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Synthesis and protein conjugation studies of vitamin K analogues

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Abstract—Two vitamin K analogues bearing a carboxylic acid side chain (**9a** and its deuterated analogue **9b**) were each synthesised in six steps from commercially available menadione. Analogue **9b** was conjugated to lysozyme and bovine serum albumin (BSA) using EDCI/HOBT and by prior formation of its activated succinimidyl ester **11**. Quantification of the thus formed conjugates by ESMS and LC–MS revealed that the number of equivalents of the analogue used in the couplings systematically controls the number of analogues that conjugate to the protein.
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1. Introduction

The K vitamins are a family of essential lipid soluble vitamins that are widely distributed in nature. Examples include the plant-derived phylloquinone (**1**), also known as vitamin K_1, and the bacterial derived menaquinones (**2**), (see Fig. 1).[1] These natural products are associated with a number of important biological functions including blood clot formation (the vitamin K dependant clotting factors VII, IX, X and prothrombin having been identified in the 1950s), early skeletal development,[2,3] bone metabolism,[2,4,5] cellular growth[6] and hence some cancers.[7–9] For these reasons it is important to be able to accurately detect and measure, the levels of K vitamins in biological samples. Currently available HPLC-based physiochemical assays for this purpose are, however, problematic since they involve laborious sample preparation, long run times and have problems with sensitivity of detection for low plasma levels.[2,10,11] Hence, there is a clear need for a more efficient and high-throughput method for the measurement of vitamin K levels in food and plasma samples. In this paper we present initial studies on the development of an enzyme-linked immunosorbent assay (ELISA) for this purpose. Such a method offers advantages of short sample preparation times and the potential for cheap and efficient high-throughput screening.

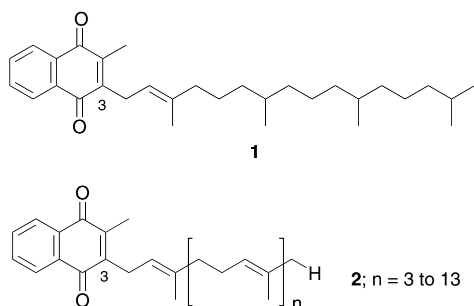

Figure 1. Two major forms of vitamin K.

2. Chemistry

The first step in the development of an ELISA assay is to produce monoclonal antibodies that recognise and bind vitamin K. This process requires attachment of a suitably substituted analogue of vitamin K to a protein in order to render it water soluble, and foreign to the species into which it is injected for monoclonal antibody production. In this paper we present the synthesis of two such vitamin K analogues (**9a** and **9b**) and a study of their conjugation with *N*-acetyl-Gly-L-Lys methyl ester, BSA and lysozyme. We chose to incorporate a terminal carboxylic acid into the analogues to allow conjugation to the lysine side chains of a protein using standard peptide coupling procedures.[12] Lysine residues are commonly located on the surface of proteins and hence are ideal targets for conjugation studies. The corresponding

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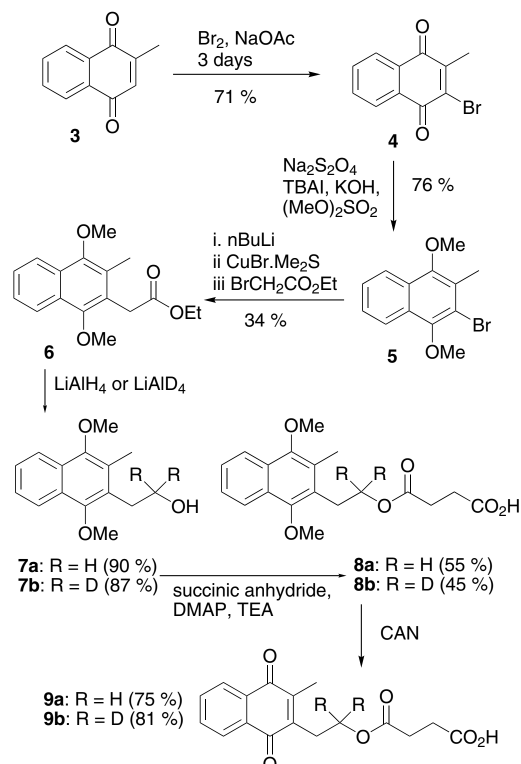
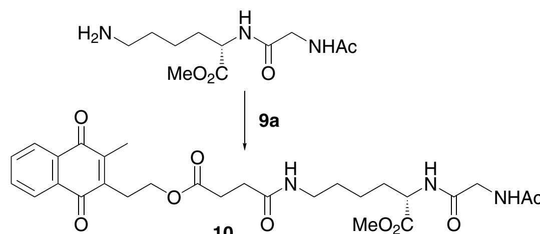


Scheme 1.



