Comparison of the Labelling Characteristics of Mercaptoacetyltriglycine (MAG3) with different S-Protective groups.

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SUMMARY

A number of different thiol protective groups have been synthesized and attached to mercaptoacetyltriglycine (MAG3) ligand. The newly made MAG3 analogues were labelled with ^{99m}Tc by direct labelling under alkaline condition and by stannous tartrate exchange labelling method. In the latter method, the amount of the ligand, reaction temperature and pH varied and their effects on the labelling efficiencies were studied.

Radiochemical purities of 51% to 70% , 58% to 75% and 46% to 81% respectively, were obtained by radio-HPLC analysis for the studied MAG3 precursors when, 0.1 mg, 0.4 mg and 1.6 mg of the ligand was used and labelling was performed at both low temperature (70°C) and pH (pH 3). All the studied ligands were efficiently labelled with ^{99m}Tc (up to 99%) when heated for 10 min at pH 9 and 100°C. The labelling efficiency obtained by the direct labelling method for MAG3 analogues varied from 32% to 94% and was in all cases

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CCC 0362–4803/97/010853–22$17.50

lower than after the exchange labelling at pH 9 and at 100°C. It was observed that the radiochemical purities can be improved significantly by heating the "direct labelling mixture" at elevated temperature.

Key Words : Mercaptoacetyltriglycine , thiol protective groups ,
labelling with technetium-99m

INTRODUCTION

Technetium-99m mercaptoacetyltriglycine (^{99m}Tc-MAG3) is currently the radiopharmaceutical of choice for routine renal function studies. The instability of the sulphydryl group compromises the synthesis of MAG3 ligand in a highly pure form and is an obstacle to prolonged storage. Therefore, the thiol group of mercaptoacetyltriglycine is usually protected by a suitable group. An S-protected precursor is used in commercially available kits for the convenient preparation of ^{99m}Tc-MAG3 in hospitals. This S-benzoyl group prevents the rapid oxidation of the thiol function during chemical synthesis and storage of MAG3 ligand, and it can easily be removed to a sufficient degree during the exchange labelling process at elevated temperature.

During this labelling procedure, the S-protected MAG3 is heated for 5 to10 minutes at 100°C with a mixture of stannous chloride, tartaric acid and [99m]-pertechnetate solution. Pertechnetate (valence of Tc = +7) is reduced by the stannous ions to an oxotechnetium core (TcO^{3+}, valence of Tc = +5) which is weakly bound by tartrate. Meanwhile a fraction of the S-benzoyl protective groups are split off by the heating to generate MAG3. This exchange reaction is also promoted by the high temperature. The unprotected SN3 tetraligand has much stronger chelating properties than tartrate and likely withdraws technetium from its weak ^{99m}Tc-tartrate complex to form ^{99m}Tc-MAG3 (Fig. 1).

The benzoylmercaptoacetyl thio-ester group is not ideal for the study of different derivatives of MAG3. It is also relatively unstable and the synthesis of S-benzoyl protected derivatives has often been found to be very difficult or unsuccessful. An ideal S-protective group for the study of derivatives of MAG3 which may require relatively complex synthesis

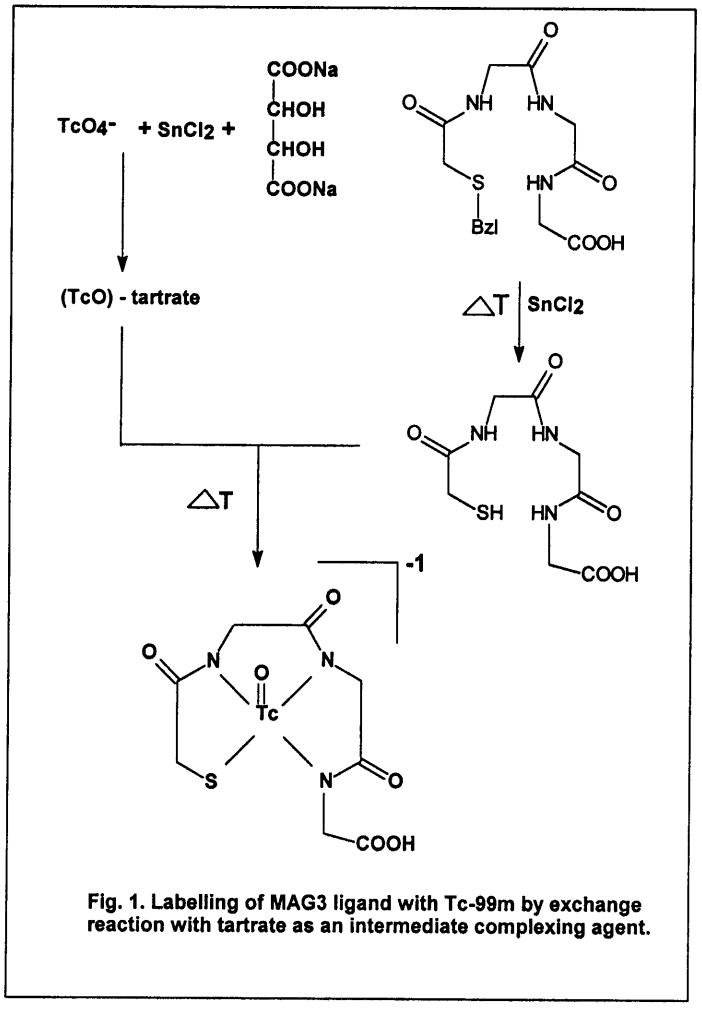

Fig. 1. Labelling of MAG3 ligand with Tc-99m by exchange reaction with tartrate as an intermediate complexing agent.

procedures, should be stable during chemical synthesis and withstand the reagents used for removal of amine or carboxylate protective groups, e.g. CBZ, BOC, etc. In addition, it should also be sufficiently removable, either prior to, or during the radiolabelling process. A limited number of alternative sulphydryl protective groups for MAG3 have already been described, namely the acetyl, benzyl and benzamidomethyl groups (1,2).

