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MINI REVIEW

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Biotechnological production of cyclic dinucleotides—Challenges and opportunities

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Abstract

Cyclic dinucleotides (CDNs) are widely used secondary signaling molecules in prokaryotic and eukaryotic cells. As strong agonists of the stimulator of interferon genes, they are of great interest for pharmaceutical applications. In particular, cyclic-GMP-AMP and related synthetic CDNs are promising candidates in preclinical work and even some in clinical phase 1 and 2 studies. The comparison of chemical and biocatalytic synthesis routes elucidated that biological CDN synthesis offers some advantages, such as shorter synthesis time, avoiding complex protective group chemistry, and the access to a new spectrum of CDNs. However, the synthesis of CDNs in preparative quantities is still a challenge, since the chemical synthesis of CDNs suffers from low yields and complex synthetic routes and the enzymatically catalyzed synthesis is limited by low product titers and process stability. We aim to review the latest discoveries and recent trends in chemical and biocatalytic synthesis of CDNs with a focus on the synthesis of a huge variety of CDN derivatives. We furthermore consider the most promising biotechnological processes for CDN production by evaluating key figures of the currently known processes.

KEYWORDS
c-di-GMP, cGAMP, cGAS, cyclic dinucleotide (CDN), DncV, nucleotidyltransferase

1 | INTRODUCTION

Cyclic dinucleotides (CDNs) are heterocyclic compounds consisting of two ribonucleoside monophosphates that are linked via canonical (3′–5′) or noncanonical (2′–5′) phosphodiester bonds (Figure 1). In recent years, their function as ubiquitous second messengers in eukaryotes as well as in prokaryotes has been elucidated and a vast spectrum of cellular processes that are under the control of CDNs has been revealed (Dias da Purificação et al., 2020). In prokaryotes, three prevalent CDNs are known, which are cyclic-di-GMP (c-di-GMP, Figure 1a), cyclic-di-AMP (c-di-AMP, Figure 1b), and 3′3′-cyclic-GMP-AMP (3′3′-cGAMP, Figure 1c). In higher eukaryotes, 2′3′-cyclic-GMP-AMP (2′3′-cGAMP, Figure 1d) containing mixed phosphodiester bonds with a rare noncanonical (2′–5′) phosphodiester bond serves as the first line of cell defense against pathogens and is, therefore, part of the innate immune system (L. Sun et al., 2013). Further classes of CDNs were recently identified in bacteria, such as cyclic-UMP-AMP, cyclic-di-UMP, as well as cyclic-CMP-UMP, and most recently the cyclic trinucleotide cyclic-AMP-AMP-GMP (Whiteley et al., 2019).

CDN signaling is tightly regulated by phosphodiesterases and nucleotidyltransferases (Zaver & Woodward, 2020). While

