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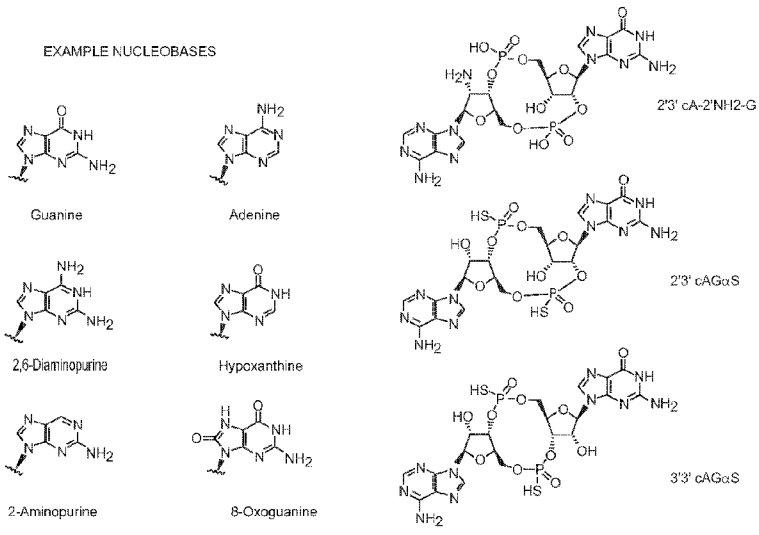
(54) Title: METHODS OF PRODUCING CYCLIC DINUCLEOTIDES

FIG. 11



(57) Abstract: The present disclosure provides cyclic dinucleotides (CDNs), and methods of preparing and using the same. The subject methods can include contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN. In some embodiments, the CDN producing-enzyme is a Hypr GGDEF enzyme. The methods can be performed in vitro or in a genetically modified host cell. Also provided are CDN compositions that find use in a variety of applications. Methods of modulating an immune response in an individual and methods of inhibiting a bacterial biofilm in an industrial system are also provided.

[Continued on next page]

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METHODS OF PRODUCING CYCLIC DINUCLEOTIDES

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/438,127, filed December 22, 2016, which application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. OD008677 awarded by National Institutes of Health. The government has certain rights in the invention.

INTRODUCTION

[0003] Cyclic dinucleotides (CDNs) are ubiquitous signaling molecules in bacteria. CDNs control bacterial processes including biofilm formation, intestinal colonization, cell division, cell wall homeostasis, and exoelectrogenesis. A role for bacterial cyclic AMP-GMP (cAG) in the deltaproteobacterium Geobacter includes controlling genes associated with Fe(III) reduction.

[0004] GGDEF domain-containing enzymes are diguanylate cyclases that produce cyclic di-GMP (cdiG), a second messenger that modulates the key bacterial lifestyle transition from a motile to sessile biofilm-forming state. The ubiquity of genes encoding GGDEF proteins in bacterial genomes has established the dominance of cdiG signaling in bacteria. A subfamily of GGDEF enzymes synthesizes the asymmetric signaling molecule cyclic AMP-GMP (cAG or 3′, 3′-cGAMP). Hybrid CDN-producing and promiscuous substrate-binding (Hypr) GGDEF enzymes are widely distributed and found in other deltaproteobacteria and have roles that include regulation of cAG signaling. GGDEF enzymes that produce cyclic dinucleotides are of interest.

SUMMARY

[0005] The present disclosure provides cyclic dinucleotides (CDNs), and methods of preparing and using the same. The subject methods can include contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN. In some embodiments, the CDN producing-enzyme is a Hypr GGDEF enzyme. The methods can be performed *in vitro* or in a genetically modified host cell. Also provided are CDN compositions that find use in a variety of applications. Methods of modulating an immune response in an individual and methods of inhibiting a bacterial biofilm in an industrial system are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] **FIG. 1** shows enzyme substrate heat maps for production of various CDNs by three exemplary Hypr GGDEF enzymes, GSU1658, Mxan2643 and Bd0367.

[0007] **FIG. 2** shows enzyme substrate heat maps for production of various CDNs by exemplary enzymes cGAS and DncV.

[0008] **FIG. 3** shows enzyme substrate heat maps for production of various CDNs by the exemplary enzymes WspR and DisA.

[0009] **FIG. 4** illustrates the structure, production conditions and identification of 3',3'-cyclic di-2APMP.

[0010] **FIG. 5** illustrates the structure, production conditions and identification of 2',3'-cyclic 2APMP-GMP.

[0011] **FIG. 6** illustrates the structure, production conditions and identification of 3',3'-cyclic di-IMP.

[0012] **FIG. 7** illustrates the structure, production conditions and identification of 3',3'-cyclic di-GMPαS.

[0013] **FIG. 8** illustrates the structure, production conditions and identification of 3',3'-cyclic AMPαS-GMPαS.

[0014] **FIG. 9** illustrates the structure, production conditions and identification of 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

[0015] **FIG. 10** illustrates the structure, production conditions and identification of 3',3'-cyclic di-AMP – 2'-NH_2.

[0016] **FIG. 11** illustrates the structures of exemplary cyclic dinucleotide analogs (CDNs) of interest.

[0017] **FIG. 12** shows data illustrating the probing RNA : ligand binding interactions with CDN biosensors.

[0018] **FIG. 13** illustrates the relative fluorescence of 2AP triphosphate versus cyclic di-2AP.

[0019] **FIG. 14** shows thin layer chromatography analysis of experiments which illustrates a method to activate CDN production of a Hypr GGDEF enzyme (MXAN_2643) through addition of cAMP as the activating agent. +cAMP exhibited 19.9 fold activation of CDNs while +cGMP exhibited 2.2 fold activation of CDN production. Further details are provided in the experimental section below.

[0020] **FIG. 15** illustrates a schematic diagram of signaling pathways in bacterium Geobacter showing how two CDNs of interest can act is response to different environmental stimuli.

[0021] **FIG. 16** shows that deletion of the Hypr GGDEF GSU1658 in Geobacter induces a noticeable growth defect specifically under insoluble Fe(III) Oxide.

[0022] **FIG. 17** shows that deletion of the diguanylate cyclase EsnD produces a defect on electrodes.

[0023] **FIG. 18** illustrates that the CDN producing-enzymes have wide nucleobase acceptance in their substrates and can generate a wide array of unnatural CDNS of interest. In contrast WspR, a canonical GGDEF, only accepts 8-oxoGTP in its substrate.

[0024] **FIG. 19** shows kinetic data for an exemplary Hypr GGDEF enzyme Mx2643 with four unique NTP substrates.

[0025] **FIG. 20** shows a schematic of steps of a pyrophosphatase assay for evaluating Hypr GGDEF enzymes of interest.

[0026] **FIG. 21** shows a summary of the kinetic data of FIG. 19 for an exemplary Hypr GGDEF enzyme Mx2643 with four unique NTP substrates fit to two different kinetic models.

DEFINITIONS

[0027] The following terms have the following meanings unless otherwise indicated. Any undefined terms have their art recognized meanings.

[0028] "Alkyl" refers to monovalent saturated aliphatic hydrocarbyl groups having from 1 to 10 carbon atoms and such as 1 to 6 carbon atoms (e.g., "an alkyl of 1 to 6 carbons atoms"), or 1 to 5 (e.g., "an alkyl of 1 to 5 carbons atoms"), or 1 to 4 (e.g., "an alkyl of 1 to 4 carbons atoms"), or 1 to 3 carbon atoms (e.g., "an alkyl of 1 to 3 carbons atoms"). This term includes, by way of example, linear and branched hydrocarbyl groups such as methyl (CH_3-), ethyl (CH_3CH_2-), n-propyl ($CH_3CH_2CH_2$-), isopropyl (($CH_3)_2CH$-), n-butyl ($CH_3CH_2CH_2CH_2$-), isobutyl (($CH_3)_2CHCH_2$-), sec-butyl ((CH_3)(CH_3CH_2)CH-), t-butyl (($CH_3)_3C$-), n-pentyl ($CH_3CH_2CH_2CH_2CH_2$-), and neopentyl (($CH_3)_3CCH_2$-).

[0029] The term "substituted alkyl" refers to an alkyl group as defined herein wherein one or more carbon atoms in the alkyl chain have been optionally replaced with a heteroatom such as -O-, -N-, -S-, -$S(O)_n$- (where n is 0 to 2), -NR- (where R is hydrogen or alkyl) and having from 1 to 5 substituents selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, aminoacyl, aminoacyloxy, oxyaminoacyl, azido, cyano, halogen, hydroxyl, oxo, thioketo, carboxyl, carboxylalkyl, thioaryloxy, thioheteroaryloxy, thioheterocyclooxy, thiol, thioalkoxy, substituted thioalkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclyl, heterocyclooxy, hydroxyamino, alkoxyamino, nitro, -SO-alkyl, -SO-aryl, -SO-heteroaryl, -SO_2-alkyl, -SO_2-aryl, -SO_2-heteroaryl, and -NR^aR^b, wherein R^a and R^b may be the same or different and are chosen from hydrogen, optionally substituted alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, heteroaryl and heterocyclic. In some instances, a "substituted alkyl" refers to an alkyl group as

defined herein having from 1 to 5 substituents selected from the group consisting of alkoxy, cycloalkyl, cycloalkenyl, acyl, acylamino, acyloxy, amino, aminoacyl, aminoacyloxy, oxyaminoacyl, azido, cyano, halogen, hydroxyl, carboxyl, carboxylalkyl, thiol, thioalkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclyl, heterocyclooxy, sulfonamido, and -NR^aR^b, wherein R^a and R^b may be the same or different and are chosen from hydrogen, alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, heteroaryl and heterocyclic.

[0030] "Alkoxy" refers to the group –O-alkyl, wherein alkyl is as defined herein. Alkoxy includes, by way of example, methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, t-butoxy, sec-butoxy, n-pentoxy, and the like. The term "alkoxy" also refers to the groups alkenyl-O-, cycloalkyl-O-, cycloalkenyl-O-, and alkynyl-O-, where alkenyl, cycloalkyl, cycloalkenyl, and alkynyl are as defined herein.

[0031] The term "substituted alkoxy" refers to the groups substituted alkyl-O-, substituted alkenyl-O-, substituted cycloalkyl-O-, substituted cycloalkenyl-O-, and substituted alkynyl-O- where substituted alkyl, substituted alkenyl, substituted cycloalkyl, substituted cycloalkenyl and substituted alkynyl are as defined herein.

[0032] The term "assessing" any form of measurement, and includes determining if an element is present or not. The terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, as well as determining whether it is present or absent.

[0033] As used herein, the terms polynucleotide and oligonucleotide are used interchangeably to refer to a compound containing a plurality of nucleoside moiety subunits or nucleoside residues that are linked by internucleoside bonds or internucleosidic linkages. Whenever a polynucleotide is represented by a sequence of letters, such as "ATGUCCTG," it is understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, "T" denotes thymidine, and "U" denotes deoxyuridine, unless otherwise noted.

[0034] As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described generally by Scheit, Nucleotide Analogs (John Wiley, New York, 1980). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g. stability, specificity, or the like, such as disclosed by Uhlmann and Peyman (Chemical Reviews, 90:543-584, 1990). As used herein,

"nucleotide" refers to a nucleoside or analog thereof linked to a phosphate group, thiophosphate group or analog thereof.

[0035] The terms "base" and "nucleobase" are used interchangeably and defined herein to include (i) conventional DNA and RNA bases (uracil, thymine, adenine, guanine, and cytosine), and (ii) modified bases or base analogs (e.g., 5-methyl-cytosine, 5-bromouracil, or inosine). A base analog is a chemical whose molecular structure mimics that of a conventional DNA or RNA base.

[0036] As used herein, "pyrimidine" means the pyrimidines occurring in natural nucleosides, including cytosine, thymine, and uracil, and common analogs thereof, such as those containing oxy, methyl, propynyl, methoxy, hydroxyl, amino, thio, halo, and like, substituents. The term as used herein further includes pyrimidines with common protecting groups attached, such as N4-benzoylcytosine. Further pyrimidine protecting groups of interest include but are not limited to, those protecting groups are disclosed by Beaucage and Iyer Tetrahedron 48: 2223-2311 (1992).

[0037] As used herein, "purine" means the purines occurring in natural nucleosides, including adenine, guanine, and hypoxanthine, and common analogs thereof, such as those containing oxy, methyl, propynyl, methoxy, hydroxyl, amino, thio, halo, and like, substituents. The term as used herein further includes purines with common protection groups attached, such as N2-benzoylguanine, N2-isobutyrylguanine, N6-benzoyladenine, and the like. Further common purine protection groups are disclosed by Beaucage and Iyer Tetrahedron 48: 2223-2311 (1992). As used herein, the term "-protected-" as a component of a chemical name refers to art-recognized protection groups for a particular moiety of a compound, e.g. "5'-protected- hydroxyl" in reference to a nucleoside includes triphenylmethyl (i.e., trityl), *p*-anisyldiphenylmethyl (i.e., monomethoxytrityl or MMT), di-*p*-anisylphenylmethyl (i.e., dimethoxytrityl or DMT), and the like; and a protected nucleobase in reference to a nucleobase including a heteroatom protected with a group such as a dimethylaminoformamidine (DMF), benzoyl (Bz), isobutyryl, and the like. Art-recognized protecting groups include those described in the following references: Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); Amarnath and Broom, Chemical Reviews, 77:183-217, 1977; Pon et al., Biotechniques, 6:768-775, 1988; Ohtsuka et al., Nucleic Acids Research, 10:6553-6570, 1982; Eckstein, editor, Oligonucleotides. and Analogues: A Practical Approach (IRL Press, Oxford, 1991), Greene and Wuts, Protective Groups in Organic Synthesis, Second Edition, (John Wiley & Sons, New York, 1991), Narang, editor, Synthesis and Applications of DNA and RNA (Academic Press, New York, 1987), Beaucage and Iyer Tetrahedron 48: 2223-2311 (1992), and like references.

[0038] The term "pharmaceutically acceptable salt" means a salt which is acceptable for administration to a patient, such as a mammal (salts with counterions having acceptable mammalian safety for a

given dosage regime). Such salts can be derived from pharmaceutically acceptable inorganic or organic bases and from pharmaceutically acceptable inorganic or organic acids. "Pharmaceutically acceptable salt" refers to pharmaceutically acceptable salts of a compound, which salts are derived from a variety of organic and inorganic counter ions well known in the art and include, by way of example only, sodium, and the like; and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, and the like. Pharmaceutically acceptable salts of interest include, but are not limited to, aluminium, ammonium, arginine, barium, benzathine, calcium, cholinate, ethylenediamine, lysine, lithium, magnesium, meglumine, procaine, potassium, sodium, tromethamine, N-methylglucamine, N,N′-dibenzylethylene-diamine, chloroprocaine, diethanolamine, ethanolamine, piperazine, zinc, diisopropylamine, triethylamine, diisopropylethylamine and triethanolamine salts.

[0039] The term "salt thereof" means a compound formed when a proton of an acid is replaced by a cation, such as a metal cation or an organic cation and the like. Where applicable, the salt is a pharmaceutically acceptable salt, although this is not required for salts of intermediate compounds that are not intended for administration to a patient. By way of example, salts of the present compounds include those wherein the compound is protonated by an inorganic or organic acid to form a cation, with the conjugate base of the inorganic or organic acid as the anionic component of the salt. Salts of interest include, but are not limited to, aluminium, ammonium, arginine, barium, benzathine, calcium, cesium, cholinate, ethylenediamine, lithium, magnesium, meglumine, procaine, N-methylglucamine, piperazine, potassium, sodium, tromethamine, zinc, N,N′-dibenzylethylene-diamine, chloroprocaine, diethanolamine, ethanolamine, piperazine, diisopropylamine, triethylamine, diisopropylethylamine and triethanolamine salts. It is understood that for any of the polynucleotide structures depicted herein that include a backbone of internucleoside linkages, such polynucleotides may also include any convenient salt forms. In some embodiments, acidic forms of the internucleoside linkages are depicted for simplicity. In some instances, the salt of the subject compound is a monovalent cation salt. In certain instances, the salt of the subject compound is a divalent cation salt. In some instances, the salt of the subject compound is a trivalent cation salt.

[0040] "Solvate" refers to a complex formed by combination of solvent molecules with molecules or ions of the solute. The solvent can be an organic compound, an inorganic compound, or a mixture of both. Some examples of solvents include, but are not limited to, methanol, *N*,*N*-dimethylformamide, tetrahydrofuran, dimethylsulfoxide, and water. When the solvent is water, the solvate formed is a hydrate.

[0041] "Stereoisomer" and "stereoisomers" refer to compounds that have same atomic connectivity but different atomic arrangement in space. Stereoisomers include cis-trans isomers, *E* and *Z* isomers, enantiomers, and diastereomers.

[0042] "Tautomer" refers to alternate forms of a molecule that differ only in electronic bonding of atoms and/or in the position of a proton, such as enol-keto and imine-enamine tautomers, -O-P(=S)(OH)-O- and -O-P(=O)(SH)-O-, or the tautomeric forms of heteroaryl groups containing a -N=C(H)-NH- ring atom arrangement, such as pyrazoles, imidazoles, benzimidazoles, triazoles, and tetrazoles. A person of ordinary skill in the art would recognize that other tautomeric arrangements of the groups described herein are possible.

[0043] It will be appreciated that the term "or a salt or solvate or stereoisomer thereof" is intended to include all permutations of salts, solvates and stereoisomers, such as a solvate of a pharmaceutically acceptable salt of a stereoisomer of subject compound. It is understood that the term "or a salt thereof" is intended to include all permutations of salts. It is understood that the term "or a pharmaceutically acceptable salt thereof" is intended to include all permutations of salts. It is understood that the term "or a solvate thereof" is intended to include all permutations of solvates. It is understood that the term "or a stereoisomer thereof" is intended to include all permutations of stereoisomers. It is understood that the term "or a tautomer thereof" is intended to include all permutations of tautomers. Thus for example it follows that it is intended to include a solvate of a pharmaceutically acceptable salt of a tautomer of a stereoisomer of subject compound.

[0044] The term "sample" as used herein relates to a material or mixture of materials, typically, although not necessarily, in fluid, i.e., aqueous, form, containing one or more components of interest. Samples may be derived from a variety of sources such as from food stuffs, environmental materials, a biological sample or solid, such as tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, semen, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components).

[0045] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular

embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0046] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0047] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0048] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a CDN" includes a plurality of such CDNs and reference to "the CDN" includes reference to one or more CDN and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0049] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0050] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0051] As summarized above, the present disclosure provides cyclic dinucleotides (CDNs), and methods of preparing and using the same. The subject methods can include contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN. In some embodiments, the CDN producing-enzyme is a Hypr GGDEF enzyme. The methods can be performed *in vitro* or in a genetically modified host cell. Also provided are CDN compositions that find use in a variety of applications. Methods of modulating an immune response in an individual and methods of inhibiting a bacterial biofilm in an industrial system are also provided.

METHODS OF PRODUCING A CYCLIC DINUCLEOTIDE

[0052] The present disclosure provides a method of preparing a cyclic dinucleotide (CDN) from precursor nucleotides via the action of a CDN-producing enzyme. Aspects of the present disclosure include a variety of CDN-producing enzymes that are capable of acting on nucleotide substrates to produce a variety of asymmetric and symmetric CDNs.

[0053] In some cases, the CDN-producing enzyme belongs to the family of diguanylate cyclase enzymes (DGC) which can be characterized by the active site amino acid sequence motif GGDEF. In certain cases, the CDN-producing enzyme is a diadenylate cyclase. In certain instances, the CDN-producing enzyme is a Cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) Synthase. The CDN-producing enzymes of the present disclosure can be naturally occurring enzymes that are identified to have a substrate specificity that provides for the combination of nucleotide precursors of interest in a CDN. For example, FIG.s 1-3 illustrate substrate heat maps that indicate the compatibility of particular nucleotide triphosphate substrates with particular CDN-producing enzymes of interests that provide for the production of CDN from those nucleotide precursors. The present disclosure provides for the production of various CDNs of interest, including non-naturally occurring CDNs, by manipulating the action of such enzymes on various precursor nucleotide substrates.

[0054] In certain instances, the CDN-producing enzyme is an enzyme of the sub-class termed Hybrid promiscuous substrate-binding (Hypr) GGDEF enzymes. Hypr GGDEF enzymes are described

by Hallberg et al. "Hybrid promiscuous (Hypr) GGDEF enzymes produce cyclic AMP-GMP (3′, 3′-cGAMP)", PNAS, pp 1790–1795, February 16, 2016, vol. 113, no. 7 , the disclosure of which is herein incorporated by reference in its entirety. Exemplary Hypr GGDEF enzymes of interest include, but are not limited to, those enzymes described in Hallberg et al, including the Supporting Information Appendix, e.g., Fig. S1, the disclosure of which is herein incorporated by reference. The CDN-producing enzyme can be mutated, e.g., in the ligand binding inhibitory site of the enzyme, to provide for increased reactivity with a particular nucleotide substrates of interest. Naturally occurring DGCs can be engineered to include a mutation in the enzyme active site that provides for promiscuous substrate binding by the enzyme and imparts a capability to produce various CDNs of interest.

[0055] An aspartate (D) to serine (S) mutation in the enzyme active site, naturally occurring in ~0.1% of GGDEF enzymes, can serve as a bioinformatic marker to identify Hypr GGDEFs. For example. in the enzyme GSU1658, this D to S mutation is at residue 347, which corresponds to D344 in PleD, a canonical GGDEF. Enzymes containing this mutation can exhibit Hypr GGDEF activity and can produce various CDNs of interest. Outside the active site, a naturally occurring arginine (R) to tyrosine (Y) mutation can also co-vary with the serine mutant, as indicated through bioinformatic analysis. In some cases, these mutations contribute to Hypr GGDEF activity through contacts across the protein dimerization interface.

[0056] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:1, below. In some cases, the CDN-producing enzyme comprises a Ser at amino acid 347.

[0057] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:2, below.

[0058] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:3, below.

[0059] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:4, below.

[0060] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:5, below.

[0061] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:6, below.

[0062] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:7, below.

[0063] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:8, below.

[0064] In some cases, a suitable CDN-producing enzyme comprises an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence designated SEQ ID NO:9, below.

[0065] In certain instances, the CDN-producing enzyme is a Hypr GGDEFs that produces 3',3'-CDNs. In certain instances, the CDN-producing enzyme is a cGAMP synthase (cGAS) that produces 2'-3'-CDNs. Exemplary CDN-producing enzymes of interest include, but are not limited to, Hypr GGDEFs such as GSU1658 (HyprA), Mxan 2643 or Bd0367, cGAMP synthases (cGAS), DncV (a bacterial cGAMP synthase from V. cholera, see e.g., Davies et al., "Coordinated Regulation of Accessory Genetic Elements Produces Cyclic Di-Nucleotides for V. cholerae Virulence" Cell, Volume 149, Issue 2, p358–370, 13 April 2012), the diguanylate cyclase enzyme WspR (see e.g., Malone et al., (December 2006). "The structure-function relationship of WspR, a Pseudomonas fluorescens response regulator with a GGDEF output domain". Microbiology. 153 (Pt 4): 980–15) and the diadenylate cyclase enzyme DisA.

