# Analysis and Experimental Inhibition of Distal Cholesterol Biosynthesis

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REVIEW

# Analysis and Experimental Inhibition of Distal Cholesterol Biosynthesis

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**Abstract** During the last decade, our understanding about the function and biological activity of metabolites has drastically changed. Metabolites previously seen as sole bystanders of biochemical reactions without unique activity are becoming more and more recognized for their biological functions. One class of metabolites for which such a paradigm shift has become evident is cholesterol and its precursors. Here, we will give a brief overview about the post-squalene pathway of cholesterol biosynthesis, its inhibition, the biological functions of its intermediates and put special emphasis on the analysis of the occurring metabolites. We will compare liquid chromatography and gas chromatography-based analysis platforms, describe mass spectrometric fragmentations and explain different derivatization strategies. In addition, we will present a full spectral data set of 27 analytes of which 24 are sterols, synthesized, isolated and characterized in several studies in our laboratories. Finally, with our overview, we hope to assist researchers in the field of cholesterol biosynthesis with the chemical analysis of cholesterol and its physiological as well as non-physiological precursors.

**Keywords** Gas chromatography (GC) · Liquid chromatography (LC) · Mass spectrometry (MS) · Cholesterol biosynthesis · Biosynthesis inhibitors

**Abbreviations**

| | |
|---|---|
| 7-DHC | 7-Dehydrocholesterol |
| ABS | Antley–Bixler Syndrome |
| APCI | Atmospheric pressure chemical ionization |
| BSTFA | *N,O*-Bis(trimethylsilyl)trifluoroacetamide |
| DMES | Dimethylethylsilyl |
| DTE | 1,4-Dithioerythritol |
| ECNI | Electron capture negative ionization |
| EI | Electron ionization |
| ER | Estrogen receptor |
| ESI | Electrospray ionization |
| FAME | Fatty acid methyl ester |
| FF-MAS | Follicular fluid meiosis-activating sterol |
| FID | Flame ionization detection |
| GC | Gas chromatography |
| GC–MS(MS) | Gas chromatography (tandem) mass spectrometry |
| IT | Ion trap |
| LC | Liquid chromatography |
| LC–MS(MS) | Liquid chromatography (tandem) mass spectrometry |
| LXR | Liver X receptor |
| MSTFA | *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide |
| MtBSTFA | *N*-Methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide |
| ODS | Octadecylsilyl |
| PCI | Positive chemical ionization |

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| | |
|---|---|
| RRT | Relative retention time |
| SC | Side chain |
| SLOS | Smith–Lemli–Opitz syndrome |
| SPE | Solid-phase extraction |
| SIM | Selected ion monitoring |
| SIS | Single ion storage |
| SREBP | Sterol response element-binding protein |
| SRM | Selected reaction monitoring |
| *t*BDMS | *tert*-Butyl dimethylsilyl |
| *t*BME | *tert*-Butyl methyl ether |
| T-MAS | Testis meiosis-activating sterol |
| TMCS | Trimethylchlorosilane |
| TMIS | Trimethyliodosilane |
| TMS | Trimethylsilyl |
| TMSiOH | Trimethylsilanol |
| TSIM | *N*-(Trimethylsilyl)imidazole |

## Introduction

The post-genome era is characterized by a paradigm shift in our understanding about the function of metabolites. For several small molecules originally solely seen as biochemical intermediates, important biological functions have been revealed [1, 2]. With respect to cholesterol (**10**) and its precursors, T-MAS (**15**) and FF-MAS (**16**) are known since the 90's to play an important role as meiosis-activating sterols in testes and follicular fluid [3]; however, bioactivity of cholesterol (**10**) and its precursors has long been underestimated (Fig. 1; Tables 1, 2). Conversely, just recently desmosterol (**11**) has been revealed as an anti-inflammatory lipid intrinsically counteracting pro-inflammatory features of foam cells [4, 5] by interacting with the liver X receptors (LXR) as well as the sterol response element-binding protein (SREBP). The LXR as well as the SREBP are two well-characterized transcription factors partially controlled by cholesterol (**10**) and its precursors such as desmosterol (**11**) [6]. Besides this direct involvement in transcription control, it has also become evident during the last decade that particularly cholesterol (**10**) and possibly to some extent its precursors play crucial roles in the formation of lipid rafts controlling protein–protein interactions, thereby influencing important signaling processes [7]. Other important aspects of cholesterol biosynthesis include the role of certain precursors during viral infections [8, 9] and its apparent involvement for example in prostate cancer, possibly rendering cholesterol biosynthesis a feasible drug target [10]. Besides these molecular biology-related aspects, it is evident that cholesterol (**10**) and its precursors also serve as important diagnostic markers. Elevated plasma cholesterol levels are possibly correlated with the occurrence of atheroma [11], while its precursors are valuable and important clinical markers for genetic diseases such as for example the Smith–Lemli–Opitz syndrome

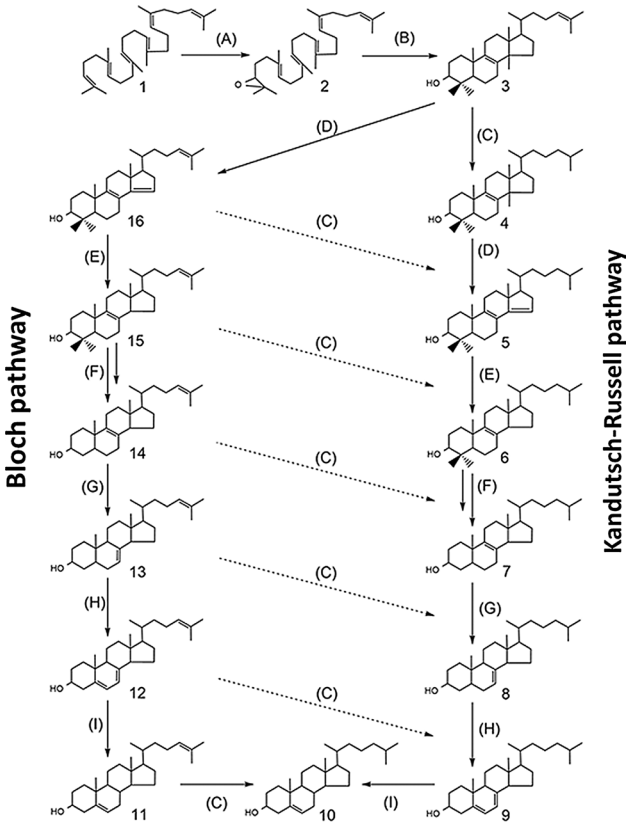


**Fig. 1** Distal cholesterol biosynthesis, enzymes and intermediates are explained in the text (see also Table 2). Taken with permission from [19] and modified

(SLOS), a mutation of the gene encoding 7-dehydrocholesterol reductase (**I**) leading to abnormally high levels of 7-dehydrocholesterol (7-DHC, **9**) and decreased levels of cholesterol (**10**) [12]. In this way, increased levels of 7-DHC (**9**) usually measured by gas chromatography–mass spectrometry (GC–MS) serve as diagnostic marker for SLOS. Other SLOS-like genetic defects such as lathosterolosis or desmosterolosis can be diagnosed and differentiated by the accumulation of the respective cholesterol precursors. Taken together, the above given facts underline the importance of cholesterol and its precursors as bioactive lipids and diagnostic markers, demanding dedicated analytical solutions allowing their highly sensitive and selective analysis. Here, we will give an overview about the most recent techniques for the analysis of cholesterol and its biochemical precursors. We will also address the formation of non-physiological sterols which have for example been described in 3T3L3 preadipocytes upon treatment with haloperidol (Fig. 2) [10] and present spectral data and briefly discuss compound-specific fragmentations allowing the reader to identify a broad set of cholesterol precursors as well as non-physiological sterols (Fig. 3). The conventional numbering system of sterols is depicted in Fig. 4.

**Table 1** Name, CAS number, chemical formula, molecular weight, and chromatographic data of analyzed compounds, (n.d.) not determined

| IUPAC name | Trivial name | Code | CAS number | Chemical formula | $M_R$ | $M_R$ (TMS) | RRT TMS ether (cholestane) | RRT TMS ether (cholesterol) | RRT TMS ether (cholestanol) |
|---|---|---|---|---|---|---|---|---|---|
| (6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene | Squalene | **1** | 111-02-4 | $C_{30}H_{50}$ | 410.7 | (–) | 0.95 | 0.76 | 0.75 |
| 2,2-Dimethyl-3-((3*E*,7*E*,11*E*,15*E*)-3,7,12,16,20-pentamethylhenicosa-3,7,11,15,19-pentaenyl)-oxirane | Monoepoxysqualene | **2** | 7200-26-2 | $C_{30}H_{50}O$ | 426.7 | (–) | 1.04 | 0.83 | 0.81 |
| Lanosta-8,24-dien-3β-ol | Lanosterol | **3** | 79-63-0 | $C_{30}H_{50}O$ | 426.7 | 498.9 | 1.45 | 1.16 | 1.14 |
| 5α-Lanosta-8-en-3β-ol | Dihydrolanosterol | **4** | 79-62-9 | $C_{30}H_{52}O$ | 428.8 | 500.9 | 1.40 | 1.12 | 1.10 |
| 4,4-Dimethylcholesta-8,14-dien-3β-ol | | **5** | 19456-83-8 | $C_{29}H_{48}O$ | 412.7 | 484.9 | 1.42 | 1.13 | 1.11 |
| 4,4-Dimethylcholesta-8(9)-en-3β-ol | | **6** | 5241-24-7 | $C_{29}H_{50}O$ | 414.7 | 486.9 | 1.43 | 1.14 | 1.12 |
| Cholesta-8-en-3β-ol | Zymostenol | **7** | 566-97-2 | $C_{27}H_{46}O$ | 386.7 | 458.9 | 1.27 | 1.02 | 1.00 |
| Cholesta-7-en-3β-ol | Lathosterol | **8** | 80-99-9 | $C_{27}H_{46}O$ | 386.7 | 458.9 | 1.31 | 1.04 | 1.03 |
| Cholesta-5,7-dien-3β-ol | 7-Dehydrocholesterol | **9** | 434-16-2 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.29 | 1.03 | 1.02 |
| Cholesta-5-en-3β-ol | Cholesterol | **10** | 57-88-5 | $C_{27}H_{46}O$ | 386.7 | 458.9 | 1.26 | 1.00 | 0.99 |
| Cholesta-5,24-dien-3β-ol | Desmosterol | **11** | 313-04-2 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.29 | 1.03 | 1.02 |
| Cholesta-5,7,24-trien-3β-ol | | **12** | 1715-86-2 | $C_{27}H_{42}O$ | 382.6 | 454.8 | 1.33 | 1.06 | 1.05 |
| Cholesta-7,24-dien-3β-ol | | **13** | 651-54-7 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.36 | 1.09 | 1.07 |
| Cholesta-8,24-dien-3β-ol | Zymosterol | **14** | 128-33-6 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.32 | 1.06 | 1.04 |
| 4,4-Dimethylcholesta-8,24-dien-3β-ol | T-MAS | **15** | 7448-02-4 | $C_{29}H_{48}O$ | 412.7 | 484.9 | 1.48 | 1.18 | 1.16 |
| 4,4-Dimethylcholesta-8,14,24-trien-3β-ol | FF-MAS | **16** | 64284-64-6 | $C_{29}H_{44}O$ | 410.7 | 482.9 | 1.47 | 1.17 | 1.15 |
| Cholesta-8,14-dien-3β-ol | | **17** | 17608-73-0 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.27 | 1.02 | 1.00 |
| 4,4-Dimethylcholesta-8(14)-en-3β-ol | | **18** | 14772-51-1 | $C_{29}H_{50}O$ | 412.7 | 486.9 | 1.41 | 1.13 | 1.11 |
| Cholesta-8,14,24-trien-3β-ol | | **19** | 64284-65-7 | $C_{27}H_{42}O$ | 382.7 | 454.8 | 1.31 | 1.05 | 1.03 |
| 4α-Methylcholesta-7-en-3β-ol | Lophenol | **20** | 481-25-4 | $C_{28}H_{48}O$ | 400.7 | 472.9 | 1.37 | 1.09 | 1.08 |
| 4α-Methylcholesta-7,24-dien-3β-ol | | **21** | 24778-51-6 | $C_{28}H_{46}O$ | 398.7 | 470.9 | 1.40 | 1.12 | n.d. |
| Cholesta-5,8-dien-3β-ol | 8-Dehydrocholesterol | **22** | 70741-38-7 | $C_{27}H_{44}O$ | 384.7 | 456.8 | 1.26 | n.d. | n.d. |
| Cholesta-5,8,24-trien-3β-ol | | **23** | (–) | $C_{27}H_{42}O$ | 382.6 | 454.8 | 1.30 | 1.04 | n.d. |
| Cholesta-5,7,22,24-tetraen-3β-ol | | **24** | 34298-92-5 | $C_{27}H_{40}O$ | 380.6 | 452.8 | n.d. | n.d. | 1.09 |
| 4,4-Dimethylcholesta-5,7-dien-3β-ol | | **25** | 53296-71-2 | $C_{29}H_{48}O$ | 412.7 | 484.9 | 1.43 | 1.14 | 1.12 |
| 5α-Cholestan-3β-ol | Cholestanol | **26** | 80-97-7 | $C_{27}H_{48}O$ | 388.7 | 460.9 | 1.27 | 1.02 | 1.00 |
| Cholestane | | **27** | 481-20-9 | $C_{27}H_{48}$ | 372.7 | (-) | 1.00 | 0.80 | 0.79 |

