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Structural modifications of antisense oligonucleotides

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Abstract

Antisense oligonucleotides are efficient tools for the inhibition of gene expression in a sequence specific way. Natural oligonucleotides are decomposed rapidly in biological systems, which strongly restrict their application. In contrast, artificial oligonucleotides are designed to be more stable against degradation than the target mRNA, which results in a catalytic effect of the drug. Modification of the phosphate linkage has been the first successful strategy for antisense drug developments and Fomivirsene the first antisense drug in therapy. The launch of Fomivirsene has resulted in a revolutionary spin off to antisense research leading to a second generation of antisense oligonucleotides, which are stable against oligonucleotide cleaving enzymes. Among these, oligonucleotides bearing an alkoxy substituent in position 2′ were the most successful ones. The third generation of antisense oligonucleotides contains structure elements, which enhance the antisense action. Zwitterionic oligonucleotides show remarkable results, first, because the stability against ribozymes is largely increased, and secondly, because the electrostatic repulsion between the anionic sense and the zwitterionic antisense cords is minimized. Promising new target molecules in antisense reseach are oligonucleotide chimäres, which enhance the antisense action (chimäres with intercalators, chelators or polyamines) or enable an application as sequence specific detectors (chimäres with biotin, fluorescein or radioligands).

Keywords: Artificial oligonucleotides; Backbone modified oligonucleotides; 2′-Modified oligonucleotides; Zwitterionic oligonucleotides; Peptide nucleic acids

1. Introduction

The essential steps in rational drug design are the identification of an appropriate target responsible for a certain disease and the development of a drug with a specific affinity to that target. One of the most general approaches of drug targeting is the specific manipulation of gene expression at the DNA or RNA stage of protein synthesis. Recently, the elucidation of the pathogenic role of individual target proteins for certain diseases is rapidly progressing, most notably in cancer research. This development causes a large demand on specific tools for manipulating the synthesis of proteins involved in pathogenic processes. Certainly, antisense oligonucleotides are recognized to be very efficient tools for the inhibition of gene expression in a sequence specific way [1,2].

2. The antisense principle

The antisense principle is based on a specific recognition of certain DNA and RNA regions by an antisense oligonucleotide, which inhibits the translation by a selective pairing of the ‘sense’ with the complementary ‘antisense oligonucleotide strand. For construction of an efficient antisense oligonucleotide a sequence of twelve to twenty bases is sufficient to guarantee a specific recognition of the target DNA region [3]. The effects of an antisense oligonucleotide aims at a selective suppression of certain genes, which are responsible for the synthesis of undesired proteins. When the affinity of the antisense oligonucleotide towards the natural DNA or RNA strand exceeds a certain level, a suppression of the corresponding protein synthesis results [1,2] (Scheme 1).

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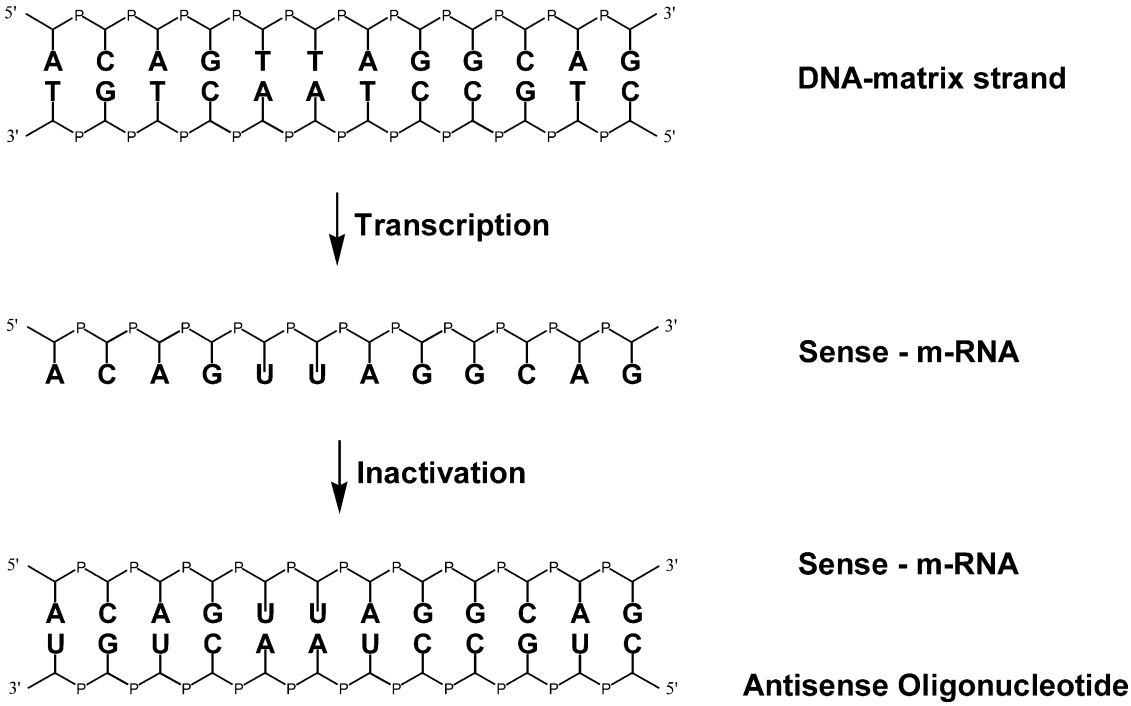
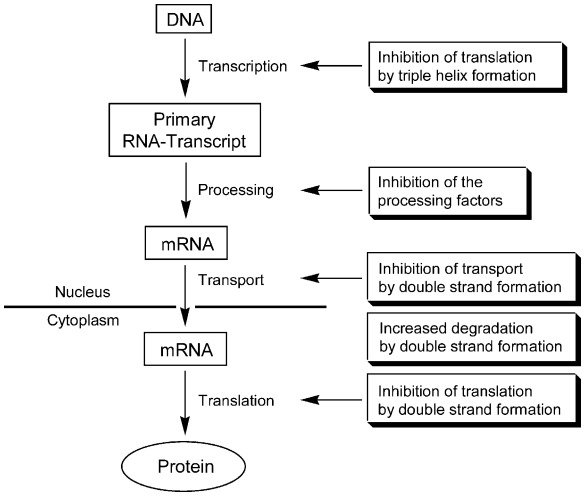


Scheme 1. Recognition of DNA and RNA regions by an antisense oligonucleotide.

3. Targets of antisense oligonucleotides

The first step of the protein synthesis may be inhibited by triple helix formation and successively blockade of transcription. Secondly, the antisense oligonucleotide may interfere with the processing proteins, which are responsible for the transformation of the primary DNA transcript into the maturated mRNA. Formation of a dual strand between the antisense oligonucleotide and the mRNA may disable the transport of the mRNA from the nucleus to the cytoplasm [3,4] (Scheme 2).

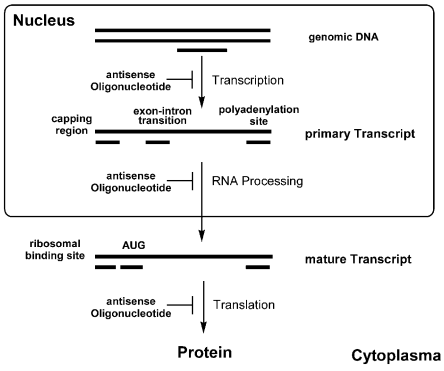


Scheme 2. The inhibition of the protein synthesis by antisense oligonucleotides.

Special targets on the mRNA are the capping region, the exon–intron-transition and the polyadenylation site. Dual strand formation in the cytoplasm results in a blockade of protein synthesis in the ribosomes. Finally, the aggregate formation between an antisense oligonucleotide and the mRNA enhances the degradation by ribozymes. If the antisense oligonucleotide is more stable against degradation than the mRNA, a catalytic effect results which multiplies the effect of the antisense drug [3,4] (Scheme 3).

4. Artificial antisense oligonucleotides

Disadvantages of natural oligonucleotides are the unsatisfactory binding affinity, the instability against



Scheme 3. Special targets on the mRNA for antisense oligonucleosides.

cellular nucleases, the insufficient membrane penetration and the low bioavailability.

One strategy to overcome these problems is the development of a suitable application system, which protects the oligonucleotide, enhances the penetration and enables a targeting to the compartment of action [5–8].

Another strategy is the structural modification of natural oligonucleotides leading to artificial structures. But structural modifications may lead to a loss of the base pairing ability. Thus, the most important test for a new artificial oligonucleotide is the measurement of the base pairing ability with natural DNA. The method of choice for this purpose is the study of the duplex stability by circular dichroism spectroscopy [9,10]. Certainly, the transition temperature (T_m) is the most common parameter characterizing the transformation of a double strand helix to a mixture of two single strand oligonucleotides. For the determination of the transition temperature, a series of circular dichroism spectra has to be measured at different temperatures (Scheme 4).

A second demand for an artificial antisense oligonucleotide is a sufficient stability against DNA and RNA cleaving enzymes. Natural oligonucleotides are decomposed rapidly in biological systems, which strongly restrict their application. In contrast, artificial oligonucleotides reveal in many cases increased stability towards the cleaving enzymes [1].

In principle structural modifications of antisense oligonucleotides may concern the oligonucleotide backbone, the sugar moiety and the nucleic bases (Scheme 5).

