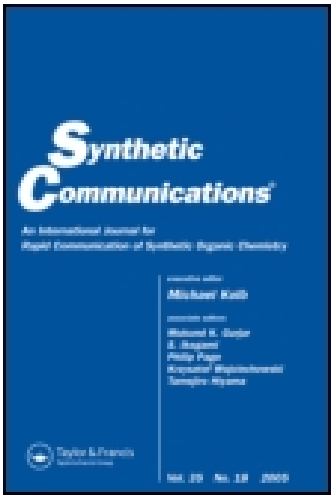

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SYNTHESIS OF N-α-FMOC-N-ε-TETRABUTYL ESTER-EDTA-L-LYSINE: AN AMINO ACID ANALOG TO PREPARE AFFINITY CLEAVING PEPTIDES

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Abstract: Synthesis of an amino acid analog, N-α-Fmoc-N-ε-tetrabutyl ester EDTA-L-Lysine, suitable for incorporating a strong metal binding site at any internal sequence position in a peptide is described. To overcome the solubility and purification problems during the synthesis, we prepared esters of N-α-Fmoc-N-ε-Boc-L-Lysine. Since the *t*- butyl group on the amino acid is acid labile and Fmoc group at α-amino group is base sensitive, protection of N-α-Fmoc-N-ε-Boc-L-Lysine requires an ester that can be prepared and removed under neutral conditions. A scheme for selective protection and deprotection of lysine is reported.

With the advent of affinity cleaving technology, transition metal complexes provide an ever increasing array of molecules designed to react specifically with nucleic acid or protein targets. The metal chelate attachment converts sequence-specific DNA-binding molecules to sequence-specific DNA-cleaving molecules that

function under physiological pH, temperature, and salt conditions. Cleavage of DNA and RNA by metal chelates is an important new approach to characterize specific structural features of nucleic acids and their complexes in solutions. Calender and Cech used Fe-EDTA to study the solution folding of the *T. thermophila* group I intron as a function of different divalent cations.[1] Moser and Dervan reported an experiment showing the sequence-specific cleavage of double helical DNA by oligonucleotides tagged with EDTA-Fe, which cleaved the DNA backbone by oxidation of the deoxyribose.[2] Recently, a sequence-specific DNA-cleaving metalloprotein has been designed by attaching a copper binding tripeptide (Gly-Gly-His) to the amino-terminus of the DNA binding domain of Hin recombinase.[3] In all of these studies, the binding moiety determines the site of cleavage simply by localizing the production of the reactive species.

As with nucleic acids, site-specific cleavage of proteins has been achieved by metal ions or chelates bound at particular sites.[4-6] Site-specific cleavage of proteins is achieved by introducing a metal-binding site at one position in a polypeptide chain. Artificial proteolytic reagents would be extremely useful to characterize important structural features of proteins and their complexes under physiological conditions.

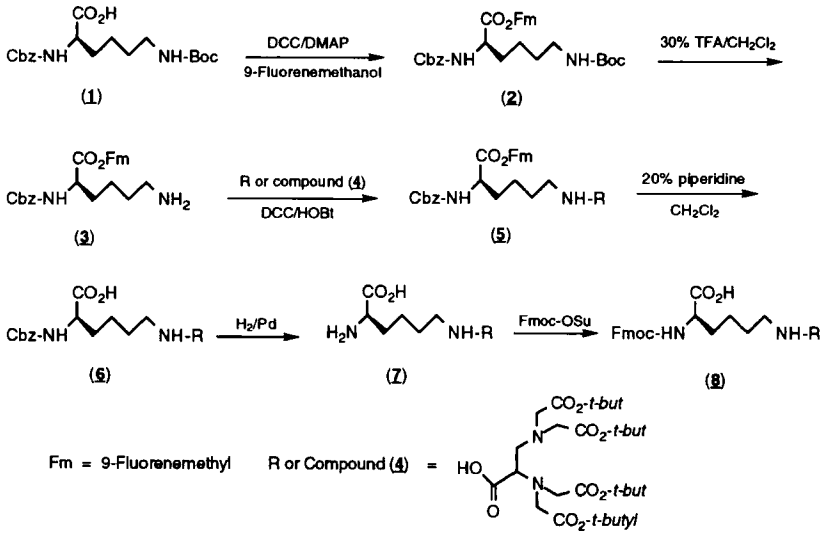
To accurately probe structure function relationships in nucleic acids, proteins, and nucleic acid-protein complexes by affinity cleaving methods, the development of general and specific methods for single site modification of peptide sidechain is required. Reagents potentially useful for such experiments are the "bifunctional chelating agents," so called because they incorporate a strong metal-chelating group and a chemically reactive functional group. Bifunctional chelating agents have been synthesized and used to provide biological molecules with the nuclear, physical, or chemical properties of chelated metal ions.[7, 8]

