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## Toward a benign strategy for the manufacturing of betulinic acid†

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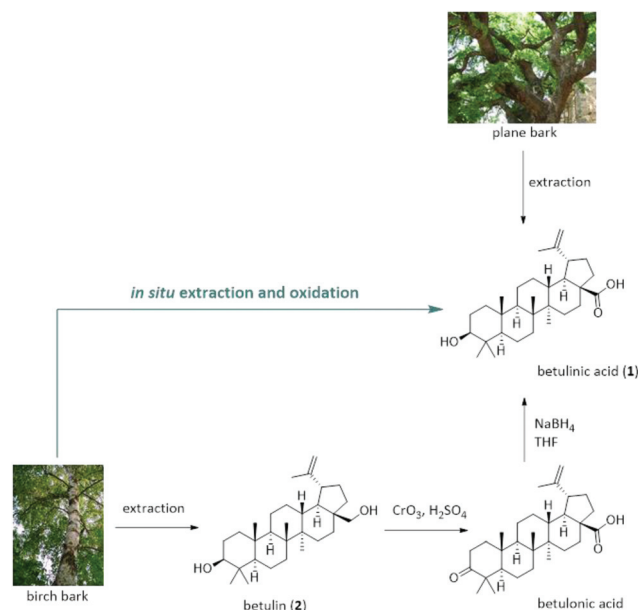
We report a novel and efficient strategy for the preparation of the high-value triterpenoid betulinic acid based on extraction and streamlined oxidation of betulin from the industrial by-product birch bark. The initial extraction of betulin relies on a biphasic system and allowed extracting betulin in short times at room temperature. The crude extract could be directly oxidized, thereby providing a chromium-free, time- and energy saving strategy for the manufacturing of betulinic acid in high yield.

### Introduction

Betulinic acid (**1**) is a naturally occurring pentacyclic triterpene which is widely found in the stem bark of a variety of tree species. Like many members of the lupane family, betulinic acid displays a number of biological and medicinal properties, and betulinic acid or its reduced species betulin (**2**) have been used in folk remedies since ancient times.<sup>1</sup>

Recent studies have shown that betulinic acid exhibits potent cytotoxic activity specific for melanoma cells both *in vitro* and *in vivo*.<sup>2</sup> Numerous papers over the past years aimed for the elucidation of the mode of action, as its anti-cancer activity is linked to its ability to induce apoptotic cell death in cancer cells.<sup>3</sup> Betulinic acid and its derivatives have also been shown as potent inhibitors of the human immunodeficiency virus (HIV), while other sources report anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, anti-nociceptive and anti-HSV-1 activities.<sup>4</sup>

To date, two strategies exist for the manufacturing of betulinic acid that rely either on the direct extraction from plant sources or on a semi-synthetic approach based on its precursor triterpenoid betulin (**2**) (Fig. 1). While betulinic acid (**1**) may be directly isolated from a number of plants, its low content renders the extraction from most sources tedious and unsuitable for a reliable supply on industrial scale. The plane tree



**Fig. 1** Conventional production of betulinic acid **1** relying either on extraction from plane bark (top) or semi-synthesis from betulin **2** (bottom).

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(*platanus acerifolia*) is one of the most widely reported sources of betulinic acid with a comparably high content of 2.4–3.3 g per 100 g dry bark.<sup>5</sup> However, its isolation suffers from the excessive use of mostly toxic solvents and time-consuming extraction or maceration strategies.<sup>6</sup> Recent patent literature suggests the use of dichloromethane, methanol or toluene/2-pentanol/methanol mixtures as extractant.<sup>7–9</sup> In any case, the obtained crude extract needs purification using chromatography techniques or multiple crystallization steps.



As alternative strategy, betulinic acid (**1**) can be prepared in a semi-synthetic pathway relying on its reduced species betulin (**2**). In contrast to betulinic acid, the triterpene betulin can be found in the white birch tree (*betula alba*) in significantly larger quantities of 10–35 wt% of the total dry weight of the outer bark.<sup>10–12</sup> Due to the readily availability of birch bark as by-product and waste material from pulp and paper mills all over the world, this offers an attractive, cost-efficient and more reliable supply for the production of expensive triterpenes from an industrial waste stream that is usually burnt for energy production.

Yet, the consecutive conversion of betulin (**2**) to betulinic acid (**1**) is problematic and restricts its sustainable production: betulinic acid (**1**) is commonly synthesized *via* Jones oxidation of betulin (**2**) with CrO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub> to betulonic acid followed by reduction to betulinic acid (**1**) using NaBH<sub>4</sub>.<sup>13,14</sup> This is not only a two-step synthesis with only moderate yield, low atom efficiency and several purification steps but also relies on the use of stoichiometric Cr(vi) reagents that are extremely toxic and a serious safety and environmental concern. As a consequence, alternative strategies have been increasingly investigated, including a catalytic oxidation with (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO) or its derivatives in BuOAc in combination with sub-stoichiometric amounts of NaOCl and NaClO<sub>2</sub> in a buffered system.<sup>15,16</sup> An oxidation method using [bis(acetoxy)i]benzene (BAIB) with TEMPO was recently disclosed.<sup>17</sup> Chromic oxide in combination with potassium permanganate,<sup>18</sup> as well as a ruthenium based oxidation using either a ruthenium catalyst *e.g.* RuCl<sub>2</sub>(PPh<sub>3</sub>)<sub>3</sub> and TEMPO under O<sub>2</sub><sup>19</sup> or TEMPO in combination with tetrapropylammonium perruthenat and *N*-methylmorpholin-*N*-oxide<sup>20,21</sup> as well as an electrochemical<sup>22</sup> and palladium based oxidation strategies<sup>23</sup> have been also described. Despite this renewed interest in benign and chromium-free synthetic strategies for the oxidation of betulin, many of these procedures suffer from low yields and selectivities for betulinic acid, require multistep procedures, and rely typically on highly purified betulin (**2**) as starting material to obtain betulinic acid (**1**) in reasonable yield and purity.

