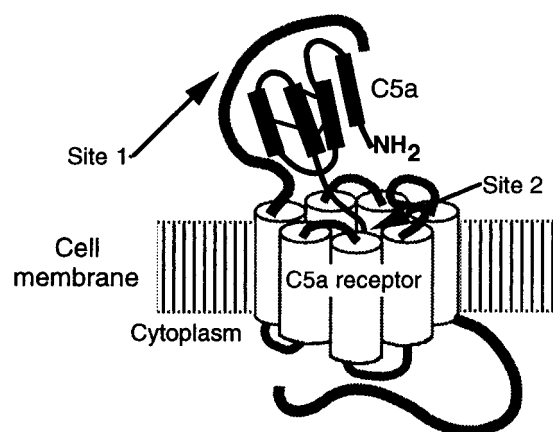
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Activation of the human complement system of plasma proteins in response to infection or injury produces a 4-helix bundle glycoprotein (74 amino acids) known as C5a. C5a binds to G-protein-coupled receptors on cell surfaces triggering receptor–ligand internalization, signal transduction, and powerful inflammatory responses. Since excessive levels of C5a are associated with autoimmune and chronic inflammatory disorders, inhibitors of receptor activation may have therapeutic potential. We now report solution structures and receptor-binding and antagonist activities for some of the first small molecule antagonists of C5a derived from its hexapeptide C terminus. The antagonist NMe-Phe-Lys-Pro-D-Cha-Trp-D-Arg-CO<sub>2</sub>H (**1**) surprisingly shows an unusually well-defined solution structure as determined by <sup>1</sup>H NMR spectroscopy. This is one of the smallest acyclic peptides found to possess a defined solution conformation, which can be explained by the constraining role of intramolecular hydrogen bonding. NOE and coupling constant data, slow deuterium exchange, and a low dependence on temperature for the chemical shift of the D-Cha-NH strongly indicate an inverse  $\gamma$  turn stabilized by a D-Cha-NH $\cdots$ OC-Lys hydrogen bond. Smaller conformational populations are associated with a hydrogen bond between Trp-NH $\cdots$ OC-Lys, defining a type II  $\beta$  turn distorted by the inverse  $\gamma$  turn incorporated within it. An excellent correlation between receptor-affinity and antagonist activity is indicated for a limited set of synthetic peptides. Conversion of the C-terminal carboxylate of **1** to an amide decreases antagonist potency 5-fold, but potency is increased up to 10-fold over **1** if the amide bond is made between the C-terminal carboxylate and a Lys/Orn side chain to form a cyclic analogue. The solution structure of cycle **6** also shows  $\gamma$  and  $\beta$  turns; however, the latter occurs in a different position, and there are clear conformational changes in **6** vs **1** that result in enhanced activity. These results indicate that potent C5a antagonists can be developed by targeting site 2 alone of the C5a receptor and define a novel pharmacophore for developing powerful receptor probes or drug candidates.

### Introduction

Activation of the human complement system,<sup>1,2</sup> a cascade of plasma proteins involved in immunological defense against infection and injury, contributes significantly to the pathogenesis of numerous acute and chronic diseases.<sup>3</sup> Although broad features of complement activation are known, mechanistic details remain poorly understood. A very potent inflammatory mediator generated during complement activation is a 74-residue glycoprotein known as C5a.<sup>4</sup> This peptide binds to a G-protein-coupled receptor (C5aR)<sup>4,5</sup> of the rhodopsin superfamily of GTP-linked binding proteins, with the characteristic seven transmembrane helices (Figure 1).<sup>5</sup> C5a receptors are found on a variety of tissues and cells including mast cells, neutrophils, monocytes, macrophages, and vascular endothelial cells.<sup>4</sup> C5a recruits



**Figure 1.** Two-site model for binding of C5a to its G-protein-coupled receptor C5aR.

neutrophils and macrophages to sites of injury and alters their morphology, induces degranulation, increases calcium mobilization, vascular permeability (edema), and neutrophil adhesiveness, contracts smooth muscle, stimulates release of inflammatory mediators

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**Table 1.**  $^1\text{H}$  NMR Assignments<sup>a</sup> for **1** in  $d_6$ -DMSO

residue	HN <sup>b</sup>	H $\alpha$	H $\beta$	H $\gamma$	others
MePhe		4.06	3.09, 3.06		7.17, 7.29; <sup>c</sup> 2.46; <sup>d</sup> 8.98 <sup>g</sup>
Lys	8.83	4.54	1.74, 1.55	1.32	1.51; <sup>e</sup> 2.74, <sup>f</sup> 7.76 <sup>g</sup> (NH <sub>2</sub> )
Pro		4.30	2.04, 1.74	1.88, 1.78	3.61, 3.48 <sup>e</sup>
D-Cha	7.91	4.35	1.19, 1.06	0.76	1.43, 1.08; <sup>e</sup> 1.61, 1.58; <sup>f</sup> 0.73
Trp	8.01	4.65	3.11, 2.94		6.97, 7.06, 7.13, 7.32, 7.65; <sup>c</sup> 10.80 <sup>g</sup>
D-Arg	8.44	4.20	1.73, 1.58	1.42	3.08; <sup>e</sup> 7.60 <sup>g</sup>

<sup>a</sup> Referenced to residual  $d_5$ -DMSO at 2.50 ppm. <sup>b</sup> Amide NHs, <sup>3</sup> $J_{\text{NH-C}\alpha\text{H}}$  values (Hz): 7.91 (Lys), 7.77 (D-Arg), 8.34 (Trp), 8.53 (D-Cha). <sup>c</sup> Aromatics. <sup>d</sup> N-Me. <sup>e</sup> Hd. <sup>f</sup> He. <sup>g</sup> NH/NH<sub>2</sub> amine.

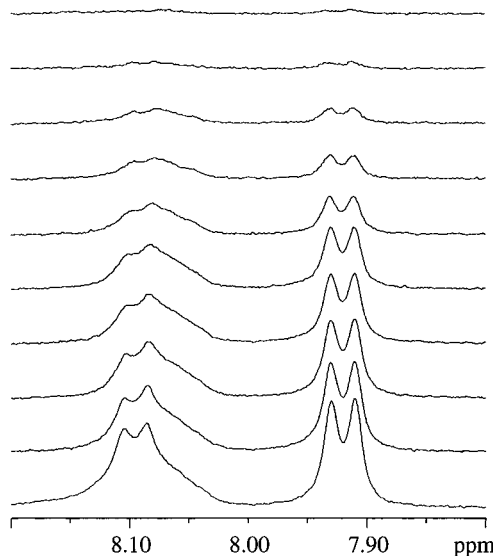
(histamine, TNF- $\alpha$ , IL-1, IL-6, IL-8, prostaglandins, and leukotrienes) and lysosomal enzymes, promotes formation of oxygen radicals, and enhances antibody production.<sup>3</sup> Overexpression or underregulation of C5a is implicated in the pathogenesis of immunoinflammatory diseases such as rheumatoid arthritis, adult respiratory distress syndrome (ARDS), systemic lupus erythematosus, tissue graft rejection, ischemic heart disease, reperfusion injury, septic shock, psoriasis, gingivitis, atherosclerosis, lung injury, and extracorporeal post-dialysis syndrome.<sup>3</sup> One strategy for developing therapeutics for such diseases is to interfere with the action of C5a, and we now describe chemical and structural studies aimed at the design of small molecule probes that inhibit the interactions of C5a with its receptor.