Besides its usefulness as a renal imaging agent, recently MAG3 is also being used as a potential chelating moiety for coupling of peptides and antibodies (2-5). This study was also undertaken to find a model thiol protective group for MAG3 which can be remove

under relatively mild labelling conditions i.e., at room temperature and close to neutral pH during radiolabelling process. These mild conditions are particularly important when labelling certain bioactive peptides which can not withstand harsh labelling conditions which might alter the biological properties of the particular peptide. We have also studied ^{99m}Tc-MAG3 as a potential bifunctional ligand for coupling of peptides, the results of which will be presented in a separate paper.

In this study we have synthesized and attached a number of different S-protective groups, mainly originating from peptide chemistry, to MAG3 in order to compare the labelling characteristics of the resulting MAG3 precursors. Comparisons have been made between direct labelling and exchange labelling using tartrate as the intermediate complexing agent.

In the latter labelling method, the amount of ligand, the heating time, reaction temperature, and the pH were varied to study their effect on labelling efficiency. The suitability of the respective labelling groups was evaluated by determining the relative amount of ^{99m}Tc-MAG3 in the reaction mixtures by RP-HPLC analysis.

Chemistry

The S-protective groups used in this study for the synthesis of intermediates and thiol protected MAG3 analogues are summarized in scheme 1.

S-Benzoylmercaptoacetyltriglycine (3a) was synthesized in two ways: reaction of triglycine with N-hydroxysuccinimidyl benzoylthioglycolate as described by Schneider *et al.* (6) or the reaction of triglycine with chloroacetyl chloride followed by treatment with sodium thiobenzoate as reported by Fritzberg *et al.* (7). S-Benzyl-MAG3 (3b) was prepared by reacting S-benzylmercaptoacetyl chloride with triglycine under Schotten-Baumann conditions.

To synthesize the other thiol protected MAG3 analogues, mercaptoacetic acid was first converted to the required S-protected derivative (1c to 1j), then reacted with N-hydroxysuccinimide (NHS) and 1,3-dicyclohexyl carbodiimide (DCC) to form the active ester (2c to 2j), which finally was coupled with triglycine (3c to 3j ; Scheme 1).

R	name	R-S-CH_2COOH (1)	NHS, DCC → R-S-CH_2-COOSuc (2)	Triglycine → R-S-CH_2CO-(gly)$_3$ (3)
C_6H_5-CO-	benzoyl	1a	2a	3a
C_6H_5-CH_2-	benzyl	1b	2b	3b
C_6H_5-CO-NH-CH_2-	benzamidomethyl	1c	2c	3c
CH_3-CO-NH-CH_2-	acetamidomethyl	1d	2d	3d
CH_3-CO-	acetyl	1e	2e	3e
CH_3-(OC_2H_5)CH-	1-ethoxyethyl	1f	2f	3f
C_6H_9O-	tetrahydropyranyl	1g	2g	3g
CH_3O-C_6H_4-CH_2-	p-methoxybenzyl	1h	2h	3h
(C_6H_5)$_2$-CH-	diphenylmethyl	1i	2i	3i
(C_6H_5)$_3$C-	triphenylmethyl	1j	2j	3j

Scheme 1. Synthesis of different thiol protected MAG3 analogues.

For the coupling of benzamidomethanol and mercaptoacetic acid in order to prepare benzamidomethylmercaptoacetic acid (1c), the method of Papsuevich *et al.* (8) was used. Acetamidomethyl mercaptoacetic acid (1d) was synthesized in the same manner.

S-Acetyl-2-mercaptoacetic acid (1e), and its activated ester (2e), are commercially available or can be prepared by published methods (12,13).

1-Ethoxyethylmercaptoacetic acid (1f) was obtained by condensation of ethyl vinyl ether and mercaptoacetic acid with p-toluenesulphonic acid as a catalyst (9,14).

Tetrahydropyranylmercaptoacetic acid (1g) was prepared by reaction of dihydro- 2H-pyran and mercaptoacetic acid according to the method of Gustavson *et al.* (15).

p-Methoxybenzyl chloride, a necessary reagent for the preparation of ethyl p-methoxybenzylmercaptoacetate and the corresponding acid (1h), was obtained from p-methoxybenzyl alcohol and thionyl chloride in 80% yield (16).

The synthesis of diphenylmethylmercaptoacetic acid (1i) (yield: 68%) followed the scheme used for the preparation of the corresponding triphenylmethyl compound (1j) (17), i.e., reaction of the alcohol with mercaptoacetic acid in the presence of trifluoroacetic acid. The latter could also be prepared from the same reagents using acetic acid and boron trifluoride etherate in a yield of 54% (18).

EXPERIMENTAL PART

Materials and Methods

Technical grade chloroform and dichloromethane were distilled from phosphorus pentoxide before use. Commercially available chemicals were of reagent grade and were used without purification except for S-benzylthioglycolic acid (which was recrystallized twice from ethyl acetate) and mercaptoacetic acid (fractionated under nitrogen, bp. 96°C, 5 mm). Triglycine was obtained from Fluka Chemie AG, (Buchs, Switzerland).

Melting points were determined using open capillaries with a Büchi-Tottoli apparatus and were uncorrected.

Analytical thin-layer chromatography (TLC) was carried out using precoated TLC silica gel plates (Merck, 60F-254). Compounds were detected with U.V. light at 254 nm, or by exposing the TLC chromatograms to iodine vapours.

Column chromatography was performed on silica gel 60 Merck (230-400 mesh). Solvents were passed through the column by suction with a water pump. Gradient elution was always used and was guided and monitored by TLC.

^1H nuclear magnetic resonance (NMR) spectra were obtained with a Jeol FX-90Q spectrometer using DMSO-d_6 as solvent with $Si(CH_3)_4$ as an internal standard. Infrared spectra (IR) were acquired using potassium bromide pellets on a Nicolet Magna-IR® 550 spectrometer. Elemental analyses were within ± 0.4% of theoritical values for all elements listed, unless otherwise stated.

