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Synthesis of 5″-branched derivatives of cyclic ADP-carbocyclic-ribose, a potent Ca^{2+}-mobilizing agent: The first antagonists modified at the N1-ribose moiety

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ABSTRACT

The 5″-branched cyclic ADP-carbocyclic-ribose derivatives were designed and synthesized. These target compounds were identified as the first antagonists of cADPR without a substituent at the adenine 8-position, and were shown to be stable due to the N1-carbocyclic-ribosyl structure.

Cyclic ADP-ribose (cADPR, **1**, Figure 1), a naturally occurring metabolite of NAD^+,[1] is now recognized as a general mediator involved in Ca^{2+} signaling.[2] Since cADPR plays important physiological roles, cADPR analogues are important in proving the mechanism of cADPR-mediated Ca^{2+} signaling pathways and are also expected to be lead structures for the development of new drugs.[2,3] Therefore, the synthesis of cADPR analogues has been extensively investigated.[3–6]

Under neutral conditions, cADPR is in a zwitterionic form with a positive charge around the N(1)–C(6)–N^6 moiety[7] (pK_a = 8.3), making the molecule unstable, since the charged adenine moiety attached to the anomeric carbon of the N1-ribose can be an efficient leaving group. Accordingly, cADPR is readily hydrolyzed at the unstable N1-ribosyl linkage of its adenine moiety to produce ADP-ribose (ADPR), even in neutral aqueous solution.[8] Under physiological conditions, cADPR is also hydrolyzed at the N1-ribosyl linkage by cADPR hydrolase to give the inactive ADPR.[2,3] This kind of chemical and biological instability is undesirable for using cADPR or its analogues as biological tools and/or drug leads. Thus, we designed and synthesized cyclic ADP-carbocyclic-ribose (cADPcR, **3**) as a stable equivalent of cADPR, in which the oxygen atom in the N1-ribose ring of cADPR is replaced by a methylene group.[6a–c] Biological evaluation has shown that, in fact, it acts as a biologically and chemically stable equivalent of cADPR.[6] We also disclosed that cADPcR is the first cell-type selective analogue of cADPR.[6d]

Because of the highly potent and stable feature of cADPcR, it can be useful as a prototype for developing biological tools and/or drug leads. Therefore, we synthesized cADPcR analogues and investigated the structure–activity relationship[6] to result in finding several useful compounds, such as 3″-deoxy-cADPcR (**4**) with the most potent Ca^{2+}-mobilizing effect in this series of compounds.[6d]

It is known that modification of cADPR at the 8-position of the adenine ring with NH_2, N_3, or halogen substituent converts it from agonist to antagonist, and therefore, 8-NH_2-cADPR (**2**) has been effectively used as a potent cADPR antagonist in biological studies.[4a] In addition, 7-deaza-cADPR, in which the N7 atom of the adenine ring is replaced by a methine, has been shown to behave as a partial agonist.[4f] Thus, it is clear that the adenine ring plays an important role in the agonist/antagonist switching of the activity.

We recently found that the 8-substituted cADPcR analogues, that is, 8-Cl, 8-N_3, and 8-NH_2-cADPcR (**5**, **6**, **7**, respectively), behaved as full agonists, in spite of their substituting at the adenine-8-position.[6b] In addition, we showed that the modification at the 3″-position affects the agonistic activity. Deletion of the 3″-hydroxyl of the full agonistic 8-Cl, 8-N_3, and 8-NH_2-cADPcR converted them into partial agonists; the average maximal levels of 3″-deoxy-8-Cl-cADPcR (**8**), 3″-deoxy-8-N_3-cADPcR (**9**), and 3″-deoxy-8-NH_2-cADPcR (**10**) were 14%, 16%, and 21% of that of cADPcR, respectively.[6e] These findings suggest that deletion of the 3″-hydroxyl of the N1-ribose may reduce the maximal Ca^{2+} releasing activity of the compounds and shifted the compound toward becoming more antagonistic. Thus, the N1-ribose moiety