[0066] In certain instances, the CDN-producing enzyme is one that has been isolated and/or purified, e.g., from a cell sample, or a recombinant production source. As used herein, the terms "isolated" and "purified" refer to isolation of a substance (e.g., CDN, compound, polynucleotide, protein, polypeptide, polypeptide composition, enzyme) such that the substance comprises the

majority percent of the sample in which it resides. In some cases in a sample, a substantially purified component comprises 50%, preferably 80%-85%, more preferably 90-95% of the sample. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

CDN Precursors

[0067] Any convenient precursor nucleotides that can act as a substrate of a CDN-producing enzyme (e.g., as described herein) can be utilized in the subject methods to provide for a target CDN. A CDN-producing enzyme can cyclize two nucleotides precursor compounds to produce a CDN of interest (e.g., a CDN of formulae (I)-(II), as described herein). A first precursor and a second precursor of the CDN can be cyclized by the enzyme to produce the cyclic dinucleotide. The first and second precursors can be the same or different. In some cases, a non-naturally occurring nucleotide is selected that includes a substituent group (e.g., a nucleobase substituent or a sugar substituent) which is desired to be incorporated into a CDN of interest. In certain cases, the nucleotide precursor compound is a nucleotide triphosphate. The nucleotide precursor compound can be selected to include a thiophosphate or dithiophosphate group that is subsequently incorporated into the CDN. Thiophosphate (or phosphorothioate) linkages, among other analogs, are of interest in some applications for providing increased stability and protecting the CDN against enzymatic degradation. Nucleotide triphosphates (NTP) of interest include, but are not limited to, adenosine triphosphate (ATP), guanosine triphosphate (GTP), inosine triphosphate (INP), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate, and thiophosphate or dithiophosphate analogs thereof, e.g., ATP(S), GTP(S) or CTP(S). In certain embodiments, the CDN precursor is a triphosphate functional analog that includes a phosphorus group suitable for undergoing a phosphorylation reaction via action of the CDN-producing enzyme.

[0068] Any convenient NTPs can be selected for use in the subject methods to provide for a desired CDN. Any convenient combination of two NTPs can be utilized, e.g., a first and a second NTP. It is understood that the CDNs of Tables 1 and 2 can be derived from particular NTP(s), whose disclosure is included herein. In certain instances, the CDN precursor is 2-aminopurine triphosphate. In certain instances, the CDN precursor is inosine triphosphate (ITP).

[0069] The CDN-producing enzymes and nucleotide precursors of a CDN can be combined under conditions sufficient to produce the CDN. Any convenient medium may be utilized, e.g., buffer, water and/or organic solvent, in performing the subject methods to produce a CDN *in vitro*. As used herein, *in vitro* means outside of a living cell, regardless of the location of the cell. Any convenient methods of addition may be adapted for use in the subject methods. The medium may

be supplemented with a sufficient amount of the starting materials of interest (e.g., CDN precursors as described herein), e.g., a mM to μM amount such as between about 1-5 mM of the precursor compound(s). It is understood that the amount of precursor added, the timing and rate of addition, the form of material added, etc., may vary according to a variety of factors. The precursor may be added neat or pre-dissolved in a suitable solvent (e.g., buffer, water or an organic solvent). The starting precursor may be added in concentrated form (e.g., 10x over desired concentration) to minimize dilution of the enzyme incubation medium upon addition. The precursor may be added in one or more batches, or by continuous addition over an extended period of time (e.g., hours or days).

[0070] The subject methods may also include recovering the CDN from the medium. Any convenient methods of separation and isolation (e.g., chromatography methods or precipitation methods) may be adapted for use in the subject methods to recover the CDN of interest from the medium. Filtration methods may be used to separate soluble from insoluble fractions. In some cases, liquid chromatography methods (e.g., reverse phase HPLC, size exclusion, normal phase chromatography) are used to separate the CDN from other soluble components of the enzyme medium.

Methods of Synthesizing a CDN in a Host Cell

[0071] The present disclosure provides a method of synthesizing a cyclic dinucleotide (CDN) in a host cell. Any convenient host cells can be utilized in the subject methods for preparing a CDN. In some instances of the method, the CDN is synthesized in a genetically modified host cell in a culture medium comprising a precursor of the CDN. The genetically modified host cell comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN. The CDN that is produced can then be recovered from the culture medium.

[0072] The term "host cells," as used herein, are cells that harbor one or more heterologous coding sequences which encode activity(ies) that enable the host cells to produce desired CDN(s), e.g., as described herein. The heterologous coding sequences could be integrated stably into the genome of the host cells, or the heterologous coding sequences can be transiently inserted into the host cell. As used herein, the term "heterologous coding sequence" is used to indicate any polynucleotide that codes for, or ultimately codes for, a peptide or protein or its equivalent amino acid sequence, e.g., an enzyme, that is not normally present in the host organism and can be expressed in the host cell under proper conditions. As such, "heterologous coding sequences" includes multiple copies of coding sequences that are normally present in the host cell, such that the cell is expressing additional copies of a coding sequence that are not normally present in the cells. The heterologous coding sequences can be RNA or any type thereof, e.g., mRNA, DNA or

any type thereof, e.g., cDNA, or a hybrid of RNA/DNA. Examples of coding sequences include, but are not limited to, full-length transcription units that comprise such features as the coding sequence, introns, promoter regions, 3'-UTRs and enhancer regions.

[0073] As used herein, the term "heterologous coding sequences" also includes the coding portion of the peptide or enzyme, i.e., the cDNA or mRNA sequence, of the peptide or enzyme, as well as the coding portion of the full-length transcriptional unit, i.e., the gene comprising introns and exons, as well as "codon optimized" sequences, truncated sequences or other forms of altered sequences that code for the enzyme or code for its equivalent amino acid sequence, provided that the equivalent amino acid sequence produces a functional protein. Such equivalent amino acid sequences can have a deletion of one or more amino acids, with the deletion being N-terminal, C-terminal or internal. Truncated forms are envisioned as long as they have the catalytic capability indicated herein. Fusions of two or more enzymes are also envisioned to facilitate the transfer of metabolites in the pathway, provided that catalytic activities are maintained.

[0074] Aspects of the present invention also relate to heterologous coding sequences that code for amino acid sequences that are equivalent to the native amino acid sequences for the various enzymes. An amino acid sequence that is "equivalent" is defined as an amino acid sequence that is not identical to the specific amino acid sequence, but rather contains at least some amino acid changes (deletions, substitutions, inversions, insertions, etc.) that do not essentially affect the biological activity of the protein as compared to a similar activity of the specific amino acid sequence, when used for a desired purpose. The biological activity refers to, in the example of a decarboxylase, its catalytic activity. Equivalent sequences are also meant to include those which have been engineered and/or evolved to have properties different from the original amino acid sequence. Mutable properties of interest include catalytic activity, substrate specificity, selectivity, stability, solubility, localization, etc. In certain embodiments, an "equivalent" amino acid sequence contains at least 80%-99% identity at the amino acid level to the specific amino acid sequence, in some cases at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% and more in certain cases, at least 95%, 96%, 97%, 98% and 99% identity, at the amino acid level. In some cases, the amino acid sequence may be identical but the DNA sequence is altered such as to optimize codon usage for the host organism, for example.

[0075] Any convenient tags may be utilized. In some embodiments, the host cell includes one or more of the enzymes that comprise a localization tag. In some embodiments, the host cell includes one or more of the enzymes that comprise a purification tag. In some cases, the localization or purification tag is a peptidic sequence that is attached at the N-terminal and or C-terminal of the enzyme. The purification tag can be a maltose binding protein (MBP). Any convenient methods may be utilized for attaching a tag to the enzyme.

[0076] In some cases, the localization tag is derived from an endogenous yeast protein. Such tags may provide route to a variety of yeast organelles: the endoplasmic reticulum (ER), mitochondria (MT), plasma membrane (PM), and vacuole (V).

[0077] The engineered host cell medium may be sampled and monitored for the production of CDN compounds of interest. The CDN compounds may be observed and measured using any convenient methods. Methods of interest include, but are not limited to, LC-MS methods (e.g., as described herein) where a sample of interest is analyzed by comparison with a known amount of a standard compound. Identity may be confirmed, e.g., by m/z and MS/MS fragmentation patterns, and quantitation or measurement of the compound may be achieved via LC trace peaks of know retention time and/or EIC MS peak analysis by reference to corresponding LC-MS analysis of a known amount of a standard of the compound.

[0078] As such, aspects of the present disclosure include culturing a host cell under conditions suitable for protein production such that the heterologous coding sequences are functionally expressed and convert precursor compounds of interest into product CDNs of interest.

[0079] Any convenient methods of culturing host cells may be employed for producing the CDNs of interest. The particular protocol that is employed may vary, e.g., depending on host cell, the heterologous coding sequences, the desired CDNs, etc. The cells may be present in any convenient environment, such as an environment in which the cells are capable of expressing one or more functional heterologous enzymes. In vitro, as used herein, simply means outside of a living cell, regardless of the location of the cell. As used herein, the term in vivo indicates inside a cell, regardless of the location of the cell. In some embodiments, the cells are cultured under conditions that are conducive to enzyme expression and with appropriate substrates available to allow production of CDNs in vivo. In some embodiments, the functional enzymes can be extracted from the host for production of CDNs under in vitro conditions. In some instances, the host cells can be placed back into a multicellular host organism. The host cells can be in any phase of growth, including, but not limited to, stationary phase and log-growth phase, etc. In addition, the cultures themselves may be continuous cultures or they may be batch cultures.

[0080] Any convenient cell culture conditions for a particular cell type may be utilized. In certain embodiments, the host cells that comprise the various heterologous coding sequences can be cultured under standard or readily optimized conditions, with standard cell culture media and supplements. As one example, standard growth media when selective pressure for plasmid maintenance is not required may contain 20 g/L yeast extract, 10 g/L peptone, and 20 g/L dextrose (YPD). Host cells containing plasmids can be grown in synthetic complete (SC) media containing 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, and 20 g/L dextrose

supplemented with the appropriate amino acids required for growth and selection. Alternative carbon sources which may be useful for inducible enzyme expression include, but are not limited to, sucrose, raffinose, and galactose. Cells can be grown at any convenient temperature (e.g., 30°C) with shaking at any convenient rate (e.g., 200 rpm) in a vessel, e.g., in test tubes or flasks in volumes ranging from 1-1000 mL, or larger, in the laboratory. Culture volumes can also be scaled up for growth in larger fermentation vessels, for example, as part of an industrial process.

[0081] Any convenient codon optimization techniques for optimizing the expression of heterologous polynucleotides in host cells may be adapted for use in the subject host cells and methods, see e.g., Gustafsson, C. et al. (2004) Trends Biotechnol, 22, 346-353, which is incorporated by reference in its entirety.

[0082] The subject method may also include adding a precursor compound to the cell culture. Any convenient methods of addition may be adapted for use in the subject methods. The cell culture may be supplemented with a sufficient amount of the starting materials of interest (e.g., as described herein), e.g., a mM to μM amount such as between about 1-5 mM of starting compound. It is understood that the amount of starting material added, the timing and rate of addition, the form of material added, etc., may vary according to a variety of factors. The starting material may be added neat or pre-dissolved in a suitable solvent (e.g., cell culture media, water or an organic solvent). The starting material may be added in concentrated form (e.g., 10x over desired concentration) to minimize dilution of the cell culture medium upon addition. The starting material may be added in one or more batches, or by continuous addition over an extended period of time (e.g., hours or days).

[0083] The subject methods may also include recovering the CDN from the cell culture. Any convenient methods of separation and isolation (e.g., chromatography methods or precipitation methods) may be adapted for use in the subject methods to recover the CDN of interest from the cell culture. Filtration methods may be used to separate soluble from insoluble fractions of the cell culture. In some cases, liquid chromatography methods (e.g., reverse phase HPLC, size exclusion, normal phase chromatography) are used to separate the CDN from other soluble components of the cell culture.

[0084] Also included are methods of engineering host cells for the purpose of producing CDNs of interest. Inserting DNA into host cells may be achieved using any convenient methods. The methods are used to insert the heterologous coding sequences into the host cells such that the host cells functionally express the enzymes and convert precursor compounds of interest into product CDNs of interest.

[0085] Any convenient promoters may be utilized in the subject host cells and methods. The promoters driving expression of the heterologous coding sequences may be constitutive promoters or inducible promoters, provided that the promoters can be active in the host cells. The heterologous coding sequences may be expressed from their native promoters, or non-native promoters may be used. Such promoters may be low to high strength in the host in which they are used. Promoters may be regulated or constitutive. In certain embodiments, promoters that are not glucose repressed, or repressed only mildly by the presence of glucose in the culture medium, are used. Promoters of interest include but are not limited to, promoters of glycolytic genes such as the promoter of the *B. subtilis* tsr gene (encoding fructose bisphosphate aldolase) or GAPDH promoter from yeast S. cerevisiae (coding for glyceraldehyde-phosphate dehydrogenase), the ADH1 promoter of baker's yeast, the phosphate-starvation induced promoters such as the PHO5 promoter of yeast, the alkaline phosphatase promoter from *B. licheniformis*, yeast inducible promoters such as Gal1-10, Gal1, GalL, GalS, repressible promoter Met25, tetO, and constitutive promoters such as glyceraldehyde 3-phosphate dehydrogenase promoter (GPD), alcohol dehydrogenase promoter (ADH), translation-elongation factor-1-α promoter (TEF), cytochrome c-oxidase promoter (CYC1), MRP7 promoter, etc. Autonomously replicating yeast expression vectors containing promoters inducible by hormones such as glucocorticoids, steroids, and thyroid hormones may also be used and include, but are not limited to, the glucocorticoid responsive element (GRE) and thyroid hormone responsive element (TRE). These and other examples are described U.S. Pat. No. 7,045,290, which is incorporated by reference, including the references cited therein. Additional vectors containing constitutive or inducible promoters such as α factor, alcohol oxidase, and PGH may be used. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of genes. Any convenient appropriate promoters may be selected for the host cell, e.g., E. coli. One can also use promoter selection to optimize transcript, and hence, enzyme levels to maximize production while minimizing energy resources.

[0086] Any convenient vectors may be utilized in the subject host cells and methods. Vectors of interest include vectors for use in yeast and other cells. Yeast vectors can be broken up into 4 general categories: integrative vectors (YIp), autonomously replicating high copy-number vectors (YEp), autonomously replicating low copy-number vectors (YCp) and vectors for cloning large fragments (YACs). Vector DNA can be introduced into prokaryotic or eukaryotic cells via any convenient transformation or transfection techniques.

Host Cells

[0087] One aspect of the present disclosure is a host cell that produces one or more CDNs. The host cell can be a naturally occurring cell, or can be genetically engineered (e.g., as described herein).

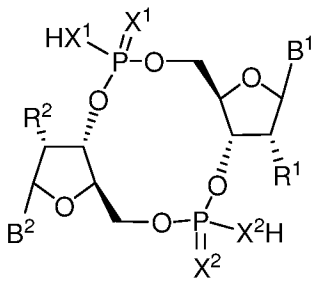
Any convenient type of host cell may be utilized in producing the subject CDN-producing cells. In some cases, the host cells are non-plant cells. In certain cases, the host cells are insect cells, mammalian cells, fungal cells, bacterial cells or yeast cells. Host cells of interest include, but are not limited to, bacterial cells, such as *Bacillus subtilis*, *Escherichia coli*, Streptomyces and *Salmonella typhimurium* cells and insect cells such as *Drosophila melanogaster* S2 and *Spodoptera frugiperda* Sf9 cells. In some embodiments, the host cells are yeast cells or *E. coli* cells. In certain embodiments, the yeast cells can be of the species *Saccharomyces cerevisiae* (*S. cerevisiae*). Yeast is of interest as a host cell because cytochrome P450 proteins, which are involved in some biosynthetic pathways of interest, are able to fold properly into the endoplasmic reticulum membrane so that their activity is maintained. Yeast strains of interest that find use in the invention include, but are not limited to, those described by in US2008/0176754.

[0088] In some cases, the host cell is a yeast strain. In some cases, the host cell is a prokaryotic cell. In certain instances, the host cell is a bacterial cell. In certain cases, the host cell is a eukaryotic cell. In some embodiments, the host cell is a yeast cell or a fungal cell.

[0089] The subject cells find use in a variety of applications where the instruction of a CDN to a sample or subject is of interest. Aspects of the present disclosure include a food product including a host cell (e.g., as described herein). In certain cases, the food product is a probiotic, such as a yogurt. The host cell can be a cell that produces a CDN of interest (e.g., as described herein). Any convenient components of the subject methods, e.g., CDN precursors, CDN-producing enzymes, etc. can be added to the cells in such food products to provide a CDN of interest in the subject composition.

CYCLIC DINUCLEOTIDE COMPOSITIONS

[0090] The present disclosure provides methods of synthesizing a CDN. Any convenient CDN's can be prepared from nucleotide precursor compounds according to the subject methods. In some cases, the CDN is naturally occurring. In some instances, the CDN is non-naturally occurring. In some embodiments, the CDN is a 3',3-CDN described by formula (I):



(I)

wherein:

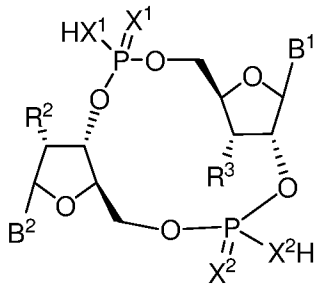
B^1 and B^2 are each independently a nucleobase;

each X^1 and each X^2 is independently O or S; and

R^1 and R^2 are independently H, OH, alkoxy, substituted alkoxy, alkyl, substituted alkyl, NH_2, fluoro, thiol or azido;

or a salt thereof.

[0091] In some embodiments, the CDN is a 2',3-CDN described by formula (II):



(II)

wherein:

B^1 and B^2 are each independently a nucleobase;

each X^1 and each X^2 is independently O or S; and

R^2 and R^3 are independently H, OH, NH_2, fluoro, thiol or azido;

or a salt thereof.

[0092] In some embodiments of formulae (I)-(II), each X^1 is O. In some embodiments of formulae (I)-(II), each X^2 is O. In some embodiments of formulae (I)-(II), each X^1 is S. In some embodiments of formulae (I)-(II), each X^2 is S. In some embodiments of formulae (I)-(II), one and only one X^1 is S. In some embodiments of formulae (I)-(II), one and only one X^2 is S. In some embodiments of formulae (I)-(II), B^1 and B^2 are independently selected from guanine, adenine, 2,6-diamino purine, 2-amino purine, hypoxanthine, xanthine, theobromine, isoguanine, uric acid, purine, and 8-oxo-guanine. In certain instances, B^1 and B^2 are the same. In certain instances, B^1 and B^2 are different.

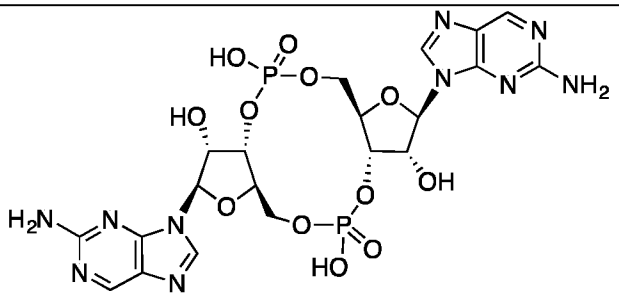
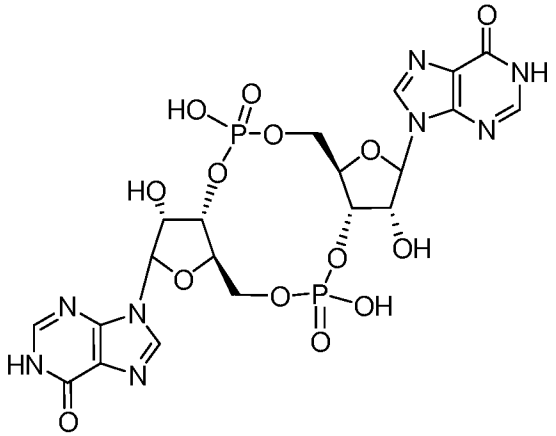
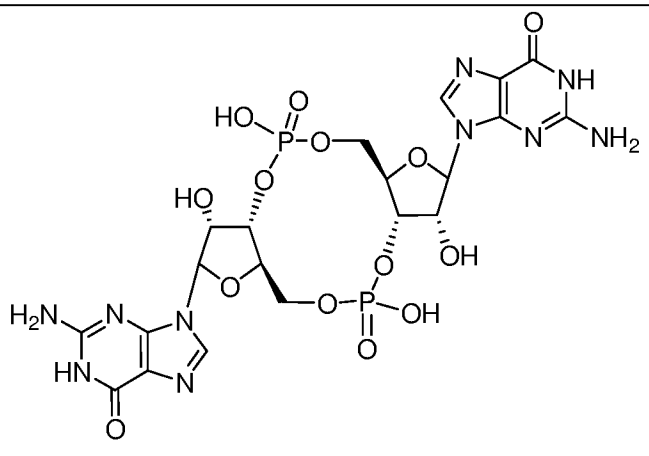
[0093] In some embodiments of formula (I), R^1 and R^2 are each independently H, OH or NH_2. In some embodiments of formula (I), R^1 and R^2 are H. In some embodiments of formula (I), R^1 and R^2 are OH. In some embodiments of formula (I), R^1 is OH. In some embodiments of formula (I), R^2 is OH. In some embodiments of formula (I), R^2 is NH_2. In some embodiments of formula (I), R^1 is NH_2. In some embodiments of formula (I), R^1 and/or R^2 are fluoro. In some embodiments of formula (I), R^1 and/or R^2 are azido.

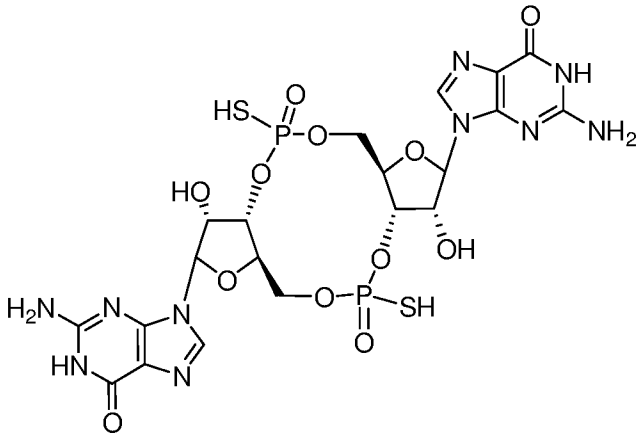
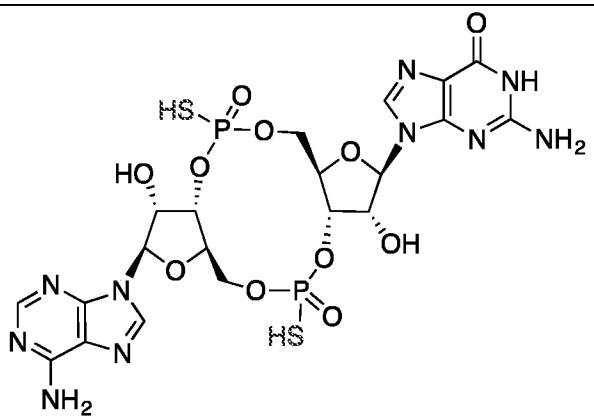
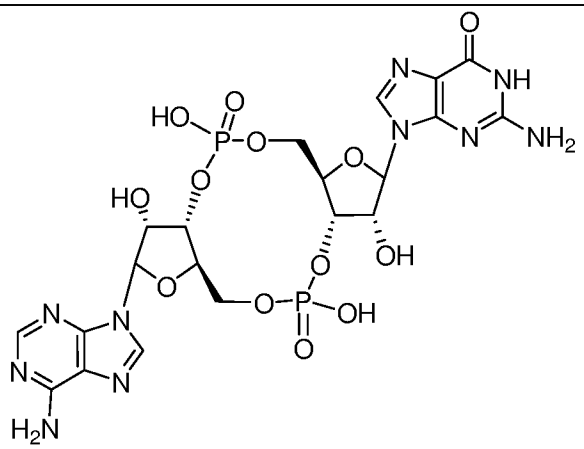
[0094] In some embodiments of formula (II), R^1 and R^3 are each independently H, OH or NH_2. In some embodiments of formula (II), R^1 and R^3 are H. In some embodiments of formula (II), R^1 and R^3

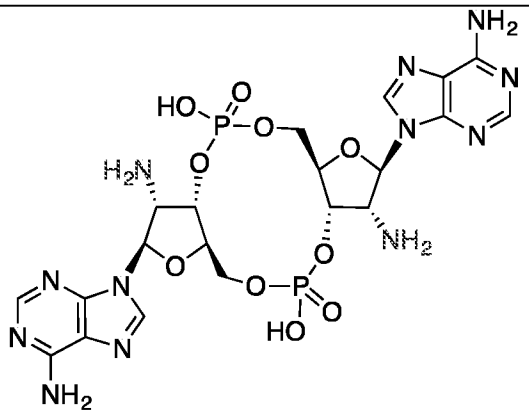
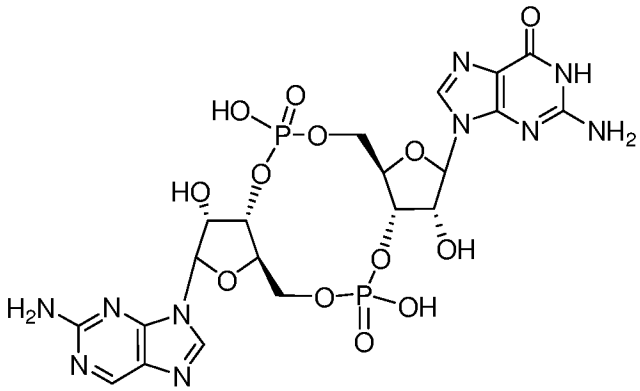
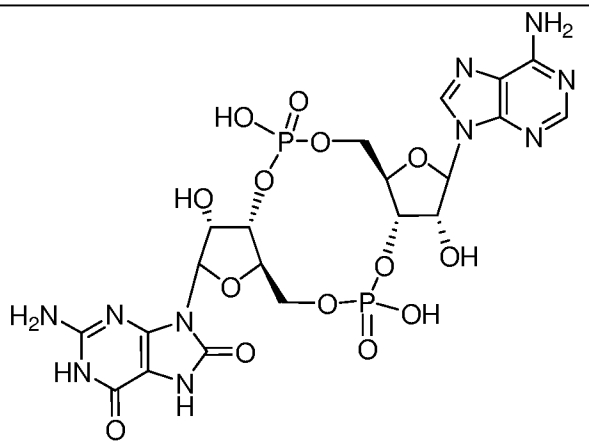
are OH. In some embodiments of formula (II), R^1 is OH. In some embodiments of formula (II), R^3 is OH. In some embodiments of formula (II), R^3 is NH_2. In some embodiments of formula (II), R^1 is NH_2. In some embodiments of formula (II), R^1 and/or R^3 are fluoro. In some embodiments of formula (II), R^1 and/or R^3 are azido.