Labelling of the different S-protected derivatives of MAG3 ligand with ^{99m}Tc was carried out either by the stannous tartrate exchange method or by the direct labelling method. In the exchange labelling method, the ligand (0.1, 0.4 or 1.6 mg) was dissolved in 0.5 mL of phosphate buffer 0.5M (pH 3 or pH 9), followed by the addition of 15 mg of sodium tartrate, 100 μg $SnCl_2.2H_2O$ in 25 μL of 0.05N hydrochloric acid and 1 to 3 mL eluate of a commercial generator (Ultratechnekow FM™, Mallinckrodt Medical, Holland) containing 370 to 740 MBq (10 to 20 mCi) ^{99m}Tc in the form of sodium pertechnetate. Finally the reaction mixture was heated in a water-bath for 5 min at 70°C, or 10 min at 100°C and cooled to room temperature. For the direct labelling, 1.6 mg of the ligand was mixed with 0.5 mL of 0.5M phosphate buffer pH 12, followed after 15 min by the addition of 100 μg of $SnCl_2.2H_2O$ and 1 mL of $^{99m}TcO_4^-$ (370 MBq). The reaction mixture was kept at room temperature for 1 hour before HPLC analysis.

Analyses of the radiolabelled reaction mixtures were performed by reverse phase HPLC on a column filled with Hypersil™ 5 μm ODS (Shandon) and eluted with a ternary gradient mixture of ethanol, 0.025 M phosphate buffer pH 5.85 and water at a flow rate of 1 mL / min. From 0 min to 20 min, a mixture of phosphate buffer (A) and ethanol (B) was used

in the following concentration: 0% to 50% B. From 20.1 min to 30 min, a mixture of water (C) and ethanol (B) (50% to 90% B) was used to avoid the precipitation of phosphate salts at high ethanol concentrations. The radio-HPLC system consisted of a Merck-Hitachi L 6200 intelligent pump, a Valco N6 injector and a 250 mm x 4.6 mm column. 25 μL of the reaction mixture after labelling with ^{99m}Tc was applied on the column. Radioactivity in the column effluent was monitored by a 2 inch NaI (Tl) scintillation detector coupled via a single channel analyzer to a Rachel- 5 - LS integrator (Raytest).

Synthesis of the S-protected MAG3 analogues

Benzylmercaptoacetyltriglycine (3b)

A solution of triglycine (5.01 g, 26.5 mmol) in water (80 mL) was adjusted to pH 10 with 2N sodium hydroxide solution. The mixture was cooled to 0°C and stirred vigorously during the dropwise addition of S-benzylmercaptoacetyl chloride (5.83 g, 29.1 mmol ; obtained by refluxing S-benzylmercaptoacetic acid (1b) with thionyl chloride followed by distillation at reduced pressure, yield: 93%) in dioxane (100 mL). The pH of 10 was maintained by the addition of 2N sodium hydroxide solution. The mixture was stirred at room temperature for 3 h and then acidified to pH 2 with 2N hydrochloric acid. Evaporation of the solvents resulted in the isolation of the product which was recrystallized from ethanol.

Yield: 4.44 g (47%); mp: 185-189°C; TLC: chloroform-methanol-acetic acid 60:40:1; R_f: 0.7; ^{1}H NMR (DMSO-d_6): δ 3.2 (s, 2H, SC$\underline{H_2}$CO), 3.8 (s, 2H, C$\underline{H_2}C_6H_5$), 3.9 (s, 6H, 3xNHC$\underline{H_2}$CO), 7.5 (s, 5H, C_6H_5), 8.1-8.4 (m, 3H, 3 x CONH). IR (KBr): 1099, 1160, 1431, 1449, 1623, 1650, 1667, 2855, 2925, 2995-3538, 3031, 3301 cm^{-1} ; Elemental analysis calculated for $C_{15}H_{19}N_3O_5S$: C, 50.98; H, 5.42; N, 11.89; S, 9.07. Found: C, 51.11; H, 5.38; N, 11.74; S, 9.19.

Benzamidomethylmercaptoacetic acid (1c)

Benzamidomethanol (22.65 g, 150 mmol) and mercaptoacetic acid (15.20 g, 165 mmol) were dissolved in water (100 mL) and the solution was cooled to 0°C. To this solution

concentrated hydrochloric acid (100 mL) was added dropwise. The mixture was stirred for 30 min and the precipitate which formed was collected by filtration and recrystallized from hot water.

Yield: 27.0 g (80%); mp: 220-223°C; TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.6; ^{1}H NMR (DMSO-d_6): δ 3.3 (s, 2H, SC$\underline{H}_2$CO), 4.5 (d, 2H, NHC$\underline{H}_2$S), 7.3-7.8 (m, 5H, C_6H_5), 9.2 (t, 1H, C_6H_5CON$\underline{H}$CH$_2$), 10.3 (s, 1H, COOH). Elemental analysis calculated for $C_{10}H_{11}NO_3S$: C, 53.32; H, 4.92; N, 6.22; S, 14.23. Found: C, 53.47; H, 5.19; N, 6.10; S, 14.29.

N-Hydroxysuccinimidyl S-benzamidomethyl-2-mercaptoacetate (2c)

Benzamidomethylmercaptoacetic acid (1c) (2.25 g, 10 mmol) and NHS (1.15 g, 10 mmol) were dissolved in tetrahydrofuran (50 mL). A solution containing DCC (2.15 g, 10.5 mmol) in tetrahydrofuran (10 mL) was added to the mixture and stirring was continued at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration, washed with hot tetrahydrofuran and the filtrate was evaporated. The active ester was purified by recrystallization from ethyl acetate.

