Interstrand Cross-Link and Bioconjugate Formation in RNA from a Modified Nucleotide

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ABSTRACT: RNA oligonucleotides containing a phenyl selenide derivative of 5-methyluridine were chemically synthesized by solid-phase synthesis. The phenyl selenide is rapidly converted to an electrophilic, allylic phenyl selenate under mild oxidative conditions. The phenyl selenate yields interstrand cross-links when part of a duplex and is useful for synthesizing oligonucleotide conjugates. Formation of the latter is illustrated by reaction of an oligoribonucleotide containing the phenyl selenide with amino acids in the presence of mild oxidant. The products formed are analogous to those observed in tRNA that are believed to be formed posttranslationally via a biosynthetic intermediate that is chemically homologous to the phenyl selenate.

INTRODUCTION

The known roles of RNA in biology and biochemistry continue to grow. Its newly discovered features include novel primary and tertiary structures. One way in which organic chemistry can contribute to this important area is by developing methods for synthesizing and analyzing these novel structures. In this report we describe the chemistry that can be used to synthesize RNA-containing CS-substituted uridines and for producing interstrand cross-links in RNA. The latter function may prove useful for detecting tertiary interactions in RNA.

A variety of enzymatic and chemical tools that are used for studying DNA structure are also employed for examining the more complex structure of RNA molecules. These tools are being applied in the development of high-throughput methods for RNA structure elucidation. The tools employed include hydroxyl radical cleavage and chemical reagents that exploit the differential reactivity of nucleotides based upon their solvent exposure. Dimethyl sulfate and diethyl pyrocarbonate are examples of the latter. We have pioneered the development of reagents that exploit the accessibility of the 2′-hydroxy group to extract RNA structural information from differential reactivity.

In contrast, cross-linking agents detect proximal functional groups. A number of cross-linking methods are available, including photolabile nucleotides (e.g., 4-thiothymidine, 6-thioguanosine) and exogenous reagents (e.g., 1,4-phenyl diglyoxal). Researchers have also utilized photochemistry to produce reactive intermediates, such as carbenes and nitrenes, although cross-link yields are often low in these experiments. In another approach, cisplatin was elegantly used in conjunction with phosphorothioated RNA to detect short-range interactions.‡ Recently, mild methods for cross-linking RNA via modified nucleotides that contain furan or vinyl sulfides have been reported. Oxidation of the furan appended through the 2′-hydroxyl of uridine or introduced as a nonnucleoside spacer in an oligonucleotide produces an electrophilic 1,4-dicarbonyl that reacts with a cytidine in the opposing strand. Similarly, oxidation of the vinyl sulfide that is an analogue of adenosine produces a more electrophilic vinyl sulfoxide that reacts with the exocyclic amine of cytidine.

We previously reported on the mild oxidation of 1 in duplex DNA by a variety of oxidants, such as singlet oxygen, H2O2, and NaIO4 (Scheme 1). The resulting phenyl selenoxide 2 undergoes rapid [2,3]-sigmatropic rearrangement to an electrophilic species (3) that rearomatizes upon reaction with nucleophiles. The 2′-deoxycytidine analogue 5 undergoes a similar reaction to form 6. In duplex DNA electrophiles 3 and 6 react with nucleotides in the opposing strand to produce cross-linked products (e.g., 4). Water slowly traps the electrophiles in the absence of an appropriate nucleophilic partner in DNA, and other nucleophiles such as azide can be used as well. We anticipated that this reactivity pattern could be extended to RNA where it might be useful for probing tertiary interactions, as well as providing a convergent approach for preparing RNA molecules containing CS-functionalized uridines, a common modification in bacterial RNA.

RESULTS AND DISCUSSION

Synthesis of RNA Containing a Latent Electrophilic CS-Modified Uridine. Phosphoramidite 12 was synthesized in a manner similar to that previously described for 1 starting from 7 (Scheme 2). Although the intermediate bromide en route to 8 could be isolated, this proved to be impractical, and it was the bromination step that was responsible for the low...
Phosphitylated to produce millimole quantities and carried forward to indicate a 2′ product yielded only the desired 2′ product.

