Chapter 2

Recent Developments in a Radio-labeling of Brassinosteroids

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Additional information is available at the end of the chapter

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Abstract

The chapter provides a comprehensive overview on methodologies used for radio-labeling of brassinosteroids as one of the newest class of phytohormones. Discussed labeling strategies are lined up in terms of reached specific activities (SA) of brassinosteroids (BRs) as a key parameter for further utilization of such labeled drugs. The chapter is focused on two key natural radio-isotopes (tritium and carbon-14) used for drug tracing in pharmaceutical research.

Keywords: brassinosteroids, radio-isotope labeling, tritium, carbon-14

1. Introduction

1.1. Radio-labeling

Radioactive labels used for tracing of studied ligands have long been a part of the biological laboratory repertoire. Radioactivity gives a clear, unmistakable signal, and its use is fairly straightforward. Because of smooth traceability, visualization in organ tissues, quantification (liquid scintillation counting [LSC]), and unsurpassed sensitivity of radio-labeled molecules the radio-labeling is a powerful and practical tool to closely follow accurate mass balance and monitor the fate of a molecule on the molecular level and its biochemically transformed derivatives. Pharmacokinetic studies have traditionally used radio-labeled target compounds as a means for evaluating body absorption, distribution, metabolism, and excretion (ADME) [1, 2]. The use of radioligands is essential tool in binding assays aimed at ligand–receptor structure–activity relationship studies, which however requires high-specific activity (SA—a qualitative parameter) of studied ligands (because of, in general, very low concentration of

receptors in tissues). Weak β-emitters such as tritium (^{3}H, T) and carbon-14 (^{14}C) are by far the most versatile and convenient natural labels available [3]. These two isotopes preserve molecular structure (no added tags or pendant groups that alter or change the structure). The advantages of ^{3}H compared to ^{14}C are much higher specific activity, significantly lower cost of starting material, and environment friendly radioactive waste management (shorter half time, **Table 1**). Also, an introduction of radio-isotope in a later stage of synthetic sequence (often in the last step) is a critical benefit in terms of synthetic yield, safety handling, and waste disposal. In general, all above-mentioned issues come out in favor of tritium over carbon-14. On the other hand, a relevant advantage of carbon-14 is lower potential of label loss. Label selection is usually at the discretion of the investigator and studies can be reported using either ^{3}H or ^{14}C label. For instance, tritium label could be applied to earlier stage development studies and then switched to a ^{14}C label for the later stage development studies, for example, an advanced human ADME [4]. Each compound radio-labeled at the non-exchangeable and metabolically stable position need to possess radiochemical purity (RCP) basically over 97%. Instability of all radioligands caused by self-radiolysis requires a need to check a radiochemical purity of studied radio-labeled material before a particular experiment is carried out. Such instability can be significantly suppressed by appropriate storage of labeled material. In general, samples stored at −196°C (Cryoflex-sealed vial immersed in liquid nitrogen) in alcohol-reached medium last over 1–2 years in acceptable quality. Radiochemical purity (RCP) is then usually still over 95%. Samples in such conditions can be often used immediately for biological experiments and no further purification is needed. On the contrary, radio-labeled drugs

	Tritium	**Carbon-14**
Radioactive half-life	12.33 years	5730 years
Specific activity–labeled drugs (1 atom per molecule)	1.066 TBq/mmol	2.309 GBq/mmol
	29.1 Ci/mmol	0.0624 Ci/mmol
Specific activity—element	3.56×10^{14} Bq/g	1.66 × 1011 Bq/g
	2.57 Ci/mL	
Type of radiation (emission probability, %)	β^- (100%)	β^- (100%)
Energy	E_{max} = 18.6 keV E_{avg} = 5.7 keV	E_{max} = 156 keV E_{avg} = 49 keV
Maximum penetration air/ water(tissue)/glass	6 mm/6 μm/2 μm	24 cm/0.250 um/170 μm
Decay product	$^{3}He^{+}$ (stable)	$^{14}N^{+}$ (stable)
Detection and measurement	LSC (undetectable by portable survey meters)	LSC Geiger-Mueller [10% efficiency]
Shielding	None required—not an external radiation hazard	None required—mCi quantities not an external radiation hazard

Table 1. Nuclear characteristics of discussed radio-isotopes.

stored in a refrigerator (+4°C) over 1 year rarely show better than 90% RCP. Foremost advantage of using radio-labeled drugs is that the radioactivity is easily detected and quantified using liquid scintillation techniques in a very low limit of detection (technically <1 Bq/L) [5, 6].

1.2. Brassinosteroids: a newest class of phytohormones

The entire evolutionary process in plants is regulated by the changes of hormonal concentration, tissue sensitivity, and their interaction during the entire life cycle of plants. One of the most recent groups of phytohormones represents brassinosteroids. They occur at low levels distributed throughout the plant kingdom [7]. The ability of plants to biosynthesize a large variety of such steroids were discovered in 1970 by Mitchell et al. who first from 40 kg of bee-collected rape pollen of *Brassica napus L.* isolated and characterized a steroidal lactone named brassinolide [8]. Few years later, castasterone was found in insect galls of *Castanea crenata spp*. Up to date, more than 70 structurally and functionally related polyhydroxylated sterol derivatives are known and this group of compounds has been identified as the natural brassinosteroids (BRs) [9]. All BRs have a 5α-cholestane skeleton, with functional variations due to differences in orientations of oxygenated functions on the skeleton [10, 11].

The highest concentration of BRs in plants is detected in the reproductive organs (pollen and seeds; 1–100 ng/g). They were also detected in other plant organs from roots to leaves. They are involved in various kinds of regulatory actions on growth and development, that is, stimulation of cell expansion, cell division, stress tolerance, accretion of biomass, yield and quality of seeds, and plant adaptability [9]. The metabolism of nucleic acids and proteins and the gene expression is changed by BRs at the molecular level.

The extremely high activity of brassinosteroids has attracted the attention of many specialists in the field of analytical and synthetic chemistry, biochemistry, plant physiology, and agriculture. Recently, it has been reported that some natural BRs have (besides early reported, e.g., antibacterial, antiviral, antifungal, and neuroprotective) potent cell growth inhibitory activities in animal and human cancer cell lines without affecting the normal cell growth (BJ fibroblasts) [12, 13]. The presence of a lactone or ketone moiety in ring B and diol functions (2α, 3α- and 22*R*, 23*R*-) turned out to be an essential factor for high biological activity of such BRs (**Figure 1**).

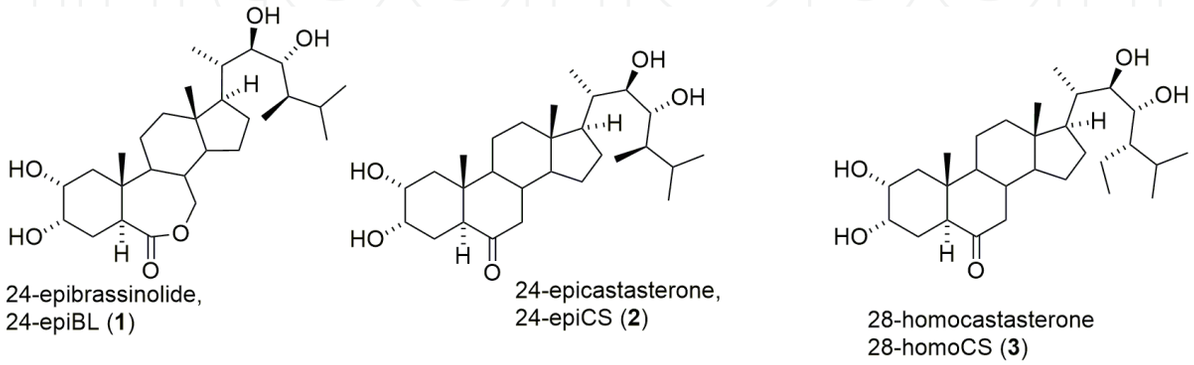

Figure 1. 24-epiBL (**1**) is the 24-(*R*)-epimer of the first isolated brassinosteroid, 24-epiCS (**2**), 28-homoCS (**3**).

This chapter is engaged in the recent developments in the synthesis of ^{3}H- and ^{14}C-radio-labeled analogs of the brassinolide.

