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Highly convergent synthesis and antiviral activity of (E)-but-2-enyl nucleoside phosphonoamidates

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Article info
Article history:
Received 17 November 2017
Received in revised form 11 January 2018
Accepted 26 January 2018
Available online 31 January 2018

Keywords:
Ultrasound
Aqueous cross-metathesis
Phosphonoamidate
Acyclic nucleoside phosphonates
DNA viruses

Abstract
Several hitherto unknown (E)-but-2-enyl nucleoside phosphonoamidate analogs (ANPs) were prepared directed with nitrogen reagents by cross-metathesis in water-under ultrasound irradiation. Two diastereoisomers were formally identified by X-ray diffraction. These compounds were evaluated against a large spectrum of DNA and RNA viruses. Among them, the phosphonoamidate thymine analogue 19 emerged as the best prodrug against varicella-zoster virus (VZV) with EC50 values of 0.33 and 0.39 mM for wild-type and thymidine kinase deficient strains, respectively, and a selectivity index >200 μM. This breakthrough approach paves the way for new purine and pyrimidine (E)-but-2-enyl phosphonoamidate analogs.

1. Introduction
Modified nucleosides represent a major class of therapeutics for cancer and viral diseases [1]. Among them, acyclic nucleoside phosphonates (ANPs) pioneered with ((S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (((S)-HPMPA) [2] in 1986 by Antonín Holý and Erik De Clercq, forms a key class of drugs active against various DNA viruses as well as against retroviruses. However, those compounds suffer from limitations such as their reduced cell penetration (the free phosphonic acid form is negatively charged at physiological pH) as well as from nephrotoxicity. This has led to extensive search for new ANPs as well as to the development of prodrug approaches [3,4] for enhanced bioavailability and cell internalisation [5–8]. Several ANP prodrugs were marketed, such as adeovir dipivoxyl (bis-POC PMEA) [9,10] for the treatment of hepatitis B virus (HBV), tenefovir disoproxyl (bis-POC PMPA) [11] or the newly tenofovir alafenamide [12–14] for the treatment of human immunodeficiency virus (HIV) and HBV [15,16].

Over the last decade, our group has developed a new family of ANPs based on a trans-but-2-enyl phosphate scaffold [17]. Compounds were directly obtained as prodrugs by a highly convergent and modular approach based on the powerful olefin cross metathesis (CM) between various allylphosphonate synthons bearing biolabile groups and N1- (or N9) crotyl (or allyl) pyrimidines or purines. This approach showed a remarkable breakthrough for the synthesis of nucleoside prodrugs compared to the known linear approaches, which suffer from low yields. It is also clear from the literature that the choice of a prodrug has a direct impact on its targeting and cell release and greatly influences the overall outcome and efficiency of the parent drug [18–20]. Thus, following this synthetic pathway, we have obtained several prodrugs [21–25] including the most commonly used carbon-xoxymethyl pronucleotides (pivaloyloxymethyl- or POM, isopropylxocarbonyloxymethyl- or POC), but also the alkoxylalkyl

Abbreviations: VZV, varicella zoster virus; VV, vaccinia virus; HSV, herpes simplex virus; VSV, vesicular stomatitis virus; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; CC50, compound concentration affording 50% inhibition of cell growth; EC50, compound concentration affording 50% inhibition of the viral cytopathicity; MCC, minimum cytotoxic concentration required to afford a microscopically detectable alteration of cell morphology; ACN, acetonitrile; DCM, dichloromethane.
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monooester (hexadecyloxypropyl or HDP, octadecyloxyethyl or ODE) [26]. Several of these (E)-but-2-ethyl ANPs prodrugs exhibited remarkable antiviral activity against DNA and RNA viruses in sub-micromolar concentrations. The bis-(POM)-(E)-TbutP (1) and the prodrugs 2–4 were all very active against several herpesviruses [i.e. herpes simplex virus 1 (HSV-1) and 2 (HSV-2), and varicella-zoster virus (VZV)], representing a new potential antiviral lead.

Despite a significant amount of research and development on aryl phosphonoamidate prodrugs reported by McGuigan [27,28], the development of aryl phosphonoamidates, especially in the field of ANPs, has been very sparsely investigated [29]. Thus, in this article, we describe the in-water ultrasound promoted synthesis and antiviral evaluation of hitherto unknown (E)-but-2-ethyl nucleoside phosphonoamidates with high yields, (Fig. 1).

