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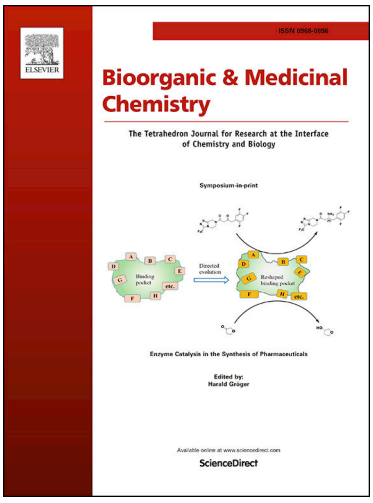

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Synthesis and biological evaluation of all possible inosine-mixed cyclic dinucleotides that activate different hSTING variants

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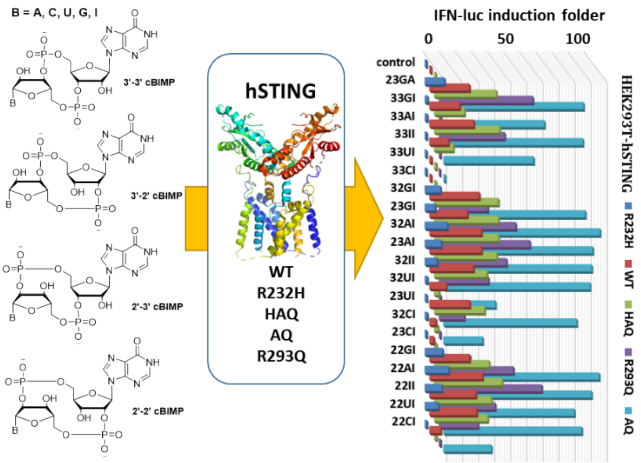
Abstract

Cyclic dinucleotides (CDNs) could activate stimulator of interferon genes (STING) protein to produce type I interferon and other pro-inflammation cytokines in mammalian cells. To explore new types of potentially efficient STING activators targeting all five major hSTING variants (WT, R232H, HAQ, AQ and R293Q), we here reported the synthesis of a total of 19 inosine-containing CDNs based on the combinations of hypoxanthine with four natural bases (A, G, C and U) and three phosphodiester linkage backbones (3′-3′, 2′-3′, 2′-2′). The IFN-β induction results showed that all of the 2′-3′ and 2′-2′ CDNs linked by inosine and purine nucleosides favored the stacking interaction with Y167 and R238 residues of hSTING protein, and several CDNs constructed by hypoxanthine and pyrimidine like c[I(2′,5′)U(2′,5′)] could also activate all five hSTING variants. The molecular dynamic simulation and the isothermal titration calorimetric (ITC) assay further demonstrated the potential of cAIMP isomers with 2′-5′ phosphate to form the hydrogen binding with R232 and R238 residues of hSTING in an entropically driven manner compared to cGAMP isomers. It would be promising to exploit novel inosine-mixed CDNs as activators of hSTING variants in immune therapy.



Keywords: Cyclic dinucleotides, Inosine, STING, Interferon-β

1. Introduction

Immunotherapy including cell therapies, antibody therapies and small molecule immune regulators has been viewed an efficient strategy in the clinical cancer treatment. Especially, cyclic dinucleotides (CDNs) were one of the most potent small molecule immune regulators. Since CDNs were discovered as second messengers in bacteria such as 3′,3′ c-di-GMP,[1] 3′,3′ c-di-AMP,[2] 3′,3′ cGAMP,[3] many categories of CDNs with different linkage directions were increasingly identified as the second messenger in other species.

In mammalian cells, 2′,3′ cGAMP was found be the only second messenger synthesized by cyclic GMP-AMP synthase (cGAS) when stimulated by cytosolic dsDNA.[4, 5, 6, 7] Stimulator of interferon genes (STING),[8] also known as ERIS,[9] MITA,[10] was identified as the main receptor protein of CDNs in mammalian cells.[7, 11, 12] The activation of STING led to innate immune responses accompanied with the fast production of type I interferon, NF-κB and other pro-inflammation cytokines. As for the efficient immune cell stimulation, there has been growing interest in combining CDNs with other small molecular drugs [13, 14] or antibody drugs [15, 16, 17] in the treatment of cancer and infections. Recently, several CDN-based STING agonists have entered into clinical trials.[18] However, the kinds of highly-efficient and common human population-targeting CDNs were still limited, especially for patients with different human STING (hSTING) variants. Hence, the further exploration of many more kinds of unnatural CDNs with all possible bases and linkage directions applicable to all patients with different hSTING variants were still waiting to be developed.

As known, all bacterial 3′-3′ CDNs were formed by two 3′-5′ phosphodiester linkages while the CDNs in mammalian cells was linked by 2′-5′ phosphodiester and 3′-5′ phosphodiester. Until now, no 2′-2′ CDNs linked with two 2′-5′ phosphodiester bonds were found in natural life. Based on the interaction relationship of CDNs and hSTING proteins, the purine base of CDNs from guanine and adenosine had more potential to activate hSTING through the stronger stacking interaction with Y163 and R238 residues of hSTING than pyrimidine rings.[7] Therefore, it suggested that the adenine or guanine bases might be replaced with hypoxanthine, which would enhance the binding capacity with different hSTING variants. It was noteworthy that patients with different hSTING genotype responded to commonly-used CDNs in a different degree.[19, 20] The coverage ratio of wild type hSTING (WT), R71H-G230A-R293Q (HAQ), R232H, G230A-R293Q (AQ) and R293Q in human population was 57.9%, 20.4%, 13.7%, 5.2% and 1.5%, respectively.[20] Our previous work also demonstrated that hSTING WT, AQ and HAQ variants showed stronger response to CDNs than R232H and R293Q variants.[21] The development of CDNs containing inosine might help to find a kind of novel CDNs targeting all patients with five hSTING variants.

As for the universality of inosine, several studies have attempted to explore the bioactivity of CDNs containing inosine. Ching *et al* reported that 3′,3′-c-di-IMP and 3′,3′ cIUMP could inhibit diguanylate cyclase (DGC) for biofilm formation in bacteria.[22] Shanahan *et al* also demonstrated that 3′-3′ CDNs containing inosine like 3′,3′ cGIMP and 3′,3′ c-di-IMP targeted the c-di-GMP riboswitches.[23] Before the cGAS-STING pathway was discovered, Libanova *et al* firstly reported

that the 3′,3′ c-di-IMP synthesized from 3′,3′ c-di-AMP catalyzed by adenosine-deaminase could effectively elicit immune response.[24] Lioux *et al* also demonstrated the CDNs containing adenosine and inosine had stronger stimulation to STING pathway than the endogenous 2′,3′ cGAMP.[25] However, these CDNs were limited to the combination of hypoxanthine and adenine bases rather than the combination of hypoxanthine and other natural bases like G, U and C. Furthermore, whether CDNs containing the different combination of hypoxanthine and natural bases (A, U, C, G) responded to five hSTING variants differently remains unknown.

To have a systemic biological evaluation of CDNs containing inosine in activating all five hSTING variants, we synthesized a total of 19 possible cyclic dinucleotides containing inosine based on the combinations of hypoxanthine with four bases (A, G, C and U) and three phosphodiester linkage backbones (3′-3′, 2′-3′, 2′-2′) (**Figure 1**). The IFN-β induction activity in HEK293T cell with different hSTING variants expression and THP-1 cell lines were evaluated. The results showed that all of the 2′-3′ and 2′-2′ CDNs linked by hypoxanthine and purine bases exhibited strong activation to all five hSTING variants (WT, R232H, HAQ, R293Q and AQ) besides several CDNs linked by hypoxanthine and pyrimidine like c[I(2′,5′)U(2′,5′)]. The introduction of inosine into CDNs could further enhance the activity to all five hSTING variants. The IFN-luc induction activity in THP-1 cell lines exhibited that CDNs with inosine could also initiate immune responses. The isothermal titration calorimetric (ITC) binding assay and molecular dynamic simulation indicated CDNs with inosine and 2'-5' phosphate diester linkage would have more potential to activate hSTING protein. It would be promising to exploit cyclic dinucleotides containing inosine as a common activator for different hSTING variants in immune therapy.

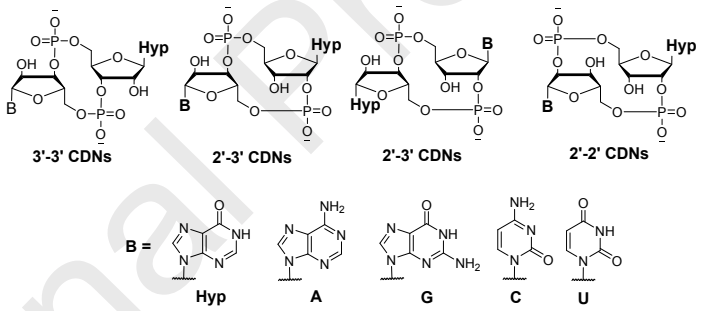


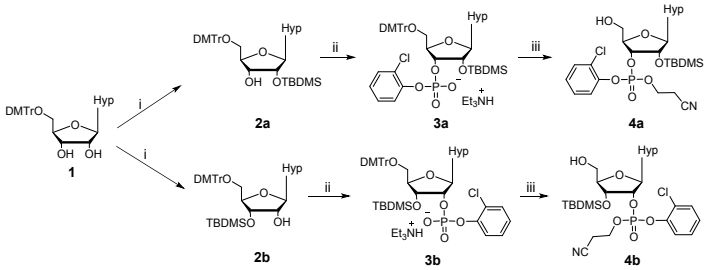
Figure 1. General structure of CDNs containing inosine