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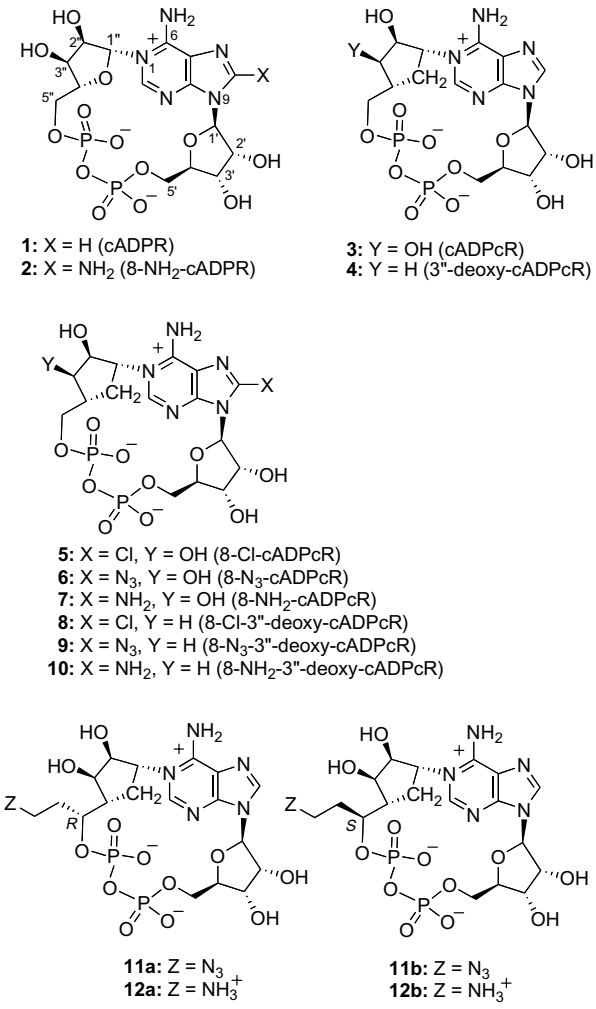


Figure 1. cADPR and its analogues.

may also be critically important in agonist/antagonist switching of the activity, and therefore, we thought that a new type of antagonists might be developed by the modification of the N1-ribose moiety of cADPcR, which can be useful because of the stability due to the carbocyclic-ribosyl structure.

On the other hand, although accumulating evidence indicates that cADPR mobilizes Ca^{2+} via ryanodine receptor (RyR) activation,[2,9] it is unclear whether cADPR directly interacts with RyRs or does so via any of the accessory proteins that interact with the cytoplasmic domains of ryanodine receptor.[10] In order to identify the target proteins of cADPR, the biologically and also chemically stable analogues of cADPR, which are able to bind specifically to the target proteins, can be effectively used. These analogues need to be conjugated with a functional residue, such as a fluorescent or a photo-reactive residue, and therefore, identification of a site for the modification of cADPR or its analogues without reducing the binding affinity is required. However, this kind of conjugation often reduced its affinity for the target proteins, in fact, it has been disclosed that modification of the pyrophosphate[4h] or N9-ribose moiety of cADPR reduced the activity.[3] Although introduction of a small group such as NH_2 at the 8-position does not reduce the affinity for the target proteins, the affinity reduced when a somewhat bulkier group is introduced at the 8-position.[3b,6b] We recently synthesized 2″ or 3″-substituted analogues of cADPcR to result in reducing the activity.[6e] Thus, the site of cADPcR suitable for introducing the functional groups to develop biological tools has not been identified, and accordingly, the 5″-substituted analogues of cADPcR would be of interest from this viewpoint.

