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Isolation and identification of peptide conformers by reversed-phase high-performance liquid chromatography and NMR at low temperature

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Abstract

Peptide conformers with one or more rotationally hindered peptide bonds due to the presence of proline and/or another N-substituted amino acid residue in the molecule were separated by reversed-phase chromatography at low temperatures, isolated and identified by NMR. The scope of this investigation included the *cis–trans* isomers of the dipeptides Leu-Pro, Phe-Pro and Tyr-Pro as well as conformers of opioid peptides containing proline and/or the proline-like Tic (1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid) residues: Tyr-Pro-Phe (β-casomorphin 1–3 fragment), Tyr-Tic-Phe-Phe, Tyr-Pro-Phe-Pro-Gly (β-casomorphin-5), Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 and Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2. Chromatography with micropellicular and totally porous octadecylated silica stationary phases and aqueous methanol under isocratic elution conditions resulted in well separated peaks of the rotational isomers at sufficiently low temperatures. Preparative RP-HPLC was carried out with eluents containing water and methanol, both deuterated, and the effluent fractions containing each isomer were collected for further investigation. The conformational states of the peptide isomers upon separation were conserved by storing the effluent fractions in liquid nitrogen. The Leu-Pro, Phe-Pro, Tyr-Pro and Tyr-Pro-Phe conformers were identified by one- and two-dimensional NMR spectroscopy at −15°C. Upon comparing the NMR spectra of the isomers, for these peptides the retention order of the conformers was unambiguously established: in each case the *trans* conformer is eluted before the *cis* conformer. On the basis of NMR data obtained with β-casomorphin-5, which contains two proline residues, the elution order of its four conformers was established by NMR spectroscopy of the fractions obtained by RP-HPLC at low temperature as *trans–trans* (least retained), *trans–cis*, *cis–cis* and *cis–trans* (most retained).

Keywords: Preparative chromatography; Nuclear magnetic resonance spectrometry; Structural analysis; Peptides; Peptide conformers; Proline peptides; β-casomorphin-5

1. Introduction

Peptides containing proline residues are known to exist in both the *cis* and *trans* conformation due to the rotationally hindered peptidyl-proline bond and they can be relatively stable at temperatures above 0°C [1–4]. Peptides with n rotationally hindered peptide bonds are expected to have 2^n relatively stable *cis* and *trans* conformers that interconvert at room temperature at a rate that depends on the number and the sequence of the amino acid residues and the solvent. The isolation and identification of such peptide conformers is of growing interest to obtain authentic isomers and thus to facilitate the

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study of various biological processes such as protein folding/refolding [5], immune response [6–13] and the opioid receptor recognition [3,14–17].

The relatively slow *cis–trans* interconversion [18] permits the separation of the conformers by HPLC at subambient temperatures. Reversed-phase chromatography at sufficiently low temperatures has received early attention and was used for the separation of the *cis* and *trans* conformers of dipeptides having a Xaa-Pro peptide bond [1,19–22]. More recently, the separation of conformers of peptides having more than one peptidyl-proline bond was reported on octadecylated silica and cyclodextrin-bonded stationary phases [14,22–28]. It was shown that on-column *cis–trans* isomerization interferes with the chromatographic separation, when the characteristic times of the interconversion and the separation process are commensurate [1,19–22]. By lowering the temperature to slow down the reaction, which has a relatively high activation energy [18,29,30], this interference could be attenuated and as a result the conformers of small proline peptides were separated at subambient temperatures by reversed-phase chromatography. In the past the conformer peaks of certain Xaa-Pro dipeptides were tentatively identified by using relatively crude molecular models to estimate the hydrophobic contact area upon binding to the non-polar surface of the stationary phase. It was found to be greater with the *cis* than with the *trans* isomer [19] and it was concluded that the *cis* conformer is retained stronger in reversedphase chromatography. The same approach was used to predict the elution order of opioid tetra- and pentapeptides [22,27,28].

Structural elucidation of species differing only in their stereochemical arrangement is greatly facilitated by NMR spectroscopy that yields different signal sets for the *cis* and *trans* conformers of molecules having bonds with partially double-bond character like the C-N bond [5]. Recently the complete assignment of the ^{1}H-NMR spectrum of β-casomorphin-5 in dimethyl sulfoxide-d_6 solution was described and the relative populations of the four conformers estimated [15]. NMR spectroscopy is also suitable for the investigation of fast equilibria with rate constants from 10 to 10^5 s^{-1} [31] that are much faster, than the interconversion of *cis–trans* dipeptides containing proline for which the rate constant is in the range from 10^{-3} to 10^{-2} s^{-1} at 298 K [1].

The goal of the present study was to examine the conditions for the separation of peptide conformers containing one or two rotationally hindered peptide bonds by RP-HPLC at temperatures from −25 to 25°C, followed by their isolation as well as identification by one- and two-dimensional NMR. The relatively low-molecular-mass peptides used in this study were chosen based on their availability and potential biological activity.

2. Experimental

2.1. Chemicals

Monobasic sodium phosphate, sodium hydroxide and trifluoroacetic acid were obtained from J.T. Baker (Phillipsburg, NJ, USA) and reagent-grade orthophosphoric acid, HPLC-grade methanol, acetonitrile, chloroform and dichlormethane were obtained from Fisher (Springfield, NJ, USA). Methanol-d_4, acetonitrile-d_3 and dimethyl sulfoxide-d_6 were purchased from Aldrich (Milwaukee, WI, USA). Deuterated water was obtained from Cambridge Isotope Laboratory (Woburn, MA, USA). Deionized water prepared by the NANOpure system from Sybron (Boston, MA, USA) was used throughout the experiments.

The peptides under investigation are listed in Table 1. The optically pure dipeptides were purchased from Sigma (St. Louis, MO, USA). 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic) was obtained through condensation of phenylalanine with paraformaldehyde as reported elsewhere [32]. The opioid peptide analogues were prepared with N-*t*-butyloxycarbonyl-protected amino acids and with diisopropylcarbodiimide (DIP)–1-hydroxybenzotriazole (HOBt) as coupling reagents on a *p*-methylbenzhydrylamine resin (MBHA) following usual solid-phase protocols. Cleavage of the peptides from resin was performed by HF/anisole treatment in the usual manner [32,33]. The crude products were purified and desalted by preparative reversed-phase chromatography [34] and final purification to homogeneity was achieved by HPLC. All opioid peptides were obtained as lyophilizates and char-

Table 1
List of the peptides investigated and the conditions employed in ^{1}H-NMR spectroscopy

Peptides	Solvent mixture (v/v)	Temp. (K)	Chemical shift (ppm)	Assignment	
				Signals	Conformation
Leu-**Pro**	D_2O–CD_3OD (90:10)	268	4.05, 0.91	Leu_α, Leu_δ	*cis*
			0.96	Leu_δ	*trans*
Phe-**Pro**	D_2O–CD_3OD (65:35)	248	3.90	Phe_α	*cis*
			4.46, 4.27	Phe_α, Pro_α	*trans*
Tyr-**Pro**	D_2O–CD_3OD (50:50)	258	7.11	$Tyr_{\phi o}$	*cis*
			7.22	$Tyr_{\phi o}$	*trans*
Tyr-**Pro**-Phe	D_2O–CD_3OD (50:50)	258	7.03	$Tyr_{\phi o}$	*cis*
			7.12	$Tyr_{\phi o}$	*trans*
Tyr-**Pro**-Phe-Asp-Val-Val-Gly-NH_2	D_2O–CD_3OD (30:70)	258	7.00	$Tyr_{\phi o}$	*cis*
			7.12	$Tyr_{\phi o}$	*trans*
Tyr-**Pro**-Phe-Pro-Gly	D_2O–CD_3OD (50:50)	258	7.22	$Tyr_{\phi o}$	*trans–cis*
			7.09	$Tyr_{\phi o}$	*trans–trans*
			7.07	$Tyr_{\phi o}$	*cis–cis*
			7.01	$Tyr_{\phi o}$	*cis–trans*
Tyr-**Tic**[a]-Phe-Gly	DMSO–CD_3CN (80:20)	273	6.65	$Tyr_{\phi m}$	*cis*
			6.75	$Tyr_{\phi m}$	*trans*
Tyr-**Tic**[a]-Phe-Phe	D_2O–CD_3OD (30:70)	258	6.63	$Tyr_{\phi m}$	*cis*
			6.84	$Tyr_{\phi m}$	*trans*
Tyr-**Tic**[a]-Phe-Phe-Val-Val-Gly-NH_2	D_2O–CD_3OD (30:70)	258	6.65	$Tyr_{\phi m}$	*cis*
			6.83	$Tyr_{\phi m}$	*trans*
Tyr-**Tic**[a]-Phe-Gly-Tyr-**Pro**-Ser-NH_2	DMSO–CD_3CN (80:20)	273	9.26, 9.28	Tyr^1-OH[b]	–
			9.32, 9.33		

