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# Kinase-Independent Phosphoramidate S1P<sub>1</sub> Receptor Agonist Benzyl Ether Derivatives

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## Key words

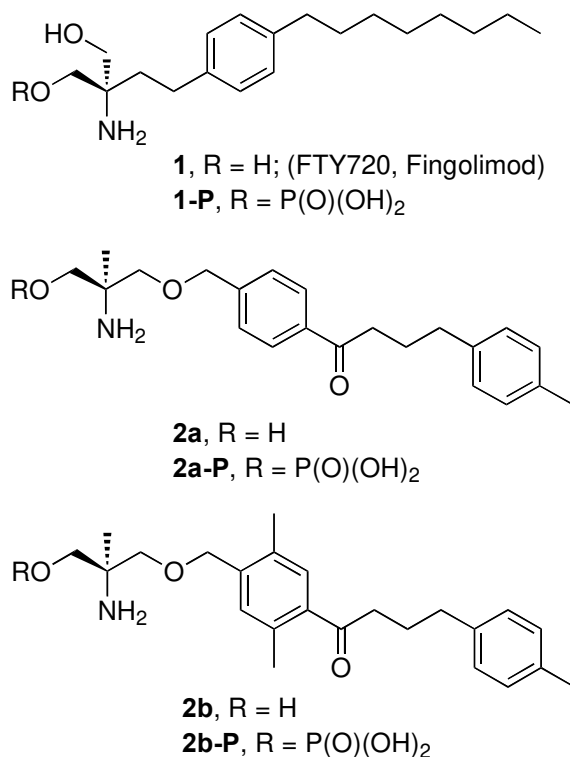
S1P<sub>1</sub> receptor agonist, Benzyl ether derivative, Phosphoramidate, ProTide, Sphingosine kinase, Enzymatic processing

## Abstract

Previously published S1P receptor modulator benzyl ether derivatives have shown potential as being viable therapeutics for the treatment of neurodegenerative diseases, however, two of the most S1P<sub>1</sub>-selective compounds are reported as being poorly phosphorylated by kinases *in vivo*. Phosphoramidates of BED compounds (2a, 2b) were synthesised with the aim of producing kinase-independent S1P receptor modulators. Carboxypeptidase, human serum and cell lysate processing experiments were conducted. ProTide BED analogues were found to have an acceptable level of stability in acidic and basic conditions and *in vitro* metabolic processing experiments showed that they are processed to the desired pharmacologically active monophosphate. The research describes the development of an entirely novel family of therapeutic agents.

Sphingosine 1-phosphate (S1P) receptor modulators have been the subject of significant interest since the emergence of FTY720 (fingolimod, **1**) as the world's first FDA approved oral treatment for relapsing-remitting multiple sclerosis.<sup>1</sup> S1P receptors are G protein-coupled receptors expressed in a variety of tissues and cover a broad range of different physiological functions including lymphocyte trafficking.<sup>2</sup> Sphingosine is phosphorylated by sphingosine kinase to form the natural S1P receptor ligand sphingosine 1-phosphate.<sup>3</sup> **1** is phosphorylated similarly to fingolimod-phosphate (**1-P**) by sphingosine kinase.<sup>4</sup> Agonism of the S1P<sub>1</sub> receptor by **1-P** induces sequestration of T-cells in the lymphocytes and thereby reduces the infiltration of lymphocytes into the central nervous system.<sup>5</sup>

Achieving a high level of S1P<sub>1</sub> selectivity for novel S1P receptor modulators has been the focus for a number of academic and industrial groups.<sup>6,7,8</sup> **1-P** is reported to have comparable affinities to the S1P<sub>1</sub> and S1P<sub>3</sub> receptors (Table 1) and it is the agonism of the S1P<sub>3</sub> receptor which has been attributed to some of FTY720's unwanted side effects such as bradycardia and hypertension.<sup>1,2</sup> Benzyl ether derivatives **2a-P** and **2b-P** previously published by Tsuji *et al* were reported to have far greater S1P<sub>1</sub> selectivity than **1-P** with EC<sub>50</sub> values for S1P<sub>3</sub> significantly higher than for S1P<sub>1</sub> (Table 1).<sup>9</sup> However, the analogues reported as being the most S1P<sub>1</sub>-selective such as **2b-P** were found to be poorly phosphorylated by kinases in rat blood and were not progressed.



**Figure 1.** Structures of FTY720 (Fingolimod, **1**), benzyl ether derivatives (**2a** and **2b**) and their phosphates (**1-P**, **2a-P** and **2b-P**).<sup>9</sup>

Phosphoramidate (ProTide) technology is a phosphate delivery technology which is typically applied to nucleosides.<sup>10,11</sup> Often the rate-limiting step of nucleoside activation is phosphorylation by kinases and therefore phosphoramidate technology is applied to nucleosides to bypass the rate-limiting step.<sup>12</sup> ProTide technology masks the charges of the phosphate motif and increases the lipophilicity of the nucleoside and aids passive diffusion of the compound across the cell membrane.<sup>13</sup> Once inside the cell ProTide nucleosides have been found to be processed to the monophosphate by enzymes such as cathepsin A and Hint.<sup>13,14,15</sup>

**Table 1.** In vitro agonist-evoked GTP $\gamma$ S binding of phosphates of FTY720 (**1-P**) and benzyl ether derivatives **2a-P** and **2b-P** to human S1P<sub>1</sub> and S1P<sub>3</sub> as reported by Tsuji *et al.*<sup>9</sup>

Compound	$\gamma$ GTP EC <sub>50</sub> (nM)		Selectivity (S1P <sub>3</sub> / S1P <sub>1</sub> )
	S1P <sub>1</sub>	S1P <sub>3</sub>	
<b>1-P</b>	7.0	2.0	0.29
<b>2a-P</b>	7.0	200	29
<b>2b-P</b>	6.5	>20,000	>3077

It was decided that the application of phosphoramidate technology to previously published benzyl ether derivatives<sup>9</sup> might be an effective way of improving the efficacy of the parent compounds particularly in kinase deficient environments. The synthesis of benzyl ether derivatives **2a** and **2b** was attempted and successfully achieved. The aim was then to synthesise phosphoramidate analogues and determine using *in vitro* testing whether ProTide BED analogues can be processed to the pharmacologically active monophosphate.

The synthesis of benzyl ether derivatives **2a** and **2b** was successfully achieved by carefully following the reported experimental procedures in the original paper<sup>9</sup> and a number of other sources.<sup>16-22</sup> Some minor modifications to the synthetic procedure previously reported by Tsuji *et al* were made. The most significant alterations to the reported synthetic procedure were made to the synthesis of **2b**. The synthesis of the compounds in the early stages of the total synthesis (compounds **4** – **7**) were developed using experimental procedures from a number of sources and repeated experimentation was required in order to synthesise the desired compounds with acceptable yields.

An amalgamation of synthetic procedures found in three papers<sup>9,16,17</sup> was eventually developed to yield **4** in acceptable yields (>80%). The synthesis of the **5** was eventually tuned after a similar process of experimentation and a combination of different experimental procedures from different papers.<sup>9,17,18</sup> Notably a Dean Stark trap was used for the synthesis of **4** and **5**. The synthesis of **6** also underwent a number of variations and the final procedure was also devised from a

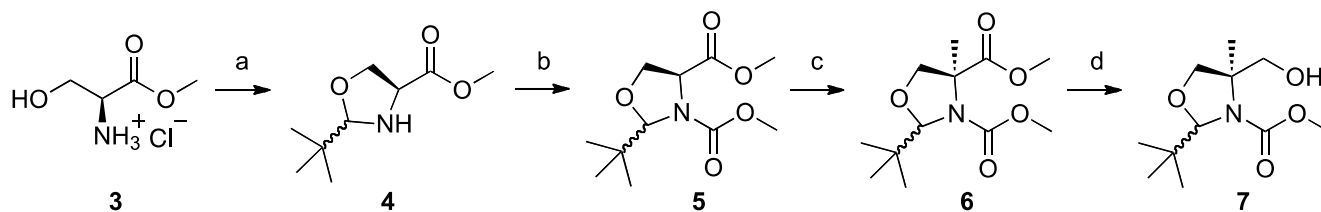
combination of procedures from different papers<sup>9,19,20</sup> and fine tuning of the experimental technique. An unusual quirk of the synthesis, as shown in scheme 1, is that if the reaction is done at -78 °C the whole mixture congeals and forms a hard solid but if the mixture is kept at -74 °C the reaction is effective. At temperatures much warmer than -74 °C the yield is compromised. Achieving this delicate balance in temperature was accomplished by simply conducting the reaction in a dry ice bath with no added acetone. Synthesis of **7** underwent fewer variations than the preceding three compounds. The synthesis was developed principally using two papers.<sup>9,20</sup> The key modifications made to the original procedure devised were an increase in temperature to 45 °C from room temperature, addition of toluene to the reaction mixture and the use of LiCl and KBH<sub>4</sub> as opposed to the more moisture sensitive LiBH<sub>4</sub>.

Once achieving satisfactory yields of **4**, **5**, **6**, and **7** was attained the rest of the synthetic process for the synthesis of **2a** was more straightforward to accomplish as the later stages are detailed in the experimental section of the lead paper.<sup>9</sup> The final **2a** compound synthesised was analysed using polarimetry to measure the optical rotation giving an optical rotation value of  $[\alpha] = 25.12$  ( $[\alpha]_D^{24} = 0.5$ ) suggesting that the enantioselective chirality of the starting amino acid ester was retained and the final product is not a 50/50 racemic mixture.

