

Antioxidant activity of O-protected derivatives of (-)-epigallocatechin-3-gallate: inhibition of soybean and rabbit 15-lipoxygenases

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Dedicated to Professor Berhanu M. Abegaz on the occasion of his 60th birthday

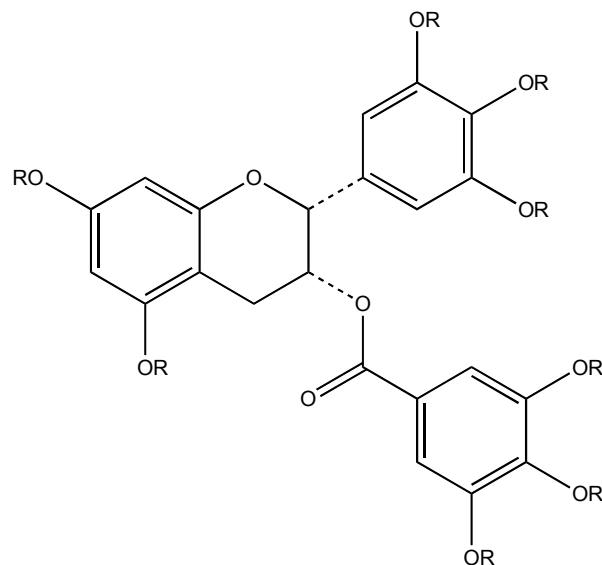
Abstract

(-)-Epigallocatechin-3-gallate (EGCG) was protected at all eight phenolic groups as the methyl ether or acetate, propionate or butyrate esters. The synthetic derivatives were examined for antioxidant activities. The compounds were tested as potential diphenylpicrylhydrazyl (DPPH) radical scavengers and as inhibitors of 15-lipoxygenase (15-LO) enzymes from soybeans and rabbit reticulocytes. The O-protected EGCG derivatives were essentially inactive as DPPH scavengers but showed profound inhibiting effects on both types of 15-LO, and the inhibitory effects towards the two enzymes were significantly correlated. The O-protected EGCG derivatives inhibited 15-LO more strongly than EGCG itself. EGCG and its O-protected derivatives were also screened for antibacterial activity against *M. tuberculosis*.

Keywords: Flavonoids, (-)-Epigallocatechin-3-gallate, 15-Lipoxygenase, Radical scavenging

Introduction

Lipoxygenases (LO) have been found in a large number of higher plants.¹ It is believed that this enzyme class is involved in plant wound healing and it has been suggested that lipoxygenase metabolites are involved in plant hormonal activity,^{2,3} growth regulation and senescence induction.³ We have previously reported that 6-substituted purines as well as indolizines act as soybean 15-lipoxygenase (15-LO) inhibitors.^{4,5} Nevertheless, our knowledge of effective 15-LO inhibitors is limited. From our ongoing studies on different new classes of 15-LO inhibitors we now wish to report that fully O-protected (-)-epigallocatechin-3-gallate derivatives (Figure 1) strongly inhibit both soybean and rabbit reticulocyte 15-LO.



- I** R=H
II R=COCH₃
III R=COCH₂CH₃
IV R=COCH₂CH₂CH₃
V R=CH₃

Figure 1

In recent years, there has been considerable interest in the antioxidant, antitumor and antimutagenic properties of green tea and its constituents, particularly the flavan-3-ols.⁶ The main flavan-3-ol in green tea, epigallocatechin 3-gallate (EGCG), has been implicated as a major factor in the physiological effects of green tea consumption. Several *in vivo* experiments have shown chemopreventive effects of EGCG against cancer initiation, promotion and progression in animal models of oral, lung, duodenal, prostate, liver and colon cancers.⁷ Recently, it has been suggested that the peracetate of EGCG could be useful as a prodrug due to the limited stability and bioavailability of EGCG itself.⁸

