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Terpenoids. III: Synthesis and biological evaluation of 23-hydroxybetulinic acid derivatives as novel inhibitors of glycogen phosphorylase

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ABSTRACT

A series of 23-hydroxybetulinic acid derivatives were prepared and tested in vitro as a new class of inhibitors of glycogen phosphorylase (GP). Within this series of compounds, **12b** (IC_{50} = 3.5 μM) is the most potent GPa inhibitor. The preliminary SAR results of the 23-hydroxybetulinic acid derivatives are discussed.

Glycogen phosphorylase (GP), the enzyme which catalyzes glycogen breakdown to produce glucose and related metabolites for energy, plays a key role in the glycogenolytic pathway.[1] Because of its key role in the modulation of glycogen metabolism, pharmacological inhibition of GP has been regarded as a promising therapeutic approach for treating diseases caused by abnormalities in glycogen metabolism. Inactivation of GP rather than inhibition of glycogen kinase synthase-3 (GKS-3) could mimic insulin stimulation of hepatic glycogen synthesis.[2] Several structural classes of GP inhibitors have been described[3] and a few have been studied in clinical trials[4] for treatment of type 2 diabetes, which is associated with disorders in glucose metabolism by the liver and periphery.[5] At the same time, the identification of several potential regulatory binding sites in GP[6] have furthered inhibitor studies and promoted the therapeutic research of diabetes.

Pentacyclic triterpenes are widely distributed throughout the plant kingdom. Some naturally occurring triterpenes (oleanolic acid, betulinic acid, and maslinic acid) have been shown to possess a wide spectrum of biological and pharmacological activities.[7] During the past few years, a new class of inhibitors of glycogen phosphorylase including many oleanane-type and ursane-type pentacyclic triterpenes (corosolic acid, oleanolic acid, and maslinic acid to name a few) have been reported. Some derivatives of these triterpenes were found to be potent GPa inhibitors.[8]

Our efforts to improve the biological activity of 23-hydroxybetulinic acid (**1**), a lupane-type triterpene isolated from the root of *Pulsatilla chinensis*[9] which has similar biological activities (anti-tumor and anti-HIV)[10] as betulinic acid,[11] are ongoing. Besides the structural similarity and pharmacological comparability of **1** with other pentacyclic triterpenes, the promising therapeutic potential of triterpenes in controlling glycogen metabolism became the main reason which led us to discover the probable GP inhibitory activity of **1**. Using **1** as the lead compound, we sought to identify derivatives of 23-hydroxybetulinic acid as novel GP inhibitors. Initial synthetic efforts were focused on structural modifications at the C-3, C-23 and C-28 positions of **1**. Herein, we report the synthesis of a series of derivatives of 23-hydroxybetulinic acid and their GP inhibition assay results.

First, starting from **1**, a series of C-28 ester derivatives were synthesized. The synthetic route is outlined in Scheme 1. Treatment of **1** with Ac_2O in pyridine afforded 3, 23-*O*-diacetyl compound **2** in 98% yield. Reaction of **2** with $(COCl)_2$ in CH_2Cl_2 yielded the C-28 acyl chloride intermediate, which was followed by esterification with hexanol, cyclohexanol and 1, 4-dihydroxy-2-butyne to give **3a–c** in yields of 81–90%. Esterification of **1** with 1,2-dibromoethane in the presence of K_2CO_3 in DMF furnished bromide compound **4** in 88% yield, which was followed by amination with hexane diamine in the presence of K_2CO_3 in DMF and acylation with Ac_2O in pyridine to afford **5** in 45% yield. Reaction of C-28 acyl chloride

