Abstract: The metabolism of brassinosteroid leads to structural modifications in the ring skeleton or the side alkyl chain. The esterification and glycosylation at C-3 are the most common metabolic pathways, and it has been suggested that conjugate brassinosteroids are less active or inactive. In this way, plants regulate the content of active brassinosteroids. In this work, the synthesis of brassinosteroid 24-norcholane type analogs conjugated at C-3 with benzoate groups, carrying electron donor and electron attractant substituents on the aromatic ring, is described. Additionally, their growth-promoting activities were evaluated using the Rice Lamina Inclination Test (RLIT) and compared with that exhibited by brassinolide (used as positive control) and non-conjugated analogs. The results indicate that at the lowest tested concentrations (10^{-8}–10^{-7} M), all analogs conjugated at C-3 exhibit similar or higher activities than brassinolide, and the diasteroisomers with S configuration at C-22 are the more active ones. Increasing concentration (10^{-6} M) reduces the biological activities of analogs as compared to brassinolide.

Keywords: synthesis; brassinosteroids; analogs; 24-norcholane; benzoate esters; Rice Lamina Inclination Test; conjugated in C-3

1. Introduction

Brassinosteroids (BRs) are an important group of polyhydroxylated sterol plant growth regulators in multiple developmental processes, at nanomolar to micromolar concentration, including cell division, cell elongation, vascular differentiation, reproductive development, and modulation of gene expression [1]. BRs also influence various other developmental processes such as the germination of seeds, rhizogenesis, flowering, senescence, abscission, and maturation. They also confer resistance to plants against various abiotic and biotic stresses [2–5].

Since the discovery of brassinolide (1) (Figure 1) [6], 70 BRs, among them 65 unconjugated (free) and 5 conjugated BRs, have been isolated from 60 plant species including 51 angiosperms (12 monocotyledons and 39 dicotyledons), 6 gymnosperms, 1 pteridophyte (Equisetum arvense), 1 bryophyte (Marchantia polymorpha), and chlorophyte, the alga (Hydrodictyon reticulatum). Thus, BRs are widely distributed in the plant kingdom, including higher and lower plants [7].
Figure 1. Structure of natural occurring brassinolide (1), 24-epibrassinolide (2), metabolites conjugated in C-3 formed by esterification (compounds 3–8), and metabolites conjugated in C-2 and C-3 formed by glycosylation (compounds 9–12).

On the other hand, a study of the miscellaneous pathways of BRs metabolism in plants reported the existence of around 19 conjugated metabolites in positions C-2, C-3, C-23, C-25, or C-26 [8]. Eight out of nineteen correspond to conjugates formed by esterification in C-3 [9–14]. The other eleven conjugated metabolites are formed by glycosylation at C-2, C-3, C-23, C-25, or C-26 [8,13–20]. Some examples of these structures are shown in Figure 1. It seems that conjugated compounds are used by plants to store inactive BRs that can be converted to active forms by de-conjugation reactions. Additionally, the natural conjugates 3, 4 (Figure 1) were synthesized from 24-epibrassinolide (2) [21].

On the other hand, a series of C-3 esterified derivatives of 24-epibrassinolide (13–15) and synthetic BRs analogs (16–19) (Figure 2) have been reported [21,22].

Figure 2. Structure of synthetic brassinosteroids (BRs) analogs conjugated in C-3 (compounds 13–19b) and free synthetic analogs 20a and 20b.

However, biological evaluations in the Bean Second-Internode Bioassay (BSIB) for compounds 16 and 17 indicated that these analogs are less active than 24-epibrassinolide [22]. These results are in line with previously established structure-activity relationships ob-
tained for natural BRs. These structure-activity relationship (SAR) studies have been made using BSIB and the Rice Lamina Inclination Test (RLIT) [23–25], and their main goal is to define general structural requirements for the growth-promoting activity of BRs [24,26–29]. These results have been used to guide the synthesis of BRs analogs with a variety of structural modifications but keeping those considered essential for biological activity.

Several studies have proved that synthetic BRs analogs with significant structural changes and different substituents, both in the ring and the alkyl chain, can induce similar or even higher biological effects in plants as compared to natural BRs [30–36]. Some recent reviews of the growth-promoting activity of BRs and their analogs have established novel structural requirements for the existence of biological activity [23,37–39]. For example, it has been shown that methyl ethers at C-3 are more active than 1 in the RLIT [40], whereas benzoate esters in the C-3 position were found to be less active than 24-epibrassinolide in the BSIB test [22].

In a previous in silico study, we have assessed the effect on activity of different groups attached to position C-3 of BRs analogs. The results suggest that bulky groups reduce the activity, whereas functionalization with electronegative and hydrophobic groups would increase it [29]. Thus, in this work, we present the synthesis of four new BR 24-norcholane type analogs conjugated with benzoate groups in C-3 (Figure 2, compounds 18a, 18b, 19a, and 19b). The aromatic ring of the benzoate group contains electron-donor and electron-withdrawing substituents. Their growth-promoting activities were evaluated using RLIT, and the results were compared with those reported for other structurally similar analogs (Figure 2, compounds 20a and 20b) [38,41,42].

The synthesis and evaluation of biological activity of these BRs analogs, conjugated in C-3 with benzylic esters, are studied either to get new active molecules or to elucidate if esterification could be a metabolic path for exogenous BRs.

2. Results and Discussion

2.1. Chemistry

To obtain the new BR analogs conjugated in C-3 (18a, 18b, 19a, and 19b, Figure 2), the synthetic strategy shown in Scheme 1 was developed. The synthesis of the key intermediate alkene 28 has been previously reported [43], but herein, we have introduced some modifications in the synthesis steps to increase the yields of reactions. In addition, more clear spectroscopic evidence (1H- and 13C-NMR) is provided [43–45].

The standard acetylation (Ac2O/N,N-dimethylaminopyridine(DMAP)/CH2Cl2) of hyodeoxycholic acid (21) leads to known diacetylated derivative (22) in 91.1% yield (ref. 80% yield, [44,45]). In the 1H-NMR spectrum of compound 22 (Figure S1, Supplementary Materials), the protons of both acetate groups appear at δH = 2.02 ppm (3H, s, CH3CO2-C6) and 1.99 ppm (3H, s, CH3CO2-C3) [44,45]. While in the 13C-NMR spectrum (Figure S1, Supplementary Materials), the observed signals at δC = 170.56 ppm (CH3C=O-C6), 170.52 ppm (CH3C=O-C3), 21.36 ppm (CH3CO2-C6), and 21.32 ppm (CH3CO2-C3) confirm the presence of both acetate groups.

Oxidative decarboxylation of the side chain of compound 22, with the Phl(OAc)2/Cu(OAc)2 system [44,45], leads to olefin 23 in 99.6% yield (yield data were not reported by other authors). In the 1H-NMR of compound 23 (Figure S2, Supplementary Materials), the protons H-22, Htrans23, and Hcis23 appear at δH = 5.65 ppm (dd, j = 17.1, 10.2 and 8.4 Hz), 4.90 ppm (dd, j = 17.1 and 2.0 Hz), and 4.81 ppm (dd, j = 10.2 and 2.0 Hz), respectively [44,45]. Meanwhile, in the 13C-NMR (Figure S2, Supplementary Materials), the carbons C-22 and C-23 appear at δC = 145.06 and 111.69 ppm, respectively. These signals confirm the presence of terminal alkene.
Scheme 1. Synthesis of hyodeoxycholic acid derivatives 22–30 and C-3 conjugated brassinosteroid analogues 18a, 18b, 19a, and 19b. Reagents and conditions: (a) Ac₂O/DMAP, CH₂Cl₂, rt, 48 h, 91.1% yield; (b) PhI(OAc)₂/Cu(OAc)₂, C₆H₆, reflux, 5 h, 99.6% yield; (c) K₂CO₃ (15% p/v, H₂O)/(CH₃)₂CO/CH₃OH, reflux, 7 h, 97.1% yield; (d) PCC/CH₂Cl₂, rt, 48 h, Column Chromatography (C.C.) separation, 25 (2.4% yield), 26 (19.1% yield), and 27 (40.2% yield); (e) NaBH₄/MeOH, 0–5 °C, 1 h, 76.3% yield; (f) HCl/CH₂OH 2.5% v/v, rt, 48 h, 74.8% yield; (g) p-CH₃C₆H₄COCl or o-FC₆H₄COCl/DMAP/CH₂Cl₂, rt, 2 h, 29 (87.9% yield), 3 h, 30 (56.0% yield); (h) Dihydroquinidine-Chlorobenzoate(DHQD-CLB)/CH₃SO₂NH₂, K₂CO₃/K₃[Fe(CN)₆], OsO₄/(CH₃)₂COH/H₂O, rt, 5 h, 18a/18b (1.0:1.0), 91.6% yield; 19a/19b (1.0:1.0), 80.8% yield.

