Accelerated dereplication of crude extracts using HPLC-PDA-MS-SPE-NMR

Stærk, Dan; Kesting, Julie Regitze; Sairafianpour, Majid; Witt, Matthias; Asili, Javad; Emami, Seyed A.; Jaroszewski, Jerzy W.

Published in:
Phytochemistry

DOI:
10.1016/j.phytochem.2009.05.004

Publication date:
2009

Document version
Publisher’s PDF, also known as Version of record

Citation for published version (APA):
Accelerated dereplication of crude extracts using HPLC–PDA–MS–SPE–NMR: Quinolinone alkaloids of Haplophyllum acutifolium

Dan Staerk a,b,*, Julie R. Kesting a, Majid Sairafianpour a, Matthias Witt c, Javad Asili d, Seyed A. Emami d, Jerzy W. Jaroszewski a

a Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
b Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark
c Bruker Daltonik GmbH, Fahrenheitstrasse 4, D-28359 Bremen, Germany
d Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran

Abstract

Direct hyphenation of analytical-scale high-performance liquid chromatography, photo-diode array detection, mass spectrometry, solid-phase extraction and nuclear magnetic resonance spectroscopy (HPLC–PDA–MS–SPE–NMR) has been used for accelerated dereplication of crude extract of Haplophyllum acutifolium (syn. Haplophyllum perforatum). This technique allowed fast on-line identification of six quinolinone alkaloids, named haplacutine A–F, as well as of acutine, haplamine, eudesmine, and 2-nonylquinolin-4(1H)-one. Acutine and haplacutine E, isolated by preparative-scale HPLC, showed moderate antimalarial activity with IC50 values of 2.17 ± 0.22 μM and 3.79 ± 0.24 μM, respectively (chloroquine-sensitive Plasmodium falciparum 3D7 strain).

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Selecting the strategy for isolation of constituents from plant extracts and other natural sources is of utmost importance with respect to the outcome as well as time and efforts spent on discovery of pharmacologically active natural products. Prior to preparativescale isolation, it is convenient to gain as much knowledge as possible about chemical entities present, both in order to make qualified decisions about worthwhileness of isolation of a particular constituent, and to choose optimal isolation methodology. Although some information about extract constituents can be obtained from thin layer chromatography combined with the use of compound class-specific spray reagents, by gas chromatography combined with mass spectrometry (MS), and by high-performance liquid chromatography (HPLC) combined with photodiode array detection (PDA) or MS, all these techniques suffer from the limited structural information they provide as compared to nuclear magnetic resonance (NMR) spectroscopic techniques.

Within recent years, hyphenation of HPLC, PDA, MS, and solid-phase extraction (SPE), with NMR spectroscopy (HPLC–PDA–MS–SPE–NMR) has emerged as an attractive analytical technique for fast and reliable dereplication of crude plant extracts (Jaroszewski, 2005a,b; Staerk et al., 2006; Sturm and Seger, 2007). This technique has been used for identification of known compounds as well as for rigorous structure elucidation of new natural products directly from crude extracts. Examples include sesquiterpene lactones (Lambert et al., 2007), quinic acid derivatives (Sprogøe et al., 2005a, b), diterpenes and caffeyl glycosides (Clarkson et al., 2006a, 2006b), flavonoid glycosides (Clarkson et al., 2005, Exarchou et al., 2006; Tatsis et al., 2007), cardenolide glycosides (Clarkson et al., 2005), isoflavonoids (Lambert et al., 2005a), and alkaloids (Bieri et al., 2006; Lee et al., 2007; Sturm et al., 2007). One advantage of the HPLC–SPE–NMR technique compared to direct HPLC–NMR methods (Albert, 2002; Jaroszewski, 2005a) is the possibility of linear analyte accumulation by repetitive adsorption on SPE cartridges (Clarkson et al., 2006a; Lambert et al., 2005a, b). Subsequent elution of the analyte with a deuterated organic solvent, resulting in focusing of the analyte in the detection cell, adds further increase in sensitivity of this hyphenated NMR technique (Jaroszewski, 2005b; Staerk et al., 2006). Here, we describe application of HPLC–PDA–MS–SPE–NMR to fast dereplication of an extract of aerial parts of Haplophyllum acutifolium, which showed activity (IC50 < 12 μg/ml) in our in vitro Plasmodium falciparum toxicity screening assay.
from the Mediterranean parts of Europe and Africa to Eastern parts of Siberia. Around 30 of these species are represented in Iran, 14 of which are endemic to the country. *Haplophyllum* is known as a rich source of quinoline alkaloids, and several phytochemical investigations of different species have been reported in recent years (Michael, 2008), including investigations of *H. acutifolium* collected in Turkmenistan (Razzakova et al., 1975, 1986; and references cited therein). Extracts of various *Haplophyllum* species are used in traditional medicine as analgesics, antispasmodics, diuretics, and sedatives, as well as topical agents against skin diseases (Nazrullaev et al., 2001). Quinoline alkaloids isolated from *Haplophyllum* and other Rutaceous species have shown estrogenic (Nazrullaev et al., 2001), antifungal (Cantrell et al., 2005), antialgal (Cheng et al., 2005), antibacterial (Hanawa et al., 2004), and antiparasitic activity (Michael, 2003). In spite of the existence of an abundant literature about quinoline alkaloids of *Haplophyllum*, dereplication of the extract of *H. acutifolium* using HPLC–PDA–MS–SPE–NMR allowed fast identification of several previously unknown minor alkaloids present in the plant.

