SUPPORTING INFORMATION
Electronic Supplementary Material (ESI) for Medical Chemical Communications

Water soluble flavonol prodrugs that protect against ischaemia-reperfusion injury in rat hindlimb and sheep heart

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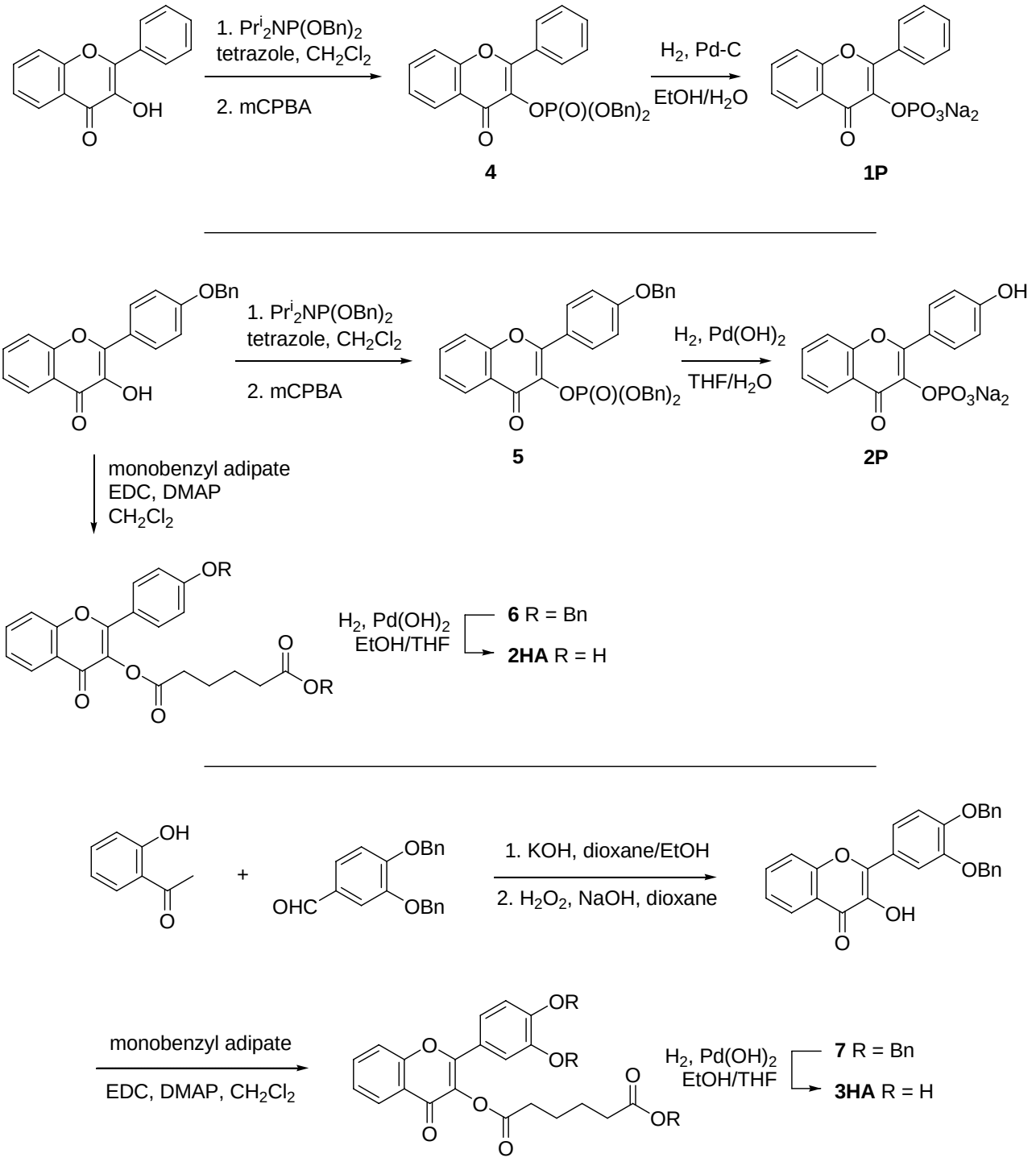
Synthetic chemistry



General chemical methods. Thin layer chromatography (t.l.c) was performed with Merck Silica Gel 60 F_{254}, using mixtures of petroleum spirits-ethyl acetate. Detection was effected by visualization in UV light. NMR spectra were obtained on a Unity 400 machine (Melbourne, Australia) operating at 400 MHz for ^{1}H, 100.5 MHz for ^{13}C, and 161.8 MHz for ^{31}P. Flash chromatography was performed according to the method of Still *et al*. with Merck Silica Gel 60

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(1). Solvents were evaporated under reduced pressure using a rotary evaporator. Melting points were obtained using an Electrothermal melting point apparatus (uncorrected) or a Reichert-Jung hot-stage (corrected). Elemental analyses were performed by C.M.A.S. (Belmont, Victoria). High resolution mass spectra were performed by Sally Duck at the Chemistry Department, Monash University, or by Hadi Loie at the University of Melbourne on an FT-ICR MS. IR spectra were obtained using a PerkinElmer Spectrum One FT-IR spectrometer with a zinc selenide/diamond Universal ATR Sampling Accessory as a thin film. Flavonol (99%) and 3',4'-dihydroxyflavonol (99%) were obtained from Indofine Chemical Company. 4'-Hydroxyflavonol (2) was prepared as reported (2).

