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Linear and Cyclic Peptides As Substrates For Lyn Tyrosine Kinase

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Abstract: Two Tyr residues are supposed to play a crucial role in the regulation of protein tyrosine kinases of the Src family. Autophosphorylation of Src Tyr416 correlates with enzyme activation, while phosphorylation of C-terminal Tyr527 by Csk gives rise to inactive forms of Src kinases.

It has previously been demonstrated that the Src-like tyrosine kinase expressed by the oncogene *lyn* displays a particularly high affinity (K_m 20 μm) toward the dimeric linear and cyclic derivatives of the heptapeptide H-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-OH which reproduces the main autophosphorylation site of most of the Src enzymes. Under the experimental conditions used only one Tyr residue of the dimeric sequence can be phosphorylated [P. Ruzza, A. Calderan, B.Filippi, B. Biondi, A. Donella Deana, L. Cesaro, L. A. Pinna & G. Borin (1995) *Int. J. Peptide Protein Res. 45*, 529–539].

The present study addresses the problem of the efficiency displayed by Lyn towards the two Tyr residues located at positions 5 and 12 of the dimeric peptide. To this purpose, two tetradecapeptides were synthesized by the classical solution method, each containing one of the two Tyr residues alternatively replaced by Phe, and the corresponding univocal cyclic form. A possible correlation between the different structural properties induced by the modifications of the native sequence and the ability of the peptides to act as Lyn substrates was noted. The kinetic data obtained indicate that Lyn phosphorylates the residues located at different positions in the two linear analogues differently. In particular, while the Tyr5, Phe12 derivative presents a K_m value similar to those obtained for the dimeric linear and cyclic unmodified analogues, the K_m value of the Phe5, Tyr12 derivative is two-fold higher than those found for the above-mentioned peptides. Moreover, as previously reported for the linear and cyclic dimeric forms of the native sequence, in the mono-tyrosine containing series of dimers the still conformationally flexible cyclic derivative shows a phosphorylation efficiency two-fold higher than those found for the linear derivatives. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: conformational studies; -OBg esters; protein tyrosine kinase; *src*–PTK; synthetic peptides; tyrosine phosphorylation

Abbreviations: AcOEt, ethyl acetate; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; tBu, *tert*-butyl; CD, circular dichroism; DIEA, *N,N*-diisopropylethylamine; DPPA, diphenylphosphoryl azide; Et_2O, diethyl ether; HOBt, 1-hydroxybenzotriazole; HOSu, N-hydroxysuccinimide; HPLC, high-performance liquid chromatography; LDMS, laser-desorption mass spectrometry; MeCN, acetonitrile; OBg, *N*-benzhydryl-glycolamide-oxy; OtBu, *tert*-butyl-oxy; PE, petroleum ether; SDS, sodium dodecyl sulphate; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TEAP, triethylamine phosphate; TFE, 2,2,2-trifluoroethanol; TLC, thin-layer chromatography; Z, benzyloxycarbonyl. Other abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem. 138*, 9–37.

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INTRODUCTION

The phosphorylation degree of specific Tyr residues in a variety of cellular proteins plays a crucial role in many biochemical processes under both normal and pathological conditions. Protein tyrosine kinases (PTKs) are involved in signal transduction systems that control cell proliferation, differentiation and/or cell–cell communication. Aberrant expression of PTKs has been associated with a number of tumour viruses and neoplastic disorders. The PTKs encoded by the cellular genes of the Src family contain a phosphoacceptor site that is homologous to Tyr416 of $pp60^{c\text{-}src}$ and represents its main autophosphorylation site. The phosphorylation of this Tyr residue correlates with increased kinase activity and is typically affected by an intermolecular autophosphorylation reaction. In contrast, the site homologous to $pp60^{c\text{-}src}$ Tyr527, close to the C-terminus, which can be phosphorylated by a protein tyrosine kinase, named Csk, is responsible for the down regulation of the Src-like kinases (reviewed in [1, 2]). The mechanism by which the negative regulation occurs involves an interaction between the phosphorylated Tyr527 and the SH2 and SH3 domains of the kinase itself [3–6], giving rise to an inactive conformation of the enzyme. Viral homologues of the Src-like kinases, which lack the terminal Tyr527, fail to assume this conformation, resulting in fully active enzymes autophosphorylated in Tyr416 [7]. By use of synthetic peptides with modified sequences around Tyr416 (EDNEYTA), the influence of the side-chain charged functions on the Src-like enzyme activity and the propensity of the different synthetic peptides to assume an ordered structure were previously studied [8]. Since the analysis of several proteins suggested that the phosphorylated Tyr residues exist on surface loops or β-turns [9], to examine the effect of these two structural factors better, we have previously 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 [10]. The N^{α}- and C^{α}-blocked, and the cyclic, derivatives resulted in being poor substrates for Lyn, a PTK belonging to the Src family; however, the detrimental effect due to either cyclization or N^{α}-,C^{α}-modification of the EDNEYTA sequence was overcome by dimerization. Specifically, the cyclic dimer was found to be especially suitable as a substrate, displaying a K_m value of 20 μM, the lowest reported for peptide substrates for Lyn, and a V_{max} value of 1036 nmol/min/mg. This dramatic increase of activity cannot simply be accounted for by the presence of two Tyr residues in the sequence.

On the basis of our results and from the conformational data obtained, it was concluded that flexible cyclic analogues as the cyclic dimer, in which the conformer population can be shifted towards the required geometry for the identification and binding by the enzymes, can result in a better substrate than either the linear or the cyclic parent heptapeptide.

In this paper, the modelling studies on two dimeric cyclic analogues, both favouring turn sequences, but being differently affected by Lyn, are reported. Moreover, to gain more information on the possible different efficiency displayed by Lyn towards the two Tyr residues located in positions 5 and 12 of the dimer, two linear dimeric analogues were synthesized, in which a Tyr residue is alternatively substituted by a Phe residue, and the EDNEFTAEDNEYTA cyclic analogue (Table 1). Whereas in the cyclic sequence the two Tyr residues are equivalent owing to the symmetry of the peptide, the linear analogue displays different length and amino acid sequence surrounding the phosphorylatable residues.