Scheme 2.

deuterated analogue **9b** was prepared to facilitate ^{2}H NMR studies.

The key naphthoquinone-based ester **6**, used in the preparation of analogues **9**, was synthesised in three steps from commercially available menadione **3**, Scheme 1. In particular, treatment of **3** with bromine and sodium acetate for three days gave a 78% yield of **4**.[13] Reduction and in situ methylation with sodium dithionite and dimethylsulfate, in the presence of the phase-transfer catalyst tetrabutylammonium iodide (TBAI), gave the dimethoxynaphthalene **5** in 76% yield. This was then treated with nBuLi and copper bromide dimethylsulfide to give the diaryl cuprate, which was alkylated with ethyl bromoacetate to give **6** in 34% yield. Reduction of **6** with lithium aluminium hydride gave alcohol **7a** (90%) to which was appended an acid functionalised linker by reaction with succinic anhydride in the presence of DMAP and triethylamine to give **8a** in 55% yield. Oxidative deprotection of the quinone with ceric ammonium nitrate (CAN), gave the desired vitamin K analogue **9a** in 75% yield. The deuterated analogue **9b** was similarly prepared from **6**; reduction with lithium aluminium deuteride gave **7b**, which was reacted with succinic anhydride, followed by CAN oxidation, to give **9b**.

3. Coupling studies

The analogue **9a** was coupled with BSA (66.5 kDa protein) using a standard mixed anhydride protocol as a first step to monoclonal antibody production. The resulting crude conjugate was then used to inoculate mice, in the hope of producing monoclonal antibodies specific for vitamin K. After six weeks a blood sample was obtained from these mice via a tail bleed. Antibodies specific to the analogue moiety of the conjugates were detected, however, attempts to competitively bind these antibodies with menadione (vitamin K_3) were unsuccessful.[14]

Given these difficulties, we decided to study and optimise, the protein conjugation reaction conditions and the results of these studies are discussed below. Initial model studies were carried out in which **9a** was reacted with *N*-acetyl-Gly-L-Lys methyl ester under the EDCI/HOBT conditions (Scheme 2) and the resulting conjugate **10** was isolated in 85% and fully characterised by NMR spectroscopy. With the knowledge that a simple lysine containing dipeptide could be efficiently coupled with **9a** we set about studying its coupling with lysozyme—with a mass of 14,305 g mol^{-1} lysozyme is over four times smaller than BSA and hence it is easier to analyse and characterise the conjugates by mass spectrometry. Lysozyme also has the advantage that it exists as a single isoform as opposed to BSA, which exists as four different isoforms. The deuterated analogue **9b** was thus reacted with lysozyme using the EDCI/HOBT methodology and the product analysis by LCMS after 5 days reaction, to reveal a peak at 14,606 g mol^{-1}, consistent with coupling of one molecule of **9b** to lysozyme (Fig. 2).

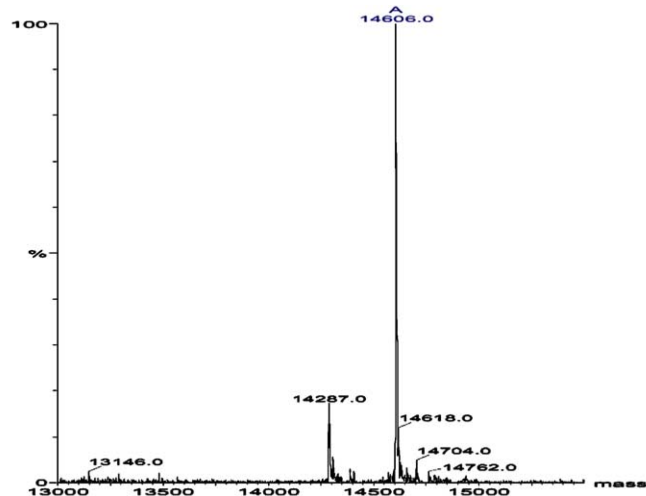
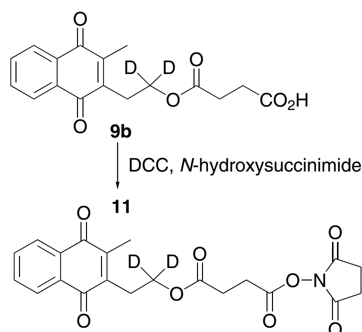


Figure 2. Maxent deconvoluted spectrum of product from EDCI coupling **9b** with lysozyme.



Scheme 3.

