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Tuning the pH sensitivities of orthoester based compounds for drug delivery applications by simple chemical modification

Hélène Bruyère, Andrew D. Westwell, Arwyn T. Jones *

Welsh School of Pharmacy, Cardiff University, Redwood Building, Cardiff CF10 3NB, Wales, UK

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ABSTRACT

Orthoesters are acid-sensitive moieties that allow substantial structural diversity for biological applications including drug delivery. Here, the pH-sensitivity of a range of novel orthoester based compounds was compared in the range 7.5–4.5 that is characteristic of the increased acidification during endocytosis. We find that simple modifications close to the orthoester had major effects on both the rate and extent of hydrolysis, suggesting this could be exploited for activating drug delivery systems on endocytic pathways.

Polymer based architectures have been extensively studied as backbones for drug delivery systems.[1,2] The strategy is often based on their conjugation to a therapeutic cargo that is then delivered to the inside of cells by endocytosis.[3–5] There is often a necessity for the therapeutic to be released from the polymer backbone for it to then reach the cytosol and possibly other intracellular targets. An approach to this design is to use the considerable drop in pH that molecules encounter as they are trafficked through endocytic pathways.[6] These are used by cells to internalize molecules that will then encounter a drop from extracellular pH 7.3–7.5 to 4.5–5.0 in lysosomes.[7] These organelles and to a lesser extent late endosomes constitute a strong hydrolytic environment, thus there may be a requirement to release the therapeutic early during endocytosis (pH 5–6) if it is labile to these conditions.

Several pH sensitive linkers have been studied in this context, including hydrazones,[8] *cis*-aconityl,[9] vinyl ethers,[10] and acetals.[11] Also included here are orthoesters, and to date these are shown to be one of the most acid-labile functionalities available.[12] As noted, drug delivery systems may need to be activated at a specific pH, considerably higher than that encountered in lysosomes. A slightly acidic pH has also been observed in tumours[13] and inflammatory tissues[14] thus there may also be an interest in designing pH specific systems for mediating effects in these environments. Orthoesters are of particular interest in attempting to achieve this goal because they can be modified with respect to structural patterns or stereochemical, stereoelectronic and steric factors.[15–17] There are numerous reports describing the behaviour of these in acidic conditions and examples include poly(orthoesters) and other orthoester- or diorthoester–lipid conjugates, designed for delivery of biologically active cargo through endocytic pathways.[18–21] Pure chemical and mechanistic studies tend to be performed in organic or mixed organic/aqueous solvents and rarely consider the efficiency of the system in an aqueous environment or with respects to time and temperature. Whereas those interested in drug delivery rarely focus on characterizing a range of different structures.

Specifically towards the design of a polymer-peptide based drug delivery system that is sensitive to acidic pHs found on endocytic pathways,[22] we designed, synthesized and measured the rates of hydrolysis of different and novel model compounds containing an orthoester linker. For proof of concept towards the design of polymer-peptide conjugates, selected structures were linked to diethylene glycol as a template polymer for polyethylene glycol (PEG) and phenylalanine as a peptide substitute. Our studies highlight that simple modifications on the orthoester backbone have a major influence on hydrolysis profiles that is then transferable to more complete drug delivery systems. This may allow a high degree of flexibility with respects to pH activation that may, depending on the biological application, be a requirement.

The synthetic template is based on a previous publication[21] and is outlined in Scheme 1. The amino groups of commercially available amino-diols **1, 4, 5** and **6** were protected as their trifluoroacetate derivatives using ethyltrifluoroacetate. Reaction of these protected amino-diols **2** and **7–9** with trimethyl orthoformate under *p*-toluene sulfonic acid (PTSA) catalysis provided the orthoester building blocks (**3** and **10–12**) primed for diethylene glycol and phenylalanine functionalisation. Transetherification using diethyl-

* Corresponding author. Tel.: +44 0 2920 876431.
E-mail address: jonesat@cardiff.ac.uk (A.T. Jones).

