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Preparation of ^{99m}Tc-N_2S_2 conjugates of chrysamine G, potential probes for the beta-amyloid protein of Alzheimer's disease

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SUMMARY

Chrysamine G is known to bind *in vitro* to the β-amyloid protein (Aβ 10-43) and to homogenates of several regions of brain of Alzheimer's patients. The purpose of this study was to develop a ^{99m}Tc-labelled derivative of chrysamine G, in which the structural requirements for β-amyloid affinity are preserved.
Bis-S-trityl protected monoamide-monoaminedithiol (MAMA-Tr_2), a chelating system used to incorporate ^{99m}Tc, was coupled with 2-(chloroacetylamino)-chrysamine G diethyl ester (*4*) yielding 2-(MAMA-Tr_2-acetylamino)-chrysamine G diethyl ester (*5*-Tr_2). To prepare *4*, 4,4'-dinitro-2-biphenylamine was treated with chloroacetyl chloride to obtain 2-(chloroacetylamino)-4,4'-dinitrobiphenyl, of which the nitro functions were reduced by catalytic hydrogenation. Diazotation of 2-(chloroacetylamino)-4,4'-diaminobiphenyl, followed by coupling with ethyl salicylate provided *4* in an overall yield of 4.3 %. Alkaline hydrolysis of *5*-Tr_2 resulted in the monoethyl ester derivative (*6*-Tr_2) and diacid derivative (*7*-Tr_2). Detritylation and labelling with ^{99m}Tc was performed in the presence of Sn^{2+} and $^{99m}TcO_4^-$ solution at pH 2-3 with heating. RP-HPLC analysis showed one peak for both the diester derivative (^{99m}Tc-*5*) and the diacid derivative (^{99m}Tc-*7*), while two peaks (A and B) were present for the monoethyl ester derivative (^{99m}Tc-*6*), probably isomers with respect to the ester position. The order of elution from RP-HPLC reflected the lipophilicity of the ^{99m}Tc-complexes as determined by partitioning between 1-octanol and phosphate buffer pH 7.4 (log P: ^{99m}Tc-*5* = 2.15, ^{99m}Tc-6_A = 1.79, ^{99m}Tc-6_B = 1.93, ^{99m}Tc-*7* = 1.08).

Key-words: Chrysamine G, Technetium-99m, Beta-amyloid, Diagnosis, Alzheimer's disease

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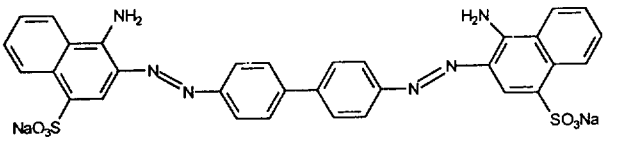
INTRODUCTION

Alzheimer's disease (AD) is a brain disorder characterised by a progressive dementia that occurs in middle or late life with a prevalence increasing with advancing age (1). Estimates reach as high as 40-50 % by ages 85-90 (2). Neuropathologically, the disease is characterised by degeneration of specific nerve cells and the presence of neuritic plaques and neurofibrillary tangles. Amyloid angiopathy is a constant accompaniment of AD, although its severity varies widely (3). The pathologic diagnostic criteria for AD, established by the National Institute of Health, are based on the number of senile plaques found in the brain (4). Senile plaques and cerebrovascular amyloid are composed of β-sheet fibrillar deposits of a 4-kD peptide, 39 to 43 amino acids in length, called beta-amyloid or Aβ protein (5,6,7). A histological dye used to visualise the amyloid deposits in AD is Congo red (Figure 1). The basis for the interaction of Congo red with β-amyloid is the presence of two positively charged amino acid residues (such as lysine-16 in Aβ protein) of two or more separate amyloid molecules of which the orientation is determined by virtue of the β-sheet fibril (8). The two anionic sulfonate groups of Congo red interact with these positively charged amino acid residues. Also the presence of the nearly planar biphenyl group in Congo red is supposed to be important for the hydrophobic interactions of the dye with β-amyloid (9). Chrysamine G (CG, Figure1), a derivative of Congo red that is more lipophilic and crosses the blood-brain barrier (BBB) in normal mice, has also affinity for β-amyloid (9). Recently, it was demonstrated that carbon-14 labelled CG binds to homogenates of several regions of AD brain as compared to control brain and the binding of ^{14}C-CG was correlated with the number of senile plaques and neurofibrillary tangles (10).

