Inhibition of cholesterol biosynthesis by Δ^{22}-unsaturated phytosterols via competitive inhibition of sterol Δ^{24}-reductase in mammalian cells

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Dietary phytosterols are cholesterol-lowering agents that interfere with the intestinal absorption of cholesterol. In the present study, we have studied their effects on cholesterol biosynthesis in human cells, particularly in the sterol-conversion pathway. For this, both Caco-2 (intestinal mucosa) and HL-60 (promyelocytic) human cell lines were incubated with [^{14}C]acetate, and the incorporation of radioactivity into sterols was determined using HPLC and radioactivity detection online. Sterols containing a double bond at C-22 in the side chain (stigmasterol, brassicasterol and ergosterol) dramatically inhibited the activity of sterol Δ^{24}-reductase, as indicated by the decrease in radioactivity incorporation into cholesterol and the accumulation of its precursors (mainly desmosterol). Phytosterols with the saturated side chain (β-sitosterol and campesterol) were inactive in this regard. The inhibition of sterol Δ^{24}-reductase was confirmed in rat liver microsomes by using ^{14}C-labelled desmosterol as the substrate. The Δ^{22}-unsaturated phytosterols acted as competitive inhibitors of sterol Δ^{24}-reductase, with K_i values (41.1, 42.7 and 36.8 μM for stigmasterol, brassicasterol and ergosterol respectively) similar to the estimated K_m for desmosterol (26.3 μM). The sterol 5,22-cholestedien-3β-ol, an unusual desmosterol isomer that lacks the alkyl groups characteristic of phytosterols, acted as a much stronger inhibitor of Δ^{24}-reductase ($K_i = 3.34$ μM). The usually low intracellular concentrations of the physiological substrates of Δ^{24}-reductase explains the strong inhibition of cholesterol biosynthesis that these compounds exert in cells. Given that inhibition of sterol Δ^{24}-reductase was achieved at physiologically relevant concentrations, it may represent an additional mechanism for the cholesterol-lowering action of phytosterols, and opens up the possibility of using certain Δ^{22}-unsaturated sterols as effective hypocholesterolaemic agents.

Key words: Caco2 cell line, HL-60 cell line, human cells, hypolipidaemic agent, liver microsomes.

INTRODUCTION

Phytosterols are the naturally occurring equivalents of mammalian sterol (cholesterol) in plants. They have a structure similar to cholesterol, but with some modifications in the side chain, such as the addition of a double bond at C-22 and/or an alkyl (methyl or ethyl) group at C-24 (Figure 1). In fungi and yeast, the natural sterol is ergosterol, which contains additional double bonds at C-7 and C-22, and a methyl group at C-24, as compared with cholesterol. The most common dietary sources of plant sterols are nuts, seeds, unrefined plant oils and legumes [1,2]. β-Sitosterol and campesterol account for up to 95% of all dietary phytosterol, the remaining 5% of the plant sterols comprising mainly stigmasterol [1]. The consumption of phytosterols is very variable; for example, a typical Western omnivorous diet contains less than 100 mg of phytosterols/day, whereas vegetarian and Japanese diets exceed 400 mg/day [3].

Under normal conditions, the intestinal absorption of phytosterols is very limited (less than 5% of that ingested is absorbed; [4]). Once in the blood, they are rapidly cleared, so that their concentration in plasma rarely exceeds 1 mg/dl [5–7]. A mechanism involving the ATP-binding cassette protein G (ABCG) proteins ABCG5 and ABCG8 has been described recently, which accounts for the reduced absorption of phytosterols through the intestinal barrier and rapid secretion into the bile [8]. Mutations in these proteins cause sitosterolaemia, a rare disorder characterized by hyperabsorption of both cholesterol and plant sterols, and reduced secretion of sterols from the liver into the bile [9,10]. These patients have very high plasma levels of phytosterols and moderately raised cholesterol levels as compared with controls, which lead to the formation of xanthomas and premature atherosclerosis [11–13].

Consumption of phytosterols has been associated with protection from cancer [14] and cardiovascular disease [15]. In the intestinal tract, phytosterols appear to displace cholesterol from the lipidic micellae, thus reducing the absorption of cholesterol [16–18]. This, in conjunction with the stimulation of the synthesis of the aforementioned ABCG transporters, may account for the decrease in the low-density-lipoprotein/cholesterol plasma concentration, as observed in both humans and animals fed on diets supplemented with phytosterols [16–18]. Phytostanols, which are the saturated counterparts of phytosterols, also lead to a decrease in low-density-lipoprotein/cholesterol plasma concentration [19,20]. Therefore interest is growing in these compounds and their possible role in the prevention of arteriosclerosis.

Phytosterols are transported in plasma bound to lipoproteins and, like cholesterol, they are esterified with fatty acids by lecithin:cholesterol acyltransferase ('LCAT'), albeit at lower rates [21]. As mentioned above, the liver secretes phytosterols into the bile very efficiently, but other tissues may accumulate them, especially the adrenal gland, ovary and testis [22]. Moreover, these tissues might convert phytosterols into steroid hormones [23,24]. Supplementing the diet with phytosterols has been reported to stimulate cholesterol biosynthesis in the whole organism [25], an effect that may be attributed to the decrease in intestinal cholesterol absorption, rather than to a direct stimulation of cholesterol biosynthesis. In fact, feeding rats with β-sitosterol did not affect the expression of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis, in ileal mucosa [26], and intravenous

Abbreviations used: ABCG, ATP-binding cassette protein G; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LPDS, lipoprotein-deficient serum.

