

Site-Specific Modification of Enzymatically Synthesized RNA: Transcription Initiation and Diels-Alder Reaction

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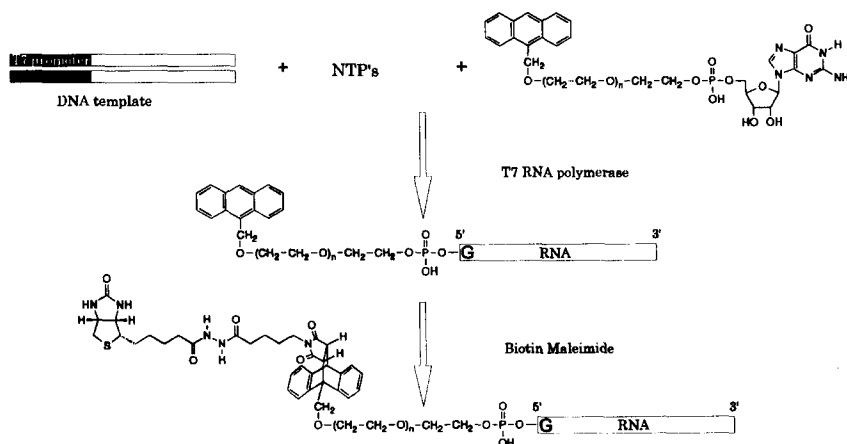
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Abstract: Initiator nucleotides consisting of an anthracene ring system coupled to the 5'-phosphate of guanosine monophosphate via a polyethylene glycol (PEG) linker were chemically synthesized. When added to transcription reactions, these initiators were site-specifically incorporated into RNA transcripts yielding RNA conjugates with a fluorophore selectively attached to the 5'-end via a flexible linker. Besides sensitive fluorescence detection, the anthracene allowed the convenient modification with maleimides by Diels-Alder reaction.

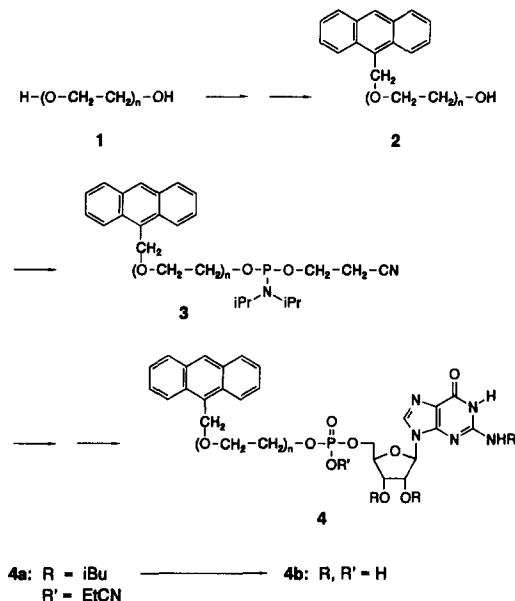
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Numerous strategies exist for the site-specific incorporation of modified nucleotides, labels and cross-linkers during chemical synthesis of oligonucleotides^{1,2}. While short RNA fragments can be easily prepared by phosphoramidite chemistry, the method of choice for the preparation of longer and biologically significant RNAs is enzymatic transcription of DNA templates by DNA-dependent RNA polymerases³. To better understand the mechanisms of ribozymes, RNA splicing or RNA-protein interactions it is highly desirable to have methods available for the site-specific chemical modification of RNA transcripts. The possibilities to achieve this are so far very limited. The only position in RNA that is distinctive during transcription is the 5'-terminus where transcription starts. While nucleoside triphosphates (NTP's) are required for chain elongation, initiation can be performed using various nucleosides, nucleoside mono-, di- and triphosphates as well as short oligonucleotides⁴⁻⁷.

We describe here a new type of initiator nucleotides where a guanosine monophosphate is attached to the aromatic hydrocarbon anthracene via a long flexible polyethylene glycol spacer. This initiator is incorporated by T7 RNA polymerase exclusively at the 5'-terminus and serves as both a fluorescence label⁸ and a reactive moiety for the attachment of maleimide-derivatized conjugate groups by Diels-Alder reaction⁹.



For the synthesis of initiator nucleotide **4b**, PEG 600 **1** was converted into the alcoholate and reacted with 9-chloromethylanthracene. The resulting mixture was chromatographically separated, and monoanthracene-PEG **2** was reacted with (2-cyanoethyl-N,N-diisopropyl)chlorophosphoramidite to give phosphoramidite **3**. 2'-O, 3'-O, N²-Triisobutylguanosine was prepared from 5'-dimethoxytrityl-N²-isobutylguanosine and reacted with **3** in the presence of tetrazole. After coupling, the reaction mixture was oxidized and purified by column chromatography on silica gel. **4a** was deprotected using aqueous ammonia giving initiator nucleotide **4b**, which was fractionated by reversed phase HPLC¹⁴.



