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# Inhibitory Effect of Ursolic Acid Derivatives on Recombinant Human Aldose Reductase[1]

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**Abstract**—Aldose reductase (AR) is the first enzyme in the polyol pathway. AR has been reported to play an important role in the pathogenesis of diabetic complications. Ursolic acid and fourteen synthetic derivatives with ursane skeleton were tested for recombinant human aldose reductase (rhAR) inhibitory activity for development of diabetic complications. Among them, *N*-(3β-hydroxyurs-12-en-28-oyl)-4-aminobutyric acid (**XV**) showed most potent rhAR inhibitory activity in vitro. Inhibition mode of *N*-(3β-hydroxyurs-12-en-28-oyl)-4-aminobutyric acid (**XV**) was tested uncompetitively by kinetic analysis using the Lineweaver-Burk plots. Ursolic acid derivative *N*-(3β-hydroxyurs-12-en-28-oyl)-4-aminobutyric acid is able to inhibit rhAR uncompetitively and could be offered as a lead compound for AR inhibition.

*Keywords: aldose reductase, N-(3β-hydroxyurs-12-en-28-oyl)-4-aminobutyric acid, ursolic acid derivatives, diabetic complication*s

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## INTRODUCTION

Diabetes is a group of metabolic disorders characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin [1]. Prolonged diabetes may develop diabetic complications such as retinopathy, nephropathy, and neuropathy.

The mechanism underlying complications of diabetes still remains unclear, but increased AR activity, increased advanced glycation endproducts (AGEs) formation, activation of protein kinase C isoforms and increased hexosamine pathway flux have been known as important factors [2].

AR (EC 1.1.1.21) is the first enzyme of the polyol pathway which reduces excess *D*-glucose into *D*-glucitol with concomitant conversion of NADPH to $NADP^+$ [3, 4], which results in progressive irreversible complications for lens, kidneys or nerves [5–8].

A large variety of structurally diverse compounds have been reported to have potent aldose reductase inhibitory potency in vitro and in vivo studies. Several studies of naturally occurring compounds have also been reported [9–11].

Ursolic acid (**I**) is a pentacyclic triterpene acid, present in many food, medicinal herbs and other plants. Ursolic acid (**I**) and its derivatives have been reported to possess antioxidant [12], anti-inflammatory [13], anti-HIV, and anti-tumor activities [14, 15]. In addition, these compounds are expected to be potent bioactive compounds, such as anti-cancer agents. Although ursolic acid derivatives have demonstrated anti-diabetic activity [16], there have been no reports on inhibitory potency for AR.

The purpose of the present study was therefore to investigate the inhibitory effects of ursolic acid (**I**) and its derivatives against rhAR. Ursolic acid (**I**) and its synthetic derivatives were tested as possible rhAR inhibitors for treatment for diabetic complications.

## RESULTS AND DISCUSSION

We synthesized ursolic acid esters and amides (**III**)–(**VII**), (**XII**)–(**XV**) starting from ursolic acid (**I**) and 3β-acetoxy-urs-12-en-28-oic acid (**II**). Synthesized compounds (**III**)–(**VII**), (**XII**)–(**XV**) together with accessible ursonic acid (**IX**), benzyl ursonate (**VIII**), and methyl corosolate (**X**) were tested for rhAR inhibition.

Alkylation of ursolic acid (**I**) with alkyl chlorides in DMF in the presence of inorganic base ($K_2CO_3$) was found to be the convenient route to the ursolic acid esters (**III**)–(**VII**) (Scheme 1, yields 84–86%). Reaction of ursolic acid (**I**) with racemic epichlorohydrine

