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# A Photocleavable Linker for the Chemoselective Functionalization of Biomaterials

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The functionalization of matrices with "caged" functional groups and their subsequent selective uncaging is a promising approach for generating patterns of bioactive molecules to guide cell growth or recreate *in vivo* microarchitectures. To date, this has been limited to caged carboxylic acids, amines and thiols, functional groups found within biological systems. We present a bifunctional caged carbonyl linker as an alternative approach for the chemoselective functionalization of biomaterials. This linker was readily coupled to collagen, employed as a model biomaterial, and underwent rapid uncaging in aqueous media upon irradiation with ultraviolet light to yield free carbonyl groups. Modified surfaces proved to be non-adhesive to cells until the chemoselective reintroduction of adhesion following incubation of uncaged carbonyls with gelatin hydrazide, with native gelatin failing to elicit a cellular response.

## Introduction

Natural tissues consist of multiple cell types arranged in specific three-dimensional (3D) configurations. It is now widely accepted that, in order to promote optimal cell proliferation, migration, differentiation and, ultimately, survival *in vitro*, this microenvironment should be replicated as closely as possible.[1-4] Such biomimetic culture systems are of great importance in a number of biomedical applications such as tissue engineering, biosensors and the development of cellular models of disease. Numerous techniques for the spatial deposition of proteins and other bioactive molecules for the patterning and guidance of cells in both two and three dimensions have been described, including microcontact printing,[5-7] photo- and nanolithography,[8-10] microfluidic patterning,[11] inkjet printing,[12,13] and photoimmobilization.[14,15] However, these techniques, as a whole, suffer from a number of drawbacks, including a lack of cytocompatibility (precluding subsequent introduction of biomolecules in the presence of living cells) and poor spatial resolution. Perhaps the most promising approach for patterning biological cues, particularly in 3D, is the use of matrices where reactive functional groups are caged by a photolabile protecting group (PPG). Upon irradiation, photolysis of the PPG uncages the chemical moiety, thus enabling the subsequent coupling of biomolecules to the exposed functional group. With the use of photomasks or a focused laser, patterned bioactive matrices can be generated, while uncaging using multiphoton excitation extends this to potentially complex three-dimensional arrangements. To date, this approach has been pursued with caged amine,[16-18] carboxylic acid[19,20] and thiol linkers[21,22] which, following irradiation, are coupled with biomolecules possessing complementary functionality (e.g. succinimide esters, amines and maleimides, respectively). However, these functional groups are commonly found in biological systems so, for the sequential functionalization of matrices with multiple bioactive molecules or biopatterning in the presence of live cells, specificity could be lost due to cross-reactivity with existing functional groups.

Aldehydes and ketones are not normally found within biological systems and can react chemoselectively with suitably functionalized molecules such as hydrazides, thiosemicarbazides or aminooxy compounds in the presence of biologically prevalent groups.[23-25] Hence, in order to pattern biomaterial matrices with multiple bioactive molecules while eliminating the potential of cross-reactivity, a caged aldehyde or ketone linker would be highly desirable. This paper describes the development and evaluation of such a molecule. The strategy for the design and application of this linker is depicted in **Fig 1**. This molecule consists of three key components (**Fig 1a**): a carbonyl group caged with PPG **2**,[26] a poly(ethylene glycol) (PEG)-based linker, and a terminal primary amine. PEG incorporation is desirable for two reasons; firstly to provide a physical spacer between the caged carbonyl and the material surface and, secondly, to render the modified biomaterial cell-repulsive until chemoselective ligation of an adhesive biomolecule, preventing non-specific adhesion.[27-29] A primary amine was chosen as the terminal functionality in order to confer the ability to couple the linker to the wide range of biomaterials that possess carboxylic acid groups, either naturally (e.g. proteins, alginate) or following minor chemical modification (e.g. hydrolysis of polyesters). The strategy for the application of this linker is shown in **Fig 1b**. The biomaterial surface, in this case with accessible carboxylic acids (I), is activated with *N*-(3-dimethylaminopropyl)-*N*′-

ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), then coupled with linker **1** to decorate the surface with caged carbonyls (II). Exposure of the surface to ultraviolet light under aqueous conditions results in photolysis of the PPG, uncaging the protected carbonyl (III). The biomaterial surface in both II and III is cell-repulsive, and will only promote cell adhesion following chemoselective ligation of a cell-adhesive molecule with a hydrazide group, or other suitable functionality, to the exposed carbonyl (IV), in this case *via* a hydrazone bond.

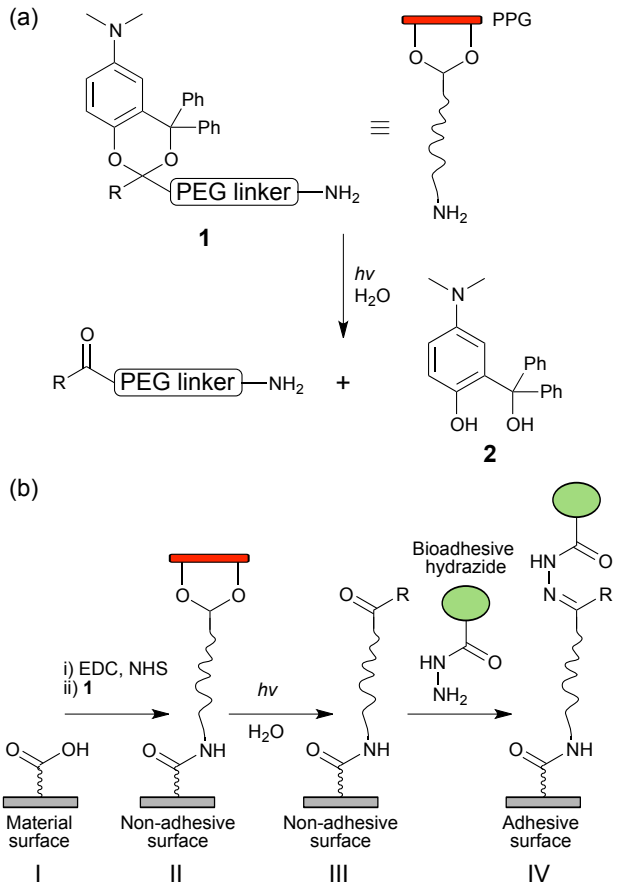



**Figure 1** Strategy for the chemoselective functionalization of materials with a caged aldehyde or ketone. (a) Structure of the proposed caged carbonyl and its photolysis upon UV irradiation under aqueous conditions. (b) Schematic of site- and chemoselective decoration of biomaterials with cell-adhesive molecules following coupling of linker **1**. Carboxylic acids on the surface of a material (I) are activated and covalently coupled to the caged carbonyl linker *via* an amide bond (II). Photolysis of the photolabile protecting groups (PPG) by irradiation with ultraviolet light yields free carbonyls on the biomaterial (III) and this functionality is subsequently used to chemoselectively ligate a cell-adhesive hydrazide, or other suitably reactive species, to the surface (IV). R = H or alkyl.

## Experimental

### Materials

All reagents were purchased from Sigma-Aldrich (Dorset, U.K.) unless stated otherwise and were used without further purification.

### Synthesis

The synthetic route to caged aldehyde **1'** is shown in **Scheme 1**. Detailed synthetic procedures and NMR spectra for **1'** and compounds **3** to **7** are provided in the ESI†.

### Photolysis and UV/Vis Spectroscopy of Caged Aldehyde 1' in Solution

Photolysis was performed on **1'** (20 μM) in 50% v/v aqueous methanol for various times by exposure to light with $\lambda_{max}$ = 365 nm (26 mW $cm^{-2}$) using a 400W UVA lamp (UV Light Technology, Ltd.). Following photolysis, solutions were analysed by RP-HPLC using a Jasco HPLC system equipped with a Phenomenex Gemini 5 μm C-18 column (150 x 4.4 mm) with a flow rate of 1 mL/minute and a mobile phase of 1:1 acetonitrile:water. The peak area of **1'** at each time point was determined to assess the extent of photolysis. UV/Vis spectra of **1'** (20 μM) in methanol were obtained using a Fluostar Omega multi-mode microplate reader (BMG Labtech).

### Functionalization and Analysis of Collagen-Coated Surfaces with Caged Aldehyde 1'

Depending on the experiment, either 24 well cell culture plate or circular glass coverslips (12 mm diameter) were incubated with collagen (0.01% w/v in 0.02 M acetic acid) for 1 hour, after which the solution was aspirated and surfaces allowed to air dry. Following washing with phosphate-buffered saline (PBS), surfaces were incubated with a solution of EDC and NHS in PBS (36.5 and 17.4 mM, respectively) for 1 hour. After rinsing with PBS, activated surfaces were incubated with **1'** in 1:1 methanol/water for 1 hour. Surfaces were then rinsed with PBS prior to use. For UV/Vis analysis, collagen-coated 24 well plates were functionalized with various concentrations of **1'** and each well immersed in ultrapure water. UV/Vis spectra were obtained using a Fluostar Omega multi-mode microplate reader. Surfaces functionalized with **1'** (30 μM) were then irradiated with light at $\lambda_{max}$ = 365 nm (26 mW $cm^{-2}$) for 20 minutes to photolyze the PPG, washed three times with ultrapure water and the UV/Vis spectroscopy repeated.

### Static Water Contact Angle Measurements

Collagen-coated circular glass coverslips functionalized with **1'** (30 μM) were placed on microscope slides and 5 μl drops of ultrapure water applied to the surface. Images of the drops were obtained using a Veho VMS-004 USB microscope and a similar experimental setup as that described by Lamour *et al.*[30] Drop analysis was performed using the "drop_analysis" plugin for ImageJ (NIH, Bethesda, Maryland, USA) and contact angles reported as the mean ± standard deviation of three independent experiments.

