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Synthesis and Interaction Studies of ^{13}C Labeled Lactone Derivatives with a Model Protein Using ^{13}C NMR

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Abstract—Two molecules **9** and **14**, representatives of two series of electrophilic lactone derivatives, have been synthesised, and labeled with carbon 13 at their reactive sites. The mechanism and the products of the reaction of these two molecules with human serum albumin (HSA) under various reaction conditions have been studied by ^{13}C NMR using DEPT 135 sequences. Results using the protein dissolved in aqueous medium or butylamine (a model nucleophile) dissolved in organic solvent were very similar. These results are entirely consistent with the *in vivo* allergising activity of these molecules. The validity of the Relative Alkylation Index (RAI) as a predicative model in contact allergy is discussed.

Introduction

Hapten–protein interactions have been shown to be one of the key steps in the induction and elicitation mechanism of allergic contact dermatitis[1] (ACD). The processing of the hapten protein complex by skin immunocompetent antigen-presenting cells[2] (Langerhans cells) and the subsequent transmission of this information to T-cells in the lymphatic nodes lead to the biological and clinical aspects of ACD[3] (erythema and edema). The formation of the hapten–protein complex occurs mainly through a covalent bond between the hapten or allergen and nucleophilic functionalities on proteins and, therefore, most skin allergens are electrophiles or "pro-electrophiles", i.e. they can be transformed *in vivo* to true electrophiles.[4]

There is a growing interest in the development of a structure–skin sensitisation relationship which can rationalise patterns of varying sensitisation potential among groups of chemicals and, ideally, predict the sensitisation potential of compounds not yet tested. To date the Relative Alkylation Index (RAI) model has proved the most successful approach to the quantitative interpretation of skin sensitisation data.[5] In brief the basis of the model is that the extent of covalent binding (alkylation) to carrier protein in the skin at the induction and challenge stages of skin sensitisation testing determines the magnitude of the sensitisation response, and that the extent of this binding can be quantified by the RAI, a composite parameter made up of dose, chemical reactivity and hydrophobicity terms.

$$\text{RAI} = \log D + a \log k_{\text{rel}} + b \log P$$

The values of the coefficients a and b should be constant for a given series of compounds all sensitising by the same molecular mechanism and all tested by the same protocol (except that where the compounds range from hydrophilic ($\log P$ negative) to hydrophobic ($\log P$ positive) the b coefficient is not constant).

†Deceased, accidentally on 20 January 1992.

In this model, the value P represents the partition coefficient of the product in an octanol/water mixture and the value k the kinetic constant of the product with respect to *n*-butylamine used as a model nucleophile.[6] In order to develop the use of the RAI, two series of compounds of structure **1a–i** and **2a–i** were synthesised for structure–skin sensitisation studies. We have found[7] that all of the compounds of series **1** react with butylamine, used as a simple chemical model for biological nuleophiles in skin, by elimination of HX to produce the intermediate α-methylene-γ-butyrolactone **3** (Scheme I), which can then react with amino groups to give the adduct **4**. Contrary to this, compounds of series **2** react directly with butylamine to give the adduct **5**.

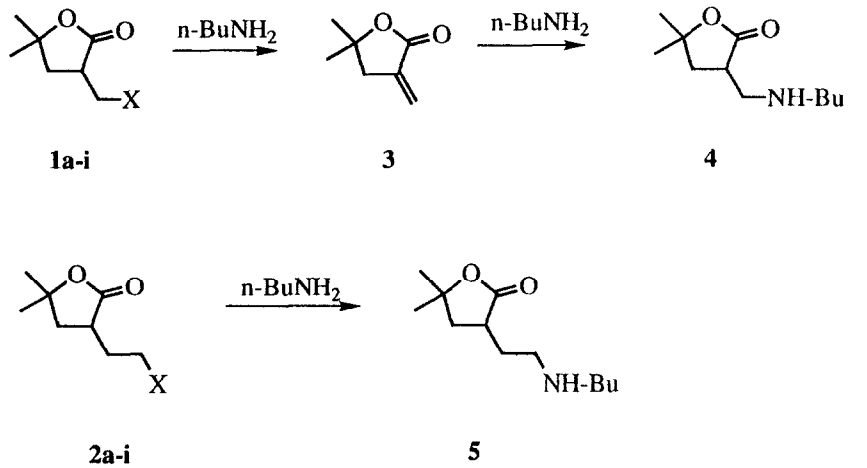
The difficulty with such a theoretical model is to know whether the reactivity and reaction mechanisms seen in an organic solvent reflects the reactivity of the same molecule towards a protein in an aqueous medium. We have recently shown that NMR studies of carbon 13-labeled molecules are able to provide much information on the nature of hapten–protein interactions,[8] especially the identification of the amino acids involved in these bonds. We have therefore synthesised a representative model from each series, as well as the reference lactone, labeled at the reactive centre with carbon 13, and have studied the reactivity and nature of the adducts formed between these molecules and a model protein, human serum albumin (HSA).

