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# Synthesis of original Cyclic Dinucleotide analogues using the sulfo-click reaction

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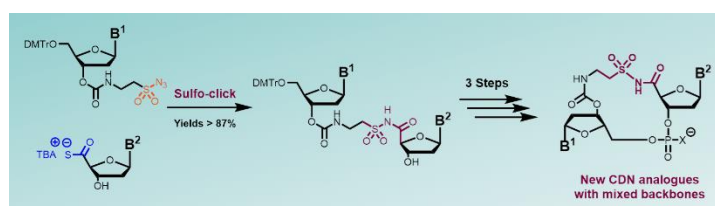
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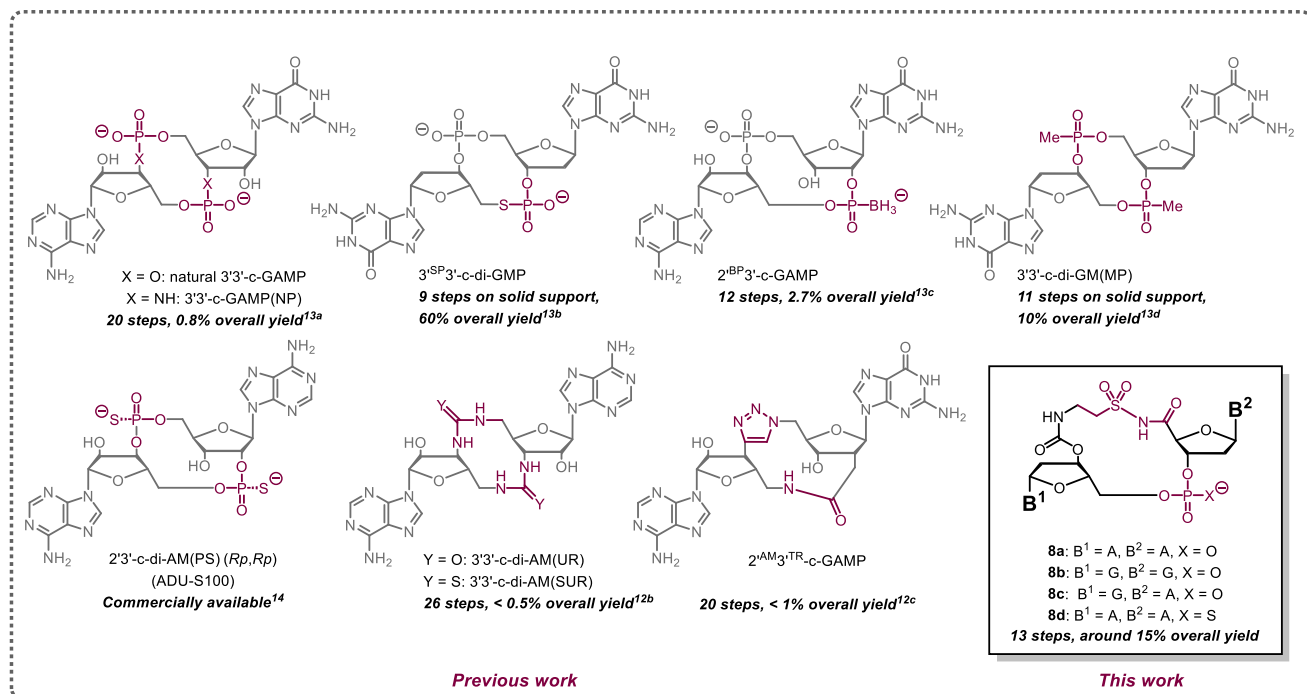
**ABSTRACT:** The Stimulator of interferon genes (STING) protein plays a crucial role in the activation of the innate immune response. Activation of STING is initiated by cyclic dinucleotides (CDNs) which prompted the community to synthesize structural analogues to enhance their biological properties. We present here the synthesis and biological evaluation of four novel CDN analogues composed of an *N*-acylsulfonamide linkage. These CDNs were obtained in high overall yields via the sulfo-click reaction as a key step.