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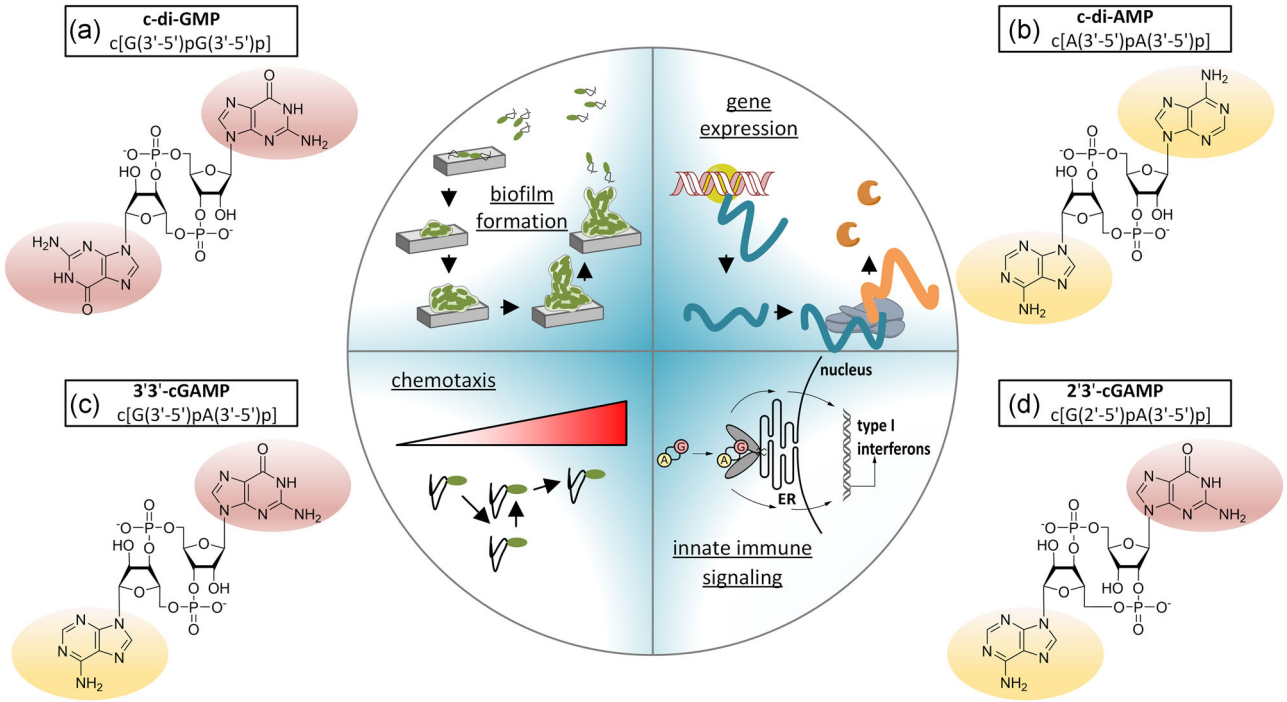

FIGURE 1 Chemical structure of the four prevalent natural cyclic dinucleotides; (a) cyclic-di-GMP, (b) cyclic-di-AMP, (c) 3′3′-cyclic-GMP-AMP, (d) 2′3′-cyclic-GMP-AMP. The adenine base is shaded yellow and the guanine base is shaded red. Examples for their role in prokaryotic and eukaryotic cells are displayed, including biofilm formation, gene expression, regulation of chemotaxis, and the innate immune signaling and antiviral response in eukaryotes

phosphodiesterases catalyze the hydrolysis of phosphodiester bonds and hence the degradation of CDNs, nucleotidyltransferases catalyze the synthesis of CDNs by cyclization of two ribonucleoside triphosphates. The dinucleotide cyclase in *Vibrio cholerae* (DncV) and its metazoan homolog cyclic GMP-AMP synthase (cGAS) are the first described members of the cGAS/DncV-like nucleotidyltransferases (CD-NTases) family and form the basis for the biocatalytic synthesis of cGAMP. While the prokaryotic CDNs are mainly described as key second messengers whose underlying signaling networks control for example biofilm formation, gene expression, DNA repair, cell wall synthesis, and chemotaxis, the metazoan analog 2′3′-cGAMP functions as an endogenous second messenger activating the innate immune system of higher eukaryotes (Wang & Xi, 2021). Cytosolic DNA, which can originate from infection with foreign DNA, damaged mitochondria or nuclei, activates cGAS that catalyzes the synthesis of 2′3′-cGAMP. 2′3′-cGAMP has a strong affinity to the stimulator of interferon genes (STING) receptor that is located at the endoplasmic reticulum and initiates two cascades that trigger the transcription of type I interferon antiviral genes to promote an antiviral state (Sun et al., 2013). Therefore, 2′3′-cGAMP is a potent candidate for pharmaceutical applications and has been extensively studied in recent years. Thus, the development of vaccines utilizing STING-activating CDN adjuvants started years ago. The other prevalent CDNs also show an affinity to the STING receptor, even if it is significantly lower (Li et al., 2014). The interest for symmetric homodimeric CDNs, such as c-di-GMP as a pharmaceutical target, has hence waned after the discovery of cGAMP. In different studies, 2'3'-cGAMP has demonstrated significant antitumor activity and may therefore be used in the immunotherapy of cancer (Corrales et al., 2015; Schadt et al., 2019).