The saponification of diacetate 23 with the system K₂CO₃/acetone/methanol/reflux leads to diol 24 in 97.1% yield (ref. 98% yield, [43]). Although compound 24 was previously reported, no NMR spectroscopic data were reported [43,45]. So, the observed signals in the ¹H-NMR spectrum (Figure S3, Supplementary Materials) at δ_H = 4.02–3.96 ppm (1H, m) and 3.48–3.42 ppm (1H, m) were assigned to carbinolic hydrogens H-6 and H-3, respectively (Table 1). While in the ¹³C-NMR (Figure S3, Supplementary Materials), the carbons C-6 and C-3 appear at δ_C = 67.63 and 71.72 ppm, respectively (Table 1). The assignments for the H-6 and H-3 signals were confirmed by the 2D HSQC spectrum of compound 24.

Table 1. Differences in ¹H- and ¹³C-NMR chemical shifts for H-3, H-6, C-3, and C-6 observed for compounds 24–27.

| Compound | H-3 (δ_H ppm) | H-6 (δ_H ppm) | C-3 (δ_C ppm) | C-6 (δ_C ppm) |
|----------|---------------|---------------|---------------|---------------|
| 24       | 3.48–3.42     | 4.02–3.96     | 71.72         | 67.63         |
| 25       | -             | 4.15–4.10     | 212.63        | 67.73         |
| 26       | -             | -             | 208.65        | 210.82        |
| 27       | 3.70–3.62     | -             | 70.18         | 213.89        |

The subsequent oxidation of compound 24 with the PCC/CH₂Cl₂ system produces a mixture of three oxidation products (Scheme 1), which were efficiently separated by flash chromatographic column. So, the least polar product was identified as diketone 26 (19.1% yield), a product of intermediate polarity identified as monoketone 25 (2.4% yield), and the most polar product identified as the desired monoketone 27 (40.2% yield). Diketone 26 was previously obtained by the oxidation of glycol with Jones reagent in...
95% yield [45]. Meanwhile, diketone 26 and monoketone 27 were obtained by oxidation with the PDC/CH₂Cl₂ system, with 21% and 61.7% yields, respectively [43]. The IR and ¹H-NMR spectroscopic data for compounds 26 and 27 were consistent with those reported (Figures S5 and S6, Supplementary Materials) [43,45]. However, none of these previous works reported obtaining monoketone 25. In the ¹H-NMR spectrum of this compound (Figure S4, Supplementary Materials), the observed signal at δ_H = 4.15–4.10 ppm (1H, m) was assigned to carbinolic hydrogen H-6, whereas in the ¹³C-NMR spectrum (Figure S4, Supplementary Materials), the observed signal at δ_C = 67.73 ppm corresponds to C-6.

Table 1 shows the differences detected for the main signals observed in the ¹H- and ¹³C-NMR spectra of compounds 24 to 27. All this information was confirmed by the 2D HSQC correlation spectra of compounds 25–27.

Diketone 26 was conveniently converted to the desired monoketone 27 by selective reduction with NaBH₄/MeOH [43] at low temperature (0–5 °C) with 76.3% yield (step e, Scheme 1). The spectroscopic data of this compound and 27, which was obtained by direct oxidation from 24 (step d, Scheme 1), were identical.

Then, compound 27 was easily isomerized under acid condition (2.5% v/v HCl/MeOH) to give the derivative 28 possessing 5α-cholestan-6-one skeleton (74.8% yield) [43,44,46–49]. The IR, ¹H- and ¹³C-NMR spectroscopic data registered for compound 28 were consistent with those reported (Figure S7, Supplementary Materials) [43,46]. The ¹H and ¹³C spectroscopy. For derivative 29, the presence of aromatic signals at δ_H = 8.00 ppm (2H, d, J = 9.0 Hz) and 6.93 ppm (2H, d, J = 9.0 Hz) were assigned to the hydrogens HAr-2' and HAr-3', respectively, whereas the signals appearing at δ_C = 163.36, 131.58, 123.35, and 113.70 ppm were assigned to the aromatic carbons C4', C2'/C6', C1', and C3'/C5' (Figure S8, Supplementary Materials). For derivative 30, the presence of the aromatic signals at δ_H = 7.92 ppm (1H, td, j = 7.6 and 1.8 Hz); 7.54–7.48 ppm (1H, m); 7.21 ppm (1H, td, j = 7.6 and 1.2 Hz), and 7.13 ppm (1H, ddd, j = 10.7, 7.6 and 0.9 Hz) were assigned to the hydrogens HAr-6', HAr-4', HAr-3', and HAr-5', respectively (Figure S9, Supplementary Materials). In the ¹³C-NMR spectrum (Figure S9, Supplementary Materials), the observed signals at δ_C = 161.92 ppm (d, 1 J_CF = 259.1 Hz); 134.44 ppm (d, 3 J_CF = 8.4 Hz); 132.32 ppm (d, 3 J_CF = 0.9 Hz); 124.11 ppm (d, 4 J_CF = 3.6 Hz); 119.38 (d, 2 J_CF = 9.6 Hz); and 117.05 (d, 2 J_CF = 21.6 Hz) were assigned to the aromatic carbons C2', C4', C6', C5', C1', and C3', respectively (Figure 3).

Figure 3. Structures of derivatives 29 and 30 and numbering of aromatic carbon atoms used in this study.

Recently, the synthesis of glycols C22/C23 in steroids with the shortest side chain of 24-nor-5α-cholane type by a Sharpless dihydroxylation reaction has been reported [42]. The results showed that this type of hydroxylation leads to a mixture of C-22 glycols (R/S) with an approximate 1:1 ratio of both diastereomers [42]. Thus, both olefins 29 and 30 were dihydroxylated following this method and using dihydroquinidine p-chlorobenzoate.
(DHQD-CLB) as a chiral ligand (Scheme 1) [32,42]. The Sharpless dihydroxylation of derivative 29 produced the 18a/18b diastereoisomer mixture with a total 91.6% yield. The diastereomeric ratio of each glycol in the mixture can be established by the integration of $^1$H-NMR signals assigned to the C-21 methyl group, which appear at $\delta_H = 0.921$ and 0.953 ppm in 18a and 18b diastereoisomers, respectively. Based on these NMR measurements, the relative ratio of 18a:18b was determined as 1:0.1:0. Subsequently, the diastereoisomers mixture was separated by a semi-preparative HPLC system, allowing obtaining the analogs 18a and 18b.

The structure and stereochemistry at C-22 of compounds 18a and 18b was established by simple comparison of $^1$H- and $^{13}$C-NMR spectra obtained for derivatives 20a and 20b, which were previously reported [41,42]. These comparisons considered chemical shifts ($\delta$), coupling constants ($J$), and multiplicities of signals corresponding to H-22, H-23a, H-23b, and CH$_3$-21 ($^1$H-NMR) and chemical shifts ($\delta$) in $^{13}$C-NMR of both epimers. The main differences in these spectroscopic parameters are listed in Table 2.