2. Synthesis of tritium-labeled BRs

2.1. BRs with very high SA of tritium (~99 Ci/mmol)

For binding assays, study aimed at ligand-receptor-activity relationship is a high specific activity (SA), a bottom line requirement. The SA of such radio-labeled drugs need to be in scale of tenths of Ci/mmol. This critical precondition used to be an obstacle in the way of BR's studies for decades. The state-of-art strategy for such a labeling was reported by Marek et al. [14]. The methodology yields tritium-labeled BRs bearing a very high SA of 99.4 and 98 Ci/mmol (approx. 3.4 tritium enrichment per molecule), respectively. Convenient, a six-step synthetic sequence starting with the brassinosteroid *to be labeled* provides the desired tritium-multi-labeled product in sufficient yield (up to 40 mCi) with satisfactory radiochemical purity (>97%). The work is focused on the 24-[^{3}H]epibrassinolide [^{3}H]-**1** and 24-[^{3}H]epicastasterone [^{3}H]-**2**, both labeled in the side chain of BR skeleton on positions of carbon C-24, -25, -26, and -27 (**Figure 2**). The labeling strategy is designed to employ a radio-labeling step at the later stage of the synthetic sequence.

In 1998, Seto et al. described a fairly elegant strategy for the deuterium-multi-labeling of brassinolide in its side-chain [15]. The five-step reaction sequence was started by full protection of hydroxyl groups on the BR. The C-25 carbon was oxidized by freshly generated trifluoromethyldioxirane (TFD) that yielded appropriate hydroxy derivative. Its consecutive dehydration led to 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, 1 h) yielded [24, 25, 26, 27-^{2}H]brassinolide with 60% deuterium enrichment calculated from MS data. The ratio of the individual multi-deuterated species in the cluster was $^{2}H_2$:$^{2}H_3$:$^{2}H_4$:$^{2}H_6$:$^{2}H_7$ = 3:8:14:15:60. The basic idea of this methodology for usage at labeling with radioactive isotope tritium was waiting almost for two decades—then 24-[^{3}H] epiBL (**1**) and 24-[^{3}H]epiCS (**2**) was synthesized [14].

The protocol of Seto et al. paved the way for the synthesis of an unsaturated precursor for the intended synthesis of ^{3}H-labeled 24-epiCS [15, 16]. First, the 2,3-22,23-bisisopropylidene derivative **4** was prepared in a 96% yield by a reaction of 24-epiCS (**2**) with 2,2-dimethoxypropane catalyzed by *p*-toluenesulfonic acid [14]. Having 2,3,22,23-diisopropylidene-24-epicastasterone **4** available, the TFD hydroxylation of C-25 carbon with this particular derivative was studied. Unfortunately, the desired hydroxylation was accompanied by oxidation of alcohol C-3 affording appropriate hydroxyketone. Hence, more resistant 2,3,22,23-tetra-O-acetyl-24-epiCS was prepared. However, its C-25 hydroxylation did not proceed at all under the conditions used for the hydroxylation of **4**. Finally, 2,3-di-O-acetyl-22,23-isopropylidene-24-epicastasterone (**6**) was prepared which upon TFD hydroxylation at low temperature gave 2,3-di-O-acetyl-22,23-isopropylidene-25-hydroxy-24-epiCS (**7**) in 58% yield (**Figure 2**). The dehydration of **7** using thionyl chloride in pyridine at 0°C for 30 min afforded a mixture of prevailing

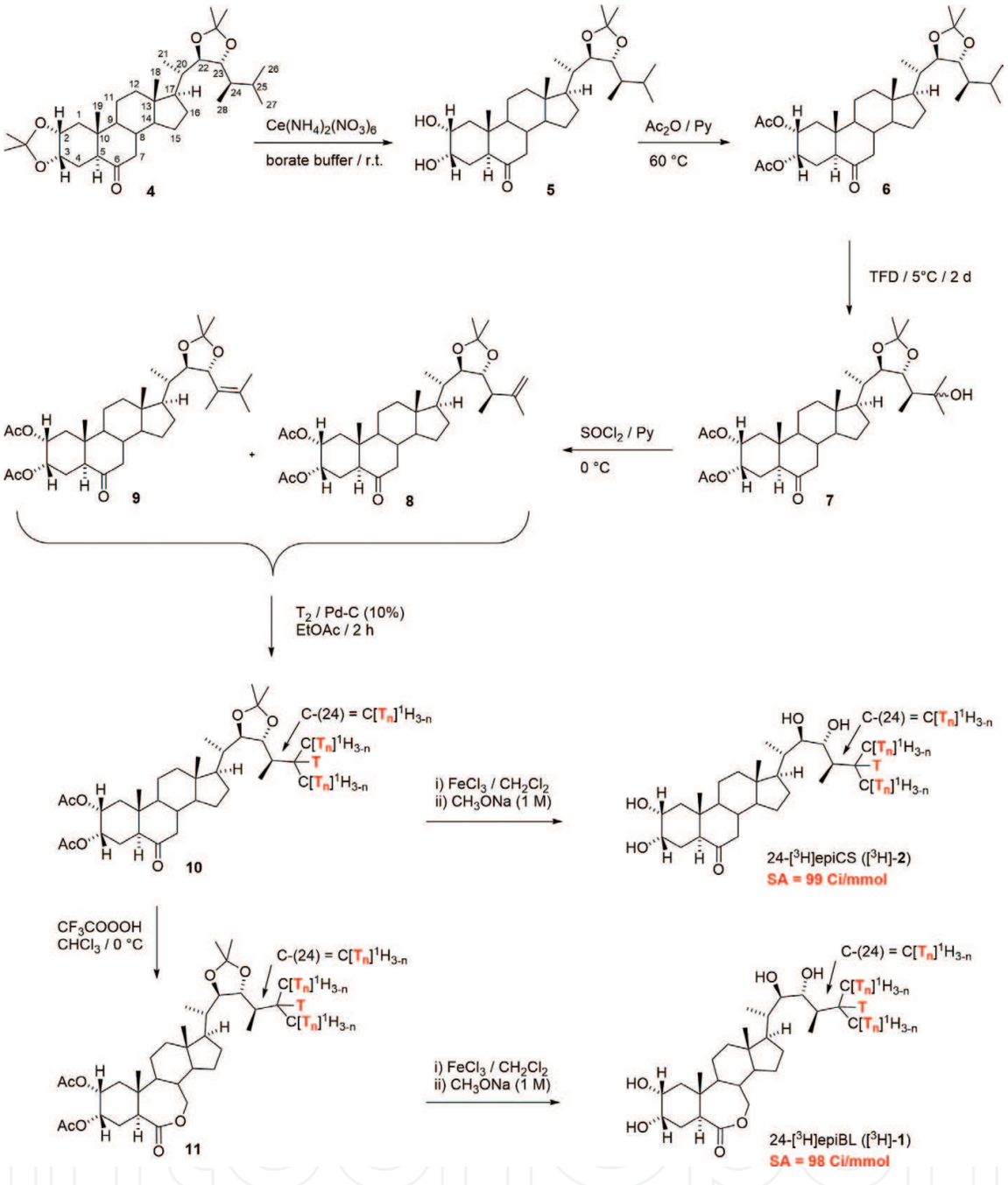

Figure 2. The successful approach for the synthesis of 24-[^{3}H]epiCS and 24-[^{3}H]epiBL with a high SA.

(22*R*,23*R*,24*R*)-2*α*,3*α*-diacetoxy-22,23-isopropylidenedioxy-24-methyl-5*α*-cholestan-25-ene-6-one (**8**) accompanied by its 24-ene regioisomer **9**. The separation of unsaturated regioisomers **8** and **9** from each other turned out to be infeasible using various conditions on high-performance liquid chromatography (HPLC). Importantly, it was possible to separate unsaturated derivatives **8** and **9** from 24-epicastasterone derivative **6** by HPLC. This fact eventually enabled the isolation of 261 mCi of (22*R*,23*R*,24*R*)-2*α*,3*α*-diacetoxy-22,23-isopropylidenedioxy-24-[24, 25, 26, 27-^{3}H]epicastasterone (**10**) after the catalytic tritiation of the mixture of the unsaturated derivatives **8** and **9** over Pd/C (10%) in ethyl acetate under carrier-free tritium gas (998 mbar)

for 2 h. In one-pot synthesis, derivative **10** was deisopropylinated and deacetylated provided by HPLC purification, 40 mCi of 24-[24, 25, 26, 27-^{3}H]epicastasterone ([^{3}H]-**2**) with RCP >97% and SA_{MS} = 99.4 Ci/mmol [14].

To get [24, 25, 26, 27-^{3}H]epibrassinolide ([^{3}H]-**1**), the Baeyer-Villiger oxidation on fully protected 24-[24, 25, 26, 27-^{3}H]epicastasterone **11** was carried out by freshly prepared chloroform solution of trifluoroperoxyacetic acid [30% H_2O_2 (20 ml), trifluoroacetic acid (100 ml), $CHCl_3$ (1 mL)] cooled to 0°C by an ice bath [14] . The de-isopropylidation of the (22*R*,23*R*,24*R*)-2α,3α-diacetoxy-22,23-isopropylidenedioxy-24-[24, 25, 26, 27-^{3}H]epiBL (**11**) was carried out by wet $FeCl_3$, afterwards full deacetylation was accomplished by methanol solution of CH_3ONa. This conditions was made possible to obtain 3.5 mCi of pure 24-[24, 25, 26, 27-^{3}H]-epiBL ([^{3}H]-**1**) with RCP > 97% and SA_{MS} = 98 Ci/mmol. The ^{3}H NMR spectra of both [^{3}H]-**1** and [^{3}H]-**2** show tritium signals in C-25, C-26, and -27, and is in accordance with the determined SA, indicating 3.4 tritium atoms per molecule.

