Design, synthesis and evaluation of furanocoumarin monomers as inhibitors of CYP3A4

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A number of furanocoumarins isolated from grapefruit juice have been found to inhibit CYP3A4 activity *in vitro*. In this study, we have designed and synthesised a range of analogues based on bergamottin to investigate the relationship between chemical structure and inhibition of CYP3A4 activity. Studies were performed using human liver microsomes and human intestinal S9 fraction, with testosterone as the marker substrate. With the exception of the coumarin and phenolic furanocoumarin derivatives, which were inactive, the alkyloxy-furanocoumarin analogues were found to inhibit CYP3A4 activity in a dose dependent manner, with observed IC_{50} values ranging from 0.13 ± 0.03 to 49.3 ± 1.9 μM. The unsaturated furan derivatives were found to exhibit time-dependent inhibition, showing a 2-, 4- and 14-fold increase in potency for 6′,7′-epoxybergamottin, 6′,7′-dihydroxybergamottin and bergamottin, respectively after a preincubation period of ten minutes. Reduction of the furan moiety resulted in an 11-fold decrease in inhibitory potency, suggesting that this functional group is key to the interaction between these compounds and CYP3A4.

Introduction

Grapefruit juice has been found to cause a marked increase in the oral bioavailability of many therapeutic agents,[1–4] in some cases leading to toxicity. Such interactions are believed to result from the mechanism-based inhibition of CYP3A4 activity in the intestine, by a series of bergamottin furanocoumarins present in the juice.[5] However, very little is known about the structural features of the furanocoumarins that are associated with enzyme inactivation. The aim of this work was to design, synthesise and test a series of compounds structurally related to bergamottin [**1**] (Fig. 1), by modifying specific functional groups postulated to be involved in CYP3A4 inhibition. After synthesis, the effect of these compounds on the 6β-hydroxylation of testosterone, an index of CYP3A4 activity, was assessed in human liver and intestine.

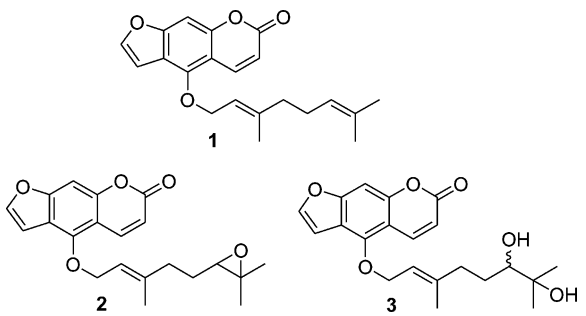

Fig. 1 Furanocoumarin monomers bergamottin [**1**], 6′,7′-EB [**2**] and 6′,7′-DHB [**3**] isolated from grapefruit juice.

Results and discussion

Design of inhibitors

The design of a series of putative inhibitors of CYP3A4 was based on the structure of bergamottin [**1**] and its analogues 6′,7′-epoxybergamottin [**2**, 6′,7′-EB] and 6′,7′-dihydroxybergamottin [**3**, 6′,7′-DHB] (Fig. 1). Four possible sites of interaction with the enzyme were proposed, which are highlighted in Fig. 2.

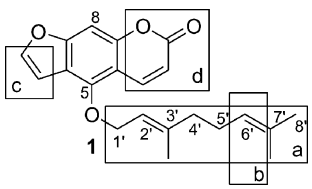

Fig. 2 Proposed sites of interaction between bergamottin and CYP3A4.

These are:

a. The olefin region. Alkene and alkyl groups are capable of fitting into hydrophobic pockets on the active site and then interacting with the enzyme. Modifying the chain length of the olefin region may result in an analogue with increased or decreased binding efficiency.

b. The 6′,7′-position. By incorporating groups such as an alcohol, an epoxide or an ether in the alkyl chain, the effect of hydrogen bond donors or acceptors can be examined.

c. The 2,3-furan moiety. It is believed that the furanocoumarin is converted to the reactive epoxide intermediate at this position before irreversibly binding to CYP3A4.[6] If this is the case, by

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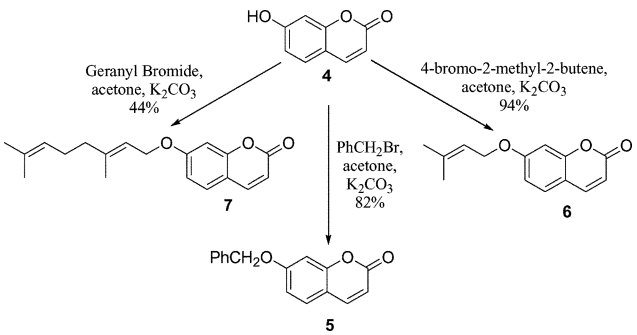
reducing the furan ring it may be possible to prevent the formation of the epoxide, and in turn the inactivation of the enzyme.

d. The lactone moiety may contribute towards enzyme inactivation with the carbonyl group acting as a hydrogen bond acceptor or interacting *via* dipole–dipole interactions. The lactone may also be susceptible to nucleophilic attack at the carbonyl or the unsaturated β-position.

