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Original article

Antiproliferative and quinone reductase-inducing activities of withanolides derivatives

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ABSTRACT

Two new and five known withanolides (jaborosalactones 2, 3, 4, 5, and 24) were isolated from the leaves of *Jaborosa runcinata* Lam. We also obtained some derivatives from jaborosalactone 5, which resulted to be the major isolated metabolite. The natural compounds as well as derivatives were evaluated for their antiproliferative activity and the induction of quinone reductase 1 (QR1; NQ01) activity. Structure–activity relationships revealed valuable information on the pharmacophore of withanolide-type compounds. Three compounds of this series showed significantly higher antiproliferative activity than jaborosalactone 5. The effect of these compounds on the cell cycle was determined. Furthermore, the ability of major compounds to induce QR1 was evaluated. It was found that all the active test compounds are monofunctional inducers that interact with Keap1. The most promising derivatives prepared from jaborosalactone 5 include (23*R*)-4β,12β,21-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**18**) and (23*R*)-21-acetoxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-lactame (**20**).

1. Introduction

Withanolides comprise a group of naturally occurring C28 steroids based on an ergostane skeleton in which C-26 and C-22, or C-26 and C-23, are oxidized in order to form a δ- or γ-lactone. They are known for the diversity of structures involving the steroid nucleus and the side chain, including the formation of additional rings. Their chemistry and occurrence has been the subject of several reviews [1–4]. Among the nearly 100 genera included in Solanaceae, ca. 50% have been investigated [4]. *Jaborosa* is an interesting South American genus growing from southern Peru to Argentina in very diverse habitats [5]. *Jaborosa* Juss. represents one of the four major contributors of withanolide structures.

A family of withanolides spiranic at C-23 has been isolated from *Jaborosa runcinata* Lam., *Jaborosa odonelliana*, and *Jaborosa araucana* [6], and many of these compounds exhibit interesting biological activities such as antiproliferative [7] and cancer chemopreventive properties [8]. In addition, some studies have reported structural modifications of withanolides and structure–activity relationships. Fundamentally, the ring A of withanolides has been modified with various nucleophiles and the derivatives have been evaluated for antiproliferative activity [9,10].

Continuing our search for bioactive compounds, we evaluated the chemical content of *J. runcinata* based on its high content of withanolides. One objective was to obtain enough jaborosalactone 5 to synthesize derivatives and introduce changes at various positions of the molecule. Two new and five previously reported withanolides were isolated. From jaborosalactone 5, a set of derivatives was synthesized, and their antiproliferative and chemopreventive activities were assessed. Preliminary mechanistic studies were performed, and the effects on the cell cycle were

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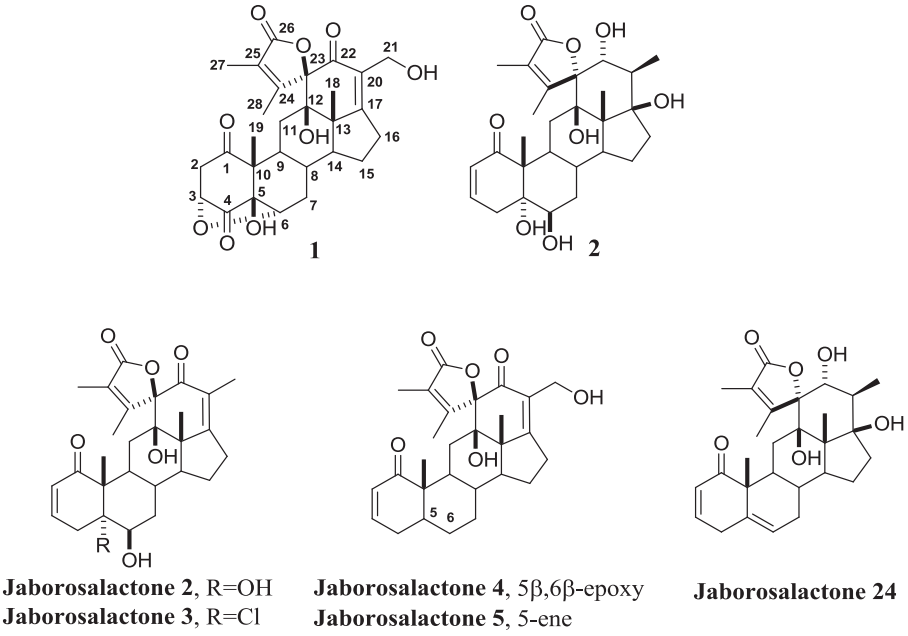


Fig. 1. Structures of isolated withanolides.

examined. With respect to quinone reductase (QR1; NQ01)-inducing compounds, their potential was determined for regulating gene expression through interaction with Keap1 protein.

The antioxidant response element (ARE) controls the expression of cytoprotective enzymes including QR1, UDP-glucuronyl transferases and glutathione *S*-transferases and is regulated, at least in part, by levels of the transcription factor NrF2 [11]. The cytosolic protein Keap1 binds Nrf2 and the enzyme, Cul3, which attaches ubiquitin to Nrf2 thereby marking it for hydrolysis by the proteosome. Ubiquitination of Nrf2 can be reduced or prevented by covalent modification of one or more of the 37 cysteine residues on Keap1 [12]. By blocking ubiquitination of Nrf2, cytosolic levels of Nrf2 increase leading to activation of the ARE. Most ARE inducers acting through modification of Keap1 cysteine sulfhydryl residues are electrophilic in nature and include Michael acceptors and isothiocyanates [13,14]. We have developed a mass spectrometry-based screening assay suitable for the identification of these Keap1 modifying agents [15].

2. Results and discussion

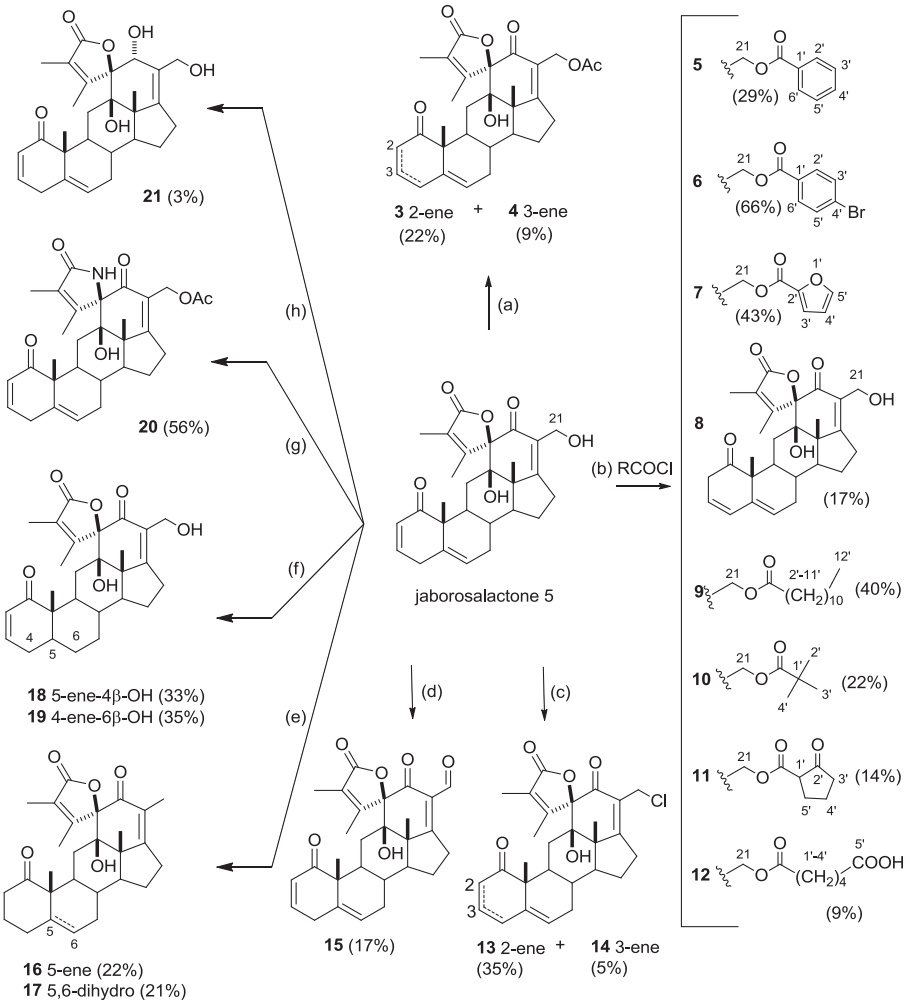
2.1. Chemistry

2.1.1. Natural products

The aerial parts of *J. runcinata* were air-dried and extracted with ethanol. After concentration and defatting, the residue was fractionated by a combination of several chromatographic techniques, ultimately giving two new withanolides, compounds **1** and **2**, and the known spiranic-type withanolides, jaborosalactones 2, 3, 4, 5 and 24 (Fig. 1).

The molecular formula of compound **1** was determined by high resolution electrospray mass spectrometry (HRESIMS) as $C_{28}H_{32}O_9Na$ (*m/z* 535.1946 $[M+Na]^+$). In its ^1H NMR spectrum, the presence of two methyl signals at δ 1.82 and δ 2.16 and the absence of a lactonic hydrogen in the 4–5 ppm region corresponding to the carbynolic hydrogen H-22 indicated a spiranoid dimethyl-substituted α,β-insaturated γ-lactone ring comprising C-23 to C-28. The typical pattern of the spiranoid arrangement was confirmed from the resonances of carbons 23–28; a similar arrangement has been described previously [6]. The absence of a singlet at the high field region of the ^1H NMR spectrum and the appearance of two doublets at 4.15 and 4.24 ppm suggested the presence of an isolated C-21 hydroxymethylene group. The ^{13}C NMR spectrum showed only four methyl groups at 14.6, 17.2, 8.6, and 15.9 ppm corresponding to C-18, C-19, C-27, and C-28, respectively. The methylene signal at 58.4 ppm (C-21) confirmed the presence of a hydroxyl group at C-21. The presence of a tetrasubstituted double bond at C-17-C-20 was ratified by the downfield shift for the signals at δ 166.1 and δ 127.5, respectively. The CH_2-21 signal was correlated with a signal of conjugated carbonyl carbon at 193.0 ppm in the HMBC experiment, confirming a 17(20)-en-22-keto functionality. The configuration at the spiranoid center (C-23*R*) was established by comparison with spectral data of jaborosalactones 1–6 and 25 [6].

Regarding the A/B rings, the ^{13}C NMR spectrum showed two carbonyl groups at δ 209.2 (C-1) and δ 207.4 (C-4), three carbynolic carbons, two signals of methine carbons at δ 74.7 and δ 72.6 (assigned to C-3 and C-6, respectively) and one signal of quaternary carbon at δ 77.6 (C-5). These data provided evidence for an oxygen ether bridge between the A/B rings through the C-3 and C-6 positions. This assumption was supported by the following cross-correlation peaks in the HMBC experiment: between H_3-19 (δ 0.77) and C-5 (δ 77.6) and C-10 (δ 50.6), confirming an oxygenated function at C-5 position; between H_2-2 (δ 3.04 and δ 2.42) and C-1 (δ 209.2), C-3 (δ 74.7), and C-4 (δ 207.4), indicating the presence of an oxygenated methine located at C-3 and two non-conjugated ketone groups located at C-1 and C-4; and finally the weak but diagnostic HMBC correlation observed between H-3 (δ 4.20) with C-6 (δ 72.2) validating C-3/C-6 epoxy functionality. The epoxy group orientation was established by analysis of the coupling constants of H_2-2, H-3, and H-6 and confirmed by comparison with spectral data of subtrifloralactone K, withanolide reported from *Deprea subtriflora* having the same A/B ring substitution pattern [16]. Compound **1** (jaborosalactone 47) was finally established as (23*R*)-3α,6α-epoxy-5β,12β,21-trihydroxy-1,4,22-trioxo-12,23-cycloergostan-17,24-dien-26,23-olide.



Scheme 1. Withanolide derivatives obtained from jaborosalactone 5 (**3**–**21**). [a]General reagents and conditions: (a) Ac_2O, pyridine, DMAP; (b) RCOCl, $(C_2H_5)_3N$, DMAP, CH_2Cl_2; (c) $SOCl_2$, CH_2Cl_2, N_2, Δ; (d) PCC, CH_2Cl_2; (e) H_2, 10% Pd/C, THF; (f) SeO_2, CH_2Cl_2, Ar, Δ; (g) NH_4OAc/AcOH, Δ; (h) $LiAlH_4$, THF-Et_2O, Δ.