**Table 2** Summary of selected inhibitors of distal cholesterol biosynthesis

| Enzyme | EC number | Inhibitor(s) | Possible indication(s) | Comment | Refs |
|---|---|---|---|---|---|
| Squalene epoxidase (**A**) | 1.14.13.132 | NB598 | Hypercholesterolemia | Developed by Banyu Pharmaceuticals Co., LtD. in the 1990s, skin irritation of unknown origin detected in dogs | [13] |
| | | TU-2078 | | | [14] |
| Oxidosqualene cyclase (**B**) | 5.4.99.7 | BIBX79 | Hypercholesterolemia, breast cancer | Dual-action drug | [15] |
| | | Ro 48-8071 | | ERβ/ERα ratio increases | [16, 17] |
| | | Aminopropylindenes | | Novel chemotype of oxidosqualene cyclase inhibitors | [18] |
| $\Delta^{24}$-Reductase (**C**) | 1.3.1.72 | Triparanol, DR-258, MGI-21 | Inflammation? | | [5, 19–21] |
| C14-Demethylase (**D**) | 1.14.13.70 | Azalanstat, clotrimazole, fluconazole | Hypercholesterolemia, trypanasoma infections? | Target of azole antifungals, however, these also pose varying affinity towards the mammalian enzyme. Lanosterol (**3**) involved in HMGCoA regulation. Long-term and high-dose application risky during pregnancy (ABS) | [19, 22] |
| $\Delta^{14}$-Reductase (**E**) | 1.3.1.70 | AY-9944, haloperidol | | Dose dependent behavior | [10, 23] |
| C4-Demethylase complex (**F**) | 1.14.13.72<br>1.1.1.170<br>1.1.1.270 | Aminotriazole | | Complex of microsomal enzymes | [24] |
| $\Delta^{8/7}$-Isomerase (**G**) | 5.3.3.5 | AY-9944, haloperidol | | Dose dependent behavior | [10, 23] |
| | | Tamoxifen | | Not selective, upregulation of low-density lipoprotein receptor | [25, 26] |
| | | Aminoindenols | | Novel chemotype; highly selective and potent | [26] |
| | | Aminomethyl spiroacetals | | Novel chemotype; selective | [27] |
| Lathosterol oxidase (**H**) | 1.14.21.6 | Lathosterol side chain amides (i.e., MGI-39) | | First inhibitors | [20] |
| Dehydrocholesterol reductase (**I**) | 1.3.1.21 | BM15.766<br>AY-9944, haloperidol | | Dose dependent behavior | [10, 23, 28] |
| | | Phenethyltetrahydroisoquinolines | | First highly selective and potent inhibitors | [29] |

EC enzyme code according to http://www.BRENDA.com [5, 10, 13–29]

## Enzymes and Inhibitors

Distal cholesterol biosynthesis is an intriguing biochemical route (Fig. 1). Starting from a straight chain sesquiterpene (squalene, **1**), the first cyclic intermediate lanosterol (**3**) is formed by epoxidation catalyzed by the enzyme squalene epoxidase (**A**) followed by one of nature's most remarkable enzymatic reactions, the cyclization of monoepoxysqualene (**2**) by the enzyme oxidosqualene cyclase (**B**). The cyclization of **2** in a single enzymatic reaction is highly stereo- and regioselective yielding lanosterol (**3**), the first sterol intermediate in the biosynthesis of cholesterol [16]. This first sterol is also the branching point of the biochemical route into either the so-called Kandutsch–Russell pathway (right side Fig. 1) or the Bloch pathway (left side Fig. 1). Both pathways are interconnected by the enzyme $\Delta^{24}$-reductase (**C**). Chemically speaking, both pathways basically involve the same biochemical reactions and intermediates, with, however, the $\Delta^{24}$-unsaturated intermediates in the Bloch branch (Fig. 1). The favored pathway for cholesterol biosynthesis is the Kandutsch–Russell pathway [26, 30]. We will explain the biochemical route based on the Kandutsch–Russell pathway: Following the generation of **3**, $\Delta^{24}$-reduction by **C** yields dihydrolanosterol (**4**), which undergoes oxidative C14-demethylation by an enzyme of the cytochrome P450 family, C14-demethylase (**D**). The reaction yields 4,4-dimethylcholesta-8,14-dien-3β-ol (**5**), which subsequently is transformed into zymosterol (**7**) by $\Delta^{14}$-reduction carried out by the respective enzyme $\Delta^{14}$-reductase (**E**) and iterative double demethylation by the C4-demethylase complex (**F**). The remaining double bond at $\Delta^{8(9)}$ is isomerized by $\Delta^{8/7}$-isomerase (**G**) yielding lathosterol (**8**) possessing a $\Delta^{7(8)}$ double bond. The further processing of **8** by the action of lathosterol oxidase (**H**) yields 7-DHC (**9**), the direct precursor of cholesterol. Conjugated diene **9** is reduced at the $\Delta^{7(8)}$-position by 7-DHC reductase (**I**), finally yielding cholesterol (**10**).

It is obvious that the sheer number of enzymes involved in the biosynthesis of cholesterol offers numerous targets for potential drugs with the main goal pursued in recent years being inhibition of endogenous cholesterol biosynthesis. While some of the enzymes involved are still actively pursued as possible cholesterol-lowering drug targets, such as for example **A** and **B** [31], have others like **F** and **H** not yet been investigated for their potential. Particularly, inhibition of oxidosqualene cyclase (**B**) has attracted much interest due to the fact that substances acting on this enzyme display a dual-action mechanism. On one hand, inhibition of **B** lowers overall cholesterol levels; on the other hand, accumulating 24(*S*),25-epoxycholesterol is a potent activator of the liver X receptor, crucial to cholesterol homeostasis [16, 32]. Importantly, blockage of endogenous cholesterol biosynthesis has not only been pursued in the context of hypercholesterolemia but also in front of the fact that inhibition of this important biochemical pathway showed growth arrest of cancer cells or induces differentiation of human leukemia cells [17, 33]. Nonetheless, the aforementioned effect is mainly restricted to situations where cells are dependent on endogenous cholesterol biosynthesis, rendering the approach restricted to tissues with low cholesterol content [17]. However, just recently, the oxidosqualene cyclase (**B**) inhibitor Ro 48-8071 (Fig. 2) has proven highly effective in the treatment of estrogen receptor (ER) positive breast cancer in a mouse xenocraft model, the inhibitory effect was coined to be mainly based on an increase in the ratio ERβ/ERα [34]. The same inhibitor led to an increased binding of p53 to DNA in BT-474 cells [35]. Besides the indications, hypercholesterolemia and cancer has new research into the potential of cholesterol biosynthesis as a drug target mainly been fueled by studies such as the report by Spann et al. [5] describing the anti-inflammatory potential of desmosterol (**11**) in foam cells or the finding that cholesterol biosynthesis is involved in hepatitis virus infections [8, 9, 36, 37]. Di Stasi et al. [38] discussed the application of $\Delta^{24}$-reductase inhibitors against melanoma metastases with up-regulated $\Delta^{24}$-reductase genes. Table 2 gives an overview about small molecules interfering with cholesterol biosynthesis and their therapeutic potential. An important issue targeting cholesterol biosynthesis with small molecule inhibitors is the possible formation of non-physiological sterols. This has for example been shown for haloperidol in SH-SY5Y cells, leading mainly to the accumulation of cholesta-8,14-dien-3β-ol (**17**) [10, 23]. As the biological effects of many cholesterol precursors and particularly non-physiological sterols are only partially investigated, as for example reported by Xu et al. [39] defining structural requirements for mammalian cancer cell growth, the formation of non-physiological sterols might possess beneficial as well as detrimental effects. In case of genetic defects of cholesterol biosynthesis [40], the fact that cholesterol precursors or non-physiological sterols accumulate in the patient's blood has been widely exploited as diagnostic tool [41] supporting diagnosis on the molecular level. A very important example is the accumulation of **9** and cholesta-5,8-dien-3β-ol (**22**) in the blood of SLOS patients [42]. The oxidized product of 7-DHC (**9**) is toxic and jointly responsible for the malformations [43]. The rare autosomal recessive Antley–Bixler syndrome (ABS) is also associated with a deficiency in cholesterol biosynthesis. It was observed that a long-term and high-dose treatment of pregnant women with fluconazole (Fig. 2), an inhibitor of the C14-demethylase (**D**) used against systemic fungal infections, induces the same malformations as congenital ABS [44].

