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(*R*)-2,4-Dihydroxybutyramide *seco*-Pseudonucleosides: New Versatile Homochiral Synthons for Synthesis of Modified Oligonucleotides

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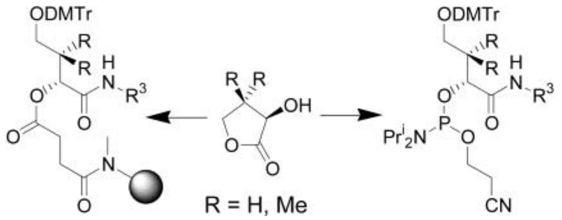
ABSTRACT



Two series of *seco*-pseudonucleoside synthons were synthesized from (*R*)-(+)-α-hydroxy-γ-butyrolactone and (*R*)-(−)-pantolactone by aminolysis, side-chain protection, dimethoxytritylation, and phosphitylation or solid-phase attachment. The phosphoramidites and solid supports were used in automated DNA synthesis to prepare oligonucleotides modified with one or more 2,4-dihydroxybutyramide units bearing side-chain reporter groups. These new oligonucleotide modification reagents allow the introduction of a label into any desired position within an oligonucleotide chain during solid-phase assembly.

Synthetic nonisotopically labeled oligonucleotide probes have found widespread application in molecular biology and various nucleic acids-based assays in medicine.[1] The most expeditious way to obtain these materials is machine-assisted solid-phase synthesis. Despite significant progress made over the last two decades in the area of oligonucleotide technology,[2] there is still a need to develop new modified reagents for oligonucleotide labeling during solid-phase assembly, which may allow introduction of various reporter groups into any predetermined position of a nucleic acid chain.

A number of nonnucleoside reagents have been suggested to allow site-specific incorporation of various functional groups and nonradioactive labels into an oligonucleotide chain during solid-phase synthesis.[4−11] Frequently, the precursors were shaped to be spatial surrogates of nucleo-

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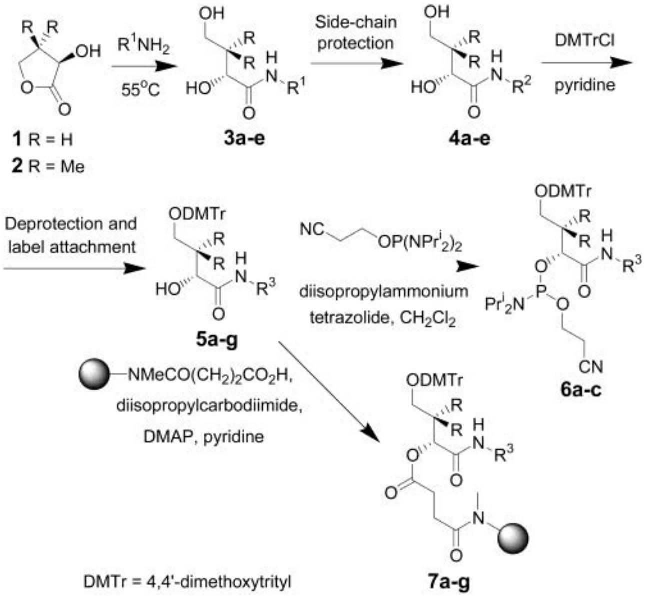
sides, e.g. maintain a three-carbon atom spacing between the phosphate groups[3] essential in the case of internal incorporation to minimize duplex destabilization. Among the reagents containing a 1,3-propanediol backbone are *racemic* derivatives of 2-(4-aminobutyl)-1,3-propanediol,[4] serinol (2-amino-1,3-propanediol),[5] 4-amino-1,3-butanediol,[6] and some 1-substituted 1,3-propanediols,[7] as well as O^2-alkylated glycerols.[8] However, most of the compounds, while simple and readily obtainable, suffer from several disadvantages. First, β-cyanoethyl phosphoramidites derived from primary alcohols are generally less stable than those from secondary alcohols. Second, use of racemic reagents leads to a mixture of diastereomeric oligonucleotides, which may complicate their isolation and purification. Few attempts to obviate the second problem by using *achiral* synthons based on N-substituted diethanolamine[9] gave even less close nucleoside mimics. Most successful approaches so far made use of natural materials, *trans*-4-hydroxy-L-prolinol[10] and L-threoninol.[11] Several other homochiral skeletons were suggested recently, including 2-hydroxymethyl-3-hydroxypyrrolidine[12] and abasic 2′-deoxynucleoside,[13] but these compounds proved to be much more expensive to synthesize.

