Phosphorylation Hot Paper

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Combined Phosphoramidite-Phosphodiester Reagents for the Synthesis of Methylene Bisphosphonates

Sander B. Engelsma, Nico J. Meeuwenoord, Hermen S. Overkleeft, Gijsbert A. van der Marel, and Dmitri V. Filippov**

Abstract: *A new class of phosphanylmethylphosphonate reagents has been developed to enable the controlled synthesis of methylene bisphosphonate mono- and diesters. Condensation of such reagents with an alcohol of choice through azole-mediated phosphoramidite chemistry followed by in situ oxidation provides orthogonally protected methylene bisphosphonate tetraesters. Global deprotection of the tetraester leads to terminal methylene bisphosphonates. Alternatively, selective deprotection at the terminal phosphonate followed by a condensation between the acquired methylene bisphosphonate triester and a second alcohol leads to methylene bisphosphonates diesters.*

Pyrophosphates are present in numerous natural products that are involved in a wide variety of fundamental physiological processes, including cell metabolism as well as immunity and genome maintenance.[1–5] Consequently, pyrophosphates are important components of molecular tools or probes that aim to study and influence these events.[6–8] Pyrophosphates are inherently susceptible to hydrolysis, transesterification, and enzymatic cleavage.[9,10] If a higher stability is desired, methylene bisphosphonates are attractive pyrophosphate bioisosteres that are less prone to undergo hydrolysis both during synthesis and in physiological surroundings.[11] The use of methylene bisphosphonate isosteres of sugar nucleotides and nucleoside di- and triphosphates in studies on the function of a wide variety of enzymes is well documented.[8,12,13] Terminal methylene bisphosphonates have been synthesized in the past using either methylene bisphosphonic dichloride or partially protected monochloridite derivatives as the phosphonylation agent.[14,15] Unsymmetric methylene bisphosphonates can be accessed using Mitsunobu chemistry or by condensing the hydroxy group of a target (bio)molecule with an independently prepared terminal methylene bisphosphonate.[16,17] The published methods use unselective reagents, which limits the regioselectivity of substitution reactions at the bisphosphonate core. Moreover, no generic strategy exists that readily allows for the introduction of methylene bisphosphonate moieties in structurally diverse pyrophosphate-containing (bio)molecules. The development of such a strategy and its application to methylene bisphosphonate analogues of natural products (ATP, NAD, FAD) is reported here.

We envisioned that generic reagents capable of providing access to both terminal and unsymmetric methylene bisphosphonates had to be orthogonally protected to allow two sequential condensations under conditions compatible with common biomolecules, and that these requirements could be met through the application of phosphanylmethylphosphonate reagents (**3**; Figure 1). Azole-mediated condensation between an alcohol and **3** followed by in situ oxidation would give fully protected methylene bisphosphonate tetraester **4**. Global deprotection of **4** would provide terminal methylene bisphosphonate molecules. Alternatively, selective deprotection of **4** to bisphosphonotriester **5** and consecutive P^V condensation with a second alcohol would give bisphosphonotetraester **6**, and removal of the protecting groups in **6** would provide unsymmetric methylene bisphosphonates. We herein describe several reagents of type **3** and their application in the synthesis of terminal and unsymmetric methylene bisphosphonates, both in solution and on solid support.

To evaluate the proposed method, phosphanylmethylphosphonate **9** was chosen as a reagent suitable for the introduction of terminal methylene bisphosphonate monoesters (Scheme 1). Lithiation of di-*tert*-butyl methylphosphonate (**7**) with 1 equiv of *n*BuLi followed by the addition of phosphoramidite **8** provided the desired phosphanylmethylphosphonate **9** in 37% yield.[18] The phosphorylating properties of reagent **9** were investigated by exploring the synthesis of known adenosine-5′-methylene bisphosphonate (**13**).[19] Condensation of phosphoramidite **9** with partially protected adenosine **10**[20] was accomplished using 1*H*-tetrazole as the activator. Subsequent in situ *t*BuOOH-mediated oxidation of the phosphonite-phosphonate intermediate afforded a methylene bisphosphonate of adenosine **11** in 37% yield. Treatment of **11** with thiophenol and TEA resulted in complete demethylation within 16 hours, as determined by LCMS and ^{31}P NMR spectroscopy. After extractive work-up, consecutive deprotection of the *tert*-butyl groups was accomplished with 10% trifluoroacetic acid (TFA) in dichloromethane (DCM) within 30 min (Supporting Information, Figure S2). Final ammonia-mediated deacylation provided me-ADP (**13**) in quantitative yield. It is of interest to note that demethylation proceeded sluggishly when the *tert*-butyl groups were removed beforehand, taking up to 48 hours to complete.