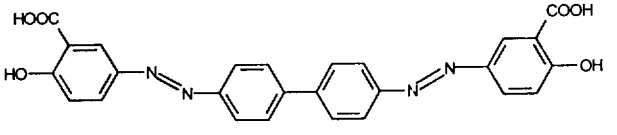
A method for a non-invasive *in vivo* quantification of amyloid deposits in AD brain could be the use of molecules with affinity for the amyloid deposits that are labelled with a γ-ray emitting radionuclide (SPECT). Attempts have been made to develop radiolabelled monoclonal antibodies as probes for *in vivo* imaging of Aβ protein (11,12,13,14). However, a drawback of antibodies is the poor diffusion of these large molecules across the BBB. So far, there is no convincing evidence of abnormalities in the BBB in AD that could enhance access of antibodies to the brain (15). Therefore, the use of small molecules that are relatively selective for Aβ protein and can readily cross a normal BBB may be the only way to monitor parenchymal deposition of amyloid. Recently, Han and

co-workers described a positively charged ^{99}Tc-complex of CG that binds to β-amyloid fibrils (Aβ 1-40) *in vitro* (16). The basic structure of this complex is the CG backbone in which the biphenyl moiety is replaced by a bipyridyl moiety capable of complexing ^{99}Tc. Mathis and co-workers described an iodine-125 labelled derivative of CG (3'-iodo CG) with a higher *in vitro* binding affinity for amyloid protein and a higher lipophilicity than the prototypical amyloid specific marker Congo red (17). Klunk and co-workers described X_{34} (1,4-bis(2-(3-carboxy-4-hydroxyphenyl)ethen-1-yl)-benzene), a fluorescent derivative of CG which is not an azo compound, but still has *in vitro* affinity for Aβ (18,19). To our knowledge, none of these compounds has been tested *in vivo* yet.

In this paper, we describe the development of a ^{99m}Tc-complex of CG, in which the biphenyl and acid moieties of CG are preserved and in which the CG backbone is coupled to a neutral ^{99m}Tc-complex.



Congo red



Chrysamine G

Figure 1. Structure of Congo red and chrysamine G

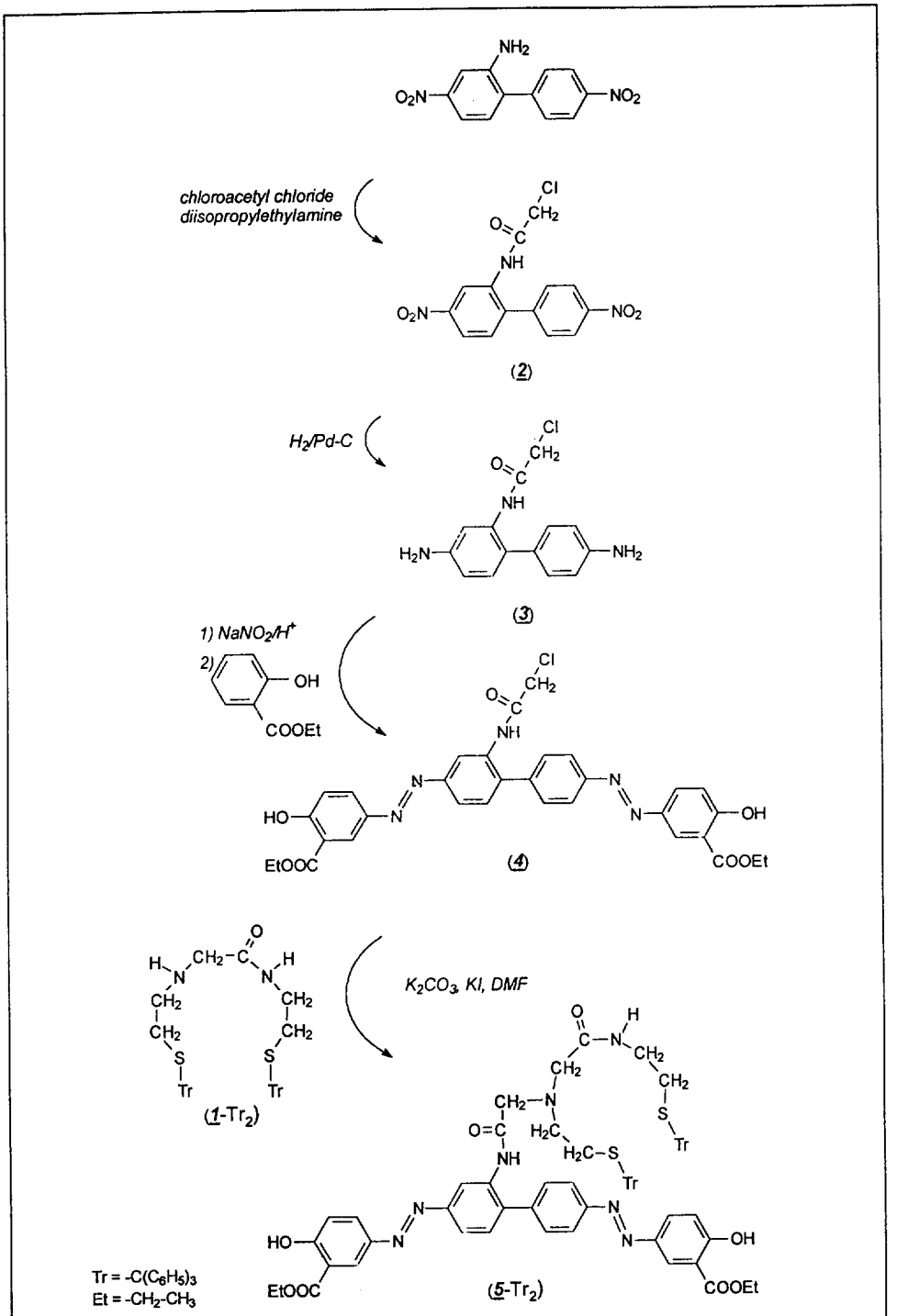
RESULTS AND DISCUSSION

Synthesis

To obtain a derivative of chrysamine G that can be labelled efficiently with ^{99m}Tc, we designed a strategy to synthesise a conjugate of CG and MAMA-Tr_2 (bis-S-trityl protected monoamide-monoaminedithiol, a ^{99m}Tc chelating agent). We first investigated the possibility to use a 2-amino derivative of CG and couple it to MAMA-Tr_2 via a