Table 2. Comparison between signals of $^1$H- (500.1 MHz, CDCl$_3$) and $^{13}$C- (125.8 MHz, CDCl$_3$) NMR for H/C21, H/C22, and H/C23a-b, for the epimers 18a and 18b.

| H/C Signal | Compound 18a | Compound 18b |
|------------|--------------|--------------|
| H-21       | 0.921 ppm (3H, d, $J = 6.7$ Hz) | 0.953 ppm (3H, d, $J = 7.0$ Hz) |
| H-22       | 3.66–3.61 ppm (1H, m) | 3.51 ppm (1H, t, $J = 10.2$ Hz) |
| H-23a      | 3.80 ppm (1H, ddd, $J = 8.9, 3.3$ and 1.2 Hz) | 3.83–3.76 ppm (1H, m) |
| H-23b      | 3.52 ppm (1H, dd, $J = 10.8$ and 3.3 Hz) | 3.69–3.57 ppm (1H, m) |
| C21        | 12.73 ppm | 13.15 ppm |
| C22        | 74.14 ppm | 73.92 ppm |
| C23        | 66.16 ppm | 62.54 ppm |

Similarly, a Sharpless dihydroxylation of derivative 30 produced the 19a/19b diastereoisomers mixture with a total 80.8% yield. The diastereomeric ratio of each glycol in the mixture was 1.0:1.0 (established by the integration of $^1$H-NMR signals assigned to the C-21 methyl group, which appear at $\delta_H = 0.917$ and 0.954 ppm in 19a and 19b diastereoisomers, respectively). The diastereoisomers mixture was separated by semi-preparative HPLC system, allowing obtaining analogs 19a and 19b. Similar to the above, the main differences in spectroscopic parameters of epimers are listed in Table 3.

Table 3. Comparison between signals of $^1$H- (500.1 MHz, CDCl$_3$) and $^{13}$C- (125.8 MHz, CDCl$_3$) NMR for H/C21, H/C22, and H/C23a-b, for the epimers 19a and 19b.

| H/C Signal | Compound 19a | Compound 19b |
|------------|--------------|--------------|
| H-21       | 0.917 ppm (3H, d, $J = 6.4$ Hz) | 0.954 ppm (3H, d, $J = 6.7$ Hz) |
| H-22       | 3.75–3.39 ppm (1H, m) | 3.51 ppm (1H, t, $J = 10.2$ Hz) |
| H-23a      | 3.87–3.76 ppm (1H, m) | 3.84–3.76 ppm (1H, m) |
| H-23b      | 3.75–3.39 ppm (1H, m) | 3.71–3.59 ppm (1H, m) |
| C21        | 12.73 ppm | 13.13 ppm |
| C22        | 75.56 ppm | 73.90 ppm |
| C23        | 61.15 ppm | 62.54 ppm |

In summary, four new BRs 24-norcholane type analogs conjugated at the C-3 position with benzoate groups substituted with electron donor and electron-withdrawing groups in the p-position (compounds 18a, 18b, 19a and 19b) have been synthesized and characterized.

2.2. Biological

In this work, the activity of new BR 24-norcholane type analogs conjugated at the C-3 position was evaluated using the Rice Lamina Inclination Test. The results of this test were compared with those obtained for other free analogs of 24-norcholane type (analogs 20a and 20b [38]) and with brassinolide. This assay was used because of its specificity and high
sensitivity for 1 and their analogs [31,52,53]. The bending angles were measured as the difference between the induced angle produced by treatment with each compound and that found for the negative control. Results obtained for 1, which was used as positive control, and BR analogs 18a, 18b, 19a, 19b, 20a, and 20b are listed in Table 4.

Table 4. Comparison between BRs C-3 conjugated 24-norcholane and free 24-norcholane type analogs on lamina inclination of rice seedlings.

| Compounds | 1 × 10⁻⁸ M | 1 × 10⁻⁷ M | 1 × 10⁻⁶ M |
|-----------|------------|------------|------------|
| 1 (C⁺)    | 31 ± 11    | 41 ± 4.5   | 70 ± 7.6   |
| 18a       | 61 ± 6.3 * | 68 ± 9.6 * | 46 ± 7.5 * |
| 18b       | 64 ± 3.3 * | 48 ± 2.9   | 14 ± 4.8 * |
| 19a       | 43 ± 5.0 * | 58 ± 2.4 * | 68 ± 9.6   |
| 19b       | 68 ± 5.0 * | 61 ± 2.5 * | 30 ± 0.0 * |
| 20a †     | 45 ± 9.5 * | 31 ± 5.0   | 24 ± 5.8 * |
| 20b †     | 35 ± 3.0   | 60 ± 3.0 * | 62 ± 12    |
| Control (C⁻) | 7 ± 5.0   |            |            |

† Data previously obtained and reported in reference [38]. Brassinolide (1) was used as positive control. The negative control only contained sterile distilled water. These values represent the mean ± standard deviation of two independent experiments with at least six replicates each (n = 12). (*) Represents experiments with a significant difference between positive control (1) and analog treatments at p < 0.05 significance level (least square differences (LSD) t-test).

Interestingly, these data clearly indicate that 24-nor-5α-cholane type analogs conjugated at C-3 exhibit interesting growth-promoting activity. These results are in line with previous studies for other analogs of 24-norcholane type [38,48]. All C-3 conjugated analogs exhibit higher activity than brassinolide at the lowest concentrations (1 × 10⁻⁸ and 1 × 10⁻⁷ M) (Figure 4 and Table 4).

To simplify the data analysis, we will consider the data obtained at 1 × 10⁻⁸ and 1 × 10⁻⁷ M to analyze the correlation between chemical structure and biological activity. The results indicate that at these concentrations, 18b and 19b are the most active in the series of conjugated analogs (19b was the most active at the concentration of 1 × 10⁻⁸ M, whereas 18a was the most active at the concentration of 1 × 10⁻⁷ M) (see Table 3), and they were more active than the free analogs 20a and 20b. Another important effect to consider is related to the configuration on the C-22 carbon of the side chain. Thus, at the concentration of 1 × 10⁻⁸, analogs 18a and 19a with C-22(R) configuration are less active than analogs 18b and 19b with C-22(S) configuration. However, an opposite effect is observed for analogs 20a and 20b. Similarly, at the concentration of 1 × 10⁻⁷ M, the analogs 19b and 20b with C-22(S) configuration are more active than analogs 19a and 20a with C-22(R) configuration. However, an opposite effect is observed for analogs 18a and 18b. The results observed for the pairs 18a/18b (at 1 × 10⁻⁷ M) and 20a/20b (at 1 × 10⁻⁸ M) would be aligned with those reported for natural occurring BRs with an intact side chain, which indicates that glycol function with C-22(R) and C-23(R) configuration appears essential for a high biological activity and are more active than those with C-22(S) and C-23(S) configuration [3,54]. However, these apparently contradictory structural effects of BRs analogs could be explained in attributed to the shorter side chains. This structural feature could give a greater rotational freedom degree.
Rice Lamina Inclination (Angle Opening, Degrees)

BR Analogs

1 × 10⁻⁸ M

Negative Control (C-)

Figure 4. Rice-lamina assays using the second leaf lamina joints of excised leaf segments treated with BR analogs (18b, 19b, and 20a) at 1 × 10⁻⁸ M. Brassinolide (I) was used as positive control at the same concentrations. The negative control only contained sterile distilled water.