2.1.1. Stability of BRs possessing very high SA

The free BRs with a high SA are extremely sensitive to radiolysis if stored improperly. Authors reported one representative example—when a sample was evaporated to dryness and used for the NMR analysis (DMSO-d_6), the signals in ^{3}H NMR spectrum were difficult to assign [14]. Hence, the particular NMR sample was re-checked for purity by radio-HPLC, and indeed, only 12% of activity of desired 24-[24, 25, 26, 27-^{3}H]epiCS ([^{3}H]-**2**) was left whereas 88% of radioactivity was found in a broaden peak with higher retention on the column (**Figures 3** and **4**). Nevertheless, authors reported secure procedure to remove chromatographic solvents and formulate the high-SA BRs for application in biochemical experiments. Combined HPLC fractions (each about 2 mL) were first enriched with glycerol (300 mL) (acts as an antioxidant and also prevents risk of getting dryness of labeled samples) and 10 mg of (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (acts as additional antioxidant with no harm in eventual biological experiments) [14]. When methanol and water evaporated on

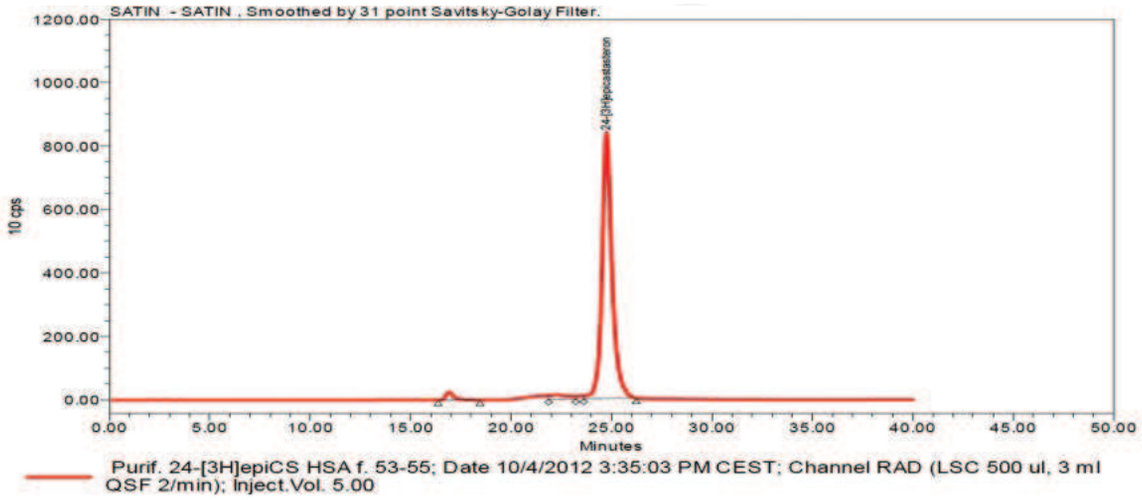

Figure 3. HPLC radiodetector chromatogram of [^{3}H]-**2** after purification.

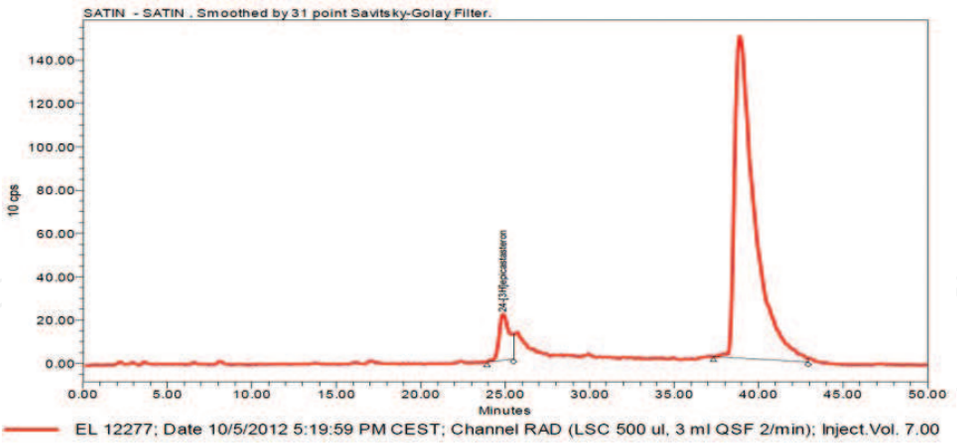

Figure 4. HPLC radiodetector chromatogram; fast decomposition of [3H]-2 when handled improperly - after its simple evaporation to dryness and storage in DMSO-d_6 overnight.

CentriVap, the residual glycerol solution was further diluted with water (2 mL), the radioactivity was determined and the concentration of 24-[24, 25, 26, 27-^{3}H]epiCS ([^{3}H]-**2**) was afterwards adjusted to 1 mCi/mL with a glycerol/water (1:1) mixture. The concentration of Trolox in the final formulation needed to be adjusted to 0.5%. For maximal stability of prepared samples, storage of 1 mCi aliquots in liquid nitrogen is recommended.

2.2. BRs with reasonable high SA of tritium

2.2.1. Reductive tritium-dehalogenation of generated chlorocarbonates (6 Ci/mmol)

To get polyhydroxylated steroid regio- and enantio-specifically labeled on the un-exchangeable position of C-3, a general procedure can be effectively used (**Figure 5**) [17]. A suitable precursor for the introduction of tritium, 3β-chloro-2,3-carbonate derivative, is synthetically affordable by a short-reaction sequence from a 2α,3α-dihydroxy steroid *to be labeled* (**Figure 6**) [18]. Chlorocarbonate undergoes reductive tritium dechlorination catalyzed by the [Pd0]/Et_3N system, providing 28-[3β-^{3}H]homoCS, 24-[3β-^{3}H]epiCS, and 24-[3β-^{3}H]epiBL, respectively, in good yield and with high SA (5.8 Ci/mmol; 0.2 tritium per molecule) (**Figure 5**) [19]. A crucial aspect in the reductive dehalogenation of the chloro derivative is the choice of a solvent that would provide a reasonable yield [17]. The optimized reaction conditions turned out to be PdO/$CaCO_3$ (5%)/Et_3N/chlorocarbonate (2:6:1) dissolved in dry EtOAc for all investigated steroids. The successful ^{3}H-labeling experiments have proven the stereo selectivity of the reductive dehalogenation and afforded a product with high specific activity (5.8 Ci/mmol).

The synthetic procedure starts with transformation of the vicinal 2α,3α-diols of appropriate BR to α-hydroxy ketone by oxidation with a freshly generated dimethyldioxirane (DMD). Such α-hydroxy ketone moiety proved to be an excellent substrate for high-yield enantiospecific formation of 3β-chloro-2,3-carbonate by a reaction with easy-to-handle triphosgene. The key substrate for reductive dechlorination—3β-chloro-2,3-carbonate—was synthesized by a three-step reaction sequence in an overall yield of 46–55% (**Figure 6**; representative synthesis of 24-[^{3}H]epiCS). To improve the solubility of the starting steroid *(to be labeled)* in a

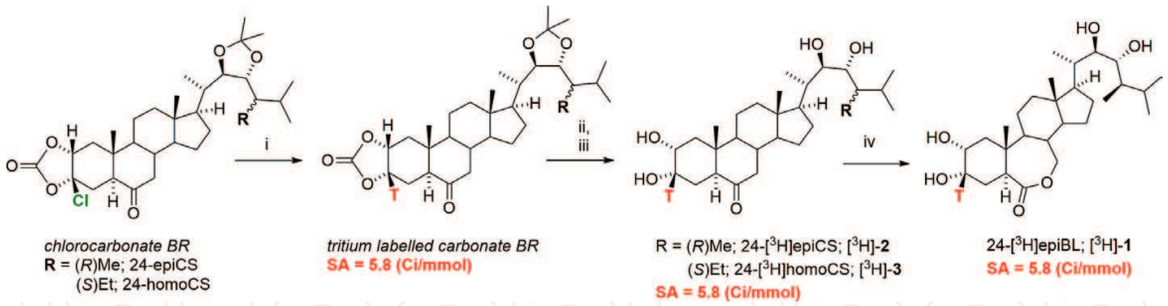

Figure 5. Tritium Pd-catalyzed reductive dehalogenation; (i) T_2/PdO/$CaCO_3$/Et_3N; (ii) Fe(III), CH_2Cl_2; (iii) NaOH, 1,4-dioxane; (iv) H_2O_2/TFA, 0°C, 30 min, r.t., 4 h, $CHCl_3$.