2. Results and discussion

HPLC–PDA–MS–SPE–NMR experiments with crude ethyl acetate extract of aerial parts of *H. acutifolium* (crude extract A, see Section 4) were performed using UV trace at 235 nm for threshold-based adsorption of constituents on SPE cartridges containing poly(divinylbenzene)-based resin (Clarkson et al., 2007). Four or six repetitive trappings of material eluted in peaks 1–9 (Fig. 1) were performed. The trapped compounds were subsequently eluted with CD3CN for acquisition of NMR data. This led to identification of five new and two already known 4-quinolinone alkaloids (1, 2a, 3, 4, 6, 8, and 9) along with a tricyclic hemiterpenoid alkaloid haplamine (5) and the lignan 7 as detailed below; the identified structures (Fig. 2) were numbered according to elution order of the HPLC peaks (Fig. 1). Parallel HPLC–PDA–MS–SPE–NMR experiments with an alkaloid-enriched fraction (fraction II) from crude extract B, see Section 4) showed the existence of one additional, new quinoline alkaloid (2b) as a minor constituent.

The constituent eluted as peak 1 (compound 1) was assigned the molecular formula C18H23NO3 as determined by off-line HRE-SIFTMS. The 1H NMR spectrum acquired in the HPLC–SPE–NMR mode showed the presence of a 1,2-disubstituted benzene with a characteristic downfield shift of H-5 (δ 8.11, J3,6 = 8.0 Hz and J5,7 = 1.4 Hz) attributable to a peri-oriented carbonyl group, a one-proton singlet of H-3 (δ 5.98), and a broad singlet of H-1 (δ 9.55). This information, together with 1H–1H correlations for H-4 and H-6, H-7, and H-8 found in a COSY spectrum and 13C NMR resonances identified in a HSQC spectrum, both acquired in the HPLC–SPE–NMR mode, proved the presence of a 2-substituted 4-quinolinone moiety. In the COSY spectrum, a spin system corresponding to a (3Z,5E)-7-hydroxyocta-3,5-dien-1-yl moiety was observed. The configuration of the two conjugated double bonds was determined by analysis of the coupling patterns of H-3' and H-4' (H-3': δ 5.44, dt, J4,5 = 10.4 Hz, J2,3 = 7.7 Hz; H-4': δ 6.03 br t, J3,4 ≈ J4,5 = 11.1 Hz) and of H-5' and H-6' (H-5': δ 6.45, ddt, J5,6 = 15.2 Hz, J4,5 = 11.2 Hz, J5,7 = J5,8 = 1.2 Hz; H-6': δ 5.62, dd, J5,6 = 15.2 Hz, J6,7 = 6.3 Hz) as Z and E, respectively. The hydroxyl- C-7 was established by HSQC correlation of the 13C resonance at δ 73.7 with H-7 (δ 3.90), identified by its COSY correlations. Preparative-scale isolation afforded 4 mg of 1, which showed no optical rotation. Since chiral allylic alcohols and 2,4-diene-1-ols display appreciable specific rotation at the sodium D-line (Airs et al., 1942; Burgess and Jennings, 1991; Takaiishi et al., 1991; Tsai et al., 2001), it is concluded that 1 isolated from *H. acutifolium* is a racemate. Compound 1 is a new alkaloid for which the name haplacutine A is suggested.