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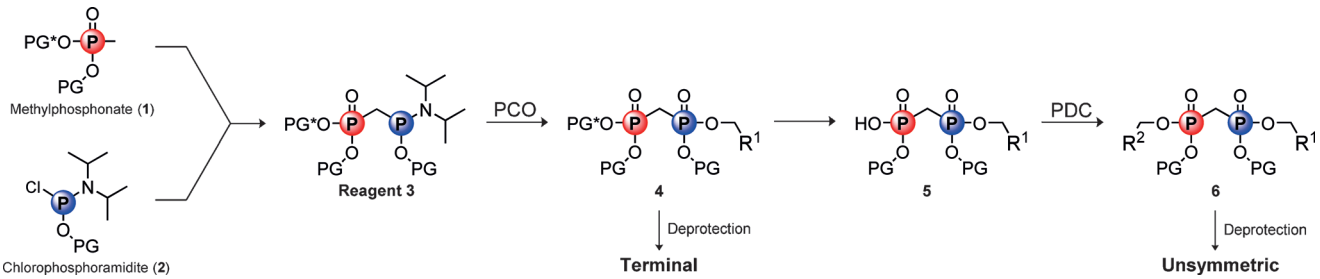
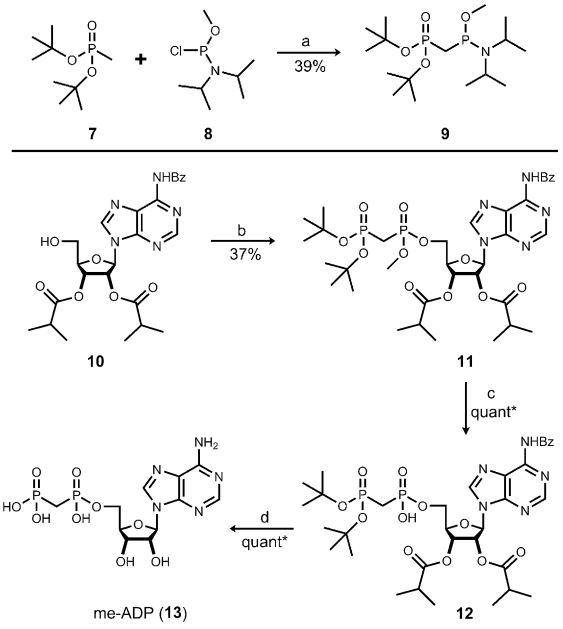
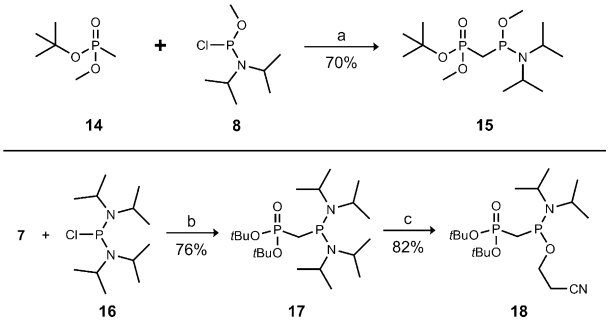


Figure 1. Method for the synthesis of terminal and unsymmetric methylene bisphosphates. Reagent **3** features orthogonally removable protecting groups PG and PG* and can be prepared from methylphosphonate **1** and chlorophosphoramidite **2**. The reagent is coupled to an alcohol-containing substrate (R^1OH) in a P^{III} phosphoramidite coupling/oxidation sequence (PCO) to give protected intermediate **4**, a precursor for terminal methylene bisphosphonates. Alternatively, after selective PG* removal (**5**), a second alcohol (R^2OH) can be introduced through phosphodiester condensation (PDC), leading to protected intermediate **6**, a precursor of unsymmetric methylene bisphosphates.



