Synthesis of a Carbon-14 Labelled Peptidyl Carbamate Human Leukocyte Elastase Inhibitor and Its Immobilization on a Tritiated Water Soluble Polymer

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SUMMARY

A carbon-14 labelled hemisuccinate peptidyl human leukocyte elastase inhibitor, p-nitrophenyl-N-(1,4-[^{14}C])-succinyl-L-alanyl-L-alanyl-prolylmethyl-N-isopropyl carbamate, and a tritium labelled hydrophilic polymer, poly-α, β-[N(2-hydroxy-1-[^{3}H]-ethyl)]-D,L aspartamide were prepared and are reported here. The immobilization of the [^{14}C]-peptidyl carbamate onto the tritiated polymer to afford a double labelled macromolecular inhibitor of human leukocyte elastase is also illustrated.

Key Words: Peptidyl carbamate, polyhydroxyethyl aspartamide, human leukocyte elastase, macromolecular inhibitor, double label.

INTRODUCTION

In continuation of our efforts concerned with the rational design of active site directed inhibitors of human leukocyte elastase (HLE), an enzyme partially responsible for tissue destruction associated with such pathological situations as emphysema, arthritis, and inflammation, it was of interest to achieve a potent inhibitor that possessed a long residency in plasma[1]. Effective small molecular weight peptidyl carbamate

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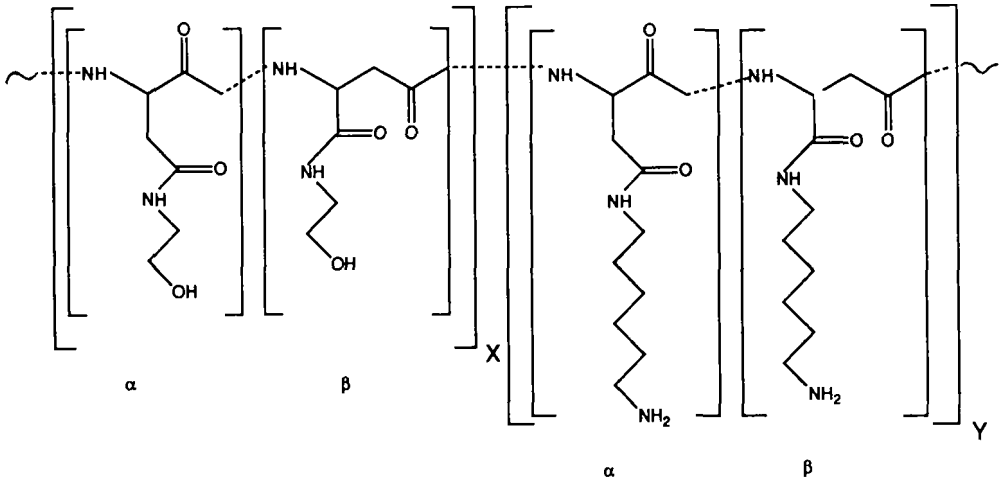
inhibitors (PC) of HLE have been reported that efficiently react with the enzyme in a stoichiometric (1:1) ratio[1]. These substrate mimicking peptidyl carbamates are protected oligopeptides into which amino acid isosteres have been placed. Unfortunately, their in-vivo use is limited since the advantage of a low dose requirement is overshadowed by their rapid elimination from plasma.

The covalent immobilization of a low-molecular-weight enzyme inhibitor on a soluble hydrophilic polymer proved to increase its residence time in plasma, providing for a sustained elevated level of the inhibitory activity in the blood and lymph compartments[2,3]. The increase of the plasma residence time is due to the slower renal clearance by the glomerular filtration of macromolecules with the size above a certain limit[4,5]. Polymer bound peptidyl-carbamate inhibitors (PPC) of human leukocyte elastase have been prepared containing multiple inhibitory moieties bound to the average polymer chain of a hydrophilic water-soluble polymer[6,7]. Thus, one molecule of the resulting macromolecular inhibitor can, in theory, react with more than one molecule of the enzyme. In vitro, the polymer-bound peptidyl-carbamate inhibitor containing 1-2 inhibitory PC per average polymer chain has been shown to be at least as active as the natural inhibitor of HLE, i.e. α_1-antitrypsin.