2. Results and discussion

2.1. Chemistry

Aryl phosphonoamidates are generally obtained by treatment of the parent dimethylphosphonate nucleoside with TMSBr into the corresponding silyl esters, followed by a subsequent treatment with an excess of phenol and L-alanine benzyl-L-alanine esters [11–15], in order to compare the influence of the ester group or the phosphorus chirality, on the activity and toxicity of final ANPs. Compounds 11-14 and 15 were obtained as a mixture of diastereomers (from 6:4 to 1:1). Only the diastereomers of compound 11 (R = Bn) were separated by careful column chromatography on silica gel (twice) and compounds 12 and 13 were isolated as single isomers, respectively. 31P NMR spectroscopy confirmed the isolation of each products with the presence of single peak, while a mixture of isomers provide two peaks. The sitting drop crystallization allows to obtain a crystal of both molecules and their structures were unambiguously determined by X-ray to be P(R) for 12 and P(S) for 13, respectively, (Fig. 2).

Next, the silylation of thymine was obtained in 5 min at room temperature in presence of bistrimethylsilylacetamide BSA. The intermediate was directly engaged in nucleophilic substitution reaction with crotyl bromide, chlorotrimethylsilane and sodium iodide [34]. This reaction is performed under ultrasonic activation to afford after seven hours the desired compound 16 in quantitative yield. 16 was then converted to the N3-Boc thymine derivative 17 in quantitative yield, (Scheme 3) [35].

With all partners in hand (16, 17 and 11–15), we turned our attention to the H-phosphonoamidate chemistry. The dimethylphosphite 7 was reacted with allyl bromide under Michaelis–Becker conditions to give the dimethylallylphosphonate 8 with 78% yield. It is important to quote than this reaction was scaled-up to 50 g. After substitution of a OMe group of 8 by a chlorine in presence of oxaly chloride, this position was then substituted by a phenolate generated in situ to give 9 (as a mixture of enantiomers) in 65% yield. Compound 9 was treated by bromotrimethylsilane in dichloromethane for 24 h at room temperature, to give the phosphonic acid monoester derivative 10 in excellent 91% yield (Scheme 2).

Following a procedure described by Gajda et al. [33], phosphonate 10 was then converted to various methyl-, isopropyl- and benzyl-L-alanine esters 11–15, in order to compare the influence of the ester group or the phosphorus chirality, on the activity and toxicity of final ANPs. Compounds 11, 14 and 15 were obtained as a mixture of diastereomers (from 6:4 to 1:1). Only the diastereomers of compound 11 (R = Bn) were separated by careful column chromatography on silica gel (twice) and compounds 12 and 13 were isolated as single isomers, respectively. 31P NMR spectroscopy confirmed the isolation of each products with the presence of single peak, while a mixture of isomers provide two peaks. The sitting drop crystallization allows to obtain a crystal of both molecules and their structures were unambiguously determined by X-ray to be P(R) for 12 and P(S) for 13, respectively, (Fig. 2).

Next, the silylation of thymine was obtained in 5 min at room temperature in presence of bisttrimethylsilylacetamide BSA. The intermediate was directly engaged in nucleophilic substitution reaction with crotyl bromide, chlorotrimethylsilane and sodium iodide [34]. This reaction is performed under ultrasonic activation to afford after seven hours the desired compound 16 in quantitative yield. 16 was then converted to the N3-Boc thymine derivative 17 in quantitative yield, (Scheme 3) [35].

With all partners in hand (16, 17 and 11–15), we turned our attention to the olefin CM reaction using either the 2nd generation Grubbs catalyst [36] (G-II), the more reactive Hoveyda–Grubbs (HG-II) catalyst [37] or its derivative, the Zhan catalyst-1B, (Table 1).

This specific CM reaction with a phosphonoamidate is challenging and needs optimization since, as stated previously, it is well
established in the literature that compounds containing basic nitrogen atoms can poison the CM ruthenium catalysts and are thus problematic substrates for olefin metathesis. It was shown that the presence of electron withdrawing groups next to the nitrogen decreases the electron density and the deactivation of the catalyst can be attenuated as well as the use of microwave irradiations [38]. It was shown also that the presence of Lewis acid or Cu(I) salt can improved the yields of RCM of amino acids [39]. The influence of ultrasonication was also tested. For this optimization, we used the allylphosphonoamidate 11 and the crotylthymine 16 or 17, taken as model.

