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FULL PAPER

Synthesis of α-Branched Acyclic Nucleoside Phosphonates as Potential Inhibitors of Bacterial Adenylate Cyclases

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Abstract: Inhibition of *Bordetella pertussis* adenylate cyclase toxin (ACT) and *Bacillus anthracis* edema factor (EF), the key virulence factors with adenylate cyclase (AC) activity, represents a potential method how to treat or prevent toxemia related to whooping cough and anthrax, respectively. A novel series of α-branched acyclic nucleoside phosphonates (ANPs) having a hemiaminal ether moiety was synthesized as potential inhibitors of bacterial adenylate cyclases. ANPs prepared as bisamidates were not cytotoxic but did not exhibited any profound activity (IC_{50} > 10 μM) towards ACT in J774A.1 macrophage cells. The apparent lack of activity of the bisamidates was speculated to be due to the inefficient formation of the biologically active species (ANPpp) in the cells. On the other hand, two 5-haloanthraniloyl-substituted ANPs in the form of diphosphates (ANPpp) were shown to be potent ACT and EF inhibitors with IC_{50} values ranging from 55 to 360 nM.

Introduction

Pertussis, also called whooping cough, is a severe disease of the respiratory tract caused by *Bordetella pertussis*, characterized by persistent cough. Despite a relatively high level of vaccination coverage, pertussis remains a serious health problem, especially for children in developing countries.[1,2] *B. pertussis* is a Gram-negative pleomorphic, aerobic coccobacillus[3] with strictly human pathogenicity.[4] Pertussis symptoms are a result of cooperation of several *B. pertussis* virulence factors.[5] Adenylate cyclase toxin (ACT)[5a] – a member of the RTX (repeat in toxin) family of bacterial pore-forming toxins – represents one of the key *B. pertussis* virulence factors.[6]

Bacillus anthracis is a Gram-positive spore-forming bacterium and the etiologic agent of anthrax, a highly contagious disease.[7] Anthrax is a common disease in livestock, only occasionally affecting the humans, but it may spread as a result of biological warfare. *B. anthracis* secretes three main virulence factors, protective antigen (PA), lethal factor (LF) and edema factor (EF).[8] EF, just as *B. pertussis* ACT, exhibits adenylate cyclase activity and is responsible for the rapid ATP break down in infected cells leading to enormously high levels of cyclic AMP (cAMP) and, subsequently, to cell homeostasis disruption.[5a] Although *B. pertussis*[9] and *B. anthracis*[10] are, in general, sensitive to antibiotics, antibiotic treatment does not affect the bacterial toxaemia.[11] In addition, several antibiotic-resistant *B. pertussis*[12] and *B. anthracis*[13] strains have been reported. Thus, a development of novel strategies for prophylaxis and/or treatment of *B. pertussis* and *B. anthracis* infections is urgently needed.

Acyclic nucleoside phosphonates (ANPs)[14] are structural analogues of natural nucleotides that are known especially for their antiviral,[15] antibacterial,[16] and antiparasitic[17] properties. Adefovir dipivoxil (bis(POM)PMEA, **I**, Fig. 1), for example, is an efficient antiviral drug currently approved for treatment of hepatitis B infections.[18] In 1999 Shoshani et al.[19] reported that adefovir diphosphate (PMEApp, **II**, Fig. 1), the active metabolite of adefovir dipivoxil, is a powerful inhibitor of rat brain adenylate cyclase. Later, it was found that PMEApp is also a potent inhibitor of bacterial adenylate cyclases,[20] and crystal structures of EF-calmodulin[20] and ACT-calmodulin[21] with PMEApp were resolved. Recently, Česnek et al.[22] studied a series of amidate prodrugs of PMEA and selected the corresponding isopropyl ester L-phenylalanyl derivative **III** (Fig. 1) as a promising type of prodrug considering its *in vitro* activity, bioavailability and synthetic accessibility. Later, a series of C-2 substituted PMEA analogues was studied by our research group in order to improve the ACT inhibitory properties of these nucleotide analogues.[23]

Studies carried out by the Seifert group[24] revealed that *O*-anthraniloyl (ANT) and *O*-(*N*-methylanthraniloyl) (MANT) derivatives of nucleoside triphosphates (NTPs), i.e. ANT-NTPs and MANT-NTPs, respectively, had low micromolar to submicromolar inhibitory activity on *B. pertussis* ACT. Furthermore, Geduhn et al.[24b] studied a large series of mono- and bis-ANT-substituted NTPs and identified the first potent ACT inhibitor bis-Cl-ANT-ATP (**IV**, Fig. 2) with high selectivity relative to mammalian ACs. In addition to that, numerous ANT-NTPs and MANT-NTPs derivatives were shown to be potent competitive EF inhibitors.[25]

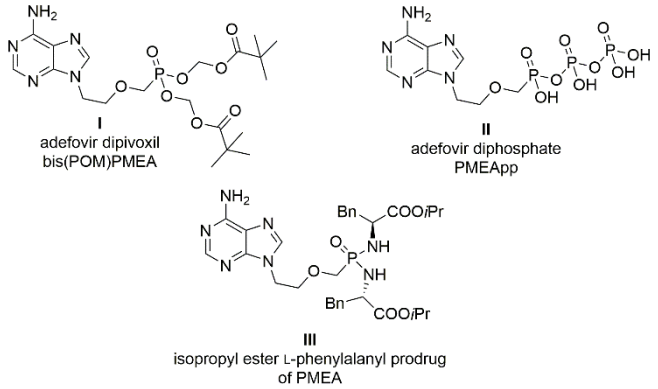


Figure 1. Structures of adefovir dipivoxil (**I**), adefovir diphosphate (**II**), and PMEA bisamidate prodrug **III**.

