Electronic Supplementary Information

Efficient Post-synthesis Incorporation and Conjugation of Reactive Ketones in RNA via 2′-Acylation

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Materials and instrumentation

Instrumentation

NMR Spectroscopy

All NMR spectra were recorded at the Stanford University Department of Chemistry NMR facility. Varian 300 MHz, 400 MHz and 500 MHz NMR instruments were used to record the 1H and 13C spectra. The spectra were analyzed using MNova software.

ESI-MS: All ESI-MS spectra were recorded at the Stanford University Mass Spectrometry Facility. The instrument used was a Waters 2795 HPLC system with dual wavelength UV detector, and ZQ single quadrupole MS with electrospray ionization source.

MALDI-TOF MS

All MALDI-TOF mass spectra were recorded at the Stanford University Mass Spectrometry facility, using a Bruker Daltonik Microflex MALDI-TOF spectrometer equipped with an N_2 laser. All spectra were recorded in linear negative mode and samples were plated on an MSP Anchorchip 96 target plate. 0.3 M trihydroxyacetophenone in EtOH (matrix) and 0.1 M aqueous ammonium citrate (co-matrix) were mixed in a 2:1 ratio by volume to be used as a matrix mix for MALDI. This mix was always freshly prepared before analysis. After RNA precipitation, the RNA pellet was redissolved in RNAse-free water to prepare a 10 μM sample solution. 1 μL of this solution was transferred to the target plate and dried under an Ar stream. 1 μL of the matrix mix was then added directly on top of the dried sample and completely dried under Ar stream. The spectral data were then recorded using Flex Control software (Bruker), and analyzed using MestReNova™. Previous experiments have documented the quantitative linearity of this method for calculating yields with RNA.[1]

Ketone reagents

Reagents for preparing double acylating agents were purchased from Sigma-Aldrich unlessspecified otherwise. 2-Oxo-2-phenylacetic acid, 5-formylfuran-2-carboxylic acid, 2-(4-formylphenyl)acetic acid, 2-(furan-2-yl)-2-oxoacetic acid, 3,3-dimethyl-2-oxobutanoic acid, 4-oxopentanoic acid, 4-oxocyclohexane-1-carboxylic acid, 3-oxocyclobutane-1-carboxylic acid, 1-methyl-3-oxocyclobutane-1-carboxylic acid, 6-oxospiro[3.3]heptane-2-carboxylic acid, 4-nminomorpholine, nicotinic hydrazide, ethylhydrazine hydrochloride, acetohydrazide, 3-hydrazinopyridine hydrochloride, O-[(3-yridyl)methyl]hydrpoxylamine, O-(carboxymethyl)hydroxylamine hemihydrochloride, O-ethylhydroxylamine hydrochloride, O-(*tert*-butyl)hydroxylamine hydrochloride, O-(4-nitrobenzoyl)hydroxylamine, 2-amino-5-methoxybenzoic acid were purchased from Ambeed, Inc.. 2-Hydrazinopyridine, Azido-PEG_2-CH2COOH were purchased from ChemScene. 2-(2-Chlorophenyl)-2-oxoacetic acid, 2-(1-methyl-1H-pyrrol-2-yl)-2-oxoacetic acid, 2-oxo-2-(2-(trifluoromethyl)phenyl)acetic acid, 2-hydrazinobenzoic acid hydrochloride, NPIA, P-anisidine, (1H-Imidazol-4-yl)methanamine hydrochloride, histamine,

(2-amino-5-methylphenyl)phosphonic acid were purchased from A2B Chem. 5,5,5-Trifluoro-4-oxopentanoic acid, 5-formylpicolinic acid, 6-formylpicolinic acid were purchased from Combi-Blocks. Biotin-ONH_2 was purchased from QUANTA BIODESIGN (Product No.: 11100). Biotin LC hydrazide (Catalog: BP-22113), BP Fluor 488 Hydroxylamine (Catalog: BP-25556) were purchased from BroadPharm. TAMRA DBCO was purchased from Click Chemistry Tools.

Biochemical reagents and buffers

MOPS buffer was purchased from Alfa Aesar. PBS buffer was purchased from Gibco. Sodium chloride solution was purchased from Promega. $MgCl_2$ solution was purchased from Quality Biological. NaOAc and glycogen were purchased from Thermo Scientific. Trihydroxyacetophenone was purchased from TCI. Ammonium citrate was purchased from Chem-Impex Int'l. Inc.

General RNA acylation procedure and MALDI-TOF analysis

RNA acylation reactions with conjugable acylation reagents

In a sterile 200 µL PCR tube, 3.3 µL of acylation 3.3X Buffer (333 mM MOPS pH 7.5, 333 mM NaCl, 20 mM $MgCl_2$ in water) was added to 4.7 µL of 21.3 µM RNA stock solution on ice. Fresh stocks of conjugable acylation reagents were prepared in DMSO; 2 µL of stock solution was added to the RNA reactions. The reactions were incubated for 2 hr at 0 °C and the RNA was subsequently purified by ethanol precipitation. The level of RNA modification was measured by MALDI-TOF M/S and analyzed using MestReNova™ software. Conversion yields were assessed by MALDI-TOF MS after ethanol precipitation; % conversion was calculated by measuring total peak intensities of all adducted RNAs and unreacted RNA, and dividing reacted RNA by the total. Median adduct number was calculated using intensity-weighted peaks. For the initial screening, we observed that four acylating compounds (**2**,**3**,**4**,**5**) showed side products with CDI in DMSO. For those four cases, we employed the same molar quantity of precursor reagent as for the other compounds, although concentrations of active species were likely lower.

Ethanol precipitation of RNA reactions

For an RNA reaction with total volume 10 µL, 90 µL RNA precipitation solution (0.33 M NaOAc (pH 5.2) in water containing 0.2 mg/mL glycogen) was added and mixed well. 300 µL of ice-cold absolute ethanol was then added, and the solution mixed by vortexing for at least 30 s. After storage at -80 °C for ca. 2 h, the mixture was centrifuged at 14.8k RPM for 60 min at 4 °C. The supernatant was discarded to obtain a pellet, which was washed with cold 70% ethanol. The obtained pellet was dried by a stream of air and subsequently either stored at -80 °C for future use, or immediately dissolved in water/PBS for direct use in further experiments.

General methods for conjugation of ketone-containing acylated RNA with nucleophiles

In a sterile 200 µL PCR tube, 3.3 µL of conjugation buffer (1x PBS pH 7.5) was mixed with 4.7 µL of 10.65 µM of stock solution containing acylated RNA with conjugable group. Fresh stocks of 100 mM nucleophile reagents were prepared in DMSO; 2 µL of stock solution was added to the RNA reactions. This reaction was incubated for 2 h at 37 °C (or 24 h at 23 °C in presence of 1 mM **19**) and the RNA was subsequently purified by ethanol precipitation. The level of RNA modification was measured by MALDI-TOF MS and analyzed using MestReNova™ software.

General de-acylation of ketone-containing acylated RNA with imidazole reagents

In a sterile 200 µL PCR tube, 3.3 µL of conjugation buffer (1x PBS pH 7.5) was mixed with 4.7 µL of 10.65 µM of stock solution containing acylated RNA with ketone. Fresh stocks of 250 mM imidazole reagents were prepared in DMSO; 2 µL of stock solution was added to the RNA reactions. This reaction was incubated for 24 h at 37 °C and the RNA was subsequently purified by ethanol precipitation. The relative quantities of unmodified and modified RNA were measured by MALDI-TOF MS and analyzed using MestReNova™ software.

Site-selective fluorescent labeling of RNA.[2]

In a sterile 200 µL PCR tube, 3.3 µL of acylation 3.3X Buffer (333 mM MOPS pH 7.5, 333 mM NaCl, 20 mM $MgCl_2$ in water) was mixed with 4.7 µL of 21.3 µM RNA and 23.43 µM DNA stock solution (see Table S1 for sequences). Fresh stocks of conjugable acylating reagents were prepared in DMSO; 2 µL of stock solution was added to the RNA reactions, vortexed well and incubated for 2 h at 0 °C. The RNA was subsequently isolated by ethanol precipitation, then the isolated acylated RNA was dissolved in PBS (1x, pH7.5) with final concentration 10.65 µM; 1 µL of 50 mM nucleophile stock solution and 10 mM **19** respectively were added to the RNA reactions and incubated for 24 h at 23 °C and the conjugated RNA was subsequently purified by ethanol precipitation. The level of RNA modification was measured by MALDI-TOF MS and analyzed using MestReNova™ software.

Testing DNA/RNA nondestructive enrichment via acylation and conjugation

Selective conjugation of RNA/DNA mixture with aminooxy biotin via conjugable acylation.

In a sterile 200 µL PCR tube, 3.3 µL of acylation 3.3X Buffer (333 mM MOPS pH 7.5, 333 mM NaCl, 20 mM $MgCl_2$ in water) was added to 2.35 µL of 42.6 µM ssRNA stock solution and 2.35 µL of 42.6 µM single-stranded DNA 2 (see Table 1) on ice. Fresh stocks of conjugable acylation reagents were prepared in DMSO; 2 µL of reagent **14** stock solution was added to the RNA reactions. The reactions were incubated for 30 minutes at 0 °C and the RNA/DNA mix was subsequently isolated by ethanol precipitation. Dissolving RNA/DNA precipitate with water, in a sterile 200 µL PCR tube, 3.3 µL of conjugation buffer (1x PBS pH 7.5) was mixed with 4.7 µL of

21.3 μM of stock solution including acylated RNA with conjugable group. Fresh stocks of 50 mM aminooxy biotin reagent and 10 mM reagent **19** were prepared in DMSO; 2 μL of stock solutions were added to the RNA/DNA reactions. This mixture was incubated for 24 h at 23 °C and the RNA/DNA was again isolated by ethanol precipitation. The levels of RNA/DNA modification were measured by MALDI-TOF M/S and analyzed using MestReNova™ software. Conversion yields were assessed by MALDI-TOF MS after ethanol precipitation; % conversion was calculated by measuring total peak intensities of all adducted RNAs/DNA and unreacted RNA/DNA, and dividing reacted RNA/DNA by the total.

Enrichment of DNA via streptavidin-mediated pulldown of biotinylated RNA.

100 μL of Dynabeads MyOne streptavidin C1 beads were washed with 1 mL of Binding Buffer (100 mM Tris, pH 7.0, 1 M NaCl, 10 mM EDTA, 0.2% v/v Tween-20) for pre-equilibration. The supernatant was aspirated, and 50 μL of Binding Buffer was added to resuspend the beads. 10 μL of 20 μM biotinylated RNA/DNA mixture from the previous step was transferred to the beads. 2 μL of RiboLock RNase inhibitor was added to the biotinylated RNA solution. The resulting suspension was incubated for 2 hours at 20 ˚C on a sample revolver (20 rpm). After incubation, the supernatant containing enriched DNA was carefully aspirated. The drained beads were then washed with Washing Buffer (100 mM Tris, pH 7.0, 4 M NaCl, 10 mM EDTA, 0.2% v/v Tween-20) three times (the first wash also contained small amounts of off-target DNA). The supernatant was aspirated carefully.

Elution of unmodified RNA. To elute RNA, the drained beads after washing were resuspended with 26.4 μL of 1xPBS buffer, 37.6 μL water, 16 μL of 250 mM Histamine in DMSO, and 2 μL of RiboLock. The bead suspension was incubated at 37˚C for 12 hours, then transferred to a magnetic rack. The supernatant was purified with an Amicon filter (cut-off MW: 3,000 Da) following the manufacturer's instructions.

Orthogonal (Click) fluorescent labeling of RNA by TAMRA.

In a sterile 200 μL PCR tube, 35 μL of conjugation buffer (1x PBS pH 7.5) was mixed with 5 μL of 40 μM acylated RNA. Fresh stock of TAMRA DBCO reagent was prepared in DMSO; 10 μL of 2.5 mM stock solution was added to the RNA reactions, vortexed well and incubated for 3 h at 37 ˚C. The RNA was subsequently purified by ethanol precipitation, then re-dissolved in water for analysis. The level of RNA modification was measured by MALDI-TOF MS and analyzed using MestReNova™ software.

Orthogonal (Oxime ligation) fluorescent labeling of RNA by BP Fluor 488.

In a sterile 200 μL PCR tube, 3 μL of conjugation buffer (1x PBS pH 7.5) was mixed with 5 μL of 20 μM acylated RNA. Fresh stock of aminooxy-BP Fluor 488 reagent was prepared in DMSO; 1 μL of 50 mM stock solution and 1 μL of 10 mM reagent **19** were added to the RNA reactions, vortexed well and incubated for 24 h at 23 ˚C. The RNA was subsequently purified by ethanol

precipitation, then conjugated RNA with TAMRA was dissolved in water. The level of RNA modification was measured by MALDI-TOF MS and analyzed using MestReNova™ software.