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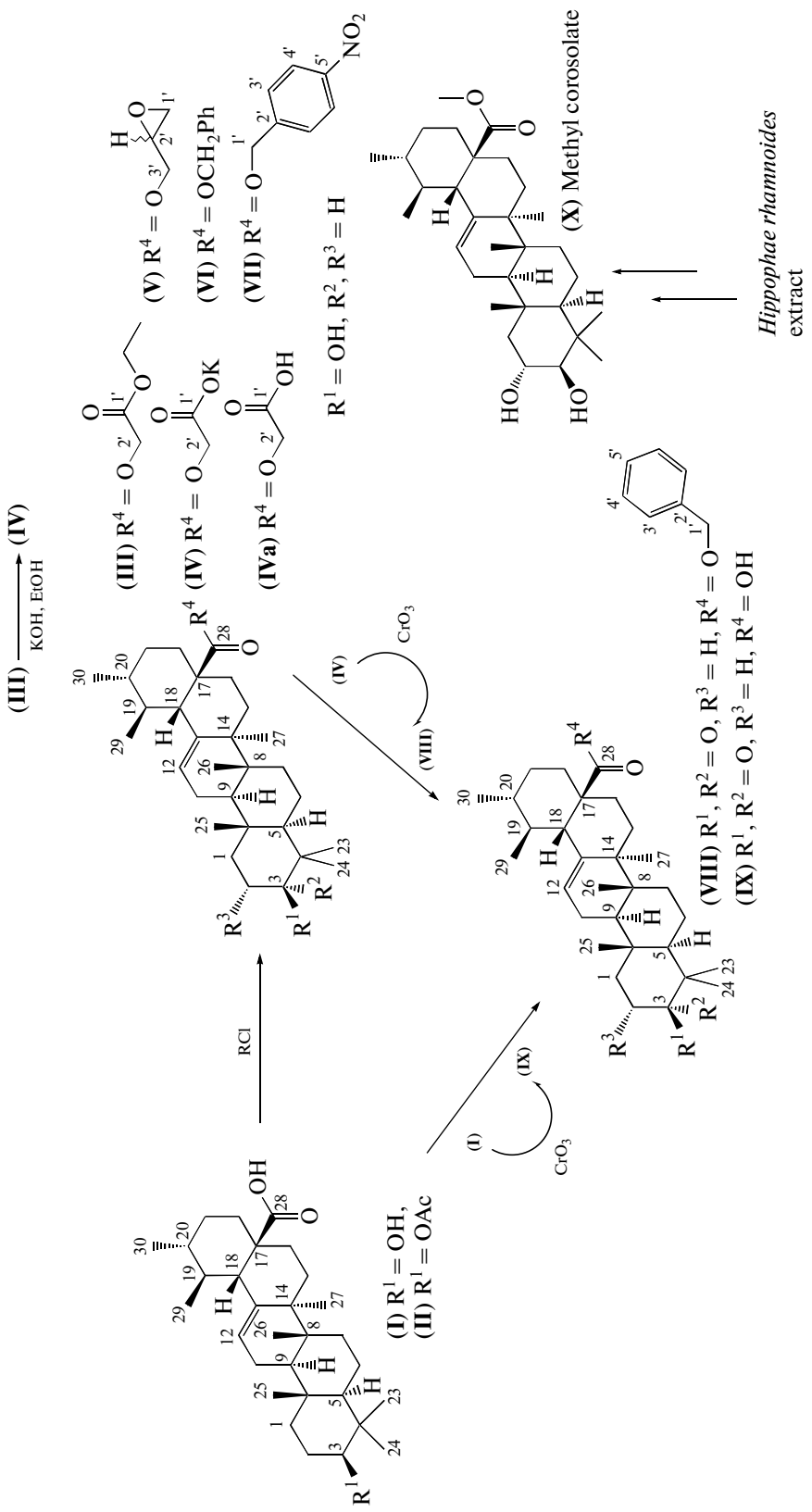
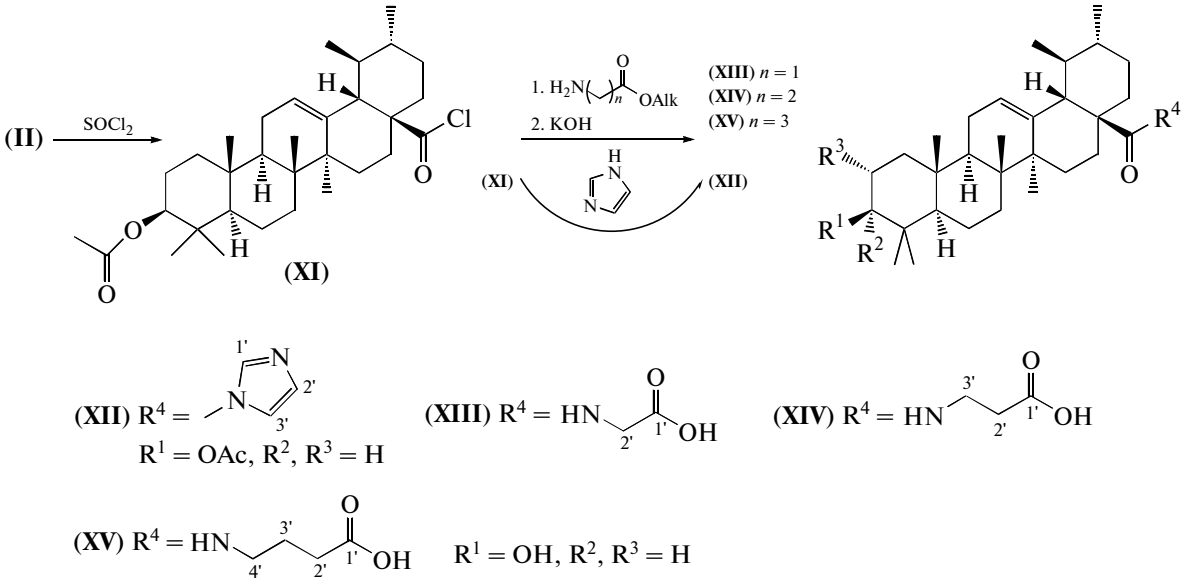



**Scheme 1.** Synthesis of alkyl 3β-hydroxyurs-12-en-28-oates and benzyl 3-oxours-12-en-28-oate.




**Scheme 2.** Synthesis of amide derivatives of ursolic acid.

resulted in glycidyl ursolate (**V**) as a 1 : 1 mixture of two diastereomers. Reactions of pharmacophore amines (imidazole and esters of amino acids) with 3β-acetoxyurs-12-en-28-oyl chloride (**XI**) were used to prepare the amides of ursolic acid 3-acetate (Scheme 2). *N*-(3β-hydroxyurs-12-en-28-oyl) aminoacids (**XIII**)–(**XV**) were obtained by one-pot deprotection of carboxy-group of acylated aminoacid and 3-hydroxy-group in the ursane moiety with ethanolic alkali (Scheme 2). The same method of hydrolysis was used to prepare ursolic acid derivative (**IV**) from 2-ethoxycarbonylmethyl ursolate (**III**) (Scheme 1). 3-Oxo-derivatives: ursonic acid (**IX**) and benzyl ursonate (**VIII**), were prepared by oxidation of ursolic acid (**I**) and benzyl ursolate (**VI**) with $CrO_3$ in acetone-$CH_2Cl_2$ (Scheme 1).

