Journal of Peptide Science, Vol. 2, 325–338 (1996)

An Exploration of the Effects of Constraints on the Phosphorylation of Synthetic Protein Tyrosine Kinase Peptide Substrates

Paolo Ruzza[1], Arianna Donella-Deana[3], Andrea Calderan[1], Bruno Filippi[2], Luca Cesaro[3], Lorenzo A. Pinna[3] and Gianfranco Borin[1]

[1]CNR, Biopolymers Research Centre, Department of Organic Chemistry, University of Padua, Padua, Italy
[2]Department of Organic Chemistry, University of Padua, Padua, Italy
[3]CNR, Centre for the Study of Mitochondrial Physiology, Department of Biological Chemistry, University of Padua, Padua, Italy

Received 28 December 1995
Accepted 23 February 1996

We synthesized by classical solution methods three conformational constrained analogues of EDNEYTA, a heptapeptide sequence that represents the common major autophosphorylation site of the protein tyrosine kinases (PTKs) of the Src family. The correlation between the different structural properties induced by the modifications of the native sequence and the propensity of the peptides to act as PTK substrates was examined. The kinetic data obtained indicate that the introduction of the tyrosine-analogue constraints Tic(OH) and MeTyr, which block the ring flexibility, completely prevents the phosphorylation catalysed by the kinases Lyn and Fgr. On the other hand PTKIIB/p38^{syk} can phosphorylate the two derivatives albeit with an efficiency lower than that found with the native sequence. A third derivative contained side chain to side chain cyclization. This analogue, in which the freedom of the phenolic moiety is not altered, can be phosphorylated by all the PTKs tested with kinetic constants comparable to the parent peptide.

Keywords: conformational constraints; CD spectroscopy; fluorescence quenching; synthetic peptides; tyrosine phosphorylation

INTRODUCTION

The use of conformational, topographical and cyclic constraints constitutes a powerful approach to peptide ligand design [1], with benefits such as increased potency, receptor selectivity, or increased stability against biodegradation as compared to non-constrained molecules. For these reasons we introduced this approach to the study of the biological properties of protein tyrosine kinase (PTK) substrates. These enzymes are involved in signal transduction systems, that control cell proliferation, differentiation and/or cell-to-cell communication, and can be divided into receptor-type kinases and non-receptor-type kinases such as the Src-like kinases [2]. In particular, aberrant expression of PTKs has been directly or indirectly associated with the aetiology of a number of tumour viruses and neoplastic processes. Therefore, the modulation of their signalling pathways is a potential area of study in the development of antiproliferative and anti-cancer therapeutics.

We postulated that it should be possible to affect significantly the phosphorylating capability of these enzymes by using competitive peptide substrates as inhibitors. At present, our attention is focused on the non-receptor-type kinases of the Src family. All the

Abbreviations: AcOEt, ethyl acetate; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; DPPA, diphenylphosphoryl azide; Et_2O, diethyl ether; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; LDMS, laser desorption mass spectrometry; MeCN, acetonitrile; MeTyr, *N*-methyl tyrosine; -OBg, *N*-benzhydrylglycolamide ester; OcHx, cyclohexyl ester; $OpNo_2Bzl$, p-nitrobenzyl ester; TEA, triethylamine; TEAP, triethylamine phosphate; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran; Tic(OH), 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TMSOTf, trimethylsilytrifluoromethansulfonate.

Other abbreviations used are those recommended by the IUPAC-IUB Commission (*European J. Biochem.* 1984, *138*, 9–37).

Address for correspondence: Prof. G. Borin, Centro di Studio sui Biopolimeri del CNR, Dipartimento di Chimica Organica, Università di Padova, via Marzolo, 1, 35131 Padova, Italy.

© 1996 European Peptide Society and John Wiley & Sons, Ltd.
CCC 1075-2617/96/050325-14

PTKs encoded by cellular genes of this family contain two major phosphoacceptor sites which are homologous to the Tyr-416 and Tyr-527 of Src. The former represents the main autophosphorylation site and its phosphorylation correlates with increased kinase activity [3,4]. By using synthetic peptides with modified sequence around Tyr-416 (EDNEYTA) the influence of the side-chain charged functions and of the propensity to assume a structural conformation on the activity of different splenic PTKs was previously studied [5]. Moreover, as reported by Tinker *et al.* [6], analysis of phosphorylation sites of several proteins suggested that the phosphorylated tyrosine residues exist on surface loops or β-turns. To better examine the effect of these two structural factors we have synthesized by the classical solution method the simultaneously or alternatively N-terminal acetylated and C-terminal amidated analogues of the parent heptapeptide, its dimeric form and their corresponding cyclic analogues [7]. By testing these peptides as phosphoacceptor substrates for Fgr, an enzyme belonging to the Src family, and for PTKIIB/p68^{syk}, we have found that the N^{α}, C^{α}-blocked and cyclic monomer are poor substrates for the employed PTKs. The detrimental effect due to either cyclization or N^{α}, C^{α}-modification of the EDNEYTA is overcome by dimerization. The dramatic increase of activity cannot simply be accounted for by the presence of two tyrosyl residues in the sequence. The aim of our research was the synthesis of a very suitable peptide-substrate to be used for the specific detection of Src-like PTK either in crude extracts or in purified PTK preparations. Since a highly active peptide substrate can act as an inhibitor itself it can be used as a starting compound to further develop inhibitors and potential anticancer therapeutics. For this purpose we have synthesized by the classical method in solution three EDNEYTA analogues (Scheme 1), containing Tic(OH) and MeTyr as local constrained analogues of the tyrosine residue and a cyclic constraint obtained through side-chain to side-chain cyclization. The presence of constraints decreases the number of possible conformations of the backbone or of the sidechain providing useful information on the relation between structure and biological activity.

H · Glu · Asp · Asn · Glu · Tic(OH) · Thr · Ala · OH **A**

H · Glu · Asp · Asn · Glu · MeTyr · Thr · Ala · OH **B**

H · Glu · Asp · Asp · Glu · Tyr · Orn · Ala · OH **C**

Scheme 1 Sequences of synthetic heptapeptide analogues to the sequence 412–418 pp60$^{c\text{-}src}$.

The synthetic peptides were tested as phosphoacceptor substrates for two Src-like tyrosine kinases, Lyn and Fgr and for PTKIIB/p88^{syk}, a non-receptor tyrosine kinase implicated in the regulation of Src PTKs [8]. Lyn is expressed by the proto-oncogene *lyn* present in normal B lymphocytes and T lymphocytes transformed by either HTLV-I or herpesvirus saimiri [9], whereas Fgr is expressed in normal and malignant B lymphocytes infected with Epstein–Barr virus [9].

To study the possible correlation existing between the conformational propensities of the peptides and their capability to serve as substrates for the enzymes, CD measurements were performed both in water and in hydrophobic environments such as TFE and SDS or CTAB micelles, assumed to mimic the situation at the binding site. Further conformational information was obtained by fluorescence studies in aqueous and micellar media, using the tyrosine analogue as an intrinsic fluorescence label. In order to obtain information on the degree of exposure of the tyrosine analogues in synthetic peptides, we have performed a quenching study using a positively charged ion (Cs^+ as CsCl) and a negatively charged ion (I^- as KI).

MATERIALS AND METHODS

Synthesis and Characterization of Peptides

Melting points were determined by a Tottoli capillary apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer model 141 polarimeter equipped with a Haake model D thermostat.

Thin-layer chromatography was carried out on silica gel 60 plates F-254 using the following eluent systems: (a) cyclohexane/chloroform/acetic acid/ethanol (9:9:2:2); (b) 2-butanol/ethyl acetate/water (14:12:5); (c) chloroform/acetic acid/benzene (17:2:1); (d) methanol/chloroform (3:17).

The amino acid derivatives and peptides were located by spraying the chromatograms with ninhydrin and fluorescamine reagents for free amino groups, a modified chlorine reagent for all peptide derivatives and Pauly's reagent for free tyrosine.

