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Discovery of Water-Soluble Antioxidant Flavonols without Vasorelaxant Activity

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3′,4′-Dihydroxyflavonol (DiOHF) is a cardioprotective flavonol that can decrease ischaemia/reperfusion injury in the heart. DiOHF exhibits antioxidant and vasorelaxant properties that are thought to underlie its cardioprotective activity. A major limitation to its use for the treatment or prevention of cardiovascular disease is its poor water solubility, preventing intravenous administration at the required dosage. In this study, three novel flavonols were synthesised that bear an ionisable succinamic acid substituent at the 6-position of the A ring with zero, one, or two hydroxy groups on the B ring. The ionised compounds possess improved aqueous solubility, dissolving at concentrations up to 10^{-1} M, whereas DiOHF is insoluble in water ($< 10^{-7}$ M). Pharmacological analysis revealed that the DiOHF-6-succinamic acid derivative was the best antioxidant, possessing activity similar to DiOHF, whereas vasorelaxant activity was attenuated. This compound was able to effectively scavenge superoxide from the autoxidation of pyrogallol, preventing oxidant-induced endothelial dysfunction. DiOHF-6-succinamic acid represents the first antioxidant flavonol that lacks vasorelaxant activity and in the future will enable studies to cast light on the specific biological activity required for cardioprotection.

Introduction

Cardiovascular disease (CVD), most commonly ischaemic heart disease and stroke, is the leading cause of mortality worldwide[1] as well as the major cause of chronic disability.[2] The major underlying cause of CVD is atherosclerosis, which results in the blockage of blood vessels leading to ischaemic heart disease. In the last two decades, acute revascularisation with the use of thrombolytic agents or surgical procedures have emerged as the standard treatment in the event of an acute myocardial infarction. This is necessary to resuscitate the ischaemic myocardium but results in a worsened secondary condition: reperfusion injury. Reperfusion injury is characterised by increased production of reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$).[3] ROS are normally present at low levels and are scavenged by endogenous scavenging enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (SOD).[4] However, when reperfusion follows ischaemia, increased amounts of ROS are produced that overwhelm the endogenous antioxidant defences, exacerbating and accelerating the injury already produced by the ischaemia. For example, $O_2^{\bullet-}$ reacts with nitric oxide (NO), an important radical species required for maintenance of normal blood vessel function. Upon reaction, NO is consumed, and the toxic peroxynitrite anion ($ONOO^-$) is formed, resulting in impairment of endothelial function and promotion of thrombosis. Furthermore, ROS can react indiscriminately with DNA, proteins, and lipids, ultimately leading to cellular damage and death.[5]

Wang et al. have shown that the simple flavonol 3′,4′-dihydroxyflavonol (DiOHF) (Figure 1) decreases infarct size in sheep by approximately 40% after myocardial ischaemia followed by reperfusion.[6] Furthermore, administration of DiOHF shortly before reperfusion decreased adverse events associated with ischaemia/reperfusion, such as animal mortality, tachycardia, and myocardial enzyme release, and resulted in improved regional myocardial contractility. This level of protection by DiOHF was similar to that achieved by ischaemic preconditioning (IPC).[6] IPC is currently the most effective preventative of ischaemia/reperfusion injury and is performed by subjecting experimental animals to short periods of ischaemia followed by reperfusion prior to the extended period of ischaemia.[7,8]

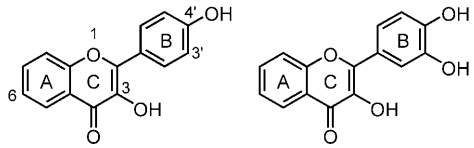

Figure 1. 4′-Hydroxyflavonol and DiOHF: two of the most potent vasorelaxant and antioxidant flavonols described to date.

While the exact mechanism through which DiOHF exerts its cardioprotective effects are obscure, previous studies have shown certain hydroxylated flavonols possess vascular and an-

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tioxidant properties that might account for this activity.[9–11] Structure–activity relationship studies have identified substituents on the flavonol ring system that are important for these biological activities. Chan et al. found that the 3-OH group of the C ring is essential for endothelium-dependent vasorelaxant activity[12,13] and additional hydroxy groups at the 3′ and 4′ positions of the B ring further improve this activity.[10] For antioxidant activity, the 3-OH of the C ring, together with either a 4′-hydroxy or a 3′,4′-catechol moiety on the B ring, were shown to be important.[14–17] In addition, Qin et al. have demonstrated that 4′-hydroxyflavonols with a variety of other substituents at the 3′ position are not antioxidant but retain vasorelaxant activity. In particular, 4′-hydroxy-3′-methoxyflavonol possesses only vasorelaxant activity and represents the first single-acting flavonol.[17] Thus, the two most potent flavonols for antioxidant and vasorelaxant activities described to date are 4′-hydroxyflavonol and DiOHF (Figure 1).[9,10,12]

The potent cardioprotection observed upon its administration means that DiOHF is an important lead compound for the treatment of CVD. However, its poor water solubility presents a major limitation for its use as a drug. Most flavonols are poorly water soluble, a feature that can be attributed to their highly crystalline nature. Thus, even for flavonols that bear multiple hydroxy groups (e.g., DiOHF and quercetin), there is insufficient energy released upon solvation to overcome the intermolecular forces present in the crystal. Additionally, the cardioprotective flavonol, DiOHF, possesses both vasorelaxant and antioxidant activity, and it is not known which is required for cardioprotection. Hence, the aim of this project was to develop a chemical approach to introduce an ionisable substituent into a biologically active flavonol to improve water solubility and to investigate the biological activity of the resultant conjugate to see if vasorelaxant and antioxidant activities are retained.

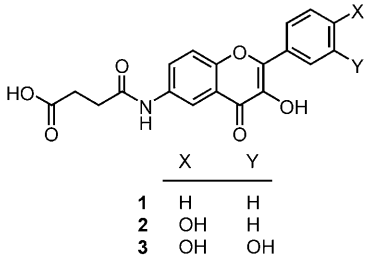


Figure 2. Ionisable flavonol targets **1**, **2**, and **3**.