It has been reported that overexpression of 15-LO in human prostate cancer cells increases tumorigenesis.⁹ Furthermore it is generally hypothesized that dietary polyphenols may reduce the risk of disorders associated with enhanced production of reactive oxygen and nitrogen species, such as coronary heart disease, stroke and inflammatory diseases as well as cancer.¹⁰ 15-LO has also been implicated in oxidation of low density lipoproteins which is believed to be linked to the development of atherosclerosis.¹¹⁻¹⁴ Recently, it has been shown that some catechins may have

preventive effects in regard to coronary heart disease, but that the activity may differ for different structures.¹⁵

Results and discussion

We have previously synthesized a number of indolizines and azaindolizines and screened their ability to act as scavengers of lipid peroxidation *in vitro*,¹⁶⁻¹⁸ and also recently investigated their role as 15-LO inhibitors.⁵ We found that an oxygen atom connected to the indolizine C-1 was required for antioxidant activity, but that a variety of oxygen substituents were tolerated. It has been shown previously that radical scavenging and 15-LO inhibition are unrelated properties.¹⁹ We have now investigated if the 15-LO inhibiting and radical scavenging properties are related to the free phenolic functionalities of EGCG or to other properties of the molecule. Consequently EGCG was permethylated by dimethyl sulfate,²⁰ or peracylated with acetic anhydride, propionic anhydride or butyric anhydride in the presence of *N,N*-dimethylaminopyridine in dichloroethane, and purified by flash chromatography on silica gel giving molecules **II-V** in moderate to good yields. (Figure 1) Substances **II**^{21,22} and **V**^{23,24} have been reported previously, but substance **V** has been characterized only incompletely. Substances **III** and **IV** have been prepared by us for the first time.^{25,26}

The radical scavenging properties of catechins towards diphenylpicrylhydrazyl have been reported and oxidation products identified.²⁷ Furthermore the reaction of EGCG with diphenylpicrylhydrazyl has been investigated using EPR spectroscopy.²⁸

Our results are summarized in Tables 1-2.

Table 1. Scavenging of the DPPH radical by epigallocatechin gallate (I) and derivatives. Final concentration of test substance was 667 μ M. Results are shown \pm SD.

Compound	DPPH scavenged (%)
I	99.0 \pm 1.2
II	3.1 \pm 3.6
III	1.2 \pm 0.7
IV	5.0 \pm 0.6
V	1.4 \pm 2.8

Table 2. Inhibition of soybean and rabbit reticulocyte 15-lipoxygenase by epigallocatechin gallate (**I**) and derivatives. Results are shown \pm SD. IC₅₀ denotes the concentration needed for 50% inhibition of the enzyme.

Compound	Inhibition of soybean 15-lipoxygenase		Inhibition of rabbit reticulocyte 15-lipoxygenase	
	Inhibition at 83 μ M ^a (%)	IC ₅₀ (μ M)	Inhibition at 87 μ M ^a (%)	IC ₅₀ (μ M)
I	62.6 \pm 1.7	59 \pm 4	40.7 \pm 4.4	ca 100 ^b
II	92.8 \pm 0.8	46 \pm 2	77.7 \pm 3.9	61 \pm 8
III	97.7 \pm 1.0	28 \pm 1	103.8 \pm 2.2	31 \pm 2
IV	99.1 \pm 0.7	35 \pm 2	99.6 ^c	33 \pm 4
V	100.1 \pm 0.5	37 \pm 4	103.7 \pm 3.1	30 \pm 3

^aAt this concentration, all compounds inhibit the enzyme significantly ($P < 0.001$)

^bDetermined by linear extrapolation

^cAverage of two measurements

Linear regression gives the equation $y = 2.428x - 48.55$ where y is the IC₅₀ value for the rabbit reticulocyte enzyme and x is the IC₅₀ value for the soybean enzyme. The R value for this correlation is 0.957. If substance **I** is omitted from the calculation (since its IC₅₀ value for the rabbit reticulocyte enzyme is determined by extrapolation), the correlation is still present, although somewhat weakened ($R=0.850$).