The conformational properties of these new analogues were studied using CD spectroscopy in four different media: 5 mM Tris/HCl (pH 6.8), 20 mM phosphate buffer (pH 3.5), 98% TFE and 30 mM SDS. Their capability to function as substrates for Lyn was also compared.

Table 1 Sequence of Synthetic Linear and Cyclic Dimer Analogues of the Heptapeptide 412–418 of $pp60^{src}$

A	H-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala-OH
B	c(Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala)
C	H-Glu-Asp-Asn-Glu-**Phe**-Thr-Ala-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala-OH
D	H-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala-Glu-Asp-Asn-Glu-**Phe**-Thr-Ala-OH
E	c(Glu-Asp-Asn-Glu-**Phe**-Thr-Ala-Glu-Asp-Asn-Glu-**Tyr**-Thr-Ala)

MATERIALS AND METHODS

General Methods

Melting points were determined by using a Tottoli's capillary apparatus and are not corrected. 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 (Merck) using the following eluent systems:

(a) chloroform/acetic acid/benzene (17 : 2 : 1)
(b) cyclohexane/chloroform/acetic acid/ethanol (9 : 9 : 2 : 2)
(c) methanol/chloroform (1 : 19)
(d) 2-butanol/ethyl acetate/water (14 : 12 : 5)
(e) methanol/chloroform (3 : 17)

The amino acid derivatives and peptides were located by spraying the chromatograms with the ninhydrin and fluorescamine reagents for compounds having a free amino group, the modified chlorine-iodide reagent for all peptide derivatives, and the Pauly's reagent for side-chain unprotected tyrosine residues.

The amino acid intermediates used in the synthesis were prepared according to standard procedures [11].

Analytical and preparative HPLC were performed using a Shimadzu liquid chromatographic system [10].

The acid hydrolyses were carried out in azeotropic hydrochloric acid containing 0.25% phenol for 22 h at 110 °C in sealed evacuated vials. The enzymatic 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 was determined with a Carlo Erba 3A30 amino acid analyser. LDMS spectra of peptides were obtained using a REFLEX time-of-flight mass spectrometer (Brücker-Fronzen Analytik). Some 1 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 α-cyano-4-hydroxycinnamic acid in 1 : 1 v/v MeCN–water) were dried on the flat surface of a cylindrical stainless steel probe tip and irradiated with the output of a nitrogen laser (337 nm). Ions were accelerated to an energy of 20 keV. Signal transients were recorded at a time resolution of 3 ns. Masses were assigned using angiotensin II as the external standard. The masses were measured as peak centroids.

Coupling and Deprotection Procedures

The main procedures used for the synthesis of the peptides may be summarized as follows.

Method A: BOP Procedure. One equiv. of BOP was 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 the 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% $KHSO_4$, 0.5 M Na_2CO_3, brine and dried over Na_2SO_4, filtered and evaporated to dryness. The residue was crystallized or precipitated from appropriate solvents.

Method B: Rudinger Azide Procedure [12]. The heptapeptide hydrazide **VIE** (0.54 g, 0.38 mmol) was dissolved in dry DMF and 2.9 N HCl in dioxane (0.52 ml, 1.52 mmol) was added at − 15 °C followed by *tert*-butyl nitrite (0.05 ml, 0.41 mmol). After 10 min the solution was cooled to − 60 °C, neutralized with TEA (0.21 ml, 1.52 mmol), and combined with a solution in DMF of the N^{α}-,C^{α}-free heptapeptide H-Glu(OtBu)-Asp(OtBu)-Asn(Trt)-Gly(OtBu)-Tyr-Thr(tBu)-Ala-OH [10] (0.51 g, 0.38 mmol) containing 0.05 ml (0.38 mmol) of TEA. Then, 0.04 g (0.38 mmol) of HOSu were added. The mixture was adjusted to pH 7.5–8 with TEA and stirred for four days at 4 °C and one day at room temperature. The solution was concentrated to a small volume and the product precipitated with water, collected and dried over P_2O_5. Yield 0.95 g (94.0%) of crude product.

Method C: DPPA Procedure. One equiv. of dipeptide **IIA** or heptapeptide Boc-Glu(OtBu)-Asp(OtBu)-Asn(Trt)-Glu(OtBu)-Tyr-Thr(tBu)-Ala-OH [10] was dissolved in anhydrous DMF, and 1 equiv. of N^{α}-free dipeptide **IC** or **ID** or heptapeptide **VID**, 2 equiv. of TEA and 1.5 equiv. of DPPA were added to the solution. The reaction was stirred for one night at room temperature and concentrated, and the product was precipitated with water. The crude peptide 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 desired peptide was precipitated from appropriate solvents.

Method D: Cyclization Procedure. The N^{α}-,C^{α}-free tetradecapeptide **VIIA** (0.44 g, 0.17 mmol) was dis-

solved in dry DMF (175 ml) in an inert N_2 atmosphere, the solution was cooled to 0 °C, and TBTU (0.16 g, 0.51 mmol), HOBt (0.08 g, 0.51 mmol) and 0.5% DIEA were added. The mixture was stirred for one night at room temperature. The crude cyclic tetradecapeptide (0.43 g) was precipitated with water from the concentrated solution, collected and dried over P_2O_5.

Method E: Hydrogenolysis. Hydrogenations were carried out in MeOH at atmospheric pressure and room temperature in the presence of 10% palladized 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 was precipitated with Et_2O. The resulting solid peptide was collected and dried over NaOH pellets.

Method F: OBg Ester Hydrazinolysis. To the heptapeptide OBg ester **VIA** (1.03 g, 0.62 mmol), dissolved in DMF (2 ml), hydrazine hydrate (0.12 ml, 2.50 mmol) was added. The solution was stirred for 45 min at room temperature, concentrated to dryness and the residue triturated with Et_2O. The white solid was collected, washed extensively with Et_2O and dried over H_2SO_4. Yield 0.61 g (67.8%) (**VIE**).

Method G: TFA treatment. The side-chain protecting groups, as well as the carboxy- and amino-terminal protecting groups of **VIII**, 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 Et_2O, dried over NaOH pellets and purified by chromatographic procedures.

