Tetrahedron Letters 53 (2012) 5891–5894

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Synthesis of novel tetrazole C5-linked C_0- and C_2-ribonucleoside phosphoramidites using MePOM and POM groups for probing RNA catalysis

Shinya Harusawa [a,*], Hiroki Yoneyama [a], Daiki Fujisue [a], Masayoshi Nishiura [a], Mihoyo Fujitake [a], Yoshihide Usami [a], Zheng-yun Zhao [b], Scott A. McPhee [c], Timothy J. Wilson [c], David M. J. Lilley [c]

[a] Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan
[b] School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[c] CR-UK Nucleic Acid Structure Group, MSI/WTB Complex, University of Dundee, Dundee DDI 5EH, UK

ARTICLE INFO

Article history:
Received 1 June 2012
Revised 14 August 2012
Accepted 22 August 2012
Available online 29 August 2012

Keywords:
Tetrazole
Phosphoramidite
Protecting group
Chemogenetic approach
Ribozyme

ABSTRACT

Novel C5-linked C_0- and C_2-tetrazole ribonucleoside phosphoramidites were designed and synthesized via tetrazole C-nucleosides. Pivaloyloxymethyl (POM) and methyl-substituted POM (MePOM) groups were introduced as N-protecting groups in the tetrazole ring that can be readily removed under mild basic conditions. The phosphoramidites were successfully incorporated into the VS ribozyme substrate and hence providing a chemogenetic approach to determine which nucleobases of ribozymes function as the acid or base, in the studies of ribozyme general acid and base catalysis.

RNA catalysis occurs in cells for the processing of RNA molecules, the control of gene expression, and even the synthesis of proteins; yet the catalysis mechanisms are still unclear in most cases.[1] The Varkud satellite (VS) ribozymes are the largest of a group of nucleolytic ribozymes that include hammerhead, hairpin, HDV, and GlmS ribozymes.[2] While no crystal structure of VS ribozymes has been determined yet, a small-angle X-ray scattering-derived model places G638 and A756 in proximity to the scissile phosphate.[2d]

We have recently developed a novel chemogenetic approach for the analysis of general acid–base catalysis by nucleobases in ribozymes.[3] This involves substitution of a C4 linked-ribonucleoside with imidazole (pK_a: 7.1)[4] being a good donor and acceptor of proton in place of an important nucleobase in VS and hairpin ribozymes.[5] In this study, *N*-pivaloyloxymethyl (POM) 2′-*O*-*t*BDMS-imidazole C_0-phosphoramidite (PA) **2**[5a,b] was first employed to yield an imidazole-substituted VS ribozyme by covalently placing imidazole as a pseudonucleobase at position 756 (replacing adenine) of the VS ribozyme (Fig. 1).[3a] The imidazole ribozyme (A756Imz) catalyzed the almost complete cleavage of substrate stem-loops at the correct position; this confirms the direct role of the nucleobase at position 756 in the chemistry of the natural VS ribozyme.[3] The overall yield of **2** tends to be rather limited due to the lack of selective introduction of the *t*BDMS group for 2′-hydroxy group as well as due to its acid-labile character.[5a,b] Therefore, a new combination of protecting groups was introduced for the carbon-elongated homologs PAs **3** (n = 0–3): POM for imidazole-*N* and cyanoethyl (CE) groups for the 2′-hydroxy group (Fig. 1).[5c,e] In addition, PAs **3a–d** are much more stable than PA **2**, making the purification steps easier. Further, it is of particular interest that the C_2-imidazole VS ribozyme ($G638C_2Imz$) incorporated from PA **3c** (n = 2) showed a 15-fold greater catalytic activity than the C_0-imidazole ribozyme ($G638C_0Imz$) [5c] These results definitely demonstrated that the key functionalities in the catalytic mechanism of VS ribozyme are G638 and A756 that concertedly act in general acid–base catalysis. However, it is impossible by this approach to determine which nucleobases specifically function as the acid or base when imidazole is employed as a nucleobase.

