Novel Applications of Biocatalysis to Stereochemistry Determination of 2′,3′-cGAMP Bisphosphorothioate (2′,3′-cG\textsuperscript{S}A\textsuperscript{S}MP)

Jongwon Lim* and Hai-Young Kim

ABSTRACT: The metazoan second messenger 2′,3′-cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) is a cyclic dinucleotide (CDN) that induces secretion of type I interferons and activates the immune system and has thus attracted significant interest as a vaccine adjuvant or immunotherapeutic. In the observation has led to intense interest in CDNs as vaccine adjuvants or immunotherapeutics.10 In the field of therapeutics, CDN bisphosphorothioates (CDNSSs) are of particular interest, because the phosphorothioate linkages in CDNSSs confer increased resistance to hydrolysis by phosphodiesterases.11 In addition to their increased hydrolytic stability, CDNSSs are more lipophilic and cell permeable than CDNs, thereby requiring improved cell activities. In our work with CDN bisphosphorothioates, we sought a method for systematic determination of the absolute stereochemistry of their phosphorothioate stereocenters. A novel biocatalytic method employing snake venom phosphodiesterase (svPDE) and nP1 has been developed and successfully applied to stereochemistry determination of 2′,3′-cGAMP bisphosphorothioates. This method unambiguously assigned the phosphorothioate stereochemistry of four diastereomers of 2′,3′-cGAMP bisphosphorothioate by analyzing distinct hydrolysis patterns of the bisphosphorothioate diastereomers upon incubation with svPDE and nP1. Furthermore, the regiospecificity as well as stereospecificity of both svPDE and nP1 toward 2′,3′-cGAMP bisphosphorothioate has been elucidated.

INTRODUCTION

Canonical cyclic dinucleotides (CDNs) that possess two 3′−5′ internucleotide linkages, such as cyclic diguanosine monophosphate (c-di-GMP, 1) and cyclic diadenosine monophosphate (c-di-AMP, 2), are ubiquitous second messenger molecules in bacteria (Figure 1). The CDNs have been reported to participate in a myriad of signaling pathways in bacteria for the past three decades.1,2 In 2013, cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) was reported as the first metazoan second messenger produced by DNA-activated cGAMP synthase (cGAS).3,4 In contrast to the bacterial CDNs, the metazoan cGAMP was identified to have one 2′−5′ and one 3′−5′ internucleotide linkage, [G(2′−5′)pA(3′−5′)p] (2′,3′-cGAMP, 3).5−8 The second messenger 3 induces secretion of type I interferons and activates the immune system; this observation has led to intense interest in CDNs as vaccine adjuvants or immunotherapeutics.9,10 In the field of therapeutics, CDN bisphosphorothioates (CDNSSs) are of particular interest, because the phosphorothioate linkages in CDNSSs confer increased resistance to hydrolysis by phosphodiesterases.11 In addition to their increased hydrolytic stability, CDNSSs are more lipophilic and cell permeable compared to CDNs, thereby offering improved cell activities.11−15

Chemical and biocatalytic syntheses of CDNSSs provide up to four bisphosphorothioate diastereomers depending upon the stereospecificity of the syntheses and the molecular symmetry of CDNSSs.11,16−18 Typically, bisphosphorothioate diastereomers are either chromatographically separated and characterized individually12,14,15,19 or evaluated as a diastereomeric mixture without further purification.11,13 Stereochemistry assignment of the three diastereomers of c-di-GMP bisphosphorothioate, R\textsuperscript{p}R\textsuperscript{p}, R\textsuperscript{p}S\textsuperscript{p}, and S\textsuperscript{p}S\textsuperscript{p}, was reported based on their retention times on reverse-phase high-performance liquid chromatography (HPLC) and \textsuperscript{31}P NMR chemical shifts.19 This method was also applied in assigning the stereochemistry of 2′,3′-CDNSS diastereomers by another group, who confirmed the stereochemistry of a 2′,3′-CDNSS diastereomer via an X-ray single-crystal structure.12,20 However, retention times on reverse-phase HPLC and \textsuperscript{31}P NMR chemical shifts of CDNSSs are often too close from one to another, making unambiguous stereochemistry assignments difficult. In addition, this method relies on relative values of retention times on reverse-phase HPLC and \textsuperscript{31}P NMR chemical shifts of CDNSSs are too close from one to another, making unambiguous stereochemistry assignments difficult. In addition, this method relies on relative values of retention times on reverse-phase HPLC and \textsuperscript{31}P NMR chemical shifts of CDNSSs are too close from one to another, making unambiguous stereochemistry assignments difficult.
syntheses fail to provide all of the possible bisphosphorothioate diastereomers due to stereoselective chemical steps and difficulty in purification.\textsuperscript{13} Moreover, biocatalytic syntheses of CDNSSs do not provide all diastereomers because of the intrinsic specificity of the enzymes.\textsuperscript{11,18,21} Therefore, development of a novel method that enables the unequivocal assignment of the stereochemistry of a given CDNSS would be highly desirable. Herein, a novel unambiguous method of stereochemistry determination of 2′,3′-cGAMP bisphosphorothioate (2′,3′-cG\textsuperscript{3}A\textsuperscript{3}MP, 4)\textsuperscript{21,22} employing snake venom phosphodiesterase (svPDE) and nP1 is reported. In addition, the stereospecificity as well as regiospecificity of svPDE and nP1 toward 4 is discussed.

