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Bile Acid–Oligodeoxynucleotide Conjugates: Synthesis and Liver Excretion in Rats

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Abstract—The synthesis of bile acid–oligodeoxynucleotide conjugates via the 3-OH group of the bile acids is described. When used in vivo in rats, covalent conjugation of an oligodeoxynucleotide via a linker to cholic acid resulted in an increased biliary excretion of bile acid–oligodeoxynucleotide conjugates compared to unconjugated oligodeoxynucleotides.

In the treatment of cancer or viral infections, oligodeoxynucleotides (ODNs) represent a new approach of therapeutic interventions by specifically affecting different target structures: by triplex formation, by acting as ribozymes, antisense agents, or as aptamers.[1] A major and still unresolved problem concerns their poor cellular uptake and insufficient drug targeting of different organs and tissues. Previous investigations confirmed these unfavourable properties also for hepatocytes.[2,3] In order to generate oligonucleotides with a higher specificity for the hepatic parenchyma cells we synthesized ODN-derivatives conjugated via the 3-OH group of the bile acid cholic acid. Bile acids are taken up selectively by hepatocytes and by ileocytes due to the presence of specific bile acid transport systems in these cells.[4] The synthesis of cholic acid–oligodeoxynucleotides was described in an earlier report,[5] but uptake and binding of these esteratic compounds tested in non-hepatic cells were not superior to unmodified ODNs. The present paper describes a method of coupling 3β-(2-aminoethoxy)-cholanoic acid methyl ester[6–8] to the 3-OH group via amide bonding and their biological testing in situ.

Synthesis of Bile Acid–Oligodeoxynucleotide Conjugates

Bile acid derivative 3β-(2-aminoethoxy)-7α,12α-dihydroxy-5β-cholan-24-oic acid methylester **1** was treated with one equivalent of succinic anhydride in tetrahydrofuran and one equivalent of triethylamine at room temperature for 10 min. The solution was added to ice-cold 0.05 M HCl and the precipitate **2** was dried.

Monoamide **2** was treated with amino-linked oligodeoxynucleotides **3**, **4**, **5** and **6** to achieve coupling via amide bonds. The amino linked mixed phosphorothioate oligodeoxynucleotides were dissolved in water: 0.5 M sodium carbonate 0.5 M/DMF 16.8:3.2:80 (v/v/v). One equivalent of **2** was treated with one equivalent of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate TBTU[9] and four equivalents of diisopropylethylamine, which were previously dissolved in dimethylformamide. The mixture was allowed to stand for 15 min at room temperature forming TBTU-activated bile acid monoamide. The respective ODNs (**3**, **4**, **5** or **6**) to be coupled were treated with a 20-fold excess of TBTU-activated bile acid monoamide and allowed to stand for 45 min at room temperature. Addition of freshly TBTU-activated bile acid monoamide was repeated twice in case of 3′-amino linked ODN **4**, **5**, and **6**, and 8 to 10 times in case of the 3′,5′-amino linked compound **3** as described above. Bile acid–oligodeoxynucleotide conjugates were precipitated with *n*-butanol and redissolved in water. Degree of coupling was controlled by means of gel electrophoresis of an aliquot (polyacrylamide gel: 20% acrylamide, 2% bisacrylamide, 7 M urea).

Deprotection of the bile acid carboxylic acid side chain of the conjugates was achieved by incubation with an