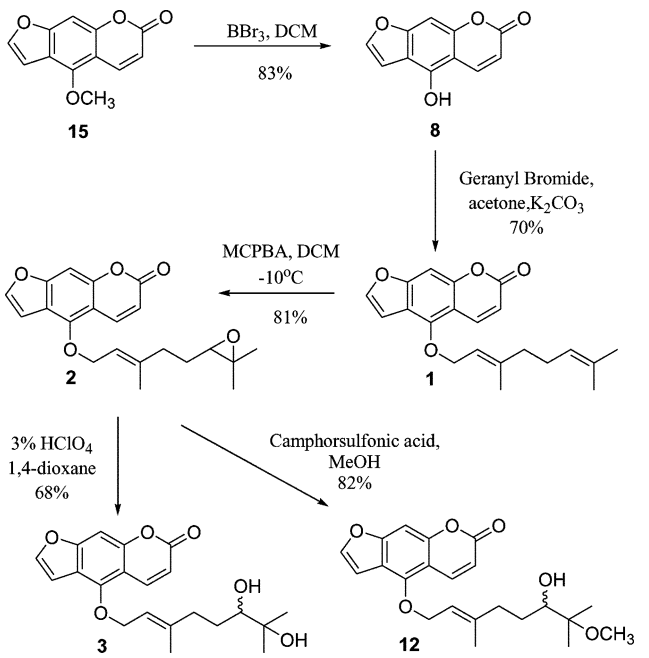
Synthetic aspects

Coumarin compounds. The three coumarin compounds [**5–7**] (Fig. 3) were synthesised by the alkylation of 7-hydroxycoumarin [**4**] with benzyl bromide, geranyl bromide or 4-bromo-2-methyl-2-butene. The reactions were performed under basic conditions in acetone to give the desired compounds in good yields (Scheme 1).



Scheme 1 Alkylation of 7-hydroxycoumarin under basic conditions.

Furanocoumarin compounds. Furanocoumarin monomers (Fig. 3) were prepared using the methods outlined in Schemes 2 and 3, where the initial step was the deprotection of the commercially available starting material bergapten [**15**]. Treatment with



Scheme 2 Synthetic routes employed in the synthesis of bergamottin and analogues from bergapten.

boron tribromide (BBr_3) in dichloromethane (DCM)[7] afforded the phenolic derivative bergaptol [**8**] in good yields. Alkylation under conditions previously employed for the coumarins, with either geranyl bromide or 4-bromo-2-methyl-2-butene resulted in the desired compounds bergamottin [**1**] and isoimperatorin [**9**]. Synthesis of the epoxide derivatives **2** and **10** was achieved by the treatment of **1** and **9** with MCPBA in DCM.[8] In the case of bergamottin [**1**], the reaction was performed at −10 °C, resulting in

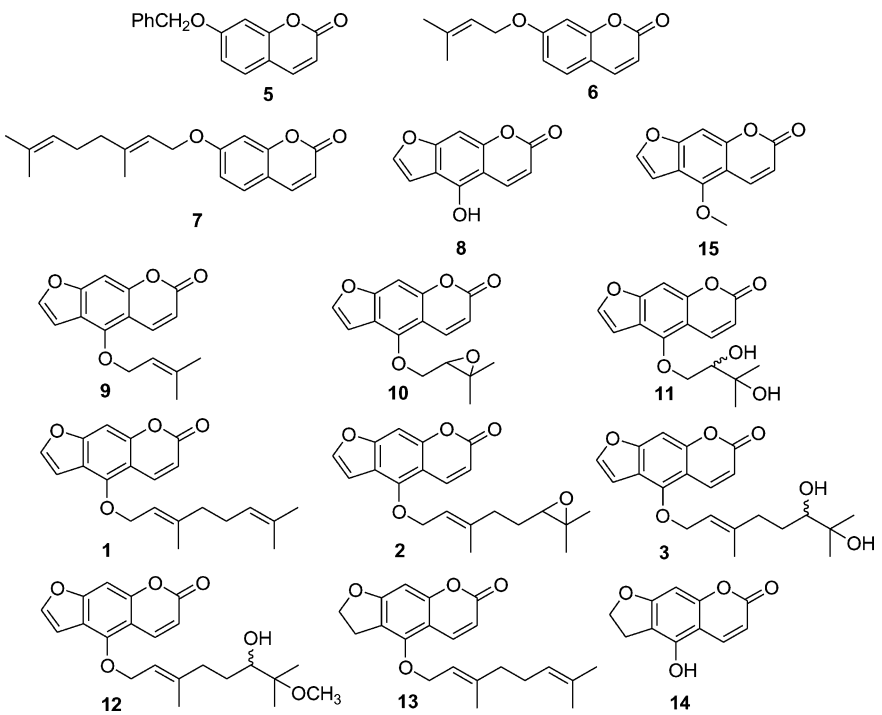
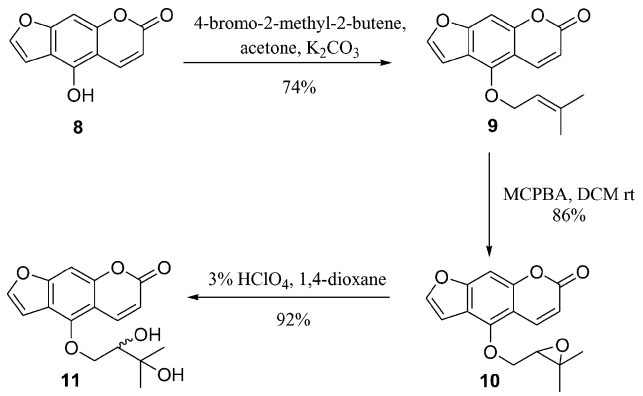


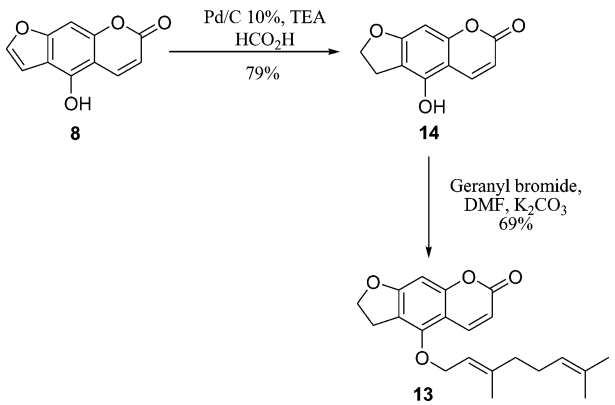
Fig. 3 The coumarin and furanocoumarin analogues that were synthesised.



