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Original article

Evaluation of ursolic acid isolated from *Ilex paraguariensis* and derivatives on aromatase inhibition

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Abstract

The inhibitory potency of ursolic acid extracted from *Ilex paraguariensis*, a plant used in South American population for a tea preparation known as maté, and its derivatives to inhibit aromatase activity was assessed and compared to a phytoestrogen apigenin and a steroidal aromatase inhibitor 4-hyroxyandrostenedione (4-OHA). Among all compounds tested only ursolic acid **1** showed an efficient and dose-dependent aromatase inhibition with IC_{50} value of 32 μM as did apigenin ($IC_{50} = 10$ μM), whereas IC_{50} value of 4-OHA was 0.8 μM. Our results show that the incorporation of a metallocene moiety into the ursolic acid derivatives decreases the aromatase inhibition. Moreover, comparison of the structure/inhibitory potency relationship of compounds indicates that the presence of cycle A and the configuration of C3-OH and C17-COOH seems to be more favourable to recognize the active site of aromatase and to block its activity.

Keywords: Aromatase; Ursolic acid; Breast cancer; Pentacyclic triterpene hemisynthesis; Ferrocene; *Ilex paraguariensis*; Maté

1. Introduction

The irreversible conversion of androgens in estrogens is assumed by an enzymatic complex called aromatase formed by the specific cytochrome P-450 aromatase and an ubiquitous flavoprotein NADPH cytochrome P-450 reductase. Expression of aromatase occurs in different species of both sexes in many tissues namely in gonads [1,2]. However, tumours from numerous sites such as breast [3,4] and prostate [5] have been shown to express aromatase. This is why the development of selective aromatase inhibitors to specifically block estrogens synthesis may be useful for the control of pathologic conditions associated with estrogens dependency [6]. Aromatase inhibitors are generally used in clinic in metastatic hormone-dependent breast cancers in often postmenopausal women when anti-estrogens become inefficient or not well tolerated. Besides steroidal and non-steroidal aromatase inhibitors, some natural and synthetic flavonoids were found to inhibit aromatase activity [7].

Ilex paraguariensis is a South American native tree belonging to the Aquifoliaceae family. The dried leaves and twigs from *I. paraguariensis* are used to prepare a tea known as maté, being one of the most commonly consumed beverages in several Southern American countries, including Brazil, Uruguay, Paraguay and Argentina. The leaves of *I. paraguariensis* contain xanthines (mainly caffeine), flavonoid glycosides (as rutin), caffeoylquinic acid derivatives (chlorogenic acids) and a significant amount of triterpenoid saponins (around 10%) having as main aglycone the ursolic acid.

The natural pentacyclic triterpenoid compounds, ursolic or oleanolic acid and several closely related derivatives, have

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exhibited biological and pharmacological properties, such as anti-HIV, hepatoprotective, anti-inflammatory, cytotoxic or anti-microbial activities, which have been summarized in some reviews [8,9]. Mezzetti et al. [10] had previously described the studies of derivatives obtained from ursolic acid for the purpose of pharmacological testing or for use as intermediates in the synthesis of more complex products. Since then, the chemistry of these triterpenoids has been developed and the semi-synthesis of several triterpene derivatives with the A-ring functionalized from oleanolic and maslinic acids has described [11]. The anti-tumour activity of the triterpenoid is well known since the first cytotoxicity report of the betulinic acid [12]. Oleanolic acid has been first used in traditional Asian medicine due to its anti-inflammatory activity [13]. Synthesis and anti-HIV activity of oleanolic acid derivatives have been described by Zhu et al. [14] while other oleanolic acid derivatives like 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) have a potential to be used as a chemo-preventive and chemo-therapeutic agent [15]. Honda et al. [16] have previously reported that CDDO shows high inhibitory activity against production of nitric oxide. CDDO displays potent anti-proliferative, differentiating and apoptosis-inducing activity. Indeed, CDDO induces apoptosis of human acute myeloid leukaemia cells, lung cancer, osteosarcoma, multiple myeloma, chronic lymphocytic leukaemia cells and breast cancer [17]. It has been suggested that ursolic acid of some plant extracts exhibited inhibitory effect against aromatase activity [18,19].

In this work we aimed, first, to better understand the role of C3-OH, C17-COOH and the size of cycle A of the ursolic acid **1** (Fig. 1) extracted from *I. paraguariensis* against aromatase activity previously described [18,19]; second, to obtain derivatives with enhanced inhibitory properties compared to ursolic acid. For the first purpose, the role of the hydroxyl group, carboxylic acid group and the size of cycle A were evaluated by pharmaco-modulation such as oxidation, acetylation, amidation or esterification and by a ring extension Beckmann reaction. To test the second issue, the ursolic acid **1** was covalently linked to a ferrocene moiety at the hydroxyl or carboxyl group position since metallocene derivatives were shown to inhibit certain metabolizing enzymes [20]. Furthermore, metallocene is recognized by cytochrome P-450 system [21], this is why we expected that ferrocene derivatives could interact with the cytochrome P-450 aromatase. In our previous paper, we have shown that some indolizinone or indane derivatives strongly inhibit human aromatase [22]. It was postulated that the phenyl group of these compounds could occupy an extra hydrophobic pocket located in the active site according to models; this interaction could reinforce the interaction between the nitrogen atom of these inhibitors and the hem iron atom of the enzyme. We thus considered that ursolic acid metallocene derivatives could interact with the human aromatase with enhanced activity compared to ursolic acid **1** due to possible interaction between metallocene moiety and this extra hydrophobic pocket.

The structure/inhibitory potency relationship of ursolic acid **1** and derivatives to inhibit aromatase is then discussed.

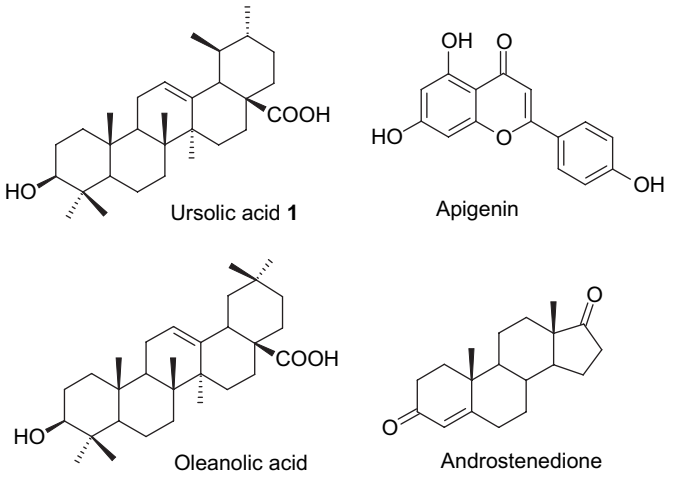


Fig. 1. Structures of ursolic acid **1** and its isomer oleanolic acid (pentacyclic triterpen), apigenin (flavone) and androstenedione (steroid), the substrate of aromatase.

2. Chemistry

2.1. Chemicals

THF was dried by distillation from sodium-benzophenone. Thin-layer and column chromatography were carried out on silica gel $60F_{254}$ and silica gel 70–35 μm, respectively (SDS, Peypin, France). High-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadruple/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 l/h, respectively. The capillary voltage was 3 kV, the cone voltage was 100 V and the RF lens1 energy was optimised for each sample (30–150 V). Lock mass correction, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in 50/50 acetonitrile/water) or of a sodium iodide solution (2 μg/μl in 50/50 propan-2-ol/water + 0.05 μg/μl caesium iodide), was applied for accurate mass measurements. The mass range was typically 50–2050 Da and spectra were recorded at 2 s/scan in the profile mode at a resolution of 10 000 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software. Melting points were determined on a Kofler plate and are given uncorrected. Infrared spectra (IR) were recorded on a NICOLET-210 spectrometer using KBr pellets or a Jasco FT/IR 4200. Optical rotations were measured on a Perkin-Elmer 241 polarimeter; values are given in 10^{-1} deg cm^2 g^{-1}. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a BRUKER AVANCE 500 spectrometer (500 MHz) and tetramethylsilane (TMS) was used as an internal standard. ^{1}H NMR analyses were obtained at 500 MHz (s: singlet, d: doublet, t: triplet, dd: double doublet, td: triple doublet, m: multiplet),

whereas ^{13}C NMR analyses were obtained at 125 MHz. *J* values are given in Hertz. The chemical shifts (δ) are given in parts per million relative to TMS ($\delta = 0.00$).

2.2. Plant material

Aerial parts of *I. paraguariensis* A. St. Hilaire, Aquifoliaceae, were collected in Mato Leitão, RS, Brazil, and they were authenticated by Marcos Sobral (Programa de Pós-Graduação em Ciências Farmacêuticas/UFRGS). A herbarium specimen is on deposit in the Botany Department Herbarium of Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

2.3. Extraction and isolation of ursolic acid from *I. paraguariensis*

In this work, air-dried powdered leaves of *I. paraguariensis* were submitted, to extraction by maceration using 70% EtOH using a plant:solvent relationship of 15%. After evaporation of ethanol under vacuum, the aqueous solution was extracted using cyclohexane in order to eliminate the lipophilic compounds (for example chlorophylls). The resulting aqueous solution was submitted to acid hydrolysis in order to obtain the aglycone that was purified through crystallization. The purification and acid hydrolysis of the hydroethanolic extract of *I. paraguariensis* led to isolation of 8 g of ursolic acid (compound **1**, Fig. 1) with 10% yield. Its purity was determined by HPLC in which ursolic acid presented $R_t = 15$ min and purity >95% [23]. Ursolic acid **1** was obtained as a white amorphous solid and had the molecular formula $C_{30}H_{47}O_3$ as deduced from the HRMS (observed *m/z* 455.3522 $[M + H]^+$), with mp 237–240 °C and optical rotation +72.5 (*c* 0.4, MeOH). Its IR spectrum showed characteristic absorption bands of one hydroxyl group at 3562 cm^{-1} and a carbonyl group at 1697 cm^{-1}. NMR spectral data of **1** showed signals corresponding to a triterpenic skeleton. The ^1H NMR spectrum displayed signals for five methyl groups (δ 0.73, 0.76, 0.87, 0.93 and 1.04) together with signals corresponding to methyl protons of H-30 (δ 0.82, 3H, d, $^3J = 6.4$ Hz) and H-29 (δ 0.91, 3H, d, $^3J = 6.0$ Hz). The signal at δ 2.14 was attributed to H-18 (1H, d, $^3J = 11.3$ Hz) while H-3α signal was observed at δ 3.20 (1H, dd). Finally, at δ 5.40 was observed a large triplet attributed to H-12. Attribution of all resonances displayed on the ^{13}C NMR spectrum of **1** was accomplished by direct comparison with literature data. Ursolic acid **1** was identified as the major aglycone of *I. paraguariensis*. The inhibitory potency of ursolic acid and derivatives (see below for their synthesis) was assessed on aromatase activity and compared to apigenin (Sigma, France), one of the most potent phytoestrogens known to inhibit aromatase activity in vitro [24,25] and to 4-hydroxyandrostenedione (4-OHA, Sigma, France) a known steroidal aromatase inhibitor [26]. Their structures are compared to androstenedione (Sigma, France), the substrate of aromatase (Fig. 1).