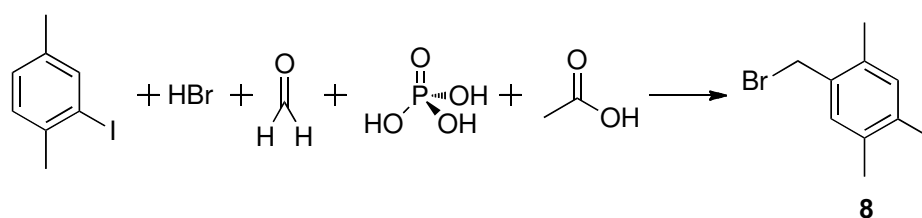
The synthesis of **2b** was done in a different manner to that reported in the original paper.<sup>9</sup> Originally 1-bromo-4-(bromomethyl)-2,5-dimethylbenzene as opposed to **8** was synthesised<sup>21</sup> but a complication was encountered in the synthesis of **2b** when coupling with **9** was unsuccessfully attempted. It has been suggested that the butyl lithium reagent successfully used in the synthesis of **2a** deprotonates the adjacent ortho methyl group on the bromo benzene ring. As iodine has been reported as being a much more reactive leaving group than bromine in Weinreb amide coupling reactions,<sup>22</sup> the synthesis of 1-iodo-4-(bromomethyl)-2,5-dimethylbenzene was attempted (scheme 2). Using a reported synthetic strategy as a basis for trying an alternative isopropylmagnesium chloride base for the Weinreb amide coupling step (step b in scheme 4) proved to be an effective way of forming the bond.<sup>22</sup>

Some alterations were made to the final stages of the synthesis of **2a** and **2b**. The final two steps (as shown in steps c and d in scheme 4) are reported in the same experimental procedure and no

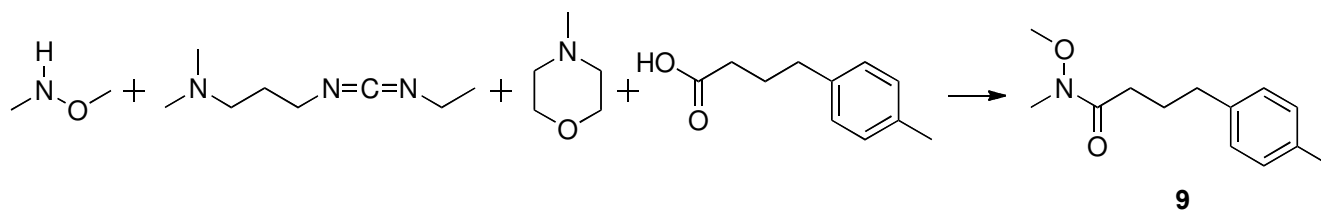
column is reportedly used to purify the product.<sup>9</sup> These stages were conducted separately and a column was used.



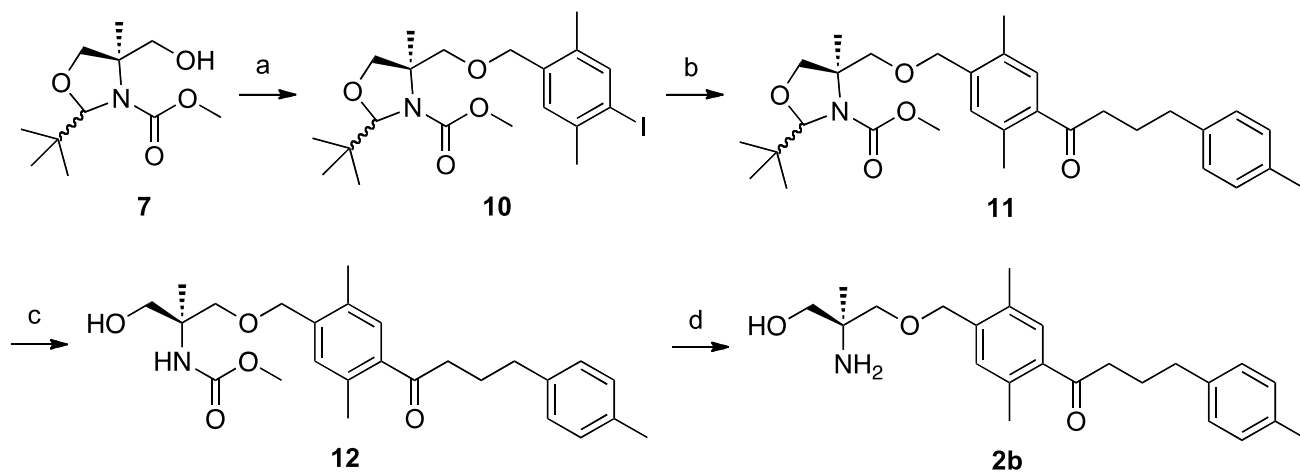
**Scheme 1.** Reagents and conditions: (a) *t*-BuCHO, Et<sub>3</sub>N, toluene, 150 °C, Dean Stark, 24 h, 83%; (b) CH<sub>3</sub>CO<sub>2</sub>Cl, Et<sub>3</sub>N, toluene, 150 °C, Dean Stark, 24 h, 77%; (c) MeI, LiHDMS, DMPU, THF, hexane, -74 °C, 4.5 h, 86%; (d) LiCl, KBH<sub>4</sub>, toluene, THF, 45 °C, 24 h, 80%.



**Scheme 2.** Conditions: Reflux, 24 h, 42%.

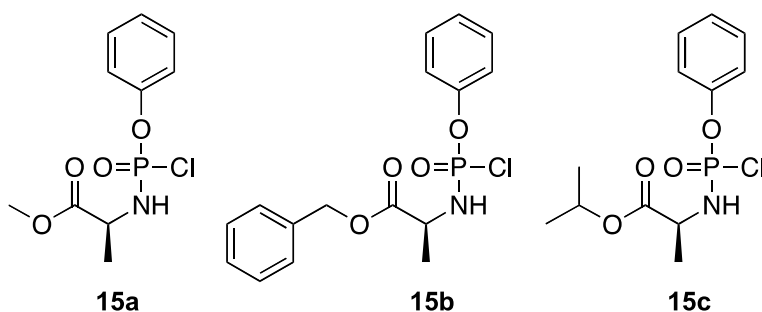


**Scheme 3.** Conditions: DCM, 0 °C – RT, 24 h, 77%.



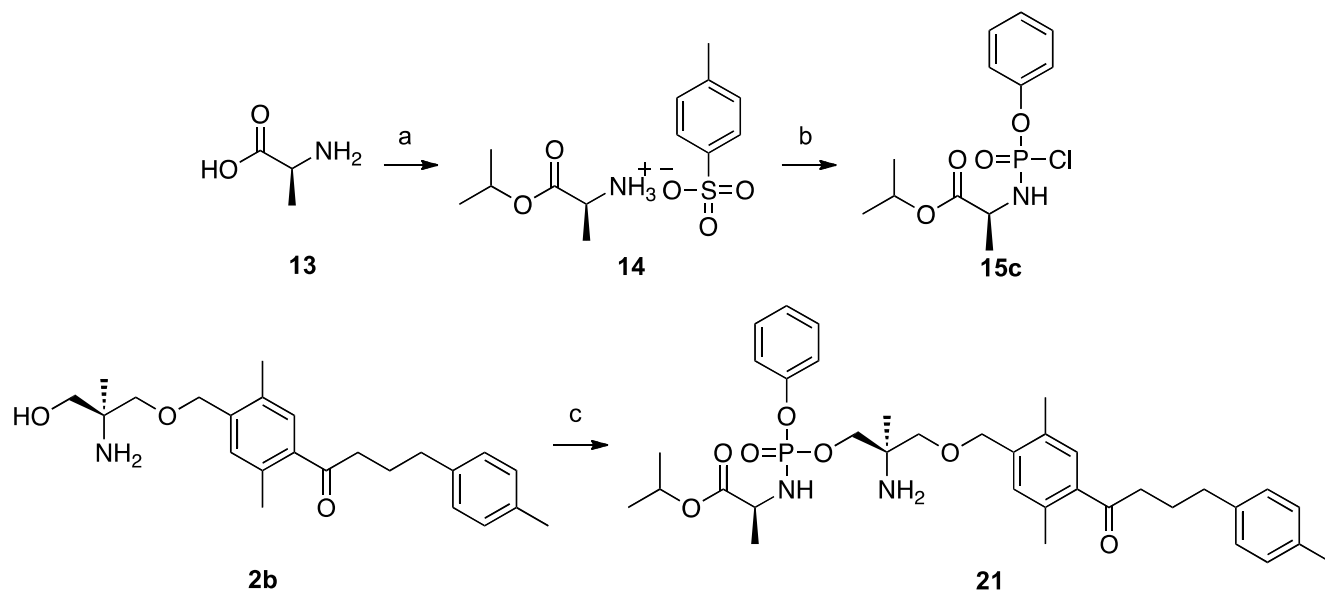
**Scheme 4.** Reagents and conditions: (a) **8**, NaH, DMF, 24 h, 77%; (b) **9**, *i*PrMgCl, THF, -78 °C – RT, 3 h, 42%; (c) p-TsOH monohydrate, MeOH, 50 °C, 24 h, 85%; (d) KOH, EtOH, 90 °C, 24 h, 56%.