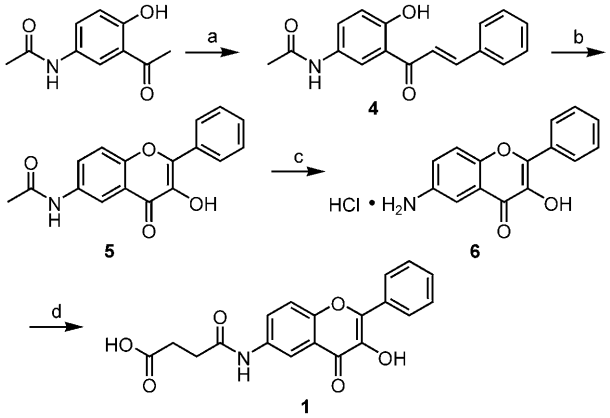
Results and Discussion

Design

We envisaged that a flavonol, modified with a carboxylic acid functional group, should exhibit improved water solubility, as the carboxylic acid can form a salt upon reaction with base. As structure–activity relationships suggest the pharmacophore that determines vasorelaxant and antioxidant activities is located on the B and C rings,[9,12,17] we targeted the A ring for modification. The succinamic acid modified flavonols **1**, **2**, and **3**, respectively bearing zero, one, and two hydroxy groups on the B ring, were selected as targets (Figure 2). The hydroxylation patterns of **2** and **3** were chosen, as they represent the most vasoactive flavonols described to date; **1** was chosen as a control compound for biological studies.

Synthesis of ionisable flavonols 1, 2, and 3

Claisen–Schmidt condensation of 5-acetamido-2-hydroxyacetophenone[18] and benzaldehyde using aqueous sodium hydroxide in ethanol afforded chalcone **4**,[19] which was converted into 6-acetamidoflavonol **5**[20] by Algar–Flynn–Oyamada reaction using alkaline hydrogen peroxide (Scheme 1). Treatment of **5** with 5 M HCl in ethanol at reflux afforded 6-aminoflavonol, isolated as the hydrochloride **6**. The salt **6** was treated with succinic anhydride in pyridine to yield the first target flavonol



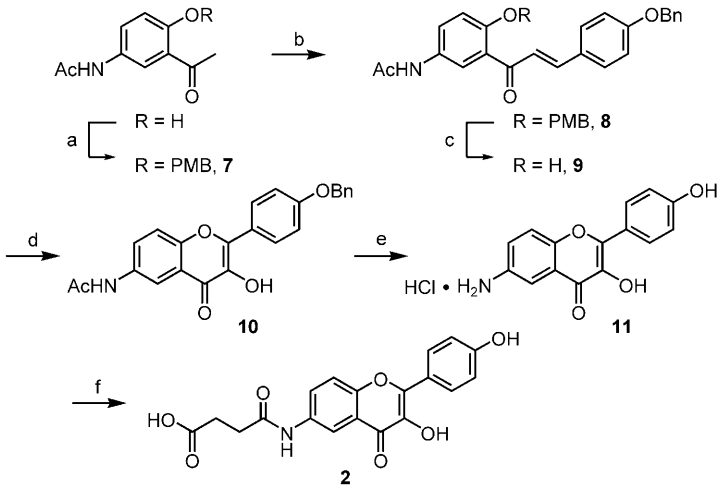
Scheme 1. Reagents and conditions: a) benzaldehyde, NaOH, EtOH, 71%; b) NaOH, H_2O_2, EtOH, 50%; c) 5 M HCl, EtOH; d) succinic anhydride, pyridine, 42% over two steps.

1. The same approach was attempted towards the synthesis of **2**; however, the Claisen–Schmidt condensation of 5-acetamido-2-hydroxyacetophenone and 4-benzyloxybenzaldehyde could not be effected with the same conditions used for the preparation of chalcone **4**. Attempts to use more vigorous conditions (aqueous potassium hydroxide, dioxane co-solvent) as reported by van Acker et al.[21] provided no improvement. Attempts using methanolic sodium hydroxide[22] led to some improvement, however, the reaction was irreproducible and either starting material or a flavanone were recovered.

It has been reported that tetrahydropyranyl (THP) protection of the hydroxy group of a 2-hydroxyacetophenone allows clean conversion into a chalcone without the complication of the formation of a flavanone.[23,24] THP groups are typically installed by treatment of a phenol with 3,4-dihydro-2*H*-pyran and catalytic acid. However, we were unable to install the THP group into 5-acetamido-2-hydroxyacetophenone using this method. While our results are at odds with those of Hsieh et al.,[24] Adams and Main[25] reported they were unable to protect a hydroxy group *ortho* to an acetophenone; indeed, it was demonstrated that under prolonged reaction times, *C*-tetrahy-

dropyranylacetophenones formed more readily than *ortho*-*O*-tetrahydropyranylacetophenones.

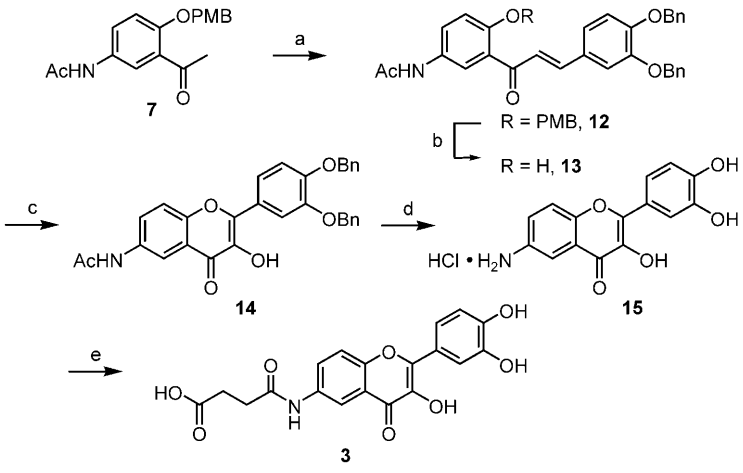
Given these difficulties, we investigated the *p*-methoxybenzyl (PMB) group for protection of the hydroxy group of 5-acetamido-2-hydroxyacetophenone. Heating a solution of 5-acetamido-2-hydroxyacetophenone and *p*-methoxybenzyl chloride at reflux in the presence of anhydrous K_2CO_3 in butanone smoothly afforded the *p*-methoxybenzyl ether **7** in good yield (82%) (Scheme 2). Gratifyingly, treatment of **7** and 4-benzyloxybenzaldehyde with aqueous NaOH, conditions used previously for the synthesis of **1**, afforded the protected chalcone **8** in excellent yield (86%). This significant result confirmed that the 2-hydroxy group of the acetophenone was responsible for the poor outcome noted in the previously attempted Claisen–Schmidt condensation.