Scheme 1. Synthesis of me-ADP **13**. Reagents and conditions: a) 1) *n*BuLi, THF, −78 °C, 0.5 h, 2) **8**, 15 min; b) 1) **9**, 1*H*-tetrazole, MeCN, 10 min, 2) *t*BuOOH, 15 min; c) PhSH/Et_3N/dioxane (1:2:2), 16 h; d) 1) 10% TFA in DCM, 0.5 h, 2) NH_4OH, 16 h. [*] Yields based on ^{31}P NMR and LCMS analysis. Bz = benzoyl.



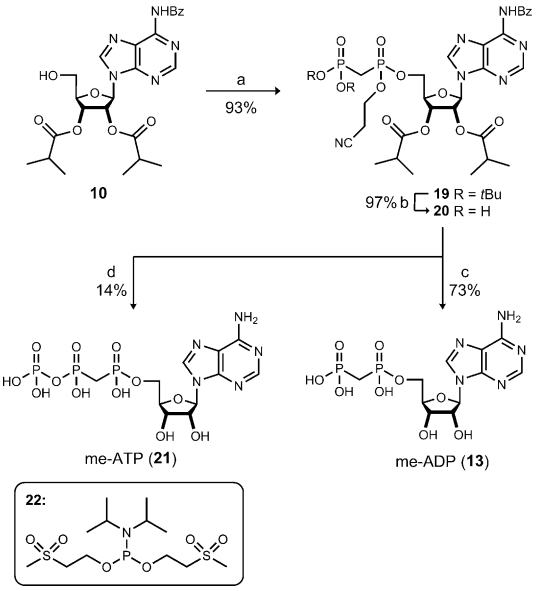
Scheme 2. Synthesis of phosphanylmethylphosphonates **15** and **18**. Reagents and conditions: a) 1) *n*BuLi, THF, −78 °C, 0.5 h, 2) **8**, −78 °C, 15 min; b) 1) LDA, THF, −78 °C, 0.5 h, 2) **16**, −78 °C→RT, 16 h; c) DCI, 2-cyanoethanol, DCM, 3.5 h.

With the proposed method validated, efforts were directed towards phosphanylmethylphosphonate reagents **15** and **18** (Scheme 2). The terminal *tert*-butyl group in **15** can be selectively cleaved to allow for consecutive P^V condensation, leading to unsymmetric methylene bisphosphonate diesters. Reagent **18** contains the base-labile 2-cyanoethanol protecting group to enable the synthesis of terminal methylene bisphosphonate monoesters both in solution and on solid supports.

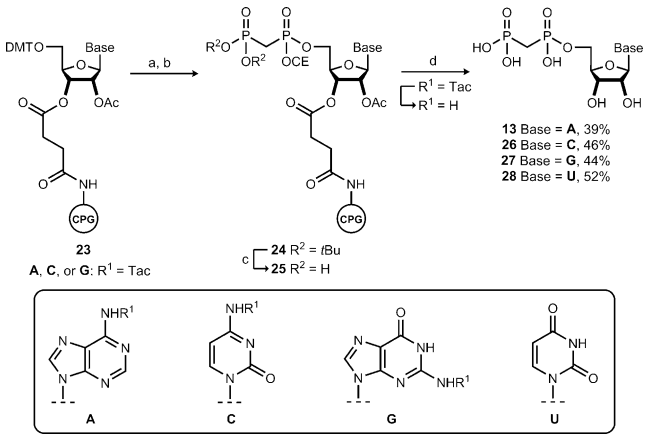
Lithiation of methylphosphonate **14** with 1 equiv of *n*BuLi was followed by the addition of chlorophosphine **8**, providing target phosphoramidite **15** in 32% yield. During refinement of this reaction, we found that the relatively low yield could be attributed to two forms of quenching. The diminished yield was partially caused by proton exchange between emerging bisphosphonate **15** and lithiated methylphosphonate **14**, neutralizing the reactant. This problem was circumvented by switching to an excess of lithium diisopropylamide (LDA; 2.1 equiv), which increased the yield to 45%. Second, we found that commercially available **8** was contaminated with diisopropylamine hydrochloride (DIPA·HCl), another species capable of quenching the lithiated methylphosphonate **14**. Removal of DIPA·HCl, by precipitation in *n*-hexane prior to addition, further increased the yield of **15** to 70% (Figure S3).