2. Results and Discussion

2.1 Chemistry

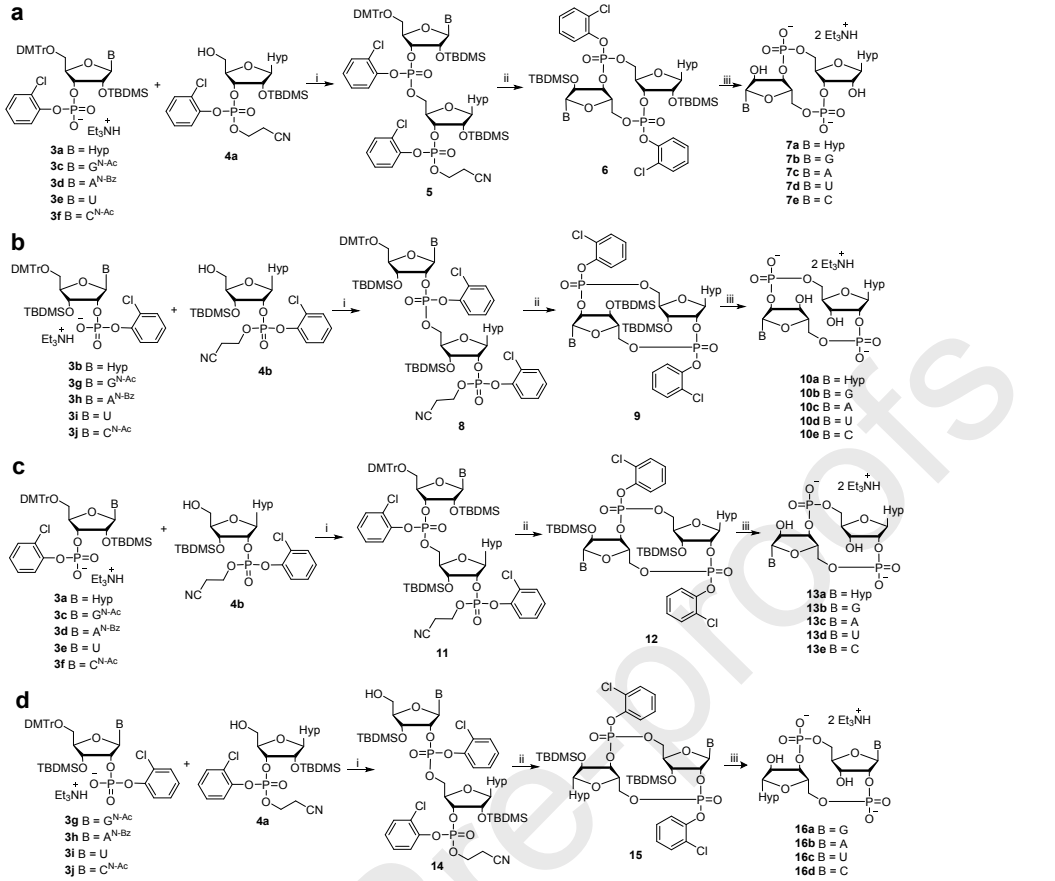
All of CDNs containing inosine were synthesized through phosphotriester approach. As shown in Scheme 1, we started from the commercial 5′-DMTr-rI nucleoside (**1**). Firstly, we introduced the *tert*-butyldinethylsilyl (TBDMS) group onto the 3′-OH or 2′-OH through the TBDMSCl in pyridine and THF mixture catalyzed by $AgNO_2$ to obtain the intermediate compounds **2a** and **2b**. The chemical structure of **2a** and **2b** were distinguished by NMR. As shown in **Figure S1**, the duplex peak at 5.48 ppm was disappeared while the D_2O was added, which indicated this peak was the proton of OH group. The ^{1}H-^{1}H COSY NMR spectra reflecting the proton correlation between 2′-H and OH indicated that the OH was the 2′-OH (**Figure S2**). The chemical structure of **2a** was also confirmed by ^{1}H-^{1}H COSY NMR spectra (**Figure S3**). The phosphate group was then introduced to obtain the compounds **3a**-**3b** as triethylamine salts by using 2-chlorophenylphosphorodichloridate.

Finally, the 3-hydroxypropionitrile was attached to the phosphodiester group with the help of coupling reagents 1-(2-Mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT), and the 5′-DMTr group was removed in a solution of 3% dichloroacetic acid (DCA) in CH_2Cl_2 to acquire the intermediates **4a**-**4b**.



Scheme 1: Synthesis of intermediates **4a**-**4b**. Reagents and conditions: i. TBDMSCl, $AgNO_2$, THF, pyridine; ii. 1*H*-tetrazole, Et_3N, 2-chlorophenylphosphorodichloridate, CH_2Cl_2, 1 M TEAB buffer; iii. 3-hydroxypropionitrile, MSNT, pyridine, DCA, CH_2Cl_2.

As shown in **Scheme 2a**, a combination of **3a** (or **3c**-**3f** [21]) and **4a** in the presence of coupling reagent MSNT in pyridine and treatment with 3% DCA solution could format the linear dinucleotides **5**. After removing the cyanoethyl with *t*$BuNH_2$/MeCN mixture, the cyclization was achieved with MSNT in pyridine to get the fully protected CDNs **6**, which was then treated with 0.5 M *N*1,*N*1,*N*3,*N*3-tetramethlyguanidinium (TMG)/*syn*-pyridine-2-carboxaldoximate (PBO) mixture in water and dioxane to remove the 2-chlorophenyl group. 33% wt $MeNH_2$/EtOH solution was subsequently added to remove base protection like Ac and Bz. After Et_3N•3HF mixture was used to remove TBDMS group, the target 3′-3′ cBIMP **7a**-**7c** as triethylamine salts were achieved through the preparative RP-LC system. As shown in **Scheme 2b**, following the same cyclization and deprotection steps as mentioned above, the combination of **3b** (**3g**-**3j** [21]) and **4b** could form the 2′-2′ cBIMP (**10a**-**10e**). Through the same steps, the combination of **3a** (or **3c**-**3f**) and **4b** could obtain the 3′-2′ cBIMP (**13a**-**13e**) (**Scheme 2c**) and the combination of **3g**-**3f** and **4a** could obtain the 2′-3′ cBIMP (**16a**-**16d**) (**Scheme 2d**).



Scheme 2: Synthesis of CDNs containing inosine. (a) 3′-3′ CDNs, (b) 2′-2′ CDNs, (c, d) 2′-3′ CDNs. Reagents and condition: i. MSNT, pyridine, DCA, CH_2Cl_2; ii. *t*$BuNH_2$, MeCN, MSNT, Py; iii. TMG, PBO, H_2O, 1,4-dioxane, $MeNH_2$, EtOH, Et_3N•3HF, pyridine, Et_3N

2.2 Dual luciferase reporter-based biological evaluation of CDNs containing inosine in HEK293T cells

In mammalian cells, hSTING recruited Tank Bind Kinas I (TBK1) and Interferon Regulator Factor 3 (IRF3) when activated by CDNs. The phosphorylated IRF3 then translocated into the nucleic and bound to the IFN-β gene promoter to induce the IFN-β production.[26] According to this mechanism, the biological evaluation of CDNs was measured through a dual-luciferase reporter which was inserted with IFN-β promoter upstream of the firefly luciferase gene.[12] In this way, the phosphorylated IRF3 when stimulated by CDNs could bind to the IFN-β promoter to express the firefly luciferase, which could be a quantitative measurement of CDN activity. HEK293T cells was co-transfected with different hSTING expression plasmids (WT, HAQ, AQ, R232H or R293Q) [21] and IFN-β-responsive luciferase reporter plasmids, followed by the stimulation with CDNs (5 μM) through digitonin permeabilization.[12, 27, 28]

As shown in **Figure 2**, the 3′-3′ CDNs containing hypoxanthine and purine bases (**7a**, **7b**, **7c**) could boost the production of the firefly luciferase when HEK293T cells transiently expressed hSTING-WT, HAQ and AQ. The CCK8 assay has revealed that all 3′-3′ inosine mixed CDNs has no significant influence on the growth of HEK293T cells (**Figure S4**). It indicated that the detectable activity of IFN-β promoter was a direct result of CDN stimulation rather than the cytotoxicity-induced IFN-β activation. The relative activity of CDNs was **7c** > **7b** > **7a**, in which **7c** showed the

comparable level to the 2′, 3′-cGAMP. Intriguingly, only **7c** could activate hSTING-R293Q variant and all of them (**7a**, **7b**, **7c**) lost the potency to activate hSTING-R232H variant. In fact, **7c** strongly induce IFN-β induction in blood [22] and also in mouse model[29], which was in accordance with our results. In contrast with the 3′-3′ CDNs containing hypoxanthine and purine, all the 3′-3′ CDNs containing hypoxanthine and pyrimidine bases (**7d** and **7e**) could not induce the production of the firefly luciferase mediated by any of five hSTING variants.

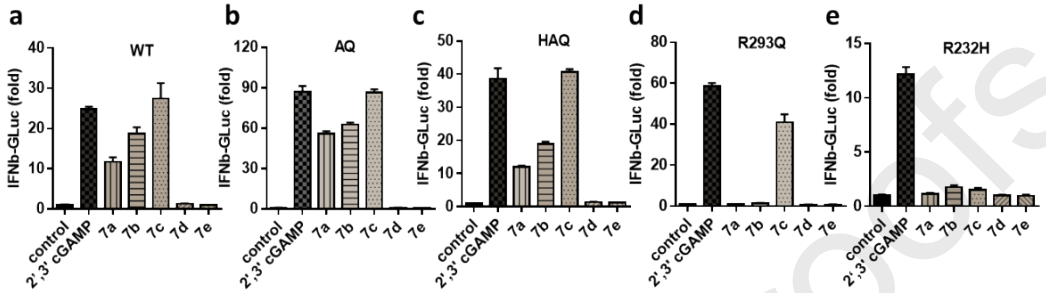


Figure 2. Dual luciferase reporter-based biological evaluation of 3′-3′ CDNs containing inosine that activated five hSTING variants. HEK293T cells were transiently transfected with (a) hSTING-WT, (b) hSTING-AQ, (c) hSTING-HAQ, (d) hSTING-R293Q, (e) hSTING-R232H and IFN-β-responsive luciferase reporter plasmids. After 24 h of transfection, cells were stimulated for 12 h with CDNs (5 μM) and then lysed for luciferase assay. Data were presented as mean ± s.e.m, n = 5.