[a] **Tic** is the acronym of 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid.
[b] Conformers were not assigned.

acterized by TLC, RP-HPLC, amino acid analysis, FAB mass spectrometry and ^{1}H-NMR.

2.2. Apparatus

HPLC unit

The analytical chromatography of the peptides was performed using a HP 1090 liquid chromatograph equipped with a diode-array detector and a 25 μl automatic injector (Hewlett-Packard, Palo Alto, CA, USA). Preparative separations were carried out by a unit assembled from a ConstaMetric III pump, a SpectraMonitor variable wavelength detector from LDC/Milton Roy (Riviera Beach, FL, USA) and an injection valve equipped with a 100 μl sample loop from Rheodyne (Cotati, CA, USA). Chromatograms were recorded and processed using LDC/Milton Roy CI 10 integrator. The column effluent was monitored at 210, 220 or 280 nm. The columns were jacketed and connected to a RTE-4DD refrigerated circulating bath from NESLAB (Portsmouth, NH, USA) suitable for operation at temperatures down to −30°C. Ethylene glycol was used as the circulating coolant.

Columns

Columns, 250×4.6 mm I.D., were packed with 5 μm Spherisorb C-18 (E. Merck, CherryHill, NJ, USA) or 5 μm UltraSphere ODS (Beckman, Fullerton, CA, USA). Another 250×4.6 mm I.D. column packed with 5 μm Vydac C-18 particles was from The Separations Group (Hesperia, CA, USA). Columns 105×4.6 mm I.D., 75×4.6 mm I.D., 50×4.6 mm I.D. and 30×4.6 mm I.D. packed with 2 μm micropellicular RP-18 particles were supplied by Glycotech (Hamden, CT, USA). Silica gel columns of 105×4.6 mm I.D., 75×4.6 mm I.D. and 50×4.6 mm I.D. were packed in our laboratory with 3 μm or 5 μm Spherisorb particles with a pore diameter of 100 Å (PhaseSeparations, Norwalk, CT, USA).

NMR

^{1}H and ^{13}C-NMR measurements were carried out using a Bruker AM-500 unit (Karlsruhe, Germany)

that was operated at 500 MHz and 125 MHz and the standard pulse sequences offered by Bruker. The Bruker AM-500 was equipped with an ASPECT 3000 computer and a variable temperature unit used for measurement at low temperatures. Peptides were dissolved in dimethyl sulfoxide-d_6–acetonitrile-d_3 or in a methanol-d_4–D_2O mixture of the same composition as that of the mobile phase in the corresponding HPLC experiments. Preliminary proton NMR experiments were carried out at a concentration of 10 mg/ml, the carbon data were observed at a concentration of 20 mg/ml. ^{1}H-NMR spectra were recorded in the Fourier transform mode with HDO signal presaturation (15–25 L decoupler power), 32 K data points and 8, 16 or 32 scans depending on signal intensity. For the 2D-COSY and NOESY experiments 8 or 16 transients were accumulated for each t_1-value. The spectral size in time domain was 1 K×512 words with zero filling in t_1 dimension. ^{13}C spectra were obtained with broadband proton decoupling, number of scans $10–20 \cdot 10^3$. Chemical shifts are reported in ppm from tetramethylsilane (TMS).

2.3. Chromatographic procedures

All chromatographic experiments were performed isocratically. For reversed-phase chromatography mobile phases were prepared by mixing methanol and near aqueous 50 m*M* phosphate buffer, pH 2.5 or 6.0. The mixture was filtered and degassed with helium prior to use. A Model pHM 82 pH meter (Radiometer America, Cleveland, OH, USA) was used for pH measurements. It was calibrated at pH 4.0 and 7.0 with buffer solutions of pH 4.0 (biphthalate) and pH 7.0 (phosphate) from J.T. Baker.

The peptides were dissolved in the eluent at a concentration of 0.5 and 10 mg/ml for analytical runs and preparative chromatography, respectively. The solutions were incubated for 1 h at 25°C to let the conformers reach the equilibrium concentration. Prior to injection the sample was incubated for 15 min at column temperature to let the solution reach thermal equilibrium.

HPLC on silica gel

The peptides under investigation were subjected to analytical chromatography on silica gel stationary phases at −25°C in order to attempt the separation of the *cis–trans* isomers of peptides at −25°C with silica gel columns. The mobile phase was *n*-hexane containing 5 to 20% (v/v) of dichlormethane, chloroform or carbontetrachloride and in all cases the column inlet pressure was kept at 350 bar. Under these conditions the conformers were not separated but the peptides eluted as single peaks with k' values ranging from 6 to 8 despite of NMR studies that indicated the presence of both the *cis* and *trans* isomers in non-polar solvents under similar conditions [39].

Analytical RP-HPLC

In the analytical chromatography of the peptide conformers columns packed with non-polar micropellicular stationary phases were used. The relatively low mass transfer resistances, enhanced mechanical and chemical stability as well as the high recovery of biological molecules characteristic for micropellicular stationary phases has made them particularly advantageous in this application that required relatively high speed of separation at low temperatures.

Preparative RP-HPLC

Preparative chromatography of the conformers was carried out to obtain the pure isomers in amounts sufficient for NMR measurements. Columns packed with totally porous octadecylated silica stationary phases, e.g., 5 μm Spherisorb C-18, 5 μm UltraSphere ODS and 5 μm Vydac C-18, were used with deuterated eluents. First the column was purged with the appropriate deuterated mobile phase at a flow-rate of 0.1 ml/min at room temperature. The process was terminated after 30 min when non-deuterated solvents were still present in the effluent but at a level that did not palpably interfere with NMR spectroscopy of the peptides. Then the circulation of the cooling fluid was started and after the proper column temperature was reached, about 1 mg peptide dissolved in 100-μl deuterated solvent was injected. Fractions of 500 μl containing the separated conformers were collected either in 2-ml Eppendorf vials (Fisher, Pittsburgh, PA, USA) or 1.2-ml cryogenic vials (Corning Science Products, Corning, NY, USA) containing 1 and 0.6 ml liquid nitrogen, respectively, to prevent interconversion.

The vials with the frozen fractions were kept in a Dewar flask (Matheson Scientific, Stoneham, MA, USA) filled with liquid nitrogen. Experiments were carried out to confirm that the conformational states of the peptides are indeed frozen at liquid nitrogen temperature. For instance the conformational integrity of the *cis* and *trans* forms of the three dipeptides containing proline, as measured by analytical HPLC at −5°C, was preserved for at least eight days upon collecting them in liquid nitrogen and storing at −70°C in a Sub-Zero temperature test chamber (Cincinnati Sub-Zero Products, Cincinnati, OH, USA).

