Targeted Natural Product Isolation Guided by HPLC–SPE–NMR: Constituents of *Hubertia* Species

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The hyphenated technique, high-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance spectroscopy (HPLC–SPE–NMR), has been applied for rapid identification of novel natural products in crude extracts of *Hubertia ambavilla* and *Hubertia tomentosa*. The technique allowed full or partial identification of all major extract constituents and demonstrated the presence of unusual quinic acid derivatives containing the (1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetyl residue that exhibit strongly coupled ABXY patterns, the parameters of which were obtained by spin simulations. Using homo- and heteronuclear 2D NMR data acquired in the HPLC–SPE–NMR mode, complete structure determination of three new natural products, i.e., 3,5-di-*O*-caffeoyl-4-*O*-[(1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetyl]quinic acid (**1**), its 2-hydroxy derivative (**2**), and 3,5-di-*O*-caffeoyl-4-*O*-[(4-hydroxyphenyl)acetyl]quinic acid (**3**), was performed. Finally, targeted isolation of **1** was achieved by SPE fractionation and preparative HPLC, followed by evaluation of its antioxidant and antimicrobial activity. In contrast to chlorogenic acid and 3,5-di-*O*-caffeoylquinic acid, which act as antioxidants, compound **1** proved at the same conditions to possess prooxidant activity in an assay evaluating the oxidation of human low-density lipoprotein induced by Cu^{2+}.

One of the bottlenecks in traditional natural-products chemistry is the purification and structure elucidation of constituents from pharmacologically active or traditionally used extracts. In natural-product-based drug-discovery programs, accelerated generation of hits and leads might be achieved using a technology capable of providing comprehensive structural information about extract constituents without actually isolating them. The identified structures could then be subjected, for instance, to chemical-diversity assessment or virtual screening. This could constitute the basis for making qualified decisions regarding targeted isolation of selected constituents for pharmacological evaluation. Waste of time and efforts on the re-isolation of known or otherwise unwanted compounds from pharmacologically active extracts could thus be avoided.

An alternative to the traditional, bioactivity-guided fractionation approach to natural-product-based drug discovery is the construction of natural-product libraries.[1] In this approach, natural products are isolated irrespective of the presence of known pharmacological activity of the extract. Here, the purpose is to build up a collection of pure compounds having novel and diverse chemical structures. The library can subsequently be screened using a broad range of pharmacological assays. This approach alleviates the problem of incompatibility of crude natural-products extracts with industrial high-throughput screens,[2] as well as the problems with following a spurious biological activity of an extract, e.g., an activity because of nonspecific, additive, or synergistic effects. Efforts toward construction of natural-product libraries would be greatly simplified if knowledge about chemical structures present in an extract could be acquired at a very early stage.

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Although high-performance liquid chromatography–mass spectrometry (HPLC–MS) is broadly used to gain an early insight into extract composition,[3,4] the only technology capable of providing truly comprehensive information about structures of previously unknown natural products is nuclear magnetic resonance (NMR) spectroscopy. This has fueled efforts to hyphenate NMR with chromatographic separations, resulting in the development of direct HPLC–NMR techniques.[5,6] Subsequently, coupling of HPLC–NMR with automated postcolumn solid-phase extraction (SPE)[7,8] allowed for an increase of the amount of material available for NMR analysis to tens of micrograms, compensating for the intrinsically lower sensitivity of NMR spectroscopy as compared to other HPLC detection techniques. Further and substantial increases in the quality of NMR data resulted from a solvent change from a nondeuterated HPLC solvent to a deuterated NMR solvent. As a result, HPLC–SPE–NMR has been shown to be capable of providing rigorously determined structures of new natural products directly from crude or slightly prepurified extracts.[9–13]

In this study, the concept of targeted isolation of new natural products, guided by HPLC–SPE–NMR, is illustrated by the isolation of a novel quinic acid derivative from *Hubertia ambavilla* Bory and *Hubertia tomentosa* Bory (Asteraceae) and subsequent evaluation of its bioactivity. The two *Hubertia* species are endemic to Réunion Island in the Indian Ocean. *H. ambavilla* has been used by the indigenous people for treating various conditions, including renal infections, eczema, and asthma.[14–16] The prior knowledge of the phytochemistry of these *Hubertia* species comprises thin-layer chromatography (TLC) investigations, suggesting the presence of flavonoids and tannins.[17,18]

Results and Discussion

Reversed-phase HPLC analysis of ethanolic extracts of the leaves of the two species showed the presence of numerous constituents

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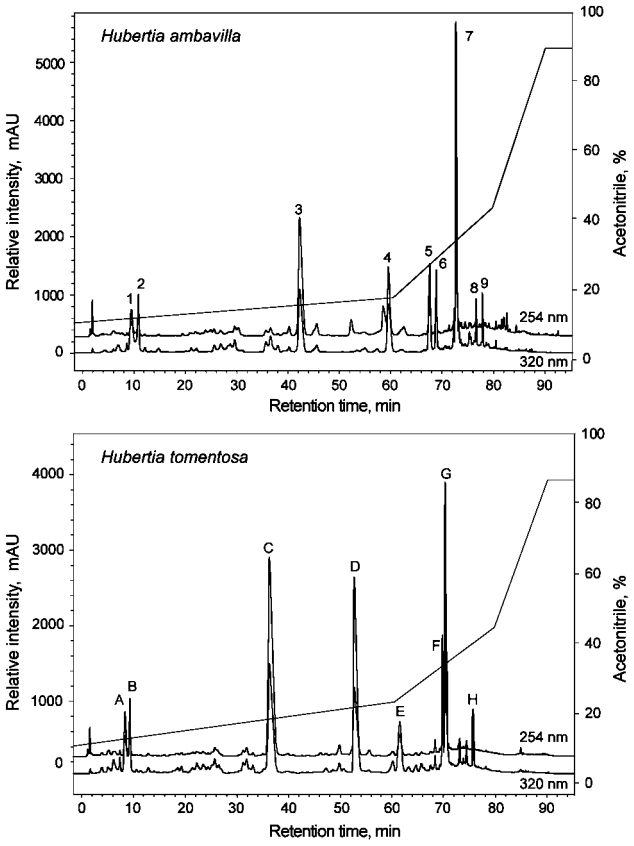


Figure 1. HPLC traces (254 and 320 nm) for ethanolic extracts of *H. ambavilla* (top) and *H. tomentosa* (bottom) [150 × 4.6 mm i.d., Phenomenex Luna C_{18}(2) column, 3 μm, eluted at 0.8 mL/min with acetonitrile gradient in water (0.1% TFA) as shown]. Peaks selected for the HPLC–SPE–NMR analysis are labeled with numbers or letters.

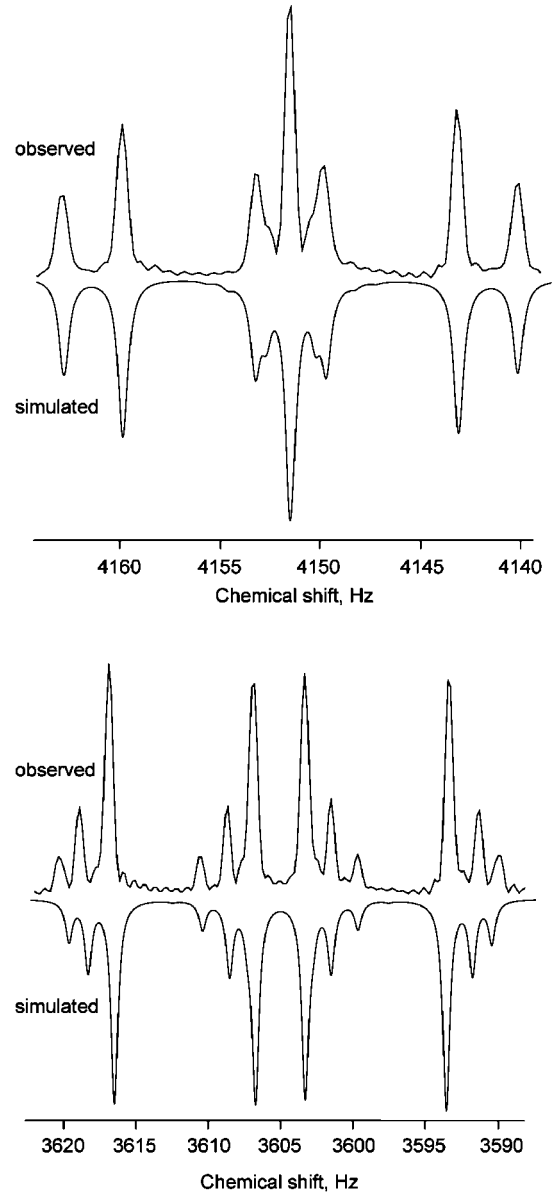


Figure 2. Observed and simulated ^{1}H NMR spectra of the 2,5-cyclohexadienone moiety of **1** (600 MHz spectra recorded in the HPLC–SPE–NMR mode with the crude extract of *H. ambavilla* and acetonitrile-d_3). The observed spectra were resolution-enhanced by Lorentz–Gauss transformation. The simulation parameters are reported in Table 1.

(Figure 1). All major HPLC peaks, nine for *H. ambavilla* and eight for *H. tomentosa*, were selected for HPLC–SPE–NMR investigations. The initial analysis included the recording of 1D ^{1}H NMR spectra (600 MHz, acetonitrile-d_3) following five cumulative SPE trappings of each peak using poly(divinylbenzene) stationary phase (GP resin). The majority of peaks corresponded to common plant constituents, such as chlorogenic acid and related mono- and dicaffeoyl derivatives of quinic acid (peaks 1, 2, 4, 6, A, B, and D) and flavonol glycosides (peaks 3 and C). The occurrence of chlorogenic acid and congeners was apparent from the presence of ABX patterns displaying characteristic couplings of a quinic acid moiety and AB doublets of trans double bonds, along with ABX patterns of 1,2,4-trisubstituted benzene moieties of caffeoyl groups.[19–21] The spectra recorded with peaks 3 and C displayed a characteristic spin pattern of 3,3′,4′,5,7-pentahydroxyflavone. In addition, the compound eluted in peak 3 displayed a single anomeric doublet (δ 5.01, d, $J_{ax,ax}$ = 7.9 Hz) and was identified as quercetin 3-*O*-β-D-galactopyranoside (hyperin), whereas the compound eluted as peak C exhibited characteristic resonances of a rutinose residue (δ 5.07, d, $J_{ax,ax}$ = 7.5 Hz; δ 4.53, d, $J_{ax,eq}$ = 1.5 Hz; δ 1.09, d, J_{vic} = 6.2 Hz) and was identified as rutin. Because flavonoids, as well as chlorogenic acid and its analogues and derivatives, are ubiquitous in plants, no further work was considered worthwhile with these constituents. No useful spectra were obtained with peaks 8, 9, and F.

The ^{1}H NMR spectrum obtained from the main peak 7 (t_R = 72.8 min) of the extract of *H. ambavilla* was identical to that obtained from peak G of the extract of *H. tomentosa* (t_R = 70.4 min, eluted using a slightly different solvent-gradient profile). The spectrum exhibited the presence of two caffeoyl residues as well as resonances attributable to the quinic acid moiety. However, in addition to these signals, the spectrum showed a higher order four-spin coupling pattern, demonstrating that the compound is not a common quinic acid derivative. Spin simulations of this ABXY spectrum allowed for the determination of individual chemical-shift values and coupling constants, which were compatible with a cyclohexa-2,5-dien-1-one system (Figure 2). Subsequently, the HPLC–SPE–NMR experiment was repeated using eight SPE trappings of peak 7 of the extract of *H. ambavilla* to record a series of 2D spectra [correlation spectroscopy (COSY), heteronuclear multiple-bond correlation (HMBC), and heteronuclear single-quantum coherence (HSQC)]. The spectra allowed for assignment of all individual ^{1}H resonances (Table 1) and identification of the

Table 1. ^{1}H NMR (600 MHz) Spectroscopic Data for **1–3** Obtained in the HPLC–SPE–NMR Mode from Crude Extracts of *Hubertia* Species

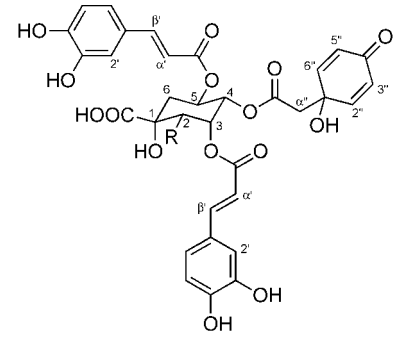
	chemical shift (δ)[a]		
position	**1**	**2**	**3**
H-2_{ax}	2.38, dd (14.8, 3.6)	4.32, d (3.5)	2.36, dd (14.2, 4.4)
H-2_{eq}	2.13, ddd (14.8, 5.5, 1.9)		
H-3	5.54, td (5.5, 3.6)	5.69, t (3.5)	5.55, td (4.3, 3.7)
H-4	5.24, dd (8.8, 3.6)	5.25, dd (10.4, 3.5)	5.20, dd (8.8, 3.7)
H-5	5.50, td (8.9, 4.4)	5.52, ddd (11.5, 10.4, 4.7)	5.50, td (8.8, 4.6)
H-6_{ax}	2.20, dd (13.7, 9.0)	2.05, dd (13.7, 11.5)	
H-6_{eq}	2.25, ddd (13.7, 4.4, 1.9)	2.34, dd (13.7, 4.8)	
H-2′	7.14 and 7.16, d (2.1)[c]	7.10, 7.17, d (2.1)[c]	7.12 and 7.15, d (1.8)[c]
H-5′	6.87 and 6.88, d (8.2)[c]	6.85 and 6.89, d (8.2)[c]	6.87 and 6.88, d (8.1)[c]
H-6′	7.05 and 7.07, dd (8.2, 2.1)[c]	7.02 and 7.08, dd (8.2, 2.1)[c]	7.03 and 7.05, dd (8.1, 1.8)[c]
H-α′	6.24 and 6.34, d (15.9)[c]	6.28 and 6.39, d (15.9)[c]	6.18 and 6.28, d (15.9)[c]
H-β′	7.56 and 7.61, d (15.9)[c]	7.54 and 7.67, d (15.9)[c]	7.52 and 7.59, d (15.9)[c]
H-α″	2.66 (A) and 2.69 (B), each d (14.7)	2.60 (A) and 2.63 (B), each d (15.1)	3.46 (A) and 3.50 (B), each d (15.4)
H-2″	6.95, m[d]	6.87, m[d]	7.03
H-3″	6.04, m[d]	6.00, m[d]	6.63
H-5″	6.02, m[d]	5.98, m[d]	6.63
H-6″	6.93, m[d]	6.89, m[d]	7.03

[a] In CD_3CN, chemical-shift values are standardized to the residual CD_2HCN signal set to δ 1.94. Multiplicity of signals is given as follows: d, doublet; t, triplet; m, multiplet. Coupling constants are given in parentheses as apparent splittings in Hz, unless otherwise stated. [b] Signals obscured by solvent peak suppression. [c] No individual assignment of resonances of the two caffeoyl groups was performed. [d] Coupling constants derived from spin simulations: $J_{2'',3''} = J_{5'',6''} = 10.3$ Hz, $J_{2'',6''} = 3.0$ Hz, $J_{3'',5''} = 1.9$ Hz, and $J_{2'',5''} = J_{3'',6''} = -0.2$ Hz (see Figures 2 and 3).

