New routes to mercaptoacetylpeptide ligand precursors utilizing carboxy terminus pentaamminecobalt(III) protection

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Abstract

Various synthetic routes to peptide ligand precursors bearing the *S*-benzoyl protected mercaptoacetyl group at the N-terminus were evaluated. Of particular interest, the *S*-benzoyl protected mercaptoacetylglycylglycylglycine (MAG_3) is a ligand precursor for the synthesis of ^{99m}Tc-MAG_3 which has been successfully used as a radiopharmaceutical imaging agent for renal studies. The inorganic carboxy terminus protecting group, $(NH_3)_5Co^{III}$, was evaluated in the preparation of MAG_3 analogues containing bulky amino acids at the N-terminus. Use of this inorganic protecting group appears to be a useful new synthetic route for such analogues.

Introduction

Because of superior qualities of the ^{99m}Tc radionuclide, a number of ligands have been synthesized and radiolabeled with the aim of replacing ^{131}I labeled *o*-iodohippuric acid (^{131}I-OIH) for renal function studies [1]. The most successful of these agents has been ^{99m}Tc labeled mercaptoacetylglycylglycylglycine (^{99m}Tc-MAG_3, Fig. 1) [1, 2], however, the clearance of ^{99m}Tc-MAG_3 is only 50–65% that of ^{131}I-OIH [1–7]. Several attempts have been made, therefore, to improve radiopharmaceutical properties of ^{99m}Tc-MAG_3 by making structural changes in the MAG_3 ligand core [8–15]. Most of these changes have focused on positions X and R_1 (Fig. 1) [8–10, 15]. Although structural changes at positions R_2 (–CH_3 [10] and –CH_2OH [13]) and R_4 (–CH_3 [10] and –CH_2COOH [14]) have also been reported, biodistribution studies have shown that changes at positions X, R_1, R_2 and R_4, in general, did not improve radiopharmaceutical properties [1, 8–15]. Consequently, synthetic procedures for changing position R_3 were the prime target for our investigation. There is only one brief report describing a structural change at this position (R_3 = –CH_3 and $R_1 = R_2 = R_4 = H$), although no synthetic details are available [10].

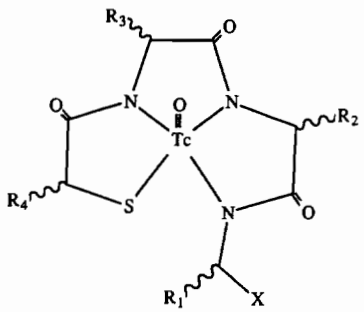

Fig. 1. General structure of ^{99m}Tc-MAG_3 type compounds; MAG_3:X = COOH; $R_1 = R_2 = R_3 = R_4 = H$.

In radiopharmaceutical labeling, it is convenient to use the *S*-benzoyl protected compound, *S*-benzoyl-MAG_3 [1]. Our initial work indicated that the general synthetic schemes suitable for the synthesis of *S*-benzoyl-MAG_3 and related compounds did not provide satisfactory yields when $R_3 \neq H$. This problem is probably the result of steric hindrance [16, 17]. Therefore, we explored alternative synthetic methods.

Isied and coworkers [18–24] have demonstrated the use and merits of Co(III) complexes in peptide synthesis. In this approach, a monodentate cobalt complex is used to selectively protect C-terminus, N-terminus, or side chain of an amino acid or peptide. The rest of the synthesis is similar to traditional solution phase and solid phase peptide synthesis. The deep color, kinetic inertness, charge, and facility and selectivity of introduction and removal of the $(NH_3)_5Co^{III}$ group under mild conditions and sol-

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ubility properties of these metallo–peptide complexes are salient features which facilitate the synthesis and purification of less tractable peptides [18–24]. Therefore, we decided to use this versatile peptide synthesis technique, developed by Isied and coworkers [18–22].

In the present work, we have used the $(NH_3)_5Co^{III}$ group to protect the C-terminus in the solution phase synthesis [18–21, 24] of [*S*-benzoyl-MAG_3–$Co(NH_3)_5$] $(BF_4)_2$ and some analogues in which the R_3 position has been changed to $-CH_3$, i.e. a Gly residue has been replaced with an Ala. We also explored the synthesis of analogues in which the α-H of the Ala is replaced with a $-CH_3$ to incorporate an α-aminoisobutyric acid residue.

Experimental

Materials

All chemicals used were of the highest purity available. Glycylglycine (Gly-Gly), chlorotrimethylsilane ($(CH_3)_3SiCl$), benzoyl chloride and thioglycolic acid were purchased from Aldrich. Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHSuc) were purchased from Chemical Dynamics Corporation and Aldrich. 1-Hydroxybenzotriazole monohydrate (HOBT) was obtained from Pierce. D-Ala and Boc-D-Ala (Boc = *N*-tert-butyloxycarbonyl) were purchased from Bachem, Inc. and Gly and α-aminoisobutyric acid (AIBA) were obtained from Sigma. *N*-Methylmorpholine (NMM) was purchased from Eastman Kodak and Sigma and stored over 4 Å molecular sieves. Trifluoroacetic acid (HTFA) was a product of Chemical Dynamics Corporation and fluoroboric acid (HBF_4) and perchloric acid were purchased from Fisher Scientific. Solvents including methylene chloride (CH_2Cl_2), dimethylformamide (DMF), tetrahydrofuran (THF) and ethyl acetate (EtOAc) were HPLC grade and were kept dry over 4 Å molecular sieves. Ether (Baker Analyzed Reagent) was a product of J. T. Baker and ethyl alcohol (EtOH) (absolute-200 proof) that of Aaper Alcohol and Chemical Company. HPLC grade methanol (MeOH) and acetonitrile (CH_3CN) were purchased from J. T. Baker. Deuterated dimethylsulfoxide (DMSO-d_6) was obtained from Aldrich. Water used for this work was distilled and deionized.

