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Design, synthesis, and anti-inflammatory activity both in vitro and in vivo of new betulinic acid analogues having an enone functionality in ring A

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Abstract—Fifteen new betulinic acid analogues were designed, synthesized, and tested for anti-inflammatory activity. Many of these analogues effectively suppress nitric oxide (NO) production in RAW cells stimulated with interferon-γ. Analogue **10** is highly and orally active in vivo for induction of the anti-inflammatory and cytoprotective enzyme, heme oxygenase-1.

Betulinic acid (BA, **1**) is a pentacyclic lupane-type triterpene isolated from various plants.[1,2] Betulinic acid selectively inhibits the growth of human melanoma cells in vitro and in vivo,[3] suppresses proliferation of neuroblastoma[4] and ovarian carcinoma cells,[5] and enhances differentiation and apoptosis of primary leukemia cells.[6] Despite a lack of toxicity in animal studies even at high concentrations,[3] the relatively low potency of betulinic acid itself lessens its clinical utility as an anti-cancer drug.

Our ongoing efforts to improve the anti-inflammatory and anti-proliferative activity of oleanolic acid (**2**), a naturally occurring oleanane-type triterpene, led us to discover 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and related compounds (e.g., CDDO–Me, CDDO–Im, and TP-222, see Fig. 1).[7–9] CDDO and CDDO–Me are currently being evaluated in phase I clinical trials for the treatment of leukemia and solid tumors. In these investigations, we discovered two

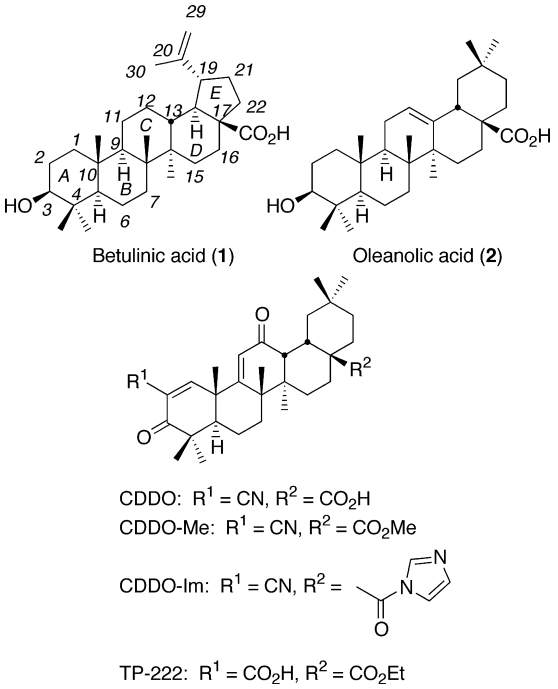

Figure 1. Structures of betulinic acid, oleanolic acid, CDDO, and CDDO analogues.

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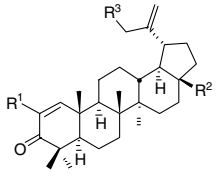
important structure–activity relationships (SARs). The combination of enone functionalities with cyano and carboxyl groups in ring A and an enone functionality in ring C is an essential structural feature for high potency in various bioassays related to inflammation and cancer,[8] and modifications of the C-17 carboxyl group affect both potency and pharmacokinetics.[9] Thus, we envisioned incorporating the same structures into betulinic acid for increasing its anti-inflammatory activity.

We initially designed and synthesized methyl ester **3**[10] and 15 new betulinic acid analogues **4**–**18**[11] without the enone functionality in ring C (Table 1) because betulinic acid differs from oleanolic acid and is devoid of functionality in ring C. We evaluated the potency of the new analogues for inhibition against nitric oxide (NO) production in RAW 264.7 cells (in vitro) and induction of the anti-inflammatory, cytoprotective enzyme, heme oxygenase-1 (in vivo).[12] We report here that several new semi-synthetic betulinic acid analogues display highly potent anti-inflammatory activity in vitro, and we show that **10** is also highly and orally active in vivo.