Chemistry

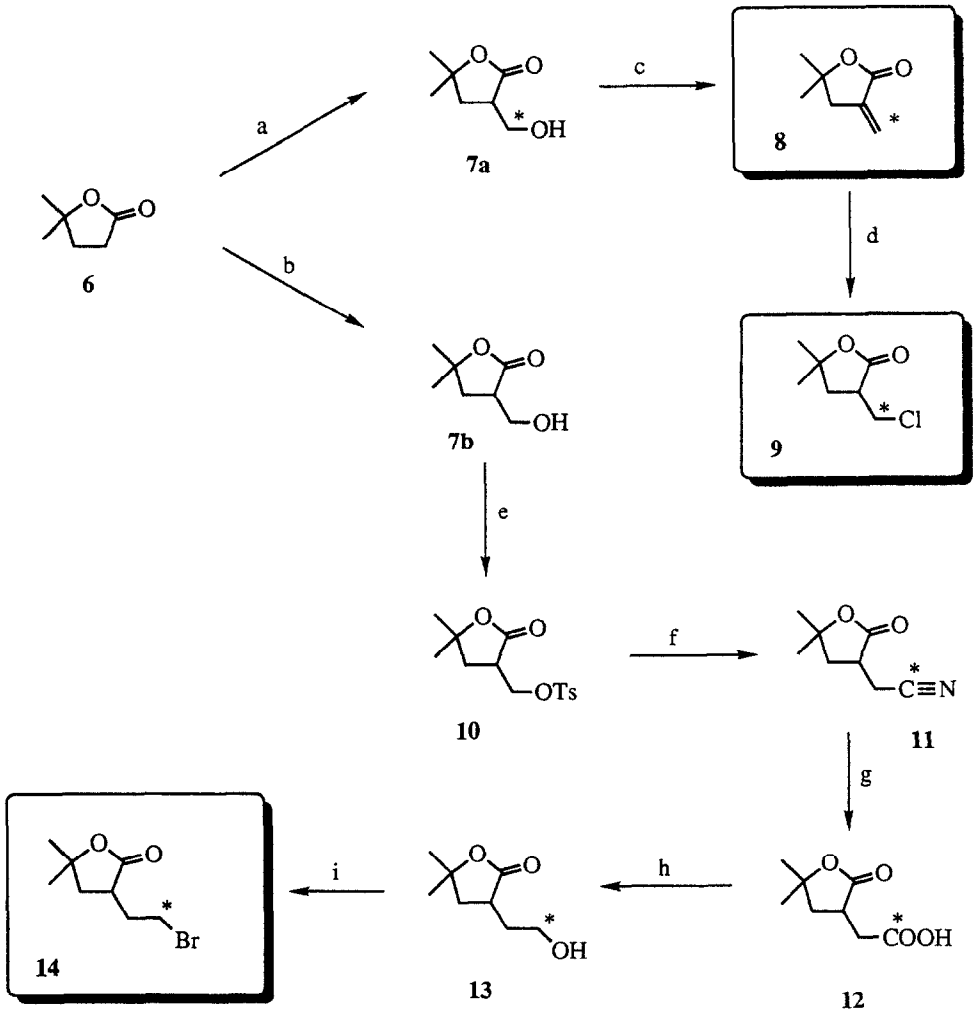
Synthesis of labeled molecules

γ,γ-Dimethyl-γ-butyrolactone **6** was converted to the alcohol **7a** (Scheme II) by treatment with one equivalent of LDA at -78 °C in THF, followed by the addition of LiCl (3 equivalents), and subsequent trapping of the intermediate anion with gaseous formaldehyde[9] (generated by thermal decomposition of carbon 13-labeled paraformaldehyde under a stream of dry nitrogen). In preliminary tests carried out in

the absence of lithium salts, a secondary product of dialkylation was formed. It is known that lithium enolates exist in solution as dimers or tetramers and that the addition of lithium salts can cleave such aggregates, making the reaction more selective.[10] In the presence of LiCl, no such dialkylation product was found. The labeled alcohol **7a** was then converted to α-methylene-γ-butyrolactone **8** by treatment with mesyl chloride in triethylamine, forming the mesylated intermediate which was eliminated in the presence of DBU in methylene chloride at room temperature, lactone **8** being obtained in an overall yield of 57%. The chlorinated derivative **9** was prepared directly, in a yield of 75%, by simple treatment of lactone **8** with HCl gas in ether.



Scheme I. Reaction mechanism of lactone derivatives **1a–i** and **2a–i** with *n*-butylamine. **a**: X = Cl; **b**: X = Br; **c**: X = OSO_2Me; **d**: X = OSO_2ArMe; **e**: X = OSO_2ArOMe; **f**: X = OSO_2Ar; **g**: X = OSO_2ArCl; **h**: X = OSO_2ArNO_2; **i**: X = SCN.



Scheme II. Synthesis of ^{13}C labeled derivatives **9** and **14**. Reagents and conditions: (a) LDA, LiCl, $H_2{}^{13}CO$, THF, -78 °C; (b) LDA, LiCl, H_2CO, THF, -78 °C; (c) MsCl, Et_3N; DBU, CH_2Cl_2; (d) HCl, Et_2O; (e) TsCl, py, DMAP, CH_2Cl_2; (f) $K^{13}CN$, 18C6, DMF, reflux; (g) HCl conc. reflux; (h) BH_3·Me_2S, THF; (i) CBr_4, PPh_3, CH_2Cl_2.

The labeled molecule representative of series **2** was also prepared from γ,γ-dimethyl-γ-butyrolactone **6**. The lactone was first converted into the alcohol **7b** by the series of reactions described above (LDA, LiCl, gaseous formaldehyde) with a yield of 78%. The alcohol was then tosylated using tosyl chloride in methylene chloride in the presence of pyridine and DMAP at ambient temperature to give **10**.

It should be noted that the same reaction carried out directly using pyridine as solvent leads to the formation of 25% of the chlorinated derivative. The tosyl group was then displaced by ^{13}C-labeled potassium cyanide in refluxed DMF,[11] yielding the cyano derivative **11** with a recovery of 94%. The cyano group was then hydrolysed[12] in concentrated refluxed HCl for 12 h, giving the acid **12** with a yield of 80%. This was then reduced using one equivalent of BH_3·Me_2S[13] in THF, giving the alcohol **13** (87% yield), which was then converted with a yield of 90% into the bromide **14** using CBr_4 in the presence of triphenylphosphine.

Preparation of model adducts with butylamine and cysteine

Initially, we prepared model adducts (Figure 1) for each series using butylamine (a model for lysine) and the methyl ester of *N*-acetylcysteine to check the carbon 13 chemical shifts of carbon bearing such amines or thiols. The adducts of series **1** were prepared directly from α-methylene-γ-butyrolactone **3** by reaction with an excess of butylamine in ethanol to give, quantitatively, product **4**. During previous studies on the modification of human serum albumin by methyl alcane sulfonates,[8] we found that a lysine group appeared reactive enough to bind two allergen molecules. In order to test if such a diadduct was formed with a lactone hapten, we prepared **15** from **4** by reaction in ethanol with an excess of lactone **3**. The cysteine derivative **16** was prepared using the sodium thiolate methyl ester of *N*-acetylcysteine at 0 °C in ethanol. The reaction was rapid and yielded, quantitatively, the expected adduct.

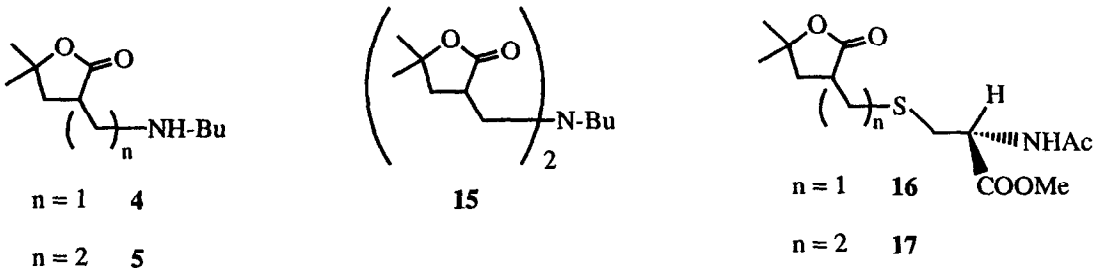
The adducts of the second series were prepared under similar conditions. The adduct **5** was quantitatively obtained by the action of an excess of butylamine on the mesylate **2c** in dichloromethane. The cysteine derivative **17** was prepared by the action of sodium thiolate methyl ester of *N*-acetylcysteine in ethanol on the bromide derivative **2b**. The ^{13}C chemical shifts of the carbons bearing the heteroatom are shown in Table 1.

