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## CHEMOENZYMIC SYNTHESIS OF $P^{\alpha}$ - METHYL DEOXYNUCLEOSIDE TRIPHOSPHATES

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**Abstract:** 5'-*O*-(methylphosphonyl)-*N*-(phenylacetyl)-2'-deoxycytidine, deoxyadenosine and deoxyguanosine were pyrophosphorylated and the resulting *N*-protected  $P^{\alpha}$ -methyl nucleoside triphosphates were deblocked by treatment with penicillin amidase at pH 7.8, 25°C to give  $P^{\alpha}$ -methyl nucleoside triphosphates.

The emergence of antisense technology brought up the need for asymmetric synthesis of P-chiral oligonucleotides<sup>1</sup>. The use of homochiral catalysts such as enzymes is a viable alternative to the nonstereoselective classical chemical methods for P-chiral nucleotide bond formation. Recently, we reported<sup>2</sup> a template-directed enzymatic synthesis of a chimeric DNA, containing 42 S-configured methyl-phosphonodiester bonds using  $P^{\alpha}$ -methyl thymidine triphosphate as a DNA polymerase substrate. Some of the incorporated thymidylic residues were consecutive suggesting that a similar approach could be applied to the synthesis of all P-methyl DNA derivatives.

The synthesis of  $P^{\alpha}$ -methyl deoxycytidine, deoxyadenosine and deoxyguanosine triphosphates, however, is not a simple extension of the method we<sup>2</sup> and others<sup>3-5</sup> used for the preparation of  $P^{\alpha}$ -methyl thymidine triphosphate. The protection of the nucleobase amino group is essential for specific 5'-*O*-phosphorylation<sup>6</sup>. Unfortunately, standard deprotection procedures require strongly basic conditions incompatible both with the methylphosphonate modification<sup>7</sup> and the phosphoanhydride functions.

The enzymatic deprotection offers a desired alternative to the classical chemical methods. Quite recently, we reported<sup>8</sup> that the phenylacetyl group can be successfully used as an enzyme-cleavable aminoprotecting group of purine nucleosides. Here we report that this approach can be used for the efficient synthesis of  $P^{\alpha}$ -methyl deoxycytidine, deoxyadenosine and deoxyguanosine triphosphates.

## RESULTS AND DISCUSSION

A chemoselective *O*-phosphorylation of *N*-unprotected nucleosides has been achieved recently<sup>9</sup> via metal alkoxide formation. The base lability of the methylphosphonyl group prevents the use of this approach for the methylphosphonylation of nucleosides, such as dCyd, dAdo or dGuo, containing hydroxy and amino groups in the same molecule. Under ordinary conditions, phosphorylation takes place competitively both at the amino and hydroxy functions<sup>6</sup>. Thus the selective aminoprotection is an unavoidable step in the synthesis of amino group-containing  $P^{\alpha}$ -methyl nucleoside triphosphates.

