to provide maximum adherence of macrophages as a monolayer. The cells were then washed 3 times with PBS to remove nonadherent cells. Five milliliters of DMEM, containing 50 μg of KLH (Keyhole limpet hemocyanine)/mL was added to each petri dish. In the experimental groups, the medium also contained tuftsin or its tested analogue at various concentrations. After 4 h at 37 °C, excess free antigen was washed out with three successive washes with an excess of PBS. Ten milliliters of spleen cell suspension (10^7 cells/mL) was added on top of the antigen-fed monolayers, and incubation proceeded for 18 h at 37 °C, in an atmosphere of 5% CO_2 in air. The nonadherent cells were then collected gently, reseeded on another set of petri dishes, and incubated for 90 min to adsorb residual adherent cells. The spleen cells were then irradiated with 1000 rad, using a Dermavolt X-ray machine (Siemens X-ray tube, 56 kV, 20 mA, 0.5 Al filter, 750 rad/min). The suspension was collected, centrifuged, and washed twice with PBS. The cells were resuspended in DMEM (10^8 cells/mL). Fifty microliters of this suspension was injected in the hind foot pads of syngeneic mice. On the seventh day, the popliteal lymph nodes were removed and a cell suspension was placed in culture medium (RPMI 1640, Gibco, supplemented with 0.5% syngeneic mouse serum and 50 μM 2-mercaptoethanol) adjusted to a concentration of 5×10^6 cells/mL. Samples (0.1 mL) of these cells were cultured in tissue microtiter plates (Costar 96 wells/tray) in the presence of antigen or control reagents at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 72 h, 1 μCi of tritiated thymidine (Israel AEC, Negev, Israel) was added and the cells were incubated for 16 h. Cells were then collected by a Titertek cell harvester (Skatronas, Liebyen, Norway) on glass filter, washed twice with saline, dried, and placed in a Bray's scintillation fluid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Competitive Binding Assay. All binding studies were performed in PBS at 22 °C for 30 min at a final volume of 0.5 mL. Macrophage suspensions were incubated with gentle agitation in plastic tubes (NUNC 70 × 12 mm; 1×10^6 cells/tube), with tritiated tuftsin([^{3}H-Arg^4]tuftsin; specific activity, 20.8 Ci/mmol; 5×10^{-8} M) and varying concentrations of unlabeled tuftsin or the tested peptide analogue. Binding was terminated by dilution with PBS (3 mL), followed by centrifugation and removal of the supernatant by aspiration. Cells were subsequently washed once more with PBS and similarly isolated. The cell pellets were then dissolved in 0.3 mL of SDS (0.2%), and the solutions obtained were collected and added to vials, each containing 5 mL of Triton–toluene mixture. Radioactivity was measured in a Beckman 7500 liquid scintillation spectrophotometer. Each point on the resulting binding curves derives from duplicate tubes, and the standard error of the mean (SEM) did not exceed 5%. Nonspecific binding was defined as the amount of tritiated tuftsin not inhibited by 10 nM tuftsin.

Role of the C-Terminal Carboxylate in Angiotensin II Activity: Alcohol, Ketone, and Ester Analogues of Angiotensin II†

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[Ac-Asn^1,Val^5]angiotensin II analogues containing a C-terminal alcohol (Phe-ol), methyl ketone (Pmk), methyl ester (Phe-OMe), or α-methyl methyl ester (Phe(αMe)-OMe) were prepared in order to examine the relative importance of COOH-mediated ionic vs. hydrogen bonding interactions in angiotensin activities. Based on the observation that only [Ac-Asn^1,Phe-OMe^8]AII (AII, angiotensin II) had significant activities (20% oxytocic and 13% pressor) in the rat, with all other analogues having negligible agonistic and antagonistic effects, it is concluded that ionic interaction of the C-terminal carboxylate with the receptor is necessary for angiotensin binding and that hydrogen bonding has little effect. Thus, the different potencies observed for the AII methyl ester and for various C-terminal analogues previously reported may simply reflect their relative abilities to generate the active carboxylate species in situ.