Reagent **18**, which features a cyanoethyl protecting group, was prepared by a similar method. Di-*tert*-butyl methylphosphonate (**7**) was deprotonated and reacted with bis(diisopropylamino)chlorophosphoramidite **16** to afford bis(amidite) **17** in 76% yield. Selective substitution of one of the diisopropylamine groups with 2-cyanoethanol in the presence of 4,5-dicyanoimidazole (DCI, 0.6 equiv) provided reagent **18** in 82% yield. It should be noted that bis(amidite) **17** is a versatile precursor that allows for other relevant protecting groups (such as benzyl) to be installed, and thus a variety of reagents of type **3** (Figure 1) are within reach.

With the reagents in hand, the synthesis of the methylene bisphosphonate analogues of adenosine di- and triphosphate[21] (me-ADP (**13**) and me-ATP (**21**); Scheme 3) with reagent **18** was undertaken. Condensation of **18** with adenosine derivative **10** in the presence of DCI and subsequent oxidation of the P^{III}/P^V intermediate gave protected me-ADP **19** in 93% yield after isolation. Removal of the terminal *tert*-



Scheme 3. Solution-phase synthesis of me-ADP (**13**) and the methylene analogue of ATP **21**. Reagents and conditions: a) 1) **18**, DCI, MeCN, 15 min, 2) *t*BuOOH, 15 min; b) HCl (1.2 equiv, 0.5 M), HFIP, 4 h; c) ammonia, 16 h; d) 1) **22**, ETT, MeCN, 15 min, 2) *t*BuOOH, 15 min, 3) DBU, 0.5 h, 4) ammonia, 16 h.

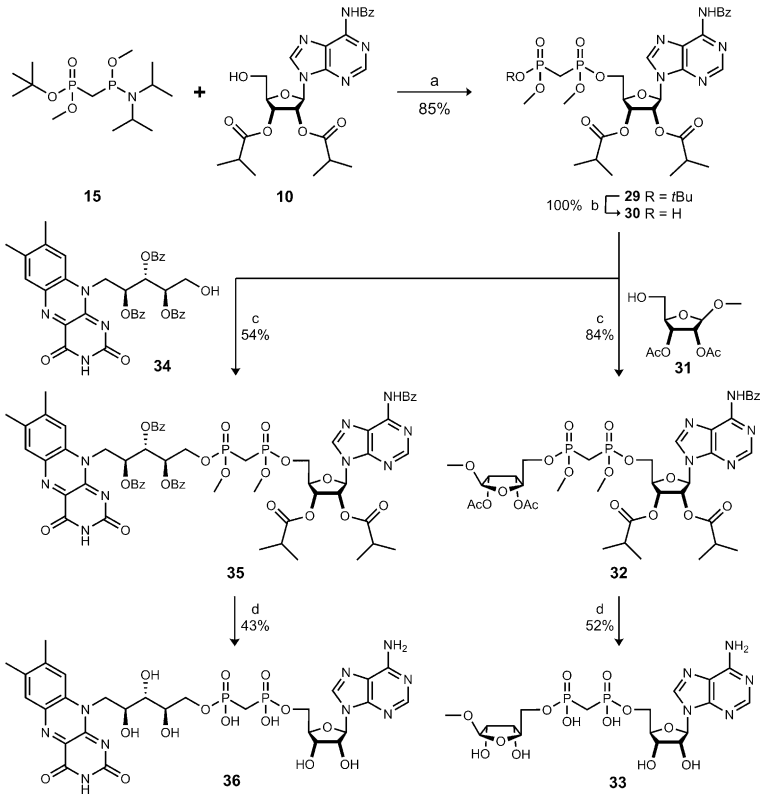


Scheme 4. Automated solid-phase synthesis of methylene bisphosphonate nucleotides. Reagents and conditions: a) 4×HCl (50 mM), HFIP, 1 min; b) 1) 2×ETT and **18**, MeCN, 5 min, 2) 2×CSO, MeCN, 5 min; c) 1) 4×HCl (50 mM), HFIP, 1 min, d) 1) 2×DBU, DMF, 2 min, 2) NH_4OH (35%), 1 h. CE = 2-cyanoethanol, Tac = (4-*tert*-butylphenoxy)acetyl.