Below, we will describe how such sterols are to be separated from physiological precursors and identified by

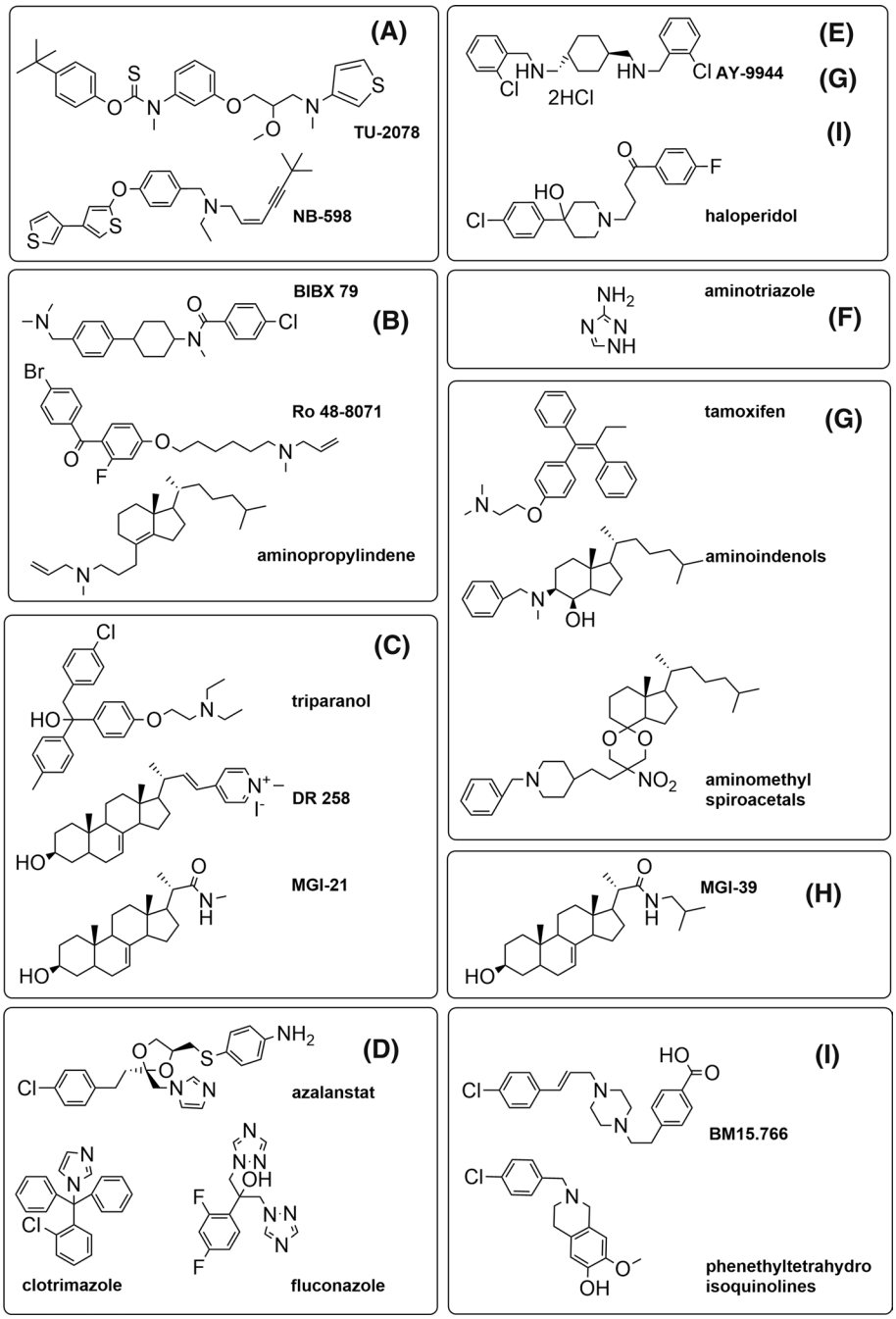


**Fig. 2** Distal cholesterol biosynthesis inhibitors (see also Table 2)

analytical techniques. With respect to the screening for novel enzyme inhibitors and effectors of distal cholesterol biosynthesis, it is important to realize that many of the enzymes involved in distal cholesterol biosynthesis are membrane bound [45], hence difficult to isolate and furthermore undergo rapid deactivation when isolated [46]. Therefore, several reports have described the investigation of distal cholesterol biosynthesis in cellular systems by the use of $^{14}$C-acetate in combination with liquid chromatography (LC) and scintillation counting [33, 47], or $^{13}$C-acetate in combination with GC–MS [19, 29].

## Analysis of Cholesterol and Its Precursors

### Sample Preparation

Cholesterol precursors and cholesterol itself can occur as conjugates with fatty acids and as glycosides in different tissues and fluids [48–51]. Hydrolysis of these conjugates must be arranged because the conjugates are in particular not suitable for GC-based analysis. The cleavage of the conjugates can be accomplished under acidic or basic conditions, or enzymatically. Under acidic conditions, bile

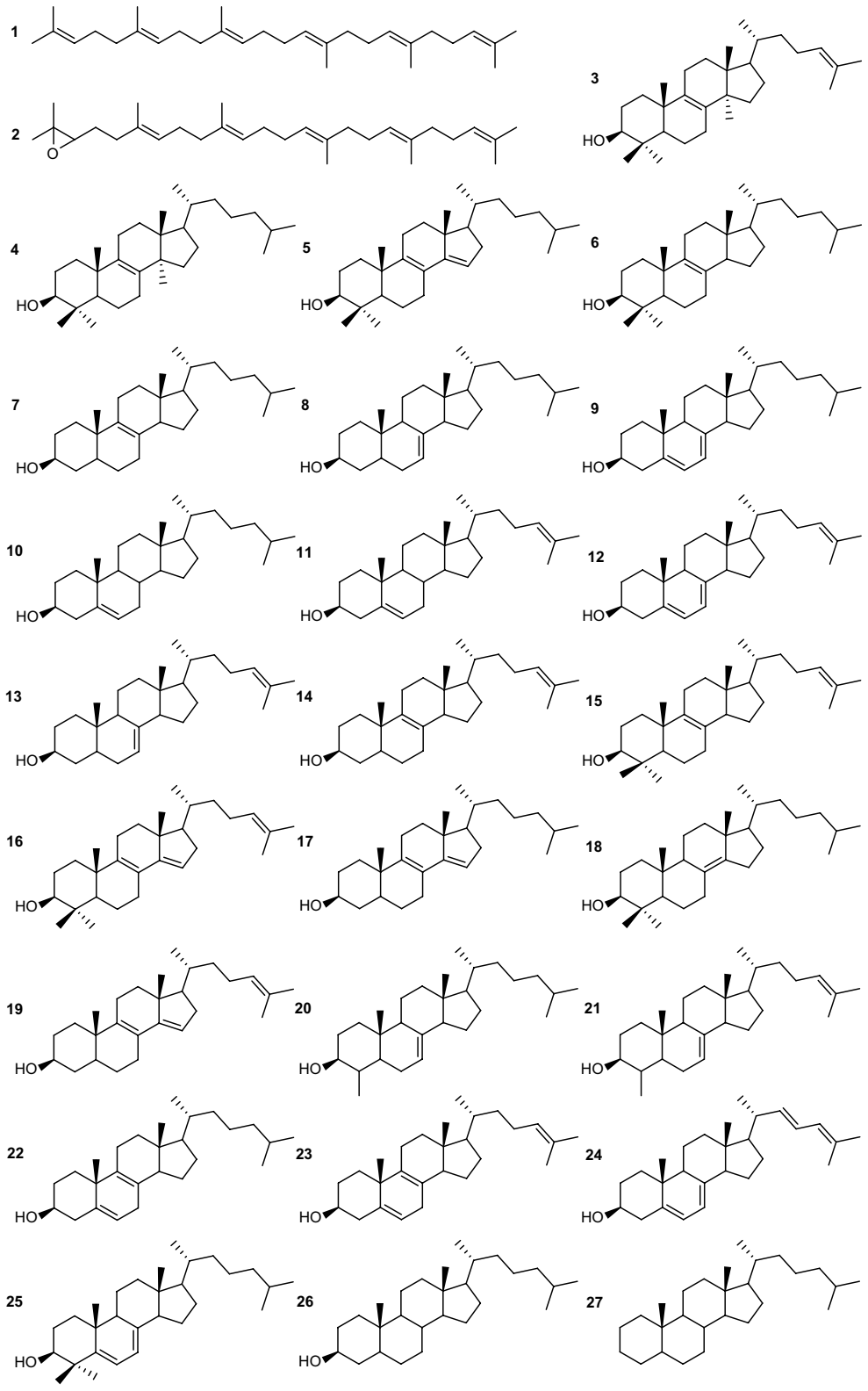


**Fig. 3** Overview of analyzed compounds (see also Table 1)

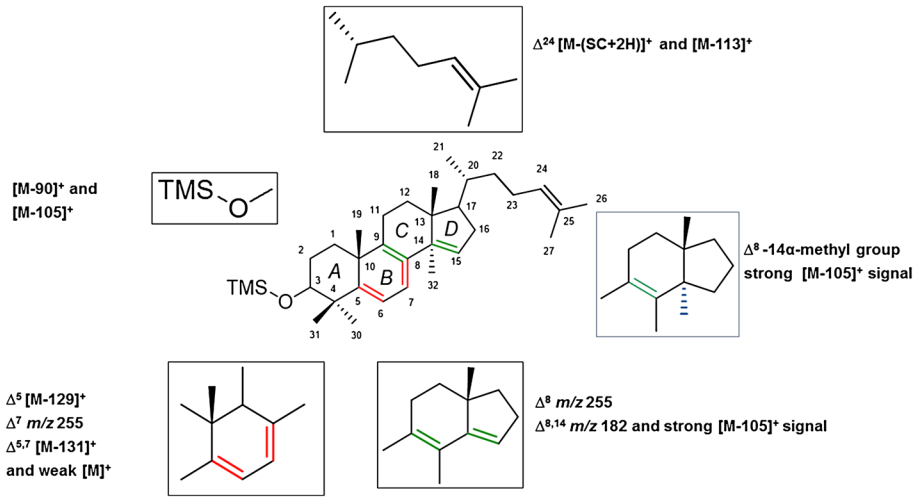


**Fig. 4** Conventional numbering of the sterol backbone and characteristic fragment ions for depicted structural features. The *letters A–D* denominate the rings of the sterol backbone

acids undergo partial dehydration [52], which is also conceivable for oxysterols. Further disadvantages of acidic ester cleavage are the reversibility of this reaction [53] and the risk of isomerizations of double bonds [54]. Hence, the standard ester hydrolysis for free sterol analysis is managed under basic conditions. Various mixtures of potassium and sodium hydroxide in water, methanol or ethanol have been used for saponification [19, 48, 55–59]. These reactions have to be conducted under heating (50–100 °C) conditions which are disadvantageous for thermolabile sterols (e.g., $\Delta^{5,7}$-sterols). Enzymatic hydrolysis offers a gentle opportunity for hydrolysis of labile compounds [49, 51]. Furthermore, particularly for $\Delta^{5,7}$-sterols, great care has to be taken to prevent autoxidation processes, as for example, 7-DHC (**9**) has been shown to be one of the most oxidizable lipids known being even more unstable than poly-unsaturated fatty acids such as for example arachidonic acid [60].

