Synthesis of Fluorine-Containing Peptides.[1a,b] Analogues of Angiotensin II Containing Hexafluorovaline

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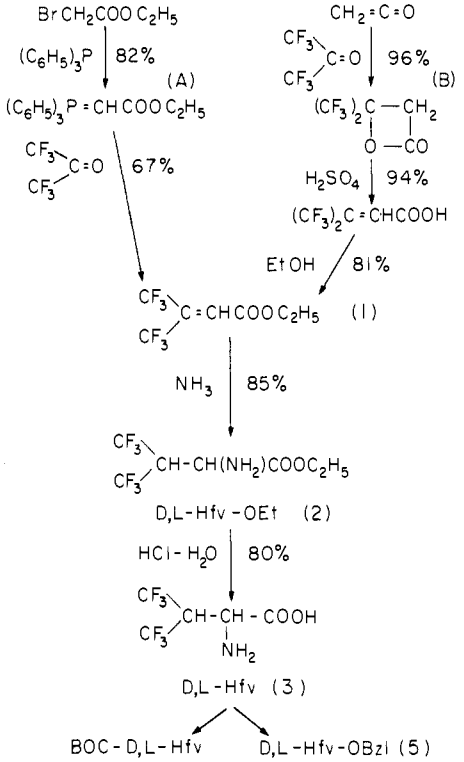
γ,γ,γ,γ′,γ′,γ′-Hexafluorovaline and derivatives have been prepared and incorporated into angiotensin II by fragment condensation and solid-phase peptide synthesis. Hexafluorovaline derivatives showed general resistance toward various enzymatic digestions and the tendency to racemize extensively upon carboxyl activation. When the angiotensin II analogues were assayed on rat uterus, [Hfv^5]AII had 133% activity, [D-Hfv^5]AII was inactive, and [Ac-Asn^1,DL-Hfv^8]AII was a potent inhibitor of angiotensin II in vitro and in vivo.

Due to the physicochemically distinct yet sterically similar characteristics of hydrogen and fluorine, fluorine substitution for hydrogen has been employed in a wide variety of compounds,[2] including steroids,[3] pyrimidines,[4,5] phenothiazines, and butyrophenones,[6] and has resulted in the development of many important new drugs. In the case of peptide hormones and proteins, fluorine substitution, such as in *p*-fluorophenylalanine, has been reported for bradykinin,[7,8] physalaemin,[9] oxytocin,[10] gastrin,[11] and ribonuclease *S*-peptide,[12] and has resulted in many highly active analogues. In addition, 5-fluorotryptophan, 4-fluorohistidine, or *m*-fluorotyrosine has been incorporated into luliberin (LH-RH),[13] ribonuclease,[14] *lac* repressor,[15] alkaline phosphatase,[16] and M13 coat protein.[17]

Scheme I. Synthesis of Hexafluorovaline[a]



[a] Route B according to Knunyants and Cherburkov.[27] Route A a simplified procedure through Wittig's reaction.

Aside from the possibility of significantly modifying the lipophilic and electronegative characteristics of a peptide with minimal accompanying steric perturbation, fluorine replacement of hydrogen also conveniently introduces a reporter group for ^{19}F NMR.[18] Since fluorine-containing molecules have a wide range of chemical shifts (about 1000 ppm vs. 15 ppm for protons), which are more sensitive to the environment than those of protons, fluorinated peptides can be valuable probes for conformational analysis of the peptide. More recently, sensitive and specific intracellular localization of the fluorine tracer of 4,6-difluoroserotonin in platelets was accomplished by a combination of electron microscopy and electron energy-loss spectroscopy.[19] Thus, fluorinated peptides are potentially

(1) (a) The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *J. Biol. Chem.*, **247**, 977 (1972), and *Biochemistry*, **14**, 449 (1975). Other abbreviations used are as follows: AII, angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe); Hfv, γ,γ,γ,γ′,γ′,γ′-hexafluorovaline or 3,3-bis(trifluoromethyl)-2-aminopropionic acid; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate. (b) This report has been presented in part. See "Abstracts of Papers", 172nd National Meeting of the American Chemical Society, San Francisco, CA, Aug 1976, American Chemical Society, Washington, DC, 1976, Abstr MEDI 015. (c) Department of Pathology, The Jewish Hospital and Washington University School of Medicine.
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useful for localizing the cellular binding sites of their parent hormones.

