RSC Advances

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Journal:	*RSC Advances*
Manuscript ID:	RA-ART-06-2015-010966.R1
Article Type:	Paper
Date Submitted by the Author:	09-Jul-2015
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The labeling of brassinosteroids by tritium

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A convenient method for the synthesis of tritium-labeled brassinosteroids with very high specific activity is reported. A ^{3}H-labeled 24-epicastasterone was isolated in high yield (40 mCi), radiochemical purity (>97%) and a specific activity up to 99 Ci/mmol. The labeling strategy was designed to employ a radiolabeling step at the late stage of the synthetic sequence.

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1. Introduction

Brassinosteroids (BRs) are polyhydroxylated sterol phytohormones with a structural resemblance to animal steroid hormones. They occur at low levels throughout the plant kingdom and have been shown to be essential for normal growth and development. They were first isolated and characterized from the pollen of *Brassica napus* (brassinolide) in 1979.[1] More than 70 of these plant-growth regulators have been discovered so far.[2–5] Recently, the pioneer work of Strnad et al. has revealed that some natural BRs induce cell-growth-inhibitory responses in several human cancer cell lines at micromolar concentration without affecting normal non-tumor cell growth (BJ fibroblasts).[6] The highest biological activity has been found in BRs containing 2α,3α- and 22*R*,23*R*-diol functions and a lactone or ketone moiety in ring B (Figure 1). Studies on the mechanism of action of biologically active compounds on the molecular level require that these substances be radioactively labeled.[7]

The first approach to BR radiolabeling was reported by Seo et al.[8] Carbon-14 was implanted into position C-(4) of the steroid skeleton at an early stage of a synthetic sequence of more than ten steps. This led to low radiochemical yields of 3.2% and 4.5%, respectively, of synthesized (22*R*,23*R*)- and (22*S*,23*S*)-24-[4-^{14}C]epibrassinolide. Kolbe et al. have published two papers dealing with the introduction of an alternative isotope – tritium – into the enolizable positions of a BR skeleton via a based-catalyzed exchange with tritiated water.[9,10] However, this method has provided only low specific activities (about 6.10^{-3} Ci/mmol) of labeled BRs; moreover, the label placed in exchangeable positions will most probably not be stable under physiological conditions. Recently, we have published the results of studies that would afford ^{3}H-labeled BRs with high specific activities of the order of tens of Ci/mmol.[11–13]

A detailed study of regio-specifically tritium-labeled brassinosteroids with a very high specific activity (SA) is reported in this paper.

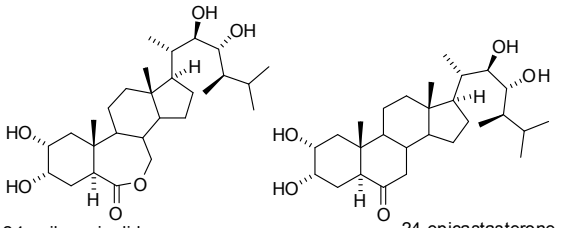


Fig. 1 24-epiBL (**1**) is the 24-(R)-epimer of the first isolated brassinosteroid, 24-epiCS (**2**).

