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Regulation of HMGCoA Reductase Activity by Policosanol and Octacosadienol, a New Synthetic Analogue of Octacosanol

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ABSTRACT

The octacosa-10,19-dien-1-ol is a newly synthesized long-chain alcohol, an unsaturated analogue of 1-octacosanol, the major component of policosanol, the purified natural mixture of different higher aliphatic alcohols obtained from sugarcane wax. Our efficient synthetic protocol (5 steps with 50% overall yield) is well suited for gram scale preparations and a rapid generation of analogues with different degrees of unsaturation. Beneficial effects of policosanol in the prevention of atherosclerosis and thromboembolic disorders have been reported and related to the inhibition of sterol biosynthesis possibly by the regulation of the activity of HMGCoA reductase mediated by AMP-dependent kinase AMPK. We have compared the effect of octacosadienol and policosanol on the regulation of HMGCoA reductase in HUVEC and HepG2 human hepatoma cells. Octacosadienol was as effective as policosanol in inhibiting the up regulation of HMGCoA reductase, in inducing the phosphorylation of AMPK and in down regulating the HMGCoA reductase mRNA.

Keywords: long-chain alcohols synthesis · octacosadienol · octacosanol · HMGCoA reductase · policosanol · AMPK phosphorylation

Abbreviations:

AMPK: AMP-activated protein kinase

DMEM: Dulbecco-modified Eagle medium

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

HMGR: HydroxyMethylGlutaryl-CoenzymeA reductase

HUVEC: Human endothelial cells from umbilical cord veins

LDL: low density lipoproteins

LDM: lipid-depleted medium

PBS: phosphate-buffered saline

RT-PCR: real time-polymerase chain reaction

SDS: sodium dodecyl sulfate

THF: tetrahydrofurane

Introduction

For many years higher aliphatic primary alcohols have been a subject of research. Early works were mainly focused on the biological activities of 1-triacontanol ($C_{30}H_{61}OH$) and 1-octacosanol ($C_{28}H_{57}OH$) [1-7]. The former was mainly isolated from beeswax [8], whereas 1-octacosanol from germ oils, wax sources (sugarcane, rice bran) and *Euphorbiaceae* [8-10]. 1-Triacontanol was highlighted as a plant growth promoter and an anti-inflammatory substance [1-3], while 1-octacosanol showed ergogenic, neurological and antioxidant properties [4, 5]. The C_{28} linear aliphatic primary alcohol is the major component (60-70%) of policosanol, the purified natural product, first described by Cuban researchers [11], that consists of a mixture of 8 higher aliphatic alcohols obtained from the wax of sugarcane (*Saccharum officinarum* L.). Policosanol was found to inhibit cholesterol biosynthesis by indirect regulation of the activity of HydroxyMethylGlutaryl-CoenzymeA reductase (EC: 1.1.1.34) (HMGR) and by an enhancement of receptor-dependent LDL processing [12-15]. The relevant literature dealing with cholesterol and serum lipids lowering, antiplatelets and antioxidant properties of policosanol has been extensively reviewed [16, 17]. More recently several authors have raised doubts on the cholesterol and serum lipids lowering, as well on the antioxidant properties of policosanol, on the basis of clinical studies on hypercholesterolemic patients [18-21].

In spite of the recent negative results as a cholesterol and lipid-lowering drug in clinical practice, policosanol seems able to affect some interesting cellular targets.The most important biochemical effect observed is the down regulation of HMGR activity, the regulatory enzyme of cholesterol biosynthesis, in homogenates of Vero fibroblasts grown for 24 h in lipid-depleted medium (LDM) in the presence of policosanol[13]. The decrease of HMGCoA reductase activity parallels the decrease of incorporation of labelled acetate in cholesterol (scheme 1). Similar results have been observed in rat hepatoma cells grown in the presence of policosanol [22]. In a recent metabolic study the possibility of a reduced cholesterol synthesis in the liver has been suggested to explain the observed decrease of faecal cholesterol end products after treatment with policosanol [23]. In neither Vero fibroblasts nor hepatoma cells could a direct inhibition of HMGR be found. Therefore the suppression of the up-regulation of HMGR that occurs when the cells are grown in LDM [24] could be caused by the interference of policosanol with the regulation of HMGR. One of the mechanisms of negative regulation of HMGR is the phosphorylation by the AMP kinase (AMPK) [25]. In rat hepatoma cells grown in the presence of policosanol, the decrease of sterol biosynthesis and HMGR activity was accompanied by a significant increase of phosphorylated AMPK, detected by immunoblot [22] (scheme 1). The increase in phosphorylated AMPK is very close to the one obtained in the presence of metformin, a glucose-lowering drug which activates AMPK phosphorylation. Phosphorylation by AMP kinase also inactivates acetylCoA carboxylase, the enzyme catalysing the first and regulatory step in fatty acid synthesis, suggesting the possibility of a coordinated regulation of cholesterol and fatty acid biosynthesis [25]. As a more general function, AMPK is involved in the control of the energy balance within the cell, acting as a metabolic sensor of the cellular AMP/ATP ratio [25, 26].

