142. Synthesis of β-Pyrazinyl-L-alanine (Paa)[1]) and of Peptide Derivatives

by **Claudia Petermann** and **Jean-Luc Fauchère**

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, CH-8093 Zürich

(11.V.83)

Summary

The title compound was prepared *via* a malonic ester synthesis starting with α-chloromethylpyrazine [2], and ending, after an asymmetric enzymatic hydrolysis of the racemic *N*-acetyl-β-2-pyrazinylalanine to the L-form of the new amino acid. The optical purity was ascertained by ^{1}H-NMR analysis at 360 MHz of the diastereoisomeric dipeptides L-pyrazinylalanine-L-leucine and D-pyrazinylalanine-L-leucine. Hydrophobic, steric and electronic parameters for its side chain were also estimated, which can be useful for the quantitative study of structure-activity relationships of biologically active peptide derivatives. The new amino acid could be introduced in the place of phenylalanine in the enkephalin-like pentapeptide [D-alanyl^2, leucine^5]enkephalin, thus showing good stability towards the classical methods of peptide synthesis.

The folding of a biologically active peptide is controlled by the structural features of the amino acid side chains, and chemical modifications of individual side chains thus modulate its biological activity. It is therefore desirable to have selected series of residues where only one of the electronic, steric or hydrophobic properties would vary, while the others would remain approximately constant. Such series would be extremely useful for quantitative structure-activity relationships (QSAR) studies, helping to vary each structural parameter independently and to select the optimized combination of them. However, at present such series can only be put together from the known list of natural or synthetic amino acids and must therefore be completed by the synthesis of new representatives.

The purpose of this work was to prepare an amino acid which would be comparable in size to phenylalanine but would display extreme electronic features. We chose β-pyrazinyl-L-alanine (**3**, abbreviated as Paa) because of the strong inductive electron-withdrawing effect of the pyrazinyl group. While the parent

[1]) Abbreviations for amino acids and protected derivatives according to the IUPAC-IUB recommendations [12]. Further abbreviations: Paa = β-pyrazinyl-L-alanine, DMF = *N*, *N'*-dimethylformamide, DCHA = dicyclohexylamine, TFA = trifluoroacetic acid, TLC = thin layer chromatography, r.t. = room temperature.

compound β-(2-pyridyl)-L-alanine (Pya) has already been described [1], **3** is a new amino acid. We describe in the following the synthesis of the free L-enantiomer **3**, of its *tert*-butyloxycarbonyl derivative and of the dipeptide L-pyrazinylalanyl-L-leucine. For use in QSAR studies, we estimated three structural parameters which characterize the side chain of **3**.

Synthesis. Starting material was α-chloromethylpyrazine obtained according to *Hirschberg & Spoerri* [2] by treatment of 2-methylpyrazine with one equivalent of *N*-chlorosuccinimide using benzoyl peroxide as catalyst. This highly unstable lacrymatory oily intermediate was not characterized but immediately condensed with the sodium salt of diethyl-α-acetamidomalonate to yield crystalline diethyl α-acetamido-α-(pyrazinylmethyl)malonate (**1**). This was converted by hydrolysis and decarboxylation into the racemic D,L-*N*-acetyl-β-(pyrazinyl)alanine (**2**). Stereospecific hydrolysis of **2** with porcine kidney acylase I afforded β-pyrazinyl-L-alanine (**3**). Protection of the N^{α}-function and coupling of the amino acid was achieved by the usual methods of peptide synthesis [3] and resulted in moderate-to-high yields of products, thus tending to show that pyrazinylalanine can be introduced into polypeptide chains without major problems. The enkephalin-like pentapeptide Tyr-D-Ala-Gly-Paa-Leu (**8**) was prepared *(cf. Scheme)* in which the new amino acid replaced natural phenylalanine in position 4. Analytical and physical data of **3**, its precursors and its peptide derivatives are given in the *Table.*

Optical Purity. Although the enzymatic hydrolysis of *N*-acetylated amino acids by acylase I is found to be stereospecific for the large number of substrates used

Scheme. *Synthesis of β-Pyrazinyl-L-alanine (Paa) and its incorporation into a leucine-enkephalin containing* D-*alanine (ala) in position 2*