### Ligation of Fluorescein-5-Thiosemicarbazide to Uncaged Aldehydes

Collagen-coated glass coverslips were functionalized with various concentrations of **1'** and the photolabile protecting group photolyzed by irradiation for 20 minutes as described above to uncage the aldehydes. As a positive, aldehyde-rich control, collagen coated coverslips were incubated with sodium periodate (2 mM) in ultrapure water for one hour at room temperature to oxidize carbohydrate moieties. Coverslips were washed three times with ultrapure water and then incubated with fluorescein-5-

thiosemicarbozide (1 mg/mL) in PBS for 20 minutes at room temperature. Following washing with ultrapure water to remove unbound thiosemicarbazide, the coverslips were imaged using fluorescence microscopy (Leica DMI400B) to determine the relative extent of fluorescein ligation to the surfaces. Images were converted to grayscale and analysed using ImageJ to determine the image area, mean grey value and integrated density. The corrected fluorescence intensity (CFI) of each image was calculated using the equation CFI = integrated density – (area x mean grey value of control images). Surfaces functionalized with **1'** (30 μM) and treated with fluorescein-5-thiosemicarbozide without photolysis were used as controls. Data were collected from three coverslips per condition and are presented as the mean ± standard deviation of three independent experiments.

### Functionalization of Gelatin with Pendant Hydrazides

Gelatin from porcine skin (type B, 0.05 g) was dissolved in ultrapure water (10 mL, pH 4) and stirred for 48 hours at room temperature with the trifluoroacetate salt of 2,5-dioxopyrrolidin-1-yl 3-(4-hydrazinyl-4-oxobutanamido)propanoate (0.50 g), previously synthesized as described by Scott and Cwi.[31] The solution was then dialyzed for 72 hours against ultrapure water and lyophilized to yield gelatin hydrazide. To confirm the presence of the hydrazide functionality, the modified gelatin (10 mg) was treated with 4-hydroxybenzaldehyde (100 mg) in ultrapure water (5 mL; pH 5.5) for 18 hours at room temperature. The resultant modified protein was dialyzed and lyophilized as described above and analysed by UV/Vis spectroscopy.

### Cell Adhesion to Functionalized Surfaces

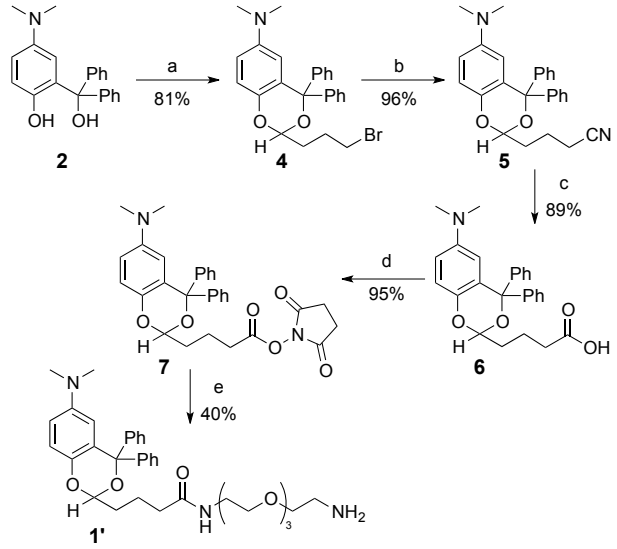
Murine C2C12 myoblasts (ECACC, #91031101) were cultured in Dulbecco's modified Eagle's medium supplemented with foetal bovine serum (10% v/v), penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL). Cells were maintained in a humidified atmosphere at 37 ºC in an atmosphere containing 5% $CO_2$.

Collagen-coated circular glass coverslips functionalized with **1'** (30 μM) were either left untreated or subjected to photolysis. Photolyzed surfaces were incubated in PBS adjusted to pH 5.5 in the absence or presence of gelatin or gelatin hydrazide (3 mg/mL) for 20 minutes at 37 ºC. Coverslips were then washed with PBS and placed in the wells of a 6 well plate. Each well was seeded with $1x10^5$ C2C12 cells and their growth monitored by phase contrast microscopy for 72 hours. Cells were then stained with Hoechst 33342 in PBS (Invitrogen; 1 μg/mL) for 20 minutes at 37 ºC, and fixed in methanol at -20 ºC for 10 minutes prior to imaging using fluorescence microscopy.

## Results and Discussion

### Synthesis of a Caged Aldehyde Linker

The synthetic route to caged aldehyde linker **1'** is depicted in **Scheme 1**. Following the synthesis of the photolabile protecting group, 4-(dimethylamino)-2-(hydroxydiphenylmethyl)phenol **2**,[26] a carbonyl with a suitable terminal functionality for subsequent installation of the PEG spacer was required. After numerous unsuccessful attempts to protect a variety of aldehydes and ketones with **2**, the key step in the synthesis of **1'** proved to be the formation of novel acetal **4**, derived from **2** and 4-bromo-butanal **3**, synthesized from 4-bromobutanol using Swern conditions.




**Scheme 1** a) 4-Bromobutanal (**3**), $Bi(OTf)_3$, toluene, 80 °C; b) $NEt_4CN$, MeCN, reflux; c) KOH, 2-methoxyethanol, reflux; d) *N*-hydroxysuccinimide, *N,N'*-diisopropylcarbodiimide, $CH_2Cl_2$, rt; e) $H_2N(CH_2CH_2O)_3(CH_2)_2NH_2$ (**8**), $CH_2Cl_2$ rt.

Unexpectedly, conversion of the bromo substituent to the corresponding carboxylic acid proved initially challenging. Original attempts to form a Grignard reagent through the reaction of **4** with activated magnesium followed by the addition of solid carbon dioxide were unsuccessful. However, an alternative route *via* conversion of bromide **4** to the corresponding nitrile **5** using tetraethylammonium cyanide, followed by hydrolysis to the acid **6** was successful, although severe hydrolysis conditions were required. Subsequent activation of acid **6** with *N*-hydroxysuccinimide and *N,N'*-diisopropylcarbodiimide gave compound **7** in high yield. Reaction of activated ester **7** with carbamate **8**, prepared from tetraethylene glycol (TEG) using three literature synthesis steps,[32] led to the isolation of caged aldehyde **1'** in 40% yield following purification.

### Functionalization of Biomaterial Surfaces with Caged Aldehydes

For its application in the chemoselective modification of materials with bioactive molecules, it was essential to demonstrate that caged aldehyde **1'** undergoes successful uncaging in an aqueous environment. This was initially demonstrated in solution by irradiation of **1'** at $\lambda_{max}$ = 365 nm (26 mW $cm^{-2}$) and subsequent HPLC determination of the remaining linker with respect to exposure time. Uncaging under these conditions was rapid, with complete photolysis of the PPG within 15 minutes (**Fig. 2**). We then proceeded to demonstrate the utility of this approach in material functionalization by employing collagen films as a model biomaterial substrate. Activation of exposed carboxylic acids with EDC and NHS followed by incubation with linker **1'** resulted in its successful coupling to the films. This was confirmed by UV/Vis spectroscopy of the modified surfaces, where the presence of the surface-bound PPG chromophore was clearly visible and concentration-dependent (**Fig. 3**), matching the UV/Vis spectrum observed in solution (**Fig. 3**, dotted line).

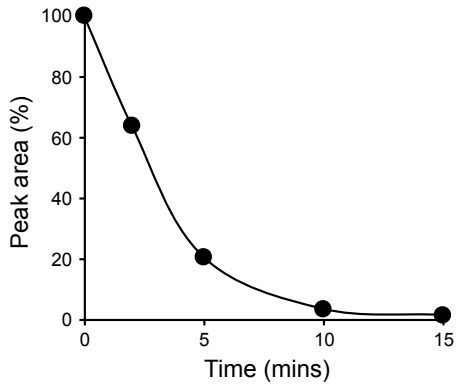



**Figure 2** Progress of uncaging of **1'** (20 μM) in 1:1 methanol/water determined by monitoring HPLC peak area of the remaining compound.

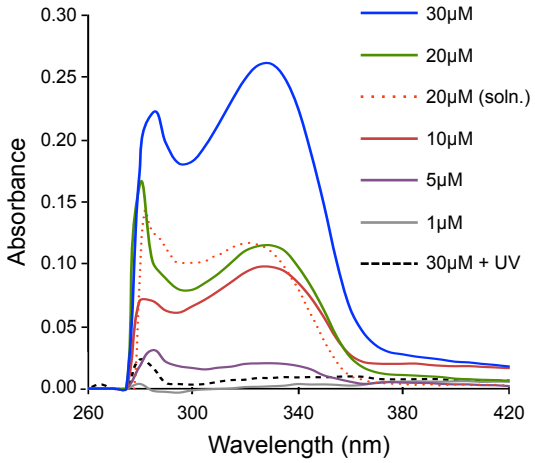



**Figure 3** UV/visible spectra of collagen films covalently modified with different concentrations of caged aldehyde **1'**. The dotted line represents the solution spectrum of **1'** in methanol (20μM), while the dashed line represents the spectrum of a film modified with **1'** (30 μM) and then irradiated at $\lambda_{max}$ = 365 nm (26 mW $cm^{-2}$).

Successful photolysis of the PPG from the functionalized collagen in an aqueous environment was demonstrated following irradiation for 20 minutes at $\lambda_{max}$ = 365 nm (26 mW $cm^{-2}$), as evidenced by the dashed line in **Fig. 3**, indicating the disappearance of the PPG chromophore. The successful coupling of **1'** to collagen films and the subsequent uncaging of the aldehyde was further demonstrated by examining the static contact angle of water droplets on the functionalized surfaces (**Fig. 4**). The contact angle for unmodified collagen films was 37.55 ± 3.61° (**Fig. 4a**), in agreement with literature values and reflecting its hydrophilic nature.[33] Upon coupling linker **1'** to collagen, the water contact angle increased to 78.17 ± 0.54°, indicating a significantly more hydrophobic surface as might be expected from the presence of the three phenyl groups in the PPG (**Fig. 4b**). Successful photolysis of the PPG was demonstrated by a further shift in the water contact angle to 28.56 ± 0.97°, resulting from the presence of the hydrophilic aldehyde and TEG spacer on the surface of the collagen (**Fig. 4c**).

