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Carbonylbisphosphonate and (diazomethylene)bisphosphonate analogues of AZT 5′-diphosphate☆

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Abstract

A novel nucleotide analogue is described, in which the α, β-phosphoric anhydride oxygen of a nucleoside 5′-diphosphate is replaced by a carbonyl group: the carbonylbisphosphonate analogue **5** of 2′,3′-dideoxy-3′-azidothymidine 5′-diphosphate (AZT 5′-diphosphate). **5** was synthesized from tetramethyl (diazomethylene)bisphosphonate **1** via the trimethyl ester **4** of the corresponding AZT 5′-(diazomethylene)bisphosphonate **6**, which is also a new type of nucleotide analogue. The ultimate product **5** was isolated by reverse-phase HPLC, and characterized by ^{31}P, ^{13}C, and ^{1}H NMR; and by high-resolution mass spectrometry. The ketone group of **5** is a visible chromophore (yellow) and reversibly forms a colorless hydrate. The ketone hydrate 'pK' is about 4.2 when excess of magnesium ion is present. The potential of such analogues as novel inhibitors of enzymes mediating nucleotide-dependent biochemical processes is discussed.

Keywords: Nucleotide analogue; Carbonylbisphosphonate; (Diazomethylene)bisphosphonate; AZT

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1. Introduction

Nucleotides modified by replacing a bridging pyrophosphate (PPi) P–O–P oxygen with a carbon atom have often proven useful in probing the structure and function of naturally occurring biomolecules. α, β-Methylene analogues of nucleoside diphosphates [3–5] and β, γ-methylene analogues of nucleoside triphosphates [6–10] provide examples of nucleotides in which a diphosphate moiety has been replaced by a dephosphorylation-resistant methylenebisphosphonate (MBP) group (Fig. 1). Adjustment of the P–OH pK_a values of such analogues to better approximate the P–OH acidities of their parent nucleotides can be accomplished by methylene substitution with electronegative atoms such as fluorine [6,11–14].

Replacement of a nucleotide phosphoric anhydride oxygen by a chemically *reactive* and electron-deficient carbon, ideally without introducing excessive steric perturbation, is of interest as a basis for the design of novel nucleotide analogues. We have investigated the synthesis of nucleotides in which a bridging P–O–P oxygen is replaced by a potentially reactive *keto carbon*. The bisphosphonate moiety in such analogues, carbonylbisphosphonate (COBP, earlier referred to as COMDP), was first prepared by Quimby as a tetrasodium salt [15]. We previously showed that COBP inhibits HIV-1 reverse transcriptase (RT) whereas underivatized and α-halo-substituted methylenebisphosphonates were non-inhibitory [16]. COBP also selectively inhibits proliferating cell nuclear antigen-independent DNA polymerase δ derived from calf thymus [17]. In addition, COBP inhibits the pyrophosphate-dependent phosphofructokinase from *Toxoplasma gondii* and its tetraisopropyl ester exhibited an anti-replicative effect against proliferation of this parasite in human foreskin fibroblasts [18]. COBP inhibition of other enzymes has been reported [19,20]. These independent and selective inhibitory properties of COBP, particularly with respect to nucleic acid polymerases, further urged exploration of preparative routes to carbonylbisphosphonate analogues of nucleotides.

Initial attempts to obtain such analogues via direct condensation of COBP with various nucleosides promoted by DCC were unencouraging (unpublished data) [21]. However, our recent achievement of practical methods for converting (diazomethylene)bisphosphonic acid tetraalkyl esters to the corresponding ketones allowed us to consider the use of protected [22] bisphosphonate intermediates. As noted above, tetrasodium COBP was prepared many years ago (by alkaline hydrolysis of a tetraalkyl (dichloromethylene)bisphosphonate [15]), but the potentially more versatile tetraalkyl esters eluded synthesis in pure form until we discovered that tetraalkyl (diazomethylene)bisphosphonates can be facilely converted to corresponding α-ketones by oxidation with *t*-butyl hypochlorite/water in ethyl acetate [23,24], or

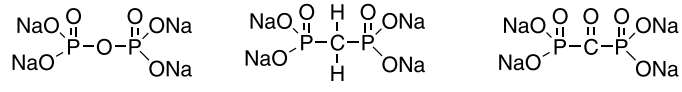


Fig. 1. Structures of pyrophosphate (PPi) analogues (shown as tetrasodium salts).

alternatively by oxidation with an epoxide, catalyzed by $Rh_2[NHCOC_3F_7]_4$ in refluxing toluene [24,25].