The difference in ^{13}C chemical displacement of the carbons bearing the heteroatom (amine or thiol) seemed great enough to suggest that it would be possible to identify and distinguish them easily after binding to HSA.

The Series 1 Derivative

Mechanism and kinetics of the reaction between derivative 9 and HSA

In the first instance, we wished to check whether the mechanism observed with butylamine in organic milieu — elimination with α-methylene lactone formation — also held for a protein in aqueous medium at pH 7.4. We therefore dissolved HSA in a phosphate buffer/D_2O mixture. The labeled derivative **9** was then added and the ^{13}C spectrum recorded at various time intervals, using a signal amplification sequence,[14] DEPT 135. As seen in Figure 2, at time zero (A), the only signal found was that of labeled carbon (43.3 ppm). After 2 h (B), a second peak appeared at 124.1 ppm, characteristic of the exomethylene of compound **8**. Between 1 and 3 days, the chief change was the increase in the α-methylene lactone peak, together with a diminution of the signal from the starting chloride derivative **9**. At day 5 (C), the chloride derivative signal had almost disappeared, and an intense peak at 124.1 ppm (lactone) was formed, with the quasitotality of the chloride derivative **9** being converted into lactone **8**. In addition, a very faint signal at 47.6 ppm began to appear. At day 12 (D), in addition to the major residual peak of the lactone, two further signals at 47.6 and 50.6 ppm were found, resulting from the reaction of **8** with the protein.



n = 1 **4**

n = 2 **5**

15

n = 1 **16**

n = 2 **17**

Figure 1. Model molecules for amino and thio adducts

Table 1. Chemical shift[a] of S-(CH_2)- and N-(CH_2)- groups in model lactone derivatives

Compound	δ ^{13}C	Compound	δ ^{13}C
4	49.8	**16**[c]	32.9
5	45.6	**17**[c]	30.36 and 30.40
15[b]	54.9 and 55.3		

[a]Spectra were run on a Bruker 200 MHz instrument. $CDCl_3$ was used as solvent.
[b]Non equivalent carbones.
[c]Diastereomers.

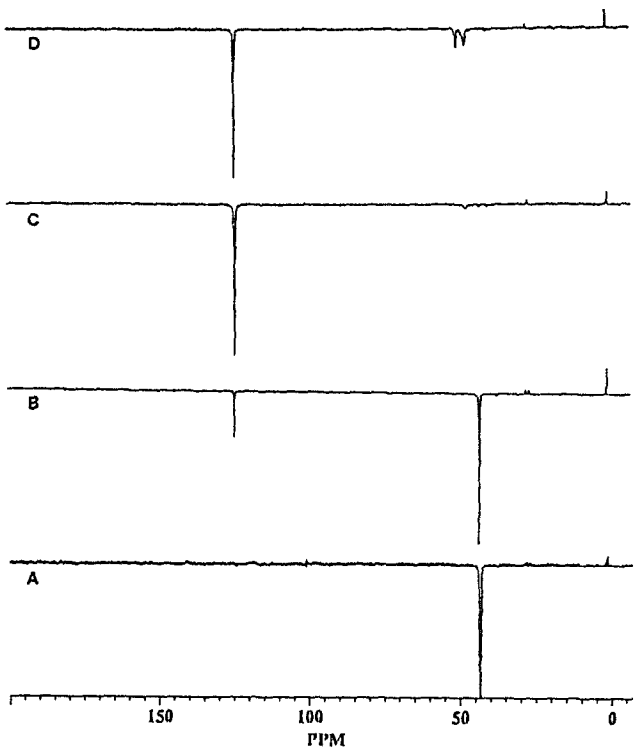


Figure 2. DEPT $^{13}C\{^1H\}$ NMR spectra (Θ = 135°) of HSA incubated with labeled compound **9**. **A**: 1mM HSA incubated with **9** at pH 7.4, t = 0; **B**: t = 2 h: **C**: t = 5 days; **D**: t = 12 days

From this experiment, we can draw two conclusions. Firstly, the chloride derivative **9** in aqueous medium at pH 7.4 in the presence of HSA undergoes an elimination reaction, similar to that seen with butylamine in organic medium, forming the lactone **8**. This reaction is relatively slow and apparently complete. Secondly, the α-methylene lactone formed reacts slowly with the protein to form one or two adducts giving two peaks on ^{13}C NMR.

Butylamine therefore seems to be an acceptable model from the mechanistic point of view for the study of the reactivity of series **1** molecules towards biological nucleophiles, the reaction mechanism paralleling that seen with a protein in aqueous medium.

Identification of series 1 adducts

As the reaction mechanism between derivative **9** and butylamine or HSA involves the formation of the α-methylene-γ-butyrolactone **8**, we have studied the adducts formed between the lactone **8** and HSA under various reaction conditions. We first of all used an excess of lactone with respect to the lysine groups theoretically present in phosphate buffer pH 7.4. We then performed the same experiment under denaturing conditions (8M urea, pH 8.8), with or without reduction of disulfide bridges, using dithiothreitol at pH 8.8. The lactone **8** was shown to be stable under the above conditions in the absence of HSA. After one week of reaction, the mixtures were dialysed (3 x 4.5 L) and lyophilised. The modified proteins obtained were then dissolved in D_2O and studied by ^{13}C NMR, using a signal amplification sequence, DEPT 135, which permits the amplification of the difference of the CH_2 signal with respect to CH and suppresses the signals of quaternary carbons, abundant in proteins. It is thus possible to obtain directly the spectrum of the labeled carbons (Figure 3) without having to subtract the residual protein spectrum.

