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POSTSYNTHETIC CONJUGATION OF RNA TO CARBOXYLATE AND DICARBOXYLATE MOLECULES

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Carboxylates and dicarboxylates are important phosphate mimics. Herein, we present a simple synthetic route for the preparation of RNA carboxylate/dicarboxylate conjugates, starting from suitably protected NH₂- and COOH-containing molecules that are coupled to the RNA on the solid support. The key point in our method was the use of trimethylsilyl ethanol (TMSE-OH) protecting group, which is removed simultaneously with the silyl protecting group on the 2'-OH of the RNA ribose (e.g. t-Butyldimethylsilyl) during the final RNA cleavage/deprotection steps. The usefulness of this method was demonstrated by preparing different RNA-phosphate mimics oligos.

Keywords: Phosphate mimics; carboxylate; dicarboxylate; solid phase RNA synthesis; TMSE

INTRODUCTION

DNA and RNA molecules that contain phosphate moieties are susceptible to enzymatic degradation. Nonetheless, the negative charge of the phosphate group is often critical for many biological processes, such as kinase signaling, enzymatic activity, etc.[1] Replacement of the phosphate group with a different negatively charged group may preserve or even enhance, in some cases, the biological activity. This change could also be accompanied with improved metabolic stability.

For example, in many cancers, the Raf/MAPK signaling cascade is broken, leading to uncontrolled growth. The biological ‘On/Off’ switch is controlled by phosphorylation or dephosphorylation of the active species. Rossner et al. showed that mutation of the PAK and Src phosphorylation sites to aspartate group (dicarboxylate) inhibited Raf-1 signaling.[2] Another example is related to siRNA modification. It has been reported that modification at the 5’ terminus of the passenger strand prevents or decreases significantly siRNA activity.[3,4] A recent study supports these findings by analysis of the

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Ago2 crystal structure, which indicates that interaction between the 5’ phosphate negative charge and Mg$^{+2}$ positive charge is important in order to stabilize the passenger strand in the Ago2 complex.\cite{5} This analysis may explain why post modifications at the 5’ decrease dramatically the siRNA activity. However, phosphate mimics which present the negative charge at a desired position in RISC may facilitate a stable interaction with the magnesium ion leading to increased activity of the siRNA due to improved RISC loading. In addition, such a phosphate mimic is anticipated to improve RNA bio-stability. In order to improve the metabolic stability of nucleic acids and at the same time preserve their biological functions, different phosphate mimics groups were developed, such as amides,\cite{6} sulfonates,\cite{7,8} carboxylates,\cite{8,9} dicarboxylates,\cite{10} alkyl phosphates,\cite{11} etc. We chose to focus on the carboxylate/dicarboxylate groups as phosphate mimics for three main reasons. First, they provide a large synthetic variability in fine-tuning the distance of the carboxylate group from the terminus of the nucleic acid. Second, there are many commercially available amino acids having the different length of the chain and already containing carboxylic function(s). Third, many of the catalytic clefts contain positive ions and the carboxylate/dicarboxylate groups may interact strongly with those cations.\cite{12–14} We chose the RNA as a target for introducing a phosphate mimic at its 5’ end as such molecules may possess interesting properties and be potential therapeutics.

RESULTS AND DISCUSSION

Conjugation of RNA to Amino Acids and Molecules Containing Terminal NH$_2$ and COOH Groups

