Solid-Phase Synthesis of Phosphorothioate Oligonucleotides Using Sulfurization Byproducts for *in situ* Capping

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HPLC chromatograms and MS spectra.

This section provides the UPLC-UV-MS chromatograms and spectra of the DMT-on products obtained from the syntheses with (4-reaction synthesis) and without (3-reaction synthesis) Ac_2O capping step. Zoomed-in chromatograms and spectra are provided to show details of the failure sequences (the small peaks eluting before the main peaks) and the (n-1) impurities. For a visual inspection of product quality, a representative full-scale LC chromatogram and a mass spectrum of product peak of crude DMT-on **9** are provided in Figure S1 and S2, respectively.

When a reference sample was available, the DMT-on product yield was obtained by the UPLC-UV-MS analysis and reported. For other compounds that we do not have reference standards, overlaid mass spectra are provided for MS purity comparison and optical density per μmol measured at 260 nm (OD_{260}) and UV purity were reported for yield comparison. The HRMS data of these compounds are also provided.

Figure S1. A Representative Full-Scale UPLC UV Chromatogram (Crude DMT-on 9)

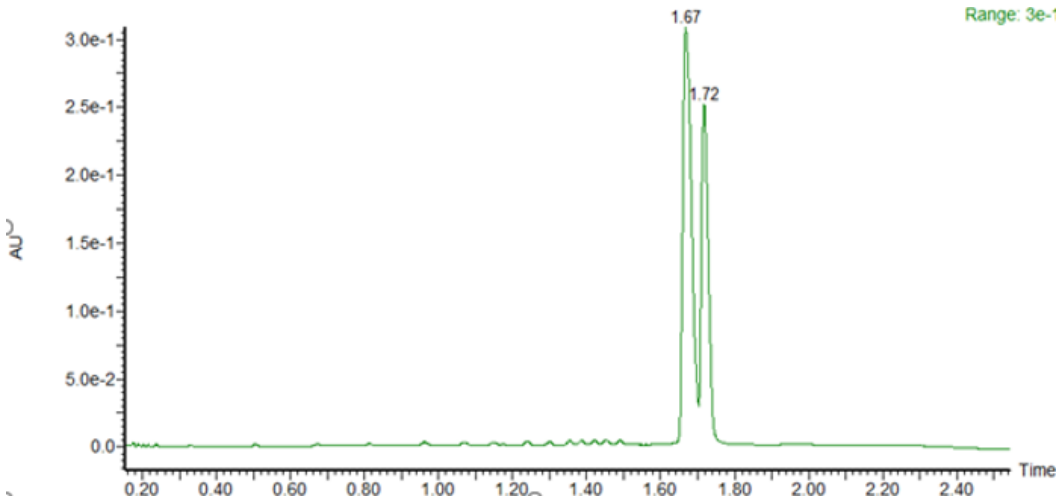
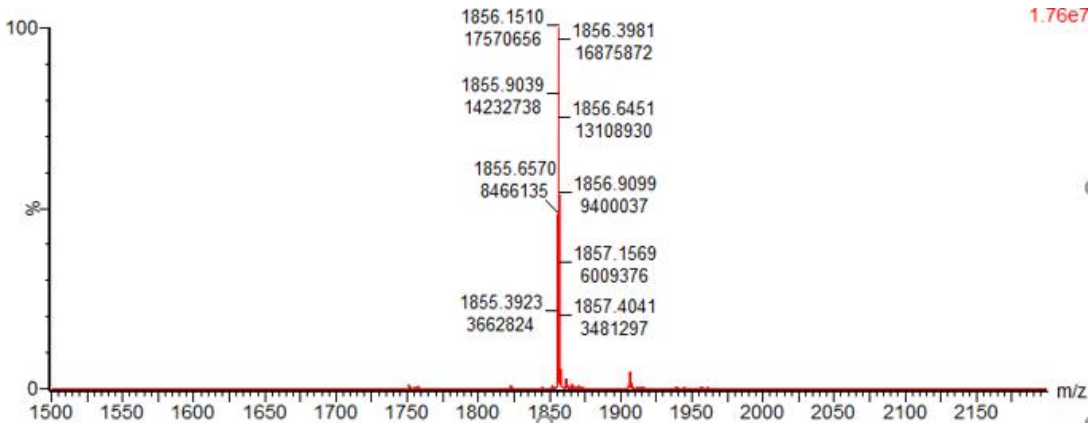
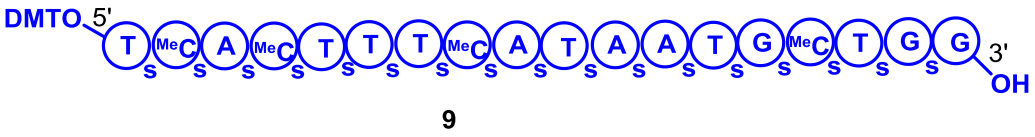


Figure S2. A Representative Full-Scale Mass Spectrum of the Product Peak (Crude DMT-on 9)



DMT-on phosphorothioate oligonucleotide 9

Sequence:



9

^{Me}C = 5-Me Cytosine

s for PS linkage

a 18-mer 2’ methoxyethoxy (MOE) ribose phosphorothioate oligonucleotide.

Figure S3. UPLC UV chromatograms of crude DMT-on 9 synthesized with PADS as the sulfurization agent

Black 3-reaction process; red: 4-reaction process

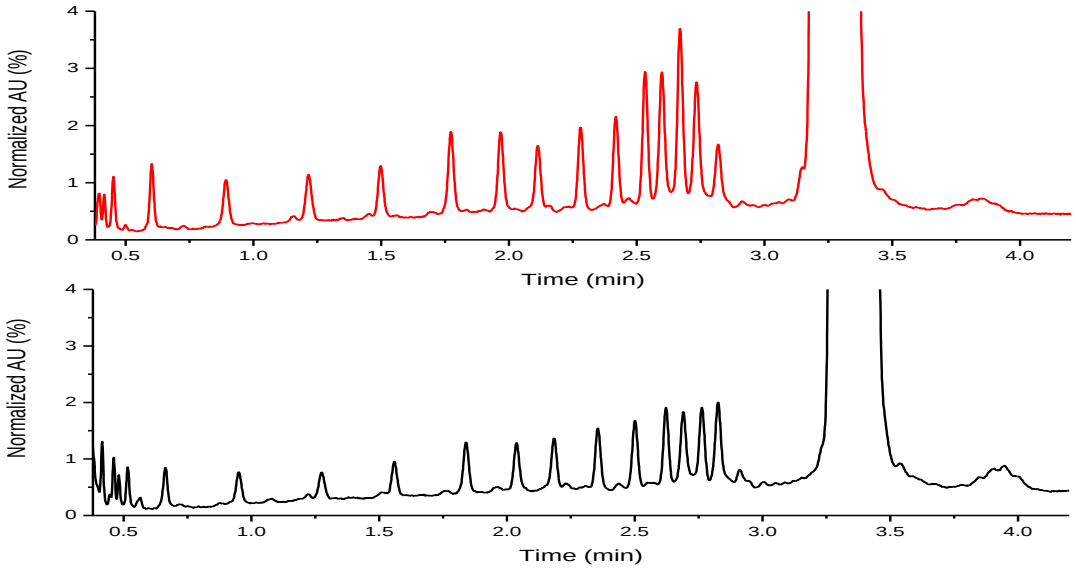


Figure S4. Overlaid MS spectra of crude DMT-on 9 synthesized with PADS as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

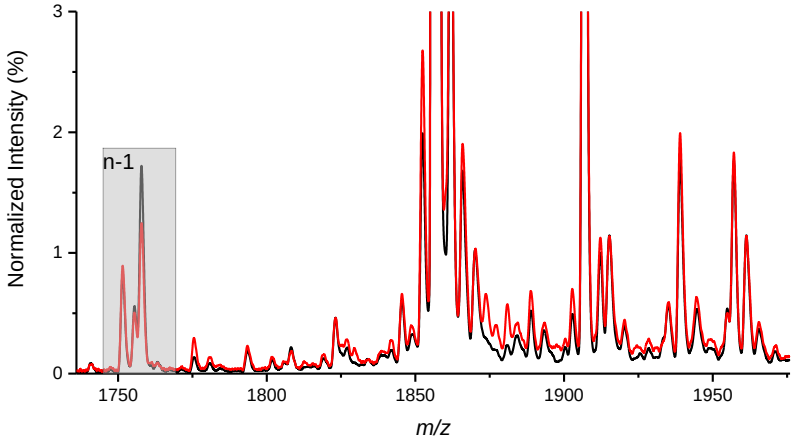


Figure S5. UPLC UV chromatograms of crude DMT-on 9 synthesized with ADTT as the sulfurization agent