Compounds **4**–**12** with a cyano enone functionality in ring A were synthesized by the sequence shown in Scheme 1. Acid **4** was obtained in 61% yield by cleavage of methoxycarbonyl group of **3** with $AlBr_3$ in Me_2S.[13] Ethyl ester **5** was prepared in 78% yield from **4** using EtI and DBU in toluene.[14] Reaction of **4** with oxalyl chloride gave acyl chloride **19** in quantitative yield. Amides **6**–**9** and imidazolides **10**–**12** were synthesized in moderate yields by condensation reactions between **19** and the corresponding amines and imidazoles (Scheme 1 and Table 1).

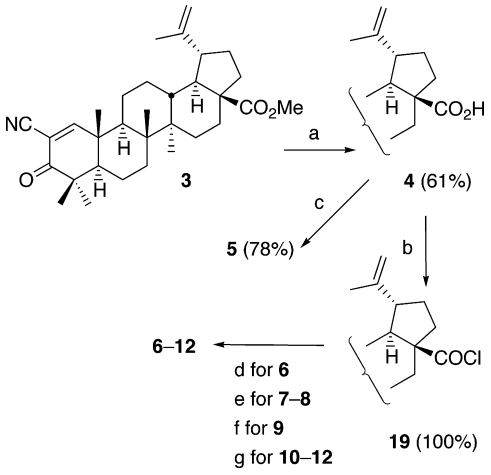
Compounds **13**–**18** with a carboxyl or methoxycarbonyl enone functionality in ring A were synthesized by the sequence shown in Scheme 2. Methyl ester **21** was prepared in 85% yield from methyl betulonate (**20**), which was easily synthesized in 95% yield in 2 steps (methylation with CH_2N_2 and Jones oxidation) from **1**, by Stiles' reagent (methoxymagnesium methyl carbonate) in DMF,[15] followed by methylation with CH_2N_2. The ^1H NMR spectrum showed that **21** is the single tautomer in $CDCl_3$ as depicted in Scheme 2. Initially, we expected that addition of phenylselenyl chloride (PhSeCl, 2 equiv-

Table 1. Inhibition of NO production in RAW cells stimulated with interferon-γ by methyl ester **3** and new betulinic acid analogues **4**–**18**

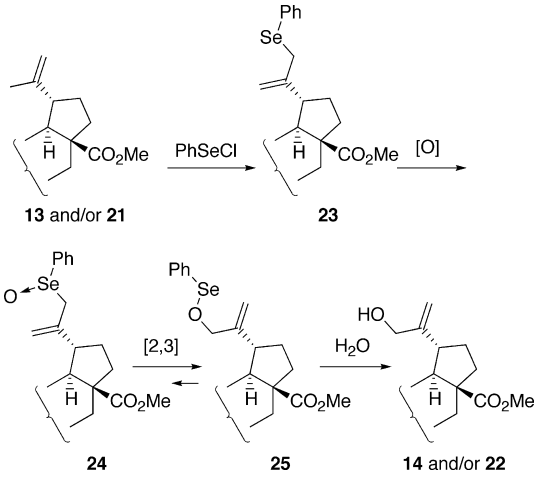


Compound	R^1	R^2	R^3	Yield (%)	Inhibition of NO IC_{50}[c] (μM)
3	CN	CO_2Me	H	Ref. 10	0.03
4	CN	CO_2H	H	[a]	0.2
5	CN	CO_2Et	H	[a]	0.02
6	CN	$CONH_2$	H	74[b]	0.03
7	CN	CONHMe	H	70[b]	0.05
8	CN	CONHEt	H	79[b]	0.07
9	CN	$CONMe_2$	H	77[b]	0.03
10	CN		H	91[b]	0.03
11	CN		H	62[b]	0.05
12	CN		H	60[b]	0.03
13	CO_2Me	CO_2Me	H	[a]	0.6
14	CO_2Me	CO_2Me	OH	[a]	1
15	CO_2Me	CO_2Me	F	[a]	0.3
16	CO_2H	CO_2Me	H	[a]	0.3
17	CO_2H	CO_2Me	OH	[a]	0.9
18	CO_2H	CO_2Me	F	[a]	0.3
BA (**1**)					Inactive
CDDO					0.02

[a] Yield is shown in the text.
[b] Yield from **19**.
[c] RAW 264.7 cells were treated with various concentrations of compounds and interferon-γ (10 ng/mL) for 24 h. Supernatants were analyzed for NO by the Griess reaction.



