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# Oxime derivatives of betulonic acid and platanic acid as novel cytotoxic or antiviral agents†

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Less frequently studied plant triterpenoids betulonic acid and platanic acid were selected to design, synthesize and investigate their oxime derivatives as novel and potentially effective cytotoxic and/or antiviral agents. The target compounds were subjected to cytotoxicity screening tests in several human cancer cell lines. Several of them displayed cytotoxicity against T-lymphoblastic leukemia (CCRF-CEM), breast adenocarcinoma (MCF7), malignant melanoma (G-361) and cervical cancer (HeLa) cell lines. Betulonic acid oxime (**3**) displayed cytotoxicity against CCRF-CEM ( $IC_{50} = 18.9 \pm 1.1 \mu\text{M}$ ;  $SI = 2.4$ ) and G-361 ( $IC_{50} = 21.3 \pm 2.8 \mu\text{M}$ ;  $SI = 2.2$ ) cancer cell lines. The benzyl ester of platanic acid oxime (**17**) displayed an anti-HIV-1 effect ( $EC_{50} = 3.2 \pm 0.43 \mu\text{M}$ ;  $SI > 31$ ) and no anti-HSV-1 effect ( $EC_{50} > 100 \mu\text{M}$ ), while platanic acid oxime (**15**) showed lower effects,  $EC_{50} = 36 \pm 4.0 \mu\text{M}$  (HIV-1) and  $EC_{50} = 48 \pm 6.0 \mu\text{M}$  (HSV-1), respectively. Compound **17** showed high selectivity in antiviral effects, being effective in HIV-1 and inactive in HSV-1. A side product **18** showed antiviral activity with  $EC_{50} = 15 \pm 1.3 \mu\text{M}$  (HIV-1) and  $EC_{50} = 12 \pm 0.21 \mu\text{M}$  (HSV-1), while another side product **19** was cytotoxic to HeLa ( $IC_{50} = 24.5 \pm 1.8 \mu\text{M}$ ).

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## 1. Introduction

Cancer and viral infections are among the most important human diseases affecting a large part of the human population.<sup>1,2</sup> Therefore, a search for new agents capable of treating either of these threats has permanently been required. Plants have always served as sustainable sources of important natural products in drug discovery, and recent review articles have clearly shown the potential of plant products, namely plant triterpenoids, to be applied in cancer and viral infection treatments.<sup>1–5</sup>

Viral infections are the main infectious diseases worldwide,<sup>6</sup> representing more than two thirds of infectious

diseases. Viruses affecting human bodies are divided into two categories, either viruses representing long-term parasites in the human body, or viruses representing long-term parasites in animals, which live in vicinity to humans. These viruses can also infect humans across species.<sup>6</sup>

Human immunodeficiency virus type 1 (HIV-1) features a common mechanism to catalyse membrane fusion as other enveloped viruses with class I fusion proteins. The envelope glycoproteins of HIV-1 play important roles in the early stage of HIV-1 entry. The surface glycoprotein subunit of HIV-1 engages in receptor binding, and the transmembrane subunit mediates the fusion of the virus with the target cell.<sup>6</sup> Therefore, anti-HIV-1 agents can be designed by targeting different steps of the enveloped virus replication cycle (maturation inhibitors).<sup>7</sup> Among the various life cycle events, the entry of enveloped viruses into host cells by fusion of the viral membrane with the cellular membrane is the first step.<sup>6</sup>

Herpes simplex virus (HSV) is a double-stranded DNA virus.<sup>8</sup> It exists in two types of HSV strains: HSV-1 causing infection of the oral mucosa, and HSV-2 affecting the genital mucosa and causing neonatal infections.<sup>9</sup> According to the latest report of the WHO, more than 65% of the world population under the age of 50 is infected with HSV-1, and the incidence of herpes simplex virus encephalitis (HSE) caused by HSV-1 infection is increasing every year.<sup>6</sup> Moreover, HSV-1 infections are associated with neurodegenerative diseases, e.g., Alzheimer's disease.<sup>10–12</sup> HSV-1 has also been identified for its association with

