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Reactions of some cyclopentenones with selected cysteine derivatives and biological activities of the product thioethers

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Abstract—The conjugate addition reaction between glutathione, *N*-Boc-cysteine methyl ester, *N*-acetyl cysteine methyl ester and *N*-acetyl cysteine and several substituted cyclopentenones is described. The reversibility of this process was demonstrated by thio-adduct metathesis on treatment of the adduct with a different cysteinyl derivative. The levels at which these compounds inhibit the function of nuclear factor kappa B (NF-κB) and potentiate heat shock factor (HSF) are reported and the possible relevance of these studies concerning the antiviral and anti-inflammatory activities of the cyclopentenone prostanoids is discussed.

1. Introduction and background information

For some time we have been interested in the chemistry[1] and biology of cyclopentenone prostaglandins-A (PG-As, **1**) and -J (PG-Js, **2**). These arachidonic acid metabolites are potent bioactive molecules and have been shown to possess anticancer, antiviral and anti-inflammatory activity.[2] Evidence indicates that these compounds act by turning on an intracellular defence response, interfering with the activity of two key cellular transcription factors, namely, the heat shock factor (HSF), which switches on the synthesis of cytoprotective heat shock proteins (HSP), and nuclear factor kappa B (NF-κB), which has a critical role in regulating the inflammatory and immune responses and in controlling cell proliferation and virus replication.[2] In particular, the IκB kinase, a key signalling protein responsible for the activation of NF-κB, has been identified as one of the molecular targets for cyclopentenone prostanoids.

The α,β-unsaturated carbonyl moiety in these molecules render them reactive as Michael acceptors. Consideration of the possible use of these PGs or derivatives as therapeutic agents must take into account the propensity of the enones to react with nucleophiles in vivo, particularly thiols, which can be present as small molecules (such as cysteine and glutathione) or embedded as reactive functional groups in protein structures.

1 PG-A_1 ..... X-Y = $(CH_2)_2$ ..... PG-J_1 **2**
PG-A_2 .. X-Y = (*Z*)-(CH=CH) .. PG-J_2

This issue has been the focus of earlier studies, particularly by Noyori and co-workers.[3] These investigators found that PG-A_1 methyl ester and Δ^7-PG-A_1 methyl ester **3** react readily and reversibly with thiols such as cysteine and glutathione under physiological conditions. Competition experiments between these two PG-A derivatives showed that the cross-conjugated dienone **3** reacts faster with thiols, but under thermodynamic conditions **3** spent more time in its free state. For the cross-conjugated dienone **3** reaction with 1 equiv of thiol occurred exclusively at the *endo*-cyclic alkene unit; the bis-thio adduct was formed only after reaction with excess thiol over a longer time period.

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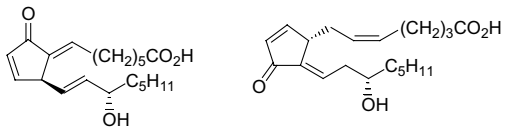

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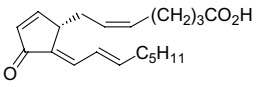

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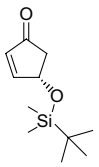
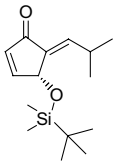
Noyori and co-workers have also observed that cyclopentenone prostaglandins are actively imported into cancer cells where they inhibit proliferation. This effect can be reversed by conjugation of the cyclopentenones with glutathione (GSH) and subsequent expulsion of the resulting metabolites from the cells. PG-J_2, Δ^{12}-PG-J_2 **4** and 15-deoxy-$\Delta^{12,14}$-PG-J_2 **5** also form GSH conjugates in vitro.[4,5] On a related theme there is debate as to whether 15-deoxy-$\Delta^{12,14}$-PG-J_2 **5** is a *bona fide* natural product based on the difficulty in detecting **5** in vivo. It has been suggested that in fact this may be due to its rapid nuclear uptake or its conjugation and excretion as polar NAC/GSH metabolites in urine.[2,6]

Taking a broader view, many other cyclopentenones have been shown to react with glutathione and other thiols.[7,8] In two studies[3,8] it was found that the stability of the thio-adduct was inversely proportional to the pH value. Thus, an equilibrium between the enone and the adduct was observed to predominate at physiological pH, while at lower pH values the thio-adduct was more stable.

Consequently, a picture emerges, which suggests that any initial view that cyclopentenone prostaglandins will indiscriminately scavenge nucleophiles in an irreversible manner in vitro and in vivo is an over-simplification of the true situation. A prevalent intracellular thiol such as glutathione (physiological concentration range 5–10 mM[9]) is likely to bind to the enone, 'protect' the electrophilic unit, and possibly help to transport the enone to a site of action. A more stable thiol adduct may then be formed when the glutathione or cysteine adduct releases the parent enone close to a protein where the microenvironment close to a cysteine residue experiences a low pH value.



(*S*)-**6**

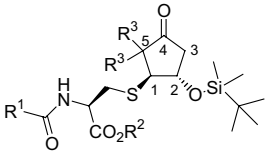


(*R*)-**7**

In our own work, we have shown that the simple cyclopentenones **6** and **7** behave in a similar manner to cyclopentenone prostaglandins in inhibiting NF-κB, potentiating the production of heat shock proteins (HSPs), especially HSP-70, and exhibiting potent activity against herpes simplex virus.[10,11] We were interested to investigate the result of reacting **6** and **7** with cysteine derivatives and glutathione in addition to determining whether the adducts so-formed possessed interesting biological activity.