Resins and packings

Reverse phase-octadecylsilane (C_{18}) packing material (40 μ) was obtained from J. T. Baker and soaked in 80% aqueous methanol for 24 h before packing the column. Bio-Gel P-2 (200–400 mesh) was purchased from Bio-Rad Laboratories and soaked in water for 24 h before packing the column. Chelex-100 (200–400 mesh, sodium form) was a product of Bio-Rad Laboratories. Before use, the column was washed with an excess of water until neutral. For preparative liquid chromatography (LC), glass columns purchased from Bio-Rad Laboratories were used.

Methods

HPLC was performed on a Beckman liquid chromatography system equipped with a fixed wavelength UV-detector operating at 254 nm. An Altex Ultrasphere-ODS (4.6 mm (i.d.) × 25 cm, 5 μm C_{18} packing) column was used and chromatograms were recorded on a Hewlett Packard integrator (model HP 3394A). All solvents were filtered through Nylon-66 (0.2 μm) filters and degassed before use. Preparative LC columns were equilibrated with the same solvent(s) used to load the sample. Columns were eluted using either a peristaltic pump (Rabbit-Plus, Rainin Instrument Company, Inc.) or by gravity flow as suited for each individual experiment. The concentration of the organic solvent in the mobile phase was changed gradually. Rotary evaporation of solvents was achieved under reduced pressure ($\sim$25 mm Hg) at 30–35 °C. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a GE-QE 300 spectrometer operating at 300 MHz. The solvent signal was used as an internal reference and chemical shifts are reported in parts per million (ppm) downfield from TMS. Elemental analyses were performed commercially by Atlantic Microlab, Inc. (Norcross, GA) and Galbraith, Inc. (Knoxville, TN). All moisture-sensitive reactions (e.g. HOBT ester coupling, silyl ester formation and coupling, etc.) were performed under moisture-free atmosphere produced using freshly oven-dried glassware and drying tubes and performing all transfers using gas-tight syringes. All products were dried at ambient temperature under vacuum (25–30 mm Hg) unless noted otherwise. All yields reported are isolated yields.

Syntheses

[Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$

Glycylglycine (37.14 g, 281.13 mmol) and $[(NH_3)_5CoOH_2](ClO_4)_3$ (8.64 g, 18.74 mmol), synthesized using a published procedure [19], were mixed with 10 ml of water, and the suspension stirred at 70 ± 5 °C. The progress of the reaction was monitored by HPLC analysis (MeOH:H_2O:HTFA (10:90:0.2); pH = 3.28 (w/NaOH); flow rate = 1.0 ml/min). A decrease in the height of the peak corresponding to $[(NH_3)_5CoOH_2]^{3+}$ (3.31 min retention time) and increase in height of a new peak corresponding to the product (4.84 min retention time) was observed

as the reaction progressed. The reaction was found to be >99% complete, as judged by HPLC analysis, within 2 h, but stirring and heating were continued for 3 h, after which no change in peak ratio of starting material and product was observed. The heat source was removed and stirring was continued until the mixture cooled to ambient temperature. The reaction mixture was maintained at 4 °C overnight and excess free peptide was removed by filtration. The filtrate was loaded onto a Chelex-100 column and a red compound adsorbed at the top of the column bed. The column was eluted with water until no free peptide was detected in the effluent with ninhydrin. The red compound, [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$, was then eluted with 0.3 M aqueous HTFA and the solvent removed by rotary evaporation. The residue was dissolved in a minimum volume of ethanol and the product precipitated by adding 10–11 ml of concentrated HBF_4 and an excess of ether. The pink compound, [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$, was collected by filtration and air-dried. This crude product was dissolved in water and loaded, in small fractions, onto a Bio-Gel P-2 column (1.5 (i.d.)×42 cm). The column was eluted with water and the major band collected. The solvent was removed by rotary evaporation and the product dissolved in ethanol and precipitated by adding an excess of ether. The precipitate was collected on a filter and dried under vacuum and weighed 8.5 g (85% yield). UV–Vis (water): λ_{max} = 504 nm (ϵ = 79.0 M^{-1} cm^{-1}) and 352 nm (ϵ = 63.6 M^{-1} cm^{-1}) [18]. ^1H NMR (DMSO-d_6): 2.7 ppm (3H, s, *trans*-NH_3), 3.57 (2H, s, ^+H_3N–*CH_2*–CO–), 3.61 (2H, d, –NH–*CH_2*–CO–), 3.73 (12H, s, 4×*cis*-NH_3), 7.9 (3H, s (broad), *^+H_3N*–CH_2–CO–), 8.17 (1H, t, –CO–*NH*–CH_2–). *Anal.* Calc. for $C_4H_{23}N_7O_3B_3F_{12}Co$: C, 8.95; H, 4.33; N, 18.27; Found: C, 9.09; H, 4.37; N, 18.21%.