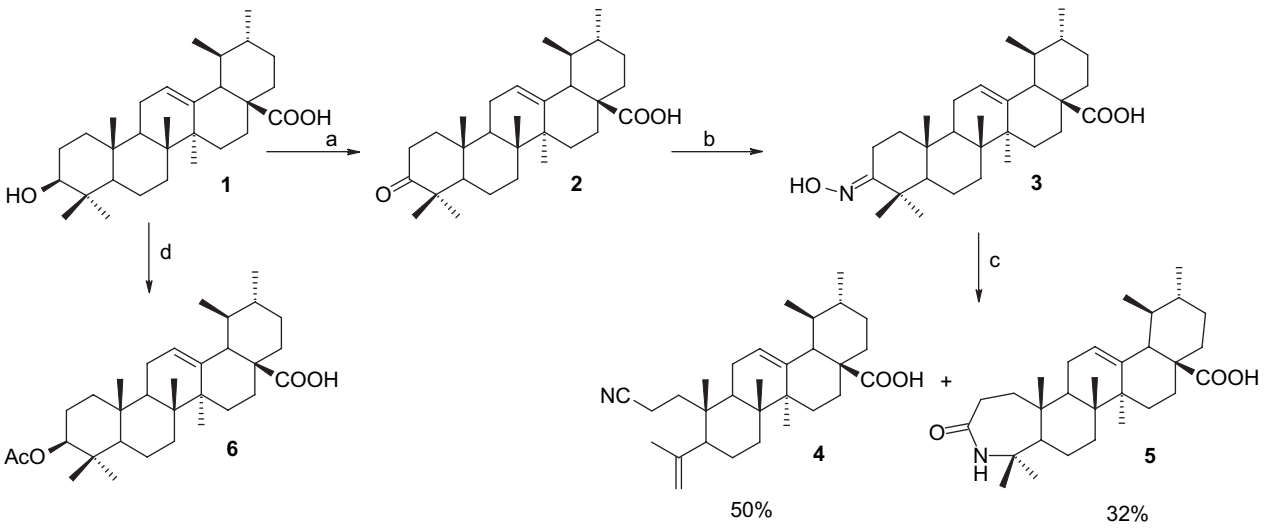
2.4. Ursolic acid *1*

Ursolic acid (8 g) was obtained from *I. paraguariensis* extract in 10% yield. Mp: 237–240 °C. $[\alpha]_D^{20}$ +72 (*c* 0.4, MeOH); IR (ATR, cm^{-1}): 3562 (OH alcohol), 2947 (OH acid), 2866, 1697 (CO), 1460, 1385, 1306, 1030; ^1H NMR (500 MHz, $CDCl_3$:CD_3OD, 9:1): 0.68 (d, $^3J = 11.4$, 1H, CH-5), 0.73 (s, 3H, CH_3-25), 0.76 (s, 4H, CH_3-24 and $CH_{2(H\alpha)}$-1), 0.81 (d, $^3J = 6.4$, 3H, CH_3-30), 0.87 (s, 3H, CH_3-26), 0.90 (d, $^3J = 6.0$, 3H, CH_3-29), 0.93 (m, 4H, CH_3-23 and CH_2-11), 0.98 (m, 1H, CH-20), 1.04 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.33 (m, 3H, CH_2-2, CH-19), 1.47 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, $CH_{2(H\alpha)}$-9, $CH_{2(H\alpha)}$-21), 1.58 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.67 (td, $^3J = 13.9$, 6.8, 1H, $CH_{2(H\alpha)}$-6), 1.81 (td, $^3J = 13.6$, 7.2, 1H, $CH_{2(H\alpha)}$-16), 1.86 (dd, $^3J = 12.6$, 7.3, 2H, CH_2-22), 1.96 (td, $^3J = 13.4$, 4.9, 1H, $CH_{2(H\beta)}$-15), 2.13 (d, $^3J = 11.3$, 1H, CH-18), 3.20 (dd, $^3J = 11.7$, 4.5, 1H, CH-3), 5.40 (m, 1H, CH-12); ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OH-d_4, 9:1): 15.7 (C-25), 15.9 (C-26), 17.1 (C-24), 17.3 (C-29), 18.6 (C-6), 21.5 (C-30), 23.6 (C-11), 23.8 (C-27), 24.5 (C-16), 27.0 (C-15), 28.3 (C-2), 28.3 (C-23), 30.9 (C-21), 33.3 (C-7), 37.1 (C-10), 37.2 (C-22), 38.9 (C-8), 39.0 (C-1), 39.2 (C-4), 39.4 (C-19), 39.7 (C-20), 42.3 (C-14), 47.8 (C-9), 48.1 (C-17), 53.1 (C-18), 55.5 (C-5), 79.1 (C-3), 125.8 (C-12), 138.5 (C-13), 181.1 (C-28); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{30}H_{47}O_3$: 455.3525, found: 455.3522.

2.5. Pharmaco-modulation of ursolic acid

The oxidation of the secondary hydroxyl group of **1** proceeded with enough selectivity to afford the required product **2** in 80% yield, after purification in silica gel column chromatography, together with the starting material (Scheme 1). It was used Jone's reagent in acetone, during 1 h at room temperature [27].

The 3-oxo-ursolic acid **2** was successfully converted to its oxime **3** under mild conditions (hydroxylamine hydrochloride, in absolute ethanol and pyridine at room temperature for 36 h). The oxime **3** was the only product (in 90% yield) observed in HPLC analysis. By HMQC analysis the compound was characterized as anti-oxime. NMR data led us to the conclusion that only one of the oxime isomers was obtained or was stable. The crucial step, in this procedure, was the rearrangement of oxime **3** to the lactam derivative. We used the conditions described in literature [28] using dropwise addition of freshly distilled $SOCl_2$ in THF at 0 °C over the oxime. However, as shown below, Beckmann reaction of compound **3** resulted in **4** as the main product (50%) and **5** as a second product (32%) (Scheme 1). The compound **4** was identified as the product of Beckmann fragmentation and the compound **5** as the product of Beckmann rearrangement. In addition, the crucial step of the Beckmann rearrangement of the triterpene cycle A was the formation of the lactam ring. Thus, treatment of **1** with acetic anhydride and pyridine at room temperature gave the 3-acetylursolic acid **6** in quantitative yield. The synthesis of the amide **11** possessing a ferrocene moiety at C-28



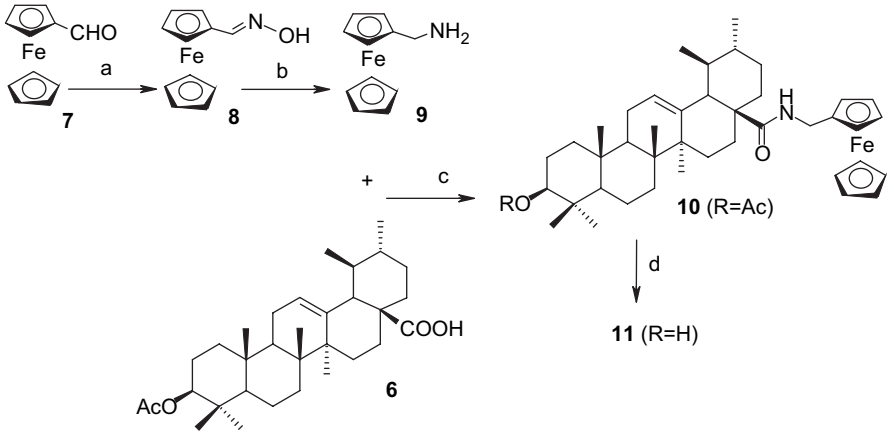
Scheme 1. *Reagents and conditions*: (a) Jone's reagent/acetone **2** (80%); (b) NH_2OH, HCl/pyridine/EtOH **3** (90%); (c) $SOCl_2$/THF **4** (50%), **5** (32%); and (d) acetic anhydride/pyridine **6** (100%).

began with the preparation of the ferrocenylmethylamine **9** [29]. Initially, the oxime **8** was synthesized from formylferrocene **7** by condensation of hydroxylamine at the aldehyde function in 98% yield (Scheme 2). Following, a reduction with $LiAlH_4$ afforded the amine **9** in 65% yield. Finally, a coupling reaction between 3-acetylursolic acid **6** and ferrocenylmethylamine **9** gave the compound **10** in 95% yield. Compound **11** was obtained in 72% yield by deprotection of **10** with 1 N NaOH in refluxing ethanol.

Initially, the commercially available bis(3-aminopropyl) piperazine **12** was protected to afford its *N*-Boc derivative **13**. Five different reaction conditions were evaluated in order to obtain **13** with improved yield (Table 1). When di-*tert*-butyl dicarbonate was used in equimolar concentration to *N*-1,4-bis(3-aminopropyl)piperazine, the compound bi-protected was the only obtained product (Table 1, Entries 1 and 3). We found that conditions similar to those reported by Zheng et al. [30] gave the best selectivity in the *N*-protection, furnishing the desired monoprotected-*N*-1,4-bis(3-aminopropyl)-piperazine **13** in 28% yield (Table 1, Entry 5).

The coupling reaction between 3-acetylursolic acid **6** and piperazine **13** afforded the compound **14** in 80% yield using oxalyl chloride in dichloromethane, at 0 °C to room temperature, during 24 h. Deprotection of **14** using trifluroacetic acid yielded the *N*-1,4-bis(3-aminopropyl)piperazinyl-ursolamine **15** in 72% yield. After deacetylation with 1 N NaOH in refluxing ethanol, **16** was obtained with 50% yield (Scheme 3).