In here we describe our efforts towards a benign and scalable manufacturing of betulinic acid directly from birch bark, aiming to combine the extraction of betulin at room temperature with the direct oxidation towards betulinic acid while avoiding chromium reagents and the use of toxic solvents.

## Materials and methods

### General

All chemicals were purchased from commercial suppliers and used as received unless otherwise stated. White birch bark was collected in Vienna in summer 2012, frozen with liquid nitrogen and milled on a Retsch® CryoMill to a particle size of <1 mm.

<sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded from CDCl<sub>3</sub>, CD<sub>2</sub>Cl<sub>2</sub>, d<sub>4</sub>-MeOD or d<sub>6</sub>-DMSO solutions on a Bruker Advance UltraShield 400 (400 MHz) spectrometer. Chemical shifts ( $\delta$ )

are reported in ppm using tetramethylsilane as internal standard and coupling constants (*J*) are given in hertz (Hz). Melting points were measured on an automated melting point system OPTI MELT of Stanford Research Systems. Scanning electron microscopy (SEM) pictures were taken with a FEI Inspect F50 at 15 kV. All samples were coated with a 3.5 nm thick gold-layer using a Leica Cool Sputter Coater EM SCD005. HPLC analysis was performed on a Jasco HPLC unit equipped with a PDA detector. For the quantification of betulin (**2**) a Maisch ReproSil 100 C18 250 × 4.6, 5  $\mu$ m was used with MeOH:H<sub>2</sub>O (0.1% TFA) = 87:13 as solvent and a flow of 1 ml min<sup>-1</sup>; detection was done at 210 nm, at 30 °C column oven temperature. Retention times were 7.3 min for the internal standard and 15.7 min for betulin (**2**). The same HPLC set-up used for the simultaneous quantification of betulin, betulinic aldehyde and betulinic acid in the oxidation process. Retention times were 7.3 min for the internal standard, 15.7 min for betulin (**2**), 16.5 min for betulinic acid (**1**) and 28.0 min for betulinic aldehyde. Microwave (MW) reactions were performed on a BIOTAGE Initiator™ sixty microwave unit. The reported times are hold times.

### Screening of betulin extraction from birch bark

**Extraction using microwave irradiation.** In a 10 ml microwave vial 100 mg ( $\pm$ 5 mg) ground birch bark and 900 mg ( $\pm$ 50 mg) of solvent were heated for 15 min at 100 °C using high absorption level. Water (2 ml) and BuOAc (3 ml) were added and the mixture was again heated for 15 min at 100 °C. The organic layer was separated and diluted to 10 ml with HPLC-grade MeOH. A sample of 100  $\mu$ l was taken, 900  $\mu$ l of HPLC-grade MeOH and 200  $\mu$ l of internal standard (200 mg 1-methyl-1-cyclohexen in 100 ml MeOH) were added. The solution was filtered and analyzed *via* HPLC. Results are based on three independent experiments.

**Extraction at room temperature.** In a 20 ml screw-cap vial 100 mg ( $\pm$ 5 mg) ground birch bark and 900 mg ( $\pm$ 50 mg) of the solvent were stirred for 24 h at room temperature. Water (2 ml) and *n*-BuOAc (3 ml) were added and the mixture was again stirred for 1 h at room temperature. The organic layer was separated and diluted to 10 ml with HPLC-grade MeOH. A sample of 100  $\mu$ l was taken, 900  $\mu$ l of MeOH and 200  $\mu$ l of internal standard solution (200 mg 1-methyl-1-cyclohexen in 100 ml MeOH) were added. The solution was filtered over a 20  $\mu$ m syringe filter and analysed *via* HPLC. Results are based on three independent experiments.

### Isolation of betulin from birch bark

**Isolation of betulin at room temperature.** In a 100 ml flask birch bark (1 g) and 9 g [P<sub>4444</sub>]OH as 40 wt% solution in water were stirred for 1 h at room temperature. Water (20 ml) and *n*-BuOAc (30 ml) were added and the mixture was again stirred for 1 h at room temperature. The organic layer was separated and concentrated under reduced pressure. After purification *via* column chromatography (30 g SiO<sub>2</sub>, PE:EtOAc = 20:1–2:1) 230 mg betulin were obtained as colorless crystals. (23 wt% corresponding to birch bark) in >95% purity according to NMR.



Alternatively, betulin could be isolated *via* crystallization: the procedure was performed as described above using 300 mg birch bark. The crude product was crystallized from a mixture of methanol/water to yield betulin (62.5 mg, corresponding to 20.8 wt% yield) as colorless crystals with a purity of >90% according to NMR, containing traces of lupeol. M.p. 230.0–231.1 °C. <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>): δ<sub>H</sub> = 0.67 (m, 1H), 0.76 (s, 3H), 0.82 (s, 3H), 0.90 (s, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.05 (m, 3H), 1.18–1.28 (m, 4H), 1.39 (m, 5H), 1.51–1.70 (m, 12H), 1.85 (m, 1H), 1.94 (m, 2H), 2.38 (m, 1H), 3.19 (dd, *J* = 11.5, 4.8, 1H), 3.33 (dd, *J* = 10.9, 1.3, 1H), 3.80 (dd, *J* = 10.8, 1.6, 1H), 4.58 (s, 1H), 4.68 (s, 1H). <sup>13</sup>C-NMR (150 MHz, CDCl<sub>3</sub>): δ<sub>C</sub> = 15.1, 15.7, 16.3, 16.5, 18.6, 19.4, 21.2, 25.5, 27.4, 27.7, 28.3, 29.5, 30.1, 34.3, 34.6, 37.5, 37.6, 39.0, 39.2, 41.3, 43.1, 48.1 (2C), 49.1, 50.7, 55.6, 60.9, 79.3, 110.1, 150.8.