[D-Ala–Gly-Gly–Co(NH_3)$_5$]$(BF_4)_3$

Three solutions, Boc-D-Ala (3.13 g, 16.74 mmol) in 12 ml of CH_2Cl_2, DCC (3.45 g, 16.74 mmol) in 6 ml of CH_2Cl_2 and HOBT (2.56 g, 16.74 mmol) in 6 ml of DMF, were separately cooled to 0 °C. Then, the solution of HOBT was added to the solution of Boc-D-Ala followed by the addition of DCC and the mixture stirred for 1 h at 0 °C and 1 h at ambient temperature. Then, [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ (3.0 g, 5.58 mmol) in 7 ml of DMF was added. The mixture was stirred for a few minutes before adding NMM (0.755 ml, 6.89 mmol) and stirring for 1 h at ambient temperature. A few minutes after addition of several drops of conc. HBF_4 and stirring, dicyclohexylurea (DCU) was removed by filtration and washed with DMF (3×2–3 ml). Filtrate and washings were combined and concentrated by rotary evaporation. The red solution was mixed with an equal volume of EtOAc and added dropwise to a vigorously stirred large excess of ether. The suspension was allowed to stand overnight at ambient temperature, whereupon a red precipitate formed. The solvent was decanted, the 'wet' residue shaken with 25 ml of EtOAc, and the suspension stirred with 500 ml of ether. The precipitate was allowed to settle for 15 min and the solvent decanted. The residue was dissolved in water and loaded onto a C_{18} column (2.5 (i.d.)×16 cm). The column was eluted with water until a small band (unreacted [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$) was removed. The major band was eluted with 40% aqueous methanol. The solvent was removed by rotary evaporation and the red fluffy compound, [Boc-D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$, dried under vacuum. ^1H NMR (DMSO-d_6): 1.15 ppm (3H, d, –CH(*CH_3*)–CO–), 1.33 (9H, s, (*CH_3*)$_3$–O–CO–), 2.60 (3H, s, *trans*-NH_3), 3.4–3.62 (4H, 4d, 2×–NH–*CH_2*–CO–), 3.7 (12H, s, 4×*cis*-NH_3), 3.92 (1H, q, –NH–*CH*(CH_3)–CO–), 7.05 (1H, d, –(CH_3)$_3$C–OCO–*NH*–CH(CH_3)–), 7.6 (1H, t, –CO–*NH*–CH_2–), 8.10 (1H, t, –CO–*NH*–CH_2–), and 2.68, 2.85, 7.90 (1H, s, CH_3/$_3$; and 0.3H, s, –CO–H of 0.3 DMF). *Anal.* Calc. for $C_{12}H_{37}N_8O_6B_2F_8Co \cdot 0.3DMF \cdot 0.5H_2O$: C, 23.90; H, 5.92; N, 17.86. Found: C, 24.10; H, 5.89; N, 17.43%.

[Boc-D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$, obtained in the above reaction, was dissolved in 25 ml of HTFA:CH_2Cl_2 (1:1) and the solution stirred for 1 h at 0 °C [24]. Then, solvents were removed immediately by rotary evaporation. The residue was dissolved in water and loaded onto a Bio-Gel P-2 column (1.5 (e.d.)×42 cm). The column was eluted with water and the major band collected and the solvent removed completely by rotary evaporation. The residue was dissolved in a few ml of ethanol and treated with 1 ml of conc. HBF_4. The product precipitated upon addition of an excess of ether. The pink precipitate was filtered, dried under vacuum and weighed 2.73 g (81% yield, based on [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$). ^1H NMR (DMSO-d_6): 1.31 ppm (3H, d, ^+H_3N–CH(*CH_3*)–CO–), 2.61 (3H, s, *trans*-NH_3), 3.64 (4H, d, 2×–NH–*CH_2*–CO–), 3.69 (12H, s, 4×*cis*-NH_3), 3.83 (1H, q, ^+H_3N–*CH*(CH_3)–CO–), 7.83 (1H, t, –CO–*NH*–CH_2–CO–), 8.0 (3H, s (broad), *^+H_3N*–CH(CH_3)–), 8.53 (1H, t, –CO–*NH*–CH_2–). *Anal.* Calc. for $C_7H_{28}N_8O_4B_3F_{12}Co.1.5\ H_2O$: C, 13.24; H, 4.93; N, 17.66. Found: C, 13.40; H, 4.85; N, 17.60%.

[R-D-Ala–Gly-Gly–Co(NH_3)$_5$]$(BF_4)_2$ (where R = $C_6H_5COSCH_2CO$–)

This compound was synthesized by two different methods.

