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Synthesis and characterization of steroidal, anellated aminothiophenes by Gewald reaction

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Abstract: Gewald 3-component reactions (G-3CR), i.e., reactions of a carbonyl compound with an activated nitrile in the presence of a secondary amine and sulfur, lead straightforwardly to anellated 2-aminothiophenes. Interestingly, their application to steroidal hydrocarbons has been limited to a single example. We were able to show in this work that Gewald 3-component reactions can be performed successfully for molecules holding a cholesterol or sitostanol skeleton, such as 5α-cholestan3-one (**8**) and 5α-sitostan-3-one (**11**), thus leading in good yields to the corresponding anellated steroidal 2-amino-thiophenes **12-15**. Gewald reaction proved to be an excellent method to access heterocyclic steroids.

Keywords: Aminothiophenes; Gewald reaction; steroids.

1. Introduction

Heterocyclic steroids demonstrated potent bioactivity due to the incorporation of a fused heterocyclic ring into the steroid skeleton [1]. Many routes have been devised for their synthesis [1-4]. Thereby, the synthesis of 2-aminothiophenes [5-11] has been the focus of organic chemists for many years. They found applications as pharmaceuticals [6,12-17] but also in agriculture and for dying processes [18]. Albeit many different synthetic schemes have been described for their synthesis; it was the merit of K. Gewald [19-24] as early as 1966 to describe a multi-component reaction [25-32] (MCR, G-3CR as an abbreviation for Gewald-3-component reaction) [13,15,18, 33-36]. Thereby a carbonyl compound **A** (Scheme 1) is allowed to react with an activated nitrile **B** and sulfur in the presence of a secondary amine to yield a poly-substituted 2-aminothiophene **C**. MCRs allow the convergent synthesis of a wide variety of compounds thus improving the efficacy to explore chemical space in a fast, cost-effective and convergent manner with a minimum of synthetic effort. Although many Gewald reactions have been described, a close inspection of the literature revealed only one example of a Gewald reaction using a steroidal hydrocarbon. A different scheme has been prepared [37]. Two molecules of a similar structure have shown some anti-bacterial and antifungal activity [38]. This is all the more surprising as the interest in sterols, especially phytosterols, has increased. This class of compounds might be used to lower blood cholesterol levels, reducing the risk of cardiovascular disease [39-42]. Some phytosterols have been reported to have prophylactic anticancer and cytotoxic activity [43] on lung, stomach, ovarian, and breast cancer cell lines [44-48].

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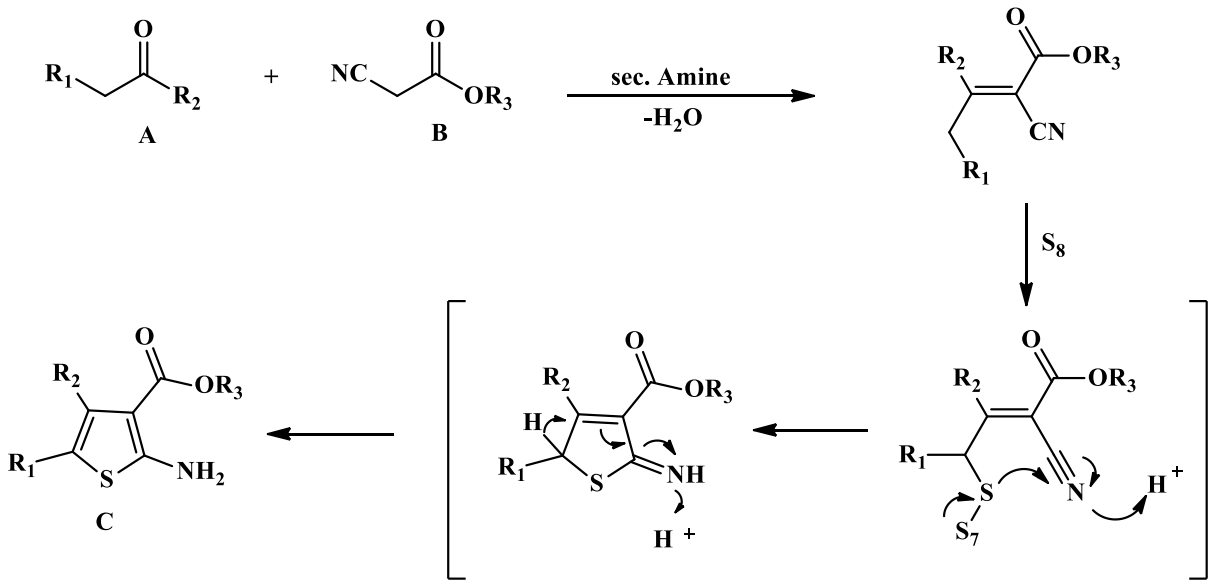
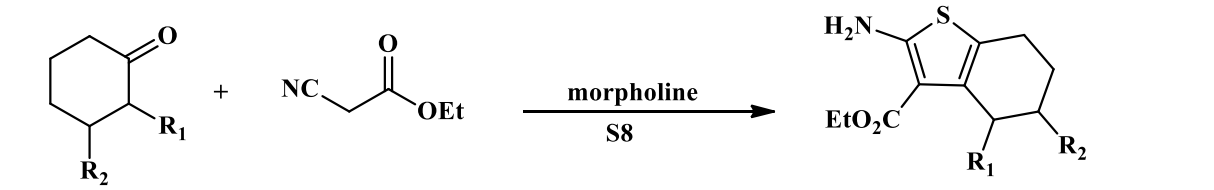


Scheme 1. Mechanism of the Gewald-3-component (G-3CR) reaction. The reaction of a carbonyl compound (**A**) with a cyano ester (**B**) in the presence of a secondary amine and sulfur leads to a 2-aminothiophene (**C**)

2. Results and Discussion

Mechanistically, as depicted in Scheme 1, the first step of a G-3CR reaction is a Knoevenagel condensation between the carbonyl compound and the α-cyanoester to yield an α-alkylidenenitrile, which cyclizes in a subsequent step when reacted with elemental sulfur to afford the target product, 2-amino-thiophene [49,50]. Since no reaction occurred in a reaction of 3-oxo-cholesterol with ethyl α-cyanoacetate in the presence of morpholine and sulfur at room temperature, deterioration and rearrangement reactions became dominant under reflux or in a microwave-assisted synthesis. The reaction was studied in more detail on a few model compounds.