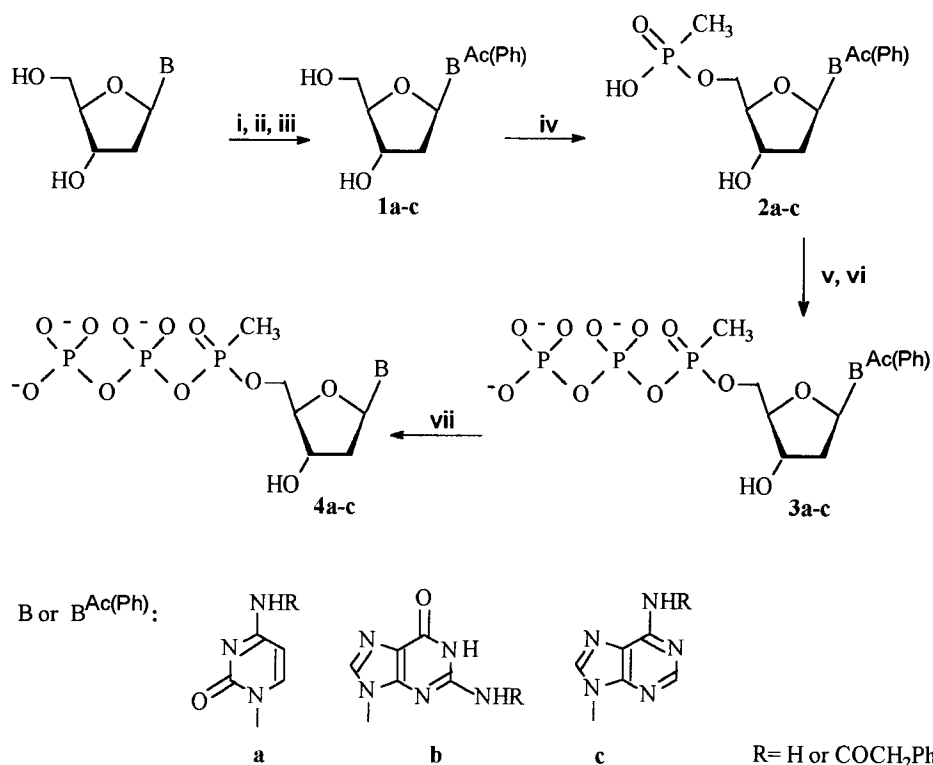
*N*<sup>2</sup>-phenylacetylation of dGuo has been used to increase the solubility and lability of *N*<sup>2</sup>-acylated deoxyguanosine derivatives in concentrated ammonia at 50°C<sup>10</sup>. The protecting group, however, is easily removable from *N*-phenylacetylated dGuo and dAdo in penicillin amidase-catalysed reaction at pH 7.8 and 25°C<sup>8</sup>. Phenylacetylation proceeds smoothly using transient trimethylsilylation of the 3' and 5'-OH functions of dCyd, dGuo and dAdo, and benzotriazol-1-yl phenylacetate as a mild acylating reagent. HPLC-pure *N*-phenylacetylated dCyd (**1a**) dGuo (**1b**) and dAdo (**1c**) were obtained after purification on XAD-2 resin column chromatography, the yields ranging from 55 to 75 %.

When dAdo and methylphosphonic acid react in anhydrous pyridine with dicyclohexylcarbodiimide (DCC), HPLC and TLC analyses reveal the formation of three major products: 5'-*O*-, *N*<sup>6</sup>- and 3'-*O*-methylphosphonylated derivatives. Under the same conditions methylphosphonylation of *N*-phenylacetylated nucleosides gave 5'-*O*-methyl phosphonyl derivatives (**2a-c**) (SCHEME) as the only product.

A chemoselective pyrophosphorylation has been observed when 5'-*O*-methylphosphonyl, *N*-phenylacetyl deoxynucleosides (**2a-c**) were coupled with pyrophosphate using carbonyldiimidazole as a condensing agent. Chromatographic purification resulted in HPLC-pure *N*-phenylacetylated  $P^{\alpha}$ -methyl deoxynucleoside triphosphates (**3a-c**), the yields ranging from 80 to 85 %.

The treatment of the protected  $P^{\alpha}$ -methyl deoxynucleoside triphosphates (**3a-c**) with immobilized penicillin amidase at pH 7.8 and 25°C results in a complete cleavage of the phenylacetyl group. The deprotection time  $t_{\max}$  depends on the nature of the nucleotide (TABLE). This observation is in agreement with the broad leaving group specificity of penicillin amidase varying from 6-aminopenicillanic acid to peptide amines, glucoses and nucleosides<sup>8, 11-13</sup>. The catalytic parameters of penicillin amidase-catalyzed deprotection, derived from the initial rate of the phenylacetic acid formation, are shown in the TABLE together with similar data for phenylacetyl derivatives, obtained previously.

As judged from the values of the substrate specificity constants  $k_{\text{cat}}/K_M$ , the reactivity in enzymatic deprotection reactions of phenylacetylated  $P^{\alpha}$ -methyl deoxynucleoside



**Reagents:** (i) trimethylchlorosilane, pyridine; (ii) phenylacetyl chloride, 1-hydroxy-benzotriazole; (iii) water, 25% aq. ammonia; (iv) methylphosphonic acid, DCC, pyridine; (v) *N,N'*-carbonyldiimidazole, DMF; (vi) tributylammoniumpyrophosphate; (vii) Penicillin amidase, phosphate buffer.

