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Anti-cancer ProTides: tuning the activity of BVDU phosphoramidates related to thymectacin

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Abstract—Based on our wide ranging knowledge of phosphoramidate ProTides as anti-viral agents we have tuned the lead anti-cancer agent thymectacin in the ester and amino acid regions and revealed a substantial enhancement in in vitro potency versus colon and prostate cancer cell lines. Twelve analogues have been reported, with yields of 29–78%. The compounds are fully characterised and data clearly reveal the presence of two phosphate diastereoisomers, as expected, in roughly equi-molar proportions. The compounds were evaluated in tissue culture versus three different tumour cell lines, using thymectacin as the control. It is notable that minor structural modification of the parent phenyl methoxyalaninyl structure of thymectacin leads to significant enhancements in potency. In particular, replacement of the methyl ester moiety in the lead by a benzyl ester gave a 175-fold boost in potency versus colon cancer HT115. This derivative emerges as a low micromolar inhibitor of HT115 cells and a new lead for further optimisation.

1. Introduction

All chemotherapeutic nucleoside analogues, for viral infections and cancer, exert their effect only after metabolic activation in the target cell to their 5′-phosphate (nucleotide) forms. Often the efficiency of intracellular phosphorylation of nucleoside analogues can limit their therapeutic potential, but the pre-formed phosphates are of limited utility on account of their poor membrane permeation. This has lead to a wide variety of phosphate pro-drug approaches,[1] known collectively as 'ProTide' methods.

One such approach which we introduced in 1992 is based on aryloxy phosphoramidates,[2] of general structure (**1**) (Fig. 1).

The application of our phosphoramidate approach was highly successful with the anti-HIV agent d4T, the phenyl methoxyalanyl phosphate (**2**) being 10-fold more potent than the parent agent versus HIV in vitro.[3] Moreover (**2**) retained full activity in thymidine kinase deficient cells, providing compelling evidence of its action via intracellular nucleoside monophosphate release.[4]

Subsequent applications of this technology by both our lab and others demonstrated significant potency boosts on phosphoramidate formation for a wide range of nucleosides and also phosphonates: >25-fold for 8-aza-isoddA,[5] ca. 400-fold for iso-ddA,[5] ca. 2000-fold for d4A,[6] >100-fold for ddA,[7] 10–20-fold for adenallene[8] and 30–100-fold for the phosphonates PMPA and PMEA.[9,10]

However, the approach did not appear to be successful in every case. The phosphoramidates derived from acycloguanosine (**3**) were found to be less active than the parent agent versus HSV-2, and only slightly more active versus HCMV.[11]

Application of our technology to the anti-VZV agent BVDU (brivudin) (**4**) has proved to be more intriguing. Thus, we reported that phosphoramidates, such as (**5a**)

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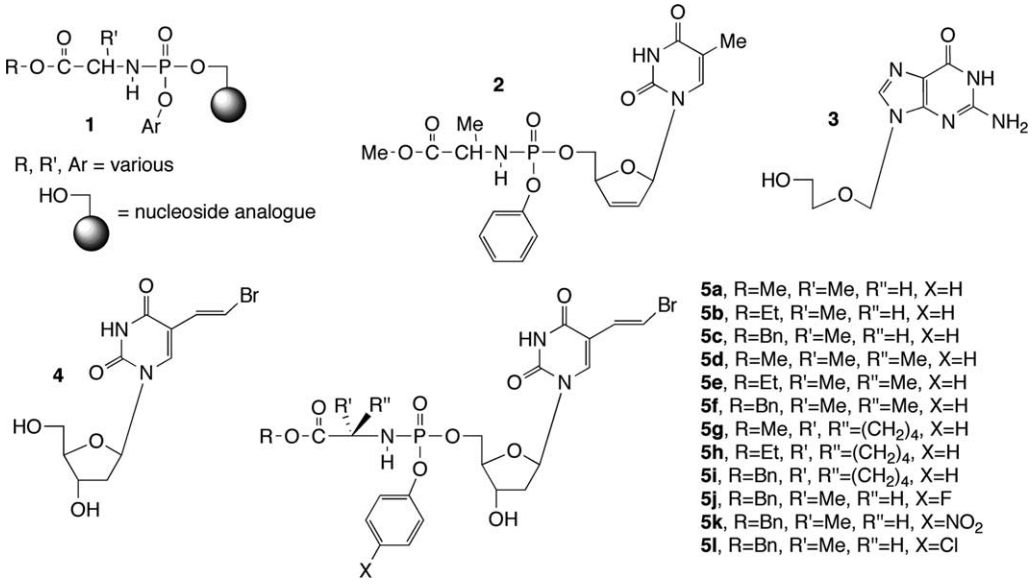


Figure 1. Some nucleosides and nucleotides.

derived from BVDU were 5–25-fold *less* potent than the parent agent versus VZV in tissue culture.[12] This was interpreted as corresponding to poor intracellular delivery of BVDU monophosphate (BVDUMP), rapid degradation to BVDU or poor onward phosphorylation of BVDUMP to the bio-active triphosphate.[12] However, at about the same time the NewBiotics group had independently prepared (**5a**) and found it was a potent and effective anti-cancer agent.[13] Further studies on (**5a**) revealed that it to be selectively toxic to tumour cells expressing elevated levels of thymidylate synthase (TS), a key enzyme in DNA synthesis.[14] Compound (**5a**) entered clinical evaluation against colon cancer, with only phase I data emerging to date and indicating the compound to be well tolerated.[15]