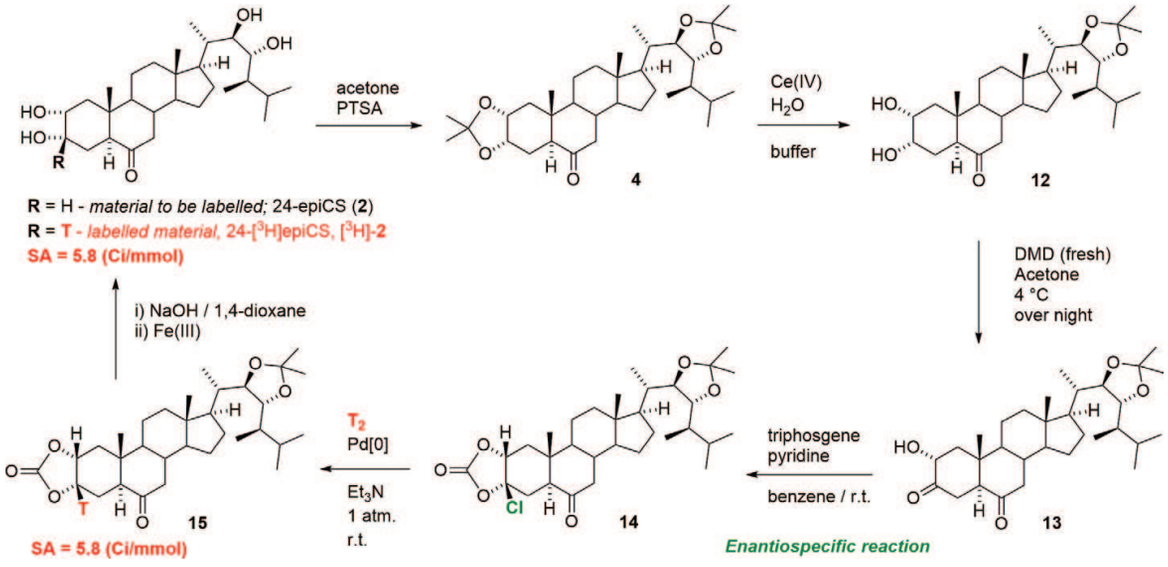

Figure 6. Reaction sequence of the synthesis of 24-[^{3}H]epiCS; [^{3}H]-**2**.

non-polar solvent, 2,3- and 22,23-vicinal diols were protected. The isopropylidation of both vicinal diol groups by 2,2-dimethoxypropane (10 eqv.) catalyzed by *p*-toluenesulphonic acid in dry CH_2Cl_2 turned out to be an elegant protecting strategy. Full conversion was reached in 30 h and the pure product **4** was isolated. Acetonide **4** was further oxidized at the position of C(3)-OH by freshly synthesized dimethyldioxirane (DMD) [20]. The reaction carried out in CH_2Cl_2 overnight at 4°C in dark afforded the desired α-hydroxy ketone **13** accompanied by its regioisomer 3-hydroxy ketone, in a ratio of 10:1 in favor of isomer **13**. In general, α-hydroxy ketone **13** is supposed to be isolated in a higher yield when the oxidation of 2,3-unprotected diols takes place; a selective deprotection of acetonide by $Ce(NH_4)_2(NO_3)_6$ in borate buffer followed by the DMD oxidation of the vicinal 2,3-diol group used to provide a higher overall yield of about 5% [21]. However, in the case of the 28-HCS derivative, a partial deprotection-oxidation reaction sequence afforded a drop of an isolated yield of about 15% compared to direct oxidation of the protected derivative. The 3β-chloro-2,3-carbonate **14** was synthesized by a stereospecific reaction of **13** with triphosgene in dry benzene providing a quantitative yield in 3 h (**Figure 7**).

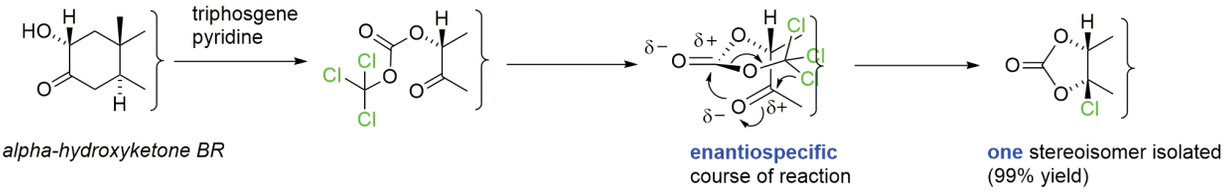

Figure 7. Mechanism of enantiospecific formation of 3β-chloro-2,3-carbonate derivatives of BR.

The catalytic reductive dehalogenation of BR–chlorocarbonates was studied with deuterium in the system of $^{2}H_2$/Pd[0]/Et_3N providing appropriate 3β-deutero-2,3-carbonates with 70–80% deuterium enrichment (based on ^{1}H NMR) at the C-3 position and with an isolated yield of up to 65% (initially on a cheap pregnane analog available in multigame scale). The best results for deuterium dehalogenation were achieved with the molar ratio of PdO/$CaCO_3$(5%)/Et_3N/chlorocarbonate being 2:6:1 for every BR derivative at a short period of time (6 h). When the amount of the base was too high, it diminished the yield of [^{2}H]-labeled ethylene carbonate. Various catalysts such as Pd/C (either 5 or 30%), PdO/$BaSO_4$ (10%) used for reduction yielded lower yield of desired carbonate (15–19%). Authors disclosed very significant solvent effect with an impact on the isolated yield as well as the by-products formation. Briefly, the best results provided EtOAc (dry), giving up to a 65% yield of labeled carbonate with 80% ^{2}H-enrichment. Other solvents used for reduction provided both low conversion and isolated yield of carbonate (0–19%). Androstane chlorocarbonate employed for reductive dehalogenation under similar condition as for pregnane analog provided analogous results (58% yield, 75% ^{2}H-enrichment at C-3). Surprisingly, in addition to desired labeled carbonate two by-products were detected, isolated, and afterwards characterized in that experiment—the multi-labeled ketone and the multi-labeled alcohol, both in the yield of 15%. The reaction course toward formation of both by-products was further accelerated while protic solvent (MeOH) was used in the reaction [43% (ketone) and 40% (alcohol)]. The reaction conditions used for the labeling of 24-epiCS were the same as described above for the other two steroids. A full conversion of appropriate chlorocarbonate was obtained after a 6-h reaction [PdO/$CaCO_3$ (5%)/Et_3N/substrate 2:6:1] in dry EtOAc. The isolated yield of labeled carbonate was determined to be 31% [D/H at C-3 = 70:30]. Both the by-products, ketone (20%) and alcohol (13%), were isolated too. The use of DMF as solvent reduced the conversion of chlorocarbonate to 45%, the yield of labeled carbonate down to 19% and the formation of by-products to 11 and 10%, respectively.

As was already mentioned, traces of water (partly synthesized by the reduction of PdO with D_2 gas) play a crucial role in the suggested mechanism of by-product formation (**Figure 8**) [17]. The initial oxidative addition of *in situ* generated Pd[0] into the chlorocarbonate C(3)-Cl bond forms an organopalladium compound **16**. Such palladium could be partly substituted by traces of water to form appropriate hydroxyl carbonate **17**, which afterwards undergoes ring opening, leading to ketone **18**. Unsaturated ketone **19** is formed by an elimination of carbonic acid from ketone **18** (Pathway A), which could be *in situ* reduced by a system of D_2/Pd[0] providing 1,2-deuterium-labeled ketone **20** and **21**, respectively. The other discussed pathway (B) involves an elimination of carbon dioxide, affording α-hydroxyketone, and the subsequent

