SYNTHESIS, CONFORMATIONAL FEATURES AND BIOLOGICAL ACTIVITY OF [Pro^3] ANTIARRHYTHMIC PEPTIDE*

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Synthesis of Gly-Pro-Pro-Gly-Ala-Gly([Pro^3]AAP) in solution has been carried out. Solvent dependent conformational distribution of [Pro^3]AAP has been studied using CD and NMR spectroscopy. The CD spectra in water, methanol and trifluoroethanol show predominance of the polyproline II-like structure. The CD curve of [Pro^3]AAP in aqueous solution suggests an absence of any intramolecular H-bonded conformation whereas in polar organic solvents like methanol and trifluoroethanol, a possible contribution from an intramolecularly H-bonded γ-turn around the Pro^3 residue has been proposed. Solvent titration experiments in methanol–water system using ^{13}C NMR suggest that all the carbonyls are exposed and are not involved in any ordered stucture. Further, the $\Delta\delta_{\beta\gamma}$ values for both the prolines corresponded to the dihedral angle of 150° for polyproline II-like structure, thereby suggesting that in solution [Pro^3]AAP exists predominantly in polyproline II-like structure. Temperature coefficients of NH chemical shifts in $(CD_3)_2SO$ also indicate that all the NH's are exposed and are not involved in any intramolecular H-bonded structure. The antiarrhythmic, as well as antithrombotic activity of [Pro^3]AAP have also been discussed.

Antiarrhythmic peptide (AAP, Gly-Pro-Hyp-Gly-Ala-Gly) isolated from bovine atria has evoked interest due to its unique feature exhibiting both antiarrhythmic and antithrombotic activity[1-3]. During the course of structure–activity studies with the antiarrhythmic peptide Gly-Pro-Pro-Gly-Ala-Gly (ref.[4]) ([Pro^3]AAP) it was found to be more active than AAP. Since [Pro^3]AAP contains two imino acids at position 2 and 3 respectively, it is possible that the manifestation of its specific biological activity may be a function of some well defined secondary structure(s) in solution. Moreover, oligopeptides containing Pro are useful models from the conformational point of view, as on the one hand, the conformational requirement inherent in the pyrrolidine ring simplifies the structural analysis and yet on the other hand, the presence of a x-Pro linkage can introduce structural heterogeneity corresponding to two possible configurations around x-Pro bonds, i.e. *cis–trans* isomerism. Earlier conformational studies on bradykinin[5] which contains Pro-Pro sequence have shown that it exists predominantly in a (all *trans*) random structure

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whereas studies on a protected hexapeptide[6] also containing Pro-Pro sequence have shown that a type VI β-turn exists in the *cis* isomer of Pro-Pro bond. As there is a small stability difference between *cis* and *trans* isomers, it may be possible that more than one of spatial arrangements of Pro^2-Pro^3 may be biologically important. We have therefore decided to investigate conformational features associated with the biologically active [Pro^3]AAP in solution using CD and NMR spectroscopy. This paper deals with the synthesis, biological activity and solution conformation of [Pro^3]AAP.

EXPERIMENTAL

Chemicals: All amino acids were of the L configuration. Protected amino acid derivatives were prepared in our laboratory by standard procedure and were characterized by thin layer chromatography, melting points and optical rotation.

Spectral measurements: Spectra were recorded on a Bruker WM 400 FT NMR spectrometer, chemical shifts are expressed in δ-scale downfield (i) from TMS in $(CD_3)_2SO$; (ii) from $CHCl_3$ in $CDCl_3$; and (iii) from 1,4-dioxane in D_2O. ^1H NMR spectra were recorded at 400 MHz. Variable temperature measurements were made in $(CD_3)_2SO$ over the range of 30—70°C. Optical rotations of the peptides were determined in the solvents indicated in parentheses on a JASCO DIP-180 automatic polarimeter. Molecular weight of the final peptide has been determined by FD mass spectrometry using Jeol-01SG-2 mass spectrometer.

The circular dichroism spectra were recorded on Jobin Yvon Mark III dichrograph. All spectra were recorded at room temperature in solutions of 1 mg/ml. A cell with 0·05 cm path length was used.