The majority of the tested compounds manifested no inhibitory effect on rhAR. However compound (**XV**) exhibited 81.27% inhibition (at 20 μM concentration), which surpassed the positive control value (Table 1). The rhAR inhibition for 4-aminobutyric acid derivative (**XV**) is superior over the same for β-alanine derivative (**XIV**) (20.3% at 20 μM concentration) and glycine derivative (**XIII**) (3.3% at 20 μM concentration). It seems probable, that carbon chain elongation in the tested series of aminoacids derivatives results in rhAR inhibition enhancing.

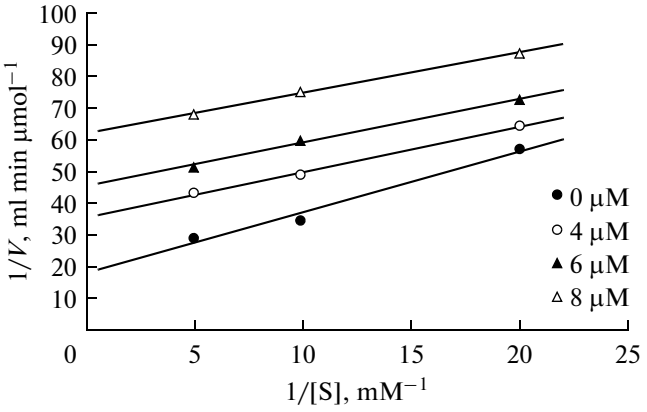
The inhibitory potency of compound (**XV**) on rhAR as expressed by $IC_{50}$ values, was 4.44 μM, which exceeded significantly the same for the so-called tetramethyleneglutaric acid (TMG) ($IC_{50}$ = 8.75 μM) as a positive control.

To determine kinetic mode of action, the inhibitory activity of compound (**XV**) against rhAR using the Lineweaver-Burk plots was tested as shown in Figure. Inhibition of rhAR by compound (**XV**) was uncompetitive, which implies that compound (**XV**) can bind to neither substrate binding region nor the nucleotide binding region of rhAR.

Thus, compound (**XV**) could be offered as a lead compound for AR inhibition.

**Table 1.** Inhibitory effects of the ursolic acid derivatives on rhAR

| Compound | Concentration, μM | % inhibition | $IC_{50}$, μM |
|---|---|---|---|
| **(I)** | 20 | 0.78 ± 2.1 | >20 |
| **(II)** | 20 | 4.69 ± 4.11 | >20 |
| **(III)** | 20 | 3.09 ± 3.57 | >20 |
| **(IV)** | 20 | 3.56 ± 4.07 | >20 |
| **(V)** | 20 | 5.64 ± 2.45 | >20 |
| **(VI)** | 20 | 3.56 ± 1.56 | >20 |
| **(VII)** | 20 | 0.36 ± 4.11 | >20 |
| **(VIII)** | 20 | 5.77 ± 2.05 | >20 |
| **(IX)** | 20 | 3.90 ± 5.02 | >20 |
| **(X)** | 20 | 11.14 ± 2.88 | >20 |
| **(XII)** | 20 | 8.31 ± 4.80 | >20 |
| **(XIII)** | 20 | 3.34 ± 1.76 | >20 |
| **(XIV)** | 20 | 20.31 ± 4.59 | >20 |
| **(XV)** | 20 | 81.27 ± 1.54 | 4.44 |
| | 10 | 71.20 ± 2.57 | |
| | 5.0 | 54.50 ± 2.14 | |
| | 2.5 | 34.88 ± 1.88 | |
| TMG | 20 | 68.40 ± 1.48 | 8.75 |
| | 10 | 53.12 ± 0.81 | |
| | 5.0 | 37.08 ± 3.20 | |
| | 2.5 | 22.51 ± 2.54 | |




Lineweaver-Burk plots from the kinetic studies of rhAR in the presence of compound (**XV**). Results are the representatives of three independent experiments.

## EXPERIMENTAL

$^1$H and $^{13}$C NMR spectra were registered on a Bruker AV-300 (300.13 MHz for $^1$H, 75.48 MHz for $^{13}$C) and Bruker AM-400 (400.13 MHz for $^1$H, 100.62 MHz for $^{13}$C) instruments at room temperature in $CDCl_3$ or pyridine-$d_5$. The chemical shifts are given in ppm relative to signals of the solvents used as internal standards: in $^1$H NMR spectra $\delta_H$ 7.24 ($CHCl_3$) or $\delta_H$ 7.15 (pyridine) and in $^{13}$C NMR spectra $\delta_C$ 76.90 ($CDCl_3$) or $\delta_C$ 123.50 (pyridine-$d_5$). *J* values are given in Hertz. Signals in the NMR spectra were assigned by correlation with those of ursolic acid (**I**) [17, 18], 3β-acetoxyurs-12-en-28-oic acid (**II**) [19], benzyl ursolate (**VI**) [20], and methyl 3-oxours-12-en-28-oate [21].

Melting points were measured on a Kofler melting point apparatus and are uncorrected. Optical rotation was measured on a PolAAR 3005 polarimeter (Great Britain) at 589 nm in a 5 cm long tube. TLC was carried out on Sorbfil plates (Russia). Purity of the tested compounds was ≥90% (by $^1$H NMR).