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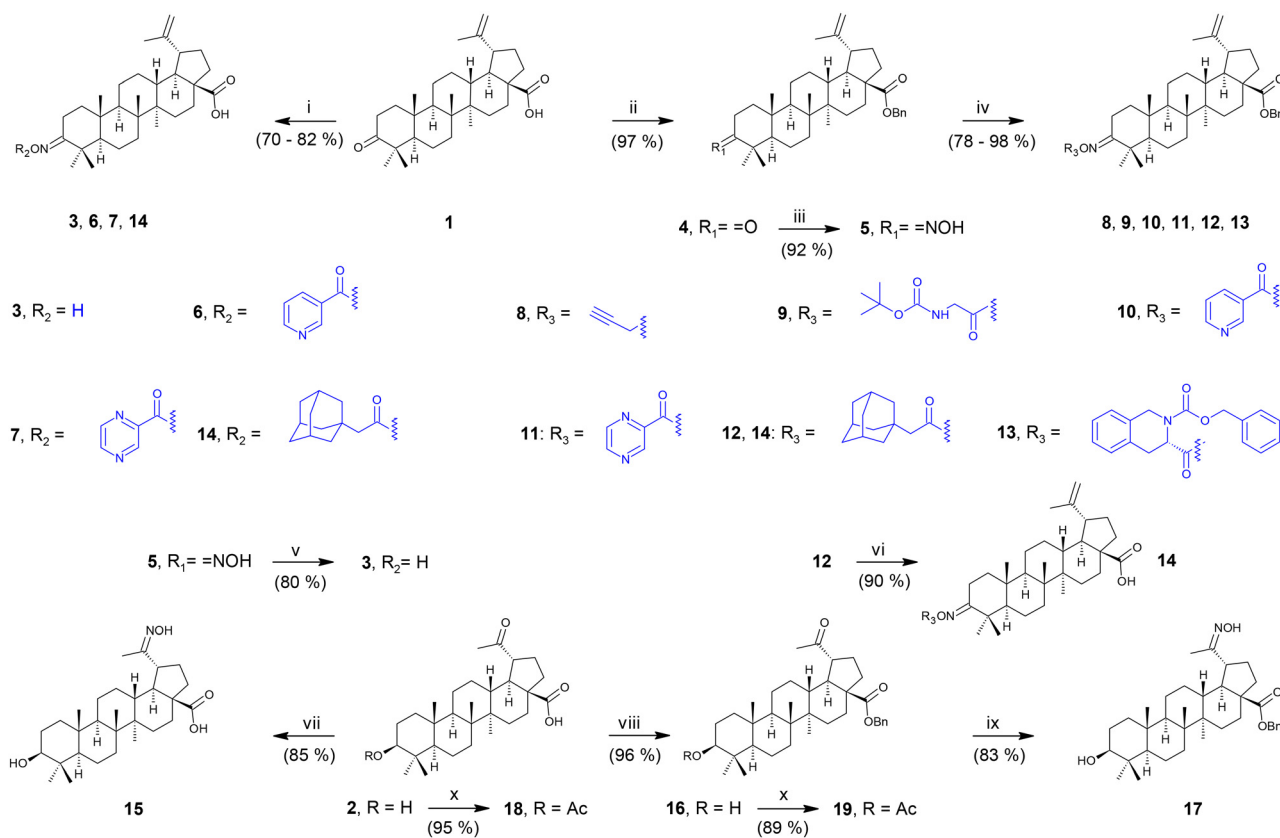
certain types of cancers, *e.g.*, cervical carcinoma or acute lymphocytic leukemia.<sup>13</sup> At present, anti-HSV-1 drugs mainly belong to nucleoside drugs (*e.g.*, acyclovir, ganciclovir, valacyclovir, *etc.*), capable of inhibiting viral replication by interfering with viral DNA polymerase. Based on the wide use of these drugs, drug-resistant strains of the virus appeared within a relatively short time period.<sup>12</sup> In order to cope with the problem of drug resistance, the investigation has been focused on developing novel anti-HSV agents targeting different viral life cycle steps.<sup>14</sup>

Triterpenoids are important plant metabolites. Studies reported that triterpenoids have a wide range of pharmacological effects and important biological activities, especially anti-inflammatory, hypoglycemic, anti-tumour, and antiviral activities. Triterpenoids have been proven to be important in the defence mechanisms of plants against pathogenic infection. Recently, an increasing number of studies have shown that triterpenoids exhibit excellent antiviral activity. These compounds can prevent viral adsorption and invasion into host cells in the early stage and thereby inhibit the viral replication process at the entry step.<sup>6</sup>

In this partial investigation, attention has been focused on two less explored plant triterpenoids, betulonic acid<sup>15</sup> and platanic acid.<sup>16</sup>

Betulonic acid, 3-oxo-lup-20(29)-en-28-oic acid (**1**; Scheme 1), belongs to the lupane family of plant triterpenoids and is found in different plant species.<sup>17,18</sup> Mostly, the content of betulonic acid in the plant sources is low, and, therefore a semisynthetic oxidation procedure from betulin often takes place to obtain betulonic acid in a higher quantity.<sup>19</sup> The solubility of betulonic acid in aqueous media is low, and so is its bioavailability. Despite this fact, betulonic acid shows important biological effects, such as antiviral, antimicrobial, anti-inflammatory, hepatoprotective, anticancer, *etc.*<sup>17</sup> To enhance its bioavailability, and thus, to enhance the biological effects, structural modifications have taken place to prepare its amides and other nitrogen-group-bearing derivatives or compounds with an extended number of cycles, either heteroaromatic or heterocyclic ones.<sup>17</sup> Betulonic acid derivatives bearing excessive ring(s) have been found to display ability to interfere with human corona virus 229E replication *via* the nsp15 endoribonuclease.<sup>20</sup> Aryl pyrimidine amides of betulonic acid have been developed as anti-inflammatory agents,<sup>21</sup> and piperazine-based amides exhibited cytotoxicity.<sup>22</sup>

Platonic acid, (3 $\beta$ )-3-hydroxy-20-oxo-30-norlupan-28-oic acid (**2**; Scheme 1), belongs to the norlupane family of pentacyclic plant triterpenoids. It is mostly extracted from



**Scheme 1** Synthetic procedures. Reaction conditions: i: a)  $\text{NH}_2\text{OH}\cdot\text{HCl}$ , pyridine, 96% EtOH (to prepare **3**), b) selected reagent, 50% T3P in ethyl acetate, pyridine (to prepare **6**, **7** and **14**); ii:  $\text{BnBr}$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Et}_3\text{N}$ , DMF; iii:  $\text{NH}_2\text{OH}\cdot\text{HCl}$ , pyridine, 96% EtOH; iv: a)  $\text{NaH}$ , DMF, b) propargyl bromide, DMF (to prepare **8**); or selected reagent, 50% T3P in ethyl acetate, pyridine (to prepare **9**); v: 10% Pd/C, 1,4-cyclohexadiene, EtOH: THF (1:1); vi: 10% Pd/C, 1,4-cyclohexadiene, EtOH: THF (1:1); vii:  $\text{NH}_2\text{OH}\cdot\text{HCl}$ , pyridine, 96% EtOH; viii:  $\text{BnBr}$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Et}_3\text{N}$ , DMF; ix:  $\text{NH}_2\text{OH}\cdot\text{HCl}$ , pyridine, 96% EtOH; x:  $\text{Ac}_2\text{O}$ , DMAP, THF, argon atmosphere.



