Bioorganic & Medicinal Chemistry 20 (2012) 2353–2361

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

2-Morpholinoisoflav-3-enes as flexible intermediates in the synthesis of phenoxodiol, isophenoxodiol, equol and analogues: Vasorelaxant properties, estrogen receptor binding and Rho/RhoA kinase pathway inhibition

Andrew J. Tilley [a], Shannon D. Zanatta [a], Cheng Xue Qin [b], In-Kyeom Kim [c], Young-Mi Seok [c], Alastair Stewart [b], Owen L. Woodman [d], Spencer J. Williams [a,*]

[a] School of Chemistry and Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, Australia
[b] Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia
[c] Cardiovascular Research Institute and Department of Pharmacology, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[d] School of Medical Sciences and Health Innovations Research Institute, RMIT University, Bundoora, Victoria, Australia

ARTICLE INFO

Article history:
Received 20 December 2011
Revised 26 January 2012
Accepted 2 February 2012
Available online 11 February 2012

Keywords:
Phytoestrogens
Kinase inhibitor
Vascular pharmacology
Polyphenols

ABSTRACT

Isoflavone consumption correlates with reduced rates of cardiovascular disease. Epidemiological studies and clinical data provide evidence that isoflavone metabolites, such as the isoflavan equol, contribute to these beneficial effects. In this study we developed a new route to isoflavans and isoflavenes via 2-morpholinoisoflavenes derived from a condensation reaction of phenylacetaldehydes, salicylaldehydes and morpholine. We report the synthesis of the isoflavans equol and deoxygenated analogues, and the isoflavenes 7,4′-dihydroxyisoflav-3-ene (phenoxodiol, haganin E) and 7,4′-dihydroxyisoflav-2-ene (isophenoxodiol). Vascular pharmacology studies reveal that all oxygenated isoflavans and isoflavenes can attenuate phenylephrine-induced vasoconstriction, which was unaffected by the estrogen receptor antagonist ICI 182,780. Furthermore, the compounds inhibited U46619 (a thromboxane A_2 analogue) induced vasoconstriction in endothelium-denuded rat aortae, and reduced the formation of GTP RhoA, with the effects being greatest for equol and phenoxodiol. Ligand displacement studies of rat uterine cytosol estrogen receptor revealed the compounds to be generally weak binders. These data are consistent with the vasorelaxation activity of equol and phenoxodiol deriving at least in part by inhibition of the RhoA/Rho-kinase pathway, and along with the limited estrogen receptor affinity supports a role for equol and phenoxodiol as useful agents for maintaining cardiovascular function with limited estrogenic effects.

1. Introduction

Epidemiological studies have correlated high consumption of leguminous foodstuffs, such as soy, with a reduced rate of cardiovascular disease.[1] While early attention focussed on isoflavones such as genistein and daidzein (**1**) as the major contributors to the positive biological activities, in recent years focus has moved to the metabolites formed from ingestion of these isoflavones (Fig. 1).[2] Metabolism of **1** or the related isoflavone formonetin **2** leads to the production of the isoflavan (*S*)-equol (**3d**),[2] and possibly the isoflavene phenoxodiol (**4**) (also known as dehydroequol and haganin E).[3] There is growing realization that **3d** has a range of beneficial effects on vascular function including as an antioxidant and as a vasorelaxant.[4] However, inter-individual variation in intestinal microflora between humans results in a greatly varying ability to produce **3d** upon isoflavone consumption. 30–50% of the adult human population cannot produce **3d** upon isoflavone consumption[5] and there is evidence that equol-producer status correlates with improved responses to isoflavone consumption in the treatment of cardiovascular disease, menopause and osteoporosis.[6] Phenoxodiol itself possesses significant anticancer activities and can act as a chemosensitizer for a variety of anticancer agents.[7]

Isoflavones and isoflavans possess structural similarities with steroidal hormones such as estrogen and estradiol and are known to bind to estrogen receptors.[8] Alternative sources of estrogenic compounds that do not possess the hormonal properties of estrogen are of interest for their therapeutic potential in the prevention of cardiovascular disease, particularly in men and in postmenopausal women as long term hormone-replacement therapy for prevention of coronary heart disease is no longer recommended.[9,10] Given the metabolism of dietary daidzein (**1**) to equol (**3d**), it is likely that many of the cardiovascular benefits ascribed to soy and red clover isoflavones in fact result from equol.[4] A recent clinical trial demonstrated that (*S*)-equol can alleviate hot flushes and

* Corresponding author.
E-mail address: sjwill@unimelb.edu.au (S.J. Williams).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2012.02.008

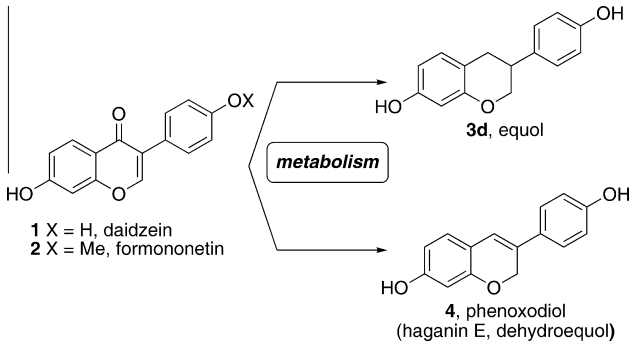


Figure 1. Equol, dehydroequol and their metabolism from dietary isoflavones.

other menopausal symptoms in postmenopausal equol-non-producing Japanese women.[11] Equol can inhibit noradrenaline-induced contraction of rat aorta and can relax pre-contracted aorta[12] and cerebral arteries both in vitro and in vivo.[13] In addition, equol has superior superoxide-scavenging activity to the isoflavones genistein and daidzein, and can preserve NO levels, thereby preventing oxidation of low density lipoprotein.[14] Phenoxodiol shares with daidzein the capacity to antagonize the contractile effects of noradrenaline, enhance vasodilatation and protect against endothelium damage by oxidized low density lipoprotein, and is comparable to 17β-estradiol in this application.[12] In rat carotid artery the vasorelaxant activity of equol is endothelium independent[13] whereas phenoxodiol causes endothelium-dependent relaxation of rat aorta.[12] In the present study we sought to develop a new route to equol and phenoxodiol allowing the synthesis of structural analogues, and to investigate structure–activity relationships for effective vasodilatation and the mechanism of vasodilatation compared to the hormone estrogen. As previous vascular studies with isoflavones have highlighted the importance of the presence of hydroxyl groups on the isoflavone core, this study focussed on the importance of hydroxylation on activity.

2. Results and discussion

2.1. Chemical synthesis

Published routes to homochiral isoflavan derivatives suffer from lengthy routes and the requirement for air and moisture-sensitive reagents.[15–18] Semi-synthetic routes to equol (**3d**) and phenoxodiol (**4**) rely upon partial hydrogenation of daidzein (**1**) and are limited in their ability to access analogues by the availability of the necessary isoflavone precursors.[19] Other de novo syntheses also suffer from problems with long synthetic routes.[20,21] Our attention was drawn to the work of Dean and Varma who reported a two-step synthesis of 2-morpholinoisoflav-3-ene (**9a**) in 55% overall yield.[22,23] In this approach reaction of morpholine and phenylacetaldehyde (**5a**) affords *N*-styrylmorpholine (**6**), which in a second step reacts with salicylaldehyde (**7a**) (Fig. 2; upper pathway). Given the structural similarity of such 2-morpholinoisoflav-3-enes and isoflavans, this route seemed a promising avenue for synthesis of equol and analogues. One-pot reaction of **5a**, **7a** and morpholine afforded **9a** directly in 62% yield (Fig. 2; middle pathway). This yield is a modest improvement on that obtained through the stepwise approach. Under these conditions it is possible that the reaction proceeds by an alternative pathway benefitting from organocatalysis by morpholine (Fig. 2; lower pathway). Thus, activation of phenylacetaldehyde by morpholine leads to **6**; reaction of morpholine with salicylaldehyde generates an iminium ion (**8**); and in situ Mannich-type condensation of these reactive species, with elimination of morpholine, affords **9a**.

Application of our one-pot approach to oxygenated substrates required the synthesis of the corresponding phenylacetaldehydes. The oxidation of 2-arylethanols using chromium reagents has been reported to be capricious, being complicated by carbon–carbon bond cleavage.[24] Instead, Dess–Martin oxidation of 4-benzyloxyphenylethanol (**10**) or IBX oxidation of 4-*tert*-butyldimethylsilyloxyphenyl)ethanol (**11**) afforded the respective phenylacetaldehydes **5b** and **5c** quantitatively (Fig. 3). One-pot synthesis of 2-morpholinoisoflav-3-enes **9b–d** bearing electron-donating benzyloxy substituents on either the phenylacetaldehyde or salicylaldehyde components, in the presence of 4 Å molecular sieves proceeded in good yield (Table 1). In particular, use of 4-benzyloxysalicyladehyde (**7b**) and 4-benzyloxyphenylacetaldehyde (**5b**)

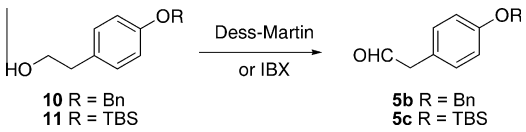


Figure 3. Preparation of phenylacetaldehydes.