The COOH-terminus of biologically active peptides is frequently a target for drug design, in that the COOH group predisposes its parent peptides to metabolic breakdown by carboxypeptidase and converting enzyme/dipeptidase. In addition, peptides with a carboxylate group are often poorly absorbed through gastrointestinal membranes due to their ionic nature at physiological pH. Consequently, conversion of the COOH group to a proteolytic-resistant and nonionic alcohol in enkephalin or to a monoester in enalapril, a converting enzyme inhibitor, significantly improved their oral effectiveness.[1,2] Since the COOH group acts primarily through ionic and hydrogen bondings, hormone–receptor and enzyme–substrate interactions involving the peptide COOH-terminus would require a counterion or nucleophile on the enzyme/receptor. This concept has been utilized in the design of selective and irreversible affinity labels, such as peptide chloromethyl ketone[3,4] and aldehyde.[5]

In angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe, AII), C-terminal esterification or amidation significantly reduces its biological activities,[6-12] suggesting that a free

† The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC Commission on Nomenclature: *Biochemistry* **1975**, *14*, 449, and *Biochemistry* **1967**, *6*, 362. Other abbreviations are as follows: AI, angiotensin I (Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu); AII, angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe); Phe-ol, phenylalaninol; Pmk, phenylalanine methyl ketone or 4-phenyl-L-3-aminobutan-2-one; Phe(αMe), α-methylphenylalanine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate; PMSF, phenylmethylsulfonyl fluoride.

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Table I. Relative Activities of C-Terminal Analogues of Angiotensin II

analogue/structure of C-terminal substituent	potential interaction: hydrogen donor	potential interaction: hydrogen acceptor	potential interaction: ionization	potential conversion to carboxylate	biological activities in rat, %: oxytocic	biological activities in rat, %: pressor
[Asn^1]AII/NHCH($CH_2C_6H_5$)C(=O)OH	+	+	+	+	100	100
[Ac-Asn^1,Phe-ol^8]AII/NHCH($CH_2C_6H_5$)CH_2OH	+	+	−	−	0.09	0.001
[Ac-Asn^1,Pmk^8]AII/NHCH($CH_2C_6H_5$)C(=O)CH_3	−	+	−	−	0.4	0.03
[Ac-Asn^1,Phe(αMe)-OMe^8]AII/NHC(CH_3)($CH_2C_6H_5$)C(=O)OCH_3	−	+	−	−	0.002	0.001
[Ac-Asn^1,Phe-OMe^8]AII/NHCH($CH_2C_6H_5$)C(=O)OCH_3	−	+	−	+	20	13
[Phe(αMe)^8]AII/NHC(CH_3)($CH_2C_6H_5$)C(=O)OH	+	+	+	+	100[a]	80[a]

[a] Reported in ref 14.

COOH group is important for receptor interaction. In accord with this interpretation, Aiken and Vane showed that various vascular beds and smooth muscle preparations contained different amounts of converting enzyme activity,[13] thus accounting for the different potencies of angiotensin I (AI) on these tissues as a result of intramural generation of angiotensin II. On the other hand, the origin of the low but definite residual effect (0.8–4%)[13] of AI observed in the presence of SQ 20475, a converting enzyme inhibitor, remains controversial. Coincidently, Oparil, Haber, and associates reported that D-Leu^{10}-AI and des-Leu^{10}-AI, which were not converted to AII when incubated with converting enzyme or with plasma, also had about 5% activity in vivo.[12]

In order to determine whether these residual activities originate from direct action of the C-terminal modified analogues on angiotensin receptor or are a result of their proteolytic conversion to AII by alternative pathways, we compared the in vitro and in vivo effects of angiotensin II methyl ester with those of a series of C-terminal angiotensin II analogues that are incapable of conversion into the carboxylate species. The results are presented in Table I.

Results and Discussion

Evidence for the Carboxylate Terminus as a Binding Site for Angiotensin II. As the COOH group can participate in hydrogen bonding as either a H donor or acceptor (Table I), initial studies were conducted to determine the relative contribution of hydrogen bonding to angiotensin activities. For this purpose, the C-terminal alcohol (-CH_2OH) analogue, which can accept as well as donate hydrogen, and the methyl ketone (-$COCH_3$) analogue, which is a hydrogen acceptor, were tested in rats for oxytocic (data not shown) and pressor effects. As shown in Table I, both the alcohol and the ketone analogues had negligible agonistic activities (Figure 1a, top) comparable to that (0.05%) of the [des-Phe^8]AII, which lacks the 8-Phe activation site.[7] Neither inhibited the effects of AII in vitro or in vivo (data not shown). Thus, it appears that although the COOH-terminus is essential for receptor binding, this event is mediated by interactions other than hydrogen bonding.