3-(Dibenzyloxyphosphoryloxy)flavone 4. Dibenzyl *N*,*N*-diisopropylphosphoramidite (12.5 ml, 38.0 mmol) and 1*H*-tetrazole (74.0 ml, 31.7 mmol) was added to a solution of 3-hydroxyflavone (3.00 g, 12.6 mmol) in dry dichloromethane (150 ml). The reaction mixture was stirred under N_2 at room temperature for 2 h. The mixture was then cooled to –78 ºC and *m*-CPBA (8.72 g, 50.6 mmol) was added. The mixture was allowed to return to room temperature and stirred for a further 45 min. The reaction mixture was washed with 0.25 M $Na_2S_2O_4$ (3 × 100 ml), saturated $NaHCO_3$ (3 × 100 ml) and water (2 × 100 ml). The organic extract was dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield a crude white solid. The crude material was purified by flash chromatography (20-50% EtOAc in toluene) followed by crystallization for EtOAc/petroleum spirits to give the protected phosphate as a white fluffy solid (5.28 g, 84 %); mp = 85-88 ·C; ^{1}H NMR (399.7 MHz, $CDCl_3$) ς 5.09-5.17 (m, 4H, C*H*$_2$Ph), 7.22-7.30, 7.38-7.47 (2 „ m, 14H, 2 × Ph,H6,3',4',5'), 7.51 (d, $J_{7,8}$ = 8.5 Hz, H8), 7.69 (ddd, $J_{5,7}$ = 1.5, $J_{6,7}$ = 7.2, $J_{7,8}$ = 8.5 Hz, 1H, H7), 7.93-7.96 (m, 2H, H2',6'), 8.29 (dd, $J_{5,6}$ = 7.5, $J_{5,7}$ = 1.5 Hz, 1H, H5); ^{13}C NMR (100.5 MHz, $CDCl_3$) ς 78.1 (2C, *C*H$_2$Ph), 119.1, 124.9, 126.2, 127.2, 128.9, 129.4, 129.5, 129.6, 130.1, 131.0, 132.3, 135.1, 157.0, 157.1 (25C, Ar), 136.9 (1C, $J_{C,P}$ = 8.0 Hz, C-O-phosphate), 173.9 (1C, C=O); ^{31}P NMR (161.8 MHz, $CDCl_3$) ς –7.45 (s, P=O); Anal.: Found: C, 69.81; H, 4.60; $C_{29}H_{23}O_6P$

requires C, 69.88; H, 4.65%; HRMS (ESI$^+$) *m/z* 521.1126, $C_{29}H_{23}NaO_6P$ $[M + Na]^+$ requires 521.1130.

3-Hydroxyflavone-3-phosphate disodium salt 1P. A solution of 3-(dibenzyloxyphosphoryloxy)flavone (2.05 g, 4.09 mmol) and palladium on carbon (10%, 0.25 g) in EtOH:water (4:1, 250 ml) was treated with H_2 at atmospheric pressure for 3.5 h. The reaction mixture was filtered (Celite) and the filtrate treated with NaOH (0.50 g in 100 ml water). The aqueous mixture was concentrated under reduced pressure then crystallized from water/acetone to yield the phosphate as pale yellow crystals (1.03 g, 87% yield). ^{1}H NMR (499.7 MHz, D_2O) ɡ 7.33 (dd, 1H, $J_{5,6}$ = 8.0, $J_{5,7}$ = 1.2 Hz, H6), 7.40-7.46 (m, 3H, H3',4',5'), 7.52 (d, 1H, $J_{7,8}$ = 8.5 Hz, H8), 7.64 (ddd, 1H, $J_{5,7}$ = 1.2, $J_{6,7}$ = 7.5, $J_{7,8}$ = 8.5 Hz, H7), 7.97 (dd, 1H, $J_{5,6}$ = 8.0, $J_{5,7}$ = 1.2 Hz, H5), 8.10 (m, 2H, H2',6'); ^{13}C NMR (100.5 MHz, D_2O) ɡ 118.3, 122.9, 125.0, 125.1, 128.5, 129.2, 130.9, 131.3, 134.2, 155.1, 156.7 (13C, Ar), 136.1 (1C, $J_{C,P}$ = 6.8 Hz, *C*-O-P), 177.2 (1C, C=O); ^{31}P NMR (161.8 MHz, D_2O) ɡ 2.98 (s, P=O); Anal.: Found: C, 49.68; H, 2.51; $C_{15}H_{11}Na_2O_6P$ requires C, 49.74; H, 2.50%.

4'-Benzyloxy-3-(dibenzyloxyphosphoryloxy)flavone 5. 1*H*-Tetrazole (483 mg, 6.89 mmol) was added to a mixture 4'-benzyloxy-3-hydroxyflavone (3) (1.00 g, 2.72 mmol), dibenzyl *N*,*N*-diisopropylphosphoramidite (1.5 ml, 1.6 g, 4.4 mmol) in dichloromethane (30 ml) and the reaction was stirred at rt for 2 h. Additional dibenzyl *N*,*N*-diisopropylphosphoramidite (1.0 ml, 1.1 g, 1.5 mmol) was added and the reaction stirred for a further 1 h. The reaction mixture was then cooled to –78 ºC and *m*-CPBA (3.00 g, 12.1 mmol, 70% w/w) was added. The reaction was then warmed to rt and stirred for 45 min. The organic layer was washed with 0.25 M $Na_2S_2O_3$ (× 3), sat $NaHCO_3$ (× 3), brine (× 3), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (50% EtOAc/petrol) to give a yellow solid, which was recrystallized from EtOAc/petrol to afford the phosphate as a colourless solid (1.01 g, 58%); mp 101 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) ɡ 5.02