Scheme 2. Reagents and conditions: a) *p*-methoxybenzyl chloride, K_2CO_3, butanone, reflux, 82%; b) 4-benzyloxybenzaldehyde, NaOH, EtOH, 73%; c) 2 M HCl, EtOH, 70 °C, 1 h; d) NaOH, H_2O_2, EtOH, 1,4-dioxane, 36% over two steps; e) 36% HCl, AcOH, reflux, 2 h; f) succinic anhydride, pyridine, 38% over two steps.

Selective deprotection of the PMB group of **8** was achieved by careful reflux in 2 M aqueous HCl (Scheme 2). The crude 2′-hydroxychalcone **9** was subjected to alkaline hydrogen peroxide to effect the Algar–Flynn–Oyamada reaction, in a procedure similar to that used for the synthesis of **1**, but with the inclusion of 1,4-dioxane to improve the solubility of starting materials and intermediates, affording the desired flavonol **10** in 57% yield from **8**. Next, **10** was heated at reflux in concentrated HCl in acetic acid to simultaneously remove the benzyl and acetamido groups, affording the deprotected flavonol, isolated as hydrochloride **11**. Finally, reaction of **11** with succinic anhydride in pyridine afforded the succinamic acid derivative of 4′-hydroxyflavonol **2**, which was purified by recrystallisation from DMF/water.

The synthesis of **3** was carried out using the procedure established for the synthesis of **2**, as outlined in Scheme 3, and proved mostly uneventful. Thus, while the condensation of the protected acetophenone **7** with 3,4-dibenzyloxybenzaldehyde did not proceed at room temperature, upon heating the mixture to 40 °C, an excellent yield of the protected chalcone **12** was obtained (71%). The PMB group of **12** was selectively removed by careful treatment with 2 M HCl at reflux in ethanol, and the crude 2′-hydroxychalcone **13** was treated immediately with alkaline hydrogen peroxide to effect an Algar–Flynn–Oyamada reaction to afford flavonol **14** in 50% yield. The benzyl ethers and acetamido group of **14** were hydrolyzed with concentrated aqueous HCl in acetic acid, and the microcrystalline hydrochloride **15** was isolated by centrifugation. Finally, the succinamic acid moiety was introduced by treating **15** with succinic anhydride in pyridine to afford the final target compound **3** in 44% yield (Scheme 3), purified by recrystallisation from 50% DMSO/water.



Scheme 3. Reagents and conditions: a) 3,4-dibenzyloxybenzaldehyde, NaOH, EtOH, 40 °C, 71%; b) 2 M HCl, EtOH, 70 °C, 1 h; c) H_2O_2, NaOH, EtOH, 1,4-dioxane 50% over two steps; d) 36% HCl, AcOH, reflux, 3 h; e) succinic anhydride, pyridine, 55% over two steps.

Solubility studies of 1, 2, and 3

The succinamic acids **1**, **2**, and **3** were converted into the sodium salts by stoichiometric treatment with a solution of Na_2CO_3. The solution was then evaporated to dryness, and the resultant residue was dissolved in water. The sodium salts of **1**–**3** dissolve readily in water, at concentrations of up to 10^{-1} M, whereas DiOHF is essentially water insoluble ($< 10^{-7}$ M).

Pharmacological studies of 1, 2, and 3

The vascular activities of the flavonols **1**–**3** were examined in standard organ bath assays using rat isolated thoracic aorta. The compounds were tested for their ability to inhibit phenylephrine (PE)-induced contraction, as flavonols are known functional antagonists of PE.[12] Endothelium intact aortic rings were incubated with the flavonols or vehicle, and a concentration–response curve to PE was constructed. Compounds **1**–**3** were less effective than DiOHF in inhibiting PE contraction (Figure 3). Most notably, **3** exhibited essentially no antagonism

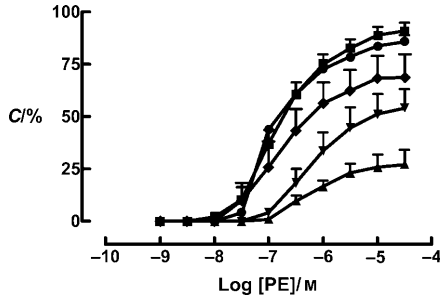


Figure 3. Effect of **1**, **2**, and **3** on the inhibition of PE-induced contraction ($n=4$). C = contraction of aortic rings as a percentage of KPSS-induced contraction. ■ = vehicle, ▲ = DiOHF (10^{-5} M), ▼ = **1** (10^{-5} M), ◆ = **2** (10^{-5} M), ● = **3** (10^{-5} M).

toward PE-induced contraction and was indistinguishable from vehicle.

The efficacy of the synthesised flavonols was determined in a direct relaxation assay using pre-contracted rat isolated thoracic aorta to determine potency (pEC_{50}) and maximum response (R_{max}). Endothelium intact aortic rings were pre-contracted with a combination of PE and U46619 (a thromboxane A_2 receptor agonist) to similar levels, and concentration–response curves to the flavonols were generated (Figure 4).

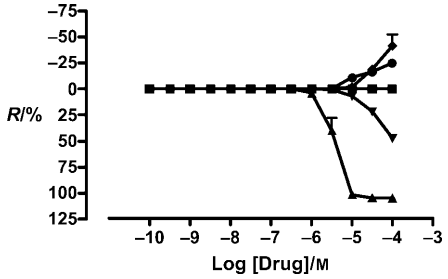


Figure 4. Comparison of concentration–response curves of **1**, **2**, and **3** after pre-contraction to similar levels with PE/U46619 ($n=4$–6). R = Relaxation of aortic ring as a percentage of the pre-contraction. ■ = vehicle, ▲ = DiOHF (10^{-5} M), ▼ = **1** (10^{-5} M), ◆ = **2** (10^{-5} M), ● = **3** (10^{-5} M).

DiOHF is the most potent vasorelaxant flavonol described to date[10,11,13] and **1**, **2**, and **3** were less effective than DiOHF at relaxing pre-contracted aorta. In the cases of **2** and **3**, relaxation activities were completely abolished, and slight contraction was observed at the highest concentrations. The pEC_{50} value for DiOHF is 5.33 ± 0.07 ($n=4$), but pEC_{50} values for the synthesised compounds could not be accurately calculated, as the results did not fit to a sigmoidal curve. The maximum relaxations of the various flavonols are shown in Table 1, and are significantly different from each other ($p<0.001$).