In angiotensin II, Asp-Arg-Val-Tyr-Val-His-Pro-Phe- (AII), fluorine substitution for the para hydrogen of 8-phenylalanine and for the hydroxyl group of 4-tyrosine resulted in a potent agonist and the only reported position-4 inhibitor of angiotensin, respectively.[20] Both analogues have been analyzed by ^{19}F NMR. In order to increase the sensitivity of the fluorinated probe, we incorporated more fluorine atoms into an amino acid residue, prepared $\gamma,\gamma,\gamma,\gamma',\gamma',\gamma'$-hexafluorovaline [Hfv, 3,3-bis(trifluoromethyl)-2-aminopropionic acid], and further investigated its resolution, derivatization, and incorporation into angiotensin II. Since native angiotensin II has valine in positions 3 and 5, whereas [Val^8]AII is a potent angiotensin antagonist,[21-23] we substituted both positions 5 and 8 with hexafluorovaline.

Results and Discussion

Synthesis of Hexafluorovaline and Derivatives. Several fluorinated analogues of valine, including 3-fluorovaline,[24] 4,4'-difluorovaline,[25] and 4,4,4-trifluorovaline,[26] have been reported. In this study, an earlier synthesis[27] of DL-hexafluorovaline (Scheme I, route B) was simplified (Scheme I, route A).

Subsequent resolution of DL-Hfv was not satisfactorily achieved by a number of standard methods. For instance, separation of DL-Hfv by L-ephedrine[28] resulted in low yields. Similarly, enzymatic digestion of F_3Ac-DL-Hfv by carboxypeptidase A[20] and by hog renal acylase,[29] as well as digestion of DL-Hfv-OEt by α-chymotrypsin,[29] resulted in the unhydrolyzed substrates only.[30]

Further attempts to protect DL-Hfv with the widely used *tert*-butyloxycarbonyl (Boc) group by the azide procedure,[31] by the *tert*-butylphenyl carbonate,[32] or the *tert*-butyl-*p*-nitrophenyl carbonate[33] procedure gave low yields. Preparation of the benzyl ester[29] by *p*-toluenesulfonic acid catalyzed or anhydrous HCl catalyzed esterification of DL-Hfv in benzyl alcohol was equally unsatisfactory. When DL-Hfv was reacted with the *p*-nitrophenyl ester of Boc-Tyr(Bzl) in the presence of 1-hydroxybenzotriazole,[34] Boc-Tyr(Bzl)-DL-Hfv was obtained in 88% yield. Subsequent separation by silica gel chromatography resolved Boc-Tyr(Bzl)-L-Hfv from Boc-Tyr(Bzl)-D-Hfv. Thus, both problems of resolution and protection of L-Hfv were circumvented.

Table I. Relative Biological Activities of Angiotensin II Analogues Containing Hexafluorovaline

	rat oxytocic assay, %
[Asn^1]AII	100
[Hfv^5]AII	133
[D-Hfv^5]AII	<1
[Ac-Asn^1,DL-Hfv^8]AII	antagonist (pA_2 = 7.88 ± 0.35)
[Ac-Asn^1,DL-Hfv-OEt^8]AII	antagonist
[Ac-Asn^1,DL-Hfv-OBzl^8]AII	antagonist

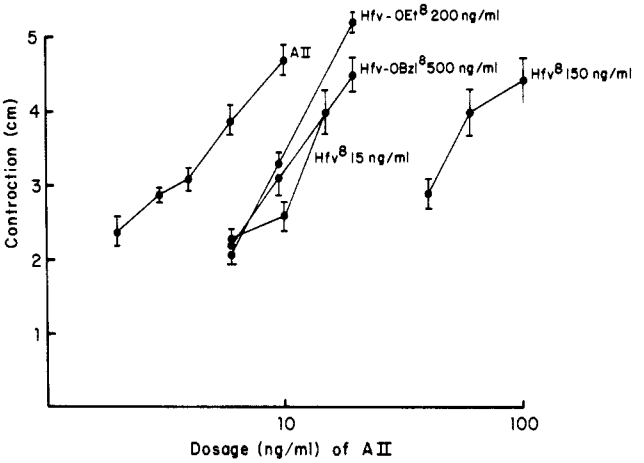

Figure 1. Inhibition of [Asn^1]angiotensin II induced rat uterine contraction by analogues containing hexafluorovaline in position 8: [Ac-Asn^1,DL-Hfv^8]AII, [Ac-Asn^1,DL-Hfv-OEt^8]AII, and [Ac-Asn^1,DL-Hfv-OBzl^8]AII.

