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Design of novel synthetic MTS conjugates of bile acids for site-directed sulfhydryl labeling of cysteine residues in bile acid binding and transporting proteins

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Abstract—The purpose of this study was to design bile acid-containing methanethiosulfonate (MTS) agents with appropriate physical attributes to effectively modify the cysteine residues present in the human apical sodium-dependent bile acid transporter. Four physical properties including surface area, molecular volume, $C\log P$, and dipole moment were calculated for each semiempirically optimized structure of MTS compounds. The specificity of the synthesized bile acid–MTS conjugate toward native cysteines and putative bile acid interacting domains of hASBT was supported by the effect of 1 mM cholyl-MTS, cholylglycyl-MTS, and 3-amino-cholyl-MTS on uptake activity, that displayed a significant decrease in TCA affinity ($K_T = 69.9 \pm 4.5$, 69.01 ± 6.2, and 63.24 ± 0.26 μM and $J_{max} = 35.8 \pm 0.3$, 24.03 ± 1.22, 46.49 ± 5.01 pmol mg protein min^{-1}, respectively). These compounds prove to be effective tools in probing the structural and functional effects of cysteine residues in bile acid binding and transporting proteins.
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Bile acids play a crucial role in the homeostasis of cholesterol. The human bile acid pool is maintained by the enterohepatic circulation,[1] wherein the re-absorption of bile acids in hepatic and intestinal cells is achieved effectively by two related sodium-dependent bile acid transporters. These include the apical sodium-dependent bile acid transporter, ASBT (SLC10A2), a 41 kDa glycoprotein[2,3] in the intestine and the Na^+-taurocholate co-transporting polypeptide, Ntcp (SLC10A1), present selectively on the canalicular domain of the hepatocytes. As a result of these highly efficient transporters, bile salts are recycled between 6 and 7 times a day circulating an estimated 20 g during this time.[4] Thus, bile acid transporters become very attractive targets in the drug discovery of: (a) lipid-lowering agents by the manipulation of the bile pool in the enterohepatic circulation[5] and (b) pro-drugs for improved oral bioavailability.[6] To achieve either of these two objectives, a more detailed understanding of the structure of bile acid transporters is required. However, polytopic membrane transporters are notoriously resistant to the determination of high resolution structure by traditional means owing to their hydrophobicity and, in many instances, because of their metastable nature. Thus, the absence of a definitive crystal structure makes it difficult to exploit this pharmaceutically significant target to any tangible gain in drug design.

Sulfhydryl-active reagents have been used effectively in the past to probe the structural and functional aspects of membrane proteins.[7,8] However, proteins are known to alter conformation in the presence of their substrates. The purpose of this study was to design bile acid-containing MTS agents with appropriate physical attributes to modify the cysteine residues present in the bile acid binding site of ASBT that mimics the binding with its natural substrate. This should afford a more accurate insight into the role of cysteine residues in the overall transport of bile salts. The presence of the MTS moiety accounts for the rapid oxidation of the sulfhydryl group in the presence of a thiol group to form a disulfide bond, thereby shielding the cysteine thiol group.[9] However, the physical properties, which include surface area, volume, lipophilicity (partition coefficient), and dipole moment, could change drastically with the alterations of functional groups in bile salts. Two challenges in this study are the identification of contributing physical properties of the conjugated bile acids in locating functionally

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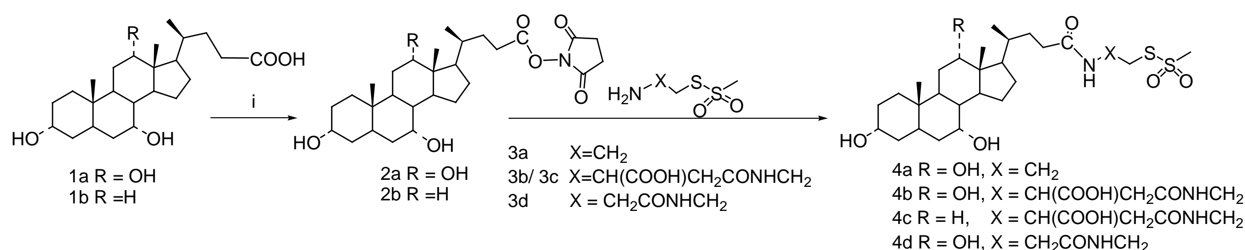
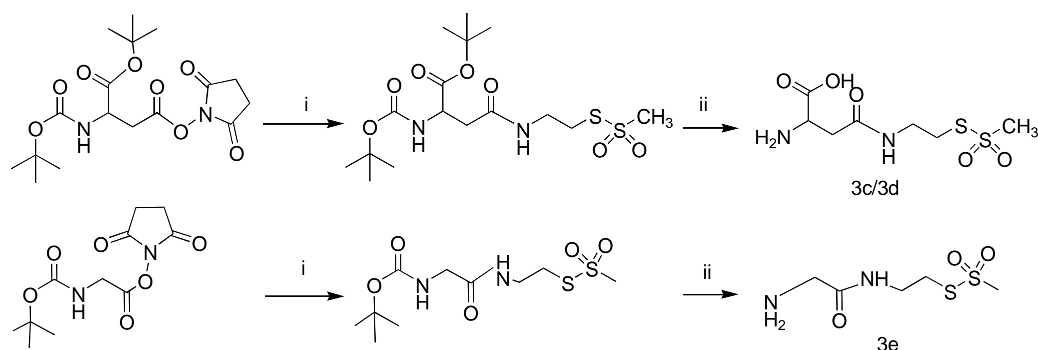
important cysteine residues and the synthesis of these conjugates.

