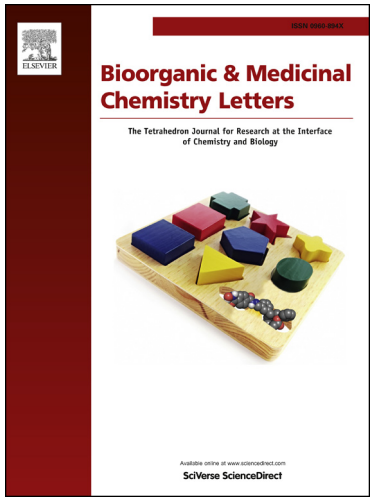

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Targeting cancer cells with oleanolic and ursolic acid derived hydroxamates

Jana Wiemann, Lucie Heller and René Csuk*

Martin-Luther Universität Halle-Wittenberg, Bereich Organische Chemie, Kurt-Mothes-Str. 2, D-06120 Halle (Saale), Germany; rene.csuk@chemie.uni-halle.de

Keywords: triterpenes; oleanolic acid; ursolic acid; hydroxamates; SRB assay; cancer

Abstract

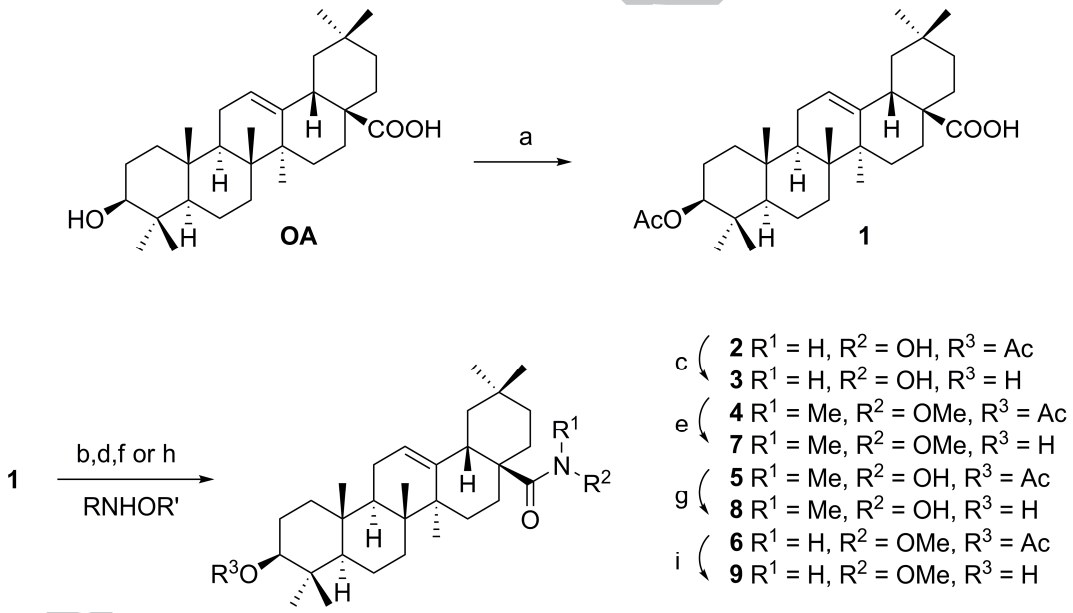
*Oleanolic and ursolic acid derived hydroxamates were easily obtained from their parent compounds; they were screened for their cytotoxicity applying SRB assays employing several human tumor cell lines. Low EC_{50} values were determined for compounds in which the nitrogen as well as the oxygen in the hydroxamic acid part still holds acidic hydrogens. Thus, ursolic acid derived compounds having at least an OH and/or NH moiety in the hydroxamate part of the molecule showed good cytotoxicity but they are significantly less selective for the tumor cells than oleanolic acid derived compounds. Good results were determined for oleanolic acid derived **7** for tumor cell lines 518A2 (melanoma, EC_{50} = 3.3 μM), A2780 (ovarian carcinoma, EC_{50} = 3.4 μM) and HT29 (colon adenocarcinoma, EC_{50} = 5.6 μM) while being significantly less cytotoxic for fibroblasts (EC_{50} = 20.4 μM).*