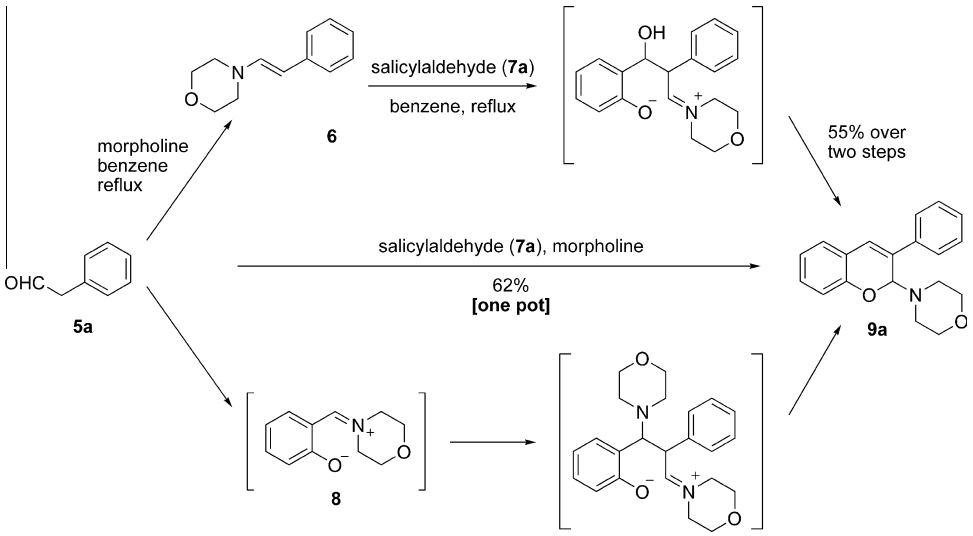
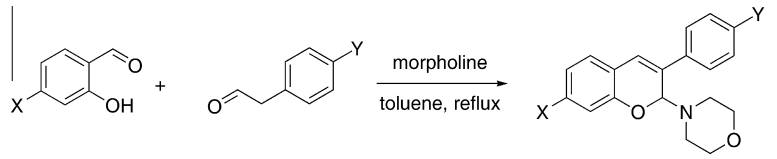


Figure 2. Upper pathway: Two-step synthesis of 2-morpholinoisoflav-3-ene **9a**. Middle pathway: one-pot synthesis of **9a**. Lower pathway: proposed mechanism of one-pot approach.

Table 1
Synthesis of 2-morpholinoisoflav-3-enes



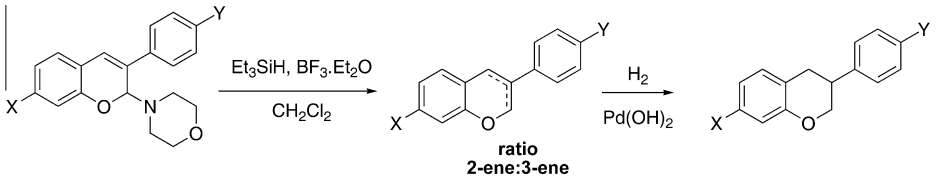
7a X = H	**5a** Y = H	**9a** X = Y = H	62%
7a X = H	**5b** Y = OBn	**9b** X = H, Y = OBn	58%
7b X = OBn	**5a** Y = H	**9c** X = OBn, Y = H	61%
7b X = OBn	**5b** Y = OBn	**9d** X = Y = OBn	80%
7c X = OTBS	**5c** Y = OTBS	**9e** X = Y = OTBS	30%

afforded the 4′,7-dibenzyloxyisoflavan (**9d**) in excellent yield (80%), isolated by recrystallization from the crude reaction mixture. On the other hand, condensation of the *tert*-butyldimethylsilyl protected salicylaldehyde (**7c**) and phenylacetaldehyde (**5c**) afforded the 4′,7-di-*tert*-butyldimethylsilyl isoflavan (**9e**) in low yield (30%), and required the omission of molecular sieves, most likely owing to the acid sensitivity of the silyl protecting groups.

Reduction of the hemiaminal of the 2-morpholinoisoflav-3-ene (**9a**) by Pd-catalyzed hydrogenolysis met with only limited success.[22] By contrast, ionic hydrogenation of **9a**, via an intermediate isoflavylium ion,[25] proved more effective. While this transformation could be effected by warming **9a** in formic acid in pyridine[26] to give **12a**, a mixture of isoflav-2-ene and 3-ene (83%, 1:2), this procedure was less successful on the oxygenated analogues. Alternatively, the reduction could be achieved by treatment with triethylsilane and $BF_3{\cdot}Et_2O$ affording 86% of a 1:4 mixture of the 2-ene and 3-ene **12a** (Table 2). These latter conditions proved effective for the reduction of the oxygenated congeners, affording mixtures of the isoflav-2-enes and -3-enes **12b–d** in good yields with isolation effected by recrystallization. Reduction of the isoflavene mixtures with H_2 and Pd–C or $Pd(OH)_2$–C effected double bond saturation and, in the cases of **12b–d**, protecting group removal, affording the isoflavans **3a–d** in good yields (Table 2). Notably, in the case of equol (**3d**) the product was purified simply by recrystallization.

Initially, it was anticipated that phenoxodiol (**4**) and isophenoxodiol (**15**) could be prepared from the isomeric benzyl-protected isoflavenes **9d**. However, these compounds possessed poor solubility in organic solvents suitable for chromatographic resolution. Instead, *tert*-butyldimethylsilyl protecting groups were investigated with the aim of improving their organic solubility. Reduction of **9e** using triethylsilane and $BF_3{\cdot}Et_2O$ afforded a readily separable mixture of the 2-ene **13** and 3-ene **14** (1:5) (Fig. 4). Finally, individual deprotection of each isomer with HF·pyridine afforded **15** and **4**.

Table 2
Synthesis of isoflavans



9a X = Y = H	**12a** X = Y = H	1:4	86%	**3a** X = Y = H	86%
9b X = H, Y = OBn	**12b** X = H, Y = OBn	1:4	80%	**3b** X = H, Y = OH	63%
9c X = OBn, Y = H	**12c** X = OBn, Y = H	1:3	74%	**3c** X = OH, Y = H	71%
9d X = Y = OBn	**12d** X = Y = OBn	1:3	80%	**3d** X = Y = OH	75%

Dashed bonds indicate the presence of two regioisomeric alkenes, in the ratio shown in the table.

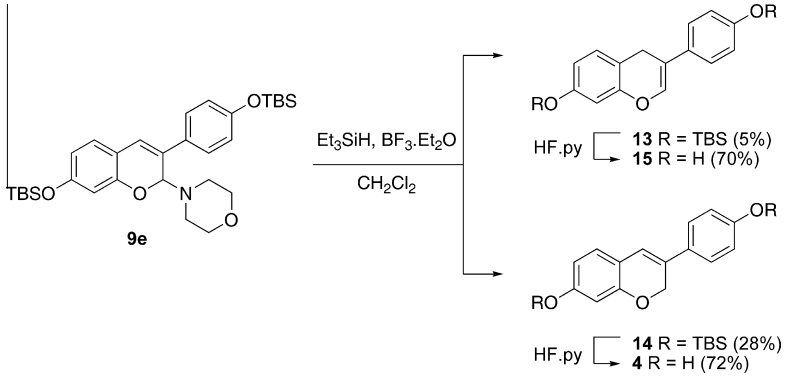


Figure 4. Synthesis of 7,4′-dihydroxyisoflav-2-ene (isophenoxodiol) **15** and -3-ene (phenoxodiol) **4**.

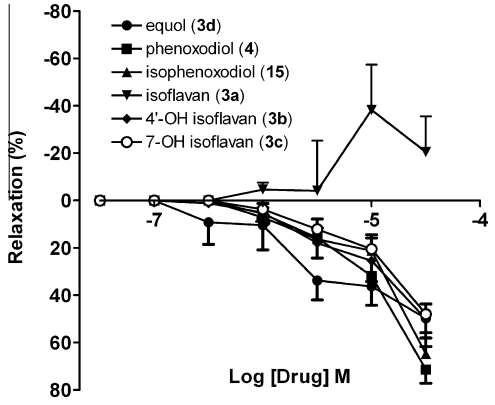


Figure 5. Effect of equol and analogues on tension in rat isolated aortae precontracted with phenylephrine ($n = 3–5$). Error bars represent standard error mean.

2.2. Vascular activity

Vasorelaxant activity was assessed by measuring inhibition of contraction of rat thoracic aorta induced by phenylephrine (PE), as it has been shown that isoflavones can act as functional antagonists of PE.[27] Equol (**3d**), phenoxodiol (**4**), isophenoxodiol (**15**) and both 7-hydroxy (**3c**) and 4′-hydroxy (**3b**) isoflavans caused concentration-dependent relaxation of rat isolated aortae, whereas isoflavan (**3a**) caused only contraction at high concentrations (Fig. 5). The responses to each of the compounds were unaffected by the estrogen receptor antagonist ICI 182,780 (0.1 μM, data not shown), demonstrating that the vasorelaxant activity is estrogen-receptor independent.

