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Novel Alkynylphosphonate Analogue of Calcitriol with Potent Antiproliferative Effects in Cancer Cells and Lack of Calcemic Activity

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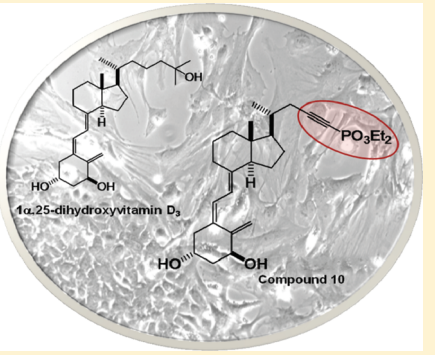
Supporting Information

ABSTRACT: Here, we describe the design and synthesis of diethyl [(5*Z*,7*E*)-(1*S*,3*R*)-1,3-dihydroxy-9,10-secochola-5,7,10(19)-trien-23-in-24-yl] phosphonate (compound **10**), which combines the low calcemic properties of phosphonates with the decreased metabolic inactivation due to the presence of a triple bond in C-24 and studied its in vitro effects on several cancer cell lines and its in vivo effects on blood calcium levels. We demonstrate that this compound is a potent antiproliferative vitamin D analogue, showing lack of calcemic effects in vivo.

KEYWORDS: 1α,25-(OH)$_2$ vitamin D$_3$, alkynylphosphonate analogues, cancer, hypercalcemia, cellular proliferation



1α,25-Dihydroxyvitamin D$_3$ (calcitriol) has been shown to exert control over a multitude of biological processes related to calcium and phosphorus homeostasis, cell proliferation, differentiation, and apoptosis.[1−3] The potent growth inhibitory effect, combined with the presence of the vitamin D receptor (VDR) in a wide variety of cells, makes calcitriol an ideal compound to treat hyperproliferative disorders such as cancer. However, major side effects such as hypercalcemia have severely hampered its therapeutic application. One way to overcome this problem is to design structural analogues of calcitriol with the same or even increased antiproliferative and pro-differentiating activities and with reduced undesired effects on calcium and bone metabolism. Several of these analogues have been synthesized and tested in various cell lines and animal models, in some cases with promising results.[3−5] Nevertheless, considerable variation in the antitumoral response to analogues has been observed among different types of cells and different type of tumors. The differential response of tumor cells to these analogues might be explained by the different level of interaction of VDR with coactivators upon analogue binding,[3] differences in binding to VDR or to vitamin D binding protein (DBP), or differences in drug metabolism.[4,6] Although the molecular pathways involved in the antitumor effects of calcitriol and analogues are not clear, substantial preclinical data support the hypothesis that vitamin D compounds may play an important role in cancer therapy and prevention. The preclinical data were rapidly followed by clinical trials in humans, and although many trials with vitamin D analogues have been conducted in cancer patients, the results have sometimes been disappointing.[5] For example, although initial phase II data suggested some beneficial effects of vitamin D analogue EB1089, a large trial in patients with hepatocellular carcinoma was negative.[7] Most studies have administered vitamin D analogues orally on a continuous daily dosing schedule, and hypercalcemia or hypercaliuria was encountered, thus limiting dose escalation. These concerns about induction of hypercalcemia by the analogues and the desire for more potent agents have prompted the development of less calcemic vitamin D analogues. These have to achieve high concentrations in cancer cells to be biologically active, this being greatly controlled by their cellular catabolism initiated by C-24 hydroxylation. The design of new analogues employs structural modifications on the side chain or A ring to prevent the inactivating hydroxylation, oxidation, and epimerization, which are characteristic of calcitriol catabolism. In this regard,