The synthesis of phosphoramidate compounds was achieved using previously published procedures.<sup>23,24</sup> A total of six different phosphoramidate benzyl ether derivatives (**16** – **21**) were synthesised. More than two different primary peaks were observed in the <sup>31</sup>P NMR spectra for compounds **16** – **21** likely due to racemisation of the chiral carbon centre of the benzyl ether derivatives under high temperature. The aryl group of the phosphoramidate chosen was simple phenol as opposed to naphthol as the lipophilicity of the parent BED analogues is already comparatively high. L-alanine was chosen as the amino acid component as all the different ProTide prodrugs which have made it to clinical trials have possessed this functionality.<sup>11</sup> The three different ester components chosen were methyl, benzyl and isopropyl groups (figure 2). A benzyl L-alaninyl phosphoramidate moiety is used in Acelarin or NUC-1031<sup>12</sup> and an isopropyl L-alaninyl phosphoramidate moiety is used in the FDA approved Sofosbuvir used to treat hepatitis C.<sup>24</sup>



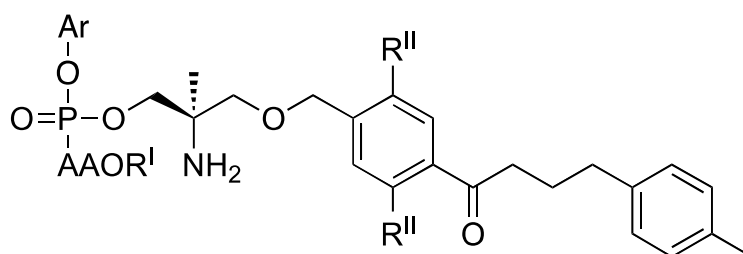


**Figure 2.** Structures of the phosphorochloridates synthesised with methyl (**15a**), benzyl (**15b**) and isopropyl (**15c**) ester groups.



**Scheme 5.** Reagents and conditions: (a) Isopropanol, p-TsOH monohydrate, toluene, 120 °C, 24 h, 88%; (b) Phenyl dichlorophosphate, Et<sub>3</sub>N, DCM, -78 °C – RT, 2 h, 55%; (c) **15c**, tBuMgCl, THF, 24 h, 15%.

**Table 2.** Synthesised phosphoramidate BED analogues.

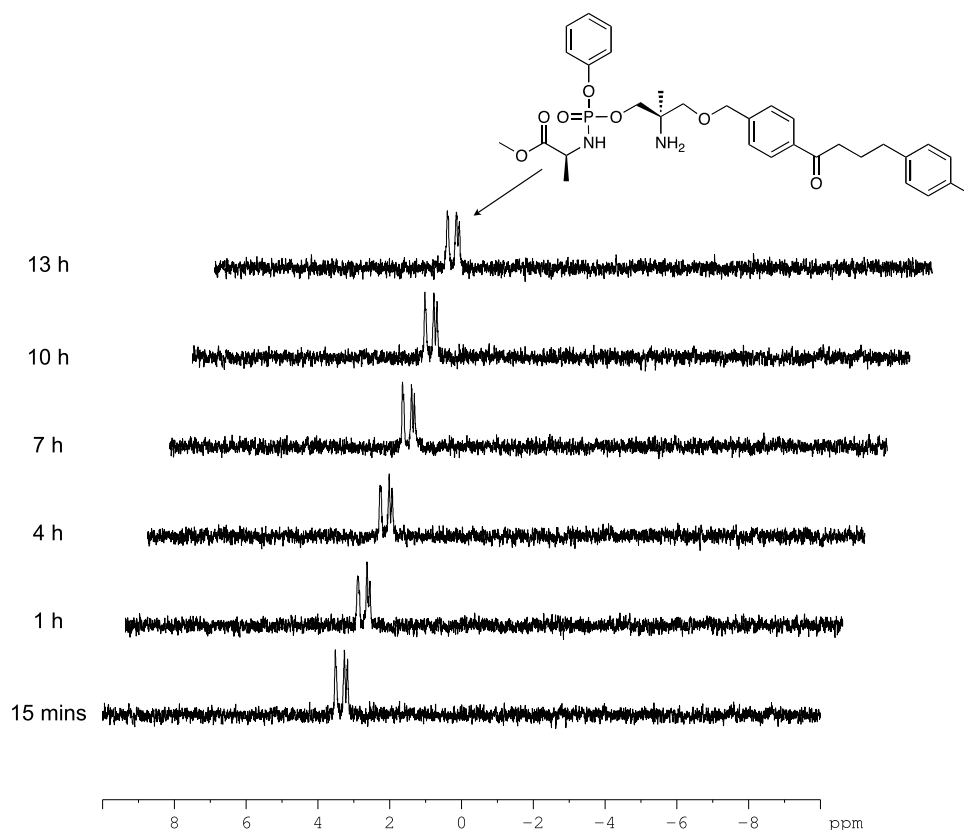


<b>Compound</b>	<b>Ar</b>	<b>AA</b>	<b>R<sup>I</sup></b>	<b>R<sup>II</sup></b>	<b>% Yield</b>	<b><sup>31</sup>P NMR <math>\delta</math> (ppm)</b>
<b>16</b>	Ph	L-Ala	CH <sub>3</sub>	H	44%	2.55, 2.52, 2.47, 2.17
<b>17</b>	Ph	L-Ala	Bzl	H	65%	3.14, 2.67, 2.26
<b>18</b>	Ph	L-Ala	iPr	H	50%	3.13, 2.77, 2.41
<b>19</b>	Ph	L-Ala	CH <sub>3</sub>	CH <sub>3</sub>	99%	3.72, 3.37, 3.30
<b>20</b>	Ph	L-Ala	Bzl	CH <sub>3</sub>	87%	3.60, 3.51, 3.08, 3.05
<b>21</b>	Ph	L-Ala	iPr	CH <sub>3</sub>	15%	2.58, 2.52, 2.29, 2.27

We performed a series of stability and metabolic assays to assess the ability of the prepared ProTides to be appropriately processed to the pharmacologically active monophosphate. As a representative example, we are showing results for **16**, as all the different analogues reported produced very similar results.

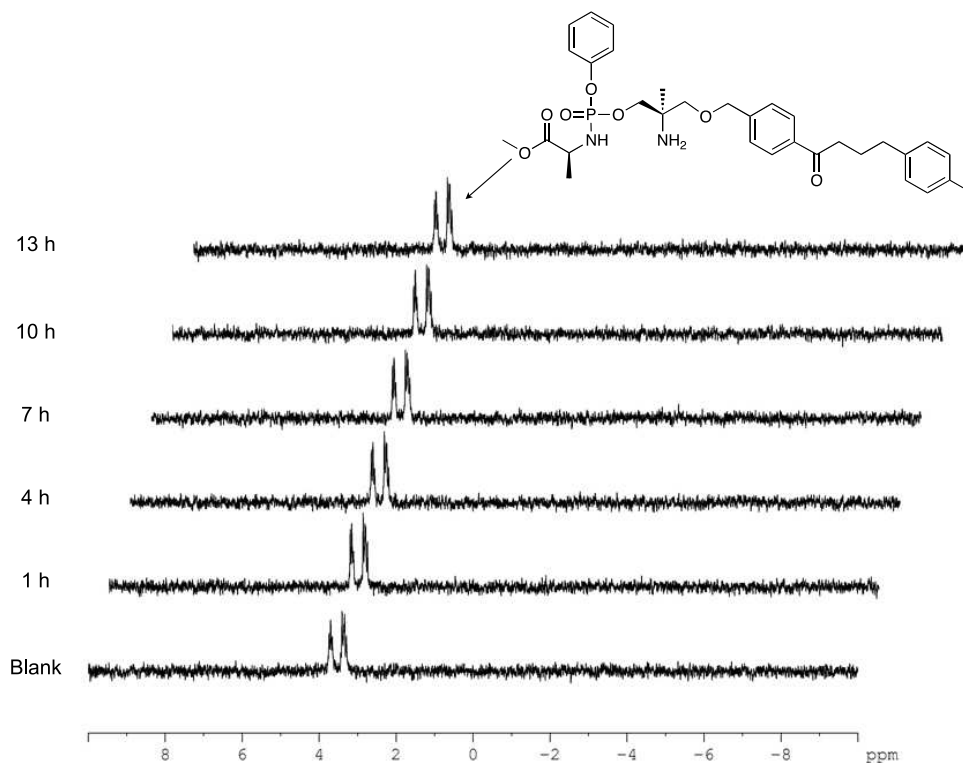
### **Acid and Base Stability**

**16** was subjected to a pH 8 stability experiment in pH 8 buffer over 13 hours.<sup>25</sup> No degradation is discernable in the NMR spectra confirming that ProTide BED analogue **16** is stable in mildly basic conditions. This result is relevant as the lumen has been reported to reach a pH of 7.6.<sup>26</sup>



**Figure 3.** Stacked <sup>31</sup>P NMR spectra showing **16** remaining stable and not degrading in the presence of pH 8 buffer over 13 hours.

An acid stability experiment was conducted involving **16** in pH 1.5 buffer over 13 h.<sup>25</sup> There was no discernable change over 13 hours at pH 1.5 suggesting that ProTide BED analogues are able to withstand the acidic conditions of the stomach.<sup>25,26</sup> Other phosphoramidate benzyl ether derivative analogues tested follow the same profile of stability in acidic and basic conditions.



