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Introduction of Lanthanide(III) Chelates to Oligopeptides on Solid Phase

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The synthesis of oligopeptide building blocks for the introduction of nonluminescent and luminescent lanthanide(III) chelates to the oligopeptide structure on the solid phase is described. The oligopeptide conjugates synthesized were used in DELFIA-based receptor binding assay (motilin) as well as in LANCE time-resolved fluorescence quenching assay (caspase-3).

INTRODUCTION

Because of their unique luminescence properties, lanthanide(III) chelates are often used as nonradioactive markers in a wide variety of routine and research applications (*1*−*4*). Because lanthanide(III) chelates give strong, long decay-time luminescence, they are ideal labels for assays where high sensitivity is required. Time-resolved fluorometric assays based on lanthanide chelates have found increasing applications in diagnostics, research, and high throughput screening. The heterogeneous DELFIA technique is applied in assays requiring exceptional sensitivity, robustness, and a multilabel approach. Development of highly luminescent stable chelates extends the use of time resolution to homogeneous assays, based on fluorescence resonance energy transfer (TR-FRET), fluorescence quenching (TR-FQA), or changes in the luminescence properties of a chelate during a binding reaction.

Most commonly, the conjugation reaction is performed in solution between an amino or mercapto group of a bioactive molecule (such as a protein, a peptide, a nucleic acid, an oligonucleotide, or a hapten) and the isothiocyanato, haloacetyl, 3,5-dichloro-2,4,6-triazinyl derivatives of lanthanide(III) chelates (*5*), as well as other reporter groups. Because, in all of the cases, the labeling reaction is performed with an excess of an activated label, laborious purification procedures cannot be avoided. Especially, when attachment of several label molecules, or site-specific labeling in the presence of several functional groups of similar reactivities is required, the isolation and characterization of the desired biomolecule conjugate is extremely difficult and often practically impossible.

Quite recently, we reported our strategy for the introduction of lanthanide(III) chelates to an oligonucleotide structure on a solid phase (*6*). We extend here our approach of biomolecule derivatization to oligopeptides. We describe the synthesis of oligopeptide building blocks that allow for the introduction of nonluminescent and luminescent lanthanide(III) chelates to synthetic oligopeptides using standard machine-assisted chemistry. Also, the suitability of these blocks to oligopeptide derivatization as well as some application data is demonstrated.

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EXPERIMENTAL PROCEDURES

General Methods. Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). Reagents for oligopeptide synthesis were purchased from Nova Biochem, Molecular Probes, and Applied Biosystems. The oligopeptides were assembled on an Applied Biosystems 433A instrument in 10 μmol scale using Fmoc chemistry and recommended protocols (Fmoc-Off synthesis). The time-resolved fluorometer (Victor^{2}V), DELFIA assay buffer, DELFIA wash solution, DELFIA enhancement solution, and the caspase buffer were from PerkinElmer Life Sciences. NMR spectra were recorded on a Brucker 250 or a JEOL GX-400 spectrometers operating at 250.13 MHz and 399.8 for ^{1}H, respectively. The signal of TMS was used as an internal reference. Coupling constants are given in hertz. Electrospray ionization time-of-flight mass spectra (ESI-TOF-MS) were recorded on an Applied Biosystems Mariner instrument. HPLC analyses were performed on a PerkinElmer LC 2000 instrument. The mobile phase used was the following: (Buffer A) 0.02 M TEAA (pH 7.5); (Buffer B) A in 50% (v/v) acetonitrile. IR spectra were recorded on a PerkinElmer Spectrum One spectrophotometer.

Tetra-*tert*-butyl-1-(4-nitrobenzyl)diethylenetriaminetetrakis(acetate), 2. 1-(4-Nitrobenzyl)diethylenetriamine (5.0 g, 21.0 mmol) was dissolved in dry dimethylformamide (DMF) (120 mL). DIPEA (34 mL), potassium iodide (3 g), and *tert*-butylbromoacetate (22 mL, 0.15 mol) were added, and the mixture was stirred for 2 h at ambient temperature and concentrated in vacuo. The residue was partitioned between water and diethyl ether. The organic phase was washed with water and brine and was dried. After concentration, the crude product was purified on silica gel column (eluent MeOH/CH_2Cl_2, 5:95, v/v). Yield was 17.2 g (95%) of compound **2**. ^{1}H NMR ($CDCl_3$, δ): 8.17 (2H, d, J = 8.5 Hz), 7.56 (2H, d, J = 8.5), 3.94 (2H, s), 3.75 (2H, s), 3.43 (4H, s), 3.32 (2H, br s), 2.79 (7H, br s), 1.46 (9H, s), 1.44 (18H, s), 1.43 (9H, s). IR (film): 1731, 1552, 1367, 1345, 1152 cm^{-1}. ESI-TOF-MS mass for $C_{35}H_{59}N_4O_{10}$ $(M + H)^+$: calcd, 695.42; found, 695.42.