2. Results and discussion

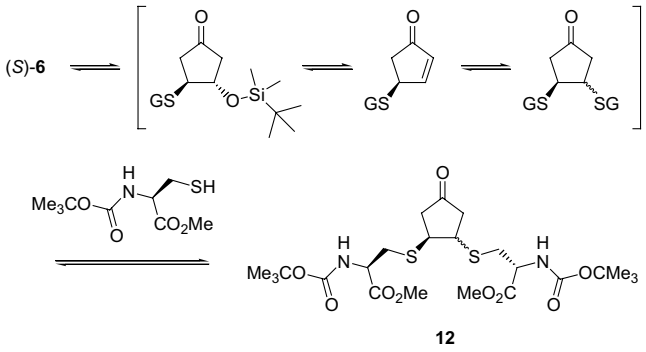
Enone **6** reacted with *N*-Boc-cysteine methyl ester smoothly, under conditions recommended by Castelli et al.,[12] to afford the adduct **8** in 80% yield. The expected *trans*-orientation of the substituents was confirmed by X-ray analysis of the crystalline product.[13] *N*-Acetylcysteine methyl ester and *N*-acetylcysteine react with the enone **6** in a similar fashion to give the adducts **9** and **10** in 97% and 71% yield, respectively. The reversibility of the latter addition process was demonstrated by modifying Castelli's conditions to include D_2O as a co-solvent instead of water. The di-deuterio compound **11** was identified by ^{1}H NMR spectroscopy and by accurate mass spectrometry. (No H/D exchange was observed at C-3.)



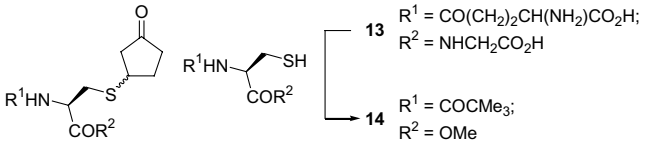
8 R^1 = Me_3CO; R^2 = Me; R^3 = H;
9 R^1 = R^2 = Me; R^3 = H;
10 R^1 = Me; R^2 = H; R^3 = H
11 R^1 = Me; R^2 = H; R^3 = D

Brief treatment of the enone **6** with glutathione (0.5 mol equiv) in pH 7.4 buffer gave a crude product, which gave mass spectrometric evidence for the formation of a mono-adduct. (Found $[M+H]^+$ 520.2153, $C_{21}H_{38}N_3O_8SiS$ requires $[M+H]^+$ 520.2149.) However, purification of the crude product proved impossible. Prolonged reaction gave no starting material but instead a mixture of polar compounds that proved difficult to characterise, although NMR evidence suggested loss of the *tert*-butylsilyloxy group from the five-membered ring. The aqueous phase was extracted with organic solvent (ether or ethyl acetate) to remove excess cyclopentenone **6** and the aqueous solution (containing the glutathione/**6** adduct) was evaporated. To the residue was added excess *N*-Boc-cysteine methyl ester in pH 7.4 buffer to afford after 15 h the bis-adducts **12** (in 16% yield) allowing the tentative proposal of a reaction pathway featured in Scheme 1. Whatever the exact structure of the intermediates the reversibility of thiol addition to cyclopentenones was indicated.

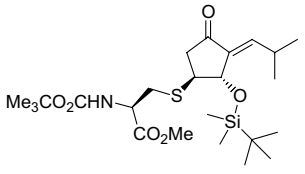
A simpler, more conclusive experiment pointing to thiol exchange under physiological conditions involved the preparation of the glutathione cyclopentenone adduct **13** (52% yield) and the conversion of **13** into the cysteine derivative **14** in 63% yield using excess *N*-Boc-cysteine methyl ester in pH 7.4 buffer.

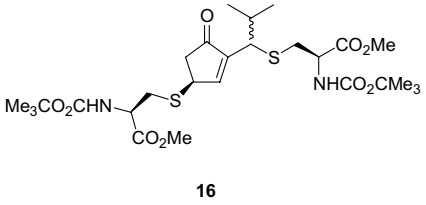


Scheme 1. Reaction of enone **6** with glutathione, then *N*-Boc-cysteine methyl ester.

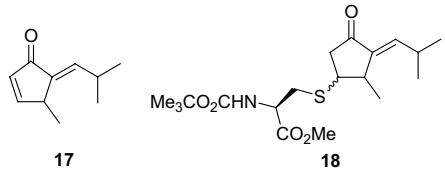


Treatment of the dienone **7** with 1 equiv of *N*-Boc-cysteine methyl ester over 2.5 h under the usual reaction conditions furnished the adduct **15** as a single diastereoisomer (88%). Interestingly, reaction of **7** with the same thiol (1 equiv) over an extended period afforded the bis-adduct **16** (49%).





The S_N2'-type reaction observed for the second addition renders the molecule intrinsically more stable; since loss of the *endo*-cyclic thiol moiety is unlikely (cyclopentadienone formation) and ready loss of the *exo*-cyclic unit is not envisaged. In this instance the *tert*-butyldimethylsilyl group is a driving force for the second addition. Thus, under identical conditions the dienone **17** gave the mono-adduct **18** (75%) over a reaction period of 4 h; extending the reaction period to 72 h showed no evidence of further reaction, in accord with the previous findings of Noyori[4] on related systems.



