Enzymatically Degradable Prodrugs: A Novel Methodology for Drug Linkage

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ABSTRACT: We have synthesized a novel enzymatically degradable prodrug system based on poly(ethylene glycol) (PEG) and tyrosine units by employing a synthetic methodology which eliminated the use of conventional blocking and deblocking methodology used for chemical linkage of drug molecule to the pendant —NH_2 group of amino acid. A diester of PEG (6 kDa) and tyrosine hydrochloride was synthesized by dicyclohexyl carbodiimide (DCC)-mediated condensation. In the second stage, oligomers were prepared by condensing phenolic —OH groups of tyrosine in the diester with sebacic acid, using DCC. Finally, the hydrochloride salt of tyrosine in the oligomer was treated with triethylamine to activate —NH_2 groups, which were reacted with benzoyl chloride to obtain a model prodrug system. The products synthesized were characterized by IR, ^{1}H-NMR, and GPC. The spectral data were in accordance with the proposed structures of products. Chymotrypsin-catalyzed degradation of the oligomers was characterized by both MW measurements and Ninhydrin assay for free tyrosine. Degradation studies indicated that the rate of main-chain degradation (ester hydrolysis) is higher than that of the side chain (amide hydrolysis). This new, simple methodology should be useful for conjugating a variety of bioactive molecules to enzymatically degradable PEG–amino acid based polymers. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 2108–2118, 2002

Key words: poly(ethylene glycol); L-tyrosine; prodrug; chymotrypsin

INTRODUCTION

Polymeric prodrugs are gaining increasing importance as promising therapeutic agents in tumor treatment as they can provide site-specific drug delivery, longevity in blood circulation, lower drug dosage levels, biocompatibility, and biodegradability.[1–5] Prodrugs based on poly(methacrylic acid-*co*-2-methylsulfinyl) ethyl methacrylate,[4] poly-[methacrylic acid-*co*-*N*-(2-hydroxypropyl] methacrylamide,[5–7] and poly(*N*-vinyl pyrrolidone-*co*-vinylamine)[8] were investigated in the past. Drug molecules, such as chlorambucil, daunomycin, bis-(2 chloroethyl) amine, and methotrexate, were linked to these polymers. Also, prodrugs of 5-fluorouracil and taxol based on high molecular weight poly(oxyethylene dicarboxylic acid) were investigated. Although biocompatible, the above-mentioned polymers are not biodegradable and tend to accumulate in the body.[4] Moreover, for easy release, the drug linkage to the polymer backbone through a spacer and biodegradable oligopeptide is needed. Among biodegradable poly amino acids, poly(L-lysine) was investigated, but its *in vivo* application is not recommended in view of its antigenicity and rapid degradation.[9] The

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need to develop newer forms of polymer–drug conjugates was recently emphasized.[2]

Excellent biocompatibility of poly(ethylene glycol) (PEG) has resulted in its applications in medicine and biotechnology, and some have received FDA approval.[10–14] Because proteolytic enzymes in the body (e.g., trypsin, chymotrypsin, and cathepsin B hydrolyze ester and amide derivatives of specific amino acids arginine, tyrosine, and glutamic acid, respectively[15–16]), polymers based on PEG and amino acids are expected to be biodegradable as well as biocompatible.

Kohn et al.[17] reported water-soluble polyurethanes comprising PEG and L-lysine in which pendant carboxyl group was protected in the ester form. Ulbrich et al.[18] synthesized enzymatically degradable oligomers containing PEG and L-glutamic acid. In this, two blocks of PEG monomethyl ether 2000 were condensed together via L-glutamic acid based oligopeptides, which were synthesized by using *N*-tertiary butyloxycarbonyl protecting group. Recently, Won et al.[19] reported synthesis of poly(ethylene glycol-*co*-L-aspartic acid), in which *N*-carbobenzoxy group was used for blocking —NH_2 group. The use of protecting groups and subsequent deprotection in prodrug design seems to be unavoidable, although laborious. This is a practical difficulty, which if addressed, would help design newer forms of prodrugs much more efficiently. In this article, we report a novel synthetic methodology for the preparation of PEG and L-tyrosine-containing oligomeric prodrugs that employ hydrochloride salt as amino protecting group, which can be readily neutralized to link drug molecules to the free amino groups. This novel prodrug was hydrolyzed by chymotrypsin to its building blocks (i.e., PEG, tyrosine, and the drug). Pechar et al.[18] have synthesized prodrugs in which the drug was linked to the oligopeptide linker through another oligopeptide spacer. Our approach involves the use of a trifunctional amino acid such as tyrosine for binding the main chain as well as to conjugate the drug. The incorporation of tyrosine enables cleavage of main chain and also the hydrolysis of the drug.

EXPERIMENTAL

Materials

L-tyrosine hydrochloride (Tyr.HCl), benzoyl chloride, triethylamine, and poly(ethylene glycol) 6000 (PEG 6 kDa) were purchased from local suppliers and used as received. Tetrahydrofuran (THF) and dimethylformamide (DMF) were distilled and dried following standard procedures.[20] Dicyclohexyl carbodiimide (DCC) was purchased from Aldrich Chemical Co., Milwaukee, WI. Chymotrypsin and *N*-benzyloxycarbonyl tyrosyl *p*-nitro phenyl ester (*N*-cbz-Tyr-PNP) were purchased from Sigma Chemical Co., St. Louis, MO.

Instrumentation

^{1}H-NMR spectra were recorded on a Bruker (Bruker, Karlsruhe, Germany) 200 MHz spectrometer,[21] with NMR tube, WILMAD 5 mm, Ultra-Imperial grade, 7 in. length; temperature: 24°C; scan number: 80. IR spectra were recorded on a Perkin–Elmer 1600 FTIR spectrophotometer (Perkin Elmer, Norwalk, CT). Melting points were recorded on a Mettler melting point apparatus (Mettler, Greifensee, Switzerland). Oligomer melting temperatures (T_m) were determined on a Perkin–Elmer DSC 2C calorimeter. The following specifications were used: heating rate: 5°C/min; temperature range: 50–100°C; heating cycle, 0–8.00 mCAL/s. Molecular weights of PEG 6 kDa samples used, oligomers prepared, and degradation products were determined by gel permeation chromatography (GPC) on a Waters 590 programmable GPC/HPLC chromatograph (Waters, Milford, MA) connected to a Waters 410 differential refractometer, using THF solvent at flow rate of 1.0 mL/min and 530 mbar pressure. PEG standards were used as a reference for plotting the calibration plot of retention time versus molecular weights.

