Aryloxy Pivaloyloxymethyl Prodrugs (POMtides) as Nucleoside Monophosphate Prodrugs

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ABSTRACT: Intracellular phosphorylation of therapeutic nucleoside analogues into their active triphosphate metabolites is a prerequisite for their pharmacological activity. However, the initial phosphorylation of these unnatural nucleosides into their monophosphate derivatives can be a rate-limiting in their activation. To address this, we herein report the development of the aryloxy pivaloyloxymethyl prodrugs (POMtides) as a novel and effective nucleoside monophosphate prodrug technology and its successful application to the anticancer nucleoside analogue 5-fluoro-2'-deoxyuridine (FdUR).

Keywords: Nucleoside, Prodrug, Anticancer, POMtide, Prodrug.

INTRODUCTION

Nucleoside analogues have been the cornerstone of antiviral and anticancer chemotherapy for decades.[1, 2] These drugs are activated *in vivo* by three sequential phosphorylation steps that eventually generate the active nucleoside triphosphate metabolites (**Figure 1a**).[1, 2] Such phosphorylation (activation) of these therapeutic nucleoside analogues is essential for them to exert their therapeutic effects. However, the *in vivo* phosphorylation of therapeutic nucleoside analogues by cellular nucleoside and nucleotide kinases is often inefficient, and this ultimately limits their efficacy. In particular, the first phosphorylation step through which nucleoside analogues are phosphorylated into their 5'-*O*-monophosphates by nucleoside kinases is often the rate-limiting step in their activation. To address this, a series of nucleoside analogue monophosphate approaches have been developed and some of these have already yielded FDA-approved prodrugs.[3] However, as the intellectual property landscape of these existing prodrug technologies is getting more congested,[4] novel, effective and safe nucleoside monophosphate prodrug approaches are needed to maintain interest in the development of this class of drugs.

With the aim of developing a novel prodrug approach for the intracellular delivery of nucleoside analogue monophosphates, we designed a hybrid prodrug approach that combines distinct parts from two of the most successful nucleoside monophosphate prodrug approaches, the *bis*POM[3, 5] and the ProTide[3, 6] (**Figure 1b**), which have both delivered FDA-approved drugs. Indeed, we hypothesized that the masking of the monophosphate groups of nucleoside analogues by one aryl motif, akin to the ProTides, and one pivaloyloxymethyl (POM), akin to the *bis*POM, would generate a new and useful monophosphate prodrug approach, which we termed the POMtide. Similar to the ProTide and *bis*POM prodrugs, POMtides would be neutral at physiological pH and thus would have improved lipophilicity (logP) as compared to the parent nucleoside analogues or its monophosphate derivatives, which are often hydrophilic. Ultimately, this is likely to translate into better cellular uptake and hence improved pharmacological activity akin to what is observed with the ProTide and *bis*POM prodrugs.
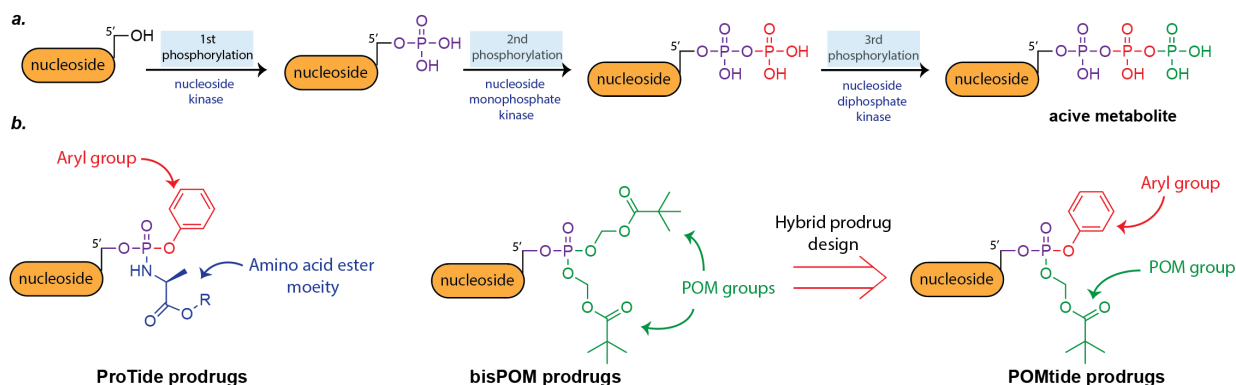
Figure 1. Nucleoside analogues and their monophosphate prodrugs. **A**. Three intracellular phosphorylation steps required for the activation of nucleoside analogues (referred to in the figure as 'nucleoside'). **B**. General chemical structures of ProTide and *bis*POM prodrugs and their components that have been used in a hybrid prodrug design to generate the POMtide prodrugs.
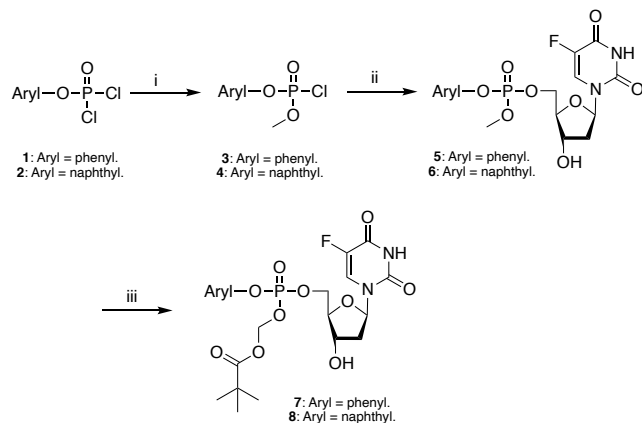
RESULTS AND DISCUSSION

Initially, bioinformatic calculations comparing the physico-chemical properties of the ProTides, *bis*POMs and POMtides of the same nucleoside analogues showed the POMtides to have calculated physicochemical properties comparable to those of the ProTides and far better than those of the *bis*POM prodrugs (**Supporting Table S1**). Encouraged by this observation, we applied the POMtide prodrug approach to 5-fluoro-2'-deoxyur-idine (FdUR), a chemotherapeutic agent that is used to treat mainly colorectal cancer.[7] This drug produces its anticancer effects via a dual mechanism of action that involves the inhibition of thymidylate synthase via its diphosphate species while the triphosphate metabolite of FdUR inhibits DNA synthesis.[8] Critically, the choice of this nucleoside analogue was driven by the fact that ProTide prodrugs of this compound have been reported[9, 10] and one of them, named NUC-3373, is currently undergoing Phase I clinical trials for solid tumors and colorectal cancer (www.clinicaltrials.gov; identifiers: NCT02723240 and NCT03428958). Thus, the ProTides of FdUR will be used in this study to compare to our FdUR POMtides.

FdUR requires phosphorylation to its active triphosphate species *in vivo*.[2] Among the key resistance mechanisms to FdUR are mutations in nucleoside transporters that limit its active uptake into cells and mutations in the nucleoside kinases, e.g., deoxycytidine kinase, which convert FdUR into its monophosphate species.[2, 11] Previously, it was shown that the application of the ProTide monophosphate prodrug technology to FdUR overcomes these resistance mechanisms,[11] and this made FdUR an ideal candidate for our proof of concept studies of the new POMtide prodrug approach.

In the design of FdUR POMtides, the monophosphate group is masked by one pivaloyloxymethyl group and an aryl motif. For the aryl motif, we explored the use of phenyl and 1-naphthyl groups. These are the two most commonly used aryl motifs in the ProTide prodrugs and a series of clinical candidates contain these groups.[3, 6, 12] The designed POMtides had excellent calculated physiochemical properties that compare to the ProTides (**Supporting Table S1**), which have for the last decade been successful in delivering clinical candidates and FDA-approved drugs.[3, 6, 12]

The synthesis of the POMtides (**Scheme 1**) started by the mono-methylation of the commercially available phenyldichlorophos-phate **1**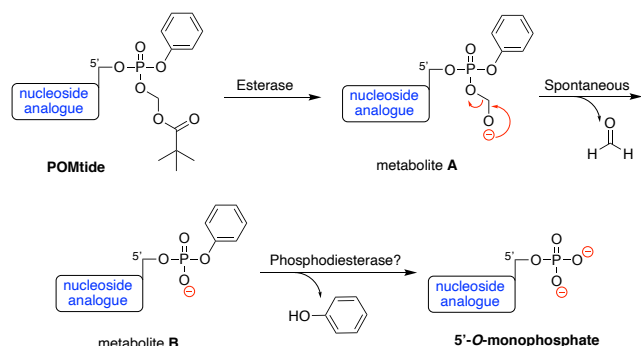 or naphthyl dichlorophosphate **2**[13] in the presence of methanol and pyridine in diethylether. The desired compounds **3** and **4** were obtained in excellent yields, 89% and 73%, respectively. Subsequently, these compounds were coupled to FdUR in THF and in the presence of 1-methylimidazole (NMI). This reaction gave the desired coupling of **3** and **4** to the 5'-*O*-position of FdUR in low to moderate yields (32% and 48%). Finally refluxing compounds **5** and **6** with the commercially available pivaloyloxymethyl chloride in acetonitrile and in the presence of sodium iodide gave the desired POMtides **7** and **8** in 41% and 40% yields respectively.