Analytical data was in accordance with literature values.<sup>14</sup>

**Isolation of betulin *via* microwave extraction.** In a 10 ml microwave vial birch bark (101.0 mg) and 900 mg 40% [P<sub>4444</sub>]OH solution were stirred for 15 min at 100 °C under MW irradiation. Water (2 ml) and 3 ml of *n*-BuOAc were added and the mixture was again subjected to MW irradiation. The organic layer was separated and concentrated under reduced pressure.

After purification *via* column chromatography (7 g SiO<sub>2</sub>, PE:EtOAc = 20:1–2:1) 25.4 mg betulin were obtained as colorless crystals in 25 wt% yield corresponding to birch bark.

#### Oxidation of betulin to betulinic acid

Betulin (2, 99.5 mg, 0.22 mmol), TEMPO (7.0 mg, 0.04 mmol, 0.2 eq.) and BAIB (217 mg, 0.67 mmol, 3 eq.) were dissolved in 10 ml *n*-BuOAc, 0.2 ml water and 42 μl *t*-BuOH. After 6 h stirring at room temperature TLC showed full conversion. To the clear yellow solution 0.2 ml water, 2-methyl-2-butene (72 μl, 1.03 mmol, 4.6 eq.), NaH<sub>2</sub>PO<sub>4</sub> (70 mg, 0.58 mmol, 2.6 eq.) and NaClO<sub>2</sub> (66 mg with 80% purity, 0.58 mmol, 2.6 eq.) were added and a colour change to brown was observed while a white solid was precipitating. The solution was quenched with saturated sodium thiosulfate solution and extracted with EtOAc three times, washed with saturated NaHCO<sub>3</sub> solution and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was filtered and concentrated under reduced pressure. The off-white residue was crystallized from MeOH to obtain betulinic acid in 89% yield as colourless crystals.

M.p.: 284.5–285.5 °C. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ<sub>H</sub> = 0.67 (m, 1H), 0.75 (s, 3H), 0.8 (s, 3H), 0.93 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 1.18–1.69 (m, 15H), 1.69 (s, 3H), 1.97 (m, 2H), 2.19 (m, 1H), 1.27 (m, 5H), 2.26 (m, 1H), 3.00 (m, 1H), 3.19 (dd, *J* = 11.3, 5.0, 1H), 4.60 (s, 1H), 4.74 (s, 1H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ<sub>C</sub> = 15.1, 15.7, 16.4, 16.5, 18.6, 19.7, 21.2, 25.8, 27.8, 28.3, 30.1, 30.9, 32.5, 34.7, 37.4, 37.6, 38.7, 39.1, 39.2, 41.0, 42.8, 47.2, 49.6, 50.9, 55.7, 56.6, 79.4, 110.1, 150.8, 180.2.

Analytical data was in accordance with literature.<sup>14</sup>

#### Streamlined extraction and oxidation process

**Isolation of betulinic acid *via* chromatography.** In a 20 ml screw-cap vial 200.2 mg ground birch bark and 1800 mg of a 40% [P<sub>4444</sub>]OH solution in H<sub>2</sub>O were stirred for 1 h at room

temperature. Water (2 ml) and *n*-BuOAc (3 ml) were added and the mixture was stirred vigorously for 1 h at room temperature. The organic layer was separated and filtered over a Pasteur pipette filled with SiO<sub>2</sub> and Na<sub>2</sub>SO<sub>4</sub>. The crude extract was concentrated to obtain 10 mg betulin per ml. TEMPO (3 mg, 0.02 mmol, 0.2 eq.), BAIB (99.5 mg, 0.31 mmol, 3 eq.) and 19 μl *t*-BuOH, 93 μl water and 1236 μl HOAc were added and the solution was stirred at room temperature for 6 h. Pinnick reagents 2-methyl-2-butene (47 μl, 33 mg, 0.47 mmol, 4.6 eq.), NaClO<sub>2</sub> (30 mg of 80% purity, 0.27 mmol, 2.6 eq.) and NaH<sub>2</sub>PO<sub>4</sub> (32 mg, 0.27 mmol, 2.6 eq.) were added and a color change to brown was observed while a white solid was precipitating. The solution was quenched with saturated sodium thiosulfate solution and extracted with EtOAc three times, washed with sat. NaHCO<sub>3</sub> solution and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was filtered and evaporated to dryness. The off-white residue was purified using column chromatography (3 g SiO<sub>2</sub>, PE:EtOAc = 20:1–2:1) to yield betulinic acid (1) in 37.5 mg corresponding to 18 wt% yield as colorless crystals with a purity of >95% according to <sup>1</sup>H NMR.

Analytical data was in accordance with literature.<sup>14</sup>

**Isolation of betulinic acid *via* crystallization.** The procedure was performed as described above using 507.5 mg birch bark, 4.5 g 40% [P<sub>4444</sub>]OH solution, 10 ml water and 15 ml BuOAc. Oxidation reagents were calculated on 130 mg betulin (2). The crude product was crystallized from a mixture of methanol/water to yield betulinic acid (111 mg, corresponding to 21.9 wt% yield) as colorless crystals with a purity of >90% according to NMR. A second fraction of betulinic acid (36.9 mg, corresponding to 7.0 wt% yield) was obtained with a purity <90% according to NMR.

## Results and discussion

### Biphasic extraction of betulin at room temperature

The extraction of betulin (2) from birch bark is typically performed using conventional Soxhlet extraction with a number of solvents such as dichloromethane, chloroform, ethanol or methanol.<sup>24–26</sup> However, these conventional extraction strategies typically require large solvent volumes and suffer from long extraction times at elevated temperatures, resulting in high time and energy consumption. As for betulinic acid, the purity of the raw extracts is typically low, and additional purification steps are usually required.