(s, 2H, C*H*$_2$Ph), 5.16 (s, 2H, C*H*$_2$Ph), 5.17 (s, 2H, C*H*$_2$Ph), 6.97 (app. d, 2H, *J* = 8.8 Hz, H3',5'), 7.29-7.36 (m, 15H, 3 × Ph), 7.41 (t, 1H, $J_{5,6} = J_{6,7}$ = 8.0 Hz, H6), 7.51 (d, $J_{7,8}$ = 8.5 Hz, H8), 7.68 (dd, 1H, $J_{6,7}$ = 8.0, $J_{7,8}$ = 8.5 Hz, H7), 7.96 (app. d, 2H, *J* = 8.8 Hz, H2',6'), 8.30 (dd, 1H, $J_{7,8}$ = 8.5, $J_{6,8}$ =1.5 Hz, H8); ^{13}C NMR (100 MHz, $CDCl_3$) ɡ 69.9, 70.0 (CH_2), 144.7, 117.9, 122.3, 123.7, 125.0, 126.1, 127.4, 127.8, 128.2, 128.4, 128.7, 130.7, 133.8, 135.8, 135.9, 136.1, 155.2, 155.7, 161.0 (Ar), 172.6 (1C, C=O); ^{31}P NMR (162 MHz, $CDCl_3$) ɡ -5.3 (s, P); IR ɤ 3063, 3031, 1647, 1601, 1506, 983 cm^{-1}; Anal.: Found C, 72.60; H, 5.05%, $C_{36}H_{29}O_7P$ requires C, 71.52; H, 4.83%.

3,4'-Dihydroxyflavone-3-phosphate disodium salt 2P. A mixture of 4'-benzyloxy-3-(dibenzyloxyphosphoryloxy)flavone (1.00 g, 1.65 mmol) and $Pd(OH)_2$ (120 mg) in THF (10 ml) and water (15 ml) was treated with hydrogen for 3 d. The mixture was filtered (Celite) and the pad washed with THF and water, and the filtrate was concentrated. The solid residue was dissolved in THF (20 ml) and water (10 ml). Triethylamine (600 ɒL, 4.3 mmol) was added and stirred at rt for 30 min. The mixture was concentrated and the residue was dissolved in water. Insoluble material was removed by filtration and the solution passed through an ion exchange column (Dowex 50WX8-400, Na^+ form). The eluant was concentrated to afford a solid, which was recrystallised from acetone/water to afford the phosphate as a brown solid (226 mg, 36%); mp 182-184 °C; ^{1}H NMR (500 MHz, D_2O) ɡ 7.07 (app. d, 2H, *J* = 8.5 Hz, H3',5'), 7.62 (t, 1H, $J_{5,6} = J_{6,7}$ = 7.5 Hz, H6), 7.72 (d, $J_{7,8}$ = 8.5 Hz, H8), 7.92 (t, 1H, $J_{6,7} = J_{7,8}$ = 7.5 Hz, H7), 8.18 (d, 1H, $J_{5,6}$ = 7.5 Hz, H5), 8.19 (app. d, 2H, *J* = 8.5 Hz, H2',6'); ^{13}C NMR (100 MHz, D_2O) ɡ 118.4, 122.5, 122.6, 124.9, 125.3, 131.2, 134.4, 155.0, 157.1, 157.2, 158.5 (Ar), 176.3 (1C, C=O); ^{31}P NMR (162 MHz, $CDCl_3$) ɡ 0.60 (s, P); IR ɤ 3281, 1597, 1579, 1542, 1393, 903 cm^{-1}; HRMS (ESI$^-$) *m/z* 333.0160, $C_{15}H_{10}O_7P$ $[M + H]^-$ requires 333.0159.

Adipic acid monobenzyl ester. A suspension of adipic acid (20.0 g, 137 mmol), benzyl alcohol (21.5 ml, 22.3 g, 206 mmol) and *p*-toluenesulfonic acid (260 mg, 1.37 mmol) in toluene (55 ml)

was heated under reflux (Dean-Stark) for 5 h. The solution was allowed to cool to rt and water was added (30 ml). Cresol red (1-2 mg) was added to the mixture to give an orange/yellow colour. 2 M NaOH was then added dropwise, with shaking after each addition, until a red colour (pH 8.8) was obtained. The aqueous phase was separated and washed with ether (× 1). The aqueous layer was acidified with 2 M HCl until an orange/yellow colour (pH 1.8) was obtained. The aqueous layer was extracted with ether (× 2), and the combined organic phases dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (15% EtOAc/petrol) to give the half ester as a colourless oil (10.8 g, 33%); ^{1}H NMR (400 MHz, $CDCl_3$) ɡ 1.68-1.70 (m, 4H, CH_2CH_2), 2.35-2.43 (m, 4H, C*H*$_2$COOH, C*H*$_2$COOBn), 5.12 (s, 2H, C*H*$_2$Ph), 7.34-7.37 (m, 5H, Ph). These data are consistent with that reported in the literature (4).