Tetra-*tert*-butyl-1-(4-aminobenzyl)diethylenetriaminetetrakis(acetate), 3. Compound **2** (12.0 g, 17.2

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mmol) was dissolved in dry methanol (240 mL) containing Pd/C (10%, 1.2 g). $NaBH_4$ (0.72 g, 19.0 mmol) was added portionwise (caution: exothermic reaction with foaming), and the reaction was allowed to proceed for 3 h at ambient temperature. The reaction mixture was filtered through Celite and neutralized with aq HCl. The filtrate was diluted with dichloromethane, washed with saturated $NaHCO_3$, and dried. Purification was performed on silica gel (eluent MeOH/CH_2Cl_2, 1:9, v/v) giving 10.0 g (87%) of compound **3**. ^{1}H NMR ($CDCl_3$, δ): 7.09 (2H, d, J = 8.5 Hz), 6.61 (2H, d, J = 8.5 Hz), 3.66 (2H, s), 3.45 (4H, s), 3.34 (2H, s), 3.20 (2H, s), 2.78 (8H, br s), 1.45 (36H, 2 s). ESI-TOF-MS mass for $C_{35}H_{61}N_4O_8$ $(M + H)^+$: calcd, 665.45; found, 665.44.

Tetra-*tert*-butyl-1-[4-(fluorenylmethyloxycarbonylaminoacetamido)benzyl]diethylenetriaminetetrakis(acetate), **4.** Compound **3** (9.8 g, 14.7 mmol) was dissolved in CH_2Cl_2 (100 mL) and deaerated with argon. Dicyclohexylcarbodiimide (DCC) (3.34 g, 16.2 mmol), DMAP (184 mg, 1.47 mmol), and Fmoc-Gly-OH (4.37 g, 14.7 mmol) were added, and the mixture was stirred at room temperature for 4 h. Precipitation formed was removed by filtration, and the filtrate was evaporated to dryness to give 11.4 g (81%) of the Fmoc derivative. Compound **4** was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, δ): 7.97 (1H, s), 7.75 (4H, m), 7.59 (2H, m), 7.38 (3H, m), 7.30 (3H, m), 5.61 (1H, s), 4.48 (2H, d, J = 6.9 Hz), 4.24 (1H, t, J = 6.8 Hz), 4.00 (2H, d, J = 4.0 Hz), 3.75 (2H, s), 3.43 (4H, m), 3.33 (2H, m), 3.22 (2H, m), 2.76 (8H, s), 1.45 (36H, 3 s). ESI-TOF-MS mass for $C_{52}H_{74}N_5O_{11}$ $(M + H)^+$: calcd, 944.54; found, 944.56.

Tetra-*tert*-butyl-1-[4-(aminoacetamido)benzyl]diethylenetriaminetetrakis(acetate), **5.** Compound **4** (10 g, 10 mmol) was dissolved in the mixture of piperidine and DMF (300 mL; 20%, v/v), stirred at room temperature for 3 h, and evaporated to dryness. Purification was performed on silica gel (eluent initially petroleum ether/ethyl acetate/triethylamine, 5:2:1, v/v/v, and then MeOH/CH_2Cl_2, 1:4, v/v). Yield was 6.0 g (79%) of compound **5**. ^{1}H NMR ($CDCl_3$, δ): 9.43 (1H, br s), 7.55 (2H, d, J = 8.4 Hz), 7.29 (2H, d, J = 8.4 Hz), 3.75 (2H, s), 3.48 (4H, s), 3.44 (2H, s), 3.33 (2H, s), 3.21 (2H, s), 2.77 (8H, m), 1.70 (36H, s). ESI-TOF-MS mass for $C_{37}H_{64}N_{45}O_9$ $(M + H)^+$: calcd, 722.47; found, 722.47.