Following the successful demonstration of collagen functionalization and subsequent uncaging of the protected aldehydes, we then investigated the utilization of the exposed aldehydes for chemoselective ligation to the collagen surface. Collagen films were activated with EDC and NHS and then incubated with various concentrations of linker **1'**. Modified films were irradiated under the conditions described above and fluorescein-5-thiosemicarbazide ligated to the resultant free aldehydes. To generate a control, aldehyde-rich surface, collagen films were treated with sodium periodate in order to oxidize available sialic acids within the glycoprotein.[34] The fluorescence of the films, a measure of the number of available aldehydes that had undergone chemoselective ligation, was then determined and shown to be dependent on linker concentration (**Fig. 5**), demonstrating the successful uncaging of the surface-bound linker and the availability of the aldehydes for further functionalization.

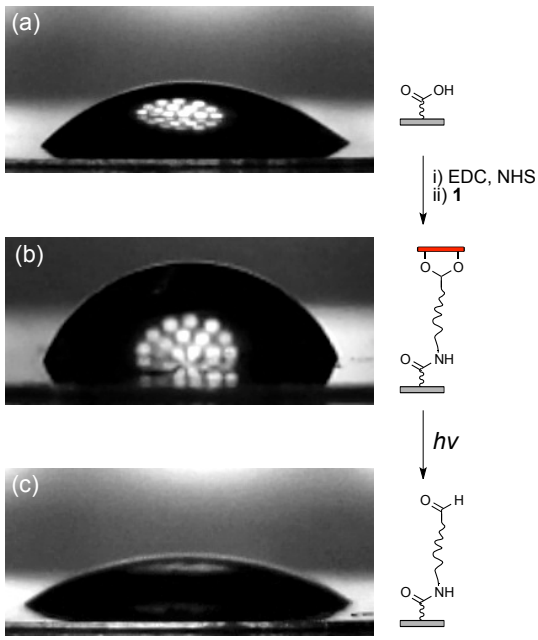



**Figure 4** Successful modification and subsequent photolysis of collagen films demonstrated by static water contact angle. Untreated collagen (a), collagen modified with caged aldehyde **1'** (b) and uncaged aldehydes on modified collagen following photolysis (c).

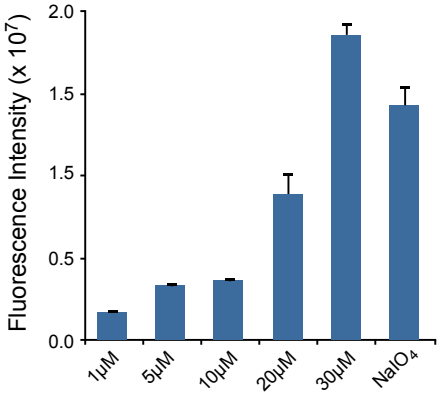



**Figure 5** Effect of linker concentration on the fluorescence of collagen films following coupling with linker **1'**, photolysis for 20 minutes and chemoselective ligation of fluorescein-5-thiosemicarbazide. Collagen films oxidized with sodium periodate ($NaIO_4$) to generate aldehydes were used as a comparison.

### Chemoselective Reintroduction of Cell Adhesion Using Caged Aldehydes

For linker **1'** to have future applications in cell patterning and guidance, it was essential to demonstrate that cell adhesivity and growth on modified biomaterials could be selectively introduced with no non-specific binding to regions that had not been irradiated (**Fig. 1b II**) or chemoselectively functionalized with an adhesive species (**Fig. 1b III**). To achieve this, we chose gelatin as a suitable cell-adhesive biomolecule. To incorporate hydrazide functionalities within gelatin, i.e. generating "gelatin hydrazide", we synthesized a short bifunctional linker containing a hydrazide trifluoroacetate salt at one end and an *N*-hydroxysuccinimide ester at the other.[31] Upon incubation with gelatin, free amine groups within the protein reacted with the NHS ester to form amide bonds, thus decorating the gelatin with pendant hydrazides (**Fig. 6a**). To confirm the incorporation of hydrazide moieties, the modified protein was incubated with 4-hydroxybenzaldehyde and, following purification, subsequently analysed using UV/Vis spectroscopy.

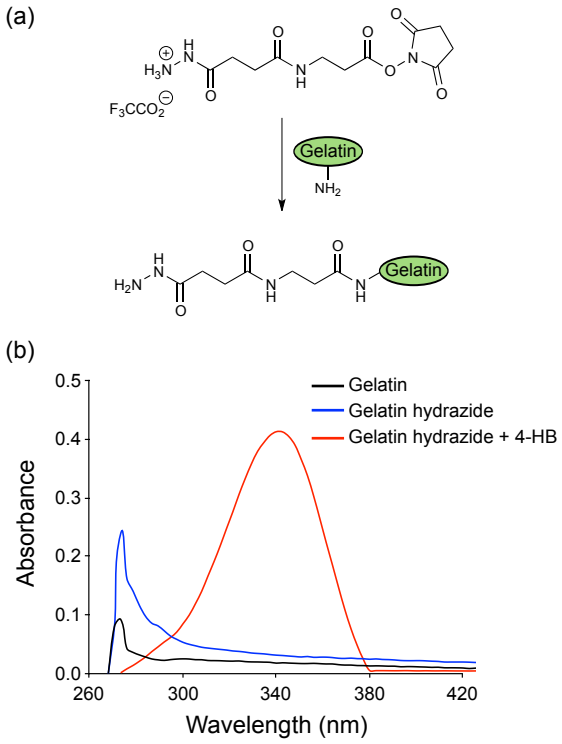



**Figure 6** (a) Decoration of gelatin with hydrazide groups *via* the reaction of free amines with the TFA salt of 2,5-dioxopyrrolidin-1-yl 3-(4-hydrazinyl-4-oxobutanamido) propanoate. (b) UV/Vis spectra of unmodified gelatin, gelatin hydrazide and gelatin hydrazide following incubation with 4-hydroxybenzaldehyde (4-HB) in PBS.

A characteristic peak at ~340 nm, corresponding to the resultant aromatic hydrazone,[35] was observed following its reaction with gelatin hydrazide, confirming the successful chemoselective reaction of the aldehyde with the pendant hydrazides. Similar peaks were absent in native gelatin and untreated gelatin hydrazide (**Fig. 6b**) and no evidence of aromatic imine formation was observed following the reaction of unmodified gelatin with 4-hydroxybenzaldehyde under the same conditions (not shown), confirming the chemoselectivity of this reaction.

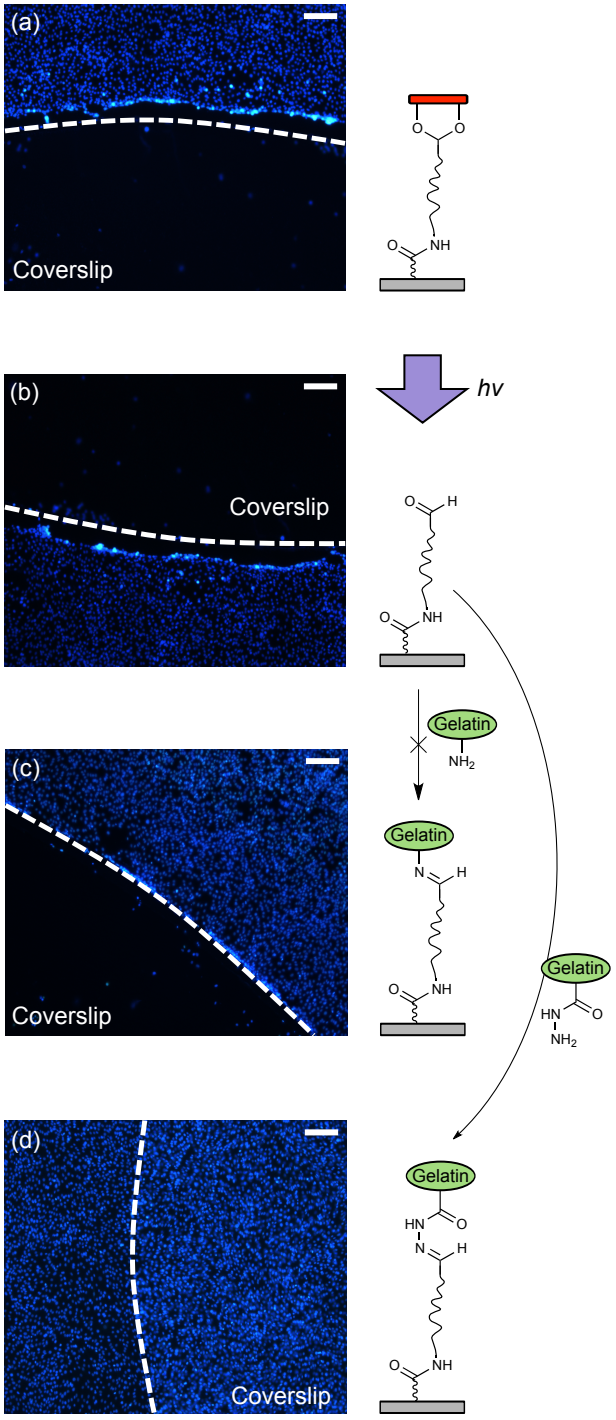



**Figure 7** Fluorescence microscopy of C2C12 myoblasts 72 hours after seeding on collagen-coated 12 mm diameter coverslips with different surface modifications within 6 well culture dishes. (a) Collagen modified with linker **1'** (30 μM) and (b) collagen modified with linker **1'** (30 μM) and then UV irradiated to uncage protected aldehydes. (c) Uncaged aldehydes incubated with gelatin (3 mg/mL) for 20 minutes at 37 ºC, pH 5.5 and (d) uncaged aldehydes incubated with gelatin hydrazide (3 mg/mL) under the same conditions as (c). Cell nuclei stained with Hoechst 33342 are shown in blue. Scale bars = 200 μm.