The HRESIMS of compound **2** showed a sodium adduct $[M+Na]^+$ at *m/z* 525.2469 corresponding to a formula of $C_{28}H_{38}O_8Na$. The ^1H and ^{13}C NMR of **2** showed a typical chemical shift for C23–C28 of a C-23-spiranoid γ-lactone, such as compound **1**. Despite these similarities, marked differences can be found for A/B rings; the ^1H NMR spectrum of compound **2** exhibited signals at δ 5.76 and δ 6.49 typical of a 2-en-1-one system without substituent at C-4. These chemical shifts, plus the presence of a carbonyl carbon signal at δ 203.4 and two methine carbon signals at δ 128.5 and 141.1 in the ^{13}C NMR spectrum are in agreement with a conjugated double bond in ring A. The downfield shift of H-6, which appeared at δ 3.60 as a broad singlet, revealed a small coupling constant with H_2-7, evidencing an equatorial orientation (α) of the hydrogen H-6. The presence of two carbons at δ 77.2 (C-5) and δ 74.2 (C-6) in the ^{13}C NMR spectrum were consistent with a 5α,6β-diol substitution typical of many withanolides [17]. The presence of five methyl groups in the ^1H NMR spectrum and the doublet signal at δ 1.11 assigned to H_3-21 confirmed the absence of a hydroxyl group at C-20. The correlation observed in the COSY experiment between δ 1.11 (H_3-21), δ 2.03 (H-20) and δ 4.01 (H-22), and the absence of carbonyl carbon corresponding to C-22, suggested a hydroxyl functionality at C-22, which was assigned in the ^{13}C NMR at δ 69.2. This assumption was confirmed for HMBC correlations between H-22 (δ 4.01) and C-20 (δ 40.1) and C-21 (δ 10.7). The α-orientation of OH-22 was determined by the nOe observed between H-22 (δ 4.01) and H_3-18 (δ 1.03). The signals of two oxidized quaternary carbons at 79.1 and 83.7 ppm were assigned to C-12 and C-17, respectively. The assignment of C-23*R* configuration was carried out by analysis of NOESY spectra, which showed strong correlation between H-22 and H_3-28 (δ 2.08). The structure of compound **2** (jaborosalactone 48) was finally established as (20*R*,23*R*)-5α,6β,12β,17β,22α-pentahydroxy-1-oxo-12,23-cycloergostan-2,24-dien-26,23-olide.

2.1.2. Derivatization of jaborosalactone 5

An considerable amount (482 mg) of jaborosalactone 5 was isolated from *J. runcinata*. The structural arrangement of jaborosalactone 5 is closely related to those of jaborosalactone P isolated from *J. odonelliana*, one of the most promising inducers of QR1 in terms of potency and low toxicity *in vitro* and *in vivo* [8]. On the other hand, compounds with the same skeleton have been evaluated as antiproliferative agents against tumor cell lines. In view of the activity reported by the spiranoid at C-23 withanolides and the amount of jaborosalactone 5 isolated, a set of derivatives (**3**–**21**, Scheme 1) was prepared. Chemical modifications were introduced at the C-21 hydroxyl group, the double bonds in the A/B rings, the lactone of the side chain, and carbonyl groups.

Compounds **3** and **4** were obtained by acetylation of jaborosalactone 5 with Ac_2O/Py, while compounds **5**–**12** were prepared by acylation with several acyl chlorides of different size, lipophilicity and stereoelectronic properties. The reaction of

jaborosolactone 5 with $SOCl_2$ afforded the chlorinated compounds **13** (35%) and **14** (5%) while the oxidation of the hydroxyl group at C-21 with PCC yielded the aldehyde **15** (17%). Compounds **16** (22%) and **17** (21%) were obtained by hydrogenation of jaborosalactone 5 in the presence of 10% Pd/C. The treatment with SeO_2 gave the derivatives with a hydroxyl group at C-4 **18** (33%) and at C-6 **19** (35%). The β orientations of the corresponding hydroxyl groups were determined on the basis of the signal splitting and the coupling constant value of the proton on C-4 or C-6.

Jaborosalactone 4 was obtained in low yield (18%) by epoxidation with MCPBA. Transformation of the lactone into lactam was made by using ammonium acetate in acetic acid. Thus, compound **20** which also presented an acetate group at C-21 was obtained in 56% yield. When jaborosalactone 5 was treated with $LiAlH_4$ the carbonyl at C-22 was chemoselectively reduced to compound **21** (3%).

2.2. Biological activity

2.2.1. Antiproliferative activity

The *in vitro* antiproliferative activities of natural jaborosalactones 2, 3, 4, 5, 24, 47 (**1**), and 48 (**2**), and semi-synthetic derivatives (**3**–**7**, **9**–**13**, **15**, **16**–**21**) were evaluated using the well-established protocol of the National Cancer Institute (NCI) of the United States [18]. As models, the representative panel of human solid tumor cell lines HBL-100 (breast), HeLa (cervix), SW1573 (non-small-cell lung), and WiDr (colon) was used. Table 1 summarizes the results as 50% growth inhibition (GI_{50}).

Viewed as a whole, these results allowed classifying the compounds into three groups. A first group, comprising jaborosalactone 3 and derivatives **5**, **7**, and **13**, caused significant growth inhibition with GI_{50} values below 10 μM in most cell lines. The second set of compounds, consisting of jaborosalactones 4 and 5 and derivatives **3**, **4**, **6**, **9**–**11**, **15**–**18**, **20**, and **21**, exhibited moderate activity. The third group, consisting of jaborosalactones 2, 24, 47 (**1**), and 48 (**2**) and derivative **19**, failed to show growth inhibition.

The influence of the substitution pattern on the activity of the withanolides tested in this work was examined. Overall, withanolides spiranic at C-23 showed moderate antiproliferative activity. A few trends were apparent in the structure–activity relationships (SAR) at the side chain. i) If one compares the activity of jaborosalactone 5 to that of the ester derivatives at C-21 position, for most of the esters obtained, the activity increased (**3**, **5**–**7**, **9**–**11**), except for adipoyl ester (**12**), which showed less activity than its precursor, jaborosalactone 5. Concerning the activity showed that compounds with aromatic substituents (**5**–**7**) are significantly more active than compounds with aliphatic substituents (**3**,**9**,**10**). ii) No change of activity was observed when the lactone was replaced by the lactame functionality at the side chain (**3** *vs* **20**). iii) Compound **13** (GI_{50} 2.1–16 μM) was the most active product, in which the hydroxyl group at C-21 position was replaced by chlorine. iv) The presence of a 17,22-dihydroxy-2-methyl system proved deleterious for activity, regardless of the A/B ring substituent types [jaborosalactones 24 and 48 (**2**)].

The A/B rings also appeared to play a role in determining activity. i) For compounds with 17 (20)-ene-22-keto-21-hydroxy system, similar and moderate activity values for 2,5-diene (jaborosalactone 5), 2,5-diene-4β-hydroxy (**18**) and 2-ene-5β,6β-epoxy (jaborosalactone 4) compounds were observed. Lower activities were observed for the 2,4-diene-6β-hydroxy derivative (**19**) and the 1,4-dioxo-5β,hydroxyl-3α,6α-epoxy compound (**1**). ii) For compounds with a methyl group at the C-21 position, moderate activity for 1-oxo-5α-chloro,6β-hydroxy compound (jaborosalactone 3) was observed, while 1-oxo-2-en-5α,6β-dihydroxy (jaborosalactone 2), 1-oxo-5-ene (**16**), and a compound with totally saturated A/B rings (**17**) showed weak or no activity. These considerations, based upon substitution patterns, were partly consistent with previous SAR findings [7], and otherwise provide valuable information on the pharmacophore of withanolide-type compounds and will be helpful for the rational design of more potent and selective anticancer drugs.

Table 1
In vitro antiproliferative activity of withanolides and analogues against human solid tumor cell lines.[a]

Compound	HBL-100	HeLa	SW1573	WiDr
Jaborosalactone 2	>100	76 ± 34	90 ± 14	>100
Jaborosalactone 3	2.9 ± 0.7	11 ± 2.2	9.8 ± 1.0	16 ± 6.2
Jaborosalactone 4	33 ± 2.5	22 ± 2.8	31 ± 3.1	40 ± 0.2
Jaborosalactone 5	38 ± 4.9	49 ± 15	42 ± 6.3	50 ± 16
Jaborosalactone 24	>100	>100	>100	>100
Jabrosalactone 47 (**1**)	82 ± 26	41 ± 5.7	72 ± 12	>100
Jabrosalactone 48 (**2**)	>100	>100	>100	>100
3	31 ± 4.3	19 ± 0.8	32 ± 3.3	51 ± 8.3
4	27 ± 8.3	20 ± 1.0	34 ± 3.9	90 ± 14
5	3.5 ± 0.3	2.40 ± 0.01	3.5 ± 0.6	13 ± 7.4
6	4.4 ± 1.8	13 ± 3.4	14 ± 0.3	41 ± 10
7	3.7 ± 0.2	4.5 ± 2.3	4.0 ± 0.4	34 ± 3.6
9	18 ± 0.9	17 ± 1.7	16 ± 0.8	29 ± 1.6
10	19 ± 2.8	12 ± 2.8	23 ± 7.5	31 ± 2.1
11	26 ± 3.2	16 ± 0.6	30 ± 6.0	41 ± 10
12	60 ± 5.5	26 ± 0.5	58 ± 12	>100
13	2.9 ± 0.7	2.1 ± 0.1	3.1 ± 0.4	16 ± 6.2
15	13 ± 4.9	8.6 ± 2.4	16 ± 2.7	26 ± 0.1
16	53 ± 10	>100	50 ± 17	96 ± 5.3
17	43 ± 20	>100	39 ± 13	92 ± 11
18	39 ± 5.8	77 ± 32	37 ± 7.9	77 ± 32
19	>100	>100	>100	>100
20	28 ± 6.5	18 ± 0.7	31 ± 3.8	50 ± 7.4
21	69 ± 4.3	52 ± 6.0	56 ± 12	29 ± 20

[a] Expressed as GI_{50} values given in μM and determined as means of two to five experiments, standard deviations are given in parentheses.

2.2.2. Cell cycle disturbances

When cells are exposed to cytotoxic agents, damaged cells may suffer a block in their cell cycle. If the cell cannot recover from the damage, cell death results from apoptosis. This blockage in cell division is known as cell cycle arrest and can take place at any of the cell cycle phases, namely G_0/G_1, S, or G_2/M phase. The cell cycle phase distribution by flow cytometry was studied to determine whether cell growth inhibition involved cell cycle changes. Three compounds from this series (**5**, **7**, and **13**) exhibited potent antiproliferative effects on tumor cells and were selected accordingly for cell cycle studies.

Cells were exposed to each agent at two different drug concentrations (high and low), which were chosen based on two premises [19]: The GI_{50} values of the compounds (Table 1), and the sensitivity of the cell line to drug treatment, since at higher drug doses cell death prevents examination of the cell cycle phase distribution. The most sensitive cells (HBL-100, HeLa and SW1573 cell lines) were exposed to 5 (low) and 10 μM (high), whilst the drug-resistant WiDr cells were treated with 15 (low) and 30 μM (high). Control cells were incubated in the absence of test drug.

The results varied according to the cell line tested (Fig. 2). In general, test compounds altered the cell cycle with a decrease in the G_1 phase, which was concomitant with an increase in the S or G_2/M compartment. Significant cellular death was observed for the HBL-100 (7–23%) and HeLa (15–35%) cell lines after exposure to the compounds. In contrast, reduced cell death was detected in SW1573 (5–15%) and WiDr (7–12%) treated cells. In HBL-100 and WiDr cells, there was an accumulation in the G_2/M phase whilst for HeLa and SW1573 cell lines, accumulation was observed in the S phase of the cell cycle. These differences cannot be explained in

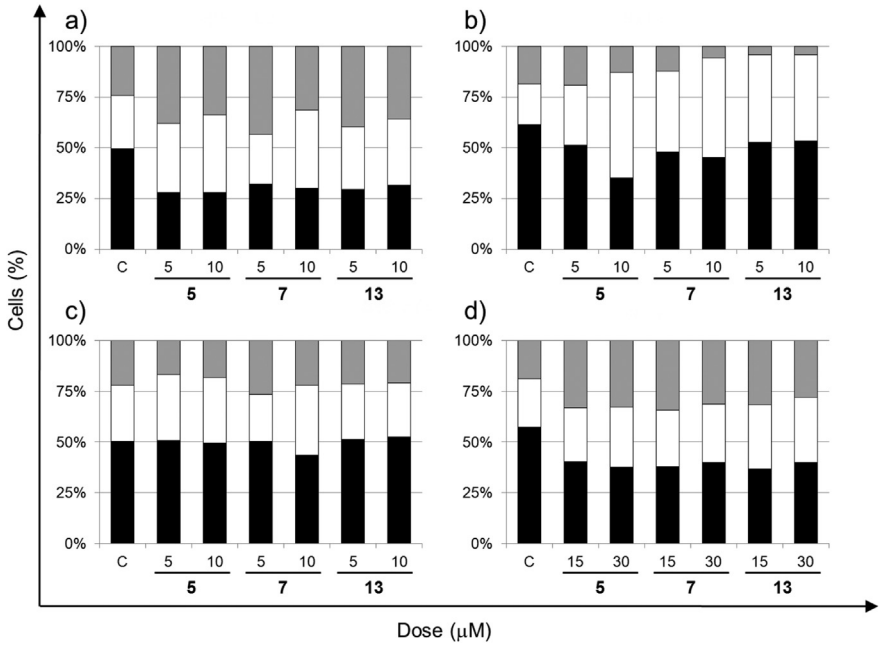


Fig. 2. Cell cycle phase distribution in untreated cells (C) and cells treated for 24 h with compounds **5**, **7** and **13** at low and high dose concentrations. a) HBL-100; b) HeLa; c) SW1573; d) WiDr; Areas: black = G_0/G_1, white = S, gray = G_2/M.

terms of GI_{50} values. Although preliminary, these results suggest that derivatives **5**, **7**, and **13** might induce cell death in these cell lines by different pathways.