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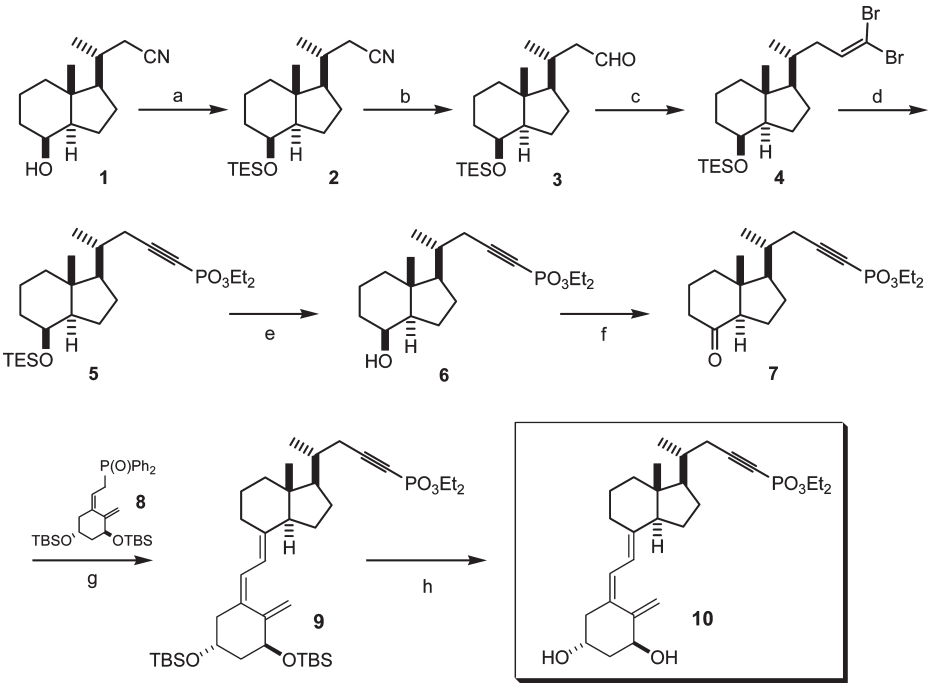
Scheme 1. Synthesis of Alkynylphosphonate Vitamin D_3 Analogue 10[a]



[a] Reagents and conditions: (a) TESCl, Im., DMAP, CH_2Cl_2, 0 °C, (97%). (b) DIBAL-H, CH_2Cl_2, −10 °C. (c) CBr_4, PPh_3, CH_2Cl_2, 0 °C to room temperature. (d) (i) LDA, THF, −78 °C; (ii) $ClPO(OEt)_2$, −78 °C (50% three steps from **2**). (e) HF, CH_3CN, room temperature, (92%). (f) PDC, PPTS, CH_2Cl_2, room temperature, (97%). (g) (i) Compound **8**, *n*-BuLi, THF, −78 °C; (ii) compound **7**, −78 °C (60%). (h) HF, CH_3CN, room temperature, (84%).

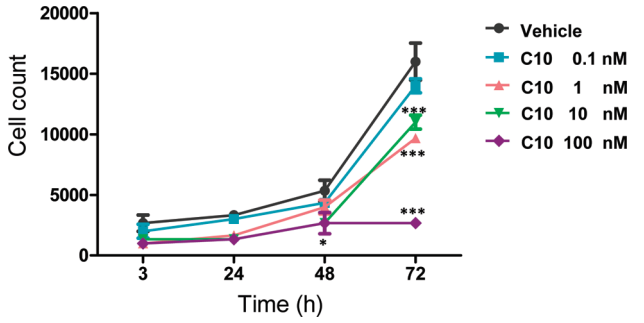


Figure 1. Time−course response analysis of compound **10** on cellular survival of human head and neck squamous cell carcinoma HN12 cell line. Cells were exposed to the indicated concentrations (nM) of compound **10** (C10) over a total time of 72 h. The experiment was repeated twice. *P* values from Bonferroni post-test of ANOVA analysis are shown.