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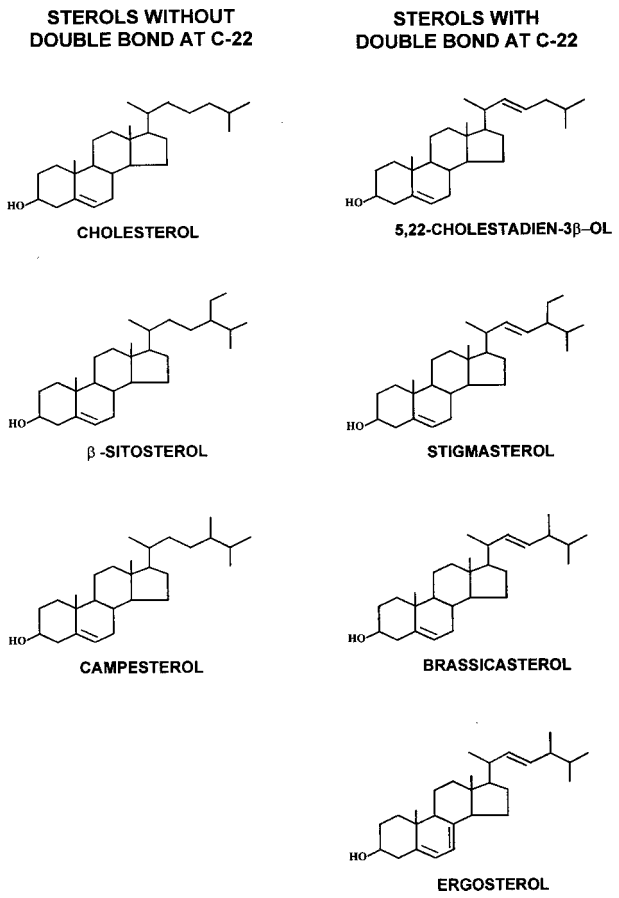
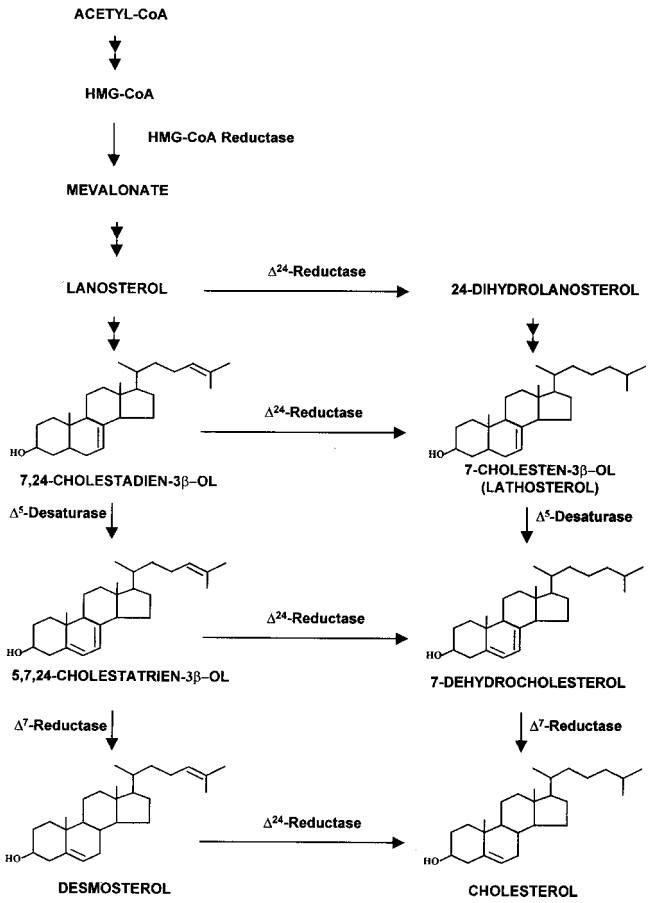


Figure 1 Chemical structures of the sterols and phytosterols used



Scheme 1 Key reactions in the conversion of lanosterol into cholesterol

administration of β-sitosterol was not accompanied by a decrease in this enzyme activity in rat liver [27,28]. Nevertheless, in Caco-2 cells, β-sitosterol has been reported to lower the activity and expression of HMG-CoA reductase [29]. Therefore the actions of phytosterols on cholesterol biosynthesis and the activity of the relevant enzymes are not clear.

Cholesterol biosynthesis from acetyl-CoA is a multi-step pathway involving 19 well-characterized enzyme reactions. The conversion of lanosterol (the first sterol formed) into cholesterol requires, among several other steps, the saturation of the double bond at C-24, which is catalysed by the sterol Δ^{24}-reductase (Scheme 1). This membrane-bound enzyme, which has been cloned recently [30], is able to act upon different intermediates containing the C-24,25 double bond. This enzyme has also been termed lanosterol reductase or desmosterol reductase, depending on the sterol substrate in question. The specificity of this enzyme has also been elucidated previously [31], showing the highest affinity for 5α-cholestan-7,24-dien-3β-ol, followed, in decreasing order, by zymosterol, desmosterol and lanosterol. This enzyme shows a strict specificity for the saturation of the double bond at C-24, being unable to reduce double bonds at other positions, e.g. C-22; it is notable that there is no detectable Δ^{22}-reductase activity in animal cells [32].

On the basis of the presence of a double bond at C-22 in many of the dietary phytosterols, in the present study we have sought to determine whether this different structural characteristic might influence the actions of phytosterols in human cells. We demonstrate that phytosterols containing this double bond in the side chain competitively inhibit sterol Δ^{24}-reductase in microsomal preparations and, consequently, the synthesis of cholesterol in living cells.

MATERIALS AND METHODS

Materials

HL-60 (human promyelocytic), Caco2 (human intestinal mucosa) and J774 (mouse macrophage) cell lines were obtained from the A.T.C.C. (Rockville, MD, U.S.A.). Fetal bovine serum, horse serum, RPMI 1640 medium, Dulbecco's modified Eagle's medium and antibiotics were purchased from Gibco BRL (Barcelona, Spain). Lipoprotein-deficient serum (LPDS) was obtained from fetal bovine serum by ultracentrifugation at a density of 1.21 kg/l. Cholesterol-free medium (DCCM-1) was from Biological Industries (Kibbtutz Beit Haemek, Israel). Desmosterol (24-dehydrocholesterol), stigmasterol, brassicasterol, ergosterol and 5,22-cholestadien-3β-ol (*trans*-22-dehydrocholesterol) were from Steraloids, Inc. (Newport, RI, U.S.A.). Cholesterol, β-sitosterol and an MEM non-essential amino acid solution were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2-^{14}C]Acetate (53 mCi/mmol) and [1α,2α(n)-^{3}H] cholesterol (45.6 Ci/mmol) were from Amersham Biosciences

(Barcelona, Spain). The other chemical products were of analytical grade.

Cell cultures

HL-60 cells were maintained in DCCM-1 supplemented with antibiotics (100 units of penicillin/ml, 100 μg of streptomycin/ml and 10 μg of gentamicin/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. J774 macrophages were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum and antibiotics. Caco2 cells were maintained in Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum, 5% (v/v) horse serum, non-essential amino acids and antibiotics. The sterols in the present study (β-sitosterol, campesterol, stigmasterol, ergosterol, brassicasterol and 5,22-cholestadien-3β-ol) were dissolved in ethanol, and added to the culture at a maximum concentration of 30 μg/ml [final concentration of ethanol of 0.44% (v/v)], owing to cytotoxicity and solubility reasons.

Metabolic labelling and HPLC analysis of cholesterol-biosynthesis intermediates

HL-60 cells (7.5×10^6) were pre-incubated for 2 h in 10 ml of DCCM-1 medium containing the sterols under investigation, and then supplemented with 40 μCi of [2-^{14}C]acetate and incubated for a further period of 8 h. Similarly, Caco2 cells were seeded at a density of 1.2×10^7 cells/flask (75 cm^2) in Dulbecco's medium containing 10% (v/v) LPDS and the sterols under investigation. After a 2 h pre-incubation, the medium was supplemented with [2-^{14}C]acetate, and the cells were incubated for an additional 24 h. At the end of the incubation, the cells were washed with ice-cold PBS and resuspended in 0.5 ml of 10% (v/v) KOH. [^{3}H]Cholesterol was added as an internal standard. The samples were treated sequentially with chloroform/methanol (2:1, v/v) and distilled water to obtain the lipidic and aqueous-soluble fractions, as reported previously [33]. The lipid extract was further sub-fractionated into the saponifiable and non-saponifiable fractions. The fraction containing the non-saponifiable lipids was resuspended in hexane and used for radioactivity counting and sterol separation by HPLC. Recovery of [^{3}H]cholesterol was greater than 60% in every case (64.9 $\pm$ 0.7%; mean $\pm$ S.D.). Sterol separation was accomplished by reverse-phase HPLC with a Luna 5 μm-pore-size C_{18} column (250 mm $\times$ 4.60 mm; Phenomenex, Torrance, CA, U.S.A.). Lipids were eluted with acetonitrile/water (95:5, v/v) during the first 37 min, and then with methanol at a flow rate of 1.2 ml/min. The column effluent was monitored simultaneously by UV-absorption spectroscopy (Beckman 168 variable-wavelength detector; Beckman Instruments, Palo Alto, CA, U.S.A.) and online radioactivity detection (using a LB-506 C-1 radioactivity detector; Berthold, Bad Widbad, Germany) [34]. The eluted sterols were identified by comparison of the retention time and the UV spectrum against those of pure standards. In the case of 5,7,24-cholestatrien-3β-ol, for which no standard is available, the identification was achieved on the basis of its retention time and, specifically, by its having a maximum of absorbance at 282 nm, which corresponds to the presence of homoannular conjugated double bonds.