However, a major drawback of CDNs is their rapid degradation or export from cells limiting their application as a vaccine adjuvant or cancer therapeutic. 2′3′-cGAMP shares this drawback and is degraded by the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) in cells, tissue extracts, and blood (Li et al., 2014). As phosphorothioate diester linkages are known to be nonhydrolyzable, α-S-ATP and α-S-GTP were used as building blocks for the enzymatic synthesis of nonhydrolyzable 2′3′-cGAMP analogs. The substitution of one or both phosphodiester bonds with phosphorothioate diesters yielded two analogs, 2′3′-cGA^SMP and 2′3′-cG^SA^SMP, which were stable for at least one day in the presence of ENPP1. Furthermore, 2′3′-cG^SA^SMP also has a high affinity to the STING receptor and showed a ~10-fold higher cell-based activity in cell cultures due to the increased biostability. Various other CDNs that function as STING agonists were already investigated in preclinical studies with promising results for the therapy of medium and advanced cancers (Jiang et al., 2020). The field of application for these CDNs included coadministration with other cancer immunotherapies, such as cancer vaccines, immune checkpoint inhibitors, and adoptive T cell transfer therapies.

There is great interest in CDNs, especially in cGAMP and cGAMP-related synthetic CDNs, because of their vast potential in

pharmaceutical applications. However, the synthesis in preparative quantities is still challenging. In this context, this review addresses the current chemical and biocatalytic synthesis routes with a major focus on cGAMP. Moreover, recently published biocatalytic processes and their key figures are compared. Finally, the remaining challenges and limitations are discussed.

2 | CURRENT CHEMICAL AND BIOCATALYTIC SYNTHESIS ROUTES

Various routes exist for the chemical synthesis of CDNs starting with phosphate triester coupling, amidite or H-phosphonate coupling (Gaffney et al., 2010; Wang & Xi, 2021). The phosphoramidite based one-pot synthesis route was developed in 2010 for the production of c-di-GMP (Gaffney et al., 2010) and is the commonly used synthesis route for the production of 2′3′-cGAMP. As shown in Figure 2a, the synthesis starts with the deprotection of the *N*-benzoyl-2′-*O*-TBS-protected adenosine@@@@ phosphoramidite **1**. After hydrolysis, removal of the cyanoethyl group and subsequent detritylation, the H-phosphonate **2** is obtained. To prevent the reverse reaction of detritylation, coupling with the guanosine phosphoramidite **3** follows immediately. The product is subsequently oxidized and detritylated forming a linear dimer **5**. The linear dimer is afterwards cyclized and oxidized to form the protected cyclic dinucleotide **6**. The CDN is then extracted from the reaction mixture and the protection group at the phosphodiester bond is removed. The final product **2′3′-cGAMP** is crystallized as triethylammonium salt in acetone within 10 min. It should be emphasized that this reaction route is comparably fast for such a complex synthesis and can be performed as one-pot

