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Angiotensin II Analogs. 11.[1] Further Studies on Des-aspartyl-angiotensin II †

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Replacement of the amino terminal arginine residue in the heptapeptide, des-Asp[1]-[Ile[5]]angiotensin II, produced analogs with pressor activities in the rat (compared to [Asn,[1]Val[5]]angiotensin II = 100%) as follows: acetyl, 3%; propionyl, 2%; L-Ala, 3%; D-Ala, 48%; valeryl, 3%; L-Nva, 1%; D-Nva, 13%. Des-Asp[1]-[D-Ala[2]]angiotensin II is the most potent heptapeptide analog of angiotensin II yet described. Quantitative values related to pressor potency were assigned to individual characteristics of the residues used to acylate the hexapeptide, Val-Tyr-Ile-His-Pro-Phe. The most important features for enhancement of pressor potency were (a) formation of a new amide bond, (b) (+)-charged ionic character, (c) minimal nonpolar character, and (d) resistance to aminopeptidase metabolism.

The angiotensin II C-terminal hexapeptide sequence, Val-Tyr-Ile(or Val)-His-Pro-Phe, contains the minimal structural features for significant pressor and myotropic activity.[3-5] The low activity of the hexapeptide (0.3%) is greatly enhanced by addition of the Arg residue (15–50%) to the amino terminus. Further addition of the Asp residue, or a variety of its structural and optical isomers and polar analogs, further enhances activity.[6] Asp-Arg is the amino terminus of the natural octapeptide. The present study focuses on the structural features of the Arg residue which contribute to its 100-fold enhancement of activity of the primary active hexapeptide sequence.

Analogs of Arg-Val-Tyr-Ile-His-Pro-Phe containing a D-amino acid at the N terminus have been shown to be more active in elevating blood pressure than those containing an L-amino acid in this position.[7,8] On the basis of comparisons with a limited number of des-amino analogs, it was initially concluded that the N-terminal L-α-amino group was exerting an unfavorable effect which was less pronounced with the D-α-amino group.[8] Working on the assumption that in the D- and des-amino series, the observed effects were due to the functional group in the side chain, Havinga and Schattenkerk[8] concluded that the potentiating effect of the guanido group is due to its capacity for hydrogen donation in hydrogen bond formation and not primarily to its positive charge.

More extensive studies on des-amino heptapeptides have disproved the assumption that the L-α-amino group acts to decrease the activity which would otherwise result from the effects of the side-chain functional groups.[4,9] Furthermore, the finding that the replacement of Arg in the heptapeptide by D-α-aminobutyric acid, a compound devoid of side-chain functional groups, gave an analog with high pressor activity (23%) compared with its L isomer or des-amino analog (each 1%), showed that the D-α-amino group was capable of exerting a profoundly favorable effect.[4] When glycine was used as a replacement for arginine, activity was somewhat lower (10%).[4] While its shorter duration of action suggested that this lower activity might be due to its more rapid destruction, it was of interest to investigate the influence of the hydrocarbon side chain. To this end, analogs have been prepared containing hydrogen, methyl, or *n*-propyl side chains in place of the ethyl side chain contributed by butyric and D- and L-α-aminobutyric acids. Thus, the argininyl residue of des-Asp[1]-[Ile[5]]angiotensin II has been replaced by acetyl, L-alanyl, D-alanyl, propionyl, L-norvalyl, D-norvalyl, and valeryl moieties.

Chemistry. The peptides were synthesized by the solid-phase method of Merrifield[10] as described previously.[11] Histidine was protected by the dinitrophenyl group[12] rather than the benzyl group which was previously employed since the former entails considerably less risk of racemization.[13] Purification was accomplished by ion-exchange chromatography on microcrystalline carboxymethylcellulose or by countercurrent distribution. The des-amino analogs were prepared by acylation of the purified hexapeptide with the corresponding fatty acid *p*-nitrophenyl esters.

