Synthesis of Novel γ-Alkenyl L-Glutamate Derivatives Containing a Terminal C−C Double Bond To Produce Polypeptides with Pendent Unsaturation

Drew S. Poché,† Stefan J. Thibodeaux,‡ Victor C. Rucker,† Isiah M. Warner,‡ and William H. Daly‡

Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803, and Department of Chemistry and Physics, Southeastern Louisiana University, Hammond, Louisiana 70402

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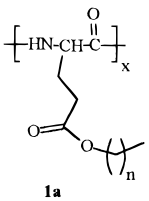
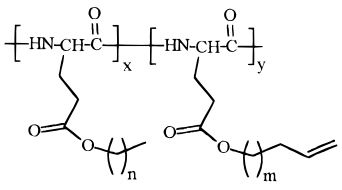
Interest in various applications of poly(γ-alkyl α,L-glutamates), **1a**, led us to develop suitable methods for the facile substitution of the alkyl side chains by functional pendent groups. For example, to produce stable, solvent swollen gels requires that the polymer must be readily cross-linked without disruption of the peptide links forming the polymeric backbone. Cross-linking by reaction of diamines at the pendent ester carbonyl of **1a** was reported,[1] but conditions that cause cleavage of the peptide backbone must be avoided. Reaction with trimethylsilyl iodide to prepare the reactive silyl ester has been used[2] but is limited to the benzyl ester or short alkyl side chains. Application of poly(L-glutamate)s as stationary phases in certain HPLC techniques is suggested by the ability of lyotropic liquid crystals of poly(γ-benzyl α,L-glutamate) to aid in visualizing enantiomers in NMR measurements.[3] One of the purposes of producing water soluble poly(L-glutamate) derivatives was to investigate their usefulness as pseudostationary phases in separation techniques such as electrokinetic capillary chromatography, ECC.[4]

We found that a pendent C−C double bond, a versatile handle for cross-linking and other chemical functions, could easily be incorporated into the alkyl side chain of the monomer without degradation during monomer or polymer preparation. An esterification of L-glutamic acid to make γ-ester derivatives using primary alcohols with long, saturated carbon chains (8-21 C atoms) was patented in 1966 by Wasserman *et al.*[5] This method works with primary alcohols containing a terminal C−C double bond without deleterious side reactions at the unsaturated position in the final product, **2**. The successful synthesis of L-glutamates with a terminal double bond in the side chain led to the production of polypeptides with the general structure **1b**, where the comonomers may be randomly distributed or in blocks.



1a

n can be 0-21



1b (**4a** or **4b**, when x = 0)

m = 0 or 7

Scheme 1 shows the routes to the various copolymers, starting with L-glutamic acid. The structures presented were all verified by FTIR and 200 or 250 MHz ^{1}H NMR. The γ-esters, **2a** and **2b**, were prepared from the reaction of either allyl alcohol or 9-decen-1-ol with L-glutamic acid using *tert*-butyl alcohol as the solvent. In both cases, a 6−8-fold molar excess of the alcohol was used with a molar equivalent of sulfuric acid, each with respect to the quantity of L-glutamic acid used. After 1−1.5 h at 60 °C, the reaction was stopped by neutralization with triethylamine. The γ-(9-decenyl) L-glutamate, **2b**, precipitated from solution upon cooling to room temperature. The γ-allyl L-glutamate, **2a**, crystallized from the reaction after cooling at 10 °C overnight. Compound **2a** has been reported previously[6] as a blocking group for the carboxylate function in amino acids. To our knowledge, **2b** is a new glutamic acid derivative. Table 1 gives a brief summary of some of the physical properties of **2a** and **2b**. In addition to the spectroscopic evidence for the intact C═C group presented in Table 1, we observed that a drop of bromine in carbon tetrachloride applied to solid **2b** was decolorized. The yields (25−30%) for both after recrystallization were lower than those reported by Wasserman *et al.*[5] using saturated alcohols (typically 50−60% according to Wasserman *et al.* and our own experience) and have not been optimized to date.