butyryl spacer. However, while 2-(γ-chlorobutyrylamino)-CG was successfully prepared (yield 12.2 %, unpublished results) we were unsuccessful in coupling it to MAMA-Tr_2 (*1*-Tr_2) using a procedure described by Kung and co-workers (20). Analysis of the reaction products demonstrated that under the slightly alkaline conditions cyclisation of the γ-chlorobutyryl spacer to a γ-lactam had occurred.

As an alternative, the acetyl moiety was chosen as a spacer because intramolecular cyclisation would not be possible (Scheme 1). This pathway, namely reaction of chloroacetyl chloride with 4,4'-dinitro-2-biphenylamine yielded the intended chloroacetyl derivative (*2*) in 61 % yield, with recuperation of 23 % of 4,4'-dinitro-2-biphenylamine. Whereas the above mentioned 2-(γ-chlorobutyrylamino)-4,4'-dinitrobiphenyl was successfully reduced with Sn/HCl to the 4,4'-diamino derivative, the N-chloroacetyl moiety of *2* was dehalogenated under these circumstances. Catalytic hydrogenation with H_2/Pd-C reduced the nitro functions without affecting the N-chloroacetyl moiety, although in moderate yields (24 %). One of the side products formed in minor quantities was 2,4,4'-triaminobiphenyl. Diazotation of 2-(chloroacetylamino)-4,4'-diaminobiphenyl (*3*), followed by coupling with ethyl salicylate (instead of salicylic acid as in the reported synthesis of CG (9)) provided 2-(chloroacetylamino)-chrysamine G diethyl ester (*4*) (yield 35%). The diethyl ester (*4*) was prepared to allow an easier and more efficient purification using column chromatography. 2-(Chloroacetylamino)-chrysamine G diethyl ester (*4*) was finally coupled (20) with MAMA-Tr_2 (*1*-Tr_2) resulting in 2-(MAMA-Tr_2-acetylamino)-chrysamine G diethyl ester (*5*-Tr_2) in a yield of 21 %. Acetylation of the non-reacted MAMA-Tr_2 with acetyl chloride was carried out to increase the difference in retention times of the conjugate and non-reacted bifunctional chelator on column chromatography, allowing a more efficient purification. The overall yield of the 4-step synthesis starting from 4,4'-dinitro-2-biphenylamine was 0.9 %. Alkaline hydrolysis of the ethyl esters of *5*-Tr_2 followed by preparative reversed phase HPLC yielded the monoethyl ester derivative *6*-Tr_2 in 27 % yield and the diacid derivative *7*-Tr_2 in 24 % yield.



Scheme 1. Synthesis of 2-(MAMA-Tr_2-acetylamino)-chrysamine G diethyl ester (5-Tr_2)

Deprotection and labelling with ^{99m}Tc

Standard deprotection methods for S-trityl groups consist of treating the compound in trifluoroacetic acid (TFA)/dichloromethane with triethylsilane and mercaptoethanol. Under appropriate acidic conditions triethylsilane can be considered as a hydride donor (24) that reacts with the trityl carbocation, making the deprotection irreversible and preventing the reverse reaction. 2-Mercaptoethanol is added to prevent the oxidation of the thiol groups during the work up of the deprotected ligand. Using MAMA-Tr_2 (*1*-Tr_2) as the test compound, it was found that residual triphenylmethane interfered with the labelling and therefore, it was removed by extraction. The resulting aqueous solution of MAMA (*1*) could then be labelled efficiently with ^{99m}Tc at pH 12 and room temperature by adding stannous ions and ^{99m}Tc-pertechnetate. However, whereas MAMA was successfully labelled under these conditions, the method failed for the MAMA-CG derivatives. RP-HPLC analysis of the solutions obtained after deprotection demonstrated the presence of a series of degradation products with UV absorbance at 254 and 380 nm. Therefore, an alternative deprotection and labelling method was developed. Instead of a two-step procedure, deprotection and labelling was performed in a single step. We envisaged that boiling the CG derivatives under mild acidic conditions would result in sufficient amounts of deprotected ligand to allow efficient labelling. Experimental evaluation of this hypothesis confirmed that under these deprotection conditions only limited deprotection without formation of a series of degradation products occurred. Although alkaline conditions are considered more favourable to label N_2S_2 bifunctional chelators, labelling of the partially deprotected mixture was nevertheless successfully performed at pH 2-3 in the presence of Sn^{2+} and ^{99m}Tc-pertechnetate at elevated temperature. Tartaric acid was added to prevent the formation of $^{99m}TcO_2$.
Once the ^{99m}Tc-complexes were formed, they remained stable for at least four hours in a pH range of 2-10. However, at pH 12 the ^{99m}Tc-complexes degraded to ^{99m}Tc-pertechnetate and a yet unknown hydrophilic compound.