As observed for enone **6**, reaction of the dienone **7** with glutathione gave a mixture of polar products. Curtailing the reaction after 30 min and analysing the mixture by high-resolution mass spectrometry gave a mass ion for a mono-adduct (found $[M+Na]^+$, 596.2443, $C_{25}H_{43}N_3O_8SSiNa$ requires $[M+Na]^+$, 596.2438) but further characterisation was not achieved.

A selection of the thiol adducts were tested for activation of heat shock transcription factor (HSF AC_{200}) and inhibition of NF-κB (IC_{50}) as well as the toxicity of the compounds as monitored in the Alamar blue test (LC_{50}).

Most of the mono-adducts tested (**8**, **9**, **15** and **18**) showed good to excellent biological activity and in some cases (**15**, **18**) displayed a better therapeutic ratio than their parent cyclopentenone compounds. Interestingly, the stable bis-adduct **16** is almost devoid of biological activity (Table 1).

One explanation for the potent biological activities of some of the thiol adducts is as follows. The thiol appendage such as that present in compound **8** protects the electrophilic enone unit from adventitious (reversible) attack by hydrophilic intracellular thiols such as glutathione. Retro-Michael reaction and release of the hydrophobic *N*-Boc-cysteine methyl ester unit from **8** into an aqueous medium will be strongly disfavoured. Release of the enone **6** would be more favoured on interaction of **8** with a hydrophobic environment such as those present on the surfaces of protein. The enone, for example, **6** would be free to react with a cysteine residue in the vicinity. The ease of cleavage of this new C-bond will depend on the microenvironment; in normal circumstances thiol/cyclopentenone interactions are readily reversible. The C–S bond is stabilised if the pH is low in the vicinity of the adduct. Perhaps significantly, it is known that cyclopentenone prostaglandins (and no doubt PG-analogues) inhibit NF-κB by binding to a thiol unit on the activation loop of the β subunit of the IκB kinase IKK,[14] close to a serine unit, which is phosphorylated during the activation process. Selective nonreversible binding of the enone may be the result of this circumstance.

Table 1. Biological activities of thiol adducts

Compound	HSF AC_{200} (μM)	NF-κB IC_{50} (μM)	LC_{50} (μM)
1 (PG-A_1)	35	28	>800
6	20	30	800
7	5	5	200
8	30	51	>800
9	27	66	>800
10	>100	>100	>800
12	>100	>100	>800
13	>100	>100	>800
14	>100	>100	>800
15	10	14	>800
16	>100	>100	>800
17	29	27	>800
18	18	21	>800

It is not possible to put forward a similar tentative postulate for the activation of heat shock factor since the molecular mechanisms regulating this process have not been elucidated as yet.

3. Experimental

3.1. General directions

Starting materials were purchased from commercial sources and were used without further purification. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded using a Bruker AMX400 spectrometer; individual assignments were aided using correlation spectroscopic methods. Infrared spectroscopy was performed on a Perkin–Elmer Paragon 1000 FTIR spectrometer. Optical rotation measurements were recorded using a Optical Activity, Polaar 2001 polarimeter at 589 nm and are quoted in units of 10^{-1} deg cm^2/g. Flash column chromatography, under moderate pressure was performed using silica gel–ICN 32-63, 60 Å.

3.2. (−)-(*R*)-2′-*tert*-Butoxycarbonylamino-3′-[(1*S*,2*S*)-2-(*tert*-butyldimethylsilanyloxy)-4-oxo-cyclopentylsulfanyl]-propionic acid methyl ester (8)

A solution of enone (−)-**6**[15] (0.30 g, 1.41 mmol) in anhydrous chloroform (5 cm^3) was added to a solution of *N*-*tert*-butoxycarbonyl-L-cysteine methyl ester (0.33 g, 1.41 mmol) and a catalytic amount of triethylamine (5.1 mg, 0.050 mmol) in anhydrous chloroform (5.0 cm^3). The reaction was stirred for 4 h at room temperature under nitrogen. The solvent was removed under reduced pressure giving a pale yellow oil. Purification of the crude product by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:4)] afforded the title compound **8** as a colourless oil (0.50 g, 80% yield), which became crystalline (mp 52–53 °C) when stored at 0 °C; ν_{max} (film/cm^{-1}): 3470 and 3054 (carbamate N–H stretch), 2955, 2950 and 2857 (saturated CH_2 and CH_3 stretch), 1747 (OC=O) and 1713 (C=O ring), 1538 (NC=O); $[\alpha]_D^{22}$ −40.4 (*c* 0.05, MeOH); δ_H (400 MHz, $CDCl_3$) 0.09 (3H, s, CH_3SiCH$_3$), 0.12 (3H, s, CH$_3$SiCH_3), 0.88 (9H, s, SiC(CH_3)$_3$), 1.45 (9H, s, OC(CH_3)$_3$), 2.14 (1H, dd, *J* 18.8 and 4.4 Hz, C(5)H^{syn}), 2.16 (1H, dd, *J* 18.2 and 2.5 Hz, C(3)H^{syn}), 2.68 (1H, dd, *J* 18.2 and 5.3 Hz, C(3)H^{anti}), 2.84 (1H, dd, *J* 18.8 and 7.5 Hz, C(5)H^{anti}), 2.99 (1H, dd, *J* 13.5 and 5.5 Hz, C*H*H-Cys), 3.13 (1H, dd, *J* 13.5 and 5.1 Hz, CH*H*-Cys), 3.30–3.36 (1H, m, SC(1)*H*), 3.77 (3H, s, OCH_3), 4.25 (1H, m, C(2)*H*O), 4.59 (1H, m, C*H*-Cys), 5.34 (1H, d, *J* 7.5 Hz, N*H*); δ_C (100 MHz, $CDCl_3$) −4.39 (*C*H$_3$SiCH$_3$), −4.39 (CH$_3$Si*C*H$_3$), 17.95 ((*C*H$_3$)$_3$*C*Si), 26.03 ((*C*H$_3$)$_3$CSi), 28.66 ((*C*H$_3$)$_3$CO), 34.57 (*C*H$_2$-Cys), 43.96 (*C-5*), 46.44 (*C-3*), 48.90 (*C-1*), 52.01 (O*C*H$_3$), 53.46 (*C*H-Cys), 75.11 (*C-2*), 80.68 (O*C*(CH$_3$)$_3$), 155.50 (N*C*=O), 178.40 (O*C*=O) and 214.50 (*C*=O); *m*/*z* (FAB) 470 ([M+Na]$^+$, 26%), 448 ([M+H]$^+$); Found: ([M+H]$^+$), 448.21721; $C_{20}H_{37}NO_6SiS{\cdot}H$ requires ([M+H]$^+$), 448.21891.