Table 1. Half Maximal Inhibitory Concentration (IC_{50}) of the Cell Lines That Responded to Analogue Treatment[a]

	IC_{50}	
cell line	compound **10**	calcitriol
HN12	22.3	553
SVEC vGPCR	113	0.52
T98G	36.1	
LM05e	1.68	0.03
T47D	4.78	1.30

[a] Values are the means of at minimum three experiments and are given in the nanomolar range.

phosphonate analogues have been shown to display a certain degree of dissociation between the vitamin D activity in vitro and undesired hypercalcemia in vivo.[8] The first reference to vitamin D analogues possessing phosphorus atoms in the side chain can be found in the work of Dauben et al.[9] Since then, few of such derivatives have been described until Steinmeyer worked on vitamin D phosphonate hybrids.[8]

We have now designed a convergent route for the synthesis of a novel vitamin D analogue **10** bearing an alkynylphosphonate moiety. This analogue combines the low calcemic properties of phosphonates[8] with the decreased metabolic inactivation due to the presence of a triple bond in C-24.[10] The synthetic approach is also useful for the preparation of structurally related phosphonate analogues from the corresponding alkynylphosphonate precursor **5**. The synthesis of such analogues is currently under way in our laboratory with a view to their biological evaluation.

Our synthetic approach involves the construction of the vitamin D triene system employing the convergent Wittig−Horner coupling between the ketone **7** and the phosphine oxide **8**. The synthesis of the key precursor **7** takes advantage of the readily available Inhoffen diol to afford the nitrile **1** (Scheme 1).[11,12]

Protection of the hydroxyl group in **1** as a triethylsilyl ether under standard conditions gave compound **2**.[13] This was treated with diisobutylaluminium hydride in dichloromethane and yielded the aldehyde **3**, which was submitted as a crude to the ylide prepared from CBr_4 and PPh_3 using Corey−Fuchs conditions to give a dibromoalkene **4**.[14,15] Treatment of **4** with an excess of lithium diisopropyl amide in THF led to the corresponding acetylenic anion, which was trapped with diethyl chlorophosphate

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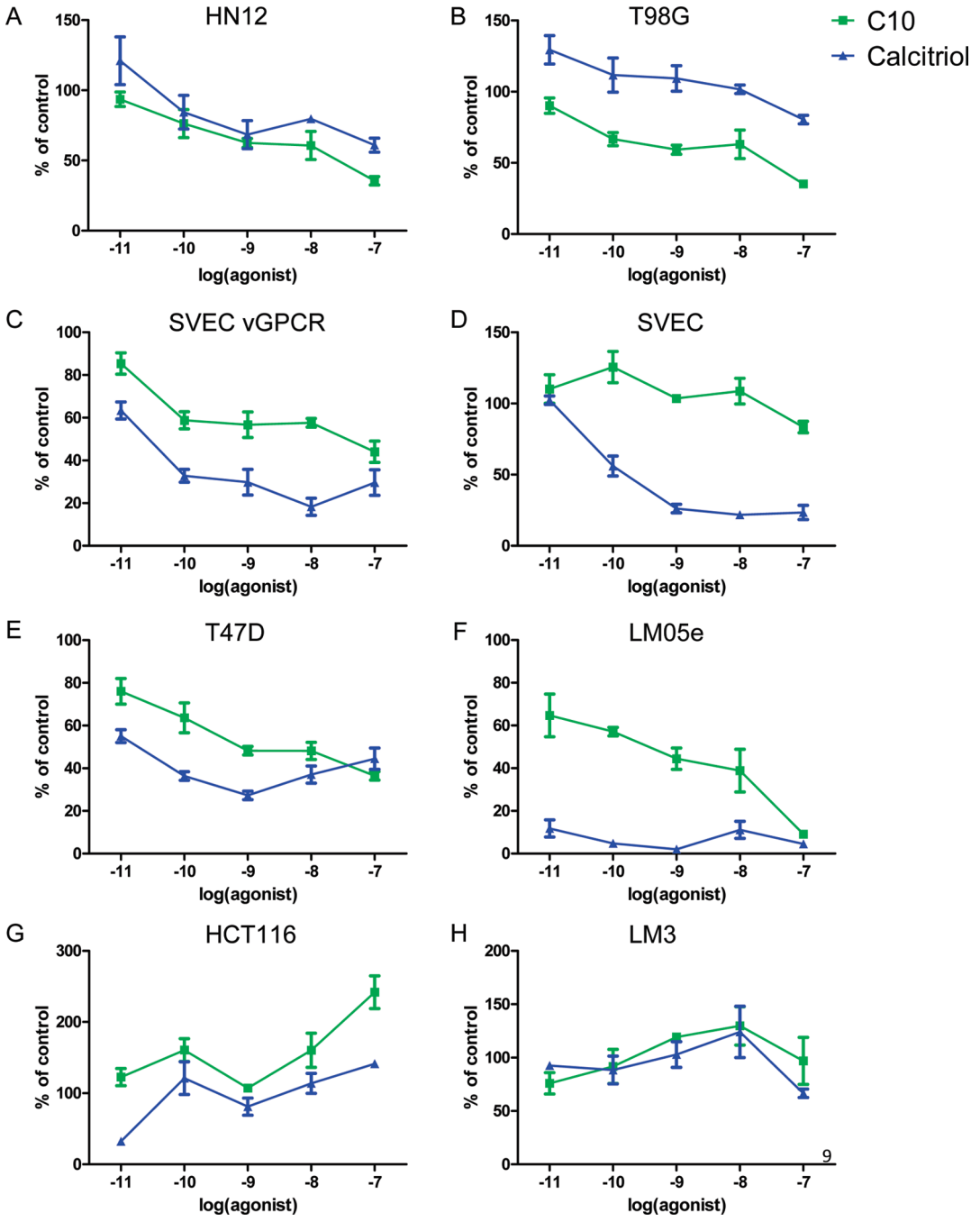