Isolation of the hepatic microsomal fraction

Male Sprague–Dawley rats (250 g) were fed on Purina rodent chow and killed at 09:30 h. Livers were homogenized with a Potter homogenizer in a buffer solution consisting of 20 mM Tris/HCl, pH 7.6, 4 mM magnesium acetate, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMFS, 2 mM benzamidine, 2 mM EGTA and 320 mM sucrose. The microsomal fraction was isolated from the cytosolic fraction by differential ultracentrifugation at 200000 g for 30 min [35]. Protein concentration was determined by the method of Lowry [36].

Synthesis and purification of [^{14}C]desmosterol from J774 macrophages

J774 cells are deficient in Δ^{24}-reductase so that they are unable to synthesize cholesterol and accumulate desmosterol [37]. In order to obtain labelled desmosterol, these cells were incubated in macrophage-serum-free medium (Gibco BRL) for 2 days to stimulate sterol biosynthesis, and subsequently were supplemented with [2-^{14}C]acetate and incubated for a further 5 days at 37 °C in an atmosphere containing 5% CO_2. At the end of the incubation, the cells were washed with ice-cold PBS and resuspended in 0.5 ml of 10% (v/v) KOH. The lipids were extracted by washing with chloroform/methanol (2:1, v/v) and distilled water [33]. The non-saponifiable lipids were then separated by reverse-phase HPLC, as described above, with a mobile phase of acetonitrile/water (95:5, v/v). Fractions containing radioactive desmosterol were collected, concentrated under a vacuum and resuspended in n-hexane.

Sterol Δ^{24}-reductase assay

Sterol Δ^{24}-reductase activity was assayed by the method of Bae and Paik [31] with slight modifications. Microsomes (0.75 mg of protein) were incubated in a final volume of 500 μl of buffer containing 20 mM Tris/HCl, pH 7.6, 1 mM dithiothreitol, 1 mM PMSF, 2 mM EGTA and an NADPH-generating system composed of 3.4 mM $NADP^+$, 30 mM glucose 6-phosphate and 0.5 unit of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of [^{14}C]desmosterol (specific radioactivity of 3160 d.p.m./nmol) solubilized in 25 μl of 0.1% (v/v) Tween 80. The incubation was performed for 30 min at 37 °C. The reaction was stopped by the addition of 1 ml of 1 M ethanolic NaOH, and the mixture was allowed to stand for 1 h at 37 °C. After adding 0.833 ml of water, the products were extracted twice with 3.33 ml of n-hexane, separated by HPLC as described above, and radioactivity appearing in both cholesterol and desmosterol was measured by liquid-scintillation spectroscopy. Under our assay conditions, the enzyme was saturated with the substrate and the formation of [^{14}C]cholesterol was linear over a period of more than 30 min (results not shown).

Inhibition of sterol Δ^{24}-reductase by different sterols

The activity of Δ^{24}-reductase in microsomes was analysed in the presence of different concentrations of the sterols under investigation. The sterols were added in 25 μl of 0.1% Triton (w/v) X-100/0.05% (w/v) sodium cholate solution. The reaction was initiated by the addition of the substrate ([^{14}C]desmosterol), and the incubation and analysis of the [^{14}C]cholesterol formed were performed as described above.

To determine the mechanism of inhibition, rat hepatic microsomes were assayed for sterol Δ^{24}-reductase activity after incubation with increasing concentrations of [^{14}C]desmosterol (12.5, 17.5, 25 and 50 μM) as substrate, in the absence or the presence of the different sterols (10, 30 and 50 μM). The data were plotted in a Lineweaver–Burk double-reciprocal plot for the analysis of the type of inhibition and the calculation of both V_{max} and K_m. The K_i values were obtained graphically using the Dixon plots for competitive inhibition.