The chemistries of RNA and peptide synthesis are completely different. RNA is synthesized by an automated synthesizer under an inert atmosphere using sensitive phosphoramidite chemistry and protecting groups such as t-butyldimethylsilyleneether (TBDMS) and cyanoethyl. As opposed, peptides are synthesized by using building blocks and coupling reagents that are less sensitive to air. To obtain RNA/amino acids conjugates one can introduce an amino acid onto the phosphoramidite or to perform a post synthetic modification on the RNA oligomer. The phosphoramidite pathway requires the preparation of a new sensitive phosphoramidite and adjustment of the automated synthesis process. The postsynthetic methodology avoids complicated and expensive adjustments of a new process, but has two major limitations: first, RNA is highly sensitive to classical chemistry; it usually degrades quickly under acidic or basic conditions. Second, the protecting groups of the peptides are typically cleaved under conditions that are not compatible for RNA.
The high efficiency of the automated process resulted in increased activity at the past years, developing many DNA/RNA analogs with different functional groups. However, the simplicity of the postsynthetic route prompted us to look for a suitable protecting group (PG) for C-protected amino acids that would be compatible with RNA chemistry. For example, 4-\(N\)-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino/ benzyl (Dmab) is a PG that is readily removed under 2% hydrazine in DMF. In practice, even these low concentrations of hydrazine degrade RNA significantly after a short exposure to hydrazine solution. We have found that trimethylsilylethyl (TMSE) is most suitable as a carboxylic-acid PG for RNA post-synthetic conjugation of amino acids or molecules that contain NH2 and COOH functional groups (Scheme 1). During the final step of de-protection, RNA is treated with triethylamine-trihydrofluoride (Et\(_3\)N·3HF) in order to cleave the TBDMS group, and the TMSE PG is also efficiently removed under these conditions (Scheme 1). Thus, the deprotection step of the amino acid does not add an additional procedure to the RNA cleavage-deprotection protocol.

![Scheme 1](#)

**SCHEME 1** Conjugation of RNA to amino acids or amine and carboxyl terminated molecules protected with TMSE.
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SCHEME 2 Synthesis of TMSE-protected molecules suitable for postsynthetic conjugation to RNA.

Synthesis

Preparation of RNA with carboxylate/dicarboxylate phosphate mimic group was demonstrated by conjugation of L-Glutamic acid (Glu), β-alanine (β-ala), and 6-amino hexanoic acid (ε-Ahx). The TMSE-protected amino acids were synthesized starting from commercially available protected amino acids (Scheme 2). NH₂-Glu-L-(TMSE)TMSE [4] was prepared in three simple steps. Deprotection of O-benzyl (OBz) was done by standard hydrogenation with using H₂ and Pd/C catalyst leading to N-(t-butyloxycarbonyl)-L-glutamic-acid (BOC-Glu) [2] in quantitative yields. The carboxylic groups of compound [2] were protected with TMSE using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) leading to compound [3]. The last step was a non-aqueous selective cleavage of the BOC group using an HCl-dioxane solution yielding compound [4].[17] L-Glu with one TMSE group (α) was commercially available and used as is. ε-Ahx-TMSE [7] was prepared by the introduction of TMSE into benzylxycarbonyl-hexanoic acid (Z-ε-Ahx) [5] followed by deprotection of Z using Pd(OH)₂/C under acidic conditions. β-ala-TMSE [10] was synthesized by similar conditions using EDC for TMSE introduction and an HCl-Dioxane solution for selective BOC deprotection. Consequently, resin-bound RNA with TBDMS and cyanoethyl protecting groups was activated at the 5’ terminus using carbonyl di-imidazole (CDI) and then reacted with the TMSE protected molecules (Scheme 1). The RNA was cleaved from the resin using aqueous ammonia and methyl amine in ethanol and the silyl groups (TBDMS, TMSE) were
cleaved simultaneously by treating the RNA with a Et₃N·3HF solution for 3 h at 65 °C.

We have encountered some difficulties with the conjugation of L-Glu to RNA. Reaction of L-Glu with two TMSE PGs did not result in any significant conjugation, not even with excess reagents. In comparison, a control reaction with methyl amine under the same conditions gave a single product in the HPLC that was confirmed by MALDI-TOF MS. Consequently, we postulated that steric hindrance may prevent the conjugation so we tried to use a less bulky molecule; L-Glu with only one α-TMSE group. Reaction with L-Glu-α-TMSE gave the product at low yield encouraging us further to optimize the reaction conditions. Temperature adjustment to 65°C improved the yields significantly (ca. 7 fold) (figure 1). The strong influence of temperature supported our assumption that steric hindrance is a dominant factor. Consequently, we attempted to react NH₂-L-Glu(TMSE)-TMSE at higher temperatures, but no product was detected by HPLC.