Figure 2. Dose−response effects of compound **10** (C10) on cellular survival and its comparison with calcitriol. (A) Human head and neck squamous cell carcinoma HN12 ($p = 0.0499$), (B) human glioma T98G ($p = 0.0014$), (C) Kaposi sarcoma SVEC vGPCR ($p \leq 0.0001$), (E) human mammary adenocarcinoma T47D ($p \leq 0.0001$), and (F) murine mammary adenocarcinoma LM05e ($p \leq 0.0001$) responded to compound **10** treatment with reduced survival, whereas (D) the nonmalignant cell line SVEC, (G) human colorectal carcinoma HCT116, and (H) murine mammary adenocarcinoma LM3 were not responsive to analogue treatment. Cells were exposed to the indicated doses of vehicle (isopropanol), compound **10**, or calcitriol over a total time of 72 h. Cellular proliferation was expressed as percentage of the vehicle. The experiments were repeated at least three times for each cell line. *P* values from Bonferroni post-test of one-way ANOVA analysis for comparisons of reduction in cellular survival with compound **10** are shown.

to afford the alkynylphophonate **5**.[16] This was deprotected by treatment with HF in acetonitrile to give alcohol **6**, which afforded ketone **7** after further oxidation with pyridinium dichromate.

The Wittig−Horner reaction of **7** with the anion derived from the phosphine oxide **8** yielded the protected analogue **9**. Subsequent removal of the silyl protecting groups afforded the desired analogue **10**.[11,12] Following this eight-step synthetic sequence, **10** was achieved in 22% overall yield from **1**.

The synthesized vitamin analogue (compound **10** hereafter) was tested for its antiproliferative effects on several human and murine tumor cell lines. In preliminary testing, we performed a time−course response experiment (0−72 h) in the human squamous cell carcinoma cell line HN12 with different

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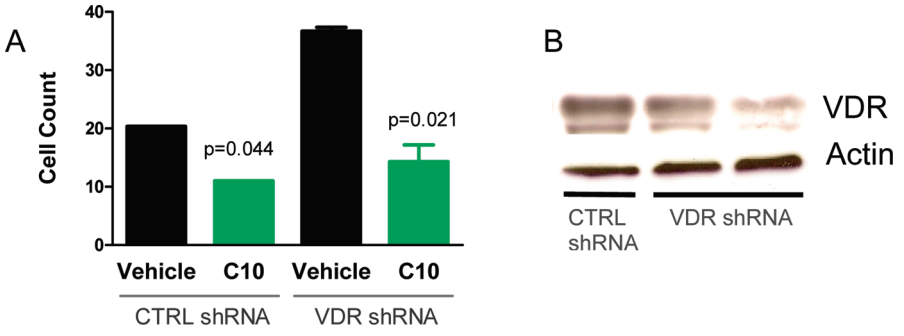