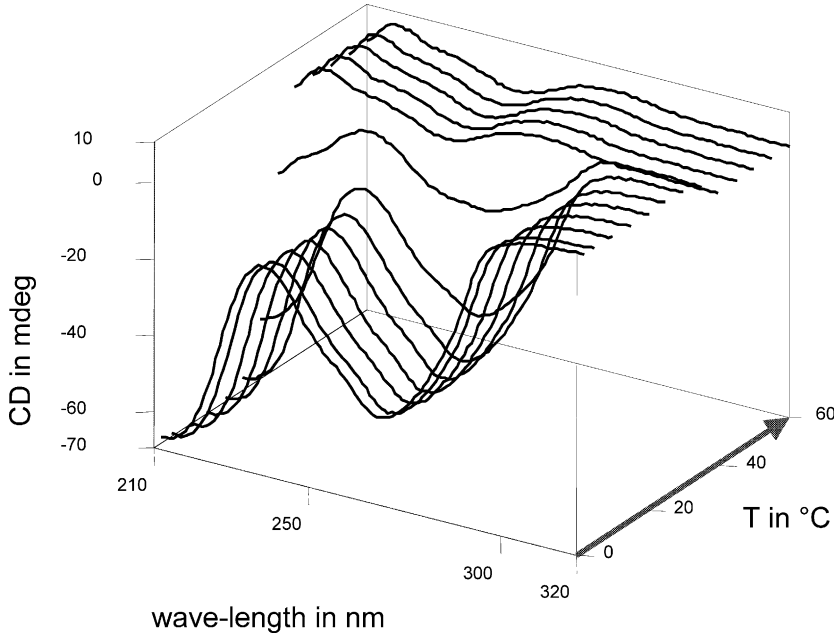
5. Backbone modified oligonucleotides (first generation)

Natural oligonucleotides are rapidly cleaved by nucleases attacking at the phosphoric ester bonds. Therefore, it is no surprise that modifications of the phosphate linkage were the first targets of antisense drug developments [1,11,12] (Scheme 6).

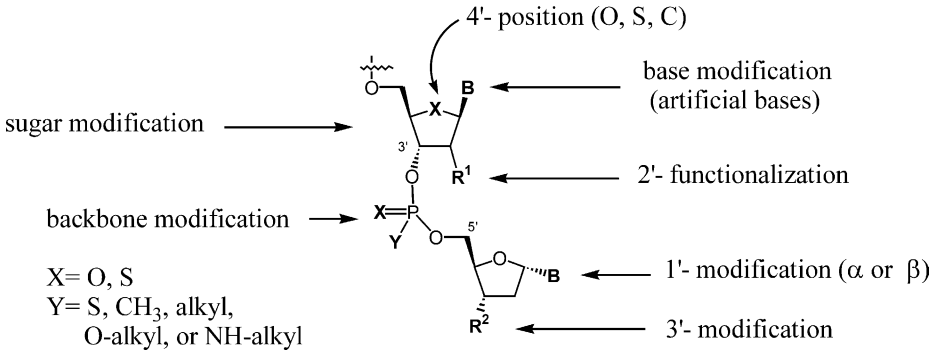
While synthesis of macromolecular nucleotides is nowadays is exclusively a field of the polymerase chain reaction, synthesis of natural (DNA and RNA) and of all types of modified oligonucleotides has remained a domain of chemical synthesis. Solid support based techniques, by which an oligomer is built up adding building blocks step by step, have been shown to be the most efficient approach in oligonucleotide synthesis. Out of a series of competitive methods, the so-called phosphoramidite method is by far the most important method for making the phosphorodiester bond [1,13] (Scheme 7).

Although the phosphoramidite method has been developed for the synthesis of natural oligonucleotides, it can proceed for the synthesis of backbone modified oligonucleotides as well, if modified protocols are used. Scheme 8 shows a selection of sulfurization reagents suitable for the synthesis of phosphorothioates. Remarkable are the shorted sulfurization times of recently developed reagents [14–16].

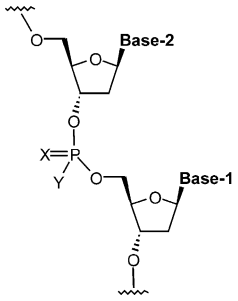
With respect to the stereochemistry, substitution of one unbonded oxygen atom of the prochiral phosphorus linkage in an oligonucleotide by sulfur, nitrogene or borane creates a chiral center leading to diastereomeric mixtures of R_P and S_P compounds. Since the number of



Scheme 4. Observation of the transformation of a double strand helix to a mixture of two single strand oligonucleotides by circular dichroism spectroscopy.



Scheme 5. Structural modifications of artificial antisense oligonucleotides.



X	Y	Trivial names
O	SH	Phosphorothioates
S	SH	Phosphorodithioates
O	Me	Methylphosphonates
O	OMe	Phosphoric acid triesters
O	NR_2	Phosphoramidates

Scheme 6. The structure of 'classical' backbone modified antisense oligonucleotides.

diastereomers increases by 2^n, this sums up to the formation of about half a million of diastereomeric compounds during the synthesis of a 20-mer. Although methods have been developed for the enantioselective synthesis of phosphorothioates [17–20], the majority of backbone modified oligonucleotides is prepared with unselective methods. Nevertheless, antisense activity remains intact in such diastereomeric mixtures [1,2] (Scheme 9).

5.1. Fomivirsene (Vitravene®)

(P-thio)-G-C-G-T-T-T-G-C-T-C-T-T-C-T-T-C-T-T-G-C-G-desoxy-ribonucleic acid Fomivirsene (ISIS 2922) is a phosphorothioate oligonucleotide which has been constructed to inhibit the replication of the human cytomegalo virus by an antisense mechanism. The nucleotide sequence of Fomivirsene is complementary to the major immediate early region 2 (IE2) of the human cytomegalo virus. This part of the viral genome is encoding a series of proteins, which are responsible for the virus replication.

In 1998 Fomivirsene has been approved by the US Food and Drug Administration (FDA) for the treatment of cytomegalo virus induced retinitis in patients with an acquired immune deficiency syndrome (AIDS). The antisense drug inhibits the virus replication in the target organ in submicromolar concentrations ($EC_{50} = 0.03 \pm 0.02$ μM) and proved to be active even when a therapy with other antiviral drugs like ganciclovir failed due to the development of a drug resistance. Side effects of Fomivirsene are limited to the treated eye(s), because the drug is administered locally [2,21].

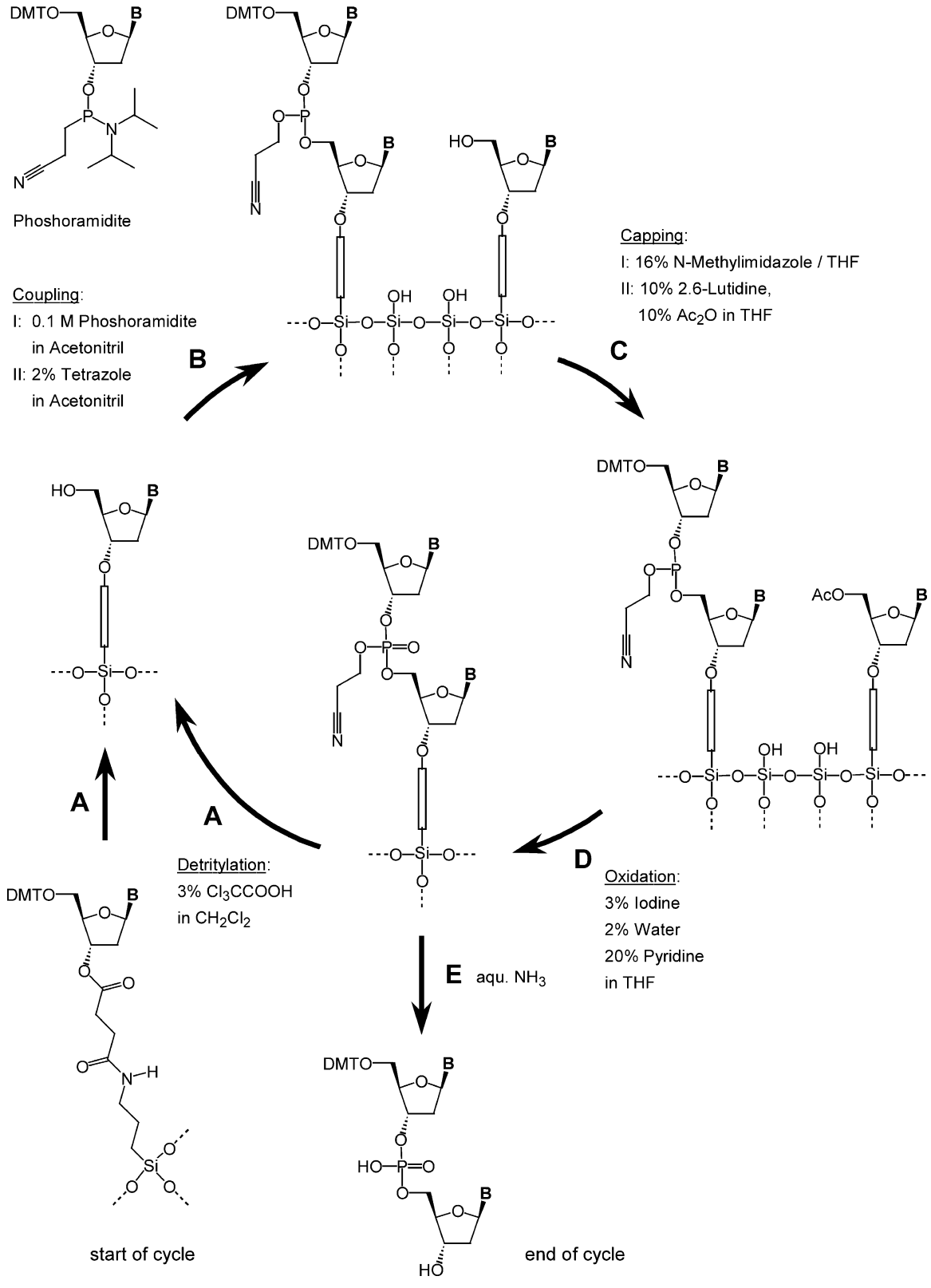
Although the field of application for Fomivirsene is rather limited in therapy, the launch of the first antisense drug resulted in a revolutionary spin off to antisense research. A sometimes seriously questioned innovative drug concept with gene therapeutic background has at once become an established drug therapy. This situation has generated a unique chance to look at this topic. Bearing in mind that antisense action may prevent synthesis of any pharmacologically relevant protein, this new type of drugs have been applicated to several novel disease targets (Table 1).