5-Aliphatic-1*H*-tetrazoles with pK_a of 4.5–4.9 are often used as metabolism-resistant isomeric replacements for carboxylic acids in medicinal chemistry.[6] Given that, the synthesis of tetrazole C-nucleoside PAs may allow the incorporation of an acid surrogate at the G638 or A756 position to specify the role of the nucleobase as a general acid in the chemistry of natural VS ribozyme. However, the tetrazole-related C-nucleosides have not been extensively studied so far.[7] We herein describe the synthesis of a novel C5-linked C_0- and C_2-tetrazole ribonucleoside PAs **1a** and **1b** (Fig. 1). The latter, with a flexible linker, may lead to greater catalytic activity at the G638 position of VS ribozyme. In this study,

* Corresponding author. Tel./fax: +81 72 690 1086.
E-mail address: harusawa@gly.oups.ac.jp (S. Harusawa).

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http://dx.doi.org/10.1016/j.tetlet.2012.08.082

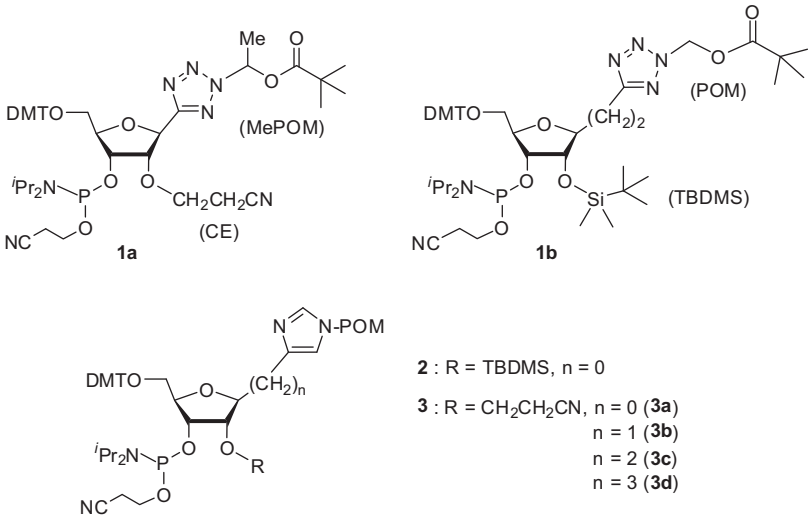
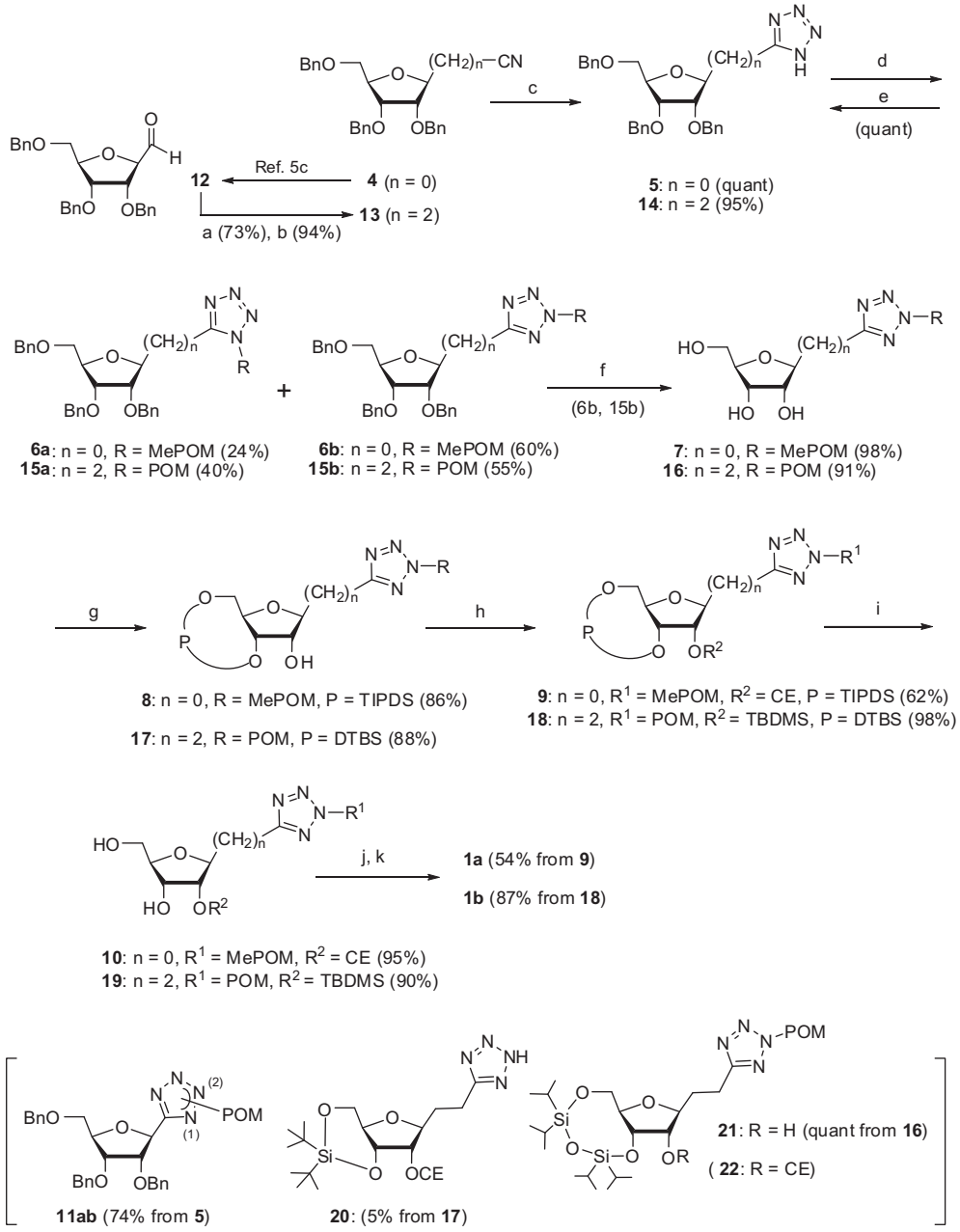