**Reagents.** *DL*-Glyceraldehyde, NADPH were purchased from Sigma Chemical (USA). Ampicillin, IPTG and imidazole were purchased from USB Corporation (USA). Human ALR2 cDNA clone was purchased from 21C Frontier Human Gene Bank (Rep. of Korea). Ursolic acid (**I**) was isolated from the extracts of lingonberry (*Vaccinium vitis-idaea*) fruits peels as described [22]. 3β-Acetoxyurs-12-en-28-oic acid (**II**), benzyl ursolate (**V**), ursonic acid (**IX**), and 3β-acetoxyurs-12-en-28-oyl chloride (**XI**) were prepared from ursolic acid (**I**) according to the published methods [23–25]. Methyl corosolate (**X**) was isolated from sea buckthorn (*Hippophae rhamnoides*) leave methanolic extract as described [26]. All other chemicals were of analytical grade. All the solvents used were reagent quality.

**Syntheses.** *Method A. General procedure for the synthesis of alkyl 3β-hydroxyurs-12-en-28-oates (III)–(VII)* (Scheme 1). To a suspension of ursolic acid (**I**) (1.0 g, 2 mmol) in DMF (10 ml) anhydrous powdered $K_2CO_3$ (0.45 g, 3.3 mM) and corresponding alkyl chloride (2.0–2.1 mmol) were added. The mixture was stirred at ambient temperature for 3–7 h (TLC monitoring). The reaction mixture was poured in water and the precipitate was washed with water (3 × 20 ml) to remove residual solvent and salts. The precipitate was dried on open air and percolated through a short alumina column to give after concentration the ursolic acid ester as a white powder, which was crystallized from an appropriate solvent.

*Method B. General procedure for the synthesis of N-[3β-hydroxyurs-12-en-28-oyl]-ω-amino acids (XIII)–(XV)* (Scheme 2). To a suspension of amino acid ester hydrochloride (4.3–4.5 mmol), pyridine 0.6 g (7.4 mmol) and triethylamine (0.6 g, 6 mmol) in $CH_2Cl_2$ (10 ml), 3β-acetoxy-urs-12-en-28-oyl chloride (**XI**) (1 g, 1.9 mmol) was added. The mixture was kept at ambient temperature for 5 h and evaporated in vacuum to give esters of *N*-[3β-acetoxyurs-12-en-28-oyl]-ω-amino acid. A solution of KOH (0.4 g, 7.2 mM) in ethanol (20 ml) was added to the obtained residue. The mixture was refluxed for 1 h, cooled to ambient temperature and concentated in vacuum. The residue was washed with 1 N aqueous HCl (30 ml), water (3 × 20 ml) and dried on open air to give *N*-[3β-hydroxyurs-12-en-28-oyl]-ω-amino acid as powders, which were crystallized from an appropriate solvent.

**Ethyl (3β-hydroxyurs-12-en-28-oyl-oxy)acetate (III)** was prepared in reaction of ursolic acid (**I**) with ethyl 2-chloroacetate (Method A). Yield 0.92 g (84%), white powder, mp 176–177°C (MeCN), $[\alpha]^{25}$ +68 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 2 and 5.

**Potassium 2-(3β-hydroxyurs-12-en-28-oyl-oxy)acetate (IV).** To a solution of KOH (0.5 g) in EtOH (20 ml), ethyl(3β-hydroxyurs-12-en-28-oyl-oxy)acetate (**III**) (1.0 g, 1.8 mM) was added. The mixture was refluxed for 0.5 h, and left to cool to ambient temperature. The needles of K salt (**IV**) were filtered and washed with cold EtOH.

**(3β-Hydroxyurs-12-en-28-oyl-oxy)acetic acid (IVa).** Washing of (**IV**) with 10% HCl and distilled water and drying on air resulted in acid (**IVa**). Yield 73%, white powder, mp 175°C (dec.), $[\alpha]^{25}$ +52 (*c* 1.0, MeOH). $^1$H NMR and $^{13}$C NMR data are given in the Tables 2 and 5.

**Glycidyl 3β-hydroxyurs-12-en-28-oates (V)** were prepared as diastereomeric mixture (1 : 1) in reaction of ursolic acid (**I**) and racemic epichlorohydrine (3.02 g, 23.8 mM) (Method A). Yield 0.92 g (84%), white powder, mp 102–103°C (MeCN), $[\alpha]^{25}$ +44 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 2 and 5.

**4-Nitrobenzyl 3β-hydroxyurs-12-en-28-oate (4-nitrobenzyl ursolate) (VII)** was prepared in reaction of ursolic acid (**I**) with 4-nitrobenzyl chloride