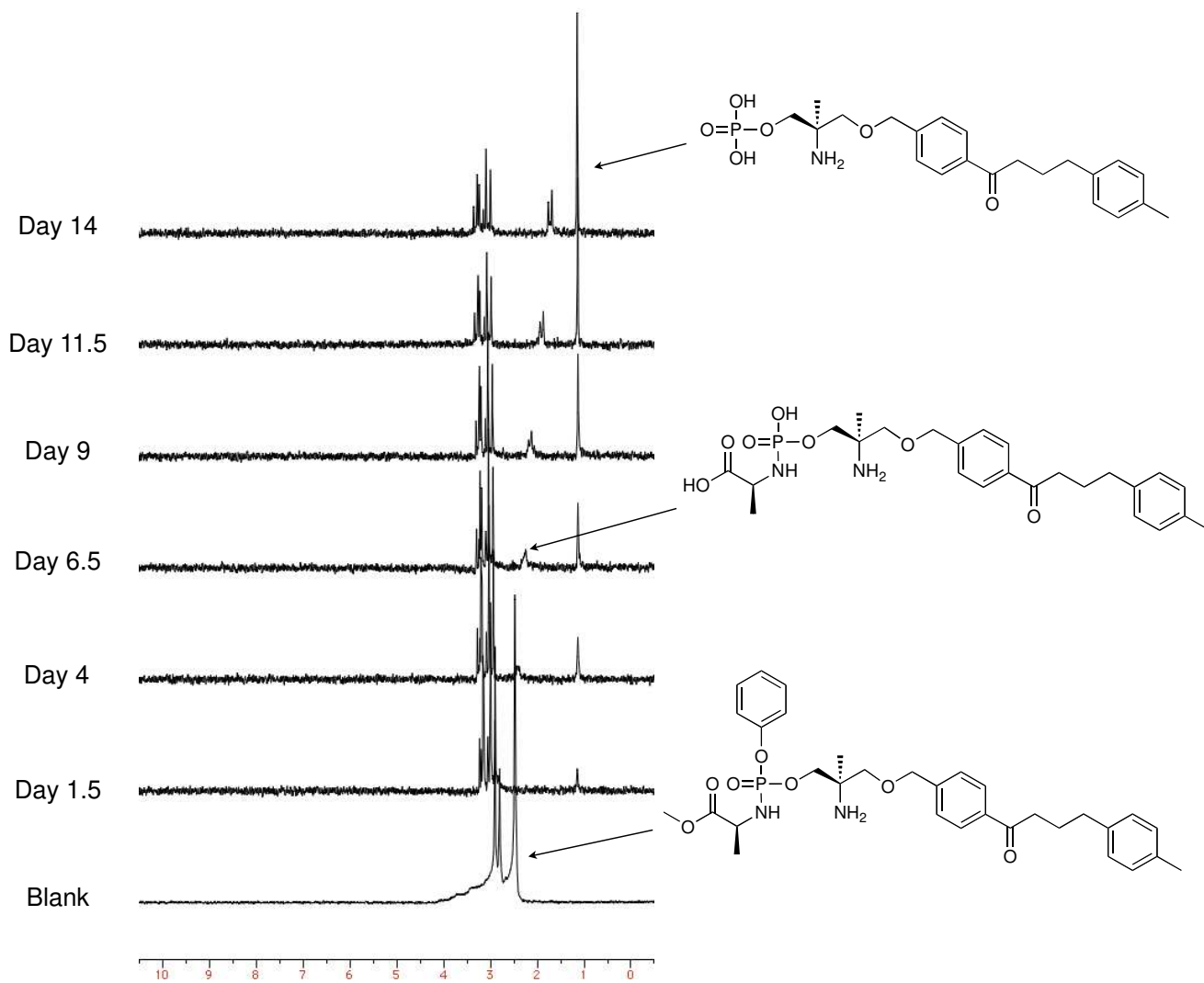
**Figure 4.** Stacked  $^{31}\text{P}$  NMR spectra showing **16** remaining stable and not degrading in the presence of pH 1.5 buffer over 13 hours.

### Carboxypeptidase Y Processing

A carboxypeptidase Y processing experiment of **16** was conducted following a previously reported experimental procedure.<sup>12</sup> Carboxypeptidase Y is used as a model for native enzymes in the human body such as Cathepsin A<sup>27</sup> which initiates phosphoramidate processing *in vivo*. Processing was

slow as the experiment was conducted over two weeks as opposed to the expected timescale of minutes or hours. After two weeks the reaction mixture was filtered and subjected to mass spectrometric analysis and unexpectedly a mass corresponding to the pharmacologically active monophosphate was detected. The complete processing to the monophosphate was unexpected, as carboxypeptidase has not been previously reported as being capable of fully processing phosphoramidates to the monophosphate. The complete processing to the monophosphate is perhaps due to a chemical difference of the benzyl ether derivatives when compared with nucleosides. The adjacent amine group may be responsible for the processing from the intermediate shown in figure 5 to the monophosphate and may also contribute to the atypical processing speed when compared to previously reported phosphoramidates of nucleosides.<sup>12</sup>

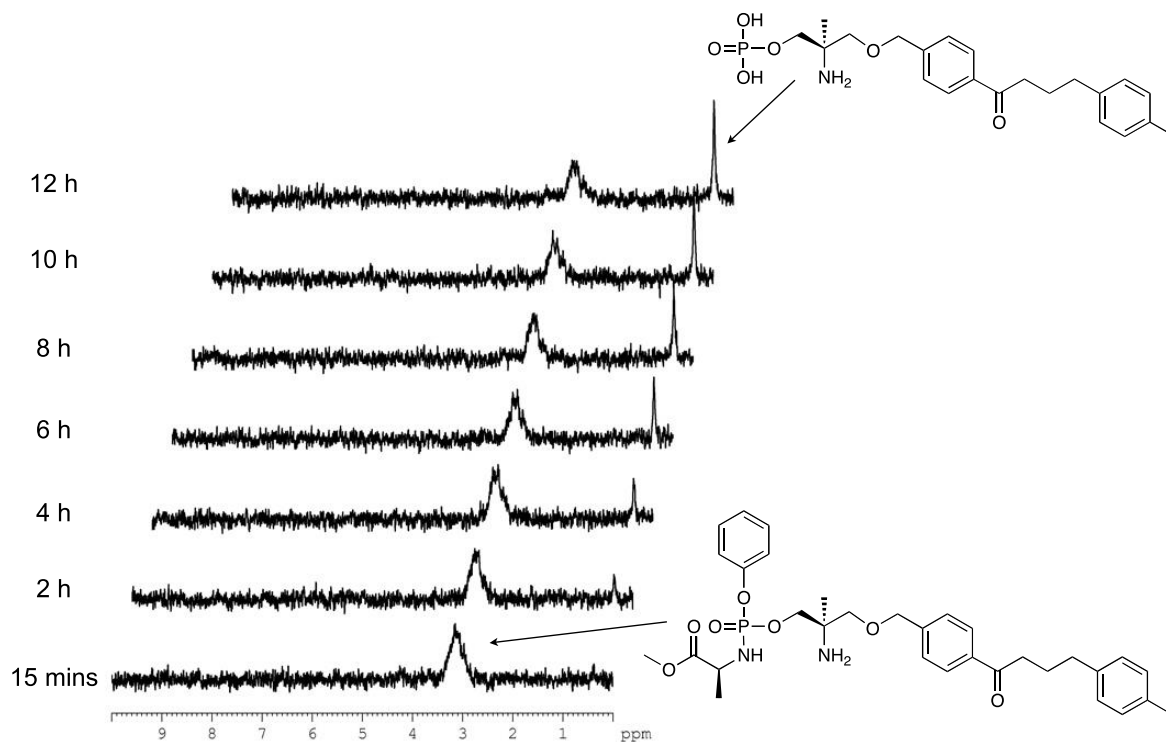
It was considered that the unusually slow processing rate was perhaps due to the fact that the experiment was conducted at ambient temperature as opposed to 37 °C, however, a repetition of the experiment conducted at 37 °C gave comparable results. Carboxypeptidase processing experiments of phosphoramidate BED analogues at ambient temperature and 37 °C consistently resulted in comparable processing speeds suggesting that analogues **16** – **21** are poor substrates of carboxypeptidase relative to previously reported phosphoramidates of nucleosides.<sup>12</sup>



**Figure 5.** Stacked  $^{31}\text{P}$  NMR spectra showing metabolism of **16** by carboxypeptidase in acetone- $d_6$  and Trizma buffer to the desired monophosphate over 2 weeks.