As our initial target nucleotide COBP analogue, we chose AZT 5′-carbonylbisphosphonate, **5**. Despite the advent of potent new anti-HIV nucleotide RT inhibitors (NRTI's) such as abacavir [26,27], AZT (2′,3′-dideoxy-3′-azidothymidine) continues to play an important role in the chemotherapy of AIDS [28,29], particularly in combination with other NRTI's, non-nucleoside reverse transcriptase inhibitors, and HIV protease inhibitors [30]. After stepwise phosphorylation to AZT 5′-triphosphate by cellular kinases, AZT is incorporated into the DNA strand synthesized by RT from its viral RNA template, causing abnormal termination [31–33]. AZT 5′-$(\text{phosphate})_x$ ($x = 1$–3) nucleotides do not constitute deliverable drugs, but long-chain alkyl thioesters of AZT 5′-phosphate have been shown in clinical trials to be effective against HIV infection [34].

In this paper we provide details of our previously presented [1,2] synthesis of AZT 5′-COBP (**5**), representing a new type of nucleotide analogue, and discuss some remarkable spectroscopic and reactive properties of this compound. We also briefly describe preparation of another novel AZT nucleotide analogue, AZT 5′-(diazomethylene)bisphosphonate (**6**).

2. Materials and methods

NMR spectra were obtained on Bruker AM360 or AMX500 spectrometers. ^1H, ^{13}C and ^{31}P chemical shifts were calibrated with reference to $CHCl_3$($CDCl_3$, δ 7.24), $CDCl_3$(δ 77.0) and external H_3PO_4 (δ 0.00), respectively, except for **5** which used HDO ($^1H, \delta$ 4.66) and $CH_3CO_2^-$ ($^{13}C, \delta$ 20.0) or $(CH_3)_2CO$ ($^{13}C, \delta$ 30.7) as references. High-resolution mass spectra (HRMS) were obtained at the UC Riverside Analytical Chemistry Instrumentation Facility on a VG ZAB2SE FAB spectrometer (Opus V3.1, DEC 3000 Alpha Station). Low-resolution MS were obtained at UC Los Angeles Center for Molecular and Medical Sciences Mass Spectrometry on a Perkin-Elmer Sciex API III ESI spectrometry. Gradient HPLC purification and analysis were carried out using a Rainin Dynamax SD-200 solvent delivery system (dual PEEK pumps) equipped with a Dynamax UV-DII UV–Vis absorbance detector. Preparative centrifugal flow thin-layer chromatography (PCFTLC) was performed on an Analtech Cyclograph operated at a rotor speed of 1000–1400 rpm with a eluent flow of 1 mL/min through the spinning silica gel plates and UV detection of eluting bands within the plates.

Acetone, ethyl acetate, dichloromethane, and dioxane were, respectively, 99.9, 99.5, 99.9, and 99.3% pure, as obtained from Van Waters and Rogers. All were further purified by distillation from calcium hydride except dioxane, which was refluxed and then distilled from sodium. Methanol from the same source was AR grade and used directly. DOWEX 50W*200-8 ion-exchange resin, trimethyl orthoformate (anh., 99.8%), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 98%), diisopropylazodicarboxylate (DIAD, 95%), diisopropylethylamine (DIEA, 99%), triphenylphosphine (PPh_3, 99%), and *tert*-butanol (*t*-BuOH, 99%) were obtained from Sigma–Aldrich. DIAD was distilled

under reduced pressure (108 °C at 20 μm Hg). Triethylamine (Fisher Scientific, AR) was redistilled before use. *Tert*-butyl hypochlorite (*t*-BuOCl) was prepared from *t*-BuOH and commercial bleach (Chlorox) [35] by treating 500 mL of the latter at 10°C (dark) with 37 mL *t*-BuOH (0.39 mol) in glacial acetic acid (24.5 mL, 0.43 mol) for 3 min with stirring. The organic layer was washed with 50 mL 10% Na_2CO_3, then 50 mL H_2O, dried over $CaCl_2$ and filtered, yield 29.6 g (70%). Tetramethyl (diazomethylene)bisphosphonate **1** [36] was synthesized from tetramethyl methylenebisphosphonate (redistilled in vacuo, >99% by ^{31}P NMR), available from Sigma–Aldrich but in this instance synthesized [37] from the tetraisopropyl ester, which was a gift from Albright and Wilson. 3-Azido-3′-deoxythymidine (AZT, >99%) was a gift from Burroughs–Wellcome.

