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# Design and Synthesis of a Novel Site-directed Reducing Agent for the Disulfide Bond Involved in the Acetylcholine Binding Site of the AChoR

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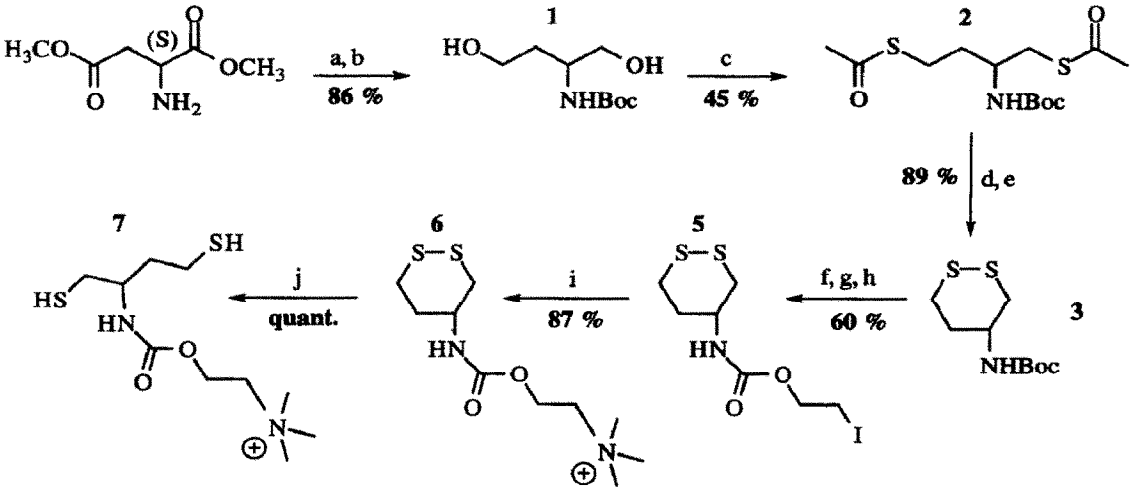
***Abstract:*** The 2-trimethylammonioethyloxycarbonylamino-1,4-butanedithiol **7** was synthesized and tested as a site-directed reducing agent for the disulfide bond involved in the acetylcholine binding site of the AChoR, which was then specifically labeled by an undecagold cluster.

The AChoR[1] is a pentamer of stoichiometry $\alpha_2\beta\gamma\delta$ bearing one acetylcholine binding site on each $\alpha$ subunit.[2,3] At present, no crystal for X-ray crystallography has been obtained with this integral protein. However, it spontaneously forms two dimensional crystalline arrangements which were exploited in electron microscopy experiments.[2,4] Some attempts were made for localizing the snake $\alpha$-bungarotoxin (competitive antagonist) binding site on the AChoR surface,[5-7] but no direct electron dense labeling of the acetylcholine binding site has been attempted. An easily reducible cystine ($C_{192}$-$C_{193}$) on the $\alpha$ subunit was shown to be involved in this site.[8,9] When the AChoR is treated with dithiothreitol (0.2 to 0.3 mM), a disulfide reducing agent, the labeling of the cysteines was shown to be specific when reacted with affinity labeling reagents.[9,10] However, non-specific labeling occured with N-ethylmaleimide,[9] a sulfhydryl sensitive compound, indicating that dithiothreitol reduces, in a certain proportion, other disulfides in the protein. The specific reduction of this disulfide bond and subsequent labeling of the cysteine(s) with an undecagold cluster should provide an appropriate approach for the three dimensional localization of the acetylcholine binding site by electron microscopy.

In this paper, we describe the synthesis of an affinity reducing agent for the disulfide involved in the agonist binding site of the AChoR. Its specificity is demonstrated and preliminary results of labeling with maleimido undecagold cluster are given.

We decided to build an analogue of carbamylcholine bearing a readily oxidizable dithiol, which would not impair the recognition of the quaternary ammonium. For this purpose, we have introduced a 1,4-butanedithiol moiety on the carbamyl end of the molecule. The synthesis started with the reduction of aspartic acid dimethylester with $LiAlH_4$ in dry THF. After hydrolysis of the aluminate, the amine was protected with ditertiobutyldicarbonate affording diol **1** with an overall yield of 86 %.[11] 2-(tBoc)amino-1,4-butanediol was then converted in the dithioacetate **2** by activation of the alcohol functions with 2-fluoro-methylpyridinium tosylate in dry $CHCl_3$, and nucleophilic substitution with thioacetic acid (45 %).[12,13] Transesterification of the 2-(tBoc)amino-1,4-dithioacetate with NaOMe in MeOH afforded a dithiol which was oxidized with iodine into the corresponding 1,2-dithiane **3** (overall yield 89 %).[14] This compound was treated with TFA to regenerate the free amino group and reacted with 2-bromoethylchloroformate in dry THF to obtain 4-(2'-bromoethyloxycarbonylamino)-1,2-dithiane **4** in 60 % yield.[15] The bromide **4** was then quantitatively