Methods

Estimation of Acid Values

A substance weighing 0.2 g was dissolved in 10 mL distilled water. To this solution, two drops of phenolphthalein indicator was added and the solution was titrated against 0.01680*N* KOH (normality calibrated against potassium hydrogen phthalate) until a colorless to faint pink end point was obtained. Acid values were calculated by using the formula:

$$\text{Acid value (mg KOH/g)} = 56.1 \times (\text{ml of KOH required}) \times \text{normality of KOH/weight of the material (g)}$$

The acid value so calculated is in terms of milligrams of KOH required to neutralize 1 g of the substance. We have used this as a measure of amount of hydrochloride salt in the polymer. Hence, we report the acid value in terms of the millimoles of HCl as

$$\text{Acid value (milimoles HCl/g)} = \text{acid value (mg KOH)}/56.1$$

Synthesis of Bis-(Tyrosyl Hydrochloride) Poly(Ethylene Glycol) 6000 Diester (Bis-Tyr.HCl–PEG 6 kDa) (diester)

In a 100-mL capacity conical flask, 6 g PEG 6 kDa (1 mmol) (molecular weight of PEG 6 kDa: M_w = 6500; M_n = 4200; M_w/M_n = 1.54), 0.435 g Tyr.HCl (2 mmol), and 10 mL DMF were taken. The contents of the flask were gently heated to dissolve the solids and to obtain a clear solution. To this solution, 0.412 g DCC (2 mmol) dissolved in 5 mL DMF was added in a single portion. The reaction mixture was stirred at room temperature (25°C) for 24 h. It was then filtered to separate out dicyclohexyl urea (DCU) formed and the clear solution was poured into 200 mL diethyl ether to precipitate out a white powdery product. The product was isolated and purified by reprecipitation from methanol into diethyl ether.

Yield: 78%. Melting point: 58°C. Acid value: Found: 0.296 mmol HCl/g; calculated: 0.2–0.3 mol HCl/g (PEG has MW range of 6–7.5 kDa). IR (nujol): 1720 cm^{-1} (ester carbonyl), 3500 cm^{-1} (—OH, NH stretching). ^{1}H-NMR (DMSO-d$_6$): 3.3 δ doublet (Tyr-CH_2), 3.8 δ triplet (Tyr-CH-COO-PEG), 3.9 δ triplet (—OCH_2 of PEG next to terminal –OCH$_2$—), 4.2 δ broad singlet (—CH_2—CH_2—O—)$_n$ of PEG chain, 4.6 δ triplet (terminal —CH_2—O— of PEG), 7.4 δ doublet (Tyr-phenyl ring protons), 7.8 δ doublet (Tyr-phenyl ring protons), 8.8 δ singlet (—NH_2.HCl), 9.6 δ singlet (phenolic —OH). Molecular weight: M_w = 7200; M_n = 4800; M_w/M_n = 1.5.

Synthesis of Oligo(Bis-Tyr.HCl-PEG 6 kDa–Sebacate) (oligomer)

In a 100-mL capacity conical flask were placed 5 g bis-Tyr.HCl-PEG 6 kDa (~ 1.48 mmol Tyr-OH groups), 0.15 g sebacic acid (1.48 mmol —COOH groups), and 10 mL DMF. Contents of the flask were gently heated to obtain a clear solution. To this solution, 0.30 g DCC (1.48 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. It was then filtered to remove DCU and the clear solution was poured into 100 mL diethyl ether to precipitate out the polymer. The polymer was purified by reprecipitation from DMF into diethyl ether.

Yield: 3.5 g (68%). T_m: 65°C. IR (nujol): 1720 cm^{-1} (ester carbonyl), 3500 cm^{-1} (—OH, —NH stretching). Acid value: 0.253 mmol HCl/g. ^{1}H-NMR (DMSO-d$_6$): 1.9 δ broad singlet (C_4 and C_7 methylene protons of sebacic acid), 2.0 δ multiplet (C_3 and C_8 methylene protons of sebacic acid), 2.3 δ triplet (C_2 and C_9 methylene protons of sebacic acid), 3.3 δ doublet (Tyr-CH_2), 3.8 δ triplet (Tyr-CH-COOR), 3.9 δ triplet (—OCH_2 of PEG next to terminal —OCH$_2$—), 4.2 δ broad singlet (—CH_2-CH_2-O—)$_n$ of PEG chain, 4.6 δ triplet (terminal —CH_2—O— of PEG), 7.4 δ doublet (Tyr-phenyl ring protons), 7.8 δ doublet (Tyr-phenyl ring protons), 8.8 δ singlet (—NH_2.HCl). Molecular weight of poly(bis-Tyr-PEG 6 kDa–sebacate): M_w = 14,117; M_n = 5251; M_w/M_n = 2.68.

Synthesis of Oligo(Bis-N-Benzoyl Tyrosyl–PEG 6 kDa–Sebacate) (oligomeric model prodrug)

In a 100-mL capacity round-bottom flask, 2.5 g poly(bis-Tyr.HCl–PEG 6 kDa–sebacate) (~ 0.63 mmol —NH$_2$.HCl groups) and 20 mL THF were placed and stirred with a magnetic needle. To this suspension, 0.18 mL triethylamine (1.26 mmol) was added. After stirring for a few minutes, 0.073 mL benzoyl chloride (0.63 mmol) dissolved in 5 mL THF was added and the reaction mixture was stirred 3 h at room temperature. It was then filtered to remove triethylamine hydrochloride salt and the clear solution was poured into 200 mL petroleum ether to precipitate out the polymer. Polymer was purified by reprecipitation from THF into petroleum ether.