Fluorescent (M)ANT-NTPs were also shown to be suitable candidates for detailed studies of their binding interactions with ACT using direct fluorescence and/or fluorescence resonance energy transfer (FRET),[24a] but clearly were not therapeutically applicable due to their rapid degradation *in vivo*. For this reason, and in order to increase the flexibility of the ribose moiety of natural NTPs, novel fluorescent 5-chloroanthraniloyl-substituted ANPs were recently synthesized either as their diphosphates, i.e. Cl-ANT-ANPpp analogues (e.g. compound **V**, Fig. 2) or as their bisamidate prodrugs to improve their bioavailability.[26] These compounds were shown to be potent competitive ACT and EF inhibitors with sub-micromolar potency (IC_{50} values: 11–622 nM).[26]

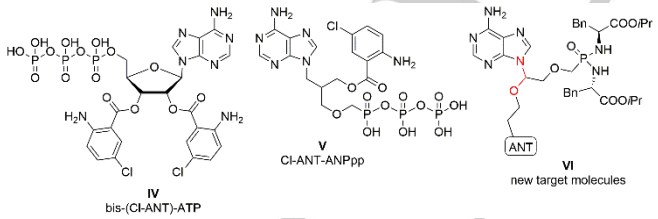


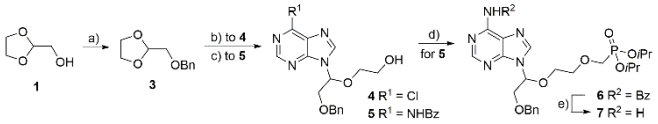
Figure 2. Structures of bis-Cl-ANT-ATP[24b] (**IV**), Cl-ANT-ANPpp derivative (**V**),[26] and new target molecules **VI**.

Herein, we report a synthesis of novel halo-ANT-ANPs as acyclic analogues of above mentioned halo-ANT-NTPs.[24,25] Compared to recently reported β-branched (branched at C-2' position) analogues (e.g. compound **V**, Fig. 2),[26] the current series represents α-branched ANPs (general structure **VI**, Fig. 2), that are branched at C-1' position of the acyclic linker connecting adenine and the phosphonate moiety in the form of the bisamidate prodrug. In this case, the C-1' atom is a part of a hemiaminal ether moiety. Although such compounds were expected to be considerably less stable and their synthesis more demanding compared to standard ANPs, the hemiaminal ether linker may represent a better mimic of the (deoxy)riboside moiety of natural NTPs than simple N^9-alkyl chain of classic ANPs. The new ANPs have two aliphatic arms, one bearing the phosphonate group and the second bearing various substitutions, including the 5-haloanthraniloyl moiety attached either through the ester or amide bond.

Results and Discussion

Chemistry

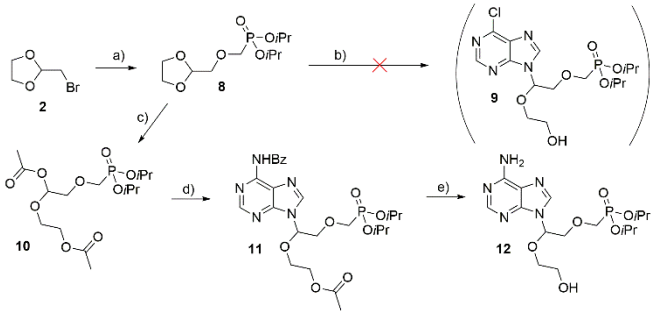
Commercially available 1,3-dioxolanes **1** and **2** were chosen as convenient starting materials. First, (1,3-dioxolan-2-yl)methanol (**1**, Scheme 1) was treated with benzylbromide and sodium hydride in DMF to give 2-(benzyloxymethyl)-1,3-dioxolane (**3**) in a 65% yield. Compound **3** was then used as a model compound for optimization of alkylation of purines with 1,3-dioxolane **1**. Reaction of compound **3** with 6-chloropurine, pre-treated with sodium hydride in DMF, afforded alkylated 6-chloropurine derivative **4** (Scheme 1) in a 52% yield. Unfortunately, subsequent attempts to alkylate intermediate **4** at the free hydroxyl group with diisopropyl (bromomethane)phosphonate were not successful and only decomposition of starting material **4** was observed. Instead, benzoyl-protected adenine derivative **5** (Scheme 1) was prepared by the reaction of N^6-benzoyladenine (*N*-(9*H*-purin-6-yl)benzamide) with compound **3** in a 30% yield. Subsequently, the alkylation of adenine derivative **5** with diisopropyl (bromomethane)phosphonate and sodium hydride in DMF afforded phosphonate **6** (in 13% yield), that was further converted by methanolysis to the desired adenine compound **7** (Scheme 1) in a 53% yield.



Scheme 1. Optimization of alkylation of the purine bases. Reagents and conditions: a) benzylbromide, NaH, DMF, 0 °C to rt, 24 h; b) TMSI, cyclohexene, -78 °C to -60 °C, then a mixture of 6-chloropurine, NaH, DMF, -60 °C to rt; c) TMSI, cyclohexene, -78 °C to rt, then a mixture of N^6-benzoyladenine, NaH, DMF, -60°C to rt; d) $BrCH_2P(O)(OiPr)_2$, NaH, DMF 0 °C to rt, 24 h; e) 1 M NaOMe/MeOH, MeOH, rt, 24 h.