The isoflavones daidzein (**1**) and genistein attenuate vascular contraction through inhibition of the RhoA/Rho-kinase signalling pathway.[28] In order to investigate attenuation of vascular contraction by this pathway, we studied the effects of the isoflavans and isoflavenes in endothelium-denuded rat aortic rings. After pretreatment with the isoflavenes or isoflavans, or vehicle for 30 min, the addition of 11,9-epoxymethano-prostaglandin F2α (U46619), a thromboxane A2 analog, increased vascular tension, which was inhibited by pretreatment with 100 μM of each of the compounds (Fig. 6A). We utilized 300 μM flavone as a positive control as this compound has previously been shown to be an inhibitor of U46619-induced vasoconstriction.[29] Limited inhibition of contraction was seen for isoflavan (**3a**) and the monohydroxylated isoflavans **3b** and **3c**, moderate inhibition for equol (**3d**), and the greatest effect was seen for phenoxodiol (**4**).

In order to examine the effect of the compounds on the RhoA/Rho-kinase pathway, we examined the levels of guanosine triphosphate (GTP)-RhoA. In its active GTP-bound form, RhoA activates Rho-kinase, which then phosphorylates and inactivates the downstream target myosin light chain (MLC) phosphatase.[30] The increase in GTP-RhoA over control therefore serves as a marker of Rho-kinase activation. The rank order of inhibition of U46619-induced GTP RhoA largely follows the trend in inhibition of U46619-induced vasoconstriction (Fig. 6A). Limited inhibition is seen for isoflavans **3b** and **3c**, moderate inhibition for isoflavan (**3a**) and equol (**3d**), with the greatest effect being seen for phenoxodiol (**4**).

2.3. Displacement of [^{3}H]-estradiol binding

In order to assess the potential hormonal properties of equol and analogues we examined their ER affinity using estrogen receptor isolated from rat uterine cytosol (Fig. 7). The rank order of potency of the analogues in displacing [^{3}H]-estradiol binding from rat uterine cytosol preparations was isophenoxodiol (**15**) > phenoxodiol (**4**) > equol (**3d**) > 7-OH isoflavan (**3c**) ~ 4-OH-isoflavan (**3b**) ≫ isoflavan (**3a**) (Table 3). Affinities for the ER were several orders of magnitude lower than for the ER antagonist ICI 182,780. It is well established that the presence and distance between the hydroxyls of estradiol and analogues are key determinants of ER binding affinity.[8] This data is in accord with these established trends, with the dihydroxy compounds isophenoxodiol (**15**), phenoxodiol (**4**) and equol (**3d**) possessing the highest potencies, the mono-hydroxy isoflavans **3b** and **3c** showing less affinity, and isoflavan (**3a**), without hydroxy substituents, having no detectable affinity at 10 μM. The affinity of equol (**3d**) determined here is similar to that previously reported for rat uterine cytosol preparations.[31]

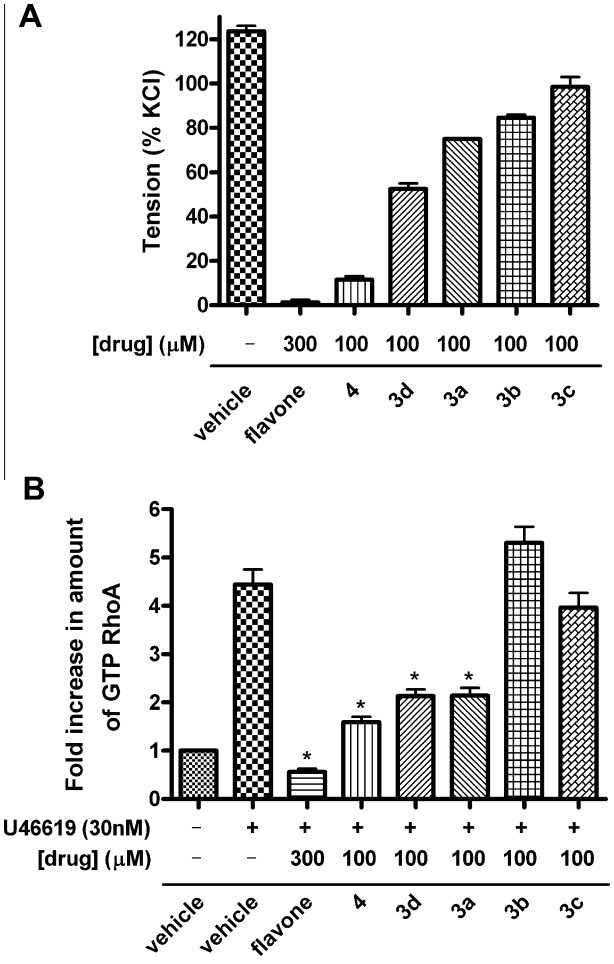


Figure 6. (A) Inhibitory effect of isoflavans and an isoflavene on U46619-induced vasoconstriction in rat aorta ($n = 2$). All experiments including vehicle contained 30 nM U46619. (B) Effect of compounds on U46619-induced increase in GTP-RhoA ($n = 3$). Error bars represent standard error mean. * significance of $p < 0.01$ (ANOVA, Dunnett's post test) relative to vehicle plus U46619.

3. Conclusion

We have described an exceptionally simple method for the synthesis of equol **3d** and related isoflavans **3a–c**. This method was used to synthesize the isoflavenes phenoxodiol (**4**) and isophenoxodiol **15.** The key step is a hybrid organocatalytic three-component coupling wherein morpholine acts both as an organocatalyst in a Mannich-type reaction, and stoichiometrically, affording 2-morpholinoisoflav-3-enes, which can be reduced to isoflavene mixtures, and thence to the isoflavans.

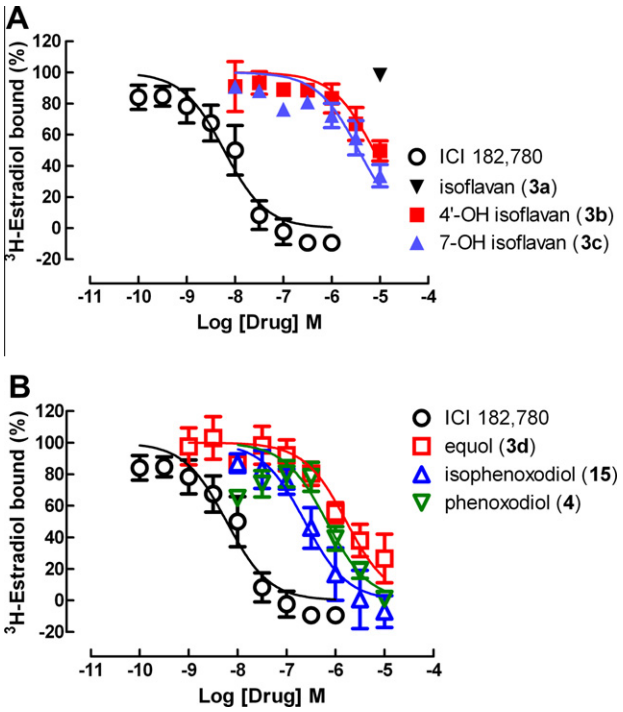


Figure 7. [^{3}H]-Estradiol displacement by the ER antagonist, ICI 182,780 and by isoflavans and isoflavenes in rat uterine cytosol estrogen receptor (n = 3). Error bars represent standard error mean.

Table 3
Log IC_{50} values for displacement of [^{3}H]-estradiol in rat uterine cytosol estrogen receptor by isoflavans and isoflavenes (n = 3)

Compound	Log IC_{50} mean ± SEM
Equol (**3d**)	5.78 ± 0.11
Phenoxodiol (**4**)	6.18 ± 0.13
Isophenoxodiol (**15**)	6.63 ± 0.13
Isoflavan (**3a**)	>5
4-OH isoflavan (**3b**)	5.12 ± 0.10
7-OH isoflavan (**3c**)	5.44 ± 0.10
ICI 182,780	8.23 ± 0.12

All mono- and dihydroxylated isoflavans and isoflavenes caused concentration-dependent relaxation of rat isolated aortae, whereas the unsubstituted isoflavan caused only contraction at high concentrations. Vasorelaxation was ER-independent, as demonstrated by the lack of effect of the ER alpha and beta antagonist ICI 182,780 on vasorelaxant responses to the isoflavenes and isoflavans. RhoA/Rho-kinase activation plays an important role in the tonic component of U46619-induced contraction and MLC_{20} phosphorylation.[32,33] U46619 activates RhoA, and was inhibited by both equol and phenoxodiol. ER receptor binding assays revealed moderate affinities for the dihydroxylated compounds equol, phenoxodiol and isophenoxodiol. This study suggests that the vascular activity of the most potent analogues, equol, phenoxodiol and isophenoxodiol, is ER independent and derives, at least in part, from inhibition of the RhoA/Rho-kinase pathway. In this context it is noteworthy that the vasorelaxant activity of other polycyclic aromatic compounds such as the isoflavones daidzein and genestein,[28] the flavones flavone[29] and 3′,4′-dihydroxyflavonol,[34] and the polyphenols honokiol,[35] glyceollin I[36] and gomisin A[37] derive in part or wholly from inhibition of the RhoA/Rho-kinase pathway.