Cell adhesion and growth was studied using C2C12 myoblasts as a model cell line. Collagen films were generated on circular glass coverslips, activated with EDC/NHS and functionalized with caged aldehyde **1'**. These coverslips were then placed within

standard multiwell cell culture plates and seeded with C2C12s following exposure of the films to various conditions. After 72 hours of culture, cell growth on the different coverslip surfaces was compared with that on the surrounding tissue culture plastic. As might be expected for a substrate rich in hydrophobic phenyl groups, surfaces functionalized with **1'** did not support cell adhesion and growth (**Fig. 7a**), thus fulfilling the criterion of non-irradiated surfaces being non-adhesive. Cells on the surrounding plastic became confluent and grew up to the edge of the coverslip but did not cross the boundary. Photolysis of the PPGs, resulting in exposure of aldehydes on the coverslip surfaces, elicited similar results, with no adhesion or growth of cells on these surfaces (**Fig. 7b**). Again, this was expected due to the design of the linker, where the TEG spacer generates a cell- and protein-repulsive layer on the biomaterial surface. The final two surface treatments demonstrated the chemoselective ligation of gelatin hydrazide to the uncaged aldehydes. The reaction of a hydrazide with a carbonyl to generate a hydrazone bond is pH-dependent and favoured under acidic conditions.[24] Hence, we incubated collagen films, following functionalization with **1'** and subsequent irradiation, with either gelatin or gelatin hydrazide for 20 minutes at pH 5.5 prior to cell seeding. These surfaces did not promote cell adhesion and growth following incubation with gelatin, suggesting that the native protein did not react with the aldehydes under these conditions (**Fig. 7c**), resulting in a surface similar to that in **Fig. 7b**. While gelatin possesses free amine groups, which may be expected to react with aldehydes, the susceptibility of any resultant imines to hydrolysis results in a shift of the equilibrium of this reaction away from imine formation.[36] Conversely, however, aldehyde rich surfaces that were incubated with gelatin hydrazide promoted cell adhesion and growth in excess of the surrounding tissue culture plastic, clearly demonstrating the successful chemoselective ligation of this protein to uncaged aldehydes under these reaction conditions (**Fig. 7d**). This is a versatile approach, which could readily be employed for the hydrazide functionalization of other bioactive proteins and peptides, and their subsequent chemoselective ligation to biomaterial surfaces and matrices.

## Conclusions

A bifunctional caged aldehyde linker that can be coupled to a number of different biomaterials and readily undergoes photolysis in aqueous media has been synthesized, and chemoselective ligation of biomolecules to the resulting uncaged aldehydes has been demonstrated. To our knowledge, this is the first reported use of caged aldehydes to functionalize material surfaces. Chemoselective ligation of hydrazides, thiosemicarbazides or aminooxy compounds to uncaged aldehyde groups offers the possibility of decorating functional materials with multiple biologically active molecules without unwanted cross-reactivity towards existing functional groups of biomolecules already present on the material. By making small changes to the design of the linker's terminal functionality, this general approach is applicable to a large number of biomaterials other than those with accessible carboxylic acids and can potentially be utilized with both two- and three-dimensional substrates, either alone or in combination with other caged functional groups. Amongst the possible applications of this approach is the recapitulation of tissue microarchitectures and cell niches for the advancement of areas such as biosensing, tissue modelling, tissue engineering and regenerative medicine.

## Notes and references

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† Electronic Supplementary Information (ESI) available: Detailed synthetic procedures and NMR spectra for all novel compounds. See DOI: 10.1039/b000000x/


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# A Photocleavable Linker for the Chemoselective Functionalization of Biomaterials

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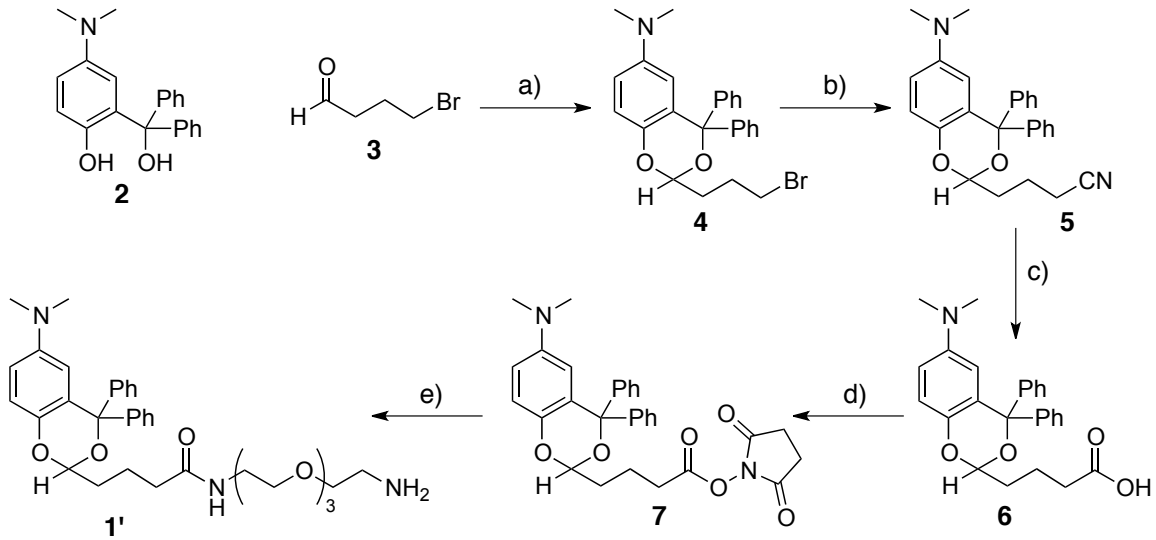
## General Experimental

All reagents were purchased from Sigma-Aldrich (Dorset, U.K.) and used without further purification unless otherwise specified. All reactions were carried out in freshly dried and distilled solvents under a dry nitrogen atmosphere apart from those involving aqueous solutions. NMR spectra were recorded on Bruker Avance III 400 MHz or 500 MHz NMR spectrometers. Data are expressed in parts per million downfield from $SiMe_4$ as an internal standard or relative to $CHCl_3$. NMR assignments were supported by $^1H$-$^{13}C$ and $^1H$-$^1H$ NMR 2D spectra and DEPT for compound **4-7** and caged aldehyde **1'**. J vales are given in Hz. IR spectra were measured on a Perkin Elmer Spectrum RXI FT-IR spectrophotometer and reported as $cm^{-1}$. Mass spectra were obtained using Bruker micrOTOF spectrometers in electrospray positive ion mode.

## Reaction Scheme




a) $Bi(OTf)_3$, toluene, 80 °C; b) $NEt_4CN$, MeCN, reflux; c) KOH, 2-methoxyethanol, reflux; d) *N*-hydroxysuccinimide, *N*,*N'*-diisopropylcarbodiimide, $CH_2Cl_2$, rt; e) $H_2N(CH_2CH_2O)_3(CH_2)_2NH_2$ (**8**), $CH_2Cl_2$ rt.

## Synthesis

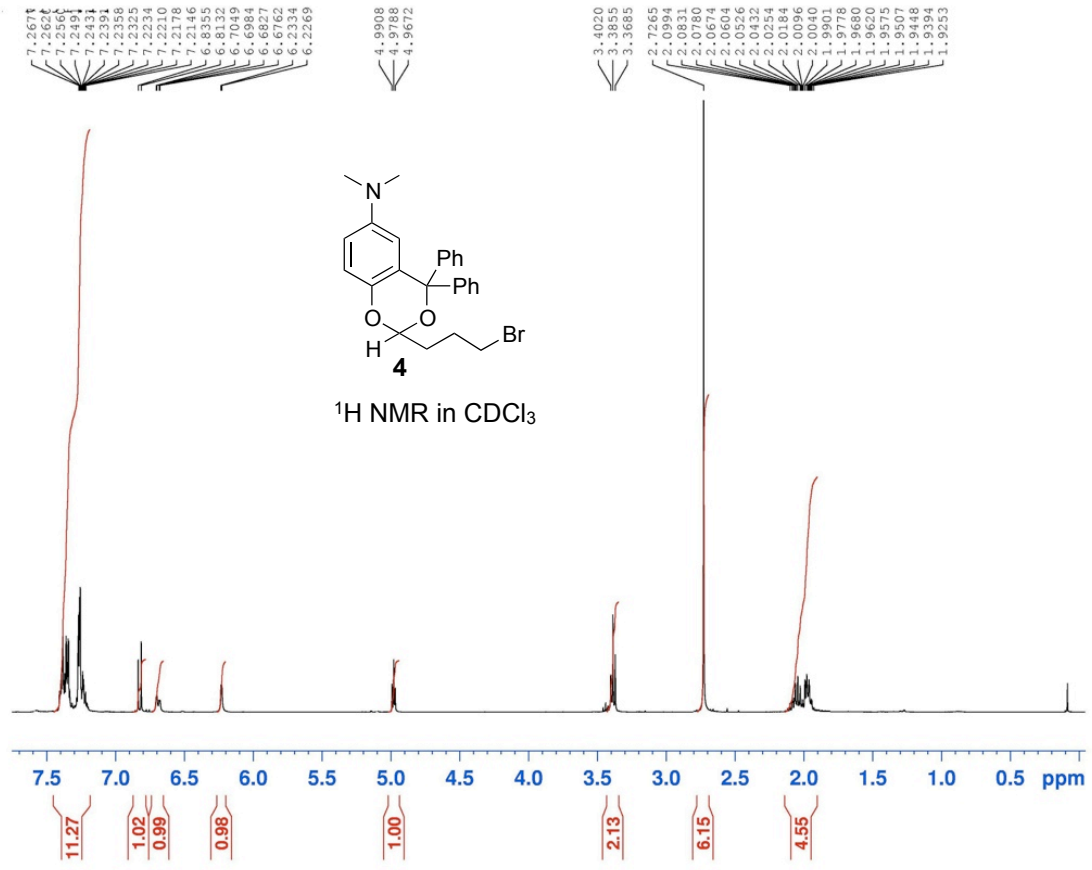
*4-(Dimethylamino)-2-(hydroxydiphenylmethyl)phenol* (**2**) was prepared according to the synthetic route described by Wang *et al.*[1]