The crude peptides were dissolved in water and chromatographed on a VYDAC 218TP1022 C18 10 μm column (250 × 22 mm), flow rate 15 ml/min, detection at 216 and 275 nm. The column was equilibrated and eluted using 0.05% TFA in water (A) and 0.05% TFA in 9:1 v/v MeCN/water as organic modifier (B). Isocratic elution at 15% B for 15 min and then a linear gradient from 15% to 20% B in 25 min for cyclic peptide **E**, whereas isocratic elution at 17% B for 3 min and then a linear gradient from 17% to 20% B in 27 min for the linear tetradecapeptide **D** were used.

Alternatively, the linear tetradecapeptide **C** was chromatographed on a Sephadex G-25 SF column (155 × 2.5 cm) equilibrated and eluted with 0.1 M ammonium acetate. The fractions containing the desired peptide were collected and lyophilized in the presence of acetic acid up to ammonium acetate elimination.

Finally, the expected linear and cyclic peptides were lyophilized to a constant weight in the presence of 0.01 M HCl.

Molecular Dynamics Simulations

The molecules used in the simulation were built as linear peptides and manually cyclized using the program Insight (Biosym Technologies). After minimization, the peptide was included in a sphere of radius 25 Å, centred on its centre of mass, and filled with water for a total of 368 molecules. The system was subjected to a Langevin dynamics, starting from a temperature of 1000 K and cooled to 300 K in steps of 25 K. A time step of 0.5 fs was used and, at every decrease in temperature, the system was equilibrated for 200 steps. At the end of the simulation, a minimization was performed with the conjugate gradient algorithm. The result of the minimization procedure was taken as the final model. Simulations were carried out with the program X-PLOR V3.0, using the following conditions: the dielectric constant was set to 1.0 and held constant; the temperature was controlled by a weak coupling to an external bath ($\tau = 12$ fs); a cut-off of 7.5 Å was used for electrostatic interactions; only hydrogens connected to polar groups were treated explicitly. The version of the CHARMM force field included in the X-PLOR program was used, with the TIP3p parameters for water molecules. Periodic boundary conditions were applied to avoid the solvent escaping at high temperature.

Circular Dichroism

The synthetic peptides were investigated by CD spectroscopy in 5 mM Tris–HCl, pH 6.8, in 20 mM phosphate buffer, pH 3.5, in 98% TFE–water, and in 30 mM SDS solutions. The CD measurements were carried out with a Jasco model J-600A spectropolarimeter equipped with a data processor. The spectra are the average of at least four scans.

Assay for Peptide Phosphorylation

Lyn was obtained as previously described [13]. Tyrosine kinase activity was checked by incubating

the peptides at 30 °C in 50 μl of a medium containing 50 mM Tris–HCl, pH 7.5, 5 mM $MnCl_2$, 10 μM Na-vanadate, 20 μM [γ^{32}P]ATP (specific activity 1000 c.p.m./pmol), 1 μM polyLys and 10 units of PTK (one unit being defined as the amount of enzyme transferring 1 pmol phosphate per min to 2 mM angiotensin II). Reactions were terminated by addition of 1 ml of a 1N HCl solution and labelled phosphopeptides were quantitated as previously described [14]. Briefly: samples were heated for 15 min at 100 °C in order to convert [γ^{32}P]ATP into ADP and ^{32}Pi, which was removed by conversion into phosphomolybdic complex and extracted with 2-butanol-toluene. The radioactivity present in the aqueous phase due to phosphopeptides was then measured in a scintillation counter.

RESULTS AND DISCUSSION

Peptide Synthesis

The synthetic route followed for the preparation of the linear heptapeptide corresponding to the sequence 412–418 of pp60$^{c\text{-}src}$ and its analogue containing a Phe residue in place of Tyr 416 is reported in Figures 1 and 2.

The standard procedure for peptide synthesis in solution was employed, using the combination of benzyloxycarbonyl as the protecting group of the α-amino function during the stepwise elongation of the peptide chain, the *tert*-butyl ester group for the selective protection of the side-chain carboxyl function of the Asp and Glu residues, the *tert*-butyl ether