Once the POMtides were synthesized, we initially studied their serum stability and metabolism to release FdUR monophosphate (FdUR-MP). For the serum stability, we incubated the FdUR POMtide with human serum at 37 ºC *in vitro* and monitored the reaction by ^{31}P NMR as reported previously with the ProTide prodrugs.[14, 15] At the start of the incubation, $t = 0$ min, one ^{31}P NMR (broad) peak corresponding to the parent POMtide (δ_P -8.03) was present (**Figure 2**). Typically, the ^{31}P NMR spectrum of POMtides show two distinct and sharp singlets (**Supporting Figure S1**), but in the aqueous media of this experiment the ^{31}P NMR peak for the FdUR POMtide appeared as one broad peak. The second ^{31}P NMR peak (δ_P 1.9 ppm) that was present at $t = 0$ min of this experiment originates from the human serum sample (**Supporting Figure S2**). Following 2 h incubation with human serum at 37 ºC *in vitro*, a new peak at δ_P -5.02 ppm appeared and increased in intensity throughout this 12 h study and became a prominent peak at 12 h (**Figure 2**). Given that it is known that *bis*POM prodrugs have limited stability in human serum since one of the two POM masking groups is rapidly cleaved off due to the esterases present in this medium,[16] we noted that this new peat at δ_P -5.02 ppm corresponds to the FdUR POMtide following the removal of the POM motif (metabolite **B**, **Figure 3**). This notion is supported by the fact that the synthesized[17, 18] version of compound **B** has a ^{31}P NMR peak at $\delta_P \sim$ -5 ppm (see **Supporting Figure S3**). Despite the presence of metabolite **B** following the incubation of POMtide in human serum, the intact FdUR POMtide peak was still relatively the major peak and indicates a POMtide's $t_{1/2}$ ≥ 12 h in human serum (**Figure 2**).
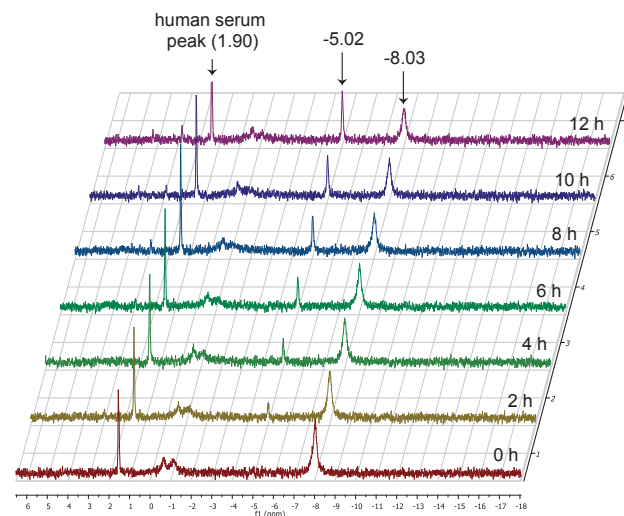
Figure 2. Stability of POMtide **7** in human serum at 37 ºC for 12 h as monitored by ^{31}P NMR.

In terms of the metabolism of the POMtides, we hypothesized that the ester group of the POM group will be metabolized first

Scheme 1. Synthesis of FdUR POMtides. Reagents and conditions: **i)** MeOH, pyridine, Et$_2$O 0 ºC, 1 h, 89% yield for **3** and 73% yield for **4**; **ii)** FdUR, NMI, THF, 24 h, 32% yield for **5** and 48% yield for **6**; **iii)** POMCl, NaI, MeCN, reflux, 24 h, 41% yield for **7** and 40% yield for **8**.

Figure 3. Proposed mechanism of the metabolism of nucleoside monophosphate POMtides.

by esterases as per the established metabolism of *bis*POM prodrugs to generate metabolite **A** (**Figure 3**).[3, 19] This will then be followed by the spontaneous breakdown of the remaining POM group that involves the release of the formaldehyde (CH_2O) and the generation of metabolite **B** (**Figure 3**). The final step will be the cleavage of the phenyl group, likely by phosphodiesterase enzymes, to generate the desired unmasked monophosphate species.

To probe the metabolism of FdUR POMtides, we first explored the ester motif cleavage of FdUR POMtide **7** by carboxypeptidase Y, which is known to cleave the ester moiety of the ProTide prodrugs.[20] FdUR POMtide **7** was incubated with recombinant carboxypeptidase Y at 37 ºC for 12 h, and the sample was monitored by ^{31}P NMR. The results showed that the ester group of POMtide **7** was not processed by carboxypeptidase Y as there were no changes to the ^{31}P NMR spectra and, critically, no new peaks were observed (**Supporting Figure S4**). This indicated that the ester motif of the POMtides is metabolized by an esterase that is different from that that metabolizes the ester motif of the ProTide prodrugs. We subsequently studied the metabolism of POMtide **7** by porcine liver esterase where the POMtide was incubated with this enzyme at 37 ºC and the sample was monitored by ^{31}P NMR for 24 h. Although a very minor ^{31}P NMR appeared at δ_P -5.14 ppm, most of the POMtide **7** was stable in the presence of porcine liver esterase (**Supporting Figure S5A-B**). We anticipated that this new minor peak was that of metabolite **B** (**Figure 3**), and therefore to confirm this we added synthesized metabolite **B** to the sample. As a result of this

addition, the intensity of the existing peak at δ_P -5.14 ppm in the ^{31}P NMR spectra increased (**Supporting Figure S5C**), confirming this new minor product was metabolite **B**. Although the rate of metabolism observed in these studies is slow, it still provided preliminary insights that support our hypothesis for the metabolism of POMtide prodrugs presented in **Figure 3**.

Finally, we embarked upon investigating the ability of the POMtide prodrugs to induce pharmacological effects. Initially, we tested the ability of the FdUR POMtide **8** to exert cytotoxic activity in the lung carcinoma cell line A549. For this, A549 cells were treated with the nucleoside FdUR or its monophosphate prodrugs POMtide **8** and the naphthyl *L*-alanine benzyl ester phosphoramidate (ProTide) for 24 h and 48 h at different concentrations, as shown in **Figure 4**. Cell proliferation was measured using Promega CellTiter 96® Non-Radioactive Cell Proliferation assay according to the manufacturer's protocol. The results showed that the parent nucleoside FdUR lacked activity (IC_{50} > 100 μM) against A549 at the two timelines studied, 24 h and 48 h (**Figure 4A and B**). In terms of the FdUR POMtide **8** and ProTide prodrugs studied, they exhibited limited cytotoxicity after 24 h treatment (IC_{50} = 52.49 μM (POMtide **8**) and 69.49 μM (ProTide) (**Figure 4A**) and this was more significant at the 48 h treatment (IC_{50} = 34.46 μM (POMtide **8**) and 35.95 μM (ProTide) (**Figure 4B**). Encouragingly, both the FdUR POMtide **8** and ProTide showed comparable cytotoxic activity in A549. The reason for the difference in the pharmacological profiles of the FdUR POMtide and ProTide at 24 h and 48 h can be accounted for by the time needed to metabolize the prodrugs into their active triphosphate species as well as the time needed for such active metabolites to reach a concentration level that can induce detectable pharmacological effect. In terms of the lack of activity seen with FdUR on A549 cells this could be rationalized by the poor uptake of the nucleoside analogue in this cell line as well as its possible poor phosphorylation to the active metabolite, especially the first phosphorylation step. Such limitations are circumvented by the application of the POMtide and ProTide prodrug approaches.