Owing to the involvement in the regulation of important signalling cascades controlling the cell proliferation and differentiation, AMPK has been suggested as a therapeutic target for atherosclerosis and cancer [27,28]. An effect of policosanol on the activity of AMPK could interfere with the complex regulatory net of this kinase and explain the controversial results obtained in different experimental models or clinical trials. Moreover an effect on the regulation of sterol or fatty acid biosynthesis can open very important therapeutic opportunities in different fields, such as angiogenesis control. The antiangiogenic activity of the HMGR inhibitors statins is known [27, 29] and recently some common inhibitors of enzymes of the late steps of sterol biosynthesis such as itraconazol, an inhibitor of sterol C-14 demethylase, and naftifine, an inhibitor of squalene epoxidase, were shown to inhibit both the growth of HUVEC cells

and angiogenesis [30,31]. More recently octacosanol purified from the medicinal plant *Tinospora cordifolia*, showed antiangiogenic and antitumoral activity both *in vitro* and *in vivo* [32].

In an attempt to study a possible effect of analogues of policosanol mixture on the angiogenic process, we synthesized octacosa-10,19-dien-1-ol, a new unsaturated derivative of octacosanol. The effect on HMGR activity and expression of this synthetic compound was studied in HUVEC cells and, for comparison, in hepatoma cells and compared to the effect, in the same experimental condition, of a commercial mixture of policosanol (Source Naturals, Inc., Scotts Valley, CA). Our results showed that, in both cell types, in the presence of octacosadienol the up-regulation of HMGR is decreased and the phosphorylation of AMP kinase increased. In HUVEC cells we could also observe by RT-PCR a decrease in HMGR mRNA. Octacosa-10,19-dien-1-ol showed almost the same activity as the natural policosanol mixture.

Experimental Procedures

Chemistry

Chemicals were purchased from Carlo Erba Reagenti and Acros Organics.

Buffers and cultural media were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise specified.

3-Hydroxy-3-methyl[3-^{14}C]glutaryl-coenzymeA (57 mCi/mmol) were obtained from Amersham Biosciences (U.K). policosanol 10 mg tablets were manufactured by Source Naturals, Inc. (Scotts Valley, CA) and purchased from a local health food store. Two 10-mg policosanol tablets were dissolved in 10 ml of absolute ethanol with the aid of a mortar and pestle. The insoluble excipients were removed by low-speed centrifugation (5000 x g for 5 min), and the supernatant was aliquoted and stored at -20°C or added directly to media upon use. Extracts were analysed by GC/MS using an Agilent 6850 gas chromatograph equipped with a 5973 MS detector and fitted with an HP-5 MS fused silica column (length 30 m; i.d. 0.25 mm; film thickness 0.25 mm).

Reactions were monitored by TLC on Fluka F_{254} (0.25 mm) plates, which were visualized by spraying with a molibdic acid and heating.

Silica gel Merck 60 was used for column chromatography (CC). IR: Shimadzu FT-IR 8001 spectrophotometer. NMR: Bruker 300 Avance spectrometer (for 1H NMR $CDCl_3$ was used as solvent, $CHCl_3$ at δ = 7.27 as reference. Low-resolution mass spectra: Finnigan-MAT TSQ70 in chemical ionization (CI-MS) with isobutane as reactant gas; ESI-MS were recorded on a Waters Micromass ZQ.

Methyl undec-10-enoate (1): A solution of undecylenic acid (1.5 g, 8.14 mmol), anhydrous methanol (8 ml) and *p*-toluensulfonic acid (50 mg, 0.29 mmol) in anhydrous toluene, placed in a 100 ml two-necked round-bottomed flask was heated under reflux for 5 h using a Dean-Stark apparatus and a condenser to remove esterification water. All glassware had been previously dried in an oven at 120°C. The reaction progress was monitored by TLC on silica gel plates with hexane/ethyl acetate 7:3 as eluent (Rf ester = 0.64). Work-up: the product was diluted with ethyl acetate, washed twice with a 1:1 $NaHCO_3/H_2O$ mixture, then with H_2O and brine; finally dried over Na_2SO_4. 1 was isolated as a transparent oil (1.5 g, 7.57 mmol, 93% yield). Residual traces of undecylenic acid could be removed by filtration on a pad of basic alumina. FT-IR (neat, cm^{-1}): ν = 2926, 1740, 1466, 1180, 1037, 742, 721, 669. 1H NMR (300 MHz, $CDCl_3$): δ = 1.30 (m, 10 H, H-4-8), 1.68 (m, 2 H, H-3), 1.96 (m, 2 H, H-9), 2.31 (t, 2 H, J=7.5 Hz, H-2), 3.6 (s, 3 H, -$COOCH_3$), 4.99 (m, 2 H, H-11), 5.70 ppm (m, 1 H, H-10). CI-MS m/z 199 $[M+H]^+$ $C_{12}H_{22}O_2$.