Despite this, the reaction was scalable and attempts to push it further resulted in even lower yields.

**Scheme 1**

![Scheme 1 Diagram]

**Scheme 2**

![Scheme 2 Diagram]

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Key: (a) NBS, AIBN, benzene; (b) (PhSe)2, NaBH4, DMF; (c) NH3, MeOH; (d) DMTCl, pyridine; (e) TBDMSCl, AgNO3, THF; (f) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DIPEA, CH2Cl2.

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5'-AGA TGG AT9 TAG CTA C
5'-aga ugg au9 uag gua C
5'-aga ugg aa9 aag gua C

Figure 1. Sequences of oligonucleotides containing 9. Capital letters indicate a 2′-deoxyribonucleotide and small letters a ribonucleotide.

Coupling yields for 12, as measured using dimethoxytrityl cation detection, varied widely from as low as 40% to almost quantitative. Oligonucleotides were deprotected using a mixture of concentrated aqueous ammonia and methylamine (25 °C, 4 h), followed by desilylation using Et3N, CH2Cl2 for 20 min as previously described.28

Carrying out the deprotection at higher temperatures (50–60 °C) led to decomposition of the phenyl selenide (9). Anhydrous tert-butyl hydroperoxide was substituted for iodine in a mixture of water and pyridine as the oxidant to minimize premature oxidation of the phenyl selenide.31 Despite this safeguard, it was necessary to purify oligonucleotides containing the phenyl selenide 9 by a two-step procedure. Following denaturing polyacrylamide gel electrophoresis, full-length oligonucleotide containing 9 was separated from its more polar product(s) (e.g., 17), believed to result from adventitious oxidation, by reverse-phase HPLC. Purified oligonucleotides were characterized by MALDI-TOF MS. We prepared three oligonucleotides containing 9 for this study (Figure 1). In each instance, a 2′-deoxynucleotide is introduced at the 3′-terminus. This was performed for convenience, so that NaIO4 could be used as an oxidant. However, H2O2 (vide infra) is also a satisfactory oxidant and is compatible with 3′-terminal ribonucleotides.

**Rapid Formation of an Electrophile from 9 upon Mild Oxidation.** Mild oxidation of phenyl selenides 1 and 5 rapidly produces the corresponding allylic phenyl selenenates (3, 6) via the respective selenoxides (e.g., 2). Sodium periodate was the oxidant of choice for the 2′-deoxyribonucleosides, but this reagent is incompatible with the vicinal diol in ribonucleosides. The oxidation of ribonucleosides with NaIO4 is so facile that this reagent is used in conjunction with reductive amination to produce 3′- oligonucleotide conjugates.32 Consequently, H2O2 was used.1H NMR analysis (with water suppression) of the oxidation carried out in deuterated phosphate buffer (50 mM, pH 7.4) showed that 9 behaves in a very similar manner to 1 and 5 (Figure 2).20,22 The phenyl selenide 9 was consumed within minutes, giving rise to a diastereomeric mixture of the allylic phenyl selenenate (16, Figure 2B). The corresponding selenoxide was not detected. In the absence of a nucleophile, 16 reacts slowly with H2O to form 17, which was only a minor product after 18 h at 25 °C (Figure 2C). Phenyl selenenate 16 reacts more slowly with H2O in buffer than does 3 or 6, both of which showed complete conversion to their respective hydroxymethyl species (e.g., 17) after 18 h.20,22

