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A New Class of Selective and Potent 7-Dehydrocholesterol Reductase Inhibitors

Aline Horling,[†] Christoph Müller,[‡] Richard Barthel,[†] Franz Bracher,[‡] and Peter Imming*[,†]

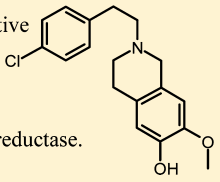
[†]Institut für Pharmazie, Martin-Luther-Universität Halle−Wittenberg, Wolfgang-Langenbeck-Strasse 4, 06120 Halle, Germany

[‡]Department für Pharmazie, Zentrum für Pharmaforschung, Ludwig-Maximilians-Universität München, Butenandtstrasse 5-13, D-81377, Germany

Supporting Information

ABSTRACT: We prepared a number of *N*-phenethyltetrahydroisoquinolines structurally related to protoberberines. They were tested for activity against bacteria, fungi, and human leukemia HL-60 cells and also for inhibition of biosynthesis: ergosterol in yeasts and cholesterol in human cells. In the latter assay panel, several of the compounds were distinguished by a strong and selective inhibition of 7-dehydrocholesterol reductase (7-DHCR, EC 1.3.1.21), an enzyme responsible for the conversion of 7-dehydrocholesterol to cholesterol in the last step of cholesterol biosynthesis. In a whole-cell assay, the most active compound **5f** showed a much stronger inhibition of overall cholesterol biosynthesis (IC_{50} 2.3 nM) than BM 15.766 (IC_{50} 500 nM), presently the most selective known inhibitor of 7-DHCR. Since a defect of 7-dehydrocholesterol reductase is associated with Smith−Lemli−Opitz syndrome (SLOS), the potent and selective inhibitors reported here will enable more detailed investigation of the pathogenesis of SLOS.

A new phenethyltetrahydroisoquinoline, distinguished by selective and potent inhibition of human 7-dehydrocholesterol reductase.



INTRODUCTION

Cholesterol has different functions in human physiology. It is an important structural component of the mammalian cell membrane and also a precursor molecule for glucocorticoids, mineralocorticoids, neurosteroids, and steroid sex hormones. A known autosomal recessive trait, the Smith−Lemli−Opitz syndrome (SLOS), is associated with the pivotal role of cholesterol, especially during embryonic development and morphogenesis.[1] The worldwide incidence of SLOS is 1 in 10 000−80 000 births, depending on ethnic group. It is accompanied by severe abnormalities, observed in most of the patients, such as microcephaly, short nose with anteverted nares, retrognathia, cutaneous syndactyly of second to third toe, feeding difficulties in the gastrointestinal tract, mental retardation, and brain growth retardation.[1] The syndrome was first described by Smith, Lemli, and Opitz in 1964.[2] As recently as 1994, a defect of 7-dehydrocholesterol reductase (7-DHCR, EC 1.3.1.21), the enzyme that catalyzes the reduction of 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) to cholesterol (cholesta-5-en-3β-ol) in the last step of cholesterol biosynthesis (Scheme 1), was identified as the biochemical cause of this clinically complex syndrome.[3−5] The route of cholesterol biosynthesis from lanosterol via 7-dehydrocholesterol is called the Kandutsch−Russell pathway.[6,7] A defect of 7-DHCR leads to a deficit in cholesterol and an accumulation of 7-dehydrocholesterol, which together with its oxidized derivatives is embryotoxic, also having a negative influence on total cholesterol transfer.[5,8,9] An early diagnosis of SLOS with external substitution of cholesterol will alleviate the symptoms of SLOS.[10,11]

For a better understanding of SLOS, the molecular mechanisms, including details of the effects of 7-dehydrocholesterol and its oxidation products, have to be further investigated.[12] This needs selective 7-DHCR inhibitors that inhibit cholesterol biosynthesis at the stage of 7-dehydrocholesterol, simulating aspects of SLOS in vitro. Several 7-DHCR inhibitors (AY 9944, BM 15.766, and YM 9429; Scheme 2) have been published, but all of them have severe disadvantages. AY 9944 inhibits several enzymes of cholesterol biosynthesis including upstream enzymes, most notably Δ8,7-isomerase.[8,13,14] This inhibition of two enzymes leads to an accumulation of other sterols, mainly zymostenol (cholesta-8-en-3β-ol), blurring the similarity with the situation in SLOS. The inhibitor BM 15.766 is a known selective inhibitor of 7-dehydrocholesterol reductase but not a potent one.[15] In rat hepatocytes, cholesterol synthesis was reported to have been almost totally inhibited by BM 15.766 at a concentration of as much as 10 μM.[15] BM 15.766 has lower toxicity than AY 9944, the latter displaying higher lethality toward rats.[16] YM 9429 was investigated only in a few studies.[17] Just as with AY 9944, inhibition of related enzymes was not completely excluded, and inhibition of 7-DHCR was even weaker than with BM 15.766.[17] Furthermore, YM 9429 is considered to be potentially teratogenic.[17] Consequently, no suitable 7-DHCR inhibitor for investigation of the SLOS pathobiochemistry mechanism is available at present.

In various pharmacological studies, protoberberines have shown antibacterial, antifungal, antileishmanial, and antispasmodic effects.[18−21] Protoberberines are characterized by many reported activities and many possibilities of binding to targets.[22] Berberine was recently found to inhibit the hepatic HMG-CoA