Dietary isoflavones and their metabolites may have potential benefits in the maintenance of human health. Owing to the risks of long-term administration of estrogen therapy, phytoestrogens have emerged as a possible alternative that may provide similar benefits with reduced side-effects. However, variability in metabolism between individuals suggests that administration of metabolites or their structural variants may prove a more reliable therapeutic approach. This study shows that structural variation of the metabolic products of isoflavones can tune their vascular activities, and therefore that modified isoflavans and/or isoflavenes may prove to be alternative agents for maintaining cardiovascular function while limiting direct hormonal activities.

4. Experimental

4.1. General chemical methods

All solvents were distilled prior to use. Petroleum spirits refers to the fraction boiling range 40–60 °C. Thin layer chromatography (t.l.c.) was performed on aluminium sheets pre-coated with Merck Silica Gel 60, using mixtures of EtOAc/petroleum spirits and EtOAc/petroleum spirits/Et_3N. Detection was achieved via irradiation by UV light. Flash chromatography was performed using the method of Still et al. using Scharlau Silica Gel 60.[38] NMR data was recorded on Varian Inova 400 and 500 instruments. Chemical shifts are expressed in parts per million (δ) using residual solvent (^{1}H NMR δ 7.26 ppm for $CDCl_3$, δ 2.50 ppm for DMSO-d_6; ^{13}C NMR δ 77.36 ppm for $CDCl_3$, 39.52 ppm for DMSO-d_6) or TMS as an internal standard (δ 0.00 ppm), and J values are reported in Hz. Melting points were obtained using a Riechert-Jung hot-stage melting point apparatus and are corrected. High resolution electrospray ionization mass spectra were obtained on a Finnigan LTQ FT mass spectrometer (The University of Melbourne). Microanalysis was performed by CMAS (Belmont, Victoria).

4.2. Chemical synthesis

4.2.1. 2-Morpholinoisoflav-3-ene (9a)

A mixture of phenylacetaldehyde **5a** (0.29 mL, 2.5 mmol), salicylaldehyde **7a** (0.27 mL, 2.5 mmol), morpholine (0.22 mL, 2.5 mmol) and 4 Å molecular sieves in toluene (2 mL) was stirred at reflux for 24 h. The mixture was filtered and the filtrate diluted with toluene (15 mL). The organic phase washed with 1 M NaOH (5 × 10 mL), satd aq $NaHCO_3$ (4 × 10 mL) and brine (3 × 10 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a light brown crystalline solid. The solid was recrystallized to afford compound **9a** as a yellow crystalline solid (0.457 g, 62%), mp 101–103 °C (EtOAc/petroleum spirits; lit.[22] 105–106 °C). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.65 (dt, 2H, ^3J = 4.6, ^2J = 11.8, CH_2NCH_2), 3.10 (dt, 2H, ^3J = 4.6, ^2J = 11.9, CH_2NCH_2), 3.63 (t, 4H, J = 4.8, CH_2OCH_2), 5.78 (s, 1H, H2), 6.91–6.96 (m, 2H, H3′,5′), 7.04–7.46 (m, 6H, Ar), 7.70–7.73 (m, 2H, H2′,6′). ^{13}C-NMR (500 MHz, $CDCl_3$) δ 47.1, 67.2, 90.7, 114.9, 120.8, 121.2, 123.5, 126.1, 127.1, 127.8, 128.3, 128.5, 129.7, 137.7, 153.7.

4.2.2. Mixture of isoflav-2-ene and isoflav-3-ene (12a)

$BF_3{\cdot}Et_2O$ (1.12 mL, 8.80 mmol) was added dropwise to a stirred solution of 2-morpholinoisoflav-3-ene **9a** (1.17 g, 4.00 mmol) and Et_3SiH (1.28 mL, 8.00 mmol) in CH_2Cl_2 (20 mL) at 0 °C. After 30 min, the reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 22 h. The reaction was quenched with satd aq $NaHCO_3$ (20 mL), diluted with CH_2Cl_2 (20 mL) and the organic phase was separated and washed with satd aq $NaHCO_3$ (3 × 30 mL), brine (3 × 30 mL) and the combined aqueous layers re-extracted with additional CH_2Cl_2 (50 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow solid. The yellow solid was purified by passing through a plug of silica (20%

EtOAc/petroleum spirits + 1% NEt_3) to afford a crystalline yellow powder (0.718 g, 86%). A small portion was recrystallized from EtOAc/petroleum spirits to afford a mixture of isoflav-2-ene and -3-ene **12a** (1:4) as a colourless powder. Partial ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.79 (s, 2H, H4,4, 2-ene), 5.18 (d, 2H, $J_{2,4}$ 1.4, H2,2, 3-ene).

4.2.3. 3-Phenylchroman (3a)

An isomeric mixture of isoflavenes **12a** (157 mg, 0.754 mmol) and $Pd(OH)_2$ (20 mg) in EtOAc (5 mL) was treated with H_2 at atmospheric pressure for 28 h. The mixture was filtered (Celite), and the filtrate concentrated under reduced pressure to give a yellow oil. The yellow oil was recrystallized to afford the chroman **3a** as a colourless solid (135 mg, 86%), mp 52–54 °C (petroleum spirits; lit.[22] 53–54 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.97–3.10 (m, 2H, H4,4), 3.19–3.28 (m, 1H, H3), 4.03 (t, 1H, J = 10.6, H2), 4.27–4.33 (m, 1H, H2), 6.83–6.89 (m, 2H, H3′,5′), 7.06–7.14 (m, 2H, Ar), 7.21–7.28 (m, 3H, H2′,4′,6′), 7.29–7.37 (m, 2H, Ar). ^{13}C NMR (500 MHz, $CDCl_3$) δ 32.6, 38.8, 71.0, 116.7, 120.5, 122.1, 127.2, 127.5, 127.6, 128.9, 129.9, 141.5, 154.5.

4.2.4. 4-Benzyloxyphenylacetaldehyde (5b)

Dess–Martin periodinane (1.103 g, 2.60 mmol) was added to a solution of 2-(4-benzyloxyphenyl)ethanol (0.457 g, 2.00 mmol) in CH_2Cl_2 (15 mL) at room temperature, and the resultant mixture allowed to stir for 0.5 h. A 1:1 mixture of $Na_2S_2O_3$ (1.5 M) and satd aq $NaHCO_3$ (30 mL) was added and the mixture stirred until two clear layers formed. The organic phase was taken up in ether (20 mL), washed with water (3 × 20 mL) and brine (3 × 20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford the crude aldehyde **5b** as a clear yellow oil which was used in the subsequent step without purification.

4.2.5. 4′-Benzyloxy-2-morpholinoisoflav-3-ene (9b)

A mixture of salicylaldehyde **7a** (0.21 mL, 2.0 mmol), morpholine (0.18 mL, 2.0 mmol), 2-(4-benzyloxyphenyl)acetaldehyde **5b** (0.453 g, 2.00 mmol) and 4 Å molecular sieves in toluene (10 mL) was stirred at reflux for 24 h. The mixture was cooled, filtered and the filtrate was diluted with toluene (25 mL). The organic phase was washed with 1 M NaOH (4 × 25 mL), satd aq $NaHCO_3$ (4 × 25 mL) and brine (2 × 25 mL). The organic extract was dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford the isoflavene **9b** as an off-white crystalline solid (0.460 g, 58%), mp 134–136 °C (EtOAc/petroleum spirit); ($C_{26}H_{25}NO_3$ requires C, 78.2; H, 6.3; N, 3.5. Found: C, 78.2; H, 6.3; N, 3.6). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.63 (dt, 2H, ^3J = 4.4, ^2J = 11.8, CH_2NCH_2), 3.05 (dt, 2H, ^3J = 4.4, ^2J = 12.0, CH_2NCH_2), 3.61 (t, 4H, J = 5.1, CH_2OCH_2), 5.11 (s, 2H, CH_2Ph), 5.73 (s, 1H, H2), 6.90–6.94 (m, 2H, Ar), 6.98–7.01 (m, 3H, H4,3′,5′), 7.11 (dd, 1H, J = 1.6, J = 7.4, Ar), 7.15–7.19 (m, 1H, Ar), 7.33–7.46 (m, 5H, Ph), 7.64 (d, 2H, J = 9.0, H2′,6′). ^{13}C NMR (500 MHz, $CDCl_3$) δ 47.2, 67.6, 70.2, 90.80, 90.81, 115.0, 120.9, 121.5, 122.1, 127.0, 127.5, 128.0, 128.1, 128.2, 128.8, 129.5, 130.7, 137.0, 153.7, 158.8. HRMS (ESI+) m/z 400.1906 ($C_{26}H_{26}NO_3$ $[M+H]^+$ requires 400.1913).