The synthesis of the ester **22** began with the preparation of the 3-ferrocenylpropanoic acid **19** with conditions similar to those reported by Debroy et al. [31]. Ethyl 3-ferrocenylpropenoate **17** was prepared by the Wittig reaction of formylferrocene **7** with ethoxycarbonylmethylene-triphenylphosphorane in dry toluene, in 51% yield (Scheme 4). We found that the *E*/*Z* ratio was 94/6; *E*-isomer was predominantly formed due to the stabilizing yield used. Ethyl 3-ferrocenylpropanoate **18** was prepared by the selective reduction of the corresponding unsaturated analogue **17** using CuBr and $NaBH_4$ in methanol, in 97% yield. Then, saponification of **18** with 1 N NaOH in refluxing ethanol afforded the 3-ferrocenylpropanoic acid **19** in 82% yield. After protection of the carboxylic group of



Scheme 2. *Reagents and conditions*: (a) NH_2OH, HCl/pyridine/EtOH/NaOH **8** (98%); (b) $LiAlH_4$/THF **9** (65%); (c) ClCOCOCl/CH_2Cl_2 **10** (95%); and (d) 1 N NaOH **11** (72%).

Table 1
Results of *N*-Boc protection of piperazine **12** under various conditions

Entry	Conditions			Yield (%) monoprotected
	Solvent	*N*-1,4-bis (3-aminopropyl) piperazine	Di-*tert*-butyl dicarbonate	
1	Dioxane	1.0	1.0	0
2	Dioxane	1.0	0.5	10
3	Dichloromethane	1.0	1.0	0
4	Dichloromethane	1.0	0.5	10
5	Methanol	1.0	0.5	28

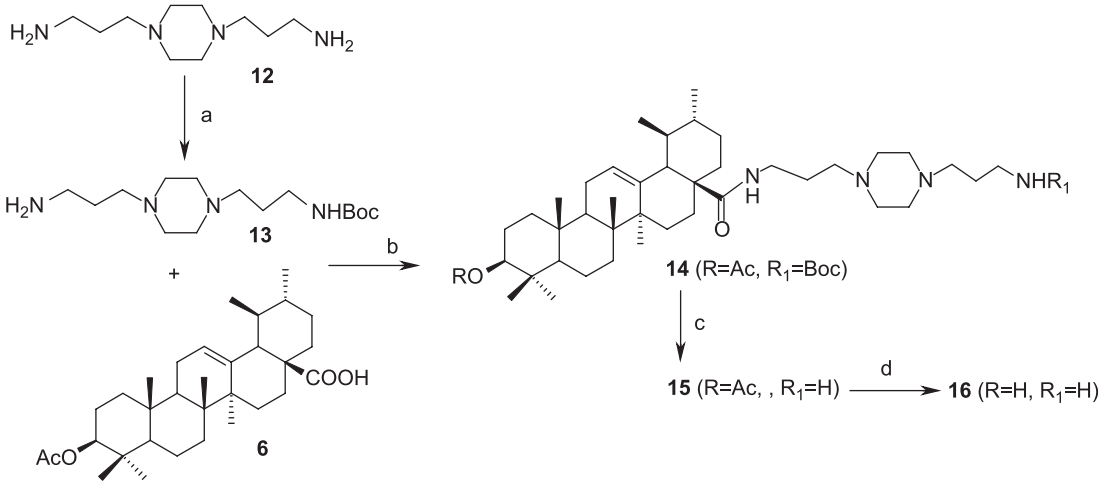
ursolic acid **1** with benzoyl chloride, the ester **20** was obtained in 94% yield. The coupling reaction between the 3-ferrocenylpropanoic acid **19** and **20** afforded the diester **21** in 51% yield using oxalyl chloride in dichloromethane, at 0 °C to room temperature, during 24 h. Hydrogenolysis of the benzyl ester with black palladium afforded the compound **22** in 42% yield after 48 h of reaction.

2.6. 3-Oxo-urs-12-en-28-oic acid *2*

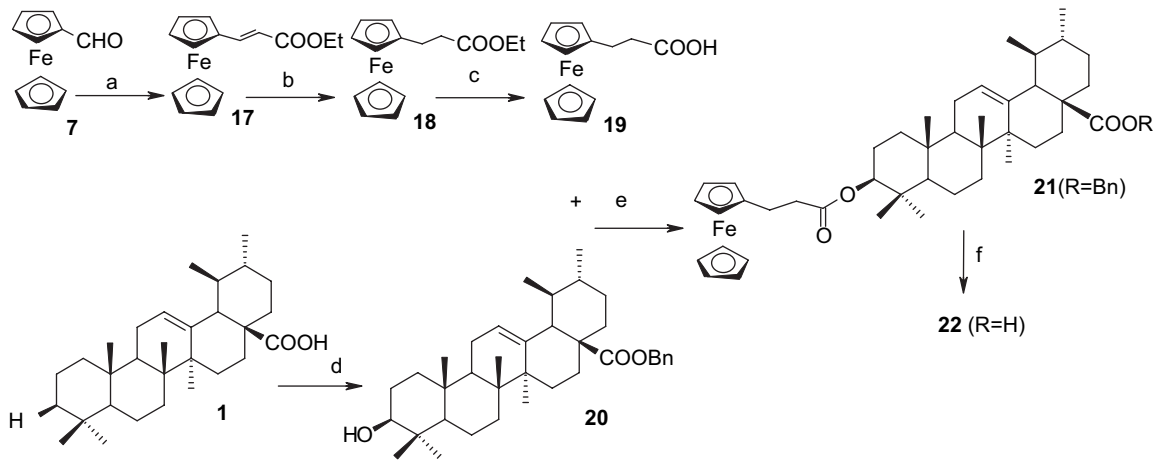
To a solution of **1** (0.455 g, 1 mmol) in acetone (5 ml) at 0 °C, Jone's reagent (1.5 ml) was slowly added. The reaction mixture was then allowed to warm up to room temperature and stirred for 1 h (TLC control). After cooling at 0 °C, *i*-propanol (20 ml) was added and the solution stirred at room temperature for 30 min. The precipitate was collected by filtration, the filtrated solution was then isolated. Final purification was achieved by means of SiO_2 chromatography using dichloromethane as an eluent to afford a white powder in 80% yield. Mp: 172 °C. $[\alpha]_D^{20}$ +83 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 3562 (OH alcohol), 2948 (OH acid), 2866, 1705 (CO), 1697 (CO), 1460, 1385, 1306, 1030; ^1H NMR (500 MHz, $CDCl_3$): 0.87 (s, 3H, CH_3-25 and 1H, CH-5), 0.91 (d, $^3J = 6.3$, 3H, CH_3-30), 0.95 (m, 2H, CH_2-11 and CH-20), 0.99 (d, $^3J = 6.0$, 3H, CH_3-29), 1.07 (s, 3H, CH_3-26), 1.11 (s, 3H, CH_3-24), 1.13 (s, 6H, CH_3-23 and CH_3-27), 1.36 (m, 5H, CH_2-2, CH_2-7 and CH-19), 1.52 (m, 5H, CH_2-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.64 (td, 1H, $^3J = 14.3$, 7.0, $CH_{2(H\alpha)}$-6), 1.76 (dd, $^3J = 13.6$, 7.0, 1H, $CH_{2(H\alpha)}$-16), 1.94 (m, 2H, $CH_{2(H\beta)}$-15 and CH_2-22), 2.24 (d, $^3J = 11.2$, 1H, CH-18), 5.31 (m, 1H, CH-12); ^{13}C NMR (125 MHz, $CDCl_3$): 15.6 (C-25), 15.9 (C-26), 17.3 (C-29), 19.6 (C-6), 19.9 (C-2), 21.6 (C-30), 21.8 (C-24), 23.8 (C-11 and C-27), 24.5 (C-16), 26.9 (C-15), 28.4 (C-23), 31.0 (C-21), 33.7 (C-7), 37.1 (C-10 and C-22), 39.2 (C-1), 39.4 (C-4), 39.7 (C-19), 39.8 (C-20), 40.6 (C-8), 42.7 (C-14), 47.8 (C-9), 48.4 (C-17), 52.9 (C-18), 55.6 (C-5), 125.9 (C-12), 138.5 (C-13), 184.1 (C-28), 217.7 (C-3); HRMS (ESI-MS, *m/z*), $(M + H + Na)^+$ calc. for $C_{30}H_{45}O_3Na$: 477.3345, found: 477.3339.

2.7. (E)-3-Oximeurs-12-en-28-oic acid *3*

NH_2OH, HCl (28 mg, 0.4 mmol) was added to a solution of **2** (77 mg, 0.17 mmol) in dry ethanol (1 ml) and pyridine (0.6 ml). The reaction mixture was stirred for 36 h at room temperature under nitrogen atmosphere. After addition of ice water (20 ml), a solid was filtered and washed with water (twice) to give a white powder in 90% yield. Mp: 188 °C. $[\alpha]_D^{20}$ +48 (*c* 0.2, $CHCl_3$); IR (ATR, cm^{-1}): 2943 (OH acide), 1685 (CO), 1657 (CN), 1460, 1385, 1277, 1045; ^1H NMR (500 MHz, $CDCl_3$ and CD_3OH-d_4): 0.78 (m, 1H, CH-5), 0.73 (s, 3H, CH_3-25), 0.83 (s, 3H, CH_3-24), 0.92 (d, $^3J = 6.4$, 3H, CH_3-30), 0.94 (s, 3H, CH_3-26), 1.02 (d, $^3J = 6.0$, 3H, CH_3-29), 1.06 (m, 4H, CH_3-23 and CH_2-11), 1.05 (m, 1H, CH-20), 1.14 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.32 (m, 2H, $CH_{2(H\alpha)}$-1, CH-19), 1.46 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.60 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.72 (m, 2H, $CH_{2(H\alpha)}$-6, $CH_{2(H\alpha)}$-16), 1.87–2.01 (m, 3H, $CH_{2(H\beta)}$-15, CH_2-22), 2.07 (d, $^3J = 11.0$, 1H, CH-18), 2.17 (m, 2H, CH_2-2), 3.42 (s, 1H, OH), 5,31 (m, 1H, H-12); ^{13}C NMR (125 MHz, $CDCl_3$ and CD_3OH-d_4): 14.3 (C-24), 15.3 (C-25), 17.1 (C-26), 17.5 (C-29), 19.4 (C-6), 21.5 (C-30), 23.4 (C-2), 23.6 (C-11), 23.8 (C-27), 24.5 (C-16),



Scheme 3. *Reagents and conditions*: (a) Boc_2O/MeOH **13** (28%); (b) ClCOCOCl/CH_2Cl_2/Et_3N **14** (80%); (c) 10% TFA/CH_2Cl_2 **15** (72%); and (d) 1 N NaOH **16** (50%).