### SCHEME

triphosphates is much lower than that of *N*<sup>6</sup>-phenylacetyl penicillanic acid (Penicillin G) - the natural substrate of penicillin amidase. It is close, however, to the reactivity of phenylacetylated nucleosides suggesting that their 5'-*O*-triphosphorylation is tolerated by the leaving group binding site of penicillin amidase. It is noteworthy, that this modification is better tolerated in the transition state (similar *k*<sub>cat</sub>-values), than in the ground state (higher *K*<sub>M</sub>-values).

During the deprotection reactions the base labile methylphosphonyl group and anhydride bonds remain intact even in the case of longer deprotection time (adenosine derivative). Thus the use of an enzymatic deprotection technique provides access to P<sup>α</sup>-

**TABLE.** Kinetic Parameters for Penicillin Amidase-Catalyzed Deprotection of *N*-Phenylacetylated Nucleosides, P $\alpha$ -Methyl Nucleoside Triphosphates and Related Compounds at pH 7.8 (*I*=0.2) and 25°C. S.D. less than 10%.

Substrates	$k_{\text{cat}}$ , s <sup>-1</sup>	$K_{\text{M}}$ , mM	$k_{\text{cat}} / K_{\text{M}}$ , s <sup>-1</sup> mM <sup>-1</sup>	$\tau_{1/2}$ , min	$t_{\text{max}}^{\text{a}}$ , min
<i>N</i> <sup>4</sup> -Ac(Ph)-dCyd <b>(1a)</b>	13	0.680	19	50	72
<i>N</i> <sup>2</sup> -Ac(Ph)-dGuo <sup>b</sup> <b>(1b)</b>	18	0.800	23	40	60
<i>N</i> <sup>6</sup> -Ac(Ph)-dAdo <sup>b</sup> <b>(1c)</b>	2	0.460	4	75	120
<i>N</i> <sup>4</sup> -Ac(Ph)-dCTP <sub>Me</sub> <b>(3a)</b>	31	2.680	11	84	300
<i>N</i> <sup>2</sup> -Ac(Ph)-dGTP <sub>Me</sub> <b>(3b)</b>	39	4.650	11	85	240
<i>N</i> <sup>6</sup> -Ac(Ph)-dATP <sub>Me</sub> <b>(3c)</b>	16	2.690	6	276	960
Ac(Ph)-Aspartame <sup>c</sup>	35	0.670	52	-	150
Ac(Ph)-Gly-OH <sup>c</sup>	47	0.005	9000	-	25
<i>N</i> <sup>6</sup> -Ac(Ph)-APAc <sup>c,d</sup>	48	0.004	10000	-	25

<sup>a</sup> Deprotection time; <sup>b</sup> Ref. 8; <sup>c</sup> Ref. 14; <sup>d</sup> Penicillin G

methyl deoxynucleoside triphosphates and their further use as substrates for DNA polymerase-catalyzed incorporation of P-methylnucleosidic residues into the growing DNA chain.

## EXPERIMENTAL SECTION

### General methods and materials

<sup>1</sup>H NMR spectra were recorded on a Bruker WM 250 spectrometer at 250 MHz with TMS as internal standard.

IR spectra were recorded on a Bruker IFS 113 V spectrometer.

HPLC analyses were performed on a Merck RP-8 (5 $\mu$ m) column, WATERS delivery system 6000 A, flow rate 1 ml/min and UV detection at 214 nm or 254 nm. The nucleotides were analyzed using one of the following eluting systems:

(I) 30% MeOH/KH<sub>2</sub>PO<sub>4</sub> buffer 0.067M pH 4.8

(II) 15% MeOH/NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> buffer 0.05M pH 5.0

(III) 5% MeOH/NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> buffer 0.05M pH 5.0

Column chromatography purification was carried out on a standard Pharmacia chromatography system using DEAE-cellulose column (1.6 x 25 cm), XAD-2 (1.4 x 75 cm) and XAD-4 (1.4 x 75 cm) resin column (Sigma), UV detection at 254 nm.

Thin layer chromatography was carried out on Silica sheets (Kieselgel 60) using system A: CHCl<sub>3</sub>/MeOH (9:1) or system B: iPrOH/H<sub>2</sub>O/25% aq. NH<sub>3</sub> (7:2:1).