Tetra-*tert*-butyl-4′-{2-[4-allyloxycarbonyl-4-(fluorenylmethyloxycarbonylamino)-1-oxo-1-butylamino]-1-oxo-1-ethylamino}benzyldiethylenetriaminetetrakis(acetate), 6. Compound **5** (3.2 g, 4.38 mmol) was dissolved in dichloromethane (80 mL) and deaerated with argon. DCC (1.0 g, 4.67 mmol) and Fmoc-Glu-OAll (1.8 g, 4.25 mmol) were added, and the mixture was stirred at room temperature for 2 h. DCU formed was removed by filtration, and the filtrate was concentrated in vacuo. The allyl derivative was purified by flash chromatography using petroleum ether/ethyl acetate (2:5, v/v) as the eluent. Yield was 4.9 g (67%) of compound **6**. ^{1}H NMR ($CDCl_3$, δ): 8.39 (1H, s), 7.76 (2H, m), 7.56 (2H, m), 7.46 (2H, m), 7.40 (2H, m), 7.31 (2H, m), 7.24 (2H, m), 6.55 (1H, m), 5.89 (1H, m), 5.69 (1H, d, J = 7.9 Hz), 5.29 (2H, m), 4.64 (2H, d, J = 5.6 Hz), 4.41 (2H, m), 4.18 (1H, t, J = 6.8 Hz), 3.73 (2H, s), 3.43 (4H, m), 2.77 (8H, m), 2.32 (2H, m), 2.05 (4H, m), 1.91 (1H, m), 1.74 (4H, m), 1.44 (36 H, 2s). ESI-TOF-MS mass for $C_{60}H_{85}N_6O_{14}$ $(M + H)^+$: calcd, 1113.12; found, 1113.61.

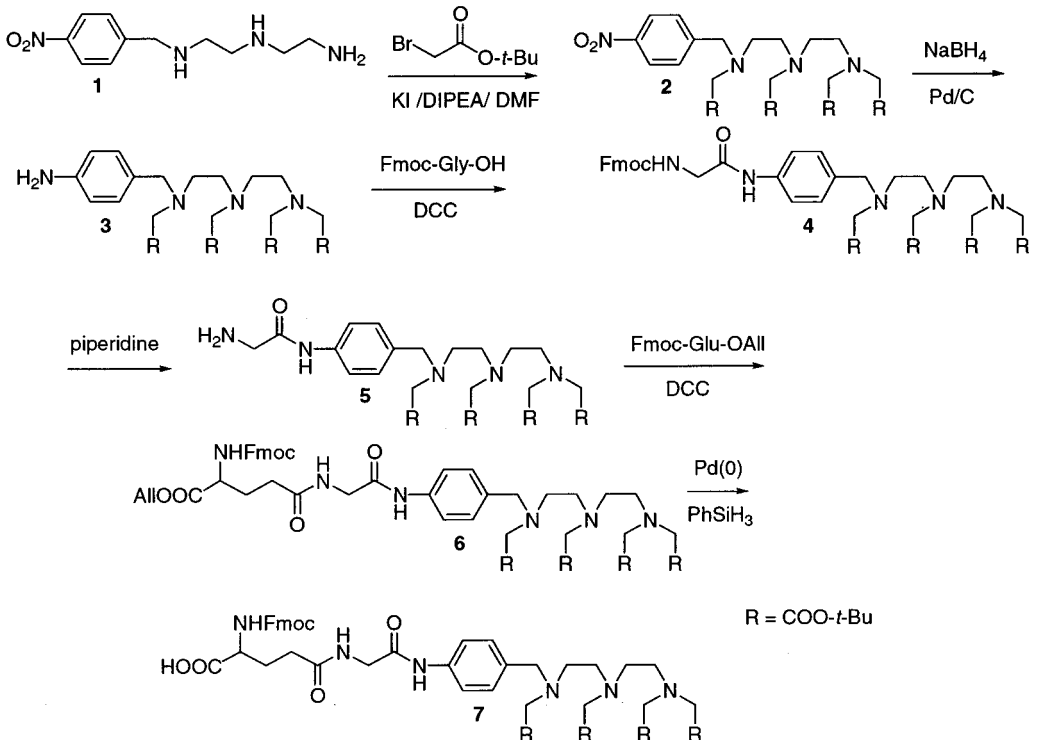
Tetra-*tert*-butyl-4′-{2-[4-carboxy-4-(fluorenylmethyloxycarbonylamino)-1-oxo-1-butylamino]-1-oxo-1-ethylamino}benzyldiethylenetriaminetetrakis(acetate), 7. Compound **6** (2.0 g, 1.8 mmol) was dissolved in dichloromethane (120 mL) and deaerated for 10 min with argon. $Pd(PPh_3)_4$ (41.5 mg, 35.9 μmol) and $PhSiH_3$ (0.5 mL, 3.6 mmol) were added, and the mixture was stirred at room temperature for 1 h. The solution was washed with citric acid (100 mL, 10%, w/v), dried over 4 Å molecular sieves, and evaporated to dryness. Yield of compound **7** was quantitative. ESI-TOF-MS mass for $C_{57}H_{81}N_6O_{14}$ $(M + H)^+$: calcd, 1073.58; found, 1073.57.