Nevertheless, both the methyl ester ($COOCH_3$) which resembles the ketone and the amide ($CONH_2$) which resembles the alcohol in their capacities for hydrogen bonding, are at least several hundredfold more active than their corresponding ketone and alcohol counterparts. One possibility for this discrepancy may arise from the ability of the ester and amide to form multiple hydrogen bonds via the additional oxygen and nitrogen atom present in their C-terminus. Alternatively, the ester and amide

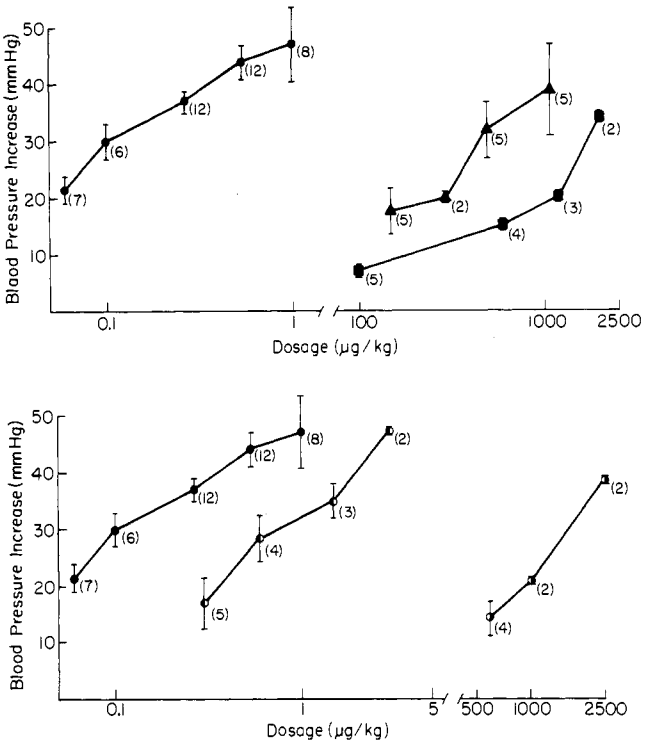

Figure 1. Dose–response curves (a, top) [Asn^1]AII (●), [Ac-Asn^1,Pmk^8]AII (▲), and [Ac-Asn^1,Phe-ol^8]AII (■) and (b, bottom) [Asn^1]AII (●), [Ac-Asn^1,Phe-OMe^8]AII (◐), and [Ac-Asn^1,Phe-(αMe)-OMe^8]AII (◑). Blood pressures were measured in urethane-anesthetized Zivic-Miller male rats by recording from the left femoral artery with a Physiograph linear core transducer. Angiotensin II or analogues were injected into the cannulated right jugular vein by means of a Hamilton syringe automatic dispensing apparatus. An AII dose–response curve was determined before and after the administration of each analogue so that each animal served as its own control. Standard errors of the mean are indicated by bars, with the number of animals tested indicated in parentheses.

analogues, similar to angiotensin I, may be converted in situ into the fully active angiotensin II, an interpretation inapplicable to the alcohol and ketone analogues.

To differentiate these alternatives, we prepared the methyl ester analogue of angiotensin II and of the highly active [α-methylphenylalanine^8]AII (100% rat oxytocic, 80% rat pressor).[14] This is based on the rationale that while both methyl ester analogues can form multiple hydrogen bonds, the α-methyl group confers considerable resistance toward endopeptidase and exopeptidase proteolysis.[15,16] Thus, these ester analogues differ markedly

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in their ability to generate a COOH-terminus in situ.

Comparison of their in vitro and in vivo (Figure 1b, bottom) activities showed that whereas [Ac-Asn^1,Phe-OMe^8]AII is 13–20% as active as AII, [Ac-Asn^1,Phe-(αMe)-OMe^8]AII has negligible effects (0.001–0.002%), comparable to those observed for the alcohol and ketone analogues. These results indicate that multiple hydrogen bonds are not important for angiotensin affinity. Instead, the significantly different potencies of the proteolytic-resistant vs. the proteolytic-susceptible methyl ester analogues strongly suggest an enzymatic role in converting [Ac-Asn^1,Phe-OMe^8]AII into the fully active angiotensin II, with the proteolytic-resistant [Ac-Asn^1,Phe(αMe)-OMe^8]AII unable to undergo a similar biotransformation into the highly active [Ac-Asn^1,Phe(αMe)^8]AII. Preliminary studies showed that treatment of the tissues with phenylmethylsulfonyl fluoride (PMSF), to eliminate serine proteases activity, reduced the oxytocic activities of both angiotensin II and its methyl ester. Although a 10-fold reduction for AII methyl ester relative to AII was observed, this approach is of limited value due to the tissue toxicity of PMSF.

