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The Synthesis of Allyl- and Allyloxycarbonyl-Protected RNA Phosphoramidites. Useful Reagents for Solid-Phase Synthesis of RNAs with Base-Labile Modifications.

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Abstract: The synthesis of allyl- and allyloxycarbonyl (AOC)-protected RNA phosphoramidites is reported. The use of allyl and AOC groups allows the chemistry of solid-phase RNA synthesis to be expanded to include base-labile modified nucleosides, such as acetylcytidine. The allyl- and AOC-protective chemistry can be further expanded for the construction of RNAs on solid supports and affinity columns. © 1998 Elsevier Science Ltd. All rights reserved.

N^4-acetylcytidine (ac^4C), **2a**, is one of >100 known modified nucleosides found in RNA.[1] This polar modification is present at conserved positions in tRNAs from numerous phylogenetic sources[2] and in the small ribosomal subunit of eukaryotes.[3] ac^4C is also present in RNAs isolated from hyperthermophilic archaea such as *Pyrodictium occultum* which grow at temperatures as high as 100 °C.[3] As an isolated nucleoside, ac^4C confers conformational rigidity to the ribose moiety and stabilizes the *C3'-endo* conformation.[4,5] Acetylation of the amino groups in poly(C) and poly(C-U) inhibits the incorporation of specific amino acids in a cell-free protein system.[6] Together, these studies suggest an important role for ac^4C, particularly with regard to maintaining local structures within RNA or providing stability and fidelity to the translational components.

The ability to incorporate ac^4C into specific locations of RNA would greatly enhance the determination of its structural and functional roles. Acetylation of cytidine residues in RNA can be achieved by reactions with acetic anhydride in DMF, however, this reaction does not discriminate between C's at different positions.[7] Similarly, biochemical methods that are available for the site-specific incorporation of modified nucleosides into RNA have certain limitations (*e.g.*, only specific residues may be modified, or unusual base-pairing schemes may be required).[8,9] For our purposes, the phosphoramidite approach[10] has proven to be a desirable method for generating milligram quantities of small RNAs modified site-selectively with acetylated cytidine.

The solid-phase approach for oligoribonucleotide synthesis involves a series of reactions on a controlled-pore glass (CPG) support with phosphoramidite monomers.[10] The standard procedures typically involve protection of the exocyclic amines and phosphate linkages with base-labile groups. For example, the cyanoethyl-protected phosphoramidite of acetylcytidine has been synthesized previously, but for the purpose of having extremely base-labile protective groups.[11] In general, the standard procedures impose a limitation to the variety of the modifications that can be introduced into RNA. The ability to incorporate and retain the acetyl group on cytidine requires the use of a scheme that would allow removal of the amine and phosphate protective groups under neutral conditions. For this purpose, Pd(0)-removable allyloxycarbonyl (AOC) and allyl groups