2.4. NMR procedures

Preliminary NMR investigations included the assignment of ^{1}H-NMR signals, the assignment of the signal sets to the conformations and the selection of appropriate signals for the identification of the isomers in the fractions obtained by HPLC. Spectra of the peptides in methanol-d_4–D_2O mixtures used as the mobile phase were recorded at both room temperature and at the column temperature in the HPLC experiments.

Low peptide concentrations of about 0.25 mg/ml in the HPLC fractions and the short measurement times, to avoid changes in the isomeric composition of the sample, mandated the use of one-dimensional ^{1}H-NMR. Furthermore it was necessary to suppress the non-deuterated water signal. The cryogenic vials containing the frozen fractions at −78°C were heated to −25°C in a bath containing an acetone–dry ice mixture. The molten sample was filled with a precooled 2-ml glass pipet (Fisher) into a 5-mm NMR tube (Wilmad Glass, Buena, NJ, USA) that was precooled to −25°C. Then the NMR tube was placed into the NMR instrument, which was thermostatted at the experimental temperature ranging from −25 to −5°C.

In the measurements with Tyr-Tic-Phe-Gly and Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2 dimethyl sulfoxide-d_6–acetonitrile-d_3 mixture was employed as the solvent. ^{1}H-NMR, two-dimensional methods like COSY and NOESY as well as literature data were used for signal assignment. In addition ^{13}C-NMR spectroscopy was employed when the above methods failed to prove an unambiguous conformational assignment.

3. Results and discussion

3.1. On-column reaction

The *cis–trans* isomerization of the peptides under investigation is a first order reversible reaction. The reaction scheme in the column containing the mobile and stationary phases and the rate constants appropriate for the interconversion of the *cis* and *trans* isomers are shown in Fig. 1.

The interplay of on-column reaction and chromatographic separation can be characterized by the dimensionless Damköhler number, *Da* [1]. It represents the ratio of the characteristic time of the separation to the characteristic time of the reaction and can be expressed as:

$$Da = Lk_{ct}(1 + 1/K_m)/u_o = L(k_{ct} + k_{tc})/u_o \quad (1)$$

where L is the column length, K_m is the equilibrium constant for the interconversion in the mobile phase and u_o is the mobile phase velocity. The overall *cis* to *trans* and *trans* to *cis* rate constants k_{ct} and k_{tc} are defined as:

$$k_{ct} = k_{s,ct}k'_c + k_{m,ct} \quad (2)$$

$$k_{tc} = k_{s,tc}k'_t + k_{m,tc} \quad (3)$$

where k_s and k_m are the rate constants for the reaction on the stationary and the mobile phases, and k'_c and k'_t are the retention factors of the *cis* and *trans* isomers, respectively.

When the Damköhler number is small ($Da<0.1$)

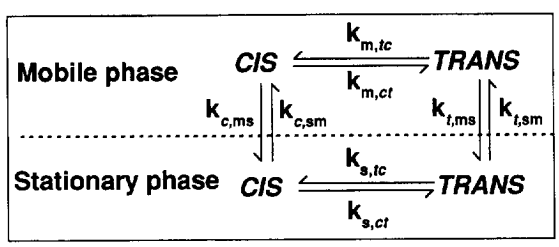

Fig. 1. Scheme for the on-column interconversion of the *cis* and *trans* isomers in the mobile and stationary phases and for the distribution of the conformers between the two phases.

the reaction does not interfere palpably with the chromatographic process and the conformers are separated, provided the efficiency and selectivity of the chromatographic system is sufficiently high. When the Damköhler number is large ($Da>10$) the reaction is so fast on the time scale of the chromatography that the conformers are not separated at all but elute as a single peak with a retention factor representing the weighted average of those of the *cis* and *trans* isomers. In light of Eq. 1, short columns, high flow velocities and low temperatures should be used to obtain a *Da* value so low that the interference by the reaction does not affect the separation appreciably. However, short columns, high velocities and low temperatures with concomitantly low eluite diffusivity are not favorable to obtain high separation efficiency [35]. Furthermore the relatively high viscosity of the mobile phase at low temperatures may preclude the use of high flow velocities at a column inlet pressure below its maximum permissible value. In practice lowering the column temperature is the most effective means to decrease *Da* and thus bring about the separation of the interconverting species. This is because the activation energy for the reaction is usually much greater than that for the diffusivity and the viscosity. Similar considerations apply also to the capillary zone electrophoresis of such conformers [36].

3.2. Solvent effects

The effect of temperature on the viscosity of liquids is approximated by the Andrade equation [37]:

$$\ln \eta = A + B/T \quad (4)$$

where η is the viscosity, T is the absolute temperature and A and B are constants. For methanol–water mixtures, which were used as eluents in this study, the dependence of viscosity on the composition and the temperature has been calculated from literature data and is illustrated in Fig. 2. It is seen that the viscosity goes through a maximum at a methanol concentration of about 40% (v/v) in the temperature range illustrated. The viscosity of methanol–water mixtures increases by a factor of about two when the

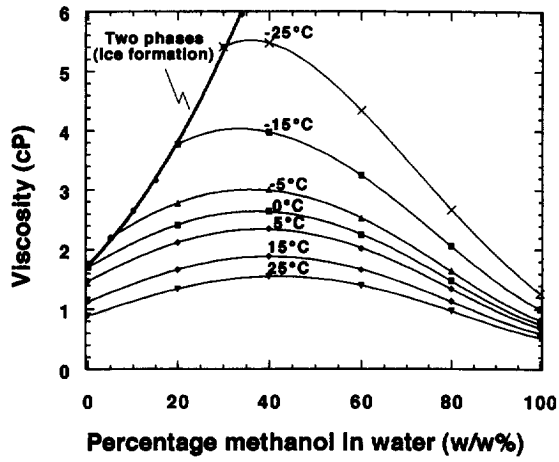

Fig. 2. Viscosity of methanol–water mixtures at and below ambient temperature as a function of the composition. Viscosities below 25°C were estimated from those of methanol and water [52] by the Grunberg and Nissan equation [53]. The viscosities of methanol–water mixtures of different compositions at 25°C were taken from Ref. [54].

temperature decreases from 25 to −5°C. Concomitantly to lowering the column temperature the organic modifier concentration in the mobile phase has to be increased in order to reduce the freezing point of the eluent and to avoid an unduly increase in the retention factors. As seen in Fig. 2, above 40% (v/v) methanol in water, the increase in the organic modifier concentration results in lower viscosity at any temperature illustrated and thus some of the problems associated with the reduction of the column temperature are mitigated.

For the proline dipeptides under investigation the effect of temperature on the equilibrium constant K_m is negligible. The concentration ratio of the *cis* and *trans* conformers varied not more than 3% in the temperature range from −50°C to 50°C. On the other hand, K_m, is known to be dependent on the amino acid composition of the peptide, pH, the solvent and the temperature [20,38,39]. The use of deuterated solvents did not affect appreciably the rate of the prolyl isomerization [30]. However, the effect of solvent polarity on the isomerization rate was found to be significant and as a rule of thumb the rate of interconversion decreases with solvent polarity [40]. Preliminary measurements in our laboratory by NMR

and CZE [41] revealed that the interconversion rate of proline dipeptides is an order of magnitude slower in water than in methanol or acetonitrile. Therefore our results are in good agreement with the observations published [40].

4. HPLC

In the analytical chromatography of the peptide conformers the use of the micropellicular stationary phase afforded more efficient and faster separations as well as higher eluite concentrations in the effluent, than the porous stationary phases under comparable conditions. Fig. 3. shows a typical analytical separation on a micropellicular octadecylated silica column at 0°C of the *cis* and *trans* conformers of Leu-Pro.