Black: 3-reaction with ADTT; Red: 4-reaction process with PADS

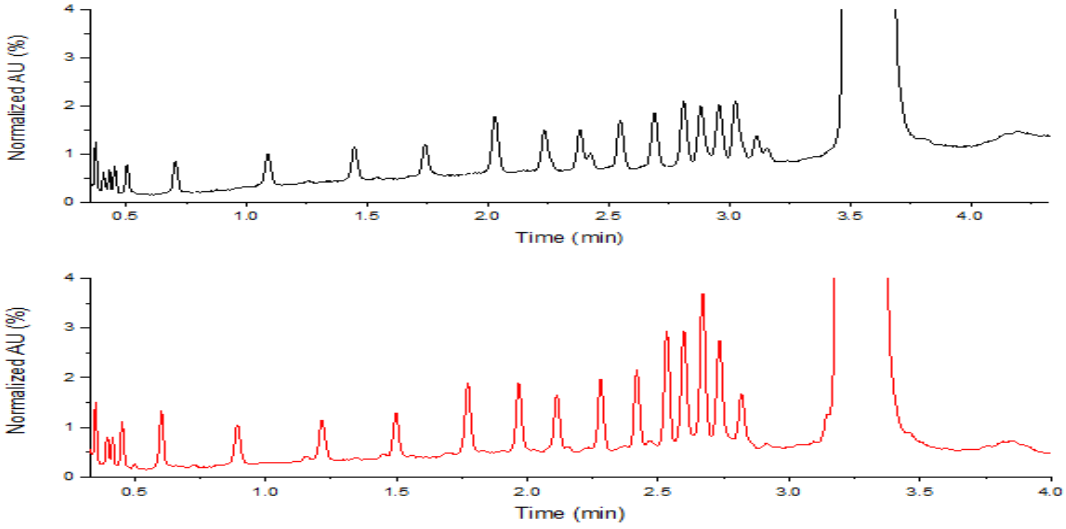
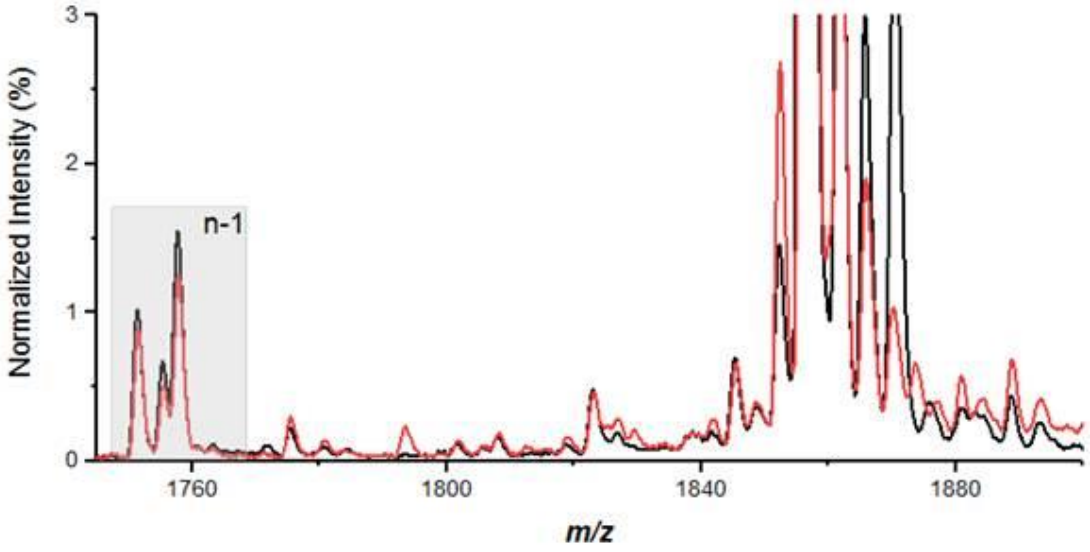


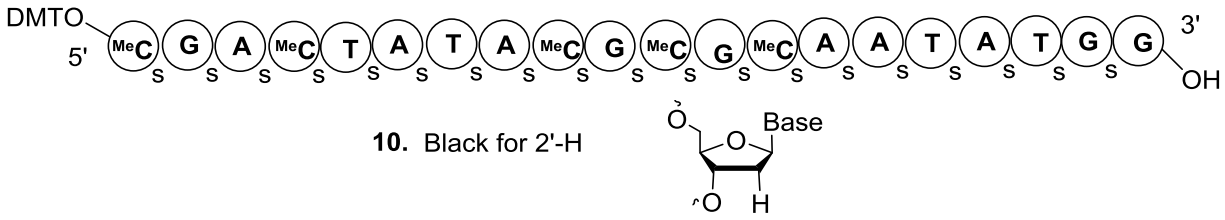
Figure S6. Overlaid MS spectra of crude DMT-on 9 synthesized with ADTT as the sulfurization agent

Black: 3-reaction with ADTT; Red: 4-reaction process (with PADS as the sulfurization agent)



DMT-on phosphorothioate oligonucleotide 10

Sequence:



a 20-mer 2’H ribose phosphorothioate oligonucleotide

n-1 impurities appear between m/z: 1613- 1620.

Figure S7. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 10 synthesized with ADTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

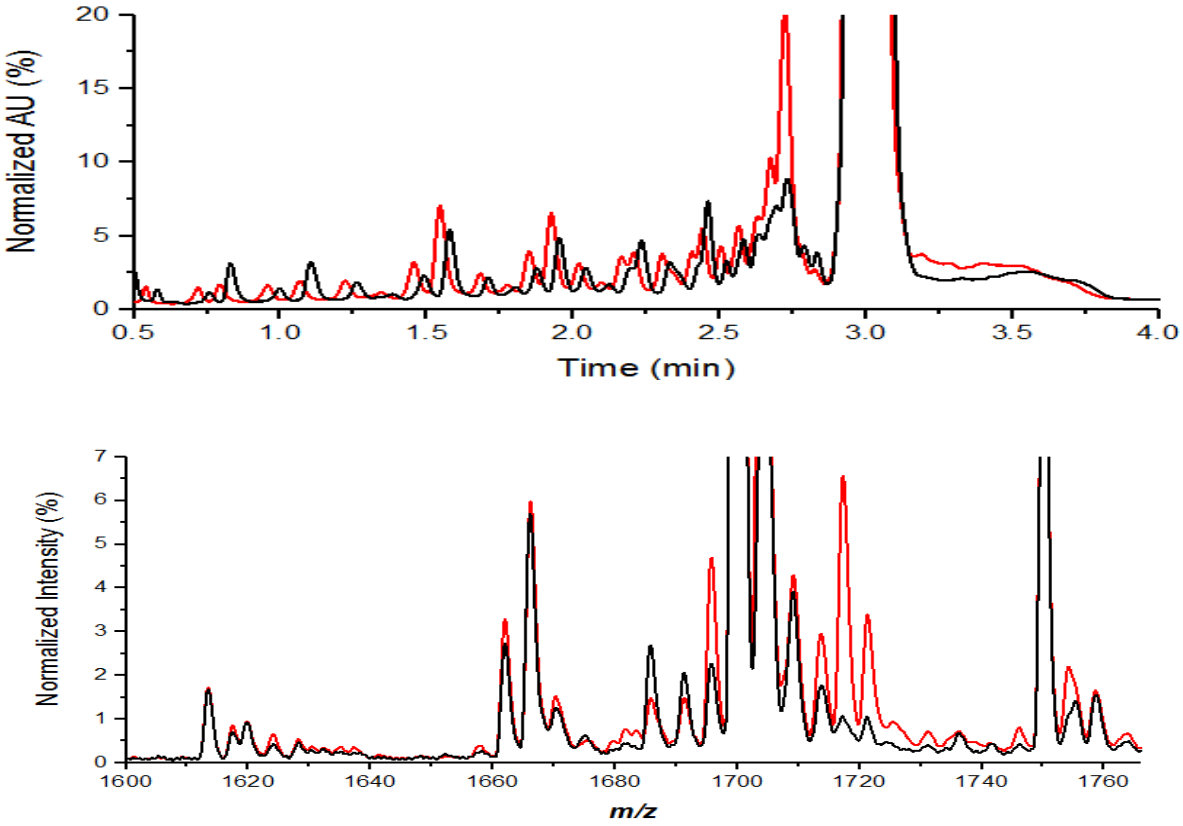


Figure S8. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 10 synthesized with PADS as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