The original goal was to develop an efficient synthesis of PMEA derivatives branched at the C-1' position (α-position) of the aliphatic linker (general structure **VI**, Fig. 2). The synthesis started with alkylation of 2-(bromomethyl)-1,3-dioxolane **2** (Scheme 2) with diisopropyl (hydroxymethane)phosphonate and sodium hydride in DMF to obtain phosphonate **8** in a 47% yield. An attempt to prepare acyclic nucleoside phosphonate **9** (Scheme 2) directly from compound **8** using previously reported conditions (treatment of 2-(benzyloxymethyl)-1,3-dioxolane with trimethylsilyl iodide (TMSI) in cyclohexene at -78 °C, then addition of the mixture to the sodium salt

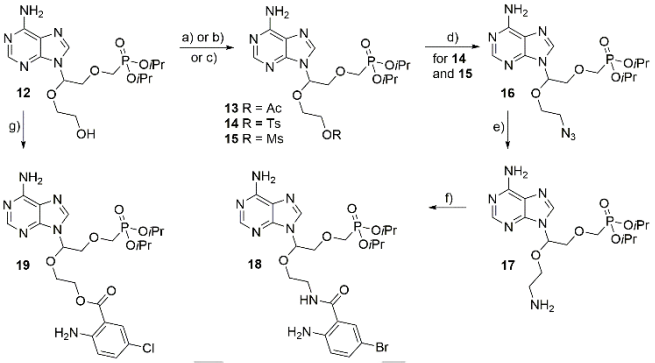
of 6-chloropurine in dry DMF at -60 °C)[27] was not successful. Albeit TMSI is commonly used for the preparation of free phosphonic acids from the corresponding dialkyl esters,[28] it was anticipated that decomposition of diisopropyl phosphonate moiety under the above conditions (-60 °C)[27] would be prevented, or at least slowed down. Unfortunately, this was not the case and decomposition of compound **8** led to formation of a complex reaction mixture and the desired product **9** was not observed.



Scheme 2. Synthesis of α-branched ANPs. Reagents and conditions: a) $HOCH_2P(O)(OiPr)_2$, NaH, DMF, -15 °C to rt, 16 h; b) TMSI, cyclohexene, -78 °C to -60 °C, then a mixture of 6-chloropurine, NaH, DMF, -60 °C to rt; c) Ac_2O, H_2SO_4 (cat.), rt, overnight; d) N^6-benzoyladenine, $SnCl_4$, MeCN, 2 h; e) 1 M NaOMe/MeOH, MeOH, rt, 24 h.

To overcome this problem, we decided to employ a modified methodology for synthesis of α-branched acyclic nucleosides developed by Zavgorodnii et al.[29] First, 1,3-dioxolane derivative **8** (Scheme 2) was – after some optimization of previously reported acetolysis of cyclic acetals[30] – converted into acyclic diacetylated phosphonate **10** in a 44% yield. An application of the modified Zavgorodnii conditions,[29] i.e. treatment of N^6-benzoyladenine and diacetyl intermediate **10** with tin(IV) chloride in acetonitrile at room temperature, afforded fully protected phosphonate **11** in a 51% yield. The removal of both acyl groups from phosphonate **11** using a catalytic amount of sodium methoxide in anhydrous methanol yielded desired acyclic nucleoside phosphonate **12** (Scheme 2) in 91%.

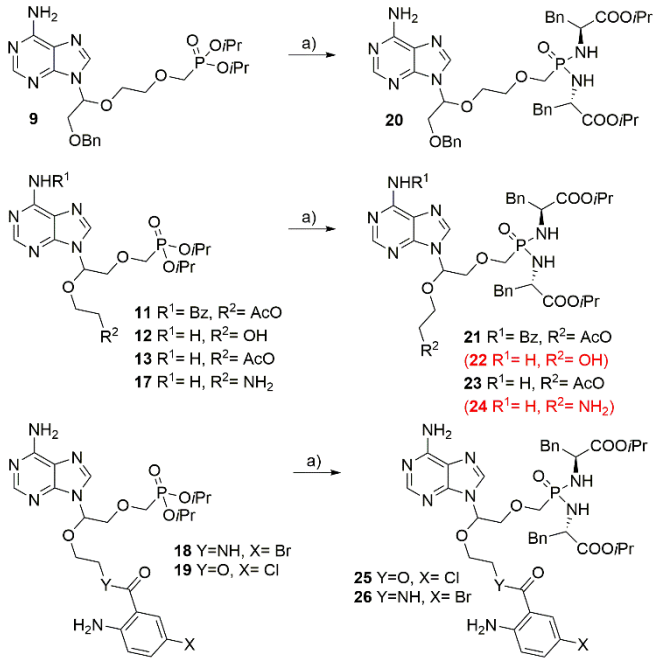
Key intermediate **12** was then converted to the corresponding acetyl, tosyl, and mesyl derivatives **13** (28%), **14** (58%), and **15** (76%), respectively (Scheme 3). Both tosyl and mesyl derivatives **14** and **15**, respectively, were utilized for the preparation of amino derivative **17** via azido compound **16** (Scheme 3). The azidation showed to be quite a problematic step due to a cleavage of one isopropyl group from the diisopropyl phosphonate moiety during heating of compound **14** or **15** with sodium azide in DMF, as reported before by Holý.[31] Thus, the azidation conditions had to be optimized and the best results were obtained when compound **14** or **15** was treated with sodium azide in DMF at room temperature for a prolonged period of time (96 h). These reaction conditions afforded azido derivative **16** (Scheme 3) in high yields: 64% from **14** and 84% from **15**. Clearly, the reaction sequence exploiting mesyl derivative **15** is advantageous since both reaction steps, i.e. mesylation and azidation, are more efficient and provided azido compound **16** in higher overall yield. Subsequent treatment of azido derivative **16** with hydrogen on Pd/C in methanol at room temperature yielded 84% of desired amino derivative **17** (Scheme 3).