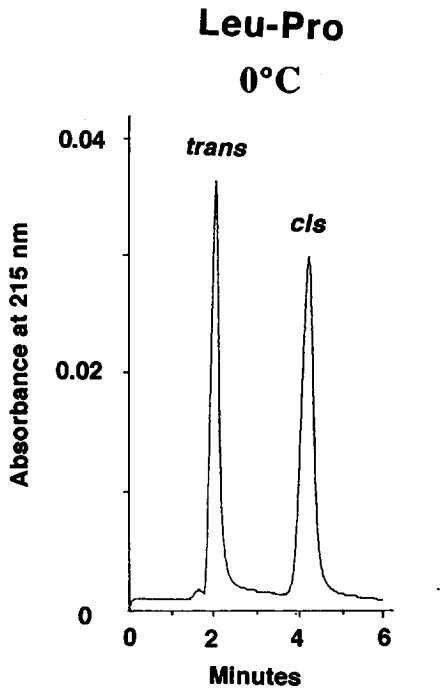

Fig. 3. Typical analytical chromatogram illustrating the separation of the *cis* and *trans* conformers of Leu-Pro by reversed-phase chromatography at 0°C. Column, 105×4.6 mm, 2-μm pellicular ODS-silica; eluent, 25 m*M* phosphate buffer; pH 6.0; flow-rate, 0.4 ml/min; sample, 10 μg of Leu-Pro in 20 μl of the mobile phase; injection volume, 20 μl.

The fractions obtained by preparative RP-HPLC at low temperature were rechromatographed under analytical conditions at both −5°C and 30°C and a schematic illustration of the chromatographic testing procedure for the reversible interconversion of the *cis* and *trans* peptide conformers is presented in Fig. 4. The two peaks obtained at low-temperature HPLC are believed to represent the two conformational states when the same sample gives only a single peak at high temperature under otherwise identical conditions. This is confirmed as follows. Upon rechromatography at high temperature of the fractions containing the isomers, each chromatogram shows the same single peak representing the mixture of both conformers. Then rechromatography at low temperature of the fractions containing the individual isomers stored in liquid nitrogen, showed a single peak, representing either the *cis* or the *trans* isomer.

Efforts were made to isolate the *cis–trans* isomers by RP-HPLC on totally porous stationary phase using non-deuterated solvents and to freeze dry the conformer fractions. The lyophilized fractions were dissolved in the precooled deuterated solvents, and subsequently analyzed by HPLC on the micropellicular octadecylated silica stationary phase and by NMR, both at low temperatures. Since the isomeric composition of the samples did change significantly in the freeze-drying process the results allowed only to estimate the elution order of the *cis–trans* conformers. For this reason the following approach was

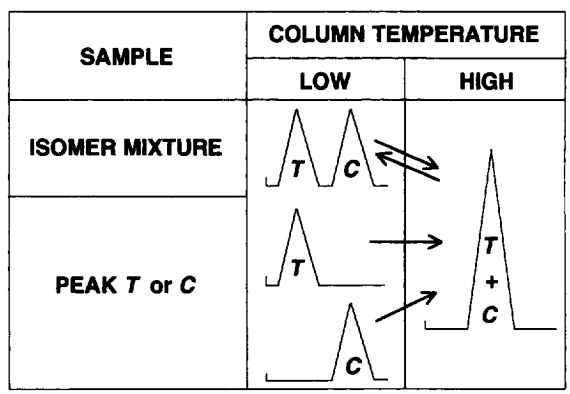

Fig. 4. Schematic illustration of chromatographic testing for the reversible interconversion of *cis* and *trans* peptide conformers by rechromatography at low and elevated temperatures.

taken. The separation of conformers was carried out using low-temperature chromatography with deuterated solvents, the fractions were collected in liquid nitrogen and subsequently subjected to low-temperature NMR. Using the appropriate signals listed in Table 1 the *cis* and *trans* conformers of peptides under investigation were identified.

4.1. Identification of the conformers by NMR spectroscopy

In earlier chromatographic studies [19] the peaks of the *cis* and *trans* isomers of Ala-Pro were tentatively identified by estimating the relative magnitude of the hydrophobic contact areas of the isomers upon binding to the stationary phase. Comparison of the appropriate molecular models showed that whereas in the *cis* conformer a plane through the two α-carbons separates non-polar residues from the polar amino and carboxyl groups, no such a plane has been found in the *trans* form of Ala-Pro. According to the solvophobic theory, which has widely been used to treat the retention behavior in reversed-phase chromatography, the *cis* conformer, which has a larger contact area upon binding to the hydrocarbonaceous ligates of the stationary phase, will be retained longer than the *trans* form, which has a smaller hydrophobic contact area.

In the present study low-temperature NMR spectroscopy was used for the identification of *cis–trans* conformers isolated by HPLC at low temperatures. NMR spectroscopy is widely used for structural elucidation of stereoisomers. However, the conformational study of short linear peptides is beset with difficulties arising from their large conformational flexibility that makes the NMR parameters represent population-weighted averages of the rapidly interconverting conformers [42]. When *cis–trans* isomerization around the peptide bond involving the proline nitrogen is slow enough, the NMR spectrum will show both conformations [43].

Since the nuclei far away from the peptide bond of interest are hardly affected by the isomerization, the signals of the nuclei near the rotationally hindered bond should exhibit the greatest differences. In earlier studies various approaches were taken to distinguish between the *cis* and *trans* conformational states of peptides containing peptidyl-proline (-Xaa-Pro-) bond. The three most important methods are as follows. (i) Different NOESY cross peaks: $XaaH_{\alpha}$-$ProH_{\alpha}$ for the *cis* and $XaaH_{\alpha}$-$ProH_{\delta}$ for the *trans* conformer [44]. (ii) Characteristic differences in the ^{13}C chemical shifts for $ProC_{\beta}$ and $ProC_{\gamma}$ [44,45]. (iii) Upfield shift of $XaaH_{\alpha}$ signal for the *cis* conformer [44,46].

The Tic residue has often been incorporated in synthetic peptides as a conformationally restricted phenylalanine analogue [32,47]. It is also a proline analogue, however, because the Tic nitrogen in a peptide bond can give rise to isomers that interconvert as slowly as those of peptides that contain proline [14]. No comparative studies have been reported on the *cis–trans* isomerization of peptides containing either proline or Tic residues.

Since all the opioid peptides under investigation share the starting sequence Tyr-Pro or Tyr-Tic, the aromatic protons of tyrosine have been useful and convenient markers as it was reported that the $Tyr_{\phi ortho}$ signals are shifted upfield in the case of the *cis* conformation of Tyr-Pro-Leu-Gly-NH_2 [48], morphiceptin [49] and β-casomorphin-5 [15]. An explanation for this behavior rests with possible 'stacking' of the aromatic moieties and the proline side chain, which has been observed with the *cis* conformation [50]. For Tyr-Tic moieties we have found that also the $Tyr_{\phi meta}$ signal is shifted upfield in the *cis* conformation, probably due to the bulkier side chain of the Tic residue.

In most cases such analysis of the spectra and comparison of the results to literature data have facilitated the assignment of the signal sets to the conformations. Due to the interference by the second tyrosine an unambiguous conformational assignment was only precluded with the heptapeptide Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2, despite the well observable signals of four conformers. The signals selected for easy recognition of the conformers are listed in Table 1. With peptides in general, the best chance to obtain well separated signals is in the region of the α-protons around 4 ppm and/or the NH-protons around 8 ppm. However, with the opioid peptides the use of the aromatic signals of the tyrosine mentioned above offers a preferable alternative. The signals

arise from two protons and are only splitted in a dublett, thus, a fourfold improvement in signal-to-noise ratio can be obtained with respect to the use of an α-proton.