The NMR solution structure of the protein C5a has been determined,<sup>6</sup> and although it is essentially a 4-helix bundle, the C-terminal end was found to be unstructured. Indeed, this conformational flexibility in the C-terminus has made structure–function studies extremely difficult to interpret. C5a is thought to interact with its receptor at two sites (Figure 1).<sup>7,8</sup> An N-terminal interhelical positively charged region of C5a binds to a negatively charged extracellular domain of C5aR (site 1), while the C-terminal “effector” region of C5a is thought to bind with an interhelical domain of the receptor (site 2) and is responsible for receptor activation leading to signal transduction. Numerous short peptide derivatives of the C terminus of C5a have been found to be agonists<sup>9–20</sup> of C5a, while only one small molecule (**1**, MeFKP-D-Cha-Wr) is reported to be a full antagonist with no agonist activity<sup>20</sup> in isolated cells and membranes.

We now report the surprising observation that the small hexapeptide **1** and a new constrained cyclic analogue **6** have well-defined solution structures. These compounds and several analogues have been examined for their receptor-binding affinities and antagonist activities using intact human polymorphonuclear (PMN) cells. The results demonstrate for the first time that certain turn conformations are responsible for antagonist activity, providing new insight to the binding of C5a to its receptor. This information should be useful for the design and development of even more potent C5a antagonists or agonists as molecular probes of the complement system or as potential drug candidates. The results may also be valuable for the design of small molecules that bind to other G-protein-coupled receptors which are important drug targets due to their crucial roles in signal transduction.<sup>21</sup>

## Results

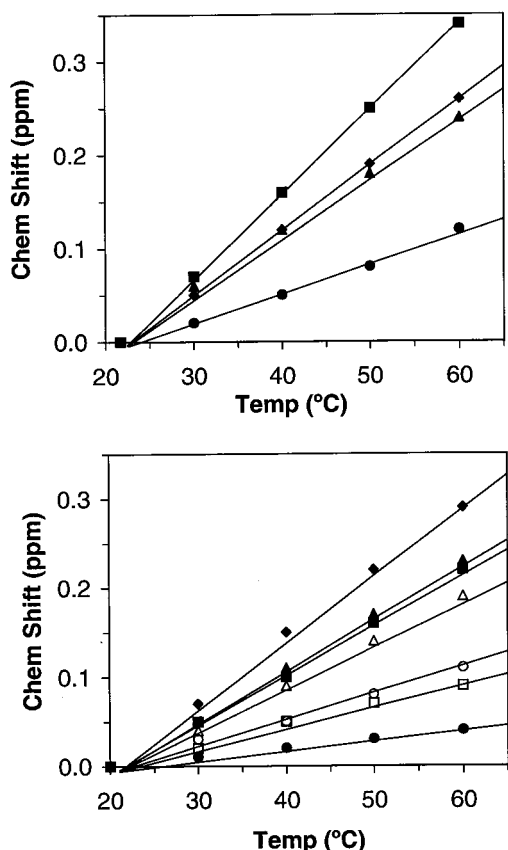
**NMR Structure of 1.** The 1D  $^1\text{H}$  NMR spectrum of peptide **1** in  $d_6$ -DMSO at 24 °C shows four distinct



**Figure 2.** Stacked plots of  $^1\text{H}$  NMR spectra showing time-dependent decay of amide-NH resonances for Trp (8.1 ppm) and D-Cha (7.9 ppm), as well as Lys-NH<sub>3</sub><sup>+</sup> (8.07 ppm), residues of **1** in  $d_6$ -DMSO containing D<sub>2</sub>O after 10 min (bottom) and then 25, 40, 55, 70, 130, 190, 250, 385, and 520 min.

resonances for amide-NH protons (Table 1). To establish their possible involvement in intramolecular hydrogen bonds, a deuterium exchange experiment was performed by adding a 10-fold excess of D<sub>2</sub>O to the solution. Two of the amide-NH doublets disappeared immediately along with the resonance attributable to the N-terminal methylamine-NH protons. However, the other two amide-NH resonances, as well as a broad resonance at 8.07 ppm, persisted for up to 6.5 h (Figure 2). These three slowly exchanging protons are assigned (Table 1) to the amide NHs of Trp and D-Cha and to the side-chain amine of Lys. The amine assignment was established from the TOCSY spectrum where cross-peaks were observed between the protonated amine and the  $\epsilon$ ,  $\delta$ , and  $\gamma$  CH<sub>2</sub> protons. The slow exchange behavior for the three NH protons indicates a degree of protection from solvent that is indicative of intramolecular hydrogen bonding.

The temperature independence of the amide chemical shifts (<3 ppb/deg)<sup>22</sup> is another useful marker of hydrogen bonding. A temperature dependence (20–60 °C) study of the amide-NH chemical shifts ( $\Delta\delta/T = 2.5$  ppb/deg, D-Cha-NH; 6.0 ppb/deg, Trp-NH; 6.5 ppb/deg, Lys-NH; 8.7 ppb/deg, Arg-NH; Figure 3, top) unambiguously confirms the involvement of only the D-Cha-NH in intramolecular hydrogen bonding. Thus the D-Cha-NH hydrogen bond appears to be present in a majority population of conformers. On the other hand, the slow exchange behavior but high-temperature coefficient ( $\Delta\delta/T$ ) of the amide Trp-NH most likely reflects solvent



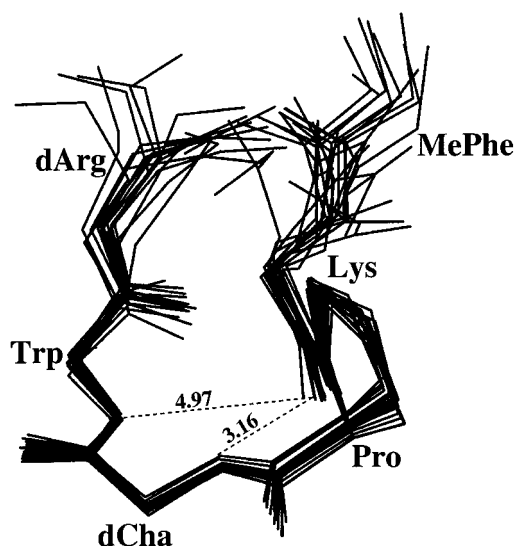
**Figure 3.** Temperature dependence (20–65 °C) of chemical shifts for amide-NH protons of **1** (top) and **6** (bottom) in  $d_6$ -DMSO. Legend: (■) D-Arg, (□) D-Arg (side chain), (▲) Trp, (●) D-Cha, (◆) Lys, (◆) Orn, (○) Orn-(CH<sub>2</sub>)<sub>3</sub>-NH, (△) Phe.

protection/intramolecular hydrogen bonding in only a small population of solution conformers.