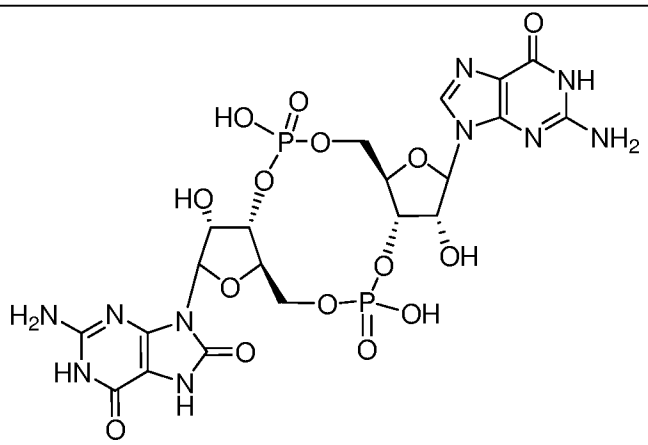
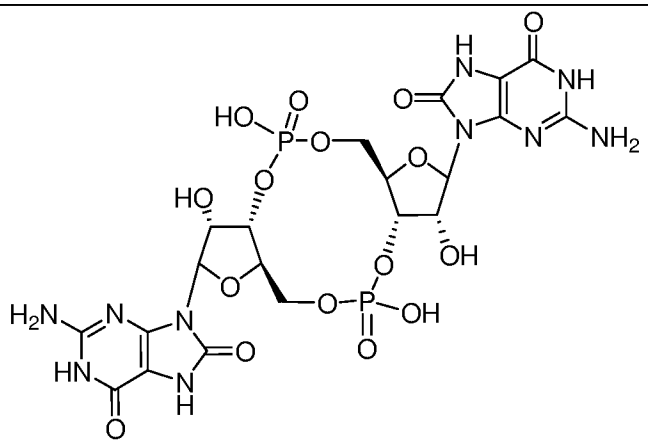
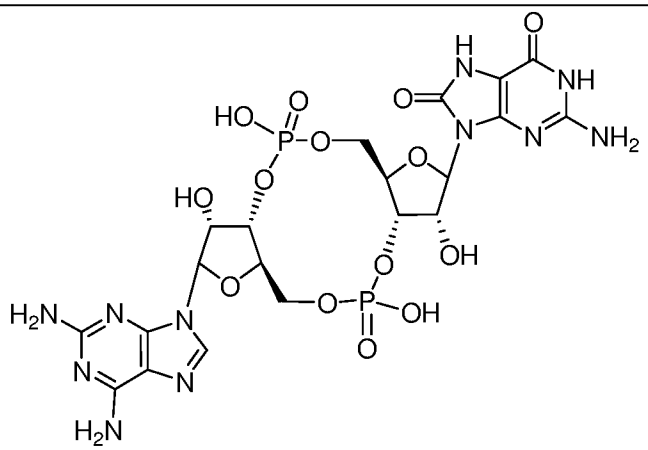
[0095] In some instances, the CDN is described by one of the compounds of Table 1, or a salt thereof.

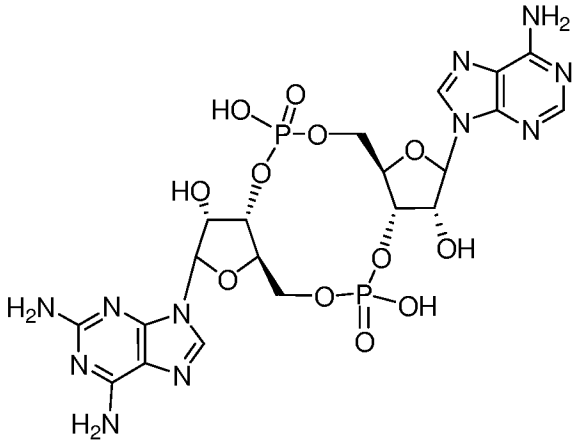
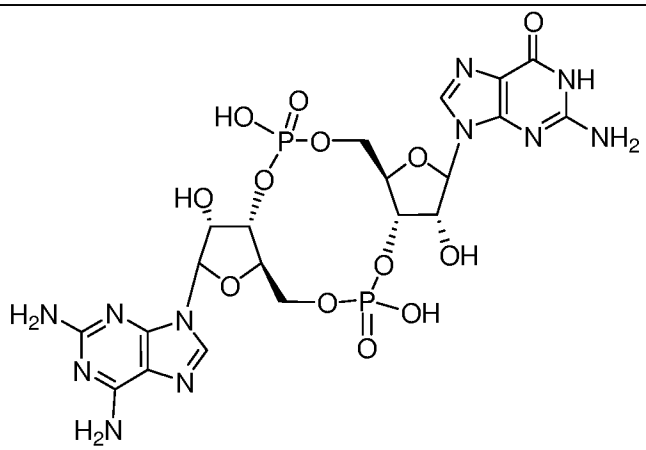
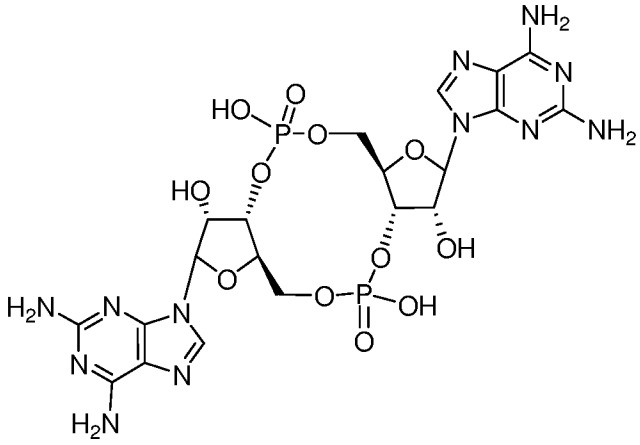
[0096] Table 1: 3',3'-CDNs of interest

Name	Abbreviation	Structure
3',3'-cyclic di-2-aminopurine monophosphate	c-di-2APMP	
3',3'-cyclic di-inosine monophospate	c di-IMP	
3',3'-cyclic-di-guanosine monophosphate	c-di-GMP	

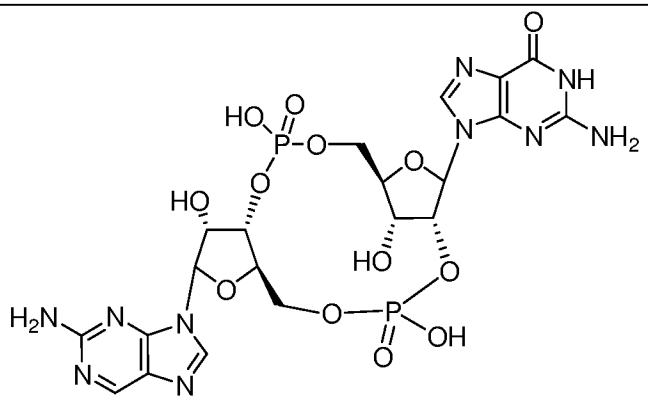
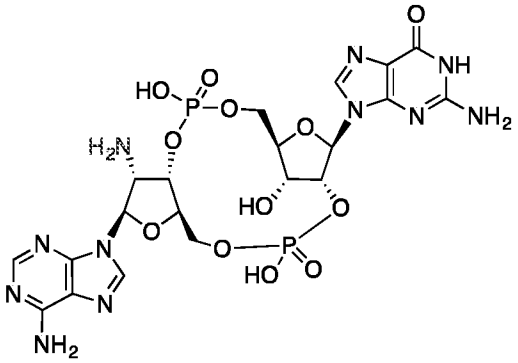
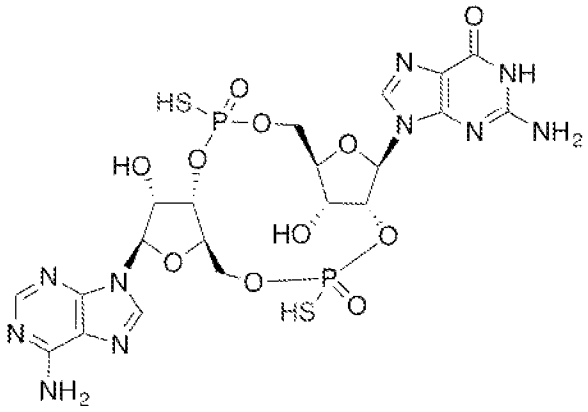
Name	Abbreviation	Structure
3',3'-cyclic-di-guanosine monothiophosphate	c-di-GMP (S)	
3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate	c-AMP-GMP (S)	
3',3'cyclic-adenosine monophosphate-guanosine monophosphate	c-AMP-GMP	

Name	Abbreviation	Structure
3',3-cyclic-di-(2'amino)-adenosine monophosphate	c-di-2'AAMP or 3',3'-cyclic di-AMP – 2'-NH_2	
3',3-cyclic-2-aminopurine monophosphate – guanosine monophosphate	3'3' c2AP-G	
3',3-cyclic-8-oxo-guanosine monophosphate – adenosine monophosphate	3'3' c8oxoG-A	

Name	Abbreviation	Structure
3',3-cyclic-8-oxo-guanosine monophosphate – guanosine monophosphate	3'3' c8oxoG-G	
3',3-cyclic di-8-oxo-guanosine monophosphate	3'3' cdi8oxoG	
3',3-cyclic diaminopurine monophosphate – 8-oxo-guanosine monophosphate	3'3' cDAP-8oxoG	

Name	Abbreviation	Structure
3',3-cyclic-diaminopurine monophosphate – adenosine monophosphate	3'3' cDAP-A	
3',3-cyclic-diaminopurine monophosphate – guanosine monophosphate	3'3' cDAP-G	
3',3'-cyclic-di-diaminopurine monophosphate	3'3' cdiDAP	

[0097] Table 2: 3',3'-CDNs of interest

Name	Abbreviation	Structure
2',3'-cyclic-2-aminopurine monophosphate-guanosine monophosphate	2',3'-c-2APMP-GMP	
2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate	c-2'AAMP-GMP or 2'3' cA-2'NH_2-G	
2',3'cyclic- adenosine monothiophosphate-guanosine monothiophosphate	2'3' cAGαS	

[0098] CDNs of interest which can be prepared according to the subject methods include, but are not limited to, 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP);

3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3',3'-cGAMP, 2',3'-cGAMP, 3',3'-c-di-AMP.

PHARMACEUTICAL COMPOSITIONS

[0099] Also provided are pharmaceutical preparations that include CDN compositions prepared according to the subject methods. Pharmaceutical preparations are compositions that include a compound (either alone or in the presence of one or more additional active agents) present in a pharmaceutically acceptable vehicle. In some embodiments, the pharmaceutical composition includes a CDN composition (e.g., as described herein) formulated in a pharmaceutically acceptable excipient.

[00100] The choice of excipient will be determined in part by the particular compound, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present disclosure.

[00101] The dosage form of CDN employed in the methods of the present disclosure can be prepared by combining the CDN composition with one or more pharmaceutically acceptable diluents, carriers, adjuvants, and the like in a manner known to those skilled in the art of pharmaceutical formulation.

[00102] The subject compositions can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

[00103] In some embodiments, formulations suitable for oral administration can include (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, or saline; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient (e.g., CDN of the present disclosure), as solids or granules; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can include the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles including the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients as are described herein.

[00104] The subject formulations can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They may also be formulated as pharmaceuticals for non-pressured preparations such as for use in a nebulizer or an atomizer.

[00105] In some embodiments, formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[00106] Formulations suitable for topical administration may be presented as creams, gels, pastes, or foams, containing, in addition to the active ingredient, such carriers as are appropriate. In some embodiments the topical formulation contains one or more components selected from a structuring agent, a thickener or gelling agent, and an emollient or lubricant. Frequently employed structuring agents include long chain alcohols, such as stearyl alcohol, and glyceryl ethers or esters and oligo(ethylene oxide) ethers or esters thereof. Thickeners and gelling agents include, for example, polymers of acrylic or methacrylic acid and esters thereof, polyacrylamides, and naturally occurring thickeners such as agar, carrageenan, gelatin, and guar gum. Examples of emollients include triglyceride esters, fatty acid esters and amides, waxes such as beeswax, spermaceti, or carnauba wax, phospholipids such as lecithin, and sterols and fatty acid esters thereof. The topical formulations may further include other components, e.g., astringents, fragrances, pigments, skin penetration enhancing agents, sunscreens (e.g., sunblocking agents), etc.

[00107] For an oral pharmaceutical formulation, suitable excipients include pharmaceutical grades of carriers such as mannitol, lactose, glucose, sucrose, starch, cellulose, gelatin, magnesium stearate, sodium saccharine, and/or magnesium carbonate. For use in oral liquid formulations, the composition may be prepared as a solution, suspension, emulsion, or syrup, being supplied either in solid or liquid form suitable for hydration in an aqueous carrier, such as, for example, aqueous saline, aqueous dextrose, glycerol, or ethanol, e.g., water or normal saline. If desired, the composition may also contain minor amounts of non-toxic auxiliary substances such as wetting agents, emulsifying agents, or buffers.

[00108] By way of illustration, the CDN composition can be admixed with conventional pharmaceutically acceptable carriers and excipients (i.e., vehicles) and used in the form of aqueous solutions, tablets, capsules, elixirs, suspensions, syrups, wafers, and the like. Such pharmaceutical compositions contain, in certain embodiments, from about 0.1% to about 90% by weight of the active compound, and more generally from about 1% to about 30% by weight of the active compound. The pharmaceutical compositions may contain common carriers and excipients, such as corn starch or gelatin, lactose, dextrose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, and alginic acid. Disintegrators commonly used in the formulations of this invention include croscarmellose, microcrystalline cellulose, corn starch, sodium starch glycolate and alginic acid.

[00109] Particular formulations of the present disclosure are in a liquid form. The liquid may be a solution or suspension and may be an oral solution or syrup which is included in a bottle with a pipette which is graduated in terms of milligram amounts which will be obtained in a given volume of solution. The liquid solution makes it possible to adjust the solution for small children which can be administered anywhere from 0.5 mg to 15 mg and any amount between in half milligram increments and thus administered in 0.5, 1.0, 1.5, 2.0 mg, etc.

[00110] A liquid composition will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s), for example, ethanol, glycerine, sorbitol, non-aqueous solvent such as polyethylene glycol, oils or water, with a suspending agent, preservative, surfactant, wetting agent, flavoring or coloring agent. Alternatively, a liquid formulation can be prepared from a reconstitutable powder.

Adjuvants

[00111] In some cases a composition of the present disclosure will comprise: a) a CDN as described herein; and b) an adjuvant (e.g., an adjuvant other than a CDN of the present disclosure. Suitable adjuvants include, but are not limited to, alum, aluminum phosphate, aluminum hydroxide, MF59 (4.3% w/v squalene, 0.5% w/v Tween 80™, 0.5% w/v Span 85), CpG-containing nucleic acid (where the cytosine is unmethylated), QS21, MPL, 3DMPL, extracts from Aquilla, ISCOMS, LT/CT mutants, poly(D,L-lactide-co-glycolide) (PLG) microparticles, Quil A, interleukins, and the like. For experimental animals, one can use Freund's, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dip- almitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

[00112] Further examples of suitable adjuvants include, but are not limited to: (1) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59TM (see, e.g., WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing MTP-PE) formulated into submicron particles using a microfluidizer, (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RIBI™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, Mont.) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components such as monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), e.g., MPL+CWS (Detox TM); (2) saponin adjuvants, such as QS21 or Stimulon™ (Cambridge Bioscience, Worcester, Mass.) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMS may be devoid of additional detergent e.g. WO 00/07621; (3) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (4) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), etc.), interferons (e.g. gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (5) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) e.g. GB-2220221, EP-A-0689454, optionally in the substantial absence of alum when used with pneumococcal saccharides e.g. WO 00/56358; (6) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions (see, e.g. EP-A-0835318, EP-A-0735898, EP-A-0761231); (7) oligonucleotides comprising a CpG motif containing at least one CG dinucleotide, where the cytosine is unmethylated (see, e.g., WO 96/02555, WO 98/16247, WO 98/18810, WO 98/40100, WO 98/55495, WO 98/37919 and WO 98/52581); (8) a polyoxyethylene ether or a polyoxyethylene ester (see, e.g. WO 99/52549); (9) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol (WO 01/21207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (WO 01/21152); (10) a saponin and an immunostimulatory oligonucleotide (e.g. a CpG oligonucleotide) (WO 00/62800); (11) an immunostimulant and a particle of metal salt (see, e.g. WO 00/23105); (12) a saponin and an oil-in-water emulsion (see e.g. WO 99/11241); (13) a saponin (e.g. QS21)+3dMPL+IM2 (optionally including a sterol) (see, e.g. WO 98/57659); (14) other substances that act as immunostimulating agents to enhance the efficacy of the composition. Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-25 acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutarninyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE), etc. Also suitable for use is Matrix-M™; Matrix-

M™ is an adjuvant that comprises 40 nm nanoparticles comprising *Quillaja* saponins, cholesterol, and phospholipid. Adjuvants suitable for administration to a human are of particular interest in some cases. In some cases, the adjuvant is one that enhances a CD4+ T helper response to the immunogen.

[00113] In some instances, the adjuvant is MF59, with or without a CpG-containing oligonucleotide. In other instances, the adjuvant is alum, with or without a CpG-containing oligonucleotide. In other instances, the adjuvant is poly(D,L-lactide-co-glycolide), with or without a CpG-containing oligonucleotide. In other instances, the adjuvant is MPL, with or without a CpG-containing oligonucleotide.

METHODS OF ENHANCING AN IMMUNE RESPONSE

[00114] The subject CDN compositions can be utilized in a variety of applications where inhibition of bacteria is of interest. Cyclic dinucleotides (CDNs) are important secondary signaling molecules in bacteria and mammalian cells. CDNs can be secondary messenger molecules that affect numerous responses of a prokaryotic cell. CDNs can also act as agonists of the innate immune response in mammalian cells. Applications of interest include any convenient application where modulation (e.g., enhancement) of an immune response is of interest. The present disclosure provides methods and pharmaceutical compositions for use in such applications.

Methods of modulating an immune response

[00115] Aspects of the present disclosure include a method of modulating an immune response in an individual. The method can include administering to the individual a CDN composition (e.g., as described herein) in an amount effective to modulate an immune response in the individual. Any convenient CDN can be included in the CDN composition. In some cases, the CDN is selected from c-2'AAMP-GMP and c-di-2'AAMP. In certain instances, the CDN composition further includes an antigen component. Any convenient antigen can be included in the subject CDN compositions. The antigen component has a purity of 50 wt% or more, such as 60 wt% or more, 70 wt% or more, 80 wt% or more, 90 wt% or more, 95 wt% or more, 98 wt% or more, or 99 wt% or more. As noted above, in some cases, the composition comprises an adjuvant (e.g., an adjuvant other than a CDN as described herein).

[00116] In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, induces a humoral immune response. In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, induces cellular immune response. In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, induces production of helper T cells (e.g., $CD4^+$ T cells). In some cases, a CDN

composition of the present disclosure, when administered to an individual in need thereof in one or more doses, induces production of cytotoxic T cells (e.g., $CD8^+$ T cells).

[00117] As noted above, in some cases, a CDN composition of the present disclosure comprises: a) a CDN as described herein; and b) an antigen. In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, induces production of antibodies; for example, where the CDN composition comprises an antigen, the CDN composition induces production of antibodies specific for the antigen. In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, where the composition comprises an antigen, induces production of helper T cells specific for the antigen. In some cases, a CDN composition of the present disclosure, when administered to an individual in need thereof in one or more doses, where the composition comprises an antigen, induces production of cytotoxic T cells specific for the antigen.

[00118] Suitable antigens include, but are not limited to, polypeptide antigens, glycoprotein antigens, glycolipid antigens, lipopolysaccharide antigens, and the like. Suitable antigens include, e.g., bacterial antigens, viral antigens, protozoan antigens, cancer-related antigens, and the like. An antigen can be naturally occurring. An antigen can be non-naturally-occurring, e.g., a recombinant or synthetic antigen (e.g., made in a laboratory). In some cases, the antigen is a cancer-related antigen that finds use in an immunotherapy application.

[00119] A CDN composition of the present disclosure, is generally administered in an amount effective to elicit an immune response, e.g., a humoral immune response (e.g., an antibody response) and/or a CTL response, in a mammalian subject. Effective amounts for immunization will vary, and can generally range from about 1 μg to 100 μg per 70 kg patient, e.g., from about 5 μg/70 kg to about 50 μg/70 kg. Substantially higher dosages (e.g. 10 mg to 100 mg or more) may be suitable in oral, nasal, or topical administration routes. The initial administration can be followed by booster immunization of the same CDN composition or a different immunogenic composition. In some instances, a subject method of inducing an immune response involves an initial administration of a CDN composition of the present disclosure, followed by at least one booster, and in some instances involves two or more (e.g., three, four, or five) boosters. The interval between an initial administration and a booster, or between a give booster and a subsequent booster, can be from about 1 week to about 12 weeks, e.g., from about 1 week to about 2 weeks, from about 2 weeks to about 4 weeks, from about 4 weeks to about 6 weeks, from about 6 weeks to about 8 weeks, from about 8 weeks to about 10 weeks, or from about 10 weeks to about 12 weeks.

[00120] In general, immunization can be accomplished by administration a CDN composition of the present disclosure by any suitable route, including administration of the composition orally, nasally, nasopharyngeally, parenterally, enterically, gastrically, topically, transdermally, subcutaneously, intramuscularly, in tablet, solid, powdered, liquid, aerosol form, locally or systemically, with or without added excipients. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980).

[00121] In some embodiments, the subject CDN compositions may be administered in combination with one or more additional compounds or therapies. Combination therapy includes administration of a single pharmaceutical dosage formulation which contains the subject CDN compound and one or more additional agents; as well as administration of the subject compound and one or more additional agent(s) in its own separate pharmaceutical dosage formulation. For example, a subject compound and an antigen agent or immune system modulatory agent can be administered to the patient together in a single dosage composition such as a combined formulation, or each agent can be administered in a separate dosage formulation. Where separate dosage formulations are used, the subject CDN compound and one or more additional agents can be administered concurrently, or at separately staggered times, e.g., sequentially.

