Synthesis of a Bis(aminoethanethiol) Ligand with an Activated Ester Group for Protein Conjugation and ^{99m}Tc Labeling

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Summary

The N-hydroxysuccinimide (NHS) ester of the bis(aminoethanethiol) (BAT) ligand, 6-(4'-(4''-carboxyphenoxy)butyl)-2,10-dimercapto-2,10-dimethyl-4,8-diazaundecane, was synthesized by reacting 7-(4'-bromobutyl)-3,3,11,11-tetramethyl-1,2-dithia-5,9-diazacycloundecane with the sodium salt of 4-hydroxybenzoic acid ethyl ester, followed by ester hydrolysis, reductive disulfide cleavage with dithiothreitol (DTT) and activation of the carboxyl group with NHS and dicyclohexylcarbodiimide (DCC). The resulting NHS-BAT ester was conjugated to monoclonal antibodies at pH 8.5 with about 40% yield. 2-4 BAT ligands conjugated to the antibody did not change the binding characteristics significantly. Proof of the amount of covalent bound BAT ligands was attained by UV-laser desorption/ionization (UV-LD/I) mass spectrometry. Complexation with ^{99m}Tc was accomplished by using the tin-reduction method. The labeling efficiency was >90%.

Key Words: Bis(aminoethanethiol) (BAT) ligand, N-hydroxysuccinimide ester, monoclonal antibody, ^{99m}Tc complex, FAB mass spectrometry, UV-laser desorption/ionization mass spectrometry.

Introduction

Several efforts were undertaken to improve the stability of reduced ^{99m}Tc bound in complexed form to DTPA-conjugated monoclonal antibodies or enzyme degraded antibody fragments. Two simple but effective methods concerning the formation of more stable ^{99m}Tc-binding sites were published recently. One approach uses the intramolecular formation of thiol groups with mercaptoethanol[1,2] and

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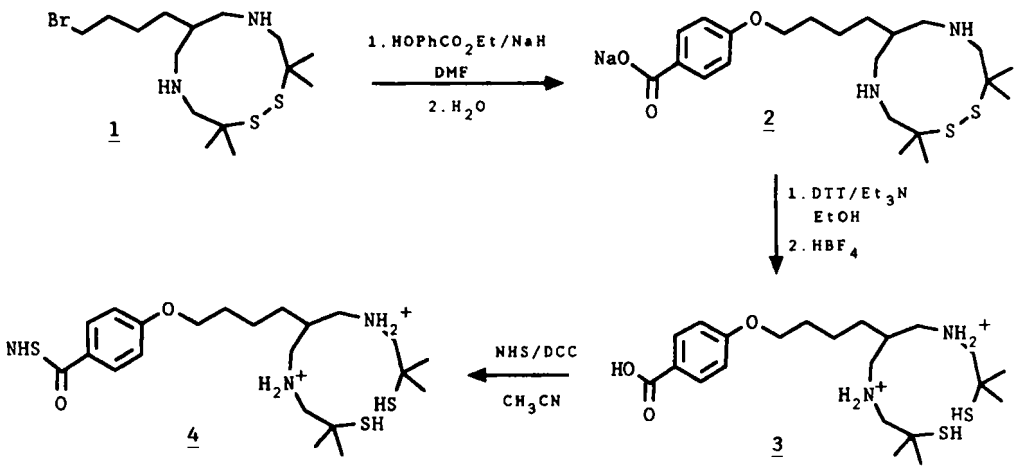
the other introduces thiols into the antibody with 2-iminothiophene[3]. However, besides mercapto groups, complexed technetium uses other electron donating heteroatoms from the protein yielding undefined complexes. In respect to smaller proteins like Fab-fragments the conjugation of a bifunctional thiol bearing ligand should therefore be more desirable.