Yield: 2.35 g (73%); mp: 101-103°C; TLC: chloroform-acetonitrile 90:10; R_f: 0.7; ^{1}H NMR (DMSO-d_6): δ 2.7 (s, 4H, C$\underline{H}_2$-C$\underline{H}_2$), 3.2 (s, 2H, SC$\underline{H}_2$CO), 4.5 (d, 2H, NHC$\underline{H}_2$S), 7.2-7.8 (m, 5H, C_6H_5), 9.3 (t, 1H, C_6H_5CON$\underline{H}$CH$_2$). Elemental analysis calculated for $C_{14}H_{14}N_2O_5S$: C, 52.17; H, 4.38; N, 8.69; S, 9.95. Found: C, 52.11; H, 4.45; N, 8.79; S, 9.99.

Benzamidomethylmercaptoacetyltriglycine (3c)

A solution of the active ester (2c) (1.60 g, 5 mmol) in acetonitrile (25 mL) was added to triglycine (0.95 g, 5 mmol) in 1N sodium hydroxide (5 mL). After 1 h, the pH of the solution was adjusted to pH 2 with 1N hydrochloric acid. The resulting precipitate was collected by filtration and recrystallized from acetone-water.

Yield: 1.41 g (72%); mp: 192-194°C; TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.5; ^{1}H NMR (DMSO-d_6): δ 3.3 (s, 2H, SC$\underline{H}_2$CO), 3.6-3.9 (m, 6H, 3xCOC$\underline{H}_2$NH), 4.5 (d, 2H, NHC$\underline{H}_2$S), 7.3-8.0 (m, 5H, C_6H_5), 8.1-8.5 (m, 3H, 3xCON$\underline{H}$CH$_2$), 9.2 (t, 1H, C_6H_5CON$\underline{H}$CH$_2$). IR (KBr): 776, 1222, 1239, 1396, 1422, 1466, 1536, 1580, 1611, 1650,

1676, 2925, 3030, 3162-3493, 3319 cm^{-1}; Elemental analysis calculated for $C_{16}H_{20}N_4O_6S$: C, 48.47; H, 5.09; N, 14.14; S, 8.08. Found: C, 48.56; H, 4.99; N, 14.33; S, 8.11.

Acetamidomethylmercaptoacetic acid (1d)

Acetamidomethanol (44.5 g, 500 mmol) and mercaptoacetic acid (50.6 g, 550 mmol) were dissolved in water (330 mL) and the solution was cooled to 0°C. Concentrated hydrochloric acid (330 mL) was added dropwise and the reaction mixture was cooled in the refrigerator for 72 h. The solution was evaporated and the resulting semi-solid mass was purified by silica gel column chromatography using a gradient of acetone in dichloromethane-acetic acid (0.5%). The product was recrystallized from ethyl acetate - petroleum ether.

Yield: 9.0 g (11%); TLC: chloroform-methanol-acetic acid 60:40:1; R_f: 0.7; ^1H NMR (DMSO-d_6): δ 2.2 (s, 3H, CH_3CO), 3.3 (s, 2H, SCH_2CO), 4.4 (d, 2H, SCH_2NH), 6.2 (t, 1H, $CH_3CONHCH_2$), 11.3 (s, 1H, COOH). Elemental analysis calculated for $C_5H_9NO_3S$: C, 36.80; H, 5.56; N, 8.59; S, 19.62. Found: C, 36.99; H, 5.63; N, 8.74; S, 19.71.

N-Hydroxysuccinimidyl S-acetamidomethyl-2-mercaptoacetate (2d)

The active ester (2d) was obtained by reaction of acid (1d) (3.26 g, 20 mmol) with NHS (2.53 g, 22 mmol) using DCC (4.22 g, 20.5 mmol) in tetrahydrofuran (30 mL), followed by the recrystallization from ether-petroleum ether.

Yield: 1.9 g (36%); TLC: chloroform-acetonitrile 90:10; R_f: 0.8; ^1H NMR (DMSO-d_6): δ 2.3 (s, 3H, CH_3CO), 2.8 (s, 4H, CH_2-CH_2), 3.2 (s, 2H, SCH_2CO), 4.5 (d, 2H, SCH_2NH), 6.2 (t, 1H, $CH_3CONHCH_2$). Elemental analysis calculated for $C_9H_{12}N_2O_5S$: C, 41.53; H, 4.65; N, 10.76; S, 12.32. Found: C, 41.65; H, 5.53; N, 11.01; S, 12.38.

Acetamidomethylmercaptoacetyltriglycine (3d)

N-Hydroxysuccinimidyl ester (2d) (1.30 g, 5 mmol) in acetonitrile (50 mL) and triglycine (0.95 g, 5 mmol) in 1N sodium hydroxide (7.5 mL) and water (10 mL) were mixed together and stirred for 18 h. Evaporation of acetonitrile and acidification of the residue to pH 2 resulted in the formation of a precipitate, which was collected by filtration.

Yield: 0.72 g (43%); mp: 238-240°C (dec); TLC: chloroform-methanol-acetic acid 60:40:1;

R_f: 0.5; ^1H NMR (DMSO-d_6): δ 2.2 (s, 3H, CH_3CO), 3.1 (s, 2H, $COC\underline{H}_2S$), 3.8 (m, 9H, CH_3CO; 3xCH_2 glycine), 4.5 (d, 2H, $SC\underline{H}_2NH$), 6.2 (t, 1H, $CH_3CON\underline{H}CH_2$), 8.0-8.3 (m, 3H, 3x$CON\underline{H}CH_2$). IR (KBr): 1220, 1242, 1400, 1420, 1470, 1575, 1650, 1680, 2920, 3160-3490, 3320 cm^{-1} ; Elemental analysis calculated for $C_{11}H_{18}N_4O_6S$: C, 39.51; H, 5.43; N, 16.77; S, 9.59. Found: C, 39.47; H, 5.56; N, 16.65; S, 9.63.

Acetylmercaptoacetyltriglycine (3e)

N-Hydroxysuccinimidyl S-acetyl-2-mercaptoacetate (2e) (1.15 g, 5 mmol ; prepared from S-acetyl-2-mercaptoacetic acid (1e) and NHS by activation with DCC) in tetrahydrofuran (15 mL), was mixed with a solution of triglycine (0.95 g, 5 mmol) in 1N sodium hydroxide (7 mL) and water (13 mL). The mixture was stirred at room temperature for 48 h. After the evaporation of tetrahydrofuran and dilution with water (70 mL), the mixture was acidified to pH 2 with 2N hydrochloric acid solution. After cooling overnight in the refrigerator, the formed precipitate was filtered off.