4'-Benzyloxy-3-(benzyloxycarbonylbutylcarbonyloxy)flavone 6. EDC hydrochloride (843 mg, 4.40 mmol) was added to a solution of 4'-benzyloxy-3-hydroxyflavone (3) (1.00 g, 2.90 mmol), adipic acid monobenzyl ester (1.30 g, 5.50 mmol) and DMAP (354 mg, 2.89 mmol) in dichloromethane (110 ml) and the mixture was stirred at rt overnight. The reaction mixture was then concentrated and the residue dissolved in ethyl acetate. The organic phase was washed with water (× 3), 1 M HCl (× 3), sat $NaHCO_3$ (× 3), brine (× 3), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (50% EtOAc/petrol) to give the benzyl ester as a brown oil, which was crystallized from EtOAc/petrol to give a colorless solid (900 mg, 55%), mp 93 °C; ^{1}H NMR (500 MHz, $CDCl_3$) ɡ 1.77-1.83 (m, 4H, CH_2CH_2), 2.42 (t, 2H, J = 7.5 Hz, CH_2CO), 2.67 (t, 2H, J = 6.5 Hz, CH_2CO), 5.13 (s, 2H, C*H*$_2$Ph), 5.15 (s, 2H, C*H*$_2$Ph), 7.09 (d, 1H, $J_{7,8}$ = 8.5 Hz, H8), 7.35 (app. d, 2H, J = 8.5 Hz, H2',6'), 7.40-7.46 (m, 12H, 2 × Ph, H6,4'), 7.69 (ddd, 1H, $J_{5,7}$ = 1.5, $J_{6,7}$ = 7.0, $J_{7,8}$ = 8.5 Hz, H7), 7.85 (app. d, 2H, J = 8.5 Hz, H3',5'), 8.25 (d, 1H, $J_{5,6}$ = 8 Hz, H5); ^{13}C NMR (125 MHz, $CDCl_3$) ɡ 24.2 (× 2), 33.6, 33.8 (4C, CH_2), 66.2, 70.1 (2C, CH_2Ph), 115.0, 117.9, 122.4, 123.5, 125.0, 126.0, 127.4, 128.1, 128.2, 128.5, 128.7, 130.0, 133.0, 133.7, 135.9, 136.2,

155.5, 156.1, 161.1 (Ar), 170.4, 172.0, 173.0 (3C, C=O); IR x 2937, 1760, 1730, 1646, 1602, 1507, 1468, 899 cm^{-1}; Anal.: Found C, 74.67; H, 5.29, $C_{35}H_{30}O_7$ requires C, 74.72; H, 5.37%.

3,4'-Dihydroxyflavone 3-hemiadipate 2HA. A mixture of 4'-(benzyloxy)-3-(benzyloxycarbonylbutylcarbonyloxy)flavone (400 mg, 0.711 mmol) and $Pd(OH)_2$ (56 mg) in THF (10 ml), ethanol (1.2 ml) and AcOH (100 pL) was treated with hydrogen (50 psi) for 18 h. The reaction mixture was then filtered (Celite) and the pad washed with THF. The filtrate was concentrated and the solid residue was purified by flash chromatography (70% THF/toluene + 1% AcOH) and the resultant solid recrystallised from THF/petrol to afford the acid as a colorless solid (150 mg, 55%), mp 177-180 ºC; ^{1}H NMR (500 MHz, d_6-DMSO) g 1.56-1.66 (m, 4H, CH_2CH_2), 2.25 (t, 2H, J = 7.0 Hz, CH_2CO), 2.64 (t, 2H, J = 7.0 Hz, CH_2CO), 6.96 (app. d, 2H, J = 8.5 Hz, H2',6'), 7.52 (t, 1H, $J_{6,7} = J_{7,8}$ = 7.5 Hz, H7), 7.80 (app. d, 2H, J = 7.5 Hz, H3',5'), 7.78 (m, 1H, H8), 7.85 (t, 1H, $J_{5,6} = J_{6,7}$ = 7.5 Hz, H6), 8.06 (d, 1H, $J_{5,6}$ = 8.0 Hz, H5); ^{13}C NMR (125 MHz, d_6-DMSO) g 23.8, 23.9, 32.9, 33.3 (4C, CH_2), 115.9, 118.5, 119.7, 122.7, 125.0, 125.6, 130.1, 131.9, 134.5, 154.9, 155.8 160.6 (Ar), 170.4, 170.9, 174.3 (3C, C=O); IR x 3257, 2944, 2869, 1765, 1706, 1595, 854 cm^{-1}; HRMS (ESI^+) *m/z* 383.1123, $C_{21}H_{19}O_7$ $[M + H]^+$ requires 383.1131.

3',4'-Dibenzyloxy-3-hydroxyflavone. A suspension of 3,4-dibenzyloxybenzaldehyde (18.2 g, 57.2 mmol) and 2-hydroxyacetophenone (7.92 g, 58.1 mmol) in ethanol (120 ml) and dioxane (75 ml) was cooled to 10 ·C, potassium hydroxide solution (40% w/v in H_2O, 45 ml) was added dropwise, then the mixture was stirred at room temperature for 6 d. The resulting red solution was diluted with dichloromethane (200 ml), washed with distilled water (4 „ 100 ml) then the organic layer was dried ($MgSO_4$) and concentrated to give a brown residue. This residue was re-dissolved in dioxane (200 ml) and ethanol (450 ml) and treated with 2 M NaOH (140 ml) then the solution cooled to 0 ·C and H_2O_2 (30% w/v, 40 ml) was added dropwise. The reaction mixture was stirred at 0 ·C for 2 h then at room temperature for a further 17 h. The resulting yellow suspension was acidified with 2

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M HCl (40 ml), filtered and the solid washed with ethanol. The crude solid was recrystallised from hot EtOAc to give the flavonol as a pale yellow fluffy solid. The filtrates from the crude and recrystallized materials were combined, concentrated under reduced pressure and a second crop of the flavonol extracted with EtOAc (120 ml). The organic extract was dried ($MgSO_4$), filtered and the filtrate concentrated and crystallized from EtOAc/petroleum spirits to give a second crop of the pure flavonol (15.8 g, 61% yield); mp 146-150 ·C (lit. (3) mp = 145.8-146.8 ·C). The ^{1}H NMR spectrum was in agreement with that reported in the literature (3).