Figure 1. Structures of imidazole and tetrazole C_n-ribonucleoside PAs employed for solid-phase RNA synthesis.

POM and a novel methyl-substituted POM (MePOM) groups were used as protecting groups for the tetrazole-*N*. In addition, we demonstrate initial results about the efficient syntheses of RNA oligonucleotides using PAs **1a** and **1b**.

We previously reported an efficient synthesis of β-D-ribofuranosyl cyanide **4**, in 56% overall yield from commercially available 2,3,5-tri-*O*-benzyl-D-ribose in two steps (Scheme 1).[5c] Tetrazole C-ribonucleoside **5** ($n = 0$) was prepared in quantitative yield by the treatment of nitrile **4** with sodium azide and triethylammonium chloride in DMF. This reaction proceeded sufficiently rapidly due to the activation of the neighboring electron-withdrawing oxygen in the sugar moiety. We first examined the use of the POM group as a protecting group for tetrazole-*N* of **5**. The POM group was used in the synthesis of imidazole C_n-PAs and was removed in the final step of RNA synthesis by a mixture of ammonia and ethanol with other base-protecting groups.[5a,b] However, the introduction of the POM group into tetrazole **5** resulted in a 1:1 inseparable mixture **11ab** (74%) of *N*-1 and *N*-2 POM-protected tetrazoles (Scheme 1, the bottom left). The subsequent synthetic processes from **11ab** suffered a setback owing to the formation of further complicated mixtures. We then tried MePOM protection via 1-chloroethyl pivaloate (MePOMCl),[8] with expectation of *N*-2 protection caused preferentially by stereo hindrance of the methyl group. Indeed, the reaction of tetrazole C-nucleoside **5** with MePOMCl in the presence of DMAP in DMF afforded *N*-1 and *N*-2 MePOM derivatives **6a** (24%) and **6b** (60%), which could be isolated as diastereoisomers by column chromatography because of the additional stereogenic center present in the MePOM group (which, of course, could easily be identified from NMR spectra). The location of the MePOM group on the tetrazole ring was assigned, since the ^{13}C NMR signal from the only carbon in tetrazole was generally about 10 ppm deshielded in 2,5-disubstituted derivatives relative to the corresponding 1,5-disubstituted isomers [^{13}C NMR shift of δ 5-C (ppm) in $CDCl_3$: **6a**, 153.4; **6b**, 165.0].[6d] MePOM groups of **6a** and **6b** could be removed quantitatively by aqueous ammonia/EtOH (MeOH) (1:3, v/v) at room temperature (rt) in 17 h to convert back into **5**. Removal of the MePOM group from tetrazole-*N* proceeded somewhat slowly compared to the removal of the POM group at imidazole-*N* (rt, 3 h).[5a,b] In contrast, stirring of **6b** in 2*N* HCl–EtOH (1:3, v/v) at rt for 17 h resulted in recovery of **6b** in 94% yield, indicating the stability of the *N*-MePOM group under an aqueous acidic condition. Subsequent treatment of **6b** with $Pd(OH)_2$-C/cyclohexene in refluxing ethanol produced *N*-MePOM-tetrazole C-nucleoside **7** (98%). It should be noted that MePOM group tolerates the debenzylation-condition. In addition, to our knowledge, the MePOM group has not been employed as an *N*-protecting group. 