**Analysis**

Purification of the products was carried out on a semi-preparative RP-HPLC column using 0.1M triethylammonium acetic acid (TEAA) buffer and
acetonitrile. The products were identified using MALDI-TOF MS analysis (Table 1, SI figures S1–S3). Counter ions for the carboxylate groups were often triethylammonium originating from the Et$_3$N·3HF solution and/or the buffer solution of the HPLC (TEAA). Replacement of the triethylammonium cation by sodium can be done by passing the aqueous solution of the product through an ion exchange column (Akta purifier, HiPREP 26/10) followed by a desalting procedure (Akta purifier, SOURCE 15Q) to remove excess sodium salt.

### CONCLUSIONS

In this report, we have presented a simple method for conjugation of carboxylate- or dicarboxylate-containing moieties to RNA on the solid support. The advantage of this method was the use of TMSE as a PG which can be removed following the standard RNA deprotection protocol without any additional step. Demonstration of the method was done by conjugation of three different molecules: L-glutamic acid, β-alanine, and 6-amino hexanoic acid. This approach can be easily extended to other amino acids or other amine- and carboxyl-containing molecules, providing chemical variability and simplicity.

### EXPERIMENTAL

#### Chemicals and Instrumentation

BOC-Glu-OBz (GL Biochem), Z-ε-AhX-OH (GL Biochem), BOC-β-ala-OH (GL Biochem), NH$_2$-Glu-TMSE (merck), Pd/C 10%w (sigma), Pd(OH)$_2$/C 20%w (sigma), HOBT (Hydroxybenzotriazole, Molekule), Dioxane-HCl 4 M (Sigma), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (EDC·HCl, GL Biochem), 1-Hydroxy-7-azabenzotriazole (HOAt, GL Biochem), Trimethylsilylethanol (TMSE-OH, Sigma), 1,1′-Carbonyldiimidazole (CDI, Sigma), Methylamine in Ethanol 33% (Sigma), Ammonia in water 33% (Sigma), Et$_3$N·3HF (Sigma), N,N-Diisopropylethylamine (DIPEA, Merck), Triethylammonium acetate (TEAA, Sigma), n-butanol (Sigma), RNAse free water (Biological industries or Sigma).
NMR spectra were recorded on a 300 MHz Bruker NMR using deuterated solvents as internal standards. MS measurements were measured on a ThermoQuest Finnigan LCQ-Duo ESI mass spectrometer. Mass analysis of RNAs was acquired on a MALDI-TOF MS (Voyager De Pro, Applied Biosystems, CA, USA).

**BOC-L-Glu [2]**

BOC-L-Glu-OBz [1] was dissolved in THF and the benzoyl group was removed by hydrogenation using 10% w/w Pd/C catalyst in the atmosphere of H2 at 30 Psi for 3 h. The solution was filtered and evaporated to dryness. Yield 98%. $^1$HNMR (300MHz, CDCl3) $\delta$ 1.43 (s, 9H), 2.14–2.28 (m, 2H), 2.43–2.54 (m, 2H), 4.30–4.39 (m, 1H). ESI-MS m/z 247.4 [M]+.