Similar to the 3′-3′ CDNs containing hypoxanthine and purine bases, all the 2′-2′ CDNs containing hypoxanthine and purine bases still induced strong IFN-luc production mediated by five hSTING variants (**Figure 3**). Especially for **10c**, it showed higher activity than 2′,3′ cGAMP. The introduction of uracil into CDNs (**10d**) also retained strong activation to all five hSTING variants while the combination of hypoxanthine and cytosine (**10e)** showed weaker activation to all five hSTING variants.

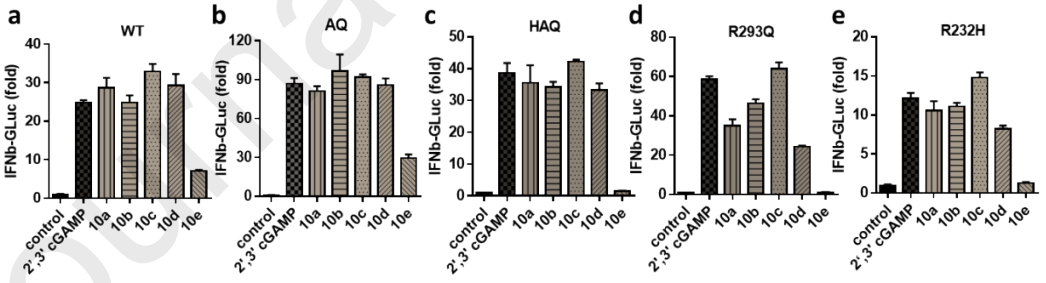


Figure 3. Dual luciferase reporter-based biological evaluation of 2′-2′ CDNs containing inosine that activated five hSTING variants. HEK293T cells were transiently transfected with (a) hSTING-WT, (b) hSTING-AQ, (c) hSTING-HAQ, (d) hSTING-R293Q, (e) hSTING-R232H and IFN-β-responsive luciferase reporter plasmids. After 24 h of transfection, cells were stimulated for 12 h with CDNs (5 μM) and then lysed for luciferase assay. Data were presented as mean ± s.e.m, n = 5.

We subsequently detected the activity of 2′-3′ CDNs containing inosine. As shown in **Figure 4**, all the 2′-3′ CDNs containing hypoxanthine and purine bases (**13a**, **13b**, **13c**, **16a** and **16b**) similarly showed excellent activity to all the five hSTING variants compared to 2′,3′ cGAMP. Unlike **7c**， **13c** also showed the strongest activity to induce IFN-luc production when HEK293T cells transiently expressed R232H or R293Q variants. Like **10d**, the combination of hypoxanthine

and uracil (**16c**) also showed strong activation to hSTING, especially for hSTING-WT, AQ and HAQ. Consistent with previous results, the combination of hypoxanthine and cytosine (**13e** and **16d**) still had weak activation to hSTING variants except the hSTING-AQ variants.

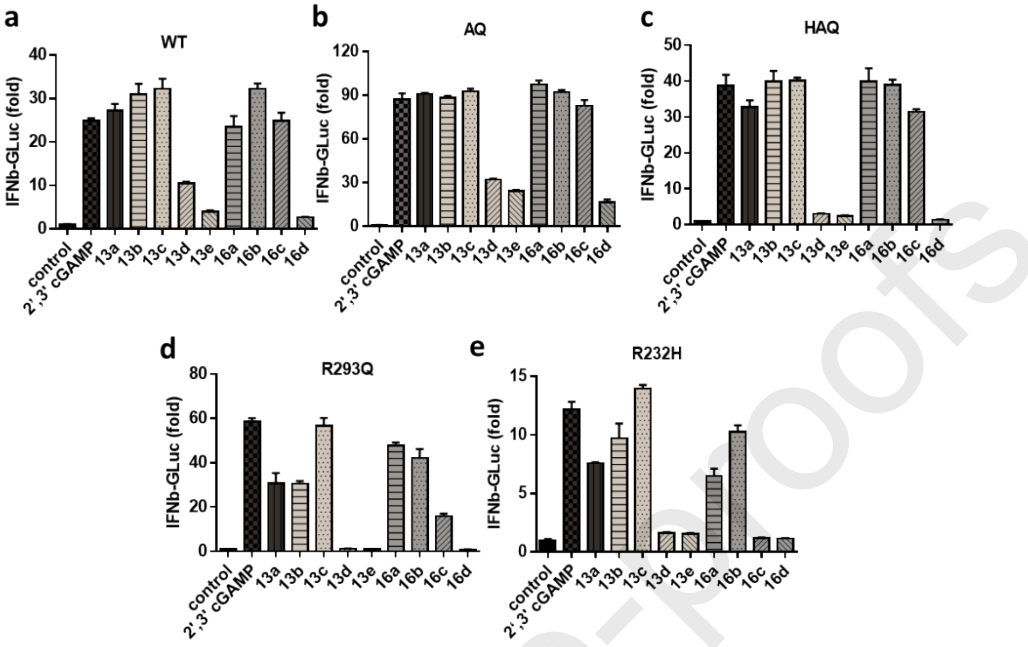


Figure 4. Dual luciferase reporter-based biological evaluation of 2′-3′ CDNs containing inosine that activated five hSTING variants. HEK293T cells were transiently transfected with (a) hSTING-WT, (b) hSTING-AQ, (c) hSTING-HAQ, (d) hSTING-R293Q, (e) hSTING-R232H and IFN-β-responsive luciferase reporter plasmids. After 24 h of transfection, cells were stimulated for 12 h with CDNs (5 μM) and then lysed for luciferase assay. Data were presented as mean ± s.e.m, n = 5.

According to the above results, we found that all 3′-3′ CDNs containing hypoxanthine and purine rings (G, A, or I) showed excellent ability to activate hSTING-WT, HAQ and AQ variants and all 3′-3′ CDNs containing inosine could not activate hSTING-R232H. For hSTING-R293Q, only **7c** could have a strong activation. For 2′-3′ and 2′-2′ CDNs containing inosine, the introduction of purine rings still strongly activated all the five hSTING variants and exhibited much higher activity compared to the natural bases constituted CDNs.[21] It was in accordance with the previous finding that CDNs with purine bases had more potential to activate hSTING for the higher potential to form stacking interaction with Y167 and R238 residues of hSTING protein.[12]

Compared with CDNs containing hypoxanthine and purine rings, CDNs containing hypoxanthine and pyrimidine base had the weaker activation except some compounds. For example, 3′-3′ CDNs containing hypoxanthine and cytosine or uracil did not activate hSTING while both 2′-3′ CDNs and 2′-2′ CDNs containing hypoxanthine and uracil could activate the IFN-β production mediated by hSTING. Especially, **10d** activated all the five hSTING variants and **16c** could also activate WT, HAQ, R293Q and AQ variants except R232H variant, which was consistent with our previous results that c[G(2′,5′)U(2′,5′)] showed good activity to all the five hSTING variants.[21] These results indicated that uracil base could be a good choice for the CDN-based STING agonist.

According to our previous findings that the stacking interactions between bases of 3′-3′ CDNs containing inosine and residues (Y167 and R238) of hSTING protein through molecular dynamic simulation,[21] we could confirm that the bases of CDNs containing inosine (such as adenine, guanine

and hypoxanthine) could also form good stacking interactions with Y167 and R238 residues (**Figure S5-S10**, **Table S1**). However, both cytosine and hypoxanthine bases could not form stacking reaction with Y167 residues when c[I(3′,5′)C(3′,5′)] bounded to hSTING. In contrast, c[I(3′,5′)U(3′,5′)] could form stacking interactions with both R238 and Y167 residues. It indicated that CDNs containing hypoxanthine and uracil bases could also have better potential binding capacity to hSTING protein.

2.3 Binding affinity of CDNs containing inosine with hSTING protein

To investigate the interaction relationship between CDNs and hSTING protein, we performed the isothermal titration calorimetry (ITC). The results showed that the Kd value of 3′-3′ CDNs containing inosine bound to wild-type hSTING-CTD was ranked as **7c** (0.65 μM) < **7b** (9.65 μM) < **7a** (19.5 μM). It indicated that the 3′-3′ CDNs linked with adenosine and inosine showed higher binding affinity to hSTING protein than 3′-3′ CDNs linked with non-adenosine and inosine (**Figure 5a**, **Table 1**). We next compared the binding affinity of different cAIMP linkage isomers with wild-type hSTING-CTD protein (**Figure 5b**, **Table 1**). The relative Kd value was ranked as **13c** (0.065 μM) < **10c** (0.311 μM) < **7c** (0.65 μM) < **16b** (3.01 μM). The only difference among these cAIMP isomers was the phosphate backbone. The hydrogen bonding of these phosphate groups were evaluated through molecular dynamic simulation. As shown in **Table S2**, cAIMP with 2′-5′ phosphodiester linkage would have more potency to form hydrogen bonding with R238 and R232, endowing these compounds more potential to activate hSTING protein. For CDNs containing uridine and inosine (**10d**, **13d**, **16c**), **10d** could bind to wild-type hSTING-CTD protein with a Kd value of 3.86 μM (**Figure 5c**, **Table 1**). It suggested that the CDNs containing inosine and uridine by 2′-5′ phosphodiester linkages had considerable potency to bind to hSTING protein for immune responses.

