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ORIGINAL PAPER

Improved isolation of betulin and lupeol from birch bark and oxidation of their acetylated derivatives with chromyl chloride

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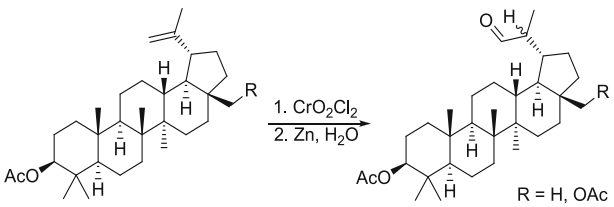
Abstract

An improved method of isolation of betulin and lupeol from birch bark is developed and reported. The method afforded triterpenes with purity of 98.2% (betulin) and 96.3% (lupeol), respectively. Chromyl chloride was also investigated as an oxidating agent of *O*-acetylated betulin and lupeol. The transformation of isopropenyl moiety to aldehyde group was observed.

Graphical abstract



Keywords Aldehyde group · Betulin · Lupeol · Oxidating agent

Introduction

The outer bark of *Betula pendula* is a source rich in triterpenes such as betulin, betulinic acid, oleanolic acid, and lupeol [1], which possess interesting biological activities [1, 2]. Lupeol (lup-20(29)-ene-3β-ol) is the most lipophilic triterpene that occurs in the birch bark. It exhibits antileishmanial, immunomodulatory [3], or antineoplastic activities [4]. Anticancer activities are also intensively studied in the case of triterpene carboxylic acids: betulinic (3β-hydroxy-lup-20(29)-en-28-oic acid) and oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) [5–7]. Betulinic acid and its derivatives as bevirimat (3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid) exhibit significant anti-HIV activity [8]. Bevirimat can be easily synthesised from betulinic acid and 2,2-dimethylsuccinic anhydride [9].

The betulin (lup-20(29)-ene-3β,28-diol) is the most abundant triterpene in birch bark. Therefore, it is a source for the preparation of betulinic acid or other triterpenes. A lot of chemical transformations of betulin were performed by oxidating agents, from which Cr(VI) reagents such as pyridinium dichromate, pyridinium chlorochromate, CrO_3, or $K_2Cr_2O_7$ in different environments were the most studied [10–14]. Chromyl chloride is another strong oxidating agent containing chromium in oxidation state VI. CrO_2Cl_2 is capable of oxidizing 2,2-disubstituted-1-alkenes