Yield: 2.5 g (77%). T_m: 61°C. Acid value: 0.07 mmol HCl/g (drug linkage, 73%). IR (nujol): 1640 cm^{-1} (amide carbonyl), 1700 cm^{-1} (ester carbonyl), 3500 cm^{-1} (—OH, —NH stretching). ^{1}H-NMR (DMSO-d$_6$): 1.9 δ broad singlet (C_4 and C_7 methylene protons of sebacic acid), 2.0 δ multiplet (C_3 and C_8 methylene protons of sebacic acid), 2.3 δ triplet (C_2 and C_9 methylene protons of sebacic acid), 2.5 δ doublet (Tyr—CH_2—), 2.8 δ triplet (Tyr—CH-COOR), 3.4 δ triplet (—OCH_2 of PEG next to terminal —OCH$_2$), 3.5 δ broad singlet (—CH_2-CH_2—O—)$_n$ of PEG chain, 3.6 δ triplet (terminal —CH_2—O— of PEG), 6.7 δ doublet (Tyr-phenyl ring protons), 7.1 δ broad multiplet (*N*-benzoyl ring protons), 7.5 δ doublet (Tyr-phenyl ring protons), 8.1 δ singlet (—NH). Poly(*N*-benzoyl tyrosyl-PEG 6 kDa–sebacate): molecular weight: M_w = 14,772; M_n = 5486; M_w/M_n = 2.69.

Degradation Studies: By Ninhydrin Assay

In a stoppered test tube, 1 g oligo(bis-*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate) was dissolved in 10

mL 0.01*M* phosphate buffer (pH 7.4). To this solution, 0.1 g chymotrypsin was added and the solution was maintained at 37°C for 8 days. After every 24 h, 1 mL aliquot of the solution was taken out and liberated free tyrosine was estimated by Ninhydrin assay.[22] An amount of 1 mL of aliquot was diluted with 4 mL water, and 1 mL Ninhydrin reagent was added. [To prepare the reagent, 2 g Ninhydrin was dissolved in 25 mL acetone and 25 mL acetate buffer (0.2*M*, pH 5.5) was added to it.] This solution was heated in a test tube to 90°C in a water bath for half an hour and then cooled to room temperature. From the absorbance, a purple color developed (550 nm), and the amount of liberated free tyrosine was estimated. In a similar manner, chymotrypsin-catalyzed hydrolyses of bis-Tyr.HCl–PEG 6 kDa, oligo(bis-Tyr.–PEG 6 kDa–sebacate) and *N*-benzoyl tyrosine were monitored.

As control, degradation of the above-mentioned polymers by phosphate buffer was also monitored. Stability of chymotrypsin during these 8 days was tested in an independent experiment. Chymotrypsin solution in phosphate buffer was incubated at 37°C for 8 days. After every 24 h, hydrolytic activity of chymotrypsin against standard substrate *N*-cbz-Tyr-PNP was estimated.

Degradation Studies by GPC

In a stoppered test tube, 1 g oligo(bis-*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate) was dissolved in 10 mL 0.01*M* phosphate buffer. An amount of 0.1 g chymotrypsin was added in the test tube and incubated at 37°C for 5 days. After every 24 h, 2 mL of the release medium was pipetted out and freeze dried. The freeze-dried powder was extracted with anhydrous THF and the THF solution analyzed for molecular weight determination by using GPC as described under Instrumentation.

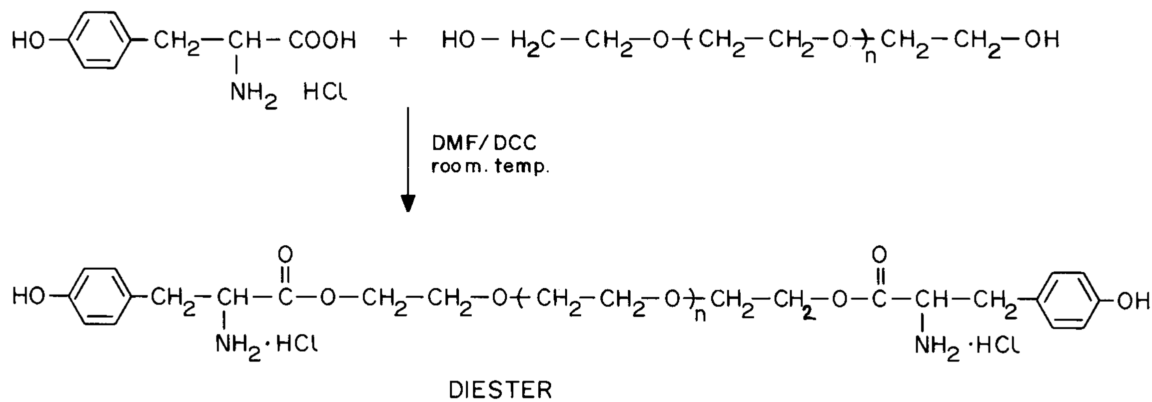
RESULTS AND DISCUSSION

As described in the introduction, the objective of the present work was to develop a simple methodology that would eliminate the use of conventional —NH_2 protecting groups of amino acids in the prodrug synthesis. We have recently reported a new method of DCC-mediated direct esterification of PEG with amino acid hydrochlorides, using hydrochloride as the —NH_2 protecting group.[23] In this article, we report the application of this basic reaction in the synthesis of enzymatically degradable prodrugs. Our synthetic methodology can be summarized as follows: (1) synthesis of PEG–Tyr.HCl diester so as to obtain PEG macromer with reactive phenolic —OH end groups; (2) oligomerization of PEG–Tyr macromer via condensation with a dicarboxylic acid; (3) neutralization of tyrosine.hydrochloride salt and drug linkage to the free amino groups (see Fig. 1). In the following sections the three stages are described in detail.