3. Materials and Methods
3.1. Chemistry

All reagents were purchased from commercial suppliers and used without further purification. Melting points were measured on a SMP3 apparatus (Stuart-Scientific, now Merck KGaA, Darmstadt, Germany) and are uncorrected. ¹H-, ¹³C-, ¹³C-DEPT-135, gs 2D HSQC, and gs 2D HMBC NMR spectra were recorded in CDCl₃ and MeOD solutions, and they are referenced to the residual peaks of CHCl₃ at δ = 7.26 ppm and δ = 77.00 ppm for ¹H and ¹³C, respectively and CD₂OD at δ = 3.30 ppm and δ = 49.00 ppm for ¹H and ¹³C, on an Avance 400 Digital NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 400.1 MHz for ¹H and 100.6 MHz for ¹³C, and JEOL JNM-ECA 500 NMR spectrometer (JEOL, Tokyo, Japan) operating at 500.16 MHz for ¹H, 125.77 MHz for ¹³C, and 470.62 MHz for ¹⁹F. Chemical shifts are reported in ppm and coupling constants (J) are given in Hz; multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet (t), quartet (q), multiplet (m), and broad singlet (bs). IR spectra were recorded as KBr disks in a Fourier Transform Infrared (FT-IR) 6700 spectrometer (Nicolet, Thermo Scientific, San Jose, CA, USA) and frequencies are reported in cm⁻¹. High-resolution mass spectra (HRMS) were recorded in an API HRMS instrument, and the samples were dissolved in chloroform (or chloroform: methanol; 1:1; v/v, in the case of hydroxylated compounds) to a concentration of 10 µg mL⁻¹. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into
the ion source, and analyzed in full scan mode. The source of the Synapt G2-Si mass spectrometer (Waters, Manchester, UK) was operated in positive ionization mode (ASAP), if not stated otherwise, at a source temperature of 120 °C. The corona needle current was kept at 5 µA and the collision energy was kept at 4 V. The probe temperature was ramped up from 50 to 600 °C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in high-resolution mode. The data were processed using the Masslynx 4.1 software (Waters, Milford, MA, USA). A mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. For analytical TLC, silica gel 60 in a 0.25 mm layer was used, and TLC spots were detected by heating after spraying with 10% H2SO4 in H2O. Chromatographic separations were carried out by conventional column on silica gel 60 (230–400 mesh) using EtOAc-hexane gradients of increasing polarity. All organic extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure, below 40 °C. The HPLC system consisted of a Waters semi-preparative HPLC system including a quaternary pump, a liquid handler, and UV-Vis and Evaporative Light Scattering Detector (ELSD) detectors. The semi-preparative column was filled with silica gel.

3.1.1. Synthesis

3α,6α-Diacetoxy-5β-cholan-24-oic acid (22)

To a solution of hyodeoxycholic acid (21) (25.4 g, 64.62 mmol) in 400 mL of CH2Cl2 (DCM), 150 mg of DMAP, 2 mL of pyridine, and 24.4 mL (257.6 mmol) of Ac2O were added. The reaction mixture was kept under constant stirring and room temperature for 48 h. The end of the reaction was verified by TLC; then, the mixture was concentrated to a volume approximately 50 mL under reduced pressure. Then, EtOAc (200 mL) and 200 mL of HCl solution (1 × 10−5 M) were added. The organic layer was separated and washed with water (4 × 50 mL), with saline (NaCl) solution (3 × 50 mL) until pH = 5, dried over Na2SO4, and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH2Cl2 (16 mL) and chromatographed on silica gel with EtOAc/hexane (20%, 200 mL). Compound 22 (28.06 g 91.1% yield) was obtained as a colorless solid, m.p. = 109.7–110.9 °C (106–110 C [44]). IR νmax (cm⁻¹): 3527 (O-H); 2948; 2899 and 2870 (C-H); 1738 (C=O); 1722 (C=O); 1681 (CH2); 1364 (CH3); 1256 (C-O); 1242(C-O); 1027 (C-O). 1H-NMR (400.1 MHz, CDCl3) (Figure S1, Supplementary Materials): δ (ppm) = 5.14–5.10 (1H, m, H-6); 4.71–4.65 (1H, m, H-3); 2.37 (1H, ddd, J = 15.3, 10.1 and 5.0 Hz, H-23a); 2.23 (1H, ddd, J = 16.0, 10.1, and 6.4 Hz, H-23b); 2.02 (3H, s, CH3CO2-C6); 1.99 (3H, s, CH3CO2-C3); 1.97–1.94 (1H, m, H-5); 0.95 (3H, s, H-19); 0.90 (3H, d, J = 6.4 Hz, H-21); 0.62 (3H, s, H-18). 13C-NMR (100.6 MHz, CDCl3) (Figure S1, Supplementary Materials) δ (ppm) = 180.09 (C-24); 170.56 (CH2CO2-C6); 170.52 (CH2CO2-C3); 73.66 (C-3); 70.92 (C-6); 56.06 (C-14); 55.84 (C-17); 45.28 (C-9); 42.81 (C-13); 39.78 (C-5 and C-12); 35.97 (C-10); 35.18 (C-14); 34.96 (C-7); 34.54 (C-8); 31.20 (C-1); 30.93 (C-22); 30.64 (C-23); 28.01 (C-4); 26.35 (C-16); 26.15 (C-2); 24.00 (C-15); 23.19 (C-19); 21.36 (CH3CO2-C6); 21.32 (CH3CO2-C3); 20.61 (C-11); 18.17 (C-21); 11.95 (C-18).

24-Nor-5β-cholan-22-ene-3α,6α-diy diacetate (23)

To a solution of 22 (2.00 g, 4.20 mmol) in dry benzene (150 mL) were added Cu(OAc)2·H2O (200 mg, 1.0 mmol) and pyridine (2.5 mL). Then, under reflux, PhI(OAc)2 (7.04 g, 21.5 mmol) was added in four portions at hourly intervals. After the addition was completed, the reaction was continued for 1 h. The end of the reaction was verified by TLC, and then, the mixture was filtered, and the solvent was evaporated under reduced pressure. The crude was re-dissolved in DCM (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 → 15.8:4.2). The reaction was repeated 5 times under identical conditions. Compound 23 (9.0 g, 99.6% yield) was obtained as a colorless solid, m.p. = 89.0–90.9 °C (88–89 °C [45]). IR νmax (cm⁻¹): 3082 (CH=CH2); 2940; 2887 and 2867 (C-H); 1740 (C=O); 1727 (C=O); 1633 (C=C); 1460 (CH2); 1366 (CH3); 1244 (C-O); 1026 (C-O); 908 (CH=CH2). 1H-NMR (400.1 MHz, CDCl3) (Figure S2, Supplementary Materials): δ (ppm) = 5.65 (1H, ddd, J = 17.1, 10.2, and 8.4 Hz, H-22); 5.16–5.13 (1H, m, H-6); 4.90 (1H,
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dd, \( J = 17.1 \) and 2.0 Hz, H-23a); 4.81 (1H, dd, \( J = 10.2 \) and 2.0 Hz, H-23b); 4.71–4.69 (1H, m, H-3); 2.07–2.03 (1H, m, H-20); 2.04 (3H, s, CH\(_3\)CO\(_2\)-C6); 2.01 (3H, s, CH\(_3\)CO\(_2\)-C3); 2.00–1.94 (1H, m, H-5); 1.02 (3H, d, \( J = 6.6 \) Hz, H-21); 0.97 (3H, s, H-19); 0.67 (3H, s, H-18). 13C-NMR (100.6 MHz, CDCl\(_3\)) (Figure S2, Supplementary Materials) \( \delta \) (ppm) = 170.47 (CH\(_3\)CO\(_2\)-C6); 170.45 (CH\(_3\)CO\(_2\)-C3); 145.06 (C-22); 111.69 (C-23); 73.70 (C-3); 70.95 (C-6); 56.21 (C-14); 55.59 (C-17); 45.39 (C-9); 42.83 (C-13); 41.13 (C-20); 39.93 (C-5); 39.79 (C-12); 36.07 (C-10); 35.06 (C-1); 34.63 (C-8); 31.30 (C-7); 28.36 (C-16); 26.44 (C-2); 26.25 (C-4); 24.09 (C-15); 23.27 (C-19); 21.41 (CH\(_3\)CO\(_2\)-C6); 21.37 (CH\(_3\)CO\(_2\)-C3); 20.68 (C-11); 20.07 (C-21); 12.18 (C-18).