Tetra-*tert*-butyl-2,2′,2″,2‴-{[4′-(4″-bromophenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}tetrakis(acetate), 9. Compound **8** (3.3 g, 5.25 mmol, pentahydrochloride) was suspended in dry DMF (25 mL). Diisopropylethylamine (13.7 mL), *tert*-butylbromoacetate (7 mL, 47.4 mmol), and potassium iodide (1.0 g, 6.0 mmol) were added, and the mixture was stirred overnight at room temperature and concentrated. The residue was dissolved in dichloromethane (150 mL), washed with saturated $NaHCO_3$ (3 × 40 mL), and dried. Purification was performed on silica gel (eluent petroleum ether/ethyl acetate/triethylamine, 5:1:1, v/v/v). Yield of compound **9** was 3.8 g (80%). ^{1}H NMR ($CDCl_3$, δ): 8.70 (2H, s), 8.55 (2H, dd, J = 1.0 and 7.8 Hz), 7.86 (2H, t, J = 7.8 Hz), 7.77 (2H, d, J = 8.6 Hz), 7.70 (2H, dd, J = 1 and 7.6 Hz), 7.64 (2H, d, J = 8.6 Hz), 4.17 (4H, s), 3.56 (8H, s), 1.44 (36H, s). ESI-TOF-MS mass for $C_{47}H_{61}BrN_5O_8$ $(M + H)^+$: calcd, 902.38; found, 902.41.

Tetra-*tert*-butyl-2,2′,2″,2‴-{[4′-(4″-(3-amino-1-propyn-1-yl)phenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}tetrakis(acetate), 10. A mixture of compound **9** (1.0 g, 1.1 mmol), bis(triphenylphosphine)-palladium(II) chloride (15 mg, 0.022 mmol), and CuI (9 mg, 0.16 mmol) in dry THF (6 mL) and triethylamine (4 mL) was deaerated with argon. 3-(Methoxytrityl)aminopropyne was added, and the mixture was stirred overnight at 55 °C. The cooled solution was filtered, and the filtrate was evaporated and redissolved in dichloromethane. The solution was washed with water, dried, and concentrated. It was then dissolved in dichloromethane containing 1% (v/v) of TFA, stirred for 30 min at room temperature, and concentrated. The residue was dissolved in dichloromethane, washed with saturated $NaHCO_3$, dried, and concentrated. Purification on silica gel (eluent petroleum ether/ethyl acetate/triethylamine, 3:5:1, v/v/v) gave the title compound (compound **10**) (45%) as an oil. ^{1}H NMR ($CDCl_3$, δ): 8.70 (2H, s), 8.55 (2H, d, J = 8 Hz), 7.87 (2H, t), 7.77 (2H, d, J = 7.5 Hz), 7.70 (2H, d, J = 7.5 Hz), 7.55 (2H, d, J = 7.5 Hz), 4.17 (4H, s), 3.71 (2H, s), 3.55 (8H, s), 1.46 (36H, s). ESI-TOF-MS mass for $C_{50}H_{64}N_6O_8Na$ $(M + Na)^+$: calcd, 899.47; found, 899.51.

Tetra-*tert*-butyl-4′-{3-[4-allyloxycarbonyl-4-(fluorenylmethyloxycarbonylamino)-1-oxo-1-butylamino]-1-propyn-1-yl}-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}tetrakis(acetate), 11. Compound **10** (200 mg, 0.23 mmol) and Fmoc-Glu-OAll (104 mg, 0.25 mmol) were dissolved in dichloromethane (6 mL). DCC (52 mg, 0.25 mmol, predissolved in 1 mL of dichloromethane) was added, and the mixture was stirred overnight at room temperature. DCU formed was removed by filtration, and the filtrate was concentrated in vacuo. The residue was filtered through silica gel using diethyl ether as eluent and concentrated to give the allyl derivative (compound **11**) (yield 250 mg, 90%). ^{1}H NMR ($CDCl_3$, δ): 8.71 (2H, s), 8.54 (2H, d, J = 8 Hz), 7.87−7.30 (tot, 16H), 6.15 (1H, br s), 5.67 (1H, br s), 5.64 (1H, br s), 5.36 (1H, m), 5.30 (2H, m), 5.27 (1H, m), 4.65 (2H, m), 4.45 (2H, m), 4.32 (2H, m), 4.25 (1H, m), 4.17 (4H, s), 3.55 (8H, s), 2.33 (2H, m), 1.46 (36H, s). ESI-TOF-MS mass for $C_{73}H_{84}N_7O_{13}$ $(M - H)^-$: calcd, 1266.61; found, 1266.61.

Scheme 1. Synthesis of the Nonluminescent Block



Tetra-*tert*-butyl-4′-{3-[4-carboxy-4-(fluorenylmethyloxycarbonylamino)-1-oxo-1-butylamino]-1-propyn-1-yl}-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}tetrakis(acetate), 12. Cleavage of the allyl group from compound **11** and workup was performed as described for compound **7**. (Compound **12**) ESI-TOF-MS: mass for $C_{70}H_{80}N_7O_{13}$ $(M - H)^-$: calcd, 1226.58; found, 1226.61.