Activated esters of ^{99m}Tc complexes using the bifunctional diamido disulfur (DADS) type ligands, 4,5-bis(thioacetamido)propanoate and 4,5-bis(thioacetamido)pentanoate, were synthesized, conjugated to antibody and successfully used for the radioimmunoscintigraphy of tumors[4a-c]. The linkage of an isothiocyanate group to ^{99m}Tc-BATO complexes and the subsequent labeling of the antibody by conjugation was also realized[5]. The major disadvantage of both methods, however, is the necessity of elevated temperatures for complex formation which restricts these methods to the precomplexation route. For routine kit application these methods are therefore of limited value.

In order to provide the formation of stable ^{99m}Tc complex at ambient temperature, we have chosen the bifunctional bis(aminoethanethiol)-ligand structure (BAT-ligand) for conjugation with antibodies. This paper describes the synthesis of the N-hydroxysuccinimide ester of 6-(4'-(4''-carboxyphenoxy)butyl)-2,10-dimercapto-2,10-dimethyl-4,8-diazaundecane (NHS-BAT ester), its reaction with antibody and ^{99m}Tc complexation.

Chemistry

7-(4'-bromobutyl)-3,3,11,11-tetramethyl-1,2-dithia-5,9-diazacycloundecane (1) recently described as a precursor for technetium-ligand syntheses[6] was used for the development of a bifunctional BAT ligand with an activated ester group for protein conjugation. As depicted in the Reaction Scheme the bromine of compound (1) was displaced by nucleophilic substitution with the sodium salt of ethyl p-hydroxybenzoate. During workup the ester hydrolyzed and the sodium salt (2) crystallized in pure form. As described earlier[6] the reductive disulfide cleavage of compound (2) was achieved with threo-1,4-dimercapto-2,3-dihydroxybutane (DTT) affording the desired BAT ligand (3).



REACTION SCHEME

The formation of the activated ester was accomplished by the reaction of BAT ligand (3) with NHS and DCC in CH_3CN. In order to obtain optimum yields the molar ratios of the reactants NHS, DCC and ligand were varied. Best results were received by reacting 0.05 M BAT ligand with 0.1 M NHS and 0.1 M DCC in MeCN. Isolated yields exceeding 23% were, however, not obtained. Although the

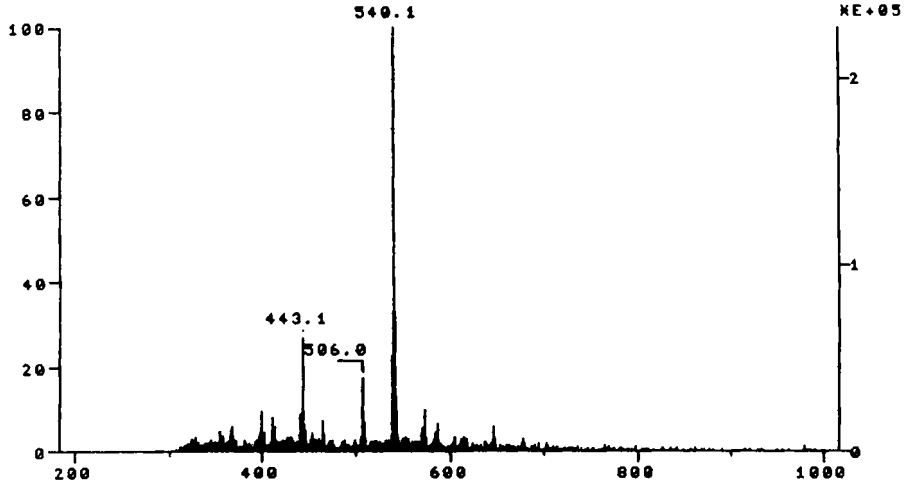


Figure 1. FAB mass spectrum of the NHS-BAT ester (4) showing the parent $(M+H)^+$ ion, fragment $(M+H)^+-H_2S$ and parent $(M'+H)^+$ of the hydrolyzed NHS-BAT ester (4)

ligand embodies nucleophilic heteroatoms FAB-MS measurement of the reaction mixture did not reveal sideproducts due to intra- or intermolecular reactions.