Free and immobilized in polyacrylamide gel penicillin amidase (penicillin amidohydrolase, E. C. 3.5.1.11 from *Escherichia coli*) were provided by Antibiotic® - Razgrad (Bulgaria). The enzyme concentration was determined by active site titration with phenylmethylsulphonyl fluoride (PMSF) inhibitor using 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) as a substrate<sup>8</sup>.

#### General procedure for the phenylacetyl protection of amino functions of nucleosides

The nucleoside (4 mmol) was dried by evaporation from dry pyridine and subsequently suspended in dry pyridine (20 ml). Chlorotrimethylsilane (20 mmol) was added and the mixture was stirred at ambient temperature until TLC analysis indicated complete silylation. Phenylacetyl chloride (6mmol) was mixed with 1-hydroxybenzotriazole (6.4 mmol) and suspended in dry acetonitrile (2 ml). Pyridine (2 ml) was added to dissolve any precipitated salt. This colourless solution was added dropwise at 0°C to the reaction mixture containing the trimethylsilylated nucleoside. The reaction mixture was stirred overnight at ambient temperature. The mixture was cooled to 0°C and the reaction was quenched by the addition of water (5 ml). After 5 min at 0°C, 25% aqueous ammonia (8 ml) was added. The aminolysis of the silyl ethers was completed in ca 30 min at 0°C (TLC). The solvents were removed by rotary evaporation and the residue was dissolved in water and extracted several times with ethyl acetate. The aqueous phases were evaporated until the product began to crystallize. The crystals were filtered off, washed with water and dried. Further purification was achieved by XAD-2 resin column chromatography using a gradient of MeOH (25%-70%) in H<sub>2</sub>O (v/v).

#### 4-(phenylacetyl)-2'-deoxycytidine (1a)

Pure product was eluted from XAD-2 resin column with 55% MeOH. Yield: 51%. R<sub>f</sub> (system A): 0.45; <sup>1</sup>H NMR (DMSO): δ 2.01 & 2.28 (m, 2'H/2"H, 2H), δ 3.58 (m, 5'H/5"H, 2H), δ 3.73 (s, CH<sub>2</sub>(Ac(Ph)), 2H), δ 3.86 (m, 4'H, 1H), δ 4.20 (m, 3'H, 1H), δ 5.01 (t, 5'OH, 1H), δ 5.30 (m, 3'OH, 1H), δ 6.10 (dd, 1'H, 1H), δ 7.32 (m, Ph(Ac(Ph)), 5H), δ 8.31 (d, H<sup>6</sup>, 1H); IR (KBr, cm<sup>-1</sup>) ν: 3317 (NH), 1690, 1664, 1492 (C=O, C=N, C=C); HPLC (system I): R<sub>T</sub> = 6 min.

**2-*N*-(phenylacetyl)-2'-deoxyguanosine (1b)**

Yield: 75%. Rf (system A): 0.50;  $^1\text{H}$  NMR (DMSO):  $\delta$  2.13 & 2.43 (m, 2'H/2''H, 2H),  $\delta$  3.55 (s,  $\text{CH}_2(\text{Ac}(\text{Ph}))$ , 2H),  $\delta$  3.75 (m, 5'H/5''H, 2H),  $\delta$  4.20 (m, 4'H, 1H),  $\delta$  4.35 (m, 3'H, 1H),  $\delta$  5.27 (m, 3'OH, 1H),  $\delta$  5.73 (m, 5'OH, 1H),  $\delta$  6.13 (dd, 1'H, 1H),  $\delta$  7.26 (m,  $\text{Ph}(\text{Ac}(\text{Ph}))$ , 5H),  $\delta$  7.79 (s,  $\text{H}^8$ , 1H); IR (KBr,  $\text{cm}^{-1}$ )  $\nu$ : 3452 (NH), 1702, 1677, 1607 (C=O, C=N, C=C); HPLC (system I):  $RT$ =6.5 min.