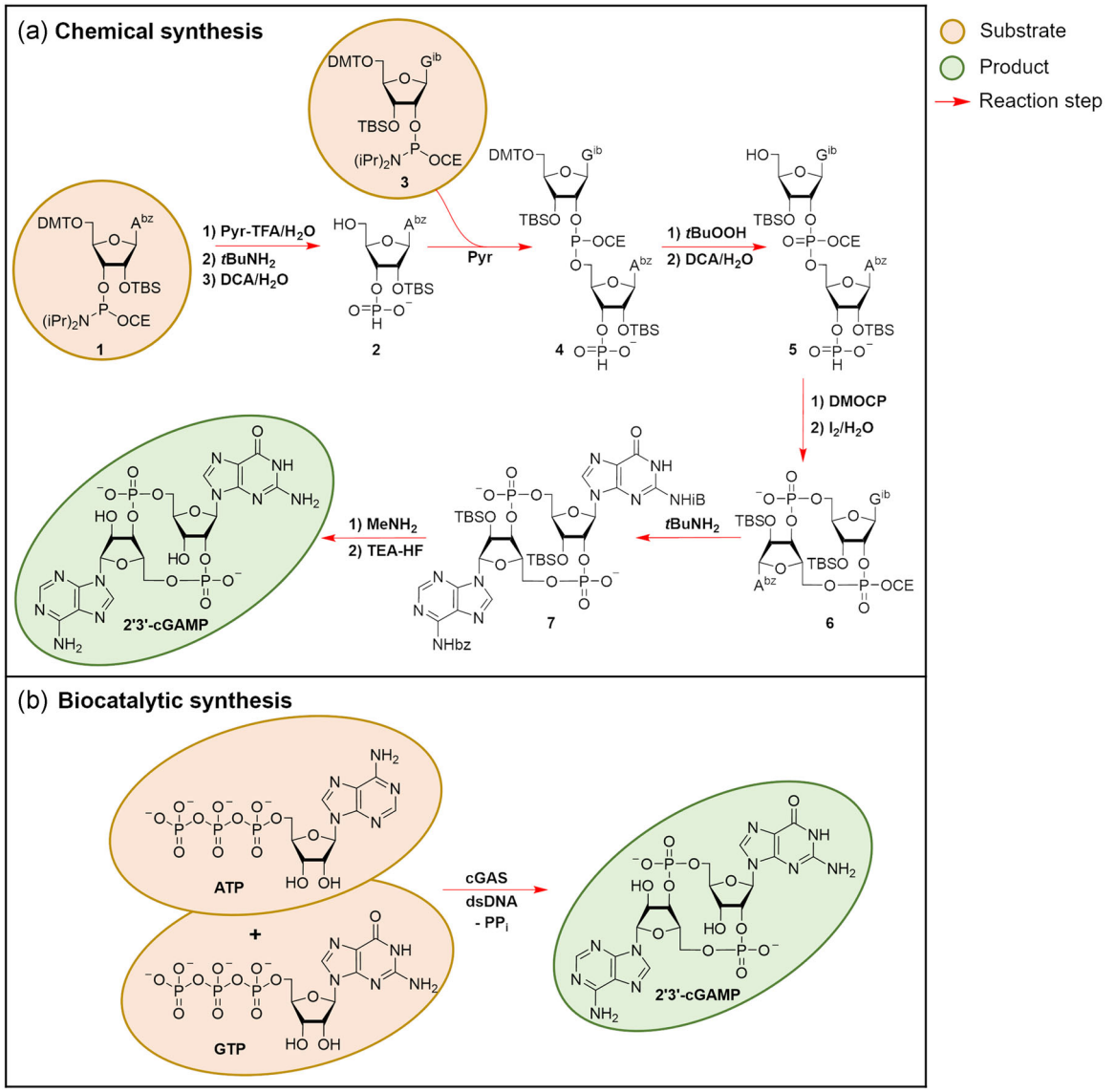

FIGURE 2 Comparison of chemical and biocatalyzed synthesis of 2′3′-cyclic GMP-AMP (2′3′-cGAMP). (a) Chemical synthesis of 2′3′-cGAMP starting from *N*-benzoyl-2′-*O*-TBS-protected adenosine phosphoramidite in eight steps. (b) Biocatalyzed one-step synthesis of 2′3′-cGAMP from ATP and GTP via cyclic GMP-AMP synthase (cGAS)

TABLE 1 Published data for chemical CDN synthesis

CDN	Synthesis route	Yield (%)	Absolute product amount (mg)	Reference
2'3'-cGAMP	Phosphoramidite	5	1.7	Zhang et al. (2013)
2'3'-cGAMP analog	Click chemistry	1	109	Dialer et al. (2019)
c-di-GMP	Phosphoramidite	30	1320	Gaffney et al. (2010)
c-di-GMP	Solid-phase	-	-	Kiburu et al. (2008)

Note: The following synthesis routes were considered: The synthesis of 2′3′-cGAMP or c-di-GMP by the phosphoramidite based one-pot synthesis, synthesis of a 2′3′-cGAMP analog using click chemistry and c-di-GMP synthesis trough solid-phase synthesis.

Abbreviation: CDN, cyclic dinucleotide.