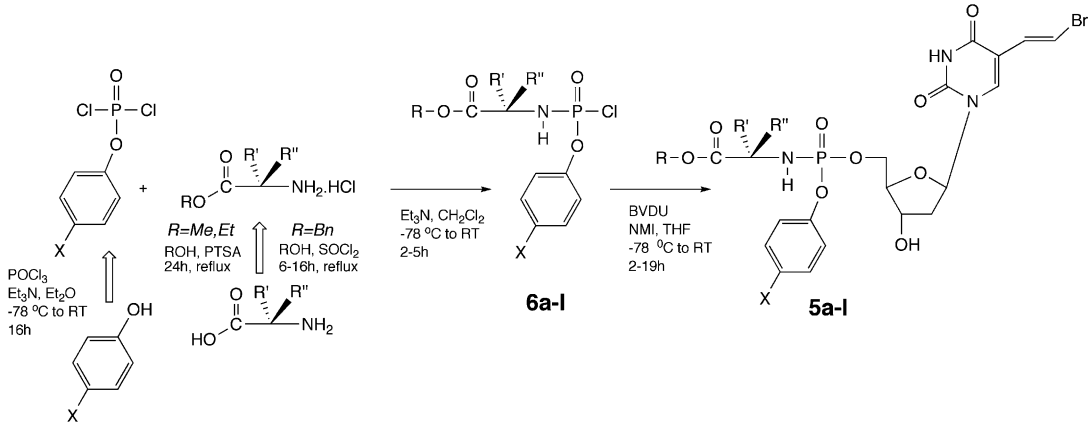
These data are intriguing on account of the detrimental effect of phosphoramidate formation on anti-VZV potency, implying poor phosphate delivery. Indeed, recent data from NewBiotics[16] indicates that, unusually, cellular protein is the eventual target for (**5a**) and not DNA as might have been expected.

In order to further pursue this intriguing situation, and given our experience of widespread phosphoramidate modification, we sought to prepare a small family of analogues of (**5a**) with modification in the aryl, ester and amino acid regions, and to evaluate them against a small panel of tumour cell lines. We herein report the first data from this project.

2. Chemistry

The target phosphoramidates were all prepared in one step from BVDU (**4**) using the phosphorochloridate chemistry we have extensively described (Scheme 1).[17,18]

Thus, according to Scheme 1 the appropriate aryl phosphorochloridates was reacted with the requisite amino



Scheme 1. Synthetic route to target phosphoramidates.

acid ester hydrochloride to give the phosphorochloridates (**6**) as key synthons. These were used as crude oils without further purification. The reagents (**6**) were allowed to react with (**4**) in THF in the presence of N-methylimidazole (NMI) at low temperature, rising to room temperature over 18 h. Evaporation, extraction and flash column chromatography on silica yielded the desired compounds (**5a–l**) in yields of 29–78%. In every case the products were isolated as mixtures of diastereoisomers corresponding to roughly 50:50 mixed stereochemistry at the phosphate centre. These isomers do not easily separate by column chromatography, but are readily distinguished by P-31 NMR (e.g., **5a**, δ_P 4.72, 4.40). The compounds were further characterised by H-1 and C-13 NMR. The presence of phosphate diastereoisomers was also apparent in the H-1 and, particularly C-13 data where several peaks were 'split' in a 1:1 ratio due to the diastereoisomeric mixture. Common examples include the H-6 signal (δca. 7.6 and the C-2′ signal (δca. 42). Phosphorus-31 coupling was also noted in the C-13 NMR to several of the carbon atoms within three bonds of the phosphate.

3. Results and discussion

The phosphoramidates **5a–l** were evaluated for their anti-cancer effect against a panel of three tumour cell lines in vitro. These were breast MDA MB231, colon (HT115) and prostate (PC-3) tumour cells. Data are presented in Table 1.

In our hands the lead clinical candidate thymectacin (**5a**) is only moderately active in vitro, with EC_{50} values of 79–245 μM in our assays. However, relatively modest tuning of the structure reveals a significant boost in potency. For example, replacement of the methyl ester (**5a**) by a benzyl ester (**5c**) gave a 2-fold boost in potency versus breast, on 8-fold boost versus prostate and a 175-fold boost in potency versus the colon cancer cell line. Use of the alternative, often highly effective, amino acid moieties α,α-dimethylglycine (**5d–f**) and cyclopentyl glycine (**5g–i**) did not yield any further improvement in potency. However, the *para*-chloro phenyl analogue of alanine (**5l**) did show increased potency versus the breast cancer cell line. Further assays are underway to probe the full biological and therapeutic potential of the derivatives herein described. However, it does appear that the biological profile of parent phosphoramidates may be tuned on a nucleoside by nucleoside basis to enhance potency and perhaps selectivity for particular cellular and/or tumour targets.

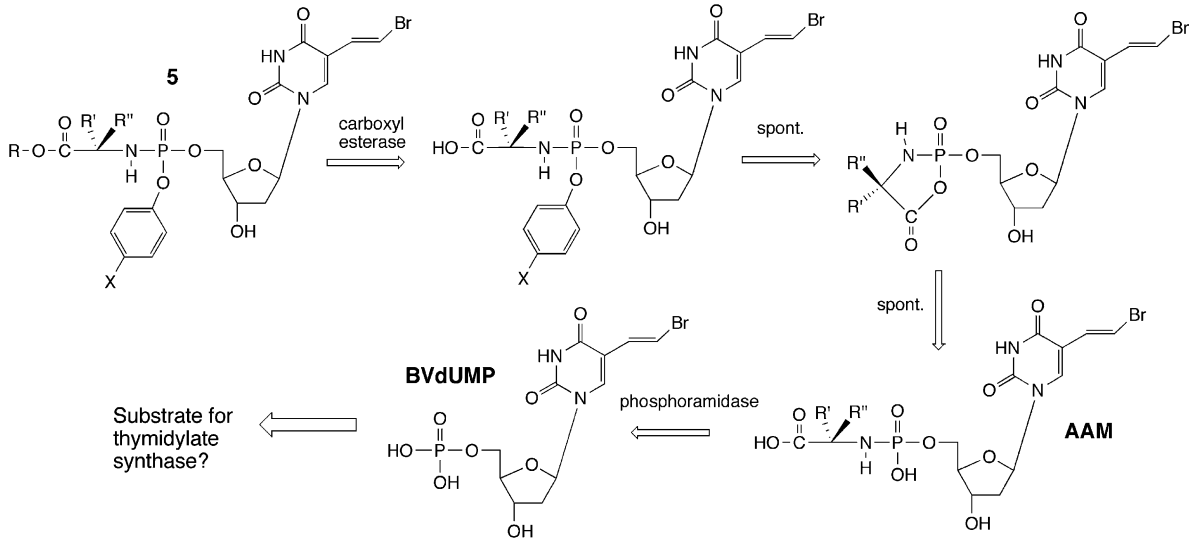
The proposed mechanism of action of the ProTides (**5**) is outlined in Scheme 2. Thus, following esterase-mediated carboxyl ester hydrolysis, a spontaneous cyclisation, loss of phenol and ring opening would yield the putative amino acid metabolite (AAM).[17,18] Structures of the latter general type are known to arise from phosphoramidates of a range of nucleosides on exposure to carboxyl esterase[18] and in tissue culture. Moreover, the replacement of a methyl by a benzyl ester leads to an enhancement in the anti-viral potency of dideoxynucleoside phosphoramidates and to more rapid esterase-mediated hydrolysis.[19] Whether this is the origin of the enhanced activity we note for **5c** over **5a** is currently unclear. However, it is also notable that **5c** is estimated to be roughly 50-fold more lipophilic than **5a** (*C*log *P*: **5a** 0.11, **5c** 1.82, Chemdraw 7.0), which may lead to more rapid membrane permeation for **5c**.