Synthesis of [D-Hfv^5]AII and [L-Hfv^5]AII. Although fragment condensation in combination with the solid-phase procedure has been successfully applied in peptide synthesis,[35-39] condensation of Boc-Tyr(Bzl)-D-Hfv with His-(Tos)-Pro-Phe-resin by sequential *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline[37,40] (EEDQ) and dicyclohexylcarbodiimide/hydroxybenzotriazole[34] (DCC/HOBt) couplings resulted in incomplete incorporation of the dipeptide (40% incorporated as shown by amino acid analysis of a peptide-resin hydrolysate). In addition, significant racemization of the hexafluorovaline residue occurred, which was indicated by the generation of Boc-Tyr(Bzl)-L-Hfv from Boc-Tyr(Bzl)-D-Hfv in the reaction supernatant when analyzed by TLC and by the isolation of [L-Hfv^5]AII and [D-Hfv^5]AII in the ratio of 38:100.

Synthesis of [Ac-Asn^1,DL-Hfv^8]AII and its ethyl and benzyl esters was carried out by solution coupling of the appropriate ethyl or benzyl ester of DL-Hfv with the angiotensin II heptapeptide (Ac-Asn-Arg-Val-Tyr-Val-His-Pro), using DCC/HOBt to suppress racemization and to accelerate coupling.[34] This approach stemmed from our difficulty in obtaining Boc-Hfv for peptide synthesis,[41] whereas the heptapeptide was readily accessible through

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the solid-phase procedure. Furthermore, the C-terminal proline of the heptapeptide is resistant to racemization during coupling,[42] and the N-terminal acetyl protecting group is compatible with angiotensin II activity.[43] Catalytic hydrogenation of the benzyl ester analogue gave [Ac-Asn^1,DL-Hfv^8]AII.

Biological activity was examined in rat uterine preparations.[43] Whereas one diastereomer containing Hfv in position 5 was 133% as active as [Asn^1]AII, the other diastereomer had less than 1% activity. Based on the extensively studied structure–activity relationship of angiotensin II analogues,[44] among which [D-Val^5]AII was shown to have no pressor activity,[45] the fully active analogue was assigned as [L-Hfv^5]AII and the inactive analogue was assigned as [D-Hfv^5]AII.

In rat oxytocic assays, [Ac-Asn^1,DL-Hfv^8]AII had 0.5% agonistic activity (data not shown) and inhibited the uterine contraction induced by angiotensin II with a pA_2 value of 7.88 (Figure 1). Further evaluation of [Ac-Asn^1,DL-Hfv^8]AII in other smooth-muscle preparations showed that this analogue was inactive as either an agonist or antagonist in isolated rabbit aorta but had ~1–2% agonistic activity in rat stomach strips and rat colon preparations. At the inhibitory dosage of 50 ng/mL, it caused a 3-fold shift of the AII dose–response curve in either tissue. Preliminary studies in rat pressor assays[43] suggested that [Ac-Asn^1,DL-Hfv^8]AII, similar to [Val^8]-AII,[21-23] was a potent angiotensin inhibitor in vivo. For instance, when rats (n = 5) were given bolus injections of the inhibitors, [Ac-Asn^1,DL-Hfv^8]AII or [Sar^1,Ala^8]AII (saralasin),[46] followed by angiotensin II (6 ng), the pressor response toward AII was reduced. When the duration required for this pressor response (6 ng of AII at 15-min intervals) to return to 50% of pretreatment level was evaluated, bolus injections of 10, 30, and 100 ng of the aminopeptidase-resistant saralasin gave 50% recovery times of 11.0 ± 1.2, 13.7 ± 1.2, and 18.0 ± 1.8 min, whereas equal dosages of [Ac-Asn^1,DL-Hfv^8]AII produced a more prolonged inhibition, with 50% recovery times of 13.2 ± 2.3, 17.6 ± 1.2, and 27.3 ± 4.3 min.

Since our previous results suggested that the ionic carboxylate terminus might be an important binding site of angiotensin II to the receptor,[47] the biological activities of the ester derivatives of [DL-Hfv^8]AII were interesting in that the ethyl ester of DL-Hfv could not be converted by chymotrypsin to the ionic carboxylate. However, in isolated rat uterine preparations, both the ethyl and benzyl ester analogues of [Ac-Asn^1,DL-Hfv^8]AII showed some activity (approximately one-tenth and one-thirtieth of the antagonistic effect of [Ac-Asn^1,DL-Hfv^8]AII in Figure 1). These results suggest that either nonchymotryptic conversion in tissue or spontaneous hydrolysis of the ester analogues to [Ac-Asn^1,DL-Hfv^8]AII under the bioassay conditions could have occurred. Since rapid and spontaneous hydrolysis of hexafluorovaline benzyl ester was observed in aqueous base,[41] it is possible that the biological activity of the ester analogues might reflect their relative ability to generate [Ac-Asn^1,DL-Hfv^8]AII prior to proteolytic inactivation at other sites of the angiotensin II molecule.