2.2.3. Induction of quinone reductase 1 (QR1)

QR1 is a flavoprotein which catalyzes two- or four-electron reduction of a wide variety of quinones, quinone imines, and other nitrogenous compounds. This enzyme has been demonstrated to reduce oxidative stress, particularly in the context of chemoprevention of carcinogenesis [20]. An MTT assay with Hepa 1c1c7 murine hepatoma cells was employed to determine the expression of QR1 following treatment with 13 compounds included in this study. An identical assay in mutant Hepa cells was used to determine whether the observed induction was monofunctional (acting through activation of the ARE only) or bifunctional (acting through activation of both phase I and phase II enzymes through alternate pathways). TAOc1 and BP^rc1 mutant cells are defective in a functional *Ah* receptor or unable to translocate the receptor–ligand complex to the nucleus, respectively. Induction of QR1 seen in these two cell lines indicates the sample does not work through the XRE pathway.

Ten compounds out of the 13 investigated were found to induce QR1 with an induction ratio (IR) greater than 2.0. Some compounds did not demonstrate induction at the highest concentration tested (20 μg/ml) due to cytotoxicity, yet they were seen to significantly induce QR1 without killing cells at lower concentrations (e.g., jaborosalactone 3). The most active compound was **7**, with a CD (concentration required to double QR1 induction) of 0.59 μM. This substance was also found to be toxic to cultured cells (Table 1). All 10 active compounds also modulated QR1 activity in both mutant cell lines. These data indicate that the compounds in this study induce QR1 monofunctionally, and therefore up-regulate the ARE, with moderate potency. Therefore, they are promising as cancer chemopreventive agents.

Jaborosalactones 3, 5, and 24, and derivatives **7**, **15**, **18**, and **20** were promising inducers of QR1 activity with CI (IC_{50}/CD) values of 9.21, 16.85, >6.78, 13.64, 8.11, >20.4, and 22.93 respectively. Jaborosalactones 2 and 4 and derivative **13** failed to show QR1 induction, and three additional compounds, **6**, **9**, and **19**, exhibited weak activity, with CD values of 16.1, 21.0, and 31.7 μM, respectively. The current results indicate that the substitution pattern in A/B rings is relevant for the activity according to the following observations. i) The most active compounds have a 1-oxo-2,5-diene system (**7** and **20**). ii) When 5-ene is replaced by a 5β,6β-epoxy system, activity decreases (jaborosalactone 5 *vs* jaborosalactone). iii) Similarly, when 5α-chloro,6β-hydroxy is replaced by a 5α,6β-dihydroxy system, the activity disappears (jaborosalactone 3 *vs* jaborosalactone). The low activity observed in compounds with α-oxygenated substituents at C-5 has been reported previously for several withanolides [8]. Regarding the side chain, the presence of oxygenated functions at C-21 positively contributed to inducing activity. However, highly hydrophobic and/or halogen substituents show a decrease in activity. Jaborosalactone 24 is a C-23 spiranoid withanolide with a 17,22-dihydroxy system. This compound has the same skeleton and substitution patterns as those of jaborosalactone P; they only differ in their configuration at C-23. Despite their close structural similarity, these compounds have shown different inducing activity for QR1, with CD values of 6.29 μM and 0.75 μM for jaborosalactone 24 and jaborosalactone P [8], respectively. These results suggest that the configuration of the carbon spiranic is relevant for inducing activity.

Overall, the compounds demonstrating the most promising profiles of activity are **18** and **20**. The compounds are not significantly cytotoxic (Table 1) and CD values are relatively low (2.04 and 0.82 μM, respectively) (Table 2), yielding relatively high CI values (>20) (Table 2). The compounds are monofunctional inducers (Table 2), presumably working through interaction with Keap1 (see below).

2.2.4. Keap1 modification by withanolides

The induction of enzymes such as QR, glucuronyl transferases, glutathione *S*-transferases, and sulfotransferases can protect cells against the toxic and neoplastic effects of carcinogens. An increase in the concentration of Nrf2 in the nucleus of a cell up-regulates the ARE and induces the expression of these chemopreventive enzymes. Based on the hypothesis that ubiquitination and

Table 2
Concentration required to double induction of QR1 (CD, μM) and chemopreventive index (CI; IC_{50}/CD) for withanolides isolated from *Jaborosa runcinata* and derivatives in wild-type Hepa 1c1c7 cells and two mutant cell lines, TAOc1 and $BP^{r}c1$. Sulforaphane was used as a monofunctional control, and 4′-bromoflavone was used as a control in wild-type cells.

Compound	Hepa 1c1c7			TAOc1	$BP^{r}c1$
	CD (μM)	IC_{50} (μM)	CI (IC_{50}/CD)	CD (μM)	CD (μM)
Jaborosalactone 2	N/A	–	N/A	N/A	N/A
Jaborosalactone 3	1.26 ± 0.14	11.57	9.21	0.96 ± 0.16	1.44 ± 0.16
Jaborosalactone 4	N/A	–	N/A	N/A	N/A
Jaborosalactone 5	2.39 ± 0.90	40.3	16.85	2.52 ± 0.75	2.75 ± 0.30
Jaborosalactone 24	6.29 ± 0.79	>42.7	>6.78	9.62 ± 1.98	12.46 ± 0.64
6	16.1 ± 1.82	>30.9	>1.92	26.50 ± 2.69	25.99 ± 2.56
7	0.59 ± 0.19	8.1	13.64	1.66 ± 0.69	2.34 ± 0.95
9	21.00 ± 1.92	24.6	1.17	9.03 ± 1.34	24.40 ± 1.87
13	N/A	–	N/A	N/A	N/A
15	3.55 ± 1.32	28.8	8.11	4.15 ± 0.39	5.32 ± 0.56
18	2.04 ± 0.29	>41.6	>20.41	0.69 ± 0.13	3.22 ± 0.92
19	31.7 ± 3.04	>41.6	>1.31	29.11 ± 2.16	31.05 ± 1.91
20	0.82 ± 0.18	18.6	22.93	0.91 ± 0.22	1.13 ± 0.32
4′-Bromoflavone	0.012 ± 0.005	>66.4	>5197	>66.4	>66.4
Sulforaphane	7.87 ± 0.43	>170	>21.65	8.95 ± 0.26	15.09 ± 0.71

proteosome-mediated degradation of Nrf2 in the cytoplasm decrease upon the covalent modification of 1 or more of the 27 cysteine sulfhydryl groups on Keap1 (a protein that sequesters Nrf2 in the cytoplasm), resulting in higher Nrf2 levels both in the cytoplasm and in the nucleus, a high-throughput mass spectrometry-based screening assay was used to detect covalent modification of sulfhydryl groups of human Keap1.

Unmodified Keap1 (negative control) was observed at *m/z* 70 998, and this mass spectrum was compared with that of each withanolide after incubation with Keap1 (Fig. 3 and Table 3). After incubation with the positive control isoliquiritigenin (a chalcone containing an α,β-unsaturated ketone), the average mass of Keap1 increased to *m/z* 71 184. Isoliquiritigenin is known to react with Keap1 cysteines, especially C151 [21]. Table 3 summarizes the MALDI mass spectra of Keap1 after incubation with each withanolide derivative. It should be noted that mass spectrometry-based assays do not suffer from interferences such as cytotoxicity that are common to cell-based assays.

After incubation with each withanolide, the mass of Keap1 increased due to covalent attachment of one or more of these electrophilic molecules. Since Keap1 contains 27 reduced cysteine residues, covalent attachment of multiple withanolide molecules to Keap1 is possible. For example, the MALDI mass spectra in Fig. 3 indicate that an average of 4 molecules of **18** or **20** bound to Keap1. Studies are in progress to identify the sites of covalent attachment of each compound. In conclusion, all of the assayed compounds contain electrophilic groups that reacted with Keap1 (Table 3).

3. Conclusions

In the present study, seven natural withanolides were isolated from *J. runcinata* and 20 derivatives, including jaborosalactone 4, were synthesized from jaborosalactone 5. Previous studies fundamentally changed withanolide A/B rings; this research aimed at introducing variability to the side chain, functionalizing the C-21 position with different residues (aliphatic, aromatic and halogenated), and replacing the side-chain lactone with a lactam function. Regarding antiproliferative activity, previous research has determined that the most active compounds are those with a 1-oxo-2-ene-5β,6β-epoxy system or a 1-oxo-3β-XR system on A/B rings. Here, the most active derivatives (**5**, **7**, and **13**) show a 1-oxo-2,5-diene system in the A/B rings. In addition, if we take the antiproliferative activity of the natural precursor (jaborosalactone 5) as a reference, activity is largely increased by replacing the hydroxyl group at the C-21 position by a chlorine atom, or by esterification with furanoic or benzoic acid.

Cell cycle studies of the most potent compounds indicated that cell death is occurring and its mechanism may vary depending on the cell line. Although the mechanism of growth alteration at the cellular level is still unclear, the biological activity and structure–activity relationships reported provide the basis for further studies, especially in relation to the search for new derivatives with enhanced antiproliferative activities.

All of the withanolides reacted with Keap1, probably due to their α,β-unsaturated ketone functional groups. To differentiate their activities in terms of up-regulating the ARE, cell-based function assays were carried out using QR1 as a marker enzyme. We observed, once more, that changes in the side chain result in drastic changes in the activity; the most notable result being the difference in the inducing activity between jaborosalactone P and jaborosalactone 24, compounds which only differ in their configuration at the C-23 position. We demonstrated that all those derivatives which acted as QR1 inducers contained electrophilic groups that react with Keap1. Overall, the most favorable profiles of activity were observed with compounds **18** and **20**.

Based on all these results, the preparation of new withanolide derivatives as valuable chemopreventive agents is in progress.

4. Experimental

4.1. General experimental procedures

Melting points were measured on a mercury thermometer apparatus and are uncorrected. Optical rotations were measured on JASCO P-1010 and Perkin-Elmer-343 polarimeters. UV spectra were obtained using Shimadzu-260 and Jasco V-560 spectrophotometers. IR spectra were obtained in a Nicolet 5-SXC and Bruker IFS 55-FTIR spectrophotometers. NMR experiments were performed on Bruker AVANCE II 400 MHz and AMX 500 MHz instruments. Multiplicity determinations (DEPT) and 2D spectra (COSY, HSQC, HMBC, and NOESY) were obtained using standard Bruker software. Chemical shifts are expressed in ppm (δ) units using tetramethylsilane as the standard. ESIMS and HRESIMS were measured on an LCT Premier XE Micromass (Manchester, UK) mass spectrometer. Positive ion MALDI-TOF mass spectra were acquired using an Applied Biosystems (Foster, CA) Voyager DE-PRO time-of-flight mass spectrometer. Chromatographic separations were performed

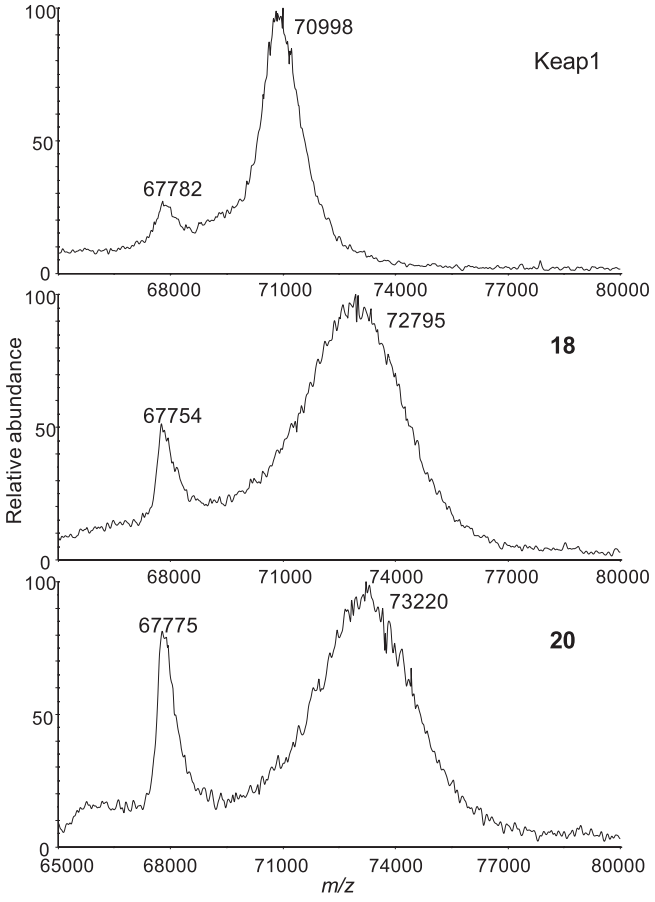


Fig. 3. Positive ion MALDI time-of-flight mass spectra of cysteine-rich Keap1 after incubation with methanol (negative control; top) or withanolides **18** and **20**. Note that the average mass of Keap1 increased due to covalent attachment of multiple withanolide molecules.

by molecular exclusion chromatography (Sephadex LH-20), column chromatography on silica gel 60 (0.063–0.200 mm), and preparative TLC on silica gel 60 F_{254} (0.2 mm thick) plates.

4.2. Plant material

A voucher specimen of *J. runcinata* is deposited at the herbarium of Museo Botánico Córdoba (CORD), Universidad Nacional de Córdoba. *J. runcinata* was collected in Departamento Paraná, Entre Ríos, Argentina, in December 2008 (Barboza et al. 2129).

Table 3
Covalent modification of Keap1 by electrophile withanolides measured using positive ion MALDI time-of-flight mass spectrometry.

Compound	*m/z*[a]	ΔM[b]
Jaborosalactone 2	71 289	291
Jaborosalactone 3	72 097	1099
Jaborosalactone 4	72 350	1352
Jaborosalactone 5	71 807	809
Jaborosalactone 24	72 212	1214
6	71 455	457
7	73 069	2071
9	73 095	2097
13	75 087	4089
15	73 131	2133
18	72 945	1947
19	71 824	826
20	73 220	2222
Isoliquiritigenin, positive control	71 184	186
Keap1, negative control	70 998	–

[a] Mass was measured after 2 h incubation with Keap1 at room temperature.
[b] Change in mass relative to Keap1 control.