Quercetin was employed as positive control in both experiments, giving IC₅₀ values of 51 \pm 3 μ M for the soybean enzyme and 37 \pm 4 μ M for the rabbit reticulocyte enzyme. Our compounds are thus seen to compare well in activity with quercetin, a known 15-LO inhibitor.⁵ From our results, it seems that derivatives without free hydroxyl groups of EGCG **I** are nearly devoid of radical scavenging activity in the DPPH assay, while **I** itself is an excellent scavenger as expected from previous knowledge. The derivatives are, however, considerably better inhibitors of 15-LO than EGCG. Furthermore, the correlation between inhibition of mammalian and soybean 15-LO shows that, at least for this type of compounds, the inexpensive and easily available soybean enzyme can be used for screening purposes, and that results obtained with this enzyme can be predictive for inhibition of the mammalian enzyme.

Most previous investigations have shown a good correlation between the inhibitory activities for soybean and mammalian 15-LO.²⁹⁻³¹ Our results are in accord with this. Recently, however, a lack of correlation was reported, based on the inability of epicatechin and caffeic acid to inhibit soybean 15-LO.³² This lack of inhibition is in contrast to other reports.^{30,33-36} Differences in methodology could conceivably be the reason for this discrepancy.

Inhibition of soybean 15-LO by epicatechin, epicatechin gallate, epigallocatechin and EGCG has been reported.³⁴ Apparently, the 3,4,5-trihydroxyphenyl moiety gave much higher inhibitory activity than the 3,4-dihydroxyphenyl one; epicatechin being one order of magnitude

less active than the others. The stronger inhibitory effect of epigallocatechin compared to epicatechin was also observed by Goupy et al,³⁷ although differences in methodology makes it difficult to compare results directly. The effects of alkylation or esterification on the lipoxygenase inhibiting effect of EGCG or related compounds such as catechin or epicatechin have apparently not been reported previously.

The purpose of this part of the work was to synthesize derivatives of EGCG, the major flavonoid in tea, and to study the lipoxygenase inhibiting activity of EGCG and its derivatives in two different systems, soybean 15-LO and mammalian 15-LO. The results reported are *in vitro*, and at present, there are no data on *in vivo* activity of these substances. Since the fate of these substances in the GI tract and their bioavailability is not well known,⁸ studies of their bioactivity *in vivo* would seem to be worth while in view of previously reported epidemiological results on the prevention of coronary heart disease by catechins.¹⁵

Tuberculosis caused by *Mycobacterium tuberculosis* (*Mtb*) is the leading cause of death in the world, greater than cancer or cardiovascular disease.³⁸ As early as in 1949 flavonoids were reported to inhibit *Mtb* and it was reported that the action of streptomycin against the tubercle bacillus was markedly potentiated by the investigated catechins.³⁹ Flavonoids have also very recently been reported as anti-tuberculosis agents.^{40,41} These recent reports prompt us to include our recent results for molecules **I-V**, (Table 3) in the present publication.

Table 3. Inhibition of *M. tuberculosis* H₃₇Rv at 6.5 µg/ml in BACTEC 12B medium using the microplate alamar blue assay.^{42,43}

Compound	Inhibition at 6.5 µg/ml (%)
I	0
II	0
III	38
IV	0
V	38

It was reported⁴⁰ that some *O*-methylated flavonoids inhibit *Mtb*, which is in accordance with the inhibition (38 % at 6.5 µg/ml) shown by the polymethylated compound **V** (Table 3). EGCG (**I**) itself shows 0 % inhibition. The finding that the polypropionylated compound **III** exhibits similar activity seems to have no precedent in the literature.

Conclusions

We conclude that EGCG and alkyl and acyl derivatives of EGCG are inhibitors of 15-LO from soybean and mammals *in vitro*. The derivatives are better inhibitors than the parent substance, but in contrast to EGCG, they are not radical scavengers. The permethylated and

perpropionylated derivatives of EGCG also show inhibitory activity against *Mycobacterium tuberculosis in vitro*.