We describe here a novel family of reagents for nucleic acid labeling and modification based on (*R*)-2,4-dihydroxybutyramide *seco*-pseudonucleosides, which are easily obtainable, chirally pure, and suitable for incorporation on both 3′- and 5′-ends, internal and multiple substitutions within an oligonucleotide chain. Advantages of our approach compared to, e.g., threoninol functionalization,[11] are that it offers a wider range of a functional group incorporation and, furthermore, is stereochemically simpler, since only one chiral center is involved.

We decided to use readily available homochiral precursors, (*R*)-(+)-α-hydroxy-γ-butyrolactone (**1**, R = H) and (*R*)-(−)-pantolactone (**2**, R = Me) as starting materials since they possess the required three-carbon chain in a configuration analogous to natural ribose, with a chiral carbon atom with a secondary hydroxy group representing 3′-OH and a carbonyl group replacing the ribose C-2′. In addition, introduction of geminal methyl groups into acyclic backbone in pantolactone series offers restraint of its flexibility and adds favorably to the solubility of these derivatives.

Lactones **1** and **2** were treated with an excess (1.1−5.0 equiv) of one of the primary amines (ethylenediamine, 1-pyrenemethylamine, or 4,7,10-trioxa-1,13-tridecanediamine) at 55 °C for 24−48 h. Then the side-chain amino group, if present, was masked by reaction with 9-fluorenylmethyl succinimidyl carbonate (**3a**,**b**) or methyl trifluoroacetate (**3e**). The primary hydroxy group of intermediates **4a**−**e** was selectively 4,4′-dimethoxytritylated,[14] and the resulting alcohols **5a**−**e** were purified by column chromatography and converted into phosphoramidites[15] (**6a**−**c**) and solid-supported reagents[16] (**7a**−**e**), respectively, by known procedures. A reporter group (biotin or fluorescein) may be introduced at this stage by transient N-protecting group cleavage by aqueous Na_2CO_3 (**5e**) or piperidine (**5a**) treatment and subsequent reaction with *O*,*O*′-diisobutyryl-5(6)-carboxyfluorescein pentafluorophenyl ester[17] or D-(+)-biotin pentafluorophenyl ester[10b] to afford labeled precursors **5f** and **5g**, correspondingly. After column purification, these compounds were used to produce two additional solid supports, **7f**,**g**. The loading range of these polymeric reagents was found to be 16−50 μmol/g. Attachment yields in the case of pantolactone-derived supports **7b**,**d** were lower, and the reaction took longer to proceed, which indicates a steric hindrance introduced by two extra methyl groups. All the phosphoramidites are white solids that can be stored at −20 °C for at least several months without any loss of reactivity.

Scheme 1. Synthesis of (*R*)-2,4-Dihydroxybutyramide *seco*-Pseudonucleoside Reagents



(*R*)-2,4-Dihydroxybutyramide reagents **6a**−**c** and **7a**−**g** were evaluated in machine-assisted oligodeoxyribonucleotide synthesis by standard 2-cyanoethyl phosphoramidite chem-

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Table 1. Side-Chain Substituents of (*R*)-2,4-Dihydroxybutyramide *seco*-Pseudonucleoside Reagents (Scheme 1)

no.	R	$R^1 - R^3$
a	H	$-CH_2CH_2NHX$, R^1 (X = H), $R^2 = R^3$ (X = Fmoc)
b	Me	$-CH_2CH_2NHX$, R^1 (X = H), $R^2 = R^3$ (X = Fmoc)
c	H	$R^1 = R^2 = R^3 = -CH_2$(1-pyrenyl)
d	Me	$R^1 = R^2 = R^3 = -CH_2$(1-pyrenyl)
e	H	$-(CH_2)_2(CH_2OCH_2)_3(CH_2)_2NHX$, R^1 (X = H), $R^2 = R^3$ (X = trifluoroacetyl)
f	H	$R^3 = -(CH_2)_2(CH_2OCH_2)_3(CH_2)_2NH$(biotin)
g	H	$R^3 = -(CH_2)_2NHCO$-5(6)-(fluorescein diisobutyrate)