The reaction of **2a** or **2b** with triphosgene[7] gave the *N*-carboxyanhydride (NCA) monomer, **3**, without loss of the C−C double bond. NCA monomers **3a** and **3b** were isolated as oils. This result is consistent with earlier reports[8] of other L-glutamates with similar R group carbon chain lengths. Monomers **3a** and **3b** can be polymerized with a variety of strong bases or nucleophiles,[8] although triethylamine or a primary amine was more commonly used. The molecular weight was controlled by the initiator chosen. Low molecular weight materials, the focus of the current work, were produced using *n*-butylamine with monomer:initiator molar ratios less than 50. Matrix-assisted laser desorption ionization (MALDI) data indicated polymer molecular weights of about 5000−6000 (a degree of polymerization of about 20). A random copolymer was produced by dissolving the monomers in the polymerization solvent prior to addition of the initiator. For example, **1b** was synthesized where $y = 0.1x$ by initiating a THF solution containing a 10:1 weight ratio of γ-decyl L-glutamate NCA to γ-(9-decenyl) L-glutamate NCA (**3b**). Incorporation of the monomers into the polymer in this ratio was verified by ^{1}H NMR. Polymer **4b** is readily soluble in THF, chloroform, or dichloromethane. Polymer **4a** is poorly soluble, even "gelling" during its synthesis in THF or chloroform.

Next, **4a** or **4b** was reacted with *m*-chloroperoxybenzoic acid (MCPBA) to produce **5a** or **5b**, a polymer with pendent epoxy groups. The epoxidation reaction gave a quantitative conversion (250 MHz ^{1}H NMR) when run 2−3 h at reflux in chloroform. Polymer **5a** or **5b** can be isolated by evaporating the reaction to near dryness, dissolving the residue in methanol/$CHCl_3$, and precipitating into an aqueous 5% $NaHCO_3$ solution to remove *m*-chlorobenzoic acid. Polymer **5b** was readily cross-linked in solution by addition of trifluoroacetic acid (TFA). For example, when TFA was added to a chloroform solution of **5b**, a gelatinous, solvent swollen mass formed in the previously homogeneous solution within 1−2 min, a qualitative indication of polymer chain cross-linking. We presume that acid catalyzed ring opening of the epoxy group occurred to form a hydroxyl group, which may then attack an activated epoxy group on

† Southeastern Louisiana University.
‡ Louisiana State University.

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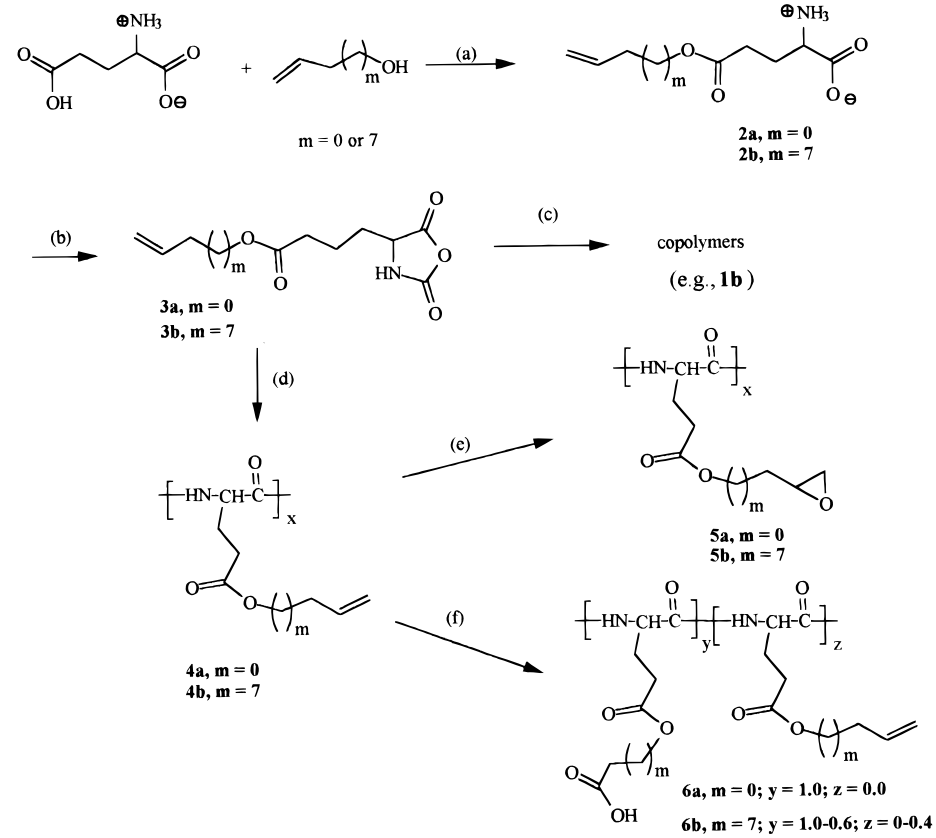
Scheme 1. Synthetic Methodology for Producing Derivatized Poly(l-glutamates)[a]



[a] The procedures used for each labeled step may be found in the Experimental Section.