Electrophoresis of labeled RNAs via denaturing PAGE gel.

1.5 pmole of labeled RNA was mixed in 1x gel loading buffer containing 4M urea, Bromophenol Blue and Orange G dye. The mixture was heated at 95˚C for 3 min and loaded to 18% denaturing PAGE gel. The labeled RNAs were separated in the gel with running buffer composed of 1x TBE, pH 8.3 (25 mA, ~1.5 h). The RNAs were visualized by fluorescence imaging under Alexa 488 and Alexa 546 channel via imager iBright FL1500.

Table S1. Oligonucleotides used in this work

Name	Sequence (left to right: 5′ to 3′)
RNA oligo	
ss RNA	ACAAUUAUCCUAUGAGCGGU
BL RNA	AGACAGCCUUUUUGGCGUCU
HP RNA	UGGCGGCCGACUACGCCA
ss RNA 5'phos	*ACAAUUAUCCUAUGAGCGGU
DNA oligo	
ss DNA	ACAATTATCCTATGAGCGGT
ss DNA 2	GCACCTTCCAAAGTGCCT
ss DNA 3'5'phos	*ACAATTATCCTATGAGCGGT*
ss DNA3'5'AAA	AAAACCGCTCATAGGATAATTGTAAA
ss DNA3'6'AAA-deAU	AAAACCGCTCACGGATAATTGTAAA

* RNA protected by phosphate at 3’ or 5’-hydroxyl group

Supplementary Figures

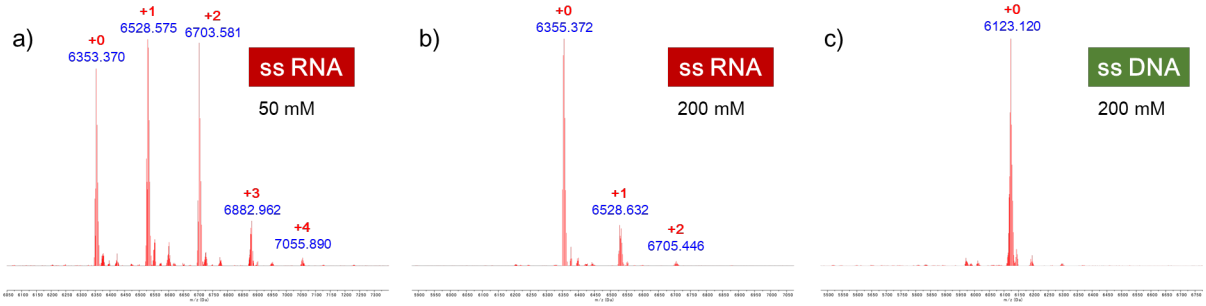
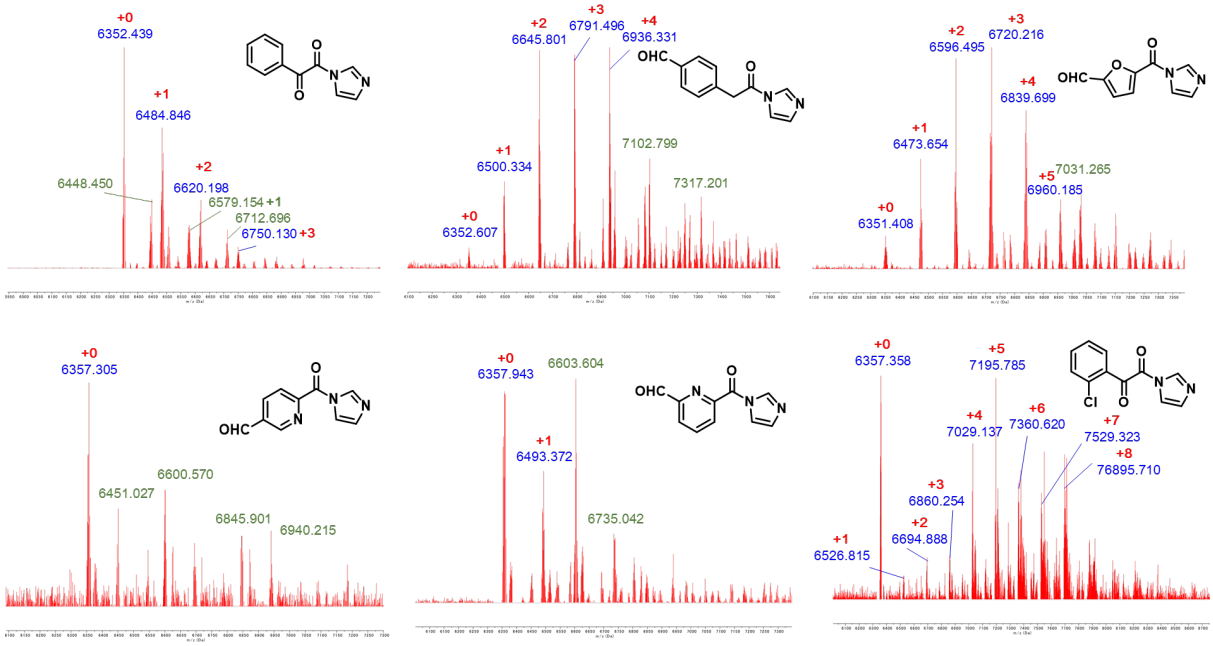


Figure S1. Testing RNA and DNA reactivity of commercial ketone-containing reagent NPIA for comparison with new reagents in this study. Moderate reaction was seen at 50 mM and very low reaction at 200 mM; see Fig. S2 for comparison with new reagents. MALDI-TOF MS data for RNA and DNA reactions for reagents shown; the RNA and DNA are single-stranded 20mers with the same sequence (Table S1). Conditions: 10 μM RNA (DNA), 50 mM for (a) 100 mM for (b, c) reagent, MOPS buffer (100 mM) pH 7.5, 37 °C, 1 h. Red numerals indicate number of adducts per RNA strand.



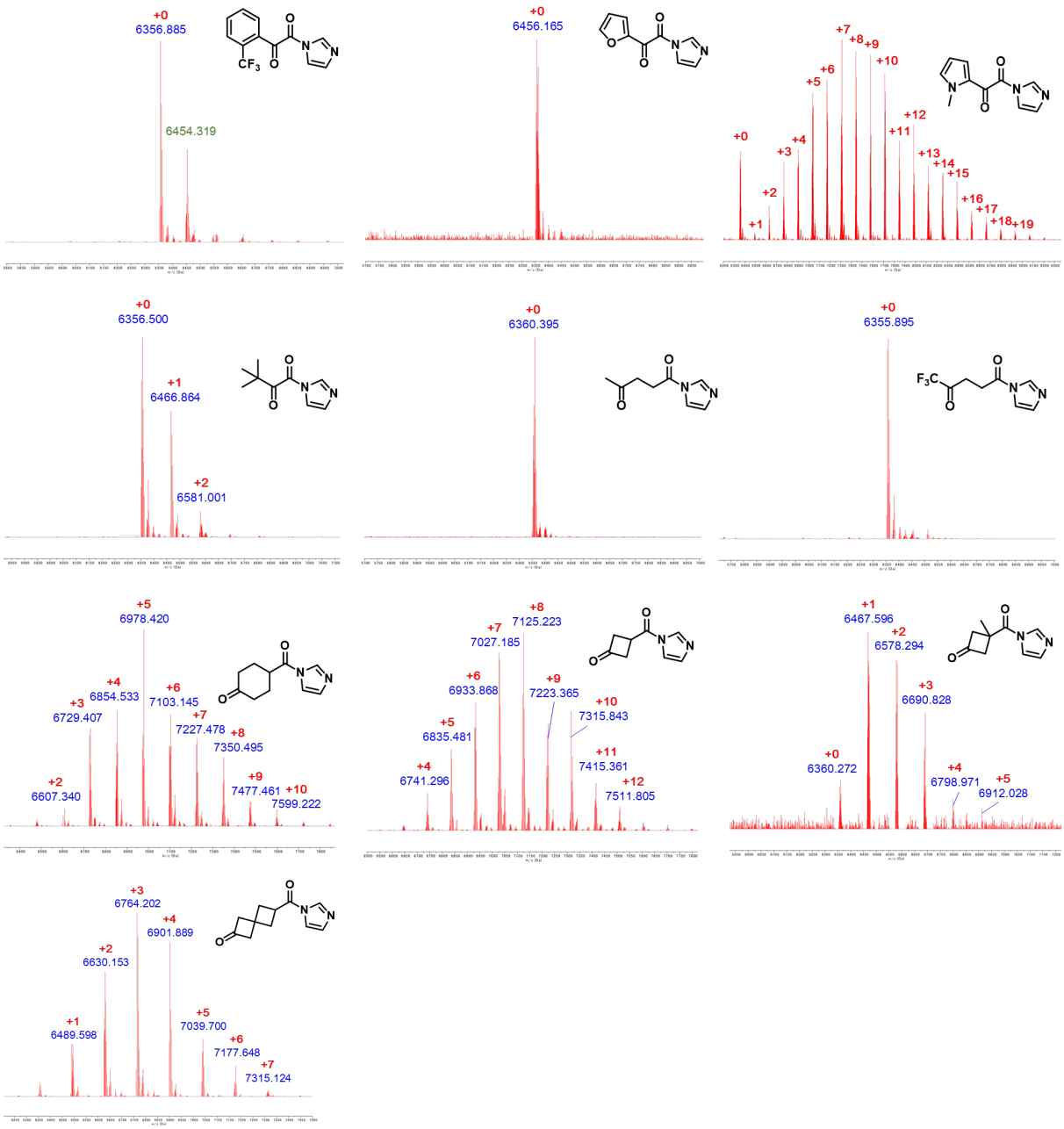


Figure S2. Representative MALDI-TOF mass spectrometry adduct data for reaction of 20nt single-stranded RNA with conjugable ketone/aldehyde acylimidazole reagents **1**-**16**. Conditions: 10 μM RNA, 2 h, 200 mM reagent, MOPS buffer (100 mM) pH 7.5, 0 °C. Red numerals indicate number of adducts per RNA strand, gray peaks show sodium or potassium associated with RNA, and green labels indicate side products such as an imidazolecarbonyl group (+95 molecular weight).

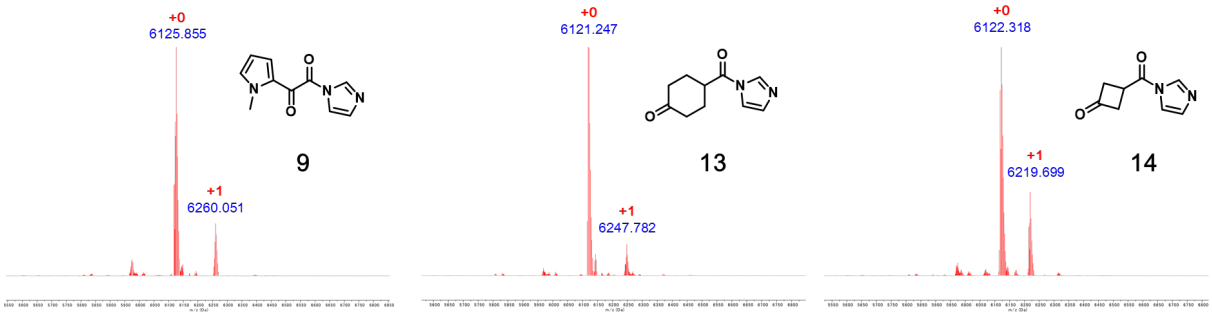


Figure S3. Testing DNA reactivity of conjugable acylating reagents **9**, **13**-**16** for ssDNA, showing little reaction due to lack of 2'-OH groups. The DNA is a single-stranded 20mer of the same sequence as the RNA in Fig. S2. The data confirm primary reaction at 2′-OH groups of RNA and not exocyclic amine groups that are present both on DNA and RNA. Conditions: 10 μM RNA, 200 mM reagent, MOPS buffer (100 mM pH, 7.5), 0 °C, 2 h. Red numerals indicate number of adducts per DNA strand.