2.1. Sodium trimethyl (diazomethylene)bisphosphonate, **2**

Tetramethyl (diazomethylene)bisphosphonate, **1** (1.03 g, 4 mmol) was dissolved in dry acetone (4 mL) and sodium iodide (0.3 g, 2 mmol) in dry acetone (2 mL) was added dropwise over 10 min. After 50 min at rt, a white precipitate appeared and two additional portions of sodium iodide in acetone (0.15 g, 1 mmol and 0.075 g, 0.5 mmol) were added at 1 h intervals. The product was washed with acetone and dried under reduced pressure, yield 0.71 g (74%). ^1H NMR ($CDCl_3$): δ 3.90 (6H, d, $^3J_{HP} = 12$ Hz), 3.70 (3H, d, $^3J_{HP} = 12$ Hz). ^{13}C NMR ($CDCl_3$): δ 54.11 ($^2J_{CP} = 5.3$ Hz), 52.67 ($^2J_{CP} = 6.3$ Hz), ~2:1. ^{31}P NMR ($CDCl_3$): δ 7.79 (1P, d, $^2J_{PP} = 32$ Hz; coupled ~d of q, $^3J_{PH} = 12$ Hz), 24.59 (1P, d, $^2J_{PP} = 32$ Hz; coupled ~d of sept, $^3J_{PH} = 12$ Hz). HRMS (glycerol, positive ion FAB): calcd. for $[C_4H_9N_2O_6P_2 + 2Na]^+$: 288.9731. Found: 288.9731.

2.2. Disodium dimethyl (diazomethylene)bisphosphonate, **3**

To **2** prepared as above in 20 mL acetone and 20 mL methanol was added a large excess of NaI and the mixture refluxed for 48 h. The solvent was evaporated and the residue thoroughly washed with acetone, leaving a white solid, yield 100%. ^1H NMR (CD_3OD): δ 3.37–4.46 (m). ^{31}P NMR (CD_3OD): δ 12.7 (s, coupled m). HRMS (glycerol, negative ion FAB): calcd. for $[C_3H_6O_6N_2P_2 + 2Na + H]^+$: 274.9575. Found: 274.9584.

2.3. Trimethyl 3′-azido-3′-deoxythymidine 5′-(diazomethylene)bisphosphonate, **4**

2.3.1. Using PyBOP as the coupling reagent

Trimethyl (diazomethylene)bisphosphonate, **2** (75 mg, 0.28 mmol) dissolved in 5 mL of methanol was stirred with DOWEX 50W*200-8 ion-exchange resin (H^+, 1 g, 4.8 meq) for 1 h. After filtration, the filtrate was evaporated under vacuum to constant weight, and the residue taken up in DMF (8 mL). AZT (102 mg, 0.38 mmol) was dissolved in the mixture and PyBOP (198 mg, 0.38 mmol) was added, followed immediately by addition of DIEA (130 mg, 1.01 mmol). After 4 h, the reaction mixture was checked by ^{31}P NMR, and **4** (25%, full characterization data given in next section) was identified together with multiple byproducts.

2.3.2. Via Mitsunobu coupling

2 (266 mg, 1 mmol) was converted to the free acid as described above, and the residue taken up in dioxane (8 mL). AZT (401 mg, 1.5 mmol) was dissolved in the mixture and triphenylphosphine (393 mg, 1.5 mmol) was added, followed immediately by dropwise addition over 30 min of DIAD (303 mg, 1.5 mmol) in 2 mL of dioxane. After 4 h (100% conversion to **4** by ^{31}P NMR), the solvent was removed by rotatory evaporation at reduced pressure, and the residue purified by flash chromatography (3 mL/min) or PCFTLC (4 mm silica gel plate), pre-eluent diethyl ether, eluent diethyl ether:methanol, 9:1 under N_2. The diastereomeric product mixture was a viscous liquid (403 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 1.93 (3H, s, C5–CH_3), 2.40, (2H, m, H2′), 3.81, 3.84, 3.87 (9H, 3d, $J = 3$ Hz, POCH_3), 3.99 (1H, s, H4′), 4.26–4.48 (3H, m, H5′ and H3′), 6.20 and 6.22 (1H, 2t, $J = 6$ Hz, H1′), 7.40 and 7.44 (1H, 2s, H6), 9.44 (s, N*H*). ^{13}C NMR (CD_3OD): δ 12.40 and 12.42 (C5–*C*H$_3$), 37.45 and 37.60 (C2′), 54.68–54.96 (3 PO*C*H$_3$, unsym. m), 61.53 (C3′), 67.53 and 67.83 (C5′), 83.35, 83.41 (*C*=N$_2$), 86.52 (C4′), 86.59 (C1′), 111.94 (C5), 137.82 and 138.14 (C6), 152.11 (C2), 166.20, 166.48 (C4). ^{31}P NMR (CD_3OD): δ 16.16 (d, $^2J_{pp} = 35.0$ Hz), 16.66 (d, $^2J_{pp} = 35.0$ Hz), 15.97 (d, $^2J_{pp} = 35.6$ Hz), 17.06 (d, $^2J_{pp} = 35.6$ Hz). HRMS (glycerol, positive ion FAB): calcd. for $[C_{14}H_{21}N_7O_9P_2 + H]^+$: 494.0954. Found: 494.0957.