The amino acid intermediates used in the synthesis were prepared according to standard procedures [10].

Analytical and preparative HPLC were performed using a Shimadzu liquid chromatographic system consisting of two LC-8A pumps, an SLC-8A system control and a SPD-10A UV detector. The sample was

injected in preparative mode with a 7125 Rheodyne injector, whereas in analytic mode by means of auto injector Shimadzu SIL-9A.

Acid hydrolyses were carried out in azeotropic hydrochloric acid containing 0.25% phenol for 22 h at 110°C in sealed evacuated vials. The enzymic hydrolysates were obtained by treatment with aminopeptidase-M in 0.1 M TRIS–HCl pH 7.73 at 37°C for 48 h. The amino acid composition of the hydrolysates were determined with a Carlo Erba 3A30 amino acid analyser.

LDMS spectra of peptides were obtained using a Bruker (Reflex™) time-of-flight mass spectrometer. One μl of liquid samples (5 μl of 0.01 mM solution in 0.1% TFA in water of synthetic peptides were mixed with 5 μl of 0.01 M dihydroxybenzoic acid in 1 : 1 v/v MeCN–water) was dried on the flat surface of a cylindrical stainless steel probe tip and irradiated with the output of an nitrogen laser (337 nm). Ions were accelerated to an energy of 20 keV in deflector mode with a positively charge of 21 keV applied to the deflector. Signals were recorded at a time resolution of 3 ns. Masses were assigned using angiotensin II as external standard. The masses were measured as peak centroid.

Coupling and Deprotection Procedures

The main procedures used for the synthesis of the peptides are as follows:

Method A: BOP Procedure. One equiv. of BOP is added at room temperature to a solution of N^{α}-protected amino acid (1 equiv.) and C^{α}-protected amino acid (1 equiv.) in dry THF in presence of 1 equiv. of DIEA (2 equiv. if the amino component is protonated). After 8 h the bulk of the solvent was evaporated and the residue distributed between AcOEt and water. The organic phase was washed consecutively with 2% $KHSO_4$, 10% $NaHCO_3$, brine and dried over Na_2SO_4, filtered and evaporated to dryness. The residue was crystallized or precipitated from appropriate solvents.

Method B: DPPA Procedure. 1.05 g (1.86 mmol) of tripeptide Z-Asp(O*t*Bu)-Glu(OcHx)-Tyr-OH was dissolved in anhydrous DMF, 1.30 g (1.86 mmol) of H-Orn(Boc)-Ala-OBg*HCl, 0.40 ml (1.87 mmol) of DPPA and 0.64 ml (3.74 mmol) of DIEA were added to the solution. The reaction was stirred for one night at room temperature, concentrated and product precipitated with water. The crude pentapeptide was dissolved in ethyl acetate–water and washed as previously reported. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was precipitated from ethyl acetate with a mixture of diethyl – petroleum ether. Yield 1.51 g (67.4%).

Method C: Cyclization Procedure. The pentapeptide **IVA** (Figure 2) (0.50 g, 0.43 mmol) was dissolved in dry DMF (430 ml), the solution was cooled to 0°C, DPPA (0.32 ml, 1.50 mmol) and K_2HPO_4 (0.37 g, 2.12 mmol) were added. The mixture was stirred three days at 4°C and one day at room temperature, after addition of 0.10 ml (0.50 mmol) of DPPA. The crude cyclic pentapeptide was precipitated with water from the concentrate solution, collected and dried over P_2O_5. The crude product was reprecipitated from MeOH with diethyl ether. Yield 0.28 g (63.7%).

Method D: Fmoc Cleavage. (a) With piperidine: Fmoc-peptides were dissolved in dry THF (10 ml) and piperidine (10% v/v) was added to the solution. After 45 min the solution was concentrated under reduced pressure and the desired N^{α}-free peptides were precipitated with diethyl ether. The solids were collected, washed extensively with ether and dried over P_2O_5. (b) With piperazine: Fmoc-peptides were dissolved in 6% piperazine in dry THF (10 ml), the mixture was stirred at room temperature for 45 min and then concentrated under reduced pressure to a small volume. The N^{α}-free peptides and the unreacted piperazine were precipitated with diethyl ether, the solid was collected and washed extensively first with water and then with diethyl ether and dried over P_2O_5.

Method E: Hydrogenolysis. Hydrogenation was carried out in MeOH at atmospheric pressure and room temperature in the presence of 10% Pd on charcoal. Routinely, the catalyst was removed by filtration through a bed of Filter Cel, the filtrate was concentrated to a small volume and the desired product precipitated with Et_2O. The resulting solid peptide was collected and dried over NaOH pellets.

Method F: TFA Treatment. The carboxy and amino terminal protecting groups as the side-chain protecting groups were removed by treating the peptide with 98% aqueous TFA containing 10% of anisole for 45 min. The crude free peptide obtained by addition of Et_2O was collected by filtration, washed extensively with diethyl ether, dried over NaOH pellets and purified by chromatographic procedures.

Method G: OBg Ester Cleavage. An aqueous solution (15 ml) of K_2CO_3 (3 equiv.) was added to a solution of peptide OBg ester 0.37 g (0.27 mmol, 1 equiv.) in DMF (15 ml) and the mixture was stirred for 90 min at room temperature. The solution was diluted with water (90 ml) and extracted with Et_2O (3 × 25 ml). The aqueous phase was collected and the C^{α}-free peptide extracted with AcOEt upon addition of 2% $KHSO_4$ to pH 2.5–3. The organic phase was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was precipitated from AcOEt with diethyl ether, yielding 0.30 g (96.1%).

Method H: OcHx Ester Cleavage. The C^{α}-free heptapeptide C was dissolved in 1 M TMSOTf in TFA in presence of 1 M thioanisole cooled in an ice bath. After 2 h the crude free peptide was precipitated with diethyl ether, collected and dried over NaOH pellets. Yield 0.19 (91.6%).

Method K: $OpNO_2Bzl$ Ester Cleavage. 1.51 g (1.81 mmol) of fully protected tripeptide Asp-Glu-Tyr **III** (Fig. 2) were treated with five-fold molar excess of zinc dust in 90% AcOH for 1.5 h at 0°C. The residue was filtered off and the filtrate was distributed between AcOEt/water. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was precipitated from ethyl acetate with diethyl ether. Yield 1.19 g (94.3%).

Purification Procedure: the crude peptides **A** and **B** were chromatographed on Sephadex G15 (145 × 2 cm) equilibrated and eluted with HCl 0.01 M, flow rate 36 ml/h, detection at 206 nm. Peptide **C** was purified using preparative HPLC. Column VYDAC C18 218TP1022 (200 × 22 mm), eluent A – 0.05% TFA in water, B – 0.05% TFA in MeCN–water 9 : 1 v/v, isocratic elution at 10% B, flow rate 15 ml/min, detection at 216 and 275 nm.

The fractions containing the desired product were collected and lyophilized to constant weight in presence of 0.01 M HCl.

Circular Dichroism and Fluorescence

The conformation equilibria of the synthetic peptides were investigated by CD spectroscopy in the far-UV in 5 mM TRIS–HCl pH 6.8, in 20 mM phosphate buffer pH 3.5 and in micellar SDS (30 mM) or CTAB (1 mM) solutions, and in the near-UV in TRIS–HCl pH 6.8 and in micellar SDS or CTAB solutions. The CD measurements were recorded with a Jasco model J-500 automatic spectropolarimeter equipped with a personal computer. The spectra are the average of at least four scans. Fluorescence measurements were performed on a Perkin–Elmer model MPF66 spectrofluorimeter equipped with a Perkin–Elmer 7300 computer. Emission in the spectral range of 290–390 nm was determined with excitation at 275 nm. With excitation at 275 nm, quenching of tyrosine emission in the spectral range of 290–390 nm due to I^- or Cs^+ interaction was also determined. The fluorescence of the solvent was always subtracted from the observed total fluorescence. The peptide concentration in fluorescence experiments never exceeded 1×10^{-6} M. In aqueous medium, fluorescence experiments were performed in 5 mM Tris–HCl. Tyrosine analogue fluorescence experiments were also performed in micellar media, i.e. SDS 30 mM and CTAB 1 mM.