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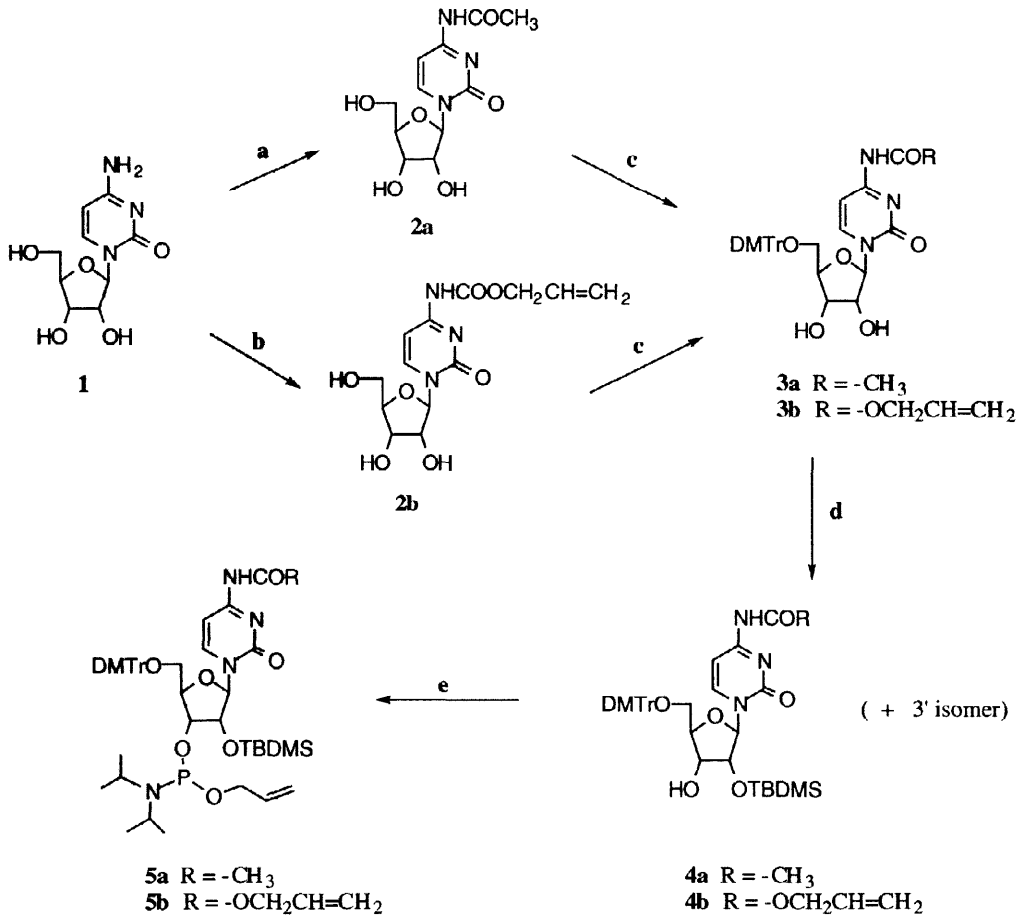
have been employed. These groups have been applied in DNA synthesis by Noyori and others,[12] but to our knowledge have not been applied in modified RNA syntheses. Here, we report the synthesis of N^4-(acetyl)cytidine and N^4-[(allyloxy)carbonyl]cytidine allyl phosphoramidites, shown in Figure 1. These new allyl phosphoramidite monomers offer an advantage over the existing cyanoethyl phosphoramidites because they allow for the site-specific incorporation of both base-labile ac^4C and unmodified cytidines into RNA.

Figure 1. The synthetic scheme for ac^4C and AOC^4C allyl phosphoramidites is shown.



Reagents and Conditions: a) Ac_2O/MeOH, reflux, 5 h; b) TMSCl/pyr, 0 °C, 1 h, followed by AOCOBT, 40 °C, 12 h; c) DMTrCl/pyr, 25 °C; d) TBDMSCl/$AgNO_3$/pyr in THF, 25 °C; e) allyl N,N,N',N'-tetraisopropylphosphorodiamidite/$iPrNH_2$/1*H*-tetrazole in CH_3CN, 25 °C.

N^4-Acetylcytidine, **2a**, was synthesized according to literature procedures.[13] In order to determine the stability of the acetamide moiety to the conditions required for the removal of the base- and phosphate-protective groups (AOC and allyl), **2a** was subjected to a mixture of $Pd_2(dba)_3$, PPh_3, and $nBuNH_3^+HCO_2^-$ (1.2 M in THF).[14] No decomposition was observed after 1 h as indicated by TLC analysis. **2a** is also stable to TBAF/THF which is used for deprotection of the TBDMS groups. The allylcarbamate derivative of cytidine was prepared by first protecting at the ribose hydroxyls of cytidine with trimethylsilyl chloride in pyridine and

then reaction with allyl 1-benzotriazolyl carbonate (AOCOBT) at 40 °C for 12 h.[12] Hydrolysis of the silyl ethers with an aqueous solution of sodium hydrogen carbonate furnished **2b** as a white solid in quantitative yield following purification. Further derivatization of **2a** and **2b** at the 5' position by standard dimethoxytritylation afforded **3a** and **3b** as yellow foams in 61% and 50% yields, respectively, after silica-gel purification.