Analysis

Reversed phase HPLC. Analysis of the labelled reaction mixtures of respectively *5*-Tr_2 and *7*-Tr_2 with RP-HPLC demonstrated a single main peak while labelling of *6*-Tr_2 resulted in two main peaks, A and B (in order of their elution from RP-HPLC), with a

ratio of peak B/peak A = 1.21. The separation of peak A and B could be optimised by adjusting the gradient system (system 2) (Figure 2).

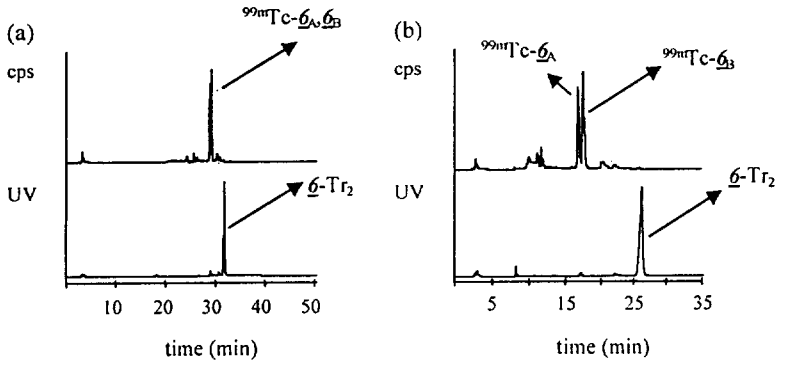


Figure 2. Chromatograms on RP-HPLC (C18) of ^{99m}Tc-MAMA-chrysamine G monoethyl ester (^{99m}Tc-6A and ^{99m}Tc-6B) and 2-(MAMA-Tr-acetylamino)-chrysamine G monoethyl ester (6-Tr2) (a = system 1A, b = system 2)

The order of elution from RP-HPLC reflected the lipophilicity of the compounds as determined by partitioning between 1-octanol and phosphate buffer (see further). ^{99m}Tc-MAMA eluted even before ^{99m}Tc-7 (Table 1). Decreasing the pH of the mobile phase to pH 2.5 increased the retention time of ^{99m}Tc-7 and of both components of ^{99m}Tc-6, indicating the presence of one or more acid functions in the ^{99m}Tc-complex. On the other hand, the retention time of the diethyl ester ^{99m}Tc-5 was not affected.

Formation of TcO complexes with mono-N-substituted N_2S_2 ligands can lead to four isomers (21). Two enantiomers (R and S) are formed from the quaternary amine nitrogen and two isomers (syn and anti) are possible by the orientation of the N-substituent relative to the Tc-oxygen bond. In general, TcO complexes with mono-N-substituted N_2S_2 ligands preferentially form the syn diastereoisomers (21). As we could observe only one peak for ^{99m}Tc-5 and ^{99m}Tc-7, it is likely that the two peaks of ^{99m}Tc-6 are structural isomers with respect to the ester position.

Octanol/buffer partition coefficient. The log P values for the partition of the ^{99m}Tc-complexes between 1-octanol and phosphate buffer pH 7.4 are listed in Table 1.

Table 1. Labelling yield, RP-HPLC (C18) retention times (t_R) and log (octanol/buffer partition coefficient) values (log P)

compound	*labelling yield*	*t_R, system 1_A (pH 7.0)*	*t_R, system 1_B (pH 2.5)*	*log P*
MAMA-Tr_2		34'19''		
5-Tr_2		38'28''	38'31''	
6-Tr_2		31'47''	33'48''	
7-Tr_2		28'57''	31'47''	
^{99m}Tc-*MAMA*	> 95 %	20'51''		0.56 (22)
^{99m}Tc-*5*	59 %	33'14''	33'19''	2.15
^{99m}Tc-6_A	67 %	28'51''	30'40''	1.79
^{99m}Tc-6_B		29'10''	30'55''	1.93
^{99m}Tc-*7*	75 %	26'12''	28'28''	1.08

EXPERIMENTAL

4,4'-Dinitro-2-biphenylamine was obtained from Aldrich Chemical Company. Residues of reaction mixtures were purified by column chromatography on silica gel with a particle size varying between 0.060 mm and 0.200 mm (Chromatographiegel C-gel C-560, CU Chemie Uetikon AG, Uetikon, Switzerland). Thin-layer chromatography (TLC) was carried out using precoated silica TLC plates (Alugram SIL G/UV_{254}, Macherey-Nagel, Düren, Germany). The structure of the synthesised ligands was confirmed with ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and ^{13}C-NMR on a Gemini 200 MHz or Unity 500 MHz spectrometer (Varian, Palo Alto, CA). Chemical shifts are reported in ppm relative to tetramethylsilane (δ=0). The splitting patterns are designed as follows: br s (broad singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). Assignments of the peaks were based mainly on literature values and by comparison with values of previous compounds in the synthetic chain. Protons in the salicyl moiety of the chrysamine G derivatives are indicated by the abbreviation 'SA'. For the ^{13}C-NMR, the assignments were confirmed by selective irradiations. Liquid secondary ion mass spectra (LSIMS) were recorded on a Kratos Concept IH mass spectrometer (Kratos Analytical, Manchester, UK) equipped with a Masspec II data system (MSS, Manchester, UK). Samples were dissolved in thioglycerol (thgly) or 3-nitrobenzylalcohol (nba). If necessary, the matrix was doped with sodium acetate (NaOAc). Spectra were obtained in positive (+LSIMS) or negative (-LSIMS) ion mode at 10 seconds/decade. The secondary ion acceleration voltage was set at + or – 7 kV.

