Tetrahedron Letters 50 (2009) 1771–1776

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Proanthocyanidin glycosides from the leaves of *Quercus ilex* L. (Fagaceae)

Anastasia Karioti [a,b,*], Anna Rita Bilia [b], Chiara Gabbiani [c], Luigi Messori [c], Helen Skaltsa [a]

[a] Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis, Zografou, 157 71 Athens, Greece
[b] Department of Pharmaceutical Sciences, University of Florence, via Ugo Schiff 6, Polo Scientifico, Sesto Fiorentino, 50019 Florence, Italy
[c] Laboratory of Metals in Medicine, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Firenze, Italy

ARTICLE INFO

Article history:
Received 20 November 2008
Revised 20 January 2009
Accepted 28 January 2009
Available online 7 February 2009

Keywords:
Quercus ilex
Fagaceae
Oak-leaves
Proanthocyanidin glycosides
ROESY
Circular dichroism

ABSTRACT

From the polar extracts of the leaves of *Quercus ilex* L., two new proanthocyanidin glycosides, namely afzelechin-(4α→8)-catechin-3-*O*-β-glucopyranoside (**1**) and afzelechin-(4α→8)-catechin-3-*O*-α-rhamnopyranoside (**2**), were isolated in addition to catechin (**3**), proanthocyanidin B_3 (**4**), prodelphinidin C (**5**), dehydrodicatechin A (**6**), quercetin (**7**) and six known flavonol glucosides with their acylated derivatives (**8**–**13**) and ellagic acid (**14**). The structures of all isolated compounds were established by spectroscopic means, mainly 1D and 2D NMR, as well as LC/MS and HR-MS spectrometric analyses. The absolute configuration of compound **1** was determined by CD measurements. The proanthocyanidin glycosides are especially interesting, as they possess the sugar in the upper unit of the dimer, which is rare for this type of compounds.

Quercus ilex, also called holly oak or evergreen oak, is a common Mediterranean, medium-size, evergreen tree which is widely distributed along the Balkan Peninsula and the Mediterranean region to N. Spain and W. France.[1] Species of the genus *Quercus* have been for long used in traditional medicine as haemostatic, in the treatment of gastrointestinal disorders,[2] inflammations of the oral, genital and anal mucosa and externally against inflammation of the skin.[3] Polar extracts of the leaves, bark, wood and galls have shown antibacterial[4,5] and antiinflammatory activities[2,6] that explain their ethnopharmacological uses[7] and are attributed to their high phenolic content.

Due to the use of *Quercus* sp. in the construction of wine barrels and the interactions between wine and oak wood during the maturation of wine, these plants have been a subject of intensive research and have shown to possess a rich load of lignins, hydrolysable tannins, ellagitannins, flavano-ellagitannins, catechins, flavan and proanthocyanidin glycosides, flavonoids and simple phenols.[8–10] *Quercus* sp. have been used in the past in diverse ecological studies.[11–13] This work has been conducted in the framework of a project aiming at investigating possible qualitative and quantitative alterations in the phenolic content of healthy and mite-infected *Q. ilex* abaxial hairs. The lack of available standards of proanthocyanidins and acylated flavonol glucosides in the market made necessary the creation of such a database by extensive phytochemical investigations in the initial plant material so that reliable LC–DAD–MS analyses could be carried out. During the isolation process, 14 substances were isolated and characterised, among them two were naturally occurring proanthocyanidin glycosides. In this Letter, we report on the isolation and structure elucidation of the proanthocyanidin and flavonoid content of *Q. ilex* leaves.