Cyclic dinucleotides (CDNs) are crucial molecular cellular second messengers present in various organisms. In bacteria, they regulate diverse processes such as quorum sensing, signal transduction or control of biofilm formation.<sup>1</sup> While purine containing CDNs such as 3'5'-c-di-GMP, 3'5'-c-di-AMP, 2'3'-c-GAMP and 3'3'-c-GAMP (Figure 1), are the most studied, the recent discovery of c-di-UMP and c-UAMP in *E. coli* suggests that CDNs containing pyrimidine nucleobase are also biosynthesized. However, their specific functions remain to be elucidated.<sup>2</sup> The Stimulator of Interferon Genes (STING) protein in mammalian cells functions as an endoplasmic reticulum transmembrane receptor for CDNs. It has the ability to directly recognize the presence of bacteria through its interaction with bacterial 3'3'CDNs.<sup>3</sup> Upon activation by CDNs, the STING protein triggers the production of Type I interferons, thus activating the innate immune system response. The protective role of the STING protein is not limited to bacteria and can also be indirectly activated in response to the presence of exogenous genetic material. Indeed, during viral infection, the STING pathway is activated upon the detection of cytosolic double-stranded DNA by the cyclic GMP-AMP synthase (c-GAS) thus triggering the synthesis of endogenous 2'3'-c-GAMP.<sup>4</sup> Numerous studies have demonstrated that regulation of the STING pathway may have therapeutic applications

against various diseases. For example, CDNs act as agonists to stimulate the innate immune response against exogenous agents,<sup>5</sup> including the SARS-CoV-2 virus.<sup>6</sup> Furthermore, STING-activating CDNs may be beneficial in the treatment of cancers<sup>7</sup> or as vaccine adjuvants.<sup>8</sup>

Because of their substantial therapeutic potential, numerous research teams have contributed to the development of novel synthetic CDNs. These efforts aim to manipulate their properties, enhance their efficacy in activating the STING protein, bolster their resistance to nucleases, and facilitate their cellular internalization and bioavailability.<sup>9</sup> Consequently, many CDN analogues containing nucleobase,<sup>10</sup> ribose<sup>11</sup> or internucleoside linkage modifications have been described and evaluated.<sup>12</sup> Among these various possible structural modifications, the most crucial one is without contest the internucleoside linkage due to its negative charge which greatly influences solubility, cell permeation and the resistance to nucleases of CDNs. Consequently, several phosphorus derived modifications previously reported for therapeutic linear oligonucleotides have been considered over the years for the synthesis of CDN analogues.<sup>13</sup> Noteworthy, the PS analogue of c-di-AMP (2'3'-c-di-AM(PS) (*Rp,Rp*), ADU-S100)<sup>14</sup> and the PS fluorinated analogue of 2'3'-c-GAMP (MK-1454)<sup>15</sup> are currently in clinical trials for the treatment of various cancers.

**Figure 1.** Selected chemical structures of CDNs with modified internucleoside linkages.



Moreover, CDN derivatives comprising non-phosphate backbones have also been described. These include modifications like (thio)urea ((S)UR),<sup>12b</sup> as well as amide (AM) and triazole (TR)<sup>12c</sup> internucleoside linkages (Figure 1). While neutral backbones greatly enhance the ability of CDNs to penetrate cell membranes and increase their resistance to nucleases, they also decrease their aqueous solubility. Furthermore, despite promising advancements, the synthesis of CDNs is still limited by intricate synthetic pathways and low overall yields with the exception of solid supported CDN synthesis, which has its own limitations in terms of scalability (3<sup>SP</sup>3'-c-di-GMP and 3'3'-c-di-GM(MP), Figure 1). Thus, despite the large number of modified CDNs recently disclosed, efficient access to new CDN analogues carrying unprecedented linkages is still required.

The sulfo-click reaction between a sulfonyl azide and a thioacid allows the synthesis of *N*-acylsulfonamide derivatives with high efficiency.<sup>16</sup> We recently exploited this reaction for the preparation of 5'-bioconjugated nucleosides<sup>17</sup> and for the synthesis of potential drugs acting as enzyme inhibitors.<sup>18</sup> Interestingly, the *N*-acylsulfonamide moiety has recently appeared in an increasing number of pharmaceutical drugs for various therapeutic applications, mostly due to its ideal physico-chemical properties.<sup>19</sup> In addition, the *N*-acylsulfonamide group has also been used as a substitute for carboxylic acids or phosphates.<sup>20</sup> Considering the importance of modified CDNs and the high performance of the sulfo-click reaction to generate *N*-acylsulfonamide linkages in the context of nucleic acid chemistry, we envisioned a straightforward access to a new class of CDN analogues. We describe here the synthesis and biological evaluation of four original purine based 2'-deoxy-3'3'-CDNs exhibiting an *N*-acylsulfonamide and either a PO or a PS internucleoside linkage required to maintain important aqueous solubility. Noteworthy, all these compounds were validated beforehand by predictive molecular simulations with the STING C-terminal domain to evaluate their binding potential. We took into account the potential synthetic constraints