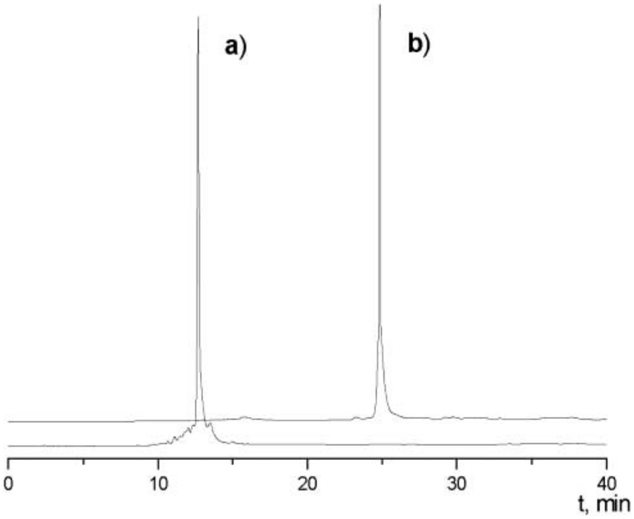

Figure 1. Typical RP-HPLC traces of crude (*R*)-2,4-dihydroxybutyramide *seco*-pseudonucleoside-containing oligodeoxyribonucleotides: (a) 2-aminoethyl **V** and (b) 1-pyrenemethyl **XIV** (Table 2). For HPLC conditions, see Supporting Information.

istry.[18] Use of modified *seco*-pseudonucleoside phosphoramidites **6a**−**c** allows the introduction of substituted 2,4-dihydroxybutyramide unit(s) most preferably on the 5′- or 3′-end of an oligonucleotide chain, while multiple incorporation and/or introduction at the internal position(s) are also possible. The 3′ case requires a special solid-phase linker, e.g., a support generating a 3′-phosphate group[19] or one of **7a**−**g** to ensure subsequent liberation of an oligonucleotide during ammonia deprotection. The polymeric reagents **7a**−**g** can also be used independently to append the modification to the 3′-terminus. The average coupling efficiency of the modified phosphoramidites **6a**−**c** at a 0.15 M concentration in dry MeCN and 10−15 min reaction time was found to be greater than 97%. Several model oligonucleotides were thus assembled, cleaved from their respective solid supports, deprotected by concentrated aqueous ammonia treatment at 55 °C overnight, and analyzed by reverse-phase HPLC and MALDI-TOF MS.[20] Examples of typical RP-HPLC traces are shown in Figure 1. Sequences and properties of the oligodeoxyribonucleotides incorporating various (*R*)-2,4-dihydroxybutyramide modifications are summarized in Table 2. Yields obtained were good, and all the molecular masses correspond to the expected values within the accuracy of the measurement.

Next, we studied the influence of substituted (*R*)-2,4-dihydroxybutyramide modification(s) on thermal stability of the duplexes formed by modified oligodeoxyribonucleotides with complementary DNA (Table 3). It is evident that many of the modifications analyzed, whether 3′- or 5′-terminal, caused very little disturbance of the corresponding duplex (ΔT_m from −0.4 to +0.1 °C), while pyrene *seco*-pseudonucleoside was in fact quite stabilizing in terminal positions (from +2.4 to +4.0°), which is in good agreement with

Table 2. Properties of Oligodeoxyribonucleotides Bearing (*R*)-2,4-Dihydroxybutyramide *seco*-Pseudonucleoside Units

no.[a]	sequence, 5′ to 3′, (modification)[b]	R	MALDI-TOF, calcd/found[c]	RP-HPLC retention time, min[c]	purity, %[c,d]
III	CTCCCAGGCTCAAAT(**6a**)p[e]	H	4801.1/4796.8	15.2	89.0
IV	CTCCCAGGCTCAAAT(**7a**)	H	4718.8/4718.7	12.8	82.1
V	CTCCCAGGCTCAAAT(**6b**)p[e]	Me	4826.8/4824.8	12.7	84.5
VI	(**6b**)CTCCCAGGCTCAAAT	Me	4749.2/4750.7	12.5	83.9
VII	CTCCCAGGCTCAAAT(**6b**)(**6b**)p[e]	Me	5078.9/5077.1	13.2	82.3
VIII	CTCCCAGGCTCAAAT(**7b**)	Me	4749.2/4747.9	12.5	81.4
IX	CTCCCAGGCTCAAAT(**6c**)p[e]	H	4969.8/4970.1	22.2	81.3
X	(**6c**)CTCCCAGGCTCAAAT	H	4892.3/4892.2	21.0	86.1
XI	CTCCCAGGC(**6c**)CAAAT	H	4588.1/4586.7	19.4	85.1
XII	C(**6c**)CCCAGGCTCAAAT	H	4588.1/4589.5	20.3	84.6
XIII	C(**6c**)CCCAGGC(**6c**)CAAAT	H	4677.0/4676.7	23.6	79.7
XIV	CTCCCAGGCTCAAAT(**7c**)	H	4892.3/4890.5	24.9	89.1
XV	ATTTGAGCCTGGGAG(**7d**)	Me	5071.4/5070.4	25.3	84.4
XVI	CTCCCAGGCTCAAAT(**7e**)	H	4881.3/4884.2	13.2	83.7
XVII	CTCCCAGGCTCAAAT(**7f**)	H	5097.6/5105.1	17.1	81.8
XVIII	CTCCCAGGCTCAAAT(**7g**)	H	5076.9/5076.8, 5079.2	13.9, 14.9	24.8, 57.7