## Stability in Human Serum

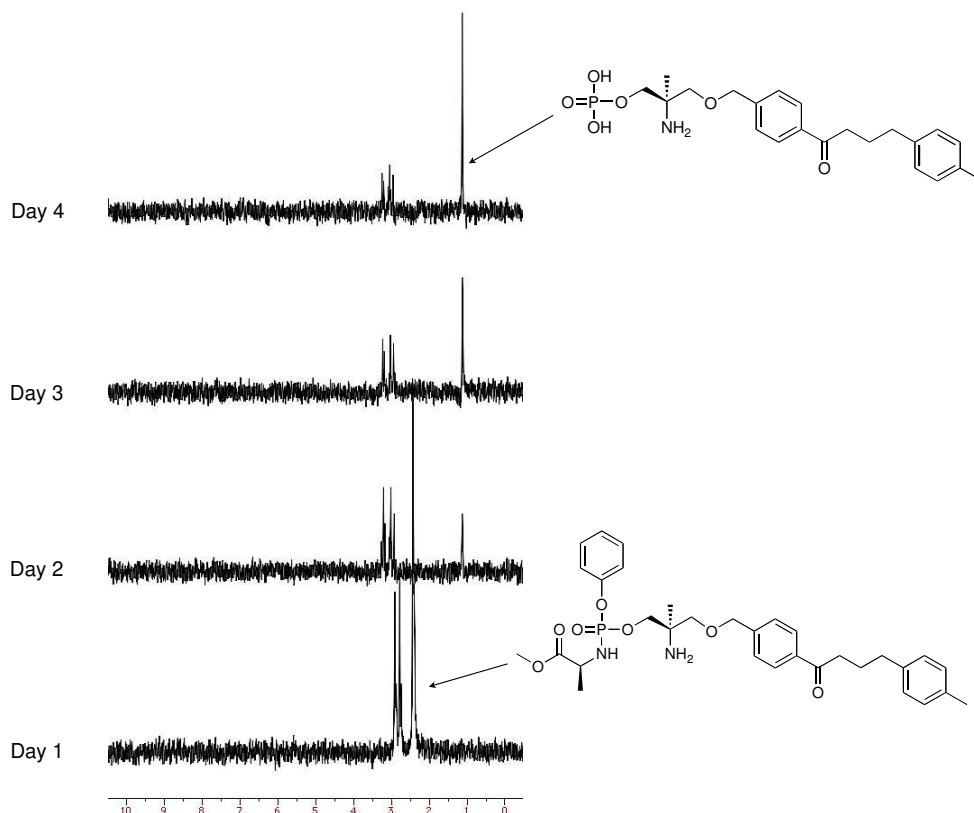
A stability study of **16** in human serum was also conducted following a previously reported procedure.<sup>12</sup> The primary product was the therapeutically relevant monophosphate confirmed by mass spectrometry by analysing the reaction mixture after 12 h and using <sup>31</sup>P NMR (figure 6). The estimated half-life for this reaction is around 14 hours and this is the case for all the phosphoramidate BED analogues tested. This figure was calculated from the change of the integrations of the two peaks over time. An accurate half-life cannot be calculated due to the margin of error implicit in determining changes in relative concentrations *via* changes in the area of <sup>31</sup>P NMR peaks. The data shown in figure 6 are a qualitative assessment of the capacity of human serum to process phosphoramidate BED analogues to the desired pharmacologically active monophosphate as opposed to an accurate quantitative measure of the rate of processing.



**Figure 6.** Stacked <sup>31</sup>P NMR spectra showing metabolism of **16** in human serum to the desired monophosphate over 12 hours

## Neuronal Cell Lysate Experiment

Neuronal cell lysate and activator was used to conduct a cell lysate experiment following previously described experimental procedures.<sup>23,28,29</sup> The main product of the processing of **16** was the desired monophosphate. Mass spectrometric analysis of the purified reaction mixture after 4 days in positive mode gave peaks of  $m/z$  436.20 and  $m/z$  459.30 which correspond to monophosphate peaks of  $[M+H]$  and  $[M+Na^+]$  respectively. The estimated half-life for the experiment is around 48 hours which is consistent with the data obtained from the other phosphoramidate BED analogues tested in neuronal cell lysate. The results of the experiment shown in figure 7 are suggestive that phosphoramidate BED analogues can be processed to the pharmacologically active monophosphate in neurons. The multiple peaks that can be seen for the parent compound are likely due to racemisation of the 2 chiral carbon centres of the benzyl ether derivative and L-alanine amino acid of the phosphoramidate.



**Figure 7.** Stacked  $^{31}\text{P}$  NMR spectra showing the metabolism of **16** in neuronal cell lysate to the desired monophosphate over 4 days.



Processing Experiment	Approximate Processing Half-Lives
Carboxypeptidase Y	14 Days
Neuronal cell lysate	48 Hours
Human serum	14 Hours

**Table 3.** Representative estimated processing rates for all the phosphoramidate BED analogues tested in carboxypeptidase Y, neuronal cell lysate and human serum processing experiments.

The synthesis of phosphoramidate analogues of non-nucleoside sphingosine 1-phosphate receptor modulators was achieved. Phosphoramidate benzyl ether derivatives have been shown to be processed to the pharmacologically active monophosphate in a range of metabolic processing experiments including neuronal cell lysate and have an acceptable stability profile in acidic and basic conditions. The application of ProTide technology to **2b** can bypass the poor phosphorylation problem reported in the original paper.<sup>9</sup> Interestingly these compounds seem to be poor substrates of carboxypeptidase, the enzyme generally indicated as responsible for the first step in the activation process of ProTides. These data suggest a possible alternative mechanism of activation for this class of compounds. The ProTide BED analogues synthesised are promising agents and the previously reported biological data<sup>9</sup> suggest that the novel compounds reported herein are suitable for *in vivo* testing.

## Acknowledgements

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## Supplementary Material

Supplementary data and information associated with this article can be found in the online version, at XXXXXXXX

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# Supplementary Information

## Kinase-Independent Phosphoramidate S1P<sub>1</sub> Receptor Agonist Benzyl Ether Derivatives

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## Experimental

### General

All experiments involving water-sensitive compounds were carried out under anhydrous conditions. Dry solvents were purchased from Sigma-Aldrich.

Pre-coated, aluminium backed plates supplied by Merck (60 F<sub>254</sub>, 0.2 mm thickness) were visualised under both short and long wave ultraviolet light (254 nm and 366 nm), or by burning using TLC indicators such as vanillin and potassium permanganate solution.

Column chromatography was carried out using silica gel supplied by Fisher (60A, 35-70 µm). Glass columns were slurry packed using the appropriate eluent with the sample being loaded pre-adsorbed onto silica gel. Fractions containing the desired product were identified by TLC, combined and the solvent removed *in vacuo*.

$^1\text{H}$  NMR (500 MHz),  $^{13}\text{C}$  NMR (125 MHz),  $^{31}\text{P}$  NMR (202 MHz) were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Spectra were calibrated to the deuterated solvent used. Chemical shifts are detailed in parts per million (ppm) downfield from tetramethylsilane. For  $^{31}\text{P}$  NMR experiments, chemical shifts are quoted in parts per million relative to an external phosphoric acid standard. Coupling constants are described in Hertz ( $J$ ). The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), b (broad), m (multiplet) and dd (doublet of doublets).

Analytical and preparative HPLC were conducted by Varian Prostar (LC Work Station- Varian Prostar 335 LC detector, Varian fraction collector (model 701), Prostar 210 solvent delivery system, using Agilent Eclipse Plus C18 (5  $\mu\text{m}$ ) as an analytical column and a Varian Pursuit XRs 5 C18 (150  $\times$  21.2 mm) model as a preparative column. The software used was Galaxie Chromatography Data System.

Low resolution mass spectrometry was performed on a Bruker Daltonics MicroTOF device (electrospray ionisation) in either positive and/or negative mode.

## Syntheses

### *Standard procedure for phosphorochloridate synthesis*

To a stirred solution of the appropriate amino acid ester salt (1 eq) and the appropriate aryl dichlorophosphate (1 eq) in anhydrous DCM was added dropwise at -78 °C anhydrous  $\text{Et}_3\text{N}$  (2 eq). Following the addition, the reaction mixture was stirred at -78 °C for 30 min and then at room temperature for 1 h. Formation of the desired compound was monitored by  $^{31}\text{P}$ NMR. After this period the solvent was removed under reduced pressure to give an oil. Most of the aryl phosphorochloridates synthesised were purified by flash column chromatography (eluting with hexane - ethyl acetate 70:30 v/v).

*(2S)-methyl 2-((((R)-2-amino-2-methyl-3-((4-(4-(p-tolyl) butanoyl) benzyl) oxy) propoxy) (phenoxy) phosphoryl) amino) propanoate (16)*