The rearrangement reactions observed in the conversion of 3-oxo-cholesterol paralleling previous results during Willgerodt-Kindler reactions [51]. Thus, for optimization, cyclohexanone (**1**), methylcyclohexanones (**2** and **3**) were each reacted with ethyl α-cyanoacetate in the presence of morpholine and sulfur (Scheme 2). While cyclohexanone (1) and 2-methyl-cyclohexanone (**2**) gave products **4** and **5** in excellent yield in the Gewald reaction, 3-methyl-cyclohexanone (**3**) gave only the 5-methyl isomer **6.** This finding reflects an apparent stereo-electronic influence of substituents on the reaction and its course. This is probably also why, for example, the pentacyclic triterpenes 3-oxo-oleanolic acid and 3-oxo-platanic acid gave no isolable reaction products in Gewald reactions.

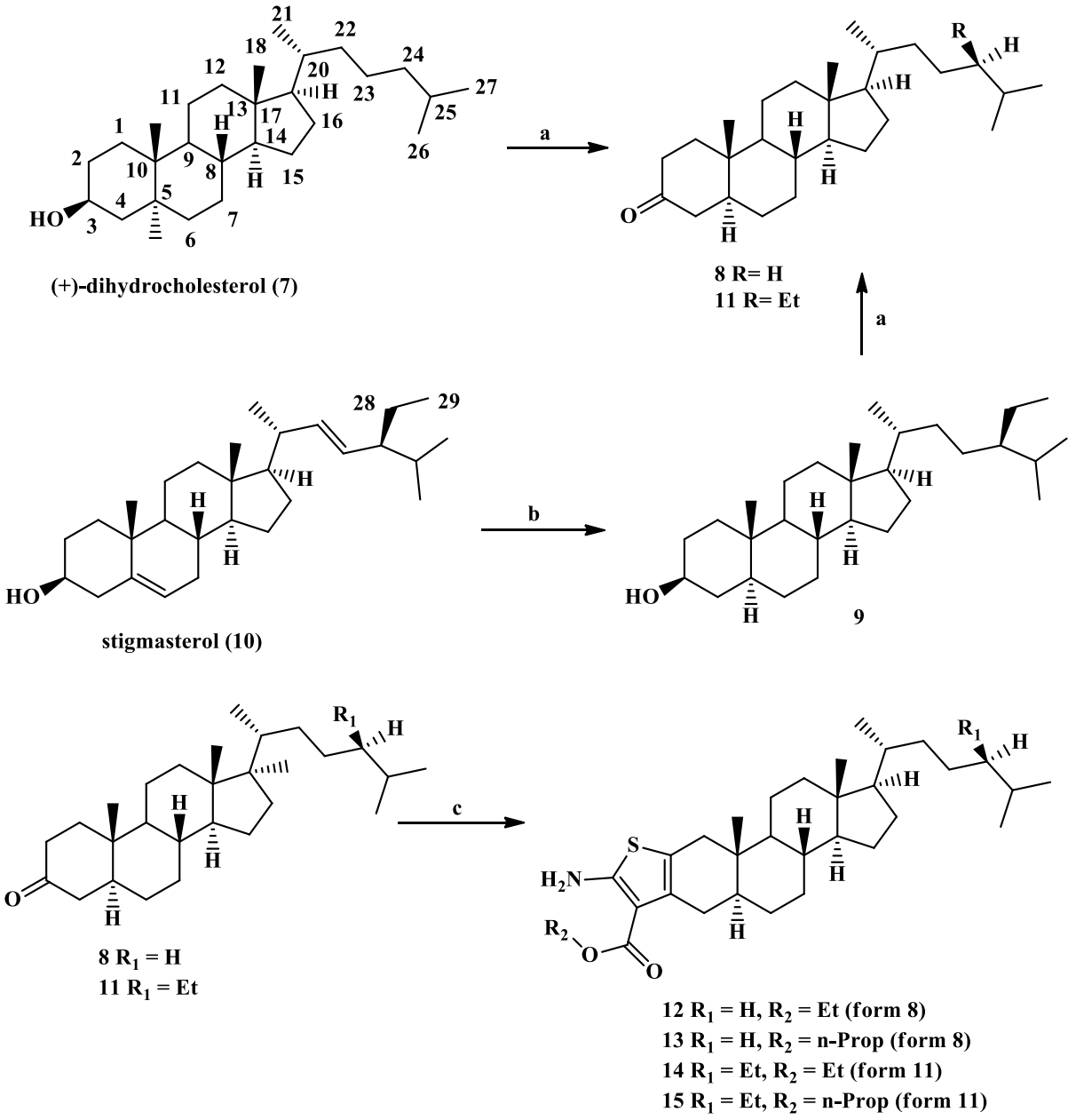


1 R_1 =H, R_2 =H
2 R_1 =Me, R_2 =H
3 R_1 =H, R_2 =Me

4 R_1 =H, R_2 =H, R_3 =H
5 R_1 =Me, R_2 =H, R_3 =H
6 R_1 =H, R_2 =Me, R_3 =H

Scheme 2. Gewald reaction of cyclohexanone and methyl-cyclohexanones following General Procedure GP1: **1**-**3**, ethyl cyanoacetate, S_8, morpholine, 21°C, 1d; yields: **4** (54.5% from **1**), **5** (83.2% from **2**), **6** (84.6% from **3**)