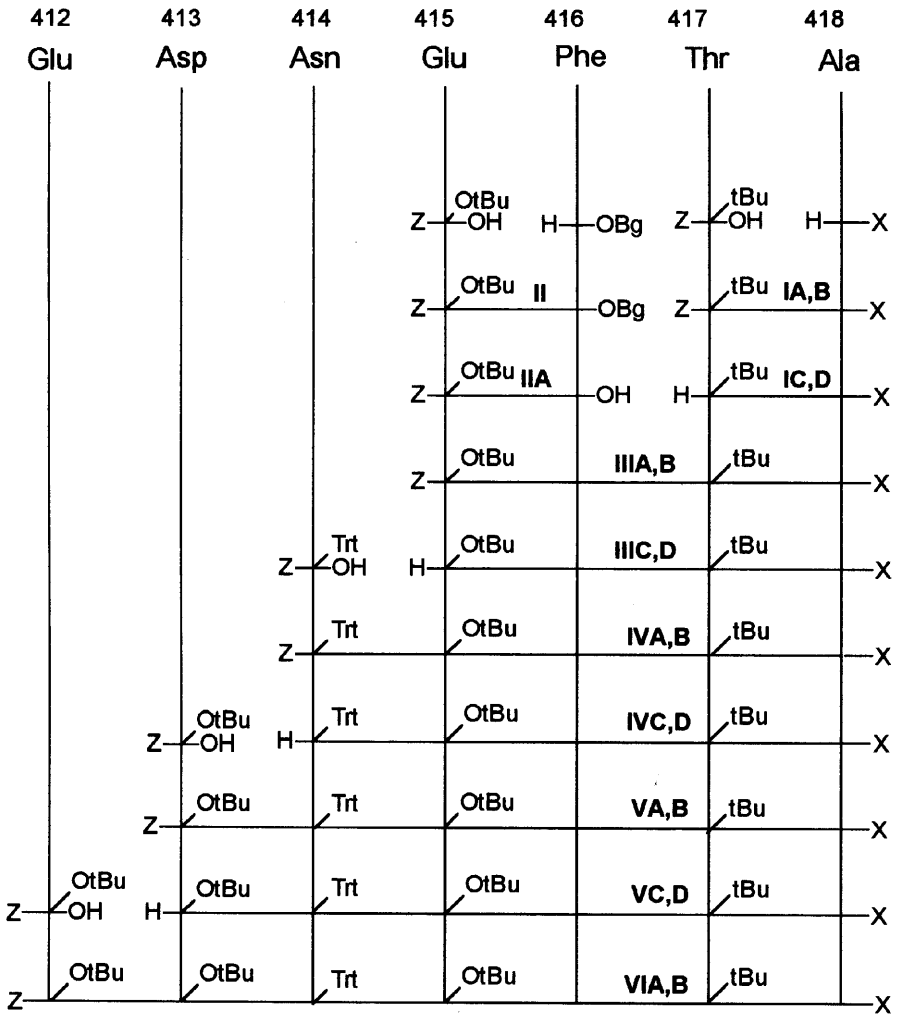

Figure 1 Synthetic scheme for the synthesis of the heptapeptide corresponding to the sequence 412–418 of pp60^{src} where Tyr 416 is replaced by a Phe residue. X = -OBg or -OtBu.

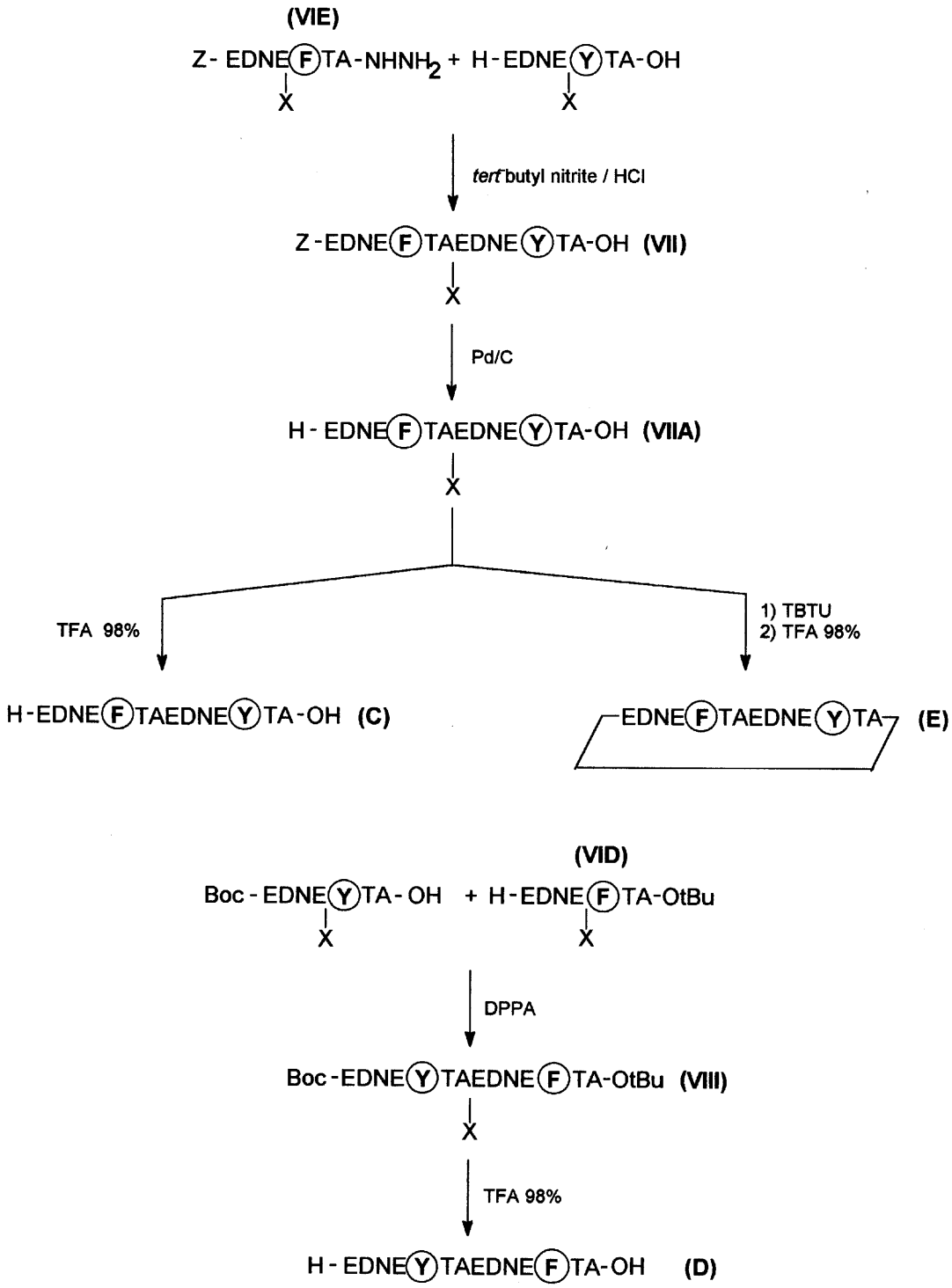

Figure 2 Synthetic routes for the synthesis of the tetradecapeptides **C**, **D** and **E**. The first route was also employed for the synthesis of dimers **A** and **B**. X = side-chain protections.

for the protection of the hydroxyl function of the Thr residue, the trityl group for the carboxamide protection of the Asn residue. The *N*-benzhydryl-glycolamide ester [15] was used as a three-dimensional orthogonal protecting group for the C^{α}-terminal carboxylic function up to the heptapeptide derivative. The ester (**VIA**) was subsequentially transformed into the corresponding hydrazide (**VIE**) by treatment with hydrazine hydrate.

For the stepwise condensation the BOP reagent was used, whereas the fragment condensation to tetrapeptides **IIIA** and **B** was performed with DPPA, known to yield coupling with a very low degree of racemization.

