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Oleanolic acid and its derivatives: New inhibitor of protein tyrosine phosphatase 1B with cellular activities

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ABSTRACT

Protein tyrosine phosphatase 1B is a key factor in the negative regulation of insulin pathway and a promising target for treatment of diabetes and obesity. Herein, a series of competitive inhibitors were optimized from oleanolic acid, a natural triterpenoid identified against PTP1B by screening libraries of traditional Chinese medicinal herbs. Modifying at 3 and 28 positions, we obtained compound 13 with a K_i of 130 nM, which exhibited good selectivity between other phosphatases involved in insulin pathway except T-cell protein tyrosine phosphatase. Further evaluation in cell models illustrated that the derivatives enhanced insulin receptor phosphorylation in CHO/*h*IR cells and also stimulated glucose uptake in L6 myotubes with or addition of without insulin.

1. Introduction

Protein tyrosine phosphatase 1B (PTP1B) has been identified as an encouraging target against type 2 diabetes for about a decade.[1] Anchored to endoplasmic reticulum, PTP1B is involved in the insulin receptor (IR) dephosphorylation process, negatively regulating the signal of insulin pathway. Two independent laboratories have generated PTP1B knockout mice, which showed increased insulin sensitivity in liver and muscle tissues and were resistant to weight gain compared with normal ones. These results apparently confirmed the relationship between PTP1B and diabetes.[2,3] Along with the elucidation of its protein structure,[4–6] many synthetic inhibitors with submicro-, even nano-molar activity were discovered through high-throughput screening (HTS) and structure-based design.[7–10] However, their further development to clinical trials was further restricted mainly for two reasons comparing with emergence of several new drugs in kinases family.[11,12] First, the selectivity between T-cell protein tyrosine phosphatase (TCPTP) and PTP1B[13] was still low because of their high conserved catalytic domain, despite the importance of which still need to be proven. Second, low cell permeability quenched most competitive inhibitors that always had strong negative chemical nature mimicking the phosphate group in IR substrate. In addition, only several of recent emerged inhibitors[14–16] were derived from natural products,[17–21] which had been considered as a great diverse and drug-like library.

In the history of traditional Chinese medicine (TCM), medicinal plants and their extracts were used to treat various diseases. Nowadays, compounds derived from natural products with the unique and diverse chemical entities still constituted a considerable resource for developing novel medicaments. Diabetes, referred as "thirsty disease" in ancient China, was recorded on different pharmacopoeia and treated with different kinds of herbs. According to the old prescription, we have established a TCM extracts library and also a library of pure natural products isolated from TCM extracts since 2001. More than 200 anti-diabetic TCM extracts as well as hundreds of isolated natural products were prepared for HTS against PTP1B.[22–24] As expected, several of them were found to possess strong positive activities, such as ursolic acid,[25] corosolic acid,[26] and oleanolic acid isolated from *Cornus officinalis*. A quantitative structure–activity relationships (QSARs) on the inhibition activities of oleanolic acid analogues against PTP1B were recently studied[27] based on our patent.[28]

In the work described herein, we focus on increasing the inhibitory activity of oleanolic acid, which was relatively easier to obtain from plant extracts than ursolic acid and others. As shown in Figure 1, oleanolic acid has two positions which can be modified, C-3 and C-28. Besides these two positions, variation of pentacyclic moiety was ineffective according to the corresponding triterpene **4–9**. Therefore, we reported the synthesis and PTP1B inhibitory activity of oleanolic acid derivatives such as **10a–10p** and **11a–11s** at C-28 position (Scheme 1) and **12a–12m** at C-3 position

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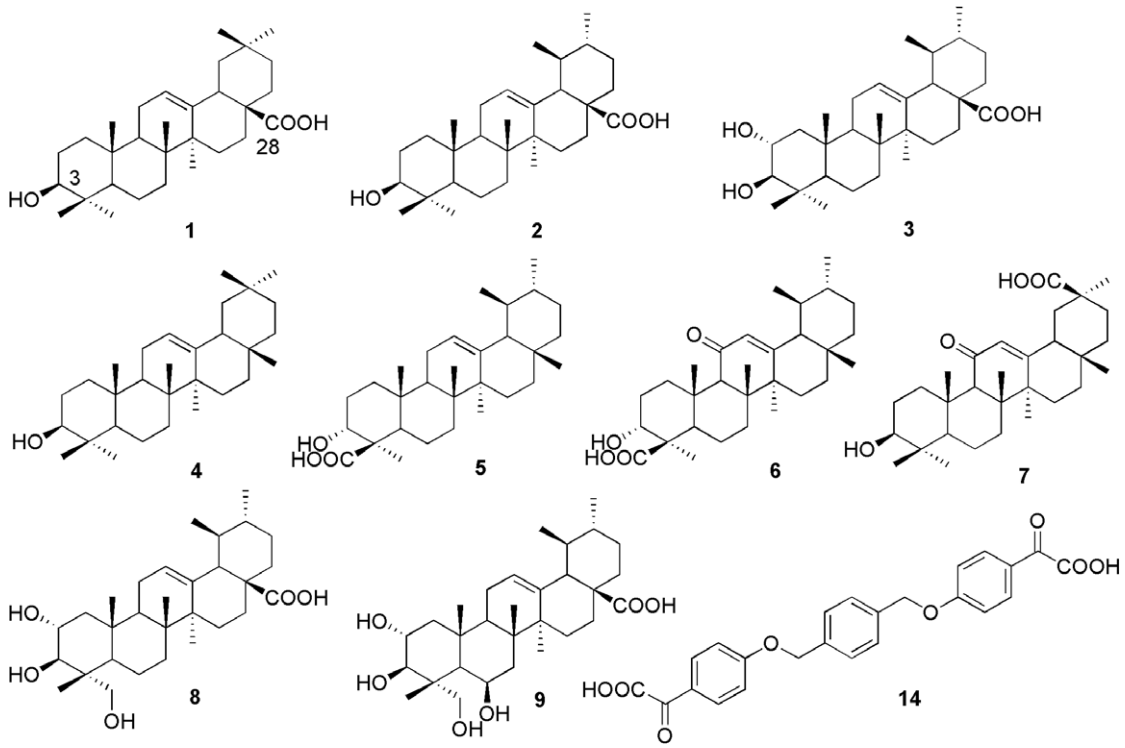


Figure 1. Structures of compounds **1**–**9** and **14**.

(Scheme 2), respectively. Moreover, selected compounds in the series were evaluated in kinetics, selectivity and cell activities to estimate if their biological properties were suitable for further development.