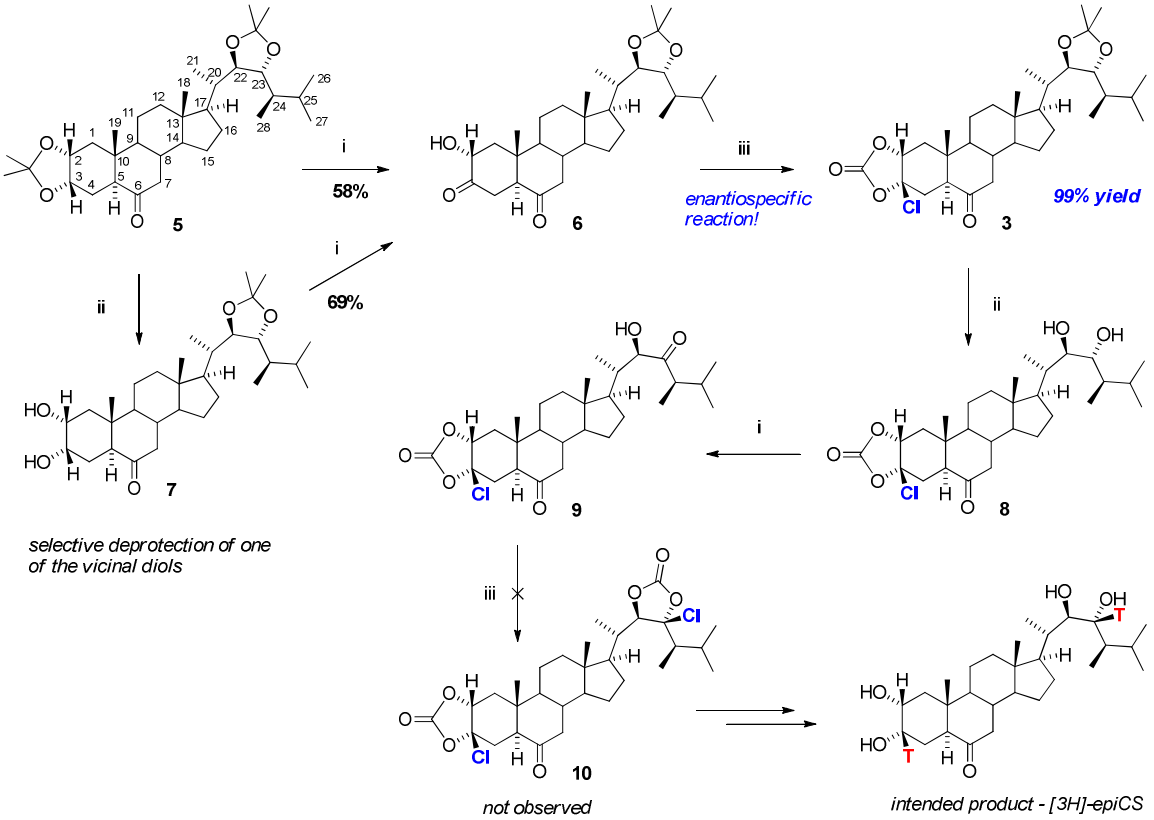
2. Results and discussion

We have recently reported the conversion of the vicinal 2α,3α-diols **2** to appropriate α-hydroxy ketones by oxidation with a freshly generated dimethyldioxirane (DMD).[12–14] An endiol form of such an α-hydroxy ketone is a suitable substrate for synthetic transformations leading to an appropriate bis-substituted endiol[11] or chlorocarbonate.[12,13] As we have recently described, the catalytic hydrogenation of bis-silylated endiol was not successful under various reaction conditions.[12,13] Moreover, the other attempted methods of the creation of a reducible double bond on the steroid skeleton from the α-hydroxy ketone group failed.[13] On the other hand, we have shown that it is possible to perform catalytic reductive dehalogenation of chlorocarbonate **3** obtained by the reaction of the corresponding α-hydroxy ketone with triphosgene in a nearly quantitative yield. The reduction of **3** by gaseous carrier-free tritium catalyzed by PdO/$CaCO_3$ in the presence of Et_3N followed by the removal of protecting groups provided 24-[3β-^{3}H]epicastasterone (**[^{3}H]-2**) with a specific activity of 5.8 Ci/mmol.[5,6] The 24-[3β-^{3}H]epicastasterone (**[^{3}H]-2**) was oxidized by trifluoroperoxyacetic acid to 24-[3β-^{3}H]epibrassinolide **[^{3}H]-1** with the retention of specific activity (5.8 Ci/mmol)(Scheme 1).

Scheme 1 i) T_2/PdO/$CaCO_3$/Et_3N; ii) Fe(III), CH_2Cl_2; iii) NaOH, 1,4-dioxane; iv) H_2O_2/TFA, 0°C 30 min, r.t. 4h, $CHCl_3$.

To obtain a product with an even higher specific activity, we attempted to synthesize derivative **10**, possessing two chlorocarbonate groups in the BR skeleton. Chlorocarbonate **3** was chosen as the starting molecule for such a synthetic

transformation.[12,13] The vicinal diol **8**, obtained after selective deprotection of **3** by $FeCl_3$ in a yield of 87% (Experimental section, 4.1.), was oxidized with dimethyldioxirane (DMD) under general conditions (Experimental section, 4.2.). After the reaction was left at 4°C for two days, α-hydroxyketon **9** was isolated in a moderate yield of 26%. The subsequent conversion of such hydroxyketon **9** into an appropriate chlorocarbonate **10** failed under the reaction conditions[12,13] used for an otherwise very efficient synthesis of chlorocarbonate **3**, in other cases always affording a quantitative yield of an appropriate chlorocarbonate. Not even enhanced reaction temperature (80°C) promoted the formation of **10**. The reaction was characterized by a low conversion of the starting material **9**, and no desired carbonate **10** was formed (Scheme 2).



Scheme 2 The unsuccessful method of obtaining a labeled epiCS with a high SA: i) fresh dimethyldioxirane, aceton, 20h, 4°C; ii) Fe(III), CH_2Cl_2; iii) triphosgene, pyridine, benzene.

Seto et al. have described a five-step reaction strategy for the side-chain deuterium-multilabeling of brassinolide.[15] After the protection of all hydroxyl groups on the BR skeleton, the C-25 carbon was hydroxylated by trifluoromethyldioxirane (TFD). The dehydration of the tertiary alcohol provided a mixture of $\Delta^{25(26)}$ and $\Delta^{24(25)}$ regiomers in the 65:35 ratio that was possible to separate after deprotection. The deuteration of $\Delta^{25(26)}$ regioisomer by deuterium gas catalyzed by Pd/C (1 atm, 25°C, 1h) yielded [24,25,26,27-^{2}H]brassinolide with 60% deuterium enrichment calculated from MS data. The ratio of the individual multideuterated species in the cluster was $^2H_2$:$^2H_3$:$^2H_4$:$^2H_6$:$^2H_7$ = 3:8:14:15:60.

We have decided to follow the protocol of Seto et al. in order to synthesize an unsaturated precursor of the intended synthesis of ^{3}H-labeled 24-epicastasterone. Having 2,3,22,23-diisopropylidene-24-epicastasterone[12] **5** available, we tried the TFD hydroxylation of C-25 carbon with this particular derivative. The hydroxylation proceeded well, but it was shown that the 2,3-isopropylidene group was not resistant under these reaction conditions and, moreover, the deprotected 2,3-hydroxyl group was further oxidized to an appropriate hydroxyketone. Therefore, we prepared 2,3,22,23-tetra-O-acetyl-24-epicastasterone; surprisingly enough, its C-25 hydroxylation did not proceed at all under the conditions used for the hydroxylation of **5**. In the end, we prepared 2,3-di-O-acetyl-22,23-isopropylidene-24-epicastasterone (**11**) (Experimental section, 4.3.) which upon TFD hydroxylation at low temperature gave 2,3-di-O-acetyl-22,23-isopropylidene-25-hydroxy-24-epicastasterone **12** in 58% yield (Experimental section, 4.4.). The dehydration of **12** using thionyl chloride in pyridine at 0°C for 30 min afforded a mixture of prevailing (22*R*,23*R*,24*R*)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5α-cholestan-25-ene-6-one (**13**) accompanied by its 24-ene regioisomer **14** (Experimental section, 4.5.). The separation of unsaturated regioisomers **13** and **14** using HPLC turned out to be infeasible; on the other hand, it was possible to separate unsaturated derivatives **13** and **14** from 24-epicastasterone derivative **11** by HPLC. This fact

