Simplified Silvestrol Analogues with Potent Cytotoxic Activity

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ABSTRACT

The complex natural products silvestrol (**1**) and episilvestrol (**2**) are inhibitors of translation initiation through binding to the DEAD-box helicase eukaryotic initiation factor 4A (eIF4A). Both compounds are potently cytotoxic to cancer cells in vitro and silvestrol (**1**) has demonstrated efficacy in vivo in several xenograft cancer models. Here we show that episilvestrol (**2**) has limited plasma membrane permeability and is metabolized in liver microsomes in a manner consistent with that reported for silvestrol (**1**). In addition we have prepared a series of analogues of these compounds where the complex pseudo-sugar at C6 has been replaced with chemically simpler moieties to improve drug-likeness. Selected compounds from this work possess excellent activity in biochemical and cellular translation assays with potent activity against leukemia cell lines.

INTRODUCTION

The cyclopenta[*b*]benzofuran family of natural products, exemplified by silvestrol (**1**) and rocaglamide (**4**) (Figure 1), possess potent cytotoxicity against a range of cancer cell lines.[1-3] Isolated from plants of the *Aglaia* genus and known variously as flavaglines or aglains, their complex stereochemically dense structure has lead to numerous synthetic studies and total syntheses.[4,5] The most potent compound of this class, silvestrol (**1**), has been shown to inhibit the initiation phase of translation through binding to the DEAD-box helicase eIF4A, in turn disrupting formation of the protein complex eIF4F.[6] A consequence of protein synthesis inhibition by silvestrol (**1**) is most notably apparent in a rapid reduction in levels of short-lived proteins such as the pro-survival protein Mcl-1. The loss of Mcl-1 and related proteins has been postulated as the primary mechanism of cell death elicited by silvestrol,[7] although more recent studies have demonstrated that multiple mechanisms of cytotoxicity are induced upon compound treatment.[8,9] A variety of molecular mechanisms underpinning the cytotoxicity of rocaglamide and analogues have also been identified [10-13]

Silvestrol (**1**) has been reported to be particularly potent against leukemia cell lines[7] and has demonstrated activity in vivo in solid tumor xenograft studies both alone and in combination with doxorubicin.[6,14] Despite this promising anti-cancer activity and unusual mechanism of action, there are limited structure-activity studies on silvestrol (**1**), with most focusing on the functionally less active, and structurally simpler, rocaglates.[3,15] The naturally occurring episilvestrol (**2**), which is the C-5''' epimer of silvestrol, is comparable to silvestrol (**1**) in a range of cytotoxicity and protein translation assays, whereas 2''',5'''-diepisilvestrol **4** is significantly weaker.[16-19] These data indicate that the dioxanyloxy pseudo-sugar moiety[20] of silvestrol (**1**) has a significant beneficial impact on the activity of **1** and **2** presumably through discrete binding interactions with their molecular target(s).

In this paper we disclose our preliminary SAR studies on changes to the pseudo-sugar moiety of silvestrol (**1**) to improve drug-likeness and ease of synthesis. Several compounds potently inhibit protein synthesis and display promising activity against a panel of human leukemia cell lines.

RESULTS

Stability and permeability studies with episilvestrol (2). Published cytotoxicity data for silvestrol (**1**) and episilvestrol (**2**) clearly indicate that these compounds are more potent (IC_{50} < 1 nM) than compounds where the dioxanyloxy group is absent or replaced with a methyl ether (IC_{50} 10-100 nM),[16] confirmed by our own data (vide infra). Nonetheless, the presence of the dioxanyloxy moiety reduces the compounds' drug-likeness considerably by contributing to the high molecular weight and polar surface area (PSA), as well as increasing its structural complexity. In addition, the presence of two acetal moieties in the dioxanyloxy groups of **1** and **2** could lead to chemical instability whilst the high polarity of the group would be expected to limit cellular penetration of the compounds.

We have found episilvestrol (**2**) to be stable in pH 3, 5 and 7 isotonic aqueous buffers, and in simulated gastric fluid, over 24 h at 37°C, with no appreciable acetal or ester hydrolysis apparent over this timeframe (see Supporting Information). This stability to acidic conditions is in contrast to metabolic stability, which was assessed using human and mouse liver microsomes, where episilvestrol exhibited a moderate rate of clearance (14-18 µL/min/mg protein; **Table 1**). In these experiments the compound was shown to be metabolized to the acid (**5**) (Figure 2) in the absence of cofactors with both preparations, suggesting contribution of non-NADPH mediated metabolism to the overall degradation rate, presumably via microsomal esterase activity. Importantly, no cleavage of the dioxanyloxy group under these conditions was observed (Table 1).

To assess the effect the dioxanyloxy moiety has on cellular penetration we used PAMPA (Parallel Artificial Membrane Permeability Assay)[21] to measure the passive permeability of

episilvestrol (**2**) across a lipid membrane. No compound diffusion across the artificial membrane could be detected over 4 h by our analytical methods, whilst controls performed as expected; however, a significant loss of material from the donor chamber was apparent (see Supporting Information). As stability control samples indicated that episilvestrol (**2**) was stable under the assay conditions, the loss is likely due to re-distribution into the lipid membrane, consistent with the amphiphilic nature of the compound. As episilvestrol (**2**) differs from silvestrol (**1**) only in the chirality at C5"', we anticipate that silvestrol (**1**) would also exhibit this apparent low cellular permeability, given that this chirality inversion would have minimal impact on the compound's physicochemical properties.

Chemistry. We have prepared a series of analogues of silvestrol (**1**) where the dioxanyloxy moiety has been replaced by benzylic and heterocyclic moieties. The cyclopenta[*b*]benzofuran framework of these compounds were prepared in racemic form using a photocycloaddition reaction of 3-hydroxyflavones with cinnamates originally developed by Porco and co-workers.[22] The requisite 3-hydroxyflavones **7** were prepared using the classical Algar-Flynn-Oyamada reaction[23] or via lithiation of flavones **6** followed by quenching with trimethylborate[24] and subsequent oxidation and hydrolysis of the intermediate boronate (Scheme 1). The photocycloaddition of the hydroxyflavones **7** with methyl cinnamate followed by an α-ketol rearrangement and chromatographic separation of the unwanted *exo*- isomer, generated the β-keto esters **8**. Anti-selective reduction of the β-keto ester function[25] afforded cyclopenta[*b*]-benzofurans **9** as the major product. The benzyl or *p*-methoxybenzyl ether of **9** was removed by hydrogenolysis using Pearlman's catalyst to give the phenols **10** which in turn were alkylated with various alkyl halides to furnish the desired series of analogues **11**.

The success of the hydrogenolysis of **9** was highly dependent on the batch of Pearlman's catalyst used with certain batches repeatedly furnishing the ethers **13** (Scheme 2) on extended reaction times (>6 h), a reaction that could be suppressed by buffering with acetic acid. These ethers therefore presumably arise from hydrogenolysis of the benzyl group and

subsequent base-induced elimination of the tertiary hydroxyl and solvolysis of the intermediate quinone methide. The identity of the products **13a**/**13b** from this unusual reaction was confirmed by spectroscopic analysis and a single crystal X-ray structure determined on the derived *p*-bromobenzoate derivative **14a** of **13a** (Figure 3). Selected compounds from this series, a functionalization that has hitherto not been widely explored for the cyclopenta[*b*]benzofurans, were also profiled in our assays (vide infra*)*.

Following published work for related cyclopenta[*b*]benzofurans,[26,27] the β-keto ester **8b** was decarboxylated under Krapcho conditions and the resultant α-hydroxyketone **15** stereoselectively reduced to the *trans* diol **16a** with $NMe_4BH(OAc)_3$ or the *syn* diol with $NaBH_4$ **16b** (Scheme 3).

We also prepared methyl rocaglate **17** following literature procedures[28] for use as an assay benchmark. The hydroxamate **18** has been reported to possess potent cytotoxicity and inhibitory activity in translation assays.[29,30] We prepared **18** and **19** (the hydroxamate analogue of **11f**), from the corresponding methyl esters by hydrolysis to the respective acids and coupling with *O*-methylhydroxylamine under conventional conditions (Scheme 4).

Assessment of Biological Activity. All compounds prepared in this work were profiled for effects on protein translation in biochemical and cellular assays (see Supplemental material for tabulated data). The biochemical assay consisted of a translation assay in rabbit reticulocyte lysates using an expression construct allowing for determination of effects on cap-dependent translation, as previously described.[31] In addition, the effect on protein synthesis in cells was determined in a separate assay measuring the metabolic incorporation of radioactive methionine and cysteine, with the correlation between the biochemical and cellular inhibition of translation giving a determination of compound permeability into the cell. The effect of the compounds on the proliferation and viability of mouse embryonic fibroblasts (MEFs) and, for the most active compounds, against selected leukemia cell lines, was also evaluated.

The effect of the compounds in the in vitro translation inhibition assays is presented in Figure 4 along with data for episilvestrol (**2**) and methyl rocaglate (**18**). As depicted in Figure

3, the compounds display varying degrees of selectivity for cap-dependant translation inhibition, with the compounds **9b**, **11c** and **11f** showing the greatest potency and selectivity.

The effects that episilvestrol (**2**) and the compounds prepared here have on translation in cells and on cellular proliferation and viability are shown in Table 2. Compounds which show inhibition of translation in the cellular assay at 35% of vehicle control or greater all show potent inhibition of mouse embryonic fibroblast (MEF) proliferation, and conversely those compounds that are weak in the cellular translation assay are also less potent against MEF viability. The exception is the rocaglaol derivative **16a** and the hydroxamate **19** which appear weaker in the translation assay but display sub-micromolar potency in the cellular proliferation assay.

Three of the most potent compounds from the studies above (**9b**, **11c** and **11f**) were profiled against a panel of human leukemia cell lines to determine if the activity observed in the translation assays and against MEFs would be apparent in disease-relevant cells. For comparison, the recently described hydroxamate **18** was included in the assay, as was **19**, the hydroxamate derivative of **11f** and one of the most potent compounds prepared in this work. The data is tabulated in Table 3 and demonstrates that both **11f** and its hydroxamate derivative **19** show excellent activity against these cell lines.