2. Results and discussion

2.1. Chemistry

The extracts of *C. officinalis* showed good inhibitory activity against PTP1B in HTS assay, in which oleanolic acid **1**, ursolic acid **2**, and corosolic acid **3** were the main components. In our compound library against diabetes, several other stored natural products with same scaffold were also selected to test, such as oleanane **4** separated from *Caesalpinia minax* Hance, boswellic acid **5** and 11-keto boswellic acid **6** from *Boswellia carteri*, glycyrrhetinic acid **7** from *Glycyrrhiza yunnanensis*, asiatic acid **8** and madecassic acid **9** from *Centella asiatica* (L.) Urban.[29–32]

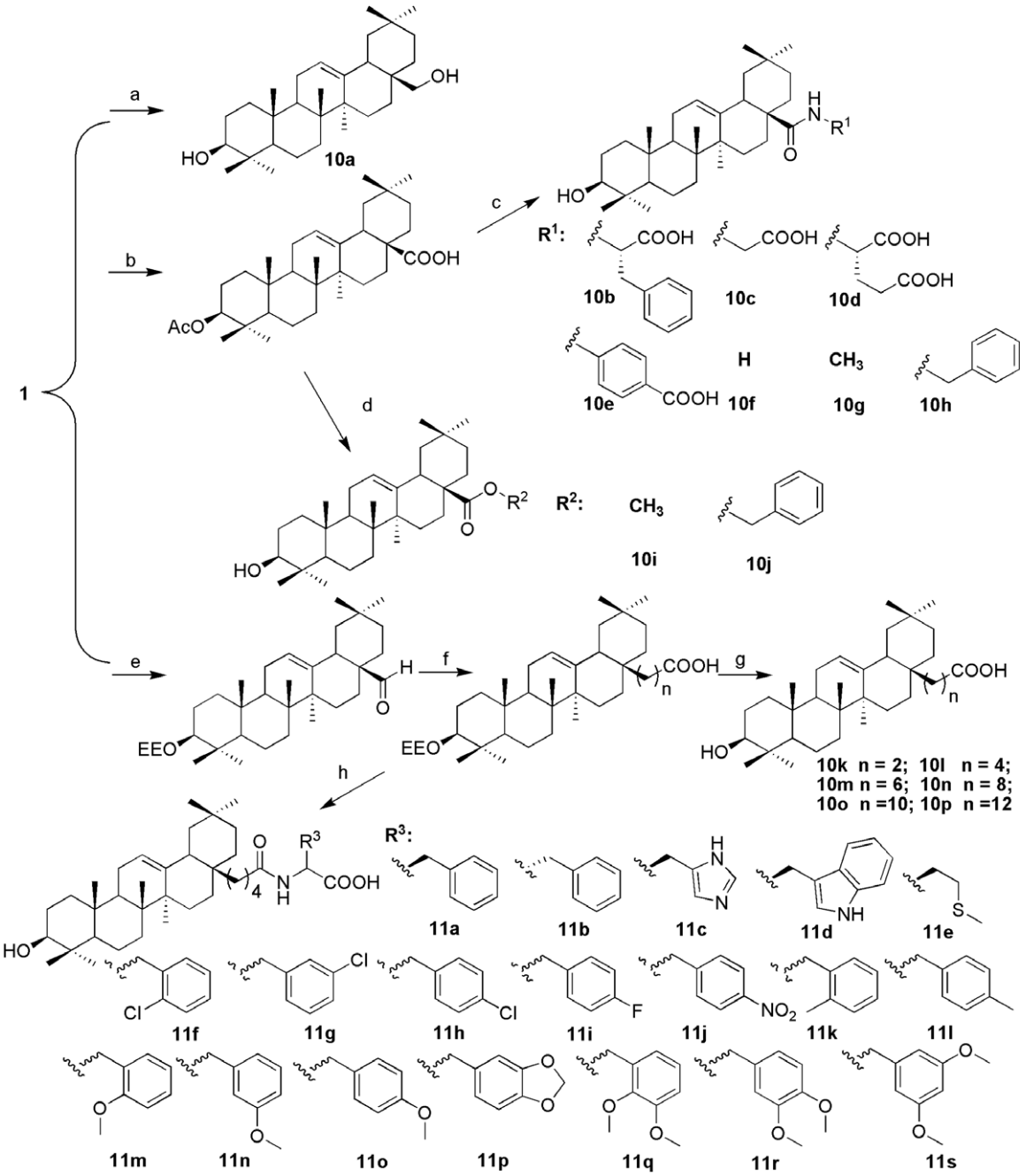
Compound **10a** was achieved by reduction of **1** with $LiAlH_4$, and **10b**–**10e** were also synthesized from **1** via coupling the 3-acetyl oleanolic acyl chloride with corresponding amino acid esters, then hydrolyzing the protective groups. Esterification and amidization of **1** with different alcohols and amines yielded **10f**–**10j**. Compounds **10k**–**10p** were synthesized through repetitious Wittig reaction between proper quaternary phosphonium salt or triethyl phosphonoacetate and oleanolic aldehyde obtained from Swern oxidation of protected **10b** at C-3 position. Amide intermediates of **11a**–**11s** were gotten through coupling EE protected **10l** with different amino acid esters, among which the unnatural ones (**11f**–**11s**) were synthesized following O'Donnell's procedure.[33] Deprotection were carried out with global method which used pyridinium *p*-toluenesulfonate for acetals and aqueous base solution for esters.

The starting material of **12a**–**12i** was oleanolic acid, and of the others (**12j**–**12m**) was oleanolic acid methyl ester. Adding 2-iodoxybenzoic acid, oleanolic acid was oxidated to **12a**, which was then converted to **12b**–**d** via Corey–Bakshi–Shibata pyrrolidine catalystic reduction,[34] Wolff-Kishner reduction,[35] and Wittig reaction, respectively. Esterification and etherification of **1** at 3-position were relatively more rigorous than common reaction occurred on hydroxyl groups due to the steric hindrance around it. **12e** was esterified with ethyl oxalyl chloride and then hydrolyzed to get **12f**. **12g**–**12i** were conducted by adding the corresponding acid anhydride to oleanolic acid in refluxing toluene overnight. While **12j** could be easily prepared with benzyl bromide and **10i** in DMF solution at room temperature, etherifying with different carboxyl substituted benzyl bromides should be conducted under refluxing dioxane with NaH overnight to prepare **12k**–**12m**. Finally, **12j**–**12m** were achieved from their methyl ester using LiI in refluxing DMF solution.

Though several reagents and different conditions were tried, the synthetic process of compound **13** was blocked at the step of etherification of **10l**'s 3-position previously. Eventually, our target was carried out through a reversal route (Scheme 3). Ether **13a** was first synthesized according to the procedure mentioned in synthesis of **12m**, then the carboxyl group was protected to 2-alkyl-1,3-oxazoline **13b** in two steps. Following the method gained **11a**, **13b** was turned to elongated carboxyl acid **13c** at 28-position via $LiAlH_4$ reduction, Swern oxidation, and Wittig reaction successively. After coupling with L-phenylalanine, the oxazoline, and methyl ester were deprotected to obtain carboxyl **13d**. Finally, we achieved compound **13** through selective hydrogenation in the presence of benzyl group by adding Na_2CO_3 to poison palladium catalyst.