eventually enabled the isolation of 261 mCi of (22*R*,23*R*,24*R*)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-[24,25,26,27-^{3}H]epicastasterone (**15**) after the catalytic tritiation of the mixture of the unsaturated derivatives **13** and **14** over Pd/C (10%) in ethylacetate under carrier-free tritium gas (998 mbar) for 2 hours (Experimental section, 4.6.). In one-pot synthesis, derivative **15** was deisopropylinated and deacetylated (Experimental section, 4.7.). After radio-HPLC purification, 40 mCi of 24-[24,25,26,27-^{3}H]epicastasterone (**[3H]-2**) with R.C.P. >97% and SA_{MS} = 99.4 Ci/mmol were obtained. Unfortunately, it was shown that the free BRs with a high SA are extremely sensitive to radiolysis. The aliquot of the column eluate was evaporated to dryness and the residue was dissolved in DMSO-d_6 for the ^{3}H NMR measurement. As the NMR spectrum was more complicated than anticipated, the solution from the NMR tube was assessed for purity by radio-HPLC. Only 12% of the activity of 24-[24,25,26,27-^{3}H]epicastasterone (**[3H]-2**) was left while 88% of the activity was found in a broad peak with considerably higher retention. By this finding, we eventually discovered the only secure procedure of how to remove chromatographic solvents and formulate the high-specific-activity BRs for application in biochemical experiments. Combined fractions (2 mL) were enriched with glycerol (300 μL) (as an antioxidant and to prevent the high concentration and dryness of the residue) and 10 mg of (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (as another antioxidant utilizable in biological experiments). After the evaporation of methanol and water on CentriVap, the residual glycerol solution was diluted with water (2 mL), the activity was determined and the concentration of 24-[24,25,26,27-^{3}H]epicastasterone (**[3H]-2**) was afterwards adjusted to 1 mCi/mL with a glycerol–water (1:1) mixture. The concentration of Trolox in the final formulation was adjusted to 0.5% and 1 mCi aliquots were stored in liquid nitrogen.

In view of this instability of free BRs with a high SA, we decided to reverse the reaction sequence for obtaining 24-[24,25,26,27-^{3}H]epibrassinolide (**[3H]-1**), i.e. to perform the Baeyer-Villiger oxidation on fully protected 24-[24,25,26,27-^{3}H]epicastasterone **15** and then to deprotect the (22*R*,23*R*,24*R*)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-[24,25,26,27-^{3}H]epibrassinolide (**16**) (Experimental section, 4.8.). This made it possible to obtained 3.5 mCi of pure 24-[24,25,26,27-^{3}H]epibrassinolide (**[3H]-1**) with R.C.P.>97% and SA_{MS} = 98 Ci/mmol from 26 mCi of **15**. The ^{3}H NMR spectra of both **[3H]-2** and **[3H]-1** show a higher substitution of hydrogens by tritium in methyls 26 and 27, which does not correspond to the intensity of the tritium signal at C-25 and is in good agreement with the SA, indicating 3.4 tritium atoms per molecule as average. The tritium substitution at C-24 can be the result of either the allylic exchange before the reduction of the double bond, like for C-26 and C-27, or the reduction of 24-ene regioisomer **14**, which is less likely but cannot be excluded. Answering this question would first require a distinction between the 24-ene and 25-ene regioisomers **13** and **14**. The slight upfield shift and broadening of the tritium multiplet of tritons at C-26 an C-27 in 24-[24,25,26,27-^{3}H]epibrassinolide (**[3H]-1**) when compared to the 24-[24,25,26,27-^{3}H]epicastasterone (**[3H]-2**) spectrum is in accordance with literature data.[16]

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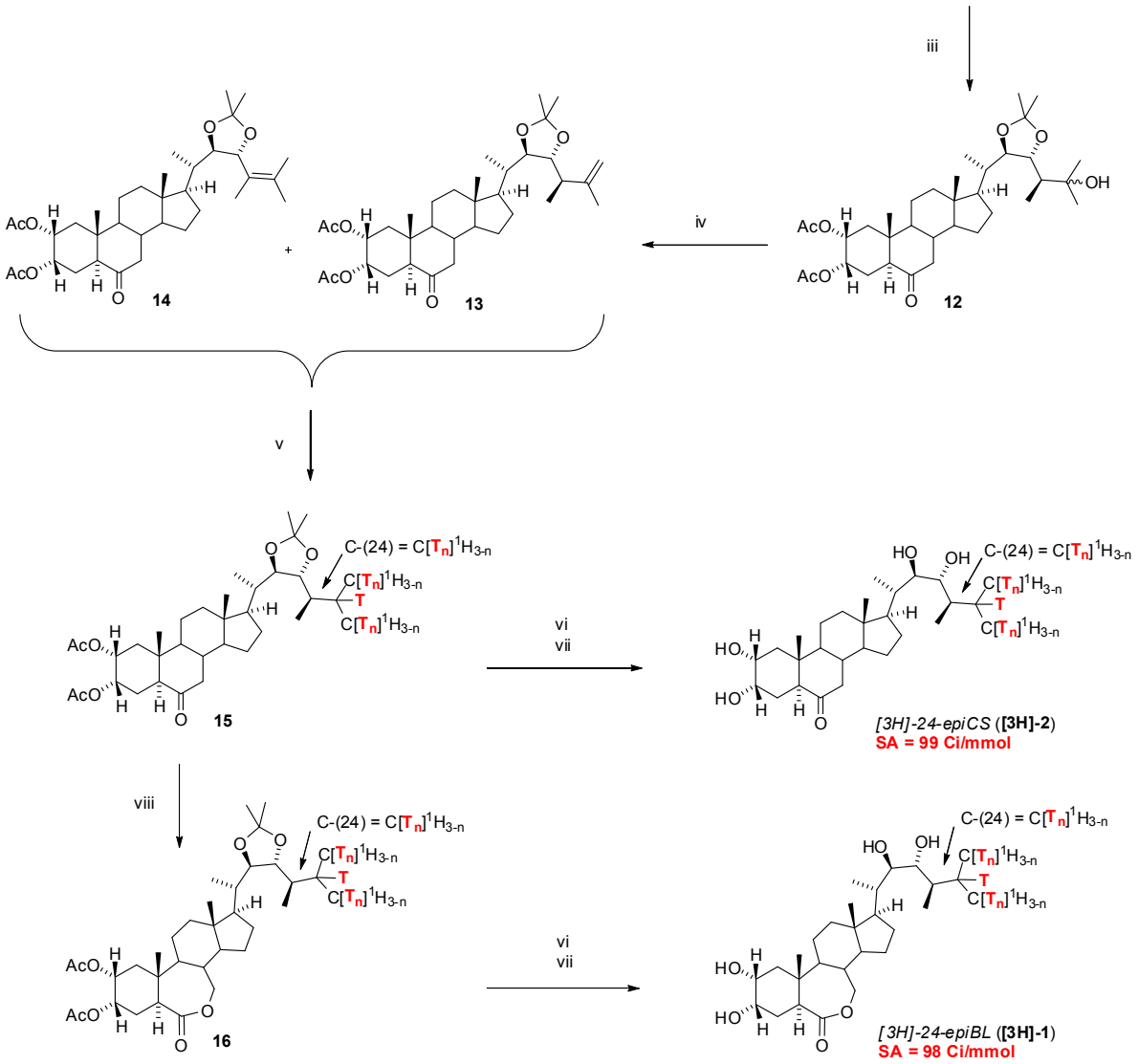

Scheme 3 The successful approach for the synthesis of [3H]-24-epiCS and [3H]-24-epiBL with a high SA: i) $Ce(NH_4)_2(NO_3)_6$, borate buffer, r.t.; ii) Ac_2O, Py, 20h, 60°C; iii) TFD, 2d, -5°C; iv) $SOCl_2$, Py, 30 min, 0°C; v) T_2, Pd/C (10%), EtOAc, 2h; vi) $FeCl_3$, CH_2Cl_2; vii) CH_3ONa (1M), r.t.; viii) TFPAA, $CHCl_3$, 30 min, 0°C, 5h, r.t.

3. Conclusion

This paper provides a new method for the synthesis of ^{3}H-labeled brassinosteroids with a high SA. 24-[24,25,26,27-^{3}H]epicastasterone and 24-[24,25,26,27-^{3}H]epibrassinolide were synthesized with a high SA – 99.4 and 98 Ci/mmol, respectively. The six-step synthetic sequence starting with the brassinosteroid *to be labeled* provided the desired tritium multilabeled product in sufficient yield with satisfactory radiochemical purity. The radiolysis of such multi-labeled brassinosteroids was substantially suppressed by the use of glycerol as a non-volatile matrix when their solutions were highly concentrated. The best formulation of the stock solution proved to be a glycerol–water (1:1) mixture containing 0.5% of the antioxidant Trolox.

4. Experimental section

General

The ^{1}H-, ^{3}H- and ^{13}C-NMR spectra were recorded at 300 MHz, 325 MHz and 75 MHz, respectively, with a Bruker Avance II 300 MHz instrument at 25 °C (the solvents are indicated in parentheses). Chemical shifts are reported in ppm relative to TMS. The ^{3}H NMR spectra were measured in D_2O, with the signal of water at 4.7 ppm being taken as an external standard. The mass spectra were obtained by the Bruker Daltonics Esquire 4000 system with direct input (ESI, stream AcCN-H_2O, a mass range of 50–1200 Da, Esquire Control Software). The HR-mass spectra were obtained in the ESI mode either on a Waters-Micromass Q-TOF Micro mass spectrometer or on a Thermo Fisher Scientific LTQ Orbitrap XLc. The mass spectra of labeled compounds were measured on a Thermo Finnigan LCQ classic spectrometer using electrospray ionization (ESI). Column chromatography was carried out with SiO_2 60 (a particle size of 0.040–0.063 mm, 230–400 mesh; Merck) and commercially available solvents. Thin-layer chromatography (TLC) was conducted on aluminum sheets coated with SiO_2 60 F_{254} obtained from Merck, with visualization by UV lamp (254 or 360 nm). The HPLC was performed on a system consisting of a WATERS Delta 600 Pump and Controller, a WATERS 2487 UV detector and a RAMONA radio chromatographic detector from Raytest (Germany) with interchangeable fluid cells. For the preparative runs, the cell with a single small crystal of a solid scintillator was used; for analytical runs, the column effluent was mixed with a Zinsser Quickszint Flow 302 cocktail at a 1:3 ratio. The data were collected and processed using Empower 2.0 software. The activities were measured on a Perkin-Elmer Tri-Carb 2900 liquid scintillation counter (LSC) in a Zinsser Quicksafe A cocktail. The evaporations were done using a CentriVap Concentrator from Labconco.

4.1. The deprotection of the 22,23-diol group of 3

An excess of $FeCl_3$ (35 mg, 215 µmol) was added to a solution of chlorocarbonate **3** (25 mg, 44 µmol) (prepared following ref. 12,13) in CH_2Cl_2 (5 ml) and the reaction mixture was stirred for one hour. The organic layer was washed with water (5 mL) and a saturated solution of $NaHCO_3$. The combined organic layers were dried (Mg_2SO_4) and the solvent evaporated. The crude product was purified by column chromatography (SiO_2; EtOAc-*n*-hexane, 1:4), affording a pure product **8** in a yield of 87%.