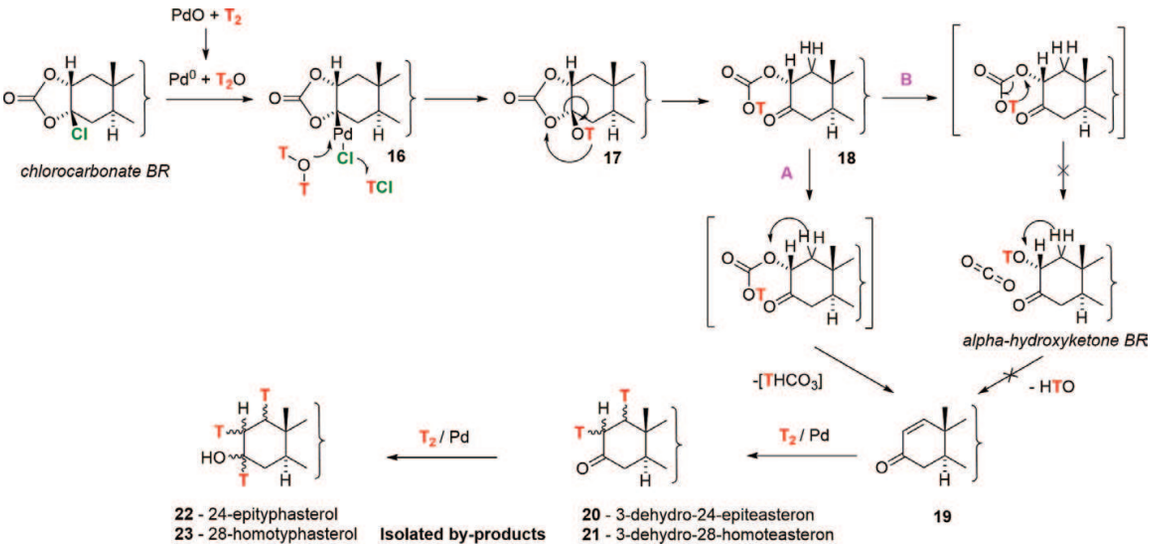

Figure 8. Mechanism of multi-labeled by-products formation.

elimination of H_2O leading to **19**. Authors declare a statement that theoretical pathway B was not supported by any analytical findings. First, the α-hydroxyketone was not detected in the reaction mixture. Moreover, the single experiment using synthesized α-hydroxyketone and reaction conditions simulating conditions used for reductive deuterium dehalogenation, yielded ultimately 2α,3β-dihydroxy-3α[^{2}H] derivative in an isolated 85% yield and no other product was identified in the reaction mixture. In advance, the absolute configuration at C-2 and C-3 of 2α,3β-dihydroxy derivative was confirmed by was confirmed by X-ray analysis affording ORTEP diagram. A regular ESI-MS analysis of **20** and **21** measured in CH_3OH has confirmed the structure of multi-labeled ketones with the distribution of deuterium 1D/2D being 70:30. The second by-product, multi-labeled alcohols **22** and **23**, are most likely formed by a Pd-catalyzed reduction of the ketones **20** and **21** by D_2. The structure of **22** and **23** was confirmed by ^{1}H NMR as well as ESI-MS with a deuterium distribution 1D/2D/3D being 10:50:40.

In view of the favorable results of deuterium experiments, this protocol was followed using tritium gas. Tritium dehalogenation experiment was designed following the optimized reaction conditions [PdO/$CaCO_3$ (5%)/Et_3N/substrate 2:6:1, dry EtOAc]. ^{3}H-labeled 24-epiCS and 24-epiBL, was synthesized when the appropriate 3β-chloro-2,3-carbonate **14** was used for a reaction with carrier-free tritium gas (600 mbar), PdO/$CaCO_3$ (5%), and in presence of base Et_3N. The reaction proceeded at 25°C for 17 h (**Figure 6**). The reduction yielded 5.9 mCi of the desired ^{3}H-labeled carbonate **15**, and two further unidentified by-products (21.3 and 12.4 mCi) were detected. By ^{1}H NMR was determined a specific activity of **15** at 5.8 Ci/mmol (which accounts for 0.2 tritium atom per molecule). Compared to previous deuterium comprehensive study, the obtained tritium enrichment was lower than expected. Authors speculate that the significant drop in SA was caused by the reduced pressure of tritium gas (600 mbar $^3H_2$) compared to deuterium gas (950 mbar of $^2H_2$) used for the reduction. The only signal in the ^{3}H NMR spectrum (the singlet at δ 4.8 ppm) explicitly determined the regio- and stereo-specificity of the reduction. The deprotection of the isopropylidene group in the side chain was

carried out by wet $FeCl_3$ in CH_2Cl_2. The last step—hydrolysis of ^3H-labeled carbonate **15**—was accomplished by NaOH (0.5 M) in 1,4-dioxane (1:1). Desired [^3H]-**2** (3.8 mCi, 5.8 Ci/mmol) was purified by preparative radio-HPLC. Aliquot of [^3H]-**2** was further employed for Baeyer-Villiger oxidation leading to [^3H]-**1** (0.3 mCi, SA 5.8 Ci/mmol).

The SA of isolated [^3H]-**2** was about four times lower than was predicted based on the previous comprehensive deuterium-using study. To further investigate the influence of a metal catalyst on the SA and the yield of the labeled product, it was considered the use of the Pd[0] catalyst instead of the Pd[II]O catalyst, where the generation of Pd[0] is accompanied by the formation of tritiated water. The suggested mechanism of the by-products formation explained how the traces of water significantly reduce the yield of the labeled product desired (**Figure 8**). Hence, to suppress synthesis of multi-labeled by-products Pd on charcoal was employed at synthesis of 28-[3β-^3H]homocastasterone [19]. The catalyst/base/substrate ratio for the tritium experiment was kept identical to the previous experiment of the tritium labeling of 24-epiCS—2:6:1. Carrier-free tritium released over the reaction mixture (738 mbar, 11.5 Ci, 180 μmol) was left to react for 24 h at room temperature. Then, the analytical radio-HPLC proved the formation of the desired ^3H-labeled carbonate (13.3 mCi) and two other by-products (42.8 and 24.7 mCi) were detected. The SA of ^3H-carbonate was determined by ^1H NMR as 5.8 Ci/mmol (based on the decrease of the corresponding ^1H signal intensity in the labeled position). The deprotection of the 22,23-isopropylidene group by treatment with wet $FeCl_3$ in CH_2Cl_2 was completed within 10 min. The crude 3β-tritio-2,3-carbonate was directly hydrolyzed by 0.5 M aqueous NaOH in 1,4-dioxane. The crude product was purified on the semi-prep radio-HPLC, affording 5.3 mCi of [^3H]-(**3**) of SA 5.8 Ci/mmol with radiochemical purity (RCP) >97%. Both by-products were isolated, and ^3H and ^1H NMR measured. The structure of multi-labeled by-products is believed to be similar to those that were recently described (**Figure 9**) for androstane and 24-epiCS (i.e., labeled 24-epityphasterol and 3-dehydro-24-epiteasteron, respectively) derivatives. In this case, these by-products are supposed to be 22,23-isopropylidene-protected multi-labeled [1, 2, 3-^3H]-28-homotyphasterol and [1, 2-^3H]-3-dehydro-28-homoteasterone derivatives (**Figure 9**).

2.2.2. Catalytic reduction of 24-methylene BRs (SA = 2 Ci/mmol)

An elegant and fast strategy to get BRs labeled by tritium was briefly communicated by Yokota et al. [22]. [^3H]-BRs synthesized on demand at Amersham International (Amersham, UK) were used for comparative analysis in stems and seeds by radioimmunoassay. The labeling strategy was based on platinum-catalyzed reduction of 24-methylene position of available BR (dolichosterone and dolicholide) in carrier-free tritium atmosphere (**Figure 10**). Reduction of dolichosterone afforded two epimers 24-[24, 28-^3H]castasterone (8.1 mCi, SA = 2.2 Ci/mmol) and 24-[24, 28-^3H]epicastasterone [^3H]-(**2**) (6.5 mCi, SA not determined), respectively, which were then separated on HPLC. Analogically, reduction of dolicholide provided 24-[24, 28-^3H] brassinolide (10.6 mCi, SA = 2.3 Ci/mmol) and 24-[24, 28-^3H]epibrassinolide [^3H]-(**1**) (4.0 mCi, SA not determined). Authors stated unexpectedly low SA of gained products (theoretical SA was supposed to be over 40 Ci/mmol). Detailed synthetic procedure and analysis were not reported.