Conclusions

Substitutions of hexafluorovaline into angiotensin II resulted in analogues resembling their valine-containing parents in biological activity. These findings suggest that the lipophilic and steric characteristics of the β-branched chain[48] of valine are more important than its other structural features for the angiotensin–receptor interaction and that hexafluorovaline may be a valid substitute for valine in other peptide hormones. Moreover, the hexafluorovaline analogues covered the whole spectrum of biological activity in angiotensin II, i.e., the very agonistic [Hfv^5]AII, the inactive [D-Hfv^5]AII, and the antagonistic [Ac-Asn^1,DL-Hfv^8]AII. Thus, these compounds offer an unusual opportunity to investigate possible conformational differences between angiotensin II agonists and antagonists by ^{19}F NMR, especially since hexafluorovaline has a relatively simple resonance pattern derived from its two trifluoromethyl groups. The results can be particularly relevant in that the fluorine probe is placed either in the primary activating site (position 8) or adjacent to the secondary activating site (position 4) of angiotensin II. For therapeutic applications, introduction of hexafluorovaline at strategic positions may be useful in reducing degradation of angiotensin and other peptides, since derivatives of hexafluorovaline resisted several enzymatic digestions.

Present limitations for incorporating hexafluorovaline into peptides are the inefficient synthesis of suitable derivatives and the lack of general methods to resolve hexafluorovaline and derivatives. Because hexafluorovaline can racemize extensively during peptide synthesis, careful characterization of the optical purity of the products as well as methods capable of resolving complex peptide diastereomers are important for further application of hexafluorovaline to other peptides.

Experimental Section

[Asn^1]AII was the generous gift of Dr. W. Rittel of Ciba-Geigy, Basel, Switzerland, and hexafluoroacetone was a generous gift from Dr. Joseph Lann of E. I. du Pont de Nemours & Co., Wilmington, DE. *tert*-Butyloxycarbonyl-protected amino acids were obtained from Bachem., Torrance, CA. Thin-layer chromatography on silica gel G plates from Analtech, Inc., or from E. Merck (60 F254) was performed in (i) 4:1 chloroform–methanol, (ii) 90:4:20 chloroform–acetic acid–pyridine, (iii) 4:1:5 1-butanol–acetic acid–water, (iv) 15:10:3:12 1-butanol–pyridine–acetic acid–water, and (v) 8:1:2:9 1-butanol–pyridine–acetic acid–water. IR spectra were obtained on a Perkin-Elmer Model 237 spectrometer, ^{1}H NMR spectra on a Varian HA-100 at 100 MHz or on a Varian T-60 at 60 MHz, and ^{19}F NMR spectra at 54.6 MHz on a Varian T-60. Mass spectra were obtained on a LKB 9000 at 70 eV by direct inlet. Melting points (Thomas-Hoover, Unimelt) were uncorrected. Elemental analyses were performed by PCR, Inc., or Robertson Laboratories. Amino acid analysis was performed on a Beckman amino acid analyzer Model 120 C after hydrolysis of the peptide in 6 N HCl at 100 °C in sealed, evacuated

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tubes for 24 h or after hydrolysis of the peptide-resin in 1:1 HCl–propionic acid at 120 °C for 2 h.

Ethyl 4,4,4-trifluoro-3-(trifluoromethyl)crotonate (1) was prepared by saturating the suspension of triphenylcarbethoxymethylenephosphorane[49] (97.4 g, 0.28 mol) in anhydrous ether (250 mL) with hexafluoroacetone (60 g, 0.36 mol) at −80 °C for 1.5 h. The mixture was stirred at room temperature for another 5 h, followed by precipitation with petroleum ether and filtration. The filtrate was evaporated in vacuo and the product was distilled through a 10-cm Vigreux column to give 43.7 g (67%) of a colorless liquid: bp 124–127 °C [lit.[27] 128 °C, (747 mm)]; IR (neat) 2980 (C═CH), 1780 (COO), 1670 (C═C), 1395 (CF), 1295 (COO), 1220, (CF) and 1175 (CF) cm^{-1}; ^1H NMR (neat) δ 1.88 (t, 3 H, J = 7 Hz, CH_3), 3.90 (q, 2 H, J = 7 Hz, CH_2), 6.57 (d, 1 H, J = 1.5 Hz, C═CH); ^{19}F NMR ϕ 62.02 (q, 3 F, J = 6.7 Hz, CF_3), 66.52 (q, 3 F, $J_{F,F'}$ = 6.7 Hz, $J_{F,H}$ = 1.2 Hz, CF_3); MS, m/e 191 ($M^+ - OCH_2CH_3$), 163 ($M^+ - COOCH_2CH_3$).