Scheme 3 Synthetic route employed in the synthesis of isoimperatorin and analogues from bergapten.

the selective epoxidation of the terminal double bond. Generally epoxides are readily opened by nucleophiles under both acidic and basic conditions. This allowed the use of the epoxide intermediates as a diversification point, theoretically enabling the introduction of a variety of functional groups. Subsequent treatment of both epoxide derivatives [**2** and **10**] with dilute perchloric acid resulted in the ring opening of the epoxide to give the dihydroxylated products **3** and **11**.[8] 6′-Hydroxy-7′-methoxybergamottin [**12**] was synthesised by acid catalysed ring opening of the epoxide [**2**] in methanol affording the ether substituent at the more substituted position as determined by ^{1}H NMR.

The two 2,3-dihydro derivatives were prepared from bergaptol [**8**]. Reduction under transfer hydrogenation conditions with Pd/C, formic acid and triethylamine afforded the phenolic intermediate [**14**], and subsequent alkylation resulted in the dihydrobergamottin derivative [**13**] (Scheme 4).



Scheme 4 Synthesis of the 2,3-dihydro derivatives **14** and **13**.

Inhibition of CYP3A4 activity by synthetic coumarins and furanocoumarins. The three coumarin derivatives containing either the benzyloxy [**5**], prenyloxy [**6**] or geranyloxy [**7**] side chains were found to be weak inhibitors of testosterone 6β-hydroxylation in human liver microsomes. IC_{50} values were greater than 100 μM, the highest inhibitor concentration used. The maximum inhibition of CYP3A4 activity was 15%, 46% and 45% for the benzyloxy [**5**], prenyloxy [**6**] and geranyloxy [**7**] derivatives, respectively. A range of furanocoumarin monomers based on the structure of bergamottin were investigated as inhibitors of CYP3A4 activity

Table 1 IC_{50} values for the inhibition of CYP3A4 activity in human liver and intestine by furanocoumarin derivatives. Data are means ± s.d. from triplicate experiments. No results (NR) were obtained when the test compounds co-eluted with the 6β-hydroxytestosterone peak

Compound number	HL7 IC_{50}/μM	Intestine S9 IC_{50}/μM
12	0.13 ± 0.03	0.06 ± 0.01
2	0.33 ± 0.04	0.22 ± 0.01
3	0.65 ± 0.07	0.64 ± 0.01
15	1.52 ± 0.18	NR
9	1.89 ± 0.26	0.46 ± 0.09
11	3.34 ± 0.10	3.23 ± 0.19
1	4.48 ± 0.42	3.11 ± 0.90
10	5.18 ± 0.22	1.33 ± 0.17
13	49.3 ± 1.9	NR
14	>100	NR
8	NR	NR

in both human liver microsomes (HL7) and human intestinal S9. With the exception of the phenolic derivatives [**8** and **14**], all furanocoumarins showed dose-dependent inhibitory effects on testosterone 6β-hydroxylation (Table 1). The most potent inhibitor evaluated was the hydroxy-ether derivative [**12**]. The introduction of more polar substituents was found to increase the inhibitory potency by approximately one order of magnitude within the geranyloxy series [**1–3** and **12**]. In general the latter were found to be more potent inhibitors than the prenyloxy series [**9–11**]. Bergamottin [**1**] showed a 10-fold increase in inhibition potency, compared to its saturated counterpart [**13**], indicating an important interaction at the furan site.

Three furanocoumarins, bergamottin [**1**], 6′,7′-EB [**2**] and 6′,7′-DHB [**3**] were evaluated as time-dependent inhibitors of CYP3A4 activity. The effects of preincubation compared to co-incubation of these compounds on 6β-hydroxytestosterone formation in human liver microsomes (HL7) and human intestinal S9 by furanocoumarins are shown in Table 2.

Preincubation with bergamottin showed the greatest increase in potency with IC_{50} values decreasing by a mean of 14-fold for both liver and intestine. The potencies of 6′,7′-EB [**2**] and 6′,7′-DHB [**3**] increased by 2- and 4-fold, respectively, when these compounds were preincubated.

During the course of this study Guo *et al.*[9] published data on the effects of compounds **1–3**, **9–11** and **15** on human liver microsomal CYP3A4. The results obtained in the present study broadly confirm those of Guo *et al.*[9] We then extended the work to include an investigation of the effects of these and the remaining newly synthesised coumarins **5–8** and **12–14** on human intestinal CYP3A4 activity.

The weak inhibition of CYP3A4 activity observed with coumarins **4**, **5** and **6** suggests that the lactone portion of the tricyclic ring system plays little or no role in the binding of these compounds to the active site and that an intact furan moiety is essential for the interaction with the enzyme.