DISCUSSION

The natural products silvestrol (**1**) and episilvestrol (**2**) are the most potent and selective inhibitors of the DEAD-box helicase eIF4A identified to date.[6,9] By virtue of their activity against eIF4A, these compounds potently inhibit translation in a cap-dependent manner. Furthermore, the compounds are strongly cytotoxic in vitro against a range of cell types and in vivo studies with silvestrol (**1**) have shown promising anti-cancer activity against several tumor models xenografted into mice.[6,14] Despite this promising activity profile, in depth pharmacological studies with silvestrol (**1**) and episilvestrol (**2**) have been hampered by limited availability of the natural products from either the natural source or by total synthesis.

There is strong biochemical evidence for the binding of these compounds to eIF4A,[6] and recent affinity chromatography studies with episilvestrol derivatives have identified only eIF4AI/II as the molecular targets of the compound.[19] Furthermore, a very recent paper has identified key amino acids involved in the binding of silvestrol to the yeast homolog of eIF4A, TIM2, however, the precise binding mode of the compound remains unknown.[32] Importantly, minor changes to the pseudo-sugar moiety of silvestrol (**1**) and episilvestrol (**2**) have profound effects on compound activity clearly indicating that this moiety is important to the compounds' interaction with eIF4A. Despite this, most SAR studies on compounds of this class have focused on modifications to the cyclopenta[*b*]benzofuran moiety where typically a methyl ether replaces the pseudo-sugar. The recent disclosure by Infinity Pharmaceuticals, where a comprehensive SAR exploration of the pseudo-sugar moiety was undertaken, is the only report of which we are aware that has systematically investigated the pseudo-sugar region of the molecule in relation to translation inhibition and cellular activity.[33] In the work reported here we have chosen to explore structurally simple replacements of the pseudo-sugar moiety with benzylic and heterocyclic functionality that can be readily introduced and that improve drug-likeness.

Initially we investigated the passive, transcellular permeability of episilvestrol (**2**) using an in vitro PAMPA model, where we observed the diffusion was limited and there was apparent retention of the compound in the lipid membrane. These results are consistent with the high PSA for episilvestrol (**2**) and amphiphilic nature of the compound, indicating that cellular potency of compounds of this class should be improved by improving cellular permeability. We have also shown that whilst episilvestrol (**2**) is chemically stable under simulated physiological conditions, the compound was susceptible to hydrolysis to the corresponding acid (**5**) with both human and mouse liver microsomes. This data mirrors earlier findings for silvestrol (**1**).[34]

A series of analogues of silvestrol (**1**)/episilvestrol (**2**) were then prepared where the chemically complex pseudo-sugar moiety was replaced with less polar benzylic and heterocyclic species. The effect these compounds have on translation was determined in a

biochemical assay and the data indicate that most compounds exhibit inhibitory effects on cap-dependent translation with varying degrees of selectivity over global non-specific inhibition of translation (Figure 3). Inhibition of translation was also apparent in the cellular translation assay and importantly, there is a good correlation between the biochemical and cellular assays (Figure 5), indicating that the compounds possess acceptable cellular permeability. The exception is compound **19** which displays good activity in the biochemical assay but is weak in the cellular translation assay. The reason for this anomalous activity is unclear though notably the compound shows potent cytotoxic activity against MEFs and leukemia cell lines. Compounds lacking a methoxy group at the C8 position of the cyclopenta[*b*]benzofuran (**9b** and **9c**) are significantly weaker in both biochemical and cellular translation assays. The importance of substitution at the C8 position for potent cellular activity has been reported previously in studies of rocaglates.[27] Likewise, compounds **14a-c** that bear an ether at C3a of the tricyclic nucleus were also inactive in the biochemical and cellular translation assays and were also inactive against MEF viability indicating the importance of a hydrogen bond donor at this site. We are aware of only one report of cyclopenta[*b*]benzofurans with this same substitution and notably these compounds were inactive when assessed in cellular cytotoxicity assays.[35]

Compounds with good potency in the cellular translation assay (>35% inhibition of DMSO control) also showed strong cytotoxicity against MEFs, the only exception being the rocaglaol analogue **16a** which was weak in the cellular translation assay but cytotoxic against MEFs (IC_{50} 143 nM). This data may indicate that **16a** possesses activities that contribute to its cytotoxicity in addition to effects on translation. Previous studies have shown that the natural product rocaglaol (**20**) and a synthetic analogue (**21**) have potent activity against a range of cancer cell lines though activity against translation was not determined (Figure 6).[28,37,38] In our series, the presence of the benzyl ether in **16a** may be detrimental to effects on translation initiation in cells compared to the methyl ether present in **20** and **21**. Indeed, the *syn*-diol **16b** is only moderately active in all our assays whereas the *syn*-diol **22**

disclosed by Désaubry and co-workers is reported to be extremely potent against a range of cancer cell lines.[27]

Three of the most active compounds (**9b**, **11c** and **11f**) from our translation assays, along with episilvestrol (**2**), the known hydroxamate **18** and the methyl hydroxamate derivative (**19**) of **11f** were tested against a panel of leukemia cell lines, to assess their activity against leukemia cell lines which have been reported to be highly sensitive to silvestrol (**1**).[7] These data demonstrate that our most potent compounds do indeed possess significant activity against leukemia lines (IC_{50}'s 12-135 nM), with the acute lymphoblastic leukemia cell line Molt4 being the most sensitive to our compounds. Importantly, the activity reported above is for the compounds tested as racemates. Data reported for silvestrol (**1**) and episilvestrol (**2**) indicates that the potent anti-proliferative and cytotoxic activity resides in a single diastereomer,[10b] therefore we anticipate the activity reported for our compounds underestimates their likely bioactivity. Nonetheless, the activity observed is notably weaker when compared to episilvestrol (**2**) in the same assay (IC_{50}'s ~1 nM) indicating that the moieties we have introduced at C6, in place of the pseudo-sugar of silvestrol (**1**) and episilvestrol (**2**), do not fully replicate the binding interactions that the parent compounds make with their target(s). The most potent compound identified in this work is the hydroxamate **19** supporting the earlier findings from the Porco group that the hydroxamate moiety confers additional potency to compounds of this class.[29,30]

CONCLUSION

Despite the promising preliminary data published on the activity of silvestrol (**1**) and episilvestrol (**2**), extensive biochemical and in vivo studies have been problematic due to their limited availability and poor metabolic stability and permeability. Structurally simpler, synthetically accessible analogues with similar potency against eIF4A therefore have great value to allow further study of translation inhibition as a therapeutic approach in the treatment of cancer. The recent work from Porco and co-workers, where the hydroxamate **18** was shown to exhibit similar activity to silvestrol (**1**) in both protein synthesis and cellular

cytotoxicity assays,[29,30] and the disclosure from researchers from Infinity Pharmaceuticals who demonstrated that the nitrile derivative **23** (Figure 7) also possesses potent cytotoxic and eIF4A inhibitory activity,[33] indicates that the pseudo-sugar moiety of silvestrol (**1**)/episilvestrol (**2**) is not essential for potent activity in compounds of this class. Data for compounds prepared in this work indicates that replacing the pseudo-sugar with chemically less complex functionality at C6 of the cyclopenta[*b*]benzofuran nucleus also leads to highly active translation initiation inhibitors with potent activity in cells and most notably significant cytotoxicity against leukemia cell lines, albeit at reduced potencies compared to the parent compounds and the hydroxamate **18**. It should be noted, however, that the most active compounds tested all show significant potency against MEFs indicating that these compounds are highly cytotoxic and may therefore possess a narrow therapeutic window. Further study with these simplified analogues will allow assessment of their activity in disease-relevant models as well as determination of on-target toxicity and tolerability, and thus therapeutic window.

EXPERIMENTAL SECTION

All NMR spectra were performed at 300K with the $CDCl_3$ as the solvent, unless otherwise indicated, on a Bruker Avance DRX 300 (^{1}H NMR at 300 MHz, ^{13}C NMR at 75 MHz). Chemical shifts are reported in ppm on the δ scale and referenced to the appropriate solvent peak. Electrospray mass spectroscopy (MS) was carried out on a Finnigan LCQ advantage MAX. High resolution mass spectra were measured using a Waters Q-TOF high resolution mass spectrometer with ESI. The purity of all compounds described below was determined by HPLC as >95% on a Waters 2525 Binary Gradient Module HPLC coupled to a Waters 2996 Photodiode Array Detector (@254 nm) with a Xbridge C18, 4.6 x 100 mm 5 micron column, eluting with water-acetonitrile (90:10 → 0:100, 1% formic acid). Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 aluminum-packed plates and visualized with short wavelength UV (254 nm) or by staining with permanganate (potassium permanganate 1% v/w, potassium carbonate 20% v/w, and sodium hydroxide

1% v/w in water). Flash chromatography was performed with silica gel 60 (particle size 0.040-0.063 mm). All non-aqueous reactions were performed in oven-dried glassware under an atmosphere of dry nitrogen. Anhydrous solvents were dried using an automated solvent purification system (MBraun SPS) based upon a technology originally described by Grubbs et al.[38] All commercial reagents were used as received.

3-Hydroxy-5-methoxy-7-(4-methoxybenzyloxy)-2-(4-methoxyphenyl)-4*H*-chromen-4-one (7)

To a solution of diisopropylethylamine (4.24 mL, 30.0 mmol) in THF (10 mL) was added a solution of nBuLi in hexanes (17.6 mL, 1.70 M, 30.0 mmol) dropwise at –78 °C. The generated LDA was allowed to warm to 0 °C for 15 min before being cooled to –78 °C. A solution of 5-methoxy-7-(4-methoxybenzyloxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (**6**) (9.00 g, 21.5 mmol) in THF (100 mL) was cannulated to the LDA solution, while maintaining the bath at –78 °C. After 5 min, a solution of trimethylborate (2.7 mL, 24.0 mmol) in THF (20 mL) was added and the mixture was stirred for 40 min at –78 °C. Glacial acetic acid (2.1 mL, 36.0 mmol) was added followed by 30% hydrogen peroxide (2.7 mL). The reaction was allowed to warm to RT for 1 h and quenched with sat. $NaHCO_3$. Ethyl acetate (150 mL) was then added and the aqueous layer was extracted with ethyl acetate (2 x 150 mL), the combined organic fractions were washed with water (2 x 200 mL), brine (200 mL) dried ($MgSO_4$) and concentrated to provide the crude hydroxyflavone which was purified by flash chromatography with 50% ethyl acetate/cyclohexane as eluent to yield the desired hydroxyflavone 8a (1.46 g, 16%) as an orange crystalline solid; ^{1}H NMR: δ=3.83 (s, 3H) , 3.88 (s, 3H), 3.96 (s, 3H), 5.07 (s, 2H), 6.41 (s, 1H), 6.63 (s, 1H), 6.92 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.9 Hz, 2H), 7.39 (d, *J* = 8.5 Hz, 2H), 8.16 (d, *J* = 8.9 Hz, 2H); MS (ES+), *m/z* 419 $[M+H]^+$.