One of the drug candidates with a more general field of application is the antisense oligonucleotide Augmerosen.

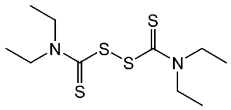
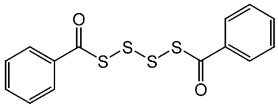
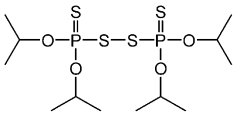
5.2. Augmerosen (Genasense, G3139)

(P-thio)-T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T-desoxy-ribonucleic acid Augmerosen (Genasense, G3139) is a phosphorothioate oligonucleotide which has been designed to inhibit the mRNA of the BCL2 group proteins. The 18-mer phosphothioate oligonucleotide is complementary to the first six codons of the open reading frame of BCL2 gene. BCL2 is an apoptosis inhibitor, which is implicated in the pathogenesis of cancer. High concentrations of this protein are observed in lymphatic and myelotic leukaemia, several types of lymphomas, human melanoma cells and other malignant tumors. Furtheron, the BCL2 group proteins are implicated not only in the pathogenesis of cancer but also in resistance to cancer treatment, because anticancer drugs and radiation ultimately destroy cells by induction of apoptosis [2,22] (Table 2).

Recently, several phase I and phase II studies have been started investigating the therapeutical utility of combinations of Augmerosen and classical anticancer drugs in patients with different cancer types (Table 3).



Scheme 7. The reaction cycle of the phosphoramidite method for the synthesis of DNA.

Reagent	Description	Sulfurization Time
	**Tetraethylthiuramdisulfide** (= TETD Reagent) 0.5 M in anhydrous acetonitril	15 min
	**Benzoyltetrasulfide** 0.4 M in THF or dichloromethane	1 min
	**Tetrakis-(2-methylethyl)-thioperoxy-diphosphate** 0.5 M in acetonitril	15 sec

Scheme 8. Some sulfurization reagents suitable for the synthesis of phosphorothioates.

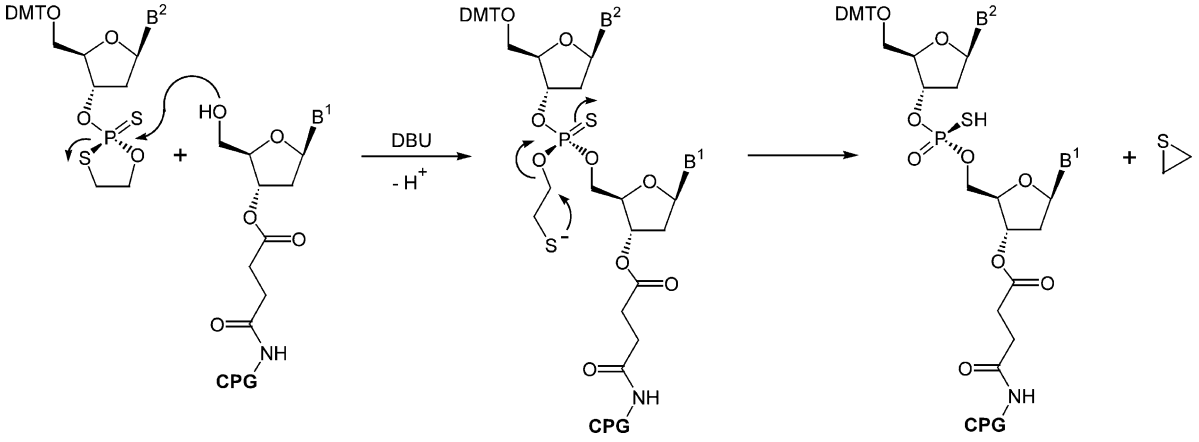
Generally, systemic treatment with phosphorothioate oligonucleotides is well tolerated and side effects are dose dependent [2].

6. 2′-Modified oligonucleotides (second generation)

Nevertheless, some fundamental requirements for an unrestricted use of antisense oligonucleotides in drug therapy have not been resolved satisfactorily till now. Responsible for this main insufficiency of the known antisense oligonucleotides is the structural similarity of phosphorothioate based antisense oligonucleotides to the natural DNA and RNA. This has caused the development of a second generation of structurally modified antisense oligonucleotides, which are based on more rigorous modifications of the oligonucleotide structure. One of the most attractive targets for variation of oligonucleotides is the sugar moiety, especially the 2′-position [23–27] (Scheme 10).

Table 1
Currently running antisense programs in pharmaceutical companies

Companies	Program
AVI BioPharma	restenosis
Calgene	antisense technology with plants
CV Therapeutics	restenosis
Cytoclonal Pharmaceutics	antisense screening
Enzo Biochem	antisense technology with plants, virustatics
Genta	restenosis, cardiovascular, oncology
Gilead Sciences	gene block technology
Hoffmann–La Roche	virustatics, HBV, HCV etc.
Human Genome Sciences	antisense technology via genomics
Hybridon	virustatics, HIV, HBV, HCV etc.
Immune Response Corp.	TGF-b antisense, oncology
INEX Pharmaceuticals	liposomal technology for antisense
Isis Pharmaceuticals	CMV, virustatics, oncology
Japan Tobacco	antisense technology with plants
Rhone–Poulenc	oncology



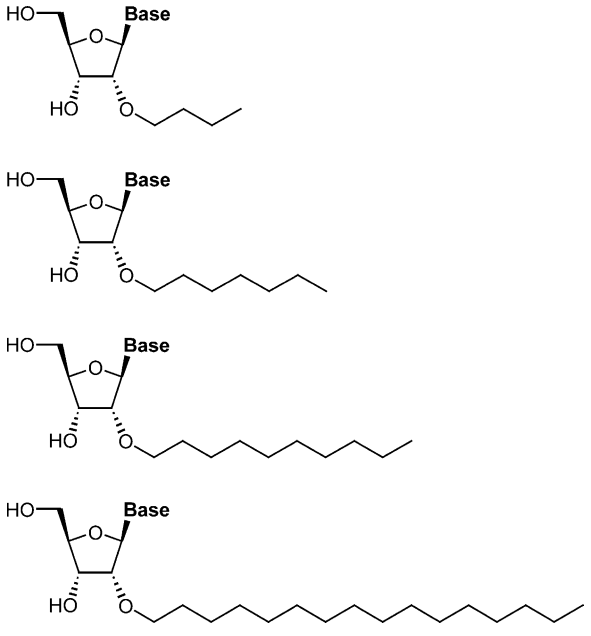
Scheme 9. A method for the enantioselective synthesis of phosphorothioates.

Table 2
Clinical trials investigating the therapeutical utility of combinations of Augmerosen and classical anticancer drugs

Type of cancer	Trial location	Status	Partner drug
Melanoma	University of Vienna, Austria	phase II	Dacarbazine
Prostate	Memorial Sloan-Kettering Cancer Center, NY, USA	phase I–II	Taxotere®
Prostate	University of Texas, TX, USA	phase II	Taxotere®
Lung (small cell)	Ohio State University, OH, USA	phase I–II	Taxotere®
Colorectal	University of Texas, TX, USA	phase II	Camptosar®
Breast	Georgetown University, Washington, DC, USA	phase I–II	Taxotere®
Acute leukaemia	Ohio State University, OH, USA	phase I–II	Fludara®

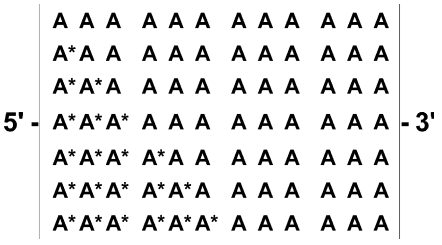
Table 3
Dosage dependence of side effects of phosphorothioates

Dosage (mg/kg)	Side effects
100	immune stimulation and hepatotoxicity in mice
80	clotting and complement effects in monkeys proximal tubular degradation
20	lymphoid hyperplasia in mice
10	inhibition of clotting times Complement activation atrophic and regenerative changes in proximal tubules
3	slight inhibition of clotting times
2.5	highest dosage in humans



Scheme 10. Structure of artificial 2′-*O*-alkyl-oligonucleotides [1].

Oligonucleotides bearing an alkoxy substituent in position 2′ have prove to be stable against DNA or RNA cleaving enzymes. This increased stability can be explained by a sterical hinderance due to the substituent in position 2′, which inhibits an attack of the DNA degrading enzymes at the phosphate groups [25–27] (Scheme 11).

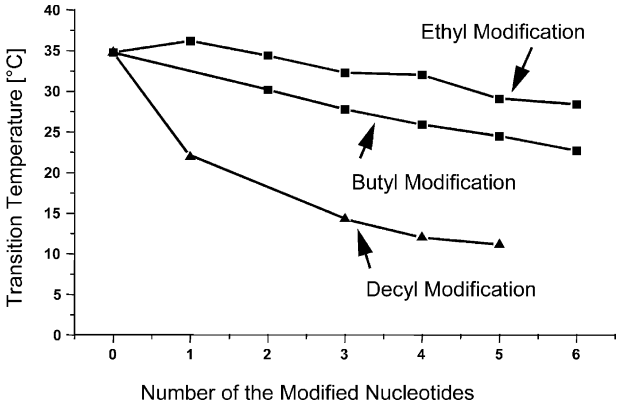


Scheme 11. Sequence of the model nucleotides of 2′-*O*-alkyl- and 2′-*O*-ethylenoxy-oligonucleotides.