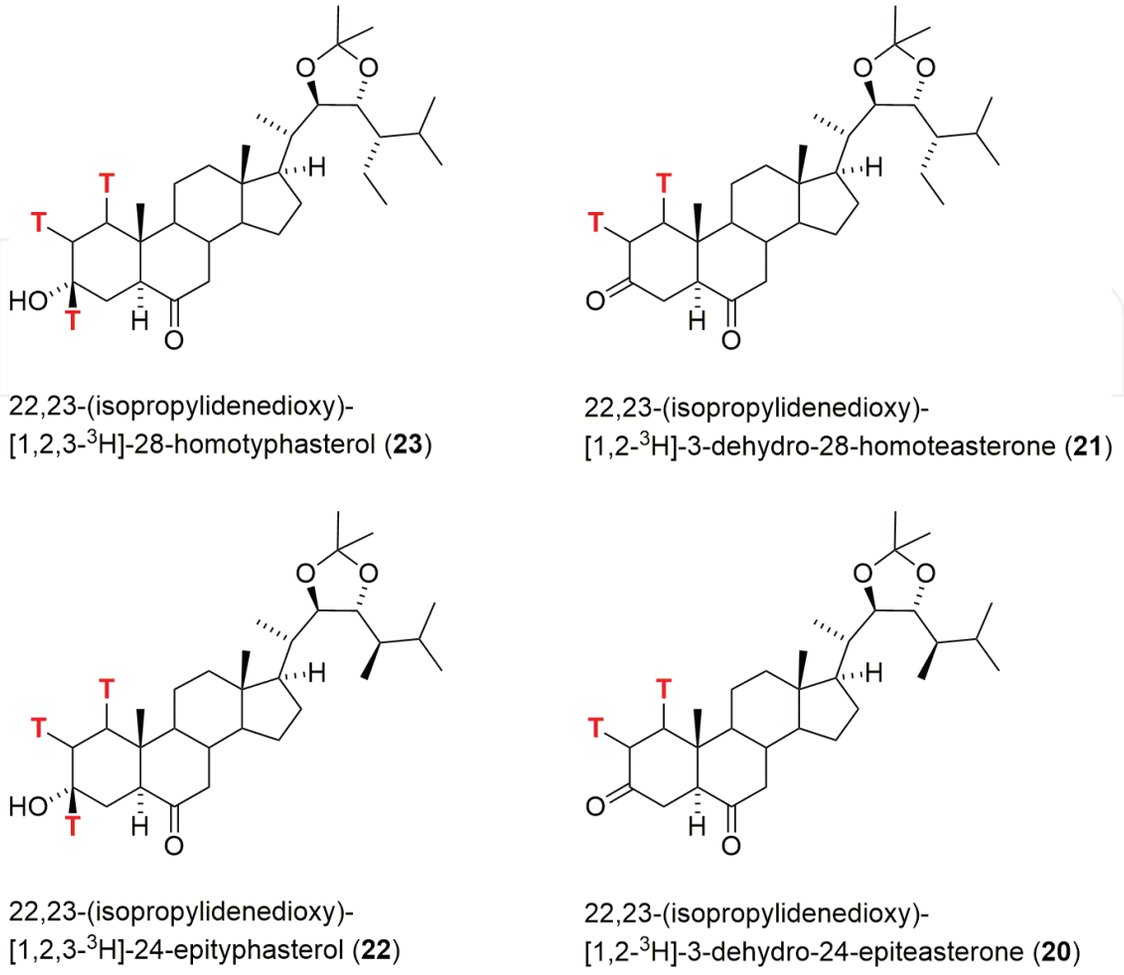

Figure 9. Multi-labeled by-products.

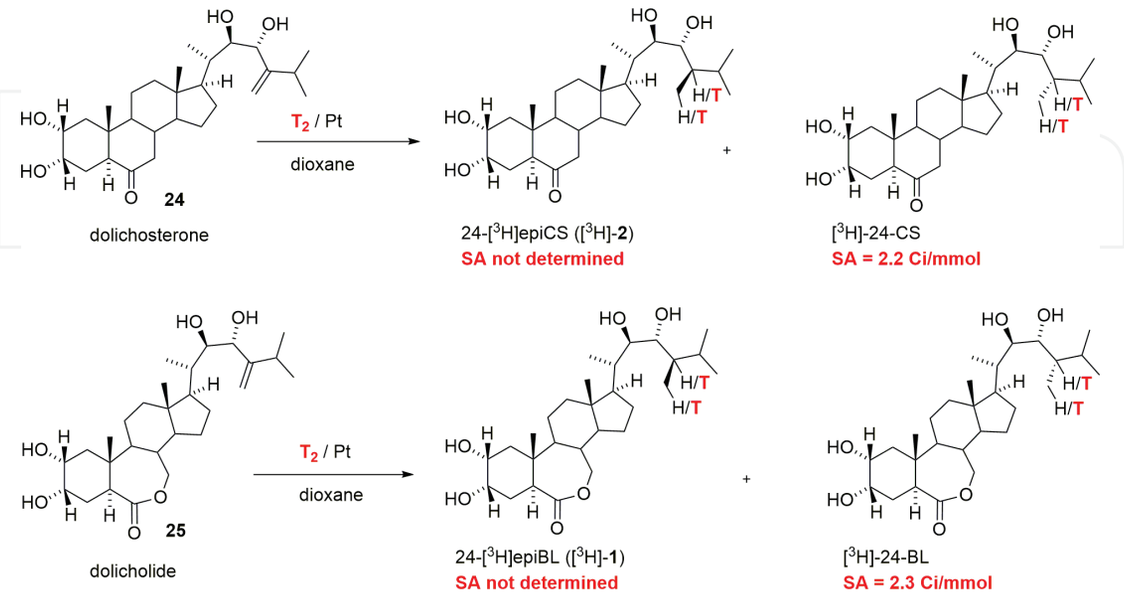

Figure 10. Metal-catalyzed 24-methylene BR tritiation.

2.3. BRs with very low SA of tritium (10^{-3} Ci/mmol)

Tritium-labeled protected 24-epiCS and 24-epiBL [^{3}H]-**1** were for the first time prepared by Kolbe et al. [23]. By very simple procedure of base-catalyzed exchange reaction with tritiated water (SA = 20.10^{-3} Ci/mmol—accounts for 0.7 × 10^{-3} tritium atom per molecule HTO) to the enolizable alpha positions of C-6 ketone of tetracetate 24-epiCS **27** (**Figure 11**). By this method, it is theoretically possible to exchange hydrogen on three distinctive positions (C-5α, C-7α, and C-7β). However, the obtained SA of BRs was very low (6.10^{-3} Ci/mmol), about 3-4 orders lower than that provided by methods described above. Because of low SA of such prepared material cannot be used in receptor studies at all. The other drawback of this procedure is chemical exchangeability of labels, which inevitably leads to loss of label in protic solvents (water) during biological experiments and disqualifies this approach from use in ADME studies. Three-step reaction sequence provided 24-[5, 7, 7-^{3}H]epiBL with SA 6.10^{-3} Ci/mmol; labeling step leading to protected 24-[^{3}H]epiCS **27**, followed by a Baeyer-Villiger oxidation with CF_3COOOH and sequence was accomplished by hydroxy group deprotection [20] . The same sequence was also performed with 2,3,22,23-bis-isopropylidenedioxy-24-epiCS **29** as starting compound. The advantage of the use of isopropylidene protecting groups over acetate group is the deprotection step. The lactone ring is stable under acidic hydrolysis conditions used for isopropylidene deprotection, which occurs simultaneously with Baeyer-Villiger oxidation. The basic hydrolysis needed for the acetate group cleavage leads to lactone hydrolysis and acid catalyzed re-lactonization is needed (**Figure 11**).

A base-catalyzed exchange was used for labeling of biogenetic brassinosteroids precursors [24]. 24-[5, 7, 7-^{3}H]epiteasterone (SA = 1.5 10^{-3} Ci/mmol), 6-oxo-24β-methyl-22-dehydro[5, 7, 7-^{3}H]cholestenol (SA = not indicated), and 6-oxo-24-[5, 7, 7-^{3}H]epicampestanol (SA = 3.5 10^{-3} Ci/mmol), respectively, were partly labeled on positions of C-5 and C-7 by reaction in sealed

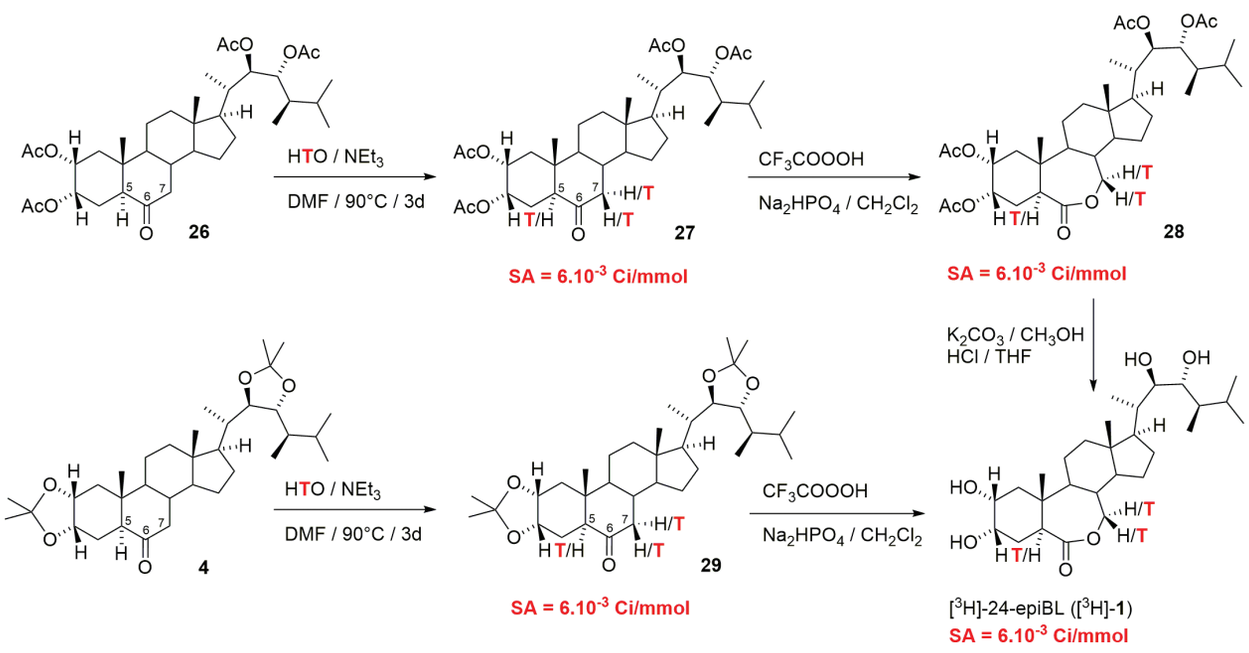

Figure 11. Low SA possessing 24-[^{3}H]-epiBL.