***rac*-Methyl (3*S*,3a*R*,8b*R*)-8b-hydroxy-8-methoxy-6-((4-methoxybenzyl)oxy)-1-oxo-3,3a-diphenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (8b)**

To a solution of **7** (702 mg, 1.68 mmol) in acetonitrile (27 mL) and MeOH (17 mL), was added methyl *trans*-cinnamate (3.42 g, 21.1 mmol). After degassing for 5 min, the mixture was irradiated (450 W Hanovia UV lamp, Pyrex filter) at –10 °C under a nitrogen atmosphere for 10 h. The solvent was removed in vacuo and the crude was purified by flash chromatography with 20% Ethyl acetate /cyclohexane followed by 50% ethyl acetate /cyclohexane as eluent to yield a bright orange foam which was dissolved in MeOH (30 mL) and to the ensuing solution was added NaOMe solution (7.0 mL, 0.5 M, 3.5 mmol). The reaction was heated to reflux for 40 min and was quenched with sat. NH_4Cl. Ethyl acetate (3 x 30 ml) was added and the organic phase collected, combined, dried ($MgSO_4$), filtered and concentrated to provide the β-keto ester **8b** as a mixture of keto-enol tautomers (706 mg, 71%) as a brown glassy oil which could be used in subsequent reactions without further purification.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-6-((4-methoxybenzyl)oxy)-3,3a-diphenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (9b)**

A solution of tetramethylammonium triacetoxyborohydride (1.02 g, 3.87 mmol) in acetonitrile (20 mL) and acetic acid (380 µL, 6.64 mmol) was stirred under argon for 5 min. A solution of keto-enol tautomers **8b** (381 mg, 0.64 mmol) in acetonitrile (12 mL) was cannulated and the mixture was stirred at RT for 16 h. The reaction was quenched with sat. NH_4Cl and 0.5 M sodium tartrate. The usual workup with dichloromethane and purification by flash chromatography with 40% ethyl acetate/cyclohexane as eluent gave the *endo* isomer (±)-**9b** (172 mg, 45%) as a colorless oil; ^{1}H NMR: δ=3.65 (s, 3H), 3.70 (s, 3H), 3.83 (s, 3H), 3.85 (s, 3H), 3.90 (dd, *J* = 6.6, 14.2 Hz, 1H), 4.32 (d, *J* = 14.2 Hz, 1H), 5.00 (s, 2H), 5.02 (d, *J* = 6.6 Hz, 1H), 6.19 (d, *J* = 1.9 Hz, 1H), 6.36 (d, *J* = 1.9 Hz, 1H), 6.68 (d, *J* = 9.0 Hz, 2H), 6.88 (m, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 7.07 (m, 3H), 7.11 (d, *J* = 9.0 Hz, 2H), 7.38 ppm (d, *J* = 8.8 Hz, 2H); MS (ES+), *m/z* 599 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,6-dihydroxy-8,8b-dimethoxy-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (14a)**

To a solution of the known benzyl ether **9a**[5c] (15.4 mg, 0.027 mmol) in methanol (1 mL) was added $Pd(OH)_2$ (20% wt., 4.0 mg, 0.0057 mmol) and Pd/C (10% wt., 8.0 mg, 0.0075 mmol). The reaction was vigorously stirred under a hydrogen atmosphere for 3 h and then filtered through Celite™. After removal of the solvent, the crude material was purified by flash chromatography with 60% ethyl acetate/cyclohexane as eluent to afford the known phenol **11**[10b] (11.9 mg, 92%) as a white solid.

If reaction times were extended (6 h) the side-product **14a** was isolated exclusively (10.6 mg, 80%) as a white solid; ^{1}H NMR ([D_6]acetone): δ=2.38 (s, 3H), 3.53 (s, 3H), 3.71 (s, 3H), 3.90 (m, 1H), 3.91 (s, 3H), 4.13 (d, *J* = 14.2 Hz, 1H), 4.24 (d, *J* = 2.5 Hz, 1H), 5.02 (dd, *J* = 2.5, 7.2 Hz, 1H), 6.24 (d, *J* = 1.5 Hz, 1H), 6.29 (d, *J* = 1.5 Hz, 1H), 6.71 (d, *J* = 8.6 Hz, 2H), 6.81 (m, 2H), 7.03 (m, 3H), 7.20 (bd, *J* = 7.7 Hz, 2H), 8.85 (s, 1H); ^{13}C NMR ([D_6]acetone) δ=50.8, 51.6, 51.9, 55.3, 56.1, 80.7, 92.4, 93.4, 100.3, 100.7, 103.6, 113.1, 127.1, 128.3, 128.5, 128.8, 129.0, 138.4, 159.2, 159.6, 162.2, 162.9, 170.3 ppm; MS (ES+), *m/z* 493 $[M+H]^+$.

In an analogous procedure, the ethyl ether **13b** was obtained in 83% when using ethanol as the solvent; ^{1}H NMR ([D_6]acetone): δ=0.67 (t, *J* = 7.0 Hz, 3H), 2.41 (m, 1H), 2.64 (m, 1H), 3.63 (s, 3H), 3.75 (s, 3H), 3.83 (dd, *J* = 7.9, 14.4 Hz, 1H), 3.87 (s, 3H), 4.21 (d, *J* = 14.4 Hz, 1H), 4.46 (s, 1H), 5.17 (d, *J* = 7.9 Hz, 1H), 6.06 (d, *J* = 1.6 Hz, 1H), 6.28 (d, *J* = 1.6 Hz, 1H), 6.40 (bs, 1H), 6.71 (d, *J* = 8.4 Hz, 2H), 6.80 (d, *J* = 7.2 Hz, 2H), 7.09 – 6.98 (m, 3H), 7.17 ppm (bs, 2H); ^{13}C NMR ([D_6]acetone): δ=15.1, 50.8, 51.5, 55.3, 56.1, 60.1, 81.0, 92.5, 93.3, 100.2, 100.5, 113.1, 127.1, 128.2, 128.7, 128.8, 129.1, 138.4, 159.1, 159.6, 162.0, 162.7, 170.3 ppm; MS (ES+), *m/z* 507 $[M+H]^+$.

General procedure for the alkylation of phenol derivatives.

To a solution of the phenol (namely, one of either **10**, **13**) (0.022 mmol) in DMF (0.5 ml) was added potassium carbonate (6.9 mg, 0.05 mmol) followed by the alkyl halide (0.05

mmol) and TBAI (1.0 mg, 0.003 mmol). The reaction mixture was stirred at RT for 16 h before water (2 mL) and ethyl acetate (2 mL) was added. The organic layer was collected and the aqueous layer extracted with ethyl acetate (2 x 5 mL). The organic fractions were combined and washed with water (3 x 5 mL), brine, dried ($MgSO_4$) and concentrated to afford a crude residue which was subjected to flash silica gel chromatography. Elution with ethyl acetate/cyclohexane provided the alkylated cyclopentabenzofuran.

***r*ac-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-6-((3-methoxybenzyl)oxy)-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11a)**

Colourless foam (56%); ^{1}H NMR: δ=3.65 (s, 3H), 3.71 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 3.90 (dd, *J* = 6.5, 13.9 Hz, 1H), 4.31 (d, *J* = 13.9 Hz, 1H), 5.02 (dd, *J* = 1.2, 6.5 Hz, 1H), 5.06 (s, 2H), 6.21 (d, *J* = 1.9 Hz, 1H), 6.35 (d, *J* = 1.9 Hz, 1H), 6.67 (d, *J* = 9.0 Hz, 2H), 6.89 (m, 3H), 7.01 (m, 2H), 7.05 (m, 3H), 7.11 (d, *J* = 9.0 Hz, 2H), 7.33 ppm (dd, *J* = 8.0, 8.0 Hz, 1H); MS (ES+), *m/z* 599 $[M+H]^+$; HRMS (ES+) *m/z* $[M+H]^+$ calcd for $C_{35}H_{35}O_9$: 599.2281, found 599.2284.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-6-((2-methoxybenzyl)oxy)-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11b)**

Colourless oil (65%); ^{1}H NMR: δ=3.67 (s, 3H), 3.71 (bs, 1H), 3.74 (s, 3H), 3.89 (s, 3H), 3.92 (m, 4H), 4.33 (d, *J* = 14.1 Hz, 1H), 5.05 (d, *J* = 6.5 Hz, 1H), 5.15 (s, 2H), 6.26 (d, *J* = 2.0 Hz, 1H), 6.40 (d, *J* = 2.0 Hz, 1H), 6.70 (d, *J* = 9.0 Hz, 2H), 6.90 (m, 2H), 7.09-6.95 (m, 5H), 7.13 (d, *J* = 9.0 Hz, 2H), 7.35 (dt, *J* = 1.8, 7.8 Hz, 1H), 7.48 ppm (dd, *J* = 1.8, 7.8 Hz, 1H); MS (ES+), *m/z* 599 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-6-(pyridin-3-ylmethoxy)-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11c)**