sycamore trees, *Platanus occidentalis* L. and *Platanus x hybrida* Brot. (Platanaceae).<sup>23</sup> It is also found in *Melaleuca leucadendra* L. (Myrtaceae)<sup>24</sup> and in *Leptospermum scoparium* J. R. Forst. & G. Forst. (Myrtaceae).<sup>25</sup> The solubility in aqueous media and bioavailability of platonic acid are also low. Nevertheless, it exhibits important anti-HIV activity<sup>26</sup> and cytotoxicity.<sup>27,28</sup> We have reviewed platonic acid, besides other triterpenoids, and its selected nitrogen-group-bearing derivatives recently.<sup>3</sup> Nevertheless, before the review paper has been published, several new papers have appeared in the literature: pentacyclic triterpenoid sulphamates used for radiotherapy of breast cancer,<sup>29</sup> nitrogen-group-bearing bicyclic compounds used as inhibitors of butyrylcholinesterase,<sup>30,31</sup> pyridinylidene derivatives developed for targeting apoptosis,<sup>32</sup> platonic acid amides<sup>33,34</sup> and even more complex derivatives of platonic acid<sup>35</sup> for investigating cytotoxicity.

The objectives of this investigation have been set, and the attention has been focused on: (a) synthesis and analysis of oximes, oxime-ethers and oxime-esters of betulonic acid and oximes of platonic acid; (b) determination and evaluation of the cytotoxicity and antiviral activity of the target compounds and selected intermediate compounds; (c) evaluation and comparison of the significance of the parent triterpenoids in the target compounds, regarding the type of their pharmacological effect.

## 2. Results and discussion

### 2.1. Synthetic protocol and structure elucidation

To prepare the oxime of betulonic acid directly, **1** was subjected to a reaction with  $\text{NH}_2\text{OH}\cdot\text{HCl}$  in 96% ethanol in the presence of pyridine as a base to obtain **3** (Scheme 1, path i). To synthesize three compounds of the variety of oxime esters (**6**, **7** and **14**), **3** was allowed to react with the appropriate carboxylic acid, using 1-propanephosphonic acid anhydride (T3P) as the condensation agent, in pyridine (path i).<sup>36</sup> To synthesize further the variety of oxime ether and oxime esters of betulonic acid, **1** was converted into its benzyl ester (**4**) to protect the carboxyl group (path ii). Subsequently, the C(3) keto group was converted into the oxime using again  $\text{NH}_2\text{OH}\cdot\text{HCl}$  in 96% ethanol in the presence of pyridine as a base yielding **5** (path iii). The Bn-protected oxime **5** was further etherified to obtain oxime ether **8** in a two-step procedure: oxime **5** was first converted into its sodium salt using sodium hydride in DMF, and the salt was then reacted with propargyl bromide affording the oxime ether **8** (path iv).<sup>36</sup> To synthesize other target oxime ester derivatives of **1**, *i.e.*, compounds **9–13**, oxime **5** was reacted with the appropriate carboxylic acids, using either T3P in pyridine as the condensation reagent (to prepare **9**; path iv) or *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl) as the condensation reagent, in DCM with DMAP (to prepare **10–13**; path iv).<sup>37</sup> The benzyl protecting group in **5** was removed by transfer hydrogenation, using 10% Pd on

charcoal as a catalyst and 1,4-cyclohexadiene as a hydrogen donor, in an ethanol/THF (1:1) mixture yielding **3** by the indirect way to compare the yields of **3** (path v). Last but not least, product **14** was prepared from **12** by the same transfer hydrogenation as described above (path vi), which was more effective than that mentioned above (path i).

The first step in the modification of platonic acid (**2**; Scheme 1) consisted of conversion of the keto group into the oxime group by  $\text{NH}_2\text{OH}\cdot\text{HCl}$  in ethanol in the presence of pyridine (**15**; path vii). The other way consisted of protection of the carboxyl group of **2** in the form of benzyl ester of platonic acid (**16**; path viii) to investigate the effect of the free carboxyl group *vs.* esterified carboxyl group in the synthesis of the oxime derivatives (**15 vs. 17**). The esterified platonic acid **16** gave a higher yield of the oxime derivative (**15 vs. 17**; path ix). Finally, a side product **18** was prepared from **2** using acetic anhydride and DMAP in THF under an argon atmosphere (path x).<sup>38</sup> Using the same procedure (path x), **19** was prepared from **16**. Compounds **18** and **19** were originally considered to become the intermediates in the synthesis of **17**; however, the protection of the C(3)-OH group in **2** was finally not required. Nevertheless, **18** and **19** were subjected to biological screening tests, displaying their antiviral effects on HIV-1 and HSV-1 cell culture models. We also tried to synthesize a series of platonic acid derivatives to be analogous to **6–14**; however, the platonic acid oxime ether and oxime ester derivatives were more difficult to prepare in comparison with the betulonic acid oxime derivatives. The reactions either gave very low yields of the required products or did not proceed at all, and, therefore, the synthetic effort to engage **2** was stopped.

To elucidate the configuration of the platonic acid oximes and their derivatives, we have studied the so far published results from the literature. The search has been based on the analysis of the <sup>1</sup>H and <sup>13</sup>C NMR spectra of compounds **15** and **17** (ESI,† Table S1). The chemical shift of the C(29) carbon atom of the (20*E*)-isomer of the earlier published oxime derivative was reported at  $\delta = 11.5$  ppm, while the same signal of the (20*Z*)-isomer was at  $\delta = 16.1$  ppm.<sup>39</sup> These values of the corresponding chemical shift in the <sup>13</sup>C NMR spectra, found for **15** and **17**, are  $\delta = 13.1$  ppm and  $\delta = 11.9$  ppm, respectively. Similarly, much earlier published data of the <sup>1</sup>H NMR chemical shifts of the H-C(19) hydrogen atom were found at  $\delta = 3.44$  ppm [(20*E*)-isomer of the earlier published compound] and  $\delta = 4.63$  ppm [(20*Z*)-isomer of the same compound].<sup>40</sup> The corresponding chemical shifts of the H-C(19) hydrogen atom in compounds **15** and **17** were found at  $\delta = 3.43$  ppm and  $\delta = 3.32$  ppm, respectively. The search resulted in a finding that compounds **15** and **17** bear the (20*E*)-absolute configuration of the oxime group. Table S1† also shows chemical shifts of several additional signals.