24-Nor-5β-chol-22-ene-3α,6α-diol (24)

To a solution of 23 (8.06 g, 18.71 mmol) in a mixture 1:1 of acetone/MeOH (60 mL), a 15% aqueous solution of K\(_2\)CO\(_3\) (37.4 mmol) was added. The suspension was stirred and refluxed for 7 h. The end of the reaction was verified by TLC. Then, the solvent was removed, the residue was diluted with EtOAc (80 mL), and the mixture was washed with 3 \( \times \) 80 mL of HCl solution (1 \( \times \) 10\(^{-3} \) M). The organic layer was dried over Na\(_2\)SO\(_4\) and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH\(_2\)Cl\(_2\) (15 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 \( \rightarrow \) 7.2:12.8). Compound 24 (6.3 g; 97.1% yield) was obtained as a colorless solid, m. p. = 154.4–155.2 °C (150–152 °C [45]). IR \( \nu_{\text{max}} \) (cm\(^{-1}\)) = 3381 (O-H); 3088 (CH=CH\(_2\)); 2934, 2889, and 2868 (C-H); 1637 (C=C); 1462 (C-H\(_2\)); 1336 (CH\(_3\)); 1269 (C-O); 1043 (C-O); 912 (CH=CH\(_2\)). 1H-NMR (400.1 MHz, Acetone) (Figure S3, Supplementary Materials): \( \delta \) (ppm) = 5.66 (1H, ddd, \( J = 17.4, 10.0, \) and 8.6 Hz, H-22); 4.90 (1H, dd, \( J = 17.1 \) and 2.0 Hz, H-23a); 4.82 (1H, ddd, \( J = 10.2 \) and 2.0 Hz, H-23b); 4.02–3.96 (1H, m, H-6); 3.48–3.42 (1H, m, H-3); 1.02 (3H, d, \( J = 6.6 \) Hz, H-21); 0.91 (3H, s, H-19); 0.66 (3H, s, H-18).

13C-NMR (100.6 MHz, Acetone) (Figure S3, Supplementary Materials): \( \delta \) (ppm) = 146.00 (C-22); 112.05 (C-23); 71.72 (C-3); 67.63 (C-6); 57.20 (C-14); 56.40 (C-17); 49.70 (C-9); 43.51 (C-13); 42.09 (C-20); 40.89 (C-5); 40.80 (C-12); 36.65 (C-1); 36.55 (C-10); 35.97 (C-7); 35.70 (C-8); 31.43 (C-4); 30.05 (C-2); 29.15 (C-16); 24.90 (C-15); 24.10 (C-19); 21.54 (C-11); 20.55 (C-21); 12.55 (C-18).

6α-Hydroxy-24-nor-5β-chol-22-ene-3-one (25), 24-nor-5β-chol-22-ene-3,6-dione (26) and 3α-hydroxy-24-nor-5β-chol-22-ene-6-one (27)

A solution of 24 (6.0 g, 17.3 mmol) in DCM (100 mL) with 3.76 g (17.3 mmol) of Pyridinium chlorochromate (PCC) in 60 mL of DCM (added by slow drip) was slowly stirred for 48 h at room temperature. The end of the reaction was verified by TLC; then, the reaction mixture was filtered on alumina and washed with ethyl acetate (20 mL). The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH\(_2\)Cl\(_2\) (10 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 \( \rightarrow \) 8.8:11.2). Four fractions were obtained: Fraction I, 1.14 g (19.1% yield) of compound 26; Fraction II, 0.141 g (2.4% yield) of compound 25; Fraction III, 2.39 g (40.2% yield) of compound 27; and Fraction IV, 3.58 g of unreacted 24. The reaction was repeated with compound 24 twice with another 5.5 g each and 26 (2.08 g), 25 (4.28 g), and 27 (0.258 g) were obtained.

Compound 25 was obtained as a colorless solid. 1H-NMR (400.1 MHz, CDCl\(_3\)) (Figure S4, Supplementary Materials): \( \delta \) (ppm) = 5.68 (1H, ddd, \( J = 17.1, 10.2, \) and 8.4 Hz, H-22); 4.93 (1H, ddd, \( J = 17.1 \) and 2.0 Hz, H-23a); 4.84 (1H, ddd, \( J = 10.2 \) and 2.0 Hz, H-23b); 4.15–4.10 (1H, m, H-6); 2.42–2.39 (2H, m, H-4); 1.05 (3H, d, \( J = 6.6 \) Hz, H-21); 1.03 (3H, s, H-19); 0.72 (3H, s, H-18). 13C-NMR (100.6 MHz, CDCl\(_3\)) (Figure S4, Supplementary Materials): \( \delta \) (ppm) = 212.63 (C-3); 145.00 (C-22); 111.77 (C-23); 67.73 (C-6); 56.17 (C-14); 55.55 (C-17); 50.16 (C-9); 42.79 (C-13); 41.15 (C-20); 40.31 (C-5); 39.74 (C-12); 37.08 (C-1); 37.06 (C-7); 36.24 (C-10); 36.02 (C-4); 34.56 (C-8); 34.40 (C-2); 28.35 (C-16); 24.17 (C-15); 22.85 (C-19); 21.08 (C-11); 20.10 (C-21); 12.23 (C-18).

Compound 26 was obtained as a colorless solid, m. p. = 177.5–178.9 °C (197–200 °C [45]). IR \( \nu_{\text{max}} \) (cm\(^{-1}\)) = 3073 (CH=CH\(_2\)); 2964, 2947, 2873, and 2855 (C-H); 1716 (C=O); 1693 (C=O); 1632 (C=C); 1466 (CH\(_2\)); 1382 (CH\(_3\)); 1245 (C-O); 1216 (C-O); 908 (CH=CH\(_2\)). 1H-NMR (400.1
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11.9 mg (3.15 mmol) of NaBH$_4$ was slowly added to a suspension of compound 24 (6.08 g, 17.65 mmol) in 100 mL of 2.5% HCl-MeOH mixture. This solution was placed in a bath of ice-water between 0 and 5 °C. Subsequently, 117.9 mg (3.15 mmol) of NaBH$_4$ was added in four portions (approximately 29.5 mg each) to maintain the temperature and with slow stirring. The end of the reaction was verified by TLC. The reaction mixture was concentrated by evaporation under reduced pressure, then EtOAc (30 mL), dried over MgSO$_4$, and filtered. The solvent was evaporated under reduced pressure. The crude was redissolved in CH$_2$Cl$_2$ (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (9.8:2 -> 5.8:4.2). Two fractions were obtained: Fraction I, 0.753 g of unreacted compound 24; Fraction II, 2.47 g (76.3% yield) of compound 26. The melting point and spectroscopic properties ($^1$H- and $^{13}$C-NMR) of compound 26 and 27 were identical to those reported above for the direct oxidation of compound 24.

$^{3a}$-Hydroxy-24-nor-5β-chol-22-ene-3,6-dione (26)