The material eluted with peak 2 (compound 2a) was assigned the molecular formula C18H23NO3 as determined by off-line HRE-SIFTMS. Based on the 1H NMR and COSY spectra acquired in the HPLC–SPE–NMR mode, it contained a 4-quinolinone moiety and a spin system corresponding to a (3Z)-7-hydroxyocta-3,5-dien-1-yl moiety. The configuration of the C-4′–C-5′ double bond was established as E (H-4′: δ 5.70, br dd, J4,5 = 15.2 Hz, J4,6 = 6.4 Hz; H-5′: δ 6.53, ddt, J5,6 = 15.2 Hz, J5,7 = 11.1 Hz, J5,8 = J5,9 = 1.2 Hz) and that of the C-6′–C-7′ double bond as Z (H-6′: δ 5.95, m; H-7′: δ 5.44, dt, J6,7 = 10.8 Hz, J7,8 = 7.5 Hz). When the HPLC–PDA–MS–SPE–NMR analysis was performed with the corresponding peak of an alkaloid-enriched fraction of the extract (fraction III obtained from crude extract B, see Section 4), 2a was accompanied by a closely related analogue 2b (approximately 60% relative to the major constituent 2a as determined from the integrals of the 1H NMR resonances, see Supplementary Fig. 3). The configuration of both double bonds in the minor constituent was E (H-4′: δ 5.61, dd,
the coupling patterns observed for H-4. Any information about the configuration of the double bond, but Razzakova et al., 1975). Acutine was initially formulated as 2-
lium with those reported for acutine, previously isolated from
H-2' and H-5' (δ 3.10 and 7.57, respectively) confirmed that the two moieties are connected by the C-3' carbonyl group. Analysis of the coupling pattern of H-4' to H-7 (see Section 4.9) established the E- and Z-configuration of the C-4--C-5' and C-6--C-7' double bonds, respectively. Compound 3 is a new alkaloid for which the name haplacutine D is suggested.

The constituent eluted as peak 4 (compound 4) was assigned the molecular formula C16H19NO (HRESIFTMS), and the 1H NMR spectrum showed signals corresponding to a 4-quinolinone moiety and a hept-4-en-1-yl moiety. The 1H NMR data were in agreement with those reported for acutine, (syn. 6-methoxyflindersine) (Campbell et al., 1990; Cantrell et al., 2005) and 7-methoxyflindersine (Bradner et al., 1996). However, correlations found in a NOESY spectrum acquired in the HPLC–SPE–NMR mode (see Supplementary Fig. 1) proved methylation at C-6.