4.2. Proline dipeptides

Because of the profuse literature on their behavior in NMR [51] and HPLC [19] proline dipeptides served as suitable model compounds in the study of the HPLC conditions leading to the isolation of the conformers of biologically active opioid peptides. In our study all proline dipeptides, Leu-Pro, Phe-Pro and Tyr-Pro had non-polar side chains.

Preparative separation at low temperature with deuterated eluent components was carried out using columns packed with octadecylated totally porous silica gel and the fractions were isolated as described in the experimental part. A typical chromatogram of the two Phe-Pro conformers obtained under overloaded conditions is shown in Fig. 5 together with the ^{1}H-NMR spectra of the *cis* and *trans* conformers. The results show that the *trans* form is retained less than the *cis* conformer. The same elution order was found also with the isomers of Leu-Pro and Tyr-Pro in reversed-phase chromatography: *trans* leads and *cis* follows. It should be noted that the identification of the conformers of proline dipeptides by NMR spectroscopy confirmed the earlier predictions based on an estimation of the relative magnitude of the hydrophobic contact areas of the two isomers that determines in reversed-phase chromatography their relative retention according to the solvophobic theory [19].

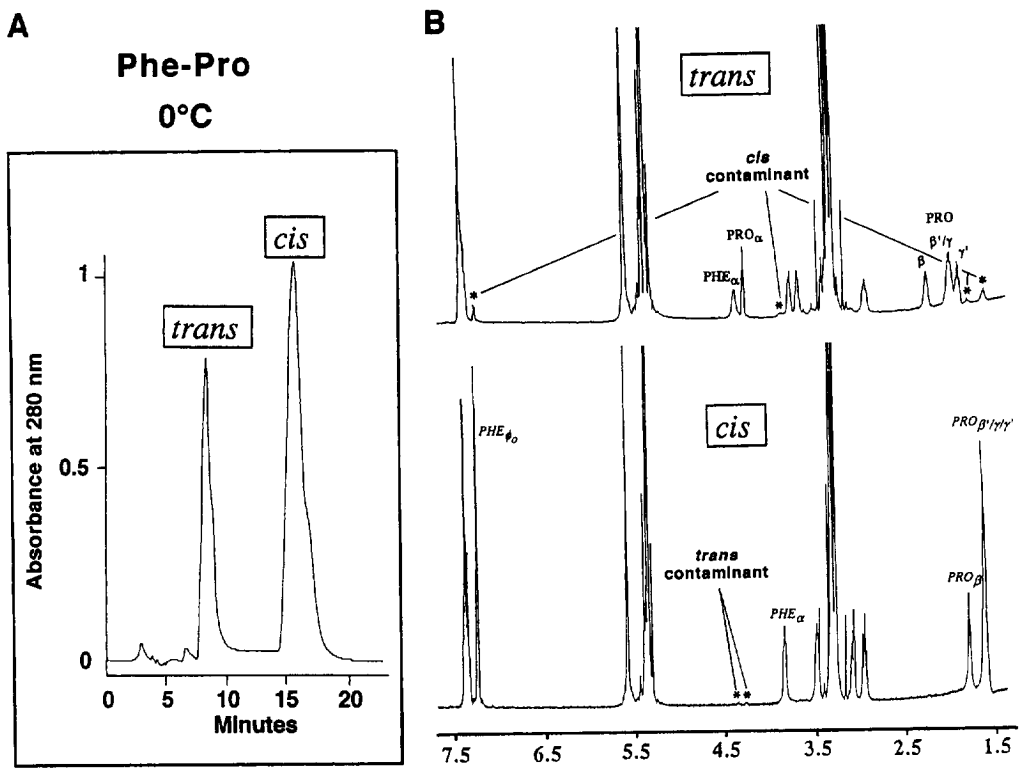

Fig. 5. Preparative separation of the *cis* and *trans* conformers of Phe-Pro by reversed-phase chromatography with deuterated eluent and their identification by NMR spectroscopy. (A) Chromatogram. Column, 250×4.6 mm, 5 μm UltraSphere ODS; eluent, 65% (v/v) 50 m*M* phosphate buffer and 35% (v/v) methanol; pH 6.0; flow-rate, 1 ml/min; temperature, 0°C; sample, 1 mg of Phe-Pro in 100 μl of the mobile phase; injection volume, 100 μl. (B) ^{1}H-NMR spectra of the isolated Phe-Pro conformers at −25°C.

4.3. Oligopeptides containing a single rotationally hindered residue

Tyr-Pro-Phe

The *cis* and the *trans* conformers of the biologically active tripeptide Tyr-Pro-Phe were isolated by RP-HPLC and identified by NMR. The chromatograms of the conformers obtained under overloaded conditions together with the corresponding NMR spectra of the aromatic region in the isolated conformers are shown in Fig. 6. The first and the second spectrum manifest the predominant presence of the *trans* and the *cis* conformer, respectively. Thus, the β-casomorphin 1–3 fragment exhibits the same elution order which was found with the dipeptides described above and the conformers of tripeptides containing a tyrosyl-proline bond are also eluted in reversed-phase chromatography so that the *trans* precedes the *cis* isomer.

Tyr-Pro-Phe-Asp-Val-Val-Gly-NH_2

The separation of conformers of this peptide by reversed-phase chromatography at −5°C is illustrated in Fig. 7. By rechromatography of the fractions it was ascertained that the two peaks represent the two conformers of the heptapeptide. The results indicate that the two conformers of this peptide can be resolved at −5°C as illustrated in Fig. 7. The $Tyr_{\phi ortho}$ signal served in this case also admirably well for the identification of the *cis* and *trans* conformers by their NMR spectra as seen in Table 1.

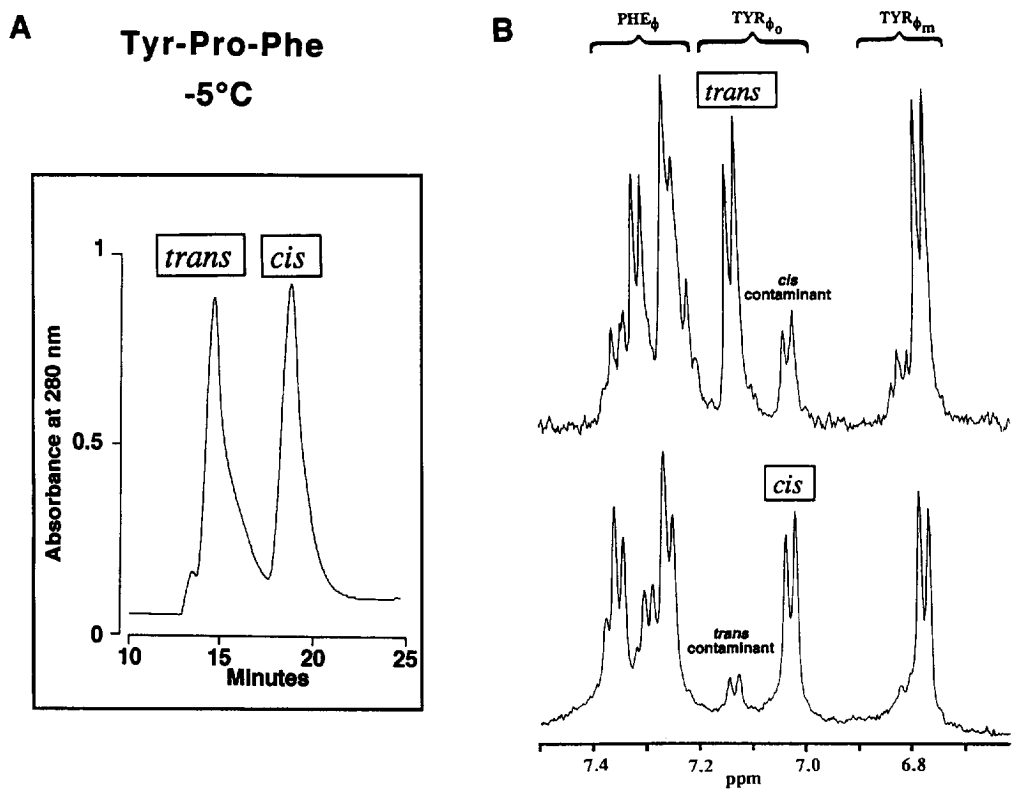

Fig. 6. Preparative separation and identification of the conformers of β-casomorphin 1–3 fragment. (A) Chromatogram. Column, 250×4.6 mm I.D., 5 μm Vydac ODS; eluent, 50% (v/v) deuterated methanol and 50% (v/v) deuterated water; flow-rate, 0.5 ml/min; temperature, −5°C; sample, 1 mg of peptide in 100 μl of the mobile phase; injection volume, 100 μl. (B) Parts of the ^{1}H-NMR spectra showing the signals of the aromatic moieties of the fractions at −15°C.