Compound **16** was prepared from **2a** (254 mg, 0.72 mmol), tBuMgCl in THF (1 M, 0.72 ml, 0.72 mmol) and phosphorochloridate **15a** (199 mg, 0.72 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed in vacuo and the desired product was isolated using flash chromatography (methanol – ethyl acetate 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (44%, 0.188 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) giving the desired product (6%, 0.026 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.74, 3.37, 3.30. <sup>1</sup>H NMR (MeOD, 500 MHz): δ 7.84 (2H, m, ArH), 7.41 (2H, m, ArH), 7.27 (2H, t, J = 8.5 Hz, ArH), 7.12 (3H, m, ArH), 6.97 (4H, s, ArH), 4.57 (2H, m, PhCH<sub>2</sub>O), 4.09 (2H, m, CH<sub>2</sub>OP), 3.88 (1H, m, CH<sub>3</sub>CH), 3.56 (3H, m, OCH<sub>3</sub>), 3.46 (2H, m, CCH<sub>2</sub>O), 2.90 (2H, m, COCH<sub>2</sub>), 2.55 (2H, t, J = 7.5 Hz, PhCH<sub>2</sub>), 2.19 (3H, s, PhCH<sub>3</sub>), 1.88 (2H, quin, J = 7 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.24 (3H, m, CHCH<sub>3</sub>), 1.21 (3H, m, CCH<sub>3</sub>). <sup>13</sup>C NMR (MeOD, 125 MHz): δ 200.62 (C=O), 175.04 (C=O), 142.74 (2d, <sup>2</sup>J<sub>C-P</sub> = 3.3 Hz, POC-Ar), 138.57 (C-Ar), 136.51 (C-Ar), 136.0 (C-Ar), 129.52 (CH-Ar), 128.64 (CH-Ar), 128.02 (CH-Ar), 127.59 (CH-Ar), 127.57 (CH-Ar), 125.08 (CH-Ar), 120.03 (2d, <sup>3</sup>J<sub>C-P</sub> = 4.9 Hz, CH-Ar), 72.55 (OCH<sub>2</sub>C), 70.81 (d, <sup>2</sup>J<sub>C-P</sub> = 3.6 Hz, CH<sub>2</sub>OP), 70.66 (PhCH<sub>2</sub>O), 51.50 (OCH<sub>3</sub>), 50.11 (CHCH<sub>3</sub>), 41.71 (NH<sub>2</sub>C), 37.31 (COCH<sub>2</sub>), 34.30 (PhCH<sub>2</sub>), 25.94 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 19.69 (PhCH<sub>3</sub>), 19.04 (m, CH<sub>3</sub>C), 17.14 (CHCH<sub>3</sub>). MS (ES+) m/z 597.3 (MH<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 263 nm showed a peak with a t<sub>R</sub> = 14.84 min (99.1%).

*(2S)-benzyl 2-(((R)-2-amino-2-methyl-3-((4-(4-(p-tolyl) butanoyl) benzyl) oxy) propoxy) (phenoxy) phosphoryl) amino) propanoate (17)*

Compound **17** was prepared from **2a** (300 mg, 0.84 mmol), tBuMgCl in THF (1 M, 0.84 ml, 0.84 mmol) and phosphorochloridate **15b** (297 mg, 0.84 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed *in vacuo* and the desired product was isolated using flash chromatography (methanol – ethyl acetate 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (65%, 0.365 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) twice giving the desired product (3%, 0.015 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.71, 3.31, 3.32. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz): δ 7.78 (2H, m, ArH), 7.30-6.94 (17H, m, ArH), 5.02 (2H, m, PhCH<sub>2</sub>O), 4.39 (2H, m, PhCH<sub>2</sub>O), 3.94 (2H, m, CH<sub>2</sub>OP), 3.89 (1H, m, CHCH<sub>3</sub>), 3.26 (1H, m, NH), 3.19 (2H, s, CCH<sub>2</sub>O), 2.83 (2H, t, J = 7 Hz, COCH<sub>2</sub>), 2.58 (2H, t, J = 7.5 Hz, PhCH<sub>2</sub>), 2.22 (3H, s, PhCH<sub>3</sub>), 1.95 (2H, quin, J = 7 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.21 (3H, m, CHCH<sub>3</sub>), 1.02 (3H, m, CCH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): δ 199.80 (C=O), 173.42 (C=O), 150.85 (d, <sup>2</sup>J<sub>C-P</sub> = 6.75 Hz, POC-Ar), 143.42 (C-Ar), 138.61 (C-Ar), 136.34 (C-Ar), 135.81 (C-Ar), 135.39 (C-Ar), 129.69 (CH-Ar), 129.65 (CH-Ar), 129.12 (CH-Ar), 128.68 (CH-Ar), 128.65 (CH-Ar), 128.52 (CH-Ar), 128.43 (CH-Ar), 128.27 (CH-Ar), 128.24 (CH-Ar), 128.21 (CH-Ar), 128.05 (CH-Ar), 127.98 (CH-Ar), 127.29 (CH-Ar), 126.02 (CH-Ar), 124.93 (CH-Ar), 122.93 (CH-Ar), 120.81 (CH-Ar), 120.33 (CH-Ar), 74.77 (OCH<sub>2</sub>C), 72.73 (d, <sup>2</sup>J<sub>C-P</sub> = 6.88 Hz, CH<sub>2</sub>OP), 71.40 (PhCH<sub>2</sub>O), 67.18 (PhCH<sub>2</sub>O), 60.43 (NH<sub>2</sub>C), 50.33 (CHCH<sub>3</sub>), 37.76 (COCH<sub>2</sub>), 34.79 (PhCH<sub>2</sub>), 25.85 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 21.05 (PhCH<sub>3</sub>), 20.85 (CH<sub>3</sub>C), 14.26 (CHCH<sub>3</sub>). MS (ES+) *m/z* 673.3 (MH<sup>+</sup>) and 695.3 (MNa<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 263 nm showed a peak with a *t<sub>R</sub>* = 17.67 min (99.2%).



(2*S*)-isopropyl 2-(((*R*)-2-amino-2-methyl-3-((4-(4-(*p*-tolyl) butanoyl) benzyl) oxy) propoxy)(phenoxy) phosphoryl) amino) propanoate (**18**)

Compound **18** was prepared from **2a** (265 mg, 0.75 mmol), tBuMgCl in THF (1 M, 0.75 ml, 0.75 mmol) and phosphorochloridate **15c** (229 mg, 0.75 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed *in vacuo* and the desired product was isolated using flash chromatography (methanol – ethyl acetate 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (50%, 0.234 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) giving the desired product (14%, 0.065 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.79, 3.49, 3.41. <sup>1</sup>H NMR (MeOD, 500 MHz): δ 7.83 (2H, m, ArH), 7.39 (2H, m, ArH), 7.26 (2H, m, ArH), 7.12 (3H, m, ArH), 6.97 (4H, s, ArH), 4.91 (1H, m, CH(CH<sub>3</sub>)<sub>2</sub>), 4.56 (2H, m, PhCH<sub>2</sub>O), 4.11 (2H, m, CH<sub>2</sub>OP), 3.81 (1H, m, CHCH<sub>3</sub>), 3.50 (2H, m, CCH<sub>2</sub>O), 2.89 (2H, m, COCH<sub>2</sub>), 2.54 (2H, t, *J* = 7.5 Hz, PhCH<sub>2</sub>), 2.18 (3H, s, PhCH<sub>3</sub>), 2.05 (1H, s, NH), 1.88 (2H, quin, *J* = 7.5 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.25 (3H, m, CHCH<sub>3</sub>), 1.19 (3H, m, CCH<sub>3</sub>), 1.11 (3H, m, CHCH<sub>3</sub>), 1.10 (3H, m, CHCH<sub>3</sub>). <sup>13</sup>C NMR (MeOD, 125 MHz): δ 200.62 (C=O), 172.99 (C=O), 142.73 (2d, <sup>2</sup>*J*<sub>C-P</sub> = 2 Hz, POC-Ar), 138.56 (C-Ar), 136.47 (C-Ar), 135.06 (C-Ar), 129.52 (CH-Ar), 128.65 (CH-Ar), 128.03 (CH-Ar), 127.58 (CH-Ar), 127.55 (CH-Ar), 125.08 (CH-Ar), 125.04 (CH-Ar), 120.04 (2d, <sup>3</sup>*J*<sub>C-P</sub> = 4.75 Hz, CH-Ar), 72.54 (OCH<sub>2</sub>C), 70.77 (PhCH<sub>2</sub>O), 70.61 (d, <sup>2</sup>*J*<sub>C-P</sub> = 1.63 Hz, CH<sub>2</sub>OP), 68.98 (OCH(CH<sub>3</sub>)<sub>2</sub>), 56.21 (NH<sub>2</sub>C), 50.54 (CHCH<sub>3</sub>), 37.31 (COCH<sub>2</sub>), 34.30 (PhCH<sub>2</sub>), 25.94 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 20.60 (OCHCH<sub>3</sub>), 20.52 (OCHCH<sub>3</sub>), 19.72 (PhCH<sub>3</sub>), 19.14 (m, CH<sub>3</sub>C), 17.11 (CHCH<sub>3</sub>). MS (ES<sup>+</sup>) *m/z* 625.3 (MH<sup>+</sup>) and 647.3 (MNa<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 263 nm showed a peak with a *t*<sub>R</sub> = 17.30 min (95.5%).