Scheme 4. *Reagents and conditions*: (a) $Ph_3P{=}CHCO_2Et/PhCH_3$ **17** (*E*/*Z*: 94/6) (51%); (b) $NaBH_4$/CuBr/MeOH **18** (97%); (c) 1 N NaOH/EtOH **19** (82%); (d) BnCl/DMF/K_2CO_3 **20** (94%); (e) $ClCOCOCl/CH_2Cl_2$ **21** (51%); and (f) H_2 Pd/C/MeOH **22** (42%).

27.0 (C-15), 27.9 (C-23), 31.0 (C-21), 32.9 (C-7), 34.2 (C-10), 37.4 (C-22), 38.7 (C-8), 39.6 (C-19), 39.7 (C-20), 40.5 (C-1), 41.5 (C-4), 42.2 (C-14), 47.8 (C-9), 48.1 (C-17), 53.1 (C-18), 55.5 (C-5), 125.8 (C-12), 138.5 (C-13), 168.0 (C-3), 181.4 (C-28).

*2.8. 2-Cyano-2,3-seco-4-yliden-olean-12-enoic acid **4** and 4-aza-A-homo-3-oxo-ursolic acid **5***

To a solution of **3** (0.068 g, 1.12 mmol) in dry THF (0.5 ml) at 0 °C, a solution of $SOCl_2$ (72 μl) in dry THF (0.25 ml) was slowly added under nitrogen atmosphere. The reaction mixture was stirred for 4 h at 0 °C and then ice water was added. After neutralisation with a NH_4OH aqueous solution, the mixture was extracted with CH_2Cl_2 (3 × 2 ml). The organic layer was washed with water, dried, and concentrated. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH: 98/2) to give **4** (32%) and **5** (50%).

*2.9. 2-Cyano-2,3-seco-4-yliden-olean-12-enoic acid **4***

White powder, mp: 130 °C. $[\alpha]_D^{20}$ +37 (*c* 0.15, $CHCl_3$); IR (ATR, cm^{-1}): 2924 (OH acid), 2867, 2246 (CN), 1692 (CO), 1635 ($H_2C{=}C$), 1454; ^1H NMR (500 MHz, CD_3OD): 0.89 (s, 3H, CH_3-26), 0.89–0.98 (m, 3H, CH_2-11 and CH-20), 0.93 (d, $^3J = 6.3$, 3H, CH_3-30), 0.99 (d, $^3J = 6.4$, 3H, CH_3-29), 1.00 (s, 3H, CH_3-25), 1.16 (s, 3H, CH_3-27), 1.20 (m, 1H, $CH_{2(H\alpha)}$-7), 1.35 (m, 1H, CH-19), 1.52 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.71 (m, 5H, $CH_{2(H\alpha)}$-6, $CH_{2(H\alpha)}$-15, CH_2-16, $CH_{2(H\beta)}$-21), 1.78 (s, 3H, CH_3-23), 1.94 (dd, $^3J = 12.5$, 7.3, 2H, CH_2-22), 2.07 (m, 2H, $CH_{2(H\beta)}$-15, CH-18), 2.29 (m, 3H, CH_2-1 and CH-5), 2.39 (m, 2H, CH_2-2), 4.69 (d, $^2J = 0.8$, 1H, CH_2-24), 4.95 (d, $^2J = 0.8$, 1H, CH_2-24), 5.35 (m, 1H, H-12); ^{13}C NMR (125 MHz, CD_3OH-d_4): 12.0 (C-2), 17.4 (C-25), 17.5 (C-26 and C-29), 19.5 (C-6), 21.6 (C-30), 23.8 (C-11), 23.9 (C-27), 24.4 (C-16), 28.3 (C-15), 30.9 (C-21), 32.8 (C-7), 37.0 (C-22), 38.1(C-10), 39.2 (C-19), 39.6 (C-20), 39.8 (C-8), 41.5 (C-1), 42.9 (C-14), 46.9 (C-17), 48.4 (C-9), 51.1 (C-18), 53.1 (C-5), 114.6 (C-24), 120.6 (C-3), 125.9 (C-12), 138.7 (C-13), 147.2 (C-4), 183.2 (C-28); HRMS (ESI-MS, *m*/*z*), $(M + Na)^+$ calc. for $C_{30}H_{45}NO_2Na$: 474.3348, found: 474.3352.

*2.10. 4-Aza-A-homo-3-oxo-ursolic acid **5***

White powder, mp: 180 °C. $[\alpha]_D^{20}$ +33 (*c* 0.2, $CHCl_3$); IR (ATR, cm^{-1}): 3027 (NH), 2931 (OH acid), 2855, 1694 (CO), 1680 (CO), 1457; ^1H NMR (500 MHz, $CDCl_3$): 0.87 (m, 1H, CH-5), 0.89 (s, 3H, CH_3-26), 0.90 (d, $^3J = 6.4$, 3H, CH_3-30), 0.92 (m, 3H, CH_2-11, CH-20), 1.00 (d, $^3J = 6.0$, 3H, CH_3-29), 1.14 (s, 3H, CH_3-27), 1.18 (m, 1H, $CH_{2(H\alpha)}$-7), 1.19 (s, 3H, CH_3-25), 1.34 (m, 1H, CH-19), 1.37 (s, 3H, CH_3-24), 1.48 (s, 3H, CH_3-23), 1.50 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.60 (m, 5H, CH_2-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.67 (td, $^3J = 13.9$, 6.8, 1H, $CH_{2(H\alpha)}$-6), 1.74 (td, $^3J = 13.6$, 7.0, 1H, $CH_{2(H\alpha)}$-16), 1.90 (dd, $^3J = 12.0$, 7.3, 2H, CH_2-22), 2.03 (td, $^3J = 13.8$, 4.8, 1H, $CH_{2(H\beta)}$-15), 2.27 (d, $^3J = 11.3$, CH-18), 2.58 (td, $^3J = 14.0$, 6.4, 2H, CH_2-2), 5.35 (m, 1H, CH-12), 8.70 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 17.0 (C-25), 17.4 (C-26), 17.5 (C-29), 21.5 (C-30), 21.9 (C-6), 23.8 (C-11), 23.9 (C-27), 24.5 (C-24), 26.0 (C-16), 28.3 (C-15), 30.1 (C-2), 30.9 (C-21), 33.0 (C-7), 34.7 (C-23), 37.0 (C-22 and C-10), 39.2 (C-19), 39.4 (C-20), 40.0 (C-8), 41.3 (C-1), 42,7 (C-14), 47.4 (C-17), 48.3 (C-9), 52.9 (C-18), 55.3 (C-5), 59.4 (C-4), 125.7 (C-12), 138.5 (C-13), 179.4 (C-3), 182.1 (C-28); HRMS (ESI-MS, *m*/*z*), $(M + Na)^+$ calc. for $C_{30}H_{47}NO_3Na$: 492.3454, found: 492.3466.

*2.11. 3-Acetylursolic acid **6***

To a solution of **1** (0.091 g, 0.2 mmol) in dry pyridine (2.5 ml) at room temperature, anhydride acetic (2.5 ml) was added slowly. The reaction mixture was stirred for 24 h at room temperature and then poured in ice water. After filtration, the residue was washed with water to give **6** (100%) as

a white powder. Mp: 170 °C. $[\alpha]_D^{20}$ +99 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 3562 (OH alcohol), 2947 (OH acid), 2866, 1732 (CO), 1697 (CO), 1460, 1385, 1306, 1248; ^1H NMR (500 MHz, $CDCl_3$): 0.83 (s, 3H, CH_3-25), 0.87 (m, 1H, CH-5), 0.90 (s, 3H, CH_3-24), 0.91 (s, 3H, CH_3-23), 0.92 (d, $^3J = 5.2$, 3H, CH_3-30), 0.93 (m, 1H, CH_2-1), 1.00 (d, $^3J = 6.8$, 3H, CH_3-29, CH_2-20), 1.01 (s, 3H, CH_3-26), 1.13 (s, 3H, CH_3-27), 1.32 (m, 1H, CH-19), 1.39 (m, 4H, CH_2-2, $CH_{2(H\beta)}$-6, $CH_{2(H\alpha)}$-21), 1.56 (m, 6H, $CH_{2(H\beta)}$-1, CH_2-7, CHα-9, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.67 (td, $^3J = 13.9$, 6.8, 1H, $CH_{2(H\alpha)}$-6), 1.84 (m, 1H, $CH_{2(H\alpha)}$-16), 1.96 (m, 2H, CH_2-22), 2.07 (td, $^3J = 13.5$, 5,0, 1H, $CH_{2(H\beta)}$-15), 2.10 (s, 3H, H_3CCOO), 2.13 (d, $^3J = 11.3$, 1H, CH-18), 4.55 (dd, $^3J = 13.3$, 7.5, 1H, CH-3), 5.30 (m, 1H, CH-12); ^{13}C NMR (125 MHz, $CDCl_3$): 15.7 (C-25), 17.5 (C-26), 17.7 (C-29), 18.7 (C-6), 21.4 (C-24), 21.6 (C-30 and H_3CCOO), 23.4 (C-2), 23.9 (C-11), 23.9 (C-27), 25.1 (C-16), 28.2 (C-23), 28,5 (C-15), 31.3 (C-21), 33.4 (C-7), 37.3 (C-10), 37.8 (C-22), 38.1 (C-4), 38.8 (C-1), 39.2 (C-19), 39.8 (C-20), 40.9 (C-8), 42.2 (C-14), 46.3 (C-9), 47.9 (C-17), 54.0 (C-18), 55.7 (C-5), 80.3 (C-3), 124.9 (C-12), 140.1 (C-13), 171.7 (H_3CCOO-), 182.1 (C-28); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{32}H_{49}O_4$: 497.3631, found: 497.3635.

2.12. Oximeferrocenylcarboxaldehyde **8**

A mixture of ferrocenecarboxaldehyde (500 mg, 2.3 mmol), NaOH (550 mg, 14.7 mmol) and hydroxylamine chlorhydrate in dry ethanol (25 ml) was heated under reflux for 3 h. Then, water (25 ml) was added and the mixture was stirred for 1 h at room temperature. The solution was extracted with CH_2Cl_2 (10 ml × 3). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo to give an orange powder (98%) which was used without further purification in the next step. Mp: 105 °C. IR (ATR, cm^{-1}): 3209 (OH), 2926, 2856, 1648 (CN), 1565 (C=C), 1106 and 1001 (Cp-ferrocene); ^1H NMR (500 MHz, $CDCl_3$): 4.28 (s, 5H, Cp′-ferrocene), 4.39 (m, 2H, Cp-ferrocene), 4.60 (m, 2H, Cp-ferrocene), 8.06 (s, 1H, CH=N); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{11}H_{12}NOFe$: 230.0124, found: 230.0141.

2.13. N-Ferrocenylmethyl-3-O-acetylursolamide **10**

To a solution of 3-acetylursolic acid **6** (49.6 mg, 0.1 mmol) in dry CH_2Cl_2 (1 ml), under nitrogen atmosphere, oxalyl chloride (26 μl, 0.3 mmol) was added at 0 °C. The reaction was stirred at room temperature for 3 h. After the mixture was cooled at 0 °C, TEA (84 μl, 0.6 mmol) and amine **9** (64.5 mg, 0.3 mmol) were added. After stirring for 24 h at room temperature, water (1 ml) was added. The solution was extracted with CH_2Cl_2 (3 ml) (three times). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo to give a yellow oil. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH: 95/5) to give a yellow oil (95%). $[\alpha]_D^{20}$ +53 (*c* 0.2; $CHCl_3$); IR (ATR, cm^{-1}): 3096 (NH), 2870, 1731 (CO), 1648 (CO), 1508 (C=C), 1457, 1368, 1243, 1106 and 1001 (Cp-ferrocene); ^1H NMR (500 MHz, $CDCl_3$): 0.76 (m, 1H, CH-5), 0.83 (d, $^3J = 6.5$, 3H, CH_3-30), 0.91 (d, $^3J = 6.3$, 3H, CH_3-29), 0.93 (m, 1H, CH_2-1), 0.97 (s, 3H, CH_3-25), 0.95 (m, 1H, CH_2-11), 1,00 (m, 4H, CH_3-24, CH-20), 1.03 (s, 3H, CH_3-23), 1.05 (s, 3H, CH_3-26), 1.13 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.38 (m, 3H, CH-19, CH_2-2), 1.48 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.60 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.69 (m, 1H, $CH_{2(H\alpha)}$-6), 1.78 (td, $^3J = 14.0$, 7.2, 1H, $CH_{2(H\alpha)}$-16), 1.91 (d, $^3J = 10.3$, 1H, CH-18), 1.99 (m, 3H, $CH_{2(H\beta)}$-15, CH_2-22), 2.04 (s, 3H, H_3CCOO), 3.92 (d, $^3J = 13.4$, 1H, CH_2-ferrocene), 4.23 (m, 11H, Cp-ferrocene, CH-ferrocene, CH-3), 5.31 (m, 1H, CH-12), 6.10 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 16.2 (C-25), 17.1 (C-26), 17.4 (C-24), 17.9 (C-29), 18.6 (C-6), 21.6 (C-30), 21.7 (H_3CCOO), 23.7 (C-11), 23.9 (C-27), 24.2 (C-2), 25.4 (C-16), 28.4 (C-23), 28.5 (C-15), 31.3 (C-21), 33.2 (C-7), 37.3 (C-10), 37.7 (C-22), 38.1 (C-4), 38.7 (C-1), 39.8 (C-19), 39.9 (C-20), 40.1 (C-8), 43.0 (C-14), 47.9 (C-9, C-17), 53.4 (C-18), 55.7 (C-5), 69.1 (Cp), 69.6 (Cp′), 70.0 (Cp, CH_2-ferrocene), 81.3 (C-3), 125.9 (C-12), 139.0 (C-13), 171.8 (H_3CCOO), 178.2 (C-28); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{43}H_{61}NO_3Fe$: 695.4001, found: 695.4020.

2.14. N-Ferrocenylmethylursolamide **11**

To acetylursolamide **10** (40 mg, 0.057 mmol) in ethanol (2 ml), 1 N NaOH (1 ml) was added. The reaction was refluxed for 2 h. Ethanol was then removed under reduced pression, the aqueous layer was extracted with CH_2Cl_2 (1 ml × 3). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo to give a yellow oil. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH: 95/5) to give a yellow powder (72%). Mp: 148 °C. $[\alpha]_D^{20}$ +50 (*c* 0.3, $CHCl_3$); IR (ATR, cm^{-1}): 3096 (NH, OH), 2868, 1638 (CO), 1518 (C=C), 1457, 1377, 1271, 1106 and 999 (Cp-ferrocene); ^1H NMR (500 MHz, $CDCl_3$): 0.77 (m, 1H, CH-5), 0.83 (d, $^3J = 6.5$, 3H, CH_3-30), 0.91 (d, $^3J = 6.3$, 3H, CH_3-29), 0.93 (m, 1H, CH_2-1), 0.97 (s, 3H, CH_3-25), 0.95 (m, 1H, CH_2-11), 1.00 (m, 4H, CH_3-24, CH-20), 1.04 (s, 3H, CH_3-23), 1.05 (s, 3H, CH_3-26), 1.14 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.38 (m, 3H, CH-19, CH_2-2), 1.48 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.59 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.69 (m, 1H, $CH_{2(H\alpha)}$-6), 1.78 (td, $^3J = 14.0$, 6.0, 1H, $CH_{2(H\alpha)}$-16), 1.91 (d, $^3J = 10.3$, 1H, CH-18), 2.00 (m, 3H, $CH_{2(H\beta)}$-15, CH_2-22), 3.26 (dd, $^3J = 12.0$, 5.5, 1H, CH-3), 3.92 (d, $^3J = 13.4$, 1H, CH_2-ferrocene), 4.23 (m, 10H, Cp-ferrocene, CH-ferrocene), 5.35 (m, 1H, CH-12), 6.10 (m, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 16.0 (C-25, C-26), 17.5 (C-24), 17.8 (C-29), 18.7 (C-6), 21.7 (C-30), 23.7 (C-11), 23.8 (C-27), 25.3 (C-16), 27.5 (C-2), 28.2 (C-23), 28.5 (C-15), 31.3 (C-21), 33.1 (C-7), 37.3 (C-10), 37.7 (C-22), 38.9 (C-1), 39.3 (C-4), 39.6 (C-19), 39.9 (C-20), 40.1 (C-8), 42.9 (C-14), 47.9 (C-9), 48.0 (C-17), 54.5 (C-18), 55.5 (C-5), 68.2 (Cp), 68.5 (Cp′), 69.2 (Cp, CH_2-ferrocene), 79.3 (C-3), 126.0 (C-12), 140.4 (C-13), 177.7 (C-28).

2.15. Terbutyl 3-[4-(3-aminopropyl)piperazinylpropyl] carbamate **13**

To a solution of 1,4-bis(3-aminopropyl)piperazine **12** (444 mg, 2,2 mmol) in dry MeOH (5 ml) was added dropwise a solution of Boc_2O (293 mg, 1.3 mmol) in dry MeOH (2.5 ml) at 0 °C. The reaction was stirred at room temperature for 48 h. The solvent was then removed under reduced pressure and the residue dissolved in diethylether (20 ml). The organic layer was extracted with 5% aq citric acid (5 ml × 3). The aqueous phase was washed with ethylacetate and Na_2CO_3 (satd) was added until pH: 11. The solution was extracted with ethylacetate (10 ml × 3). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo to give a yellow oil (28%) which was used without further purification in the next step. IR (ATR, cm^{-1}): 3365 (NH_2), 2940, 2813, 1685 (CO), 1390, 1250; ^1H NMR (500 MHz, $CDCl_3$): 1.48 (s, 9H, CH_3-Boc), 1.72 (m, 4H, CH_2-γ, CH_2-γ′), 2.50 (m, 12H, CH_2-α1, CH_2-α1′, CH_2-α2, CH_2-α2′, CH_2-β and CH_2-β′), 2.80 (t, $^3J = 6.9$, 2H, CH_2-δ); 3.12 (t, $^3J = 6.7$, 2H, CH_2-δ′); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{15}H_{33}N_4O_2$: 301.2604, found: 301.2622.

2.16. N-{3-[4-(3-aminopropyl)piperazinopropyl] terbutylcarbamate}-3-O-acetylursolamide **14**

To a solution of 3-*O*-acetylursolic acid **6** (1.48 g, 3 mmol) in dry CH_2Cl_2 (30 ml) at 0 °C was added dropwise oxalyl chloride (7.8 ml, 9 mmol). The reaction was stirred at room temperature for 3 h and then TEA (25 ml, 18 mmol) and *N-tert*-butoxycarbonyl-1,4-bis(3-aminopropyl)piperazine **13** (2.7 g, 9 mmol) were added. The mixture was stirred for 24 h and then water (10 ml) and CH_2Cl_2 (30 ml × 3) were added. The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH: 95/5) to give a white powder (80%). Mp: 152 °C. $[\alpha]_D^{20}$ +60 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 3325 (NH), 2873, 2807, 1731 (CO), 1683 (CO), 1640 (C═C), 1460, 1365, 1310, 1245, 1010; ^1H NMR (500 MHz, $CDCl_3$): 0.79 (d, $^3J = 6.9$, 3H, CH_3-30), 0.86 (m, 1H, CH-5), 0.89 (d, $^3J = 5.2$, 3H, CH_3-29), 0.93 (m, 1H, $CH_{2(H\alpha)}$-1), 0.94 (s, 3H, CH_3-25), 0.96 (m, 1H, CH_2-20), 0.98 (s, 3H, CH_3-24), 1.00 (s, 6H, CH_3-26, CH_3-23), 1.12 (s, 3H, CH_3-27), 1.19 (m, 1H, $CH_{2(H\alpha)}$-7), 1.33 (m, 3H, CH_2-2, CH-19), 1.48 (s, 9H, CH_3-Boc), 1.56 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.66 (m, 10H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-6, $CH_{2(H\alpha)}$-15, CH_2-16, $CH_{2(H\beta)}$-21, CH_2-γ, CH_2-γ′), 1.83 (m, 2H, CH_2-22), 1.99 (m, 1H, $CH_{2(H\beta)}$-15), 2.10 (s, 3H, H_3CCOO), 2.56 (m, 13H, CH_2-α1, CH_2- α1′, CH_2-α2, CH_2-α2′; CH_2-β, CH_2-β′, CH-18), 3.23 (m, 2H, CH_2-d′), 3.49 (m, 2H, CH_2-d), 4.54 (dd, $^3J = 11.6$, 6.5, 1H, CH-3), 5.35 (m, 1H, CH-12), 6.56 (m, 1H, NH); ^{13}C NMR (125 MHz, CDCl3): 15.9 (C-25), 17.2 (C-26), 17.3 (C-26), 17.8 (C-29), 18.5 (C-6), 21.7 (C-30), 21.8 (H_3CCOO), 23.8 (C-11), 23.9 (C-27), 24.1 (C-2), 25.1 (C-16), 26.0 (C-γ), 28.2 (C-23), 28.5 (C-15), 28.9 (3 × CH_3-Boc), 31.2 (C-21), 33.0 (C-7), 34.4 (C-γ′), 37.2 (C-10), 37.8 (C-22), 38.1 (C-4), 38.6 (C-1), 39.2 (C-d), 39.4 (C-19), 39.9 (C-20), 40.0 (C-8), 42.7 (C-14), 47.8 (C-9), 47.9 (C-17), 53.4 (C-α1, C-α1′, C-α2, C-α2′), 53,7 (C-δ′), 53,9 (C-18), 55.6 (C-5), 57.1 (C-β), 57.6 (C-β′), 79.3 (Cq-Boc), 81.2 (C-3), 125.7 (C-12), 140.0 (C-13), 156.5 (CO Boc), 171.6 (H_3CCOO), 178.3 (C-28); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{47}H_{81}N_4O_5$: 781.6207, found: 781.6224.