As observed with oligonucleotide-PEG conjugates^{10,11}, the degree of polymerization (number of CH₂CH₂O-units) of the coupled PEG influences the molecular properties. For initiator nucleotides **4b**, single degrees of polymerization were eluted sequentially in reversed phase HPLC, giving a characteristic elution pattern (figure 1). The UV spectra show the typical features of both anthracene (absorbance maximum at 254.5 nm,

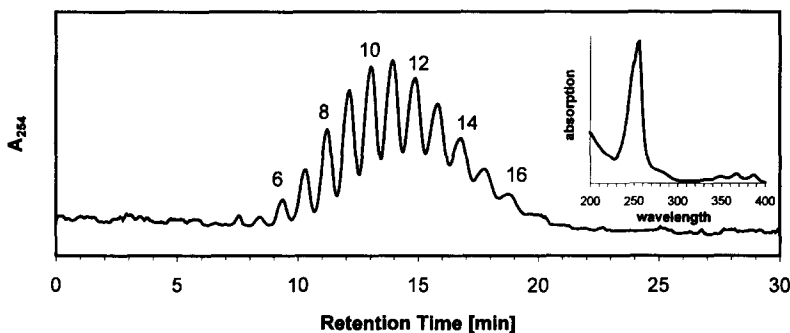
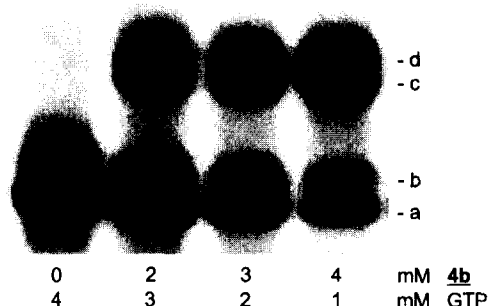


Figure 1. Reversed phase HPLC of initiator nucleotides **4b**. Eluent: 38 % acetonitrile in 100 mM triethylammonium acetate, pH 7.0. The figures at the peaks indicate the respective degree of polymerization of coupled PEG, as determined by mass spectrometry. Insert: UV spectrum of the peak eluted at 13.2 min.

“fingers” at 348, 365, and 385 nm) and guanosine (shoulder at 277 nm, see insert in figure 1). Upon UV excitation, the initiator nucleotides show a typical blue anthracene fluorescence. The molecular weights of neighboring peaks in the HPLC chromatograms differ by 44 Dalton, i.e., by the size of one $\text{CH}_2\text{CH}_2\text{O}$ -unit, as established by MALDI mass spectrometry. Electrophoretic mobility decreases with increasing size of the PEG chain.

To investigate whether the initiator nucleotide is accepted by T7 RNA polymerase, HPLC-purified **4b** (with $n_{\text{PEG}} = 10$) was added to standard transcription reactions from a synthetic DNA template, while the concentration of GTP was reduced accordingly (Figure 2). Since T7 RNA polymerase always generates a mixture of correct transcripts and molecules with an additional nucleotide appended to the 3'-end, RNA transcripts of 25 (n-mer, band a) and 26 nucleotides (n+1-mer, band b), respectively, were generated. With increasing concentration of **4b** two new bands with lower electrophoretic mobility appeared in the autoradiogram (Fig. 2). Upon excitation with 254 nm UV light, these bands showed the typical blue anthracene fluorescence. After elution from the gel, the n-mer conjugate (band c) was found to have a molecular weight of 8623.3 (calculated 8623.7 for MH^+), indicating that no other modifications had taken place. A series of transcripts of different length (up to 157 nucleotides) was prepared, always yielding more than 50 % yield of fluorescence-labeled RNA molecules. Incorporation appears to be independent on n_{PEG} , at least in the investigated range from 6 to 15.

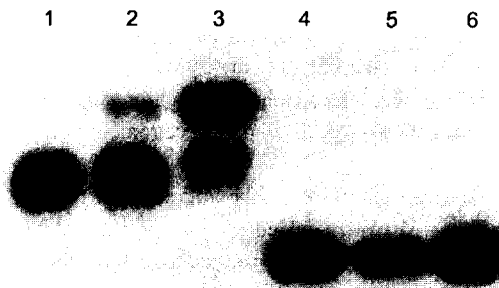
Figure 2. T7 transcriptions of a short DNA oligonucleotide duplex using initiator nucleotides **4b** ($n_{\text{PEG}} = 10$). Autoradiogram of a 15 % denaturing polyacrylamide gel. Nucleotide sequence of transcripts a-d: $5'(\text{anthracene-PEG})\text{-G GAG CUC AGC CUU CAC UGC UCC ACC (N)}_3'$ (N = A, C, G oder U), a: unmodified n-mer transcript, b: n+1-mer, c: anthracene-PEG-conjugate, n-mer, d: anthracene-PEG-conjugate, n+1-mer.