**6-*N*-(phenylacetyl)-2'-deoxyadenosine (1c)**

Pure product was eluted from XAD-2 resin column with 60% MeOH. Yield: 80%. Rf (system A): 0.54;  $^1\text{H}$  NMR (DMSO):  $\delta$  2.34 & 2.73 (m, 2'H/2''H, 2H),  $\delta$  3.46 (s,  $\text{CH}_2(\text{Ac}(\text{Ph}))$ , 2H),  $\delta$  3.55 (m, 5'H/5''H, 2H),  $\delta$  3.88 (s, 4'H, 1H),  $\delta$  4.42 (m, 3'H, 1H),  $\delta$  5.00 (m, 5'OH, 1H),  $\delta$  5.34 (m, 3'OH, 1H),  $\delta$  6.44 (dd, 1'H, 1H),  $\delta$  7.34 (m,  $\text{Ph}(\text{Ac}(\text{Ph}))$ , 5H),  $\delta$  8.63 (s,  $\text{H}^8$ , 1H),  $\delta$  8.65 (s,  $\text{H}^2$ , 1H); IR (KBr,  $\text{cm}^{-1}$ )  $\nu$ : 3440 (NH), 1731, 1690, 1587 (C=O, C=N, C=C); HPLC (system I):  $RT$ = 6.8 min.

**General procedure for methylphosphonylation of phenylacetyl protected nucleosides**

Methylphosphonic acid (2 mmol) and phenylacetylated nucleoside (1 mmol) were dried and suspended in dry pyridine (10 ml). DCC (4 mmol) was added and the solution was stirred for 24 h at room temperature. When the reaction was completed (TLC) the mixture was diluted with water (10 ml) and the solid was filtered off and washed thoroughly with water. The combined filtrate and washings were extracted three times with ether. After concentration, the aqueous solution was applied on a XAD-2 resin column (1.4x75 cm). The product was eluted with 5% - 10% MeOH/H<sub>2</sub>O (v/v).

**5'-*O*-(methylphosphonyl)-4-*N*-(phenylacetyl)-2' deoxycytidine (2a)**

Yield 31% (106 mg); Rf (system B): 0.63;  $^1\text{H}$  NMR (DMSO):  $\delta$  1.31 (d,  $\text{CH}_3\text{-P}$ ,  $J$ =17 Hz, 3H),  $\delta$  2.22-2.48 (m, 2'H/2''H, 2H),  $\delta$  3.56 (s,  $\text{CH}_2(\text{Ac}(\text{Ph}))$ , 2H),  $\delta$  3.73 (m, 5'H/5''H, 2H),  $\delta$  4.30 (m, 4'H, 1H),  $\delta$  4.86 (m, 3'H, 1H),  $\delta$  5.87 (d, 3'OH, 1H),  $\delta$  6.13 (dd, 1'H, 1H),  $\delta$  7.3 (m,  $\text{Ph}(\text{Ac}(\text{Ph}))$ , 5H),  $\delta$  7.86 (d,  $\text{H}^6$ , 1H), HPLC (system II):  $RT$ = 2.9 min.

**5'-*O*-(methylphosphonyl)-2-*N*-(phenylacetyl)-2'-deoxyguanosine (2b)**

Yield 44% (200 mg); Rf (system B): 0.72;  $^1\text{H}$  NMR (DMSO):  $\delta$  1.06 (dd,  $\text{CH}_3\text{-P}$ ,  $J$ =16.12 Hz, 3H),  $\delta$  2.31 & 2.98 (m, 2'H/2''H, 2H),  $\delta$  3.17 (s,  $\text{CH}_2(\text{Ac}(\text{Ph}))$ , 2H),  $\delta$  3.85 (m, 5'H/5''H, 2H),  $\delta$  4.19 (m, 4'H, 3'H, 2H),  $\delta$  5.17 (d, 3'OH, 1H),  $\delta$  6.24 (dd, 1'H, 1H),  $\delta$  7.32 (m,  $\text{Ph}(\text{Ac}(\text{Ph}))$ , 5H),  $\delta$  8.13 (s,  $\text{H}^8$ , 1H); HPLC (system II):  $RT$ = 3.4 min.