A solution of compound 26 (3.22 g, 9.4 mmol) was prepared in 100 mL of 1:1 MeOH/THF mixture. This solution was placed in a bath of ice-water between 0 and 5 °C. Subsequently, 117.9 mg (3.15 mmol) of NaBH$_4$ were added in four portions (approximately 29.5 mg each) maintaining the temperature and with slow stirring. The end of the reaction was verified by TLC. 10 mL of acetone, and subsequently, 5 mL of HCl 2.5% were added, maintaining the reaction temperature. The reaction mixture was concentrated by evaporation under reduced pressure to a volume of about 15 mL, and then, EtOAc (50 mL) was added. The organic layer was washed with saturated solution of NaHCO$_3$ and then, EtOAc (20 mL) and water (2 × 30 mL), dried over anhydrous MgSO$_4$, and filtered. The solvent was evaporated under reduced pressure. The crude was obtained as a colorless solid, m.p. = 151.9-153.6 °C. IR $\nu_{\text{max}}$ (cm$^{-1}$): 3288 (O-H); 3074 (CH=CH$_2$); 2970, 2949, and 2867 (C-H); 1702 (C=O); 1637 (C=C); 1458 (CH$_2$); 1379 (CH$_3$); 1248 (C-O); 1064 (C-O); 912 (CH=CH$_2$). $^1$H-NMR (400.1 MHz, CDCl$_3$) (Figure S6, Supplementary Materials): $\delta$ (ppm) = 5.67 (1H, ddd, $J$ = 17.1, 10.1, and 8.4 Hz, H-22); 4.93 (1H, dd, $J$ = 17.1 and 2.0 Hz, H-23a); 4.85 (1H, dd, $J$ = 10.1 and 2.0 Hz, H-23b); 3.70-3.62 (1H, m, H-3); 2.20–2.18 (2H, m, H-7); 2.14 (1H, dd, $J$ = 12.1 and 5.1 Hz, H-5); 1.05 (3H, d, $J$ = 6.6 Hz, H-21); 0.86 (3H, s, H-19); 0.70 (3H, s, H-18). $^{13}$C-NMR (100.6 MHz, CDCl$_3$) (Figure S6, Supplementary Materials): $\delta$ (ppm) = 213.89 (C-6); 144.89 (C-22); 111.87 (C-23); 70.18 (C-3); 59.41 (C-5); 56.89 (C-14); 55.41 (C-17); 43.04 (C-13); 42.93 (C-7); 41.11 (C-20); 40.07 (C-9); 39.53 (C-12); 37.99 (C-10); 37.07 (C-8); 34.86 (C-1); 34.38 (C-4); 28.26 (C-16); 23.99 (C-15); 23.17 (C-19); 20.84 (C-11); 20.08 (C-21); 12.15 (C-18).

$^{3a}$-Hydroxy-24-nor-5α-chol-22-ene-3,6-dione (28)

Compound 27 (6.08 g, 17.65 mmol) was dissolved in 100 mL of 2.5% v/v HCl-MeOH at room temperature and constant agitation for 48 h. The end of the reaction was verified by TLC. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in 60 mL of EtOAc. The organic layer was washed with saturated solution of NaHCO$_3$ (2 × 15 mL) and water (2 × 30 mL), dried over anhydrous MgSO$_4$, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH$_2$Cl$_2$ (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (9:8:2 -> 5.8:4.2). Two fractions were obtained: Fraction I, 0.753 g of unreacted compound 26; Fraction II, 2.47 g (76.3% yield) of compound 27. The melting point and spectroscopic properties ($^1$H- and $^{13}$C-NMR) of compounds 26 and 27 were identical to those reported above for the direct oxidation of compound 24.
212.67 (C-6); 144.96 (C-22); 111.81 (C-23); 65.47 (C-3); 56.84 (C-14); 55.37 (C-17); 53.88 (C-9); 51.69 (C-5); 46.86 (C-7); 42.96 (C-13); 41.57 (C-10); 41.12 (C-20); 39.41 (C-12); 37.98 (C-8); 31.69 (C-1); 28.19 (C-2 and C-4); 27.71 (C-16); 23.93 (C-15); 21.07 (C-11); 20.07 (C-21); 12.32 (C-19); 12.20 (C-18). HRMS (API+) (Figure S14, Supplementary Materials): m/z calculated for C23H37O2 ([M + H]+) 345.2794; found 345.2795.

6-Oxo-24-nor-5α-chol-22-en-3α-y1 4-methylbenzoate (29)

A solution of compound 28 (150 mg, 0.44 mmol) and DMAP (44 mg, 0.36 mmol) was prepared in 3 mL of anhydrous pyridine. To this solution, 4-methylbenzoyl chloride 174 µL (1.32 mmol) was added by slow dripping, and the reaction was maintained at room temperature with constant stirring for 2 h. The end of the reaction was verified by TLC. After completion of the reaction, 3 mL of hot water was added. After an additional 20 min of stirring, the mixture was extracted with EtOAc (20 mL) and washed successively with saturated NaHCO3 solution (2 × 10 mL) and water (2 × 10 mL), dried over anhydrous MgSO4, and filtered. The solvent was evaporated under reduced pressure, and the crude was redissolved in CH2Cl2 (5 mL) and chromatographed on silica gel with EtOAc/cyclohexane (10:19) mixture. Compound 29 (117 mg, 87.9% yield) was obtained as a colorless solid, m.p. = 133.3–135.2 °C. 1H-NMR (500.16 MHz, CDCl3) (Figure S8, Supplementary Materials): δ (ppm) = 7.90 (2H, d, J = 8.0 Hz, HAr-2′); 7.24 (2H, d, J = 8.0 Hz, HAr-3′); 5.65 (1H, ddd, J = 17.1, 10.1 and 8.6 Hz, H-22); 5.36–5.34 (1H, m, H-3); 4.91 (1H, ddd, J = 17.1, 18.0 and 0.9 Hz, H-23a); 4.82 (1H, dd, J = 10.1 and 1.8 Hz, H-23b); 2.66 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.41 (3H, s, CH3-C); 2.32 (1H, dd, J = 13.1 and 4.6 Hz, H-7a); 1.03 (3H, d, J = 6.7 Hz, H-21); 0.786 (3H, s, H-19); 0.695 (3H, s, H-18). 13C-NMR (125.77 MHz, CDCl3) (Figure S8, Supplementary Materials): δ (ppm) = 211.92 (C-6); 165.78 (CO2-Ar); 145.01 (C-22); 143.61 (C4′-Ar); 129.60 (C2′-Ar and C6′-Ar); 129.18 (C3′-Ar); 128.18 (C1′-Ar); 111.95 (C-23); 69.37 (C-3); 56.86 (C-14); 55.50 (C-17); 54.09 (C-5); 53.01 (C-9); 46.85 (C-7); 42.96 (C-13); 41.57 (C-10); 41.12 (C-20); 39.44 (C-12); 38.06 (C-8); 32.81 (C-1); 28.31 (C-16); 25.57 (C-2); 24.32 (C-4); 24.02 (C-15); 21.79 (CH3-Ar); 21.22 (C-11); 20.16 (C-21); 12.57 (C-19); 12.31 (C-18). HRMS (API+) (Figure S15, Supplementary Materials): m/z calculated for C31H43O3 ([M + H]+) 463.3212; found 463.3212.

6-Oxo-24-nor-5α-chol-22-en-3α-y1 2-fluorobenzoate (30)