Methyl 10-oxodecanoate (2): A two necks 250 ml round-bottomed flask containing methyl undec-10-enoate (2.43 g, 12.27 mmol) and H_2O/acetone 1:1 (50 ml) was placed in a high-power ultrasound bath (with ice-water) with an emitting titanium surface fixed to two sonotrodes (19.0 kHz). A 0.2 M solution of OsO_4 in toluene (305 µl; 0.061 mmol) and *N*-methylmorpholine-*N*-oxide (1.44 g, 12.27 mmol) were added to the mixture under sonication (70 W). After 5 min $NaIO_4$ (7.59 g, 35.46 mmol) was added in small portions over a time of 20 min at 20°C. The reaction course was monitored by TLC on silica gel plates with hexane/ethyl acetate 7:3 as eluent (Rf of **2** = 0.5). After 3 h sonication the conversion was complete. Work-up: the reaction mixture was filtered on a porous synthered glass funnel, washing the residue with ethyl acetate. The filtrate was washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The product was purified by column chromatography using hexane/ethyl acetate 9:1 as eluent. 2 was obtained as a pale yellow oil (2.11 g, 10.55 mmol, yield 86%). FT-IR (neat, cm^{-1}): ν = 2928, 2675, 1745, 1464, 1242, 1047, 943, 725. 1H NMR (300 MHz, $CDCl_3$): δ = 1.29 (m, 8 H, H-3, 4, 5, 6), 1.56 (m, 2 H, H-8), 1.42 (m, 2 H, H-7), 2.31 (t, J=7.5 Hz, 2 H, H-2), 2.4 (td, J_t=8.1 Hz, J_d=1.8 Hz, 2 H, H-9), 3.60 (s, 3 H, -OCH_3), 9.74 ppm (t, J=1.8 Hz, 1 H, -CHO). CI-MS m/z 201 $[M+H]^+$ $C_{11}H_{20}O_3$.

(*E*) Octadec-9-en triphenylphosphonium bromide (3): The phosphonium salt was prepared from (*E*)-1-bromooctadec-9-ene (1.3 g, 3.9 mmol) heated under reflux overnight (N_2 atmosphere) in a toluene (30 ml) solution with triphenylphosphine (1.02 g, 3.9 mmol). After cooling down to 0°C and addition of diethyl ether (30 ml), the precipitate of phosphonium salt was collected on a paper filter. 3 was obtained as a whitish-pink powder (1.87 g, 3.16 mmol, 81% yield). 1H NMR (300 MHz, $CDCl_3$): δ = 0.87 (t, J=6.4 Hz, 3H, H-18), 1.24 (m, 30 H, H-3-17), 1.65 (m, 4 H, H-1, 2), 7.85 ppm (m, 15 H, -ArH). CI-MS (m/z): 593 $[M+H]^+$ $C_{36}H_{50}BrP$.

Methyl octacosa-10,19-dienoate (4): Glassware was previously dried at 120°C for 2 h. The phosphonium bromide (3) (3.32 g, 5.6 mmol) was dissolved in 30 ml of anhydrous THF in a 100 ml two-neck, round-bottomed flask. To the stirred solution under N_2 atmosphere 1.6 N BuLi (3.4 ml; 1.05 equiv) in hexane was added dropwise. Due to the formation of phosphorus ylide the mixture turned orange-red. After 20 minutes, 5 ml of a solution of 2 (1.07 g, 5.35 mmol) were added dropwise and the solution turned yellow-orange. After the addition was complete, the mixture was allowed to stir overnight. TLC with hexane/ethyl acetate 9:1 as eluent (Rf 4 = 0.69). The crude product (4) was diluted with a 0.1 N solution of HCl, extracted with ethyl acetate, washed with brine and dried over sodium sulfate. Methyl octacosa-10,19-dienoate (4) was obtained in good yield as an oil (90%) (2.09 g, 4.82 mmol, 90% yield). FT-IR (neat cm^{-1}): 3005, 2926, 1709, 1288, 1120, 964, 721. 1H NMR (300 MHz, $CDCl_3$): δ = 0.87 (t, J=6.0 Hz, 3 H, H-28). 1.31 (m, 34 H, H-3-8, 13-17, 22-27), 2.01 (m, 8 H, H-9, 12, 18, 21), 2.36 (t, J=6.5 Hz, 2 H, H-2), 3.5 (s, 3 H, -$COOCH_3$), 5.35 ppm (m, 4 H, H-10, 11, 19, 20). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 13.77 (C-28), 22.48 (C-27), 27.14 (C-9,11,18, 21), 29.05 ($(CH_2)_n$), 31.50 (C-26), 34.17 (C-2), 51.4 (-OCH_3), 130.03 (C-10, 11, 19, 20), 174.3 ppm (C-1). CI-MS m/z 435 $[M+H]^+$ $C_{29}H_{54}O_2$. ESI-HRMS (m/z): 435.4117 ($M + H^+$), (calcd. 435.4124).