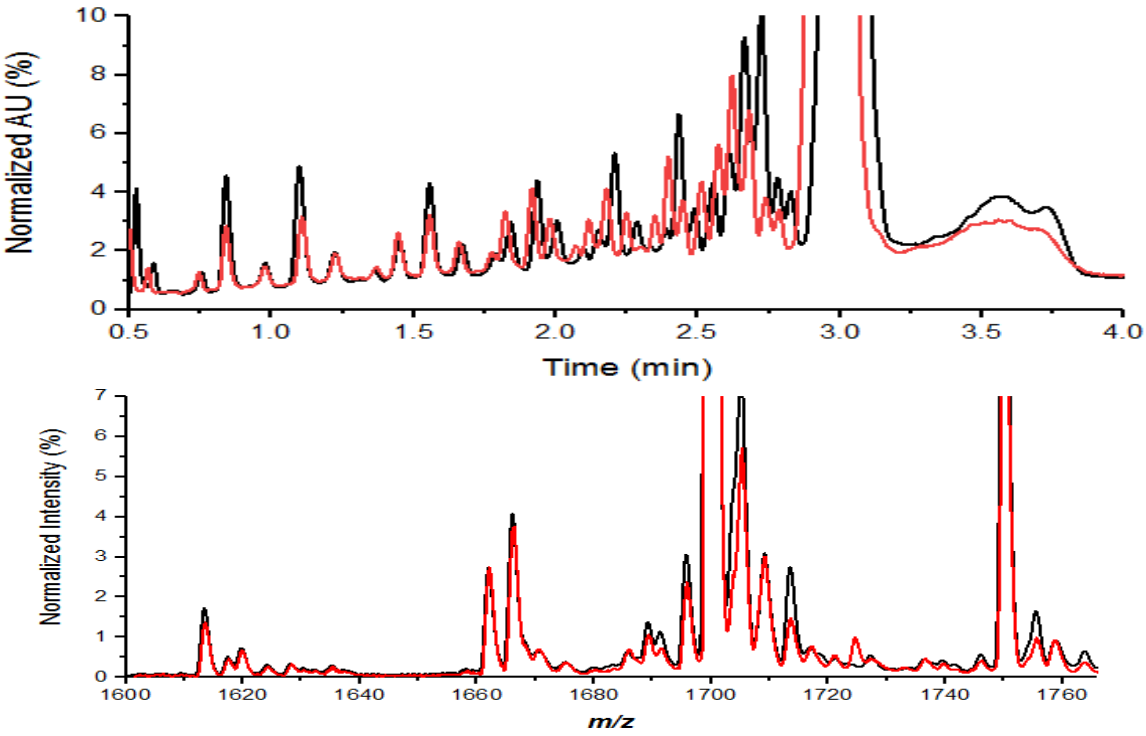
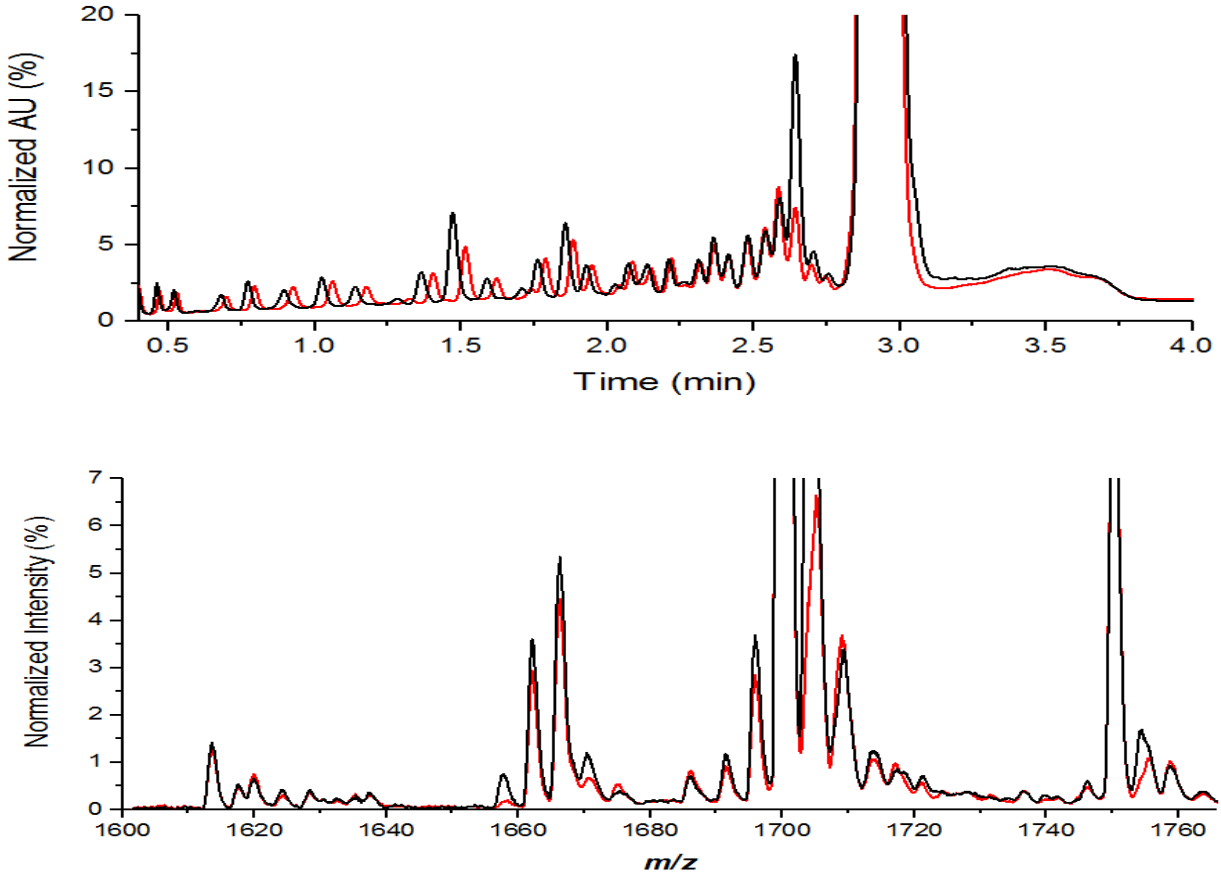


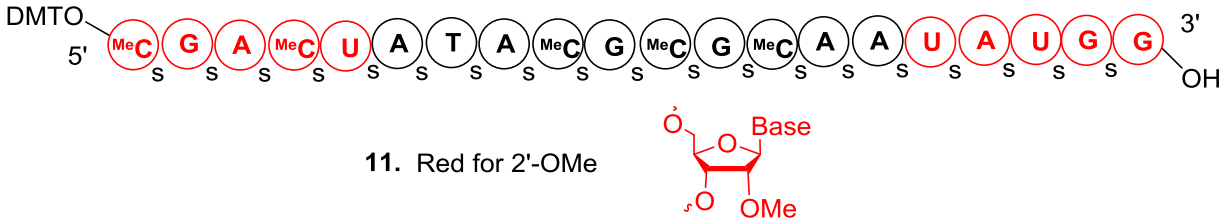
Figure S9. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 10 synthesized with DDTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process



DMT-on phosphorothioate oligonucleotide 11

Sequence:



a 20-mer 2'H (black) and 2' OMe (red) ribose phosphorothioate oligonucleotide

n-1 impurities appear between m/z: 1671-1690.

Figure S10. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 11 synthesized with ADTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