To further strengthen the case of the POMtides as a viable monophosphate prodrug technology, we subsequently studied the effect of FdUR, FdUR POMtides **7** and **8**, and the phenyl *L*-alanine benzyl ester phosphoramidate (ProTide) on the proliferation of MCF7 cells. The cells were treated with these compounds for 24 h and 48 h at different concentrations as shown
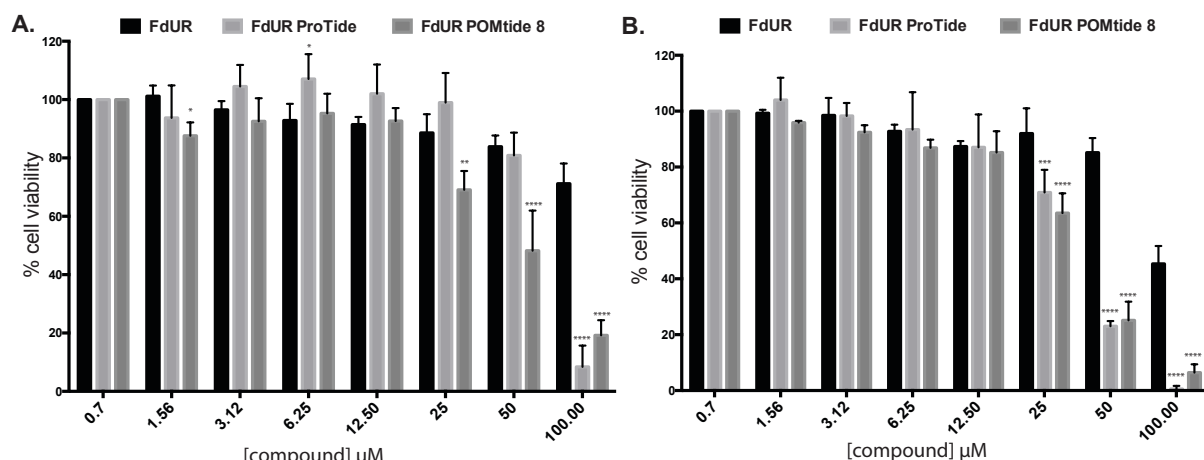
Figure 4. Effect of FdUR, its ProTide and POMtide **8** on A549 cell viability. Cell viability was determined by standard MTT assay. The compounds were incubated with the A549 lung cancer cell line for 24 h (**A**) or 48 h (**B**) at the indicated concentrations. The percentage of cell viability was calculated and is presented as a normalized value to the control DMSO. The samples were run in triplicates and the values are shown as mean ± SEM from each replicate data set. Two- ANOVA followed by Dunnett's test were used to compare the results. *P < 0.1, **P < 0.05, ***P < 0.01, ****P < 0.001.
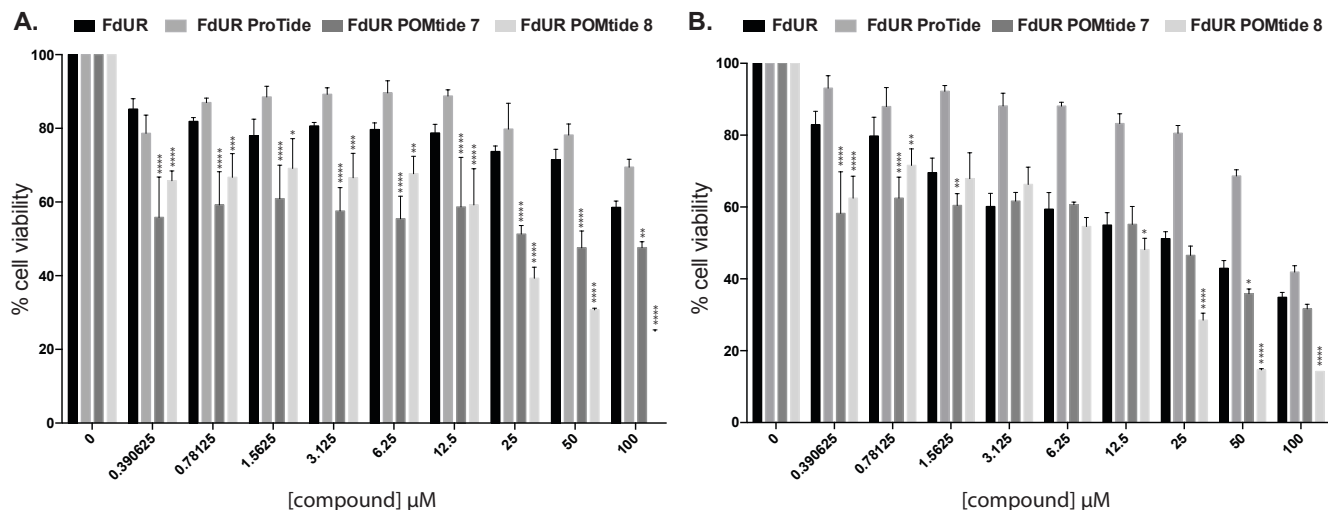
Figure 5. Effect of FdUR, its ProTide and two POMtides **7** and **8** on MCF7 cell viability. Cell viability was determined by standard MTT assay. The compounds were incubated with the A549 lung cancer cell line for 24 h (**A**) or 48 h (**B**) at the indicated concentrations. The percentage of cell viability was calculated and is presented as a normalized value to the control DMSO. The samples were run in four replicates and the values are shown as mean ± SEM from each replicate data set. Two- ANOVA followed by Dunnett's test were used to compare the results showing reduction in cell viability. $*P < 0.1$, $**P < 0.05$, $***P < 0.01$, $****P < 0.001$.

in **Figure 5**. Intriguingly, in this cell line, the parent nucleoside, and the two POMtides showed significant reduction of MCF7 proliferation compared to the FdUR ProTide, which did not exhibit any effect on MCF7 proliferation. After 24 h treatment (**Figure 5A**), only the two FdUR POMtides showed inhibition of MCF7 proliferation ($IC_{50} = 63.64$ μM for POMtide **7** and $IC_{50} = 28.75$ μM for POMtide **8**), while FdUR and the FdUR ProTide did not show any significant inhibition of MCF7 proliferation ($IC_{50} > 100$ μM in both cases). The data also shows that after 48 h incubation (**Figure 5B**), the parent nucleoside, FdUR, showed some level of inhibition of MCF7 proliferation ($IC_{50} = 46.94$ μM), while POMtides **7** and **8** exhibited more potent inhibition especially POMtide **8** ($IC_{50} = 35.51$ μM for POMtide **7** and $IC_{50} = 13.11$ μM for POMtide **8**) while the FdUR ProTide lacked significant inhibition of MCF7 proliferation ($IC_{50} = 84.76$ μM).

Considering the data in **Figures 4** and **5**, it is apparent that the FdUR POMtides exhibited promising biological activity in both cell lines, A549 and MCF7. However, the FdUR ProTide had comparable potency as POMtide **8** in A549, but no significant effect on MCF7 cell viability. The lack of activity observed with the FdUR ProTide in the MCF7 cell line may be attributed to the lack or low expression of enzymes required for the efficient metabolism of the ProTide prodrugs, primarily the carboxypeptidase and Hint-1.[6] In order to investigate this, we examined the gene expression of the carboxypeptidase cathepsin A (gene name CSTA)[20] and the phosphoramidase Hint-1, which are established as key enzymes in the metabolism of the ProTide prodrugs, in 1,019 cancer cell lines in the Cancer Cell Line Encyclopedia (Broad Institute and Novartis).[6] Out of these, we chose MCF7 and A549, which we used in the proliferation assay as well as four more cancer cell lines (SW480, HCT116, RKO and HT29) that expressed both enzymes and were available for us in the laboratory to perform Western blotting for the expression of these enzymes. Analyzing the mRNA expression of CSTA and Hint-1 in these six cancer cell lines showed that cathepsin A is widely expressed in these cell lines with the lowest expression observed in the MCF7 cell line (**Figure 6A**). In terms of Hint-1 expression, this was also shown to be expressed well across the six cell lines studied, with the lowest expression seen,

again, in the MCF7 cell line. To verify this further, we subsequently investigated the protein expression of these two enzymes in the aforementioned six cell lines plus HEK293 and HeLa cells, which are widely used in studying potential small molecule therapeutics. Thus, cell lysates from these cells underwent Western blotting for cathepsin A and Hint-1 as shown in **Figure 6B**. The results indicated that cathepsin A was expressed in four out of the eight cell lines (HT29, HeLa, A549 and SW480), while in the HEK293 and HCT116 cell lines, the expression of this enzyme was lacking or was at a very low level. In MCF7, the band detected with the cathepsin A antibody was not of the same molecular weight as that observed in the other cells, indicating a possible truncated version of cathepsin A, which may have different catalytic activity compared to the full length version of this enzyme that has been detected in the other cell lines covered in **Figure 6B**. In terms of the phosphoramidase enzyme Hint-1, it was expressed to good levels in all of the cell lines studied apart from HEK293, MCF7 and HeLa cells, which had a comparatively lower expression.
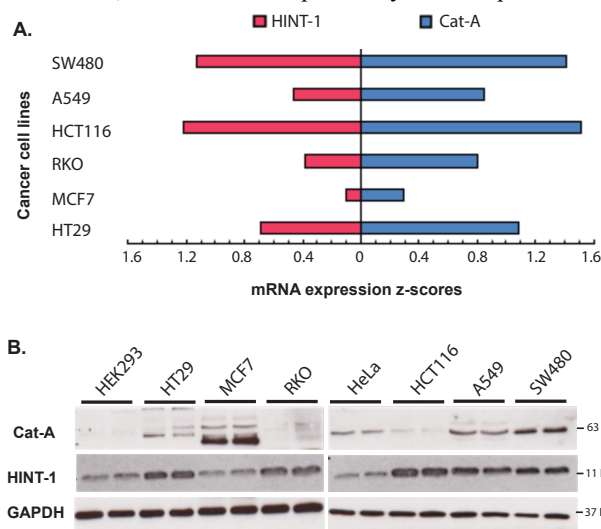
Figure 6. Expression of the enzymes needed for the ProTides metabolism in human cancer cells. **A**. mRNA expression levels of cathepsin A and Hint-

1 in the indicated cell lines. **B**. Protein expression of cathepsin A and Hint-1 in the indicated cell lines.

As the mRNA and protein expression of the two enzymes, cathepsin A and Hint-1, seem to be lower in MCF7, this may explain the lower level of FdUR activity in this cell line compared to A549 in which there are better levels of these two enzymes that are needed in the metabolism of the ProTide prodrugs. In fact, the dependency of the ProTide metabolism on two distinct enzymes may limit the activity of these prodrugs to cells where these enzymes are expressed and active to a certain level. Compared to the POMtide metabolism, the enzymes needed for metabolizing this type of prodrug, especially the esterases for the first step, are ubiquitously expressed. This may make the POMtide prodrugs exhibit pharmacological activity in cell lines and tissues where the ProTide prodrugs lack activity.