Peptide concentrations were determined either by absorption spectroscopy in the near-UV or by the average recovery of amino acids from acid hydrolysates.

Protein Tyrosine Kinase Assays

Lyn, Fgr and PTKIIB/$p38^{syk}$ were obtained as previously described [5,11,12]. Tyrosine kinase assays were performed by incubating at 30°C 50 μl of a medium containing 50 mM Tris/HCl, pH 7.5, 5 mM $MnCl_2$ (Fgr was assayed in the presence of 5 mM $MgCl_2$ instead of $MnCl_2$), 20 μM [γ^{32}P]ATP (specific activity 1000 c.p.m./pmol) and 10 units of enzyme (one unit being defined as the amount of enzyme transferring 1 pmol phosphate per min to 2 mM angiotensin II). 1 μM polyLys was added in the assays containing Lyn and Fgr. Reactions were terminated by the addition of 1 ml of 1 N HCl and labelled phosphopeptides were quantified as previously described [13]. Briefly: samples were heated for 15 min at 100°C in order to hydrolyse [γ^{32}P]ATP into ^{32}Pi, which was removed by conversion into a phosphomolybdic complex and extraction with isobutanol–toluene. The radioactivity due to phosphopeptides present in the aqueous phase was then measured in a scintillation counter.

In the inhibition experiments, the derivative of angiotensin II, DRVYIHPFR, containing an additional Arg residue at the C-terminus, was used as substrate. This peptide, containing two basic residues in the sequence, can be adsorbed on phosphocellulose paper [14], while EDNEYTA analogues, used as inhibitors, cannot.

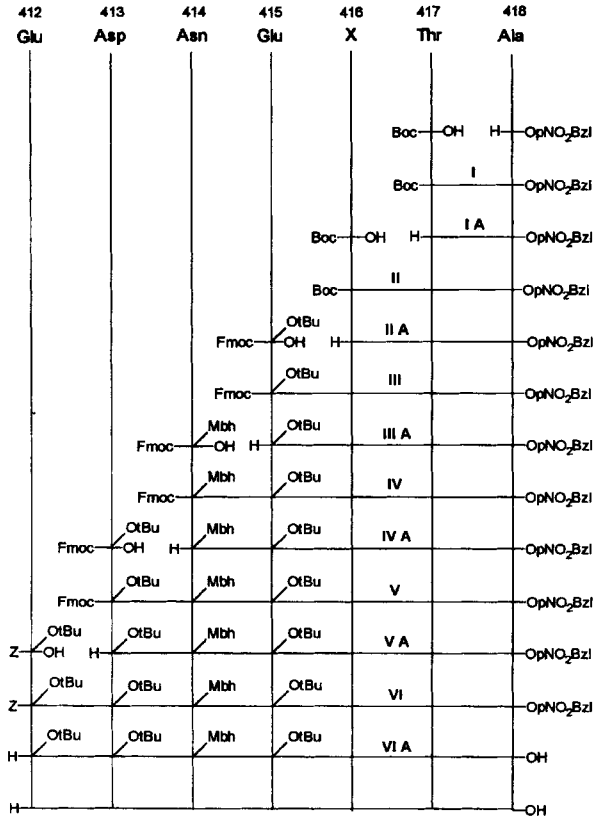

Figure 1 Synthetic scheme for the synthesis of heptapeptide analogues corresponding to the sequence 412–418 of $pp60^{c\text{-}src}$ (X = Tyr or Tic(OH) or MeTyr).

K_m and V_{max} values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis–Menten equation.

RESULTS AND DISCUSSION

Peptide Synthesis

The peptides **A**, **B** and **C** were prepared by classical solution synthetic methodologies using different strategies (Figures 1 and 2). Thus **A** and **B** were assembled using a Fmoc/*t*Bu strategy, whereas the peptide **C** was assembled using a Z/*t*Bu strategy, both with a BOP-mediated coupling in THF.

The tyrosine residue in peptide **C**, its modified residues and the threonine residue in peptides **A** and **B** were incorporated with their side-chain functions unprotected. Under the experimental conditions used we have not found appreciable acylation of the unprotected phenolic or alcoholic functions [15–17].

For the synthesis of peptides **A** and **B** the N- and C-terminal protecting groups were incorporated as Z- and -$OpNO_2Bzl$ derivatives respectively, so that cleavage of these groups could be effected concomitantly, by catalytic hydrogenation, without modifying the side-chain protecting group. In this way it is possible to synthesize the corresponding cyclic peptides.

For the synthesis of peptide **C** (Figure 2), the *p*-nitrobenzyl group was used as protection for the carboxyl group of tyrosine residue which was selectively removed with zinc dust in 90% AcOH [18]. The C-terminal amino acid was incorporated as -OBg ester. In this mode it is possible to selectively cleave the side-chain protecting group without interfering with the carboxylic protection. The fragment condensation and the side-chain to side-chain cyclization in peptide **C** were performed using the DPPA method as described under Materials and Methods. This procedure was known to yield fragment coupling with a very low degree of racemization. In peptide **C** the residues Asn-414 and Thr-417 were always substituted with Asp and Orn respectively, and the side chains of Asp-413 and Glu-415 were protected as cyclohexyl esters (-OcHx) which is undeleted under the usual conditions of cleavage of the *t*-Bu group. The 4,4′-dimethoxybenzhydryl group (-Mbh) was employed for the carboxamide protection of the asparagine residue.

The cyclization of aspartate to form aspartimide has been recognized as a substantial side reaction occurring during both synthesis and storage of peptides [19–21]. Aspartimide formation during peptide synthesis can be either acid or base-catalysed, with the kinetics of ring closure depending upon the nature and strength of the acid or base, the structure of the aspartate side-chain protecting group, and the aspartate carboxyl neighbouring residue. It had been assumed that for Fmoc-based synthesis, *tert*-butyl side-chain protection of aspartate inhibits aspartimide formation. However, an unexpectedly high extent of aspartimide and piperidide formation during the synthesis of Asp(O*t*Bu)-Asn(X)-containing peptides was recently observed when piperidine in Fmoc-chemistry was used [22,23]. This side reaction is present only in the presence of piperidine; all other bases prevented the undesired reaction [24]. Using 6% piperazine in THF for Fmoc cleavage [24] in the presence of Asp(O*t*Bu)-Asn(Mbh) we isolated the target peptides **A** and **B** as main products without any traces of aspartimide or piperidide as confirmed by MALDI-MS and enzymic hydrolyses. By enzymic hydrolyses also the possible α–β shift was excluded.

The physicochemical properties of peptides are listed in Tables 1 and 2.

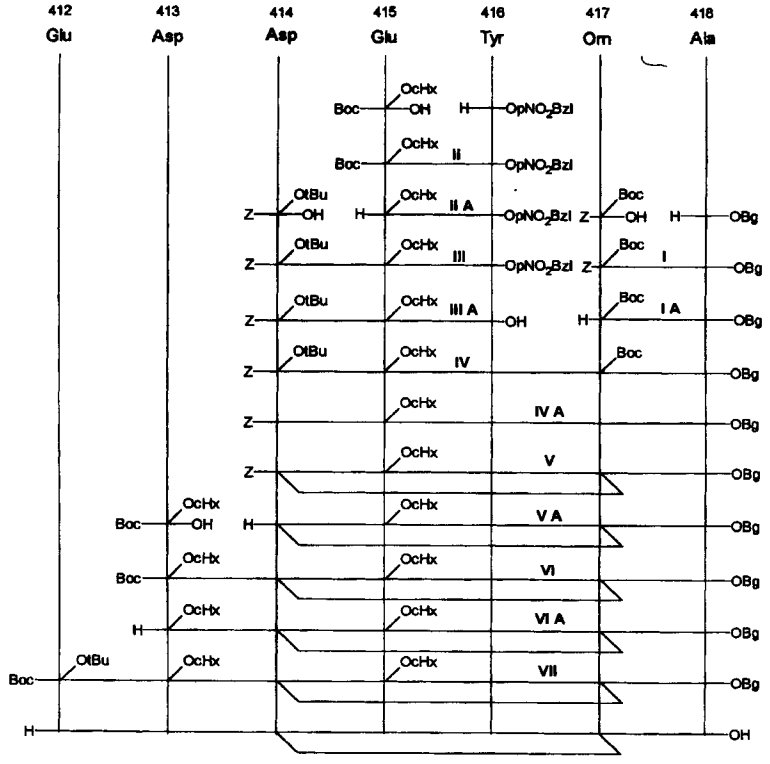

Figure 2 Synthetic scheme for the synthesis of cyclic lactam bridge analogue corresponding to the sequence 412–418 of pp60$^{c\text{-}src}$.