Yield: 0.3 g (20%); mp 210°C (dec); TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.5; ^1H NMR (DMSO-d_6): δ 2.5 (s, 3H, CH_3CO), 3.7 (m, 8H, CH_2COS), 3xCH_2 glycine), 8.0-8.5 (bm, 3H, 3x $CON\underline{H}CH_2$). IR (KBr): 1224, 1235, 1395, 1416, 1462, 1581, 1648, 1673, 2922, 3158-3492 cm^{-1} ; Elemental analysis calculated for $C_{10}H_{15}N_3O_6S$: C, 39.34; H, 4.96; N, 13.77; S, 10.49. Found: C, 39.25; H, 5.11; N, 13.70; S, 10.56.

1-Ethoxyethylmercaptoacetyltriglycine (3f)

Triglycine (1.89 g, 10 mmol) in 1N sodium hydroxide (10 mL) was mixed with a solution of N-Hydroxysuccinimidyl S-1-ethoxyethyl-2-mercaptoacetate (2f) (2.61 g, 10 mmol) in acetonitrile (25 mL). The pH of the resulting solution was maintained at 8.5 with 1N sodium hydroxide solution. After stirring at room temperature for 30 min, TLC analysis indicated the complete formation of the protected peptide. The organic solvent was evaporated and the residue was diluted with water (100 mL), saturated with ammonium sulphate and acidified to pH 2 with 2N hydrochloric acid. The solution was then extracted with ethyl acetate (3 x 50 mL) and n-butyl alcohol (2 x 50 mL). The organic layer was dried over anhydrous sodium sulphate and evaporated.

Yield: 1.7 g (51%); mp: 222°C (dec); TLC: dichloromethane-acetone-acetic acid 60:40:1; R_f: 0.5; ^{1}H NMR (DMSO-d_6): δ 1.2 (t, 3H, $CH_2C\underline{H}_3$), 1.6 (d, 3H, $CHC\underline{H}_3$), 3.3 (s, 2H, $COC\underline{H}_2S$), 3.6 (q, 2H, $C\underline{H}_2CH_3$), 3.8 (m, 6H, 3xCH_2 glycine), 4.9 (q, 1H, $C\underline{H}CH_3$), 8.0-8.5 (b, 3H, $CON\underline{H}CH_2$). IR (KBr): 1105, 1163, 1430, 1447, 1655, 1670, 2852, 2929, 2990-3535, 3300 cm^{-1} ; Elemental analysis calculated for $C_{12}H_{21}N_3O_6S$: C, 42.97; H, 6.32; N, 12.54; S, 9.55. Found: C, 43.11; H, 6.38; N, 12.47; S, 9.61.

Tetrahydropyranylmercaptoacetyltriglycine (3g)

N-Hydroxysuccinimidyl S-tetrahydropyran-2-mercaptoacetate (2g) (1.37 g, 5 mmol) in acetonitrile (30 mL) was added to triglycine (0.95 g, 5 mmol) dissolved in 1N sodium hydroxide (7 mL) and water (10 mL). The mixture was stirred at room temperature overnight and the organic solvent was evaporated. The residue was diluted with water (40 mL), acidified to pH 2 with 1N hydrochloric acid and cooled in ice. A small amount of the precipitate was filtered off and the filtrate was evaporated to dryness. The oily residue was triturated with acetone-ether (1:3). The formed white product was filtered off and dried.

Yield: 1.25 g (72%); mp: 180-184°C (dec); TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.4; ^{1}H NMR (DMSO-d_6): δ 1.6 (m, 6H, 3,4,5 CH_2), 3.3 (s, 2H, $SC\underline{H}_2CO$), 3.5 (t, 2H, $OC\underline{H}_2$), 3.7-3.9 (m, 6H, 3xCH_2 glycine), 5.7 (t, 1H, $OC\underline{H}CH_2$), 8.3 (m, 3H, 3x$CH_2N\underline{H}$ CO). IR (KBr): 1098, 1158, 1433, 1454, 1622, 1653, 1672, 2856, 2930, 2999-3538, 3030, 3296 cm^{-1} ; Elemental analysis calculated for $C_{13}H_{21}N_3O_6S$: C, 44.95; H, 6.10; N, 12.10; S, 9.23. Found: C, 45.14; H, 5.92; N, 12.22; S, 9.29.

p-Methoxybenzylmercaptoacetic acid (1h)

Ethyl 2-mercaptoacetate (52.20 g, 434 mmol) was added to a solution of sodium ethoxide, prepared from sodium (9.98 g, 434 mmol) in absolute ethanol (500 mL), followed by the addition of p-methoxybenzyl chloride (67.97 g, 434 mmol) in absolute ethanol (100 mL). The mixture was refluxed overnight and the precipitated sodium chloride was removed by filtration. After evaporation of the filtrate, the oily residue was taken up in ether (250 mL) and filtered to remove the last traces of sodium chloride. The solvent was removed by evaporation, and the ethyl ester was obtained as a colourless oil.

Yield: 98.6 g (94%); TLC: Chloroform; R_f: 0.7

The ethyl ester was dissolved in a mixture of methanol (200 mL) and 2N sodium hydroxide (210 mL). The solution was stirred at room temperature for 1 h, at which time TLC indicated the complete formation of the acid. The methanol was evaporated, the residue was diluted with water (300 mL), acidified to pH 2 and the resulting precipitated product was collected.