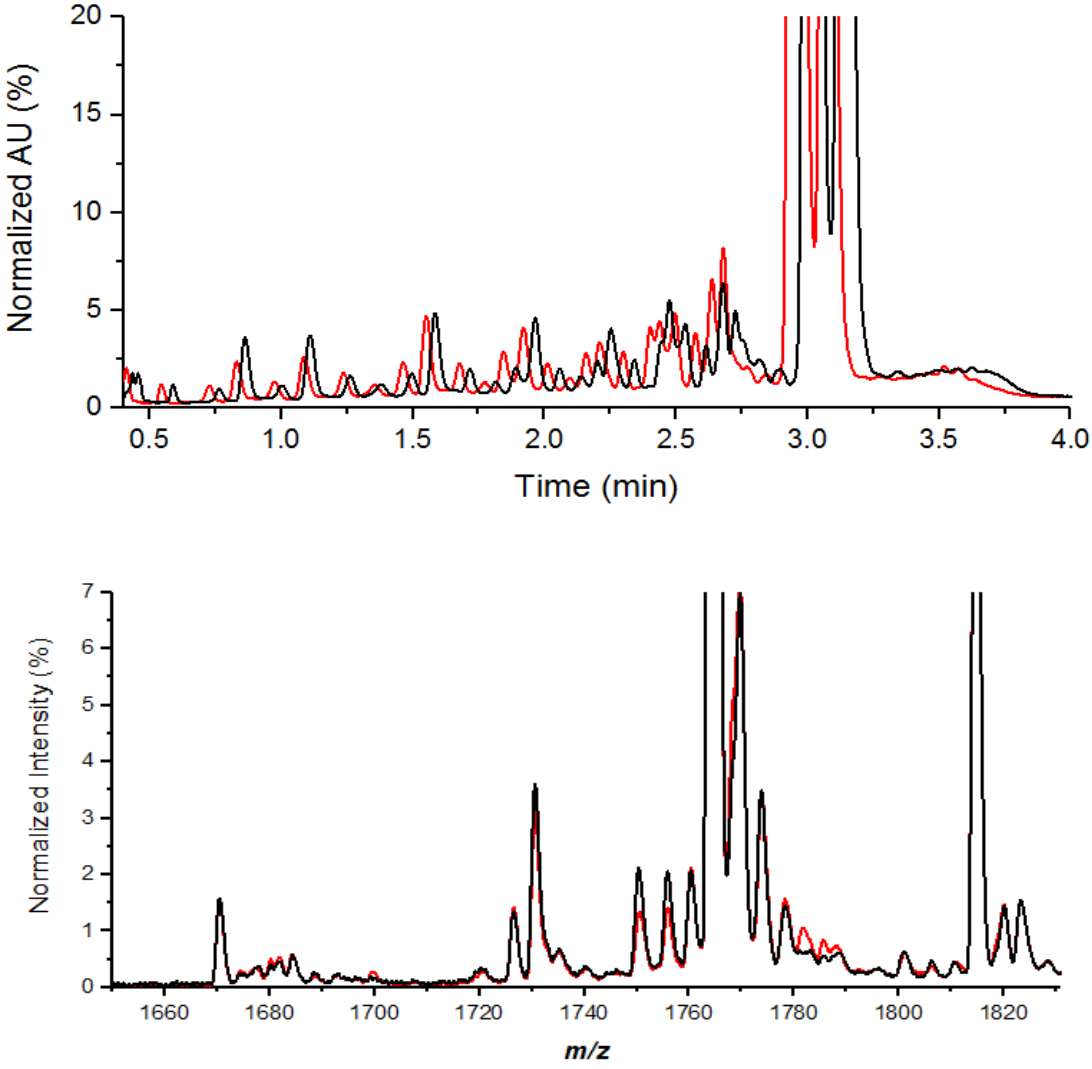
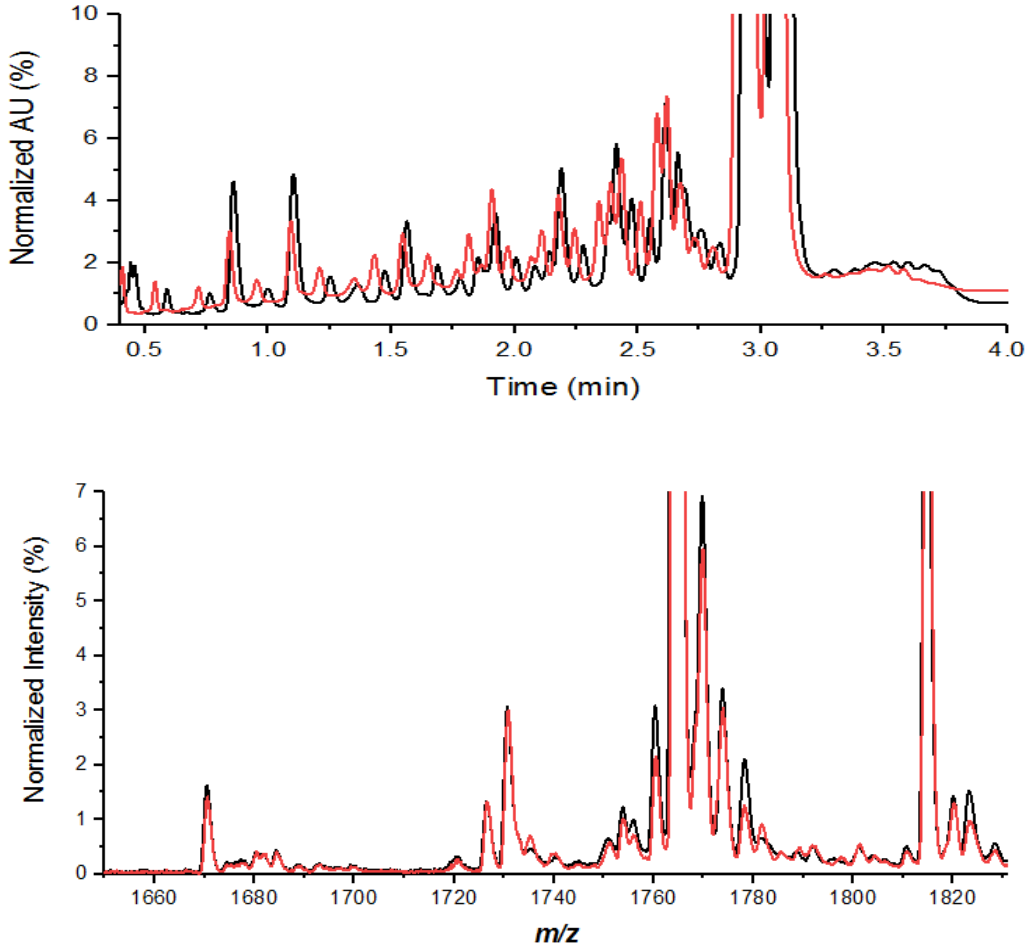


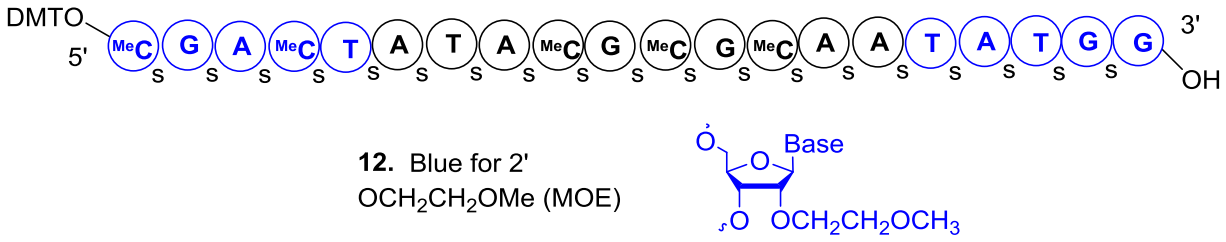
Figure S11. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 11 synthesized with PADS as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process



DMT-on phosphorothioate oligonucleotide 12

Sequence:



a 20-mer 2'H (black) and 2' methoxyethoxy (MOE) (blue) ribose phosphorothioate oligonucleotide

n-1 impurities appear between m/z: 1780-1804.

Figure S12. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 12 synthesized with ADTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