**BOC-L-Glu(TMSE)-TMSE [3]**

BOC-L-Glu [2] (210 mg, 0.90 mmol), EDC$^*$HCl (383 mg, 2.0 mmol), and HOAt (272 mg, 2.0 mmol) were added into a 20 mL glass vial. Dry DCM (10 mL), DIPEA (708 $\mu$L, 4.0 mmol), and TMSE-OH (420 $\mu$L, 2.9 mmol) were added and the solution was stirred for 48 h. The organic phase was washed with a saturated solution of sodium bicarbonate (2 $\times$ 20 mL) followed by brine (1 $\times$ 20 mL). The organic phase was concentrated in vacuum and left overnight in high vacuum in order to remove excess TMSE-OH. The crude material was relatively pure according to $^1$H NMR and therefore taken as is to the following reaction. Yield 95%. $^1$HNMR (300MHz, CDCl3) $\delta$ 0.01–0.02 (s+s, 18H), 0.93–1.01 (m, 4H), 1.43 (s, 9H), 2.32–2.51 (m, 4H), 4.13–4.21 (m, 4H), 4.55–4.58 (m, 1H). ESI-MS m/z 447.5 [M]+.

**NH\textsubscript{2}-Glu(TMSE)-TMSE [4]**

BOC-L-Glu(TMSE)-TMSE [3] (180 mg, 0.40 mmol) and 4 M HCl solution in dry dioxane (1 mL) were mixed for 1 hr. The dioxane solution was diluted with 50 mL DCM and then washed with saturated NaHCO$_3$ solution (2 $\times$ 50 mL) and brine (1 $\times$ 50 mL). The organic layer was concentrated in vacuum to afford the free amine product as a yellow oil. Yield 82%. $^1$HNMR (300MHz, CDCl3) $\delta$ 0.03–0.04 (s+s, 18H), 0.95–1.03 (m, 4H), 1.43 (s, 9H), 2.32–2.51 (m, 4H), 4.13–4.21 (m, 4H), 4.55–4.58 (m, 1H). ESI-MS m/z 348.5 [M+1].

**Z-\varepsilon-AhX-TMSE [6]**

Z-\varepsilon-AhX-OH [5] (2000 mg, 7.54 mmol), EDC$^*$HCl (1591 mg, 8.29 mmol), and HOAt (1127 mg, 8.29 mmol) were added into a 20 mL glass vial. Dry DCM (15 mL), DIPEA (2665 $\mu$L, 2.0 mmol), and TMSE-OH (1085 $\mu$L, 7.54 mmol) were added and the solution was mixed for 48 h.
The organic phase was concentrated in vacuum and 30 mL of diethyl ether was added. The organic phase was washed with water (2 × 30 mL), sodium bicarbonate (2 × 20 mL), and brine (1 × 20 mL) and concentrated under vacuum. Yield = 78%. 1HNMR (300MHz, DMSO-d6) δ 0.00 (s, 9H), 0.88–0.94 (m, 2H), 1.17–1.29 (m, 2H), 1.31–1.56 (m, 4H), 2.16–2.25 (t, 2H, J = 7.5Hz), 2.88–3.00 (q, 2H, J = 6.3Hz), 3.30 (s, 2H), 7.16–7.39 (m, 5H). ESI-MS m/z 351.8 [M]+.

**NH₃Cl-ε-AhX-TMSE [7]**

Z-ε-AhX-TMSE [6] (600 mg, 17.05 mmol) was dissolved in ethanol (5 mL) to which Pd(OH)₂/C (0.15 g, 20% w/v) and formic acid (0.5 mL) were added. The solution was heated to 60°C for 2 h until the UV spot of the starting material disappeared. The solution was filtered and concentrated under vacuum. The crude material was dissolved in a minimal amount of DCM and the ammonium salt product was precipitated and washed with hexane (2 × 20 mL) and dried under vacuum. Yield = 81%. 1HNMR (300MHz, DMSO-d6) δ 0.00 (s, 9H), 0.85–0.95 (m, 2H), 1.19–1.56 (m, 6H), 1.86 (s, 2H), 2.17–2.28 (t, 2H, J = 7.5Hz), 3.03–3.11 (t, 2H, J = 7.5Hz), 4.03–4.11 (m, 2H). ESI-MS m/z 232.6 [M+1].