Broad band ^1H decoupled ^{13}C NMR spectra at 25°C of [Pro^3]AAP in different solvents, all at 60 mg/ml, were obtained at 100 MHz on WM 400 FT NMR spectrometer. pH titration studies were accomplished with minimum sample dilution with 6M-NaOH and 6M-HCl stock solutions. pH values were not changed monotonically but were varied somewhat randomly between the extreme values of 1·0 and 11·0.

Analytical procedures: Melting points were determined by the capillary method and are uncorrected. High performance liquid chromatography (HPLC) was carried out in a Waters system using a μ Bondapak C_{18} column (10 m; 30 cm × 0·39 cm i.d.) also from Waters. Purity of the samples was determined using methanol–water–trifluoroacetic acid (0·025%) as a mobile phase (k' values are given). Detection was done at 220 nm. The enantiomeric purity and amino acid composition of peptides were monitored by ^{13}C NMR at each step of the synthesis. Homogeneity of all the peptides was confirmed by silica gel thin layer chromatography employing the following solvent systems: (A) chloroform—methanol (8 : 2) and (B) 1-butanol–acetic acid–water (4 : 1 : 5).

Boc-Gly-Ala-Gly-OBzl (*I*)

This tripeptide was synthesized as published earlier[7]; yield 1·7 g (90%), m.p. 68—70°C, $[\alpha]_D^{25}$ −21·56° (*c* 0·25, MeOH); k' 6·5 (MeOH–H_2O, 3 : 2).

Boc-Pro-Gly-Ala-Gly-OBzl (*II*)

The protected tripeptide *I* (0·5 g; 1·27 mmol) was dissolved in a mixture of methylene chloride–trifluoroacetic acid (2 ml, 1 : 1 v/v). The solution was left at room temperature for 30 min and

then evaporated to dryness. The residue after the double precipitation with methanol–ether was isolated and dried over P_2O_5 and NaOH. The trifluoroacetate salt was neutralized with N-methylmorpholine (0·14 ml; 1·27 mmol) in dry dimethylformamide (4 ml) and coupled with the mixed anhydride formed from Boc-Pro-OH (0·28 g; 1·27 mmol) in the presence of N-methylmorpholine (0·14 ml; 1·27 mmol) and isobutylchloroformate (0·16 ml; 1·27 mmol) in tetrahydrofurane (3 ml) at −15°C. The reaction mixture was stirred for 1 h at −10°C and for 2 h at room temperature. The solvent was evaporated to dryness and the residue was taken up in ethyl acetate and washed three times each with 5% citric acid, water and 5% $NaHCO_3$ and water. The organic layer was dried over Na_2SO_4 and concentrated. The residue was crystallized from ethyl acetate–ether. Yield 0·5 g (80%), m.p. 140°, $[\alpha]_D^{25}$ −41·4° (*c* 0·25, MeOH), R_F 0·68 (A), *k'* 9 (MeOH–H_2O, 3 : 2). ^{13}C NMR ($CDCl_3$): 41·46, 43·31 ($2C^{\alpha}$-Gly), 49·49 (C^{α}-Ala), 17·78 (C^{β}-Ala), 58·58 (C^{α}-Pro), 28·51 (C^{β}-Pro), 24·62 (C^{γ}-Pro), 46·34 (C^{δ}-Pro). For $C_{24}H_{34}N_4O_7$ (490·5) calculated: 58·77%C, 6·99%H, 11·42%N; found: 58·72%C, 6·9%H, 11·44%N.