Jones oxidation (Scheme 3) of (+)-dihydrocholesterol (**7**) gave 87% of **5**. Sitostanol (**9**; obtained in 83% yield by hydrogenolysis from stigmasterol (10) with Pd/C and H_2, 48 h, 5 bar) was also converted to ketone **11** by Jones oxidation. The stereochemical course of hydrogenolysis of **10** is directed by the position of the methyl group C-19 [52]. The physicochemical data obtained for **8** and **11** are in excellent agreement with those reported in the literature. Thus, the vibrations of the carbonyl group for **5** and **7** were detected at ν = 1713 and 1716 cm^{-1}, respectively. In the ^{13}C NMR spectrum, each case finds the corresponding signal at δ = 212.0 ppm. The reaction of **8** and **11** with ethyl α-cyanoacetate or propyl α-cyanoacetate gave the anellated 2-aminothiophenes **12**–**15** in 58-67% isolated yield.



Scheme 3. Synthesis of steroidal scaffolds **8** and **11** and their Gewald reactions leading to products **12**–**15**. Reactions and conditions: a) Jones oxidation (CrO_3, H_2O, H_2SO_4, silica gel, 0°C, 2h); yields: 87.5% of **8**, 59.7% of **11**; b) H_2 (5 bar), Pd/C, THF/MeOH, 2:1, 48h, 83.4%; c) following GP1 (ethyl cyanoacetate or propyl cyanoacetate, S_8, morpholine, 21 °C, 1d: yields: **12** (57.6%), **13** (66.9%), **14** (61.8%), **15** (64.3%)

These compounds are characterized by a UV/Vis maximum λ_{max} = 230 nm; the carbonyl group of the ester shows up in the IR spectra at ν = 1663-1665 cm^{-1}; the carboxyl group is found in the ^{13}C NMR spectrum at δ = 165.9-166.1 ppm. The quasi-molecule ions *m/z* = 514 (for **12**), 528 (for **13**), 542 (for **14**) and 556 (for **15**) further confirm the structure of the Gewald products.

The study of phytochemical activity is currently the subject of further investigation; of particular interest will be the activity of these compounds and analogs thereof onto the enzyme anandamide amidohydrolase to treat anxiety disorders [53-57] onto the orexin receptor [58-64] to treat narcolepsy or insomnia. A mandatory prerequisite for these applications is that the compounds are not (cyto)-toxic. Sulforhodamine B assays employing several human cancer cell lines [A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (pharynx carcinoma)] as well as fibroblasts (NIH 3T3) resulted in EC_{50} values > 30 µM; thus, these compounds are to be considered as non-cytotoxic. Furthermore, potential neurotoxicity can also be excluded with high probability since compounds **12-15** showed no inhibitory effect on the enzymes acetylcholinesterase (eeAChE) and butyrylcholinesterase (BChE).

3. Conclusion

The Gewald reaction had hardly been applied to steroidal systems before We were now able to. Moreover, we could show in this work that Gewald 3-component reactions can also be carried out successfully for this class of compounds, thus leading in good yields to the corresponding anellated 2-amino-thiophenes.

4. Experimental

NMR spectra were recorded using the Varian spectrometers (Darmstadt, Germany) DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on an Advion expression LCMS mass spectrometer (Ithaca, NY, USA; positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 µA, capillary temperature: 250°C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel (Düren, Germany). IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin Elmer (Rodgau, Germany). The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin Elmer (Rodgau, Germany); optical rotations were measured at 20°C using a JASCO-P2000 instrument (JASCO Germany GmbH, Pfungstadt, Germany) The melting points were determined using a Leica hot stage microscope Galen III (Leica Biosystems, Nussloch, Germany) and are uncorrected. The solvents were dried according to usual procedures. Microanalyses were performed with an Elementar Vario EL (CHNS) instrument (Elementar Analysensysteme GmbH, Elementar-Straße 1, D-63505, Langenselbold, Germany).

General procedure for the Gewald reaction (GP1)
To a suspension of the starting material (**1**-**3**, **8**, **11**, 1 eq.) in dry EtOH (15 mL), the cyanoester (1 eq.), and sulfur (1 eq.) were added; freshly distilled morpholine (1 eq) was added dropwise, and the mixture was stirred at room temperature for 1 day. Then, the volatiles were removed under reduced pressure, and the residue was purified either by re-crystallization or column chromatography to yield products **4**–**6** and **12**–**15**, respectively.

General procedure for the Jones oxidation (GP2)
To a suspension of the sterol (**7** or **9**, 1 eq.) in acetone (125 mL), silica gel (20 mL) was added, and the mixture was heated under reflux for 20 min and after cooling to 0°C, freshly prepared Jones reagent [from CrO_3 (0.78 g, 7.76 mmol), dist. H_2O (2.5 mL), conc. H_2SO_4 (0.75 mL)] was added, and the reaction was stirred for 2 h at 0°C. For work-up, MeOH (1 mL) was added, and stirring at room temperature continued for another 90 min. Finally, the volatiles were removed under reduced pressure, the residue extracted (Et_2O, Soxhlet apparatus, 5 h), the solvent was removed, and the residue subjected to chromatography to yield **8** and **11**, respectively.

2-Amino-4,5,6,7-tetrahydro-benzo[b]-thiophene-3-carboxylic acid ethyl ester (4)
Following GP1, from cyclohexanone (**1**,5.2 mL, 0.05 mol), ethyl cyanoacetate (5.7 mL, 0.05 mol), sulfur (1.6 g, 0.05 mol), morpholine (4.4 mL, 0.05 mol) and EtOH (15 mL) followed by re-crystallization of the crude product from EtOH, **4** (6.14 g, 54.5%) was obtained as an off-white solid; R_f = 0.43 (SiO_2, *n*-hexane/ethyl acetate, 9:1); m.p.: 115 – 117°C (lit.:[65] 115°C).
IR (ATR): ν = 3402*m*, 3296*m*, 2939*w*, 1645*s*,1595*s* , 1575*s*, 1490*s*, 1273*vs*, 1152*s* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 209 nm (4.01);
^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.94 (*s*, 2H, -NH_2, H-2), 4.25 (*q*, *J* = 7.1 Hz, 2H, H-11), 2.77 – 2.65 (*m*, 2H, H-7), 2.54 – 2.44 (*m*, 2H, H-4), 1.81 – 1.71 (*m*, 3H, H-5 + H-6), 1.33 (*t*, *J* = 7.1 Hz, 3H, H-12) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 166.0 (C-10), 160.8 (C-2), 132.5 (C-9), 118.2 (C-8), 106.4 (C-3), 59.4 (C-11), 26.9 (C-7), 24.5 (C-4), 23.2 (C-5), 22.8 (C-6), 14.4 (C-12) ppm;
MS (ESI, MeOH/$CHCl_3$ = 4:1): *m/z* = 226.1 ($[M+H]^+$, 77%), 248.1 ($[M+Na]^+$, 15%).

2-Amino-4-methyl-4,5,6,7-tetrahydro-benzo[b]-thiophene-3-carboxylic acid ethyl ester (5)
Following GP1 from 2-methylcyclohexanone (**2**, 5.2 mL, 0.05 mol), ethyl cyanoacetate (5.7 mL, 0.05 mol), sulfur (1.6 g, 0.05 mol), morpholine (4.4 mL, 0.05 mol) and EtOH (15 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **5** (9.96 g, 83.2%) was obtained as a yellowish solid; R_f = 0.25 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 74 – 76°C (lit.:[66] 73°C) ;
IR (ATR): ν = 3411*m*, 3304*m*, 2942*m*, 1641*vs*, 1591*vs*, 1568*vs*, 1486*s*, 1277*vs*, 1149s cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 230 nm (4.39);
^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.20 (*s*, 2H, -NH_2, H-2), 4.25 – 4.05 (*m*, 2H, H-11), 3.19 – 3.09 (*m*, 1H, H-4), 2.47 – 2.30 (*m*, 2H, H-7), 1.84 – 1.47 (*m*, 4H, H-6 + H-5), 1.24 (*t*, *J* = 7.1 Hz, 3H, H-12), 1.07 (*d*, *J* = 6.7 Hz, 3H, H-13) ppm;
^{13}C NMR (101 MHz, DMSO-d_6): δ = 165.2 (C-10), 163.8 (C-2), 137.0 (C-9), 115.6 (C-8), 102.5 (C-3), 59.1 (C-11), 29.9 (C-4), 29.4 (C-5), 24.4 (C-7), 21.95 (C-12), 18.4 (C-6), 14.7 (C-13) ppm;
MS (ESI, MeOH): *m/z* = 240.4 ($[M+H]^+$, 52%), 262.4 ($[M+Na]^+$, 100%).

2-Amino-5-methyl-4,5,6,7-tetrahydro-benzo[b]-thiophene-3-carboxylic acid ethyl ester (6)
Following GP1 from 3-methylcyclohexanone (**3**, 5.2 mL, 0.05 mol), ethyl cyanoacetate (5.7 mL, 0.05 mol), sulfur (1.6 g, 0.05 mol), morpholine (4.4 mL, 0.05 mol) and EtOH (15 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **6** (10.12 g, 84.6%) was obtained as a yellowish solid; R_f = 0.25 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 71 – 73°C (lit.:[67] 70 – 71°C);

IR (ATR): ν = 3424*m*, 3312*m*, 2946*m*, 1644*s*, 1578*s*, 1487*vs*, 1274*vs*, 1163*s* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 229 nm (4.39);
^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.87 (*s*, 2H, -NH_2, H-2), 4.26 (*q*, *J* = 7.1, 0.9 Hz, 2H, H-12), 2.97 – 2.86 (*m*, 2H, H-4), 2.58 – 2.48 (*m*, 2H, H-7), 2.18 (*ddt*, *J* = 17.2, 9.5, 2.4 Hz, 1H, H-5), 1.93 – 1.70 (*m*, 1H, H-6a), 1.46 – 1.34 (*m*, 1H, H-6b), 1.33 (*t*, *J* = 7.1 Hz, 3H, H-12), 1.04 (*d*, *J* = 6.6 Hz, 3H, H-13) ppm;
^{13}C NMR (101 MHz, $CDCl_3$): δ = 165.9 (C-10), 161.0 (C-2), 132.5 (C-9), 117.8 (C-8), 106.2 (C-3), 59.4 (C-11), 35.3 (C-6), 31.3 (C-4), 28.9 (C-5), 24.3 (C-7), 21.7 (C-13), 14.5 (C-12) ppm;
MS (ESI, MeOH): *m/z* = 240.1 ([M+H]$^+$, 60%), 262.1 ([M+Na]$^+$, 14%).

5α-Cholestan-3-one (8)
Following GP2 from (+)-dihydrocholesterol (**7**, 2.51 g, 6.47 mmol), acetone (125 mL), CrO_3 (0.78 g, 7.76 mmol), dist. H_2O (2.5 mL), conc. H_2SO_4 (0.75 mL) and silica gel (20 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **8** (2.19 g, 87.5%) was obtained as a colorless solid; R_f = 0.32 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 127 – 129°C (lit.:[68] 128 – 129°C);
IR (ATR): ν = 2931*s*, 2866*s*, 1713*vs*, 1444*m*, 1454*m*, 1376*m*, 1384*m* cm^{-1};
$[\alpha]_D^{20}$= +40.83° (*c* = 0.49, $CHCl_3$);
^{1}H NMR (500 MHz, $CHCl_3$): δ = 2.41 – 2.32 (*m*, 1H, H-2a), 2.32 – 2.21 (*m*, 2H, H-2b + H-4a), 2.06 (*ddd*, *J* = 15.0, 3.9, 2.3 Hz, 1H, H-4b), 2.03 – 1.95 (*m*, 2H, H-1a + H-24a), 1.86 – 1.76 (*m*, 1H, H-16a), 1.72 – 1.65 (*m*, 1H, H-7a), 1.60 – 1.46 (*m*, 5H, H-15 + H-11a + H-25 + H-5), 1.42 – 1.29 (*m*, 8H, H-1b + H-6 + H-11b + H-20 + H-22a + H-23a + H-8), 1.29 – 1.19 (*m*, 1H, H-16b), 1.17 – 1.06 (*m*, 5H, H-12 + H-23b + H-24 + H-14), 1.07 – 0.92 (*m*, 2H, H-22b, H-17), 0.99 (*s*, 3H, H-19), 0.92 – 0.87 (*m*, 4H, H-7b + H-21), 0.85 (*dd*, *J* = 6.6, 2.2 Hz, 6H, H-26 + H-27), 0.80 – 0.68 (*m*, 1H, H-9), 0.67 (*s*, 3H, H-18) ppm;
^{13}C NMR (125 MHz, $CDCl_3$): δ = 212.0 (C-3), 56.3 (C-14), 56.3 (C-17), 53.8 (C-9), 46.7 (C-5), 44.7 (C-4), 42.6 (C-13), 39.9 (C-24), 39.5 (C-12), 38.6 (C-1), 38.2 (C-2), 36.1 (C-22), 35.8 (C-20), 35.6 (C-10), 35.4 (C-8), 31.7 (C-7), 29.0 (C-6), 28.2 (C-16), 28.0 (C-25), 24.2 (C-15), 23.8 (C-23), 22.8 (C-26), 22.5 (C-27), 21.4 (C-11), 18.7 (C-21), 12.1 (C-18), 11.4 (C-19) ppm;
MS (ASAP): *m/z* = 387.4 ([M+H]$^+$, 100%).

3β, 5α-Sitostanol (9)
Hydrogenation of stigmasterol (**10**, 5.0 g, 12.12 mmol) in THF:MeOH (2:1, 150 mL) with Pd/C (10%, 500 mg) for 48 h and 5 bar H_2 pressure followed by usual work-up and chromatography (SiO_2, *n*-hexane/ethyl acetate, 85:15) gave **9** (4.17 g, 83.4%) as a colorless solid; R_f = 0.29 (SiO_2, *n*-hexane/ethyl acetate, 85:15); m.p.: 129 – 131°C (lit.:[69] 134 – 136°C);
IR (ATR): ν = 3271*br*, 2956*s*, 2932*vs*, 1466*m*, 1449*m*, 1375*s*, 1044*s* cm^{-1};
$[\alpha]_D^{20}$= +17.16° (*c* = 0.40, $CHCl_3$); ^{1}H NMR (500 MHz, $CHCl_3$): δ = 3.59 (*m*, *J* = 10.7, 4.8 Hz, 1H, H-3), 2.34 – 2.24 (*m*, 1H, OH), 1.95 (*dt*, *J* = 12.7, 3.5 Hz, 1H, H-12a), 1.86 – 1.75 (*m*, 2H, H-6a + H-7a), 1.73 – 1.61 (*m*, 3H, H-1a + H-4a + H-25), 1.60 – 1.51 (*m*, 2H, H-2a + H-15a), 1.51 – 1.37 (*m*, 2H, H-7b + H-11a), 1.37 – 1.19 (*m*, 9H, H-2b + H-8 + H-11b + H-16 + H-20 + H-22a + H-28), 1.19 – 1.06 (*m*, 6H, H-5 + H-12 + H-14 + H-23), 1.06 – 0.94 (*m*, 3H, H-15b + H-17 + H-22b), 0.94 – 0.87 (*m*, 5H, H-1b + H-21 + H-24), 0.85 (*s*, 1H, H-4b), 0.84 – 0.80 (*m*, 9H, H-18 + H-26 + H-27), 0.80 (*s*, 3H, H-19), 0.70 – 0.57 (*m*, 4H, H-9 + H-29) ppm;
^{13}C NMR (125 MHz, $CDCl_3$): δ = 71.4 (C-3), 56.5 (C-17), 56.2 (C-14), 54.4 (C-9), 45.8 (C-24), 44.9 (C-5), 42.6 (C-13), 40.0 (C-12), 38.1 (C-2), 37.0 (C-1), 36.2 (C-20), 35.5 (C-8), 35.5 (C-10), 33.9 (C-22), 32.1 (C-4), 31.4 (C-7), 29.2 (C-25), 28.7 (C-16), 28.3 (C-6), 26.1 (C-23), 24.2 (C-15), 23.1 (C-28), 21.3 (C-11), 19.8 (C-27), 19.0 (C-26), 18.7 (C-21), 12.3 (C-19), 12.1 (C-29), 12.0 (C-18), ppm;
MS (ASAP): *m/z* = 397.4 ([M-H_2O-H]$^-$, 80%).

5α-Sitostan-3-one (11)
Following GP2, from **9** (2.70 g, 6.47 mmol), acetone (125 mL), CrO_3 (0.78 g, 7.76 mmol), dist. H_2O (2.5 mL), conc. H_2SO_4 (0.75 mL) and silica gel (20 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 85:15 und 9:1) **11** (1.60 g, 59.7%) was obtained as a colorless solid; R_f = 0.23 (SiO_2, *n*-hexane/ethyl acetate, 85:15); m.p.: 153 – 155°C (lit.:[70] 155°C);
IR (ATR): ν = 2931*s*, 2959*s*, 1716*vs*, 1443*m*, 1435*m* cm^{-1};
$[\alpha]_D^{20}$= +40.52° (*c* = 0.42, $CHCl_3$);
^{1}H NMR (500 MHz, $CHCl_3$): δ = 2.41 – 2.32 (*m*, 1H, H-2a), 2.31 – 2.21 (*m*, 2H, H-2b + H-4a), 2.10 – 2.04 (*m*, 1H, H-4b), 2.03 – 1.95 (*m*, 2H, H-1a + H-12a), 1.87 – 1.78 (*m*, 1H, H-16a), 1.72 – 1.61 (*m*, 2H, H-7a + H-25), 1.60 – 1.47 (*m*, 3H, H-5 + H-11a + H-15a), 1.43 – 1.22 (*m*, 9H, H-1b + H-6 + H-8 + H-11b + H-16b + H-20 + H-22a + H-28a), 1.21 – 1.08 (*m*, 5H, H-12b + H-14 +H-23 + H-28b), 1.07 – 0.95 (*m*, 3H, H-15b + H-17 + H-22b), 0.99 (*s*, 3H, H-19), 0.95 – 0.86 (*m*, 5H, H-7b + H-21 + H-24), 0.86 – 0.79 (*m*, 9H, H-26 + H-27 +H-29), 0.79 – 0.68 (*m*, 1H, H-9), 0.67 (*s*, 3H, H-18) ppm;
^{13}C NMR (125 MHz, $CDCl_3$): δ = 212.0 (C-3), 56.3 (C-17), 56.2 (C-14), 53.8 (C-9), 46.7 (C-5), 45.8 (C-24), 44.7 (C-4), 42.6 (C-13), 39.9 (C-12), 38.6 (C-1), 38.2 (C-2), 36.1 (C-20), 35.6 (C-10), 35.4 (C-8), 33.9 (C-22), 31.7 (C-7), 29.2 (C-25), 29.0 (C-6), 28.2 (C-16), 26.1 (C-23), 24.2 (C-15), 23.1 (C-28), 21.4 (C-11), 19.8 (C-26), 19.0 (C-27), 18.7 (C-21), 12.0 (C-18), 12.0 (C-29), 11.4 (C-19) ppm;
MS (ASAP): *m/z* = 415.4 ([M+H]$^+$, 100%).

5'-Amino-cholest-2-eno[2,3-b]thiophene-4′-carboxylic acid ethyl ester (12)
Following GP1 from **8** (402 mg, 1.04 mmol), ethyl cyanoacetate (0.12 mL, 1.04 mmol), sulfur (32 mg, 1.04 mmol), morpholine (0.1 mL, 1.04 mmol) and

EtOH (10 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **12** (308 mg, 57.6%) was obtained as a colorless solid; R_f = 0.28 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 177 – 180°C (lit.:[38] 181°C);
IR (ATR): ν = 3485*w*, 3356*w*, 2927*m*, 1665*vs*, 1598*m*, 1497*m*, 1268*w*, 1160*m* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 229 nm (4.01);
$[\alpha]_D^{20}$= +69.52° (*c* = 0.35, $CHCl_3$); ^{1}H NMR (500 MHz, $CHCl_3$): δ = 5.90 (*s*, 2H, -NH_2, H-5‘), 4.34 – 4.19 (*m*, 2H, H-29), 2.79 – 2.70 (*m*, 1H, H-4a), 2.46 – 2.39 (*m*, 1H, H-1a), 2.23 – 2.13 (*m*, 2H, H-1b + H-4b), 2.00 (*dt*, *J* = 12.6, 3.5 Hz, 1H, H-12a), 1.87 – 1.76 (*m*, 1H, H-16a), 1.73 – 1.66 (*m*, 1H, H-6a), 1.63 – 1.46 (*m*, 3H, H-7a + H-15a + H-25), 1.49 – 1.20 (*m*, 8H, H-5 + H-7b + H-8 + H-11 + H-16b + H-20 + H-23a), 1.33 (*t*, *J* = 7.1 Hz, 3H, H-30), 1.20 – 1.02 (*m*, 6H, H-12b H-15b + H-17 + H-23b + H-24), 1.05 – 0.92 (*m*, 3H, H-14 + H-22), 0.95 – 0.84 (*m*, 10H, H-6b + H-21 + H-26 + H-27), 0.83 – 0.73 (*m*, 1H, H-9), 0.78 (*s*, 3H, H-19), 0.67 (*s*, 3H, H-18) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 165.9 (C-28), 160.1 (C-5‘), 131.2 (C-3), 118.2 (C-2), 106.5 (C-4‘), 59.5 (C-29), 56.4 (C-14), 56.3 (C-17), 53.8 (C-9), 42.5 (C-13), 41.7 (C-5), 39.9 (C-12), 39.5 (C-24), 38.7 (C-1), 36.2 (C-22), 36.2 (C-10), 35.8 (C-20), 35.7 (C-8), 31.9 (C-4), 31.8 (C-6), 28.9 (C-7), 28.2 (C-16), 28.0 (C-25), 24.2 (C-15), 23.9 C (C-23), 22.8 (C-27), 22.6 (C-26), 21.1 (C-11), 18.7 (C-21), 14.5 (C-30), 12.0 (C-18), 11.6 (C-19) ppm;
MS (ESI, MeOH/$CHCl_3$ = 4:1): *m/z* = 514.3 ([M+H]$^+$, 100%);
analysis calcd for $C_{32}H_{521}NO_2S$ (513.83): C 74.80, H 10.00, N 2.73, S 6.24; found: C 74.57, H 10.16, N 2.52, S 6.01.

5’-Amino-cholest-2-eno[2,3-b]thiophene-4′-carboxylic acid *n*-propyl ester (13)
Following GP1, from **8** (402 mg, 1.04 mmol), propyl cyanoacetate (0.13 mL, 1.04 mmol), sulfur (32 mg, 1.04 mmol), morpholine (0.1 mL, 1.04 mmol) and EtOH (15 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) gave **13** (367 mg, 0.70 mmol, 66.9%) as an off-white solid; R_f = 0.33 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 161 – 163°C;
IR (ATR): ν = 3484*w*, 3354*w*, 2928*m*, 1665*vs*, 1599*m*, 1587*m*, 1497*s*, 1268*w*, 1160*m* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 230 nm (4.01); $[\alpha]_D^{20}$= +70.91° (*c* = 0.41, $CHCl_3$);
^{1}H NMR (500 MHz, $CHCl_3$): δ = 5.90 (*s*, 2H, -NH_2, H-5‘), 4.33 – 4.11 (*m*, 2H, H-29), 2.80 – 2.71 (*m*, 1H, H-6a), 2.43 (*d*, *J* = 15.4 Hz, 1H, H-1a), 2.23 – 2.15 (*m*, 2H, H-1b + H-6b), 2.00 (*dt*, *J* = 12.6, 3.4 Hz, 2H, H-12), 1.87 – 1.77 (*m*, 1H, H-7a), 1.77 – 1.67 (*m*, 3H, H-4a + H-30), 1.63 – 1.56 (*m*, 2H, H-15), 1.56 – 1.49 (*m*, 2H, H-16a + H-25), 1.48 – 1.40 (*m*, 3H, H-5 + H-11), 1.39 – 1.29 (*m*, 5H, H-8 + H-16b + H-20 + H-22a + H-23a), 1.29 – 1.19 (*m*, 1H, H-7b), 1.19 – 1.03 (*m*, 4H, H-17 + H-23b + H-24), 1.03 – 0.95 (*m*, 5H, H-14 + H-22b + H-31), 0.95 – 0.84 (*m*, 10H, H-4b + H-21 + H-26 + H-27), 0.84 – 0.72 (*m*, 1H, H-9), 0.78 (*s*, 3H, H-19), 0.67 (*s*, 3H, H-18) ppm;
^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.1 (C-28), 159.7 (C-5‘), 131.2 (C-3), 118.4 (C-2), 106.8 (C-4‘), 65.3 (C-29), 56.4 (C-14), 56.3 (C-17), 53.8 (C-9), 42.5 (C-13), 41.7 (C-5), 39.9 (C-12), 39.5 (C-24), 38.7 (C-1), 36.2 (C-10), 36.2 (C-22), 35.8 (C-20) , 35.7 (C-8), 31.9 (C-6), 31.7 (C-4), 29.0 (C-16), 28.2 (C-7), 28.0 (C-25), 24.2 (C-15), 23.9 (C-23), 22.8 (C-27), 22.6 (C-26), 22.2 (C-30), 21.1 (C-11), 18.7 (C-21), 12.0 (C-18), 11.6 (C-19), 10.8 (C-31) ppm;
MS (ESI, MeOH/$CHCl_3$ = 4:1): *m/z* = 528.1([M+H]$^+$, 100%);
analysis calcd for $C_{33}H_{53}NO_2S$ (527.38): C 75.09, H 10.12, N 2.65, S 6.07; found: C 74.86, H 12.40, N 2.46, S 5.83.

