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ANALYSIS OF POST-SYNTHETICALLY MODIFIED OLIGODEOXYNUCLEOTIDES BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY.

Robert S. Coleman,*,[1a] Edward A. Kesicki, John C. Arthur,[1b] and William E. Cotham

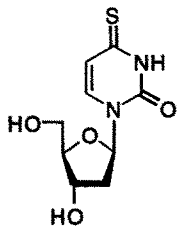
Department of Chemistry and Biochemistry
University of South Carolina
Columbia, South Carolina 29208

ABSTRACT: *Electrospray-ionization mass spectrometry is used for the effective analysis of post-synthetically modified oligodeoxynucleotides, wherein thiol groups present on 4-thio-2′-deoxyuridine residues within the oligomers are used as site-specific points of attachment for functionalized tethers.*

INTRODUCTION

Complementary addressed modification of DNA[2] uses Watson-Crick base-pairing to deliver chemically reactive functionality to targeted sequences of nucleic acids, thereby enabling sequence-specific chemical modification of DNA. This procedure has relied on the development of methods for the synthetic incorporation of functionalized nucleosides into oligonucleotides. There have been a number of recent reports describing the synthesis and incorporation of non-natural nucleic acids into oligonucleotides, and this methodology has provided a variety of loci upon which to tether reactive or reporter groups;[3] the most flexible approaches have used a post-synthetic modification strategy.[4]

We have taken an approach to template-directed covalent cross-linking of DNA in which synthetic incorporation of non-natural *thionucleic acids* into oligodeoxynucleotides is followed by site-specific chemical modification of the non-natural bases. The thiol groups of modified nucleosides allow for *chemoselective* incorporation of tethered functionality. This strategy permits the *divergent* incorporation of reactive functionality that would otherwise be incompatible with solid-phase synthesis conditions. Our work in this area includes the first description of the *S*-cyanoethyl group for effective thiocarbonyl protection,[5] the incorporation of 4-thio-2′-deoxyuridine (d^{4S}U; **1**) into oligonucleotides,[6] and an efficient synthesis of the purine surrogate *N*-(2-deoxy-β-D-ribosyl)-4-(thiomethyl)indole.[7]



d^{4S}U (1)

In our studies on post-synthetic modification of thionucleic acids contained within oligomers, we were faced with the problem of determining the extent and selectivity of covalent transformation. We have examined electrospray-ionization mass spectrometry (ESIMS)[8,9] as a method for analysis of covalently modified oligodeoxynucleotides, which complements the standard, but tedious enzymatic digestion methods for analysis of modified oligonucleotides.[10] Electrospray ionization (ESI) is a liquid-phase, atmospheric pressure ionization interface to mass spectrometry that has made mass analysis a viable analytical tool for large biomolecules. In this technique, an aqueous solution of the analyte flows through the tip of a metal capillary that is held at a 3-4 kV potential. The high induced electric field (10^6 V/m) causes the emerging liquid to disperse into a fine aerosol of highly charged droplets that are transformed into gas-phase ions, which are sampled by the mass spectrometer.

A unique characteristic of ESI is that it creates multiply charged ions through proton attachment (positive ions) or proton abstraction (negative ions). This feature allows for the analysis of very large molecules (*e.g.*, proteins, oligonucleotides, polymers) because the mass-to-charge ratio (m/z) is in the range of typical mass spectrometers. Deconvolution algorithms are used to determine the actual molecular weight from the spectrum of the multiply charged ions. This technique has proved useful in our studies, and herein we disclose our results on ESIMS analysis of small oligodeoxynucleotides containing covalently appended groups.

METHODS

Solid-phase oligodeoxynucleotide synthesis protocols using standard protecting groups[11] were implemented on an Applied Biosystems 380B or a Milligen Cyclone Plus Oligonucleotide Synthesizer. We have described the synthesis and phosphitylation of modified nucleoside **1** previously. For the synthesis of 4-thio-2′-deoxyuridine-containing oligomers, a 1 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in CH_3CN was used to remove the *S*-cyanoethyl protecting group, immediately before cleavage from the support with NH_4OH. Trityl-on oligomers were purified by reverse-phase HPLC using a Hamilton PRP-1 column (4.1 x 250 mm, 10 µm, 5-60% CH_3CN in 0.1 M pH 6.5 triethylammonium acetate buffer), and were deprotected by treatment with 80% AcOH. HPLC analyses of digests containing **1** were monitored at 332 nm.