4.3. Extraction and isolation

The air-dried powdered aerial parts of *J. runcinata* (233 g) were exhaustively extracted with EtOH (3 × 500 mL) and the solvent was evaporated at reduced pressure. The residue was defatted by partition in hexane-EtOH-H_2O (10:3:1), the resultant EtOH–H_2O phase was washed with hexane (3 × 100 mL), and EtOH evaporated at reduced pressure. The residue was diluted with H_2O and extracted with CH_2Cl_2 (5 × 300 mL). The CH_2Cl_2 extract was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness at reduced pressure. Finally, 3.49 g of crude extract was obtained.

The residue was fractionated initially by molecular exclusion chromatography. Elution with MeOH afforded 15 fractions. Fractions 7–8 (850 mg) were column chromatographed over silica gel 60 G using hexane:EtOAc and EtOAc:MeOH mixtures of increasing polarity. Jaborosalactone 5 (482 mg) was precipitated from fractions corresponding to Hexane:EtOAc (4:6 to 2:8) elution. Remaining fractions were regrouped and chromatographed on silica gel 60 G, using CH_2Cl_2:MeOH (10:0 to 8:2) as elution solvent mixture. The collected eluates were pooled in eleven fractions containing withanolides which were then purified. Fractions III-IV were purified by CC with hexane:EtOAc and EtOAc:MeOH mixtures of increasing polarity, yielding jaborosalactone 4 (28.2 mg). The remaining fractions were purified by preparative TLC (EtOAc:hexane, 8:2). 39.5 mg of crystallized jaborosalactone 24 was obtained from fraction V; fraction VI yielded 2.5 mg of compound **1** and fraction VII, 6.0 mg of compound **2**. Jaborosalactone 2 (30 mg) and jaborosalactone 3 (16 mg) were isolated from fractions VIII-IX. The purification of fractions X-XI allowed isolation of 35 mg of jaborosalactone 24.

4.3.1. Jaborosalactone 47 (23R)-3α,6α-epoxy-5β,12β,21-trihydroxy-1,4,22-trioxo-12,23-cycloergostan-17,24-dien-26,23-olide (*1*)

White amorphous solid; $[\alpha]_{21}D$: −82.7 (c 0.15, MeOH). UV (MeOH) λmax (log ε): 221.3 (4.12) nm. IR (film) ν_{max}: 3454, 2957, 2920, 1756, 1677, 1119, 1009, 757 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 4.24 d (1H, J = 12.7 Hz, H-21a), 4.20 brd (1H, J = 4.4 Hz, H-3), 4.15 d (1H, J = 12.7 Hz, H-21b), 3.97 d (1H, J = 3.9 Hz, H-6), 3.04 dd (1H, J = 20.0,4.4 Hz, H-2a), 2.55 m (2H, H_2-16), 2.42 dd (1H, J = 20.0, 1.3 Hz, H-2b), 2.31 m (1H, H-14), 2.16 s (3H, H_3-28), 1.817 s (3H, H_3-27), 1.815 m (1H, H-11α), 1.79 m (1H, H-7β), 1.75 m (1H, H-15α), 1.73 m (1H, H-9), 1.728 m (1H, H-11β), 1.61 m (1H, H-7α), 1.60 m (1H, H-8), 1.58 m (1H, H-15β), 1.03 s (3H, H_3-18), 0.77 s (3H, H_3-19). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 209.2 (C, C-1), 207.4 (C, C-4), 193.0 (C, C-22), 172.3 (C, C-26), 166.1 (C, C-17), 161.1 (C, C-24), 127.5 (C, C-20), 127.2 (C, C-25), 91.2 (C, C-23), 77.6 (C, C-5), 74.7 (CH, C-3), 74.5 (C, C-12), 72.2 (CH, C-6), 58.4 (CH_2, C-21), 50.6 (C, C-10), 48.9 (C, C-13), 45.8 (CH, C-14), 42.5 (CH, C-9), 39.5 (CH_2, C-2), 32.1 (CH_2, C-11), 31.2 (CH, C-8), 29.7 (CH_2, C-7), 25.1 (CH_2, C-16), 22.7 (CH_2, C-15), 17.2 (CH_3, C-19), 15.9 (CH_3, C-28), 14.6 (CH_3, C-18), 8.6 (CH_3, C-27). HRESIMS *m/z* 535.1946 $[M+Na]^+$ (calcd for $C_{28}H_{32}O_9Na$, 535.1944).

4.3.2. Jaborosalactone 48 (20R,22R,23R)-5α,6β,12β,17β,22-pentahydroxy-1-oxo-12,23-cicloergostan-2,24-dien-26,23-olide (*2*)

White amorphous solid; $[\alpha]_{21}D$: −70.8 (c 0.24; MeOH), UV (MeOH) λmax (log ε): 307.3 (3.69), 220 (4.27) nm. IR (film) ν_{max}: 3418, 2966, 2930, 1738, 1668, 1387, 994, 656 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.49 m (1H, H-3), 5.76 dd (1H, J = 10.0, 2.4 Hz, H-2), 4.01 d (1H, J = 11.2 Hz, H-22), 3.60 brs (1H, H-6), 3.15 brd (1H, J = 19.0 Hz, H-4β), 2.47 m (1H, H-14), 2.34 dd (1H, J = 13.7,4.0 Hz, H-11α), 2.19 m (1H, H-9), 2.08 s (3H, H_3-28), 2.03 m (1H, H-4α), 2.03 m (1H, H-20), 1.97 m (1H, H-16α), 1.96 s (3H, H_3-27), 1.80 m (1H, H-8), 1.73 m (1H,

H-7β), 1.52 m (2H, H_2-15), 1.502 m (1H, H-7α), 1.504 m (1H, H-16β), 1.23 m (1H, H-11β), 1.19 s (3H, H_3-19), 1.11 d (3H, J = 6.6 Hz, H-21), 1.03 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 203.4 (C, C-1), 174.5 (C, C-26), 162.7 (C, C-24), 141.1 (CH, C-3), 128.5 (CH, C-2), 127.3 (C, C-25), 94.1 (C, C-23), 83.7 (C, C-17), 79.1 (C, C-12), 77.2 (C, C-5), 74.2 (CH, C-6), 69.2 (CH, C-22), 52.0 (C, C-10), 50.5 (C, C-13), 43.3 (CH, C-14), 40.1 (CH, C-20), 38.2 (CH, C-9), 35.2 (CH_2, C-4), 34.8 (CH_2, C-16), 32.8 (CH_2, C-7), 32.2 (CH_2, C-11), 29.5 (CH, C-8), 23.4 (CH_2, C-15), 15.3 (CH_3, C-28), 14.5 (CH_3, C-19), 12.1 (CH_3, C-18), 10.7 (CH_3, C-21), 8.8 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 525.2469 (calcd for $C_{28}H_{38}O_8Na$, 525.2464).

4.4. Preparation of jaborosalactone 5 derivatives

4.4.1. Preparation of (23R)-21-acetyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (3) and (23R)-21-acetoxy-12β-hydroxy-1,22-dioxo-12,23-cicloergostan-3,5,17,24-tetraen-26,23-olide (4)

DMAP in catalytic amount and excess of acetic anhydride (1 mL, 0.0106 mol) were added to a solution of jaborosalactone 5 (14.3 mg, 0.031 mmol) in pyridine (100 μL). The reaction mixture was stirred for 24 h at room temperature. The reaction mixture was concentrated and the residue was subjected to preparative TLC purification using EtOAc to yield 3.5 mg of compound **3** (22.4%) and 1.4 mg of compound **4** (9%).

4.4.1.1. (23R)-21-acetyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (3). White amorphous solid; $[\alpha]_{21}D$: −86.2 (c 0.23, MeOH), UV (MeOH) λmax (log ε): 209 (3.77) nm., IR (film) ν_{max}: 3364, 2960, 2922, 1737, 1670, 1237, 1010, 542, 515 cm^{-1}. ^{1}H NMR ($CDCl_3$. 400.13 MHz): 6.67 ddd (1H, J = 10.0, 5.0, 2.5 Hz, H-3), 5.75 dd (1H, J = 10.0,2.5 Hz, H-2), 5.48 brd (1H, J = 5.9 Hz, H-6), 4.72 d (1H, J = 12.2 Hz, H-21a), 4.64 d (1H, J = 12.2 Hz, H-21b), 3.19 brd (1H, J = 21.0 Hz, H-4β), 2.78 dd (1H, J = 21.0,5.0 Hz, H-4α), 2.59 m (2H, H_2-16), 2.43 m (1H, J = 14.0, 3.6 Hz, H-11α), 2.35 m (1H, H-14), 2.20 s (3H, H_3-28), 1.96 s (3H, H_3-27), 1.96 m (1H, H-7β), 1.95 s, (3H, CH_3COO-21), 1.80 m (1H, H-15α), 1.80 m (1H, H-9), 1.67 m (1H, H-7α), 1.63 m (1H, H-15β), 1.45 m (1H, H-8), 1.45 m (1H, H-11β), 1.09 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 203.0 (C, C-1), 191.6 (C, C-22), 172.9 (C, C-26), 171.0 (C, CH_3COO-21), 168.7 (C, C-17), 159.5 (C, C-24), 145.0 (CH, C-3), 135.3 (C, C-5), 128.4 (C, C-25), 127.7 (CH, C-2), 124.1 (CH, C-6), 123.5 (C, C-20), 91.1 (C, C-23), 75.6 (C, C-12), 58.4 (CH_2, C-21), 49.8 (C, C-10), 48.9 (C, C-13), 46.7 (CH, C-14), 40.7 (CH, C-9), 35.1 (CH_2, C-11), 33.3 (CH_2, C-4), 32.0 (CH, C-8), 29.1 (CH_2, C-7), 25.7 (CH_2, C-16), 23.3 (CH_2, C-15), 20.9, (CH_3, CH_3CO-21), 18.4 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 529.2219 (calcd for $C_{30}H_{34}O_7Na$, 529.2226).

4.4.1.2. (23R)-21-acetyloxy-12β-hydroxy-1,22-dioxo-12,23-cicloergostan-3,5,17,24-tetraen-26,23-olido (4). White amorphous solid; $[\alpha]_{21}D$: −51.4 (c 0.09, MeOH), UV (MeOH) λmax (log ε): 231 (4,51) nm., IR (film) ν_{max}: 3424, 2916, 1737, 1709, 1681, 1239, 1010, 848 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400.13 MHz): 5.96 brd (1H, J = 9.8 Hz, H-4), 5.53 m (1H, H-6), 5.50 m (1H, H-3), 4.72 d (1H, J = 12.2 Hz, H-21a), 4.63 d (1H, J = 12.2 Hz, H-21b), 3.11 brd (1H, J = 21.0 Hz, H-2a), 2.65 m (1H, H-2b), 2.60 m (2H, H_2-16), 2.37 m (1H, H-14), 2.15 s (3H, H_3-28), 2.12 m (1H, H-7β), 1.99 m (1H, H-11α), 1.97 s (3H, H_3-27), 1.94 s, (3H, CH_3COO-21), 1.93 m (1H, H-9), 1.79 m (1H, H-15α), 1.74 m (1H, H-7α), 1.63 m (1H, H-15β), 1.53 m (1H, H-8), 1.30 m (1H, H-11β), 1.21 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 209.3 (C, C-1), 191.4 (C, C-22), 172.9 (C, C-26) 170.8 (C, CH_3COO-21), 168.6 (C, C-17), 159.3 (C, C-24), 140.0 (C, C-5), 129.1 (CH, C-4), 128.4 (C, C-25), 126.2 (CH, C-6), 123.8 (C, C-20), 121.5 (CH, C-3), 90.8 (C, C-23), 75.4 (C, C-12), 58.2 (CH_2, C-21), 51.1 (C, C-10), 49.1 (C, C-13), 46.9 (CH, C-14), 39.2 (CH, C-2), 39.1 (CH, C-9), 34.0 (CH_2, C-11), 30.7 (CH, C-8), 29.3 (CH_2, C-7), 25.6 (CH_2, C-16), 23.1 (CH_2, C-15), 20.9 (CH_3, CH_3COO-21), 19.7 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 529.2219 (calcd for $C_{30}H_{34}O_7Na$, 529.2226).