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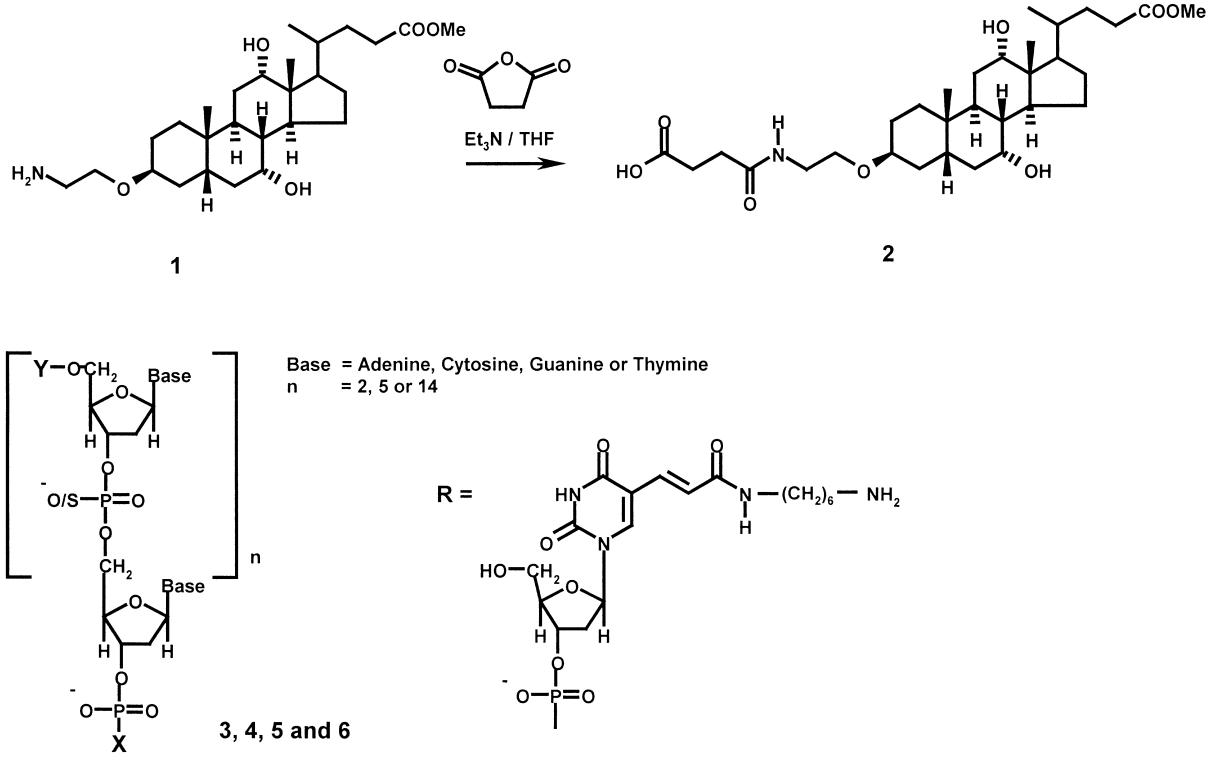
equal volume of 0.05 M NaOH for 30 min at room temperature. Subsequent treatment with 0.1 volumes of 3 M sodium acetate solution pH 4.8 arrested ester cleavage. Addition of 2.5 volumes of ethanol followed by removal of the supernatant gave the crude bile acid–oligodeoxynucleotide conjugate which was purified by gel electrophoresis as described above. The products were extracted from the gels with 0.2 M triethylammonium bicarbonate solution. Each extract was desalinated via solid-phase extraction (C_{18} Bond Elut Column). Treatment with methanol/water 6:4 (v/v) cleaved the pure bile acid–oligodeoxynucleotide conjugate from the column. The solvent was removed by rotary evaporation and the residue was redissolved in water.