4.2.6. Mixture of 4′-benzyloxyisoflav-2-ene and 4′-benzyloxyisoflav-3-ene (12b)

$BF_3{\cdot}Et_2O$ (0.31 mL, 2.4 mmol) was added dropwise to a stirred solution of 4′-benzyloxy-2-morpholinoisoflav-3-ene **9b** (0.439 g, 1.10 mmol) and Et_3SiH (0.35 mL, 2.2 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 30 min, the reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 16 h. The reaction was quenched with satd aq $NaHCO_3$ (10 mL), diluted with CH_2Cl_2 and the organic phase was washed with satd aq $NaHCO_3$ (3 × 25 mL), brine (2 × 25 mL) and the combined aqueous layers re-extracted with additional CH_2Cl_2 (30 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a light yellow solid, which was recrystallized from EtOAc/petroleum spirits to afford a mixture of isoflav-2-ene and -3-ene **12b** (1:4) as a colourless solid (0.277 g, 80%). Partial ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.75 (s, 2H, H4,4, 2-ene), 5.14 (d, 2H, $J_{2,4}$ = 1.4, H2,2, 3-ene).

4.2.7. 3-(4′-Hydroxyphenyl)chroman (3b)

The isomeric mixture of isoflavenes **12b** (126 mg, 0.400 mmol), Pd/C (25 mg) and AcOH (0.1 mL) in 1:1 THF/EtOAc (10 mL) was treated with H_2 (10 atm) for 24 h. The mixture was filtered (Celite), and the filtrate concentrated under reduced pressure to give a light brown solid which was purified by flash chromatography (20% EtOAc/petroleum spirits + 1% AcOH), affording **3b** as a colourless solid (57 mg, 63%), mp 102–103 °C (EtOAc/petroleum spirit; lit.[39] 120–122 °C); ($C_{15}H_{14}O_2$ requires C, 79.6; H, 6.2. Found: C, 79.5; H, 6.2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.96–3.06 (m, 2H, H4,4), 3.17–3.23 (m, 1H, H3), 3.99 (t, 1H, J = 10.6, H2), 4.31–4.34 (m, 1H, H2), 4.78–4.80 (br s, 1H, OH), 6.81–6.89 (m, 4H, H3′,5′,Ar), 7.08–7.15 (m, 4H, H2′,6′,Ar). ^{13}C NMR (500 MHz, $CDCl_3$) δ 32.7, 37.9, 71.2, 115.8, 116.7, 120.5, 122.2, 127.6, 128.7, 129.9, 133.7, 154.4, 154.7. HRMS (ESI+) m/z 249.0886 ($C_{15}H_{14}NaO_2$ $[M+Na]^+$ requires 249.0891).

4.2.8. 7-Benzyloxy-2-morpholinoisoflav-3-ene (9c)

A mixture of phenylacetaldehyde **5a** (0.18 mL, 1.5 mmol), 4-benzyloxysalicylaldehyde **7b** (0.343 g, 1.50 mmol), morpholine (0.13 mL, 1.5 mmol) and 4 Å molecular sieves in toluene (2.0 mL) was stirred at reflux for 24 h. The mixture was filtered and the filtrate was diluted with toluene (25 mL). The organic phase was washed with 1 M NaOH (4 × 20 mL), satd aq $NaHCO_3$ (4 × 20 mL) and brine (2 × 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow/brown crystalline solid. The solid was recrystallized to afford **9c** as a light-brown crystalline solid (0.37 g, 61%), mp 143–144 °C (EtOAc/petroleum spirits); ($C_{26}H_{25}NO_3$ requires C, 78.2; H, 6.3; N, 3.5. Found: C, 78.2; H, 6.4; N, 3.5). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.65 (dt, 2H, ^3J = 4.8, ^2J = 11.9, CH_2NCH_2), 3.05 (dt, 2H, ^3J = 4.5, ^2J = 12.1, CH_2NCH_2), 3.62 (t, 4H, J = 4.8, CH_2OCH_2), 5.06 (d, 1H, J = 11.4, CH_2Ph), 5.09 (d, 1H, J = 11.4, CH_2Ph), 5.75 (s, 1H, H2), 6.55–6.60 (m, 2H, H3′,5′), 7.06–7.07 (m, 2H, Ar), 7.27–7.47 (m, 8H, Ar), 7.68 (d, 2H, J = 8.4, H2′,6′). ^{13}C NMR (500 MHz, $CDCl_3$) δ 47.2, 67.4, 70.3, 91.1, 101.4, 108.1, 115.1, 123.3, 125.8, 126.0, 127.6, 127.7, 128.0, 128.2, 128.6, 128.7, 136.9, 138.0, 155.2, 160.5. HRMS (ESI+) m/z 422.1727 ($C_{26}H_{25}NNaO_3$ $[M+Na]^+$ requires 422.1732).

4.2.9. Mixture of 7-benzyloxyisoflav-2-ene and 7-benzyloxyisoflav-3-ene (12c)

$BF_3{\cdot}Et_2O$ (0.28 mL, 2.2 mmol) was added dropwise to a stirred solution of 7-benzyloxy-2-morpholinoisoflav-3-ene **9c** (0.398 g, 0.996 mmol) and Et_3SiH (0.32 mL, 2.0 mmol) in CH_2Cl_2 (7 mL) at 0 °C. After 30 min, the reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 21 h. The reaction was quenched with satd aq $NaHCO_3$ (10 mL), diluted with CH_2Cl_2 (20 mL) and the organic phase was separated and washed with satd aq $NaHCO_3$ (3 × 25 mL), brine (2 × 25 mL) and the combined aqueous layers re-extracted with additional CH_2Cl_2 (50 mL). The combined organic extract was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow solid. The yellow solid was purified by passing through a plug of silica (20% EtOAc/petroleum spirits + 1% NEt_3) to afford a mixture of isoflav-2-ene and -3-ene **12c** (1:3) as a yellow powder (0.23 g, 74%). Partial ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72 (s, 2H, H4,4, 2-ene), 5.15 (d, 2H, $J_{2,4}$ 1.4, H2,2, 3-ene).

4.2.10. 7-Hydroxy-3-phenylchroman (3c)

An isomeric mixture of isoflavenes **12c** (157 mg, 0.499 mmol) and $Pd(OH)_2$ (25 mg) and AcOH (0.1 mL) in 1:1 THF/EtOAc (5 mL) was treated with H_2 (10 atm) for 24 h. The mixture was filtered (Celite), and the filtrate was concentrated under reduced pressure to give a colourless solid. The solid was recrystallized to afford the chroman **3c** as a colourless powder (80 mg, 71%), mp 132–134 °C (EtOAc/petroleum spirits; lit.[40] 130 °C); ($C_{15}H_{14}O_2$ requires C, 79.6; H, 6.2. Found: C, 79.8; H, 6.4). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.92–3.02 (m, 2H, H4,4), 3.19–3.25 (m, 1H, H3), 4.02 (t, 1H, J = 10.6, H2), 4.32–4.35 (m, 1H, H2), 4.63–4.88 (br s, 1H, OH), 6.37–6.41 (m, 2H, Ar), 6.95 (d, 1H, J = 8.2, H5), 7.24–7.37 (m, 3H, Ar), 7.34–7.37 (m, 2H, Ar). ^{13}C NMR (500 MHz, $CDCl_3$) δ 31.9, 38.9, 71.0, 103.4, 108.2, 114.5, 127.2, 127.5, 128.9, 130.6, 141.5, 155.1, 155.2. HRMS (ESI+) m/z 227.1067 ($C_{15}H_{15}O_2$ $[M+H]^+$ requires 227.1072).

4.2.11. 4′,7-Dibenzyloxy-2-morpholinoisoflav-3-ene (9d)

A mixture of morpholine (0.14 mL, 1.5 mmol), 2-(4-benzyloxyphenyl)acetaldehyde **5b** (0.339 g, 1.50 mmol), 4-benzyloxysalicylaldehyde **7b** (0.342 g, 1.50 mmol) and 4 Å molecular sieves in toluene (10 mL) was stirred at reflux for 20 h. The mixture was filtered and the filtrate was diluted with toluene. The organic phase was washed with 1 M NaOH (4 × 30 mL), satd aq $NaHCO_3$ (3 × 30 mL) and brine (30 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a light brown solid. The solid was recrystallized to afford **9d** as a light brown crystalline powder (0.606 g, 80%), mp 162–164 °C (EtOAc/petroleum spirits); ($C_{33}H_{31}NO_4$ requires C, 78.4; H, 6.2; N, 2.8. Found: C, 78.4; H, 6.1; N, 2.7). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.64 (dt, 2H, ^3J = 4.5, ^2J = 11.8, CH_2NCH_2), 3.05 (dt, 2H, ^3J = 4.6, ^2J = 11.9, CH_2NCH_2), 3.61 (t, 4H, J = 4.8, CH_2OCH_2), 5.05 (d, 1H, J = 11.5, CH_2Ph), 5.08 (d, 1H, J = 11.6, CH_2Ph), 5.10 (s, 2H, CH_2Ph), 5.70 (s, 1H, H2), 6.56 (dd, 1H, $J_{6,8}$ = 2.5, $J_{5,6}$ = 8.3, H6), 6.59 (d, 1H, $J_{6,8}$ = 2.5, H8), 6.95 (s, 1H, H4), 6.98 (d, 2H, J = 9.0, H3′,5′), 7.02 (d, 1H, J = 8.3, H5), 7.32–7.45 (m, 10H, Ph). ^{13}C NMR (500 MHz, $CDCl_3$) δ 31.1, 47.2, 67.4, 70.2, 70.3, 91.2, 101.4, 108.0, 115.0, 115.2, 121.8, 125.4, 127.3, 127.6, 127.7, 127.8, 128.2, 128.7, 128.8, 130.9, 136.9, 137.1, 154.9, 158.5, 160.3. HRMS (ESI+) m/z 506.2324 ($C_{33}H_{32}NO_4$ $[M+H]^+$ requires 506.2331).