butyl protecting groups was carried out with HCl in hexafluoroisopropanol (HFIP) to obtain the partially protected ADP analogue **20** in 97% yield. Global deprotection of **20** by treatment with aqueous ammonia provided adenosine methylene bisphosphonate (**13**) in 73% yield. The synthesis of adenosine methylene triphosphate **21** was accomplished by the modification of a method developed by us[22–24] and others for native pyrophosphates.[25] Partially protected methylene bisphosphonate **20** was first condensed with known bis[(methylsulfonyl)ethyl]diisopropylphosphoramidite (**22**)[26] in the presence of 5-ethylthiotetrazole (ETT) followed by in situ oxidation of the phosphonite-phosphonate anhydride intermediate by *t*BuOOH to give the protected precursor of **21**. All steps could be monitored by ^{31}P NMR spectroscopy. Subsequent elimination of the (methylsulfonyl)ethyl and cyanoethyl protecting groups with DBU was followed by deacylation with ammonia. Purification by size-exclusion and anion-exchange chromatography gave me-ATP (**21**).

Next, we decided to test our newly developed method in an automated solid-phase synthesis to allow for future extension of the phosphonite-phosphonate chemistry to the synthesis of biomolecules containing multiple pyrophosphate bridges. In addition, the solid-phase approach could prove useful for poorly soluble compounds, such as guanosine derivatives. For this purpose, the methylene bisphosphonate analogues of all nucleosides (me-ADP (**13**), me-CDP (**26**), me-GDP (**27**), and me-UDP (**28**); Scheme 4) were selected as target molecules. Commercially available controlled pore glass (CPG), preloaded with the respective suitably protected nucleoside through a succinyl linker (**23**),[27] was loaded into an automated solid-phase oligonucleotide synthesizer. The four-cycle procedure started with coupling of the immobilized nucleoside with phosphanylmethylphosphonate reagent **18**, with ETT as the activating agent. Subsequent oxidation with (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) yielded protected, resin-bound methylene bisphosphonate nucleotides **24**. The terminal *tert*-butyl groups in intermediate **24** were cleaved with HCl in HFIP (**25**), which was followed by DBU-mediated removal of the 2-cyanoethyl group. Final aminolysis of the acyl protecting groups with concomitant release from the solid support was effected by treatment with aqueous ammonia. The crude products were purified by gel filtration, providing methylene bisphosphonate nucleotides **13** (39%), **26** (46%), **27** (44%), and **28** (52%).

As a final research objective, the synthesis of two unsymmetric methylene bisphosphonates, namely adenosine bisphosphonate ribose (ADPR) analogue **33** and flavin adenine dinucleotide (FAD) analogue **36** (Scheme 5), was undertaken. The condensation efficiency of reagent **15** with adenosine derivative **10** was evaluated using various activators. Based on ^{31}P NMR spectroscopic analysis and the obtained yields, ETT and 1*H*-tetrazole performed equally well whereas DCI gave cleaner and more reproducible results. Subsequent oxidation gave clean conversion into me-ADP (**29**) as monitored by ^{31}P NMR spectroscopy (Figure S4). Unexpectedly, using DCI, me-ADP (**29**) was isolated in yields not exceeding 55%. Exclusion of product loss during column purification and washing steps led to the tentative conclusion that the methylene bisphosphonate moiety in **29** binds Mg^{2+} from magnesium sulfate. Indeed, switching to the use of sodium sulfate as a drying agent resulted in the isolation of **29** in significantly higher yields (76–85%). Subsequent removal of the *tert*-butyl group in **29** to give **30** would permit the next condensation with the 5-hydroxy group from ribose **31**. Cleavage of the *tert*-butyl group using 10% TFA in DCM cleanly provided phosphonic acid **30** as determined by ^{31}P and ^{1}H NMR spectroscopy. Surprisingly, all attempts to condense **30** with **31** in the presence of 2-mesitylenesulfonyl chloride (TMBSC) and 4-methoxypyridine *N*-oxide (MNO) were unsuccessful.[28] Based on model studies, it became evident



Scheme 5. Synthesis of Me-ADPR (**33**) and Me-FAD (**36**). Reagents and conditions: a) 1) DCI, MeCN, 15 min, 2) *t*BuOOH, 15 min; b) HCl (1.5 equiv, 0.2 M), HFIP; c) PyNTP, **31**, 2,6-lutidine, MeCN, 2 h **or** PyNTP, **34**, 2,6-lutidine, MeCN, 1 h; d) 1) PhSH/Et_3N/MeCN (3:2:3), 35 °C, 4.5 h, 2) 30% NH_4OH, 16 h.