In LC for analysis of free sterols, only pH adjustment after saponification and a solvent/matrix ratio >10 is necessary for direct injection [50]. Of course, for sophisticated LC approaches and GC analysis in general removing salts, proteins and other hydrophilic compounds with a (micro-) liquid–liquid extraction are recommended [55]. Diethyl ether [49, 61, 62], *n*-hexane [55–57, 61, 63, 64], and *tert.*-butyl methyl ether (*t*BME) [19, 48, 49, 59] are common extraction solvents. Acceptable recovery was shown for urinary oxysterols and bile acids with *t*BME, but not with methanol, chloroform/methanol and dichloromethane/isopropanol [49]. Approaches for neutral sterol analysis were managed normally without solid phase extraction (SPE). Hydrophobic SPE materials [e.g., octadecylsilyl cartridges (ODS)] failed for the analysis of bile acids [49, 50], steroid hormones [51] and oxysterols [50]. Prepurification of extracts with silver-ion chromatography (see LC–MS(MS) analysis) has been described [62]. A comparison of the chromatographic properties of a large number of sterols is given by Xu et al. [65] as well as in [66].

## LC–MS(MS) Analysis

### *Label-free Analysis*

While LC–MS(MS) analysis certainly is one of the most prominent techniques for the analysis of hydroxylated sterols [67], bile acids [50] as well as the D-series vitamins [68], the most common technique for the analysis of cholesterol and its precursors certainly is GC–MS. However, in recent years also, some methods have been developed analyzing the latter sterols using LC–MS(MS) [69–71]. Separation of most sterols can be achieved using reversed-phase columns [70]; nonetheless, due to the isomeric (mainly double bond related) character many cholesterol precursors present, dedicated methods have been developed over the years. A technique particularly suited for the separation of double bond isomers is silver-ion chromatography, which has successfully been applied to the separation of several sterol isomers [66, 72]. For silver-ion chromatography, usually strong cation-exchange columns (i.e., Nucleosil SA columns) coated with silver ions are used [73]. While it is accepted that one of the main interactions responsible for compound separation is π-cation interaction between the silver cation and double bonds present in the analytes, other factors have also been discussed. A detailed description of silver-ion chromatography and the underlying mechanism can be found in [66, 74]. An important implication is the fact that silver-ion chromatography is usually performed in the normal-phase mode employing eluents such as hexane or acetone. New stationary phases which are used for the separation of corticosteroids (pentafluorophenyl phase) [75] and endogenous or synthetic estrogens (biphenyl phase) [76] might be an alternative. The mode of separation

of both stationary phases (pentafluorophenyl and biphenyl) is a π–π interaction with steroidal double bonds. These phases seem to be an alternative to silver-ion chromatography but they have not yet been described for the analysis of cholesterol precursors.

Due to the fact that the sterol intermediates in cholesterol biosynthesis are neutral molecules, they are generally analyzed using atmospheric pressure chemical ionization (APCI) [70], as electrospray ionization (ESI) is considered too mild for effective ionization. A drawback related to both APCI and ESI is the fact that usually only non-specific fragmentation such as a neutral loss of water is found. An application presented by Shui et al. [77] used the neutral loss fragment of *m/z* 369 in the analysis of cholesterol for further fragmentation, thereby generating somewhat more component specific fragments for analysis. While the aforementioned fact has implications with respect to selectivity, the rather weak ionization efficiency of sterols during ESI in particular has over the years led to the development of several derivatization agents enhancing sensitivity when analyzing sterols using LC–MS(MS)-based platforms. The main derivatization techniques described in recent years will briefly be discussed in the following section.

*Derivatization Strategies*

Chemically speaking, the only functional group available for derivatization common to all precursors of cholesterol is the secondary alcohol at position 3. Logically, this strongly influences the applicable derivatization strategies. An interesting method for cholesterol and its precursors was described by Honda et al. [78] making use of picolinic acid as derivatization reagent. The reaction makes use of 2-methyl-6-nitrobenzoic anhydride as a coupling promotor and 4-dimethylaminopyridine as a base. The resulting picolinic acid ester derivatives can be analyzed in the ESI+ mode making use of selected reaction monitoring (SRM). An application using pentafluorophenyl isocyanate forming the corresponding carbamates was described by Kuo et al. [79]. The authors applied the derivatization procedure for the analysis of cholesterol in atherosclerotic plaques using APCI in the negative mode as an ionization technique. An application combining nano-LC and negative mode ESI (ESI-) was described by Sandhoff et al. [80]. The method is based on transforming the available alcohol at position 3 into a sulfate group using sulfur trioxide pyridine complex. Due to the high ionization efficiency of sulfate groups, this procedure should result in derivatives producing high response in ESI-. Other derivatization strategies targeting at the 3-hydroxy function are: esterification using acetyl chloride [81], generation of a *N*-methylpyridyl ether [82], a ferrocenecarbamate ester [83], a mono-(dimethylaminoethyl) succinyl ester [84], and a dimethylglycine ester [85]. Most of the aforementioned derivatization strategies have been developed for the detection of cholesterol (**10**) and 7-DHC (**9**) in the context of screening for metabolic changes in SLOS patients. In contrast, the derivatization approach employing picolinic acid has been developed and tested for cholesterol and its biosynthetic precursors in particular. Therefore, this technique seems to be the most reliable one within the field of derivatization based LC–MS(MS) analysis of cholesterol and its precursors. An approach developed particularly for the detection of **9** (and possibly other $\Delta^{5,7}$-diene sterols) making use of a different kind of derivatization chemistry, namely a Diels–Alder reaction between the $\Delta^{5,7}$-diene system and 4-phenyl-1,2,4-triazoline-3,5-dione was described by Liu et al. [86]. This approach might possibly be advantageous in stabilizing the particularly easily oxidizable $\Delta^{5,7}$-diene sterols.

GC–MS(MS) Analysis

*General Aspects*

Regarding GC separation, the 5 % phenyl polymethylsiloxane fused-silica capillary columns are considered the standard stationary phases for sterol analysis [18–20, 26, 27, 29, 48, 52, 55–57, 59, 61, 63, 86–88], but also dimethyl polysiloxane [49, 87] and polyethylene glycol phases (WAX) are used [88]. An interesting comparison of a 5 % phenyl polymethylsiloxane (DB-5) and a WAX column has been presented by Gerst et al. [88] in an important overview about the analysis of sterols using GC. The separation on a non-polar phenyl polymethylsiloxane column is mainly based on volatility, while polar WAX columns show multiple retention mechanisms. Hence, both column types show some selectivity for certain sterols making them complementary in the analysis. However, for routine analysis, phenyl polymethylsiloxane should be preferred over polar columns due to their high separation efficiency combined with higher robustness and ease of use in combination with silylation reagents. With respect to the retention behavior of cholesterol (**10**) and its precursors, the following can be remarked: The higher the degree of unsaturation the longer usually the retention time, the same also counts for the addition of methyl and hydroxyl groups. Of particular interest also for sterol identification is the fact that the presence of a $\Delta^{24}$ double bond causes an increase in the relative retention time (RRT) of approximately 3 % under the here described conditions (see online resource 2 and Table 1).

More recent developments in the field of GC–MS analysis such as the use of narrow-bore columns for high-throughput analysis [89] or the application of vacuum outlet GC analysis [90] have to our knowledge only been

investigated to some extent [19]. The two main detection techniques coupled with GC for the analysis of various classes of sterols, bile acids, cholesterol/ergosterol precursors, steroid hormones, and phytosterols in a variety of biological matrices are flame ionization detection (FID) [42, 62, 87, 88, 91] and MS detection [51, 52, 55–57, 63, 88, 92]. While FID is a robust technique, its limited sensitivity and its lack of structural information make MS detection an attractive alternative in the field of sterol analysis. The two main types of mass spectrometers used in the field are ion trap (IT) [19, 59] and quadrupole-based instruments [56, 57]. Depending on the aim of the study, both types of mass spectrometers can be used in different modes of operation. The scan mode is mainly used if structural or isotopologue analysis is to be carried out [19, 20, 26, 59, 63, 87, 92] with IT instruments in our experience having an approximately 10 times higher sensitivity in scan mode when compared to quadrupole instruments (see also [93]). Both types of instruments can be used in the selected ion monitoring (SIM) mode [49, 52, 57, 61, 63], respectively, sometimes called single-ion storage (SIS) mode in IT instruments, selectively monitoring only a limited number of ions. This mode of operation is usually applied for quantification purposes as higher sensitivities are achieved. IT as well as triple-quadrupole instruments are also capable of being operated in the tandem MS mode with applications up to date being rather limited [56, 94]. With respect to the applied ionization techniques, positive chemical ionization (PCI) with ammonia as reagent gas was applied for the analysis of TMS ethers of 24-, 25-, and 27-hydroxycholesterol in human plasma and electron capture negative ionization (ECNI) for the TMS ether of 7-hydroxycholesterol [56]. ECNI might be a particularly useful technique when combined with (per)fluorinated or other suitable derivatization reagents [51] (see below). However, electron ionization (EI) is still the most commonly applied ionization technique in GC–MS analysis of sterols [18–20, 26, 27, 29, 52, 57, 59, 63, 92, 95]. The main reasons for this are the ease of use particularly in combination with silylation, the highly comparable mass spectrometric data obtained, and the universal applicability of this ionization technique. As already emphasized, sterols are mainly analyzed by GC–MS after derivatization (see below), the main reason for this is the fact that underivatized sterols tend to show breakdown catalyzed by active sites mainly in the GC inlet system [63, 95]. Furthermore, improved stability of thermolabile sterols (e.g., $\Delta^{5,7}$-sterols), increased volatility accompanied by improved chromatographic properties (peak shape, resolution, sensitivity), and characteristic fragmentations (Table 3 and online resource 1) [51, 63, 87, 92, 95] are observed after derivatization. Nevertheless, some examples provide data on the analysis of underivatized sterols [52, 88, 91].

*Derivatization Strategies and Fragmentation Characteristics*

As lined out above, the main purpose of sterol derivatization for GC-based analysis is stabilization of the analytes as well as improvement of chromatographic and spectrometric analyte characteristic. In general, the following possibilities are pursued: alkylation, acylation and silylation of the secondary alcohol at C3. This free hydroxyl group usually allows convenient and entire derivatization [49, 87]. Nevertheless, derivatization is still one of the crucial steps in GC analysis [63].

With respect to alkylation has particularly *O*-methylation with 10 % boron trifluoride methanol solution ($BF_3$/MeOH) at 100 °C, a reaction frequently used for the preparation of fatty acid methyl esters (FAME), been used for the analysis of sterols [58, 96]. However, the reaction conditions may be too drastic for labile analytes [97]. MS analysis of the resulting sterol methyl ethers showed an intense molecular ion $[M]^+$ and a prominent $[M\text{-MeOH}]^+$ peak [95]. Hwang et al. [58] provide an overview of different methylation strategies. Yet, reports about sterol methylation (alkylation) preceding GC–MS analysis are limited in the literature.