3′,5′-*O*-TIPDS-protection (1,1,3,3-tetraisopropyldisiloxanediyl) of **7** gave **8** (86%), and this allowed selective introduction of the 2′-hydroxy protecting group. Cyanoethylation of **8** with acrylonitrile gave fully protected intermediate **9** (62%).[9] The TIPDS group of **9** was selectively removed by treatment with $Et_3N{\cdot}3HF$ to give 3′,5′-*O*-unprotected ribonucleoside derivative **10** (95%). Standard 5′-*O*-dimethoxytritylation (80%) of **10** followed by phosphitylation of 3′ hydroxy afforded tetrazole C_0-PA **1a** as a mixture of four diastereomers (67%).[10]

C_2-tetrazole ribonucleoside PA **1b** was synthesized from aldehyde **12** that was prepared in two steps (92%) from **4** (Scheme 1).[5c] Wittig olefination of **12** using (cyanomethyl)triphenylphosphonium chloride gave the vinylnitrile (73%) and then reduction of the formed double bond yielded the two-carbon-elongated nitrile **13** (94%). Conversion of the inactive alkylcyanide **13** into tetrazole **14** in the presence of sodium azide and triethylammonium chloride proceeded slowly (40 h, 130 °C, DMF) in a moderate yield (57%), while microwave (MW) irradiation (130 °C, DMF) led to both a shorter reaction time (2 h) and an improved yield (95%). The treatment of **14** with POMCl (DBU, 100 °C, 0.5 h, DMF, MW) yielded *N*-1 and *N*-2 POM derivatives **15a** (40%) and **15b** (55%), respectively, which could be easily separated by column chromatography. Meanwhile, MePOM-protection of **14** also gave almost the similar result. The *N*-2 POM isomer **15b** was used for the subsequent synthetic process because it showed a clearer ^1H NMR peak pattern than **15a** did. The base-labile POM **15a** could be transformed back to tetrazole **14** quantitatively for reuse, while **15a** remained intact under 2*N* HCl/EtOH (1:2, v/v; rt, 5 h). There is little information regarding *N*-POM tetrazoles in the literature,[11] and their role as a protecting group has not yet been fully clarified. Debenzylation of **15b** produced *N*-2-POM-tetrazole C_2-ribonucleoside **16** (91%) and subsequent 3′,5′-*O*-DTBS-protection (88%, DTBS = di-*tert*-butylsilanediyl) afforded **17** (88%). Although 2′-*O*-TBDMS protection of **17** provided 2′-*O*-silylated derivative **18** (98%), cyanoethylation of **17** afforded only a POM removed tetrazole **20** (5%) (Scheme 1). Alternatively, 3′,5′-*O*-TIPDS protection of **16** gave **21** (quant), but the cyanoethylation of **21** to the corresponding **22** could not be obtained at all (Scheme 1). Selective removal (90%) of the DTBS group in **18**, DM-tritylation (quant), and phosphitylation (87%) proceeded successfully to give the final 2′-*O*-TBDMS tetrazole C_2-PA **1b**. [12]



