The peptidyl fluoromethyl ketones were synthesized in view of earlier observations that they were sometimes significantly more effective than the corresponding diazomethyl ketones. The in vitro results summarized in Table I show that this was true in some comparisons in the present series. For example, the second-order rate constant for inactivation of calpain by Z-Leu-Tyr-CH_2F (2) is at least 1 order of magnitude greater than that determined for Z-Leu-Tyr-CHN_2 (5). On the other hand, the tripeptide derivatives 1 and 4 have the converse relationship. However, these pairs of inhibitors were comparable in their inhibition of cathepsin L. The difference between the corresponding diazomethyl ketones and fluoromethyl ketone is much less. However, the change in length from dipeptide to tripeptide has a pronounced effect, increasing the rate of inactivation 50–100-fold.

Although Z-Leu-Leu-Tyr-CHN_2 (4) is more effective in inactivating calpain in vitro than the fluoromethyl ketone 1, in intact platelets the latter was more effective[25] under the conditions studied. A preincubation with 10 μM of fluoromethyl ketone 1 was able to protect components of the cytoskeleton from calcium-triggered degradation, whereas diazomethyl ketone 4 at this concentration was not effective. The fluoromethyl ketone 1 may penetrate platelets more readily.

Z-Leu-NLe-H has been shown to inhibit the calcium-activated proteinase in vitro and in intact platelets.[9] We expected this inhibitor also to be a powerful cathepsin L inhibitor in view of the results described here and in ref 5. In fact, during the present studies we have shown that this material will produce a graded inhibition in the presence of 2×10^{-5} M Z-Phe-Arg-AMC. From a plot (Figure 2) it was established that at pH 5.4, the concentration required for 50% inhibition is 7.2 nM. The choice of leucine in the penultimate position has been based on the observations of Sasaki.[22] A calpain inhibitor with greater selectivity would be desirable. Meanwhile, the use of two irreversible inhibitors permits the exploration of the cellular role of the calcium-activated proteinase.

Registry No. 1 (diasteromer 1), 137490-22-3; 1 (diasteromer 2), 133410-84-1; 2, 123392-26-7; 3 (diastereomer 1), 137362-97-1; 3 (diastereomer 2), 123392-25-6; 4, 116614-45-0; 7 (diastereomer 1), 137362-98-2; 7 (diastereomer 2), 137490-23-4; 9 (diastereomer 1), 137362-99-3; 8 (diastereomer 2), 137393-18-1; Cbz-Leu-Leu-Tyr-OH, 137363-00-9; Cbz-Tyr-Ala-OH, 23018-09-9; $(FCH_2CO)_2O$, 407-33-0; calpain, 78990-62-2; cathepsin L, 60616-82-2.

Structure–Function Studies in a Series of Carboxyl-Terminal Octapeptide Analogues of Anaphylatoxin C5a

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The synthesis and structure–activity relationships of C-terminal octapeptide analogues of anaphylatoxin C5a have been studied. The introduction of hydrophobic amino acids into the N-acetylated native octapeptide (N-Ac-His-Lys-Asp-Met-Gln-Leu-Gly-Arg-OH) (1) has led to an analogue with 100 times more activity than the native octapeptide in inhibiting the binding of ^{125}I-labeled anaphylatoxin C5a to human neutrophil membrane receptors. The observed apparent binding K_i's for the compounds (8–10) are in the range of 1–3 μM, and they possess nearly full agonist activity, despite the fact that these analogues are one-eighth or -ninth the size of the natural ligand anaphylatoxin C5a.