A more efficient method of coupling the analogues to lysozyme was sought due to the long reaction times required with the EDCI/HOBT method and also to minimise by-products and reagent-based impurities when dealing with a protein. We also wanted to develop conditions that would allow the reproducible conjugation of more than one molecule of the vitamin K analogue to a protein. Thus, the succinimidyl ester **11**, prepared by reaction of **9b** with *N*-hydroxysuccinimide and DCC (Scheme 3), was reacted with varying amounts of lysozyme for 18 h, and the resulting conjugates analysed by LC–MS. From these studies it was found that the number of analogues that conjugate to the protein is determined, predictably, by the number of equivalents of analogue **9b** used in the reaction. In particular, reaction of lysozyme with 1.1 equiv of **11** gave conjugates with masses of 14,306.0, 14,606.0, 14,906.0 and 15,207.0, corresponding to native lysozyme, and lysozyme coupled to one, two and three analogues, respectively (Fig. 3). A Deuterium NMR spectrum of the conjugate gave a broad singlet at 4.77 ppm, a chemical shift that is 0.52 ppm downfield relative to free **9b**. Analysis of the product from an analogous reaction of lysozyme with 6 equiv of **11**, revealed up to seven analogues bound to lysozyme (Fig. 4). Deconvoluted ESI/LC–MS

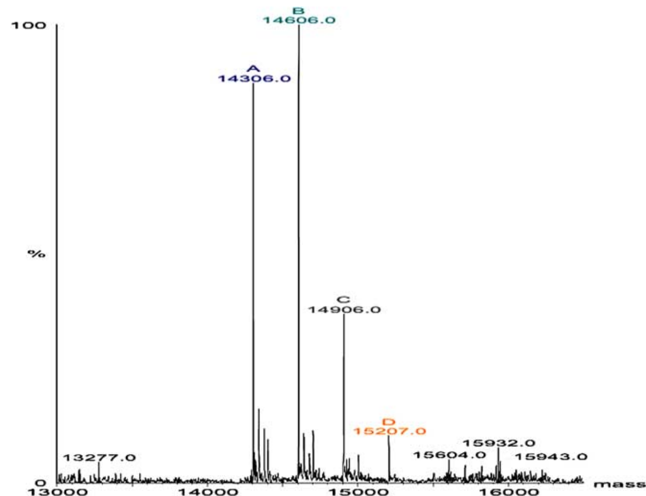


Figure 3. Maxent deconvoluted spectrum of the product from coupling **11** (1.1 equiv) to lysozyme.

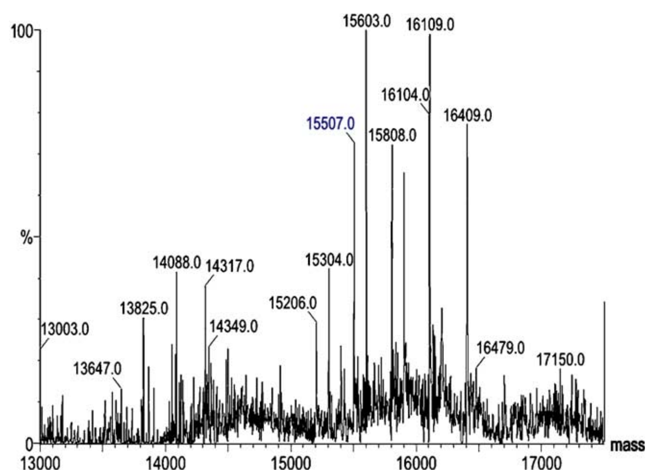


Figure 4. Maxent deconvoluted spectrum of the product from coupling **11** (6 equiv) to lysozyme.

revealed peaks of masses 15,507.0, 15,808.0, 16,109.0 and 16,409.0, consistent with lysozyme coupled to four, five, six and seven molecules of **9b**, respectively.

The reaction of 1.1 equiv of **11** with BSA was then attempted and the resulting conjugate analysed by Electrospray mass spectrometry with LC–MS to reveal that coupling had occurred with up to three conjugates per isoform of BSA (Fig. 5). Deconvolution of the ESI/LC–MS data gave peaks at mass 66,384.0, 66,475.0 and 66,577.0 corresponding to unreacted BSA isoforms and peaks at mass 66,672.0, 66,772.0, 66,876.0, 66,978.0, 67,076.0, 67,181.0, 67,276.0, 67,380.0 and 67,456.0 consistent with 1, 2 and 3 equiv of **11** conjugating to the three observed isoforms of BSA.