Figure 1. Structures of c-di-GMP, c-di-AMP, and 2′,3′-cGAMP.

Figure 2. Enzymatic hydrolysis of 2′,3′-cG\textsuperscript{3}A\textsuperscript{3}MP with svPDE and nP1. LC profiles of 4a (A), 4b (B), 4c (C), and 4d (D) incubated with svPDE and nP1. SvPDE conditions: 2′,3′-cG\textsuperscript{3}A\textsuperscript{3}MP (0.05 μmol), svPDE (0.008 unit), pH 8.5 buffer solution (0.2 mL), 37 °C, 20 h; nP1 conditions: 2′,3′-cG\textsuperscript{3}A\textsuperscript{3}MP (0.05 μmol), nP1 (11 unit), pH 7.0 buffer solution (0.2 mL), 37 °C, 20 h. The liquid chromatography (LC) spectra of the starting materials, the reactions after incubation with svPDE, and the reactions after incubation with nP1 are in green, red, and blue, respectively. A fixed amount of 2′,3′-isopropylideneadenosine was added to each LC-mass spectrometry (LCMS) sample as a reference (ref). The minor peak on the LC spectrum of 4c incubated with nP1 was from an inseparable impurity in the sample. GMP = guanosine monophosphate, GMPS = guanosine monophosphorothioate, AMP = adenosine monophosphate, AMPS = adenosine monophosphorothioate, MP = monophosphate, and MPS = monophosphorothioate.
RESULTS AND DISCUSSION

Determination of the absolute stereochemistry of phosphorothioate centers utilizing various enzymes has been well established for linear dinucleotide phosphorothioates (DNSs) in the literature. Two of the most widely used enzymes are snake venom phosphodiesterase (svPDE) and nuclease P1 (nP1). SvPDE cleaves $2'$-$5'$ and $3'$-$5'$ phosphodiester bonds and hydrolyzes $R_p$ phosphorothioates stereospecifically. On the other hand, nP1 specifically hydrolyzes $3'$-$5'$ phosphodiester bonds and $S_p$ phosphorothioates. These biocatalytic transformations have been successfully applied in determining the absolute stereochemistry of numerous linear DNSs for decades. However, enzymatic determination of the stereochemistry of phosphorothioate centers in CDNSS has not been reported in the literature. Application of hydrolytic enzymes to CDNSS has been limited to demonstrating their resistance to enzymatic hydrolysis and higher cellular activities compared to CDNs. In fact, application of biocatalysis to CDNSSs in the context of the relative hydrolytic stability between $R_p$ and $S_p$ let alone the stereochemistry determination, has not been reported. Given that svPDE and nP1 differentially hydrolyze $R_p$ and $S_p$ stereocenters in linear DNSs, we hypothesized that the enzymes might hydrolyze the phosphorothioate centers in CDNSSs regioselectively and stereoselectively, offering an opportunity to develop a novel method of stereochemistry determination of CDNSSs.

To test the hypothesis, four diastereomers of 4 ($4a$, $4b$, $4c$, and $4d$, whose retention times on HPLC are 6.0, 5.4, 5.3, and 4.9 min, respectively; 50 pmol each) were subjected to svPDE and nP1 hydrolysis conditions (Figure 2). Interestingly, the four diastereomers displayed distinct LC profiles when they were incubated with the enzymes. Diastereomer $4a$ was hydrolyzed to the monophosphorothioates (MPSs) and the monophosphates (MPs) by svPDE and was resistant to nP1 (Figure 2A). On the other hand, $4b$ was hydrolyzed to two different sets of linear DNSs by either svPDE or nP1 (Figure 2B). While $4c$ was resistant to both enzymes, $4d$ was hydrolyzed to linear DNSs by nP1 and was resistant to svPDE (Figure 2C,D).