3',4'-Dibenzyloxy-3-(benzyloxycarbonylbutylcarbonyloxy)flavone 7. Adipic acid monobenzyl ester (1.91 g, 5.04 mmol) followed by EDC hydrochloride (0.764 g, 3.98 mmol) and DMAP (0.324 g, 2.65 mmol) were added to a stirring solution of 3',4'-dibenzyloxyflavonol (1.21 g, 2.68 mmol) in dry dichloromethane (100 ml) and the resultant mixture was stirred at room temperature under N_2 for 3 h. The reaction mixture was concentrated under reduced pressure and re-suspended in ethyl acetate (100 ml). The suspension was then washed with water (3 × 50 ml), 1M HCl (3 × 50 ml), saturated $NaHCO_3$ (3 × 50 ml) and brine (3 × 50 ml). The organic extract was dried ($MgSO_4$), filtered, concentrated under reduced pressure and the yellow residue crystallized from EtOAc/ petroleum spirits to yield the benzyl ester as a fluffy yellow solid (1.58 g, 88%); mp = 84-85 °C; ^{1}H NMR (399.8 MHz, $CDCl_3$) ɡ 1.70-1.80 (m, 4H, C*H*$_2$C*H*$_2$), 2.38 (t, 2H, *J* = 6.8 Hz, C*H*$_2$CO), 2.55 (m, 2H, C*H*$_2$CO), 5.10 (s, 2H, *CH*$_2$Ph), 5.20 (s, 2H, *CH*$_2$Ph), 5.24 (s, 2H, *CH*$_2$Ph), 7.01 (d, 1H, $J_{7,8}$ = 8.4 Hz, H8), 7.26-7.49 (m, 19H, Ar, H6,2',5',6'), 7.62 (ddd, 1H, $J_{5,7}$ = 1.2, $J_{6,7}$ = 7.2, $J_{7,8}$ = 8.4 Hz, H7), 8.22 (dd, 1H, $J_{5,6}$ = 7.6, $J_{5,7}$ = 1.2 Hz, H5); ^{13}C NMR (100.5 MHz, $CDCl_3$) ɡ 25.3 (2C, *C*H_2CH_2CO_2), 34.6, 34.9 (2C, *C*H_2CO_2), 67.3, 71.9, 72.6 (2C, *C*H_2Ph), 114.8, 115.8, 119.1, 123.8, 123.8, 124.6, 126.2, 127.1, 128.2, 128.4, 129.2, 129.3, 129.6, 129.7, 130.1, 134.2, 134.9, 137.0, 137.5, 137.8, 149.6, 152.6, 156.5, 157.0 (32C, Ar), 171.5, 173.1, 174.2 (3C, C=O); Anal.: Found: C, 75.39; H, 5.47; $C_{42}H_{36}O_8$ requires C, 75.43; H, 5.43%; HRMS (ESI^+) *m/z* 691.2303, $C_{42}H_{36}NaO_8$ $[M + Na]^+$ requires 691.2308.

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3,3',4'-Trihydroxyflavone-3-hemiadipate 3HA. A mixture of 3',4'-dibenzyloxy-3-(benzyloxycarbonylbutylcarbonyloxy)flavone (2.12 g, 3.16 mmol) and $Pd(OH)_2$ (107 mg) in 9:1 THF:EtOH containing 0.05% acetic acid (50.0 ml) was treated with H_2 under high pressure (40 psi) for 5 h. The reaction mixture was filtered (Celite) and concentrated to give a dark green solid. The green residue was purified by flash chromatography (30-90% THF/toluene + 1% acetic acid) followed by crystallization from THF/petroleum spirits to yield the pure hemiadipate as a pale brown solid (0.70 g, 56%); mp = 194-197 ·C; ^{1}H NMR (399.8 MHz, $CDCl_3$) ɡ 1.44-1.62 (m, 4H, CH_2CH_2), 2.10 (t, 2H, J = 6.8 Hz, CH_2CO), 2.44 (t, 2H, J = 6.8 Hz, CH_2CO), 6.73 (d, 1H, $J_{5',6'}$ = 8.4 Hz, H5'), 7.09 (dd, 1H, $J_{2',6'}$ = 2.0, $J_{5',6'}$ = 8.4 Hz, H6'), 7.16-7.22 (m, 2H, H6,2'), 7.32 (d, 1H, $J_{7,8}$ = 8.0 Hz, H8), 7.48 (ddd, 1H, $J_{5,7}$ = 1.6, $J_{6,7}$ = 6.8, $J_{7,8}$ = 8.0 Hz, H7), 7.94 (dd, 1H, $J_{5,6}$ = 8.4, $J_{5,7}$ = 1.6 Hz, H5); ^{13}C NMR (100.5 MHz, d_6-DMSO) ɡ 25.3, 25.6 (2C, CH_2CH_2CO_2), 34.4, 34.8 (2C, CH_2CO_2), 116.6, 117.4, 120.0, 121.4, 122.1, 124.1, 126.5, 127.1, 133.3, 136.0, 147.0, 150.6, 156.4, 157.3 (14C, Ar), 172.0, 173.5, 180.0 (3C, C=O); Anal.: Found: C, 63.30; H, 4.50; $C_{21}H_{18}O_8$ requires C, 63.32; H, 4.55 %; HRMS (ESI^+) *m/z* 389.1000, $C_{21}H_{18}NaO_6$ $[M + Na]^+$ requires 389.1001.