Experimental Section

(-)-Epigallocatechin gallate (**I**) (from green tea, minimum 80% (HPLC)) was purchased from Sigma (St. Louis, MO, USA) and used as received or isolated from Formosa Gunpowder Tea.⁴⁴ Diphenylpicrylhydrazyl (DPPH) and soybean 15-lipoxygenase type 1-B (EC 1.13.11.12) were purchased from Sigma (St. Louis, MO, USA). Rabbit reticulocyte 15-lipoxygenase (Catalog No. PL-015, lot B2559, 5000 U/ml, solution in 10 mM potassium phosphate buffer (pH 6.0), supplied frozen) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Butyric anhydride and propionic anhydride were purchased from Fluka (Buchs, Switzerland). Silica gel for flash chromatography was available from Merck (Darmstadt, Germany) (Merck No. 9385) or Fluka (Fluka No 60752). HPLC grade acetone and HPLC grade acetonitrile were used as obtained from supplier, dichloroethane was distilled from CaH₂. HPLC was performed on a Spectra System P 2000 equipped with an LC-18 Semi-Prep column, length 25 cm, ID-10 mm and a Spectra System UV 3000 detector. ¹H NMR spectra were recorded at 500 MHz with a Bruker Avance DRX 500 instrument or at 300 MHz with a Bruker Avance DPX 300 instrument. The ¹³C NMR spectra were recorded at 125 or 75 MHz using the instruments mentioned above. Unless otherwise stated, the spectra were recorded at ambient temperature. Chemical shifts (δ) are referenced to solvent shifts. Mass spectra were recorded with a Micromass QTOF2 instrument using electrospray ionization (solution for III and IV: MeCN + 2% formic acid, for II and V MeOH:H₂O 50/50 + 2% formic acid) and presented as m/z (% rel. int.). Melting points are uncorrected. All measurements of DPPH scavenging and 15-LO activity were carried out in a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

(-)-5,7-O-Diacetyl-3',4',5'-O-triacetylepigallocatechin-3-O-(3'',4'',5''-O-triacetyl)gallate (II)

(-)-EGCG 70 mg (0.15 mmol) in dry dichloroethane (30 ml) under N₂ was cooled to 0 °C before adding *N,N*-dimethylaminopyridine 186 mg (1.52 mmol) and acetic anhydride 0.14 ml (1.52 mmol) dissolved in dichloroethane. The resulting mixture was stirred at 0 °C for 1 h and at room temperature for 4 h, diluted with CHCl₃ (50 ml) and washed with saturated aqueous CuSO₄ (4×30 ml), sat. aq. NaHCO₃ (2×30 ml) and brine (30 ml). The resulting mixture was dried (MgSO₄) and concentrated under reduced pressure. The product was redissolved in acetonitrile and purified by HPLC, using a mixture of acetonitrile:water (90:10) as a mobile phase at a flow rate of 4 ml/min and monitored at 280 nm. The product was finally freeze-dried. Yield 66 mg (55%); white crystals, mp 110-111 °C. ¹H NMR (CDCl₃, 300 MHz): δ 2.20-2.28 (8 s, 24H, Me); 2.98 (dd, J = 2.3, 17.9 Hz, 1H, H-4b); 3.04 (dd, J = 4.6, 17.9 Hz, 1H, H-4a); 5.18 (br s, 1H, H-2); 5.63 (m, 1H, H-3); 6.62 (d, J = 2.15 Hz, 1H, H-8); 6.74 (d, J = 2.15 Hz, 1H, H-6); 7.21 (s, 2H, H-2'(6')); 7.62 (s, 2H, H-2''(6'')). ¹³C NMR (CDCl₃, 125 MHz): δ 20.06 (CH₃), 20.07 (CH₃), 20.49 (CH₃), 20.50 (CH₃), 20.71 (CH₃), 21.01 (CH₃); 25.4 (C-4); 67.96 (C-3); 77.02 (C-2); 108.23 (C-