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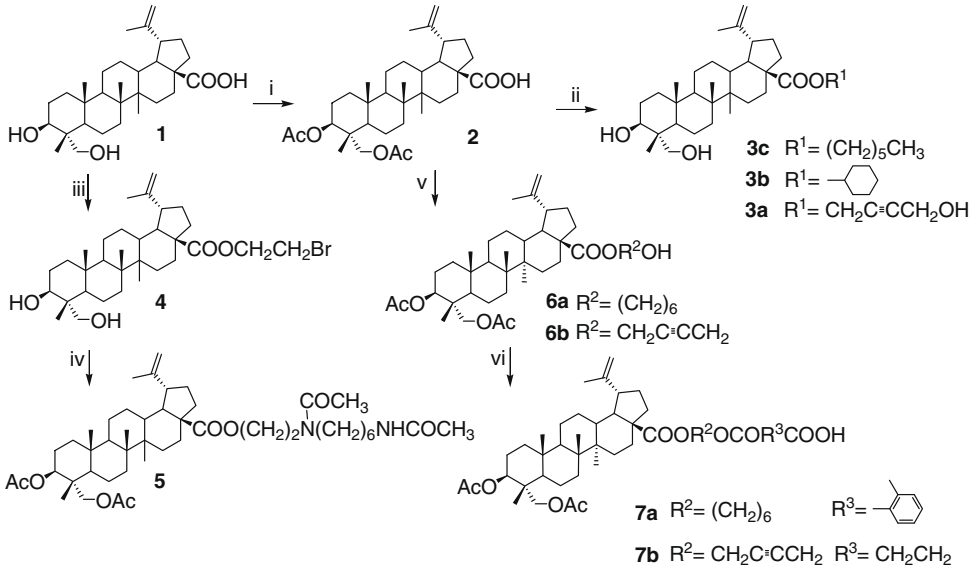
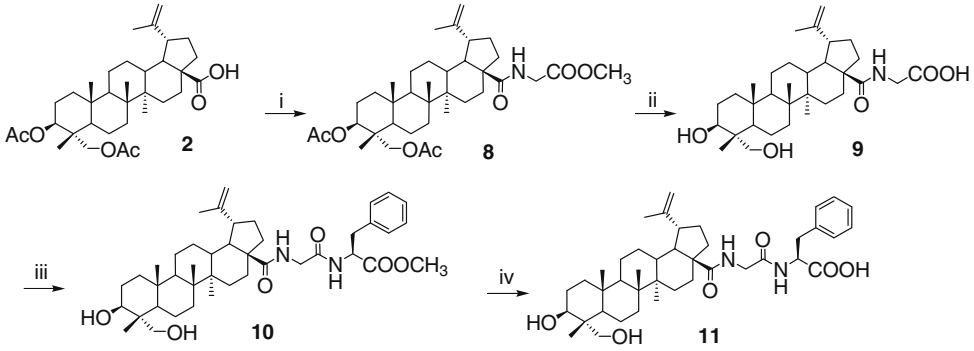
with diols in CH_2Cl_2 gave hydroxyesters **6a**–**b** in yields of 52–58%, then compounds **7a**–**b** were prepared by reaction of **6a**–**b** with $R^3(CO)_2O$ in the presence of DMAP in pyridine in yields of 59–64%.