All oligodeoxynucleotide samples were analyzed by negative-ion electrospray on a VG Trio-3 triple quadrupole mass spectrometer. Initial mass calibration was performed in the EI mode using perfluorotributylamine. Subsequent calibrations were made using synthetically prepared unmodified oligomers. The samples were analyzed by direct infusion using a Harvard Apparatus Model 22 syringe pump and a Rheodyne injection valve equipped with a 10 µL sample loop. The mobile phase consisted of a 20:80 *i*-PrOH/H_2O solution made basic to pH 9.5 with NH_4OH. It has been shown that *i*-PrOH greatly reduces corona discharge problems common to negative ion electrospray MS.[12] Mobile phase flow rate was 7 µL/min. Nitrogen was used as the source bath and nebulizing gas, and the source temperature was held at 95 °C. Mass spectra were acquired in profile mode from 300-900 amu at 6 s/scan. The data were transferred to a PC equipped with VG's Lab-Base mass spectral analysis software for final analysis and mass determination.

RESULTS

The dodecamer d(GACTCT^{4S}UTTGAC), where d^{4S}U = 4-thio-2′-deoxyuridine, was synthesized via the phosphoramidite of **1** as described above. A small amount of the corresponding dC-containing oligomer d(GACTCTCTTGAC), which results from ammonolysis of the thiocarbonyl group, could be separated by HPLC. Post-synthetic modification of the thiocarbonyl group as the (3-chloropropyl)disulfide was accomplished by treatment with *N*-((3-chloropropyl)thio)phthalimide (10 mM) in 0.1 M phosphate buffer (pH 8.0, 10% DMF, 25 °C, 1 h), to afford d(GACTCT^{RS}UTTGAC), R = $SCH_2CH_2CH_2Cl$. Similarly, S-alkylation of the thiocarbonyl group with methyl iodide or iodoacetamide (100-300 mM, pH 8.0, 10% DMF, 25 °C, 2 h) occurred with complete chemoselectivity to afford d(GACTCT^{RS}UTTGAC), R = CH_3 or CH_2CONH_2, respectively. The 17-mer d(TAATACGAC^{4S}UCACTATA) was modified chemoselectively with methyl iodide to afford oligomer d(TAATACGAC^{MeS}UCACTATA). Electrospray-ionization mass spectrometric results with these oligonucleotides are tabulated below. In all instances, accuracy was better than ± 1 amu.

ESIMS DATA ON 4-THIO-2′-DEOXYURIDINE OLIGOMERS

Oligonucleotide Sequence	Calculated MW	Observed MW
d(GACTCT^{4S}UTTGAC)	3613.38	3613.79 ± 0.50
d(GACTCTCTTGAC)	3596.41	3596.94 ± 0.29
d(GACTCT^{MeS}UTTGAC)	3627.41	3626.94 ± 0.24
d(GACTCT^{RS}UTTGAC) [R = CH_2CONH_2]	3670.43	3670.82 ± 1.21
d(GACTCT^{RS}UTTGAC) [R = $SCH_2CH_2CH_2Cl$]	3721.48	3721.50 ± 0.37
d(TAATACGAC^{MeS}UCACTATA)	5153.43	5153.14 ± 0.46

For comparison, a sample of dodecamer d(GACTCT^{RS}UTTGAC), R = $SCH_2CH_2CH_2Cl$, was treated with a mixture of snake venom phosphodiesterase and alkaline phosphatase (10 mM phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 37 °C, 3 h). Analysis of the hydrolysis mixture by HPLC using a Hamilton PRP-1 column[13] gave a ratio of dC/dG/T/dA/d^{RS}U of 3.2 : 2.0 : 3.9 : 1.9 : 1.1, in excellent agreement with the expected ratio of 3.0 : 2.0 : 4.0 : 2.0 : 1.0. Neither d^{4S}U or dU were detected at significant levels by HPLC. All oligomers were digested in the same manner, and experimentally determined nucleoside compositions were in agreement with expected values.

CONCLUSION

We have demonstrated the utility of electrospray-ionization mass spectrometry (ESIMS) in the analysis of post-synthetically modified oligonucleotides. In our studies, we have used ESIMS to determine the selectivity of covalent modification of 4-thio-2′-deoxyuridine residues contained within short oligomers (12-17 mers). This technique has proven complementary to traditional enzymatic methods for analysis of covalently modified nucleosides.

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REFERENCES AND NOTES

1. (a) Recipient of a Dreyfus Foundation Distinguished New Faculty Award (1989-94), an American Cancer Society Junior Faculty Research Award (1990-93), and the American Cyanamid Young Faculty Award (1993-96). (b) Recipient of a U. S. Department of Education Graduate Fellowship (1991-92).
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13. Eluent: 5-75% CH_3CN over 25 min, 0.1 M triethylammonium acetate buffer, pH 6.5. Detector: 254 nm (t = 0), 332 nm (t = 9 min), 308 nm (t = 14 min).

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