A. Ten grams (16.45 mmol) of [D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ were dissolved in 27 ml of DMF and 7.23 g (24.67 mmol) of succinimidyl-*S*-benzoylthioglycolate (SucBTG), prepared by a published method [25], were dissolved in 35 ml of CH_2Cl_2. The two solutions were combined and the mixture stirred until homogeneous. Then NMM (2.194 ml, 19.74 mmol) was added and the mixture stirred at ambient temperature. The progress of the coupling reaction was monitored by HPLC analysis (CH_3CN:H_2O:HTFA (45:55:0.3); pH = 3.64 (w/ NaOH); flow rate = 0.3 ml/min) of aliquots drawn at different times. Gradually, the peak corresponding to [D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ (retention time, 7.28 min) decreased, and a new peak corresponding to the product (retention time, 9.37 min) increased in height. After 30 min, no change in peak height was observed, but stirring was continued for 1 h. Then, 1 ml of HTFA was added and the mixture stirred for 10 min. The solution was concentrated by rotary evaporation and the crude product precipitated in a manner similar to that described for [D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$. The precipitate was dissolved in 2.5% aqueous acetic acid (200 ml) and the solution extracted with CH_2Cl_2 (4 × 100 ml). The aqueous phase was concentrated by rotary evaporation and loaded onto a C_{18} column (2.5 (i.d.) × 17 cm) in two batches. The column was eluted with water. A small band of unchanged [D-Ala–Gly-Gly–$Co(NH_3)_5$)]$(BF_4)_3$ separated and was washed off the column. On further elution another small band of hydrolyzed product separated and was eluted from the column. The product was retained much longer by the column and eluted with 60% aqueous methanol. This solvent was removed by rotary evaporation. The product was dried further under vacuum and weighed 6.75 g (59% yield). ^1H NMR (DMSO-d_6): 1.20 ppm (3H, d, –NH–CH(*CH*$_3$)–Co–), 2.61 (3H, s, *trans*-NH_3), 3.4–3.67 (4H, 4d, 2 × –NH–*CH*$_2$–CO–), 3.7 (12H, s, 4 × *cis*-NH_3), 3.88 (2H, s, –S–*CH*$_2$–CO–), 4.21 (1H, q, –NH–*CH*(CH$_3$)–CO–), 7.55 (3H, t, –CO–*NH*–CH_2– and aromatic), 7.63 (1H, t, aromatic), 7.9 (2H, d, aromatic), 8.21 (1H, t, –CO–*NH*–CH_2–), 8.58 (1H, d, –CO–*NH*–CH(CH$_3$)–). *Anal.* Calc. for $C_{16}H_{33}N_8O_6SB_2F_8Co.1.5\ H_2O$: C, 26.50; H, 5.01; N, 15.45; Co, 8.13. Found: C, 26.44; H, 4.84; N, 15.27; Co, 8.23%.

B. A mixture of *N*-(*S*-benzoylthioglycolyl)–D-alanine (R-Ala, preparation described below) (80 mg, 0.3 mmol, in 5 ml of CH_2Cl_2), HOBT (36 mg, 0.3 mmol, in 0.5 mL of DMF) and DCC (47 mg, 0.3 mmol, in 1 mL of CH_2Cl_2) was stirred for 1 h at 0 °C and 1 h at room temperature. Then, [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ (85 mg, 0.15 mmol, in 1 ml of DMF) was added, followed by the addition of NMM (19 μl = 0.17 mmol) and the mixture stirred for 45 min at ambient temperature. The workup was similar to that in method A. The crude product was purified using a C_{18} column (0.5 (i.d.) × 7 cm) and weighed 60 mg (57% yield). The ^1H NMR spectrum (DMSO-d_6) was similar to that in method A.

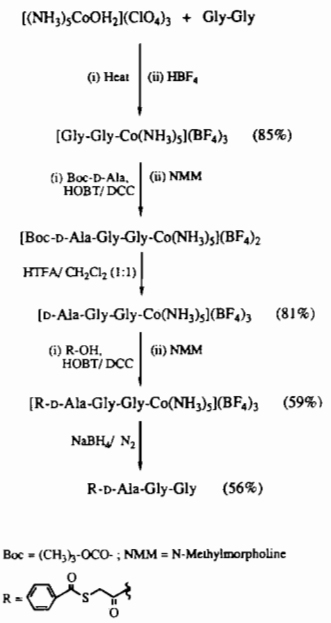
N-(S-Benzoylthioglycoly)–D-alanine

Solutions of SucBTG (4.12 g, 14.06 mmol, in 150 ml of THF) and R-Ala (1.0 g, 11.24 mmol, in 10 ml of water) were mixed at 50 °C. The resulting milky suspension, which became clear during reflux, was heated under reflux for 2.5 h and then stirred for 14.5 h at ambient temperature. The solvents were removed by rotary evaporation and the yellowish oily residue mixed with 15 ml of CH_3CN. The colorless precipitate that formed was collected by filtration, washed with acetone, and air-dried. This crude product was recrystallized from water, dried under vacuum and weighed 1.06 g (35% yield). m.p. 178–179 °C; ^1H NMR (DMSO-d_6): 1.23 ppm (3H, d, –NH–CH(*CH*$_3$)–COO); 3.80 (2H, s, –S–*CH*$_2$–CO–) 4.17 (1H, q, –NH–*CH*(CH$_3$)–COO); 7.53, 7.65, 7.9 (2,1,2H; t,t,d; aromatic), 8.50 (1H, d, –CO–*NH*–CH(CH$_3$)–). *Anal.* Calc. for $C_{12}H_{13}NO_4S$: C, 53.89; H, 4.91; N, 5.29; S, 11.99. Found: C, 53.97; H, 4.91; N, 5.30; S, 11.91%.

Similar yields were also obtained under the following conditions: using a THF/H_2O solution (16.5 h reflux and stirring for 1 h at ambient temperature) gave a 37%, (24 h reflux) 35%, and (48 h reflux) 33% yield and an acetone/H_2O solution refluxed for 24 h gave a 35% yield.