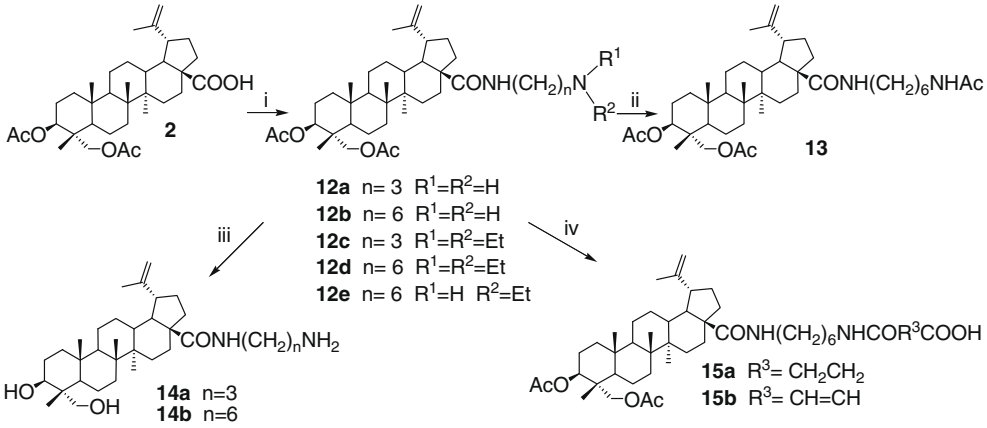
As depicted in Schemes 2 and 3, several C-28 amide derivatives of **1** were synthesized. Treatment of diacetate **2** with $(COCl)_2$ and then glycine methyl ester in the presence of DMAP, both in CH_2Cl_2, afforded **8** in 62% yield. Hydrolysis of **8** in the presence of 4 N NaOH in THF and CH_3OH gave acid **9** in 84% yield. Reaction with α-phenylalanine methyl ester in the presence of EDC and HOBT in CH_2Cl_2 converted **9** into **10** in 76% yield, which was followed by hydrolysis



Scheme 1. Reagents: (i) Ac_2O, pyridine; (ii) (a) $(COCl)_2$, then R^1OH, CH_2Cl_2; (b) 4 N NaOH, CH_3OH, THF; (iii) $BrCH_2CH_2Br$, K_2CO_3, DMF; (iv) (a) $H_2N(CH_2)_6NH_2$, K_2CO_3, DMF; (b) Ac_2O, pyridine; (v) $(COCl)_2$, CH_2Cl_2, then HOR^2OH; (vi) $R^3(CO)_2O$, DMAP, pyridine.



Scheme 2. Reagents: (i) $(COCl)_2$, CH_2Cl_2, then $H_2NCH_2COOCH_3{\cdot}HCl$, DMAP; (ii) 4 N NaOH, CH_3OH, THF; (iii) $H_2NCH(CH_2Ph)COOCH_3{\cdot}HCl$, EDC, HOBT; (iv) 4 N NaOH, CH_3OH, THF.



Scheme 3. Reagents: (i) for **12a** and **12b**: $(COCl)_2$, CH_2Cl_2, then $H_2N(CH_2)_nNH_2$; for **12c** or **12d**: C_2H_5Br (2.5 equiv), K_2CO_3, DMF, **12a** or **12b**; for **12e**: C_2H_5Br (1.2 equiv), K_2CO_3, DMF, **12b**; (ii) Ac_2O, pyridine; (iii) 4 N NaOH, CH_3OH, THF; (iv) $R(CO)_2O$, DMAP, pyridine.

with 4 N NaOH in THF and CH_3OH to give **11** in 82% yield. Treatment of diacetate compound **2** with $(COCl)_2$ and then diamines in CH_2Cl_2, produced monoamides **12a–b** in yields of 40–53%. Reaction of **12a–b** with ethyl bromide in the presence of K_2CO_3 in DMF afforded **12c–e** in 40–70% yield. Hydrolysis of **12a–b** with 4 N NaOH in THF and CH_3OH afforded **14a–b** in 70–75% yield,[12] then acylation of **12b** and with $R(CO)_2O$ gave **15a–b** in yields of 59–64%.

The semi-synthesis of 23-hydroxybetulin (**17**) was completed through the reduction of 23-hydroxybetulinic acid ester with $LiAlH_4$ in THF, which was obtained by esterification of **1** with ethyl bromide in the presence of K_2CO_3 in DMF. Both **1** and **17** were treated with $(COCl)_2$, and then reacted with different amines or alcohols as shown in Scheme 4 to afford **16** and **18a–d** in yields of 34–67%. Acylation of **17** with succinic anhydride or acetic anhydride in pyridine gave **19a** and **19b** in yields of 71 and 39%, respectively.