4. Conclusions

We have successfully applied the phosphoramidate based ProTide methodology to the preparation of a series of analogues of thymectacin, the BVDU-derived phosphoramidate of NewBiotics. Twelve analogues have been reported, with yields of 29–78%. The compounds are fully characterised and data clearly reveal the presence of two phosphate diastereoisomers, as expected, in roughly equi-molar proportions. The compounds were evaluated in tissue culture versus three different tumour cell lines, using thymectacin as the control. It is notable that minor structural modification of the parent phenyl methoxyalaninyl structure of thymectacin leads to significant enhancements in potency. In particular, replacement of the methyl ester moiety in the lead by a benzyl ester gave a 175-fold boost in potency versus colon cancer HT115. This derivative, **5c**, emerges as a low micromolar inhibitor of HT115 cells and a new lead for further optimisation.

Table 1. Anti-cancer effect of test compounds

Compound	Aryl	Ester	Amino	EC_{50}/μM		
				Breast MDAMB2.31	Colon HT115	Prostate PC-3
5a (Thymectacin)	Ph	Me	Ala	79	245	155
5b	Ph	Et	Ala	56	52	36
5c	Ph	Bn	Ala	34	1.4	19
5d	Ph	Me	Me_2Gly	41.1	77.9	1.5
5e	Ph	Et	Me_2Gly	218	39.7	76.1
5f	Ph	Bn	Me_2Gly	19	14.5	5.1
5g	Ph	Me	cPntGly	79	77	16
5h	Ph	Et	cPntGly	44	81.3	41
5i	Ph	Bn	cPntGly	78	9.7	33
5j	*p*-FPh	Bn	Ala	17	3.5	16
5k	*p*-NO_2Ph	Bn	Ala	28	ND	9
5l	*p*-ClPh	Bn	Ala	6.2	3.4	2.4



Scheme 2. Proposed mechanism of action of BVDU ProTides.

5. Experimental

Note on NMR multiplicity descriptions: standard descriptors are used (s, d, t, etc.) with a '2' prefix if a signal is split due to the presence of phosphate diastereomers, if this is clear from the signals.

5.1. Standard procedure: synthesis of phosphorodichloridate

Phosphorus oxychloride (1.0 mol equiv) and the appropriate substituted phenol (1.0 mol) were stirred with anhydrous diethylether (31 mol equiv). To this was added anhydrous triethylamine (1.0 mol equiv) at −78 °C and allowed to warm to room temperature over 16 h. The triethylamine hydrochloride salt was filtered off, and the filtrate reduced to dryness to give the crude product as a clear liquid.

5.2. Synthesis of *p*-chlorophenyl-phosphodichloridate

This was synthesised according to Standard procedure 5.1 using phosphorus oxychloride (1533 mg, 10.00 mmol, 932 μL), 4-chlorophenol (1.29 g, 10.03 mmol) and TEA (1.01 g, 9.98 mmol, 1394 μL) in ethylether (40 mL) to give an oil (1.90 g, 78%).

5.3. Synthesis of *p*-fluorophenyl-phosphodichloridate

This was synthesised according to Standard procedure 5.1, using phosphorus oxychloride (2.07 g, 13.50 mmol, 1259 μL), 4-fluorophenol (1.52 g, 13.56 mmol, 1884 μL) and TEA (1.37 g, 13.54 mmol, 1692 μL) in ethylether (40 mL) to give an oil (2.59 g, 84% yield). ^{31}P NMR ($CDCl_3$, 121 MHz): δ 5.49.

5.4. Standard procedure: synthesis of phosphochloridate

Phosphodichloridate (1.0 mol equiv) and the appropriate amino ester hydrochloric salt (1.0 mol equiv) were suspended in anhydrous DCM (123 mol equiv). Anhydrous triethylamine was added dropwise at −80 °C and after 1 h the reaction was left to rise to room temperature. The formation of phosphochloridate was monitored by ^{31}P NMR. After 2–5 h the solvent was removed under reduced pressure and the solid obtained washed with anhydrous ether (2 × 20 mL), filtered and the filtrate reduced to dryness to give the products as crude oil. These oils were usually used without further purification.

5.5. Synthesis of phenyl-(methoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine methyl ester hydrochloride (2.00 g, 14.3 mmol), phenyldichlorophosphate (3.02 g, 2.14 mL, 14.3 mmol) and TEA (2.9 g, 4.0 mL, 28.7 mmol) in DCM (60 mL), to yield 3.91 g (98%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.28, 8.97.

5.6. Synthesis of phenyl-(ethoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine ethyl ester hydrochloride (770 mg, 5.01 mmol), phenyldichlorophosphate (1.12 g, 5.01 mmol, 749 μL) and TEA (1.4 mL, 10.02 mmol) in DCM (40 mL). The crude was purified by flash chromatography (ethyl acetate/petroleum ether 7:3) affording 1.02 (69%) of oil. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.49, 9.07.

5.7. Synthesis of phenyl-(benzoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine benzyl ester hydrochloride (1.00 g, 4.64 mmol), phenyldichlorophosphate (980 mg, 0.69 mL, 4.64 mmol) and TEA (0.94 g, 1290 μL, 9.27 mmol) in DCM (40 mL). The crude was purified by flash chromatography (ethyl acetate/petroleum ether 6:4) affording 1.61 (98%) of oil. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.41, 9.23.