Introduction

Numerous investigations have been performed studying the inflammatory role of anaphylatoxin C5a (hereafter abbreviated C5a), a glycopeptide generated by proteolytic cleavage of the fifth complement component C5 during complement activation.[1,2] C5a, a relatively large molecule containing 74 amino acids, is a potent inflammatory mediator and potentially plays an important role in the pathogenesis of a number of inflammatory diseases.[3] In addition, recent studies have suggested that C5a may also be important in mediating inflammatory effects of phagocytic mononuclear cells that accumulate at sites of chronic inflammation[4] and may have a further proinflammatory role by enhancing the local production of antibodies at inflammatory sites.[5] Thus, a C5a antagonist would have significant inhibitory effects on these events, and therefore the discovery of a C5a antagonist is of particular

(1) Abbreviations follow the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature for the amino acids and peptides: *Eur. J. Biochem.* **1984**, *158*, 9. Additional abbreviations used herein are Cha, cyclohexylalanine and Ac, acetyl.

(2) (a) Johnson, R. J.; Tamerius, J. D.; Chenoweth, D. E. Identification of an Antigenic Epitope and Receptor Binding Domain of Human C5a. *J. Immunol.* **1987**, *138*, 3856–3862. (b) Chenoweth, D. E.; Erickson, B. E.; Hugli, T. E. Human C5a-Related Synthetic Peptides as Neutrophil Chemotactic Factors. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 227–234. (c) Yancey, K. B. Biological Properties of Human C5a: Selected in vitro and in vivo Studies. *Clin. Exp. Immunol.* **1988**, *71*, 207–210. (d) Martin, S. E.; Chenoweth, D. E.; Engler, R. L.; Roth, D. M.; Longhurst, J. C. C5a Decreases Regional Coronary Blood Flow and Myocardial Function in Pigs: Implications for a Granulocyte Mechanism. *Circ. Res.* **1988**, *63*, 483–491. (e) Regal, J. F. C5a-Induced Bronchoconstriction: Absence of a Role of Circulating White Blood Cells and Platelets. *Int. Arch. Allergy Appl. Immunol.* **1988**, *86*, 196–200.

(3) (a) Hugli, T. E.; Muller-Eberhard, H. J. Anaphylatoxins: C3a and C5a. *Adv. Immunol.* **1978**, *26*, 1–53. (b) Hugli, T. E. The Structural Basis for Anaphylatoxin and Chemotactic Functions of C3a, C4a, and C5a. *Crit. Rev. Immunol.* **1981**, *1*, 321–366.

(4) Allison, A. C.; Ferluga, J.; Prydz, H.; Scherlemmer, H. U. The Role of Macrophage Activation in Chronic Inflammation. *Agents Actions* **1978**, *8*, 27–35.

(5) (a) Morgan, E. L.; Weigle, W. O.; Hugli, T. E. Anaphylatoxin-mediated Regulation of Human and Murine Immune Responses. *Fed. Proc.* **1984**, *43*, 2543–2547. (b) Weigle, W. O.; Morgan, E. L.; Goodman, M. G.; Chenoweth, D. E.; Hugli, T. E. Modulation of the Immune Response by Anaphylatoxin in the Microenvironment of the Interacting Cells. *Fed. Proc.* **1982**, *41*, 3099–3103.

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Table I. Amino Acid Sequences of C-Terminal Octapeptides of C5a from Various Species and Other Anaphylatoxin Sequences

	67	68	69	70	71	72	73	74
human	His	Lys	Asp	Met	Gln	Leu	Gly	Arg
porcine	His	Lys	Asn	Ile	Gln	Leu	Gly	Arg
bovine	His	Lys	Asp	Met	Gln	Leu	Gly	Arg
mouse	His	Lys	Pro	Val	Gln	Leu	Gly	Arg
rat	His	Lys	Gly	Met	Leu	Leu	Gly	Arg
C3aH[a]	Ala	Ser	His	Leu	Gly	Leu	Ala	Arg
C4aH[a]	Lys	Gly	Gln	Ala	Gly	Leu	Gln	Arg

[a] Human C3a and C4a amino acid sequences. Exact numbering of these amino acids are from residue 70 to 77 (see ref 10).

