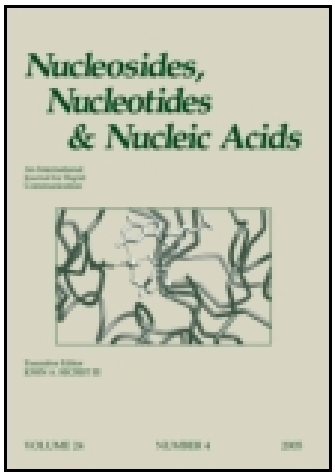

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SYNTHESIS OF 3′-THIOAMIDO-MODIFIED 3′-DEOXYTHYMIDINE-5′-TRIPHOSPHATES AND THEIR USE AS CHAIN TERMINATORS IN SANGER-DNA SEQUENCING

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ABSTRACT

The thioamide derivatives 3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-[(2-methyl-1-thioxo-propyl)amino]thymidine **1** and 3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′- {{6-{[(9*H*-(fluo-ren-9-ylmethoxy)carbonyl]-amino}-1-thioxohexyl}amino} thymidine **2** were synthesized by regioselective thionation of their corresponding amides **7** and **8** with 2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (*Lawesson*'s reagent). The thioamides were converted into the corresponding 5′-triphosphates **3** and **4**. Compound **3** was chosen for DNA sequencing experiments and **4** was further labelled with fluorescein.

INTRODUCTION

Dye-terminator sequencing is characterized by an enzymatic incorporation of chain-terminating, fluorescent nucleotides. These terminators are attached to fluorescent dyes at their base moiety. Apart from this labeling strategy, there are other potential positions within nucleosides that may be used for labeling. We have shown that 3′-amino- and amido-modified nucleoside-5′-triphosphates are excellent terminators (1,2). However, the ability of DNA polymerases to hydrolyze ester- and amide-bonds at the 3′-position limits the use of these 3′-dye-terminators for DNA sequencing since the label is lost during the incorporation process (3). Therefore, we

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decided to alter the link between dye and sugar moiety to find a bond that is stable against enzymatic degradation. In this context, we describe an efficient synthesis of 3′-thioamido-modified thymidines. We synthesized the thioamide derivative **1** as a model compound for the investigation of the thionation reaction with *Lawesson*'s reagent. In a second synthetic route we optimized the thionation reaction. The addition of exact amounts of pyridine to the reaction mixture proved to be essential for an efficient transformation. The corresponding 5′-triphosphate **4** was further labeled with fluorescein and **3** was chosen for DNA sequencing experiments.

RESULTS AND DISCUSSION

A broad spectrum of reactions exists for the synthesis of thioamides, only the conversion of amides into thioamides seemed to be suitable for nucleoside chemistry. Therefore, we synthesised the readily accessible amide **7** as a model compound for the investigation of the thionation reaction (Fig. 1). Direct action of hydrogen sulfide or phosphorus pentasulfide on **7**/**8** would result in an undesired exchange of the two carbonyl oxygen atoms at C(2) and C(4) of the base moiety. A more sophisticated thionation reagent is *Lawesson*'s reagent, which converts carbonyl groups into thiocarbonylgroups in high yield (4–6). Therefore, 7 was reacted with *Lawesson*'s reagent for 30 minutes at RT.

No by-products were observed under these reaction conditions, but the yield of **1** was rather poor (22%). 33% of the reactant was recovered unchanged. The synthesis of **2** under the same conditions resulted in even lower yields caused mainly by 5′-hydroxy deprotection. Therefore we tested basic solvents as scavengers for the phosphoric acids.