Bioassay. The compounds were tested for pressor activity in nephrectomized, pentolinium treated male rats anesthetized with pentobarbital.[14,15] The peptides were dissolved in normal saline containing 0.1% polyvinylpyrrolidone in siliconized glass containers. The molarities of the peptide solutions, including the angiotensin standard, were calculated from amino acid analyses. Pressor activities calculated on a molar basis for the compounds from the present study and for related analogs[4,9] are listed in Table I. Estimates of duration of response were obtained by measuring the width of blood pressure peaks at half-height for equipressor doses of test compound and of angiotensin standard.

Discussion

As can be seen from Table I (α-alkyl, α-$^+NH_3$ acyl residues), the pressor activity of the D-α-aminobutyryl heptapeptide (D-Abu, 23%) has been halved by extending the side chain (D-Nva, 13%) and doubled by shortening the side chain (D-Ala, 48%). The corresponding L-α-amino acids and des-α-$^+NH_3$ acyl analogs (Table I, acyl residues) also generally fit a regular pattern of reduced activity with extension of the aliphatic side chain. The roles of such structural features of the residues replacing arginine in des-Asp[1]-[Arg,[2]Ile[5]]angiotensin II may be segregated and identified by assigning and testing quantitative values for each. In Table II, numerical values are assigned

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Table I. Pressor Activities of Heptapeptide Analogs of Angiotensin II in the Rat

R—Val—Tyr—Ile—His—Pro—Phe		Pressor activity[a]		
	R	Calcd[b]	Found	Duration[a]
Acyl residues	Acetyl[c]	3	3	55
	Propionyl[c]	1.5	2	60
	Butyryl[d]	0.75	1	70
	Valeryl[c]	0.4	3	55
α-Alkyl, α-$^+NH_3$ acyl residues	Gly[d]	9	10	35
	L-Ala[c]	2.2	3	20
	D-Ala[c]	45	48	80
	L-Abu[d]	1.1	1	30
	D-Abu[d]	22.5	23	100
	L-Nva[c]	0.6	1	15
	D-Nva[c]	11	13	100
	L-Leu[e]	0.3	0.4	
	D-Leu[e]	5.6	3	
α,ω-$^+NR_3$ acyl residues	L-Orn[e]	4	3	
	D-Orn[e]	22.5	23	
	L-Lys[e]	2	3	
	D-Lys[e]	11	23	
	L-Hly[e]	1	2	
	D-Hly[e]	6	7	
	L-Ath[e]	0.3	0.2	
Des-α-$^+NH_3$, ω-$^+NR_3$ acyl residues	δ-Avl	7.5	7,[d] 6[e]	100[d]
	ε-Ahx	4	5,[d] 4[e]	90[d]
	ε-Thx[e]	0.5	1.5	
ω-Guanidino acyl residues	L-Arg[e]	11	15	
	D-Arg[e]	38	33	
	δ-Gvl[e]	38	38	
	L-Har[e]	6	2	
	D-Har[e]	19	11	
Neutral polar acyl residues, α-$^+NH_3$	L-Cit[e]	0.6	0.5	
	D-Cit[e]	11	11	
	L-Arg(NO$_2$)[e]	0.6	1	
	D-Arg(NO$_2$)[e]	11	4	
Neutral polar acyl residues, des-α-$^+NH_3$	Ac-Gly[d]	1.5	1	100
	δ-Uvl[e]	0.4	1	
	δ-Nvl[e]	0.4	8	
	ω-Pmn[e]	0.4	0.7	
Acetylenic side-chain acyl residues, α,ω-$^+NR_3$	L-A$_2$hy[e]	15.8[f]	15	
	D-A$_2$hy[e]	7.9[f]	8	
	L-Adh[e]	3.9[f]	4	
	D-Adh[e]	2[f]	2	

[a] Relative to [Asn^1, Val^5]angiotensin II = 100. [b] Calculated using the parameters of Table II, see text. [c] Present investigation. [d] Reference 4. [e] Reference 9. [f] -C≡C- considered equivalent to $-CH_2-$ (×0.5); L-α-$^+NH_3$ (×0.7), D-α-$^+NH_3$ (×0.35). The normal parameters of Table II for the α-asymmetric center give calculated values of L-A$_2$hy, 4.5%; D-A$_2$hy, 22.5%; L-Adh, 1.1%; D-Adh, 5.6%. See Discussion section of text.