Peptide Design

Incorporation of the constraints into PTK substrates modifies the backbone conformation and topological properties of one or more specific amino acid residues such as to restrict one or several ϕ, ψ, ω and/or χ angles.

The Tic(OH) amino acid can be viewed as a constrained analogue of Tyr, the major difference being that it is cyclic, and as result χ_1 can exist only in the *gauche* (−) or *gauche* (+) conformations. Toniolo and coworkers [25] showed that internal L- or D-Tic residues are compatible with β-turn and α-helix structures, and in particular that the L-Tic residue has the *gauche* (+) side-chain conformation. This topology may be applied also to Tic(OH). Peptides containing this analogue possess a completely different topology in comparison with the tyrosyl

Table 1 Physicochemical Properties of Final Free Heptapeptides

Compound	[MH]$^+$	t_r[c] (min)	Amino acid ratios in acidic and enzymatic hydrolysate						
			Asp	Thr	Asn	Glu	Ala	X	Orn
A	877[d]	18.7	[a]1.97	1.03	n.d.	1.97	1.03	1.09	–
			[b]1.05	n.d.	1.02	1.01	n.d.	n.d.	
B	878[d]	20.5	[a]2.09	1.05	n.d.	1.92	1.03	1.00	–
			[b]1.05	n.d.	1.02	1.05	n.d.	n.d.	
C	837	19.6	[a]1.95	–	–	2.08	1.03	1.03	0.98

X = Tic(OH) or MeTyr.
[a]In acid hydrolysates.
[b]In enzymic hydrolysates.
[c] Elution conditions are: eluent A, 0.05% TFA in water; B, 0.05% TFA in 9 : 1 v/v MeCN–water; column VYDAC 218TP104, 250 × 4.6 mm; flow rate 1 ml/min; detection at 216 and 275 nm; isocratic elution at 5% B for 3 min and then linear gradient from 5% to 35% B in 30 min.
[d]As [MNa]$^+$.

Table 2 Physicochemical Properties of Peptide Intermediates

Peptide	m.p. (°C)	$[\alpha]^{D}_{25}$ (°)[a]	Recryst. solvent[b]	TLC R_f (system)	Amino acid ratios Asp	Thr	Glu	Ala	X	Orn
I	119	−10.10	Et_2O–PE	0.14(a) 0.64 (b) 0.82 (d)	–	1.01 (1)	–	0.99 (1)	–	–
II Tic(OH)	64	−23.95	AcOEt–IE	0.77 (a) 0.40 (c)	–	1.00 (1)	–	1.00 (1)	1.15 (1)	–
III Tic(OH)	116	−40.30	AcOEt	0.65 (a) 0.58 (d)	–	0.94 (1)	1.11 (1)	0.90 (1)	1.06 (1)	–
IV Tic(OH)	84	−19.02	AcOEt–Et_2O	0.60 (a) 0.88 (b)	1.08 (1)	0.96 (1)	1.03 (1)	0.93 (1)	1.00 (1)	–
V Tic(OH)	132	−18.00	AcOEt–Et_2O	0.67 (a)	2.05 (2)	0.97 (1)	1.05 (1)	0.96 (1)	0.98 (1)	–
VI Tic(OH)	123	−48.93	AcOEt–Et_2O	0.60 (a)	2.01 (2)	1.04(1)	1.96 (2)	1.02 (1)	1.14 (1)	–
II MeTyr	69	−15.64	AcOEt-IE	0.53 (a)	–	0.96 (1)	–	1.10 (1)	0.93 (1)	–
III MeTyr	84	−32.60	AcOEt–Et_2O	0.68 (a) 0.46 (c)	–	1.00 (1)	1.00 (1)	0.98 (1)	0.98 (1)	–
IV MeTyr	117	−28.61	AcOEt–Et_2O	0.67 (a) 0.45 (c)	0.98 (1)	0.99 (1)	1.04 (1)	0.97 (1)	0.99 (1)	–
V MeTyr	123	−35.50	AcOEt–Et_2O	0.68 (a)	1.95 (2)	1.02 (1)	1.07 (1)	1.02 (1)	0.99 (1)	–
VI MeTyr	101	−39.47	AcOEt–Et_2O	0.61 (a) 0.31 (c)	2.08 (2)	0.98 (1)	1.99 (2)	0.97 (1)	0.99 (1)	–
I Lactam	95	−9.35	Et_2O–PE	0.86 (a) 0.62 (c)	–	–	–	0.99 (1)	–	1.02 (1)
II Lactam	–	–	Oil	0.81 (a)	–	–	1.02 (1)	–	0.99 (1)	–
III Lactam	112	−3.98	Et_2O–PE	0.62 (a) 0.58 (c)	1.05 (1)	–	1.08 (1)	0.99 (1)	–	–
IV Lactam	91	−12.50	AcOEt–Et_2O/PE	0.75 (a) 0.96 (d)	1.08 (1)	–	1.07 (1)	0.92 (1)	1.00 (1)	0.95 (1)
V Lactam	218	−25.55	MeoH–Et_2O	0.48 (c)	1.09 (1)	–	1.09 (1)	1.00 (1)	1.01 (1)	0.99 (1)
VI Lactam	–	–	Oil	0.58 (a) 0.90 (d)	1.95 (2)	–	1.01 (1)	1.01 (1)	0.99 (1)	0.99 (1)
VII Lactam	215	−26.58	AcOEt–Et_2O/PE	0.79 (a)	1.98 (2)	–	2.07 (2)	1.05 (1)	0.98 (1)	0.97 (1)

[a]Solvent DMF, c = 1.
[b]AcOEt, ethyl acetate; Et_2O, diethyl ether; PE, petroleum ether; IE, diisopropyl ether.

analogue: the χ_1 and χ_2 values of Tic(OH) are in fact different from those of a Tyr residue in that position [26].

The introduction of a methyl group to the tyrosine amino group restricts its conformation and also the conformation of the preceding residue [27]. Studies carried out on model dipeptides by Marraud and coworkers have shown the tendency of *N*-methyl residues to participate in different turn conformations, depending on the chirality of the sequence and the *trans* or *cis* configuration of the tertiary amide bond [28]. In analogy with MePhe [29], for an internal MeTyr residue all the three side-chain conformations, $g(-)$, $g(+)$ and t are possible, even if the t and $g(-)$ are more probable.

Among the various methods that are available for preparing constrained peptides, cyclization is a major possibility since cyclic peptides are expected to adopt conformations which are better defined than their linear counterparts. As previously reported [7], end-to-end cyclization of the heptapeptide EDNEYTA induces a rigid conformation with a loss of biological activity. Another type of cyclization is constituted by the side-chain lactam bridge, where a covalent bond between the side chain of aspartic or glutamic acid and the side chain of lysine or ornithine is present. Side-chain cyclizations not involving disulphides generally are not found in nature, but they can be important tools in structure–function studies with synthetic peptides. For this purpose, the native sequence EDNEYTA was modified to Glu-Asp-**Asp**-Glu-Tyr-**Orn**-Ala and the i, $i+3$ lactam bridge was obtained between aspartic acid in position 3 and ornithine in position 6 (shown in bold characters). This lactam forms a 16-member ring which may impart steric strain into the peptide with consequent side-chain 'protrusions' of the amino acids involved in the ring.