Next, the reactions were scaled up for full characterization of the products to decipher the distinct LC patterns. Hydrolysis products of $4b$ were studied first since $4b$ was hydrolyzed by both svPDE and nP1, which would offer an insight into the stereospecificity of the enzymes. Incubation of $4b$ with svPDE and nP1 afforded the distinct linear DNSs $5$ and $6$, respectively (Scheme 1). The structure of $5$ was unambiguously determined to be $5'$-$p$A($3'$-$5'$)-$p$G via two-dimensional (2-D) NMR experiments, displaying correlations from $H^{5A}$ and
incubated with the other enzyme, svPDE, to determine the phosphorothioate stereochemistry. AMPS by nP1 and svPDE, respectively, which confirmed that the stereochemistry of 4b was determined to be (2′−S′)−pGS(2′−S′)P in 4b, which was hydrolyzed by svPDE, while the 3′−S′ phosphorothioate bond was hydrolyzed by nP1. Linear DNSs 5 and 6 were then incubated with the other enzyme, svPDE with nP1 and svPDE, to determine the phosphorothioate stereochemistry. Both 5 and 6 were hydrolyzed to GMP/GMPs and AMP/AMPs by nP1 and svPDE, respectively, which confirmed the stereochemistry of 5 and 6 to be (3′−S′)S_p and (2′−S′)R_p, respectively (Scheme 1). Taken together, the stereochemistry of 4b was determined to be (2′−S′)R_p(3′−S′)S_p, which indicates that (2′−S′)R_p in 4b was hydrolyzed by svPDE to afford 5 and (3′−S′)S_p in 4b was hydrolyzed by nP1 to afford 6.

Biocatalytic hydrolysis products of 4a and 4d by svPDE and nP1 were characterized next. The hydrolysis products of 4a by svPDE were confirmed to be a mixture of mononucleotides 7 and 8 (Scheme 2). The outcome indicates that svPDE hydrolyzed either the (2′−S′)R_p or the (3′−S′)R_p phosphorothioate linkage in 4a first and then subsequently rapidly hydrolyzed the linear DNS product to the mononucleotides. The resistance of 4a to nP1 also supports the assignment of the 3′−S′ phosphorothioate of 4a to be R_p. Taken together, the stereochemistry of 4a was determined to be (2′−S′)R_p(3′−S′)R_p. Incubation of 4d with nP1 afforded linear DNS 9, whose 5′−pG(2′−S′)pA structure was confirmed via 2-D NMR experiments, indicating that (3′−S′)S_p in 4d was hydrolyzed by nP1. While 6, the biocatalysis product of 4b by nP1, was hydrolyzed by svPDE, 9 was resistant to svPDE. The results confirmed that the stereochemistry of 9 is (2′−S′)S_p and that of 4d is (2′−S′)S_p(3′−S′)S_p.

Structural determination of the hydrolysis products of 4a, 4b, and 4d demonstrated that (2′−S′)R_p and (3′−S′)S_p of 4 are hydrolyzed by svPDE and nP1, respectively. Diastereomer 4c was resistant to both enzymes (Figure 2C), which confirms that the stereochemistry of 4c is (2′−S′)S_p(3′−S′)R_p. The results also indicate that the 3′−S′ phosphorothioate linkage and the 2′−S′ phosphorothioate linkage in 4 are resistant to svPDE and nP1, respectively, regardless of the phosphorothioate stereochemistry. Given the regiospecificity of svPDE, when 4a was incubated with svPDE, the (2′−S′)R_p phosphorothioate linkage was hydrolyzed first to afford 5′−pA(2′−S′)pG, and then, the (3′−S′)R_p phosphorothioate of the resultant linear DNS was subsequently hydrolyzed to afford the mononucleotides.