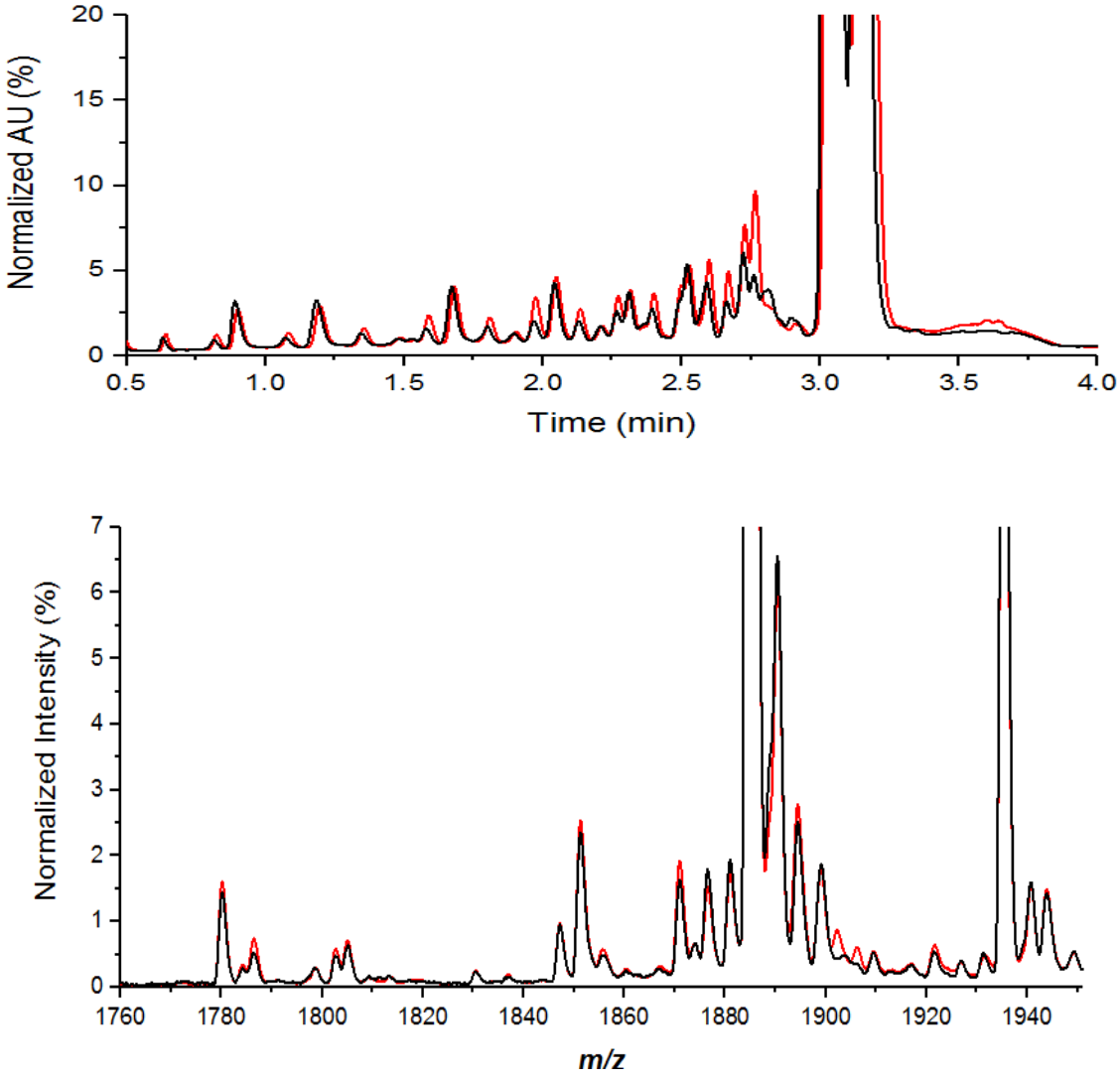
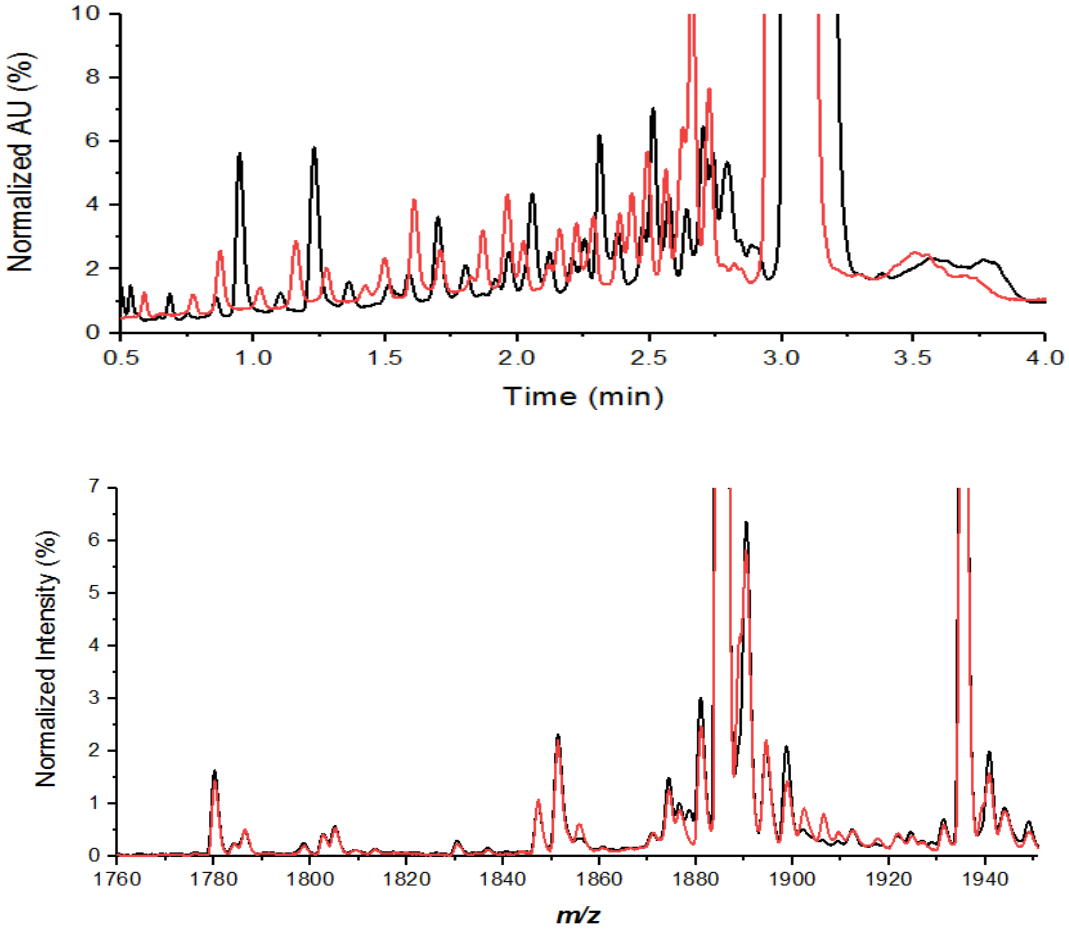


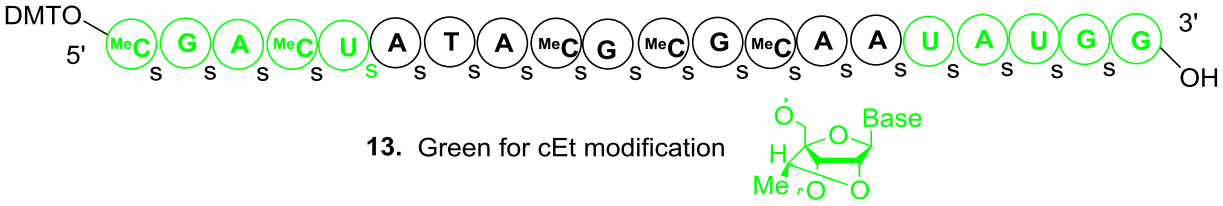
Figure S13. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 12 synthesized with PADS as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process



DMT-on phosphorothioate oligonucleotide 13

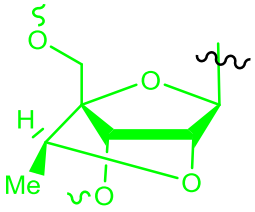
Sequence:



13. Green for cEt modification

a 20-mer 2'H (black) and 2' cEt (green) ribose phosphorothioate oligonucleotide

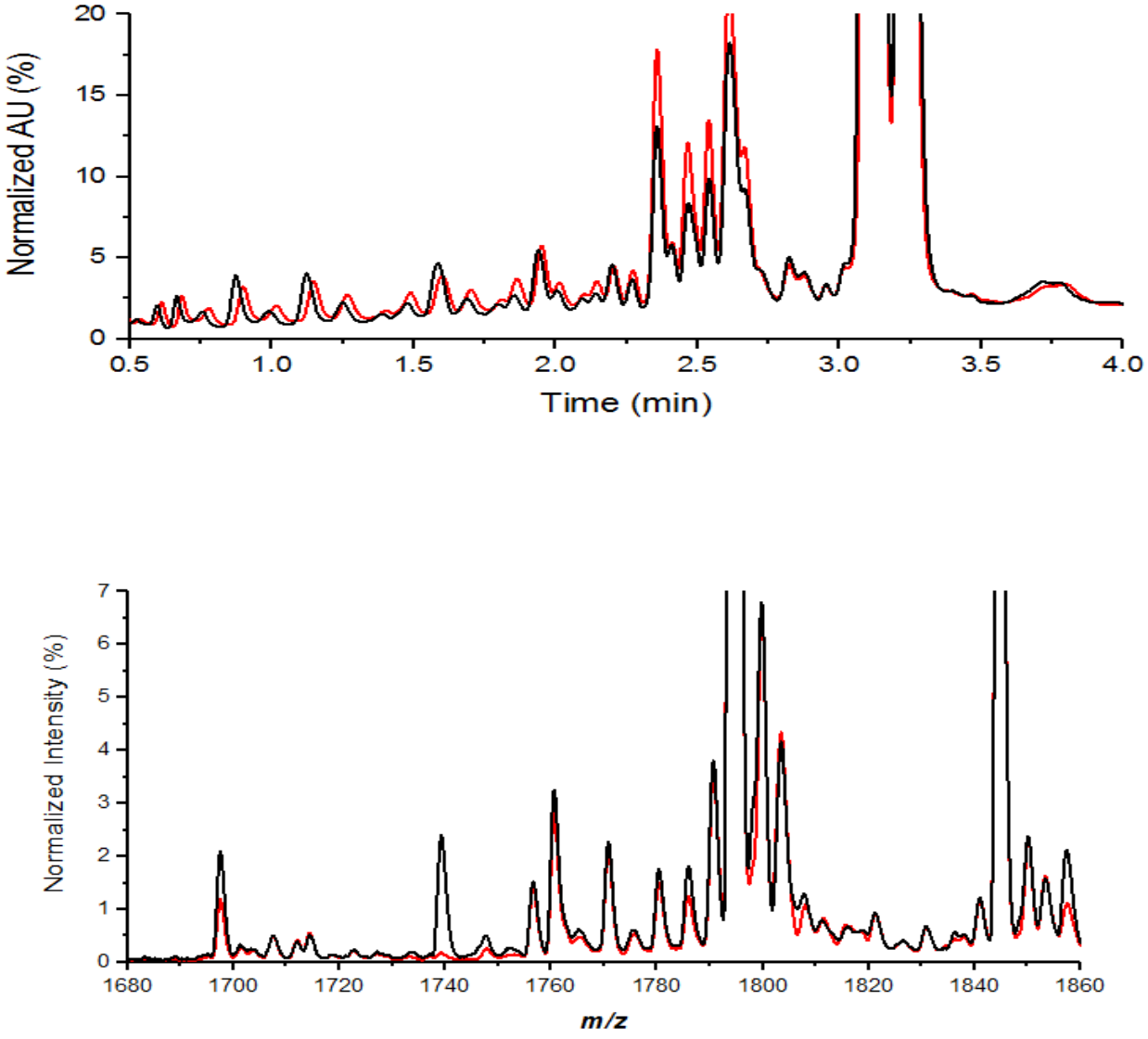
cEt ribose



n-1 impurities appear between m/z: 1699-1718.