Scheme 3. Synthesis of α-branched ANPs and their anthraniloyl derivatives. Reagents and conditions: a) AcCl, pyridine, 0 °C to rt, 2 h; b) TsCl, **DMAP, pyridine,** 0 °C to rt, 12 h; c) MsCl, pyridine, -15 °C to 0 °C, 2 h; d) NaN_3, DMF, rt, 96 h; e) H_2 (760 torr), Pd/C (10%), MeOH, rt, 96 h; f) 5-bromoisatoic anhydride, TEA, DMF, 50 °C, 1.5 h; g) 5-chloroisatoic anhydride, TEA, DCM/DMF, rt, 8 h.

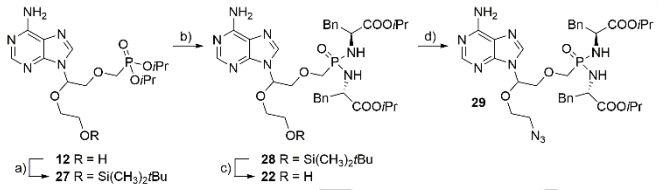
Once we had synthesized compounds **12** and **17** we were able to proceed with the synthesis of target fluorescent halo-anthraniloyl-substituted acyclic nucleoside phosphonates (halo-ANT-ANPs). Amino derivative **17** was converted to compound **18** (64%) by its treatment with 5-bromoisatoic anhydride and triethylamine in DMF at 50 °C. Product **19** (Scheme 3), containing an ester bond, was prepared in the same yield (64%) by the reaction of compound **12** with 5-chloroisatoic anhydride and triethylamine in a dichloromethane/DMF mixture at room temperature.

Finally, the selected diisopropyl phosphonates were converted into the corresponding bisamidates using transsilylation (trimethylsilylbromide (TMSBr) in pyridine at room temperature) and subsequent treatment of silyl esters with L-phenylalanine isopropyl ester under standard reaction conditions (trimethylamine, 2,2′-dipyridyldisulfide (aldrithiol-2), triphenylphosphine, pyridine, 55 °C) developed in our laboratory.[32] Since our α-branched ANPs with hemiaminal ether moiety were expected to be labile under acidic conditions, pyridine is the preferred solvent over acetonitrile during the bisamidate formation as pyridine is able to scavenge HBr which may be present in commercial TMSBr and is released during the reaction course. Under the above reaction conditions, phosphonates **9**, **11**, **13**, **18** and **19** (Scheme 4) afforded final bisamidate prodrugs **20**, **21**, **23**, **25** and **26** in 17-35% yields. Analogous treatment of compounds **12** and **17** did not afford desired bisamidates **22** and **24** (Scheme 4), respectively. High reactivity of the free hydroxyl and amino groups might be the reason for the failure to prepare compounds **22** and **24** by the above procedure and another synthetic approach had to be developed.



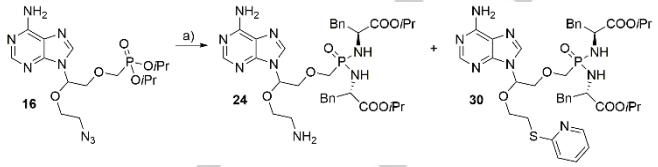
Scheme 4. Synthesis of bisamidate prodrugs of α-branched ANPs. Reagents and conditions: a) TMSBr, pyridine, rt, 12 h, then L-phenylalanine isopropyl ester hydrochloride, Et_3N, 2,2′-dipyridyldisulfide, PPh_3, pyridine, 55 °C, 24 h. Compounds **22** and **24** were not obtained.

In order to prepare the final product **22**, *t*-butyldimethylsilyl (TBDMS) group was selected as suitable protecting group of the hydroxyl in derivative **12**, as TBDMS can be efficiently removed later under non-acidic conditions. Thus, compound **12** was treated with TBDMSCl and imidazole in DMF at room temperature to give TBDMS derivative **27** (66%), which was then converted by the standard procedure[32] to bisamidate **28** in a 35% yield (Scheme 5). The target hydroxyl derivative **22** (Scheme 5) was obtained in a 46% yield upon removal of the TBDMS group from compound **28** using silica gel-supported tetrabutylammonium fluoride (TBAF) in dry DMF.



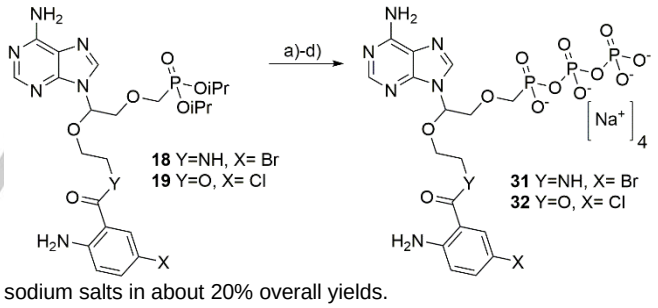
Scheme 5. Synthesis of bisamidate prodrugs of α-branched ANPs. Reagents and conditions: a) TBDMS-Cl, imidazole, DMF, rt, 12 h; b) TMSBr, pyridine, rt, 12 h, then L-phenylalanine isopropyl ester hydrochloride, Et_3N, 2,2′-dipyridyldisulfide, PPh_3, pyridine, 55 °C, 24 h; c) TBAF on silica gel (~1.5 mmol/g F- loading), DMF, rt, 12 h; d) PPh_3, DIAD, DPPA, THF, 1 °C to -15 °C, 20 min, 0 °C to rt, 12 h.