When N1 alkylated thymines 16 or 17 were reacted in DCM with diastereomeric mixture of phosphonoamidate synthon 11 under classical heat activation (Δ) (entries 1–3), no desired compounds were found in all conditions tested (equivalents, substrate, Ru catalyst, co-catalyst (Lewis acid, entry thymines derivatives). The use of microwaves irradiation (MW) (entries 4, 5) and sonication (J)) (entries 6 to 10) failed also. Only the use of water on sonication led the expected phosphonoamidate ANP 18 in moderate 41% yield (entry 11) [40]. The modulation of the conditions in a sealed tube and with an addition of a surfactant (2.5% of polyoxyethanyl-tocopheryl sebacate) (entries 12 and 13) does not improve significantly the yield of the reaction [41].

Thanks to this breakthrough approach, some (E)-but-2-enyl C5-substituted thymidine phosphonoamidates 19 and 20 were obtained in yields ranging from 35% to 36% yield, respectively.

2.2. Biological evaluation

Among all the tested compounds, bearing different biolabile group (POM, POC, HDP, phosphonoamidate), the diastereomeric single phosphonoamidate forms 19 and 20 were the most potent and selective against both wild-type (TK⁺) and thymidine kinase deficient (TK⁻) varicella-zoster (VZV) strains with EC₅₀ (50% effective concentration) values in the range of 0.3–0.6 μM (Table 2). The cytostatic activity (CC₅₀) decreased by a factor 2 when comparing compound 19 with the bis-(POM)-(E)-TbutP (1) resulting in a selectivity index (SI, ratio CC₅₀ to EC₅₀) superior to 200. The selectivity of 20 against VZV was about half of that calculated for 19. These prodrugs showed also activity against herpes simplex virus 1 (HSV-1), TK⁻/HSV-1 and herpes simplex virus 2 (HSV-2) strains (EC₅₀ in the range of 3–12 μM), which was comparable to the EC₅₀’s obtained for cidofovir (Table 3). The diastereomeric single phosphonoamidate forms 19 and 20 had weak activity against human cytomegalovirus (HCMV) or no activity at the higher concentration tested (100 μM) against vaccinia virus and adenovirus.

The (E)-but-2-enyl C5-substituted pyrimidine phosphonoamidates 21 and 22 inhibited VZV replication with EC₅₀’s in the range of 1–8 μM and did not affect cell growth or morphology at the highest tested concentration (100 μM). In contrast to 22, compound 21 had some anti-HSV activity while both were able to reduce HCMV.
multiplication.

The introduction of these biolabile prodrugs revealed the potential of our ANPs to inhibit HCMV replication (EC50's in the range of 13–70 μM for compounds 19, 20, 21, 22), hitherto undetected under other pronucleotide forms. Several hypotheses can support these results, as a better bioavailability of these molecules under the phosphonoamidate form compared to the other prodrug forms. The activity can also be increased by a better half-life and less toxic side-products. However, the newly synthesized prodrugs did not showed activity against vaccinia virus in contrast to bis-(POM)-(E)-TbutP (1).

The phosphonoamidate ANPs 23 and 24 were not active against various viral strains; only the chlorine analog 24 shown a moderate activity against human coronavirus (EC50 8.9 μM).

The compounds were also evaluated against different RNA viruses, but no activity was found.

3. Conclusion

We have described herein the synthesis of (E)-but-2-enyl nucleoside phosphonoamidates using the cross-metathesis in water-under ultrasound irradiation. The overall yield obtained from commercial dimethylallylphosphonate is >15%, well above the datas reported in the literature for the preparation of phosphonoamidates (~3%). Two diastereoisomers were formally identified by X-ray diffraction. All those compounds were evaluated against various DNA viruses for their antiviral properties. Among them, the thymine analogue 19 showed to be the best prodrug tested against VZV with an EC50 = 0.33–0.39 μM and a selectivity index increased up to >200, compared to its other prodrugs 1–4. This breakthrough approach paves the way for new purine and pyrimidine (E)-but-2-enyl phosphonoamidates.

4. Experimental section

4.1. Chemistry

General.

Commercially available chemicals were of reagent grade and used as received. All reactions requiring anhydrous conditions were carried out using oven-dried glassware and under an atmosphere of dry Ar or N2. All reactions under microwave irradiation were performed using the Microwave Biotage Initiator in 2–5 mL sealed tubes. The reactions under ultrasound were carried out with Elmasonic P30H apparatus with a frequency of 80 kHz and effective