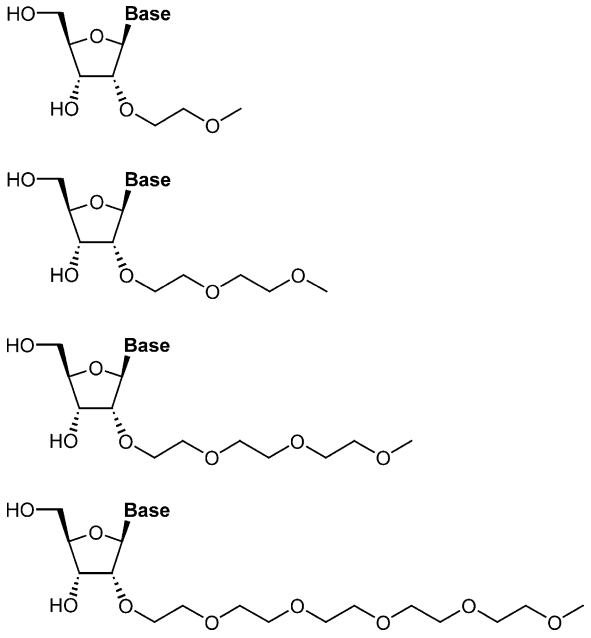
The base pairing ability of 2′-*O*-alkyl derivatives was investigated using artificial nucleotides, which were constructed with an increasing number of modified adenosine derivatives. A comparison with the unmodified 12-mer of adenosine revealed the influence of the substituents in position 2′ of the oligonucleotides [25–27] (Scheme 12).

In principle, any modification of the oligonucleotide backbone may result in a loss of the base pairing ability, but 2′-modified oligonucleotides have proved to form stable duplices. Stability of duplices with the complementary DNA or RNA strands decrease with the length of alkyl chain and with an increasing number of modified nucleotides in a given sequence [25–27] (Scheme 13).

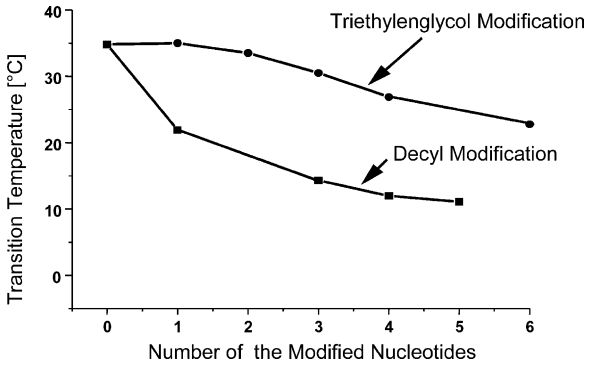
One reason for the low T_m values of oligonucleotides bearing long alkyl residues may be the high lipophility



Scheme 12. Impact of the alkyl substituents on the transition temperatures (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.0, 9 μM).



Scheme 13. Structure of artificial 2′-*O*-ethylenoxy-oligonucleotides.



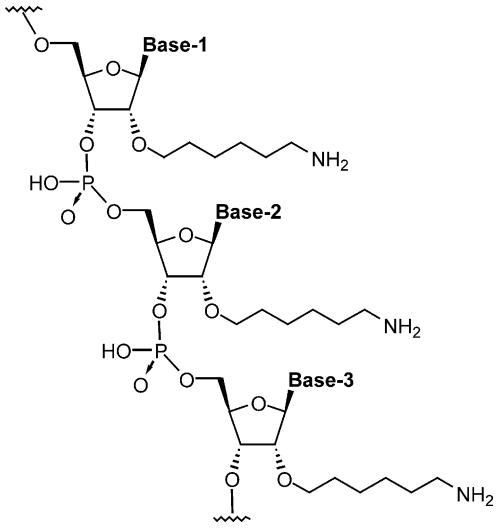
Scheme 14. Impact of the oxygen atoms on the transition temperatures (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.0, 9 μM).

of these alkyl substituents. Thus, the alkyl residues were substituted by ethylenoxy groups with an increasing number of ethylenglycol units [25–27] (Scheme 14).

The base pairing ability of 2′-*O*-ethylenoxy derivatives have been investigated by circular dichroism spectroscopy. Stability of duplices of 2′-*O*-ethylenoxy derivatives with complementary DNA strands proved to be more stable than duplices of the corresponding 2′-*O*-alkyl derivatives. Nevertheless, the duplex stability decreases with the length of alkyl chain and with an increasing number of modified nucleotides in a given sequence, but replacement of alkyl groups by an 2′-*O*-ethylenoxy moiety reduces this negative effect [25–27].

7. Zwitterionic oligonucleotides (third generation)

One of the most successful strategies for the design of artificial antisense oligonucleotides is the modification at the sugar moiety by introduction of a 6-aminohexyl



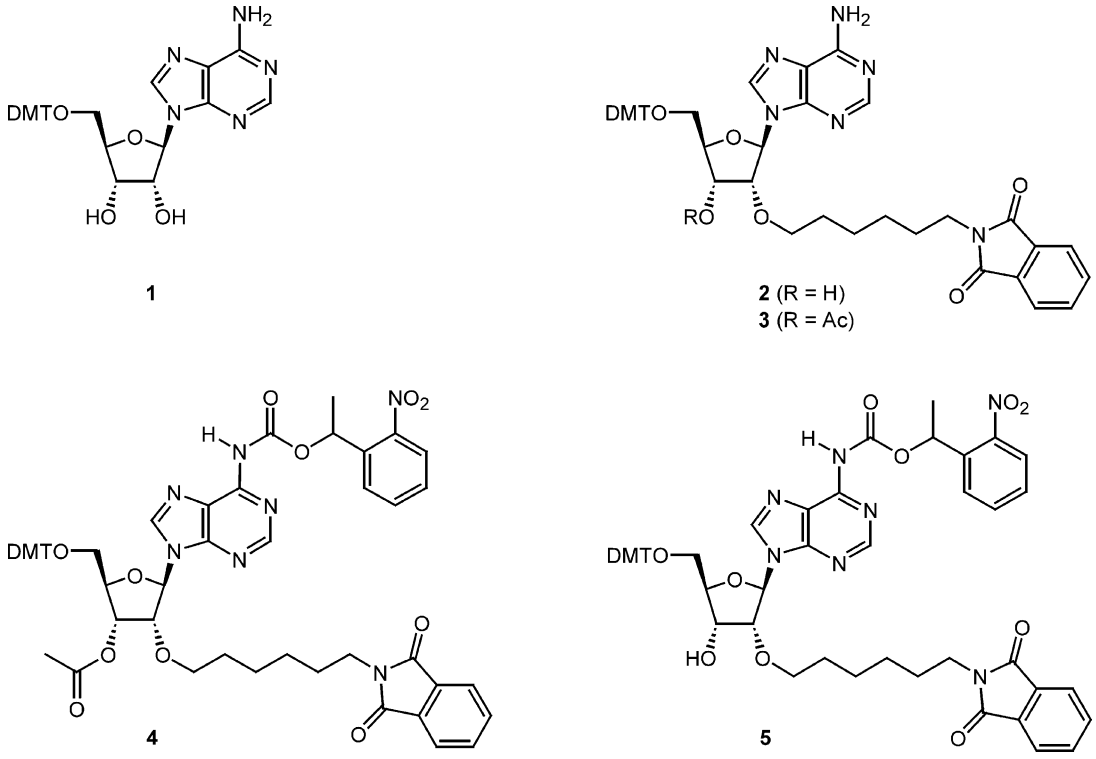
Scheme 15. Zwitterionic 2′-*O*-(6-aminohexyl)-modified oligonucleotides.

group, first, because the stability of the 6-aminohexyl-modified oligonucleotides against the catalytic degradation effect of ribozymes is largely increased, and secondly, because the electrostatic repulsion between the anionic sense and the zwitterionic antisense cords is minimized. The flexible alkyl group between the oxygen of the ribose and the terminal amino function acts as a spacer enabling an optimal arrangement of the amino group for a linkage to the complementary DNA or RNA strands [28,29] (Scheme 15).

For the synthesis of 6-aminohexyl-modified oligonucleotides suitably protected 6-aminohexyl-modified nucleotides are necessary. A representative strategy for the versatile choice of protecting groups can be deduced from a recent report on the synthesis of a 6-aminohexyl-modified adenosine derivative [30] (Scheme 16).

Thus, in the first step of the synthesis adenosine was reacted to the 5′-dimethoxytrityl derivative **1**. This readily soluble adenosine derivative proved to be the optimal intermediate for the introduction of a phthaloyl protected 6-aminohexyl group in position 2′. Acetylation of **2** under mild conditions resulted in a selective formation of the 3′-*O*-acetyl derivative **3**, which was protected at the amino function to yield the urethane derivative **4**. Finally, deprotection of **4** by methanolysis gave the key intermediate **5**, which is a versatile building block for the synthesis of zwitterionic oligonucleotides. Protective groups of the nucleoside **5** have been chosen according to state of the art to enable a versatile solid phase synthesis of artificial oligonucleotides by the phosphor-amidite method [30] (Scheme 17).

Studies on the acidity of uridine showed, that the imide NH can easier be deprotonated ($pK_a = 9.38$) than the hydroxy groups at C-2′ ($pK_a = 12.5$) or C-3′ and C-5′ ($pK_a = 14.5–16.5$). Thus, for the synthesis of 6-aminohexyl modified uridine derivatives the NH group



Scheme 16. Synthesis of the protected 2′-*O*-(6-aminohexyl)-adenosine derivative **5**.

must be protected before alkylation. Using strong bases like sodium hydride for deprotonation, DMF as a solvent and alkyl halide **7** as alkylating agent leads preferable (**8:9** = 70:30) to the 2′-*O*-alkyl nucleoside **8**, which was isolated by chromatography.

Reacting the stanyl derivative **10** and the alkyl halide **11** at high temperatures (130 °C) leads to the formation of equal amounts (**12:13** = 55:45) of 2′- and 3′-*O*-alkyluridines, which may be separated by medium pressure chromatography after dimethoxy-tritylation [29].

A comparison of the two alkylation methods revealed, that the stanyl-process is significantly superior, because no protection of the imido group is necessary, the yield in the alkylation step is higher, the chromatographic separation is easier and finally the protecting group at the side chain is removable by standard protocols from the oligonucleotides [29].