Table 1. Properties[a] **of γ-Alkenyl L-Glutamates**

2	recrystallization solvent	mp, °C	^{1}H NMR, ppm ($CDCl_3$/TFA)	NCA, **3** ^{1}H NMR, ppm ($CDCl_3$)
γ-(9-decenyl)	1:1 2-propanol−water	188−190	α-C**H**, 4.2 (t); β-C**H$_2$**, 2.3 (m); γ-C**H$_2$**, 2.7 (t); −CH=C**H$_2$**, 4.9 (m); −C**H$_2$**CH=CH$_2$, 2.0 (m); −C**H**=CH$_2$, 5.9 (m); −(C**H$_2$**)$_6$−,1.3 (s, b); O−C**H$_2$**, 4.1 (t); −N**H$_3^+$**, 7.8 (s, b)	oil: α-C**H**, 4.4 (t); β-C**H$_2$**, 2.2 (m); γ-C**H$_2$**, 2.5 (t); −CH=C**H$_2$**, 4.9 (m); −C**H$_2$**CH=CH$_2$, 2.1 (m); −C**H**=CH$_2$, 5.8 (m); −(C**H$_2$**)$_6$−, 1.3 (s, b); O−C**H$_2$**, 4.1 (t); −N**H** (ring), 6.8 (s)
γ-allyl	9:1 methanol−water	193−194	α-C**H**, 4.3 ppm (t); β-C**H$_2$**, 2.3 (m); γ-C**H$_2$**, 2.8 (t); −CH=C**H$_2$**, 5.3 (m); −C**H**=CH$_2$, 5.9 (m); O−C**H$_2$**, 4.6 (d); −N**H$_3^+$**, 7.8 (s, b)	oil: α-C**H**, 4.4 ppm (t); β-C**H$_2$**, 2.2 (m); γ-C**H$_2$**, 2.6 (t); −CH=C**H$_2$**, 5.3 (m); −C**H**=CH$_2$, 5.9 (m); O−C**H$_2$**, 4.6 (d); −N**H** (ring), 6.6 (s)

[a] Both displayed IR bands at 1640 cm^{-1} (C=C) and 1739 cm^{-1} (ester). NCA derivatives showed characteristic bands at 1790 and 1861 cm^{-1}.

another polymer chain. Although an extra synthetic step is necessary to produce the epoxy group, cross-linking by this method may be superior to cross-linking **4a** or **4b** by a free radical process where the radicals are generated from reagents such as BPO or AIBN. Other workers[9] attempted free radical cross-linking using these initiators with a polyurethane containing pendent C−C double bonds. They reported poor cross-linking efficiency apparently due to the stability of the resulting allyl radical.

Polymer **4** was converted to **6** by modifying a method[10] that used $KMnO_4$ in the presence of $NaHCO_3$ to oxidize and cleave the C−C double bond. Polymer **6a** was soluble in H_2O/Na_2CO_3 (pH > 8) and acetone insoluble. Polymer **6b** suspended well in H_2O/Na_2CO_3 and was acetone soluble. MALDI data of **4a,b** and **6a,b** showed that there was no significant molecular weight reduction after the oxidation (i.e., the backbone remained intact) and also revealed repeat units still containing the C−C double bond for **6b**. ^{1}H NMR in acetone-d_6 revealed the residual double bonds that were estimated to be in about 30% of the side chains in **6b**. However, use of potassium bicarbonate instead of sodium bicarbonate in the oxidation of **4b** resulted in complete conversion of the pendent double bonds to the carboxylic acid derivative, **6b**. These polymers, even with 30−40% of the double bonds unconverted, were soluble in water at pH values of 8 or greater. However, care must be exercised in exposing the compounds to high pH for extended periods. Attempts to remove inorganic impurities from **6a** or **6b** using dialysis at pH = 9 for 2 days resulted in hydrolysis of the ester groups that link the side chains to the polymeric backbone. This was confirmed by observation of mass loss after hydrolysis and ^{1}H NMR of the isolated polymer, which revealed that the remaining material was poly(L-glutamic acid).