Yield: 73.4 g (84%); mp: 60-62°C; TLC: chloroform-acetone-acetic acid 90:10:1; R_f: 0.7; ^{1}H NMR (DMSO-d_6): δ 3.2 (s, 2H, $COC\underline{H_2}S$), 3.7 (s, 2H, $SC\underline{H_2}C_6H_4OCH_3$), 3.8 (s, 3H, CH_3O), 6.8-7.4 (m, 4H, C_6H_4), 11.4 (s, 1H, COOH). Elemental analysis calculated for $C_{10}H_{12}O_3S$: C, 56.59; H, 5.70; S, 15.09. Found: C, 56.70; H, 5.83; S, 15.19.

N-Hydroxysuccinimidyl p-methoxybenzylmercaptoacetate (2h)

To a solution of p-methoxybenzylmercaptoacetic acid (5.32 g, 25 mmol) and NHS (3.45 g, 30 mmol) in acetonitrile (30 mL), was added DCC (5.36 g, 26 mmol) in acetonitrile (10 mL). The reaction mixture was stirred at room temperature overnight, then filtered and evaporated. The residual oil was crystallized from ether-petroleum ether.

Yield: 7.3 g (94%); TLC: chloroform-acetonitrile 90:10; R_f: 0.6; ^{1}H NMR (DMSO-d_6): δ 2.7 (s, 4H, $C\underline{H_2}$-$C\underline{H_2}$), 3.2 (s, 2H, $COC\underline{H_2}S$), 3.7 (s, 2H, $SC\underline{H_2}C_6H_4OCH_3$), 3.8 (s, 3H, CH_3O), 7.0-7.2 (m, 4H, C_6H_4). Elemental analysis calculated for $C_{14}H_{15}NO_5S$: C, 54.36; H, 4.89; N, 4.53; S, 10.34. Found: C, 54.30; H, 5.11; N, 4.60; S, 10.39.

p-Methoxybenzylmercaptoacetyltriglycine (3h)

The active ester (2h) (1.55 g, 5 mmol) in acetonitrile (50 mL) was added to triglycine (0.95 g, 5 mmol) in 1N sodium hydroxide (7 mL) and water (10 mL). After stirring overnight followed by evaporation of the acetonitrile, the product was precipitated at pH 2 and then collected by filtration.

Yield: 1.25 g (65%); mp: 197-201°C (dec); TLC: chloroform-acetone-acetic acid 80:20:1; R_f: 0.6; ^{1}H NMR (DMSO-d_6): δ 3.1 (s, 2H, $COC\underline{H_2}S$), 3.7-3.9 (m, 11H, $SC\underline{H_2}C_6H_4O\underline{CH_3}$; 3x$CH_2$ glycine), 6.8-7.4 (m, 4H, C_6H_4), 8.3 (m, 3H, 3 x NHCO). IR (KBr): 1103, 1156, 1432, 1448, 1618, 1649, 1666, 2858, 2929, 3000-3541, 3033, 3302 cm^{-1}; Elemental analysis

calculated for $C_{16}H_{21}N_3O_6S$: C, 50.12; H, 5.52; N, 10.97; S, 8.35. Found: C, 50.03; H, 5.65; N, 11.11; S, 8.30.

Diphenylmethylmercaptoacetyltriglycine (3i)

A solution of N-Hydroxysuccinimidyl diphenylmethylmercaptoacetate (0.41 g, 1.2 mmol) (a solution of benzhydrol and mercaptoacetic acid in trifluroacetic acid afforded diphenylmethylmercaptoacetic acid, from which the N-Hydroxysuccinimidyl active ester was prepared via DCC) in tetrahydrofuran (10 mL) was added to triglycine (0.218 g, 1.2 mmol) in 1N sodium hydroxide (1.5 mL) and water (8 mL). The reaction mixture was stirred overnight at room temperature and the organic solvent was removed by evaporation. The aqueous residue was acidified to pH 2 and the resulting precipitate was collected by filtration.

Yield: 0.25 g (50%); mp: 145-150°C; TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.7; ^{1}H NMR (DMSO-d_6): δ 3.1 (s, 2H, COC$\underline{H_2}$S), 3.7-3.9 (m, 6H, 3 x CH_2 glycine), 5.5 (s, 1H, CH), 7.4 (m, 10H, 2 x C_6H_5), 8.3 (m, 3H, 3 x NHCO). IR (KBr): 1100, 1165, 1429, 1455, 1620, 1647, 1670, 2850, 2921, 3000-3540, 3035, 3305 cm^{-1} ; Elemental analysis calculated for $C_{21}H_{23}N_3O_5S$: C, 58.72; H, 5.40; N, 9.79; S, 7.45. Found: C, 58.97; H, 5.29; N, 9.70, S, 7.41.

Triphenylmethylmercaptoacetyltriglycine (3j)

A solution of N-Hydroxysuccinimidyl triphenylmethylmercaptoacetate (17,18) (1.5 g, 3.5 mmol) in acetonitrile (12 mL) and tetrahydrofuran (15 mL) was added to triglycine (0.662 g, 3.5 mmol) in 1N sodium hydroxide (5 mL) and water (10 mL). After stirring for two days at room temperature, the organic solvents were evaporated. The peptide was obtained from the aqueous layer by acidification to pH 2.

Yield: 0.5 g (28%); mp: 165-170°C (dec); TLC: chloroform-methanol-acetic acid 70:30:1; R_f: 0.6; ^{1}H NMR (DMSO-d_6): δ 2.9 (s, 2H, COC$\underline{H_2}$S), 3.7-3.9 (m, 6H, 3xCH_2 glycine), 7.3 (s, 15H, 3xC_6H_5), 8.2 (m, 3H, 3xNHCO). IR (KBr): 1095, 1161, 1433, 1450, 1622, 1651, 1668, 2856, 2924, 2996-3543, 3033, 3304 cm^{-1} ; Elemental analysis calculated for

$C_{27}H_{27}N_3O_5S$: C, 64.14; H, 5.39; N, 8.32; S, 6.33. Found: C, 63.92; H, 5.22; N, 8.47; S, 6.45.