3.3. (−)-(*R*)-2′-Acetyl-amino-3′-[(1*S*,2*S*)-2-(*tert*-butyl-dimethylsilanyloxy)-4-oxo-cyclopentylsulfanyl]propionic acid methyl ester (9)

The compound was prepared from (−)-**6** and *N*-acetyl-L-cysteine methyl ester, using the general procedure with acetone–water (3:1) as the solvent. The crude product was purified by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:3)] to give the title compound **9** (0.104 g, 92%) as a white solid, mp 207 °C (dec); Found: C, 52.5; H, 7.9; N, 3.8; $C_{17}H_{31}NO_5SSi$ requires C, 52.4; H, 8.0; N, 3.6; ν_{max} (film/cm^{-1}): 2953 (NH carbamate), 2929 and 2856 (saturated CH_3 and CH_2 stretch), 1749 (OC=O acid), 1690 (C=O ring), 1537 (NC=O carbamate); $[\alpha]_D^{25}$ −29.7 (*c* 0.54, MeOH); δ_H (400 MHz, $CDCl_3$) 0.01 (3H, s, CH_3SiCH$_3$), 0.03 (3H, s, CH$_3$SiCH_3), 0.79 (9H, s, SiC(CH_3)$_3$), 1.93 (3H, s, CH$_3$-acetyl), 1.85–1.96 (1H, dd, partially obscured C(5)H^a or C(5)H^b), 2.01–2.12 (1H, dd, partially obscured C(3)H^a or C(3)H^b), 2.60–2.63 (1H, dd, *J* 18.3 and 5.4 Hz, C(3)H^a or C(3)H^b), 2.70–2.77 (1H, dd, *J* 18.8 and 7.7 Hz, C(5)H^a or C(5)H^b), 2.91–2.96 (1H, dd, *J* 13.5 and 5.4 Hz, C*H*H-Cys), 3.03–3.09 (1H, dd, *J* 13.5 and 5.1 Hz, CH*H*-Cys), 3.23–3.28 (1H, m, SC(1)*H*), 3.70 (3H, s, OCH_3), 4.24 (1H, m, SiOC(2)*H*), 4.83 (1H, m, C*H*-Cys), 6.35 (1H, d, *J* 7.16 Hz, N*H*); δ_C (100 MHz, $CDCl_3$) −4.46 (*C*H$_3$SiCH$_3$), −4.43 (CH$_3$Si*C*H$_3$), 18.26 ((CH$_3$)$_3$*C*Si), 23.39 (*C*H$_3$ acetate), 26.01 ((*C*H$_3$)$_3$CSi), 34.01 (*C*H$_2$-Cys), 43.01 (*C-5*), 46.29 (*C-3*), 48.87 (*C-1*), 51.16 (O*C*H$_3$), 53.04 (*C*H-Cys), 74.85 (*C-2*), 170.20 (NH*C*=O), 171.40 (O*C*=O) and 214.20 (*C*=O ring); *m*/*z* (CI) 407 ([M+NH$_4$]$^+$); Found: ([M+H]$^+$), 390.17714; $C_{17}H_{31}NO_5SSi{\cdot}H$ requires ([M+H]$^+$), 390.17706.

3.4. (−)-(*R*)-2′-*tert*-Butoxycarbonylamino-3′-[(1*S*,2*S*)-2-(*tert*-butyldimethylsilanyloxy)-4-oxo-cyclopentylsulfanyl]propionic acid (10)