[00122] Individuals suitable for administration of a CDN composition of the present disclosure include any individual (e.g., a mammal, such as a human, a non-human primate, a rodent (e.g., a mouse; a rat), a lagomorph (e.g., a rabbit), an ungulate, a dog, a cat, etc.) in whom it is desired to modulate (e.g., increase) an immune response. Individuals include immunologically naïve individuals; individuals who are in need of a booster immunization; etc.

METHODS OF INHIBITING A BACTERIAL BIOFILM IN AN INDUSTRIAL SYSTEM

[00123] The subject CDN compositions can be utilized in a variety of applications where inhibition of bacteria is of interest. Applications of interest include biological sulfate reduction in the oil and gas industry. The generation of hydrogen sulfide (H_2S) as a metabolic end-product of microbial sulfate respiration results in a variety of oil recovery problems, including oil reservoir souring, contamination of crude oil, metal corrosion, and the precipitation of metal sulfides which can subsequently plug pumping wells. Sour service metallurgy for wells, pipelines, and pump systems carry an estimated cost premium of 2% of total project costs at project initiation but may be an order of magnitude higher if retrofitting is required. Sour production facilities also entail additional risks associated with prevention of operator exposure to toxic H_2S; control of oil-wet iron sulfide pads that reduce oil-water separator performance, management of iron

sulfide solids that interfere with produced water cleanup and recycle, and accumulation of iron-sulfide deposits that may foul equipment and enhance equipment corrosion.

[00124] In some instances, CDN compositions produced using the subject methods find use in biocide and inhibitor cocktails that are used in oil recovery systems. A CDN composition of interest can be selected having a defined relative potency and selectivity against a sulfate reducing microorganism (SRM). In such cases the CDN of interest can be termed a CDN inhibitor of a target microbe. The subject methods can be applied to a variety of microbial metabolisms, including any convenient microbial systems of industrial or medical importance e.g. denitrification, sulfur oxidation, dechlorination, methanogenesis, or clinical pathogens to name a few.

[00125] As such, the present disclosure provides a method of inhibiting a bacterial biofilm in an industrial system. Any convenient industrial systems can be targeted by the subject methods. In some embodiments, the method comprises contacting the industrial system with a biocidally-effective amount of a biocide composition comprising a CDN inhibitor of a target microbe, thereby inhibiting formation of bacterial biofilm in the system. In certain cases, the subject CDN composition can inhibit microbial sulfate reduction. In certain cases, the CDN inhibitor sensitizes the microbe to an additional antimicrobial agent, e.g., an antibiotic.

[00126] In certain instances, the CDN inhibitor of a microbe is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3'3' cdiDAP (diaminopurine monophosphate); 3'3' cDAP-G (diaminopurine monophosphate – guanosine monophosphate); 3'3' cDAP-A (diaminopurine monophosphate – adenosine monophosphate); 3'3' cDAP-8oxoG (diaminopurine monophosphate – 8-oxo-guanosine monophosphate); 3'3' cdi8oxoG (8-oxo-guanosine monophosphate); 3'3' c8oxoG-G (8-oxo-guanosine monophosphate – guanosine monophosphate); 3'3' c8oxoG-A (8-oxo-guanosine monophosphate – adenosine monophosphate); 3'3' c2AP-G (2-aminopurine monophosphate – guanosine monophosphate); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); and 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

[00127] The industrial system can be one that is used in the production, transportation, storage, and separation of crude oil and natural gas (e.g., an oil reservoir). In certain instances, the system is a storage system or container, e.g., a vat. In some cases, the industrial system is one that is

used in a hospital, such as a storage system, or medical equipment, or any other system where the inhibition of growth of a biofilm is of interest.

[00128] In certain instances, the method comprises combining the biocide composition (e.g., a CDN composition as described herein) with a water flow in connection with recovery of oil by injection of water. In certain instances, the biocide composition further comprises an additional active agent (e.g., a non-selective biocidal agent or a biofilm dispersant). Aspects of the present disclosure include CDN composition that include a non-naturally occurring CDN (e.g., as described herein) that finds use in the subject methods. The CDN compositions can further include a surfactant, e.g., a biofilm dispersant. The CDN compositions can further include an addition active agent, such as an antibacterial agent. In certain instances, the additional active agent is selected from a peracetic acid, acrolein, formaldehyde, an isothiazolinon-derived biocide (e.g., methylisothiazolinones and their derivatives (e.g., 5-chloro-2-methyl-4-isothiazolin-3-one or 2-methyl-4-isothiazolin-3-one), and a bromide-based biocide. In certain instances, the industrial system comprises a complex microbial ecosystem comprising the SRM. In certain instances, the SRM (e.g., as described herein) produces hydrogen sulfide and the method provides for a reduction in toxicity, risk of explosion and/or corrosion in the system.

[00129] Aspects of the subject methods are described by Yeo, Hammond et al. in "In Vivo Biochemistry: Single-Cell Dynamics of Cyclic Di-GMP in Escherichia coli in Response to Zinc Overload", Biochemistry, Oct 20, 2017, the disclosure of which is herein incorporated by reference in its entirety.

METHODS OF DETECTION

[00130] The subject methods and compositions can be adapted for a variety of diagnostic and research applications, such as any application where the detection of a target bacteria is of interest. The substrate specificity of the CDN-producing enzymes described herein that is utilized to produce CDNs can be applied in an assay to identify the presence of a CDN-producing enzyme, e.g., an enzyme that is present, or suspected of being present in a sample or a target bacteria. Similarly, the CDNs can be applied in an assay or method to detect a CDN-degrading enzyme or other biomolecules of interest which bind to a CDN of interest, such as a CDN-binding protein or nucleic acid (e.g., a CDN binding RNA riboswitch). As such, aspects of the present disclosure include a method of detecting target bacteria in a sample. In some embodiments, the method comprises: contacting a sample suspected of comprising target bacteria with a precursor of a CDN under conditions sufficient to convert the precursor to a CDN in the presence of a CDN-producing enzyme. The CDN precursor(s) which are utilized can be selected according to the target enzyme and CDN product to provide for detection of the CDN-producing enzyme in the sample. In some cases, the sample includes, or is suspected of

including, a target bacteria can comprise the CDN-producing enzyme. The subject methods can be adapted to provide a detectable signal upon production of the CDN by the CDN-producing enzyme.

[00131] Any convenient methods of detection of one or more components of the subject methods can be utilized in the subject methods of detection, such as spectroscopic methods (e.g., fluorescence), mass spectrometry methods. In some cases, one or more of the components of the subject methods (e.g., the CDN precursor) is directly or indirectly detected, e.g., via an inherent spectroscopic property of the component, or via the introduction of a detectable label or a chemoselective functional group. A variety of detection methods can be adapted for use in conjunction with the CDN precursors and products.

[00132] In some cases, the CDN precursor and/or the CDN that is produced has a fluorescence property that can be detected. For example, FIG. 13 illustrates the fluorescence emission spectra (excitation 310nm) of the CDN 3',3'-cyclic di-2AP and its CDN nucleotide precursor 2APTP (2-aminopurine triphosphate), where the precursor is more fluorescent than the CDN produced. In some cases, the precursor is fluorescent and the CDN is non-fluorescent. In certain instances, the precursor and the CDN product have different fluorescent properties. In some instances, a detectable decrease in fluorescence would provide for detection and/or quantitation of the enzyme activity in the sample.

[00133] In some cases, detecting a change in fluorescence would correlate to the conversion of the precursor to the CDN, in the presence of the target enzyme, and in some cases target bacteria. The subject method can further comprise quantitating the change in fluorescence and correlating the change to an amount of target enzyme, and thus target bacteria. The subject methods can be applied to any convenient bacteria and bacterial samples, e.g., that include a CDN-producing enzyme. In some cases, the target bacterium is a Geobacter. In certain cases, the CDN producing-enzyme is a Hypr GGDEF.

Examples of Non-Limiting Aspects of the Disclosure

[00134] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure numbered 1-56 are provided below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

[00135] Aspect 1. An in vitro method of preparing a cyclic dinucleotide (CDN), the method comprising:

a) contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN; and b) isolating the CDN.

[00136] Aspect 2. The method of aspect 1, wherein the contacting comprises contacting an isolated and purified CDN producing-enzyme.

[00137] Aspect 3. The method of any one of aspects 1-2, wherein the method comprises contacting the CDN producing-enzyme with a first precursor of the CDN and a second precursor of the CDN.

[00138] Aspect 4. The method of aspect 3, wherein the first and second precursors of the CDN are each a nucleotide triphosphate.

[00139] Aspect 5. The method of any one of aspects 1-4, wherein the precursor of a CDN is a nucleotide triphosphate (NTP) that is selected from ATP, GTP, ITP, ATP(S), GTP(S), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate.

[00140] Aspect 6. The method of any one of aspects 1-5, wherein the CDN producing-enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN.

[00141] Aspect 7. The method of aspect 6, wherein the Hypr GGDEF enzyme comprises an active site mutation.

[00142] Aspect 8. The method of any one of aspects 6-7, wherein the Hypr GGDEF enzyme is selected from GSU1658 (HyprA), Mxan 2643 and Bd0367.

[00143] Aspect 9. The method of any one of aspects 1-5, wherein the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN.

[00144] Aspect 10. The method of any one of aspects 1-5, wherein the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

[00145] Aspect 11. The method of any one of aspects 1-10, wherein the CDN is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3'3' cdiDAP (diaminopurine monophosphate); 3'3' cDAP-G (diaminopurine monophosphate – guanosine monophosphate); 3'3' cDAP-A (diaminopurine monophosphate – adenosine monophosphate); 3'3' cDAP-8oxoG (diaminopurine monophosphate – 8-oxo-guanosine monophosphate); 3'3' cdi8oxoG (8-oxo-guanosine monophosphate); 3'3' c8oxoG-G (8-oxo-guanosine monophosphate

– guanosine monophosphate); 3'3' c8oxoG-A (8-oxo-guanosine monophosphate – adenosine monophosphate); 3'3' c2AP-G (2-aminopurine monophosphate – guanosine monophosphate); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); and 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

[00146] Aspect 12. The method of aspect 11, wherein the CDN is 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP).

[00147] Aspect 13. The method of aspect 11, wherein the CDN is 3',3'-cyclic di-inosine monophospate (c di-IMP).

[00148] Aspect 14. The method of aspect 11, wherein the CDN is 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)).

[00149] Aspect 15. The method of aspect 11, wherein the CDN is 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)).

[00150] Aspect 16. The method of aspect 11, wherein the CDN is 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP).

[00151] Aspect 17. A method of synthesizing a cyclic dinucleotide (CDN), the method comprising:

a) culturing a genetically modified host cell in a culture medium comprising a precursor of the CDN, wherein the genetically modified host cell comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN; and b) recovering the CDN from the culture medium.

[00152] Aspect 18. The method of aspect 17, further comprising contacting the cell culture with a first precursor of the CDN and a second precursor of the CDN.

[00153] Aspect 19. The method of aspect 18, wherein the first and second precursors of the CDN are each a nucleotide triphosphate.

[00154] Aspect 20. The method of any one of aspects 17-19, wherein the precursor of a CDN is a nucleotide triphosphate (NTP) that is selected from ATP, GTP, ITP, ATP(S), GTP(S), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate.

[00155] Aspect 21. The method of any one of aspects 17-20, wherein the CDN is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S));

2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP); and 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP).

[00156] Aspect 22. The method of any one of aspects 17-21, wherein the CDN producing-enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN.

[00157] Aspect 23. The method of aspect 22, wherein the Hypr GGDEF enzyme is selected from GSU1658 (HyprA), Mxan 2643 and Bd0367.

[00158] Aspect 24. The method of any one of aspects 17-21, wherein the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN.

[00159] Aspect 25. The method of any one of aspects 17-21, wherein the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

[00160] Aspect 26. The method of any one of aspects 17-25, wherein the genetically modified host cell is a prokaryotic cell.

[00161] Aspect 27. The method of any one of aspects 17-25, wherein the genetically modified host cell is a bacterial cell.

[00162] Aspect 28. The method of any one of aspects 17-25, wherein the genetically modified host cell is a eukaryotic cell.

[00163] Aspect 29. The method of any one of aspects 17-21, wherein the genetically modified host cell is a yeast cell or a fungal cell.

[00164] Aspect 30. A genetically modified host cell that produces a cyclic dinucleotide (CDN), the cell comprising a heterologous coding sequence encoding a CDN-producing enzyme within a pathway that converts a precursor for the CDN to a CDN.

[00165] Aspect 31. The cell of aspect 30, wherein the a CDN-producing enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN.

[00166] Aspect 32. The cell of aspect 30, wherein the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN.

[00167] Aspect 33. The cell of aspect 30, wherein the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

[00168] Aspect 34. The cell of any one of aspects 30-33, wherein the genetically modified host cell is selected from a prokaryotic cell, a bacterial cell, a eukaryotic cell, a yeast cell and a fungal cell.

[00169] Aspect 35. A composition comprising: a CDN selected from c-2'AAMP-GMP and c-di-2'AAMP.

[00170] Aspect 36. The composition of aspect 35, further comprising an antigen.

[00171] Aspect 37. The composition of aspect 36, wherein the antigen component of the composition has a purity of 50 wt% or more.

[00172] Aspect 38. A pharmaceutical composition comprising the composition of any one of aspects 35-37 and a pharmaceutically acceptable excipient.

[00173] Aspect 39. A method of modulating an immune response in an individual, the method comprising administering to the individual a CDN composition in an amount effective to modulate an immune response in the individual.

[00174] Aspect 40. The method of aspect 39, wherein the CDN composition comprises: a CDN selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3'3' cdiDAP (diaminopurine monophosphate); 3'3' cDAP-G (diaminopurine monophosphate – guanosine monophosphate); 3'3' cDAP-A (diaminopurine monophosphate – adenosine monophosphate); 3'3' cDAP-8oxoG (diaminopurine monophosphate – 8-oxo-guanosine monophosphate); 3'3' cdi8oxoG (8-oxo-guanosine monophosphate); 3'3' c8oxoG-G (8-oxo-guanosine monophosphate – guanosine monophosphate); 3'3' c8oxoG-A (8-oxo-guanosine monophosphate – adenosine monophosphate); 3'3' c2AP-G (2-aminopurine monophosphate – guanosine monophosphate); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); and 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

[00175] Aspect 41. The method of aspect 40, wherein the CDN composition further comprises an antigen.

[00176] Aspect 42. The method of aspect 41, wherein the antigen component of the composition has a purity of 50 wt% or more.

[00177] Aspect 43. A method of inhibiting a bacterial biofilm in an industrial system, the method comprising: contacting the industrial system with a biocidally-effective amount of a biocide composition comprising a CDN inhibitor of a target microbe, thereby inhibiting formation of bacterial biofilm in the system.

[00178] Aspect 44. The method of aspect 43, wherein the CDN inhibitor inhibits microbial sulfate reduction.

[00179] Aspect 45. The method of any one of aspects 43-44, wherein the CDN inhibitor is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S));

3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3'3' cdiDAP (diaminopurine monophosphate); 3'3' cDAP-G (diaminopurine monophosphate – guanosine monophosphate); 3'3' cDAP-A (diaminopurine monophosphate – adenosine monophosphate); 3'3' cDAP-8oxoG (diaminopurine monophosphate – 8-oxo-guanosine monophosphate); 3'3' cdi8oxoG (8-oxo-guanosine monophosphate); 3'3' c8oxoG-G (8-oxo-guanosine monophosphate – guanosine monophosphate); 3'3' c8oxoG-A (8-oxo-guanosine monophosphate – adenosine monophosphate); 3'3' c2AP-G (2-aminopurine monophosphate – guanosine monophosphate); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); and 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

[00180] Aspect 46. The method of any one of aspects 43-45, wherein the industrial system is used in the production, transportation, storage, and separation of crude oil and natural gas (e.g., an oil reservoir).

[00181] Aspect 47. The method of any one of aspects 43-46, wherein the industrial system is used in a hospital.

[00182] Aspect 48. The method of any one of aspects 43-44, wherein the method comprises combining the biocide composition with a water flow in connection with recovery of oil by injection of water.

[00183] Aspect 49. The method of any one of aspects 43-48, wherein the biocide composition further comprises an additional agent selected from a non-selective biocidal agent and a biofilm dispersant (e.g., a surfactant).

[00184] Aspect 50. The method of aspect 49, wherein the additional active agent is selected from a peracetic acid, acrolein, formaldehyde, an isothiazolinon-derived biocide (e.g., methylisothiazolinones and their derivatives (e.g., 5-chloro-2-methyl-4-isothiazolin-3-one or 2-methyl-4-isothiazolin-3-one), and a bromide-based biocide.

[00185] Aspect 51. A method of detecting target bacteria in a sample, the method comprising: contacting a sample suspected of comprising target bacteria with a precursor of a CDN under conditions sufficient to convert the precursor to a CDN in the presence of a CDN-producing enzyme, wherein the target bacteria comprises a CDN-producing enzyme; and detecting whether a change in fluorescence correlating to the conversion of the precursor to the CDN occurs.

[00186] Aspect 52. The method of aspect 51, further comprising quantitating the change in fluorescence and correlating the change to an amount of target bacteria.

[00187] Aspect 53. The method of any one of aspects 51-52, wherein the precursor of a CDN is a nucleotide triphosphate (NTP) that is selected from ATP, GTP, ITP, ATP(S), GTP(S), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate.

[00188] Aspect 54. The method of any one of aspects 51-53, wherein the precursor is fluorescent and the CDN is non-fluorescent.

[00189] Aspect 55. The method of any one of aspects 51-54, wherein the target bacteria is a Geobacter.

[00190] Aspect 56. The method of any one of aspects 51-55, wherein the CDN producing-enzyme is a Hypr GGDEF.

EXAMPLES

[00191] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Enzymatic Synthesis of Cyclic Dinucleotide Analogs

Protein expression and purification

[00192] Briefly, a protein expression vector containing the MBP- and His-tagged enzyme was transformed into chemically competent BL21* *E. coli* with the pRARE vector. Hypr GGDEF enzymes were mutated at an arginine residue in the inhibitory site to promote increased activity. Proteins were expressed overnight in autoinducing media and cells were pelleted for storage at -80 °C until purification.

[00193] Frozen cells were thawed and lysed by sonication. His-tagged protein was purified from clarified lysate by Ni-NTA chromatography. Eluted protein was concentrated and flash frozen for storage at -80 °C for use in later in vitro assays.

In vitro enzymatic synthesis

[00194] In vitro enzyme assays performed as described previously in Hallberg et al. with modifications. Enzyme (5-10 μM) and NTPs (100 μM-1 mM) were combined in buffered solution containing 40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 5 mM DTT. Mouse cGAS conditions were adopted from Mitchison and colleagues "Hydrolysis of 2'3'-cGAMP by ENPP1 and design of nonhydrolyzable analogs", Nature Chemical Biology, pp 1043-1048, October 26, 2014, vol. 10, which includes addition of 0.1 mg/mL HT-DNA for cGAS activation. 50 μL scale reactions were used in pilot experiments and scaled up to 1 mL total volume in preparation for product purification. Reactions were performed for 16-20 h overnight and protein was heat inactivated post-reaction for 3 min at 95 °C. Precipitated protein was pelleted at 13,000 rpm for 3 min and supernatant was used for purification. Success of reaction was determined by LC-MS.

High-performance liquid chromatography (HPLC) purification of CDN analogs

[00195] 100 μL of enzymatic reaction were injected into an Agilent 1260 HPLC. A C18-amide column (4.6 x 250 mm) was used for separation with a gradient of 100% A, 0% B to 0% A, 100% B over 20-30 minutes. Solvent A was H_2O +0.05% TFA and Solvent B was MeCN +0.05% TFA. Small adjustments to the slope of the gradient were required to improve separation of some analogs. Separation was monitored by UV at 254 nm. 0.25-0.5 mL fractions were collected and product fractions combined. Solvent was removed by rotary evaporation and cyclic dinucleotides were resuspended in H_2O.

Thin layer chromatography analysis of activation of CDN production of a Hypr GGDEF enzyme

[00196] CDN production from a Hypr GGDEF enzyme (MXAN_2643) was investigated under the following experimental conditions: 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 5 mM DTT; 500 nM MXAN_2643 R292A, 100 μM GTP (+GTPα^{P32}), and +/- 50 μM cAMP or cGMP. Thin layer chromatography analysis of these experiments is shown in FIG. 14 and illustrates the activation of CDN production of a Hypr GGDEF enzyme (MXAN_2643) through addition of cAMP as the activating agent. +cAMP exhibited 19.9 fold activation of CDNs while +cGMP exhibited 2.2 fold activation of CDN production.