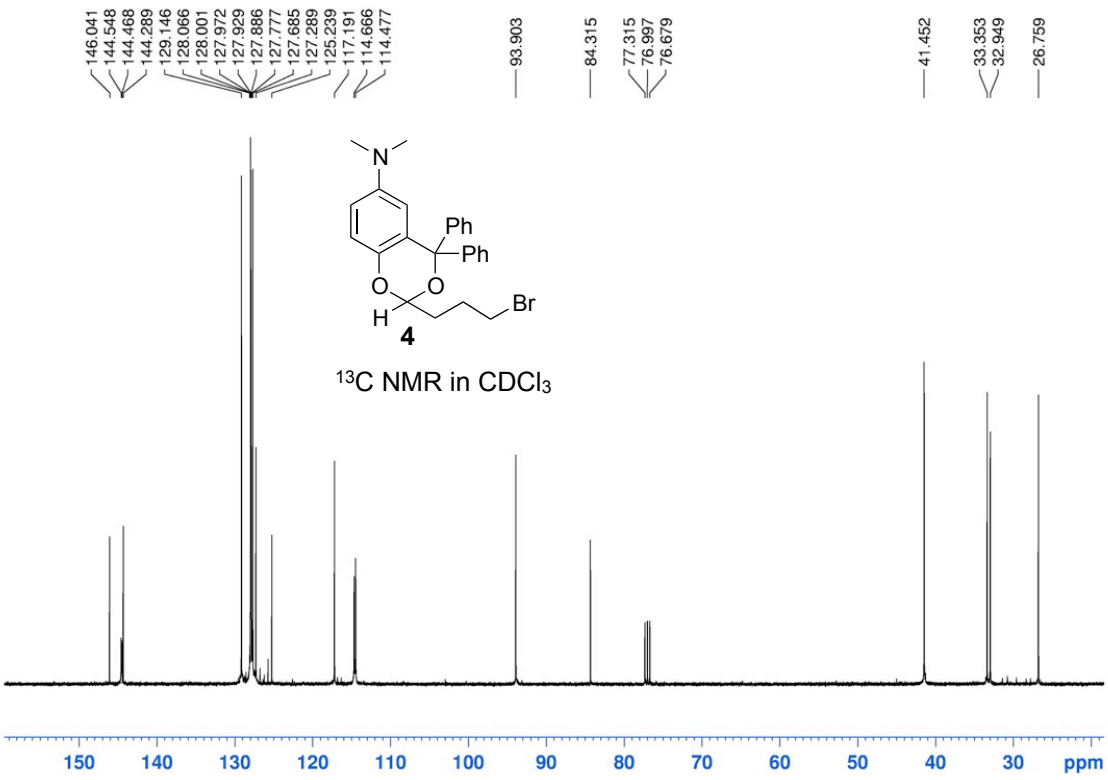
*4-Bromobutanal* (**3**)

DMSO (0.50 mL, 6.44 mmol) in dichloromethane (4 mL) was added to oxalyl chloride (0.41 mL, 4.57 mmol) in dichloromethane (4 mL) at -78 °C. After 5 minutes a solution of 4-bromo-1-butanol (0.68 g, 4.44 mmol) in dichloromethane (8 mL) was added to the mixture and after 10 minutes, *N,N*-diisopropylethylamine (2.50 mL, 14.33 mmol) was added. The solution was stirred at -65 °C for 15 minutes and for an additional 15 minutes at room temperature, poured into 10% citric acid and extracted with dichloromethane (3 x 30 mL). The organic layer was washed with saturated $NaHCO_3$ (30 mL) and dried over $MgSO_4$. The solution was evaporated to dryness and the residue purified by silica column chromatography (1:4 dichloromethane/hexane) to yield compound **3** (0.54 g, 80%) as a colourless oil; $R_f$ 0.45 (1:1 dichloromethane/hexane); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 2.15-2.22 (2 H, quintet, $CH_2$), 2.65-2.68 (2 H, m, $CH_2$), 3.44-3.47 (2 H, t, $J$ = 6.0, $CH_2$), 9.81 (1 H, s, CHO). Spectroscopic data are consistent with those described in the literature.[2]

*2-(3-Bromopropyl)*-N,N-*dimethyl-4,4-diphenyl*-4H-*benzo[*d*][1,3]dioxin-6-amine* (**4**)

Photolabile protecting group **2** (0.70 g, 2.20 mmol), 4-bromobutanal **3** (1.00 g, 6.60 mmol), Bismuth (III) trifluromethanesulfonate (0.015 g, 0.023 mmol), in toluene (8 mL) were heated at 80 ºC under nitrogen for 72 hr. The reaction was quenched with saturated aqueous sodium hydrogen carbonate solution (10 ml) and extracted with EtOAc (2 x 20 mL). The organic extracts were combined and dried ($MgSO_4$). The solution was evaporated to dryness and the residue purified by silica column chromatography (0.5:9.5 EtOAc/hexane) to yield compound **4** as a colourless oil (0.80 g, 81%); $R_f$ 0.48 (0.5:9.5 EtOAc/hexane); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.93-2.10 (4 H, m, $(CH_2)_2$), 2.73 (6 H, s, 2 x $CH_3$), 3.37-3.40 (2 H, t, $J_3$ 6.7, $CH_2Br$), 4.97-4.99 (1 H, t, $J_3$ 4.7, CH), 6.22-6.23 (1 H, d, $J_5$ 2.6, Ar-H), 6.68-6.70 (1 H, dd, $J_3$ 8.9, $J_5$ 2.6, Ar-H), 6.81-6.84 (1 H, d, $J_3$ 8.9, Ar-H), 7.21-7.48 (10 H, m, Ph-H); $\delta_C$ (300 MHz; $CDCl_3$; $Me_4Si$) 26.8 ($CH_2$), 32.9 ($CH_2$), 33.4 ($CH_2$), 41.5 (2 x $CH_3$), 84.3 (C), 93.9 (CH), 114.5 (Ar-CH), 114.7 (Ar-CH), 117.2 (Ar-CH), 125.2, 127.3, 127.7, 127.8, 127.9, 128.0, 129.1 (all Ph-CH), 144.3, 144.5, 144.6, 146.0 (*ipso*-C); $\nu_{max}/cm^{-1}$ 3468, 3417, 1650, 1501, 1439; HRMS (ESI): found $MH^+$, 452.1208 ($C_{25}H_{27}BrNO_2$ requires 452.1225); m/z (ES1) 452 ($C_{25}H_{27}BrNO_2$, 45%), 302 (100), 289 (8).



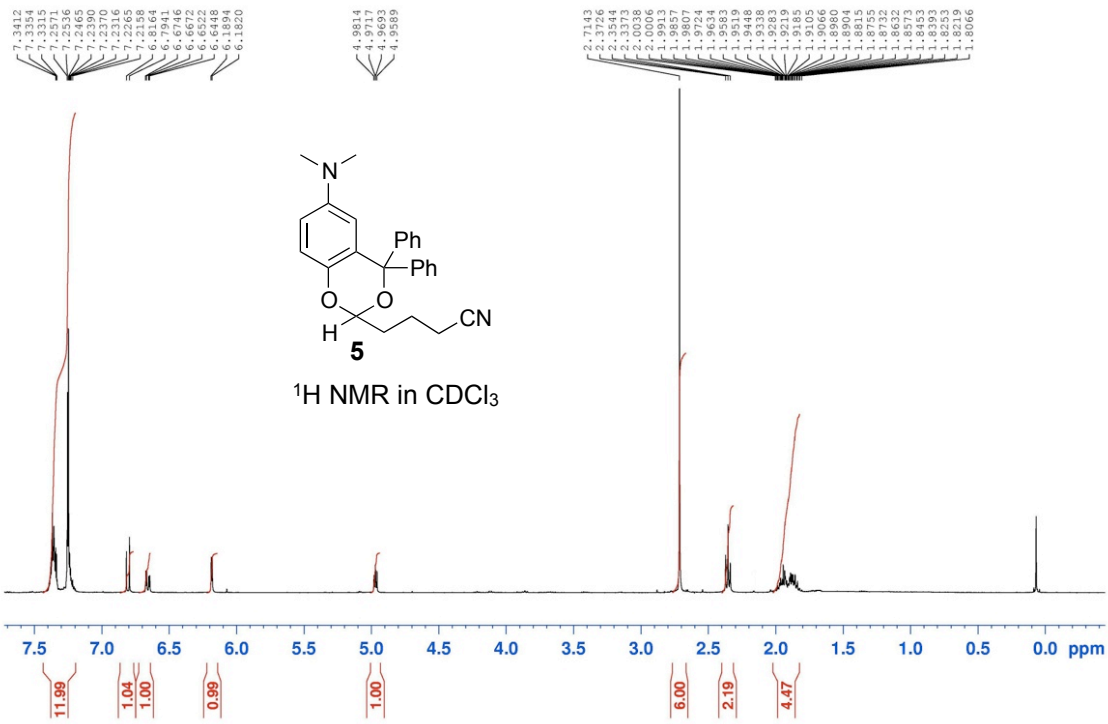



*4-(6-(Dimethylamino)-4,4-diphenyl*-4H-*benzo[*d*][1,3]dioxin-2-yl)butanenitrile* (**5**)