Table II. Inhibition of ^{125}I-C5a Receptor Binding by Synthetic C5a Analogues

no.	analogues	PMNL membrane binding, K_i, μM	chemokinetic efficacy (% of rC5a max)
1	Ac-His^1-Lys^2-Asp^3-Met^4-Gln^5-Leu^6-Gly^7-Arg^8-OH	150	107
2	Nle^4-Nle^5-Nle^6	210	*c*
3	Cha^4-Cha^5-Cha^6	25	75
4	Cha^4-Cha^5-Cha^6-D-Ala^7	10	67
5	Phe^4-Phe^5-Phe^6-D-Ala^7	750	109
6	Tyr^4-Tyr^5-Tyr^6-D-Ala^7	inac[a]	*c*
7	Cha^6	30%[b]	*c*
8	Cha^4-Cha^5 D-Ala^7	2.9	57

[a] Less than 20% inhibition was observed at 1 mM peptide concentration. [b] Percent inhibition at 1 mM peptide concentration. [c] Not tested.

Table III. Inhibition of ^{125}I-C5a Receptor Binding by Synthetic C5a Analogues: Modifications of Residue 6 and Chirality Studies at Residues 4 and 5

no.	analogues	PMNL membrane binding, K_i, μM	chemokinetic efficacy (% of rC5a)
8	Ac-His^1-Lys^2-Asp^3-Cha^4-Cha^5-Leu^6-D-Ala^7-Arg^8-OH	2.9	57
9	Ile^6	2.0	56
10	Val^6	1.6	60
11	Nva^6	5.3	55
12	D-Cha^4	31%[a]	*b*
13	D-Cha^5	26%[a]	*b*
14	D-Cha^4-D-Cha^5	600	71

[a] Percent inhibition at 1 mM peptide concentration. [b] Not tested.

interest for treatment of inflammation and tissue damage. Several regions of C5a have been postulated to be receptor binding domains.[2a,b,6] The importance of the carboxyl-terminal region of C5a was first shown by Fernandez et al., who reported that biological activity is markedly reduced by carboxypeptidase N treatment which converts C5a to C5a des-Arg.[7] The other anaphylatoxins C3a and C4a are essentially inactivated by the loss of the C-terminal arginine residue.[7] Our earlier investigations with synthetic peptides demonstrated that the C-terminal octapeptide (compound 1) inhibits ^{125}I-C5a binding to its receptor.[8] To develop a therapeutically useful compound, our next focus was to initiate structure–activity relationship studies of the octapeptide in hopes of improving the binding affinity of synthetic peptides. We describe here structure–activity relationship studies of the lead octapeptide. All of the peptides were synthesized by solid-phase peptide synthesis. The synthetic peptides were evaluated for their ability to inhibit the binding of ^{125}I-C5a to polymorphonuclear leukocyte (PMNL) membrane receptors and to stimulate PMNL chemokinetic migration.

Results and Discussion

It is interesting to note that C5a from all species sequenced to date, with the exception of the rat, contains the unique carboxyl-terminal tetrapeptide Gln-Leu-Gly-Arg (Table I). Moreover, comparison of the five sequences available for C5a and those for other human anaphylatoxins C3a and C4a suggests that conserved regions of C5a may not only be receptor binding sites but also be important for imparting selectivity for the C5a receptor over other receptors. With this information in mind, as well as our finding of a bioactive fragment of C5a (octapeptide 1, Table II), we closely examined the amino acid composition among the different species. Only two out of the eight amino acids vary (i.e., residues 69 and 70). However, residue 70 and half the conserved amino acids are neutral residues (Gln^{71}, Leu^{72}, Gly^{73}). Therefore, since the residues spaning 70–73 are neutral, we felt this would be an appropriate region to use to study the effect of hydrophobicity on binding. The results are summarized in Table II. Triple substitution at positions 4–6 by norleucine (Nle) maintained binding affinity (2), whereas a triple replacement by the more hydrophobic residue cyclohexylalanine (Cha) at positions 4–6 drastically increased binding affinity (3). D-Ala replacement at the 7-position, a modification found to increase affinity slightly in other related series,[9]