5.8. Synthesis of phenyl-(methyl-2-amino-2-methylpropanoate)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using 2-aminoisobutyrate methyl ester hydrochloride (583.5 mg, 3.75 mmol), phenyl dichlorophosphate (791.1 mg, 3.75, 560 μL) and TEA (759 mg, 7.5 mmol, 1045 μL) in DCM (20 mL), to yield 1.04 g (95%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 6.99.

5.9. Synthesis of phenyl-(ethyl-2-amino-2-methylpropanoate)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using 2-aminoisobutyrate ethyl ester hydrochloride (629 mg, 3.75 mmol), phenyl dichlorophosphate (791 mg, 3.75, 560 μL) and TEA (759 mg, 7.50 mmol, 1045 μL) in DCM (20 mL), to yield 1.018 g (89%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 7.02.

5.10. Synthesis of phenyl-(benzyl-2-amino-2-methylpropanoate)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using 2-aminoisobutyrate benzyl ester hydrochloride (861 mg, 3.75 mmol), phenyl dichlorophosphate (791 mg, 3.75, 560 μL) and TEA (759 mg, 7.50 mmol, 1045 μL) in DCM (30 mL). The crude was purified by flash chromatography (ethyl acetate/petroleum ether 6:4) affording 580 mg (42%) of colourless oil. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 6.79

5.11. Synthesis of phenyl-(methoxy-α,α-cycloleucinyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using methyl-1-amino-1-cyclopentanoate hydrochloride salt (0.885 g, 5.01 mmol), phenyldichlorophosphate (1.12 g, 0.749 mL, 5.01 mmol) and TEA (1.4 mL, 10 mmol) in DCM (40 mL), to yield 1.266 g (81%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 7.90.

5.12. Synthesis of phenyl-(ethoxy-α,α-cycloleucinyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using ethyl-1-amino-1-cyclopentanoate hydrochloride salt (955 mg, 5.01 mmol), phenyldichlorophosphate (1.12 g, 5.01 mmol, 749 μL) and TEA (1.4 mL, 10.02 mmol) in DCM (40 mL). The crude was purified by flash chromatography (ethyl acetate/petroleum ether 7:3) affording 1.457 g (89%) of oil. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 8.04, 7.97.

5.13. Synthesis of phenyl-(benzoxy-α,α-cycloleucinyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using benzyl-1-amino-1-cyclopentanoate hydrochloride salt (0.984 g, 3.84 mmol), phenyldichlorophosphate (0.577 mL, 3.84 mmol) and TEA (1.08 mL, 7.69 mmol) in DCM (30 mL), to yield 1.485 g (98%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 7.85.

5.14. Synthesis of *p*-fluorophenyl-(benzoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine benzyl ester hydrochloride (1.08 g, 5.01 mmol), *para*-fluorophenyl-dichlorophosphate (1.210 mg, 5.01 mmol) and TEA (1.4 mL, 1.4 mmol) in DCM (40 mL). The crude was purified by flash chromatography (ethyl acetate/petroleum ether 7:3) affording 1.599 (86%) of oil. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.15, 9.06.

5.15. Synthesis of *p*-nitrophenyl-(benzoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine benzyl ester hydrochloride (1.08 g, 5.01 mmol), *para*-nitrophenyl-dichlorophosphate (1.362 g, 5.01 mmol) and TEA (1.4 mL, 10.02 mmol) in DCM (40 mL), to yield 1.85 g (93%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.15, 9.06.

5.16. Synthesis of *p*-chlorophenyl-(benzoxy-L-alaninyl)-phosphorochloridate

This was synthesised according to Standard procedure 5.4, using L-alanine benzyl ester hydrochloride (1.00 g, 4.63 mmol), 4-chlorophenylphosphodichloridate (1.14 g, 4.63 mmol) and TEA (937 mg, 9.26 mmol, 1290 μL) in DCM (40 mL), to yield 1534 mg (87%) of crude product used without further purification. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 9.43, 9.16.

5.17. Standard procedure: synthesis of phosphoramidate derivatives

To a stirring solution of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (1.0 mol equiv) and the appropriate phosphorochloridate (2.0–3.0 mol equiv) in anhydrous THF at −80 °C was added dropwise over 1 min NMI (5.0 mol equiv). After 15 min the reaction was left to rise to room temperature and stirred at room temperature for 2–19 h. The solvent was removed under reduced pressure and the yellow oil obtained was dissolved in DCM, washed with 0.5 M HCl, and water. The organic layer was dried over $MgSO_4$, filtered, reduced to dryness and purified by flash chromatography (chloroform/methanol 97/3, dichloromethane/methanol 97/3).

5.18. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl methoxy-L-alaninyl]-phosphate, 5a

This was synthesised according to Standard procedure 5.17 using BVdU (300 mg, 0.90 mmol), phenyl methoxy-L-alaninyl phosphorochloridate (472 mg, 1.7 mmol), NMI (4.73 mmol, 378 μL) in THF (9 mL) for 2 h. The crude product was purified by column chromatography,

eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (356 mg, yield 69%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.72, 4.40. ^{1}H NMR ($CDCl_3$; 300 MHz): δ 9.90 (1H, br s, H-3), 7.64 (1H, 2s, H-6), 7.44, 7.39 (1H, 2d, ^{3}J = 14 Hz, H-5b), 7.37–7.15 (5H, m, O*Ph*), 6.75, 6.67 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.30, 6.21 (1H, 2t, ^{3}J = 6 Hz, H1′), 4.57–4.29 (3H, m, H-5′ + H-3′), 4.20–3.96 (3H, H-4′, NH, C*H*CH_3), 3.72 (3H, s, C*H*$_3$O), 2.49–2.40 (1H, m, one of H-2′), 2.12–2.01 (1H, m, one of H-2′), 1.38 (3H, d, ^{3}J = 7 Hz, C*H*$_3$CH). ^{13}C NMR (DMSO; 75 MHz): δ 22.4 (*C*H_3CH), 41.9, 41.8 (C-2′), 51.9 (*C*HCH_3), 54.3 (*C*H_3O), 67.5 (C-5′), 72.3, 71.9 (C-3′), 87.3, 87.2, 86.9, 86.8 (C-1′, C-4′), 110.6 (C-5b), 113.1 (C-5), 121.7 (‘*o*’, O*Ph*), 127.0 (‘*p*’, O*Ph*), 130.1 (C-5a), 131.5 (‘*m*’, O*Ph*), 139.2 (C-6), 150.9 (‘*ipso*’, O*Ph*) 151.9 (C-4), 163.2 (C-2), 175.7 (*C*OOCH_3).