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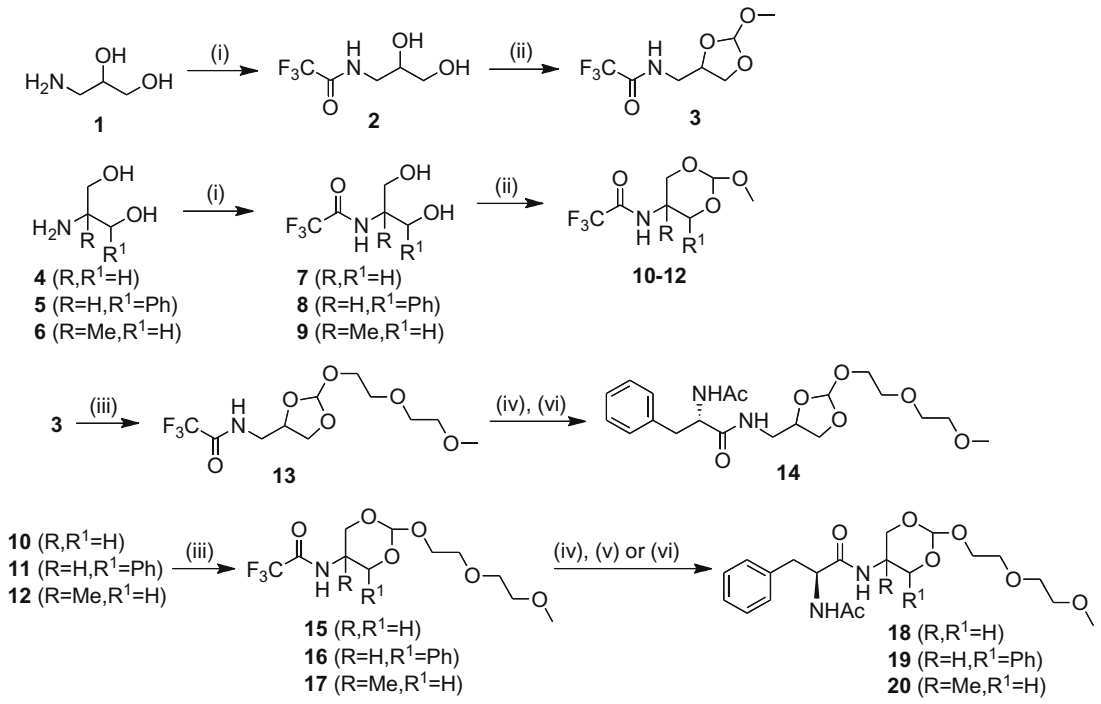
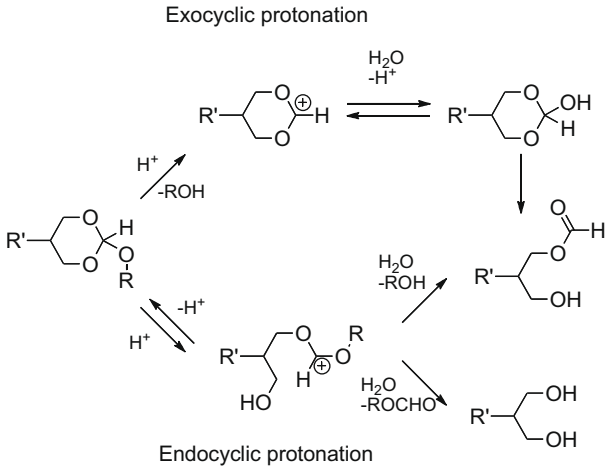


Scheme 1. Synthesis of orthoester model compounds **14** and **18–20**. Reagents and conditions: (i) ethyltrifluoroacetate, THF, room temperature; (ii) trimethylorthoformate, PTSA, CH_2Cl_2, room temperature; (iii) diethylene glycol monomethyl ether, PPTS, toluene, reflux; (iv) 1 M NaOH, THF, room temperature; (v) *N-Ac*-L-phenylalanine, EDCI, DMAP, CH_2CL_2, THF, room temperature; (vi) *N-Ac*-L-phenylalanine, EDCI, CH_2Cl_2, THF, room temperature.

ene glycol monomethyl ether gave the intermediate orthoesters **13** and **15–17**. The trifluoroacetyl protecting group was then removed by the action of aqueous NaOH in THF, and pseudo-peptidic coupling with *N*-acetyl phenylalanine provided the necessary orthoesters **14** and **18–20**. The specified conditions for synthesizing compound **18** were not suitable for generating the other model compounds unless DMAP was omitted as a catalyst. Due to acid-sensitivity and the fact that no starting material was detectable by ^{1}H NMR after work-up and concentration, the intermediate compounds were not purified. Indeed, attempts at purification after *trans*-etherification did not lead to significant improvements in yield of the final compound. Similarly, purification of the final compounds (**14** and **18–20**) by flash chromatography on silica gels was difficult thus neutral aluminum oxide was used as an alternative. Of note for NMR analysis is that several isomers were identified at different steps as the orthoester cycle is formed. From this, four different orthoester model compounds were prepared (**14, 18, 19, 20**).