Fluorescence and CD Studies

All four peptides interact with the cationic CTAB micelle system, as monitored by the strong increment of emission of the phenolic rings, in comparison with the emission in aqueous solution (Figure 3). With the anionic SDS system, however, only the cyclic analogue **C** (Figure 3 shows a significant increment of phenolic emission, compared to the aqueous medium. The forces governing the binding of the peptides to the micellar systems may be constituted by hydrophobicity and ionic interactions. The anionic character of the peptides may be the determining

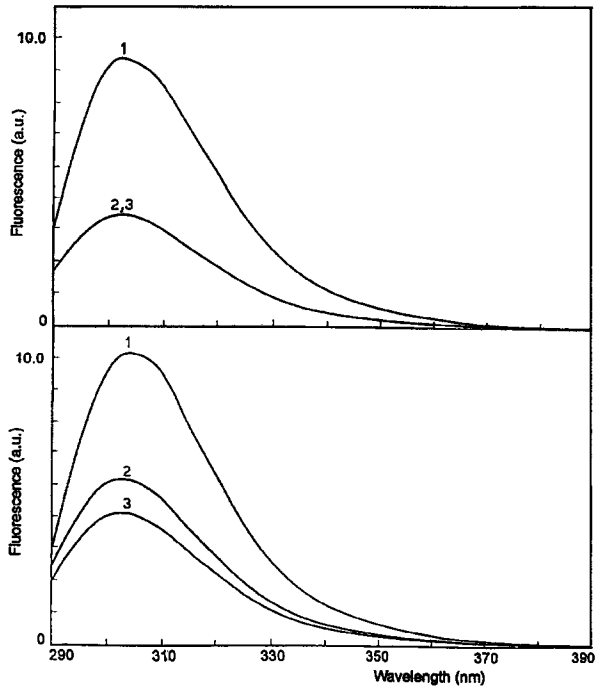

Figure 3 Fluorescence emission spectra of Tic(OH) peptide (top) and of cyclic lactam bridge heptapeptide **C** (bottom) in: (1) CTAB 1 mM; (2) SDS 30 mM; (3) *Tris*–HCl 5 mM, pH 6.8. Experimental conditions are described in Materials and Methods.

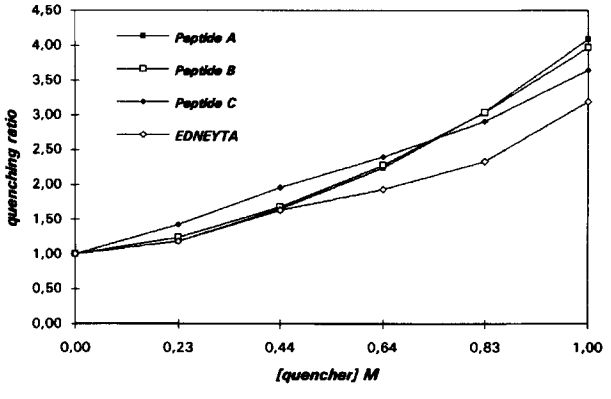

Figure 4 Iodide–caesium quenching ratio at increase of quencher concentration.

parameter for the interaction with CTAB, and is also responsible for the small or null effect with SDS.

To further investigate the environment of the aromatic residues, fluorescence quenching experiments were performed with Cs^+ and I^- aqueous solutions. With both quenching agents, the efficiency follows the order: Tyr > Tyr (**C**) > MeTyr > Tic(OH).

The parallelism of the quenching efficiency with the two ions, which bear opposite charges, indicates

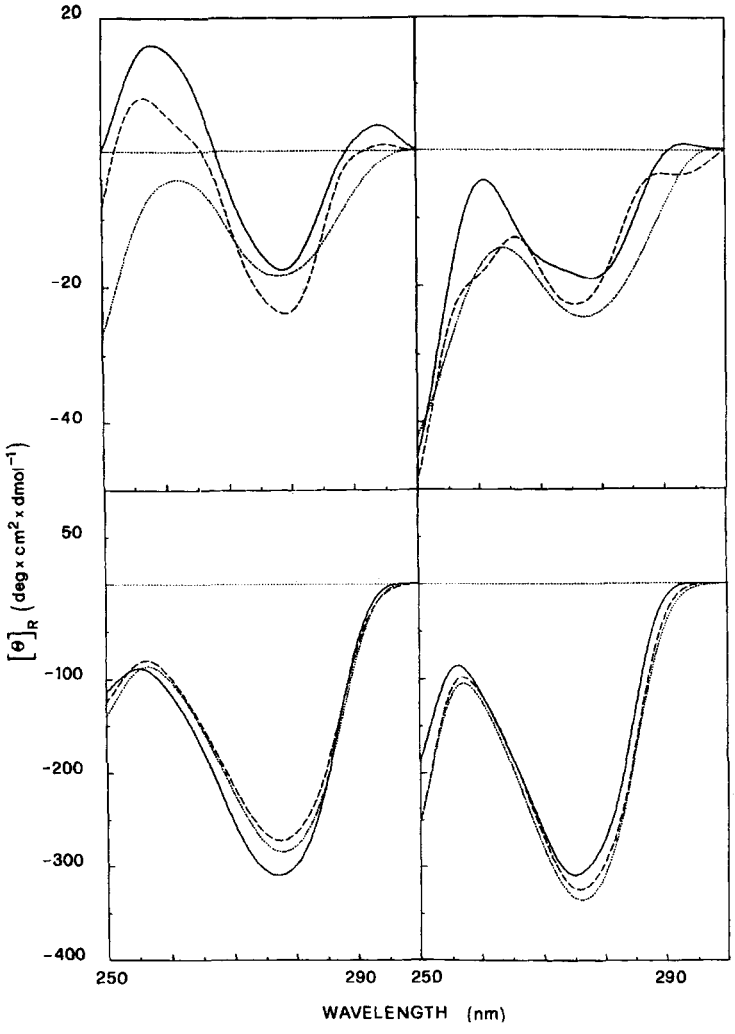

Figure 5 Near-UV CD spectra of Tyr (2.6×10^{-4} M) (top left), cyclic lactam bridge (1.7×10^{-4} M) (top right), Tic(OH) (3.1×10^{-4} M) (bottom left), and MeTyr (4.7×10^{-4} M) (bottom right) heptapeptides. — TRIS–HCl 5 mM, pH 6.8; ---- SDS 30 mM; ···· CTAB 1 mM. Experimental procedures are described in Materials and Methods.

that no phenolic rings are influenced by surrounding charges in a significantly different way from all the others (Figure 4).

The properties of the aromatic ring have been further investigated by circular dichroism measurements in the near-UV absorption region, in aqueous solutions and in the two micellar systems (Figure 5).

In aqueous solution, the CD spectra of the two tyrosyl rings, EDNEYTA and peptide **C**, are characterized by a negative maximum at 275–280 nm, and by a positive maximum (or a negative minimum) at 250 nm. As a consequence of the interaction with the SDS and CTAB micelles, the biggest changes in the CD spectra are between the two tyrosyl peptides; in contrast, the three CD spectra of the Tic(OH) and MeTyr peptides are very similar, with a well-defined negative maximum at 275 nm, and seem practically insensitive to the micellar systems.