Boc-Pro-Pro-Gly-Ala-Gly-OBzl (*III*)

The Boc group was removed from the tetrapeptide *II* (0·4 g; 0·81 mmol) as described for *I*. The trifluoroacetate salt was treated with HCl/tetrahydrofurane followed by precipitation of the hydrochloride salt with dry ether. The white solid was filtered, washed with ether and dried over P_2O_5 and NaOH in vacuo. It was dissolved in dimethylformamide (3 ml) and neutralized with N-methylmorpholine (0·09 ml; 0·81 mmol) at 0°C, followed by addition of symmetrical anhydride of Boc-Pro-OH prepared by the treatment of Boc-Pro-OH (1·4 g; 6·5 mmol) with dicyclohexylcarbodiimide (0·67 g; 3·2 mmol) in methylene chloride (10 ml) for 2 h. The reaction mixture was processed as described for *II*. The product was crystallized from ethyl acetate to obtain a chromatographically homogeneous solid. Yield 0·44 g (92%), m.p. 148°, $[\alpha]_D^{25}$ −54·1° (*c* 0·25; MeOH); R_F 0·57 (A), *k'* 6·2 (MeOH–H_2O; 13 : 7). ^{13}C NMR ($CD_3)_2SO$: 40·66, 42·00 ($2C^{\alpha}$-Gly), 47·46 (C^{α}-Ala), 17·93 (C^{α}-Ala), 57·38, 58·11, 59·89 ($2C^{\alpha}$-Pro), 28·41, 28·76, 30·50, 30·01 ($2C^{\beta}$-Pro), 23·62, 23·06, 24·18, 24·79 ($2C^{\gamma}$-Pro), 46·39, 46·46 ($2C^{\delta}$-Pro). For $C_{29}H_{41}N_5O_8$ (587·7) calculated: 59·28% C, 6·98% H, 11·92% N; found: 59·25% C, 6·97% H, 11·95% N.

Z-Gly-Pro-Pro-Gly-Ala-Gly-OBzl (*IV*)

The Boc group was removed from the pentapeptide *III* (0·2 g; 0·34 mmol) as described for *I*. The trifluoroacetate salt obtained in this manner was then treated with HCl/tetrahydrofurane, followed by precipitation of the hydrochloride salt with dry ether. The white solid was filtered, washed with ether and dried over P_2O_5 and NaOH in vacuo. It was dissolved in dimethylformamide (2·5 ml) and treated with N-methylmorpholine (0·038 ml; 0·34 mmol) and coupled with the symmetrical anhydride[8] of Z-Gly-OH prepared by treatment of Z-Gly-OH (0·56 g; 3·4 mmol) with dicyclohexylcarbodiimide (1·7 mmol; 0·3 g) in methylene chloride (10 ml). The reaction mixture was processed as described for *II*. The product was crystallized twice from ethyl acetate to obtain a chromatographically homogeneous solid; yield 0·2 g (87%), m.p. 131–133°C, $[\alpha]_D^{25}$ −41·2° (*c* 0·25; MeOH); R_F 0·6 (B), *k'* 10 (MeOH–H_2O, 13 : 7). ^{13}C NMR ($CD_3)_2SO$: : 40·92, 42·01, 42·21 ($3C^{\alpha}$-Gly), 57·41, 58·23, 59·12, 56·91 ($2C^{\alpha}$-Pro), 27·73, 28·83, 29·70, 30·70 ($2C^{\beta}$-Pro), 23·45, 23·91, 24·40, 24·43 ($2C^{\gamma}$-Pro), 46·49, 46·40 ($2C^{\delta}$-Pro), 47·31 (C^{α}-Ala), 18·01 (C^{β}-Ala). For $C_{34}H_{42}N_6O_9$ (678·7) calculated: 60·17% C, 6·24% H, 12·38% N; found: 59·90% C, 6·12% H, 12·34% N.

Gly-Pro-Pro-Gly-Ala-Gly (*V*)

The peptide *IV* (0·2 g; 0·3 mmol) was dissolved in methanol (20 ml) and subjected to catalytic hydrogenation over 10% Pd/C in the presence of formic acid (0·3 ml) for 10 h. After removing