The reaction of the heptapeptide hydrazide (**VIE**) with the N^{α}-,C^{α}-free and side-chain protected heptapeptide H-Glu(OtBu)-Asp(OtBu)-Asn(Trt)-Glu(OtBu)-Tyr-Thr(tBu)-Ala-OH [10], to obtain the linear N^{α}- and side-chain protected, C^{α}-free dimer **VII**, was carried out using the Rudinger's acyl azide procedure. After hydrogenolysis of the N^{α}-benzyloxycarbonyl protecting group cyclizations were performed according to Zimmer *et al.* [16] in dilute DMF (1 mM) solution using TBTU as the coupling reagent in the presence of HOBt and DIEA. The fully protected tetradecapeptide **VIII** was obtained by condensation of Boc-Glu(OtBu)-Asp(OtBu)-Asn(Trt)-Gly(OtBu)-Tyr-Thr(tBu)-Ala-OH [10] with the N^{α}-free, C^{α}- and side-chain protected heptapeptide **VID** in the presence of DPPA. Then, the linear and cyclic analogues were side-chain deprotected with TFA and chromatographically purified as described above.

The physical properties of the final peptides and their intermediates are listed in Tables 2 and 3, respectively.

Molecular Dynamics Simulations

The results of the simulations for cyclic peptides **B** and **E** are shown in Figures 3 and 4. Both molecules can be roughly described as formed by two strands, running antiparallel, connected by turns. Both Tyr residue in **B**, and the Tyr and Phe residues in **E** are present in the turns. However, it is interesting to note that in the two cases, despite the symmetry (or pseudosymmetry) of the sequence, equivalent residues do not present a similar conformation, and also that the overall conformation of the two peptides is not identical, as illustrated in Figure 4. Possibly because of steric constraints due to the relatively short sequence and/or to the presence of the solvent (water), the two antiparallel strands are not able to form all the possible regular hydrogen bonds among main-chain atoms. Both Tyr residues of cyclopeptide **B** are present in the $i+2$ position of a turn, but while the first one, involving residues from 3 to 6, can be roughly assimilated to a β-turn, the second one is more difficult to classify. The conformation of cyclopeptide **E** is different from that of **B**, possibly because of the presence of a more hydrophobic residue (Phe) replacing Tyr. Specifically, while Tyr5 is helical, Phe12 assumes a conformation similar to that of an inverse γ-turn. The values of the torsion angles ϕ and ψ for the turn residues in positions 4 and 5 (Glu4, Tyr5 in peptides **B** and **E**) and 11 and 12 (Glu11, Tyr12 in peptide **B** and Glu11, Phe12 in peptide **E**) are reported in Table 4.

Circular Dichroism

The CD spectra of peptides **A**, **C** and **E** in aqueous solution at pH 6.8 exhibit a negative band at 190–

Table 2 Physicochemical Properties of Linear and Cyclic Tetradecapeptides **C**, **D** and **E** (Data for peptides **A** and **B** are Quoted in [10])

Compound		$[MH]^+$	t_r^a	Amino acid ratios in acid and enzymatic hydrolysates						
				Asp	Thr	Asn	Glu	Ala	Phe	Tyr
EDNE***F***TAEDNE***Y***TA	(**C**)	1648	23.7[b]; 4.9[c]	3.93[d] (4)	2.09 (2)	–	3.99 (4)	2.07 (2)	0.98 (1)	1.03 (1)
				2.00[e] (2)	2.09 (2)	1.99 (2)	3.99 (4)	2.07 (2)	0.98 (1)	1.03 (1)
EDNE***Y***TAEDNE***F***TA	(**D**)	1648	28.3[b]; 5.5[c]	4.07[d] (4)	2.08 (2)	–	3.98 (4)	2.01 (2)	1.02 (1)	0.98 (1)
				1.96[e] (2)	2.05 (2)	2.03 (2)	3.91 (4)	1.92 (2)	0.98 (1)	1.01 (1)
c(EDNE***F***TAEDNE***Y***TA)	(**E**)	1630	23.8[b]; 18.9[c]	3.95[d] (4)	1.94 (2)	–	3.95 (4)	2.07 (2)	0.98 (1)	1.04 (1)

[a] 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.
[b] Isocratic elution at 8% B for 8 min and then a linear gradient from 8% to 35% in 27 min.
[c] Isocratic elution at 15% B for 3 min and then a linear gradient from 15% to 30% in 15 min.
[d] In the acid hydrolysate.
[e] In the enzymatic hydrolysate.

Table 3 Physicochemical Properties of the Protected Fragments Listed in Figure 1

Compound	m.p. (°C)	Recryst. solvent	$[\alpha]_D^{20}$ (°)[a]	TLC		Amino acid ratios					
				R_F (system)		Asp	Thr	Glu	Ala	Tyr	Phe
II	117–120	Et_2O–PE	− 10.2	0.86 (a)	0.94 (b)	–	–	1.02 (1)	–	–	0.98 (1)
IIA	88	Et_2O–PE	+ 2.0[b]	0.76 (a)	0.96 (b)	–	–	0.96 (1)	–	–	1.05 (1)
IIIA	178	AcOEt	− 8.5	0.66 (a)	0.84 (b)	–	0.97 (1)	1.01 (1)	1.01 (1)	–	1.01 (1)
IVA	185	AcOEt–Et_2O	− 10.3	0.39 (a)	0.92 (b)	0.95 (1)	1.05 (1)	0.95 (1)	1.06 (1)	–	1.01 (1)
VA	180	DCM–Et_2O	− 12.48	0.54 (a)	0.87 (b)	1.95 (2)	1.05 (1)	1.03 (1)	1.09 (1)	–	1.07 (1)
VIA	82	DCM–Et_2O	− 4.92	0.88 (b)	0.57 (c)	1.96 (2)	1.00 (1)	1.98 (2)	1.04 (1)	–	1.00 (1)
VIE	208	DCM–Et_2O	− 2.3	0.26 (c)	0.96 (d)	1.94 (2)	1.04 (1)	2.00 (2)	1.04 (1)	–	1.01 (1)
VII	195	DMF–5%AcOH	− 6.8	0.44 (e)		4.06 (4)	2.11 (2)	4.06 (4)	2.12 (2)	1.03 (1)	1.08 (1)
IIIB	200	AcOEt–Et_2O	− 21.3[b]	0.93 (a)	0.87 (b)	–	1.00 (1)	1.03 (1)	0.96 (1)	–	1.00 (1)
IVB	178–180	AcOEt–Et_2O	− 14.9	0.73 (a)	0.85 (b)	0.99 (1)	0.98 (1)	1.00 (1)	1.01 (1)	–	1.01 (1)
VB	199	AcOEt–Et_2O	− 14.9	0.31 (a)	0.88 (b)	1.93 (2)	1.03 (1)	1.03 (1)	1.02 (1)	–	1.10 (1)
VIB	202	AcOEt–PE	− 11.7	0.80 (a)	0.88 (b)	1.91 (2)	1.03 (1)	2.04 (2)	1.04 (1)	–	0.98 (1)
VIIB	215	AcOEt–Et_2O	− 10.5	0.16 (a)	0.83 (b)	3.93 (4)	2.09 (2)	4.02 (4)	2.09 (2)	0.97 (1)	1.01 (1)