The duplex stability of 2′-*O*-(6-aminohexyl) derivatives was investigated using artificial nucleotides, which were constructed with an increasing number of modified uridine derivatives. A comparison with the unmodified 12-mer revealed the influence of the substituents in position 2′ of the oligonucleotides [29] (Scheme 18).

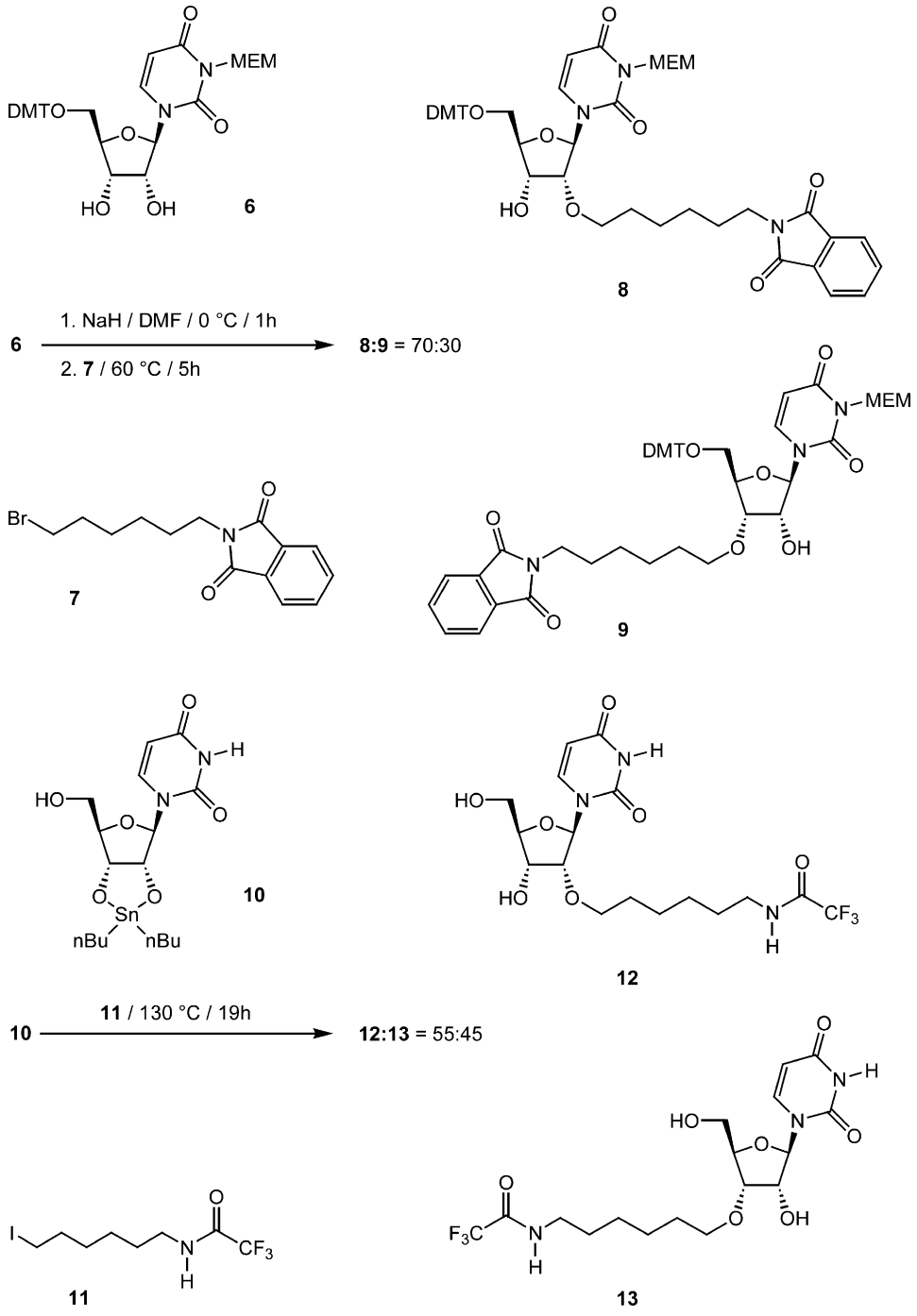
The base pairing ability of 6-aminohexyl modified uridine derivatives have been investigated by circular dichroism spectroscopy. Stability of duplices of 6-aminohexyl modified uridine derivatives with complementary DNA strands have proved to be more stable than duplices of the corresponding 2′-*O*-alkyl derivatives. Nevertheless, the duplex stability decreases with the length of the alkyl chain and with an increasing number of modified nucleotides in a given sequence, but replacement of alkyl groups by an 6-aminohexyl moiety reduces this negative effect [29] (Scheme 19).

Zwitterionic 2′-*O*-[L-lysyl-(6-aminohexyl)] oligonucleotides have been chosen as target molecules, because we expected, that an additional amino function should improve the linkage between the antisense and the complementary sense strands [29] (Scheme 20).

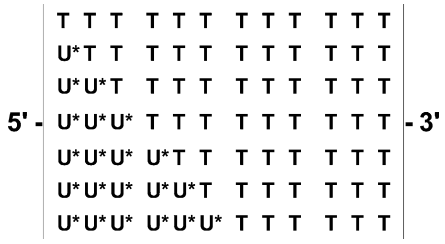
Synthesis of the required nucleoside building block **17** started from the phthaloyl-protected (6-aminohexyl)-uridine **14**. Deprotection by hydrazinolysis and subsequent acylation by the activated lysine derivative **16** gave the desired nucleotide **17** [29] (Scheme 21).

The base pairing ability of 2′-*O*-(6-aminohexyl) and 2′-*O*-[L-lysyl-(6-aminohexyl)] oligonucleotides has been investigated by circular dichroism spectroscopy. Stability of duplices with complementary DNA strands have been reported to decrease with an increasing number of modified nucleotides in a given sequence.

Generally, the linkage of additional lysyl residues does not affect the duplex stability of the model oligonucleotides-with one exception-the oligonucleotide, which contains six 2′-*O*-[L-lysyl-(6-aminohexyl)]-uridine



Scheme 17. A comparison of the two methods for the aminoalkyation of uridines the 'direct alkylation approach' vs. the 'stanylene process'.

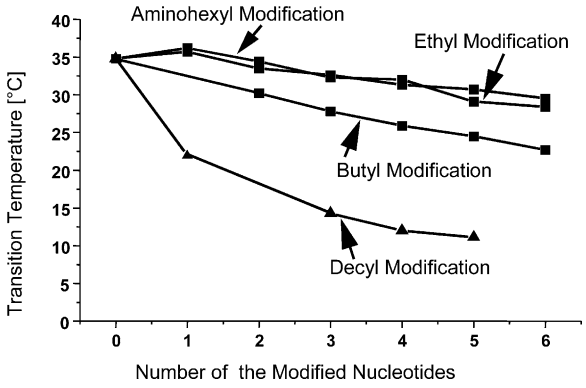


Scheme 18. Sequence of the model nucleotides.

units, shows a significant higher duplex stability than the other zwitterionic oligonucleotides [29] (Scheme 22).

8. Oligonucleotides with an amide type backbone

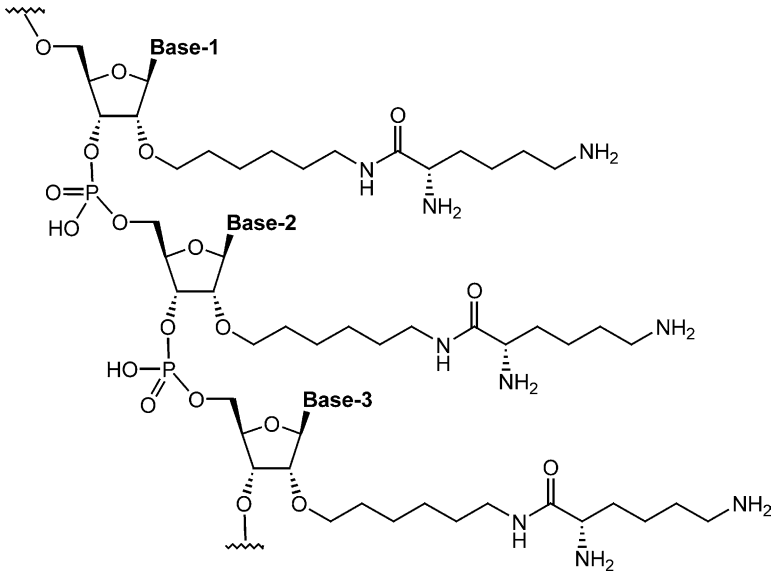
Replacement of natural structures by artificial bioisosteric elements is a general strategy in drug synthesis also valuable for nucleic acids. Peptide type nucleic acids have had a significant impact on antisense drug devel-