Hexafluorovaline ethyl ester (DL-**Hfv-OEt**, 2) was synthesized according to reported procedure[27] by reacting 1 (41.7 g, 0.18 mol) in anhydrous ether (200 mL) with liquid NH_3 (13.6 g, 0.8 mol) at −80 °C for 40 min. The mixture was stirred at room temperature for 3 h and filtered. The filtrate was evaporated in vacuo, and the product was distilled through a 10-cm Vigreux column to give 38.2 g (85%) of a colorless liquid: bp 80 °C (22 mm) (lit.[27] 77.0–77.2 °C (20 mm)]; IR (neat) 3400 (NH) 2998 (CH), 1750 (COO), 1400 (CF), 1205 (CF), 1100 (CN) cm^{-1}; ^1H NMR (neat) δ 1.07 (t, 3 H, J = 7 Hz, CH_3), 1.62 (s, 2 H, NH_2), 2.85 (m, 4 H, $C_\alpha H$, $C_\beta H$, and CH_2); ^{19}F NMR ϕ 62.22 (m, 3 F, $J_{F,F'}$ = 8.5 Hz, $J_{F,H}$ < 1 Hz, CF_3), 65.95 (p, 3 F, J = 8.6 Hz, CF_3); MS, m/e 253 (M^+), 180 ($M^+ - COOCH_2CH_3$). Anal. ($C_7H_9NF_6O_2$) C, H; N: calcd, 5.5; found, 4.9.

Hexafluorovaline (DL-**Hfv**, 3) was obtained by hydrolyzing the ethyl ester (2; 30.7 g, 0.12 mol) in 400 mL of 6 N HCl at 90 °C for 7 h. The hydrolysate was evaporated to dryness, and the residue was recrystallized from 2-propanol–petroleum ether to give 21.7 g (80%): mp 211 °C dec (lit.[27] 204 °C); IR (KBr) 3000 (NH_3^+), 2600 (NH_3^+), 2060 (NH_3^+), 1600 (NH_3^+), 1600 (CO_2^-), 1390–1255 (CF) cm^{-1}; ^{19}F NMR ϕ (H_2O, pH 2.93) 62.00 (p, 3 F, J = 8 Hz, CF_3), 64.78 (p, 3 F, J = 8 Hz, CF_3). Anal. ($C_5H_5NF_6O_2$) C, H, N, F.

Trifluoroacetylhexafluorovaline hemihydrate (Tfa-DL-Hfv·0.5H_2O, 4) was prepared according to standard procedure by reacting trifluoroacetic anhydride (39.6 g, 0.18 mol) with hexafluorovaline (23.4 g, 0.10 mol) in trifluoroacetic acid (125 mL) at 3 °C for 1 h and at 20 °C for 30 min. The reaction mixture was evaporated to dryness in vacuo, and the residue was extracted with methylene chloride and ethyl acetate. The organic solutions were combined, dried ($MgSO_4$), and evaporated to dryness. Recrystallization from ethyl acetate–ligroin gave 26.0 g (82%): mp 123.5–125.5 °C; IR (KBr) 3270 (NH), 1755 (CF_3CO–NH), 1730 (CO_2H), 1560 (NH), 1400–1100 (NH and CF_3) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 4.69 (m, 1 H, $J_{H,F}$ = 8.6 Hz, $J_{H,H'}$ = 3.5 Hz, $C_\beta H$), 5.30 (q, 1 H, $J_{H,H'}$ = 3 Hz, $J_{H,NH}$ = 9 Hz, $C_\alpha H$), 10.24 (d, 1 H, $J_{H,H'}$ = 9 Hz, NH); ^{19}F NMR (acetone) ϕ 62.62 (p, 3 F, $J_{F,F'}$ = 8.6 Hz, CF_3), 66.52 (q, 3 F, $J_{F,F'}$ = 8.6 Hz, $J_{F,H}$ = 8.6 Hz, CF_3), 75.79 br s, 3 F, CF_3CO); MS, m/e 276 ($M^+ - CO_2H$), 256 ($M^+ - CF_3$), 69 (CF_3^+). Anal. ($C_7H_4NF_9O_3$·0.5H_2O) H, N, F; C: calcd, 25.4; found, 26.0.