ampoule with tritiated water (SA = 14.10^{-3} Ci/mmol—accounts for 0.5×10^{-3} tritium atom per molecule HTO) in presence of base Et_3N (**Figure 12**). As mentioned above this simple methodology affords poor SA (in order of 10^{-3} Ci/mmol) of BRs labeled on an exchangeable positions thus prone to loss of label later on. On the other hand, this approach provides high yield (>45%) of desired material.

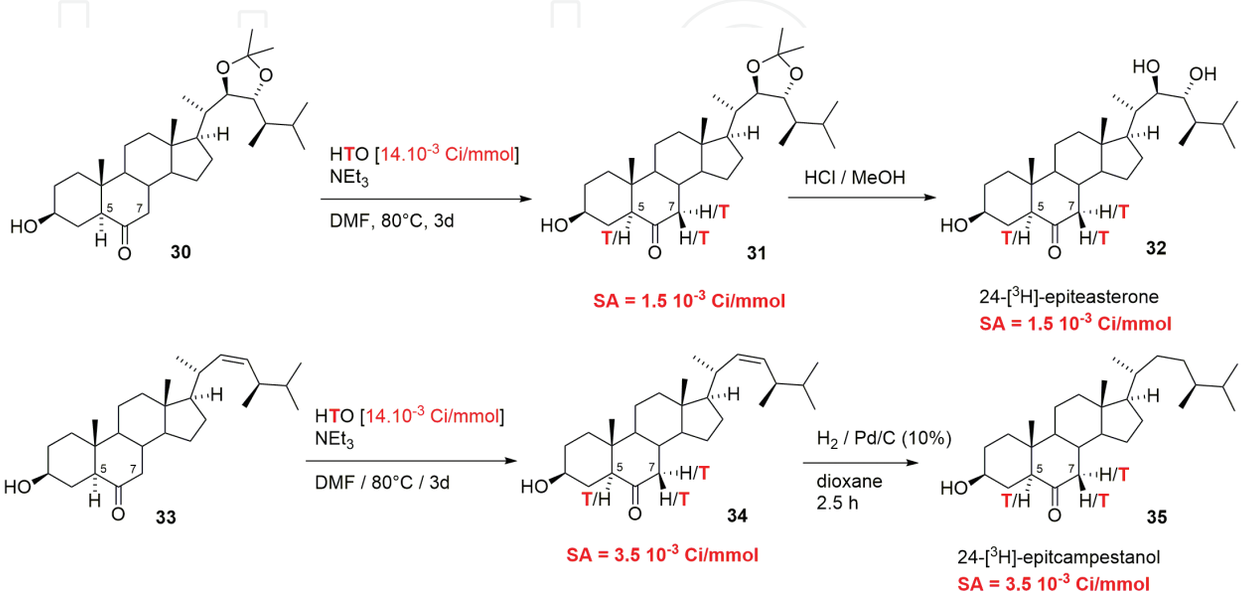

Figure 12. 24-[^{3}H]epiteasterone and 6-oxo-24-[^{3}H]epicampestanol.

2.4. BRs labeled by carbon-14 (SA = 56.8×10^{-3} Ci/mmol)

Till now, only one report on ^{14}C-labeled brassinosteroids is available in the literature. In 1989, Seo et al. described the synthesis of [^{14}C]-labeled (22*R*,23*R*)- and (22*S*,23*S*)-epiCS **50** and **51**, and epiBL **52** and **53**, respectively [25]. The obtained BRs possessed top SA (56.8×10^{-3} Ci/mmol) possible to reach while doing labeling by carbon-14. Because of multi-step synthetic sequence (>10 steps) needed, the reported overall yield of labeled products is 3.2 and 4.5%. The C-4 position in 24-epiBL **52** was selected for [^{14}C]-labeling because of its stability to metabolic loss and easy way to do the preparation. According to the well-established method for ^{14}C incorporation into the C-4 position of steroids, the enol lactone **41** was synthesized from the starting material brassicasterol **36** in five steps (**Figure 13**). Bridged ketone **42** was prepared from lactone **41** after its treatment with [^{14}C]methyl iodide. Alkaline treatment of **42** provided [4-^{14}C] enone **43**. Gentle acid catalyzed acetylation of enone **43** with isopropenyl acetate led to the enol acetate **44** that was afterwards reduced by $NaBH_4$ to give [4-^{14}C]brassicasterol **45**. Simple mesylation of **45** afforded **46** which was treated with sodium carbonate providing the major product 3,5-cyclo-6-ol **47** isolated in 92% yield (**Figure 14**). Jones oxidation of **47** then gave 3,5-cyclo-6-one **48**. Rearrangement of **48** moderated by lithium bromide and camphor sulfonic acid in dimethyl acetamide afforded 2,22-diene-6-one **49** in quantitative yield. Oxidation of

49 with osmium tetroxide led to a stereo isomeric mixture of 2,3,22,23-tetraols–24-(22*R*,23*R*)-[^{14}C]epiCS **50** and 24-(22*S*,23*S*)-[^{14}C]epiCS **51** which were separated. The final Baeyer-Villiger oxidation of ketone C-6 with TFA in dichloromethane afforded the (22*R*,23*R*)-7-oxa-lactone **52** accompanied with its 6-oxa isomer **56** as a minor product. Analogically, (22*S*,23*S*)-tetraol provided the (22*S*,23*S*)-7-oxa-lactone **53** accompanied with its 6-oxa isomer **57**. The ultimate products (22*R*,23*R*)-24-[^{14}C]epiBL **52** and (22*S*,23*S*)-24-[^{14}C]epiBL **53** were isolated in overall 3.20 and 4.46% radiochemical yield (toward $Ba^{14}CO_3$ used as an initial source or radio-label), respectively. SA of prepared BRs was 56.8×10^{-3} Ci/mmol.