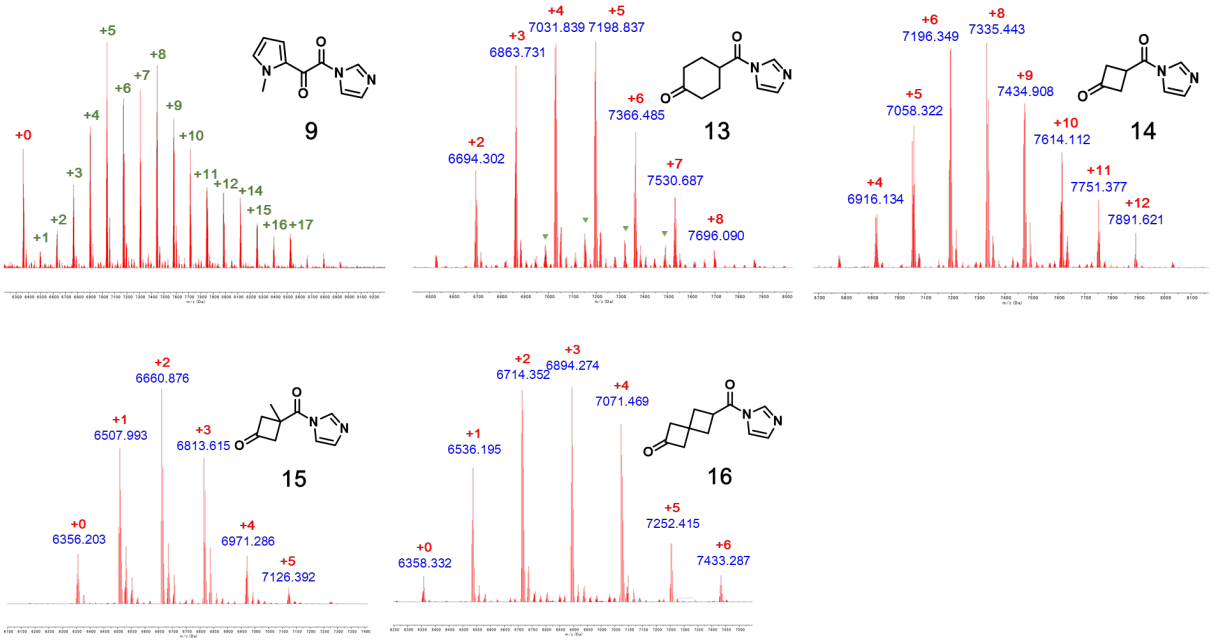


Figure S4. Representative MALDI-TOF mass spectrometry oxime conjugation data for reaction of 20nt single-stranded ketone-conjugated RNA with ethoxyamine. The RNA was acylated with

reagent **9, 14-16** (structures shown on the spectra) as described above, and then precipitated (2-step protocol) prior to re-dissolving for conjugation reactions. Conjugation conditions: 5 μM RNA, 100 mM ethoxyamine, PBS buffer (1x, pH 7.5), 2 h, 37 °C. Note that conjugated RNA with **9** showed only acyl adduct peaks with no oxime adduct. Compound **13** shows major oxime formation with minor peaks for less-conjugated RNA. RNAs with **14-16** show essentially complete oxime formation. Red numerals indicate number of adducts per RNA strand, Green numerals and ▼ show unreacted ketone adduct of RNA or the number of adducts.

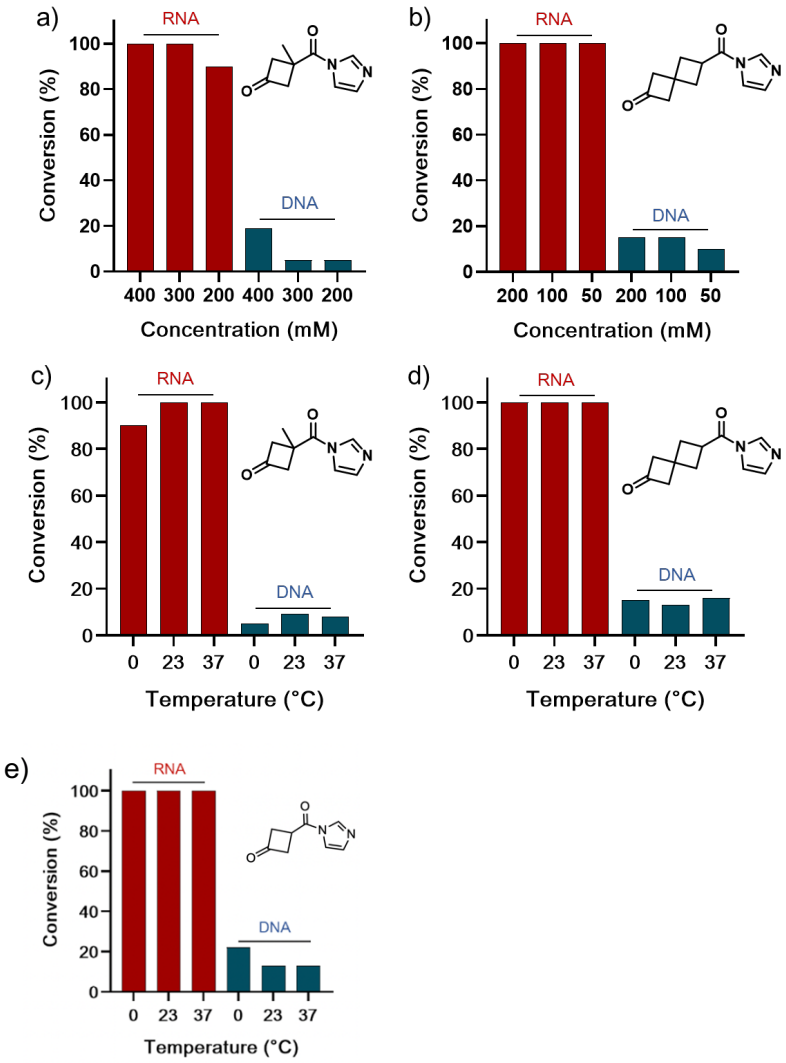


Figure S5. Optimizing reaction conditions with RNA acylating reagents **15** and **16**. (a, b) Concentration, (c, d, e) temperature effects on RNA acylation (red bars). Also shown (blue bars) is the reactivity with ssDNA. General conditions: RNA 10 μM, acylimidazole 200 mM, MOPS buffer pH 7.5, 0°C, 2 h; concentrations as shown. Note that higher concentrations were required to achieve full conversion for the more hindered compound in (a).

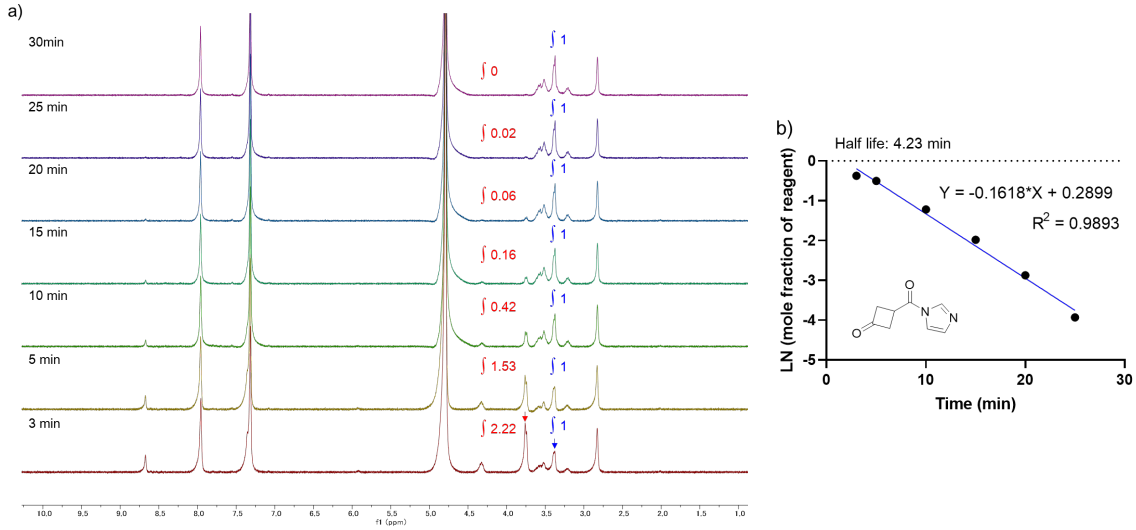


Figure S6. Time course for hydrolysis of conjugable acylating reagent **14** as measured by NMR at 23°C in deuterated aqueous buffer (pD=7.5). Measured half-life is 4.2 min.

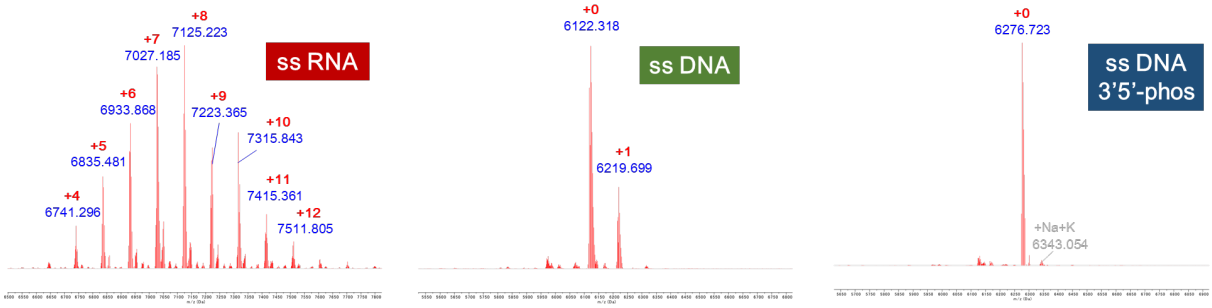


Figure S7. Comparison of acylation reactivity of ssRNA (left) and ssDNA (center and right) with cyclobutanone acylating reagent **14**. The tests show that **14** is OH group selective, and does not react with exocyclic groups on bases. The small amount of reactivity for DNA (center) is absent when the 5′ and 3′ OH groups on the ends are blocked (right). Conditions: RNA (DNA) 10 μM, acylimidazole 200 mM, MOPS buffer pH 7.5, 0°C, 2 h.

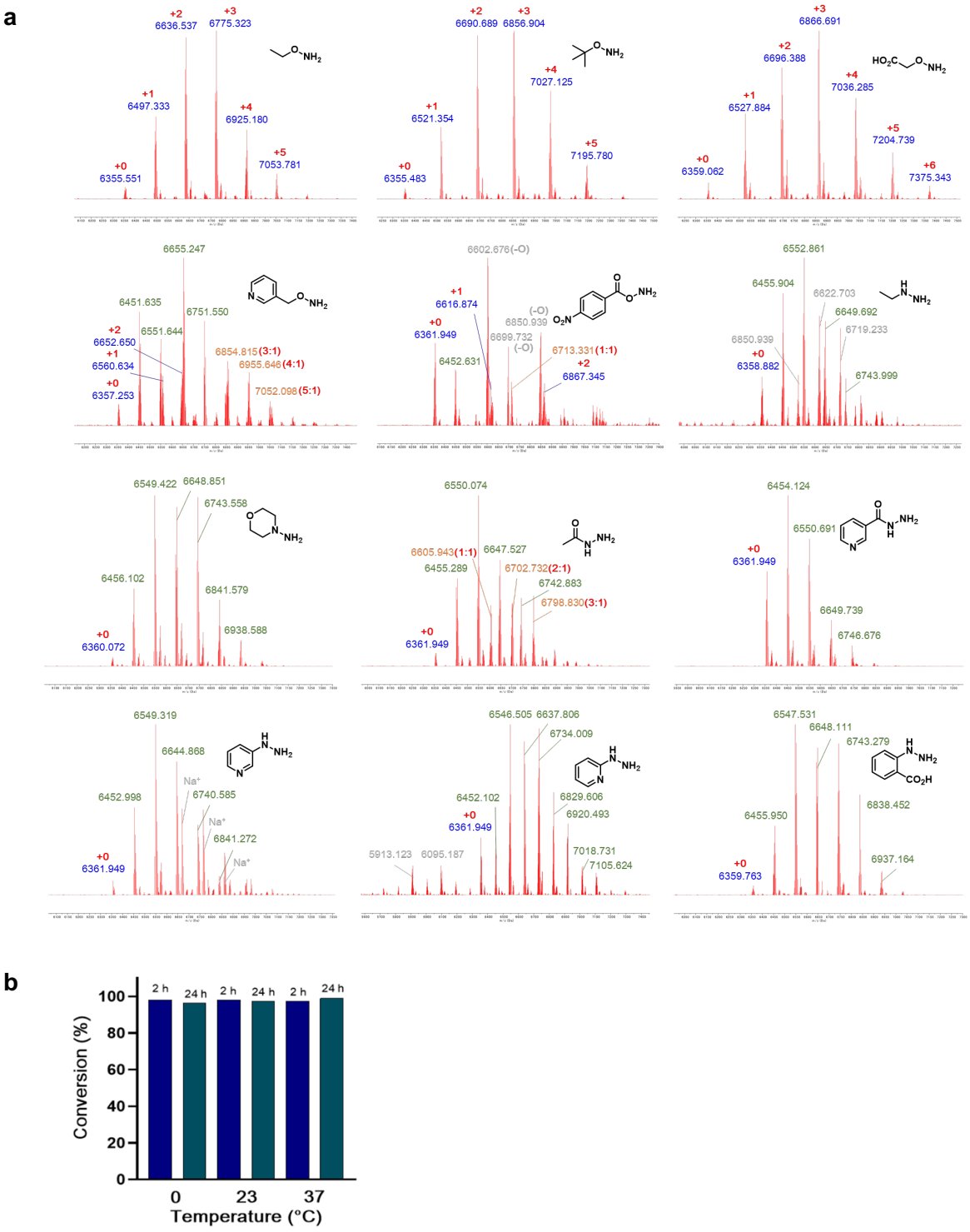


Figure S8. (a) Representative MALDI-TOF mass spectrometry conjugation data for reaction of 20nt single-stranded conjugable acylated RNA with aminooxy and hydrazine nucleophile reagents. The RNA was pre-acylated with reagent **14** as described above, and then precipitated prior to re-dissolving for conjugation reactions. Conjugation conditions: 5 μM RNA, 100 mM nucleophile reagent, PBS buffer (1x, pH 7.5), 2 h, 37 °C. Red numerals indicate number of adducts per RNA strand, gray show unknown adduct of RNA. Green numerals show unreacted adduct of **14** on RNA. The combination of

unreacted ketone and target conjugate are shown as orange numerals, with adduct ratio as unreacted: reacted (X:Y) respectively. (b) Effect of temperature on oxime conjugation with ethoxyamine, reacting with RNA modified with ketone **14** at 2 or 24 h. Conversions (%) are shown.

