Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1827–1835

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Properties of Bile Acid Phosphoramidites 5′-Tethered to Antisense Oligodeoxynucleotides Against HCV

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Received 21 December 2000; accepted 14 March 2001

Abstract—Recently, we synthesized antisense oligonucleotides (AS-ODNs) directed against the non-coding-region (NCR) and the adjacent core region of the hepatitis C virus (HCV) RNA. Backbone modifications like phosphorothioates, methyl- and benzylphosphonates were introduced three at each end of the sequence. For improvement of liver specific drug targeting and/or hepatocellular uptake efficient AS-ODNs were covalently conjugated to biomolecules such as cholesterol or bile acids. The use of base-labile alkylphosphonates afforded mild conditions for deprotection of bile acid conjugated AS-ODNs. Here, we describe a convenient synthesis of new cholic acid and taurocholic acid phosphoramidites. Derivatization to taurocholic acid was effected directly before phosphitylation reaction, which is the last step of the phosphoramidite synthesis. These building blocks were coupled to the 5′-position of AS-ODNs in the last step of solid-phase synthesis. After mild deprotection, purification and characterization the properties of these modified AS-ODNs like their lipophilicity or their ability to form stable duplices to DNA and RNA were investigated. Enhanced lipophilicity and formation of stable duplices and heteroduplices makes bile acid conjugated AS-ODNs interesting as antiviral antisense therapeutics against HCV.

Introduction

To enhance organ specific drug targeting, absorption and distribution, several macromolecular carriers like liposomes, nanoparticles or dendrimers have been developed. Bile acids are natural ligands specifically recognized by hepatitic cells and are amphiphilic molecules that undergo a biological recycling during enterohepatic circulation. After formation from cholesterol in the liver, bile acids are secreted into the bile and stored in the gall-bladder. After food ingestion, the bile is secreted into the duodenum and is mainly involved in the digestion and resorption of fats in the small intestine. Passing the end of the small intestine, the majority of the bile acids is reabsorbed and transported with portal blood back to the liver. This enterohepatic circulation is repeated six to 15 times a day. This demonstrates the high transport capacity and organ specifity of bile acids and makes them attractive tools to improve liver specific drug targeting.[1]

To couple AS-ODNs to bile acids two different strategies have been followed so far. The first successful attempt reacted an amino-linked oligonucleotide with an activated carboxyl group of cholic acid. For activation, the carboxyl group was converted into its *N*-hydroxysuccinimide ester or its pentafluorophenyl ester.[2,3] Alternatively, activation occurred with the dicyclohexylcarbodiimide/1-hydroxy-benzotriazole system to a 5′-branched amino group.[4] As clarified in the following paragraphs, the carboxyl function seems to be essential for an efficient intestinal and hepatic uptake. Furthermore, this strategy is not useful to synthesize taurocholic acid derivatives of oligonucleotides.

The second strategy utilized derivatization of the 3-hydroxy group of the steroid scaffold to combine bile acids with oligonucleotides. In this case, protection of the carboxyl group of cholic acid or the sulfonic acid group of taurocholic acid was indispensable. Introduction of an ω-aminoalkoxy-spacer at the 3-position of protected cholic acid was followed by amidation with dicarboxylic acid.[5–8] After activation with TBTU or ethyl chloroformate conjugation to an amino-linked oligonucleotide yielded the esterified bile acid-oligonucleotide product. Deprotection of the carboxylester required sodium hydroxide as the last step. Oligonucleotides containing methyl- or benzyl modified phosphodiesters are very sensitive towards hydroxyl bases. Therefore, the synthesis of such modified oligonucleotides requires a new deprotection strategy.

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0968-0896/01/$ - see front matter
PII: S0968-0896(01)00079-7

The latter approach of derivatization via the 3-hydroxy position of bile acids offers several advantages. In contrast to the carboxyl group, the 3α-position, which is present in all natural bile acids, seems not to be essential for high affinity interaction to ileal or hepatic bile acid transporters. Rather, removal or exchange of the 3α-hydroxy group leads to an increased affinity to bile acid transporters. Additionally a delayed hepatobiliary secretion across the hepatocyte canalicular membrane into bile is observed for bile acids lacking a free 3α-hydroxy group.[9,10] In summary, conjugation of bile acids to therapeutics via the 3α-hydroxy group results in liver specific drug targeting, improved basolateral uptake into hepatocytes and delayed secretion into bile.

A large number of therapeutics lack sufficient penetration through biological membranes. For drugs of low molecular weights like the alkylating cytostatic drug chlorambucil and the fluorescent prolyl-4-hydroxylase inhibitor 4-nitrobenzo-2-oxa-1,3-diazol-β-Ala-Phe-5-oxaproline-Gly attached to bile acids, a specific transport into the liver and the biliary system is observed.[11] Peptides up to 10 amino acid residues coupled to bile acids also show an improved intestinal and hepatic uptake. Moreover, they show an enhanced enzymatic stability against peptidases.[1,12] Recently, the first investigation of a mixed 15mer phosphorothioate phosphodiester ODN coupled to cholic acid via the 3-hydroxy position led to a significantly improved targeting of the hepatocytes compared to unmodified oligonucleotides. However, no significant improvement of hepatocellular uptake for these macromolecules could be determined yet.[8] For specific treatment of liver infections like hepatitis or malaria, bile acid conjugated oligonucleotides seem to be useful candidates for a better targeting of hepatocytes.

HCV is the major cause of more than 90% of transfusion-associated, chronic non-A non-B hepatitis worldwide. Acute infections progress to a chronic state in greater than 50% of patients. Chronic infection can lead to liver cirrhosis and associated development of hepatocellular carcinoma within 10–15 years.[13] Treatment of chronic HCV infection with a combination of interferons and the nucleoside analogue ribavirin is not satisfactory. Therefore several attempts have been undertaken to fight HCV with antisense oligonucleotides.[14–19] HCV is an enveloped positive strand RNA virus with a genome size of approximately 9.4 kb, which encodes a single large open reading frame of about 3010 amino acids.[20] The highly conserved 5′-noncoding region (NCR) forms a characteristic secondary structure[21,22] that exerts the function of an internal ribosomal entry site (IRES).[23,24] Thus the 5′-NCR of HCV represents a promising target to inhibit viral gene expression by an antisense approach.

Recently, we synthesized terminally backbone modified phosphorothioates, methyl- and benzylphosphonates targeting stem/loop structures within the 5′-NCR of the hepatitis C virus (HCV) genome and the adjacent core region (nucleotides 326–348). These 23mer AS-ODNs contained three modifications at each end of the sequence and inhibited viral gene expression in a dose dependent manner combined with experimental evidence for RNase H activity.[15,25–27] Best inhibition in cell culture was affected with terminally modified benzylphosphonates.[26] After discovering a 17mer (nucleotides 326–342) as the minimal inhibitory sequence, our efforts concentrated on improving liver specific drug targeting and hepatocellular uptake by coupling effective AS-ODNs to biomolecules such as cholesterol or bile acids. Within this paper, the synthesis of phosphoramidites of cholesterol, cholic acid and taurocholic acid is described. Their solid-phase coupling to base labile methyl- and benzylphosphonates followed by mild deprotection led to modified oligonucleotides with enhanced lipophilic properties and stable duplex formation to complementary DNA and RNA target strands.