To exploit the chemistry of anthracene for the site-specific derivatization of RNA transcripts by Diels-Alder reaction, bands a and c from figure 2 were excised from the gel, eluted and mixed with a 10- and 1000-fold excess of biotin-maleimide (fig. 3). More than 60 % of the anthracene-linker-RNA conjugates appear in a band with lower electrophoretic mobility after overnight reaction (lanes 1-3). The molecular weights of different RNA conjugates obtained by this reaction found by MALDI-MS (e.g., $m/z = 9208$ for the 25mer RNA transcript with $n_{\text{PEG}} = 14$) corroborate the identity as RNA molecules linked to biotin via a polyethylene glycol linker and the respective Diels-Alder product. The presence of biotin in the reaction product was verified by incubation with streptavidin agarose causing immobilization. There seem to be no side reactions of the maleimide with the nucleotide part of the conjugates. No reaction at all could be detected with the unmodified reference transcript with the same nucleotide sequence (fig. 3, lanes 4-6), and longer RNA-anthracene conjugates derivatized by this protocol were accurately copied by reverse transcriptase, indicating that the products are not modified at internal positions.

The approach described here for coupling of anthracene derivatives allows the incorporation of a single label at the 5'-position of a transcript. The guanosine part of the initiator nucleotides represents the minimum substrate for T7 RNA polymerase⁴, and the polyethylene glycol linker does obviously not interfere with the transcription reaction. Combined with data from other publications, the results suggest that a flexible linker attached to the 5'-phosphate of GMP could be used to incorporate other small molecules into RNA,

Figure 3. Reaction of ^{32}P -labeled anthracene-PEG-RNA conjugates (lanes 1-3) and unmodified RNA transcripts (lanes 4-6) with biotin maleimide (N-biotinoyl-N'-[6-maleimidohexanoyl]-hydrazide). Autoradiogram of a 15% denaturing polyacrylamide gel; lanes 1 and 4: without biotin maleimide, lanes 2 and 5: 10-fold excess, lanes 3 and 6: 1000-fold excess of biotin maleimide. samples: excised bands a and c from fig. 2; conditions: RNA concentration 1 μM , 20°C, 16 hours, pH 7.4.



too^{4,6}. This type of initiator nucleotides could therefore be useful for non-radioactive labeling of RNA, for the investigation of interactions between biopolymers, the incorporation of cross-linkers or the assembly of supramolecular structures. Additionally, terminal coupling of PEG has been reported to increase the resistance of oligonucleotides to degradation in biological media and also to alter pharmacokinetic properties, so enzymatic incorporation of PEG might provide an elegant alternative to the chemical methods described^{12,13}.

The described derivatization of the anthracene moiety is to our knowledge the first use of the Diels-Alder reaction for site-specific modification of unprotected oligonucleotides. Besides enzymatic incorporation of **4b**, phosphoramidite **3** can be used in automated synthesis to add a linker-coupled anthracene to the 5'-end of a chemically synthesized DNA or RNA oligonucleotide (data not shown). Thus, incorporation of the diene is straightforward and may expand the methodological repertoire of oligonucleotide conjugation, especially in situations where different conjugate groups have to be attached at specific sites.

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- 2:** ^1H NMR (250 MHz, $[\text{D}_1]\text{CHCl}_3$, 25°C) δ = 3.5-3.8 (m, 56 H; $\text{CH}_2\text{-O}$ für n = 14), 5.6 (s, 2H; $\text{CH}_2\text{-Ar}$), 7.48 (m, 4H; H-2/H-3/H-6/H-7), 8.0 (d, 2H; H-1/H-8), 8.4 (d, 2H; H-4/H-5), 8.42 (s, 1H; H-10); ^{13}C NMR (63 MHz, $[\text{D}_1]\text{CHCl}_3$, 25°C): δ = 131.2, 130.8, 128.8, 128.6, 128.1, 125.9, 124.7, 124.3; ESI⁺-MS: m/z: 649.7 (MH^+ (n=10), calculated for $\text{C}_{35}\text{H}_{52}\text{O}_{11}$ 649.8). **3:** ^{31}P NMR (202 MHz, $[\text{D}_1]\text{CHCl}_3$, 20°C): δ = 146.05 ppm. **4b:** MALDI⁺-MS: m/z: 994; MH^+ (n=10), calculated for $\text{C}_{45}\text{H}_{65}\text{O}_{18}\text{N}_5\text{P}$ 994.4; UV (H_2O): λ_{max} = 254.5, 348, 365, 385; 277 nm (shoulder).

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