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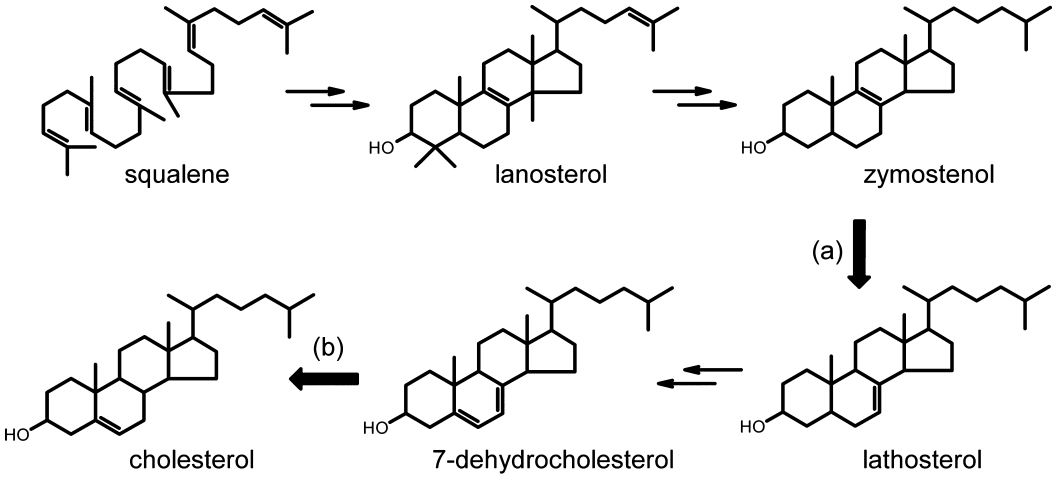
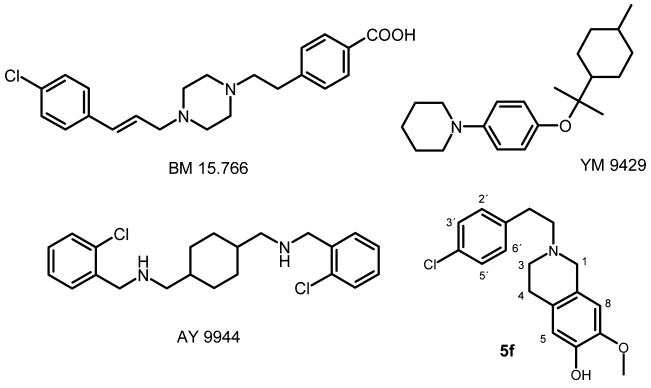
Scheme 1. Post-Squalene Pathway of Cholesterol Biosynthesis[a6]



[a](a) Catalyzed by Δ8,7-isomerase. (b) Catalyzed by 7-dehydrocholesterol reductase (7-DHCR).

Scheme 2. Structures of Known Inhibitors of 7-Dehydrocholesterol Reductase and the Herein-Reported Potent and Selective Inhibitor 5f

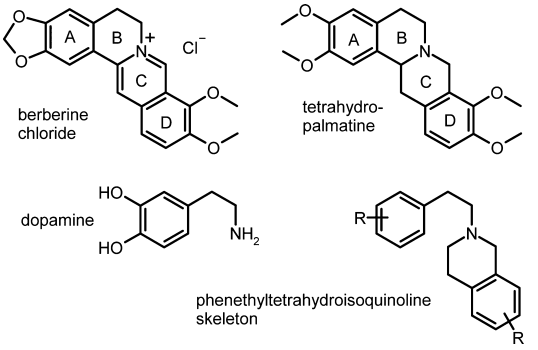


reductase in hyperhomocysteinemic rats and to reduce the cholesterol level in liver.[23] HMG-CoA reductase catalyzes the rate-limiting step in cholesterol biosynthesis. This prompted us to take a closer look at the effect of some protoberberine alkaloids on cholesterol biosynthesis in mammalian cells and on ergosterol biosynthesis in fungal cells. Since the molecular targets for inhibition (and thus the structural requirements for inhibitors) were unknown, we decided to go for a broader approach, including not only berberine but also the related alkaloid tetrahydropalmatine (nonquarternary) and phenethyltetrahydroisoquinolines (formal seco analogues having higher conformational flexibility). The test compounds were selected without taking the constitution of known 7-DHCR inhibitors into account, as we did not know at the outset where the effect on cholesterol catabolism and metabolism might reside. We rather relied on the effectiveness of the assay method in identifying a molecular target through a whole-cell approach. In order to keep undesired effects in view, the compounds were also screened for antibacterial and antifungal activities and general cytotoxicity for human cells. The investigations led to the discovery of the synthetic phenethyltetrahydroisoquinoline **5f** as a highly potent and selective inhibitor of 7-DHCR in cholesterol biosynthesis, displaying no effect on ergosterol biosynthesis in fungi and very low cytotoxicity.

RESULTS

Chemistry. In the course of studies with protoberberines and related alkaloid analogues,[24] we prepared a series of phenethyltetrahydroisoquinolines. Since the phenethylamine moiety seems to be essential for the various pharmacological effects of protoberberines, we decided to include this motif in all synthetic analogues. Compared to the parent alkaloids berberine and palmatine, ring B has formally been opened, whereas ring C has been reduced to the tetrahydro form in the synthetic analogues in order to avoid getting quaternary ammonium compounds (Scheme 3). Drugs containing

Scheme 3. Target Phenethyltetrahydroisoquinoline Skeleton in Comparison with Parent Alkaloids and Dopamine



quaternary ammonium groups are characterized by high hydrophilicity, causing low permeability through biological membranes and restricted in vivo distribution. Several investigations document the low absolute bioavailability of berberine.[25,26] Its log *P* value was determined to be −1.5.[27] In contrast, the nonquaternary tetrahydropalmatine was reported to cross biological membranes rapidly. Its maximum concentration in plasma after oral administration was reached within 30 min.[28,29] The phenethyltetrahydroisoquinoline skeleton retains the dopamine moiety included in (proto)berberine alkaloids.

The phenethyltetrahydroisoquinoline **5a** was synthesized in four steps (Scheme 4). The synthetic strategy builds on protocols previously described;[30−32] however, the literature contains many discrepancies and contradictions in experimental

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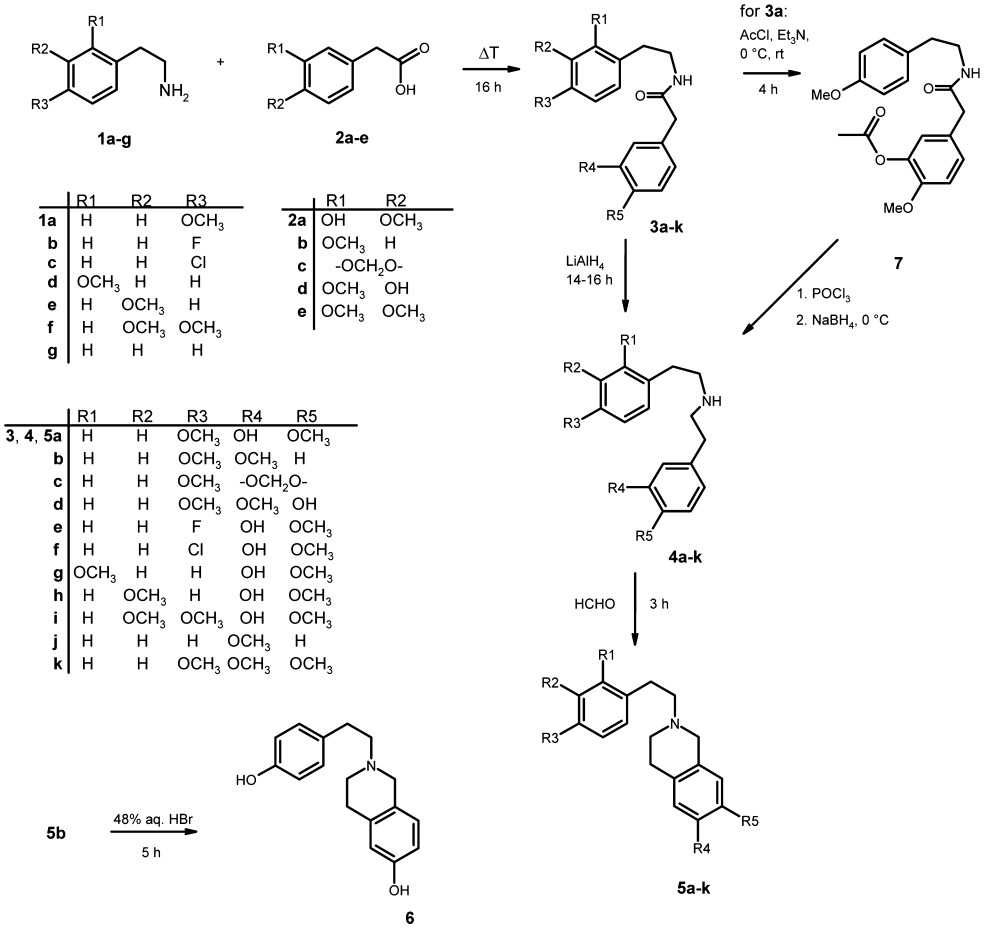
Scheme 4. Syntheses of Phenethyltetrahydroisoquinolines



	R1	R2	R3
1a	H	H	OCH_3
b	H	H	F
c	H	H	Cl
d	OCH_3	H	H
e	H	OCH_3	H
f	H	OCH_3	OCH_3
g	H	H	H

	R1	R2
2a	OH	OCH_3
b	OCH_3	H
c	$-OCH_2O-$	
d	OCH_3	OH
e	OCH_3	OCH_3

	R1	R2	R3	R4	R5
3, 4, 5a	H	H	OCH_3	OH	OCH_3
b	H	H	OCH_3	OCH_3	H
c	H	H	OCH_3	$-OCH_2O-$	
d	H	H	OCH_3	OCH_3	OH
e	H	H	F	OH	OCH_3
f	H	H	Cl	OH	OCH_3
g	OCH_3	H	H	OH	OCH_3
h	H	OCH_3	H	OH	OCH_3
i	H	OCH_3	OCH_3	OH	OCH_3
j	H	H	H	OCH_3	H
k	H	H	OCH_3	OCH_3	OCH_3

detail. We have investigated each step extensively in order to find the best strategy for different substitution patterns. In the Experimental Section and Supporting Information, detailed procedures are provided for the derivatives reported here. The first step comprises a condensation reaction of the phenylethylamine **1a** and the phenylacetic acid **2a** without activation of the carboxylic acid to give the amide **3a** in excellent yield. Normally, amines and carboxylic acids would form only a salt, but in refluxing xylenes with azeotropic removal of the reaction water, we were able to get the amides. This straightforward procedure also relies on the thermostability of the educts and products.

Due to its incompatibility with phosphorus oxychloride used in a later step of the sequence, the phenolic moiety of **3a** had to be protected by acetylation. The protected amide **7** was treated with phosphorus oxychloride to give an imidoyl chloride, which in turn was smoothly converted to the amine **4a** by a mild reduction protocol.[33] In the course of this reduction, the acetyl protecting group was removed quantitatively. Mannich reaction of **4a** with formalin in methanol finally yielded **5a**. For the ring to be closed in the desired way, the solvent, concentration of the starting material, and pH (1−2) had to be diligently chosen. For the Mannich-type cyclization, forming ring C of phenethyltetrahydroisoquinolines, an activated phenyl ring is absolutely necessary. Derivatives that bear no +M or +I substituents at the phenyl ring annulated to the future piperidine ring cannot be closed by the Mannich reaction. We have investigated this reaction under a variety of pH conditions. The formation of phenethyltetrahydroisoquinolines that were substituted only with a methylenedioxy group or methoxy groups was achieved by Mannich reaction in methanol independent of pH value. C-6-hydroxylated compounds, in contrast, needed to be synthesized at pH 1−2. At higher pH, two products were furnished by C-6 and C-8 hydroxylation.

In order to reduce the steps of the synthesis of further phenethyltetrahydroisoquinolines and avoid the possible formation of phosphoric acid esters, reduction of the amide with phosphorus oxychloride followed by sodium borohydride was replaced by a reduction with lithium aluminum hydride, resulting in easier workup and better yields. With this method, only three steps were needed to prepare the target compounds **5b−k** since protection of the phenolic groups was no longer required (Scheme 4). Again, the Mannich-type cyclizations occurred regioselectively if reaction conditions were adjusted properly (see Experimental Section). Demethylation of dimethoxy compound **5b** was achieved in refluxing 48% aqueous hydrobromic acid, yielding the diphenol **6**.

Pharmacology. *Cytotoxic Activity.* For the detection of undesired cytotoxic activities, the substances were subjected to a preliminary screening for unspecific cytotoxicity in an MTT assay on HL-60 cells (human promyelocytic leukemia cells).[34] The reference inhibitor cisplatin showed an IC_{50} value of 5 μM in this assay, whereas none of the compounds under investigation here showed significant cytotoxicity (IC_{50} values >50 μM; data not shown).

Antibacterial and Antifungal Activity. The compounds were subjected to a standard disk diffusion antimicrobial sensivity test (agar diffusion assay) with different strains of

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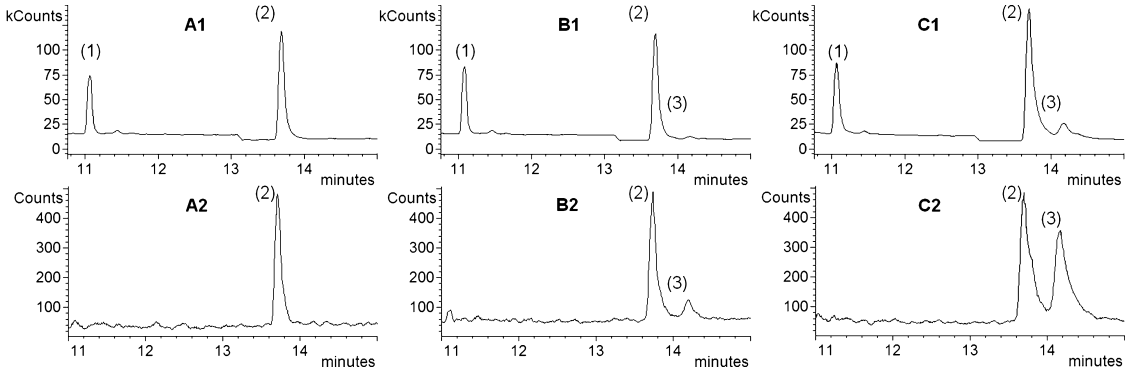
Scheme 5. Chromatograms of Sterol Fractions Isolated after Treatment with or without Inhibitor[a]



[a](A1−C1) Full scan mode (*m/z* 100−650); (A2−C2) selected ion mode for identification of 7-dehydrocholesterol (*m/z* 351, 325, 366). (A1) Blank sample, full scan; (A2) blank sample, selected ion scan; (B1) BM 15.766, 1 μM, full scan; (B2) BM 15.766, 1 μM, selected ion scan; (C1) **5f**, 1 μM, full scan; (C2)**5f**, 1 μM, selected ion scan. (Peak 1) Cholestane (internal standard); (peak 2) cholesterol; (peak 3) 7-dehydrocholesterol.

Gram-positive or Gram-negative bacteria, yeasts, and fungi. The activities, expressed as diameters of zone of inhibition, were compared to those found for reference inhibitors, viz. the antibacterial tetracycline and the antimycotic clotrimazole.[35] None of the compounds showed significant antibacterial activity, and in the screening for antifungal activity, only compounds **5a**, **5e**, and **5i** showed some moderate effects against *Hyphopichia burtonii* (data not shown).

Screening for Ergosterol Biosynthesis Inhibition in Yeasts. In the first step, MIC (minimal inhibitory concentration) values against the yeasts *Candida glabrata*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* were determined according to DIN 58940-84.[36] Clotrimazole as a positive control showed an MIC value of <1 μg/mL; all other compounds gave values >20 μg/mL. These values, in combination with the data from the agar diffusion assay, showed that the antifungal potency of the substances investigated here was orders of magnitude lower than the 7-DHCR inhibition of some of them (v.i.).

However, inhibition of single enzymes of ergosterol biosynthesis need not necessarily result in cell death. Consequently, the effect of our compounds on ergosterol biosynthesis was studied in order to detect the inhibition of one or several biosynthetic steps. The compounds were subjected to a whole-cell assay with a liquid−liquid microextraction workup after cell lysis for the isolation of sterols, and subsequent gas chromatography−mass spectrometry (GC-MS) analysis of the sterol pattern. Qualitative analysis of the effects of all compounds, including the known inhibitors AY 9944 and BM 15.766, on three different yeasts (*C. glabrata*, *S. cerevisiae*, and *Y. lipolytica*) was performed at two different inhibitor concentrations (20 and 10 μg/mL). The inhibition of any enzyme of the post-squalene part of ergosterol biosynthesis would lead to a characteristic change in the sterol composition.[37] With all compounds tested, GC-MS analysis showed no significant change in the sterol pattern, and no abnormal sterols were detected compared to blank samples.

Screening for Inhibition of Cholesterol Biosynthesis. The effect on cholesterol biosynthesis was determined in an in vitro assay developed by us, based on incubation with HL-60 cells, followed by lysis of the cells, liquid−liquid microextraction, and GC-MS analysis of the resulting sterol pattern.[13,38] By simply identifying accumulating sterols and comparing the sterol patterns with those resulting from incubation with known enzyme inhibitors, this assay indicates which enzymes in the post-squalene part of cholesterol biosynthesis are inhibited (qualitative results). The inhibitory potency of a discrete inhibitor (quantitative results) has to be investigated in an additional test.

Qualitative Results. The sterol patterns resulting from incubation with our compounds were compared to blank samples obtained by incubation of HL-60 cells without inhibitor. Compounds **5a** and **5e** caused an accumulation of 7-dehydrocholesterol (7-DHC; see Scheme 1) at low concentration levels, and at higher levels an accumulation of both 7-DHC and zymostenol. Consequently, these compounds inhibit both Δ8,7-isomerase (marker sterol zymostenol) and 7-dehydrocholesterol reductase (marker sterol 7-dehydrocholesterol).[4,38] In contrast, compounds **5b**, **5c**, **5j**, and **5k** caused an accumulation of zymostenol only (see Supporting Information), indicative of a selective inhibition of the enzyme Δ8,7-isomerase. Compound **5d** had no effect on cholesterol biosynthesis.

Most importantly, compounds **5f**, **5g**, **5h**, **5i**, and **6** displayed a sterol pattern characteristic of selective 7-dehydrocholesterol reductase inhibition. Compounds **5g** and **6** caused an accumulation of 7-dehydrocholesterol at higher concentration levels only, whereas **5f**, **5h**, and **5i** effected this at very low levels.

The results obtained with known inhibitors of 7-DHCR as reference compounds agreed with the literature, leading to the expected accumulation effects and thus validating the data found for the new compounds. AY 9944 inhibited both Δ8,7-isomerase and 7-dehydrocholesterol reductase.[7,8,13,14] Giera et al.[38] observed that AY 9944 inhibits 7-dehydrocholesterol reductase at 0.1 μM and two upstream enzymes, Δ14-reductase and Δ8,7-isomerase, at 10 μM. Similar results were published by Fernández et al.[9] BM 15.766 was reported to be more selective for 7-DHCR[9,15] (see Scheme 5). The phenethylamines **5f**, **5h**, and **5i** showed the same effects on HL-60 cells that BM 15.766 had (accumulation of 7-dehydrocholesterol) but led to significantly stronger accumulation of the substrate sterol at the inhibitor concentrations investigated here.

Quantitative Results. The next step was quantitative determination of the inhibitory potency of the most promising substances. They were selected by visual inspection of the peaks of accumulating 7-dehydrocholesterol in the chromatograms obtained in the qualitative assay described above. In order to avoid having to use isolated enzymes of each step of cholesterol

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biosynthesis, we recently established a whole-cell assay for determination of the effect of inhibitors on total cholesterol biosynthesis.[38] The IC_{50} values were determined for phenethyltetrahydroisoquinolines **5f**, **5h**, and **5i** and for BM 15.766. In order to distinguish newly synthesized cholesterol from the large amounts of matrix cholesterol already present in the cells, incubation of the cells was performed in the presence of 2-^{13}C-acetate in this assay. Due to the incorporation of several labeled acetate units into cholesterol, newly synthesized sterol can be determined selectively by GC-MS.[38] The IC_{50} values determined in this way correspond to percent inhibition of cholesterol biosynthesis in the whole-cell assay. The results are shown in Table 1. Compound **5f** displayed exceptionally high inhibitory activity.

Table 1. IC_{50} Values for Inhibition of Total Cholesterol Biosynthesis through Determination of ^{13}C Incorporation into Newly Synthesized Cholesterol

compd	relative molecular mass	IC_{50} value (nM)	confidence interval[a] (nM)	R^2 [b]
BM 15.766 sulfate	483.0	500	430−570	0.975
5f	317.8	2.3	2.0−2.6	0.979
5h	313.4	120	95−150	0.908
5i	343.4	7300	6,100−8,700	0.958

[a]Confidence interval for the IC_{50} value was 95%. [b]R^2 = goodness of fit of the dose−response curves.

DISCUSSION

Since none of the new phenethyltetrahydroisoquinolines showed considerable cytotoxicity toward HL-60 cells in a MTT colorimetric assay (IC_{50} values >50 μM), the compounds were considered to be nontoxic against human cells. In the agar diffusion assay, all compounds of interest were found to be not toxic against bacteria. Only compounds **5a**, **5e**, and **5i** showed poor inhibitory activity against one of the yeasts tested (*H. burtonii*). Despite the poor antifungal activity found in the agar diffusion assay, we investigated the effect of the phenethyltetrahydroisoquinolines on ergosterol biosynthesis in three yeast strains. None of the compounds gave rise to detectable changes in the sterol pattern of the yeasts, indicating that the enzymes of the post-squalene part of ergosterol biosynthesis are not targets of the compounds. Particularly with regard to the results obtained in the screening for inhibition of cholesterol biosynthesis in human cells, this is not surprising because no enzyme similar to the human 7-dehydrocholesterol reductase (v.i.) exists in yeasts and fungi.[39,40] Inhibition of other fungal enzymes in ergosterol biosynthesis had nevertheless been conceivable but was excluded with this assay.

In a whole-cell screening assay for inhibition of cholesterol biosynthesis in human cells, two established inhibitors of cholesterol biosynthesis from different chemical classes, AY 9944 and BM 15.766 (Scheme 2), were used as reference substances. Biochemically, AY 9944 was known to inhibit both Δ14-reductase and Δ8,7-isomerase at higher concentrations and 7-dehydrocholesterol reductase at lower concentrations.[9,41] BM 15.766 inhibits 7-dehydrocholesterol reductase selectively but with low potency. By reproducing these results with our screening system, we confirmed the validity of data obtained in the first step, namely, identification of the target enzymes. Phenethyltetrahydroisoquinolines **5a** and **5e** were found to inhibit both Δ8,7-isomerase and 7-dehydrocholesterol reductase at higher concentration levels, while they exhibited selective inhibition of 7-DHCR at lower concentrations. Compounds **5b**, **5c**, **5j**, and **5k** selectively inhibited the enzyme Δ8,7-isomerase. Compound **5d** showed no effect on cholesterol biosynthesis. Five compounds (**5f−i** and **6**), were found to inhibit 7-DHCR only, with **5f**, **5h**, and **5i** showing the strongest inhibitory activity (Table 1). The IC_{50} values presented in Table 1 refer to inhibition of overall cholesterol biosynthesis in a whole-cell assay, but since 7-DHCR is the very last enzyme in this multistep biosynthesis, in all likelihood they reflect the selective inhibition of this enzyme. This is corroborated by two observations: (1) even at the much higher inhibitor concentration of 50 μM, no other steroids were found to accumulate; and (2) nonselective inhibitors (**5a**, **5e**) were detected by our method. Furthermore, 7-DHCR is the very last enzyme in cholesterol biosynthesis, and for this reason downstream effects concealing the inhibition of additional enzymes in this biosynthesis can be excluded.

In view of the low number of compounds tested, structure−activity relationships (SAR) have a very tentative nature at this stage. However, all inhibitors of 7-DHCR (**5f−i**, **6**), including the nonselective inhibitors **5a** and **5e**, have a hydroxy group at C-6 of the tetrahydroisoquinoline moiety in common. Additionally, all these compounds except **6** bear a methoxy group in the 7-position.

None of the selective inhibitors of Δ8,7-isomerase (**5b**, **5c**, **5j**, and **5k**) showed this substitution pattern. Derivatives that lacked a hydroxy group at C-6 and methoxy group at C-7 did not inhibit 7-DHCR. Hydroxylation at C-7 and introduction of a methoxy group in position 6 of the phenethyltetrahydroisoquinoline scaffold (**5d**) resulted in a loss of effects on cholesterol biosynthesis. So for the phenethyltetrahydroisoquinoline scaffold, the 6-hydroxy group appears to be a requirement for inhibition of 7-DHCR. The known inhibitors BM 15.766 and AY 9944 resemble **5f** in that they all bear a chlorine on one of the phenyl rings, but since **5h**, which does not bear an electron-attracting substituent on either phenyl ring, also has higher activity than BM 15.766, the importance of the substitution pattern of the phenyl ring annulated to the piperidine ring appears to be much higher for (selective) 7-DHCR inhibition than the pattern on the other aromatic moiety.

Further studies will provide more inhibitory data for an SAR analysis of the scaffold. This will address two important points, closely connected to each other, that were out of the scope of the present study: (1) determine the inhibition of isolated enzymes of the 7-DHC pathway and (2) on the basis of this with clogP and permeability data, detect the influence lipophilicity and cell membrane permeability of the compounds have on the effect seen in the present study. With some compounds showing selective inhibition of one or two enzymes of the human cholesterol biosynthesis, the phenethyltetrahydroisoquinoline scaffold holds promise for development of selective inhibitors even for other enzymes of this pathway.

CONCLUSION

We have identified a new class of very active and selective inhibitors of 7-dehydrocholesterol reductase, the last enzyme in cholesterol biosynthesis. The compound **5f** showed 200 times stronger inhibition of cholesterol biosynthesis in a whole-cell assay than the established (commercially available) inhibitor BM 15.766 did under the same conditions. It was devoid of

inhibitory activity in fungal ergosterol biosynthesis and showed negligible cytotoxicity. The new chemotype of selective inhibitors of 7-DHCR, phenethyltetrahydroisoquinolines, is characterized by facile synthetic access and stability. Through blocking 7-dehydrocholesterol reductase selectively, **5f** will be a useful tool for studying molecular details of the pathogenesis of Smith−Lemli−Opitz syndrome, a disease with debilitating effects on patients for which presently there is no cure.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on an electrically heated Boëtius microscope hot stage and are uncorrected. NMR spectra were recorded on a Varian Gemini 2000 spectrometer, with 400 MHz operating frequency for ^{1}H NMR and 100 MHz operating frequency for ^{13}C NMR, or on a Varian Unity Inova 500, with 500 MHz operating frequency for ^{1}H NMR and 125 MHz operating frequency for ^{13}C NMR. The spectra were recorded in trichloromethane-*d* or methanol-d_4 as solvent. The chemical shifts are reported in parts per million (ppm, δ) and rounded to two decimal places for ^{1}H NMR and one decimal place for ^{13}C NMR. *J* values are given in hertz.

Mass spectra (MS) were recorded on an AMD Intectra DP 10 instrument operating at an ionizing potential of 70 eV (EI-MS) or on a Thermo Finnigan LCQ-Classic instrument (ESI-MS).

Most chemicals were purchased from Sigma−Aldrich (Schnelldorf, Germany) or Acros Organics (Geel, Belgium). All solvents were commercial-grade and were distilled before use. Berberine chloride was purchased from Alfa Aesar (Karlsruhe, Germany). Palmatine iodide belonged to an old substance collection. Its identity and purity was ascertained by NMR, MS, elementary analysis, and melting-point determination. Reactions were monitored by thin-layer chromatography (TLC) on precoated aluminum sheets of TLC silica gel 60 F_{254} from Merck (Darmstadt, Germany). Detection of the compounds on TLC plates was achieved by using UV light at 254 nm or with iodine vapor. Merck silica gel 60 (40−63 μm) was used as the stationary phase for flash column chromatography. Elementary analysis was performed with a LECO CHNS-932 instrument. Data are given as percentages and rounded to two decimal places as the average of duplicate determinations. Elementary analytical results were within 0.4% of the theoretical values, except when noted otherwise. The purity of compounds **5h**, **5i**, and **5f** was determined by HPLC (Agilent 1100 Series, Waldbronn, Germany) and found to be >97%.

*Synthesis of Phenylacetamides **3a−k**: General Procedure.* The phenylacetic acid derivative and 1.1 mol equiv of the respective phenylethylamine were suspended in dry xylene and refluxed under argon for 16 h. After evaporation of xylene, an oily residue was chromatographed and a solid residue was recrystallized from ethyl acetate/heptane 2:1, providing the amides (**3a−k**) as bright fluffy crystals. Details and spectroscopic data can be found in the Supporting Information; representative examples are given below.

*N-[2-(4-Chlorophenyl)ethyl]-2-(3-hydroxy-4-methoxyphenyl)-acetamide **3f**.* 3-Hydroxy-4-methoxyphenylacetic acid **2a** (1.6 g, 8.6 mmol) and 4-chlorophenethylamine **1c** (1.3 g, 8.5 mmol) in 25 mL of xylene gave 1.8 g of **3f** (65%) as pale beige crystals. ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.17 (dd, *J* = 1.83/8.24 Hz, 2H), 6.94 (dd, *J* = 1.83/8.55 Hz, 2H), 6.74 (d, *J* = 7.94 Hz, 1H), 6.71 (s, 1H), 6.59 (d, *J* = 7.94 Hz, 1H), 5.32 (br s, 1H), 3.89 (s, 3H), 3.43 3.38 (m, 4H), 2.67 (t, *J* = 6.71 Hz, 2H).

*N-[2-(3,4-Dimethoxyphenyl)ethyl]-2-(3-hydroxy-4-methoxyphenyl)acetamide **3i**.* 3-Hydroxy-4-methoxyphenylacetic acid **2a** (1.0 g, 5.5 mmol) and 2-(3,4-dimethoxyphenyl)ethylamine **1f** (1.0 g, 5,5 mmol) in 17 mL of xylene gave 1.4 g of **3i** (75%) as an oily residue. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 6.75−6.71 (m, 3H), 6.62−6.54 (m, 3H), 5.57 (br s, 1H), 3.87 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.50 (s, 2H), 3.44 (dd, *J* = 5.87/6.26 Hz, 2H), 2.67 (t, *J* = 6.65 Hz, 2H).

*Synthesis of **4b−k** by Reduction with Lithium Aluminum Hydride: General Procedure.* For reduction of the amides (**3b−k**), the respective substance was dissolved in dry tetrahydrofuran (THF) or dry *tert*-butyl methyl ether (TBME), stirred under argon, and cooled to 0 °C. A 2.5−5.0-fold molar amount of lithium aluminum hydride was added in one portion. The reaction was allowed to reach room temperature and stirred for 14−16 h. The mixture was cooled to 0 °C, and water was added until no more hydrogen was released. The solid was separated and extracted five times with trichloromethane/methanol (9:1). The liquid layer was also extracted with trichloromethane/methanol (9:1). The organic layers were combined, washed with water, and dried over anhydrous magnesium sulfate. Removal of the solvent gave an oily residue, which was purified by silica column chromatography (gradient, trichloromethane 100% to trichloromethane/methanol 93:7) to give the amine. Details and spectroscopic data can be found in the Supporting Information; representative examples are given below.

*5-{2-[2-(4-Chlorophenyl)ethylamino]ethyl}-2-methoxyphenol **4f**.* Amide **3f** (1.4 g, 4.3 mmol), dissolved in 50 mL of dry TBME, and lithium aluminum hydride (1.0 g, 26.4 mmol) gave 0.4 g of **4f** (33%) as white crystals. The compound was used for the next step without further purification.

*5-{2-[2-(3,4-Dimethoxyphenyl)ethylamino]ethyl}-2-methoxyphenol **4i**.* Amide **3i** (1.4 g, 4.1 mmol), dissolved in 50 mL of dry TBME, and lithium aluminum hydride (0.3 g, 6.8 mmol) gave 0.2 g of **4i** (14%) as white crystals. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 6.80−6.61 (m, 6H), 3.85 (s, 3H), 3.83 (s, 3H), 3 0.82 (s, 3H), 3.50 (s, 2H), 3.22−3.13 (m, 8H).

*Synthesis of **5a−k** by Mannich Reaction: General Procedure.* For preparation of the phenethyltetrahydroisoquinolines, the amines (**4a−k**) and a few drops of concentrated aqueous hydrochloric acid were stirred in dry methanol. An aqueous methanal solution (37%) was added, and the mixture was heated under reflux for 3 h. Methanol was distilled off, and the residue was basified with 10% aqueous ammonia and extracted with trichloromethane three times. The extract was washed with water, dried over anhydrous magnesium sulfate, and evaporated to leave either a solid or an oily residue. Phenethyltetrahydroisoquinolines **5a−k** were finally purified by silica column chromatography (gradient, trichloromethane 100% to trichloromethane/methanol 95:5). Details and spectroscopic data can be found in the Supporting Information; representative examples are given below.

*2-[2-(4-Chlorophenyl)ethyl]-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline **5f**.* Amine **4f** (0.4 g, 1.4 mmol), dissolved in 15 mL of dry methanol, and 15 mL of aqueous methanal solution (37%) gave 0.19 g (43%) of **5f** as a yellow solid, mp 168−171 °C. ^{1}H NMR (CD_3OD, 400 MHz) δ = 7.28 (dd, *J* = 2.35/8.61 Hz, 2H), 7.24 (dd, *J* = 2.35/8.61 Hz, 2H), 6.62 (s, 1H), 6.56 (s, 1H), 3.81 (s, 3H), 3.64 (s, 2H), 2.92−2.88 (m, 2H), 2.80 (s, 4H), 2.76−2.72 (m, 2H). ^{13}C NMR (CD_3OD, 125 MHz) δ = 145.0, 144.2, 138.8, 131.8, 130.1, 128.5, 126.8, 125.6, 114.3, 108.8, 59.8, 56.0, 55.8, 51.0, 33.3, 28.3. MS (ESI) *m*/*z* 318.2 (100, M^+ + 1). Anal. calcd for $C_{18}H_{20}ClNO_2$: C, 68.03; H, 6.34; N, 4.41. Found: C, 68.22; H, 6.21; N, 4.35.

*2-[2-(3,4-Dimethoxyphenyl)ethyl]-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline **5i**.* Amine **4i** (210 mg, 0.6 mmol), dissolved in 15 mL of dry methanol, and 15 mL of aqueous methanal solution (37%) gave 79 mg (36%) of **5i** as a pale yellow solid, mp 142−145 °C. ^{1}H NMR (CD_3OD, 400 MHz) δ = 6.87 (d, *J* = 7.83, 1H), 6.86 (s, 1H), 6.79 (dd, *J* = 1.96/7.83 Hz, 1H), 6.62 (s, 1H), 6.56 (s, 1H), 3.83 (s, 3H), 3.81 (s, 3H), 3.80 (s, 3H), 3.65 (s, 2H), 2.87−2.72 (m, 8H). MS (ESI) *m*/*z* 344.2 (100, M^+ + 1); Anal. calcd for $C_{20}H_{25}NO_4$: C, 69.95; H, 7.34; N, 4.08. Found: C, 69.60; H, 7.30; N, 4.13.

The new selective inhibitors **5h**, **5i**, and **5f** were subjected to HPLC purity testing (see Supporting Information).

*Synthesis of 2-(3-Acetoxy-4-methoxyphenyl)-N-[2-(4-methoxyphenyl)ethyl]acetamide (**7**), 2-Methoxy-5-{2-[2-(4-methoxyphenyl)ethylamino]ethyl}phenol (**4a**), and 2-[2-(4-Hydroxyphenyl)ethyl]-1,2,3,4-tetrahydroisoquinolin-6-ol Hydrobromide (**6**).* Synthetic details and spectroscopic data can be found in the Supporting Information.

Biological Assays, General. All cell cultures were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and cultivated according to the recom-

mendations of DSMZ. The following strains were used: *Aspergillus brasiliensis* (DSM 1988), *Candida glabrata* (DSM 11226), *Escherichia coli* (DSM 426), *Hyphopichia burtonii* (DSM 70663) *Pseudomonas antimicrobia* (DSM 8361), *Saccharomyces cerevisiae* (DSM 1333) *Staphylococcus equorum* (DSM 20675), *Streptococcus entericus* (DSM 14446), and *Yarrowia lipolytica* (DSM 1345).

HL 60 cells (DSM ACC3) were cultivated in RPMI 1640 medium with 10% FBS (fetal bovine serum, both from PAA Laboratories, Cölbe, Germany) without the addition of antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2.

Stock solutions of all compounds and references were diluted with absolute ethanol according to the SANCO guideline.[42]

The assays were performed under laminar flow cabinets: HeraSafe 12 from Heraeus Instruments (Hanau, Germany) for microorganisms, and MSC Advantage from Thermo Fisher (Schwerte, Germany) for HL 60 cells.

Assay for antibacterial and antifungal activities (agar diffusion assay), screening assay for ergosterol biosynthesis inhibition, and MTT assay are described in Supporting Information.

Screening Assay for Cholesterol Biosynthesis Inhibition. This assay is based on a method described by us previously,[38] but workup was slightly modified in analogy to the ergosterol biosynthesis assay.