Bis-Tyr.HCl–PEG 6 kDa (diester preparation)

In esterification or amidation of tyrosine, its phenolic —OH group does not need protection. For example, Kohn et al.[24] synthesized tyrosine dipeptides (diphenols) by reacting *N*-acyl tyrosine with tyrosyl alkyl ester in the presence of DCC. Also, amino groups of tyrosine and serine as well as amino sugars could be selectively acylated without protection of the hydroxyl groups.[25,26] (Note that in the present case, amino groups are protected in the hydrochloride form.) Unlike basic nucleophiles, hydroxyl groups of PEG cannot neutralize Tyr.HCl salt. We, therefore, envisaged DCC-mediated condensation between PEG 6 kDa and Tyr.HCl to synthesize the diester having tyrosyl phenolic —OH end groups free and amino groups protected as hydrochloride salt (Fig. 1, step 1). Bis-Tyr.HCl–PEG 6 kDa was synthesized as described above. IR spectrum of bis-Tyr.HCl–PEG 6 kDa in Figure 2 shows a peak at 1720 cm^{-1}, which corresponds to carbonyl of ester formed between PEG and Tyr.HCl. The spectrum does not contain an amide carbonyl peak. This indicates that amino groups remained protected in hydrochloride form. This was quantified and substantiated from the acid value measurements. Acid value of bis-Tyr.HCl–PEG 6 kDa was found to be 0.29 mmol HCl/g, which is in close agreement with its theoretical value of 0.31 mmol HCl/g. This confirms complete esterification of carboxyl groups of Tyr.HCl with PEG. ^{1}H-NMR spectrum of bis-Tyr.HCl–PEG 6 kDa in Figure 3 shows that the assigned peaks are in agreement with the proposed structure. Particularly, peaks for —NH_2.HCl and phenolic —OH are seen in the spectrum at 8.8 δ and 9.6 δ, respectively. GPC analysis revealed the following MW data for the diester: M_w = 7200; M_n = 4800; M_w/M_n = 1.5. We also determined the MW of commercial PEG 6 kDa samples used, which showed M_w = 6500; M_n = 4200; and M_w/M_n = 1.54 and confirmed the

STEP-1



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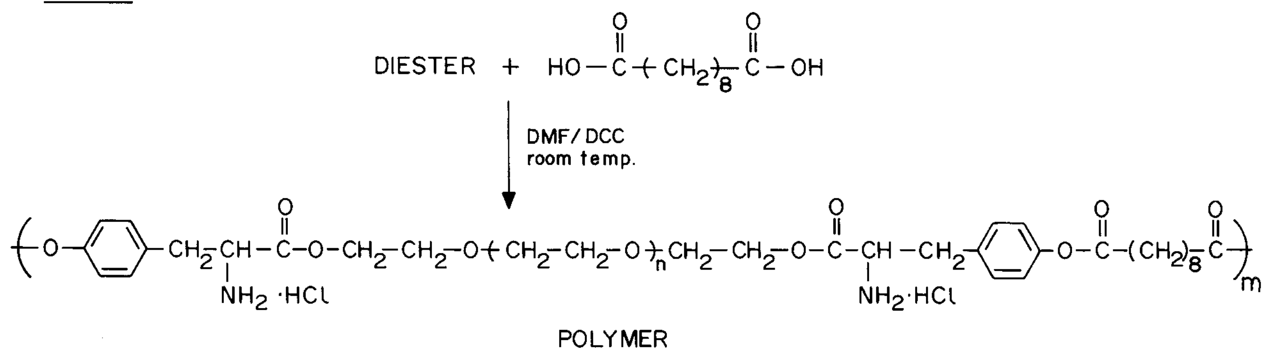

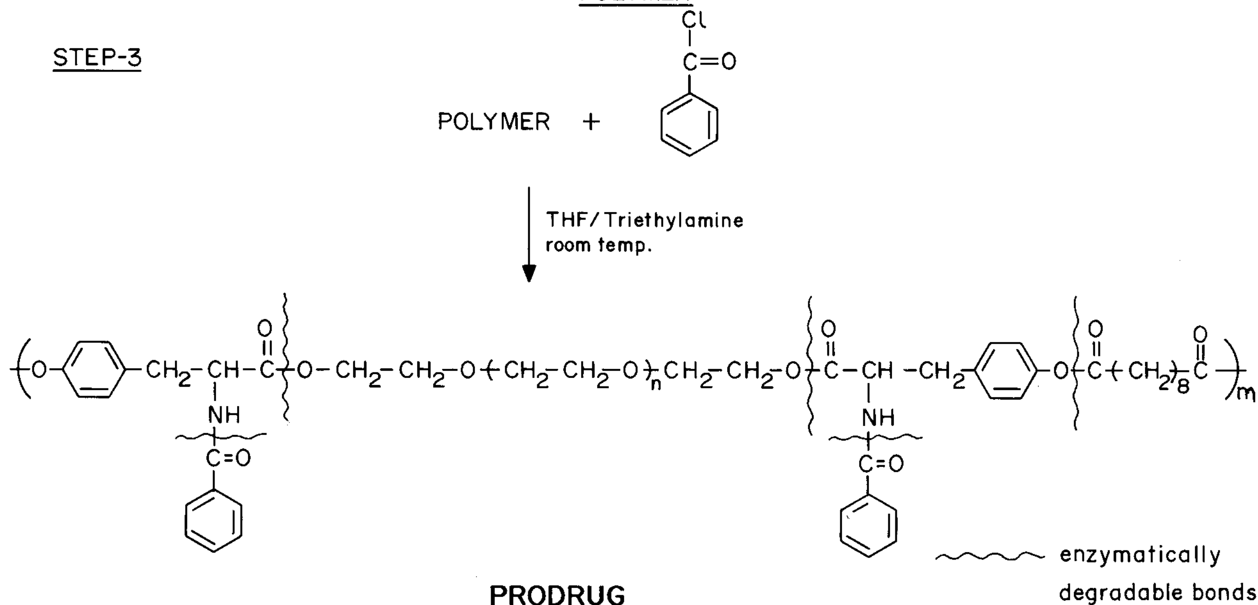

Figure 1 Schematic representation of the synthetic methodology.

synthesis of bis-Tyr.HCl–PEG 6 kDa by this reaction.