CONCLUSION

Aryloxy pivaloyloxymethyl prodrugs (POMtides) of the cytotoxic nucleoside analogue FdUR were designed and synthesized. These prodrugs showed reasonable stability in human serum ($t_{1/2} \geq 12$ h), and initial metabolic studies suggested that the metabolism of these prodrugs proceeds by the cleavage of the POM masking group followed by that of the phenyl ring to release the nucleoside analogue monophosphate species. Additionally, in A549 and MCF7 cell lines, FdUR POMtides showed improved potency in inhibiting cell proliferation compared to the parent nucleoside FdUR. Critically, these POMtides retained activity in MCF7 cells, where FdUR ProTides lacked significant pharmacological activity likely due to the low expression and activity of the enzymes responsible for metabolizing the ProTide prodrugs. Collectively, this work presents the POMtide prodrug approach as a viable approach for the intracellular delivery of nucleoside analogue monophosphates, which may prove useful in the discovery of new antiviral and anticancer nucleotide therapeutics.

EXPERIMENTAL SECTION

General information. All reagents and solvents were of general purpose or analytical grade and were purchased from Sigma-Aldrich Ltd. (Merck), Fisher Scientific, Fluorochem, or Acros. ^{31}P, ^{1}H, ^{19}F and ^{13}C NMR data were recorded on a Bruker AVANCE DPX500 spectrometer operating at 202, 500, and 125 MHz, respectively. Chemical shifts (δ) are quoted in ppm, and J values are quoted in Hz. In reporting spectral data, the following abbreviations were used: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), td (triplet of doublets), and m (multiplet). All of the reactions were carried out under a nitrogen atmosphere and were monitored using analytical thin layer chromatography on precoated silica plates (kiesel gel 60 F254, BDH). Compounds were visualized by illumination under UV light (254 nm) or by the use of KMnO4 stain followed by heating. Flash column chromatography was performed with silica gel 60 (230-400 mesh) (Merck). HPLC was carried out on a SHIMADZU Prominence-i quaternary low-pressure gradient pump with a Prominence-i UV detector (190 to 700 nm). All solvents for HPLC were HPLC grade purchased from Fisher Scientific. HPLC data analysis was performed using the SHIMADZU Lab solutions software package. The purity of the tested prodrugs was determined by HPLC, and they were all of ≥95% purity, except where specified.

Methyl phenyl phosphorochloridate (3). Phenyl phosphorodichloridate **1** (0.28 mL, 1.896 mmol) was added to anhydrous diethyl ether (5 mL) and stirred at 0 °C, under an atmosphere of nitrogen. Anhydrous methanol (0.08 mL, 1.896 mmol) and pyridine (0.15 mL, 1.896 mmol) were carefully added dropwise and left to stir for 30 minutes at 0 °C. The white suspension was then stirred at room temperature for 30 minutes. Upon reaction completion, the white precipitate was filtered and triturated several times with anhydrous diethyl ether. The filtrate was concentrated under reduced pressure and purified by flash column chromatography (6:4 ethyl acetate:hexane) to yield **3** as a colorless liquid (351 mg, 89%). The product was isolated as a mixture of enantiomers. ^{1}H NMR (500 MHz, CDCl$_3$): δ 7.32 (dd, J = 8.1, 7.4 Hz, 2H, *H*-3), 7.23-7.16 (m, 3H, *H*-2 + *H*-4), 3.95 (d, J = 13.9 Hz, 3H, *H*-5). ^{31}P NMR (202 MHz, CDCl$_3$): δ 1.06.

Methyl naphthalen-1-yl phosphorochloridate (4). Naphthyl phosphorodichloridate **2** (1.00 g, 3.831 mmol) was added to anhydrous diethyl ether (30 mL) and stirred at 0 °C, under an atmosphere of nitrogen. Anhydrous methanol (0.15 mL, 3.831 mmol) and pyridine (0.34 mL, 3.831 mmol) were carefully added dropwise and left to stir for 30 minutes, at 0 °C. The white suspension was then stirred at room temperature for 30 minutes. Upon reaction completion, the white precipitate was filtered and triturated several times with anhydrous diethyl ether. The filtrate was concentrated under reduced pressure and purified by flash column chromatography (6:4 ethyl acetate:hexane) to yield **4** as a light brown liquid (717 mg, 73%). The product was used in the next step without purification and isolated as a mixture of enantiomers. ^{1}H NMR (500 MHz, CDCl$_3$): δ 8.11 (d, J = 4.6 Hz, 1H, *H*-9), 7.88 (d, J = 8.2 Hz, 1H, *H*-6), 7.75 (d, J = 8.2 Hz, 1H, *H*-4), 7.62-7.49 (m, 3H, *H*-3 + *H*-7 + *H*-8), 7.45 (d, J = 7.9 Hz, 1H, *H*-2), 4.09 (d, J = 14.0 Hz, 3H, *H*-11). ^{31}P NMR (202 MHz, CDCl$_3$): δ -1.46.

((3S,5R)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl methyl phenyl phosphate (5). To a stirring solution of methyl phenyl phosphorochloridate **3** (547 mg, 2.648 mmol) in anhydrous THF (12 mL) was added FdUR (550 mg, 2.207 mmol), under a nitrogen atmosphere. The mixture was then cooled to -78 °C and NMI (0.90 mL, 11.034 mmol) was added dropwise and left to stir for 10 minutes. The reaction mixture was then left to warm to room temperature and stirred for 12 h. The solvent was then removed under reduced pressure and the resultant colorless oil was dissolved in water (30 mL) and the product was extracted into DCM (3 x 30 mL). The combined organic layers were washed with 0.5N HCl (30 mL), dried over MgSO$_4$, filtered and concentrated under reduced pressure to afford a colorless oil. The oil was purified by flash column chromatography (5:95 MeOH:DCM) to yield nucleotide **5** as a white solid (297 mg, 32%). The product was used without purification and isolated as a mixture of diastereoisomers. ^{1}H NMR (500MHz, CDCl$_3$): δ 7.65 (d, J = 6.2 Hz, 1H, *H*-2), 7.33 (dd, J = 7.5, 6.8 Hz, 2H, *H*-14), 7.22-7.13 (m, 3H, *H*-13 + *H*-15), 6.99-6.94 (m, 1H, *H*-4), 6.26 (d, J = 6.5 Hz, 1H, *H*-6), 4.52-4.42 (m, 1H, *H*-8), 4.40 (dd, J = 10.6, 4.1 Hz, 2H, *H*-11), 4.14-4.07 (m, 1H, *H*-9), 3.89 (d, J = 5.5 Hz, 3H, *H*-16), 3.69 (s, 1H, *H*-10), 2.40 (dd, J = 7.8, 2.8 Hz, 1H, *H*-7a/b), 2.02 (dd, J = 14.0, 7.0 Hz, 1H, *H*-7a/b). ^{13}C NMR (126MHz, CDCl$_3$): δ 176.6 (C=O), 156.8 (C-Ar), 150.3 (C=O/C=C), 148.9 (C=O/C=C), 130.0 (CH-Ar), 125.7 (CH-Ar), 124.0 (CH=C), 119.8 (CH-Ar), 85.4 (CH), 84.9 (CH), 70.4 (CH), 67.1 (CH$_2$), 55.4 (CH$_2$), 40.4 (CH$_3$). ^{19}F NMR (471 MHz, CDCl$_3$): δ -164.09, -164.16. ^{31}P NMR (202 MHz, CDCl$_3$): δ -4.88, -4.98. MS (ES+) m/z 439.1 [M+Na]$^{+}$.

((3S,5R)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl methyl naphthalen-1-yl phosphate (6). To a stirring solution of methyl naphthyl phosphorochloridate **4** (990 mg, 3.858 mmol) in anhydrous THF (24 mL) was added FdUR (791 mg, 3.215 mmol), under a nitrogen atmosphere. The mixture was then cooled to -78 °C and NMI (1.28 mL, 16.075 mmol) was added dropwise and left to stir for 10 minutes. The reaction mixture was then left to warm to room

temperature and stirred for 12 h. The solvent was then removed under reduced pressure and the resultant colorless oil was dissolved in water (30 mL) and the product was extracted into DCM (3 x 30 mL). The combined organic layers were washed with 0.5N HCl (30 mL), dried over MgSO$_4$, filtered and concentrated under reduced pressure to afford a brown oil. The oil was purified by flash column chromatography (5:95 MeOH: DCM) to yield nucleotide **6** as a white solid (720 mg, 48%). The product was used without purification and isolated as a mixture of diastereoisomers. ^{1}H NMR (500 MHz, CDCl$_3$): δ 9.93 (bs, 1H, *H*-2), 8.09 (d, *J* = 7.9 Hz, 1H, *H*-20), 7.83 (d, *J* = 7.3 Hz, 1H, *H*-17), 7.66 (dd, *J* = 13.1, 6.4 Hz, 1H, *H*-15), 7.60-7.45 (m, 3H, *H*-14 + *H*-18 + *H*-19), 7.47-7.33 (m, 2H, *H*-4 + *H*-13), 6.17 (dt, *J* = 13.3, 6.4 Hz, 1H, *H*-6), 4.40 (dd, *J* = 10.2, 6.7 Hz, 2H, *H*-11), 4.27 (d, *J* = 6.1, 3.0 Hz, 1H, *H*-9), 4.07 (dd, *J* = 8.5, 2.7 Hz, 1H, *H*-8), 3.92 (d, J = 11.8 Hz, 3H, *H*-22), 2.28 (dd, *J* = 6.4 Hz, 1H, *H*-7a/b), 1.84 (dd, *J* = 13.8, 6.8 Hz, 1H, *H*-7a/b). ^{13}C NMR (126MHz, CDCl$_3$): δ 156.9 (C=O), 149.0 (C), 146.1 (C), 141.5 (C), 139.6 (C), 134.7 (C), 128.0 (CH-Ar), 127.0 (CH-Ar), 126.8 (CH-Ar), 125.6 (CH-Ar), 125.5 (CH-Ar), 123.7 (CH=C), 121.1 (CH-Ar), 115.0 (CH-Ar), 85.3 (CH), 85.0 (CH), 70.9 (CH), 67.7 (CH$_2$), 55.6 (CH$_3$), 40.3 (CH$_2$). ^{19}F NMR (471 MHz, CDCl$_3$): δ -163.84, -164.03. ^{31}P NMR (202 MHz, CDCl$_3$): δ -4.46, -4.61. MS (ES+) m/z 489.1 [M+Na]$^+$.

