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ALPHITOL, A PHENOLIC SUBSTANCE FROM *ALPHITONIA ZIZYPHOIDES* WHICH INHIBITS PROSTAGLANDIN BIOSYNTHESIS *IN VITRO*

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Key Word Index—*Alphitonia zizyphoides*; Rhamnaceae; prostaglandin biosynthesis; phenyl ethanoid; alphitol; 3,5-dihydroxy-4-methoxy phenethyl alcohol; betulinic acid.

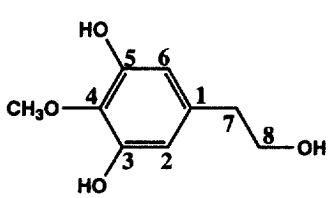
Abstract—The new phenolic compound, 3,5-dihydroxy-4-methoxy phenethyl alcohol, named alphitol, and betulinic acid were isolated from the bark of *Alphitonia zizyphoides*. The chemical structure of alphitol was determined by mass spectrometry in combination with one and two dimensional NMR, including HMBC. Both compounds inhibited prostaglandin biosynthesis *in vitro*, alphitol with an IC_{50} value of 0.66 mM, which is of the same magnitude as acetyl salicylic acid. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Alphitonia zizyphoides, (Spreng.) A. Gray is used medicinally to treat inflammation, gastro-intestinal and uro-genital disorders and as a tonic [1, 2]. We previously reported that an extract of *A. zizyphoides* inhibited prostaglandin biosynthesis *in vitro* as well as the formation of rat ear oedema after treatment with ethyl phenyl propiolate (EPP) *in vivo* [2]. The aim of this study was to use bio-assay guided fractionation to isolate and characterise the compounds responsible for the previously noted *in vitro* inhibition of prostaglandin biosynthesis.

RESULTS AND DISCUSSION

From the crude EtOH extract prepared from the dried bark of *A. zizyphoides* two compounds were isolated, alphitol (**1**) and betulinic acid, by using a combination of Accelerating Gradient Chromatography (AGC) [3] on silica and Centrifugal Partition Chromatography (CPC) [4]. Betulinic acid was identified by comparison with reference spectral data from the literature [5] and with an authentic sample.



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On HREIMS **1** gave an $[M]^+$ peak at *m/z* 184.0732 indicating its molecular formula to be $C_9H_{12}O_4$ (calculated value: 184.0736) with four double bond equivalents. The molecular ion gave rise to a fragment with *m/z* 153 formed by the loss of CH_2OH, a fragmentation typical for primary alcohols. This fragment then rearranged to give a stable tropylium ion. Loss of CH_2 from the phenolic methoxy group gave rise to a peak at *m/z* 139. Thus from the mass spectrum compound **1** was shown to contain an aromatic ring substituted with a 2-hydroxyethyl group, two hydroxyl groups and a methoxy group.

The ^1H NMR spectrum taken in deutero methanol showed two coupled triplets ($J = 7.1$ Hz) at δ 2.62 and 3.68 arising from two methylene groups of a 2-ethanol fragment. The former corresponded to the benzylic methylene and the latter to the alcoholic methylene. A singlet at δ 3.75 corresponded to an aromatic methoxy group. Finally, a singlet at δ 6.23 integrating for two protons showed the presence of two magnetically equivalent aromatic protons, suggesting a degree of symmetry in the aromatic substitution pattern. The symmetrical disposition of the substituents was further supported by the presence of only seven signals in the ^{13}C NMR spectrum. Signals corresponding to the two methylene groups and the

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methoxy group were confirmed as such by DEPT experiments. The aromatic region showed signals between δ 109.3 and 151.5. The signal at δ 109.3 was shown by DEPT to represent two carbons each bound to one proton. The relatively high field shift showed they were *ortho* to one or more hydroxy groups. The HMBC spectrum showed a correlation between the benzylic methylene protons and the ^{13}C signal at δ 136.3, thus establishing this signal was due to C-1. The same carbon showed correlation with the proton signal at δ 6.23 showing that these protons occupied positions 2 and 6. The symmetry of the molecule placed the hydroxyl groups at positions 3 and 5 with the methoxy at position 4. Thus the entire structure of **1** was established. Comparison with literature data for solorinin and meso-2,3-bis(3,4,5-trimethoxybenzyl)-1,4-butanediol confirmed the final structure [6, 7].

Compound **1** inhibited *in vitro* prostaglandin biosynthesis in a dose dependent manner, having an IC_{50} value of 0.66 mM (123 μg/ml), which is comparable to acetyl salicylic acid (0.95 mM, 150 μg/ml) but less potent than indomethacin (0.25 μM) [8].

Betulinic acid inhibited prostaglandin biosynthesis by 52% at 200 μg/ml. However, due to its low solubility no accurate dose-response curve was obtained. Betulinic acid has been shown to inhibit EPP-induced rat ear oedema, giving a 30% inhibition at 0.5 mg/ear [9]. This effect is suggested to be due to a glucocorticoid like effect rather than inhibition of prostaglandin biosynthesis [10]. Therefore, the isolation of betulinic acid from *A. zizyphoides* can at least partly explain the high inhibitory activity on EPP induced rat ear oedema that *A. zizyphoides* exhibited in our first evaluation for anti-inflammatory activity (42% inhibition after 1 h, 1 mg/ear) [2].