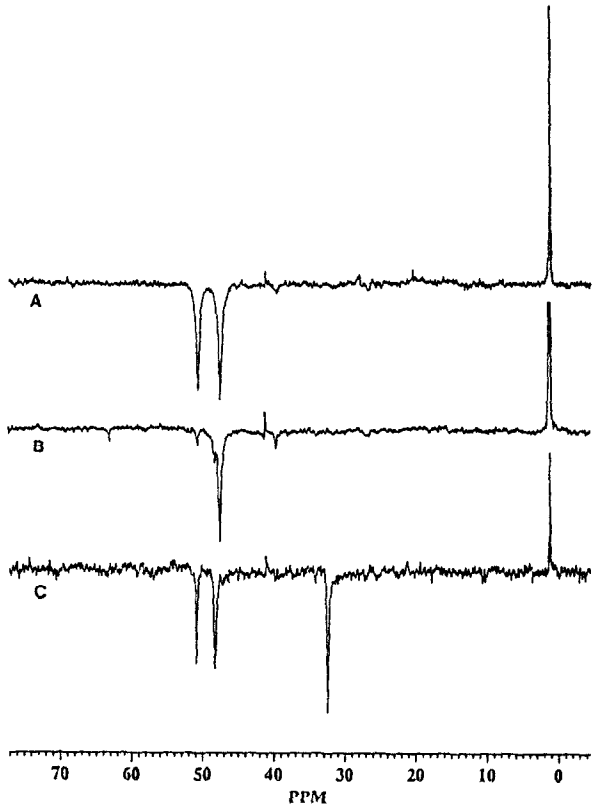


Figure 3. DEPT $^{13}C\{^1H\}$ NMR spectra (Θ = 135°) of HSA incubated with labeled compound **8**. **A**: 1 mM HSA incubated with **8** at pH 7.4; **B**: 1 mM HSA incubated with **8** under denaturing conditions (8M urea); **C**: 1 mM HSA incubated with **8** after reduction of the disulfide bridges.

Under normal conditions (pH 7.4), the ^{13}C NMR spectrum showed two signals characteristic of CH_2 at 47.6 and 50.6 ppm (A). These chemical displacements are compatible with a mono- or a diadduct with lysine. This hypothesis is supported by our previous kinetic study in which we found a signal at 47.6 ppm appearing prior to that at 50.6 ppm.

Under denaturing conditions (B), the main product was the monoadduct, only a faint diadduct signal being present. The more complex structure of the signal between 47.6 and 48.3 ppm probably indicates the formation of several closely-related adducts.

Under reducing conditions (C), two signals at 48.3 and 50.9 ppm corresponding to adducts on nitrogen were found, together with an intense signal at 32.5 ppm characteristic of a lactone–cysteine bond, resulting from the formation of several adducts on the free cysteines generated by the breaking of the disulfide bridges.

Lactone **9** therefore reacts in aqueous medium with lysine residues to form monoadducts, or even diadducts in the case of especially reactive lysines. When cysteine thiol groups are available, lactone **9** can also form covalent bonds with this amino acid.

The Series 2 Derivative

Mechanism of reaction between derivative 14 and butylamine

In the first instance, we wished to check whether the series **2** derivatives reacted with nitrogen nucleophiles by a type S_N2 reaction or if an elimination–addition reaction with the formation of a cyclopropane intermediate **18** might be involved (Scheme III). If the reaction of the ^{13}C-labeled derivative **14** with butylamine involved a type S_N2 reaction, we would expect only a single product labeled on the carbon bearing the nitrogen (45.6 ppm), whereas, if a cyclopropyl intermediate was involved, we would obtain two products labeled either on the α or β position.

Scheme III. Possible reaction mechanisms of **2a–i** derivatives with *n*-butylamine.

The reaction of the labeled derivative **14** in deuterated chloroform with 10 equivalents of butylamine produced, after 12 h, only the amine adduct labeled on the carbon bearing the nitrogen (45.4 ppm). The reaction therefore seems to occur in a single step by a type S_N2 mechanism. On following the reaction with time, no intermediate product was found.

The reaction between derivative 14 and HSA

We then reacted the bromide derivate **14** with HSA under normal conditions (phosphate buffer, pH 7.4), then under denaturing conditions after disulfide bond reduction at pH 8.8. Despite an incubation period of more than 2 weeks, no product of binding to the protein was formed under physiological conditions (pH 7.4). Under denaturing conditions and after disulfide bridge reduction, we obtained a weak signal at 29.7 ppm corresponding to the formation of an adduct on cysteine. Despite the more basic conditions of pH used, no signal corresponding to a nitrogen adduct was found. These results support the kinetic results obtained using butylamine. In effect, we have found that series **2** derivatives are 100-fold less reactive than series **1** derivatives with respect to butylamine.

Conclusion

The results obtained with HSA in aqueous medium confirm the validity of butylamine as a model for a biological nucleophile in RAI modelling of contact allergy. We have found that the mechanism of the reaction between either the amine in organic milieu or HSA in aqueous medium and the lactone derivatives tested is identical. In addition, the results obtained by NMR confirm those obtained *in vivo* on the allergenising activity of these molecules. Series **1** molecules react rapidly with butylamine (via the lactone intermediate **3**), form amino or sulfur adducts with a protein in aqueous medium and are good allergens in animal experiments. In contrast, series **2** molecules are 100-fold less reactive with butylamine,[7] form adducts with proteins only with great difficulty and are very mediocre allergens in animals. This confirms that there is a quasidirect relationship between the chemical reactivity of allergens for skin proteins and their allergising capability. RAI therefore seems a very promising approach for modelling the contact allergy reaction and butylamine an acceptable model of a biological nucleophile.

Experimental Section

General procedures

^1H and ^{13}C NMR spectra were recorded on a Bruker 200-MHz spectrometer in $CDCl_3$ unless otherwise specified. Chemical shifts are reported in ppm (δ) with respect to TMS, and $CHCl_3$ was used as internal standard (δ = 7.27 ppm). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), m (multiplet). Infrared spectra were obtained on a Perkin-Elmer spectrometer; peaks are reported in reciprocal centimetres. Melting points were determined on a Buchi Tottoli 510 apparatus and are uncorrected.

Dried solvents were freshly distilled before use. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone. Triethylamine and hexamethylphosphoramide (HMPA) were distilled from powdered calcium hydride. Methylene chloride (CH_2Cl_2) was dried over P_2O_5 before distillation. All air- or moisture-sensitive reactions were conducted in flame-dried glassware under an atmosphere of dry argon. Chromatographic purifications were conducted on silica gel column according to the flash chromatography technique.

DEPT $^{13}C\{^1H\}$ NMR spectra (Θ = 135°) of HSA–lactone adducts were obtained by the method of Doddrell *et al.* on a Bruker 200 MHz instrument using the following conditions: 600 scans, sweep width 15,150 Hz, acquisition time 0.54 s, memory size 16K (digital resolution 1.8 Hz/pt). Exponential multiplication was used for Fourier Transform spectra using a line broadening factor of 5 Hz. Samples were dissolved in 2 mL deuterium oxide (99.8 atom % excess 2H) and the methyl signal of a trace of acetonitrile was used as internal reference at 1.3 ppm.