The 23-hydroxybetulinic acid derivatives were evaluated in the enzyme inhibition assay against rabbit muscle glycogen phosphorylase a (RMGPa), which shares considerable sequence similarity with human liver GPa. The activity of rabbit muscle GPa was measured by detecting the release of phosphate from glucose-1-phosphate in the direction of glycogen synthesis.[13] As shown in Table 1, the results indicated that most target compounds exhibited moderate inhibitory activity against rabbit muscle GPa with IC_{50} values in the range of 3.5–289 μM.

The structural diversity of the 23-hydroxybetulinic acid derivatives tested in the enzyme inhibition assay insured that a wide variety of modifications were analyzed. As the assay results showed, C-28 ester derivatives exhibited moderate GPa inhibitory potency with hydrophilic groups (**3c**, **7a** and **7b**) providing slightly more potency than hydrophobic groups (**3a** and **4**) at the end of the C-28 side chain. However, compound **3b** was an exception with an IC_{50} of 50.4 μM. This result is promising and further studies with cyclic group substitution at the end of the C-28 side chain are currently in progress.

Introduction of an amino group at the end of C-28 side chain resulted in a significant change in potency. Compound **12a** (IC_{50} = 15.2 μM), **12b** (IC_{50} = 3.5 μM), **14a** (IC_{50} = 23.8 μM) and **14b** (IC_{50} = 10.2 μM) were potent GPa inhibitors and it is possible that the terminal amino group might contribute to the enhancement of the inhibitory activity. While N-substitution at the end of the amino group analogs **12c**, **12d** and **12e** resulted in slightly less potent inhibitory activity, and acylation of the amino group derivatives **13**, **15a** and **15b** also showed a decrease in potency, indicating that an amino group at the end of the C-28 side chain might be crucial and deserves more attention during the process of further inhibitor design and lead optimization.

Table 1
Inhibition assay results of target compounds against rabbit muscle GPa

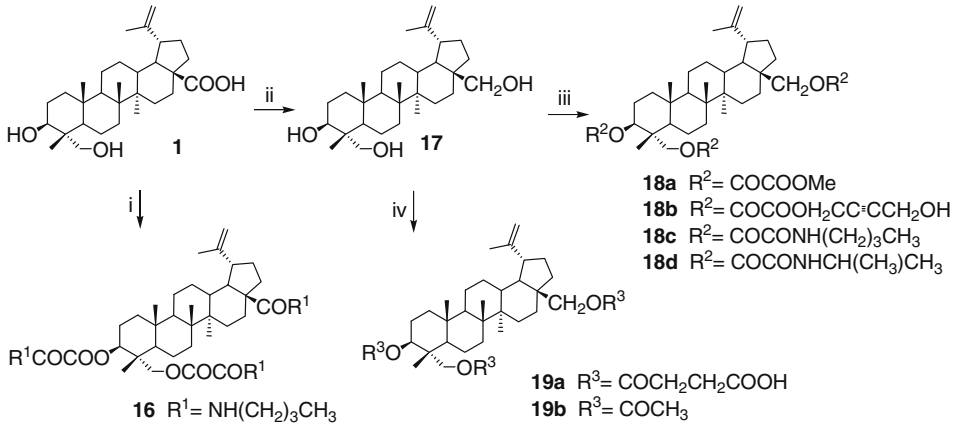
Compound	RMGPa [IC_{50} (μM) ± SD][a]
Caffeine	74.9 ± 7.2
1	103 ± 1.7
3a	194 ± 11.8
3b	50.4 ± 9.4
3c	69.1 ± 5.9
4	289 ± 21.0
5	96.6 ± 30.1
7a	69.2 ± 11.3
7b	94.5 ± 24.2
8	103 ± 13.3
9	67.7 ± 10.6
10	40.6 ± 7.1
11	129 ± 9.0
12a	15.2 ± 5.0
12b	3.5 ± 0.8
12c	72.8 ± 3.6
12d	55.6 ± 6.1
12e	29.5 ± 5.0
13	34.1 ± 9.3
14a	23.8 ± 0.9
14b	10.2 ± 1.8
15a	194 ± 10.9
15b	54.8 ± 7.5
16	na[b]
18a	30.1 ± 5.5
18b	19.1 ± 4.1
18c	34.7 ± 7.2
18d	35.9 ± 3.0
19a	97.8 ± 11.6
19b	143 ± 11.3

[a] Values are means of three experiments.
[b] na = no activity.