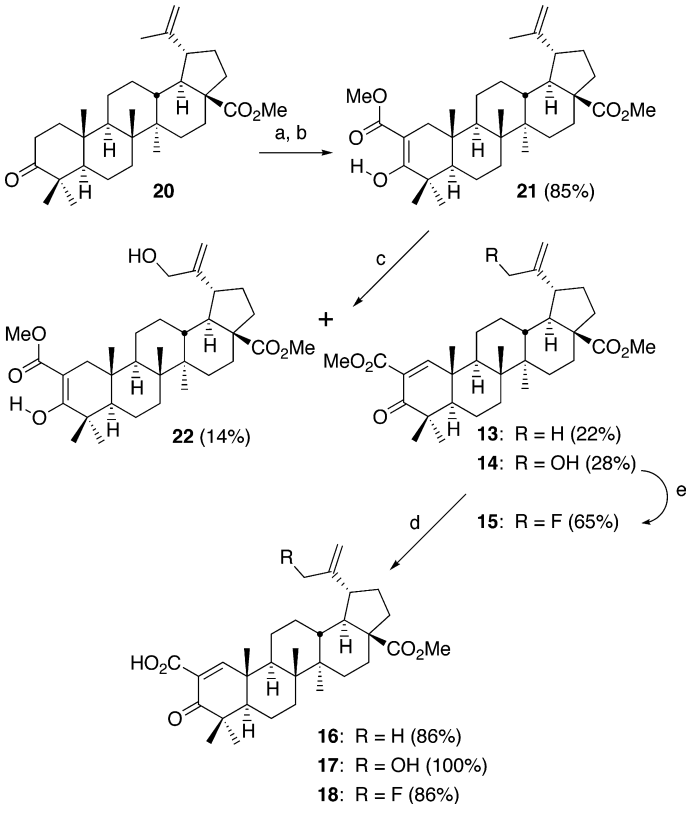
Scheme 1. Reagents and conditions: (a) $AlBr_3$, Me_2S (rt); (b) $(COCl)_2$, CH_2Cl_2 (rt); (c) EtI, DBU, toluene (reflux); (d) NH_3, PhH (rt); (e) amine hydrochloride, $NaHCO_3$, PhH, water (reflux); (f) Me_2NH, PhH (reflux); (g) imidazole, PhH (rt).



Scheme 3. Conversion of **13/21** to **14/22** using PhSeCl–H_2O_2.

alents) to **21** and subsequent oxidation/elimination of the selenated intermediate with H_2O_2 would give only **13**. However, these conditions[16] afforded **14** (28% yield) and **22** (14%) in addition to **13** (22%). We believe that PhSeCl produces the rearranged allylic selenide **23** from **13** and/or **21** (Scheme 3).[17] Oxidation of **23** with H_2O_2 forms allylic selenoxide **24**, which is converted to allylic selenenic ester **25** by a [2, 3]sigmatropic rearrangement, followed by hydrolysis of **25** to give allylic alcohol **14** and/or **22**.[18] Allylic fluoride **15** was obtained in 65% yield from **14** with DAST in CH_2Cl_2.[19] Acids **16**–**18** were prepared from the corresponding methyl esters **13**–**15** by alkaline hydrolysis (86%, 100%, and 86% yield, respectively).

We have evaluated the potency of methyl ester **3** and new analogues **4**–**18** for inhibition of NO production in RAW 264.7 cells stimulated with interferon-γ, and induction of the anti-inflammatory, cytoprotective



Scheme 2. Reagents and conditions: (a) Stiles' reagent, DMF (at 110 °C); (b) CH_2N_2, Et_2O, THF (rt); (c) PhSeCl, pyr., CH_2Cl_2 (at 4 °C); 30% H_2O_2, CH_2Cl_2 (at 4 °C); (d) aq KOH, MeOH (reflux); (e) DAST, CH_2Cl_2 (−78 °C).