**5'-*O*-(methylphosphonyl)-6-*N*-(phenylacetyl)-2'-deoxyadenosine (2c)**

Yield 49 % (221 mg); Rf (system B): 0.75;  $^1\text{H}$  NMR (DMSO):  $\delta$  1.22 (d,  $\text{CH}_3\text{-P}$ ,  $J$ =16.25 Hz, 3H),  $\delta$  2.34 & 2.75 (m, 2'H/2''H, 2H),  $\delta$  3.49 (m, 5'H/5''H, 2H),  $\delta$  3.58 (s,

CH<sub>2</sub>(Ac(Ph)), 2H), δ 3.93 (m, 4'H, 1H), δ 4.42 (m, 3'H, 1H), δ 5.31 (s, 3'OH, 1H), δ 6.56 (dd, 1'H, 1H), δ 7.34 (m, Ph(Ac(Ph)), 5H), δ 8.27 (s, H<sup>8</sup>, 1H), δ 8.46 (s, H<sup>2</sup>, 1H); HPLC (system II): RT= 3.6 min.

#### General procedure for synthesis of triphosphate analogues

Phenylacetylated 5'-methylphosphonate nucleoside (0.2 mmol) and tributylamine (0.2 mmol) were stirred in methanol (5 ml) until a clear solution was obtained. The residue was dried and suspended in dry DMF (5 ml). Carbonyldiimidazole (1 mmol) was added and the solution was stirred at room temperature for 2 h (TLC). Dried tributylammonium pyrophosphate (2 mmol) was suspended in DMF (5 ml) and added to the activated methylphosphonate analogues. The precipitate was filtered off after stirring at room temperature for 4 h (TLC). The filtrate was diluted with water (50 ml) and chromatographed on a DEAE-cellulose column (1.6x25) with a linear gradient of NH<sub>4</sub>HCO<sub>3</sub> 0.025-0.3 M, pH 7.5 (total volume 500 ml). The fraction eluted with 0.2-0.3 M buffer was evaporated in vacuum in the presence of ethanol several times to remove the buffer and was freeze dried.

#### 5'-O-(α-methylphosphonyl)-β,γ-pyrophosphoryl-4-N-(phenylacetyl)-2-deoxycytidine (3a)

Yield 79% (98 mg); R<sub>f</sub> (system B): 0.52; <sup>1</sup>H NMR (DMSO): δ 1.66 (dm, CH<sub>3</sub>-P, J=18 Hz, 3H), δ 2.13 & 2.29 (m, 2'H/2''H, 2H), δ 3.42 (s, CH<sub>2</sub>(Ac(Ph)), 2H), δ 3.75 (m, 5'H/5''H, 2H), δ 4.77 (m, 3'H, 1H), δ 5.93 (m, 3'OH, 1H), δ 6.23 (dd, 1'H, 1H), δ 7.32 (m, Ph(Ac(Ph)), 5H), δ 7.91 (d, H<sup>6</sup>, 1H); HPLC (system II): RT=1.9 min.

#### 5-O-(α-methylphosphonyl)-β,γ-pyrophosphoryl-2-N-(phenylacetyl)-2-deoxyguanosine (3b)

Yield 83% (110 mg); R<sub>f</sub> (system B): 0.54; <sup>1</sup>H NMR (DMSO): δ 1.22 (dd, CH<sub>3</sub>-P, J=17.85 Hz, 3H), δ 2.26 & 2.85 (m, 2'H/2''H, 2H), δ 3.54 (s, CH<sub>2</sub>(Ac(Ph)), 2H), δ 3.70 (m, 5'H/5''H, 2H), δ 4.10 (m, 4'H, 1H), δ 4.90 (m, 3'OH, 1H), δ 6.27 (dd, 1'H, 1H), δ 7.27-7.38 (m, Ph(Ac(Ph)), 5H), δ 8.23 (s, H<sup>8</sup>, 1H); HPLC (system II): RT=2.2 min.