R–D-Ala–Gly-Gly

One gram (1.43 mmol) of [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$ was dissolved in 200 ml of water and N_2 gas was bubbled through for 30 min. Then, solid $NaBH_4$ was slowly added, with concomitant stirring and bubbling of N_2 gas, until the color changed from red to black [19]. The black solution was stirred for an additional 15 min and enough HTFA was added to make the solution 2.5% in acid. The color became faint pink. After stirring for a few minutes, the solution was extracted with CH_2Cl_2 (total 500 ml) and then with ether (total 600 ml). The aqueous phase was rotary evaporated to dryness and the purplish residue was dissolved in ~50 ml boiling isopropanol. Some insoluble material was removed by filtration and discarded. The alcohol solution was left overnight in a fume hood at ambient temperature, during which the solvent evaporated. The purplish residue thus obtained was washed with water (3 × 20 ml). The resultant colorless product was dried under vacuum and weighed 0.31 g (56% yield) (Scheme



Scheme 1.

1). ^{1}H NMR (DMSO-d_6): 1.18 ppm (3H, d, –NH–CH(*CH_3*)–CO–), 3.6–3.7 (4H, t or d+d, 2×–NH–*CH_2*–CO–), 3.83 (2H, s, –S–*CH_2*–CO–), 4.25 (1H, q, –NH–*CH*(CH_3)–CO–); 7.53, 7.67, 7.90 (2,1,2H; t,t,d; aromatic), 8.03 (1H, t, –CO–*NH*–CH_2–CO–), 8.20 (1H, t, –CO–*NH*–CH_2–CO–), 8.47 (1H, d, –CO–*NH*–CH(CH_3)–). *Anal.* Calc. for $C_{16}H_{19}N_3O_6S \cdot 0.75H_2O$: C, 48.65; H, 5.24; N, 10.64; S, 8.12. Found: C, 48.57; H, 5.27; N, 10.46; S, 8.04%.

[R-Gly-Gly-Gly-Co(NH_3)$_5$](BF_4)$_2$

Solutions of N-(S-benzoylthioglycolyl)glycine (S-benzoyl-MAG) [25] (1.01 g, 4.0 mmol, in 18 ml of THF), HOBT (0.61 g, 4.0 mmol, in 2 ml of DMF) and DCC (0.83 g, 4.0 mmol, in 5 ml of THF) were mixed and stirred for 1 h at 0 °C and 1 h at ambient temperature. Then, [Gly-Gly–Co(NH_3)$_5$](BF_4)$_3$ (1.07 g, 2.0 mmol, in 3 ml of DMF) was added followed by NMM (0.278 ml, 2.5 mmol). The mixture was stirred for 1.25 h at ambient temperature. The workup was similar to that described for [R–D-Ala–Gly-Gly–Co(NH_3)$_5$](BF_4)$_2$. The crude product was purified by using a C_{18} column (2.5 (i.d.)×15 cm) as described for [R–D-Ala–Gly-Gly–Co(NH_3)$_5$](BF_4)$_2$ and weighed 1.08 g (79% yield based on [Gly-Gly–Co(NH_3)$_5$](BF_4)$_3$). ^{1}H NMR (DMSO-d_6): 2.70 ppm (3H, s, *trans*-NH_3), 3.53 (2H, d, –NH–*CH_2*–CO–), 3.65 (4H, d, 2×–NH–*CH_2*–CO–), 3.73 (12H, s, 4×*cis*-NH_3), 3.83 (2H, s, –S–*CH_2*–CO–), 7.53 (3H, t, –CO–*NH*–CH_2–CO– and aromatic), 8.27 (1H, t, –CO–*NH*–*CH_2*–CO–), 8.60 (1H, t, –CO–*NH*–CH_2–CO–). HPLC analysis: (CH_3CN:H_2O:HTFA (45:55:0.3); pH = 3.78 (w/NaOH); flow rate = 0.3 ml/min): single peak, retention time = 17.94 min (reference: [Gly-Gly–Co(NH_3)$_5$](BF_4)$_3$, retention time = 14.95 min). *Anal.* Calc. for $C_{15}H_{31}N_8O_6SB_2F_8Co \cdot H_2O$: C, 25.66; H, 4.75; N, 15.96. Found: C, 25.65; H, 4.66; N, 15.85%.

N-(S-benzoylthioglycolyl)–α-aminoisobutyric acid (R–AIBA)