| Entry | Solvent | Equivalents (of 11 and nucleobase) | Catalyst | Activation | R1 | Yield |
|-------|---------|---------------------------------|----------|------------|----|-------|
| 1     | DCM     | 1–2                             | HG-II    | Δ, 50 °C, 24h | H  | / |
| 2     | DCM     | 1.3–1                           | HG-II    | Δ, 50 °C, 24h | H  | / |
| 3     | DCM     | 1–1                             | HG-II, Cy2BCl | Δ, 50 °C, 24h | Boc | / |
| 4     | DCM     | 1.3–1                           | G-II     | MW, 100 °C, 1h | H  | / |
| 5     | DCM     | 1.3–1                           | G-II, Cul| MW, 100 °C, 1h | H  | / |
| 6     | DCM     | 1.3–1                           | HG-II    | ⌜, 55 °C, 20h | H  | / |
| 7     | DCM     | 1.3–1                           | G-II, Cul| ⌜, 55 °C, 20h | H  | / |
| 8     | DCM     | 1.3–1                           | G-II, BCl2,SMc2 | ⌜, 55 °C, 20h | H  | / |
| 9     | DCM     | 1.3–1                           | HG-II    | ⌜, 55 °C, 20h | Boc | / |
| 10    | DCM     | 1–2                             | Zhan 1B  | ⌜, 55 °C, 20h | H  | 41% |
| 11    | H2O     | 1–2                             | G′II     | ⌜, 55 °C, 20h | H  | 40% |
| 12    | H2O     | 1–2                             | G′II     | ⌜, 55 °C, 20h | H  | 35% |

Bold character represent in the table the best yield obtained in water.

\(^a\) 2.5% of Polyoxyethanyl-α-tocopheryl Sebacate PTS.

\(^b\) Sealed tube.

\(^c\) Catalyst introduced in 3 × 6 mol%.

Table 1
Cross-metathesis optimization.
power of 100 W. The reactions were monitored by thin layer chromatography (TLC) analysis using silica gel plates (Kieselgel 60F254, E. Merck). Column chromatography was performed on Silica Gel 60 M (0.040–0.063 mm, E. Merck). The 1H and 13C NMR spectra were recorded on Bruker Avance DPX 250 or Bruker Avance 400 Spectrometers using deuterated solvents as internal standard. Chemical shifts are given in ppm and multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quartet), bs (broad signal) and m (multiplet). High Resolution Mass spectra were performed on a Bruker Q-TOF MaXis mass spectrometer by the “Fédération de Recherche” ICO/CBM (FR2708) platform. LC-MS data was acquired on a Thermo-Fisher UHPLC-MSQ system equipped with an electron spray ionization source (ESI), in positive mode. After 20h, three compounds were observed the allylphosphonoamidate 11, ([M + H]+, 360), its homodimer form ([M + H]+, 690), and phosphonoamidate ANP 18 product ([M + H]+, 498). Electronic extraction of ions was performed and the subsequent areas under the corresponding chromatographic peaks determined. The conversion yield was determined as the ratio of the concentration of the allylphosphonooamide 11 transformed to its initial concentration.
4.11. Dimethyl allylyphosphonate (8)

Under inert atmosphere, allyl bromide (51 mL, 1.25 eq., 0.57 mol) was dissolved in THF (400 mL). To this mixture potassium carbonate (94 g, 1.5 eq., 0.68 mol), tert-butylammonium bromide (2.9 g, 2 mol%, 9.1 mmol) and finally dimethylphosphite (7 (412 mL, 1 eq., 0.45 mol) were added. The resulting solution was stirred for 36 h at room temperature, followed by the filtration of all solids present in the flask. The filtrate was then evaporated under reduced pressure, and the crude product was then distilled at 130 °C under 40 mm/Hg. After collection of the different fractions, the clean product 8 was obtained as a colorless liquid. (53 g, 75%). 1H NMR (250 MHz, CDCl3): δ 5.80 (m, 1H, CH=CH2), 5.23 (m, 2H, CH2=CH2), 3.77 (s, 3H, OMe), 3.72 (s, 3H, OMe), 2.62 (ddt, J = 22.0, 7.4, 1.3 Hz, 2H, CH2-P). CAS #: 757-54-0.