All ^{13}C labeled compounds were first prepared in a non labeled form and were fully characterised (^1H and ^{13}C NMR). They gave satisfactory microanalysis.[7]

3-(2'-Azahexyl)-5,5-dimethyl-dihydro-2(3H)furanone 4

To a solution of methylene lactone **3** (223.3 mg; 1.77 mmol) in ethanol (15 mL) was added an excess of *n*-butylamine (0.4 mL). The mixture was stirred at room temperature for 24 h and the excess of amine removed under reduced pressure. The crude adduct was purified by column chromatography (AcOEt 100%) to give 352.8 mg (quantitative yield) of **4** as a liquid. ^1H NMR ($CDCl_3$) δ 0.90 (t, 3H, CH_3CH_2, J = 7.0 Hz), 1.24–1.61 (m, 4H, $CH_2CH_2CH_3$), 1.38 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 1.89 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.5 Hz, J_{AX} = 10.8 Hz), 2.21 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.5 Hz, J_{BX} = 8.7 Hz), 2.59 (t "like", 2H, $NHCH_2CH_2$, J = 6.8 Hz), 2.75–3.05 (m, 3H, $CHCH_2N$ + $CHCH_2N$), 5.29–5.30 (m, 1H, N–H); ^{13}C NMR ($CDCl_3$) δ 13.8, 20.2, 27.0, 28.7, 32.0, 39.4, 41.2, 49.5, 49.8, 82.5, 177.8; IR (CCl_4) ν 3321 (N–H), 1760 (C=O).

N-*Butyl-bis(3'-(5',5'-dimethyl-2-oxo-dihydro-(3H)furanyl)-methyl) amine 15*

To a solution of **4** (200 mg; 1 mmol) in ethanol (2 mL) was added an excess of methylene lactone **3** (504.6 mg; 4 mmol) and the reaction mixture was stirred at room temperature for 1 week. The solvent was removed under reduced pressure and the crude product purified by column chromatography (AcOEt 30%, hexane) to give 194 mg (60% yield) of amine **15** as a white solid. ^1H NMR ($CDCl_3$) δ 0.91 (t, 3H, CH_2CH_3, J = 6.9 Hz), 1.24–1.47 (m, 4H, $CH_2CH_2CH_3$), 1.39 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 1.85 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 13.9 Hz, J_{AX} = 10.8 Hz), 1.89 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.8 Hz, J_{AX} = 10.9 Hz), 2.24 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 13.9 Hz, J_{BX} = 9.2 Hz), 2.29 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.8 Hz, J_{BX} = 8.8 Hz), 2.45 (t, 2H, NCH_2CH_2, J = 6.9 Hz), 2.56–2.69 (m, 2H, 2 x $CHNCH_2CH_2$), 2.81–3.08 (m, 4H, 2 x CHCO + 2 x $CHNCH_2CH_2$); ^{13}C NMR ($CDCl_3$) δ 13.9, 20.4, 27.2, 28.9, 39.8, 40.3, 40.9, 41.0, 54.0, 54.9, 55.3, 82.4, 177.5; IR (CCl_4) ν 1770 (C=O). Anal. calcd for ($C_{18}H_{31}NO_4$): C, 66.43; H, 9.60; N, 4.30. Found: C, 66.51; H, 9.71; N, 4.11.

3-(3'-Azaheptyl)-5,5-dimethyl-dihydro-2(3H)furanone 5

To a solution of mesylate **2c** (100 mg; 0.42 mmol) in dichloromethane (2 mL) was added *n*-butylamine (0.5 mL; 4.8 mmol) and the mixture was stirred at room temperature for 12 h. The reaction was hydrolysed with a saturated solution of NH_4Cl and extracted with CH_2Cl_2. Organic layers were dried over $MgSO_4$ and solvents removed under reduced pressure. The crude product was purified by column chromatography (AcOEt 80%, hexane) to give 89.6 mg (quantitative yield) of **5** as a yellow liquid. ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H, CH_2CH_3, J = 7.2 Hz), 1.21–1.95 (m, 7H, $CH_2CH_2CH_3$ + $CHCMe_2O$ + $CHCH_2$), 1.25 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.89 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 14.5 Hz, J_{BX} = 9.9 Hz), 2.16–2.31 (m, 1H, NH), 2.69–2.86 (m, 1H, CH), 3.23–3.38 (m, 4H, CH_2NHCH_2); ^{13}C NMR ($CDCl_3$) δ 13.7, 20.1, 27.2, 28.6, 29.3, 31.4, 39.0, 42.8, 45.6, 46.0, 69.2, 177.7; IR (CCl_4) ν 3321 (N–H), 1760 (C=O).

Cysteine adduct 16

To a freshly prepared solution of sodium methanolate, prepared from sodium (50.6 mg; 2 mmol) in MeOH (7 mL), was added at 0 °C *N*-acetylcysteine methylester (326.4 mg; 2 mmol). The methylene lactone **3** (252.3 mg; 2 mmol) in ethanol (7 mL) was then added at 0 °C and the reaction stirred for 1 h. The mixture was acidified with a 2N solution of HCl and extracted with methylene chlonde. Organic layers were dried over $MgSO_4$, filtered and solvents removed under vacuum. The crude adduct was purified by column chromatography (AcOEt 80%, hexane) to give 542.8 mg (quantitative yield) of **16** as a mixture of diastereomers. ^1H NMR ($CDCl_3$) δ (mixture of diastereomers) 1.39 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.83–1.96 (m, 1H, $CHCMe_2$), 2.06 and 2.07 (s, 3H, HNCOMe), 2.23–2.36 (1H, m, $CHCMe_2O$), 2.64–2.79 (m, 1H, CH_2CHCH_2S), 2.96–3.15 (m, 4H, CH_2CHCH_2S + $CHCOOCMe_2$ + $HNCHCH_2S$), 3.78 and 3.79 (s, 3H, COOMe), 4.81–4.89 (m, 1H, CHNHCOMe), 6.30–6.39 and 6.45–6.50 (m, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 26.8, 28.5, 32.9, 34.5, 34.6, 39.7, 40.1, 41.2, 51.9, 52.3, 76.4, 77.7, 82.5, 169.7, 170.6, 176.3.