As part of a mechanistic study of the inactivation of HLE by the polymer bound peptidyl carbamates (PPC), as well as the disposition of the peptidyl carbamates themselves, an isotopically labelled form of a model PPC was required. Efforts were focused, therefore, on the production of a carbon-14 and tritium double labelled PPC. Having the polymer labelled with a different isotope than the peptidyl carbamate should allow experiments aimed at assessing whether the carbon-14 peptidyl carbamate remains intact, or is released during the enzyme inactivation process, to be undertaken. Furthermore, since the carbon-14 to tritium ratio

would be known, the stability of the polymer-peptidyl carbamate covalent linkage in aqueous or biological media could be studied.

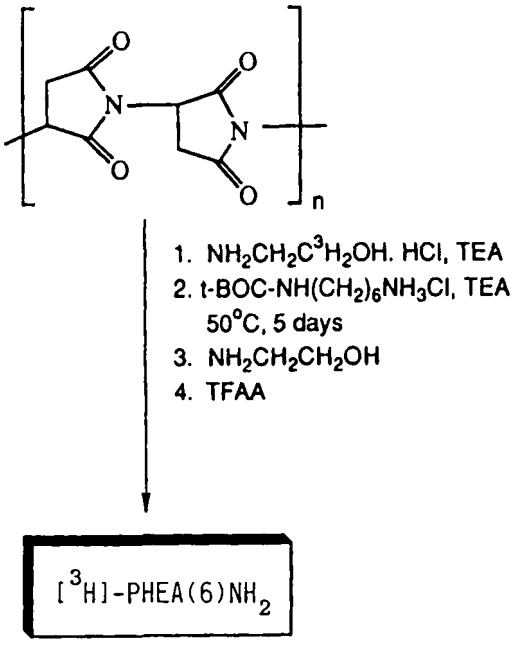
A synthetic polypeptide, i.e. poly-α,β-[N^4-(2-hydroxyethyl)-aspartamide], (PHEA) (Figure 1) is known as a linear, water-soluble



X= Amount of Hydroxyl Side Chains
Y=1-X = Amount of Amino Side Chains

Figure 1: POLY-α,β-[N^4-12-hydroxyethyl]-D,L ASPARTAMIDE PHEA-(6) NH_2

neutral polymer with very low immunogenicity and good biocompatibility[8]. It has been chosen for preparation of the polymer-bound PC inhibitor. In summary, a method for double labelling of PPC was developed which consists of the following steps. At first, the tritiated PHEA was prepared containing t-butoxy carbonyl (tboc)-protected aminohexyl aspartamide units by successive reactions of polysuccinimide with [1-^{3}H]-ethan-1-ol-2-amine, N-tboc-1,6-diaminohexane, and carrier ethanolamine (Scheme 1). After purification by dialysis, the deprotection of amino hexyl groups with trifluoroacetic acid afforded the poly-α,β-[N^4-(2-hydroxy-1-[^{3}H]-ethyl)-D,L-aspartamide] with amino hexyl side chains ([^{3}H]-PHEA(6)NH_2). Because Poly-α,β-[N^4-(2-hydroxyethyl)-D,L-aspartamide containing the aminohexyl moeity