RESULTS AND DISCUSSION

Results of the labelling experiments are summarized in table 1. For the preparation of ^{99m}Tc-MAG3, two labelling methods have been described by Fritzberg (8), both starting from the S-benzoyl protected MAG3 precursor. In the first method, S-benzoyl-MAG3 is heated at 95°C in the presence of sodium hydroxide, sodium dithionite and the generator eluate. The role of dithionite is to reduce the pertechnetate ions ($^{99m}TcO_4^-$) to pentavalent technetium in the form of a TcO^{3+} species which is then bound by deprotected MAG3 ligand, generated by heating. An advantage of the alkaline medium is that it prevents the precipitation of reduced technetium in colloidal form (TcO_2) and the subsequent oxidation of the deprotected thiol to a disulphide. However, it results also in a reaction mixture that can not be used as such for biological studies in view of the high pH and the presence of a relatively high percentage of radiochemical impurities.

The second labelling method, which provides a higher radiochemical yield, involves exchange labelling at neutral pH during which the technetium atom is transferred from the weak complexing agent gluconate to the in situ deprotected MAG3 ligand.

For this purpose, a solution of gluconate, $SnCl_2$, S-benzoyl MAG3 and sodium pertechnetate ($^{99m}TcO_4^-$) is heated for 5 min at 95°C. The commercially available cold kits of MAG3 (Mallinckrodt Medical, Holland) contain tartrate as an intermediate complexing agent together with S-benzoyl MAG3 and stannous chloride. Labelling can be achieved (yield : >95 %) by addition of the generator eluate and heating the labelling mixture for 10 min at 100°C.

An S-protected MAG3 precursor is preferentially labelled with ^{99m}Tc by heating the ligand in the presence of a weak complexing agent such as tartrate. Exchange of technetium from the ^{99m}Tc-tartrate complex towards the more stable thiol containing ligand can occur as soon as the thiol protecting group is removed and this is enhanced by heating the labelling mixture.

Protective group	Exchange Labelling: 5 min at 70°C. (pH 3) 0.1 mg	0.4 mg	1.6 mg	Exchange Labelling: 10 min at 100°C. (pH 9) 0.1 mg	0.4 mg	1.6 mg	Direct Labelling: 60 min at room temperature. (pH 12) 1.6 mg
S-Benzoyl	65	75	81	98	96	91	83
S-Benzyl	51	65	78	98	98	97	71
S-Benzamidomethyl	61	66	79	99	97	98	94
S-Acetamidomethyl	18	36	59	89	89	87	69
S-Acetyl	67	73	64	91	86	81	59
S-1-Ethoxyethyl	65	58	46	93	93	92	75
S-Tetrahydropyranyl	69	66	46	96	95	91	79
S-p-Methoxybenzyl	70	71	78	98	97	93	83
S-Diphenylmethyl	67	69	74	86	68	64	73
S-Triphenylmethyl	63	74	66	96	96	90	32

Table 1. Percentage of ^{99m}Tc-MAG3 in the labelling reaction mixtures.

Radio-HPLC analyses of the exchange labelling mixtures revealed, beside ^{99m}Tc-tartrate and ^{99m}Tc-MAG3, the presence of a radiochemical species eluting somewhat earlier on HPLC than ^{99m}Tc-MAG3.

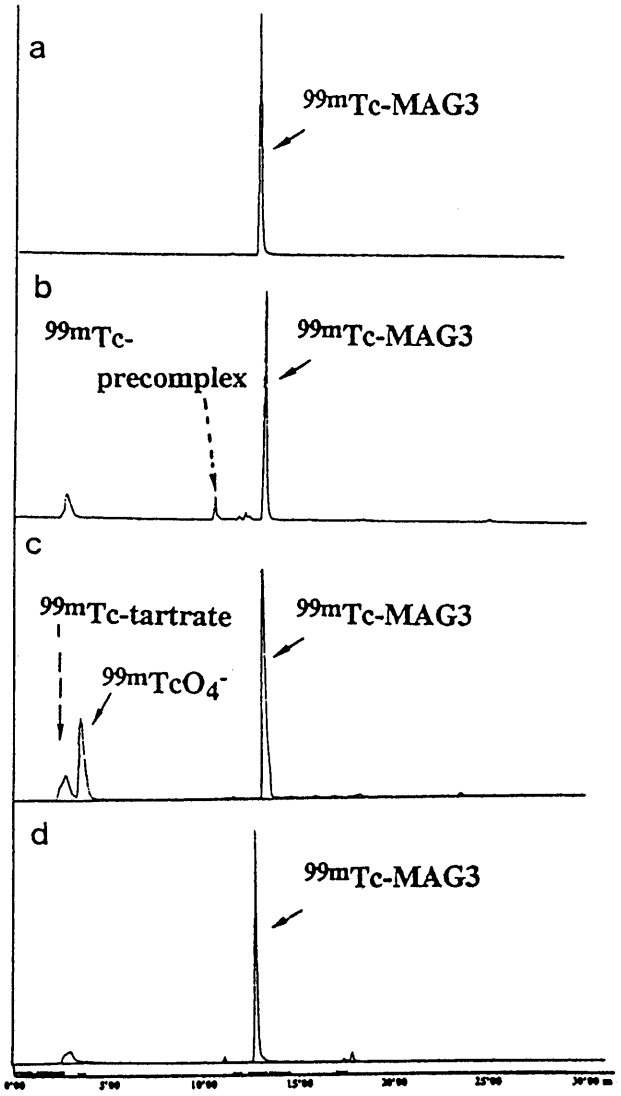

Fig. 2. Radio-HPLC chromatograms after exchange and direct labelling of S-protected MAG3 precursors.

a. S-Benzyl MAG3 (0.1 mg), labelled at pH 9, 100°C for 10 min.
b. S-Benzoyl MAG3 (0.4 mg), labelled at pH 3, 70°C for 5 min.
c. S-Acetamidomethyl MAG3 (1.6 mg), labelled at pH 3, 70°C for 5 min.
d. S-Diphenylmethyl MAG3 (1.6 mg), labelled at ambient temperature, pH 12 for 15 min.