Results and Discussion

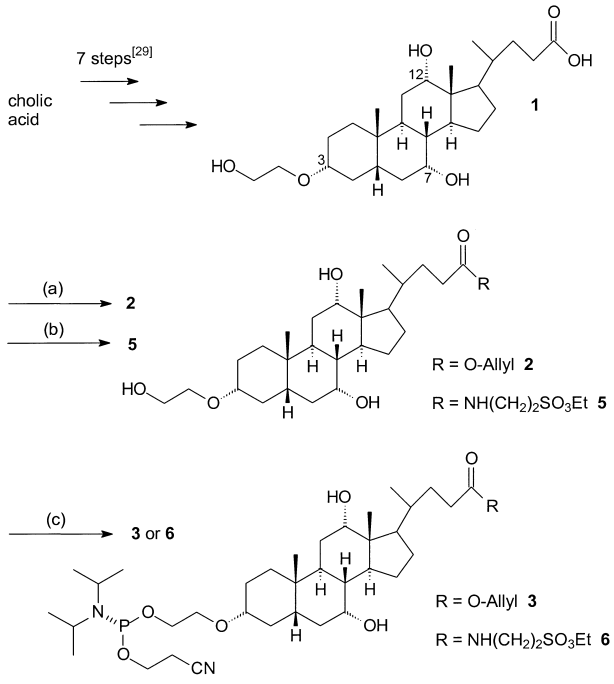
Synthesis of bile acid phosphoramidites

For the synthesis of bile acid conjugated AS-ODNs containing base labile alkylphosphonate linkages, mild conditions for the deprotection of the esterified carboxyl group or esterified sulfonic acid must be found. A suitable protection of the carboxyl group located in the side chain of cholic acid proved to be the allyl ester. Mild deprotection of allyl esters by treatment with tetrakis(triphenylphosphine)-palladium(0), triphenylphosphine and diethylammonium hydrogencarbonate liberated the carboxylic acid effectively.[28] Earlier allyl esters have been used in nucleotide chemistry to protect the internucleotide bond.[29]

Starting from 3α-(2-hydroxyethoxy)cholic acid **1**, which was obtained in seven steps from inexpensive cholic acid,[30] the allyl ester **2** has been easily prepared by reaction of **1** with acidic allyl alcohol in 80% yield.[31] Allyl ester **2** was converted into its phosphoramidite **3** by reaction of **2** with 1.1 equiv *rac*-chloro-(2-cyanoethyl)-*N,N*-diisopropylphosphine in the presence of 4 equiv diisopropylethylamine within 2 h. Previously, we carried out the same sequence of reactions with 7α- and 12α-acetylated derivatives of **1** and **2**. Surprisingly deacetylation of acetylated 7α- and 12α-hydroxyl groups failed with conc ammonia at 55 °C overnight. Looking for another protection/deprotection strategy we found, that the 7α- and 12α-hydroxyl groups could remain unprotected due to their low reactivities. The phosphitylation reaction occurred with high regioselectivity at the primary 2-hydroxyethoxy-spacer. Only a small amount of bisphosphitylated product could be obtained if a small excess of the phosphitylating reagent was used. The strategy to modify oligonucleotides at their 5′-ends via phosphoramidite chemistry with non nucleotidic groups or biomolecules was developed by Uhlmann and Engels.[32]

Synthesis of taurocholic acid conjugated AS-ODNs also started from 3α-(2-hydroxyethoxy)cholic acid **1**. Before the amidation of **1** was carried out, taurine derivative **4**

has been synthesized. Esterification of the sulfonic acid succeeded in refluxing 2-aminoethanesulfonic acid (taurine) in triethylorthoacetate for 48 h.[33] Unreacted taurine was filtered and the solvents were evaporated. The crude resulting liquid contained 2-aminoethanesulfonic acid ethylester **4** as shown by NMR and mass spectrometry. Further purification failed as well as esterification of taurine silver salts with ethyl iodide.[34] Synthesis of the esterified taurine derivative **5** was effected by treatment of **1** with *n*-tributylamine and ethyl chloroformate. For amidation, the mixed anhydride was reacted with a solution of crude 2-aminoethanesulfonic acid ethylester **4** in water/dioxan (1:1) to yield **5** in 82%.[35] Amidation to taurocholic acid derivatives directly before the phosphitylation made an independant synthesis of amidated cholic acid derivatives unnecessary. Phosphitylation to obtain phosphoramidite **6** was carried out analogously to the cholic acid derivative. The new phosphoramidites **3** and **6** were obtained as mixtures of two diastereomers. Purification by flash chromatography led to decomposition of **3** and **6**. Therefore, the well-dried crude phosphoramidites were used in solid-phase oligonucleotide synthesis (Scheme 1).



Scheme 1. Synthesis of monomeric phosphoramidites of cholic- and taurocholic acid **3** and **6 [R/S]**. Reagents and conditions: (a) 1% HCl in allyl alcohol, 20 h, rt; (b) *n*-tributylamine, ethyl chloroformate, 2-aminoethanesulfonic acid ethylester **4**, dioxane, 2 h, rt; (c) *rac*-chloro-(2-cyanoethyl)-*N,N*-diisopropylphosphine, DIPEA, CH_2C1_2, 2 h, rt; compounds **3** and **6** are mixtures of two diastereomers.

Purities up to 85% analyzed by ^{31}PNMR could be obtained. Due to the high electrophilicity of the α-carbon atom of sulfonic acid alkylesters, ammonolysis with ammonia or other nitrogen nucleophiles generates the free sulfonic acid during the deprotection procedure for AS-ODNs.[36] For the synthesis of cholesterol phosphoramidite **8**, 3β-(2-hydroxyethoxy)cholesterol **7** was phosphitylated as described for the bile acids.

Synthesis of backbone modified oligonucleotides 5′-tethered to bile acids

Scheme 2 shows the modifications which were introduced into a 17mer AS-ODN directed against nucleotides 326–342 of the non-coding-region of the HCV genome. For future determination of cell uptake, the AS-ODNs were partly labelled with fluorescein at their 3′-position. At each end of the sequence three phosphodiesters were modified as phosphorothioates, methyl- or benzylphosphonates[37] to obtain sufficient nucleolytic stability. The 5′-end was coupled to cholesterol or bile acids.