As a consequence, efforts to reduce the exhaustive use of undesirable solvents as extraction media for betulin have been made, and recent trends include the use of bio-derived solvents such as limonene,<sup>27</sup> pressurized solvent<sup>10</sup> and supercritical fluid extraction<sup>28</sup> or ionic liquid-based extraction strategies.<sup>29</sup> In a previous paper, we reported the microwave-assisted extraction of betulin (2) using several hydrophilic and hydrophobic ionic liquids. Ionic liquids as solvents for the extraction of value-added compounds may result in increased yields compared to conventional solvents through fractionation of the lignocellulosic matrix.<sup>30,31</sup> In case of betulin, the



best extraction yield was obtained after dissolution of birch bark in the ionic liquid 1-ethyl-3-methylimidazolium acetate ( $[\text{C}_2\text{mim}]\text{OAc}$ ) at 100 °C for 15 min. An isolation strategy relying on the stepwise precipitation of biopolymers with ethanol followed by the addition of water to crystallize betulin was developed and allowed isolating betulin in good yields and purity.

However, aiming for the consecutive *in situ* oxidation of betulin (**2**) this strategy had to be modified in order to avoid ethanol as solvent and to remove any other biomass fragments that would interfere with the further conversion.

The search for an efficient extraction procedure of betulin that would allow the consecutive oxidation to betulinic acid lead us to the development of a biphasic extraction strategy relying on the dissolution or pretreatment of birch bark in ionic liquids or their aqueous solutions and an organic co-solvent, *n*-butyl acetate. This biphasic concept should enable not only a fast and efficient extraction of betulin from birch bark under mild conditions, but the simultaneous separation of the biopolymers or their fragments dissolved in the aqueous ionic liquid layer, while a relatively pure stream of the hydrophobic triterpene betulin (**2**) would be obtained in the organic phase (Fig. 2).

In 2013, Ohno *et al.* reported the efficient dissolution of woody biomass at 60 °C and of pine, cedar and poplar wood even at room temperature in aqueous solution of onium ionic liquids, *e.g.* in a 40% solution of tetrabutylammonium hydroxide ( $[\text{P}_{4444}]\text{OH}$ ) in water.<sup>32,33</sup> The extraction degree of polysaccharides from poplar wood reached up to 37 wt% after 1 hour treatment with  $[\text{P}_{4444}]\text{OH}$  solution at room temperature, suggesting that this phosphonium salt solution might also provide a powerful pretreatment medium for birch bark and could facilitate the extraction of betulin under mild conditions.

To evaluate the extraction performance birch bark was initially pretreated in the aqueous  $[\text{P}_{4444}]\text{OH}$  solution for 15 min at 100 °C under microwave irradiation. Water and *n*-BuOAc were added to extract betulin in the organic phase. Several aspects contributed to the selection of *n*-butyl acetate as organic solvent at this stage. As key criteria, *n*-butyl acetate has been positively evaluated in the revised GSK solvent selection guide, suggesting that it might act as sustainable alterna-

**Table 1** Evaluation of extraction conditions and salt concentration for the biphasic extraction of betulin

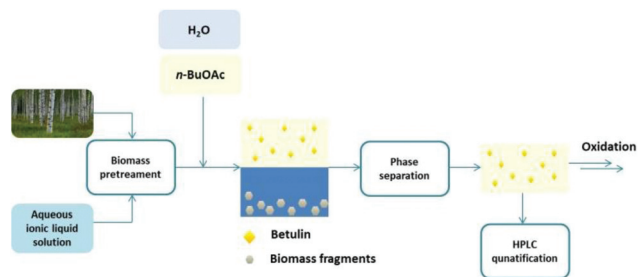
Entry <sup>a</sup>	Salt concentration in H <sub>2</sub> O	Conditions	Yield betulin ( <b>2</b> ) <sup>b,c</sup> [wt%]
1	40% $[\text{P}_{4444}]\text{OH}$	100 °C, MW, 15 min	29.2 ± 0.6
2	20% $[\text{P}_{4444}]\text{OH}$	100 °C, MW, 15 min	25.1 ± 1.8
3	10% $[\text{P}_{4444}]\text{OH}$	100 °C, MW, 15 min	24.4 ± 0.9
4	5% $[\text{P}_{4444}]\text{OH}$	100 °C, MW, 15 min	22.1 ± 1.0
5	0 (pure H <sub>2</sub> O)	100 °C, MW, 15 min	22.3 ± 1.1
6	0 (pure H <sub>2</sub> O)	r.t., 24 h	15.0 ± 1.0
7	40% $[\text{P}_{4444}]\text{OH}$	r.t., 24 h	28.6 ± 1.3
8	40% $[\text{P}_{4444}]\text{OH}$	r.t., 6 h	27.0 ± 0.1
9	40% $[\text{P}_{4444}]\text{OH}$	r.t., 3 h	27.5 ± 1.2
10	40% $[\text{P}_{4444}]\text{OH}$	r.t., 1 h	26.5 ± 0.6
11	40% $[\text{P}_{4444}]\text{OH}$	r.t., 30 min	20.4 ± 0.8
12	20% $[\text{P}_{4444}]\text{OH}$	r.t., 1 h	24.5 ± 0.6
13	10% $[\text{P}_{4444}]\text{OH}$	r.t., 1 h	20.9 ± 0.8
14	8% NaOH	r.t., 1 h	19.7 ± 1.5
15	6% NaCl	r.t., 1 h	19.1 ± 0.9

<sup>a</sup> Performed using 100 mg ground birch bark and 900 mg aqueous  $[\text{P}_{4444}]\text{OH}$  solution or H<sub>2</sub>O at the indicated conditions, followed by addition of 2 ml water and 3 ml BuOAc and stirring of 15 min, 100 °C under MW irradiation (entries 1–5) or 1 h at room temperature (entries 6–15). <sup>b</sup> Yields determined *via* HPLC using 1-methyl-1-cyclohexene as internal standard. <sup>c</sup> Mean ± STD, *n* = 3.

tive to the more volatile ethyl acetate.<sup>34</sup> Moreover, our studies showed that *n*-butyl acetate is a suitable solvent for the consecutive oxidation of betulin – even in the presence of water traces – which is of fundamental importance for the development of the envisaged streamlined extraction and oxidation process (*vide infra*, see also ESI Fig. S2 and Table S3†).