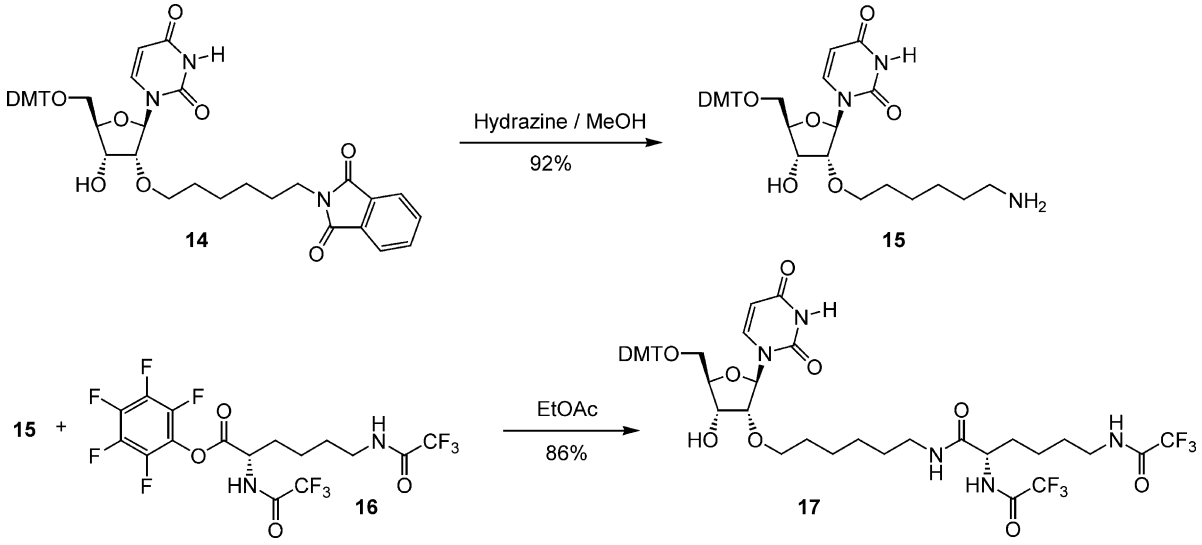
Scheme 19. Impact of the amino group on the transition temperatures (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.0, 9 μM).

opment, because they are achiral and neutral molecules [31,32]. Hybridization with DNA and RNA and formation of triple helices have been shown [33]. The oligomers are resistant to *endo*- and *exo*-nuclease mediated degradation as well as protease digestion [34]. Nevertheless, all drug candidates presently in the stage of clinical development exhibit only minor modifications of natural oligonucleotides [1,2]. One of the main reasons for the lower importance of peptide type nucleic acids is the lack of a well established method for the oligomerization reaction (Scheme 23).

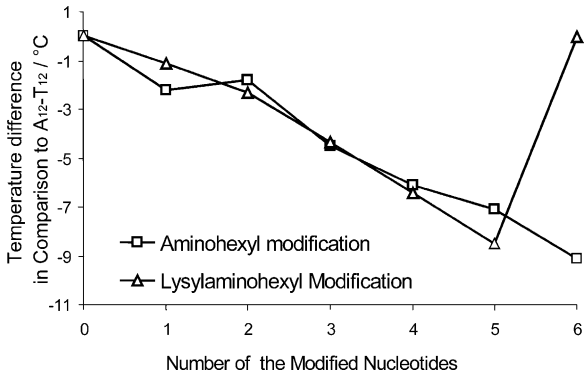
The peptide type oligonucleotide **B** has a backbone containing both phosphate and amide linkages in alternating sequence. Thus, hybrid **B** may exhibit both the properties of an artificial peptide type nucleic acid



Scheme 20. Zwitterionic 2′-*O*-[L-lysyl-(6-aminohexyl)] oligonucleotides.



Scheme 21. Synthesis of the protected 2′-*O*-[L-lysyl-(6-aminohexyl)]-uridine **17**.

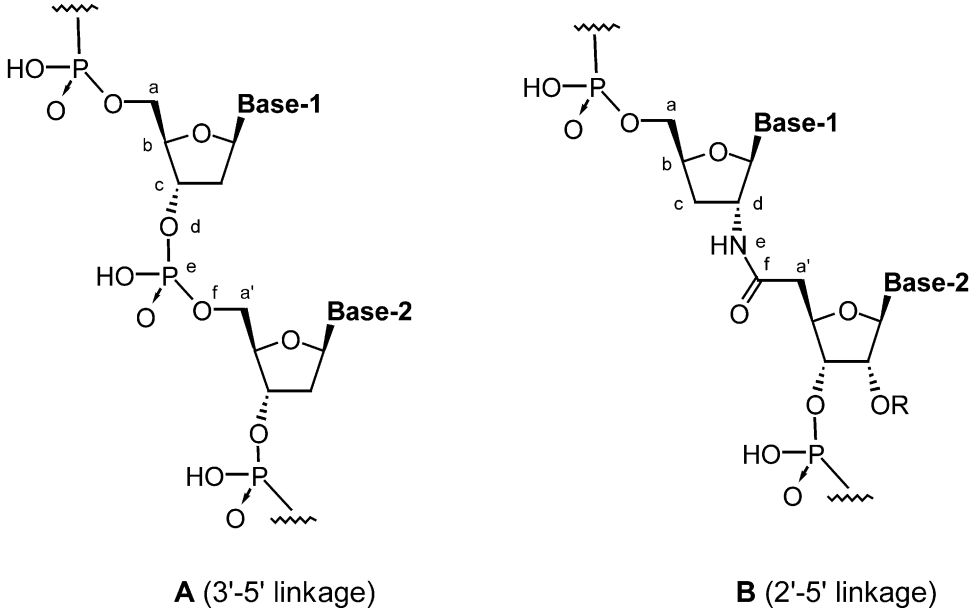


Scheme 22. Transition temperatures of zwitterionic oligonucleotides.

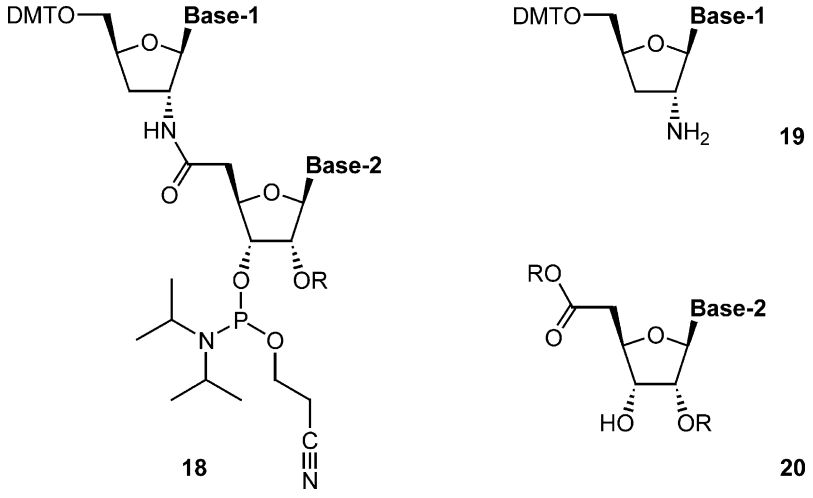
and the advantage of accessibility by the well established phosphoramidite method. Molecular dynamics calculations on models of **B** indicate, that double helices with natural DNA should be stable [35,36]. Nuclease resistance may be anticipated due to the character of substituents in position 2′ (Scheme 24).

Dinucleotide **18** is the logical building block for the synthesis of the oligonucleotide **B**. The preparation of dinucleotides **18** requires the 2-amino-2,3-didesoxyribose **19** and the 5-desoxy-allofuranose **20** as synthetic precursors [37,38].

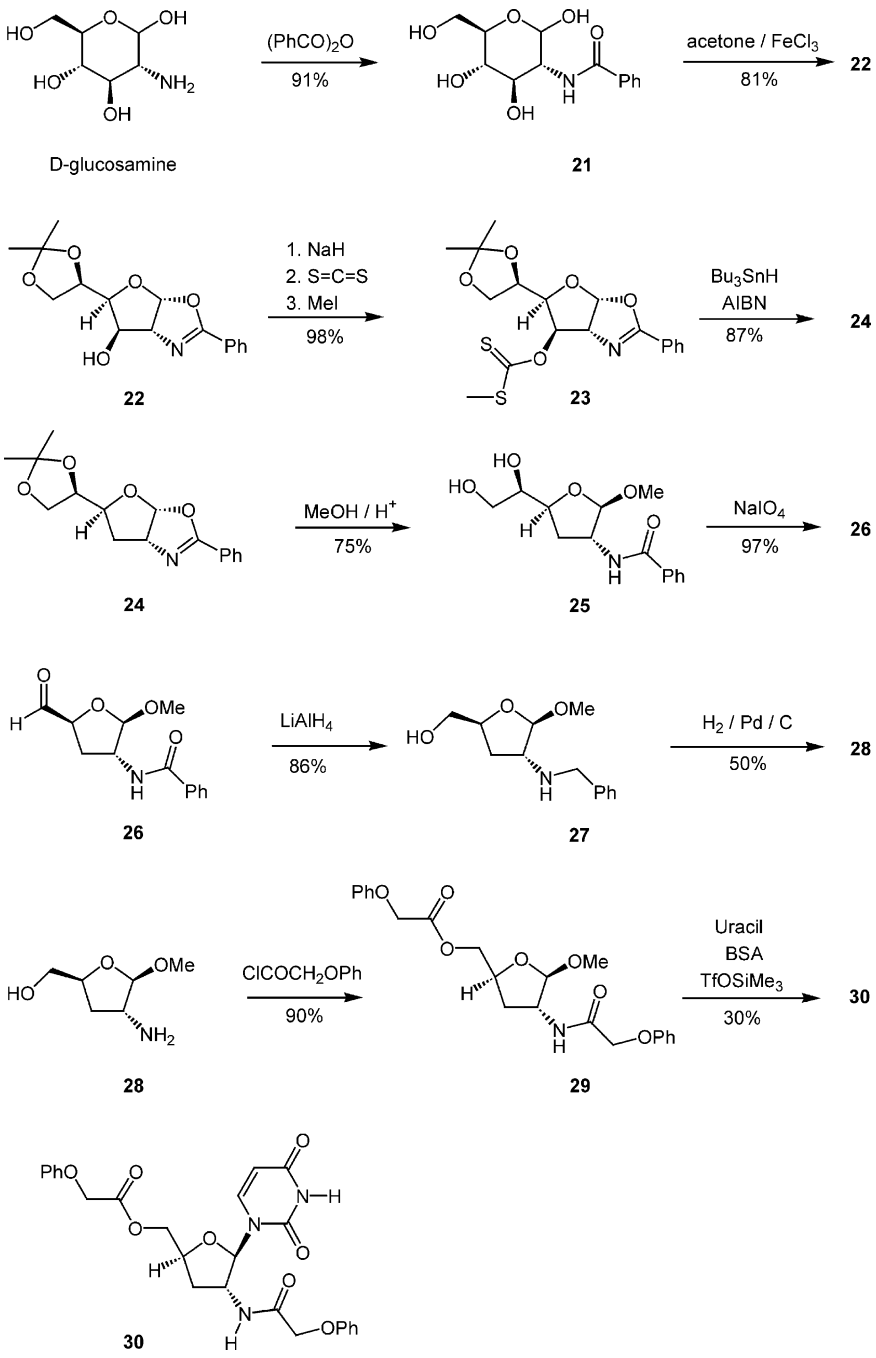
The unnatural 2-amino-ribose derivative **19** is not directly accessible from the chiral pool, but has to be prepared by a multi step reaction sequence from an available chiral starting material. We decided to use easily available D-glucosamine as adduct for the preparation of **19** [37], since the configurations of all important asymmetric carbon atoms were as desired



Scheme 23. A comparison of DNA (**A**) to an artificial peptide oligonucleotide bearing both phosphate and amide linkages in alternating sequence (**B**).