2.4. 3′-Azido-3′-deoxythymidine 5′-carbonylbisphosphonate, ***5***

The triester **4** (286 mg, 0.58 mmol) in 1 mL of CH_2Cl_2 under dry nitrogen was stirred with excess BTMS (459 mg, 3.0 mmol) for 1 h. Volatiles were removed under reduced pressure (N_2) and the residue dissolved in 4 mL of ethyl acetate. Upon addition of *t*-BuOCl (76 mg, 0.70 mmol) in 2 mL ethyl acetate containing a slight excess of water (20 μL, 1.20 mmol), the solution turned yellow and gas evolution was observed. After 3 min, the solvent was evaporated, the residue dissolved in a minimum amount of water and the pH quickly adjusted to 7.5 with Na_2CO_3. Water was evaporated under vacuum and the product reprecipitated from methanol/diethyl ether, then purified as a triethylammonium salt by semipreparative reverse phase gradient HPLC on a Varian Dynamax 100 Å 25 cm × 21.4 mm C-18 column eluted with 2–15% acetonitrile in 0.1 M triethylammonium acetate, 6 mL/min, pH 7.5, UV detection at 266 and 210 nm. A byproduct (77 min) was identified by MS as AZT 5′-phosphonate, **8**. (M^-, m/z 330).

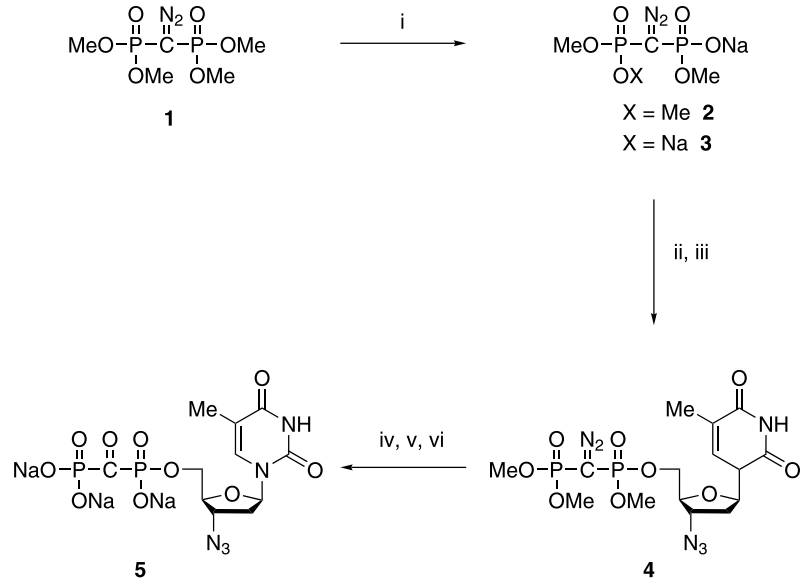
Lyophilization of the peak eluting at 92 min left the triethylammonium salt as a bright yellow solid (film, 150 mg, 0.30 mmol, 50%). ^{1}H NMR (D_2O, ext. ref. $CDCl_3$): δ 1.11 (12H. t, $J = 8$ Hz, CH_3–CH$_2$–N), 1.76 (3H, s, C5–CH$_3$), 2.34 (2H, m, $J = 6$ Hz, H2′), 2.91 (8H, q, $J = 8$ Hz, CH$_3$–CH_2–N), 4.04 (1H, s, H4′), 4.48 (1H, m, H3′), 4.6–5.5 HDO (b), 6.13 (1H, t, $J = 6$ Hz, H1′), 7.65 (1H, s, H6). ^{13}C NMR (D_2O, reference AcO^-): δ 12.35 (C5–*C*H$_3$), 38.05 (C2′), 62.62 (C3′), 67.09 (C5′), 85.03 (C4′), 86.54 (C1′), 113.64 (C5), 139.19 (C6), 153.60 (C2), 168.55 (C4), 238.4 ($^1J_{CP} = 118$, 124 Hz) C=O). ^{31}P NMR (D_2O, $pH_{obs} = 7.5$): −2.13 (1P, d, $^2J_{PP} = 196$ Hz), δ −1.29 (1P, d, $^2J_{PP} = 196$ Hz). HRMS (glycerol, negative ion FAB): Calcd. for $[C_{11}H_{14}N_5O_{10}P_2]^-$: 438.0216. Found: 438.0220.