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Animal studies

Animal studies followed current NIH guidelines for the Use of Laboratory Animals. Rat studies were performed within the guidelines of the National Health & Medical Research Council of Australia and were approved by the Pharmacology and Physiology subcommittee of the University of Melbourne Animal Experimentation Ethics Committee. Sheep studies were performed with approval of the Howard Florey Institute Animal Ethics Committee.

Pharmacological analysis

Preparation of rat aorta. Male Sprague-Dawley rats (250-300 g) were housed 3 per cage, with free access to tap water and food pellets. Rats were killed by exposure to halothane for 5 min. The descending thoracic aorta was rapidly dissected and placed in Krebs-bicarbonate solution of the following composition (mM): NaCl 118.0, $NaHCO_3$ 25.0, D-glucose 11.0, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, and $MgSO_4$ 1.18. Superficial connective tissue and fat surrounding the aorta was removed and the aorta was cut into segments 2-3 mm long. The rings were mounted between two stainless steel hooks with one hook linked to an isometric force transducer (WPI Fort 5000), which was connected to a Mac Lab. The other hook was connected to a glass rod in a 10 ml organ bath chamber containing Krebs-bicarbonate solution maintained at 37·C and pH 7.4 and continuously aerated with 95% O_2 and 5% CO_2. The aortic rings were allowed to equilibrate for 90 min at a resting tension of 1 g, with the bath media being changed every 20 min.

Effect of Flavonols on Ca^{2+}-Induced Contraction. To determine the effect of flavonols on responses to the influx of extracellular Ca^{2+}, contractile responses to exogenous application of Ca^{2+} were examined in the presence of flavonols in Ca^{2+}-free high-K^+ solution (60 mM, K^+-PSS). Aortic rings were initially equilibrated at a resting tension of 1 g in normal Ca^{2+}-free PSS for 45 min. The bath medium was then replaced with Ca^{2+}-free high-K^+-PSS for 45 min to determine a reference contraction to Ca^{2+} (3×10^{-3} M). Following a 30 min re-equilibration period with Ca^{2+}-free PSS, the

cumulative contractile responses to Ca^{2+} (10^{-5}-3 × 10^{-3} M) were determined in K^{+}-PSS in the presence of a range of concentrations (10^{-7}-10^{-4} M) of vehicle (0.1% DMSO), **1**, **1P**, **2**, **2HA**, **2P**, **3**, or **3HA**. A 20 min incubation period was allowed for the flavonols before examining the responses to Ca^{2+}. Experiments were conducted in the presence and absence of 1 U/mL butyrylcholinesterase to cleave the hemiadipate in **2HA** and **3HA**. In experiments using **1P** and **2P**, 1 U/mL phosphatase was used in some experiments to cleave the phosphate group. In some experiments the appropriate drug and butyrylcholinesterase or phosphatase were incubated together at 37°C for 1 h prior to the Ca^{2+} response curve being measured.

Effect of Flavonols on Superoxide Levels in an *In vitro* Assay. Superoxide anion production in rat isolated aortic segments was determined using lucigenin-enhanced chemiluminescence. Aortic rings were prepared as described above and then placed in ice-cold Krebs-(*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES buffer)) (composition (mM): NaCl 99.0, KCl 4.7, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 1.2, D-glucose 11.0, $NaHCO_3$ 25.0, $CaCl_2.2H_2O$ 2.5, Na-HEPES 20.0) bubbled with O_2/CO_2 (95/5). Aortic rings were preincubated for 45 min at 37°C with a pH 7.4, in Krebs-HEPES buffer containing diethylthiocarbamic acid (DETCA, 10^{-3} M) to irreversibly inactivate superoxide dismutase and e-nicotinamide adenine dinucleotide phosphate (NADPH, 10^{-4} M) as a substrate for NADPH oxidase and one of the following treatments diphenyliodonium chloride (5 „ 10^{-6} M) to inhibit NADPH oxidase, vehicle (0.1% DMSO), **1**, **1P**, **2**, **2HA**, **2P**, **3**, or **3HA**. Each well of a 96-well Optiplate contained 0.3 mL of Krebs-HEPES buffer together with lucigenin (5 „ 10^{-5} M), NADPH (10^{-4} M) and vehicle (0.1% DMSO) or flavonols (10^{-7}-10^{-4} M) and background photon emissions were measured for 12 cycles. After background readings were completed the incubated aortic rings were transferred to the appropriate wells and photon emissions were recounted as described above with one ring per well and at 37 °C. The aortic rings were then placed in a 65 ·C oven for 48 h to allow superoxide production to be normalised to dry tissue weight. Experiments were conducted in the presence and absence of 1 U/mL

butyrylcholinesterase to cleave the adipate in **2HA** and **3HA**. In experiments using **1P** and **2P**, 1 U/mL phosphatase was used in some experiments to cleave the phosphate. In some experiments the flavonol prodrugs and butyrylcholinesterase or phosphatase were mixed and pre-incubated for 1 h prior to adding wells of the Optiplate.