2.2. Biological evaluation

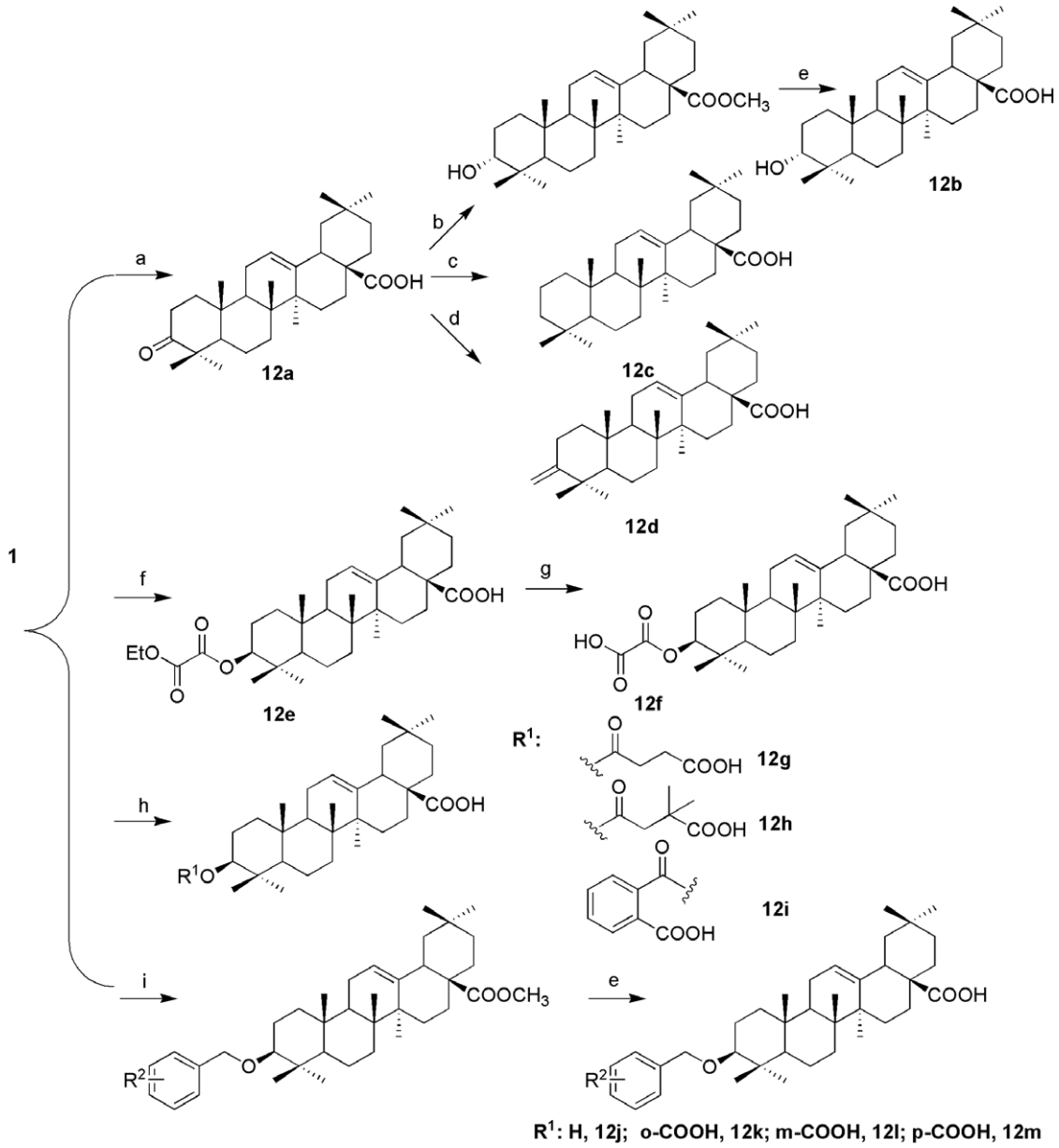
To examine the potential ability of the natural and artificial pentacyclic triterpene derivatives, their PTP1B inhibitory activities were assayed by the method of *p*NPP using compound **14** as reference compound.[36] Moreover, their TCPTP inhibitory activities were examined simultaneously by the same method for further selectivity studying.



Scheme 1. Synthesis of 28-modified oleanolic acid. Reagents and conditions: (a) $LiAlH_4$, THF, RT, 4 h; (b) Ac_2O, pyridine, RT, overnight; (c) 1: $(COCl)_2$; 2: different methyl amino acid esters or amines, DCM, Et_3N, 0 °C to RT, 2 h; 3: 5 N NaOH (aq.), THF, MeOH, RT; (d) MeOH or benzyl alcohol, DMAP, EDCI, reflux, overnight; (e) 1: ethyl vinyl ether, PPTS, DCM, 2 h; 2: $LiAlH_4$, THF, RT, 4 h; 3: $(COCl)_2$, DMSO, DCM, −60 °C to RT, 1 h, then Et_3N, 1 h; (f) 1: $BrPh_3P^+(CH_2)3COOH$, KOtBu or $BrPh_3P^+(CH_2)_5COOH$, KOtBu or triethyl phosphonoacetate, NaH; THF, RT, 2 h; 2: H_2, EtOAc, Pd/C, RT, overnight; (g) PPTS, MeOH, 50 °C, 1 h; if n = 8, 10, 12, repeated $LiAlH_4$ reduction and step f; (h) 1: different amino acid esters, EDCI, HOBt, Et_3N, DCM, 4A MS, RT, 8 h; 2: PPTS, MeOH, 50 °C, 1 h, then 5 N NaOH (aq.), THF, MeOH, RT.

Primarily, the core structure of pentacyclic triterpenoids shared by **1**–**3** reminded us if the other compounds with the oleanane or ursane scaffolds had the same effect. Compounds **4–9** from our library were tested for this purpose, but only **6** and **9** had weaker effect. After careful consideration of the unsatisfying results varied at pentacyclic core, we selected oleanolic acid as the lead in the following optimization, because of its cheapness, commercial availability and safety. It is noticed that oleanolic acid has been used as an over-the-counter drug for treatment of hepatitis in China more than 20 years.[37]

Simply changing the carboxyl group at C-28 position into methyl (**4**) and hydroxyl group (**10a**) decreased the activity below the threshold value (>20 μM), which indicated that the presence of carboxyl group was vital to the PTP1B inhibitory potency. It was also proved by amide (**10f**–**h**) or ester (**10i** and **10j**) substitution compounds. Demonstrated by previous research,[11] keeping proper elongated phosphate mimics entering the catalytic domain with an aryl group nearby was one important principle in designing competitive PTP1B inhibitor. This rule was verified by **10b** and **10k**, where compound **10c** with a removed benzyl and replaced amide was definitely less potent. So, compounds **10k**–**10p** with the carbon chain length at 2, 4, 6, 8, 10, and 12 were synthesized, respectively, to find the proper length. The corresponding results suggested that the inhibitory potency of elongating series were