Table II. Relative Contributions to Pressor Activity by Structural Features of Heptapeptide Analogs of Angiotensin II

R–(CH$_2$)$_n$–($^+NH_3$)CH–CO–Val-Tyr-Ile-His-Pro-Phe

e d c_1 c_2 b a

Structural features	Contribution to pressor activity
(a) Primary active hexapeptide sequence	0.3%
(b) Acetyl residue	×10
(c_1) α-$^+NH_3$, regardless of stereochemistry	×30
(c_2) α-D-Asymmetric center	×1
α-L-Asymmetric center	
Alkyl or nonionic polar group on side chain (*e.g.*, Ala, Cit)	×0.05
H side chain (Gly)	×0.10
ω-$^+NR_3$ (*e.g.*, Lys, Orn, Ath)	×0.20
ω-Guanidino (*e.g.*, Arg, Har)	×0.30
(d) *n* = no. of methylene or methyl groups in side chains, including methyl groups or methylene groups of R [*e.g.*, $-(CH_2)_4-{}^+N(CH_3)_3$, ×0.5[7]]	×0.5^n
(e) R = (+)-charged ionic groups at pH 7	
ω-$^+NR_3$, if there is also an α-$^+NH_3$ (*e.g.*, Lys)	×2
ω-$^+NR_3$, if there is no α-$^+NH_3$ (*e.g.*, δ-Avl)	×20
ω-Guanidino group, if there is also an α-$^+NH_3$ (*e.g.*, Arg)	×3.3
ω-Guanidino group, if there is no α-$^+NH_3$ (*e.g.*, δ-Gvl)	×100
ω-polar nonionic group with or without α-$^+NH_3$ (*e.g.*, Cit, δ-Uvl)	×1
Examples	
Des-Asp^1-[D-Orn^2]angiotensin II	
H_3N^+–(CH$_2$)$_3$–($^+NH_3$)CH–CO–Val-Try-Ile-His-Pro-Phe	
(2) (×0.5)^3 (×30) (×1) (×10) ×0.3% = 22.5%	
Des-Asp^1-[Cit^2]angiotensin II	
H_2NCONH–(CH$_2$)$_3$–($^+NH_3$)CH–CO–Val-Tyr-Ile-His-Pro-Phe	
(1) (×0.5)^3 (×30) (×0.05) (×10) ×0.3% = 0.56%	

to each structural feature present in all known heptapeptide analogs of angiotensin II. These values have been selected to give the best fit with reported pressor activities. Values calculated using the parameters of Table II, reported pressor activities, and relative duration of response values are presented in Table I.

The basal hexapeptide sequence, $^+H_2$-Val-Tyr-Ile-His-Pro-Phe-O$^-$ has its 0.3% activity enhanced tenfold by acetylation (acetyl, 3%). This does not separate the relative contributions of the two features which were altered, loss of the (+) charge and addition of a new amide bond, but it appears likely that the primary activating step is the formation of the new peptide bond since loss of a (+) charge on the amino terminus generally results in decreased activity.