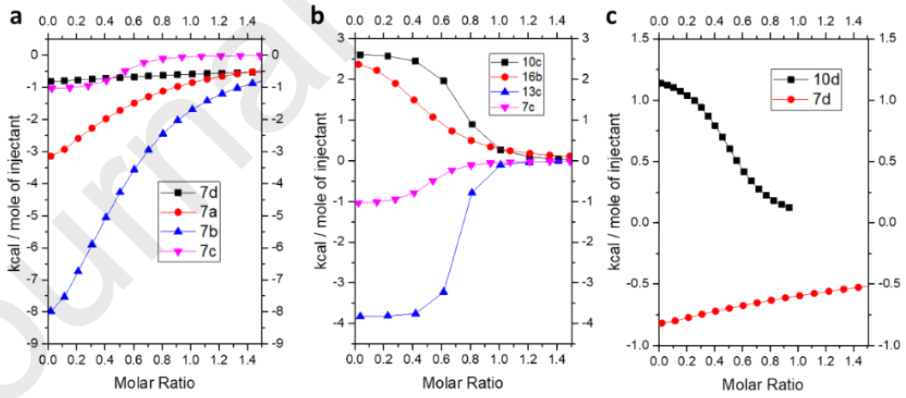


Figure 5. ITC binding curve of CDNs containing inosine to wild-type hSTING-CTD (aa 139-389). (a) 3′-3′ CDNs (**7a**, **7b**, **7c**, **7d**), (b) cAIMP isomers, (c) **10d** and **7d**.

Table 1. ITC parameters for binding of CDNs containing inosine to wild-type hSTING-CTD (aa 139-379)

	7a	**7b**	**7c**	**10c**	**13c**	**16b**	**10d**
[Cell] (μM)	40	40	30	20	20	30	80
[Syr] (μM)	400	400	400	400	400	400	400
N (sites)	0.462	0.511	0.481	0.652	0.628	0.465	0.532
Kd (μM)	19.5	9.65	0.650	0.311	0.064	3.01	3.86

ΔH (kcal/mol)	-6.54	-11.8	-1.08	2.66	-3.85	2.90	1.24
ΔG (kcal/mol)	-6.43	-6.84	-8.44	-8.88	-9.81	-7.53	-7.40
-TΔS(kcal/mol)	0.118	5.00	-7.36	-11.5	-5.94	-10.4	-8.63

Among all of the tested CDNs, **13c** had the lowest Kd value indicative of the most effective hSTING activation. When we decreased the concentration of **13c** to 50 nM in HEK293T, **13c** showed almost 2-fold higher IFN-luc induction activity than 2′,3′-cGAMP (**Figure 6a**), which had lower Kd value (5.91 nM).[21] The structural difference between **13c** and 2′,3′-cGAMP was the missing of the exocyclic amino group of guanine. According to the previous reports, the exocyclic NH_2 group of guanine could directly form hydrogen binding with E260 and T263 while 2′,3′-cGAMP bound to hSTING protein, which was absent for the hypoxanthine binding (**Figure S5**).[7, 28] Based on the ITC binding assay, we supposed that the hydrogen binding formation of exocyclic amino group of 2′,3′-cGAMP showed the lower ΔH changes (-8.04 kcal/mol) while binding to hSTING-CTD compared to **13c** (-3.85 kcal/mol). In contrast, **13c** showed lower -TΔS value (-5.94 kcal/mol) compared to 2′, 3′-cGAMP (-3.91 kcal/mol), which displayed a larger entropically change. We also found that **7c** had lower –TΔS value and higher ΔH value than 3′,3′-cGAMP while binding to hSTING-CTD protein (**Figure 6b**).[29] It indicated that both **13c** and **7c** bound to the hSTING in an almost exclusively entropically driven manner, which were assumed to be entropically favorable.

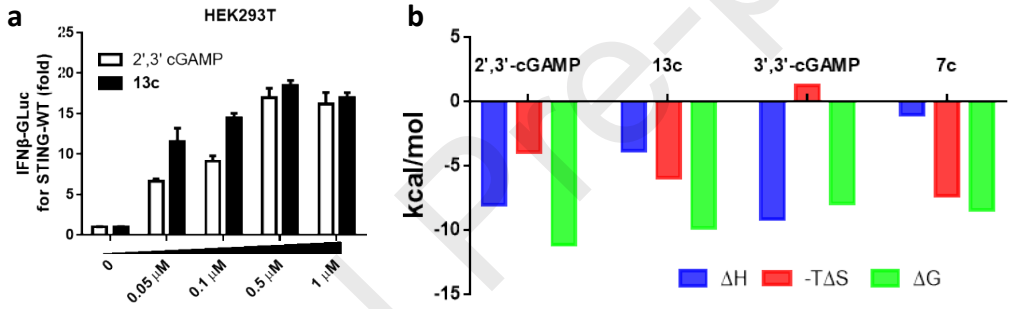


Figure 6. Comparison of cGAMP and cAIMP. (a) IFN-luc induction by 2′,3′-cGAMP and **13c** in HEK293T cells pre-transfected with hSTING-WT expression plasmids. (b) Thermodynamic signature of cGAMP and cAIMP bound to hSTING protein.

2.4 Biological evaluation of CDNs containing inosine in THP-1 cell lines

To further confirm the efficacy of tested CDNs, we used THP-1 monocytes for the evaluation of immune stimulation by the production of IFN-β. THP-1-Lucia cells were directly stimulated with 10 μM CDNs in cell culture medium for 24 h. The luciferase activity was monitored with the Quanti-Luc assay. The results showed that the **7c** showed highest activity to induce IFN-luc production (**Figure 7**). The previous reports also demonstrated that **7c** showed higher activity to induce IFN-β production in human blood than **10c**, **13c** and **16b**. [25] Furthermore, **7c** also showed strong activation to STING in humanized NOG mice through single systemic administration because of the stability towards phosphodiesterase.[29] The lower activity of these CDNs containing inosine in THP-1 cell lines than that in HEK293T cells through digitonin permeabilization was possibly associated with the transmembrane efficiency and serum stability.[28, 30, 31] The two negatively charged phosphodiester linkages retard CDNs from penetrating cell membrane which may influence the cellular uptake by THP-1-Lucia cells. Our recent work also demonstrated that the 3′-3′ CDNs with two 3′-5′ phosphodiester linkages have most stability in serum and ENPP1 than 2′-3′ and 2′-2′ CDNs.[21] We then compared the stability of cAIMP isomers in 20% FBS and found that **7c** exhibited

the highest stability as expected (**Figure S11**). But on the other hand, **7c** exhibited low ability to activate hSTING-R232H, which occupied 13.7% population of people. It would limit the usage in clinical practice. Similar to **7c**, **13c** also showed strong IFN-luc induction activity in THP-1-lucia cells. For CDNs containing inosine and uracil, **10d** and **13d** also showed considerable ability to induce the production of IFN-luc.

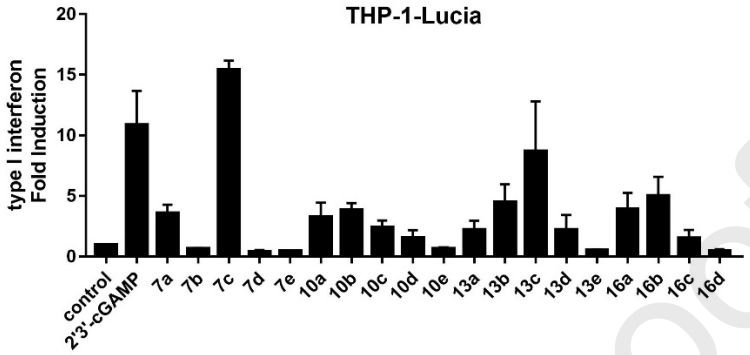


Figure 7. Type I interferon production of CDNs containing inosine in THP-1 cell lines. THP-1-Lucia cells were directly stimulated with 10 μM CDNs in the cell culture medium. After 24 h, the luciferase activity was measured with Quanti-Luc reagent. Data were presented as mean ± s.e.m, n = 3.

3. Conclusion

CDNs act as important second messengers by eliciting immune responses, which was viewed good candidates for the treatment of tumor and infectious diseases. Due to the common occurrence of five hSTING variants (WT, HAQ, AQ, R293Q and R232H) and limited CDN categories, we firstly synthesized a total of 19 possible cyclic dinucleotides containing inosine based on the combinations of hypoxanthine with four bases (A, G, C and U) and three phosphodiester linkage backbones (3′-3′, 2′-3′, 2′-2′). Among these CDNs, we found that the introduction of inosine into CDNs greatly enhanced IFN-β production mediated by hSTING. Except cytosine, CDNs containing inosine and other nucleosides (I, G, A, and U) showed strong activation activity to hSTING. All of the 2′-3′ and 2′-2′ CDNs linked by inosine and purine nucleosides (G, A or I) exhibited excellent activity to all five hSTING variants. Among four cAIMP isomers, the 3′-2′ cAIMP showed the highest activity in HEK23T cells and binding affinity to hSTING-WT-CTD protein, which indicated that the CDNs with 2'-5' phosphate diester linkage would have more potential to activate hSTING. Furthermore, we also found that CDNs linked by inosine and uridine (**10d**, **13d**, **16c)** could also activate the hSTING-mediated immune response. Especially, **10d** could activate all five hSTING variants. Collectively, the development of novel CDNs containing inosine targeting all five hSTING variants would help to further improve the treatment efficacy in immune therapy.