Colourless foam (74%); ^{1}H NMR: δ=3.65 (s, 3H), 3.72 (s, 3H), 3.87 (s, 3H), 3.88 (m, 1H), 4.30 (d, *J* = 14.2 Hz, 1H), 5.05 (m, 3H), 6.21 (d, *J* = 1.9 Hz, 1H), 6.33 (d, *J* = 1.9 Hz, 1H), 6.67 (d, *J* = 9.0 Hz, 2H), 6.85 (m, 2H), 7.05 (m, 3H), 7.10 (d, *J* = 9.0 Hz, 2H), 7.36 (bs, 1H), 7.78 (d, *J* = 7.7 Hz, 1H), 8.53 ppm (bs, 2H); MS (ES+), *m/z* 570 $[M+H]^+$; HRMS (ES+) *m/z* $[M+H]^+$ calcd for $C_{33}H_{32}NO_8$: 570.2128, found: 570.2130.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-6-((3-fluorobenzyl)oxy)-1,8b-dihydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11d)**

Colourless oil (30%); ^{1}H NMR: δ=3.67 (s, 3H), 3.73 (s, 3H), 3.90 (s, 3H), 3.92 (dd, *J* = 6.7, 14.2 Hz, 1H), 4.33 (d, *J* = 14.2 Hz, 1H), 5.05 (d, *J* = 6.7 Hz, 1H), 5.10 (s, 2H), 6.23 (d, *J* = 2.0 Hz, 1H), 6.35 (d, *J* = 2.0 Hz, 1H), 6.70 (d, *J* = 9.0 Hz, 2H), 6.88 (m, 2H), 7.08 (m, 4H), 7.13 (d, *J* = 9.0 Hz, 2H), 7.22 (m, 2H), 7.39 ppm (m, 1H); MS (ES+), *m/z* 587 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-6-((4-isopropylbenzyl)oxy)-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11e)**

Colourless oil (46%); ^{1}H NMR: δ=1.29 (d, *J* = 7.0 Hz, 6H), 2.96 (p, *J* = 7.0 Hz, 1H), 3.67 (s, 3H), 3.73 (s, 3H), 3.91 (s, 3H), 3.92 (dd, *J* = 6.5, 14.4 Hz, 1H), 4.34 (d, *J* = 14.4 Hz, 1H), 5.04 (m, 3H), 6.23 (d, *J* = 1.9 Hz, 1H), 6.39 (d, *J* = 1.9 Hz, 1H), 6.70 (d, *J* = 9.0 Hz, 2H), 6.89 (m, 2H), 7.07 (m, 3H), 7.13 (d, *J* = 9.0 Hz, 2H), 7.31 (d, *J* = 8.3 Hz, 2H), 7.41 ppm (d, *J* = 8.3 Hz, 2H); MS (ES+), *m/z* 611 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-6-(pyrimidin-2-ylmethoxy)-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (11f)**

White solid (63%); ^{1}H NMR: δ=3.67 (s, 3H), 3.73 (s, 3H), 3.89 (s, 3H), 3.91 (dd, *J* = 6.8, 14.2 Hz, 1H), 4.31 (d, *J* = 14.2 Hz, 1H), 5.04 (d, *J* = 6.8 Hz, 1H), 5.33 (s, 2H), 6.34 (m, 2H), 6.69 (d, *J* = 9.0 Hz, 2H), 6.86 (m, 2H), 7.06 (m, 3H), 7.12 (d, *J* = 9.0 Hz, 2H), 7.31 (d, *J* = 4.9 Hz, 1H), 8.82 ppm (d, *J* = 4.9 Hz, 2H); MS (ES+), *m/z* 571 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-8b-ethoxy-1-hydroxy-8-methoxy-6-((2-methoxy-benzyl)oxy)-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzo-furan-2-carboxylate (14b)**

Light yellow solid (93%); ^{1}H NMR: δ=0.69 (t, *J* = 7.0 Hz, 3H), 2.42 (m, 1H), 2.65 (m, 1H), 3.59 (s, 3H), 3.74 (s, 3H), 3.78 (dd, *J* = 7.4 , 14.5 Hz, 1H), 3.85 (s, 3H), 3.88 (s, 3H), 4.11 (m, 2H), 5.07 (s, 2H), 5.11 (d, *J* = 7.4 Hz, 1H), 6.24 (d, *J* = 1.9 Hz, 1H), 6.38 (d, *J* = 1.9 Hz, 1H), 6.70 (m, 4H), 6.91 (m, 1H), 7.04 (m, 5H), 7.15 (bs, 2H), 7.34 ppm (dd, *J* = 8.4, 8.4 Hz, 1H); MS (ES+), *m/z* 628 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-8b-ethoxy-1-hydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-6-(pyridin-4-ylmethoxy)-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (14c)**

Light yellow solid (93%); ^{1}H NMR: δ=0.68 (t, *J* = 7.0 Hz, 3H), 2.42 (m, 1H), 2.65 (m, 1H), 3.59 (s, 3H), 3.74 (s, 3H), 3.77 (dd, *J* = 7.4, 14.2 Hz, 1H), 3.90 (s, 3H), 4.10 (m, 2H), 5.12 (bs, 3H), 6.24 (d, *J* = 2.0 Hz, 1H), 6.32 (d, *J* = 2.0 Hz, 1H), 6.68 (m, 4H), 7.04 (m, 3H), 7.14 (bs, 2H), 7.38 (d, *J* = 4.6 Hz, 2H), 8.61 ppm (s, 2H); MS (ES+), *m/z* 598 $[M+H]^+$.

***rac*-Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-6-((4-bromobenzoyl)oxy)-1-hydroxy-8,8b-dimethoxy-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylate (14a)**

To a solution of phenol, **13a** (20.4 mg, 0.0414 mmol) in dichloromethane (1 mL) at 0 °C was added triethylamine (13.9 µL, 0.100 mmol) followed by 4-bromobenzoyl chloride (11.0 mg, 0.050 mmol). The reaction was warmed to RT and stirred for a further 14 h, the reaction was quenched with sat. aqueous $NaHCO_3$ (2 mL). The aqueous layer was extracted with dichloromethane (2 x 5 mL) and the organic fractions combined, washed with water (10 mL), Sat. $NaHCO_3$ (10 mL), brine, dried ($MgSO_4$) and concentrated. The crude residue was then subjected to flash chromatography, eluted with 40 – 60% ethyl acetate/cyclohexane provided the title compound **14a** (23.2 mg, 83%) as a white crystalline solid; ^{1}H NMR: δ=2.50 (s, 3H), 3.62 (s, 3H), 3.77 (s, 3H), 3.83 (dd, *J* = 7.4, 14.3 Hz, 1H), 3.95 (s, 3H), 4.16 (d, *J* = 14.3 Hz, 1H), 5.17 (d, *J* = 7.4 Hz, 1H), 6.50 (d, *J* = 1.8 Hz, 1H), 6.72 (m, 5H), 7.06 (m, 3H), 7.16 (bd, *J* = 7.9 Hz, 2H), 7.70 (d, *J* = 8.6 Hz, 2H), 8.09 ppm (d, *J* = 8.6 Hz, 2H); MS (ES+), *m/z* 697 $[M+Na]^+$ Recrystallisation from dichloromethane/heptanes provided crystals suitable for x-ray crystallography.

Single crystal X-ray structure determination. Intensity data were collected with an Oxford Diffraction SuperNova CCD diffractometer using Mo-Kα or Cu- Kα radiation, the temperature during data collection was maintained at 130.0(1) using an Oxford Cryosystems cooling device. The structure was solved by direct methods and difference Fourier synthesis.[39] Thermal ellipsoid plots were generated using the program ORTEP-3[40] integrated within the WINGX[41] suite of programs. The asymmetric unit consists of two enantiomerically related molecules of **14a** and a molecule of heptane solvate. The crystal was inversion twinned with Flack parameter refined to 0.36(1).Crystal data for **14a**. $2(C_{35}\ H_{30}\ Br\ O_9)\ .(C_7H_{18})$ *M* = 1451.21, *T* = 130.0 K, λ= 1.54180, Monoclinic, space group C2, *a* = 19.6904(2)*, b* = 15.5914(1), *c* =24.3002(3) *Å,* β = 113.469(1)°. *V* 6843.0(1) *Å*^{3}, Z = 4, D_c = 1.409 Mg M^{-3} µ (Cu-Kα) 2.107 mm^{-1}, F(000) = 3016, crystal size 0.44 x 0.19 x 0.15 mm^3, 25796 reflections measured, 11508 independent reflections [R(int) = 0.0174], the final R was 0.0267 [I > 2σ(I)] and *w*R(F^2) was 0.0726 (all data).

***rac*-(3*S*,3a*R*,8b*R*)-8b-hydroxy-8-methoxy-6-((4-methoxybenzyl)oxy)-3,3a-diphenyl-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-1-one (15)**

To a solution of the β-keto esters **8b** (370 mg, 0.618 mmol) in DMSO (3 mL) was added LiCl (39.5 mg, 0.93 mmol) followed by water (33.2 µL, 1.85 mmol). The reaction mixture was heated at 100 °C for 14 h before being cooled to RT and diluted with ethyl acetate (10 mL) and water (10 mL). The aqueous phase was extracted with ethyl acetate (2 x 10 mL) and the combined organic fractions were washed with water (3 x 10 mL), brine, dried ($MgSO_4$) and concentrated to afford a crude residue which was subjected to flash silica gel chromatography. Elution with 20 – 30% ethyl acetate/cyclohexanes provided the ketone **15** (85.1 mg, 26%) as a light yellow oil;^{1}H NMR: δ=3.01 (m, 2H), 3.70 (s, 3H), 3.80 (s, 3H), 3.82 (s, 3H), 3.85 (m, 1H), 5.02 (s, 2H), 6.18 (d, *J* = 2.0 Hz, 1H), 6.42 (d, *J* = 2.0 Hz, 1H), 6.67 (d, *J* = 8.8 Hz, 2H), 6.96 (m, 6H), 7.10 (m, 3H), 7.38 ppm (d, *J* = 8.8 Hz, 2H); MS (ES+), *m/z* 539 $[M+H]^+$.