4.4.2. Preparation of (23R)-21-benzoyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (5)

Triethylamine (45 μL, 0.34 mmol) and benzoyl chloride (30 μL, 0.25 mmol) were added to a solution of jaborosalactone 5 (19.5 mg, 0.042 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred until disappearance of the starting withanolide (24 h). After removal the solvent, the residue was purified by preparative-TLC using EtOAc-Hexane (7:3) to obtain 6.9 mg of compound **5** (29%) as a white amorphous solid; $[\alpha]_{21}D$: −165.6 (c 0.15, MeOH), UV (MeOH) λmax (log ε): 226 (4.51) nm., IR (film) ν_{max}: 3439, 2969, 2921, 1763, 1726, 1689, 1601, 1272, 1019, 717 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400.13 MHz): 7.91 brd (2H, J = 8.4 Hz, H-2′/H-6′), 7.46 m (1H, H-4′), 7.33 m (2H, H-3′/H-5′), 6.67 dd (1H, J = 10.0,5.0,2.5 Hz, H-3), 5.75 dd (1H, J = 10.0,2.5 Hz, H-2), 5.47 brd (1H, J = 6.0 Hz, H-6), 4.98 d (1H, J = 12.2 Hz, H-21a), 4.92 d (1H, J = 12.2 Hz, H-21b), 3.19 brd (1H, J = 21.0 Hz, H-4β), 2.77 dd (1H, J = 21.0,5.0 Hz, H-4α), 2.64 m (2H, H_2-16), 2.44 dd (1H, J = 14.0, 4.0 Hz, H-11α), 2.37 m (1H, H-14), 2.20 s (3H, H_3-28), 1.961 m (1H, H-7β), 1.960 s (3H, H_3-27), 1.81 m (1H, H-9), 1.80 m (1H, H-15α), 1.67 m (1H, H-7α), 1.61 m (1H, H-15β), 1.47 m (1H, H-11β), 1.44 m (1H, H-8), 1.09 s (3H, H_3-19), 1.087 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100,03 MHz): 202.9 (C, C-1), 191.7 (C, C-22), 172.8 (C, C-26), 168.7 (C, C-17) 166.5 (C, COO-21), 159.5 (C, C-24), 144.9 (CH, C-3), 135.2 (C, C-5), 133.6 (CH, C-4′), 130.0 (C, C-1′), 129.5 (CH, C-2′/C-6′), 128.2 (CH, C-3′/C-5′), 128.2 (C, C-25), 127.6 (CH, C-2), 124.0 (CH, C-6), 124.1 (C, C-20), 91.1 (C, C-23), 75.6 (C, C-12), 58.8 (CH_2, C-21), 49.7 (C, C-10), 48.8 (C, C-13), 46.7 (CH, C-14), 40.6 (CH, C-9), 34.9 (CH_2, C-11), 33.1 (CH_2, C-4), 31.9 (CH, C-8), 28.9 (CH_2, C-7), 25.7 (CH_2, C-16), 23.1 (CH_2, C-15), 18.3 (CH_3, C-19), 15.8 (CH_3, C-28), 14.4 (CH_3, C-18), 8.8 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 591.2361 (calcd for $C_{35}H_{36}O_7Na$, 591.2359).

4.4.3. Preparation of (23R)-21-p-bromobenzoyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (6)

Triethylamine (153 μL, 1.1 mmol), *p*-bromobenzoyl chloride (15 mg, 0.07 mmol) and DMAP in catalytic amount were added to a solution of jaborosalactone 5 (20.5 mg, 0.044 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature under nitrogen until disappearance of the starting material (1 h). After removing the solvent, the residue was purified by preparative-TLC using EtOAc to obtain 18.7 mg of compound **6** (65.5%) as a white amorphous solid; $[\alpha]_{21}D$: −104.3 (c 0,30, MeOH); UV (MeOH) λmax (log ε): 242 (4.35), 228 (4.31) nm.; IR (film) ν_{max}: 3484, 2957, 2926, 2857, 1732, 1668, 1268, 1012, 757 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400.13 MHz): 7.83 d (2H, J = 8.5 Hz, H-2′/H-6′), 7.54 d (2H, J = 8.5 Hz, H-3′/H-5′), 6.74 ddd (1H, J = 9.6,4.5,2.2 Hz, H-3), 5.82 brd (1H, J = 9.6 Hz, H-2), 5.54 brs (1H, H-6), 5.04 d (1H, J = 12.2 Hz, H-21a), 4.96 d (1H J = 12.2 Hz, H-21b), 3.25 brd (1H, J = 21.0 Hz, H-4β), 2.84 dd (1H, J = 21.0,5.0 Hz, H-4α), 2.27 s (3H, H_3-28), 2.03 s (3H, H_3-27), 1.17 s (3H, H_3-19), 1.15 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.8 (C, C-1), 191.6 (C, C-22), 172.7 (C, C-26), 168.8 (C, C-17), 165.7 (C, COO-21), 159.4 (C, C-24), 145.0 (CH, C-3), 135.3 (C, C-5), 131.7 (CH, C-2′/C-6′), 131.2 (CH, C-3′/C-5′), 128.9 (C, C-1′), 128.3 (C, C-25), 128.0 (C, C-4′), 127.7 (CH, C-2), 124.0 (CH, C-6), 123.8 (C, C-20), 91.1 (C, C-23), 75.5 (C, C-12), 59.1 (CH_2, C-21), 49.8 (C, C-10), 48.9 (C, C-13), 46.8 (CH, C-14), 40.6 (CH, C-9), 35.1 (CH_2, C-11), 33.2 (CH_2, C-4), 32.0 (CH, C-8), 29.0 (CH_2, C-7), 25.8 (CH_2, C-16), 23.3 (CH_2, C-15), 18.4 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 9.0

(CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 669.1468 (calcd for $C_{35}H_{35}O_7NaBr$, 669.1464).

4.4.4. Preparation of (23R)-21-furoyloxy-12β-hydroxy-1,22-dioxo-12,23-cicloergostan-2,5,17,24-tetraen-26,23-olide (**7**)

Triethylamine (240 μL, 1.7 mmol), furoyl chloride (134 μL, 1.3 mmol) and catalytic amount of DMAP were added to a solution of jaborosalactone 5 (19.8 mg, 0.043 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature under nitrogen until disappearance of the starting material (1 h). After removal of the solvent, the residue was purified by preparative-TLC using EtOAc to obtain 10.2 mg of compound **7** (43%) as a white amorphous solid; $[\alpha]_{21}D$: −165 (c 0.20, MeOH), UV (MeOH) λmax (log ε): 250 (4.38), 226 (4.34) nm., IR (film) ν_{max}: 3473, 2963, 1736, 1684, 1296, 1118, 1013, 758 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 7.58 dd (1H, *J* = 1.8, 0.9 Hz, H-5′), 7.14 dd (1H, *J* = 3.7, 0.9 Hz, H-3′), 6.76 ddd (1H, *J* = 10.0, 5.0, 2.6 Hz, H-3), 6.49 dd (1H, *J* = 3.7, 1.8 Hz, H-4′), 5.81 dd (1H, *J* = 10.0, 3.3 Hz, H-2), 5.54 dt (1H, *J* = 6.1, 2.1 Hz, H-6), 5.01 dd (1H, *J* = 12.4, 1.3 Hz, H-21a), 4.95 d (1H, *J* = 12.4 Hz, H-21b), 3.25 bd (1H, *J* = 21.0 Hz, H-4β), 2.84 dd (1H, *J* = 21.0, 5.0 Hz, H-4α), 2.78 m (1H, H-16α), 2.71 dd (1H, *J* = 11.2, 2.4 Hz, H-16β), 2.53 dd (1H, *J* = 13.8, 3.9 Hz, H-11α), 2.45 m (1H, H-14), 2.29 bs (3H, H_3-28), 2.050 bs (3H, H_3-27), 2.047 m (1H, H-7β), 1.93 m (1H, H-15α), 1.90 m (1H, H-9), 1.756 m (1H, H-7α), 1.75 m (1H, H-15β), 1.56 m (1H, H-11β), 1.54 m (1H, H-8), 1.19 s (3H, H_3-19), 1.17 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.9 (C, C-1), 191.4 (C, C-22), 172.8 (C, C-26), 169.3 (C, C-17), 159.4 (C, C-24), 158.5 (C, COO-21), 146.2 (CH, C-5′), 144.8 (CH, C-3), 144.2 (C, C-2′), 135.2 (C, C-5), 128.1 (C, C-25), 127.5 (CH, C-2), 124.0 (CH, C-6), 123.5 (C, C-20), 118.0 (CH, C-3′), 111.4 (CH, C-4′), 90.9 (C, C-23), 75.4 (C, C-12), 58.6 (CH_2, C-21), 49.7 (C, C-10), 48.9 (C, C-13), 46.5 (CH, C-14), 40.5 (CH, C-9), 34.8 (CH_2, C-11), 33.0 (CH_2, C-4), 31.9 (CH, C-8), 29.1 (CH_2, C-7), 25.6 (CH_2, C-16), 23.0 (CH_2, C-15), 18.2 (CH_3, C-19), 15.7 (CH_3, C-28), 14.3 (CH_3, C-18), 8.7 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 581.2158 (calcd for $C_{33}H_{34}O_8Na$, 581.2151).

4.4.5. Preparation of (23R)-12β,21-dihydroxy-1,22-dioxo-12,23-cycloergostan-3,5,17,24-tetraen-26,23-olide (**8**)

Dimethyl carbamoyl chloride (6.2 μL, 0.07 mmol), triethylamine (18.8 μL, 0.14 mmol), and catalytic amount of DMAP were added to a solution of jaborosalactone 5 (21.1 mg, 0.045 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature under nitrogen for 24 h. Then, an additional amount of triethylamine (67 μL, 0.5 mmol) and acid chloride (27 μL, 0.3 mmol) were added, and the reaction mixture was left for 24 h. The solvent was removed and the residue was purified by preparative-TLC using CH_2Cl_2:MeOH (9.7:0.3) to obtain 5 mg of a mixture which could not be separated neither by normal TLC phase nor by reversed TLC phase. The ^1H NMR spectrum of this mixture indicated that it consisted of jaborosalactone 5 and **8** in a 1:2 ratio. ^1H NMR ($CDCl_3$, 400.13 MHz): 5.96 brd (1H, *J* = 9.7 Hz, H-4), 5.54 m (1H, H-3), 5.51 m (1H, H-6), 4.27 d (1H, *J* = 12.5 Hz, H-21a), 4.14 d (1H, *J* = 12.5 Hz, H-21b), 3.12 brd (1H, *J* = 20.5 Hz, H-2a), 2.65 dd (1H, *J* = 20.5,4.6 Hz, H-2b), 2.55 m (2H, H_2-16), 2.43 dd (1H, *J* = 13.9, 3.9 Hz, H-11α), 2.32 m (1H, H-14), 2.18 s (3H, H_3-28), 1.97 s (3H, H_3-27), 1.96 m (1H, H-7β), 1.94 m (1H, H-9), 1.77 m (1H, H-15α), 1.65 m (1H, H-7α), 1.62 m (1H, H-15β), 1.45 m (1H, H-11β), 1.43 m (1H, H-8), 1.22 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 209.1 (C, C-1), 193.4 (C, C-22), 172.8 (C, C-26), 166.3 (C, C-17), 159.4 (C, C-24), 140.2 (C, C-5), 129.1 (CH, C-4), 128.4 (C, C-25), 127.4 (C, C-20), 126.2 (CH, C-3), 121.4 (CH, C-6), 91.1 (C, C-23), 75.5 (C, C-12), 58.7 (CH_2, C-21), 51.5 (C, C-10), 48.4 (C, C-13), 46.9 (CH, C-14), 39.2 (CH_2, C-2), 39.1 (CH, C-9), 35.0 (CH_2, C-11), 32.0 (CH, C-8), 29.1 (CH_2, C-7), 25.2 (CH_2, C-16), 23.3 (CH_2, C-15), 19.8 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 9.0 (CH_3, C-27).

4.4.6. Preparation of (23R)-21-lauroyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**9**)

Triethylamine (188 μL, 1.4 mmol), lauroyl chloride (159 μL, 0.7 mmol) and catalytic amount of DMAP in were added to a solution of jaborosalactone 5 (20.7 mg, 0.045 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature under nitrogen until disappearance of the starting material (1 h). After removing the solvent, the residue was purified by preparative-TLC using CH_2Cl_2:MeOH (9.7:0.3) to obtain 11.5 mg of compound **9** (40%) as a white amorphous solid; $[\alpha]_{21}D$: −336.7 (c 0.15, $CHCl_3$), UV ($CHCl_3$) λ_{max} (log ε): 229 (5.51), IR (film) ν_{max}: 3497, 2923, 2854, 1742, 1712, 1463, 1111, 723 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.67 ddd (1H, *J* = 10.0, 5.0, 2.6 Hz, H-3), 5.74 dd (1H, *J* = 10.0,3.4 Hz, H-2), 5.47 m (1H, H-6), 4.72 d (1H, *J* = 12.2 Hz, H-21a), 4.64 d (1H, *J* = 12.2 Hz, H-21b), 3.18 brd (1H, H-4β), 2.76 m (1H, H-4α), 2.57 m (2H, H-16), 2.41 m (1H, H-11α), 2.32 m (1H, H-14), 2.19 s (3H, H_3-28), 1.95 m (1H, H-7β), 1.94 brs (3H, H_3-27), 1.78 m (1H, H-9), 1.78 m (1H, H-15α), 1.66 m (1H, H-7α), 1.61 m (1H, H-15β), 1.44 m (1H, H-11β), 1.43 m (1H, H-8), 1.22−2.18 m (20H, CH_2 × 10, H_2-2′-11′), 1.09 s (3H, H_3-19), 1.05 s (3H, H_3-18), 0.81 t (3H, H_3-12′). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.8 (C, C-1), 191.5 (C, C-22), 173.8 (C, COO-21), 172.7 (C, C-26), 168.6 (C, C-17), 159.4 (C, C-24), 145.0 (CH, C-3), 135.2 (C, C-5), 128.3 (C, C-25), 127.7 (CH, C-2), 124.1 (CH, C-6), 124.1 (C, C-20), 91.1 (C, C-23), 75.4 (C, C-12), 58.2 (CH_2, C-21), 49.8 (C, C-10), 48.9 (C, C-13), 46.9 (CH, C-14), 40.7 (CH, C-9), 35.0 (CH_2, C-11), 34.1−22.6 (CH_2x10, C-2′-11′), 33.3 (CH_2, C-4), 31.9 (CH, C-8), 29.1 (CH_2, C-7), 25.7 (CH_2, C-16), 23.3 (CH_2, C-15), 18.4 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 14.0 (CH_3, C-12′), 8.8 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 669.3758 (calcd for $C_{40}H_{54}O_7Na$, 669.3767).