α-Aminoisobutyric acid (AIBA) (12.73 g, 122.2 mmol) was suspended in 100 ml of dry CH_2Cl_2. After addition of $(CH_3)_3SiCl$ (15.9 ml, 122.2 mmol) with a syringe, the mixture was heated at reflux for 4 h and then allowed to cool to ambient temperature [27]. Then solid SucBTG (28.65 g, 97.80 mmol) was added and stirred until it dissolved. After addition of NMM (14.95 ml, 134.4 mmol), the mixture was stirred for 45 min at ambient temperature. Then concentrated HCl (2–3 ml) was added and stirring was continued for additional 10 min. The solvent was removed by rotary evaporation and the residue mixed with 150 ml of methanol [27]. The resulting suspension was maintained at 4 °C for two days and the colorless material collected by filtration and washed with methanol (2–3 ml). The filtrate and washings were combined and concentrated, by rotary evaporation, to a thick oil. This oil was mixed with water (65 ml) and acetone (25 ml), whereupon a colorless precipitate formed. The precipitate was collected by filtration. The filtrate was concentrated to 70 ml, whereupon some more precipitate formed which was also collected by filtration. These two batches of precipitate were combined and dried under vacuum. The crude product was crystallized from EtOAc and the colorless flaky crystals (m.p. 160–163 °C) were collected by filtration, dried under vacuum and weighed 12.1 g (44% yield). An analytical sample was obtained by recrystallization from water and drying under vacuum. ^{1}H NMR (DMSO-d_6): 1.31 ppm (6H, s, –NH–C(*CH_3*)$_2$–COO); 3.78 (2H, s, –S–*CH_2*–CO–); 7.52, 7.67, 7.89 (2,1, 2H; t,t,d; aromatic); 8.40 (1H, s, –CO–*NH* –*C*(CH_3)$_2$–). *Anal.* Calc. for $C_{13}H_{15}NO_4S$: C, 55.49; H, 5.38; N, 4.98; S, 11.40. Found: C, 55.65; H, 5.45; N, 4.97; S, 11.34%.

[R–AIBA–Co(NH_3)$_5$](BF_4)$_2$

This compound was synthesized by two methods.

A. A suspension of R–AIBA (12.0 g, 42.7 mmol, in 140 ml of CH_2Cl_2 and solutions of NHSuc (4.91 g, 42.7 mmol, in 9 ml of DMF) and DCC (9.79 g, 47.0 mmol in 7 ml of CH_2Cl_2) were cooled to 0 °C. Then, the solution of NHSuc was added to the stirred suspension of R–AIBA followed by the addition of the DCC solution, and the mixture stirred for 2 h at 0 °C and 1.5 h at ambient temperature. After

addition of 2 ml of glacial acetic acid the mixture was stirred for 30 min at ambient temperature and the DCU removed by filtration. The residue was triturated with boiling CH_2Cl_2 (2×100 ml) which was combined with the filtrate and the CH_2Cl_2 removed by rotary evaporation. The residue (in DMF) was mixed with 40 ml of water. The colorless precipitate that formed was collected on a filter and washed with water (3–4 ml). The wet precipitate was stirred with 20 ml of THF and the solvent was removed by rotary evaporation. The gummy residue was then stirred with an excess of ether. The colorless grainy precipitate (9 g) that formed was collected by filtration. A suspension of this crude succinimidyl-*N*-(*S*-benzoylthioglycolyl)–α-aminoisobutyrate (SucBTG–AIBA) (0.50 g, 1.32 mmol, in 7 ml of CH_2Cl_2) and a solution of $[(NH_3)_5CoOH](ClO_4)_2$ [19] (0.95 g, 2.64 mmol, in 4.5 ml of DMF) were mixed and stirred for 1 h at ambient temperature. Then, conc. HBF_4 (1 ml) was added, the mixture stirred for 5 min, and the CH_2Cl_2 removed by rotary evaporation. The workup was similar to that described for [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$. The crude product was purified by using a C_{18} column (2.5 (i.d.)×16 cm) and dried under vacuum and weighed 0.58 g (74% yield). ^{1}H NMR (DMSO-d_6): 1.23 ppm (6H, s, –NH–C(*CH*$_3$)$_2$–CO–); 2.60 (3H, s, *trans*-NH_3); 3.67 (12H, s, 4×*cis*-NH_3); 3.77 (2H, s, –S–*CH*$_2$–CO–); 7.57, 7.65, 7.92 (2,1,2H; t,t,d; aromatic); 8.23 (1H, s, –CO–*NH*–C(CH$_3$)$_2$–). HPLC analysis [CH_3CN: H_2O:HTFA (45:55:0.3); pH = 3.73 (w/NaOH); flow rate = 0.3 ml/min]: >99% purity; retention time = 13.29 min. *Anal.* Calc. for $C_{13}H_{29}N_6O_4SB_2F_8Co \cdot 0.5HBF_4$: C, 24.32; H, 4.64; N, 13.09. Found: C, 24.43; H, 4.76, N, 13.35%.

B. Solutions of SucBTG (0.53 g, 1.81 mmol, in 6 ml of CH_2Cl_2) and [AIBA–$Co(NH_3)_5$]$(BF_4)_3$ (1.0 g, 1.97 mmol, preparation described below, in 4–5 ml of DMF) were mixed and then NMM (0.27 ml, 2.46 mmol) was added. The mixture was stirred for 1 h at ambient temperature. Then, conc. HBF_4 (0.2 ml) was added and stirring continued for additional 10 min. The workup and purification were similar to those described under method A; yield 0.76 g (70%). The ^{1}H NMR spectrum (DMSO-d_6) was similar to that of the product in method A.

[AIBA–Co(NH$_3$)$_5$](BF$_4$)$_3$

A suspension of AIBA (22.6 g, 216.92 mmol) and $[(NH_3)_5CoOH_2](ClO_4)_3$ [19] (10.0 g, 21.69 mmol) in 25 ml of water was stirred for 3.5 h at 70±5 °C. The reaction mixture was then allowed to cool to ambient temperature. The excess of amino acid was removed by filtration and the crude product purified by Chelex-100 (2.5 (i.d.)×16 cm) and Bio-Gel P-2 (1.5 (i.d.)×42 cm) column chromatography in a manner similar to that described for [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$; yield 8.33 g (75%). ^{1}H NMR (DMSO-d_6): 1.22 ppm (6H, s, $^+$H$_3$N–C(*CH*$_3$)$_2$–CO–); 2.63 (3H, s, *trans*-NH_3); 3.67 (12H, s, 4×*cis*-NH_3); 7.67 (3H, s (broad), $^+$*H*$_3$*N*–C(CH$_3$)$_2$–CO–). *Anal.* Calc. for $C_4H_{24}N_6O_2B_3F_{12}Co$: C, 9.46; H, 4.77; N, 16.56. Found: C, 9.58; H, 4.78; N, 16.65%.