4. Experimental

4.1 Chemical Materials

All solvents and reagents were purchased from commercial sources and used without further purification. NMR spectra were recorded on Bruker AVANCE 400 M instruments at 298K. Chemical shits are relative to TMS (0.00). The following abbreviations were used to explain the multiplicities: s (singlet), d (doublet), t (triplet), q (quarter), m (multiplet). The number of protons (n) for a given resonance was indicated as nH. HRMS (ESI) were obtained from Agilent 6520 Q-

TOF LC/MS. HPLC was using an Agilent 1260 equipped with Agilent ZORABX SB-C18 5 μm [4.6 × 150 mm] column. The preparative LC was achieved with Agela OCTOPUS purification system equipped with Agela ASB-C18 10 μm 21.2 × 250 mm column. Reaction was monitored by TLC on silica gel GF254 with detection under UV light. 300-400 mesh silica gel was purchased from Qingdao Haiyang Huagong for silica gel purification. 1 M TEAB buffer was prepared by bubbling CO_2 through a 1 M Et_3N solution in H_2O until pH ~ 8.5. 1 M TEAA buffer was prepared by adding CH_3COOH to Et_3N in water until pH 7.0 and then filtering through a 0.45 μm membrane filter. Both TEAA and TEAB buffer were kept in 4 ℃. 0.5 M PBO-TMG buffer was freshly prepared through dissolving PBO and TMG in water and 1,4-dioxane.

4.2 Biological Materials

pGL3-IFN-β plasmid was a gift from Prof. Nicola Manel (addgen, 102597). THP-1-lucia cells were gift from Prof. Junmin Quan from Peking University. GTP and ATP were purchased from MCE. The following cell culture regents, buffers, and screening solvents were purchased elsewhere: Digitonin (Abcam, ab141501); polyjet (signagen, SL100688); FBS-Austrialian Originecl (Life, 10099-141); DMEM (Gibco, 670087); Opti-DMEM (Gibco, 31985088); RPMI1640 (Cell, 10-040-CVR); Dual-Luciferase Reporter Assay System (Promega, E1960); QUANTI-Luc system (InvivoGen, rep-qlc1).

4.3 Dual-luciferase reporter assay in HEK293T cell

5×10^4 HEK293T cells were seeded in 24-well plates. pcDNA3.1-hSTING (50 ng), pGL3-IFN-β (400 ng) and pGL4.74-Rluc-TK (50 ng) were transfected with polyjet. In the following day, cells were stimulated with CDNs (5 μM) using digitonin permeabilization (50 mM HEPES, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM DTT, 85 mM surcrose, 0.2% BSA, 1 mM ATP, 0.1 mM GTP, and 10 μg/mL digitonin). After 30 min, stimulation mixture were removed and normal media was added. After 12 h, cell lysates were prepared and dual-luciferase reporter assay was measured according to the manufacturer's instruction.

4.4 Type I interferon activity assay in THP-1 cell

5×10^4 THP-1-lucia cells were seeded in 24-well plates. In the following day, cells were directly stimulated with CDNs (10 μM) in cell culture medium. After 24 h, the culture medium were prepared and luciferase assay was measured using the QUANTI-Luc according to the manufacturer's instruction.

4.5 Expression and purification of wild-type hSTING-CTD

The partial gene of wild-type hSTING-CTD (139-379) were cloned into pET28b(+) vector between NdeI and XhoI sites. Overexpression of hSTING-CTD was induced in *E. coli* BL21(DE3) with 0.5 mM IPTG overnight at 20 ℃. Cells were collected, resuspended in a buffer A (20 mM Tris-HCl pH 7.5, 300 mM NaCl and 20 mM imidazole) and lysed by sonication on ice. After centrifugation, the soluble proteins were first purified using Ni^{2+}-NTA resin with elution buffer B (20 mM Tris-HCl pH 7.5, 300 mM NaCl and 300 mM imidazole). Subsequently, imidazole was removed using PD-10 (GE-Health) with buffer C (20 mM Tris-HCl pH 7.5 and 150 mM NaCl). The concentration of protein was monitored using absorbance at 280 nm by Nanodrop (Thermos Scientific). Protein were diluted to 0.6 ~ 2.4 μg/μL (20-80 μM) for ITC experiment.

4.6 Isothermal titration calorimetry

ITC were employed to measure the binding affinities between hSTING-CTD and CDNs using a PEAQ-ITC (Malvern). Prior to titration, both protein and CDNs were centrifuged at 13000 rpm for 10 min to remove any debris and air bubbles. The titration were performed at 25 ℃ in the buffer

C. The reference offset was at 10 μcal/s. 18 successive injections were performed with 150 s spacing time.

4.7 FBS digestion assay

0.1 μg/μL CDNs were separately incubated with either a solution (total 30 μL) of 20 % FBS, 20 mM PBS pH7.4 buffer, 5mM $MgCl_2$ or water (as control) in an air bath incubator at 37 ℃. Aliquots of the reaction mixture were collected at various time points and stopped with addition of 5 μL 0.5 M EDTA pH 8.0 buffer. Extra 80 μL water was added to the mixture and centrifuged at 10000 rpm for 10 min. Each 100 μL mixture was removed into the sampling bottles for HPLC auto-injection. 20 μL of each aliquot was injected directly in the HPLC equipped with a UV detector (detection at 254 nm, column temperature 25 ℃). 10 mM TEAA buffer (solvent A) and MeCN (solvent B) were used as the mobile phase with a flow rate of 1 mL/min.

4.8 Molecular dynamic simulation

We carried out an investigation on the equilibrium conformations of hSTING in the presence and absence of ligands by molecular dynamics simulations using Amber 14 molecular modelling package. [32, 33] The X-ray structure of hSTING was retrieved from the Protein Data Bank (PDB code: 4F5D). Ala230 in the crystal structure of hSTING cytoplasmic domain was mutated back to the wild type residues Gly230. Amber ff14SB force field [34] was used to parameterize amino acids residues of hSTING. The force field parameters of CDNs come from previous work [35]. Each system was immersed in a truncated octahedral box of explicit TIP3P water molecules. Particle Mesh Ewald (PME) [36] and periodic boundary condition was employed to deal with the long-range electrostatic interactions. SHAKE method [37] was used to constrain bonds involving hydrogen atoms in order to tolerate a time step of 2 fs. The system was gradually heated from 0 to 300 K over 50 ps. The equilibrating calculation was executed at 1 atm and at 300 K for 50 ps. Then, 15 ns MD simulations were performed under 300 K and 1 atm. The snapshot of the system was taken every 1 ps.

4.9 Synthesis of inosine mixed CDNs

4.9.1 General procedure of the synthesis of 2a and 2b

In a 250 mL three neck flask, compound **1** (30 g, 52.58 mmol) and AgNO3 (10.72 g, 63.1 mmol) was in 40 mL dry pyridine and 40 mL dry THF under Ar atmosphere. TBDMSCl was dissolved in 20 mL dry THF and added into the mixture dropwise under ice-water bath. 30 min later, the reaction was removed under room temperature overnight. Filtered with diatomite, solvent was removed under reduced pressure. Compound **2a** was achieved with crystalized with dichloromethane (15 g, yield 42%). Compound **2b** was purified with silica gel column chromatography (dichloromethane: methanol = 200:1 ~ 20:1) (10 g, yield 28%).

2a: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.45 (s, 1H), 8.24 (s, 1H), 8.01 (s, 1H), 7.40 (d, J = 7.6 Hz, 2H), 7.35 – 7.14 (m, 7H), 6.86 (d, J = 8.3 Hz, 4H), 5.95 (d, J = 4.9 Hz, 1H), 5.21 (d, J = 5.9 Hz, 1H), 4.70 (t, J = 4.9 Hz, 1H), 4.20 (q, J = 5.2 Hz, 1H), 4.13 (t, J = 4.8 Hz, 1H), 3.73 (s, 6H), 3.36 – 3.14 (m, 2H), 0.76 (s, 9H), -0.04 (s, 3H), -0.13 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 158.11, 156.60, 148.16, 145.82, 144.92, 138.73, 135.51, 135.41, 129.78, 127.84, 127.69, 126.73, 124.64, 113.17, 87.91, 85.60, 83.66, 75.54, 70.23, 63.54, 55.04, 25.57, 17.88, -4.81, -5.29. HRMS：calculated: 684.2979, found: $[M+Na]^+$ 707.2874.

2b: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (s, 1H), 8.29 (s, 1H), 8.01 (d, J = 1.9 Hz, 1H), 7.37 (d, J = 7.2 Hz, 2H), 7.31 – 7.19 (m, 7H), 6.84 (dd, J = 8.9, 3.4 Hz, 4H), 5.91 (d, J = 5.2 Hz, 1H), 5.50 (d, J = 6.1 Hz, 1H), 4.71 (q, J = 5.4 Hz, 1H), 4.41 (t, J = 4.6 Hz, 1H), 4.03 (q, J = 4.5 Hz, 1H), 3.72 (s, 6H), 3.34 (dd, J = 10.6, 4.3 Hz, 1H), 3.15 (dd, J = 10.6, 5.0 Hz, 1H), 0.82 (s, 9H), 0.07 (s, 3H),

0.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.12, 156.64, 148.20, 145.68, 144.79, 139.34, 135.50, 135.46, 129.74, 129.69, 127.80, 127.69, 126.72, 124.78, 113.13, 87.95, 85.71, 83.63, 72.65, 72.19, 63.17, 54.95, 25.78, 18.02, -4.45, -5.09. HRMS: calculated: 684.2979, found：[M+Na]$^+$ 707.2875.

4.9.2 General procedure of the synthesis of 4a and 4b

In a 250 mL three-necked flask, 1*H*-1,2,4-triazole (4.04 g, 58.4 mmol) and Et_3N (5.95 g, 58.4 mmol) was dissolved in 50 mL dichloromethane under Ar atmosphere. 2-Chlorophenylphosphoryl Dichloride (5.73 g, 23.4 mmol), dissolved in 10 mL dichloromethane, was added into the mixture slowly under 0 ℃ in 30 min. The reaction was stirred for another 1 h. A solution of **2a** or **2b** (10 g, 14.6 mmol) in 20 mL dichloromethane was added dropwise into the mixture in 30 min under -5 ℃ and then the temperature was increased to 0 ℃. Two hours later, 40 mL 1 M TEAB was added into the flask and stirred for another half of an hour. The organic phase was separated and washed with 1 M TEAB buffer and water. After the drying over anhydrous sodium sulfate, filtering and the further concentration. **3a** or **3b** was obtained as the white solid without further purification.