Solutions were filtered through a 0.22-μm membrane filter containing a polysulfone HT Tuffryn® membrane (Acrodisc, Gelman Sciences, Ann Arbor, MI), except if they contained DMF, in which case 0.22-μm membrane filters containing a modified polyvinylidene fluoride membrane (Millex-GV, Millipore, Molsheim, France) were used. HPLC-systems consisted of a Merck-Hitachi ternary gradient pump (model L-6200 intelligent pump, Merck, Overijse, Belgium), a Valco N6 injector (Alltech, Laarne, Belgium) and a column filled with Hypersil BDS (Alltech, Laarne, Belgium). The column eluate was monitored for radioactivity with a 2-in. NaI(Tl) scintillation detector coupled to a single channel analyser and for UV absorbance at 254 or 380 nm (L-4000 UV detector, Merck, Overijse, Belgium). Radiometric and UV detected signals were fed into a Rachel integration system (LabLogic, Sheffield, UK) installed on a personal computer. Generator eluate containing ^{99m}Tc in the form of sodium pertechnetate was obtained from an Ultratechnekow generator (Mallinckrodt Medical, Petten, Holland). Labelling solutions were heated in a microwave oven (Radorange model RS520A, Amana Refrigerations, Amana, IA) at 1500 Watt.

Synthesis of intermediates and final products

N-[2-(Triphenylmethylmercapto)ethyl]-2-[(2-(triphenylmethylmercapto)ethyl) amino] acetamide (1-Tr_2), (MAMA-Tr_2)

Bis-S-trityl protected monoamide-monoaminedithiol (MAMA-Tr_2) chelating agent was synthesised based on a reported procedure (23).

2-(Chloroacetylamino)-4,4'-dinitrobiphenyl (2)

To a stirred solution of 4,4'-dinitro-2-biphenylamine (18.66 g, 72 mmol) and N,N-diisopropylethylamine (21.8 ml, 125 mmol) in a mixture of THF (300 ml) and acetonitrile (240 ml) at 0°C was added dropwise over 30 minutes a solution of chloroacetyl chloride (7.5 ml, 94.2 mmol) in acetonitrile (60 ml). The mixture was allowed to warm to room temperature and stirred for 24 hours. After evaporation of the organic solvents, the residue was dissolved in 500 ml of a methanol/dichloromethane mixture (1:1, V/V) and washed successively with 1N hydrochloric acid (4 x 200 ml), water (2 x 200 ml), saturated sodium hydrogen carbonate solution (2 x 200 ml), water (2 x 200 ml) and brine (200 ml). The organic layer was dried over anhydrous sodium sulphate, filtered and

evaporated under reduced pressure. The residue was purified by column chromatography to yield 14.83 g (61.4 %) of (2) as a light yellow powder. ^{1}H-NMR (DMSO-d_6, 200 MHz): δ 4.2 (2H, s, C$\underline{H}_2$Cl); 7.6-7.8 (3H, m, 6-H, 2'-H, 6'-H); 8.2 (1H, dd, ^4J = 2.2 Hz, ^3J = 8.8 Hz, 5-H); 8.35 (2H, d, ^3J = 8.8 Hz, 3'-H, 5'-H); 8.5 (1H, d, ^4J = 2.2 Hz, 3-H); 10.1 (1H, s, ArN$\underline{H}$COCH$_2$). mp 163.3-166.3°C.

2-(Chloroacetylamino)-4,4'-diaminobiphenyl (3)

To a solution of 2 (14 g, 41.7 mmol) in THF (200 ml) were added successively acetic acid (20 ml) and 10 % palladium-on-activated carbon (3 g). The flask was rinsed with hydrogen (three times) and then the suspension was shaken for 30 min under a H_2 atmosphere at 1378 hPa. Next, the catalyst was filtered off and the filter was rinsed with dichloromethane. Water was added to the filtrate and the mixture was neutralised with sodium hydrogen carbonate. The organic layer was separated and the aqueous layer was extracted two times with dichloromethane. The combined organic layers were washed with water (2 x 100 ml) and brine (100 ml), dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography to yield 2.3 g (20.0 %) of (3) as a dark yellow oil. ^{1}H-NMR (DMSO-d_6, 200 MHz): δ 4.2 (2H, s, C$\underline{H}_2$Cl); 5.2 (4H, br s, 2 x ArN$\underline{H}_2$); 6.5 (1H, dd, ^4J = 2.2 Hz, ^3J = 8.7 Hz, 5-H); 6.6 (2H, d, ^3J = 8.8 Hz, 3'-H, 5'-H); 6.9-7.1 (4H, m, 3-H, 6-H, 2'-H, 6'-H); 9.2 (1H, s, ArN$\underline{H}$COCH$_2$).