The separation of the NHS-BAT ligand (4) was performed with RP_{18}-TLC plates using completely volatile solvents for development. HPLC separation proved to be less suitable since the eluants contain non volatile buffer salts. The application of neat solvents caused peak broadening. The identity of the activated-BAT ester (4) was proved by HPLC, FAB-MS and its aminolytic reactivity. The FAB-MS spectrum of NHS-BAT ester (4) is depicted in Figure 1. Besides the parent $(M+H)^+$ two other main peaks were observed. The peak at 506 can be referred to the fragment $(M+H)^+-H_2S$ which is generally observed in FAB-MS measurements with BAT-type ligands[7]. The other peak at 443 belongs to the parent $(M'+H)^+$ ion of the hydrolyzed ligand (4). Hydrolysis must have occurred during FAB-MS since no free acid could be detected by HPLC before or after measurement. The NHS-BAT ester was stored in liquid N_2 and thawed when needed. As long as the ester (4) is protonated, CH_3CN solutions are stable for days.

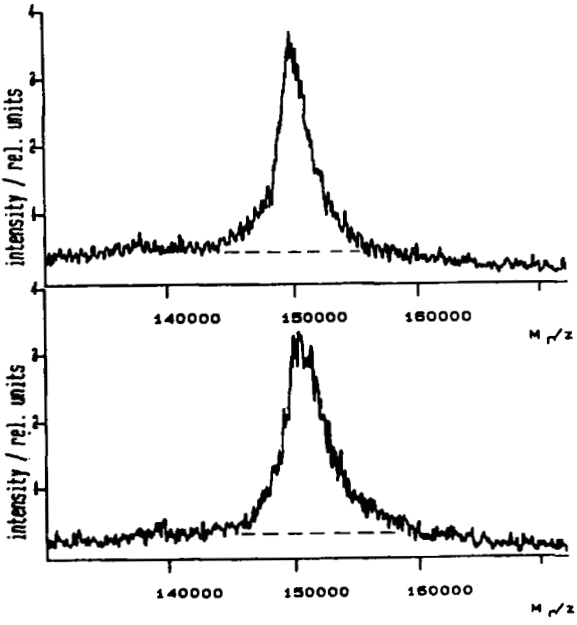


Figure 2. UV-LD/I mass spectra of A 2.6 antibody (upper) and NHS-BAT conjugated A 2.6 (lower panel).

The activated-BAT ester (4) was conjugated with monoclonal antibodies at pH 8.5. The conjugation yield was estimated from the number of SH-groups measured per IgG using Ellman's reagent. Molar ratios of BAT ligand/IgG of 2 - 4 were found to be sufficient for technetium labelling without affecting antibody properties significantly[8,9].

An alternative method to Ellman's test for the analysis of the amount of BAT ligands conjugated to antibody was made possible by UV-laser desorption/ionization mass spectrometry (UV-LD/I MS). Figure 2 shows as an example UV-LD/I mass spectra of unconjugated IgG (upper panel) and BAT-conjugated IgG. The centroids of both peaks differ by 730 indicating that about 1.7 BAT ligands were bound to one IgG. In this case the molar excess of NHS-BAT ester reacted in relation to IgG was 4. The broader peak width of the lower spectrum was due to the statistical distribution of conjugation yielding un-, mono- and disubstituted products.

Figure 3 shows the result of UV-LD/I MS measurements where antibody molecular masses were determined with varying NHS-BAT ligand/antibody ratios. The formation of conjugation products