Analogues of 23-hydroxybetulin also showed somewhat potent GPa inhibitory activity. Tri-oxalate derivatives (**18a–d**) exhibited slightly potent GPa inhibitory activity, while the derivatives acylated with acetic anhydride (**19b**) or succinic anhydride (**19a**) resulted in a decrease in potency.

The modification of the hydroxy groups at C-3 and C-23 did not provide clear SAR information that could be used for further GPa inhibitor design. Further modifications at these two positions and SAR studies are underway in our laboratory.

In summary, a series of 23-hydroxybetulinic acid derivatives have been prepared and evaluated as a new class of GP inhibitors, among which **12b** was the most potent GPa inhibitor (IC_{50} = 3.5 μM). This discovery has afforded a novel lead compound for developing potent GP inhibitors. As a possible outcome from the present findings, 23-hydroxybetulinic acid derivatives may,



Scheme 4. Reagents: (i) $(COCl)_2$, CH_2Cl_2, then R^1H; (ii) (a) BrC_2H_5, K_2CO_3, DMF; (b) $LiAlH_4$, THF; (iii) $(COCl)_2$, CH_2Cl_2, then amines or alcohols; (iv) for **19a**: $(CH_2CO)_2O$, DMAP, pyridine; for **19b**: Ac_2O, pyridine.

in the future, become potential drug candidates for treating diseases caused by abnormalities in glycogen metabolism.[14] Further research on GPa inhibition in vivo and SAR studies of 23-hydroxybetulinic acid are ongoing in our laboratory and the results will be reported in due course.

Acknowledgments

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12. *Analytical data for compound* **12b**: mp 80–82 °C; IR (KBr, cm^{-1}): ν 3472, 2936, 2859, 1731, 1633, 1535, 1448, 1369, 1247, 1045, 882; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.80, 0.87, 0.93, 0.96, 1.68 (s, 3H each, 24, 25, 26, 27, 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 2.17 (m, 2H, 28-$CONH(CH_2)_6NH_2$), 2.47 (m, 1H, 19-CH), 3.17 (m, 4H, 28-$CONHCH_2(CH_2)_4CH_2NH_2$), 3.68, 3.84 (dd, 1H each, $J_A = J_B$ = 11.52 Hz, 23-CH_2), 4.59, 4.73 (d, 1H each, 29=CH_2), 4.76 (m, 1H, 3-CH), 5.65 (m, 1H, 28-$CONH(CH_2)_6NH_2$); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 12.7, 14.3, 15.9, 16.3, 17.6, 19.2, 20.7, 20.8, 21.0, 22.9, 25.3, 26.3, 26.5, 28.9, 29.4, 30.5, 32.6, 33.3, 33.7, 36.6, 36.8, 37.7, 37.8, 38.3, 40.0, 40.4, 41.6, 42.0, 46.3, 47.9, 49.8, 50.2, 55.0, 64.9 (23-C), 73.9 (3-C), 109.3 (29-C), 151.1 (20-C), 170.0(CH_3CO), 170.2(CH_3CO), 175.4 (28-C). EIMS: $[M+H]^+$ 655.5.
13. The biological assay procedure for target compounds: Each compound was dissolved in DMSO and diluted at different concentrations for IC_{50} determination. The enzymes were added into the 100 μl buffer with compounds dissolved in containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate, and 1 mg/ml glycogen in 96-well microplates. After the addition of 150 μl of 1 M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green, reactions were run at 22 °C for 25 min. And then the phosphate absorbance was measured at 655 nm. The IC_{50} values were estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation.
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