2.5. 3′-Azido-3′-deoxythymidine 5′-(diazomethylene)bisphosphonate, **6**

The α-diazo bisphosphonate AZT triester **4** (100 mg, 0.203 mmol) in 2 mL of CH_2Cl_2 under dry nitrogen was stirred with BTMS (112 mg, 0.731 mmol) for 1 h at rt, the reaction being followed by ^{31}P NMR. Volatiles were removed under reduced pressure (N_2) and the residue treated with aq. Na_2CO_3 until the pH was 9.5, yield ~95% by NMR. The product solution was frozen for storage at <0 °C. ^{31}P NMR (D_2O, $pH_{obs} = 9$): δ 6.3 (1P, d, $^2J_{PP} = 28$ Hz), 15.3 (1P, d, $^2J_{PP} = 28$ Hz, dt, $^3J_{pH} = 6$ Hz). The stability to hydrolysis was assessed in aq. $NaHCO_3$, 0.5 M, at 25 °C, pH 9.5 and 6.9. Purification (20 μL samples) by semipreparative HPLC on a Rainin PureGel 7 μ 10 cm × 15 mm SAX column eluted with 30% methanol in 0.1 M TEA carbonate, pH 10 at 5 mL/min gave the product at 5.0 min, detected by flow UV scanning (λ_{max} ~ 223, 244, 267 nm). Evaporation of the pooled eluates in vacuo at 0–4 °C (3 × 2 mL methanol) left a residue, taken up in H_2O (D_2O) adjusted to pH 10.5 with dilute NaOH. ^{31}P NMR: δ 6.3 (d, $J = 28$ Hz), 15.6 (dt, $J = 28$, 6 Hz). MS (negative ion ESI, aq. alc.): calcd for $[C_{11}H_{14}N_7O_9P_2]$: 450.0. Found: 450.1.

3. Results and discussion

Our successful route to **5** is outlined in Scheme 1. Tetramethyl (diazomethylene)bisphosphonate **1** is monodealkylated to **2**, which is coupled to AZT at the



Scheme 1. Synthesis of AZT 5′-carbonylbisphosphonate via AZT 5′-(diazomethylene)bisphosphonate trimethyl ester. Reagents and conditions: (i) to 0.88 eq. NaI/acetone (**2**); then xs NaI/MeOH–acetone, Δ (**3**); (ii) **2**, Dowex-H^+/MeOH; (iii) PPh_3/DIAD/AZT/dioxane; (iv) BTMS/CH_2Cl_2; (v) *t*-BuOCl/EtOAc/H_2O; and (vi) aq. Na_2CO_3 (**5** shown as tetrasodium salt; isolated as triethylammonium salt).

5′ position, giving the conjugate **4**. Then, in a one-pot process, the protecting methyl ester groups in **4** are converted to tris(trimethylsilyl) (TMS) groups, the diazo function is oxidized, and the TMS triester hydrolyzed to yield an aqueous solution of **5**.

Tetraester **1** is readily available by diazo transfer from an aromatic sulfonyl azide to tetramethyl methylenebisphophonate [36]. Monodemethylation of dimethyl α-diazobenzylphosphonate by NaI in acetone [38] proved easily adaptable to synthesis of the bisphosphonate trimethyl ester monosodium salt **2** by the simple expedient of adding less than one equivalent of NaI in portions (0.5, 0.25, 0.13 eq.), which minimized formation of the symmetrical disodium salt byproduct **3**. The latter compound was prepared by treatment of **2** (or **1**) with excess NaI in refluxing methanol–acetone.

Our investigations of the stability and photochemistry in protic solvents of compounds such as **2** and **3** [39] will be reported separately [40].

We examined two different approaches to couple **2** with the 5′-OH of AZT to form the conjugate **4**. Use of PyBOP [41] gave only a modest yield (25%, NMR) of **4**. In contrast, under Mitsunobu conditions [42,43], the coupling reaction proceeded quantitatively (^{31}P NMR). Success in this reaction was critically dependent on (a) the choice of solvent system and (b) maintaining perfectly anhydrous reaction conditions. Pure **4** was conveniently and rapidly isolated by flash chromatography or PCFTLC on silica gel under dry N_2, the yield being somewhat reduced by demethylation on this chromatographic medium. Conjugation of the bisphosphonate with AZT generates a new R/S chiral center at P_α, predicting diastereotopic doubling of the ^{31}P NMR $P_\alpha P_\beta$ ab multiplet, which is observed (Figs. 2a and b). The structure was confirmed by ^{1}H and ^{13}C NMR and by FAB-HRMS.