Cancer remains one of the most threatening diseases in the world. Worldwide, 8.2 million people died of cancer in 2012, with lung cancers (with bronchus and trachea cancers) rising to become the 5th leading cause of death in 2012 killing 1.1 million men and 0.5 million women [1]. Expenses for cancer-related drugs increased from 80.8 billion US$ in 2010 to 100.2 billion US$ in 2014 [2]. Although over the past decades notable progress has been achieved on developing new antitumor active drugs, there is still an increasing demand to develop new and innovative drugs to target tumor cell specific mechanisms. Recently, the importance of molecular pathways regulating the cellular epigenome has been pointed out [3-8], and their dysregulation has been related to cancer [9-11].

Among others, histone deacetylases play a key-role in these processes [12, 13], and hydroxamate substituted inhibitors have been recognized as potent antitumor active drugs. Several of them

are already applied in clinical trials for cancer treatment [17, 18]. In addition, hydroxamic acids have again entered the focus of scientific interest reflecting the fact that they show various biological activities [16, 19-23].

Although many derivatives of pentacyclic triterpenoic acids have been prepared and screened for their cytotoxic activity [24-29] hydroxamate substituted triterpenoids have only scarcely been described so far. There are only two reports on glycyrrhetinic acid derived hydroxamates, and they acted as inhibitors of 11β hydroxysteroid-dehydrogenase 2 but these compounds have not been screened for any cytotoxic activity [30, 31]. Recently, we [32] described the synthesis and antiproliferative properties of betulinic acid derived hydroxamates.

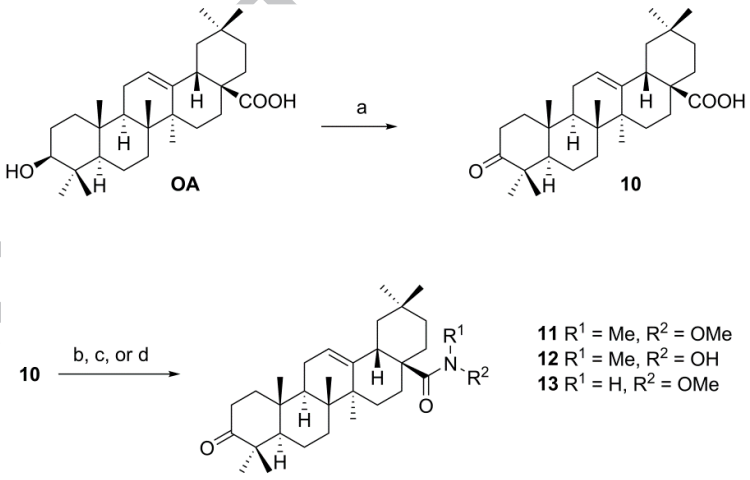


Scheme 1. Synthesis of hydroxamates **2–9** from 3-*O*-acetyl-oleanolic acid (**1**): (**a**) Ac_2O, pyridine, NEt_3, DMAP, 25 °C, 12 h, 86 %; **(b)** $(COCl)_2$, DCM, 25 °C, 2 h then $NH_2OH.HCl$, NEt_3, DCM, 25 °C, 3 h, 74 %; **(c)** KOH, MeOH, 25 °C, 4 d, 98 %; **(d)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOMe.HCl, NEt_3, DCM, 25 °C, 12 h, 84 %; **(e)** KOH, MeOH, 25 °C, 3 d, 99 %; **(f)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOH.HCl, NEt_3, DCM, 25 °C, 1 d, 48 %; **(g)** KOH, MeOH, 25 °C, 5 d, 62 %; **(h)** $(COCl)_2$, DCM, 25 °C, 2 h then $NH_2OMe.HCl$, NEt_3, DCM, 25 °C, 30 min, 75 %; **(i)** KOH, MeOH, 25 °C, 4 d, 93 %.