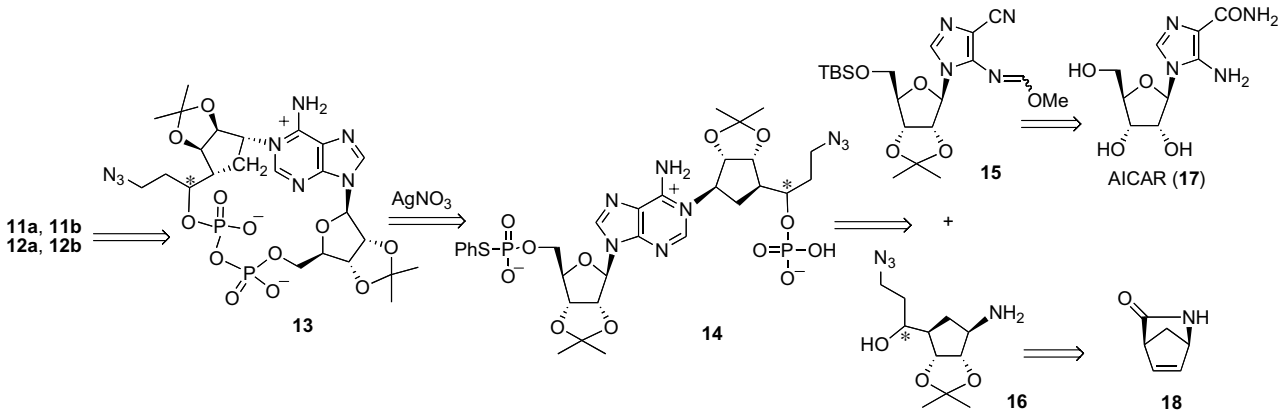
Taking these results and considerations into account, we designed the target 5″-branched analogues of cADPcR to develop the antagonists and/or the prototype compounds for biological tools. As the substituent at the 5″-position, azidoethyl and aminoethyl groups, which can be easily conjugated with a photo-reactive or a fluorescent group, were selected. Both of the (5″*R*)-diastereomers **11a** and **12a** and the (5″*S*)-diastereomers **11b** and **12b** were planned to synthesize for examining the effect of the stereochemistry on the biological activity.

We have developed an efficient total synthetic method for cADPR analogues,[5] which have been effectively used in the synthesis of a variety of cADPR analogues. Thus, we set out to synthesize the target compounds based on the total synthetic method. The synthetic plan is shown in Scheme 1 as a retrosynthetic analysis. The 5-branched chiral carbocyclic-ribosyl amines **16**, composing the N1-substituted moiety in the target compounds, were planned to prepare from commercially available (1*R*)-(−)-2-azabicyclo[2.2.1]hept-5-en-3-one (**18**). From the carbocyclic amines **16** and the known imidazole nucleoside derivative **15** derived from 5-amino-4-carbamoylimidazole-riboside (AICAR, **17**),[6a] the 5′-phenylthiophosphate-type substrate **14** for the key intramolecular condensation could be prepared. Treatment of **14** with $AgNO_3$/MS 3A as a promoter[5b,6a] was expected to form the cyclized product **13**, from which the desired 5″-branched cADPcR analogues **11a**, **11b**, **12a**, and **12b** would be obtained.