The constituent eluted as peak 6 (compound 6) was assigned the molecular formula C18H21NO2 (off-line HRESIFTMS). This is in agreement with the presence of a 4-quinolinone moiety and a nona-3,6-dien-1-yl moiety, as confirmed by connectivities observed in a COSY spectrum. The Z-configuration of the C-6'--C-7' double bond was identified based on 1H NMR coupling patterns (H-6': δ 5.20, dtt, J6'.7' = 10.7 Hz, J6'.8' = 7.3 Hz, J7'.8' = 1.6 Hz; H-7': δ 5.31, dtt, J7'.8' = 10.7 Hz, J7'.7' = 7.2 Hz, J7'.8' = 1.6 Hz), whereas the configuration of the C-3'--C-4' double bond was not immediately clear because H-3' and H-4' appeared as a multiplet (δ 5.42). However, the simulated coupling pattern of H-3' and H-4' using J3'.4' coupling constant corresponding to the Z-configuration was practically identical to the experimentally observed coupling pattern (simulation parameters: δH3'.7' = 5.43, δH3'.8' = 5.41, J3'.4' = 10.7 Hz, J4'.5' = 7.1 Hz, J5'.6' = 6.8 Hz, J4'.5' = J6'.7' = 1.1 Hz), whereas simulated spectrum using an E coupling constant was distinctly different (data not shown; cf. however structure of 8 and Fig. 4). In addition, the observed chemical shifts of C-2' and C-5' (δ 27.2 and δ 26.0, respectively, obtained from a HSQC spectrum), were in agreement with the Z-configuration of the C-3'--C-4' double bond, whereas chemical shift values above δ 30 would be expected for the E-configuration, as shown by chemical shift predictions and literature data (Ivaniciuc et al., 1997). Thus, compound 6 is formulated as 2-[(3Z,6Z)-nona-3,6-dien-1-yl]quinolin-4(1H)-one. Full assignment of all 1H and 13C NMR resonances was possible based on 1H, COSY, HSQC, and HMBC experiments obtained in the HPLC–SPE–NMR mode. In order to illustrate the use of HPLC–SPE–NMR methodology, the HSQC and HMBC spectra used for assignment of 13C resonances of 6 are shown in Fig. 3. A compound with a similar structure has previously been isolated from Vepris ampody (Kan-Fan et al., 1970). However, the authors were only able to identify the compound as either 2-[(nona-3,6-dien-1-yl)quinolin-4(1H)-one or 2-[(nona-2,6-dien-1-yl)quinolin-4(1H)-one, and without any information about configuration of the double bonds. Later, Razzakova et al. (1986) isolated a constituent named 2-nonadie-
nyl-4-quinoline, but without any information about the position and configuration of the two double bonds. Compound 6 is therefore considered a new compound, for which the name haplacutine is proposed.

The constituent eluted as peak 7 was identified as (±)-eudesmin (7) on the basis of 1H NMR spectroscopic data recorded in HPLC–SPE–NMR mode (see Supplementary Fig. 2), as well as the identity of 1H and 13C NMR data and optical rotation data (see Supplementary material) of isolated material with those previously reported (Iida et al., 1982; Miyazawa et al., 1995; Roy et al., 2002). Eudesmin has previously been isolated from Haplophyllum species (Razakova et al., 1972).

The constituent eluted as peak 8 (compound 8) was assigned the molecular formula C19H21NO (HRESIFTMS), and the presence of a 4-quinolinone moiety and a non-3-en-1-yl side chain was revealed by comparing the experimental coupling pattern of the double bond hydrogens with simulated spectra (Fig. 4). This, and the chemical shift values of C-2' and C-5' (δ 27.4 and 27.7, respectively), established the structure of 8 as 2-[(3Z)-non-3-en-1-yl]quinolin-4(1H)-one, for which the name haplacutine F is proposed.

2-Nonylquinolin-4(1H)-one (9) was identified as the compound eluted as peak 9 based on HRESIFTMS data and comparison of 1H NMR spectra with those reported in literature (Kostova et al., 1999).

NMR and HRMS data for new compounds are reported in Section 4. The finding of six new 2-alkylquinolinones in extract of aerial parts of H. acutifolium shows that reinvestigation of plants with new and more sensitive technology can add considerable new phytochemical information to already well-investigated species. In continuation of the HPLC–PDA–MS–SPE–NMR investigation, two of the alkaloids were isolated and were shown to inhibit growth of malaria parasites in the low micromolar range. On the basis of this finding, the observed antiplasmodial activity of crude extract of H. acutifolium is concluded to represent combined effect of quinoline alkaloids present in this plant.

4. Experimental

4.1. General

Optical rotations were measured using a Perkin–Elmer 241 polarimeter. NMR spectra of isolated compounds were recorded on a Bruker Avance 400 spectrometer (proton frequency 400.13 MHz) at 25 °C, using TMS as internal standard. HRMS measurements were performed using a Bruker APEX Qe Fourier-transform mass spectrometer equipped with a 9.4 T superconducting cryomagnet and an external ESI source (Apollo II source). The spectra were externally calibrated with arginine cluster in positive and negative ion mode. The samples were dissolved in MeOH, further diluted in 50% MeOH with 0.2% HCOOH, and introduced into the ion source using a syringe pump with a flow of 2 µl/min. Preparative-scale HPLC separations were performed with a 250 × 16 mm Lichrosorb RP18 (5 µm) column at a flow rate of 8 ml/min, using a chromatograph consisting of a Gynkotek P 580 pump, a Rheodyne 7725 injector, and a Shimadzu SPD–10AV spectrophotometric detector operating at 254 nm, or with a 250 × 21.2 mm Luna 2 C18 (5 µm) column at a flow rate of 10 ml/min, using a Waters 590 pump, a Rheodyne 7125 injector, and a Lambda-Max Model 481 LC UV detector operating at 235 nm. Spin coupling pattern simulations were performed with gNMR ver. 4.12 software (Adept Scientific, Herts, UK). Chemical shift predictions were performed with ChemNMR software as implemented in ChemBioDraw Ultra ver. 11.0 (CambridgeSoft, Cambridge, UK).