To gain a deeper insight into this class of neglected compounds we decided to synthesize representative examples of hydroxamates derived from oleanolic acid (**OA**) and ursolic acid (**UA**) and to investigate their cytotoxic activity employing several human tumor cell lines and nonmalignant mouse fibroblasts (NIH 3T3) in photometric sulforhodamine B (SRB) assays [33].

Commercially available oleanolic acid (**OA**) was acetylated, and 3-*O*-acetyl-oleanolic acid (**1**) was obtained in 86 % yield (Scheme 1). Reaction of **1** with oxalyl chloride followed by reaction with hydroxylammonium chloride/triethylamine for 3 h furnished a hydroxamate **2** whose deacetylation with potassium hydroxide in methanol for 4 days gave an almost quantitative yield of **3**. Hydroxamic acid **2** is characterized in its ^{13}C NMR spectrum by a signal at δ = 176.7 ppm being assigned to the **C**ONHOH moiety (C28) of **2**. In the IR spectrum the C-O stretch vibration was detected at ν = 1646 cm^{-1}.

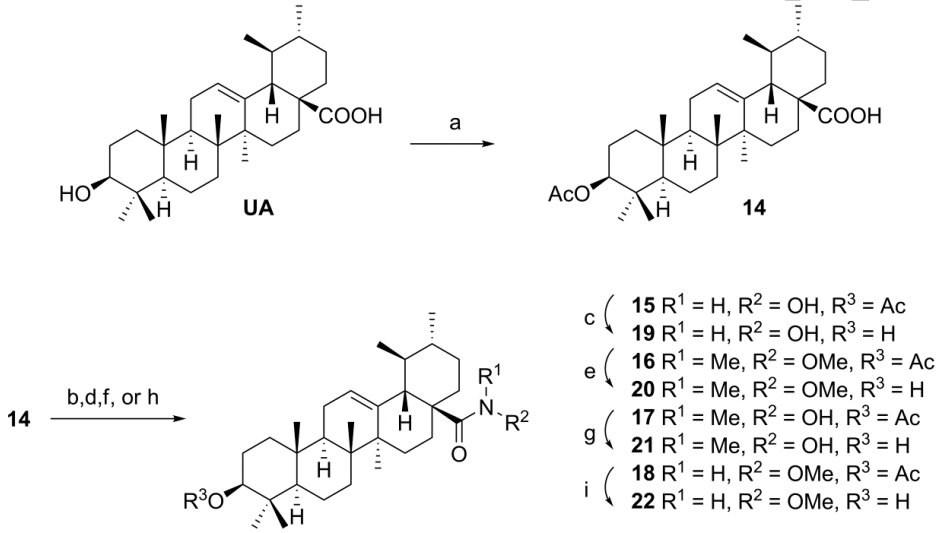
Reaction of **1** with oxalyl chloride followed by reaction with *N*,*O*-dimethylhydroxylammonium chloride or *N*-methylhydroxylammonium chloride or *O*-methylhydroxylammonium chloride in the presence of triethylamine gave products **4**–**6** whose deacetylation yielded compounds **7**– **9**, respectively. Under similar conditions, 3-oxo-oleanolic acid (**10**, obtained by Jones-oxidation from **OA** in 81 % yield) gave substituted hydroxamic acids **11**–**13** (Scheme 2).



Scheme 2. Synthesis of hydroxamates **11**–**13** from 3-keto-oleanolic acid (**10**): **(a)** CrO_3, H_2SO_4, H_2O, acetone, 25 °C, 2 h, 81 %; **(b)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOMe.HCl, NEt_3, DCM, 25 °C, 12 h, 43 %; **(c)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOH.HCl, NEt_3,

DCM, 25 °C, 12 h, 44 %; **(d)** $(COCl)_2$, DCM, 25 °C, 2 h then $NH_2OMe.HCl$, NEt_3, DCM, 25 °C, 2 h, 80 %.

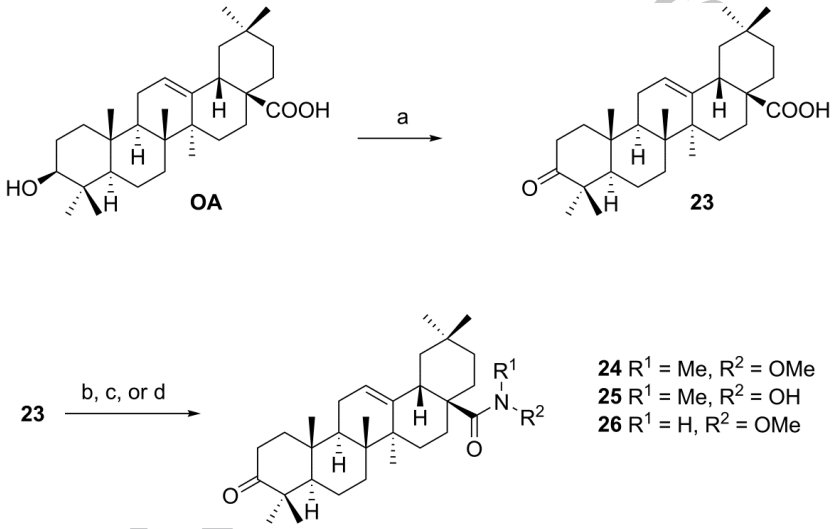
Similarly, commercially available ursolic acid (**UA**) was acetylated to yield 3-*O*-acetate **14** (Scheme 3) that was transformed into acetylated hydroxamates **15**–**18**; deacetylation as described above gave compounds **19**–**22**. Jones oxidation of **UA** yielded 3-oxo-usolic acid (**23**, Scheme 4) that was converted into hydroxamic acids **24**–**26**.



Scheme 3. Synthesis of hydroxamates **15**–**22** from 3-*O*-acetyl-ursolic acid (**14**): **(a)** Ac_2O, pyridine, NEt_3, DMAP, 25 °C, 12 h, 97 %; **(b)** $(COCl)_2$, DCM, 25 °C, 2 h then $NH_2OH.HCl$, NEt_3, DCM, 25 °C, 3 h, 68 %; **(c)** KOH, MeOH, 25 °C, 6 d, 89 %; **(d)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOMe.HCl, NEt_3, DCM, 25 °C, 12 h, 43 %; **(e)** KOH, MeOH, 25 °C, 6 d, 92 %; **(f)** $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOH.HCl, NEt_3, DCM, 25 °C, 12 h, 78 %; **(g)** KOH, MeOH, 25 °C, 5 d, 89 %; **(h)** $(COCl)_2$, DCM, 25 °C, 2 h then $NH_2OMe.HCl$, NEt_3, DCM, 25 °C, 1 h, 73 %; **(i)** KOH, MeOH, 25 °C, 4 d, 93 %.

The oleanolic acid derived hydroxamates **2**–**9**/**11**–**13** and ursolic acid derived compounds **15**–**22**/**24**–**26** were evaluated for their cytotoxic activity using photometric sulforhodamine B assays employing several human tumor cell lines (518A2, A2780, A549, FaDu, HT29, MCF-7) and nonmalignant mouse fibroblasts (NIH 3T3) [33]. For comparison, **OA**, **UA** as well as standard compound betulinic acid (**BA**) were included into this screening. The results of these assays are summarized in Table 1.

The results from the SRB assays showed ursolic acid (**UA**) as cytotoxic as betulinic acid (**BA**); oleanolic acid (**OA**) was significantly less cytotoxic. This displays the potential of **OA** and **UA** as starting materials for the development of cytotoxic/antitumor active drugs. All hydroxamates derived from **OA** or **UA** were more cytotoxic than their parent compounds but most of the compounds were as cytotoxic for cancer cells as for non-malignant mouse fibroblasts. Low EC_{50} values were determined for compounds **OA** derived **2** and **3** – in these compounds the nitrogen as well as the oxygen in the hydroxamic acid part still holds acidic hydrogens.