Reaction of BSA with 60 equiv of **11**, (the number of lysine residues on BSA) gave a complex mixture from which clear resolution of the conjugates was not possible, but conjugation had clearly taken place. Electrospray analysis of this mixture gave a spectrum with an increase in the mass distribution of charge states, but due to the number of BSA isoforms and the increased number of conjugated forms, no individual charge distributions could be identified either manually or with Maxent. However, the change in the mass of the distribution indicates that approximately 30 equiv of **11** had been conjugated to BSA.

4. Conclusion

The first step towards the production of an ELISA assay for vitamin K has been achieved, that is the synthesis and coupling of vitamin K analogues **9a** and **9b** to a protein. An efficient method for the characterisation of the conjugates by mass spectrometry is also presented. This methodology has been used to demonstrate that the number of vitamin K analogues that bind to the protein is determined by the number of equivalents of **11** used in the coupling reaction.

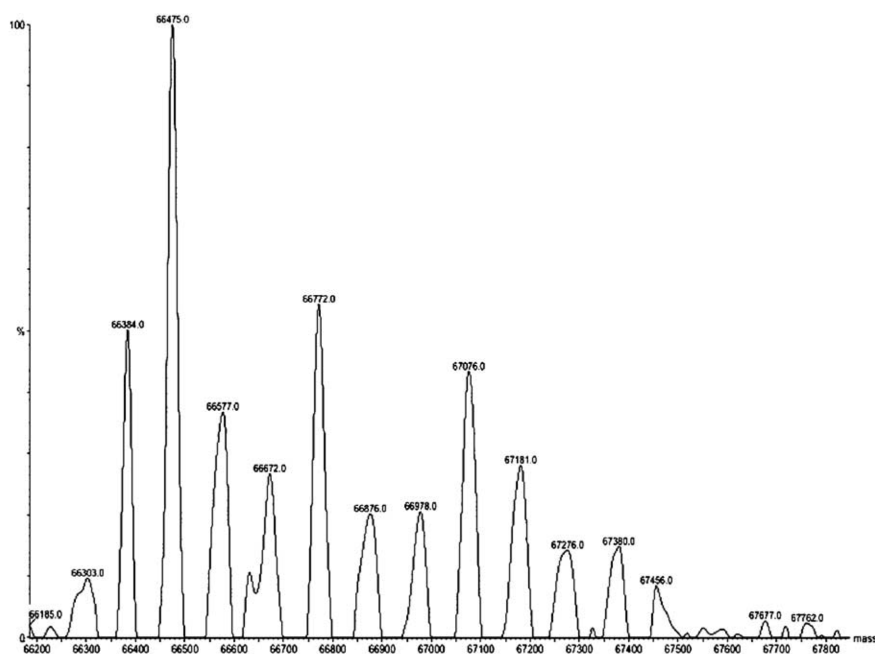


Figure 5. Maxent deconvoluted spectrum of the product of coupling **11** (1.1 equiv) to BSA.

5. Experimental

Proton and Deuterium NMR spectra were recorded on an Inova 500 spectrometer operating at 500 and 46 MHz, respectively. Deuterium NMR spectra were obtained using a broad band probe, referencing to 1% deuterated $CHCl_3$ or 1% deuterated DMSO. Carbon NMR spectra were recorded on a Varian Unity XL 300 MHz Fourier Transform spectrometer operating at 75 MHz. High resolution electron impact, 70 eV, (HREI) and fast atom bombardment (FAB) mass spectral data were acquired on a Kratos MS80RFA equipped with an Iontech ZN11NF ion gun using xenon as reagent gas. Electrospray ionisation (ESI) mass spectral data was acquired using a Micromass LCT mass spectrometer operating with MassLynxV3.5 or V4.0 and with Transform and Maxent1 software packages, with the source held at 80 °C, probe temperature 150 °C, nebuliser gas flow of 170 L/h and desolvation gas flow of 520 L/h. Liquid chromatography mass spectrometry (LC–MS) was carried out by coupling the Micromass LCT to a Waters 2790 separation module, with a Waters 996 PDA detector, fitted with a zorbax 2.1 mm id × 150 mm 1 C3 column maintained at 40 °C with solvent flow of 0.2 mL/min, this flow was split post column with 20 μL/min to the mass spectrometer. The gradient used to elute the protein conjugates had a starting condition of 100% water (0.5% formic acid) changing to 40% water (0.5% formic acid) and 60% acetonitrile over 80 min then to 100% acetonitrile over a further 5 min, held for 10 min before resetting to starting condition after 100 min, total run time 115 min.

IR spectra were obtained using on a Shimadzu 8201PC series FT-IR using a KBr disk. Lysozyme, bovine serum albumin (BSA) and *N*-acetyl-Gly-L-Lys-methyl ester were obtained from Sigma chemical company®.