The  $^3J_{\text{NH-H}\alpha}$  coupling constants for the amide-NH protons were measured from the 1D spectrum and are reported in Table 1. In small linear peptides it is unusual for backbone coupling constants to differ from the conformationally averaged value of  $\sim 7$  Hz,<sup>23</sup> and thus it is significant that the two constants for D-Cha and Trp exceed this value (Table 1). This observation strongly indicates the presence of a well-defined local structure near these residues, and the existence of this structure is supported by the involvement of the D-Cha residue in hydrogen bonding interactions.

Medium range NOEs often provide the strongest evidence for the presence of folded conformers of peptides in solution,<sup>23</sup> and thus a series of 2D <sup>1</sup>H NMR spectra were recorded to assign all resonances and to determine if such NOE interactions are present. TOCSY and DQ-COSY experiments were used to identify residue types, while sequential assignments were made from the analysis of NOESY data. Among the identified NOEs for this small peptide are several that indicate the presence of a folded conformer. These include  $i, i + 2$  NOEs for D-Cha to Lys and Pro to Trp, and a D-Cha-Trp NH-NH NOE which suggest a turn in the peptide centered on the Pro-D-Cha residues.

Other significant NOEs were observed between Lys- $\alpha$ H and the two Pro- $\delta$ H protons. These confirm that the Lys-Pro bond exists in the trans conformation. Although the presence of cis Pro bonds is not uncommon in small peptides, in this case there is no evidence of

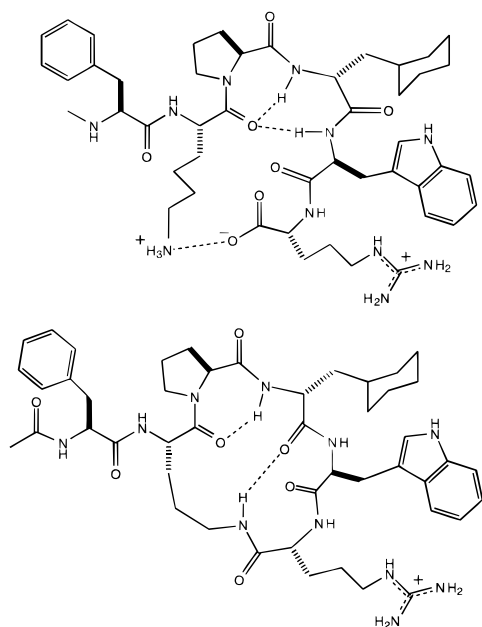


**Figure 4.** Backbone and proline heavy atoms of twenty lowest energy minimized NMR structures of **1** in  $d_6$ -DMSO (24 °C). Residues are labeled at C $\alpha$  positions. Distances between heavy atoms discussed in text: D-Cha-NH...OC-Lys (3.16 Å) and Trp-NH...OC-Lys (4.97 Å).

any minor populations containing a cis form. This lack of evidence further supports the conclusion that peptide **1** exists in a well-defined conformation in solution and that there are not multiple slowly exchanging conformers present.

The remaining NOEs detected in **1** also supported the existence of a well-defined conformer and were used to derive a set of 55 interproton distance restraints. These data, combined with the dihedral angle restraints for the D-Cha and Trp  $\phi$  angles based on the observed coupling constants shown in Table 1, were used to generate a set of 50 structures, using a simulated annealing algorithm within the program X-PLOR. It is remarkable that, in the absence of any explicit hydrogen bonding restraints, the majority of these structures showed a good convergence to a folded conformation. This conformation is represented in Figure 4 by a superposition of 20 of the lowest energy structures. The pairwise rmsd is 1.01 Å for all of the backbone C $\alpha$ , N, and C atoms in this family of structures, but this reduces to 0.30 Å for the turn region Lys to Trp. This difference reflects the mobility of the terminal residues Phe and Arg, as seen in Figure 4. In these structures, the D-Cha-NH and Lys-CO are within hydrogen bonding distance, consistent with the slow exchange and temperature coefficient data for this amide proton.

The hydrogen bond suggested by the NOE, the coupling constant data, the temperature-independent chemical shift, and the slow exchange behavior defines a seven-membered ring or an inverse  $\gamma$  turn formed by the Lys-Pro-D-Cha residues. The D-Cha-NH...OC heavy atom distance is 3.16 Å and the N-H...O angle is 142°. Hydrogen bonds in  $\gamma$  turns are often weak,<sup>24</sup> and thus it is remarkable that such a bond is detected in this small peptide. The other NH proton, Trp-NH, that showed evidence for partial involvement in hydrogen bonding is relatively well aligned with a suitable acceptor group, Lys-CO, in the family of structures in Figure 4. However, it is not within hydrogen bonding distance (4.97 Å). Trial calculations in which hydrogen



**Figure 5.** Possible H bonding in **1** (top) and **6** (bottom) from NMR spectra in  $d_6$ -DMSO.

bonding restraints were included showed that a Trp-NH...OC-Lys hydrogen bond can be readily accommodated within the experimental restraint set. This is consistent with the evidence presented earlier that such a hydrogen bond is present in a small population of solution conformers. Hydrogen bonding involving the Lys-amine side chain was also implicated from the H/D exchange experiment, but a potential acceptor group could not be distinguished from the structures. The hydrogen bonds for the proposed  $\beta$  and  $\gamma$  turns are indicated in Figure 5.

The fact that the Trp-NH and Lys-NH<sub>3</sub><sup>+</sup> hydrogen bonds were not formally detected in the structures may reflect the well-known difficulty of defining solution conformers of small peptides because of the relative weakness of NOEs due to correlation time effects<sup>25</sup> or may reflect a degree of conformational averaging. In any case, a hydrogen bond is not required to define a  $\beta$  turn, and analysis of the C $\alpha$ -C $\alpha$  distances (~4.6 Å) in the structures in Figure 4 confirms the fact that the geometry of the four residues Lys-Pro-D-Cha-Trp does satisfy the criteria<sup>26</sup> for a  $\beta$  turn.