Diesters of this type are conventionally synthesized as follows. In the first step, *p*-toluene sulfonate salt of the diester is synthesized by Dean–Stark esterification by using stoichiometric amounts of amino acid, PEG, and *p*-toluene sulfonic acid monohydrate. In the second step, *p*-

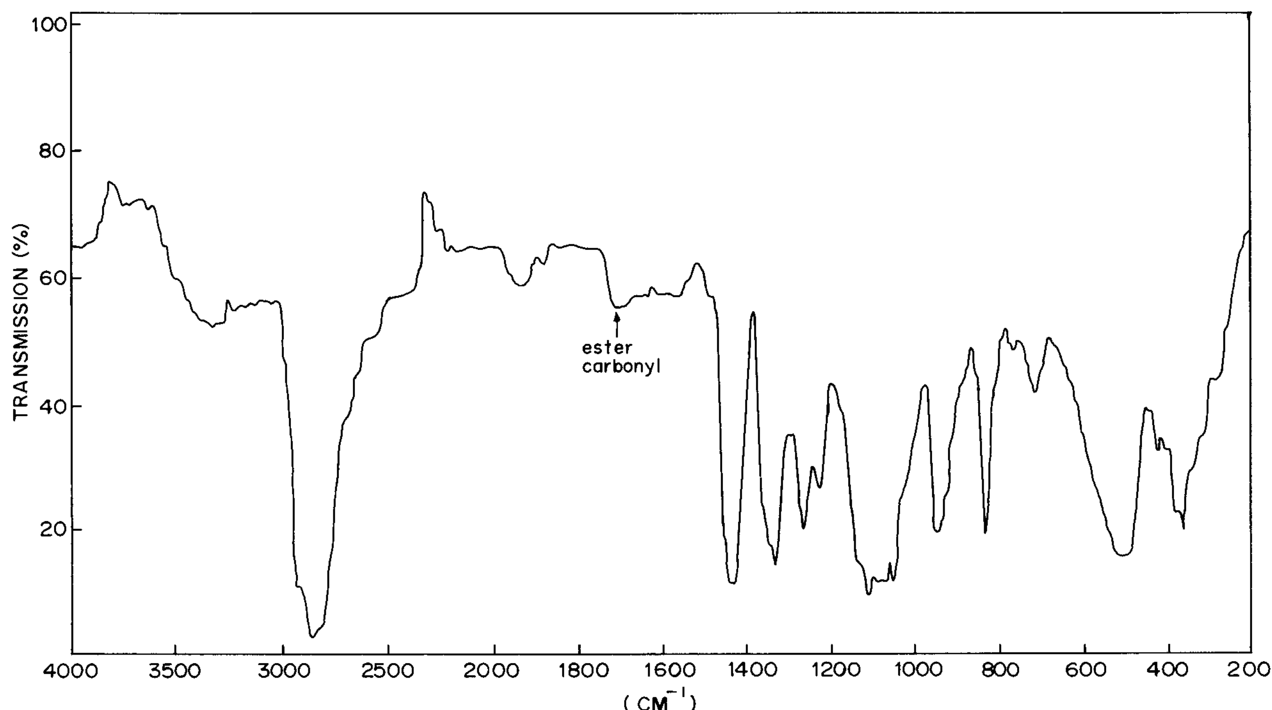

Figure 2 IR spectrum of bis-Tyr.HCl–PEG 6 kDa.

toluene sulfonate is deblocked by the treatment of triethylamine or sodium bicarbonate. For stability purposes, the free ester so synthesized is then converted into hydrochloride salt by passing dry HCl into the ether solution of the ester. The present reaction route has the advantage of eliminating such tedious steps.

Oligo(Bis-Tyr.HCl–PEG 6 kDa–Sebacate) (oligomerization)

Enzymatic hydrolysis of a prodrug is affected by its molecular weight [e.g., poly(*N*-2-hydroxypropyl methacrylamide) and poly(maleic anhydride-*co*-vinylpyrrolidone)-based prodrugs of MW 20–30 kDa exhibited slower chymotrypsin-catalyzed hydrolysis of *p*-nitoanilide in the side-chain oligopeptide].[6,27] This was attributed to the higher steric hindrance for the access of the enzyme to the oligopeptide substrate in high MW polymers.[28] Another point to be considered is the MW threshold for the elimination of macromolecules via glomerular filtration, which is $\sim$ 50 kDa.[29] Thus, in principle, high MW polymers can also be eliminated from the body. However, attachment of drug-targeting moiety can increase the uptake of prodrug by targeted cells. Therefore, degradable oligomers containing PEG blocks were used for prodrug design.[18] The active site for these oligomers is easily accessible to the enzyme. Thus, no steric hindrance is observed in the enzymatic degradation of these oligomeric prodrugs.

For oligomerization of PEG blocks, phenolic —OH end groups of the diester were condensed with sebacic acid in the presence of DCC as described above by using nearly equal molar quantities of phenolic —OH groups of the diester and carboxyl groups of sebacic acid. In this case, because the amino and carboxyl groups of tyrosine are blocked, condensation between —OH of tyrosine and —COOH of sebacic acid took place. This is consistent with the reactions of tyrosine diphenols with various dicarboxylic acids in the presence of DCC.[30] IR spectrum of this polymer was similar to that of bis-Tyr.HCl–PEG 6 kDa. In the ^{1}H-NMR spectrum, peak for phenolic —O*H* proton at 9.6 δ was absent, as it was consumed during condensation. Remaining peaks were identical with the diester, except that the additional peaks for sebacic acid moiety were observed. (See ^{1}H-NMR data in the experimental section.) Because of the presence of strongly polar —NH_2.HCl groups, the oligomer was insoluble in THF, the solvent used for GPC. For determination of the molecular weight, the polymer was treated with triethylamine to obtain free-base,