2.17. N-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-O-acetylursolamide **15**

To a solution of acetylursolamide **14** (1 g, 1.28 mmol) in CH_2Cl_2 (20 ml) at room temperature was added dropwise 10% TFA in CH_2Cl_2 (20 ml). The reaction was stirred at room temperature for 3 h. The solution was washed with H_2O (40 ml × 2), K_2CO_3 (satd), and H_2O (40 ml × 2). The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. A white powder (72%) was obtained, which was used without further purification in the next step. Mp: 138 °C. $[\alpha]_D^{20}$ +54 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 3325 (NH_2), 2873, 2807, 1731 (CO), 1683 (CO), 1637 (C═C), 1462, 1367, 1308, 1245, 1008; ^1H NMR (500 MHz, $CDCl_3$): 0.81 (d, $^3J = 6.9$, 3H, CH_3-30), 0.87 (m, 1H, CH-5), 0.91 (d, $^3J = 6.6$, 3H, CH_3-29), 0.93 (m, 1H, $CH_{2(H\alpha)}$-1), 0.94 (s, 3H, CH_3-25), 0.96 (m, 1H, CH_2-20), 0.94 (m, 1H, CH-11), 0.99 (s, 3H, CH_3-24), 1.00 (s, 3H, CH_3-23), 1.01 (s, 3H, CH_3-26), 1.14 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.33 (m, 1H, CH-19), 1.41 (m, 2H, CH_2-2), 1.55 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.62 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.70 (m, 5H, CH_2-γ, CH_2-γ′ and $CH_{2(H\alpha)}$-6), 1.77 (td, $^3J = 14.0$, 6.6, 1H, $CH_{2(H\alpha)}$-16), 1.83 (m, 2H, CH_2-22), 2.00 (m, 1H, $CH_{2(H\beta)}$-15), 2.10 (s, 3H, H_3CCOO), 2.51 (m, 13H, CH_2-α1, CH_2-α1′, CH_2-α2, CH_2-α2′, CH_2-β, CH_2-β′ and CH-18), 3.03 (m, 2H, CH_2-δ′), 3.49 (m, 2H, CH_2-δ), 4.54 (dd, $^3J = 13.8$, 6.4, 1H, CH-3), 5.34 (m, 1H, CH-12), 6,46 (m, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 15.9 (C-25), 17.1 (C-26), 17.3 (C-24), 17.6 (C-29), 18.6 (C-6), 21.7 (C-30, H_3CCOO), 23.8 (C-11), 23.9 (C-27), 24.1 (C-2), 25.1 (C-16), 26.2 (C-γ), 28.2 (C-23), 28.5 (C-15), 31.2 (C-21), 31.8 (C-γ′), 33.1 (C-7), 37.2 (C-10), 37.8 (C-22), 38.1 (C-4), 38.5 (C-1), 39.2 (C-δ), 39.4 (C-19), 39.9 (C-20), 40.0 (C-8), 40.2 (C-δ′), 42.6 (C-14), 47.8 (C-9), 47.9 (C-17), 53.4 (C-18), 54.7 (C-α1, C-α1′, C-α2, C-α2′), 55.6 (C-β and C-β′), 55.7 (C-5), 81.2 (C-3), 123.1 (C-12), 139.5 (C-13), 171.4 (H_3CCOO), 179.5 (C-28); HRMS (ESI-MS, *m/z*), $(M + H)^+$ calc. for $C_{42}H_{73}N_4O_3$: 681.5683, found: 681.5667.

2.18. N-{3-[4-(3-aminopropyl)piperazinyl]propyl}-ursolamide **16**

This compound was prepared using the general method described for **11**. Reaction of acetylursolamide **15** (0.2 g, 0.29 mmol) and 1 N NaOH (5 ml) gave 93.8 mg (50%) of **16** as white powder. Mp: 140 °C. $[\alpha]_D^{20}$ +56 (*c* 0.2, $CHCl_3$); IR (ATR, cm^{-1}): 3385 (NH_2, OH), 2869, 1698 (CO), 1636 (C═C), 1454, 1385, 1306, 1254, 1029, 999; ^1H NMR

(500 MHz, $CDCl_3$): 0.78 (m, 1H, CH-5), 0.82 (s, 3H, CH_3-24), 0.83 (d, $^3J = 7.0$, 3H, CH_3-30), 0.91 (d, $^3J = 6.6$, 3H, CH_3-29), 0.93 (m, 1H, CH_2-1), 0.94 (s, 3H, CH_3-25), 0.96 (m, 1H, CH-20), 0.94 (m, 1H, CH_2-11), 0.98 (s, 3H, CH_3-23), 1.02 (s, 3H, CH_3-26), 1.14 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.33 (m, 1H, CH-19), 1.41 (m, 2H, CH_2-2), 1.55 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.62 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16,$CH_{2(H\beta)}$-21), 1.70 (m, 5H, CH_2-γ, CH_2-γ′ and $CH_{2(H\alpha)}$-6), 1.77 (td, $^3J = 14.0$, 6.0, 1H, $CH_{2(H\alpha)}$-16), 1.83 (m, 2H, CH_2-22), 2.00 (m, 1H, $CH_{2(H\beta)}$-15), 2.52 (m, 13H, CH_2-α1, CH_2-α1′, CH_2-α2, CH_2-α2′, CH_2-β, CH_2-β′ and CH-18), 3.03 (m, 2H, CH_2-δ′), 3.25 (dd, $^3J = 13.8$, 6.4, 1H, CH-3), 3.34 (m, 2H, CH_2-d), 5.35 (m, 1H, CH-12), 6.46 (m, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): 15.8 (C-25), 15.9 (C-26), 16.8 (C-24), 17.4 (C-29), 18.7 (C-6), 21.7 (C-30), 23.8 (C-11, C-27), 25.1 (C-16), 27,2 (C-γ), 28.3 (C-23), 28.4 (C-2), 28.5 (C-15), 31.3 (C-21), 33.2 (C-7), 31,5 (C-γ′), 37.4 (C-10), 37.8 (C-22), 38.9 (C-8), 39.0 (C-1), 39.2 (C-δ), 39.3 (C-4), 39.5 (C-19), 39.9 (C-20), 40.1 (C-δ′), 42.8 (C-14), 47.9 (C-9, C-17), 53.4 (C-18), 53.7 (C-α1, C-α1′, C-α2, C-α2′), 55.6 (C-5), 56.5 (C-β, β′), 79.3 (C-3), 123.0 (C-12), 140.1 (C-13), 178.2 (C-28).

2.19. Ethyl 3-ferrocenylpropenoate **17**

A mixture of formylferrocene **7** (1.5 g, 7 mmol) and ethoxycarbonylmethylene-triphenylphosphorane (3.15 g, 9 mmol) was refluxed in dry benzene (20 ml). Following solvent removal; the residue was triturated with dichloromethane. The organic extract was washed with water and brine, and dried over anhydrous magnesium sulphate. After removal of solvent, the mixture was chromatographed over silica gel (cyclohexane/AcOEt: 90/10) to afford **17** (51%) as red crystalline solid. Mp: 65 °C. IR (ATR, cm^{-1}): 1701 (CO), 1633 (C=C), 1438, 1366, 1313, 1104, 998; ^{1}H NMR (500 MHz, $CDCl_3$): 1.34 (t, $^3J = 7.0$, 3H, CH_3), 4.17 (s, 5H, Cp′), 4.23 (q, $^3J = 7.0$, 2H, $-OCH_2$), 4.41 (m, 2H, Cp), 4.49 (m, 2H, Cp), 6.69 (d, $^3J_{trans} = 16.0$, 1H, *CH*=CH), 7.57 (d, $^3J_{trans} = 16.0$, 1H, CH=*CH*); HRMS (ESI-MS, *m/z*), $(M + Na)^+$ calc. for $C_{15}H_{16}FeO_2Na$: 307.0397, found: 307.0387.

2.20. Ethyl 3-ferrocenylpropanoate **18**

To a stirred solution of **17** (284 mg, 1 mmol) and CuBr (215 mg, 1,5 mmol) in dry methanol (20 ml) was added $NaBH_4$ (378 mg, 10 mmol) in small portions over a period of 30 min at 0 °C and allowed to stir for 1 h. The resulting black precipitate was then removed by filtration and the filtrate was acidified with 5% aqueous HCl and extracted with Et_2O. The extract was washed successively with saturated aqueous bicarbonate solution and water followed by drying over anhydrous $MgSO_4$. The solvent was evaporated in vacuo, and the product was isolated by column chromatography in 97% yield as orange oil. IR (ATR, cm^{-1}): 1726 (CO), 1632 (C=C), 1443, 1103, 999; ^{1}H NMR (500 MHz, $CDCl_3$): 1.31 (t, $^3J = 7.1$, 3H, CH_3), 2.60 (m, 4H, CH_2), 4,34 (s, 9H, Cp and Cp′), 4.39 (q, 2H, $^3J = 7.0$ Hz, $-OCH_2$); HRMS (ESI-MS, *m/z*), $(M)^+$ calc. for $C_{15}H_{18}FeO_2$: 286.0656, found: 286.0648.