A mixture of **3a** or **3b** (8 g, 8.19 mmol), 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT, 8.52 g, 28.67 mmol) and 3-hydroxypropionitrile (1.16 g, 16.38 mmol) in 30 mL anhydrous pyridine were mixed and stirred under room temperature in a 100 mL flask. Two hours later, TLC showed that reaction was finished, 5 mL water was added into the flask to stop the reaction. The solvent was removed under the reduced pressure and dissolved in 50 mL dichloromethane again, followed by the addition of 5% oxalic acid aqueous solution until pH 3 ~ 4. The organic phase was separated and the water phase was extracted with dichloromethane. The organic phase was combined and washed with water twice (2 × 50 mL). After the drying over anhydrous sodium sulfate and filtering, the solvent was removed under the reduced pressure and the residue was dissolved in 50 mL dichloromethane for stirring under the ice-water bath. A 50 mL solution of 6% dichloroaceticacid in dichloromethane was added into the mixture and continuously stirred for 5 min. Several drops of methanol were added. Saturated solution of sodium bicarbonate was added to regulate the pH to the neutral. Organic phase was separated and the water phase was extracted with dichloromethane. The organic phase was combined and washed with sodium bicarbonate saturated solution and water. After the drying over anhydrous sodium sulfate, filtering and concentration, the residual compound was purified by the chromatography on silica gel (dichloromethane: methanol = 100:1 ~ 100:30) to give compounds **4a-4b** as white solid.

4a (4.5 g, yield 87.8%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.57 – 12.44 (m, 1H), 8.41 (d, J = 9.5 Hz, 1H), 8.15 (t, J = 3.4 Hz, 1H), 7.60 (t, J = 6.7 Hz, 1H), 7.52 (dd, J = 17.2, 8.2 Hz, 1H), 7.41 (td, J = 7.8, 1.6 Hz, 1H), 7.29 (t, J = 7.7 Hz, 1H), 6.00 (dd, J = 13.6, 7.2 Hz, 1H), 5.45 (s, 1H), 5.14 – 5.03 (m, 1H), 4.97 (dddd, J = 9.7, 7.2, 4.7, 2.0 Hz, 1H), 4.50 – 4.16 (m, 3H), 3.82 – 3.54 (m, 2H), 3.01 (q, J = 5.9, 4.4 Hz, 2H), 0.65 (d, J = 14.2 Hz, 9H), -0.13 (d, J = 14.6 Hz, 3H), -0.27 (d, J = 11.3 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 156.91, 156.90, 148.70, 148.65, 146.75, 146.32, 146.26, 146.20, 146.13, 139.11, 131.20, 131.18, 129.18, 129.08, 127.38, 127.31, 125.07, 125.03, 124.96, 124.93, 124.89, 124.86, 122.13, 122.11, 122.07, 122.05, 118.46, 118.36, 86.98, 86.95, 84.84, 84.81, 84.77, 79.51, 79.45, 79.24, 79.18, 74.82, 74.76, 64.38, 64.33, 64.19, 64.13, 61.36, 61.31, 55.36, 25.71, 25.66, 19.61, 19.53, 18.04, 17.97, -4.87, -4.92, -5.36, -5.38.^{31}P NMR (162 MHz, DMSO-d_6) δ -7.66, -7.79. HRMS: calculated: 625.1525, found: [M+Na]$^+$ 648.1418.

4b (3.8g, yield 74.1%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.42 (t, J = 5.2 Hz, 1H), 8.35 (d, J = 13.9 Hz, 1H), 8.05 (dd, J = 6.9, 3.1 Hz, 1H), 7.48 (ddt, J = 25.1, 7.8, 1.2 Hz, 1H), 7.32 – 7.13 (m,

3H), 6.18 (dd, *J* = 11.4, 6.0 Hz, 1H), 5.55 (ddd, *J* = 8.5, 5.9, 4.6 Hz, 1H), 5.32 (td, *J* = 5.3, 2.1 Hz, 1H), 4.63 (td, *J* = 4.3, 2.7 Hz, 1H), 4.23 (ddt, *J* = 13.1, 7.3, 4.8 Hz, 2H), 4.05 (q, *J* = 3.6 Hz, 1H), 3.72 (ddt, *J* = 12.4, 8.4, 4.6 Hz, 1H), 3.59 (dp, *J* = 8.1, 5.0, 4.2 Hz, 1H), 2.93 – 2.81 (m, 2H), 0.89 (d, *J* = 12.9 Hz, 9H), 0.17 – 0.05 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.05, 148.03, 146.18, 146.11, 145.62, 145.55, 145.40, 145.34, 138.80, 138.75, 130.64, 130.55, 128.46, 128.37, 126.75, 126.70, 124.54, 124.18, 124.11, 120.99, 120.95, 120.93, 117.71, 86.31, 86.18, 85.21, 85.16, 84.99, 78.67, 78.62, 71.24, 71.10, 71.05, 64.04, 63.98, 63.85, 63.79, 60.47, 60.40, 25.66, 25.63, 19.06, 19.02, 18.97, 18.94, 17.82, 17.79, -4.88, -4.95, -5.00, -5.07. ^{31}P NMR (162 MHz, DMSO-d_6) δ -7.93, -7.98. HRMS: calculated: 625.1525, found: $[M+Na]^+$ 648.1421.

4.9.3 General procedure of the synthesis of 7a-7e, 10a-10e, 13a-13e and 16a-16d.

A mixture of **3a-3j** (2.22 mmol), **4a-4b** (1.85 mmol.), and MSNT (3.70 mmol.) in 20 mL anhydrous pyridine was stirred under room temperature in a 100 mL flask. Several drops of water was added into the flask to stop the reaction when the TLC indicated the reaction was completed. Afterwards, the solvent was removed under the reduced pressure and resolved with 20 mL dichloromethane again. Oxalic acid solution was added into the mixture. The organic phase was separated and the water phase was extracted with dichloromethane. The organic phase was combined and washed with water twice (2 × 50 mL). After the drying over anhydrous sodium sulfate and filtering, the solvent was removed under the reduced pressure and the residue was redissolved with 15 mL dichloromethane and stirred under ice-water bath. A solution of 6% dichloroaceticacid in dichloromethane (15 mL) was added into the mixture and continuously stirred for 5 min. Several drops of methanol were added. The saturated solution of sodium bicarbonate was added to regulate the pH to the neutral. Organic phase was separated and the water phase was extracted with dichloromethane. The organic phase was combined and washed with sodium bicarbonate saturated solution and water. After the drying over anhydrous sodium sulfate, filtering and concentration, the residual compound was purified by chromatography on silica gel (dichloromethane: methanol = 100:1 ~ 100:30) to give compounds **5**，**8**，**11** and **14** as the white solid (75 ~ 85% yields within two steps) .

In a 100 mL flask, **5** (or **8**，**10**，**11**) (0.6 mmol) was dissolved in 8 mL acetonitrile and 8 mL *tert*-butylamine and then stirred under room temperature for 20 min. Solvent was evaporated under reduced pressure. Acetonitrile was added to the residual compounds again and evaporated. 100 mL anhydrous pyridine was added after the residual was fully dried. MSNT (3.0 mmol) was added in batches. The mixture was stirred under room temperature for 6 h. The reaction was stopped with the addition of several drops of water. The solvent was removed under the reduced pressure and the residual was dissolved in 30 mL dichloromethane, which was then treated with acidic water, dried over anhydrous sodium sulfate, filtered and concentrated. Further purification with chromatography on silica gel (dichloromethane: methanol = 80:1 ~ 20:1) give the fully protected cyclic dinucleotides **6**, **9**, **12 or 15** as the white or yellow solid. (50 ~ 60% yields within two steps).

Fully protected cyclic dinucleotide **6**, **9**, **12** or **15** (200 mg) was dissolved in 5 mL 0.5 M PBO-TMG buffer in solution of 1,4-dioxane/water (1:1, v/v) and then pyridine-2-carboxaldoxime and *N*1,*N*1,*N*3,*N*3-tetramethylguanidine was added. The mixture was stirred under room temperature for 24 h. Afterwards, the solvent was removed under the reduced pressure. 5 mL 33 wt% $MeNH_2$ in EtOH was added and the system was stirred at room temperature for 90 min and then the solvent was removed. (If the two bases are UI and II combination, this step was absent). 2 mL Et_3N, 1 mL Et_3N•3HF and 400 μL anhydrous pyridine were added and the mixture was stirred at 55 ℃ for 4 h.

Then the solvent was evaporated and then recrystallized in acetone. The final deprotected cyclic dinucleotides **7a-7e**, **10a-1e**, **13a-13e** and **16a-16d** as triethylammonium salts were purified with the preparative reverse phase HPLC, A = water with 50 mM TEAA, B = MeCN; gradient: 0 - 2 min: 98% A/2% B; 2 - 32 min: 98% A to 70% A/2% B to 30% B; 32 - 36 min: 70% A to 0% A/30% B to 100% B; 36 - 48 min: 100% B.

7a c[I(3',5′)I(3',5′)] (total yield 6.4%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.18 (s, 2H), 7.92 (s, 2H), 6.02 (s, 2H), 5.17 (td, *J* = 8.2, 4.4 Hz, 2H), 4.90 (s, 2H), 4.49 (d, *J* = 8.9 Hz, 2H), 4.45 (d, *J* = 12.6 Hz, 2H), 4.12 (dd, *J* = 11.9, 4.5 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 156.69, 146.57, 145.52, 138.79, 123.58, 90.83, 80.56, 80.45, 80.34, 73.72, 70.09, 70.05, 62.29, 62.24. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.52. HRMS: calculated: 660.0731, found: $[M-H]^-$ 659.0656.