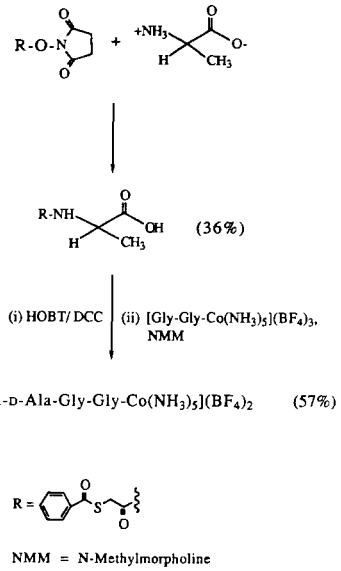
Discussion

Synthesis of R–AA, where R = S-benzoylthioglycolyl and AA = amino acid

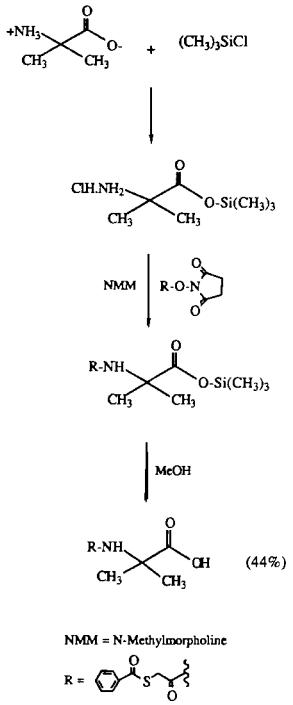
Fritzberg *et al.* [2] and Brandau *et al.* [26] published the synthesis of *S*-benzoyl-MAG_3, the precursor for the synthesis of ^{99m}Tc-MAG_3 imaging agent, in high yield (>80%) in aqueous EtOH. The method of Brandau *et al.* [26] for the synthesis of *S*-benzoyl-MAG_3 is essentially the same as that for *S*-benzoyl-MAG reported by Schneider *et al.* [27] except that the former authors used NaOH to dissolve triglycine. This requirement of a base indicates that, as the hydrophobicity of the amino acid or the peptide increased, solubility in the mixed solvent system became a problem.

Although Schneider *et al.* [27] were successful in the synthesis of *S*-benzoyl-MAG (59%) and its β-Ala analogue (84%), under identical conditions we obtained only 6% of the Ala analogue. The presence of (*S*-benzoyltyhioglycolyl) ethyl ester in the product suggested that transesterification (ϕ–CO–SCH$_2$CO–NHSuc $\xrightarrow{EtOH}$ ϕ–CO–SCH$_2$CO–OEt) was the major side reaction. This poor yield is due to low solubility and/or increased steric hindrance of Ala compared to Gly and β-Ala. Nevertheless, we successfully circumvented this problem by using more inert and less polar solvents than aqueous EtOH, such as aqueous THF or acetone. R–Ala was obtained in an average yield of 36% (Scheme 2). Attempts to increase this yield by prolonged heating were unsuccessful.

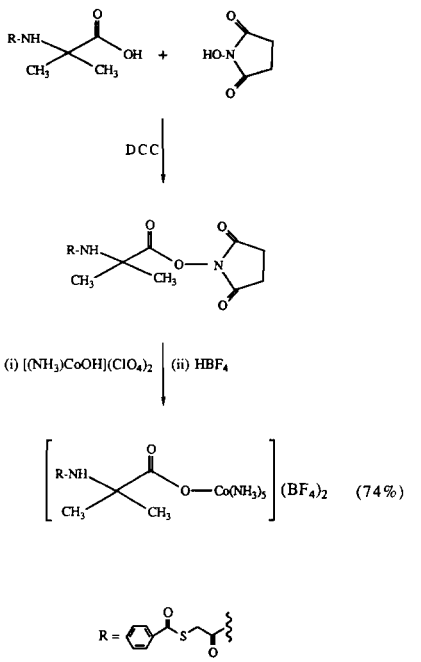
We attempted to synthesize R–AIBA under our improved conditions for R–Ala but only trace amounts of R–AIBA were detected in the reaction mixture. We hypothesized that, if the amino acid could be derivatized at the C-terminus, it could be dissolved in a more inert solvent, e.g. CH_2Cl_2, instead of an aqueous mixed solvent system. Then, a mild base (e.g. NMM) could be used for deprotonation of the amino terminus. However, this approach required a temporary C-terminus protecting group that could be cleaved selectively under mild conditions, leaving the benzoyl group intact. Silyl ester [25] or $(NH_3)_5Co^{III}$ group [18–21, 24] protection were the