Antioxidant activities of the synthesised flavonols towards superoxide were examined in a tissue-based lucigenin-enhanced chemiluminescence assay. This assay provides a realistic context for the production of ROS by using endogenous enzymes present in tissue samples as the source of oxidant species. Isolated rat aortic rings in Krebs-HEPES buffer were incubated with NADPH as the substrate for NADPH oxidase in the

Table 1. Vasorelaxation of rat thoracic aorta by flavonols.

Compound	n	R_{max} [%]
1	6	47 ± 2[a]
2	2	-42 ± 3[a]
3	4	-25 ± 3[a]
DiOHF	4	105 ± 2

[a] Significant difference from DiOHF, $p<0.001$, Newman–Keuls multiple comparison test.

vasculature, diethylthiocarbamic acid (DETCA) to deactivate endogenous superoxide dismutase, and either vehicle or flavonols. Superoxide produced by NADPH oxidase reacts with lucigenin, leading to the emission of photons, which can be quantified to give a measure of superoxide levels.[26] Superoxide levels are decreased if flavonols possess antioxidant activity. Figure 5 shows that DiOHF and **3** are able to significantly decrease superoxide levels (by 71 ± 3 and 56 ± 10%, respectively ($p<0.01$)). By contrast, compounds **1** and **2** were not able to decrease superoxide levels significantly. Thus, while 4′-hydroxyflavonol is an effective antioxidant,[10] introduction of the 6-succinamic acid moiety to afford **2** results in a decrease in antioxidant activity. Likewise, introduction of the 6-succinamic acid moiety into DiOHF to give **3** results in a partial attenuation of antioxidant activity.

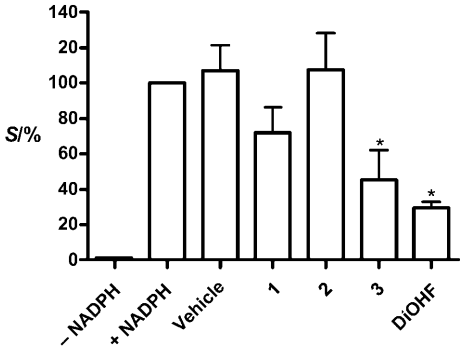


Figure 5. Antioxidant activity of **1**, **2**, and **3** (each at 10^{-4} M) using lucigenin-enhanced chemiluminescence. **3** and DiOHF (also 10^{-4} M) can significantly decrease superoxide production by NADPH/NADPH oxidase in rat aorta *-($p<0.01$). Compounds **1** and **2** are not able to decrease superoxide production significantly ($n=4$–6). S = Superoxide levels as a percentage of the positive control (+NADPH).

We next sought to investigate the ability of the synthesised flavonols to preserve vascular function in the presence of oxidative stress through intercepting ROS before they can react with the endogenous vasodilator NO.[27] This assay extends the results of the tissue-based lucigenin-enhanced chemiluminescence assay by examining a functional consequence of oxidative stress. The combination of the chemiluminescence assay and the functional assay is therefore superior to widely used in vitro assays for the assessment of antioxidant activity, which do not provide functional assessments of antioxidant ability. As an example, L-ascorbic acid is an excellent antioxidant in vitro,

but is only partially able to restore endothelial dysfunction caused by superoxide, even at very high ascorbic acid concentrations (10 mM),[27] possibly because its rate of scavenging superoxide is too low (3.3×10^{-5} M^{-1} s^{-1}).[28] In contrast, flavonols scavenge superoxide an order of magnitude faster (for quercetin: 2.4×10^{-6} M^{-1} s^{-1}),[29] and DiOHF at a much lower concentration (10 μM) can completely reverse endothelial dysfunction caused by superoxide.[9] In addition, the antioxidant capacity of quercetin is fivefold that of ascorbate.[30] The ability of **1**, **2**, and **3** to maintain vascular NO bioactivity in the presence of oxidative stress was determined by measuring relaxant responses to acetylcholine (ACh) in rat isolated aortic rings in the presence of pyrogallol. Pyrogallol autoxidises to form superoxide radical, which results in endothelial dysfunction (≥ 50% decrease in R_{max} to ACh). Rat aortic rings were incubated with pyrogallol and flavonol or vehicle, and concentration–response curves to ACh were constructed (Figure 6). Compounds **1** and **3** were able to prevent endothelial dysfunction, but were marginally poorer than DiOHF.

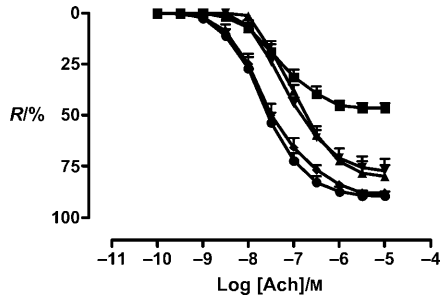


Figure 6. Effect of compounds **1** and **3** on superoxide-induced vascular dysfunction by pyrogallol ($n=4$–6). R = Relaxation as a percentage of the precontraction. ■ = pyrogallol + vehicle, ▲ = pyrogallol + **1** (10^{-4} M), ▼ = pyrogallol + **3** ($10^{-4.5}$ M), ◆ = pyrogallol + DiOHF (10^{-5} M), ● = vehicle.

Conclusions

Three ionisable flavonols bearing succinamic acid side chains were synthesised using a Claisen–Schmidt condensation followed by Algar–Flynn–Oyamada reaction. Notably a *p*-methoxybenzyl group was used to protect the 2-hydroxy group of 5-acetamidoacetophenone, thereby providing smooth conversion into the chalcone in the Claisen–Schmidt condensation with electron-rich benzaldehydes. Whilst vasoactive flavonols in current use such as DiOHF possess low solubility in water (< 10^{-7} M), the sodium salts of **1**, **2**, and **3** dissolve in water at concentrations up to 10^{-1} M. The approach outlined herein uses a succinamic acid substituent as a permanent modification to the parent drug DiOHF, and led to changes in the pharmacological profile of **3** relative to DiOHF. As such, this approach differs from that used for the development of water-soluble cardioprotective carotenoid "soft-drugs" where the parent carotenoid is modified to the succinate or phosphate ester, enhancing their solubility and providing drugs that are active in both the derivatised and free forms.[31]

Very recently we discovered that 4′-hydroxy-3′-methoxyflavonol possesses vasorelaxant activity similar to that of DiOHF, but lacks functional antioxidant activity, the first known flavonol in which these two activities have been separated.[17] As reported herein, through the introduction of a succinamic acid substituent at the 6-position of the A ring of DiOHF to obtain **3**, we have discovered a flavonol derivative with the complementary biological profile that lacks vasorelaxant activity, but that retains functional antioxidant activity only marginally poorer in potency relative to DiOHF. This flavonol is now available for future studies to determine whether vasorelaxant or antioxidant activities are required for cardioprotection. Moreover, with improved solubility achieved by incorporation of an ionisable carboxylic acid substituent, this compound can be administered intravenously at high concentrations in aqueous solution.