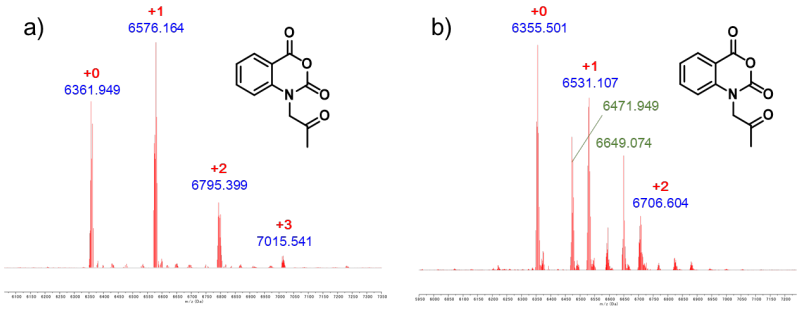


Figure S9. Oxime conjugation properties of RNA modified with commercial reagent NPIA. (a) Ethoxyamine adducts with NPIA. Conditions: 10 μM RNA, 100mM ethoxyamine, PBS buffer (1x, pH 7.5), 37 °C, 2 h. Red numerals indicate number of adducts per RNA strand. (b) Stability in aqueous buffer after 11 days, showing significant degradation.

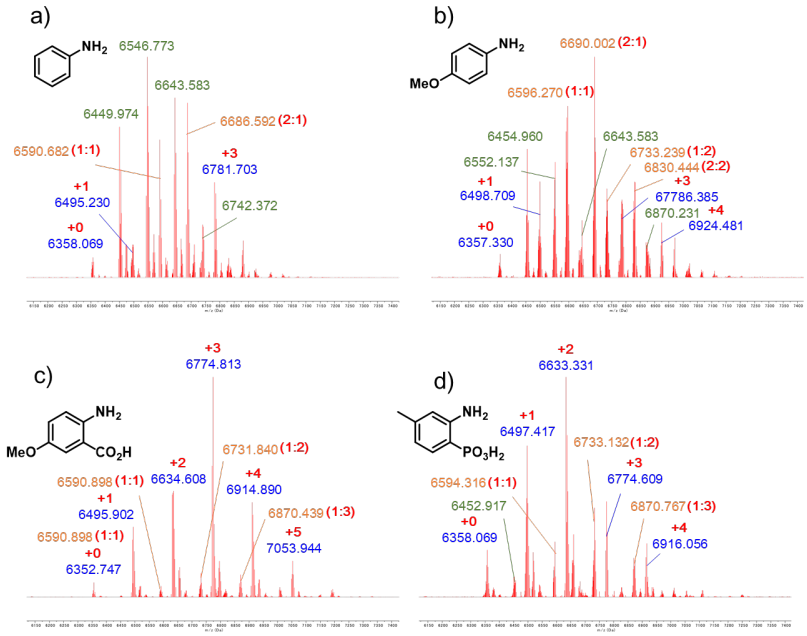


Figure S10. Catalyst-promoted oxime conjugation of RNA adducted with **14**. Representative MALDI-TOF mass spectrometry adduct data for conjugation of 20nt single-stranded RNA fragment with ethoxyamine in presence of four different catalysts. Results show that catalyst **19** (4-methoxy-2-carboxy-aniline, see c)) gives the cleanest products and high conjugation yields. Conditions: 5μM RNA, 500 μM ethoxyamine, 1 mM catalyst, PBS buffer (1x, pH 7.5), 23 °C, 24 h. Red numerals indicate number of adducts per RNA strand. Green numerals show unreacted conjugable acylated adduct of RNA. The combination of target adduct and imidazole carbamate is shown as orange numerals, with adduct ratio as unreacted: reacted (X:Y) respectively.

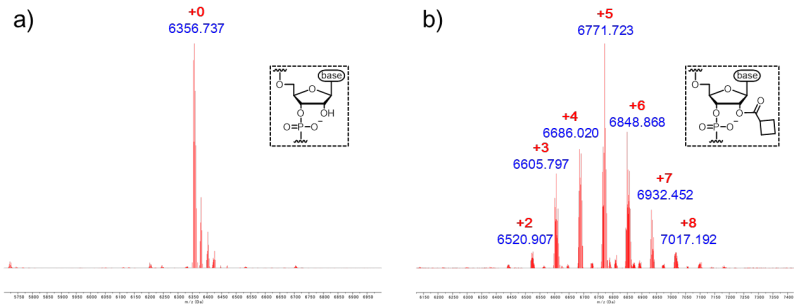
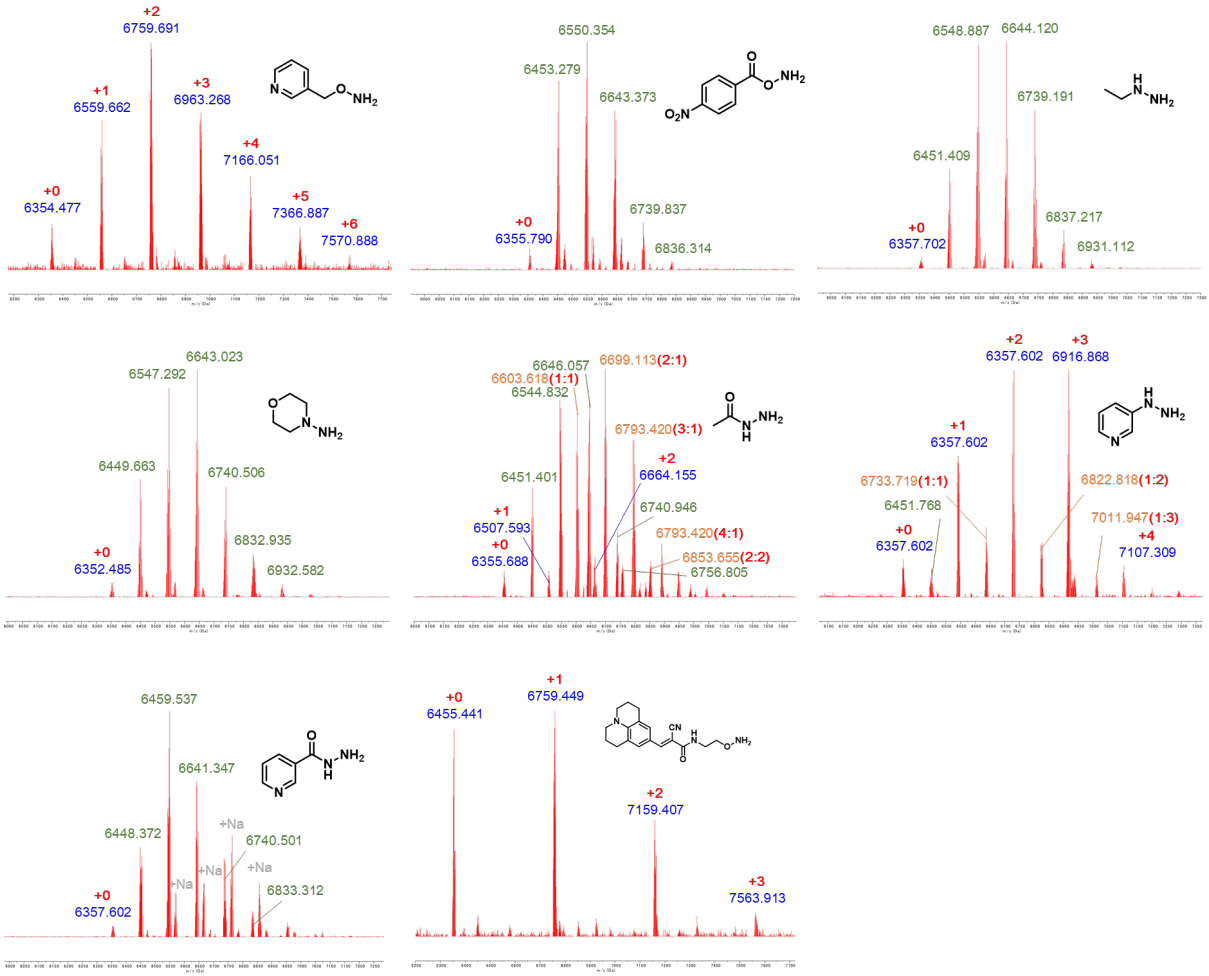


Figure S11. Confirmation of that ethoxyamine does not react with (a) ssRNA or (b) RNA acylated by reagent **21** which lacks a ketone group. Conditions for a: 10 μM RNA, 5 mM ethoxyamine, 1 mM catalyst **3**, PBS buffer (1x, pH 7.5), 23 °C, 24 h. Conditions for b: 5 μM RNA with cyclobutane, 5 mM ethoxyamine, 1 mM **19**, PBS buffer (1x, pH 7.5), 23 °C, 24 h.



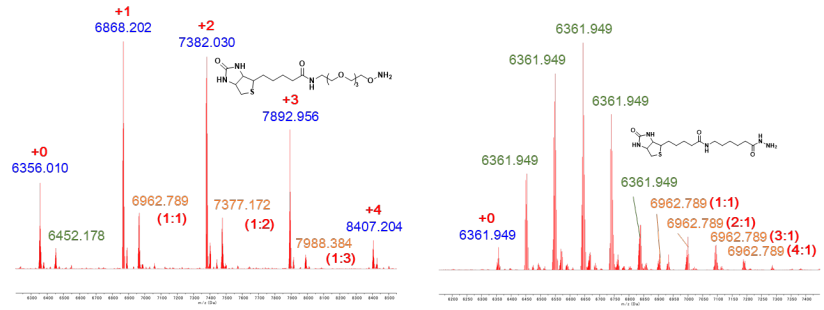


Figure S12. Representative MALDI-TOF mass spectrometry conjugation data for reaction of 20nt single-stranded RNA acylated with reagent **14** with low concentration of nucleophile reagents in presence of catalyst **19**. Conditions: 5 μM RNA, 5 mM reagent, PBS buffer (1x, pH 7.5), 24 h, 23 °C. Red numerals indicate number of adducts per RNA strand, gray show sodium adduct of RNA. Green numerals show unreacted conjugable acylated adduct of RNA. The combination of unreacted ketone and target conjugate are shown as orange numerals, with adduct ratio as unreacted: reacted (X:Y) respectively.

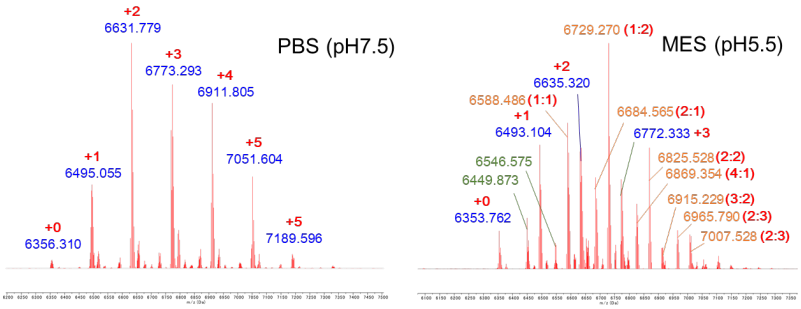


Figure S13. Effect of pH on oxime conjugations, using PBS (pH 7.5) and MES (pH5.5) buffers, containing the same concentration of sodium chloride, indicating that pH 7.5 is suitable for conjugation with ethoxyamine. Conditions: 5 μM RNA, 5 mM reagent, PBS buffer (1x, pH 7.5, 137mM NaCl) or MES buffer (50mM, pH5.5, 137mM NaCl), 24 h, 23 °C. Red numerals indicate number of adducts per RNA strand, gray show sodium adduct of RNA. Green numerals show unreacted conjugable acylated adduct of RNA. The combination of unreacted ketone and target conjugate are shown as orange numerals, with adduct ratio as unreacted: reacted (X:Y) respectively.