Thionation of **8** in pure anh. pyridine instead of anh. THF did not at all indicate any formation of **2** even when the amount of *Lawesson*'s reagent was drastically increased. Thus, the reaction of **8** with *Lawesson*'s reagent was accomplished in mixtures of anh. THF/pyridine. We observed that lowering the amount of

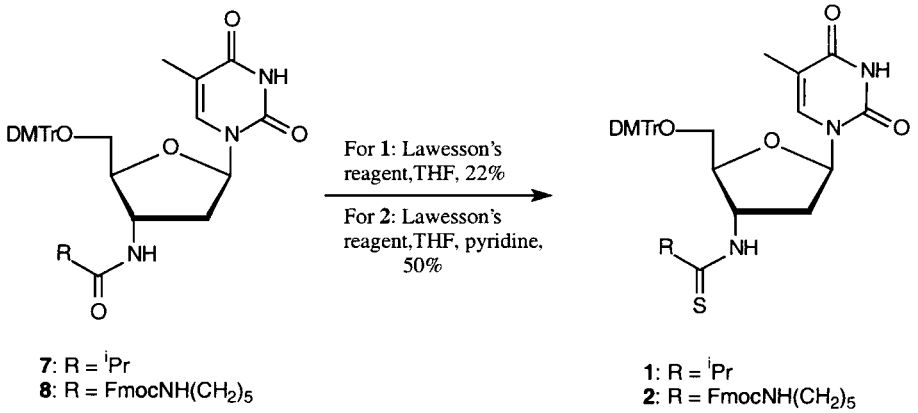

Figure 1. Synthesis of the thioamide derivatives **1** and **2**.

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pyridine gradually resulted in an increased yield. Finally, the addition of **1** equiv. of pyridine and 4 equiv. of *Lawesson*'s reagent in anh. THF as solvent yielded **2** in 50%; almost 50% of the intact educt was recovered. **1** and **2** were deprotected in 80% aq. AcOH solution at room temperature. The syntheses of the triphosphates **3** and **4** were achieved according to the procedure of Ludwig and Eckstein (7). The aminofunction of **4** was deprotected with 20% piperidine in pyridine and DMF at room temperature and succeedingly coupled with fluoresceinisothiocyanate (FITC) in aq. 0.1M $NaHCO_3$ (pH = 9.3) and DMF at room temperature for 24 hours. Both thioamides **1/2** were clearly identified by mass spectrometry and NMR spectroscopy. The electrospray-ionization MS (ESI-MS) showed formation of only one product with an increased weight of 16 Da compared with the educt, representing the exchange of an O-atom against a S-atom. The ^{13}C-NMR spectrum revealed the identity of the carbonyl group involved. The ^{13}C-chemical shifts of the carbon atoms C(2) and C(4) of the base moiety remained unchanged, whereas the carbonyl signal at the 3′-terminal moved downfield as expected.

The thioamides **1** and **2** were converted into the corresponding 5′-triphosphates **3** and **4**. Compound **4** was further labelled with fluorescein and **3** was investigated in DNA sequencing experiments (8). No band pattern was detected with Sequenase or with Thermosequenase. With Taq DNA polymerase, we obtained a band pattern which did not correlate with the standard ddTTP sequence. It was, however, identical with the band pattern obtained with a 3′-thioether 3′-deoxy-thymidine-5′-triphosphate. Although **3** can obviously not be defined as specific terminator for the enzymatic DNA synthesis under the chosen conditions, the result can not be called nonselective, since two structurally different nucleotides were incorporated at exactly the same positions within the DNA. This excludes a random process. Variation of the terminator concentration only resulted in a different fragment length distribution. The reason for this could be a conformationally driven selection process.

CONCLUSION

We demonstrated that 3′-thioamido modified nucleosides can efficiently be synthesised from 3′-amido tethered nucleosides by a regioselective thionation strategy applying *Lawesson*'s reagent. Addition of 1 equiv. of pyridine to the reaction mixture was necessary to prevent degradation of the nucleoside and ensure good yields at the same time. Thioamide **3** was accepted as a substrate by the Taq DNA polymerase. However, specific incorporation did not occur. Rather the band pattern showed a distinct correlation with a thioether tethered nucleoside triphosphate.

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