***rac*-(1*R*,3*S*,3a*R*,8b*S*)-8-methoxy-6-((4-methoxybenzyl)oxy)-3,3a-diphenyl-1,2,3,3a-tetrahydro-8b*H*-cyclopenta[*b*]benzofuran-1,8b-diol (16a)**

A solution of tetramethylammonium triacetoxyborohydride (93.5 mg, 0.356 mmol) and acetic acid (35 µL, 0.58 mmol) in acetonitrile (1.5 mL) was stirred for 5 min. A solution of the ketone **15** (20.8 mg, 0.039 mmol) in acetonitrile (1 mL) was cannulated and the mixture was stirred at RT for 16 h. The reaction was quenched with sat. aqueous NH_4Cl (2 mL) and sodium/potassium tartrate (2 mL, 0.5 M). Dichloromethane (5 mL) was added and the organic layer was collected and the aqueous layer was extracted with dichloromethane (2 x 5 mL). The combined organic phases were washed with water (2 x 10 mL), brine (10 mL), dried ($MgSO_4$) and concentrated to provide a crude residue which was subjected to flash chromatography. Elution with 20 – 40% ethyl acetate/cyclohexane provided the title compound **16a** (15.9 mg, 76%) as a white solid; 1H NMR: δ=2.22 (ddd, J = 1.2, 6.9, 14.1 Hz, 1H), 2.76 (dt, J = 6.4, 14.1 Hz, 1H), 3.73 (s, 3H), 3.85 (s, 3H), 3.91 (s, 3H), 4.02 (dd, J = 6.4,

14.1 Hz, 1H), 4.83 (d, J = 6.4 Hz, 1H), 5.03 (s, 2H), 6.24 (d, J = 2.0 Hz, 1H), 6.39 (d, J = 2.0 Hz, 1H), 6.70 (d, J = 9.0 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 7.03 (m, 2H), 7.13 (m, 5H), 7.39 pp (d, J = 8.8 Hz, 2H); MS (ES+), m/z 563 [M+Na]+; HRMS m/z (ES+) [M+Na]+ calcd for $C_{33}H_{32}O_7Na$: 563.2040, found: 563.2039.

***rac*-(1*S*,3*S*,3a*R*,8b*S*)-8-methoxy-6-((4-methoxybenzyl)oxy)-3,3a-diphenyl-1,2,3,3a-tetrahydro-8b*H*-cyclopenta[*b*]benzofuran-1,8b-diol (16b)**

To a solution of ketone **15** (27.4 mg, 0.051 mmol) in methanol (3 mL) was added $NaBH_4$ (19.3 mg, 0.510 mmol). The solution was stirred at RT for 16 h before the solvent was removed in vacuo and the crude residue subjected to flash chromatography. Elution with 20 – 40% ethyl acetate/cyclohexanes provided title compound **16b** (16.9 mg, 61%) as a light yellow solid; ^{1}H NMR: δ=2.60 (m, 1H), 2.65 (m, 1H), 3.48 (dd, *J* = 7.0, 14.0 Hz, 1H), 3.73 (s, 3H), 3.85 (s, 3H), 3.86 (m, 1H), 4.83 (dd, *J* = 7.8, 7.8 Hz, 1H), 5.02 (s, 2H), 6.20 (d, *J* = 1.9 Hz, 1H), 6.35 (d, *J* = 1.9 Hz, 1H), 6.71 (d, *J* = 9.0 Hz, 2H), 6.95 (d, *J* = 8.8 Hz, 2H), 7.02 (m, 2H), 7.10 (m, 3H), 7.22 (d, *J* = 9.0 Hz, 2H), 7.40 ppm (d, *J* = 8.8 Hz, 2H); MS (ES+), *m/z* 563 $[M+Na]^+$; HRMS *m/z* (ES+) $[M+Na]^+$ calcd for $C_{33}H_{32}O_7Na$: 563.2040, found: 563.2041.

***rac*-(1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-*N*,8-dimethoxy-3a-(4-methoxyphenyl)-3-phenyl-6-(pyrimidin-2-ylmethoxy)-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxamide (19)**

To a stirred solution of pyrimidine **12f** (42.0 mg, 0.087 mmol) in THF (2.5 mL) and H_2O (0.5 mL) at 20 °C was added $LiOH.H_2O$ (37 mg, 0.87 mmol). The mixture was heated at 50 °C under N_2 for 4 h. The reaction mixture was then cooled to RT and the pH was adjusted to ~ 3 with HCl (1 M). The aqueous phase was extracted with ethyl acetate (3 x 3 mL) and the combined extracts were dried (Na_2SO_4), filtered and concentrated. The crude material was subjected to flash chromatography, elution with 0 – 30% MeOH/dichloromethane gave *rac*-(1*R*,2*R*,3*S*,3a*R*,8b*S*)-1,8b-dihydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-6-(pyrimidin-2-ylmethoxy)-2,3,3a,8b-tetrahydro-1*H*-cyclopenta[*b*]benzofuran-2-carboxylic acid

(16.0 mg, 36%) as a white solid; ^{1}H NMR (600 MHz, [D_6]DMSO): δ=3.57 (s, 3H), 3.76 (dd, *J* = 13.8, 5.4 Hz, 1H), 4.07 (d, *J* = 13.8 Hz, 1H), 4.64 (d, *J* = 5.4 Hz, 1H), 5.00 (s, 1H), 5.25 (s, 2H), 6.16 (d, *J* = 1.8 Hz, 1H), 6.27 (d, *J* = 1.8 Hz, 1H), 6.55 (d, *J* = 9.0 Hz, 2H), 6.85 (d, *J* = 7.2 Hz, 2H), 6.95-6.96 (m, 3H), 7.01 (m, 2H), 7.47 (t_{app}, *J* = 4.8 Hz, 1H), 8.85 ppm (d, *J* = 4.8 Hz, 2H); MS (ES+), *m/z* 557 $[M+H]^+$

To a stirred solution of acid generated as described above (17.0 mg, 0.0305 mmol) in dichloromethane (4 mL) was added EDC (10.0 mg, 0.0501 mmol) followed by HOBt•H_2O (10.9 mg, 0.0728 mmol). After stirring for 5 min, methoxyamine hydrochloride (12.2 mg, 0.144 mmol) was added followed by triethylamine (20.1 µL, 14.6 mmol). The reaction was stirred at 20 ^{o}C for 16 h then quenched with HCl (1 M) and diluted with water (2 mL). The aqueous phase was separated, then extracted with ethyl acetate (2 x 3 mL) and the organic phases were combined and dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to flash chromatography, elution with 0 – 20% MeOH/dichloromethane provided the title compound **19** (9.5 mg, 56%) as a white solid; ^{1}H NMR ([D_6]DMSO): δ=3.49 (s, 3H), 3.61 (s, 3H), 3.73 (s, 3H), 4.17 (d, *J* = 12 Hz, 1H), 4.55 (m, 1H), 4.69 (d, *J* = 3 Hz, 1H), 5.03 (s, 1H), 5.28 (s, 2H), 6.21 (d, *J* = 1.8 Hz, 1H), 6.30 (d, *J* = 1.8 Hz, 1H), 6.59 (d, *J* = 9.0 Hz, 2H), 6.88 (d, *J* = 8.0 Hz, 2H), 6.98-7.04 (m, 5H), 7.50 (t_{app}, *J* = 6.0 Hz, 1H), 8.88 (d, *J* = 6.0 Hz, 2H), 11.14 (s, 1H); MS (ES+), *m/z* 586 $[M+H]^+$; HRMS *m/z* (ES+) $[M+Na]^+$ calcd for $C_{32}H_{31}N_3O_8$: 608.2009, found: 608.2003.

In Vitro Translation Assays

In vitro translation assays were performed in rabbit reticulocyte lysate (Promega, Alexandria, Australia) for 1h at 30 °C as previously described.[19] Firefly luciferase was translated in a cap-dependent manner while *Renilla* luciferase production was driven by HCV IRES-mediated translation, which occurs independent of eIF4A, from capped pSP/(CAG)33/FF/HCV/Ren.pA51 mRNA. Compounds were tested at 50 µM with a final concentration of 135 mM KCl. The Dual Glo Luciferase Assay kit (Promega) was used to measure luciferase activity.

Translation in Mouse Embryonic Fibroblasts (MEFs)

Protein synthesis inhibition by silvestrol and its analogs was performed as previously described in mouse embryonic fibroblasts.[19] Briefly, cells were treated with 1 μM compound for 1 h and 35S-labeled methionine and cysteine was added in the last 20 min. Cells were then washed, and lysed. Proteins in the cell extract were precipitated with trichloroacetic acid and the radiolabeled protein/total protein content calculated.

Cellular Viability Assays

Cell viability and proliferation assays were performed as previously described[9,42] using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Alexandria, Australia). Briefly, cells (MEFs or leukaemia lines) were seeded in opaque 96-well plates and treated with 11 point dilution of indicated compounds, incubated at 37 °C/10% CO_2 for 72 h and analyzed via the manufacturer's instructions.

Chemical Stability determination

Stability experiments were conducted at a nominal episilvestrol (2) concentration of 10 μM in simulated gastric fluid (SGF; USP23, p2053), pH 3 (50 mM isotonic phosphate buffer), pH 5 (50 mM isotonic acetate buffer) and pH 7 (50 mM isotonic phosphate buffer) media at 37 °C. Samples were analysed by LC/MS at the beginning of the study and after 1, 4 and 8 h by diluting 1:20 with quench solution (prepared at a ratio of 0.9 (50 mM phosphate buffer, pH 7) to 1 (acetonitrile)) containing verapamil as an internal standard.

LC/MS analysis was conducted using a Waters Acquity UPLC coupled to a Waters Micromass Quattro Ultima Pt triple quadrupole mass spectrometer. Separation was performed using a Supelco Ascentis Express RP-C18 (50 x 2.1 mm) column maintained at a column temperature of 40 °C.

Microsomal Stability

The metabolic stability assay was performed by incubating episilvestrol (**2**) (1 μM) with human (XenoTech) or mouse (BD Gentest) liver microsomes at 37 °C and 0.4 mg/mL

protein concentration. The metabolic reaction was initiated by the addition of a NADPH-regenerating system and quenched at various time points over the 60 min incubation period by the addition of acetonitrile containing verapamil as an internal standard. Control samples (devoid of NADPH) were included (and quenched at 2, 30 and 60 min) to monitor for potential degradation and formation of metabolites in the absence of cofactors. Incubations were conducted in triplicate. Sample analysis by LC/MS was conducted as described above.