converted into the iodide **5** with NaI in dry acetone. The iodide was dissolved in dry toluene saturated with dry trimethylamine. After three days, the 2-trimethylammonioethyloxycarbonylamino-1,2-dithiane **6** precipitated as a white solid (87 %).[16] The 2-trimethylammonioethyloxycarbonylamino-1,4-butanedithiol **7** was obtained quantitatively by overnight reduction of the dithiane **6** in water, by hydrogene (4 bars) in the presence of 10 equivalents of Pd black.[17]




**Scheme 1:** Synthesis of the affinity reducing agent.

a) $LiAlH_4$, THF, Δ; b) $Boc_2O$, THF; c) 2-fluoro-1-methylpyridinium *p*-toluenesulfonate, $Et_3N$, AcSH, $CHCl_3$; d) NaOMe, MeOH; e) $I_2$, MeOH; f) TFA; g) $ClCO_2CH_2CH_2Br$, $Et_3N$, THF; h) NaI, acetone; i) $Me_3N$, toluene; j) Pd black, $H_2O$, $H_2$, 4 bars.

Dithiol **7** reduces a disulfide bond involved in the agonist binding site, modifying the affinity of the ligand for the receptor. On the other hand, partial oxidation of compound **7** leads to the competition between two cholinergic ligands (**6** and **7**). Thus, assuming the affinities of **6** and **7** are similar, we determined the affinity constants of the oxidized form **6** for the agonist and for the non competitive antagonist binding sites of the receptor. A value of 20 μM was determined on the cholinergic binding site. Compound **6** was shown to share very little affinity for the non competitive antagonist site ($K_i$ = 1 mM), proving its specificity on the acetylcholine binding site.

Reduction experiments were carried out on alkali-treated membranes[19] after alkylation of the free sulfhydryls with N-ethylmaleimide. The amount of reduction was followed after labeling of the liberated cysteine residues with [$^3$H]-N-ethylmaleimide, by sodium dodecylsulfate polyacrylamide gel electrophoresis. Optimal concentration of reducing agent **7**, which specifically reduced a disulfide bond on the α subunit (figure 1) was shown to be as low as 20 μM. In this concentration range, dithiothreitol was almost inefficient in reducing disulfide bonds on the AChoR. Moreover, the agonist carbamylcholine (200 μM) protected the disulfide bond from the reduction by compound **7** (figure 1). These two arguments establish an affinity process in the reduction with our ligand, and the specificity for the acetylcholine binding site.

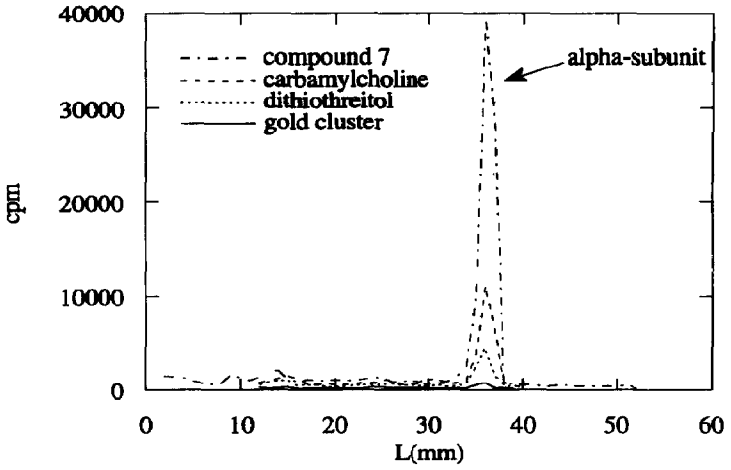
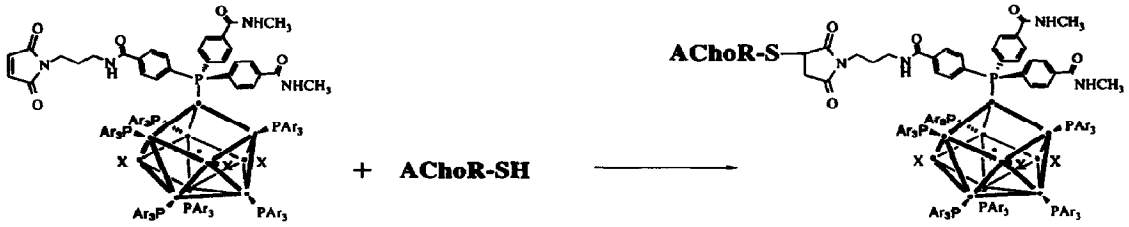



**Figure 1:** [$^3$H]-N-ethylmaleimide labeling profile on reduced AChoR.
(– · –) Reduction by affinity reducing agent **7** (20 μM); (- - -) protection of the affinity reduction with carbamylcholine (200 μM); (·····) reduction with dithiothreitol (20 μM); (——) protection with maleimido undecagold cluster (20 μM) after affinity reduction.