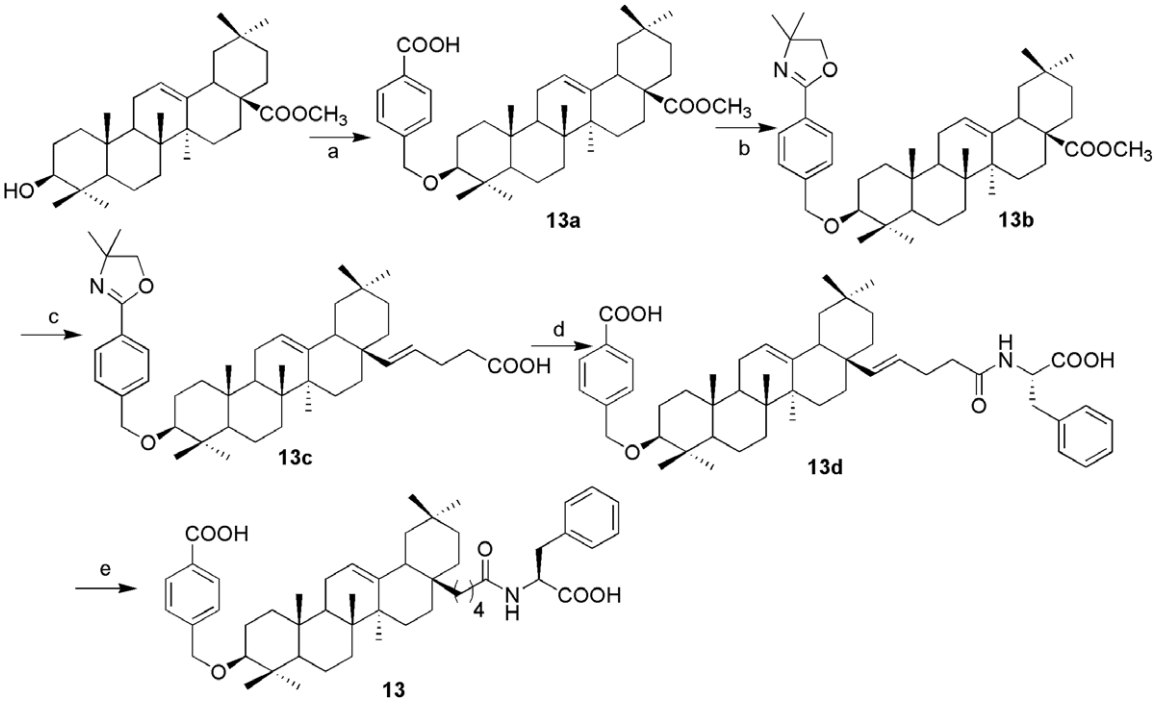


Scheme 2. Synthesis of 3-variated oleanolic acid. Reagents and conditions: (a) 2-iodoxybenzoic acid, toluene, DMSO, RT, 4 h; (b) 1: CH_2N_2, Et_2O, 0 °C to RT; 2: (*s*)-(−)-2-(diphenylhydroxymethyl)pyrrolidine, $BH_3 \cdot SMe_2$, THF, RT, 1 h; (c) diethyl glycol, NH_2NH_2, KOH, 200 °C, 4 h; (d) $BrPh_3P^+CH_3$, KOtBu, THF, RT, 2 h; (e) LiI, DMF, reflux, overnight; (f) ethyl oxalyl chloride, Et_3N, DCM, 0 °C to RT, 4 h; (g) $NaHCO_3$ (aq), THF, MeOH, 1 h; (h) corresponding acid anhydride, toluene, pyridine, reflux, overnight; (i) if R_2 = H, DMF, NaH, RT, 4 h; if R_2 = COOH, dioxane, NaH, reflux, overnight.

low acceleration along with the increasing length gradually, which suggested that the compounds with the length of 4–6 carbon chain were suitable for further optimization due to their biological activities and synthetic facilities.

In further study, different substituted α-amino acids were coupled with elongated oleanolic acid (**10l**) to examine the impact of vicinal aryl group. Primarily, similar activities shown by the diasteromers **11a** and **11b** linking with L- and D-phenylalanine, respectively, meant that the stereochemistry of amino acids was less important. Three essential amino acid derivatives (**11c**–**11e**) and fourteen racemic artificial amino acid derivatives (**11f**–**11s**) were obtained to enrich the complexity. Interestingly, piperonyl substituent (**11p**) had the best activity comparing with others, in which the electron donating 1,3-benzodioxole may had the more affinitive π-bond with Phe182 and less steric hindrance to rigid catalytic domain.[7] In general, all of these analogues showed only some nuance in activities (IC_{50} = 0.44–0.82 μM) except **11c** possessed an electron-positive imidazole group, which might hardly enter the catalytic domain surrounded by alkyl amino acids.[8]

Retaining the pentacyclic scaffold was also the principle of exploration at C-3 position, because hydroxyl substitution (**2**, **8**, **9**) at neighbor positions did not get any valuable improvement. Furthermore, activities did not ameliorate when we oxidating parent compound **1** to carbonyl (**12a**), converting configuration (**12b**), removing hydroxyl group (**12c**) and changing to hydrophobic groups (**12d**, **12j**). Stronger inhibitors **12e**, **12f**, and **12h** suggested that introducing carboxyl increased the activities. However, **12g** and **12i** showed weaker effect than oleanolic acid. Fortunately, benzyl group derivatives **12k**–**12m** with different carboxyl replacement gave us satisfied results, which displayed 7-fold increase at para position, 6-fold at meta and retainment at ortho. On the basis of preceding results, we combined **12m**'s moiety at



Scheme 3. Synthesis of compound **13**. Reagents and conditions: (a) *p*-COOH benzyl bromide, dioxane, NaH, reflux, overnight; (b) 1: 2-amino-2-methylpropan-1-ol, EDCI, HOBt, Et_3N, DCM, 4 Å MS, RT, 8 h; 2: Ph_3P, diisopropyl azodicarboxylate, THF, 0 °C to RT, 2 h; (c) 1: $LiAlH_4$, THF, RT, 4 h; 2: $(COCl)_2$, DMSO, DCM, −60 °C to RT, 1 h, then Et_3N, 1 h; 3: $Br^-Ph_3P^+(CH_2)_3COOH$, KOt-Bu, NaH; THF, RT, 2 h; (d) 1: L-phenalanine methyl ester hydrochloride, EDCI, HOBt, Et_3N, DCM, 4 Å MS, RT, 8 h; 2: 2 N HCl, EtOH, reflux, 2 h, 3: then 5 N NaOH (aq.), THF, MeOH, RT; (e) H_2, EtOAc, Pd/C, Na_2CO_3, RT, overnight.

C-3 position and **11a**'s at C-28 position to design compound **13**, which showed the best activity against PTP1B in our derivatives.

Most of the compounds (except **10a**–**10j**) were evaluated in TCPTP inhibitory assay (Tables 1 and 2). Unfortunately, none of them exhibited any obvious distinction (above 2-fold) between these two homogeneous enzyme. The conclusion was similar to most published PTP1B inhibitors, whereas the importance of selectivity between PTP1B and TCPTP still need to be proved for their remarkable differences in expressive amounts, endocellular distribution, and biological functions.[38]

Besides TCPTP, there are several other critical protein tyrosine phosphatases (PTPs) negatively regulating insulin dephosphorylation, such as src homology phosphatase-1 (SHP-1), leukocyte antigen-related phosphatase (LAR), protein tyrosine phosphatases α and ε (PTPα and PTPε).[39] So, we tested the inhibitory activity of our derivatives on the purified recombinant human SHP1, LAR D1, PTPα D1 and PTPε D1. As listed in Table 3, we concluded that our inhibitors had no visible activities against receptor-like transmembrane PTPs and possessed about 2–10-folds selectivity between PTP1B and SHP1.

To elucidate the characteristics of our modified compounds, the K_i values of three nodal compounds (**11a**, **12m**, and **13**) were at 0.64, 0.97, and 0.13 μM, respectively, coinciding with the active trend described in SAR study. As also elucidated in Supplementary material, the increasing K_m and retaining V_{max} demonstrated that our derivatives were competitive inhibitors, which probably had good binding affinity at catalytic domain like other published competitive inhibitors. And we also adopted a time-course inhibition assay showed in supplementary material to verify that the inhibitory effect was not related to irreversible binding, such as oxidation mechanism.

We tested the cellular effects of compounds **1**, **11a**, and **13** on the phosphorylation level of IR and Akt in Chinese hamster ovary cell line transfected with an expression plasmid encoding human IR (CHO/*h*IR). As illustrated in Figure 2A–C, sodium vanadate (1 mM) was used as a positive control after treating the cell with 10 nM insulin (vehicle, lane 1), while DMSO as negative control (lane 2), compound **13** significantly increased the level of IR phosphorylation in a dose dependent manner (Fig. 2C). However,

Table 1
Inhibitory activity of compounds **1**–**10p** against PTP1B and TCPTP

Compounds	IC_{50} (μM)[a]	
	PTP1B	TCPTP
1	3.37 ± 0.20	3.40 ± 0.26
2	3.08 ± 0.26	3.33 ± 0.19
3	5.49 ± 0.12	11.31 ± 0.19
4	>20	>20
5	>20	>20
6	8.04 ± 1.04	9.45 ± 0.77
7	>20	>20
8	>20	>20
9	12.38 ± 3.19	>20
10a	>20	NT[b]
10b	3.31 ± 1.11	NT
10c	15.44 ± 0.80	NT
10d	16.35 ± 1.20	NT
10e	3.19 ± 0.08	NT
10f	4.76 ± 0.63	NT
10g	9.20 ± 0.90	NT
10h	8.10 ± 1.08	NT
10i	4.44 ± 0.05	NT
10j	8.61 ± 0.50	NT
10k	2.10 ± 0.36	2.77 ± 0.10
10l	1.33 ± 0.23	1.11 ± 0.13
10m	0.88 ± 0.06	0.85 ± 0.04
10n	0.78 ± 0.08	0.79 ± 0.03
10o	0.72 ± 0.07	0.63 ± 0.05
10p	0.59 ± 0.07	0.53 ± 0.05
14[c]	5.73 ± 0.32	5.35 ± 0.21

[a] The *p*NPP assay. IC_{50} values were determined by regression analyses and expressed as means ± SD of three replications.
[b] Not tested.
[c] Positive control.

Table 2
Inhibitory activity of compounds **11a**–**13** against PTP1B and TCPTP

Compounds	IC_{50} (μM)[a]	
	PTP1B	TCPTP
11a	0.57 ± 0.15	0.63 ± 0.09
11b	0.74 ± 0.07	0.68 ± 0.03
11c	4.62 ± 0.20	4.31 ± 0.21
11d	0.59 ± 0.10	0.59 ± 0.15
11e	0.55 ± 0.07	0.75 ± 0.12
11f	0.56 ± 0.07	0.55 ± 0.07
11g	0.51 ± 0.04	0.52 ± 0.04
11h	0.61 ± 0.20	0.48 ± 0.05
11i	0.57 ± 0.02	0.65 ± 0.05
11j	0.45 ± 0.01	0.36 ± 0.03
11k	0.55 ± 0.33	0.50 ± 0.03
11l	0.65 ± 0.04	0.66 ± 0.05
11m	0.53 ± 0.07	0.45 ± 0.07
11n	0.52 ± 0.08	0.52 ± 0.04
11o	0.60 ± 0.10	0.55 ± 0.07
11p	0.44 ± 0.02	0.60 ± 0.05
11q	0.66 ± 0.25	0.75 ± 0.15
11r	0.82 ± 0.20	0.70 ± 0.19
11s	0.63 ± 0.17	0.85 ± 0.20
12a	5.32 ± 0.09	5.63 ± 0.31
12b	5.05 ± 0.69	5.10 ± 0.31
12c	2.61 ± 0.52	3.00 ± 0.24
12d	2.85 ± 0.47	2.53 ± 0.35
12e	2.89 ± 0.36	3.66 ± 0.25
12f	2.86 ± 0.23	4.55 ± 0.42
12g	5.97 ± 0.32	5.36 ± 0.57
12h	2.33 ± 0.24	1.38 ± 0.15
12i	4.58 ± 0.58	5.00 ± 0.80
12j	2.67 ± 0.20	2.75 ± 0.30
12k	2.72 ± 0.24	2.22 ± 0.21
12l	0.62 ± 0.04	0.49 ± 0.04
12m	0.54 ± 0.09	0.47 ± 0.04
13	0.15 ± 0.02	0.16 ± 0.01
14[b]	5.73 ± 0.32	5.35 ± 0.21

[a] The *p*NPP assay. IC_{50} values were determined by regression analyses and expressed as means ± SD of three replications.
[b] Positive control.