The conformational properties of the peptides were investigated by CD spectroscopy in the far-UV region. In this region, both the peptide group and the aromatic ring contribute to the CD spectra, and the two contributions cannot be estimated separately. In spite of this limitation, some qualitative information can be obtained. In aqueous solution, the CD spectra of all the peptides are characterized by a negative maximum at 195 nm, which is assumed to monitor the presence of unordered conformations. In addition, a further band is observed at 220–230 nm, containing both the CD of the La band of the phenolic

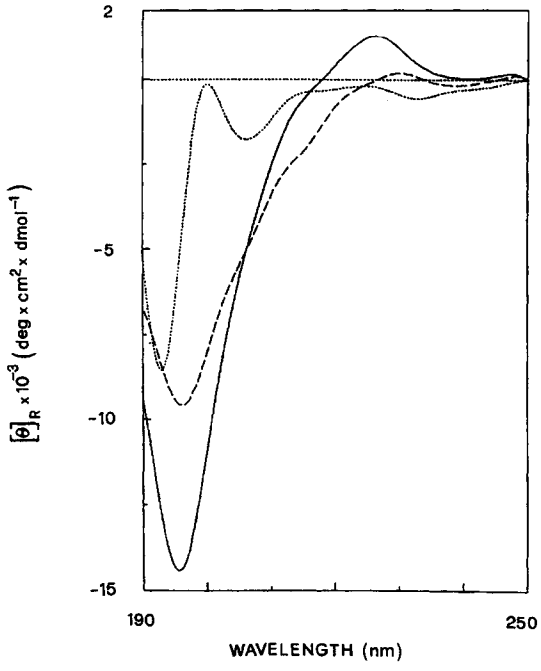

Figure 6 Far-UV CD spectra of EDNEYTA, peptide concentration was 4.7×10^{-5} M. — TRIS–HCl 5 mM, pH 6.8; ---- SDS 30 mM; ···· CTAB 1 mM. Experimental procedures are described in Materials and Methods.

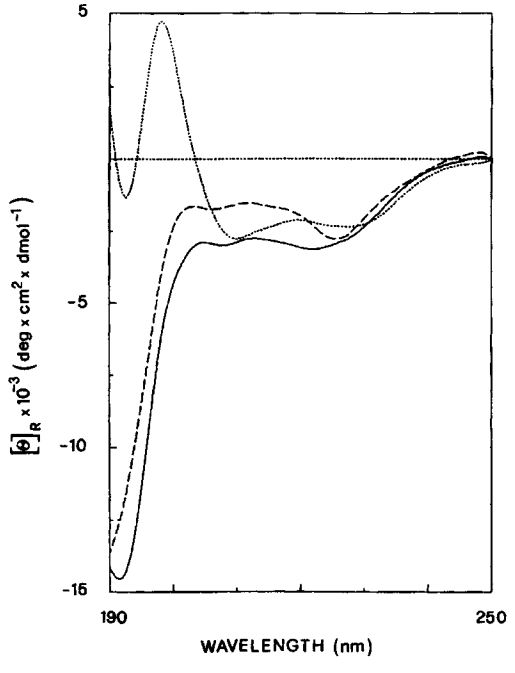

Figure 7 Far-UV CD spectra of lactam bridge heptapeptide, peptide concentration was 4.7×10^{-5} M. — TRIS–HCl 5 mM, pH 6.8; ---- SDS 30 mM; ···· CTAB 1 mM. Experimental procedures are described in Materials and Methods.

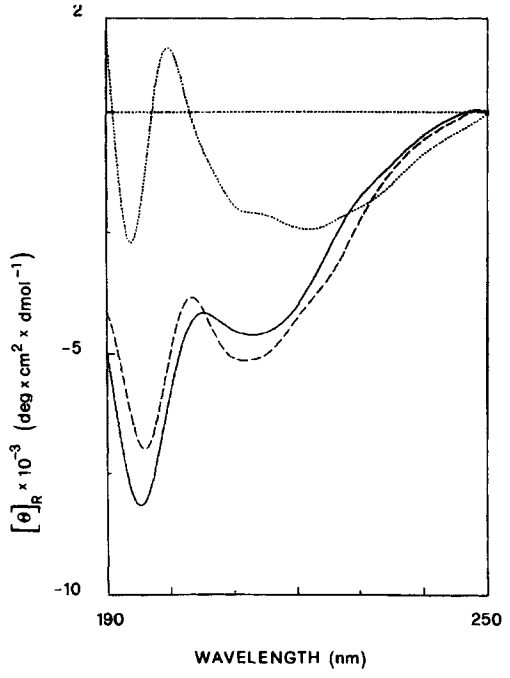

Figure 8 Far-UV CD spectra of Tic(OH) heptapeptide, peptide concentration was 5.9×10^{-5} M. — TRIS–HCl 5 mM, pH 6.8; ---- SDS 30 mM; ···· CTAB 1 mM. Experimental procedures are described in Materials and Methods.

ring and the n–π^* band of peptide chromophores. This band has the opposite sign in EDNEYTA (Figure 6) and in the cyclic lactam analogue (Figure 7), and is much more pronounced in both the Tic(OH) (Figure 8) and MeTyr (Figure 9) analogues. Minor variations are observed between pH 6.8 to 3.0 (data not shown) and in SDS, while in the presence of CTAB micelles all the CD spectra undergo significant changes, mainly consisting in the disappearance or reduction of the negative band at 200 nm. The resulting spectra may indicate the presence of some locally ordered structures.

Synthetic Peptides as substrates for PTKs

The peptide EDNEYTA and its derivatives have been assayed for their ability to serve as phosphoacceptor substrates for Lyn and Fgr, two PTKs belonging to the Src family as well as for the non-receptor tyrosine kinase PTKIIB/p38^{syk}. The last enzyme, which corresponds to the catalytic domain of p72^{syk}, exhibits a specific activity six-fold higher than that of p72^{syk} (Brunati *et al.*, manuscript submitted) and has been involved in the recruitment of Src PTK substrates ([8] and Ruzzene *et al.*, manuscript submitted). The time courses and the kinetics of peptide phosphorylation

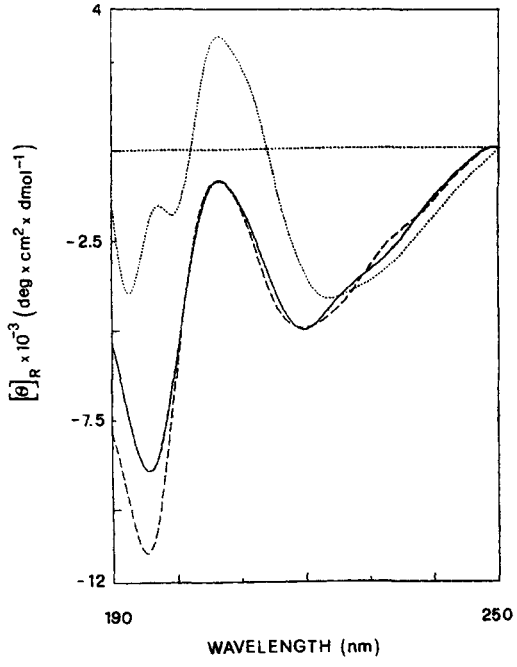

Figure 9 Far-UV CD spectra of MeTyr heptapeptide, peptide concentration was 4.3×10^{-5} M. — TRIS-HCl 5 mM, pH 6.8; ---- SDS 30 mM; ···· CTAB 1 mM. Experimental procedures are described in Materials and Methods.

indicate that the activities displayed by the tyrosine kinases vary depending on the different conformational constraints included in the sequence. The Src-like PTKs Lyn and Fgr can phosphorylate only the lactam bridged analogue (Figure 10) with an efficiency similar to that displayed towards the parent peptide (Table 3). Lyn and Fgr could not transfer the γ^{32}P from the ATP donor to the Tic(OH) and MeTyr derivatives nor could their catalytic domain recognize the two peptides, despite the presence in the sequences of positive determinants specific for the two Src-like PTK substrate recognition [5,30]. Indeed Tic(OH) and MeTyr analogues do not act as inhibitors of Lyn and Fgr activities tested on the peptide angiotensin II (data not shown).