The remaining synthetic problem was then to choose the best sequence for deprotecting and oxidizing the α-diazo bisphosphonate moiety. Silyldealkylation of dialkyl phosphonates with BTMS followed by mild, rapid hydrolysis provides a facile path to the corresponding acids [44] and is generally compatible with alkyl or aryl ketones and esters [45]. However, it was previously found that the unusually electrophilic carbonyl group in triethyl α-oxophosphonoacetate (triethyl phosphonoglyoxylate) tended to react with BTMS, whereas the α-diazo precursor of this compound cleanly underwent BTMS silyldeethylation without affecting the diazo group [46]. The resulting P,P-bis(trimethylsilyl) C-ethyl triester could be oxidized in refluxing benzene by $Rh_2(OAc)_4$-propylene oxide, and the distillable ketone bis(trimethylsilyl ester) was then hydrolyzed by brief contact with water. Accordingly, the three methyl ester groups of **4** were first removed by BTMS, and hydrolysis of the resultant silyl triester intermediate was carried out concurrently with diazo oxidation, using *t*-butyl hypochlorite in EtOAc containing a slight excess of water at room temperature [24,47]. It should be noted that the $Rh_2(OAc)_4$-propylene oxide system is ineffective in oxidizing (diazomethylene)bisphosphonate esters to ketones, even in refluxing toluene [24].

Rapid removal of unreacted BTMS after the silyldealkylation step, while avoiding premature hydrolysis of the silyl ester groups, was essential for success. Ketone formation was extremely rapid, characterized by sudden appearance of a bright yellow color with gas evolution (N_2). Another important reaction parameter was the

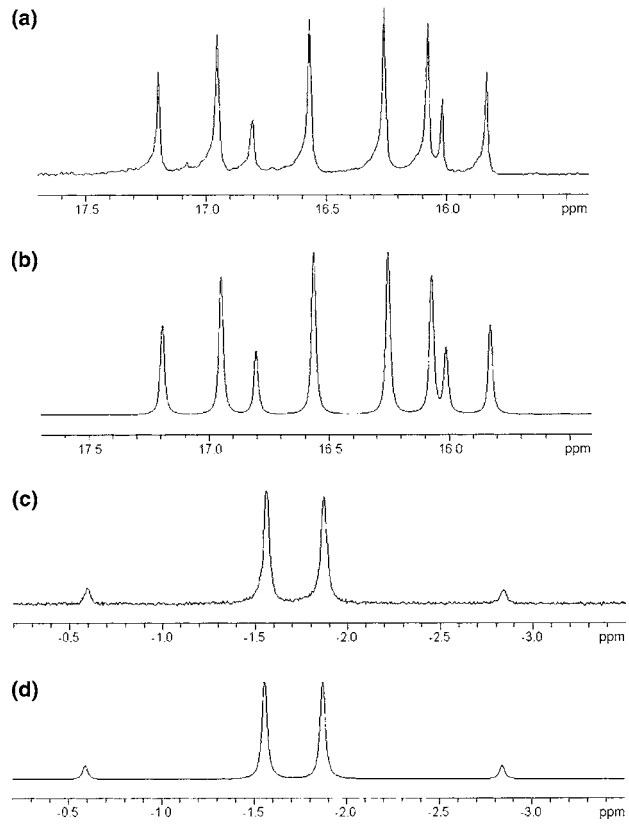


Fig. 2. ^{31}P NMR (^{1}H-decoupled) spectra of: (a) **4** diastereomers in CD_3OD, recorded at 202 MHz; (b) same, simulation: parameters: δ 16.16 (d, $^{2}J_{pp} = 35.0$ Hz), 16.66 (d, $^{2}J_{pp} = 35.0$ Hz). 15.97 (d, $^{2}J_{pp} = 35.6$ Hz), 17.06 (d, $^{2}J_{pp} = 35.6$ Hz); (c) **5** in D_2O, $pH_{obs} = 7.5$, as recorded; (d) same, simulated as an ab multiplet: δ −1.29 (d, $^{2}J_{PP} = 196$ Hz), −2.13 (d, $^{2}J_{PP} = 196$ Hz).

excess of water present, which had to be carefully controlled. 'Anhydrous' conditions produced a very slow reaction and favored formation of α-chlorinated side product, but too great an excess of water also decreased the yield by promoting product decomposition [47]. Finally, adjustment of the reaction pH to slightly above neutral (7.5) promptly after hydrolysis proved critical to avoid decomposition of the product, which was isolated using semipreparative HPLC with UV detection (Fig. 3). We have not investigated the competition between hydrolytic silyldealkylation of **4** (to the novel diazoconjugate salt **6**) and hydrolytic oxidation of **4**, preceding conversion to **5**. However **6** (Fig. 4) can be prepared in situ from **4**: BTMS treatment of **4** effects a gradual decrease in the **4** ^{31}P NMR multiplet at δ 17 ppm with appearance of two broad resonances (~1:1) at δ 5.5 (assigned to the P-mono(trimethylsilyl) ester group of the silylated intermediate) and δ −5.5 (assigned to the P′,P′ bis(trimethylsilyl) ester group of the silylated intermediate) ppm. Quenching with aqueous Na_2CO_3 to obtain a pH above 7.5 quantitatively gave **6**, identified by its characteristic monoalkyl