(6) (a) Gerard, C.; Showell, H. J.; Hoeprich, P. D., Jr.; Hugli, T. E.; Stimler, N. P. Evidence for a Role of the Amino-terminal Region in the Biological Activity of the Classical Anaphylatoxin, Procine C5a-des-Arg-74. *J. Biol. Chem.* **1985**, *260*, 2613–2616. (b) Hahn, G. S. Peptide Antagonists for the C5a Anaphylatoxin. US Patent 4,692,511, Sept 1987.

(7) Fernandez, H. N.; Henson, P. M.; Otani, O.; Hugli, T. E. Chemotactic Response to Human C3a and C5a Anaphylatoxins: I. Evaluation of C3a and C5a Leukotaxis in vitro and under Simulated in vivo Conditions. *J. Immunol.* **1978**, *120*, 109–115.

(8) (a) Kawai, M.; Quincy, D. A.; Lane, B.; Mollison, K. W.; Luly, J. R.; Carter, G. W. Identification of a Receptor Binding Region of the Human Anaphylatoxin, C5a. *Abstracts of Papers*, 199th National Meeting of the American Chemical Society, Boston, MA, April 1990. Part II, MEDI 87. (b) Kawai, M.; Quincy, D. A.; Lane, B.; Mollison, K. W.; Luly, J. R.; Carter, G. W. Identification and Synthesis of a Receptor Binding Site of Human Anaphylatoxin C5a. *J. Med. Chem.* **1991**, *34*, 2068–2071.

Table IV. Characterization Data of C5a Analogues[a]

no.	calcd M_r	obsd FAB-MS	amino acid analysis							
			Asx	Ala	Leu	His	Lys	Arg	Gly	other
2	992	993	0.96	–	–	0.84	1.01	1.05	0.99	Nle, 3.07
3	1112	1113	0.99	–	–	3.70[b]	0.99	1.04	0.99	
4	1126	1127	0.98	1.01	–	3.82[b]	1.02	1.03		
5	1108	1109	0.98	0.98	–	1.03	1.04	1.10	–	Phe, 2.89
6	1156	1157	0.96	0.99	–	1.00	1.00	1.10	–	Tyr, 3.07
7	1065	1066	0.99	–	–	1.88[b]	0.98	1.04	0.96	Glx, 1.06; Met, 0.98
8	1086	1087	0.97	0.96	1.02	2.90[b]	1.03	1.02	–	
9	1086	1087	0.97	0.98	–	1.02	1.01	1.02	–	Cha, 1.88; Ile, 1.02
10	1072	1073	1.85	1.04	1.08	–	0.96	1.07	–	Cha, 1.89; Val, 0.96
11	1072	1073	0.98	0.93	1.01	–	1.01	1.01	–	Cha, 1.87; Nva, 1.03
12	1086	1087	0.99	0.97	1.03	1.00	1.21	0.98	–	Cha, 1.58
13	1086	1087	0.99	0.99	1.04	1.00	1.13	0.99	–	Cha, 1.33
14	1086	1087	0.99	0.95	1.02	2.87[b]	1.03	1.02	–	

[a] Data for compound 1 has been reported previously (see ref 8). [b] Depending on the column or experimental conditions, the observed retention times of His and Cha residues are often too close to make a clear distinction.

led to an analogue with slightly better binding (4 vs 3). Since hydrophobic residue replacements at positions 4–6 improved binding affinity, other hydrophobic amino acids were incorporated (Phe in 5, and Tyr in 6). It was found that aliphatic residues were preferred over aromatic (4 vs 5 and 6) ones. Another interesting observation was that the substitution of Cha for Leu at position 6 in the original peptide (1) caused a loss of activity, indicating that a less hydrophobic side chain, such as isobutyl, is preferred. Therefore, only replacement of Met-Gln by Cha-Cha in positions 4 and 5 together with D-Ala in position 7 was required to produce an analogue (8) with 100 times greater binding affinity than the original compound (1).