In view of this possible protease activation, it is interesting to note that the rank order and relative potencies for these analogues (13–20% for AII ester > 3% for AII amide > 0.001–0.002% for α-methyl AII ester) are in excellent correlation with their susceptibilities toward biotransformation into the carboxylate species[15,17] (ester > amide > α-methyl amino acid ester at an approximate rate of $1:10^{-3}:10^{-5}$).[18] As the lack of biological activity for the alcohol and ketone analogues unequivocally shows that the angiotensin II C-terminal carboxylate is essential for receptor binding, the markedly different potencies of AII methyl ester and its α-methyl analogue (13–20% vs. 0.001–0.002%) may simply reflect their relative abilities to generate the active species in situ.

For the design of affinity labeling probes, the necessity of the COOH-terminus suggests the presence of a counter nucleophile on the receptor, the irreversible alkylation of which is useful for receptor localization and prolonged blockade. On the other hand, the requirement of a free carboxylate for receptor binding would preclude C-terminal modification of AII, i.e., into the affinity labels of chloromethyl ketone and aldehyde. For the development of orally effective inhibitors, C-terminal esterification converts the ionic carboxylate into a nonionic species more conducive to membrane transport, but subsequent regeneration of the bioactive carboxylate is not always feasible for long-acting antagonists like [Sar^1,Hfv^8]AII and [2-Ind^8]AII.[19,20] In these inhibitors, the proteolytic resistant hexafluorovaline and 2-amino-2-indanecarboxylic acid responsible for their potent inhibitory activity may also prevent the enzymatic activation of their ester prodrug.

Synthesis and Characterization of the C-Terminal Analogues of Angiotensin II. Boc-phenylalaninol and phenylalaninol can be prepared readily from their corresponding amino acid by $NaH_2Al(OCH_2CH_2OCH_3)_2$ reduction. Both Phe-ol and ZPCK (*N*-carbobenzoxyphenylalanine chloromethyl ketone) are commercially available. Catalytic hydrogenation of the latter gave phenylalanine methyl ketone with a 3-proton singlet at 2.20 ppm in NMR analysis. Esterification of α-methylphenylalanine by standard procedure[21] gave the appropriate methyl ester with a 3-proton singlet at 3.80 ppm in NMR.

Solution coupling of the Ac-Asn-Arg-Val-Tyr-Val-His-Pro heptapeptide with phenylalanine alcohol, methyl ketone, methyl ester, or α-methyl methyl ester resulted in angiotensin II analogues that showed different thin-layer electrophoretic mobilities from those for the heptapeptide at pH 8.0 but not at pH 1.8, suggesting the lack of a COOH-terminus in these analogues. Positive identification of phenylalaninol in peptide hydrolysate can be accomplished by TLC analysis in the basic solvent system of *s*-Buol-NH_4OH followed by ninhydrin detection. Under these conditions, Phe-ol has a much higher R_f value than amino acids due to its lack of a COOH group. Direct identification of the methyl ketone group in angiotensin II is possible with a 2,4-dinitrophenylhydrazine spray[22] on TLC.

In addition, fast atom bombardment mass spectrometry (FABMS) analyses indicated the correct molecular weight for each analogue. In conjunction with amino acid analyses, which indicated the appropriate amino acid ratio present in each peptide hydrolysate, these results provide a definitive verification of the identity of these C-terminal analogues.