Two different hydrolysis pathways have been described for orthoesters depending on whether the first protonation occurs on an exocyclic or endocyclic oxygen atom.[23] The stages are detailed in Scheme 2 using a six-membered ring orthoester as an example. It is expected that any stereoelectronic or stereochemical/conformational effect on a model molecule would influence the nature of the hydrolysis products, their stability and their endocyclic or exocyclic pathway ratio.[16,24]

In order to directly measure the hydrolysis ratio between the parent orthoester and the hydrolysis products by NMR, we used deuterated solvents to prepare all buffers. These were prepared from a mixture of 0.5 M citric acid and 0.5 M Na_2HPO_4 solutions in deuterium oxide. Two similar ^{1}H NMR methods were then used to evaluate the stability of the orthoester-containing products under a range of pH values from 7.5 to 4.5. Initially, hydrolysis studies were carried out for 90 min at 37 °C in single tubes inside the NMR spectrometer that was previously equilibrated to this temperature.



Scheme 2. Orthoester hydrolytic pathways.

Alternatively the reactions were performed in a 37 °C water bath and aliquots were removed at different time periods for NMR analysis. To stop further hydrolysis these samples were quenched with 0.5 M Na_2HPO_4 in deuterium oxide (pH 8.5). We confirmed that this completely inhibited any further hydrolysis irrespective of the pH of the initial solution and experiments performed in the laboratory and on the NMR instrument yielded identical data (not shown). Some experiments were performed at room temperature whereby the same reaction tubes were periodically placed on the NMR instrument.

The extent of hydrolysis was determined by relative integration of specific orthoester and newly formed hydrolysis peaks (Fig. 1). Several peaks were visible in each specific area because of the creation of different diastereoisomers as mentioned above. The pH of the hydrolysis mixtures did not change during the reaction

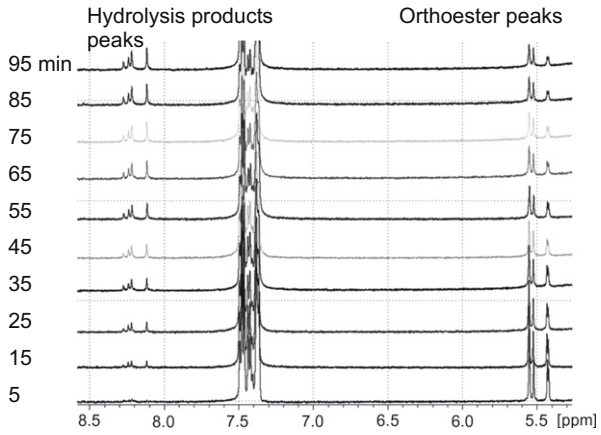


Figure 1. Labeled NMR profiles for orthoester hydrolysis of compound **18** at pH 5.5.

periods. For substituted compounds **19** and **20** that suffered from low aqueous solubility, it was necessary to use DMSO as a co-solvent for hydrolysis studies. For this, the orthoester compound was dissolved in DMSO, prior to adding deuterated buffer solutions to achieve the necessary pH. Using water soluble compound **14** as a reference we confirmed that DMSO by itself did not influence hydrolysis (see Supplementary Fig. S1).

Initially, we compared, at a range of pH, the time dependent hydrolysis of the six-membered ring **18** at ambient temperature. As seen in Figure 2, these results confirmed that the rate and extent of hydrolysis were pH dependent. At pH 2.3, 100% hydrolysis occurred in less than 10 min and the same extent of hydrolysis was observed after 60 min at pH 4.1. We were unable to see greater than 20% hydrolysis at pH 5.2 and no hydrolysis was observed at pH >6.4.