Example 2: Sequences of Exemplary CDN-Producing Enzymes

[00197] **GSU1658**

[00198] Genbank WP_010942302; Uniprot Q74CL4

```
         10         20         30         40         50
MERILVVEDD RFFRQMYVDL LKEEGYEVDT VASGTEGLKR LEKQEYHLVI
         60         70         80         90        100
TDLVMPGMSG IEVLSRVKQK APNVDVILVT GHANLESAVY ALKNGARDYI
        110        120        130        140        150
LKPFNHDEFK HTVALCFEQR RLINENYELK ELLNLFQVGQ NIANCIDLER
        160        170        180        190        200
LSAVVVDAFC KEVGVSRAIG LFPEKSEPHA LKELRGLEPE VAAALAEKAL
        210        220        230        240        250
TLCSDAAETA GGFRRLDGSH FSDGLLRTAG INGALVVSIR QRTLLQGVLL
        260        270        280        290        300
LVNDQGKPFP AVFKHKSIQF LLEQASLAFD NALRYSSARD MLYVDELTGL
        310        320        330        340        350
FNYRYLDISL DRELKRADRF GSVVSMIFID MDHFKGVNDT HGHLFGSQVL
        360        370        380        390        400
HEVGQLLKKS VREVDVIIRY GGDEFTIILV ETGEKGAATV AERIRRSIED
        410        420        430        440        450
HHFLASEGLD VRLTASLGYA CYPLDTQSKM ELLELADKAM YRGKEEGKNR
     458
VFRATAIR   (SEQ ID NO:1)
```

[00199] **Mxan2643**

[00200] Genbank WP_011552713.1; UniProt Q1D911

```
    10         20         30         40         50
MNPADLLSAM KRTVEQLAAF NEMAKALTST LELREVLALV MQKVSSLLLP
         60         70         80         90        100
RNWSLILQDE RTGKLYFEIA VGDGADVLKG LQLNPGEGIA GAVFTSGAAR
        110        120        130        140        150
LVHDVGGDPS FSPRFDEASA FHTRSILAVP LLARGRVLGI IELVNGPMDP
        160        170        180        190        200
PFTNEDLTIL TAIADYAAIA IENARNFRRV QELTITDEHT GCYNARHLRA
        210        220        230        240        250
LLDQEVKRSE RFSHPLSLVF LDLDHFKSIN DTHGHLVGSA TLKEVGDLLM
        260        270        280        290        300
TLGRQNLDAV FRYGGDEFAM LLVETDPEGA AVIGQRVCEA FRGRGFLLEQ
        310        320        330        340        350
GLDVRLTASV GVATYPDHAS SALDLIRAAD FAMYAAKARG RDALCIAEPI
        360
APNGGTGSHE FPER   (SEQ ID NO:2)
```

[00201] **Mxan4463**

[00202] Genbank WP_011554461.1 ; UniProt Q1D3Y9

```
  10         20         30         40         50
MARILLVDDE KIARTLYGDY LTAVGHAVTA VGTLQEAKEA LAGDRFDAVV
         60         70         80         90        100
TDLILPGGDG MEVLRHVREH HPGVEVVVIT GLEKVDPAVR AIKSGAAEYL
        110        120        130        140        150
VKPVAPEALQ HAVRRALTTR DLMQENASLR RHVAMLEAGQ RIATTLDREK
```

```
       160        170        180        190        200
LASATASALQ SMASASAVVL LERDSAFALR RHGTSGLSTA LEEPLIAELI
       210        220        230        240        250
ERLTNERGPR ELDGMDAPFP RAISFPALEG DAVLGHAVLF FGGTGAEWAG
       260        270        280        290        300
ETASFLVRNW ALALRNLGRF AAVEDLAYVD DLTRLFNTRY LHLVVDREVQ
       310        320        330        340        350
DALQSQRTFS LLFLDLDHFK SINDTHGHLV GSKVLVEAAR VVKGCVRDHD
       360        370        380        390        400
VVARYGGDEY VVVLRNTDSG GALKVAERIR RTMETHNFLA REGLSLKLTT
       410        420        430        440        450
CIGVASFPEH AQDKATLLDL SDRAMYRGKR GSRNVVYMAA KDLEAPPAER

RQAHSAS (SEQ ID NO:3)
```

[00203] **Ddes1475**

[00204] Genbank WP_000425918.1; UniProt B9J0V0

```
10         20         30         40         50
MEMRHVKTFC AIVKYGSFSK AAHALGYAQS TVTAHMKALE NDLHIPLFDR
        60         70         80         90        100
LGKKVLLTKA GHQFHPYALE LLAIYEKAQE IPQNTDQLEG TLSITSNESL
       110        120        130        140        150
AVYRLPQLLR TYKQKNPKVN IVLETNTNEQ ALQKLREGET DVVFIIGESI
       160        170        180        190        200
EHNDFITRTF SNETFGWILP PHYCIHSNPF YLLKDTQFIF TEQSCGYRPM
       210        220        230        240        250
VDRFLRQSGN IPAKTFETSN VEVIKQSVMC ELGISILPYI VVQESCQKEQ
       260        270        280        290
LCFQPIETPT VIQSHVIYHK SRWISPVVQS FLSLLKVMKV  (SEQ ID NO:4)
```

[00205] **Bd0367 WT ***

[00206] Genbank WP_011162957.1;UniProt Q6MQU2

```
10         20         30         40         50
MSRAEVTLVC KMSFEVSPKQ PKSRRILVID DDKDSLEILL EPLRWEGYDA
        60         70         80         90        100
RGVTTEAEAH KLIESWIPHI VILDWMAPSM AGLRVLKSVR ERLSHVSCVF
       110        120        130        140        150
VSENSSTEAI IEALDSGADD YIVKPFVPLE LLARIRSQLR IRDLHEQLLF
       160        170        180        190        200
ANEKLKELVD TDDLTGLYNM RSLYQRLDFE MERGRRFHRD VCVVMMDMDY
       210        220        230        240        250
FKTVNDGHDH LFGSYVLSEV GKIIRANTRN IDIPARYGGD EFLMVLTETN
       260        270        280        290        300
HAGAMYFCER LRENIEKTTF RNGEDSMKLT ASLGFAITIP GENISARELV
       310        320        330        340
RRADHALYQA KRAGRNQVAH YKPESAPVVE IKSAVHKRRK AAG (SEQ ID NO:5)
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[00207] **CabtherA_1065 WT ***

[00208] Genbank WP_014099557.1; UniProt G2LH77

```
10          20          30          40          50
MNLKLGAILR PVNSLNQTQK LQANPLARPR QARPALVHMR GDYLGSSFRI
         60          70          80          90         100
EHAITRIGRG SDAELRLEND DEASRLHARI ERLETPTGHF QYWLTDLRST
        110         120         130         140         150
NGTQLNGIPL VPGEAVLLHD GDKFSIGRHI LKFTFLDDID EEFHRRITEL
        160         170         180         190         200
ITHDDLTGLL NRKSFILEMQ REMARSNRYG HPFGLLMMDI DHFKRVNDTY
        210         220         230         240         250
GHLVGSQVLR EVATVIRETL RDSDIAGRYG GEEYIALLPE TDRLRAHEAA
        260         270         280         290         300
ERIRQAIERT PFTASLNVPH HKLRLTISIG IASYPGDAAQ INDLIQRADE
        310         320         330         340         350
AMYEAKRRGR NLVQTTGQSA ANRATPPSLP LPPPSGDDSP TEHLTVEQPQ

PVKP  (SEQ ID NO:6)
```

[00209] **DEFDS_0689 R248A ***

[00210] Genbank WP_013007417.1; UniProt D3PC46

```
  10          20          30          40          50
MYESLKRNIF VILTSILLIY ETYNKTNENL LLLTSLLLTC YIAATLIKKV
         60          70          80          90         100
ELDEVLFALF VILIGYLSIA NREFIYFQIL AITFLVFDSK FYVIKVILAI
        110         120         130         140         150
LLILFDLFYL NISILSTFSL MILYSLFFSI FIKLLIDRLE EEIDELSITD
        160         170         180         190         200
DLTGLLNQKG FLKKFEEEYY RSVRYKKNFT VIMLDSDDLK KVNDTYGHKY
        210         220         230         240         250
GTKVILFIAD EIKKNIRRTD FACRYGGDEF MICLVETPIN NGKIFAERLK
        260         270         280         290         300
NNIAMKPVFT DKGRGFNVTV SVGVVGYPHT SEKSFELLDL VDKALYEAKN
        310
KGKNRVEILT KNSSL (SEQ ID NO:7)
```

[00211] **CaIni_1629 R268A ***

[00212] Genbank WP_013451747.1; UniProt E4TFG3

```
10          20          30          40          50
MIDNKIKHFQ YKIAEVLYLF AFTIIIASIK FLDSTSNKAN YAILVFFLIF
         60          70          80          90         100
IILKFSIDDN LFSSKILSYY LLFQSQNIFA AFINGSTPNL LIFMSMLGLL
        110         120         130         140         150
IFSIVLYDKK YLVVHFIVTG ILAFVFFTTF DSKESFVFFI SLPFIFIISL
        160         170         180         190         200
NFNKIYITTR NLITELSITD EMTGLLNQSG FMKKIEEEFY RSQRYQKTFS
        210         220         230         240         250
VLMIDSDNLK LINDTYGHKY GSIVIKSIAE VIKTNIRRTD FAARYGGDEF
        260         270         280         290         300
ILCLVETDLD GALEVAERIR KQFELKSFFT KDEKKFTITI SIGVSNYPKS
```

```
        310         320         330
GDSLMDVIEL ADKAMYHSKN SGKNKTSFLL KN (SEQ ID NO:8)
```

[00213] **ACP_2467 WT ***

[00214] Genbank WP_015897549.1; UniProt C1F1G0