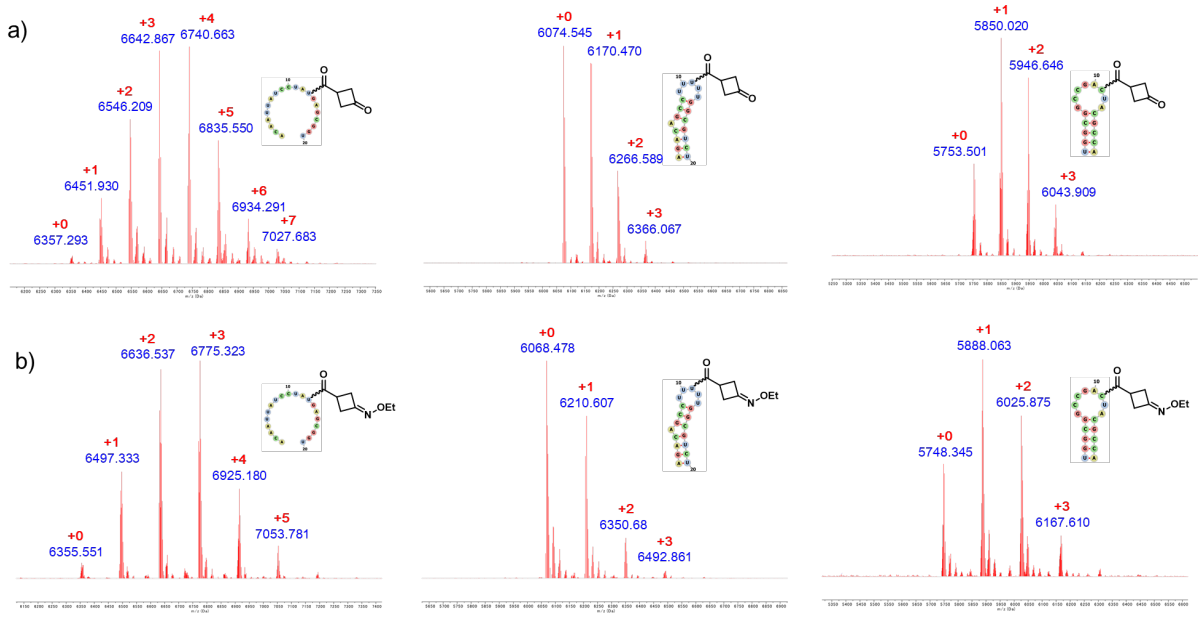


Figure S14. Survey of activation and conjugation with RNAs having varied folding: ssRNA, hairpin RNA and loop RNA (see Table S1). (a) MALDI-TOF mass spectra of RNA acylation reactions with reagent **14**, shown as ssRNA (left), loop RNA (middle) and hairpin RNA (right). The results show that the level of acylation varies with the number of unpaired nucleotides in the RNA. Conditions: 10 μM RNA, 50 mM reagent, MOPS buffer (100 mM) pH 7.5, 0 °C, 2 h (b) MALDI-TOF MS of RNA conjugation with ethoxyamine. Conditions: 10 μM RNA, 500 μM reagent, PBS buffer (1x, pH 7.5), 23 °C, 24 h. Red numerals indicate number of adducts per RNA strand, gray show sodium adduct of RNA.

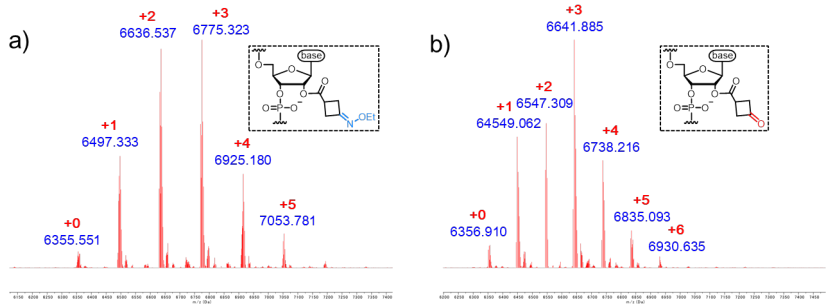


Figure S15. Catalyzed release of ethoxyamine from oxime adducts in the presence of catalyst **19**. (a) Starting oxime conjugates; (b) restored ketone product. Conditions: 5 μM RNA, 50 mM catalyst **19**, PBS buffer (1x, pH 7.5), 24 h, 37 °C. Red numerals indicate number of adducts per RNA strand.

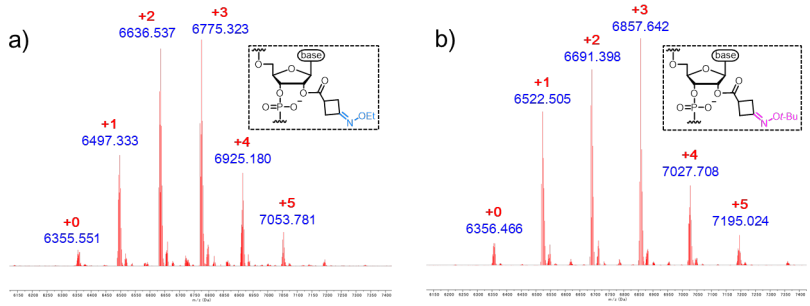


Figure S16. Catalyzed oxime exchange from ethoxyamine conjugate (a) to *t*-butoxyamine conjugate (b). Conditions: 5 μM ethoxyamine-conjugated RNA, 50 mM *t*-butoxyamine, 1 mM catalyst **19**, PBS buffer (1x, pH 7.5), 24 h, 37 °C. Red numerals indicate number of adducts per RNA strand.

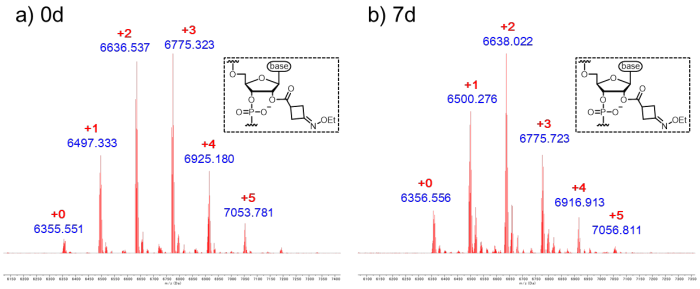


Figure S17. Stability of RNA conjugated with ethoxyamine for 7 days in aqueous solution. (a) Initial RNA adduct (b) confirmation of stability in PBS buffer for 7 days at 23 °C, showing conjugated RNA adducts are relatively stable. Conditions: 5 μM RNA, PBS buffer (1x, pH 7.5), 7 d, 23 °C. Red numerals indicate number of adducts per RNA strand.

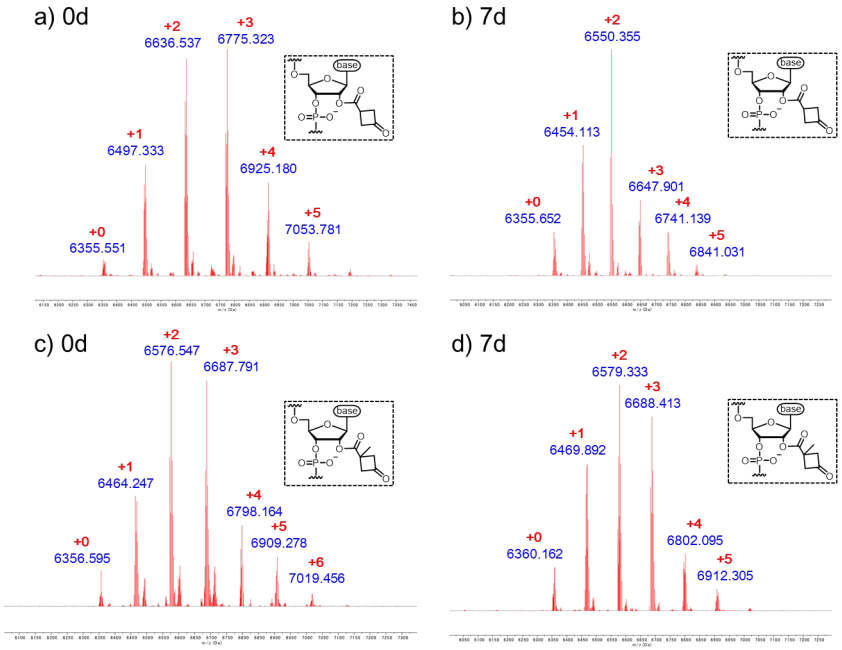


Figure S18. Stability of acyl ester adducts of **14** and **15** on RNA in PBS buffer for 7 days at 37 °C, showing that these RNA adducts remain intact. Conditions: 5 μM RNA, PBS buffer (1x, pH 7.5), 7 d, 37 °C. Red numerals indicate number of adducts per RNA strand.

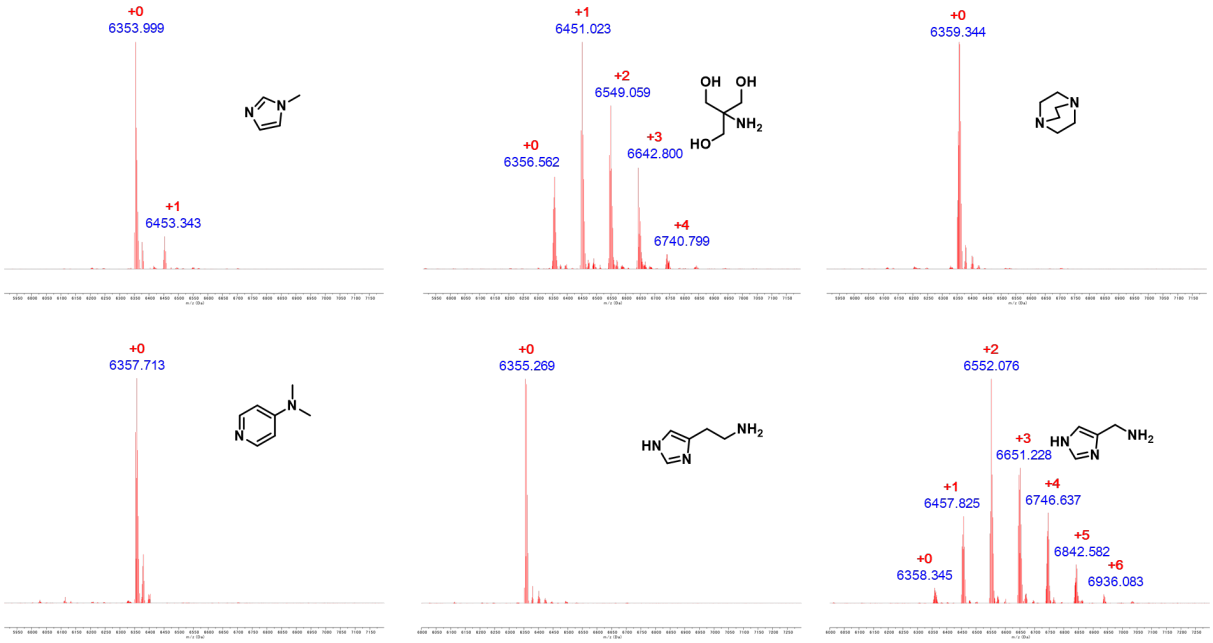


Figure S19. Testing catalyzed hydrolysis of esters on polyacylated RNA (acylated with **14**) using varied nucleophilic bases. Polyacylated RNA was treated with 200 mM of methylimidazole, Tris, DABCO, DMAP, histamine and 4-(2-aminoethyl)imidazole respectively in PBS buffer. Conditions:

5 μM RNA, 200 mM base, PBS buffer (1x, pH 7.5), 24 h, 37 °C. Red numerals indicate number of adducts per RNA strand.