After these stepwise dissolution and extractions steps, the betulin (**2**) content in the upper organic phase was quantified *via* HPLC. Results showed that the phosphonium-based salt solution was indeed a suitable pretreatment media for birch bark, allowing a fast and efficient extraction of betulin with a high extraction yield of 29 wt% (Table 1, entry 1 and ESI Fig. S1†).‡ The influence of the concentration of tetrabutylammonium hydroxide in water was further investigated using more dilute solutions (Table 1, entries 2–4). A 20% solution of  $[\text{P}_{4444}]\text{OH}$  in water still gave a good yield of 25%; however, if the concentration decreased to 5%, the yield dropped again until the same level as in the biphasic extraction with pure water/*n*-BuOAc was obtained (Table 1, entry 5).

In order to develop an energy saving process, the extraction efficiency of the salt solution/*n*-BuOAc system was further evaluated at room temperature, as it was previously shown that the phosphonium ionic liquid  $[\text{P}_{4444}]\text{OH}$  in aqueous solution is able to dissolve wood even at room temperature.<sup>32</sup> The effect



**Fig. 2** Biphasic system for the isolation of betulin (**2**) and the continued oxidation to betulinic acid (**1**).

‡ Several other hydrophilic ionic liquids, either in pure form or as aqueous solution, were also tested for the pretreatment of birch bark. Imidazolium derivatives  $[\text{C}_2\text{mim}]\text{OAc}$ ,  $[\text{C}_2\text{mim}]\text{Me}_2\text{PO}_4$ ,  $[\text{C}_4\text{mim}]\text{Cl}$ , and  $[\text{C}_4\text{mim}]\text{MeSO}_3$  as well as the low-prize ammonium protic ionic liquid  $[\text{HNEt}_3]\text{HSO}_4$  were evaluated for the biphasic extraction of birch bark using the aqueous solution and *n*-BuOAc; however, extraction yields with these ionic liquids remained significantly below the values obtained with the phosphonium hydroxide solution. See ESI Fig. S1† for details.



of pretreatment with the aqueous solution is even more evident at room temperature. Only 15 wt% betulin could be extracted in biphasic extraction with pure water/*n*-BuOAc (Table 1, entry 6). In contrast, pretreatment with a 40% [P<sub>4444</sub>]OH solution in water followed by extraction with *n*-BuOAc dramatically increased the extraction yield to 28.6 wt%, indicating that the extraction yield can be doubled by the mere addition of phosphonium salt. Moreover, the extraction time could be dramatically reduced: while 30 min extraction time were not sufficient, 26.5 wt% betulin were already extracted after 1 hour extraction time, providing an ideal compromise between extraction yield and time consumption of the process (Table 1, entries 7–10). Further studies on the impact of phosphonium salt concentration at room temperature showed a similar effect as we observed in case of microwave treatment at 100 °C. With decreasing concentration of the phosphonium salt, the yield of betulin also decreased. A 20% solution of [P<sub>4444</sub>]OH in water resulted in a lower extraction yield of 24.5 wt% betulin. With a 10% solution the loss of extraction efficiency is even more pronounced and the extraction yield is reduced to 20.9 wt% betulin (Table 1, entries 12 and 13).

It is worthwhile noticing that this efficient pretreatment and strong increase in extraction yield obtained with a 40% solution of [P<sub>4444</sub>]OH in water is a unique effect arising from the combination of cation and anion in this system: when comparing the extraction yield with solutions of 40% [P<sub>4444</sub>]Br or 40% NaOH in water to mimic the impact of cation or anion separately, we did not observe the strong enhancement of extraction yield, and values remained drastically below those obtained with [P<sub>4444</sub>]OH solution (Fig. 3). Similarly, a saturated solution of sodium chloride did not result in a strong increase in comparison to pure water/*n*-BuOAc, thereby ruling out the presence of a pure salt effect on extraction and phase transfer. To exclude that the extraction of betulinic was inhibited by a kosmotropic effect of the 40% NaOH solution, experiments were also performed with a 8% solution NaOH that has the same molarity as 40% [P<sub>4444</sub>]OH in water (1.45 M). As it can be seen in Table 1, entries 14 and 15 yields remained significantly below the value obtained with the [P<sub>4444</sub>]OH solution. A solution of sodium chloride with the same molarity (1.45 M, corresponding to 6% NaCl in water) gave also only low yields (Table 1, entries 14–16).