The  $i + 1$   $\phi$  and  $\psi$  angles for **1** ( $\phi_2 = -86^\circ$ ,  $\psi_2 = 33^\circ$ ) are consistent with those reported for inverse  $\gamma$  turns ( $\phi_2 = -70$  to  $-85^\circ$ ,  $\psi_2 = 60$ - $70^\circ$ )<sup>27</sup> and both  $i + 1$  and  $i + 2$   $\phi$  and  $\psi$  angles for **1** (Table 4) most closely match a type II  $\beta$  turn,<sup>28</sup> the differences being attributed to distortion by the presence of the  $\gamma$  turn wholly within the  $\beta$  turn (Figure 5). To our knowledge, this is the first example of an intramolecular hydrogen bond between residues within a  $\beta$  turn, although there are many examples of hydrogen bonds between a residue within the "10-membered ring" of a  $\beta$  turn and a residue outside of it.<sup>28</sup>

**Structure-Activity Relationships.** Others<sup>9-13,19,20</sup> have previously claimed nanomolar affinities of C5a agonist peptides for the PMN C5aR, but their assays used membranes, isolated from PMN cells, for which affinities of both C5a and peptide analogues are typically<sup>9-13,30</sup> 2 orders of magnitude greater than those

**Table 2.** Receptor Binding Affinities and Antagonist Activities in Human PMNs<sup>a</sup>

compd	receptor affinity <sup>b</sup>	IC <sub>50</sub> , $\mu$ M	
		antagonist potency <sup>c</sup>	agonist activity <sup>d</sup>
<b>1</b> MeFKP(D-Cha)Wr	1.8 (15)	0.085 (9)	no
<b>2</b> MeFKP(D-Cha)Wr-CONH <sub>2</sub>	14 (5)	0.5 (3)	no
<b>3</b> MeFKP(D-Cha)WR	11 (5)	0.7 (3)	no
<b>4</b> MeFKPLWR	144 (1)	> 1000 (3)	nd
<b>5</b> AcF[KP-D-Cha-Wr] <sup>e</sup>	3.2 (4)	0.090 (5)	no
<b>6</b> AcF[OP-D-Cha-Wr] <sup>e</sup>	0.28 (6)	0.012 (4)	no
<b>7</b> C5a <sub>65-74</sub> , ISHKDMLGR	> 1000 <sup>f</sup>		
<b>8</b> C5a	0.0008 (9)		yes

<sup>a</sup> Number of experiments in parentheses. Corrected for amino acid content. nd = not determined. <sup>b</sup> Fifty percent reduction in binding of <sup>125</sup>I-C5a to intact human PMNs. <sup>c</sup> Fifty percent reduction in myeloperoxidase secretion from human PMNs mediated by 100 nM C5a. <sup>d</sup> Agonist activity in dose range 0.1 nM to 1 mM. <sup>e</sup> Cyclic compounds (Figure 5). O is symbol for ornithine. <sup>f</sup> Ref 10.

observed for intact cells. Thus, although the hexapeptide **1** has previously been reported to compete with C5a binding to receptors on isolated PMN membranes (IC<sub>50</sub> 70 nM),<sup>19</sup> we decided to reexamine this competition using intact PMN cells since this is physiologically more relevant. Under these conditions we establish that **1** binds with a much lower receptor affinity of IC<sub>50</sub> 1.8  $\mu$ M (Figure 6a, Table 2) but confirm that it is indeed a full antagonist with no agonist properties. The relative affinity of **1** for the C5aR in intact PMNs was, however, similar to that previously reported for isolated PMN membranes.<sup>19</sup>

We find that **1** shows antagonist activity against both C5a (Figure 6b) and a C-terminal peptide agonist decapeptide analogue YSFKPMPLaR (receptor affinity IC<sub>50</sub> 6  $\mu$ M)<sup>17</sup> of the C terminus of C5a<sub>65-74</sub> (antagonism not shown), suggesting that it acts on site 2 of the receptor (Figure 1). These two small molecules have similar micromolar affinities for the receptor C5aR on intact polymorphonuclear leukocytes despite their opposing modes of action. A striking feature of the data in Table 2 is the excellent linear correlation ( $r = 0.93$ ) between the log of the binding affinities and the log of the antagonist potencies for this limited set of site 2 antagonists (compounds **1-6**, Table 2). Since receptor affinity and antagonist activity are directly proportional here, the experimentally simpler approach of measuring receptor binding can also be used to quickly estimate the antagonist activity, provided that there is no evidence of agonist activity. We have also tested some compounds in human umbilical artery, and our preliminary results indicate that they are also antagonists in this preparation (Finch and Taylor, unpublished).

It has been proposed that the C-terminal carboxylate of C5a and of agonist peptides is essential for activity due to its interaction with a positively charged Arg206 of the receptor.<sup>20</sup> We confirm here (Table 2) that the C-terminal carboxylate is indeed important for affinity, since the amide **2** shows a 5-fold reduction in both receptor affinity and antagonist activity (**2** vs **1**, Table 2). Changing chirality of the Arg-C $\alpha$  (**3** vs **1**) causes a similar reduction in activities, and replacing D-Cha with the L-Leu residue (**4**) is also detrimental to receptor binding (Table 2). Clearly the hydrophobic D-cyclohexylalanine residue is not only important in defining the  $\gamma$  turn through hydrogen bonding but also directs its

**Table 3.**  $^1\text{H}$  NMR Assignments<sup>a</sup> for **6** in  $d_6$ -DMSO

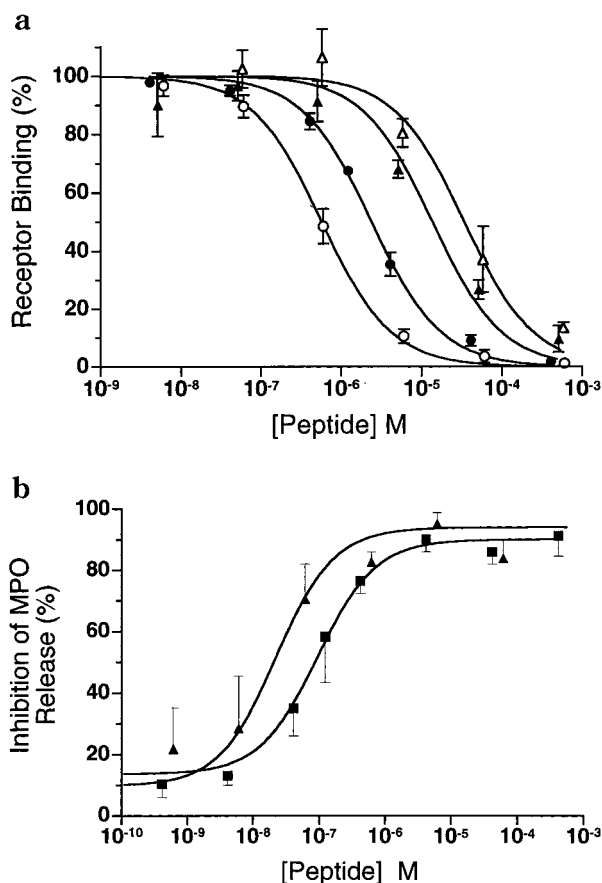
residue	HN <sup>b</sup>	H $\alpha$	H $\beta$	H $\gamma$	others
AcPhe	8.06	4.55	2.71, 2.94		1.76 (Me); 7.20, 7.29 <sup>c</sup>
Orn	8.23	4.47	1.56, 1.70	1.42	2.85, 3.27; <sup>d</sup> 7.37 (NH $\epsilon$ )
Pro		4.55	1.71, 2.26	1.88	3.48, 3.59 <sup>d</sup>
D-Cha	7.38	4.50	1.41, 1.43	1.06	0.78, 0.84; <sup>d</sup> 1.62, 1.65, 1.48, 1.58
Trp	8.50	4.30	3.0		6.98, 7.05, 7.33, 7.58; <sup>c</sup> 10.83 (NH)
D-Arg	8.51	4.02	1.34, 1.81	1.20	2.97, 2.97; <sup>d</sup> 7.39 (NH $\epsilon$ )