The compound was prepared from (−)-**6** and *N*-acetyl-L-cysteine using the general procedure with acetone–water (3:1) as the solvent. Purification by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether–acetic acid, 11:88:1)] afforded the title compound **10** (0.51 g, 71% yield) as a colourless oil; Found: C, 51.0; H, 7.8; N, 3.8; $C_{16}H_{29}NO_5SSi$ requires C, 51.2; H, 7.8; N, 3.8; ν_{max} (film)/cm^{-1}: 3393 (OH stretch), 3055 (carbamate N–H stretch), 2986 and 2932 (saturated CH_2 and CH_3 stretch), 1730 (OC=O) and 1715 (C=O ring), 1492 (NC=O); $[\alpha]_D^{19}$ −31.4 (*c* 0.06, MeOH); δ_H (400 MHz, $CDCl_3$) 0.04 (3H, s, CH_3SiCH$_3$), 0.07 (3H, s, CH$_3$SiCH_3), 0.83 (9H, s, SiC(CH_3)$_3$), 2.09 (4H, m, CH_3 acetate and C(5)H^{syn}), 2.17 (1H, m, C(3)H^{syn}), 2.68 (1H, dd, *J* 18.3 and 5.5 Hz, C(3)H^{anti}), 2.82 (1H, dd, *J* 17.7 and 7.7 Hz, C(5)H^{anti}), 3.04 (1H, dd, *J* 13.7 and 5.75 Hz, C*H*H-Cys), 3.15 (1H, dd, *J* 13.7 and 4.75 Hz, CH*H*-Cys), 3.34 (1H, m, SC(1)*H*), 4.33 (1H, m, SiOC(2)*H*), 4.78 (1H, m, C*H*-Cys), 6.99 (1H, d, *J* 7.5 Hz, N*H*), 8.65 (1H, br s, *OH* acid); δ_C (100 MHz, $CDCl_3$) −4.46 (*C*H$_3$SiCH$_3$), −4.41 (CH$_3$Si*C*H$_3$), 18.27 ((CH$_3$)$_3$*C*Si), 23.12 (*C*H$_3$ acetate), 25.93 ((*C*H$_3$)$_3$CSi), 33.68 (*C*H$_2$-Cys), 42.91 (*C-5*), 46.40 (*C-3*), 48.92 (*C-1*), 52.55 (*C*H-

Cys), 74.80 (*C-2*), 172.10 (NC=O), 172.80 (OC=O) and 215.60 (C=O); *m/z* (ES^-) 749 ($[2M-H]^+$, 5%), 374 ($[M-H]^+$); Found: ($[M-H]^+$), 374.1469; $C_{16}H_{28}NO_5SiS$ requires ($[M-H]^+$), 374.1457.

3.5. (*R*)-2′-*tert*-Butoxycarbonylamino-3′-[2-((*R*)-2′-*tert*-butoxycarbonylamino-2-methoxycarbonylethylsulfanyl)-4-oxo-cyclopentylsulfanyl]propionic acid methyl ester (12)

A solution of glutathione (1.45 g, 4.71 mmol) in pH 7.4 buffer (20 cm^3) was degassed using a sonicator for 20 min. A solution of (−)-**6** (2.00 g, 9.42 mmol) in ethanol (4 cm^3) was added and the mixture was stirred in the dark at room temperature under nitrogen for 10 h. The solvent was removed under reduced pressure and the residue was re-dissolved in water (15 cm^3) and extracted with ethyl acetate (6×10 cm^3). The aqueous layer was separated and the water removed under reduced pressure. The orange residue was re-dissolved in pH 7.4 buffer (25 cm^3) and the solution was degassed for 20 min. The degassed solution was added to a solution of *N-tert*-butoxycarbonyl-L-cysteine methyl ester (2.95 g, 12.53 mmol) in ethanol (10 cm^3). The reaction was stirred at room temperature and in the dark for 15 h under argon. The solvent was removed under reduced pressure and the residue was re-dissolved in water (15 cm^3). The aqueous solution was extracted with ethyl acetate (6×10 cm^3), and the organic layers were combined and dried ($MgSO_4$). Purification of the crude product by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:2)] gave title compound **12** (0.426 g 16%, an inseparable mixture of diastereoisomers) as a colourless oil; ν_{max} (film)/cm^{-1} 3366 (carbamate NH stretch), 2977 and 2953 (saturated CH_2 and CH_3 stretch), 1747 (OC=O), 1711 (C=O ring), 1512 (NC=O); δ_H (400 MHz, $CDCl_3$) 1.46 (18H, s, 2×OC(CH_3)$_3$), 2.22 (2H, m, C(5)H^a or C(5)H^b and C(3)H^a or C(3)H^b), 2.91 (2H, m, C(5)H^a or C(5)H^b and C(3)H^a or C(3)H^b), 2.89–3.17 (4H, m, 2×CH_2-Cys), 3.46 (1H, m, SC(1)*H* or SC(2)*H*), 3.52 (1H, m, SC(1)*H* or SC(2)*H*), 3.79 (6H, s, 2×OCH_3), 4.59 (2H, m, br, 2×C*H*-Cys), 5.43 (1H, d, *J* 5.7 Hz, N*H*), 5.53 (1H, d, *J* 6.1 Hz, N*H*); δ_C (100 MHz, $CDCl_3$) 28.65 (2×C(*C*H_3)$_3$), 34.29 and 34.79 (2×*C*H_2-Cys), 43.09, 43.67, 44.06 and 44.15 (*C-3* and *C-5*), 46.56 and 47.15 (*C-1* and *C-2*), 53.04 (2×O*C*H_3), 53.42, 53.69 and 53.92 (2×*C*H-Cys), 80.71 (2×*C*(CH$_3$)$_3$), 155.5 (2×N*C*=O), 171.4 and 171.6 (2×O*C*=O), 213.1 and 213.2 (2×*C*=O ring); *m/z* (ES^+) 573 ($[M+Na]^+$); Found: ($[M+Na]^+$), 573.1920; $C_{23}H_{38}N_2O_9S_2$·Na requires ($[M+Na]^+$), 573.1916.