Permeability Assay

Standard 96-well PVDF filter plates were coated with a solution of lipid material (dioleoyl-3-phosphocholine (DOPC)) to prepare the artificial membrane. The filter plate was sandwiched with a 96-well acceptor plate filled with transport buffer (Hanks balanced salt solution containing 20 mM HEPES, pH 7.4). Episilvestrol (**2**) (10 μM; n=8) and verapamil (20 μM; n=4) included as a high membrane permeability marker, were prepared in transport buffer and transferred to the donor wells of the filter plate. Nadolol (n=12) was added to all wells as low permeability marker for assessment of membrane integrity. The acceptor compartment of the filter plate was filled blank transport buffer and samples were allowed to permeate across the artificial membrane for 4 h at 37 °C with 200 rpm orbital shaking. Parallel samples were incubated at 37°C to assess the chemical stability under the conditions of the PAMPA assay. At the end of the incubation period, stability samples and the donor and acceptor wells of the PAMPA sandwich were quenched with an equal volume of acetonitrile and the concentrations of test and control compounds were quantitated by LC/MS. The lower limits of quantitation (LLQ) were found to be 0.02 μM for episilvestrol and 0.005 μM for verapamil and nadolol.

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LEGENDS FOR FIGURES AND SCHEMES

Figure 1. Chemical structures of silvestrol (**1**) and analogues, and rocaglamide (**4**)

Figure 2. Structure of episilvestric acid (**5**)

Scheme 1. Reagents and conditions: a) LDA, THF, $B(OMe)_3$, AcOH, H_2O_2. b) methyl *trans*-cinnamate, hu, MeOH, CH_3CN. c) $Me_4NBH(OAc)_3$, AcOH, CH_3CN. d) H_2, $Pd(OH)_2$, Pd/C, MeOH. e) alkyl halide, K_2CO_3, TBAI, DMF.

Scheme 2. Reagents and conditions: a) H_2, $Pd(OH)_2$, Pd/C, R_1OH. b) (**14a**) *p*-Br-benzoyl chloride, Et_3N, DCM. c) (**14b** and **14c**) alkyl halide, K_2CO_3, TBAI, DMF.

Figure 3. Thermal ellipsoid plot for one of the molecules of 14a. The ellipsoids are at the 30% probability level. The heptane solvate molecule has been removed for clarity.

Scheme 3. Reagents and conditions: a) LiCl, DMSO, 100 ^oC. b) (for **16a**) $Me_4NBH(OAc)_3$, AcOH, CH_3CN. c) (for **16b**) $NaBH_4$, MeOH.

Scheme 4. Reagents and conditions: a) i) LiOH, MeOH, THF. ii) $MeONH_2$•HCl, EDC, HOBt, Et_3N, DCM.

Figure 4. Biochemical assessment of cap-dependent activity and selectivity determined for compounds at 50 μM. For assay details see Experimental section.

Figure 5. Comparison of cap-dependant translation inhibition in the biochemical and cellular assays.

Figure 6. Rocaglaol (**20**) and known[28] synthetic analogues (**21**, **22**)

Figure 7. Rocaglamide analogue disclosed in ref 33.

TABLES

Table 1. In vitro stability of episilvestrol in mouse and human microsomal preparations

Species	**Degradation half-life (min)**	**In vitro CL_{int} (μL/min/mg protein)**	**Metabolites detected**
Human	151 ± 42	14 ± 2	episilvestric acid
Mouse	104 ± 19	18 ± 2	episilvestric acid

Table 2. Cellular activity of silvestrol analogues.

Compound number	**Relative translation rate at 1 μM (% vehicle treated control)[a]**	**IC_{50} (nM) of cell viability and cytotoxicity[b]**
2	3.35 ± 0.31	0.66 ± 0.12
9a	27.7 ± 1.63	123 ± 7.25
9b	34.4 ± 1.72	53.1 ± 3.26
9c	81.0 ± 3.02	1360 ± 283
9d	116 ± 36.2	303 ± 112
11a	35.6 ± 2.42	248 ± 9.03
11b	36.0 ± 2.80	81.5 ± 24.2
11c	28.0 ± 3.60	32.2 ± 5.51

11d	79.6 ± 5.09	182.8 ± 93.48
11e	97.3 ± 6.99	987 ± 141
11f	17.6 ± 0.54	12.3 ± 6.55
11g	17.9 ± 0.60	10.7 ± 4.24
14a	ND	> 10000
14b	97.0 ± 4.85	>10000
14c	88.6 ± 1.93	>10000
15	102 ± 5.87	2070 ± 207
16a	63.2 ± 4.91	142.9 ± 23.0
16b	85.9 ± 5.17	659 ± 107
17	11.4 ± 0.36	2.43 ± 0.32
18	4.78 ± 0.15	0.96 ± 0.06
19	93.5 ± 3.96	44.9 ± 7.2

[a] Translation inhibition in MEFs at 1 μM after 1h (% vehicle ± s.e.m.). ND: not determined

[b] Inhibition of proliferation of MEFs. The average IC_{50} + s.e.m. is shown (n = 3-4)

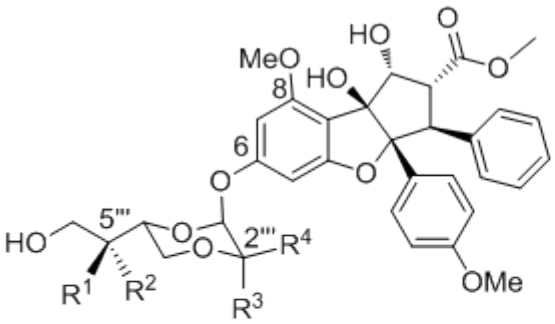
Table 3. Anti-proliferative activity of selected silvestrol analogues against leukemia cell lines[a]

Cell line	2	9b	11f	11c	18	19
CCRF-CEM	1.3 ± 0.3	109.0 ± 18.9	58.7 ± 14.1	137.3 ± 35.3	3.7 ± 0.8	28.4 ± 5.8

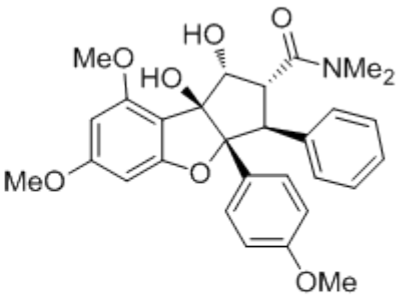
HL-60	1.7 ± 0.1	130.7 ± 2.2	70.7 ± 1.2	168.7 ± 7.7	3.6 ± 1.2[b]	36.7 ± 4.3
K562	1.7 ± 0.1	135.0 ± 3.1	77.3 ± 1.5	155.7 ±2.9	4.4 ± 0.8	29.2 ± 2.4
Molt4	1.0 ± 0.2	53.0 ± 3.5	27.3 ± 1.3	57.3 ± 5.0	2.1 ± 0.1	13.3 ± 1.2
RPMI-8226	1.3 ± 0.1	91.3 ± 10.1	41.7 ± 5.7	100.3 ± 14.8	1.8 ± 0.2	13.9 ± 3.3
[a] The average IC_{50} (nM) $^{\pm}$ s.e.m. shown, n = 3, except [b] where n = 2						

FIGURES AND SCHEMES

Figure 1

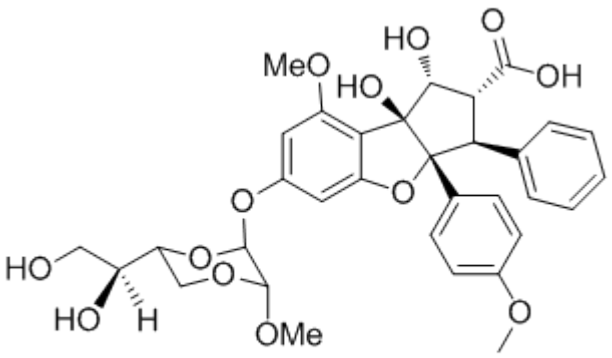


1: $R^1 = R^4 = H$, $R^2 = OH$, $R^3 = OMe$: Silvestrol
2: $R^1 = OH$, $R^2 = R^4 = H$, $R^3 = OMe$: 5'''-Episilvestrol
3: $R^1 = OH$, $R^2 = R^3 = H$, $R^4 = OMe$, 2''',5'''-Diepisilvestrol



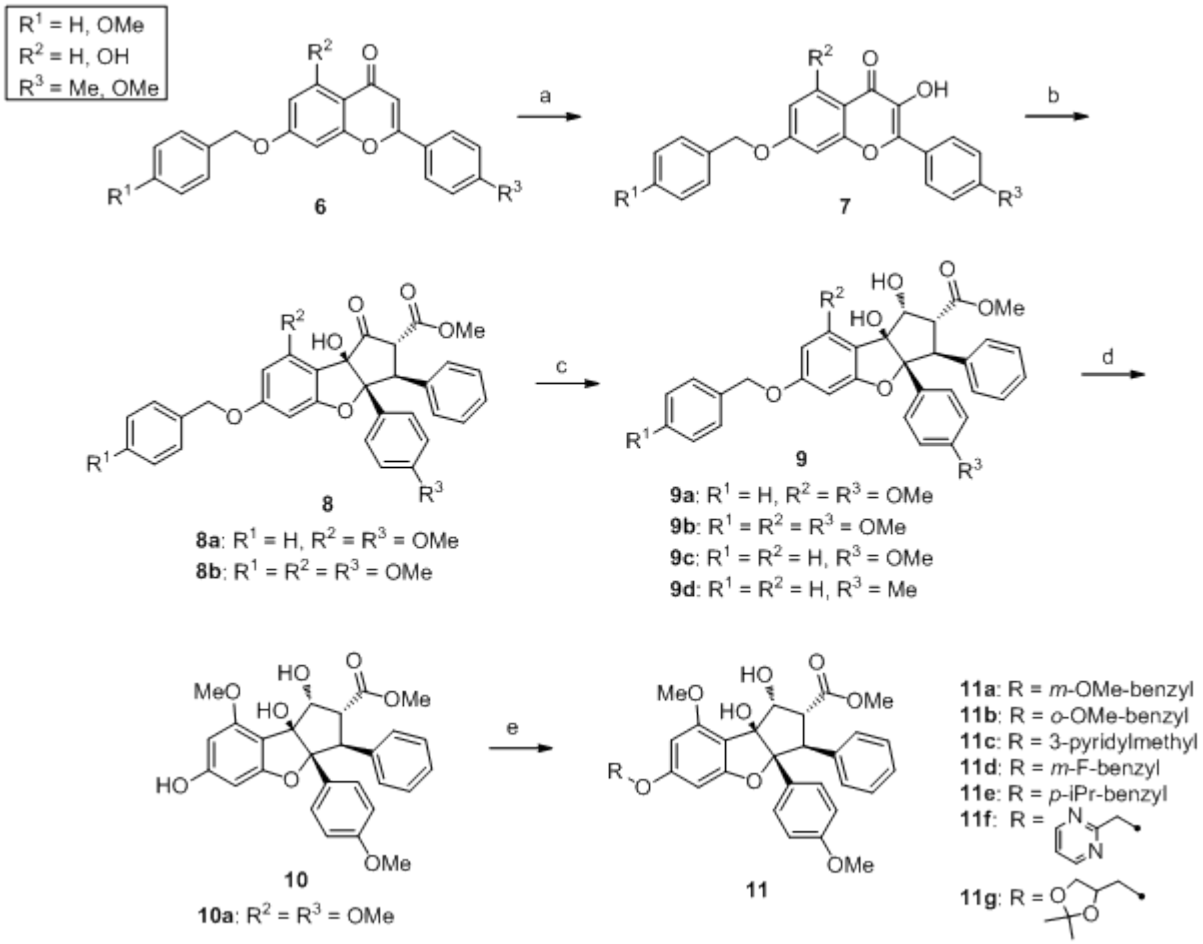
Rocaglamide (**4**)