(((((3S,5R)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)oxy)methyl pivalate (7). To a stirring solution of nucleotide **5** (250 mg, 0.601 mmol) in anhydrous acetonitrile (12 mL), sodium iodide (91 mg, 0.601 mmol), and chloromethyl pivalate (0.15 mL, 0.661 mmol) were added and reflux at 82 ºC under nitrogen for 16 h. Upon reaction completion, the mixture was cooled at room temperature. The solvent was then removed under reduced pressure and the resultant oil was dissolved in water (30 mL) and the product was extracted into DCM (3 x 30 mL) and sodium thiosulphate. The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure to afford an oil. The oil was purified by flash column chromatography (5:95 MeOH: DCM) to yield POMtide **7** which isolated as a white fluffy solid (127mg, 41%). Product isolated as a mixture of diastereoisomers. ^{1}H NMR (500 MHz, CDCl$_3$): δ 7.61 (s, 1H, *H*-2), 7.34 (dd, *J* = 8.1, 7.5 Hz, 2H, *H*-14), 7.24-7.15 (m, 4H, *H*-13 + *H*-15 + *H*-4), 6.30-6.19 (m, 1H, *H*-6), 5.83-5.62 (m, 2H, *H*-16), 4.49 (ddd, *J* = 17.1, 8.5, 3.7 Hz, 1H, *H*-8), 4.41 (dd, *J* = 6.3, 2.8 Hz, 2H, *H*-11), 4.11 (dt, *J* = 6.1, 3.2 Hz, 1H, *H*-9), 3.76-3.71 (m, 1H, *H*-10), 2.46-2.30 (m, 1H, *H*-7a/b), 2.02 (dt, *J* = 13.7, 6.8 Hz, 1H, *H*-7a/b), 1.18 (d, *J* = 5.0 Hz, 9H, *H*-19). ^{13}C NMR (126MHz, CDCl$_3$): δ 177.1 (C=O), 156.9 (C), 148.9 (C), 141.5 (C), 139.6 (C), 130.0 (CH-Ar), 125.9 (CH-Ar), 123.8 (CH=C), 119.9 (CH-Ar), 85.4 (CH), 84.8 (CH), 83.3 (CH$_2$), 70.6 (CH), 67.4 (CH$_2$), 40.2 (CH$_2$), 38.8 (C), 26.8 (CH$_3$). ^{19}F NMR (471 MHz, CDCl$_3$): δ -163.77, -163.81. ^{31}P NMR (202 MHz, CDCl$_3$): δ -7.59, -7.82. MS (ES+) m/z 539.1 [M+Na]$^+$. HRMS (ESI) C$_{21}$H$_{26}$FN$_2$NaO$_{10}$P calcd. 539.1201 [M+Na]$^+$, found 539.1208. HPLC (reverse-phase) MeOH/H$_2$O 0:100 to 100:0 in 12 min, λ = 254 nm, t_R = 4.55 min (94%).

(((((3S,5R)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)oxy)methyl pivalate (8). To a stirring solution of nucleotide **6** (100 mg, 0.214 mmol) in anhydrous acetonitrile (12 mL), sodium iodide (32 mg, 0.214 mmol), and chloromethyl pivalate (0.03 mL, 4.516 mmol) were added and reflux at 82 ºC under nitrogen for 16 h. Upon reaction completion, the mixture was cooled at room temperature. The solvent was then removed under reduced pressure and the resultant oil was dissolved in water (30 mL) and the product was extracted into DCM (3 x 30 mL) and sodium thiosulphate. The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure to afford

an oil. The oil was purified by flash column chromatography (5:95 MeOH: DCM) to yield POMtide **8** which isolated as a fluffy white solid (48 mg, 40%). Product isolated as a mixture of diastereoisomers. ^{1}H NMR (500 MHz, CDCl$_3$): δ 9.42 (bs, 1H, *H*-2), 8.09 (d, *J* = 8.5 Hz, 1H, *H*-20), 7.84 (dd, *J* = 6.2, 4.4 Hz, 1H, *H*-17), 7.69 (dd, J = 8.0, 3.7 Hz, 1H, *H*-15), 7.59 – 7.47 (m, 3H, *H*-14 + *H*-18 + *H*-19), 7.49-7.34 (m, 2H, *H*-4 + *H*-13), 6.16 (dd, *J* = 9.6, 3.6 Hz, 1H, *H*-6), 5.91-5.64 (m, 2H, *H*-22), 4.50-4.35 (m, 2H, *H*-11), 4.29 (dd, *J* = 8.0, 4.8 Hz, 1H, *H*-9), 4.07 (dt, *J* = 6.0, 4.5 Hz, 1H, *H*-8), 3.34 (bs, 1H, *H*-10), 2.40-2.11 (m, 1H, *H*-7a/b), 1.77 (dt, J = 13.9, 6.8 Hz, 1H, *H*-7a/b), 1.14 (d, *J* = 9.8 Hz, 9H, *H*-25). ^{13}C NMR (126MHz, CDCl$_3$): δ 177.0 (C=O), 156.7 (C), 148.7 (C), 141.4 (C), 139.6 (C), 134.8 (C), 127.9 (CH-Ar), 127.0 (CH-Ar), 126.8 (CH-Ar), 125.8 (CH-Ar), 125.4 (CH-Ar), 123.7 (CH=C), 121.2 (CH-Ar), 115.3 (CH-Ar), 85.4 (CH), 84.8 (CH), 83.4 (CH$_2$), 70.7 (CH), 67.5 (CH$_2$), 40.1 (CH$_2$), 38.7 (C), 26.7 (CH$_3$). ^{19}F NMR (471 MHz, CDCl$_3$): δ -163.52, -163.64. ^{31}P NMR (202 MHz, CDCl$_3$): δ -7.13, -7.37. MS (ES+) m/z 589.1 [M+Na]$^+$. HRMS (ESI) C$_{25}$H$_{28}$FN$_2$NaO$_{10}$P calcd. 589.1358 [M+Na]$^+$, found 589.1366. HPLC (reverse-phase) MeOH/H$_2$O 0:100 to 100:0 in 12 min, λ = 254 nm, t_R = 6.27 min (96%).

MTT assay. This was carried out using according to our previously reported procedure.[21]

Western blotting. Immunoblotting was performed according to the reported procedure.[22] The primary antibodies used in this work were human Cathepsin A (Thermo Fisher, USA) [1:1,000 dilution], Hint-1 (Atlas Antibodies AB, Sweden) [1:500 dilution], and GAPDH (Cell Signalling Technology) [1:5,000 dilution].

Gene expression. The cBIO Cancer Genomics Portal was used to assess the expression of cathepsin A and Hint-1 across different cancers. The database chosen from this portal was the Cancer Cell Line Encyclopedia (Broad Institute and Novartis) which contained 1,019 different tumour types. Values obtained were given mRNA expression z-Scores following microarray analysis.

Statistical analysis. The data obtained in these experiments were evaluated using two-way ANOVA followed by Dunnett test. The values were compared to the control (FdUR). The samples were run in triplicates or replicates as stated in the figure legends and the values are shown as mean ± SEM from each replicate data set. All statistical analyses were performed using Graphpad Prism 6.2 software (GraphPad Software Inc., San Diego, CA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting Table S1 (PDF)
Supporting Figures S1-S5 (PDF)
Synthesis of FdUR ProTide (PDF)
HPLC chromatograms of 7, 8 and FdUR ProTide (PDF)
Molecular Formula Strings (CSV)

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Author Contributions

A.S.A. and A.M. synthesized the compounds reported in this work and performed the stability and metabolism studies. A.S.A. carried

out the cell cytotoxicity experiments. A.S.A., A.M. and Y.M. designed the experiments. Y.M. supervised the work. The manuscript was written by Y.M. and all authors have given approval to the final version of the manuscript.

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Notes

ABBREVIATIONS

*bis*POM, *bis*-pivaloyloxymethyl; DCM, dichloromethane; FdUR, 5-fluoro-2'-deoxyuridine; NMI, 1-methylimidazole; POM, pivaloyloxymethyl; POMCl, pivaloyloxymethyl chloride.