We can select bifunctional chelating agents which will modify the cysteine residue (bromoacetamido derivative) or amino groups on lysine side chains (isothiocyanato derivative). Unfortunately, these chelating agents cannot distinguish a specific amino acid when it is present more than once in the sequence of the protein. Secondly, the efficiencies of the coupling reactions between bifunctional chelating agents and peptides usually are not very high. To overcome these problems, protected derivatives of EDTA compatible with Merrifield solid-phase protein synthesis using N-tert-butyloxycarbonyl-(Boc) protected amino acids were developed.[9-11] There are two major concerns about this synthetic strategy:

(a) repetitive TFA acidolysis in Boc-group deprotection could lead to acid catalyzed side-reactions, (b) cleavage and deprotection of peptides requires HF and specific laboratory set up which is not available to many researchers. Due to these concerns Fmoc (9-fluorenylmethyl carbamate) solid phase peptide synthesis was developed which employs N-α-Fmoc amino acids.[12] In this strategy, the Fmoc group is deprotected with piperidine and TFA is required only for the final cleavage and deprotection step. We have synthesized an amino acid analog N-α-Fmoc-N-ε-tetrabutyl ester EDTA-L-Lysine (**8**).[13] However, the synthesis of this compound posed two major problems in the final step of synthesis: (i) very poor solubility of N-α-Fmoc-L-Lysine in all available solvents, (ii) purification of the final product required reverse phase HPLC procedures. We report here a new methodology for the preparation of this amino acid analog (**8**) which is designed to be compatible with the Fmoc solid phase peptide synthesis strategy. Synthetic route for this synthesis is outlined in Scheme I. During the development of these methods, we found that two commonly used carboxylic acid protective groups cannot be employed to protect amino acids analogous to N-α-Fmoc-N-ε-Boc-L-Lysine.

EDTA analog (**4**) was synthesized as reported earlier.[13] The final step to prepare the Fmoc protected amino acid (**8**) requires the coupling of EDTA analog (**4**) to the ε-amino group of the lysine.[13] The poor solubility of N-α-Fmoc-L-Lysine in various solvents required for its coupling with compound (**4**) prompted us to make a more hydrophobic derivative of N-α-Fmoc-L-Lysine by preparing the corresponding ester, which will help in increasing its solubility and overall yield of the reaction. To implement this idea, we tried different strategies which appeared quite suitable according to the published procedures. Since the *t*-butyl group on compound **8** is acid labile and Fmoc group at α-amino group is base sensitive, protection of N-α-Fmoc-N-ε-Boc-L-Lysine requires an ester that can be prepared and removed under neutral conditions. The complexity of selective protection in this synthesis left us with very limited choices of protective groups. As an initial effort towards this goal, we selected benzyl ester to protect carboxylic group of N-α-Fmoc-N-ε-Boc-L-Lysine because deprotection of this ester requires neutral hydrogenolysis conditions.[14] We prepared the corresponding benzyl ester of N-α-Fmoc-N-ε-Boc-L-Lysine, treated it with 30% TFA/CH_2Cl_2 to remove boc group and coupled it with tetrabutyl ester protected EDTA (**4**). The product was isolated in high yield, however, removal of benzyl group by hydrogenolysis was very

Scheme I



sluggish and during the course of the reaction Fmoc group was also cleaved.[15] During our second attempt to protect N-α-Fmoc-N-ε-Boc-L-Lysine, we decided to replace benzyl ester with dimethoxy benzyl ester that can be cleaved oxidatively by stirring it with DDQ at room temperature.[16] Dimethoxybenzyl group was found to be cleaved under 30% TFA/CH_2Cl_2 conditions necessary for the removal of Boc group.