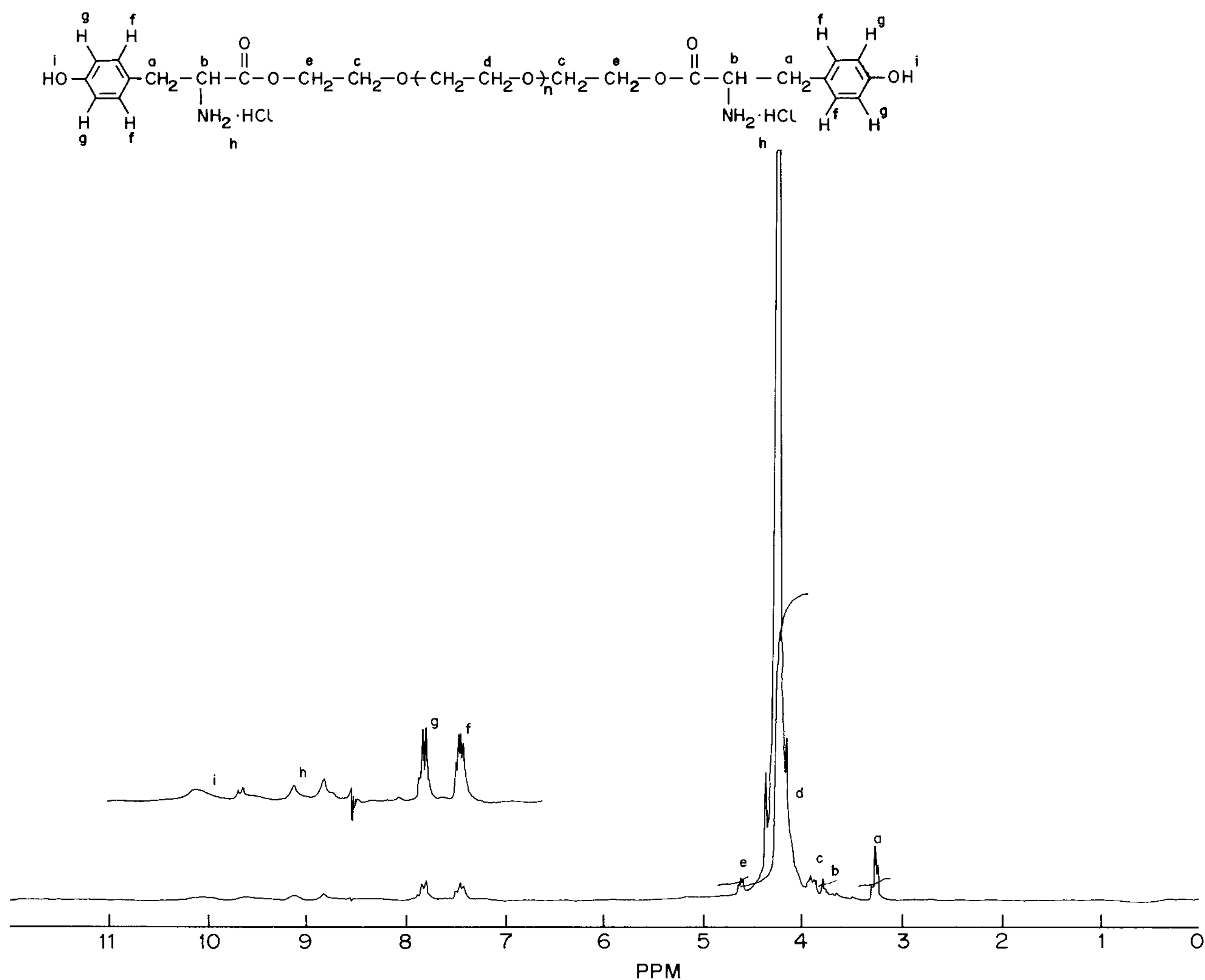

Figure 3 ^{1}H-NMR spectrum of bis-Tyr.HCl–PEG 6 kDa.

oligo(bis-Tyr–PEG 6 kDa–sebacate), where $M_w = 14{,}117$; $M_n = 5251$; $M_w/M_n = 2.68$. M_w indicates that it is a dimer or trimer of bis-Tyr–PEG 6 kDa connected via sebacic acid.

Oligo(Bis-*N*-Benzoyl Tyrosyl–PEG 6 kDa–Sebacate) (oligomeric prodrug)

To demonstrate the reactivity of pendant amino groups in oligo(bis-Tyr–PEG 6 kDa–sebacate), benzoyl chloride was selected as a model reagent so that the resulting prodrug would contain benzoic acid chemically linked via amide bond. *N*-benzoylation was carried out as described above.

IR spectrum of this model prodrug in Figure 4 shows a sharp peak at 1640 cm^{-1} that corresponds to carbonyl of amide bonds formed and a small shoulder at 1700 cm^{-1} for ester carbonyl. From the acid value, it was estimated that 73% of the amino groups were benzoylated. This shows the accessibility and reactivity of pendant amino groups in the oligomer. ^{1}H-NMR spectral data of oligo(bis-*N*-benzoyl Tyr–PEG 6 kDa–sebacate) were in accordance with its proposed structure (Figure 5). Molecular weight data for poly(*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate) were as follows: $M_w = 14{,}772$; $M_n = 5486$; $M_w/M_n = 2.69$. T_m of the prodrug was 61°C. This decrease in the T_m (from 65 to 61°C) can be attributed to conversion of high melting hydrochloride salts to *N*-benzoyl derivatives, which support benzoylation of the pendant amino groups of the oligomer. Thus, we have eliminated the conventional use of *N*-benzyloxycarbonyl or *N*-tertiary butyloxycarbonyl group for protection of —NH_2 and its subsequent deprotection by hydrogenation or hydrolysis, re-