the catalyst by filtration, methanol was evaporated under reduced pressure and the residue dried in a vaccum dessicator over P_2O_5 and NaOH. The oily residue was dissolved in methanol and passed through a column of IR 45 resin. The solvent was then removed in vacuo and the residue triturated with dry ether to obtain white solid. The product thus obtained was filtered and precipitated twice from methanol–ether to get *V*; yield 0·14 g (87%), $[\alpha]_D^{25}$ −66·0° (*c* 0·5; MeOH), R_F 0·22 (B); *k'* 6 (MeOH–H_2O, 2 : 3). ^{13}C NMR (D_2O): 42·53, 43·49, 44·19 (3C$^{\alpha}$-Gly), 58·87, 60·69 (2C$^{\alpha}$-Pro), 28·11, 29·06 (2C$^{\beta}$-Pro), 24·15, 24·56 (2C$^{\gamma}$-Pro), 47·12, 47·71 (2C$^{\delta}$-Pro), 49·95 (C$^{\alpha}$-Ala), 16·72 (C$^{\beta}$-Ala); FDMS: 455 $(M + H)^+$ and 477 $(M + Na)^+$. For $C_{19}H_{30}N_6O_7$ (454·2) calculated: 50·22% C, 6·60% H, 18·50% N; found: 50·31% C, 6·69% H, 18·62% N.

Biological Methods

[Pro^3]AAP has been screened for its antiarrhytmic and antithrombotic activities in rats by the method described earlier by Dixit et al.[4] The antiarrhythmic activity has been expressed in the terms of aconitine amount required for the onset of early arrhythmia (EA), ventricular tachycardia (VT), ventricular fibrillation (VF) and cardiac arrest (CA). The results have been compared with commonly used antiarrhythmic drugs Quinidine and Verapamil. On the other hand, the antithrombotic effect of the compound has been measured in the terms of the percentage of animals protected from death or paralysis.

RESULTS AND DISCUSSION

Synthesis of [Pro^3]AAP

Acylation of the α-imino group of Pro-Gly-Ala-Gly-OBzl and Pro-Pro-Gly-Ala--Gly-OBzl with Boc-Pro and Z-Gly, respectively, has been carried out by symmetrical anhydride procedure[8] which gave chromatographically pure products in reasonable yields (87−92%). The mixed anhydride procedure using isobutylchloroformate[9] or pivaloyl chloride[10] and DCC/HOBt method gave impure products thereby diminishing the coupling yields. The side reactions observed can be attributed to the ring structure of Pro which causes severe steric hindrance. Such side reactions resulting from steric restriction in Pro have been also reported by Savrda[11]. Thus, the procedure reported herein can be widely utilized in solution for acylation of the α-imino group of Pro present at the N-terminal region of peptides.

CD *Studies*

Linear peptides are well known to be present in solution, mostly as a mixture of conformers. However, Pro has a unique position among the naturally occurring amino acids, as it limits the number of the possible conformers available to the molecule. This has been ascribed to the rotational constraints induced by conformationally restricted pyrrolidine ring. These factors, in turn, simplify the structural analysis using CD.

Moreover, recent reports concerning CD studies on Pro containing dipeptides[12−14], tripeptides[15] and tetrapeptides[16] have clearly demonstrated the power

of CD spectroscopy to detect the presence of intramolecularly H-bonded conformations in the equilibrium of small peptides in solution. Thus, in order to investigate the presence of these minor conformations in the equilibrium of [Pro^3]AAP, solvent dependent conformational distribution of [Pro^3]AAP has been studied.

The CD spectrum of [Pro^3]AAP in methanol is characterized by a negative $n - \pi^*$ CD band at 230 nm and a large negative $\pi - \pi^*$ trend with an anticipated band around 200 nm. The band near 230 nm may be due to a γ-turn (C_7) fixed by an intramolecular H-bond ($3 \rightarrow 1$) between the CO of Pro^2 and the NH of Gly^4 (Fig. 1). This assumption was based on earlier reports on bradykinin[17] and proline derivatives[18] where this bond has been atributed to the γ-turn involving a bridged Pro residue. On the other hand, strong negative band around 200 nm is characteristic of proline II-like structure[14,16]. In trifluoroethanol, however, the negative $n - \pi^*$ band undergoes a blue shift by 3 nm and appears at 227 nm along with a negative $\pi - \pi^*$ trend around 200 nm. Thus, in methanol and trifluoroethanol, the CD pattern of [Pro^3]AAP may be explained on the basis of a conformational equilibrium existing between a polyproline II-like extended structure and, probably a (C_7) γ-turn. The spectra in methanol and trifluoroethanol show practically no concentration dependence in the 0·2–2 mg/ml range. This behaviour suggests that there is no intermolecular association present and the $n - \pi^*$ band may be due to the intramolecularly H-bonded structure. On the contrary, in water there is no minimum in the $n - \pi^*$ region and the spectrum is dominated by a negative $\pi - \pi^*$ trend around 200 nm which suggests that the intramolecular H-bonded (C_7) structure