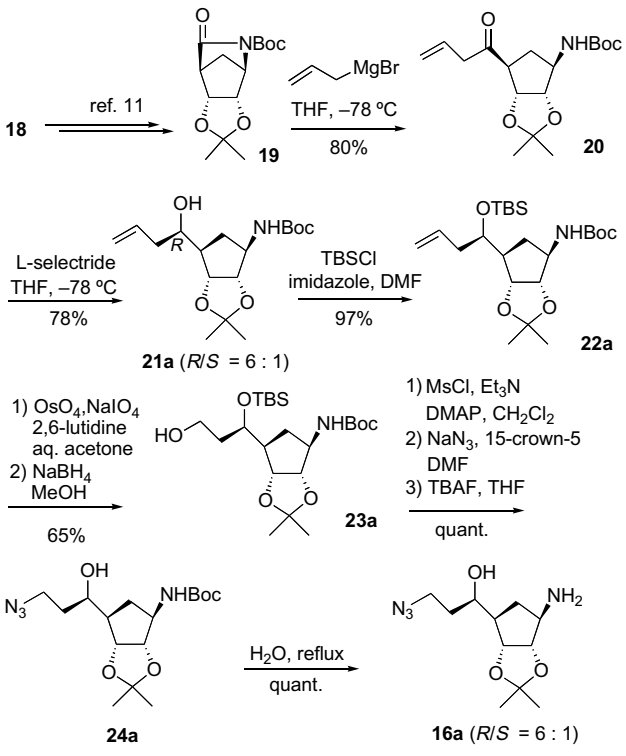
Synthesis of (5*R*)-branched carbocyclic-ribose **16a** is shown in Scheme 2. Grignard reaction of a known bicyclic amide **19**[11] with $CH_2{=}CHCH_2MgBr$ afforded the ring-opened ketone **20**, treatment of which with L-Selectride gave the (5*R*)-alcohol **21a** as the major product with the minor (5*S*)-alcohol **21b** (*R*/*S* = 6:1).[12] Although the diastereomers **21a** and **21b** were inseparable at this stage, the minor diastereomer was effectively removed at a later stage. After protection of the hydroxyl of **21a** with a silyl group, oxidative cleavage of the unsaturated bond followed by reduction with $NaBH_4$ gave the primary alcohol **23a**. The resulting hydroxyl of **23a** was replaced with an azido group via *O*-mesylation, and then the TBS group was removed to give **24a**. Heating **24a** in refluxing H_2O gave the (5*R*)-branched carbocyclic-ribosyl amine **16a** (*R*/*S* = 6:1).

Mitsunobu reaction of **21a** including the minor diastereomer **21b** (*R*/*S* = 6:1) with a Ph_3P/DIAD/*p*-NO_2 $PhCO_2H$ system, followed by removal of the *p*-nitorobenzoyl group and silylation of the resulting hydroxyl gave the silyl ether **22b** including **22a** as the minor diastereomer (*R*/*S* = 1:6) (Scheme 3), which was converted into **16b** (*R*/*S* = 1:6) by the same procedure as for preparing **16a**.

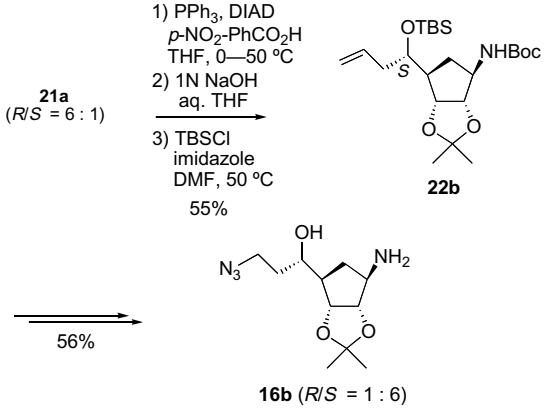
The target (5″*R*)-branched cADPcR analogues **11a** and **12a** were successfully synthesized from the carbocyclic amine **16a** and the imidazole nucleoside **15**, as shown in Scheme 4. The N1-substituted adenosine derivative **25a** was obtained in high yield by treating a mixture of **16a** and **15** with K_2CO_3 in MeOH at room temperature. The 5′-*O*-TBS group of **25a** was removed with TBAF to give **26a**. Treatment of **26a** with a *S*,*S*′-diphenylphosphorodithioate (PSS)/2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl)/pyridine system[13] gave the 5′-bis(phenylthio)phosphate **27a**. At this stage, (5″*S*)-minor diastereomer was removed by silica gel flash column chromatography and **27a** was obtained in a diastereomerically pure form. A phosphoryl group was introduced at the 5″-secondary hydroxyl of **27a** by Yoshikawa's method with $POCl_3$/$(EtO)_3PO$,[14] and the product was treated with H_3PO_2 and Et_3N[15] in pyridine to afford the corresponding 5′-phenylthiophosphate **14a**. The key intramolecular condensation reaction of **14a** was investigated with the $AgNO_3$/Et_3N/MS 3A/pyridine method.[5b,6a] The reaction was first carried out under 0.5 mM concentration of