Hexafluorovaline benzyl ester hydrochloride salt (DL-**Hfv-OBzl·HCl**, 5) was prepared by azeotropic (Dean-Stark) distillation[29] of a suspension containing hexafluorovaline (8 mmol), benzyl alcohol (8 mL, 80 mmol), and p-toluenesulfonic acid monohydrate (3.2 g, 16 mmol) in benzene (50 mL) for 10 days. Evaporation of the mixture, followed by chromatography of the residue on a dry column (2 × 50 cm) of silica gel, removed the toluenesulfonic acid through successive washings with petroleum ether and chloroform. Subsequent elution with chloroform–methanol (9:1), followed by evaporation in vacuo, gave an oil, which was acidified with anhydrous HCl in dioxane. Removal of the solvent in vacuo and recrystallization of the residue from ether–petroleum ether gave 159 mg (6%): mp 147–148 °C; IR (KBr) 3400 (NH), 2900 (CH), 1750 (CO) cm^{-1}; ^1H NMR ($CDCl_3$–Me_2SO-d_6) δ 4.63 and 4.93 (m, 2 H, $C_\beta H$ and $C_\alpha H$), 5.25 (s, 2 H, benzylic CH_2), 7.05 (s, 5 H, benzylic C_6H_5). Anal. ($C_{12}H_{11}NF_6O_2$·HCl) C, H, N, F.

Catalytic hydrogenation of a sample of **5** with 10% Pd on charcoal resulted in **3**.

Boc-Tyr(Bzl)-DL-Hfv (6) was prepared by condensing **3** (0.83 g, 3.7 mmol) with *tert*-butyloxycarbonyl-*O*-benzyltyrosine *p*-nitrophenyl ester (2.0 g, 4.2 mmol) and 1-hydroxybenzotriazole monohydrate (0.62 g, 4.0 mmol) in 80 mL of dimethylformamide and 0.36 mL of pyridine at room temperature for 12 h. The mixture was added to 0.5 mL of acetic acid and evaporated. The residue was extracted with ether and ethyl acetate, and the organic solutions were evaporated to an oil, which was chromatographed on a column (100 × 2.5 cm) of silica gel in chloroform–methanol (12:1) to give 2.0 g (88%) of **6**. Analysis of the product by TLC showed a single spot in chloroform–methanol (4:1) (R_f 0.47) and two spots in chloroform–acetic acid–pyridine (90:4:20) (R_f^1 0.46, R_f^2 0.31): IR (KBr) 3400 (NH), 2940 (CH), 1650 (CO_2H), 1510 (CON), 1395–1175 (CF) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.18, 1.28 [br s, 9 H, $(CH_3)_3$], 3.0 (br s, 2 H, Tyr $C_\beta H_2$), 3.5 (br s, 1 H, NH), 4.16 (m, 1 H, Tyr $C_\alpha H$), 4.9 (br s, 3 H, NH, Hfv $C_\alpha H$ and $C_\beta H$), 6.8 (d, 2 H, $J_{H,H'}$ = 7 Hz, Tyr *o*-H), 7.0 (d, 2 H, $J_{H,H'}$ = 8 Hz, Tyr *m*-H), 7.28 (s, 5 H, benzyl H). Anal. ($C_{26}H_{28}N_2F_6O_7$) C, H, N.

Amino acid analysis of a hydrolysate gave tyrosine and hexafluorovaline. Hfv was eluted from the long column in regular amino acid analysis at 16 min before Asp. The color constants of Hfv prepared from hydrogenation of Hfv-OBzl[41] were 1.62 of that of Pro at 440 nm and had a ratio of 2.42 between the values at 440 and 570 nm.

Separation of Boc-Tyr(Bzl)-L-Hfv (L-6) from Boc-Tyr(Bzl)-D-Hfv·3H_2O (D-6). The above dipeptide **6** (1.95 g, 3.3 mmol) was chromatographed on a column (100 × 2.5 cm) of silica gel in chloroform–acetic acid–pyridine (90:4:20) at 40 mL/h to give two fractions: 648–1092 mL (L-**6**) and 1200–2568 mL (D-**6**).[50] Analysis of the two fractions by TLC indicated that each fraction was devoid of the other diastereomer. Recrystallization of D-**6** from ether–hexane gave 0.82 g (82%): mp 140 °C dec; IR (KBr) 3320 (NH), 2990 (CH), 1680–1645 (OCON, CON, and CO_2H), 1510 (CON), 1395 (CF), 1295 (CON), 1240–1080 (CF) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.22 [s, 9 H, $(CH_3)_3$], 2.90 (m, 3 H, NH and Hfv $C_\alpha H$ and $C_\beta H$), 6.90 (d, 2 H, $J_{H,H'}$ = 8 Hz, Tyr *o*-H), 7.20 (d, 2 H, $J_{H,H'}$ = 8 Hz, Tyr *m*-H), 7.29 (s, 5 H, benzyl H). Anal. ($C_{26}H_{28}N_2F_6O_7$·3H_2O) C, H, N.