3.6. (*S*)-2′-Amino-4′-[(*R*)-1-(carboxymethyl-carbamoyl)-2′-(3-oxo-cyclopentylsulfanyl)ethylcarbamoyl]butyric acid (13)

A solution of cyclopentenone (0.54 g, 6.59 mmol) in acetone (6.5 cm^3) was added to a solution of glutathione (2.02 g, 6.59 mmol) in distilled water (11.5 cm^3). The reaction was sonicated for 10 min at 60 °C, and the solution was stirred under nitrogen for 14 h. The solvent was evaporated under reduced pressure to afford a cream coloured residue (2.55 g). The crude product was purified by recrystallisation (85% aqueous EtOH) to give the title compound **13** (1.34 g, 52%, an inseparable mixture of diastereoisomers) as a white solid, mp (dec) 170–173 °C (from 85% aqueous EtOH); $R_f = 0.40$ (butanol–H_2O–acetic acid, 4:4:2); δ_H (400 MHz, D_2O) 1.93 (1H, m, C(5)H^a or C(5)H^b), 2.10–2.17 (2H, m, C(β)H_2-Glu) 2.17-2.25 (1H, dd, partially obscured C(2)H^a or C(2)H^b), 2.23–2.30 (1H, m, partially obscured C(4)H^a or C(4)H^b), 2.32–2.42 (1H, m, partially obscured C(5)H^a or C(5)H^b), 2.40–2.50 (1H, m, partially obscured C(4)H^a or C(4)H^b), 2.52 (2H, m, partially obscured C(γ)H_2-Glu) 2.63 (1H, m, C(2)H^a or C(2)H^b), 2.91 (1H, dt *J* 14.2 and 8.6 Hz, C*H*H-Cys), 3.00 (1H, dt, *J* 14.2 and 5.3 Hz, CH*H*-Cys), 3.59 (1H, m, SC(1)*H*), 3.78 (1H, t, *J* 6.3 Hz, C(α)*H*-Glu), 3.91 (1H, s, CH_2-Gly), 4.58 (1H, m, C*H*-Cys); δ_C (100 MHz, D_2O) 26.44 and 26.62 (*C*(γ)H_2-Glu), 29.45 and 29.46 (*C*(β)H_2-Glu), 31.59 (*C-5*), 32.43 and 32.51 (*C*H_2-Cys), 37.41 and 37.46 (*C-4*), 42.21 and 42.32 (*C*H_2-Gly), 40.49, 40.67 and 40.69 (*C-1*), 45.79 and 45.81 (*C-2*), 53.77 (*C*H-Cys), 55.92 (*C*H-Glu), 172.50, 172.70, 172.80, 174.30, 175.10 and 175.10 (2*C*=O carbamate and 2×*C*=O acid), 223.90 and 224.10 (*C*=O ring); *m/z* (ES^+) 412 ($[M+Na]^+$), 390 ($[M+H]^+$); Found: ($[M+Na]^+$), 412.1143; $C_{15}H_{23}N_3O_7S$·H requires ([M+Na]), 412.1154.

3.7. (*R*)-2′-*tert*-Butoxylcarbonylamino-3′-(3-oxo-cyclopentylsulfanyl)propionic acid methyl ester (14)

3.7.1. Method A. The compound was prepared from cyclopentenone using the general procedure described above. Purification by flash column chromatography [$R_f = 0.15$ (SiO_2, ethyl acetate–petroleum ether, 1:2)] afforded the title compound **14** (9.14 g, 92%, an inseparable mixture of diastereoisomers) as a colourless wax; Found: C, 52.4; H, 7.1; N, 4.2; $C_{14}H_{23}NO_5S$ requires C, 52.9; H, 7.3; N, 4.4; ν_{max} (film)/cm^{-1} 3355 (carbamate NH stretch), 2976 and 2940 (saturated CH_2 and CH_3 stretch), 1745 (OC=O), 1713 (C=O ring), 1506 (NC=O); δ_H (400 MHz, $CDCl_3$) 1.45 (9H, s, OC(CH_3)$_3$), 1.89 (1H, m, C(5)H^a or C(5)H^b), 2.19–2.23 (1H, dd, partially obscured C(2)H^a or C(2)H^b), 2.23–2.30 (1H, ddd, partially obscured C(4)H^a or C(4)H^b), 2.32–2.38 (1H, m, partially obscured C(5)H^a or C(5)H^b), 2.38–2.48 (1H, ddd, partially obscured C(4)H^a or C(4)H^b), 2.63 (1H, dd, *J* 18.5 and 7.5 Hz, C(2)H^a or C(2)H^b), 2.93–3.13 (2H, m, CH_2-Cys), 3.47–3.55 (1H, m, SC(1)*H*), 3.77 (3H, s, OCH_3), 4.55 (1H, br m, C*H*-Cys), 5.36 (1H, br m, N*H*); δ_C (100 MHz, $CDCl_3$) 28.32 (C(*C*H_3)$_3$), 30.21 and 30.24 (*C-5*), 33.93 and 34.05 (*C*H_2-Cys), 37.33 (*C-4*), 41.13 and 41.17 (*C-2*), 45.88 and 45.94 (*C-1*), 52.97 (O*C*H_3), 53.07 and 53.08 (*C*H-Cys), 80.29 (*C*(CH$_3$)$_3$), 155.10 (N*C*=O), 171.30 (O*C*=O), 214.80 (*C*=O); *m/z* (CI) 335 ($[M+NH_4]^+$, 25%), 318 ($[M+H]^+$); Found: ($[M+H]^+$), 318.13747; $C_{14}H_{23}NO_5S$·H requires ($[M+H]^+$), 317.13754.