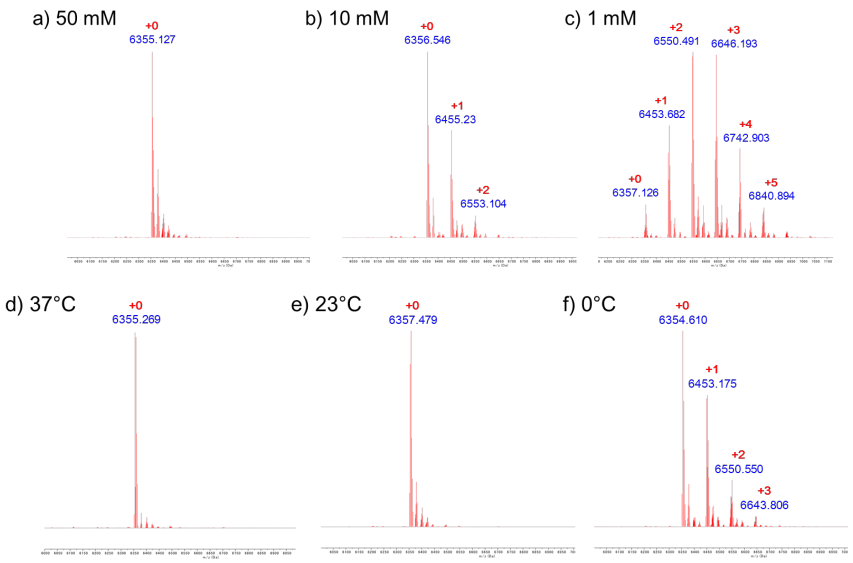


Figure S20. Optimization of conditions for de-acylation of RNA with histamine. (a-c) Concentration and (d-f) temperature effects. General conditions: 5 μM RNA acylated with **14**, 50 mM base, PBS buffer (1x, pH 7.5), 24 h, 37 °C. Red numerals indicate number of adducts per RNA strand.

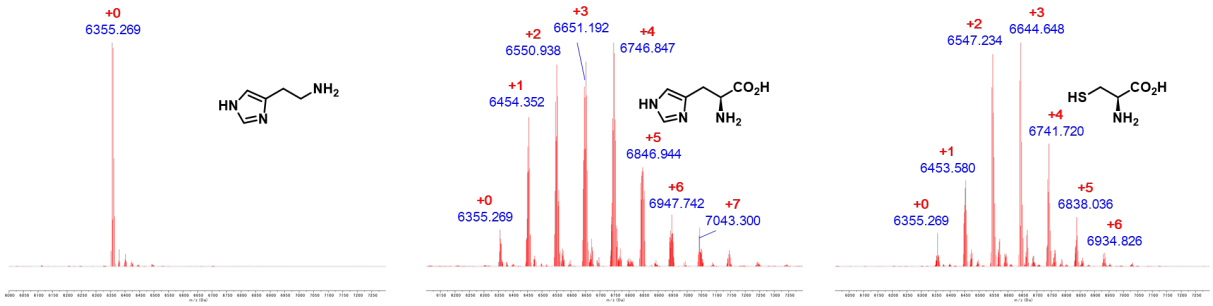


Figure S21. Scope of de-acylating RNA promoted by histamine and amino acids. RNA acylated with **14** was treated with 50mM of histamine, histidine and cysteine respectively in PBS buffer. Conditions: 5 μM RNA, 50 mM base, PBS buffer (1x, pH 7.5), 24 h, 23 °C. Red numerals indicate number of adducts per RNA strand.

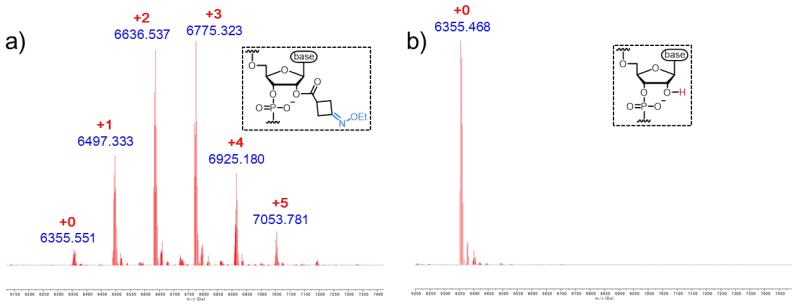


Figure S22. Test of de-acylating RNA with ethoxyamine oxime adduct of **14** using histamine. Conditions: 5 μM RNA, 50 mM histamine, PBS buffer (1x, pH 7.5), 24 h, 37 °C. Red numerals indicate number of adducts per RNA strand. (a) Starting conjugated RNA; (b) product RNA showing complete loss of adducts.

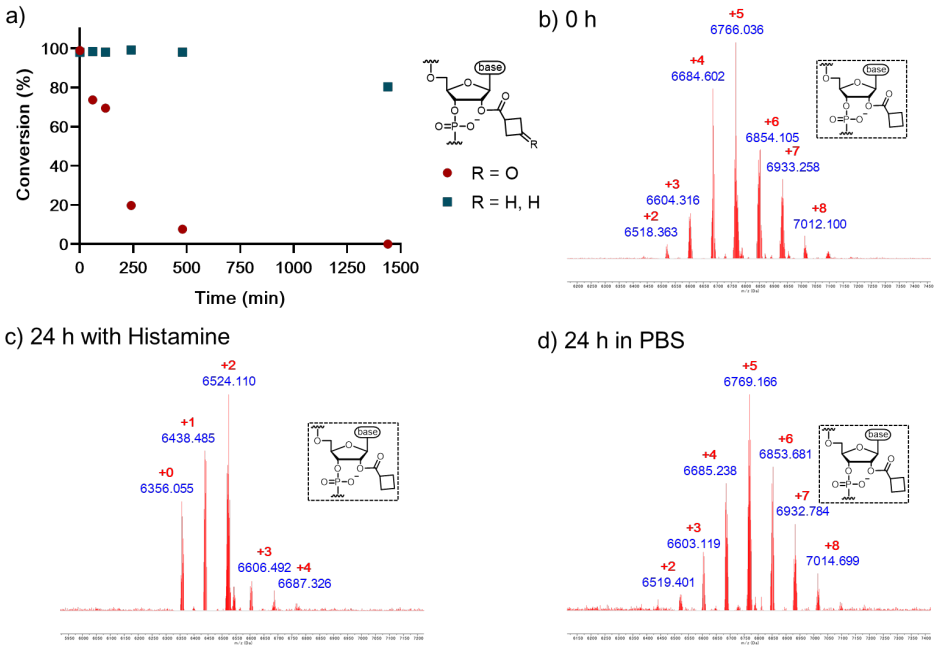


Figure S23. Investigation of mechanism of de-acylation of RNA with histamine, using a cyclobutane adduct (reagent **21**) as control. (a) Time course of de-acylating RNA for cyclobutanone (reagent **14**) and cyclobutane acyl adducts, showing that reaction depends strongly on the presence of the ketone. Conditions: 5 μM RNA, 50 mM histamine, PBS buffer (1x, pH 7.5), 24 h, 23 °C. (b-d) Representative MALDI-TOF mass spectrometry conjugation data at three time points of the curve for RNA adducted with 21. Conditions: for b, Conditions: 10 μM RNA, 200 mM reagent, MOPS buffer (100 mM pH, 7.5), 2 h, 0 °C; for c, Conditions: 5 μM RNA, 50 mM histamine, PBS buffer (1x, pH 7.5), 24 h, 23 °C; for d, Conditions: 5 μM RNA, PBS buffer (1x, pH 7.5), 24 h, 23 °C.

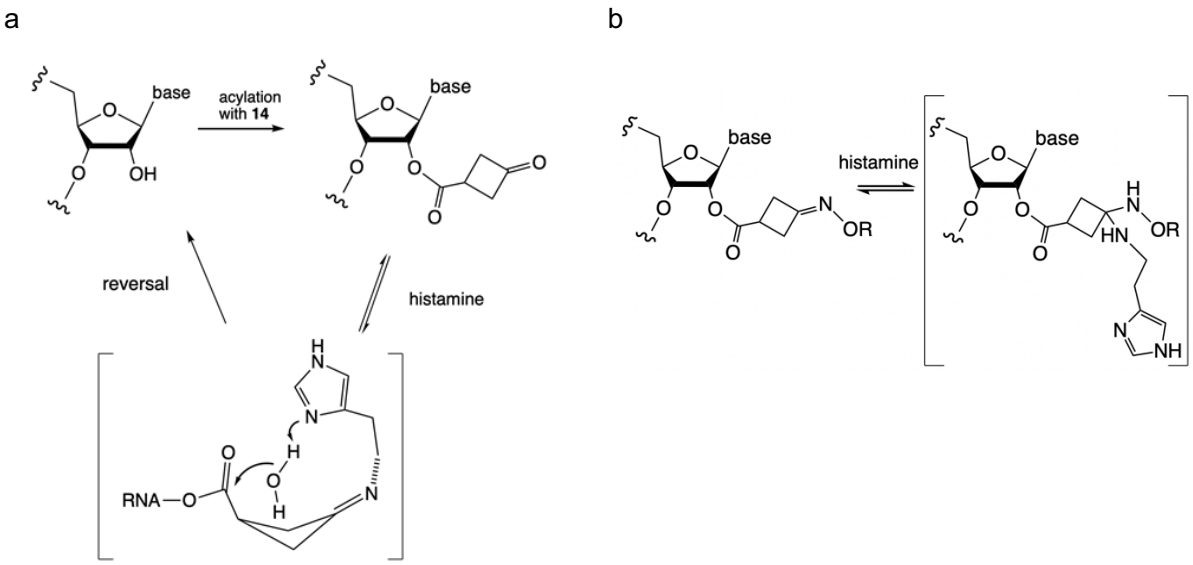
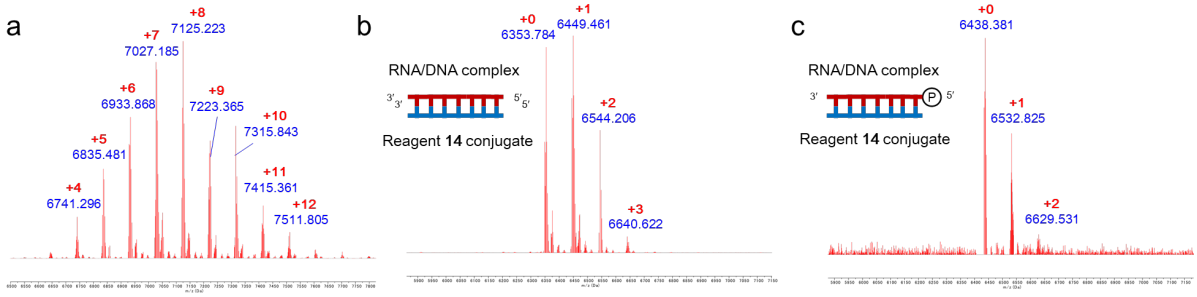


Figure S24. (a) Proposed mechanism for histamine-induced de-acylation of cyclobutanone ester adducts of **14** on RNA. Histamine rapidly forms an imine intermediate with the ketone, bringing imidazole into proximity with the ester carbonyl. General base catalysis delivers a nucleophilic water molecule, promoting hydrolysis. The proposed mechanism is supported by the fact that histamine is much less effective in the absence of the ketone, and by the observation that the absence of the terminal amine or a shorter chain leads to loss of activity. (b) Proposed structure of analogous intermediate with oxime-conjugated ester adducts, which are also reversed in ht presence of histamine (see data in Fig. S22, S27).