**Table 2.** $^1$H NMR data for compounds (**II**), (**III**), (**IVa**), (**V**) ($\delta_H$ (*J*))

| Atom No. | (**II**) (pyridine-$d_5$) | (**III**) ($CDCl_3$) | (**IVa**) (pyridine-$d_5$) | (**V**) ($CDCl_3$) |
|---|---|---|---|---|
| 3 | 4.61 dd (10.7, 5.0) | 3.19 dd (10.9, 5.2) | 3.39 dd (9.3, 6.8) | 3.18 dd (10.2, 4.5); 3.18 dd (10.2, 4.5) |
| 11 | 1.81 dd (8.4, 2.7) (2H) | 1.87 dd (8.8, 3.6) (2Í) | | 1.88 dd (8.6, 3.7); 1.88 dd (8.6, 3.7) (2H) |
| 12 | 5.39 dd (3.1, 3.1) | 5.22 dd (3.6, 3.6) | 5.36 dd (3.5, 3.5) | 5.24 dd (3.8, 3.8); 5.24 dd (3.8, 3.8) |
| 15 | 2.06§ ddd (13.2, 13.2, 3.3) ($H^{ax}$) | | | |
| 16 | 2.23§ ddd (13.2, 13.2, 4.4) ($H^{ax}$) | | | |
| 18 | 2.55 d (11.4) | 2.23 d (11.3) | 2.47 d (11.3) | 2.22 d (11.7); 2.21 d (10.9) |
| 23 | 0.84# s | 0.96§ s | 1.13§ s | 0.89§ s; 0.89§ s (3H) |
| 24 | 0.81# s | 0.75§ s | 0.88§ s | 0.75§ s; 0.75§ s (3H) |
| 25 | 0.94 s | 0.89§ s | 0.97§ s | 0.96§ s; 0.96§ s (3H) |
| 26 | 0.76# s | 0.71§ s | 0.87§ s | 0.73§ s; 0.72§ s (3H) |
| 27 | 1.16 s | 1.05§ s | 1.18§ s | 1.06 s; 1.06 s (3H) |
| 29 | 0.94‡ d (6.4) | 0.83‡ d (6.5) | 0.89# d (6.4) | 0.83# d (6.4); 0.83# d (6.4) (3H) |
| 30 | 0.92‡ d (6.4) | 0.91‡ d ($A\underline{B}_3$, $J_{BA} = 6.0$ (3H)) | 0.87# m | 0.91# d ($A\underline{B}_3$, $J_{BA} = 5.8$); 0.91# d ($A\underline{B}_3$, $J_{BA} = 5.8$ (3H)) |
| 1' | – | – | – | 2.80 dd (4.9, 2.9); 2.78 dd (5.0, 2.9) ($H^{1'trans}$); 2.59 dd (4.9, 2.6); 2.61 dd (5.0, 2.6) ($H^{1'cis}$) |
| 2' | – | 4.49 (<u>AB</u>, $\Delta\nu_{AB} = 19.6$ Hz, $J_{AB} = 15.7$ (2H)) | 4.95 (<u>AB</u>, $\Delta\nu_{AB} = 29.4$ Hz, $J_{AB} = 15.6$ (2H)) | 3.15 m; 3.13 m |
| | 1.99 br.s (OC(O)<u>$CH_3$</u>) | 4.16 q (7.2) (O<u>$CH_2$</u>$CH_3$) | | 4.31 dd (12.2, 3.3); 4.29 dd (12.2, 3.3) ($H^{3'a}$); 3.82 dd (12.2, 6.0); 3.80 dd (12.2, 6.3) ($H^{3'b}$) |
| | | 1.24 t (7.2) (O$CH_2$<u>$CH_3$</u>) | | |

§, #, ‡ Signals marked with the same symbols may be exchanged within the column.

(Method A). Yield 1.03 g (86%), white powder, mp 214–215°C (MeCN), $[\alpha]^{25}$ +36 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 3 and 5.

**Benzyl 3-oxours-12-en-28-oate (benzyl ursonate) (VIII).** To a stirred suspension of benzyl ursolate (**VI**) (5 g, 9.1 mmol) in $CH_2Cl_2$ (20 ml) and acetone (20 ml), a solution of Jones reagent (9 ml, 3 equiv.) was added at 5°C in 0.5 h. Reaction was monitored by TLC and after 1 h a mixture of isopropyl alcohol (20 ml) and water (20 ml) was added. The mixture was stirred for 1 h, diluted with water (100 ml), and extracted with $CH_2Cl_2$ (3 × 50 ml). The organic extract was concentrated, percolated ($Al_2O_3$, $CCl_4$) and evaporated to give benzyl ursonate (**VIII**) 4.6 g (83%), white crystals, mp 158–160°C (MeCN), $[\alpha]^{25}$ +76 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 3 and 5.

***N*-(3β-Acetoxyurs-12-en-28-oyl)imidazole (XII).** To a solution of imidazole 0.2 g (2.9 mmol) and pyridine 0.1 g (1.2 mmol) in $CH_2Cl_2$ (20 ml), 3β-acetoxyurs-12-en-28-oyl chloride (**XI**) 1 g (1.9 mmol) was added. The mixture was kept at ambient temperature for 3 h, washed with water (2 × 50 ml), dried ($Na_2SO_4$), and concentrated to give after percolation ($Al_2O_3$, $CCl_4$) imidazolide derivative (**XII**) (0.82 g, 87%), mp 204–205°C (EtOH), $[\alpha]^{25}$ +18 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 3 and 5.

***N*-(3β-Hydroxyurs-12-en-28-oyl)glycine (XIII)** was prepared by reaction of 3β-acetoxyurs-12-en-28-oyl chloride (**XI**) with ethyl glycinate hydrochloride and subsequent hydrolysis (Method B). Yield 0.72 g (82%), white powder, mp 192–194°C (EtOH), [192–194°C (EtOH), $[\alpha]^{25}$ +53 (*c* 1.0, $CHCl_3$). $^1$H NMR and $^{13}$C NMR data are given in the Tables 4 and 5.

***N*-(3β-Hydroxyurs-12-en-28-oyl)-β-alaninate (XIV)** was prepared by reaction of 3β-acetoxyurs-12-en-28-oyl chloride (**XI**) with methyl β-alaninate hydrochloride and subsequent hydrolysis (Method B). Yield 0.8 g (78%), white amorphous powder, mp 182–204°C (dec.), $[\alpha]^{25}$ +53 (*c* 1.0, EtOH). $^1$H NMR and $^{13}$C NMR data are given in the Tables 4 and 5.