3.7.2. Method B. To a solution of **13** (0.15 g, 0.473 mmol) in pH 7.4 buffer (5 cm^3) was added a solution of *N-tert*-butoxycarbonyl-L-cysteine methyl ester

(1.11 g, 4.73 mmol) in acetone (5 cm^3) and the reaction was stirred for 16 h. The solvent was removed under reduced pressure and the residue was re-dissolved in distilled water (5 cm^3), and extracted with ethyl acetate (5 × 20 cm^3). The solvent was removed under reduced pressure and the residue was purified using column chromatography [$R_f = 0.15$ (SiO_2, ethyl acetate–petroleum ether, 1:2)] to afford the title compound **14** (0.85 g, 63%, an inseparable mixture of diastereoisomers) as a colourless wax.

3.8. (−)-(*R*)-3′-{(1*S*,2*S*)-2-(*tert*-Butyldimethylsilanyloxy)-3-[2″-methyl-prop-(*E*)-ylidene]-4-oxo-cyclopentylsulfanyl}-2′-isopropoxycarbonylaminopropionic acid methyl ester (15)

The compound was prepared from (−)-**7**[16] using the general procedure described above. The reaction was stirred for 3 h before the solvent was removed under reduced pressure. Purification of the residue by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:4)] afforded the title compound **15** as a colourless oil (0.64 g, 88%); ν_{max} (film)/cm^{-1}: 3420 and (carbamate N–H stretch), 2977 and 2928 (saturated CH_2 and CH_3 stretch), 1728 (OC=O ring) and 1653 (C=O ring), 1506 (C=O carbamate); $[\alpha]_D^{22}$ −32 (*c* 1.00, MeOH); δ_H (400 MHz, $CDCl_3$) 0.12 (3H, s, CH_3SiCH_3), 0.15 (3H, s, CH_3SiCH_3), 0.84 (9H, s, SiC(CH_3)$_3$), 1.05 (6H, t, *J* 5.9 Hz, isopropyl), 1.45 (9H, s, OC(CH_3)$_3$), 2.27 (1H, d, *J* 18.2 Hz, C(5)H^{syn}), 2.59 (1H, m, C=CHCH(CH_3)$_2$), 2.97 (1H, dd, partially obscured C(5)H^{anti}), 2.90–3.50 (2H, 2×dd, partially obscured CH_2-Cys), 3.32 (1H, d, *J* 6.8 Hz, SC(1)*H*), 3.79 (3H, s, OCH_3), 4.55 (1H, m, C*H*-Cys), 4.75 (1H, s, C(2)*H*OS*i*), 5.33 (1H, d, *J* 7.5 Hz, N*H*), 6.52 (1H, d, *J* 10.5 Hz, C=C*H*CH(CH_3)$_2$); δ_C (100 MHz, $CDCl_3$) −5.78 (*C*H_3SiCH_3), −5.25 (CH_3Si*C*H_3), 16.77 ((CH_3)$_3$*C*Si), 20.82 and 21.08 (C=CHCHC(*C*H_3)$_2$), 24.56 and 24.57 ((*C*H_3)$_3$CSi), 27.26 ((*C*H_3)$_3$CO), 28.05 (C=CH*C*H(CH_3)$_2$), 32.20 and 32.54 (*C*H_2-Cys), 40.81 (*C-5*), 45.32 and 45.71 (*C-1*), 51.61 and 51.75 (OCH_3), 52.48 (*C*H-Cys), 72.02 and 75.06 (*C-2*), 79.27 (O*C*(CH_3)$_3$), 134.7 (*C*=CH-CH(CH_3)$_2$), 147.1 (C=*C*HCH(CH_3)$_2$), 153.90 and 154.10 (N*C*=O), 170.20 (O*C*=O) and 202.70 (*C*=O); *m*/*z* (ES^+) 1025 ($[2M+Na]^+$, 3%), 524 ($[M+Na]^+$); Found: ($[M+Na]^+$), 524.2470; $C_{24}H_{43}NO_6SiS{\cdot}Na$ requires ($[M+Na]^+$), 524.2478.

3.9. (*R*)-2′-*tert*-Butoxycarbonylamino-3′-{(*S*)-3′-[1-((*R*)-2′-*tert*-butoxycarbonylamino-2-methoxycarbonyl-ethylsulfanyl)-2-methyl-propyl]-4-oxo-cyclopent-2-enylsulfanyl}propionic acid methyl ester (16)

The compound was prepared from (−)-**7** using the procedure described above; the solution was stirred for 14 h. Purification of the crude product by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:2)] gave the title compound **16** (96 mg, 49%, an inseparable mixture of diastereoisomers) as a colourless oil; ν_{max} (film)/cm^{-1} 3357 (carbamate NH stretch), 2934 and 2897 (saturated CH_2 and CH_3 stretch), 1751 (OC=O), 1714 (C=O ring), 1550 (NC=O); δ_H (400 MHz, $CDCl_3$) 0.96 (6H, m, CH(CH_3)$_2$), 1.47 (18H, s, 2×OC(CH_3)$_3$), 2.05 (1H, m, C(5)H^{syn}), 1.73 (1H, m, CH(CH$_3$)$_2$), 2.91–3.15 (5H, m, C(5)H^{anti} and 2×CH_2-Cys), 3.42 (1H, m, C(3)SC(1′)*H*), 3.79 (6H, s, 2×OCH_3), 4.08 (1H, m, SC(1)*H*), 4.51 (2H, m, 2×C*H*-Cys), 5.42 (2H, m, 2×N*H*); δ_C (100 MHz, $CDCl_3$) 20.23 and 20.70 (CH(*C*H$_3$)$_2$), 28.62, 28.69, 29.37 (2×C(*C*H$_3$)$_3$), 33.61, 33.95, 34.58 and 34.83 (2×*C*H$_2$-Cys), 42.03 (*C-1′*), 43.82 and 43.91 (*C-5*), 47.93 (*C-1*), 52.81 and 53.01 (2×OCH_3), 53.12 and 53.78 (2×*C*H-Cys), 80.48 and 80.48 (2×*C*(CH$_3$)$_3$), 146.90 and 147.20 (*C-2*), 155.40 (2×N*C*=O), 158.10 and 158.30 (*C-3*), 171.40, 171.50, 171.60 and 171.70 (2×O*C*=O), 204.90 and 205.10 (2×*C*=O ring); *m*/*z* (ES^-) 604 ($[M-H]^+$); Found: ($[M-H]^+$), 604.2487; $C_{27}H_{43}N_2O_9S_2$ requires ($[M-H]^+$), 604.2488.