An attempt to prepare bisamide prodrug directly from the azido derivative **16** (Scheme 6) was not successful. Instead, under the standard reaction conditions for the formation of bisamidate prodrugs,[32] only amino derivative **24** (2%) and 2-(pyridin-2-ylthio) analogue **30** (5%) were isolated in low yields. Compound **24** was most likely formed by Staudinger[33] reduction of azido group with triphenylphosphine (or its complex) and side product **30** probably arisen as a result of nucleophilic attack of pyridine-2-sulfide (released from the aldrithiol-2/triphenylphosphine complex) on an activated intermediate during the reaction course. The azido analogue **29** (Scheme 5) was successfully prepared in a 40% yield directly from bisamidate **22** by its treatment with diphenoxyphosphoryl azide (DPPA)[34] under the Mitsunobu reaction conditions.[35]



Scheme 6. Synthesis of bisamidate prodrugs of α-branched ANPs. Reagents and conditions: a) TMSBr, pyridine, rt, 12 h, then L-phenylalanine isopropyl ester hydrochloride, Et_3N, 2,2′-dipyridyldisulfide, PPh_3, pyridine, 55 °C, 24 h.

In order to study inhibitory potential of novel halo-ANT-ANPs directly on ACT and EF enzymes, the selected analogues **18** and **19** were converted to their diphosphate derivatives (halo-ANT-ANPpp), i.e. analogues of natural NTPs. Since the standard morpholidate methodology for the NTPs synthesis[36] failed, the method using 1,1′-carbonyldiimidazole (CDI) was employed instead.[37] Thus, diisopropyl phosphonates **18** and **19** (Scheme 7) were stepwise treated with TMSBr in pyridine (to obtain silyl esters), with tetraethylammonium bicarbonate (TEAB) buffer (to form tetraethylammonium salts), CDI in DMF (to form activated esters), and finally with tetrabutylammonium pyrophosphate in DMF to obtain, after purification on DOWEX (50WX8 Na^+) and lyophilisation, desired NTP analogues **31** and **32** as



sodium salts in about 20% overall yields.

Scheme 7. Synthesis of phosphonato-diphosphates. Reagents and conditions: a) TMSBr, pyridine, rt, 12 h, then 2 M TEAB buffer; b) CDI, DMF, rt, 1 h; c) $(Bu_3N)_2P_2O_7$, DMF, rt, 12 h; d) DOWEX (50WX8 Na^+).

Biological activity

Inhibition of ACT in the cell-based assay

All prepared bisamidate prodrugs were tested for their ability to inhibit ACT activity in J774A.1 macrophage cells. Murine macrophage cells J774A.1 were preincubated with various concentrations of the tested compounds for 5 h and subsequently exposed to *Bordetella pertussis* ACT for 0.5 h. The cells were lysed, and the amount of cAMP was determined. None of the final prodrugs tested (**20** - **26**, **29**, and **30**) showed any appreciable activity towards ACT (IC_{50} > 10 μM) and none exhibited any cytotoxic effect in J774A.1 cells at concentrations of 10 μM for 5 h.

Inhibition of ACT activity in the cell-free assay

The triphosphate analogues **31** and **32** were tested, along with PMEApp as a control, for their inhibitory activity towards recombinant adenylate cyclase toxin from *B. pertussis* provided by two distinct suppliers (Sigma and Enzo Life Sciences), as well as towards edema factor (EF) from *Bacillus anthracis* (Table 1). Compounds **31** and **32** inhibited both ACTs with IC_{50} values in a range of 100 – 360 nM and had slightly better inhibitory activity towards EF with IC_{50} values 55 and 81 nM, respectively.

Table 1. IC_{50} values of halo-ANT-ANPpp for ACT and EF.

Compound	IC_{50} [nM][a]		
	ACT Sigma	ACT Enzo	EF
PMEApp	16.0 ± 0.2	13.6 ± 4.7	11.5 ± 2.6
31	214 ± 26	102 ± 17	55.1 ± 4.5
32	103 ± 30	362 ± 36	81.2 ± 25.8

[a] Data are the mean ± SD of at least three independent experiments.

Inhibition of mACs

Furthermore, the ability of the prepared bisamidate prodrugs (**21** – **24**, **26** and **30**) to inhibit host mammalian adenylate cyclases (mACs) was examined (Table 2). The assays were carried out using HEK293 cells stably expressing mAC1, mAC2, or mAC5, and each compound was tested in two independent experiments at 30 μM. The mACs tested are representatives of the three major mAC families. Specific activation of the mACs overexpressed in the cells was accomplished by previously reported methodology,[38] in which the calcium ionophore A23187 was employed to stimulate AC1, a PKC activator – phorbol 12-myristate 13-acetate (PMA) – was used to stimulate AC2, and a low forskolin concentration was used to selectively stimulate AC5. Most compounds had no or minimal effects on the activity of the mACs. Exceptions included compound **22**, which inhibited AC2 by 50% and for two compounds that slightly potentiated the selectively-stimulated cAMP response at AC5 (compound **26**) and at both AC2 and AC5 (compound **30**, Table 2).

Table 2. Mammalian AC1, AC2 and AC5 inhibition with prodrugs at 30 μM.