<sup>a</sup> Referenced to residual  $d_5$ -DMSO at 2.50 ppm. <sup>b</sup> Amide NHs, <sup>3</sup> $J_{\text{NH-C}\alpha\text{H}}$  (Hz): 8.41 (Phe), 6.99 (Orn), 8.02 (D-Cha), 4.81 (Trp), 8.28 (D-Arg). <sup>c</sup> Aromatic CHs. <sup>d</sup> H $\delta$ .

**Table 4.** Backbone  $\phi$  and  $\psi$  Angles<sup>a</sup> (deg) for **1** and **6**

compd	Pro		D-Cha		Trp		D-Arg	
	$\phi$	$\psi$	$\phi$	$\psi$	$\phi$	$\psi$	$\phi$	$\psi$
<b>1</b>	-86	33	97	-45	-144	124	111	
<b>6a</b>	-68	-8	165	-54	-58	90	69	-3
<b>6b</b>	-64	-10	170	-98	-77	158	69	-2

<sup>a</sup> For the average structure, restrained by NOE data and dihedral angles but no hydrogen bond restraints.



**Figure 6.** (a, top) Receptor binding. Inhibition of binding of  $^{125}\text{I}$ -C5a to human PMNs by **1** (●), **2** (△), **3** (▲), **6** (○). (b, bottom) C5a antagonist potency as inhibition of myeloperoxidase (MPO) release from human PMNs by **1** (■,  $n = 9$ ) and **6** (▲,  $n = 4$ ). All data are means  $\pm$  SEM.

bulky side chain into specific receptor space that is critical for antagonism.

To further examine the importance of the C-terminal carboxylate in **1** for receptor binding, a side-chain-to-main-chain cyclization from the amino acid side-chain amine of Lys (**5**) and Orn (**6**, Figure 5) to the C terminus was carried out. Despite the fact that both **5** and **6** now have an amide bond at the C terminus as in **2**, compound **5** is equipotent with **1**, while **6** is 1 order of magnitude more potent. To understand the structural

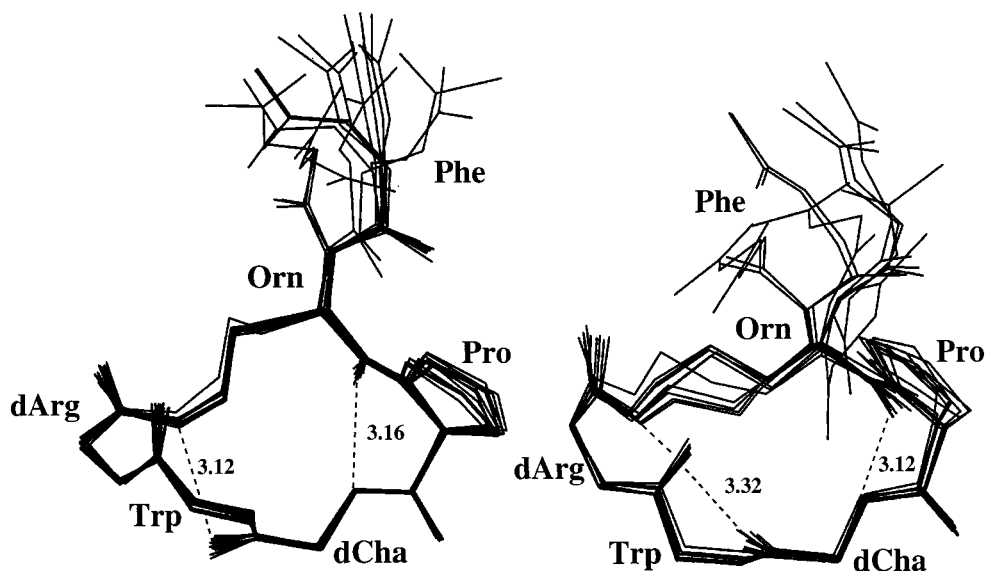
basis for the improved receptor affinity and antagonist activity of cyclic **6** over acyclic **1**, we determined the solution structure of **6** for comparison with **1**.

**NMR Structure of 6.** The 1D  $^1\text{H}$  NMR spectral data for **6** (Table 3) showed three NH protons which have a chemical shift/temperature coefficient ( $\Delta\delta/T$ )  $< 3$  ppb/deg, characteristic of hydrogen bonding (Figure 3). These NH protons are assigned to the side chain NH protons of D-Arg and Orn, as well as to that of the backbone amide of D-Cha. The latter proton has a significantly lower temperature coefficient ( $\Delta\delta/T = 1$  ppb/deg) for **6** than for the open chain derivative **1** (Figure 3), suggesting a strengthening of the hydrogen bond (Lys-CO $\cdots$ HN-D-Cha) which defines a  $\gamma$  turn for the Orn-Pro-D-Cha residues. Four of the five backbone  $^3J_{\text{NH-H}\alpha}$  coupling constants are either  $> 8$  Hz or  $< 5$  Hz (Table 3), suggesting a well-defined backbone conformation and a lack of significant conformational averaging.

2D TOCSY and NOESY spectra of **6** were recorded and assigned as described for **1**. The NOE data are consistent with the cyclic derivative adopting a well-defined solution conformation. In combination with the coupling constant data, several highly characteristic medium range NOEs confirm the presence of a  $\gamma$  turn seen in **1** and also suggest a new  $\beta$  turn involving the residues D-Cha-Trp-D-Arg-Orn. In particular, the presence of small and large coupling constants for the two central residues, Trp and D-Arg, respectively, together with an NH-NH $\epsilon$  NOE between D-Arg and Orn and an  $\alpha\text{H}$ -NH $\epsilon$  NOE between Trp and Orn suggest a type II  $\beta$  turn as illustrated for **6** in Figure 5. This fits well with the observed low-temperature coefficient of the Orn-N $\epsilon$  proton.

From a set of 89 NOE-based distance restraints and four dihedral angle ( $\phi$ ) constraints, a family of 50 structures was calculated. The structures converged with high precision, although there were two distinct conformational populations which are shown in Figure 7. For conformer **6a** the rmsd over all of the backbone atoms of the cycle is 0.15 Å, while for conformer **6b** it is 0.28 Å. These conformers differ mainly in the backbone conformation of the Trp residue; however, both contain the  $\gamma$  turn, and this region of the molecule is similar to that of the open chain analogue **1**.