*(2S)-methyl 2-((((R)-2-amino-3-((2,5-dimethyl-4-(4-(p-tolyl) butanoyl) benzyl) oxy)-2-methylpropoxy) (phenoxy) phosphoryl) amino) propanoate (19)*

Compound **19** was prepared from **2b** (500 mg, 1.3 mmol), tBuMgCl in THF (1 M, 1.3 ml, 1.3 mmol) and phosphorochloridate **15a** (360 mg, 1.3 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed *in vacuo* and the desired product was isolated using flash chromatography (methanol – ethyl acetate 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (99%, 0.803 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) twice giving the desired product (11%, 0.087 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.73, 3.43, 3.31. <sup>1</sup>H NMR (MeOD, 500 MHz): δ 7.44 (1H, m, ArH), 7.38 (2H, t, *J* = 8 Hz, ArH), 7.27 (4H, m, ArH), 7.07 (4H, m, ArH), 4.63 (2H, m, PhCH<sub>2</sub>O), 4.25 (2H, m, CH<sub>2</sub>OP), 3.99 (1H, m, CH<sub>3</sub>CH), 3.68 (3H, m, OCH<sub>3</sub>), 3.60 (2H, m, CCH<sub>2</sub>O), 3.33 (1H, m, NH), 2.90 (2H, m, COCH<sub>2</sub>), 2.63 (2H, t, *J* = 7.5 Hz, PhCH<sub>2</sub>), 2.40 (3H, m, PhCH<sub>3</sub>), 2.35 (3H, m, PhCH<sub>3</sub>), 2.30 (3H, s, PhCH<sub>3</sub>), 1.96 (2H, quin, *J* = 7 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.40 (3H, m, CHCH<sub>3</sub>), 1.35 (3H, m, CCH<sub>3</sub>). <sup>13</sup>C NMR (MeOD, 125 MHz): δ 205.25 (C=O), 173.89 (C=O), 150.50 (2d, <sup>2</sup>*J*<sub>C-P</sub> = 4.88 Hz, POC-Ar), 138.51 (C-Ar), 138.31 (C-Ar), 137.71 (C-Ar), 135.08 (C-Ar), 134.81 (C-Ar), 134.19 (C-Ar), 131.92 (CH-Ar), 130.07 (CH-Ar), 129.52 (CH-Ar), 128.66 (CH-Ar), 128.05 (CH-Ar), 125.08 (CH-Ar), 120.07 (2d, <sup>3</sup>*J*<sub>C-P</sub> = 4.75 Hz, CH-Ar), 71.17 (OCH<sub>2</sub>C), 70.72 (d, <sup>2</sup>*J*<sub>C-P</sub> = 2.6 Hz, CH<sub>2</sub>OP), 70.57 (PhCH<sub>2</sub>O), 56.18 (NH<sub>2</sub>C), 51.50 (OCH<sub>3</sub>), 50.34 (CHCH<sub>3</sub>), 40.31 (COCH<sub>2</sub>), 34.25 (PhCH<sub>2</sub>), 26.00 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 19.73 (PhCH<sub>3</sub>), 19.44 (PhCH<sub>3</sub>), 19.06 (PhCH<sub>3</sub>), 18.87 (CH<sub>3</sub>C), 17.12 (CHCH<sub>3</sub>). MS (ES+) *m/z* 625.3 (MH<sup>+</sup>) and 647.3 (MNa<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 245 nm showed a peak with a *t<sub>R</sub>* = 17.03 min (94.6%).

(2*S*)-benzyl 2-((((*R*)-2-amino-3-((2,5-dimethyl-4-(4-(*p*-tolyl) butanoyl) benzyl oxy)-2-methylpropoxy) (phenoxy) phosphoryl) amino) propanoate (**20**)

Compound **20** was prepared from **2b** (500 mg, 1.3 mmol), tBuMgCl in THF (1 M, 1.3 ml, 1.3 mmol) and phosphorochloridate **15b** (459 mg, 1.3 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed *in vacuo* and the desired product was isolated using flash chromatography (methanol – ethyl acetate 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (87%, 0.791 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) giving the desired product (8%, 0.07 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.73, 3.37, 3.29. <sup>1</sup>H NMR (MeOD, 500 MHz): δ 7.42 (1H, m, ArH), 7.33 (7H, m, ArH), 7.23 (4H, m, ArH), 7.07 (1H, m, ArH), 5.11 (2H, m, PhCH<sub>2</sub>O), 4.61 (2H, m, PhCH<sub>2</sub>O), 4.22 (2H, m, CH<sub>2</sub>OP), 4.04 (1H, m, CHCH<sub>3</sub>), 3.60 (2H, m, CCH<sub>2</sub>O), 3.37 (1H, m, NH), 2.88 (2H, t, *J* = 7 Hz, COCH<sub>2</sub>), 2.63 (2H, t, *J* = 7.5 Hz, PhCH<sub>2</sub>), 2.40 (3H, s, PhCH<sub>3</sub>), 2.34 (3H, s, PhCH<sub>3</sub>), 2.30 (3H, s, PhCH<sub>3</sub>), 2.04 (2H, s, NH<sub>2</sub>), 1.95 (2H, quin, *J* = 7.5 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.34 (3H, m, CHCH<sub>3</sub>), 1.33 (3H, m, CCH<sub>3</sub>). <sup>13</sup>C NMR (MeOD, 125 MHz): δ 207.18 (C=O), 175.67 (C=O), 152.48 (d, <sup>2</sup>*J*<sub>C-P</sub> = 6.5 Hz, POC-Ar), 140.50 (C-Ar), 140.28 (C-Ar), 139.69 (C-Ar), 137.76 (C-Ar), 137.07 (C-Ar), 137.81 (C-Ar), 136.16 (C-Ar), 133.89 (CH-Ar), 133.84 (CH-Ar), 132.07 (CH-Ar), 131.50 (CH-Ar), 130.66 (CH-Ar), 130.25 (CH-Ar), 130.04 (CH-Ar), 130.03 (CH-Ar), 129.94 (CH-Ar), 129.86 (CH-Ar), 129.84 (CH-Ar), 127.08 (CH-Ar), 127.03 (CH-Ar), 122.11 (2d, <sup>3</sup>*J*<sub>C-P</sub> = 4.63 Hz, CH-Ar), 73.17 (OCH<sub>2</sub>C), 72.70 (PhCH<sub>2</sub>O), 69.50 (d, <sup>2</sup>*J*<sub>C-P</sub> = 5.38 Hz, CH<sub>2</sub>OP), 68.68 (PhCH<sub>2</sub>O), 58.23 (NH<sub>2</sub>C), 50.50 (CHCH<sub>3</sub>), 42.29 (COCH<sub>2</sub>), 36.24 (PhCH<sub>2</sub>), 27.98 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 21.73 (PhCH<sub>3</sub>), 21.45 (PhCH<sub>3</sub>), 21.05 (PhCH<sub>3</sub>), 20.85 (CH<sub>3</sub>C), 19.13 (CHCH<sub>3</sub>). MS (ES<sup>+</sup>) *m/z* 701.5 (MH<sup>+</sup>) and 723.4 (MNa<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 245 nm showed a peak with a *t*<sub>R</sub> = 19.88 min (97.3%).

*(2S)-isopropyl 2-((((R)-2-amino-3-((2,5-dimethyl-4-(4-(p-tolyl) butanoyl) benzyl) oxy)-2-methylpropoxy) (phenoxy) phosphoryl) amino) propanoate (21)*

Compound **21** was prepared from **2b** (499 mg, 1.3 mmol), tBuMgCl in THF (1 M, 1.3 ml, 1.3 mmol) and phosphorochloridate **15c** (397 mg, 1.3 mmol) in anhydrous THF (20 ml) under anhydrous conditions. The reaction was left to stir for 24 hours. After 24 hours the solvent was removed *in vacuo* and the desired product was isolated using flash chromatography (methanol – dichloromethane 0:100 v/v increasing to 10:90 v/v) giving the desired oil product as a mixture of 2 diastereoisomers (15%, 0.129 g). The resulting oil was further purified using preparative reverse-phase HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 100:0 v/v in 35 min) giving the desired product (8%, 0.065 g).

<sup>31</sup>P NMR (MeOD, 202 MHz): δ 3.78, 3.51, 3.43. <sup>1</sup>H NMR (MeOD, 500 MHz): δ 7.31 (1H, d, *J* = 3.5 Hz, *ArH*), 7.26 (2H, m, *ArH*), 7.15 (1H, m, *ArH*), 7.12 (3H, m, *ArH*), 6.96 (4H, s, *ArH*), 4.85 (1H, m, *CH*(CH<sub>3</sub>)<sub>2</sub>), 4.51 (2H, m, PhCH<sub>2</sub>O), 4.13 (2H, m, CH<sub>2</sub>OP), 3.80 (1H, m, CHCH<sub>3</sub>), 3.51 (1H, m, CCH<sub>2</sub>O), 3.47 (1H, m, CCH<sub>2</sub>O), 2.78 (2H, t, *J* = 7.5 Hz, COCH<sub>2</sub>), 2.51 (2H, t, *J* = 7.5 Hz, PhCH<sub>2</sub>), 2.28 (3H, s, PhCH<sub>3</sub>), 2.23 (3H, s, PhCH<sub>3</sub>), 2.18 (3H, s, PhCH<sub>3</sub>), 1.84 (2H, quin, *J* = 7.5 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.18 (6H, m, CHCH<sub>3</sub>), 1.12 (3H, m, CCH<sub>3</sub>), 1.11 (3H, m, CHCH<sub>3</sub>). <sup>13</sup>C NMR (MeOD, 125 MHz): δ 205.24 (C=O), 173.54 (C=O), 150.53 (2d, <sup>2</sup>*J*<sub>C-P</sub> = 2 Hz, POC-Ar), 138.51 (C-Ar), 138.32 (C-Ar), 137.70 (C-Ar), 135.08 (C-Ar), 138.56 (C-Ar), 134.80 (C-Ar), 134.17 (C-Ar), 131.88 (CH-Ar), 130.07 (CH-Ar), 129.52 (CH-Ar), 128.66 (CH-Ar), 128.05 (CH-Ar), 125.09 (CH-Ar), 120.14 (2d, <sup>3</sup>*J*<sub>C-P</sub> = 4.75 Hz, CH-Ar), 71.18 (OCH<sub>2</sub>C), 70.74 (PhCH<sub>2</sub>O), 68.98 (OCH(CH<sub>3</sub>)<sub>2</sub>), 67.59 (2d, <sup>2</sup>*J*<sub>C-P</sub> = 5.13 Hz, CH<sub>2</sub>OP), 56.22 (NH<sub>2</sub>C), 50.52 (CHCH<sub>3</sub>), 40.31 (COCH<sub>2</sub>), 34.25 (PhCH<sub>2</sub>), 26.00 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 20.58 (OCHCH<sub>3</sub>), 20.53 (OCHCH<sub>3</sub>), 19.73 (PhCH<sub>3</sub>), 19.44 (PhCH<sub>3</sub>), 18.92 (PhCH<sub>3</sub>), 18.84 (CH<sub>3</sub>C), 17.13 (CHCH<sub>3</sub>). MS (ES+) *m/z* 653.3 (MH<sup>+</sup>) and 675.3 (MNa<sup>+</sup>). Reverse-phase analytical HPLC (0.1% TFA in H<sub>2</sub>O – ACN 20:80 v/v increasing to 0:100 v/v in 35 min), 1 ml/min, λ = 245 nm showed a peak with a *t*<sub>R</sub> = 18.64 min (98.2%).