Table 3
Inhibitory activity of selected compounds against related PTPs

Compounds	IC_{50} (μM)			
	SHP-1	LAR	PTPα D1	PTPε D1
1	31.33 ± 5.39	>40	>40	>40
2	25.78 ± 2.30	>40	>40	>40
3	24.56 ± 0.56	>40	>40	>40
10b	12.64 ± 1.46	>40	>40	>40
10k	27.38 ± 4.00	>40	>40	>40
10l	5.20 ± 0.77	>40	>40	>40
11a	4.08 ± 0.25	>40	>40	>40
11p	0.71 ± 0.17	>40	>40	>40
12h	12.36 ± 0.49	>40	>40	>40
12m	4.05 ± 0.24	>40	>40	>40
13	0.89 ± 0.12	>40	>40	>40

compound **11a** and oleanolic acid **1** only exhibited unclear effect in the same assay, which successfully verified our SAR strategy on cell model in qualitative analysis. The phosphor-Akt (Ser473), a downstream protein of IR, was also stimulated by treatment of compound **13**, which demonstrated that inhibition of PTP1B in CHO/*h*IR cells enhanced insulin signal (Fig. 2C). In cytotoxicity experiment, no changes in viability were observed when the CHO/*h*IR cells were incubated with **13** (20 μM) for 48 h using MTT method (data not shown). We also studied the effect of compound **13** on glucose uptake in L6 myotubes either in the absence or presence of insulin. In the absence of insulin, the rate of basal glucose uptake increased in a dose-dependent manner and reached a flat roof with about 40% increment at 1 μM (Fig. 3A) compared with 75% under only insulin-stimulated condition.[40] Simultaneously, compound **13** was also tested in insulin-stimulated glucose uptake assay at three different insulin concentrations (1–100 nM, Fig. 3B). Unfortunately, our inhibitor had about 20% increment compared to submaximal insulin concentrations, which was lower than the condition without insulin cooperating. More experiment results in vivo are going to support that if our inhibitors are therapeutically meaningful.

The cell results demonstrated that our synthesized compounds had well cell permeablity, due to their good lipophilic property provided by pentacyclic core. Furthermore, showed in glucose uptake assay in L6 myotubes the effect of **13** appeared significant because the skeletal muscles accounted for majority of glucose uptake.[40] In addition, the elevated tyrosine phosphorylation level of IR and Akt indicated that our compounds stimulated the sensitivity of cell to insulin through inhibition of PTP1B and other phosphatases activities.

3. Conclusion

Oleanolic acid, as far as we know, was first developed in SAR research as PTP1B inhibitor derived from natural products. After varying its structure at C-3 and C-28 positions, we found a series of derivatives more potent than the primary hit, such as **13**, 22-fold more effective than the parent compound. Although their inhibitory toward TCPTP and PTP1B still shared little difference, our competitive and reversible inhibitors exhibited good selectivity against other phosphatases involving in the insulin pathway. Moreover, **13** exhibited cell activities in enhancing IR and downstream Akt phosphorylation and in increasing the glucose uptake assay. The cellular results provided evidence that this type of inhibitor can be potent and cell permeable. Further optimization will focus on improving activity in vitro in order to choosing a PTP1B inhibitor suitable for animal studies in diabetes and obesity.

4. Experimental

4.1. General experimental procedure

^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian Mercury-VX300 Fourier transform spectrometer. The chemical shifts were reported in δ (ppm) using the δ 7.26 signal of $CDCl_3$ (^1H NMR) and the δ 77.23 signal of $CDCl_3$ (^{13}C NMR) as internal standards. ESI-MS was run on a Bruker Esquire 3000 plus spectrometer in MeOH and HR-ESI-MS was run on a Bruker Atex III spectrometer in MeOH, respectively. All commercially available reagents were used without further purification. The solvents used were all AR grade and were redistilled under positive pressure of dry nitrogen atmosphere in the presence of proper desiccant when necessary. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) on $HSGF_{254}$ precoated silica gel plates. Details of analytical HPLC profile of compounds in two diverse systems were described in the Supplementary material.

4.2. Synthesis of compound 13

This synthetic procedure represented a typical method for the variation at C-3 and C-28 positions, besides which, what was not described herein was referred to the Supplementary material.

4.2.1. 3-(4-Carboxy-benzyloxy)-oleanolic methyl ester (13a)

NaH (800 mg, 20 mmol, 60% suspension in mineral oil) was added to a solution of oleanolic methyl ester (940 mg, 2 mmol) in dry dioxane (10 mL), then *p*-carboxy-benzyl bromide (430 mg, 2 mmol) was plunged into the mixture after stirring for 20 min

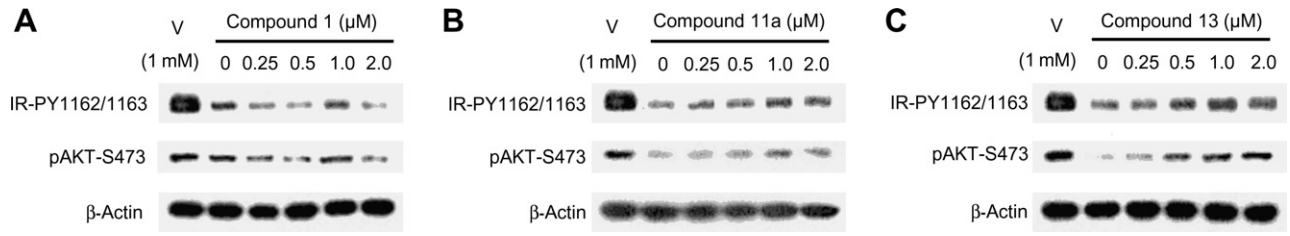


Figure 2. Effect of compounds **1** (A), **11a** (B) and **13** (C) on tyrosine phosphorylation of IRβ and Akt in CHO/*h*IR celts. Sodium vanadate (1 mM) and DMSO were used as postitive and negative controls (each lane 1 and 2). The levels of IRβ and β-actin were used to normalization.

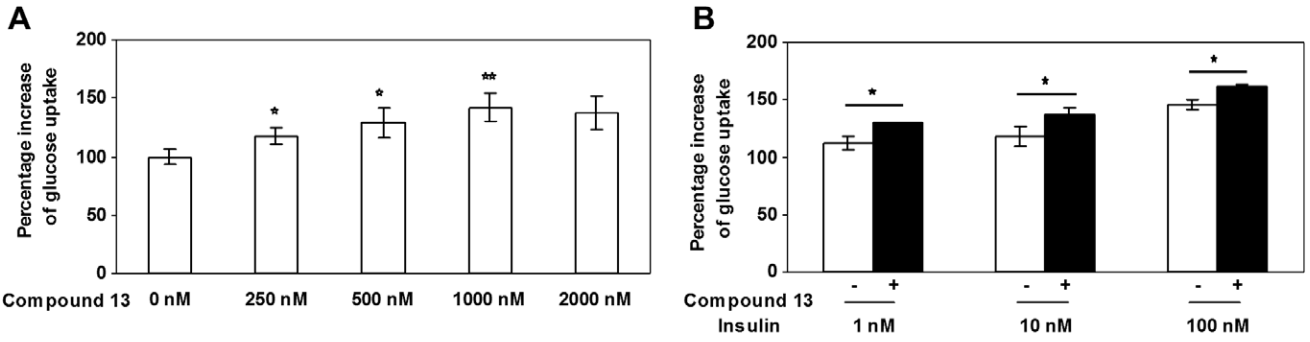


Figure 3. Effect of compound **13** on glucose uptake in **L6** myotubes. **L6** myotubes were starved for 2 h. Subsequently, the cell was treated with 250 nM, 500 nM, 1 μM and 2 μM for 3 h in the absent of insulin (A); or incubated with 1.0 μM compound **13** for 2.5 h and stimulated with a range of insulin concentrations for 30 min (B). The rate of glucose uptake was expressed as the percentage of the basal value or cells treated with 0.2% DMSO. The figures showed the means ± standard error (SE) of triplet. ($^{*}P < 0.05$, $^{**}P < 0.01$).