Despite the retention of the hydrogen bond that defines the three-residue  $\gamma$  turn, there are some differences between the three-dimensional structures of **1** and **6**. There are subtle differences in the backbone dihedral angles associated with the  $\gamma$  turn (Table 4) and some significant changes in the rest of the molecule. Instead of the Trp-NH hydrogen bonding with the Orn (Lys in **1**) CO, there is a preference for a new hydrogen bond between D-Cha-CO $\cdots$ H $\epsilon$ N-Orn (Figure 7). The backbone  $\phi$  and  $\psi$  angles for the Trp and D-Arg residues shown



**Figure 7.** Backbone and proline heavy atoms of twenty lowest energy minimized NMR structures of **6** in  $d_6$ -DMSO (24 °C). Two sets of conformations **6a** (left) and **6b** (right) are shown along with the putative H-bond lengths: D-Cha-NH $\cdots$ OC-Orn (**6a** 3.16 Å; **6b** 3.12 Å), Orn-NH $\cdots$ OC-D-Cha (**6a** 3.12 Å; **6b** 3.32 Å).

for **6** in Table 4 are qualitatively consistent with typical values reported<sup>27</sup> for type II  $\beta$  turns ( $\phi_2$   $-60^\circ$ ,  $\psi_2$   $120^\circ$ ,  $\phi_3$   $80^\circ$ ,  $\psi_3$   $0^\circ$ ). The cyclization and Lys/Orn substitution in **6** thus induces a new  $\beta$  turn that restricts the positions of residues in **6** that were relatively disordered in **1**.

## Discussion

There are very few short *acyclic* peptides that have been found to have substantial turn structure in solution.<sup>33–35</sup> The general consensus is that short acyclic peptides tend to adopt a myriad of solution conformations that may include small populations of turn structures which are frequently responsible for bioactivity. Here we have presented strong evidence based upon NMR constraint data,  $^3J_{\text{NH-CH}\alpha}$  values, and deuterium exchange and temperature dependence data for an acyclic hexapeptide **1** in which a high population of conformers adopt an inverse  $\gamma$  turn stabilized by a D-Cha-NH $\cdots$ OC-Lys hydrogen bond. Structure calculations suggest that the well-defined  $\gamma$  turn coexists within a  $\beta$  turn encompassing the residues Lys-Pro-D-Cha-Trp. Slow exchange and temperature coefficient data suggest that a hydrogen bond associated with this  $\beta$  turn, Trp-NH $\cdots$ OC-Lys, is populated to a much lesser extent than that of the  $\gamma$  turn hydrogen bond.

To compare antagonist structures under conditions in which all compounds are soluble, we performed our studies using DMSO rather than water as solvent. For some peptides, DMSO is thought to disrupt local structure via intermolecular hydrogen bonding interactions, and therefore, the finding of defined solution conformations in this structure-destroying solvent makes it highly likely that an even higher structural order is present under physiological conditions. Antagonist structures in nonaqueous solvents are also more likely to be relevant than in water since the antagonists likely interact with a hydrophobic region (site 2) of the C5a receptor.

The probability of a turn in **1** is, of course, enhanced by the presence of the Pro residue, which, of all the

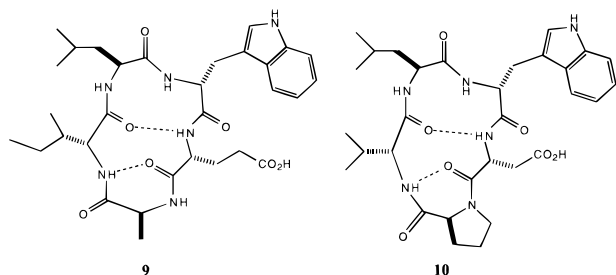
amino acids, is the one with the highest propensity for turn formation. With a Pro at position  $i + 1$  of the  $\beta$  turn, it would be expected<sup>33–35</sup> to be either a type I or a type II turn, and the latter is discerned from an analysis of the backbone dihedral angles in the calculated family of structures. The preference for this form is readily seen from the NOE data and, in particular, from the strong  $\alpha\text{N}$  sequential NOE between residues  $i + 1$  (Pro) and  $i + 2$  (D-Cha) of the turn relative to a weak  $\delta\text{N}$  NOE which would be more diagnostic of type I.<sup>33–35</sup> Type II turns are often associated with a Gly residue at position  $i + 3$  to accommodate the requirement of a positive  $\phi$  angle; however, in this case this steric requirement is met by the presence of a D-amino acid (D-Cha). The D-amino acid has implications for the dihedral angle defining the  $^3J_{\text{NH-H}\alpha}$  coupling constant, and in this case a large coupling (8.53 Hz) is observed, consistent with the type II geometry. This contrasts with the small coupling (5 Hz) expected in a type II turn involving only L-amino acids. The observed large coupling is particularly significant because coupling constants are susceptible to conformational averaging<sup>23</sup> and thus detection of a value  $> 8$  Hz strongly suggests a lack of conformational flexibility.

Cyclic derivatives of **1** were constructed with the aim of further limiting conformational flexibility of the hexapeptide residues, particularly the flexible N and C termini, and increasing the stability of a turn motif. With a view to ultimately developing bioavailable drug candidates, cyclization to **5** and **6** was expected to offer a number of advantages on the basis of the fact that cyclic peptides in general are known to (i) more effectively stabilize turn conformations by restricting conformational freedom, (ii) increase resistance to proteolytic cleavage by degradative enzymes which destroy peptides, (iii) improve bioavailability, and (iv) increase target selectivity.<sup>31–33</sup> These features tend to make hydrophobic cyclic peptides more attractive drug candidates than their acyclic analogues.

Unfortunately, cycle **5** showed no improvement in activity over **1**, and thus its structure was not investi-

gated in detail. However, cycle **6** was a much more potent antagonist than **1** or **5**; therefore, its structure was determined for comparison with that of **1**. There is strong NMR evidence for the presence of a hydrogen bond across the Orn-Pro-D-Cha segment of the cyclic derivative **6**, thereby defining a  $\gamma$  turn in the identical position to that in **1**. By contrast, the putative type II  $\beta$  turn that is described above for **1** does not appear to be prevalent in **6**, this cycle being further conformationally constrained by an additional amide bond between the Orn side-chain amine and the Arg C terminus. This new amide NH in **6** is strongly implicated in a hydrogen bond to D-Cha CO. The effect of such a H bond is to distort the conformation of the Trp-(D-Arg)-Orn segment, and to some extent also the Orn-Pro-D-Cha region, of the cycle **6** compared to corresponding residues of **1**. The enhanced antagonist activity for **6** may in part be attributable to the increased stabilization of a  $\gamma$  turn, but most likely also reflects the part played by the new type II  $\beta$  turn in positioning the critical D-Cha, Trp, D-Arg, and Phe residues for receptor binding.