The synthesis of oligonucleotides was carried out on a PerSeptive Biosystems DNA-synthesizer under standard conditions. Coupling times for methyl- or benzyl phosphoramidites, cholesterol- or bile acid phosphoramidites were enhanced to 300 s. For mild deprotection of solid phase bound alkyl modified oligonucleotides the 5′-conjugated cholic acid allylester was treated for 18 h with a solution of tetrakis(triphenylphosphine)-palladium(0), triphenylphosphine and diethylammonium hydrogencarbonate in dichloromethane.[28] After removal of the palladium reagent with aqueous diethyldithiocarbamate, cleavage from the solid support was affected with ammonia within 1 h. Deprotection of the nucleobases in the presence of alkylphosphonates afforded treatment with a mixture of ethylenediamine/ethanol/acetonitrile/water (50.0:23.5:23.5:3.0; v:v:v:v).[38] Benzyl modified oligonucleotides were also permitted to be deprotected by careful treatment with ammonia at room temperature for 24 h. Cleavage and deprotection of base labile oligonucleotides conjugated to esterified taurocholic acid- or cholesterol-modified oligonucleotides is identical with no necessity for the palladium(0) mediated ester hydrolysis (Scheme 3).

Oligonucleotides were purified by RP-HPLC with gradients of acetonitrile in 0.1 M TEAA. Fluorescein labeled oligonucleotides were additionally purified with preparative polyacrylamide gel chromatography. Characterization was achieved by analytical RP-HPLC, analytical PAGE and electrospray ionization (ESI) mass spectrometry as shown in Table 1.

5′- **X,Y,Z** - T#G#G#T G C A C G G T C T A#C#G#A - **F** -3′

= phosphodiester modified as phosphorothioate, methyl- or benzylphosphonate

F = 3′-fluorescein; **X** = 5′-cholesterol; **Y** = 5′-cholic acid; **Z** = 5′-taurocholic acid

Scheme 2. Design of AS-ODNs directed against the HCV genome.

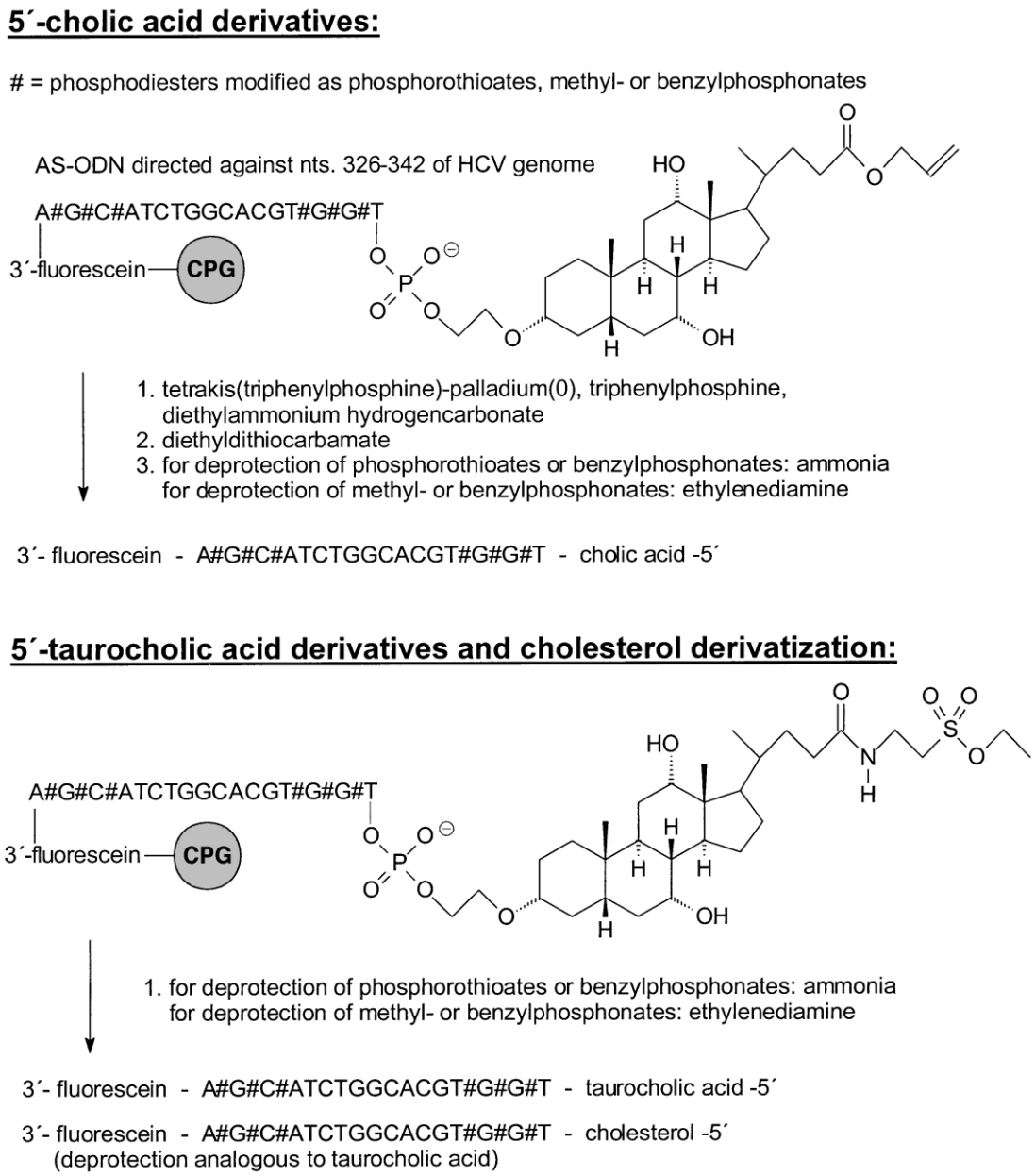
Lipophilicity of modified oligonucleotides

The lipophilic character of different modified AS-ODNs directed against nucleotides 326–342 of the hepatitis C virus genome was investigated by RP-HPLC. An acetonitrile gradient from 0 to 60% in 0.1 M TEAA from 8 to 28 min was used to determine the elution times of cholesterol-, cholic acid-, taurocholic acid- and fluorescein-modified AS-ODNs. In addition, the influence on lipophilicity of terminally modified benzylphosphonates compared to phosphorothioates was examined (Table 2).

The modification of the phosphodiester backbone with three benzylphosphonates at each end of the sequence enhances the lipophilicity equally like the conjugation of a bile acid to a terminally modified phosphorothioate. Coupling of a bile acid to a benzylphosphonate yields in addition to the lipophilicities as represented by the delayed elution times. As expected, coupling of cholesterol leads to the largest enhancement of lipophilicity, whereas the effect of the fluorescein dye is negligible.

Binding properties of AS-ODNs to DNA and RNA target sequences

UV melting curves of different modified AS-ODNs hybridized to DNA and RNA target sequences of different length were measured. The concentration of each strand was 2 μM in phosphate buffer (140 mM sodium chloride, 10 mM phosphate (NaH_2PO_4/Na_2HPO_4), pH = 7.0). The target strands were of the same length compared to the AS-ODNs or had an overlap of three nucleotides at each end (Table 3).