5.1. 2-Bromo-3-methyl-1,4-dimethoxynaphthalene 5

To a solution of **4**[13] (1 g, 3.97 mmol) and tetrabutylammonium iodide (176 mg, 0.48 mmol) in tetrahydrofuran (10.9 mL) and water (3.6 mL) was added to a solution of sodium dithionite (4.16 g, 23.88 mmol) in water (10.9 mL). The mixture was stirred at rt, under a nitrogen atmosphere, for 15 min at which time a solution of potassium hydroxide (5.14 g, 91.54 mmol) in water (5.8 mL) was added slowly. The reaction was stirred for a further 5 min. Dimethylsulfate (4.5 mL, 47.76 mmol) was added and the solution was stirred at rt for 18 h at which time it appeared dark red in colour. The reaction mixture was extracted with dichloromethane (2 × 40 mL) and the combined organic fractions washed with water, dried ($MgSO_4$) and solvent removed under reduced pressure to give an orange oil, which was crystallised from hot methanol to give **5** (72 mg, 64%) as pale orange crystals. Mp = 82–84 °C (lit. mp = 84–85 °C).[13] ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.53 (s, 3H, CH_3), 3.88 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 7.51 (m, 2H, ArH), 8.06 (m, 2H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.9, 61.5, 61.9, 117.5, 122.6, 122.6, 126.4, 126.7, 127.5, 127.7, 128.0, 150.1, 150.7. HRMS calculated for $C_{13}H_{13}O_2Br$: 280.0099 g mol^{-1}. Found: 280.0096 g mol^{-1}.

5.2. Ethyl 2-(3-methyl-1,4-dimethoxynaphthalen-2-yl)acetate 6

A solution of **5** (1 g, 3.56 mmol) in ether (5.5 mL) was stirred in an ice bath under an argon atmosphere and *n*-butyllithium (1.5 M solution in hexanes, 2.7 mL, 4.09 mmol) was added slowly. After stirring for 30 min in ice, copper bromide dimethylsulfide complex (512 mg, 2.50 mmol) was added and the reaction stirred for 2.5 h. A solution of ethyl bromoacetate (0.44 mL,

3.9 mmol) in ether (3.7 mL) was added and the reaction stirred in ice for a further 2 h. The ice bath was removed and the mixture allowed to warm to rt with stirring over 3 h. The reaction was quenched with 10% aqueous HCl and the ether layer separated. The aqueous phase was back extracted with ether and the combined organic fractions washed with water, saturated aqueous $NaHCO_3$, water, dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by flash chromatography on silica (ethyl acetate/petroleum ether 5:95) to give **6** (0.33 g, 34%) as an orange oil. R_f (1:4 v/v ethyl acetate–petroleum ether) = 0.61. FT-IR 2988, 2941, 2837, 1732, 1595 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.26 (t, J = 5.4 Hz, 3H, OCH_2CH_3), 2.36 (s, 3H, $ArCH_3$), 3.87 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.92 (s, 2H, $ArCH_2$), 4.19 (q, J = 7.3 Hz, 2H, OCH_2CH_3), 7.48 (m, 2H, ArH), 8.06 (m, 2H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.54, 14.13, 32.97, 60.80, 61.30, 62.23, 122.13, 122.36, 124.01, 125.37, 125.92, 126.54, 126.93, 128.17, 150.01, 150.66, 171.64. HRMS calcd for $C_{17}H_{20}O_4$: 288.1362. Found: 288.1366. Anal. Calcd for $C_{17}H_{20}O_4$: C, 70.81; H, 6.99. Found C, 70.51; H, 7.03.

5.3. 2-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)ethanol 7a and 2-(3-methyl-1,4-dimethoxynaphthalen-2-yl)-ethan-[2,2-D]-ol 7b