Scheme 1. Synthesis of tetrazole C_0 and C_2-PAs **1** and related intermediates. Reagents and conditions: (a) BuLi, (cyanomethyl)triphenylphosphonium chloride, toluene, −14 °C, 30 min, then rt, 2 h; (b) 10% Pd/C (cat.), H_2, 2Kg/cm^2, AcOEt, 1 h; (c) for **4**: NaN_3 (3.0 equiv), $Et_3N{\cdot}HCl$ (3.0 equiv), DMF, 130 °C, 2 h; for **13**: NaN_3 (3.0 equiv), $Et_3N{\cdot}HCl$ (3.0 equiv), DMF, MW, 130 °C, 2 h; (d) for **5**: MePOMCl (4 equiv), DMAP (4 equiv), DMF, 50 °C, 10 h; for **14**: POMCl (2 equiv), DBU (2 equiv), DMF, MW, 100 °C, 0.5 h; (e) 28% aq. NH_3/EtOH (MeOH) (1:3, v/v), rt, 14–17 h; (f) for **6b**: 20% $Pd(OH)_2$-C, cyclohexene , EtOH, reflux, 25 h; for **15b**: 20% $Pd(OH)_2$-C, cyclohexene, EtOH, MW, 80 °C, 0.5 h; (g) for **7**: TIPDSCl2, py, 0 °C then rt, 2 h; for **16**: ^{t}Bu$_2$Si(OTf)$_2$, py., rt, 10 min; (h) for **8**: CH_2=CHCN, Cs_2CO_3, ^{t}BuOH, 40 °C, 10 h; for **17**: TBDMSCl, imidazole, DMF, 50 °C 5 h; (i) for **9**: $Et_3N{\cdot}3HF$, Et_3N, THF, rt, 1.5 h; for **18**: py·HF, py, CH_2Cl_2, 0°C, 1h; (j) DMTCl, Et_3N, DMAP, py, rt, 2.5 - 3.0 h; (k) $Et_3N{\cdot}3HF$, $(^iPr_2N)_2POCH_2CH_2CN$, 4,5-DCI, $ClCH_2CH_2Cl$, 40 °C, 20–30 min.

Purification of crude PAs **1a** and **1b** could be easily carried out by medium-pressure flash column chromatography (MPLC) using a standard silica gel column (Fuji Silysia Chemical FL60D, pH 6.6). This suggests that **1a** and **1b** are considerably more stable than the acid-sensitive imidazole PAs. Base-protecting groups of RNA are conventionally removed in the final step of RNA synthesis by ammonia and ethanol mixture [28% aq NH_3/EtOH (3:1, v/v)] at 55 °C for 16 h. As the POM and MePOM groups can be removed under milder basic conditions at rt, it is particularly attractive to sensitive RNA such as Cy5 labeled RNA where deprotection should preferably be carried out at rt in order to minimize the destruction of cyanine dye. In addition, MS measurements of tetrazole C_n-PAs **1a** and **1b** were problematic owing to their labile properties, but we recently reported that the molecular weight of PAs may be accurately determined by using a novel matrix system [triethanolamine (TEOA)–NaCl] on FABMS equipped with a double-focusing mass spectrometer.[13]

The present method successfully revealed the sodium adduct ions $[M+Na]^+$ of **1a** and **1b**, and thus, their composition formulas could be determined.[10,12] The overall yields of **1a** is 16.0% in 8 steps from nitrile **4**, and that of **1b** is 22.0% in 10 steps from aldehyde **12**; making them feasible approaches for the scaling up of their synthesis.