Scheme 3. Synthesis and deprotection of different modified AS-ODNs.

sense DNA 17: 5′-TCGTAGACCGTCCACCA -3′
sense DNA 23: 5′-GTCTCGTAGACCGTGCACCA TGA-3′
sense RNA 17: 5′-UCGUAGACCGUGCACCA-3′
sense RNA 23: 5′-GUCUCGUAGACCGUGCACCA UGA-3′

AS-ODNs directed against the HCV genome form stable duplices and heteroduplices to complementary target strands also with 3′- and 5′-overlaps. In DNA/DNA duplices the T_m values of benzylphosphonates are up to 2.4 °C lower than those of their phosphorothioate analogues. This can be due to steric hindrance of the

Table 1. Characterization of oligonucleotides by ESI mass spectrometry[a]

Compound	Derivatives of AS-ODN tS-13 directed against nucleotides 326-342 of HCV-RNA	Mass calculated (Da)	Mass found (Da) ESI $(M-H)^-$
Nucleotides of HCV-RNA:	342 326		
tS-13	T°G°G°TGCACGGTCTA°C°G°A	5322,8	5322,0
tS-13K	T°G°C°TGCACGCTCTA°G°G°A	5282,8	5282,3
tB-13	T*G*G*TGCACGGTCTA*C*G*A	5671,2	5669,7
tB-13K	T*G*C*TGCACGCTCTA*G*G*A	5631,2	5630,4
tS-13X	**X**- T°G°G°TGCACGGTCTA°C°G°A	5814,5	5814,4
tS-13KX	**X**- T°G°C°TGCACGCTCTA°G°G°A	5774,5	5774,3
tB-13X	**X**- T*G*G*TGCACGGTCTA*C*G*A	6162,9	6163,4
tB-13KX	**X**- T*G*C*TGCACGCTCTA*G*G*A	6122,9	6122,9
tS-13Y	**Y**- T°G°G°TGCACGGTCTA°C°G°A	5836,4	5836,8
tS-13KY	**Y**- T°G°C°TGCACGCTCTA°G°G°A	5796,4	5795,9
tB-13Y	**Y**- T*G*G*TGCACGGTCTA*C*G*A	6184,8	6185,4
tB-13KY	**Y**- T*G*C*TGCACGCTCTA*G*G*A	6144,8	6145,3
tS-13Z	**Z**- T°G°G°TGCACGGTCTA°C°G°A	5943,6	5944,8
tS-13KZ	**Z**- T°G°C°TGCACGCTCTA°G°G°A	5903,5	5904,0
tB-13Z	**Z**- T*G*G*TGCACGGTCTA*C*G*A	6291,9	6292,3
tB-13KZ	**Z**- T*G*C*TGCACGCTCTA*G*G*A	6251,9	6252,3
tS-13F	T°G°G°TGCACGGTCTA°C°G°A- **F**	5921,4	5920,8
tS-13FX	**X**- T°G°G°TGCACGGTCTA°C°G°A- **F**	6413,1	6415,6
tS-13FY	**Y**- T°G°G°TGCACGGTCTA°C°G°A- **F**	6435,0	6436,8
tS-13FZ	**Z**- T°G°G°TGCACGGTCTA°C°G°A- **F**	6542,1	6544,9

[a]°, phosphorothioate; *, benzylphosphonate; **F**, fluorescein; **X**, cholesterol; **Y**, cholic acid; **Z**, taurocholic acid; **K**, control AS-ODN; _, mismatches in control AS-ODNs.

Table 2. RP-HPLC elution times[a]

Compound	Derivatives of AS-ODN tS-13	Elution time (min)	Acetonitrile (%)
tS-13	T°G°G°TGCACGGTCTA°C°G°A	15.0	21
tB-13	T*G*G*TGCACGGTCTA*C*G*A	18.3	31
tS-13X	**X**-T°G°G°TGCACGGTCTA°C°G°A	25.7	53
tB-13X	**X**- T*G*G*TGCACGGTCTA*C*G*A	25.7	53
tS-13Y	**Y**-T°G°G°TGCACGGTCTA°C°G°A	18.0	30
tB-13Y	**Y**- T*G*G*TGCACGGTCTA*C*G*A	20.7	38
tS-13Z	**Z**- T°G°G°TGCACGGTCTA°C°G°A	18.0	30
tB-13Z	**Z**- T*G*G*TGCACGGTCTA*C*G*A	20.8	38
tS-13F	T°G°G°TGCACGGTCTA°C°G°A- **F**	16.0	24
tS-13FX	**X**-T°G°G°TGCACGGTCTA°C°G°A- **F**	25.7	53
tS-13FY	**Y**-T°G°G°TGCACGGTCTA°C°G°A- **F**	18.2	30
tS-13FZ	**Z**- T°G°G°TGCACGGTCTA°C°G°A- **F**	18.4	31

[a]°, phosphorothioate; *, benzylphosphonate; **F**, fluorescein; **X**, cholesterol; **Y**, cholic acid; **Z**, taurocholic acid.

Table 3. UV-melting curves of DNA and RNA targets with modified oligonucleotides[a]

Compound	Derivatives of AS-ODN tS-13	Sense DNA 17 (sense RNA 17)	sense DNA 23 (sense RNA 23)
tS-13	T°G°G°TGCACGGTCTA°C°G°A	65.1 (65.1)	64.6 (63.7)
tB-13	T*G*G*TGCACGGTCTA*C*G*A	62.7 (n.d.)	63.1 (58.2)
tS-13X	**X**- T°G°G°TGCACGGTCTA°C°G°A	65.3 (62.7)	66.4 (61.9)
tS-13Y	**Y**- T°G°G°TGCACGGTCTA°C°G°A	65.7 (n.d.)	65.2 (62.6)
tS-13Z	**Z**- T°G°G°TGCACGGTCTA°C°G°A	66.6 (64.9)	66.2 (61.6)
tS-13F	T°G°G°TGCACGGTCTA°C°G°A- **F**	65.2 (n.d.)	64.1 (64.3)

[a]°, phosphorothioate; *, benzylphosphonate; **F**, fluorescein; **X**, cholesterol; **Y**, cholic acid; **Z**, taurocholic acid; T_m, values in °C; n.d., not determined.

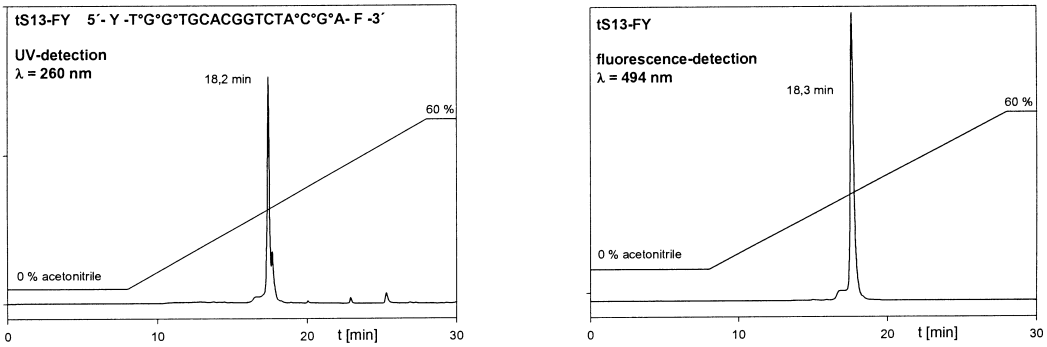


Figure. 1. Analytical RP-HPLC of fluorescein and cholic acid coupled AS-ODNs, Purospher® STAR RP 18(3μm), gradient from 0 to 60% acetonitrile in 0.1 M TEAA from 8 to 28 min, measured at $\lambda = 260$nm and $\lambda = 494$nm, elution time at 18.2.

large uncharged benzyl residues in the duplex. In heteroduplices the difference rises to 5.5 °C. The influence of 3′-fluorescein and/or 5′-steroid modification is negligible in both cases- hybridization to the 17mers and the 3′-5′-overlapping 23mers. Melting points of the investigated DNA/RNA heteroduplices are somewhat lower.