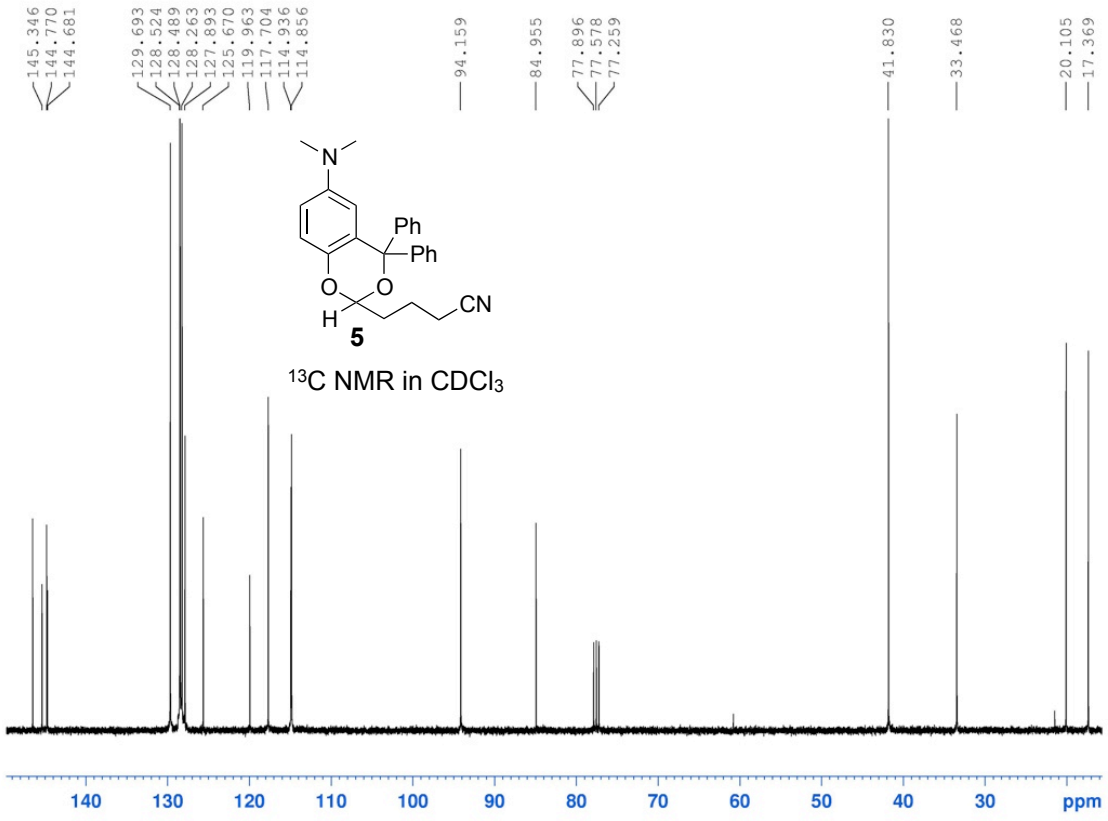
Bromide **4** (0.130 g, 0.29 mmol) and tetraethylammonium cyanide ($NEt_4CN$) (0.067 g, 0.426 mmol) were dissolved in MeCN (15 mL). The solution was heated at reflux for 5.5 hr, cooled and reduced to dryness. The resultant orange residue was purified by silica column chromatography (2:8 EtOAc/hexane) to yield compound **5** as a colourless oil (0.11 g, 96%) $R_f$ 0.5 (2:3 EtOAc/hexane); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.81-2.00 (4 H, m $(CH_2)_2$), 2.34-2.37 (2 H, t, $J_3$ 7.0, $CH_2CN$), 2.71 (6 H, s, 2 x $CH_3$), 4.96-4.98 (1 H, t, $J_3$ 4.5, CH), 6.18-6.19 (1 H, d, $J_5$ 3.0, Ph-H), 6.64-6.67 (1 H, dd, $J_3$ 9.0, $J_5$ 3.0, Ph-H), 6.79-6.82 (1 H, d, $J_3$ 9.0, Ph-H), 7.22-7.44 (10 H, m Ph-H); $\delta_C$ (300 MHz; $CDCl_3$; $Me_4Si$) 17.4 ($CH_2$), 20.1 ($CH_2$), 33.5 ($CH_2$), 41.8 (2 x $CH_3$), 85.0 (CH), 94.2 (C), 114.9 (Ar-CH), 117.7 (Ar-CH), 119.0 (C), 125.7, 127.9, 128.3, 128.5, 128.5, 128.7, 144.7, 144.8, 145.3 (all Ar-CH); $\nu_{max}/cm^{-1}$ 2923, 1623, 1516, 1446, 1238; HRMS (ESI): found $MH^+$, 399.2076 ($C_{26}H_{27}N_2O_2$ requires 399.2073); m/z (ES1) 399 ($C_{26}H_{27}N_2O_2$, 73%), 302 (100), 288 (55).

*4-(6-(Dimethylamino)-4,4-diphenyl*-4H-*benzo[*d*][1,3]dioxin-2-yl)butanoic acid* (**6**)

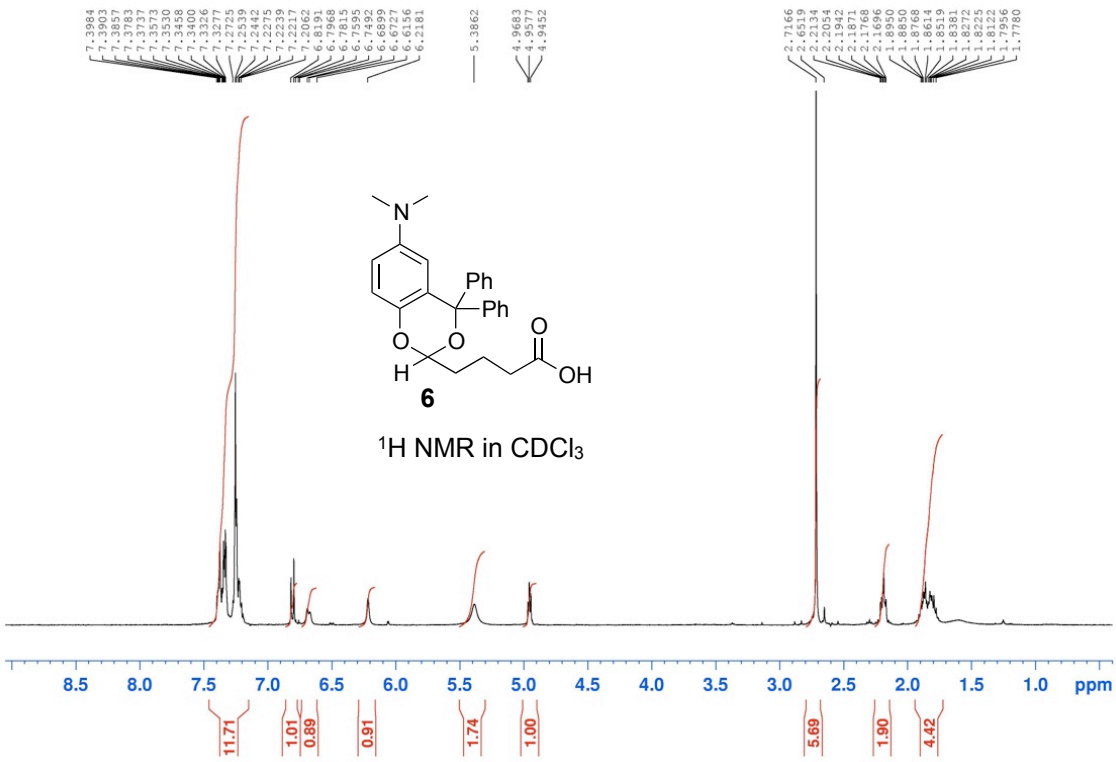
Nitrile **5** (0.30 g, 0.75 mmol), was dissolved in a saturated solution of KOH in 2-methoxy methanol (2 mL). The solution was heated at reflux for 18 hr, cooled and HCl (2 M) was added dropwise until the pH was adjusted to 2. The solution was extracted with $CH_2Cl_2$ (2 x 20 mL), the organic extracts were combined, dried over $MgSO_4$ and reduced to dryness to yield compound **6** as a brown oil (0.28 g, 89%); $R_f$ 0.2 (1:1 EtOAc/hexane); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.78-1.86 (4 H, m $(CH_2)_2$), 2.17-2.21 (2 H, m, $CH_2CO$), 2.71 (6 H, s, 2 x $CH_3$), 4.95-4.97 (1 H, t, $J_3$ 4.6, CH), 5.35-5.39 (1 H, br s, OH), 6.19-6.21 (1 H, br s, Ph-H), 6.61-6.68 (1 H, m, Ph-H), 6.75-6.82 (1 H, m, Ph-H), 7.20-7.40 (10 H, m Ph-H); $\delta_C$ (300 MHz; $CDCl_3$; $Me_4Si$) 19.5 ($CH_2$), 33.4 ($CH_2$), 35.3 ($CH_2$), 41.6 (2 x $CH_3$), 84.4 (C), 94.4 (CH), 114.9 (Ar-CH), 117.2 (Ar-CH), 125.4 (C), 127.3, 127.7, 127.8, 127.9, 128.0, 129.3, 144.3, 146.1, 174.9 (C=O); $\nu_{max}/cm^{-1}$ 3074, 1706, 1565, 1367; HRMS (ESI): found $MH^+$, 418.2013 ($C_{26}H_{28}NO_4$ requires 418.2018); m/z (ES1) 418 ($C_{26}H_{28}NO_4$, 100%), 302 (67.9), 217 (37).