7b c[G(3',5′)I(3',5′)] (total yield 6.6%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.16 (s, 1H), 7.89 (s, 1H), 7.87 (s, 1H), 5.98 (s, 1H), 5.78 (s, 1H), 5.06 (qd, *J* = 7.6, 4.4 Hz, 2H), 4.84 (d, *J* = 4.7 Hz, 2H), 4.47 – 4.42 (m, 1H), 4.41 – 4.32 (m, 3H), 4.08 - 4.02 (m, 2H). ^{13}C NMR (101 MHz, D_2O--d_2) δ 156.91, 153.41, 149.67, 149.25, 146.61, 145.35, 138.80, 136.19, 128.07, 123.75, 90.74, 90.31, 80.33, 80.18, 80.07, 73.63, 73.17, 70.14, 70.09, 70.04, 62.23. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.56, -1.60. HRMS: calculated: 675.0840, found: $[M-H]^-$ 674.0765.

7c c[A(3',5′)I(3',5′)] (total yield 13.5%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.19 (s, 1H), 8.08 (s, 1H), 7.85 (s, 1H), 7.83 (s, 1H), 5.93 (s, 2H), 5.06 – 4.99 (m, 2H), 4.91 (d, *J* = 4.5 Hz, 1H), 4.845 – 4.84 (m, 1H), 4.59 – 4.40 (m, 4H), 4.10 – 4.04 (m, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 156.59, 153.82, 151.67, 146.37, 146.33, 145.06, 139.17, 138.57, 123.24, 117.84, 91.26, 91.09, 81.02, 80.91, 80.79, 80.63, 73.46, 72.93, 70.26, 70.21, 70.05, 62.40, 62.19. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.75, -1.86. HRMS: calculated: 659.0891, found: $[M-H]^-$ 658.0815.

7d c[I(3',5′)U(3',5′)] (total yield 10.7%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.38 (s, 1H), 8.15 (s, 1H), 7.98 (d, *J* = 8.2 Hz, 1H), 6.18 (s, 1H), 5.78 (s, 1H), 5.68 (d, *J* = 8.1 Hz, 1H), 4.97 – 4.92 (m, 1H), 4.77 (s, 1H), 4.68 (td, *J* = 8.9, 4.7 Hz, 1H), 4.51 (d, *J* = 4.9 Hz, 1H), 4.48 (d, *J* = 8.9 Hz, 1H), 4.43 (s, 1H), 4.40- 4.37 (m, 2H), 4.12 – 4.06 (m, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 166.01, 158.12, 151.14, 147.66, 145.87, 141.25, 139.17, 123.87, 101.25, 91.11, 89.94, 79.93, 79.82, 79.71, 79.56, 79.45, 73.83, 73.63, 70.16, 70.11, 69.70, 69.66, 62.26, 62.21, 62.12, 62.07. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.47, -1.66. HRMS: calculated: 636.0618, found: $[M-H]^-$ 635.0544.

7e c[I(3',5′)C(3',5′)] (total yield 11.0%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.30 (s, 1H), 8.02 (s, 2H), 6.13 (s, 1H), 5.74 (s, 2H), 4.95 (s, 1H), 4.75 (s, 1H), 4.67 (s, 1H), 4.49 (d, *J* = 8.4 Hz, 2H), 4.45 – 4.35 (m, 2H), 4.08 (t, *J* = 12.7 Hz, 2H). ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.26, -1.76. HRMS: calculated: 635.0778, found: $[M-H]^-$ 634.0703.

10a c[I(2',5′)I(2',5′)] (total yield 1.7%): ^{1}H NMR (400 MHz, D_2O) δ 8.02 (d, *J* = 3.7 Hz, 2H), 7.76 (d, *J* = 6.6 Hz, 2H), 5.87 (s, 2H), 5.14 (s, 1H), 5.04 (s, 1H), 4.76 (s, 3H), 4.45 – 4.35 (m, 3H), 4.33 (d, *J* = 13.7 Hz, 1H), 3.94 (td, *J* = 11.7, 5.0 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.44, 149.16, 146.08, 145.95, 145.77, 85.09, 85.01, 83.93, 83.83, 77.69, 77.63, 71.77, 65.37. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.07, -1.20. HRMS: calculated: 660.0731, found: $[M-H]^-$ 659.0655.

10b c[G(2',5′)I(2',5′)] (total yield 3.3%): ^{1}H NMR (400 MHz, D_2O) δ 8.16 (s, 1H), 8.10 (s, 1H), 7.75 (s, 1H), 6.02 (s, 1H), 5.82 (d, *J* = 8.5 Hz, 1H), 5.55 (d, *J* = 4.6 Hz, 1H), 5.09 (td, *J* = 8.7, 4.1 Hz, 1H), 4.62 (d, *J* = 4.1 Hz, 1H), 4.46 (d, *J* = 4.0 Hz, 1H), 4.40 – 4.28 (m, 4H), 4.03 – 3.93 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 158.85, 152.84, 151.75, 147.43, 140.60, 140.59, 138.60, 138.58, 118.44, 117.24, 89.65, 86.03, 83.10, 82.99, 79.89, 74.06, 71.16, 70.49, 70.40, 66.94, 62.01. HRMS: calculated: 675.0840, found: $[M-H]^-$ 674.0765.

10c c[A(2',5')I(2',5')] (total yield 3.3%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.35 (s, 1H), 8.11 – 8.02 (m, 2H), 7.97 (s, 1H), 6.13 (d, J = 8.3 Hz, 1H), 6.00 (d, J = 2.4 Hz, 1H), 5.14 (td, J = 8.7, 4.2 Hz, 1H), 4.93 – 4.82 (m, 1H), 4.78 (d, J = 3.9 Hz, 1H), 4.55 (dd, J = 8.9, 3.9 Hz, 1H), 4.42 – 4.32 (m, 2H), 4.30 – 4.14 (m, 2H), 4.05 (d, J = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.36, 154.89, 151.91, 149.36, 145.99, 140.10, 139.05, 118.50, 89.21, 84.33, 84.25, 84.17, 80.71, 77.70, 73.44, 72.21, 71.76, 66.30, 62.95, 46.61, 8.17. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.15, -1.62. HRMS: calculated: 659.0891, found: $[M-H]^-$ 658.0820.

10d c[I(2',5')U(2',5')] (total yield 1.6%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.26 (d, J = 31.8 Hz, 1H), 8.05 (d, J = 25.0 Hz, 1H), 7.73 (dd, J = 44.0, 8.1 Hz, 1H), 6.20 – 6.15 (m, 1H), 6.14 – 6.02 (m, 1H), 5.53 (dd, J = 8.0, 1.6 Hz, 1H), 4.94 (td, J = 7.6, 4.2 Hz, 1H), 4.59 (dd, J = 8.8, 4.2 Hz, 1H), 4.43 (d, J = 4.5 Hz, 1H), 4.40 – 4.34 (m, 1H), 4.30 – 4.19 (m, 2H), 4.16 (ddd, J = 12.1, 6.2, 1.8 Hz, 1H), 4.09 – 4.04 (m, 1H), 4.04 – 3.90 (m, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 165.64, 165.45, 158.49, 151.90, 149.28, 146.08, 145.99, 140.92, 140.84, 139.86, 103.29, 102.99, 89.24, 84.54, 84.20, 84.13, 84.01, 83.93, 80.53, 76.75, 76.70, 73.40, 72.02, 71.66, 71.44, 66.42, 46.63, 8.19. ^{31}P NMR (162 MHz, D_2O-d_2) δ -0.77, -1.04, -1.23, -1.42. HRMS: calculated: 636.0618, found: $[M-H]^-$ 635.0544.

10e c[I(2',5')C(2',5')] (total yield 1.4%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.22 (s, 1H), 7.87 (s, 1H), 7.58 (d, J = 7.5 Hz, 1H), 6.26 (d, J = 8.3 Hz, 1H), 6.12 (d, J = 8.2 Hz, 1H), 5.59 (d, J = 7.6 Hz, 1H), 5.03 (ddd, J = 8.1, 6.0, 4.2 Hz, 1H), 4.56 – 4.50 (m, 2H), 4.43 (dd, J = 10.5, 4.1 Hz, 1H), 4.38 (d, J = 3.2 Hz, 1H), 4.31 – 4.26 (m, 1H), 4.24 (d, J = 3.3 Hz, 1H), 4.18 – 4.08 (m, 2H), 4.06 (d, J = 5.0 Hz, 1H), 4.03 – 3.94 (m, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 165.13, 158.43, 149.83, 149.10, 146.02, 145.53, 140.92, 140.76, 138.68, 128.30, 122.62, 97.08, 89.10, 85.36, 84.91, 84.14, 84.04, 83.45, 83.36, 78.11, 78.05, 77.76, 77.71, 73.40, 71.69, 71.50, 65.58. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.01, -1.13, -1.25. HRMS: calculated: 635.0778, found: $[M-H]^-$ 634.0693.

13a c[I(2',5')I(3',5')] (total yield 2.1%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.51 (s, 1H), 8.21 (s, 2H), 8.08 (s, 1H), 6.27 (d, *J* = 8.2 Hz, 1H), 6.14 (d, *J* = 2.8 Hz, 1H), 5.29 (td, *J* = 8.7, 4.2 Hz, 1H), 4.99 (td, *J* = 6.8, 4.8 Hz, 1H), 4.90 (dd, *J* = 4.9, 2.8 Hz, 1H), 4.67 (d, *J* = 4.2 Hz, 1H), 4.50 (dt, *J* = 8.1, 2.4 Hz, 2H), 4.34 (ddt, *J* = 11.9, 8.3, 3.1 Hz, 2H), 4.17 (dddd, *J* = 16.5, 11.8, 4.4, 2.1 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 159.09, 158.98, 149.92, 148.84, 146.80, 146.70, 140.52, 139.95, 124.71, 124.36, 89.82, 85.32, 85.24, 85.16, 81.61, 81.51, 81.41, 78.40, 78.35, 74.09, 73.05, 73.00, 72.55, 72.51, 67.01, 66.96, 63.77, 63.72. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.12, -1.48. HRMS: calculated: 660.0731, found: $[M-H]^-$ 659.0654.