5.19. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl ethoxy-L-alaninyl]-phosphate, 5b

This was synthesised according to Standard procedure 5.17, using BVdU (150 mg, 0.45 mmol), phenyl ethoxy L-alaninyl phosphorochloridate (249 mg, 0.9 mmol), NMI (2.38 mmol, 190 μL) in THF (4 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (145 mg, yield 55%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.48, 4.86. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.68, 7.64 (1H, 2s, H-6), 7.47, 7.41 (1H, 2d, ^{3}J = 13 Hz, H-5b), 7.35–7.10 (5H, m, O*Ph*), 6.73, 6.69 (1H, 2d, ^{3}J = 13 Hz, H-5a), 6.31, 6.27 (1H, 2t, ^{3}J = 6 Hz, H1′), 4.62–3.95 (8H, m, H-5′, H-3′, H-4′, C*H*CH_3, NH, CH_3C*H*$_2$O), 2.49–2.40 (1H, m, one of H-2′), 2.10–2.00 (1H, m, one of H-2′), 1.40 (3H, d, ^{3}J = 7 Hz, C*H*$_3$CH), 1.25 (3H, 2t, ^{3}J = 7 Hz, C*H*$_3$CH$_2$O). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.5 (*C*H_3CH$_2$O), 21.2, 21.1 (*C*H_3CH), 40.9, 40.7 (C-2′), 50.8, 50.7 (*C*HCH$_3$), 62.2, 62.1 (CH_3*C*H$_2$O), 66.5, 66.3 (C-5′), 70.9, 70.6 (C-3′), 86.0, 85.6 (C-1′, C-4′), 110.1 (C-5b), 111.8 (C-5), 120.6 (‘*o*’, O*Ph*), 125.0 (‘*p*’, O*Ph*), 129.0 (C-5a), 130.2 (‘*m*’, O*Ph*), 138.2 (C-6), 149.9 (C-4), 150.7 (‘*ipso*’, O*Ph*), 162.3 (C-2), 174.2, 174.1 (*C*OOCH$_2$CH$_3$).

5.20. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl benzoxy-L-alaninyl]-phosphate, 5c

This was synthesised according to Standard procedure 5.17, using BVdU (150 mg, 0.45 mmol), phenyl benzyloxy-L-alaninyl phosphorochloridate (249 mg, 0.9 mmol), NMI (2.38 mmol, 190 μL) in THF (5 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (228 mg, yield 78%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.74, 4.44. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.31 (1H, br s, H-3), 7.63 (1H, 2s, H-6), 7.45–7.14 (11H, m, O*Ph*, CH_2*Ph*, H-5b), 6.75, 6.66 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.30–6.25 (1H, m, H-1′), 5.18–5.09 (1H, m, C*H*$_2$Ph), 4.70–4.04 (6H, m, H-3′, H-5′, H-4′, NH, C*H*CH_3), 2.42 (1H, m, one of H-2′), 2.02 (1H, m, one of H-2′), 1.40 (3H, d, ^{3}J = 7 Hz, C*H*$_3$CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 20.7, 20.8 (*C*H_3CH), 40.4 (C-2′), 50.4 (*C*HCH$_3$), 66.0 (C-5′), 67.4 (*C*H$_2$Ph), 70.6 (C-3′), 85.4, 85.5, 85.6, 85.8 (C-1′, C-4′), 109.9 (C-5b), 111.5 (C-5b), 120.2 (‘*o*’, O*Ph*), 125.4 (‘*p*’, O*Ph*), 128.5, 128.6, 129.9 (‘*m*’ O*Ph*, CH_2*Ph*, C-5a), 135.1 (‘*ipso*’, CH_2*Ph*) 137.8 (C-6), 149.8 (C-4) 150.2 (‘*ipso*’, O*Ph*), 161.8 (C-2), 173.6 (*C*OOCH$_2$Ph).

5.21. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl methoxy-α,α-dimethylglycinyl]-phosphate, 5d

This was synthesised according to Standard procedure 5.17, using BVdU (200 mg, 0.60 mmol), phenyl (methyl-2-amino-2-methylpropanoate) phosphorochloridate (437.5 mg, 1.5 mmol), NMI (3.0 mmol, 239 μL) in THF (5 mL) for 4 h. The crude product was purified by column chromatography, eluting with chloroform/methanol 97/3 to give the pure product as a white foamy solid (117 mg, yield 33%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 3.36, 3.14. ^{1}H NMR ($CDCl_3$; 300 MHz): δ 9.91 (1H, br s, H-3), 7.73, 7.65 (1H, 2s, H-6), 7.48–7.45 (1H, 2d, ^{3}J = 14 Hz, H-5b), 7.41–7.02 (5H, m, O*Ph*), 6.81, 6.71 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.34–6.28 (1H, m, H1′), 4.55–4.17 (6H, m, H-5′, H-4′, H-3′, NH, OH-3′), 3.78 (3H, s, C*H*$_3$O), 2.53–2.39 (1H, m, one of H-2′), 2.25–1.99 (1H, m, one of H-2′), 1.60 (6H, s, [C*H*$_3$]$_2$C). ^{13}C NMR ($CDCl_3$; 75 MHz): δ 27.5, 27.4, 27.2 ([*C*H$_3$]$_2$C), 40.7, 40.6 (C-2′), 53.5 (*C*H$_3$O), 57.6 (*C*[CH$_3$]$_2$), 66.5, 66.2 (C-5′), 70.7, 71.1 (C-3′), 85.4, 85.6, 85.5, 85.9 (C-1′, C-4′), 110.4 (C-5b), 111.9 (C-5), 120.5, 120.6 (‘*o*’, O*Ph*), 125.7 (‘*p*’, O*Ph*), 128.9 (C-5a), 130.3 (‘*m*’, O*Ph*), 138.0, 138.3 (C-6), 149.8 (‘*ipso*’, O*Ph*) 150.9, 150.8 (C-4), 162.0, 162.1 (C-2), 176.4, 176.2 (*C*OOCH$_3$).