4.2. Plant material

Aeriel parts of H. acutifolium (DC.) G. Don [syn. Haplophyllum perforatum (M. Bieb.) Kar. & Kär., H. flexuosum Boiss., H. sieversii
Fisch., *Ruta perforata* M. Bieb. (Rutaceae) were collected on August 20, 2004, at a location 40 km west of Mashhad, Iran (altitude 1650 m) and identified by Mr. Ali Ahl. The plant material was dried immediately after collection and powdered before extraction. Voucher specimen (DHJ556) was deposited in Herbarium C (Botanical Museum, University of Copenhagen, Copenhagen, Denmark).

### 4.3. Extraction and sample preparation

Extract A was prepared by macerating 117 g of the plant material with 3 × 0.5 l of EtOAc, and the combined extracts were evaporated to dryness under reduced pressure to give 2.27 g of residue. A small amount (7.5 mg) of extract A was used for HPLC–PDA–MS–SPE–NMR analysis by making solutions of 28 mg/ml in H2O–MeCN 2:7, and the rest was used for prep. HPLC. Extract B was prepared by extraction of 500 g of powdered material with 4 × 1 l of EtOAc and evaporated.

### 4.4. HPLC–PDA–MS–SPE–NMR experiments

The HPLC–PDA–MS–SPE–NMR instrument consisted of an Agilent 1100 HPLC system (degasser, quaternary solvent delivery pump, autosampler, PDA detector), a Knauer K100 Wellchrom pump for post-column H2O delivery, a Spark Propekt 2 solid-phase extraction device, a Bruker EsquireLc mass spectrometer equipped with electrospray ionization (ESI) interface, and a Bruker Avance 600 NMR spectrometer equipped with a 30-μl inverse \(^{1}H\) \(^{1}C\) flow-probe operating at 25 °C. HPLC separations were performed at 40 °C on a 150 × 4.6 mm i.d., 3 μm particle size, Phenomenex Luna 2 C18 column, using mixtures of H2O–MeCN 95:5 + 0.1% HCOOH (eluent A) and H2O–MeCN 5:95 + 0.1% HCOOH (eluent B). The eluate from the column was split with an A W splinter, directing 5% of the flow to the mass spectrometer and 95% to the PDA detector. MS spectra were acquired in positive-ion mode, using drying temperature of 350 °C, nebulizer pressure of 15 psi, and drying gas flow of 15 ml/min. The HPLC solvent flow (0.8 ml/min) from the PDA detector was diluted with H2O (2 ml/min) and directed to the SPE unit. GP phase (general-purpose poly(divinylbenzene)-based resin) SPE cartridges, 10 × 2 mm i.d. from Spark Holland, were used for trapping of selected compounds based on UV absorption levels at 235 nm. Four cumulative trapplings of analyses from separation of 1.4 mg of the raw extract per injection were performed using the following linear gradient elution profile: 0 min, 10% B; 30 min, 32.2% B; 60 min, 100% B; 80 min, 100% B; and 8 min conditioning with 10% B. Two extended separations, allowing separation of up to 2.8 mg extract per injection, were performed with four or six adsorptions using the following linear gradient elution profile: 0 min, 10% B; 30 min, 32.2% B; 60 min, 100% B; 70 min, 100% B; 72 min, 10% B, and 8 min conditioning with 10% B. Prior to use, the SPE cartridges were conditioned with 500 μl of MeCN at 6 ml/min and flushed with 500 μl H2O at 1 ml/min. After trapping, the cartridges were dried with a stream of dry N2 gas for 45 min and the analytes eluted to the NMR flow cell with CD3CN. The HPLC–PDA–MS–SPE–NMR experiments were controlled with Bruker HyStar ver. 2.3 software. NMR data acquisition and processing were performed using Bruker XWINNMR ver. 3.1 software. After acquisition of NMR data, the samples were removed from the probe and subjected to HRESIFTMS analysis.