Acetylation of sterols has found a much wider application than alkylation [95]. The reaction is usually carried out using pyridine and acetic anhydride (1:1) [88, 96]. Gerst et al. [88] have described the EI spectral data for a large set of sterol actetates, while such data are basically absent for the methyl ethers. Rahier and Benveniste presented a seminal review describing in detail the fragmentation patterns of free and *O*-acetylated phytosterols [98]. As the described sterols share structural features with the here discussed cholesterol precursors, this work can be regarded as excellent reference particularly for the *O*-acetylated and free forms of the here described analytes [98]. *O*-acetylated cholesterol precursors with $\Delta^5$ structure (**9**, **10**, **11**, **12**, **22**, **23**, **24**) would not show a strong $[M]^+$ ion, instead a strong $[\text{M-acetic acid}]^+$ fragment occurs [88, 95]. For more details on the fragmentation of *O*-acetylated sterols, please refer to ref. [98]. An alternative and highly sensitive detection could be accomplished applying ECNI when sterols were esterified with fluorinated reagents, e.g., pentafluorobenzoyl bromide, trifluoroacetic or perfluorpropionic anhydride [51, 64, 95]. Alternatively, Řimnáčová et al. [64] just recently described the use of fluorinated chloroformates for the analysis of sterols in human serum and amniotic fluid. The hydroxyl groups were converted into mixed carbonates using different chloroformates. The derivatization reaction is fast, combination with a clean-up step is possible (liquid–liquid (micro-)extraction) and the products were described to be stable. Although the authors in their study only used EI, the formed mixed sterol carbonates containing several fluorine atoms might as well ideally be suited

**Table 3** MS fragments of sterol TMS ethers, relative abundances for diagnostic ions; bold base peak (100 %)

| | $[M]^+$ | $[M-(CH_3)]^+$ | $[M-TMSiOH]^+$ | $[M-(CH_3 + TMSiOH)]^+$ | $[M-(SC)]^+$ | $[M-(SC + 2H)]^+$ | $[M-(TMSiO + C_3H_4)]^+$ | $[M-(TMSiO + C_3H_6)]^+$ | $[M-(TMSiO + C_3H_8)]^+$ | $[M-(TMSiOH + SC)]^+$ | $[M-(TMSiOH + SC + 2H)]^+$ | $[M-(TMSiOH + C_3H_6 + SC)]^+$ | $[M-(CH_3 + TMSiOH + SC\text{-}H)]^+$ | $[M-(TMSiOH + C_3H_6 + SC\text{-}H)]^+$ | $M-(CH_3 + TMSiOH + C_3H_6 + SC\text{-}H)]^+$ | | $[TMSiO\text{-}C_3H_4]^+$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **3** | 498 (12) | 483 (10) | 408 (0) | **393** (100) | 387 (0) | 385 (0) | 369 (0) | 367 (0) | 365 (0) | 297 (10) | 295 (3) | 285 (4) | 271 (7) | 255 (10) | 241 (21) | 135 (8) | 129 (15) |
| **4** | 500 (0) | 485 (4) | 410 (0) | **395** (100) | 387 (0) | 385 (0) | 371 (0) | 369 (0) | 367 (0) | 299 (0) | 297 (0) | 285 (0) | 271 (0) | 255 (4) | 241 (5) | 135 (5) | 129 (5) |
| **5** | 484 (55) | 469 (6) | 394 (25) | **379** (100) | 371 (6) | 369 (5) | 355 (25) | 353 (12) | 351 (40) | 281 (25) | 279 (0) | 269 (0) | 255 (5) | 239 (6) | 225 (5) | 135 (0) | 129 (5) |
| **6** | **486** (100) | 471 (13) | 396 (92) | 381 (68) | 373 (5) | 371 (6) | 357 (0) | 355 (0) | 353 (25) | 283 (25) | 281 (0) | 271 (5) | 257 (8) | 241 (45) | 227 (7) | 135 (68) | 129 (30) |
| **7** | **458** (100) | 443 (37) | 368 (23) | 353 (45) | 345 (15) | 343 (0) | 329 (0) | 327 (0) | 325 (0) | 255 (25) | 253 (0) | 243 (14) | 229 (25) | 213 (47) | 199 (10) | 135 (15) | 129 (20) |
| **8** | **458** (100) | 443 (37) | 368 (23) | 353 (38) | 345 (13) | 343 (0) | 329 (0) | 327 (0) | 325 (0) | 255 (57) | 253 (0) | 243 (13) | 229 (25) | 213 (50) | 199 (10) | 135 (12) | 129 (8) |
| **9** | 456 (12) | 441 (0) | 368 (26) | **351** (100) | 343 (5) | 341 (0) | 327 (4) | 325 (72) | 323 (0) | 253 (15) | 251 (3) | 241 (0) | 227 (6) | 211 (15) | 197 (8) | 135 (2) | 129 (12) |
| **10** | 458 (41) | 443 (12) | **368** (100) | 353 (48) | 345 (5) | 343 (3) | 329 (68) | 327 (7) | 325 (2) | 255 (34) | 253 (0) | 243 (5) | 229 (2) | 213 (20) | 199 (10) | 135 (12) | 129 (67) |
| **11** | 456 (21) | 441 (8) | 366 (27) | 351 (45) | 345 (37) | 343 (17) | 327 (10) | 325 (2) | 323 (2) | **253** (100) | 251 (2) | 243 (7) | 229 (5) | 213 (22) | 199 (10) | 135 (12) | 129 (70) |
| **12** | 454 (16) | 439 (0) | 364 (30) | **349** (100) | 343 (0) | 341 (0) | 325 (2) | 323 (47) | 321 (0) | 253 (8) | 251 (20) | 241 (5) | 227 (7) | 211 (5) | 197 (10) | 135 (2) | 129 (15) |
| **13** | 456 (3) | 441 (19) | 394 (0) | 379 (0) | 345 (10) | **343** (100) | 327 (0) | 325 (0) | 323 (0) | 253 (24) | 251 (0) | 243 (5) | 229 (8) | 213 (18) | 199 (5) | 135 (0) | 129 (5) |
| **14** | 456 (48) | 441 (89) | 366 (33) | **351** (100) | 345 (22) | 343 (27) | 327 (0) | 325 (2) | 323 (5) | 255 (12) | 253 (20) | 243 (10) | 229 (22) | 213 (60) | 199 (16) | 135 (10) | 129 (2) |
| **15** | 484 (48) | 469 (21) | 394 (82) | 379 (97) | 373 (2) | 371 (6) | 355 (11) | 353 (4) | 351 (35) | 283 (20) | 281 (12) | 271 (6) | 257 (17) | 241 (60) | 227 (12) | **135** (100) | 129 (52) |
| **16** | **482** (100) | 467 (27) | 392 (7) | 377 (71) | 371 (2) | 369 (0) | 353 (0) | 351 (8) | 349 (13) | 281 (12) | 279 (14) | 269 (2) | 255 (0) | 239 (10) | 225 (5) | 135 (2) | 129 (20) |
| **17** | 456 (39) | 441 (17) | 366 (11) | **351** (100) | 343 (2) | 341 (0) | 327 (0) | 325 (0) | 323 (0) | 253 (5) | 251 (15) | 241 (3) | 227 (0) | 211 (7) | 197 (3) | 135 (0) | 129 (5) |
| **18** | **486** (100) | 471 (7) | 396 (63) | 381 (48) | 373 (0) | 371 (0) | 357 (0) | 355 (0) | 353 (32) | 283 (20) | 281 (0) | 271 (0) | 257 (10) | 241 (33) | 227 (2) | 135 (44) | 129 (17) |
| **19** | **454** (100) | 439 (65) | 364 (4) | 349 (40) | 343 (8) | 341 (38) | 325 (0) | 323 (0) | 321 (3) | 253 (0) | 251 (10) | 241 (0) | 227 (5) | 211 (15) | 197 (2) | 135 (0) | 129 (7) |
| **20** | **472** (100) | 457 (21) | 382 (27) | 367 (49) | 359 (8) | 357 (10) | 343 (0) | 341 (0) | 339 (0) | 269 (23) | 267 (0) | 257 (8) | 243 (10) | 227 (52) | 213 (7) | 135 (10) | 129 (10) |
| **21** | 470 (66) | 455 (45) | 380 (43) | **365** (100) | 359 (3) | 357 (18) | 341 (0) | 339 (3) | 337 (12) | 269 (12) | 267 (18) | 257 (13) | 243 (28) | 227 (68) | 213 (17) | 135 (15) | 129 (20) |
| **22** | 456 (21) | 441 (5) | 366 (17) | **351** (100) | 343 (2) | 341 (0) | 327 (5) | 325 (97) | 323 (0) | 253 (12) | 251 (2) | 241 (2) | 227 (2) | 211 (2) | 197 (10) | 135 (0) | 129 (8) |
| **23** | 454 (33) | 439 (8) | 364 (24) | **349** (100) | 343 (0) | 341 (0) | 325 (0) | 323 (75) | 321 (0) | 253 (7) | 251 (31) | 241 (0) | 227 (5) | 211 (15) | 197 (8) | 135 (0) | 129 (5) |
| **24** | 452 (16) | 437 (0) | 361 (20) | **347** (100) | 343 (0) | 341 (7) | 323 (0) | 321 (3) | 319 (2) | 253 (16) | 251 (26) | 241 (12) | 227 (5) | 211 (2) | 197 (5) | 135 (0) | 129 (7) |
| **25** | 484 (2) | 469 (1) | 394 (14) | **379** (100) | 371 (0) | 369 (0) | 355 (4) | 353 (30) | 351 (12) | 281 (5) | 279 (0) | 269 (2) | 255 (5) | 239 (5) | 225 (6) | 135 (0) | 129 (2) |
| **26** | 460 (13) | 445 (51) | 370 (32) | 355 (72) | 347 (0) | 345 (0) | 331 (0) | 329 (0) | 327 (0) | 257 (5) | 255 (0) | 245 (3) | 231 (5) | **215** (100) | 201 (20) | 135 (18) | 129 (0) |

for highly selective ECNI analysis. As expected, fluorinated ester derivatives show a somewhat higher volatility when compared to their non-fluorinated counterparts, with fragmentation being basically identical [95, 96].

Although alkylation and acetylation have been described in the field of sterol analysis, silylation and particularly trimethylsilylation still is the most wide-spread derivatization strategy [51, 63, 95–97, 99]. This has to do with several facts: (a) when applying silylation, usually no further purification step is required after derivatization [99] (b) conversion of sterols into the corresponding TMS ethers usually is fast and quantitative (c) mild derivatization conditions can be applied (d) prolonged shelf life of the GC capillary column [95], and (e) characteristic fragmentations of sterol TMS ethers are observed [62, 88, 92, 95] (Table 3; online resource 1, Fig. 4).

Some practical aspects have to be emphasized: (a) TMS ethers are moisture-sensitive (b) they should be handled in aprotic solvents (c) basic additives (pyridine, imidazole) or catalysts (1–2 % trimethylchlorosilane (TMCS) or trimethyliodosilane (TMIS)) are required for some reactions and (d) clean-up is mandatory in combination with polyethylene glycol-coated columns (otherwise derivatization of stationary hydroxyl groups) [51, 63, 87, 99]. The standard silylation reagents for the formation of TMS ethers are BSTFA (*N,O*–bis(trimethylsilyl)trifluoroacetamide) and MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) [99]. Other common silylation reagents are TMCS [99] and TSIM (*N*-(trimethylsilyl)imidazole) [19, 62, 99]. Besides transformation into TMS ethers, also the formation of dimethylethylsilyl (DMES) and *tert*-butyldimethylsilyl (*t*BDMS) ethers has been described with the latter one usually requiring catalysts such as ammonium iodide while still being described as incomplete [63]. The retention times of sterol silyl ethers typically are: *t*BDMS > DMES > TMS.