4.4.7. Preparation of (23R)-21-pivaloyloxy-12β-hydroxy-1,22-dioxo-12,23-cicloergostan-2,5,17,24-tetraen-26,23-olide (**10**)

Triethylamine (30 μL, 0.2 mmol), pivaloyl chloride (17 μL, 0.13 mmol) and catalytic amount of DMAP were added to a solution of jaborosalactone 5 (20.9 mg, 0.045 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature for 48 h, and then additional aliquots of triethylamine (300 μL, 2 mmoL) and pivaloyl chloride (136 μL, 1 mmol) were added. After completion of reaction, the solvent was removed and the residue was purified by preparative-TLC using EtOAc:Hexane (7:3) to obtain 5.4 mg of compound **10** (22%) as a white amorphous solid; $[\alpha]_{21}D$: −116.0 (c 0.12, MeOH), UV (MeOH) λmax (log ε): 225.4 (3.82) nm., IR (film) ν_{max}: 3442, 2961, 2930, 1732, 1457, 1156, 1009 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.67 ddd (1H, *J* = 10.0, 5.0, 2.5 Hz, H-3), 5.75 dd (1H, *J* = 10.0, 2.5 Hz, H-2), 5.48 m (1H, H-6), 4.69 m (2H, H_2-21), 3.19 brd (1H, *J* = 21.0 Hz, H-4β), 2.77 (1H, *J* = 21.0,5.0 Hz, H-4α), 2.61 m (2H, H_2-16), 2.43 (1H, *J* = 14.0, 4.0 Hz, H-11α), 2.32 m (1H, H-14), 2.20 s (3H, H_3-28), 1.95 s (3H, H_3-27), 1.95 m (1H, H-7β), 1.791 m (1H, H-9), 1.790 m (1H, H-15α), 1.68 m (1H, H-7α), 1.61 m (1H, H-15β), 1.53 m (1H, H-8), 1.44 m (1H, H-11β), 1.09 s (3H, H_3-19), 1.07 s (9H, H_3-2′/H_3-3′/H_3-4′), 1.06 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.9 (C, C-1), 191.5 (C, C-22), 178.3 (C, COO-21), 172.7 (C, C-26), 167.7 (C, C-17), 159.4 (C, C-24), 144.9 (CH, C-3), 135.2 (C, C-5), 128.2 (C, C-25), 127.6 (CH, C-2), 124.4 (C, C-20), 124.0 (CH, C-6), 91.0 (C, C-23), 75.4 (C, C-12), 58.0 (CH_2, C-21), 49.7 (C, C-10), 48.5 (C, C-13), 46.4 (CH, C-14), 40.5 (CH, C-9), 38.7 (C, C-1′), 35.0 (CH_2, C-11), 33.1 (CH_2, C-4), 30.4 (CH, C-8), 29.9 (CH_2, C-7), 26.9 (CH_3 × 3, C-2′/C-3′/C-4), 25.6 (CH_2, C-16), 23.2 (CH_2, C-15), 18.3 (CH_3, C-19), 15.8 (CH_3, C-28), 14.2 (CH_3, C-18), 8.9 (CH_3, C-27).

HRESIMS *m/z* $[M+Na]^+$ 571.2666 (calcd for $C_{33}H_{40}O_7Na$, 571.2672).

*4.4.8. Preparation of (23R)-21-(2-oxo-cyclopentane-carboxyl)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**11**) and (23R)-21-adipoyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**12**)*

Triethylamine (20 μL, 0.14 mmol), adipoyl chloride (4.3 μL, 0.03 mmol), and DMAP in catalytic amount were added to a solution of jaborosalactone 5 (26.7 mg, 0.057 mmol) in dry CH_2Cl_2 (5 mL). The reaction was carried out under argon atmosphere. The mixture was stirred for 22 h at room temperature. Then, the reaction mixture was refluxed for 4 h and an aliquot of acid chloride (4.3 μL, 0.03 mmol) were added. After 1 h of additional reaction, the solvent was removed and the residue was purified by preparative TLC using CH_2Cl_2:MeOH (9.7:0.3) to obtain 4.6 mg of **11** (14%) and 3 mg of **12** (9%).

*4.4.8.1. (23R)-21-(2-oxo-cyclopentane-carboxyl)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**11**).* White amorphous solid, $[\alpha]_{21}D$: −156.5 (c 0.34, MeOH), UV (MeOH) λmax (log ε): 226 (3.64) nm; IR (film) ν_{max}: 3360, 2922, 2851, 1733, 1659, 1632, 1382, 1251, 1011, 771 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz: 6.67 ddd (1H, *J* = 10.0, 5.0, 2.5 Hz, H-3), 5.74 dd (1H, *J* = 10.0,2.5 Hz, H-2), 5.47 d (1H, *J* = 5.7 Hz, H-6), 4.82 dd (1H, *J* = 12.0, 9.7 Hz, H-21a), 4.69 d (1H, *J* = 12.0 Hz, H-21b), 3.17 brd (1H, *J* = 21.0 Hz, H-4β), 3.06 t (1H, *J* = 8.9 Hz, H-1′), 2.77 dd (1H, *J* = 21.0, 5.0 Hz, H-4), 2.61 m (2H, H_2-16), 2.40 dd (1H, *J* = 14.0, 3.7 Hz, H-11α), 2.31 m (1H, H-14), 2.21 m (2H, H_2-3′), 2.17 m (2H, H_2-5′), 2.19 s (3H, H_3-28), 2.02 m (2H, H_2-4′), 1.95 s (3H, H_3-27), 1.94 m (1H, H-7β), 1.80 m (1H, H-15α), 1.78 m (1H, H-9), 1.65 m (1H, H-7α), 1.60 m (1H, H-15β), 1.45 m (1H, H-11β), 1.43 m (1H, H-8), 1.09 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 212.8 (C, C-2′), 202.8 (C, C-1), 191.2 (C, C-22), 172.8 (C, C-26), 169.7 (C, C-17), 169.6 (C, COO-21), 159.6 (C, C-24), 144.9 (CH, C-3), 135.3 (C, C-5), 128.4 (C, C-25), 127.7 (CH, C-2), 124.1 (CH, C-6), 123.6 (C, C-20), 91.1 (C, C-23), 75.5 (C, C-12), 59.1 (CH_2, C-21), 54.9 (CH, C-1′), 49.8 (C, C-10), 48.9 (C, C-13), 46.7 (CH, C-14), 40.6 (CH, C-9), 38.0 (CH_2, C-3′), 34.9 (CH_2, C-11), 33.2 (CH_2, C-4), 32.0 (CH, C-8), 29.1 (CH_2, C-7), 27.4 (CH_2, C-5′), 25.9 (CH_2, C-16), 23.3 (CH_2, C-15), 20.9 (CH_2, C-4′), 18.4 (CH_3, C-19), 15.9 (CH_3, C-28), 14.5 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 597.2469 (calcd for $C_{34}H_{38}O_8Na$, 597.2464).

*4.4.8.2. (23R)-21-adipoyloxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**12**).* White amorphous solid, $[\alpha]_{21}D$: −167.6 (c 0.25, MeOH); UV (MeOH) λmax (log ε): 225 (4.36) nm; IR (film) ν_{max}: 3363, 2923, 2854, 1730, 1660, 1411, 1261, 1013, 758 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz: 6.67 ddd (1H, *J* = 10.0, 5.0, 2.5 Hz, H-3), 5.74 dd (1H, *J* = 10.0, 2.5 Hz, H-2), 5.48 brd (1H, *J* = 5.9 Hz, H-6), 4.72 d (1H, *J* = 12.0 Hz, H-21a), 4.67 d (1H, *J* = 12.0 Hz, H-21b), 3.18 brd (1H, *J* = 21.0 Hz, H-4β), 2.78 dd (1H, *J* = 21.0, 5.0 Hz, H-4α), 2.64 m (1H, H-16α), 2.55 m (1H, H-16β), 2.41 dd (1H, *J* = 14.0, 4.0 Hz, H-11α), 2.31 m (1H, H-14), 2.26 m (2H, H-1′), 2.23 m (2H, H_2-4′), 2.19 s (3H, H_3-28), 1.96 s (3H, H_3-27), 1.96 m (1H, H-7β), 1.84 m (1H, H-15α), 1.77 m (1H, H-9), 1.68 m (1H, H-7α), 1.64 m (1H, H-15β), 1.56 m (4H, H_2-2′/H_2-3′), 1.44 m (1H, H-11β), 1.42 m (1H, H-8), 1.09 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz: 202.8 (C, C-1), 191.5 (C, C-22), 175.0 (C, C-5′), 173.3 (C, C-26), 173.2 (C, COO-21), 168.8 (C, C-17), 159.8 (C, C-24), 145.1 (CH, C-3), 134.7 (C, C-5), 128.2 (C, C-25), 127.4 (CH, C-2), 123.9 (CH, C-6), 123.7 (C, C-20), 90.8 (C, C-23), 75.4 (C, C-12), 57.7 (CH_2, C-21), 49.6 (C, C-10), 48.6 (C, C-13), 46.3 (CH, C-14), 40.3 (CH, C-9), 34.7 (CH_2, C-11), 33.7 (CH_2, C-4′), 33.3 (CH_2, C-1′), 32.9 (CH_2, C-4), 31.6 (CH, C-8), 28.6 (CH_2, C-7), 25.3 (CH_2, C-16), 24.3[a] (CH_2, C-2′), 24.1[a] (CH_2, C-3′), 22.8 (CH_2, C-15), 18.1 (CH_3, C-19), 15.6 (CH_3, C-28), 14.0 (CH_3, C-18), 8.6 (CH_3, C-27). [a]Assignments may be interchanged. HRESIMS *m/z* $[M+Na]^+$ 615.2561 (calcd for $C_{34}H_{40}O_9Na$, 615.2570).

*4.4.9. Preparation of (23R)-21-chloro-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23- olide (**13**) and (23R)-21-chloro-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-3,5,17,24-tetraen-26,23-olide (**14**)*

Thionyl chloride (84 μL, 1.14 mmol) was added to a solution of jaborosalactone 5 (26.4 mg, 0.057 mmol) in dry CH_2Cl_2 (3 mL). The reaction was carried out under argon atmosphere. The reaction mixture was refluxed during 20 h and an aliquot of acid chloride (3 μL, 0.03 mmol) were added. After 1 h of additional reaction, the solvent was removed and the residue was purified by preparative TLC using CH_2Cl_2:MeOH (9.7:0.3) to obtain 9.7 mg (35.3%) of compound **13** and 1.5 mg (5.4%) of compound **14**, which could not be obtained in pure form and was decomposed after NMR characterization.

*4.4.9.1. (23R)-21-chloro-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**13**).* White amorphous solid, $[\alpha]_{21}D$: −258.7 (c 0.2, MeOH); UV (MeOH) λmax (log ε): 224 (4.58) nm; IR (film) ν_{max}: 3469, 2965, 2925, 1742, 1666, 1382, 1250, 1009, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.74 ddd (1H, *J* = 10.0, 5.0, 2.5 Hz, H-3), 5.80 dd (1H, *J* = 10.0,2.4 Hz, H-2), 5.54 brd (1H, *J* = 5.4 Hz, H-6), 4.28 d (1H, *J* = 11.2 Hz, H-21a), 4.12 d (1H, *J* = 11.2 Hz, H-21b), 3.25 brd (1H, *J* = 21.0 Hz, H-4β), 2.84 dd (1H, *J* = 21.0; 5.0 Hz, H-4α), 2.64 m (2H, H_2-16), 2.49 m (2H, H_2-11), 2.41 m (1H, H-14), 2.26 s (3H, H_3-28), 2.02 s (3H, H_3-27), 1.16 s (3H, H_3-19), 1.14 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.7 (C, C-1), 190.9 (C, C-22), 172.6 (C, C-26), 168.5 (C, C-17), 159.2 (C, C-24), 144.9 (CH, C-3), 135.2 (C, C-5), 128.3 (C, C-20), 127.6 (CH, C-2), 125.6 (C, C-25), 124.0 (CH, C-6), 90.9 (C, C-23), 75.4 (C, C-12), 49.8 (C, C-10), 48.9 (C, C-13), 46.6 (CH, C-14), 40.6 (CH, C-9), 37.1 (CH_2, C-21), 35.0 (CH_2, C-11), 33.2 (CH_2, C-4), 31.9 (CH, C-8), 29.1 (CH_2, C-7), 25.4 (CH_2, C-16), 23.3 (CH_2, C-15), 18.4 (CH_3, C-19), 15.9 (CH_3, C-28), 14.3 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 505.1749 (calcd for $C_{28}H_{31}O_5NaCl$, 505.1758).