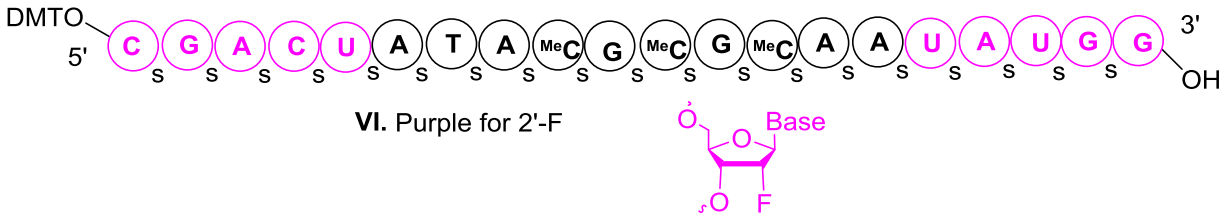
Figure S14. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 13 synthesized with ADTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process



DMT-on phosphorothioate oligonucleotide 14

Sequence:



a 20-mer 2'H (black) and 2' F (purple) ribose phosphorothioate oligonucleotide

n-1 impurities appear between m/z: 1636- 1645.

Figure S15. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 14 synthesized with ADTT as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process

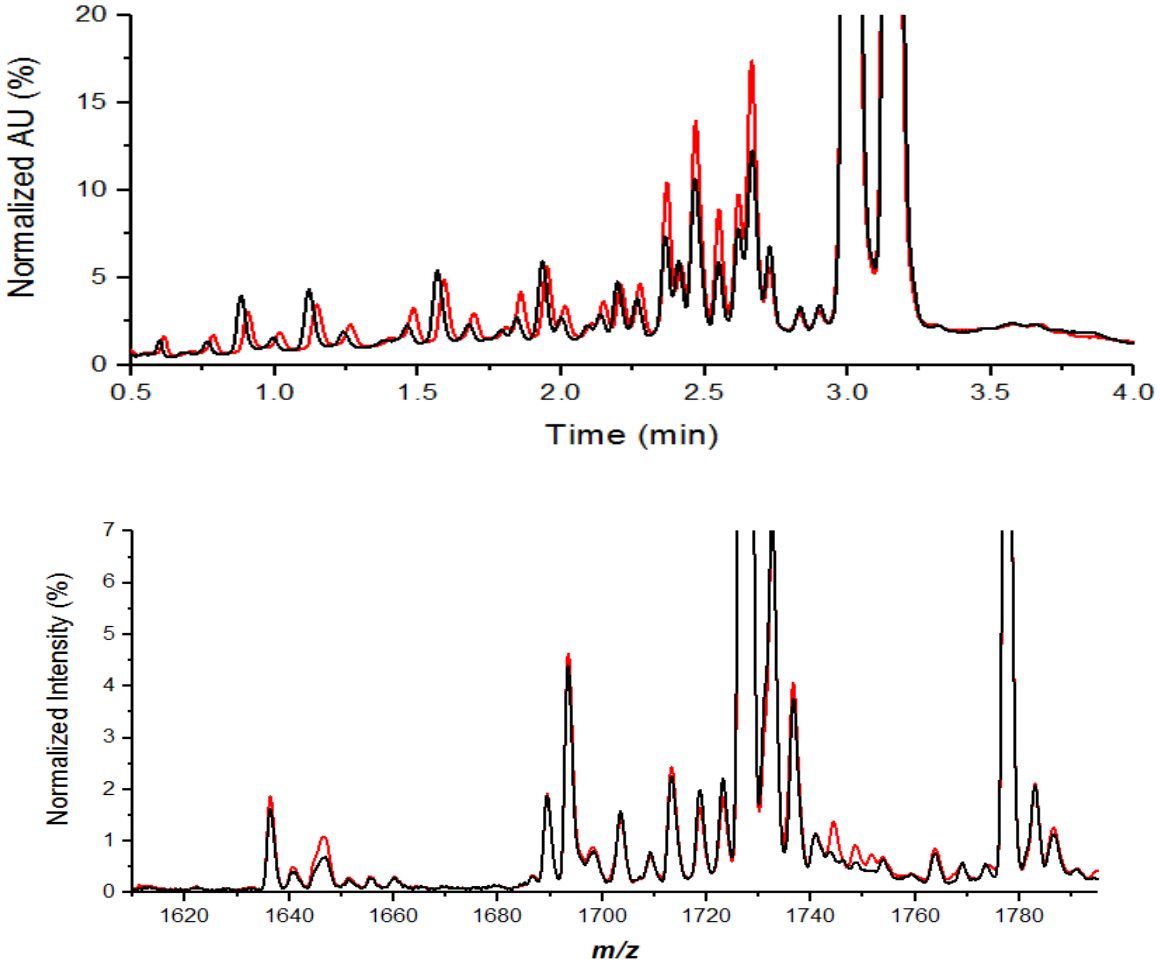
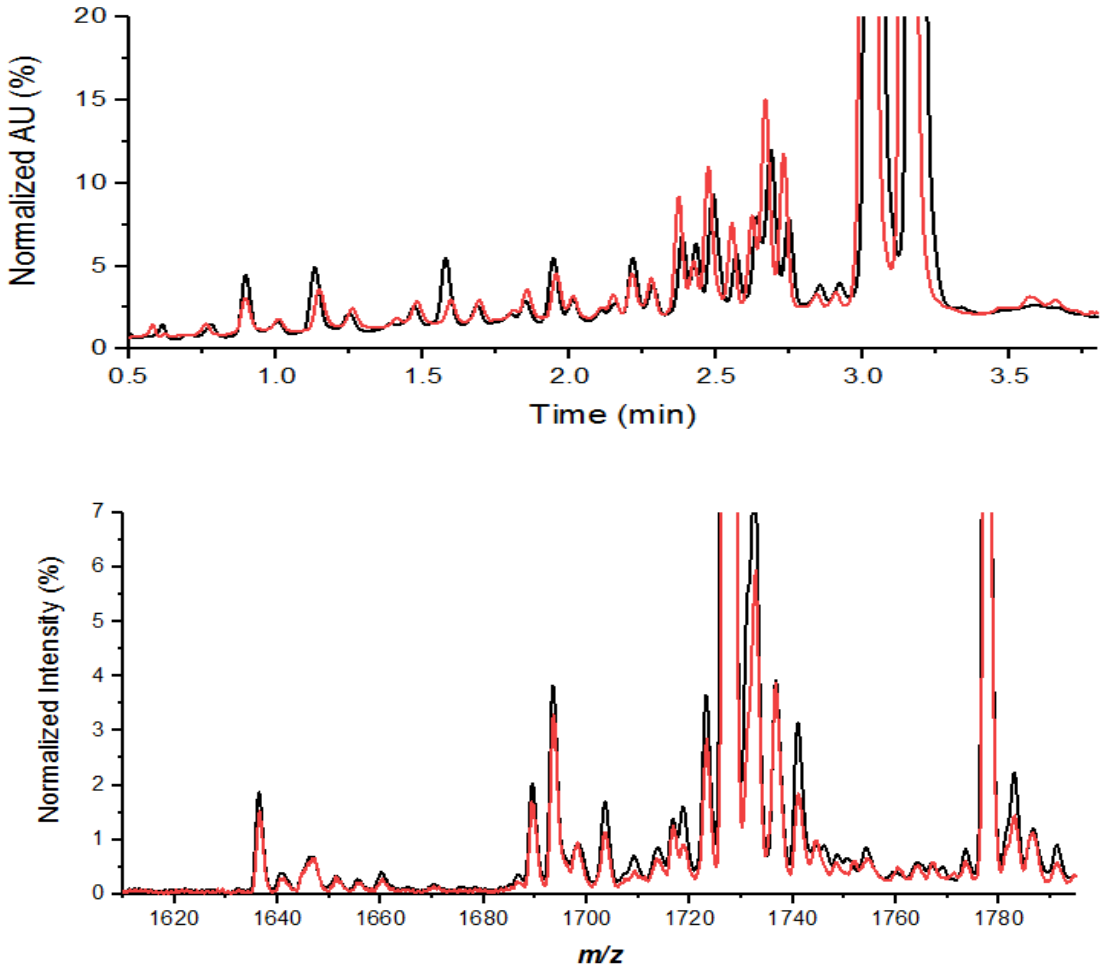


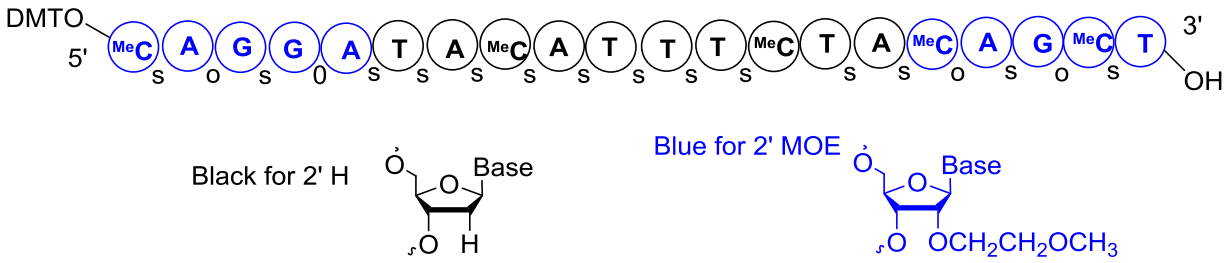
Figure S16. Overlaid UPLC UV Chromatograms and Mass Spectra of crude DMT-on 14 synthesized with PADS as the sulfurization agent

Black: 3-reaction; Red: 4-reaction process



DMT-on phosphorothioate oligonucleotide 15

Sequence:



15. s for PS linkage and o for PO linkage

a 20-mer 2'H (black) and 2' MOE (blue) ribose oligonucleotide; s: PS linkage, o: PO linkage

PADS used as sulfurization agent.