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Table of Contents graphic:
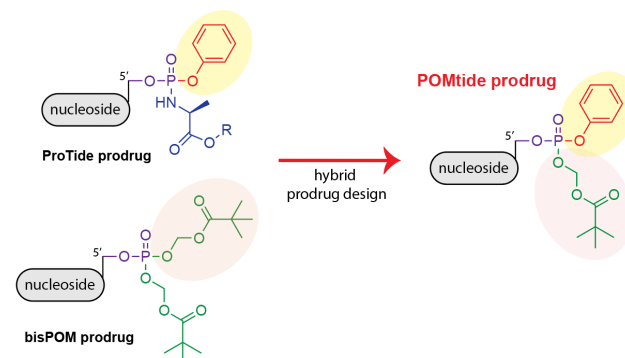
Supporting Information

Aryloxy Pivaloyloxymethyl Prodrugs (POMtides) as Nucleoside Monophosphate Prodrugs

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Contents:

Supporting Table S1. Calculated physicochemical properties of FdUR, FdUR POMtides **7** and **8**, FdUR ProTide and FDUR *bis*POM. These were calculated using SwissADME (http://www.swissadme.ch/).

| Compound	Molecular Weight (g/mol)	cLogP*	Bioavailability Score**
FdUR	246.19	-0.53	0.55
FdUR POMtide 7	516.41	1.82	0.17
FdUR POMtide 8	566.47	2.61	0.17
FdUR ProTide (*L*-alanine benzyl ester)	563.47	1.89	0.17
FdUR *bis*POM	554.46	1.46	0.17

*ClogP values presented in the table are consensus logP values that are the average of five different logP values obtained using five different logP calculation systems. See http://www.swissadme.ch/ for further details.
**The bioavailability score is the probability of F > 10% in rat. See http://www.swissadme.ch/ for further details.
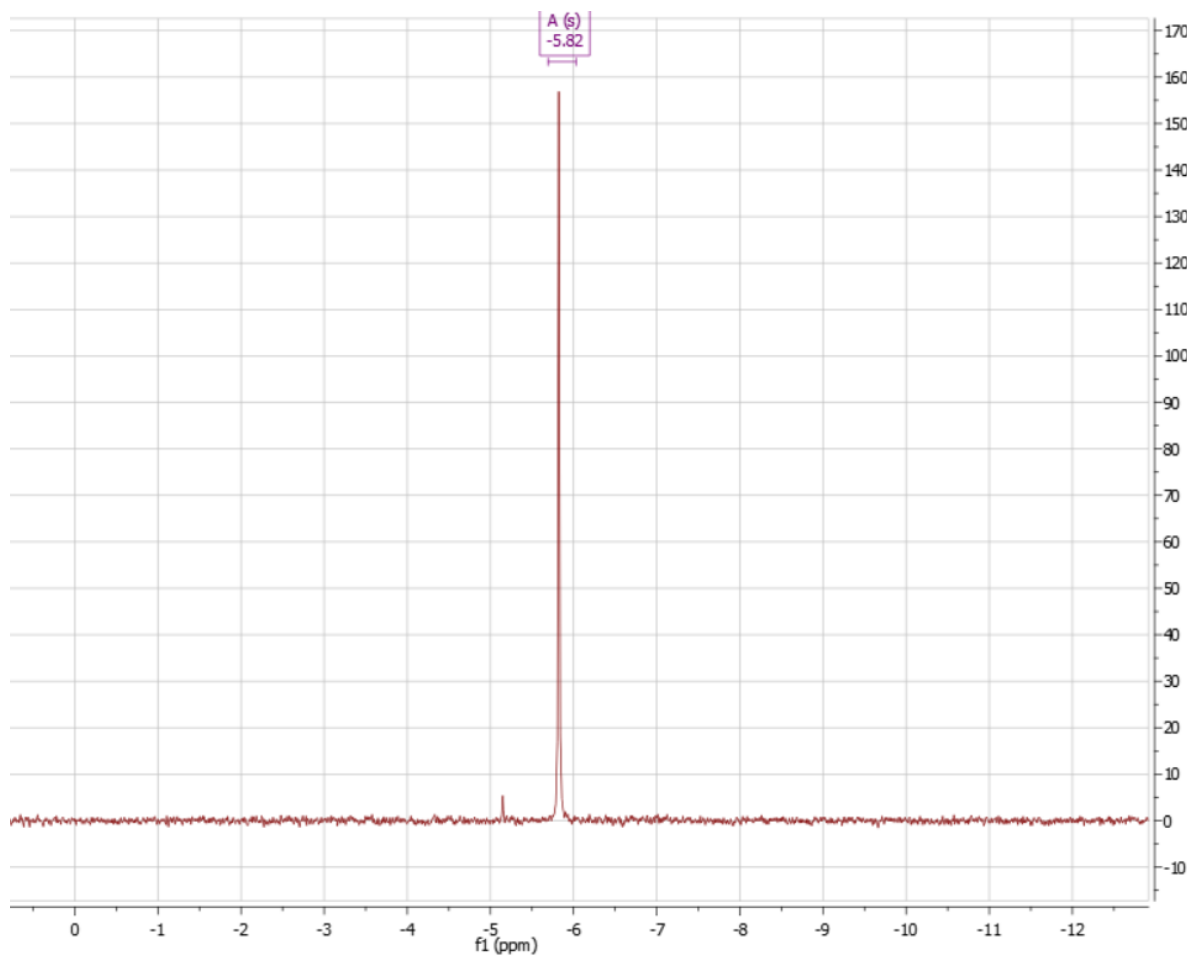
Supporting Figure S1. ^{31}P NMR of POMtide **7** showing the two distinct ^{31}P peaks corresponding to the two FdUR POMtide diastereoisomers. The -50 to +50ppm ^{31}P NMR is shown in the top left, and the zoomed in -10 to -6ppm spectra is presented as the major spectra.
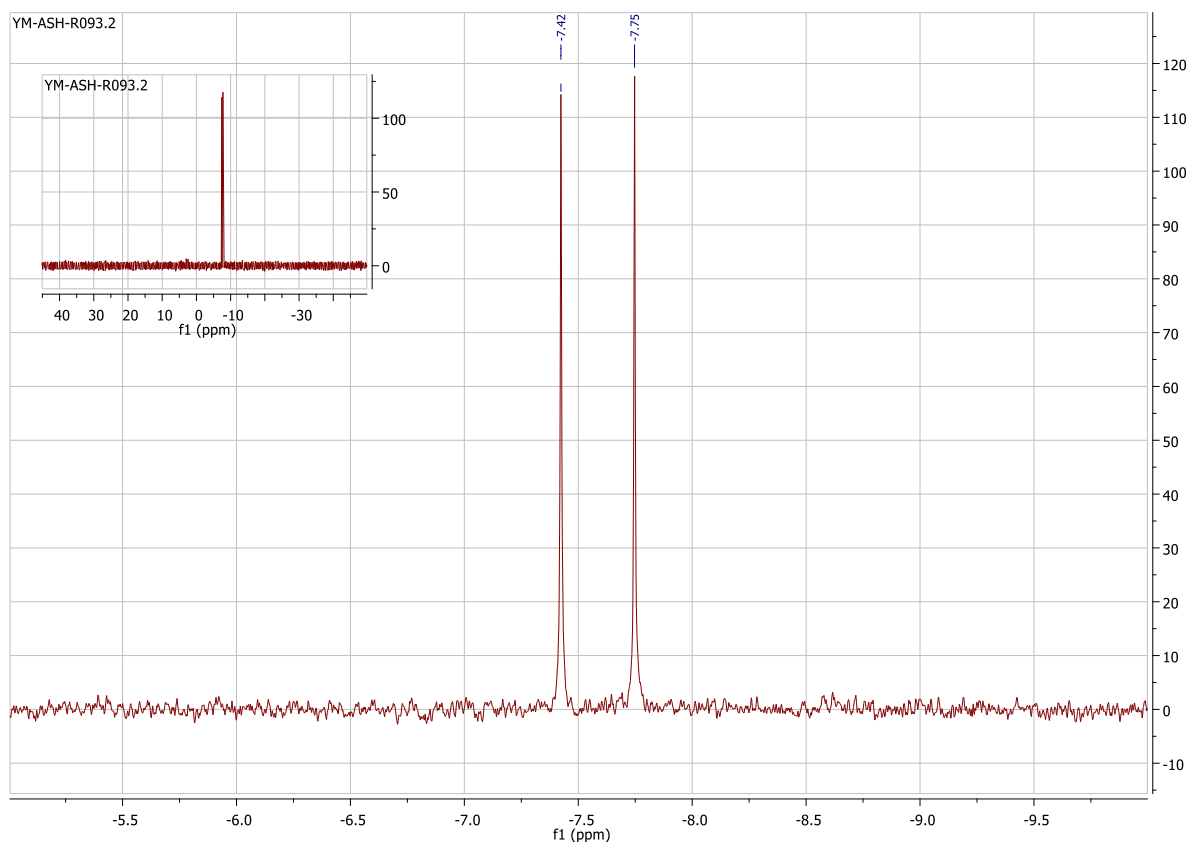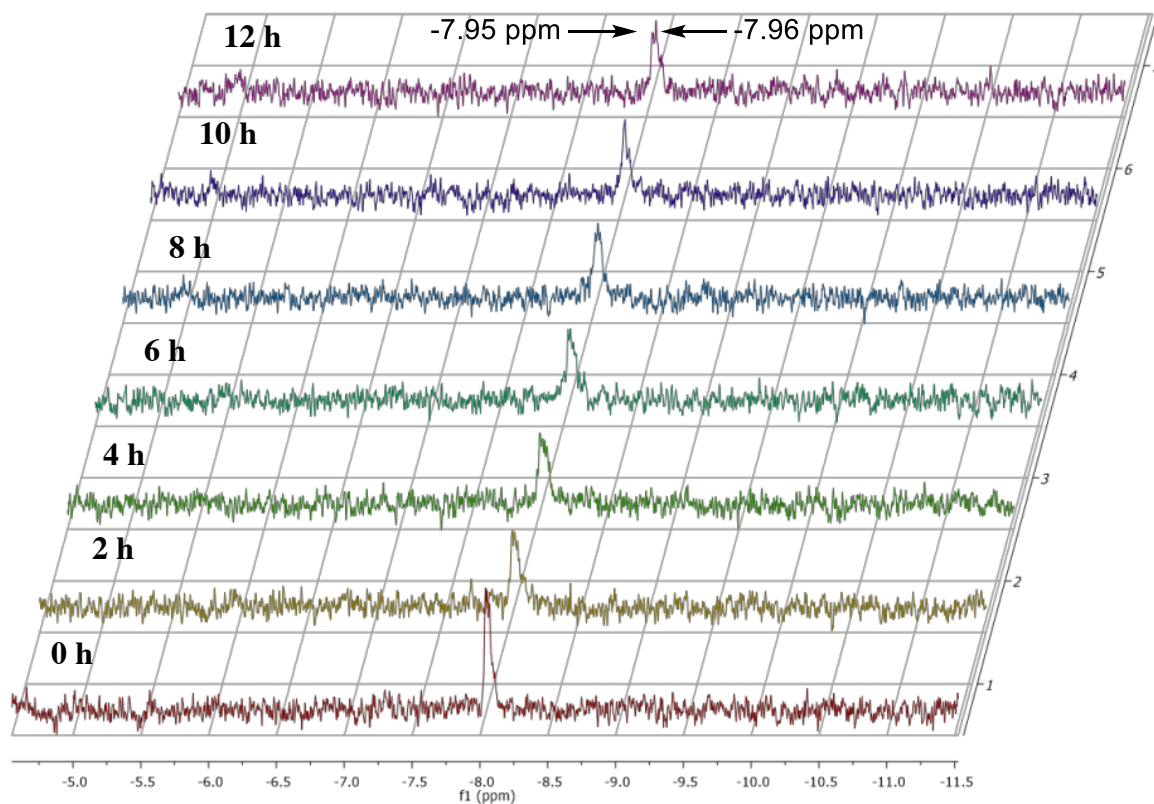
Supporting Figure S2. ^{31}P NMR of human serum showing the presence of a phosphorous peak in the serum at 1.93ppm.
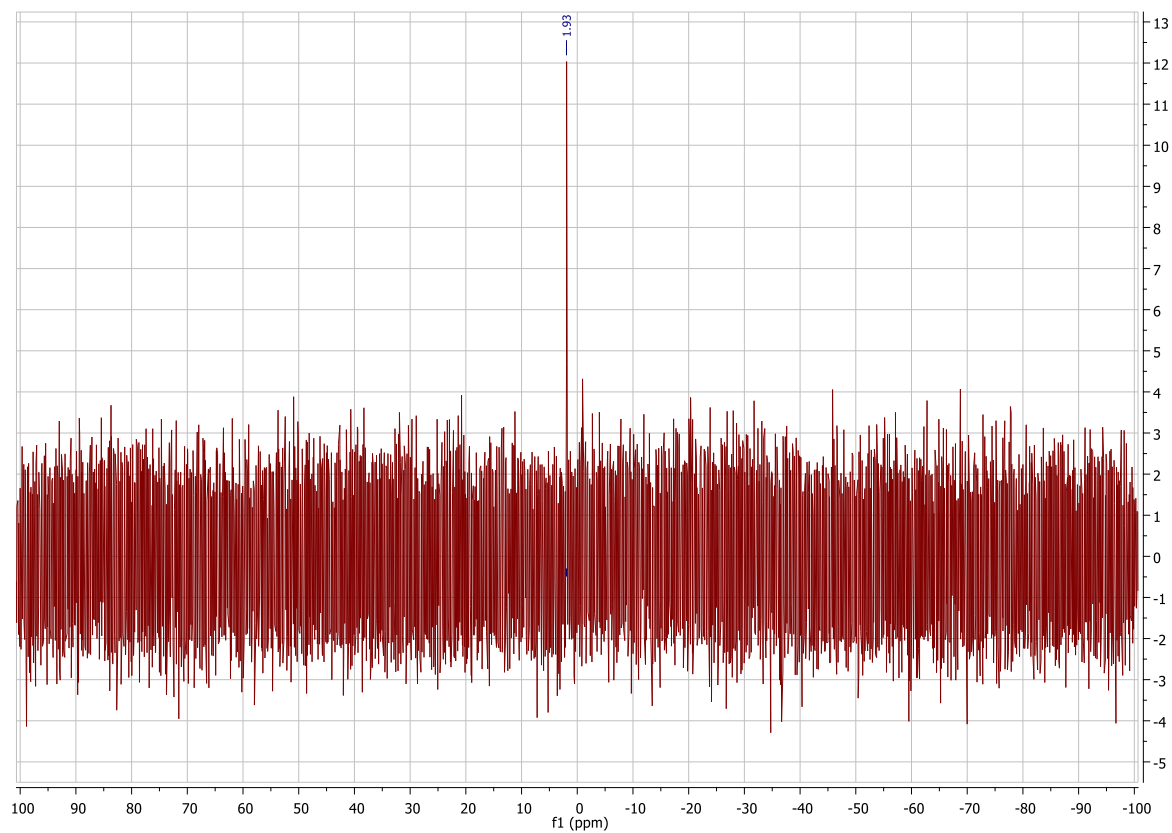
**Supporting Figure S3.** ^{31}P NMR of metabolite **B** showing a phosphorous peak at -5.82ppm.