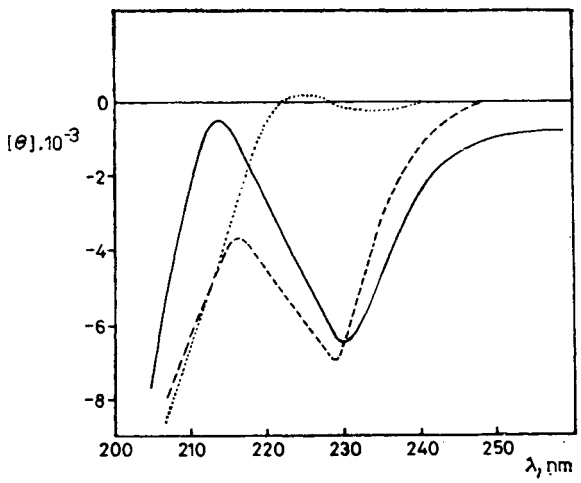

FIG. 1

CD spectra of [Pro^3]AAP in MeOH (———), TFE (– – – –) and water (. . . . . .)

becomes depopulated in water. Thus, the CD spectra of [Pro³]AAP in solution show predominance of polyproline II-like structure. The striking difference between CD patterns of [Pro³]AAP and AAP reported earlier[19] is the presence of an additional positive band at 220 nm in AAP in all the solvents which may be attributed to the possible interaction between γ OH of Hyp residue and the peptide backbone.

pH *Titration*

The pH dependence of the ^{13}C chemical shifts of [Pro³]AAP is shown in Fig. 2. A complete pH titration of the resonances was undertaken in order to facilitate resonance assignments, to study the possible pH dependence of Pro *cis–trans* isomerism. Most of the resonance assignments have been based on the pH data and comparison of the shifts with the data obtained from intermediate fragments Gly--Pro-Pro, Gly-Pro-Pro-Gly, Pro-Gly-Aly-Gly and Gly-Ala-Gly (unpublished data). In addition, signals were also assigned on the basis of the previous report[20] showing