4.2.12. Mixture of 4′,7-dibenzyloxyisoflav-2-ene and 4′,7-dibenzyloxyisoflav-3-ene (12d)

$BF_3{\cdot}Et_2O$ (0.17 mL, 1.3 mmol) was added dropwise to a stirred solution of 4′,7-dibenzyloxy-2-morpholinoisoflav-3-ene **9d** (0.290 g, 0.574 mmol) and Et_3SiH (0.19 mL, 1.2 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 30 min, the reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 15 h. The reaction was quenched with satd aq $NaHCO_3$ (15 mL), diluted with CH_2Cl_2 (20 mL) and the organic phase washed with satd aq $NaHCO_3$ (3 × 25 mL), brine (3 × 25 mL) and the combined aqueous layers re-extracted with additional CH_2Cl_2 (50 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a light brown solid, which was recrystallized from THF/petroleum spirits to afford a mixture of isoflav-2-ene and -3-ene **12d** (1:4) as a light brown powder (0.199 g, 80%). Partial ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.67 (s, 2H, H4,4, 2-ene), 5.11 (d, 2H, $J_{2,4}$ = 1.4, H2,2, 3-ene).

4.2.13. Equol (3d)

An isomeric mixture of isoflavenes **12d** (180 mg, 0.428 mmol), $Pd(OH)_2$ (50 mg) and AcOH (0.1 mL) in 1:1 THF/EtOAc (10 mL) was treated with H_2 (10 atm) for 36 h. The mixture was filtered (Celite), and the filtrate concentrated under reduced pressure to give a light brown solid, which was recrystallized to afford racemic equol **3d** as a off-white plates (78 mg, 75%), mp 157–158 °C (EtOH/water; lit.[19] 158–160 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.74–2.86 (m, 2H, H4,4), 2.98–3.04 (m, 1H, H3), 3.89 (t, 1H, J = 10.5, H2), 4.13–4.15 (m, 1H, H2), 6.19 (d, 1H, $J_{6,8}$ = 2.2, H8), 6.28 (dd, 1H, $J_{5,6}$ = 8.2, $J_{6,8}$ = 2.3, H6), 6.71 (d, 2H, $J_{2',3'}$ = 8.4, H3′,5′), 6.86 (d, 1H, $J_{5,6}$ = 8.2, H5), 7.10 (d, 2H, $J_{2',3'}$ = 8.4, H2′,6′), 9.00–9.56 (br s, 2H, OH). ^{13}C NMR (500 MHz, DMSO-d_6) δ 31.3, 37.1, 70.2, 102.5, 108.0, 112.5, 115.2, 128.3, 130.0, 131.6, 154.5, 156.1, 156.6.

4.2.14. 2-(4-*tert*-Butyldimethylsiloxyphenyl)acetaldehyde (5c)

A mixture of IBX (1.31 g, 4.69 mmol) and 2-(4-*tert*-butyldimethylsilyloxyphenyl)ethanol (**11**)[41] (847 mg, 3.35 mmol) in DMSO (8 mL) was stirred at rt for 4.5 h. EtOAc (20 mL) was then added followed by 1.5 M $Na_2S_2O_3$ (10 mL) and satd $NaHCO_3$ (10 mL). The aqueous layer was extracted with EtOAc (10 mL × 2) and the combined organic extracts washed with water (2 × 10 mL), brine (10 mL), dried ($MgSO_4$) and concentrated. The crude 2-(4-*tert*-butyldimethylsiloxyphenyl)acetaldehyde **5c** was used without purification (734 mg, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.20 (s, 12H, Si(CH_3)$_2$(C(CH_3)$_3$)), 0.98 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$)), 3.61 (d, 2H, J = 2.8, CH_2), 6.83 (app. d, 2H, J = 8.4, Ar-H), 7.07 (app. d, 2H, J = 8.4, Ar-H), 9.71 (t, 1H, J = 2.8, CHO).

4.2.15. 4′,7-Di-*tert*-butyldimethylsilyloxy-2-morpholinoisoflav-3-ene (9e)

A solution of morpholine (252 mg, 2.93 mmol), 4-[(*tert*-butyldimethylsilyl)oxy]-2-hydroxybenzaldehyde (**5c**)[42] (729 mg, 2.93 mmol) and 2-(4-*tert*-butyldimethylsiloxyphenyl) acetaldehyde (734 mg, 2.93 mmol) in toluene (10 mL) was heated at reflux for 18 h. The reaction was then cooled to rt, filtered (Celite) and concentrated. 4′,7-Di-*tert*-butyldimethylsilyloxy-2-morpholinoisoflav-3-ene **9e** was obtained as a brown oil after flash chromatography (10% EtOAc/petroleum spirits + 1% triethylamine) (483 mg, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.23 (s, 12H, Si(CH_3)$_2$(C(CH_3)$_3$) × 2), 1.00 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$)), 1.01 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$), 2.64 (m, 2H, CH_2NCH_2), 3.05 (m, 2H, CH_2OCH_2), 3.62 (t, 4H, J = 4.0, CH_2OCH_2), 5.68 (s, 1H, H2), 6.41 (dd, 1H, $J_{5,6}$ = 10, $J_{6,8}$ = 2.4, H6), 6.45 (d, 1H, $J_{6,8}$ = 2.4, H8), 6.84 (app. d, 2H, J = 8.6, H3′,5′), 6.94 (s, 1H, H4), 6.97 (d, 1H, $J_{5,6}$ = 10, H5), 7.55 (app. d, 2H, J = 8.6, H2′,6′). ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.4, −0.02, 18.2, 25.5, 25.7, 47.0, 67.2, 90.9, 106.6, 112.9, 115.5, 120.0, 126.7 121.7, 127.1, 127.4, 131.1, 154.5, 155.3, 156.9.

4.2.16. 4′,7-Di-*tert*-butyldimethylsilyloxyisoflav-2-ene (13) and 4′,7-di-*tert*-butyldimethylsilyloxyisoflav-3-ene (14)

$BF_3{\cdot}OEt_2$ (849 μL, 6.67 mmol) was added dropwise to a solution of 4′,7-di-*tert*-butyldimethylsilyloxy-2-morpholinoisoflav-3-ene **9e** (1.68 g 3.03 mmol) and triethylsilane (970 μL, 6.07 mmol) in CH_2Cl_2 (50 mL) at 0 °C. The resulting mixture was stirred at rt for 18 h. The mixture was diluted with EtOAc (10 mL) and water (10 mL) and the aqueous layer was extracted with EtOAc (2 × 10 mL) and the combined organic extracts washed with water (2 × 10 mL), brine (10 mL), dried ($MgSO_4$) and concentrated. 4′,7-Di-*tert*-butyldimethylsilyloxyisoflav-2-ene **13** was obtained as a colourless solid after flash chromatography (10% CH_2Cl_2/petroleum spirits) (288 mg) and recrystallisation from EtOAc/petroleum spirits (78 mg, 5%); mp 89 °C; ($C_{27}H_{40}O_3Si_2$ requires C, 69.18; H, 8.60. Found C, 69.30; H, 8.67). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.216 (s, 6H, Si(CH_3)$_2$(C(CH_3)$_3$), 0.22 (s, 6H, Si(CH_3)$_2$(C(CH_3)$_3$), 1.00 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$), 1.004 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$), 3.67 (s, 2H, H4), 6.45 (d, 1H, $J_{6,8}$ = 2.5, H8), 6.55 (dd, 1H, $J_{5,6}$ = 10, $J_{6,8}$ = 2.5, H6), 6.48 (app. d, 2H, $J_{5,6}$ = 9.0, H3′,5′), 6.91 (t, 1H, J = 1.5, H2), 6.99 (d, 1H, $J_{5,6}$ = 8.5, H5), 7.25 (app. d, 2H, $J_{5,6}$ = 9.0, H2′,6′). ^{13}C NMR (125 MHz, $CDCl_3$) δ −4.5, −4.4, −0.01, 18.2, 25.7, 25.8, 107.6, 112.4, 115.5, 120.1, 125.4, 129.6, 130.8, 136.7, 151.0, 154.8, 155.0. IR ν 2955, 1610, 1509, 1253, 1162, 836, 779 cm^{-1}. Further elution (15–25% CH_2Cl_2/petroleum spirits) gave 4′,7-di-