```
10          20          30          40          50
MDAHTIVSLP PTWNQGMSAE ARNQNWKDLV VFHNLARALT SSLELDSVLH
         60          70          80          90         100
AIMEQMRQFF EPETWSLLIL DETTQELYYA VAVGQSEAAL RNVRVPLGEG
        110         120         130         140         150
MAGWVAQHGE SLIVPDLEQD PRFAATSDAR TPMRSAICMP LLSRQRTLGV
        160         170         180         190         200
IQLFNCRLES MTEYTISFLH ILCDYAAIAI ENARAVEKIQ ALTITDDCTG
        210         220         230         240         250
LYNQRHLQQK IEEEVTRARR HHHPFSVIFL DLDHFKQIND QHGHLIGSRL
        260         270         280         290         300
LAGIGQCLRL HIRPGDHAFR YGGDEFILLL PETTKAEAEQ IARNLRQKLR
        310         320         330         340         350
SHVFEMGSDL RLQVSASFGV ASFPEDGRTG HQIIRMADAM MYLVKGSTRD
        360
DVAVADRNTE LLRNS  (SEQ ID NO:9)
```

Example 3

[00215] Cyclic dinucleotides (CDNs) are second messengers that dictate diverse cellular processes in bacteria and mammals and serve as promising scaffolds for the evolution of new signaling systems. Yet, limitations of natural CDN synthases and their ability to communicate orthogonally hinder progression in this field. The discovery of hybrid CDN-producing, promiscuous substrate-binding (Hypr) GGDEFs in deltaproteobacteria demonstrated a canonical cyclic di-GMP-synthesizing domain has evolved toward new functions, specifically the production of the heterodimeric cyclic AMP-GMP. Cyclic AMP-GMP can act as an intracellular signal orthogonal to cyclic di-GMP in Geobacter and controls growth on insoluble metal surfaces, whereas cyclic di-GMP can control electrode growth. Hypr GGDEF substrate selectivity may extend beyond natural nucleotides. A Hypr GGDEF from Myxococcus was profiled with a set of nucleotide analogs varying in nucleobase, ribose, and phosphate functionality. This Hypr GGDEF was able to synthesize a wide pool of unnatural CDNs, with unexpected substrate selectivity and functional group dependence. Together, this sets the stage for engineering new CDN signaling circuits with the ability to communicate in the complex cellular environment.

[00216] Cyclic dinucleotides (CDNs) are ubiquitous signaling molecules in bacteria. CDNs control bacterial processes including biofilm formation, intestinal colonization, cell division, cell

wall homeostasis, and exoelectrogenesis. A role was elucidated for bacterial cyclic AMP-GMP (cAG) in the deltaproteobacterium Geobacter, controlling genes associated with Fe(III) reduction. Deconstruction of this pathway led to the discovery of a new subclass of enzymes, Hypr GGDEFs, responsible for production of cAG in this organism. Prior to this discovery, GGDEF enzymes were known to only synthesize the CDN cyclic di-GMP (cdiG), unraveling 30 years of our understanding of this enzyme class. Interestingly, Hypr GGDEFs exhibit promiscuous enzyme activity, able to produce all three known bacterial CDNs. It was hypothesized that this unique activity may allow Hypr GGDEFs to accept unnatural substrates beyond those known to current bacterial CDN signaling.

Results and Analysis

Cyclic dinucleotides control distinct growth phenotypes in Geobacter.

[00217] The environmental bacterium Geobacter has an impressive ability to use diverse metals as its terminal electron acceptor, but how the organism senses, communicates, and transform this environmental signal to a phenotype is poorly understood. The work elucidates how CDNs can control this process. Deletion of the Hypr GGDEF GSU1658 in Geobacter induces a noticeable growth defect specifically under insoluble Fe(III) Oxide (FIG. 16), whereas deletion of the diguanylate cyclase EsnD produces a defect on electrodes (FIG. 17). This is the first evidence of two CDNs acting is response to different environmental stimuli. This work can illuminate the phosphorylation network between a hypothetical cognate histidine kinase and GSU1658, as well as identification of primary signals triggering this pathway (see FIG. 15).

Hypr GGDEFs Accept Unnatural NTP Substrates

[00218] Recent work by Hallberg et al. demonstrated the promiscuity of a new subclass of GGDEF enzymes, Hypr GGDEFs. It was hypothesized that this unique activity may allow acceptance of unnatural NTP substrates. An enzymatic activity profile of a Hypr GGDEF was obtained with a set of NTPs unknown to biologically relevant CDN synthases. Substrate dependence of the related synthase Mxan2643 from Myxococcus is demonstrated herein. This is the first demonstration of a CDN synthase able to generate a wide array of unnatural CDNs and is a promising protein class for engineering of new signaling systems.

[00219] FIG. 18 illustrates that the CDN producing-enzymes have wide nucleobase acceptance in their substrates and can generate a wide array of unnatural CDNS of interest. In contrast WspR, a canonical GGDEF, only accepts 8-oxoGTP in its substrate.

[00220] Ribose functionality influences selectivity of the enzymes. For adenosine, a 2'-OH was needed while 2'deoxy, 2'-fluoro and 2'-amino were not preferred. However, guanosine substrates permit substitutions at the 2'-position. In some cases, removal of the 3'-OH of the substrate yielded no product, and 2'-amino substitution disrupted catalysis by the enzyme.

Preliminary Kinetics Demonstrate Effect of ATP Ribose Analogs

[00221] Measured initial rates for the Hypr GGDEF Mx2643 with four unique NTP substrates (FIG. 19) fit to a two substrate model (FIG. 21). An enzyme- coupled pyrophosphatase assay was used to measure initial rates (FIG. 20). Results show a stark change in binding and catalysis when changes are made to the ATP substrate.

[00222] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. An *in vitro* method of preparing a cyclic dinucleotide (CDN), the method comprising:

a) contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN; and

b) isolating the CDN.

2. The method of claim 1, wherein the contacting comprises contacting an isolated and purified CDN producing-enzyme.

3. The method of claim 1, wherein the method comprises contacting the CDN producing-enzyme with a first precursor of the CDN and a second precursor of the CDN.

4. The method of claim 1, wherein the precursor of a CDN is a nucleotide triphosphate (NTP) that is selected from ATP, GTP, ITP, ATP(S), GTP(S), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate.

5. The method of claim 1, wherein the CDN producing-enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN.

6. The method of claim 1, wherein the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN.

7. The method of claim 1, wherein the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

8. The method of claim 1, wherein the CDN is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP); 3'3' cdiDAP (diaminopurine monophosphate); 3'3' cDAP-G (diaminopurine monophosphate – guanosine monophosphate); 3'3' cDAP-A (diaminopurine monophosphate – adenosine monophosphate); 3'3' cDAP-8oxoG (diaminopurine monophosphate – 8-oxo-guanosine