at room temperature (RT). The mixture was heated to 105 °C overnight, after which it was allowed to RT and ceased by adding saturated NH_4Cl solution. Extracting with ethyl acetate, the organic phase was collected and washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was subjected to column chromatography (CC) on silica gel 200–300 mesh using petroleum ether 60 ~ 90 °C (PE) and ethyl acetate (10:1) as eluant to get **13a** (612 mg, 51%). ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 8.18$ (d, $J = 8.1$ Hz, 2H), 7.45 (d, $J = 4.8$ Hz, 2H), 5.25 (s, 1H), 4.71 (d, $J = 12.9$ Hz, 1H), 4.45 (d, $J = 12.9$ Hz, 1H), 3.59 (s, 3H), 2.91 (dd, $J = 4.2$, 11.4 Hz, 1H), 2.82 (dd, $J = 4.5$, 14.1 Hz, 1H), 2.00–0.70 (m, 43H); ESI-LRMS, $[M+Na]^+$: 627.

4.2.2. 3-[4-(2,2-Dimethyl-oxazoline)-benzyloxy]-oleanolic methyl ester (13b)

A solution of **13a** (612 mg, 1 mmol), 1-hydroxybenzotriazole (HOBt, 148 mg, 1.1 mmol), 2-amino-2-methylpropan-1-ol (89 mg, 1.1 mmol), triethylamine, (0.28 mL, 2 mmol), 4 Å molecular sieves (200 mg) in dry CH_2Cl_2 (10 mL) was treated with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 210 mg, 1.1 mmol) and the resulting reaction mixture was stirred at RT for 8 h. After that the mixture was partitioned by dichloromethane (DCM) and water, washed by 1 N HCl (5 mL), saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Without further purification, the residue dissolved in dry THF (10 mL) with triphenyl phosphine (268 mg, 1 mmol), and diisopropyl azodicarboxylate (0.2 mL, 1 mmol) was added to the mixture under ice bath. The muddy solution was gradually clarified along with stirring for 2 h at RT. The reaction mixture was quenched by the addition of water and diluted with ethyl acetate. The organic layer was separated, washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by CC (PE:ethyl acetate = 15:1) to give the product **13b** (351 mg, 53%). ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 7.89$ (d, $J = 8.1$ Hz, 2H), 7.38 (d, $J = 8.1$ Hz, 2H), 5.27 (s, 1H), 4.69 (d, $J = 12.3$ Hz, 1H), 4.44 (d, $J = 12.3$ Hz, 1H), 4.10 (s, 2H), 3.67 (s, 3H), 2.91 (dd, $J = 4.2$, 11.4 Hz, 1H), 2.85 (dd, $J = 4.5$, 14.1 Hz, 1H), 1.38 (s, 6H), 2.00–0.70 (m, 43H); ESI-LRMS, $[M+H]^+$: 658.

4.2.3. 3-[4-(2,2-Dimethyl-oxazoline)-benzyloxy]-28-(1-ene-4-butyric acid)-Δ^{12}-oleanene (13c)

To a suspension of $LiAlH_4$ (100 mg, 2.5 mmol) in dry THF (3 mL) at 0 °C, a solution of **13b** (351 mg, 0.5 mmol) in dry THF (2 mL) was added slowly. The reaction mixture was stirred at room temperature for 4 h and then quenched cautiously by the subsequent addition of water (0.1 mL), 5 N NaOH (aq) (0.1 mL) and water (0.3 mL). The suspension was filtered through a fritted funnel and the filtrate was concentrated *in vacuo* to give alcohol, which was used in the next step without purification. A solution of DMSO (0.1 mL, 1.3 mmol) in dry DCM (1 mL) was added dropwise to a −60 °C cooled solution of oxalyl chloride (0.05 mL, 0.55 mmol) in dry DCM (2 mL). After 10 min of stirring at −60 °C, a solution of alcohol obtained from previous step in dry DCM (2 mL) was added dropwise. After stirring for another 45 min at −60 °C, triethylamine (0.35 mL, 2.5 mmol) was added. The mixture was allowed to RT and stirred for 1 h, then diluted with DCM. The organic layer was washed with water, brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to gain the colorless aldehyde. The aldehyde dissolved in dry THF (2 mL) was syringed into the orange–red yield suspension, prepared by adding 4-carboxybutyl triphenylphosphonium bromide (427 mg, 1 mmol) to the KOtBu (336 mg, 3 mmol) in dry THF (3 mL). The mixture was stirred for 2 h, quenched by 1 N HCl (5 mL) and diluted with ethyl acetate. The organic layer was extracted, washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The acid **13c** (159 mg, 46%) was purified by CC. (PE: ethyl acetate = 5:1) ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 7.85$ (d, $J = 8.1$ Hz, 2H), 7.35 (d, $J = 8.1$ Hz, 2H), 5.30–5.20 (m, 2H), 5.19 (s, 1H), 4.69 (d, $J = 12.3$ Hz, 1H), 4.44 (d, $J = 12.3$ Hz, 1H), 4.11 (s, 2H), 2.91 (dd, $J = 3.9$, 11.1 Hz, 1H), 2.45–2.35 (m, 2H), 2.00–0.70 (m, 52H); ESI-LRMS, $[M+H]^+$: 658.