For the initial extraction, which was all conducted in dark, a classical extraction scheme was applied that included organic solvents, such as cyclohexane and dichloromethane, and finally polar solvents like MeOH and MeOH/H_2O 70:30. This procedure was chosen in order to deactivate enzymes responsible for degradation, oxidation and polymerisation of the catechin/proanthocyanidin content.[14,15] Generally, the isolation protocol alternated between column chromatographies over Sephadex LH-20 and Sephadex LH-60 using hydroalcoholic mixtures or eluotropic mixtures of EtOH/MeOH/H_2O, which is a most suitable, gentle technique frequently applied for this type of secondary metabolites.[16,17] The isolation of these compounds is quite problematic and many authors[18,19] proceed to acetylation of the whole extracts and formation of the peracetates prior to chromatographic separation. In our case, the complexity of the mixtures due to the presence of rotamers of proanthocyanidins and cis and trans isomers of acylated flavonoid glycosides was evident not only in the TLC plates (tailing of spots) but also in the HPLC–DAD–MS analyses (double, triple, quadruple peaks; see Supplementary data), but the need to obtain the compounds in their natural form did not permit us for this approach.

Phytochemical investigations of the methanolic extract of the leaves of *Q. ilex* afforded two naturally occurring proanthocyanidin glycosides, namely afzelechin-(4α→8)-catechin-3-*O*-glucoside (**1**)

* Corresponding author. Tel.: +39 055457 3709; fax: +39 055457 3679.
E-mail address: anastasiakarioti@yahoo.it (A. Karioti).

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doi:10.1016/j.tetlet.2009.01.158

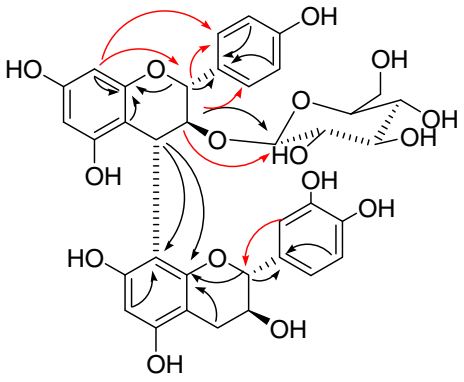

Figure 1. Selected HMBC and ROE correlations for compound **1**.

and afzelechin-(4α→8)-catechin-3-*O*-rhamnoside (**2**), catechin (**3**),[20] two known proanthocyanidins, proanthocyanidin B_3 (**4**)[19] and prodelphinidin C (**5**),[21] as well as small amounts of the oxidation product of catechin, dehydrodicatechin A (**6**).[22] The polar extracts were also abundant in flavonoids, in particular quercetin (**7**), two known flavonol glucosides, quercetin-3-*O*-glucopyranoside (**8**)[23] and isorhamnetin-3-*O*-glucopyranoside (**9**),[24] four flavonol acylated glucosides, namely kaempferol-3-*O*-(6′-galloyl)-glucopyranoside (**10**),[25] quercetin-3-*O*-(6′-galloyl)-glucopyranoside (**11**),[26] tiliroside (**12**),[27] kaempferol-3-*O*-(2″,6″-di-*E*-*p*-coumaroyl)-glucopyranoside (**13**)[28] and the phenolic ellagic acid (**14**). The known compounds **3**–**14** were identified by spectral analysis and direct comparison of their physical properties with those reported previously for these compounds.