Due to the problems of selective protection of N-α-Fmoc-N-ε-Boc-L-Lysine, we chose a different route to synthesize the final product (**8**). N-α-Cbz-N-ε–Boc-L-Lysine was protected as 9-fluorenemethyl ester (**2**) by using DCC/DMAP coupling procedures.[17] Removal of Boc group, followed by its coupling with compound **4** gave the desired product **5** in 96% yield. After Fmoc group removal, compound **6** was converted to N-ε-tetrabutyl ester EDTA-L-Lysine (**7**) by catalytic hydrogenation. Subsequently, the final product **8** was obtained by protecting α-amino group of compound **7** as Fmoc using Fmoc-OSu (9-Fluorenylmethyl-succinimidyl carbonate) in DME. Overall yields are high in this method and it effectively circumvents the problem of low solubility of N-α-Fmoc-L-Lysine and also the tedious column purification involved in the previously reported

procedure.[13] Through this methodology, flexible linkers of different lengths and various structures can be incorporated between the α-carbon backbone of peptides and metal binding moiety. Thus, these peptides will provide a variety of affinity cleaving reagents which can be directed against protein or nucleic acid targets.

Experimental Section

Anhydrous DMF, N,N-diisopropylethylamine, Fluorenemethanol, TFA, piperidine, DMAP and 10% Pd on charcoal were purchased from Aldrich. DCC and HOBt were obtained from Sigma. N-α-Cbz-N-ε-Boc-L-Lysine was purchased from Bachem. All chemicals were reagent grade unless otherwise specified. Compound **4** was prepared as described earlier.[13] ^{1}H NMR spectra were recorded at 200 MHz on a Gemini 200 spectrometer (Varian) using TMS as an internal reference. FAB MS spectrum was recorded using *m*-nitrobenzoic acid/NaI as matrix. IR spectra were obtained on a Perkin Elmer FT 1600 as thin film. TLC was performed with precoated 0.2 mm silica gel 60 F-254 TLC plates (EM Reagents, Darmstadt, FRG).

N-α-Cbz-N-ε-Boc-L-Lysine-9-Fluorenemethyl ester (2). N-α-Cbz-N-ε-Boc-L-Lysine (**1**) (380 mg, 1 mmol.) was dissolved in dry dichloromethane (5 ml) and cooled to 0°C. To this solution DCC (206 mg, 1 mmol) and DMAP (2 mg) were added. The reaction mixture was stirred for 1 h at 0°C, after which 9-Fluorenemethanol (216 mg, 1.1 mmol.) in dry dichloromethane (2 ml) was added. The reaction mixture was stirred overnight at rt. Filtration and evaporation yielded compound **2**, which was purified by column chromatography (ethyl acetate/benzene, 30:70). Yield 452 mg, 0.81 mmol., 81%. ^{1}H NMR ($CDCl_3$): δ7.78-7.20 (13H, m, aromatic), 5.10 (2H, s, benzylic), 4.60-4.45 (2H, m), 4.42-4.30 (1H, m), 4.29-4.18 (1H, m), 3.17-3.00 (2H, m), 1.80-1.20 (6H, m), 1.43 (9H, s). IR 1724 cm^{-1} (C=O, br. s). TLC (benzene/ethyl acetate, 3:1) R_f 0.75.

N-α-Cbz-L-Lysine-9-Fluorenemethyl ester (3). To an ice cooled solution of compound **2** (558 mg, 1 mmol) in dry dichloromethane (3.5 ml) was added TFA (1.5 ml) and stirring was continued for 2 h at 0°C. The reaction mixture was concentrated to dryness under reduced pressure without heating. The product was pure on TLC silica gel. Yield 430 mg, 0.96 mmol, 96%. ^{1}H NMR (DMSO d_6): δ7.93–7.36 (13H, m, aromatic), 5.05 (2H, s, benzylic), 4.60-4.38 (2H, m), 4.31-4.20 (1H, m), 4.08-3.90 (1H, m), 2.80-2.65 (2H, m), 1.60-1.20 (6H, m). IR 1724 cm^{-1} (C=O, br. s). TLC (ethyl acetate/methanol, (9:1) R_f 0.26.