The addition of an α-$^+NH_3$ group to the acetyl residue results in a 30-fold enhancement of activity, which is further modified by the stereochemistry and nature of the side chain introduced. The α-D-residue, which is metabolically stable, permits full expression of this enhancement. Pressor activity is decreased by the α-L residue, the extent being dependent upon the nature of the side chain. Alkyl or nonionic polar side chains produce the greatest decrease in activity (×0.05), presumably due to their rapid metabolism, although a detrimental steric effect in the L series may also be involved. The metabolic effect is supported by the relative durations of pressor responses (Table I) measured for analogs with α-$^+NH_3$ acyl residues. The L-Ala, L-Abu, and L-Nva peptides show only 15–30% the duration of response of either the corresponding D analog or of angiotensin II. Calculated values consistent with reported pressor data are obtained if factors for slower metabolic rates (and possibly steric factors) are assigned for glycine (×0.10) ω-ammonium residues (×0.20) and the ω-guanidino residue (×0.30).

Further support for the importance of metabolic susceptibility at the amino terminus comes from data on the myotropic activities of heptapeptide analogs.[9] In the isolated rat terminal ileum where metabolic influences are minimal, activities of the D and L isomers are much closer to equal than they are in the intact animal pressor assay. In addition, the D isomer, which is less sensitive to its

metabolic environment, shows the same order of activity relative to angiotensin II in both the myotropic and pressor assays, while the L isomer invariably shows much higher myotropic than pressor potency.

Nonpolar methylene and methyl residues contribute an exponential progression of decrease in activity. Pressor activity is halved for each methyl or methylene group present in the side chain, whether these make up an alkyl side chain, form a connective chain to a functional group, or are present as substituents on the functional group. The D-Ala (48%), D-Abu (23%), D-Nva (13%) sequence clearly shows this trend, as does the addition of two methylene groups to D-Orn (23%) to form D-Hyl (7%), although the reported value for the intermediate D-Lys (23%) peptide is anomolous and may indicate a structural specificity for the location of the (+) charge in the lysine side chain.

A (+)-charged ammonium group on the side chain is favorable, the extent being dependent upon the presence (×2) or absence (×20) of an α-ammonium group in the residue. An ω-guanidino group generates maximal activation (×100), regardless of the presence or absence of an α-ammonium group. A polar nonionic side-chain group has neither an activating nor deactivating effect (×1) in the limited series studied, although the nitroarginyl analogs D-Arg(NO_2) and δ-Nvl are somewhat anomolous. The graded activating effects of the (+)-charged residues (ω-$^+NR_3$, ×20; α-$^+NH_3$, ×30; α-$^+NH_3$ and ω-$^+NR_3$, ×60; ω-guanido, ×100) show the major role of the (+) charge in the activity of heptapeptide angiotensin analogs. The importance of this effect was previously minimized,[8] due to the low activity of the L-α-amino-ε-trimethylammoniohexanoic acid residue (L-Ath, 0.2%), which can now be seen to be due to the deactivating effects of the L-asymmetric center (×0.2) and of the four methylene and three methyl groups in the side chain (×0.5[7]). The low activity of the corresponding des-α-$^+NH_3$ analog, ε-Thx (1.5%), is similarly explained by the deactivating effects of the nonpolar methyl and methylene groups.

With the exception of the D-Lys analog, activity correlates well when a strong activating effect is assigned to (+)-charged groups located at various distances from the amide bond. However, there are presently no examples of (+)-charged groups separated by 3 or 4 atoms from the valine nitrogen of the 3–8 hexapeptide sequence of angiotensin II. Such compounds are necessary to judge whether or not specific spatial requirements exist for the N-terminal (+) charges which strongly enhance the activity of the primary hexapeptide sequence. If there is a spatial importance for the α-$^+NH_3$ or for the (+) charge on the side chain of the N-terminal residue of 2–8 heptapeptide analogs of angiotensin II, this should be revealed by heptapeptide analogs acylated at the N terminus by short-chain α,ω-diamino acids, α-amino-ω-guanidino acids, and their des-α-amino analogs. Spatial importance, related to either intermolecular ("α and ω receptors") or intramolecular (conformational) interactions, would be seen by low activities for such analogs. If, however, activity is enhanced in a spatially noncritical manner by the general physicochemical characteristics imparted by (+) charge and minimal nonpolar character to the primary active hexapeptide sequence, activities approaching and exceeding that of angiotensin II should be found among such analogs.