monophosphate); 3'3' cdi8oxoG (8-oxo-guanosine monophosphate); 3'3' c8oxoG-G (8-oxo-guanosine monophosphate – guanosine monophosphate); 3'3' c8oxoG-A (8-oxo-guanosine monophosphate – adenosine monophosphate); 3'3' c2AP-G (2-aminopurine monophosphate – guanosine monophosphate); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); and 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP).

9. A method of synthesizing a cyclic dinucleotide (CDN), the method comprising:

a) culturing a genetically modified host cell in a culture medium comprising a precursor of the CDN, wherein the genetically modified host cell comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN; and

b) recovering the CDN from the culture medium.

10. The method of claim 9, further comprising contacting the cell culture with a first precursor of the CDN and a second precursor of the CDN, wherein the first and second precursors of the CDN are each a nucleotide triphosphate.

11. The method of claim 9, wherein the precursor of a CDN is a nucleotide triphosphate (NTP) that is selected from ATP, GTP, INP, ATP(S), GTP(S), CTP(S), GTP(S), 2-aminopurine triphosphate, 2'-amino-adenosine triphosphate, 2,6-diaminopurine (DAP) triphosphate and 8-oxo-guanosine triphosphate.

12. The method of claim 9, wherein the CDN is selected from 3',3'-cyclic di-2-aminopurine monophosphate (c-di-2APMP); 2',3'cyclic-2-aminopurine monophosphate-guanosine monophosphate (2',3'-c-2APMP-GMP); 3',3'-cyclic di-inosine monophospate (c di-IMP); 3',3'cyclic-di-guanosine monothiophosphate (c-di-GMP (S)); 3',3'cyclic-adenosine monothiophosphate-guanosine monothiophosphate (c-AMP-GMP (S)); 2',3'cyclic-(2'amino)-adenosine monophosphate-guanosine monophosphate (c-2'AAMP-GMP); and 3',3'cyclic-di-(2'amino)-adenosine monophosphate (c-di-2'AAMP).

13. The method of claim 9, wherein:

the CDN-producing enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN;

the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN; or

the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

14. The method of claim 9, wherein the genetically modified host cell is selected from a prokaryotic cell, a bacterial cell, a eukaryotic cell, a yeast cell and a fungal cell.

15. A genetically modified host cell that produces a cyclic dinucleotide (CDN), the cell comprising a heterologous coding sequence encoding a CDN-producing enzyme within a pathway that converts a precursor for the CDN to a CDN.

16. The cell of claim 15, wherein the a CDN-producing enzyme is a Hypr GGDEF enzyme and the CDN is a 3',3'-CDN.

17. The cell of claim 15, wherein the CDN producing-enzyme is a cGAS enzyme and the CDN is a 2',3'-CDN.

18. The cell of claim 15, wherein the CDN producing-enzyme is a DncV enzyme and the CDN is a 3',3'-CDN.

19. The cell of claim 15, wherein the genetically modified host cell is selected from a prokaryotic cell, a bacterial cell, a eukaryotic cell, a yeast cell and a fungal cell.

FIG. 1

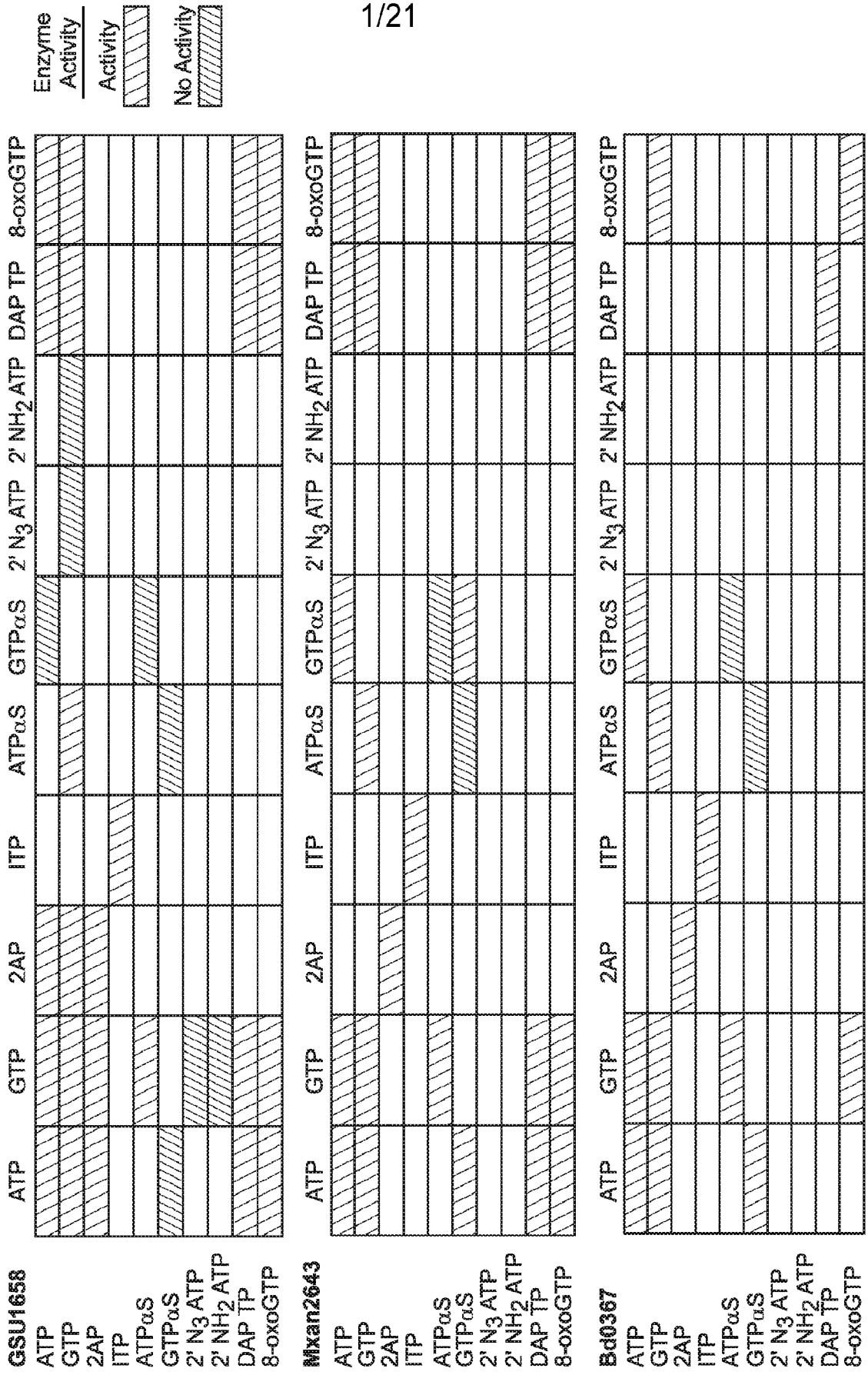

Enzyme Activity
Activity
No Activity
ATP
GTP
2AP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DAP TP
8-oxoGTP
GSU1658
ATP
GTP
2AP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DAP TP
8-oxoGTP
Mxan2643
ATP
GTP
2AP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DAP TP
8-oxoGTP
Bd0367
ATP
GTP
2AP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DAP TP
8-oxoGTP

FIG. 2

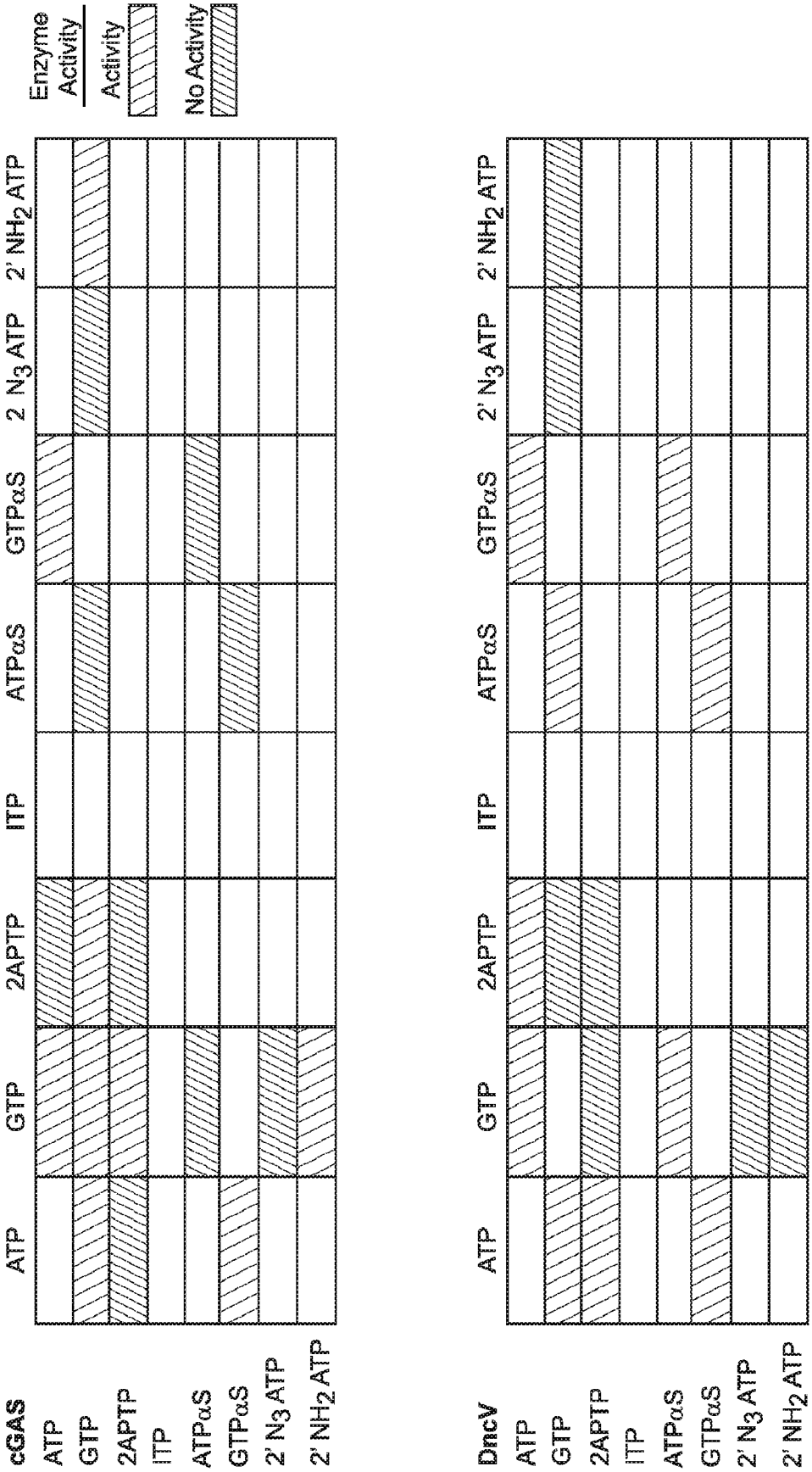
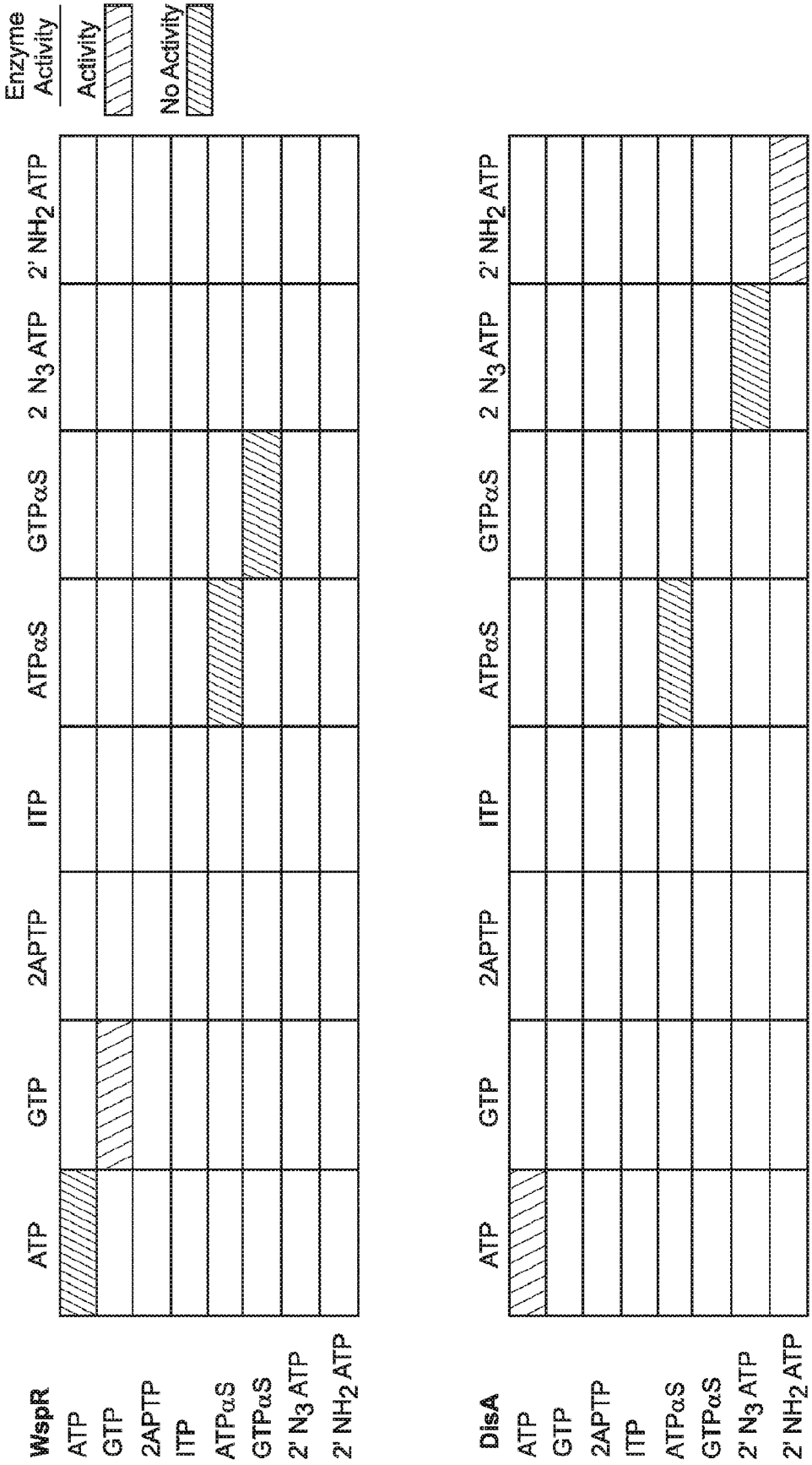

Enzyme Activity
Activity
No Activity
cGAS
ATP
GTP
2APTP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DncV
ATP
GTP
2APTP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP

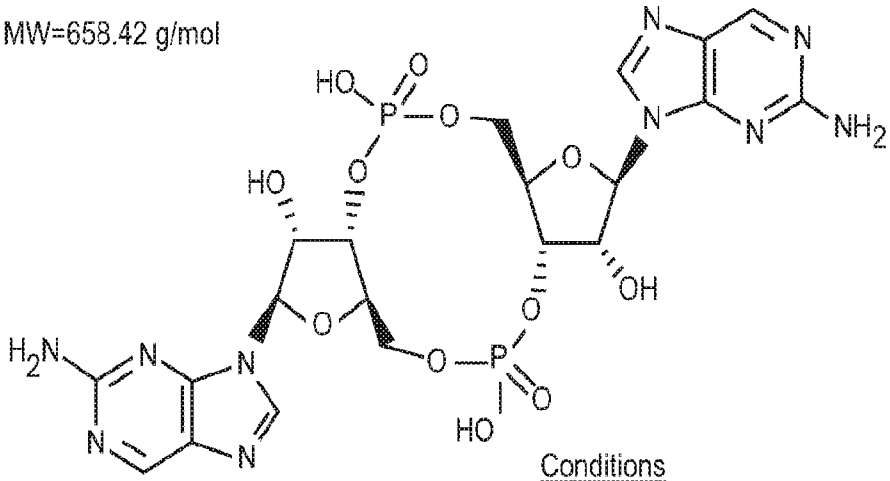
FIG. 3


Enzyme Activity
Activity
No Activity
ATP
GTP
2APTP
ITP
ATPαS
GTPαS
2 N3 ATP
2' NH2 ATP
WspR
ATP
GTP
2APTP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP
DisA
ATP
GTP
2APTP
ITP
ATPαS
GTPαS
2' N3 ATP
2' NH2 ATP

4/21

FIG. 4

MW=658.42 g/mol



Conditions

5 µM Mxan2643 200 µM 2APTP	50 mM Tris-HCl, pH 7.5 100 mM NaCl 10 mM $MgCl_2$
28 °C, 14h	5mM DTT

Mass spectrum

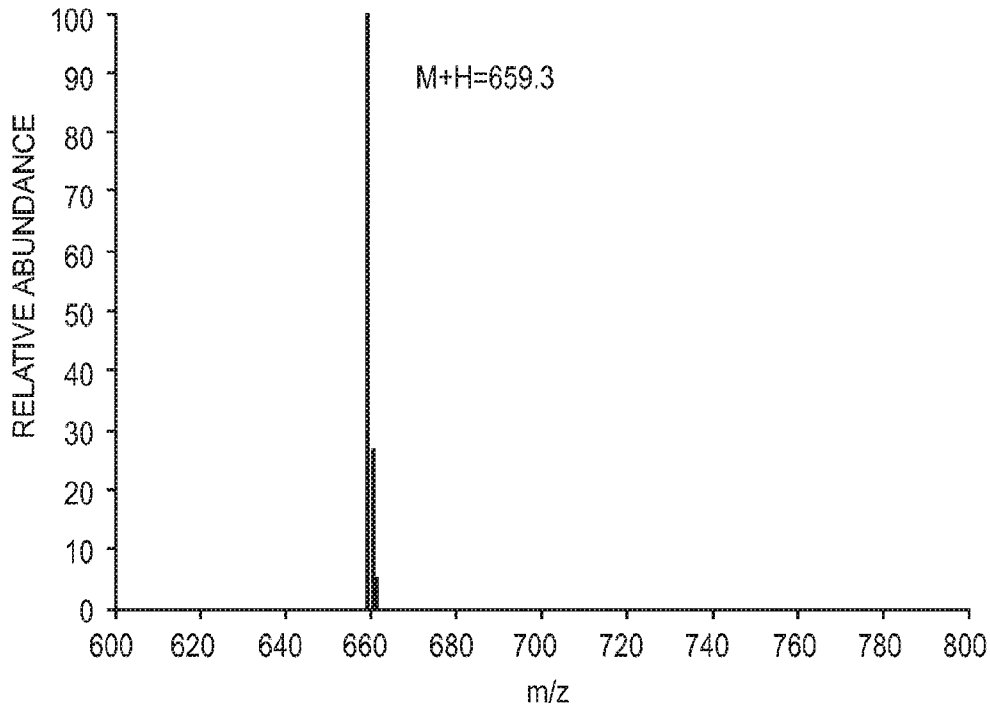
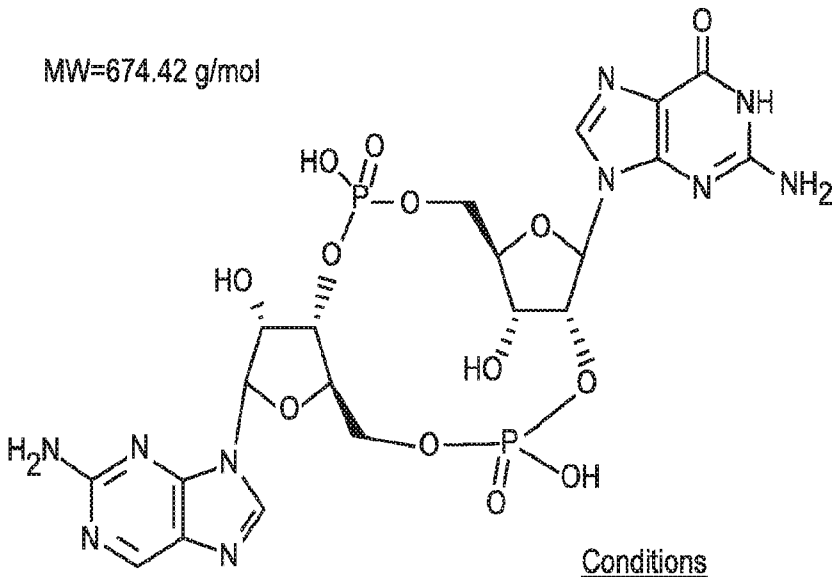


FIG. 5



Conditions

5 μM cGAS	40 mM Tris-HCl, pH 7.5
100 μM 2APTP	100 mM NaCl
100 μM GTP	10 mM $MgCl_2$
	5mM DTT
37 °C, 20 h	0.1 mg/mL HT-DNA

Mass spectrum

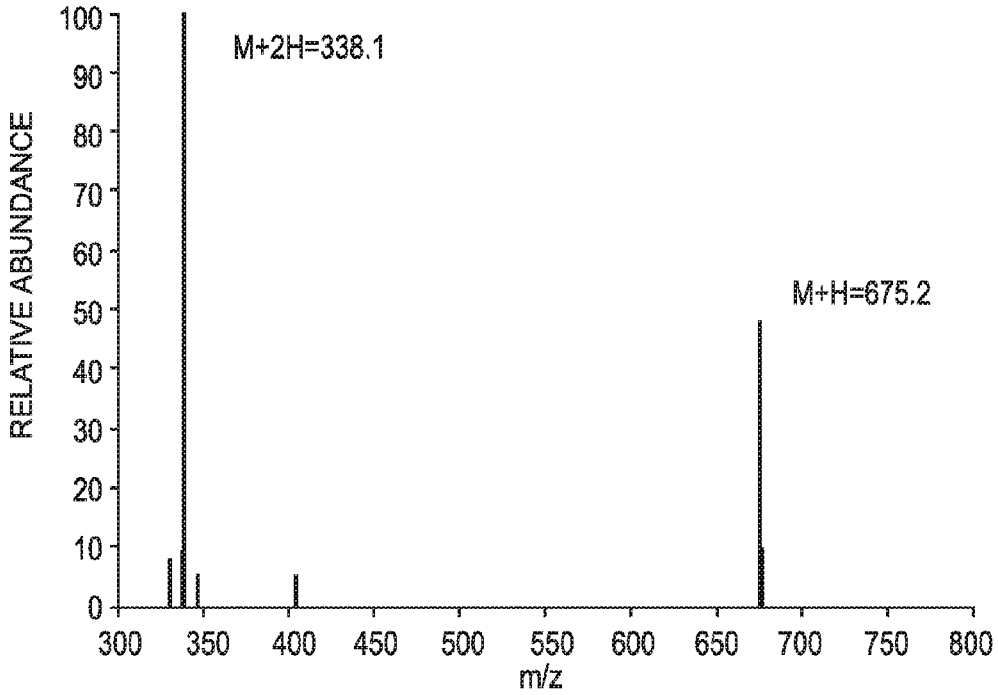
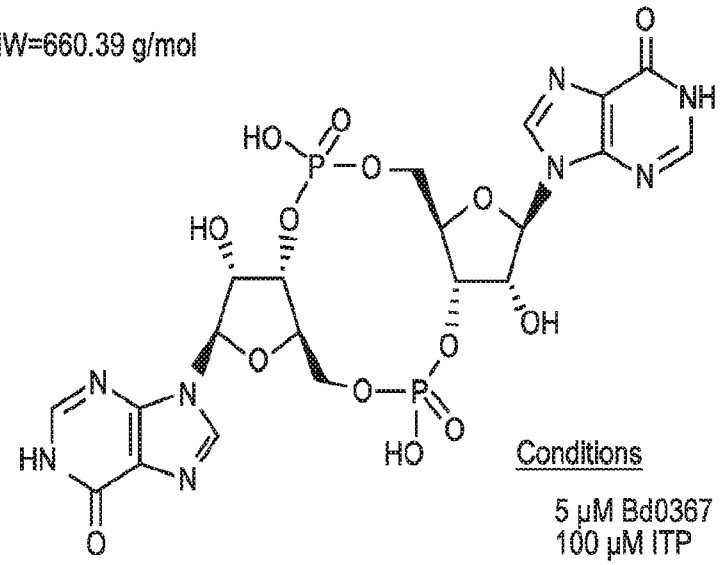


FIG. 6

MW=660.39 g/mol



Conditions

5 µM Bd0367
100 µM ITP

28 °C, 1 h

50 mM Tris-HCl, pH 7.5
100 mM NaCl
10 mM $MgCl_2$
5mM DTT

Mass spectrum

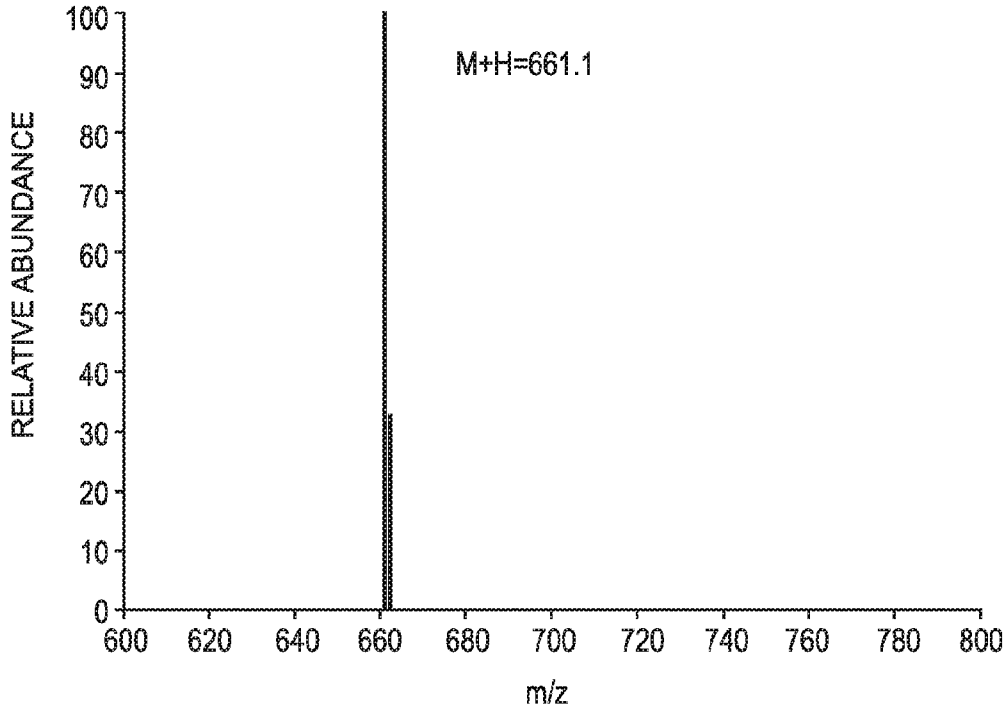
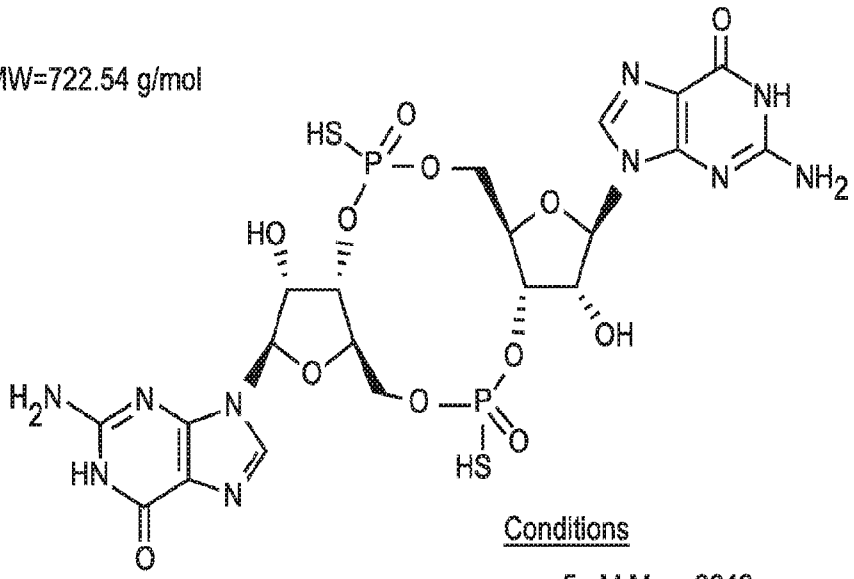


FIG. 7

MW=722.54 g/mol



Conditions

5 µM Mxan2643
200 µM GTPαS

28 °C, 20 h

50 mM Tris-HCl, pH 7.5
100 mM NaCl
10 mM $MgCl_2$
5mM DTT

Mass spectrum

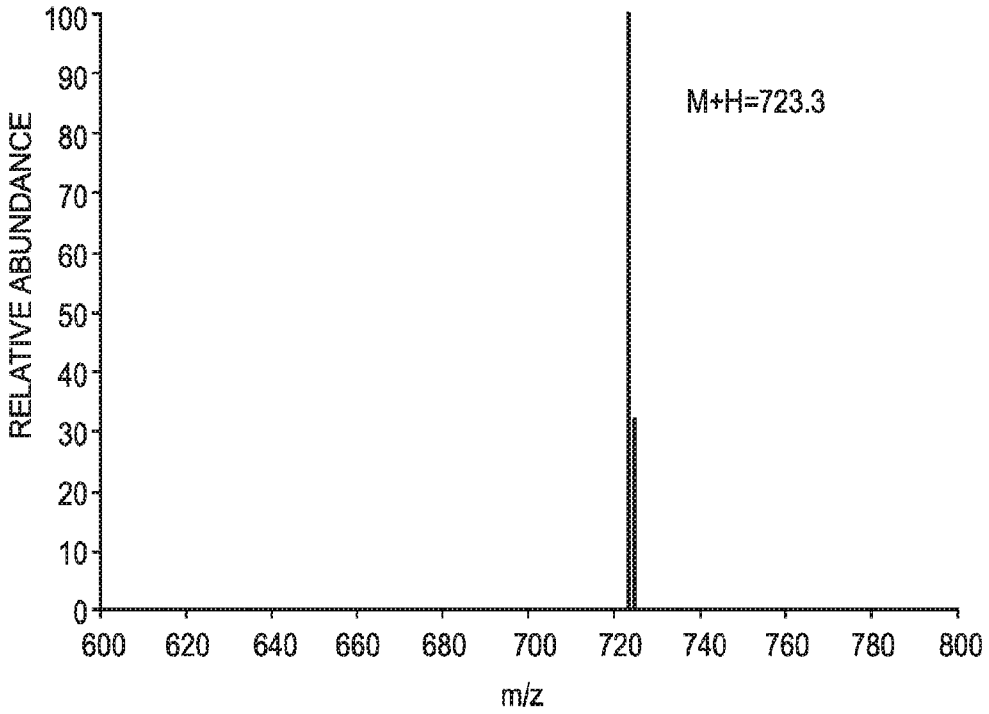
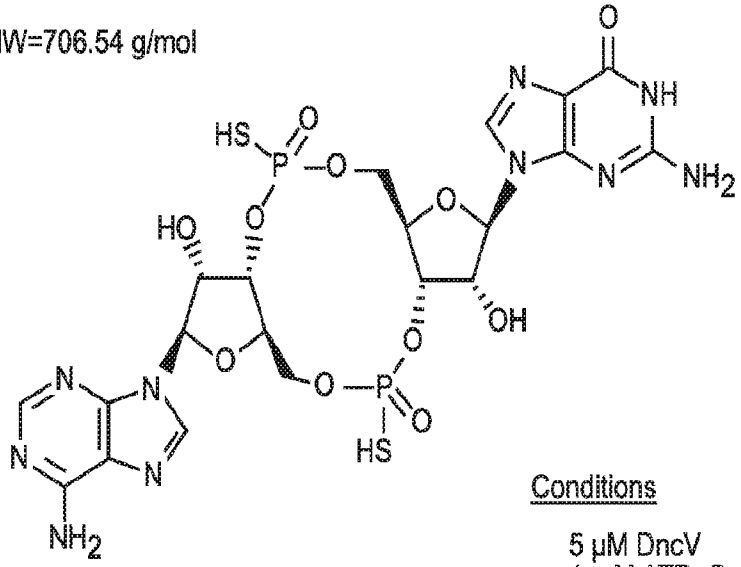


FIG. 8

MW=706.54 g/mol



Conditions

5 µM DncV
1 mM ATPαS
1 mM GTPαS

40 mM Tris-HCl, pH 7.5
100 mM NaCl
10 mM $MgCl_2$
5mM DTT

37 °C, 20 h

Mass spectrum

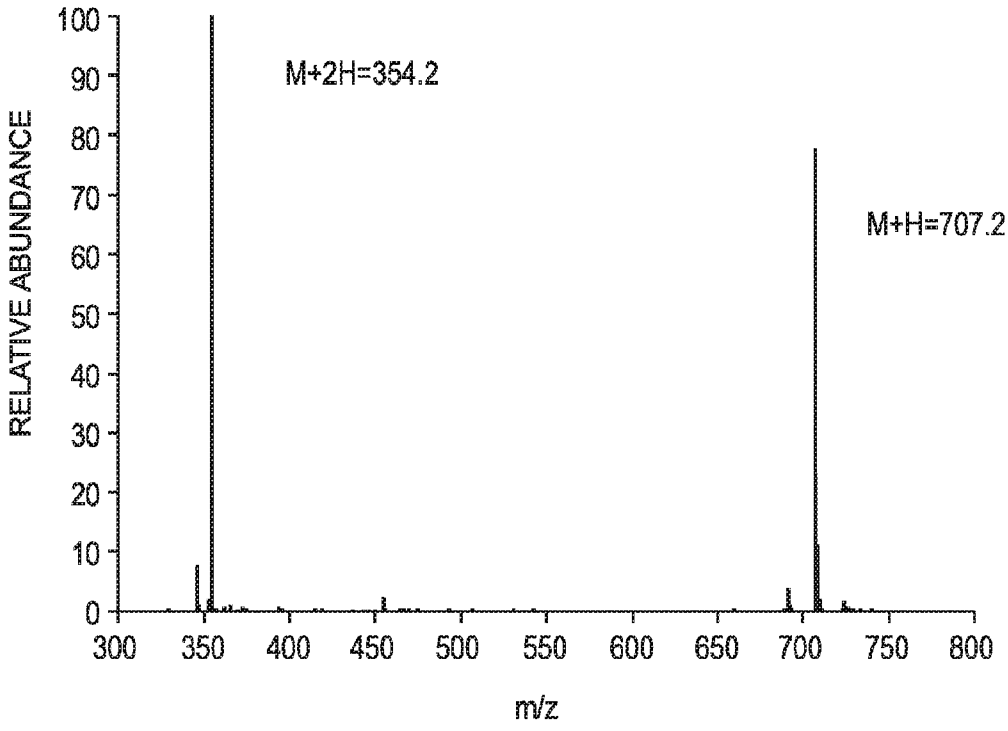
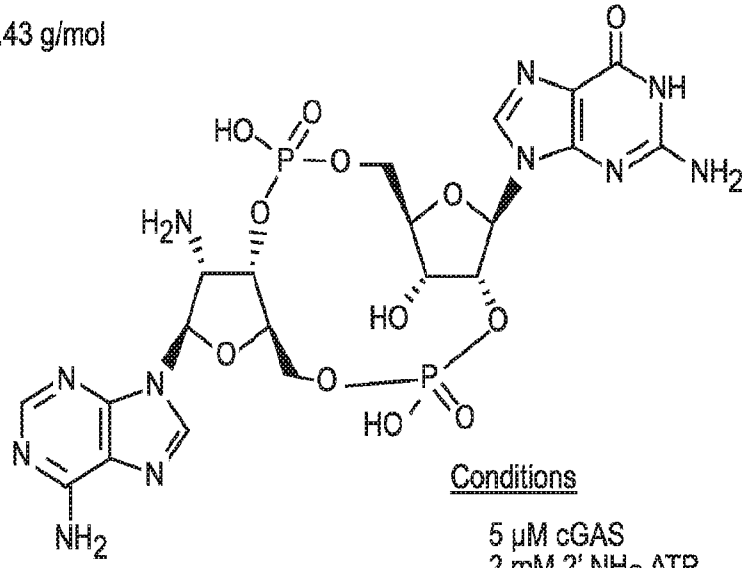


FIG. 9

MW=673.43 g/mol



Conditions

5 μM cGAS	40 mM Tris-HCl, pH 7.5
2 mM 2' NH_2 ATP	100 mM NaCl
1 mM GTP	10 mM $MgCl_2$
37 °C, 24 h	5mM DTT
	0.1 mg/mL HT-DNA

Mass spectrum

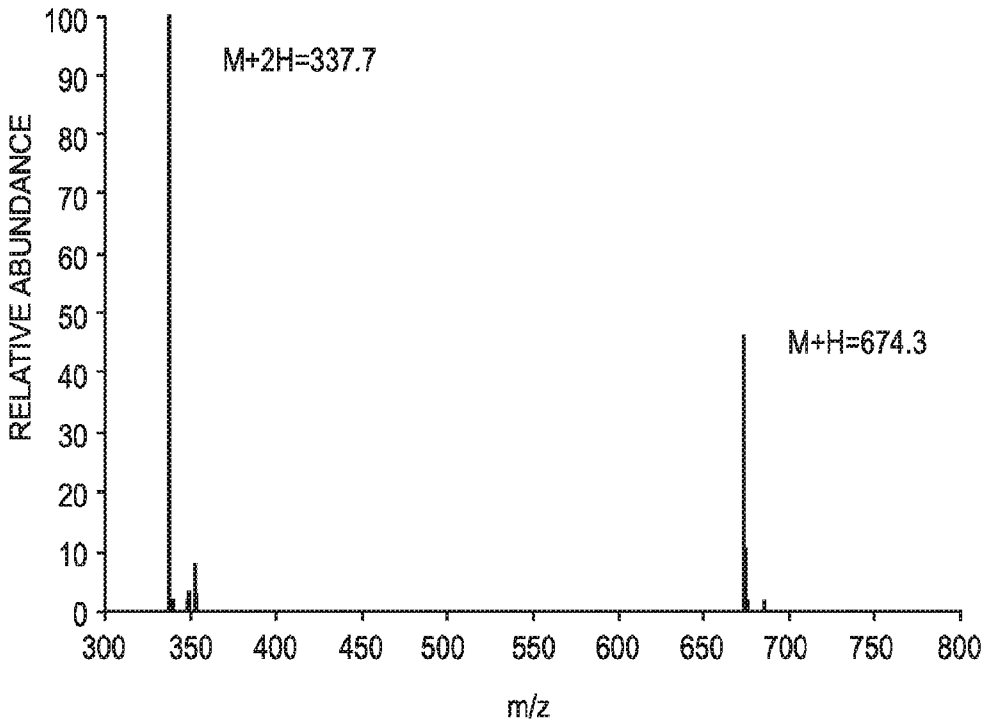
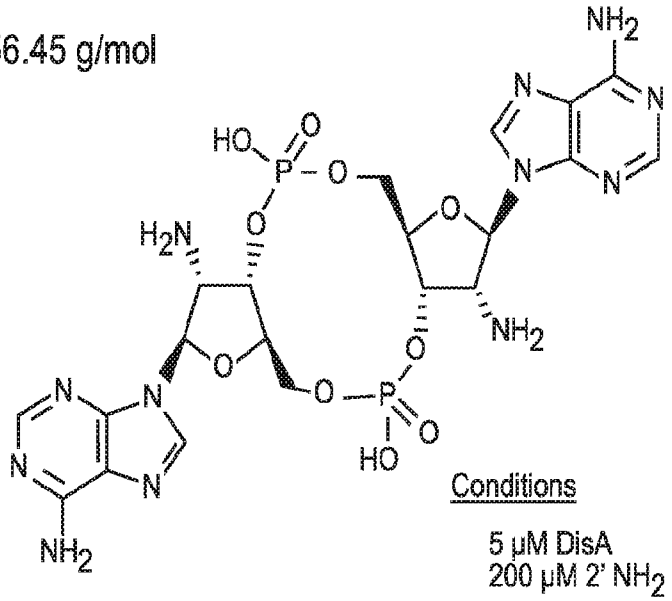


FIG. 10

MW=656.45 g/mol



Conditions

5 µM DisA
200 µM 2' NH_2 ATP

40 mM Tris-HCl, pH 7.5
100 mM NaCl
10 mM $MgCl_2$
5mM DTT

37 °C, 20 h

Mass spectrum

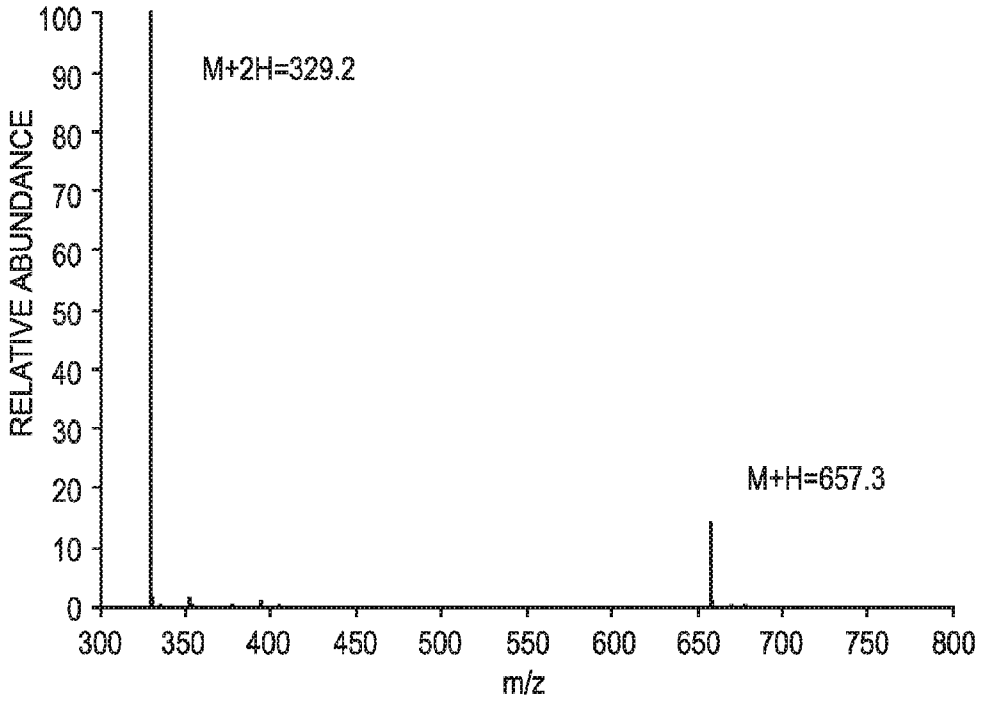
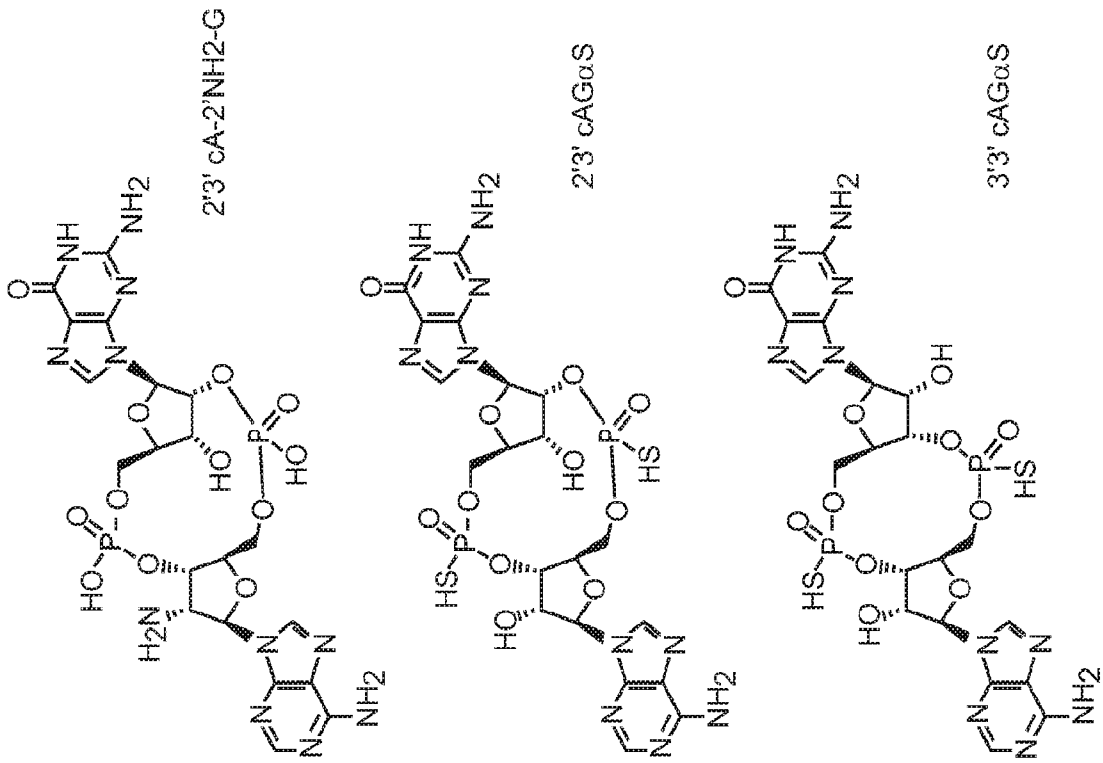
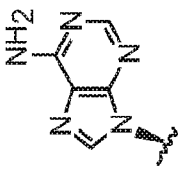
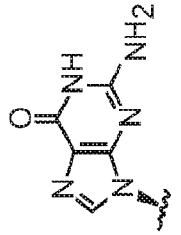


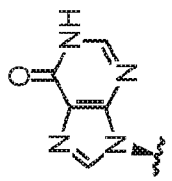
FIG. 11

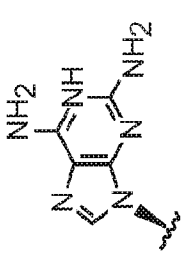


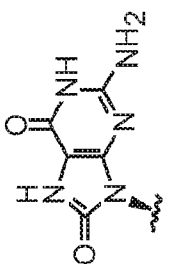
EXAMPLE NUCLEOBASES


Adenine


Guanine


Hypoxanthine


2,6-Diaminopurine


8-Oxoguanine

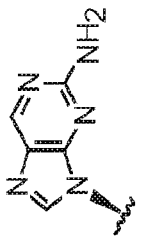

2-Aminopurine

FIG. 12

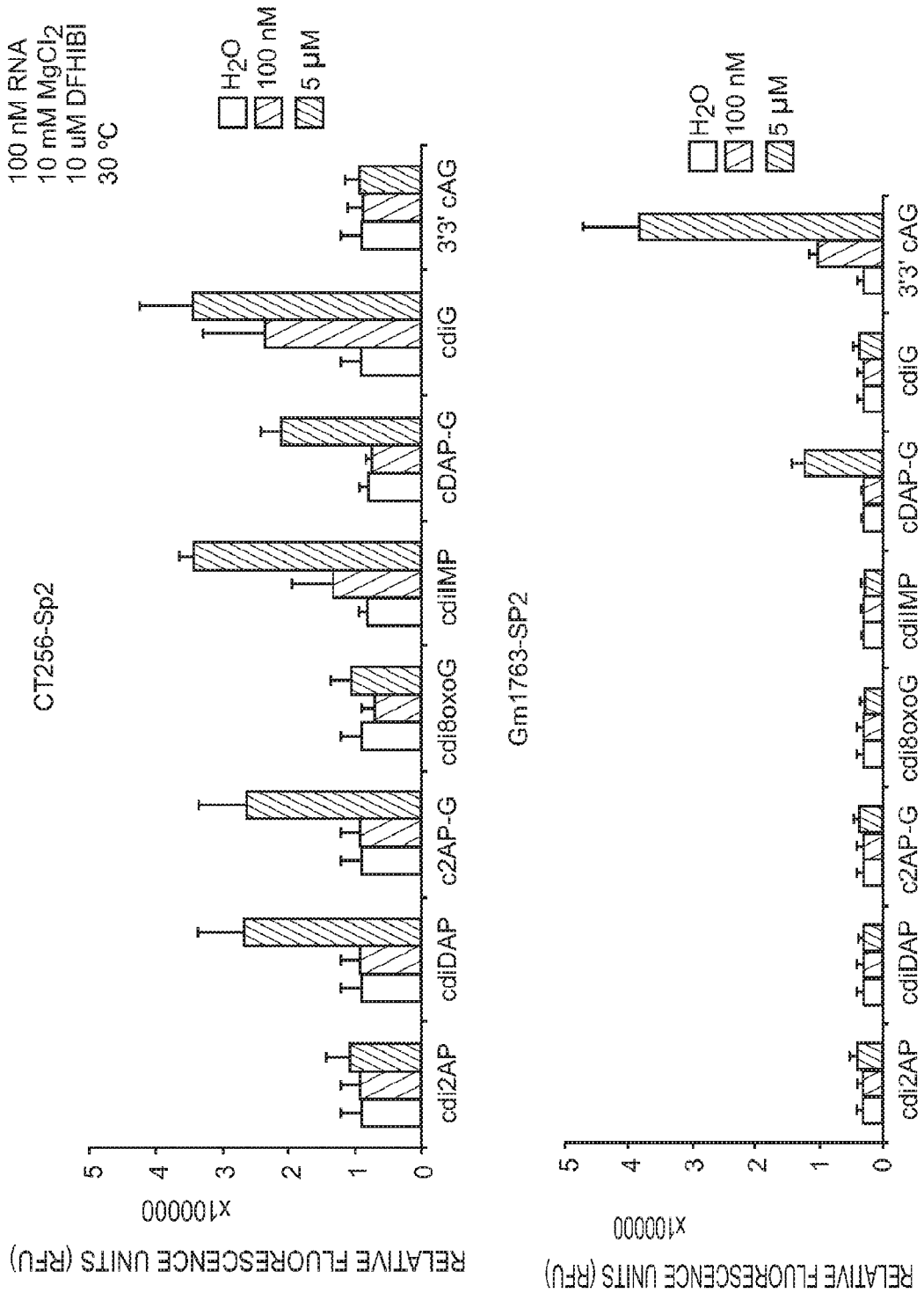
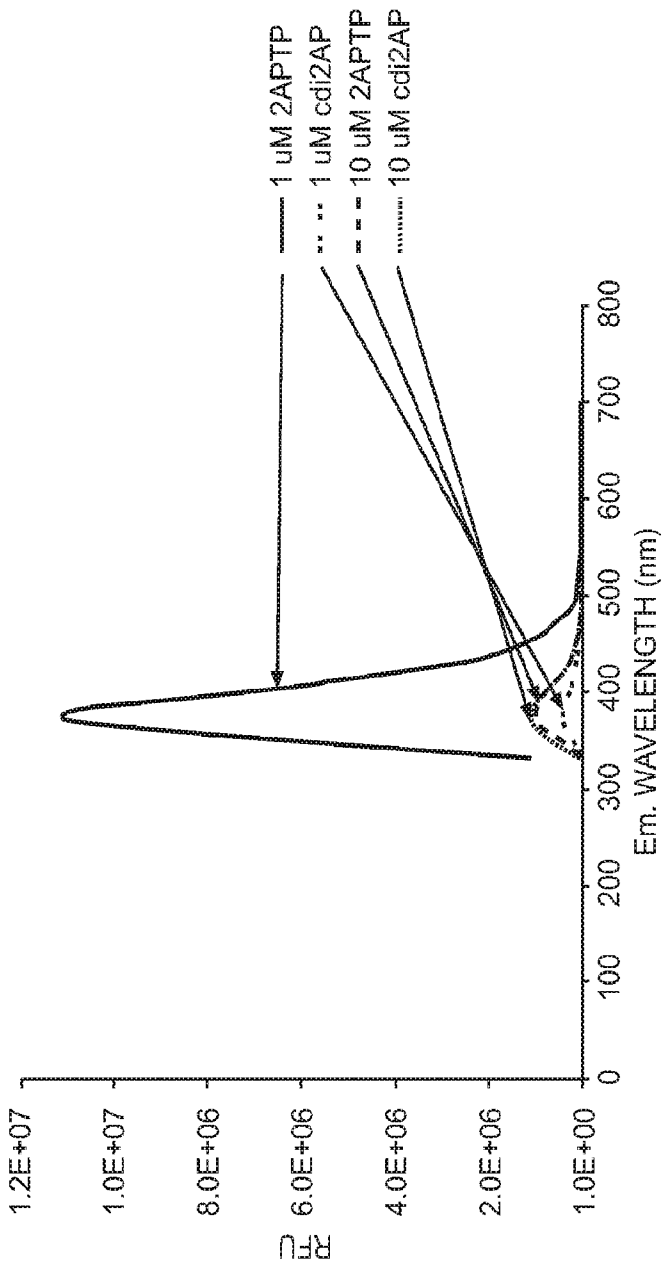

100 nM RNA
10 mM MgCl2
10 uM DFHIBI
30 °C
H2O
100 nM
5 µM
CT256-Sp2
Gm1763-SP2
cdi2AP
cdiDAP
c2AP-G
cdi8oxoG
cdiIMP
cDAP-G
cdiG
3'3' cAG
RELATIVE FLUORESCENCE UNITS (RFU)
x100000
0
1
2
3
4
5

FIG. 13



SAMPLE	Ex. WAVELENGTH (nm)	λ max Em. (nm)	RFU max
1 uM 2APTP	310	375	952169
1 uM cdi2AP	310	380	393908
10 uM 2APTP	310	375	11155673
10 uM cdi2AP	310	375	1081301

FIG. 14

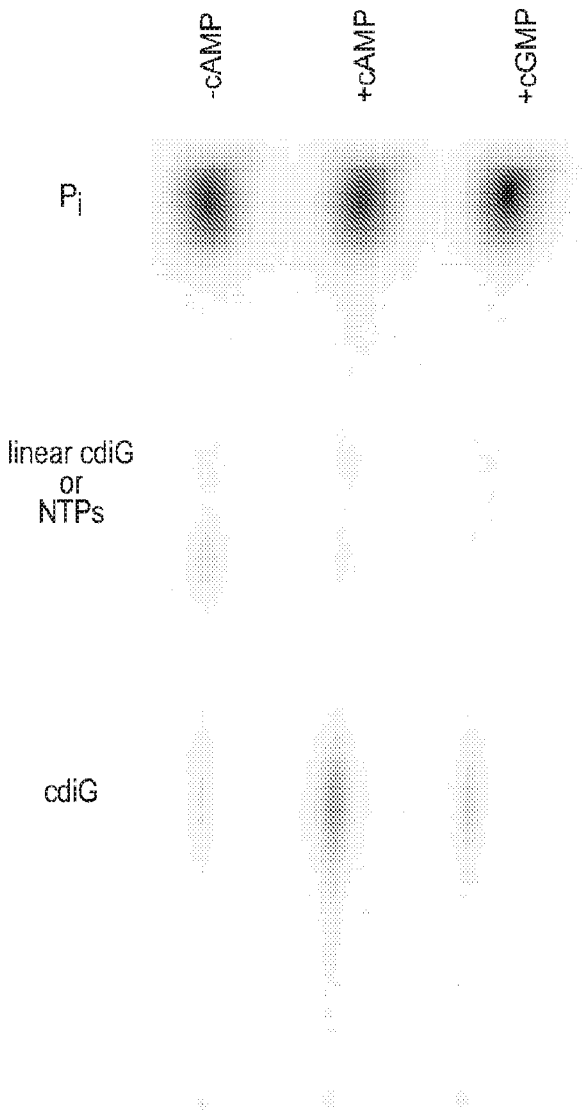


FIG. 15

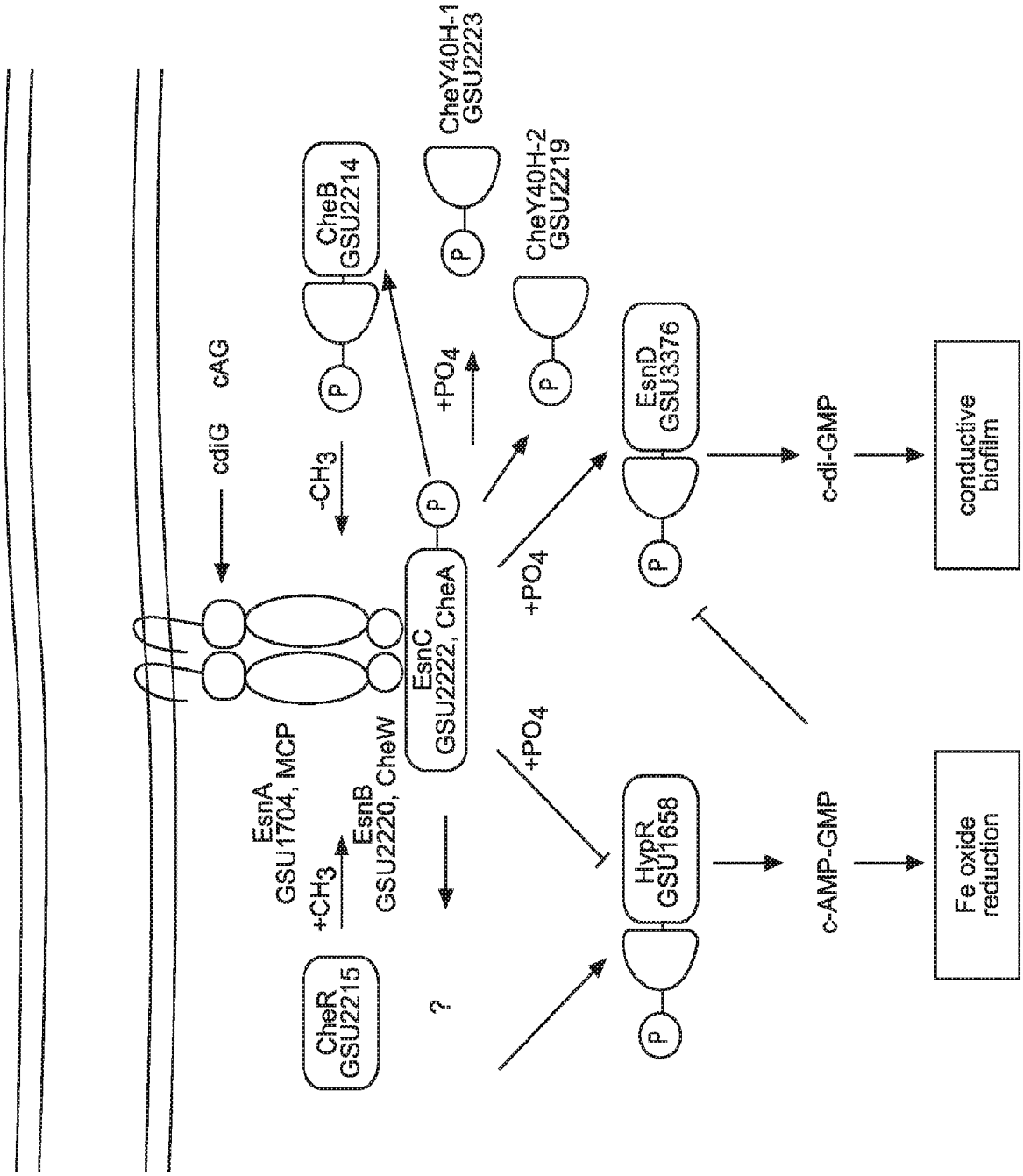

cdiG
cAG
EsnA
GSU1704, MCP
+CH3
EsnB
GSU2220, CheW
EsnC
GSU2222, CheA
-CH3
CheB
GSU2214
CheR
GSU2215
?
+PO4
+PO4
+PO4
CheY40H-1
GSU2223
CheY40H-2
GSU2219
EsnD
GSU3376
HypR
GSU1658
c-di-GMP
c-AMP-GMP
conductive biofilm
Fe oxide reduction
P

FIG. 16

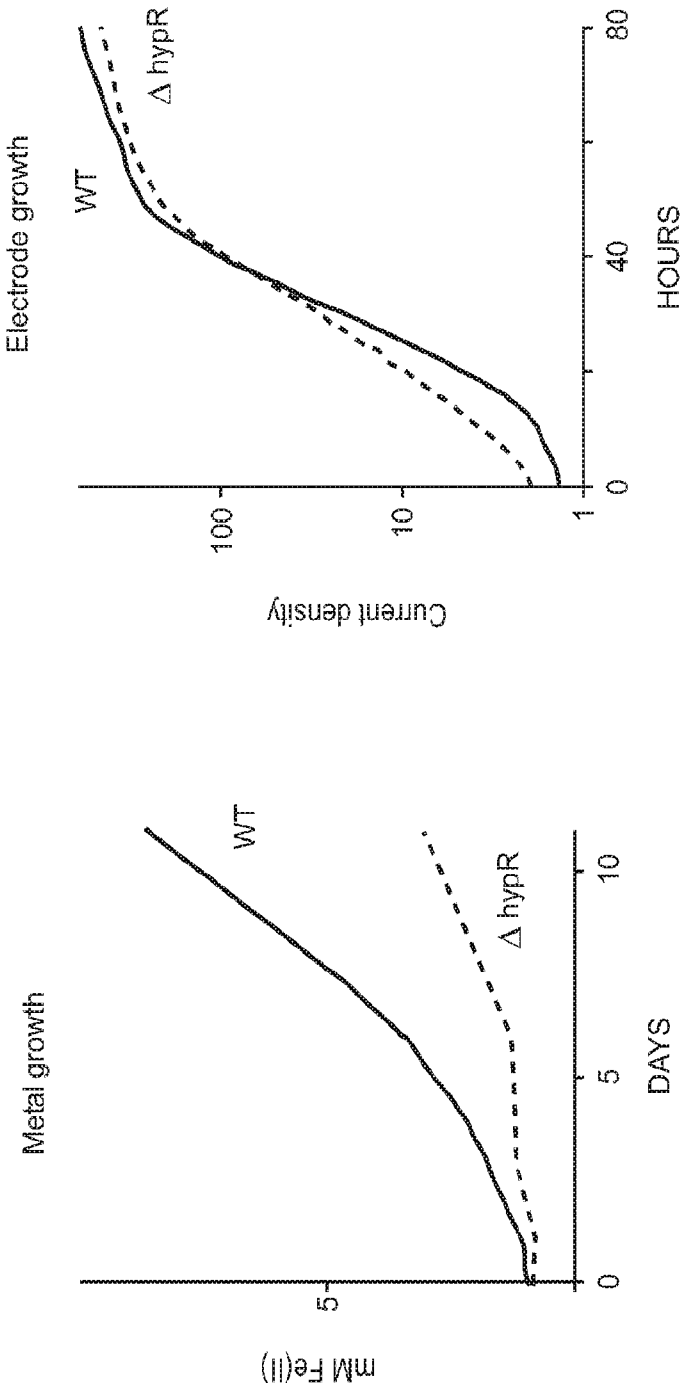

Electrode growth
WT
Δ hypR
100
10
1
Current density
0
40
80
HOURS
Metal growth
WT
Δ hypR
5
mM Fe(II)
0
5
10
DAYS

FIG. 17

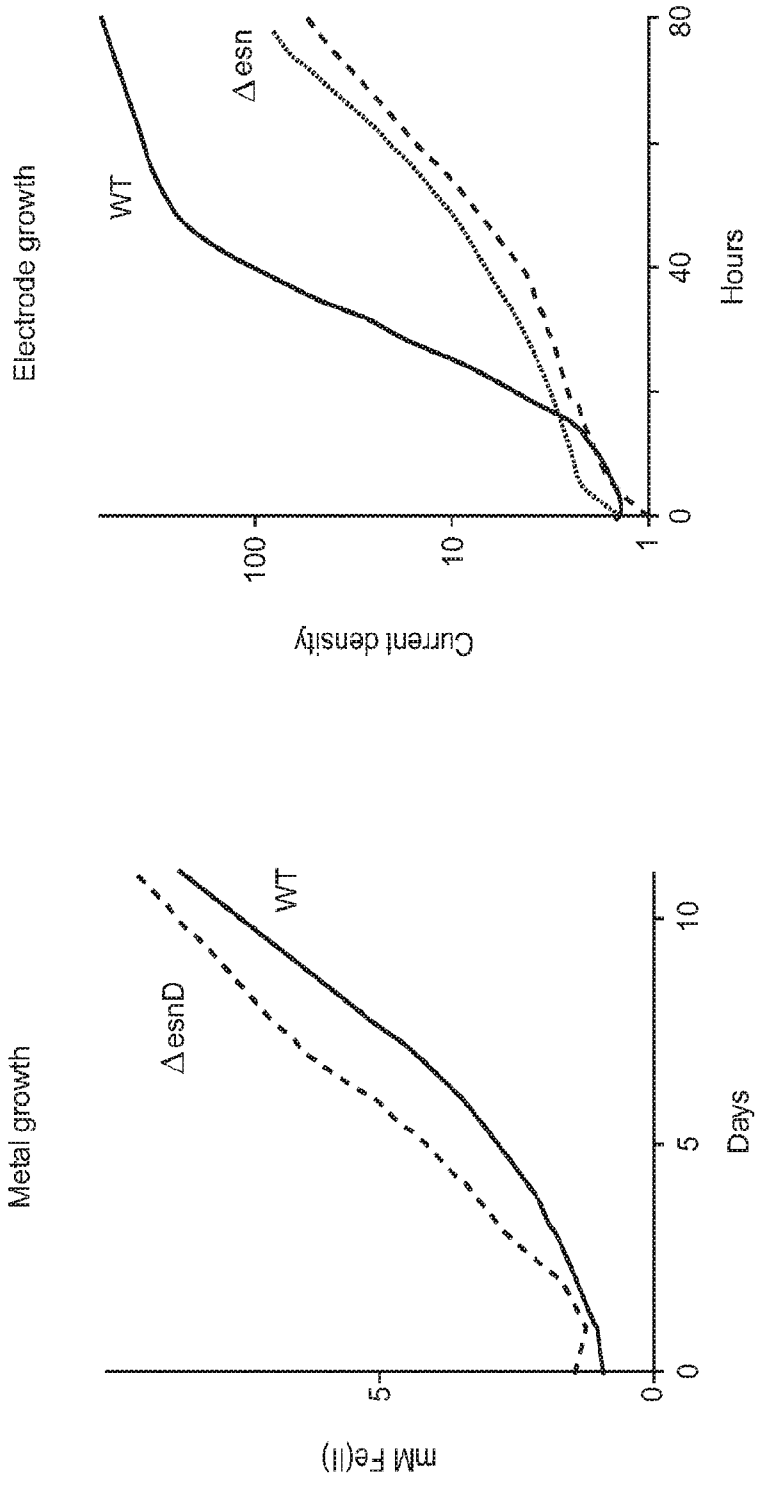
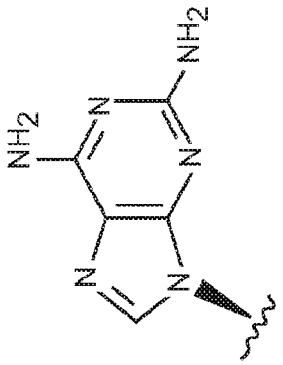
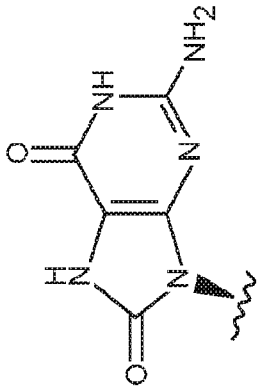

Electrode growth
WT
Δesn
100
10
1
Current density
0
40
80
Hours
Metal growth
ΔesnD
WT
5
0
mM Fe(II)
0
5
10
Days

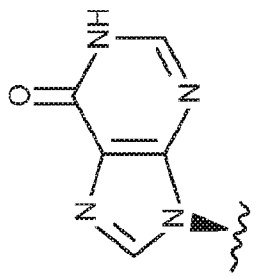
FIG. 18



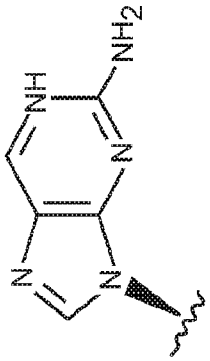
DAP TP



8-oxoGTP



ITP



