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Synthesis and search for 3β,3′β-disteryl ethers after high-temperature treatment of sterol-rich samples

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ABSTRACT

It has been proven that at increased temperature, sterols can undergo various chemical reactions e.g., oxidation, dehydrogenation, dehydration and polymerisation. The objectives of this study are to prove the existence of dimers and to quantitatively analyse the dimers (3β,3′β-disteryl ethers). Sterol-rich samples were heated at 180 °C, 200 °C and 220 °C for 1 to 5 h. Quantitative analyses of the 3β,3′β-disteryl ethers were conducted using liquid extraction, solid-phase extraction and gas chromatography coupled with mass spectrometry. Additionally, for the analyses, suitable standards were synthetized from native sterols. To identify the mechanism of 3β,3′β-disteryl ether formation at high temperatures, an attempt was made to use the proposed synthesis method. Additionally, due to the association of sterols and sterol derivatives with atherosclerosis, preliminary studies with synthetized 3β,3′β-disteryl ethers on endothelial cells were conducted.

1. Introduction

Sterols of both vegetable and animal origin are compounds that are relatively easily oxidized in autoxidation reactions. Radical or reactive forms of oxygen play a major role in reactions of this type. Sterol oxidation products are various chemical compounds that contain additional oxygen groups such as alcohol, ketone or ether. The formation of oxysterols/oxyphytosterols has been studied extensively (Derewiaka & Obiedziński, 2010; Schött & Lütjohann, 2015; Szterk & Pakuła, 2016). Various cholesterol oxidation products: 7-ketocholesterol (7-kCh), 7-hydroxycholesterol (α and β isomer), 5,6-epoxycholesterol (α and β isomer) and cholestane-3β,5,6β-triol have been proven to exhibit cytotoxicity as well as apoptotic and pro-inflammatory effects on human cells (Kulig, Cwiklik, Jurkiewicz, Rog, & Vattulainen, 2016). Additionally, after the discovery of the presence of oxysterol in atherosclerotic plaques in humans, their pro-atherosclerotic properties were postulated (Zmysłowski & Szterk, 2017, 2019). However, the formation of sterol oxidation products via oxidation is only one type of reaction that sterols can undergo at high temperatures. The reactions that occur above 180 °C are more diverse. In addition to oxidation reactions, dehydrogenation and dehydration reactions can occur (Chien, Wang, & Chen, 1998). It has also been proven that various sterols can be concatenated if subjected to high-temperature treatment, which results in polymeric structures (Derewiaka & Molińska (née Sosińska), 2015; Rudzińska, Przybylski, & Wąsowicz, 2009; Sosińska, Przybylski, Aladedunye, & Hazendonk, 2014; Struijs, Lampi, Ollilainen, & Piironen, 2010). After incubation for 3 h at 180 °C, 20% of the initially present sterols are converted into various dimers (Lampi, Kemmo, Mäkelä, Heikkinen, & Piironen, 2009; Menéndez-Carreño, Ansorena, Astiasarán, Piironen, & Lampi, 2010). Cholesterol transformations during heat treatment were studied by Derewiaka et al. (Derewiaka & Molińska (née Sosińska), 2015). The use of higher temperatures, namely, 180 °C and 220 °C, led to the formation of polymers and other products, such as cholestadienes and fragmented cholesterol molecules. Unfortunately, the structures of the analysed cholesterol polymers were not confirmed. Struijs et al. demonstrated the formation of stigmasterol polymers after heating at 180 °C (Struijs et al., 2010). Based on molecular mass obtained by the high-resolution mass spectrometry it was suggested that one of the found dimers consisted of two stigmasterol moieties linked with ether bond, which could correspond to the structure of 3β,3′β-distigmasteryl ether. A similar investigation was published in the same year by Rudzińska et al., which described the thermal oxidation of β-sitosterol and the production of dimers, trimers, and tetramers, but no results were presented on structural confirmation of the found polymers. (Rudzińska et al., 2009). Such research was done by Sosinska et al. (2013), which confirmed using combination of NMR, APCI/MS, IR and Raman spectroscopy, that the most abundant dimeric product of thermo-oxidation of sitosterol was 3β,3′β-disitosteryl ether (Sosińska, Przybylski, Hazendonk, Zhao, & Curtis, 2013). However, those reactions were studied only for heated sterols standards. Thus, a study

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should be conducted to determine whether such compounds can be formed in food or biological samples. Very little information is available on the properties of the dimers, such as their bioavailability and biological activity. Thus, it is desirable to synthesize such compounds to the search for these compounds in biological matrices using spectrometric methods and to evaluate their biological properties.

The objective of this study is to evaluate the possibility of formation of sterols dimers – 3β,3′β-disteryl ethers during thermal treatment at temperatures that are typical for food processing in a food matrix. Hence, an attempt was made to prepare 3β,3′β-disteryl ethers, which could be used as standards for an analytical method and as substances for biological studies. In addition, a probable formation mechanism was proposed based on the formation of various dimeric products. Additionally, due to the association of sterols and sterol derivatives with atherosclerosis, preliminary studies with synthetized 3β,3′β-disteryl ethers on endothelial cells were conducted.

2. Materials and methods

2.1. Materials

2.1.1. Chemical materials

All solvents for synthesis were of HPLC-grade quality. Isopropanol (IPA), tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), *n*-hexane (*n*-Hex), *n*-heptane (*n*-Hep), methanol (MeOH), diethyl ether (Et_2O), ethyl acetate (EtOAc), acetic anhydride (Ac_2O), methyl *tert*-butyl ether (MTBE), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), pyridine and acetone, along with cyclohexane (c-Hex) for gas chromatography MS SupraSolv®, were purchased from Merck Millipore. All reagents were purchased from Aldrich, except stigmasterol (Cayman Chemicals). The deuterated chloroform-d_1 (99.95 atom % D) was obtained from Dr Glaser at AG Basel. Rapeseed oil (crude, cold-pressed), corn oil (crude, cold-pressed) and butter (based on the label: 82% fat content) were purchased from a local market. Cod-liver oil was purchased from a local pharmacy. Thin-layer chromatography (TLC) was conducted on precoated silica gel plates (Merck 60 PF254). The solid phase extraction (SPE) was done using Stata silica SI-1 cartridges (500 mg, 3 mL, 55 μm particle size, 70 Å pore size) from Phenomenex (Torrance, CA, USA). Visualization of compounds on TLC plates was realized via ultraviolet (UV) (254 nm) light detection and/or iodine staining. The melting points (mp.) are uncorrected and were recorded on a capillary melting point apparatus. Dry column vacuum chromatography (DCVC) was performed using Merck Silica gel 60 (Pedersen & Rosenbohm, 2001). Montmorillonite K 10 (MK10) was dried for 2 h at 125°C before the reaction. Each compound was obtained in pure crystal form and was stored in 4 °C.

2.1.2. Biological materials

Endothelial cells TeloHAEC were purchased from ATCC. The endothelial cell growth medium for the *in vitro* culture was purchased from PAN-Biotech GmbH. The medium contains epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), vitamin C, insulin-like growth factor-1 (*R*3-IGF-1), 3% fetal bovine serum (FBS), gentamycin/amphotericin, hydrocortisone, and heparin. Trypsin TrypLe Express was purchased from Thermo Scientific. The MTT test was purchased from Sigma-Aldrich. 1,2-dioleoyl-*sn-glycero*-3-phosphocholine (DOPC) and 1,2-Distearoyl-*rac-glycero*-3-methylpolyoxyethylene (DSG-PEG 2000) were acquired from Avanti Polar.

2.2. Chemical synthesis

The synthesis methods of all used compounds (**1–33**) along with the ^1H and ^{13}C NMR data can be found in the Supplementary Data. The synthesis route is presented in Fig. 1.

2.3. Standard and sample preparation

2.3.1. Working solutions and calibration curve for the GC–MS method

The concentrations of 3β,3′β-disteryl ether were determined using a standard calibration curve with synthesized isotope-labelled standards (**13**, **14**, **15**). Isotope-labelled standards were dissolved in *n*-Hep to a stock concentration of 1.0 mg/mL. A working solution with a concentration of 1.0 μg/mL for internal standards (ISs) was prepared from the stock solution. Non-isotope-labelled standards were dissolved in *n*-Hep to a stock concentration of 1.0 mg/mL. A working solution was prepared from the 3β,3′β-disteryl ether stock solutions, which contained a mixture of ethers (except for the isotope-labelled standards) at a concentration of 1.2 μg/mL. Subsequent working solutions were prepared via suitable dilutions of the stock solution (1.2 μg/mL) to the desired concentrations. For calibration solutions, the following 3β,3′β-disteryl ether concentrations were used: 500, 250, 100, 75, 50, 25 and 10 ng/mL. To these solutions, isotope-labelled standards (100 ng/mL) were always added.

2.3.2. Sample preparation of biological origin samples

The samples were chosen based on their high native sterol concentrations (Derina et al., 2018; Verleyen et al., 2002). Butter and cod-liver oil due to high content of cholesterol were selected for analysing the 3β,3′β-dicholesteryl ether, crude rapeseed oil due to high content of β-sitosterol was selected for analysing the 3β,3′β-disitosteryl ether and crude corn oil due to high content of β-sitosterol and stigmasterol was selected for analysing the 3β,3′β-disitosteryl ether and the 3β,3′β-distigmasteryl ether. Prior to heating, the butter was clarified by melting it at low temperature (60 °C) and removing the undissolved sediment.

The prepared samples were heated in open glass vials for the durations and at the temperatures that are specified in Table 1 in an oven. The samples were taken out from the oven and were allowed to cool in room temperature, after which they were subjected to saponification. A portion of each sample (1.0 g in the cases of rapeseed oil, corn oil and cod-liver oil and 0.25 g in case of butter) after the heat treatment was mixed with 15 mL 1 M KOH in MeOH and left for 24 h in the dark. Next, all saponificated samples were extracted at least twice with 20 mL of *n*-Hex each, and the solvent was evaporated using a rotavapor. The samples were redissolved in 5 mL of *n*-Hex and extracted at least 3 times with 2 mL of MeOH. After the extraction, the solvent was evaporated in a stream of nitrogen and redissolved in 1 mL of *n*-Hex, and the samples were subjected to purification via solid-phase extraction.

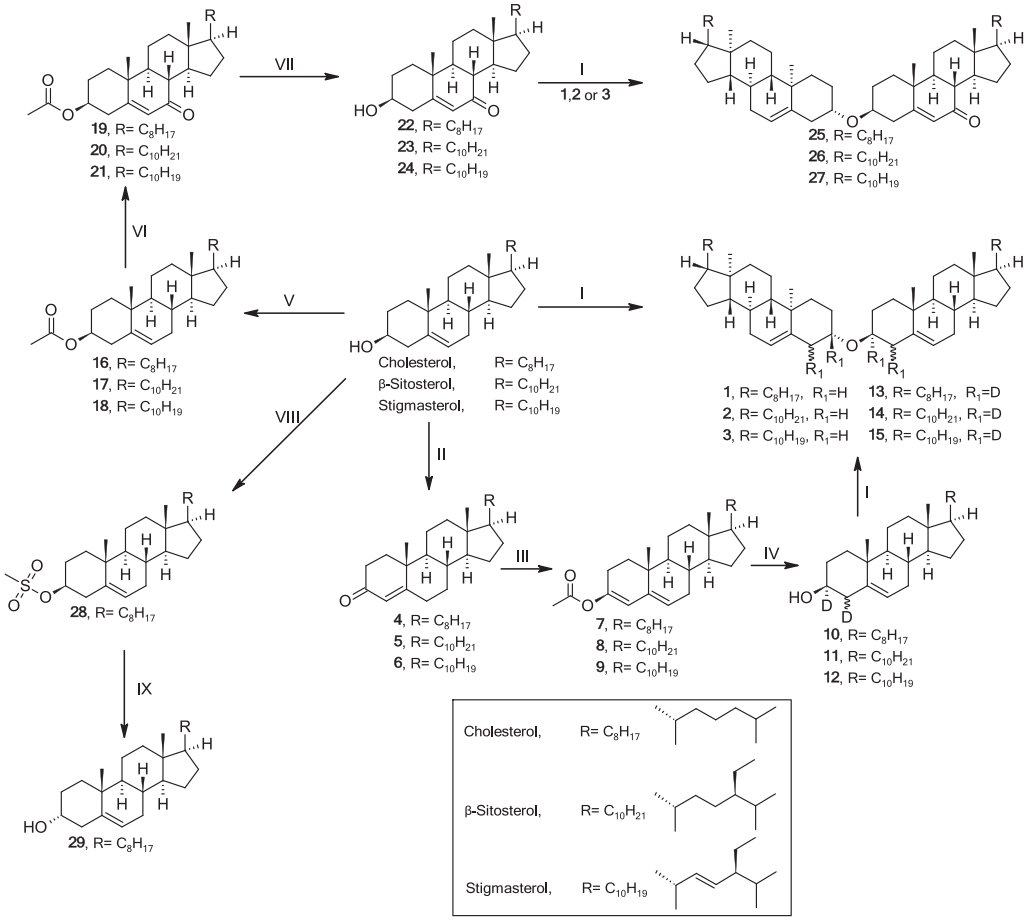
2.3.3. Solid phase extraction (SPE) for the determination of 3β,3′β-disteryl ethers

SPE cartridges were conditioned with 5 mL of *n*-Hex. On SPE, 1 mL of the *n*-Hex phase was applied. The cartridge was rinsed with 5 mL of *n*-Hex. 3β,3′β-Disteryl ether was eluted with 10 mL of *n*-Hex:CH_2Cl_2 10:1 (v/v) solution. The solvent was evaporated under a stream of nitrogen at 30 °C. The residue was dissolved in 1 mL *n*-Hep.

2.4. Chromatographic methods

2.4.1. Preparation of the silver impregnated silica column

The Zorbax Si column of dimensions 250 × 4.6 mm 5 μm was used for impregnation of silver ions. The column was flushed with 10 volumes of IPA and, subsequently, with 10 volumes of water. After column equilibration with water, it was subjected to a 200 mL solution of 5% $AgNO_3$ with a 0.5 mL/min flow rate. After the solution was consumed, the column was flushed with 2 volumes of water and, subsequently, with IPA. The lines of the HPLC solvent carrier were flushed with IPA to remove all water, and the column was flushed with *n*-Hep and stored in the same solvent prior to use.



Reagents and conditions: I) MK10, CH_2Cl_2, reflux, 24h; II) Shvo's cat., acetone, reflux, 24h; III) isopropenyl acetate, H_2SO_4, 100°C, 2h; IV) $NaBD_4$, EtOD, D_2O, 0°C, 1h; V) Ac_2O, pirydine, 37°C, overnight; VI) $Mn(Acac)_3$, t-ButOOH, molecular sieves, rt, 48h; VII) LiOH, THF:MeOH:H_2O (3:1:1), rt, 2h; VIII) $MeSO_2Cl$, TEA, CH_2Cl_2, 0°C 2h, 25°C 16h; IX) KO_2, 18-crown-6, DMSO:DMF (anhydrous) (1:1), 12h

Fig. 1. Synthesis of sterol derivatives.