Rat Hindquarters Ischaemia and Reperfusion model

Male Sprague-Dawley rats (300-400 g) were anaesthetized with pentobarbitone sodium (60 mg/kg, i.p.) and blood pressure and heart rate continuously monitored. When required, extra anaesthetic (3 mg, iv) was given to the rat. Rats were placed on a heating pad to maintain normal body temperature. The trachea was isolated and cannulated with polyethylene tracheal tube (I.D. 2.0 mm), and the rat was allowed to breathe spontaneously. The jugular vein was cannulated with a saline-filled cannula for intravenous administration of drugs or anaesthetic. Bilateral hindlimb ischaemia was induced through the application of latex O-rings (Bainbridge Supreme, Australia) above the greater trochanter of each hind limb. Following 2 h of ischaemia, the latex rings were cut and removed and limbs were allowed to reperfuse for 4 h. The sham ischaemia/reperfusion group underwent surgery and cannulation, but without hindquarter ischaemia (6 h). In the ischaemia/reperfusion group, rats were subjected to hindquarter ischaemia and the various treatments by a single dose of either vehicle or flavonol, administered via the jugular vein 5 min before reperfusion.

Measurements of Arterial Pressure and Heart Rate. The right carotid artery was cannulated with a heparinised saline filled cannula, which was connected to a pressure transducer (Spectramed, Carolina Medical Inc, King, NC, USA). Mean and phasic arterial pressure were continuously measured and recorded on a polygraph (Model 7E, Grass Instrument Company, Quincy, MA, USA). The heart rate was derived from the phasic arterial pressure using a tachometer.

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Experimental Design. Four experimental groups were chosen for this study: sham-operated, ischaemia/reperfusion (IR) only, I/R + **3HA** and I/R + **2P**. Sham-operated rats did not undergo any ischaemia or reperfusion. I/R rats had tourniquets applied for 2 h of ischaemia followed by 4 h of reperfusion. I/R + flavonols treated rats underwent the same process but were also treated with the flavonol as described below.

Drug Administration. All drugs were given as bolus injections. Rats were allowed to stabilise for approximately 15 min at the end of surgery prior to the induction of ischaemia. The flavonols were administered 5 min prior to reperfusion.

Evaluation of Lactate Dehydrogenase Levels. Circulating levels of lactate dehydrogenase (LDH) were measured in plasma samples taken immediately before and after ischaemia and following 1, 2, 3 and 4 h reperfusion. LDH levels were assayed using spectrophotometric detection with commercially available assay kits (Roche Diagnostics). Blood samples were collected, centrifuged at 3500 rpm and plasma samples were stored at –80 ·C until LDH assay. An aliquot of 200 pL of plasma was added to 700 pL or reaction buffer and 100 pL of distilled water. Samples were incubated in cuvettes and absorbance of samples measured for 3 min at 3 s intervals. LDH levels were measured as the decrease in absorbance at 340 nm due to the oxidation of NADH, which indicates the oxidation of lactate to pyruvate.

Ovine Cardiac Ischaemia/Reperfusion Injury Model

The studies were performed in adult female merino sheep (39-58 kg, 2-3 years old). The procedure for cardiac ischaemia and reperfusion in sheep was performed as previously described (5). Briefly, on the day of surgery, anaesthesia was induced by intravenous thiopentone sodium (15 mg kg^{-1}) and following intubation, was maintained by isoflurane (1.5-2%). A catheter was inserted into the right facial artery for monitoring arterial blood pressure. The heart was exposed through a left

thoracotomy performed at the fifth intercostal space. The second diagonal branch (D2) of the left anterior descending coronary artery (LAD) was dissected from the epicardium, and a transit time flow-probe (2 mm, Transonic Systems Inc, USA) was placed around it to monitor D2 blood flow. A soft rubber neoloop (2 mm width) was passed under the D2 branch proximal to the probe and both ends of the loop were threaded through a plastic tube to form a vascular snare.

Following the surgical procedure, sheep were allowed to stabilize for 30 min. Sheep in different groups were then subjected to 60min ischaemia and 3h reperfusion with intravenous administration of vehicle or flavonol prodrug (n=5-6 per group). Ischaemia was achieved by tightening the snare to stop flow. ECG and coronary flow were monitored to confirm LAD occlusion. Lidocaine (via catheter in left atria, 2 mg kg^{-1}) was used to control arrhythmias as required. DiOHF (**3**) was dissolved in 4 ml dimethylsulfoxide, then mixed in polyethylene glycol and water (1:1; total 40 ml) at a dose of 5 mg kg^{-1} and the solutions was infused intravenously for 20 min (beginning after 30 min ischaemia and ending 10 min before reperfusion). Water-soluble prodrugs **2P** and **3HA** were administered at equivalent molar doses to **3** (6.6 mg kg^{-1}, dissolved in 10 ml 0.1 M sodium carbonate). The prodrugs were administered as bolus injections into the jugular vein over 1 min, beginning at 50 min ischaemia.

At the end of the ischaemic period, the snare was loosened. After 3 h reperfusion, the D2 branch was re-occluded with the snare. Immediately after injecting sodium pentobarbitone (100 mg kg^{-1}) to arrest the heart, Evans blue dye (1.5%, 40 ml, Sigma) was injected into the heart via the left atrium. The heart was rapidly removed and the left ventricle (LV) was sliced into transverse sections 1 cm thick. After photographing the sections with the unstained risk area, the LV rings were stained with triphenyltetrazolium chloride and re-photographed. The area of myocardium at risk and infarct size was determined by computerized planimetry (MCID-M 2, Imaging Research Inc., Canada) by personnel blinded to treatment identity. The former was expressed as a percentage of total LV volume and infarct size was expressed as a percentage of the area of myocardium at risk.