Experimental Section

General methods

Thin-layer chromatography (TLC) was performed on aluminum sheets pre-coated with silica gel 60 (Merck), using mixtures of EtOAc and petroleum spirits, or mixtures of Et_2O and CH_2Cl_2. Detection was achieved by irradiation with UV light. NMR spectroscopic data were obtained on Varian Unity Plus 400 or 500 instruments in solutions of [D_6]DMSO using residual solvent as internal standard ($\delta=2.50$ ppm for ^{1}H NMR, $\delta=39.51$ ppm for ^{13}C NMR) or in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard ($\delta=0.00$ ppm). Evaporation of solvents was performed under reduced pressure using a rotary evaporator. Elemental analyses were performed by CMAS (Belmont, Victoria, Australia). Melting points were obtained using an Electrothermal melting point apparatus or Riechert–Jung Hot-stage melting point apparatus, and in the latter case, are corrected. Mass spectra were obtained by electrospray ionisation using a triple-quad Quattro II instrument (The University of Melbourne). The syntheses of 5-acetamido-2-hydroxyacetophenone[18] and 6-acetamidoflavonol **5**[20] were described previously.

Synthesis

6-(Hydroxycarbonylethylcarbonylamino)flavonol (1): A mixture of aqueous HCl (5 M, 1 mL) and 6-acetamidoflavonol (**5**, 50.0 mg, 0.169 mmol) in EtOH (1.5 mL) was heated at reflux for 1.5 h. The mixture was cooled and diluted with H_2O, and the precipitate that formed was collected by filtration to afford the hydrochloride salt **6** as a bright yellow powder. Crude **6** and succinic anhydride (14.1 mg, 0.141 mmol) were dissolved in pyridine (2 mL), and the mixture was stirred at room temperature for 4 h. H_2O (1 mL) was added, and the mixture was acidified with 2 M HCl. The mixture was left to stand at room temperature for 30 min, and the resulting suspension was filtered and recrystallised from THF/petroleum spirits to afford **1** as a yellow powder (24.5 mg, 42%); mp: 220–223 °C; ^{1}H NMR (399.7 MHz, [D_6]DMSO): $\delta=2.55$–2.61 (m, 4H, CH_2CH_2), 7.49–7.59 (m, 3H, H3′,4′,5′), 7.73 (d, 1H, $J_{7,8}=9.2$ Hz, H8), 7.87 (dd, 1H, $J_{5,7}=2.8$, $J_{7,8}=9.2$ Hz, H7), 8.20 (app. d, 2H, $J=8.4$ Hz, H2′,6′), 8.47 (d, 1H, $J_{5,7}=2.8$ Hz, H5), 9.61 (br s, 1H, OH), 10.31 (s, 1H, NH), 12.18 ppm (br s, 1H, CO_2H); Anal. calcd for $C_{19}H_{15}NO_6$: C 64.59, H 4.28, N 3.96, found: C 64.51, H 4.19, N 4.08%.

5-Acetamido-2-(4-methoxybenzyloxy)acetophenone (7): A mixture of 5-acetamido-2-hydroxyacetophenone (3.00 g, 15.5 mmol), 4-methoxybenzyl chloride (3.20 mL, 31.1 mmol), and anhydrous K_2CO_3 (3.21 g, 23.3 mmol) in butanone (45 mL) was heated at

reflux overnight. The mixture was filtered, and the filtrate was concentrated in vacuo, giving a yellow residue. The residue was triturated with petroleum spirit and immediately recrystallised from THF/petroleum spirit to afford **7** as a white powder (4.02 g, 82%); mp: 169–171 °C; ^{1}H NMR (399.7 MHz, $CDCl_3$): $\delta = 2.16$ (s, 3H, CH_3CON), 2.57 (s, 3H, CH_3COAr), 3.83 (s, 3H, CH_3O), 5.08 (s, 2H, CH_2), 6.92 (d, 2H, $J = 8.4$ Hz, H2′,6′), 7.02 (d, 1H, $J_{3,4} = 8.8$ Hz, H3), 7.35 (app. d, 2H, $J = 8.4$ Hz, H3′,5′), 7.52 (d, 1H, $J_{4,6} = 3.2$ Hz, H6), 7.97 ppm (dd, 1H, $J_{3,4} = 8.8$, $J_{4,6} = 3.2$ Hz, H4); ^{13}C NMR (100.5 MHz, $CDCl_3$): $\delta = 24.58$, 32.45, 55.52, 71.03 (4C, CH_2, OCH_3, $2\times CH_3$), 113.86, 114.28, 122.09, 126.52, 128.30, 128.43, 129.59, 131.47, 155.23, 159.82 (10C, Ar), 168.63, 199.52 ppm (2C, 2×C=O); Anal. calcd for $C_{18}H_{19}NO_4$: C 68.99, H 6.11, N 4.47, found: C 68.86, H 6.19, N 4.53%.

5′-Acetamido-4-benzyloxy-2′-(4-methoxybenzyloxy)chalcone (8): A mixture of aqueous NaOH (25.5 mL of 25.2 g/100 mL), the protected acetophenone **7** (3.00 g, 9.57 mmol), and 4-benzyloxybenzaldehyde (2.03 g, 9.57 mmol) in EtOH (25.5 mL) was stirred at room temperature overnight. The mixture was filtered to afford **8** as a light yellow solid (3.54 g, 73%); mp: 201–202 °C; ^{1}H NMR (399.7 MHz, $CDCl_3$): $\delta = 2.17$ (s, 3H, CH_3CON), 3.77 (s, 3H, CH_3O), 5.06, 5.09 (2s, 2×2H, 2×CH_2Ar), 6.82 (app. d, 2H, $J = 8.8$ Hz, BB′), 6.88 (app. d, 2H, $J = 8.8$ Hz, BB′), 7.04 (d, 1H, $J_{3',4'} = 9.0$ Hz, H3′), 7.27 (app. d, 2H, $J = 8.8$ Hz, AA′), 7.31–7.45 (m, 8H, AA′, C=CH, Ph), 7.51 (d, 1H, $J_{4',6'} = 2.8$ Hz, H6′), 7.58 (d, 1H, $J_{trans} = 15.6$ Hz, C=CH), 7.98 ppm (dd, 1H, $J_{3',4'} = 9.0$, $J_{4',6'} = 2.8$ Hz, H4′); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 23.85$, 55.05 (2C, $2\times CH_3$), 69.38, 70.13 (2C, $2\times CH_2$), 79.20, 113.82, 113.91, 115.14, 120.54, 123.94, 124.80, 127.39, 127.79, 128.00, 128.45, 128.53, 129.94, 130.30, 132.73, 136.70, 142.17, 153.06, 159.10, 160.24 (20C, Ar, CH=CH), 168.06, 190.69 ppm (2C, 2×C=O); Anal. calcd for $C_{32}H_{29}NO_5$: C 75.72, H 5.76, N 2.76, found: C 75.60, H 5.74, N 2.73%.