Octacosa-10,19-dien-1-ol (5): To a suspension of $LiAlH_4$ (0.51 g, 13.4 mmol, 3 eq) in anhydrous THF (30 ml) under N_2, a solution of 4 (2.0 g, 4.6 mmol) in THF (10 ml) was added at 0°C. The reaction was stirred at room temperature for 3 h and monitored by TLC with hexane/ethyl acetate 80:20 (Rf 5 = 0.4). Work-up: 1N H_2SO_4 (15 ml) was added and the organic phase extracted with CH_2Cl_2 washed with brine and dried over sodium sulfate. The solvent was removed under vacuum and a white powder was obtained (1.59 g, 3.91 mmol, yield 85 %). FT-IR (neat cm^{-1}): 3510, 2926, 1464, 1290, 1174, 1034, 965, 731. 1H NMR (300 MHz, $CDCl_3$): δ = 0.88 (t, J=6.0 Hz, 3 H, H-28), 1.28 (m, 34 H, H-3-8, 13-

17, 22-27), 1.61 (m, 2 H, H-2), 2.02 (m, 8 H, H-9, 12, 18, 21), 3.64 (t, J=6.0 Hz, 2 H, H-1), 4.45 (br, 1 H, OH), 5.35 ppm (m, 4 H, H-10, 11, 19, 20). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 13.77 (C-28), 22.48 (C-27), 27.14 (C-9,11,18, 21), 29.05 ($(CH_2)_n$), 31.50 (C-26), 32.64 (C-2), 63.02 (C-1) 130.03 ppm (C-10, 11, 19, 20). CI-MS m/z 407 $[M+H]^+$ $C_{28}H_{54}O$. ESI-HRMS (m/z): 407.4160 ($M + H^+$), (calcd. 407.4175).

Cell culture: Human hepatoma HepG2 cells (ATCC HB-8065, HepG2) were routinely maintained as monolayers in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% v/v adult bovine serum, 0.03% of glutamine, penicillin 100 U/mL and streptomycin 0.1 mg/mL. Human endothelial cells from umbilical cord veins (HUVEC) were prepared, characterized and grown as previously described [33]. HUVEC were routinely maintained as monolayers in M199 medium supplemented with 20% v/v adult bovine serum, 0.03% of glutamine, penicillin 100 U/mL, streptomycin 0.1 mg/mL and heparine 8.5 USP unit/mL. Cells were grown at 37 °C in a humidified atmosphere (air 95%/ CO_2 5%).

HMGR assay: The HMGR activity was determined in HepG2 and HUVEC cells seeded in 24-well plastic clusters. Each well received 25000 cells suspended in 1000 µL of complete medium. After 24 h cells were rinsed twice with phosphate-buffered saline (PBS) and the medium was replaced with 987.5 µl of fresh growth medium containing lipid-depleted serum [34] to induce the up regulation of HMGR. Cells were divided into three experimental groups and grown 24 hours after addition of 12.5 µl of absolute ethanol (control wells) or 12.5 µl of an ethanolic solution of octacosadienol or policosanol (final concentration 50 µg/mL). In this range of concentration octanosadienol, policosanol and absolute ethanol had no effect on cell viability. As a control of the up regulation obtained in the above conditions, the HMGR activity was determined also in cells grown in parallel but in a medium supplemented with normal serum plus absolute ethanol. Cells were then washed with ice-cold PBS and lysed in ice for 20 min with 100 µl/well of ice-cold phosphate buffer, pH 7.4, containing 0.1 M sucrose, 40 mM KH_2PO_4, 30 mM EDTA, 50 mM KCl, 5 mM dithiothreitol, 0.25% (v/v) Brij 96. Cell debris was removed by centrifugation at 12000 x g for 3 min and the protein concentration of the supernatant was determined with a SIGMA Protein assay Kit based on the method of Lowry modified by Peterson [35]. In order to measure the HMGR activity 80 µl of supernatant were added with 10 µl of NADPH-generating system and 10 µl of labeled substrate Hydroxy-3-methyl[3-^{14}C]glutaryl-coenzymeA. The final mixture assay (100 µl) contained 2.5 mM NADP, 20 mM glucose 6-phosphate, 1.5 units of glucose 6-phosphate dehydrogenase and 0.01 µCi of Hydroxy-3-methyl[3-^{14}C]glutaryl-coenzymeA. The enzymatic reaction was stopped after 60 min at 37°C by addition of 10 µl of 5 M HCl. After an additional incubation for 30 min at 37 °C to allow for complete lactonization of the product, the mixture was centrifuged at 12000 x g for 2 min. The aqueous supernatant was applied to a silica column that was primed with 2 ml of eluting solvent mixture toluene/acetone (3:1, v/v). The mevalonolactone was eluted from the column with 6 ml of the eluting solvent mixture and the eluate collected in a scintillation vial for measuring ^{14}C radioactivity (2500 TR Liquid Scintillation Analyzer - Packard BioScience, Waltham, MA - USA). TLC analysis (toluene/acetone, 1:1 as eluent) of the eluate with a System 200 Imaging Scanner (Canberra Packard, USA) showed that all the recovered radioactivity was in correspondence of the reference of mevalonolactone. The HMGR activity was expressed as dpm of [^{14}C]mevalonate formed per milligram of protein incubated.