Summary and Conclusion

We have synthesized new bile acid phosphoramidite building blocks for solid-phase synthesis of bile acid coupled oligonucleotides. These building blocks have been coupled to the 5′-positions of different backbone modified AS-ODNs. The mild deprotection conditions facilitated the introduction of base labile methyl- and benzylphosphonates as phosphate backbone modifications, which were hydrolized under conditions described in the literature yet. For this purpose, the carboxyl group of cholic acid derivatives was protected as its allylester, which could be liberated by pre-treatment with tetrakis(triphenylphosphine)-palladium(0), triphenylphosphine and diethylammonium hydrogencarbonate on the solid support. The 5′-derivatization with esterified taurocholic acid required no pre-treatment. Base labile methyl- and benzylphosphonates were cleaved from solid support using a short incubation with ammonia followed by treatment with ethylene diamine for deprotection of exocyclic amino functions. Benzylphosphonates were also permitted to get deprotected by careful treatment with ammonia for 24 h at room temperature. Separation of bile acid conjugated oligonucleotides from sequences that failed the last coupling step was possible due to their difference in lipophilicity (see Table 2).

The bile acid conjugated oligonucleotides were characterized by ESI–MS, analytical PAGE and analytical RP-HPLC as shown in Figure 1 for the 3′-fluorescein and 5′-cholic acid coupled AS-ODN **tS-13FY**.

The elution times of the analytical RP-HPLC spectra correspond with the lipophilicities of the different modified AS-ODNs. Bile acid conjugation or three benzylphosphonates at each end of the oligonucleotide caused the same increase of elution times. Simultaneous incorporation of bile acids and benzylphosphonates led to an addition of prelonged elution times. Enhanced lipophilicity of these AS-ODNs directed against HCV may promote their cellular uptake and consequently their biological activity.

In addition, we investigated the effect of coupling bile acids or cholesterol to the 5′-end or the incorporation of different backbone modifications on hybrid stability with complementary DNA and RNA target strands. No significant change of duplex stability could be observed when terminally modified phosphorothioates were additionally 3′- or 5′-conjugated. Incorporation of sterical demanding benzylphosphonates led to a slow decrease of $-0.4\,^{\circ}C$ per modification compared to analogous phosphorothioates. Hybridization to RNA enhanced the destabilization up to $-0.9\,^{\circ}C$ per benzyl modification. Altogether, sufficient binding properties to complementary target strands could be found to use these oligonucleotides in an antisense approach.

However, this synthesis is still lacking specific protecting groups for the 7α- and 12α-hydroxyl groups, which are cleavable with the described deprotecting conditions for the AS-ODNs. Efficient protection of 7α- and 12α-hydroxyl groups reveals the possibility of a successful purification of the resulting phosphoramidites as seen for 7α- and 12α-acetylated phosphoramidites followed by a higher yield of the coupling reaction and suppression of bisphosphitylated side products.

In conclusion, we developed a new synthesis for coupling bile acids to base labile AS-ODNs. Bile acid or cholesterol modified AS-ODNs showed an enhanced lipophilicity as investigated by RP-HPLC and no significant loss of duplex stability due to nearly identical UV melting points as compared to unmodified phosphorothioates. Additionally, enhanced nucleolytic stability and activation of RNase H could be found.[26] Cellular RNase H cleaves selectively the target-RNA portion of an RNA–DNA heteroduplex. These properties makes them useful as potential liver specific antisense therapeutics.

Experimental

General

T_m values were performed with a UV-spectrometer (Varian cary 1) in phosphate buffer (140 mM sodium

chloride, 10 mM phosphate, pH = 7.0). For electrospray ionization, a mass spectrometer (VG Platform II with quadrupole analysator) was used.

3α-(2-hydroxyethoxy)-7α,12α-dihydroxycholic acid allylester (2). $C_{29}H_{48}O_6$ [492.70]. 3α-(2-Hydroxyethoxy)-cholic acid **1** was available as its chloroform adduct. 572 mg (1.0 mmol) of **1** were dissolved in 30 mL freshly distilled allyl alcohol. After addition of 1 mL concd HCl the solution was stirred for 20 h at ambient temperature. The reaction mixture was neutralized with 5% $NaHCO_3$ solution and evaporated in vacuo. The residual solid was resolved in diethylether and 5% $NaHCO_3$ solution and transferred into a separation funnel. After washing three times with 5% $NaHCO_3$ solution, the organic layer was dried over $MgSO_4$, evaporated to dryness and purified by flash chromatography on silica gel using dichloromethane:methanol (96/4, v:v). Yield: 396 mg (80%) pale yellow foam; mp: 155–156 °C; R_f 0.41 (CH_2Cl_2/CH_3OH, 9:1, v:v); 400 MHz, ^{1}H NMR ($CDCl_3$): 0.67 (s, 3H, H-18), 0.89 (s, 3H, H-19), 0.98 (d, 3H, H-21), 0.95–2.43 (24H), 3.14 (m, 1H, H-3β), 3.68, 3.57 (2×b, 2×2H, O–$\underline{CH_2}$–CH_2OH and O–CH_2–$\underline{CH_2}$OH), 3.84 (m, 1H, H-7β), 3.96 (m, 1H, H-12β), 4.56 (d, 2H, O–$\underline{CH_2}$–CH=CH_2), 5.22 (dd, 1H, *cis*-O–CH_2–CH=$\underline{CH_2}$), 5.31 (dd, 1H, *trans*-O–CH_2–CH=$\underline{CH_2}$), 5.91 (ddt, 1H, O–CH_2–$\underline{CH}$=CH_2); 100.6 MHz, ^{13}C NMR, ($CDCl_3$): 12.5 (C-18), 17.3 (C-21), 22.6 (C-19), 23.1, 26.6, 27.2, 27.4, 28.3, 30.7, 30.8, 31.2, 34.6, 34.9, 35.1, 36.2, 39.6, 41.4, 41.9, 46.5, 47.1, 62.1 (O–$\underline{CH_2}$–CH_2OH), 64.9 (O–$\underline{CH_2}$–CH=CH_2), 68.3 (C-7), 69.0 (O–CH_2–$\underline{CH_2}$OH), 72.8 (C-12), 79.7 (C-3), 118.1 (O–CH_2–CH=$\underline{CH_2}$), 132.3 (O–CH_2–$\underline{CH}$=CH_2), 173.9 (C-24); ESI-MS $[M+H]^+$: *m*/*z* 493.4.