Biological Data

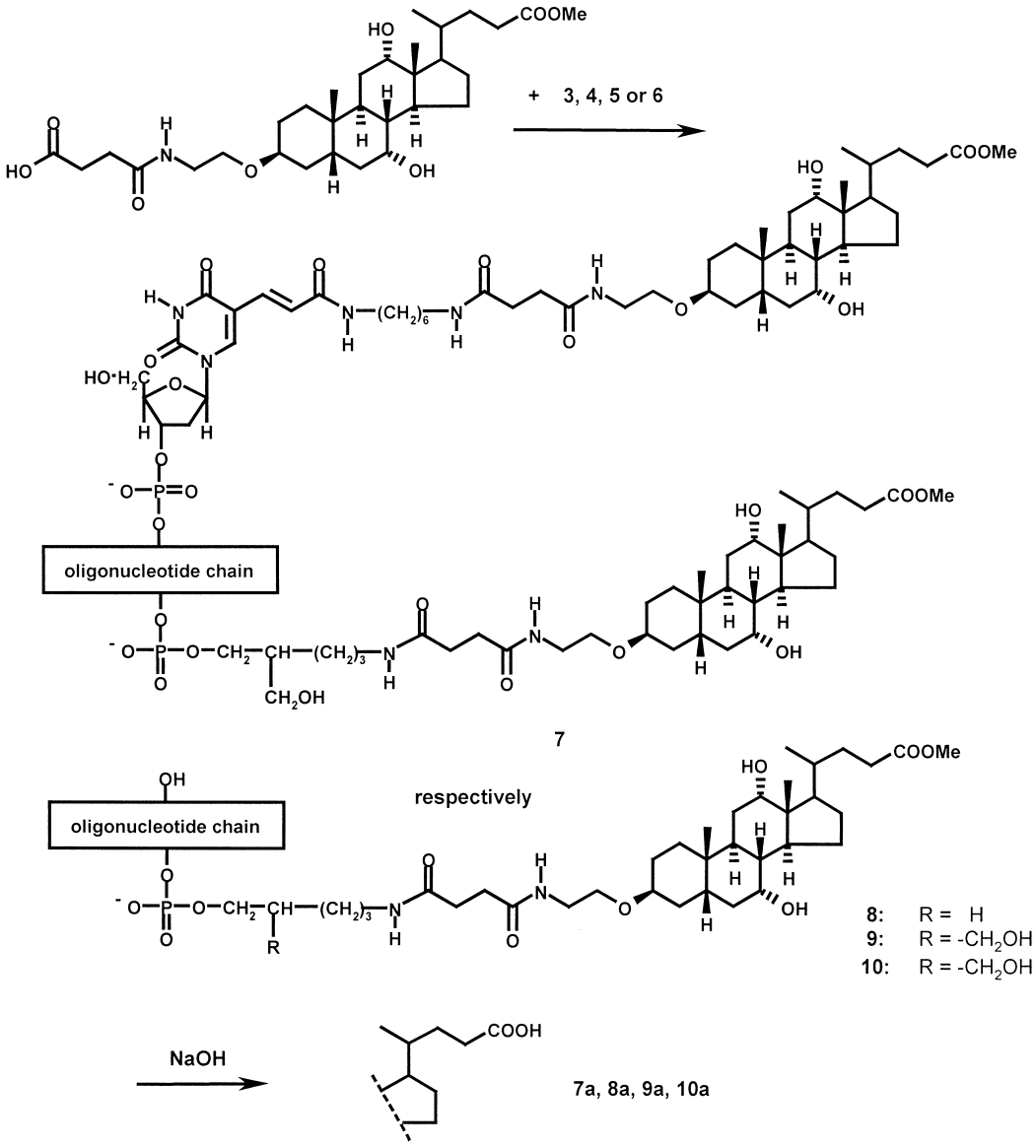
As model compounds for the biological studies we used a nuclease resistant non-hybridizing 15-mer mixed-backbone ODN with phosphodiester and phosphorothioate moieties (n-ODN = **0**; see Schemes 1 and 2; analytical data) and the derivatives of this ODN tagged with one (1C-ODN = **8a**) or two cholic acid molecules (2C-ODN = **7a**) at the 3′- and 3′- plus 5′-end position, respectively. Since solely the hepatocytes form bile fluid and because concentration readings within these cells cannot be continously analyzed, bile fluid was sampled and excreted ODN within was determined. It was anticipated that changes of ODN concentration in bile