Scheme 4. Synthesis of hydroxamates **24**–**26** from 3-keto-ursolic acid (**23**): a) CrO_3, H_2SO_4, H_2O, acetone, 25 °C, 2 h, 96 %; b) $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOMe.HCl, NEt_3, DCM, 25 °C, 5 h, 65 %; c) $(COCl)_2$, DCM, 25 °C, 2 h then HNMeOH.HCl, NEt_3, DCM, 25 °C, 1 d, 66 %; d) $(COCl)_2$, DCM, 25 °C, 2 h then NH_2OMe.HCl, NEt_3, DCM, 25 °C, 2 h, 74 %.

However, these compounds were of equal cytotoxicity for fibroblasts and for A2780 ovarian cancer cells with EC_{50} values as low as 2.6 µM (for **2**) and 3.4 µM (**3**). Low EC_{50} values were also found for compounds **7** (EC_{50} = 3.4 µM), **17** (EC_{50} = 6.5 µM), **19** (EC_{50} = 4.6 µM), **21** (EC_{50} = 4.5 µM), **24** (EC_{50} = 4.5 µM) and 25 (EC_{50} = 4.0 µM) for A2780 cells. In addition, compound **24** showed a noteworthy low EC_{50} = 8.3 µM for the multidrug resistant human tumor cell FaDu while for nonmalignant fibroblasts EC_{50} = 18.5 µM was determined.

Table 1. Cytotoxicity of oleanolic (**2–9/11–13**) or ursolic acid (**15–22/24–26**) derived hydroxamates and starting materials **OA** and **UA** as well as betulinic acid (**BA**) as standard (EC_{50} values in µM from SRB assays after 96 hours of treatment; confidence interval CI = 95%). The cell lines are human cancer cell lines: 518A2, A2780, A549, FaDu, HT29, MCF-7 and nonmalignant mouse fibroblasts (NIH 3T3).

	518A2	A2780	A549	FaDu	HT29	MCF-7	NIH 3T3
BA	9.4±0.7	8.8±0.9	17.1±1.1	15.3±2.1	14.4±2.3	10.2±1.2	16.1±1.4
OA	64.3±4.2	14.0±0.7	72.3±1.5	75.4±3.4	38.8±3.1	80.0±3.5	76.4±0.7
UA	14.7±0.1	11.7±0.6	15.5±1.3	14.2±2.0	10.6±0.3	12.7±0.1	18.7±1.6
2	4.2±0.1	2.6±0.5	3.1±0.3	10.6±0.4	4.9±0.1	9.5±0.1	2.6±0.3
3	5.5±0.6	3.4±0.6	4.7±0.3	8.2±0.1	4.7±0.1	8.2±0.2	4.3±0.2
4	8.4±0.2	6.8±1.3	17.6±2.0	9.4±1.1	13.1±1.0	7.5±0.8	13.3±0.7
5	9.4±0.3	7.5±1.1	10.8±1.9	12.1±0.2	10.6±0.4	6.2±1.2	12.3±0.7
6	14.2±0.4	10.8±2.0	18.6±1.4	12.3±0.6	17.5±0.4	9.1±0.6	13.2±1.9
7	3.3±0.5	3.4±0.1	11.8±2.0	29.8±3.1	5.6±1.0	16.2±1.2	20.4±2.1
8	11.6±0.2	3.9±0.2	9.8±0.6	5.8±0.3	8.7±0.4	8.3±1.1	11.1±0.4
9	12.8±1.1	12.3±1.5	20.9±1.1	14.3±1.1	18.8±0.8	17.0±1.8	18.2±0.6
11	20.9±2.1	8.8±0.7	5.6±0.8	24.3±2.1	15.6±1.3	16.4±1.5	13.7±0.9
12	12.9±0.6	4.8±0.7	8.1±0.4	14.1±0.9	11.9±0.4	16.4±1.1	9.2±0.3
13	17.7±0.4	6.1±1.4	4.8±0.4	23.1±0.7	18.6±0.9	20.0±1.8	12.2±1.8
15	3.6±0.1	2.7±0.1	3.9±0.1	6.4±0.4	3.5±0.3	3.3±0.2	2.5±0.6
16	12.7±0.5	9.8±0.9	9.6±0.8	15.0±1.1	17.6±1.0	15.4±0.6	23.0±1.7
17	8.4±0.2	6.5±0.2	5.7±1.0	11.3±0.7	8.7±0.3	7.9±0.8	6.6±0.4
18	15.5±0.6	10.6±1.5	6.6±0.5	12.1±0.3	13.2±1.0	6.8±0.7	14.8±0.7
19	9.4±0.1	4.6±0.7	6.7±0.4	18.1±1.1	7.5±0.2	13.1±0.2	5.5±0.5
20	22.9±0.8	21.8±3.1	11.6±2.0	26.8±2.0	18.4±0.7	18.7±1.7	23.3±1.5
21	12.5±0.9	4.5±1.3	9.7±0.6	15.1±0.3	12.3±1.4	15.4±0.3	10.1±0.2
22	14.3±1.2	12.1±1.7	8.3±0.9	11.2±0.8	15.4±2.1	10.0±2.1	16.2±1.5
24	15.5±1.8	4.5±1.5	8.7±1.1	8.3±1.2	18.7±1.3	10.7±0.3	18.5±2.3