Scheme 24. Building blocks for the synthesis of the peptide type oligonucleotide **B**.



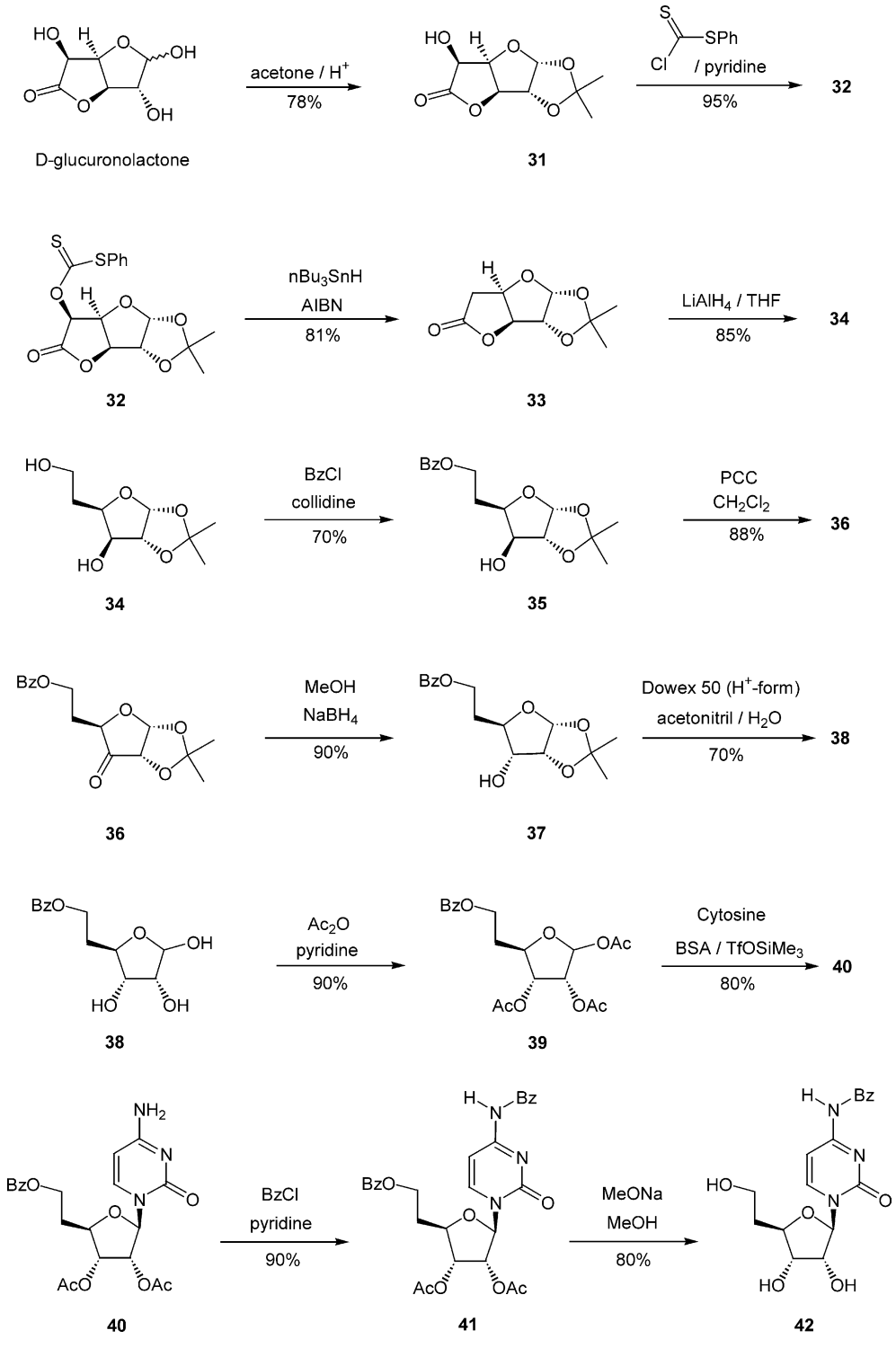
Scheme 25. Synthesis of the 2-amino-nucleoside **30**, a precursor for dinucleotide **18**.

and there was no need for introduction of the amino function.

After protecting the amino function of D-glucosamine, the resulting benzoate **21** [39] was reacted with acetone and Lewis acid to form **22** [40]. This oxazoline is an important intermediate product because the glucosamine is fixed in the desired furanose form and all reactive groups are protected with exception of the hydroxy group in 3. The free hydroxy group was transformed to the corresponding dithiocarbonate **23** by reaction with carbon disulfide and methyl iodide. Radical reaction of **23** with tributyltin hydride [41], activated by AIBN (α,α′-azoiso-butyronitril) yielded deoxygenated product **24**.

Treatment with toluenesulfonic acid opened the 5,6-acetonide ring and the oxazoline moiety too. Oxidative cleavage of **25** with sodium periodate gave ribose derivative **26**.

Reaction of **26** with lithium aluminum hydride resulted in a reduction of the aldehyde and benzylamide



Scheme 26. Synthesis of the artificial nucleoside **42**, a precursor for dinucleotide **18**.

functions to afford **27**. In the next reaction step the benzyl group was removed using catalytic hydrogenation to yield **28**. Finally, phenoxyacetylation and subsequent nucleosidation gave the desired nucleoside **30** (Scheme 25).

For the preparation of the 5-desoxy-allofuranose derivative **20** [38] we have chosen D-glucuronolactone as chiral precursor, because this sugar has the right number of carbon atoms and the configuration at the asymmetric carbons differs from D-allose only in position 3.

In the first reaction step D-glucuronolactone was transformed to the acetonide **31** according to a known procedure [40]. Acetonide **31** was recognized to be the

optimal intermediate for the following steps, because the desired furanose form is fixed by the acetal protecting group and secondly because carbon 5 was the only position bearing a free hydroxy function. The free hydroxy group was transformed to the corresponding dithiocarbonate **32** by reaction with carbon disulfide and methyl iodide. Radical reaction with tributyltin hydride [41] gave the desoxysugar **33**. Reaction with lithium aluminum hydride resulted in a reductive opening of the lactone ring to give **34**.

In the next step the primary hydroxy function at C-6 of **34** was selectively benzoylated to give **35**. Then the configuration at position 3 was inverted via oxidation of the D-configurated glucose derivative **35** to the corresponding ketone **36** and by selective reduction of ketone **36** with sodium borohydride to the L-configurated allose derivative **37**. Acid catalyzed hydrolysis resulted in a removal of the acetal protecting group to give derivative **38**. After peracetylation we obtained the 5-desoxy-allofuranose **39**, which is the key intermediate for the nucleosidation reaction. According to our expectations the nucleosidation of the 2′-*O*-acetyl derivative **39** gave high yields of the cytidine derivative **40**, which was finally protected at the amino group and selectively deprotected at the hydroxy functions leading to the desired artificial nucleotide **42** (Scheme 26).

In conclusion, we have prepared the 2-amino-nucleoside **30** [37] and the 5-desoxy-allo-furanose derivative **42** [38], which are interesting synthetic precursors for the synthesis of the dinucleotide **18**. Nevertheless, large efforts are necessary in the synthetic field of artificial nucleotides to realize an oligomerization reaction of dinucleotide **18** to oligonucleotide **B**.

9. A preview to future developments in antisense research

Finally, the question arises, what will be the next structural modifications of antisense oligonucleotides and what will be the new targets in antisense research in the near future (Table 4).

Table 4
New trends in antisense research in the near future

Effectors (enhance antisense action)
Intercalators
Ion chelators
Zwitterionic oligonucleotides
Modulators (modify biological effects)
Phosphorylation
Inhibitors of enzymatic degradation
Detectors (allow localization of oligonucleotides)
Biotinylation
Fluorescein-linked oligonucleotides
Radioactive oligonucleotides

First, antisense oligonucleotides may be combined with structure elements, which enhance the antisense action. Promising candidates are intercalators, ion chelating moieties and polyamine like structure elements, which may be bond covalently to antisense oligonucleotides.

Secondly, antisense oligonucleotides may be combined with structure elements, which modify the biological effects of oligonucleotide. Possible targets are the phosphorylation reaction and the enzymatic degradation of nucleotides.

Third, antisense oligonucleotides may be used as sequence specific detectors for the localization of certain oligonucleotides. For this purpose oligonucleotides should be coupled with biotin, with fluorescein like compounds or radioactive ligands.

Certainly, a novel generation of antisense oligonucleotides will rise up enabling an improved insight to the processes of gene regulation and affording an important contribution to modern drug development.