[R_p/S_p]-{2-cyanoethyl, 2-[3α-(7α,12α-dihydroxycholic acid allylester)]-ethyl, *N*,*N*-diisopropylamino}-phosphine (3). $C_{38}H_{65}N_2O_7P$ [692.92]. The dried cholic acid allylester derivative **2** (493 mg, 1 mmol) was dissolved in 15 mL CH_2Cl_2 under argon atmosphere. To this solution, *N*,*N*-diisopropylethylamine (680 μL, 4 mmol) and *rac*-chloro-(2-cyanoethyl)-*N*,*N*-diisopropylphosphine (245 μL, 1.1 mmol) were added. After 2 h at room temperature the solution was transferred into a separation funnel and once rapidly quenched with 5% $NaHCO_3$ solution. The organic layer was filtered over $MgSO_4$ and evaporated to dryness in vacuo. Because purification by flash chromatography on silica gel led to decomposition the well dried, crude product was used for phosphoramidite coupling. Yield: 85% of all phosphorus containing products as a pale yellow foam; R_f 0.50 (CH_2Cl_2/CH_3OH, 9:1, v:v); 400 MHz, ^{1}H NMR ($CDCl_3$): 0.63 (s, 3H, H-18), 0.84 (s, 3H, H-19), 0.93 (d, 3H, H-21), 1.12, 1.12, 1.14, 1.14 (4d, 4×3H, 4×CH_3–isopropyl), 0.91–2.47 (24H), 2.63 (t, 2H, NC–$\underline{CH_2}$–CH_2OP), 3.12 (m, 1H, H-3β), 3.51–3.67 (m, 4H, O–CH_2–$\underline{CH_2}$OP and 2×CH–isopropyl), 3.75–3.83 (m, 4H, O–$\underline{CH_2}$–CH_2OP and NC–CH_2–$\underline{CH_2}$OP), 3.81 (m, 1H, H-7β), 3.90 (m, 1H, H-12β), 4.52 (d, 2H, O–$\underline{CH_2}$–CH=CH_2), 5.20 (dd, 1H, *cis*-O–CH_2–CH=$\underline{CH_2}$), 5.29 (dd, 1H, *trans*-O–CH_2–CH=$\underline{CH_2}$), 5.89 (ddt, 1H, O–CH_2–$\underline{CH}$=CH_2); 162.0 MHz, ^{31}P NMR, ($CDCl_3$): s at 148.98 and 149.19 ppm; ESI–MS $[M+H]^+$: *m*/*z* 693.7.

2-aminoethanesulfonic acid ethylester (4). $C_4H_{11}NO_3S$ [153.20]. 2-Aminoethanesulfonic acid (2.0 g, 16 mmol) was suspended in 30 mL triethylorthoacetate and heated to reflux for 48 h. Unreacted taurine was filtered and the solvents were evaporated in vacuo. The oily residue was dried with a vacuum pump and the crude product was used for amidation reactions, because purification by flash chromatography on silica gel led to decomposition. Yield: 1.75 g of a brown oil containing unkown impurities; 400 MHz, ^{1}H NMR ($CDCl_3$): 1.33 (t, 3H, CH_3–ethylester), 3.28 (t, 2H, S–$\underline{CH_2}$–CH_2–NH_2), 3.58 (t, 2H, S–CH_2–$\underline{CH_2}$–NH_2), 4.23 (q, 2H, CH_2–ethylester); ESI–MS $[M+H]^+$: *m*/*z* 154.1.

3α-(2-hydroxyethoxy)-7α,12α-dihydroxytaurocholic acid ethylester (5). $C_{30}H_{53}NO_8S$ [587.81]. 858 mg (1.5 mmol) of **1** were suspended in 10 mL freshly distilled dioxane under argon atmosphere. After addition of 382 μL (1.6 mmol) *n*-tributylamine, the clear solution was stirred for 30 min at ambient temperature. 200 μL (2.1 mmol) ethyl chloroformate were added and the solution was stirred for another 30 min. 1 g of the crude 2-aminoethanesulfonic acid ethylester **4** was dissolved in 6 mL of dioxane/water (1:1) and was added dropwise under generation of carbon dioxide to the mixed anhydride. After stirring another 75 min the solvents were evaporated in vacuo and the oily residue was purified by flash chromatography on silica gel using a gradient of 4–12% methanol in dichloromethane (v:v). Yield: 720 mg (82%) pale yellow foam; mp 75–78 °C; R_f 0.24 (CH_2Cl_2/CH_3OH, 9:1, v:v); 250 MHz, ^{1}H NMR ($CDCl_3$): 0.69 (s, 3H, H-18), 0.90 (s, 3H, H-19), 0.99 (d, 3H, H-21), 1.42 (t, 3H, CH_3–ethylester), 1.03–2.34 (24H), 3.15 (m, 1H, H-3β), 3.33 (t, 2H, S–$\underline{CH_2}$–CH_2–NH_2), 3.55–3.60 (m, 2H, O–CH_2–$\underline{CH_2}$OH), 3.65–3.75 (m, 4H, S–CH_2–$\underline{CH_2}$–NH_2 and O–$\underline{CH_2}$–CH_2OH), 3.85 (m, 1H, H-7β), 3.96 (m, 1H, H-12β), 4.32 (q, 2H,CH_2–ethylester), 6.57 (t, 1H, NH); 100.6 MHz, ^{13}C NMR, ($CDCl_3$): 12.5 (C-18), 15.1 (CH_3–ethylester), 17.4 (C-21), 22.5 (C-19), 23.2, 26.5, 27.2, 27.5, 28.2, 31.3, 32.8, 34.1 (S–CH_2–$\underline{CH_2}$–NH_2), 34.6, 35.0, 35.1, 35.2, 36.3, 39.5, 41.4, 41.9, 46.5, 46.6, 49.7 (S–$\underline{CH_2}$–CH_2–NH_2), 62.1 (O–$\underline{CH_2}$–CH_2OH), 66.8 (CH_2–ethylester), 68.3 (C-7), 68.9 (O–CH_2–$\underline{CH_2}$OH), 72.9 (C-12), 79.6 (C-3), 174.4 (C-24); ESI–MS $[M-H]^-$: *m*/*z* 586.6.