A solution of ester **6** (110 mg, 0.38 mmol) in ether (3.60 mL) was stirred under an argon atmosphere, lithium aluminium hydride (29 mg, 0.76 mmol) was added and the reaction stirred at rt for 20 min. TLC analysis showed the reaction to be incomplete so additional lithium aluminium hydride (10 mg) was added and the reaction stirred for a further 30 min. The reaction was quenched by the addition of saturated aqueous NH_4Cl and the organic layer separated. The aqueous phase was back extracted with ethyl acetate and the combined organic fractions washed with water, brine, dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by radial chromatography eluting with 1:4 ethyl acetate–petroleum ether to 1:1 ethyl acetate–petroleum ether to give **7a** (85 mg, 90%) as a white solid, which was used subsequently without further purification. mp 66–69 °C. R_f (1:4 v/v ethyl acetate–petroleum ether) = 0.14. FT-IR 3265, 2936, 2841, 1589 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 2.43 (s, 3H, $ArCH_3$), 3.12 (t, J = 6.8 Hz, 2H, $ArCH_2$), 3.85 (t, J = 6.8 Hz, 2H, CH_2OH), 3.86 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 7.47 (m, 2H ArH), 8.04 (m, 2H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.54, 30.61, 61.32, 61.87, 62.62, 122.17, 122.19, 125.46, 125.72, 126.43, 126.91, 127.33, 127.81, 150.32, 150.50. HRMS calcd for $C_{15}H_{18}O_3$: 246.1256. Found: 246.1255. Anal. Calcd for $C_{15}H_{18}O_3$: C, 73.1; H, 7.4. Found C, 72.8; H, 7.4.

A solution of ester **6** (50 mg, 0.18 mmol) in ether (1.7 mL) was reduced with lithium aluminium deuteride (22 mg, 0.53 mmol) as described for **7a** above to give **7b** (40 mg, 87%) as a yellow oil. ^1H NMR (500 MHz, $CDCl_3$): δ 2.43 (s, 3H, CH_3), 3.12 (s, 2H, $ArCH_2$), 3.87 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3) 7.26 (m, 2H, ArH), 8.04 (m, 2H, ArH). ^2H NMR (46 MHz, $CHCl_3$ + 1% $CDCl_3$) δ 3.79 (br s, CD_2O–). HRMS calcd for $C_{13}H_{13}O_2Br$: 248.1382. Found: 248.1389.

5.4. 2-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)ethyl hydrogen succinate 8a and 2-(3-methyl-1,4-dimethoxynaphthalen-2-yl)eth-[2,2-D]-yl hydogen succinate 8b

A solution of recrystallised succinic anhydride (23 mg, 0.23 mmol), DMAP (1 mg, 0.01 mmol) and triethylamine (0.02 mL, 0.16 mmol) in CH_2Cl_2 (0.4 mL) was added drop wise to a solution of **7a** (27 mg, 0.11 mmol, 1 equiv) in CH_2Cl_2 (1 mL) and the reaction stirred at rt overnight. The solvent was removed by evaporation under reduced pressure and the resulting solid was dissolved in CH_2Cl_2. This was washed with 10% aqueous HCl, water (×2) and solvent removed to give **8a** (21 mg, 55%) as a yellow oil that was not purified further. ^1H NMR (500 MHz, $CDCl_3$): δ 2.45 (s, 3H, $ArCH_3$), 2.66 (m, 4H, 2× CH_2), 3.17 (t, J = 7.3 Hz, 2H, $ArCH_2$), 3.86 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.29 (t, J = 7.3 Hz, 2H, CH_2O), 5.29 (s, 1H, OH), 7.47 (m, 2H, ArH), 8.03 (m, 2H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.48, 26.68, 28.85 (×2), 61.30, 62.14, 63.93, 122.18, 122.27, 125.43, 125.81, 126.12, 126.49, 127.01, 127.92, 150.15, 150.89, 172.13, 177.93. HRMS calcd for $C_{19}H_{22}O_6$: 346.1416 found: 346.1420. Anal. Calcd for $C_{19}H_{22}O_6$: C, 65.9; H, 6.4. Found: C, 66.3; H, 6.8.

A solution of recrystallised succinic anhydride (23 mg, 0.23 mmol), DMAP (1 mg, 0.01 mmol) and triethylamine (0.02 mL, 0.16 mmol) in CH_2Cl_2 (0.4 mL) was reacted with **7b** (27 mg, 0.11 mmol) in CH_2Cl_2 (1 mL) as described for **8a** above to give **8b** (31 mg, 45%) as an orange oil. ^1H NMR (500 MHz, $CDCl_3$): δ 2.45 (s, 3H, CH_3), 2.66 (m, 4H, 2× CH_2), 3.16 (s, 2H, $ArCH_2$), 3.87 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 5.29 (s, 1H, OH), 7.47 (m, 2H, ArH), 8.04 (m, 2H, ArH). ^2H NMR (300 MHz, $CHCl_3$ + 1% $CDCl_3$) δ 4.22 (br s, CD_2O–). HRMS calcd for $C_{19}H_{20}D_2O_6$: 348.1542. Found: 348.1547.