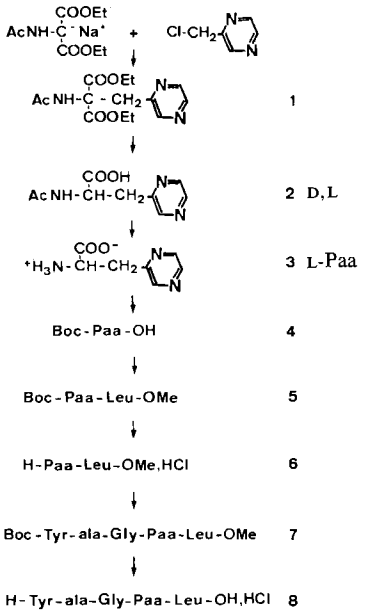

Table. *Physical and Analytical Data of β-Pyrazinyl-L-alanine, its Precursors and Derivatives*

Compound	$[\alpha]_D^{23}$ [a] ($c = 1$, MeOH)	M.p. [b]	R_f (TLC) [c]			Elemental analysis (calc./found)		
			B/A/W 72:7:21	C/M/A 95:5:3	I/P/W 36:32:32	C	H	N
1	-	110°	0.62	0.57		54.19/54.34	6.50/6.36	13.54/13.54
2	-	189°	0.12		0.62	64.58/64.59	8.77/8.90	14.34/14.30
3	−18.0 [d]) +23.5 [e])	229°	0.06		0.47	50.30/50.10	5.43/5.44	25.14/24.98
4	−16.8°	75°	0.40	0.19	0.62	53.92/53.68	6.41/6.43	15.72/15.84
5	−32.4	80°	0.65	0.57		57.85/57.80	7.67/7.69	14.20/14.09
8	8.3	154°	0.26		0.68	53.33/53.21	6.30/6.32	16.12/15.97 [f])

[a]) Specific rotation. [b]) M.p. uncorrected. [c]) R_f-values in the solvent systems BuOH/HOAc/H_2O (B/A/W), $CHCl_3$/MeOH/HOAc (C/M/A) and i-PrOH/pyridine/H_2O (I/P/W). [d]) In H_2O. [e]) In 5N HCl. [f]) Cl 5.83/5.65.

to date [4], the optical purity of the free amino acid Paa was checked by an independent method. As the dipeptide Paa-Leu was needed for the incorporation of Paa into leucine-enkephalin, we prepared the diastereoisomeric protected dipeptides Boc-L-Paa-L-Leu-OMe (**5**) and Boc-D-Paa-L-Leu-OMe (**5′**) and compared their ^{1}H-NMR spectra to detect any contamination by D-Paa in **5**. While **5** was prepared as a stereochemically pure compound, the second reference was obtained as the mixture of **5** and **5′** in the following way: racemic **2** was subjected to an acidic hydrolysis in conc. HCl at 80° for 6 h followed by the introduction of the Boc-group (→**4**) and the coupling to methyl-L-leucinate. ^{1}H-NMR spectra were then recorded separately for compound **5** and for two different mixtures of the diastereoisomers **5** and **5′** (the *Figure*). No major problems were encountered for the assignment of the signals. As expected, the chemical shifts of several protons from the same groups in each of the two diastereoisomers were significantly different. Two such clear-cut cases were the signals at $\delta = 3.64$ and 3.65 ppm of the methyl-ester protons, and the signals at $\delta = 6.90$ and 7.11 ppm of the amide proton of leucine, respectively. These two pairs of singlets were used for the estimation of the relative amounts of L- and D-Paa in the diastereoisomeric mixtures. The proportion of L/D was found to be 0.31 ± 0.01 and 0.72 ± 0.01 in the mixtures corresponding to spectrum B and C, respectively. No trace of the D-Paa could be detected in spectrum A (compound **5**), while the excellent resolution obtained at 360 MHz would reveal easily the presence of less than 1% contaminant. We therefore conclude that the optical purity of the free amino acid **3** was at least 99% and that no detectable racemization occurred in the two steps leading to the protected dipeptide **5**.