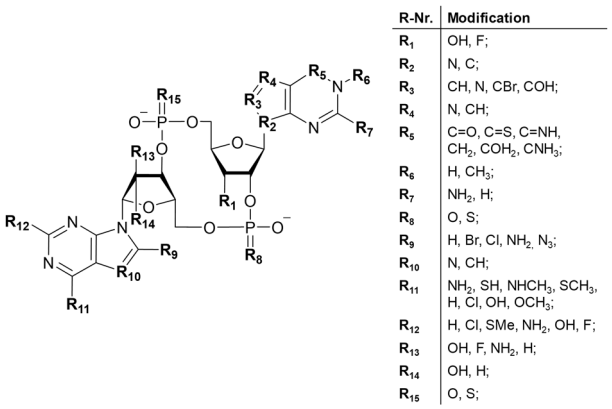

FIGURE 3 2′3′-cGAMP product scope that can be synthesized by cGAS

procedure. However, even though most of the steps take only a few minutes, the total effort is high due to a variety of solvent changes. In total, several days are required to produce a few mg of 2′3′-cGAMP with a total yield of 5% (Table 1) (Gaffney et al., 2010; Zhang et al., 2013). The synthesis of c-di-GMP yields 30% and its bisphosphorothioate analog is obtained in yields of 17%–19% (Gaffney et al., 2010). To which extent the synthesis can be transferred to a larger scale cannot be estimated from the data published to date. Another recently published synthesis route based on click chemistry reactions resulted in a 2′3′-cGAMP analog with neutral linkages that is characterized by a better cell membrane penetrability (Dialer et al., 2019). The labile and charged phosphate groups were replaced by an amide bond and a triazole linkage. The 20-step synthesis of this uncharged product resulted in a yield of 1%. Unfortunately, the product lacked affinity towards the STING receptor (Dialer et al., 2019). Beside the solution-phase synthesis, solid-phase synthesis of c-di-GMP starting from 3′-phosphate controlled pore glass as carrier material was demonstrated (Kiburu et al., 2008). Initially, P-methoxy phosphorusamidite was coupled and oxidized by an iodine-pyridine-water mixture. After coupling a second P-methoxy phosphorusamidite, the linear dimer was cleaved by trimethylamine and subsequently cyclized. The CDN was finally deprotected using ammonia and $Et_3N{\cdot}3HF$. Advantages of the method compared to the liquid-phase synthesis are the possibility of automation, simpler purification and that the coupling of the nucleotide monomers could go to completion by using an excess of coupling reagents.

The biocatalyzed synthesis of CDNs (Figure 2b) is based on the biocatalyst cGAS, which can be produced heterologously in bacterial expression hosts. The enzyme is activated with double-stranded DNA and supplied with nucleotide triphosphates as substrates in aqueous buffer. The synthesis results in a yield of 92% after incubation for 24 h at 37°C (Rolf et al., 2019) and results in an overall yield of 33% after purification (Rosenthal et al., 2020). In contrast to chemical synthesis, which requires eight steps starting from the protected phosphoramidite, biocatalyzed synthesis of 2′3′-cGAMP requires one enzyme-catalyzed reaction step. In addition to 2′3′-cGAMP, many other analogs can be synthesized enzymatically by cGAS from the corresponding nucleoside triphosphates as shown in Figure 3. In total, modifications at 15 different positions are accepted by cGAS homologs originating from human, murine, and chicken demonstrating the promiscuous properties of the enzyme (Novotná et al., 2019; Rosenthal et al., 2020). For example, the synthesis of 2′3′-cGAMP bisphosphorothioate was confirmed (Novotná et al., 2019; Rosenthal et al., 2020), which is highly interesting for medical applications due to the hydrolysis-resistance against phosphodiesterase in the cytosol (Li et al., 2014). The enzyme also accepts halogens at different positions of the molecule (Eedwin, 2019; Novotná et al., 2019; Rosenthal et al., 2020). Modifications with fluorine are generally interesting for medical applications, as it is known to increase the binding affinity to the drug-target and improves the metabolic stability. In addition, bromine and chlorine are chemically easily accessible functional groups and might be a promising starting point for consecutive reactions. This is also the case for the azide group, which could be used in click chemistry reactions. As these 2′3′-cGAMP derivatives amongst others were synthesized with different cGAS homologs, it is interesting to note that different homologs accept different substrates (Novotná et al., 2019). The same was observed for truncated or full length enzymes having a slightly different substrate scope (Rosenthal et al., 2020).