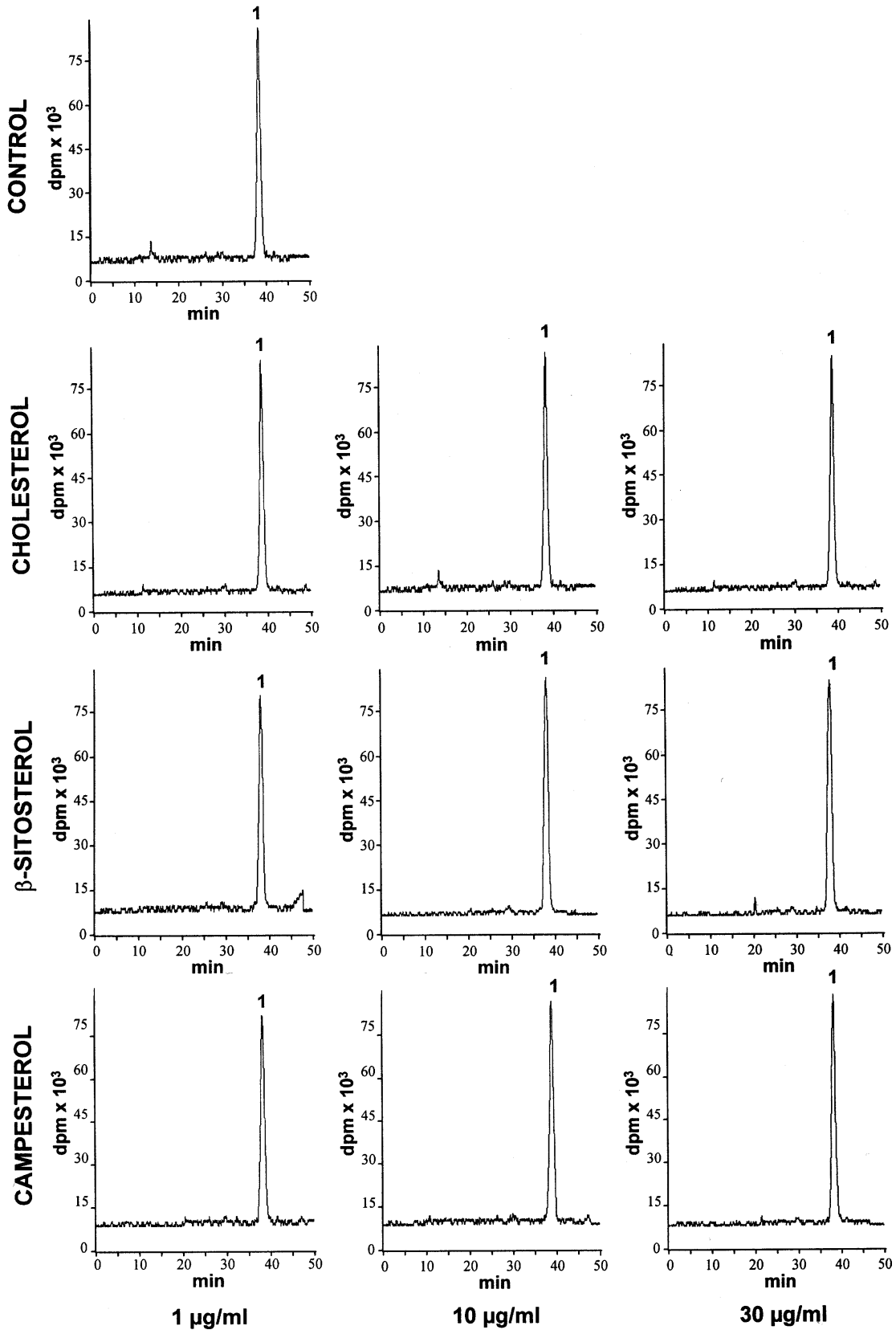


Figure 2 **For legend see facing page.**

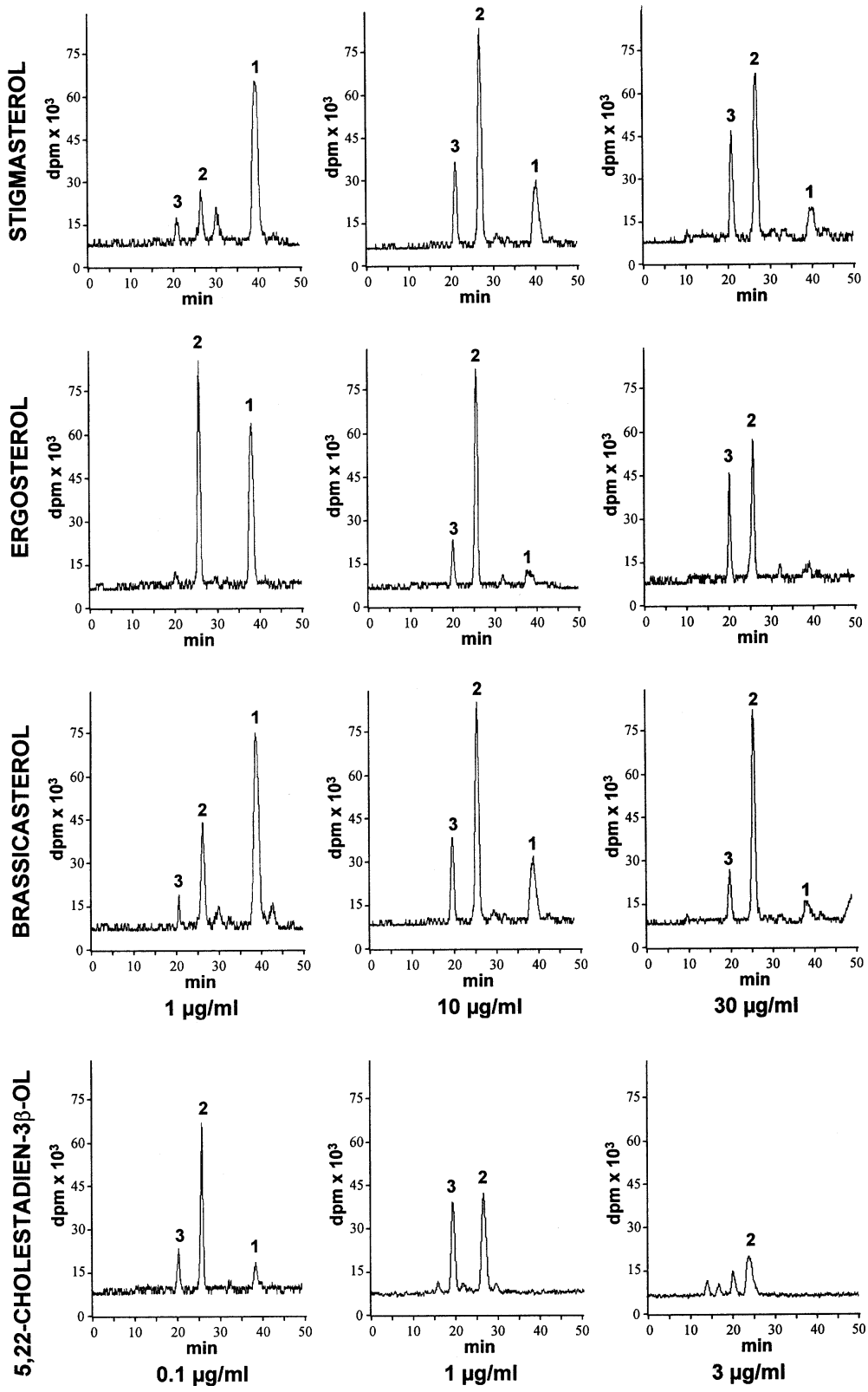
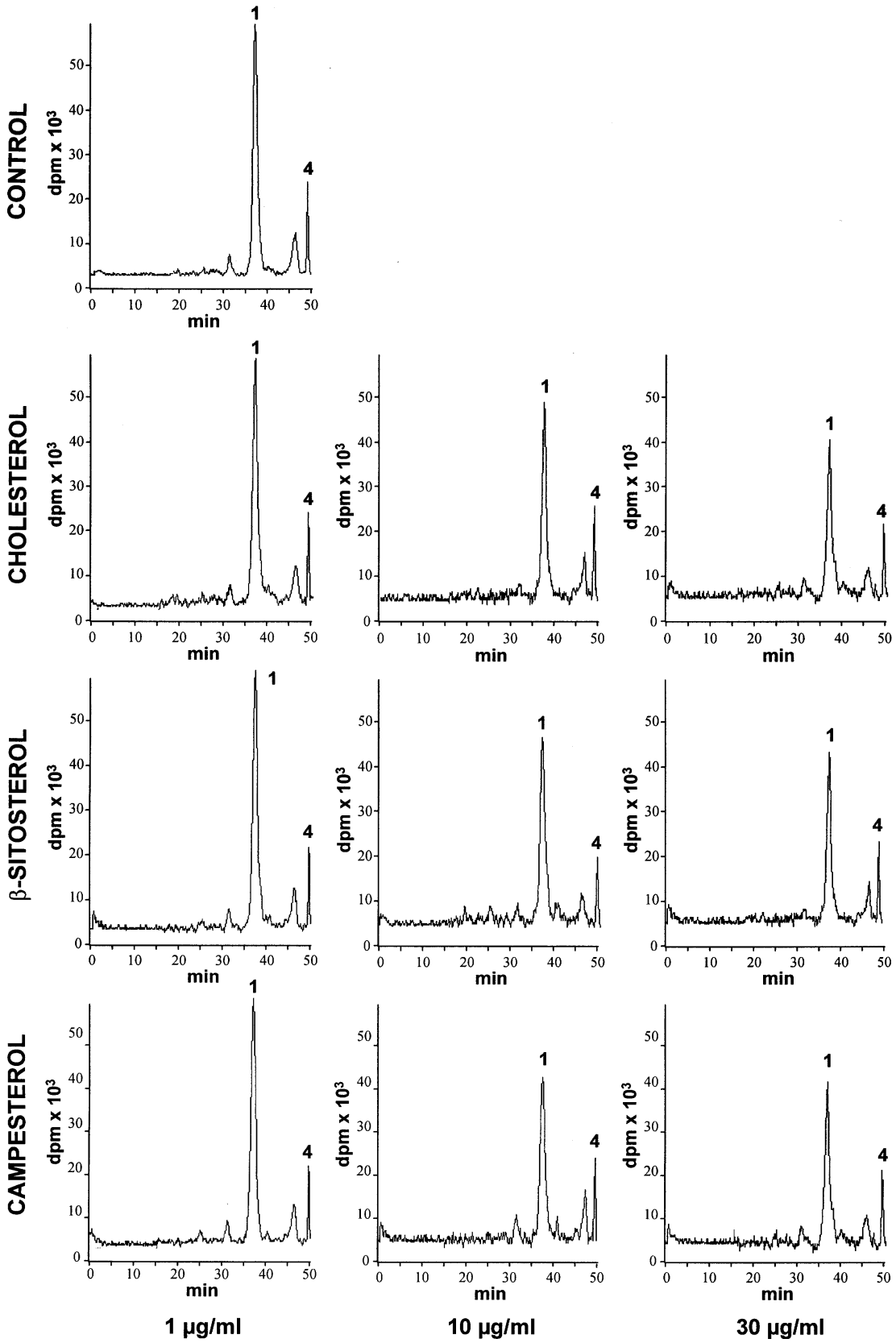