13b c[G(3',5')I(2',5')] (total yield 3.3%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.51 (s, 1H), 8.09 (s, 1H), 7.90 (s, 1H), 6.27 (d, *J* = 8.2 Hz, 1H), 5.94 (d, *J* = 3.3 Hz, 1H), 5.29 (td, *J* = 8.7, 4.1 Hz, 1H), 5.02 (q, *J* = 6.0 Hz, 1H), 4.88 (dd, *J* = 4.9, 3.5 Hz, 1H), 4.66 (d, *J* = 4.2 Hz, 1H), 4.48 (q, *J* = 2.7 Hz, 1H), 4.47 – 4.43 (m, 1H), 4.36 – 4.27 (m, 2H), 4.19 (ddd, *J* = 12.1, 4.2, 1.9 Hz, 1H), 4.12 (ddd, *J* = 11.9, 5.1, 2.9 Hz, 1H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.69, 158.35, 153.81, 151.09, 149.26, 145.99, 139.88, 137.17, 123.69, 116.30, 88.49, 84.60, 84.53, 84.45, 80.75, 77.54, 77.49, 72.99, 72.93, 72.85, 71.86, 71.83, 66.32, 66.27, 63.23, 63.18. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.02, -1.20. HRMS: calculated: 675.0840, found: $[M-H]^-$ 674.0764.

13c c[A(3',5')I(2',5')] (total yield 6.6%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.40 (s, 1H), 8.16 (s, 1H), 8.15 (s, 1H), 7.94 (s, 1H), 6.22 (d, *J* = 8.2 Hz, 1H), 6.10 (d, *J* = 2.0 Hz, 1H), 5.39 – 5.29 (m, 1H), 4.99 – 4.92 (m, 1H), 4.77 (s, 1H), 4.65 (d, *J* = 4.2 Hz, 1H), 4.50 – 4.43 (m, 2H), 4.37 (d, *J* = 12.1 Hz, 1H), 4.32 – 4.26 (m, 1H), 4.16 (s, 1H), 4.14 – 4.10 (m, 1H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.12, 152.37, 149.68, 149.03, 147.83, 145.67, 140.14, 138.70, 123.78, 118.54, 88.90, 84.89, 84.86,

84.85, 84.82, 84.28, 84.20, 80.42, 80.32, 80.22, 73.50, 71.71, 71.67, 71.61, 66.14, 62.83. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.32, -1.89. HRMS: calculated: 659.0891, found: $[M-H]^-$ 658.0818.

13d c[I(2',5′)U(3',5′)] (total yield 3.5%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.53 (s, 1H), 8.17 (s, 1H), 7.80 (d, *J* = 8.1 Hz, 1H), 6.27 (d, *J* = 8.2 Hz, 1H), 5.80 (d, *J* = 1.4 Hz, 1H), 5.51 (d, *J* = 8.1 Hz, 1H), 5.26 (td, *J* = 8.5, 4.1 Hz, 1H), 4.74 – 4.68 (m, 1H), 4.66 (d, *J* = 4.1 Hz, 1H), 4.58 (d, *J* = 4.9 Hz, 1H), 4.48 (d, *J* = 3.1 Hz, 1H), 4.43 – 4.38 (m, 1H), 4.38 – 4.29 (m, 2H), 4.23 – 4.17 (m, 1H), 4.15 – 4.09 (m, 1H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 165.80, 158.22, 151.14, 149.27, 146.07, 140.85, 139.85, 123.70, 101.16, 90.72, 84.60, 84.53, 84.49, 84.41, 80.15, 80.05, 79.95, 77.88, 77.83, 73.34, 71.69, 71.66, 71.10, 71.04, 66.21, 66.16, 62.60, 62.55. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.27, -2.01. HRMS: calculated: 636.0618, found: $[M-H]^-$ 635.0541.

13e c[I(2',5′)U(3',5′)] (total yield 3.7%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.30 (s, 1H), 8.19 (s, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 6.34 (d, *J* = 8.4 Hz, 1H), 6.16 (s, 1H), 5.87 (d, *J* = 7.6 Hz, 1H), 4.69 (s, 2H), 4.54 (d, *J* = 4.1 Hz, 1H), 4.48 (s, 1H), 4.36 (s, 2H), 4.32 – 4.23 (m, 1H), 4.11 (d, *J* = 12.1 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 164.05, 158.44, 155.72, 148.07, 146.06, 141.33, 139.18, 124.09, 96.97, 89.11, 84.40, 83.82, 83.74, 80.52, 77.10, 73.45, 72.12, 71.53, 66.54, 62.84. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.17, -1.41. HRMS: calculated: 635.0778, found: $[M-H]^-$ 634.0695.

16a c[G(2',5′)I(3',5′)] (total yield 0.7%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.26 (s, 1H), 8.19 (s, 1H), 7.92 (s, 1H), 6.16 (d, *J* = 1.1 Hz, 1H), 5.96 (d, *J* = 8.4 Hz, 1H), 5.64 (td, *J* = 8.4, 4.2 Hz, 1H), 5.05 (ddd, *J* = 8.7, 6.4, 4.3 Hz, 1H), 4.59 (d, *J* = 4.2 Hz, 1H), 4.48 (d, *J* = 8.8 Hz, 1H), 4.45 – 4.38 (m, 2H), 4.26 (ddd, *J* = 11.8, 6.0, 2.6 Hz, 1H), 4.18 (dt, *J* = 12.2, 2.6 Hz, 1H), 4.14 – 4.07 (m, 1H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.77, 158.29, 153.22, 151.92, 147.69, 146.01, 139.90, 138.76, 123.83, 122.37, 99.99, 89.57, 85.75, 83.62, 83.54, 80.23, 74.45, 74.00, 71.56, 71.51, 70.90, 70.84, 65.89, 65.83, 62.23. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.21, -1.88. HRMS: calculated: 675.0840, found: $[M-H]^-$ 674.0763.

16b c[A(2',5′)I(3',5′)] (total yield 11.5%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.46 (s, 1H), 8.14 (s, 1H), 8.14 (s, 1H), 8.07 (s, 1H), 6.24 (d, *J* = 8.3 Hz, 1H), 6.10 (d, *J* = 2.6 Hz, 1H), 5.24 (td, *J* = 8.5, 4.2 Hz, 1H), 4.99 (td, *J* = 6.9, 4.7 Hz, 1H), 4.90 (dd, *J* = 4.8, 2.7 Hz, 1H), 4.65 (d, *J* = 4.2 Hz, 1H), 4.49 (dt, *J* = 4.6, 2.2 Hz, 1H), 4.48 – 4.44 (m, 1H), 4.38 – 4.28 (m, 2H), 4.15 (tdd, *J* = 12.0, 4.2, 2.1 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 158.22, 155.03, 152.19, 149.27, 147.89, 145.92, 139.87, 138.99, 123.95, 118.36, 89.14, 84.28, 84.20, 84.07, 83.98, 80.78, 80.69, 80.59, 77.75, 77.70, 73.37, 72.21, 71.79, 66.31, 66.26, 62.98. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.10, -1.56. HRMS：calculated：659.0891，found：$[M-H]^-$ 658.0816.

16c c[I(3',5′)U(2',5′)] (total yield 1.7%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.35 (s, 1H), 8.22 (s, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 6.28 (d, *J* = 8.4 Hz, 1H), 6.16 (d, *J* = 2.0 Hz, 1H), 5.68 (d, *J* = 8.1 Hz, 1H), 4.85 (d, *J* = 6.1 Hz, 2H), 4.74 (td, *J* = 8.8, 4.3 Hz, 1H), 4.56 (d, *J* = 4.2 Hz, 1H), 4.50 (d, *J* = 6.7 Hz, 1H), 4.39 (h, *J* = 4.0 Hz, 2H), 4.33 – 4.26 (m, 1H), 4.16 (d, *J* = 3.5 Hz, 1H), 4.12 (s, 1H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 165.45, 158.48, 151.91, 148.04, 146.09, 140.85, 139.23, 124.18, 103.01, 89.22, 84.22, 84.15, 84.02, 83.95, 80.66, 80.57, 80.46, 76.75, 76.70, 73.40, 72.07, 72.01, 71.48, 71.44, 66.49, 66.43, 62.91, 62.86. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.18, -1.36. HRMS: calculated: 636.0618, found: $[M-H]^-$ 635.0543.

16d c[I(3',5′)C(2',5′)] (total yield 4.5%): ^{1}H NMR (400 MHz, D_2O-d_2) δ 8.30 (s, 1H), 8.19 (s, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 6.34 (d, *J* = 8.4 Hz, 1H), 6.16 (s, 1H), 5.87 (d, *J* = 7.6 Hz, 1H), 4.69 (s, 2H), 4.54 (d, *J* = 4.1 Hz, 1H), 4.48 (s, 1H), 4.36 (s, 2H), 4.32 – 4.23 (m, 1H), 4.11 (d, *J* = 12.1 Hz, 2H). ^{13}C NMR (101 MHz, D_2O-d_2) δ 164.05, 158.44, 155.72, 148.07, 146.06, 141.33, 139.18,

124.09, 96.97, 89.11, 84.40, 83.82, 83.74, 80.52, 77.10, 73.45, 72.12, 71.53, 66.54, 62.84. ^{31}P NMR (162 MHz, D_2O-d_2) δ -1.17, -1.41. HRMS: calculated: 635.0778, found: $[M-H]^-$ 634.0695.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: