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POTENT INHIBITION OF CHOLESTEROL BIOSYNTHESIS IN 3T3 FIBROBLASTS BY *N*-[(1,5,9)-TRIMETHYLDECYL]-4α,10-DIMETHYL-8-AZA-*TRANS*-DECAL-3β-OL, A NEW 2,3-OXIDOSQUALENE CYCLASE INHIBITOR

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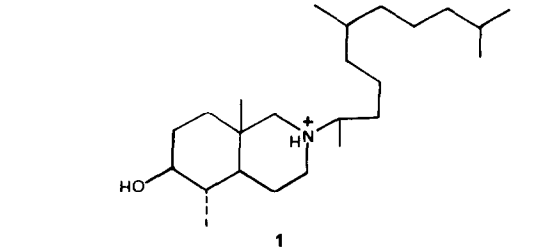
Abstract—*N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol, a new compound rationally designed to inhibit the 2,3-oxidosqualene cyclase (M. Taton *et al.*, *Biochem. biophys. Res. Commun.* **138**, 764, 1986) was studied as an inhibitor of cholesterol biosynthesis in Swiss 3T3 fibroblasts. Treatment of cells, which were grown for 2 days in a delipidated medium, resulted in a dramatic decrease of [^{14}C]acetate incorporation into the C_{27}-sterol fraction. An IC_{50} of 20 nM was calculated, which classes this drug amongst the most powerful cholesterol biosynthesis inhibitors acting at the 2,3-oxidosqualene-lanosterol cyclase tested so far on mammalian cells. The inhibition of the C_{27}-sterols synthesis was correlated with the accumulation of 2,3-[^{14}C]oxidosqualene and of 2,3:22,23-[^{14}C]dioxidosqualene indicating that the cyclase was indeed an intracellular target of the drug. A minor secondary target was identified as the sterol-8-ene isomerase. Cells treated with the inhibitor also accumulated sterols more polar than cholesterol which could originate, for example, from the metabolization of 2,3:22,23-dioxidosqualene. Treatment of the cells with increasing concentrations of the drug resulted in a progressive reduction of the HMG-CoA reductase activity (up to 50% of control). The drug affected normal growth of the fibroblasts and growth arrest was correlated with a decrease in cellular cholesterol content to less than 50% of control. This work indicates that *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol is a potent and promising new tool in the inhibition of cholesterol biosynthesis in mammalian cells.

2,3-Oxidosqualene-lanosterol cyclase is an attractive target for the design of new hypocholesterolemic drugs which affect selectively the biosynthesis of cholesterol. In addition to the direct reduction of lanosterol production, inhibitors of this enzyme also induce the accumulation of 2,3:22,23-dioxidosqualene [1–4], a precursor of 24(*S*),25-epoxycholesterol, which is a known repressor of HMG-CoA reductase [5]. § This dual function might result, at the cellular level, in an amplification of the inhibitory effects, i.e. the inhibitors of the cyclase could possess an efficiency superior to that anticipated from their inhibition constant determined *in vitro* on the enzyme. We have recently described the inhibition of cholesterol biosynthesis in 3T3 fibroblasts by 2-aza-2,3-dihydrosqualene [3], a rationally designed 2,3-oxidosqualene cyclase inhibitor. This molecule mimicked the first transient carbocationic intermediate occurring in the oxirane-ring opening during the cyclization of 2,3-oxidosqualene into β-amyrin and cycloartenol in higher plants [6] or into lanosterol in animals [7]. A series of 8-azadecalins (**3–6**; Fig. 1), which have been previously shown to be potent inhibitors of the cycloeucalenol-obtusifoliol isomerase [8], were tested without success in order to mimic the C-8 high-energy carbocationic intermediate postulated during the cyclization of 2,3-oxidosqualene [7]. Such an intermediate can be generated, in principle, during the annulation process, i.e. concomitant to the B-ring formation, and at the



	R_1	R_2	R_3
3	CH_3	CH_3	H
4	CH_3	CH_3	CH_2–C_6H_5
5	H	CH_3	H
6	H	CH_3	CH_2–C_6H_5

Fig. 1. Structure of the decalins and 8-azadecalins.

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§ Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; U 18666 A, 3β-[2-(diethylamino)-ethoxy]androst-5-en-17-one; AY-9944, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride.

penultimate step, before the final hydrogen abstraction at C-9 which leads to lanosterol. It was, however, found by Taton *et al.* that such 8-azadecalin structures, when bearing an isoprenoid-like N-substituent, e.g. *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**1**; Fig. 1), became excellent inhibitors of the 2,3-oxidosqualene-lanosterol and -cycloartenol cyclases [9]. We now report the effect of **1** (the title compound), on the biosynthesis of cholesterol in 3T3 fibroblasts. This molecule was found to be a very potent and selective inhibitor and should prove to be an interesting new tool in the control of cholesterol biosynthesis.

MATERIALS AND METHODS

Chemicals. Sodium [2-^{14}C]acetate (48 mCi/mmol) was purchased from CEA (Saclay, France), DL-[2-^{3}H]mevalonic acid lactone (1 Ci/mmol) and 3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA (60 mCi/mmol) were from Amersham (Les Ulis, France). Cholesterol, desmosterol, lanosterol, mevalonolactone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were obtained from Sigma (St. Louis, MO). U18666A was a kind gift from Dr Cenedella. The synthesis of **1** has been reported previously [9]; 4,4,10 β-trimethyl-*trans*-decal-3β-ol, compactin and mevinolin were gifts from the Laboratoires Fournier (Dijon, France). 5α-Cholesta-8-ene-3β-ol was prepared in this laboratory by M. Taton. Cell culture medium and serum were obtained from Gibco and the culture flasks were from Falcon. All other chemicals were of the highest purity available.