4.1.3. Phenylallylphosphonic acid (10)

To a mixture of dimethylallylphosphonate 8 (4.9 g, 1 eq., 32.5 mmol) and DCM (150 mL), oxalyl chloride (8.6 mL, 3 eq., 53.16 mmol) was added slowly at 0 °C, followed by the removal of the volatiles in vacuo to obtain the methyl allylphosphonochloridate. In another flask, a solution of phenol (6.12 g, 2 eq., 65 mmol), triethylamine (8.8 mL, 2 eq., 65 mmol) and DCM (200 mL) was stirred at room temperature. The phosphonate residue was then dissolved in DCM (0.2 M), and slowly added to this solution, and refluxed during 48h. After evaporation of all volatiles, the residue was purified by silica gel column chromatography, eluting Petroleum ether/Ethyl ether 9/1, to afford solution A. A second solution was prepared with aL-alanine ester chlorhydrate (1.2 eq.), freshly distilled triethylamine (8 eq.) and DCM. To this mixture, the solution A was slowly added at 0 °C, and stirred 24h at room temperature. For 1 g of starting phosphonate, the solution was successively washed with 10 mL of water, 10 mL NaOH 1 M, 10 mL water, 10 mL HCI 10%, 10 mL NaHCO3, and 10 mL water. After this work-up, the aqueous phase was extracted with 4 × 10 mL with ethyl acetate, the organic phases were washed with brine (20 mL), dried over MgSO4, filtered and evaporated. The residue was purified twice by flash column chromatography to afford the desired allylphosphonoamidates 11, 14 and 15 as diastereomeric mixture. Diastereomers of compound 11 were separated as single diastereomers 12 and 13, respectively.

4.2.1. Benzyl 2-[(S)-allylphosphoryl]amino] propanoate (11)

Titled compound was obtained following general procedure starting from compound 10 (1 g, 1 eq., 5.05 mmol). The residue was purified twice by flash column chromatography, first eluting PE/EA (7/3) and then pentane/diethyl ether (55/45); the 6:4 diastereomeric mixture of 11 was obtained as a white powder (114 g, 63%). Each diastereoisomers were crystallized from a toluene/pentane mixture, as colorless needles, respectively, and their structures were established by X-ray.

Diastereoisomer 11a: Benzyl 2-[(S)-(R)-allylphosphoryl]amino] propanoate (12): 1H NMR (400 MHz, CD2D2CO) δ 7.37 (m, 7H, Aromatic H), 7.24 (m, 2H, Aromatic H), 7.14 (t, J = 7.4 Hz, 1H, Aromatic H), 5.89 (m, 1H, CH=CH2), 5.18 (m, 4H, CH2=CH2, CH2-O), 4.60 (t, J = 11.2 Hz, 1H, NH), 4.16 (m, 1H, CH-NH), 2.76 (d(d, J = 20.7, 7.1, 2.5 Hz, 2H, CH2-P), 1.22 (d, J = 7.2 Hz, 3H, CH3). 13C NMR (101 MHz, CD2D2CO) δ 174.42 (d, J = 4.8 Hz, C=O), 151.94 (d, J = 9.1 Hz), 137.12, 130.19 (Aromatic C), 129.46 (d, J = 11.0 Hz, CH=CH2), 129.28 (Aromatic C), 128.92 (Aromatic C), 128.88 (Aromatic C), 125.09 (d, J = 1.1 Hz, Aromatic C), 121.82 (d, J = 4.6 Hz, Aromatic C), 119.86 (d, J = 14.3 Hz, CH2=CH2, 67.09 (CH2-O), 50.47 (CH-N), 35.03 (d, J = 128.7 Hz, CH2-P), 20.88 (d, J = 4.8 Hz, CH3). 31P NMR (162 MHz, CD2D2CO) δ 26.70. HRMS (ESI): m/z [M+H]+ calcd for CI9H23O3P: 316.0365, found: 316.0365.

Diastereoisomer 11b: Benzyl 2-[(S)-(S)-allylphosphoryl]amino] propanoate (13): 1H NMR (400 MHz, CD2D2CO) δ 7.19 (m, 10H, Aromatic H), 5.79 (m, 1H, CH=CH2), 5.17 (m, 2H, CH2=CH2), 5.04 (s, 2H, CH2-O), 4.06 (m, 1H, CH-NH), 3.49 (t, J = 10.6 Hz, 1H, NH), 2.69 (dd, J = 21.5, 7.4 Hz, 2H, CH2-P), 1.27 (d, J = 7.1 Hz, 3H, CH3). 13C NMR (101 MHz, CD2D2CO) δ 174.20 (d, J = 5.3 Hz, C=O), 150.52 (d, J = 9.0 Hz), 135.25, 129.65 (Aromatic C), 128.62 (Aromatic C), 128.48 (Aromatic C), 128.21 (Aromatic C), 127.45 (d, J = 11.2 Hz, CH=CH2), 124.67 (Aromatic C), 120.60 (d, J = 14.3 Hz, CH2=CH2), 120.56 (d, J = 4.7 Hz, Aromatic C), 67.12 (CH2-O), 49.61 (CH-NH), 34.25 (d, J = 128.8 Hz, CH2-P), 21.57 (d, J = 4.3 Hz, CH3). 31P NMR (162 MHz, CD2D2CO) δ 26.70. HRMS (ESI): m/z [M+H]+ calcd for C19H23O3P: 316.0365, found: 316.0354.

4.2.2. Methyl 2-[(S)-allylphosphoryl]amino] propanoate (14)

Titled compound was obtained following general procedure 1, starting from compound 10 (1.3 g, 1 eq., 6.56 mmol). The obtained residue was purified twice by flash column chromatography, eluting PE/EA (55:45 to 5:5) to afford a non-separable 6:4 mixture of diastereoisomers 14 as a colorless oil, (860 mg, 52%). 1H NMR (400 MHz, CD2D2CO) δ 7.30 (m, 2H, Aromatic H), 7.18 (m, 3H, Aromatic H), 5.87 (m, 1H, CH=CH2), 5.27 (m, 2H, CH2=CH2), 4.08 (m, 1H, CH-NH), 3.66 (d, J = 10.6 Hz, 3H, OMe), 3.32 (2 × t, J = 10.1 Hz, 1H, NH), 2.77 (m, 2H, CH2-P), 1.28 (2 × d, J = 7.2 Hz, 3H, CH3). 13C NMR (101 MHz, CD2D2CO) δ 174.53 (d, J = 6.1 Hz, C=O), 174.20 (d, J = 5.3 Hz, C=O), 150.53 (d, J = 9.5 Hz), 150.45 (d, J = 9.9 Hz), 129.65 (Aromatic C), 129.60 (Aromatic C), 127.68 (d, J = 11.4 Hz, CH=CH2), 127.52 (d, J = 11.2 Hz, CH=CH2), 124.72 (d, J = 12 Hz, Aromatic C), 124.68 (d, J = 1.1 Hz, Aromatic C), 120.70 (d, J = 4.8 Hz, Aromatic C), 120.60 (d,
4.3.1. Benzyl 2-[(S)-[(R)-[(E)-4-(thymin-1-yl)but-2-enyl]-
phosphonoamidates

Titled compound was obtained following general procedure, starting from phosphonoamide 13 (125 mg, 1 eq., 0.35 mmol) and crotylthymine (125 mg, 2 eq., 0.70 mmol). The obtained residue was purified by twice flash chromatography (DMC/MeOH 95/5 and toluene/acetone 6/4), to afford 21 as a white solid (70 mg, 41%). 1H NMR (400 MHz, CDCl3) δ 8.79 (bs, 1H, NH), 7.27 (m, 7H, Aromatic H), 7.12 (m, 3H, Aromatic H), 6.94 (d, J = 1.1 Hz, 1H), 5.67 (m, 2H, H2', H3'), 5.07 (s, 2H), 4.23 (t, J = 4.9 Hz, 2H, CH2-O), 4.03 (dq, J = 9.6, 7.6 Hz, 1H, CH-NH), 3.38 (d, J = 10.3 Hz, 1H, NH), 2.70 (dt, J = 20.3, 5.7 Hz, 2H), 1.84 (d, J = 1.1 Hz, 3H), 1.27 (s, 3H). 13C NMR (101 MHz, CDCl3) δ 173.46 (d, J = 5.7 Hz, C-O), 150.58 (d, J = 9.5 Hz, 1H), 150.45 (d, J = 9.9 Hz, 1H), 129.65 (Aromatic C), 129.59 (Aromatic C), 127.71 (d, J = 11.4 Hz, C2'), 127.53 (d, J = 11.4 Hz, C2'), 124.69 (Aromatic C), 124.65 (Aromatic C), 120.75 (C3' Aromatic C), 120.70 (C2' Aromatic C). 31P NMR (162 MHz, CDCl3) δ 39.73 (s, 1P). HRMS (ESI) m/z [M+H]+ calcd for C25H26N3O6P: 497.1842; found: 497.1840.