Cysteine adduct 17

To a freshly prepared solution of sodium methanolate (108 mg; 2 mmol) in MeOH (7 mL), was added at 0 °C *N*-acetylcysteine methylester (326.4 mg; 2 mmol). The bromoderivative **2b** (442.2 mg; 2 mmol) in methanol (7 mL) was then added at 0 °C and the reaction stirred for 2 h. The mixture was acidified with a 2N solution of HCl and extracted with methylene chloride. Organic layers were dried over $MgSO_4$, filtered and solvents removed under vacuum. The crude adduct was purified by column chromatography (AcOEt 80%, hexane) to give 174 mg (29% yield) of **17** as a mixture of diastereomers. ^1H NMR ($CDCl_3$) δ (mixture of diastereomers) 1.38 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 1.59–1.80 (m, 2H, $CHCMe_2O$ + $CHCHCH_2S$), 2.06 and 2.07 (s, 3H, NHCOMe), 2.10–2.33 (m, 2H, $CHCMe_2O$ + $CHCHCH_2S$), 2.59–2.77 (m, 2H, CH_2CH_2S), 2.85–2.98 (m, 1H, $CHCH_2CH_2S$), 2.94 (A part of an ABX system, 1H, J_{AB} = 13.8 Hz, J_{AX} = 5.6 Hz, $SCHCHNH$), 3.05 (1H, B part of an ABX system, J_{AB} = 13.8 Hz, J_{BX} = 4.6 Hz), 3.77 (s, 3H, COOMe), 4.78–4.89 (m, 1H, X part of an ABX system, J_{AX} = 5.6 Hz, J_{BX} = 4.6 Hz, J = 7.0 Hz), 6.28 and 6.39 (d, 1H, J = 7.0 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 23.3, 27.18, 27.20, 29.1, 30.36, 30.40, 30.54, 30.57, 34.20, 34.22, 39.31, 39.47, 41.30, 41.37, 52.01, 52.18, 52.83, 52.87, 82.56. 82.59, 170.10, 170.16, 171.46, 178.34, 178.23.

3-Hydroxymethyl-5,5-dimethyl-dihydro-2(3H)furanone 7a

To a solution of lithium diisopropylamine (LDA), prepared at -40 °C from diisopropylamine (1.75 mL, 12.5 mmol) in THF (40 mL) and a solution of butyllithium in THF (9.46 mL, 12.5 mmol, 1.32 M), was added dropwise at -78 °C a solution of lactone **3** (1.36 g, 11.9 mmol) in THF (40 mL). The mixture was stirred for 15 min at -78 °C and lithium chloride (1.51 g, 37.5 mmol) was added. The solution was stirred for an additional 30 min at -78 °C and gaseous ^{13}C-formaldehyde (500 mg, 16.6 mmol, generated from paraformaldehyde at 150 °C) was passed through the reaction mixture with the aid of a dry nitrogen stream. The reaction mixture was stirred for an additional 3 h at -20 °C, hydrolysed with a saturated solution of NH_4Cl (150 mL) and extracted with ethyl acetate (3 x 25 mL). Organic layers were dried over $MgSO_4$, solvents removed under reduced pressure and the crude lactone purified by column chromatography (AcOEt 75%, hexane) to give 804.4 mg (47% yield) of alcohol **7a**. ^1H NMR ($CDCl_3$) δ 1.40 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 1.99 (A part of an ABX-d system, 1H, CH, J_{AB} = 12.6 Hz, J_{AX} = 12.5 Hz, $^3J_{C-H}$ = 5.7 Hz), 2.17 (B part of an ABX system, 1H, CH, J_{AB} = 12.6 Hz, J_{BX} = 10.0 Hz), 2.31 (s, 1H, OH), 2.91–3.09 (X part of two ABX systems, m, 1H, CH), 3.76 (A part of an ABX-d system, 1H, CHO, J_{AB} = 11.2 Hz, J_{AX} = 6.0 Hz, J_{C-H} = 144.6 Hz), 3.93 (B part of an ABX-d system, 1H, CHO, J_{AB} = 11.2 Hz, J_{BX} = 4.7 Hz, J_{C-H} = 144.6 Hz); ^{13}C NMR ($CDCl_3$) δ 27.2, 28.5, 37.1, 43.3 (d, J_{C-C} = 38.4 Hz), 60.8, 83.1, 177.7.

3-Hydroxymethyl-5,5-dimethyl-dihydro-2(3H)furanone 7b

To a solution of lithium diisopropylamine (LDA), prepared at -40 °C from diisopropylamine (3.5 ml, 24.99 mmol) in THF (90 mL) and a solution of butyllithium in THF (18.93 mL, 24.99 mmol, 1.32 M), was added dropwise at -78 °C a solution of lactone **3** (2.74 g, 23.8 mmol) in THF (90 mL). The mixture was stirred for 15 min at -78 °C and lithium chloride (3.03 g, 71.4 mmol) was added. The solution was stirred for an additional 30 min at -78 °C and gaseous formaldehyde (1 g, 33.3 mmol, generated from paraformaldehyde at 150 °C) was passed through the reaction mixture with the aid of a dry nitrogen stream. The reaction mixture was stirred for an additional 3 h at -78 °C, hydrolysed with a saturated solution of NH_4Cl (150 mL) and extracted with ethyl acetate (3 x 50 mL). Organic layers were dried over $MgSO_4$, solvents removed under reduced pressure and the crude lactone purified by column chromatography (AcOEt 20%, hexane) to give 2.37 g (69% yield) of alcohol **7b**. ^1H NMR ($CDCl_3$) δ 1.41 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.98 (A part of an ABX system, 1H, CH, J_{AB} = 12.6 Hz, J_{AX} = 12.6 Hz), 2.18 (B part of an ABX system, 1H, CH, J_{AB} = 12.6 Hz, J_{BX} = 9.7 Hz), 2.92–3.08 (X part of two ABX systems, m, 1H, CH), 3.77 (A part of an ABX system, 1H, CHO, J_{AB} = 11.2 Hz, J_{AX} = 6.1 Hz), 3.93 (B part of an ABX system, 1H, CHO, J_{AB} = 11.2 Hz, J_{BX} = 4.7 Hz); ^{13}C NMR ($CDCl_3$) δ 27.2, 28.5, 37.1, 43.3, 60.9, 83.2, 177.8; IR (CCl_4) ν 3620, 3500 cm^{-1} (O–H), 1756 cm^{-1} (C=O). Anal. calcd for ($C_7H_{12}O_3$): C, 58.31; H, 8.39. Found: C, 58.06; H, 8.63.