Besides the above-mentioned reagents, also various mixtures of silylation reagents and additives have been described: MSTFA/TSIM [18, 29, 59], TSIM/TMCS/BSA (*N,O*-bis(trimethylsilyl)acetamide) [87], HMDS/TMCS/pyridine [55, 87], MSTFA/$NH_4I$/DTE (1,4-dithioerythritol) [49], MSTFA/DTE/TMIS [63], and MSTFA/TSIM/TMCS [49]. Saraiva et al. [63] compared several of the aforementioned mixtures, concluding that the use of MSTFA/DTE/TMIS shows the best relation between specificity and sensitivity when compared to BSTFA/TMCS or M*t*BSTFA/$NH_4I$ (*N*-Methyl-*N*-*tert*.-butyldimethylsilyltrifluoroacetamide) for the analysis of cholesterol precursors and phytosterols in human blood. However, it has to be critically noted that TMIS is able to produce iodinated products and further on is very sensitive to air, light and water [51]. Overall, several possibilities exist for silylating cholesterol and its precursors; however, due to the fact that only the 3-hydroxy group is to be derivatized usually BSTFA or MSTFA with the addition of small amounts of a catalyst such as TMCS, TSIM and/or pyridine result in fast and complete derivatization [57].

Below we describe characteristic fragmentations of cholesterol, its precursors, as well as several non-physiological sterols which can appear under multiple enzyme inhibition. The presented data were gathered in recent years in our laboratory analyzing TMS ethers using a 5 % phenyl polymethylsiloxane fused-silica capillary column and detection employing EI (70 eV) (for spectral data and experimental details, see online resources 1 and 2). We will discuss characteristic fragmentations of several sterols and present full spectral data for all listed analytes. The data presented here will allow the reader to identify a broad set of cholesterol precursors as well as non-physiological sterols (see Tables 1, 3, and online resource 1). The presented sterols have been synthesized in our laboratory as described elsewhere [19, 57], were isolated from large-scale yeast incubations [100], or have been observed in several studies carried out in our laboratory and identified based on characteristic fragmentations [19, 20, 59, 100] in combination with characteristic relative retention times (RRT). The latter fact is based on the observation that the sterols of the Bloch and Kandutsch–Russell pathway differ in the presence of a $\Delta^{24}$ double bond, a fact which can be translated into a characteristic RRT difference between a $\Delta^{24}$ saturated and unsaturated sterol of approximately 3 % (increment of 0.03–0.05), with the $\Delta^{24}$ unsaturated component eluting after the $\Delta^{24}$ saturated one. An early description of this concept can be found in [65]. Based on the fact that we have all sterols of the Kandutsch–Russell pathway available in our laboratory (and some of the Bloch pathway), we used the above given observation for backing up analyte identities deduced from mass spectrometric data as well as selective biosynthesis inhibition. For all analyte sources, see online resource 1.

Fragmentation of sterol TMS ethers has been studied in detail by Brooks et al. [92] and Gerst et al. [88]. Therefore, we will here focus on the precursors of cholesterol and several non-physiological sterols. Figure 4 gives an overview of characteristic fragmentations of sterol TMS ethers. All analytes give rise to fragments related to the sterol TMS ether group, namely the $[M\text{-}90]^+$ and $[M\text{-}105]^+$ ions being the $[M\text{-}TMSiOH]^+$ and $[M\text{-}(CH_3 + TMSiOH)]^+$ fragments.

$\Delta^5$-sterols give rise to a characteristic fragment of *m/z* 129 resulting from a fragmentation of the C1–C10 and C3–C4 bonds in ring **A** [92]. The di-unsaturated $\Delta^{5,7}$-sterols usually show a very low abundant $[M]^+$ ion with a relative abundance normally below 10 % [95] with the base peak being the $[M\text{-}105]^+$ fragment ion. Another characteristic ion of $\Delta^{5,7}$-sterols is a fragment ion with *m/z* $[M\text{-}131]^+$ resulting from an **A** ring fragmentation. The $\Delta^7$ and $\Delta^8$-sterols present very similar mass spectra, characteristically giving a strong abundance of the molecular ion

signal as well as a strong fragment ion at *m/z* 255, being somewhat more abundant in $\Delta^7$-sterols, resulting from a loss of the sterol side chain (SC) [95]. A characteristic fragment obtained for $\Delta^{8,14}$-di-unsaturated sterols is a fragment ion obtained at *m/z* 182 as well as a strong $[M-105]^+$ ion [95]. The here discussed $\Delta^{24}$-sterols lacking a C24 methyl group do usually not undergo fragmentation via a McLafferty rearrangement. The most characteristic fragments found for $\Delta^{24}$-unsaturated sterols are related to a cleavage of the side chain (SC), being the $[M-(SC + 2H)]^+$ and $[M-(SC + 2H + TMSiOH)]^+$ ions. The SC cleavage proceeds under simultaneous 17α-H and C18 migration as well as a 16ξ-H transfer under simultaneous C17/20 bond scission [101]. Other characteristic ions for $\Delta^{24}$-unsaturated sterols are the fragment ions at $[M-113]^+$ and $[M-203]^+$ with the latter one accompanied by a simultaneous loss of TMSiOH. While $\Delta^{5,24}$-unsaturated sterols present a characteristic ion at *m/z* 129, a signal at *m/z* 343 is observed for the $\Delta^{7,24}$ double bond isomer. Sterols possessing a C14 methyl group in combination with a $\Delta^8$ double bond, such as lanosterol (**3**) and dihydrolanosterol (**4**), usually present a very strong ion at $[M-105]^+$. In poly-unsaturated sterols, the here described fragmentation patterns overlay each other and are abundant to a different extent as can be visualized in the spectrum of cholesta-5,7,24-trien-3β-ol (**12**) presenting a weak $[M]^+$, a strong $[M-105]^+$ as well as a strong $[M-131]^+$ and ions at *m/z* 364 and 251. Overall several characteristic ions exist for defining the exact positions of double bonds within the sterol backbone; however, some sterol isomers are very difficult to be distinguished from each other. Therefore, full spectra matching as well as retention time comparison either with synthetic standards or if not available, such as in the case of the vast majority of the $\Delta^{24}$ unsaturated sterols, with their saturated counterparts are crucial for the unambiguous identification of sterols. Nonetheless, some sterol isomers might be hardly distinguished from each other by the use of GC–MS as for example described by Gerst et al. [88], and by Norton and Nes [102] for $\Delta^{6,8}$ and $\Delta^{5,7}$ sterols. In these cases, MS/MS or orthogonal chromatographic techniques such as the analysis on a different type of GC column or the use of silver-ion chromatography pose possible solutions. Further on, when working in mammalian systems, it appears justified to also take plausible biochemical pathways into account.

## Conclusion and Perspectives

Cholesterol and its precursors have for many years been the subjects of numerous studies. Several noble prices have been given to researchers dedicated to this intriguing biochemical pathway (i.e., Konrad Bloch and Feodor Lynen [103]) additionally underlining its fundamental importance for many aspects of today's life sciences research. Besides cholesterol's (**10**) tremendous importance for membrane fluidity has recent research clearly underlined the involvement of cholesterol as well as its precursors in several physiological and pathophysiological processes. In recent years several pitfalls of sterol analysis have been overcome, with GC–MS certainly being the most versatile technique for the analysis of cholesterol (**10**) and its precursors in form of their TMS ethers. Moreover, in the past decade, several reports have described important analytical characteristics of several cholesterol precursors and numerous substances have become commercially available, facilitating research in the field. Overall, the analytical techniques necessary for studying cholesterol and its precursors are largely available today. Hence, the application and adaptation of the here described analytical approaches will be highly useful in future studies unraveling new and exciting functions of cholesterol and its precursors in physiological and pathophysiological processes.

## References


1. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Di Y, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. doi:10.1038/nature08530
2. Kloos D-P, Lingeman H, Mayboroda OA, Deelder AM, Niessen WMA, Giera M (2014) Analysis of biologically-active, endogenous carboxylic acids based on chromatography-mass spectrometry. Trends Anal Chem. doi:10.1016/j.trac.2014.05.008
3. Byskov AG, Andersen CY, Nordholm L, Thogersen H, Guoliang X, Wassmann O, Andersen JV, Guddal E, Roed T (1995) Chemical structure of sterols that activate oocyte meiosis. Nature. doi:10.1038/374559a0
4. Spann NJ, Glass CK (2013) Sterols and oxysterols in immune cell function. Nat Immunol. doi:10.1038/ni.2681
5. Spann NJ, Garmire LX, McDonald JG, Myers DS, Milne SB, Shibata N, Reichart D, Fox JN, Shaked I, Heudobler D, Raetz CR, Wang EW, Kelly SL, Sullards MC, Murphy RC, Merrill AH Jr, Brown HA, Dennis EA, Li AC, Ley K, Tsimikas S, Fahy E, Subramaniam S, Quehenberger O, Russell DW, Glass CK (2012) Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. Cell. doi:10.1016/j.cell.2012.06.054
6. Rodríguez-Acebes S, de la Cueva P, Fernández-Hernando C, Ferruelo AJ, Lasunción MA, Rawson RB, Martínez-Botas J, Gómez-Coronado D (2009) Desmosterol can replace cholesterol in sustaining cell proliferation and regulating the SREBP pathway in a sterol-Δ24-reductase-deficient cell line. Biochem J. doi:10.1042/BJ20081909
7. Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. doi:10.1038/35036052
8. Rodgers MA, Villareal VA, Schaefer EA, Peng LF, Corey KE, Chung RT, Yang PL (2012) Lipid metabolite profiling identifies desmosterol metabolism as a new antiviral target for hepatitis C virus. J Am Chem Soc. doi:10.1021/ja207391q
9. Rodgers MA, Saghatelian A, Yang PL (2009) Identification of an overabundant cholesterol precursor in hepatitis B virus