By synthesising a number of analogues with differing functionality, the interaction of the furanocoumarin structure with the active site of CYP3A4 was investigated. In the alkylated series the order of inhibitory potency was 5-OMe [**15**] > isoimperatorin [**9**] > bergamottin [**1**]. This indicates that an increase in alkyl chain length is associated with decreased CYP3A4 inhibition, which suggests that as the chain length increases, the furanocoumarin is interacting with a more hydrophilic region of the binding site. The

Table 2 IC_{50} values for the inhibition of CYP3A4 activity obtained for the co-incubation and preincubation of furanocoumarin monomers with testosterone in human liver (HL7) and intestine. Data are means ± s.d. of triplicate incubations

Inhibitor	Co-incubated HL7 $IC_{50}/\mu M$	Preincubated HL7 $IC_{50}/\mu M$	Co-incubated intestine S9 $IC_{50}/\mu M$	Preincubated intestine S9 $IC_{50}/\mu M$
1	3.92 ± 0.14	0.24 ± 0.01	3.76 ± 0.85	0.31 ± 0.03
2	0.32 ± 0.04	0.11 ± 0.01	0.18 ± 0.02	0.08 ± 0.01
3	0.65 ± 0.07	0.13 ± 0.01	0.64 ± 0.01	0.21 ± 0.02

resulting decreased binding affinity is because alkane and alkene substituents interact by van der Waals forces. The hypothesis that the geranyloxy side chain is interacting with a more hydrophilic region of the binding site is supported by an increase in inhibitory potency when more polar substituents are introduced at the 6′,7′-position. The epoxy derivative [**2**] seems to be a slightly more potent inhibitor than 6′,7′-DHB [**3**]. Epoxides have the potential to react with nucleophilic amino acid residues in the active site, resulting in covalent binding to the enzyme.[10] This may explain the increased potency of inhibition of CYP3A4 compared to the diol derivative [**3**], which will interact with the binding site through weaker hydrogen bonding. However, it is possible that the epoxide may be converted to the corresponding diol before binding to the active site.

To evaluate the effect of hydrogen bonding at the binding site, an ether linkage was introduced to replace one of the hydroxyl groups. This change resulted in a 5-fold increase in the potency of inhibition of CYP3A4. To determine whether hydroxyl groups are involved in ligand binding, they are often converted to ethers, which prevents the former acting as a hydrogen bond donor. Depending on its size, an ether group can also interfere with the ability to act as a hydrogen bond acceptor, as the increased bulk of the ether substituent may sterically hinder this interaction. If a hydroxyl group is involved, through hydrogen bonding, in the interaction with the active site, a decrease in CYP3A4 inhibition by the ether derivative would be expected. However, an increase in potency was observed, suggesting that the ether group is now interacting with a more hydrophobic region of the active site.

In the case of the prenyloxy derivatives [**9–11**] a decrease in potency of CYP3A4 inhibition was observed as more polar substituents were introduced at the 2′,3′-position on the side chain, in the rank order isoimperatorin [**9**] > oxypeucedanin hydrate [**11**] > oxypeucedanin [**10**]. This decrease in potency supports the proposal that the prenyloxy side chain binds to a more hydrophobic region of the active site of CYP3A4.

To explore these structure–activity considerations in more detail, we applied two pharmacophore models to the structure of bergamottin, and in both cases a good fit was obtained. The furanocoumarin ring system can interact with three of the four binding regions suggested by Ekins *et al.*,[11] with the third hydrophobe (Fig. 4A) or hydrogen bond donor region lying immediately to the right of the ring system. Bergamottin [**1**] showed a good fit with the two proposed hydrophobic regions situated near the central aromatic ring, and the alkyl chain with the hydrogen bond acceptor region positioned around the carbonyl group of the lactone. Incorporation of these compounds into the model suggested for quinine (Fig. 4B) indicated a good fit between the two proposed hydrophilic regions on the active site and the oxygen atoms within the lactone portion of the ring system. As with the other model, the alkyl linkage lies within a hydrophobic region.

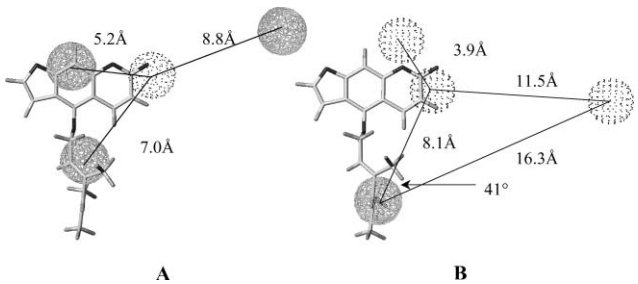


Fig. 4 Bergamottin fitted to the CYP3A4 Catalyst K_i pharmacophore produced by Ekins *et al.*[11] from a published data set derived from (**A**) the inhibition of midazolam 1-hydroxylation and (**B**) the inhibition of quinine metabolism. The pharmacophore consists of hydrophobic regions (wire frame) and hydrogen bond acceptor regions (dots). The inter-bond angles and distances between the pharmacophore features are inset.

The requirement of an alkyl chain for binding to the enzyme is supported by the good fit of bergamottin to the two pharmacophore models. One common structural feature within the models appears to be the presence of a hydrophobic binding region along the alkyl chain. The data obtained suggest that the presence of a hydrophobic substituent on the phenolic oxygen in this region is essential for binding possibly through van der Waals forces and may affect the orientation of the ring system. The integration of these monomers into the pharmacophore models has given a tentative insight into the binding of the furanocoumarin ring system to the active site of CYP3A4.