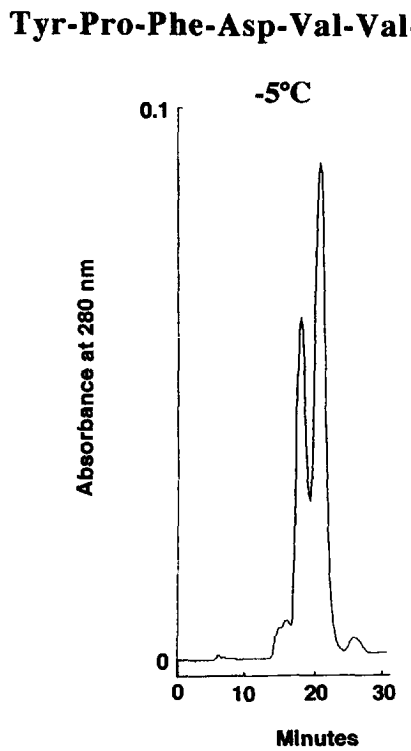

Fig. 7. Chromatogram of Tyr-Pro-Phe-Asp-Val-Val-Gly-NH_2 obtained at −5°C. Column, 250×4.6 mm I.D., 5 μm Spherisorb ODS; eluent, 35% (v/v) 50 m*M* phosphate buffer and 65% (v/v) methanol; pH 6.0; flow-rate 0.5 ml/min; sample, 20 mg of peptide in 20 μl of the mobile phase; injection volume, 20 μl.

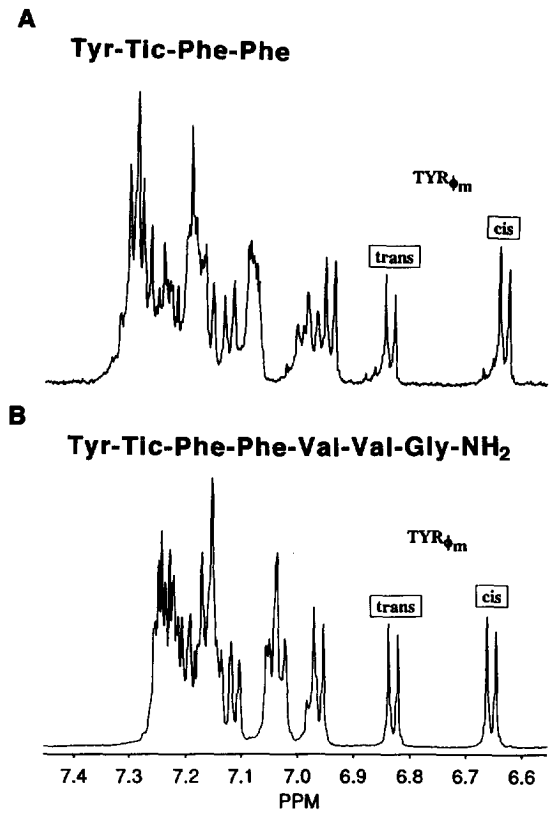

Fig. 8. Parts of the ^{1}H-NMR spectra showing the signals of the aromatic moieties in (A) Tyr-Tic-Phe-Phe and (B) Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 in 30% D_2O–70% CD_3OD (v/v) at −15°C.

Peptides containing a Tic residue

Two tetrapeptides, Tyr-Tic-Phe-Gly and Tyr-Tic-Phe-Phe as well as the heptapeptide, Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 were first purified by RP-HPLC at room temperature. NMR spectroscopy of Tyr-Tic-Phe-Gly, Tyr-Tic-Phe-Phe and Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 was carried out using 1D- and 2D-NMR methods for the assignment of signals. The results with these peptides containing Tic residue confirmed the presence of different conformers that slowly undergo interconversion. NMR measurements were carried out under conditions identical to those employed in the HPLC of these peptides, i.e., in a 30–70% D_2O–CD_3OD mixture, at temperatures 25°C, 0°C and −25°C. The ^{1}H-NMR spectra of the aromatic region in Tyr-Tic-Phe-Phe and Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 are depicted in Fig. 8. The two conformers were identified by using the proton signals $Tyr_{\phi ortho}$ and $Tyr_{\phi meta}$ since both exhibited significant differences in the chemical shifts. It is noteworthy that one of the $TicH_\beta$ signals showed a remarkable upfield shift in the case of the *cis* conformer.

For the separation of the two Tyr-Tic-Phe-Phe conformers by reversed-phase HPLC with aqueous methanol as the mobile phase the column temperature had to be lowered to −25°C as illustrated in Fig. 9. Whereas at temperatures at or above 0°C only a single peak appeared on the chromatogram, lowering the temperature to −15°C resulted in the formation of a shoulder indicating that the peak may represent several isomers. Direct evidence for the existence of two conformers has been found by chromatography at −25°C, were Tyr-Tic-Phe-Phe yielded two, albeit

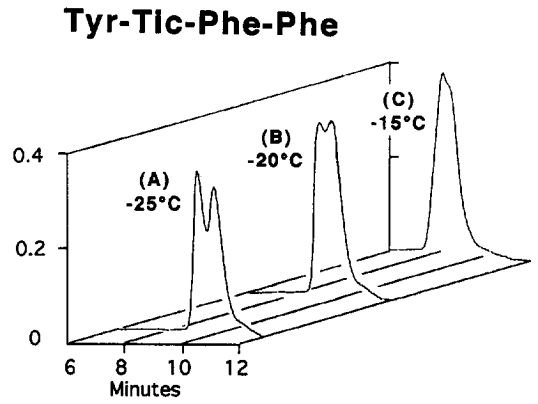

Fig. 9. Chromatograms of Tyr-Tic-Phe-Phe obtained at (A) −25°C, (B) −20°C and (C) −15°C. Column, 250×4.6 mm I.D., 5 μm Spherisorb ODS; eluent, 30% (v/v) 50 m*M* phosphate buffer and 70% (v/v) methanol; pH 2.5; flow-rate, 0.5 ml/min; sample, 20 μg of peptide in 20 μl of the mobile phase; injection volume, 20 μl.

not completely separated, peaks. The presence of two conformers was further confirmed by rechromatographing the collected fractions as described in the experimental part and shown in Fig. 4. Although not as effective as proline, the Tic residue also can make the peptide bond involving the Tic nitrogen so rotationally restricted that at sufficiently low temperatures two relatively stable conformers can be found by reversed-phase HPLC. The two conformers of Tyr-Tic-Phe-Gly and Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 were also authenticated in the same way and the chromatogram of the heptapeptide containing a single Tic residue is shown in Fig. 10. It is seen that the chromatographic pattern is similar to that obtained with Tyr-Tic-Phe-Phe.