Substrate	Amino-linked oligonucleotide	*n*	X	Y	Conjugate
3	5'-Y-GsGsCs TGCs CAsT GGTs CsCsC-X-3'	14	1-amino-(5-hydroxymethyl)-hexan-6-ol	R	**7**
4	5'-Y-GsGsCs TGCs CAsT GGTs CsCsC-X-3'	14	1-amino-hexan-6-ol	H	**8**
5	5'-Y- GGTs CsCsC-X-3'	5	1-amino-(5-hydroxymethyl)-hexan-6-ol	H	**9**
6	5'Y-CsCsC-X	2	1-amino-(5-hydroxymethyl)-hexan-6-ol	H	**10**

As, Gs, Cs, and Ts: phosphorothioate of the respective nucleotide.

Scheme 1. Synthesis of monoamide **2** (above) and scheme of different amino-linked ODNs (= **3**–**6**) and conjugated ODNs (= **7**–**10**) employed; the unmodified ODN (n-ODN = **0**) (see Biological data, Analytical data) bears the same sequence as **3**/**4** without the aminolinker.



Scheme 2. Conjugate formation and deprotection of the carboxylic group by methylester cleavage.

are preceeded by appropriate ODN concentration changes in the cells and thus this can be regarded as an indirect estimate of hepatocyte kinetics. Practically, the derivatives were labelled with ^{35}S at the 5′-end and were injected into a mesenteric vein of male Wistar rats. The excreted bile was collected fractionally. ODN contents within bile fractions were determined quantitatively by liquid scintillation counting. Previous experiments concerning stability in homogenized liver tissue showed no degradation of the 15-mer ODNs or its conjugates. As a result, biliary excretion experiments in situ revealed similar elimination profiles of all three compounds applied. However, the peak concentration in bile which is preceeded by a parallel rise of ODN concentration in the hepatocyte is much higher in case of the ODN tagged with bile acid molecules. The improvement of their availability for the liver strongly correlates with the conjugation of one, and even more with the conjugation of two bile acid molecules to the oligonucleotide chain. Whereas retrieval of unconjugated *n*-ODN in bile was about 5% of the whole applied dose this increased to about 8% regarding the 1C-ODN. Highest recovery namely 25% of the 2C-ODN was found in bile two hours after injection. A higher amount of the bile acid tagged ODNs (1C-ODN, 2C-ODN) remained in the whole organ two hours after injection (see Fig. 1).

With isolated rat hepatocytes in suspension as an additional in vitro test system hepatocellular uptake of bile acid–oligodeoxynucleotide conjugates was determined. Uptake was remarkably small for all ODNs. Although the conjugates showed slightly higher hepatocellular uptake velocity constants than the unconjugated ODNs ($p < 0.05$) the total amount of cell associated derivatives did not differ decisively over an incubation period of 20 min (less than 0.5% of the applied dose). Competition experiments with substrates of hepatic bile acid carriers such as cholate did not show altered uptake properties. These findings clearly differ from various bile acid tagged drugs; their hepatocellulare uptake is inhibited by bile acids.[10–13]