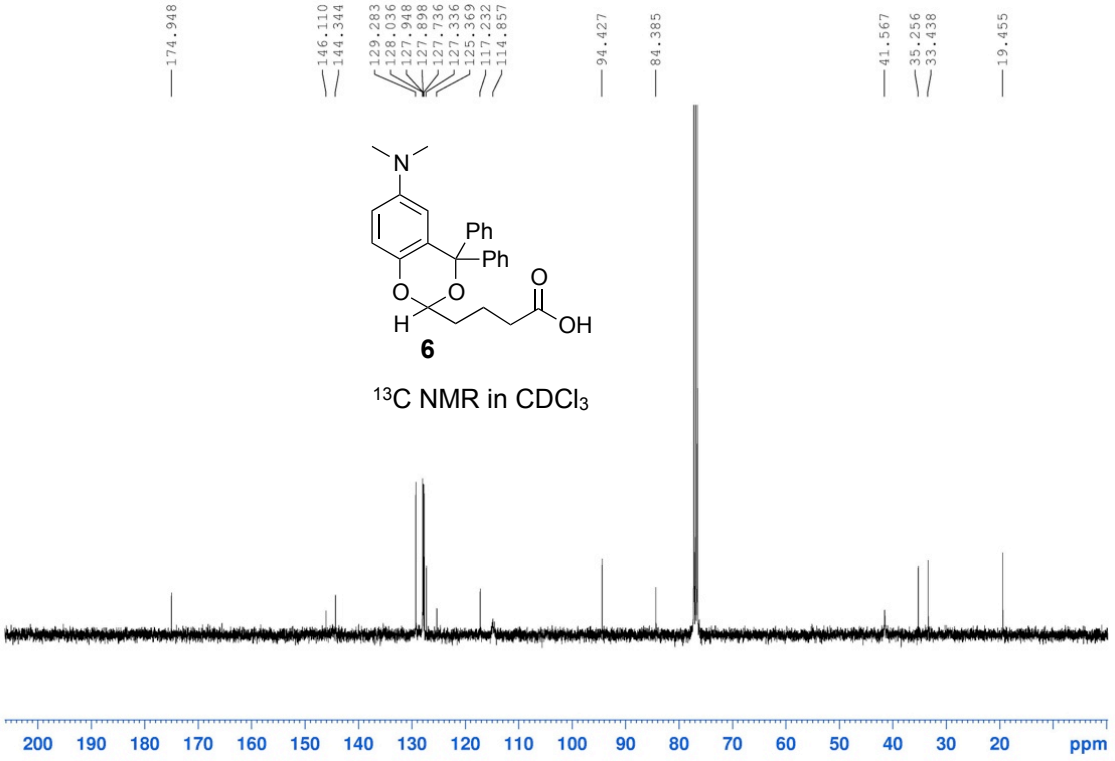



$^{1}$H NMR in $CDCl_3$



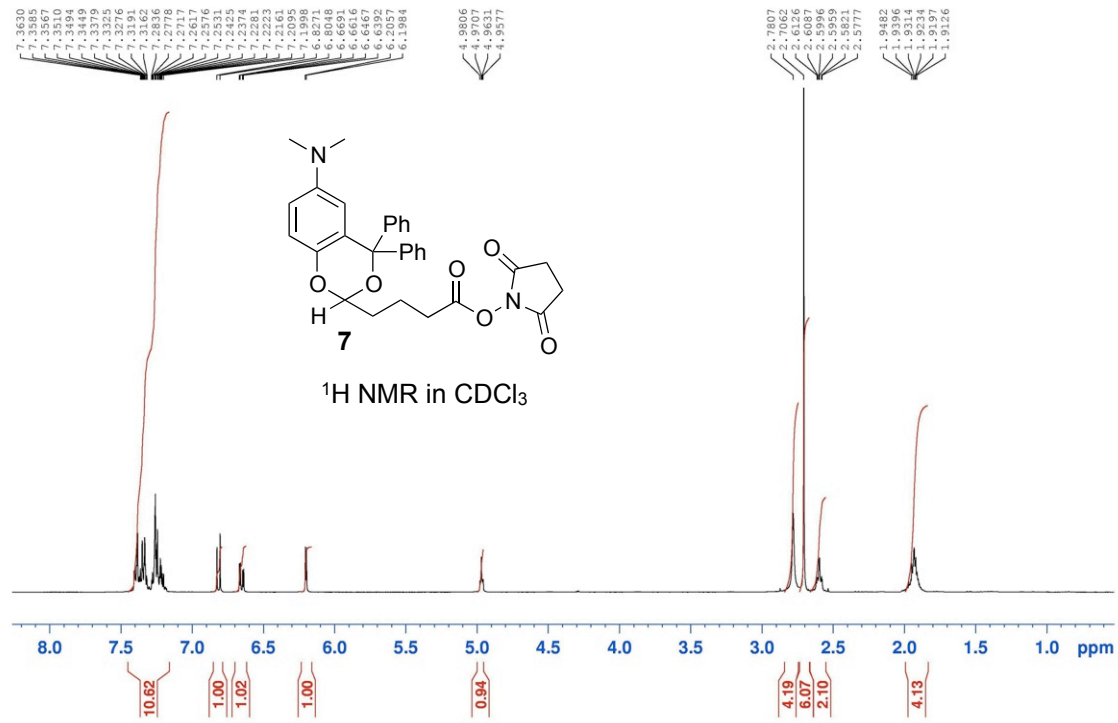

$^{13}$C NMR in $CDCl_3$

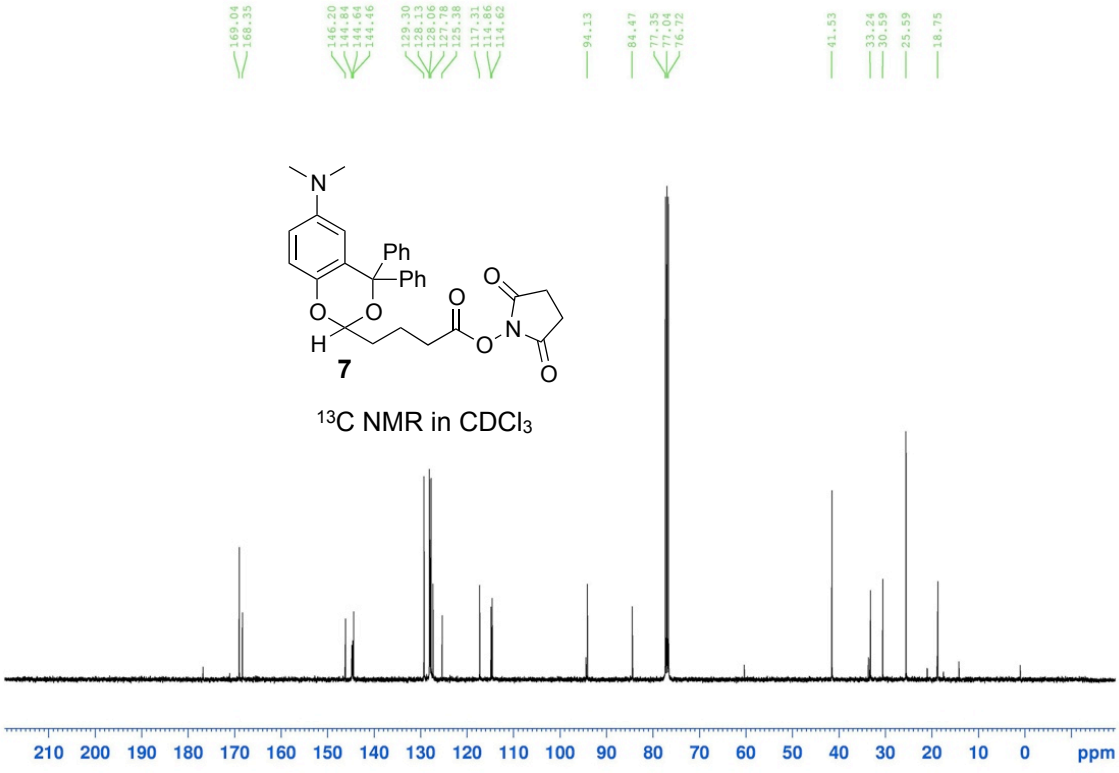



*2,5-Dioxopyrrolidin-1-yl 4-(6-(dimethylamino)-4,4-diphenyl*-4H-*benzo*[d]*[1,3]dioxin-2-yl) butanoate* (**7**)
To a solution of acid **6** (0.20 g, 0.479 mmol) and *N*-hydroxysuccinimide (0.066 g 0.574 mmol) in anhydrous $CH_2Cl_2$ (2 mL) was added *N,N'*-diisopropylcarbodiimide (0.073 g, 0.575 mmol) in anhydrous $CH_2Cl_2$ (2 mL). The solution was stirred for 72 hr at room temperature and quenched with brine. The aqueous layer was extracted with $CH_2Cl_2$ (3 x 10 mL), the organic layers were combined and dried over $MgSO_4$. The solution was evaporated to dryness and the residue purified by silica column chromatography (1:1 EtOAc/hexane) to yield compound **7** as a yellow oil (0.234 g, 95%); $R_f$ 0.60 (1:1 EtOAc/hexane); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.91-1.95 (4 H, m, $(CH_2)_2$), 2.58-2.61 (2 H, m, $CH_2$), 2.71 (6 H, s, 2 x $CH_3$), 2.78 (4 H, s, $(CH_2)_2$), 4.96-4.98

(1 H, t, $J_3$ 3.1, CH), 6.20-6.21 (1 H, d, $J_5$ 2.9, Ar-H), 6.65-6.67 (1 H, dd, $J_3$ 8.9, $J_5$ 2.9, Ar-H), 6.81-6.83 (1 H, d, $J_3$ 8.9, Ar-H), 7.21-7.44 (10 H, m, Ph-H); $\delta_C$ (300 MHz; $CDCl_3$; $Me_4Si$) 18.8 ($CH_2$), 25.6 (2 x $CH_2C$=O), 30.6 ($CH_2$), 33.2 ($CH_2$), 41.5 (2 x $CH_3$), 84.5 (C), 94.1 (CH), 114.6, 114.9, 117.3, 125.4, 127.8, 128.0, 128.1, 129.3, 144.5, 144.6, 146.2 (all Ph-CH), 168.4 (C=O), 169.0 (2 x NC=O); $\nu_{max}/cm^{-1}$ 3010, 1711, 1652, 1426, 1135 HRMS (ESI): found $MH^+$, 515.2197 ($C_{30}H_{31}N_2O_6$ requires 515.2182); m/z (ES1) 537 ($C_{30}H_{30}NaN_2O_6$, 1%), 515 ($C_{30}H_{31}N_2O_6$, 83), 418 (37.5), 302 (100).