Supporting Figure S4. ^{31}P NMR spectra of POMtide **7** after 12 h incubation with recombinant of carboxypeptidase Y at 37 °C. The ^{31}P NMR shown are at 2 h intervals.

Supporting Figure S5. ^{31}P NMR spectra of POMtide **7** with porcine liver esterase at 37 °C. (**A**) ^{31}P NMR spectra of POMtide **7** incubation with porcine liver esterase for 12 h with ^{31}P NMR spectra shown at 2 h intervals. Only the two ^{31}P NMR singlets of POMtide **7** (-7.89 and -7.95 ppm are present throughout). (**B**) ^{31}P NMR spectra of POMtide **7** incubation with porcine liver esterase after 24 and 48 h incubation. Along with the major two ^{31}P NMR singlets of POMtide **7**, a minor phosphorous singlet peak on the ^{31}P NMR spectra appeared at -5.14 ppm. (**C**) Addition of synthetic metabolite **B** (5 mg) to the sample from 48 h incubation that contains POMtide **7** and porcine liver esterase. The ^{31}P NMR peak of metabolite B overlaps perfectly with the original ^{31}P NMR peak at -5.14 that was generated following the incubation of FdUR POMtide **7** with porcine liver esterase.

B.

C.
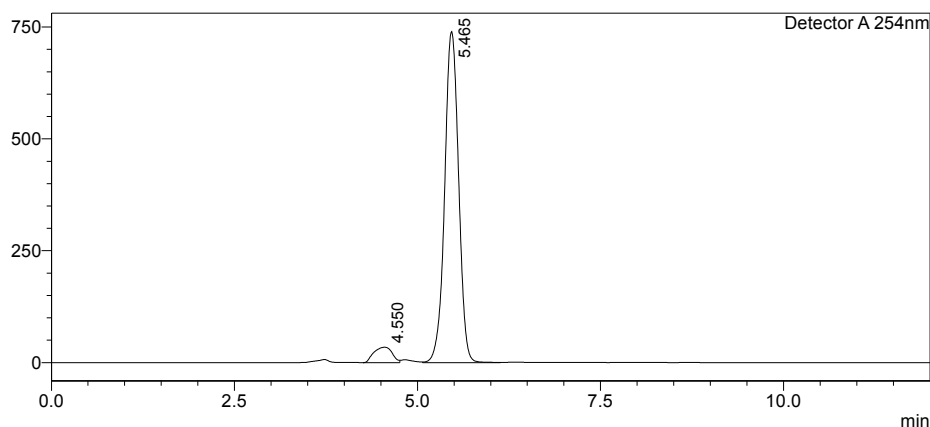

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HPLC chromatogram of POMtide 7