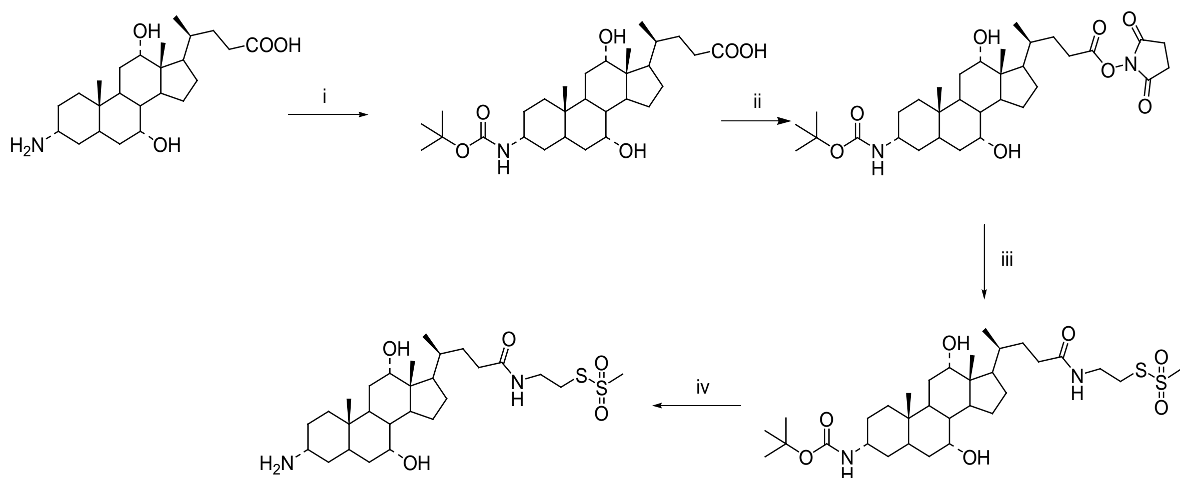
The synthesis of CA-MTS (**4a**) was carried out by activating cholic acid using *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′,-tetramethyluronium tetrafluoroborate (TSTU) to form the *O*-(*N*-succinimido) ester derivative.[10] The ratio of solvents DMF and 1,4-dioxane, 1:1 (v/v), was critical to the success of this reaction. The activated succinimido ester was then reacted with MTSEA to give the desired compound **4a**. However, the masking of the charge of cholic acid in the formation of an amide bond resulted in reduced solubility of these compounds. The reintroduction of a negative charge present in cholic acid was carried out by inserting an aspartic acid residue. The α-carboxylic acid in aspartic acid was maintained in its free, ionizable form and the MTS group was attached to the ω-position. CA-Asp-MTS (**4b**) and CDCA-Asp-MTS (**4c**) were synthesized by reacting Boc-L-aspartic acid-4-*tert*-butyl 1-(hydroxysuccinimide) ester with MTSEA and the resultant product with activated (*N*-hydroxysuccinimidyl) CA or CDCA to give the desired products. The synthesis of glycocholic acid derivative CA-Gly-MTS **(4d)** was achieved by replacing the aspartic acid in CA-Asp-MTS by glycine. The conversion of CA to its 3-amino derivative was performed as described previously.[11] The amine moiety was protected by a Boc group using di-*tert*-butyl pyrocarbonate and 1 N sodium hydroxide. The activation of the carboxylic acid followed by the reaction with MTSEA and the removal of the protecting group resulted in the formation of 3-amino-CA-MTS (**4e**) (Schemes 1–3).



Scheme 1. Reagents: (i) DMF, 1,4-dioxane, H_2O, DIEA, TSTU.