As well as confirming the work of others[5,6]—that furanocoumarin monomers cause time-dependent inhibition of human liver CYP3A4—we have also demonstrated that these compounds are active in the intestine, which is believed to be the site of the grapefruit juice interaction.[5] The preincubation of furanocoumarin monomers with human liver microsomes and human intestinal S9 fraction had variable effects on the potency of inhibition when compared to co-incubation. Preincubation with bergamottin [**1**] showed the greatest increase in potency, with IC_{50} values decreasing by 14-fold for liver and intestine. IC_{50} values for 6′,7′-EB [**2**] and 6′,7′-DHB [**3**] decreased by a mean of 2- and 4-fold, respectively. After preincubation 6′,7′-EB [**2**] and 6′,7′-DHB [**3**] abolished CYP3A4 activity at a concentration of 5 μM.

The extent of CYP3A4 inhibition by the compounds tested was comparable in the liver and the intestine, which is line with the finding that the amino acid sequences of enteric and hepatic CYP3A4 are identical.[12]

Conclusions

Reliable and reproducible synthetic routes have been developed for the synthesis of 7-alkyloxy-coumarins and substituted furanocoumarin analogues. This has allowed the investigation of

the relationship between furanocoumarin structure and inhibition of CYP3A4 activity. In total, three coumarin and ten furanocoumarin monomers were synthesised and tested *in vitro*. With the exception of the coumarin and phenolic furanocoumarin analogues, which were inactive, all the compounds synthesised inhibited CYP3A4 activity with varying degrees of potency when using testosterone as the marker substrate. The lack of interaction observed with the coumarin and phenolic furanocoumarin analogue indicates that the presence of both a furan ring and an alkyl substituent at the 5-position on the central aromatic ring are essential for the interaction with CYP3A4. The inhibitory potency of the furanocoumarin monomers was enhanced by the incorporation of geranyloxy derived moieties with hydrophilic groups at the 6′,7′-position. This suggests that these compounds interact with both lipophilic and hydrophilic regions within the enzyme active site.

Experimental

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 250 or Bruker AMX1-400 instruments. Chemical shifts (δ_H, δ_C) are reported in ppm and coupling constants (J) are in Hertz (Hz). Infrared spectra were determined by direct sample analysis using a Bruker Goldengate ATR Vector 22 Spectrometer and are reported by wave numbers (cm^{-1}). Electron Impact (EI) and Chemical Ionisation (CI) mass spectrometry were carried out on a Micromass Prospec magnetic sector instrument or a Kratos Concept 1S instrument. Melting points were determined on a hot stage microscope apparatus, and are quoted uncorrected in °C. Thin layer chromatography (TLC) was carried out on aluminium backed Merck Kiesgel plates, with detection by UV (254 nm) fluorescence, or ammonium molybdate or potassium permanganate dips. Chromatography was carried out using Merck Silica gel 60 (<63 micron) or Fisher Matrex (35–70 micron).

Chemicals

All chemicals were of analytical grade or of a higher purity and were purchased from Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK), Fisher Scientific (Loughborough, UK), Roche Diagnostics Ltd (Lewes, UK), VWR International Ltd (Poole, UK), or were donated by Ultrafine (Manchester, UK).

Synthetic procedures

7-Benzyloxychromen-2-one [5]. Benzyl bromide (1.31 mL, 11 mmol) was added to a stirred mixture of 7-hydroxycoumarin [**4**] (1.62 g, 10 mmol) and potassium carbonate (2.07 g, 14.9 mmol) in acetone (20 mL). The reaction mixture was heated under reflux until TLC analysis confirmed that the reaction was complete. Aqueous citric acid (5% w/v) was added until the potassium carbonate was neutralised and the mixture was extracted with dichloromethane (2 × 20 mL). The combined organic extracts were washed with water and brine, dried ($MgSO_4$), and the solvent was removed under reduced pressure. The title compound [**5**] was obtained as colourless crystals (2.05 g, 8.13 mmol, 82%). Mp 153–155 °C (lit.,[13] 155–156 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.65 (d, 1H, J = 9.5 Hz), 7.47–7.33 (m, 6H), 6.96–6.89 (m, 2H), 6.27 (d, 1H, J = 9.5 Hz), 5.14 (s, 2H); m/z (CI) 253 (100%, $[M + H]^+$).

Compounds **6** and **7** were prepared similarly.

7 - (3 - Methylbut - 2 - enyloxy)chromen - 2 - one [6]. (2.17 g, 9.43 mmol, 94%). Mp 77–78 °C (lit.,[14] 66–68 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.65 (d, 1H, J = 9.5 Hz), 7.38 (d, 1H, J = 8.4 Hz), 6.88–6.82 (m, 2H), 6.25 (d, 1H, J = 9.5 Hz), 5.52–5.45 (m, 1H), 4.58 (d, 2H, J = 6.7 Hz), 1.82 (s, 3H), 1.78 (s, 3H); m/z (CI) 231 (100%, $[M + H]^+$).

7-(3′,7′-Dimethylocta-2,6-dienyloxy)chromen-2-one [7]. (1.30 g, 4.36 mmol, 44%). Mp 64–65 °C (lit.,[15] 66–67 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.64 (d, 1H, J = 9.5 Hz), 7.37 (d, 1H, J = 8.3 Hz), 6.87–6.82 (m, 2H), 6.25 (d, 1H, J = 9.5 Hz), 5.47 (t, 1H, J = 6.5 Hz), 5.10–5.05 (m, 1H), 4.60 (d, 2H, J = 6.5 Hz), 2.01 (m, 4H), 1.76 (s, 3H), 1.66 (s, 3H), 1.60 (s, 3H); m/z (CI) 299 (84%, $[M + H]^+$).