Additional studies showed the possibility to recover and recycle the aqueous [P<sub>4444</sub>]OH solution after the extraction. In a straightforward process, betulin was extracted from the aqueous phase with butyl acetate without further dilution with water to maintain the concentration. Fresh biomass was added, and the extraction process was repeated. With this strategy, the aqueous [P<sub>4444</sub>]OH solution could be reused for three runs without any loss efficiency, and yields reached 29.5% and 32.5% betulin in the second and third run, respectively (see ESI Table S1†). The small increase in yield in the third run can be attributed to an incomplete phase separation. However, after the third addition of fresh biomass, extraction yields dropped drastically. Only 18.6% betulin were isolated in the 4<sup>th</sup> run, which might be related to the increasing viscosity

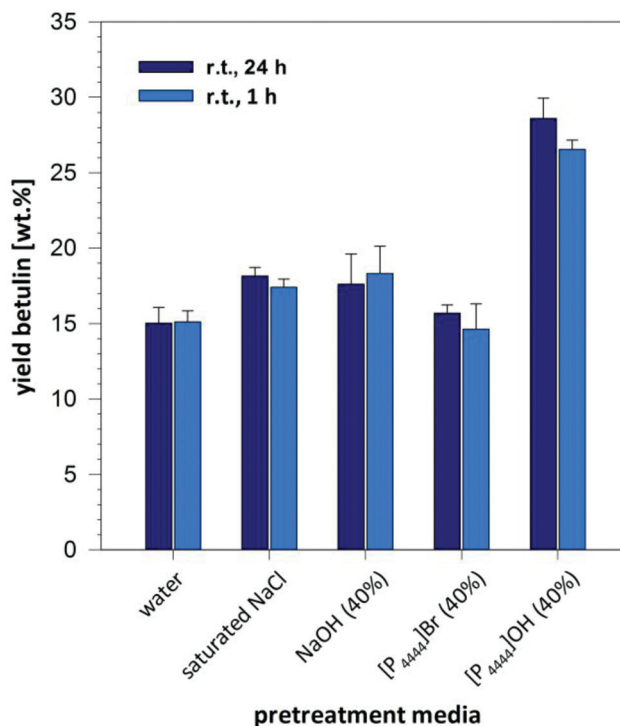


Fig. 3 Impact of different salt solutions on the extraction of betulin from birch bark at room temperature.

of the aqueous [P<sub>4444</sub>]OH slurry that became increasingly difficult to stir. In order to avoid problems with viscosity due to the accumulation of biopolymers in the aqueous solution, we evaluated the possibility of their precipitation with ethanol as anti-solvent. This approach relied on a strategy that we previously used for the extraction of betulin from birch bark with the ionic liquid 1-ethyl-3-methylimidazolium acetate.<sup>29</sup> After extraction of betulin with *n*-butyl acetate, the aqueous [P<sub>4444</sub>]OH solution was diluted with an excess ethanol. Precipitated biopolymers were removed *via* filtration and ethanol was removed *via* distillation before the aqueous [P<sub>4444</sub>]OH solution was subjected to another run. However, this strategy failed as the yield of betulin dropped already in the second run to 10.0%.

Eventually, the strong impact of lignocellulose pretreatment with a 40% [P<sub>4444</sub>]OH in water is also visible in electron microscopy. While preparing the biomass for the SEM-pictures it was already visible with the eye that pretreatment using [P<sub>4444</sub>]OH tremendously changed the biomass structure. As can be seen in Fig. 4 the morphology of the birch bark changed when treated with different solvents. The strongest effects were observed with a [P<sub>4444</sub>]OH solution, resulting in a perforated biopolymer matrix left after 1 h of pretreatment only.

After the identification of suitable extraction conditions, it was possible to isolate betulin from the organic solution after phase separation. At this stage, purification *via* column chromatography gave betulin in 23 wt% yield and excellent purity



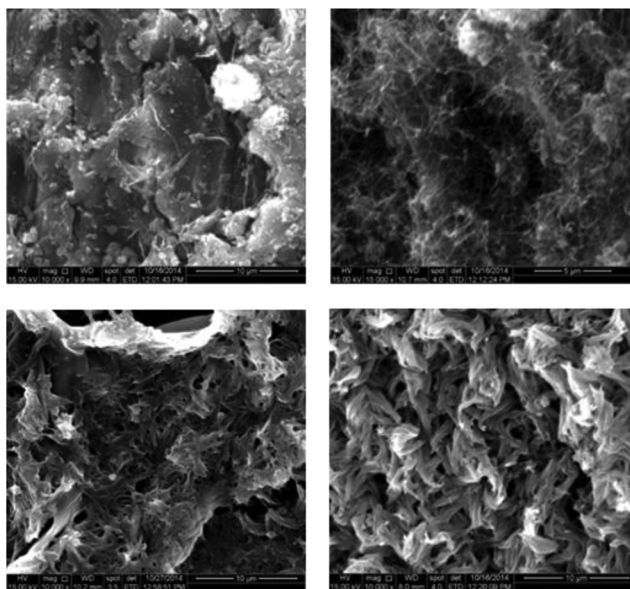


Fig. 4 SEM pictures of untreated birch bark (top, left), birch bark treated with 40% solutions of NaOH (top, right), [P<sub>4444</sub>]Br (bottom, left) and [P<sub>4444</sub>]OH (bottom, right) in water.

after 1 h extraction time at room temperature only. Alternatively, betulin could be isolated *via* crystallization using water/methanol mixtures in 20.8 wt% yield. However, we were more interested in the *in situ* extraction and oxidation towards betulinic acid and therefore the oxidation reaction was investigated in a next step (see ESI Fig. S4–S7†).

### Oxidation of betulin to betulinic acid

Once suitable extraction conditions were found, we focused on the oxidation of betulin (2) to betulinic acid (1) that was initially optimized using pure betulin rather than the biomass-derived crude extraction stream (Fig. 5). Despite being one of the most frequently used reactions in organic synthesis, the oxidation of alcohols to carboxylic acids relied for a long time on the use of metal oxides or metal salts.<sup>35</sup>

Apart from their outstanding toxicity, *e.g.* in case of notorious Cr(vi) salts, these methods suffer from the necessity of using stoichiometric or even higher amounts of the metal oxide, from the poor atom economy, and from the generation of heavy-metal waste.<sup>36</sup> As a result, research has focused on the development of more benign oxidations protocols, particularly on catalytic versions using air or oxygen as oxidants.<sup>37,38</sup>

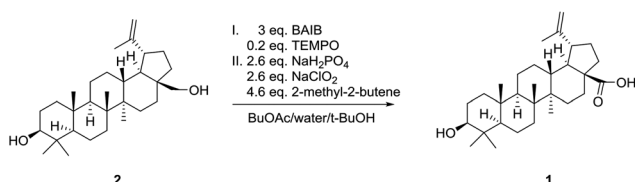


Fig. 5 Optimized oxidation of betulin (2) to betulinic acid (1).