replicating cells by untargeted lipid metabolite profiling. J Am Chem Soc. doi:10.1021/ja809949r
10. Sánchez-Wandelmer J, Dávalos A, de la Peña G, Cano S, Giera M, Canfrán-Duque A, Bracher F, Martín-Hidalgo A, Fernández-Hernando C, Lasunción MA, Busto R (2010) Haloperidol disrupts lipid rafts and impairs insulin signaling in SH-SY5Y cells. Neuroscience. doi: 10.1016/j.neuroscience.2010.01.051
11. Blake GJ, Ridker PM (2001) Novel clinical markers of vascular wall inflammation. Circ Res. doi:10.1161/hh2101.099270
12. Porter FD (2008) Smith-Lemli-Opitz syndrome: pathogenesis, diagnosis and management. Eur J Hum Genet. doi:10.1038/ejhg.2008.10
13. Chugh A, Ray A, Gupta JB (2003) Squalene epoxidase as hypocholesterolemic drug target revisited. Prog Lipid Res 42:37–50
14. Matzno S, Yamauchi T, Gohda M, Ishi N, Katsuura K, Hanasaki Y, Tokunaga T, Itoh H, Nakamura N (1997) Inhibition of cholesterol biosynthesis by squalene epoxidase inhibitor avoids apoptotic cell death in L6 myoblasts. J Lipid Res 38:1639–1648
15. Mark M, Müller P, Maier R, Eisele B (1996) Effects of a novel 2,3-oxidosqualene cyclase inhibitor on the regulation of cholesterol biosynthesis in HepG2 cells. J Lipid Res 37:148–158
16. Chuang JC, Valasek MA, Lopez AM, Posey KS, Repa JJ, Turley SD (2014) Sustained and selective suppression of intestinal cholesterol synthesis by Ro 48-8071, an inhibitor of 2,3-oxidosqualene:lanosterol cyclase in the BALB/c mouse. Biochem Pharmacol. doi:10.1016/j.bcp.2014.01.031
17. Lasunción MA, Martín-Sánchez C, Canfrán-Duque A, Busto R (2012) Post-lanosterol biosynthesis of cholesterol and cancer. Curr Opin Pharmacol. doi:10.1016/j.coph.2012.07.001
18. Lange S, Keller M, Müller C, Oliaro-Bosso S, Balliano G, Bracher F (2013) Aminoproylindenes derived from Grundmann's ketone as a novel chemotype oxidosqualene cyclase inhibitors. Eur J Med Chem. doi:10.1016/j.ejmech.2013.03.002
19. Giera M, Plössl F, Bracher F (2007) Fast and easy in vitro screening assay for cholesterol biosynthesis inhibitors in the post-squalene pathway. Steroids. doi:10.1016/j.steroids.2007.04.005
20. Giera M, Renard D, Plössl F, Bracher F (2008) Lathosterol side chain amides—a new class of human lathosterol oxidase inhibitors. Steroids. doi:10.1016/j.steroids.2007.10.015
21. Popják G, Meenan A, Parish EJ, Nes WD (1989) Inhibition of cholesterol synthesis and cell growth by 24(R,S),25-iminolanosterol and triparanol in cultured rat hepatoma cells. J Biol Chem 264:6230–6238
22. Lepesheva GI, Waterman MR (2007) Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta. doi:10.1016/j.bbagen.2006.07.018
23. Canfrán-Duque A, Casado ME, Pastor Ó, Sánchez-Wandelmer J, de la Peña G, Lerma M, Mariscal P, Bracher F, Lasunción MA, Busto R (2013) Atypical antipsychotics alter cholesterol and fatty acid metabolism in vitro. J Lipid Res. doi:10.1194/jlr.M026948
24. Beynen AC, Buechler KF, Van der Molen AJ (1981) Inhibition of lipogenesis in isolated hepatocytes by 3-amino-1,2,4-triazole. Toxicology 22:171–178
25. Suárez Y, Fernández C, Gómez-Coronado D, Ferruelo AJ, Dávalos A, Martínez-Botas J, Lasunción MA (2004) Synergistic upregulation of low-density lipoprotein receptor activity by tamoxifen and lovastatin. Cardiovasc Res. doi:10.1016/j.cardiores.2004.06.024
26. König M, Müller C, Bracher F (2013) Stereoselective synthesis of a new class of potent and selective inhibitors of human Δ8/7-sterol isomerase. Bioorg Med Chem. doi:10.1016/j.bmc.2013.01.041
27. Krojer M, Müller C, Bracher F (2014) Steroidomimetic aminomethyl spiroacetals as novel inhibitors of the enzyme Δ8,7-sterol isomerase in cholesterol biosynthesis. Arch Pharm Pharm Med Chem. doi:10.1002/ardp.201300296
28. Correa-Cerro LS, Porter FD (2005) 3β-Hydroxysterol Δ7-reductase and the Smith–Lemli–Opitz syndrome. Mol Genet Metab. doi:10.1016/j.ymgme.2004.09.017
29. Horling A, Müller C, Barthel R, Bracher F, Imming P (2012) A new class of selective and potent 7-dehydrocholesterol reductase inhibitors. J Med Chem. doi:10.1021/jm3006096
30. Nes WD (2011) Biosynthesis of cholesterol and other sterols. Chem Rev. doi:10.1021/cr200021m
31. Trapani L, Segatto M, Ascenzi P, Pallottini V (2011) Potential role of nonstatin cholesterol lowering agents. IUBMB Life. doi:10.1002/iub.522
32. Brown AJ (2009) 24(*S*),25-Epoxycholesterol: a messenger for cholesterol homeostasis. Int J Biochem Cell Biol. doi:10.1016/j.biocel.2009.05.029
33. Sánchez-Martín CC, Dávalos A, Martín-Sánchez C, de la Peña G, Fernández-Hernando C, Lasunción MA (2007) Cholesterol starvation induces differentiation of human leukemia HL-60 Cells. Cancer Res. doi:10.1158/0008-5472.CAN-06-4093
34. Liang Y, Besch-Williford C, Aebi J, Mafuvadze B, Cook M, Zou X, Hyder S (2014) Cholesterol biosynthesis inhibitors as potent novel anti-cancer agents: suppression of hormone-dependent breast cancer by the oxidosqualene cyclase inhibitor Ro 48-8071. Breast Cancer Res Treat. doi:10.1007/s10549-014-2996-5
35. Grinter SZ, Liang Y, Huang S-Y, Hyder S, Zou X (2011) An inverse docking approach for identifying new potential anti-cancer targets. J Mol Graphics Modell. doi:10.1016/j.jmgm2011.01.002
36. Nishimura T, Kohara M, Izumi K, Kasama Y, Hirata Y, Huang Y, Shuda M, Mukaidani C, Takano T, Tokunaga Y, Nuriya H, Satoh M, Saito M, Kai C, Tsukiyama-Kohara K (2009) Hepatitis C virus impairs p53 via persistent overexpression of 3β-hydroxysterol Δ24-reductase. J Biol Chem. doi:10.1074/jbc.M109.043232
37. Clark PJ, Thompson AJ, Vock DM, Kratz LE, Tolun AA, Muir AJ, McHutchison JG, Subramanian M, Millington DM, Kelley RI, Patel K (2012) Hepatitis C virus selectively perturbs the distal cholesterol synthesis pathway in a genotype-specific manner. Hepatology. doi:10.1002/hep.25631
38. Di Stasi D, Vallacchi V, Campi V, Ranzani T, Daniotti M, Chiodini E, Fiorentini S, Greeve I, Prinetti A, Rivoltini L, Pierotti MA, Rodolfo M (2005) DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. Int J Cancer. doi:10.1002/ijc.20885
39. Xu F, Rychnovsky SD, Belani JD, Hobbs HH, Cohen JC, Rawson RB (2005) Dual roles for cholesterol in mammalian cells. Proc Natl Acad Sci USA. doi:10.1073/pnas.0503590102
40. Waterham HR (2006) Defects of cholesterol biosynthesis. FEBS Lett. doi:10.1016/j.febslet.2006.07.027
41. Herman GE (2003) Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. Hum Mol Genet. doi:10.1093/hmg/ddg072
42. Liu W, Xu L, Lamberson CR, Merkens LS, Steiner RD, Elias ER, Haas D, Porter NA (2013) Assays of plasma dehydrocholesteryl esters and oxysterols from Smith-Lemli-Opitz syndrome patients. J Lipid Res. doi:10.1194/jlr.M031732
43. De Fabiani E, Caruso D, Cavaleri M, Galli Kienle M, Galli G (1996) Cholesta-5, 7, 9(11)-trien-3β-ol found in plasma of patients with Smith–Lemli–Opitz syndrome indicates formation of sterol hydroperoxide. J Lipid Res 37:2280–2287
44. Aleck KA, Bartley DL (1997) Multiple malformation syndrome following fluconazole use in pregnancy: report of an additional patient. Am J Med Genet. doi:10.1002/(SICI)1096-8628(19971031)72:3<253::AID-AJMG1>3.0.CO;2-S