Heptapeptide analogs of angiotensin II, containing in the N-terminal position an acetylenic group in the side chain as well as α- and ω-ammonium groups, show the unusual characteristic of the L isomer being more active than the D isomer[9] (Table I, A_2hy, Adh). This may be due to unique orientation of the more rigid side chain relative to the α-ammonium group. It may also be due to greater susceptibility to metabolism for the acetylenic D isomer. Assignment of appropriate factors, 0.7 for the L- and 0.35 for the D-asymmetric center, results in excellent agreement of experimental and calculated pressor data but does not identify the mechanism involved.

In addition to the contributions to polarity and resistance to metabolism identified for the amino terminus of heptapeptides in the present study, the N-terminal Asp-Arg sequence of angiotensin II must possess a high steric requirement, as indicated by octapeptides studied[5] with a D-amino acid in the 2 position. While the heptapeptide, des-Asp[1]-[D-Arg[2]]angiotensin II, is highly active (33%), the octapeptide Asn[1]-[D-Arg[2]]angiotensin II is only one-fifth as active (6%). In contrast, conversion of the less active des-Asp[1]-[L-Arg[2]]angiotensin (15%) into the parent octapeptide, angiotensin II, leads to a sevenfold increase in activity, although both octapeptides should possess approximately the same polarity. If metabolic stability is presumed to be similar, it appears that the D-Arg[2] residue in the octapeptide must impose an unfavorable steric constraint, which may be related to a receptor interaction or to the active conformation of the essential C-terminal hexapeptide.

Experimental Section

Amino acid analyses (Spinco Model 116 analyzer) were obtained using the standard 4-hr methodology. Peptides were hydrolyzed for 48 hr under N_2 in constant boiling HCl containing 1% PhOH as a scavenger and Ala or Asp as an internal standard. Peptide content was calculated as free peptide although all are salts and possibly solvates. Precoated silica gel G plates (E. Merck) were used for tlc with the following systems: I, *n*-BuOH–AcOH–H_2O (3:1:1); II, *sec*-BuOH–3% NH_4OH (100:44); III, *n*-BuOH–AcOH–H_2O (4:1:5); IV, C_5H_5N–H_2O (4:1). CCD was carried out in an automatic 200 tube E-C Apparatus machine using the solvent system, *n*-BuOH–*t*-BuOH–H_2O (5:2:5), 10 ml of each phase. The number of transfers was limited to that necessary to produce sufficient homogeneous peptide for further studies. In the interest of solvent economy, no attempt was made to maximize the yield in purification. Peptides were located on tlc and in CCD fractions by the Pauly reaction. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter.

Val-Tyr(Bzl)-Ile-His(Dnp)-Pro-Phe-polymer Trifluoroacetate (1). This intermediate was prepared from 14 g (3.4 mmol) of Boc-Phe-polymer and 13.6-mmol portions of the appropriate Boc-amino acids [Boc-Pro, Boc-His(Dnp), Boc-Ile, Boc-Tyr(Bzl), and Boc-Val] and DCC as described previously.[1] The product was dried *in vacuo* (wt 16.5 g) and stored at −20°.

Ala-Val-Tyr-Ile-His-Pro-Phe (2). A 1.5-g (0.3 mmol) portion of 1 was neutralized and acylated in 6 ml of CH_2Cl_2 with 0.23 g (1.2 mmol) of Boc-Ala and 0.25 g (1.2 mmol) of DCC. After 24 hr, the polymer was washed with CH_2Cl_2 (3 × 8 ml) and then shaken for 1 hr with 0.5 ml of Ac_2O and 0.5 ml of Et_3N in 6 ml of CH_2Cl_2. Following washes with DMF (3 × 8 ml), the polymer was shaken for 48 hr with 2 ml of $HSCH_2CH_2OH$ in 6 ml of DMF. After washing with DMF (6 × 8 ml) and CH_2Cl_2 (3 × 8 ml), the peptide was cleaved from the polymer with HBr–CF_3COOH as previously described,[1] giving 295 mg of yellow powder.