Qualitative Screening (Identification of Target Enzyme). A suspension of 1×10^6 HL 60 cells in a special HL 60 cell medium ("Medium für HL 60 Zellen, lipid free medium"; PAN Biotech, Aidenbach, Germany) was incubated with 10 μL of inhibitor solution (concentrations 50 μM and 1 μM) in 24-well plates. Blank samples were prepared with 10 μL of ethanol instead of 10 μL of inhibitor solution. AY 9944 and BM 15.766 sulfate (both from Sigma–Aldrich, Steinheim, Germany) were used as reference inhibitors for 7-DHCR.[9,15] After incubation for 24 h (conditions, 37 °C in a humidified atmosphere containing 5% CO_2), the suspensions were transferred into 2.0-mL plastic microcentrifuge safe-lock tubes. The tubes were centrifuged for 5 min at 9000*g*. The cell pellets were suspended in 1 mL of 1 M aqueous sodium hydroxide solution, vortexed for 30 s, and transferred into 4-mL glass vials with Teflon septa. The vials were flooded with nitrogen and closed tightly. The vials were stored for 1 h at 70 °C in a laboratory drying cabinet. While cooling down to room temperature, the vials were ultrasonicated. The whole solutions were transferred back to the microcentrifuge safe-lock tubes, and 700 μL of TBME and 50 μL of cholestane as internal standard (10 μg/mL in TBME) were added. After thorough shaking for 1 min, the solutions were centrifuged (5 min, 9000*g*). The organic supernatant was transferred to a second 2.0-mL plastic microcentrifuge safe-lock tube containing 40 ± 2 mg of a mixture of anhydrous sodium sulfate/PSA (primary secondary amine sorbent, Bondesil PSA, Varian, Darmstadt, Germany) 7:1. Two further extractions were performed with 750 μL of TBME each. The combined organic extracts were shaken for 1 min, followed by a last centrifugation step (5 min, 9000*g*). Each 1.0 mL of the purified extracts obtained by this dispersive solid-phase extraction was transferred into a brown glass vial. Under a gentle stream of nitrogen, the extracts were brought to dryness. The residues were taken up in 950 μL of TBME and 50 μL of a silylation reagent mixture of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and *N*-(trimethylsilyl)imidazole (MSTFA/TSIM 9:1). The samples were stored for at least 30 min at room temperature and then subjected to GC-MS analysis.

Analysis was focused on identifying 7-DHC unequivocally. This was accomplished by comparing the relative retention time and the fragmentation of the sterols. Characteristic ions for zymostenol are *m*/*z* 458, 353, and 213; for 7-dehydrocholesterol, *m*/*z* 351, 325, and 366; and for cholestane, *m*/*z* 357, 217, and 203. The reference standard, zymostenol, was synthesized from cholesta-8,14-dien-3β-ol;[38] 7-dehydrocholesterol and cholestane were purchased from Sigma-Aldrich (Steinheim, Germany). The investigations were performed in duplicate.

Quantitative Screening. Six suitable concentrations of each potent inhibitor (selected by semiquantitative analysis of the peak of accumulating 7-dehydrocholesterol in the qualitative screening) plus one blank sample, all in triplicate, were tested in a 24-well plate. The incubation procedure was identical to the one described above, but only 980 μL of the cell suspension was used; 10 μL of inhibitor solution and 10 μL of sterile sodium ^{13}C-acetate solution (6.25 mg/mL) were added to obtain a final volume of 1000 μL. A slight change in the workup procedure was necessary. After cell lysis, 3 × 25 μL of every cell lysate (total volume 1.0 mL) was not transferred back to the microcentrifuge safe-lock tubes. These suspensions were separated for a Bradford assay to determine the protein content.[38,43] Further treatment of the samples was the same as described above.

The overall inhibition of cholesterol biosynthesis was determined by integration of the relevant MS signals of newly synthesized, labeled cholesterol (quantifier ions *m*/*z* 372–379 and 462–469) and the internal standard cholestane (quantifier ions *m*/*z* 203, 217, and 357), with the results from the Bradford assay taken into account. The percentage inhibition is calculated in eq 1:

$$\%\ \text{inhibition} = \left[1 - \left(\frac{A_S \times A_{ISC} \times PC_C}{A_C \times A_{ISS} \times PC_S}\right)\right] \times 100 \quad (1)$$

where A_S is the area of the sample (^{13}C-labeled cholesterol); A_{ISC} is the area of the internal standard (cholestane); PC_C is the protein content control; A_C is the area of the control (^{13}C-labeled cholesterol); A_{ISS} is the area of the internal standard sample (cholestane); and PC_S is the protein content sample.

The percentage inhibition (eq 1) relative to untreated control samples (0% inhibition) was plotted against logarithmic inhibitor concentration by use of Graph Pad Prism 4 (Graph Pad Inc., San Diego, CA). A bottom level constant equal to zero was set as a constraint, by use of a sigmoidal dose–response model with a variable slope.[38]

GC-MS Analysis of Sterols. A Varian Saturn 2200 ion trap was coupled with a Varian 3800 gas chromatograph (Darmstadt, Germany). The gas chromatograph was linked with a CombiPal from CTC Analytics (Zwingen, Schweiz). A 1177 injector from Varian (Darmstadt, Germany) was used in splitless injection mode during injection. Data analysis and instrument control was made with Varian Workstation 6.9 SP 1 software. A Varian VF-5 ms capillary column of 30 m length, 0.25 mm i.d., and 0.25 μm film thickness with a 10 m EZ-Guard column was used for analysis. The GC-MS conditions were the following: helium 5.0 (99.9990%), constant flow of 1.3 mL/min, inlet temperature 250 °C, injection volume 1 μL, MS transfer line temperature 270 °C. The initial GC oven temperature was 50 °C (1 min hold) ramped up to 260 °C (50 °C/min), followed by a gradient of 4 °C per minute up to 310 °C (hold time 0.5 min). The total run time was 18.2 min. The MS was operated in full scan mode from 9 to 12 min at a mass range from 50 to 450 *m*/*z* and from 12 to 18.2 min at a mass range from 100 to 650 *m*/*z*. The sterols were identified by comparison with authentic standard substances or by alignment with data from NIST 2005 mass spectral database.[37,38]

■ ASSOCIATED CONTENT

Supporting Information

Additional text and one scheme with synthetic details and spectroscopic data for intermediates and final substances. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

* Phone +49-(0)345-55-25175; e-mail peter.imming@pharmazie.uni-halle.de.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

7-DHC, 7-dehydrocholesterol; 7-DHCR, 7-dehydrocholesterol reductase; DSMZ, German Collection of Microorganisms and Cell Cultures; SLOS, Smith−Lemli−Opitz syndrome; TBME, *tert*-butylmethyl ether; THF, tetrahydrofuran

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