**Interstrand Cross-Link Formation from 9 upon Mild Oxidation.** UV-melting experiments indicated that 9 did not significantly destabilize duplex RNA. The Tm (10 mM phosphate pH 7.4, 100 mM KCl, 2 mM MgCl2) for 19a containing 9 (56.3 ± 0.4 °C) was just slightly lower than that of the analogous duplex containing uridine in place of the phenyl selenide (19b, 58.7 ± 0.3 °C). Stable hybridization is a requirement for ICL formation. Initial cross-linking experiments were carried out on 9 in a duplex composed of 2′-deoxyribonucleotides (18, Figure 3). Treatment of 5′-32P-18 with NaIO4 (5 mM) yielded 51% interstrand cross-linked (ICL) product in 3 h at 25 °C and more than 45% in 30 min. This is comparable to and even higher than the ICL yields obtained from 1 and 5 under the same conditions.20,22 In contrast, oxidation of 5′-32P-19a reliably produced less than 15% of the cross-linked product at 25 °C and only 30 ± 4% at 37 °C in 5 h. Similarly, oxidation of 5′-32P-19a by H2O2 (10 mM) at 37 °C produced the cross-linked product in 26 ± 1% yield. Hydroxyl...
radical cleavage indicated that the A opposite 9 (A24) was the major cross-linking site, but a small amount of cross-linking also occurred at A25. Mass spectrometry of the ICL confirmed that the product corresponded to the mass of the duplex following the loss of benzeneselenol. Upon the basis of these observations and the precedent established by 1, we propose that 23 corresponds to the major ICL product from oxidation of 9 in 19a. Hence, 16 (produced from oxidation of 9) reacts predominantly with the opposing adenine, producing the cross-link product analogous to 4 (Scheme 1).

Kinetic analysis of the cross-linking of 19a correlated with the observed lower yields (Figure 4). The first-order rate constant was $1.0 \pm 0.2 \times 10^{-4}$ s$^{-1}$ ($t_{1/2} = 116$ min). This is more than 11 times slower than the rate constant for cross-linking from 1 in a comparable DNA duplex. The slower reactivity of 16 with water observed by $^1$H NMR is not the source of decreased cross-linking rate constant. If it were, then 9 would also have reacted more slowly in the DNA duplex (18). Subsequent cross-linking experiments were typically carried out overnight at 37 °C to ensure complete reaction of 9 (or more accurately 16). In search of an explanation for why the ICL yield from 19a was so much lower than in 18, we focused on possible differences in the rotational barrier of the glycosidic bond in 16 as a function of oligonucleotide sequence, because ICL formation requires occupation of the syn-conformation in which the allylic phenyl selenate is oriented toward the opposing strand in the duplex (Scheme 3). This implicitly assumes that the rate-limiting step in cross-linking is...

Figure 2. $^1$H NMR analysis of H$_2$O$_2$ (50 mM) oxidation of 9 (50 mM) in deuterated phosphate buffer (50 mM, pD 7.4) at 25 °C. A. Prior to H$_2$O$_2$ addition. B. Fifteen minutes after H$_2$O$_2$ addition. C. Eighteen hours after H$_2$O$_2$ addition.

Figure 3. Sequences of oligonucleotide duplexes containing 9. Capital letters indicate a 2′-deoxyribonucleotide and small letters a ribonucleotide.

Figure 4. Interstrand cross-link growth from 5′-32P-19a as a function of time at 37 °C following oxidation by NaIO$_4$ (5 mM).
adoption of the syn-conformation. This is unknown for 16 but has been shown to be the case in related studies in which ICLs are produced upon photolysis of 1,23,36

We initially examined the effects of mismatches at the adjacent base pair (20a, 20b). Duplex destabilization in the vicinity of the 9-adenosine base pair might facilitate adoption of the syn-conformation by weakening base pairing. However, in side-by-side reactions the ICL yields from 19a (22 ± 2%), 20a (21 ± 2%), and 20b (22 ± 5%) were within experimental error of one another. Consequently, we may rely upon related literature to rationalize the slower cross-linking by 16 than by 3 and the assumption. It is well established that the barrier for rotation about the glycosidic bond in pyrimidines is lower when the nucleoside is in the C2′-endo conformation than when it populates the C3′-endo conformation (Scheme 3).37 2′-Deoxynucleotides adopt the C2′-endo conformation in B-form DNA whereas RNA duplexes typically adopt a A-form structure in which the ribonucleotides are in the C3′-endo conformation. Furthermore, recent calculations predict that 2′-deoxy pyrimidine nucleosides populate the syn-conformational isomer that is required for ICL formation more readily than do the comparable ribonucleosides.38 Although ribonucleotides typically adopt the C3′-endo conformation in duplex RNA (e.g., 19a), structurally related 2′-fluorothymidine is believed to populate the C2′-endo isomer when only one molecule of it is present in a DNA duplex.39 We hypothesize that the surrounding duplex environment influences the sugar pucker of 9 (16) much the same way that the conformation of 2′-fluorothymidine is affected. Lower ICL yields and slower cross-linking are observed from 19a because 9 (16) exists predominantly in the C3′-endo conformation, whereas the C2′-endo conformation of the phenyl selenide should be relatively preferred in the DNA duplex (18).