Fig. 2. shows as an example the HPLC chromatograms of 4 experiments using a different S-protected MAG3 precursor in different labelling conditions. The presence of this species, so called ^{99m}Tc-precomplex, was generally more pronounced when the amount of the ligand was high, suggesting that in this type of radiochemical species, technetium is possibly bound to more than one ligand molecule. A relatively high amount of this precomplex (more than 8%) was observed when 1.6 mg of S-acetyl MAG3 (pH 3, 70°C) and S-ethoxyethyl MAG3 (pH 9, 100°C) respectively were used. The formation of precomplex was negligible (< 1.5 %) for all S-protected MAG3 precursors when 0.1 mg of the ligand was labelled at pH 9.

A relatively poor radiochemical purity was obtained when the labelling mixture was heated at low temperature (i.e., 5 min at 70°C). This was probably due to both insufficient removal of the protective groups and a slow and incomplete exchange rate. Radiochemical purities of 51% to 70% , 58% to 75% and 46% to 81% respectively, were obtained for the studied MAG3 precursors when 0.1 mg , 0.4 mg and 1.6 mg of the ligand was used and the labelling was performed at both low temperature (70°C) and pH (pH 3).

S-Acetamidomethyl MAG3 showed a low labelling yield (less than 20% and 40% when 0.1 mg and 0.4 mg respectively of ligand was used for exchange labelling). The reason for this poor labelling at 70°C is not yet clear, but probably this thio-ether is less easily split off than the others under these labelling conditions.

These results indicate that none of the protective groups permit high yield labelling (>95%) of typical MAG3-like ligands in relatively mild labelling conditions (70°C). Such conditions are required in some instances, e.g. when derivatives containing an activated carboxyl group have to be labelled (15). However, it appears also that for this purpose the S-benzoyl protective group is at least as suitable as other potential S-protective groups (e.g. S-benzyl, S-benzamidomethyl, p-methoxybenzyl, etc.) and therefore remains one of the attractive S-protective group when synthesis of the ligand is not compromised by its presence.

The pH of the labelling mixture can also influence the radiochemical purity as the amount of the residual ^{99m}Tc-tartrate is significantly lower for all protected ligands when the radiolabelling is performed at high pH. This is also true for radiolabelling reactions at 70°C (results not shown) at high pH. The binding of technetium-99m by MAG3 ligand requires both the deprotection of the MAG3 precursor and then the deprotonation of the sulphur and nitrogen atoms of the ligand. The process of deprotection is in each case pH dependent for the thio-ester precursors (S-benzoyl and S-acetyl) and probably also for the other groups. It seems logical that the deprotonation was also facilitated at alkaline pH and both deprotonation and deprotection will promote the formation of ^{99m}Tc-MAG3. Therefore, it is understandable that all tested ligands can be labelled efficiently when heated for 10 min at pH 9 and at 100°C. Hence the reason for using these standard labelling conditions in our investigation of new MAG3 derivatives.

From these results it is clear that the chemically very stable protective groups, i.e., the thio-ethers such as S-benzamidomethyl, S-benzyl and derivatives thereof, may be used without compromising the possibility to obtain a high labelling yield. The S-diphenylmethyl and S-triphenylmethyl protective groups are particularly useful for increasing the solubility of intermediates and final products in organic solvents. In this way the availability of a number of S-protective groups with differing chemical and physical properties enhances one's ability to obtain derivatives which require complex synthesis procedures.

Although a high labelling efficiency can be achieved by the exchange labelling procedure, the heating step may cause decomposition and formation of labelled impurities for some of the tested S-protected MAG3 analogues. In order to avoid these problems, an alternative and less aggressive method has been developed; i.e., the "direct labelling method."

In the direct labelling method, the MAG3-precursors are incubated at high pH for 15 min prior to the addition of the reducing agent ($SnCl_2$) and pertechnetate solution. During this incubation a very small amount of the S-protected ligand is converted to the form with a free thiol group. As only an extremely low mass of technetium has to be bound (370 MBq

^{99m}Tc is about 2 ng) a deprotection of less than 0.1% of the ligand molecules is generally sufficient to bind all ^{99m}Tc-atoms.

Labelling of the S-protected MAG3 precursors by the direct labelling method gave the same main radioactive species obtained by the exchange labelling, i.e., ^{99m}Tc-MAG3, but only if the pH of the labelling mixture was higher than 10. This strongly alkaline condition is necessary for the removal of a sufficient percentage of the S-protective group along with the removal of the amide protons during the chelation step. During the exchange labelling method this removal is achieved by thermal energy during the heating process.

Direct labelling yields, beside ^{99m}Tc-MAG3, different impurities (^{99m}Tc-pertechnetate, ^{99m}Tc-pre-complex, etc.). It was observed that these radiochemical impurities can be diminished to a great extent by heating the direct labelling mixture for 10 min at 100°C. In the direct labelling method, S-benzamidomethyl group exhibited superior labelling characteristics (up to 94%) compared to the other protective groups studied, when labelling with ^{99m}Tc is performed under alkaline conditions and at room temperature. The radiochemical purity obtained for the studied MAG3 precursors varied from 32% to 94% and was, in all cases lower than after exchange labelling at pH 9 and at 100°C. It was noted that the radiochemical purities can be improved significantly by heating the direct labelling mixtures at elevated temperature.

CONCLUSION

The results of this work demonstrate that all the MAG3 precursors studied may be labelled with ^{99m}Tc. Chemically unstable thio-ester precursors, such as S-benzoyl, remain one of the attractive groups in terms of the labelling characteristics when synthesis of the ligand is not compromised by its presence. It was found that chemically stable thio-ethers, like S-benzamidomethyl, S-benzyl and its derivatives, may be used without compromising a high labelling yield. The S-diphenylmethyl and S-triphenylmethyl protective groups are particularly useful for increasing the solubility of intermediates and final compounds in organic solvents. Thus, the availability of a number of useful thiol protective groups with

diverging physical and chemical properties, allow one to obtain derivatives which require complex synthetic procedures.

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