6-Acetamido-4′-benzyloxyflavonol (10): A solution of the protected chalcone **8** (1.20 g, 2.37 mmol) in aqueous HCl (2 M, 66 mL) and EtOH (290 mL) was heated at reflux for 1 h. The mixture was cooled to room temperature, and evaporated in vacuo to approximately half the volume. The resultant suspension was filtered to afford the crude deprotected chalcone **9** as a yellow solid. Crude **9** was dissolved in 1,4-dioxane (19.2 mL), EtOH (24 mL), and NaOH (5.4% *w/v*, 7.8 mL). The resultant solution was cooled in an ice bath, and H_2O_2 (30%, 1.2 mL) was added. The solution was stirred at 0 °C for 2 h, and subsequently at room temperature overnight. The solution was then acidified with 2 M HCl, and the precipitate that formed was filtered, then recrystallised from THF/petroleum spirit to afford **10** as a bright yellow solid (341 mg, 36%); mp: 255–258 °C; ^{1}H NMR (499.7 MHz, [D$_6$]DMSO): $\delta = 2.09$ (s, 3H, CH_3), 5.19 (s, 2H, CH_2), 7.19 (app. d, 2H, $J = 9.0$ Hz, H2′,6′), 7.32–7.50 (m, 5H, Ph), 7.69 (d, 1H, $J_{7,8} = 9.0$ Hz, H8), 7.86 (dd, 1H, $J_{5,7} = 2.5$, $J_{7,8} = 9.0$ Hz, H7), 8.18 (app. d, 2H, $J = 9.0$ Hz, H3′,5′), 8.41 (d, 1H, $J_{5,7} = 2.5$ Hz, H5), 10.28 ppm (brs, 1H, NH); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 24.00$ (1C, CH_3), 69.37 (1C, CH_2), 112.63, 114.88, 118.83, 121.41, 123.84, 125.27, 127.84, 127.98, 128.49, 129.35, 135.89, 136.68, 137.93, 145.41, 150.45, 159.48 (16C, Ar), 168.54, 172.46 ppm (2C, 2×C=O); Anal. calcd for $C_{24}H_{19}NO_5$: C 71.81, H 4.77, N 3.49, found: C 71.90, H 4.80, N 3.51%.

4′-Hydroxy-6-(hydroxycarbonylethylcarbonylamino)flavonol (2): A mixture of the protected flavonol **10** (600 mg, 1.49 mmol) in aqueous HCl (36%, 38 mL) and acetic acid (38 mL) was heated at reflux for 2 h. The mixture was then cooled on ice and diluted with H_2O. The resulting suspension was centrifuged, and the collected solid was washed with H_2O, then freeze-dried to afford the salt as a crude yellow solid (457 mg). A mixture of the crude yellow product and succinic anhydride (179 mg, 1.79 mmol) in pyridine was stirred at room temperature for 4 h. H_2O (1 mL) was added, and the mixture was acidified with 2 M HCl. The mixture was left to stand at room temperature for 30 min, and the resulting suspension was centrifuged and the collected solid was washed with H_2O, freeze-dried and recrystallised from DMF/H_2O to afford **2** as a brown powder (211 mg, 38%); mp: 256–257 °C; ^{1}H NMR (399.7 MHz, [D$_6$]DMSO): $\delta = 2.55$–2.60 (m, 4H, CH_2CH_2), 6.93 (app. d, 2H, $J = 9.2$ Hz, H3′,5′), 7.69 (d, 1H, $J_{7,8} = 8.8$ Hz, H8), 7.85 (dd, 1H, $J_{5,7} = 2.8$, $J_{7,8} = 8.8$ Hz, H7), 8.09 (app. d, 2H, $J = 9.2$ Hz, H2′,6′), 8.43 (d, 1H, $J_{5,7} = 2.8$ Hz, H5), 9.33 (s, 1H, NH), 10.11, 10.31 (2brs, 2×1H, 2×OH), 12.21 ppm (brs, 1H, CO_2H); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 25.16$, 28.78 (2C, CH_2CH_2), 31.06, 67.05, 112.65, 115.46, 118.80, 121.45, 122.06, 125.11, 129.55, 135.80, 137.57, 146.01, 150.38, 159.11 (14C, Ar), 170.40, 172.33, 173.86 ppm (3C, 3×C=O); Anal. calcd for $C_{19}H_{15}NO_7 \cdot 1/2\,H_2O$: C 60.32, H 4.26, N 3.70, found: C 59.99, H 4.53, N 3.90%.

5′-Acetamido-3,4-dibenzyloxy-2′-(4-methoxybenzyloxy)-chalcone (12): A mixture of aqueous NaOH (3.8 mL of 25.2 g/100 mL), the protected acetophenone **7** (500 mg, 1.60 mmol), and 3,4-dibenzyloxybenzaldehyde (500 mg, 1.60 mmol) in EtOH (3.8 mL) was stirred at 40 °C overnight. The mixture was cooled, then filtered, and recrystallised from THF/petroleum spirits to afford **12** as a yellow solid (692 mg, 71%); mp: 145–146 °C; ^{1}H NMR (399.7 MHz, $CDCl_3$): $\delta = 2.17$ (s, 3H, CH_3CON), 3.69 (s, 3H, CH_3O), 5.03, 5.06, 5.20 (3 s, 3×2H, 3×CH_2), 6.77 (app. d, 2H, $J = 8.4$ Hz, BB′), 6.85 (d, 1H, $J_{5,6} = 8.2$ Hz, H5), 6.92 (dd, 1H, $J_{2,6} = 2.4$, $J_{5,6} = 8.2$ Hz, H6), 7.04 (app. d, 2H, H2,3′), 7.26–7.48 (m, 14H, H6′, 2×Ph, AA′, C=CH), 7.51 (d, 1H, $J_{trans} = 15.6$ Hz, C=CH), 7.95 ppm (dd, 1H, $J_{3',4'} = 8.2$, $J_{4',6'} = 2.4$ Hz, H4′); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 23.88$, 54.98, 69.91, 70.01, 70.14 (5C, $3\times CH_2$, $2\times CH_3$), 113.65, 113.71, 113.88, 113.97, 120.37, 123.08, 123.65, 125.30, 127.53, 127.67, 127.77, 127.90, 128.45, 128.50, 128.56, 129.06, 129.51, 132.78, 136.93, 137.07, 142.74, 148.31, 150.46, 152.73, 158.96 (25C, Ar, CH=CH), 168.15, 191.48 ppm (2C, 2×C=O); Anal. calcd for $C_{39}H_{35}NO_6$: C 76.33, H 5.75, N 2.28, found: C 76.36, H 5.81, N 2.19%.