Synthesis of the Oligopeptide Conjugates. Oligopeptides were assembled on an Applied Biosystems 433A instrument in 10 μmol scale using Fmoc chemistry and recommended protocols (coupling time 30 min for natural amino acid analogues, and 2 h for blocks **7** and **12**). In the synthesis of the caspase-3 substrate, a commercial ϵ-dabsyl Fmoc-lysine block (Molecular Probes) was used to introduce the quencher and Fmoc-hexanoic acid (Fluka) as a spacer at the C-terminus. When the chain assembly was completed, the resin was treated with the mixture of crystalline phenol (75 mg), ethanedithiol (25 μL), thioanisole (50 μL), water (50 μL), and trifluoroacetic acid (1 mL) for 2−4 h. The resin was removed by filtration, and the solution was concentrated in vacuo. The crude oligopeptide was precipitated with diethyl ether. The precipitate was redissolved in water and treated with aqueous europium(III) citrate (5 equiv). Purification was performed on HPLC techniques (column, LiChrocart 125-3 Purospher RP-18e 5 μm; gradient, from 95% to 0% A in 30 min; flow rate was 0.6 mL min^{-1}).

Motilin Assay. Eu-motilin prepared using the ligand building block, **7**, was tested in saturation binding assay using human motilin receptor. All of the reagents were diluted in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.2% bovine serum albumin, and 25 μM EDTA. Human motilin receptor membrane preparate (0.5 μg/well; PerkinElmer Life Sciences) was incubated in the presence of Eu-motilin (0.25, 1, 4, and 16 nM) in an AcroWell filtration plate (Pall Life Sciences) in the total volume of 100 μL/well at room temperature for 90 min. To determine nonspecific binding, the assay was performed also in the presence of 800 nM motilin. Next, the wells were washed with 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 7.5) 4 times (300 μL/well) using a manifold (Millipore) and a vacuum pump. After washing, DELFIA enhancement solution was added (200 μL/well), and the plate was shaken for 15 min. Europium fluorescence was measured using a VICTOR^{2}V multilabel counter.

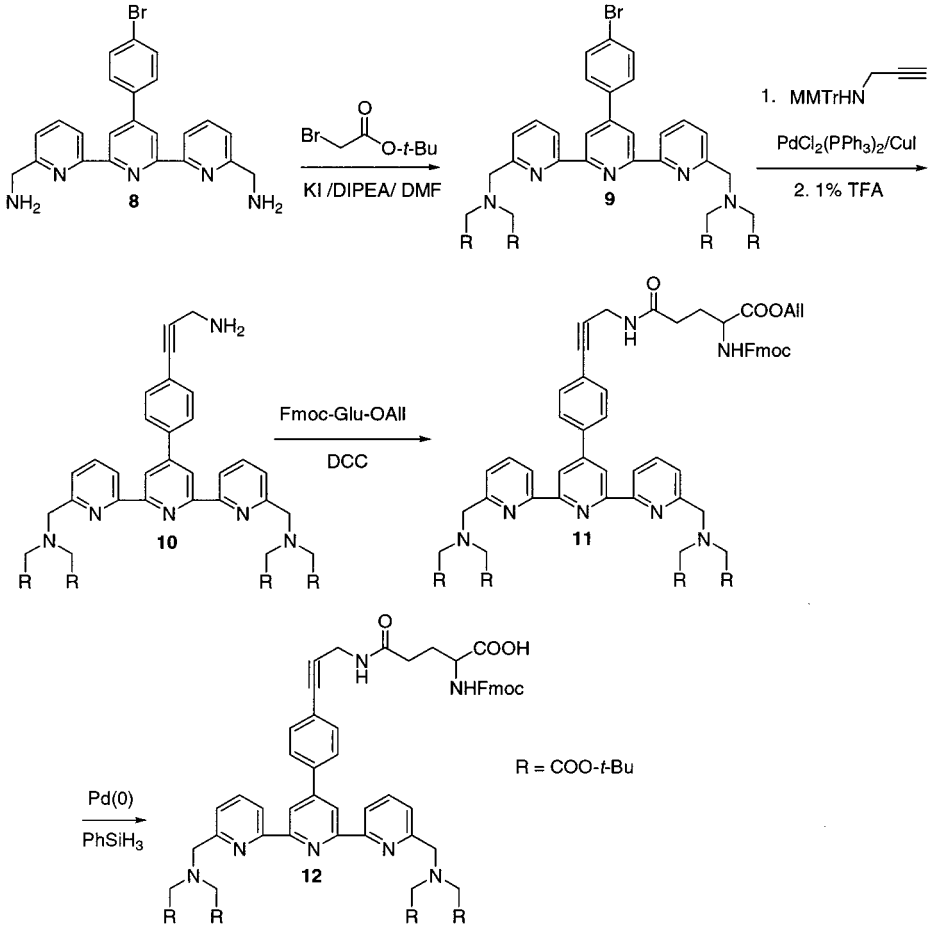
Caspase Assay. The performance of the caspase-3 substrate prepared using the building block **12** was tested in time-resolved fluorescence quenching assay (TR-FQA). The assay was performed in a Wallac 384-plate. Accordingly, caspase buffer (18 μL), substrate (2 μL, 200 pM−2 μM; final concentrations), and caspase-3 enzyme (1 μL, 5 pg/well) were pipetted to a well. In blank wells, there was buffer instead of enzyme. The plate was incubated at 37 °C. Europium fluorescence was measured with VICTOR^{2}V multilabel counter in 15 min time intervals.