2.21. Benzyl ursolate **20**

To a solution of ursolic acid **1** (90 mg, 0.2 mmol) and K_2CO_3 (25.2 mg, 0.2 mmol) in dry DMF (2 ml) was added benzoyl chloride (22.9 μl, 0.2 mmol). The reaction was refluxed for 3 h under nitrogen atmosphere. After filtration, the solvent was removed under reduced pressure and then the residue was poured into a 1 N NaOH solution. The aqueous layer was extracted with ether (3 × 2 ml). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to give a white powder (94%). Mp: 62 °C. $[\alpha]_D^{20}$ +37 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 3599 (OH), 2866 (C–H), 1722 (CO), 1457, 1387, 1260, 1030, 1011, 996; ^{1}H NMR (500 MHz, $CDCl_3$): 0.70 (s, 3H, CH_3-25), 0.78 (m, 1H, CH-5), 0.83 (m, 4H, CH_3-24 and $CH_{2(H\alpha)}$-1), 0.90 (d, $^3J = 6.4$, 3H, CH_3-30), 0.95 (s, 3H, CH_3-26), 0.99 (d, $^3J = 6.3$, 3H, CH_3-29), 1.04 (m, 4H, CH_3-23 and CH_2-11), 1.11 (m, 1H, CH-20), 1.13 (s, 3H, CH_3-27), 1.33 (m, 1H, $CH_{2(H\alpha)}$-7), 1.36 (m, 3H, CH_2-2, CH-19), 1.53 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.67 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.75 (td, $^3J = 13.9$, 6.8, 1H, $CH_{2(H\alpha)}$-6), 1.88 (td, $^3J = 13.6$, 7.2, 1H, $CH_{2(H\alpha)}$-16), 1.93 (dd, $^3J = 13.7$, 7.3, 2H, CH_2-22), 2.06 (td, $^3J = 13.2$, 4.4, 1H, $CH_{2(H\beta)}$-15), 2.32 (d, $^3J = 11.3$, 1H, CH-18), 3.26 (dd, $^3J = 11.1$, 4.7, 1H, CH-3), 5.03 (d, $^2J = 12.4$, 1H, $-CH_2$–Bn), 5.16 (d, $^2J = 12.5$, 1H, $-CH_2$–Bn), 5.29 (tl, $^3J = 6.4$, 1H, CH-12), 7.39 (m, 5H, Bn); ^{13}C NMR (125 MHz, $CDCl_3$): 15.8 (C-25), 16.0 (C-26), 16.9 (C-24), 17.4 (C-29), 18.7 (C-6), 21.6 (C-30), 23.7 (C-11), 23.9 (C-27), 24.7 (C-16), 27.6 (C-15), 28.4 (C-2), 28.5 (C-23), 31.1 (C-21), 33.4 (C-7), 37.0 (C-10), 37.4 (C-22), 39.0 (C-8), 39.2 (C-1), 39.3 (C-4), 39.5 (C-19), 39.9 (C-20), 42.5 (C-14), 47.9 (C-9), 48.5 (C-17), 53.3 (C-18), 55.6 (C-5), 66.4 ($-CH_2$–Bn), 79.5 (C-3), 126.1 (C-12), 128.3 (C-*meta*), 128.5 (C-*ortho*), 128.8 (C-*para*), 136.8 (C-*ipso*), 138.5 (C-13), 177.7 (C-28); HRMS (ESI-MS, *m/z*), $(M + Na)^+$ calc. for $C_{37}H_{54}O_3Na$: 569.3971, found: 569.3954.

2.22. Benzyl 3-O-β-(3-ferrocenylpropanoyl) ursolate **21**

To a solution of 3-ferrocenylpropanoic acid **19** (25.8 mg, 0.1 mmol) in dry CH_2Cl_2 (1 ml) at 0 °C was added dropwise oxalyl chloride (17.3 μl, 0.2 mmol). The reaction was stirred at room temperature for 3 h and then TEA (0.5 ml, 0.4 mmol) and benzyl ursolate **20** (81.2 mg, 0.15 mmol) in dry CH_2Cl_2 (1 ml) were added. The mixture was stirred for 24 h and then the solvent was evaporated in vacuo. The residue was purified by silica gel chromatography (CH_2Cl_2) to give a red powder (51%). Mp: 94 °C. $[\alpha]_D^{20}$ +49 (*c* 0.2, $CHCl_3$); IR (ATR, cm^{-1}): 1726 (CO), 1457, 1363, 1106, 1011; ^{1}H NMR (500 MHz, $CDCl_3$): 0.67 (s, 3H, CH_3-25), 0.87 (m, 1H, CH-5), 0.90 (s, 6H, CH_3-24 and CH_3-26), 0.96 (m, 6H, CH_3-29 and CH_3-30), 0.98 (s, 3H, CH_3-23), 1.10 (m, 2H,

CH_2-11 and CH-20), 1.18 (s, 3H, CH_3-27), 1.23 (m, 1H, $CH_{2(H\alpha)}$-7), 1,27 (dd, $^3J = 11.8$, 6.0, 1H, CH-19), 1.31 (m, 2H, CH_2-2), 1.43 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, $CH_{2(H\alpha)}$-9, $CH_{2(H\alpha)}$-21), 1.60 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.75 (m, 2H, CH_2-6, $CH_{2(H\alpha)}$-16), 1.90 (dd, $^3J = 12.8$, 8.0, 2H, CH_2-22), 2.03 (td, $^3J = 13.6$ and 4.5, $CH_{2(H\beta)}$-15), 2.13 (d, $^3J = 11.3$, 1H, CH-18), 2.59 (t, $^3J = 7.5$, 2H, $-CH_2CH_2-$), 2.70 (t, $^3J = 7.0$, 2H, $-CH_2CH_2-$), 4.12 (d, $^3J = 10.4$, 4H, Cp′), 4.17 (s, 5H, Cp), 4.56 (dd, $^3J = 15.9$, 4.6, 1H, CH-3), 5.09 (d, $^3J = 12.6$, 1H, CH_2–Bn), 5.12 (d, $^3J = 12.6$, 1H, CH_2–Bn), 5.34 (m, 1H, CH-12), 7.39 (m, 4H, Bn); ^{13}C NMR (125 MHz, $CDCl_3$): 15.9 (C-25), 17.3 (C-26), 17.4 (C-29), 18.6 (C-6), 21.6 (C-30), 23.7 (C-11), 23.9 (C-27), 24.1 (C-16), 24.6 (C-2), 25.5 (C-24), 27.3 (C-15), 28.4 (C-23), 31.1 (C-21), 33.4 (C-7), 37.0 (C-22), 37.3 (C-10), 38.2 (C-4), 38.7 (C-1), 39.2 (C-8), 39.5 (C-19), 39.9 (C-20), 42.4 (C-14), 47.9 (C-9), 48.5 (C-17), 53.3 (C-18), 55.7 (C-5), 66.3 ($-CH_2$–Bn), 67.7 ($2 \times CH_2$), 68.3 (Cp), 68.4 (Cp), 68.9 (Cp), 81.3 (C-3), 126.8 (C-12), 128.3 (C-*meta*), 128.4 (C-*ortho*), 128.8 (C-*para*), 136.9 (C-*ipso*), 138.5 (C-13), 173.3 (C=O), 177.8 (C-28); HRMS (ESI-MS, *m/z*), $(M)^+$ calc. for $C_{50}H_{66}FeO_4$: 786.4311, found: 786.4282.

2.23. 3-O-β-(3-Ferrocenylpropanoyl)ursolic acid **22**

To a solution of **21** (86 mg, 0.11 mmol) in MeOH (2 ml) placed under a nitrogen atmosphere, 10 mg of 10% Pd–C was added. Nitrogen was slowly replaced by hydrogen and the suspension was stirred for 48 h then filtered over celite and concentrated in vacuo, affording **22** as a red solid (42%). Mp: 110 °C. $[\alpha]_D^{20}$ +56 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}): 2924 (OH acid), 2857, 1729 (CO), 1688 (CO), 1457, 1315, 1031, 1101, 1000; ^{1}H NMR (500 MHz, $CDCl_3$): 0.81 (m, 1H, CH-5), 0.84 (s, 3H, CH_3-25), 0.91 (s, 6H, CH_3-24 and CH_3-26), 0.93 (d, $^3J = 6.3$, 3H, CH_3-30), 0.98 (d, $^3J = 6.3$, 3H, CH_3-29), 1.00 (s, 3H, CH_3-23), 1.10 (m, 2H, CH_2-11 and CH-20), 1.14 (s, 3H, CH_3-27), 1.19 (m, 1H, CH_2-7), 1.31 (m, 2H, CH_2-2), 1.36 (m, 1H, CH-19), 1.43 (m, 4H, $CH_{2(H\beta)}$-6, CH_2-7, CH-9, $CH_{2(H\alpha)}$-21), 1.59 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.73 (m, 2H, CH_2-6, $CH_{2(H\alpha)}$-16), 1.97 (dd, $^3J = 12.6$, 6.6, 2H, CH_2-22), 2.05 (td, $^3J = 14.6$, 4.5, $CH_{2(H\beta)}$-15), 2.23 (d, $^3J = 11.3$, 1H, CH-18), 2.59 (t, $^3J = 7.5$, 2H, $-CH_2CH_2$), 2.72 (t, $^3J = 7.0$, 2H, $-CH_2CH_2$), 4.12 (d, $^3J = 10.4$, 4H, Cp), 4.17 (s, 5H, Cp), 4.56 (dd, $^3J = 15.9$, 5.6, 1H, CH-3), 5.34 (m, 1H, H-12); ^{13}C NMR (125 MHz, $CDCl_3$): 15.9 (C-25), 17.2 (C-26), 17.4 (C-29), 18.6 (C-6), 21.6 (C-30), 23.7 (C-11), 23.8 (C-27), 24.0 (C-16), 25.4 (C-24), 27.3 (C-15), 24.5 (C-2), 28.4 (C-23), 31.0 (C-21), 33.3 (C-7), 37.1 (C-22), 37.3 (C-10), 39.2 (C-8), 38.2 (C-4), 38.7 (C-1), 39.4 (C-19), 39.9 (C-20), 42.4 (C-14), 47.9 (C-9), 48.3 (C-17), 53.0 (C-18), 55.7 (C-5), 67.7 ($2 \times CH_2$), 68.3 (Cp), 68.4 (Cp), 68.9 (Cp), 81.3 (C-3), 126.2 (C-12), 138.4 (C-13), 173.4 (CO), 182.8 (C-28); HRMS (ESI-MS, *m/z*), $(M)^+$ calc. for $C_{43}H_{60}FeO_4$: 696.3841, found: 696.3810.

3. Results and discussion

Estrogens play an important role in hormonal-dependent cancer promotion and growth in some instances including breast and prostate cancers [6]. Breast cancer is the most occurring cancer in women, more than one-tenth of the women develop a breast cancer in their lifespan, whereas benign prostatic hyperplasia (BPH) is the most commonly occurring neoplastic disease in the aging human male [32]. The irreversible conversion of androgens in estrogens is controlled by a unique enzyme called aromatase. Therefore, aromatase has been the target for the design of numerous inhibitors, as agents in the treatment of estrogen-dependent cancers especially breast cancer in postmenopausal women. To reach this goal, new steroidal and non-steroidal inhibitors are continuously being developed [33–36], and some of them are used as first or second line therapeutic agents. Moreover, some natural compounds such as phytoestrogens, found in a wide variety of plants especially in soybeans, were found to inhibit aromatase activity [7,24,25]. Thus, phytoestrogens may have cancer-preventive effects and their consumption might explain the low cancer rates observed in some populations consuming primarily plant-based diets.