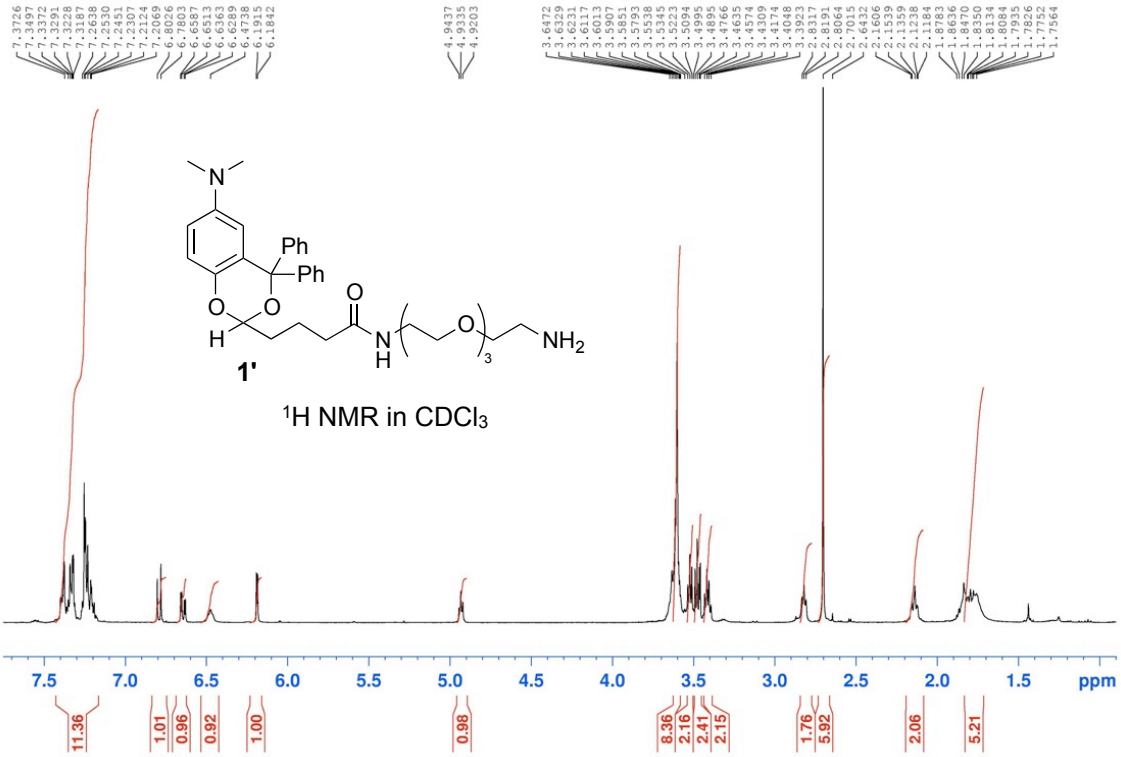


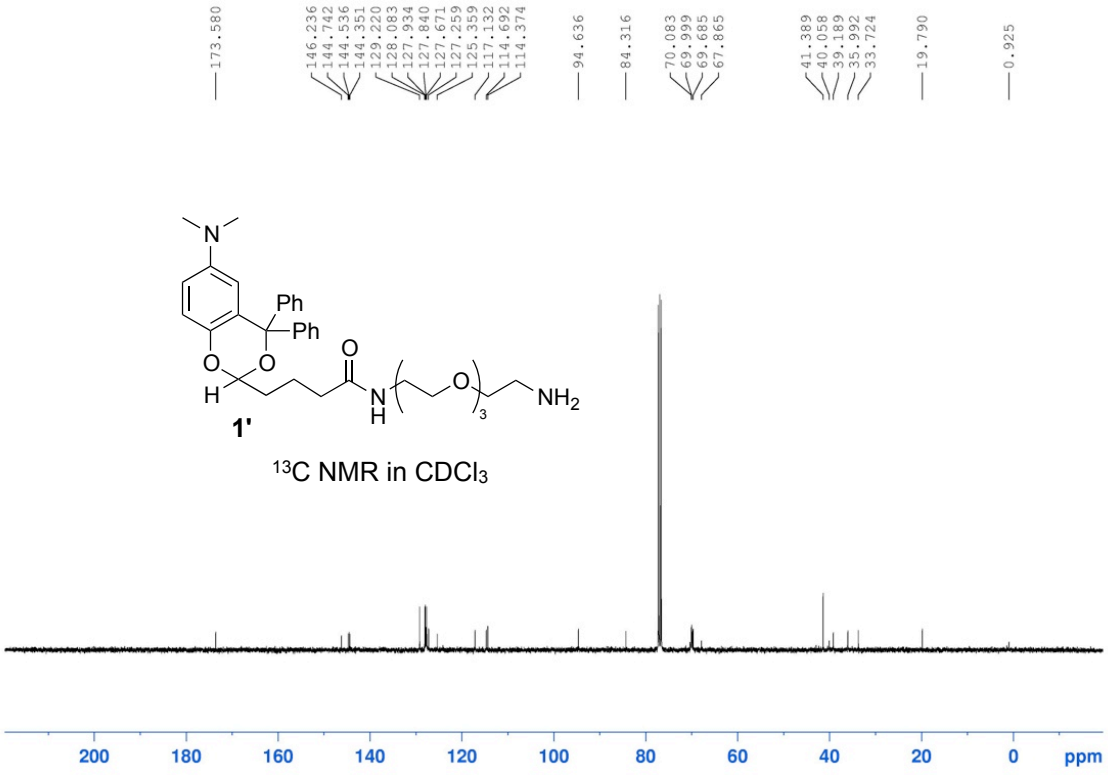



*2,2'-((Oxybis(ethane-2,1-diyl))bis(oxy))diethanamine* (**8**) was prepared according to the synthetic route described by Numata *et al.*[3]

N-*(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethyl)-4-(6-(dimethylamino)-4,4-diphenyl*-4H-*benzo[*d*][1,3]dioxin-2-yl)butanamide* (**1'**)

NHS ester **7** (0.100 g, 0.195 mmol) in dichloromethane (1 mL) was added dropwise to a solution of tetraethyleneglycol diamine **8** (0.149 g, 0.777 mmol) in dichloromethane (1 mL) over 30 minutes. The solution was stirred for 48 hours at room temperature and quenched with brine (10 mL). The aqueous layer was extracted with dichloromethane (3 x 10 mL), the organic layers were combined and dried over $MgSO_4$. The solution was evaporated to dryness and the residue purified by silica column chromatography (2:8:0.1 MeOH/$CH_2Cl_2$/TEA) to yield compound **1** as a colourless oil (0.039 g, 40%); $R_f$ 0.20 (3:7 MeOH/$CH_2Cl_2$); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.76 -1.88 (4 H, m $(CH_2)_2$), 2.12-2.16 (2 H, m, $CH_2$), 2.70 (6 H, s, 2 x $CH_3$), 2.82-2.83 (2 H, t, $J_3$ = 5.1 Hz, $CH_2$), 3.39-3.43 (2 H, m, $CH_2$), 3.46-3.50 (2 H, m, $CH_2$), 3.52-3.55 (2 H, m, $CH_2$), 3.58-3.65 (8 H, m, $(CH_2)_4$), 4.92-4.94 (1 H, t, $J_3$ 4.7, CH), 6.18-6.19 (1 H, d, $J_5$ 3.0, Ar-H), 6.47-6.49 (1 H, bs, $NH_2$) 6.63-6.66 (1 H, dd, $J_3$ 9.0 Hz, $J_5$ 3.0 Hz, Ar-H), 6.78-6.80 (1 H, d, $J_3$ 9.0 Hz, Ar-H), 7.20-7.41 (10 H, m, Ph-H); $\delta_C$ (300 MHz; $CDCl_3$; $Me_4Si$) 19.8 ($CH_2$), 33.7 ($CH_2CH$), 36.0 ($CH_2C$=O), 39.2 ($CH_2NH$) 40.1 ($CH_2NH$), 41.4 (2 x $CH_3$), 67.9 ($CH_2$), 69.7 ($CH_2$), 70.0 ($CH_2$) 70.1 ($CH_2$), 84.3 (C), 94.6 (CH), 114.4, 114.7, 117.1, 125.4, 127.3, 127.7, 127.8, 127.9, 128.1, 129.2, 129.3, 144.4, 144.5, 144.7, 146.2, (all Ph-CH), 173.6 (C=O); $\nu_{max}$/$cm^{-1}$ 2955, 2985, 1650, 1525, 1500, 1255; HRMS (ESI): found $MNa^+$, 614.3229 ($C_{34}H_{45}N_3O_6Na$ requires 614.3206); m/z (ES1) 592 ($C_{34}H_{46}N_3O_6$, 47%), 302 (45%), 242 (100).



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