Similarly, recrystallization of L-**6** from ether–hexane gave 0.67 g (67%): mp 108 °C dec; IR (KBr) 3310 (CON), 2950 (CH), 1715 (OCON), 1670 (CON and CO_2H), 1515 (CON), 1395 (CF), 1370 (CF), 1295 (CON), 1245–1095 (CF) cm^{-1}.

Asp-Arg-Val-Tyr-Hfv-His-Pro-Phe (7) and Asp-Arg-Val-Tyr-D-Hfv-His-Pro-Phe (8) were prepared through fragment condensation of Boc-Tyr(Bzl)-D-Hfv (D-**6**; 0.14 g, 0.24 mmol) to His(Tos-Pro-Phe-resin (0.62 g, 0.108 mmol), with a hydrolysate containing His-Pro-Phe in the ratio of 0.97:0.95:1.00, using EEDQ (84 mg, 0.33 mmol) in dioxane (5 mL) at room temperature for 15 h. Analysis of the reaction supernatant by TLC (in chloroform–acetic acid–pyridine) showed extensive racemization of D-**6** into L-**6**.[50] Repeated coupling of D-**6** (72 mg, 0.12 mmol) to the resin by DCC (33 mg, 0.16 mmol) and HOBt (25 mg, 0.16 mmol) in tetrahydrofuran (5 mL) for 24 h resulted in some racemization of D-**6**. Subsequent termination of the unreacted tripeptide-resin by acetic anhydride (87 mg, 0.87 mmol) in triethylamine (87 mg, 0.97 mmol) and CH_2Cl_2, followed by stepwise incorporation of

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(50) Because racemization of peptide upon carboxyl activation is mediated through an oxazolinone (azalactone) intermediate and/or α-proton abstraction (ref 42), it follows that partial racemization of the C-terminal residue of a L,L dipeptide during coupling with the amino component of X would produce a diastereomeric pair containing a major product of L,L-X and a minor product of L,D-X, whereas similar partial racemization of a L,D dipeptide would produce a major product of L,D-X and a minor product of L,L-X. Since [D-Hfv[5]]AII was the major product isolated from the diastereomeric mixture of [D-Hfv[5]]AII and [L-Hfv[5]]AII, it was concluded that Boc-Tyr(Bzl)-D-Hfv was incorporated into His(Tos)-Pro-Phe-resin. Therefore, fraction 1200–2586 mL which resulted in Boc-Tyr(Bzl)-D-Hfv was assigned as D-**6**, and fraction 648–1092 mL was assigned as L-**6**.

the remaining residues (0.69 mmol) by standard solid-phase procedure,[51-53] resulted in Boc-Asp(OBzl)-Arg(NO_2)-Val-Tyr-(Bzl)-DL-Hfv-His(Tos)-Pro-Phe-resin, a hydrolysate of which showed Asp (0.25), Arg (0.30), Val (0.36), Tyr (0.35), Hfv (0.44), His (0.83), Pro (1.04), Phe (1.00). Treatment of this peptide-resin (600 mg) with liquid HF (10 mL) in the presence of anisole (1 g, 10 mmol) at 0 °C for 20 min gave 160 mg of crude peptide, which was separated into two fractions of K values of 0.27 and 0.47 by countercurrent distribution in 1-butanol–acetic acid–water (4:1:5) for 200 passages. Removal of the solvent followed by lyophilization resulted in 10.6 mg of peptide of $K = 0.27$ and 17.8 mg of peptide of $K = 0.47$. Further purification of the respective peptides by gel chromatography on Sephadex G25-120 (2 × 100 cm) in 1% AcOH gave 5 mg of the L diastereomer (**7**, $K = 0.27$) and 13 mg of the D diastereomer (**8**, $K = 0.47$). Amino acid analysis of a hydrolysate gave Asp (0.99), Arg (1.00), Val (0.96), Tyr (1.00), Hfv (0.98), His (1.09), Pro (1.20), and Phe (1.00) for compound **7** [R_f (iii) 0.26, R_f (iv) 0.49] and Asp (1.05), Arg (0.97), Val (1.00), Tyr (1.03), Hfv (1.07), His (0.98), Pro (1.10), and Phe (1.02) for compound **8** [R_f (iii) 0.27, R_f (iv) 0.57]. In the rat uterus assay, compound **7** was 133% as active as angiotensin II, while compound **8** had less than 1% activity.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro (9) was prepared by standard solid-phase procedure, followed by sequential deprotection of the Ac-Asn-Arg(Tos)-Val-Tyr(Bzl)-Val-His(Tos)-Pro-resin with HBr/TFA and liquid HF. Purification of the peptide by countercurrent distribution in 8:1:2:9 of 1-butanol–pyridine–acetic acid–water for 1200 passages, followed by gel filtation of the appropriate fraction ($K = 0.38$) on Sephadex G25-120 (2 × 100 cm) in 10% AcOH, gave the peptide **9** in 36% yield, R_f (v) 0.17. Amino acid analysis of a hydrolysate gave Asp (1.10), Arg (0.99), Val (1.03), Tyr (0.92), Val (1.03), His (1.01), and Pro (0.92).