A solution of compound 29 (120 mg, 0.348 mmol) and DMAP (35 mg, 0.286 mmol) was prepared in 3 mL of anhydrous pyridine. To this solution, 2-fluorobenzoyl chloride 123.5 µL (1.05 mmol) was added by slow dripping, and the reaction was maintained at room temperature with constant stirring for 3 h. The end of the reaction was verified by TLC. After completion of the reaction, 3 mL of hot water was added. After an additional 20 min of stirring, the mixture was extracted with EtOAc (20 mL) and washed successively with saturated NaHCO3 solution (2 × 10 mL) and water (2 × 10 mL), dried over anhydrous MgSO4, and filtered. The solvent was evaporated under reduced pressure, and the crude was redissolved in CH2Cl2 (5 mL) and chromatographed on silica gel with EtOAc/cyclohexane (1.0:19) mixture. Compound 30 (91 mg, 56.0% yield) was obtained as a colorless solid, m.p. = 120.6–120.8 °C. 1H-NMR (500.16 MHz, CDCl3) (Figure S9, Supplementary Materials): δ (ppm) = 7.92 (1H, td, J = 7.6 and 1.8 Hz, HAr-6′); 7.54–7.48 (1H, m, HAr-4′); 7.21 (1H, td, J = 7.6 and 1.2 Hz, HAr-3′); 7.13 (1H, ddd, J = 10.7, 7.6 and 0.9 Hz, HAr-5′); 5.65 (1H, ddd, J = 17.1, 10.1, and 8.6 Hz, H-22); 5.42–5.39 (1H, m, H-3); 4.90 (1H, ddd, J = 17.1, 1.8, and 0.9 Hz, H-23a); 4.82 (1H, dd, J = 10.1 and 1.8 Hz, H-23b); 2.71 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, J = 13.1 and 4.6 Hz, H-7a); 1.03 (3H, d, J = 6.7 Hz, H-21); 0.781 (3H, s, H-19); 0.692 (3H, s, H-18). 13C-NMR (125.77 MHz, CDCl3) (Figure S9, Supplementary Materials): δ (ppm) = 212.04 (C-6); 163.85 (d, J_CF = 3.6 Hz, CO2-Ar); 161.92 (d, J_CF = 259.1 Hz, C2′-Ar); 145.02 (C-22); 134.44 (d, J_CF = 8.4 Hz, C4′-Ar); 132.32 (d, J_CF = 0.9 Hz, C6′-Ar); 124.11 (d, J_CF = 3.6 Hz, C5′-Ar); 119.38 (d, J_CF = 9.6 Hz, C1′-Ar); 117.05 (d, J_CF = 21.6 Hz, C3′-Ar); 111.93 (C-23); 70.34 (C-3′); 56.82 (C-14); 55.50 (C-17); 53.99 (C-9); 52.79 (C-5); 46.84 (C-7); 43.05 (C-13); 41.42 (C-10); 41.23 (C-20); 39.43
To a mixture of t-butanol/water (10 mL, 1:1 v/v) and alkene 29 (100 mg, 0.22 mmol), DHQD-CLB (20.1 mg; 0.043 mmol), CH₂SO₂NH₂ (41.12 mg; 0.43 mmol), K₂CO₃ (179.2 mg; 1.3 mmol), and K₃[Fe(CN)₆] (427.0 mg; 1.3 mmol) were added; then, the mixture was homogenized by magnetic stirring for 10 min. Later, 100 µL of OsO₄ solution (1.0 g, 0.562 mmol in 20 mL of t-butanol) were added, and the mixture reaction was stirred at room temperature for 5 h. The end of the reaction was verified by TLC; then, H₂O (10 mL) and a saturated solution of Na₂SO₄ 5H₂O (2 mL) were added. The mixture was filtered, and the solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (1.0 mL) and chromatographed on silica gel with EtOAc/cyclohexane (16:4) mixture. A mixture of 18a/18b = 1.0/1.0 was obtained (93 mg; 91.6% yield). Separation by HPLC of an analytical sample allowed the separation and obtaining of the pure compounds 18a and 18b.

Compound 18a was obtained as a colorless solid, m. p. = 74.1 ± 4.6 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S10, Supplementary Materials): δ (ppm) = 7.90 (2H, d, J = 8.3 Hz, HAr-2'); 7.24 (2H, d, J = 8.3 Hz, HAr-3'); 5.36–5.34 (1H, m, H-3); 3.83–3.76 (1H, m, H-8); 3.69–3.57 (1H, m, H-23A); 3.51 (1H, t, J = 10.2 Hz, H-22); 2.65 (1H, dd, J = 12.5 and 3.1 Hz, H-7a); 0.953 (3H, d, J = 7.0 Hz, H-21); 0.778 (3H, s, H-19); 0.679 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S10, Supplementary Materials): δ (ppm) = 211.91 (C-6); 165.82 (CO₂-Ar); 143.65 (C-2'-Ar); 129.61 (C-3'-Ar, C-6'-Ar); 129.20 (C-3'-Ar, C-5'-Ar); 128.15 (C-1'-Ar); 74.14 (C-22); 69.36 (C-3); 66.16 (C-23); 56.71 (C-14); 53.96 (C-17); 52.47 (C-5); 46.79 (C-7); 42.94 (C-13); 41.46 (C-10); 39.53 (C-20); 38.09 (C-12); 37.97 (C-8); 35.83 (C-6); 165.79 (CO₂-Ar); 143.65 (C-2'-Ar and C-6'-Ar); 129.18 (C-3'-Ar and C-5'-Ar); 128.16 (C-1'-Ar); 73.92 (C-22); 69.36 (C-23); 62.54 (C-14); 56.42 (C-17); 53.02 (C-9); 52.92 (C-5); 46.79 (C-7); 43.48 (C-13); 41.43 (C-10); 40.15 (C-20); 39.47 (C-12); 38.04 (C-8); 32.81 (C-1); 27.47 (C-16); 25.55 (C-2); 25.23 (C-4); 24.12 (C-15); 21.78 (CH₃-Ar); 21.22 (C-11); 12.73 (C-21); 12.55 (C-19); 12.00 (C-18). HRMS (API+) (Figure S17, Supplementary Materials): calculated for C₃₅H₃₄O₅ ([M + H]+) 947.3267, found 947.3262.

Compound 18b was obtained as a colorless solid, m. p. = 83.0 ± 5.0 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S11, Supplementary Materials): δ (ppm) = 7.89 (2H, d, J = 8.2 Hz, HAr-2'); 7.23 (2H, d, J = 8.2 Hz, HAr-3'); 5.36–5.34 (1H, m, H-3); 3.83–3.76 (1H, m, H-23A); 3.69–3.57 (1H, m, H-23B); 3.51 (1H, t, J = 10.2 Hz, H-22); 2.65 (1H, dd, J = 12.5 and 3.1 Hz, H-7a); 2.41 (3H, s, CH₃-Ar); 2.32 (1H, dd, J = 13.1 and 4.6 Hz, H-7a); 0.953 (3H, d, J = 7.0 Hz, H-21); 0.778 (3H, s, H-19); 0.679 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S11, Supplementary Materials): δ (ppm) = 211.82 (C-6); 165.79 (CO₂-Ar); 143.65 (C-2'-Ar); 129.60 (C-3'-Ar and C-6'-Ar); 129.18 (C-3'-Ar and C-5'-Ar); 128.16 (C-1'-Ar); 73.92 (C-22); 69.36 (C-23); 62.54 (C-14); 56.42 (C-17); 53.02 (C-9); 52.92 (C-5); 46.79 (C-7); 43.48 (C-13); 41.43 (C-10); 40.15 (C-20); 39.47 (C-12); 38.04 (C-8); 32.81 (C-1); 27.47 (C-16); 25.55 (C-2); 25.23 (C-4); 24.12 (C-15); 21.78 (CH₃-Ar); 21.22 (C-11); 13.15 (C-21); 12.55 (C-19); 11.85 (C-18). HRMS (API+) (Figure S18, Supplementary Materials): calculated for C₃₅H₃₄O₅ ([M + H]+) 947.3267, found 947.3262.
room temperature for 5 h. The end of the reaction was verified by TLC; then, H₂O (10 mL) and a saturated solution of Na₂S₂O₃·5H₂O (2 mL) were added. The mixture was stirred for another 20 min. Later, it was extracted with EtOAc (2 × 35 mL) and washed with water (2 × 35 mL), and both organic phases were combined, dried over MgSO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (1.0 mL) and chromatographed on silica gel with an EtOAc/cyclohexane (16:4) mixture. A mixture of 19a/19b = 1.0/1.0 was obtained (65 mg; 80.8% yield). The separation by HPLC of an analytical sample allowed the separation and obtaining of the pure compounds 19a and 19b.