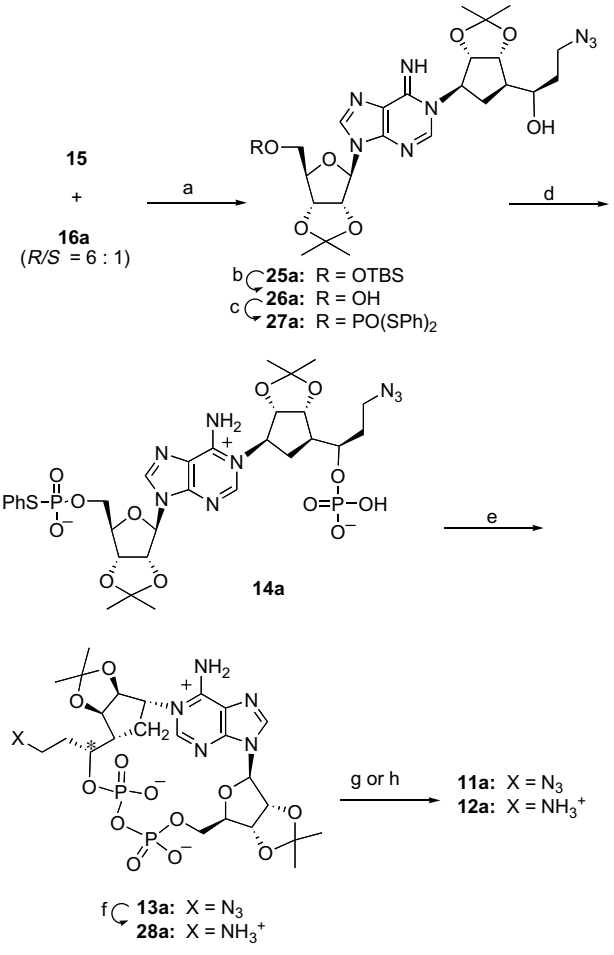
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4. Reagents and conditions: (a) K_2CO_3, MeOH, 83%; (b) TBAF, THF, quant; (c) PSS, TPSCl, py, 55%; (d) 1—$POCl_3$, $(EtO)_3PO$, 0 °C, 2—H_3PO_2, Et_3N, pyridine, 34%; (e) $AgNO_3$, MS 3A, Et_3N, py, 50%; (f) H_2, Lindar cat., H_2O, 74%; (g) 60% HCO_2H, quant (**11a**); (h) 1—60% HCO_2H; 2—28% NH_4OH, 67% (**12a**).

the substrate **14a**. Although the intramolecular pyrophosphate linkage was formed, the yield of the desired cyclization product **13a** was insufficient (27%), in which the unexpected cyclic dimer **29** (Fig. 2)[16] was concomitantly obtained in 20% yield. However, when the reaction was performed under lower than 0.1 mM substrate concentration, the yield of **13a** increased to 50%, where the dimer **29** was not obtained. Hydrogenation of **13a** with Lindlar's

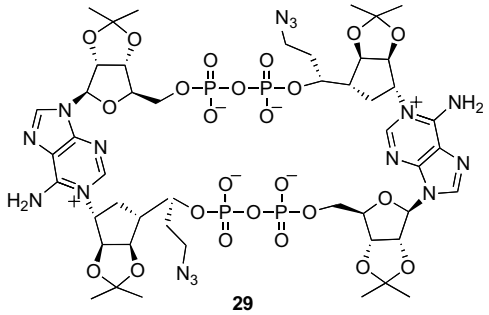


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Figure 2. Structure of the cyclic dimer **29**.