5.5. 2-(3-Methyl-1,4-naphthoquinon-2-yl)ethyl hydrogen succinate 9a and 2-(3-methyl-1,4-naphthoquinon-2-yl)-eth-[2,2-D]-yl hydrogen succinate 9b

An ice cooled solution of ceric ammonium nitrate (CAN) (230 mg, 0.42 mmol) in acetonitrile/water (0.75 mL of a 1:1 v/v mixture) was added drop wise to a cooled solution of **8a** (58 mg, 0.17 mmol) in acetonitrile/water (0.65 mL of a 2:1 v/v mixture). The reaction mixture was stirred for 20 min at 0 °C, and then at rt for a further 10 min. Water was added and the mixture was extracted with CH_2Cl_2 (×2). The combined organic fractions were washed with water, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was purified by radial chromatography eluting with dichloromethane to 1:19 MeOH–petroleum ether to give **9a** (40 mg, 76%) as a bright yellow oil. ^1H NMR (500 MHz, $CDCl_3$): δ 2.19 (s, 3H, $ArCH_3$), 2.55 (m, 4H, 2× CH_2), 2.96 (t, J = 5.9 Hz, 2H, $ArCH_2$), 4.21 (t, J = 6.4 Hz, 2H, CH_2O), 7.66 (m, 2H, ArH), 8.02 (m, 2H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.92,

26.60, 28.66 (×2), 62.88, 126.23 (×2), 131.80, 131.91, 133.44, 133.47, 142.49, 145.41, 172.06, 177.27, 184.29, 184.86. HRMS calcd for $C_{17}H_{15}O_6$: 315.0869. Found: 315.0874.

The vitamin K analogue **8b** (50 mg, 0.14 mmol) was deprotected with CAN as described for **9a** above to give **9b** (30 mg, 81%) as a bright yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.23 (s, 3H, CH_3), 2.62 (m, 4H, 2× CH_2), 3.00 (s, 2H, ArCH_2), 7.71 (m, 2H, ArH), 8.08 (m, 2H, ArH). ^{2}H NMR ($CHCl_3$ + 1% $CDCl_3$) δ 4.25 (br s, CD_2O–).

5.6. Methyl 2-(2-acetylamino-acetylamino)-6-{3-[2-(3-methyl-1,4-naphthoquinon-2-yl)ethoxycarbonyl]propionylamino}hexanoate 10

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide·HCl (EDCI) (3 mg, 0.03 mmol), 1-hydroxybenzotriazole hydrate (HOBT) (6.1 mg, 0.05 mmol), *N*-ethyldiisopropylamine (DIEA) (5.2 μL, 0.03 mmol) were added to a solution of **9a** (10 mg, 0.03 mmol) and *N*-acetyl-Gly-L-Lys methyl ester (11 mg, 0.03 mmol) in CH_2Cl_2 (1.2 mL) under an argon atmosphere with stirring. After stirring for 16 h CH_2Cl_2 was added followed by 3 M aqueous NaOH. The aqueous phase was extracted with CH_2Cl_2 (×3) and combined organic fractions dried ($MgSO_4$) and evaporated under reduced pressure to give a dark orange oil. This was purified by radial chromatography eluting with 1:19 MeOH–dichloromethane to give **10** (15 mg, 85%) as an orange oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.34 (2H, m, CH_2), 1.47 (2H, m, CH_2), 1.69 (1H, m, CHCH_{2a}), 1.84 (1H, m, CHCH_{2b}), 2.03 (3H, s, $COCH_3$), 2.24 (3H, s, ArCH_3), 2.61 (4H, m, $COCH_2CH_2CO$), 3.01 (2H, t, J = 6.8 Hz, CH_2), 3.14 (1H, m, NHCH_{2a}), 3.24 (1H, m, NHCH_{2b}), 3.72 (3H, s, CO_2CH_3), 3.90 (1H, dd, J = 5.4 and 17.1 Hz, CO-CH_{2a}NH), 4.03 (1H, dd, J = 5.4 and 17.1 Hz, COCH_{2b}NH), 4.27 (2H, t, J = 6.8 Hz, CH_2), 4.54 (1H, m, CH), 6.41 (1H, m, NH), 6.86 (1H, m, NH), 7.16 (1H, d, J = 7.8 Hz, NH), 7.71 (2H, m, ArH), 8.08 (2H, m, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.00, 22.06, 22.76, 26.63, 28.49, 29.38 (×2), 31.22, 38.65, 43.01, 51.91, 52.41, 62.88, 126.30, 126.34, 131.89, 132.05, 133.55 (×2), 142.55, 145.57, 169.25, 171.25, 171.77, 172.60, 173.16, 184.48, 184.95. HRMS (M+H) calcd for $C_{28}H_{36}N_3O_9$: 558.2452. Found: 558.2464.