in order to test only structures whose synthesis was possible with a reasonable number of steps. Based on preliminary results from 800 nanoseconds-long molecular dynamics (MD) simulations, ribose derivatives of the envisaged molecules were discarded since only the deoxyribose compounds showed a theoretical ability to be able to spontaneously interact near the entrance of the STING protein active site (Figure 2). Indeed, modified deoxyribose derivatives demonstrated a tendency to localize on the top or even in between the overhanging loop that constitutes the lead of the STING protein natural binding pocket, while the ribose analogues experienced higher fluctuations and interacted only transiently on the sides of the protein. Deoxyribose compounds were then subjected to Steered MD to pull them into the active site and generate starting protein-ligand complexes featuring the CDN in the protein binding pocket. These structures were further equilibrated along an additional 1200 ns unbiased MD to probe the stability of the cavity-bound complexes. The binding energy of each compound within the binding site was estimated using the MM/PBSA approach (Table 1 and SI). The former is an approximate strategy, which provides reasonable binding free energy trends for similar series of compounds with only a moderate computational overload, and is commonly used for ligand interaction studies.<sup>21</sup> In all cases, the formation of stable complexes (*i.e.* negative binding energy) has been previewed and the **8c** and **8d** derivatives clearly exhibit the most favorable binding free energies. Following these results from molecular simulations, we decided to engage in the synthesis and biological evaluation of 4 CDNs **8a-d**.

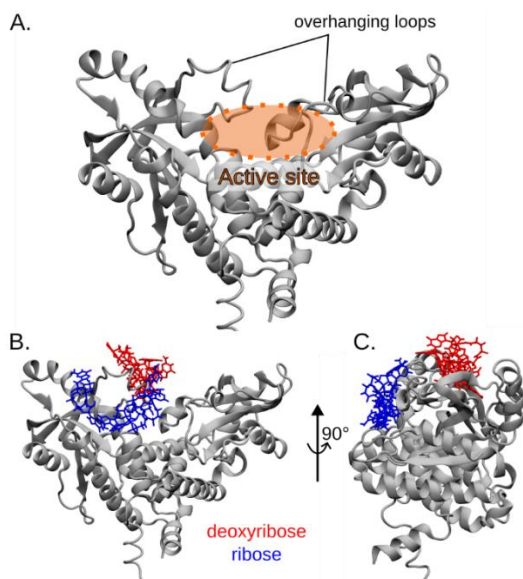
We decided to implement a convergent strategy from commercially available 5'-*O*-DMTr nucleosides (**1a,b**). Upon *in situ* activation of the 3'-OH with 4-nitrophenyl chloroformate, we successfully coupled the 2-aminoethanesulfonyl azide **2**<sup>17, 22</sup> leading to building blocks **3a,b** with moderate yields. Then, 4'-thioacid nucleosides synthons **4a,b** were synthesized as previously described (Scheme 1).<sup>17-18</sup> The assembly of nucleoside building

blocks **3a,b** and **4a,b** was performed using the sulfo-click reaction under aqueous conditions to yield nucleoside dimers **5a-c** (Scheme 2). The reaction was complete in 10 min and provided high isolated yields (87-96%) consistent with our previous work.<sup>17-18</sup> The free 3'-OH of dimers **5a-c** were then converted into the corresponding phosphoramidite derivatives **6a-c** by reaction with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite with good isolated yields (72-79%). Thereafter, the phosphoramidite function was hydrolyzed in MeCN by addition of an aqueous Pyr.TFA salt solution. Then, the cyanoethyl protecting group was removed by treatment with *t*-butylamine in one pot. After evaporation to dryness, deprotection of the 5'-*O*-DMTr group was then carried out by treatment with 3% dichloroacetic acid in DCM leading after RP chromatographic purification to the H-phosphonate derivatives **7a-c** (65-69% yields).