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-DL-Hfv-OEt (10) was prepared from the heptapeptide **9** (100 mg, 0.12 mmol) and DL-Hfv-OEt (**2**; 63 mg, 0.25 mmol) by DCC (51 mg, 0.25 mmol) coupling in the presence of HOBt (19 mg, 0.12 mmol) in 5 mL of redistilled dimethylformamide (DMF) at 60 °C for 20 h. The mixture was evaporated to dryness in vacuo, and the residue was washed with chloroform, ethyl acetate, and ether and chromatographed on a vacuum-packed dry column (1 × 12 cm) of silica gel (CC-7, neutral, 100–200 μm, from Taylor Chemical Co., St. Louis, MO), which was prewashed with the eluting solvent and dried in vacuo at room temperature overnight. The dry column was eluted with the upper phase of the solvent mixture of 1-butanol–pyridine–acetic acid–water (8:1:2:9) at 12 mL/h, and 2-min fractions were collected and analyzed by TLC. The appropriate fractions (8 to 22 mL) were combined, evaporated to dryness, and precipitated from elution solvent–dioxane–ether (1:1:40) to give 55 mg. Further purification by ion-exchange chromatography on CM-Sephadex C-25-120 (2 × 100 cm) eluted with 0.5 M ammonium acetate in 10% acetic acid gave 45 mg (39%) of peptide **10**, R_f (v) 0.32. Amino acid analysis of a hydrolysate gave Asp (1.21), Arg (1.03), Val (0.97), Tyr (0.98), Val (0.97), His (0.90), Pro (0.97), and Hfv (0.93).

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-DL-Hfv-OBzl (11) was prepared from **9** (100 mg, 0.12 mmol) and DL-Hfv-OBzl·HCl (**5**; 88 mg, 0.25 mmol) by DCC/HOBt coupling in DMF at room temperature for 3 h and at 60 °C for 3 h. The mixture was evaporated to dryness in vacuo, and the residue was washed with chloroform, ethyl acetate, and ether and chromatographed on a vacuum-packed dry column (1 × 20 cm) of silica gel as described for **10**. Isolation of the peptide, followed by precipitation from dimethylformamide–ether, gave 75 mg (61%) of **11**, R_f (v) 0.25. Amino acid analysis of a hydrolysate gave Asp (1.13), Arg (1.08), Val (0.93), Tyr (1.01), Val (0.93), His (0.92), Pro (1.10), and Hfv (0.95).

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-DL-Hfv (12) was prepared by catalytic hydrogenation of peptide **11** (69 mg) in DMF (5 mL) with 10% Pd/C under 1 atm of H_2 for 2 h. The mixture was evaporated to dryness in vacuo, and the residue including the Pd catalyst was chromatographed on a vacuum-packed dry column (1 × 20 cm) of silica gel as described for **10**. Isolation of the peptide, followed by precipitation from dimethylformamide–ether, gave 54 mg (85%) of **12**, R_f (v) 0.10. Amino acid analysis of a hydrolysate gave Asp (1.14), Arg (1.02), Val (1.00), Tyr (0.97), Val (1.00), His (0.80), Pro (1.02), and Hfv (0.92).

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Phencyclidine Metabolism: Resolution, Structure, and Biological Activity of the Isomers of the Hydroxy Metabolite, 4-Phenyl-4-(1-piperidinyl)cyclohexanol

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One of the major biotransformation pathways in the metabolism of phencyclidine is hydroxylation at C-4 of the cyclohexane ring to give 4-phenyl-4-(1-piperidinyl)cyclohexanol (1). Since the latter compound can exist as cis and trans isomers and the synthetic mixture has been reported to be biologically active, it was of interest to separate the isomers, test them for biological activity, and determine their ratio as metabolic products of phencyclidine. The synthetic mixture of 1 was separated by TLC and the individual isomers were characterized by ^{13}C and ^1H NMR and MS analyses. Preliminary testing of the isomers in the mouse rotarod assay indicates that the trans isomer (1b) is only slightly more active then the cis isomer (1a). Both isomers produced seizure activity and lethality at doses required to produce maximal ataxia.

Essential to the therapeutic management of drug overdosage is an understanding of the factors involved in the distribution and elimination of the active drug species. Metabolism may be an important consideration, particularly if the biotransformation products are active. The metabolites of 1-(1-phenylcyclohexyl)piperidine (phency-