5.22. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl ethoxy-α,α-dimethylglycinyl]-phosphate, 5e

This was synthesised according to Standard procedure 5.17, using BVdU (200 mg, 0.60 mmol), phenyl (ethyl-2-amino-2-methylpropanoate) phosphorochloridate (458.0 mg, 1.5 mmol), NMI (3.0 mmol, 239 μL) in THF (5 mL) for 5 h. The crude product was purified by column chromatography, eluting with chloroform/methanol 97/3 to give the pure product as a white foamy solid (106 mg, yield 29%).

^{31}P NMR (MeOD, 121 MHz): δ 3.91, 3.85. ^{1}H NMR (MeOD, 300 MHz): δ 7.84, 7.81 (1H, 2s, H-6), 7.44–7.20 (6H, m, O*Ph*, H-5b), 6.86, 6.84 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.34–6.28 (1H, m, H-1′), 4.50–4.34 (3H, m, H-5′, H-3′), 4.23–4.15 (3H, m, H-4′, CH_3C*H*$_2$O), 2.38–2.28 (1H, m, one of H-2′), 2.22–2.09 (1H, m, one of H-2′), 1.51 (6H, s, [C*H*$_3$]$_2$C), 1.29 (3H, t, ^{3}J = 7 Hz, C*H*$_3$CH$_2$O). ^{13}C

NMR (MeOD, 75 MHz): δ 14.9 (*C*H_3CH$_2$O) 27.9, 28.3 ([*C*H$_3$]$_2$C), 41.5 (C-2′), 58.51 (*C*[CH$_3$]$_2$), 63.1 (CH$_3$*C*H$_2$O), 68.2 (C-5′), 72.6 (C-3′), 87.1, 87.4 (C-1′, C-4′), 109.6 (C-5b), 112.7 (C-5b), 122.0, 122.1, 122.2, (‘*o*’, O*Ph*), 126.7 (‘*p*’, O*Ph*), 131.0, 131.2 (C-5a, ‘*m*’ O*Ph*), 140.4 (C-6), 151.4 (‘*ipso*’, O*Ph*), 152.5 (C-4), 164.0 (C-2), 177.2 (*C*OOCH$_2$CH$_3$).

5.23. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl benzoxy-α,α-dimethylglycinyl]-phosphate, 5f

This was synthesised according to Standard procedure 5.17, using BVdU (242 mg, 0.73 mmol), phenyl (benzyl-2-amino-2-methylpropanoate) phosphorochloridate (533.0 mg, 2.0 mmol), NMI (3.63 mmol, 289 μL) in THF (5 mL) for 4 h. The crude product was purified by column chromatography, eluting with chloroform/methanol 97/3 to give the pure product as a white foamy solid (129 mg, yield 27%).

^{31}P NMR (CDCl$_3$, 121 MHz): δ 3.39, 3.12. ^{1}H NMR (CDCl$_3$, 300 MHz): δ 9.92 (1H, br s, H-3), 7.67, 7.60 (1H, 2s, H-6), 7.46, 7.44 (1H, 2d, ^3J = 14 Hz, H-5b), 7.40–7.16 (10H, m, O*Ph*, CH$_2$*Ph*), 6.76, 6.70 (1H, 2d, ^3J = 14 Hz, H-5a), 6.31–6.25 (1H, m, H-1′), 5.18 (1H, s, C*H*$_2$Ph), 4.50–4.09 (6H, m, H-3′, H-5′, H-4′, NH, OH-3′), 2.48–2.25 (1H, m, one of H-2′), 2.16–1.82 (1H, m, one of H-2′), 1.60 (6H, s, [C*H*$_3$]$_2$C). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 27.3, 27.4, 28.5 ([*C*H$_3$]$_2$C), 40.6, 40.7 (C-2′), 57.6, 57.6 (*C*[CH$_3$]$_2$), 66.2, 66.5 (C-5′), 68.1 (*C*H$_2$Ph), 70.6, 71.1 (C-3′), 85.4, 85.5, 85.6, 85.8 (C-1′, C-4′), 110.4 (C-5b), 112.0 (C-5), 120.4, 120.5, 120.6, 125.7, 128.4, 128.5, 128.8, 128.9, 130.3 (O*Ph*, C-5a), 135.7 (‘*ipso*’, CH$_2$*Ph*) 138.1, 138.3 (C-6), 149.8, 150.8, 150.9 (‘*ipso*’ O*Ph*, C-4), 162.1 (C-2), 177.5, 175.7 (*C*OOCH$_2$Ph).

5.24. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl methoxy-α,α-cyclopentylglycinyl] phosphate, 5g

This was synthesised according to Standard procedure 5.17, using BVdU (250 mg, 0.75 mmol), phenyl methoxy-α,α-cyclopentylglycinyl phosphorochloridate (589 mg, 1.87 mmol), NMI (5.2 mmol, 415 μL) in THF (7 mL) for 3 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (234 mg, yield 51%).

^{31}P NMR (CDCl$_3$, 121 MHz): δ 3.87, 3.82. ^{1}H NMR (CDCl$_3$; 300 MHz): δ 10.28 (1H, br s, H-3), 7.75, 7.72 (1H, 2s, H-6), 7.42 (1H, d, ^3J = 13 Hz, H-5b), 7.37–7.15 (5H, m, O*Ph*), 6.73, 6.71 (1H, 2d, ^3J = 13 Hz, H-5a), 6.30 (1H, t, ^3J = 6 Hz, H1′), 4.4–4.2 (4H, m, H-5′, H-3′, NH), 4.1 (1H, m, H-4′), 3.72 (3H, s, C*H*$_3$O), 2.49–2.40 (1H, m, one of H-2′), 2.35–2.01 (5H, m, one of H-2′ + 4H cyclopentane), 1.76–1.70 (4H, m, 4H cyclopentane). ^{13}C NMR (CDCl$_3$; 75 MHz): δ 24.4, 24,3, 24.2 (2CH$_2$ cyclopentane), 39.2, 38.6, 38.5 (2CH$_2$ cyclopentane), 40.0 (C-2′), 53.2 (*C*H$_3$O), 66.4 (*Cq* cyclopentane), 66.6 (C-5′), 70.9 (C-3′), 85.8, 85.6, 85.4, 85.3 (C-1′, C-4′), 110.2 (C-5b), 111.9 (C-5), 120.7–120.6 (‘*o*’, O*Ph*), 125.7 (‘*p*’, O*Ph*), 129.0 (C-5a), 130.2 (‘*m*’, O*Ph*), 138.5 (C-6), 149.9 (C-4), 150.9, 150.8 (‘*ipso*’, O*Ph*), 162.3 (C-2), 176.3, 176.2 (*C*OOCH$_3$).