Among natural products, the terpenoid represents a unique and most important class of bioactive compounds. Ursolic acid and its isomer, oleanolic acid (Fig. 1), are pentacyclic triterpenoid compounds widely present in food, medicinal herbs and other plants. In this work, we show that *I. Paraguariensis* can be considered an excellent source of ursolic acid since we obtained, after the acid hydrolysis of saponins, 8 g of this compound per 3 kg of air-dried powdered leaves of *I. Paraguariensis*. As comparison, *Urtica dioica* roots [18] and *Isodon excisus* var. coreanus [19] give, respectively, 15 mg ursolic acid per 1 kg dry weight and 20.5 mg per 1 kg fresh plant. Ursolic acid and its isomer, oleanolic acid, have been recommended in cancer therapy in Japan [37] and a topical cosmetic preparations containing these compounds have been patented in this country for the prevention of skin cancer [38].

We evaluated here the estrogen synthesis in the presence of ursolic acid and derivatives, the steroidal aromatase inhibitor 4-OHA, and apigenin known among the phytoestrogens as one of the most potent inhibitor of aromatase activity [24,25]. Fig. 2 shows a dose-dependent aromatase inhibition which was more pronounced for 4-OHA, apigenin and ursolic acid **1** than for other compounds tested whereas Table 2 compares the IC_{50} values obtained graphically from Fig. 2. As shown in Table 2, all ursolic acid derivatives, excepting compounds **15** and **16**, presented IC_{50} values more than 500 μM. IC_{50} values of 4-OHA, apigenin and ursolic acid **1** were, respectively, 0.8, 10 and 32 μM. As expected, 4-OHA, a known steroidal aromatase inhibitor, inhibits efficiently aromatase activity at low concentration (Fig. 2 and Table 2). IC_{50} value of 4-OHA concords those reported previously which usually higher than K_i value given in nM range [6,33,35]. This can be explained by the fact that IC_{50} value is obtained in saturation conditions of experimentation whereas K_i value is reached in

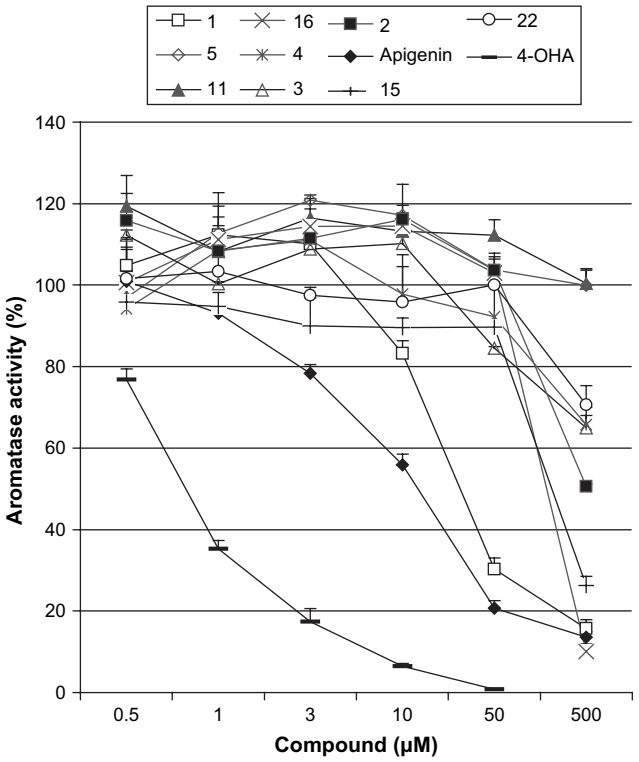


Fig. 2. Dose-depending decrease of aromatase activity by ursolic acid **1** extracted from *Ilex paraguariensis* and synthetic derivatives. Fifty micrograms of human placental microsomes were incubated with 200 nM radiolabeled androstenedione for 15 min at 37 °C in the presence of different concentrations of each compound. Aromatase activity was evaluated by tritiated water release assay as described in experimental protocols. 4-OHA and apigenin were used as controls. Results are expressed as mean ± SD of three independent experiments in triplicate.

the steady states thus in the linear phase of enzymatic reaction. Although the inhibitory potency of apigenin is not limited to aromatase and that this compound showed in the literature to interact with numerous biological target, but its case being interesting since most animal studies using phytoestrogens have shown cancer-preventive effects particularly when phytoestrogens are taken early in life; this is why several authors explain the low cancer rates observed in some populations consuming primarily plant-based diets. Albeit, the cellular and molecular actions of phytoestrogens in general and apigenin in particular depend on many factors such as the type of target organ or cell, their concentration, receptor bio-availability, the concentration of endogenous estrogens, and the activation or inhibition of different enzymatic pathways (for review see Ref. [6]).

Anti-aromatase feature of ursolic acid and derivatives extracted from different plants has also been reported. Ganβer and Spiteller [18] previously reported that oleanolic acid and ursolic acid from *Urtica diocica* L. (Urticaceae) roots were responsible of aromatase inhibition and this might explain in part the anti-tumour property of this plant when applied in the treatment of stages I and II of BPH. In that study, oleanolic acid and ursolic acid were identical to inhibit aromatase activity (about 12% of inhibition with 40.7 μM of each). More recently, it has been shown that ursolic acid inhibits more efficiently aromatase activity than its derivative ursolic acid 3-*O*-acetate ($IC_{50} = 14$ and 42.7 μg/ml, respectively) both extracted from *Isodon excisus* var. coreanus Nakai (Labiatae) [19]. Therefore, from our results and those of literature, we can hypothesize that the presence of cycle A and the free hydroxyl group at C-3 and/or the carboxyl group at C-17 of ursolic acid **1** seem to be necessary for aromatase inhibition since esterification of one of these functions led to significant decrease of the inhibitory potency. The stability of the ferrocenyl group in aqueous, aerobic media, the accessibility of a large variety of derivatives, and its favourable electrochemical properties have made ferrocene and its derivatives very popular molecules for biological applications and for conjugation with biomolecules. Structural variations of established drugs with the ferrocenyl moiety were reported, such as the anti-malarial drugs chloroquine (termed ferroquine), quinine, mefloquine, and artemisinin and the anti-cancer drug tamoxifen to give ferrocifen [39]. Some iron sandwich compound studies have demonstrated anti-tumour activity [40]. A ferrocenyl fragment was introduced into steroidal derivatives, particularly, estradiol for metalloimmunoassay [41] and radioactive labelling [42] and some ferrocenylestradiol were found to bind irreversibly to estradiol receptors [43,44]. A characteristic of metallocenes is that the top surface closely resembles a simple aromatic ring, but the metallocenes are substantially thicker, being approximately as thick as they are wide [45]. Metallocene complexes are metabolised and excreted in a manner similar to that of various aromatic compounds via the cytochrome P-450 system [46]. Metallocene derivatives offer a unique method of enzyme active and receptor site studies. However, in our study the incorporation of a metallocene moiety into the ursolic acid derivatives **11** and **22** does not increase the interaction of this triterpenoid with the human aromatase. This might be explained by the fact that ferrocene molecule is weekly recognized by cytochrome P-450 aromatase and/or once recognized it being metabolised to less potent

Table 2
IC_{50} values of ursolic acid **1**, derivatives, apigenin and 4-OHA

Compound	IC_{50} (μM)
1	**32**
2	>500
3	>500
4	>500
5	>500
11	>500
15	<500
16	<500
22	>500
Apigenin	**10**
4-OHA	**0.8**

Fifty micrograms of human placental microsomes were incubated with 200 nM radiolabeled androstenedione for 15 min at 37 °C in the presence of various concentrations of each compound. Aromatase activity was evaluated by tritiated water release assay as described in experimental protocols. Apigenin and 4-OHA were used as controls. IC_{50} values are determined graphically from Fig. 2.

metabolites by this cytochrome or by other cytochromes P-450 present in the microsomal preparation.

Thus, the configuration of ursolic acid **1**, but not its derivatives, is undoubtedly more appropriate to recognize active site of enzyme and to hamper substrate aromatisation. The structure homology between ursolic acid **1** and aromatase substrate, androstenedione, prompts this hypothesis (see Fig. 1). Moreover, it is interesting to underline that squalene is considered as the common precursor for biosynthesis of both natural steroid and triterpenoid systems which can explain the similitude of their structures [47].

4. Conclusion

Among all triterpenoid compounds tested, only ursolic acid **1** inhibits efficiently and dose-dependently aromatase activity in vitro. The incorporation of a metallocene moiety into the ursolic acid derivatives does not increase the aromatase inhibition. The ursolic acid **1** inhibitory potency was comparable to that of apigenin, one of the most potent aromatase inhibitor among phytoestrogens. A comparison of structure/activity relationship of compounds reveals that the configuration of ursolic acid (C3-OH and C17-COOH) seems to be more favourable to recognize the active site of aromatase and to block androstenedione aromatisation. This might contribute to develop new molecules derived from natural terpenoid compounds to inhibit aromatase activity.

5. Experimental protocols

5.1. Preparation of microsomes

Microsomes from full-term placentas of young healthy and non-smoking women (Centre Hospitalier Régional de Caen, France) were prepared by differential centrifugations as described previously [48]. Briefly, tissues were washed with 0.5 M KCl, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM DTT, and centrifuged at 20 000*g*. The supernatant was then ultracentrifuged at 100 000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at −70 °C until use. All steps of the preparation were carried out at 4 °C.

5.2. Aromatase assay

Aromatase activity was evaluated by tritiated water release from radiolabeled substrate [1β,^{3}H]-androstenedione as previously described [48] according to Thompson and Siiteri [49]. Human placental microsomes (50 μg protein) were incubated with 200 nM radiolabeled androstenedione at 37 °C for 15 min, in the presence or absence of various concentrations of each compound (solvent of substrate and compounds are previously evaporated), in 0.5 ml total volume of 50 mM Tris–maleate buffer (pH 7.4). The reaction was started by adding 50 μl of 0.6 mM H+-NADPH and stopped with 1 ml chloroform, and the centrifuged at 2700*g* at 4 °C for 5 min. After adding 0.5 ml 7% charcoal/1.5% dextran T-70 solution into the preparation, the centrifugation was repeated for 10 min. Aromatase activity was determined by measuring the radioactivity of the 0.5 ml aqueous phase by scintillation liquid in a Packard scintillation counter (Perkin Elmer, Courtabœuf, France).

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