Besides the biocatalyzed synthesis of 2′3′-cGAMP derivatives, symmetric 3′3′-CDNs can be synthesized with enzymes of bacterial origin. In contrast, no enzymatic synthesis route is known for 2′2′-CDNs, which nevertheless activates the STING pathway (Wang &

Xi, 2021). The well-studied synthesis of 3′3′-CDNs is catalyzed by DncV from *Vibrio cholerae* (Launer-Felty & Strobel, 2018). This enzyme showed a broad substrate scope with reaction yields between 63% and 98% for the synthesis of c-di-GMP, c-di-AMP, c-di-CMP, c-di-UMP, and c-di-purine monophosphates. Furthermore, DncV accepts the insertion of fluorine atoms and amino groups at the ribose as well as the phosphorothioate derivatives. It has been shown that the accepted substrate scope strongly depends on whether Mg^{2+}, Co^{2+}, or Mn^{2+} is used as a divalent cation as a cofactor in the reaction buffer.

This demonstrates that biological CDN synthesis might have some advantages over chemical synthesis. It is not only much faster and avoids complex protective group chemistry, it also provides access to a new spectrum of CDNs. In particular, the production of halogenated products is highly interesting due to their promising properties with regard to medical application and consecutive chemistry. However, up to now, an economic bioprocess is not available for the large-scale production of CDNs, especially for the heterodimeric CDNs.

3 | PROCESS ASPECTS OF BIOCATALYTIC CDN PRODUCTION

The discovery of various naturally occurring CDNs promoted the development of their biocatalytic production. The general procedure includes preparation of the biocatalyst by heterologous expression of the CD-NTase in an appropriate expression host, which is up to now solely *Escherichia coli*, subsequent purification, the biocatalytic reaction step, and chromatographic product purification. Table 2 summarizes the current state of key parameters of bioprocesses for the preparation of naturally occurring CDNs. For an economically feasible industrial fine chemical production process, a titer of at least 1 g L^{-1} and a space-time-yield (STY) of 0.1 g L^{-1} h^{-1} should be achieved (Straathof et al., 2002). The enzymatic processes for bacterial CDNs are mostly reaching these threshold values. The values for nonnatural CDN-derivative production, however, are generally lower compared to the synthesis of natural CDNs due to decreased production rates and maximal achievable titers.

By now, c-di-GMP and c-di-AMP can be produced at gram-scale. For biocatalytic c-di-GMP production, the thermophilic diguanylate cyclase domain (tDGC) protein was used showing a prolonged activity compared to the mesophilic equivalents (Venkataramani & Liang, 2017). Additionally, a variant optimized by site-directed mutagenesis exhibited reduced product inhibition, so that nearly 100% of the substrate GTP was converted. A production process was set up as fed-batch to overcome the substrate inhibition at GTP levels of 0.8 mM and led to the production of 800 mg of c-di-GMP with 10 mg of the biocatalyst within 10 h at 45°C. The amount of 800 mg of purified product obtained from a 30 mL scale reaction is outstanding for the biocatalytic production of CDNs. Furthermore, a technique for the immobilization of the tDGC R158A mutant enzyme on sol-gel blocks or particles was developed, which stabilized the biocatalyst for up to 6 months (Venkataramani & Liang, 2017). As the substrate GTP is very expensive, a three enzyme cascade with GMP kinase, nucleoside diphosphate kinase, and a mutated form of diguanylate cyclase (DGC) named $DgcA^{VMGG}$ was established to synthesize c-di-GMP from GMP (Spehr et al., 2011). The synthesis resulted in 1.75 g of the purified diammonium salt of c-di-GMP from a 5 L reaction mixture. A more than 40-fold reduction of costs for precursors was achieved showing additional options for the development of an economic process without GTP as an expensive substrate. Besides c-di-GMP production, efficient enzyme-based c-di-AMP synthesis processes were developed. The enzyme btDisA from *Bacillus thuringiensis* was used to synthesize 100 mg of c-di-AMP in 4 h in a 50 ml reaction system and hence proved the possibility for a gram-scale production (Zheng et al., 2013). Recently, an immobilized form of the cyclic-AMP-GMP synthetase DncV from *Vibrio cholerae* was used for the production of c-di-AMP at a gram-scale resulting in titers of up to 3.3 g L^{-1} and a process yield of 80% (Table 1) (Sun et al., 2020). The biocatalyst DncV was previously reported to catalyze the synthesis of the bacterial cGAMP containing two 3′5′-linkages (Launer-Felty & Strobel, 2018). This promiscuous enzyme catalyzes the preparation of cleavage resistant thio-dinucleotides as well.