8); 108.96 (C-6); 109.40 (C-4a); 118.76 (C-2''(6'')); 122.30 (C-2'(6')); 127.38 (C-1''); 134.31 (C-4'); 135.04 (C-1'); 138.90 (C-4''); 143.26 (C-3''(5'')); 143.35 (C-3'(5')); 149.61 (C-7); 149.69 (C-5); 154.69 (C-8a); 163.48 (CO), 166.15 (CO), 166.68 (CO), 167.38 (CO), 167.54 (CO), 168.36 (CO), 168.84 (CO). C₃₈H₃₄O₁₉ (794.66): calcd. C 57.43, H 4.31; found C 57.47, H 4.35. ¹H and ¹³C NMR spectra in good accordance with lit.^{21,22}

(-)-5,7-O-Dipropionyl-3',4',5'-O-tripropionylepigallocatechin-3-O-(3'',4'',5''-O-tripropionyl) gallate (III) (-)-EGCG 80 mg (0.17 mmol) in dry dichloroethane (30 ml) under N₂ was cooled to 0 °C before adding *N,N*-dimethylaminopyridine 181 mg (1.48 mmol) and propionic anhydride 0.19 ml (1.48 mmol) dissolved in dichloroethane. The resulting mixture was stirred at 0 °C for 1 h and at room temperature for 6 h, diluted with CHCl₃ (50 ml) and washed with saturated aqueous CuSO₄ (4×30 ml), sat. aq. NaHCO₃ (2×30 ml) and brine (30 ml). The resulting mixture was dried (MgSO₄) and concentrated under reduced pressure. The product was redissolved in acetonitrile and purified by HPLC, using a mixture of acetonitrile:water (70:30) as a mobile phase at a flow rate of 4 ml/min and monitored at 280 nm. The product was finally freeze-dried. Yield 69 mg (44%); white crystals, mp 65-67 °C. ¹H NMR (CDCl₃, 500 MHz): δ 1.12-1.21 (m, 24H, CH₃); 2.43-2.55 (m, 16H, CH₂); 2.92 (dd, J = 2.05, 17.8 Hz, 1H, H-4b); 3.01 (dd, J = 4.66, 17.8 Hz, 1H, H-4a); 5.13 (br s, 1H, H-2); 5.61 (m, 1H, H-3); 6.56 (d, J = 2.2 Hz, 1H, H-8); 6.68 (d, J = 2.2 Hz, 1H, H-6); 7.17 (s, 2H, H-2'(6')); 7.54 (s, 2H, H-2''(6'')). ¹³C NMR (CDCl₃, 75MHz): δ 8.87 (3 x CH₃), 8.99 (CH₃), 9.03 (CH₃), 9.08 (CH₃); 25.89 (C-4); 27.03 (2·CH₂), 27.25 (CH₂), 27.28 (CH₂), 27.43 (CH₂), 27.61 (CH₂); 67.87 (C-2); 107.83 (C-8); 108.87 (C-6); 109.27 (C-4a); 118.56 (C-2''(6'')); 122.13 (C-2' (6')); 127.28 (C-1''); 134.30 (C-4'); 134.88 (C-1'); 138.93 (C-4''); 143.34 (C-3''(5'')); 143.43 (C-3'(5')); 149.66 (C-7); 149.78 (C-5); 154.71 (C-8a); 163.50(CO), 169.49 (CO), 169.99 (CO), 170.75 (CO), 170.91 (CO), 171.77 (CO), 172.25 (CO). C₄₆H₅₀O₁₉ (906.87): calcd. C 60.92, H 5.55; found C 61.11, H 5.77. m/z (electrospray) 929.2785 (diff (ppm) -5.76), M+Na⁺ C₄₆H₅₀O₁₉[Na⁺] requires 929.2838.