To prepare the phosphoramidites, the 5'-protected derivatives **3a** and **3b** were silylated with *tert*-butyldimethylsilyl chloride in THF in the presence of $AgNO_3$. In both cases, mixtures of the 2'- and 3'-silylated products were generated, but the 2' derivatives were the major products. The two regioisomers were separated by silica-gel chromatography with 80% ethyl acetate:hexane (R_f's = 0.56 and 0.28 for **4a** and the 3' isomer, respectively). Similarly, **4b** and the corresponding 3' isomer were separated with 30% ethyl acetate:hexane (R_f's = 0.36 and 0.10, respectively). The 2'-silylated derivatives **4a** and **4b** were obtained as off-white foams in 57% and 40% yields, respectively. The 3'-silyl compounds were used to generate additional 2'-silyl products (**4a** and **4b**) (~15%) by repetitive isomerization with 3% triethylamine/methanol, followed by chromatographic purification. Phosphitylation of the 5', 2'-protected nucleosides with allyl N,N,N',N'-tetraisopropylphosphorodiamidite and 1*H*-tetrazole in anhydrous acetonitrile was performed under conditions similar to those described in the literature.[12] A longer reaction time (6 h) was necessary because of the steric hindrance introduced by the presence of the 2'-silyl group. The crude amidites were subjected to silica-gel purification followed by lyophilization from benzene to give pure **5a** (63%)[15] and **5b** (60%).[16] Both **5a** and **5b** are stable under all of the standard solid-phase synthesis conditions, with the exception of the oxidation step. **5a** and **5b** are stable to a modified oxidizing reagent, 1.1 M t-C_4H_9OOH-CH_2Cl_2 in dichloromethane.[12]

The modified nucleoside phosphoramidites **5a** and **5b** have been incorporated into an 18-nucleotide RNA (5'-UXCX(UX)$_3$UCUXCXUX-3', where X=ac^{4}C) by automated chemical synthesis using an extension of the reported coupling time.[12] Standard CPG supports require removal with NH_4OH and are not compatible with the ac^{4}C phosphoramidite. Therefore, a photolabile support developed by McMinn and Greenberg[17] as well as a uridine allyl phosphoramidite[18] were employed. The coupling efficiencies were >93% for **5a** and **5b**, respectively. Following the RNA synthesis, the allyl and allyloxycarbonyl groups were removed from the support-bound RNA, followed by UV photolysis at 365 nm for support cleavage. Final treatment with TBAF/THF removed the 2'-silyl protective groups, furnishing the fully deprotected RNA. The resulting RNA was purified and analyzed by reverse-phase HPLC and polyacrylamide gel electrophoresis. In order to verify the efficiency of the deprotection steps and structural integrity of ac^{4}C, the RNA was subjected to nuclease digestion and dephosphorylation followed by reverse-phase HPLC and comparison with nucleoside standards.

Phosphoramidites **5a** and **5b** enable RNA incorporation and retention of the base-sensitive ac^{4}C. Further experiments towards structural characterization of the modified RNA and synthesis of AOC-protected adenosine and guanosine are currently underway. We will also consider the use of an allyl linker developed by Zhang and Jones which should be compatible with our allyl phosphoramidites.[19] The phosphoramidites presented here should be generally applicable for use with other base-labile modifications in RNA, and can also be employed to produce support-bound RNAs for uses as affinity columns and diagnostic purposes.