[R_p/S_p]-{2-cyanoethyl, 2-[3α-(7α,12α-dihydroxytaurocholic acid allylester)]-ethyl, *N*,*N*-diisopropylamino}-phosphine (6). $C_{39}H_{70}N_3O_9PS$ [788.03]. The phosphoramidite **6** was prepared from taurocholic acid derivative **5** (350 mg, 595 μmol) as described for the preparation of **3**. Yield: 80% of all phosphorus containing products as a pale yellow foam; R_f 0.50 (CH_2Cl_2/CH_3OH, 9:1, v:v); 400 MHz, ^{1}H NMR ($CDCl_3$): 0.63 (s, 3H, H-18), 0.84 (s, 3H, H-19), 0.93 (d, 3H, H-21), 1.11, 1.11, 1.13, 1.13 (4d, 12H, 4×CH_3–isopropyl), 1.38 (t, 3H, CH_3–ethylester), 1.03–2.28 (24H), 2.63 (t, 2H, NC–$\underline{CH_2}$–CH_2OP), 3.11 (m, 1H, H-3β), 3.33 (2t, 2H, S–$\underline{CH_2}$–CH_2–NH_2), 3.51-3.67 (m, 6H, O–CH_2–$\underline{CH_2}$OP and 2×CH–isopropyl and NC–CH_2–$\underline{CH_2}$OP), 3.74–3.86 (m, 5H, H-7β and S–CH_2–$\underline{CH_2}$–NH_2 and O–$\underline{CH_2}$-CH_2OP), 3.89 (m, 1H, H-12β), 4.26 (q, 2H, CH_2–ethylester), 6.38 (t, 1H, NH); 162.0 MHz, ^{31}P NMR, ($CDCl_3$): s at 149.07 and 149.24 ppm; ESI–MS [M-H]$^-$: *m*/*z* 786.8.

[R_p/S_p]-[2-(3β-cholesterol)-ethyl, 2-cyanoethyl, *N*,*N*-diisopropylamino]-phosphine (8). $C_{38}H_{67}N_2O_3P$ [630.94]. The phosphoramidite **8** was prepared from 3β-(2-hydroxyethoxy)-cholesterol **7** (431 mg, 1.0 mmol) as described for the preparation of **3**. In contrast to the bile acid phosphoramidites **3** and **6**, purification of **8** was effected by flash chromatography on silica gel using ethylacetate/*n*-hexane (1:1, v:v). Yield: 590 mg (94%) white amorphous solid; R_f 0.65 (CH_2Cl_2/CH_3OH, 9:1, v:v); 400 MHz, ^{1}H NMR ($CDCl_3$): 0.64 (s, 3H, H-18), 0.83 and 0.84 (2d, 6H, H-26 and H-27), 0.88 (d, 3H, H-21), 0.97 (s, 3H, H-19), 1.15, 1.15, 1.16, 1.16 (4d, 12H, 4×CH_3–isopropyl), 2.35–0.91 (28 H), 2.62 (t, 2H, NC–$\underline{CH_2}$–CH_2OP), 3.17 (m, 1H, H-3α), 3.53-3.87 (m, 8H, NC–CH_2–$\underline{CH_2}$OP and O–$\underline{CH_2}$–CH_2OP and O–CH_2–$\underline{CH_2}$OP and 2×CH–isopropyl), 5.31 (m, 1H, H-6); 100.6 MHz, ^{13}C NMR, ($CDCl_3$): 11.8 (C-18), 18.7 (C-21), 19.3 (C-19), 20.3, 21.0, 22.5, 22.8, 23.8, 24.3, 24.6 (2×CH_3–isopropyl), 28.0, 28.2, 28.4, 31.9, 35.7, 36.2, 36.8, 37.2, 39.0, 39.1, 39.5, 39.8, 42.3, 43.0 (2×CH–isopropyl), 50.2, 56.1, 56.8, 58.4 and 62.9 and 67.8 (3C, O–CH_2–$\underline{CH_2}$OP and O–$\underline{CH_2}$–CH_2OP and NC–CH_2–$\underline{CH_2}$OP), 79.4 (C-3), 117.6 (NC–CH_2–CH_2OP), 121.5 (C-6), 140.9 (C-5); 162.0 MHz, ^{31}P NMR, ($CDCl_3$): s at 149.00 and 149.03 ppm; ESI-MS $[M+H]^+$: *m*/*z* 631.6.

Oligodeoxynucleotide synthesis

All oligonucleotides were synthesized at one micromolar scale on a PerSeptive Biosystems DNA-synthesizer (Expedite 8905) under standard conditions. Solutions of phosphoramidites in dry acetonitrile had a minimum of 100 mM on concentration. To solve the cholesterol phosphoramidite **8** one-third of dichloromethane was necessary. Coupling times for methyl- or benzyl phosphoramidites, cholesterol- or bile acid phosphoramidites were enhanced to 300 s. Deprotection of different modified oligonucleotides afforded special conditions. 5′-Cholic acid modified oligonucleotides still bound to the solid support were treated with a solution of tetrakis(triphenylphosphine)-palladium(0), triphenylphosphine and diethylammonium hydrogencarbonate in dichloromethane for 18 h to cleave the allylester. Removal of the palladium reagent required treatment with aqueous diethyldithiocarbamate for 30 min. For 5′-taurocholic acid conjugated oligonucleotides direct treatment with nitrogen nucleophiles was possible due to the higher electrophilicity of the α-C-atoms of alkylsulfonates compared to the electrophilicity of the sulfur. Cholesterol has no further functional groups and there was also no special treatment necessary before cleavage from solid support and deprotection.

For methyl- and benzylphosphonates cleavage from solid support was carried out by treatment with ammonia for 1 h. After evaporation to dryness, deprotection of the nucleobases afforded 1 mL of a mixture of ethylenediamine/ethanol/acetonitrile/water (50.0:23.5:23.5:3.0; v:v:v:v). After 6 h at room temperature, the solution was neutralized by careful adding of acetic acid. Phosphorothioates and surprisingly benzylphosphonates could be deprotected by careful treatment with ammonia for 18 h at room temperature. The deprotection solutions were directly soaked onto a POROS-R3 RP-HPLC column and eluted with gradients of acetonitrile in 100 mM triethylammonium acetate. Characterization was carried out with analytical HPLC, analytical PAGE (16% polyacrylamide/8 M urea) and ESI–mass spectrometry. For biological assays the oligonucleotides were precipitated as their sodium salts. The isolated yields were in the range of 10–20 A_{260}-units for cholic acid- and taurocholic acid derivatives and up to 40 A_{260}-units for cholesterol or 5′-unmodified oligonucleotides.

Acknowledgements

The authors would like to thank Dr. Gottfried Zimmermann and his coworkers for measuring NMR spectra, Hannelore Brill and Ilona Prieβ in recording mass spectra and Beate Conrady for their assistance in HPLC. Special thanks to Professor Dr. Wolfgang H. Caselmann and Dr. Matthias Serwe at the Rheinische Friedrich Wilhelms-University in Bonn, Germany for our productive cooperation on the HCV field.