**Table 3.** $^1$H NMR data for compounds (**VII**), (**VIII**), (**XII**) ($\delta_H$ ($J$), $CDCl_3$)

| Atom No. | (**VII**) | (**VIII**) | (**XII**) |
|---|---|---|---|
| 2 | | 2.56 ddd (A**B**XY, $J_{AB}$ = 16.0, $J_{AX}$ = 10.9, $J_{AY}$ = 7.2 ($H^{2ax}$)); 2.39 ddd (A**B**XY, $J_{B\grave{A}}$ = 16.0, $J_{BX}$ = 6.9, $J_{BY}$ = 3.8 ($H^{2eq}$)) | |
| 3 | 3.18 dd (11.0, 4.7) | – | 4.45 dd (10.0, 6.0) |
| 11 | | 1.93 dd (8.3, 3.6) | |
| 12 | 5.22 dd (3.3, 3.3) | 5.27 dd (3.6, 3.6) | 5.20 dd (3.5, 3.5) |
| 16 | | | 2.29 ddd (14.2, 14.2, 4.2) ($H^{16ax}$) |
| 18 | 2.23 d (11.1) | 2.30 d (11.2) | 2.43 d (11.1) |
| 23 | 0.95§ s (3H) | 1.10 s (3H) | 0.82§ s (3H) |
| 24 | 0.84§ s (3H) | 1.06§ s (3H) | 0.81§ s (3H) |
| 25 | 0.55§ s (3H) | 1.04§ s (3H) | 0.90 s (3H) |
| 26 | 0.74§ s (3H) | 0.70 s (3H) | 0.65 s (3H) |
| 27 | 1.05§ s (3H) | 1.10 s (3H) | 1.07 s (3H) |
| 29 | 0.84# d (6.0) (3H) | 0.87 d (6.4) (3H) | 0.98# d (A$B_3$, $J_{BA}$ = 6.3 (3H)) |
| 30 | 0.92# d (A$B_3$, $J_{BA}$ = 5.3 (3H)) | 0.95 d (A'$B'_3$, $J_{B'A'}$ = 5.8 (3H)) | 0.89# d (6.3) (3H) |
| 1' | 5.10 (A'B', $\Delta\nu_{A'B'}$ = 46.0 Hz, $J_{A'B'}$ = 13.4 (2H)) | 5.07 (A"B", $\Delta\nu_{A"B"}$ = 35.5 Hz, $J_{A"B"}$ = 12.4 (2H)) | 8.23 br.s |
| 2' | – | – | 7.02 br.s |
| 3' | 7.48 d (8.6) (2H) | 7.36 m (2H) | 7.52 br.s |
| 4' | 8.19 d (8.6) (2H) | 7.36 m (2H) | – |
| 5' | – | 7.36 m (1H) | – |
| | | | 2.02 s (3H) (OC(O)$CH_3$) |

§, # Signals marked with the same symbols may be exchanged within the column.

**Table 4.** $^1$H NMR data for compounds (**XIII**), (**XIV**), (**XV**) ($\delta_H$ ($J$), pyridine-$d_5$)

| Atom No. | (**XIII**) | (**XIV**) | (**XV**) |
|---|---|---|---|
| 3 | 3.39 dd (7.8, 7.8) | 3.37 dd (9.6, 6.3) | 3.38 dd (10.0, 6.0) |
| 12 | 5.50 dd (3.0, 3.0) | 5.42 dd (3.2, 3.2) | 5.42 dd (3.0, 3.0) |
| 18 | 2.34 d (10.7) | 2.21 d (10.7) | 2.31 d (10.8) |
| 23 | 1.14§ s | 1.13§ s | 1.13§ s |
| 24 | 0.93§ s | 0.95§ s | 0.95§ s |
| 25 | 0.96§ s | 0.96§ s | 0.95§ s |
| 26 | 0.85§ s | 0.87§ s | 0.90§ s |
| 27 | 1.17§ s | 1.17§ s | 1.17§ s |
| 29 | 0.89# d (6.4) | 0.88# d (5.8) | 0.88# d (6.5) |
| 30 | 0.85# m | 0.87# m | 0.87# m |
| 2' | 4.37 (A**X**Y, $\Delta\nu_{XY}$ = 73.4 Hz, $J_{XY}$ = 17.7, $J_{XA}$ = 5.1, $J_{YA}$ = 3.9 (2H)) | 2.75 ddd ((6.2, 6.2, 1.9) (2H)) | 2.54 t (7.3 (2H)) |
| 3' | – | 3.75 m (2H) | |
| 4' | – | – | 3.49 m (2H) |
| N**H** | 7.70 (A**X**Y, $J_{AX}$ = $J_{AY}$ = 4.6) | 7.33 dd (5.6, 5.6) | 7.28 dd (4.6, 4.6) |

§, # Signals marked with the same symbols may be exchanged within the column.