(22R, 23R, 24R)-3β-chloro-2α, 3α-carbonyldioxy)-22,23-dihydroxy-24-methyl-5α-cholestan-6-one (**8**). White solid. mp = 212-214 °C. $[\alpha]_D^{20}$= +12.8 (*c* 0.14, $CHCl_3$). R_f = 0.35 (SiO_2; SiO_2; EtOAc/*n*-hexane, 1:4). ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (3H, s, C*H*$_3$-18), 0.81 (3H, s, CH_3-19), 0.85 (3H, d, *J* = 7.0 Hz, CH_3-28), 0.87 (3H, d, *J* = 6.7 Hz, CH_3-26), 0.93 (3H, d, *J* = 6.9 Hz, CH_3-27), 0.98 (3H, d, *J* = 6.9 Hz, CH_3-21), 1.12–2.24 (33H, m), 2.85 (1H, dd, *J* = 14.8 Hz, *J* = 2.5 Hz, CH_2-4), 3.41 (1H, brs, *CH*-23), 3.69 (1H, brs, *CH*-22), 4.84 (1H, dd, *J* = 10.0 Hz, *J* = 7.4 Hz, C*H*-2). ^{13}C NMR (75 MHz, $CDCl_3$): 11.08 (CH_3-28), 11.97 (CH_3-18), 12.66 (CH_3-21), 13.35 (CH_3-19), 17.47 (CH_3-26), 21.55 (CH_2-11), 22.33 (CH_3-27), 24.08 (CH_2-15), 27.20 (CH-25), 27.88 (CH_2-16), 33.31 (CH_2-4), 37.48 (CH-8), 39.22 (CH_2-12), 40.42 (CH-20), 41.48 (CH_2-1), 42.37 (C-10), 41.60 (CH-24), 42.85 (C-13), 46.65 (CH_2-7), 52.44 (CH-17), 52.71 (CH-9), 56.44 (CH-14), 53.31 (CH-5), 72.75 (CH-22), 76.55 (CH-23), 82.89 (CH-2), 101.96 (C_3-Cl), 152.04, 207.96 (C-6). MS (ESI, *m/z*): 525.2 [M + 1].

4.2. The oxidation of the 22,23-diol group 8 to α-hydroxyketone 9

A solution of sterol **8** (20 mg, 28 µmol) in DCM (3 mL) was enriched with 15 mL of a freshly prepared 0.09 M solution of dimethyldioxirane in acetone (0.8 mmol, prepared according to ref. 11) and the reaction mixture was kept in the refrigerator at 4°C overnight. It was left to warm to room temperature for 1 hour, after which the solvents were evaporated. Water (20 mL) was added to the oily residue and the resulting emulsion was extracted with DCM (3 × 20 mL). The combined organic layers were dried with anhydrous $MgSO_4$ and the solvent was evaporated. The crude product was purified by column chromatography (SiO_2; EtOAc-*n*-hexane-MeOH, 10:30:1) to afford a pure product **9** (11 mg, 58% yield).

(22R, 24R)-3β-chloro-2α, 3α-carbonyldioxy)-22-hydroxy-24-methyl-5α-cholestan-6, 23-dione (**9**). Amorphous solid. R_f = 0.53 (SiO_2; EtOAc/*n*-hexane, 1:4). $[\alpha]_D^{20}$ = -39.4 (*c* 0.11, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.72 (3H, d, *J* = 6.8 Hz, C*H*$_3$-21), 0.74 (3H, s, C*H*$_3$-18), 0.83 (3H, s, C*H*$_3$-19), 0.88 (3H, d, *J* = 6.8 Hz, C*H*$_3$-26), 0.91 (3H, d, *J* = 6.8 Hz, C*H*$_3$-27), 1.03 (3H, d, *J* = 6.7 Hz, C*H*$_3$-28), 1.04–2.62 (17H, m), 2.05 (1H, ddd, *J* = 1.0 Hz, *J* = 12.6 Hz, *J* = 13.4 Hz, C*H*-7α), 2.41 (1H, dd, *J* = 4.4 Hz, *J* = 13.4 Hz, C*H*-7β), 2.48 (1H, m, C*H*-5α), 2.52 + 1.33 (2H, 2 × m, CH_2-1), 3.50 (1H, d, *J* = 5.0 Hz, OH), 4.22 (1H, m, C*H*-22), 4.84 (1H, dd, *J* = 7.4 Hz, *J* = 10.1 Hz, C*H*-2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 11.93 (*C*H_3-18), 12.25 (*C*H_3-28), 12.83 (*C*H_3-21), 13.14 (*C*H_3-19), 18.93 (*C*H_3-26), 20.96 (*C*H_3-27), 21.30 (*C*H_2-11), 23.80 (*C*H_2-15), 28.04 (*C*H_2-16), 28.41 (*C*H-20), 31.77 (*C*H-25), 33.10 (*C*H_2-4), 37.29 (*C*H-8), 38.77 (*C*H_2-12), 41.27 (*C*-10), 42.15 (*C*H_2-1), 42.58 (*C*-13), 46.40 (*C*H_2-7), 47.39 (*C*H-24), 52.10 (*C*H-17), 52.15 (*C*H-14), 53.09 (*C*H-5), 56.25 (*C*H-9), 79.57 (*C*H-22), 82.62 (*C*H-2), 101.71 (*C*-3), 151.76 (O*C*(O)$_2$), 207.62 (*C*O-6), 216.30 (*C*O-23). MS (ESI): 545.5 [M + Na], 1067.9 [2 × M + Na]. HRMS (ESI) for $C_{29}H_{43}O_6NaCl$ calculated: 545.26404, found: 545.26407.