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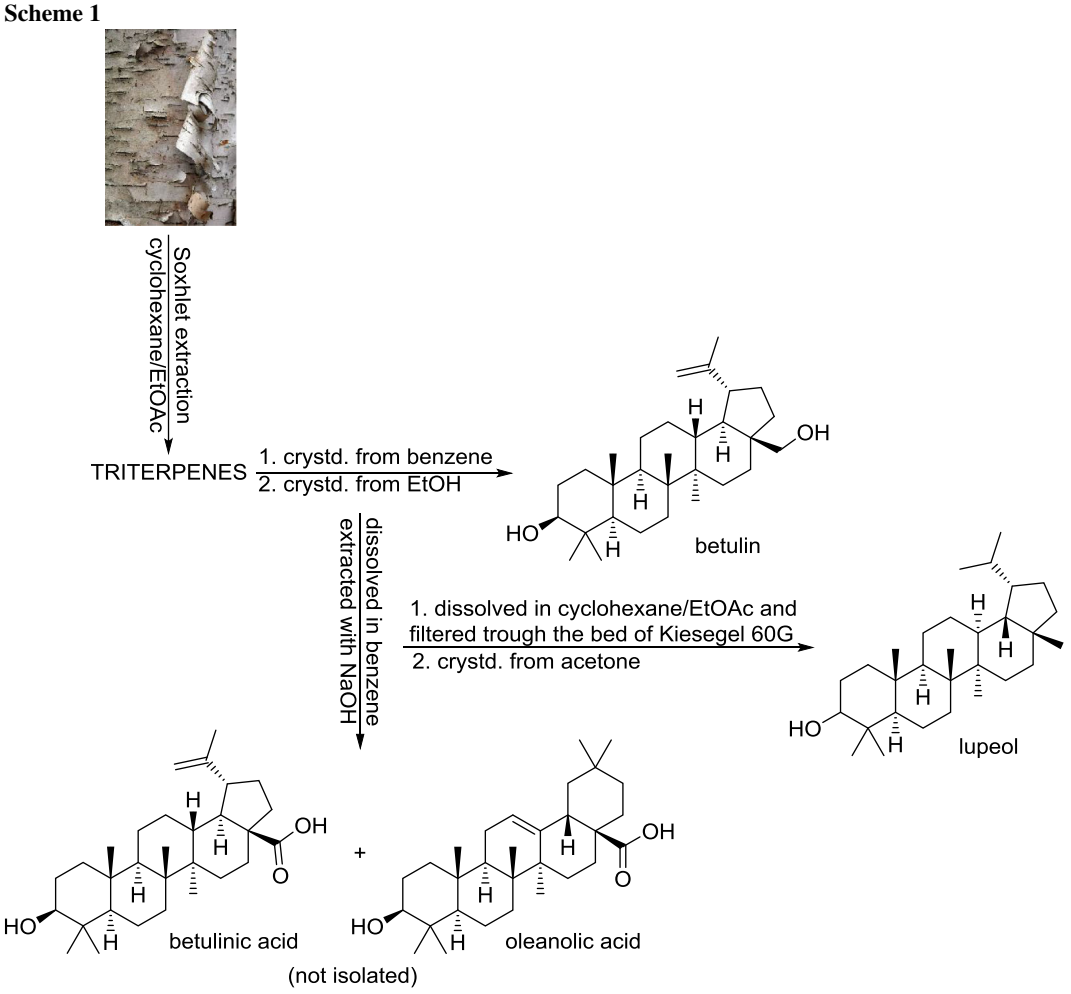
to aldehydes [15]. This moiety is present in the molecules of betulin and lupeol and its oxidation with chromyl chloride was investigated in this work. Following the results of the present study, our aim is to expand research in this area to develop an improved method for the isolation of betulin and lupeol from birch bark.

Results and discussion

Betulin and lupeol were isolated from the outer part of birch bark (Scheme 1). The isolation was performed by Soxhlet extraction with azeotropic mixture cyclohexane/ethyl acetate instead of using alcohols which are commonly used extraction agents [11, 16]. Acetone or ethyl acetate was also used as an extraction solvent as described previously [17, 18]. The choice of the aforementioned mixture is due to the minimisation of the extraction of more polar components from the bark. The raw extract was purified by crystallization from benzene and ethanol. More nonpolar triterpenes present in birch bark (lupeol, betulinic acid, and oleanolic acid) are more soluble in benzene than betulin and can be easily removed by crystallization from this solvent. A similar method of the removal of lupeol from birch bark extract was used by Šiman et al. [16]. However, they used three crystallisation cycles and crystallised betulin was isolated by centrifugation. The final crystallisation from ethanol was used for removing impurities which are more polar than betulin. These procedures led to the isolation of betulin in a relatively good yield (14.5%) and high purity, 98.2%.

According to our best knowledge, the purification of betulin from crude extract was mainly performed by column chromatography [19–24]. Column chromatography-free processes are less commonly reported [2, 16, 25]. We compared our purification process with the latest one published by Šiman [16], which provides thorough information of the efficiency of extraction, purification process, and the purity of the product. The comparison shows that the main advantage of our purification process is the

Scheme 1



simplification of the method. We reduced the number of purification steps. We used only three crystallizations instead of four crystallization steps (preparation of fractions B0 and B2) and one extraction with $Ca(OH)_2$ (preparation of fraction B1) as described Šiman et al. [16] for the preparation of betulin fraction B2. The other advantage is that the crystals were separated by filtrations only. The centrifugation was not necessary, as requested by the procedure published by Šiman et al. [16]. The purity of betulin obtained with both methods was identical, amounting to 98.2%. We obtained only slightly lower overall yield of betulin (14.5%) as compared with the yield (overall yield of B2 was 17.1%) published by Šiman [16]. However, our less time-consuming and simpler purification process provides significant benefits.