Cell cultures. Swiss 3T3 fibroblasts (obtained from Dr J. P. Beck) were routinely maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in plastic culture flasks. The cells were grown in a humidified incubator with a 5% CO_2 atmosphere at 37°. Lipid-depleted serum was prepared from fetal calf serum according to [10] and residual cholesterol determined by gas chromatography [3]. The inhibitors, cholesterol were added to the cells as solutions in absolute ethanol. Final concentration of ethanol in culture medium did not exceed 0.4% (v/v) and an equivalent amount of solvent was added to the control cultures. No significant effect of ethanol on cell growth or sterol synthesis could be observed during the time course of our experiments.

Incorporation of radiolabeled precursor into non-saponifiable sterols. Cells derived from stock cultures were seeded at about 3×10^5 cells in 60 mm dia. plastic Petri dishes in 5 ml medium containing 10% (v/v) delipidated serum. On the second day of culture, the cells were pretreated for indicated times (0.5–4 hr) with given amounts of the inhibitors. Labeled acetate (10 μCi per dish) was then added to the culture medium. After 2 hr of incubation the medium was removed and the dishes washed 3 times with phosphate-buffered saline (pH 7.4). The cells were then treated for 30 min with 2 ml of 0.1 N NaOH and aliquots removed for protein determination [11]. The remainder was saponified with 6% potassium hydroxide in methanol (w/v) and the nonsaponifiable lipids extracted with *n*-hexane, finally the C_{27}-sterol fraction was isolated by TLC essentially as described before [3].

Sterol-8-ene isomerase assay. Conversion of 5α-cholesta-8-ene-3β-ol into 5α-cholesta-7-ene-3β-ol catalyzed by rat liver microsomal $\Delta^8 \rightarrow \Delta^7$-sterol isomerase was performed essentially as described by Yamaga and Gaylor [12] under conditions where the reaction was linear with respect to time and protein amounts. Briefly, to washed rat liver microsomes [7] resuspended in 0.1 M potassium phosphate buffer pH 7.5 (5 mg proteins per assay of 2 ml), were added 60 mg D-glucose, 15 units of glucose oxidase. After a pre-incubation of 3 min at 37°, the substrate (final concentration in the assay: 100 μM, i.e. about 2 Km [12]) and varying concentrations of inhibitors (**1** or **6**), emulsified in 0.1 ml of the same buffer containing 6% (w/v) Triton X-100, were added. All these procedures were carried out under nitrogen. After an incubation time of 20 min, the reaction was stopped by addition of methanolic potassium hydroxide and worked-up as above. The C_{27}-sterol fraction was further fractionated by argentation chromatography on alumina TLC (*vide infra*). The band corresponding to the mixture of the Δ^8- and Δ^7-sterols was analyzed by gas chromatography [3, 8] in order to determine the extent of isomerization. The inhibitor concentrations which reduced the observed reaction rates by 50%, i.e. the I_{50} values, were determined using five inhibitor concentrations (duplicates) in the range of $0.1\ I_{50} \leq I \leq 5\ I_{50}$.

Measurement of HMG-CoA reductase activity. After incubation, the cells were washed 3 times with phosphate-buffered saline, harvested by scraping into buffer A (5 mM dithiothreitol, 10 mM EDTA and 50 mM potassium phosphate, pH 7.4) and frozen in liquid nitrogen. After thawing and sonication, aliquots of about 50–100 μg cell extract proteins were assayed for enzyme activity in a final volume of 140 μl containing buffer A, 20 mM glucose-6-phosphate, 5 mM $NADP^+$ and 0.5 units of glucose-6-phosphate dehydrogenase. After a pre-incubation of 15 min at 37°, the reaction was started by addition of 3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA (100,000 dpm; final concentration 5.4 μM). After 30 min, the reaction was stopped by the addition of 10 μl of 15 N HCl. Under these experimental conditions, the reduction rate was found proportional to time and protein (at least up to 150 μg) concentrations. Lactonization was performed by further incubation at 37° for 30 min after addition of [2-^{3}H]mevalonic acid (30,000 dpm) as internal standard, to monitor lactonization and recovery, and mevalonolactone (200 μg) as carrier. After centrifugation the clear supernatant was chromatographed on silica TLC plates using toluene/acetone (1:1, v/v) as eluant. The mevalonolactone ($R_f = 0.55$) was visualized in iodine vapors, scraped-off the plate and counted. The blanks were obtained similarly with boiled cell extracts.

Analytical procedures. The nonsaponifiable lipid extracts were separated by one-dimensional thin-layer chromatography on Silica Gel 60 F_{254} plates (Merck) as detailed elsewhere [3]. The radioactive bands were scraped off into scintillation vials and counted.

The composition of the C_{27}-sterol fraction was

analyzed by TLC on silver nitrate (10% in ethanol/water 70:30, v/v) impregnated silica gel or aluminum oxide 150 F_{254} (Merck) plates. Monoenic sterols, e.g. cholesterol, and sterols with two double bonds, e.g. desmosterol, were separated as acetates on the silica plates by double migration in toluene/cyclohexane (40:60, v/v) as developing solvent. Isomeric sterols containing one double bond, i.e. Δ^5- Δ^7/Δ^8-sterols, were separated by a single migration on the aluminum oxide plates using chloroform/hexane/acetone (12:6:1, v/v) as eluting system. Polar sterols were chromatographed on silica plates, previously washed with chloroform/methanol (2:1, v/v), using hexane/ethyl acetate (1:1, v/v) as eluant.

Expression of results. The results, in Figs. 3–6 and Tables 2 and 3, are expressed as the means of duplicates, which were not found to differ by more than 10%.

Gas chromatography and mass spectroscopy. Cholesterol content of cultured cells was quantified by gas chromatography using an OV-1 coated fused silica capillary column as described previously [3]. In cells treated with the inhibitors, gas chromatography coupled with mass spectrometry was used to identify the components of the C_{27}-sterol fraction [3]. 5α-Cholesta-8-ene-3β-ol was identified by the fragmentation (at 70 eV) of its acetate derivative; *m/z* (relative intensity): 428 (100), 368(50),273(12), 213(30). This fragmentation is identical to that of an authentic sample.

RESULTS

The effect of *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**1**) on the cholesterol biosynthesis was studied on 3T3 fibroblasts which have been growing for 2 days in a delipidated medium. We have shown previously [3] that under such conditions the cells, which were in the logarithmic phase of growth, incorporated actively [^{14}C]acetate into the C_{27}-sterol fraction.

Effect of **1** *on the sterol synthesis*

Treatment of the cells with **1** resulted in an important inhibition of the sterol biosynthesis, e.g. an incubation for 4 hr with 0.1 μM of this compound provoked more than 80% reduction of [^{14}C]acetate incorporation into the C_{27}-sterols. In order to determine how rapidly the drug exerted its effect a time course experiment was performed. As shown in Fig. 2, the effect of **1** is rather fast; its maximum is reached between 30 and 60 min of preincubation, i.e. under our experimental conditions, the maximum inhibition requires about 2.5–3 hr of contact with the drug. The inhibition of sterol synthesis by **1** is concentration dependent and a half-maximal inhibition was determined using a 4 hr preincubation time (Fig. 3); under these conditions the measured IC_{50} was 0.02 μM. This remarkably low value indicates that **1** is, at a cellular level, a very potent drug. Interestingly, this IC_{50} is largely inferior to the constant obtained in the inhibition of the rat liver microsomal 2,3-oxidosqualene-lanosterol cyclase, i.e. I_{50} = 2 μM [9]. The efficiency of **1** was compared to that of other drugs known to inhibit the biosynthesis of cholesterol at the 2,3-oxidosqualene-lanosterol cyclase step (**2** and U 18666 A) and at the HMG-CoA reductase step (compactin and mevinolin); the results are indicated in Table 1. Although 4,4,10 β-trimethyl-*trans*-decal-3β-ol (**2**; Fig. 1), which was originally described

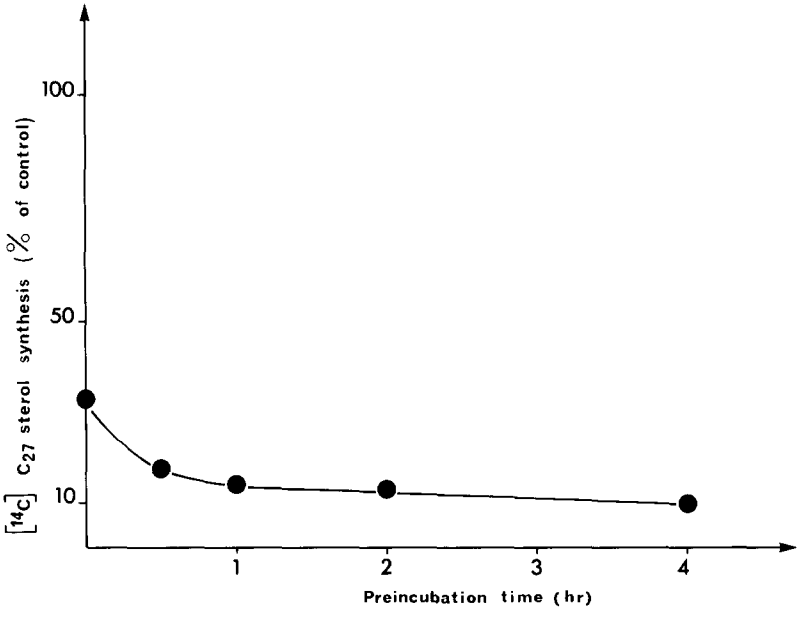

Fig. 2. Influence of drug pre-incubation time on the synthesis of the C_{27}-sterols. 3T3 fibroblasts were cultured in a delipidated growth medium for given times in the presence of 1 μM of compound **1**. After the pre-incubation, [^{14}C]acetate was added (10 μCi per dish) and, after a further 2 hr of incubation, the incorporation of label into the C_{27}-sterol fraction was determined. The results are expressed as percentages of [^{14}C]sterols measured in the absence of drug (100% corresponds to an average of 3.2×10^4 dpm/mg of cell protein).

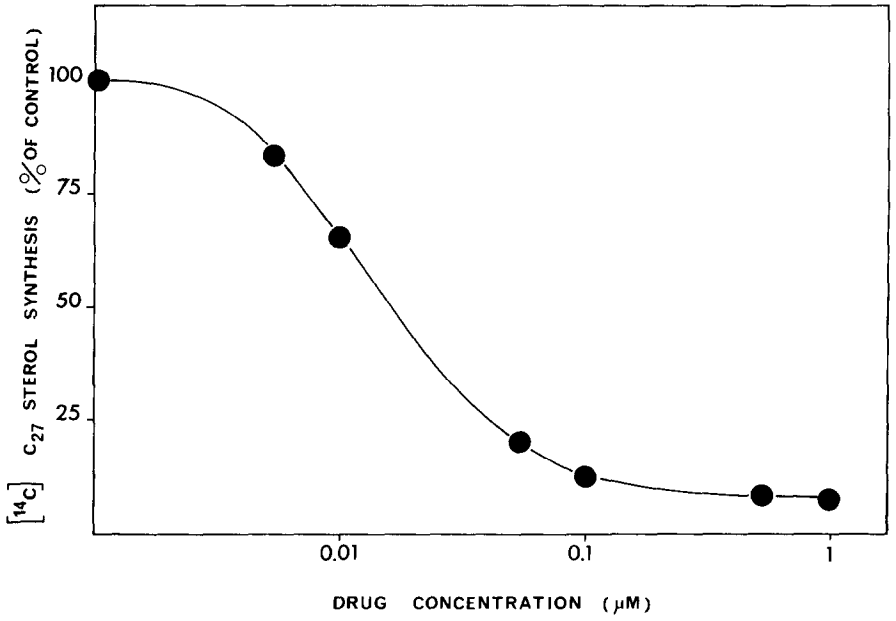

Fig. 3. Influence of the concentration of **1** on the biosynthesis of C_{27}-sterols. 3T3 cells cultured for 2 days in a delipidated growth medium were preincubated for 4 hr, in the presence of the indicated concentrations of the drug. [^{14}C]acetate was then given (10 μCi per dish) and after 2 hr the incorporation of the label in the C_{27}-sterol fraction determined. The results are expressed as percentages of [^{14}C]sterols measured in the absence of drug (100% corresponds to an average of 4.8×10^4 dpm/mg cell protein).

by Nelson *et al.* [13], is a good inhibitor of the mammalian cyclase both *in vitro* [1, 7] and in cultured cells ([1] and Table 1), the derived 8-azadecalin **1** is a far superior inhibitor of the C_{27}-sterols biosynthesis in 3T3 fibroblasts. Under similar experimental conditions, compactin and mevinolin which belong to the class of the most potent cholesterol biosynthesis inhibitors [14], were slightly less effective than **1**.

The specificity of **1** for the sterol biosynthetic pathway is suggested by the fact that the [^{14}C]label associated with non-saponifiable lipids was not much affected after the cells were treated with drug concentrations up to 0.1 μM. At 1 μM of **1** for 6 hr, which resulted in a complete blockade of C_{27}-sterols biosynthesis (Fig. 2), a 25% decrease of label incorporation was observed. The main effect of **1** was to provoke a redistribution of the label amongst the constituents of the lipid fraction (Table 2). Under similar conditions in short term preincubations **1** had no effect on [^{14}C]fatty acid biosynthesis (not shown). From these observations one can conclude that the inhibition of cholesterol biosynthesis by **1** cannot be ascribed to a general cytotoxicity of the drug. To identify the step(s) where the drug exerts its action, the nonsaponifiable [^{14}C]lipids were analyzed by thin-layer chromatography. As expected from its mode of action, incubation of the cells with **1** (1 μM) provoked an accumulation of 2,3-oxidosqualene and of 2,3:22,23-dioxidosqualene at the expense of the C_{27}-sterols (Table 2); both squalene oxides were characterized chemically as described previously [3]. Contrary to 2-aza-2,3-dihydrosqualene [3, 15], **1** did not inhibit the squalene oxidase and no formation of squalene above control was noticeable in the treated cells. Analysis of the C_{27}-sterols of the fibroblasts, which have been exposed for 4 hr to 0.05 μM of **1**, by TLC on argentinated silica plates revealed that about 96% of the fraction were monoenes the

Table 1. Comparative effects of cholesterol biosynthesis inhibitors on the incorporation of [^{14}C]acetate into the C_{27}-sterol fraction in 3T3 fibroblasts

Compound (IC_{50}, μM):					
1	**2**	2-Aza-2,3-dihydro-squalene*	U18666A	Compactin	Mevinolin
0.02	10	0.3	0.017	0.08	0.06

3T3 cells were grown in a delipidated growth medium. After 2 days, they were preincubated for 4 hr in the presence of varying concentrations of the given drugs. This treatment was followed by a 2 hr labeling period with [^{14}C]acetate (10 μCi per dish) and the IC_{50} was determined essentially as in Fig. 3. The values are means of at least two independent determinations, which did not differ by more than 20%.

* Data from Ref. 3.

Table 2. Analysis by thin layer chromatography of the effect of **1** on the distribution of nonsaponifiable [^{14}C]-lipids

	Lipids			
Supplements	Squalene	2,3-Oxido-squalene	2,3:22,23-Dioxido-squalene	C_{27}-Sterols
None	0.7	6.1	0.8	92.4
1 (1 μM)	0.8	54.2	34.7	10.3

3T3 cells were grown in delipidated medium. After 2 days they were preincubated for 4 hr in the presence or absence of **1**; this treatment was followed by a 2 hr labeling with [^{14}C]acetate (10 μCi per dish). The cells were then harvested and the nonsaponifiable lipids analyzed by thin layer chromatography as described under Materials and Methods. The values are given as percentages of the total radioactivity associated with the C_{27}-sterols and precursors fractions in the control (2.06×10^4 dpm/dish) and in the treated (1.6×10^4 dpm/dish) cells.

remainder being sterols with two double bonds. In the untreated cells this ratio was 99:1. This result indicates that contrary to 2-aza-2,3-dihydrosqualene and U 18666 A, another 2,3-oxidosqualene cyclase inhibitor [2], **1** does not inhibit the Δ^{24}-sterol reductase. The C_{27}-sterols of the treated cells, which contained one double bond, were further analyzed by TLC. About 18% of the fraction corresponded to a Δ^5-sterol, i.e. cholesterol, and the remaining 82% to Δ^7/Δ^8-C_{27}-sterols. In the control cells this ratio was inverse, i.e. 90:10; this observation indicates a possible inhibitory action of **1** either on the sterol-8-ene isomerase or the Δ^7-sterol 5-desaturase steps. It was of importance to gain a better insight on this secondary target of **1**; the Δ^7/Δ^8-C_{27}-sterol fraction available from the treated cells was too limited to be analyzed by our present techniques. Since the chemical mechanism of the sterol-8-ene isomerase also involves the formation of a transient high-energy carbocationic intermediate at C-8 [8], the most plausible alternate target of **1** would be the isomerase. Accordingly, it was reported previously by this laboratory that 8-aza-4α,10-dimethyl-*trans*-decal-3β-ol and derivatives were very potent inhibitors, i.e. high-energy intermediate analogs, of plant $\Delta^8 \rightarrow \Delta^7$-sterol isomerase; e.g. the I_{50} of **6** was 0.13 μM for the maize enzyme [8]. When tested on the rat liver microsomal sterol-8-ene isomerase, as described under Materials and Methods, **6** was also found to be a good inhibitor of the animal enzyme with an $I_{50} = 20$ μM. Similarly compound **1** had an I_{50} of 10 μM. This inhibition of the isomerase could imply that, under conditions where the fibroblasts are exposed for a short period to the 8-azadecalin **1**, the conversion of Δ^8 into Δ^7-sterols could be impaired, thus leading to the accumulation of Δ^8-sterols which should constitute the major part of the Δ^7/Δ^8-sterols which accumulate in the C_{27}-sterol fraction. This interpretation was borne out in long-term experiments (*vide infra*).

Besides these sterols, analysis of the nonsaponfiable extract revealed also the presence of polar sterols (Table 3). As shown previously [3] when analyzed by GC-MS, the polar fraction contained mainly 24,25-epoxycholesterol, but its complete analysis has not yet been performed.

In order to demonstrate a precursor–product relationship between the accumulated [^{14}C]2,3-oxidosqualene and the C_{27}-sterols, chase experiments were performed. A representative experiment, in which the cells were pretreated with 0.1 μM of **1** for 4 hr, is given in Table 3. After the chase, a decrease in labeled 2,3-oxidosqualene was observed which was correlated with a corresponding increased

Table 3. Analysis by thin layer chromatography of the distribution of the nonsaponifiable [^{14}C]-lipids after pulse and pulse-chase experiments in 3T3 fibroblasts pretreated with **1**

	Lipids			
Conditions	Polar sterols	C_{27}-sterols	2,3:22,23-Dioxido-squalene	2,3-Oxido-squalene
Pulse-label	17.1	15.1	4.2	63.6
Pulse-label and chase	19.9	53.7	2.2	24.2

Cells were grown in a delipidated medium. After 2 days they were preincubated for 4 hr with **1** (0.1 μM); after this treatment the cells were pulse-labeled for 2 hr with [^{14}C]acetate (10 μCi per dish). For the chase experiment the cells were then washed and incubated further for 4 hr in the presence of the normal growth medium supplemented with cold acetate (20 mM). The cells were then collected and the nonsaponifiable lipids analyzed by TLC. The values are given as the percentages of total activity associated with the nonsaponifiable lipid extracts. The label recovered in these fractions was 2.27×10^4 dpm/mg of cell protein (i.e. 1.4×10^4 dpm/dish) in the pulse-label experiments and 1.76×10^4 dpm/mg (i.e. 1.13×10^4 dpm/dish) in the pulse-label and chase experiments.

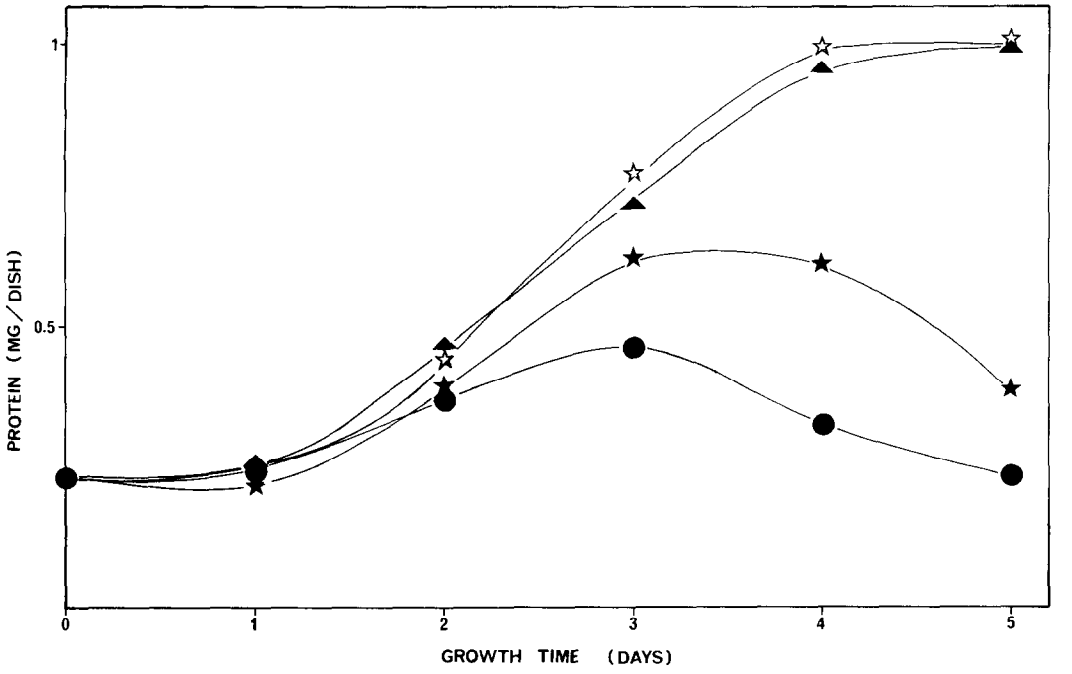

Fig. 4. Influence of **1** on growth. Effect of cholesterol and fetal calf serum. 3T3 cells were plated at a density of 2.5×10^5 cells/60 mm dish and cultured in 5 ml of delipidated growth medium in the absence (—☆—) or presence (—●—) of 0.05 μM **1**. Growth curve with 50 μg/ml cholesterol (—★—) or 10% (v/v) undelipidated fetal calf serum (—▲—) and 0.05 μM of **1** co-administered at time zero. Cells were harvested each day and cell growth determined.

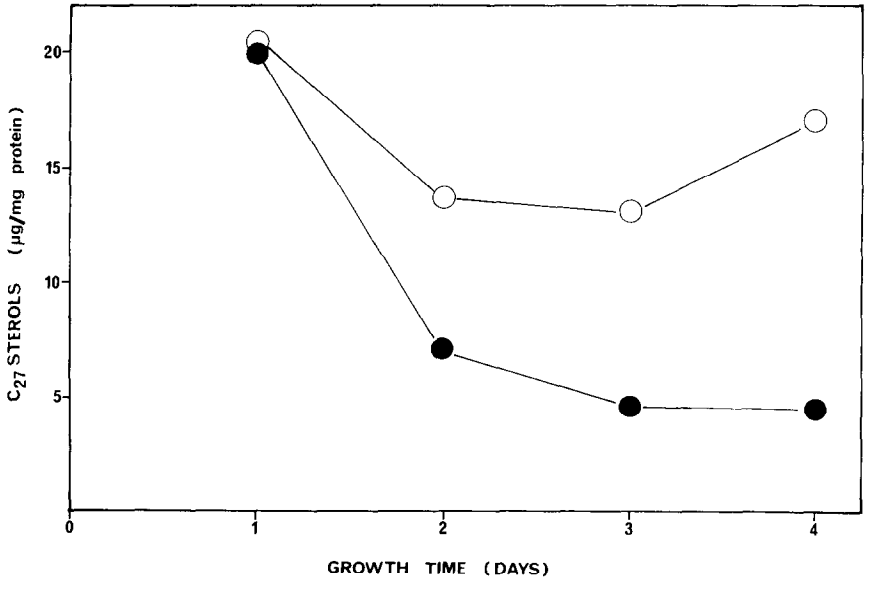

Fig. 5. Evolution of cholesterol during growth of treated and control fibroblasts. The cells were seeded at a density of 3×10^5 cells/60 mm dish and cultured in a delipidated growth medium in the absence (—○—) or the presence (—●—) of 0.05 μM of **1**. Every day dishes were carefully washed with phosphate-buffered saline and the cells harvested. The C_{27}-sterol fraction, obtained by thin layer chromatography from the nonsaponifiable lipid extract, was analyzed by gas chromatography as described in Materials and Methods. The C_{27}-sterol fraction consisted mostly in cholesterol (see text).

incorporation of the label into the C_{27}-sterol fraction. At these concentrations of drug only little 2,3:22,23-dioxidosqualene is accumulated by the cells. At higher concentrations of **1**, such as 1 μM, more 2,3:22,23-dioxidosqualene is formed (Table 2) which during the chase is transformed into polar sterols; however, under these conditions, the chase experiments are less complete probably because of an important retention of the drug by the cells (not shown).

Effect of **1** *on cell growth*

Incubation of 3T3 fibroblasts in delipidated medium with **1** at concentrations between 0.01 and 1.0 μM caused a marked inhibition of cell growth. In Fig. 4 are given the results obtained with 0.05 μM. The reversal of this effect could only be partially accomplished by complementing the medium with free cholesterol (up to 50 μg/ml), but was complete by supplementing the cells with non-delipidated fetal calf serum (Fig. 4). In order to determine the influence of the pre-incubation time of **1** with the cells on the growth inhibition, the fibroblasts were incubated between 0.5 and 24 hr with 0.5 μM of the drug, then thoroughly washed and given fresh medium containing delipidated serum. The cells were then further incubated for 48 hr and growth estimated. Under these conditions a pre-incubation of 1 hr was sufficient to provoke a decreased cell growth (residual growth: about 25% of control) which is similar to that observed with the longer preincubation times (not shown). This result is consistent with a fast action of **1** and its high retention by the cells. Under these conditions the viability of the cells remained unaffected.

Effect of **1** *on cellular sterol content*

The results obtained above indicate that compound **1** markedly affects the incorporation of [^{14}C]acetate into the C_{27}-sterols and the growth of the fibroblasts. In order to find-out if these effects are correlated, we have now studied the influence of **1** on the evolution of cellular cholesterol during growth. When treated with 0.05 μM of **1**, growth of the fibroblasts is markedly slowed down after 2 days (Fig. 4) and, compared to control cells, this corresponds to a decrease of 60% in cholesterol content (Fig. 5). Growth was less affected when the cells were treated with smaller concentrations of **1**. After 5 days at 0.01 μM, growth when estimated by cellular proteins, was about 85% of the control; however, the C_{27}-sterols content represented only 50% of the control cells. As analyzed by GC-MS the C_{27}-sterol fraction of the cells, which have been treated for several days (about 4 days) with the drug at 0.01 μM, was composed essentially of cholesterol (96%), the remainder being desmosterol (2–3%) and a compound identified as 5α-cholesta-8-ene-3β-ol (1–2%). Besides this latter sterol, this profile is identical to that found in the untreated cells. Such a result contrasts with those obtained with 2-aza-2,3-dihydrosqualene, where an important accumulation of desmosterol was observed [3].

Cellular toxicity of **1**

When given at concentrations ≥ 100 μM compound **1** provoked cell death, as judged from the trypan blue-exclusion test, within 6 hr. Under the same conditions the cells remained viable (> 95%) when incubated in the presence of the drug at

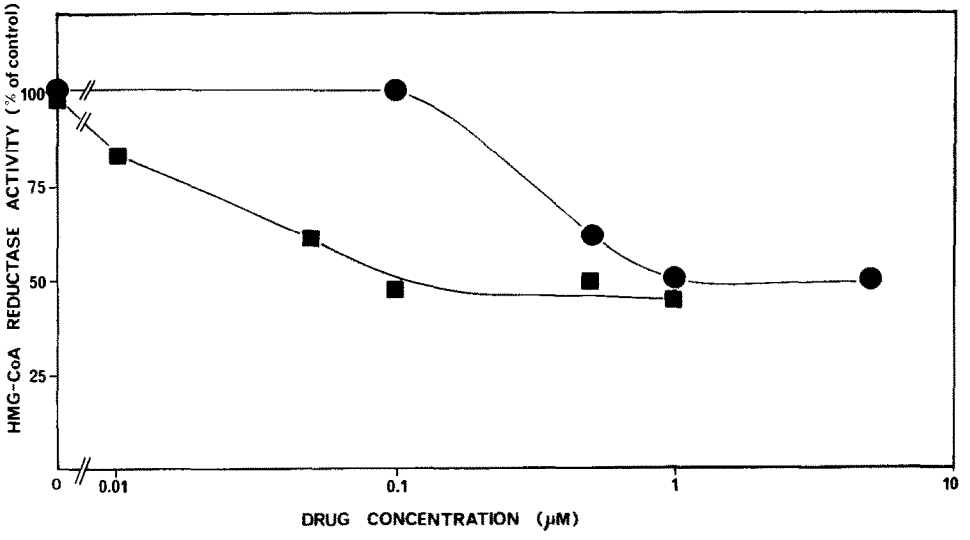

Fig. 6. Effect of varying concentrations of **1** and 2-aza-2,3-dihydrosqualene on the activity of HMG-CoA reductase in fibroblasts. The cells were seeded at 2.5×10^5 cells/60 mm dish in DMEM medium supplemented with 10% (v/v) delipidated fetal calf serum. After 48 hr of growth, the cells were treated with the indicated concentrations of the inhibitors **1** (—■—) and 2-aza-2,3-dihydrosqualene (—●—). The cells were collected after 24 hr of exposure to the drugs and the activity of the reductase was determined as described under Materials and Methods. The 100% value (control) corresponds to an average activity of 38 pmol $\times$ min^{-1} $\times$ mg^{-1} cell protein.

$\leq 10\,\mu M$. In contrast to cholesterol which had virtually no effect (up to $50\,\mu g/ml$), undelipidated serum efficiently protected the cells against this lethal effect. Previously we have shown that the cytoxicity of 2-aza-2,3-dihydrosqualene, the other inhibitor of 2,3-oxidosqualene cyclase we have studied, might be accounted for by its ability to disrupt membrane integrity [3]. Similar experiments on model systems such as liposomes, have indicated that **1** up to $150\,\mu M$, is unable to induce membrane leakiness (not shown).

Effect of **1** *on cellular HMG-CoA reductase activity*

We have investigated the effect of varying concentrations of **1** on the HMG-CoA reductase activity. The cells were grown for 2 days in a delipidated medium and, in their logarithmic phase of growth, were treated for 24 hr with the drug. A gradual inhibition of the reductase activity was observed (Fig. 6), reaching a maximum of 50% of control cells when the concentration of **1** was superior to $0.1\,\mu M$. For comparison we have also studied the effect of 2-aza-2,3-dihydrosqualene, this cyclase inhibitor [3] reduced the reductase activity to a similar extent, albeit at a higher concentration, i.e. $1\,\mu M$ (Fig. 6). Interestingly the potency of both compounds in decreasing the HMG-CoA reductase activity reflects their respective capacity to inhibit the cholesterol biosynthesis in the fibroblasts (see Table 1).

DISCUSSION

The purpose of this work was to assess the potency, at a cellular level, of *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**1**) a new inhibitor of 2,3-oxidosqualene cyclase which has been rationally designed in our laboratory [9]. *In vitro* this compound is an excellent inhibitor of the 2,3-oxidosqualene-lanosterol and -cycloartenol cyclases, but interestingly it does not inhibit the 2,3-oxidosqualene-β amyrin cyclase. This could be rationalized, according to the transition state analogs theory, by assuming that the 8-azadecalin **1** mimics a localized high-energy carbocationic intermediate occurring at C-8, at the penultimate step of the reaction process leading to lanosterol, or at C-9 in the formation of cycloartenol [9].

Compound **1** was found to exert a dramatic effect on the synthesis of cholesterol in 3T3 fibroblasts. Because of its IC_{50}, which is about 20 nM, **1** is, along with U18666A [2], one of the most powerful cholesterol biosynthesis inhibitors acting at the 2,3-oxidosqualene-lanosterol cyclase step tested so far on mammalian cells. Compared to mevinolin and compactin, the highly potent HMG-CoA reductase inhibitors [14], **1** seems even slightly more active in the Swiss 3T3 fibroblasts cell line (Table 1). This result validates our rational approach, i.e. use of transition state and high-energy intermediate analogs, in the design of new molecules capable of interfering with the sterol biosynthesis [16–18]. Treatment of the cells with **1** resulted in an accumulation of squalene oxides at the expense of the C_{27}-sterols, indicating that the major intracellular target of this compound was indeed the cyclase. At higher concentrations of **1**, large amounts of 2,3:22,23-dioxidosqualene were accumulated (Table 2) which could be converted by the cells into polar sterols, among which 24,25-epoxycholesterol is the best characterized [19]. This is now a classical observation with potent 2,3-oxidosqualene cyclase inhibitors (for a review see Ref. 4). The possibility exists that the cyclase inhibitors might act, under certain conditions, synergistically in inhibiting the biosynthesis of cholesterol, i.e. by decreasing the amount of lanosterol formed and, at up-stream, by repressing the HMG-CoA reductase through the formation of oxysterols such as 24,25-epoxycholesterol [20]. In agreement with this hypothesis we have found that treatment of the cells with increasing concentrations of **1** resulted in a progressive reduction of the expression of HMG-CoA reductase activity (Fig. 6). The concentrations of **1** which were effective in inducing such an effect were well correlated with those favoring the formation of polar sterols (Table 3) and a decreased incorporation of [^{14}C]acetate into the nonsaponifiable fractions. Other authors [20, 21] have shown a paradoxical effect of the inhibition of the 2,3-oxidosqualene-lanosterol cyclase on the activity of HMG-CoA reductase, i.e. at high concentrations of the drug (U16888A) the reductase activity increases again in a concentration-dependent manner. This was ascribed to a complete blockage of the cyclase, thus preventing the transformation of the accumulated 2,3:22,23-dioxidosqualene into the repressor 24,25-epoxycholesterol. We did not observe such an effect at the highest concentration of **1** used, i.e. $1\,\mu M$. It is possible, however, that this concentration was too low, e.g. in Hep G2 cells the reductase activity increases at U18666A concentrations higher than $3\,\mu M$ [21], or that our cell line presents a different sensitivity to the regulatory effect of the oxysterols. Work is in progress trying to correlate the reductase levels with that of cellular concentrations of 2,3:22,23-dioxidosqualene and 24,25-epoxycholesterol.