*4.4.9.2. (23R)-21-chloro-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-3,5,17,24-tetraen-26,23-olide (**14**).* ^1H NMR ($CDCl_3$, 400.13 MHz): 5.95 brdd (1H, *J* = 10.1, 2.0 Hz, H-4), 5.53 m (1H,H-6), 5.51 m (1H, H-3), 4.20 d (1H, *J* = 11.4 Hz, H-21a), 4.05 d (1H, *J* = 11.4 Hz, H-21b), 3.01 brd (1H, *J* = 20.8 Hz, H-2a), 2.65 m (1H, H-2b), 2.60 m (2H, H_2-16), 2.38 m (1H, H-14), 2.15 s (3H, H_3-28), 2.15 m (1H, H-7β), 2.00 m (1H, H-11α), 1.97 s (3H, H_3-27), 1.94 m (1H, H-9), 1.79 m (1H, H-15α), 1.76 m (1H, H-7α), 1.67 m (1H, H-15β), 1.29 m (1H, H-11β), 1.21 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 209.2 (C, C-1), 190.7 (C, C-22), 172.6 (C, C-26), 168.2 (C, C-17), 158.9 (C, C-24), 140.3 (C, C-5), 128.9 (CH, C-4), 128.5 (C, C-25), 126.1 (CH, C-6), 125.8 (C, C-20), 121.4 (CH, C-3), 90.7 (C, C-23), 75.2 (C, C-12), 51.3 (C, C-10), 49.1 (C, C-13), 46.7 (CH, C-14), 39.1 (CH_2, C-2), 38.9 (CH, C-9), 36.9 (CH_2, C-21), 34.0 (CH_2, C-11), 30.4 (CH, C-8), 29.2 (CH_2, C-7), 25.3 (CH_2, C-16), 23.3 (CH_2, C-15), 19.7 (CH_3, C-19), 15.6 (CH_3, C-28), 14.3 (CH_3, C-18), 8.9 (CH_3, C-27).

*4.4.10. Preparation of (23R)-21-carbaldehyde-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**15**)*

A solution of pyridinium chlorochromate (19.5 mg, 0.09 mmol in CH_2Cl_2) was added to jaborosalactone 5 (27.6 mg, 0.059 mmol) and dissolved in dry CH_2Cl_2 (3 mL). The reaction mixture was stirred during 0.5 h at room temperature. After completion of the reaction, the reaction mixture was filtered through florisil and washed with ether (20 mL), CH_2Cl_2 (20 mL), and EtOAc (20 mL). After the solvent elimination the residue was subjected to preparative-TLC

purification using CH_2Cl_2:MeOH (9:1) to yield 4.6 mg (16.7%) of compound **15** as a white amorphous solid; $[\alpha]_{21}D$: −270 (c 0.15, MeOH); UV (MeOH) λmax (log ε): 239 (3.71), 220 (3.73) nm; IR (film) ν_{max}: 3467, 2964, 2926, 1743, 1683, 1600, 1247, 1011, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 9.86 s (1H, H-21), 6.75 ddd (1H, J = 10.0, 4.9, 2.4 Hz, H-3), 5.81 dd (1H, J = 10.0,2.4 Hz, H-2), 5.55 brd (1H, J = 5.6 Hz, H-6), 3.26 brd (1H, J = 21.4 Hz, H-4β), 2.87 m (2H, H_2-16), 2.85 brd (1H, J = 21.4 Hz, H-4α), 2.40 ddd (1H, J = 19.0, 11.0, 7.0 Hz, H-14), 2.29 s (3H, H_3-28), 2.05 s (3H, H_3-27), 1.19 s (3H, H_3-19), 1.17 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 203.0 (C, C-1), 189.9 (C, C-22), 181.6 (C, C-21), 172.5 (C, C-26), 166.7 (C, C-17), 158.9 (C, C-24), 145.0 (CH, C-3), 135.2 (C, C-5), 128.7 (C, C-20), 127.6 (CH, C-2), 126.1 (C, C-25), 124.0 (CH, C-6), 91.0 (C, C-23), 75.4 (C, C-12), 50.6 (C, C-13), 49.6 (C, C-10), 46.3 (CH, C-14), 40.6 (CH, C-9), 35.4 (CH_2, C-11), 33.2 (CH_2, C-4), 31.9 (CH, C-8), 29.0 (CH_2, C-7), 28.2 (CH_2, C-16), 23.5 (CH_2, C-15), 18.5 (CH_3, C-19), 15.9 (CH_3, C-28), 14.4 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS m/z $[M]^+$. 462.2037 (calcd for $C_{28}H_{30}O_6$, 462.2042).

4.4.11. Preparation of (23R)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-5,17,24-trien-26,23- olide (**16**) and (23R)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-17,24-dien-26,23-olide (**17**)

Jaborosalactone 5 (25.4 mg, 0.047 mmol) was dissolved in dry THF (4 mL) and Pd/C (10%) in catalytic amount was added. An H_2-filled ball was connected to the reaction flask and the system was closed under this atmosphere for 26 h. Then the solution was filtered and the solvent was removed. The residue was purified by preparative TLC using EtOAc:hexane (7:3) as eluent to yield 5.4 mg (22%) of compound **16** and 5.1 mg (21%) of compound **17**.

*4.4.11.1. (23R)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-5,17,24-trien-26,23-olide (**16**).* White amorphous solid, $[\alpha]_{21}D$: −134.6 (c 0.13, MeOH); UV (MeOH) λmax (log ε): 220 (3.99) nm; IR (film) ν_{max}: 3411, 2928, 2869, 1739, 1706, 1677, 1249, 1011, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 5.39 brd (1H, J = 6.0 Hz, H-6), 2.42 m (2H, H_2-16), 2.42 m (1H, H-4β), 2.42 m (1H, H-2a), 2.27 m (1H, H-14), 2.17 m (1H, H-2b), 2.14 s (3H, H_3-28), 2.08 m (1H, H-4α), 1.93 m (1H, H-7β), 1.92 s (3H, H_3-27), 1.87 m (1H, H-11α), 1.82 m (1H, H-9), 1.81 m (1H, H-3a), 1.80 m (1H, H-15α), 1.67 s (3H, H_3-21), 1.61 m (1H, H-15β), 1.56 m (1H, H-3b), 1.53 m (1H, H-7α), 1.38 m (1H, H-8), 1.33 m (1H, H-11β), 1.14 s (3H, H_3-19), 1.02 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz: 213.0 (C, C-1), 193.2 (C, C-22), 173.6 (C, C-26), 162.8 (C, C-17), 160.3 (C, C-24), 140.6 (C, C-5), 127.9 (C, C-25), 124.5 (C, C-20), 122.1 (CH, C-6), 91.1 (C, C-23), 75.5 (C, C-12), 53.0 (C, C-10), 48.5 (C, C-13), 46.7 (CH, C-14), 37.5 (CH, C-2), 40.2 (CH, C-9), 33.5 (CH_2, C-11), 30.6 (CH_2, C-4), 30.3 (CH, C-8), 29.3 (CH_2, C-7), 22.9 (CH_2, C-15), 25.4 (CH_2, C-16), 25.1 (CH, C-3), 18.3 (CH_3, C-19), 11.9 (CH_3, C-21), 15.5 (CH_3, C-28), 14.1 (CH_3, C-18), 8.6 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 473.2300 (calcd for $C_{28}H_{35}O_5Na$, 473.2304).

*4.4.11.2. (23R)-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-17,24-dien-26,23-olide (**17**).* White amorphous solid, $[\alpha]_{21}D$: −134.3 (c 0.23, MeOH); UV (MeOH) λmax (log ε): 221 (4.12) nm; IR (film) ν_{max}: 3469, 2928, 2865, 1755, 1696, 1449, 1380, 1247, 1012, 757 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz: 2.43 t (2H, J = 6.7 Hz, H_2-16), 2.27 m (1H, H-14), 2.14 m (1H, H-2a), 2.08 s (3H, H_3-28), 2.07 m (1H, H-2b), 2.00 m (2H, H_2-3), 1.89 m (1H, H-9), 1.76 m (1H, H-15α), 1.74 s (3H, H_3-27), 1.66 s (3H, H_3-21), 1.62 m (1H, H-5), 1.57 m (1H, H-15β), 1.53 m (1H, H-7α), 1.50 m (1H, H-6a), 1.46 m (2H, H_2-4), 1.40 m (1H, H-6b), 1.38 m (1H, H-8), 1.29 m (1H, H-11α), 1.02 s (3H, H_3-19), 0.98 s (3H, H_3-18), 0.92 m (1H, H-11β). ^{13}C NMR ($CDCl_3$ 100.03 MHz: 215.7 (C, C-1), 192.2 (C, C-22), 173.2 (C, C-26), 163.0 (C, C-17), 162.6 (C, C-24), 126.4 (C, C-25), 124.2 (C, C-20), 91.4 (C, C-23), 74.6 (C, C-12), 52.3 (C, C-10), 48.7 (C, C-13), 46.8 (CH, C-14), 45.1 (CH, C-5), 40.0 (CH, C-9), 37.5 (CH_2, C-2), 34.4 (CH, C-8), 33.4 (CH_2, C-11), 30.2 (CH_2, C-7), 26.3 (CH_2, C-3), 26.0 (CH_2, C-6), 25.6 (CH_2, C-16), 24.7 (CH_2, C-4), 22.8 (CH_2, C-15), 15.8 (CH_3, C-28), 14.7 (CH_3, C-19), 14.5 (CH_3, C-18), 12.0 (CH_3, C-21), 8.5 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 475.2468 (calcd for $C_{28}H_{36}O_5Na$, 475.2460).

4.4.12. Preparation of jaborosalactone 4

Meta-chloroperoxybenzoic acid (18 mg, 0.05 mmol) and $NaHCO_3$ (16 mg, 0.19 mmol) were added to a solution of jaborosalactone 5 (20 mg, 0.043 mmol) in dry CH_2Cl_2 (5 mL). The reaction mixture was stirred at room temperature for 46 h. Then CH_2Cl_2 was added and organic phase was washed with saturated aqueous sodium thiosulfate. Organic phases were pooled, dried with $MgSO_4$, and concentrated to dryness. The residue was purified by preparative TLC using EtOAc to obtain 3.7 mg (18%) of jaborosalactone 4.

4.4.13. Preparation of (23R)-4β, 12β, 21-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**18**) and (23R)-6β,12β,21-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,4,17,24-tetraen-26,23-olide (**19**)

SeO_2 (29 mg, 0.26 mmol) were added to a solution of jaborosalactone 5 (35.3 mg, 0.075 mmol) in dry CH_2Cl_2 (5 mL). The reaction was carried out with stirring at room temperature under argon atmosphere. After 24 h of reaction, 0.1 mmol of SeO_2 were added and the reaction mixture was refluxed for 45 h. Then the reaction mixture was concentrated and the residue was subjected to preparative TLC purification using EtOAc to yield 12 mg of compound **18** (32.9%) and 12.8 mg (35,1%) of compound **19**.

*4.4.13.1. (23R)-4β, 12β,21-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**18**).* White amorphous solid, $[\alpha]_{21}D$: −222.1 (c 0,28, MeOH); UV (MeOH) λmax (log ε): 250 (4,31), 221 (4,50) nm; IR (film) ν_{max}: 3438, 3017, 2964, 2927, 1741, 1668, 1216, 1012, 759 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.66 dd (1H, J = 10.5, 4.3 Hz, H-3), 5.83 dd (1H, J = 5.5,1.9 Hz, H-6), 5.81 dd (1H, J = 10.5,0.5 Hz, H-2), 4.55 brd (1H, J = 4.3 Hz, H-4), 4.26 d (1H, J = 12.7 Hz, H-21a), 4.14 d (1H, J = 12.7 Hz, H-21b), 2.56 m (2H, H_2-16), 2.34 m (1H, H-11α), 2.32 m (1H, H-14), 2.18 brs (3H, H_3-28), 2.09 m (1H, H-7β), 1.95 brs (3H, H_3-27), 1.83 m (1H, H-15α), 1.75 m (1H, H-9), 1.69 m (1H, H-7α), 1.65 m (1H, H-15β), 1.51 m (1H, H-8), 1.46 m (1H, H-11β), 1.31 s (3H, H_3-19), 1.07 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz: 201.9 (C, C-1), 193.9 (C, C-22), 172.7 (C, C-26), 166.0 (C, C-17), 159.8 (C, C-24), 142.7 (CH, C-3), 138.3 (C, C-5), 130.4 (CH, C-6), 128.4 (CH, C-2), 128.3 (C, C-25), 127.6 (C, C-20), 91.1 (C, C-23), 75.3 (C, C-12), 68.9 (CH, C-4), 58.4 (CH_2, C-21), 48.7 (C, C-10), 48.5 (C, C-13), 46.7 (CH, C-14), 40.5 (CH, C-9), 34.4 (CH_2, C-11), 31.3 (CH, C-8), 29.6 (CH_2, C-7), 25.2 (CH_2, C-16), 22.8 (CH_2, C-15), 22.1 (CH_3, C-19),15.6 (CH_3, C-28), 14.3 (CH_3, C-18), 8.9 (CH_3, C-27). HRESIMS m/z $[M+Na]^+$ 503.2048 (calcd for $C_{28}H_{32}O_7Na$, 503.2046).