Figure 2 HPLC analysis of [^{14}C]sterols in HL-60 cells treated with different sterols or phytosterols

HL-60 cells were incubated with [2-^{14}C]acetate in the absence (control) or the presence of increasing concentrations of cholesterol, β-sitosterol, campesterol, stigmasterol, ergosterol and brassicasterol (1, 10 and 30 μg/ml), or 5,22-cholestadien-3β-ol (0.1, 1 and 3 μg/ml). At the end of the 8 h incubation, the cells were lysed with 10% KOH and the lipids were extracted with chloroform/methanol (2:1, v/v). The unsaponifiable lipids were separated by HPLC and the radioactivity was detected online. Identified peaks: **1**, cholesterol; **2**, desmosterol; **3**, 5,7,24-cholestatrien-3β-ol.



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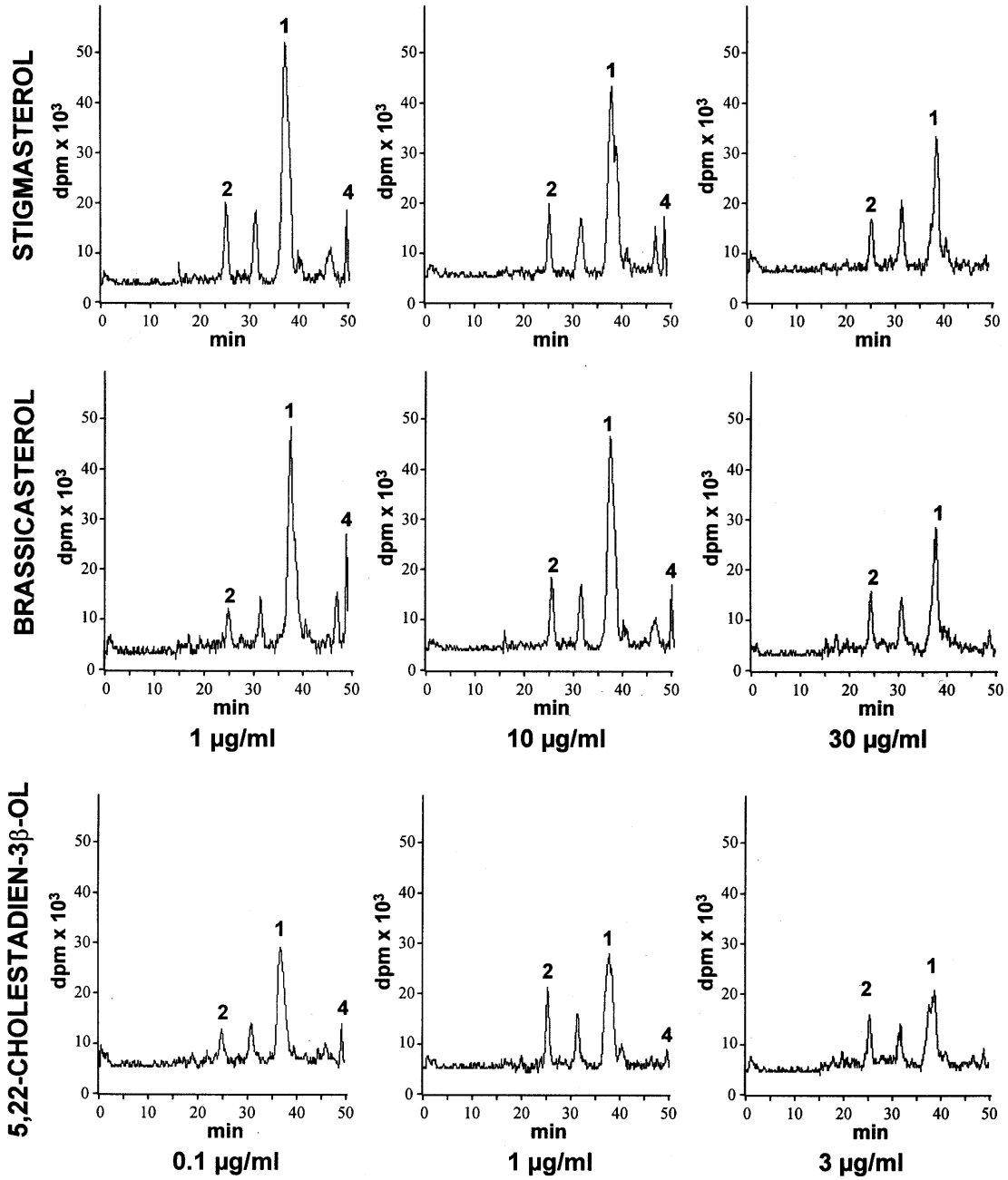

Figure 3 HPLC analysis of [^{14}C]sterols in Caco-2 cells treated with different sterols or phytosterols

Caco-2 cells were incubated with [2-^{14}C]acetate in the absence (control) or the presence of increasing concentrations of cholesterol, β-sitosterol, campesterol, stigmasterol, brassicasterol (1, 10 and 30 μg/ml) or 5,22-cholestadien-3β-ol (0.1, 1 and 3 μg/ml). At the end of the 24 h incubation, the cells were lysed with 10% KOH and the lipids were extracted with chloroform/methanol (2:1, v/v). The unsaponifiable lipids were separated by HPLC and the radioactivity was detected online. Identified peaks: **1**, cholesterol; **2**, desmosterol; **4**, dihydrolanosterol.

RESULTS

The structures of the compounds tested in the present study are shown in Figure 1. Among the phytosterols, stigmasterol and ergosterol were selected for the presence of a double bond at C-22, as opposed to β-sitosterol and campesterol; brassicasterol is the C7-saturated counterpart of ergosterol, and 5,22-cholestadien-3β-ol is an unusual isomer of desmosterol, containing the double bond at C-22 instead of C-24.