Elucidating the configuration of the C(3) oxime group in the betulonic acid derivatives is much more difficult (ESI,† Table S2). The relevant signal seems to belong to the C(24) carbon atom in the <sup>13</sup>C NMR spectra. The chemical shift of the C(24) carbon atom was found to be  $\delta = 14.3$  ppm.<sup>41</sup> We



have found these chemical shifts at  $\delta = 14.3$  ppm (**3**) and  $\delta = 14.5$  ppm (**5**), respectively. In the  $^1\text{H}$  NMR spectra, the H–C(24) and H–C(23) hydrogen atom chemical shifts were found to be  $\delta = 0.83$  ppm and  $\delta = 1.06$  ppm, respectively, for the (3*E*)-isomer.<sup>41</sup> We have found these chemical shifts at  $\delta = 0.86$  ppm and  $\delta = 0.78$  ppm (**3**) and  $\delta = 1.06$  ppm and  $\delta = 1.16$  ppm (**5**), respectively. These findings also support the (3*E*)-configuration of **3** and **5**. Table S2† also shows chemical shifts of several additional signals.

Similar values of the chemical shifts of the corresponding signals in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra can be found in the spectra of other compounds of the published series. However, we could not find relevant NMR data comparing (*E*)- and (*Z*)-isomers of the structurally identical substances in the literature. The available literature data either do not contain the determination of the stereochemistry or they establish it as (*E*)-configuration while stating the presence of the only stereoisomer. In addition, we have not identified the (*Z*)-isomers in the prepared series of compounds. It seems realistic that they appeared as minor components in the reaction mixtures that were separated from the major products during column chromatography purification.

## 2.2. Cytotoxicity

Only several compounds of this series showed cytotoxicity; however, the  $\text{IC}_{50}$  values are mostly low (Table 1). The oxime of betulonic acid (**3**) displayed cytotoxicity against T-lymphoblastic leukemia (CCRF-CEM), breast adenocarcinoma (MCF7), malignant melanoma (G-361) and cervical cancer (HeLa) cell lines comparable with those displayed by the parent betulonic acid (**1**) (Table 1). Compound **14** was partly successful in cytotoxicity screening tests showing  $\text{IC}_{50}$  values of  $7.4 \pm 0.3$   $\mu\text{M}$  (G-361; SI (selectivity index) = 2.1) and  $8.3 \pm 2.8$   $\mu\text{M}$  (HeLa; SI = 1.9), respectively. The other disadvantage of this series of compounds is the fact that the cytotoxicity of the compounds

is almost equal to their toxicity to the reference cells, normal human fibroblasts, BJ. CDDP (cisplatin) was used as a positive reference compound. It should be noted that CDDP displayed cytotoxicity comparable with **1**, **6** and **14** against the HeLa cancer cell line, and with **14** against the G-361 cancer cell line. Moreover, compounds **1**, **6** and **14** showed a better ratio between cytotoxicity to the cancer cell lines and toxicity to the reference cells (Table 1).

## 2.3. Antiviral activity

To determine the anti-HIV-1 and anti-HSV-1 activities of the prepared derivatives of **1** and **2**, the ability of the compounds to inhibit the virus-induced cytopathic effect (CPE) in MT-4 and Vero cells, respectively, was measured. The most important result of the anti-HIV-1 tests was observed with **17** displaying a high anti-HIV-1 effect ( $\text{EC}_{50} = 3.2 \pm 0.43$   $\mu\text{M}$ ; SI > 31; Fig. 1 and Table 2), while being inactive in anti-HSV-1 tests (Fig. 2 and Table 2). Compound **18**, a side product in this series of compounds, showed  $\text{EC}_{50} = 15 \pm 1.3$   $\mu\text{M}$  (HIV-1) and  $\text{EC}_{50} = 12 \pm 0.21$   $\mu\text{M}$  (HSV-1) (Table 2; Fig. 1 and 2). Inhibition of the virus-induced CPE in MT-4 and Vero cells by **17** and **18**, as well as the viability of the MT-4 or Vero cells, respectively, is also shown in Fig. 1 and 2. Only the compounds showing no or very low cytotoxicity in the screening tests (Table 1) were subjected to the antiviral tests subsequently (Table 2; Fig. 1 and 2; ESI† Fig. S1). Therefore, graphical dependence of inhibition of the virus-induced CPE in the MT-4 and Vero cells on the concentration of the less active compounds **4**, **5**, **8** and **15**, the source triterpenoids (**1** and **2**), and the positive reference compounds (acyclovir or saquinavir) is summarized in ESI† Fig. S1.

## 2.4. A comparison of the oxime derivatives of betulonic acid and platanic acid in cytotoxicity and antiviral activity

A remarkable result was achieved with the oximes **3** and **17**, in which the former compound (**3**) displayed cytotoxicity

Table 1 Cytotoxicity screening tests ( $\text{IC}_{50}$  values [ $\mu\text{M}$ ], 72 h)