5.25. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl ethoxy-α,α-cyclopentylglycinyl]-phosphate, 5h

This was synthesised according to Standard procedure 5.17, using BVdU (250 mg, 0.75 mmol), Phenyl ethoxy-α,α-cyclopentylglycinyl phosphorochloridate (642 mg, 1.87 mmol), NMI (5.2 mmol, 415 μL) in THF (7 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (258 mg, yield 55%).

^{31}P NMR (CDCl$_3$, 121 MHz): δ 4.23, 4.10. ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.22 (1H, br s, H-3), 7.73, 7.63 (1H, 2s, H-6), 7.51 (1H, d, ^3J = 14 Hz, H-5b), 7.45–7.10 (5H, m, O*Ph*), 6.74, 6.71 (1H, 2d, ^3J = 14 Hz, H-5a), 6.22 (1H, t, ^3J = 4 Hz, H1′), 4.55–4.05 (7H, m, H-5′, H-3′, H-4′, NH, CH$_3$C*H*$_2$O), 2.50–2.40 (1H, m, one of H-2′), 2.35–1.95 (5H, m, one of H-2′, 4H cyclopentane), 1.95–1.75 (4H, m, 4H cyclopentane), 1.27, 1.26 (3H, 2t, ^3J = 7 Hz, C*H*$_3$CH$_2$O). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 14.5 (*C*H$_3$CH$_2$O), 24.5, 24,4 (2CH$_2$ cyclopentane), 39.2, 38.9 38.8, 38.4 (2CH$_2$ cyclopentane), 40.6 (C-2′), 62.2, 62.1 (CH$_3$*C*H$_2$O), 66.2 (*Cq* cyclopentane), 66.6 (C-5′), 70.8 (C-3′), 85.7, 85.5 (C-1′, C-4′), 110.2 (C-5b), 111.5 (C-5), 120.7, 120.6 (‘*o*’, O*Ph*), 125.6 (‘*p*’, O*Ph*), 129.7 (C-5a), 130.2 (‘*m*’, O*Ph*), 138.5, 138.3 (C-6), 149.7 (C-4), 150.9, 150.8 (‘*ipso*’, O*Ph*), 162.3 (C-2), 176.3 (*C*OOCH$_2$CH$_3$).

5.26. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[phenyl benzoxy-α,α-cyclopentylglycinyl] phosphate, 5i

This was synthesised according to Standard procedure 5.17, using BVdU (200 mg, 0.6 mmol), phenyl benzyloxy-α,α-cyclopentylglycinyl phosphorochloridate (589 mg, 1.5 mmol), NMI (4.16 mmol, 332 μL) in THF (5 mL) for 10 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (127 mg, yield 31%).

^{31}P NMR (CDCl$_3$, 121 MHz): δ 4.11, 4.01. ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.20 (1H, br s, H-3), 7.69, 7.60 (1H, 2s, H-6), 7.45, 7.44 (1H, 2d, ^3J = 14 Hz, H-5b), 7.40–7.10 (10H. m, O*Ph*+CH$_2$*Ph*), 6.74, 6.71 (1H, 2d, ^3J = 14 Hz, H-5a), 6.20 (1H, m, H-1′), 5.15 (1H, s, C*H*$_2$Ph), 4.4–4.2 (3H, m, H-3′, H-4′, NH), 4.1 (2H, m, H-5′), 2.45–2.35 (1H, m, one of H-2′), 2.35–1.95 (5H, m, one of H-2′, 4H cyclopentane), 1.95–1.75 (4H, m, 4H cyclopentane). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 24.4, 24,3, 24.2 (2CH$_2$ cyclopentane), 39.9, 39.7 38.6, 38.5 (2CH$_2$ cyclopentane), 40.5 (C-2′), 66.2 (*Cq* cyclopentane), 66.5 (C-5′), 67.8 (*C*H$_2$Ph), 70.8, 70.7 (C-3′), 85.7, 85.6, 85.5, 85.4 (C-1′, C-4′), 110.2 (C-5b), 111.8, 118.7 (C-5b), 120.7, 120.5 (‘*o*’, O*Ph*), 125.7 (‘*p*’, O*Ph*), 130.2, 129.0, 128.8, 128.7, 128.5 (‘*m*’ OPh, CH$_2$*Ph*, C-5a), 135.8 (‘*ipso*’, CH$_2$*Ph*) 138.4, 138.2 (C-6), 149.8 (C-4), 150.9, 150.8 (‘*ipso*’, O*Ph*), 162.2 (C-2), 175.7, 175.5 (*C*OOCH$_2$*Ph*).

5.27. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[*para*-fluorophenyl benzoxy-L-alaninyl]-phosphate, 5j

This was synthesised according to Standard procedure 5.17, using BVdU (200 mg, 0.60 mmol), *para*-fluorophenyl benzyloxy-L-alaninyl phosphorochloridate (556 mg, 1.5 mmol), NMI (4.16 mmol, 332 μL) in THF (5 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (256 mg, yield 64%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.74, 4.44. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.66, 7.64 (1H, 2s, H-6), 7.45–7.00 (9H, m, O*Ph*, CH_2*Ph*, H-5b), 6.75 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.31, 6.26 (1H, 2t, ^{3}J = 6 Hz, H-1′), 5.19–5.16 (1H, 2s, CH_2Ph), 4.85–4.00 (6H, m, H-3′, H-5′, H-4′, NH, C*H*CH_3), 2.47 (1H, m, one of H-2′), 2.0–2.15 (1H, m, one of H-2′), 1.38 (3H, d, ^{3}J = 7 Hz, CH_3CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.2, 21.1 (*C*H_3CH), 40.7 (C-2′), 50.4 (*C*HCH$_3$), 66.7, 66.4 (C-5′), 67.8 (*C*H_2Ph), 71.1, 70.7 (C-3′), 86.0, 85.7, 85.4, 85.3 (C-1′, C-4′), 110.4 (C-5b), 111.9 (C-5), 117.0 (‘*o*’, O*Ph*), 122.0 (‘*m*’, O*Ph*), 128.7, 128.6 (CH_2*Ph*, C-5a), 135.4 (‘*ipso*’, CH_2*Ph*) 138.2 (C-6), 146.5 (‘*ipso*’, O*Ph*), 149.9 (C-4), 158.5 (‘*p*’ O*Ph*), 162.2 (C-2), 173.9 (*C*OOCH_2*Ph*).