Analysis Report

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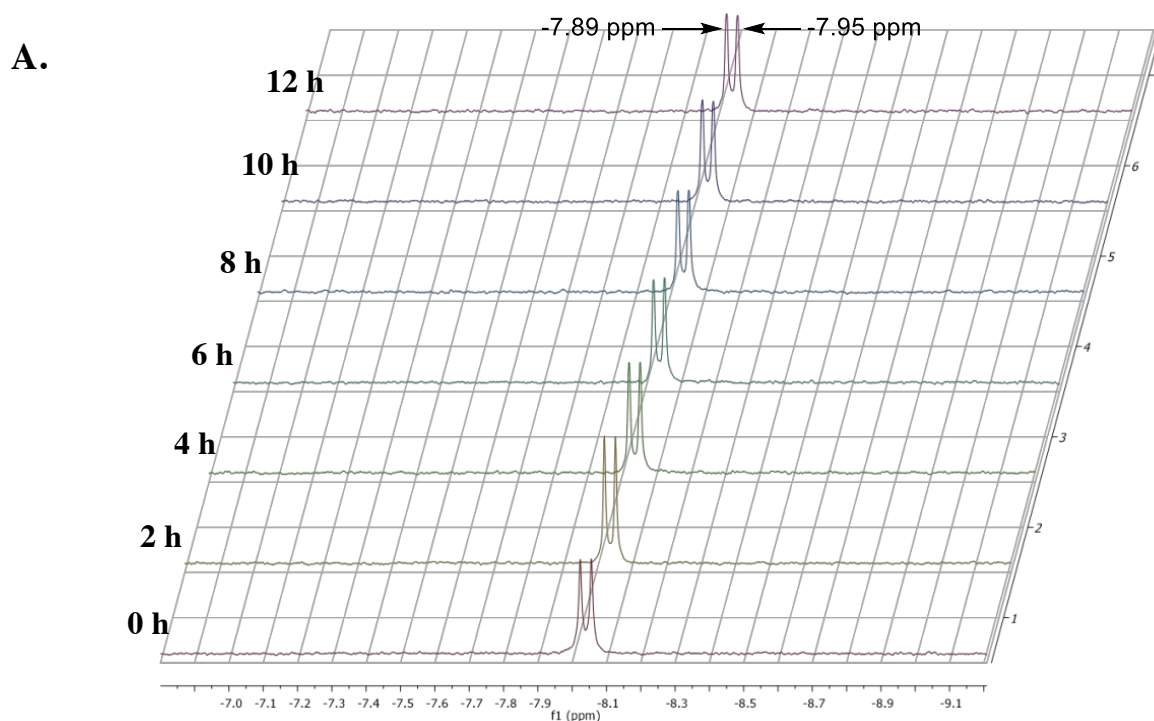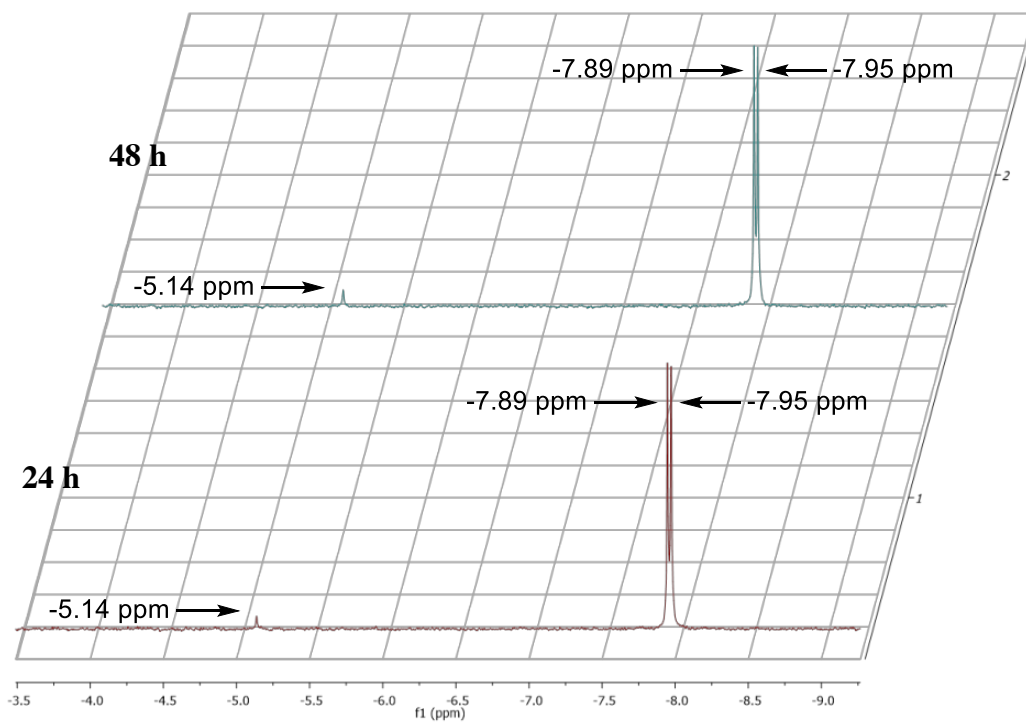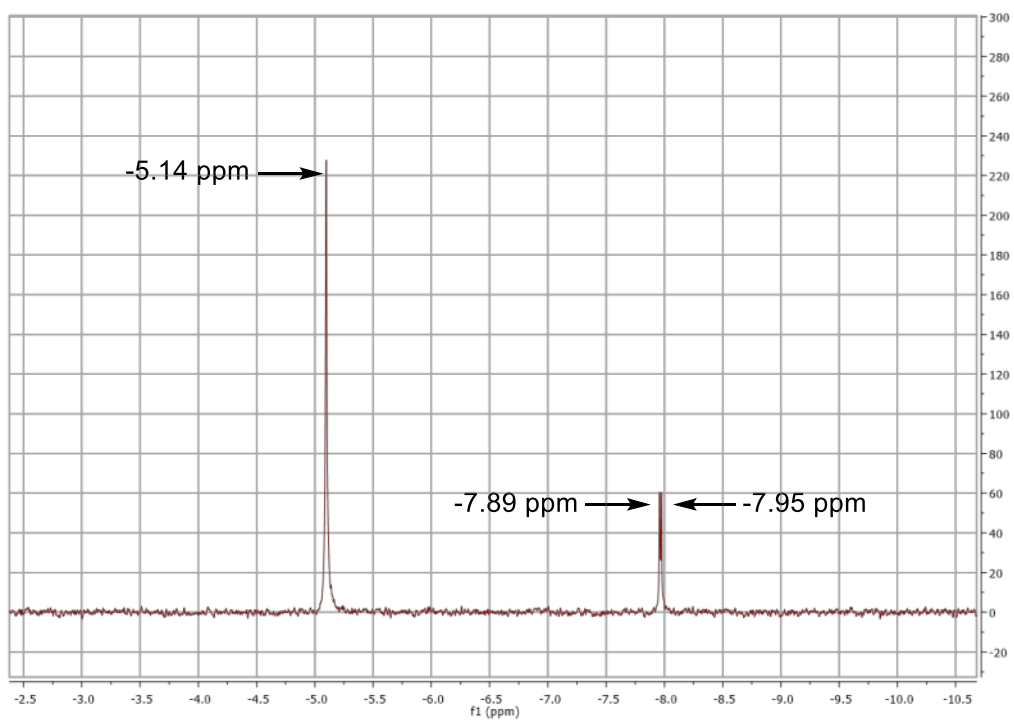
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Date Processed	: 27/03/2019 14:46:01

Sample Type   : Unknown

Acquired by   : System Administrator
Processed by   : System Administrator

\<Chromatogram\>

\<Peak Table\>

Detector A 254nm

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1	4.550	598294	34337	5.762
2	5.465	9785470	739537	94.238
Total		10383764	773874	100.000

C:\LabSolutions\Data\YM\Ashwag\YM-ASH-R094.lcd

HPLC chromatogram of POMtide 8

Analysis Report

<Sample Information>

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Batch Filename   : BATCH 0.5MLMIN 270319.lcb
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<Peak Table>

Detector A 254nm

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3	7.794	87422	6085	2.203
Total		3968168	295055	100.000

C:\LabSolutions\Data\YM\Ashwag\YM-ASH-R101.lcd

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HPLC chromatogram of FdUR ProTide

Analysis Report

\<Sample Information\>

Sample Name	: YM-ASH-R056		
Sample ID	: YM-ASH-R056		
Data Filename	: YM-ASH-R056.lcd		
Method Filename	: method 0.5mlmin80-20 051218.lcm		
Batch Filename	: YM-ASH-R052.lcb		
Vial #	: 1-2	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 19/07/2019 10:50:56	Acquired by	: System Administrator
Date Processed	: 19/07/2019 11:02:57	Processed by	: System Administrator

\<Chromatogram\>

\<Peak Table\>

Detector A 254nm

Peak#	Ret. Time	Area	Height	Conc.	Area%
1	4.589	45189	3078	0.000	0.692
2	4.889	74470	5737	0.000	1.140
3	5.212	9508	899	0.000	0.146
4	5.518	10059	699	0.000	0.154
5	5.878	16010	1306	0.000	0.245
6	6.255	6346415	455140	0.000	97.194
7	7.063	4202	314	0.000	0.064
8	7.892	2250	168	0.000	0.034
9	8.460	2817	186	0.000	0.043
10	9.787	18725	313	0.000	0.287
Total		6529645	467840		100.000

C:\LabSolutions\Data\YM\Ashwag\YM-ASH-R056.lcd