Figure 2

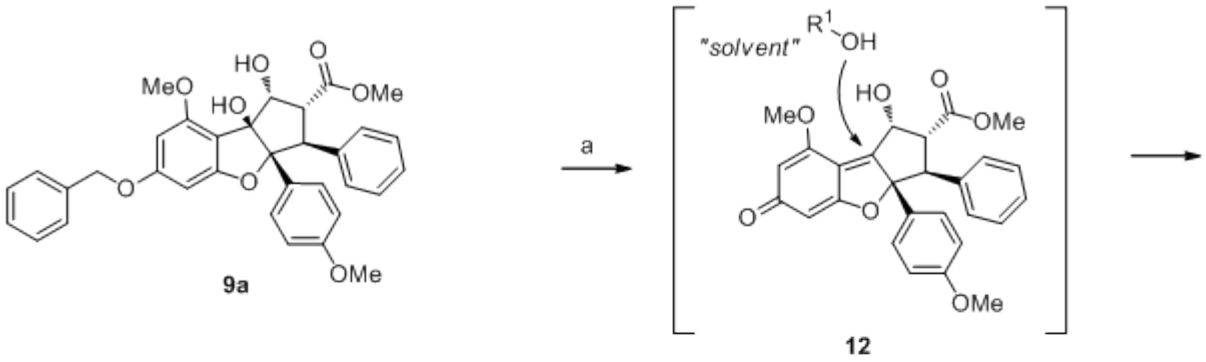


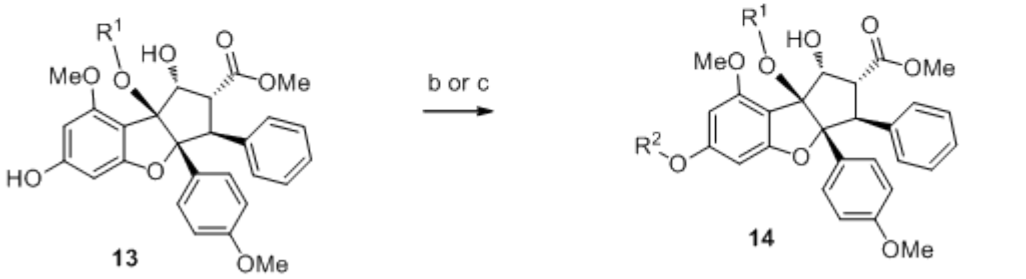
Episilvestrol acid (**5**)

Scheme 1


R1 = H, OMe
R2 = H, OH
R3 = Me, OMe
a
b
c
d
e
6
7
8
9
10
11
8a: R1 = H, R2 = R3 = OMe
8b: R1 = R2 = R3 = OMe
9a: R1 = H, R2 = R3 = OMe
9b: R1 = R2 = R3 = OMe
9c: R1 = R2 = H, R3 = OMe
9d: R1 = R2 = H, R3 = Me
10a: R2 = R3 = OMe
11a: R = m-OMe-benzyl
11b: R = o-OMe-benzyl
11c: R = 3-pyridylmethyl
11d: R = m-F-benzyl
11e: R = p-iPr-benzyl
11f: R =
11g: R =

Scheme 2

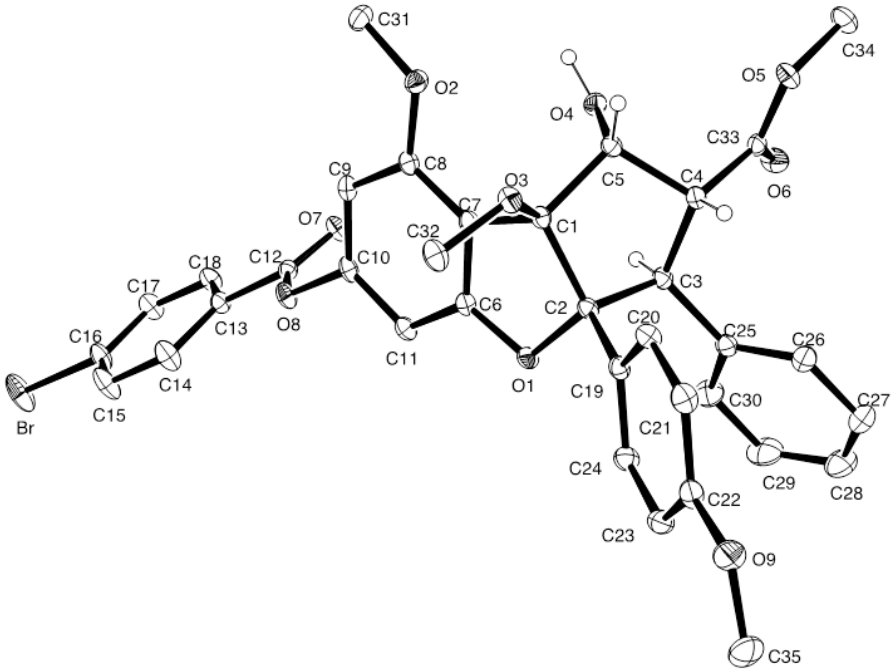




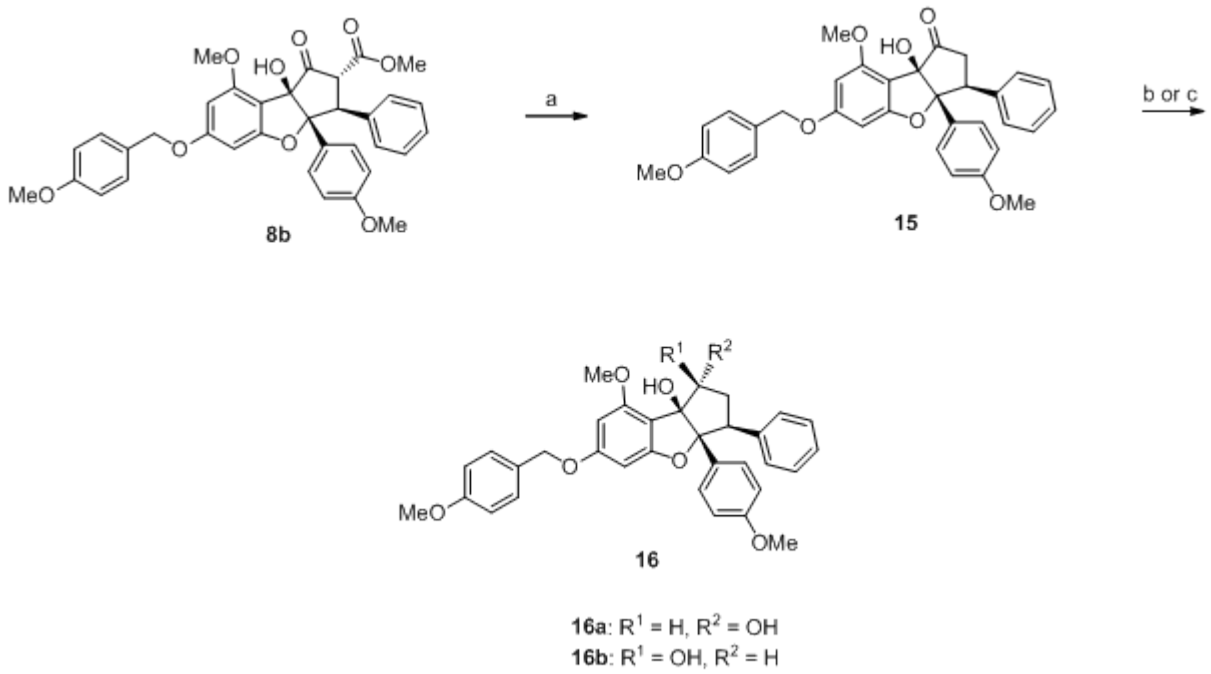
13a: R1 = Me
13b: R1 = Et

14a: R^1 = Me, R^2 = p-Br-benzoyl
14b: R^1 = Et, R^2 = m-OMe-benzyl
14c: R^1 = Et, R^2 = 4-pyridylmethyl