N-α-Cbz-N-ε-tetrabutyl ester EDTA-L-Lysine-9-Fluorenemethyl ester (5). Tetrabutyl ester EDTA **4** (336 mg, 0.6 mmol) was dissolved in dry CH_2Cl_2 (4 ml) and cooled to 0°C. To this reaction mixture were added HOBt (81 mg, 6 mmol) in dry DMF (1.5 ml), followed by DCC (124 mg, 0.6 mmol) in dry dichloromethane (1.5 ml). The reaction mixture was stirred at 0°C for 1 h and 2 h at rt, followed by filtration. The filtrate was concentrated to dryness and the activated ester was redissolved in dry dichloromethane (4 ml). A solution of compound **3** (224 mg, 0.5 mmol) in dry DMF (5 ml) was added to the activated ester solution, followed by diisopropylethylamine (100μl) to bring the apparent pH to 8.0. The reaction mixture was stirred for 4 h at rt. The reaction was stopped by removing the solvent under reduced pressure and purified by column chromatography (ethyl acetate : benzene 30:70) to provide compound **5** (411 mg, 0.4 mmol, 83%) as thick colorless oil. ^1H NMR ($CDCl_3$): δ7.78-7.24 (13H, m, aromatic), 5.10 (2H, s, benzylic), 4.58-4.45 (2H, m), 4.40-4.28 (1H, m), 4.28-4.14 (1H, m), 3.68-3.38 (9H, m), 3.50-3.12 (4H, m), 1.81-1.40 (6H, m), 1.47 (36H, s). IR 1731 cm^{-1} (C=O, br. s). TLC (Benzene/ethyl acetate, 2:1) R_f 0.67.

N-α-Cbz-N-ε-tetrabutyl ester EDTA-L-Lysine (6). Compound **5** (396 mg, 0.4 mmol) was dissolved in 20% piperidine in dry dichloromethane (5 ml) and stirred at rt for 2 h. Solvent was evaporated to dryness by rotary evaporation, followed by purification by column chromatography (methanol/ethyl acetate, 30:70). Compound **6** was obtained as thick oil (247 mg, 0.3 mmol, 76%). ^1H NMR ($CDCl_3$): δ7.28 (5H, s, aromatic), 5.00 (2H, s, benzylic), 4.18-4.00 (1H, m), 3.60-3.20 (9H, m), 3.20-2.85 (4H, m), 1.92-1.40 (6H, m), 1.42 (36H, s). IR 1731 cm^{-1} (C=O, br. s). TLC (ethyl acetate/methanol, 9:1) R_f 0.79.

N-ε-tetrabutyl ester EDTA-L-Lysine (7). To a solution of compound **6** (162 mg, 0.2 mmol.) in methanol (5 ml) was added 10% Pd on C (30 mg) and the reaction mixture was stirred under H_2 for 6 h. Filtration and evaporation afforded compound **7** which was purified by recrystallization (ethyl acetate). Yield 120 mg, 0.17 mmol, 89%. ^1H NMR ($CDCl_3$): δ4.32 (1H, m), 3.60-3.32 (9H, m), 3.25-2.80 (4H, m), 1.90-1.40 (6H, m), 1.41 (36H, s). IR 1731 cm^{-1} (C=O, br. s). TLC (ethyl acetate/methanol, 4:1) R_f 0.32.

N-α-Fmoc-N-ε-tetrabutyl ester EDTA-L-Lysine (8). To a solution of Fmoc-OSu (252 mg, 0.75 mmol.) in dry DME (5 ml) was added compound **7** (344 mg, 0.5 mmol.) in 10% Na_2CO_3 (5 ml). The reaction mixture was stirred at rt for 4 h. Reaction was stopped by removing the solvent under reduced pressure.

Residue was redissolved in water (5 ml) and pH was carefully adjusted to 7. The aqueous layer was extracted with ethyl acetate (10 x 3 ml), organic layer dried and evaporated to yield compound **1** as yellow oil, which was purified by column chromatography (methanol/ethyl acetate 1:1). Yield 405 mg, 0.44 mmol. 89% . ^{1}H NMR ($CDCl_3$): δ7.7 (2H, d, J=6.96, aromatic), 7.6 (2H, d, J=6.96, aromatic), 7.3-7.24 (4H, m, aromatic), 4.34-4.02 (4H, m), 3.75-3.40 (9H, m), 3.20-3.04 (2H, m), 2.86-2.72 (2H,m), 1.9-1.4 (6H,m), 1.40 (36 H, s). IR 1731 cm^{-1} (C=O, s). MS: *m/e* 933 $(M+Na)^+$, 956 $(M+2Na)^+$, 915 $(M+Na-H_2O)^+$. TLC (acetone/water, 7:3) R_f 0.77.

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