Structural Parameters. An electronic, a steric and a hydrophobic substituent constant were determined as descriptors of the properties of the side chain of Paa. Following the approach described previously [5] for other amino acids with a ring structure in the side chain, we used for the calculation of an *electronic* parameter S the equation $S = pK_a(\text{R-COOH}) - pK_a(\text{HCOOH})$, in which R is the substituent on C(β) of alanine. Given the literature values for the pK_a of pyrazinecarboxylic

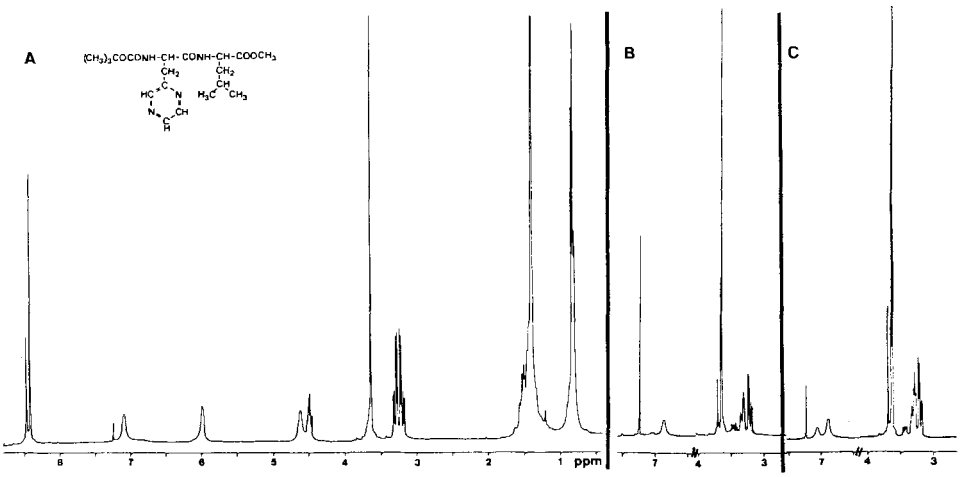

Figure. **A.** *Chemical shifts at 360 MHz of* **5** *(see formula) in $CHCl_3$ at 25° with the residual peak of the solvent proton* ($\delta = 7.24$ ppm from TMS) *taken as reference* (Decoupling experiments led to the following assignments: (δ): 0.90 (two overlapping *d*, $i = 3.0$ (3), two non-equivalent $C^{\delta}H_3$(Leu)); 1.45–1.60 (unresolved *m*, $i = 12.6$ (12), $C(CH_3)_3$ (Boc) and $C^{\beta}H_2$–$C^{\gamma}H$(Leu)); 3.28 (*AB*-part of an *ABMX*-system, $i = 1.85$ (2), two non-equivalent $C^{\beta}H_2$(Paa)); 3.68 (*s*, $i = 2.87$ (3), $COOCH_3$); 4.52 (double *t*, $i = 1.0$ (1), $C^{\alpha}H$(Leu)); 4.70 (br. *s*, $i = 1.05$ (1), $C^{\alpha}H$(Paa)); 6.02 (*d*, $i = 0.98$ (1), NH(Paa)); 7.08 (br. *s*, $i = 1.06$ (1), NH(Leu)); 8.45 (*s*, $i = 2.13$ (2), *ar*-5-CH and 6-CH); 8.53 (*s*, $i = 0.94$ (1), *ar*-3-CH(Paa)). **B** and **C**. *Selected regions of the spectra of two mixtures of the* L-L *and* D-L *diastereoisomers* (Abbreviations: *s* = singlet, *d* = doublet, *t* = triplet, *i* = normalized integral expressed in proton units with the theoretical value in parentheses. The signal at $\delta = 3.70$ in spectra **B** and **C** is from an unidentified impurity)

acid (2.92) [6], and formic acid (3.77), $S = -0.85$. A *steric* parameter v for the side chain of Paa was obtained from the *van der Waals* volume, which could be measured directly on the *CPK* space-filling molecular models (*Ealing Scientific Ltd.*, Cambridge, MA). The normalized value (*cf.* [5]) was 5.32. A *hydrophobic* parameter π was estimated from the value of the partition coefficient P of the free amino acid in the system octanol/H_2O at pH 7. P was obtained by means of a ninhydrin assay as previously described [7], and the value of the *Hansch* hydrophobicity parameter π *via* the equation $\pi = \log P(\text{pyrazinylalanine}) - \log P(\text{glycine}) = 0.42$, using the literature value $\log P(\text{glycine}) = -3.21$ [7]. Comparison with other amino acid shows that at least four side chains are characterized by more negative S-values than Paa. However, as histidine ($S = -1.80$) contains a five-atom ring, *p*-aminophenylalanine ($S = -1.48$) and (3,5-dinitrophenyl)alanine ($S = -0.94$) bear substituent(s) on the aromatic ring, and carboranylalanine ($S = -1.28$) is considerably more bulky, Paa appears to be a very unique probe when a position occupied by phenylalanine has to be tested for its sensitivity towards electronic factors. The difference in hydrophobicity ($\pi_{Phe} = 1.63$) and the possibility of Paa to form H-bonds, could however obscure the interpretation of the results.