Evaluation of Lactate Dehydrogenase Levels. Circulating levels of lactate dehydrogenase (LDH) in sheep were measured in plasma samples taken immediately before ischaemia, and following 3 h reperfusion. Venous blood samples (5 ml) were collected, centrifuged at 3500 rpm for 10 min at 4°C and plasma samples were stored at -80 °C until assay by an automated analyser (Beckman, Brea, CA, U.S.A.).

Statistics. All values are expressed as group mean ± s.e.m. Data analysis was performed with SigmaStat (version 2.03) or Prism 4.0. Differences in infarct size, LDH and superoxide levels were determined by one-way ANOVA. Concentration response curves to Ca^{2+} were assessed by one-way ANOVA with repeated measures followed, if appropriate, by Bonferroni's test for multiple comparisons. A value of $P<0.05$ was regarded as statistically significant.

Formation of 3',4'-dihydroxyflavonol following administration of 3HA to rats

The following studies were performed under contract with TetraQ (Brisbane, Queensland). The formation of 3',4'-dihydroxyflavonol (**3**) following administration of **3HA** was assessed in male Sprague-Dawley rats (approx 300 g, n = 3). Dose of **3HA** was administered over approximately 1 min by jugular vein catheter, followed by flushing with saline after completion of dose. **3HA** was dosed at 6.6 mg/kg, using a solution of **3HA** at 3.3 mg/mL in 0.1 M sodium carbonate (dosing volume = 2 mL/kg). Blood (approx. 230 μL) was sampled from jugular catheter at $t = 0$ (pre-dose), 2, 5, 10, 30, 60, 90, 120, 240, and 360 minutes after completion of dose and immediately placed on ice. Blood was centrifuged and plasma snap frozen on dry ice prior to being stored at -80°C until analysed. Plasma samples were analysed for concentration of **3** using a non-validated LC-MS/MS method. Samples were analysed alongside calibration curves for analyte prepared in drug free rat plasma.

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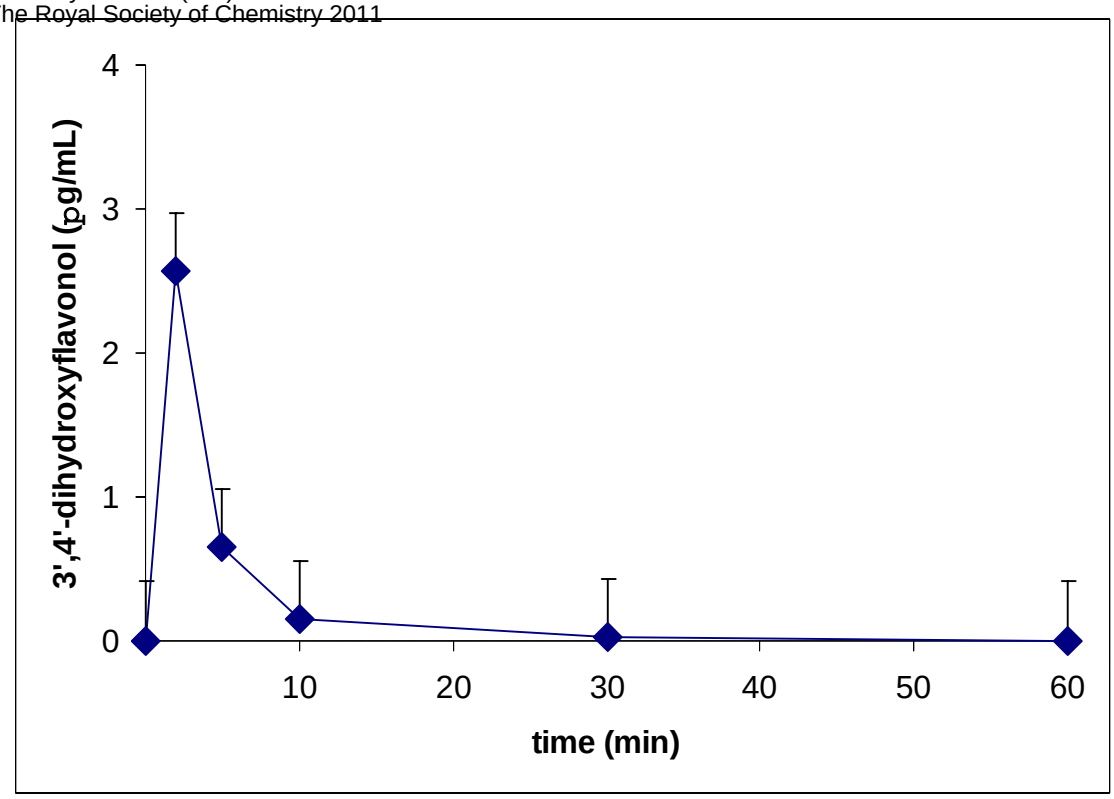

Figure S1. Concentration of **3** (3',4'-dihydroxyflavonol) following bolus intravenous administration of **3HA** to rats. Concentrations shown are the average of determinations in three different rats, with error bars denoting s.e.m. Compound **3** could not be detected in plasma after 60 min.

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