Lupeol was isolated from solutions which remained after the separation of betulin. The solutions were ethyl acetate/cyclohexane (B1) and benzene (B2). Triterpenes contained in these solutions were betulin, lupeol, betulinic acid, and oleanolic acid. Triterpene acids were separated from the raw mixture of triterpenes by washing of benzene solution with aqueous solution of sodium hydroxide. Betulinic and oleanolic acids formed sodium salts which were soluble in water. Some other impurities were precipitated from benzene solution in the form of dark brown solid compounds. Lupeol is the most lipophilic triterpene from birch bark. Therefore, it was subsequently purified by filtration of its solution (10% ethyl acetate in cyclohexane) through the bed of silica gel. The last step of purification was performed by crystallization of lupeol from acetone. Triterpene was obtained in the yield 0.98%, as calculated with respect to birch bark, and in good purity of 96.3%. Its purity was higher than that of some commercially available agents. Less time-consuming and column chromatography-free process with good purity of obtained lupeol represents the main benefit resulting from this purification method in comparison with the previously described isolation and preparation processes [21, 24, 26–28]. Our procedure provides an easy and cheap isolation method for obtaining lupeol. It is a superior alternative to expensive sources provided by commercial suppliers.

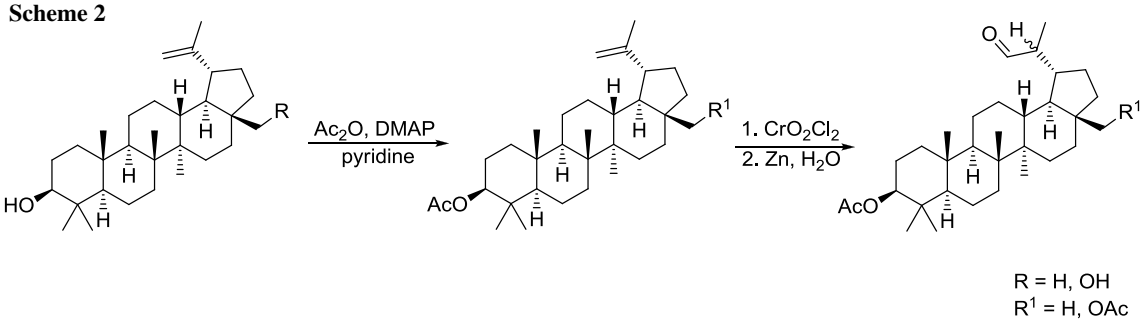
Oxidation of betulin and its diacetate with chromyl chloride was performed three times (Scheme 2). The first oxidation of unprotected betulin was unsuccessful. Betulin is very slightly soluble in dichloromethane and therefore, it is poorly oxidisable. The remaining two oxidations were made on 3β,28-di-*O*-acetylbetulin which is well soluble in dichloromethane. Acetyl groups protected hydroxyl groups before oxidation with the reagent. Acetylated betulin was prepared by conventional reaction of alcohol with acetic anhydride in basic environment of pyridine and in the presence of catalytic amount of 4-(dimethylamino)pyridine (DAMP) [2]. Oxidation of protected betulin was performed with 1 equivalent or 1.5 equivalents of chromyl chloride. Reaction was stirred for 15 or 60 min, as indicated in the experimental procedure below. Longer time and higher amount of oxidizing agents produced lower yield. The reaction provided 130 mg (23.9%) of 3β,28-diacetoxylupan-29-al-11.0% of 20*R*-epimer and 12.9% of 20*S*-epimer. Shorter time and equimolar amount of chromyl chloride afforded better yield. The isolated amount of aldehyde was 292 mg (53.8%). 3β,28-Diacetoxylupan-29-al was isolated as 20*R*-epimer (143 mg, 26.3%), 20*S*-epimer (83 mg, 15.3%). The mixture of both epimers (66 mg, 12.2%) is composed of 21.5% of 20*R*-epimer and 79.5% of 20*S*-epimer. A calculation shows that the ratio of the isolated epimers was 54%:46% (20*R*-epimer to 20*S*-epimer). The results from the both experiments show that the oxidation of 3β,28-di-*O*-acetylbetulin with chromyl chloride is not a stereospecific reaction.