Table 1
Measurements of the 3β,3′β-disteryl ethers [μg/g] that are formed during thermal processing of sterol in various matrices at various temperatures. [a,b,c])Values within the same temperature of processing with different letters are significantly different ($p < 0.05$).

Food matrix		Contents of 3β,3′β-disteryl ethers [μg/g]					
(n = 3)	Time [h]	Temperature					
		180 °C		200 °C		220 °C	
				dCh			
Cod-liver oil	1	< LOD		< LOD		0.041 ± 0.023[a]	
	2	< LOD		0.006 ± 0.009[a]		0.020 ± 0.008[b]	
	3	0.019 ± 0.005[a]		0.020 ± 0.006[b]		0.016 ± 0.004[b]	
	4	0.018 ± 0.010[a]		0.030 ± 0.013[b]		0.015 ± 0.003[b]	
	5	0.021 ± 0.005[a]		0.023 ± 0.007[b]		0.016 ± 0.007[b]	
Butter	1	0.116 ± 0.015[a]		0.199 ± 0.029[a]		0.374 ± 0.060[a]	
	2	0.134 ± 0.018[a]		0.240 ± 0.051[a]		0.231 ± 0.094[b]	
	3	0.150 ± 0.026[a]		0.254 ± 0.029[b]		0.234 ± 0.064[b]	
	4	0.210 ± 0.029[b]		0.331 ± 0.056[c]		0.257 ± 0.053[b]	
	5	0.200 ± 0.025[b]		0.325 ± 0.052[c]		0.165 ± 0.079[a]	
		dStig	dSito	dStig	dSito	dStig	dSito
Rapeseed oil	1	< LOD	< LOD	< LOD	< LOD	< LOD	0.043 ± 0.010[a]
	2	< LOD	< LOD	< LOD	< LOD	< LOD	0.022 ± 0.008[b]
	3	< LOD	< LOD	< LOD	0.040 ± 0.005[a]	< LOD	< LOD
	4	< LOD	< LOD	< LOD	0.049 ± 0.015[a]	< LOD	< LOD
	5	< LOD	0.040 ± 0.004	< LOD	0.044 ± 0.005[a]	< LOD	< LOD
Corn oil	1	< LOD	< LOD	< LOD	< LOD	0.030 ± 0.012[a]	0.034 ± 0.027[a]
	2	< LOD	< LOD	< LOD	0.026 ± 0.014[a]	0.010 ± 0.007[b]	0.017 ± 0.014[b]
	3	< LOD	< LOD	< LOD	0.028 ± 0.029[a]	< LOD	< LOD
	4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	5	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

2.4.2. HPLC method for 3β,3′β-disteryl ether synthesis optimization

The synthesis optimization was conducted using a Thermo Scientific Accela Model 430 bar liquid chromatograph, an autosampler (10 °C), a thermostat for the column (25 °C), and a diode array detector (DAD, 210 nm). 3β,3′β-Dicholesteryl ether was separated on Zorbax Si 250x4.6 mm 5 μm using *n*-Hep as the mobile phase. The sample was dissolved in 1 mL of *n*-Hep after the evaporation of the reaction solvent. The injection volume was 5 μL.

2.4.3. Preparative separation of 3β,3′β-disteryl ether

Preparative chromatography was conducted using a LC-20 Prominence (Shimadzu) system, which consisted of a pump, an autosampler (ambient temperature), a thermostat for the column (25 °C), a diode array detector (DAD, 210 nm) and a fraction collector. 3β,3′β-Disteryl ethers were separated on a silver-impregnated silica column, namely, Zorbax Si of dimensions 250 × 4.6 mm 5 μm, using 0,20% MTBE in *n*-Hep. The major fractions were collected into separate collector vials. After several injections and runs, the organic solvents in the collected fractions were removed by a gentle nitrogen stream with no heat.

2.4.4. GC–MS method for 3β,3′β-disteryl ethers

A Shimadzu GC-2010 Plus with an Optic-4 autosampler that was coupled with an MS-TQ8050 mass spectrometer was used. 3β,3′β-disteryl ethers were separated on ZB-1HT of dimensions 30 m × 0.25 mm with a film thickness of 0.1 μm. The carrier gas was helium with an inlet pressure of 318 kPa and a flow rate of 68.6 cm/s. The initial column temperature of 280 °C was held for 2 min and increased by 10 °C/min to 390 °C, where it was held for 10 min. Injections (20.0 μL) were conducted in a split program mode (Table S7). The injector temperature was 70 °C for 45 s, with the solvent valve open, which was increased with a temperature gradient of 40 °C/s to 360 °C. The transfer line temperature was 325 °C, the ion source temperature was 280 °C and the energy of the electron impact ionization was 70 eV. The 3β,3′β-disteryl ethers were analysed using selected ion monitoring (SIM). For 3β,3′β-dicholesteryl ether, 369 *m/z* were monitored, for 3β,3′β-distigmasteryl ether 394 *m/z* and for 3β,3′β-disitosteryl ether 397 *m/z*.

2.4.5. Method validation

The method validation was described in previous works (Szterk, Bus, et al., 2018; Szterk, Zmysłowski, et al., 2018). The linearity was evaluated, and the limits of detection (LOD) and limits of quantitation (LOQ) were calculated based on the standard deviation of the response and the slope of the analytical curve. Recovery tests were conducted using three concentration levels: low concentration (25 ng/mL), medium concentration (100 ng/mL) and high concentration (250 ng/mL)

2.4.6. LC-QTOF-MS methods

A UHPLC Ultimate 3000 (Dionex Thermo Fisher Scientific, Sunnyvale, California, USA) system, which consisted of a pump, a degasser, an autosampler, and a column heater instrument, was used. Data processing was conducted with Chromeleon 6.8 and Chromeleon Validation ICH software (Dionex). To determine the peak elution order, a mass spectrometer maXis 4G from Bruker Daltonic (Billerica, Massachusetts, USA) was used. The QTOF settings were as follows: atmospheric pressure chemical ionization (APCI) in positive ion mode, nebulizer 2.0 bar, dry gas (nitrogen) flow rate of 4.0 L/min, dry heater at 200 °C, vaporizer temperature 450 °C, capillary voltage of 3000 V, corona discharge 3000nA and end plate offset of −500 V. The MS data were recorded in full scan mode (from 100 to 1600 *m/z*). The mass spectrometer was used in high-resolution mode (R = 60000), and an internal calibrant (APCI/APPI calibrant) was used to obtain a precise mass measurement. Chromatographic separation was conducted using a Zorbax XBD-C18 50 × 2.1 mm with a 1.8 μm particle size in a gradient system (phase A: MeOH, phase B: CH_2Cl_2). The following gradient system was applied: 0–2 min 0% B, 2–10 min 0–60% B, 10–12 min 60% B, and 12–15 min 0% B (balancing the column until the initial conditions are restored). Chromatographic separation was conducted with a constant flow of the mobile phase (400 μL/min) at a temperature of 35 °C.

2.5. NMR spectroscopy

The NMR spectra were recorded at 298 K by either a Varian VNMRS-500 or Varian VNMRS-600 spectrometer that was equipped with a 5-mm Z-SPEC Nalorac IDG 500-5HT gradient probe or a 5-mm PFG AutoXID (1H/X15N-31P) probe, respectively. Standard pulse sequences were used. The structures of the studied steroids were determined via analysis of homonuclear (DQF-COSY, zTOCSY, and NOESY) and heteronuclear ($^1H-^{13}C$ HSQC, HSQC-TOCSY, and HMBC) spectra. The ^1H connectivities were established via analysis of the DQF-COSY and TOCSY spectra. The resonances of all carbons with directly attached protons were assigned using the HSQC and HSQC-TOCSY spectra. Then, the HMBC spectra were used to assign the quaternary carbon resonances and to evaluate the correctness of the connectivities that were established via the analysis of the other spectra. NOESY spectra were also utilized to establish the α/β deuterations of steroids **10–15**.

The experiments were conducted under the following conditions: DQF-COSY - spectral widths of 5000 Hz in both dimensions, 4096 complex points in t_2, 1024 complex points in t_1, 1 scan per increment, and a relaxation delay of 1 s; TOCSY - spectral widths of 5000 Hz in both dimensions, 2048 complex points in t_2, 1024 complex points in t_1, 2 scans per increment, a relaxation delay of 1 s and a spin-lock time of 20 ms; NOESY - spectral widths of 5000 Hz in both dimensions, 1024 complex points in t_2, 512 complex points in t_1, 4 scans per increment, a relaxation delay of 1 s and a mixing time of 400 ms; $^1H-^{13}C$ HSQC - spectral widths of 5000 Hz in F2 and 19000 Hz in F1, 1024 complex points in t_2, 512 complex points in t_1, 2 scans per increment, and a relaxation delay of 1 s; $^1H-^{13}C$ HSQC-TOCSY - spectral widths of 5000 Hz in F2 and 15000 Hz in F1, 1024 complex points in both dimensions, 2 scans per increment, a relaxation delay of 1 s and a spin-lock time of 18 ms; and $^1H-^{13}C$ HMBC - spectral widths of 5000 Hz in F2 and 25000 Hz in F1, 1024 complex points in both dimensions, and 4 scans per increment.

All the spectra were referenced indirectly (Wishart et al., 1995). When necessary, the data were processed using linear prediction in t_1, which was followed by zero-filling in both dimensions. Gaussian weighting functions were applied in both domains prior to Fourier transformation.

Prior to the analysis, 10 to 20 mg of the samples were dissolved in 600 μL of deuterated chloroform-d_1.

2.6. Biological studies

2.6.1. Cell culture

Endothelial cells were grown in a medium that consisted of the following for the *in vitro* culture congaing: EGF, FGF-2, VEGF, vitamin C, *R*3-IGF-1, 3% FBS, gentamycin/amphotericin, hydrocortisone, and heparin. The cells were kept in a 37 °C incubator with 95% humidity and 5% CO_2.

2.6.2. Liposome preparation

The DOPC: 3β,3′β-disteryl ether: DSG-PEG 2000 with a molar ratio of 3:1:0.3 was dissolved in $CHCl_3$, a Rotavapor was used to evaporate the solvent, and the thin lipid film was rewetted in 10 mL of the culture medium. The liposomes were prepared using ultrasound and an extrusion method that utilized nylon membranes (10 μm, 0.45 μm, and 0.22 μm).

The control liposomes and the liposomes that contained 7-kCh (**22**) were prepared via the same approach by exchanging the 3β,3′β-disteryl

Table 2
Validation parameters for GC–MS analysis of the studied 3β,3′β-disteryl ethers.

	Linearity range	Equation	R^2 (n = 8)	LOD [ng/mL]	LOQ [ng/mL]	Recovery	
3β,3′β-dicholesteryl ether (dCh)	15–250 ng/mL	y = 0.0188x + 0.1749	0.9996	3.7	12.3	25 ng/mL	90.5% ± 7.6%
						100 ng/mL	93.5% ± 3.4%
						250 ng/mL	92.7% ± 1.4%
3β,3′β-distigmasteryl ether (dStig)	25–250 ng/mL	y = 0.0125x + 0.0158	0.9981	7.3	24.4	25 ng/mL	89.3% ± 10.8%
						100 ng/mL	94.4% ± 4.1%
						250 ng/mL	95.0% ± 1.9%
3β,3′β-disitosteryl ether (dSito)	20–250 ng/mL	y = 0.0192x + 0.1402	0.9993	4.9	16.3	25 ng/mL	80.5% ± 9.8%
						100 ng/mL	94.6% ± 2.9%
						250 ng/mL	98.3% ± 2.0%

ether for cholesterol or 7-kCh (DOPC:sterol:DSG-PEG 2000) in a molar ratio of 3:2:0.3.

2.6.3. MTT test

The cell growth inhibition was determined via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Endothelial cells (HAEC) were plated in 96-well microtiter plates with 8000 cells/well and allowed to attach for 24 h at 37 °C. The test compounds in liposomes or dissolved in EtOH were added to the cell culture medium to the desired final concentrations. After the cells were cultured for 24 h, to the medium was added 10 μL of 5 mg/mL MTT. After incubation for 4 h at 37 °C, 100 μL of 10% SDS in 0.01 N HCl was added to the medium that contained MTT, and the plates were shaken gently for an hour at room temperature. Absorbance values were derived from the plate reading at 550 nm on a Biotek microtiter plate reader (Winooski, VT). The reading reflected the number of viable cells and was expressed as a percentage of the viable cells in the control.

3. Results

3.1. Sterol derivatives synthesis

As outlined in Fig. 1 the synthesis plan was divided into two main routes. The first route was to synthesize the deuterium-labelled sterols in order to prepare the 3β,3′β-d_4-disteryl ethers. Conversion of sterols to Δ^4-3-ketosteroids (**4**, **5** and **6**) using the Shvo's catalyst in acetone as hydrogen acceptor resulted in a simple and inexpensive alteration to the Oppenauer oxidation with aluminum isopropoxide in acetone or cyclohexanone (Almeida, Kočovský, & Bäckvall, 1996; Ishihara, Yamamoto, & Crich, 2009). In the next sequence of reaction, the enol acetates (**7**, **8** and **9**) were prepared by the reaction of the Δ^4-3-ketosteroids with isopropenyl acetate followed by reduction with sodium borodeuteride in deuterated ethanol (EtOD), which yielded 3,4-d_2-sterols (**10**, **11** and **12**) (Gruenke & Craig, 1979). Reaction of obtained 3,4-d_2-sterols in CH_2Cl_2 with MK10 catalyst resulted in 3β,3′β-d_4-disteryl ethers (**13**, **14**, and **15**).

The second route was to prepare the 7-ketosterols. Sterols were protected as acetate (**16**, **17** and **18**), which underwent an oxidation reaction by *tert*-butyl hydroperoxide (*t*-ButOOH) with manganese (III) acetate as a catalyst, to give the 7-keto derivatives (**19**, **20** and **21**) (Shing & Yeung, & Su, P. L. , 2006). The choice of the reaction was based on the fewer side reactions due to mild oxidation, higher efficiency and easier purification compared to the reactions using chromium as an oxidizer (e.g., in the form of chromium oxide complex with 3,5-dimethylpyrazole or pyridinium chlorochromate) (Wendell & Edward, 2016). At this stage, a deprotection reaction in alkaline medium led readily to 7-ketosterols (**22**, **23** and **24**).

Additionally, *epi*-cholesterol (**29**) was obtained from cholesteryl mesylate (**28**), by using KO_2 as a nucleophile in the presence of 18-crown-6 in the mixed solution of DMF and DMSO (You, Gong, & Sun, 2014).

3.2. Optimization of 3β,3′β-disteryl ether synthesis

To maximize the yield of the ether formation reaction, various solvents and molar ratios of the substrate to the catalyst were compared. The solvents that were investigated were aprotic solvents that differed in terms of polarity: CH_2Cl_2, c-Hex, *n*-Hex, EtOAc, THF and $CHCl_3$. The temperature of the reaction was the boiling temperature of the chosen solvent. The reaction was conducted until the substrate reacted, which was checked via TLC (*n*-Hex:EtOAc 3:1), or for 24 h. The mass ratios of the substrate to the catalyst that were investigated, along with the corresponding yields, are presented in Table S8. The yield of each reaction was determined via HPLC analysis. Each time after the reaction was stopped and catalyst was filtered off, a portion of 1 mL was placed in a 10 mL glass vial, and the solvent of the reaction was evaporated. The residue was redissolved in 1 mL of *n*-Hep, and the samples were injected for HPLC (Fig. S7). The yield of the reaction was calculated based on the peak area of the 3β,3′β-dicholesteryl ether that was acquired after purification. Based on the yields of the reaction, the ratio of the MK10 was chosen for the synthesis of all 3β,3′β-disteryl ethers. CH_2Cl_2 produced the best results in terms of the yield of the reaction; however, there were side products of similar polarity to 3β,3′β-dicholesteryl ether, which rendered chromatographic purification more difficult. These products did not form with c-hex.

3.3. Method validation of isotope dilution gas chromatography mass spectrometry

To analyse the 3β,3′β-disteryl ethers, gas chromatography-mass spectrometry (GC–MS) with electron impact ionization and SIM was used. The recoveries, linearity, LOD and LOQ were calculated. LOD, LOQ, and recoveries on various levels and the linearity range of the developed method are presented in Table 2. Chromatographs of the 3β,3′β-disteryl ethers are presented on Fig. 2. As was discussed, the 3β,3′β-disteryl ethers are difficult to analyse due to their instability in various ion sources. Thus, gas chromatography must be employed with high volume injection with a suitable split and temperature program in the injector.

3.4. Analysis of a high-temperature treatment samples

Using prepared and validated method the 3β,3′β-disteryl ethers were analysed in samples that were subjected to treatment at various temperatures. The contents of each 3β,3′β-disteryl ether are presented in Table 1. The highest contents of the dimers were identified in the prepared samples that were treated for 1 h at 220 °C. These contents are as follows: in cod-liver oil – 0.041 μg/g; butter – 0.374 μg/g; rapeseed oil – 0.043 μg/g; and corn oil – 0.034 μg/g. The contents of the dimers in the animal-origin samples are much higher than in the vegetable-origin samples, and most of the contents of the prepared samples were below LOD. The time-dependent 3β,3′β-disteryl formation is more readily observable in Fig. 3, which presents the 3β,3′β-dicholesteryl ether content in butter samples. The content at 180 °C is rising during

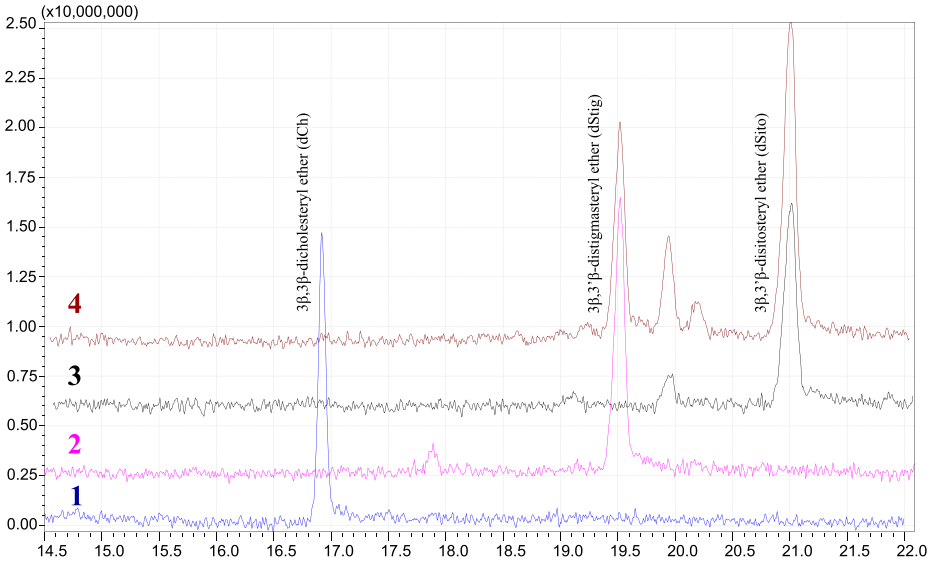


Fig. 2. Chromatograms of the studied 3β,3′β-disteryl ethers: (1) a chromatogram of the 3β,3′β-dicholesteryl ether standard; (2) a chromatogram of 3β,3′β-distigmasteryl ether; (3) a chromatogram of 3β,3′β-dististeryl ether; and (4) a chromatogram of the corn oil sample.

the time of heating from 0.116 μg/g to 0.200 μg/g, as also at 200 °C. The same tendency is observed for the cod-liver oil. The measured content at 220 °C peaks in 1 h of the treatment at 0.374 μg/g and 0.041 μg/g in butter and cod-liver oil, respectively. Additional heating time resulted in significant reductions in the 3β,3′β-disteryl ether content. The same is observed with the vegetable-origin samples; however, after 2 h of heating, the measured contents were below LOD.

3.5. Effect of 3β,3′β-disteryl ethers on endothelial cells

After incorporation into the bilayer liposomes, the synthesized compounds were tested on endothelial (TeloHAEC) cells. The effects of 3β,3′β-disteryl ethers on the percent of live cells were compared to that of the control, and they are presented in Fig. S1. Additionally, images were taken before and after staining with propidium iodide to present the changes in the morphology. To evaluate the suitability of the preparation of the liposomes, the cytotoxic properties of the liposomes that contained 7-kCh were compared to those of the same substance that were dissolved in EtOH. The cytotoxic properties of 7-kCh are comparable between EtOH dissolution and incorporation in liposomes (Fig. S2).

4. Discussion

4.1. Synthesis of the ethers and the ether formation mechanism

It was reported previously that 3β,3′β-disteryl ethers were prepared using reaction at high temperature with copper sulfate as a catalyst (Schulte & Weber, 1987; Sosińska et al., 2013), which suggests that a catalyst must be present to realize the formation of an ether bond (in this case, the metal ion). The yield of the reaction was very low (< 5%), and very tedious procedures were required for obtaining a pure compound. 3β,3′β-dicholesteryl ether was also synthesized using direct anodic oxidation of cholesterol in CH_2Cl_2 with 28% yield (Morzycki & Sobkowiak, 2015). Despite this, only the use of the sterol reaction with MK10 enabled the acquisition of 3β,3′β-disteryl ethers in pure form and

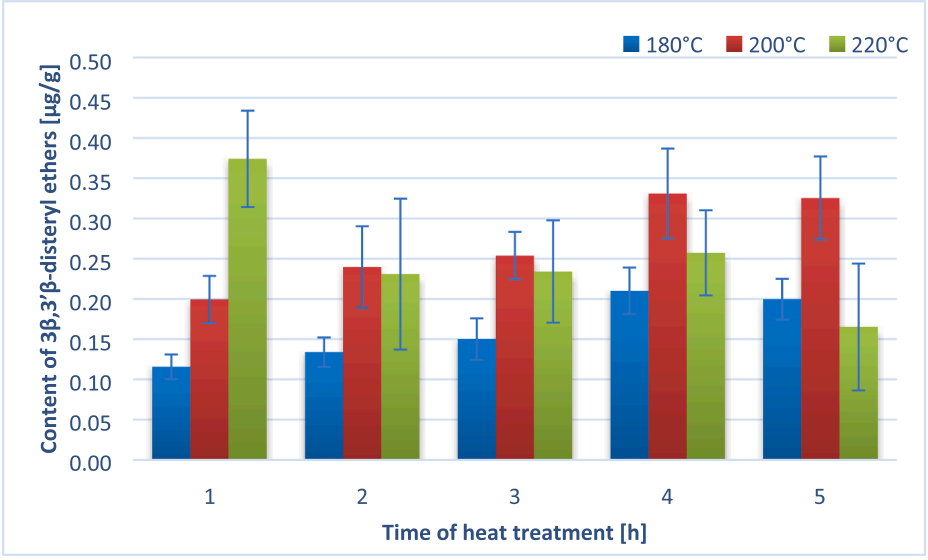


Fig. 3. Measured contents of 3β,3′β-dicholesteryl ether after treatment at various temperatures (180, 200 and 220 °C).

in large quantity (Li, Li, Li, & Yang, 1998).

To realize the highest yield of the 3β,3′β-disteryl ethers formation the various solvents were tested. The use of CH_2Cl_2 with MK10 produces the best results in terms of the yield of the reaction. The reaction in c-hex has a lower yield, but the formation of side products is limited; hence, the compounds are much easier to purify using preparative chromatography.

MK10 has been widely used in various reactions as a catalyst (Kumar, Dhakshinamoorthy, & Pitchumani, 2014). MK10 is used as a heterogeneous catalyst due to its favourable properties, such as low cost, thermal stability, the presence of Lewis and Brønsted acid sites, large surface area, and ease of separation; furthermore, it is environmentally benign. The reactions that are catalysed by MK10 are typically conducted under mild conditions with high yields and high selectivity, and the workups of these reactions are highly simple; only filtration for removing the catalyst and evaporation of the solvent are required. The structure of the catalyst is presented in Fig. S4. Based on the composition of the catalyst, the metal ion that coordinates the ether formation is likely aluminium. However, the use of aluminium chloride ($AlCl_3$) instead of MK10 in the reaction does not lead ether formation; the only product of the reaction was the elimination product – cholesta-3,5-diene. Moreover, the characteristic purple colour of the catalyst is observed, as in the MK10 reaction (Photograph S1), which suggests that aluminium or another metal ion in MK10 can be a catalyst in the ether formation mechanism. We suggest that the Lewis site of the catalyst with a metal ion is mainly responsible for the elimination of the β-hydroxyl moiety and that the *i*-steroid intermediate is stabilized by a tetrahedral sheet of $Si(OH)_4$, which enables the oxygen from another sterol molecule to attack the partially positively charged intermediate to form an ether bond.

Due to the proven 3β,3′β-disteryl ether formation at high temperatures, an attempt was made to analyse the reaction mechanism using the prepared synthesis method. The reaction does not yield the assumed product when only 5α-cholestan-3β-ol (cholestanol) is used. However, a mixture of cholesterol and cholestanol yields 2 products – 3β,3′β-dicholesteryl ether and 3β,3′β-cholestenyl-cholesterol ether (GC–MS chromatogram in Fig. S6). The ether was confirmed only via the GC–MS method due to the inability to acquire dimers in pure form. Based on that reaction, it was concluded that the double bond between carbons 5 and 6 of the sterol moiety is essential for the ether formation. Additionally, after synthesis of the *epi*-cholesterol, which was used as a substrate for ether formation, again, no product was observed. Hence, it is assumed that to form an ether, the double bond and a hydroxyl group in position β must be present. The observed lack of reaction is likely caused by the difficulty of leaving the hydroxyl group in the α position (Sun, Cai, & Peterson, 2009). Additionally, to evaluate these assumptions, the dimeric products were prepared from a few additional sterol derivatives with a double bond and a hydroxyl moiety in position β: 5-pregnen-3β-ol-20-one, 3β,21-dihydroxy-5-pregnen-20-one 21-acetate, 16α,17α-epoxy-3β,21-dihydroxy-5-pregnen-20-one 21-acetate and diosgenin (Table 3). From all substrates, it was possible to acquire 3β,3′β-disteryl ethers in pure form, with confirmation of the structure via NMR. Diosgenin did not form the dimeric product in the reaction in CH_2Cl_2; it only formed this product in c-hex. This is probably due to the increased reaction temperature. Additionally, the low yield of 3β,3′β-diosgenin ether could be due to low purity of the substrate, which might contribute to side reactions.

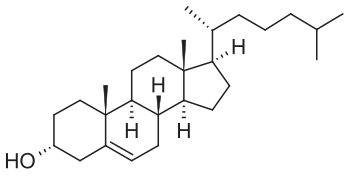
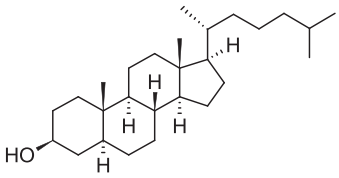
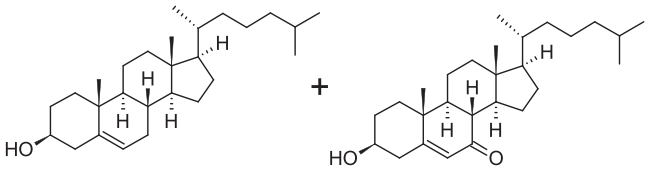
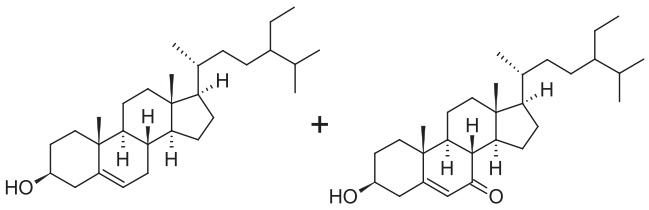
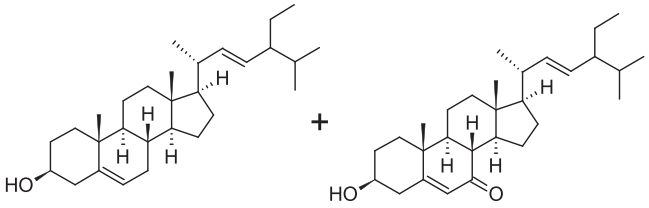
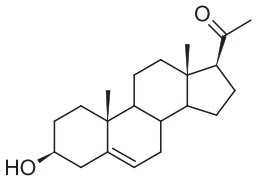
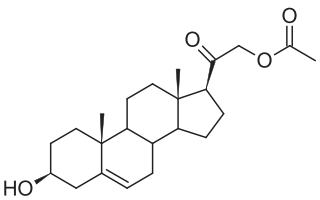
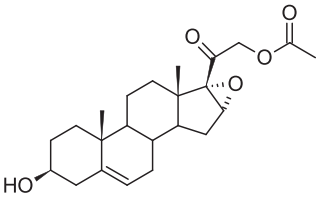
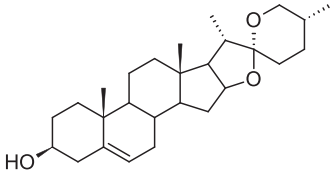
However, as discussed, a structural requirement is needed on only one particle, which is subjected to a reaction. The double bond and hydroxyl group at position β are probably required for the formation of an *i*-steroid intermediate ion, which attacks a partially negatively charged oxygen atom on another sterol particle (Fig. S5). Such an *i*-steroid intermediate product was also observed in a reaction on mesylate and in a tosylate cholesterol reaction with a strong Lewis acid by Sun et al. (Sun et al., 2009). This is why only one particle must be converted into *i*-steroid to form the ether bond. This may also be required in the biological matrix. Various metal ions (such as Al, Mg and Fe) that are present in food could catalyse the elimination of the β-hydroxyl moiety and the formation of an *i*-steroid intermediate, which could create a bond with a partially negatively charged oxygen on another sterol particle to form 3β,3′β-disteryl ether.

Based on the conclusions that were obtained on the basis of the reaction of sterols and stanols, namely, only one sterol molecule after the dehydration reaction forms an *i*-steroid intermediate to form an ether bond, an attempt was made to generate ether from the native sterol (cholesterol) and oxidized sterol in carbon position 7 (7-ketocholesterol, **22**). Reaction with MK10 was conducted, and the expected product was obtained (3β,3′β-dicholesteryl ether), along with oxidized 3β,3′β-disteryl ether (7-ketocholesteryl-cholesterol ether). The structure was confirmed using NMR and LC-MS/MS (product **25**). Additionally, the oxidized 3β,3′β-disteryl ethers from phytosterol (β-sitosterol and stigmasterol) and theirs oxidized analogues (7-ketositosterol and 7-ketostigmasterol, which correspond to **26** and **27**, respectively) were prepared. Compounds with such structure have been identified using LC-MS/MS in previous studies; however, to the best of our knowledge, they have not been obtained in pure form so far via chemical synthesis (Rudzińska et al., 2009; Struijs et al., 2010). Since one of the main reactions occurs during the high-temperature oxidation of sterols, oxidized sterol dimers may form or the resulting 3β,3′β-disteryl ether may be oxidized. Hence, oxidized 3β,3′β-disteryl ethers are a new type of compounds that could originate from high-temperature sterol treatment and could affect humans. Since sterols and oxysterols originate from reactive oxygen species that are constantly associated with atherosclerosis, oxidized 3β,3′β-disteryl ethers could have an impact on atherosclerosis.

4.2. Chromatography methods for analysing 3β,3′β-disteryl ethers

Due to the very low polarity of the 3β,3′β-disteryl ethers, a different approach is required for analysis via chromatography and mass spectrometry. The typical ion source that is used for sterol analysis that is coupled with liquid chromatography is APCI. However, chromatography of 3β,3′β-disteryl ether on C8 or C18 modified silica columns requires the use of a high-elution-strength solvent (e.g., CH_2Cl_2 or $CHCl_3$), which unfortunately suppress the ionization of 3β,3′β-disteryl ether, thereby resulting in a very poor LOD. The use of a shorter-chain modified silica (e.g., C1 or C4) does not result in satisfactory resolution between 3β,3′β-distigmasteryl and 3β,3′β-disitosteryl ether. The use of normal phase chromatography that is coupled with APCI mass spectrometry yielded similar results regarding LOD and LOQ to those of reversed-phase chromatography. Additionally, instability in various ion sources of mass spectrometers render compounds of this type very complicated to analyse. The other method for analysing dimeric products, which was used earlier, is liquid chromatography that is coupled with mass spectrometry using silver ion coupled adducts (Struijs et al., 2010). To form such adducts, a volatile silver salt must be used, such as $AgBF_4$. However, due to the occurrence of two isotopes of silver, namely, ^{107}Ag and ^{109}Ag, in a ratio of almost 50%, the abundance of molecular ions is lower by 50%; hence, this method is mostly useful for qualitative research. Therefore, based on the previously reported GC-FID method (Schulte & Weber, 1987), which demonstrated that the analysis of 3β,3′β-disteryl ethers is possible with gas chromatography, a method that uses this technique was developed. To search for 3β,3′β-disteryl ether that was formed after high-temperature treatment, gas chromatography must be coupled with mass spectrometry to realize enhanced sensitivity and selectivity. Unfortunately, due to the instability of the compounds in the electron impact (EI) ionization method, the LOD and LOQ are not high. EI-ionized sterols provide spectra that are typical for hydrocarbons, with many fragments but with no apparent precursor that could be used for MS/MS analysis (Szterk & Pakuła, 2016). In the case of 3β,3′β-disteryl ethers, no molecular ions are observed, which limits the analysis to SIM of

Table 3
Various substrates, products, and yields for 3β,3′β-disteryl formation via MK10 reaction.

Substrate		Yield (%)	Product
Structure	Name		
	cholesten-5-en-3α-ol(*epi*-cholesterol)	no reaction	–
	5α-cholestan-3β-ol(cholestanol)	no reaction	–
	cholesterol + 5-cholesten-3β-ol-7-one (**22**, 7-ketocholesterol)	67%	**25**
	β-sitosterol + 5-sitosten-3β-ol-7-one (**23**, 7-ketositosterol)	61%	**26**
	stigmasterol + 5-stigmasten-3β-ol-7-one (**24**, 7-ketostigmasterol)	64%	**27**
	5-pregnen-3β-ol-20-one	74%	**30**
	3β,21-dihydroxy-5-pregnen-20-one 21-acetate	68%	**31**
	16α,17α-epoxy-3β,21-dihydroxy-5-pregnen-20-one 21-acetate	53%	**32**
	(3β,25R)-spirost-5-en-3-ol(diosgenin)	35%	**33**

fragmented molecules. The use of a different ionization source, such as the positive or negative chemical ionization (PCI and NCI with methane as the reagent gas), yielded a very low molecular ion concentration, which resulted in very high LOQ. Further optimization of the ion source parameter did not improve the ionization; thus, the use of a chemical ionization source was discontinued. Moreover, there is no available oxygen atom for derivatization reactions for the realization of higher resolution and/or sensitivity, which is a common approach in analysing sterol compounds with GC–MS (Pizzoferrato, Nicoli, & Lintas, 1993). Additionally, the high boiling point of 3β,3′β-disteryl ethers requires the use of suitable high-temperature-resistance components (septum, liners, and ferrules) and a high-temperature GC column (ZB-1HT). Due to the low LOD and LOQ of the 3β,3′β-disteryl ethers using split/splitless analysis, it was concluded that high-volume injection is necessary for enhancing the detection performance. The most important parameters, which needed to be optimised were: the choose of the liner, the solvent of the samples and the starting temperature of the injector program. The usage of sintered glass liner with a taper in combination with *n*-Hep as a sample solvent (*n*-Hep has relatively high boiling point compared to other nonpolar solvents like CH_2Cl_2 or *n*-Hex) and optimised program temperature (Table S7) allowed to increase the volume of the injection to 20 μL to enhance the LOD and LOQ, which made possible to analyse the low amounts of the 3β,3′β-disteryl ethers in prepared samples.

4.3. Analysis of high-temperature treatment

As presented in Table 1, the content of 3β,3′β-disteryl ethers varies according to the temperature and duration of the heat treatment of the samples. Based on the presented results, there is a noticeable trend of increasing content at 180 °C and 200 °C during heating. However, the highest content of 3β,3′β-disteryl ethers was observed at 1 h with a heating temperature of 220 °C. The prolonged heating resulted in decreased content, which can be correlated with side reactions of native sterols or reactions of dimers. It was proved that at such high temperatures, fragmentation of the sterol groups and/or further polymerization occurs (Derewiaka & Molińska (née Sosińska), 2015).