4.2.4. 3-(4-Carboxy-benzyloxy)-28-[1-en-4-butyric ((s)-1-carboxy-phenylethyl)-amide]-Δ^{12}-oleanene (13d)

A solution of **13c** (159 mg, 0.23 mmol), HOBt (33 mg, 0.25 mmol), L-phenalanine methyl ester hydrochloride (54 mg, 0.25 mmol), triethylamine, (0.07 mL, 0.5 mmol), 4 Å molecular sieves (50 mg) in dry CH_2Cl_2 (2 mL) was treated with EDCI (48 mg, 0.25 mmol) and the resulting reaction mixture was stirred at RT for 8 h. After that the mixture was partitioned by DCM and water, washed by 1 N HCl (1 mL), saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product dissovled in EtOH (2 mL) containing 2 N HCl (2 mL), and the solution was heated at 90 °C for 2 h. After the reactant disappearing monitored by TLC, the mixture was allowed to RT and separated between ethyl acetate and water. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Without further purification, the residue was also directly hydrolyzed in the solution of 5 N NaOH (1 mL), MeOH (1 mL), and THF (2 mL) at RT overnight. After neutralizing the superfluous base with 2 N HCl, the mixture was partitioned between EtOAc and water, dried over anhydrous Na_2SO_4, and evaporated to afford the crude product, in which the diacid **13d** (79 mg, 43%) was purified through CC ($CHCl_3$: MeOH = 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.05 (d, J = 8.1 Hz, 2H), 7.44 (d, J = 8.1 Hz, 2H), 7.40–7.15 (m, 5H), 6.09 (d, J = 7.2 Hz, 1H), 5.30–5.20 (m, 2H), 5.19 (s, 1H), 5.10–4.90 (m, 1H), 4.73 (d, J = 13.2 Hz, 1H), 4.47 (d, J = 13.2 Hz, 1H), 3.20 (ddd, J = 5.7, 14.1, 20.1 Hz, 2H), 2.93 (dd, J = 3.9, 11.1 Hz, 1H), 2.45–2.35 (m, 2H), 2.00–0.70 (m, 46H); ESI-LRMS, $[M+Na]^+$: 815.

4.2.5. 3-(4-Carboxy-benzyloxy)-28-[4-butyric ((s)-1-carboxy-phenylethyl)-amide]-Δ^{12}-oleanene (13)

A mixture of **13d** (79 mg, 0.1 mmol), Na_2CO_3 (31 mg, 0.3 mmol), 10% Pd on carbon (20 mg) in EtOAc (1 mL) was stirred under an atmosphere of H_2 overnight. After adding several drops of formic acid (0.2 mL) for neutralizing, the mixture was filtered through a fritted funnel and the filtrate was concentrated in vacuo. The residue was subject to CC ($CHCl_3$:MeOH = 10:1) to furnish 3β-(*p*-carboxy-benzyloxy)-28-[4-butyric((*s*)-1-carboxy-phenylethyl)-amide]-Δ^{12}-oleanene **13** (40 mg, 51%) as white amorphous solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.05 (d, J = 8.1 Hz, 2 H), 7.44 (d, J = 8.1 Hz, 2H), 7.40–7.15 (m, 5H), 6.03 (m, 1H), 5.16 (s, 1H), 4.95–4.92 (m, 1H), 4.74 (d, J = 13.2 Hz, 1H), 4.48 (d, J = 13.2 Hz, 1H), 3.20 (ddd, J = 5.7, 15.0, 20.1 Hz, 2H), 2.95 (dd, J = 3.9, 11.1 Hz, 1H), 2.20 (t, J = 7.2 Hz, 2H), 2.00–0.70 (m, 50H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 176.1, 174.0, 171.9, 146.2, 145.2, 135.9, 130.4 × 2, 129.6 × 2, 128.8 × 2, 128.2, 127.4, 127.2 × 2, 122.2, 87.4, 70.9, 55.8, 53.3, 47.8, 47.5, 46.8, 41.8, 40.1, 39.9, 39.1, 38.6, 37.5, 37.1, 36.7, 34.7, 33.5, 33.4, 32.7, 31.2, 29.9, 28.5, 26.8, 26.3, 25.9, 23.9, 23.9, 23.2, 22.9, 22.3, 18.5, 17.0, 16.9, 15.7; ESI-HRMS: calcd for $C_{51}H_{71}NO_6$ $[M+Na]^+$: 816.5179, founded: 816.5185. Scanned ^{1}H and ^{13}C NMR figures were shown in the Supplementary material.

4.3. Biological assay

4.3.1. PTP1B and other phosphatases expression and biological assay

The enzymatic assays of PTP1B, TC-PTP, LAR, PTPα and PTPε were referred.[25] SHP1 (742–1710 according to BC002523) assay was carried in 50 mM Tris at pH 8.0 with 10 μM OMFP as substrates.

4.3.2. Characterization of the inhibitors' enzyme kinetics

To determine the inhibition mode of inhibitors, assay was carried out in a 100 μL assay mixture contained 50 mM MOPS at pH 6.5, 30 nM PTP1B, *p*NPP in 2-fold dilution up to 80 mM, and different concentrations of inhibitor. In the presence of the competitive inhibitor, the Michaelis–Menton equation is described as $1/v = [K_m/(V_{max}\cdot[S])](1 + [I]/K_i) + 1/V_{max}$, where v is the initial rate, V_{max} is the maximum rate, and $[S]$ is the substrate concentration. K_i value was obtained by linear re-plot of apparent K_m/V_{max} (slope) from primary reciprocal plot vs. inhibitor concentration $[I]$ according to the equation $K_m/V_{max} = 1 + [I]/K_i$.

4.3.3. Insulin receptor and Akt phosphorylation cellular assay

The Chinese hamster ovary cell line transfected with an expression plasmid encoding human IR (CHO/*h*IR) were cultured in six-well plates. Nearly confluent CHO/*h*IR cells were starved 2 h with Ham's F-12 medium without serum. Cells were incubated with compound for 3 h, followed by stimulation with 10 nM insulin for 10 min. Cells were scraped and lysed with loading buffer. The samples were resolved by 8% SDS-PAGE, eletrotransferred to nitrocellulose membranes, and probed with anti-pIR (Tyr1162/1163) antibody from Biosource, anti-pAKT (Thr473) antibody from Cell Signaling Technology and anti-β-actin antibodies from Santa Cruz Biotechnology. The bolts were developed using the ECL chemiluminescence detection system.

4.3.4. Glucose uptake assay

L6 myoblast cells were grown and maintained in alpha Dulbeco's modified Eagle' medium (α-DMEM) containing 10% FBS under 5% CO_2 environment at 37 °C. For differentiation of L6 myoblasts, cells were seeded in appropriate culture plates, and after the cells had reached subconfluency, the medium was changed to a-DMEM containing 2% FBS. The medium was then changed every 2 days until the cells were fully differentiated. Glucose uptake on L6 myotubes was measured on the 5th day. L6 myotubes were starved for 2 h with α-DMEM containing 0.2% BSA without serum. For the effect of inhibitors on basal glucose uptake, the cell was treated with differrent concentrations of inhibitors for 3 h in the absence of insulin. In the presence of insulin, L6 myotubes were treated with 1.0 μM inhibitors (2.5 h) and stimulated with 1, 10, and 100 nM of insulin concentrations for 30 min, respectively. Cells were then treated with 2-deoxy [1,2-^{3}H] glucose for 10 min (final concentration of 0.5 μCi and 0.1 mM glucose) in HEPES-buffered saline solution [20 mM HEPES (pH 7.4), 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.2% BSA]. Transport was terminated by aspirating the radioactive solution and followed by three rapid washes with cold PBS. Radioactivity inside the cells was measured after cells were lysed with 0.1% Triton and normalized with protein concentration levels.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.080.

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