RT-PCR: HepG2 and HUVEC cells were seeded in 100 mm diameter dishes and incubated in 5 ml of complete medium. At 70% confluence, cells were rinsed twice with phosphate-buffered saline (PBS) and the medium was replaced with fresh growth medium containing lipid-depleted serum [34]. At this point, cells were divided into three

experimental groups and a further 24 hours treatment was carried out in the presence of 62.5 µl of absolute ethanol (control wells) or 62.5 µl of a ethanolic solution of octacosadienol or policosanol (50 µg/mL). A further group of cells was grown in a medium supplemented with normal serum plus absolute ethanol to check the up-regulation of HMGR after incubation with lipid-depleted serum. After 24h cells were washed twice with phosphate-buffered saline, and total RNA was extracted using NucleoSpin® RNA II Kit (Machery-Nagel). The cDNA was synthesized with the RevertAid TM H Minus First Strand cDNA synthesis Kit (Fermentas). TaqMan® RT-PCR reactions (25 µl) were set up in 96-well reaction plates using a TaqMan® Gene Expression Assay Mix specific for human HMGR or a TaqMan® Endogenous Control TBP (TATA-box binding protein) Mix and a TaqMan® Universal Master Mix (Applied Biosystems). Real-time PCR was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems), using generic cycling conditions.

Immunoquantitation of AMP-Kinase: HepG2 and HUVEC cells were seeded in 100 mm diameter dishes and incubated in 5 ml of complete medium. Cells were grown to 70% confluence and then maintained overnight in serum-free medium containing 1.5% bovine serum albumin. Cells were then divided into three experimental groups for a further 6 hours culture in the presence of 62.5 µl of absolute ethanol (control wells) or 62.5 µl of an ethanolic solution of octacosadienol or policosanol (50 µg/mL). As a positive control cells were cultured as described above in the presence of metformin (360 µg/mL) and 62.5 µl of absolute ethanol. The treatment of HepG2 and HUVEC cells with metformin did not show any detectable cell toxicity. After 6 hours cells were washed twice with phosphate-buffered saline, and lysed in ice-cold buffer (20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride and 1 mM protease inhibitor cocktails (Sigma-Aldrich) [36]. Cells were scraped from the plates and collected in a micro-centrifuge tube. Cells debris was removed by centrifugation at 12000 x g for 10 min and the resulting supernatant was used for Western Blotting. Protein concentration in cell lysated was measured using a BCA protein assay kit (Pierce). For Westerrn Blotting, 20-50 µg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to a nylon membrane in a transfer buffer containing 125 mM Tris-HCl, 960 mM glycine, 0.5 % SDS and 20% methanol. The membrane was blocked with 10% nonfat dry milk in PBS buffer and then incubated in the same buffer containing 0.1% Tween 20 with rabbit antibody to total AMP-kinase (anti-AMPK α-pan, 1:2000; Upstate Biotechnology) or to phosphorylated AMP-kinase (anti-phospho-AMPK α, 1:1000; Upstate Biotechnology). The immunoblot was developed with a secondary antibody conjugated to horseradish peroxidase and visualized and quantified by chemiluminescence (Western LightningTM Plus-ECL, Medical Imaging, PerkinElmer Life Sciences) on Molecular Imager Chemidoc XRS System, Quantity One software (Biorad) .

Statistical analysis: Data of HMGR activity (% of delipidated controls) were compared by the Kruskal-Wallis rank test [37], while RT-PCR data ($2^{\Delta\Delta Ct}$) were analyzed by ANOVA.

Results

Synthesis of octacosa-10,19-dien-1-ol.

In the search of a more active synthetic analogue of policosanol and with the aim to study the effect of unsaturations on C-28 fatty alcohol, we planned the synthesis of octacosa-10,19-dien-1-ol. Our synthetic approach was studied for gram-scale production of long chain unsaturated fatty alcohols [38]. The key step was a Wittig olefination to bind two

relatively cheap building blocks: the methyl ester of the 10-oxodecanoic acid and the phosphorus ylide obtained from oleyl bromide. The whole synthetic procedure is depicted in scheme 2.

HMGR activity regulation

The effect on HMGR of the octacosadienol, the synthetic unsaturated derivative of octacosanol, was studied in HUVEC and hepatoma cells and compared with the effect of the natural policosanol mixture extracted from commercial tablets. The composition of ethanolic extracts of tablets, analysed by GC/MS after extraction and purification [39] was the same found by Singh, Li and Porter [22]. Octacosanol content was about 60%.

In agreement with previous results of different authors [13, 22], we could not observe a direct inhibition of the enzyme after the addition of octacosadienol or Policosanol to cell lysates from both cell lines.

However after a 24 hours incubation of the cells grown in a delipidated medium with either octacosadienol or the commercial policosanol, the activity of HMGR measured in cell lysates was significantly reduced with respect to the controls grown in the same conditions in the absence of octacosadienol or policosanol. Fig 1 shows the HMGR activity measured in lysates of cells grown in a complete medium, compared to cells grown in a delipidated medium, in the absence or in the presence of octacosadienol or policosanol. As shown in the figure, the HMGR activity of cells grown in a delipidated medium was significantly increased by 50% in hepatoma and by 80% in HUVEC cells ($P < 0.01$), compared with cells grown in a normal medium. In hepatoma the addition of 50 μg/ml of octacosadienol to the delipidated medium caused a 30% decrease in HMGR activity. In HUVEC cells the effect of octacosadienol was even higher and the enzymatic activity dropped by 50%. In both cell types octacosadienol was almost as effective as policosanol. The enzymatic activity did not drop to the level found in the not induced cells grown in a complete medium, but in both cell types the presence of policosanol or octacosadienol significantly reduced ($P < 0.01$) the induction of the enzymatic activity caused by the absence of uptake of sterols from the medium.