Next, we investigated small aliphatic replacements at position 6 (compounds 9–11) since analogue 8 revealed that a large lipophilic residue was not appropriate at this site. As shown in Table III, incorporation of Ile as well as shorter aliphatic analogues (Val or Nva) led to analogues that maintain binding activity. Since Cha replacements at the fourth and fifth residues produced an active analogue, we investigated the importance of the chirality of these residues and found that the L–L configuration gave an analogue with the highest binding affinity (8 vs 12, 13, and 14), suggesting that other combinations may destroy a favorable bioactive conformation.

A PMNL chemokinesis assay was employed to distinguish agonist from antagonist activity of the synthesized analogues. A basic effect of chemoattractants like rC5a is to stimulate PMNL locomotion. In the presence of a suitable concentration gradient established by diffusion, this results in directional movement or chemotaxis, while if the stimulus is present at uniform concentration, an increase in random migration, called chemokinesis, will result. In order to assess numerous compounds, measuring their chemokinetic activity has the advantages over chemotaxis of greater technical simplicity and the fact that cells respond to a defined concentration of the stimulus, rather than a gradient concentration that is constantly changing and dependent on the particular diffusion coefficient of the stimulus.

All of the synthetic peptide analogues were tested in dose–response fashion at concentrations within a range spanning their rC5a receptor binding K_i values to confirm that their biologic potency was consistent with their binding affinity and thus could be attributed to their interaction with the C5a receptor. The chemokinetic response to rC5a exhibits the same bell-shaped curve seen with chemotaxis that results from desensitization of the cells at high stimulus concentrations. Therefore, the maximal chemokinetic migration distance induced by rC5a, which typically reaches a peak at a 1 nM concentration, was considered to represent a full agonist response and was compared to the peak migration distance produced by peptide analogues as a measure of their agonist efficacy. This key parameter of chemokinetic efficacy, expressed as a percent of the maximal control response to rC5a, is given in the tables.

Disappointingly, all of the C5a analogues synthesized in this series possess agonist activity, despite the fact that the synthetic analogues are one-eighth or -ninth the size of the natural ligand C5a. At best, compounds such as 8–11 showed modest decreased efficacy in chemokinesis related to the original peptide 1 (57%, 56%, 60%, and 55%, respectively), and thus might be considered partial agonists. However, these results are clearly still short of the goal of reducing agonism to an extent that would yield a therapeutically useful antagonist.

In summary, we have described the results of an ongoing investigation of octapeptide C5a analogues with emphasis on the hydrophobicity and chirality of the central portion. Analogues with high binding affinity were prepared by simple replacement with hydrophobic amino acids at appropriate positions and with suitable chiralities. Systematic modification has led to analogues (8–10) with 100-fold greater affinity than the original octapeptide (1), having observed K_i values in the range of 1–5 μM. Additional work is now in progress to study further the structure–activity relationship of these analogues with the aim of optimizing binding affinity while reducing size and further eliminating agonism.

Experimental Section

Synthesis. Boc-protected amino acids were purchased from Bachem Inc. and Chemical Dynamics Corp. The syntheses of Boc-L-Cha-OH and Boc-D-Cha-OH were carried out according to