(n-1) impurities appear between m/z: 1750–1784

Figure S17. Overlaid RPIP HPLC UV chromatograms of the Crude DMT-on 15 Using PADS

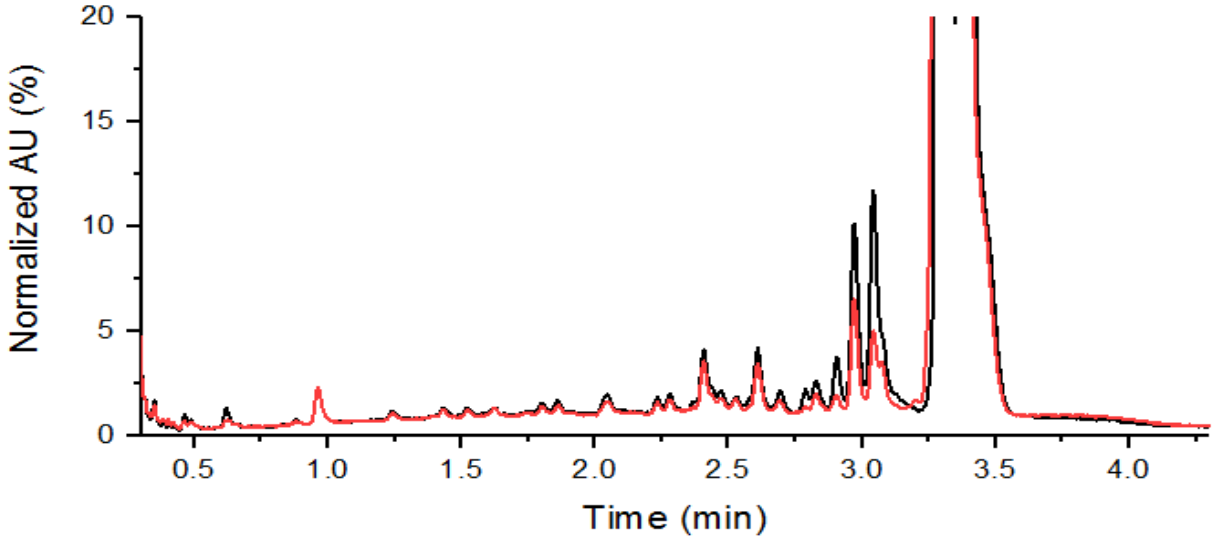
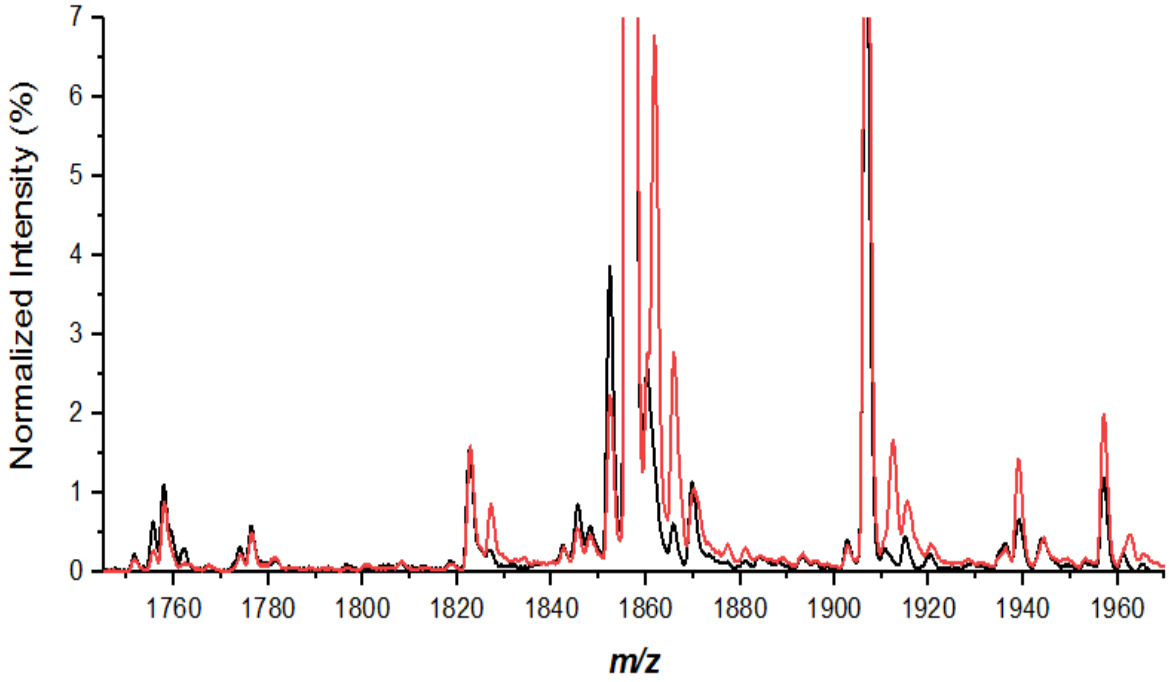


Figure S18. Overlaid MS spectra of Crude DMT-on 15



NMR Experiments

Esterification reaction of *n*-BuOH in the reaction mixture of $P(OMe)_3$ and PADS aged in the presence of 3-picoline (1.0 equiv)

Scheme S1: Conversion of n-BuOH into $PhCH_2CO_2Bu$ during the Sulfurization of $P(OMe)_3$ with PADS Aged in 3-Picoline

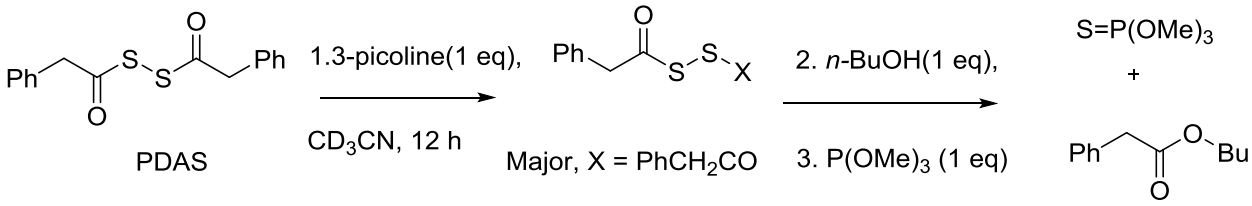


Figure S19 and **Figure S20** show the full and partial spectra obtained at different times. From the spectra, the ratios of n-BuOH to $PhCH_2CO_2Bu$ at the corresponding reaction times were obtained and listed in **Table S1**.

Figure S19. Partial 1H NMR spectra (region 4.1-0.9 ppm) of the reaction mixture of n-BuOH (1.0 equiv), $P(OMe)_3$ (1.0 equiv), PADS (1.0 equiv) aged with picoline (1.0 equiv) at different reaction time points.