Compound	Response % of Control[a]		
	AC1	AC2	AC5
21	67 ± 11	119 ± 4	116 ± 2
22	72 ± 11	50 ± 7	102 ± 6
23	80 ± 8	125 ± 1	116 ± 5
24	79 ± 18	105 ± 6	113 ± 4
26	105 ± 15	123 ± 3	137 ± 10
30	70 ± 1	144 ± 3	137 ± 21
SKF83566[b]	77 ± 7	-1 ± 5	107 ± 7
SQ22536[c]	64 ± 5	47 ± 8	70 ± 4

[a]Data are the mean ± SEM relative to the control response (100%) in two independent experiments. [b]SKF83566 is a selective inhibitor AC2.[38] [c]SQ22536 is a non-selective P-site inhibitor.

Molecular modelling

Crystal Structure Reinterpretation and Docking. The crystal structure of ACT with PMEApp bound (PDB code: 1ZOT)[21] was re-interpreted due to several problems in the active site caused by low resolution and weak electron density in that region. First, the purine core of PMEApp was shifted to form two H-bonds with Val271:N,O backbone and still fit in the electron density visualised in Coot.[39] Second, Mg903 was fitted to the electron density map and the PMEApp linker (up to the phosphonate group) was optimized by quantum mechanics to relieve any clashing and to maintain a good magnesium coordination geometry. Third, several active-site residues (Arg41, Lys58, Lys65) were fitted to the $2F_oF_c$ and F_oF_c electron density maps to achieve good β- and γ-phosphate coordination. Furthermore, the Asn304 and His298 side chains were flipped and Mg902 was changed to a water molecule to comply with H-bonding and magnesium coordination requirements. This re-interpreted crystal structure was used to redock the PMEApp ligand. The redocked ligand closely mimicked the re-interpreted crystal structure, which validated the procedure (Fig. 3a).

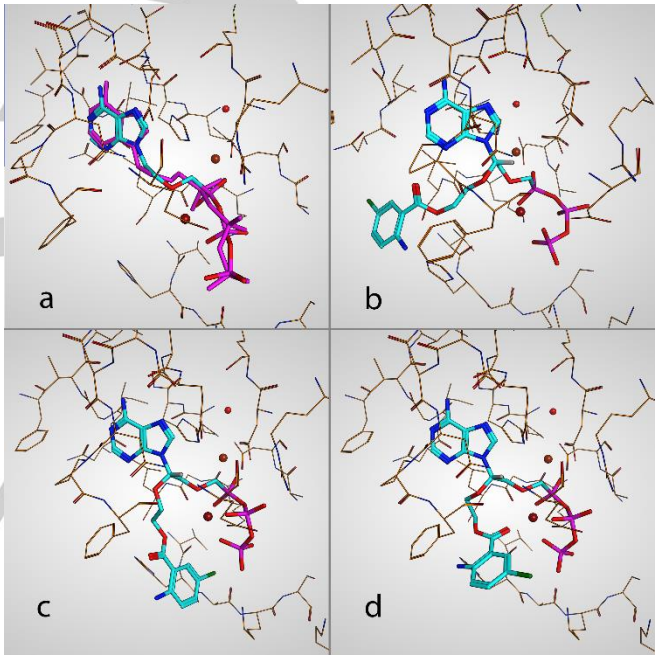


Figure 3. Docking studies of PMEApp and compound 32 to ACT. Picture description: Red sphere - Mg902 changed to a water molecule; Brown spheres - Mg901 and Mg903 ions; Purple - optimized position of original PMEApp; Cyan - docking results a) redocked PMEApp with optimized docking setup; b) first representative docking pose of **32**; c) second non-chelating docking pose of **32**; d) second chelating pose of **32**.

Subsequently, docking studies were performed to elucidate the binding properties of the ANT moiety in compound **32**. For that purpose, the template docking protocol was used due to the close structural similarity of the main aliphatic chains of PMEApp and compound **32**. This approach helped to eliminate potential false-positive results arising from the combination of quite a large and flexible ligand on one side, and a bulky binding site of ACT on the other. First, we attempted to figure out which of the two enantiomers

of **32** would be preferred by the enzyme. Although it was difficult to absolutely discriminate between the two isomers due to the high flexibility of these molecules, (*S*)-**32** exhibited better ability to relax in the ACT binding site than (*R*)-**32**. The docking of (*S*)-**32** revealed two possible binding modes in the ACT active site: a) one depicts presumed orientation of the ANT moiety in the pocket adjacent to the purine base without any specific interaction (Fig. 3b); b) another shows the ANT moiety positioned in the space nearby magnesium ion Mg903 in non-chelating (Fig. 3c) or chelating (Fig. 3d) orientations, which cannot be excluded or considered as false-positive because of the inhibitor structure. Again, because of the significant flexibility of the acyclic part of inhibitor **32**, it is difficult to determine the predominant pose.

Conclusions

We have designed and synthesized a novel series of acyclic nucleoside phosphonates (ANPs) as potential inhibitors of bacterial adenylate cyclases (ACs), namely adenylate cyclase toxin (ACT) from *Bordetella pertussis* and edema factor (EF) from *Bacillus anthracis*. The structural design of target ANPs was based on previously reported[24] 5-haloanthraniloyl derivatives of nucleoside triphosphates (halo-ANT-NTPs) as potent ACT inhibitors with high selectivity relative to mammalian ACs (mACs). It was expected that the attached aromatic halo-ANT moiety could increase the inhibitory properties of halo-ANT-ANPs due to its potential interactions with the hydrophobic pocket of bacterial adenylate cyclases.