5,5'-[2-(Chloroacetylamino)-1,1'-biphenyl-4,4'-diylbis(azo)]-bis(ethyl 2-hydroxy-benzoate) (4), (2-(chloroacetylamino)-chrysamine G diethyl ester)

To a stirred suspension of 3 (6 g, 21,8 mmol) in a mixture of methanol (185 ml) and water (280 ml) at 0°C, was added 12N hydrochloric acid (9.4 ml, 112.5 mmol). Next, a solution of sodium nitrite (3.07 g, 44.5 mmol) in water (57 ml) was added dropwise, resulting in a dark red solution at pH 1.15. A solution of ethyl salicylate (6.4 ml, 43.6 mmol) in methanol was cooled to 0°C and added to the solution of the diazonium salt at 0°C. The pH of the reaction mixture was adjusted to 10.5 by addition of 4N sodium hydroxide. After stirring for 6 hours at 0°C, the mixture was allowed to warm to room temperature, stirred overnight and neutralised with 1N hydrochloric acid. The aqueous layer was extracted with dichloromethane (3 x 400 ml) and the combined organic layers were washed with water (2 x 200 ml) and brine (200 ml), dried over anhydrous sodium

sulphate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography to yield 4.8 g (35 %) of (*4*) as an orange powder. ^{1}H-NMR (C_6D_6, 200 MHz): δ 0.9 (6H, t, 2 x $OCH_2C\underline{H}_3$); 3.4 (2H, s, $C\underline{H}_2Cl$); 3.8-4.0 (4H, q, 2 x $OC\underline{H}_2CH_3$); 7.0 (2H, d, ^3J = 9.2 Hz, 2 x 3-SA): 7.1-7.3 (3H, m, 6-H, 2'-H, 6'-H); 7.9 (1H, dd, ^4J = 2.2 Hz, ^3J = 8.4 Hz, 5-H); 8.0-8.2 (4H, m, 3'-H, 5'-H, 2 x 4-SA); 8.35 (1H, s, $ArN\underline{H}COCH_2$); 8.7 (2H, d, ^4J = 2.6 Hz, 2 x 6-SA); 9.6 (1H, d, ^4J = 2.2 Hz, 3-H); 11.65 (2H, s, 2 x Ar-O$\underline{H}$). +LSIMS (thgly) $[M+H]^+$ 630 (^{35}Cl) and 632 (^{37}Cl). mp 193.8-195.8 °C.

*5,5'-{1,1'-biphenyl-2-[N-[2-(triphenylmethylmercapto)ethyl]-N-[N-(2-(triphenylmethyl-mercapto)ethyl)-carbamoylmethyl]aminoacetamido]-4,4'-diylbis(azo)}-bis(ethyl 2-hydroxybenzoate) (*5*-Tr_2), (2-(MAMA-Tr_2-acetylamino)-chrysamine G diethyl ester)*

A solution of (*4*) (0.50 g, 0.79 mmol), (*1*-Tr_2) (0.54 g, 0.79 mmol), potassium carbonate (0.13 g, 0.94 mmol) and potassium iodide (0.01 g, 0.06 mmol) in DMF (33 ml) was stirred at 30°C for 24 hours. After addition of water (150 ml), the mixture was extracted with dichloromethane (3 x 100 ml). The combined organic layers were washed with water (2 x 100 ml) and brine (100 ml), dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. To a solution of the residue in dichloromethane (75 ml) at 0°C, acetyl chloride (45 µl, 0.63 mmol) in dichloromethane (10 ml) was added dropwise over 15 minutes. The mixture was allowed to warm to room temperature and stirred for 10 minutes, at which time the reaction was quenched by the addition of water (50 ml). The layers were separated, and the organic fraction was washed successively with water (2 x 30 ml), saturated sodium hydrogen carbonate solution (2 x 30 ml), water (2 x 30 ml) and brine (2 x 30 ml), dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The residue was purified by column chromatography yielding 0.21 g (21 %) of *5*-Tr_2 as an orange foam. ^{1}H-NMR (C_6D_6, 500 MHz): δ 0.90 (6H, t, 2 x $OCH_2C\underline{H}_3$); 2.07 (2H, t, $SC\underline{H}_2CH_2N$); 2.18 (2H, t, $SCH_2C\underline{H}_2N$); 2.31 (2H, t, $SC\underline{H}_2CH_2NHCO$); 2.50 (2H, s, $NC\underline{H}_2CONHAr$); 2.96 (2H, s, $NC\underline{H}_2CONHCH_2$); 2.99 (2H, q, $CH_2C\underline{H}_2NHCO$); 3.91-3.96 (4H, q, 2 x $OC\underline{H}_2CH_3$); 5.46 (1H, t, $CH_2N\underline{H}COCH_2$); 6.95-7.45 (35H, m, Ar); 7.87 (1H, dd, ^4J = 2 Hz, ^3J = 8 Hz, 5-H); 8.03 (2H, d, ^3J = 8 Hz, 3'-H, 5'-H); 8.12 (2H, dd, ^4J = 2.5 Hz, ^3J = 8.5 Hz, 2 x 4-SA); 8.71 (2H, d, ^4J = 2.5 Hz, 2 x 6-SA); 9.16 (1H, s, $ArN\underline{H}COCH_2$); 9.66 (1H, d, ^4J = 2 Hz, 3-H); 11.66 (2H, s, 2 x Ar-O$\underline{H}$). ^{13}C-NMR (C_6D_6, 50 MHz): δ 13.71 ($OCH_2\underline{C}H_3$); 29.70 ($S\underline{C}H_2CH_2N$); 32.19