2AP TP

FIG. 19

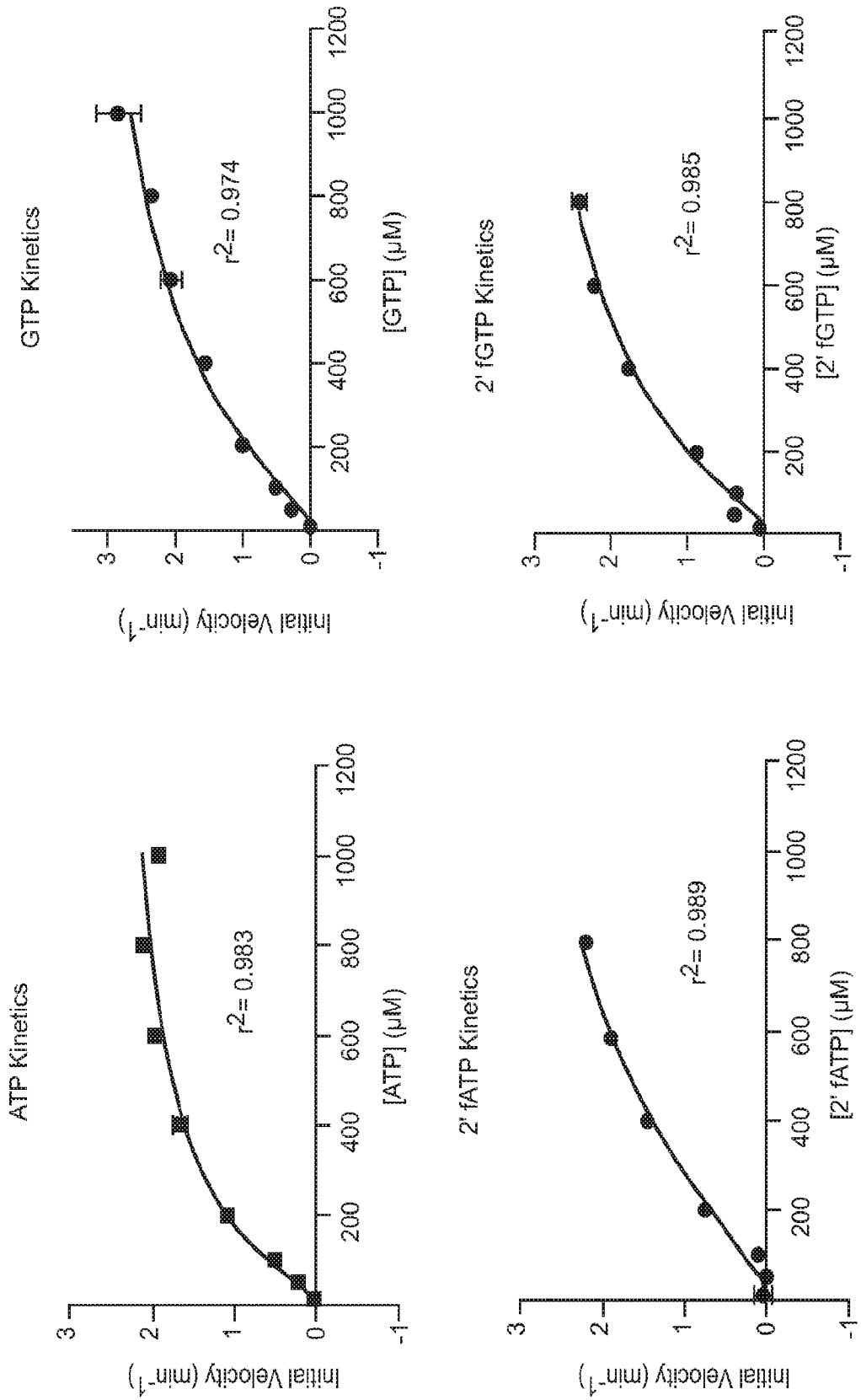

GTP Kinetics
r2= 0.974
Initial Velocity (min-1)
[GTP] (μM)
2' fGTP Kinetics
r2= 0.985
Initial Velocity (min-1)
[2' fGTP] (μM)
ATP Kinetics
r2= 0.983
Initial Velocity (min-1)
[ATP] (μM)
2' fATP Kinetics
r2= 0.989
Initial Velocity (min-1)
[2' fATP] (μM)

FIG. 20

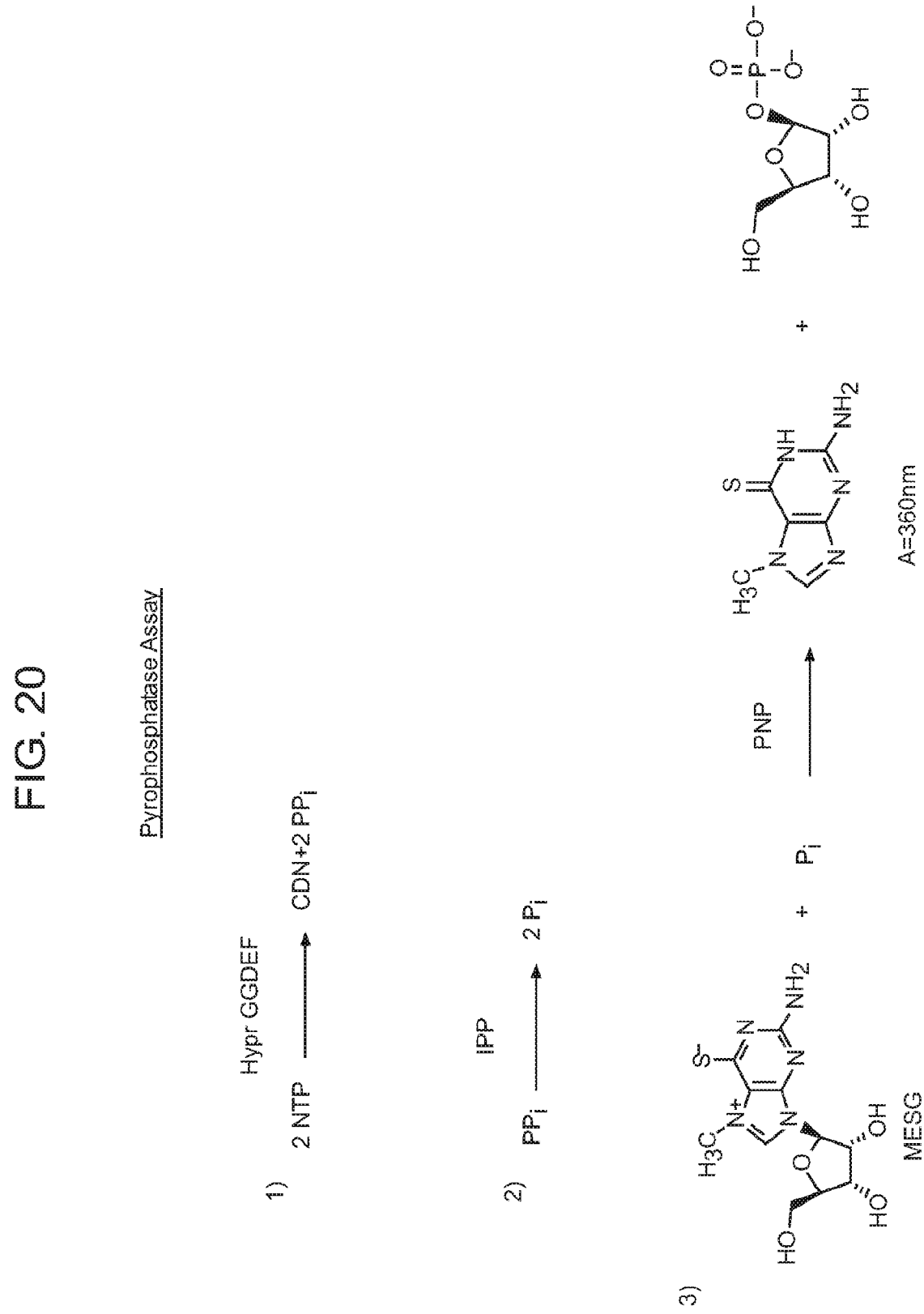
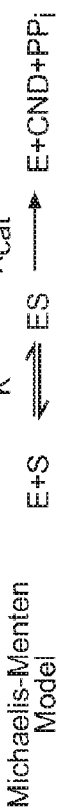
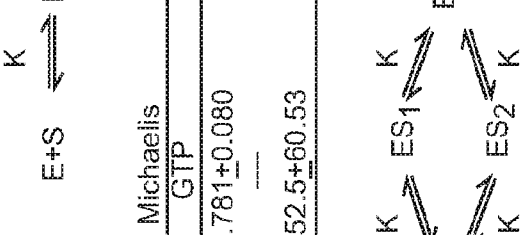

Pyrophosphatase Assay
1)
Hypr GGDEF
2 NTP
CDN+2 PPi
2)
IPP
PPi
2 Pi
3)
Pi
PNP
MESG
A=360nm

FIG. 21



	Michaelis Menten			
	ATP	GTP	2' fATP	2' fGTP
Kcat (min-1 μM-1)	1.397±0.073	1.781±0.080	3.526±1.340	2.163±0.252
K (μM)	---	---	---	---
Km (μM)	479.0±51.2	752.5±60.53	2283±1131	919.6±172.8



	Two Substrate		
	ATP	2' fATP	2' fGTP
	1.043±0.033	1.548±0.090	1.679±0.111	1.545±0.081
	109.3±9.1	210.2±23.9	294.3±28.3	200.6±18.6
	---	---	---	---

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/67669

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07H 19/207; C12N 15/09, 15/10, 15/52, 15/79, 15/80, 15/81 (2018.01)

CPC - C07H 19/207; C12N 15/09, 15/10, 15/52, 15/79, 15/80, 15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ... Y	US 2014/0170689 A1 (NESBITT, NM) 19 June, 2014; paragraphs [0049]-[0050], [0052], [0054], [0200], [0225], [0230], [0239], [0245], [0254], [0259]	1-4 --- 5, 8-16, 19
Y	(HALLBERG, ZF et al.) Hybrid Promiscuous (Hypr) GGDEF Enzymes Produce Cyclic AMP-GMP (3′, 3′-cGAMP). Proceedings of the National Academy of Sciences of the United States of America. 02 February, 2016; Vol. 113, No. 7; pages 1790-1795; abstract; page 1793, column 1, paragraph 2; DOI: 10.1073/pnas.1515287113	5, 13, 16
Y	US 2014/0205653 A1 (ADURO BIOTECH, INC.,) 24 July, 2014; figure 3- molecule 11b; paragraph [0015]	8, 12
Y	US 2016/0074507 A1 (INSTITUT CURIE, et al.) 17 March, 2016; paragraph [0163]	9-16, 19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2018 (17.04.2018)	25 APR 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/67669

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group 1+ Claims 1-5, 8-11, 12-16, 19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US17/67669

-***-Continued from Box III Observations where unity of invention is lacking -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-19; Hypr GGDEF (enzyme) and 3',3'-CDN (CDN) are directed toward methods and compositions for preparing a cyclic dinucleotide (CDN) by contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN; and a method of synthesizing a CDN by culturing a genetically modified host cell in a culture medium comprising the precursor of the CDN.

The methods and compositions will be searched to the extent they encompass a CDN-producing enzyme encompassing Hypr GGDEF (enzyme) and a CDN encompasing a 3',3'-CDN (CDN). Applicant is invited to elect additional CDN-processing enzyme(s) and corresponding CDN(s) produced thereby, to be searched. Additional enzyme(s) and corresponding CDN(s) will be searched upon the payment of additional fees. It is believed that claims 1-5, 8 (in-part), 9-11, 12 (in-part), 13 (in-part), 14-16 and 19 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass Hypr GGDEF (enzyme) and 3', 3'-CDN (CDN). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an enzyme encompassing a cGAS enzyme (enzyme), and a 2',3'-CDN (CDN).

No technical features are shared between the CDN-preocessing enyzmes and CDNs of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features of: an in vitro method of preparing a cyclic dinucleotide (CDN), the method comprising: a) contacting a CDN producing-enzyme with a precursor of a CDN under conditions sufficient to convert the precursor into a CDN; and b) isolating the CDN; a method of synthesizing a cyclic dinucleotide (CDN), the method comprising: a) culturing a genetically modified host cell in a culture medium comprising a precursor of the CDN, wherein the genetically modified host cell comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN; and b) recovering the CDN from the culture medium; and a genetically modified host cell that produces a cyclic dinucleotide (CDN), the cell comprising a heterologous coding sequence encoding a CDN-producing enzyme within a pathway that converts a precursor for the CDN to a CDN; however, these shared technical features are previously disclosed by US 2014/0170689 A1 to Nesbitt et al. (hereinafter 'Nesbitt') in view of US 2016/0074507 A1 to Institut Curie et al. (hereinafter 'Institut Curie').

Nesbitt discloses an in vitro (paragraph [0245]) method of preparing a cyclic dinucleotide (CDN) (paragraph [0049]), the method comprising: a) contacting a CDN producing-enzyme with a precursor of a CDN (contacting a dinucleotide cyclase with a purine nucleotide triphosphate (CDN precursor); paragraph [0050]) under conditions sufficient to convert the precursor into a CDN (converting GTP to pGpC or cyclic-diGMP; paragraphs [0017], [0308]); and b) isolating the CDN (isolating cyclic dinucleotide monophosphate; paragraph [0050]); a method of synthesizing a cyclic dinucleotide (CDN) (paragraph [0049]), the method comprising: a) culturing (paragraph [0254]) a genetically modified host cell (recombinant or transformed host cell; paragraphs [0240], [0282]-[0284]) using a CDN-producing enzyme (diguanylate cyclase; paragraph [0008]) within a pathway that converts a precursor for the CDN to a CDN (reactions in the synthesis of cyclic-diGMP from GTP; paragraph [0308]). Nesbitt also discloses heterologous coding sequences (paragraph [0282]) for a CDN-producing enzyme (diguanylate cyclase; paragraph [0008]), but Nesbitt does not disclose recovering the CDN from the culture medium, wherein the culture medium comprises the genetically modified host cell which comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN.

Institut Curie discloses production of viral particles comprising a CDN produced by a genetically-modified host cells (paragraphs [0023], [0040]) which comprises a heterologous coding sequence (paragraph [0064]) encoding a CDN-producing enzyme (sequence encoding a cyclic dinucleotide synthase; paragraph [0017]) in a culture medium (paragraphs [0154], [0163]) and recovery of said particles (paragraphs [0029], [0031]). It would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the disclosure of Nesbitt to provide the step of recovering the CDN from the culture medium, wherein the culture medium comprises the genetically modified host cell which comprises a heterologous coding sequence encoding a CDN-producing enzyme that converts the precursor into a CDN, because the ability to recover CDN-containing particles produced in cultured, genetically modified host cells which comprise a polynucleotide sequence encoding a CDN-producing enzyme as disclosed by Institut Curie would have enabled the isolation of the CDN derived from the CDN precursor as previously disclosed by Nesbitt directly from the culture medium, thus enhancing yield of the CDN by eliminating subsequent purification steps.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the combination of the Nesbitt and Institut Curie references, unity of invention is lacking.