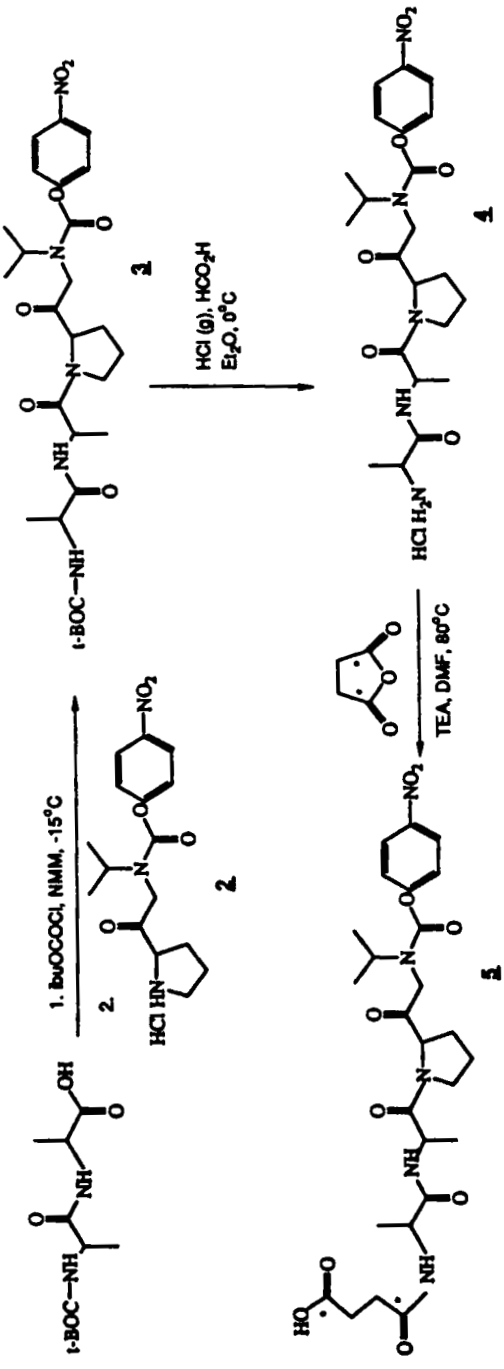
SCHEME 1

is prepared using a polymer analogue reaction of polysuccinimide with the amino functions, the amines can react with the succinimide at either the α or β site resulting in a random distribution of amino and hydroxyl side chains (α or β)[9]. The amino hexyl side chains were then used for binding of carbodiimide activated [^{14}C]-peptidyl carbamate ([^{14}C]-PC) (Scheme 2), yielding double labelled polymer-bound inhibitor ([^{3}H], [14HC]-PHEA(6)PC)[7].

This approach may also be applicable for preparation and testing of other double labelled polymer-bound experimental drugs.

MATERIALS AND METHODS

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared (ir) spectra were recorded on a Perkin-Elmer model 1430 ratio recording spectrophotometer. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta,



SCHEME 2

* Denotes Position of Carbon-14 Label

GA and were within ± 0.4% of theoretical values. Ultraviolet (uv) spectral analyses were carried out using a Cary 2200 UV-Vis spectrophotometer, Varian Associates, Walnut Creek, CA. Nuclear magnetic resonance (nmr) spectra were recorded on a Varian model Em-360 or Em-390 spectrometer Varian Associates, Walnut Creek, CA. Radiochemical assessments were made using a Packard Tricarb 3375 or CAA model liquid scintillation spectrometer, Packard Instrument Company, Downers Grove, IL. [1,4, ^{14}C-]-Succinic anhydride was purchased from Amersham International and was supplied in break seal ampules under nitrogen. [1-^{3}H]-Ethan-1-ol-2-amine hydrochloride was obtained from Amersham International as an aqueous solution in a duoseal vial. Polymer purity assessments and molecular weight distribution analysis of polymer carrier was performed by size exclusion chromatography on a mixed bed column Sepharose CL-4B, Sephacryl S200 SF, Sephadex G-25 SF (16:5:3) (13 x 350 mm). Phosphate buffer (0.05 M) pH 7.5, containing 0.15 M NaCl was used as an eluent. Calibration of the column was achieved by using standard samples of PHEA[10]. The elution profiles were monitored by flow through UV spectrophotometry (Waters Flowthrough Detector). The values of molecular weight averages (Mw and Mn) and a cumulative molecular weight distribution were calculated from size exclusion chromatography. The content of aminohexyl groups of the polymer was assessed spectrophotometrically after the reaction of amino groups with 2,3,5 trinitro-benzene sulphonic acid and was found to be 7.8%.