(9) Kawai, M.; et al., unpublished results.
(10) (a) Cui, L. X.; Ferreri, K.; Hugli, T. E. Characterization of Rat C5a, a Uniquely Active Spasmogen. *Complement* **1985**, *2*, 18–19. (b) Fernandez, H. N.; Hugli, T. E. Primary Structural Analysis of the Polypeptide Portion of Human C5a Anaphylatoxin: Polypeptide Sequence Determination and Assignment of the Oligosaccharide Attachment Site in C5a. *J. Biol. Chem.* **1978**, *253*, 6955–6964. (c) Gennaro, R.; Simonic, T.; Negri, A.; Mottola, C.; Secchi, C.; Ronchi, S.; Romeo, D. C5a Fragment of Bovine Complement: Purification, Bioassays, Amino-acid Sequence and Other Structural Studies. *Eur. J. Biochem.* **1986**, *155*, 77–86. (d) Gerard, C.; Hugli, T. E. Amino Acid Sequence of the Anaphylatoxin from the Fifth Component of Porcine Complement. *J. Biol. Chem.* **1980**, *255*, 4710–4715. (e) Wetsel, R. A.; Ogata, R. T.; Tack, B. F. Primary Structure of the Fifth Component of Murine Complement. *Biochemistry* **1987**, *26*, 737–743.

a literature method,[11] by hydrogenation of the corresponding Phe analogues. The synthesis of peptides was performed on a Biosearch 9500ex peptide synthesizer using Merrifield resin. A detailed synthetic protocol has been reported previously.[8] The crude peptide was extracted with 20% aqueous acetic acid, lyophilized, and purified by HPLC (21.4 mm i.d. × 25 cm, C18, 8-μm silica, Dynamax preparative HPLC, Rainin). The purified peptides, recovered by lyophilization of the HPLC fractions, were at least 95% pure and gave proton NMR, FAB-MS, and amino acid analyses consistent with the proposed structure (Table IV).

C5a Receptor Binding Assay. C5a receptor binding affinity of peptides was determined by competing for binding of radioiodinated C5a to human polymorphonuclear leukocyte (PMNL) membranes. Human PMNL membranes were isolated following cell lysis by nitrogen cavitation and Percoll density-gradient centrifugation.[12] Radiolabeled C5a was prepared by glucose oxidase-lactoperoxidase catalyzed radioiodination,[13] and the product was purified by affinity purification on a goat anti-human C5a resin.[14] Binding was performed in buffered balanced salts solution (pH 7.0) containing 0.25% gelatin, a cocktail of protease inhibitors, 50–250 pM ^{125}I-C5a, and 5-20 μg/mL PMNL membranes. Samples were incubated for 60 min at ambient temperature, and membrane-bound C5a was collected by filtration onto Millipore HVLP filters. The inhibitor concentration that displaced ^{125}I-C5a binding by 50% was determined by linear-regression analysis of the data and the apparent inhibition constant (K_i) was calculated by the method of Cheng and Prusoff.[15]

PMNL Chemokinesis. The assay procedure was based on the method of Smith et al.[16] Human peripheral blood PMNL were purified by density-gradient centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA), subjected to brief hypotonic lysis to remove contaminating red cells, and washed three times before use. The assay was conducted in Earle's balanced salt solution buffered with 20 mM HEPES at pH 7.0 and supplemented with 0.25% gelatin to prevent adsorptive loss of C5a proteins. PMNL were suspended at 2 × 10^8 PMNL/mL in 37 °C buffer containing 0.3% SeaPlaque agarose (Marine Colloids, Rockland, ME). The cell mixture was dispensed in 1-μL droplets in a 96-well microtiter plate (Falcon Micro-Test III, Becton Dickinson, Oxnard, CA). The plates were chilled on ice for 10 min. The droplets were layered with 100 μL of buffer, and then 11 μL of buffer with or without stimulus was added. Plates were incubated for 2 h at 37 °C, and the cell-migration distance was measured from the edge of the droplet to the leading front. Chemokinetic migration was estimated by subtracting the mean of a random migration (buffer) control and expressed as a percent of the maximal response to rC5a. The highest value obtained for each peptide is shown in the tables as chemokinetic efficacy.