A solution of 45 mg of the crude peptide in 10 ml of 0.01 *M* NH_4Ac was applied to a 2.5 × 93 cm bed of carboxymethylcellulose ($^+NH_4$ form, Whatman CM-52) packed in the same buffer (low solubility limited the amount of material which could be handled). The column was eluted at 50 ml/hr with a linearly increasing concentration of NH_4Ac ($dc/dV = 10^{-4}$ *M*/ml). The effluent was monitored at 254 and 280 mμ and chromatographically homogeneous fractions from the main peak were pooled and lyophilized giving 13 mg of white powder: R_f(I) 0.54, R_f(II) 0.39, R_f(III) 0.33. An acid hydrolysate had Ala 1.01, Val 1.01, Tyr 0.91, Ile 0.99, His 0.99, Pro 1.00, Phe 1.03, peptide content 70%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Ala 0.05, Val 0.02, Tyr 0.05, *a*Ile 0.02, His 0.10, Pro 1.00, Phe 0.05;‡ $[\alpha]^{23}D$ −41.9° (*c* 0.5, 1 *N* AcOH).

D-Ala-Val-Tyr-Ile-His-Pro-Phe (3). This was synthesized as for 2 giving 280 mg of deep yellow powder. Purification by CCD

‡ A mixture of amino acids subjected to the same hydrolytic and enzymatic procedures had Ala 0.02, Nva 0.02, Val 0.02, Tyr 0.04, *a*Ile 0.02, His 0.08, Pro 1.00, Phe 0.04.

through 300 transfers (K = 0.28) gave 63 mg of chromatographically homogeneous white powder: R_f(I) 0.55, R_f(II) 0.37, R_f(III) 0.34. An acid hydrolysate had Ala 1.01, Val 1.03, Tyr 1.04, Ile 0.95, His 0.98, Pro 0.99, Phe 0.98, peptide content 76%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Ala 1.13, Val 0.02, Tyr 0.05, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −55.6° (c 1, 1 N AcOH).

Nva-Val-Tyr-Ile-His-Pro-Phe (4). This was synthesized as for **2** giving 280 mg of deep yellow powder. Purification by CCD through 200 transfers (K = 0.43) gave 100 mg of chromatographically homogeneous white powder: R_f(I) 0.58, R_f(II) 0.41, R_f(III) 0.38. An acid hydrolysate had Nva 0.97, Val 1.01, Tyr 1.04, Ile 0.97, His 0.99, Pro 0.99, Phe 1.01, peptide content 77%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Nva 0.04, Val 0.02, Tyr 0.05, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −46.1° (c 1, 1 N AcOH).

D-Nva-Val-Tyr-Ile-His-Pro-Phe (5). This was synthesized as for **2**, giving 185 mg of yellow powder, and purified as for **4** through 200 transfers (K = 0.42) giving 33 mg of chromatographically homogeneous white powder: R_f(I) 0.60, R_f(II) 0.41, R_f(III) 0.40. An acid hydrolysate had Nva 0.96, Val 1.04, Tyr 1.04, Ile 0.99, His 0.98, Pro 0.99, Phe 1.02, peptide content 72%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Nva 0.96, Val 0.02, Tyr 0.04, aIle 0.01, His 0.05, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −65.3° (c 1, 1 N AcOH).