In addition to isolated enzymes, whole cell biocatalysts were demonstrated as promising alternative for the production of CDNs. Due to the comparably large scale of 1 L, 134 mg of crude c-di-GMP and 64 mg of crude 3′3′-cGAMP were produced with recombinant *E. coli* (Lv et al., 2019). This is also the first published attempt for the biocatalytic production of 2'3'-cGAMP at a liter scale. Unexpectedly, 2′3′-cGAMP is secreted into the culture broth from the recombinant *E. coli* BL21-CodonPlus (DE3)-RIL strain expressing murine cGAS. In total, a titer of 146 mg L^{-1} cGAMP was obtained in minimal medium.

Generally, higher CDN titers can be obtained in syntheses with free enzymes. The highest specific activity for 2′3′-cGAMP synthesis for in vitro assays were achieved using murine cGAS in comparison to various other tested cGAS homologs (Rolf et al., 2019). These assays were performed at a small-scale with 2 ml reactions containing 40 µg mL^{-1} of the corresponding cGAS homolog. One of the largest preparations of 2′3′-CDNs with the free enzyme has been performed in 20 ml reaction vessels (Rosenthal et al., 2020). Even though the reported cGAMP syntheses do not yet meet the targets for an economic process, the values for a titer of 0.31 mg L^{-1} and STY of 0.013 g L^{-1} h^{-1} are promising initial values for further process development (Table 2).

In contrast to the upstream process for CDNs, the downstream is less in the focus of current research. Chromatography is the standard purification method for CDNs. For c-di-GMP purification, reversed-phase chromatography with ion pair reagents are used at a preparative scale with 5 mL per injection (Venkataramani & Liang, 2017). Alternatively, a downstream process with ion-exchange chromatography and subsequent evaporation, precipitation, filtration, and drying has been used for the purification of c-di-GMP (Spehr et al., 2011). An overall yield of 44% has been achieved with an 82% (w/w) purity confirmed by NMR analysis and a low endotoxin contamination <0.00005% (w/w) confirmed by the Limulus test.

TABLE 2 Published data for biocatalytic processes and calculated values for key parameters

CDN	Biocatalyst	CD-Ntase concentration (μg mL^{-1})	Reaction time (h)	Scale (ml)	Titer (mg L^{-1})	STY (g L^{-1} h^{-1})	Reaction yield (%)	Process yield (%)	Absolute product amount (mg)	Reference
c-di-GMP	DgcA, (NDK, GMPK)	6.25	16	5,000	-	-	-	44	1750.0	Spehr et al. (2011)
c-di-GMP	tDGCm	166.67	10	30	-	-	-	-	800.0	Venkataramani and Liang (2017)
c-di-AMP	btDisA	86.00	4	50	2106	0.527	64	62	102.7	Zheng et al. (2013)
c-di-AMP	Immobilized DncV	65.00	over night	300	3290	0.206[a]	100	80	830.0	Sun et al. (2020)
3′3′-cGAMP	DncV	470.00	1	0.2-0.5	-	-	-	-	-	Launer-Felty and Strobel (2018)
2′3′-cGAMP	thscGAS	40.00	24	10	310	0.013	92	33	1.1	Rosenthal et al. (2020)
	whole cells									Lv et al. (2019)
c-di-GMP	tDGCm[b]	5 g L^{-1} cell wet weight	20	1000	134	0.007	-	-	36.5	
3′3′-cGAMP	DncVtm (NDK, GMK)[b]	7 g L^{-1} cell wet weight	20	1000	64	0.003	-	-	15.9	
2′3′-cGAMP	mcGAS[c]	n.a.	20	1000	146	0.007	-	-	42.8	