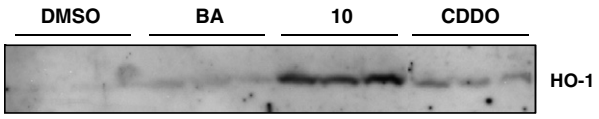


Figure 2. Analogue **10** induces heme oxygenase-1 (HO-1) in vivo in liver. Male CD-1 mice (three mice per group) were gavaged with triterpenoids (2 μM) in DMSO. After 6 h, livers were collected and analyzed by Western blot for heme oxygenase-1.[20]

enzyme, heme oxygenase-1 in the liver (in vivo). In the RAW cell assay (Table 1), compounds **3**–**12** with a cyano enone functionality in ring A are highly active, with a potency which is similar to that of CDDO, whereas betulinic acid is inactive. The series of compounds **13**–**18** with a carboxyl or methoxycarbonyl enone functionality in ring A is less active, with a potency more than 10-fold less than that of CDDO. Most importantly, we have found that one of the new analogues, **10**, is significantly more potent in vivo than both betulinic acid and the oleanolic acid analogue, CDDO. Thus, as shown in Figure 2, oral dosing of 2 μM of compound **10** caused significant induction of the anti-inflammatory, cytoprotective enzyme, heme oxygenase-1, in the liver, while betulinic acid and CDDO were both markedly less potent at this low dose. There is major interest in stimulating heme oxygenase-1 as a protective enzyme in many chronic disease conditions in which inflammation and oxidative stress play a key role.[12]

In summary, in a series of new betulinic acid analogues, we have found the following interesting SARs: (1) A cyano enone functionality in ring A is necessary for exhibiting potent inhibitory activity against NO production in RAW cells. (2) Noteworthy is that an enone functionality in ring C is not necessary for the potency. The SARs are entirely different from those of oleanolic acid analogues.[7,8] (3) The methoxycarbonyl and carboxyl enone functionalities in ring A are not important for the potency. (4) C-17 modifications do not affect the potency in the RAW cell assay. These results are also different from those of CDDO analogues.[9] However, interestingly, only the acyl imidazole increases the potency in vivo. (5) Isopropenyl side chains do not affect the potency. Overall, it is important to note that our present results are different from those of the oleanolic acid analogues that we have studied previously. Further syntheses and biological evaluation of new betulinic acid analogues are in progress.

Acknowledgments

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10. Shortly after we started this project, a Korean group reported methyl ester **3**; You, Y.-J.; Kim, Y.; Nam, N.-H.; Ahn, B.-Z. *Bioorg. Med. Chem. Lett*. **2003**, *13*, 3137. This compound was synthesized from betulinic acid (**1**) by the same method as they reported.
11. All new compounds **4**–**18** provided acceptable HRMS data (±5 ppm) and ^{1}H NMR spectra that exhibit no discernible impurities. Compound **10**: amorphous solid. $[\alpha]_D^{25}$ +0.7° (*c* 0.57, $CHCl_3$). CD (*c* 0.0016, EtOH) $\Delta\varepsilon_{265}$ +1.71 and $\Delta\varepsilon_{346}$ −2.32. ^{1}H NMR ($CDCl_3$) δ 8.30 (1H, br s), 7.82 (1H, s), 7.55 (1H, br s), 7.07 (1H, br s), 4.79 (1H, d, J = 1.46 Hz), 4.68 (1H, t, J = 1.46 Hz), 2.98 (1H, ddd, J = 4.76, 10.98, 10.98 Hz), 2.77 (1H, ddd, J = 3.66, 12.27, 12.27 Hz), 2.48 (1H, ddd, J = 3.29, 3.29, 13.91 Hz), 2.34 (1H, q, J = 6.59 Hz), 1.72, 1.18, 1.13, 1.12, 1.021, 1.016 (each 3H, s); ^{13}C NMR ($CDCl_3$) δ 198.4, 173.1, 170.8, 149.5, 137.5, 130.0, 117.6, 115.2, 114.2, 110.7, 57.7, 52.8, 51.4, 45.3, 45.2, 44.2, 42.7, 42.1, 41.0, 37.2, 34.2, 33.6, 33.2, 30.7, 29.8, 27.9, 25.4, 21.6, 19.6, 19.0, 18.6, 16.7, 14.7. MS (ESI+) *m*/*z* 528 $[M+H]^+$; HRMS (ESI+) calcd for $C_{35}H_{45}N_3O_2$ + H 528.3590, found 528.3580.
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