Direct of oxidation of 3β,28-di-*O*-acetylbetulin was previously performed by iron(III) picolinate [29]. This oxygenation results in different amounts of epimers. Their ratio was approximately 1:2 (20*R*-epimer to 20*S*-epimer). The overall yield of the reaction was lower (44.8%). 3β,28-Diacetoxylupan-29-al was prepared for the first time by an oxidation of a double bond with potassium permanganate [30]. However, the resolution of epimers was not carried out. The transformation of the double bond to an aldehyde in lup-20(29)-ene moiety was also performed by several other methods [31–34]. However, it had to be made in several reaction steps.

The oxidation of isopropenyl group of acetylated lupeol (3β-*O*-acetyllupeol) by chromyl chloride was also investigated (Scheme 2). The reaction was performed with good yield (64.5%). It was slightly higher than that observed in the case of 3β,28-di-*O*-acetylbetulin. The procedure was also nonstereo-specific. Both epimers of 3β-acetoxylupan-29-al were obtained. Their ratio was approximately 3:2 (20*R*-epimer to 20*S*-epimer). However, only small amount of epimeric pure 3β-acetoxylupan-29-al (20*R*-epimer; $m = 94$ mg) was obtained by the purification on silica gel. The main fraction consists from the mixture of both epimers. 20*S*-Epimer could not be separated because its R_f value was close to that of 20*R*-epimer.

3β-Acetoxylupan-29-als has been prepared previously [35]. Firstly, 3β-*O*-acetyllupeol was oxidised by *m*-chloroperbenzoic acid [36]. Then, the epoxide was treated with boron trifluoride [35]. This method also produced epimers in a ratio 3:2 (20*R*-epimer to 20*S*-epimer; determined by ^{1}H NMR) which is similar to our observation. The authors prepared enantiomeric pure 3β-acetoxylupan-29-als by the reduction of aldehyde to an alcohol. Epimeric 3β-acetoxylupan-29-ols were separated by multiple chromatography on silica gel. Then, enantiomerically pure alcohols were repeatedly oxidised to aldehydes.

Scheme 2



The method using oxidation of isoprenyl group with chromyl chloride to aldehyde has several advantages in comparison with previously discussed methods [29–36]. The yields of aldehydes are in the most cases higher than those cited in the literature and the aldehydes are prepared in one reaction step instead of multistep reactions.

Conclusion

We evaluated an improved separation method of betulin and lupeol from birch bark. The main benefit resulting from the application of this procedure is the isolation of lupeol in good purity and its recovery. This triterpene is also available by commercial suppliers. However, it is expensive and is often provided with lower purity than that obtained by us. Our procedure provides an easy, efficient, and cheap isolation method.

The acetylated triterpenes were also investigated in an oxidation reaction with oxidizing agent which enables a direct transformation of alkene to aldehyde. Lup-20(29)-ens were oxidised with chromyl chloride to lupan-29-als with a good yield. Reactions were not stereoselective and both enantiomers were obtained. Epimers of 3β,28-diacetoxylupan-29-als were separated by column chromatography on silica gel. However, the separation of epimeric pure 3β-acetoxylupan-29-als is more complicated.

Experimental

All chemicals used in the synthesis were obtained from commercial suppliers and were of p.a. purity. ^{1}H and ^{13}C NMR spectra were measured on a Varian MERCURY plus spectrometer operating at frequencies of 300 and 75 MHz, respectively. ^{13}C NMR spectra were decoupled against protons. The spectra were measured in $CDCl_3$. The chemical shifts were referenced with respect to an internal TMS ($\delta(^{1}H) = 0$ ppm, $\delta(^{13}C) = 0$ ppm).