Compound 19a was obtained as a colorless solid, m. p. = 56.9 ± 4.6 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S12, Supplementary Materials): δ (ppm) = 7.92 (1H, td, J = 7.6 and 1.8 Hz, HAr-6'); 7.54–7.49 (1H, m, HAr-4'); 7.21 (1H, td, J = 7.6 and 1.2 Hz, HAr-3'); 7.13 (1H, ddd, J = 10.7, 7.6, and 0.9 Hz, HAr-5'); 5.40–5.39 (1H, m, H-3); 3.84–3.76 (1H, m, H-23a); 3.71–3.69 (1H, m, H-23b); 3.51 (1H, t, J = 10.2 Hz, H-22); 2.70 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, J = 13.1 and 4.6 Hz, H-7α); 0.954 (3H, d, J = 6.7 Hz, H-21); 0.777 (3H, s, H-19); 0.681 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S12, Supplementary Materials): δ (ppm) = 211.97 (C-6); 163.89 (d, ³JC₂ = 3.6 Hz, CO₂-Ar); 161.93 (d, ³JC₂ = 259.1 Hz, C₂'-Ar); 134.49 (d, ³JC₂ = 8.4 Hz, C₄'-Ar); 132.31 (d, ³JC₂ = 0.9 Hz, C₆'-Ar); 124.13 (d, ³JC₂ = 3.6 Hz, C₅'-Ar); 117.17 (d, ²JC = 9.6 Hz, C₁'-Ar); 116.99 (d, ²JC = 21.6 Hz, C₃'-Ar); 75.56 (C-22); 70.33 (C-3); 61.15 (C-23); 56.67 (C-14); 53.85 (C-17); 52.79 (C-9); 52.45 (C-5); 46.78 (C-7); 43.47 (C-13); 42.93 (C-10); 41.41 (C-20); 39.51 (C-12); 38.09 (C-8); 32.55 (C-11); 27.72 (C-16); 25.49 (C-2); 25.20 (C-4); 23.94 (C-15); 21.21 (C-11); 12.73 (C-21); 12.61 (C-19); 12.00 (C-18). ¹⁹F-NMR (470.62 MHz, CDCl₃) δ (ppm) = −108.91 (s, 1F). HRMS (API+) (Figure S19, Supplementary Materials): calculated for C₃₀H₂₄O₅F [M + H⁺] 501.3016, found 501.3015.

Compound 19b was obtained as a colorless solid, m. p. = 75.4 ± 1.5 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S13, Supplementary Materials): δ (ppm) = 7.92 (1H, td, J = 7.6 and 1.8 Hz, HAr-6'); 7.54–7.49 (1H, m, HAr-4'); 7.21 (1H, td, J = 7.6 and 1.2 Hz, HAr-3'); 7.13 (1H, ddd, J = 10.7, 7.6, and 0.9 Hz, HAr-5'); 5.40–5.39 (1H, m, H-3); 3.84–3.76 (1H, m, H-23a); 3.71–3.69 (1H, m, H-23b); 3.51 (1H, t, J = 10.2 Hz, H-22); 2.70 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, J = 13.1 and 4.6 Hz, H-7α); 0.954 (3H, d, J = 6.7 Hz, H-21); 0.777 (3H, s, H-19); 0.681 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S13, Supplementary Materials): δ (ppm) = 212.12 (C-6); 163.89 (d, ³JC₂ = 3.6 Hz, CO₂-Ar); 161.93 (d, ³JC₂ = 259.1 Hz, C₂'-Ar); 134.49 (d, ³JC₂ = 8.4 Hz, C₄'-Ar); 132.31 (d, ³JC₂ = 0.9 Hz, C₆'-Ar); 117.17 (d, ²JC = 9.6 Hz, C₁'-Ar); 116.99 (d, ²JC = 21.6 Hz, C₃'-Ar); 75.56 (C-22); 70.33 (C-3); 61.15 (C-23); 56.67 (C-14); 53.85 (C-17); 52.79 (C-9); 52.45 (C-5); 46.78 (C-7); 43.47 (C-13); 42.93 (C-10); 41.41 (C-20); 39.51 (C-12); 38.09 (C-8); 32.55 (C-11); 27.72 (C-16); 25.49 (C-2); 25.20 (C-4); 23.94 (C-15); 21.21 (C-11); 12.73 (C-21); 12.61 (C-19); 12.00 (C-18). ¹⁹F-NMR (470.62 MHz, CDCl₃) δ (ppm) = −108.91 (s, 1F). HRMS (API+) (Figure S19, Supplementary Materials): calculated for C₃₀H₂₄O₅F [M + H⁺] 501.3016, found 501.3015.

3.2. Biological

Rice Lamina Inclination Test (RLIT)

The biological activity of the growth of the compounds was evaluated by the rice lamina inclination test [55,56], according to a previously described procedure [38], and using the same a Zafiro cultivar (Oryza sativa) provided by the Institute of Agricultural Research (INIA-Quilamapu-Chile) as previous studies.

The seeds were sown and cultivated until the seedlings presenting the second internode of the rice blade were selected for cutting. Six segments per treatment were incubated in Petri dishes containing 60 mL of distilled water, and the amount of test compound (BRs analogs 18a, 18b, 19a, 19b, 20a, and 20b and positive control (1)) needed to reach final concentrations equal to 1 × 10⁻⁸ M; 1 × 10⁻⁷ M; and 1 × 10⁻⁶ M. The negative control only contained sterile distilled water. All treatments were incubated by 48 h at 25 °C in

room temperature for 5 h. The end of the reaction was verified by TLC; then, H₂O (10 mL) and a saturated solution of Na₂S₂O₃·5H₂O (2 mL) were added. The mixture was stirred for another 20 min.
darkness, and the angles developed between the blade and the sheath were measured. Each experiment was performed by duplicate.

Results were expressed as mean ± standard deviation (SD) using twelve angle measurements. Statistical analysis was done using a statistical package Excel by applying mean values using one-way ANOVA with the post-hoc least square differences (LSD) test to determine if there was a significant difference between the positive control and the treatments. A P value of less than 0.05 was considered significant.

4. Conclusions

Brassinosteroid 24-norcholane type analogs conjugated at C-3 and configurations S and R on the C-22 carbon of the side chain have been synthesized and characterized. The synthesis uses hydeoxycholic acid as the starting material, and epimers with different configuration at C-22 are obtained. These epimers have been separated, and their growth-promoting activity was measured using RLIT. The results show that the esterification of BRs analog at C-3 has no effect on the biological activity of synthetic analogs. This suggest that reducing activity by esterification at C-3 requires a long chain carboxylic acid. In addition, the presence of a hydroxyl group at C-3 is not an essential structural feature for activity. This result confirms previous SAR where it has been proposed that activity is not determined by the presence or absence of specific groups in the BR structure.

Supplementary Materials: The following are available online, Figure S1: NMR spectra of 3α,6α-diacetoxy-5β-cholan-24-oic acid (22), Figure S2: NMR spectra of 24-nor-5β-cholan-22-ene-3α,6α-diyli diacetate (23), Figure S3: NMR spectra of 24-nor-5β-chol-22-ene-3α,6α-diol (24), Figure S4: NMR spectra of 6α-hydroxy-24-nor-5β-chol-22-ene-3-one (25), Figure S5: NMR spectra of 24-nor-5β-chol-22-ene-3,6-dione (26), Figure S6: NMR spectra of 3α-hydroxy-24-nor-5β-chol-22-en-6-one (27), Figure S7: NMR spectra of 3α-hydroxy-24-nor-5β-chol-22-en-6-one (28), Figure S8: NMR spectra of 6-oxo-24-nor-5β-chol-22-en-3α-yl 4-methylbenzoate (29), Figure S9: NMR spectra of 6-oxo-24-nor-5β-chol-22-en-3α-yl 2-fluorobenzoate (30), Figure S10: NMR spectra of (22R)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 4-methylbenzoate (18a), Figure S11: NMR spectra of (22S)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 4-methylbenzoate (18b), Figure S12: NMR spectra of (22R)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 2-fluorobenzoate (19a), Figure S13: NMR spectra of (22S)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 2-fluorobenzoate (19b), Figure S14: HRMS (API+) spectrum of 3α-hydroxy-24-nor-5β-chol-22-ene-3-one (28), Figure S15: HRMS (API+) spectrum of 6-oxo-24-nor-5β-chol-22-en-3α-yl 4-methylbenzoate (29), Figure S16: HRMS (API+) spectrum of 6-oxo-24-nor-5β-chol-22-en-3α-yl 2-fluorobenzoate (30), Figure S17: HRMS (API+) spectrum of (22R)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 4-methylbenzoate (18a), Figure S18: HRMS (API+) spectrum of (22S)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 4-methylbenzoate (18b), Figure S19: HRMS (API+) spectrum of (22R)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 2-fluorobenzoate (19a), Figure S20: HRMS (API+) spectrum of (22S)-22,23-dihydroxy-6-oxo-24-nor-5β-chol-3α-yl 2-fluorobenzoate (19b).

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