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REFERENCES

1. Limbach, P. A.; Crain, P. F.; McCloskey, J. A. *Nucleic Acids Res.* **1994**, *22*, 2183-2196.
2. Grosjean, H.; Sprinzl, M.; Steinberg, S. *Biochimie* **1995**, *77*, 139-141.
3. Bruenger, E.; Kowalak, J. A.; Kuchino, Y.; McCloskey, J. A.; Mizushima, H.; Stetter, K. O.; Crain, P. F. *FASEB J.* **1993**, *7*, 196-200.
4. Kawai, G.; Hashizume, T.; Yasuda, M.; Miyazawa, T.; McCloskey, J. A.; Yokoyama, S. *Nucleosides & Nucleotides* **1992**, *11*, 759-771.
5. Parthasarathy, R.; Ginell, S. L.; De, N. C.; Chheda, G. B. *Biochem. Biophys. Res. Comm.* **1978**, *83*, 657-663.
6. Michelson, A. M.; Grunberg-Manago, M. *Biochim. et Biophys. Acta* **1964**, *91*, 92-104.
7. Keith, G.; Ebel, J.-P. *Biochim. et Biophys. Acta* **1968**, *166*, 16-28.
8. Moore, M. J.; Sharp, P. A. *Science* **1992**, *256*, 992-997.
9. Tor, Y.; Dervan, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 4461-4467.
10. Usman, N.; Ogilvie, K. K.; Jiang, M.-Y.; Cedergren, R. J. *J. Am. Chem. Soc.* **1987**, *109*, 7845-7854.
11. Chaix, C.; Duplaa, A. M.; Molko, D.; Téoule, R. *Nucleic Acids Res.* **1989**, *17*, 7381-7393.
12. Hayakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R. *J. Am. Chem. Soc.* **1990**, *112*, 1691-1696.
13. Watanabe, K. A.; Fox, J. J. *Angew. Chem. Int. Ed.* **1966**, *5*, 579-580.
14. A 1:2.5:25:50 mixture of **2a**:$Pd_2(dba)_3$:PPh_3:1.2 M $nBuNH_3{}^+HCO_2^-$ in THF was employed. Compound **2a** was also stable towards treatment with a slightly basic solution of N,N-diethyldithiocarbamate sodium salt (ddtc), pH 9.5, which is required for removal of Pd contamination.
15. Mixture of two diastereomers. UV (EtOH): λ_{max} 238, 286, 298 nm. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.12 ($SiCH_3$, 3H, s), 0.13 ($SiCH_3$, 3H, s), 0.25 ($SiCH_3$, 3H, s), 0.24 ($SiCH_3$, 3H, s), 0.89 and 0.91 ($SiC(CH_3)_3$, 9H, s), 1.10 and 1.12 ($2CH(CH_3)_2$, 12H, two d, J=6.5 Hz), 2.21 ($NHCOCH_3$, 3H, s), 3.45-3.55 ($2CH(CH_3)_2$, 2H, m), 3.65-3.70 (H5', 5", 2H, m), 3.80 ($2OCH_3$, 6H, s), 3.90-4.10 ($POCH_2CH=CH_2$, 2H, m), 4.15 (H4', 1H, m), 4.22 (H3', 1H, m), 4.35 (H2', 1H, m), 4.90-5.12 ($POCH_2CH=CH_2$, *cis*, 2H, two dd, J=1.5 and 10.5 Hz), 5.12-5.25 ($POCH_2CH=CH_2$, *trans*, two 2H, two dq, J=1.5 and 17 Hz), 5.75 and 5.85 ($POCH_2CH=CH_2$, 2H, two m), 5.82 and 5.88 (H1', 2H, two s), 6.85 (DMTr-Ar, H3,3',5,5', 4H, 2d, J=9 Hz), 6.88 and 6.95 (H5, 2H, two d, J=7.5 Hz), 7.26-7.44 (DMTr-Ar, 9H, m), 7.41 and 7.46 (H6, 2H, two d, J=8.0 Hz), 9.59 and 9.63 (NHCO, 2H, br s, exch. with D_2O). ^{31}P NMR ($CDCl_3$): 149.34, 147.97 ppm. FAB: $[M-H]^-$ calc. 887.4, found 887.7.
16. Mixture of two diastereomers. UV (EtOH): λ_{max} 236, 284, 302 nm. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.14 ($SiCH_3$, 3H,s), 0.15 ($SiCH_3$, 3H, s), 0.24 ($SiCH_3$, 3H, s), 0.26 ($SiCH_3$, 3H, s), 0.90 and 0.91 ($SiC(CH_3)_3$, 9H, s), 1.10 and 1.12 ($2CH(CH_3)_2$, 12H, two d, J=6.5 Hz), 3.45-3.55 ($2CH(CH_3)_2$, 2H, m), 3.67-3.71 (H5', 5", 2H, m), 3.80 ($2OCH_3$, 6H, s), 3.90-4.11 ($POCH_2CH=CH_2$, 2H, m), 4.15 (H4', 1H, m), 4.30-4.35 ($COOCH_2CH=CH_2$, H2', 3H, m), 4.98-5.37 ($POCH_2CH=CH_2$, $COOCH_2CH=CH_2$, *cis*, 4H, m, J=10.5 Hz, and $POCH_2CH=CH_2$, $COOCH_2CH=CH_2$, trans, 4H, three d, J=17 Hz), 5.74-5.95 (H1', $POCH_2CH=CH_2$ and $COOCH_2CH=CH_2$, 3H, two m), 6.67 and 6.73 (H5, 2H, two d, J=7.5 Hz), 6.83 (DMTr-Ar, H3,3',5,5', 4H, d, J=9 Hz), 7.23-7.46 (DMTr-Ar, 9H, m), 7.49 (NHCO, 2H, br s, exch. with D_2O), 8.51 and 8.57 (H6, 2H, two d, J=7.5 Hz). ^{31}P NMR ($CDCl_3$): 149.3, 147.9 ppm. FAB: $[M-H]^-$ calc. 929.4, found 929.5.
17. McMinn, D. L.; Greenberg, M. M. *Tetrahedron* **1996**, *52*, 3827-3840.
18. The uridine allyl phosphoramidite was synthesized in a similar manner as the cytidine phosphoramidites.
19. Zhang, X.; Jones, R. A. *Tetrahedron Lett.* **1996**, *37*, 3789-3790.