A maleimido undecagold cluster was synthesized as previously described,[20] and allowed to react with the cysteines obtained by site-directed reduction of the cystine (scheme 2). The incorporation of radioactivity was then completely abolished when the cluster alkylated membranes were incubated with [$^3$H]-N-ethylmaleimide, indicating that the acetylcholine binding site was labeled with the electron dense cluster (figure 1).




**Scheme 2:** coupling step between undecagold cluster and site-directed reduced AChoR (AChoR-SH).

Thus, we demonstrated the efficiency of 2-trimethylammonioethyloxycarbonylamino-1,4-butanedithiol **7** to specifically reduce a disulfide bond in the close vicinity (1 nm) of the acetylcholine binding site on the AChoR. Preliminary experiments with a maleimido undecagold cluster showed the possibility of labeling this site with the ultimate view of localizing it in three dimensions by electron cryomicroscopy.

† Deceased May, 1992.

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11. mp 64°C. MS (DCI): m/z = 206 ($M + H^+$). $^1H$ NMR ($CDCl_3$): δ (ppm) 1.45 (s, 9 H), 1.56-1.87 (m, 2 H), 3.66 (m, 4 H), 3.82 (m, 1 H), 5.23 (d, J = 8.2 Hz, 1 H). $C_9H_{19}NO_4$ (205.25) calc.: C 52.66, H 9.33, N 6.82, found: C 52.70, H 9.35, N 6.83.
12. mp 91°C. MS (DCI): m/z = 322 ($M + H^+$). $^1H$ NMR ($CDCl_3$): δ (ppm) 1.44 (s, 9 H), 1.63-1.82 (m, 2 H), 2.33 (s, 3 H), 2.35 (s, 3 H), 2.74-3.13 (m, 4 H), 3.78 (m broad, 1 H), 4.59 (d broad, 1 H). $C_{13}H_{23}NO_4S_2$ (321.45) calc.: C 48.57, H 7.21, N 4.36 found: C 48.59, H 7.23, N 4.50.
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14. mp 128°C. MS (DCI): m/z = 236 ($M+ H^+$). $^1H$ NMR ($CDCl_3$): δ (ppm) 1.46 (s, 9 H), 1.77-2.24 (m, 2 H), 2.69 (dd, $J_{gem}$ = 13.2 Hz, $J_{ax.-ax.}$ = 8.2 Hz, 1 H), 2.90-3.06 (m, 2 H), 3.12 (dd, $J_{gem}$ = 13.2 Hz, $J_{eq.-ax.}$ = 2.5 Hz, 1 H), 3.87 (m broad, 1 H), 5.00 (s broad, 1 H). $C_9H_{17}NO_2S_2$ (235.36) calc.: C 45.92, H 7.28, N 5.95, O 13.60 found: C 45.61, H 7.20, N 5.82, O 13.56.
15. mp 68°C. MS (DCI): m/z = 287 ($M + H^+$). $^1H$ NMR ($CDCl_3$): δ (ppm) 1.78-2.25 (m, 2 H), 2.73 (dd, $J_{gem}$ = 13.2 Hz, $J_{ax.-ax.}$ = 8.2 Hz, 1 H), 2.83-3.07 (m, 2 H), 3.14 (dd, $J_{gem}$ = 13.2 Hz, $J_{eq.-ax.}$ = 2.5 Hz, 1 H), 3.52 (t, J = 5.9 Hz, 2 H), 3.95 (m broad, 1 H), 4.38 (t, J = 5.9 Hz, 2 H), 5.31 (s broad, 1 H). $C_7H_{12}BrNO_2S_2$ (286.22) calc.: C 29.37, H 4.23, N 4.89, O 11.18 found: C 29.40, H 4.21, N 4.86, O 11.32.
16. mp 167°C. MS (DCI): m/z = 251 ($M - CH_3I + H^+$). $^1H$ NMR ($D_2O$): δ (ppm) 0.40 (s broad, 1 H), 1.67-2.31 (m, 2 H), 2.84 (dd, $J_1$ = 8.8 Hz, $J_2$ = 6.5 Hz, 1 H), 3.07-3.11 (m, 3 H), 3.27 (s, 9 H), 3.78 (m, 3 H), 4.59 (m, 2 H).
17. The concentration of thiols was checked by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) and measurement of the optical density at 412 nm (ε = 13600 $M^{-1} . cm^{-1}$).
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