Compound **1** was obtained as amorphous yellow solid with $[\alpha]_D^{23}$ −26.9 (*c* 0.16, MeOH). The IR spectrum of **1** contained absorption bands characteristic of hydroxyl (3382 cm^{-1}), aliphatic groups (2920 cm^{-1}) and olephinic bonds (1630 cm^{-1}). The ESI mass spectrum (positive ion mode) of **1** showed molecular/pseudomolecular ion peaks at *m/z* 725.5 $[M+H]^+$, 747.5 $[M+Na]^+$, while in the negative ion mode exhibited a molecular peak at *m/z* 723.5 $[M-H]^-$ consistent with the molecular formula $C_{36}H_{36}O_{16}$. Its HR-ESI-MS spectrum exhibited pseudomolecular peak at *m/z* 747.1911 $[M+Na]^+$ (calcd for $C_{36}H_{36}O_{16}Na$ 747.1890) and at *m/z* 725.2092 $[M+H]^+$, (calcd for $C_{36}H_{37}O_{16}$ 725.2070). It gave characteristic red colour after spraying with vanillin/sulfuric acid and positive blue with ferric ion reagent. Its UV spectrum (HPLC–DAD) presented a band with maximum at 278 nm. All the above data suggested that it belonged to the group of catechins/proanthocyanidins. Accordingly, the ^{1}H NMR spectrum (Table 1) showed signals characteristic of a flavan-3-ol skeleton (aromatic signals in the area of 6.0–7.0 ppm and aliphatic signals with large coupling constants in the region of 2.50–4.50), with catechin stereochemistry. Careful analysis of the ESI-MS spectrum provided us with more information about its structure. Positive ion mode MS spectra exhibited fragments at *m/z* 273.0 and 291.0, characteristic of the presence of afzelechin and catechin units, respectively, while a peak at 563.3 [M-163] suggested the loss of an hexose unit (possibly glucose) and gave evidence of the linkage between afzelechin and catechin. Furthermore, a peak at *m/z* = 435.4 indicated the linkage of the hexose to the afzelechin unit. Further 2D NMR experiments (COSY, HSQC, HMBC and ROESY) gave substantial evidence for these speculations. The ^{1}H NMR and COSY spectra of **1** exhibited signals of a *p*-disubstituted aromatic ring (AA′BB′ system with two doublets centred at δ_H 6.91 and δ_H 6.66, J_{ortho} = 8.4 Hz) which were attributed to the B ring of afzelechin. In the same area of the ^{1}H NMR spectrum, three aromatic protons resonating at δ_H 6.76 (d, *J* = 8.4), 6.69 (d, *J* = 1.8) and 6.45 (dd, *J* = 8.4, 1.8) forming thus an ABX system were observed and were assigned as H-5″, H-2″, H-6″, respectively, of the catechin moiety. The COSY permitted assignments of the aliphatic protons of rings C and F of the two flavan moieties and displayed the following connectivities: H-2″ (δ_H 4.49)/H-3″ (δ_H 3.76)/H-4a″ (δ_H 2.86), H-4b″ (δ_H 2.51) and H-2 (δ_H 4.38)/H-3 (δ_H 4.54)/H-4 (δ_H 4.48). The latter signals belonged to the upper unit of the dimer, as shown by the simplification in the correlation pattern due to the presence of one proton at C-4 instead of two. In the HSQC spectrum, the downfield shift of C-3 at δ 81.5 indicated that this carbon was the glycosylation site. This finding was further supported in both HMBC (crosspeak H-2/C-1 of glucose) and ROESY (H-3/H-1 of glucose) experiments. The HMBC spectrum was crucial for the identification of the afzelechin and catechin units. Common crosspeaks between the *meta* protons H-6 and H-8 (ring A) and the aliphatic protons H-4 & H-2 (ring C) with carbons C-10 and C-9, respectively, proved that these signals belonged to the same flavan nucleus, while crosspeaks between H-2, H-3 (ring C) and H-2′, 6′ (ring B) with C-1′ helped in the complete assignment of afzelechin (Fig. 1). Therefore, afzelechin was the upper part of the proanthocyanidin skeleton, bearing a sugar in position 3 and being linked to catechin at C-4. The interflavanoid bond was further proved in the HMBC spectrum by a crosspeak between H-4 of afzelechin and a quaternary aromatic carbon at δ_C 109.3 (C-8″ or C-6″) belonged to catechin. The interpretation of the HMBC spectrum was very problematic, since the key correlations[19] H-4/C-9″ and H-2″/C-9″ were observed but overlapping of protons H-4 and H-2″ rendered any assignment ambiguous. This problem was solved by re-measuring the spectra in acetone-d6. Indeed, protons H-4 and H-2″ were completely separated (at δ_H 4.39 and 4.53, respectively) and common crosspeaks between H-4, H-2″, H-4″ with C-9″ proved the 4→8 interflavanoid linkage.