5.28. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[*para*-nitrophenyl benzoxy-L-alaninyl]-phosphate, 5k

This was synthesised according to Standard procedure 5.17, using BVdU (200 mg, 0.60 mmol), *para*-nitrophenyl benzyloxy-L-alaninyl phosphorochloridate (597 mg, 1.5 mmol), NMI (4.16 mmol, 332 μL) in THF (5 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 97/3 to give the pure product as a white foamy solid (228 mg, yield 55%).

^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.74, 4.44. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.29 (1H, br s, H-3), 8.20–8.14 (2H, m, O*Ph*), 7.64, 7.59 (1H, 2s, H-6), 7.38, 7.22 (1H, 2d, ^{3}J = 14 Hz, H-5b), 7.37–7.00 (7H, m, O*Ph*, CH_2*Ph*), 6.71, 6.62 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.25–6.15 (1H, 2t, ^{3}J = 6Hz, H-1′), 5.17 (2H, d, CH_2Ph), 4.87 (1H, m, H-3′), 4.6–4.2 (3H, m, H-5′, C*H*CH_3) 4.20–4.00 (2H, m, H-4′, NH), 2.55–2.45 (1H, m, one of H-2′), 2.2–2.05 (1H, m, one of H-2′), 1.38 (3H, d, ^{3}J = 7 Hz, CH_3CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.2, 21.1 (*C*H_3CH), 40.6 (C-2′), 50.9 (*C*HCH$_3$), 67.1, 67.0 (C-5′), 68.0 (*C*H_2Ph), 71.3, 70.9 (C-3′), 86.3, 86.0, 85.3, 85.2 (C-1′, C-4′), 110.4 (C-5b), 111.9, 111.8 (C-5), 121.3 (‘*o*’, O*Ph*), 126.2–126.1 (‘*m*’, O*Ph*), 129.1, 128.7, 128.6 (CH_2*Ph*, C-5a), 135.4 (‘*ipso*’, CH_2*Ph*), 138.3 (C-6), 145.1 (‘*ipso*’, O*Ph*), 149.9 (C-4), 155.6 (‘*p*’ OPh), 162.2 (C-2), 173.8, 173.7 (*C*OOCH_2*Ph*).

5.29. Synthesis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-[*para*-chlorophenyl benzoxy-L-alaninyl]phosphate, 5l

This was synthesised according to Standard procedure 5.17, using BVdU (300 mg, 0.90 mmol), *para*-chlorophenyl benzoxy-L-alaninyl phosphorochloridate (698.7 mg, 1.80 mmol), NMI (369.5 mg, 4.5 mmol, 358.7 μL) in THF (10 mL) for 2 h. The crude product was purified by column chromatography, eluting with dichloromethane/methanol 95:5 to give the pure product as a white foamy solid (310 mg, yield 50%). ^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.81, 4.53. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.10 (1H, br s, H-3), 7.65, 7.63 (1H, 2s, H-6), 7.44, 7.43 (1H, 2d, ^{3}J = 14 Hz, H-5b), 7.40–7.17 (9H, m, O*Ph*), 6.73, 6.68 (1H, 2d, ^{3}J = 14 Hz, H-5a), 6.31, 6.25 (1H, 2t, ^{3}J = 6 Hz, H-1′), 5.17 (2H, s, CH_2Ph), 4.60–4.23 (4H, m, H-3′, H-5′, NH), 4.20–3.97 (2H, m, H-4′, C*H*CH_3), 2.48–2.44 (1H, m, one of H-2′), 2.15–2.05 (1H, m, one of H-2′), 1.43, 1.40 (3H, d, ^{3}J = 7.0 Hz, CHCH_3). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.2 (CH-*C*H_3), 40.7 (C-2′), 50.8, 50.9 (*C*HCH$_3$), 66.6 (C-5′), 67.9 (*C*H_2Ph), 70.7, 71.1 (C-3′), 85.4, 85.5, 85.8, 86.1 (C-1′, C-4′), 110.5 (C-5b), 111.9, 112.0 (C-5), 122.0 (‘*o*’, O*Ph*), 128.7, 129.0, 129.1, 130.3 (‘*m*’, O*Ph* + C-5a), 131.1 (‘*ipso*’, CH_2*Ph*), 135.4 (‘*p*’, O*Ph*), 138.2 (C-6), 149.1 (‘*ipso*’, O*Ph*), 150.0 (C-4), 162.1 (C-2), 173.9, 174.0 (*C*OOCH_2Ph).

5.30. Biological evaluation

Human breast cancer cell lines MDA MB 231, human colon cancer cell line HT115 and prostate cancer cell line PC-3, were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, England). Cytoxicity assay was based on MTT assay as we previously reported.[20,21] The method is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt (3-[4,5-dimethylthiazd-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble formazan precipitate that is dissolved and quantified by spectrophotometry.[22] A 96-well culture cell culture plate was used. Cells were counted with a haemocytomete counting chamber and a specific number (4000 per well) of cells were seeded to each well with culture medium (DMEM). Compounds, dissolved in DMSO, were series diluted (1:5) in culture medium, to cover a final concentration range between 0.128 and 2000 μM. The culture plate was incubated for 72 h at 37 °C. The cells were washed twice with BSS. A solution of MTT in 0.5 mg/mL in culture medium was added into each well. The culture plate was then incubated at 37 °C for 4 h. MTT was then removed by aspiration. The crystals produced by MTT reagent within the cells were then extracted by the addition of 100 μL of Triton X100 (10% in water). The cells were incubated at 4 °C for 24 h. The absorbance of the colorimetric products was then measured at a wavelength of 540 nm using a spectrophotometer (Titertecek).

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