4.4. Peptides containing two rotationally hindered residues

Tyr-Pro-Phe-Pro-Gly

An early NMR study on the biologically active β-casomorphin-5 dissolved in dimethyl sulfoxide-d_6 revealed the existence of four conformers [15]. By examining the ^{1}H-NMR spectrum of this peptide in a methanol-d_4–D_2O mixture we also found four isomers in this solution. Preparative chromatography at −5°C yielded only a partial separation of the conformers as illustrated in Fig. 11. Eight effluent fractions

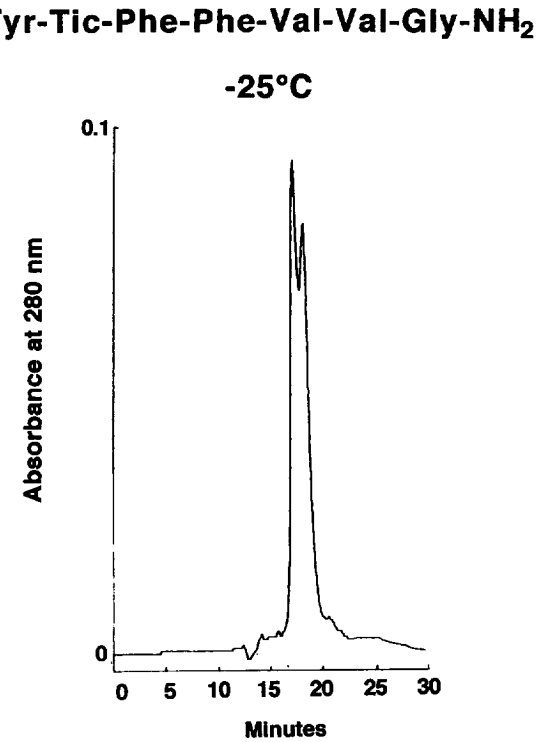

Fig. 10. Chromatogram of Tyr-Tic-Phe-Phe-Val-Val-Gly-NH_2 obtained at −25°C. Column, 250×4.6 mm I.D., 5 μm Spherisorb ODS; eluent, 30% (v/v) 50 m*M* phosphate buffer and 70% (v/v) methanol; pH 6.0; flow-rate, 0.5 ml/min; sample, 20 μg of peptide in 20 ml of the mobile phase; injection volume, 20 μl.

were collected, subjected to NMR spectroscopy and the conformers were identified by the $Tyr_{\phi ortho}$ signals listed in Table 1. The molar composition of the isomer mixtures was calculated from the height of the signals and expressed as mol fractions as shown in Fig. 12. The four effluent fractions containing the highest mol fraction of a particular conformer are shaded and numbered from I to IV in Fig. 12 and their NMR spectra are shown in Fig. 11. The mol fraction of the *trans–trans* conformer is maximal in Fraction I and then steadily decreasing whereas the mol fraction of the *cis–trans* conformer increases in the later fractions. On the other hand the mol fractions of *trans–cis* and *cis–cis* are maximal, albeit not the most abundant, in Fraction II and III, respectively. From these data we conclude that first elutes the *trans–trans* conformer followed by *trans–cis*, *cis–cis* and *cis–trans* conformers. The first major peak in the chromatogram is the *trans–trans*

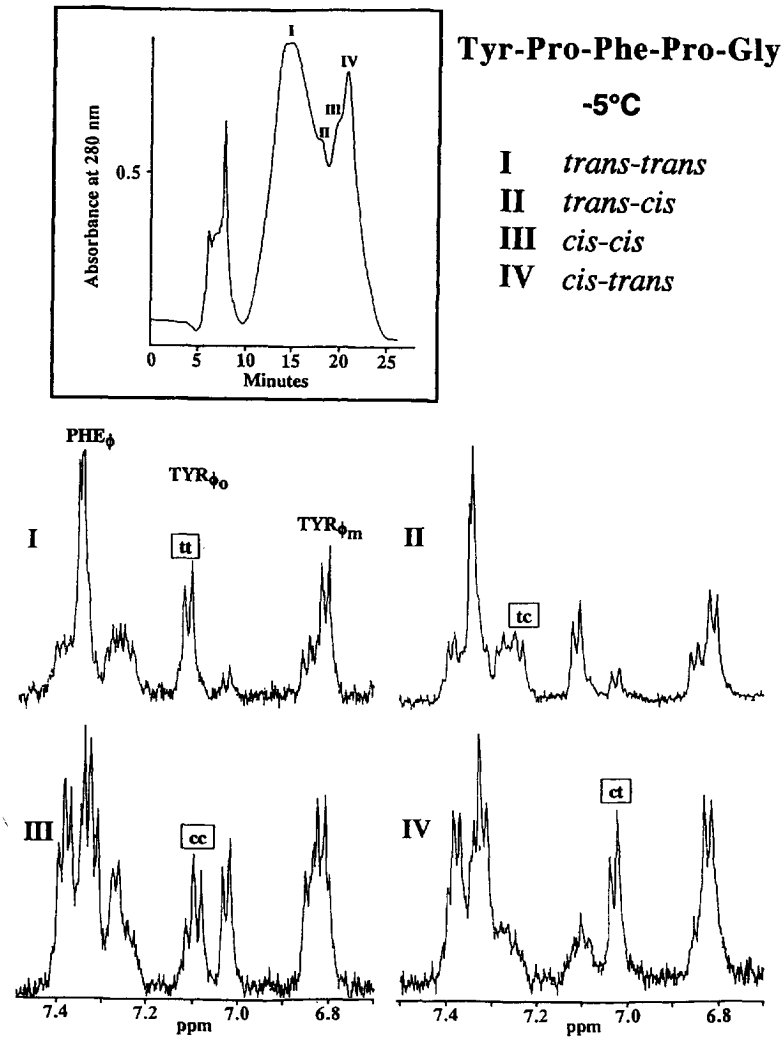

Fig. 11. Partial separation of the four conformers of β-casomorphin-5 by RP-HPLC at −5°C and their identification by ^{1}H-NMR spectroscopy. (A) Chromatogram. Column, 250×4.6 mm I.D., 5 μm Vydac ODS; eluent, 50% (v/v) deuterated methanol and 50% (v/v) deuterated water; flow-rate, 0.5 ml/min; sample, 1 mg of peptide in 100 μl of the mobile phase; injection volume, 100 μl. (B) Parts of the NMR spectra showing the signals of the aromatic moieties in the four effluent fractions containing the highest mol fraction of a particular conformer at −15°C.

conformer and its tail entails the *trans–cis* conformer. The second major peak is the *cis–trans* conformer and the *cis–cis* conformer appears as a shoulder at its front.

In the light of the above we can conclude that the two conformers having a *cis* Tyr^1-Pro^2 bond have larger hydrophobic surfaces upon binding to the stationary phase than the corresponding *trans* conformers. Furthermore the chromatographic pattern suggests that the conformational change around the Phe^3-Pro^4 bond results in a smaller difference in the hydrophobic contact surface than that around the Tyr^1-Pro^2 peptide bond.

Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH$_2$

This peptide is a dermorphin analogue and contains two rotationally hindered peptide bonds. Therefore it is expected to have, at least theoretically, four

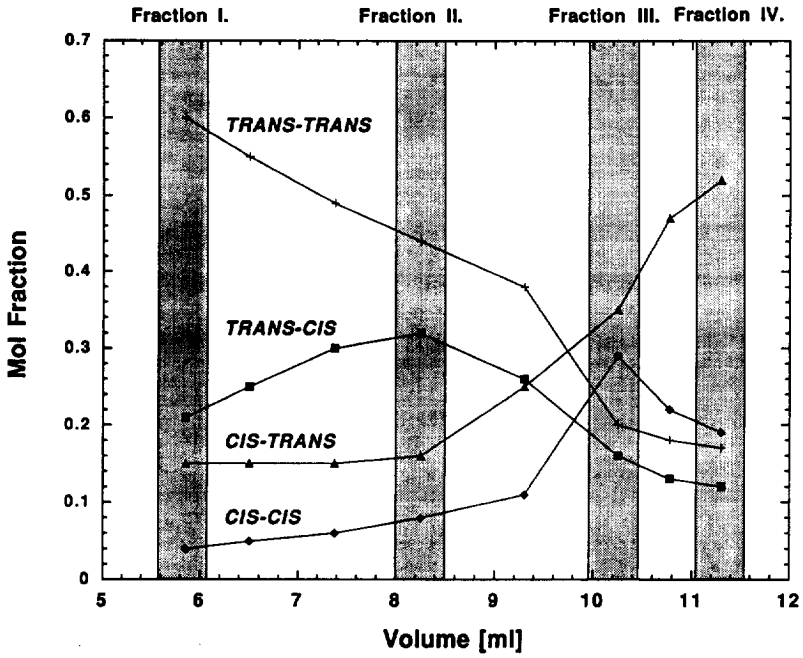

Fig. 12. Illustration of the mol fractions of β-casomorphin-5 conformers in the effluent, as determined by NMR measurements, and the fractions used in the identification of the isomers by NMR. The chromatogram and the NMR spectra of fractions I to IV are shown in Fig. 11.

relatively stable conformers and there were indeed signal sets of four conformers shown by ^{1}H-NMR. However, their identification is beset with difficulties arising from the similarity in the chemical shifts of the aromatic tyrosine protons Tyr^{1} and Tyr^{5} otherwise used throughout the study. In order to facilitate the identification of the conformers, the heptapeptide was cleaved into a tetrapeptide and tripeptide: Tyr-Tic-Phe-Gly and Tyr-Pro-Ser-NH_2. They were subjected to HPLC at −15°C and each peptide fragment yielded two peaks representing the *cis* and *trans* conformers. This finding suggests the existence of four heptapeptide conformers.

The heptapeptide Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2 was investigated by ^{1}H-NMR and ^{13}C-NMR measurements. In the ^{1}H-NMR spectrum in dimethyl sulfoxide-d_6–acetonitrile-d_3 solution four separated Tyr^{1}-OH signals can be found and the 2D-COSY in the same solvent mixture also results in four different NH-CH_α cross peaks. As seen in Fig. 13 the ^{13}C-NMR spectrum in methanol-d_4–D_2O mixture exhibits four separate signals for almost all carbons including the ProC_β and ProC_γ which facilitated the conformational assignment. Although ^{13}C-NMR spectroscopy did not allow positive identification of all four conformers of the heptapeptide it has unambiguously shown that its two major and two minor isomers have *trans* and *cis* conformation around the Tyr^{5}-Pro^{6} peptide bond, respectively.

The existence of the four conformers shown by NMR was confirmed by reversed-phase chromatography. The separation of the conformers of the heptapeptide Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2 was carried out on the Hytach column packed with 2-μm micropellicular C-18 stationary phase particles by using a mixture of 36% (v/v) methanol and 64% (v/v) 50 m*M* sodium phosphate adjusted to pH 2.5 with 60% orthophosphoric acid, as the mobile phase. The column temperature was −5°C and the flow-rate was 0.25 ml/min [14].

5. Conclusions

The isolation and analysis of the conformers of small peptides containing proline described in this

report was prompted by indications that the biological activity of the *cis* and *trans* isomers may be different. For instance, the pharmacological characterization together with structural studies by NMR spectroscopy and molecular mechanics on morphiceptin and dermorphin analogues with N-alkylated L-amino acid residues in the 2-position revealed that a *cis* Tyr-Xaa peptide bond is required for the μ receptor mediated opioid activity [14,16,17]. Evidently further progress in pursuing research in this direction mandates the development of methodologies for the separation and identification of the conformers.

In this work *cis–trans* conformers of linear peptides containing proline or another N-substituted α-amino acid, were separated at low temperatures by reversed-phase chromatography on columns packed with octadecylated silica stationary phases of different types. Preparative chromatography was carried out with columns packed with totally porous octadecylsilica stationary phases and was used to obtain the isolated peptide conformers in amounts sufficient for NMR measurements. Analytical chromatography and the test for the interconversion of the isolated conformers by rechromatography at both low and high temperatures were performed by the use of columns packed with micropellicular octadecyl silica stationary phases.

The identification of the conformers of proline dipeptides and the β-casomorphin 1–3 fragment by NMR allowed us to determine their elution order in the chromatographic experiments. It was found that in reversed-phase chromatography the *trans* conformer elutes before the *cis* conformer and this suggests that the *cis* conformer binds stronger to non-polar surfaces and therefore is more 'hydrophobic' than the *trans* isomer. This difference in the binding properties of the conformers may be of some, yet to be determined, physiological significance. Even when preparative chromatography did

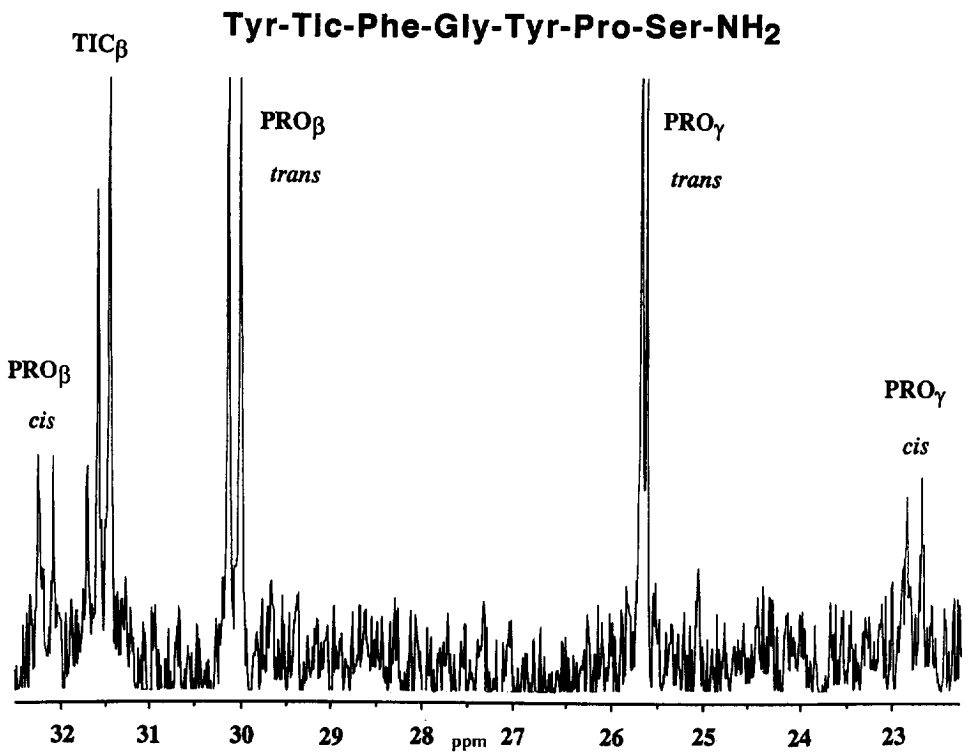

Fig. 13. Tentative identification of the Tyr-Tic-Phe-Gly-Tyr-Pro-Ser-NH_2 conformers by ^{13}C-NMR spectroscopy in 65% D_2O–35% CD_3OD (v/v) at −15°C using the $ProC_\beta$ and $ProC_\gamma$ signals.

not yield the pure conformers, e.g., with the pentapeptide β-casomorphin-5, the elution order of the four conformers could be established by NMR of eluent fractions enriched in one of the isomers as: *trans–trans*, *trans–cis*, *cis–cis* and *cis–trans*.

Acknowledgements

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