Scheme 2. Reagents: (i) DMF, 1,4-dioxane, MTSEA; (ii) CH_2Cl_2, TFA.



Scheme 3. Reagents: (i) dioxane, H_2O, 1 N NaOH, $(Boc)_2O$; (ii) DMF, dioxane, H_2O, DIEA, TSTU; (iii) MTSEA; (iv) CH_2Cl_2, TFA.

Table 1. Physical properties of bile acids and MTS agents

Compound	Name	Surface area (Å^2)	Molecule volume (Å^3)	C log *P*	Dipole moment	High resolution mass		
						Formula	Calcd	Found
	MTSEA	320.9	477.5	−0.6	1.8	—	—	—
	CA	606.0	1108.3	2.43	4.86	—	—	—
	TCA	708.3	1326.5	−0.01	6.27	—	—	—
4a	CA-MTS	757.2	1418.0	1.95	4.98	$C_{27}H_{47}NO_6S_2Cs$	678.1899	678.1874
4b	CA-Asp-MTS	872.4	1613.6	1.32	3.84	$C_{31}H_{52}N_2O_9S_2Na$	683.3012	683.2944
4c	CDCA-Asp-MTS	880.4	1606.8	3.41	2.97	$C_{31}H_{51}N_2O_8S_2$	643.3087	643.3120
4d	CA-Gly-MTS	765.1	1480.2	1.38	2.84	$C_{29}H_{50}N_2O_7S_2Na$	625.2957	625.2963
4e	3-Amino-CA-MTS	766.8	1433.7	2.05	4.71	$C_{27}H_{48}N_2O_5S_2Na$	545.3083	545.3062

C log *P* is an indicator of molecule hydrophobicity.
Dipole moment is calculated as the summation of all dipole vectors of all bonds in the molecule.
It represents the overall imbalance of electron density in a molecule.

Physicochemical properties of MTS conjugates were assessed using the Sybyl 6.9 software package (Tripos, St. Louis, MO). Structures were sketched using standard bond distances and bond angles, and subjected to conformational analysis as described previously.[12] Each structure was then evaluated in Sybyl for calculation of physicochemical descriptors including molecular surface area, molecular volume, C log *P*, and dipole moment.

The synthesis of CA-MTS was mandated by the prior knowledge that proteins frequently alter conformation in the presence of natural substrates and the requirement of possessing a thiol modifier. This molecule meets the requirements of containing a natural substrate, cholic acid (CA), and a thiol modifier along with its molecular dimensions which were similar to those of taurocholic acid (TCA) (Table 1), another natural substrate. CA-MTS and TCA share similar surface area (757.2 vs 708.3 Å^2, respectively) and molecular dimensions (1418 vs 1326 Å^3, respectively). The relative similarity of molecular dimensions and dipole moment between CA-MTS and TCA correlated well with the high effectiveness of this conjugate in significantly decreasing the transport activity of the tracer substrate, TCA (Table 2). The aspartate-containing compounds CA-Asp-MTS and CDCA-Asp-MTS had a relatively larger surface area (872.402 and 880.436 Å^2) and a larger volume (1613.55 and 1606.81 Å^3). The associated lowering of C log *P* resulted in solubilization of CA-Asp-MTS and CDCA-Asp-MTS, but this did not result in higher reactivity of these conjugates. The dipole moment of CA-Asp-MTS (3.8) and CDCA-Asp-MTS (3.0) is generally smaller than that of CA-MTS (5.0) and TCA (6.3), suggesting that a higher dipole moment may be of greater relevance than the C log *P* value. The larger dimension provided an explanation for the higher J_{max} of CA-Asp-MTS and CDCA-Asp-MTS and may be attributed to their inability to enter the critical site. Our preliminary study suggests that C log *P* values of these compounds do not seem to play a role in determining the effectiveness since TCA is highly soluble with a low C log *P* value and high affinity, whereas MTSEA has a similar C log *P* but relatively low reactivity. A glycine spacer was used as a biomimetic of glycocholic acid. The similarity of some of these physical properties of CA-MTS, CA-Gly-MTS, and 3-amino-CA-MTS appeared to have correlated well with comparable efficacy.

Table 2. Effect of MTSEA and bile acid–MTS conjugates on hASBT activity

Compound		K_T (μM)[a]	J_{max} (pmol mg protein^{-1} min^{-1})
	Control	11.2 ± 0.4	300.2 ± 8.3
	MTSEA	17 ± 2.7	140.3 ± 11.5*
4a	CA-MTS	69.9 ± 4.5*	35.8 ± 0.3*
4b	CA-Asp-MTS	72.2 ± 2.6*	114.2 ± 10.2*
4c	CDCA-Asp-MTS	11.2 ± 2.3	122.7 ± 5.4*
4d	CA-Gly-MTS	69.01 ± 6.2*	24.03 ± 1.22*
4e	3-Amino-CA-MTS	63.24 ± 0.26	46.5 ± 5.01*

[a] [^{3}H]-TCA flux (*J*) was fitted to the following equation: $J = J_{max} \cdot C/(K_T + C)$, where *C* represents the TCA concentration, K_T the concentration at half maximal flux, and J_{max} the maximal flux.