In order to find out if the decrease of the activity of HMGR in the presence octacosadienol or policosanol in HUVEC cells could be explained with an inactivation of the enzyme induced by AMPK we analyzed HUVEC cell lysates in western blot to evidence the phosphorylated form of AMPK by a specific antibody. As shown in fig. 2 the increase of 2.5 to 3.5 fold of the phosphorylated form of AMPK in lysates of HUVEC cells grown in a delipidated medium in the presence of the same amount of octacosadienol or policosanol used to measure the activity of HMGR, is comparable to that obtained in the presence of metformine, a well known activator of the phosphorylation of AMPK [40]. A comparable increase of phosphorylated AMPK was observed in hepatoma cells treated with Policosanol and octacosadienol in the same conditions. From these experiments the efficacy of octacosadienol is comparable to that of the natural mixture of policosanol.

Furthermore, in order to study the occurrence of an alternative or additional mechanism based on a decrease of the expression of the enzyme, we determined by quantitative RT-PCR the relative amount of the HMGR mRNA present in HUVEC cells grown in a delipidated medium in absence or in presence of octacosadienol. RNA was extracted from both cells grown in a complete medium and cells grown in a delipidated medium with or without the addition of 50 μg/ml of octacosadienol. The cDNA from all the samples was obtained by RT-PCR using the same starting amount of RNA. The relative amount of cDNA was determined in real time PCR with the $2^{\Delta\Delta Ct}$ method [41], using as housekeeping gene the TBP gene cDNA and as a calibrator the HMGR mRNA of HUVEC cells grown in a complete medium. By using this method the value of $2^{\Delta\Delta Ct}$ of the calibrator (cells grown in a complete medium) is 1 and the $2^{\Delta\Delta Ct}$ value of the cells grown in a delipidated medium in the presence or in the absence of octacosadienol or policosanol represents the fold change in mRNA in a delipidated medium. As shown in the histogram of figure 3, the increase of

HMGR mRNA in HUVEC cells grown in a delipidated medium is almost 6 fold. The addition of octacosadienol reduces this increase by 50%. ($P < 0.05$). By comparing in the same experiment the $2^{-\Delta\Delta Ct}$ obtained in the presence of octacosadienol and of the mixture of policosanol we have shown that octacosadienol alone has the same activity of the mixture of policosanol, as can be seen in Fig. 4. A grade of variability among the experiments was observed mainly in the amount of the induction of the expression of HMGR in a delipidated medium. The higher the induction in the control cells, the more evident was the reducing effect of the octacosadienol. In hepatoma cells we could not observe a significant effect of octacosadienol. In two different experiments we obtained no or very little (18%) reduction of the HMGR mRNA with respect to the control cells grown in a delipidated medium.

Discussion

The present paper describes an efficient synthetic procedure to obtain octacosa-10,19-dien-1-ol in gram scale, starting from commercially available cheap building blocks, namely oleyl bromide and undecylenic acid. The method is extremely versatile because by varying the chain length of the alkenyl halide as well as the number/position of the double bonds, a wide number of analogues can be prepared. The simplicity of this protocol and the excellent overall yield (about 50%) are worthy of mention.

Our results show that the naturally occurring policosanol is able to down regulate the HMGR activation that occurs when the cells are grown in a delipidated medium also in cell types different from the previously studied fibroblasts and hepatoma cells [13,22], i.e. the angiogenic HUVEC cells. Moreover, the synthetic unsaturated long-chain alcohol octacosadienol has the same activity as the natural product in both hepatoma and HUVEC cells. Both policosanol and octacosadienol increased the phosphorylation of AMPK, a kinase that phosphorylates several downstream substrates including HMGR, having the overall effect of switching off ATP-consuming pathways such as fatty acid and cholesterol synthesis and switching on ATP-generating pathways [25]. An increased phosphorylation of HMGR by AMPK could explain the down regulation of HMGR observed in hepatoma and HUVEC cells, since the phosphorylated HMGR is inactive [25]. The increased phosphorylation of AMPK can affect the activity of HMGR either by the direct phosphorylation and consequent inactivation of the enzyme, or by an indirect down regulation, mediated by transcription factors. SREBP-1, a transcription factor involved in the regulation of several lipogenic enzymes, included HMGR [27], has been recently shown to be down regulated following the activation of AMPK [26, 40]. Our quantification of HMGR mRNA by RT-PCR shows that in HUVEC cells both policosanol and octacosadienol affect the regulation of HMGR activity also at the transcription level, causing a decrease of mRNA. In hepatoma cells we could not detect a significant decrease of the level of the HMGR mRNA, and the observed down regulation of the enzyme could be mainly explained with the phosphorilation by AMPK and consequent inactivation.