**BOC-β-ala-TMSE [9]**

BOC-β-ala-OH [8] (1000 mg, 5.28 mmol), EDC⁺HCl (1116 mg, 5.81 mmol), and HOAt (791 mg, 5.81 mmol) were added to a 20 mL glass vial. Dry DCM (15 mL), DIPEA (2056 μL, 2.0 mmol), and TMSE-OH (759 μL, 5.28 mmol) were added and the solution was stirred for 48 h. The organic phase was washed with a saturated solution of sodium bicarbonate (2 × 20 mL) and brine (1 × 20 mL) and concentrated in vacuum. Yield 96%. 1HNMR (300 MHz, DMSO-d6) δ 0.01 (s, 9H), 0.90–0.95 (m, 2H), 1.35 (s, 9H), 2.34–2.39 (t, 2H, J = 7.5Hz), 3.09–3.16 (q, 2H, J = 7.0Hz), 4.05–4.10 (m, 2H), 6.80–6.83 (m, 1H). ESI-MS m/z 289.7 [M+1].

**NH₃Cl-ε -ala -TMSE ammonium salt [10]**

BOC-β-ala-TMSE [9] (400 mg, 1.38 mmol) was dissolved in a dry solution of Dioxane-HCl (1 mL, 4 M) and stirred for 0.5 h. Hexane (10 mL) was added in order to precipitate the product from the dioxane solution. The precipitate was washed with hexane (3X10 mL) and dried under vacuum. Yield 83%. 1HNMR (300MHz, DMSO-d6) δ 0.01 (s, 9H), 0.92–0.98 (m, 2H), 2.64–2.69 (t, 2H, J = 7.5Hz), 2.93–3.00 (m, 2H), 4.10–4.15 (m, 2H), 6.80–6.83 (m, 1H). ESI-MS m/z 190.5 [M+1].
RNA Synthesis

RNA sequence that includes 2′OMe modifications (underlined bases in sequence) and a phosphate group at the 3′ terminus (\text{UAGGAUACCACUUUGCACG-pi}) was synthesized on an AKTA Oligopilot 10 synthesizer (GE healthcare) on a 20 μmol scale using standard conditions. After completion, the resin was dried and used as is in the conjugation with the TMSE-protected molecules.

Coupling of NH\textsubscript{2}- and COOH- Containing Molecules to the RNA

30 mg of RNA-bound resin (∼1 μmol) were weighed into a 1 mL plastic reactor and 0.7 mL solution of CDI (90 μmol) in dry acetonitrile was added. The solution was placed in a shaker for 3 hours and the resin was washed thoroughly with ACN (3 × 0.8 mL) and DMF (3 × 0.8 mL). Solution of TMSE-protected compound (60 μmol) in DMF (0.7 mL) and DIPEA (230 μmol) were added. The reactor was heated to 65°C for 2 h and the resin was washed with DMF (4 × 0.8 mL) and DCM (0.8 mL). The pellet was placed in a to 2 mL Eppendorf and the cleavage was done as follows: a mixture of 33% methylamine in ethanol and 33% aqueous ammonia (1:1, v/v) was added and allowed to react for 2 h at 65°C. Resin was then washed with ethanol and combined washings were dried on a speed-vac under reduced pressure. The dry pellet was re-dissolved in minimal amount of DMSO (∼0.1 mL) and treated with a solution of Et\textsubscript{3}N·3HF (∼1 mL) for 3 h at 65°C. RNA conjugate was precipitated upon the addition of cooled n-butanol and further purification was done by HPLC.

HPLC conditions: 0–20 min gradient 0–20% ACN in Buffer (100 mM of TEAA, pH=7, in RNAse free water), 20–35 min gradient 20–60%, 35–40 min at a constant mixture 6:4 ACN: buffer. (Column: Phenomenex Clarity 3u oligo-RP, 50 × 4.60 mm, 3 micron).

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