The *opiate activity in vitro* of the enkephalin-like peptide **8** on the guinea-pig ileum ($IC_{50} = 4.45\ 10^{-6}$ M) and on the mouse vas deferens ($IC_{50} = 1.50\ 10^{-6}$ M) was found in preliminary measurements (Prof. Dr. *P. W. Schiller*, Clinical Research

Institute of Montreal) to be 18 and 130 times lower than for leucine-enkephalin, respectively, showing that in spite of its aromaticity, Paa cannot replace phenylalanine in position 4 without extensive loss of the biological activity.

This work was supported by the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* (grant 3.525–1.79 to Prof. Dr. *R. Schwyzer* and grant 3.327–0.82 to *J. L. F.*).

Experimental Part

TLC was carried out on *Merck* silica gel plates with fluorescence indicator, using the solvent systems indicated in the Table. Optical rotation was measured on a *Perkin-Elmer* polarimeter *141*. Elemental analyses were performed in the Laboratorium für Organische Chemie, ETHZ *(D. Manser)*. ^{1}H-NMR spectra were determined in $CDCl_3$ at 360 MHz on a *Bruker HXS* spectrometer (*A. Eugster* & Prof. Dr. *K. Wüthrich*). The opiate activity *in vitro* was measured in two classical bioassays on isolated tissues in the laboratory of Prof. Dr. *P. W. Schiller* (for methods *cf.* [8]).

Diethyl α-acetamido-α-(pyrazinylmethyl)malonate (**1**). To the solution of 9.51 g (0.1 mol) 2-methylpyrazine *(Fluka)* in 250 ml CCl_4 was added 13.6 g (0.1 mol) *N*-chlorosuccinimide and 0.1 g benzoyl peroxide. The mixture was refluxed for 16 h, then cooled at 0°. The solid material was eliminated by filtration and washed with CCl_4. The filtrate was evaporated at r.t. and the resulting oil (assumed yield 80% of 2-(chloromethyl)pyrazine) immediately reacted with the previously prepared Na-salt of diethyl α-acetamidomalonate (23.9 g, 0.1 mol) in 140 ml DMF for 16 h at 80°. After evaporation of the solvent, the solid residue was extracted with $CHCl_3$, the solvent exchanged against EtOH and the solution treated with activated charcoal. The partially decolored solution was concentrated and the product purified by chromatography on 250 g silica gel (column ∅2.5 × 80 cm) in $CHCl_3$. The pure fractions were evaporated and the residue recrystallized twice from EtOAc/diisopropyl ether. Yield 10.3 g.

D,L-N-*Acetyl-β-(pyrazinyl)alanine* (DCHA, **2**). In 70 ml 1N NaOH 6.2 g (20 mmol) **1** was dissolved and kept for 4 h at 80°. The solution was then acidified to pH 2 with 1N HCl under cooling. Upon reduction of the volume to 50 ml and addition of 100 ml isopropyl alcohol, the major part of the salt could be eliminated by filtration. The solution was then evaporated to dryness and the product precipitated from the solution in 50 ml i-PrOH by addition of 3.6 g (20 mmol) DCHA and dilution with diisopropyl ether. Yield 7.8 g (87%).

β-Pyrazinyl-L-*alanine* (Paa, **3**). The solution of 7.8 g (20 mmol) **2** in H_2O was neutralized to pH 7.5 with 1N HCl and treated with 10 mg renal hog acylase I *(Fluka)* previously dissolved in 2 ml H_2O. After 20 h at r.t., the pH was readjusted to 7.5 and a new portion of 10 mg acylase I (in 2 ml H_2O) added. The solution was kept for two days at r.t., then treated with 2 ml TFA and heated for 10 min. at 60° to denaturate the enzyme. Most of the solvent was then evaporated, a large amount of salt filtered off and the filtrate poured into 10 volumes of acetone. In addition to the free amino acid, the precipitate obtained was shown by TLC to contain a small proportion of its acetylated form as contaminant. However, recrystallization twice from H_2O/i-PrOH afforded pure **3**. Yield 940 mg (55% of L-enantiomer).