Cross-linking in duplexes containing 9 treated with NaIO4 was general and in many ways paralleled the reactivity of 1.21 For instance, the ICL yield declined to 14 ± 2% when 9 was flanked by adenosines (22) that provide greater π-stacking from 22 ± 2% in 19a. We also observed that ICLs form more efficiently when 9 is opposite adenosine and cytidine than when the phenyl selenide is opposed by guanosine or uridine (Figure 5). This could be the result of the positioning of the electrophile in syn-16 with the relatively more nucleophilic N1 and N3 of adenine (19a) and cytosine (21b), respectively, compared to the N1 (21a) and N3 (21c) of guanine and uracil. As was the case with 19a, hydroxyl radical cleavage of the isolated ICLs indicated that the nucleotide opposite 9 (16) is the major site of cross-linking.35 Salt content also had an effect on ICL yields in 19a (Figure 6). Addition of MgCl2 to NaIO4 oxidations of 19a significantly lowered the cross-link yields, and to a smaller extent so too did increasing the concentration of NaCl from 100 mM to 250 mM. One possible explanation for the reduced ICL yields with either increasing MgCl2 or NaCl concentration is that the duplex is more stable in higher salt, resulting in an increase in the barrier for rotation about the glycosidic bond in 16 (Scheme 3). The larger decrease observed by the addition of MgCl2 may also be explained by the report that magnesium rigidifies the RNA.39 This too could increase the barrier to forming the syn-conformation of 16 that is required for cross-linking.

**Synthesis of Amino Acid Conjugates from 9.** Oligonucleotide conjugates have useful applications in biotechnology and as potential therapeutic agents.41–47 In addition, RNA is often posttranslationally modified. Some amino acid modifications at the C5-position of uridine are proposed to proceed via electrophilic intermediates analogous to 16 that result from cysteine addition to the C6-position.23 Consequently, we carried out conjugation reactions between 14 and amino acids to demonstrate the utility of phenyl selenide 9 (Scheme 4). The respective conjugates of 14 (10 μM) with glycin (24a, 59%) and phenylalanine (24b, 43%) were isolated by reverse-phase HPLC and characterized by MALDI-TOF MS, following reaction in the presence of the amino acid (10 mM) and NaIO4 (5 mM) at 37 °C for 3 h.

**Figure 5.** Interstrand cross-link yield from 9 as a function of opposing nucleotide at 37 °C following oxidation by NaIO4 (5 mM).