*4.4.13.2. (23R)-6β,12β,21-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,4,17,24-tetraen-26,23-olide (**19**).* White amorphous solid, $[\alpha]_{21}D$: −310.0 (c 0.18, MeOH); UV (MeOH) λmax (log ε): 232 (4.22) nm; IR (film) ν_{max}: 3437, 2925, 1739, 1657, 1012, 769 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.82 dd (1H, J = 9.7, 5.9 Hz, H-3), 6.05 d (1H, J = 5.9 Hz, H-4), 5.85 d (1H, J = 9.7 Hz, H-2), 4.48 t (1H, J = 2.6 Hz, H-6), 4.22 d (1H, J = 12.2 Hz, H-21a), 4.13 d (1H, J = 12.2 Hz, H-21b), 2.55 t (2H, J = 6.0 Hz, H_2-16), 2.32 dd (1H, J = 14.4, 3.7 Hz, H-11α), 2.17 m (1H, H-14), 2.16 s (3H, H_3-28), 2.02 m (1H, H-8), 1.96 m (1H, H-7β), 1.79 s (3H, H_3-27), 1.77 m (1H, H-15α), 1.69 m (1H, H-15β), 1.53 t (1H, J = 14.0 Hz, H-11β), 1.34 s (3H, H_3-19), 1.30 m (1H, H-9), 1.23 m (1H, H-7α), 1.11 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 205.2 (C, C-1), 193.4 (C, C-22), 172.4 (C, C-26), 166.3 (C, C-17), 160.7 (C, C-24), 156.4 (C, C-5), 139.9 (CH, C-3), 127.4 (C, C-25), 127.2 (C, C-20), 126.1 (CH, C-2), 119.1 (CH, C-4), 91.2 (C, C-23), 75.1 (C, C-12),

73.0 (CH, C-6), 58.3 (CH_2, C-21), 52.7 (C, C-10), 48.5 (C, C-13), 46.1 (CH, C-14), 45.5 (CH, C-9), 37.5 (CH_2, C-7), 32.9 (CH_2, C-11), 29.9 (CH, C-8), 25.0 (CH_2, C-16), 23.1 (CH_2, C-15), 15.7 (CH_3, C-28), 19.0 (CH_3, C-19), 14.4 (CH_3, C-18), 8.3 (CH_3, C-27). HRESIMS *m/z* $[M+Na-H_2O]^+$ 485.1933 (calcd for $C_{28}H_{30}O_6Na$, 485.1940).

4.4.14. Preparation of (23R)-21-acetoxy-12β-hydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-lactame (**20**)

Jaborosalactone 5 (26 mg, 0.056 mmol) and NH_4AcO (86.3 mg, 1.1 mmol) were dissolved in acetic acid (5 mL). The reaction mixture was refluxed for 18 h. Then the reaction mixture was concentrated and the residue was subjected to preparative TLC purification using EtOAc to yield 15.9 mg (56.2%) of compound **20** as a white amorphous solid; $[\alpha]_{21}D$: −211.5 (c 0.26, MeOH); UV (MeOH) λmax (log ε): 224 (4.34) nm; IR (film) ν_{max}: 3459, 3367, 2924, 2854, 1740, 1666, 1246, 1015, 758 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.67 ddd (1H, *J* = 10.0, 5.0, 2.4 Hz, H-3), 5.74 dd (1H, *J* = 10.0, 2.4 Hz, H-2), 5.47 brd (1H, *J* = 5.6 Hz, H-6), 4.71 d (1H, *J* = 12.0 Hz, H-21a), 4.63 d (1H, *J* = 12.0 Hz, H-21b), 3.18 brd (1H, *J* = 21.0 Hz, H-4β), 2.77 dd (1H, *J* = 21.0,5.0 Hz, H-4α), 2.48 dd (1H, *J* = 13.7, 3.3 Hz, H-11), 2.39 ddd (1H, *J* = 19.0, 11.0, 7.0 Hz, H-14), 2.19 s (3H, H_3-28), 1.97 s (3H, C*H*$_3$COO-21), 1.94 s (3H, H_3-27), 1.09 s (3H, H_3-19), 1.06 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 202.8 (C, C-1), 191.6 (C, C-22), 172.7 (C, C-26), 170.8 (C, CH_3COO-21), 168.5 (C, C-17), 159.3 (C, C-24), 144.9 (CH, C-3), 135.2 (C, C-5), 127.7 (CH, C-2), 128.4 (C, C-20), 123.9 (C, C-25), 124.1 (CH, C-6), 90.6 (C, C-23), 75.6 (C, C-12), 58.3 (CH_2, C-21), 49.7 (C, C-10), 48.8 (C, C-13), 46.7 (CH, C-14), 40.6 (CH, C-9), 34.9 (CH_2, C-11), 31.9 (CH, C-8), 33.2 (CH_2, C-4), 29.1 (CH_2, C-7), 25.6 (CH_2, C-16), 23.2 (CH_2, C-15), 20.9 (CH_3, *C*H_3COO-21), 15.8 (CH_3, C-28), 18.3 (CH_3, C-19), 14.3 (CH_3, C-18), 9.0 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 529.2219 (calcd for $C_{30}H_{35}NO_6$ Na, 529.2202).

4.4.15. Preparation of (22R,23S)-21,12β, 22α-trihydroxy-1,22-dioxo-12,23-cycloergostan-2,5,17,24-tetraen-26,23-olide (**21**)

$LiAlH_4$ (9.2 mg, 0.02 mmol) dissolved in 0.3 mL of ether was added to a solution of jaborosalactone 5 (35 mg, 0.075 mmol) in anhydrous ether (2 mL) and THF (1.5 mL). The reaction mixture was stirred at room temperature. After 40 h of reaction, the reaction mixture was refluxed for an additional 16 h. Then, ice and 1 mL of distilled H_2O were added. The aqueous phase was partitioned with ether. The resulting organic phase was dried with anhydrous Na_2SO_4. The filtrate was evaporated to dryness and purified by preparative TLC using EtOAc to obtain 1.1 mg (3%) of compound **21** as a white amorphous solid; $[\alpha]_{21}D$: −271 (c 0.04, MeOH); UV (MeOH) λmax (log ε): 209 (4.37) nm; IR (film) ν_{max}: 3415, 2964, 2921, 1747, 1668, 1439, 1382, 1007, 757 cm^{-1}. ^1H NMR ($CDCl_3$, 400.13 MHz): 6.72 ddd (1H, *J* = 10.0, 5.0, 2.4 Hz, H-3), 5.81 dd(1H, *J* = 10.0, 2.4 Hz, H-2), 5.53 brd (1H, *J* = 5.6 Hz, H-6), 5.14 brs (1H, H-22), 4.39 d (1H, *J* = 12.1 Hz, H-21a), 4.165 d (1H, *J* = 12.1 Hz, H-21b), 3.25 brd (1H, *J* = 21.0 Hz, H-4β), 2,82 dd (1H, *J* = 21.0,5.0 Hz, H-4α), 2.51 dd (1H, *J* = 14.0, 4.0 Hz, H-11α), 2.36 m (2H, H_2-16), 2.21 s (3H, H_3-28), 2.13 m (1H, H-14), 2.02 s (3H, H_3-27), 1.98 m (1H, H-7β), 1.78 m (1H, H-9), 1.74 m (1H, H-15α), 1.66 m (1H, H-7α), 1.53 m (1H, H-15β), 1.36 m (1H, H-11β), 1.35 m (1H, H-8), 1.16 s (3H, H_3-19), 0.97 s (3H, H_3-18). ^{13}C NMR ($CDCl_3$ 100.03 MHz): 203.0 (C, C-1), 174.2 (C, C-26), 160.8 (C, C-24), 144.7 (CH, C-3), 144.6 (C, C-17), 135.2 (C, C-5), 127.7 (CH, C-2), 127.5 (C, C-25), 124.3 (CH, C-6), 123.9 (C, C-20), 93.5 (C, C-23), 76.3 (C, C-12), 69.2 (CH, C-22), 60.3 (CH_2, C-21), 49.8 (C, C-10), 47.9 (CH, C-14), 47.8 (C, C-13), 35.8 (CH_2, C-11), 41.0 (CH, C-9), 33.2 (CH_2, C-4), 31.6 (CH, C-8), 29.1 (CH_2, C-7), 23.7 (CH_2, C-16), 23.0 (CH_2, C-15), 18.3 (CH_3, C-19), 14.7 (CH_3, C-28), 12.6 (CH_3, C-18), 8.8 (CH_3, C-27). HRESIMS *m/z* $[M+Na]^+$ 489.2257 (calcd for $C_{28}H_{34}O_6Na$, 489.2253).

4.5. Biological assays

4.5.1. Materials

All starting materials were commercially available research-grade chemicals and used without further purification. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) from Gibco (Grand Island, NY), trichloroacetic acid (TCA) and glutamine from Merck (Darmstadt, Germany), and penicillin G, streptomycin, dimethyl sulfoxide (DMSO) and sulforhodamine B (SRB) from Sigma (St Louis, MO).

4.5.2. Cells, culture and plating

The human solid tumor cell lines HBL-100 (breast), HeLa (cervix), SW1573 (non-small cell lung), and WiDr (colon) were used in this study. These cell lines were a kind gift from Prof. Godefridus J. Peters (VU Medical Center, Amsterdam, The Netherlands) and Dr. Rubén P. Machín (HUGC Dr. Negrín, Las Palmas de Gran Canaria, Spain). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine in a 37 °C, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 μL per well at densities of 10 000 (HBL-100 and SW1573), 15 000 (HeLa), and 20 000 (WiDr) cells per well, based on their doubling times.

4.5.3. Chemosensitivity testing

Chemosensitivity tests were performed using the SRB assay of the NCI with slight modifications. Briefly, pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 1–100 μM. The drug treatment was started on day 1 after plating. Drug incubation times comprised 48 h, after which time cells were precipitated with 25 μL ice-cold 50% (w/v) trichloroacetic acid and fixed for 60 min at 4 °C. Then the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells containing only medium. The percentage growth (PG) was calculated in relation to untreated control cells (C) at each of the drug concentration levels based on the difference in OD at the start (T_0) and end of drug exposure (*T*), according to NCI formulas[1]. Therefore, if *T* is greater than or equal to T_0, the calculation is $100 \times [(T-T_0)/(C-T_0)]$. If *T* is less than T_0, denoting cell killing, the calculation is $100 \times [(T-T_0)/(T_0)]$. The effect is defined as percentage of growth, where 50% growth inhibition (GI_{50}) represents the concentration at which PG is +50. With these calculations, a PG value of 0 no difference from the start of drug exposure, while negative PG values denote net cell death.

4.5.4. Cell cycle analysis

Cells were seeded in six well plates at a density of $2.5–5 \times 10^5$ cells/well. After 24 h, test compounds were added to the respective well and incubated for an additional period of 24 h. Cells were trypsinized, harvested, transferred to test tubes (12 × 75 mm) and centrifuged at 1500 rpm for 10 min at 5 °C. The supernatant was discarded and the cell pellets were resuspended in 200 μL of cold PBS and fixed by the addition of 1 mL ice-cold 70% ethanol. Fixed cells were incubated overnight at −20 °C followed by

centrifugation at 1500 rpm for 10 min. The cell pellets were resuspended in 500 μL PBS. Then, 5 μL of DNAse-free RNAse (10 mg/mL) was added, and the cell suspension was incubated in the dark at 37 °C for 30 min. After incubation, 5 μL of propidium iodide (0.5%) was added. Flow cytometric determination of DNA content (25 000 cells/sample) was analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson, San José, CA, USA). The fractions of the cells in G_0/G_1, S, and G_2/M phase were analyzed using cell cycle analysis software, ModFit LT 3.0 (Verity Software House, Topsham, ME, USA).

4.6. Quinone reductase 1 assay

4.6.1. Cell cultures

Cell culture media and supplements were purchased from Gibco (Carlsbad, CA, USA). Hepa 1c1c7 cells were obtained from ATCC (Manassas, VA, USA). Mutant cell lines TAOc1 and BP^rc1 were supplied by Dr. J. P. Whitlock, Jr. (Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305, USA). All cells were maintained and passaged according to ATCC instructions in MEM-α containing 5% antibiotic-antimycotic and 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

4.6.2. Determination of QR1 activity in cell culture

The activity of quinone reductase 1 was assessed in 96-well plates using Hepa 1c1c7 murine hepatoma cells. Briefly, cells were grown to a density of 2×10^4 cells/mL in 200 μL of MEM-α containing 5% antibiotic-antimycotic (Gibco) and 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere. After a 24 h preincubation, the media were changed and cells were treated with the indicated concentrations of sample or control. The cells were incubated with test samples for an additional 48 h. QR1 activity was measured as a function of the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimetylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Protein content was determined via crystal violet staining of identical plates. Specific activity is defined as nmol of formazan formed per mg protein per min. The induction ratio (IR) of QR1 activity represents the specific enzyme activity of agent-treated cells compared with a DMSO-treated control. The concentration to double activity (CD) was determined through a dose–response assay for active substances (IR > 2). Two mutant cell lines derived from wild-type Hepa 1c1c7 cells, TAOc1 and BP^rc1, were also used for testing. These cells either lack a functional Ah receptor or are unable to translocate the receptor–ligand complex to the nucleus, respectively. Use of these cell lines according to the protocol outlined above allowed for identification of mono- and bifunctional inducers.

4.7. Modification of Keap1 protein

4.7.1. Sample preparation

Keap1 (10 μM) was incubated with 14 compounds (200 μg/mL) in 25 μL of 20 mM Tris–HCl buffer (pH 8.0) for 2 h at room temperature. Control incubations included methanol (negative control) and 100 μM isoliquiritigenin (positive control). The reaction was quenched by adding 1 mM dithiothreitol (DTT) followed by incubation for 15 min. Samples were analyzed immediately using MALDI-TOF mass spectrometry.

4.7.2. MALDI-TOF mass spectrometry

A 1 μL aliquot of the reaction mixture was mixed with 1 μL matrix solution which contained sinapinic acid (saturated) in acetonitrile/water (1:1, v/v) acidified with 0.1% trifluoroacetic acid. The mixed sample was then spotted onto the MALDI-TOF target and air dried before analysis. Linear mode TOF mass spectrometry was used to acquire mass spectra over the mass range *m/z* 65 000 to *m/z* 80 000. The signals from 300 laser shots were averaged per mass spectrum.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.045.

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