25	12.4±1.4	4.0±0.8	9.3±1.1	17.6±0.6	12.8±0.8	17.6±1.2	10.5±0.1
26	12.9±1.1	14.3±2.0	9.2±1.1	12.0±1.3	12.9±1.1	11.2±2.0	14.3±1.5

Best results, however, were determined for oleanolic acid derived **7** for tumor cell lines 518A2 (EC_{50} = 3.3 µM), A2780 (EC_{50} = 3.4 µM) and HT29 (EC_{50} = 5.6 µM) while being significantly less cytotoxic for fibroblasts (EC_{50} = 20.4 µM). Interestingly, the ursolic acid derived analogue, **20** was significantly less cytotoxic [518A2 (EC_{50} = 22.9 µM), A2780 (EC_{50} = 21.8 µM) and HT29 (EC_{50} = 18.4 µM)] but also less selective for the tumor cells [EC_{50} for NIH 3T3: **7** (20.4 µM), **20** (23.3 µM)]. Ursolic acid derived compounds possessing at least an OH and/or NH moiety in the hydroxamate part of the molecule (as in **15**, **19**, **20**) showed good cytotoxicity (as good as **OA** derived **7**) but they are significantly less selective for the tumor cells.

To sum up, oleanolic and ursolic acid derived hydroxamates were easily obtained from their parent compounds. Low EC_{50} values were determined in SRB assays for those compounds in which the nitrogen as well as the oxygen in the hydroxamic acid part still holds acidic hydrogens. Ursolic acid derived compounds showed good cytotoxicity but they were significantly less selective for the tumor cells compared to oleanolic acid derived compounds. Thus, oleanolic acid derived hydroxamate **7** showed low EC_{50} values for several tumor cell lines while being significantly less cytotoxic for fibroblasts.

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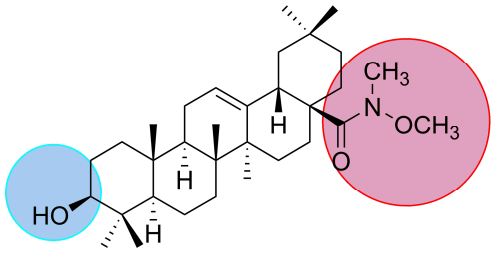
Graphical abstract



EC_{50} = 3.3μM (518A2 tumor cells)
EC_{50} = 3.4 μM (A2780 tumor cells)
EC_{50} = 20.4 μM (NIH 3T3fibroblasts)