Boc-Paa-OH (**4**). In 15 ml dioxane/H_2O 2:1 835 mg (5 mmol) **3** was dissolved and treated with 5 ml 1N NaOH followed by 1.5 g (6.9 mmol) di-*tert*-butyl dicarbonate *(Fluka)* at 0° [9]. After 1 h reaction at 0° and 16 h at r.t., dioxane was evaporated and the residual aq. solution acidified to pH 2 and immediately extracted, at 0°, into ethyl acetate. Evaporation of the solvent, dissolution in $CHCl_3$ and slow precipitation by addition of CCl_4 produced a TLC-pure compound. Yield 1.25 g (78%).

Boc-Paa-Leu-OMe (**5**). This compound was prepared from **4** (802 mg, 3 mmol) and H-Leu-OMe · HCl (545 mg, 3 mmol, *Fluka*) in 25 ml DMF using *N*-ethylmorpholine (3 mmol), 1-hydroxybenzotriazole (3 mmol) and dicyclohexylcarbodiimide (3.3 mmol) following the usual isolation procedure [10]. Repeated crystallizations from Et_2O/petrolether allowed the isolation of 725 mg (61%) of a product which was free of dicyclohexylurea.

Boc-Tyr-D-*Ala-Gly-Paa-Leu-OMe* (**7**). In 1.5 mol-equiv. of 0.4N HCl in HCOOH 433.9 mg (1.1 mmol) **5** were dissolved and kept for 15 min. at r.t. The solvents were cautiously evaporated at 30° *in vacuo* and the product suspended in Et_2O and centrifuged three times. All these operations were performed in the same tube. The residue, which was shown by TLC (R_f 0.32 BuOH/HOAc/H_2O; 0.63

i-PrOH/pyridine/H_2O) to be freed of any starting material, was then directly coupled to 409 mg (1 mmol) of Boc-Tyr-D-Ala-Gly-OH [11] in 25 ml DMF using the same reaction conditions and additives and the same isolation procedure [10] as for **5**. Recrystallization from MeOH/Et_2O yielded a first fraction of 380 mg (42%) of pure **7**.

H-Tyr-D-Ala-Gly-Paa-Leu-OH · HCl (**8**). For deprotection, 369 mg (0.5 mmol) **7** was dissolved in 10 ml dioxane and treated by 15 ml of 0.2N KOH for 20 min. at r.t. The solution was then neutralized with 0.2N HCl under cooling and the solvents were evaporated *in vacuo* at 40°. Although it contained a large amount of salt, the residue was dissolved in 12 ml of 0.1N HCl in HCOOH and kept for 30 min. at r.t. The solvents were evaporated thoroughly at 30° and the solid residue suspended in i-PrOH, thus allowing the separation of the salts by filtration. The product was precipitated three times from i-PrOH by addition of diisopropyl ether, then further purified by chromatography on *Sephadex G15* (column ⌀2 × 40 cm) in H_2O. Lyophilization of the pure fractions yielded a final product (130 mg, 42%) which was homogenous on TLC.

REFERENCES

[1] *K. Hsieh & E. C. Jorgensen*, J. Med. Chem. *22*, 1199 (1979).
[2] *A. Hirschberg & P. E. Spoerri*, J. Org. Chem. *26*, 2356 (1961).
[3] *E. Gross & J. Meienhofer*, 'The Peptides', Vol. 1-3, Academic Press New York, 1981.
[4] *J. P. Greenstein & M. Winitz*, 'Chemistry of the Amino Acids', Vol. 1, p. 744, John Wiley & Sons Inc., New York, 1961.
[5] *J. L. Fauchère*, J. Med. Chem. *25*, 1428 (1982).
[6] The Merck Index *(M. Windholz, ed.)* 9th edition, Merck & Co. Inc., Rahway (USA), 1976, p. 1032.
[7] *J. L. Fauchère, K. Q. Do, P. Y. Jow & C. Hansch*, Experientia *36*, 1203 (1980).
[8] *J. DiMaio, T. M. Nguyen, C. Lemieux & P. W. Schiller*, J. Med. Chem. *25*, 1432 (1982).
[9] *L. Moroder, A. Hallett, E. Wünsch, O. Keller & G. Wersin*, z. Physiol. Chem. *357*, 1651 (1976).
[10] *W. König & R. Geiger*, Chem. Ber. *103*, 788 (1970).
[11] *R. Schwyzer, K. Q. Do, A. N. Eberle & J. L. Fauchère*, Helv. Chim. Acta *64*, 2078 (1981).
[12] *IUPAC-IUB Commission on Biochemical Nomenclature*, J. Biol. Chem. *247*, 977 (1972).