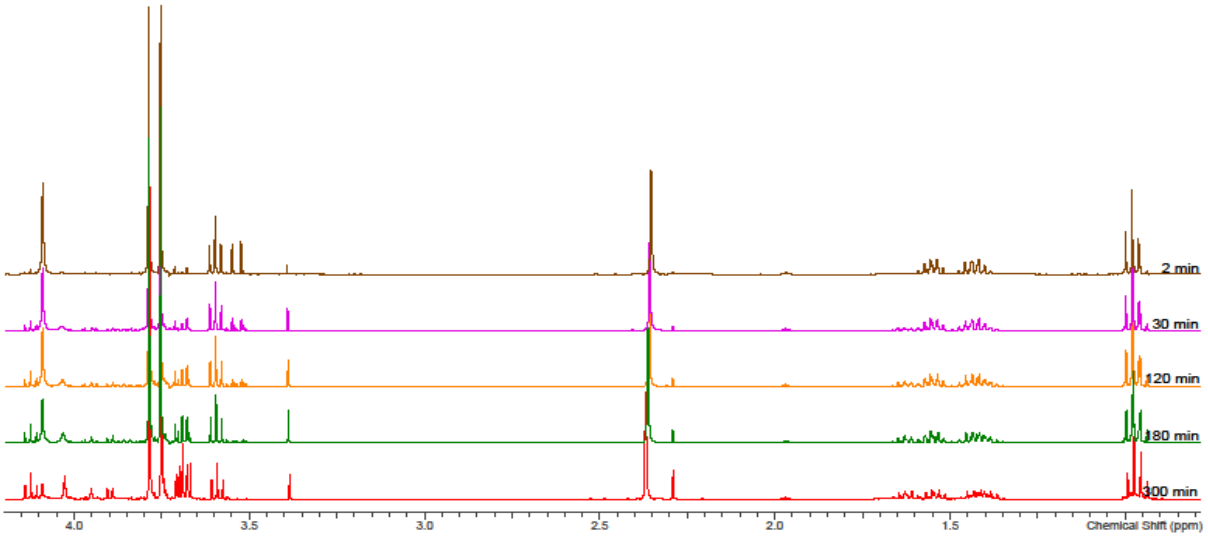
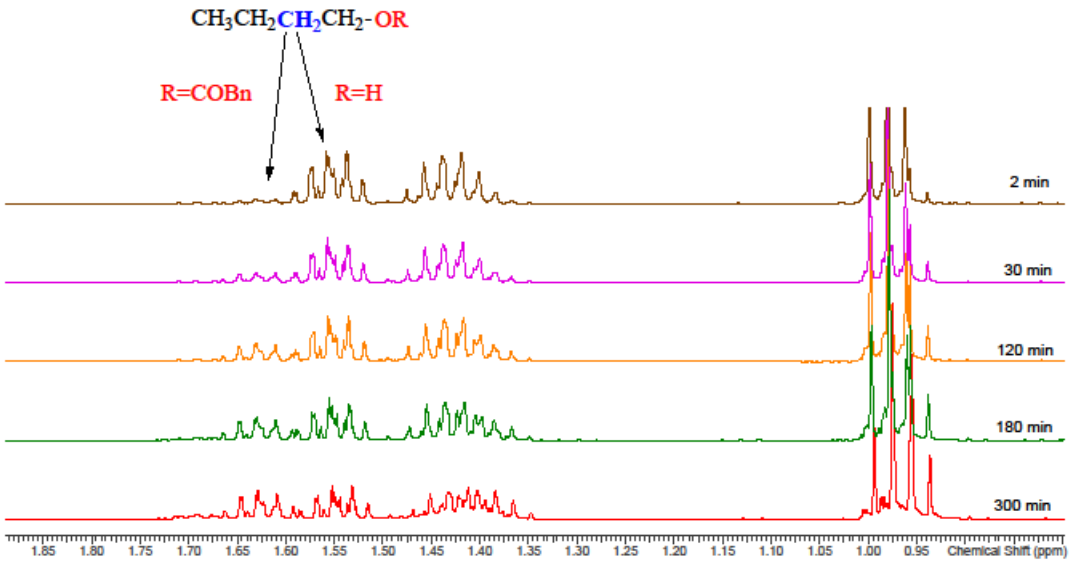


Figure S20: Zoomed-in spectrum region (1.80-0.85 ppm) showing the signals of $CH_3CH_2CH_2CH_2$- of BuOH and BuO_2CCH_2Ph.



The $P(OMe)_3$ (doublet at ~3.55 ppm) is very quickly sulfurized to $S=P(OMe)_3$ (doublet at 3.75 ppm). The n-BuOH is progressively esterified as shown by the decrease of -C<u>H₂</u>OH (t, 3.6 ppm) and increase of -C<u>H₂</u>OCO- (t, 4.1 ppm) signals.

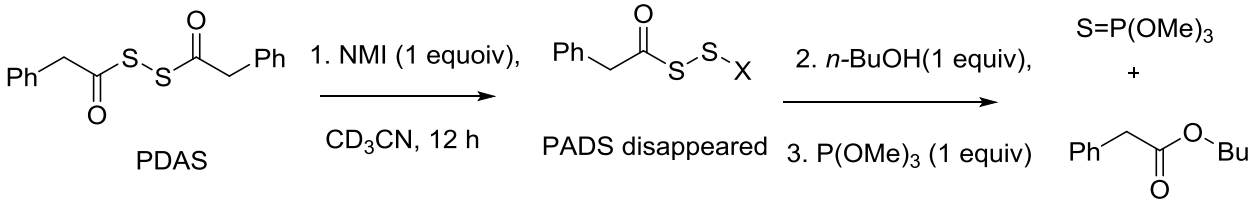
Table S1. Ratios of *n*-BuOH : $PhCH_2CO_2Bu$

Time	n-BuOH ***:*** $PhCH_2CO_2Bu$[a]
2 min	100 : 0
30 min	89 : 11
1 h	85 : 15
3 h	74 : 26
5 h	71 : 29

[a] Ratios were calculated by ^1H integration: δ 1.48-1.55 (m, 2H) for *n*-BuOH, δ 1.57-1.65 (m, 2H) for $PhCH_2CO_2Bu$.

Esterification reaction of n-BuOH in the reaction mixture of $P(OMe)_3$ and PADS aged in the presence of NMI (1.0 equiv)

Scheme S2: Conversion of n-BuOH into $PhCH_2CO_2Bu$ during the Sulfurization of $P(OMe)_3$ with PADS Aged in NMI



In this reaction, sulfurized of $P(OMe)_3$ (doublet at ~3.55 ppm) stalled quickly with about half of $P(OMe)_3$ was converted into $S{=}P(OMe)_3$ (doublet at 3.75 ppm). This result indicates the sulfurization reaction is fast and is consistent with literature data that PADS generates less than one equivalent of active sulfurization species. The n-BuOH is progressively esterified as shown by the decrease of $-CH_2OH$ (t, 3.6 ppm) and increase of $-CH_2OCO-$ (t, 4.1 ppm) signals.

Figure S21 and **Figure S22** show the full and partial spectra obtained at different times.

Figure S21: Partial ^{1}H NMR spectra (region 4.1-0.9 ppm) of the reaction mixture of n-BuOH (1.0 equiv), $P(OMe)_3$ (1.0 equiv), PADS (1.0 equiv) aged with NMI (1.0 equiv) at different reaction time points.

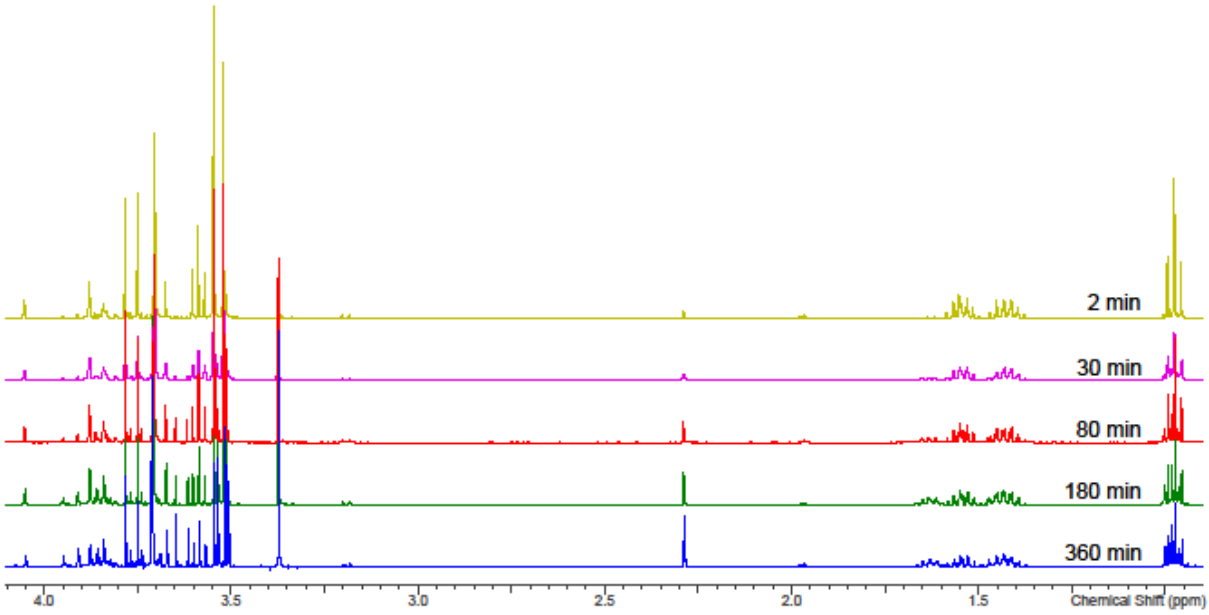


Figure S22: Zoomed-in spectrum region for the signals of $CH_3CH_2CH_2CH_2$- of n-BuOH and $PhCH_2CO_2Bu$.