These first results allowed us to progress to performing real time NMR studies at 37 °C with this compound and we also investigated the effect of the ring size on the hydrolysis rates at different pHs by comparing model compounds **14** and **18**. Within the experiment time we observed complete stability for both compounds at pH 7.5. Complete hydrolysis itself was achieved for both compounds at pH 4.5 but while compound **14** was completely hydrolysed in 15 min, the six-membered ring (**18**) required incubation for 55 min to achieve this (Fig. 3). Similarly, at pH 6.5 compound **18** was considerably less sensitive to hydrolysis than its five-membered ring counterpart. This does not appear to be a universal phenomenon as previous studies have shown a relative insensitivity to hydrolysis of a five-membered ring orthoester at pH 4 and 5.[21] However the immediate environment surrounding the six-membered ring orthoester was different in that study thus highlighting

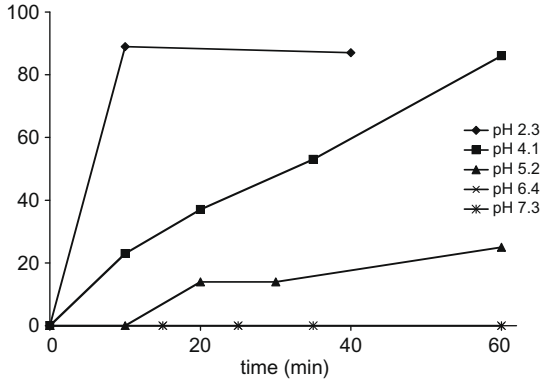


Figure 2. Hydrolysis profiles for compound **18** at ambient temperature.

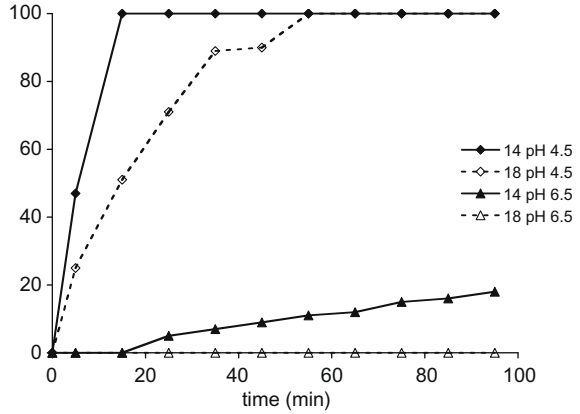


Figure 3. Hydrolysis profiles of compounds **14** and **18** at 37 °C.

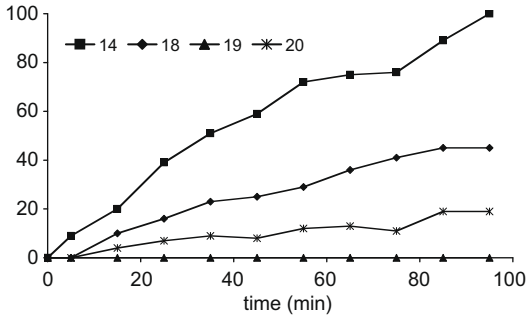


Figure 4. Time-dependent hydrolysis of **14**, **18–20** at pH 5.5.

the dramatic change in sensitivity that may be mediated by minor structural modifications.

In order to focus on pH values representing early and late endosomes (pH 6–5), the hydrolytic profiles of all model orthoesters were analyzed at pH 5.5 and at 37 °C. Results in Figure 4 showed that when comparing substitution patterns (compounds **18–20**), the addition of substituents on two different positions dramatically decreased the pH-sensitivity of the orthoester. At pH 5.5, compound **19** (R^1 = Ph) was insensitive and compound **20** (R = Me) had a maximum hydrolysis of 20% after 90 min at 37 °C compared with 45% hydrolysis for reference compound **18**. This may be due to stereoelectronic effects on the orthoester ring or decreased solubility in aqueous buffers. All six-membered ring compounds **18–20** were less sensitive to hydrolysis compared with compound **14**.

In this study, we showed that structurally diverse orthoester compounds bearing a protected amino acid and diethylene glycol (as PEG mimic) show favourable hydrolysis profiles for drug delivery applications. Our results in particular emphasise that the five-membered orthoester **14** is a promising model compound showing complete stability at neutral pH but complete hydrolysis at pH 4.5–5.5 over a relatively short time period. Overall it may be possible to design linkers that are fine tuned around the orthoester moiety to influence the pH sensitivities for a range of biological applications including drug delivery.

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Supplementary data

Supplementary data (Fig. S1 and spectroscopic and analytical data on the final compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.02.035.

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