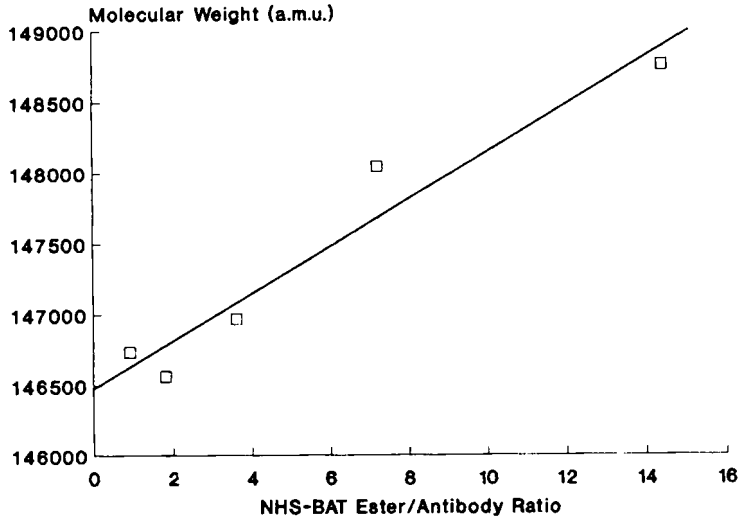


Figure 3. UV-LD/I MS masses of BAT-conjugated BW 431/26 as a function of NHS-BAT ester/antibody ratio (y=167.2x+146500; R=0.97).

appears from the increase of molecular weight measured by this method. According to the slope of the regression line the conjugation yield was calculated to be 39%. The complexation of technetium with the activated-BAT ester (4) was performed by using the tin-reduction method. Following 5 minutes reaction time at ambient temperature the labeled IgG was ready for application. The stability of the ^{99m}Tc labeled antibody was excellent. Over night storage at ambient temperature did not produce free pertechnetate and challenging with 1000 fold excess DTPA did not show transcomplexation. Proof of antigen affinity of the ^{99m}Tc labeled, BAT-conjugated antibody was presented earlier using cell binding assays[8].

Materials and Methods

The solvents were of analytical grade and used without further purification. Threo-1,4-dimercapto-2,3-butanediol (DTT) was purchased from Aldrich (Steinheim). Monoclonal antibodies used for conjugation experiments and mass spectrometry were A2.6 (rat sarcoma affine) and BW 431.26 (anti CEA). Elemental analysis was performed by Mikroanalytisches-Labor Beller (Göttingen, FRG). Analysis of BAT-Ligand concentrations was performed with Ellman's reagent at pH >8 using DTT as a standard.

Positive FAB mass spectra were measured with a MAT 95 (Finnigan, Bremen, FRG)) using glycerol or thioglycerol as a solvent matrix and a 15 eV Cs^+-ion beam. UV-laser desorption/ionization mass spectrometry used a 260 nm Nd-Yag laser for excitation and nicotinic acid as a matrix. Proton NMR spectra were recorded with a Bruker HX 90 (Karlsruhe, FRG) using TMS as an internal standard. The ^1H resonances are reported in ppm downfield (δ) from TMS.

Reverse phase high performance liquid chromatography (HPLC) was performed with 270x4 mm Nucleosil 5-C_{18} columns (Ziemer, Mannheim). The substances were eluted with methanol and Et_3N/H_3PO_4 buffer (1% Et_3N; pH 2.6). For size exclusion chromatography 250x10 mm column filled with Biogel P2 (Bio-Rad, München) was used. In order to exclude the oxidative ring closure of the conjugated BAT ligand the mobile phase, PBS pH 6.25, was purged with N_2 before use. Reverse phase TLC was performed with MKC_{18} plates (Whatman, Maidstone, England) and developed with methanol, ethanol and water of indicated ratios.