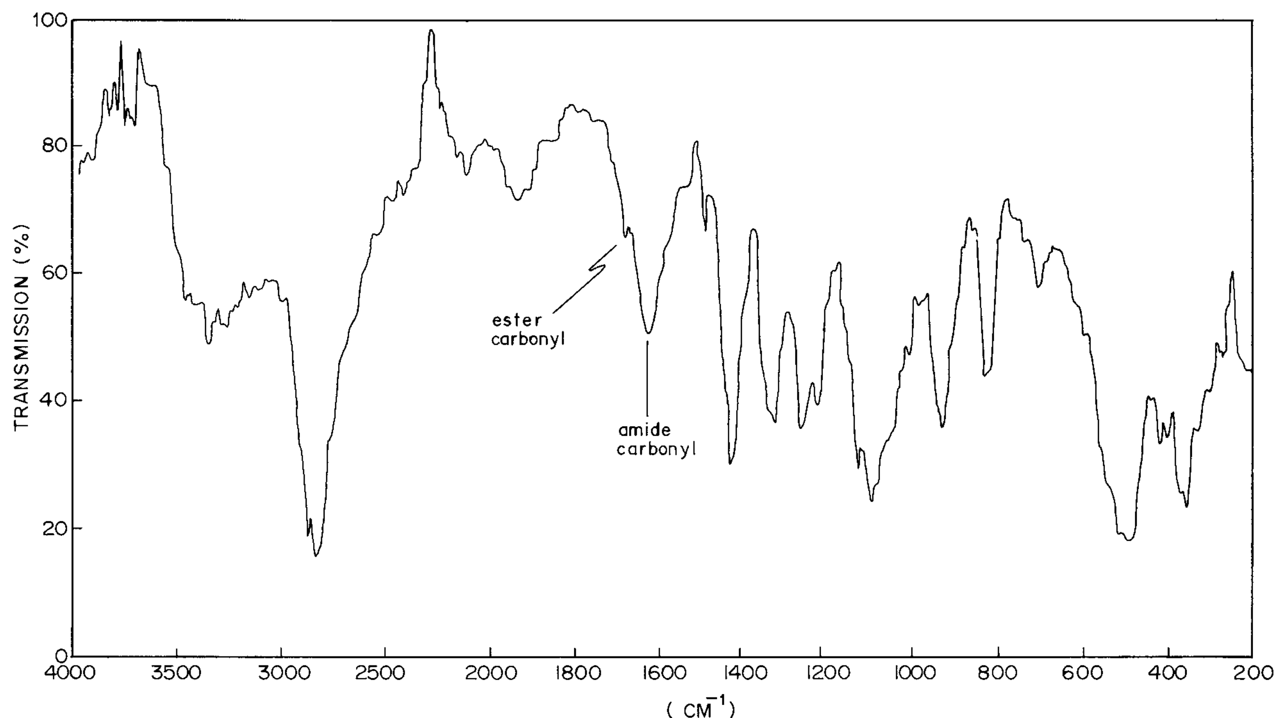

Figure 4 IR spectrum of oligo(bis-*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate).

spectively. In the present process, after neutralization of hydrochloride salt, a variety of bioactive molecules can be linked to this polymer. In this work, low molecular weight PEG blocks (PEG 6 kDa) were used because after enzymatic degradation the PEG units formed could be easily eliminated from the system.

Chymotrypsin-Catalyzed Degradation of the Prodrug

Enzymatic degradation of the prodrug was followed by GPC analysis as described above. Data for decrease in MW of the prodrug listed in Table I show that oligomeric prodrug with MW 15,400 (dimer/trimer) was hydrolyzed by chymotrypsin to the PEG 6 kDa diester stage over a period of 2 days. However, MW degradation studies did not give data on the rate of main-chain versus side-chain hydrolysis. To obtain these data, free tyrosine that is liberated should be analyzed. Ninhydrin assay was used to follow enzymatic degradation of polymers containing amino acids.[31] We therefore monitored degradation of the prodrug by Ninhydrin assay.

Table I GPC Data for Decrease in Molecular Weight of Prodrug Due to Chymotrypsin Catalyzed Degradation

Days	MW of Oligo(bis-*N*-benzoyl-tyr-PEG 6 KD-sebacate)
1	15,400
2	9200
3	7900

Enzymatic hydrolysis of the diester, oligomer, and *N*-benzoylated prodrug as well as Ninhydrin assay were conducted as described in the experimental section. It should be noted here that only free amino acids give a positive Ninhydrin test.[22] We verified this by performing Ninhydrin assay separately on diester, oligomer, and the prodrug in aqueous as well as in phosphate-buffered solutions. The assays were negative in all cases. Ninhydrin assay was also negative for pure chymotrypsin. Only in the presence of chymotrypsin did the above-mentioned substrates exhibit positive Ninhydrin assays. This supports the enzymatic hydrolysis. An independent experiment was also conducted to measure the activity of chymotrypsin during the 8 days of incubation. It was observed that there was no loss in the activity.

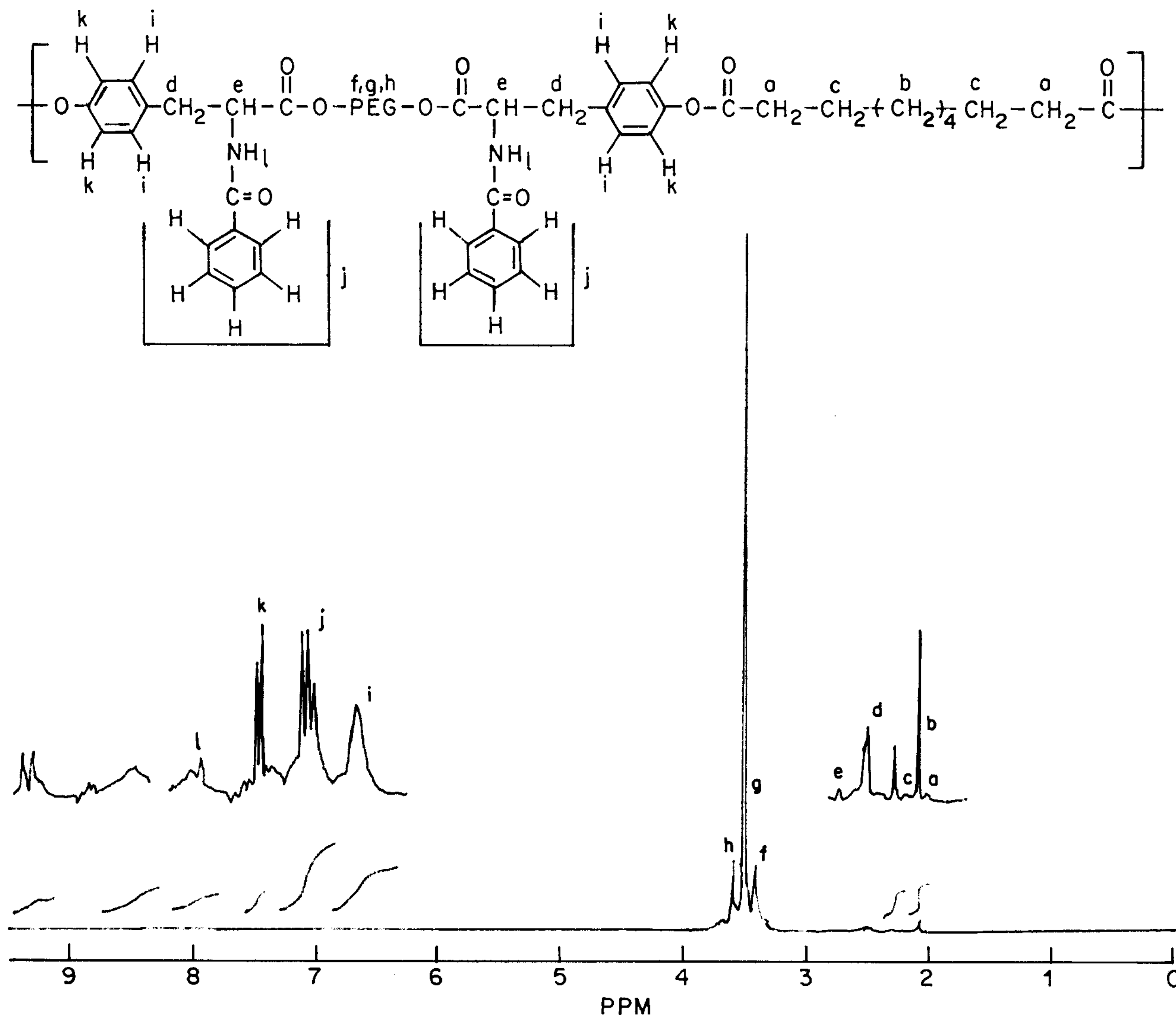

Figure 5 ^{1}H-NMR spectrum of oligo(bis-*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate).