Our first attempt to cyclize the dimers **7a-c**, using 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide (DMOCP) failed to deliver the expected cyclic product. By contrast, pivaloyl chloride (Piv-Cl) allowed the rapid cyclization out of the dimers (~85% cyclisation).<sup>23</sup> Thereafter, oxidation of the H-phosphonate to the corresponding phosphodiester or phosphorothioate internucleoside linkages was performed using I<sub>2</sub>/H<sub>2</sub>O or the Beaucage reagent respectively. Finally, the nucleobases protective groups were removed using aq. 28% ammonia solution in the presence of MeOH yielding the desired CDNs with overall yields of around 15%.

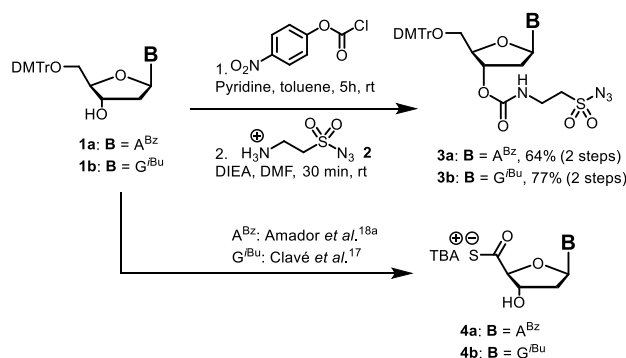
**Table 1. Predicted binding free energy for compound **8a-c** and cGAMP to the C-terminal domain binding pocket of STING.**

CDN	<b>8a</b>	<b>8b</b>	<b>8c</b>	<b>8d</b>	cGAMP
$\Delta G_{\text{bind}}$	-6.7	-8.9	-18.3	-15.6	-25.4
(kcal/mol)	$\pm 0.7$	$\pm 0.5$	$\pm 0.6$	$\pm 0.3$	$\pm 0.7$



**Figure 2.** A. C-terminal domain of the STING protein, highlighting the position of its active site (orange circle) and the overhanging loops. B. Front and C. side views of ribose derivatives (blue) and deoxyribose analogues (red) interaction with the C-terminal domain of STING (in grey), as predicted by unbiased MD simulations.

**Scheme 1. Synthesis of building blocks **3a,b** and **4a,b**.**



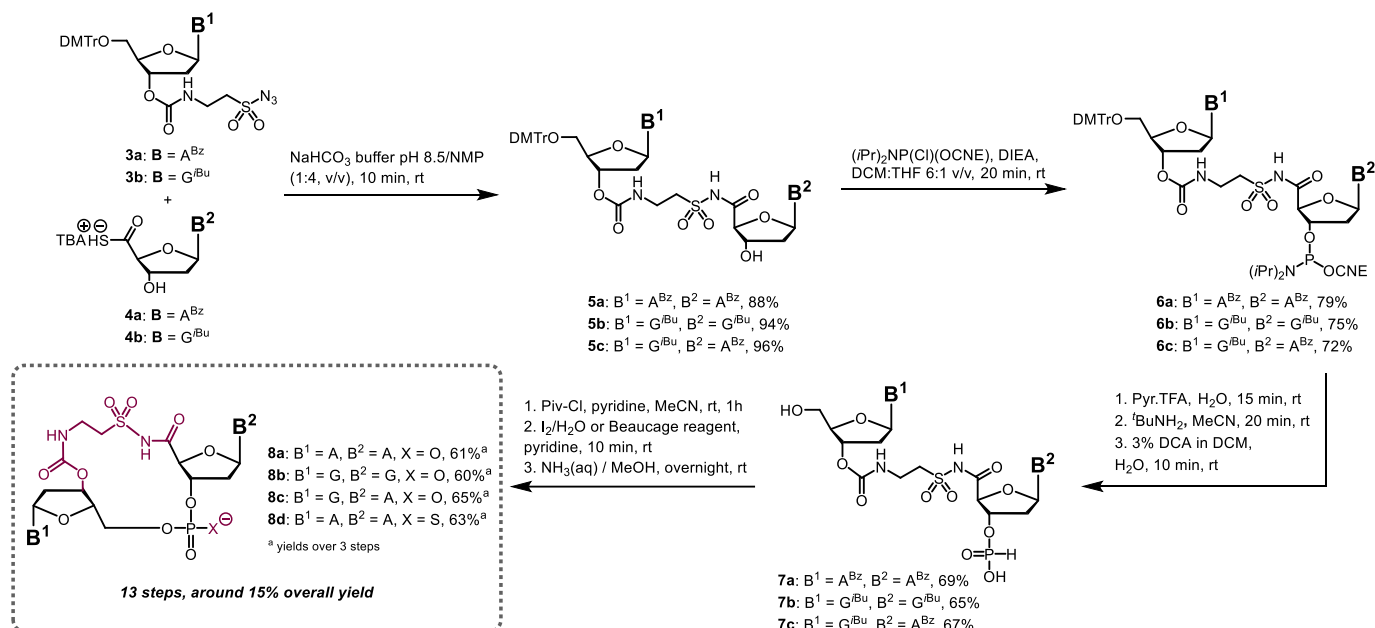
We then performed detailed NMR analysis on compound **8b** to determine its conformation. This study highlighted the presence of two conformers due to interactions between the protons of the adenine and the flexible *N*-acylsulfonamide linkage (see S45).