The effects of these compounds on [^{14}C]acetate incorporation into sterols were determined first of all in HL-60 cells, a promyelocytic cell line with a very active cholesterol biosynthesis, and the results are shown in Figure 2. Under control conditions, the radioactivity profile showed a single peak, corresponding to cholesterol (Figure 2). In our 8 h incubation assay, the presence of cholesterol in the medium at concentrations up to 30 μg/ml did not affect this profile. At higher concentrations ($\geqslant$ 60 μg/ml), however, cholesterol supplementation produced a significant decrease in [^{14}C]acetate incorporation into sterols (results not shown). Similarly to cholesterol, neither β-sitosterol nor campe-

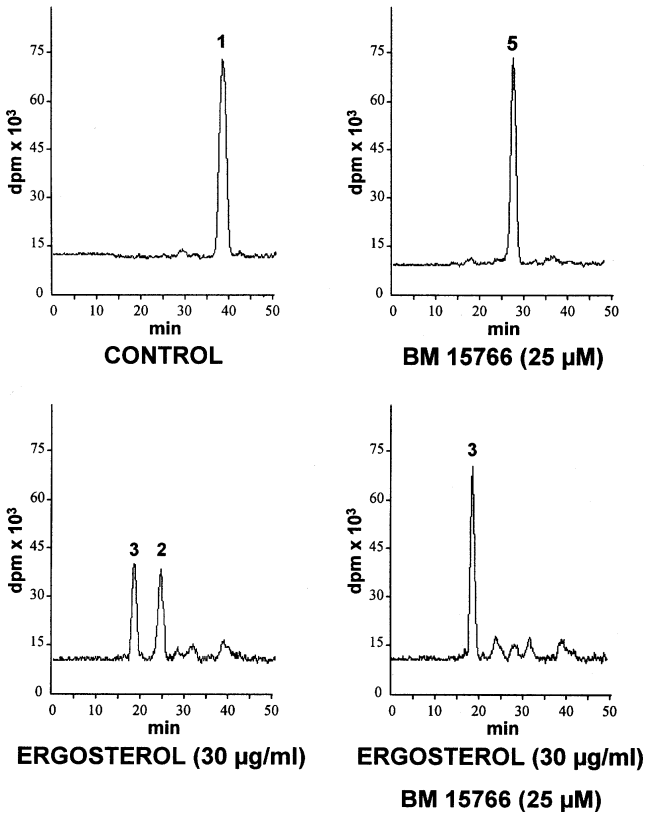

Figure 4 HPLC radioactivity profile of sterols in HL-60 cells treated with BM 15.766 and ergosterol

HL-60 cells were incubated with [2-^{14}C]acetate in the absence (control) or presence of 25 μM BM 15.766, 30 μg/ml ergosterol, or both in combination. At the end of the 8 h incubation, the cells were lysed with 10% KOH and the lipids were extracted with chloroform/methanol (2:1, v/v). The unsaponifiable lipids were separated by HPLC and the radioactivity was detected online. Identified peaks: **1**, cholesterol; **2**, desmosterol; **3**, 5,7,24-cholestatrien-3β-ol; **5**, 7-dehydrocholesterol.

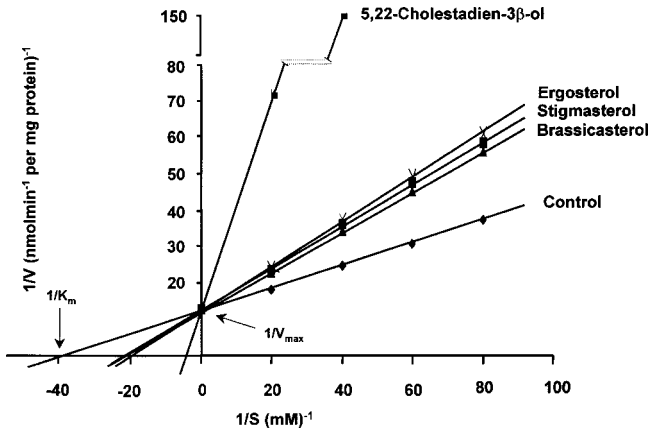

Figure 5 Lineweaver–Burk plots of the inhibition of sterol Δ^{24}-reductase activity by Δ^{22}-unsaturated sterols

Microsomal fractions from rat livers were incubated with [^{14}C]desmosterol as substrate in the absence (control) or presence of the indicated sterols (30 μM) for 45 min at 37 °C. At the end of the incubation, the reaction was stopped with ethanolic NaOH, the lipids were extracted with n-hexane, and the [^{14}C]cholesterol formed was analysed by HPLC. The results correspond to the means for three experiments.

sterol at concentrations up to 30 μg/ml (maximum concentration permitted by their solubility) altered the radioactivity incorporation into sterols. In contrast, phytosterols containing the Δ^{22}-unsaturated bond behaved very differently. Stigmasterol, brassicasterol and ergosterol inhibited cholesterol formation in a dose-dependent manner, producing an accumulation of the immediate precursor of cholesterol, desmosterol, and also (albeit to a lower extent) the immediate precursor of the latter, 5,7,24-cholestatrien-3β-ol. The compound 5,22-cholestadien-3β-ol inhibited [^{14}C]acetate incorporation into cholesterol much more strongly, with an estimated IC_{50} value lower than 0.1 μg/ml. This sterol was highly cytotoxic, cell death being evident with concentrations higher than 10 μg/ml. The accumulation of desmosterol mediated by the Δ^{22}-unsaturated sterols suggested the inhibition of the Δ^{24}-reductase.

To examine whether these results could be extrapolated to other cell types, the effects of phytosterols on [^{14}C]acetate incorporation were also determined in the human colonic adenocarcinoma Caco-2 cell line (Figure 3). The radioactivity profile of cells incubated under control conditions showed a major peak corresponding to cholesterol, and three others of minor intensity, namely dihydrolanosterol (peak 4) and two unidentified sterols (Figure 3). Supplementing the incubation medium with cholesterol produced a substantial decrease in radioactivity incorporation into sterols, indicating the inhibition of early steps in the pathway (probably, that of HMG-CoA reductase). β-Sitosterol and campesterol acted very similarly to cholesterol, decreasing total radioactivity incorporation, but without affecting the proportions between cholesterol and its sterol precursors (Figure 3). Phytosterols containing the Δ^{22}-unsaturation (stigmasterol and brassicasterol), in addition to lowering the total radioactivity incorporation into sterols in a dose-dependent manner, produced a marked change in the sterol profile, decreasing selectively the radioactivity present in cholesterol and dihydrolanosterol (products of the action of Δ^{24}-reductase), and increasing desmosterol (last of the eventual substrates of the Δ^{24}-reductase enzyme). 5,22-Cholestadien-3β-ol, a synthetic isomer of desmosterol, was very active in inhibiting cholesterol biosynthesis in Caco-2 cells, as found in HL-60 cells, with a concentration of 3 μg/ml decreasing radioactivity incorporation into cholesterol in more than 80% of the control cells and increasing the desmosterol-to-cholesterol ratio (Figure 3). These results agree with those obtained in HL-60 cells, altogether suggesting the selective inhibition of Δ^{24}-reductase activity by Δ^{22}-unsaturated sterols.