Isolation of betulin

Betulin was extracted from outer white bark of *Betula pendula*. The bark was collected from fallen trees in the locality: Detva, Slovakia, 25.12.2016 (coordinates WGS-84: 48°34′25″N, 19°23′55″E). The bark was air dried at 25 °C, then cut in small pieces in a kitchen blender. 50 g of bark were extracted in 500 cm^3 Soxhlet extractor. The extraction was performed with an azeotropic mixture of ethyl acetate and cyclohexane (cyclohexane to ethyl acetate − 46 wt% : 54 wt%). The bark was extracted for 3 h. The resulting extract was concentrated to a volume of 200 cm^3 by distillation in the rotary evaporator. The concentrate was cooled to − 18 °C, and the precipitate (fraction B1) was filtered through a filter glass with pore size 16–40 μm. The precipitate was dissolved in the boiling mixture of 110 cm^3 of benzene and 11 cm^3 of ethanol and the solution was filtered through the filter glass with pore size 16–40 μm. The filtrate was concentrated by distillation until the first precipitate was observed. This concentrate was cooled to 4 °C. The precipitate (fraction B2) was filtered through the filter glass with pore size 16–40 μm and washed by 25 cm^3 of cold benzene. The solid compound was dried at 110 °C for 2 h. B2 was obtained in the amount 8.14 g. The fraction B2 (1 g) was crystallised from 30 cm^3 of technical grade ethanol ($w = 96\%$). The solution was cooled in the fridge at − 18 °C over night. Crystals (fraction B3) were filtered through the filter glass with pore size 16–40 μm and washed with small amount (3 cm^3) of cold technical grade ethanol. The betulin was dried at 150 °C for 75 min and the final amount of 890 mg was obtained. The overall yield of betulin was 14.5%.

Isolation of lupeol

The solution after filtration of fraction B1 was evaporated and the solid rest was dissolved in benzene solution which remained after the filtration of B2. Benzene solution was washed with 10% NaOH solution (2 × 50 cm^3), H_2O (1 × 50 cm^3) and brine (1 × 25 cm^3), then dried with

anhydrous Na_2SO_4. Benzene was evaporated and the rest in the flask was suspended in a hot solution of cyclohexane and ethyl acetate (50 + 5 cm^3). The suspension was filtered through the bed of silica gel (3 cm layer of Kieselgel 60G, diameter 4 cm) and washed with 100 cm^3 of 10% ethyl acetate in cyclohexane. Then, the solution was evaporated. The fraction L1 ($m = 1.7$ g) was obtained. This fraction was crystallised from 35 cm^3 of acetone at – 18 °C. Crystals were filtered through the filter glass with pore size 16–40 μm, washed with 5 cm^3 of cold acetone and subsequently dried. Acetone solution was concentrated to a volume of 20 cm^3 and cooled to − 18 °C. The crystals were filtered through the filter glass with pore size 16–40 μm, washed with 5 cm^3 of cold acetone and dried. The obtained crystals were added to the first portion of crystals. The fraction L2 was obtained. Pure lupeol was obtained by the recrystallization of fraction L2 from acetone with the same procedure as was used for the fraction L2. The lupeol was dried at room temperature for 1.5 h and at 145 °C for 15 min. The final amount of lupeol was 480 mg (0.96%).

Determination of purity of betulin and lupeol

The purity of betulin and lupeol was determined using ^{1}H NMR spectroscopy. Dimethyl sulfone (Standard for quantitative NMR, TraceCERT®) was used as the internal standard. The purity was estimated to 90.8 ± 0.8% for betulin fraction B2, 98.2 ± 0.4% for betulin fraction B3, and 96.3 ± 0.8% for lupeol, respectively. The large differences in molecular weight of the standard and triterpenes and low solubility of betulin caused that a relatively high level of uncertainty had to be considered according the recommendation of producer.

3*β*,28-Di-*O*-acetylbetulin and 3*β*-*O*-acetyllupeol Acetylation of betulin and lupeol was performed according to the literature [2]. Analytical data of acetylated betulin were in accordance with the literature [2]. Analytical data of acetylated lupeol were in accordance with the literature [37].