Figure 3. VDR silencing does not alter the antiproliferative effects exerted by compound **10** on the glioma cell line T98G. (A) Cellular count following transfection of cells with a plasmid silencing VDR (shRNA VDR) or a scrambled shRNA (CTRL shRNA). Cells were treated for 48 h after transfection with compound **10** (C10, 10 nM) or vehicle, and cell count was performed 72 h following treatments. The experiment was repeated three times and was done in triplicate. Shown is the mean ± SE. (B) Silencing of VDR with shRNA plasmid (p FIV-H1-Puro shRNA VDR) in T98G cells: CTRL plasmid (lane 1) or 2 (lane 2) and 4 μg (lane 3) of VDR shRNA plasmid were transfected using lipoafectamine reagent, and protein lysates were performed 48 h later.

concentrations of compound **10** (0.1−100 nM) and observed a significant decrease in cell number at 72 h (Figure 1) starting at 1 nM.

We subsequently performed 72 h dose−response analyses for all of the cell lines, comparing the effects of compound **10** with those elicited by the natural hormone calcitriol. Table 1 contains the IC_{50} of cell lines that responded to compound **10** treatments. As shown in Figure 2, we observed a significant decrease in cell counting after treatment with compound **10** in human squamous cell carcinoma HN12 (Figure 2A), human glioma T98G (Figure 2B), and Kaposi sarcoma SVEC vGPCR (Figure 2C) cell lines. Furthermore, in the human glioma T98G and in the human squamous cell carcinoma HN12 cell lines, the antiproliferative effects exerted by compound **10** were greater than those elicited by calcitriol. Importantly, although calcitriol was more potent than compound **10** in inhibiting the growth of Kaposi sarcoma cells, the nonmalignant parental cell line, SVEC, did not respond to compound **10** (Figure 2D). The human T47D (Figure 2E) and the murine LM05e (Figure 2F) hormone-sensitive breast adenocarcinoma cell lines also responded to compound **10** with decreased survival. In contrast, the human colorectal carcinoma HCT116 (Figure 2G) and the murine hormone-insensitive breast adenocarcinoma LM3 (Figure 2H) cell lines did not show reduced survival following compound **10** treatment. Longer treatment periods or higher concentrations of compound **10** did not show significant antiproliferative effects.

It is known that cancer cell lines display a range of sensitivities to the antiproliferative effects of calcitriol and its derivatives, although the reason for this is largely unknown and could result from defects in any of the components in the VDR signaling pathway including VDR and 24-hidroxylase (CYP24A1). Calcitriol action is limited by its catabolism, occurring mainly by the CYP24A1 resulting in 1α,24,25-$(OH)_3$-D_3, a metabolite with substantially lower affinity for the VDR. Although this enzyme is located primarily in liver, many studies have demonstrated that it can also be expressed by many tissues.[17] The augmented expression of CYP24A1 has been shown to be detrimental to calcitriol antiproliferative effects. For example, in prostate cancer cell lines, it has been demonstrated that enzyme expression was inversely correlated to the antiproliferative effects displayed by the cells.[18] In addition, antagonists of the CYP24A1, such as azoles, have been shown to potentiate the antitumor effects of calcitriol in vitro and in vivo.[19] In this regard, compound **10** presents limitations in its metabolization through 24-hydroxylation due to the presence of a triple bond between the carbons 23 and 24, so its metabolic transformation might be reduced.[10] This is relevant in cells showing important activity of CYP24A1 such as astrocytes,[20] prostate cells,[21] and also in colon, ovary, lung tumors, [22] and glioma.[23] Importantly, in human glioma cell lines, the natural hormone either does not exert antiproliferative activity or it increases proliferation,[23,24] whereas compound **10** potently inhibits cellular survival, as shown in this report. The potential differences in the metabolic degradation between calcitriol and compound **10** might account for the differences observed in the antiproliferative response; therefore, compound **10** might be useful for the treatment of human gliomas. Preliminary experiments showed that VDR is not necessary for the antiproliferative effects observed in the glioma cell line following compound **10** treatment (Figure 3), thus suggesting the involvement, at least in part, of nongenomic effects elicited by this analogue. This is in accordance with previously published results showing no upregulation of VDR by calcitriol in several glioblastoma cell lines and the presence of low levels of the receptor mRNA in human biopsies of these tumors.[23] Our results demonstrating compound **10** antiproliferative effects on breast cancer cell lines that are hormone-responsive are also supported by previous observations showing that the sensitivity to calcitriol is higher in those mammary cancer cell lines that express estrogen receptors.[25]