Compound <sup>a</sup>	$\text{IC}_{50} \pm \text{SE}$ [ $\mu\text{M}$ ], 72 h, DMSO				
	CCRF-CEM <sup>b</sup>	MCF7 <sup>c</sup>	G-361 <sup>d</sup>	HeLa <sup>e</sup>	BJ <sup>f</sup>
<b>1</b>	20.5 ± 1.6	31.1 ± 4.2	27.2 ± 10.5	11.7 ± 2.1	>50
<b>2</b>	>50	>50	>50	>50	>50
<b>3<sup>g</sup></b>	18.9 ± 1.1	23.3 ± 0.9	21.3 ± 2.8	27.8 ± 1.8	46.2 ± 1.1
<b>6</b>	44.0 ± 4.4	37.2 ± 2.5	29.8 ± 2.2	13.5 ± 2.6	47.8 ± 3.1
<b>7</b>	44.5 ± 3.5	23.8 ± 2.5	22.0 ± 0.4	27.7 ± 5.5	>50
<b>11</b>	26.5 ± 2.5	>50	>50	25.6 ± 4.3	20.4 ± 0.8
<b>14<sup>h</sup></b>	16.2 ± 0.3	16.3 ± 0.3	7.4 ± 0.3	8.3 ± 2.8	15.6 ± 1.2
<b>16</b>	>50	47.9 ± 0.2	>50	>50	>50
<b>17</b>	33.3 ± 1.3	22.3 ± 3.4	27.1 ± 1.7	>50	>50
<b>19</b>	36.5 ± 4.5	>50	>50	24.5 ± 1.8	>50
CDDP <sup>i</sup>	0.8 ± 0.1	7.7 ± 1.7	4.5 ± 0.6	11.4 ± 3.8	6.9 ± 0.9

<sup>a</sup> Compounds **4**, **5**, **8**, **9**, **10**, **12**, **13**, **15** and **18** showed no cytotoxicity against the cancer cell lines, and no toxicity to BJ cells ( $\text{IC}_{50} > 50$   $\mu\text{M}$ ).

<sup>b</sup> CCRF-CEM, human T-lymphoblastic leukemia. <sup>c</sup> MCF7, human breast adenocarcinoma. <sup>d</sup> G-361, human malignant melanoma. <sup>e</sup> HeLa, human cervical cancer. <sup>f</sup> BJ, normal human fibroblasts, reference cells. <sup>g</sup> SI (selectivity index) =  $\text{IC}_{50}$  (BJ)/ $\text{IC}_{50}$  (cancer cell line), for **3**: SI = 2.4 (CCRF-CEM), SI = 2.0 (MCF7), SI = 2.2 (G-361), SI = 1.6 (HeLa). <sup>h</sup> SI = 2.1 (G-361), SI = 1.9 (HeLa). <sup>i</sup> *cis*-Diaminedichloridoplatinum(II), cisplatin, a pharmacologically used agent for treating cancers, a positive reference compound.



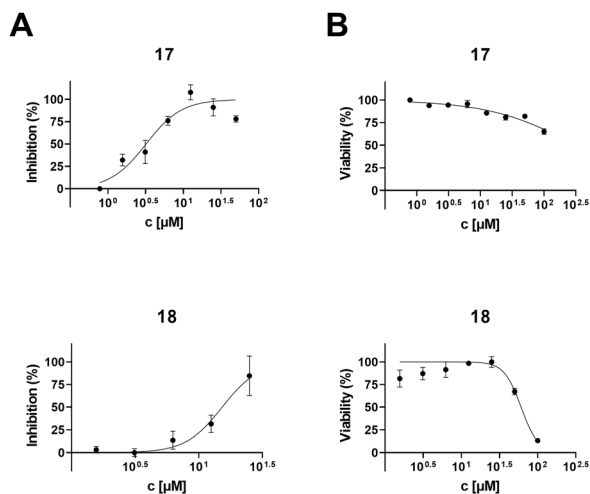


Fig. 1 HIV-1 inhibition (A) and cytotoxicity (B) of **17** and **18** against MT-4 cells.

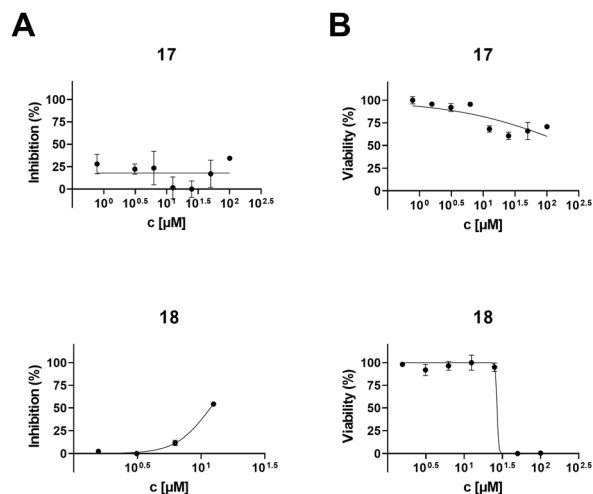


Fig. 2 HSV-1 inhibition (A) and cytotoxicity (B) of **17** and **18** against Vero cells.

against CCRF-CEM, MCF7, G-361 and HeLa cancer cell lines (Table 1), while the latter compound (**17**) showed anti-HIV-1 activity displaying  $IC_{50} = 3.2 \pm 0.43 \mu\text{M}$  ( $SI > 31$ ) (Table 2 and Fig. 1) besides low cytotoxicity (Table 1). In fact, **17** is the benzyl ester of the oxime of platanic acid. When the benzyl group was removed to obtain **15**, the anti-HIV-1 activity decreased 10 times, and the selectivity for the anti-HIV-1 effect was lost (Table 2 and Fig. 1). Compound **14** showed cytotoxicity against G-361 and HeLa cancer cell lines; however, it was also toxic to normal human fibroblasts (BJ). The side product **18** displayed no cytotoxicity; however, it showed activity both in HIV-1 and HSV-1 tests ( $EC_{50} = 15 \pm 1.3 \mu\text{M}$  and  $12 \pm 0.21 \mu\text{M}$ , respectively; Table 2; Fig. 1 and 2). Another side product **19** displayed medium to low cytotoxicity against HeLa and CCRF-CEM cancer cell lines (Table 1).