We have applied polymers **6a** and **6b** as a pseudo-stationary phase in electrokinetic capillary chromatography (ECC). Like polymerized surfactants,[11] cyclodextrin,[12] and poly(glycosaminoglycan),[13] our initial data indicate that both are capable of resolving enantiomers when added to the mobile phase in a CE analysis. In

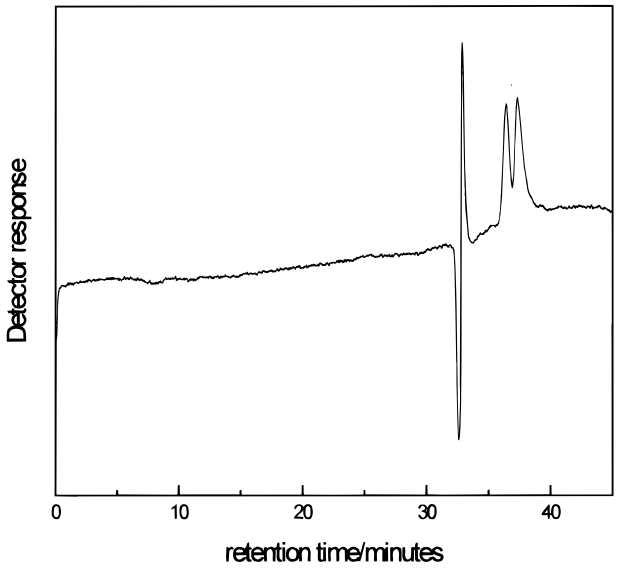


Figure 1. ECC separation of (±)-oxazepam. Chromatographic conditions are stated in the Experimental Section.

contrast, poly(L-glutamic acid) (Sigma) of a similar molecular weight and chromatographic conditions failed to resolve the same enantiomers. This observation suggests that the hydrophobic spacer between the peptide backbone and the pendent carboxylic acid group in **6a** and **6b** plays a key role in the separation mechanism. At present, the resolution observed must be interpreted cautiously since the polymer's effectiveness as a pseudostationary phase for chiral separations appears strongly related to the amount of pendent double bonds remaining in the polymer after the permanganate oxidation. For example, our preliminary ECC results indicated that when there was essentially no residual double bonds remaining in **6b**, the polymer was a poor pseudostationary phase for resolving enantiomers. When 30−40% of the side chains contained the pendent double bond, the resolution of certain enantiomers was noted. We are currently investigating whether the double bond itself is important for the selectivity or if the increase in hydrophobicity due to the unoxidized alkenyl side chains is sufficient for one of the at least three interactions[14] needed for chiral selectivity. This may be determined by making a pseudostationary phase from the product of permanganate oxidation of copolymer **1b**. Quantitative oxidation of the double bonds in **1b** will provide a polymer with only alkyl (not alkenyl) and carboxylated side chains. Also, low molecular weight (i.e., DP less than 50) polymers may be more effective for ECC applications since they are more water soluble than higher molecular weight polymers. Figure 1 shows a typical chromatogram using **6b** (about 40% of the side chains contain pendent double bond) as the pseudostationary phase (R_s = 1.0, other conditions as indicated in the Experimental Section) and racemic oxazepam as the analyte.

Experimental Section. FT-IR spectra were recorded on a Perkin-Elmer model 1720. ^{1}H NMR were recorded on a Bruker AC model at either 200 or 250 MHz and the chemical shift data were referenced to the TMS internal standard (See Table 1 for NMR data). MALDI data were collected on a Voyager model of a PerSeptive Biosystems instrument. The ECC data were collected on a Beckman PACE system. Separations were performed with uncoated fused silica capillaries of 50 mm i.d. with a column length of 55 cm (45.5 cm to the detector window) purchased from Polymicro Technologies (Phoenix, AZ). The ECC conditions were as follows: 3 s injection of a 0.1 mg/mL solution of the racemate, 200 mM TRIS, pH = 8, adjusted by addition of sodium hydroxide; applied voltage 10 kV; UV detection, 254 nm. For the data shown in Figure 1, polymer **6b** was employed in the mobile phase at approximately 0.25% w/v. The THF used for the polymerization reactions was distilled from potassium metal prior to use. When ethyl acetate was used as the solvent in the NCA monomer preparation, Aldrich Sure-Seal grade was used. Other solvents or reagents were purchased from Aldrich, Inc. and used as received.

2a and 2b, Step a. These compounds were synthesized according to the method of Wasserman *et al.* as described in ref 5. The modifications are noted in the discussion above.