Because of its significant in vitro antiproliferative activity in some tumor cells, compound **10** was evaluated for hypercalcemic effects in vivo. Previous pharmacokinetic studies performed in normal mice indicated that calcitriol at 0.125 μg/mouse (approximately 5 μg/kg body weight) results in a C_{max} >10. 0 ng/mL and AUC > 40.0 ng h/mL,[26] which exceeds the concentration needed for calcitriol antitumor activity in vitro. Therefore, we chose doses of 5 and 20 μg/kg that have antitumor effects in vivo. Mice were divided into three groups (n = 5/group) and given a daily intraperitoneal injection of calcitriol, compound **10**, or vehicle at 5 or 20 μg/kg body weight for 5 days. Blood was collected prior to dose administration, then at 24, 48, 72, and 96 h post-treatment. Plasma calcium levels were measured by reading the absorbance of metallochromic indicator Arsenazo III. Interestingly, compound **10** showed no calcemic activity as observed in Figure 4. Instead, calcitriol was effective at causing an increase in plasma calcium, as expected. Moreover, mice that were treated with calcitriol died after 3 days, whereas mice treated with

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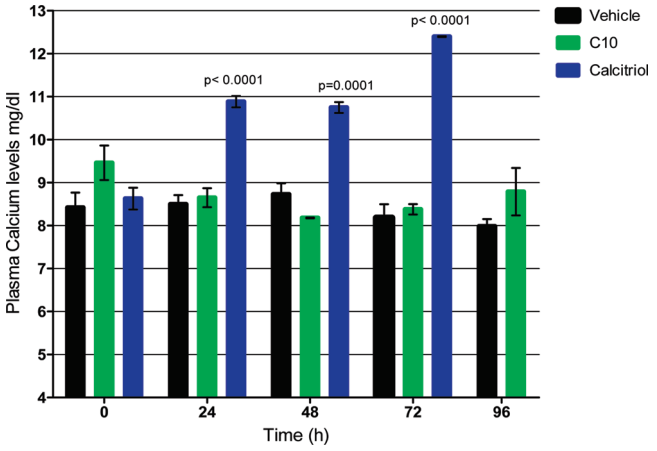

Figure 4. Plasma calcium levels in mice in response to daily intraperitoneal injections of vehicle, calcitriol, or compound **10** during a period of 5 days. Animals were injected with 5 μg/kg body weight of compound **10**, calcitriol, or vehicle (isopropanol), and plasma calcium was measured before the injection (basal levels, 0 h) and at 24, 48, 72, and 96 h. Values for calcitriol at 96 h are missing because animals died following 3 days of treatment due to hypercalcemia. Values are means ± SEs from five animals in each group. The experiment was repeated two times.

compound **10** remained alive and healthy during the entire examination period. The 20 μg/kg dose gave similar results for compound **10** (not shown). Visual observation of the internal organs of the animals such as liver, duodenum, lungs, and kidneys showed no macroscopic morphological alterations after the treatment with compound **10**. Thus, this compound appears to be well tolerated even at high doses.

Altogether, these results suggest that compound **10** exerts considerable antiproliferative activity at nonhypercalcemic dosages and may have therapeutic potential for the treatment of different hyperproliferative disorders.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, ^{1}H, ^{13}C and ^{31}P NMR spectral data of compounds **2**−**10**, HRMS data for compounds **5**, **9**−**10** and HPLC data for alkynylphosphonate analogue **10**, and experimental procedures for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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