RESULTS AND DISCUSSION

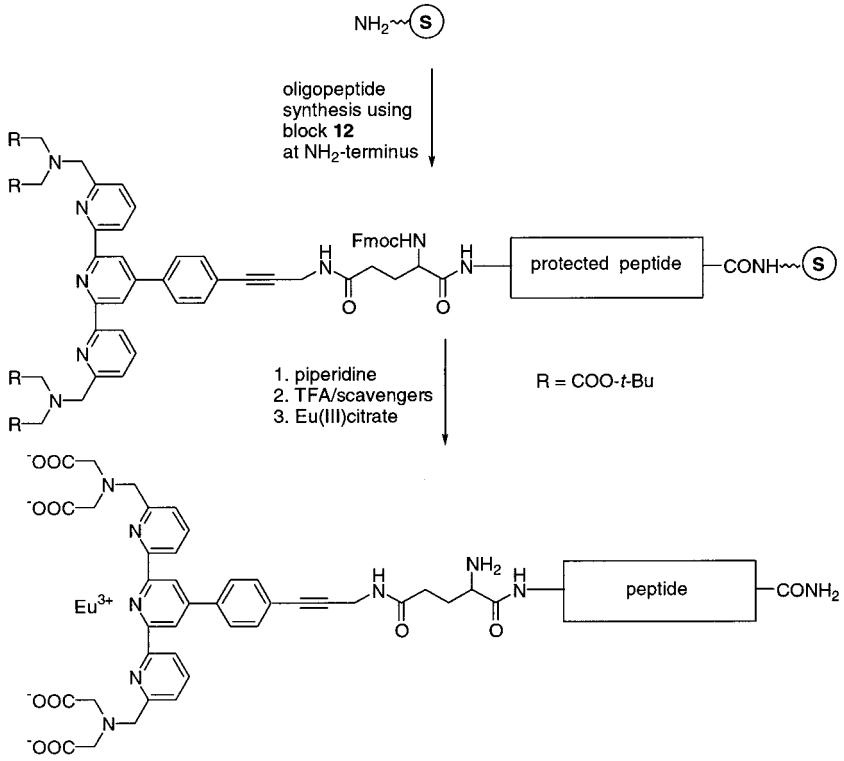
Synthesis of the Nonluminescent Oligopeptide Block. The synthesis of a nonluminescent oligopeptide building block for the introduction of lanthanide(III) chelates to the oligopeptide structure is shown in Scheme 1. Initially, 1-(4-nitrobenzyl)diethylenetriamine (**1**; *7*) was allowed to react with bromoacetic acid *tert*-butyl ester in the presence of potassium iodide as a catalyst to give the corresponding tetraester (**2**) in high yield. Reduction of the nitro group to the amino function gave **3**. It was then converted to the corresponding oligopeptide building block, **7**, by attachment of the spacer arm and the appropriately protected amino acid to the amine function. It is worth noting that elongation of the spacer arm with the glycine residue was essential to reach acceptable coupling efficiency. Transient protection of the glutamic acid residue, in turn, considerably simplified the purification procedures. Palladium-catalyzed allyl cleavage using phenylsilane as allyl scavenger (*8*) gave the final product quantitatively after washing with aq citric acid without need of further purification.

Synthesis of the Luminescent Block. The oligopeptide building block for the introduction of luminescent

Scheme 2. Synthesis of the Luminescent Block



Scheme 3. Introduction of a Luminescent Europium(III) Chelate to an Oligopeptide in the Aid of Block 12



lanthanide(III) chelates to the oligopeptide structure, **12**, was synthesized as shown in Scheme 2. In general, synthesis of the protected ligand was analogous to that published for the corresponding oligonucleotide building block (*6*). Accordingly, alkylation of the amine, **8**, with bromoacetic acid *tert*-butyl ester yielded **9**, the reaction of which with MMTr-protected propargylamine in the presence of Pd(II) and Cu(I) gave, after removal of the