[^{3}H]-PHEA with amino hexyl side-chains ([^{3}H]-PHEA(6)NH_2)

(1-[^{3}H]-1-ethan-1-ol-2-amine-HCl (29.8 Ci/mmol, 2 mCi, 67.1 μmol) in water (2 mL) was transferred to a round bottom flask, the vial washed with water (5 x 1 mL), and the washings added to the flask in preparation for removal of water at reduced pressure. The

flask was slowly evaporated to dryness and the residue (approximately 1.0 mCi) taken up in dimethyl formamide (DMF) (15 mL). Next, polysuccinimide (1.5 g, approximately 15 mmol of succinimide units, Mw-27,000), t-butoxycarbonyl-1,6 hexamethylene diamine-HCl (454 mg, 1.8 mmol, 12 mol%,). Triethylamine (TEA) (275 µL, 2.0 mmol) and carrier ethanolamine (120 µL, 2.0 mmol) were added and the mixture heated to 50°C for three days. On the third day, excess ethanolamine (1.2 mL) was added (to ensure complete reaction) and stirring continued for an additional 12 h. The solution was next transferred to dialysis tubing and was dialysed (Spectrapor cellulose tubing) against water (4 x 2 L) changing the dialysate every 6 h. The contents of the dialysis tube were transferred to a flask and concentrated in vacuo to a volume of approximately 20 mL. Trifluoroacetic acid (20 mL, 0.26 mol) was added, the mixture stirred for 20 min and then loaded into dialysis tubing. Dialysis against water (3 x 2L) followed by a concentration of the contents to approximately 30 mL afforded pure polymer. GPC on a Sephadex-G25 column indicated absence of low molecular weight impurities. Lyophilization overnight produced [^{3}H]-PHEA(6)NH_2 (1.77 g, 403.56 µCi, 0.228 µCi/mg, 44.7% radiochemical yield) as a fluffy colorless solid.

p-Nitrophenyl N-[L-prolylmethyl]-N-isopropyl Carbamate hydrochloride 2. This compound was synthesized in a manner analogous to that of Tsuji, et. al, and was found to be spectrally identical to an authentic sample[12].

p-Nitrophenyl N-[N-t-butoxycarbonyl-L-alanyl-L-alanyl-L-prolyl methyl]-N-isopropyl Carbamate 3.

To a solution of N-tboc-Boc-Ala-Ala 1 (287 mg 1.1 mmol) and N-methyl morpholine (NMM) (154.7 µL, 1.4 mmol) in THF (5 mL) cooled to -20°C was added a solution of isobutyl chloroformate (167 µL,

1.3 mmol) in THF (3 mL) with stirring. Stirring was allowed to continue for 20 min after which the temperature was reduced to -50°C and a suspension of 2 (400 mg, 1.1 mmol) and NMM (154.7 µL, 1.4 mmol) in 50% MeCN/THF (5 mL) was added. The reaction was allowed to warm to room temperature over a 5 h period after which the mixture was diluted with chloroform (10 mL) and washed with 10% citric acid (2 x 10 mL) and 4% $NaHCO_3$ (1 x 10 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to afford approximately 750 mg of an oily product. Column chromatography (15 g silica gel G 8% MeOH/CH_2Cl_2) followed by drying in vacuo over phosphorus pentoxide afforded 555.2 mg (87% yield) of 3 which melted at 168-171°C. 1H-NMR (90MHz) $CDCl)_3$ δ 1.5 (9H, S), 1.6-1.7 (21 H, m), 2.0-2.2 (4H, m), 3.5-3.7 (3H, m), 4.1-4.5 (5H, m), 7.20 (2H, d, J=9Hz), 8.20 (2H, d, J=9Hz), ir (KBr) ν_{max} 1730, 1650, 1520, 1345, 1190, 1155 cm^{-1}, Anal ($C_{26}H_{39}N_5O_8$) C,H,N.

p-Nitrophenyl N-(L-alanyl-L-alanyl-L-prolylmethyl N-isopropyl carbamate hydrochloride 4.