(-)-5,7-O-Dibutyryl-3',4',5'-O-tributyrylepigallocatechin-3-O-(3'',4'',5''-O-tributyryl) gallate (IV) (-)-EGCG 78 mg (0.17 mmol) in dry dichloroethane (30 ml) under N₂ was cooled to 0 °C before adding *N,N*-dimethylaminopyridine 176 mg (1.44 mmol) and butyric anhydride 0.23 ml (1.44 mmol) dissolved in dichloroethane. The resulting mixture was stirred at 0 °C for 1 h and at room temperature for 8 h, diluted with CHCl₃ (50 ml) and washed with saturated aqueous CuSO₄ (4×30 ml), sat. aq. NaHCO₃ (2×30 ml) and brine (30 ml). The resulting mixture was dried (MgSO₄) and concentrated under reduced pressure. The product was redissolved in acetonitrile and purified by HPLC, using a mixture of acetonitrile:water (90:10) as a mobile phase at a flow rate of 4 ml/min and monitored at 280 nm. The product was finally freeze-dried. Yield 106 mg (63%); oil, ¹H NMR (CDCl₃, 500 MHz): δ 0.95-1.02 (m, 24H, CH₃); 1.67-1.78 (m, 16H, CH₂); 2.42-2.52 (m, 16H, CH₂); 2.95 (dd, J = 1.6, 17.9 Hz, 1H, H-4b); 3.02 (dd, J = 4.6, 17.9 Hz, 1H, H-4a); 5.14 (br s, 1H, H-2); 5.63 (m, 1H, H-3); 6.57 (d, J = 2.2 Hz, 1H, H-8); 6.70 (d, J = 2.2 Hz, 1H, H-6); 7.19 (s, 2H, H-2'(6')); 7.57 (s, 2H, H-2''(6'')). ¹³C NMR (CDCl₃, 75MHz): δ 14.01 (5 x CH₃), 14.08 (CH₃); 18.67 (CH₂), 18.73 (CH₂), 18.79 (CH₂), 18.84 (3 x CH₂); 26.52 (C-4); 35.90 (2 x CH₂), 36.17 (CH₂), 36.22 (CH₂), 36.42 (CH₂), 36.59 (CH₂); 68.32 (C-3); 108.38 (C-8);

109.42 (C-6); 109.77 (C-4a); 119.1 (C-2''(6'')); 122.7 (C-2'(6')); 127.78 (C-1''); 134.84 (C-4'); 135.31 (C-1'); 139.47 (C-4''); 143.86 (C-3''(5'')); 143.96 (C-3'(5')); 150.16 (C-7); 150.25 (C-5); 155.24 (C-8a); 164.01 (CO), 169.18 (CO), 169.68 (CO), 170.43 (CO), 170.61 (CO), 171.47 (CO), 171.91 (CO). $C_{54}H_{66}O_{19}$ (1019.09): calcd. C 63.64, H 6.52; found C 63.81, H 6.40. m/z (electrospray) 1041.4152 (diff (ppm) 5.89), $M+Na^+ C_{54}H_{66}O_{19}[Na^+]$ requires 1041.4090.

(-)-5,7-O-Dimethyl-3',4',5'-O-trimethylepigallocatechin-3-O-(3'',4'',5''-O-trimethyl) gallate (V). Dimethylsulphate 0.13 ml (8.26 mmol) was added to a mixture of (-)-EGCG 80 mg (1.70 mmol) and potassium carbonate 300 mg (12.6 mmol) in dry acetone (35 ml). After stirring for 6 h in boiling acetone under N_2 , the reaction mixture was filtered and evaporated in vacuo. The product was purified by flash chromatography eluting with hexane: ethyl acetate:acetone (6:3:1) Yield 34 mg (35%); white crystals, mp 62 °C. 1H NMR ($CDCl_3$, 300 MHz): δ 3.025 (s, 1H, H-4); 3.035 (s, 1H, H-4); 3.69-3.84 (m, 24H, OCH_3); 5.07 (br s, 1H, H-2); 5.64 (m, 1H, H-3); 6.10 (d, $J = 2.18$ Hz, 1H, H-6); 6.23 (d, $J = 2.18$ Hz, 1H, H-8); 6.68 (s, 2H, H-2'(6'')); 7.15 (s, 2H, H-2''(6'')). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 25.57 (C-4); 55.03 (OCH_3), 55.08 (OCH_3), 55.64 (OCH_3), 55.89 (OCH_3), 60.36 (OCH_3), 60.48 (OCH_3); 68.50 (C-3); 91.49 (C-8); 93.01 (C-6); 99.80 (C-4a); 103.58 (C-2''(6'')); 106.82 (C-2'(6'')); 124.86 (C-1''); 133.30 (C-4'); 137.45 (C-1'); 142.19 (C-4''); 152.60 (C-3''(5'')); 152.81 (C-3'(5')); 155.30 (C-7); 158.61 (C-5); 159.49 (C-8a); 164.78(CO). $C_{30}H_{34}O_{11}$ (570.58): calcd. C 63.14, H 6.00; found C 62.88, H 5.98. m/z (electrospray) 593.2006 (diff (ppm) 2.13), $M+Na^+ C_{30}H_{34}O_{11}[Na^+]$ requires 593.1993. 1H NMR spectrum in good accordance with lit.^{23,24}

Antioxidant assays. Scavenging activity towards the diphenylpicrylhydrazyl (DPPH) radical was measured as the decrease in absorbance at 517 nm of a methanolic DPPH solution ($A_{517} = 1.0$, 2.95 ml) over a 5-min period after addition of 50 μ L of a DMSO solution of test substance.⁴⁵ Appropriate corrections were made for dilution and for absorbance of the reaction product, reduced DPPH. Calculation of radical scavenging activity was carried out as previously described.¹⁹

Soybean 15-lipoxygenase activity was measured in borate buffer solutions (0.2 M, pH 9.00) as previously described,⁴⁶ by the increase in absorbance at 234 nm from 30 to 90 s after addition of the enzyme, using linoleic acid (134 μ M) as substrate. The final enzyme concentration was 167 U/mL. Test substances were added as DMSO solutions (final DMSO concentration 1.6%); DMSO alone was added in uninhibited control experiments. Six or more parallel runs of control and three or more parallel runs for each test substance solution were measured. To ensure constant enzyme activity throughout the experiment, the enzyme solution was kept on ice, and controls were measured at regular intervals. Calculation of enzyme activity was carried out as previously described,¹⁹ and IC_{50} values were determined by linear interpolation between the measuring points closest to 50% activity. Values are expressed as means \pm SD.

The frozen rabbit reticulocyte enzyme (in phosphate buffer, used as received) was thawed immediately before experiment and subsequently kept on ice throughout the experimental period (maximum one day).

Enzyme activity was measured according to the assay procedure supplied with the enzyme, using 2-methoxyethanol (which had much less effect on enzyme activity than DMSO) as a solvent for the test substances: A mixture of substrate solution (linoleic acid in abs. ethanol; 0.4% (w/v), 20 μ L), 2-methoxyethanol (in uninhibited control experiments, 50 μ L) or a methoxyethanolic solution of test substance (50 μ L), and phosphate-buffered saline (0.1 M phosphate, pH 7.60, 2.80 mL) was stirred in a quartz cuvette. The oxidation reaction was started by addition of 8 μ L enzyme solution. After brief stirring, the increase in absorbance at 234 nm was measured from 30 to 90 s after enzyme addition.

All measurements were carried out in triplicate, and controls without test substance were measured at intervals to ensure constant enzyme activity. Per cent inhibition and IC₅₀ values were calculated as described for the soybean enzyme. Linear regression and calculation of significance for the correlation between inhibition of the two enzymes was performed with laboratory-developed software using the Lotus 1-2-3 worksheet programme. For calculation of statistical significance, Student's *t*-test was employed, taking a *P* value of less than 0.05 as a criterion of significance.

Activity against *Mycobacterium tuberculosis*. The screening against *Mycobacterium tuberculosis* H₃₇Rv (ATCC 27294) was conducted at 6.25 ug/ml in BACTEC 12B medium using Microplate Alamar Assay (MABA).^{42,43} Compounds exhibiting fluorescence were tested in the BACTEC 460-radiometric system.^{42,43,47}

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