The presence of constraints in the peptide is less detrimental for PTKIIB/p38^{syk} than for Lyn and Fgr. Indeed, PTKIIB/p38^{syk}, besides being active on the lactam-bridged peptide, can recognize also the Tic(OH) and the MeTyr analogues of the tyrosine residue as substrates (Figure 11). However the reduced chain flexibility of these derivatives induces a decrease of the enzyme affinity, as judged by the K_m values obtained for Tic(OH) and MeTyr which are about 6 and 22-fold higher respectively than that found with EDNEYTA (Table 4).

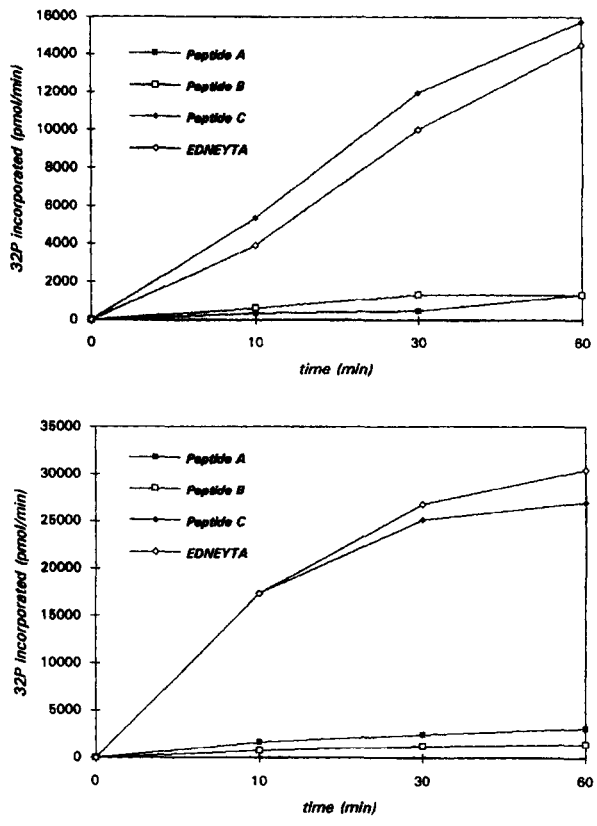

Figure 10 Time courses of synthetic peptides phosphorylation by Lyn (top) and Fgr (bottom) tyrosine kinases. Peptide concentration was 100 μM. Values are the means of three separate experiments; the SE value was always less than 17%. Other experimental conditions are described in Materials and Methods.

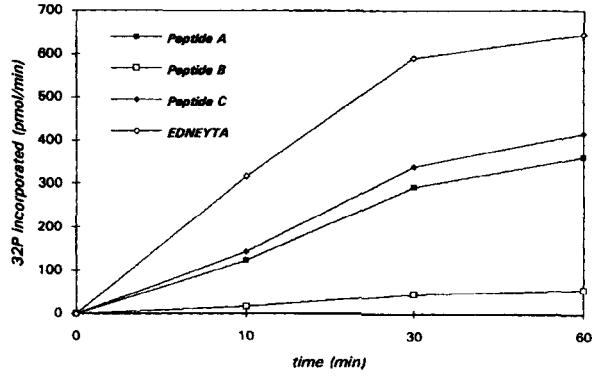

Figure 11 Time courses of synthetic peptides phosphorylation by PTKIIB/p38^{syk}. Peptide concentration was 100 μM. Values are the means of three separate experiments; the SE value was always less than 14%. Other experimental conditions are described in Materials and Methods.

Table 3 Kinetic Constants for Src-like Tyrosine Kinases with Synthetic Peptide Substrates

Peptide	Lyn			Fgr		
	V_{max} (nmol/min/mg)	K_m (mM)	Efficiency (V_{max}/Km)	V_{max} (nmol/min/mg)	K_m (mM)	Efficiency (V_{max}/Km)
EDNEYTA	810	64	12.7	1150	135	8.5
EDc(DEY-Orn)A	1451	81	17.9	1169	142	8.2

The values shown are the means of three separate determinations. For each of these means the SE value was less than 14% of the mean. Kinetic constants relative to peptides **A** and **B** were not detectable.

Table 4 Kinetic Constants for PTKIIB/p38^{syk} with Synthetic Peptide Substrates

Peptide	V_{max} (nmol/min/mg)	K_m (mM)	Efficiency (V_{max}/Km)
EDNEYTA	1907	70	27.2
EDNE-Tic(OH)-TA	2519	400	6.3
EDNE-MeTyr-TA	1229	1560	0.8
EDc(DEY-Orn)A	1846	190	9.7

The values shown are the means of three separate determinations. For each of these means the SE value was less than 16% of the mean.

DISCUSSION

Tic(OH) and MeTyr residues have successfully been substituted in opioid peptides for Tyr in order to increase the binding selectivity towards the receptors and the efficiency of the pharmacological response [29]. However, the same constraints included in a potentially phosphorylatable sequence turn out to be negative determinants for the two Src-like tyrosine kinases tested, Lyn and Fgr. By examining the phosphorylation efficiency of Lyn on synthetic peptide substrates, characterized by large modifications in the primary structure of the parent sequence EDNEYTA, Ruzza *et al.* [7] pointed out the flexibility of the peptide chain as more important than a particular secondary structure in influencing the Lyn phosphorylation process. This suggestion was further substantiated by the finding that the introduction of constraints in the peptide chain, produced by end-to-end cyclization of the linear heptapeptide, was detrimental for the Lyn-catalysed phosphorylation of the resulting cyclic analogue [7]. The present results reinforce the previous data and further indicate that the phenolic ring itself must be free from steric constraints in order to be recognized by both Lyn and Fgr, the other Src-related PTK tested. Indeed, in spite of the presence of positive recognition motives for the two kinases in the sequence, the two analogues hinder the catalytic enzyme domain from binding the peptides, which are also ineffective as inhibitors.

The phosphorylation data are supported by the conformational results obtained, which indicate that all the peptides are capable of undergoing conformational changes when the medium is perturbed. Fluorescence data show that all the phenolic side chains are influenced practically in the same manner by micelles and quenchers. The only significant difference in the behaviour of the aromatic ring is found in the CD spectra in the aromatic absorption region; in the media investigated, only the CD spectra of the Tic(OH) and MeTyr residues are insensitive to the change of solvents. Since the two residues produce inactivation of the peptides as substrates for the Src-like PTKs Lyn and Fgr, it seems reasonable to connect the biological inactivity of these enzymes to the rigidity of the aromatic residues.

Interestingly, the reduced chain flexibility of the peptide substrate is less detrimental for the non-receptor tyrosine kinase PTKIIB/p38^{syk} than for the other kinases tested. In fact, PTKIIB/p38^{syk} can phosphorylate the Tic(OH) and MeTyr analogues with V_{max} values in the same range as those shown by the parent peptide. Nevertheless, the affinity displayed by this enzyme towards the Tic(OH) analogue is favourable enough to designate this peptide as a good specific substrate for PTKIIB/p38^{syk}. Considering that signalling through the B-cell and T-cell antigen receptors (BCR and TCR, respectively) results in a rapid and almost simultaneous activation of Syk and Src-like PTKs [31,32] and that Syk is involved in the recruitment of Src substrates [8], the Tic(OH) peptide can be considered as a useful tool to assess *in vitro* the activity specifically belonging to Syk.

The kinetic constants shown by all the tyrosine kinases tested on the lactam-bridged derivative are similar to those of the parent peptide. This finding indicates that the partial peptide cyclization induces

a steric strain in the sequence which does not alter the freedom of the phenolic ring. The phosphorylability of this derivative correlates with the conformational studies, which confirm a protrusion of the tyrosyl residue.

Considering that cyclic peptides can enter the cell membrane more easily and are more resistant to metabolic degradation, the cyclic derivative can be considered a substrate more suitable than EDNEYTA for testing PTK activity in cells or crude extracts. Moreover, the cyclic sequence may serve as a more appropriate precursor than the parent peptide for the synthesis of inhibitors, which are specific for the PTK catalytic domains.