EXPERIMENTAL

General

^1H, ^{13}C, DEPT, HMBC and HETCOR NMR: JEOL-EX-270/4000 spectrometer, using MeOH-d_4 for **1** (15 mg/0.7 ml) and a mixture of $CHCl_3$-d_1 and MeOH-d_4 (4:1) for betulinic acid (40 mg/0.7 ml). TMS was used as int. standard. HREIMS: JEOL SX-102A model spectrometer (direct inlet probe); Centrifugal Partition Chromatography (CPC): Ito Multi-Layer Coil CCC Instrument, coil no. 10, flow rate of 3 ml/min; Accelerating Gradient Chromatography (AGC): Separo AB AGC equipment (Baeckström Separo AB, Lidingö, Sweden), column diameter 2.5 cm. Flow rate of 25 ml/min; TLC: silica gel with $CHCl_3$–MeOH–H_2O (9:6:1).

Plant material

Bark of *Alphitonia zizyphoides* was collected near Aleisa village, Upulu island, Western Samoa in November 1991. The plant was identified by Professor Paul Alan Cox, Brigham Young University, Provo, Utah, U.S.A. A voucher specimen, no. SA 1, is deposited at the Botanical Garden in Uppsala.

Extraction and isolation

Dried and ground bark (2.3 kg) was soaked in 23 l EtOH (70%) overnight. After filtration, the EtOH was removed by evaporation and the crude extract lyophilised. The crude extract (100 g) was extracted twice using 4.8 l of three phase liquid–liquid extraction ($CHCl_3$–MeCN–hexane–H_2O, 1:3.4:2:1). The organic solvents were evaporated and the water phase further extracted with 3×140 ml of EtOAc. The EtOAc and the $CHCl_3$–MeCN extracts were combined giving a total wt of 14.8 g.

Alphitol (**1**). The combined extract was divided into two portions and each was mixed with 14 g of silica. The mixture was applied to a SEPARO column and 14 g silica gel was added on top. The column was eluted with a nine step $CHCl_3$ gradient in hexane (0, 50, 75, 87.5, 93.8, 96.9, 98.2, 99.6 and 100% $CHCl_3$, 50 ml portions) and then eluted with a nine step accelerating gradient of MeOH in $CHCl_3$ (0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100% MeOH, 50 ml portions). Frs were collected, monitored by TLC and then combined. Fr. 3 exhibited inhibition of prostaglandin biosynthesis and was further fractionated using AGC, ten step accelerating gradient of MeOH in toluene (0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100% MeOH, 25 ml portions) giving four frs. Fr. 4 was dissolved in EtOH and applied to a LH-20 column and eluted with EtOH. Separation was monitored by TLC and five frs were collected. Final purification was achieved using CPC. The lower phase of $CHCl_3$–MeOH–BuOH–H_2O (10:10:1:6) was used as mobile phase. Frs collected were monitored by TLC and then combined. Fr. 13 contained 15 mg of compound **1**.

Alphitol (**1**). ^1H NMR (CD_3OD) δ: 2.62 (2H, *t*, $J = 7.1$, H-7), 3.75 (3H, *s*, CH_3O-), 3.67 (2H, *t*, $J = 7.1$, H-8) and 6.23 (2H, *s*, H-2 and H-6); ^{13}C NMR (CD_3OD) δ: 40.1 (*t*, C-7), 60.8 (*q*, CH_3O–), 64.3 (*t*, C-8), 109.3 (*d*, C-2 and C-6), 135.2 (*s*, C-4), 136.3 (*s*, C-1), and 151.5 (*s*, C-3 and C-5); HREIMS, 70 eV: *m/z* 184.0732; EIMS, 70 eV: *m/z* 184 $[M]^+$ (43), 154 $[M-30]^+$ (12), 153 $[M-CH_2-CH_2-OH]^+$ (100), 139 $[M-CH_2-CH_2-CH_2-OH]^+$ (15), 138 $[M-46]^+$ (11), 111 $[M-66]^+$ (3), 110 $[M-67]^+$ (10); UV λ_{max} nm: 270.

Betulinic acid

The hexane fr. of the three phase liquid–liquid extraction was mixed with silica and applied to a SEPARO column. The column was eluted by a ten step accelerating gradient of EtOAc in $CHCl_3$ (0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100% EtOAc, 25 ml portions). Fr. 2 exhibited inhibitory activity on prostaglandin biosynthesis and was further fractionated using CPC (hexane–EtOAc–H_2O, 6:7:2).

The upper phase was used as mobile phase. After fifty tubes the phases were switched and the lower phase used as mobile phase. The separation was monitored by TLC giving a total of five frs. Fr. 4 yielded 100 mg of betulinic acid.

Prostaglandin biosynthesis assay

The experiments were performed according to the method of White and Glassman [11] which has been modified by Pongprayoon *et al.* [12]. Bovine prostaglandin synthetase (cyclo-oxygenase) was prepared as described by Takeguchi *et al.* [13] from bovine seminal vesicles. Compound **1** was dissolved in 10% MeOH, betulinic acid in 20% DMSO and acetyl salicylic acid in 10% EtOH. All solvents were used as background and as untreated controls. Samples were tested in triplicate for a minimum of three experiments and indomethacin was used as a positive control (70% inhibition at 2 μg/ml). Inhibition was calculated using the following formula:

$$(1-[DPM_{sample}-DPM_{blank}/DPM_{untreated}-DPM_{blank}]) \times 100 = \% \text{ Inhibition.}$$

To calculate IC_{50} values regression analysis was used.

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