catalyst gave the corresponding (5″*R*)-5″-aminoethyl derivative **28a**. Finally, the isopropylidene groups of **13a** and **28a** were removed by acidic treatment with aqueous HCO_2H to furnish the target (5″*R*)-5″-azidoethyl-cADPcR (**11a**) and (5″*R*)-5″-aminoethyl-cADPcR (**12a**), respectively.[17]

According to the same procedure, (5″*S*)-5″-azidoethyl-cADPcR (**11b**) and (5″*S*)-aminoethyl-cADPcR (**12a**) were also synthesized from **16b**.[17]

The Ca^{2+}-mobilizing activity of 5″-branched cADPcR analogues **11a**, **11b**, **12a**, and **12b** in sea urchin egg homogenate was determined fluorometrically by monitoring Ca^{2+} with fura-2.[18] cADPR (**1**) and cADPcR (**3**) showed dose-dependent Ca^{2+} release with EC_{50} of 210 nM and 79 nM, respectively (Fig. 3A), which were consistent with the previous results.[6d] However, no Ca^{2+}-mobilizing activity was detected with all of the 5″-branched cADPcR analogues **11a**, **11b**, **12a**, and **12b** up to 30 μM of the compounds, indicating that they have no agonistic activity.

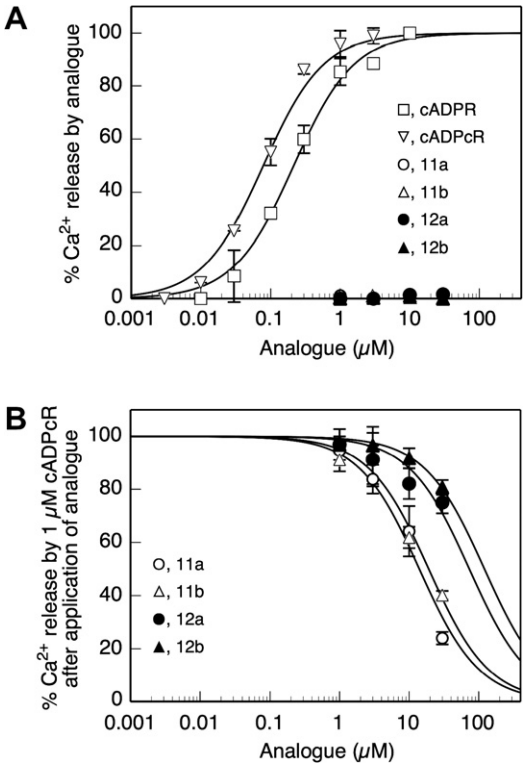

Figure 3. Ca^{2+}-mobilizing activity of compounds in sea urchin egg homogenate. (A) Dose-dependent Ca^{2+}-mobilizing activity of cADPR, cADPcR, and 5″-branched cADPcR analogues. The activity of each compound was expressed as a percent change in fura-2 fluorescence ratio (F340/F380) relative to 10 μM cADPR. (B) Dose-dependent antagonistic activity by 5″-branched cADPcR analogues. Antagonistic activity of each compound was expressed as a percent inhibition of Ca^{2+} release induced by 1 μM cADPcR after treatment of homogenate with the compound.

On the other hand, Ca^{2+} release by 1 μM cADPcR was decreased by the treatment of the 5″-branched analogues in a dose-dependent manner (Fig. 3B). Taken together, these findings suggest that the 5″-branched cADPcR analogues act as antagonists of cADPR. The IC_{50} for cADPcR-induced Ca^{2+} release of **11a**, **11b**, **12a**, and **12b** was estimated to be 14 μM, 19 μM, 73 μM, and 120 μM, respectively. Thus, azidoethyl derivatives **11a** and **11b** are more potent antagonists than the corresponding aminoethyl derivatives **12a** and **12b**, and effect of the stereochemical difference at the 5″-position on the activity is subtle.