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Registry No. 1, 133009-93-5; 2, 133214-63-8; 3, 137232-25-8; 4, 137232-26-9; 5, 137232-27-0; 6, 137232-28-1; 7, 137232-29-2; 8, 137232-30-5; 9, 133214-67-2; 10, 133214-78-5; 11, 133253-59-5; 12, 137232-31-6; 13, 137232-32-7; 14, 133214-90-1; C5a, 80295-54-1.

(11) For example: Boger, J.; Payne, L. S.; Perlow, D. S.; Lohr, N. S.; Poe, M.; Blaine, E. H.; Ulm, E. H.; Schorn, T. W.; LaMont, B. L.; Lin, T.-Y.; Kawai, M.; Rich, D. H.; Veber, D. F. Renin Inhibitors. Syntheses of Subnanomolar, Competitive, Transition-State Analogue Inhibitors Containing a Novel Analogue of Statine. *J. Med. Chem.* **1985**, *28*, 1779–1790.

(12) Borregard, N.; Heiple, J. M.; Simon, E. R.; Clark, R. A. Subcellular Localization of the b-Cytochrome Complement of the Human Neutrophil Microbicidal Oxidase: Translocation during Activation. *J. Cell Biol.* **1983**, *97*, 52–61.

(13) Gerard, C.; Chenoweth, D. E.; Hugli, T. E. Response of Human Neutrophils to C5a: A Role for the Oligosaccharide Moiety of Human C5a des Arg-74 but not of C5a in Biological Activity. *J. Immunol.* **1981**, *127*, 1978–1982.

(14) Chenoweth, D. E.; Goodman, M. G.; Wiegle, W. O. Demonstration of a Specific Receptor for Human C5a Anaphylatoxin on Murine Macrophages. *J. Exp. Med.* **1982**, *156*, 68–78.

(15) Cheng, Y.-C.; Prusoff, W. H. Relationship between the Inhibition Constant (K_i) and the Concentration of Inhibitor which causes 50 per cent Inhibition (I_{50}) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(16) Smith, M. J. H.; Walker, J. R. The Effect of Some Antirheumatic Drugs on an in vitro Model of Human Polymorphonuclear Leukocyte Chemokinesis. *Br. J. Pharmacol.* **1980**, *69*, 473–478.

Preparation and Opioid Activity of Analogues of the Analgesic Dipeptide 2,6-Dimethyl-L-tyrosyl-*N*-(3-phenylpropyl)-D-alaninamide

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A number of analogues of the recently disclosed analgesic dipeptide 2,6-dimethyl-L-tyrosyl-D-alanine-phenylpropylamide (SC-39566, **2**) were prepared. These analogues contained oxymethylene, aminomethylene, ketomethylene, bismethylene, and trans double bond (including vinyl fluoride) isosteric replacements for the amide bond between the D-alanine and phenylpropylamine units in **2**. These compounds were tested in opioid binding assays and in the mouse writhing assay for analgesic activity. Though not as potent as **2**, the oxymethylene, and trans double bond isosteres showed analgesic activity. The aminomethylene analogues also showed binding activity in subnanomolar concentrations at the μ receptor. The amide bond between 2,6-dimethyl-L-tyrosine and D-alanine units seems to be critical for opioid activity.

The Tyr tyramine and Phe phenyl moieties in enkephalins were found to be the major contributors to recognition at opioid receptors.[1-3] However, the identification of several potent opioidergic peptides (dermorphin,[4] casomorphin,[5] and morphiceptin[6]) that have Phe in the third

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(1) Morley, J. S. Structure–activity Relationships of Enkephalin-like Peptides. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 81.
(2) Morley, J. S. Chemistry of Opioid Peptides. *Br. Med. Bull.* **1983**, *39*, 5.
(3) Roques, B. P.; Fournie-Zaluski, M. C.; Gacel, G.; David, M.; Meunier, J. C.; Maigret, B.; Morgat, J. L. *Adv. Biochem. Pharmacol.* **1982**, *33*, 321.

0022-2623/92/1835-0223$03.00/0