The constraining  $\gamma$  and  $\beta$  turns proposed in the linear peptide **1** and cyclic peptide **6** have precedent in other cyclic peptides. For example, there have been many other attempts to conformationally fix putative  $\beta$  and  $\gamma$  turns in bioactive peptides, resulting in notable mimetics for RGD, somatostatin, and opioid peptides, to name a few,<sup>36–39</sup> derived through structure–activity relationships. Most of these examples preserve some type of turn structure through cyclization of the peptide. We have also previously detected overlapping  $\gamma$  and  $\beta$  turns in the cyclic octapeptide, ascidiacyclamide, found in the marine organisms ascidians.<sup>40</sup> Combinations of a  $\gamma$  and  $\beta$  turn have also been found in the backbone of cyclic penta- and hexapeptides, particularly those containing alternating D- and L-amino acids.<sup>36–39</sup> For example, a type II  $\beta$  turn and an inverse  $\gamma$  turn have been identified in cyclic antagonists **9**, *c*-[D-Glu-Ala-L-allo-Ile-Leu-L-



Trp]<sup>41–44</sup> and **10**, *c*-[D-Asp-Pro-D-Val-Leu-D-Trp]<sup>44</sup> for endothelin receptors, also members of the rhodopsin family of G-protein-coupled receptors with seven transmembrane domains.<sup>45</sup> An inverse  $\gamma$  turn also forms in **10** (Asp-CO $\cdots$ Val-NH), as in **1** and **6** (Lys-CO $\cdots$ D-Cha-NH), through hydrogen bonding between residues that flank the proline.

The preservation of a  $\gamma$  turn conformation in compounds **1** and **6** suggests that both molecules are preorganized for binding to the G-protein-coupled C5a receptor. This common feature, together with the observed linear relationship between receptor-binding affinity and antagonist potency, highlights the important role of this turn conformation in expression of antagonism. The conformational limitations placed on

the hexapeptide by the cycle **6** are responsible for a  $\geq 10^4$  fold increase in receptor-binding affinity over the conformationally flexible decapeptide C terminus of C5a (**7**, Table 2). It is interesting to note that, compared with that of a linear peptide chain as in **7**, the turn conformations of both **1** and **6** can bring the hydrophobic Phe, Trp and D-Cha side chains into closer proximity, creating a hydrophobic surface "patch". This may be the key to developing small molecules with high affinity for site 2 of the C5a receptor. Although this study does not deal with the locations of the amino acid side chains in a receptor environment, it does reveal a novel backbone scaffold (in **6**) that leads to potent antagonist activity and high receptor binding affinity.

The results presented here for **1** and **6** will assist in the design and development of even more potent, conformationally constrained, C5a antagonists that may find therapeutic applications. Such refined molecules are expected to be powerful structural probes of the C5a-binding site(s) on G-protein-coupled receptor(s) for C5a and of the mechanistic role of C5a in complement-mediated immune defense.

## Experimental Section

Abbreviations: TFA = trifluoroacetic acid; DIPEA = diisopropylethylamine; HBTU = O-benzotriazole *N,N,N,N*-tetramethyluronium hexafluorophosphate; BOP = benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate; DMF = dimethylformamide; TIPS = triisopropylsilane; TMSBr = trimethylsilyl bromide; Mtr = 4-methoxy-2,3,6-trimethyl-benzenesulfonyl; O = L-ornithine; D-Cha = D-cyclohexylalanine; amino acid symbols "i" and "a" represent D-Arg and D-Ala, respectively.

**General Methods.** Protected amino acids and resins were obtained from Novabiochem. TFA, DIEA, and DMF (peptide synthesis grade) were purchased from Auspep. All other materials were reagent grade unless otherwise stated. Preparative scale reverse-phase HPLC (RP-HPLC) separations were performed on a Vydac C<sub>18</sub> reverse-phase column (2.2  $\times$  25 cm); analytical RP-HPLC on Waters Delta-Pak PrepPak C<sub>18</sub> reverse-phase column (0.8  $\times$  10 cm); using gradient mixtures of solvent A = water/0.1% TFA and solvent B = 10% water/90% acetonitrile, 0.09% TFA. The molecular weight of the peptides was determined by electrospray mass spectrometry recorded on a triple quadrupole mass spectrometer (PE SCIEX API III) as described elsewhere.<sup>40</sup> <sup>1</sup>H NMR spectra were recorded on either Bruker ARX 500 MHz or Varian Unity 400 or 600 spectrometers. Proton assignments were determined by 2D NMR experiments (DFCOSY, TOCSY, NOESY). All assay data were analyzed by nonlinear regression and statistics using Student's *t* test (*p* < 0.05).

**Peptide Synthesis.** The linear peptides **1–4** were assembled by manual stepwise solid-phase peptide synthesis using HBTU activation and DIPEA in situ neutralization. Couplings were monitored by the quantitative ninhydrin test. Boc chemistry was employed for temporary N<sup>q</sup>-protection of amino acids with two 1 min treatments with TFA for Boc group removal. Peptide **2** was synthesized on a Peptide Institute MBHA resin, with a substitution value of 0.79 mmol/g, to give a C-terminal amide. The other peptides were synthesized on a Novabiochem Boc-D-Arg(Tos)-PAM resin, with a substitution value of 0.51 mmol/g. The peptides were fully deprotected and cleaved by treatment with liquid HF (10 mL), *p*-cresol (1 mL) at  $-5^\circ\text{C}$  for 1–2 h. Analytical HPLC (gradient; 0 to 75% B over 60 min): **1**, *t<sub>R</sub>* = 32.0 min, MS [M + H]<sup>+</sup>(calcd) = 900.5, [M + H]<sup>+</sup>(exptl) = 900.7; **2**, *t<sub>R</sub>* = 32.2 min, [M + H]<sup>+</sup>(calcd) = 899.6, [M + H]<sup>+</sup>(exptl) = 899.7; **3**, *t<sub>R</sub>* = 30.0 min, MS [M + H]<sup>+</sup>(calcd) = 900.5, [M + H]<sup>+</sup>(exptl) = 900.7; **4**, *t<sub>R</sub>* = 23.8 min, MS [M + H]<sup>+</sup>(calcd) = 860.5, [M + H]<sup>+</sup>(exptl) = 860.5.