Yet a number of challenges exist for the oxidation of betulin to betulinic acid that limits the pool of available strategies for this particular reaction. Problems are mostly caused by the presence of a secondary alcohol group in position 3 that must not be oxidized, since otherwise a consecutive reduction step of betulonic acid would be required. Additionally, the presence of the electron-rich double bond in position 20 that is likely to be attacked under various conditions complicates the oxidation step.

Our search for a mild oxidation strategy that would fulfill the desired criteria led us to the evaluation of hypervalent iodine(III) reagents, and in particular to the catalytic oxidation using (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO) in combination with [bis(acetoxy)iodo]benzene (BAIB). In general catalytic TEMPO mediated oxidations with secondary oxidants such as NaOCl,<sup>39</sup> NaOCl/NaClO<sub>2</sub><sup>40</sup> or BAIB<sup>41,42</sup> are comparatively mild and known for the selective oxidation of primary alcohols in the presence of secondary alcohols due to the bulky oxoammonium salt derived from TEMPO. Moreover, the combination of BAIB with catalytic amounts of TEMPO was previously reported to be compatible with a number of functionalities, including double and triple bonds, esters, acetals, epoxides, amides, and azides as well as protection groups. Initial attempts on the oxidation of betulin using equimolar amount of TEMPO and 3 eq. BAIB in *n*-BuOAc showed complete conversion of betulin and a yield of 24% betulinic acid. Most importantly, we did not observe any attack of the reactive double bond or any over oxidation of the secondary alcohol group but detected betulinic acid and the intermittent betulinic aldehyde as sole products. The amount of TEMPO could be further reduced to 0.2 eq. TEMPO and 3 eq. BAIB. Further investigations into the reaction conditions revealed a strong influence of the concentration of betulin in *n*-BuOAc, as the highest yield and conversion was found at a concentration of 10 mg betulin per ml *n*-BuOAc (see ESI Tables S2 and S3†). After optimization of reaction conditions it was possible to obtain betulinic acid in 43% yield. However, at this stage, the yield of betulinic acid levelled, and a longer reaction time or the addition of fresh reagents did not shift the ratio between aldehyde and acid towards a higher yield of betulinic acid (Fig. 6).

We found that the addition of acetic acid in excess can be beneficial for this oxidation and increases the yield of betulinic acid to 58%; however, it was still not possible to completely convert the intermittent aldehyde (see ESI Fig. S2†). In order for the further conversion of the crude mixture, we added a consecutive Pinnick oxidation step relying on NaClO<sub>2</sub>/

§ Although a TEMPO/metal catalyst/air-based oxidation would provide an ideal oxidation strategy in terms of sustainability and atom efficiency, this reaction is known to over-oxidise the secondary alcohol of betulin (2) towards betulonic acid.<sup>19</sup> In case of TEMPO mediated oxidations with secondary oxidants such as NaOCl, NaOCl/NaClO<sub>2</sub> we faced considerable problems when applying the protocols of Zhao,<sup>45</sup> Csuk,<sup>15</sup> Zanka<sup>46</sup> or Noula<sup>47</sup> to the oxidation of betulin and yields remained low, particularly when streams of crude betulin isolated from birch bark rather than highly purified starting materials were used.



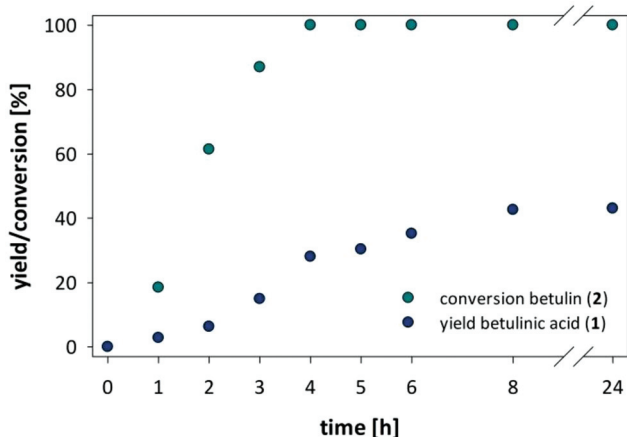


Fig. 6 Conversion of betulin and yield of formed betulinic acid against time in the TEMPO/BAIB mediated oxidation of betulin (2) to betulinic acid (1).

$\text{NaH}_2\text{PO}_4$  with 2-methyl-2-butene as chlorine scavenger to convert the intermittent betulinic aldehyde to the acid.<sup>43</sup> Eventually, after optimization of conditions the combination 0.2 eq. of TEMPO with 3 eq. BAIB followed by the addition of 2.6 eq. Pinnick reagents with a chlorine scavenger allowed to isolate betulinic acid in a reliable one-pot-two-step procedure in 89% yield without the use of chromium reagents (see ESI Fig. S8 and S9†).<sup>¶</sup>