In the course of determining the phosphorothioate stereochemistry of 4a−4d, the regiospecificity as well as stereo-specificity of both svPDE and nP1 toward 4 has been revealed (Table 1). As seen with linear DNSs, svPDE and nP1 preferentially hydrolyzed R_p and S_p phosphorothioate linkages in 4, respectively. It is interesting to note that svPDE hydrolyzes only (2′−S′)R_p phosphorothioate linkages in 4, whereas the enzyme hydrolyzes both (2′−S′)R_p and (3′−S′)R_p phosphorothioate linkages in linear DNS. On the other hand, nP1 hydrolyzes only (3′−S′)S_p phosphorothioate linkages in both 4 and linear DNS. Overall, the assignment of the 4a−4d phosphorothioate stereochemistry completely matches the stereospecificity and the regiospecificity of the svPDE and nP1 biocatalysis as well as the hydrolysis patterns (Table 2). This novel biocatalysis was then applied to ADU-S100 (R_p(2′−S′)(2′−3′)-c-di-AMPSS), whose stereochemistry had been confirmed by an X-ray single-crystal structure.20 ADU-S100 was hydrolyzed to a mixture of adenosine monophosphorothioate and

Scheme 2. Hydrolytic Products of 4a and 4d Treated with svPDE and nP1

| svPDE | nP1 |
|-------|-----|
| 2′3′-cGAApMP | (2′−S′)R_p |
| linear DNS | (2′−S′)R_p/(3′−S′)R_p |
| (3′−S′)S_p |

Table 1. Stereospecific and Regiospecific Hydrolysis of 2′3′-cGAApMP (4) and Linear DNS by svPDE and nP1

H^5^G to 2′3′-cGAApMP in 1H−31P heteronuclear multiple bond correlation (HMBC) (Figure 3). In the same manner, the structure of 6 was determined to be 5′−pG(2′−S′)pA^5^ via 2-D NMR experiments. Formation of linear DNSs 5 and 6 indicates that the 2′−5′ phosphorothioate bond of 4b was hydrolyzed by svPDE, while the 3′−5′ phosphorothioate bond was hydrolyzed by nP1. Linear DNSs 5 and 6 were then incubated with the other enzyme, svPDE with nP1 and svPDE, to determine the phosphorothioate stereochemistry. Both 5 and 6 were hydrolyzed to GMP/GMPs and AMP/AMPs by nP1 and svPDE, respectively, which confirmed the stereochemistry of 5 and 6 to be (3′−S′)S_p and (2′−S′)R_p, respectively (Scheme 1). Taken together, the stereochemistry of 4b was determined to be (2′−S′)R_p(3′−S′)S_p, which indicates that (2′−S′)R_p in 4b was hydrolyzed by svPDE to afford 5 and (3′−S′)S_p in 4b was hydrolyzed by nP1 to afford 6.

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Structural determination of the hydrolysis products of 4a, 4b, and 4d demonstrated that (2′−S′)R_p and (3′−S′)S_p of 4 are hydrolyzed by svPDE and nP1, respectively. Diastereomer
adenosine monophosphate by svPDE and was resistant to nP1, which is consistent with the \((2’-S’)/\text{R}P/(3’-S’)/\text{R}P\) stereochemistry of ADU-S100.

**CONCLUSIONS**

A novel method of stereochemistry determination of 2’3’-cGAMP (4) via svPDE and nP1 biocatalysis has been developed. This method unambiguously assigned the phosphorothioate stereochemistry of four diastereomers of 4 by analyzing distinct hydrolysis patterns of the diastereomers incubated with svPDE and nP1. Furthermore, the stereospecificity as well as regiospecificity of both svPDE and nP1 toward 4 has been delineated. This novel method was successfully applied to other 2’3’-CDNSSs for their stereochemistry determination.

**EXPERIMENTAL SECTION**

General Information. Commercial reagents were obtained from reputable suppliers and used as received. Reactions were monitored by LC/MS (100 mm × 3 mm 2.7 μm column; 1–98% MeCN/water + 100 mM triethylammonium acetate gradient over 12 min; 1.0 mL/min flow; electrospray ionization (ESI); negative ion mode; UV detection at 254 nm). The \(^1^H\), \(^13^C\), and \(^31^P\) NMR spectra were recorded on a 500 MHz, a 151 MHz, and a 202 MHz Varian spectrometer, respectively. Data for the NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet), coupling constant (Hz), and integration.