References and Notes

1. Kramer, W.; Wess, G. *Eur. J. Clin. Invest.* **1996**, *26*, 715.
2. Manoharan, M.; Johnson, L. K.; Bennett, C. F.; Vickers, T. A.; Ecker, D. J.; Cowsert, L. M.; Freier, S. M.; Cook, P. D. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1053.
3. Manoharan, M.; Tivel, K. L.; Andrade, L. K.; Mohan, V.; Condon, T. P.; Bennett, C. F.; Cook, P. D. *Nucleosides Nucleotides* **1995**, *14*, 969.
4. Chow, T. Y.-K.; Juby, C.; Brousseau, R. *Antisense Research Drug Dev.* **1994**, *4*, 81.
5. Wess, G.; Kramer, W.; Bartmann, W.; Enhsen, A.; Glombik, H.; Müllner, S.; Bock, K.; Dries, A.; Kleine, H.; Schmitt, W. *Tetrahedron Lett.* **1992**, *3*, 195.
6. Wess, G.; Kramer, W.; Enhsen, A.; Glombik, H.; Baringhaus, K.-H.; Bock, K.; Kleine, H.; Schmitt, W. *Tetrahedron Lett.* **1993**, *34*, 817.
7. Wess, G.; Kramer, W.; Schubert, G.; Enhsen, A.; Baringhaus, K.-H.; Glombik, H.; Müllner, S.; Bock, K.; Kleine, H.; John, M.; Neckermann, G.; Hoffmann, A. *Tetrahedron Lett.* **1993**, *34*, 819.
8. Petzinger, E.; Wickboldt, A.; Pagels, P.; Starke, D.; Kramer, W. *Hepatology* **1999**, *30*, 1257.
9. Kramer, W.; Stengelin, S.; Baringhaus, K.-H.; Enhsen, A.; Heuer, H.; Becker, W.; Corsiero, D.; Girbig, F.; Noll, R.; Weyland, C. *J. Lipid Res.* **1999**, *40*, 1604.
10. Baringhaus, K.-H.; Matter, H.; Stengelin, S.; Kramer, W. *J. Lipid Res.* **1999**, *40*, 2158.
11. Kramer, W.; Wess, G.; Schubert, G.; Bickel, M.; Girbig, F.; Gutjahr, U.; Kowalewski, S.; Baringhaus, K.-H.; Enhsen, A.; Glombik, H.; Müllner, S.; Neckermann, G.; Schulz, S.; Petzinger, E. *J. Biol. Chem.* **1992**, *267*, 18598.
12. Kramer, W.; Wess, G.; Neckermann, G.; Schubert, G.; Fink, J.; Girbig, F.; Gutjahr, U.; Kowalewski, S.; Baringhaus, K.-H.; Böger, G.; Enhsen, A.; Falk, E.; Friedrich, M.; Glombik, H.; Hoffmann, A.; Pittius, C.; Urmann, M.a *J. Biol. Chem.* **1994**, *269*, 10621.
13. Saito, I.; Miyamura, T.; Ohbayashi, A.; Harada, H.; Katayama, T.; Kikuchi, S.; Watanabe, Y.; Koi, S.; Onji, M.; Ohta, Y.; Choo, Q.-L.; Houghton, M.; Kuo, G. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6547.

14. Wakita, T.; Wands, J. R. *J. Biol. Chem*. **1994**, *269*, 14205.
15. Alt, M.; Renz, R.; Hofschneider, P. H.; Paumgartner, G.; Caselmann, W. H. *Hepatology* **1995**, *22*, 707.
16. Vidalin, O.; Major, M. E.; Rayner, B.; Imbach, J.-L.; Trépo, C.; Inchauspé, G. *Antimicrob. Agents Chemother*. **1996**, *40*, 2337.
17. Hanecak, R.; Brown-Driver, V.; Fox, M. C.; Azad, R. F.; Furusako, S.; Nozaki, C.; Ford, C.; Sasmor, H.; Anderson, K. P. *J. Virol*. **1996**, *70*, 5203.
18. Brown-Driver, V.; Eto, T.; Lesnik, E.; Anderson, K. P.; Hanecak, R. C. *Antisense Nucl. Acid Drug Dev*. **1999**, *9*, 145.
19. Zhang, H.; Hanecak, R.; Brown-Driver, V.; Azad, R.; Conklin, B.; Fox, M. C.; Anderson, K. P. *Antimicrob. Agents Chemother*. **1999**, *43*, 347.
20. Choo, Q.-L.; Richman, K. H.; Han, J. H.; Berger, K.; Lee, C.; Dong, C.; Gallegos, C.; Coit, D.; Medina-Selby, A.; Barr, P. J.; Weiner, A. J.; Bradley, D. W.; Kuo, G.; Houghton, M. *Proc. Natl. Acad. Sci. U.S.A*. **1991**, *88*, 2451.
21. Wang, C.; Le, S.-Y.; Ali, N.; Siddiqui, A. *RNA* **1995**, *1*, 526.
22. Honda, M.; Brown, E. A.; Lemon, S. M. *RNA* **1996**, *2*, 955.
23. Tsukiyama-Kohara, K.; Iizuka, N.; Kohara, M.; Nomoto, A. *J. Virol*. **1992**, *66*, 1476.
24. Wang, C.; Siddiqui, A. *Curr. Top. Microbiol*. **1995**, *203*, 99.
25. Caselmann, W. H.; Eisenhardt, S.; Alt, M. *Intervirology* **1997**, *40*, 394.
26. Alt, M.; Eisenhardt, S.; Serwe, M.; Renz, R.; Engels, J. W.; Caselmann, W. H. *Eur. J. Clin. Invest*. **1999**, *29*, 868.
27. Caselmann, W. H.; Serwe, M.; Lehmann, T.; Ludwig, J.; Sproat, B. S.; Engels, J. W. *World J. Gastroentero*. **2000**, *6*, 626.
28. Hayakawa, Y.; Hirose, M.; Noyori, R. *J. Org. Chem*. **1993**, *58*, 5551.
29. Hayakawa, Y.; Uchiyama, M.; Kato, H.; Noyori, R. *Tetrahedron Lett*. **1985**, *26*, 6505.
30. Wess, G.; Kramer, W.; Bartmann, W.; Enhsen, A.; Glombik, H.; Müllner, S.; Bock, K.; Dries, A.; Kleine, H.; Schmitt, W. *Tetrahedron Lett*. **1992**, *33*, 195.
31. Grand, R.; Reichstein, T. *Helv. Chim. Acta* **1945**, *28*, 344.
32. Uhlmann, E.; Engels, J. W. *Tetrahedron Lett*. **1986**, *27*, 1023.
33. Trujillo, J. I.; Gopalan, A. S. *Tetrahedron Lett*. **1993**, *34*, 7355.
34. Lepoittevin, J.-P.; Benezra, C. *Tetrahedron Lett*. **1992**, *33*, 3875.
35. Norman, A. *Ark. Kemi* **1955**, *8*, 331.
36. Musicki, B.; Widlanski, T. S. *Tetrahedron Lett*. **1991**, *32*, 1267.
37. Samstag, W.; Engels, J. W. *Ang. Chem. Int. Ed. Engl*. **1992**, *31*, 1386.
38. Hogrefe, R. I.; Vaghefi, M. M.; Reynolds, M. A.; Young, K. M.; Arnold, L. J. *Nucleic Acids Res*. **1993**, *21*, 2031.