tert-butyldimethylsilyloxyisoflav-3-ene **14** as a colourless solid after flash chromatography (10% CH_2Cl_2/petroleum spirits) (733 mg) and recrystallisation from EtOAc/petroleum spirits (400 mg, 28%) mp 81 °C; ($C_{27}H_{40}O_3Si_2$ requires C, 69.18; H, 8.60. Found C, 69.16; H, 8.65). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.221 (s, 6H, Si(CH_3)$_2$(C(CH_3)$_3$), 0.22 (s, 6H, Si(CH_3)$_2$(C(CH_3)$_3$), 0.99 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$ 1.00 (s, 9H, Si(CH_3)$_2$(C(CH_3)$_3$), 5.10 (s, 2H, H2), 6.40 (d, 1H, $J_{6,8}$ = 2.5, H8), 6.41 (dd, 1H, $J_{6,8}$ = 2.5, $J_{5,6}$ = 10, H6), 6.67 (s, 1H, H4), 6.85 (app. d, 2H, J = 8.5, H3′,5′), 6.93 (d, 1H, $J_{5,6}$ = 8.5, H5), 7.29 (app. d, 2H, J = 8.5, H2′,6′). ^{13}C NMR (125 MHz, $CDCl_3$) δ −4.4, −4.39, −0.01, 18.2, 25.7, 67.2, 107.5, 113.4, 117.0, 118.3 120.3, 125.7, 127.3, 128.7 130.2, 154.0, 155.4, 156.4. IR ν 2956, 1509, 1269, 1170, 838, 781 cm^{-1}.

4.2.17. 4′,7-Dihydroxyisoflav-2-ene (isophenoxodiol; 15)

HF·pyridine (300 μL) was added to a cooled (0 °C) solution of 4′,7-di-*tert*-butyldimethylsilyloxyisoflav-2-ene **13** (70.0 mg, 0.149 mmol) in THF (1.0 mL). The reaction was then allowed to warm to rt and stirred for 7 h. EtOAc (5 mL) was then added followed by careful addition of satd $NaHCO_3$ until bubbling ceased. The organic layer was washed with satd $NaHCO_3$ (5 mL), water (2 × 5 mL), brine (5 mL), dried ($MgSO_4$) and concentrated. The residue was recrystallised from EtOAc/petroleum spirits to give the 4′,7-dihydroxyisoflav-2-ene **15** (25 mg, 70%) as a pink solid; mp 186–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.54 (s, 2H, CH_2), 6.30 (d, 1H, J = 2.4, H8), 6.47 (m, 1H, H6), 6.74 (app. d, 2H, J = 8.8, H2′,6′), 6.98 (d, 1H, J = 8.0, H5), 7.07 (s, 1H, H2), 7.25 (d, 2H, J = 8.8, H3′,5′), 9.40 (br s, 2H, OH × 2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 24.7, 102.2, 109.5, 111.0, 112.1, 115.3, 125.3, 127.8, 130.0, 135.7, 150.5, 156.4, 156.7. IR ν 2956, 2929, 1509, 1269, 1170, 839 cm^{-1}. HRMS ESI$^+$ [M+H]$^+$ 241.0859, requires 241.0859 for $C_{15}H_{13}O_3$.

4.2.18. 4′,7-Dihydroxyisoflav-3-ene (phenoxodiol; 4)

HF·pyridine (200 μL) was added to a cooled (0 °C) solution of 4′,7-di-*tert*-butyldimethylsilyloxyisoflav-3-ene **14** (150 mg, 0.320 mmol) in THF (1.5 mL). The reaction was then allowed to warm to rt and stirred for 7 h. EtOAc (5 mL was then added followed by careful addition of satd $NaHCO_3$ until bubbling ceased. The organic layer was washed with satd $NaHCO_3$ (5 mL), water (2 × 5 mL), brine (5 mL), dried ($MgSO_4$) and concentrated. The residue was recrystallised from EtOAc/petroleum spirits to give **4** (56 mg, 72%) as a pink solid mp 211–212 °C (lit.[43] mp 230 °C dec). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.00 (s, 2H, CH_2), 6.22 (d, 1H, J = 2.2, H8), 6.31 (dd, 1H, J = 2.2, 8.0, H6), 6.74 (s, 1H, H4), 6.76 (app. d, 2H, J = 8.8, H2′,6′), 6.92 (d, 1H, J = 8.0, H5), 7.32 (d, 2H, J = 8.8, H3′,5′), 9.51 (br s, 1H, OH), 9.56 (br s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 67.0, 103.3, 109.5, 115.8, 116.4, 117.7, 126.6, 128.2, 128.3, 128.5, 154.7, 158.0, 159.0.

4.3. Pharmacology

All procedures were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes or conducted in accordance with Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kyungpook National University School of Medicine).

4.4. Vasorelaxation

Male Sprague-Dawley rats were asphyxiated by CO_2 inhalation, followed by decapitation, and their chests opened to isolate the thoracic aortae. After the removal of superficial connective tissues, the aorta was cut into ring segments of approximately 2–3 mm in length. The aortic rings were mounted between two stainless steel wires, one of which was linked to an isometric force transducer (model FT03, Grass Medical Instruments, Quincy, MA, USA) connected to a MacLab/8 (model MKIII, AD Instrument Co., Sydney, Australia), and the other end anchored to a glass rod submerged in a standard 10 mL organ bath. The organ bath was filled with Krebs-bicarbonate solution [composition (mM): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; D-glucose, 11.0; $NaHCO_3$, 25.0; $CaCl_2 \cdot 2H_2O$, 2.5]. The bath medium was maintained at 37 °C, pH 7.4 and continuously aerated with 95% O_2, 5% CO_2. Aortic rings were equilibrated for 1 h at a resting tension of 1 g, and then were precontracted with an isotonic, high potassium physiological salt solution (KPSS), in which all of the NaCl of the normal Krebs solution was replaced with KCl (122.7 mM), to achieve maximal contraction. After re-equilibration, the rings were sub-maximally contracted with phenylephrine (PE, 0.01–0.3 μM) and endothelial integrity was tested by a single dose of acetylcholine (ACh, 10 μM). Only rings that responded to ACh (>80% relaxation) were judged to be endothelium intact and were used in the subsequent experiments. After establishing a stable precontraction using PE (0.01–0.3 μM), cumulative concentration-response curves to equol and the analogues were determined in the absence and the presence of the estrogen receptor antagonist ICI 182,780 (0.1 μM). Test compounds were dissolved in DMSO and diluted to a final concentration of a maximum of 0.1% DMSO, in Krebs-bicarbonate. All measurements were conducted in groups of n = 3–5.

In a separate experiment for GTP-RhoA measurement, aortic rings were denuded of endothelium by gently rubbing the internal surface with the edge of forceps. Each ring was equilibrated in the organ bath solution for 90 min, and contracted with 50 mM of KCl to validate vascular function. After washout, the aortic rings were pretreated with 100 μM flavonoids, 300 μM flavone (positive control), or vehicle (negative control) for 30 min, and subjected to contraction with U46619. Stock solutions of isoflavans and isoflavenes were prepared at 100 μM in DMSO; stock solutions of flavone and U46619 were prepared at 300 mM and 300 μM, respectively, in DMSO. The final concentrations of DMSO in assay buffer was 0.1%. Experiments were performed in duplicate.

4.5. Assay for GTP-RhoA measurement

Aortic rings were rapidly frozen by liquid nitrogen, and stored at −80 °C. For GTP-RhoA measurement, the procedure followed the manufacturer's protocol for the RhoA G-LISA™ Activation Assay (Cytoskeleton Inc, Denver, CO, USA). Briefly, previously stored samples were homogenized in a lysis buffer, and were centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant, containing 37 μg of protein, was transferred into each well of a plate which equal volumes of ice-cold binding buffer mixtures were added into. The plate was shaken on a cold orbital microplate shaker (300 rpm) for 30 min at 4 °C and the supernatant solution removed from wells. Diluted anti-RhoA primary antibody was added and the plates were incubated on a microplate shaker (300 rpm) at room temperature for 45 min, followed by addition of secondary antibody and incubation on a microplate shaker (300 rpm) at room temperature for another 45 min. The plate was incubated with an HRP detection reagent for 15 min at 37 °C. After addition of an HRP stop buffer, the absorbance was immediately recorded at 490 nm. Experiments were performed in triplicate.