6-Acetamido-3′,4′-dibenzyloxyflavonol (14): A solution of the 4′-methoxybenzyloxychalcone **12** (300 mg, 0.489 mmol) in aqueous HCl (2 M, 16 mL) and EtOH (66 mL) was heated at reflux for 1 h. The mixture was cooled to room temperature and evaporated in vacuo to approximately half the volume. The resultant suspension was filtered to afford the crude deprotected chalcone as a dark yellow solid. The deprotected chalcone was dissolved in 1,4-dioxane (4.8 mL), EtOH (6 mL) and NaOH (5.4% *w/v*, 1.9 mL), and the resultant solution was cooled in an ice bath, and H_2O_2 (30%, 0.3 mL) was added. The solution was stirred at 0 °C for 2 h, and subsequently at room temperature overnight. The solution was then acidified with 2 M HCl, and the precipitate that formed was filtered, and then recrystallised from THF/petroleum spirits to afford **14** as a yellow solid (136 mg, 55%); mp: 229–230 °C; ^{1}H NMR (399.7 MHz, [D$_6$]DMSO): $\delta = 2.09$ (s, 3H, CH_3), 5.21, 5.24 (2 s, 2×2H, 2×CH_2), 7.26 (d, 1H, $J_{5',6'} = 8.8$ Hz, H5′), 7.33–7.52 (m, 11H, 2×Ph), 7.71 (d, 1H, $J_{7,8} = 9.2$ Hz, H8), 7.84–7.92 (m, 3H, H2′,6′,7), 8.40 (d, 1H, $J_{5,7} = 2.4$ Hz, H5), 9.48 (brs, 1H, OH), 10.27 ppm (s, 1H, NH); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 24.03$ (1C, CH_3), 69.91, 70.43, 112.65, 112.69, 113.79, 118.89, 121.38, 121.91, 124.06, 125.32, 135.91, 136.88, 137.08, 138.14, 145.14, 147.77, 149.77, 150.42 (18C, Ar), 168.59, 172.47 ppm (2C, 2×C=O); Anal. calcd for $C_{31}H_{25}NO_6$: C 73.36, H 4.96, N 2.76, found: C 73.38, H 4.98, N 2.68%.

3′,4′-Dihydroxy-6-(hydroxycarbonylethylcarbonylamino)flavonol (3): A mixture of the protected flavonol **14** (467 mg, 0.92 mmol) in aqueous HCl (36%, 35 mL) and acetic acid (35 mL) was heated at

reflux for 3 h. The mixture was then cooled on ice and diluted with H_2O. The resulting suspension was centrifuged, and the collected solid was washed with H_2O, then freeze-dried to afford the salt as a crude yellow solid. A mixture of the crude yellow product and succinic anhydride (110 mg, 1.10 mmol) in pyridine (28 mL) was stirred at room temperature for 4 h. H_2O (1 mL) was added, and the mixture was acidified with 5 M HCl. The mixture was left to stand at room temperature for 30 min, and the resulting suspension was centrifuged, and the collected solid was washed with H_2O, freeze-dried and recrystallised from 50% DMSO/H_2O to afford **3** as a yellow powder (131 mg, 44%); mp: 257–258 °C; ^{1}H NMR (399.7 MHz, [D$_6$]DMSO): $\delta = 2.54$–2.60 (m, 4H, CH_2CH_2), 6.89 (d, 1H, $J_{5',6'} = 8.5$ Hz, H5′), 7.57 (dd, 1H, $J_{2',6'} = 2.5$, $J_{5',6'} = 8.5$ Hz, H6′), 7.66 (d, 1H, $J_{7,8} = 9.0$ Hz, H8), 7.73 (d, 1H, $J_{2',6'} = 2.5$ Hz, H2′), 7.85 (dd, 1H, $J_{5,7} = 2.5$, $J_{7,8} = 9.0$ Hz, H7), 8.43 (d, 1H, $J_{5,7} = 2.5$ Hz, H5), 9.29, 9.32, 9.59 (3 br s, 3×1H, 3×OH), 10.29 (s, 1H, NH), 12.20 ppm (br s, 1H, CO_2H); ^{13}C NMR (100.5 MHz, [D$_6$]DMSO): $\delta = 28.83$, 31.11 (2C, CH_2CH_2), 112.7, 115.25, 115.65, 118.76, 120.00, 121.43, 122.39, 125.17, 135.80, 137.67, 145.12, 146.04, 147.63, 150.38 (14C, Ar), 170.46, 172.32, 173.93 ppm (3C, 3×C=O); MS (ESI) *m/z* 384.4 ($C_{19}H_{15}NO_8$ $[M-H]^+$ requires 384.08).

Assessment of water solubility of succinamic acids: a solution of Na_2CO_3 was prepared and titrated with 0.01000 M HCl to determine the exact concentration. Compounds **1**, **2**, and **3** were dissolved in stoichiometric amounts of Na_2CO_3 solution, and then evaporated to dryness. The resultant residue was redissolved in water to assess aqueous solubilities.