4-Hydroxy-7*H*-furo-[3,2-*g*]chromen-7-one [8]. To a solution of bergapten [**15**] (500 mg, 2.31 mmol) in dichloromethane (20 mL) BBr_3 (20 mL, 20 mmol, 1 M in DCM) was slowly added and the solution stirred under argon at room temperature. After 2 hours the mixture was poured slowly into a solution of saturated sodium bicarbonate (100 mL) resulting in the precipitation of a grey solid. After 30 minutes the product was recovered by filtration, washed with cold water and ether and was dried under a high vacuum, yielding the title compound [**8**] as an off-white solid (387 mg, 1.92 mmol, 83%). Mp 283–284 °C (lit.,[16] 276 °C); ^{1}H NMR (d_6-acetone, 250 MHz): δ 8.28 (d, 1H, J = 9.8 Hz), 7.82 (d, 1H, J = 2.3 Hz), 7.17 (d, 1H, J = 2.3 Hz), 7.06 (s, 1H), 6.23 (d, 1H, J = 9.8 Hz); m/z (EI) 202 (100%, M^+).

4-(3,7-Dimethylocta-2,6-dienyloxy)-furo[3,2-*g*]chromen-7-one (bergamottin) [1]. Geranyl bromide (240 μL, 1.2 mmol) was added dropwise to a stirred mixture of bergaptol [**8**] (242 mg, 1.2 mmol) and potassium carbonate (248 mg, 1.8 mmol) in acetone (20 mL) and heated under reflux for 1 hour. Aqueous citric acid (5% w/v) was added to neutralise the potassium carbonate and the solution was extracted with DCM (2 × 20 mL). The combined organic layers were washed with water and brine and were dried ($MgSO_4$). Removal of the solvent under reduced pressure furnished a pale yellow oil, which was purified by column chromatography eluting with ethyl acetate–hexane (1 : 4). Removal of the solvent under reduced pressure afforded a translucent yellow oil, which crystallised on trituration and cooling with hexane. Recovery by filtration afforded colourless crystals of the title compound [**1**] (283 mg, 0.84 mmol, 70%). Mp 55–56 °C (lit.,[17] 56–58 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.18 (d, 1H, J = 9.8 Hz), 7.61 (d, 1H, J = 2.1 Hz), 7.16 (s, 1H), 6.97 (d, 1H, J = 2.1 Hz), 6.28 (d, 1H, J = 9.8 Hz), 5.54 (t, 1H, J = 6.5 Hz), 5.07 (bs, 1H), 4.96 (d, 2H, J = 6.5 Hz), 2.10 (s, 4H), 1.69 (s, 6H), 1.60 (s, 3H); m/z (EI) 338 (17%, M^+), 202 (100%).

4-(3-Methylbut-2-enyloxy)-furo[3,2-*g*]chromen-7-one [9]. This compound was prepared using the procedure outlined above. (305 mg, 1.13 mmol, 74%). Mp 108–109 °C (lit.,[16] 108 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.18 (d, 1H, J = 9.9 Hz), 7.60 (d, 1H, J = 2.4 Hz), 7.19 (s, 1H), 6.97 (d, 1H, J = 2.4 Hz), 6.28 (d, 1H, J = 9.8 Hz), 5.54 (t, 1H, J = 6.0 Hz), 4.92 (d, 2H,

J = 6.5 Hz), 1.81 (s, 3H), 1.73 (s, 3H); m/z (EI) 270 (4%, M^+), 202 (100%).

4-[5-(3,3-Dimethyloxiranyl)-3-methylpent-2-enyloxy]-furo[3,2-*g*]chromen-7-one [2]. 3-Chloroperoxybenzoic acid (180 mg, 1.04 mmol) was added to a stirred solution of bergamottin [**1**] (250 mg, 0.74 mmol) in DCM (10 mL). The solution was stirred at −10 °C for 2 hours 30 minutes. The organic layer was washed with aqueous sodium sulfite (10% w/v) and sodium carbonate (5% w/v) (20 mL, 1 : 1), was dried and the solvent was evaporated under reduced pressure. The title compound was obtained by column chromatography, eluting with ethyl acetate–hexane (4 : 1). Removal of the solvent under reduced pressure furnished a translucent oil, which crystallised on trituration and cooling with hexane. Recovery by filtration afforded colourless crystals (212 mg, 0.60 mmol, 81%). Mp 69–70 °C (lit.,[17] 67–70 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.17 (d, 1H, J = 9.8 Hz), 7.61 (d, 1H, J = 2.3 Hz), 7.17 (s, 1H), 6.96 (d, 1H, J = 2.3 Hz), 6.28 (d, 1H, J = 9.8 Hz), 5.61 (t, 1H, J = 6.4 Hz), 4.97 (d, 2H, J = 6.4 Hz), 2.73–2.71 (m, 1H), 2.26–2.22 (m, 2H), 1.71 (s, 3H), 1.70–1.61 (m, 2H), 1.32 (s, 3H), 1.28 (s, 3H); m/z (EI) 354 (23%, M^+), 202 (100%).

4-(3,3-Dimethyloxiranylmethoxy)-furo[3,2-*g*]chromen-7-one [10]. 3-Chloroperoxybenzoic acid (160 mg, 0.93 mmol) was added to a stirred solution of **9** (250 mg, 0.92 mmol) in dichloromethane (10 mL) at −10 °C in an ice–methanol bath. After 2 hours 30 minutes the temperature was allowed to rise to room temperature and the reaction was stirred for a further hour. The solution was washed with aqueous sodium sulfite (10% w/v) and sodium carbonate (5% w/v) (20 mL, 1:1). Evaporation of the organic layer under reduced pressure yielded yellow crystals, which were purified by column chromatography eluting with ethyl acetate–hexane (3 : 7). The relevant fractions were combined and removal of the solvent under reduced pressure afforded pale yellow crystals (228 mg, 0.80 mmol, 86%). Mp 141–142 °C (lit.,[16] 142–143 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.23 (d, 1H, J = 9.8 Hz), 7.64 (d, 1H, J = 2.3 Hz), 7.23 (s, 1H), 6.97 (d, 1H, J = 2.3 Hz), 6.36 (d, 1H, J = 9.8 Hz), 4.63 (dd, 1H, J = 4.4, 10.8 Hz), 4.46 (dd, 1H, J = 6.5, 10.8 Hz), 3.26 (dd, 1H, J = 4.4, 6.5 Hz), 1.43 (s, 3H), 1.35 (s, 3H); m/z (CI) 287 (100%, $[M + H]^+$).