($S\underline{C}H_2CH_2NHCO$); 38.23 ($SCH_2\underline{C}H_2NHCO$); 54.62 ($SCH_2\underline{C}H_2N$); 57.41 ($ArNHCO\underline{C}H_2N$); 59.20 ($N\underline{C}H_2CONHCH_2$); 61.72 ($O\underline{C}H_2CH_3$); 67.33 ($(C_6H_5)_3\underline{C}S$); several signals for aromatic carbon atoms. +LSIMS (thgly/nba) $[M+H]^+$ 1272.

Hydrolysis of (5-Tr$_2$) to (6-Tr$_2$) (2-(MAMA-Tr$_2$-acetylamino)-chrysamine G monoethyl ester) and (7-Tr$_2$) (2-(MAMA-Tr$_2$-acetylamino)-chrysamine G)

A solution of (5-Tr$_2$) (0.020 g, 15.7 µmol) in a mixture of DMF, 1N sodium hydroxide and ethanol (33:53:14, V/V) was heated at 50°C for 38 hours. After neutralisation with 12N hydrochloric acid, the mixture was evaporated under reduced pressure yielding an orange residue. The residue was shaken in DMF/ethanol (1:1, V/V) and the mixture was decanted, passed over a silica SepPak column (Waters, Milford, MA) and filtered. The filtrate was purified using RP-HPLC on a Hypersil BDS C18 column (10 µm, 250 mm x 10 mm). Fractions of 1 ml were applied on the column which was eluted at a flow rate of 4 ml/min with gradient mixtures of 0.005M phosphate buffer (PB) pH 7.0 (A), water (B) and ethanol (C) (see Table 2, system 3). Combined eluates containing the respective compounds, i.e. the diester derivative (5-Tr$_2$, t_R = 22 min, yield 17.5 %), the monoethyl ester derivative (6-Tr$_2$, t_R = 12.5 min, yield 27 %) and the diacid derivative (7-Tr$_2$, t_R = 8 min, yield 24 %) were concentrated under reduced pressure and the residual aqueous solutions were lyophilised. The resulting yellow-orange powders were used as such for analysis and labelling. +LSIMS (thgly/nba) 5-Tr$_2$ $[M+H]^+$ = 1272; -LSIMS (thgly doped with NaOAc) 6-Tr$_2$ $[M-H]^-$ 1242 and -LSIMS (thgly doped with NaOAc) 7-Tr$_2$ $[M-H]^-$ 1214, $[M+Na-2H]^-$ 1236, $[M+2Na-3H]^-$ 1258.

Table 2. Composition of the mobile phase for preparative HPLC analysis (A = 0.005 M phosphate buffer pH 7.0, B = H_2O and C = EtOH)

	% (V/V)		
time (min)	*A*	*B*	*C*
0	75	-	25
2	65	-	35
2.1	-	65	35
5	-	50	50
13	-	10	90
25	-	10	90

Deprotection and labelling with ^{99m}Tc

MAMA-Tr_2 (1-Tr_2)

Method 1. *Deprotection.* The sulphur atoms of MAMA-Tr_2 (1-Tr_2) were deprotected by treatment of 14.7 μmol (10 mg) of 1-Tr_2 during 1 hour with 9.7 ml of a solution containing equal volumes of TFA and dichloromethane, 2 equivalents of triethylsilane per protecting group (24) and 2-mercaptoethanol (1% V/V). The volatile compounds like triethylsilane and TFA were removed with a stream of nitrogen, the resulting residue was dissolved in ethanol (0.5 ml) and the pH was adjusted to 12 using 1N sodium hydroxide and 0.5M PB pH 12 (total volume of aqueous layer was 4 ml). Next, the aqueous layer was extracted under nitrogen atmosphere with dichloromethane (2 x 2 ml), diluted with 0.25M PB pH 12 and finally filtered before labelling (solution 1). *Labelling procedure.* To 1 ml of solution 1 in a 10-ml labelling vial, 100 μg of $SnCl_2.2H_2O$ in 25 μl of 0.05N HCl was added, immediately followed by the addition of 1 ml generator eluate containing 370-740 MBq $^{99m}TcO_4^-$. The mixture was incubated for 15 minutes at room temperature before RP-HPLC analysis.