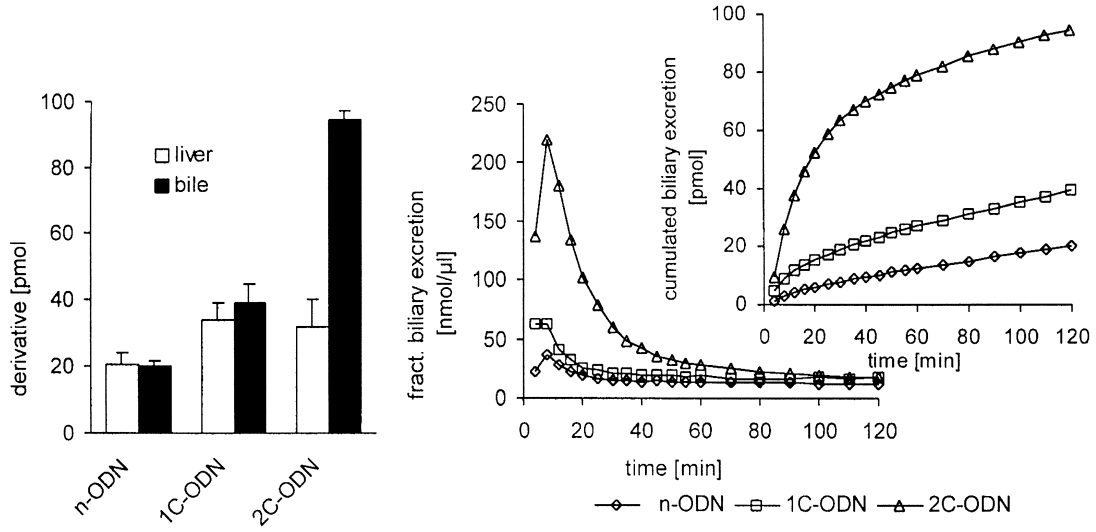


Figure 1. Amounts of derivatives found in the liver and in the collected bile fluid after 2 h; bars represent S.E.M. ($n=3$) (left); fractionated (right) and cumulated (insert) depiction of unconjugated ODN (n-ODN = **0**), tagged with one (1C-ODN = **8a**) and two (2C-ODN = **7a**) molecules of cholic acid after bolus injection of 0.4 nmol of the respective derivative into a mesenteric vein of a Wistar rat ($n=3$); $p < 0.001$.

In summary, the synthesis described above allows an efficient preparation of ODNs conjugated to the 3-OH group of bile acids via amide bonding. The conjugation was feasible with oligonucleotides of different length (see Scheme 1) with comparable yields. When a 15-mer mixed-backbone ODN was conjugated to one or two cholic acid molecules, conjugation led to a significantly increased biliary elimination. Previous results show that elongation of the ODN chain leads to a reduced elimination.[14] Consequently the enlargement of the molecule by conjugation cannot be the reason for the improved excretion. A higher lipophilicity resulting in a better membrane penetration cannot explain the observed effects because in comparison with the unmodified ODN = **0** the bile acid tagged 2C-ODN = **7a** is more hydrophilic (see analytical data). The results obtained thus indicate that targeting of ODNs to the liver tissue by means of endogenous bile acids is possible and confirm earlier results with bile acid tagged drugs. In these cases of various low molecular weight drugs it was concluded that they were transported by specific and well characterized hepatic carriers in in vivo and in vitro assays.[10–13] The transport mechanisms responsible for the hepatic uptake and elimination of ODNs differ from low molecular weight drug conjugates and are still unknown. With ODNs it appears that more than one bile acid molecule should be introduced into the nucleotide chain for liver specific targeting. Whether the higher amounts having passaged the hepatocytes indicated by improved biliary excretion lead to a better efficiency in clinical applications such as in antisense approaches will be answered by future investigations.

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Typical conjugation procedure: 20 OD of oligonucleotide in 100 μL solvent (see above).

Characteristic analytical data:

1 and **2**: see ref 5

HPLC: Waters Gempack: Fax column; solvent A: 10 mM NaH_2PO_4 in H_3CCN/water 2:8 (v/v), pH 6.8; solvent B: 10 mM NaH_2PO_4 and 3 M NaCl in acetonitrile/water 2:8 (v/v), pH 6.8; gradient: 5 to 60% B within 40 min; 1 mL/min; 260 nm

ESI-MS:	Found	Calculated
15-mer without linkers (=**0**)	M = 4673.7	4673.5
15-mer with 3′-linker (=**4**)	M = 4882	4882
15-mer after coupling (=**8**)	M = 5431	5429
8 after ester cleavage (=**8a**)	M = 5417	5415
3′,5′-linked 15-mer (=**3**)	M = 5341	5341
7 after ester cleavage (=**7a**)	M = 6411	6409

Water:lipid (phosphate buffered saline, pH 7.4: 1-octanol) distribution coefficient: **7a**: 71.2; **0** 13.1