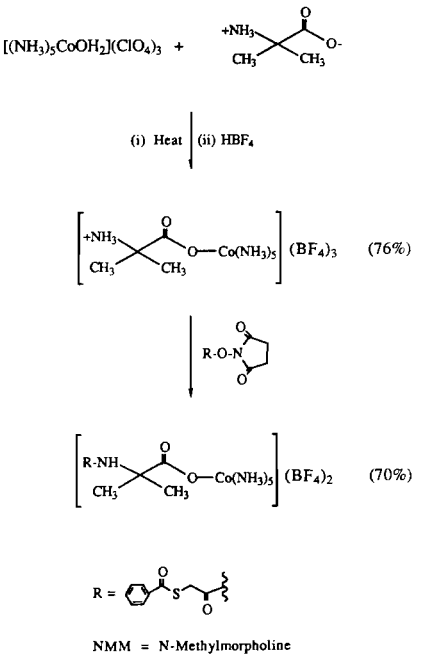
Scheme 2.



Scheme 3.



Scheme 4.



Scheme 5.

two most promising choices. We chose the silyl ester route first since it allowed us to use the less polar solvent, CH_2Cl_2. This method was useful and R–AIBA was successfully obtained in 44% yield (Scheme 3). The $(NH_3)_5Co^{III}$ route is useful also (Schemes 4 and 5) but requires deprotection and purification. Therefore, it was not pursued further in view of our success using silyl ester protection.

Synthesis of [R–Y–Co(NH$_3$)$_5$](BF$_4$)$_2$ complexes (Y = amino acid or peptide)

The coupling of SucBTG with L-Ala–Gly–Gly, under conditions similar to those employed for the synthesis of R–Ala, gave only 5% of R–L-Ala–Gly–Gly in impure form. Moreover, although L-Ala–Gly–Gly is commercially available at reasonable cost, most other peptides of interest are either expensive or

not available. Therefore, it was essential to develop a methodology to obtain pure precursors of the MAG_3 analogues in good yield. The chemistry developed by Isied and coworkers [18–24] for stepwise peptide synthesis offers a good alternative approach.

We first examined the synthesis and characterization of the MAG_3 precursor, [R–Gly–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$, in order to establish the technique. This compound was synthesized in high yield by coupling S-benzoyl-MAG with [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$. Isied and Kuehn [18] have reported the synthesis of [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ by coupling [Gly–$Co(NH_3)_5$]$(BF_4)_3$ with Boc-Gly. It has been suggested that peptide–Co(III) complexes could not be prepared by heating a peptide with $[(NH_3)_5CoOH_2]^{3+}$ because of possible hydrolysis of the peptide bond [18]. However, we have synthesized [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ in high yield by heating Gly-Gly with $[(NH_3)_5CoOH_2]^{3+}$. Thus, the direct synthesis can be successful, at least in the case of simple peptides like Gly-Gly. Nevertheless, the stepwise synthesis might be the only option when one is limited by cost, availability, and/or specific amino acid sequence of the peptide under consideration.

A MAG_3 analogue precursor, [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$, was obtained by stepwise synthesis in high yield. This compound was synthesized by two different methods. In method A (Scheme 1), [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$ was synthesized by coupling [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ with Boc–D-Ala followed by Boc deprotection and subsequent SucBTG coupling. In method B (Scheme 2), [Gly-Gly–$Co(NH_3)_5$]$(BF_4)_3$ was coupled with R–D-Ala. Although yields in the last step of the two methods are comparable, the overall yield of [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$ in method A (41% based on $[(NH_3)_5CoOH_2](ClO_4)_3$) is twice that in method B (20% based on D-Ala) because of the overall higher yield in the first two steps of the preferable method A.

We have synthesized [R–AIBA–$Co(NH_3)_5$]$(BF_4)_2$, another precursor of MAG_3 analogues, by two methods. In method A (Scheme 4), SucBTG–AIBA was coupled with $[(NH_3)_5CoOH](ClO_4)_2$ and in method B (Scheme 5), SucBTG was coupled with [AIBA–$Co(NH_3)_5$]$(BF_4)_3$. The overall yield in method A (53% based on $[(NH_3)_5CoOH](ClO_4)_2$) is about three times higher than that in method B (18% based on AIBA). As yields in the last step of the two methods are comparable, this difference arises because of the low yield in both the first and second steps of method B. Therefore, method A is certainly preferred.

Synthesis of R–D-Ala–Gly-Gly

The treatment of [R–D-Ala–Gly-Gly–$Co(NH_3)_5$]$(BF_4)_2$, obtained by method A or B (Scheme 1 or 2, respectively), with $NaBH_4$ gave a 56% yield of R–D-Ala–Gly-Gly. The overall yield (23% based on $[(NH_3)_5CoOH_2](ClO_4)_3$ in method A and 11% based on D-Ala in method B) in this multiple-step synthesis is several times higher than that in the one-step synthesis of R–L-Ala–Gly-Gly from the tripeptide.

From the above comparison it is clear that for the synthesis of [R–Y–$Co(NH_3)_5$]$(BF_4)_2$ compounds, methods making maximum use of the $(NH_3)_5Co^{III}$ group produce higher yields. Furthermore, our yields are comparable to those reported by Isied and coworkers [18–20, 24] for the synthesis of peptides or peptide–cobalt complexes of comparable chain length. Thus, our work confirms the versatility of the $(NH_3)_5Co^{III}$ group in peptide synthesis and provides an alternative approach for the synthesis of sterically hindered MAG_3 analogues in high yield.

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