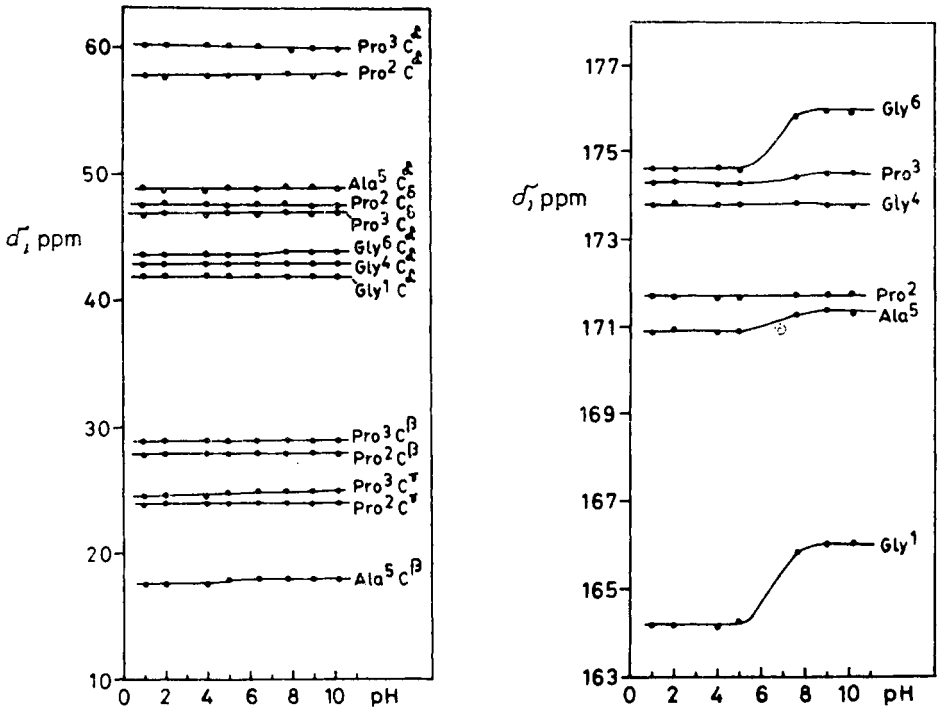

FIG. 2

^{13}C Chemical shifts of [Pro³]AAP as a function of pH; *a* upfield region, *b* downfield region

that because of the steric effect of the Pro residue, the α-carbons of the residues preceeding Pro resonate upfield by 1·0 ppm as compared to the resonance position of the α-carbons placed inside the peptide chain.

Generally, the effect of the terminal residues titration can be observed for both the terminal and the penultimate amino acid signals. Similar effect have been previously reported in other systems[5,21].

The carbonyl signals of the N and C terminal glycines are readily distinguishable by the pH behaviour as are the carbonyl signals of Pro^3 and Ala^5. Remaining carbonyl signals were assigned on the basis of the comparisons with N and C terminal fragments mentioned above. Such experiments have been carried out earlier by London et al.[5] for assigning carbonyls of bradykinin.

Solvent Dependence of Carbonyl Peaks

Numerous studies have shown that acceptance of a H-bond by a carbonyl oxygen leads to a downfield shift of the carbonyl ^{13}C resonance[22,23]. Based on this observation, solvent titration experiments have been carried out by London et al.[5] and Raj et al.[24] to obtain information about intramolecular H-bonding .The procedure is based on the expectation that peptide carbonyl which is intramolecularly H-bonded exhibits less variation in the chemical shift when passed from methanol to water which is a better H-bond donor. In other words, intramolecularly H-bonded carbonyls are more shielded from solvent interaction than the remaining carbonyls.

The ^{13}C shifts observed for carbonyl resonances of [Pro^3]AAP in methanol and water have been shown in Table I. A solvent titration experiment carried out in water–methanol mixtures of varying composition, confirmed an absence of any cross over of peaks passing from water to methanol. All the carbonyl resonances shift upfield as the solvent composition changes from water to methanol. Similar upfield shifts have been observed by other workers for the carbonyl residues in a variety of

TABLE I

^{13}C Chemical shifts (δ) of carbonyl carbons of [Pro^3]AAP

Carbonyl	Water	Methanol	Δ
Gly	164·54	163·10	1·44
Pro	171·71	170·13	1·57
Pro	174·53	172·92	1·61
Gly	174·32	172·65	1·67
Ala	170·91	169·32	1·59
Gly	174·63	173·13	1·50

peptides[25,26]. These results suggest that the secondary structure of [Pro^3]AAP in solution assumes a conformation in which all the carbonyls are exposed and hydrogen bonded primarily with the solvent. Further, we have calculated the dihedral angle ψ around the C^{α}—CO bond in order to establish the predominant conformer of [Pro^3]AAP in solution using chemical shift data obtained for Pro. Siemion, Wieland and Pook[27] proposed a correlation between the dihedral angle ψ around the Pro C^{α}—CO bond and the C^{β}—C^{γ} chemical shift difference $\Delta\delta_{\beta\gamma}$ for both *cis* and *trans* proline peptides. We measured $\Delta\delta_{\beta\gamma}$ for Pro^2 and Pro^3 of [Pro^3]AAP in methanol and water. The observed value for the two Pro residues of [Pro^3]AAP are in the range $\Delta\delta_{\beta\gamma}$ 3·76–4·20 ppm corresponding to ψ 145°–158° (Table II). The value of ψ corresponds to a polyproline II-like structure [ψ = 150°, ref.[17]], thereby ruling out any possibility of a γ-turn (ψ = 70° corresponds to a very low value of $\Delta\delta_{\beta\gamma}$ = = 1·3 ppm). The ^{13}C NMR studies, thus, indicate that [Pro^3]AAP exists predominantly in a polyproline II-like conformation which is in agreement with our CD studies. The intramolecular H-bond conformer, however, as observed by CD presence of negative band at 227 nm and 230 nm in organic solvents, may be present in the equilibrium as a minor conformer, thereby rendering it difficult to be detected by ^{13}C NMR.