Other ammonium-containing molecules known to be effective 2,3-oxidosqualene cyclase inhibitors such as U16888A [2] or 2-aza-2,3-dihydrosqualene [3] have also been found to be good inhibitors of the Δ^{24}-sterol reductase, leading ultimately to the accumulation of desmosterol. This was not found to be the case with the 8-azadecalin **1**, either in short-term labeling experiments or when the cells have been exposed to the drug for several days. Analysis of the C_{27}-sterol fraction obtained from the fibroblasts, which have been cultivated during a short period (about 4 hr) in the presence of **1**, indicated the presence of a large proportion of [^{14}C] Δ^8/Δ^7-sterols; e.g. over 78% with $0.01\,\mu M$ **1**. From our data on the inhibition of the mammalian sterol-8-ene isomerase by 8-azadecalins such as **1** and **6** (this work), we have inferred that this fraction might essentially be composed of 5α-cholesta-8-ene-3β-ol which accumulates because of the blockade of the cellular isomerase by **1**. Long-term experiments allowed us to identify in treated cells, under conditions in which cholesterol biosynthesis was inhibited by 50%, the formation, albeit in very minor quantities, of 5α-cholesta-8-ene-3β-ol. This sterol was absent in the control cells and signs the inhibition of the isomerase in fibroblasts by the compound **1**.

The fact that 5α-cholesta-8-ene-3β-ol accumulates in such small quantities in long-term experiments (less than 2% of the C_{27}-sterol fraction), indicates however that the prime cellular target of **1** is the 2,3-oxidosqualene-lanosterol cyclase and that the sterol-8-ene isomerase, although down-stream in the biosynthetic pathway, is only transiently inhibited by this compound. This result reflects well the fact that when considering the I_{50} values, determined *in vitro* on murine liver microsomal enzymes, compound **1** is a better inhibitor of the cyclase as compared to the isomerase (i.e. one order of magnitude) [9 and this work]. Inhibition of the sterol-8-ene isomerase has been documented with several other ammonium-containing molecules, e.g. morpholines such as triparanol, tridemorph [22–24] and AY-9944 [22]. At this point it might be interesting to note that the molecule **1** is a far better inhibitor of the isomerase in higher-plants systems than of the mammalian enzyme, i.e. $I_{50} = 0.2\ \mu M$ for the maize-seedling enzyme [18] instead of 10 μM. This might reflect the fact that the structure of **1**, because of the presence of a 4α-methyl substituent, is better related to 4α-methyl fecosterol, the preferred substrate of the plant isomerase. The animal enzyme catalyzes the transformation of substrates such as 5α-cholesta-8-ene-3β-ol and zymosterol, which lack this substituent. Moreover, it was shown recently by the group of Gaylor that the presence of such a methyl at position-4 of potential substrates totally forbids the isomerization [22]. In summary, compound **1** appears to be a potent inhibitor of cholesterol biosynthesis in 3T3 fibroblasts, with a high selectivity for the 2,3-oxidosqualene-lanosterol step.

Although the molecule **1** affected rapidly the biosynthesis of the sterols, its action could not be easily reversed, e.g. in the chase-experiments or by dilution of the incubation medium. This might be due to the hydrophobicity of the drug which is therefore expected to accumulate in the cells and not to diffuse out readily. At this point we also ignore the fate of the drug at a cellular level and its possible metabolization. Compound **1** had a profound effect on the growth of the fibroblasts which was correlated with a deficiency of cellular cholesterol, e.g. cell growth arrest was observed when the cholesterol level was less than 50% of control. This observation was expected since a minimal cholesterol accumulation is needed in order for mammalian cells to proliferate [25]. This growth inhibition could, however, not be fully reversed by addition of free cholesterol to the growth medium. Complete serum, i.e. undelipidated serum containing cholesterol associated to lipoprotein particles, on the contrary was able to protect the cells against the inhibitory action of **1**. In an earlier work on the mode of action of 2-aza-2,3-dihydrosqualene we made a similar observation, i.e. fibroblasts whose growth was arrested by the inhibitory action of the drug on cholesterol biosynthesis, resumed dividing only when given complete serum [3]. As speculated before it is possible that the cells when starving for cholesterol can efficiently channel LDL-associated cholesterol into cellular compartments relevant to cell growth. On the other hand, in the present case we cannot exclude that the reversal of the effect of **1** on growth by the complete serum might be due to an adsorption of the drug to the serum proteins. A cytotoxic effect of **1** unrelated to the biosynthesis of cholesterol, e.g. lysosomotropic effect [26], could also be envisaged; such an effect becomes indeed prevalent at higher concentrations of the drug (superior to $500 \times IC_{50}$).

In conclusion, *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**1**), proved to be a remarkably potent new inhibitor of the biosynthesis of cholesterol in cultured mammalian cells. This compound showed, in long term experiments, an excellent specificity for the 2,3-oxidosqualene-lanosterol cyclase. We believe that **1** constitutes an interesting leader molecule for the synthesis of new compounds presenting different bio-availabilities; it should also be a valuable tool to validate, *in vivo*, the pharmacological significance of the 2,3-oxidosqualene-lanosterol cyclase for the design of hypocholesterolemic drugs.

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