3.10. (*R*)-2′-*tert*-Butoxycarbonylamino-3′-[(1*RS*,2*SR*)-2-methyl-4-oxo-cyclopentylsulfanyl]propionic acid methyl ester (18)

The compound was prepared from (±)-**17**[17] using the general procedure described above. Purification of the residue by flash column chromatography [$R_f = 0.25$ (SiO_2, ethyl acetate–petroleum ether, 1:2)] afforded the title compound **18** (96 mg, 75%, an inseparable mixture of diastereoisomers) as a colourless oil; ν_{max} (film)/cm^{-1}: 3367 (carbamate N–H stretch), 2960, 2922 and 2868 (saturated CH_2 and CH_3 stretch), 1746 (OC=O) and 1717 (C=O ring), 1647 (NC=O); δ_H (400 MHz, $CDCl_3$) 1.05 (6H, t, *J* 13.8 Hz, C=CHCH(CH_3)$_2$), 1.22 (3H, d, *J* 7.2 Hz, CH_3), 1.46 (9H, s, OC(CH_3)$_3$), 2.33 (1H, dd, *J* 18.7 and 0.6 Hz, C(5)H^{syn}), 2.53 (1H, m, C=CHCH(CH_3)$_2$), 2.80 (1H, dd, *J* 18.7 and 4.1 Hz, C(5)H^{anti}), 2.90–2.95 (1H, m, partially obscured C(2)H), 2.92–3.06 (2H, m, partially obscured CH_2-Cys), 3.17 (1H, m, SC(1)*H*), 3.77 (3H, s, OCH_3), 4.57 (1H, m, C*H*-Cys), 5.32 (1H, d, *J* 7.5 Hz, N*H*), 6.43 (1H, dt, *J* 10.5 and 1.9 Hz, C=C*H*CH(CH$_3$)$_2$); δ_C (100 MHz, $CDCl_3$): 20.88 and 20.92 (*C*H$_3$), 22.36 and 22.54 (C=CHCH(*C*H$_3$)$_2$), 28.66 and 29.04 ((*C*H$_3$)$_3$CO), 33.75 and 33.81 (*C*H$_2$-Cys), 40.66 and 40.79 (*C-2*), 43.17 and 42.23 (*C-5*), 45.47 and 45.56 (*C-1*), 52.95 and 53.04 (OCH_3), 53.71 (*C*H-Cys), 80.65 (O*C*(CH$_3$)$_3$), 137.90 (*C*=CHCH(CH$_3$)$_2$), 145.20 (C=*C*HCH(CH$_3$)$_2$), 155.40 (N*C*=O), 171.70 (O*C* =O) and 204.30 (*C*=O); *m*/*z* (ES^+) 408 ($[M+Na]^+$); Found: ($[M+Na]^+$), 408.1801; $C_{19}H_{31}NO_5S{\cdot}Na$ requires ($[M+Na]^+$), 408.1821.

3.11. Determination of biological activity

The effect of the compounds on the activity of HSF and NF-κB transcription factors was determined in a gene reporter assay. HeLa cell clones, stably transfected with a luciferase reporter plasmid controlled by the human HSP-70 promoter or by a synthetic NF-κB construct, were maintained in DMEM medium supplemented with 10% FBS, L-glutamine (2 mM) and G418 (250 μg/mL) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were seeded at a density of 4×10^4 cells/well in 96-well plates.

After 18–20 h, the medium was removed and cells were treated for 8 h with the test compounds at the appropriate dilutions in serum-free medium. For the NF-κB-dependent reporter gene assay, cells were stimulated with TPA (25 ng/mL) 2 h after exposure to the compounds. After incubation, the medium was removed and cells were lysed in 10 μL of lysis buffer. The luciferase activity was determined by adding 100 μL of substrate and the release of light was measured using a Victor 1420 microplate reader (Wallac, Finland). The concentrations of the compounds that induced 100% increases (AC_{200}) and 50% inhibition (IC_{50}) of luciferase activity were calculated for HSF and NF-κB, respectively.

3.12. Determination of cytotoxicity

The cytotoxicity of the compounds was determined using the Alamar blue assay (Biosources Intl, Camarillo, CA). HeLa cells were plated in 96-well microtiter plates in 100 μL culture medium (4×10^4/well). After 20 h, cells were exposed to the test compounds at different dilutions and incubated for the next 8 h at 37 °C in a 5% CO_2 humidified atmosphere. After 6 h of incubation, Alamar blue was added in an amount equal to 10% of the culture volume, then 2 h after the addition of the dye, the fluorescence was measured using a Victor 1420 microplate reader. The concentrations of the compounds that caused 50% inhibition in this assay (LD_{50}) was calculated by Allfit program.

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