**Table 5.** $^{13}C$ NMR data for compounds **(II)**, **(III)**, **(IVa)**, **(V)**, **(VII)**, **(VIII)**, **(XII)**, **(XIII)**, **(XIV)**, **(XV)**

| Atom No. | **(II)** (pyridine-$d_5$) | **(III)** ($CDCl_3$) | **(IVa)** (pyridine-$d_5$) | **(V)** ($CDCl_3$) | **(VII)** ($CDCl_3$) | **(VIII)** ($CDCl_3$) | **(XII)** (pyridine-$d_5$) | **(XIII)** (pyridine-$d_5$) | **(XIV)** (pyridine-$d_5$) | **(XV)** (pyridine-$d_5$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 38.31 t | 38.51 t | 39.08 t | 38.61[§] t; 38.61[§] t | 38.49[§] t | 39.38[§] t | 38.57 t | 39.07 t | 39.16 t | 39.11 t |
| 2 | 23.86[§] t | 27.07 t | 28.02 t | 27.09 t; 27.09 t | 27.08 t | 34.04 t | 23.82[§] t | 28.01 t | 28.02 t | 28.04 t |
| 3 | 80.70 d | 78.92 d | 78.09 d | 78.86 d; 78.86 d | 78.88 d | 217.63 s | 81.05 d | 78.06 d | 78.15 d | 78.14 d |
| 4 | 37.83 s | 38.61 s | 39.32 s | 38.51[§] s; 38.51[§] s | 39.41 s | 47.26 s | 37.94 s | 39.30 s | 39.37 s | 39.36 s |
| 5 | 55.53 d | 55.09 d | 55.76 d | 55.09 d; 55.09 d | 55.08 d | 55.15 d | 55.59 d | 55.70 d | 55.74 d | 55.77 d |
| 6 | 18.45 t | 18.18 t | 18.72 t | 18.18 t; 18.18 t | 18.14 t | 19.47 t | 18.37 t | 18.68 t | 18.76 t | 18.75 t |
| 7 | 33.32 t | 32.87 t | 33.43 t | 32.91 t; 32.89 t | 32.87 t | 32.43 t | 33.03 t | 33.27 t | 33.42 t | 33.42 t |
| 8 | 39.87 s | 39.40 s | 39.92 s | 39.42 s; 39.42 s | 38.61[§] s | 39.19[§] s | 39.74 s | 39.93 s | 40.00 s | 39.98 s |
| 9 | 47.79 d | 47.43 d | 47.96 d | 47.41 d; 47.41 d | 47.37 d | 46.65 d | 47.68 d | 48.00 d | 48.04 d | 48.02 d |
| 10 | 37.01 s | 36.83 s | 37.21 s | 36.83[#] s; 36.83[#] s | 36.81[#] s | 36.48[#] s | 37.10 s | 37.17 s | 37.24 s | 37.27 s |
| 11 | 23.52[§] t | 23.17 t | 23.59 t | 23.19 t; 23.19 t | 23.14 t | 23.29 t | 23.59[§] t | 23.67 t | 23.71 t | 23.69 t |
| 12 | 125.41 d | 125.61 d | 126.00 d | 125.61 d; 125.61 d | 125.68 d | 125.35 d | 126.78 d | 126.25 d | 126.27 d | 126.87 d |
| 13 | 139.23 s | 137.76 s | 138.56 s | 137.87 s; 137.92 s | 138.00 s | 138.10 s | 137.34 s | 139.23 s | 139.10 s | 139.62 s |
| 14 | 42.45 s | 41.91 s | 42.34 s | 41.94 s; 41.94 s | 41.94 s | 42.04 s | 42.39 s | 42.45 s | 42.58 s | 42.55 s |
| 15 | 28.63 t | 27.87 t | 28.40 t | 27.89 t; 27.87 t | 27.79 t | 27.82 t | 28.03 t | 28.26 t | 28.30 t | 28.33 t |
| 16 | 24.85 t | 24.07 t | 24.64 t | 24.08 t; 24.05 t | 24.18 t | 24.11 t | 25.28 t | 24.98 t | 24.93 t | 24.81 t |
| 17 | 47.99 s | 48.00 s | 48.33 s | 48.15 s; 48.13 s | 48.15 s | 48.03 s | 51.20 s | 47.76 s | 47.90 s | 47.81 s |
| 18 | 53.48 d | 52.60 d | 53.24 d | 52.75 d; 52.75 d | 52.85 d | 52.85 d | 54.47 d | 53.56 d | 53.69 d | 53.47 d |
| 19 | 39.47[#] d | 38.65[§] d | 39.12[§] d | 38.94[‡] d; 38.94[‡] d | 38.96[‡] d | 38.99[‡] d | 39.52[#] d | 39.22[§] d | 39.27[§] d | 39.30[§] d |
| 20 | 39.38[#] d | 38.94[§] d | 39.29[§] d | 38.70[‡] d; 38.70[‡] d | 38.74[‡] d | 38.70[‡] d | 38.98[#] d | 39.79[§] d | 39.88[§] d | 39.85[§] d |
| 21 | 31.07 t | 30.53 t | 30.86 t | 30.50 t; 30.50 t | 30.47 t | 30.54 t | 30.67 t | 31.13 t | 31.23 t | 31.21 t |
| 22 | 37.40 t | 36.31 t | 36.96 t | 36.57[#] t; 36.53[#] t | 36.58[#] t | 36.53[#] t | 35.84 t | 37.75 t | 38.04 t | 38.13 t |
| 23 | 28.17 q | 28.01 q | 28.77 q | 28.01 q; 28.01 q | 28.01 q | 26.45 q | 28.34 q | 28.74 q | 28.81 q | 28.81 q |
| 24 | 16.97 q | 15.50[#] q | 16.54[#] q | 15.50[¶] q; 15.50[¶] q | 15.25[¶] q | 21.37 q | 17.01[‡] q | 16.50[#] q | 16.53[#] q | 16.54[#] q |
| 25 | 15.51 q | 15.33[#] q | 15.70[#] q | 15.34[¶] q; 15.34[¶] q | 15.49[¶] q | 15.08 q | 15.79 q | 15.67[#] q | 15.78[#] q | 15.74[#] q |
| 26 | 17.49[‡] q | 16.88[‡] q | 17.34[‡] q | 16.86[Δ] q; 16.86[Δ] q | 16.86 q | 16.87[¶] q | 17.42[‡] q | 17.48[‡] q | 17.52[‡] q | 17.52[‡] q |
| 27 | 23.89 q | 23.38 q | 23.77 q | 23.42 q; 23.40 q | 23.44 q | 23.35 q | 23.88 q | 23.77 q | 23.76 q | 23.84 q |
| 28 | 179.82 s | 176.67 s | 176.87 s | 175.15 s; 175.15 s | 176.88 s | 177.10 s | 175.05 s | 177.76 s | 177.67 s | 177.64 s |
| 29 | 17.32[‡] q | 16.79[‡] q | 17.30[‡] q | 16.92[Δ] q; 16.92[Δ] q | 16.86 q | 16.85[¶] q | 17.05[‡] q | 16.98[‡] q | 17.57[‡] q | 17.53[‡] q |
| 30 | 21.11[¶] q | 21.05 q | 21.25 q | 21.03 q; 21.03 q | 21.01 q | 21.02 q | 21.32[¶] q | 21.33 q | 21.44 q | 21.42 q |
| 1' | | 167.94 s | 170.97 s | 44.58 t; 44.58 t | 64.44 t | 65.87 t | [Δ] | 173.12 s | 175.27 s | 175.90 s |
| 2' | | 61.05[¶] t | 61.35 t | 49.33 d; 49.30 d | 143.53 s | 136.22 s | 130.00 d | 42.45 t | 34.89 t | 32.60 t |
| 3' | | | | 64.82 t; 64.48 t | 128.47[†] d | 128.05[†] d | 117.83 d | | 35.88 t | 25.44 t |
| 4' | | | | | 123.57[†] d | 128.28[†] d | | | | 39.66 t |
| 5' | | | | | [Δ] | 127.83 d | | | | |
| | 170.54 s ($O\underline{C}(O)CH_3$) | 60.29[¶] t ($O\underline{CH_2}CH_3$) | | | | | 171.14 s ($O\underline{C}(O)CH_3$) | | | |
| | 21.42[¶] q ($OC(O)\underline{CH_3}$) | 13.98 q ($OCH_2\underline{CH_3}$)21.54[¶] q | | | | | ($OC(O)\underline{CH_3}$) | | | |