[a] Roman numerals indicate the number of an oligonucleotides, starting with an unmodified 15-mer (**II**) and its complementary template (**I**) (Table 3). [b] Numbers in brackets indicate modification from a phosphoramidite (**6**) or a solid support (**7**) (Table 1). [c] Values separated by a comma refer to 5(6)-isomers of carboxyfluorescein. [d] Integrated from HPLC traces. [e] 3′-Phosphate.

Table 3. DNA Duplex Stability of Various (*R*)-2,4-Dihydroxybutyramide *seco*-Pseudonucleoside-Modified Oligodeoxyribonucleotides

no.[a]	modification[b]	R	position	T_m, °C	ΔT_m/mod[c], °C
II−I	none			57.6	
IV−I	**7a**	H	3′	57.3	−0.3
V−I	**6b**[d]	Me	3′	56.2	−1.5
VI−I	**6b**	Me	5′	57.7	+0.1
IX−I	**6c**[d]	H	3′	60.0	+2.4
XI−I	**6c**	H	int[e]	51.4	−6.3
XII−I	**6c**	H	int[e]	55.0	−2.7
XIII−I	**6c**	H	int[e]	50.7	−3.4
XIV−I	**7c**	H	3′	61.6	+4.0
XVI−I	**7e**	H	3′	57.7	+0.1
XVII−I	**7f**	H	3′	57.2	−0.4

[a] Roman numerals indicate the number of an oligonucleotide (**II−IX**) in a duplex with a complementary DNA template GAGGGTCCGAGTTTA (**I**). [b] Coming from phosphoramidite **6** or solid support **7** (Table 1). [c] ΔT_m per one modification. [d] 3′-Phosphate. [e] Internal position (Table 2).

previous findings.[21] Whereas pyrene is relatively well tolerated when substituting one of the internal thymidines close to the 5′-end (−2.7°), it is noticeably more destabilizing (−6.3°) when placed in the midst of the sequence in place of another thymidine, which is in line with our previous results on pyrene 2′-carbamate modification.[22] However, this effect is not additive, since substitution at both positions led to only −3.4° per modified residue, a marginal further decrease from a single pyrene *seco*-pseudonucleoside in the middle. Switching from a butyrolactone to a pantolactone unit in terminal positions seems to have little effect on T_m either. It should also be noted that the presence of a 3′-phosphate group led to a decrease in T_m by ca. 1.6°. Hybridization of pyrene-modified oligodeoxyribonucleotides **XI−XIV** to the complementary RNA follows the same trend both in 3′-terminal and internal positions, with slightly higher and more additive destabilization in the latter case (data not shown).

In conclusion, we describe here a new family of oligonucleotide modification reagents comprising (*R*)-2,4-dihydroxybutyramide *seco*-pseudonucleosides, which are homochiral and readily accessible from (*R*)-(+)-α-hydroxy-γ-butyrolactone and (*R*)-(−)-pantolactone. These may be either phosphoramidites for 3′-, internal, and 5′-terminal modification, or solid-supported linkers for 3′-modification of oligonucleotides during solid-phase synthesis. By choice of side-chain functional groups, one can incorporate various functional (short- or long-chain amine) or reporter groups (pyrene, biotin, fluorescein) into an oligonucleotide chain. In addition, the 3′- and/or 5′-terminal modifications do not decrease the ability of a modified oligonucleotide to form a duplex with a complementary DNA sequence.

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Supporting Information Available: Experimental details, compound characterization, HPLC traces, MALDI-TOF spectra, and melting curves for some of the oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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