Figure 3

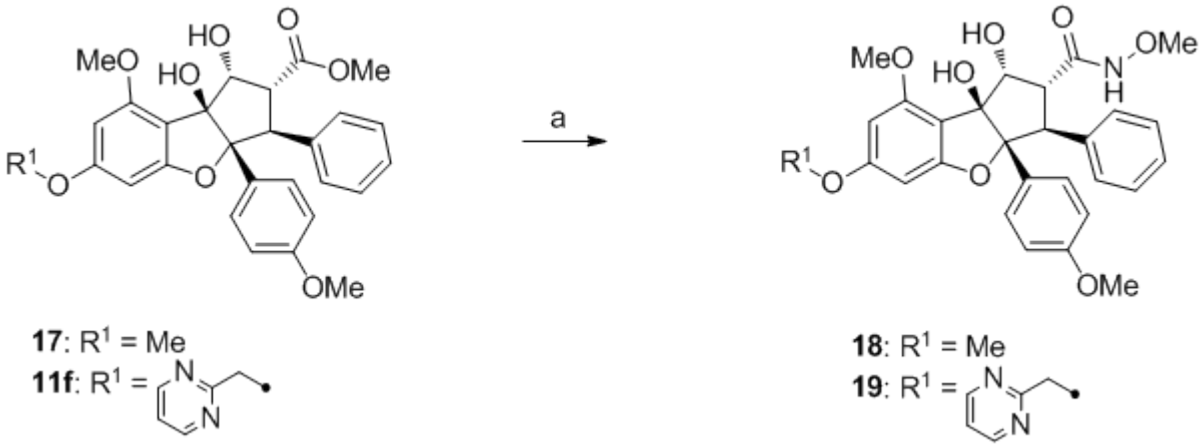

C31
O2
C34
O5
O4
C33
C8
C9
O3
C5
C4
O6
O7
C7
C32
C1
C18
C12
C10
C3
C17
C6
C2
C20
C25
C26
C16
C13
O8
C11
O1
C19
C30
C27
C14
C21
Br
C15
C24
C29
C28
C22
C23
O9
C35

Scheme 3



16a: R^1 = H, R^2 = OH
16b: R^1 = OH, R^2 = H

Scheme 4



17: R^1 = Me
11f: R^1 =

18: R^1 = Me
19: R^1 =

Figure 4

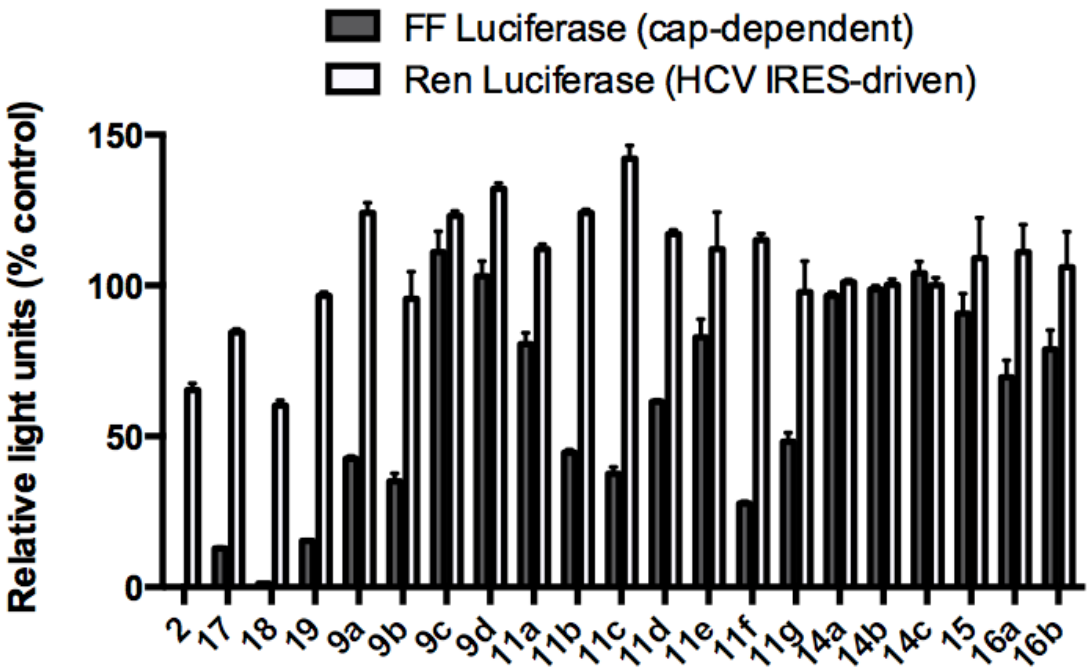

FF Luciferase (cap-dependent)
Ren Luciferase (HCV IRES-driven)
Relative light units (% control)
150
100
50
0
2
17
18
19
9a
9b
9c
9d
11a
11b
11c
11d
11e
11f
11g
14a
14b
14c
15
16a
16b

Figure 5

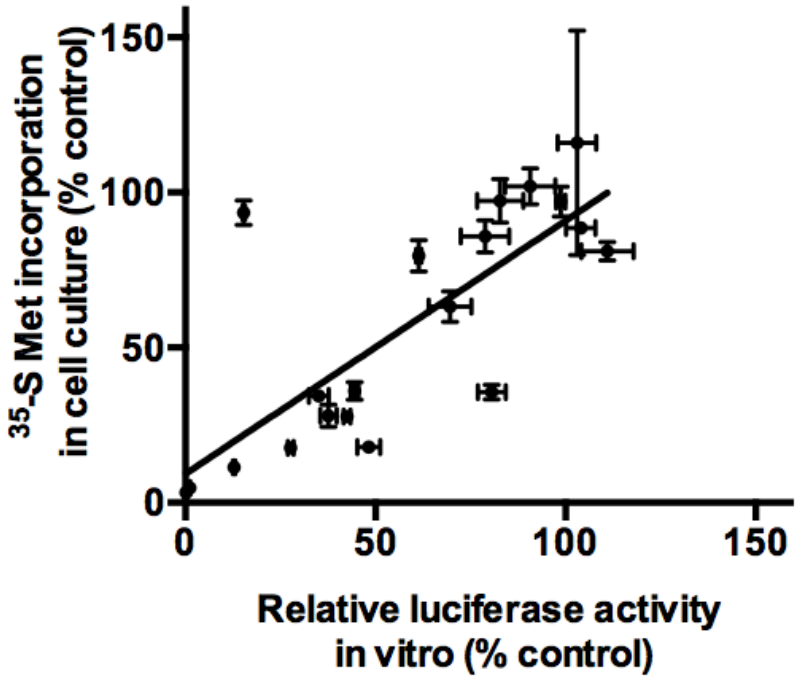
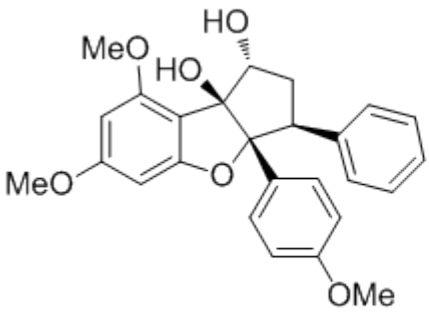
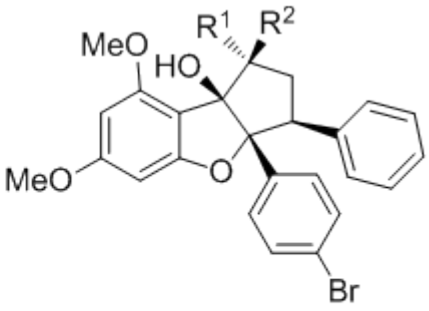

35S Met incorporation in cell culture (% control)
150
100
50
0
0
50
100
150
Relative luciferase activity in vitro (% control)

Figure 6



20



21: R^1 = OH, R^2 = H
22: R^1 = H, R^2 = OH

Figure 7

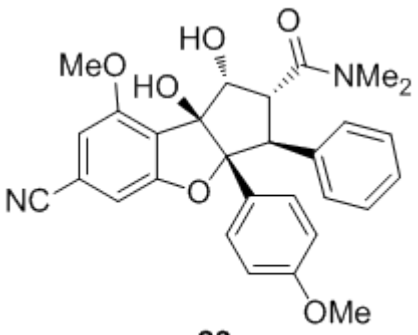


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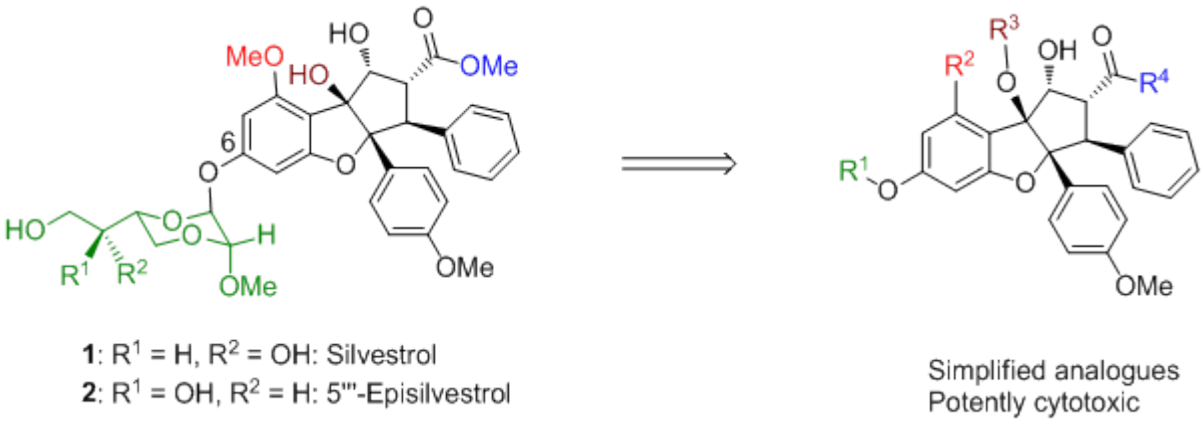
Table of Contents

The natural products silvestrol (**1**) and episilvestrol (**2**) are translation initiation inhibitors with potent anti-cancer activity. We report replacing the complex pseudo-sugar moiety at C6 with readily accessible and drug-like moieties. Selected compounds show potent anti-leukemic activity in vitro.



1: R^1 = H, R^2 = OH: Silvestrol
2: R^1 = OH, R^2 = H: 5'''-Episilvestrol

Simplified analogues
Potently cytotoxic