that the one equivalent of TFA used during *tert*-butyl deprotection remains adhered to **30**. During the condensation reaction, an unproductive mixed anhydride of TFA and the bisphosphonate is formed. Switching to 1.5 equiv of HCl in hexafluoroisopropanol (HFIP) effectively cleaved the *tert*-butyl group, providing **30** in quantitative yield. Indeed, the subsequent key P^V coupling reaction of **30** with **34** under the agency of TMBSC and MNO afforded a mixture of the fully protected target compound me-ADPR (**32**) and its monodemethylated analogue in a combined yield of 60%. Previously, Wada and co-workers had shown that phosphonium reagents are selective activators and effective agents for the condensation of methylphosphonates with alcohols.[29] Using 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyNTP) as the condensation agent further improved the yield of protected me-ADPR (**32**) to 84% (Figure S5). Two-step removal of all protecting groups in **32** (demethylation with thiophenol in MeCN/TEA followed by deacylation using aqueous ammonia) and purification of the crude product by gel filtration provided the desired product me-ADPR (**33**) in 52% yield (Figure S6). The thus determined optimal method for the installation of unsymmetric methylene bisphosphonates was implemented in the synthesis of **36**, a known bioisostere for FAD, which is an essential redox cofactor in cellular metabolism.[30] Riboflavin building block **34** was condensed with **30** under the agency of PyNTP to furnish the protected me-FAD derivative **35** in 54% yield. Ensuing two-step deprotection followed by purification by size-exclusion chromatography gave me-FAD (**36**) in 43% yield after isolation. The synthesis of me-FAD proved to be challenging as the poor solubility of the flavin moiety reduced the conversion rate and complicated the purification.

In conclusion, we have developed a new and broadly applicable strategy for the synthesis of methylene bisphosphonate esters that is based on the combination of phosphoramidite and phosphotriester chemistry. A new class of orthogonally protected phosphanylmethylphosphonate reagents was developed, which are accessible by reacting a lithiated methylphosphonate diester with a chlorophosphoramidite. Relevant examples are reagents **15**, **17**, and **18**, in which the *tert*-butyl groups are selectively removable. Bis(amidite) **17** should enable the installation of other relevant protecting groups. The efficiency of our procedure to prepare methylene bisphosphonates was demonstrated by the solution-phase synthesis of analogues of adenosine di- and triphosphates (me-ADP (**13**) and me-ATP (**21**)) as well as analogues of adenosine bisphosphonate ribose me-ADPR (**33**) and flavin adenine dinucleotide me-FAD (**36**). In addition, the method is suitable for solid-phase synthesis, as shown for nucleotide methylene bisphosphonates (**13**, **26**–**28**). Our method is based on classical methods of nucleic acid chemistry, and we expect it to be compatible with a broad range of biomolecules.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: methylene bisphosphonates · phosphanylmethylphosphonate · phosphorylation · pyrophosphate bioisosteres · solid-phase synthesis

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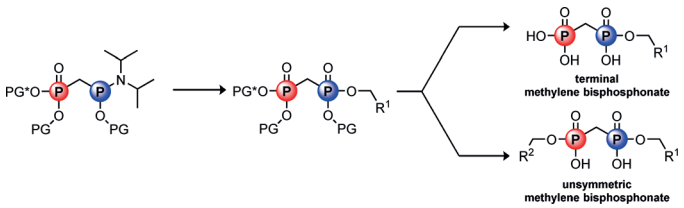
Communications

Phosphorylation

S. B. Engelsma, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel,* D. V. Filippov* _____ ■■■–■■■

Combined Phosphoramidite-Phosphodiester Reagents for the Synthesis of Methylene Bisphosphonates



The condensation of novel phosphanylmethylphosphonate reagents with an alcohol of choice followed by oxidation provides orthogonally protected methylene bisphosphonate tetraesters. Global deprotection of the tetraester leads to terminal methylene bisphosphonates whereas selective deprotection at the terminal phosphonate followed by condensation with a second alcohol yields methylene bisphosphonate diesters.