4-(6,7-Dihydroxy-3,7-dimethylocta-2-enyloxy)-furo[3,2-*g*]-chromen-7-one (6′,7′-DHB) [3]. An aqueous solution of perchloric acid (9 mL, 3% v/v) was added dropwise to a stirred solution of **2** (140 mg, 0.40 mmol) in 1,4-dioxane (15 mL) under argon at room temperature. After 1 hour water (10 mL) was added and the solution was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with water (30 mL) and dried ($MgSO_4$). Removal of the solvent under reduced pressure yielded the desired compound[3] as small colourless needles (100 mg, 0.27 mmol, 68%). Mp 112–113 °C (lit.,[17] 104–107 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.16 (d, 1H, J = 9.8 Hz), 7.60 (d, 1H, J = 2.4 Hz), 7.16 (s, 1H), 6.96 (d, 1H, J = 2.4 Hz), 6.28 (d, 1H, J = 9.8 Hz), 5.60 (t, 1H, J = 6.7 Hz), 4.96 (d, 2H, J = 6.7 Hz), 3.33 (d, 1H, J = 10.4 Hz), 2.38–2.16 (m, 3H), 1.93 (bs, 1H), 1.67 (s, 3H), 1.66–1.42 (m, 2H), 1.33 (s, 3H), 1.26 (s, 3H); m/z (EI) 372 (6%, M^+), 202 (100%).

4-(2,3-Dihydroxy-3-methylbutoxy)-furo[3,2-*g*]chromen-7-one (oxypeucedanin hydrate) [11]. This compound was prepared as outlined in the previous procedure. (78 mg, 0.26 mmol, 92%). Mp 129–131 °C (lit.,[18] 129–132 °C); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.19 (d, 1H, J = 9.8 Hz), 7.62 (d, 1H, J = 2.4 Hz), 7.16 (s, 1H), 7.01 (d, 1H, J = 2.4 Hz), 6.29 (d, 1H, J = 9.8 Hz), 4.56 (dd, 1H, J = 3.1, 9.8 Hz), 4.46 (dd, 1H, J = 7.7, 9.8 Hz), 3.93 (t, 1H, J = 3.7 Hz), 2.97 (bd, 1H, J = 3.7 Hz), 2.26 (s, 1H), 1.38 (s, 3H), 1.34 (s, 3H). Following D_2O exchange, the peaks at 2.97 and 2.26 ppm disappeared and the bt at 3.97 ppm became a dd at 3.93 ppm (J = 3.1 Hz, 7.6 Hz); m/z (EI) 304 (20%, M^+).

4-(6-Hydroxy-7-methoxy-3,7-dimethyloct-2-enyloxy)-furo[3,2-*g*]chromen-7-one (6′-OH-7′-OMeB) [12]. (1*R*)-(−)-10-Camphorsulfonic acid (66 mg, 0.28 mmol) was added to a stirred solution of **2** (100 mg, 0.28 mmol) in methanol (5 mL) under argon. On completion of the reaction (TLC) a few drops of saturated aqueous sodium bicarbonate solution were added and the methanol was evaporated under reduced pressure. The residual oil was dissolved in DCM (20 mL), then the organic layer washed with water (2 × 20 mL) and dried ($MgSO_4$). Removal of the solvent yielded a translucent oil, which upon trituration with ether–hexane (1 : 1) furnished colourless crystals (89 mg, 0.23 mmol, 82%). Mp 67–68 °C; ν_{max} cm^{-1} 3581, 1731, 1622; ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.17 (d, 1H, J = 9.8 Hz), 7.60 (d, 1H, J = 2.3 Hz), 7.18 (s, 1H), 6.98 (d, 1H, J = 2.3 Hz), 6.27 (d, 1H, J = 9.8 Hz), 5.59 (t, 1H, J = 6.7 Hz), 4.94 (d, 2H, J = 6.7 Hz), 3.36 (bd, 1H, J = 9.8 Hz), 3.24 (s, 3H), 2.50 (bs, 1H), 2.45–2.33 (m, 1H), 2.20–2.08 (m, 1H), 1.71 (s, 3H), 1.56–1.41 (m, 2H), 1.10 (s, 6H). Following D_2O exchange the bs at 2.50 ppm disappeared and the bd at 3.36 ppm became a dd at 3.36 ppm (J = 2.0 Hz, 10.2 Hz); ^{13}C NMR ($CDCl_3$, 63 MHz): δ 159.7, 156.4, 151.0, 147.2, 143.3, 141.5, 138.0, 117.4, 112.6, 110.9, 105.9, 103.4, 92.6, 75.7, 74.6, 68.0, 35.0, 27.5, 19.0, 17.0, 15.1; m/z (EI) 386 (5%, M^+), 202 (74%); found (EI) 386.1736; $C_{22}H_{26}O_6$ requires 386.1729; found C 68.40%, H 6.83%; $C_{22}H_{26}O_6$ requires C 68.38%, H 6.78%.