### Streamlined extraction and oxidation process

After finding suitable and compatible conditions for extraction and oxidation, we eventually merged the two individual processes, aiming for a one-pot extraction and *in situ* oxidation of the crude betulin (2) extract under the previously established conditions, and conversion and yield remained significantly below the values obtained for pure starting material. Part of these problems could be traced to the presence of small amounts of phosphonium salt in the organic extract after phase separation that inhibited the consecutive oxidation. When only 0.5 eq. of the phosphonium salt  $[\text{P}_{4444}]\text{OH}$  were deliberately added with regard to betulin (2), conversion dropped from 100% to 88%, whereas no conversion was observed when 2 eq. of the phosphonium salt were present. A similar inhibition of the oxidation was observed in the presence of  $[\text{P}_{4444}]\text{Br}$ . Attempts to remove undesired phosphonium salts *via* an additional extraction step of the crude betulin extract in *n*-BuOAc with water did not remove phosphonium contaminants, as <sup>31</sup>P NMR still showed signals for

<sup>¶</sup>Moreover, the change from stoichiometric chromium-based oxidations strategies towards the catalytic TEMPO/BAIB strategies also should add to the financial impact of the process in terms of disposal costs. Although these savings might be relatively minor compared to the value of the product, the prevention and replacement of a mixed liquid waste containing halides and heavy metals, *i.e.* chromium with a dilute halide waste stream should result in a reduction in disposal cost by the factor 1.5.<sup>48</sup>

the phosphonium cation in the *n*-BuOAc layer. It was however possible to remove phosphonium salts *via* a brief filtration over a batch of silica. Eventually, we were able to develop a streamlined process for the oxidation as shown in the flow scheme in Fig. 7: birch bark was pretreated in aqueous solution of  $[\text{P}_{4444}]\text{OH}$  at room temperature for a short dissolution time of 1 h. After the addition of water and *n*-BuOAc the solution was stirred for another hour to extract betulin. After separation of the organic layer and filtration over  $\text{SiO}_2$  the solution was concentrated to obtain a  $10 \text{ mg ml}^{-1}$  solution of betulin (1) in *n*-BuOAc. The oxidation reagents were added, starting with TEMPO/BAIB reagents in combination with acetic acid. After 5 h reaction time Pinnick reagents were added and the solution was stirred for another 19 h.

As a final challenge, the isolation of betulinic acid from the raw biomass-derived solution required a purification step to separate betulinic acid from lupeol, a triterpenoid whose content in birch bark varies between 1–4 wt%.<sup>44</sup> Eventually, two options exist for the isolation of betulinic acid from the crude biomass-derived solution. The raw extract can be either directly subjected to flash column chromatography, allowing to separate the two triterpenoids and to isolate betulinic acid with high purity with a final yield of 18 wt% calculated from the amount of birch bark (see ESI Fig. S10 and S11†).

Alternatively, the final isolation and separation of lupeol and betulinic acid could be achieved *via* crystallization to avoid chromatography. Fractionated crystallization of the

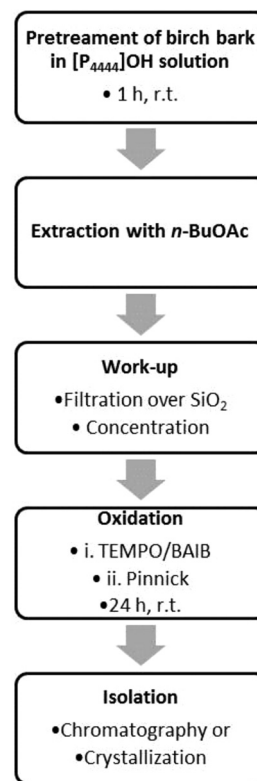


Fig. 7 Flow scheme of the streamlined extraction and oxidation process for the direct preparation of betulinic acid (1) from birch bark.



crude extract from water/methanol mixtures gave a purified fraction, and betulinic acid could be isolated in 22 wt% yield and good purity of >90% (see ESI Fig. S12 and S13†). However, this strategy could not separate betulinic acid and lupeol quantitatively, and betulinic acid was contaminated with 3 wt% lupeol. Additionally, a second fraction was obtained with 7 wt% yield betulinic acid and a purity <90%. Clearly, improvements in this final crystallization step for purification would add to the overall process efficiency and ease of manufacturing on large scale.

## Conclusions

In this paper, we report a novel and efficient strategy for the synthesis of the high-value triterpenoid betulinic acid based on the extraction and streamlined oxidation of betulin (2).

The initial extraction of betulin from the industrial by-product birch bark relies on a biphasic system using aqueous phosphonium hydroxide solution and *n*-butyl acetate. This strategy could extract betulin in high yields at room temperature after 1 hour only, thereby providing a cheap, benign and time and energy-saving strategy for the extraction of valuable ingredients from biomass compared to existing strategies (Fig. 8).

Furthermore, this biphasic approach enables the further conversion of betulin (2) after simple phase separation toward the value-added triterpenoid betulinic acid (1). The crude extract could be directly oxidized using TEMPO and hypervalent iodine(III) reagents, thereby providing a reliable and high yielding strategy for the synthesis of betulinic acid.

In summary, we presented for the first time a streamlined extraction and oxidation strategy for the manufacturing of

betulinic acid from birch bark that is running at room temperature, while simultaneously avoiding toxic chromium reagents or solvents (Fig. 8).

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	State of the art	This work
Extraction from bark	various solvents reflux, long hours 15 wt.% (birch)	[P <sub>4444</sub> ]OH/BuOAc, room temp., 1 h 23 wt.% (birch)
Oxidation of betulin	CrO <sub>3</sub> /H <sub>2</sub> SO <sub>4</sub> and NaBH <sub>4</sub> /THF 2 steps 36–56%	Chromium-free, <i>n</i> -BuOAc 1 step 89%
Streamlined process	-	<b>18 wt.%<sup>a</sup></b> <b>(22 wt.%<sup>b</sup>) betulinic acid</b>

Fig. 8 Comparison of conventional strategies and the novel approach featured in this paper for the synthesis of betulinic acid (1). <sup>a</sup> Isolated via column chromatography. <sup>b</sup> Isolated via recrystallization from water/methanol, 3 wt% lupeol present.





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