^{1}H NMR *Spectra*

The signal assignments in the proton spectra of [Pro^3]AAP in $(CD_3)_2SO$ were achieved by the use of spin decoupling techniques and by comparison with other peptides. The temperature dependence of exchangeable protons in $(CD_3)_2SO$ was examined in order to delineate the hydrogen-bonded protons in the [Pro^3]AAP. The results of variable temperature experiments are shown in Fig. 3. All the three NH's corresponding to Gly^4-Ala^5 and Gly^6 in both *cis* and *trans* isomers are clearly

TABLE II

$^{13}C^{\beta}$—$^{13}C^{\gamma}$ chemical shift difference and their corresponding ψ values for Pro^2 and Pro^3 residues for [Pro^3]AAP

Solvent	Amino acid	C^{β}—C^{γ}, ppm	ψ, deg
Water	Pro^2	3·96	149·7
Water	Pro^3	4·20	158·3
Methanol	Pro^2	3·76	145
Methanol	Pro^3	4·13	154·4
$(CD_3)_2SO$	Pro^2	3·80	145·3
$(CD_3)_2SO$	Pro^3	4·27	158·3

solvent exposed as shown by their high temperature coefficients ($d\delta/dT$) values of 6·7 to 7·5 . 10^{-3} ppm deg^{-1}. These studies, thus, further confirm that the secondary structure of [Pro^3]AAP in solution assumes a conformation in which all the NH's are exposed and are not involved in any ordered structure.

Proline cis : trans Isomerism

Phenomenon of *cis : trans* isomerism is encountered in peptides and proteins. Although the difference of energy between the *cis* and *trans* isomer is low (0·5 kJ mol^{-1}), the *trans* form generally prevails over the *cis* isomer. The shift of the most intensive resonances of the Pro^2 and Pro^3 residues is consistent with the dominance of the *trans* conformation around all the X-Pro peptide bonds. Nevertheless, some low intensity resonances do occur at approximately the positions expected for *cis* Pro, indicating a low population of *cis* peptide linkages. The content of *cis* isomers around Pro-Pro bond according to integration of ^{13}C NMR spectra was found to be 14 and 16% in water and MeOH, respectively. However, in $(CD_3)_2SO$ the situation is different as the *cis* isomer reaches about 22% and this value was found to be in agreement with the value determined by ^1H NMR spectra of

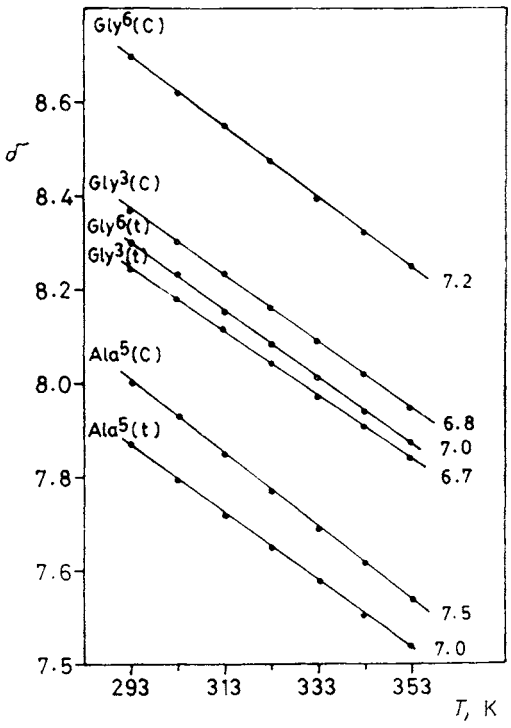

FIG. 3
Temperature dependence of NH chemical shifts (C, *cis*; t, *trans*)

[Pro³]AAP. In water, pH variations do not produce marked effects, the *cis* isomer remaining about equal to 10% within the entire range of pH. Further, because the fraction of *cis* isomer around Pro²-Pro³ is between 14 and 16%, the measurements suggest that the contribution of the *cis* isomer to the CD spectra is nearly constant. These observations are in contrast to AAP where phenomenon of *cis–trans* isomerism was not observed[19]. This may be attributed to the presence of Hyp at position 3 of AAP which leads to the stabilization of *trans* isomer in solution.