[a] Solvent: DMF, $c = 1$.
[b] Solvent: MeOH, $c = 1$.

195 nm and a positive CD absorption at about 220 nm (Figure 5). This last CD band may be determined by the ^1L_a electronic transition of Tyr. The CD spectrum of Ac-L-Tyr-NH_2 has indeed a strong positive band near 225 nm [17]. Evidence for this contribution has been noted also in different peptides where Tyr was replaced by a non-aromatic amino acid [17]. However, only a negative shoulder has been observed near 220 nm in the CD spectra of the peptides **B** and **D** (Figure 5). The lowering of the pH to 3.5 determines a change in the CD pattern at 220 nm, being negative for all the five peptides under these experimental conditions. As far as the negative CD band at wavelengths shorter than 200 nm is concerned, the spectral variation is not very marked compared with pH 6.8. The red-shift of the maximum observed for peptides **A** and **E** represents the most significant effect determined by the lowering of the pH. These changes of the CD spectra are probably due to different ionization of the peptides. In any case, the CD spectra in aqueous solution are indicative of the presence of an unordered conformation.

In a membrane-like environment (TFE) the CD spectra of the peptides show a negative band at about 200 nm (Figure 6). The shift of the band to longer wavelengths with respect to the aqueous solution is indicative of a slightly more ordered structure. However, this behaviour and the higher intensity of the negative CD around 220 nm do not allow one to define the secondary structure of the compounds examined in terms of relative contributions arising from different types of ordered structures to the observed CD spectra. In general, the intensity of the negative CD band at about 200 nm is low, being relatively high only in the case of the cyclic peptide **E**. The lowest intensity was observed for **A** and **B**, the linear and cyclic dimers of EDNEYTA. This result is in agreement with the observation of Bradley *et al.* [18] who suggested a less ordered structure for peptides containing Tyr residues.

The other hydrophobic medium examined (30 mM SDS) produces a much less intense spectral variation with respect to the aqueous solution. Thus, an unordered conformation can be hypothesized as predominant also for the peptides in this micellar system.

From these experiments and within the limits of the CD technique the only conclusion that can be drawn is that all of the linear and cyclic peptides examined have no defined conformation, no matter what solvent or pH is considered.

Kinetic Studies

The synthetic peptides were tested for their ability to behave as phosphoacceptor substrates for Lyn, a tyrosine kinase purified from spleen and belonging to the Src family. Figure 7 and Table 5 show the time course and the kinetic constants of the Lyn-catalysed peptide phosphorylation, respectively. The data obtained demonstrate that both Tyr residues located in the dimeric peptides **A** and **B** can be