7-(4'-(4''-Carboxyphenoxy)butyl)-3,3,11,11-tetramethyl-1,2-dithia-5,9-diazacycloundecane sodium salt (2):
500 mg (0.92 mmol) 7-(4'-bromobutyl)-3,3,11,11-tetramethyl-1,2-dithia-5,9-diazacycloundecane (1) and 168 mg (1.01 mmol) 4-hydroxybenzoic acid ethyl ester were dissolved in 13 ml DMF, cooled to 0°C and reacted under N_2 with 120 mg (5.00 mmol) NaH. 1 hour after the last addition of NaH the mixture was stirred for 1 hour at ambient temperature. With the addition of H_2O and CH_2Cl_2 and upon standing over night compound 2 precipitated as a colorless, crystalline solid: 195 mg (0.42 mmol; 45.8%); mp > 200°C (decomp.); Anal.: C 57.24% (theor. 57.12%), H 8.12% (7.63%), N 6.15% (6.01%). ^{1}H-NMR (CD_3OD): δ = 1.2-2.3 (m, 19H, CH_3, CH_2, CH), 2.6-3.2 (m, 8H, CH_2N), 3.9 (t, J=6 Hz, 2H, CH_2O), 6.8 (d, J=9 Hz, 2H, arom.), 7.9 (d, J=9 Hz, 2H, arom.).

6-(4'-(4''-Carboxyphenoxy)butyl)-2,10-dimercapto-2,10-dimethyl-4,8-diazaundecane tetrafluoroborate salt (3):
144 mg (0.31 mmol) sodium salt (2) was dissolved in 3 ml MeOH. To this solution 240 mg (1.56 mmol) DTT and 0.5 ml Et_3N were added. The mixture was stirred over night at ambient temperature. After complete evaporation and dissolving the residue in 2 ml 1 N HCl, 0.1 ml HBF_4 (48%) was added. The resulting precipitate was washed with cold H_2O and dried. Yield: 173 mg (0.28 mmol, 90%); mp > 200°C (decomp.). ^{1}H-NMR (CD_3CN): δ = 1.5 (s, 12H, CH_3), 1.6-2.1 (m, 7H, CH_2, CH) 2.6 (s, 2H, SH), 3.1-3.4 (m, 8H, CH_2N), 4.1 (t, J=6 Hz, 2H, CH_2O), 7.0 (d, J=9 Hz, 2H, arom.), 8.0 (d, J=9 Hz, 2H, arom.), 7.5-8.2 (broad m, 4H, NH_2^+), 11-12 (broad s, 1H, COOH).

N-hydroxysuccinimide ester of 6-(4'-(4''-Carboxyphenoxy)butyl)--2,10-dimercapto-2,10-dimethyl-4,8-diazaundecane tetrafluoroborate salt (4):
To 2 mg (3.24 μmol) tetrafluoroborate salt (3) 32 μl of 0.2 M CH_3CN solutions each of N-hydroxysuccinimide and dicyclohexylcarbodiimide, respectively, were added in a 1 ml reaction vial. After 2 hours reaction time at ambient temperature the dicyclohexylurea crystalls formed were filtered off. The filtrate was separated on RP_{18}-TLC plates with 50% EtOH, 20% MeOH and 30% H_2O. The product at R_f = 0.45 was scraped off, extracted from the support and evaporated to the desired concentration. According to Ellman's test the yield was 23%. FAB-MS (positive) thioglycerin: 540 $(M+H)^+$, 100%, 506 $(M+H)^+-H_2S$, 17% and 443 $(M'+H)^+$, 25%.

NHS-BAT ester conjugation with IgG:
For example 24.6 µl 8.45 mM solution of NHS-BAT ester (4) was added to 3.13 mg (20.8 nmol) IgG in 500 µl PBS of pH 8.5. After 3 hours at ambient temperature the mixture was separated by size exclusion chromatography using Biogel P2 and N_2 purged PBS of pH 6.25. According to SH analysis with Ellman's reagent a ligand/IgG ratio of 3.7 was obtained.

Labeling of BAT-IgG conjugate with ^{99m}Tc:
1 mg (0.3 ml) BAT-IgG conjugate in PBS pH 6.25 was mixed with 0.5 µl 0.01 M $SnCl_2$ solution (in 0.1 N HCl) and added to 100 µl $Na^{99m}TcO_4$ (200 MBq). Analysis by size exclusion chromatography showed that >90% radioactivity was complexed to BAT-IgG conjugate after 5 minutes. No free pertechnetate could be identified and <10% of technetium was associated with an unknown, low molecular weight impurity of the BAT-IgG conjugate.

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