It is important that antagonists of cADPR can be developed without modifying the adenine moiety, since all of the antagonists of cADPR known to date have a substituent at the 8-position. The activities of these 5″-branched cADPcR analogues are not so strong, compared with those of the well-known 8-substituted antagonists, such as 8-NH_2-cADPR (**2**).[4a] One possible advantage of these 5″-branched cADPcR analogues to the previous 8-substituted antagonists could be their stability due to the N1-carbocyclic-ribosyl structure. Thus, we investigated the stability in human serum with the (5″*S*)-azidoethyl derivatives**11b**; after 3 days of incubation of **11b** at 37 °C, 89% of **11b** remained.

As described, the 5″-branched cADPcR analogues have been successfully synthesized, which clearly demonstrates that the strategy of using a phenylthiophosphate-type substrate in the key intramolecular condensation reaction forming the pyrophosphate linkage is effective for total syntheses of cADPR related compounds. The 5″-branched cADPcR analogues were identified as the first antagonists of cADPR modified at the N1-ribose moiety and are shown to be stable due to the N1-carbocyclic-ribosyl structure. In addition, these results show that the azidoethyl derivatives **11a** and **11b** preserve the affinity for the target protein of cADPR, which suggests that the 5″-position may be a site suitable for introducing the functional groups to develop biological tools.