45. Fernández C, Suárez Y, Ferruelo AJ, Gómez-Coronado D, Lasunción MA (2002) Inhibition of cholesterol biosynthesis by delta22-unsaturated phytosterols via competitive inhibition of sterol delta24-reductase in mammalian cells. Biochem J. doi:10.1042/BJ20011777
46. Paik YK, Trzaskos JM, Shafiee A, Gaylor JL (1984) Microsomal enzymes of cholesterol biosynthesis from lanosterol. Characterization, solubilization, and partial purification of NADPH-dependent delta 8,14-steroid 14-reductase. J Biol Chem 259:13413–13423
47. Sánchez-Wandelmer J, Dávalos A, Herrera E, Giera M, Cano S, de la Peña G, Lasunción MA, Busto R (2009) Inhibition of cholesterol biosynthesis disrupts lipid raft/caveolae and affects insulin receptor activation in 3T3-L1 preadipocytes. Biochim Biophys Acta. doi:10.1016/j.bbamem.2009.05.002
48. Grimm C, Holdt LM, Chen C-C, Hassan S, Müller C, Jörs S, Cuny H, Kissing S, Schröder B, Butz E, Northoff B, Castonguay J, Luber CA, Moser M, Lüllmann-Rauch R, Fendel C, Klugbauer N, Griesbeck O, Haas A, Mann M, Bracher F, Teupser D, Saftig P, Biel M, Wahl-Schott C (2014) High susceptibility to fatty liver disease in two-pore channel 2 deficient mice. Nat. Commun. doi: 10.1038/ncomms5699
49. Kumar BS, Chung BC, Lee Y-J, Yi HJ, Lee B-H, Jung BH (2011) Gas chromatography-mass spectrometry-based simultaneous quantitave analytical method for urinary oxysterols and bile acids in rats. Anal Biochem. doi:10.1016/j.ab.2010.09.031
50. Griffiths WJ, Sjövall J (2010) Bile acids: analysis in biological fluids and tissues. J Lipid Res. doi:10.1194/jlr.R001941-JLR200
51. Choi MH, Chung BC (2014) Bringing GC-MS profiling of steroids into clinical applications. Mass Spectrom Rev. doi:10.1002/mas.21436
52. Keller S, Jahreis G (2004) Determination of underivatised sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography–mass spectrometry—single ion monitoring in faeces. J Chromatogr B. doi:10.1016/j.jchromb.2004.09.046
53. Vollhardt KPC, Schore NE (1999) Organic chemistry, structure and function. WH Freeman and company, New York
54. Seto H, Fujioka S, Koshino H, Takatsuto S, Yoshida S (2000) Stereo and chemical course of acid-catalyzed double bond migration of cholesta-5,7-dien-3β-ol to 5α-cholesta-8,14-dien-3β-ol. J Chem Soc, Perkin Trans 1. doi:10.1039/B001726O
55. Batta AK, Salen G, Batta P, Tint GS, Alberts DS, Earnest DL (2002) Simultaneous quantitation of fatty acids, sterols and bile acids in human stool by gas–liquid chromatography. J Chromatogr B. doi:10.1016/S1570-0232(02)00289-1
56. Matysik S, Klünemann HH, Schmitz G (2012) Gas chromatography–tandem mass spectrometry method for the simultaneous determination of oxysterols, plant sterols, and cholesterol precursors. Clin Chem. doi:10.1373/clinchem.2012.189605
57. Kloos D-P, Gay E, Lingeman H, Bracher F, Müller C, Mayboroda OA, Deelder AM, Niessen WMA, Giera M (2014) Comprehensive gas chromatography–electron ionisation mass spectrometric analysis of fatty acids and sterols using sequential one-pot silylation: quantification and isotopologue analysis. Rapid Commun Mass Spectrom. doi:10.1002/rcm.6923
58. Hwang B-S, Wang J-T, Choong Y-M (2003) A simplified method for the quantification of total cholesterol in lipids using gas chromatography. J Food Comp Anal. doi:10.1016/S0889-1575(02)00130-8
59. Müller C, Staudacher V, Krauss J, Giera M, Bracher F (2013) A convenient cellular assay for the identification of the molecular target of ergosterol biosynthesis inhibitors and quantification of their effects on total ergosterol biosynthesis. Steroids. doi:10.1016/j.steroids.2013.02.006
60. Porter NA (2013) A perspective on free radical autoxidation: the physical organic chemistry of polyunsaturated fatty acid and sterol peroxidation. J Org Chem. doi:10.1021/jo4001433
61. Paik M-J, Yu J, Hu M-B, Kim S-J,Kim K-R, Ahn Y-H, Choi S, Lee G (2008) Gas chromatographic-mass spectrometric analyses of cholesterol and its precursors in rat plasma as *tert*-butyldimethylsilyl derivatives. Clin Chim Acta. doi:10.1016/j.cca.2008.06.025
62. Nakanishi S, Nishino T, Nagai J, Katsuki H (1987) Characterization of nystatin-resistant mutants of *Saccharomyces cerevisiae* and preparation of sterol intermediates using the mutants. J Biochem 101:535–544
63. Saraiva D, Semedo R, da Conceição Castilho M, Silva JM, Ramos F (2011) Selection of the derivatization reagent—the case of human blood cholesterol, its precursors and phytosterols GC–MS analyses. J Chromatogr B. doi:10.1016/j.chromb.2011.10.021
64. Řimnáčová L, Hušek P, Šimek P (2014) A new method for immediate derivatization of hydroxyl groups by fluroalkyl chloroformates and its application for the determination of sterols and tocopherols in human serum and amniotic fluid by gas chromatography–mass spectrometry. J Chromatogr A. doi:10.2016/j.chroma.2014.03.007
65. Xu S, Norton RA, Crumley FG, Nes WD (1988). Comparison of the chromatographic properties of sterols, select addional steroids and triterpenoids: gravity-flow column liquid chromatography, thin-layer chromatography, gas-liquid chromatography and high-performance liquid chromatography. J Chromatogr. doi: 10.1016/S0021-9673(01)81462-X
66. Nes WR (1985). A comparison of methods for the identification of sterols. Methods Enzymol. doi:10.1016/S0076-6879(85)11003-7
67. Griffiths WJ, Wang Y (2011) Analysis of oxysterol metabolomes. Biochim Biophys Acta. doi:10.1016/j.bbalip.2011.05.012
68. van den Ouweland JW, Vogeser M, Bächer S (2013) Vitamin D and metabolites measurement by tandem mass spectrometry. Rev Endocr Metab Disord. doi:10.1007/s11154-013-9241-0
69. Mendiara I, Domeño C, Nerín C (2012) Development of a fast sample treatment for the analysis of free and bonded sterols in human serum by LC–MS. J Sep Sci. doi:10.1002/jssc.201200519
70. McDonald JG, Smith DD, Stiles AR, Russell DW (2012) A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma, J Lipid Res. doi:10.1194/jlr.D022285
71. Palmgrén JJ, Töyräs A, Mauriala T, Mönkkönen J, Auriola S (2005) Quantitative determination of cholesterol, sitosterol, and sitostanol in cultured Caco-2 cells by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry, J Chromatogr B. doi:10.1016/j.jchromb.2005.04.029
72. Ruan B, Shey J, Gerst N, Wilson WK, Schroepfer GJ (1996) Silver ion high pressure liquid chromatography provides unprecedented separation of sterols: application to the enzymatic formation of cholesta-5,8-dien-3β-ol. Proc Natl Acad Sci USA 93:11603–11608
73. Christie WW (1987) A stable silver-loaded column for the separation of lipids by high performance liquid chromatography. J High Res Chromatogr 10:148–150
74. Nikolova-Damyanova B (2009) Retention of lipids in silver ion high-performance liquid chromatography: facts and assumptions. J Chromatogr A. doi:10.1016/j.chroma.2008.10.097
75. Saracino MA, Iacono C, Somaini L, Gerra G, Ghedini N, Raggi MA (2014) Multi-matrix assay of cortisol, cortisone and corticosterone using a combined MEPS-HPLC procedure. J Pharm Biomed Anal. doi:10.1016/j.jpba.2013.10.008
76. Phenomenex HPLC Application ID No.: 22145 (2014) Steroid panel on Kinetex 2.6u biphenyl 50x3.0 http://www.phenomenex.com/application/print/22145 Accessed 10 July 2014

77. Shui G, Cheong WF, Jappar IA, Hoi A, Xue Y, Fernandis AZ, Tan BKH, Wenk MR (2011) Derivatization-independent cholesterol analysis in crude lipid extracts by liquid chromatography/mass spectrometry: Applications to a rabbit model for atherosclerosis, J Chromatogr A. doi:10.1016/j.chroma.2011.05.011
78. Honda A, Yamashita K, Miyazaki H, Shirai M, Ikegami T, Xu GF, Numazawa M, Hara T, Matsuzaki Y (2008) Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. J Lipid Res. doi:10.1194/jlr.D800017-JLR200
79. Kuo MS, Kalbfleisch JM, Rutherford P, Gifford-Moore D, Huang XD, Christie R, Hui K, Gould K, Rekhter M (2008) Chemical analysis of atherosclerotic plaque cholesterol combined with histology of the same tissue, J Lipid Res. doi:10.1194/jlr.D700037-JLR200
80. Sandhoff R, Brügger B, Jeckel D, Lehmann WD, Wieland FT (1999) Determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry. J Lipid Res 40:126–132
81. Liebisch G, Binder M, Schifferer R, Langmann T, Schulz B, Schmitz G (2006) High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Biochim Biophys Acta. doi:10.1016/j.bbalip.2005.12.007
82. Quirke JME, Adams CL, Van Berkel GJ (1994) Chemical derivatization for electrospray ionization mass spectrometry. 1. alkyl, halides, alcohols, phenols, thiols, and amines. Anal Chem. doi:10.1021/ac00080a016
83. Van Berkel GJ, Quirke JM, Tigani RA, Dilley AS, Covey TR (1998) Derivatization for electrospray ionization mass spectrometry. 3. Electrochemically ionizable derivatives. Anal Chem. doi:10.1021/ac00080a016
84. Johnson DW, ten Brink HJ, Jakobs C (2001) A rapid screening procedure for cholesterol and dehydrocholesterol by electrospray ionization tandem mass spectrometry. J Lipid Res 42:1699–1705
85. Jiang X, Ory DS, Han X (2007) Characterization of oxysterols by electrospray ionization tandem mass spectrometry after one-step derivatization with dimethylglycine, Rapid Commun Mass Spectrom. doi:10.1002/rcm.2820
86. Liu W, Xu L, Lamberson C, Haas D, Korade Z, Porter NA (2014) A highly sensitive method for analysis of 7-dehydrocholesterol for the study of Smith-Lemli-Opitz syndrome. J Lipid Res. doi:10.1194/jlr.D043877
87. Iida T, Hikosaka M, Goto J, Nambara T (2001) Capillary gas chromtographic behaviour of tert.–hydroxylated steroids by trialkylsilylation. J Chromatogr A. doi:10.1016/S0021-9673(01)01305-X
88. Gerst N, Ruan B, Pang J, Wilson WK, Schroepfer GJ Jr (1997) An updated look at the analysis of unsaturated C27 sterols by gas chromatography and mass spectrometry. J Lipid Res 38:1685–1701
89. Dömötörová M, Kirchner M, Matisová E, de Zeeuw J (2006) Possibilities and limitations of fast GC with narrow-bore columns. J Sep Sci. doi:10.1002/jssc.200500472
90. de Zeeuw J, Reese S, Cochran J, Grossman S, Kane T, English C (2009) Simplifying the setup for vacuum-outlet GC: using a restriction inside the injection port. J Sep Sci. doi:10.1002/jssc.200900009
91. Arca M, Montali A, Ciocca S, Angelico F, Cantafora A (1983) An improved gas–liquid chromatographic method for the determination of fecal neutral sterols. J Lipid Res 24:332–335
92. Brooks CJW, Horning EC, Young JS (1968) Characterization of sterols by gas chromatography–mass spectrometry of the trimethylsilyl ethers. Lipids 3:391–402
93. Vorce SP, Sklerov JH, Kalasinsky KS (2000) Assessment of the ion-trap mass spectrometer for routine qualitative and quantitative analysis of drugs of abuse extracted from urine. J Anal Toxicol. doi:10.1093/jat/24.7.595
94. Saraf A, Larsson L (1996) Use of gas chromatography/ion-trap tandem mass spectrometry for the determination of chemical markers of microorganisms in organic dust. J Mass Spectrom. doi:10.1002/(SICI)1096-9888(199604)31:4<389::AID-JMS312>3.0.CO;2-H
95. Goad LJ, Akihisa T (1997) Analysis of sterols. Blackie Academic & Professional, London
96. Vycudilik W (1977) Die Derivsatisierung in der gaschromatographischen Analyse. Z Rechtsmed 80:197–203
97. Macherey–Nagel Derivatization reagents for GC (2014) ftp://mn-net.com/english/Flyer_Catalogs/Chromatography/GC/KATEN200144_Deriv_Flyer_EN_220x200_web.pdf. Accessed 09 July 2014
98. Rahier A, Benveniste P (1988) Mass spectral identification of phytosterols. In: Nes WD, Parish EJ (eds). Analysis of sterols and other biologically significant isopentenoids, Academic Press, San Diego
99. Schummer C, Delhomme O, Appenzeller BMR, Wennig R, Millet M (2009) Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. Talanta. doi:10.1016/j.talanta.2008.09.043
100. Renard D, Perruchon J, Giera M, Müller J, Bracher F (2009) Side chain azasteroids and thiasteroids as sterol methyltransferase inhibitors in ergosterol biosynthesis. Biorg Med Chem. doi:10.1016/j.bmc.2009.09.037
101. Massey IJ, Djerassi C (1979) Mass spectrometry in structural and stereochemical problems. 252. Structural and stereochemical applications of mass spectrometry in the marine sterol field. Synthesis and electron impact induced mass spectral fragmentation of $\Delta^{24}$- and$\Delta^{24(28)}$-3β-hydroxy-$\Delta^{5}$-sterols. J Org Chem 44:2448–2456
102. Norton RA, Nes WD (1991). Identification of ergosta-6(7),8(14),25(27)-trien-3β-ol and ergosta-5(6),7(8),25(27)-trien-3β-ol, two new steroidal trienes synthesized by *Prototheca wickerhamii*. Lipids. doi:10.1007/BF02543980
103. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1964/bloch-facts.html