## **Assays**

### ***Cell lysate assay***

The experiment was carried out by dissolving phosphoramidate test compound (5 mg) in acetone- $d_6$  (0.3 ml) and adding 0.1 ml of Trizma buffer (pH 7.6). After the  $^{31}\text{P}$  NMR data were recorded at 25 °C as a control, 0.125 ml cell lysate and 0.02 ml activator was added to the sample. Next, the sample was shaken and submitted to  $^{31}\text{P}$  NMR experiments at 37 °C and the spectra were recorded every 7 min over 13 h.  $^{31}\text{P}$  NMR recorded data were processed and analysed with the Bruker Topspin 2.1 program. One experiment was performed for each compound tested.

### ***Human serum assay***

The experiment was carried out by dissolving test compound in DMSO (0.05 ml) and  $\text{D}_2\text{O}$  (0.15 ml). After the  $^{31}\text{P}$  NMR data were recorded at 37 °C as a control, a previously defrosted human serum (male AB plasma) (0.3 ml) was added to the sample. Next, the sample was submitted to  $^{31}\text{P}$  NMR experiments at 37 °C and the spectra were recorded every 15 min over 13 h.  $^{31}\text{P}$  NMR recorded data were processed and analysed with the Bruker Topspin 2.1 program. One experiment was performed for each compound tested.

### ***Carboxypeptidase Y assay***

The experiment was carried out by dissolving phosphoramidate compound (3 mg) in acetone- $d_6$  (0.15 ml) and adding 0.3 ml of Trizma buffer (pH 7.6). After the  $^{31}\text{P}$  NMR data were recorded at 25 °C as a control, a previously defrosted carboxypeptidase Y (0.1 mg dissolved in 0.15 ml of Trizma) was added to the sample. Next, the sample was submitted to  $^{31}\text{P}$  NMR experiments the spectra were recorded every day over 2 weeks.  $^{31}\text{P}$  NMR recorded data were processed and analysed with the Bruker Topspin 2.1 program.<sup>1</sup> Two experiments were performed for each compound tested.

### ***Acid stability***

The stability assay toward hydrolysis by aqueous buffer at pH 1.5 was conducted using *in situ*  $^{31}\text{P}$  NMR (202 MHz). The experiment was carried out by dissolving test compound (3 mg) in methanol- $d_4$  (0.1 ml) and then adding buffer of pH 1.5 (prepared from HCl and KCl). Next, the sample was subjected to  $^{31}\text{P}$  NMR experiments at 25 °C and the spectra were recorded every 12 min over 13 h.

### ***Base stability***

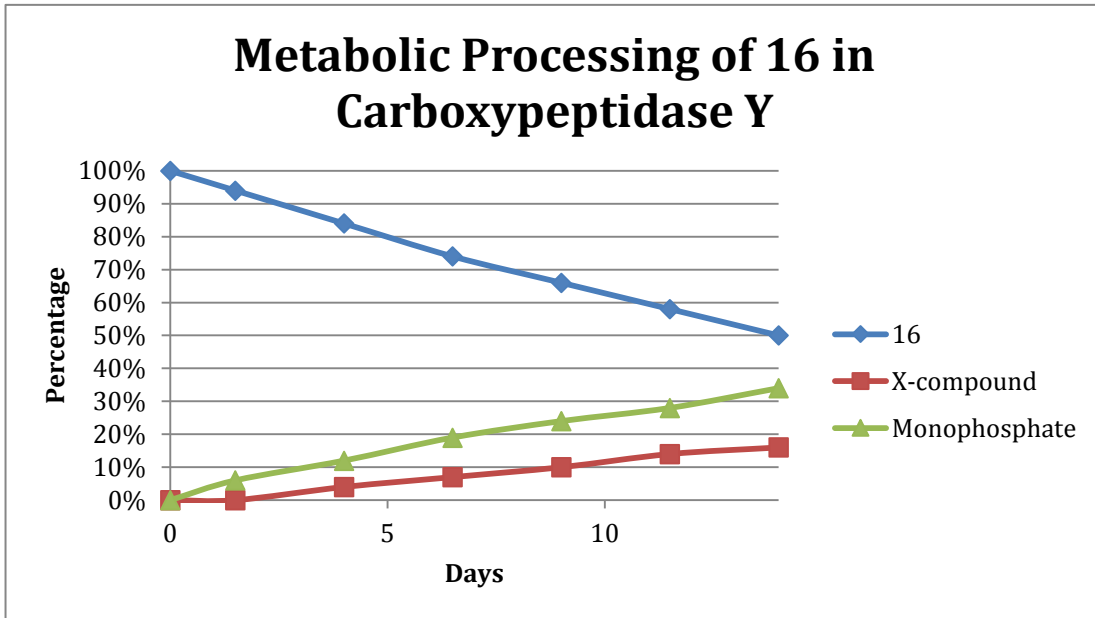
The stability assay toward hydrolysis by aqueous buffer at pH 8 was conducted using *in situ*  $^{31}\text{P}$  NMR (202 MHz). The experiment was carried out by dissolving test compound (3 mg) in methanol- $d_4$  (0.1 ml) and then adding pH 8 buffer (prepared from tris(hydroxymethyl)aminomethane and HCl). Next, the sample was subjected to  $^{31}\text{P}$  NMR experiments at 25 °C and the spectra were recorded every 12 min over 13 h.

### **Generating cell lysate**

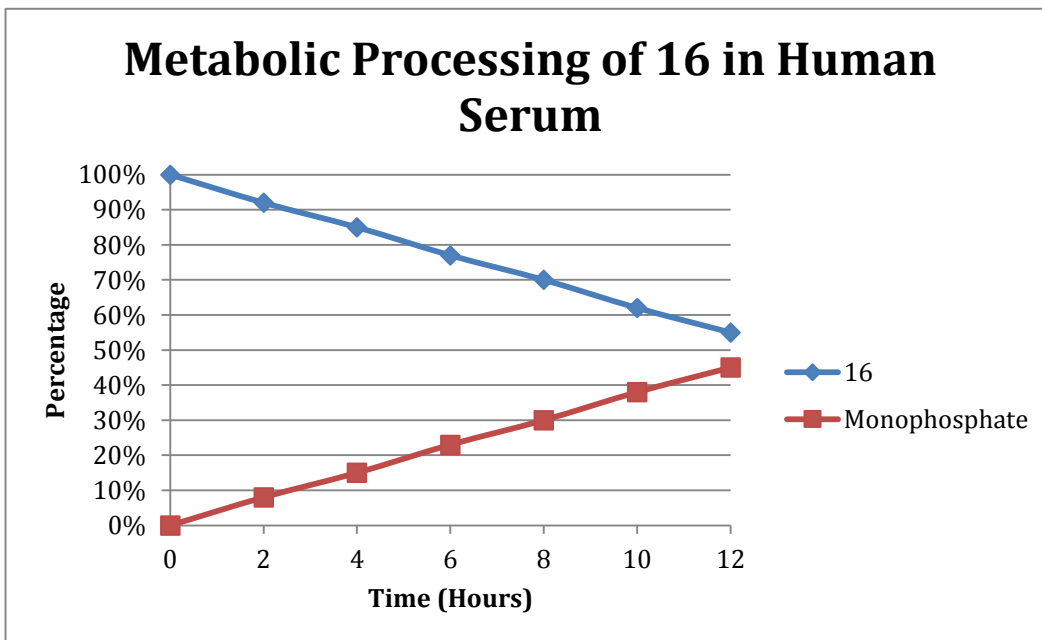
J1 ES cell-derived neurons were generated *in vitro* as previously described.<sup>28</sup> The glutamatergic-like neurons were cultured at a density of  $5.4\text{E}+5$  cells/cm<sup>2</sup> for 2 weeks before the lysis of the neurons. 6 million neurons were lysed in 300  $\mu\text{l}$  of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton, 0.2 % Sodiumdeoxycholate, 10 % Glycerol). Cell lysate was centrifuged at 3000 rpm for 10 min and transferred into a fresh tube. It was stored at -20 °C until further use. To improve enzyme performance  $\text{MgCl}_2$  and ATP was added in form of a DNA ligation buffer (T4 DNA Ligase Buffer, NEB).

### **Metabolic Processing Graphical Data**

## Carboxypeptidase Y



## Human Serum Processing



## Neuronal Cell Lysate

## Metabolic Processing of 16 in Neuronal Cell Lysate