**Figure 6.** Intersstrand cross-link yield from 9 in 19a as a function of salt at 37 °C following oxidation by NaIO4 (5 mM). Note: [NaCl] = 100 mM as [MgCl2] is varied (left), and [MgCl2] = 0 as [NaCl] is varied (right).
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The reaction was stirred under argon for 5 h. The contents of the reaction flask consisted of a slightly yellow liquid layer and gray solid collected at the bottom of the flask. The heterogeneous mixture was filtered through Celite into a stirring 20% NaHCO₃ solution (10 mL), extracted with EtOAc (3 × 10 mL), and concentrated in vacuo to yield a yellow foam (448 mg). Purification by flash chromatography (10% EtOAc in DCM) yielded 11 as a white foam (337 mg, 64%). 1H NMR (CDCl₃) δ 0.13 (s, 6H), 0.92 (s, 10H), 2.59 (d, 1H, J = 5.2 Hz), 3.12–3.10 (d, 1H, J = 11.6 Hz), 3.44–3.35 (dd, 2H, J = 19.6, J = 12.4 Hz), 3.09 (d, 1H, J = 12.4 Hz), 3.34 (ddd, 2H, J = 19.6, J = 12.4 Hz, J = 4.4 Hz), 3.31 (t, 1H, J = 4.8 Hz), 3.25–3.15 (m, 7H), 3.76 (m, 3H, J = 7.2 Hz), 4.49–4.46 (dd, 1H, J = 6.4 Hz, J = 4.8 Hz), 4.85–4.83 (s, 1H, J = 4.8 Hz), 5.95–5.93 (d, 1H, J = 5.6 Hz), 6.05–6.03 (d, 1H, J = 6 Hz), 6.84–6.79 (m, 8H), 7.16–7.12 (m, 6H), 7.33–7.26 (m, 19H), 7.73–7.41 (m, 4H), 7.70 (s, 1H), 7.73 (s, 1H), 8.62 (br, 2H). 13P NMR (CDCl₃) 149.1, 150.5; HRMS (ESI/APCI-TOF) calculated for [M + Na]+ C₇₁H₈₁N₂O₄NaSiSe 853.2394, found 853.2395.

Preparation of 12. Silylated nucleoside 11 (101 mg, 0.13 mmol) was aerotropically dried twice over pyridine (1 mL). Dried 11 was dissolved in DCM (0.8 mL), and H₂O₂ was added to the tube via pipet. The reaction was quickly agitated and placed as a white foam (102 mg, 81%): 1H NMR Study of Oxidation of 9 by H₂O₂. Nucleoside 9 was dissolved in D₂O (50 mM deuterated phosphate buffer, pH 7.4) to make a 50 mM solution in an NMR tube. The mixture was analyzed via 1H NMR with water suppression, whereupon a 20X solution of H₂O₂ was added to the tube via pipet. The final concentrations of H₂O₂ were 50 mM. The reaction mixture was quickly agitated and placed in a 30°C water bath for 20 min. To this was added 5 μL of 95% formamide loading buffer, and subjected to electrophoresis on a 20% denaturing polyacrylamide gel. The alkali ladder was generated by treating radiolabeled oligonucleotide with 0.2 M NaOH in 10 mM EDTA at 90 °C for 15 s. The reaction was quenched by addition of 5 μL of each stop buffer (9.5 M urea, 85 mM NaOAc, 1% v/v AcOH) and 95% formamide loading buffer. RNase A sequencing was performed with 1 μL of enzyme in 5 μL of reaction buffer (300 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl (pH 7.5)) at 37 °C for 20 min. To this was added 5 μL of 95% formamide loading buffer.

Conjugation Reactions with Amino Acids. The phenyl selenide-modified oligonucleotide (14, 10 μM) and the relevant nucleophile (10 mM) were incubated at 37 °C in a mixture of 10 mM potassium phosphate (pH 7.2), 100 mM NaCl, and 5 mM NaOAc (total volume 10 μL). The resulting solution was diluted with the addition of extra water (15 μL) and filtered through a 0.22 μm filter. The mixture was subjected to UPLC analyses on a RP-C18 HPLC column with monitoring carried out at 260 nm. Peaks were analyzed using the following gradient conditions: 0–18 min 10–20% B in A, 18–23 min 20–80% B in A, 23–28 min 80% B in A, 28–30 min 80–10% B in A, 30–50 min 10% B in A, at a flow rate of 1.0 mL/min [A: 0.05 M TEAA (pH 7.0)/MeCN 95:5; B: 0.05 M TEAA (pH 7.0)/MeCN 50:50]. The relevant peak of interest was collected from the LC, lyophilized to dryness, and analyzed by MALDI-TOF MS.

### ASSOCIATED CONTENT

#### Supporting Information

NMR spectra of monomers, mass spectra of oligonucleotides containing nonnative nucleotides, hydroxy radical cleavage histograms of cross-linked RNA, and full 1H NMR spectra of the oxidation of 9 (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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