The synthesis of the desired compounds was a challenging task because of the presence of the hemiaminal ether moiety (an analogy of N-glycosidic bond of natural nucleotides) that made the target compounds considerably less stable compared to standard ANPs. The methodology consisting of condensation of suitable acetal derivative, namely compound **10**, with N^6-benzoyladenine in the presence of $SnCl_4$ enabled us to prepare nine final bisamidates, including the key halo-ANT-ANP derivatives **25** and **26**, for the cell-based assays and two phosphonato-diphosphates **31** and **32** (analogues of natural NTPs) for enzymatic assays.

The bisamidates tested did not exhibited the ability to significantly inhibit ACT activity in J774A.1 macrophage cells (IC_{50} > 10 µM). The lack of potency of the prepared compounds was speculated to be due to insufficient phosphorylation in the cells to form the active species (ANPpp) or their poor stability. Therefore, we decided to prepare two analogues of natural NTPs, namely **31** and **32** (Scheme 7), to verify their direct inhibitory potential in the enzymatic assays. Both compounds were shown to be potent inhibitors of all ACTs and EF tested (IC_{50}s in a range of 55 – 360 nM). Furthermore, the known crystal structure of ACT with bound PMEApp was re-interpreted in order to obtain more reliable data from molecular modelling. The thorough docking studies based on this improved model suggested several possible docking poses for (*S*)-**32**, but it was not possible to determine the predominant pose with certainty.

In order to reveal potential selectivity of the prepared compounds, they were also evaluated as potential inhibitors of selected mammalian adenylate cyclases (mACs). No significant inhibition of mAC1, mAC2, and mAC5 in cell-based assays was observed for the novel ANPs, except for compound **22,** which selectively inhibited mAC2 by 50%. Two other compounds were shown to slightly potentiate some of the mACs, namely mAC5 (compound **26**) and both mAC2 and mAC5 (compound **30**).

In summary, variously modified ANPs represent excellent tools to study the molecular interactions between nucleotide analogues and their target enzymes, and they are potentially valuable agents with promising biological properties. This study is a part of broader structure-activity relationship (SAR) study of modified ANPs as potent inhibitors of bacterial ACs. The compounds designed as phosphonato-diphosphate derivatives were shown to be potent inhibitors of both ACT and EF in the range of hundreds of nM, but in the form of their bisamidate prodrugs they lacked activity in the cell-based assays. Thus, although the ANPs reported herein lack the potential for further development as therapeutic agents, the nucleoside triphosphate analogues are valuable compounds for further studies of their interactions with adenylate cyclases on molecular basis.

Experimental Section

General chemistry.

Starting compounds and other chemicals were purchase from commercial suppliers or prepared according to the published procedures. Solvents were dried by standard procedures. Solvents were evaporated at 40 °C/2 kPa. Analytical TLC was performed on plates of Kieselgel 60 F 254(Merck). NMR spectra were recorded on Bruker Avance 500 (^{1}H at 500 MHz, ^{13}C at 125.8 MHz, ^{31}P at 202.4 MHz,) spectrometer with TMS or dioxane (3.75 ppm for ^{1}H, 67.19 ppm for ^{13}C NMR) as internal standard or referenced to the residual solvent signal. The numbering system for ^{1}H NMR and 13C NMR spectra of the prepared compounds are shown on compounds **5**, **12**, **19**, **25**, and **26** (see Supplementary Information).

HR MS spectra were taken on a LTQ Orbitrap XL spectrometer. Flash chromatography on normal phase and deionization on reversed phase were performed on a Reveleris Flash Chromatography System. Final purification was performed on a Redisep Rf Gold C18 Teledyne ISCO column. Preparative HPLC purification of triphosphate analogues was performed on a column packed with POROS®HQ 50 mm (50 mL) with use of a gradient of TEAB in water (0.05-0.5 M). The purity of the tested compounds was determined by HPLC (H_2O-CH_3CN,linear gradient) and was higher than 95%.

Molecular Modelling.

Crystal Structure Reinterpretation. Crystal structure of *Bordetella pertussis* adenylate cyclase toxin (ACT) with calmodulin (CaM) and

bound PMEApp (PDB ID:1ZOT, resolution 2.2 Å)[21] was used for molecular modelling. A close inspection of ACT active site using Coot (version 0.8.7)[39] revealed several obvious problems with the ligand, Mg^{2+} ions and active-site residues due to low resolution and weak electron density in this region. We have therefore used model building in Coot and quantum mechanical (QM) optimizations. For the latter, we have used fast and reliable corrected semiempirical QM method PM6-D3H4[40] coupled with implicit solvent model COSMO[41] with ε_r = 78.4 using MOPAC2016 program[42] within Cuby4 framework.[43] Optimization convergence criteria were the following: the maximal energy change was below 0.006 kcal/mol, the maximum gradient change was below 1.2 kcal/mol/Å and the maximal root-mean-square of the gradient was below 0.6 kcal/mol/Å.

Docking. The pre-processed protein was prepared with MOE Structure Preparation and Protonate 3D tools with the default setup. Both enantiomers of compound **32**, (*R*)-**32** and (*S*)-**32**, were properly protonated and minimized to RMS gradient of 0.001 kcal/mol. For docking study, the Template docking protocol was selected with substructure setup where all atoms of PMEApp except hydrogens in the C-1' position (aliphatic α-carbon atom) were selected as the Query in the setup. Bond rotation of ligands was allowed and default placement and refinement methods were used with 50 retained structures after the placement and 30 retained structures after the refinement. For all calculations Amber12:EHT mixed force-field was used with R-Field solvent model.

Biological assays

Effect on the viability of J774A.1 cells. J774A.1 cells were plated onto white 96-well assay plates at 5 x 10^4 cells per well and allowed to attach overnight. Cells were then washed with HBSS and treated with 10 µM compounds for 5 h. Cell viability was then assessed with a Cell Titer-Glo Luminescent Cell Viability assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Measurement of luminescence signal was performed by use of a GENios microplate reader (Tecan Systems). Data was expressed as percentage of the control, represented by untreated cells.