### 4.5. Preparative-scale isolation

Crude extract B was dissolved in 0.5 l of CHCl3 and the solution extracted with H2O acidified to pH 2 with 1 M H2SO4 (7 × 0.5 l) to give fractions I (21 g) and II, respectively. Fraction II was alkalized to pH 11 with concentrated aq. NH3 and extracted with CHCl3, which after concentration yielded 250 mg of fraction III. Fraction I was separated by isocratic prep. HPLC (Lichrosorb RP18, MeCN–H2O 6:4 + 0.1% TFA), resulting in fractions la (20.2 mg), lb (32.7 mg), and lc (108.6 mg). These fractions were re-chromatographed on HPLC (Lichrosorb RP18, MeCN–H2O 6:4 + 0.1% TFA) and 18 mg of Tc (MeCN–H2O 6:4 + 0.1% TFA), Fraction III was separated by isocratic prep. HPLC (Lichrosorb RP18, MeCN–H2O 6:4 + 0.1% TFA), resulting in fractions IIIa–IIIc. Fractions IIIa and IIIc were re-chromatographed using MeCN–H2O 1:1 + 0.1% TFA to yield 5 mg of 4 and 10 mg of 6, respectively. Extract A was subjected to targeted isolation of 1 by repeated prep. HPLC (Luna 2 C18, MeCN–H2O 22:78) to yield 4 mg of 1.

### 4.6. Compound 1

Haplaclin A [2-[(3Z,5E)-7-hydroxyxona-3,5-dien-1-yl][quinolin-1(2H)-one]; colourless gum; [α]D

### 4.7. Compound 2a

Haplaclin B [2-[(4E,6Z)-3-hydroxyxona-4,6-dien-1-yl][quinolin-4(1H)-one]; 1H NMR spectral data (600 MHz, CD3CN, hyphenation mode): δ 0.98 (3H, t, J=7.5, H-9), 1.87 (2H, m, H-2), 2.09 (2H, m, H-8), 2.66 (2H, m, H-1), 3.11 (1H, d, br, J=3.0, 4.1), 4.18 (1H, s, br, H-3), 5.44 (1H, d, J=10.8, J=7.5, H-7), 5.70 (1H, d, br, J=15.2, J=6.4, H-4), 5.95 (1H, m, H-6), 5.98 (1H, s, br, H-3), 6.53 (1H, d, J=15.2, J=11.1, J=7.5, J=7.5, 1.2), 7.28 (1H, d, J=15.2, J=6.3, J=8.0, J=7.5, 1.4, H-5), 8.11 (1H, d, J=15.2, J=1.4, H-5), 1C NMR spectral data (150 MHz, CD3CN, from HSQC spectrum recorded in hyphenation mode): δ 10.1 (C-9), 27.4 (C-2), 30.8 (C-8), 34.1 (C-1), 73.7 (C-7), 109.2 (C-3), 118.3 (C-8), 123.9 (C-6), 125.0 (C-5), 126.0 (C-5), 130.4 (C-4), 132.5 (C-7), 138.7 (C-6); HRESIFTMS m/z 284.16467 [M+H]⁺ (calc. for [C18H22NO2]⁺, 284.16451).

### 4.8. Compound 2b

Haplaclin C [2-[(4E,6E)-3-hydroxyxona-4,6-dien-1-yl][quinolin-4(1H)-one]; 1H NMR spectral data (600 MHz, CD3CN, hyphenation mode): δ 0.99 (3H, t, J=7.5, H-9), 1.87 (2H, m, H-2), 2.19 (2H, pd, J=4.1, J=7.5, J=8.0, 1.5, H-3), 2.70 (2H, m, H-1), 3.06 (1H, d, br, J=3.0, 4.1), 4.13 (1H, s, br, H-3), 5.61 (1H, dd, J=15.3, J=6.5, H-4), 5.75 (1H, dt, J=15.3, J=6.4, H-5), 5.98 (2H, s, H-2), 5.98 (1H, m, H-3), 6.05 (1H, dd, J=15.3, J=6.4, H-3).
**Appendix A: Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.05.004.

**References**