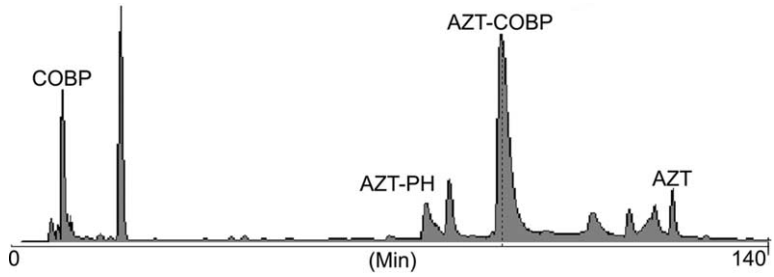


Fig. 3. Semipreparative HPLC purification of **5** (as a triethylammonium salt; UV detection, 266 nm). COBP, (eluted at 11 min); AZT-PH (**8**), (eluted at 77 min); AZT-COBP (**5**) (eluted at 92 min); AZT, (eluted at 123 min). COBP and AZT: spiked with authentic samples. HPLC conditions given in Section 2.

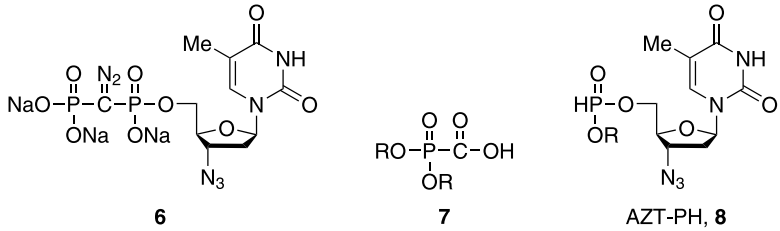


Fig. 4. Structures of 5′-(diazomethylene)bisphosphonate AZT (**6**, shown as tetrasodium salt); possible fragmentation byproducts from conversion of **4** to **5** (**7**, **8**; R = TMS, H, or Na).

bisphosphonate P_α and P_β doublets (proton-decoupled ^{31}P NMR, $J = 28$ Hz) at δ 6.3 and 15.3 ppm, respectively; in the coupled spectrum, the P_α resonances are further split into triplets with $J = 6$ Hz, demonstrating attachment to an OCH_2R group; the structure was confirmed by ESI-MS. At pH 9.5 and ~25 °C, **6** showed only slight decomposition (10–15%) after two days at rt (^{31}P NMR), but at pH 6.9 significant hydrolysis (~50%) was evident within half an hour. This behavior corresponds to progressive activation of the α-diazo group with protonation of the bisphosphonate, suggesting an interesting basis for pH-dependent inactivation of nucleotide-binding enzymes.

Minor byproducts from the oxidative hydrolysis step included AZT 5′-phosphonic acid (AZT-PH, **8**), apparently arising from C–P cleavage (which would also release a phosphonoformate fragment, **7**) (Fig. 4). Tetraalkyl COBP esters readily form hydrates that can undergo C–P bond cleavage on deprotonation of an α-OH group, resulting in analogous fragmentation products [24]. Compound **8** might derive from a product in which oxidation to the ketone preceded silyl ester hydrolysis, or alternatively from **5** in acid form after silyl ester hydrolysis, but prior to neutralization.

Evidence for the structure of **5** includes, besides the FAB-HRMS, the ^{31}P NMR spectrum which displays two non-equivalent phosphorus nuclei, both resonating upfield from δ 0 ppm and exhibiting an unusually large $^2J_{PP}$ coupling constant

(196 Hz) suggestive of spin coupling enhanced via the intervening C=O π-system (Figs. 2b and c); the ^{1}H NMR; the ^{13}C NMR which displays a strikingly downfield-shifted C=O resonance at 238.4 ppm, split by two non-equivalent, vicinal phosphorus nuclei ($^{1}J_{CP} = 118$, 124 Hz); and the visible absorption of **5** in aqueous solution, as discussed below (see Fig. 5).

A remarkable property of **5** is the pH-dependent reactivity of its ketone group, which is reversibly converted to the hydrate form (**9**) (Scheme 2).