References

[1] C.R. Noe, L. Kaufhold, Chemistry of antisense oligonucleotides, in: Methods and Principles in Medicinal Chemistry, New Trends in Synthetic Medicinal Chemistry, 2000, pp. 261–347.
[2] I. Tamm, B. Dörken, G. Hartmann, Antisense therapy in oncology: new hope for an old idea, Lancet 358 (2001) 489–497.
[3] C. Helene, J.J. Toulme, Specific regulation of gene expression by antisense, sense and antigene nucleic acids, Biochim. Biophys. Acta 1049 (1990) 99–125.
[4] C.J. Marcus-Sekura, Techniques for using antisense oligodeoxyribonucleotides to study gene expression, Anal. Biochem. 172 (1988) 289–295.
[5] A. Zimmer, S. Atmaca-Abdel Aziz, M. Gilbert, D. Werner, C.R. Noe, Synthesis of cholesterol modified cationic lipids for liposomal drug delivery of antisense oligo-nucleotides, Eur. J. Pharm. Biopharm. 47 (1999) 175–178.
[6] H.-P. Zobel, A. Zimmer, S. Atmaca-Abdel Aziz, M. Gilbert, D. Werner, C.R. Noe, J. Kreuter, F. Stieneker, Evaluation of aminoalkylmethacrylate nanoparticles as colloidal drug carrier systems. Part I: synthesis of monomers, dependence of the physical properties on the polymerization methods, Eur. J. Pharm. Biopharm. 47 (1999) 203–213.
[7] H.P. Zobel, F. Stieneker, S. Atmaca-Abdel Aziz, M. Gilbert, D. Werner, C.R. Noe, J. Kreuter, A. Zimmer, Evaluation of aminoalkylmethacrylate nanoparticles as colloidal drug carrier systems. Part II: characterization of antisense oligonucleotides loaded copolymer nanoparticles, Eur. J. Pharm. Biopharm. 48 (1999) 1–12.
[8] H.P. Zobel, D. Werner, M. Gilbert, C.R. Noe, F. Stieneker, J. Kreuter, A. Zimmer, Effect of ultrasonication on the stability of oligonucleotides absorbed on nanoparticles and liposomes, J. Microencapsulation 16 (1999) 501–509.
[9] V.I. Ivanov, L.E. Minchenkova, A.K. Schyolkina, A.I. Poletayev, Different conformations of double-stranded nucleic acid in solution as revealed by circular dichroism, Biopolymers 12 (1973) 89–100.
[10] W. Saenger, Principles of Nucleic Acid Structure, Springer, New York, 1984.

[11] C. Cazenave, C.A. Stein, N. Loreau, N.T. Thuong, L.M. Neckers, C. Subasinghe, C. Helene, J.S. Cohen, J.J. Toulme, Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides, Nucleic Acids Res. 17 (1989) 4255–4273.
[12] A. Storey, D. Oates, L. Banks, L. Crawford, T. Crook, Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16, Nucleic Acids Res. 19 (1991) 4109–4114.
[13] S. Scott, P. Hardy, R.C. Sheppard, M.J. McLean, Innovations and perspectives in solid phase synthesis, Third International Symposium, Mayflower Worldwide, 1994.
[14] H. Vu, B.L. Hirschbein, Internucleotide phosphite sulfurization with tetraethylthiuram disulfide, phosphorothioate oligonucleotide synthesis via phosphoramidite chemistry, Tetrahedron Lett. 32 (1991) 3005–3008.
[15] M.V. Rao, C.B. Reese, Z. Zhengyun, Dibenzoyl tetrasulphide—a rapid sulfur transfer agent in the synthesis of phosphorothioate analogues of oligonucleotides, Tetrahedron Lett. 33 (1992) 4839–4842.
[16] W.J. Stec, B. Uznanski, A. Wilk, B.L. Hirschbein, K.L. Fearon, B.J. Bergot, Bis(*O*,*O*-diisopropoxy phosphinothioyl) disulfide—a highly efficient sulfurizing reagent for cost-effective synthesis of oligonucleotide phosphorothioates, Tetrahedron Lett. 34 (1993) 5317–5320.
[17] M. Fuji, K. Ozaki, A. Kume, M. Sekine, T. Hata, Acylphosphonates. 5. A new method for stereospecific generation of phosphorothioate via aroylphosphonate intermediate, Tetrahedron Lett. 26 (1986) 935–938.
[18] R. Cosstick, D.M. Williams, An approach to the stereoselective synthesis of Sp-dinucleoside phosphorothioates using phosphotriester chemistry, Nucleic Acids Res. 15 (1987) 9921–9932.
[19] B. Uznanski, A. Grajkowski, B. Krzyzanowska, A. Kazmierkowska, W.J. Stec, M.W. Wieczorek, J. Blaszczyk, Stereochemistry of base-catalyzed ring opening of 1,3,2-oxathiaphospholanes. Absolute configuration of 2-{*N*-[(Rc)-1-(-naphthyl)ethyl]amino}-2-thiono-1,3,2-oxathiaphospholanes and *O*,*S*-dimethyl *N*-[(Rc)-1-(-naphthyl)ethyl]phosphoramidothioates, J. Am. Chem. Soc. 114 (1992) 10197–10202.
[20] A. Okruszek, Methods of synthesis of modified oligonucleotides and their analogs-potential inhibitors of gene expression by antisense strategy, Biotechnologia (1994) 16–39.
[21] W. Jasek, Austria-Codex Fachinformation Österr, Apotheker-Verlagsgesellschaft, Wien, 2001.
[22] B. Jansen, V. Wacheck, E. Heere-Ress, H. Schlagbauer-Wadl, C. Hoeller, T. Lucas, M. Hoermann, U. Hollenstein, K. Wolff, H. Pehamberger, Chemosensitisation of malignant melanoma by BCL2 antisense therapy, Lancet 18 (2000) 1728–1733.
[23] M. Cotten, B. Oberhauser, H. Brunar, A. Holzner, G. Issakides, C.R. Noe, G. Schaffner, E. Wagner, M.L. Birnstiel, 2′-*O*-Methyl, 2′-*O*-Ethyloligoribo-nucleotides and phosphothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event, Nucleic Acid Res. 19 (1991) 2629–2635.
[24] E. Wagner, B. Oberhauser, A. Holzner, H. Brunar, G. Issakides, G. Schaffner, M. Cotten, M. Knollmüller, C.R. Noe, A simple procedure for TBC preparation of protected 2′-*O*-methyl or 2′-*O*-ethyl ribonucleoside-3′-*O*-phosphoramidites, Nucleic Acid Res. 19 (1991) 5965–5971.
[25] H. Brunar, Herstellung, Paarungseigenschaften und antivirale Wirksamkeit von funktionalisierten Oligonukleotiden, thesis, Technische Universität Wien, 1993.
[26] D. Werner, Synthese von 2′-*O*-Alkyl- und 2′-*O*-Oxyethylen-Adenosin-Synthese von Oligonukleotiden und Untersuchung der Paarungseigenschaften, thesis, Universität Frankfurt am Main, 1997.
[27] D. Wemer, H. Brunar, C.R. Noe, Investigations on the influence of 2′-*O*-alkyl modifications on the base pairing properties of oligonucleotides, Pharm. Acta Helv. 73 (1998) 3–10.
[28] C.R. Noe, H. Brunar, Int. Appl. WO9516696 A2 950622, Österr. Patent 2505/93.
[29] M. Gilbert, Optimierung von Antisense-Wirkstoffen-zwitterionische Nukleinsäuren und Trägersystemvariationen, thesis, Universität Frankfurt am Main, 2000.
[30] C. Novak, S. Wurst, E. Urban, C.R. Noe, Sixteenth Scientific Congress of Austrian Pharmaceutical Society, Vienna, Austria, 2001. Sci. Pharm. 69 (2001) 76–77.
[31] P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide, Science 254 (1991) 1497–1500.
[32] P.E. Nielsen, M. Egholm, O. Buchardt, Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone, Bioconjug. Chem. 5 (1994) 3–7.
[33] V. Demidov, M.D. Frank-Kamenetskii, M. Egholm, O. Buchardt, P.E. Nielsen, Sequence selective double strand DNA cleavage by peptide nucleic acid (PNA) targeting using nuclease S1, Nucleic Acids Res. 21 (1993) 2103–2107.
[34] V. Demidov, V.N. Potaman, M.D. Frank-Kamenetskii, M. Egholm, O. Buchard, S.H. Sonnichsen, P.E. Nielsen, Stability of peptide nucleic acids in human serum and cellular extracts, Biochem. Pharmacol. 48 (1994) 1310–1313.
[35] C.R. Noe, N. Windhab, G. Haberhauer, Novel three-atom 2′-5′ linkages in antisense nucleotides: synthesis and pairing properties of dinucleotides with a carboxylic ester linkage, Arch. Pharm. (Weinheim, Germany) 328 (1995) 743–745.
[36] S. Jordanova, G. Haberhauer, G.F. Ecker, C.R. Noe, Sixteenth Scientific Congress of Austrian Pharmaceutical Society, Vienna, Austria, 2001. Sci. Pharm. 69 (2001) 328–329.
[37] J. Winkler, E. Urban, C.R. Noe, Sixteenth Scientific Congress of Austrian Pharmaceutical Society, Vienna, Austria, 2001. Sci. Pharm. 69 (2001) 78–79.
[38] A. Bauchinger, E. Urban, C.R. Noe, Sixteenth Scientific Congress of Austrian Pharmaceutical Society, Vienna, Austria, 2001. Sci. Pharm. 69 (2001) 74–75.
[39] Y. Inouye, K. Onodera, S. Kitaoka, S. Hirano, Some fatty-acid derivatives of D-glucosamine, J. Am. Chem. Soc. 78 (1956) 4722–4724.
[40] H. Mack, J.V. Basabe, R. Brossmer, 2-Acetamido-2-deoxy-D-gluco- and -D-manno-furanose: a simple preparation of 2-acetamido-2-deoxy-D-mannose, Carbohydr. Res. 175 (1988) 311–316.
[41] D.H.R. Barton, S.W. McCombie, New method for the deoxygenation of secondary alcohols, Perkin Trans. I (1975) 1574–1585.