Therefore, the induction of the phosphorylation of AMPK seems to be a general mechanism by which Policosanol, and also synthetic analogues of these natural compounds, contrast the up regulation of HMGR by activating different cell-specific mechanisms of regulation. The down regulation of HMGR, besides affecting cholesterol biosynthesis, could influence cell cycle progression as a consequence of the decreased synthesis of nonsterol mevalonate derivatives as farnesyl, geranyl and geranyl-geranyl intermediates which are necessary for the prenylation of proteins as Ras and Rho, involved in the signal transduction cascades controlling cell proliferation and differentiation. Antitumour properties such as induction of growth arrest and apoptosis, inhibition of metastasis and inhibition of angiogenesis, have been observed in various cell lines and in experimental animals after treatment with statins, the most known inhibitors of HMGR. The mechanism that mediates the antitumoral effects of statins, originally related to the inhibition of Ras

farnesylation, is still being discussed and possibly dissimilar in different cell lines [42]. Recently, the involvement of a long chain aliphatic alcohol in the control of cell proliferation has been described in a paper studying the effect of octacosanol purified from *Tinospora cordifolia*, on the proliferation of HUVEC and tumour cells. In this study, octacosanol has been shown to inhibit the proliferation of HUVEC and Ehrlich ascite tumour cells (EAT) [32]. Moreover, angiogenesis decreased in the peritoneum of EAT-bearing mice treated with octacosanol. The antiangiogenic activity was accompanied by a decrease of the secretion of VEGF in ascite fluid, a decreased activity of the collagenase MMP2, and the inhibition of nuclear translocation of NFkB, a transcription factor involved in the control of the inflammatory response and apoptosis and controlled by different, complex activation pathways [43, 44].

The regulation of the HMGR activity and AMPK phosphorylation in HUVEC cells by a synthetic long-chain alcohol such as octacosadienol opens promising perspectives for the design of new antiangiogenic compounds. Pathological angiogenesis is a hallmark of cancer and various ischemic and inflammatory diseases and anti-angiogenic therapy is one of the most promising approaches to cancer treatment, making the discovery of new anti-angiogenic molecules very attractive.

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Figure and scheme legends

Figure 1. Effect of octacosadienol and Policosanol on the activity of HMGR of HUVEC and HepG2 hepatoma cells grown in a delipidated medium.

The HMGR activity was measured in lysates prepared from HepG2 or HUVEC cells grown either in a delipidated or in a complete medium and compared with the activity of lysates of cells incubated with 50 μg/ml of octacosadienol or Policosanol in LDM. The HMGR activity is expressed as % of the activity found in control cells grown in LDM. Each value represents the mean and S.E. of at least two separate experiments in duplicate.

A Control cells grown in LDM

B Control cells grown in a complete medium

C Cells grown in LDM with 50 μg/ml octacosadienol

D Cells grown in LDM with 50 μg/ml Policosanol

Figure 2. Immunoblot of total and phosphorylated AMPK in HUVEC cell lysates.

Cell lysates untreated (controls) or incubated with 360 μg/mL of metformine (+ met) or 50 μg/ml of Policosanol (+ pol) or octacosadienol (+ oct) were fractionated by electrophoresis and transferred to nylon membranes for immunodetection with an antibody specific for total AMPK or for phosphorylated AMPK (AMPKP).The band density determined as arbitrary units are expressed as ratio versus the untreated control. Bars represents S.E. of duplicate experiments.

Figure 3. Effect of octacosadienol on the HUVEC cells expression of HMGR.

The cDNA obtained after extraction of total RNA from cell lysates was amplified in the presence of a TaqMan probe specific for HMGR in real-time PCR and quantified relatively to untreated controls grown in a complete medium.

A Lysates from cells grown in LDM

B Lysates from cells grown in LDM with octacosadienol

Each value represents the mean and S.E. of six separate experiments.

Figure 4. Effect of octacosadienol and policosanol on the HUVEC cells expression of HMGR.

The cDNA obtained after extraction of total RNA from cell lysates was amplified in the presence of a TaqMan probe specific for HMGR in real-time PCR and quantified relatively to untreated controls grown in a complete medium.

A lysates from cells grown in LDM

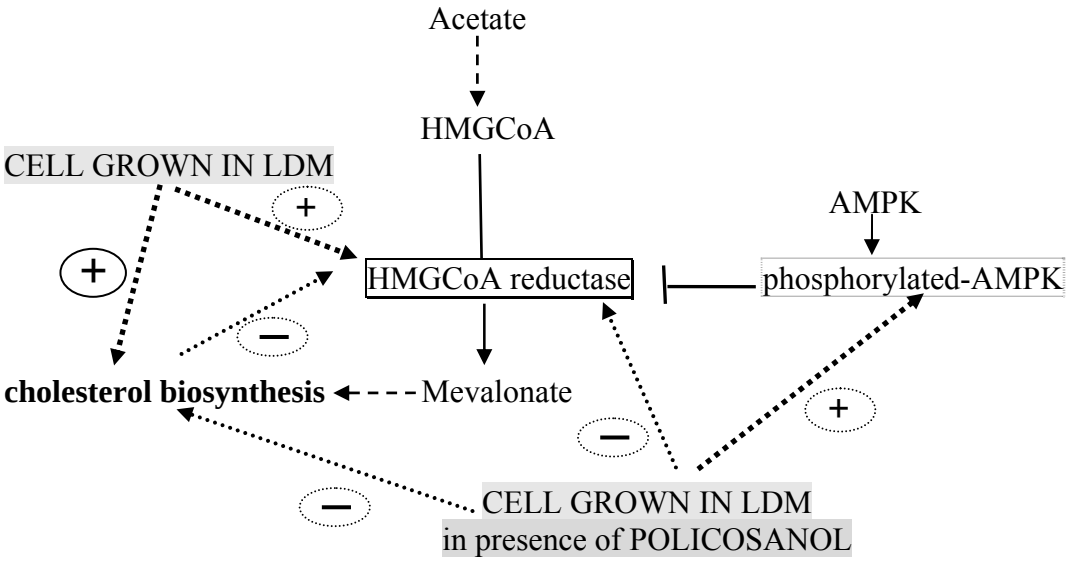
B lysates from cells grown in LDM with octacosadienol