4.3.2. Benzyl 2-[(S)-[(S)-[(E)-4-(thymin-1-yl)but-2-enyl]-
phenoxyphosphoryl][amino]propanoate (20)

Titled compound was obtained following general procedure, starting from phosphonoamide 13 (125 mg, 1 eq., 0.35 mmol) and crotylthymine (125 mg, 2 eq., 0.70 mmol). The obtained residue was purified by twice flash chromatography (DMC/MeOH 95/5 and toluene/acetone 6/4), to afford 21 as a white solid (70 mg, 41%). 1H NMR (400 MHz, CDCl3) δ 8.79 (bs, 1H, NH), 7.27 (m, 7H, Aromatic H), 7.12 (m, 3H, Aromatic H), 6.94 (d, J = 1.1 Hz, 1H), 5.67 (m, 2H, H2', H3'), 5.07 (s, 2H), 4.23 (t, J = 4.9 Hz, 2H, CH2-O), 4.03 (dq, J = 9.6, 7.6 Hz, 1H, CH-NH), 3.38 (d, J = 10.3 Hz, 1H, NH), 2.70 (dt, J = 20.3, 5.7 Hz, 2H), 1.84 (d, J = 1.1 Hz, 3H), 1.27 (s, 3H). 13C NMR (101 MHz, CDCl3) δ 173.46 (d, J = 5.7 Hz, C-O), 150.95 (C-O), 150.60 (d, J = 9.1 Hz, 1H), 135.19, 129.74 (Aromatic C), 129.17 (d, J = 14.4 Hz, C2'), 128.86 (Aromatic C), 128.56 (Aromatic C), 128.25 (Aromatic C), 125.08 (d, J = 11.1 Hz, C3'), 124.54 (Aromatic C), 123.37 (d, J = 4.9 Hz, Aromatic C), 111.00 (C5), 67.25 (C2'-O), 49.67 (CH-NH), 49.17 (d, J = 2.2 Hz, C1'), 32.73 (d, J = 128.2 Hz, 1H), 24.16 (d, J = 4.3 Hz, CH3-CH), 12.27 (CH3-C), 31P NMR (162 MHz, CDCl3) δ 25.60. HRMS (ESI) m/z [M+H]+ calcd for C25H29N3O6P: 498.1794; found: 498.1789.

4.3.3. Methyl 2-[(S)-[(E)-4-(thymin-1-yl)but-2-enyl]-
phenoxyphosphoryl][amino]propanoate (21)

Titled compound was obtained following general procedure, starting from phosphonoamide 14 (50 mg, 1 eq., 0.18 mmol) and crotylthymine (64 mg, 2 eq., 0.35 mmol). The obtained residue was purified by twice flash chromatography (DMC/MeOH 95/5 and toluene/acetone 6/4), to afford 21 as a white solid (30 mg, 41%). 1H NMR (400 MHz, CDCl3) δ 8.06 (bs, 1H, NH), 7.27 (m, 2H, Aromatic H), 7.15 (m, 3H, Aromatic H), 6.97 (bs, 1H, H6), 5.73 (m, 2H, CH=CH), 4.28 (t, J = 4.9 Hz, 2H, CH2-N), 4.00 (m, 1H, CH-NH), 3.72 – 3.59 (m, 3H), 3.42 (m, 1H, CH-NH), 2.74 (dd, J = 20.9, 6.8 Hz, 2H, CH2-P), 1.88 (s, 3H, CH3 thym), 1.26 (m, 3H, CH3-CH3). 31P NMR (162 MHz, CDCl3) δ 26.01, 25.51. HRMS (ESI): m/z [M+H]+ calcd for C19H25N3O6P: 422.1481 found: 422.1480.

4.3.4. Isopropyl 2-[(S)-[(E)-4-(thymin-1-yl)but-2-enyl]-
phenoxyphosphoryl][amino]propanoate (22)

Titled compound was obtained following general procedure, starting from phosphonoamide 15 (50 mg, 1 eq., 0.16 mmol) and crotylthymine (58 mg, 2 eq., 0.32 mmol). The obtained residue was purified by twice flash chromatography (DMC/MeOH 95/5 and toluene/acetone 6/4), to afford 22 as a white solid (32 mg, 44%). 1H NMR (400 MHz, CDCl3) δ 8.38 (bs, 1H, NH), 7.30 (m, 2H, Aromatic H), 7.16 (m, 3H, Aromatic H), 7.00 (s, 1H, H6), 5.75 (m, 2H, CH=CH), 4.96 (d, sept., J = 6.3, 2.1 Hz, 1H, CH-IPr), 4.31 (t, J = 4.7 Hz, 1H, H1'), 3.99 (m, 1H, CH-NH), 3.45 (2 × t, J = 10.8 Hz, 1H, CH2), 2.77 (m, 2H, CH2-P), 1.88 (s, 3H, CH3-C), 1.26 (m, 3H, CH3-CH3). 13C NMR (101 MHz, CDCl3) δ 173.17 (C=O), 163.76 (C=O), 150.60 (C=O), 150.54, 139.69, 139.59 (C6), 129.74 (Aromatic C), 129.67 (Aromatic C), 129.12 (d, J = 14.0 Hz, C2'), 125.20 (d, J = 11.1 Hz, C3'), 124.85 (Aromatic C), 120.65 (Aromatic C), 120.61 (Aromatic C), 120.44 (Aromatic C), 120.40 (Aromatic C), 111.03 (CH-IPr), 68.09 (CH IPr), 67.07 (CHPr), 49.77 (CH-NH), 49.64 (CH-NH), 49.19 (CH2-O), 32.70 (d, J = 129.4 Hz, CH2-P), 21.68 (CH3), 21.60 (CH3), 21.58 (CH3), 21.54 (CH3), 21.57 (CH3-C). 31P NMR (162 MHz, CDCl3) δ 26.02, 25.61. HRMS (ESI) m/z [M+H]+ calcd for C21H29N3O6P: 450.1795; found: 450.1789.
4.3.6. Benzyl 2-[(S)-{(E)-4-(5-fluorouridin-1-yl)but-2-enyl]-phenoxypophosphoryl]amino] propanoate (24)