[§], [#], [‡], [¶], [†] Signals marked with the same symbols may be exchanged within the column.

[Δ] Signal was not detected.

***N*-(3β-Hydroxyurs-12-en-28-oyl)-4-aminobutyric acid (XV)** was prepared by reaction of 3β-acetoxy-urs-12-en-28-oyl chloride (**XI**) with methyl 4-aminobutanoate hydrochloride and subsequent hydrolysis (Method B). Yield 0.79 g (75%), white powder, mp 167–170°C (EtOH), $[\alpha]^{25}$ +60 (*c* 1.0, EtOH). $^1$H NMR and $^{13}$C NMR data are given in the Tables 4 and 5.

**Preparation of rhAR.** Open reading frame of human AR was amplified by PCR and inserted into *E. coli* expression vector pET-23b (Merck, Darmstadt, Germany). Then the recombinant expression plasmid was transformed into *E. coli* BL21(DE3) host strain. The transformant was grown in LB broth containing 50 μg/ml of ampicillin at 37°C, 200 rpm. When the absorbance at 600 nm reached 0.8, the expression of recombinant AR was induced by IPTG and the culture continued at 30°C. After 4 h of additional culture, bacterial cells were harvested and stored at –20°C until further use. Further procedures were all performed at 4°C, except the lysis of bacterial cells, which was performed at room temperature. Harvested cells were lysed by adding 5 ml of BugBuster master mix lysis solution (Merck, Darmstadt, Germany) per 1 mg of wet cell. After adding about 3 volumes of binding buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, and 1 mM DTT), cell lysates were centrifuged to remove cell debris. The supernatant was mixed with 1 ml of Ni-NTA resin (Merck, Darmstadt, Germany) on a twist shaker for 1 h. Then the protein-bound resin was collected by gentle centrifugation and packed in a column. After washing with 10 column volumes of washing buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 20 mM imidazole, and 1 mM DTT), bound proteins were eluted with 4 ml of elution buffer (100 mM sodium phosphate, pH 6.2, 0.5 M NaCl, 250 mM imidazole, and 1 mM DTT). The concentration of the protein solution was determined by using Bradford dye (BioRad, Hercules, USA) and adjusted to a final concentration of 40 μM rhAR. Small aliquots of enzyme solution were frozen with liquid nitrogen and stored at –80°C until further use.

**Measurement of rhAR activity:** rhAR activities were assayed spectrophotometrically by monitoring the NADPH oxidation that accompanies the *DL*-glyceraldehyde reduction used as substrate as shown in Table 1 (measuring the decrease in absorption of NADPH at 340 nm over a 5 min period) [21].

Each 1.0 mL cell contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2), 0.3 mM NADPH with or without 10 mM substrate and inhibitor.

Inhibition rate was calculated as percentage with respect to the control value and expressed as mean ± standard deviation of triplicate experiments. The concentration of each test sample giving 50% inhibition of activity ($IC_{50}$) was estimated from the least-squares regression line of the logarithmic concentration plotted against inhibitory activity. TMG (tetramethylene glutaric acid) was used as positive control.

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