To confirm the mechanism of action, additional experiments were undertaken in HL-60 cells treated with BM 15.766, a non-competitive specific inhibitor of Δ^{7}-reductase [38,39]. As shown in Figure 4, BM 15.766 totally blocked the incorporation of [^{14}C]acetate into cholesterol, and the cells accumulated 7-dehydrocholesterol. The absence of desmosterol indicated that Δ^{24}-reduction was fully operative in this condition. In the presence of ergosterol, the cells accumulated 5,7,24-cholestatrien-3β-ol and desmosterol, as in the previous experiments. With BM 15.766 and ergosterol in combination, neither 7-dehydrocholesterol nor desmosterol were formed; instead, the cells accumulated further 5,7,24-cholestatrien-3β-ol (Figure 4). Similar results were obtained with the other Δ^{22}-unsaturated phytosterols (results not shown). These results indicated that these phytosterols inhibit the conversion of Δ^{24}-unsaturated sterols into their saturated counterparts, and therefore the main reaction affected was that catalysed by Δ^{24}-reductase, although an inhibitory effect of Δ^{22}-unsaturated phytosterols on Δ^{7}-reductase could not be ruled out.

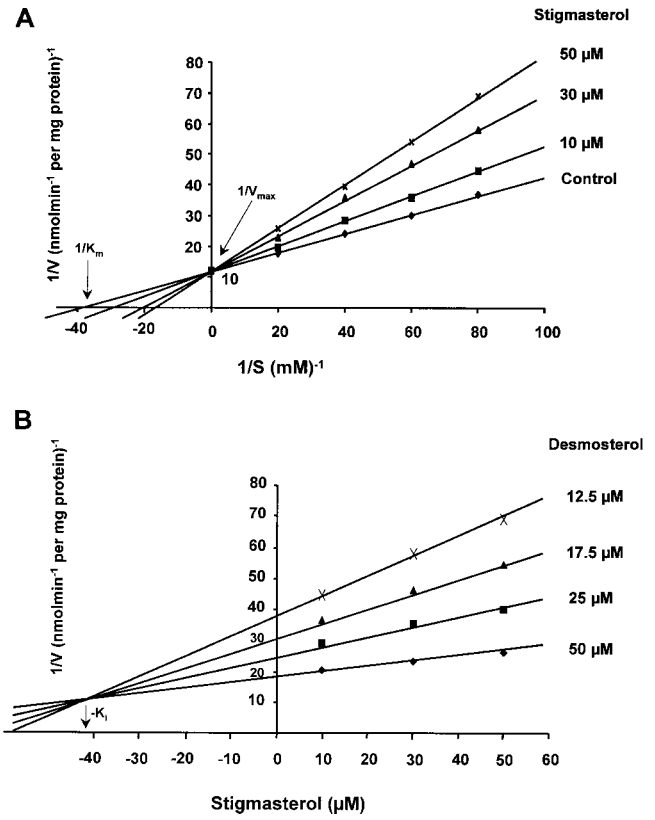

Figure 6 Competitive inhibition of sterol Δ^{24}-reductase by stigmasterol

Microsomal fractions from rat livers were incubated with [^{14}C]desmosterol as the substrate in the absence (control) or presence of increasing concentrations of stigmasterol for 45 min at 37 °C. At the end of the incubation, the reaction was stopped with ethanolic NaOH, the lipids were extracted with n-hexane, and the [^{14}C]cholesterol formed was analysed by HPLC. The results correspond to the means for three independent experiments. (**A**) Lineweaver–Burk plot; (**B**) Dixon plot.

In order to demonstrate their action on Δ^{24}-reductase, the microsomal fraction from liver homogenates was incubated with increasing concentrations of [^{14}C]desmosterol as the substrate, and the effects of the phytosterols on the formation of [^{14}C]cholesterol were determined. The results obtained with a single concentration (30 μM) of the phytosterols are shown by the Lineweaver–Burk double-reciprocal plots to illustrate the type of inhibition involved (Figure 5). In the control condition, the estimated V_{max} for the transformation of desmosterol into cholesterol was 0.083 nmol/min per mg of protein, and the K_m was 26.3 μM. β-Sitosterol and campesterol did not significantly affect these parameters (results not shown); in contrast, all the Δ^{22}-unsaturated sterols tested inhibited the enzyme reaction in a competitive manner, as indicated by the increased slope of plotted lines, as compared with the control. The compound 5,22-cholestedien-3β-ol was a much stronger inhibitor than the phytosterols (Figure 5).

To determine the inhibition constants (K_i values), enzyme assays were performed at four different concentrations of substrate and three concentrations of the sterol under investigation. Figure 6 shows the results obtained with stigmasterol, as a representative phytosterol among those containing the Δ^{22}-unsaturated bond. The double-reciprocal plot (Figure 6A) clearly illustrates that stigmasterol inhibited Δ^{24}-reductase activity competitively, as indicated by the common intersection of the four lines on the y-axis. Similar results were obtained with the other inhibitory sterols (results not shown). The K_i values were obtained graphically using Dixon plots for competitive inhibition (Figure 6B). As observed, the four straight lines, each for a desmosterol concentration, converged to a single point that represents a K_i value of $-$[I]. The K_i values ($n = 3$) for the inhibition of Δ^{24}-reductase were: stigmasterol, 41.1 ± 5.4 μM; ergosterol, 36.8 ± 5.2 μM; and brassicasterol, 42.7 ± 5.7 μM, which are similar to the K_m for the natural substrate of the enzyme (desmosterol). The K_i for 5,22-cholestedien-3β-ol was one order of magnitude lower (3.34 ± 0.76 μM), which exemplifies the strong inhibition of Δ^{24}-reductase this compound exerts.

DISCUSSION

Since the pioneering studies of Peterson [40] in chicks, the interest in phytosterols as anti-hypercholesterolaemic agents has increased notably, having been repeatedly demonstrated in humans that they efficiently reduce cholesterol levels when administered in the diet [17,41]. Indeed, phytostanols, which are the Δ^5-saturated counterparts of phytosterols, are currently used to enrich foods (margarine) in order to reduce hypercholesterolaemia [19,20,42,43]. Both phytosterols and phytostanols decrease the intestinal absorption of cholesterol [16,19,20], this being likely to be the most important mechanism contributing to their hypocholesterolaemic effect, but whether other actions on cholesterol homoeostasis also contribute to this effect remains to be investigated.

We have studied comparatively the effects of phytosterols on cholesterol biosynthesis in two human cell lines, promyelocytic HL-60 and colon adenocarcinoma Caco-2 cells. In both cases, we observed the inhibition of sterol Δ^{24}-reductase by Δ^{22}-unsaturated sterols, a finding reported here for the first time. Exposure of human cells *in vitro* to stigmasterol, brassicasterol or ergosterol at concentrations as low as 1 μg/ml resulted in a decrease in cholesterol biosynthesis, with an accumulation of desmosterol. There were slight differences in the efficiency of the phytosterols, depending on the cell line, being more active in HL-60 than in Caco-2 cells, which is attributed to the eventual presence of the ABCG5/8 transporters in the latter cells due to their intestinal origin [9]. The sterol 5,22-cholestedien-3β-ol, an unusual desmosterol isomer that lacks the alkyl groups characteristic of phytosterols, acted as a much stronger inhibitor of Δ^{24}-reductase and cholesterol biosynthesis in all of the cell types. These results raise the possibility of using certain Δ^{22}-unsaturated sterols as effective hypocholesterolaemic agents, provided that they are efficiently absorbed by the intestine.