### 3. Conclusions

A series of more than 15 oxime derivatives of betulonic acid and platanic acid that include several oxime esters and an oxime ether were synthesized and subjected to screening tests for cytotoxicity and antiviral activity. It should be stressed here that oxime derivatives of betulonic acid and platanic acid have been studied earlier by other authors.

However, with exception of the structures identical to compounds **3**, **15** and **19** that have been prepared before,<sup>39,41–43</sup> all other compounds of the prepared series of oxime derivatives of betulonic acid and platanic acid are new compounds. None of the synthesized compounds showed high cytotoxicity. However, a partly remarkable result was a finding that the betulonic acid oxime derivative **3** displayed medium cytotoxicity against CCRF-CEM, while the platanic acid oxime derivative **17** showed high anti-HIV-1 activity and was inactive against HSV-1. This finding corresponds to the cytotoxicity and antiviral activity of the parent triterpenoids **1** and **2** (*cf.* Tables 1 and 2). Compound **14** was cytotoxic against G-361 and HeLa cancer cell lines; however, it was also toxic to the reference BJ cells. The side product **18** showed medium antiviral activity against both HIV-1 and HSV-1, while another side product **19** displayed medium cytotoxicity against the HeLa cancer cell line. Based on these partial conclusions, the platanic acid oxime derivative **17** should be pointed out as the most promising and selective anti-HIV-1 compound of the studied series of the prepared oxime derivatives. It seems reasonable for future investigation of antiviral activity of a broader series of triterpenoid oxime derivatives to focus attention on less polar compounds, which were the results that revealed the advantage of ester-

Table 2 Antiviral activity and cytotoxicity of compounds **1**, **2**, **4**, **5**, **8**, **15**, **17** and **18** against the MT-4 or Vero cells

Compound <sup>a</sup>	Anti-HIV-1 activity and cytotoxicity against the MT-4 cells			Anti-HSV-1 activity and cytotoxicity against the Vero cells		
	$EC_{50} \pm SE [\mu\text{M}]$	$CC_{50} \pm SE [\mu\text{M}]$	$SI^b$	$EC_{50} \pm SE [\mu\text{M}]$	$CC_{50} \pm SE [\mu\text{M}]$	$SI^b$
<b>1</b>	>71	$71 \pm 6.7$	<1	$17 \pm 1.1$	$92 \pm 21$	5.4
<b>2</b>	$47 \pm 5.0$	>100	>2.1	$75 \pm 2.2$	>100	>1.3
<b>15</b>	$36 \pm 4.0$	>100	>2.8	$48 \pm 6.0$	>100	>2.1
<b>17</b>	$3.2 \pm 0.43$	>100	>31	>100	>100	n.d. <sup>c</sup>
<b>18</b>	$15 \pm 1.3$	$61 \pm 4.5$	4.1	$12 \pm 0.21$	~27	2.3
Saquinavir	$0.0015 \pm 0.0005$	>1	>670	n.d.	n.d.	n.d.
Acyclovir	n.d.	n.d.	n.d.	$9.4 \pm 0.90$	>100	>11

<sup>a</sup> Compounds **4**, **5** and **8** showed no antiviral activity ( $EC_{50} > 100 \mu\text{M}$ ) and no cytotoxicity ( $CC_{50} > 100 \mu\text{M}$ ). <sup>b</sup>  $SI$  (selectivity index) =  $CC_{50}/EC_{50}$ . <sup>c</sup> n.d. = not determined.



group bearing compounds over those bearing a free carboxyl group or a free C(3)-OH group in the studied series of compounds.

## 4. Experimental

### 4.1. General

The NMR measurements were performed on a Bruker AVANCE II 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe in a 5 mm tube in different solvents. The  $^1\text{H}$  NMR and the  $^{13}\text{C}$  NMR spectra were recorded at 600.13 MHz and 150.90 MHz (AVANCE II 600 MHz) in  $\text{CDCl}_3$  or  $\text{CD}_3\text{OD}$  using tetramethylsilane ( $\delta = 0.0 - \text{CDCl}_3$ ) or the signal of the solvent ( $\delta = 3.31$  or  $49.50$  for  $^1\text{H}/^{13}\text{C} - \text{CD}_3\text{OD}$ ) as internal references.  $^1\text{H}$  NMR data are presented in the following order: chemical shift ( $\delta$ ) expressed in ppm, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), number of protons, and coupling constants in Hertz. For unambiguous assignment of both  $^1\text{H}$  and  $^{13}\text{C}$  signals, 2D NMR,  $^1\text{H}$ ,  $^{13}\text{C}$  gHSQC and gHMBC spectra were measured using standard parameter sets and pulse programs delivered by the producer of the spectrometer. Infrared spectra (IR) were measured with a Nicolet iS5 FT-IR spectrometer. Mass spectra (MS) were measured with a ZMD mass spectrometer (Waters, Eschborn, Germany) in a positive ESI mode (coin voltage, CV = 10 to 20 eV). TLC was carried out on silica gel plates (Merck 60F<sub>254</sub>) and the visualization was performed by both UV detection and spraying with a methanolic solution of phosphomolybdic acid (5%) followed by heating. For column chromatography, silica gel 60 (0.063–0.200 mm) from Merck was used. All chemicals and solvents of analytical grade were purchased from regular commercial sources and the solvents were purified by general methods before use. Triterpenoids were purchased from Dr. Jan Šarek – Betulinines (<https://www.betulinines.com>). Analytical data and the scanned NMR spectra of the prepared compounds are presented in the ESI† Experimental part.

**4.1.1. (3E)-3-(Hydroxyimino)lup-20(29)-en-28-oic acid (3).** A solution of betulonic acid (**1**; 1 g; 2.2 mmol),  $\text{NH}_2\text{OH}\cdot\text{HCl}$  (1.53 g; 22 mmol) and pyridine (1.78 mL; 22 mmol) were dissolved in 96% ethanol (50 mL) and kept under stirring at 110 °C in an argon atmosphere using a Dimroth condenser for 5 h. Saturated brine was added to the cold reaction mixture, and the organic layer was extracted with chloroform. The organic solution was dried over anhydrous sodium sulphate. After evaporation of the solvent, the crude residue was purified by column chromatography on silica gel, using a chloroform/methanol mixture (100/0 to 90/10) as the mobile phase, affording product **3** in 82% yield.