5,5-Dimethyl-3-methylene-dihydro-2(3H)furanone 8

To a solution of alcohol (300 mg; 2.07 mmol) in CH_2Cl_2 (6 mL) were added at 0 °C Et_3N (0.75 mL; 5.38 mmol) and mesyl chloride (0.24 mL; 3.1 mmol). The mixture was stirred at 0 °C for 30 min, hydrolysed with brine and extracted with dichloromethane. Organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude mesylate was dissolved in CH_2Cl_2 (5 mL) and DBU (0.62 mL; 4.14 mmol) was added. The solution was stirred at room temperature for 1.5 h, hydrolysed with brine and extracted with ether. Combined organic layers were dried over $MgSO_4$, and solvents removed under vaccum. The crude lactone was purified by column chromatography (Et_2O 30%, hexane) to give 149.2 mg (57% yield) of ^{13}C-labeled lactone as a colourless oil. ^1H NMR ($CDCl_3$) δ 1.43 (s, 6H, CH_3), 2.75 (dt, 2H, CH_2, J = 2.7 Hz, $^3J_{C-H}$ = 4.2 Hz), 5.61 (dt, 1H, =CH, J = 2.7 Hz, J_{C-H} = 160.4 Hz), 6.22 (dt, 1H, =CH, J = 2.7 Hz, J_{C-H} = 160.4 Hz); ^{13}C NMR ($CDCl_3$) δ 28.3, 41.1, 81.4, 121.8, 136.0 (d, J_{C-C} = 75.1 Hz), 169.6.

3-Chloromethyl-5,5-dimethyl-dihydro-2(3H)furanone 9

In a solution of lactone **8** (68.8 mg; 0.54 mmol) in ether (20 mL) was bubbled at 0 °C for 30 min anhydrous HCl obtained by adding dropwise concentrated H_2SO_4 on aqueous concentrated solution of HCl and drying the gaseous HCl through a flask containing H_2SO_4 before conducting it into the reaction flask. The mixture was stirred for 1 h at room temperature and excess of HCl was neutralised with a saturated solution of $NaHCO_3$. The compound was extracted with ether, dried over $MgSO_4$ and the solvent was evaporated. Purification by column chromatography (Et_2O 30%, hexane) gave 66.3 mg (75% yield) of **9**. ^1H NMR ($CDCl_3$) δ 1.41 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 2.13 (A part of an ABX-d system, 1H, C<u>H</u>CMe_2O, J_{AB} = 12.9 Hz, J_{AX} = 12.2 Hz, $^3J_{C-H}$ = 7.1 Hz), 2.32 (B part of an ABX system, 1H, C<u>H</u>CMe_2O, J_{AB} = 12.9 Hz, J_{BX} = 8.9 Hz), 3.16–3.31 (m, X part of 2 ABX systems, 1H, CH), 3.79 (A part of an ABX-d system, 1H, ^{13}CH, J_{AB} = 10.4 Hz, J_{AX} = 8.9 Hz, $^1J_{C-H}$ = 152.3 Hz), 3.82 (B part of an ABX-d system, 1H, ^{13}CH, J_{AB} = 10.4 Hz, J_{BX} = 0 Hz, $^1J_{C-H}$ = 152.3 Hz); ^{13}C NMR ($CDCl_3$) δ 27.5, 28.8, 38.9, 43.3, 43.3, 82.7, 174.6.

5,5-Dimethyl-3-tosyloxymethyl-dihydro-2(3H)furanone 10

To a solution of alcohol **7b** (3 g, 20.81 mmol) and pyridine (11.76 mL) in dichloromethane (60 mL) were added at 25 °C dimethylaminopyridine (249.1 mg) and dropwise a solution of tosylchloride (5.95 mg, 31.21 mmol) in dichloromethane (10 mL). The mixture was stirred for 24 h and neutralised with a solution of HCl 10%. The organic layer was washed twice with HCl 10% and then with brine. Removal of solvents under reduced

pressure and purification by column chromatography (AcOEt 25%, hexane) gave 3.41 g (55%) of compound **10** as a solid (m.p. 53–55 °C). ^{1}H NMR ($CDCl_3$) 1.35 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 2.05 (A part of an ABX system, 1H, CH, J_{AB} = 12.9 Hz, J_{AX} = 12.4 Hz), 2.31 (B part of an ABX system, 1H, CH, J_{AB} = 12.9 Hz, J_{BX} = 9.6 Hz), 2.46 (s, 3H, CH_3–Ar), 3.04–3.19 (X part of 2 ABX systems, m, 1H, CH), 4.16 (A part of an ABX system, 1H, CHO, J_{AB} = 10.0 Hz, J_{AX} = 6.7 Hz), 4.33 (B part of an ABX system, 1H, CHO, J_{AB} = 10.0 Hz, J_{BX} = 3.9 Hz), 7.34–7.39 (m, 2H, ArH), 7.75–7.81 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$) δ 21.5, 27.1, 28.5, 37.8, 41.0, 66.1, 83.0, 127.8, 129.9, 132.2, 145.1, 174.0. IR (CCl_4) ν 1772 cm^{-1} (C=O). Anal. calcd for ($C_{14}H_{18}O_5S$): C, 56.36; H, 6.08. Found: C, 56.20; H, 6.23.

3-Cyanomethyl-5,5-dimethyl-dihydro-2(3H)furanone 11

To a solution of tosylate **10** (494.9 mg; 1.66 mmol) in DMF (16 mL) were added potassium cyanide (170 mg; 2.61 mmol) 18-6 crown ether (30 mg; 0.11 mmol). The mixture was heated to reflux for 6 h, hydrolysed with water and extracted with CH_2Cl_2. The organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude compound was purified by column chromatography (AcOEt 30%, hexane) to give 241.6 mg (94% yield) of the cyanato derivative **11** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 1.97 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.7 Hz, J_{AX} = 12.5 Hz), 2.47 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.7 Hz, J_{BX} = 8.9 Hz), 2.63 (A part of an ABX-d system, 1H, CHCN, J_{AB} = 17.1 Hz, J_{AX} = 8.3 Hz, $^2J_{C-H}$ = 9.8 Hz), 2.87 (B part of an ABX-d system, 1H, CHCN, J_{AB} = 17.1 Hz, J_{BX} = 4.5 Hz, $^2J_{C-H}$ = 9.8 Hz), 3.02–3.18 (m, X part of 2 ABX systems, 1H, CH), ^{13}C NMR ($CDCl_3$) δ 18.5 (d, $^1J_{C-C}$ = 57.6), 26.7, 28.6, 37.7, 40.3, 83.0, 117.0, 174.6. Mass spectrum (relative intensity) m/z 154 (M^+ + 1; 0.6), 138 (51), 69 (100).