5’-Amino-sitost-2-eno[2,3-b]thiophene-4′-carboxylic acid ethyl ester (14)
Following GP1 from **11** (431 mg, 1.04 mmol), ethyl cyanoacetate (0.12 mL, 1.04 mmol), sulfur (32 mg, 1.04 mmol), morpholine (0.1 mL, 1.04 mmol) and EtOH (15 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **14** (348 mg, 0.64 mmol, 61.8%) was obtained as an off-white solid; R_f = 0.27 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 196 – 198°C;
IR (ATR): ν = 3476*w*, 3346*w*, 2929*s*, 1663*vs*, 1595*m*, 1584*m*, 1494*m*, 1269*m*, 1161*m* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 229 nm (4.01); $[\alpha]_D^{20}$= +68.69° (*c* = 0.33, $CHCl_3$);
^{1}H NMR (500 MHz, $CHCl_3$): δ = 5.94 (*s*, 2H, -NH_2, H-5‘), 4.37 – 4.16 (*m*, 2H, H-31), 2.75 (*dd*, *J* = 17.9, 5.1 Hz, 1H, H-6a), 2.43 (*d*, *J* = 15.2 Hz, 1H, H-1a), 2.24 – 2.14 (*m*, 2H, H-1b + H-6b), 2.00 (*dt*, *J* = 12.6, 3.5 Hz, 1H, H-12a), 1.88 – 1.78 (*m*, 1H, H-16a), 1.73 – 1.62 (*m*, 2H, H-4a + H-25), 1.62 – 1.50 (*m*, 2H, H-7a + H-15a), 1.48 – 1.37 (*m*, 3H, H-5 + H-11), 1.33 (*t*, *J* = 7.1 Hz, 6H, H-8 + H-20 + H-22a + H-32), 1.30 – 1.21 (*m*, 4H, H-7b + H-16b + H-28), 1.21 – 1.10 (*m*, 4H, H-12b + H-17 + H-23), 1.10 – 0.97 (*m*, 3H, H-14 + H-15b + H-22b), 0.95 – 0.88 (*m*, 5H, H-4b + H-21 + H-24), 0.87 – 0.79 (*m*, 9H, H-26 + H-27 + H-29), 0.78 (*m*, 4H, H-9 + H-19), 0.67 (*s*, 3H, H-18) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ =165.9 (C-30), 159.5 (C-5‘), 131.3 (C-3), 118.6 (C-2), 106.9 (C-4‘), 59.5 (C-31), 56.4 (C-14), 56.2 (C-17), 53.8 (C-9), 45.8 (C-24), , 42.5 (C-13), 41.7 (C-5), 39.9 (C-12), 38.8 (C-1), 36.2 (C-10), 36.2 (C-20), 35.7 (C-8), 33.9 (C-22), 31.9 (C-6), 31.8 (C-4), 29.2 (C-25), 28.9 (C-7), 28.2 (C-16), 26.1 (C-23), 24.2 (C-15), 23.1 (C-28), 21.1 (C-11), 19.8 (C-26), 19.0 (C-27), 18.8 (C-21), 14.5 (C-32), 12.0 (C-18 + C-29), 11.6 (C-19), ppm; MS (ESI, MeOH/$CHCl_3$ = 4:1): *m/z* = 542.1 ([M+H]$^+$, 100%);
analysis calcd for $C_{34}H_{55}NO_2S$ (541.88): C 75.36, H 10.23, N 2.58, S 5.92; found: C 75.15, H 10.46, N 2.41, S 5.77.

5’-Amino-sitost-2-eno[2,3-b]thiophene-4′-carboxylic acid *n*-propyl ester (15)
Following GP1, from **11** (431 mg, 1.04 mmol), propyl cyanoacetate (0.13 mL, 1.04 mmol), sulfur (32 mg,

1.04 mmol), morpholine (0.1 mL, 1.04 mmol) and EtOH (15 mL) followed by chromatography (SiO_2, *n*-hexane/ethyl acetate, 95:5) **15** (372 mg, 64.3%) was obtained as a colorless solid; R_f = 0.29 (SiO_2, *n*-hexane/ethyl acetate, 95:5); m.p.: 174 – 176°C;
IR (ATR): ν = 3442*w*, 3325*w*, 2927*s*, 1663*vs*, 1593*s*, 1582*s*, 1481*s*, 1268*vs*, 1159*s* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 230 nm (4.01); $[\alpha]_D^{20}$= +67.20° (*c* = 0.32, $CHCl_3$);
^{1}H NMR (500 MHz, $CHCl_3$): δ = 5.93 (*s*, 2H, -NH_2, H-5‘), 4.17 (*qt*, *J* = 10.7, 6.6 Hz, 2H, H-31), 2.80 – 2.73 (*m*, 1H, H-6a), 2.43 (*d*, *J* = 15.4 Hz, 1H, H-1a), 2.24 – 2.14 (*m*, 2H, H-1b + H-6b), 2.00 (*dt*, *J* = 12.6, 3.4 Hz, 1H, H-12a), 1.88 – 1.78 (*m*, 1H, H-7a), 1.78 – 1.63 (*m*, 4H, H-4a + H-25 + H-32), 1.63 – 1.49 (*m*, 2H, H-15a + H-16a), 1.48 – 1.37 (*m*, 3H, 5 + H-11), 1.37 – 1.29 (*m*, 4H, H-8 + H-16b + H-20 + H-22a), 1.29 – 1.21 (*m*, 3H, H-7b + H-28), 1.21 – 1.08 (*m*, 4H, H-12b + H-17 + H-23), 1.07 – 0.97 (*m*, 6H, H-14 + H-15b + H-22b + H-33), 0.95 – 0.88 (*m*, 5H, H-4b + H-21 + H-24), 0.87 – 0.79 (*m*, 9H, H-26 + H-27 + H-29), 0.79 – 0.74 (*m*, 4H, H-9 + H-19), 0.67 (*s*, 3H, H-18) ppm;
^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.1 (C-30), 159.8 (C-5‘), 131.2 (C-3), 118.4 (C-2), 106.8 (C-4‘), 65.3 (C-31), 56.4 (C-14), 56.2 (C-17), 53.8 (C-9), 45.8 (C-24), 42.5 (C-13), 41.7 (C-5), 39.9 (C-12), 38.7 (C-1), 36.2 (C-10), 36.2 (C-20), 35.7 (C-8), 33.9 (C-22), 32.0 (C-6), 31.7 (C-4), 29.2 (C-25), 28.9 (C-16), 28.2 (C-7), 26.1 (C-23), 24.2 (C-15), 23.1 (C-28), 22.2 (C-32), 21.1 (C-11), 19.8 (C-26), 19.0 (C-27), 18.8 (C-21), 12.0 (C-18 + C-29), 11.6 (C-19), 10.8 (C-33) ppm;
MS (ESI, MeOH/$CHCl_3$ = 4:1, ASAP): *m/z* = 556.1 ($[M+H]^+$, 100%);
$C_{35}H_{57}NO_2S$ (555.91): C 75.62, H 10.34, N 2.52, S 5.77; found: C 75.34, H 10.46, N 2.37, S 5.51.

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