Preliminary incorporation of these modified tetrazole PAs **1a** and **1b** into VS ribozyme substrate stands (25nt) was carried out

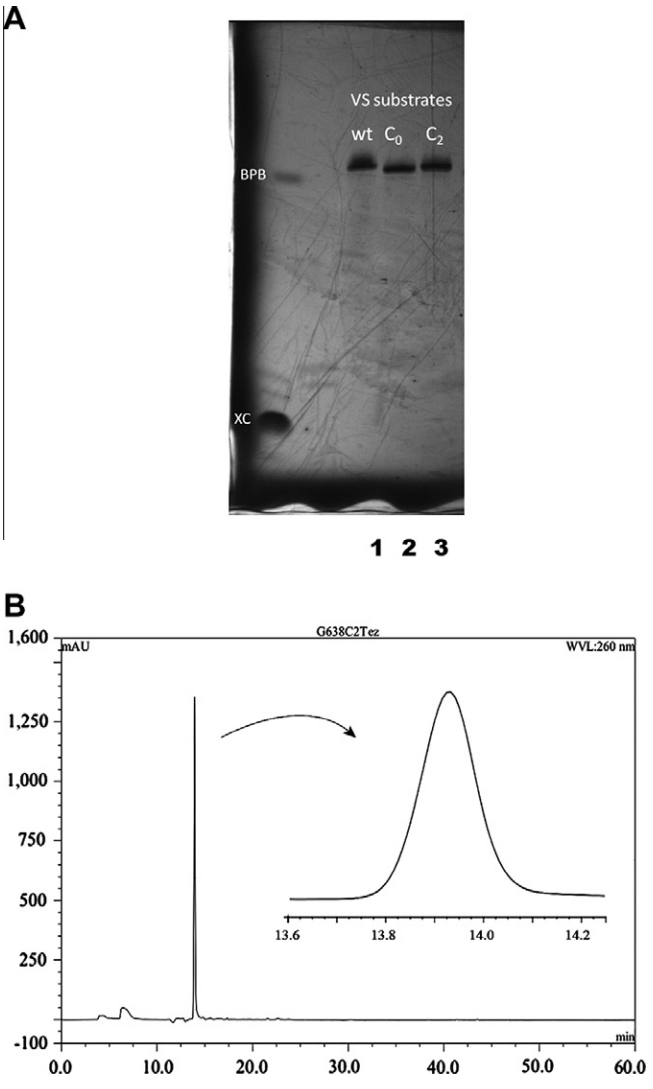


Figure 2. (A) Analysis of the products of RNA synthesis by gel electrophoresis.[14] Lane 1 VS substrate wtG638; Lane 2 G638C_0Tez; Lane 3 G638C_2Tez. (B) HPLC profile of G638C_2Tez. Desired 25mer has a retention-time of 14 min.[15]

with Applied Biosystems 394 synthesiser with stepwise coupling yield over 97%.[14] Demonstrated here is the denaturing gel image for wild type substrate and C_0- and C_2-tetrazole substituted (G638C_0Tez and G638C_2Tez) strands at the same position of the sequence as well as the HPLC profile[15] of G638C_2Tez, as shown in Figure 2.

Further investigation on the application of **1a** and **1b** in ribozyme studies is under way and will be published in due course.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research [Grant No.2159030 (to S.H.)] from JSPS, 2009–2013.