3-Carboxymethyl-5,5-dimethyl-dihydro-2(3H)furanone 12

To the cyano derivative **11** (241.2 mg; 1.56 mmol) was added a solution of concentrated HCl (2 mL) and the mixture was heated to reflux for 12 h. Water was added and the reaction extracted with ethyl acetate. Organic layers were dried over $MgSO_4$, filtered and concentrated under vacuum to give 214.1 mg (80% yield) of acid **12** as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.85 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.6 Hz, J_{AX} = 12.2 Hz), 2.41 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.6 Hz, J_{BX} = 9.0 Hz), 2.60 (A part of an ABX-d system, 1H, CHCOOH, J_{AB} = 17.4 Hz, J_{AX} = 8.8 Hz, $^2J_{C-H}$ = 7.1 Hz), 2.96 (B part of an ABX-d system, 1H, CHCOOH, J_{AB} = 17.4 Hz, J_{BX} = 4.3 Hz, $^2J_{C-H}$ = 7.1 Hz), 3.08–3.27 (m, X part of 2 ABX systems, 1H, CH); ^{13}C NMR ($CDCl_3$) δ 26.9, 28.8, 34.6 (d, $^1J_{C-C}$ = 56.3 Hz), 37.2, 41.0, 82.9, 176.8 (2C). Mass spectrum of the silylated acid (*N*-methylsilyltrifluoroacetamide) (relative intensity) m/z 230 (M^+ - 15 (CH_3); 32), 73 (100).

5,5-Dimethyl-3-(2'-hydroxyethyl)-dihydro-2(3H)furanone 13

To acid **12** (214.1 mg; 1.24 mmol) in ether (2 mL) was added at -5 °C a solution of BH_3·Me_2S (0.3 mL; 0.58 mmol; 2N in THF) and the mixture was stirred at room temperature for 4 h. Ethyl acetate (1 mL) was then added and the reaction hydrolysed with water. The aqueous layer was saturated with NaCl and extracted with ethyl acetate. Organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give the crude alcohol which was purified by column chromatography (AcOEt 75%, hexane) yielding 171.9 mg (87% yield) of **13**. ^{1}H NMR ($CDCl_3$) δ 1.39 (s. 3H, CH_3), 1.47 (s, 3H, CH_3), 1.63–1.81 (m, 1H, $CH^{13}CH_2$), 1.79 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.6 Hz, J_{AX} = 12.2 Hz), 1.98–2.15 (m, 1H, $CH^{13}CH_2$); 2.32 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.6 Hz, J_{BX} = 9.0 Hz), 2.35–2.60 (s, 1H, OH), 2.90 (m, X part of an ABX system, 1H, CH, J_{AX} = 12.2 Hz, J_{BX} = 9.0 Hz), 3.66–3.89 (m, 2H, $^{13}CH_2$, $^1J_{C-H}$ = 143.1 Hz); ^{13}C NMR ($CDCl_3$) δ 26.6, 28.8, 34.5 (d, $^1J_{C-C}$ = 37.3 Hz), 38.6, 41.6, 60.6, 82.8, 145.3. Mass spectrum of the silylated alcohol (*N*-methylsilyltrifluoroacetamide) (relative intensity) m/z 232 (M^+ + 1; 10), 216 (M^+ - 15 (CH_3); 86), 75 (100).

3-(2'-Bromoethyl)-5,5-dimethyl-dihydro-2(3H)furanone 14

To a solution of alcohol **13** (120 mg; 0.75 mmol) in dichloromethane (4 mL), were added at 0 °C CBr_4 (323.3 mg; 0.97 mmol) and triphenylphosphine (511.4 mg; 1.95 mmol). The mixture was stirred at room temperature for 14 h and CBr_4 (124.4 mg; 0.375 mmol) and triphenylphosphine (196.7 mg; 0.75 mmol) were added. After 30 min ethyl ether was added (a precipitate formed). The supernatant was recovered and the precipitate washed with ether. The crude bromide was purified by chromatography (AcOEt 10%, hexane) to give 149.6 mg (90% yield) of **14** as a colourless oil. ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 1.72 (A part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.5 Hz, J_{AX} = 12.1 Hz), 1.89–2.05 (m, 1H, $CH^{13}CH_2$), 2.34 (B part of an ABX system, 1H, $CHCMe_2O$, J_{AB} = 12.5 Hz, J_{BX} = 8.9 Hz), 2.35–2.53 (m, 1H, $CH^{13}CH_2$), 2.93–3.09 (m, X part of an ABX system, 1H, CH, J_{AX} = 12.1 Hz, J_{BX} = 8.9 Hz), 3.40–3.71 (m, 2H, $^{13}CH_2$, $^1J_{C-H}$ = 152.7 Hz); ^{13}C NMR ($CDCl_3$) δ 26.9, 28.9, 30.9, 33.8 (d, $^1J_{C-C}$ = 36.4 Hz), 39.2, 41.2, 79.9, 177.6. Mass spectrum (relative intensity) m/z = 224 (M^+ + 1; ^{81}Br; 22), 222 (M^+ + 1; ^{79}Br; 21), 114 (100).

Coupling of lactone 8 and human serum albumin (HSA)

To HSA (150 mg; 2.25 mmol) in phosphate buffer (15 mL) was added EtOH (2.5 mL). The pH was adjusted to 7.4 and lactone **8** (30 mg; 236 μmol) in EtOH (1 mL) was added. The reaction mixture was stirred at room temperature

for 1 week, dialysed against water (3 x 4.5 L) and lyophilised to give the modified protein as a white solid.

Coupling of lactone 8 and human serum albumin (denaturing conditions)

To HSA (150 mg; 2.25 mmol) in urea solution (8 M; 15 mL) was added EtOH (2.5 mL). The pH was adjusted to 8.8 and lactone **8** (30 mg; 236 μmol) in EtOH (1 mL) was added. The reaction mixture was stirred at room temperature for 1 week, dialysed against water (3 x 4.5 L) and lyophilised to give the modified protein as a white solid.

Coupling of lactone 8 and human serum albumin (reductive conditions)

To HSA (150 mg; 2.25 mmol) in phosphate buffer (15 mL) was added EtOH (2.5 mL). The pH was adjusted to 8.8, the flask purged with argon and dithiothreitol (2.5 mg; 16.2 μmol) was added. After 2 h at room temperature, lactone **8** (30 mg; 236 mmol) in EtOH (1 mL) was added. The reaction mixture was stirred at room temperature for 1 week, dialysed against water (3 x 4.5 L) and lyophilised to give the modified protein as a white solid.

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