Sterol Δ^{24}-reductase is a microsomal enzyme that catalyses the reduction of the double bond at C-24 in the cholesterol-biosynthesis pathway. This enzyme may use different Δ^{24}-unsaturated cholesterol precursors as substrates, including lanosterol and desmosterol [31]. However, the highest affinity is for 5α-cholestan-7,24-dien-3β-ol and, accordingly, it has been suggested that physiologically the C-24 reduction preferentially takes place right after the $\Delta^{8,7}$-isomerization of zymosterol [31]. In Caco-2 cells, we observed that Δ^{22}-unsaturated phytosterols produced the selective decrease in both cholesterol and dihydrolanosterol, and the accumulation of desmosterol. In HL-60 cells, these phytosterols also resulted in the inhibition of [^{14}C]acetate incorporation into cholesterol and the simultaneous accumulation of desmosterol and, to a lower extent, 5,7,24-cholestatrien-3β-ol. The accumulation of desmosterol typically takes place in cells deficient in Δ^{24}-reductase [37,44], as well as in normal cells treated with inhibitors of the Δ^{24}-reductase, such as U18666A and triparanol [45,46]. Therefore the results indicated that Δ^{22}-

unsaturated sterols inhibit Δ^{24}-reductase activity in living cells, an effect that was corroborated further in microsomal preparations.

The appearance of 5,7,24-cholestatrien-3β-ol in cells treated with the Δ^{22}-unsaturated sterols suggested that these compounds could also affect Δ^{7}-reductase activity. This is in agreement with previous results by Shefer et al. [39], who reported that ergosterol was a low affinity, competitive inhibitor of Δ^{7}-reductase in rat liver preparations. Earlier findings by Koroly and Dempsey [32], however, indicated that the unsaturation at the Δ^{22}-position of the sterol side chain does not influence the sterol Δ^{7}-reductase activity in microsomes. The results obtained from the experiments using BM 15.766 simultaneously with the phytosterols allow us to conclude that sterol transformations preceding that catalysed by Δ^{7}-reductase are not affected by the Δ^{22}-unsaturated phytosterols (Figure 4). Other potential actions on the initial steps of the cholesterol pathway (up to lanosterol) were not evaluated directly in the present study. Nevertheless, the fact that total radioactivity incorporated into sterols was decreased by the presence of all phytosterols studied in Caco-2 cells (Figure 3) indicates that these compounds might inhibit early steps in the cholesterol biosynthetic pathway in these cells. This is in agreement with Field et al. [29], who reported that phytosterols (β-sitosterol, stigmasterol and campesterol) inhibited HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, in Caco-2 cells. This effect, however, appears to be cell-type-dependent. In fact, in fibroblasts β-sitosterol appears not to affect HMG-CoA reductase activity [6]. Furthermore, as reported here with HL-60 cells, which are highly proliferating cells very active in cholesterogenesis, radioactivity incorporation into total sterols (cholesterol plus steroid precursors) was minimally affected by any phytosterol. Therefore the action of these compounds on the mevalonate pathway might depend upon the metabolic/proliferative state of the cells.

To analyse the mechanism of action of phytosterols, the inhibition constants of the Δ^{24}-reductase reaction were determined in microsome preparations. The values obtained in our preparations differ slightly from those reported by Bae and Paik [31], probably because of the different microsome sources. Specifically, we prepared the liver microsomes from rats fed on normal chow, whereas those authors used rats treated with cholestyramine and lovastatin, a procedure known to stimulate Δ^{24}-reductase activity [31]. We found that Δ^{22}-unsaturated phytosterols efficiently inhibited the conversion of [^{14}C]desmosterol into cholesterol in a competitive manner. On the basis of the distinct effect of the different sterols used, this inhibitory activity can be attributed to the presence of the double bond at C-22, and not to other modifications in the cholestane structure. Thus, while compounds containing the Δ^{22}-unsaturation were inhibitory, their saturated counterparts were inactive (stigmasterol compared with β-sitosterol; 5,22-cholestedien-3β-ol compared with cholesterol). Ergosterol and brassicasterol, which contain the C-22 double bond but which differ in a double bond in ring B, were both inhibitory. Finally, β-sitosterol and campesterol, which differ in the length of the alkyl substituent but which both have a saturated side chain, were inactive.

The estimated K_i values for the three phytosterols tested were very similar, but were much higher than the observed K_i for the unusual sterol 5,22-cholestedien-3β-ol. This is probably due to the additional alkyl substituents present in the phytosterols as compared with the desmosterol isomer, which might result in spatial hindrance with respect to recognition by the enzyme. The fact that K_i values for phytosterols were similar to the K_m value observed for desmosterol could be interpreted as Δ^{24}-reductase having a similar affinity for these sterols. This indicates that Δ^{22}-unsaturated phytosterols are efficient competitors of desmosterol for the enzyme, and, indeed, they inhibited cholesterol biosynthesis in cells very strongly, with IC_{50} values much lower than the respective K_i values estimated in microsomes. The cause of this could reside in the relative concentrations of inhibitors and natural Δ^{24}-reductase substrate(s) within the cells. In this respect, the intracellular concentrations of lanosterol, zymosterol and desmosterol in HL-60 cells incubated under control conditions were negligible when compared with that of cholesterol (results not shown), which is in accordance with other results reported previously in different cell types [47–49].

The K_i value for 5,22-cholestedien-3β-ol was 8 times lower than the K_m value for desmosterol. Even considering that the preferred substrate for Δ^{24}-reductase is 5α-cholestan-7,24 dien 3β-ol, for which the enzyme shows a slightly lower K_m than for desmosterol [31], our results clearly indicate that 5,22-cholestedien-3β-ol is a strong inhibitor of Δ^{24}-reductase. Indeed, at very low concentrations in the culture medium (0.1 μg/ml) this compound almost completely suppressed cholesterol formation (Figure 3).

The effect of Δ^{22}-unsaturated sterols on Δ^{24}-reductase might have a physiological relevance. In general, these compounds are in the minority among dietary phytosterols, although there are important differences between different foods. Centring the discussion on stigmasterol (as the most abundant Δ^{22}-unsaturated phytosterol), barley, lettuce, peach and sunflower oil are reported to be relatively the richest in this sterol within cereals, vegetables, fruits and edible oils [1,2]. Intestinal absorption of stigmasterol has been calculated to be approx. 2–5% of the ingested dose, both in humans [4] and rats [50,51]. It is therefore expected that plasma concentrations of stigmasterol will strongly depend on dietary habits and, indeed, a large variation has been reported in adult humans, ranging from 0.2 to 5 μg/ml [7,52,53]. Given that stigmasterol, at concentrations as low as 1 μg/ml, significantly inhibited Δ^{24}-reductase in any cell type, Δ^{22}-unsaturated phytosterols should be considered as potential modulators of cholesterol biosynthesis under physiological conditions. In individuals afflicted with sitosterolaemia, whose phytosterol levels are increased 100-fold [6], these unsaturated sterols may contribute to the characteristic diminished cholesterol biosynthesis of these patients [5,12]. Finally, the desmosterol isomer 5,22-cholestedien-3β-ol, which is an extraordinarily strong inhibitor of Δ^{24}-reductase, might prove to be an effective hypocholesterolaemic agent.

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