Titled compound was obtained following general procedure 2, starting from phosphonoamide 13* (50 mg 1 eq., 0.14 mmol) and N1-crotyl-5-chlorouracile (56 mg, 2 eq., 0.28 mmol). The obtained residue was purified by twice flash chromatography (DCM/MeOH 95/5 and toluene/acetone 6/4), to afford compound 24* as a white solid (33 mg, 35%). 1H NMR (400 MHz, CDCl3) δ 8.52 (d, J = 4.3 Hz, aromatic C), 7.76 (s, 1H, CH2-P), 2.13 (d, J = 4.6 Hz, minor CH3), 2.13 (d, J = 4.6 Hz, major CH3). 13C NMR (101 MHz, CDCl3) δ 173.49 (d, J = 128.8 Hz, CH2-P), 128.28 (Aromatic C), 126.50 (d, J = 10.1 Hz, major CH2), 124.94 (Aromatic C), 120.66 (d, J = 4.6 Hz, minor Aromatic C), 120.38 (d, J = 4.6 Hz, major Aromatic C), 67.30 (CH2-O), 49.73 (CH-NH), 49.19 (CH2-O), 32.51 (d, J = 128.8 Hz, CH2-P), 21.36 (d, J = 4.6 Hz, major CH3), 21.28 (d, J = 4.6 Hz, minor CH3). 31P NMR (162 MHz, CDCl3) δ 173.49 (d, J = 5.3 Hz, C=O), 159.14 (C=O), 150.38 (C=O), 150.27 (d, J = 9.1 Hz, minor C), 149.75 (d, J = 9.1 Hz, major C), 140.57, 135.21 (C6), 129.79 (Aromatic C), 129.72 (Aromatic C), 128.68 (Aromatic C), 128.62 (d, J = 4.7 Hz, Aromatic C), 128.49 (Aromatic C), 128.30 (d, J = 13.9 Hz, C37’), 128.28 (Aromatic C), 128.18 (Aromatic C), 126.50 (d, J = 10.1 Hz, minor C2’), 126.28 (d, J = 10.1 Hz, major C2’), 124.93 (Aromatic C), 120.66 (d, J = 4.6 Hz, minor Aromatic C), 120.38 (d, J = 4.6 Hz, major Aromatic C), 67.30 (CH2-O), 49.73 (CH-NH), 49.19 (CH2-O), 32.51 (d, J = 128.8 Hz, CH2-P), 21.36 (d, J = 4.6 Hz, major CH3), 21.28 (d, J = 4.6 Hz, minor CH3). 31P NMR (162 MHz, CDCl3) δ 173.49 (d, J = 5.3 Hz, C=O), 159.14 (C=O), 150.38 (C=O), 150.27 (d, J = 9.1 Hz, minor C), 149.75 (d, J = 9.1 Hz, major C), 140.57, 135.21 (C6), 129.79 (Aromatic C), 129.72 (Aromatic C), 128.68 (Aromatic C), 128.62 (d, J = 4.7 Hz, Aromatic C), 128.49 (Aromatic C), 128.30 (d, J = 13.9 Hz, C37’), 128.28 (Aromatic C), 128.18 (Aromatic C), 126.50 (d, J = 10.1 Hz, minor C2’), 126.28 (d, J = 10.1 Hz, major C2’), 124.93 (Aromatic C), 120.66 (d, J = 4.6 Hz, minor Aromatic C), 120.38 (d, J = 4.6 Hz, major Aromatic C), 67.30 (CH2-O), 49.73 (CH-NH), 49.19 (CH2-O), 32.51 (d, J = 128.8 Hz, CH2-P), 21.36 (d, J = 4.6 Hz, major CH3), 21.28 (d, J = 4.6 Hz, minor CH3).

Acknowledgment

MB is grateful to MESR for a PhD scholarship. We thank the LABEX SynOrg (ANR-11-LABX-0029) for VH PhD fellowship and partial financial support.
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