The ketone **5** and hydrate **9** at equilibrium in aqueous solution are simultaneously detectable by ^{31}P NMR over a pH range from slightly alkaline to at least 2, and **5** is independently observable via the visible absorbance (420 nm) of its ketone chromophore (yellow). At low pH, the colorless hydrate form **9** (δ 15 ppm) predominates. As the pH is raised, the ketone multiplet (δ −2 ppm) enlarges and the solution increasingly appears yellow. The pH at which 50% of the ketone is transferred to its hydrate form ('pK') is about 2.3 (rt, D_2O). Upon addition of excess $MgCl_2$, the

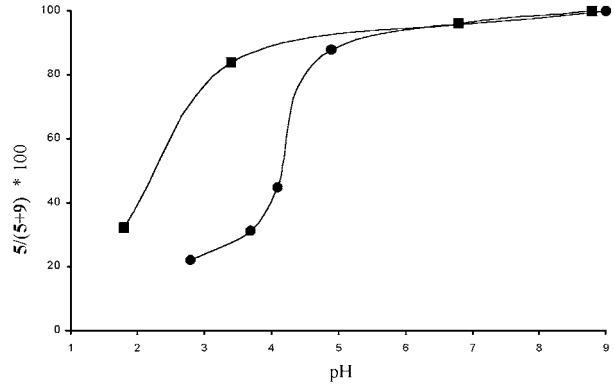
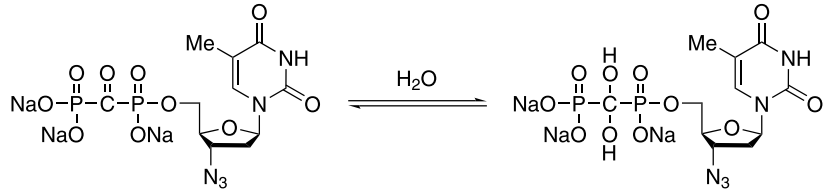


Fig. 5. Variation with pH and effect of added Mg^{2+} ($MgCl_2$) on relative amount of ketone **5** in dilute solution (D_2O) as a per cent fraction of ketone **5** + hydrate **9** (total concentration 0.1 M), observed by ^{31}P NMR. ■–■ No added Mg^{2+}. ●–● Added Mg^{2+}, >0.1 M.



5 **9**

Scheme 2. Equilibrium between AZT 5′-carbonylbisphosphonate **5** and its hydrate **9** in water. The extent of phosphonate oxygen protonation is not specified, as it depends on the pH and species. The equilibrium shifts from ketone to hydrate as the pH is lowered, and can be monitored by the change in the ketone chromophore absorption of **5** at 420 nm, or more exactly by ^{31}P NMR observing **5** and **9** simultaneously.

apparent 'pK' of the equilibrium dramatically shifts upward by nearly 2 pH units (Fig. 5).

The reactivity of the ketone group in **5** is increased by the two electronegative phosphonate substituents on its carbon atom, but at neutral to high pH, the negative charges on the phosphonate oxygens reduce this effect significantly and the stabilized ketone form predominates in aqueous solution. As the pH is decreased, stepwise protonation of the phosphonate anions diminishes their electrostatic effect, and the ketone becomes increasingly reactive to nucleophilic addition, shifting the ketone-hydrate equilibrium to the hydrate form.

Rasanen et al. have shown in a series of ab initio calculations that methylenebisphosphonate and (dichloromethylene)bisphosphonate can form bi- and tridentate complexes with one or two magnesium dications in aqueous solution [48–51]. Similar complexes may be formed when Mg^{2+} is added to **5** in water. Evidently such complexation redistributes local charges on the bisphosphonate moiety, resulting in an increase in ketone reactivity towards solvent nucleophile, and/or stabilizes **9**.

4. Conclusion

Nucleoside carbonylbisphosphonates such as **5** represent a new type of nucleotide analogue with intriguing physical and chemical properties, including their 'sp^2' P–C–P geometry [16], compact steric profile, hydrogen bond-acceptor properties and the pH-tunable chemical reactivity of the bridging C=O group. Binding to an enzyme active site which produces a downward shift in local pH or an equivalent change in the electrostatic environment of the bound, relative to the unbound analogue, could facilitate addition of an active site nucleophile to the α-ketone group of the bound analogue, resulting in a novel enzyme inactivation process.

We have demonstrated a convenient, flexible route to COBP nucleotides which postpones aqueous chemistry until the final step. Some of its other advantages include: (1) the protected bisphosphonate-nucleoside Mitsunobu-type coupling step proceeds facilely; (2) in a rapid and mild, one-pot process, regioselective (the 5′-methylene ester link is left intact) BTMS silyldemethylation, followed by treatment with *t*-butyl hypochlorite/water, deprotects all three bisphosphonate methyl ester groups while oxidizing the α-diazo group; (3) subsequent neutralization with the base of choice provides the crude desired salt directly, without the necessity of cation exchange; (4) the product is readily purified by semipreparative HPLC followed by lyophilization.

In the course of this work, we have also prepared the first example of a (diazomethylene)bisphosphonate nucleotide analogue (**6**). The pH-dependent reactivity of its α-diazo group suggests evaluation of such compounds as a potential inhibitors of enzymes mediating nucleotide-dependent biochemical processes.

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