Acknowledgments

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17. Physicochemical data of the final compounds: (5″*R*)-5″-(2-Azidoethyl)-*cyclic ADP-carbocyclic-ribose* (**11a**, *triethylammonium salt*). ^{1}H NMR (500 MHz, D_2O) δ 8.78 (s, 1H), 8.32 (s, 1H), 6.08 (d, 1H, J = 5.5 Hz), 5.00 (dd, 1H, J = 5.5, 5.5 Hz), 4.88 (m, 1H), 4.60 (dd, 1H, J = 5.5, 5.5 Hz), 4.47 (dd, 1H, J = 5.5, 5.5 Hz), 4.40–4.36 (m, 3H), 4.25 (m, 1H), 4.07 (m, 1H), 3.49 (dd, 2H, J = 7.2, 14.4 Hz), 3.19–3.04 (m, 7H), 2.48 (m, 1H), 2.24 (m, 1H), 2.05–2.01 (m, 2H), 1.24 (t, 9H, J = 7.2 Hz); ^{13}C NMR (125 MHz, D_2O) δ 151.65, 142.22, 143.86, 120.35, 91.16, 84.77 (d, J = 11 Hz), 77.95, 76.65 (d, J = 5.5 Hz), 74.53, 72.16, 70.60, 64.64, 63.76, 48.28, 45.77 (d, J = 7.3 Hz), 33.46, 30.78; ^{31}P NMR (202 MHz, D_2O) δ −10.01 (s), −10.82 (s); UV (H_2O) λ_{max} 260 nm; HRMS (ESI, negative) calcd for $C_{18}H_{25}N_8O_{12}P_2$ 607.1073 found 607.1060 [(M−H)$^-$]. (5″*R*)-5″-(2-Aminoethyl)-*cyclic ADP-carbocyclic-ribose* (**12a**). ^{1}H NMR (500 MHz, D_2O) δ 8.71 (s, 1H), 8.30 (s, 1H), 6.06 (d, 1H, J = 4.6 Hz), 4.95 (dd, 1H, J = 4.6, 4.6 Hz), 4.81 (m, 1H), 4.52 (dd, 1H, J = 4.6, 4.6 Hz), 4.44–4.40 (m, 3H), 4.32 (m, 1H), 4.15 (m, 1H), 4.05 (m, 1H), 3.13 (m, 2H), 3.01 (m, 1H), 2.39 (m, 1H), 2.25(m, 1H), 2.13 (m, 1H), 2.05 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 151.48, 147.32, 143.55, 120.43, 91.14, 84.55 (d, J = 10.7 Hz), 76.73, 75.44 (d, J = 3.6 Hz), 74.50, 71.81, 70.50, 64.74, 63.59, 46.23, 37.13, 32.29, 29.92; ^{31}P NMR (202 MHz, D_2O) δ −9.37 (d, J = 17.3 Hz), −10.63 (d, J = 17.3 Hz); UV (H_2O) λ_{max} 260 nm; HRMS (ESI, negative) calcd for $C_{18}H_{27}N_6O_{12}P_2$ 581.1162 found 581.1159 [(M−H)$^-$]. (5″*S*)-5″-(2-Azidoethyl)-*cyclic ADP-carbocyclic-ribose* (**11b**, *triethylammonium salt*). ^{1}H NMR (500 MHz, D_2O) δ 9.00 (s, 1H), 8.28 (s, 1H), 5.99 (d, 1H, J = 6.4 Hz), 5.26 (m, 1H), 4.87 (m, 1H), 4.77 (m, 1H), 4.62–4.58 (m, 2H), 4.33 (m, 1H), 4.22 (dd, 1H, J = 3.2, 8.4 Hz), 4.03 (m, 2H), 3.52 (t, 2H, J = 6.8 Hz), 3.15 (dd, H, J = 7.3, 14.3 Hz), 2.86 (m, 1H),2.44–2.41 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H), 1.22 (t, 9 H, J = 7.3 Hz); ^{13}C NMR (125 MHz, D_2O) δ 151.21, 146.82, 146.18, 144.51, 120.64, 90.93, 85.15 (d, J = 10 Hz), 76.29, 74.57 (d, J = 4.6 Hz), 74.54, 73.39, 72.18, 71.07, 64.97 (d, J = 4.6 Hz), 62.81, 48.10, 45.62 (d, J = 4.6 Hz), 34.91, 24.99; ^{31}P NMR (202 MHz, D_2O) δ −10.27 (br s), −10.50 (br s); UV (H_2O) λ_{max} 259 nm; HRMS (ESI, negative) calcd for $C_{18}H_{25}N_8O_{12}P_2$ 607.1073 found 607.1066 [(M−H)$^-$]. (5″*S*)-5″-(2-Aminoethyl)-*cyclic ADP-carbocyclic-ribose* (**12b**). ^{1}H NMR (500 MHz, D_2O) δ 9.03 (s, 1H), 8.33 (s, 1H), 5.99 (d, 1H, J = 6.4 Hz), 5.22 (dd, 1H, J = 4.9, 6.4 Hz), 4.83 (m, 1H), 4.67 (m, 1H), 4.59 (dd, 1H, J = 2.1, 4.9 Hz), 4.53 (m, 1H), 4.32 (m, 1H), 4.23 (dd, 1H, J = 3.8, 9.8 Hz), 4.05–4.01 (m, 2H), 3.19 (m, 2H), 2.88 (m, 1H), 2.47 (m, 1H), 2.37 (m, 1H), 2.14 (m, 1H), 2.00 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 151.36, 146.70, 146.05, 144.31, 120.79, 90.82, 85.18 (d, J = 11 Hz), 76.09, 73.44 (d, J = 6.5 Hz), 73.34, 72.02, 71.05, 65.21 (d, J = 4.6 Hz), 62.67, 46.16 (d, J = 4.6 Hz), 36.89, 34.42, 24.80; ^{31}P NMR (202 MHz, D_2O) δ −9.30, −9.81 (each d, J = 13.9 Hz); UV (H_2O) λ_{max} = 259 nm; HRMS (FAB, negative) calcd for $C_{18}H_{27}N_6O_{12}P_2$581.1162 found 581.1175 [(M−H)$^-$].
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