C lysates from cells grown in LDM with Policosanol

Scheme 1. Sterol biosynthesis and HMGCoA reductase activity are upregulated in cells grown in a delipidated medium (LDM). In the presence of policosanol the upregulation of HMGCoA reductase and the incorporation of acetate into sterols is decreased [13] and the phosphorylated form of AMPK is increased [22].

Scheme 2. a) MeOH, $PTSA_{cat}$, toluene, rfx. 5h; b) H_2O/acetone, OsO_4, NMMO, $NaIO_4$, US 19 kHz;
c) toluene rfx., PPh_3; d) **2**, BuLi, THF; e) $LiAlH_4$, THF.

Scheme 1



Scheme 2

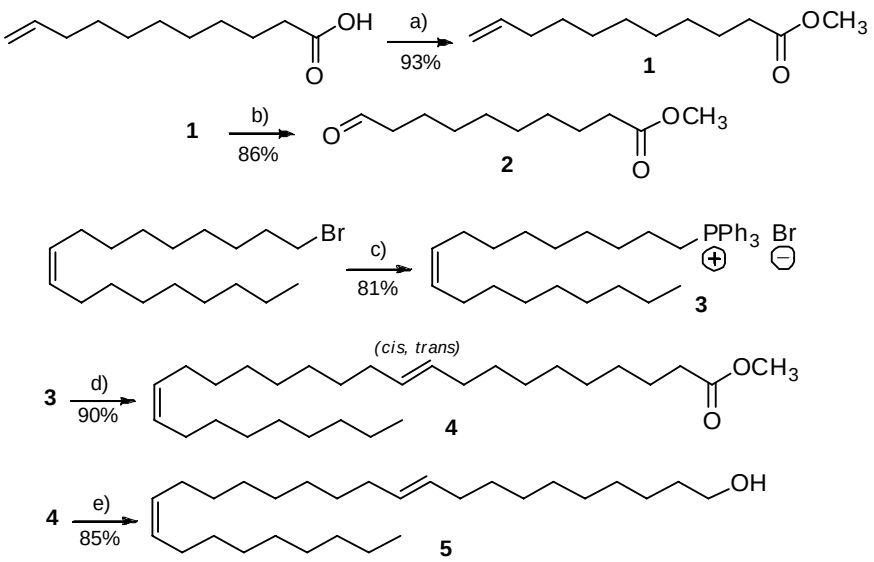


Figure 1

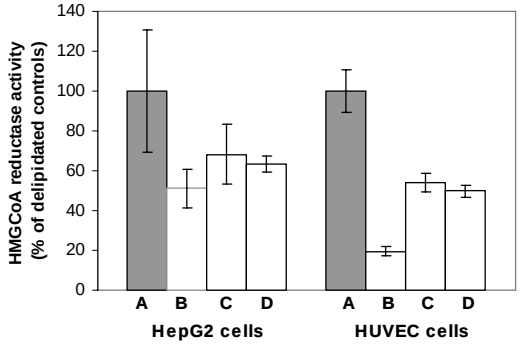


Figure 2

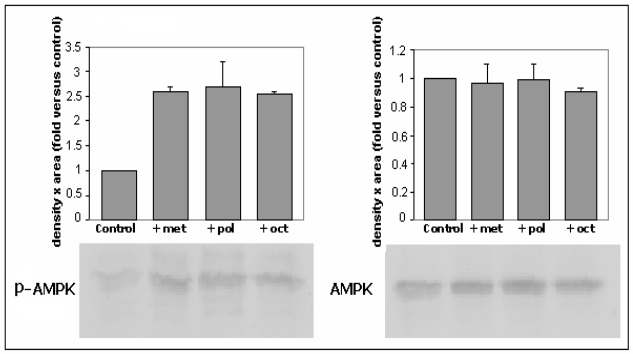


Figure 3

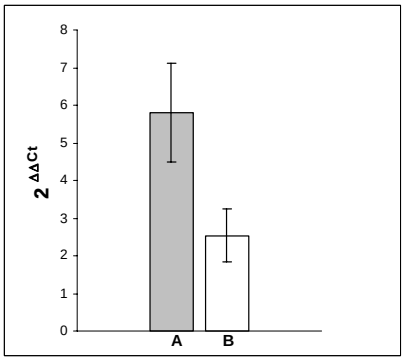


Figure 4