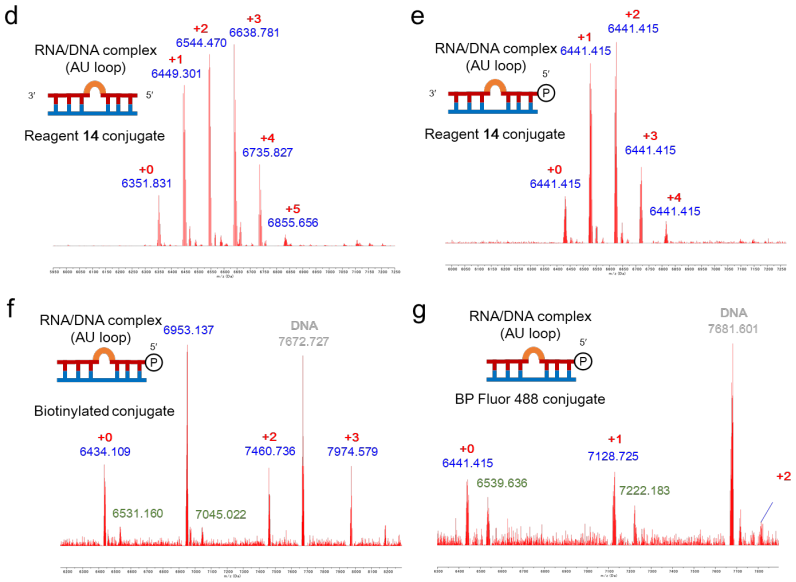
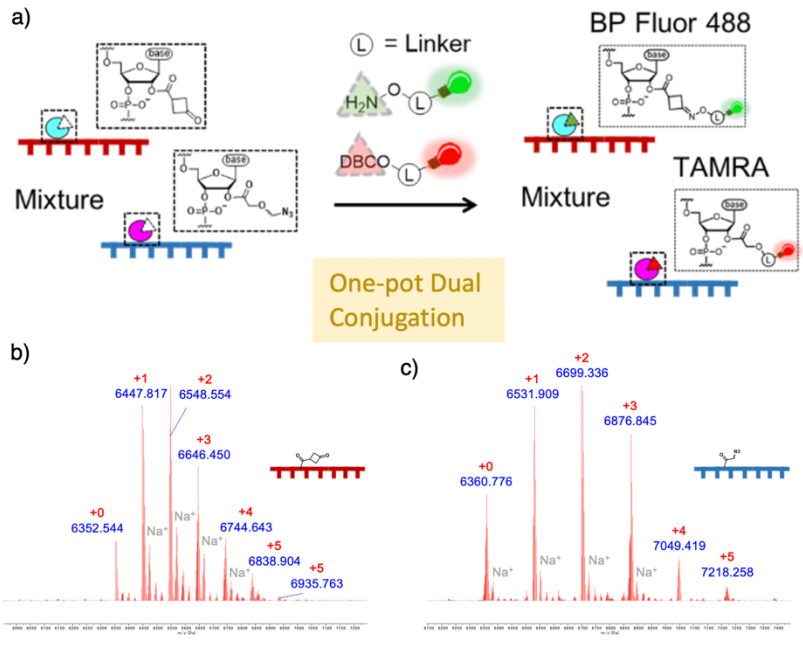


Figure S25. Representative conjugation data for site-localized reaction with an RNA induced loop (RAIL). Site-selective fluorescent labeling of ssRNA with DNA-induced (RAIL) reaction of reagent **14** and oxime conjugation with BP Fluor 488. MALDI-TOF mass spectra of (a) unmodified RNA; (b) RNA hybridized to fully complementary DNA; (c) RNA with phosphate at the end blocking 5′-OH, in the presence of fully complementary DNA; (d) DNA-induced 2 nt bulge RNA; (e) DNA-induced 2 nt bulge RNA protected by 5'-phosphate after treatment with 200 mM reagent **14** in MOPS buffer (100 mM, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$) for 2 h at 0 °C. (f, g) MALDI-TOF mass spectra after reacting with different nucleophilic reagents (aminooxy-BP Fluor 488, aminooxy biotin) after treatment with 5 mM nucleophilic reagents in PBS buffer (1x, pH 7.5) for 24 h at 24 °C in presence of 1 mM **19**. Red and blue numerals indicate number of adducts per RNA strand and the exact mass, gray show helper DNA to induce loop, and green is for unreacted ketone.



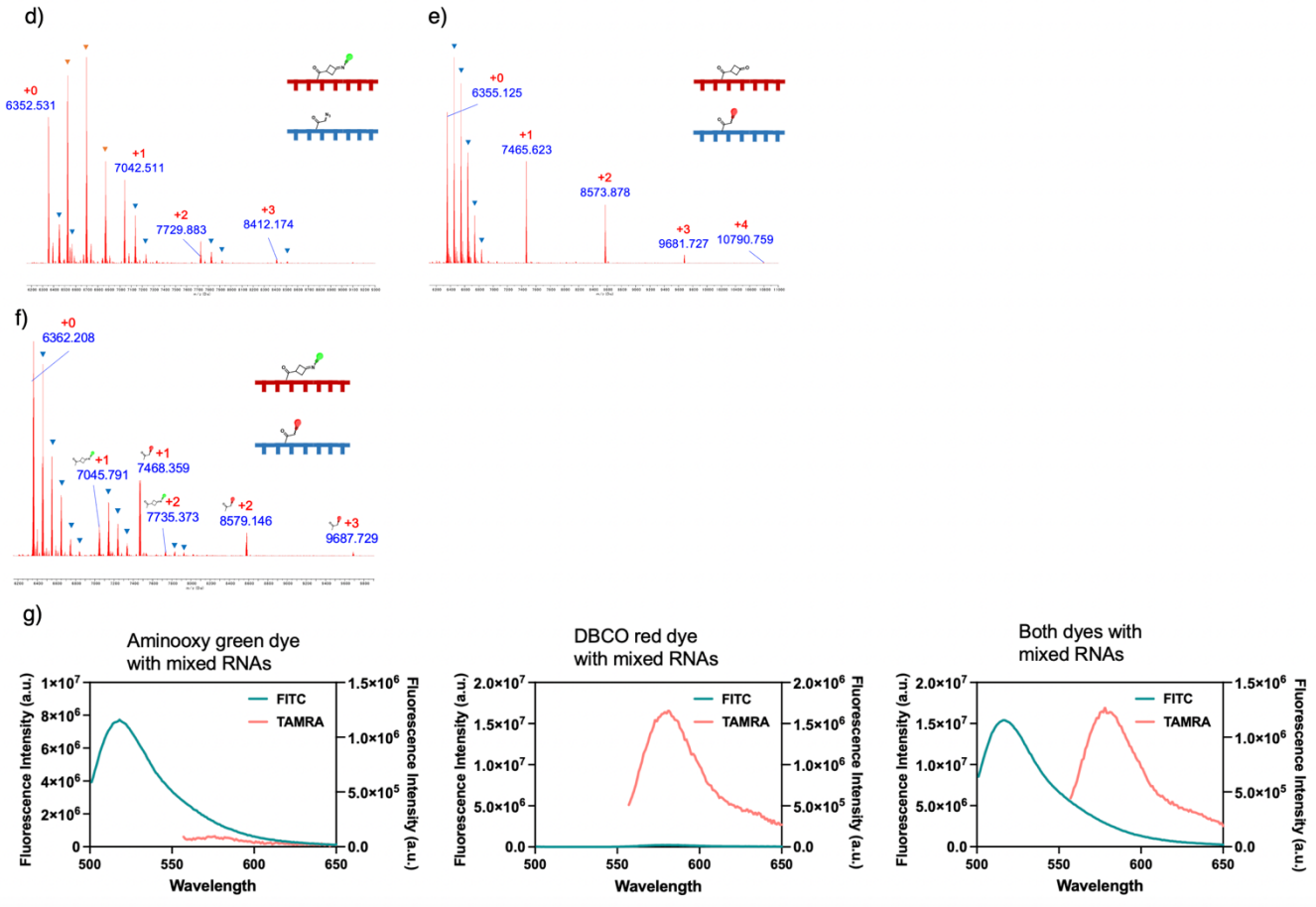


Figure S26. Conjugation data for orthogonal dual fluorescence labeling of RNAs conjugated with azide (reagent **22**) and ketone **14**. (a) Scheme for orthogonal labeling of RNA with two acylating reagents **14**, **22**, and subsequent conjugation with BP Fluor 488 (for ketone) and/or TAMRA DBCO (for azide) via acylated RNA. (b) MALDI-TOF mass spectrum of introduction of ketones on the RNA via acylation with **14** and (c) introduction of azides on the RNA via acylation with **22**; (d) reaction with BP Fluor 488 with the mixture of the two acylated RNAs, showing masses of adducts only on ketones; (e) orthogonal reaction with TAMRA DBCO for the mixture of two acylated RNAs, showing reaction only with azides; (f) simultaneous reaction of both dyes with the mixed RNAs; (g) normalized fluorescence spectra of RNAs from experiments in (d),(e),(f) respectively, confirming orthogonality. Acylation condition: 50 mM reagent **14** or **22** in MOPS buffer (100 mM, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$) for 30 min at 0 °C. Conjugation condition A: 5 mM aminooxy BP Fluor 488 and 1 mM **19** in PBS buffer (1x, pH 7.5) for 24 h at 23 °C. Conjugation condition B: 500 µM TAMRA DBCO in PBS buffer (1x, pH 7.5) for 24 h at 23 °C. Conjugation condition C: 5 mM aminooxy BP Fluor 488, 1 mM **19** and 500 µM TAMRA DBCO in PBS buffer (1x, pH 7.5) for 24 h at 23 °C. Red and blue numerals indicate number of adducts per RNA strand and the mass, gray numbers show Na or K salts, and ▼ marks unreacted ketone or azide adducts.

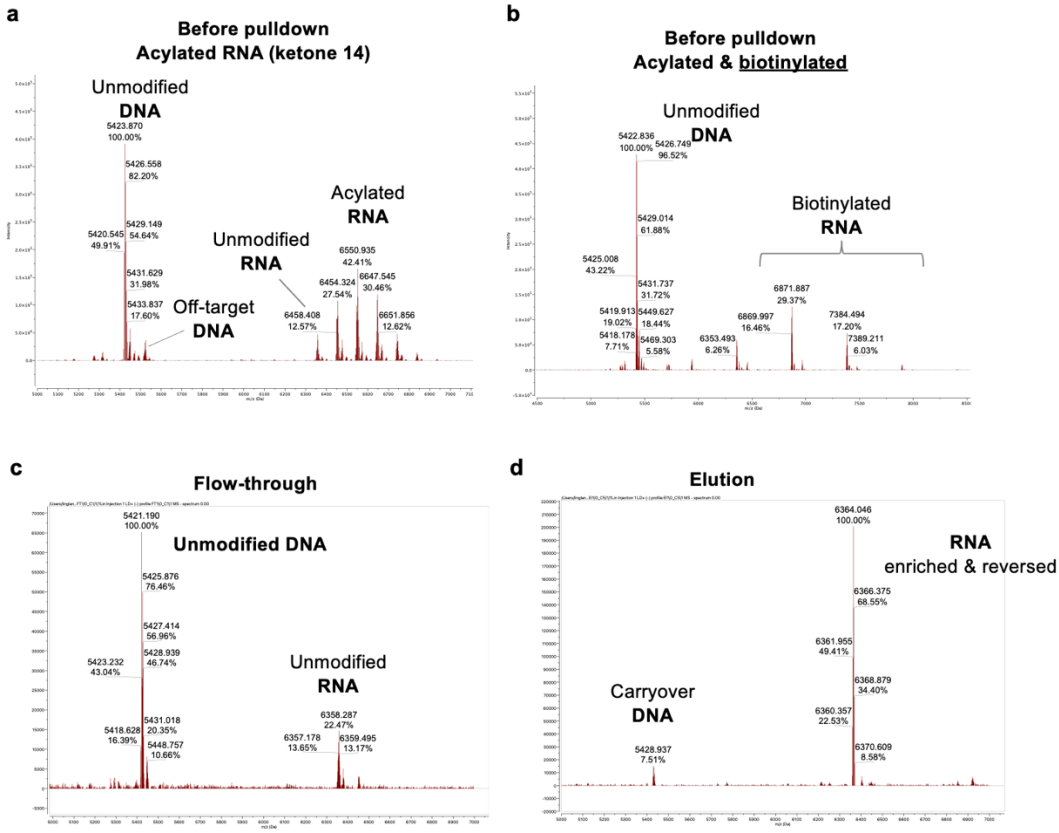
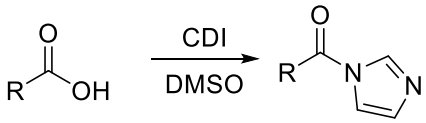


Figure S27. Employing selective acylation with ketone **14** to rapidly and nondestructively separate and enrich RNA and DNA from a mixture. MS spectra show (a) RNA/DNA mixture after acylation, showing only a small amount of unintended DNA acylation and near-complete acylation of RNA; (b) Mixture after conjugation with aminooxy-biotin; (c) elution (flow-through) of enriched DNA after capture of the RNA on streptavidin-coated magnetic beads; (d) elution fraction of unmodified native RNA after incubation with histamine to release it from the beads. Calculated masses (m/z): DNA 5419.6; RNA 6351.9.

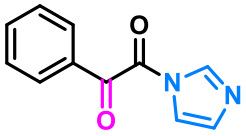
Synthetic procedures

General procedure for the preparation of conjugable acylating reagents



To a solution of carboxylic acid derivative (1 equivalent) in anhydrous DMSO (as 1 M stock solution) was added CDI (1.7 equivalent) at ambient temperature under argon. The reaction mixture was stirred at ambient temperature 30 minutes to afford the acylated reagent as mixture with imidazole. For NMR analysis, the reactions were performed in DMSO-*d6*. Note that reagent **2**, **13** were not detected by ^{1}H NMR or MS due to instability, and reagents **3**, **4** ,**5**, **6** included an unknown side product.