Oxidation of acetylated betulin and lupeol with chromyl chloride

3*β*,28-Di-*O*-acetylbetulin (1 mmol, 527 mg) or 469 mg 3*β*-*O*-acetyl-lupeol (1 mmol) was dissolved in 20 cm^3 of anhydrous dichloromethane. After cooling to 2 °C the solution of 80 mm^3 freshly distilled CrO_2Cl_2 (1 mmol) in 10 cm^3 of anhydrous dichloromethane was added dropwise over a period of 10 min. The reaction mixture was stirred at 2 °C for 15 min after adding of the whole amount of chromyl chloride solution. Subsequently, 0.7 g zinc dust was added. The mixture was stirred for 5 min, 1 cm^3 of distilled water was added, and the mixture was stirred for additional 15 min. The reaction mixture was diluted with 50 cm^3 of dichloromethane and dried with 3 spoons of anhydrous Na_2SO_4. The suspension was filtered through a bed of neutral aluminium oxide (1.5 cm high). Colourless filtrate was mixed with silica gel and evaporated to dryness.

Purification of 3*β*,28-diacetoxylupan-29-al

The adsorbed raw reaction mixture was chromatographed on silica gel (20 g). The column was eluted with 100 cm^3 of petrol ether and mixtures of ethyl acetate and petrol ether (100 cm^3 of 2%, 100 cm^3 of 4%, 100 cm^3 of 6%, 100 cm^3 of 7%, 100 cm^3 of 8%, 200 cm^3 of 10%, and 100 cm^3 of 12.5% of solution of ethyl acetate in petrol ether). The first collected fraction provided 80 mg of unreacted 3*β*,28-di-*O*-acetylbetulin. The second collected fraction afforded 143 mg (26.3%) of 3*β*,28-diacetoxy-(20*R*)-lupan-29-al ($R_f = 0.14$ in 5% solution of ethylacetate in petrol ether). The third collected fraction consisted from the mixture of 3*β*,28-diacetoxy-(20*R*)-lupan-29-al and 3*β*,28-diacetoxy-(20*S*)-lupan-29-al in the ratio 21.5%:79.5%. The ratio of epimers was determined according to the integrals of hydrogens of aldehydic groups (*R*-epimer: $\delta = 9.85$ (d, $J = 1.9$ Hz, 1H) ppm; *S*-epimer: $\delta = 9.61$ (s, 1H) ppm) in ^{1}H NMR spectra. The amount of third fraction was 66 mg (12.2%). The fourth collected fraction provided 83 mg (15.3%) of 3*β*,28-diacetoxy-(20*S*)-lupan-29-al ($R_f = 0.09$ in 5% solution of ethylacetate in petrol ether). Analytical data of 3*β*,28-diacetoxy-lupan-29-als were in accordance with the literature [29].

Purification of 3*β*-acetoxylupan-29-al

The adsorbed raw reaction mixture was chromatographed on silica gel (30 g). The column was eluted with 100 cm^3 of petrol ether and mixtures of ethyl acetate and petrol ether (100 cm^3 of 1%, 100 cm^3 of 2%, 100 cm^3 of 3%, 100 cm^3 of 4%, 400 cm^3 of 5% of solution of ethyl acetate in petrol ether). The first collected fraction provided 94 mg (19.6%) of 3*β*-acetoxy-(20*R*)-lupan-29-al ($R_f = 0.33$ in 5% solution of ethylacetate in petrol ether). The second collected fraction consisted from the mixture of 3*β*-acetoxy-(20*R*)-lupan-29-al and 3*β*-acetoxy-(20*S*)-lupan-29-al ($R_f = 0.30$ in 5% solution of ethylacetate in petrol ether) in the ratio 43.8% : 56.2%. The ratio of epimers was determined according to the integrals of hydrogens of aldehydic groups (*R*-epimer: $\delta = 9.87$ (d, $J = 1.9$ Hz, 1H) ppm; *S*-epimer: $\delta = 9.63$ (s, 1H) ppm) in ^{1}H NMR spectra. The amount of second fraction was 218 mg (44.9%). Analytical

data of 3β,-acetoxylupan-29-als were in accordance with the literature [35].

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