**4.1.2. Benzyl-3-oxolup-20(29)-en-28-oate (4).** Benzyl bromide (1.31 mL; 11.0 mmol) and potassium carbonate (912 mg; 6.61 mmol) were added to a solution of betulonic acid (**1**; 2 g; 4.41 mmol) in DMF (20 mL), and the reaction mixture was stirred at r.t. for 2 h. The reaction was stopped by addition of water (20 mL), extracted with chloroform, and dried over anhydrous sodium sulphate. The solvent was

evaporated, and the residue was purified by column chromatography on silica gel using chloroform/methanol (99.5/0.5) as the mobile phase. Product **4** was obtained with a 97% yield.

**4.1.3. Benzyl-(3E)-3-(hydroxyimino)lup-20(29)-en-28-oate (5).** Applying the procedure described above for the synthesis of **3** from **1** and using **4** as a starting compound, the target compound **5** was prepared in 92% yield.

**4.1.4. (3E)-3-[[Pyridin-3-ylcarbonyl]oxy]imino]lup-20(29)-en-28-oic acid (6).** T3P (50% solution in ethyl acetate, 1.27 mL; 2.13 mmol) was added to a solution of nicotinic acid (63 mg; 0.51 mmol) and **3** (200 mg; 0.43 mmol) in pyridine (10 mL), and the reaction mixture was stirred under an argon atmosphere for 1 day. To stop the reaction, the mixture was quenched with a saturated solution of sodium bicarbonate, and the organic layer was extracted with chloroform. The extract was dried with sodium sulphate, then the solid was filtered off, and the solvent evaporated under reduced pressure. The residue was purified by column chromatography on silica gel, using chloroform as the mobile phase, affording **6** in 72% yield.

**4.1.5. (3E)-3-[[Pyrazin-2-ylcarbonyl]oxy]imino]lup-20(29)-en-28-oic acid (7).** Applying the procedure described above for the synthesis of **6** and using **3** as a starting compound, product **7** was prepared in 78% yield.

**4.1.6. (3E)-3-[[Tricyclo[3.3.1.1<sup>3,7</sup>]dec-1-ylacetyl]oxy]imino]lup-20(29)-en-28-oic acid (14).** Applying the procedure described above for the synthesis of **6** and using **3** as a starting compound, product **14** was prepared in 70% yield.

**4.1.7. Benzyl-(3E)-3-[[prop-2-in-1-yloxy]imino]lup-20(29)-en-28-oate (8).** Sodium hydride (28.55 mg; 0.71 mmol) was added to a solution of oxime benzyl ester of betulonic acid (**5**; 50 mg; 0.09 mmol) in DMF (2 mL), and the reaction mixture was stirred at r.t. under argon for 30 min. Then propargyl bromide (11.5  $\mu\text{L}$ ; 1.07 mmol) was added, and stirring was continued for 24 h. DMF was evaporated, brine (10 mL) was added to the mixture, and the organic layer was extracted with chloroform. The solution was dried over anhydrous sodium sulphate, the solvent was evaporated, and the crude residue was purified by column chromatography on silica gel, using chloroform/petroleum ether (1:2) as the mobile phase, affording product **8** in 78% yield.

**4.1.8. Benzyl-(3E)-3-[[[tert-butoxycarbonyl]amino]acetyl]oxy]imino]lup-20(29)-en-28-oate (9).** Applying the procedure described above for the synthesis of **6** and using **5** as a starting compound, product **9** was prepared in 84% yield.

**4.1.9. Benzyl-(3E)-3-[[pyridin-3-ylcarbonyl]oxy]imino]lup-20(29)-en-28-oate (10).** The oxime benzyl ester of betulonic acid (**5**; 150 mg; 0.27 mmol) was dissolved in dry DCM (5 mL). Then nicotinic acid (40 mg; 0.32 mmol), an activation agent (EDC-HCl; 130 mg; 0.67 mmol), and, finally, DMAP (3.5 mg; 0.03 mmol) were added, and the reaction mixture was stirred at r.t., under an argon atmosphere, overnight. Then a saturated solution of sodium bicarbonate was added, the organic layer was extracted with chloroform, and the solution was dried over anhydrous sodium sulphate. After filtering the



solid phase off and evaporating the solvent, the crude residue was purified by column chromatography on silica gel, using chloroform/methanol (100:0 to 99:1) as the mobile phase, affording product **10** in 98% yield.

**4.1.10. Benzyl-(3*E*)-3-[[tricyclo[3.3.1.1<sup>3,7</sup>]dec-1-ylacetyl]oxy]imino}lup-20(29)-en-28-oate (**11**).** Applying the procedure described above for the synthesis of **10** and using **5** as a starting compound, product **11** was prepared in 96% yield.

**4.1.11. Benzyl-(3*E*)-3-[[tricyclo[3.3.1.1<sup>3,7</sup>]dec-1-ylacetyl]oxy]imino}lup-20(29)-en-28-oate (**12**).** Applying the procedure described above for the synthesis of **10** and using **5** as a starting compound, product **12** was prepared in 96% yield.

**4.1.12. Benzyl-(3*E*)-3-[[{(3*S*)-2-[(benzyloxy)carbonyl]-1,2,3,4-tetrahydroisoquinolin-3-yl}carbonyl]oxy]imino}lup-20(29)-en-28-oate (**13**).** Applying the procedure described above for the synthesis of **10** and using **5** as a starting compound, product **13** was prepared in 90% yield.