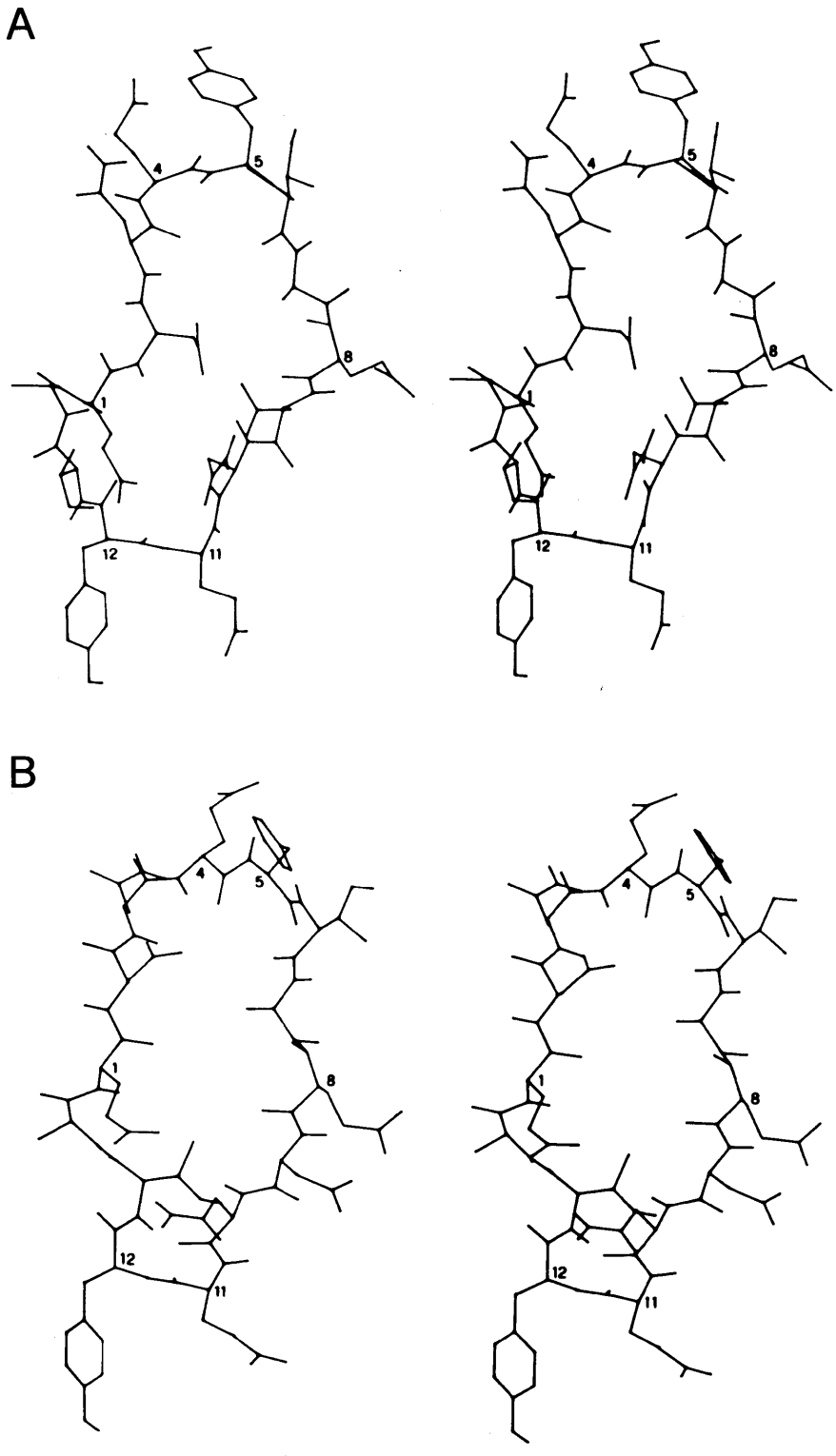

Figure 3 Stereoviews of (A) *c*(EDNE<u>Y</u>TAEDNE<u>Y</u>TA) and (B) *c*(EDNE<u>F</u>TAEDNE<u>Y</u>TA).

Figure 4 Stereoviews of the two peptides, *c*(EDNEYTAEDNEYTA) (thin line) and *c*(EDNEFTAEDNEYTA) (thick line), superimposed at residues 11 and 12.

affected by the enzyme. Indeed, peptides **C** and **D**, which contain a single phosphorylatable Tyr, can be phosphorylated by Lyn with a rather similar efficiency, even if the different position of the target residue in the two sequences gives rise to a somehow different kinetic value. Specifically, peptide **D** (which contains a Tyr residue at position 5) displays a similar K_m and a lower V_{max} than those shown by the doubly phosphorylatable peptides. On the other hand, peptide **C** (which contains a Tyr residue at position 12) can be affected by the enzyme with a lower affinity than that displayed by peptides **A** and **B**. However, the Lyn efficiency for the peptides containing a single Tyr residue (**C** and **D**) is less than half that shown by the doubly phosphorylatable peptide **A**. This finding is also evident if the

Table 4 Values of the Torsion Angles ϕ and ψ for the Turn Residues in the Cyclic Peptides **B** and **E**

	PEPTIDE **B**		PEPTIDE **E**	
Residue	ϕ	ψ	ϕ	ψ
Glu4	− 60	− 57	− 101	− 180
Tyr5	− 80	145	− 68	− 26
Glu11	− 51	139	44	− 70
Tyr/Phe12	62	− 59	− 91	70

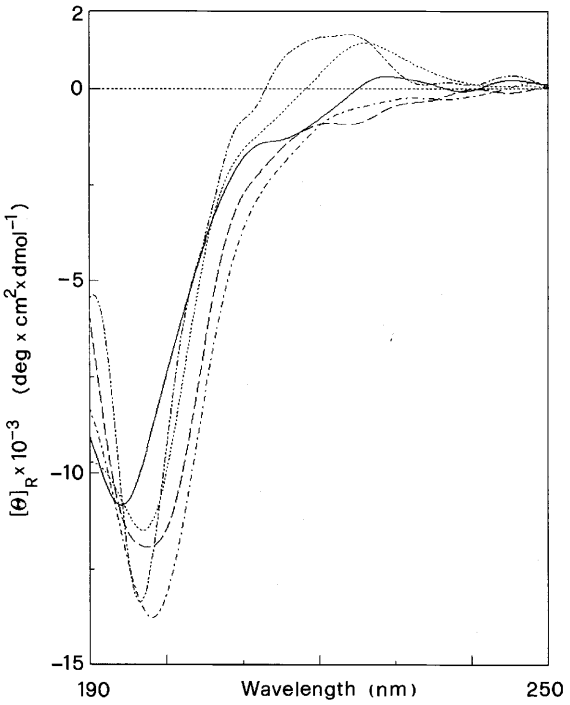

Figure 5 Far-UV CD spectra of the synthetic peptides in 5 mM Tris–HCl, pH 6.8: — **A**; --- **B**; ···· **C**; –·– **D**; ····· **E**.

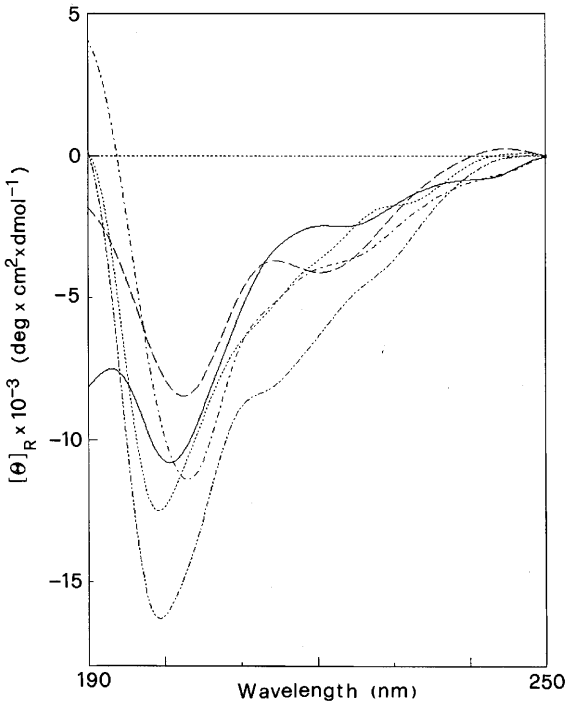

Figure 6 Far-UV CD spectra of synthetic peptides in 98% TFE–2% water: — **A**; --- **B**; -·-· **C**; –·– **D**; ····· **E**.

kinetic values obtained with the cyclic peptides **B** and **E** are compared. The derivative containing two Tyr residues (**B**) displays an efficiency that is twice that of the peptide containing a single Tyr residue (**E**). Since the K_m values of the two cyclic peptides are similar, the higher V_{max} displayed by peptide **B** could be ascribed to conformational features induced by the presence in the sequence of a second Tyr residue which positively influences the enzyme turnover. A similar effect could also be evoked in the case of the cyclic peptides (**B** and **E**) which display efficiencies higher than those of their linear analogues (**A**, **C** and **D**, respectively). We believe that the cyclic structure would force the substrate to adopt a conformation that enhances the rate of the phosphorylation process.