Experimental Section

All chemicals were of reagent grade. *N-tert*-Butyloxycarbonyl-L-α-amino acids were supplied by Bachem, Torrance, CA. Phenylalanine methyl ester hydrochloride salt and *N*-carbobenzoxyphenylalanine chloromethyl ketone (ZPCK) were obtained from Sigma, St. Louis, MO, and phenylalaninol was obtained from Nutritional Biochemicals-ICN, Cleveland, OH. Silica gel (CC-7, neutral, 100–200 μm) for dry-column chromatography of amino acid and peptide derivatives was supplied by Mallinckrodt, St. Louis, MO, and was washed with the appropriate solvents and dried in vacuo prior to column packing. Melting points (Thomas-Hoover Uni-Melt) are uncorrected. Microanalyses were performed by the Robertson Laboratory, Florham Park, NJ. Proton magnetic resonance (NMR) spectra in deuterated methanol containing tetramethylsilane were obtained on a Varian T-60 (60 MHz). Infrared measurements were obtained on a Perkin-Elmer Model 237 spectrophotometer. The homogeneity of amino acid derivatives and of the synthetic peptides was assessed by thin-layer chromatography (TLC) on Merck precoated silica gel glass plates (type G60-F254) in different solvent systems: (i) chloroform–methanol (9:1), (ii) *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase), (iii) *n*-butyl alcohol–pyridine–acetic acid–water (8:1:2:9, upper phase), and (iv) *sec*-butyl alcohol–3% NH_4OH (100:44). Thin-layer electrophoresis (TLE) was performed on Eastman precoated cellulose plates (type 13254) using (i) a pH 1.9 aqueous formic acid–acetic acid buffer and (ii) a pH 8.0 aqueous 0.2 M boric acid–NaOH buffer, with E_p indicating electrophoretic mobility relative to Phe($4NH_2$) = 1.00 at both pHs. The products were identified by a combination of UV, ninhydrin, chlorox–toluidine, and Pauly sprays. Amino acid analyses (ninhydrin procedure) were performed on a Beckman Model 119 analyzer equipped with a Beckman system AA computing integrator after hydrolysis of the peptide in 6 N HCl containing 0.2% phenol at 110 °C for 24 in sealed tubes.

Phenylalanine Methyl Ketone Hydrochloride Salt (L-α-Amino-β-phenylethyl Methyl Ketone Hydrochloride,

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(18) The relative rates of hydrolysis (k_3) by chymotrypsin were reported to be 2.6 for Ac-Tyr-OEt and 3×10^{-3} for Ac-Tyr-NH_2 in ref 17; 0.92 for Ac-Phe-OMe, 2.9 for Ac-Tyr-OEt, 2.5×10^{-5} for Ac-α-Me-Tyr-OMe, and less than 10^{-5} for Ac-α-Me-Phe-OMe in ref 15. These k_3 values give a relative hydrolysis rate of $1:10^{-3}:10^{-5}$ for ester–amide–α-methyl methyl ester.

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Pmk·HCl, 1). N^{α}-Benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (0.3 g, 0.9 mmol) was dissolved in dimethylformamide (5 mL) and hydrogenated with 10% Pd/C (100 mg) under 1 atm of H_2 for 2 h. The suspension was filtered; the filtrate was evaporated to dryness in vacuo, and the residue was crystallized from methanol–ether–petroleum ether to give 110 mg (56% yield): mp 147.5–148 °C; TLC R_f (i) 0.23, (ii) 0.43, (iii) 0.56, (iv) 0.65; ^{1}H NMR (CD_3OD) δ 2.20 (s, 3 H, $COCH_3$), 3.23 (m, 2 H, $C_\beta H_2$), 4.43 (m, 1 H, $C_\alpha H$), 7.00 (s, 5 H, C_6H_5); IR (KBr) indicated a strong carbonyl band at 1720 cm^{-1}. Anal. ($C_{10}H_{13}NO$·HCl) C, H, N, Cl.

L-α-Methylphenylalanine methyl ester hydrochloride salt (Phe(αMe)-OMe·HCl, 2) was prepared by saturating the suspension of L-α-methylphenylalanine·HCl·H_2O (1.08 g, 4.6 mmol) in methanol (10 mL) with anhydrous HCl for 2 h at room temperature.[21] The mixture was evaporated to dryness, and the residue was chromatographed on a dry column (2 × 50 cm) of silica gel. The column was washed with petroleum ether and chloroform, and the product was eluted with 9:1 chloroform–methanol. Evaporation of the solvent, followed by recrystallization of the residue from methanol–ether, gave 0.83 g (78% yield): mp 108–113 °C; R_f (i) 0.47, (ii) 0.50, (iii) 0.58, (iv) 0.64; ^{1}H NMR (CD_3OD) δ 1.65 (s, 3 H, α-CH_3), 3.22 (m, 2 H, $C_\beta H_2$), 3.80 (s, 3 H, $COOCH_3$), 4.90 ($C_\alpha H$ and NH_3^+), 7.28 (m, 5 H, C_6H_5).

Ac-Asn-Arg-Val-Tyr-Val-His-Pro (3) was prepared by the stepwise solid-phase procedure and purified as described elsewhere:[23] TLC R_f (ii) 0.15, (iii) 0.16; TLE E_p (i) 0.56, (ii) 2.1. An acid hydrolysate gave: Asp (1.03), Arg (0.98), Val (1.02), Tyr (1.01), Val (1.02), His (0.95), Pro (0.98).