Inhibition of ACT – cell-based assay. J774A.1 cells were seeded in a 96-well plate at 5x10^4 cells per well and left to attach overnight. Prior to the experiment, cells were washed with HBSS (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM glucose, 10 mM HEPES [pH 7.4]) and pre-incubated with compounds at concentrations of 0.001–30 µM for 5 h. After that, cells were exposed to ACT (2 nM) from *B.pertussis* (Enzo Life Sciences, Palo Alto, CA; SA=115 µmol/min/mg) for 30 min. Finally, the cAMP content was determined by using the CatchPoint cAMP immunoassay kit (Molecular Devices, Wokingham, UK). After the addition of the lysis buffer (50 µL per well) provided by the manufacturer, the cellular content was extracted by shaking the plate at 250 rpm for 10 min. The plate was centrifuged to remove cell debris, the supernatant was replaced to the assay plate, and immunoassays were carried out according to the manufacturer's instructions. Fluorescence signal was acquired using an Infinite M1000 plate reader (Tecan Systems Inc., San Jose, CA, USA).

Inhibition of ACT – cell-free assay. In a cell-free assay, AC enzymatic activity was measured by conversion of [^{3}H] ATP to [^{3}H]cAMP. The reaction was carried out at 30 °C for 30 min, with a final reaction volume of 50 µl. Each assay mixture contained 3 µM BSA, 20 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 1 mM EDTA, 1 µM $CaCl_2$, 0.1 mM cold ATP, 20 µCi [2,8-^{3}H]ATP (ARC, St. Louis, MO, USA; specific activity 20 Ci/mmol), 1.2 µM calmodulin and tested compound at concentrations of 0 – 100 µM. Inhibition of AC activity was determined in the presence of 3 different enzymes ACT (Sigma, specific activity 65 µmol/min/mg), ACT (Enzo, specific activity 115 µmol/min/mg) and EF (LBL, specific activity 830 µmol/min/mg) with the final enzyme concentration of 1.1 nM , 0.67 nM and 0.12 nM , respectively. The incubation was carried out for 30 min at 30 °C, in a final reaction volume of 50 µl. A 2 µL aliquot of the assay mixture was spotted on a polyethylenimine chromatographic sheet, and developed in 4M LiCl:1 M acetic acid (1:4). After developing, the spots containing ATP and cAMP were quantified using Radio-TLC scanner RITA (RAYTEST, Germany) with evaluation software GINA STAR TLC. Data were calculated from the percentage conversion of [^{3}H]ATP to [^{3}H]cAMP. K_i values were calculated using the Graphpad Prism 5 software (San Diego, CA, USA). All assays were performed in duplicate with three independent repetitions. For statistical analysis, Student's *t* test (two-sided) was used. Results are given as means ± SD.

Assays with mACs. HEK cells stably expressing AC1, AC2, or AC5 were cultured and frozen as previously described.[38,44] Cells were thawed and plated in a white bottom 384-well plate (PerkinElmer, Shelton, CT), and incubated for 1 hour at 37 °C with 5% CO_2. Inhibitor compounds (for a final concentration of 30 µM) were added to cells and incubated at room temperature for 30 min prior to the addition of the specific mAC stimulator (final concentrations: 3 µM A23187 for AC1, 100 nM PMA for AC2, and 300 nM forskolin for AC5) in 500 µM 3-isobutyl-1-methylxanthine (IBMX). Cells were incubated at room temperature for 1 h and cAMP accumulation was measured using Cisbio's dynamic 2 kit (Cisbio Bioassays, Bedford, MA) according to the manufacturer's instructions. **Materials:** 3-Isobutyl-1-methylxanthine (IBMX), and A23187 were purchased from Sigma-Aldrich (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA), and forskolin were purchased from Tocris Bioscience (Ellisville, MO). Opti-MEM, antibiotic-antimycotic 100x solution, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life technologies (Grand Island, NY). FetalClone I serum and bovine calf serum, were purchased from Hyclone (Logan, UT). G418 was purchased from InvivoGen (San Diego, CA). The homogenous time-resolved fluorescence (HTRF) cAMP kits were purchased from Cisbio Bioassays (Bedford, MA).

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Conflict of interest

The authors declare no conflict of interest.

Keywords: acyclic nucleoside phosphonates • adenylate cyclase toxin • bisamidate • *Bordetella pertussis* • prodrugs

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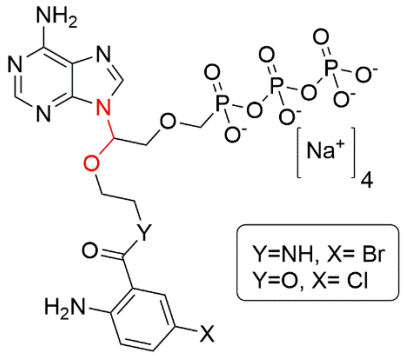

ACT and EF inhibitors with IC_{50}: 55-360 nM

Fighting bacterial toxemia, is where inhibitors of adenylate cyclases (ACs), the key virulence factors of many bacteria, may find their clinical potential. Novel α-branched acyclic nucleoside phosphonato-diphosphates (nucleoside triphosphate analogues), with a hemiaminal ether moiety and a 5-haloanthraniloyl substituent in the aliphatic part of the molecule are potent bacterial ACs inhibitors with IC_{50} values in the range of 55 and 360 nM.