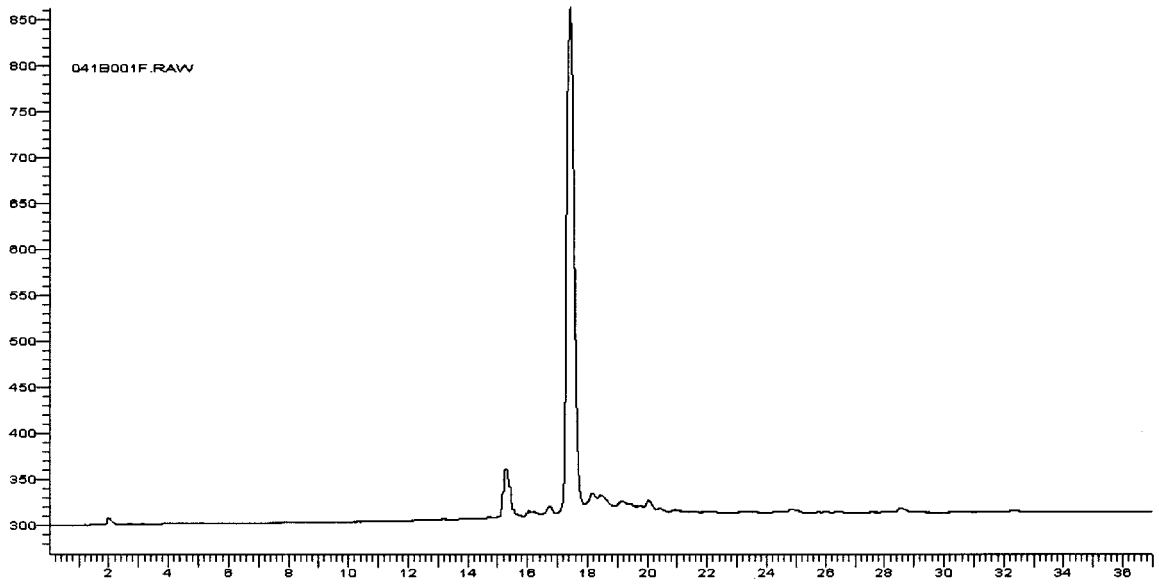


Figure 1. Reversed-phase HPLC trace of an oligopeptide conjugate X_2-Ser-Ile-Glu-Thr-Asp-Lys-$CONH_2$ labeled with a luminescent europium(III) chelate synthesized in the aid of block **12** (crude reaction mixture) detected at 270 nm. X_2 is the position of the label. For chromatographic conditions, see Experimental Procedures.

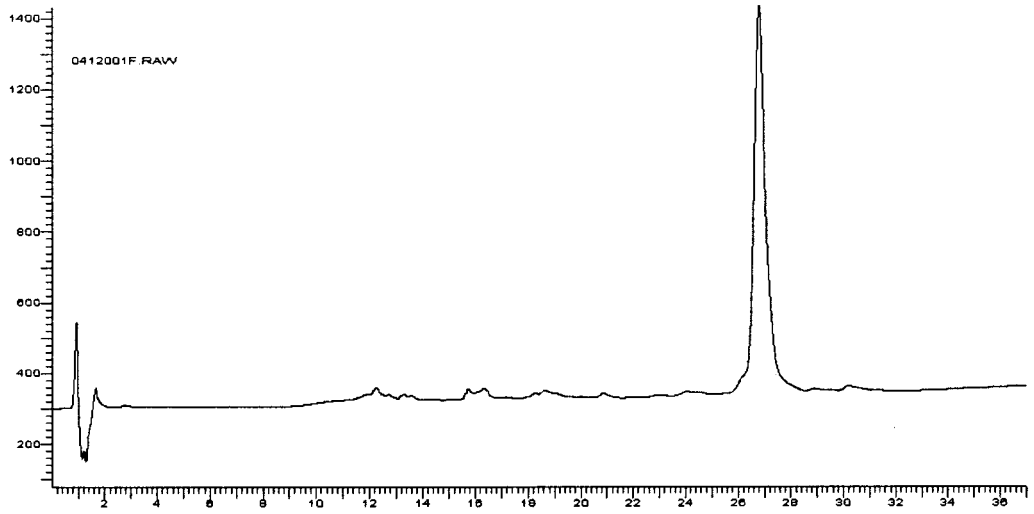


Figure 2. Reversed-phase HPLC trace of an oligopeptide conjugate Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln-X_1-$CONH_2$ (motilin) labeled with a nonluminescent europium(III) chelate synthesized in the aid of block **7** (crude reaction mixture) detected at 220 nm. X_1 is the position of the label. For chromatographic conditions, see Experimental Procedures.

amine protection, the ligand **10**. It was then converted to the oligopeptide building block, **12**, as described previously for **7** but by omitting the now unnecessary glycine spacer unit from the linker arm.