Biological Activity

The present investigation demonstrates that [Pro³]AAP delays the onset of aconitine induced arrhythmias in rats and protects against pulmonary thromboembolism in mice. [Pro³]AAP has been tested at a dose of 10 mg/kg (i.v.) in rats against early arrhythmias, as well as ventricular tachycardia, ventricular fibrillation and cardiac arrest. The activity of AAP has been reported earlier in mice at 25 mg/kg (i.v.) dose[2] against early arrhythmias. The amount of aconitine required for the induction of early arrhythmias in rats is comparable to the reported values in mice. The antiarrhythmic activity of AAP tested in rats against aconitine is comparable to that of the analogue [Pro³]AAP. In addition, this peptide has been found to delay the onset of VT, VP and CA as well (Table III). The antiarrhythmic activity of this

TABLE III

Cardiotoxic effect of aconitine in rats, pretreated with [Pro³]AAP, Quinidine and Verapamil (values are Mean ± SE; *n*, number of rats)

Pretreatment	Aconitine required, μg/kg [a]			
	EA	VT	VF	CA
Saline	78·49	101·24	190·92	258·10
$n = 16$	±8·83	±9·33	±21·16	±30·56
AAP	89·38	125·00***	331·33**	347·50
$n = 4$	±7·75	±5·52	±36·47	±46·47
[Pro³]AAP	126·63	180·38**	331·25***	463·00**
$n = 4$	±23·44	±24·99	±60·24	±56·99
Quinidine	153·19*	196·78*	367·98*	647·60*
$n = 16$	±56·01	±69·64	±167·38	±315·18
Verapamil	85·00	122·00	155·00	223·20
$n = 5$	±12·22	±18·79	±18·80	±37·44

Values are significantly different from saline treated group *$p < 0{\cdot}001$; **$p < 0{\cdot}01$; ***$p < 0{\cdot}05$.

analog is comparable to that of Quinidine (10 mg/kg) as well. However, Verapamil (1 mg/kg), a calcium antagonist has not been found to protect against the onset of arrhythmias in this model. These observations demonstrate that [Pro^3]AAP is superior to Verapamil.

It has been observed that [Pro^3]AAP (10 mg/kg, i.p.) possesses 40% antithrombotic activity where Quinidine (10 mg/kg, i.p.) and Verapamil (1 mg/kg, i.p.) do not have any antithrombotic activity at the doses used. The antithrombotic activity of the [Pro^3]AAP (40%) is higher than that of the parent peptide AAP (17%). Aonuma et al.[3] have reported the antithrombotic activity of AAP in various in vivo models. However, they have not tested AAP in the model employed for the present study.

Thus, it appears that [Pro^3]AAP does possess antithrombotic, as well as antiarrhythmic activity which makes it more effective than standard antiarrhythmic drugs Quinidine and Verapamil tested in the present investigation.

CONCLUSIONS

Our CD data indicate that the secondary structure of [Pro^3]AAP in organic solvents may be represented by a time average of two interconverting structures – one polyproline II-like type and one partially ordered, due to a hydrogen bond (C_7) configuration. In water, however, the equilibrium shifts to generate polyproline II-like structure. On the contrary, ^{13}C NMR shifts of Pro C^β and C^γ resonances and solvent dependence shifts of all the carbonyls indicate that [Pro^3]AAP exists exclusively in a polyproline II-like structure, both in methanol and water. The high temperature coefficients $d\delta/dT$ values for all the NH resonances further support an idea that in solution, [Pro^3]AAP assumes a conformation in which all the NH's are exposed. Thus, our CD and NMR studies suggest that [Pro^3]AAP exists predominantly in a polyproline II-like structure in solution. Any other ordering which exists is significant for a too low fraction of time to be reflected unequivocally in the NMR parameters.

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