Val-Tyr-Ile-His-Pro-Phe (6). A 5.0-g (1.0 mmol) portion of **1** was subjected to thiolysis by 6 ml of $HSCH_2CH_2OH$ in 18 ml of DMF; then the peptide was cleaved from the polymer as for **2** giving 600 mg of yellow powder. Purification by CCD through 500 transfers (K = 0.23) gave 240 mg of chromatographically homogeneous off-white powder: R_f(I) 0.55, R_f(II) 0.39, R_f(III) 0.35, R_f(IV) 0.65. An acid hydrolysate had Val 1.00, Tyr 1.04, Ile 0.97, His 0.99, Pro 1.02, Phe 0.98, peptide content 73%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.04, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −31.8° (c 0.4, 1 N AcOH).

Propionyl-Val-Tyr-Ile-His-Pro-Phe (7). To a solution of 25 mg (0.025 mmol) of **6** in 1.7 ml of purified DMF[16] were added 0.011 ml (0.15 mmol) of Et_3N and 10.3 mg (0.053 mmol) of p-nitrophenyl propionate.[17] After 24 hr at 25°, the peptide **7** was precipitated by the addition of 17 ml of anhydrous Et_2O. The precipitate was collected by centrifugation, washed with 17 ml of Et_2O, and lyophilized from 1 N AcOH giving 23 mg of chromatographically homogeneous white powder: R_f(I) 0.64, R_f(II) 0.43, R_f(III) 0.54, R_f(IV) 0.69. An acid hydrolysate had Val 1.02, Tyr 1.00, Ile 0.97, His 0.98, Pro 1.00, Phe 1.00, peptide content 61%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.05, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −48.3° (c 1, 1 N AcOH).

Valeryl-Val-Tyr-Ile-His-Pro-Phe (8). This was prepared in the same manner as **7** using p-nitrophenyl valerate.[18] There was obtained 22 mg of chromatographically homogeneous white powder: R_f(I) 0.68, R_f(II) 0.49, R_f(III) 0.60, R_f(IV) 0.69. An acid hydrolysate had Val 1.04, Tyr 1.00, Ile 0.98, His 0.98, Pro 1.04, Phe 1.03, peptide content 57%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.04, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −42.6° (c 0.8, 4 N AcOH).

Ac-Val-Tyr-Ile-His-Pro-Phe (9). This was prepared in the same manner as **7** using p-nitrophenyl acetate: yield, 24 mg of chromatographically homogeneous white powder; R_f(I) 0.62, R_f(II) 0.38, R_f(III) 0.52, R_f(IV) 0.60. An acid hydrolysate had Val 1.03, Tyr 1.01, Ile 1.01, His 0.99, Pro 0.96, Phe 0.97, peptide content 59%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.04, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −54.1° (c 0.5, 1 N AcOH).

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Folic Acid Antagonists. Methotrexate Analogs Containing Spurious Amino Acids. Dichlorohomofolic Acid†

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The synthesis and some biological testing results are reported for dichlorohomofolic acid (**2a**) and three methotrexate analogs in which the L-glutamic acid moiety has been replaced by D-glutamic acid, glutaric acid, and L-lysine to give **4b**, **4c**, and **4d**, respectively. Compounds **4b–d** were all like MTX in being strong inhibitors of dihydrofolate reductase, but only **4b** demonstrated activity against leukemia L1210 in the mouse.

In connection with our cancer chemotherapy research, we have long been interested in analogs of folic acid (**1**).[1] We now report the synthesis and some biological evaluations of 3′,5′-dichlorohomofolic acid (**2a**) and some methotrexate (MTX, **4a**)[2] analogs, **4b–d**.

Both MTX (**4a**) and its dichloro derivative are active antitumor agents,[3] with the latter being less toxic. Since homofolic acid (**2b**) in the tetrahydro form[4] has extremely interesting biological properties,[4,5] it seemed worthwhile to prepare the dichloro derivative **2a** for testing.

The MTX analog in which the glutamic acid moiety is replaced by aspartic acid[6] has shown antitumor activity against advanced lymphoid leukemia L1210.[3] This and the fact that natural folate derivatives may have various numbers of glutamate residues[7] suggest that MTX ana-

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