Synthesis of the Oligopeptide Conjugates. To demonstrate the applicability of the building blocks **7** and **12** for oligopeptide derivatization, model sequences (e.g., motilin, substance-P, neurokinin-A, and caspase-3) were synthesized in 10 μmol scale using Fmoc chemistry. The blocks **7** and **12** were coupled either to the amino or carboxyl terminus or to the internal position of the coding sequence using prolonged coupling time (2 h instead of 30 min) but otherwise standard conditions (5 equiv of blocks; HBTU/HOBt as an activator). According to fulvene-piperidine assay, coupling efficiencies of **7** and **12** were comparable to natural amino acid analogues. After completion of the oligopeptide synthesis, the oligopeptides were released from the resin and deprotected followed by precipitation from ether. Treatment of the deblocked oligomers with europium(III) citrate converted the oligopeptide conjugates to the corresponding europium chelates (Scheme 3).

Table 1. Oligopeptide Conjugates Synthesized and Their Observed and Calculated Molecular Weights

sequence[a]	$[M - H]^-$ calculated	$[M - H]^-$ observed
X_1-RPKPEEFFGLM-$CONH_2$ (substance P)	2103.87	2103.86
X_2-SIETDK-$CONH_2$ (Ser-caspase-8)	1602.51	1602.40
H-X_1-TDSFVGLM-$CONH_2$ (neurokinin A)	1761.62	1761.59
X_1-HKTDSFVGLM-$CONH_2$ (neurokinin A)	1889.71	1889.59
X_2-DEVD-dabcyl-hex-$CONH_2$ (caspase-3)	1879.63	1879.23
FVPIFTYGELQRMQEKERNKGQ-X_1-$CONH_2$ (motilin)	1728.26[b]	1728.29[b]

[a] X = position of the chelate; X_1 = synthesized in the aid of block **7**; X_2 = synthesized in the aid of block **12**; dabcyl = quencher synthesized in the aid of ϵ-dabcyl-Fmoc-lysine; hex = aminohexanoic acid residue. [b] $[M + 2\ H]^{2+}/2$.

The oligopeptides synthesized were purified on reversed-phase HPLC and characterized on ESI-TOF mass spectrometry. Typical HPLC profiles (crude reaction mix-

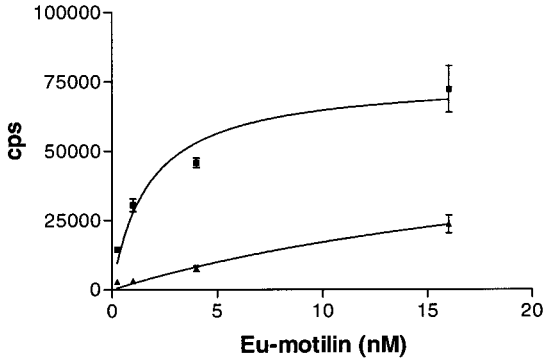


Figure 3. Saturation binding assay using Eu-motilin. Binding curves show total binding (squares) and nonspecific binding (triangles).

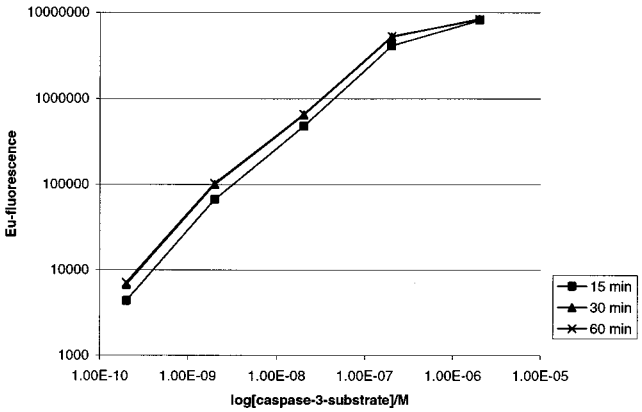


Figure 4. Concentration curve of caspase-3 substrate.

tures) are shown in Figures 1 and 2 as illustrative examples. In all of the cases, the observed molecular weights were in accordance with the proposed structures (Table 1).

Assays. Two of the oligopeptide conjugates synthesized were used in a DELFIA-based binding assay as well as in a LANCE fluorescence quenching assay. Accordingly, the motilin conjugate prepared using the ligand building block, **7**, was tested in saturation binding assay using human motilin receptor (Figure 3). Analysis of data gave a K_d value of 1.7 nM. The performance of the caspase-3 substrate prepared using the building block, **12**, in turn, was tested in TR-FQA. The substrate was cleaved by caspase-3 enzyme. The cleavage separates the quencher and the chelate and thus recovers the europium fluorescence. The results showed that the linear range of the assay is from 200 pM to 200 nM (Figure 4).

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