Note: Process yield refers to the entire production process including downstream. The following biocatalysts were considered; DgcA: diguanylate cyclase mutant + NDK: nucleoside diphosphate kinase + GMPK: guanosine monophosphate kinase as three enzyme cascade, tDGCm: thermophilic diguanylate cyclase R158A mutant, btDisA: deadenylate cyclase DisA from Bacillus thuringiensis BMB 171, DncV: Vibrio cholerae dinucleotide cyclase, thscGAS: truncated human cyclic GMP-AMP, DncVtm: DncV truncated mutant, mcGAS: mouse cyclic GMP-AMP.

Abbreviation: CDN, cyclic dinucleotide.

[a]Calculation based on assumption over night corresponds to 16 h.

[b]In *E. coli* BL21 (DE3).

[c]In *E. coli* CodonPlus (DE3) RIL.

Noteworthy is a sequential chromatography that was applied for the purification of the whole-cell catalysis products (Lv et al., 2019). Preliminary to solid phase extraction and semipreparative reversed-phase chromatography, the CDNs were purified using a column containing an affinity resin that consists of the ligand-binding domain of human STING^{R232} immobilized on the Sepharose resin. This sequential chromatography resulted in 42.8 mg purified 2′3′-cGAMP from 0.5 L culture, 53% purification yield, and a purity of 99.5% (w/w) measured via UV spectroscopy. The highest overall yield of 80% was obtained by an integrated process development of c-di-AMP production (Sun et al., 2020). The process was performed with immobilized DncV that was easily separated after reaction. The product purification required only one additional purification step with macroporous resin resulting in 1 g c-di-AMP diammonium salt after desiccation with a purity of 98% (w/w) measured via UV spectroscopy.

In general, it is favorable to reduce the application of chromatography and the loss of product in the process. While the approaches with ion-exchange chromatography and STING-immobilized affinity resin have shown a high product purity after processing reactions mixtures at a liter scale, their use in industrial scale production might be limited due to the cost of the stationary phase. However, in most publications, the purification yield is not specified and there is no sophisticated general downstream procedure for CDNs developed yet.

4 | CONCLUSION

During recent years, multiple STING agonists were developed and studied in the field of immunology research and cancer immunotherapy. Although there are a few non-nucleotidic STING agonists available, none of them was used for clinical trials (Jiang et al., 2020). In contrast, synthetic CDNs achieved promising results in preclinical work (c-di-GMP, 2′2′-cGAMP, 3′3′-cGAMP) and some of them are currently tested in clinical phase 1 and 2 studies (2′3′-c-di-AMP bisphosphorothioate and others of which the structure is not disclosed) (Ding et al., 2020; Jiang et al., 2020).

Especially 2′3′-cGAMPs induce a balanced immune response and potent antitumor immunity. Next to the natural CDNs, more than 70 2′3′-cGAMP derivatives have been synthesized, whose biological activity is often completely unknown. Accordingly, the potential to develop new derivatives with improved properties is far from being exhausted. To advance CDNs as vaccines and cancer adjuvants for humans, the development of economical production processes is necessary, because active ingredients without production capabilities are not relevant. Even though the chemical and biocatalytic synthesis of natural and various synthetic CDNs is well established, large scale production processes with economic values have not been implemented so far. While chemical synthesis of CDNs suffers from low yields and complex synthesis routes, enzymatically catalyzed synthesis is limited by low product titers and process stability. These limitations result from substrate inhibition, low biocatalyst activity and stability. Additionally, downstream processing of CDNs in aqueous phase is challenging due to the highly hydrophilic character of the products and impurities such as enzymes and DNA. Especially for cGAMP, the development of a biocatalytic production processes is still in the beginning and is mainly focused on the synthesis step itself. Nevertheless, the biocatalytic production is well developed for the earliest known CDN c-di-GMP. This process could represent a promising starting point for the production of other CDNs.

In general, the transfer from generating material in research quantities to commercially relevant amounts requires a successful scale-up with high productivity, quality, and robustness of the process. There is no doubt that this emerging and exciting field of biotechnological CDN production will continue to be in the focus of the scientific community.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable—no new data generated.

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