Table 5 Kinetic Constants for Lyn with Synthetic Peptides

	Peptide	V_{max} (nmol/min/mg)	K_m (μM)	Efficiency (V_{max}/K_m)
A	*EDNE**Y**TAEDNE**Y**TA*	633	20	32
B	*c(EDNE**Y**TAEDNE**Y**TA)*	1036	20	52
C	*EDNE**F**TAEDNE**Y**TA*	572	43	13
D	*EDNE**Y**TAEDNE**F**TA*	398	26	15
E	*c(EDNE**F**TAEDNE**Y**TA)*	614	23	27

The values shown are the means of four separate determinations. For each of these values the SE value diverged by less than 16%.

CONCLUSIONS

The results of the biochemical assays obtained using the synthetic peptides described in this paper as substrates for the tyrosine kinase expressed by the pro-oncogene *lyn*, which belongs to the *src* family, indicate that the enzyme can phosphorylate both Tyr residues located in the dimeric linear peptide **A**. However, the different position of the target residue in the sequence can slightly affect both the conformation and the phosphorylatability of the substrate, as shown by the kinetic constants of peptides **C** and **D** which contain one of the two Tyr alternatively replaced by Phe. The phosphorylation data are in agreement with the results obtained in recent studies on Src kinase substrate specificity, which suggests that for these specific kinases there are no individual determinants stringently required at any given position, apart from a hydrophobic/β-branched side chain on the N-terminal side of the target Tyr [19]. Moreover, they rather suggest that there may be certain overall peptide structures that are better suited than others to fit in the catalytic site of the Src kinases [20, 21]. This is also the case for the EDNEYTAEDNEYTA peptide (**A**), which exhibits the lowest K_m value for Lyn among the peptide substrates tested. In order to obtain a more suitable peptide substrate to be used for the specific detection of the Src PTKs, the next step of our project will be the synthesis of a cyclic dimer similar to peptide **B**, but containing the hydrophobic, β-branched residue Ile on the N-side of the Tyr residues. This change should strongly improve the phosphorylatability of the peptide by Lyn, and possibly by other Src PTKs as well, and provide a sequence useful to design analogues to be employed as active site-directed specific inhibitors for this class of enzymes. In this respect the cyclic derivatives, which are generally found to be more suitable substrates for Lyn than the linear analogues ([8] and this paper), can also improve the efficiency of potential inhibitors as a consequence of their increased resistance towards degradation by exopeptidases, a widespread phenomenon in biological systems.

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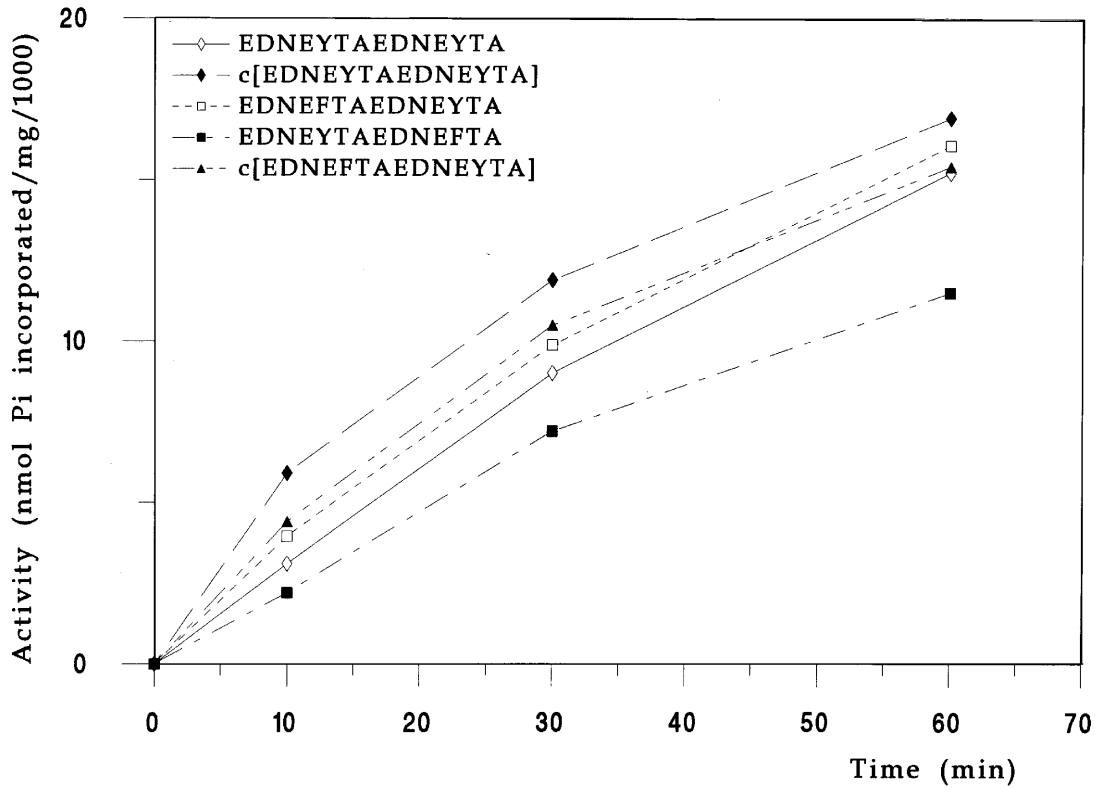

Figure 7 Time-course of synthetic peptide phosphorylation by Lyn. Peptide concentration was 50 μM. Other experimental conditions are described in detail in the Materials and Methods section. Lyn activity is expressed as nmol Pi incorporated/mg of enzyme.

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