Data for the amount of free tyrosine liberated versus time is graphically represented in Figure 6. Curve a in Figure 6 shows that chymotrypsin completely hydrolyzed oligo(bis-Tyr–PEG 6 kDa–sebacate) in only 4 days, of which, almost 80% hydrolysis (in terms of the liberated free tyrosine) was observed in just 2 days, although the data on curve c for the prodrug show that within the first 4 days of incubation only 10% of the total tyrosine present in the prodrug was liberated and in the next 4 days a rapid release of almost 90% of the total tyrosine took place. These two release profiles for free tyrosine indicate rapid hydrolysis of ester backbone of the oligomer followed by slow hydrolysis of *N*-benzoyl tyrosyl units. We verified this by monitoring chymotrypsin-catalyzed hydrolysis of *N*-benzoyl tyrosine. Curve b in Figure 5 shows that in the first 4 days only 50% hydrolysis of *N*-benzoyl tyrosine took place. We also quantified chymotrypsin-catalyzed hydrolysis rates for *N*-benzoyl tyrosine and the polymer main chain in the framework of Michaelis–Menten kinetics. V_{max} for polymer main-chain hydrolysis [i.e., poly(bis-Tyr.HCl–PEG 6 kDa–sebacate)] was 0.033 mmol tyrosine/24 h, whereas V_{max} for *N*-benzoyl tyrosine was 0.026 mmol tyrosine/24 h. This is consistent with the literature that chymotrypsin-catalyzed ester hydrolysis is faster than amide hydrolysis.[15,16]

Because chymotrypsin brings about hydrolysis of specific substrates containing amino acids such as tyrosine and phenyl alanine, the development

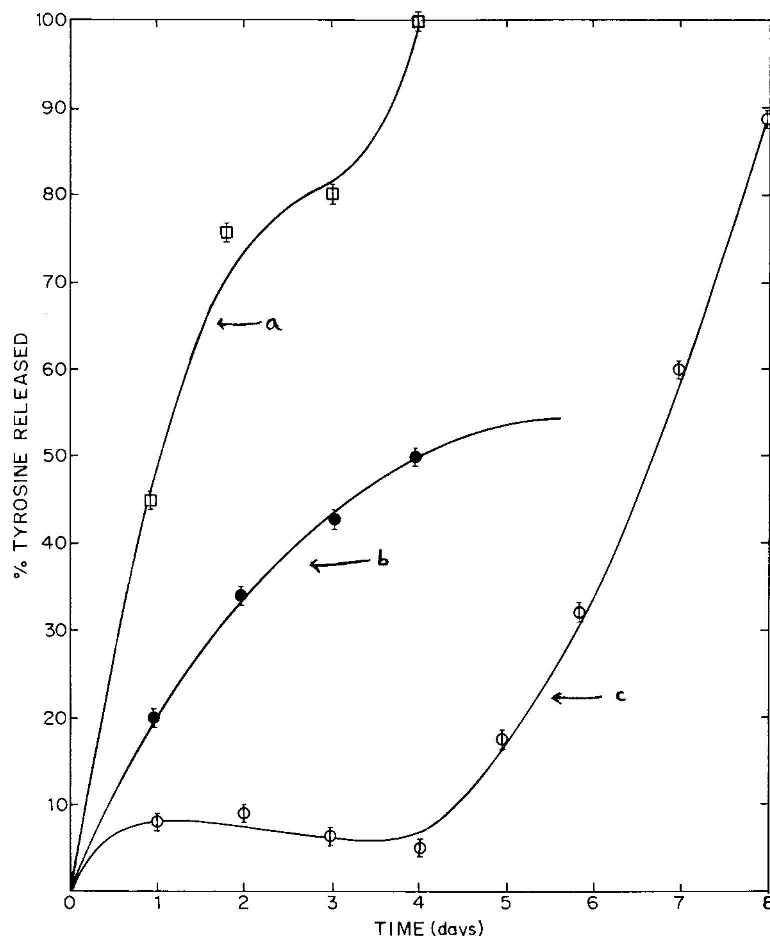

Figure 6 Free tyrosine liberated versus time. Curve a: release of tyrosine versus time for oligo(bis-Tyr–PEG 6 kDa–sebacate). Curve b: release of tyrosine versus time for the monomeric conjugate (*N*-benzoyl tyrosine). Curve c: release of tyrosine versus time from the oligomeric prodrug oligo(bis-*N*-benzoyl tyrosyl–PEG 6 kDa–sebacate).

of such specific prodrug systems and the enzymatic degradation in the presence of this enzyme is useful for developing enzymatically degradable drug carrier systems.

The formation of free tyrosine during enzymatic hydrolysis of the prodrug shows that in the absence of oligopeptide spacer also, the pendant drug release took place. This novel prodrug system reported by us should be useful as it degrades to release the drug and its nontoxic building blocks.

CONCLUSION

A model polymeric prodrug based on PEG and L-tyrosine was synthesized by employing a novel synthetic methodology. This methodology eliminates the use of conventional *N*-benzyloxycarbonyl (*N*-cbz) and *N*-tertiary butyloxycarbonyl (*N*-t boc) groups and uses simple hydrochloride salt as —NH_2 protecting group. Chymotrypsin exhibited hydrolysis of this prodrug wherein the first polymer main-chain hydrolysis and then pendant chain drug release were observed. This new methodology should be useful in synthesizing prodrugs based on PEG and various other amino acids.

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