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10. **1a**: oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.01–1.26 (21H, m), 1.84 (1.05H, d, J = 6.5 Hz), 1.85 (0.45H, d, J = 6.5 Hz), 1.88 (1.05H, d, J = 6.5 Hz), 1.90 (0.45H, d, J = 6.5 Hz), 2.35 (0.6H, t, J = 6.6 Hz), 2.60 (1.4H, t, J = 6.3 Hz), 2.56–2.68 (2H, m), 3.06–3.11 (1H, m), 3.37–3.47 (1H, m), 3.54–3.62 (2H, m), 3.64–3.86 (2H, m), 3.78 (4.2H, s), 3.79 (1.8H, s), 3.88–4.00 (2H, m), 4.28–4.30 (0.7H, m), 4.33–4.35 (0.3H, m), 4.45–4.52 (1H, m), 4.56–4.62 (1H, m), 5.26–5.30 (1H, m), 6.78–6.82 (4H, m), 7.16–7.44 (10H, m); ^{31}P NMR (202 MHz, $CDCl_3$) δ 149.8, 149.9, 150.4, 150.5; HRMS (FABMS:TEOA+NaCl) m/z calcd for $C_{46}H_{60}N_7O_9P$+Na $[M+Na]^+$ 908.4088, found 908.4085.
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12. **1b**: oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.11 (1.8H, s), 0.13 (2.1H, s), 0.14 (2.1H, s), 0.92 (8.4H, d, J = 7.2 Hz), 0.98 (3.6H, d, J = 6.8 Hz), 1.08–1.18 (9H, m), 1.19 (9H, s), 1.92–2.04 (1H, m), 2.18–2.30 (2H, m), 2.53–2.67 (1H, m), 2.98–3.14 (2H, m), 3.19–3.38 (2H, m); 3.48–3.64 (3H, m), 3.74–4.22 (5H, m), 3.78 (4.2H, s), 3.79 (1.8H, s), 6.40 (0.6H, s), 6.41 (1.4H, s), 6.80–6.86 (4H, m), 7.16–7.48 (9H, m); ^{31}P NMR (120 MHz, $CDCl_3$) δ 148.6, 151.2; HRMS (FABMS: TEOA+NaCl) m/z calcd for $C_{50}H_{73}N_6O_9PSi$+Na $[M+Na]^+$ 983.4844, found 983.4841.
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14. (a) Oligoribonucleotides were synthesized on an Applied Biosystems 394 instrument using 2′-O-tBDMS PA chemistry on Applied Biosystems LV 200 polystyrene 2′-deoxythymidine columns. A coupling time of 12 min was used. 0.5 M 5-ethylthio-1*H*-tetrazole in acetonitrile was used as an activator and 0.1 M iodine/water was used as an oxidiser.; (b) Oligoribonucleotides containing tetrazole modification were deprotected using 75% aqueous ammonia (35%) in ethanol for 17 h at rt and then evaporated to dryness. Oligoribonucleotides were redissolved in 0.3 ml of 1 M TBAF in THF to remove t-BDMS, and agitated at rt for 16 h prior to desalting by NAP-10 columns (GEHealthcare). Following ethanol precipitation, an aliquot (0.5 OD) of oligoribonucleotides was loaded on 20% denaturing polyacrylamide gel with 7 M urea and 90 mM TBE, and electrophoresed at 25 W for 2.5 h with bromophenyl blue (BPB) and xylene cyanol (XC) as makers. The gel was visualized by UV shadowing over a F254 TLC plate.; (c) Sequences of oligoribonucleotides synthesized; Wild type: GCGCGAAGGGCGUCGUCGCCCGAdT; C_0Tez-strand: GCGCGAAGGGCGUCGUCGCCCC(C_0Tet)AdT; C_2Tez-strand: GCGCGAAGGGCGUCGUCGCCCC(C_2Tez)AdT.
15. It was analyzed on an Ace C_{18}–300 column, eluted (1 mL/min) with buffer A (0.1 M TEAA, pH 7.0) and then eluted with a linear gradient from buffer A to 100% buffer B (MeCN) over a period of 60 min.