Derewiaka has studied cholesterol dimer formation, especially in cholesterol standards. The highest total dimer content was observed after 1 h of processing at 220 °C and reached 184.4 mg/g of the non-heated standard, which is in accordance with our findings (Derewiaka & Molińska (née Sosińska), 2015). Recently, it was proven that dimer cholesterol can be formed in butter after heating to 180 °C (Derewiaka, 2019). However, the method that was used to analyse the dimer formation was gel permeation chromatography (GPC), which separates the analysed substances in the samples according to differences in molecular mass. Unfortunately, the structures of the analysed substances were not confirmed. Characterization of 3β,3′β-disteryl ethers via mass spectrometry is challenging, and they can be easily mistaken for the native sterol of sterol derivative molecules.

Despite a higher concentration of phytosterols in oils per gram compared to the concentration of cholesterol in butter, the measured amounts of 3β,3′β-disteryl ethers are much lower. Different compositions of fatty acids in fats of animal and vegetable origin in selected samples may be one of the reasons for the obtained results. In animal origin samples, the content of saturated fats predominates. The butterfat mainly consists of palmitic, myristic, and stearic acid with a low concentration of unsaturated acids (Rutkowska & Adamska, 2011). Cod-liver oil contains mainly stearic acid with the addition of unsaturated acids like oleic, and eicosenoic acid (Pan, Ushio, & Ohshima, 2005). On the other hand, vegetable oils mainly consist of unsaturated fatty acids, for rapeseed oil, it is oleic, linoleic, and linolenic acid (Farahmandfar, Asnaashari, & Sayyad, 2015) and for corn oil, it is oleic and linolenic acid (Hwang et al., 2011). The higher concentration of unsaturated fatty acids in vegetable oils may cause double bond oxidation to be the main reaction occurring thought heat treatment, which may inhibit the ether bond formation between sterols moieties. However, it is also possible that ether formation reaction occurs independently of unsaturated fatty acids oxidation. Based on the hypothesis that to form 3β,3′β-disteryl ethers in food samples metal ions need to be present to catalyse the reaction of ether bond formation, the different concentration of metals in the selected samples could be another reason for obtained such different results. Copper, which was used in the salt form as a catalyst for the synthesis of 3β, 3′β-disteryl ethers, may differ 10 times in concentration between the butter (up to 1.72 ppm) and vegetable oils (up to 0.18 ppm) (Garrido, Frías, Díaz, & Hardisson, 1994; Meshref, Moselhy, & Hassan, 2014). Therefore, the different composition of metal ions could implicate the disproportion of 3β,3′β-disteryl ethers content, measured in selected samples.

Various 3β,3′β-disteryl ethers, in addition to 3β,3′β-distigmasteryl ether and 3β,3′β-disitosteryl ether, were also detected in corn oils (Fig. 2). However, due to the lack of the pure standards of the 3β,3′β-disteryl ethers and based on the results regarding the differences in the responses between 3β,3′β-distigmasteryl ethers and 3β,3′β-disitosteryl ether, the quantitative analysis could not be conducted. Based on the spectrum that was acquired of the visible peaks and of this additional peak in a standard solution of 3β,3′β-disitosteryl ether, which was formed during the synthesis with a common impurity of sitosterol - stigmasterol, it was assumed that those ethers were 3β,3′β-stigmasteryl-sitosterol ether and 3β,3′β-campesteryl-sitosterol ether.

4.4. Effect of 3β,3′β-disteryl ethers on endothelial cells

Due to the proven formation of 3β,3′β-disteryl ethers during high-temperature treatment of sterols, a preliminary study was conducted to investigate their impact on human cells. Sterols and sterol derivatives (e.g., oxysterols) are often associated with atherosclerosis. To investigate the influence of 3β,3′β-disteryl ethers on the eventual initiation of atherosclerosis, endothelial cells were selected based on their participation in plague formation (Zmysłowski & Szterk, 2017). The cell line that was used in the study was TeloHAEC, which is a clonal cell line that was immortalized by stably expressing human telomerase catalytic subunit hTERT. The selection of the cell line was based on proven involvement of the endothelium in atherosclerosis lesion initiation and progression. Due to the non-polarity of 3β,3′β-disteryl ethers, suitable sample preparation must be conducted. To evaluate the preparation of the liposomes, the oxidation product of cholesterol – 7-kCh – was incorporated into the liposomes, and the cytotoxic properties were compared to those of the control and those of oxysterol that was dissolved in EtOH. The cytotoxic properties of 7-kCh were comparable to those of HAEC cells that were either in liposomes or dissolved in EtOH (Fig. S2.).

The cells were incubated for 72 h with tested compounds in prepared liposomes; however, based on data that were acquired via the MTT test, no changes in cell viability compared to the control were observed. Additionally, the morphology of the cells was investigated. Images that were acquired after incubation with 3β,3′β-disteryl ethers and images of the control are presented in Fig. S3. Changes are observed in the morphology of the cell compared to the control, which are probably due to the incorporation of the ethers into the lipid bilayer, which impedes the fluidity of the membrane. However, based on staining with propidium iodide, the cells still maintain the continuity of the cell membrane. It also should be pointed out that the concentration of 3β,3′β-disteryl ethers tested on endothelial cells was significantly higher than concentration measured in selected samples. At a concentration comparable to that determined in the selected samples, no biological effect was found. Therefore, the concentration was increased to observe if the 3β, 3′β-disteryl ethers have any cytotoxic properties. However, based on the acquired data, it was concluded that 3β,3′β-disteryl ethers under the selected experimental conditions does not possess toxic properties to endothelial cells. It was already shown that 3β,3′β-dicholesteryl ether does not cause toxicity to rats after

subcutaneous administration (Larsen & Barrett, 1944). However, this report only discusses the effects of 3β,3′β-disteryl ethers on rats after subcutaneous administration, which is not a typical route by which compounds of this type enter the body.

5. Conclusions

Using a reaction of sterols with the MK10 catalyst, various 3β,3′β-disteryl ethers were prepared. Additionally, a reaction mechanism that is based on various sterol analogues of ether bond formation was proposed, which could explain how the 3β,3′β-disteryl ethers are formed in high-temperature-treatment samples. The GC–MS method for analysing 3β,3′β-disteryl ethers was developed and validated. It was proven that such compounds can be formed via the high-temperature treatment of biological type samples. The concentration of ethers depended on the duration and the temperature of the treatment. The most 3β,3′β-disteryl ethers are produced at 220 °C after 1 h of heating; however, it is possible that formed dimers may oxidize and that the native dimer concentration is decreasing over time. Additionally, to the best of our knowledge, this is the first report that describes the concentration of 3β,3′β-disteryl ethers (specific steryl dimers) after the thermal heating of high-concentration sterol samples. This is because most publications are focused on analysing sterol dimers, without structural confirmation of the analysed compounds. The influence of 3β,3′β-disteryl ethers was also evaluated with endothelium cells; however, for those cells, no toxicity was observed on the tested substances.

CRediT authorship contribution statement

Adam Zmysłowski: Conceptualization, Investigation, Validation, Writing - original draft. **Jerzy Sitkowski:** Investigation. **Katarzyna Bus:** Investigation. **Karol Ofiara:** Investigation. **Arkadiusz Szterk:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127132.

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