3a and 3b, Step b. These monomers were synthesized by the method of Daly and Poché as described in ref 7. Both compounds were isolated as oils. While most α-amino acid NCA derivatives show poor solubility in hexane, these NCAs are quite soluble in hexane. As such, product clean-up by washing with hexane was not done. There are two key differences we used in the work-up of these (and other) NCA monomers that differ from ref 7 and other literature procedures. Since recrystallization of the NCAs could not be done, contamination of the NCA with unreacted triphosgene was avoided by using less than 1 equiv in the reaction. Any unreacted amino acid (i.e., **2a** or **2b**) was filtered out using a Celite pad and suction filtration. Despite the well-known reactivity of NCA compounds with water, the reaction mixture (using ethyl acetate as the solvent), cooled to room temperature, was washed one time with ice-cold water in a separatory funnel and the separated organic layer immediately dried with anhydrous $MgSO_4$. This step was preceded by an aggressive Ar or N_2 sparging of the reaction. The purpose of these steps was to remove HCl from the NCA, which is known to interfere with the subsequent polymerization reaction.

4a and 4b, Steps c and d. Five grams (1.60×10^{-2} mol) of **3b** was dissolved in 20 mL of dry THF. If a copolymer was desired (e.g., **1b**), the appropriate quantity of NCA was codissolved with **3b**. An appropriate amount of *n*-butylamine initiator was added, the quantity depending upon the molar mass range desired for the polymer. For example, for a polymer with a degree of polymerization (DP) approaching 50, the [M]:[I] mole ratio was 50. Polymers with higher DP were made by adding 0.1 mL of triethylamine to the reaction (the [M]:[I] ratio does not predict DP in this case). After stirring for 5 days at room temperature, under a calcium chloride filled drying tube, the reaction was concentrated to about one-third of its volume and the polymer precipitated into methanol. The sticky solid was vacuum dried at room temperature. Typical isolated yields were 75−85%. Use of THF for the polymerization of **4a** resulted in a very gelatinous reaction, viscous enough to prevent the magnetic stirrer from moving. Instead, the polymerization of **4a** was carried out in chlorobenzene, which prevented gelation as the polymerization proceeded. FT-IR data of films cast on a NaCl plate showed amide I and II bands in positions consistent with the α-helix.

5a and 5b, Step e. The same procedure was used for both. Two grams (7.5×10^{-3} mol) of **4b** was dissolved in 60 mL of $CHCl_3$. A large molar excess, 2.0 g (0.012 mol), of *m*-chloroperoxybenzoic acid (MCPBA)

was added and the reaction refluxed, about 3 h. The cooled reaction was washed with saturated sodium bicarbonate solution and water in turn to remove *m*-chlorobenzoic acid. The chloroform layer was dried over anhydrous magnesium sulfate, evaporated to a small volume and codissolved by addition of a few milliliters of methanol. This solution was poured into 100 mL of aqueous 5% sodium bicarbonate to precipitate the polymer. The isolated yield was 75%. ^{1}H NMR indicated complete reaction of the double bonds with signals indicative of the terminal epoxy group present: 2.45 (t), 2.70 (t), 2.90 ppm (m). A chloroform solution of this polymer, about 10% w/v or greater, when mixed with a few drops of trifluoroacetic acid, formed a stiff, nonflowing "gel".

6a and 6b, Step f. The same procedure was used for each except the THF was not used in the synthesis of **6a**. Two grams (7.5×10^{-3} mol) of **4b** was dissolved in 50 mL of THF. When the polymer was dissolved, 50 mL of acetone was added. Two grams of sodium bicarbonate was added to the stirred solution followed by 1.3 g of potassium permanganate. The reaction became brown within minutes as manganese(IV) oxide formed. The suspension was stirred 10−12 h. The suspension was then poured into 50 mL of water containing 2.0 g of sodium carbonate. Then, 2.0 g of sodium bisulfite was added to reduce any remaining permanganate. Two to three small scoops of celite were added, and the suspension was suction filtered. The yellow filtrate was concentrated to remove the organic solvents. The water solution remaining was slowly acidified with concentrated sulfuric acid until an acidic pH was reached, which caused the precipitation of **6b**. The precipitate was isolated by centrifugation at 5000−7000 rpm for 15 min. The pellets were water washed in the centrifuge tubes several times and the solid again recovered by centrifugation. The wet pellets were dissolved in acetone, and the solution was gravity filtered to remove small quantities of insoluble material. **6b** was isolated by evaporation of the acetone. The isolated yield was about 50−60%. The FT-IR and ^{1}H NMR were consistent with the formation of carboxylic acid groups. About 30−40% of the side chains still contained double bonds (estimated by ^{1}H NMR). The side chains may be oxidized to near completion by increasing the quantity of permanganate or by changing the base used in the reaction to potassium bicarbonate from sodium bicarbonate.

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