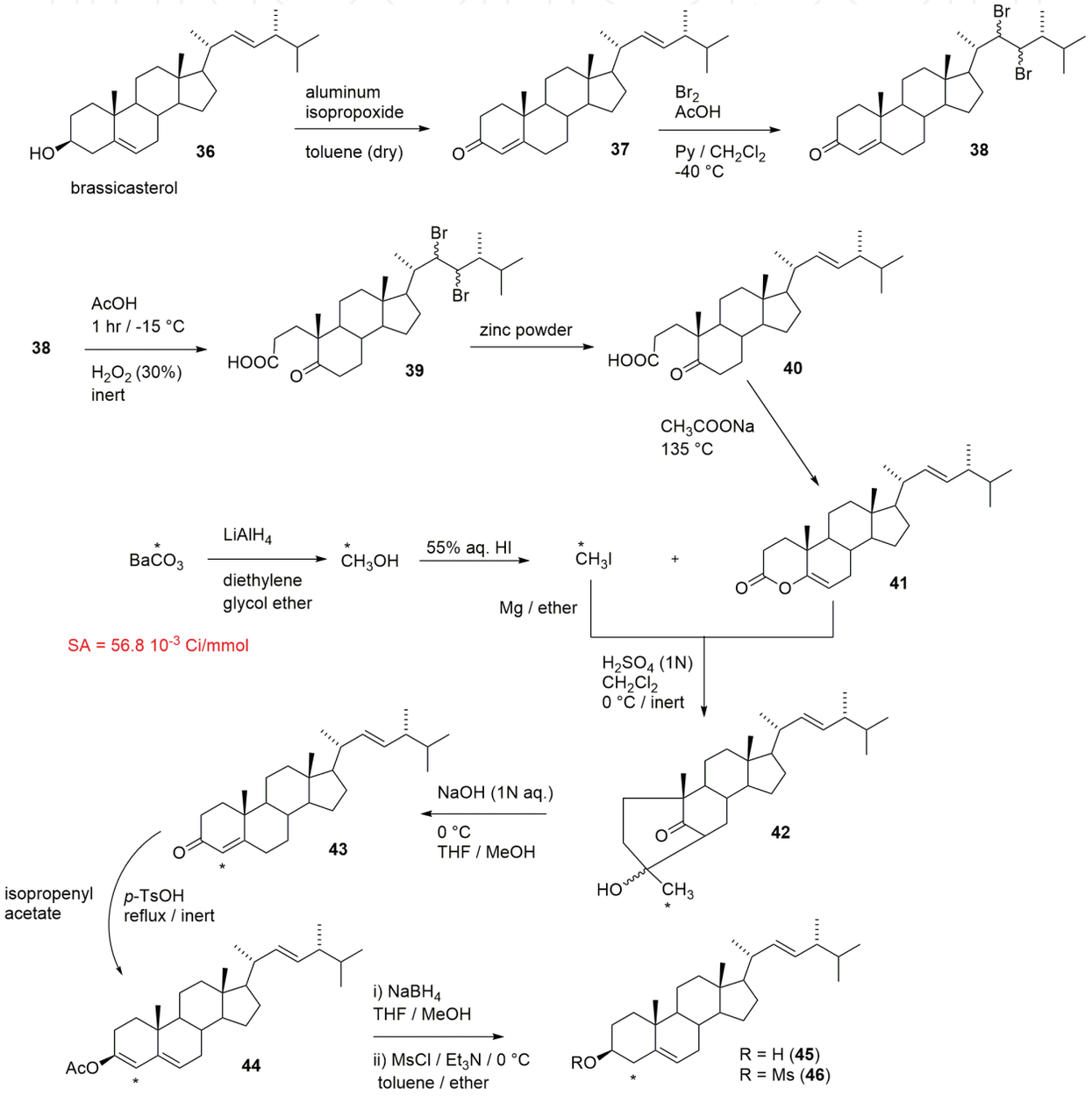

Figure 13. Synthetic pathway to 24-[^{14}C]epiCS and 24-[^{14}C]epiBL.

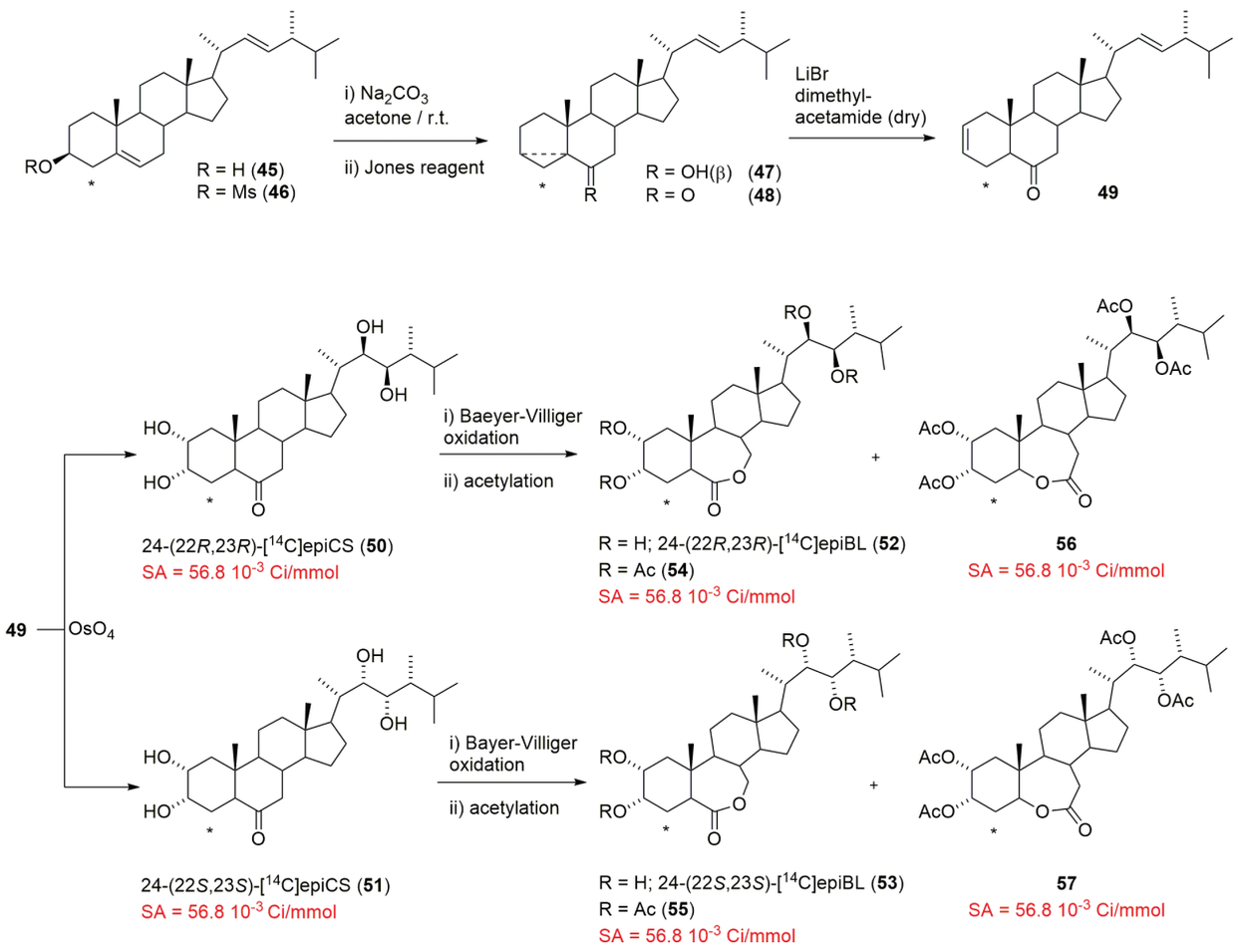

Figure 14. Synthetic pathway to 24-[^{14}C]epiCS and 24-[^{14}C]epiBL.

3. Equipment of the radio-isotope laboratory (IOCB)

The author of this chapter is a member of the Radio-isotope laboratory, a service group of the IOCB CAS, working as a synthetic radiochemist in the production of radioactive molecular tracers. The laboratory is classified for handling of the open sources of ionizing radiation in quantities authorized for laboratories of II category according to Czech bylaw 307/2002 Sb for research and development and educational purposes. It is currently authorized to work with the main radioactive isotopes used in research, for example, ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{51}Cr, ^{54}Mn, ^{55}Fe, ^{99}Tc and ^{125}I. The main purpose of the laboratory is to provide a series of highly specific facilities, equipment and services fully adapted to researchers' needs and maintained in optimum conditions, always in line with applicable legislation to ensure that all personnel is fully protected and ensuring the physical safety of the materials used and the environment. The laboratory has an equipment, stuff, and knowledge to cooperate with other chemists and biologists to provide them with custom synthesis of radio-labeled compounds, especially commercially unavailable compounds. Following time-depending stability of synthesized radio-labeled compounds is one of our basic services provided to biologists.

The key instrument of the laboratory is a glove box with tritiation manifold from RC-TRITEC AG (Teufen, Switzerland) suitable for handling 100–1000 Ci of carrier-free tritium gas (**Figure 15**). Tritiation manifold is based on U-Bed Technology to provide fresh,

Figure 15. The equipment for safe handling of tritium gas; the tritium manifold (RC-TRITEC AG) placed in the glove box; a scrubber for decontamination placed behind the glove box.

^{3}He-free tritium for tritiation by simply heating the UT_3-bed, also allowing the recovery of surplus gas after completion of a reaction (**Figure 16**). During the operation of the manifold, the internal atmosphere of the glove box is continually decontaminated by a scrubber equipped with catalytic oxidation of gaseous tritium to tritiated water, which is trapped

Figure 16. The equipment for safe handling of tritium gas; the tritium manifold (RC-TRITEC AG) placed in the glove box.

on a molecular sieve. ^{3}H NMR measurements were performed on a Bruker Avance II 300 MHz in the laboratory. Liquid scintillation analyser Tri-Carb 2900TR (Perkin Elmer) was used for detecting small amounts of α, β, and γ radioactivity. Mobile contamination monitor CoMo 170 (GRAETZ Strahlungsmeßtechnik GmbH) was used for the high-sensitive and nuclide referred measurement of surfaces with regard to α-, β-, and γ-contaminations when handling ionizing radiation. Analytical -preparative radio-HPLC (pump Waters 600, UV detector Waters 2487, radio chromatogram detector Ramona with analytical cell (LSC) and solid scintillator preparative cell (Raytest, Germany), data management software Empower 2 from Waters). Basic radiation protection equipment and waste disposal management.

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