4.3. The acetylation of the 2,3-diol group of 7

Ac_2O (0.25 mL, 2.3 mmol) and pyridine (5 mL, 64 mmol) were added to a solution of **7** (130 mg, 0.25 mmol) (prepared following ref. 12,13) and the reaction mixture was heated at 60°C overnight. The solvent was evaporated and the crude mixture was purified by column chromatography (SiO_2; EtOAc-*n*-hexane, 1:3), affording a pure compound **11** (216 mg, 88%).

(22R,23R,24R)-2α, 3α-diacetoxy-22, 23-(isopropylidenedioxy)-24-methyl-5α-cholestan-6-one (**11**). White solid. mp = 209–212°C. R_f = 0.48 (SiO_2; EtOAc/*n*-hexane, 1:3), $[\alpha]_D^{20}$ = +3.3 (*c* 0.18, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.78 (3H, s, C*H*$_3$-18), 0.82 (3H, d, *J* = 6.8 Hz, C*H*$_3$-28), 0.94 (3H, s, C*H*$_3$-19), 0.94 (3H, s, C*H*$_3$-26), 1.01 (3H, s, C*H*$_3$-27), 1.08 (3H, d, *J* = 6.9 Hz, C*H*$_3$-21), 1.20–2.25 (9H, m), 1.46 + 1.51 (6H, 2 × s, C(C*H*$_3$)$_2$), 1.96–2.00 (1H, m, C*H*-7α), 2.10 + 2.21 (6H, 2 × s, 2 × Ac), 2.42 (1H, dd, *J* = 12.9 Hz, *J* = 4.7 Hz, C*H*-7β), 2.70 (1H, dd, *J* = 13.7 Hz, *J* = 4.8 Hz, C*H*-5α), 3.66–3.70 (1H, dd, *J* = 9.3 Hz, *J* = 6.8 Hz, C*H*-23), 4.05–4.07 (1H, d, *J* = 6.9 Hz, C*H*-22), 5.04–5.08 (1H, m, C*H*-2), 5.50 (1H, d, *J* = 3.2 Hz, C*H*-3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 9.88 (*C*H$_3$-28), 10.56 (*C*H$_3$-18), 12.66 (*C*H$_3$-19), 13.52 (*C*H$_3$-27), 13.83 (*C*H$_3$-26), 16.01 (*C*H$_3$-21), 21.03 + 21.19 (*C*H$_3$Ac), 21.23 (*C*H$_2$-11), 23.82 (*C*H$_2$-15), 24.86 (*C*H$_2$-4), 27.32 + 27.73 (C(*C*H$_3$)$_2$), 27.76 (*C*H$_2$-16), 37.48 (*C*H$_2$-1), 37.63 (*C*H-8), 37.98 (*C*H-20), 39.06 (*C*H$_2$-12), 42.38 (*C*-13), 43.61 (*C*-10), 43.76 (*C*H-24), 46.52 (*C*H$_2$-7), 51.80 (*C*H-17), 53.63 (*C*H-5), 53.66 (*C*H-9), 56.28 (*C*H-14), 68.12 (*C*H-3), 69.11 (*C*H-2), 80.39 (*C*H-23), 82.36 (*C*H-22), 107.97 (*C*-3), 169.97 + 170.23 (2 × *C*OAc), 210.56 (*C*O-6). MS (ESI): 611.4 [M + Na], 1199.8 [2 × M + Na]. HRMS (ESI) for $C_{35}H_{56}O_7Na$ calculated: 611.39171, found: 611.39183.

4.4. The oxidation of the carbon C-25 of 11

A 0.5 M solution of freshly prepared TFD in trifluoroacetone (2 mL) was added to a dichloromethane solution (4 mL) of **11** (60 mg, 63 µmol) and kept at -5°C. The presence of an oxidative reagent was monitored by a potassium iodine test; after two days, the solvent was evaporated and the crude product was purified by column chromatography (SiO_2; EtOAc-n-hexane, 3:1), affording a pure product **12** in a yield of 58%.

25-hydroxy-(22R,23R,24R)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5α-cholestan-6-one (**12**). White solid. mp = 230–232°C. R_f = 0.28 (SiO_2; EtOAc/n-hexane, 3:1). $[\alpha]_D^{20}$ = -2.6 (c 0.19, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.79 (3H, s, C*H*$_3$-18), 0.89 (3H, d, *J* = 6.8 Hz, C*H*$_3$-28), 0.96 (3H, s, C*H*$_3$-19), 0.99 (3H, d, *J* = 6.9 Hz, C*H*$_3$-21), 1.05 (3H, s, C*H*$_3$-26), 1.07 (3H, s, C*H*$_3$-27), 1.20–2.25 (14H, m), 1.29 + 1.31 (6H, 2 × s, C(C*H*$_3$)$_2$), 1.40 + 2.33 (2H, 2 × m, C*H*$_2$-1), 1.99–2.04 (1H, m, C*H*-7α), 2.10 + 2.20 (6H, 2 × s, 2 × Ac), 2.43 (1H, dd, *J* = 13.1 Hz, *J* = 4.3 Hz, C*H*-7β), 2.69 (1H, dd, *J* = 13.7 Hz, *J* = 4.8 Hz, C*H*-5α), 3.69–3.71 (1H, dd, *J* = 10.4 Hz, *J* = 7.0 Hz, C*H*-23), 4.06 (1H, d, *J* = 6.9.Hz, C*H*-22), 5.05–5.08 (1H, m, C*H*-2), 5.51 (1H, brs, C*H*-3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 11.83 (*C*H$_3$-28), 12.53 (*C*H$_3$-18), 13.19 (*C*H$_3$-21), 13.55 (*C*H$_3$-19), 21.01 + 21.19 (Ac), 21.22 (*C*H$_2$-11), 23.80 (*C*H$_2$-15), 23.81 (*C*H$_3$-27), 24.85 (*C*H$_2$-4), 26.87 + 26.88 (C(*C*H$_3$)$_2$), 27.65 (*C*H$_2$-16), 28.97 (*C*H$_3$-26), 37.32 (*C*H-8), 37.52 (*C*H$_2$-1), 38.20 (*C*H-20), 38.88 (*C*H$_2$-12), 42.31 (*C*-10), 42.88 (*C*-13), 46.50 (*C*H$_2$-7), 47.96 (*C*H-24), 51.82 (*C*H-17), 52.97 (*C*H-5), 53.68 (*C*H-9), 56.19 (*C*H-14), 68.19 (*C*H-3), 68.97 (*C*H-2), 73.57 (*C*-25), 81.03 (*C*H-23), 83.65 (*C*H-22), 169.88 + 170.28 (2 × CH$_3$*C*=O), 210.46 (6-*C*O). MS (ESI): 627.3 [M + Na], 1231.6 [2 × M + Na]. HRMS (ESI) for $C_{35}H_{56}O_8Na$ calculated: 627.38656, found: 627.38674.