The final CDN products **8a-d** were then evaluated to determine their activity as STING agonists or antagonists by comparing them to the reference compounds 2'2'-c-GAMP, 2'3'-c-GAMP, 3'3'-c-diAMP, 3'3'-c-diIMP and ADU-S100 following established protocols.<sup>24</sup> First, the potential direct binding of compounds **8a-d** to STING was tested *in vitro* by DSF assays using four STING haplotypes (*i.e.* Wild type (WD), AQ, R232H and R293Q).<sup>24</sup> Unfortunately, these experiments have shown that compounds **8a-d** have no measurable affinity for the investigated STING haplotypes (see Table S1). Despite these results, STING reporter cell-based assays were also performed. Cellular membranes were permeabilized using a detergent (digitonin A) to ensure CDN penetration but as expected according to DSF results, no STING agonistic activity has been observed (see S55).

Considering that the energetic gain calculated for CDN **8c** (*i.e.* the new compound showing the most favorable binding with -18.3 kcal/mol) is much lower than the stabilization achieved by natural cGAMP (-25.4 kcal/mol), it seems that this difference is significant enough to prevent interactions with the STING protein. MD simulations suggest that the *N*-acylsulfonamide internucleoside linkages induce a significant steric hindrance, which prevents an optimal fitting within the STING binding pocket. Indeed, intramolecular interactions of the *N*-acylsulfonamide linkage occur only transiently, preventing the adoption of a specific rigid structure. Moreover, we cannot rule out that the 16-membered macrocycle prevents CDNs **8a-d** from adopting a structure similar to that of natural CDNs (see supporting information S49 for details). Therefore, new MD simulations will be engaged to design *N*-acylsulfonamide-based analogues with less steric conflicts.

In summary, following our previous work on the synthesis of 4'-thioacid nucleosides, we describe herein the synthesis of 4 new CDN analogues comprising an *N*-acylsulfonamide internucleoside linkage. The use of the sulfo-click reaction as key step efficiently led to the formation of linear dimers that were cyclized to generate new CDNs with very good overall yields of around 15% over 13 steps. This synthetic route compares favorably with the best yield ever obtained for the synthesis in solution of CDNs comprising a non-phosphorous based internucleoside linkage. Four CDN analogues have been evaluated as potential agonists or antagonists of natural CDNs on four STING haplotypes. Despite the lack of activity and given the many biological functions of CDNs, the chemistry developed in this work discloses new opportunities for the synthesis of potentially bioactive compounds and expands the application of the sulfo-click reaction in the context of nucleic acid chemistry.

## Scheme 2. Assembly of building blocks 3a,b and 4a,b using the sulfo-click reaction towards CDNs 8a-d.



## ASSOCIATED CONTENT

### Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures, copies of the NMR spectra of all new products, detailed conformational study of CDN **8b**, computational methods and biological evaluation (PDF). All input files for the MD simulations are available on Github: <https://github.com/emmanuellebignon/STING-CDN>

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