Analytical Biocatalysis with svPDE. A 2’3’-cGAMP phosphorothioate triethylammonium salt diastereomer (10 mM in dimethyl sulfoxide (DMSO), 5 μL, 50 pmol) was placed in a vial and concentrated under high vacuum for 1 h. To the residue were added the buffer solution (100 mM Tris-HCl, pH 7, 1 mM ZnCl₂, 0.2 mM) and svPDE (Sigma-Aldrich P3243, a vial of 22 mg, 0.020 mmol) in the buffer solution (100 mM Tris-HCl, pH 7, 1 mM ZnCl₂) was added. The reaction mixture was shaken at 37 °C for 2 days, filtered, and purified by HPLC, eluting acetonitrile/water gradient with 100 mM triethylammonium acetate modifier to afford 5 triethylammonium salt (12 mg, 0.012 mmol, 63% yield) as a white solid. High-resolution mass spectrometry (HRMS) (ESI) calcld for \((C_{20}H_{25}N_{10}O_{12}P_{2}S_{2})\) \([M – H]^-\) 723.0570, found 723.0570. \(^1^H\) NMR (500 MHz, D₂O) δ 8.63 (s, 1H), 8.12 (s, 1H), 8.03 (s, 1H), 6.02 (d, J = 5.0 Hz, 1H), 5.81 (d, J = 5.5 Hz, 1H), 4.99 (m, 1H), 4.86 (m, 1H), 4.71 (m, 1H), 4.53 (brs, 2H), 4.34 (brs, 1H), 4.28–4.03 (m, 4H). \(^13^C\) NMR (151 MHz, D₂O) δ 158.79, 155.39, 153.85, 152.65, 151.44, 148.80, 140.03, 137.64, 118.49, 116.23, 87.27, 87.07, 83.57, 83.20, 75.03, 73.75, 73.61, 70.36, 65.24, 63.46. \(^31^P\) NMR (202 MHz, D₂O) δ 55.46, 43.38. **Table 2.** Stereochemistry Determination of 2’3’-cGAMPs (4) via Biocatalysis

| svPDE | nP1 |
|-------|-----|
| R₆R₆ | Mono-Nuc |
| R₆S₆ | linear DNS |
| S₆R₆ | resistant |
| S₆S₆ | resistant |

To a stirred solution of 2’3’-cGAMP phosphorothioate triethylammonium salt 4b (22 mg, 0.020 mmol) in the buffer solution (100 mM Tris-HCl, pH 7, 15 mM MgCl₂, 2 mL) was added svPDE (Sigma-Aldrich P3243, a vial of ≥0.40 units in 1 mL of water, 2 mL). The reaction mixture was shaken at 37 °C for 2 days, filtered, and purified by HPLC, eluting acetonitrile/water gradient with 100 mM triethylammonium acetate modifier to afford 5 triethylammonium salt (12 mg, 0.012 mmol, 63% yield) as a white solid. High-resolution mass spectrometry (HRMS) (ESI) calcld for \((C_{20}H_{25}N_{10}O_{12}P_{2}S_{2})\) \([M – H]^-\) 723.0570, found 723.0570. \(^1^H\) NMR (500 MHz, D₂O) δ 8.63 (s, 1H), 8.12 (s, 1H), 8.03 (s, 1H), 6.02 (d, J = 5.0 Hz, 1H), 5.81 (d, J = 5.5 Hz, 1H), 4.99 (m, 1H), 4.86 (m, 1H), 4.71 (m, 1H), 4.53 (brs, 2H), 4.34 (brs, 1H), 4.28–4.03 (m, 4H). \(^13^C\) NMR (151 MHz, D₂O) δ 158.79, 155.39, 153.85, 152.65, 151.44, 148.80, 140.03, 137.64, 118.49, 116.23, 87.27, 87.07, 83.57, 83.20, 75.03, 73.75, 73.61, 70.36, 65.24, 63.46. **Table 3.** Stereochemistry Determination of 2’3’-cGAMPs (4) via Biocatalysis

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furanyl)methyl Dihydrogen Phosphate (9). Prepared in analogy to that described for 6 in 78% yield. HRMS (ESI) calcd for \( (C_{20}H_{25}N_{10}O_{13}P_{2}S) [M – H]^- \) 707.0799, found 707.0807. \(^1\)H NMR (500 MHz, D$_2$O) $\delta$ 8.28 (s, 1H), 8.15 (s, 1H), 7.97 (s, 1H), 5.98 (m, 1H), 5.93 (m, 1H), 5.25 (m, 1H), 4.65 (m, 1H), 4.43 (m, 1H), 4.36 (m, 1H), 4.27–3.92 (m, 6H). \(^{13}\)C NMR (151 MHz, D$_2$O) $\delta$ 158.18, 155.24, 153.40, 152.70, 151.31, 148.39, 134.39, 138.03, 118.28, 115.86, 87.70, 86.34, 83.45, 82.74, 76.55, 74.82, 70.01, 69.55, 64.53, 63.84. \(^{31}\)P NMR (202 MHz, D$_2$O) $\delta$ 55.57, 2.09.

### ASSOCIATED CONTENT

1. Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01942.

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### Notes

The authors declare no competing financial interest.

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