4-Hydroxy-2,3-dihydrofuro[3,2-*g*]chromen-7-one (5-DHOH) [14]. Bergaptol [**8**] (350 mg, 1.73 mmol) was dissolved in acetone (120 mL) at 40 °C under argon. Pd/C (10%, 700 mg), formic acid (653 μL, 17.3 mmol) and triethylamine (2.90 mL, 20.8 mmol) were added to the solution. After 10 minutes ^{1}H NMR indicated that the reaction was complete. The solution was filtered through celite and the solvent was removed under reduced pressure, affording a light green oil, which solidified on cooling. The solid was dissolved in acetone and pre-absorbed onto silica. Purification by column chromatography furnished the desired compound after elution with ethyl acetate–hexane (3 : 7) increasing to ethyl acetate (100%). The relevant fractions were combined and the solvent was removed under reduced pressure, yielding an off-white solid (280 mg, 1.37 mmol, 79%). Mp 200–202 °C; ν_{max} cm^{-1} 3303, 1669, 1623; ^{1}H NMR (d_6-acetone, 250 MHz): δ 7.92 (d, 1H, J = 9.7 Hz), 6.13 (s, 1H), 5.92 (d, 1H, J = 9.7 Hz), 4.54 (t, 2H, J = 8.6 Hz), 3.10 (t, 2H, J = 8.6 Hz); ^{13}C NMR (d_6-DMSO, 63 MHz): δ 164.5, 160.8, 156.3, 150.6, 139.8, 108.9, 108.8, 103.4, 89.7, 72.7, 26.5; m/z (EI) 204 (100%, M^+); found (EI) 204.0421; $C_{11}H_8O_4$ requires 204.0423; found C 64.81%, H 3.91%; $C_{11}H_8O_4$ requires C 64.71%, H 3.95%.

4-(3,7-Dimethylocta-2,6-dienyloxy)-2,3-dihydrofuro[3,2-*g*]-chromen-7-one (5-DHB) [13]. Preparation as outlined for compound **1**. (115 mg, 0.33 mmol, 69%). Mp 61–62 °C; ν_{max} cm^{-1} 1713, 1620; ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.58 (d, 1H, J = 9.7 Hz),

6.50 (s, 1H), 6.15 (d, 1H, $J = 9.7$ Hz), 5.48 (t, 1H, $J = 6.8$ Hz), 5.09 (bs, 1H), 4.68 (m, 4H), 3.39 (t, 2H, $J = 8.5$ Hz), 2.19 (bs, 4H), 2.10 (s, 3H), 1.70 (s, 3H), 1.61 (s, 3H); ^{13}C NMR ($CDCl_3$, 63 MHz): δ 163.4, 160.0, 154.8, 150.3, 141.1, 137.8, 130.4, 121.8, 117.3, 110.4, 108.9, 105.2, 91.7, 70.8, 67.2, 37.8, 26.4, 24.6, 24.0, 16.1, 15.0; *m/z* (CI) 341 (18%, $[M + H]^+$); found (CI) 341.1750; $C_{21}H_{25}O_4$ requires 341.1675; found C 73.92%, H 7.08%; $C_{21}H_{25}O_4$ requires C 74.09%, H 7.11%.

Source and preparation of tissue samples

Human liver tissue (coded HL7) was obtained with written consent from a patient undergoing surgery for the removal of a hepatocellular tumour secondary to colon cancer. Macroscopically normal tissue close to the resection line was used. Samples of upper intestine (duodenum and jejunum) were obtained with written consent from patients undergoing total gastrectomy. These studies were approved by the appropriate Hospital Ethics Committee. Human liver microsomes and intestinal supernatant S9 fractions were prepared as described previously.[19,20] Intestinal S9 samples were pooled from five patients due to limited tissue availability.

Incubation conditions

Human liver microsomes and intestinal S9 (0.2 mg protein mL^{-1}) were incubated with testosterone (37 μM) and the test compounds (0.5–100 μM) at 37 °C in the presence of KCl (1.15%), phosphate buffer (0.2 M, pH 7.4) and an NADPH generating system, for 10 min in a total volume of 1 mL. Preincubation studies were undertaken employing the same conditions, but the test compounds were incubated with intestinal S9 and liver microsomes for 15 min prior to the addition of testosterone. After 10 minutes the reaction was terminated by the addition of ethyl acetate (2 mL). 16-α-Hydroxytestosterone (1.5 μg) or 11-β-hydroxytestosterone (1 μg) was added as the internal standard. Samples were gently mixed for 15 minutes, before centrifugation at 1500 g for 15 minutes. The organic layer was evaporated to dryness under reduced pressure.

Determination of 6β-hydroxytestosterone by HPLC

Sample residues were reconstituted with the mobile phase (150 μL) and an aliquot was injected onto the HPLC. A Hypersil C_8 BDS column (150 mm × 4.6 mm; 5 μm particle size) was used. The mobile phase was methanol–water (55 : 45 v/v) delivered at a flow rate of 1 mL min^{-1}. Eluants were detected by UV at 254 nm. The lower limit of determination of the assay was 30 pmol mL^{-1}, and the coefficient of variation at 328 pmol mL^{-1} was less than 5%.

Molecular modelling

The models used were derived from the two Catalyst hypotheses generated by Ekins *et al.*[11] and based on the inhibition of midazolam 1-hydroxylation and of quinine metabolism. Alignments were performed by manual fitting of the hydrogen bond acceptor and the hydrophobic regions to bergamottin [**1**] using Sybyl (v 7.0) software.

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