1-(1H-Imidazol-1-yl)-2-phenylethane-1,2-dione (**1**)

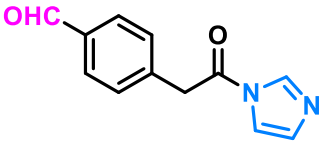


Analytical data:

Compound could not be characterized by 1H NMR due to large number of side products.

ESI-MS [M+H]: Calculated: 201.06; Observed: 201.05

4-(2-(1H-Imidazol-1-yl)-2-oxoethyl)benzaldehyde (**2**)

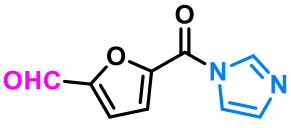


Analytical data:

Compound could not be characterized by 1H NMR due to large number of side products.

ESI-MS [M+H]: Calculated: 215.08; Observed: 214.99

5-(1H-Imidazole-1-carbonyl)furan-2-carbaldehyde (**3**)

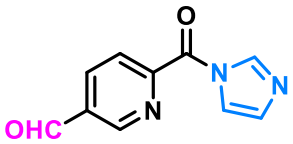


Analytical data:

Compound could not be characterized by 1H NMR due to large number of side products.

ESI-MS [M+H]: Calculated: 191.04; Observed: 190.988

6-(1H-Imidazole-1-carbonyl)nicotinaldehyde (**4**)

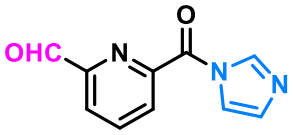


Analytical data:

Compound could not be characterized by 1H NMR due to large number of side products.

ESI-MS [M+H]: Calculated: 202.06; Observed: 201.91

6-(1H-imidazole-1-carbonyl)picolinaldehyde (**5**)

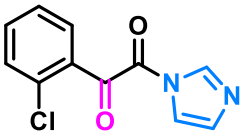


Analytical data:

Compound could not be characterized by 1H NMR due to large number of side products.

ESI-MS [M+H]: Calculated: 202.06; Observed: 202.03

1-(2-Chlorophenyl)-2-(1H-imidazol-1-yl)ethane-1,2-dione (**6**)



Analytical data:

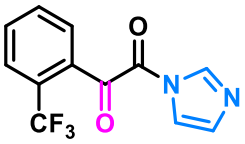
^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.58 (s, 1H), 7.99 (d, *J* = 8.8 Hz, 1H), 7.88 (s, 1H), 7.68 (d, *J* = 8.1 Hz, 1H), 7.62 (t, *J* = 7.8 Hz, 1H), 7.25 (s, 1H)

Note that a proton peak is overlapped with CDI and imidazole peak around 7.76-7.80 ppm.

^{13}C NMR (125 MHz, DMSO-*d6*) δ185.97, 161.15, 138.42, 136.05, 135.07, 133.13, 132.65, 131.68, 131.54, 128.06, 121.50.

ESI-MS [M+H]: Calculated: 235.02; Observed: 235.01

1-(1H-Imidazol-1-yl)-2-(2-(trifluoromethyl)phenyl)ethane-1,2-dione (**7**)



Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.59 (s, 1H), 8.07 (dd, *J* = 6.8, 2.9 Hz, 1H), 7.79 (dd, *J* = 7.7, 2.3 Hz, 1H), 7.92-7.89 (m, 2H), 7.87 (s, 1H), 7.23 (s, 1H).

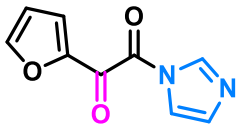
^{13}C NMR (125 MHz, DMSO-*d6*) δ 186.31, 159.23, 138.63, 135.08, 133.44, 132.64, 131.71, 129.86, 127.26, 127.22, 124.51, 122.33, 121.44.

Note that peaks around 127.26 and127.22 ppm are from trifluoromethyl coupling.

ESI-MS [M+H]: Calculated: 269.05; Observed: 269.12

Note that a proton peak overlaps with imidazole peak near 7 ppm.

1-(Furan-2-yl)-2-(1H-imidazol-1-yl)ethane-1,2-dione (**8**)



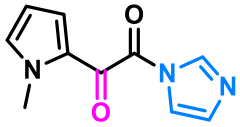
Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.48 (s, 1H), 8.29 (d, *J* = 1.8 Hz, 1H), 7.83 (d, *J* = 3.7 Hz, 1H), 7.78 (m, 1H), 7.17 (s, 1H), 6.88 (dd, *J* = 3.7, 1.8 Hz, 1H)

^{13}C NMR (125 MHz, DMSO-*d6*) δ 171.64, 159.44, 151.44, 148.83, 135.13, 130.76, 126.06, 121.53, 113.83.

ESI-MS [M+H]: Calculated: 191.04; Observed: 190.93

1-(1H-Imidazol-1-yl)-2-(1-methyl-1H-pyrrol-2-yl)ethane-1,2-dione (**9**)



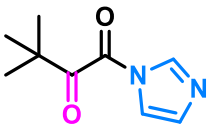
Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.42 (s, 1H), 7.76 (s, 1H), 7.53 (s, 1H), 7.18 (s, 1H), 7.13 (dd, *J* = 4.2, 1.6 Hz, 1H), 6.29 (dd, *J* = 4.5, 2.4 Hz, 1H), 4.01 (s, 3H)

^{13}C NMR (125 MHz, DMSO-*d6*) δ 174.18, 161.70, 138.29, 136.66, 135.14, 126.49, 125.63, 121.67, 110.44, 37.17.

ESI-MS [M+H]: Calculated: 204.07; Observed: 204.08

1-(1H-Imidazol-1-yl)-3,3-dimethylbutane-1,2-dione (**10**)



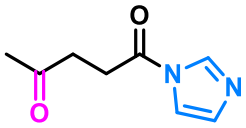
Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.45 (s, 1H), 7.74 (s, 1H), 7.20 (d, *J* = 9.2 Hz, 1H),7.15 (s, 1H), 6.74 (d, *J* = 8.5 Hz, 1H), 2.88 (s, 6H)

Note that two proton peaks overlap with CDI and imidazole peaks, which occur at 7.71 (CDI), 7.04 (imidazole) ppm respectively.

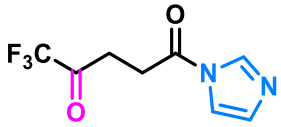
^{13}C NMR (125 MHz, DMSO-*d6*) δ 202.68, 161.95, 138.01, 135.14, 121.62, 43.04, 25.31.
ESI-MS [M+H]: Calculated: 181.09; Observed: 181.03

1-(1H-Imidazol-1-yl)pentane-1,4-dione (**11**)



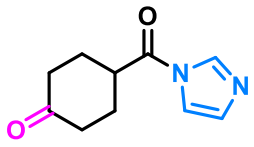
Analytical data:
^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.40 (s, 1H), 7.65 (s, 1H), 7.02 (s, 1H), 3.12 (t, *J* = 5.8 Hz, 1H), 2.82 (t, *J* = 5.7 Hz, 1H), 2.08 (s, 3H)
^{13}C NMR (125 MHz, DMSO-*d6*) δ 174.80, 135.21, 135.11, 121.73, 93.49, 33.89, 28.41, 26.78.
ESI-MS [M+H]: Calculated: 167.08; Observed: 167.02

5,5,5-Trifluoro-1-(1H-imidazol-1-yl)pentane-1,4-dione (**12**)



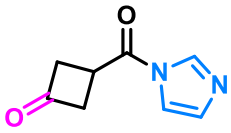
Analytical data:
^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.08 (s, 1H), 7.50 (s, 1H), 7.10 (s, 1H), 3.21-3.15 (m, 1H), 3.07-3.00 (m, 1H), 2.95-2.91 (m, 1H)
^{13}C NMR (125 MHz, DMSO-*d6*) δ 172.89, 135.18, 129.76, 124.51, 123.05, 121.66, 120.78, 90.31, 90.03, 89.76, 27.65, 26.43.
Note that three peaks around 90.31-89.76 ppm are from trifluoromethyl coupling.
ESI-MS [M+H]: Calculated: 221.05; Observed: 221.00

4-(1H-Imidazole-1-carbonyl)cyclohexan-1-one (**13**)



Analytical data:
^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.54 (s, 1H), 7.75 (s, 1H), 7.07 (s, 1H), 3.71-3.66 (m, 1H), 2.53-2.46 (m, 2H), 2.53-2.46 (m, 2H), 2.28-2.14 (m, 2H), 1.89-1.82 (m, 2H)
^{13}C NMR (125 MHz, DMSO-*d6*) δ 209.09, 172.43, 137.18, 130.52, 116.63, 39.13, 28.38.
ESI-MS [M+H]: Calculated: 193.09; Observed: 193.07

3-(1H-Imidazole-1-carbonyl)cyclobutan-1-one (**14**)



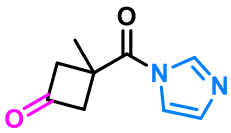
Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.46 (s, 1H), 7.74 (s, 1H), 7.11 (s, 1H), 4.18-4.11 (m, 1H), 3.51-3.48 (m, 4H)

^{13}C NMR (125 MHz, DMSO-*d6*) δ 203.73, 170.82, 137.21, 135.19, 121.71, 50.70, 36.97, 27.76.

ESI-MS [M+H]: Calculated: 165.06; Observed: 164.94

3-(1H-Imidazole-1-carbonyl)-3-methylcyclobutan-1-one (**15**)



Analytical data:

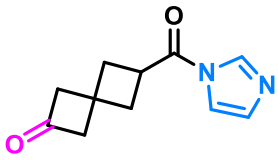
^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.35 (s, 1H), 7.11 (s, 1H), 3.81 (d, *J* = 18 Hz, 2H), 3.21 (d, *J* = 19 Hz, 2H), 1.70 (s, 3H)

Note that a proton peak overlaps with the imidazole peak, which occurs at 7.66 ppm.

^{13}C NMR (125 MHz, DMSO-*d6*) δ 204.16, 172.63, 137.42, 135.15, 121.69, 56.76, 36.07, 24.92.

ESI-MS [M+H]: Calculated: 179.08; Observed: 179.04

6-(1H-Imidazole-1-carbonyl)spiro[3.3]heptan-2-one (**16**)



Analytical data:

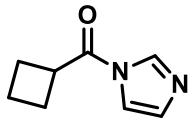
^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.07 (s, 1H), 4.40-3.98 (m, 1H), 3.32 (s, 1H), 3.06 (s, 1H), 2.58 (d, *J* = 8.4 Hz, 2H)

Note that two proton peaks overlap with CDI and imidazole peaks, which occur at 8.37 (CDI), 7.66 (imidazole) ppm respectively.

^{13}C NMR (125 MHz, DMSO-*d6*) δ 206.83, 171.48, 136.92, 135.14, 121.67, 58.67, 58.09, 35.96, 33.17, 29.21.

ESI-MS [M+H]: Calculated: 205.09; Observed: 205.07

Cyclobutyl(1H-imidazol-1-yl)methanone (**21**)



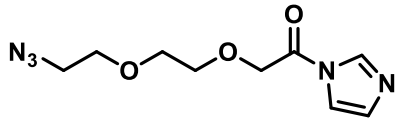
Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.31 (s, 1H), 7.62 (s, 1H), 7.06 (s, 1H), 4.04-4.00 (m, 1H), 2.32-2.30 (m, 4H), 2.05-1.99 (m, 1H), 1.86-1.83 (m, 1H)

^{13}C NMR (125 MHz, DMSO-*d6*) δ 171.65, 136.81, 130.30, 119.48, 37.83, 24.45, 17.63.

ESI-MS [M+H]: Calculated: 151.08; Observed: 151.02

2-(2-(2-Azidoethoxy)ethoxy)-1-(1H-imidazol-1-yl)ethan-1-one (**22**)



Analytical data:

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.38 (s, 1H), 7.68 (s, 1H), 7.08 (s, 1H), 4.85 (s, 2H), 3.72 (s, 2H), 3.62-3.60 (m. 4H), 3.37 (s, 2H)

^{13}C NMR (125 MHz, DMSO-*d6*) δ 167.80, 136.69, 130.16, 119.49, 70.40, 69.56, 69.50, 69.20, 50.03.

ESI-MS [M+H]: Calculated: 240.10; Observed: 240.08

References

(1) Chatterjee, S.; Shioi, R.; Kool, E. T. Sulfonylation of RNA 2′-OH Groups. *ACS Cent. Sci.* **2023**, *9* (3), 531–539. https://doi.org/10.1021/acscentsci.2c01237.

(2) Xiao, L.; Habibian, M.; Kool, E. T. Site-Selective RNA Functionalization via DNA-Induced Structure. *J. Am. Chem. Soc.* **2020**, *142* (38), 16357–16363. https://doi.org/10.1021/jacs.0c06824.