4.5. The synthesis of olefins 13 and 14

A solution of thionyl chloride (35 mg, 294 µmol) in dry pyridine (3 mL) was added to a solution of tertiary alcohol **12** (12 mg, 123 µmol) in pyridine (3 mL) at 0°C. The reaction mixture was stirred for 30 min and then quenched by the addition of water (5 mL), after which the organic solvent was separated, dried ($MgSO_4$) and evaporated. A crude mixture was purified by column chromatography (SiO_2; EtOAc-n-hexane, 1:5) to afford a mixture of olefin regioisomers $\Delta^{25(26)}$ **13** and $\Delta^{24(25)}$ **14** in a yield of 75%.

(22R,23R,24R)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5α-cholestan-25-en-6-one (**13**) and *(22R,23R)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5α-cholestan-24-en-6-one* (15). White oil. R_f = 0.41 (SiO_2; EtOAc/n-hexane, 1:5). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.69 (3H, s, C*H*$_3$-18), 0.86 (3H, s, C*H*$_3$-19), 0.90–2.00 (36H, m), 2.01 + 2.12 (6H, 2 × s, 2 × Ac), 2.32–2.42 (1H, m, C*H*-7β), 2.58–2.63 (1H, m, C*H*-5α), 3.71–3.84 (1H, m, C*H*-23), 3.93–4.06 (1H, m, C*H*-22), 4.93–5.05 (1H, m, C*H*-2), 5.40–5.43 (1H, m, C*H*-3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 11.77, 12.45, 12.68, 13.55, 16.09, 20.28, 21.03, 21.15, 21.24, 23.81, 24.84, 27.16, 27.25, 27.54, 29.68, 37.06, 37.51, 37.64, 39.08, 42.46, 42.88, 44.96, 46.52, 51.80, 53.40, 53.47, 53.55, 53.67, 56.31, 68.10, 69.10, 80.16, 81.16, 108.05, 111.72, 147.39, 170.28, 170.01, 210.72. MS (ESI): 609.6 [M + Na]. HRMS (ESI) for $C_{35}H_{54}O_7Na$ calculated: 609.37610, found: 609.37610.

4.6. The palladium-catalyzed carrier-free tritiation of 13

The synthesis of *(22R,23R,24R)-2α,3α-di-O-acetyl-22,23-(isopropylidenedioxy)-24-methyl-5α-[24,25,26,27-^{3}H]cholestan-6-one* (**15**).

The mixture of *(22R,23R,24R)-2α,3α-Di-O-acetyl-22,23-(isopropylidenedioxy)-24-methyl-5α-cholest-25-en-6-one* (**13**) and *(22R,23R)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5α-cholestan-24-en-6-one* (**14**) (6 mg, 12 µmol) with Pd/C (10%) (7 mg) was placed in a tritiation flask equipped with a Teflon-coated stir bar, and EtOAc (1 mL) was added. The tritiation flask was connected to a tritiation manifold, the reaction mixture was degassed three times in a repeated freeze–thaw cycle (liquid nitrogen), and the pressure of the carrier-free tritium over the reaction mixture was adjusted to 985 mbar. The reaction mixture was vigorously stirred for 2 hours. Afterward, the reaction mixture was frozen with liquid nitrogen and excessive tritium gas was reabsorbed in the uranium bed. The reaction mixture was then brought to room temperature and contaminated ethyl acetate and tritium residue were trapped on charcoal placed in a sealable glass tube. The residue was dissolved in ethyl acetate and the catalyst was filtered off with a PTFE syringe filter (0.45 µm). The tritiation flask and filter were washed with ethyl acetate (5 × 1 mL). To remove labile activity, 3 mL of methanol were added to combined filtrates and the solvents were evaporated. Two other evaporations from methanol (8 mL) were performed. The activity of the crude product (72% R.C.P.) was 480 mCi. One third of the crude mixture was purified by radio-HPLC on a Labio C18 210x10-mm semi-preparative column using gradient elution with A = water, B = methanol (a flow of 4.7 mL/min, a temperature of 25°C, 80% of B isocratic for 5 min, 100% of B in 30 min). This method produced 87 mCi of pure **15**.

4.7. The synthesis of 24-[24,25,26,27-^{3}H]epicastasterone ([3H]-2)

Wet $FeCl_3$ (5 mg) was added to the solution of **15** in DCM (2 mL). The reaction mixture was stirred with a magnetic stir bar and the course of deisopropylidenation was followed by radio-HPLC. After two hours, water (5 mL) was added and the product was extracted by DCM (3 × 5 mL). Combined extracts were evaporated to dryness, the residue was dissolved in methanol (2 mL) and two drops of CH_3ONa (1M) in methanol were added. The course of deacetylation was followed by radio-HPLC. After two hours, the amount of the starting compound detected decreased below 10%. Three hours later, water (5 mL) was added and the reaction mixture was acidified with two drops of 1N HCl (checked by pH reagent paper). The water phase was extracted by four 3 mL portions of DCM. The combined extracts were dried by anhydrous $MgSO_4$, filtered and evaporated. The residue was dissolved in an acetonitrile–water (1:1) mixture (0.6 mL) and the crude product separated on a Labio C18 250x10-mm semi-preparative column. Gradient elution was performed with A = water, B = acetonitrile (a flow of 4.7 mL/min, a temperature of 25°C, 30% B isocratic for 4 min, 40% of B in 5 min, 50% of B in 36 min, 50% of B isocratic for 5 min, 100% of B in 5 min). A methanolic solution (0.2 mL) of antioxidant (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 350 mg/10 mL) was placed in tubes located in a fraction collector to provide a 0.5% concentration of Trolox in each fraction. Glycerol (6 mL) was added to the combined fractions containing the product and the chromatography solvents were evaporated on a CentriVap. The glycerol solution was diluted with 6 mL of water. 35 mCi of **[3H]-2** were obtained with R.C.P. > 97%. The concentration of the solution was adjusted by the glycerol–water (1:1) mixture to 1 mCi/mL and the solution was stored in liquid nitrogen. The ^{3}H NMR spectrum was measured in a glycerol–D_2O mixture. The multiplet at 0.66–0.72 ppm of the methyls 26 and 27 with a variable number of tritium atoms and the multiplet at 1.66 ppm correspond to tritium on C-25. The small signal at 1.33 ppm (2.4% of the total integration) corresponds to tritium on C-24. The SA of **[3H]-2** was determined by MS as 99.4 Ci/mmol.

4.8. The synthesis of 24-[24,25,26,27-^{3}H]epibrassinolide ([3H]-1)

The solution of **15** (25.5 mCi) in chloroform (1 mL) was added dropwise to an intensively stirred freshly prepared chloroform solution of trifluoroperoxyacetic acid [30% H_2O_2 (20 µl), trifluoroacetic acid (100 µl), $CHCl_3$ (1 mL)] cooled to 0°C by an ice bath. The cooling bath was removed after 30 min. and the reaction mixture was stirred at r.t. for 5 hours. Another chloroform solution (4 mL) was added and the reaction mixture was washed by a saturated aqua solution of sodium bicarbonate (4 mL). The organic phase containing crude product **16** was separated and the water phase was extracted by chloroform (4 mL). The combined organic phases were concentrated to circa 2 mL, wet $FeCl_3$ (8 mg) was added and the reaction mixture was intensively stirred. In this case, deisopropylidenation did not proceed as smoothly as for **15**. Water (200 µL), conc. hydrochloric acid (20 µL) and methanol (1.3 mL) were added and the homogenous mixture was stirred overnight. Radio-HPLC revealed that the **16** was not present anymore. The reaction mixture was evaporated (with the magnetic stir bar left in a flask) and the residue was dissolved in methanol (2mL). A methanol solution of CH_3ONa (1M, 0.6 mL) was added and the reaction mixture became turbid afterwards. The completion of deacetylation was confirmed by radio-HPLC after 30 min of the reaction passed. To perform the relactonization of the ring B, the reaction mixture was acidified by 0.7 g of wet Dowex 50x8 in a H^+ cycle. After 3 hours, radio-HPLC showed **[3H]-1** as the major product. Cation exchange resin was filtered off on the short column of another 0.8 g of Dowex 50x8 in H^+ cycle. The column was washed with a methanol–water (3:1) mixture (12 mL). Glycerol (0.3 mL) was added to the elute, the solution was concentrated to circa 2.5 mL and the crude product was purified on a Synergi 4µ Fusion-RP 80 250x10-mm column (Phenomenex). Gradient elution was performed with A = water, B = acetonitrile (a flow of 6.6 mL/min, a temperature of 25°C, 30% B isocratic for 3 min, 40% of B in 33 min, 40% B isocratic for 10 min, 100% of B in 7 min). Each fraction containing the main product was enriched with 0.250 mL of the methanol solution of an antioxidant, (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), in methanol (100 mg/10 ml). Glycerol (3 mL) was added to the combined fractions and the solvents were evaporated on a CentriVap. The glycerol solution was diluted with water (3 mL). This produced 3.5 mCi of pure **[3H]-1** with R.C.P. > 97%. The concentration of the solution was adjusted by a glycerol–water (1:1) mixture to 1 mCi/mL and the solution was stored in liquid nitrogen. The ^{3}H NMR spectrum was measured in a glycerol–D_2O mixture. The multiplet signal of 0.57–0.70 ppm of the methyls 26 and 27 with a variable number of tritium atoms and the multiplet at 1.59 ppm correspond to tritium on C-25. The small signal at 1.23 ppm (2.4% of the total integration) corresponds to tritium on C-24. The SA of **[3H]-1** was determined by MS as 98 Ci/mmol.

Acknowledgments

The authors thank the Academy of Sciences of the Czech Republic for the financial support of this project within the program RVO: 61388963. The project was further supported by grant No. IAA400550801 of the Grant Agency of the Academy of Sciences of the Czech Republic. We kindly thank Dr. L. Kohout for providing us with the 24-epicastasteron and the standard of 24-epibrassinolide.

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