* Data are represented as means ± SD of at least three separate experiments and were considered statistically significant (*) with $p \leqslant 0.01$.

Sensitivity of hASBT to MTSEA and bile acid–MTS reagents was assessed in COS-1 cells transiently expressing hASBT in the absence (control) or presence of 1.0 mM MTSEA, CA-MTS, CA-Asp-MTS, CDCA-Asp-MTS, CA-Gly-MTS, and 3-amino-CA-MTS for 10 min. At this concentration, the conjugates do not disrupt the cell membrane as observed by the MTT cellular cytotoxicity assay. The specificity of these compounds was tested by generating kinetic transport parameters. The comparison with control or MTSEA of CA-MTS, CA-gly-MTS, and 3-amino-CA-MTS indicates that these conjugates may be entering the bile acid binding site; thus, they effectively reduce TCA uptake as evidenced by an increase in K_T and a decrease in J_{max}. On the other hand, CA-Asp-MTS and CDCA-Asp-MTS interact with the hASBT-transfected cells in a non-specific manner, suggesting that they may not enter the bile acid binding site (Table 2).

Following incubation with MTS modifiers, cells were washed twice in uptake medium (modified Hanks' balanced salt solution, MHBSS), pH 7.4. Uptake kinetic parameters were determined at varying concentrations of TCA (0–150 μM) by incubating the cells in MHBSS containing ^{3}H-TCA (0.2 Ci/mmol) for 12 min. Uptake

Table 3. Cholic acid inactivation of CA-Asp-MTS of hASBT ^{3}H-TCA uptake

Cholic acid (μM)	K_T (μM)	J_{max} (pmol mg protein^{-1} min^{-1})
0	70.2 ± 8.12	96.5 ± 6.2
5	20.7 ± 1.25	149.0 ± 2.1
50	9.5 ± 1.03	210.4 ± 9.4
100	11.5 ± 1.57	239.1 ± 11.59
Control (no treatment)	14.0 ± 0.75	257.7 ± 3.99

was stopped by washing the cells four times in ice-cold PBS (pH 7.4) containing 0.2% fatty acid-free bovine serum albumin (BSA) and 0.5 mM TCA. Cell-associated radioactivity was measured using an LS6500 liquid scintillation counter (Beckman Coulter, Inc, Fullerton, CA) and normalized to total protein content using the Bradford assay (Bio-Rad, Hercules, CA). The bile acid conjugates modify the internal cysteines as these molecules affect ^{3}H-TCA transport more effectively than MTSEA alone. Further, the compounds do not affect transporter integrity as the kinetic curves show saturation with increase in concentration indicating active transport.[13]

To further confirm the specificity of interaction between bile acid–MTS conjugate and the substrate binding regions, bile acid protection assays were carried out in which the transporter binding domains are presaturated with ligand (CA) prior to incubation with MTS reagents. CA-Asp-MTS (**4b**) was chosen as a representative compound as it displays similar affinity toward hASBT in comparison to the other conjugates (Table 2). However, CA-Asp-MTS distinguishes itself from other compounds by the fact that this conjugate has a relatively high J_{max}, thereby allowing the application of a broader concentration range in substrate protection assays. Cells transfected with hASBT were pre-equilibrated with increasing concentrations of cholic acid (0–100 μM) for 5 min prior to addition of CA-Asp-MTS (1.0 mM). The cells were co-incubated with or without the conjugate for 10 min, washed with uptake buffer, equilibrated in MHBSS (pH 7.4, at 37 °C) for 15 min. Following equilibration, uptake kinetics was determined at increasing concentrations of TCA as described above. Uptake activity was restored to 90% of control (absence of conjugate) at approximately 100 μM cholic acid (Table 3), suggesting probe-specificity.

We have demonstrated that: (i) bile acid–MTS conjugates can aid in defining the putative ligand binding region(s) and translocation pathway of hASBT, (ii) the surface area, molecular volume, and dipole moment of these conjugates similar to those of TCA appear to be more important than C log *P* in the design of these molecules. Furthermore, these conjugates may be used as novel probes for characterizing other bile acid transporters such as hepatic *Ntcp* and the bile salt excretory pump (BSEP), as well as bile acid binding proteins (BABP).

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