The t-boc protecting group was removed by acidolysis using the procedure described below. Formic acid (1.25 mL) was added to a stirring solution of 3 (0.6 g, 1.02 mmol) in EtoAC (7 mL) cooled to 4°C. Anhydrous HCl was bubbled through the reaction mixture while the reaction was monitored by TLC (10% MeOH/$CHCl_3$). After the total disappearance of starting material 3, the solvent was removed in vacuo. The formic acid was removed by repetitive azeotropic evaporations with n-heptane at reduced pressure. The resulting oil was used in the next step without further purification.

p-Nitrophenyl N-([1,4-^{14}C]-succinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyl carbamate 5 [7].

To a solution of 4 (0.385 g, 0.7523 mmol) in DMF (1 mL) was

added triethylamine (105 µL, 0.7523 mmol). The ampule containing [1,4-^{14}C] succinic anhydride (300 µCi, 111 mCi/mmol) was opened and DMF washes (4 x 1 mL) were added to the reaction mixture. The flask was heated to 80°C for 10 min. At this time, carrier succinic anhydride (0.075 g, 0.75 mmol) was added and the mixture heated an additional 2 h. The reaction was allowed to cool to room temperature and diethyl ether (approximately 15 mL)) was added to precipitate the triethylamine hydrochloride. Filtration was followed by azeotropic removal of the DMF using toluene in vacuo. Complete evaporation yielded a crystalline product which was triturated with 2% HCl (2X) to remove excess succinic acid. Filtration, followed by recrystallization from THF/Et_2O, afforded 380 mg (86.9%; 258 µCi; 86% radiochemical yield) which melted at (m.p. 184-198°C). The specific activity was determined to be 0.394 mCi/mmol by the standard curve method. The material was found to be chemically and radiochemically pure by TLC (TLC autoradiography using two solvents; EtoAc, 20% MeOH/$CHCl_3$). The sample was found to be spectrally identical to a non-radioactive sample[7].

Binding of p-Nitrophenyl N-(1,4-[^{14}C]-succinyl-L-alanyl-L alanyl-L-prolylmethyl)-N-isopropyl carbamate ([^{14}C]-PC) to [^{3}H]-PHEA(6)NH_2 ([^{3}H][^{14}C]-PHEA(6)PC)

[1,4-^{14}C] Peptidyl carbamate hemisuccinate 5 (74.8 mg, 0.128 mmol, 50.3 µCi) and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) (27 mg, 0.142 mmol) were reacted in DMF (1.0 mL) for 50 min at 4°C. Next, an ice cooled solution of [^{3}H]-PHEA(6)NH_2 (258 mg, 0.128 mmol-NH_2, 57.53 µCi) and TEA (20 µL, 0.15 mmol) in DMF (2.5 ml) was added, and stirring continued overnight at 4°C. The polymer product was then dialysed against water (4 x 2 L) with changing the dialysate every 4-6 h. The dialyzed solution was concentrated in vacuo to afford a viscous pale yellow oil. Lyophillization afforded a fluffy colorless

powder weighing 153 mg (40.5 μCi ^{3}H, 13.92 μCi ^{14}C). The content of peptidyl carbamate groups was determined from UV absorption data at 276 nm (ε=9800), to be 0.214 μmol peptidyl carbamate/mg polymer bound product. Thus, the molecular weight equivalent per one peptidyl carbamate unit corresponded to 4671 μg/μmol. The loading was estimated to be 5 mol % from the molecular weight equivalent. The specific activities were determined to be 0.265 μCi/mg (^{3}H) and 0.0910 μCi/mg (^{14}C). The $^{3}H/^{14}C$ ratio was determined to be 2.91. The radiochemical yields, expressed separately for tritium and carbon-14, were respectively 70.4 and 27.8 percent yield.

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