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-ol (4) was prepared form the heptapeptide (3; 50 mg, 0.05 mmol) and phenylalaninol (76 mg, 0.50 mmol) by DCC (52 mg, 0.25 mmol) coupling in the presence of HOBt (38 mg, 0.25 mmol) in dimethylformamide (12 mL) at 60 °C overnight. The mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by countercurrent distribution in 8:1:2:9 *n*-butyl alcohol–pyridine–acetic acid–water for 200 passages, followed by gel filtration of the appropriate fraction (K = 1.74) on Sephadex G-25 (2.5 × 35 cm) in 10% AcOH to give 40 mg of peptide (59% yield): TLC R_f (ii) 0.25, (iii) 0.31; TLE E_p (i) 0.55, (ii) 3.1. An acid hydrolysate gave Asp (1.03), Arg (1.01), Val (1.00), Tyr (1.06), Val (1.00), His (0.95), Pro (0.96); FABMS, MH^+ = 1060.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Pmk (5). Heptapeptide (3; 100 mg, 0.1 mmol), phenylalanine methyl ketone hydrochloride salt (1; 50 mg, 0.25 mmol), HOBt (19 mg, 0.12 mmol), and DCC (51 mg, 0.25 mmol) were suspended in dimethylformamide (5 mL) at 60 °C. After 3 days, TLC analysis of the mixture still indicated unreacted heptapeptide. The mixture was evaporated to dryness, and the residue was washed with CH_2Cl_2, EtOAc, and Et_2O and chromatographed on a dry column (1 × 20 cm) of silica gel. Elution of the column with the upper phase of the mixture of *n*-butyl alcohol–pyridine–acetic acid–water (8:1:2:9) at 1.2 mL/h (2-min fractions) separated the product from unreacted starting material. The appropriate fractions (48–66 mL) were combined, evaporated to dryness, and precipitated from elution solvent–dimethylformamide–dioxane–water to give 39 mg (36%): TLC R_f (ii) 0.23, (iii) 0.32; TLE E_p (i) 0.53, (ii) 3.2. An acid hydrolysate gave Asp (1.06), Arg (0.99), Val (1.03), Tyr (1.00), Val (1.03), His (0.93), Pro (0.96).

TLC analysis of 5 in different solvents followed by 3% dinitrophenylhydrazine spray[22] and heating at 110 °C for 10 min gave a dark-brown spot on a yellow background, indicating the presence of a ketone group. Similar treatment of other peptides without the ketone or aldehyde function gave the yellow background only: FABMS, MH^+ = 1072.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-OMe (6) was prepared from 3 (100 mg, 0.1 mmol) and phenylalanine methyl ester hydrochloride salt (54 mg, 0.25 mmol) by DCC (51 mg, 0.25 mmol) coupling in the presence of HOBt (19 mg, 0.12 mmol) in dimethylformamide (5 mL) at 60 °C overnight. The mixture was evaporated to dryness, and the residue was washed with CH_2Cl_2, EtOAc, and Et_2O and purified by gel filtration on Sephadex LH-20-120 (2 × 92 cm) in 10% AcOH to give 76 mg (70% yield): TLC R_f (ii) 0.24, (iii) 0.34; TLE E_p (i) 0.56, (ii) 3.0. An acid hydrolysate gave Asp (1.05), Arg (1.02), Val (1.00) Tyr (1.00), Val (1.00), His (0.99), Pro (0.97), Phe (0.97); FABMS, MH^+ = 1088.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Phe(αMe)-OMe (7) was prepared from 3 (100 mg, 0.1 mmol), α-methylphenylalanine methyl ester hydrochloride salt (2; 58 mg, 0.25 mmol), DCC (51 mg, 0.25 mmol), and HOBt (19 mg, 0.12 mmol) in dimethylformamide (5 mL) at 60 °C overnight. The mixture was evaporated to dryness, and the residue was washed with $CHCl_3$, EtOAc, and Et_2O and purified by ion-exchange chromatography on Sephadex C-25-120 in 10% AcOH with an NH_4OAc gradient. The appropriate fractions were combined to give 35 mg (32%): TLC R_f (ii) 0.24, (iii) 0.33; TLE E_p (i) 0.54, (ii) 2.8. An acid hydrolysate gave Asp (1.14), Arg (1.01), Val (1.01), Tyr (1.02), Val (1.01), His (0.87), Pro (0.93); FABMS, MH^+ = 1102.

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