**4.1.13. (3*E*)-3-(Hydroxyimino)lup-20(29)-en-28-oic acid (**3**).** A Pd catalyst on charcoal (10% Pd/C; 64 mg; 0.06 mmol) and 1,4-cyclohexadiene (97%; 90  $\mu$ L; 0.89 mmol) were used in transfer hydrogenation to remove the benzyl protecting group of **5** (50 mg; 0.09 mmol). The reaction was made in a THF/ethanol (1:1; 4 mL) mixture, under an argon atmosphere, with stirring at r.t. for 24 h. Then the solvents were evaporated, and the product was obtained as yellow crystalline compound **3** in 80% yield.

**4.1.14. (3*E*)-3-[[Tricyclo[3.3.1.1<sup>3,7</sup>]dec-1-ylacetyl]oxy]imino}lup-20(29)-en-28-oic acid (**14**).** Applying the procedure described above for the synthesis of **3** from **5** and using **12** as a starting compound, product **14** was prepared in 90% yield.

**4.1.15. (3 $\beta$ )-3-Hydroxy-20-hydroximino-29-norlupan-28-oic acid (**15**).** Applying the procedure described above for the synthesis of **3** from **1** and using **2** as a starting compound, product **15** was prepared in 85% yield.

**4.1.16. Benzyl-(3 $\beta$ )-3-hydroxy-20-oxo-29-norlupan-28-oate (**16**).** Applying the procedure described above for the synthesis of **4** and using **2** as a starting compound, product **16** was prepared in 96% yield.

**4.1.17. Benzyl-(3 $\beta$ )-3-hydroxy-20-hydroximino-29-norlupan-28-oate (**17**).** Applying the procedure described above for the synthesis of **3** from **1** and using **16** as a starting compound, product **17** was prepared in 83% yield.

**4.1.18. (3 $\beta$ )-3-(Acetyloxy)-20-oxo-29-norlupan-28-oic acid (**18**).** Acetic anhydride (300  $\mu$ L; 3.14 mmol) and DMAP (35 mg; 0.28 mmol) were added to a solution of platanic acid (**2**; 1 g; 2.18 mmol) in THF (10 mL) and triethylamine (1 mL), and the reaction mixture was stirred at 90  $^{\circ}$ C using a Dimroth condenser under an argon atmosphere for 3 h. The reaction was stopped by the addition of water (10 mL) and left to stir at r.t. for an additional 2 h. Brine was added, and the organic layer was extracted with chloroform. The extract was dried over anhydrous sodium sulphate, then the solid phase was filtered off and washed with chloroform and the solvent was evaporated from the collected organic solution. The crude residue was purified by column chromatography on silica gel,

using a chloroform/methanol (99.5/0.5) mixture as the mobile phase. Product **18** was obtained with 95% yield.

**4.1.19. Benzyl-(3 $\beta$ )-3-(acetyloxy)-20-oxo-29-norlupan-28-oate (**19**).** Applying the procedure described above for the synthesis of **18** and using **16** as a starting compound, product **19** was prepared in 89% yield.

## 4.2. Cytotoxicity screening tests

The cytotoxicity screening tests were performed according to the standard experimental procedure published earlier.<sup>44,45</sup> Cell viability was measured after 72 h using resazurin (Sigma-Aldrich).

## 4.3. Antiviral tests

The anti-HIV-1 and anti-HSV-1 activity and cytotoxicity against MT-4 cells or Vero cells, respectively, were determined as previously described.<sup>46,47</sup> Briefly, two-fold dilutions of compounds were added to MT-4 and Vero cells in triplicate, incubated for 1 hour at 37  $^{\circ}$ C in 5% CO<sub>2</sub> and infected with HIV-1 (laboratory strain NL4-3 at multiplicity of infection [MOI] of 0.004 IU per cell) and HSV-1 (strain HF at MOI of 0.005 IU per cell), respectively. Saquinavir and acyclovir were used as controls for HIV-1 and HSV-1, respectively. Following three (HSV-1) and five (HIV-1) days of incubation at 37  $^{\circ}$ C in 5% CO<sub>2</sub>, the cell viability was determined by the formazan-based cell viability XTT assay (Sigma-Aldrich). Briefly, 50  $\mu$ L of a 50:1 mixture of an XTT labelling reagent (1 mg mL<sup>-1</sup>) and a PMS electron-coupling reagent (0.383 mg mL<sup>-1</sup>) was added to the wells and incubated for 4 hours at 37  $^{\circ}$ C in 5% CO<sub>2</sub>. The absorbance of the newly formed orange formazan solution was measured at 450 nm using an EnVision 2105 microplate reader (Perkin Elmer). Drug concentrations required to reduce viral CPE by 50% (EC<sub>50</sub>) were calculated using nonlinear regression from plots of percentage of cell viability vs. log<sub>10</sub> drug concentration using GraphPad Prism v.9.5.1 (GraphPad Software). The cytotoxicity of the compounds was evaluated by incubating the same dilutions with uninfected MT-4 and Vero cells. After three days of incubation at 37  $^{\circ}$ C in 5% CO<sub>2</sub>, the cell viability was determined as above. The compound concentrations resulting in 50% reduction of absorbance (CC<sub>50</sub>) were calculated from plots of percentage of cell viability vs. log<sub>10</sub> drug concentration as above.

## Author contributions

Conceptualization: Z. W., J. W. and P. L.; methodology: L. C., U. B., L. R., J. T. and D. Š.; investigation: L. C., U. B., L. R., J. T. and D. Š.; resources: Z. W.; data curation: L. C., U. B., L. R., J. T. and D. Š.; writing – original draft preparation: Z. W., J. W., L. R. and P. L.; writing – review and editing: Z. W., J. W., L. R. and P. L.; project administration: Z. W.; funding acquisition: Z. W. and J. W. All authors have read and agreed to the published version of the manuscript.



## Conflicts of interest

There are no conflicts to declare.

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