Acknowledgements

We are most grateful to Mr U. Anselmi and E. De Menego for skillful technical assistance and for performing amino acid analyses. This work was supported by CNR (Target Projects on Fine Chemistry and Progetto Strategico Tecnologie Chimiche Innovative to BG and ACRO), Italian MURST, AIRC and Italian Ministry of Health (Project AIDS).

REFERENCES

1. M. L. Moore in: *Synthetic Peptides. A user's guide*, G. A. Grant, Ed., p. 9–75, W. H. Freeman, New York 1992.
2. T. Hunter and J. A. Cooper (1985). Protein-tyrosine kinases. *Annu. Rev. Biochem. 54*, 897–930.
3. T. E. Kmiecik, P. J. Johnson and D. Shalloway (1988). Regulation by the autophosphorylation site in overexpressed pp60$^{c\text{-}src}$. *Molec. Cell. Biol. 8*, 4541–4546.
4. J. A. Cooper and B. Howell (1993). The when and how of Src regulation. *Cell 73*, 1051–1054.
5. A. Donella Deana, O. Marin, A. M. Brunati and L. A. Pinna (1992). Selective effect of poly(lysine) on the enhancement of the Lyn tyrosine protein kinase activity. *Eur. J. Biochem. 204*, 1159–1163.
6. D. A. Tinker, E. A. Krebs, I. C. Feltham, S. K. Attak-Poku and V. S. Ananthanarayanan (1988). Synthetic β-turn as substrates for a tyrosine protein kinase. *J. Biol. Chem. 263*, 5024–5026.
7. P. Ruzza, A. Calderan, B. Filippi, B. Biondi, A. Donella-Deana, L. Cesara, L. A. Pinna and G. Borin (1995). Linear and cyclic synthetic peptides related to the main autophosphorylation site of Src tyrosine kinase as substrates and inhibitors of Lyn. *Int. J. Peptide Protein Res. 45*, 529–536.
8. A. M. Brunati, M. Ruzzene, P. James, B. Guerra and L. A. Pinna (1995). Hierarchical phosphorylation of a 50-kDa protein by protein tyrosine kinase TPK-IIB and c-Fgr, and its identification as HS1 hematopoietic-lineage cell-specific protein. *Eur. J. Biochem. 229*, 164–170.
9. T. Mustelin in: *Src Family Tyrosine Kinases in Leukocytes*, R. G. Landes, Ed., CRC Press, Boca Raton, FL 1994.
10. E. Wünsch in: *Houben-Weyl Methoden der Organischen Chemie*, vol. 15, E. Wünsch, Ed., Georg-Thieme Verlag, Stuttgart 1974.
11. A. M. Brunati, P. James, A. Donella-Deana, B. Matoskova, K. C. Robbins and L. A. Pinna (1993). Isolation and identification of two protooncogene products related to c-*fgr* and *fyn* in a tyrosine-protein-kinase fraction of rat spleen. *Eur. J. Biochem. 216*, 323–327.
12. A. M. Brunati, G. Allee, O. Marin, A. Donella-Deana, L. Cesaro, C. Bougeret, R. Fagard, R. Benarous, S. Fischer and L. A. Pinna. (1992). Spleen protein tyrosine kinases TPK-IIB and CSK display different immunoreactivity and opposite specificities toward c-*src*-derived peptides. *FEBS Lett. 313*, 291–294.
13. F. Meggio, A. Donella and L. A. Pinna (1976). A new procedure for the measurement of the protein-kinase catalyzed incorporation of the γ-^{32}P-phosphoryl group of ATP into phosphoproteins and phosphopeptides. *Anal. Biochem. 71*, 583–587.
14. D. B. Glass, R. A. Masaracchia, J. R. Feramisco and B. E. Kemp (1978). Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem. 87*, 566–575.
15. P. Ruzza, A. Donella Deana, A. Calderan, M. Pavanetto, L. Cesaro, L. A. Pinna and G. Borin (1995). Synthetic Tyr-phospho and non hydrolyzable phosphonopeptides as PTKs and TC-PTP inhibitors. *Int. J. Peptide Protein Res. 46*, 535–546.
16. A. Fournier, W. Danho and A. M. Felix (1989). Applications of BOP reagent in solid phase peptide synthesis. *Int. J. Peptide Protein Res. 33*, 133–139.
17. A. Fournier, C. Wang and A. M. Felix (1988). Applications of BOP reagent in solid phase synthesis. *Int. J. Peptide Protein Res. 31*, 86–87.
18. K. Suzuki, N. Endo, K. Nitta and Y. Sasaki in: *Peptide Chemistry 1976*, T. Nakajima, Ed., p. 45–48, Protein Research Fondation, Osaka 1977.
19. G. Barany and R. B. Merrifield in: *The Peptides*, Vol. 2, E. Gross and J. Meienhofer, Eds., p. 1–284, Academic Press, New York 1979.
20. M. Bodanszky and J. Martinez in: *The Peptides*, Vol. 5, E. Gross and J. Meienhofer, Eds., p. 111–216, Academic Press, New York, 1983.
21. S. Capasso, L. Mazzarella, F. Sica, A. Zagari and S. Salvadori (1992). Spontaneous cyclization of the aspartic acid side chain to the succinimide derivative. *J. Chem. Soc. Chem. Commun.* 919–921.
22. M. Bodanszky, J. C. Tolle, S. S. Deshmane and M. Bodanszky (1978). Side reactions in peptide synthesis. VI. *Int. J. Peptide Protein Res. 12*, 57–68.
23. J. L. Lauer, C. G. Fields and G. B. Fields (1994).

Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. *Lett. Peptide Sci. 1*, 197–205.

24. R. Dolling, M. Beyermann, H. Haenel, F. Kernchen, E. Krause, P. Franke, M. Brudel and M. Bienert in: *Peptides 1994*, Hernani L. S. Maia, Ed., p. 244–245, ESCOM, Leiden 1995.
25. G. Valle, W. M. Kazmierski, M. Crisma, G. M. Bonora, C. Toniolo, and V. J. Hruby (1992). Constrained phenylalanine analogues. *Int. J. Peptide Protein Res. 40*, 222–232.
26. V. J. Hruby (1993). Conformational and topographical considerations in the design of biologically active peptides. *Biopolymers 33*, 1073–1082.
27. J. Rizo and Gierasch (1992). Constrained peptides: models of bioactive and protein structures. *Annu. Rev. Biochem 61*, 387–418.
28. A. Aubry and M. Marraud (1989). Pseudopeptide and β folding: X-ray structures compared with structures in solution. *Biopolymers 28*, 109–122.
29. P. W. Schiller, T. M. D. Nguyen, G. Weltrowska, B. C. Wilkes, B. J. Marsden, C. Lemieux and N. N. Chung (1992). Differential stereochemical requirements of μ vs. δ opioid receptors for ligand binding and signal transduction: development of a class of potent and highly δ-selective peptide antagonist. *Proc. Natl Acad Sci. USA 89*, 11871–11875.
30. O. Marin, A. Donella-Deana, A. M. Brunati, S. Fischer and L. A. Pinna (1991). Peptides reproducing the phosphoacceptor sites of $pp60^{c\text{-}src}$ as substrates for TPK-IIB, a splenic tyrosine kinase devoid of autophosphorylation activity. *J. Biol. Chem. 266*, 17798–17803.
31. S. J. Corey, A. L. Burkhardt, J. B. Bolen, R. L. Geahlen, L. S. Tkatch and D. J. Tweardy (1994). Granulocyte colony-stimulating factor receptor signaling involves the formation of three-component complex with Lyn and Syk protein-tyrosine kinases. *Proc. Natl Acad. Sci. USA 91*, 4683–4687.
32. K. Nagai, M. Takada, H. Yamamura and T. Kurosaki (1995). Tyrosine phosphorylation of Shc is mediated through Lyn and Syk in B cell receptor signaling. *J. Biol. Chem. 87*, 6824–6829.