4.6. Radioligand binding

Rat uterine cytosol was prepared as previously described[44,45] and incubated with 0.2 nM [^{3}H]-estradiol in a 10 mM Tris buffer (pH 7.4, 1.5 mM EDTA, 10% w/v glycerol, 1 mM phenylmethylsulfonylfluoride) in the presence and absence of ICI 182,780 to define

non-specific binding. Increasing concentrations of either ICI 182,780 or test compounds were added to cytosol containing 0.2 nM [^{3}H]-estradiol to determine Log IC_{50} values for displacement of [^{3}H]-estradiol. Stock solutions of test compounds were prepared at 10 mM in 100% DMSO and diluted into the assay buffer resulting in a DMSO concentration of 0.1% v/v DMSO with 10 μM of each compound at the maximum concentration used in the assay. To enable direct comparisons, assay buffer for dilutions below 10 μM contained 0.1% DMSO. All measurements were performed in triplicate. Log IC_{50} values were calculated by non-linear regression using a one-site displacement model as provided in Graph Pad Prism 5™.

Acknowledgements

Funding was provided by the Australian Research Council and the National Health and Medical Research Council of Australia.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.008.

References and notes

1. Park, D.; Huang, T.; Frishman, W. H. *Cardiol. Rev.* **2005**, *13*, 13.
2. Setchell, K. D.; Clerici, C. *J. Nutr.* **2010**, *140*, 1355S.
3. Joannou, G. E.; Kelly, G. E.; Reeder, A. Y.; Waring, M.; Nelson, C. *J. Steroid Biochem. Mol. Biol.* **1995**, *54*, 167.
4. Jackman, K. A.; Woodman, O. L.; Sobey, C. G. *Curr. Med. Chem.* **2007**, *14*, 2824.
5. Atkinson, C.; Frankenfeld, C. L.; Lampe, J. W. *Exp. Biol. Med.* **2005**, *230*, 155.
6. Setchell, K. D.; Brown, N. M.; Lydeking-Olsen, E. *J. Nutr.* **2002**, *132*, 3577.
7. Silasi, D. A.; Alvero, A. B.; Rutherford, T. J.; Brown, D.; Mor, G. *Expert Opin. Pharmacother.* **2009**, *10*, 1059.
8. Minutolo, F.; Macchia, M.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. *Med. Res. Rev.* **2011**, *31*, 364.
9. Anderson, G. L.; Limacher, M.; Assaf, A. R.; Bassford, T.; Beresford, S. A.; Black, H.; Bonds, D.; Brunner, R.; Brzyski, R.; Caan, B.; Chlebowski, R.; Curb, D.; Gass, M.; Hays, J.; Heiss, G.; Hendrix, S.; Howard, B. V.; Hsia, J.; Hubbell, A.; Jackson, R.; Johnson, K. C.; Judd, H.; Kotchen, J. M.; Kuller, L.; LaCroix, A. Z.; Lane, D.; Langer, R. D.; Lasser, N.; Lewis, C. E.; Manson, J.; Margolis, K.; Ockene, J.; O'Sullivan, M. J.; Phillips, L.; Prentice, R. L.; Ritenbaugh, C.; Robbins, J.; Rossouw, J. E.; Sarto, G.; Stefanick, M. L.; Van Horn, L.; Wactawski-Wende, J.; Wallace, R.; Wassertheil-Smoller, S. *JAMA* **2004**, *291*, 1701.
10. Rossouw, J. E. *JAMA* **2002**, *288*, 321.
[11]. Aso, T.; Uchiyama, S.; Matsumura, Y.; Taguchi, M.; Nozaki, M.; Takamatsu, K.; Ishizuka, B.; Kubota, T.; Mizunuma, H.; Ohta, H. J. *Womens Health* **2012**, *21*, 92–100.
12. Chin-Dusting, J. P.; Fisher, L. J.; Lewis, T. V.; Piekarska, A.; Nestel, P. J.; Husband, A. *Br. J. Pharmacol.* **2001**, *133*, 595.
13. Jackman, K. A.; Woodman, O. L.; Chrissobolis, S.; Sobey, C. G. *Brain Res.* **2007**, *1141*, 99.
14. Hwang, J.; Wang, J.; Morazzoni, P.; Hodis, H. N.; Sevanian, A. *Free Radical Biol. Med.* **2003**, *34*, 1271.
15. Takashima, Y.; Kaneko, Y.; Kobayashi, Y. *Tetrahedron* **2010**, *66*, 197.
16. Takashima, Y.; Kobayashi, Y. *Tetrahedron Lett.* **2008**, *49*, 5156.
17. Heemstra, J. M.; Kerrigan, S. A.; Doerge, D. R.; Helferich, W. G.; Boulanger, W. A. *Org. Lett.* **2006**, *8*, 5441.
18. Versteeg, M.; Bezuidenhoudt, B.; Ferreira, D. *Tetrahedron* **1999**, *55*, 3365.
19. Lamberton, J. A.; Suares, H.; Watson, K. G. *Aust. J. Chem.* **1978**, *31*, 455.
20. Li, S.-R.; Chen, P.-Y.; Chen, L.-Y.; Lo, Y.-F.; Tsai, I.-L.; Wang, E.-C. *Tetrahedron Lett.* **2009**, *50*, 2121.
21. Gharpure, S. J.; Sathiyanarayanan, A. M.; Jonnalagadda, P. *Tetrahedron Lett.* **2008**, *49*, 2974.
22. Dean, F. M.; Varma, R. S. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1193.
23. Varma, R. S.; Dahiya, R. *J. Org. Chem.* **1998**, *63*, 8038.
24. Fernandes, R. A.; Kumar, P. *Tetrahedron Lett.* **2003**, *44*, 1275.
25. Faragalla, J.; Heaton, A.; Griffith, R.; Bremner, J. B. *Synlett* **2009**, 306.
26. Liepa, A. J. *Aust. J. Chem.* **1984**, *37*, 2545.
27. Vera, R.; Galisteo, M.; Villar, I. C.; Sanchez, M.; Zarzuelo, A.; Pérez-Vizcaíno, F.; Duarte, J. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1300.
28. Seok, Y. M.; Baek, I.; Kim, Y.-H.; Jeon, Y.-S.; Lee, I.-J.; Shin, D. H.; Hwang, Y. H.; Kim, I. K. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 991.
29. Baek, I.; Jeon, S. B.; Song, M.-J.; Yang, E.; Sohn, U. D.; Kim, I. K. *Korean J. Physiol. Pharmacol.* **2009**, *13*, 201.
30. Budzyn, K.; Sobey, C. G. *Curr. Opin. Drug Disc. Dev.* **2007**, *10*, 590.
31. Branham, W. S.; Dial, S. L.; Moland, C. L.; Hass, B. S.; Blair, R. M.; Fang, H.; Shi, L.; Tong, W.; Perkins, R. G.; Sheehan, D. M. *J. Nutr.* **2002**, *132*, 658.
32. Mita, M.; Yanagihara, H.; Hishinuma, S.; Saito, M.; Walsh, M. P. *Biochem. J.* **2002**, *440*, 431.
33. Wilson, D. P.; Susnjar, M.; Kiss, E.; Sutherland, C.; Walsh, M. P. *Biochem. J.* **2005**, *389*, 763.
34. Song, M. J.; Baek, I.; Seo, M.; Kim, S.-H.; Suk, K.; Woodman, O. L.; Williams, S. J.; Kim, I.-K. *Clin. Exp. Pharmacol. Physiol.* **2010**, *37*, 803.
35. Seok, Y. M.; Cho, H. J.; Cha, B.-Y.; Woo, J.-T.; Kim, I. K. *J. Pharm. Pharmacol.* **2011**, *63*, 1244.
36. Song, M.-J.; Baek, I.; Jeon, S. B.; Seo, M.; Kim, Y.-H.; Cui, S.; Jeong, Y.-S.; Lee, I.-J.; Shin, D. H.; Hwang, Y. H.; Kim, I. K. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2010**, *381*, 517.
37. Seok, Y. M.; Choi, Y. W.; Kim, G.-D.; Kim, H. Y.; Takuwa, Y.; Kim, I. K. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2011**, *383*, 45.
38. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
39. Abul-Hajj, Y. J.; Ghaffari, M. A.; Mehrota, S. *Xenobiotica* **1991**, *21*, 1171.
40. Deschamps-Vallet, C.; Ilotse, J.-B.; Meyer-Dayan, M. *Tetrahedron Lett.* **1983**, *24*, 3993.
41. Smith, A. B., III; Sperry, J. B.; Han, Q. *J. Org. Chem.* **2007**, *72*, 6891.
42. Seidel, J. L.; Epstein, W. W.; Davidson, D. W. *J. Chem. Ecol.* **1990**, *16*, 1791.
43. Liepa, A. J. *Aust. J. Chem.* **1981**, *34*, 2647.
44. Sutherland, T. E.; Schuliga, M.; Harris, T.; Eckhardt, B. L.; Anderson, R. L.; Quan, L.; Stewart, A. G. *Clin. Cancer Res.* **2005**, *11*, 1722.
45. Hughes, R. A.; Harris, T.; Altmann, E.; McAllister, D.; Vlahos, R.; Robertson, A.; Cushman, M.; Wang, Z.; Stewart, A. G. *Mol. Pharmacol.* **2002**, *61*, 1053.