#### 5-O-(α-methylphosphonyl)-β,γ-pyrophosphoryl-6-N-(phenylacetyl)-2-deoxyadenosine (3c)

Yield 85% (110 mg); R<sub>f</sub> (system B): 0.58; <sup>1</sup>H NMR (D<sub>2</sub>O): δ 1.43 (d, CH<sub>3</sub>-P, J=17.0 Hz, 3H), δ 2.86 (m, 2'H/2''H, 2H), δ 3.54 (s, CH<sub>2</sub>(Ac(Ph)), 2H), δ 3.79 (m, 5'H/5''H, 2H), δ 4.03 (m, 4'H, 1H), δ 4.43 (m, 3'H, 1H), δ 6.55 (dd, 1'H, 1H), δ 7.21-7.47 (m, Ph(Ac(Ph)), 5H), δ 8.47 (s, H<sup>8</sup>, 1H), δ 8.67 (s, H<sup>2</sup>, 1H); HPLC (system II): RT= 2.3 min.



### Penicillin amidase catalyzed deprotection of the phenylacetyl protecting group

300 mg of immobilized penicillin amidase (0.1 mmol/g support) were added to a 10 mM substrate solution in 0.1 M phosphate buffer (pH 7.8). The reaction mixture was shaken at room temperature till complete deprotection (HPLC). The immobilized enzyme was filtered off and the filtrate was purified on a XAD-4 resin column (1.4x70): H<sub>2</sub>O, pH 3.5 (HCl adj.), flow rate: 0.5 ml/min. The appropriate fraction (elution volume - ca 1.5x column void volume) was freeze dried.

#### 5-*O*-( $\alpha$ -methylphosphonyl)- $\beta$ , $\gamma$ -pyrophosphoryl-2-deoxycytidine (4a)

Yield 52% (48 mg); R<sub>f</sub> (system B): 0.42; <sup>1</sup>H NMR (D<sub>2</sub>O):  $\delta$  1.32 (dd, CH<sub>3</sub>-P, *J*=16.4 Hz, 3H),  $\delta$  2.34 & 2.56 (m, 2'H/2"H, 2H),  $\delta$  4.06 (m, 5'H/5"H, 2H),  $\delta$  4.35 (m, 4'H, 1H),  $\delta$  6.37 (dd, 1'H, 1H),  $\delta$  7.93 (d, H<sup>8</sup>, 1H); HPLC (system III): *RT*=1.5 min.

#### 5-*O*-( $\alpha$ -methylphosphonyl)- $\beta$ , $\gamma$ -pyrophosphoryl-2-deoxyguanosine (4b)

Yield 31% (25 mg); R<sub>f</sub> (system B): 0.44; <sup>1</sup>H NMR (D<sub>2</sub>O):  $\delta$  1.30 (dd, CH<sub>3</sub>-P, *J*=34.6 Hz, 3H),  $\delta$  2.66 & 2.90 (m, 2'H/2"H, 2H),  $\delta$  4.04 (m, 5'H/5"H, 2H), 4.38 (m, 4'H, 1H), 5.01 (d, 3'H, 1H), 6.36 (dd, 1'H, 1H),  $\delta$  8.09 (s, H<sup>8</sup>, 1H); HPLC (system III): *RT*=2.1 min.

#### 5-*O*-( $\alpha$ -methylphosphonyl)- $\beta$ , $\gamma$ -pyrophosphoryl-2-deoxyadenosine (4c)

Yield 34% (28 mg); R<sub>f</sub> (system B): 0.47; <sup>1</sup>H NMR (D<sub>2</sub>O):  $\delta$  1.38 (d, CH<sub>3</sub>-P, *J*=16.9 Hz, 3H),  $\delta$  2.93 (m, 2'H/2"H, 2H),  $\delta$  4.04 (m, 5'H/5"H, 2H),  $\delta$  4.44 (m, 4'H, 1H),  $\delta$  6.56 (dd, 1'H, 1H), 8.28 (s, H<sup>8</sup>, 1H),  $\delta$  8.47 (s, H<sup>2</sup>, 1H); HPLC (system III): *RT*=2.4 min.

### Kinetic studies

The kinetics of the penicillin amidase - catalyzed deprotection was studied under the conditions used in preparative experiments with substrate concentration ranging from 0.25 to 8x10<sup>-3</sup> M. The initial rates were followed by HPLC analysis of samples withdrawn from the reaction mixture at appropriate time intervals. The catalytic parameters were derived from the initial rate of phenylacetic acid formation using the computer program Enzfitter<sup>15</sup> for non-linear regression analysis.

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