5.7. Coupling of 9b and 11 to lysozyme

(a) A solution of **9b** (9 mg, 0.03 mmol) in DMF (0.25 mL) was added to a stirred solution of lysozyme (425 mg, 0.03 mmol, 1.1 equiv) in H_2O (8 mL) under an argon atmosphere. EDCI (7 mg, 0.04 mmol), HOBT (6 mg, 0.04 mmol) and DIEA (5.2 μL, 0.03 mmol) were added and the resulting solution vigorously stirred under an argon atmosphere for 16 h. Analysis by direct electrospray mass spectrometry and LC–MS of the resulting product indicated that **9b** had coupled to lysozyme. Deconvolution of the ESI/LC–MS data with both manual assignment and Maxent gave a peak at mass 14606.0 consistent with lysozyme coupled to one molecule of **9b** (increase of 300 amu, Fig. 2).

(b) *N*-Hydroxysuccinimide (4.4 mg, 0.04 mmol) and DCC (7.8 mg, 0.04 mmol) were added to a stirred solution of **9b** (6.1 mg, 0.02 mmol) in THF (1.5 mL, freshly distilled over Na) under an argon atmosphere. After 6 h stirring, ethyl acetate (10 mL) was added and the solution washed with water (3 × 10 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated under reduced pressure to give **11** as a yellow oil that was not purified further. ESI: MH+($C_{21}H_{17}D_2NO_8$) = 416.4. Lysozyme (chicken egg white) (49 mg, 3.04 mmol) in 500 μL of HCl/Tris buffer (pH = 8) was added to a solution of **11** (1 mg, 1.1 equiv) in DMF (150 μL) and stirring was continued under an argon atmosphere for 18 h. Analysis by Electrospray mass spectrometry and LC–MS experiments revealed that coupling had occurred and that there were up to three conjugates formed during the reaction. Deconvolution of the ESI/LC–MS data with both manual assignment and Maxent yielded peaks at 14,306.0, corresponding to native lysozyme and at 14,606.0, 14,906.0 and 15,206.0 consistent with lysozyme coupled to one, two and three molecules of **11** (each + 300 amu corresponding to **9b** – H_2O), respectively (Fig. 3). ^{2}H NMR (D_2O + 1% DMSO) δ 4.77 (br s).

(c) In a further experiment **11** (6 equiv) was reacted with lysozyme (7.49 mg, 0.52 mmol) in 500 μL of HCl/Tris buffer (pH = 8) as described in (b) above. Analysis by Electrospray mass spectrometry with LC–MS of the resulting reaction product revealed that coupling had occurred and that there were up to five conjugates formed during the reaction. Deconvolution of the ESI/LC–MS data with both manual assignment and Maxent yielded peaks at 15,206.0, 15,506.0, 15,808.0, 16,108.0, 16,409.0 and 16,709.0 consistent with lysozyme coupled to three, four, five, six, seven and eight molecules of **11** (each + 300 amu corresponding to **9b**–H_2O), respectively (Fig. 4).

5.8. Coupling of 11 to BSA

(a) A solution of **11** (0.79 mmol, approx 11 equiv) in DMF (50 μL) was added to a stirred solution of BSA (50 mg, 0.75 mmol) in 500 μL of HCl/Tris buffer (pH = 8) under argon. Stirring was continued for 18 h at which time analysis by Electrospray mass spectrometry with LC–MS of the resulting reaction product revealed that coupling had occurred and that there were up to three conjugates per isoform of BSA formed during the reaction. Deconvolution of the ESI/LC–MS data yielded peaks at mass 66,384.0, 66,475.0 and 66,577.0 corresponding to unreacted BSA isoforms and peaks at mass 66,672.0, 66,772.0, 66,876.0, 66,978.0, 67,076.0, 67,181.0, 67,276.0, 67,380.0 and 67,456.0 consistent with 1, 2 and 3 equiv of **11** (each increase by 300 amu, corresponding to **9b**–H_2O) conjugating to the three observed isoforms of BSA.

(b) A solution of **11** (approx 60 equiv) in DMF (50 L) was reacted with BSA (0.9 mg) as above. Analysis by electrospray mass spectrometry and LC–MS revealed a charge distribution that had increased in mass over that observed for the 1:1 reaction of BSA and **9b**, but due to the complexity of the result no individual species could

be deconvoluted using either manual assignment or the Maxent software package within MassLynx. As such there is no accurate determination as to the number of **9b**-BSA coupled products generated during the reaction; however, the increase in the mass of the raw electrospray data by approximately 180 amu suggests that the average number of coupled **9b** to BSA is in the order of 30 equiv.

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