Pharmacological assays

Drugs and biochemicals: Acetylcholine perchlorate was obtained from BDH Chemicals (Poole, Dorset, England). L-Phenylephrine hydrochloride, pyrogallol, propranolol, DETCA, NADPH, lucigenin, and nifedipine were purchased from Sigma–Aldrich Pty. Ltd. (Castle Hill, NSW, Australia). 9,11-Dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) was purchased from Cayman Chemical (Ann Arbor, MI, USA). DiOHF was purchased from Indofine Chemical Co. Inc. (Belle Mead, NJ, USA). All biochemicals were dissolved in distilled water, except the following: U46619 was dissolved in 1 M $NaHCO_3$, nifedipine was dissolved in absolute EtOH and subsequently in Krebs' bicarbonate solution. Lucigenin, NADPH, and DETCA were dissolved in Krebs-HEPES buffer. DiOHF was dissolved in 10% DMSO, 90% MeOH, with subsequent dilutions in 50% MeOH (10^{-3} M), and distilled water (10^{-4}–10^{-7} M). Succinamic acid-substituted flavonols were dissolved in 0.1 M Na_2CO_3 as a stock solution (**1** and **2** at 10^{-1}, and **3** at 10^{-2} M), and further diluted in distilled water as required for use in organ bath experiments, and in 100% DMSO for use in the lucigenin assay. All experimental procedures were performed within the guidelines of National Health and Medical Research Council of Australia and were approved by the Pharmacology and Physiology subcommittee of the University of Melbourne Animal Experimentation Ethics Committee.

Preparation of rat thoracic aortic rings: Male Sprague–Dawley rats (200–400 g) were euthanised by exposure to 80% CO_2, 20% O_2, 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, which were then used for the in vitro assays.

Preparation of aortic rings for organ bath experiments: Aortic rings were mounted in standard 10-mL organ baths containing Krebs-bicarbonate solution [composition (mM): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 5.0; $NaHCO_3$, 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 60 min at a resting tension of 1 g. The rings were pre-contracted with an isotonic, high-potassium physiological salt solution (Krebs' potassium salt solution, KPSS, 123 mM KCl) to achieve the maximum tension. After a washout of 30 min, the integrity of the endothelium was tested. Rings were pre-contracted with phenylephrine (PE, 10–100 nM), and only rings that responded to ACh (≥90% relaxation) were judged endothelium intact and were used in the subsequent experiments. The aortic rings were then re-equilibrated (15 min) before the subsequent experiments.

Effect of flavonols on PE-induced vasoconstriction: Aortic rings were incubated with a β-adrenoceptor antagonist, propranolol (10^{-5} M), and either vehicle or flavonol (10^{-4}–10^{-5} M) for 15 min before cumulative doses of phenylephrine (10^{-9}–$10^{-4.5}$ M) were added to generate a concentration–response curve to phenylephrine. Contraction responses were expressed as a percentage of KPSS-induced tension.

Relaxation by flavonols: Aortic rings were pre-contracted submaximally with phenylephrine (10^{-8}–10^{-7} M), and the thromboxane mimetic, 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, 10^{-10}–10^{-9} M) to approximately 50% of KPSS-induced contraction. After stabilisation of the contraction, cumulative doses of the flavonol or vehicle (10^{-10}–10^{-4} M) were added to generate a concentration–response curve. Relaxant responses were expressed as a percentage of the pre-contraction tension.

Effect of flavonols on superoxide levels: Superoxide concentrations were measured in isolated aortic rings by lucigenin-enhanced chemiluminescence. Prior to assaying, the aortic rings were incubated at 37 °C for 1 h in Krebs-HEPES buffer containing DETCA (3×10^{-5} M), which inactivates superoxide dismutase, NADPH (10^{-4} M), and either vehicle, succinamic acid-substituted flavonol (10^{-4} or 10^{-5} M), or DiOHF (10^{-4} or 10^{-5} M). Assay solutions consisting of lucigenin (5×10^{-6} M), NADPH (10^{-4} M), and either vehicle (DMSO), succinamic acid-substituted flavonol (10^{-4} or 10^{-5} M), or 3′,4′-dihydroxyflavonol (DiOHF, 10^{-4} or 10^{-5} M), as a positive control, were prepared in Krebs-HEPES buffer [composition (mM): NaCl, 99.0; KCl, 4.7; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 11.0; $NaHCO_3$, 25.0; $CaCl_2 \cdot 2H_2O$, 2.5; Na-HEPES, 20.0]. Aliquots (300 μL) of the assay solution were placed into separate wells on a 96-well Optiplate, which was loaded into a TopCount single-photon counter (Packard Bioscience) to determine the background emission (12 cycles). After background counting was completed, one aortic ring was added per well, and photon emission was counted (12 cycles). Superoxide levels were reported as a percentage of photon emission for +NADPH control. At the conclusion of the assay, the aortic rings were dried for 48 h at 80 °C for normalisation of superoxide production to dry tissue weight.

Effect of flavonols on vasorelaxation to acetylcholine in the presence of oxidative stress generated by pyrogallol: To test for the ability of the succinamic acid substituted flavonols to preserve endothelial function in the presence of oxidative stress, responses to ACh were examined in the presence of pyrogallol, a generator of superoxide radicals. Isolated aortic rings were prepared as described above in standard 10-mL organ baths. After equilibration, the maximum responses established, and the endothelium integrity tested, rings were incubated for 20 min with pyrogallol (30 μM) and vehicle, pyrogallol (30 μM) and the succinamic acid-substituted flavonols (10^{-4} or $10^{-4.5}$ M), pyrogallol (30 μM) with the positive control 3′,4′-dihydroxyflavonol (10^{-5} M), or vehicle. The L-type Ca^{2+}

channel blocker nifedipine (30 nM) was also added to all aortic rings to prevent spontaneous activity. After establishing a stable pre-contraction tone of approximately 50% of KPSS-induced contraction with U46619 (1–10 nM), cumulative doses of ACh were added to generate a concentration–response curves. Relaxant responses were expressed as a percentage of the pre-contraction tension.

Data presentation and statistical analysis: The results are expressed as the mean ± SE mean and *n* indicates the number of experiments (rats). Relaxation concentration–response curves were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 4.0) to enable calculation of the pEC_{50} values of the flavonols. For cases in which the data did not fit a sigmoidal curve, pEC_{50} values were not calculated. Maximum relaxation responses (R_{max}) were compared by using a one-way analysis of variance (ANOVA) test with post-hoc multiple comparisons using the Newman–Keuls test. Superoxide levels were compared by using ANOVA with post-hoc multiple comparison using Dunnett's test; $p < 0.05$ was considered statistically significant. Superoxide levels from rat aortic rings were expressed as average counts per second normalised to dry tissue weight. Counts in the presence of treatments were expressed as a percentage of the counts in the presence of vehicle (0.1% Krebs-HEPES buffer) using Dunnett's test (Prism version 4.0); $p < 0.05$ was considered statistically significant.

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