Method 2. *Deprotection and labelling.* To 4.1 μmol (2.8 mg) of MAMA-Tr_2 in a 10-ml labelling vial were added successively 1 mg tartaric acid dissolved in 0.15 ml of 0.05N HCl, 125 μg of $SnCl_2.2H_2O$ dissolved in 25 μl of 0.05N HCl and 370-740 MBq $^{99m}TcO_4^-$ solution. The mixture was heated for 24 seconds in a microwave oven and cooled to room temperature before RP-HPLC analysis.

2-(MAMA-Tr_2–acetylamino)-chrysamine G diethyl ester (5-Tr_2)

Deprotection and labelling. To 0.256 μmol (0.3 mg) of 5-Tr_2 in a 10-ml labelling vial were added successively 2 mg of tartaric acid dissolved in 0.20 ml of 0.05N HCl, 130 μg of $SnCl_2.2H_2O$ dissolved in 25 μl of 0.05N HCl and finally 370-740 MBq $^{99m}TcO_4^-$ solution. The mixture was heated 10 minutes on a boiling water bath and cooled to room temperature before RP-HPLC analysis.

2-(MAMA-Tr_2-acetylamino)-chrysamine G monoethyl ester (6-Tr_2) and 2-(MAMA-Tr_2-acetylamino)-chrysamine G (7-Tr_2) were labelled in a similar way.

Analysis

Reversed phase high performance liquid chromatography (RP-HPLC). RP-HPLC analysis of the labelled reaction mixtures and preparative separation of the labelled compounds was carried out using a column filled with Hypersil BDS C18 (5 μm, 250 mm x 4.6 mm). 100 to 300 μL of the filtered preparation was applied on the column that was eluted at a flow rate of 1ml/min with gradient mixtures of 0.005M PB pH 7.0 (system 1_A and 2) or pH 2.5 (system 1_B), water and ethanol. For the separation of ^{99m}Tc-5 and ^{99m}Tc-7, gradient system 1 was used: elution was started with the PB and the percentage ethanol was first increased from 0 % to 5 % (0 to 10 min) and then from 5 % to 50 % in the next 10 min. At 20.1 min, the PB was replaced by water and the amount of ethanol was increased from 50 % to 80 % in the next 5 min. Finally, from 26 min to 50 min the mobile phase was water-ethanol (1:9, V/V). For a better resolution in the analysis of ^{99m}Tc-6, gradient system 2 was used: elution was started with the PB and the percentage ethanol was first increased from 0 % to 50 % (0 to 5 min). At 5.1 min, the PB was replaced by water and the amount of ethanol was increased from 50 % to 90 % in the next 30 min.

Stability of the ^{99m}Tc-complexes. RP-HPLC-isolated peaks were reinjected on the RP-HPLC system at different time intervals after isolation and the relative amounts of the ^{99m}Tc-labelled components were calculated by integration of the resulting chromatograms.

Octanol/buffer partition coefficient. The lipophilicity of the RP-HPLC-isolated ^{99m}Tc-complexes was determined using a modification of the method described by Yamauchi and co-workers (25). In a test vial, 25 μl of the ^{99m}Tc-complexes was mixed with 3 ml of 1-octanol and 3 ml of 0.025M phosphate buffer pH 7.4. The test vial was vortexed at room temperature for 2 minutes and then centrifuged at 2046 g for 10 min. A 0.5-ml aliquot of both phases was pipetted into separate test tubes with adequate care to avoid cross contamination between the phases, and their activity was counted using a 2-in. NaI(Tl) scintillation detector, connected to a single channel analyser and scaler. The partition coefficient, P, was calculated using the following equation:

P = (cpm in 1-octanol - cpm background)/(cpm in buffer - cpm background)

Experiments were done in triplicate.

CONCLUSIONS

In this study, we have been able to synthesise a conjugate of chrysamine G with a monoamide monoamine bisthiol tetraligand (MAMA), which can be labelled efficiently with ^{99m}Tc to form a stable complex. In ^{99m}Tc-MAMA-chrysamine G (^{99m}Tc-*7*), the structural requirements for β-amyloid affinity are preserved, namely a nearly planar biphenyl moiety and the presence of two acid moieties. The lipophilicity of this ^{99m}Tc-MAMA-chrysamine G complex, as indicated by its log P value of 1.08, suggests that the compound should be able to cross the blood-brain barrier (26) by passive diffusion (27). This is an important requirement for a probe intended to be useful for showing the presence of senile plaques in the brain by radioisotopic imaging. However, net brain uptake is not only dependent on lipophilicity but also upon other factors, such as percentage of drug ionised at physiological pH, cerebral blood flow, molecular size, hydrogen bonding potential, extent of protein binding, and affinity for endogenous carriers (27). Therefore, the ^{99m}Tc-MAMA-CG complex is now being evaluated in animals for its biological properties, with a special focus on its ability to cross the blood-brain barrier. Further studies will determine the affinity of these ^{99m}Tc-labelled derivatives of chrysamine G for the β-amyloid protein (Aβ 10-43) *in vitro* and for the amyloid deposition in AD. In this way, we hope to elucidate whether ^{99m}Tc-MAMA-chrysamine G could be useful as a marker of β-amyloid deposition in patients.

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