**Synthesis of Cycle AcF[KP-D-ChaWr] (**5**).** The linear peptide Ac-Phe-Lys-Pro-D-Cha-Trp-D-Arg was synthesized by

Fmoc chemistry using HBTU/DIPEA activation on a Fmoc-D-Arg(Mtr)-Wang resin, with a substitution value of 0.32 mmol/g. Fmoc group removal was effected by two 1 min treatments with 50% piperidine/DMF. Cleavage and deprotection using 95% TFA/2.5% TIPS/2.5% H<sub>2</sub>O for 1 h gave the Mtr-protected peptide which was purified by RP-HPLC. The linear peptide (15 mg, 13.1  $\mu$ mol) and BOP (41 mg, 92.7  $\mu$ mol) were dissolved in DMF (12 mL), DIPEA (18  $\mu$ L) was added, and the mixture was stirred for 15 h at room temperature. Analysis by RP-HPLC and IS-MS indicated the completion of the reaction, and then the solvent was removed in vacuo and the cyclized peptide fully deprotected by treatment with 1 M TMSBr in TFA for 1 h. A final RP-HPLC gave the desired cyclic peptide (7.8 mg, 65%):  $t_R = 44.0$  min, MS [M + H]<sup>+</sup> (calcd) = 910.5, [M + H]<sup>+</sup> (exptl) = 910.7.

**Synthesis of Cycle AcF[OP-D-ChaWr] (6).** The linear peptide Ac-Phe-Orn-Pro-D-Cha-Trp-D-Arg was synthesized by Boc chemistry as per the procedure for peptides 1–4. Cyclization of the peptide was carried out by stirring the peptide (52 mg, 57  $\mu$ mol), BOP (126 mg, 0.28 mmol) and pyridine (460  $\mu$ L) in DMF (57 mL) for 15 h. The solvent was removed in vacuo and the cyclic peptide purified by RP-HPLC ( $t_R = 43.7$  and 44.7 min). Yield 11.3 mg, 22%: MS [M + H]<sup>+</sup> (calcd) = 896.5, [M + H]<sup>+</sup> (exptl) = 896.5. The peptide was tested as a mixture of these two diastereomers. For the NMR structure, the peptide was cyclized using the above procedure, but DIPEA was used as the base instead of pyridine, resulting in only one of these two compounds (RP-HPLC,  $t_R = 44.7$  min; [M + H]<sup>+</sup> = 896.5) which is attributed to the cycle AcF[OP-D-ChaWr] rather than to AcF[OP-D-ChaWR].

**NMR Structure Determination.** <sup>1</sup>H NMR spectra were recorded for compounds 1 and 6 (3 mg in 750  $\mu$ L *d*<sub>6</sub>-DMSO,  $\delta$  2.50) referenced to solvent on a Varian Unity 400 or 600 spectrometer at 24 °C. Two-dimensional <sup>1</sup>H NMR NOESY (relaxation delay 2.0 s, mix time 50–300 ms), DQF-COSY and TOCSY (mixing time 75 ms) experiments were acquired and recorded in phase sensitive mode. Acquisition times = 0.186 s, spectral width = 5500 Hz, number of complex points ( $t_1$  dimension) = 1024 for all experiments. Data were zero-filled and Fourier-transformed to 1024 real points in both dimensions.

NMR data were processed using TRIAD software (Tripos Assoc.) on a Silicon Graphics Indy work station. 2D NOE cross-peaks were integrated and characterized into strong (1.8–2.7 Å), medium (1.8–3.5 Å), and weak (1.8–5.0 Å). Backbone dihedral restraints were inferred from <sup>3</sup>J<sub>NH-H $\alpha$</sub>  coupling constants with  $\phi$  restrained to  $-120 \pm 40^\circ$  for a <sup>3</sup>J<sub>NH-H $\alpha$</sub>  > 8 Hz,  $-65 \pm 25^\circ$  for J < 5 Hz<sup>46</sup> for L-amino acids, and to  $+120 \pm 40^\circ$  for <sup>3</sup>J<sub>NH-H $\alpha$</sub>  > 8 Hz in D-Cha. Three-dimensional structures were calculated using a simulated annealing and energy minimization protocol in the program X-PLOR 3.1.<sup>47</sup> In the first step an ab initio simulated annealing protocol<sup>48</sup> was used, starting from template structures with randomized  $\phi$  and  $\psi$  angles and extended side chains, to generate a set of 50 structures. The simulated annealing protocol consisted of 20 ps of high-temperature molecular dynamics (1000 K) with a low weighting on the repel force constant and NOE restraints. This was followed for a further 10 ps with an increased force constant on the experimental NOE restraints. The dihedral force constant was increased prior to cooling the system to 300 K and increasing the repel force constant over 15 ps of dynamics. Refinement of these structures was achieved using the conjugate gradient Powell algorithm with 1000 cycles of energy minimization<sup>49</sup> and a refined force field based on the program CHARMM.<sup>50</sup> Structures were displayed using INSIGHT (Biosym Technologies, San Diego, CA). All structural analysis described in the text was done using structures calculated without explicit H-bond restraints. The structure calculations for 6 utilized a similar protocol except that the peptide backbone was constrained to be cyclic via an amide bond between Orn-N $\epsilon$  and Trp-CO. The final structures were examined to obtain mean pairwise rms differences over the backbone heavy atoms (N, C $\alpha$ , and C).

**Receptor-Binding Assay.** Assays were performed with fresh human PMNs, isolated as previously described,<sup>17</sup> and a buffer of 50 mM HEPES, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.5% bovine serum albumin, 0.1% bacitracin, and 100  $\mu$ M PMSF (phenylmethylsulfonyl fluoride). In assays performed at 4 °C, buffer, unlabeled human recombinant C5a (Sigma) or test peptide, Hunter/Bolton labeled <sup>125</sup>I-C5a (~50 pM) (New England Nuclear, MA), and PMNs (0.2  $\times$  10<sup>6</sup>) were added sequentially to a Millipore Multiscreen assay plate (HV 0.45) having a final volume of 200  $\mu$ L/well. After incubation for 60 min at 4 °C, the samples were filtered and the plate washed once with buffer (100  $\mu$ L). Filters were dried, punched, and counted in an LKB gamma counter. Nonspecific binding was assessed by the inclusion of 1 mM peptide or 100 nM C5a which typically resulted in 10–15% total binding.

**Antagonism Assay.** Antagonist activity was assessed by monitoring myeloperoxidase release as follows. Cells were isolated as previously described<sup>17</sup> and incubated with cytochalasin B (5  $\mu$ g/mL, 15 min, 37 °C). Hank's Balanced Salt solution containing 0.1% gelatin and test peptide were added onto a 96-well plate (total volume 100  $\mu$ L/well), followed by 25  $\mu$ L cells (4  $\times$  10<sup>6</sup>/mL). To assess the capacity of each peptide to antagonize C5a, cells were incubated for 5 min at 37 °C with each peptide, followed by addition of C5a (100 nM) or agonist peptide and further incubation for 5 min. Then 50  $\mu$ L of phosphate buffer (0.1 M, pH 6.8) was added to each well, followed by the addition of 25  $\mu$ L of a fresh 1:1 mixture of dimethoxybenidine (5.7 mg/mL) and H<sub>2</sub>O<sub>2</sub> (0.51%). The reaction was stopped at 20 min by addition of 2% sodium azide (25  $\mu$ L). Absorbances were measured at 450 nm in a Bioscan 450-plate reader and corrected for control values (no peptide).

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