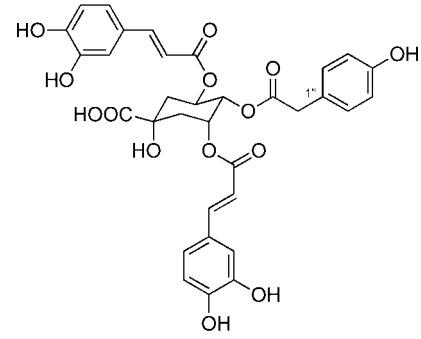
compound as **1**. In particular, the HMBC correlation between the axial hydrogen H-4 (δ 5.24, dd, $J_{ax,ax} = 8.8$ Hz, $J_{ax,eq} = 3.6$ Hz) and the carbonyl group of the (1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetyl moiety (δ 168.1) was used to establish the attachment point of the latter. The resonance of this carbonyl group could be distinguished from those of the caffeoyl carbonyl groups (δ 169.6 and 168.6) by its correlation to the methylene group (H-α″). A full HMBC connectivity network observed within the (1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetyl moiety confirmed its structure. The two caffeoyl residues present must thus be attached via the two remaining secondary hydroxy groups of the quinic acid moiety, as shown by high chemical shifts of H-3 (δ 5.54, td, $J_{eq,ax} = 5.5$ Hz, $J_{eq,eq} = 3.6$ Hz) and H-5 (δ 5.50, td, $J_{ax,ax} = 8.9$ Hz, $J_{ax,eq} = 4.4$ Hz) because of acylation-induced shifts.[19,21] The molecular formula of **1** was confirmed by negative-ion mode electrospray ionization (ESI) MS (*m/z* 665, $[M - H]^-$).

The ^{1}H NMR spectra obtained with peak 5 ($t_R = 66.6$ min) and peak E ($t_R = 61.8$ min) were identical to each other and similar to that of compound **1**, except that one of the methylene groups of the quinic acid moiety was replaced by a methine resonance (δ 4.32, d, $J_{ax,eq} = 3.5$ Hz). The presence of an equatorial hydroxy group at C-2 was confirmed by the multiplicity of H-3 (δ 5.69, t, $J = 3.5$ Hz), which couples with identical coupling constants to H-2 and H-4 and also by the disappearance of the long-range coupling between H-2_{eq} and H-6_{eq} observed in **1** ($J = 1.9$ Hz). The remaining ^{1}H NMR data obtained in the HPLC–SPE–NMR mode (Table 1) confirmed the compound to have the structure **2**, in agreement with ESI MS (*m/z* 681, $[M - H]^-$). The observed and simulated ABXY spin pattern of **2** is shown in Figure 3.

Peak H of the extract of *H. tomentosa* ($t_R = 75.9$ min) contained compound **3** (*m/z* 649, $[M - H]^-$) in which the (1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetyl moiety was converted to a (4-hydroxyphenyl)acetyl moiety, formally by reduction of the carbonyl group and elimination of water. This was shown by the replacement of the ABXY spin system observed in **1** and **2** with an AA′XX′ pattern characteristic for 1,4-disubstituted benzenes (δ 6.63 and 7.03), with only minor changes of the ^{1}H NMR parameters of the quinic acid core of the molecule. This compound was not detected in the extract of *H. ambavilla*.



1 R = H
2 R = OH



3

All three compounds (**1–3**) are new natural products. After their identification in crude extracts by HPLC–SPE–NMR, it was decided to isolate the major component **1**. The crude extracts of *H. ambavilla* and *H. tomentosa* were initially defatted by liquid–liquid partitioning between MeOH/H_2O (1:9) and petroleum ether. To diminish the amount of material to be injected to the preparative

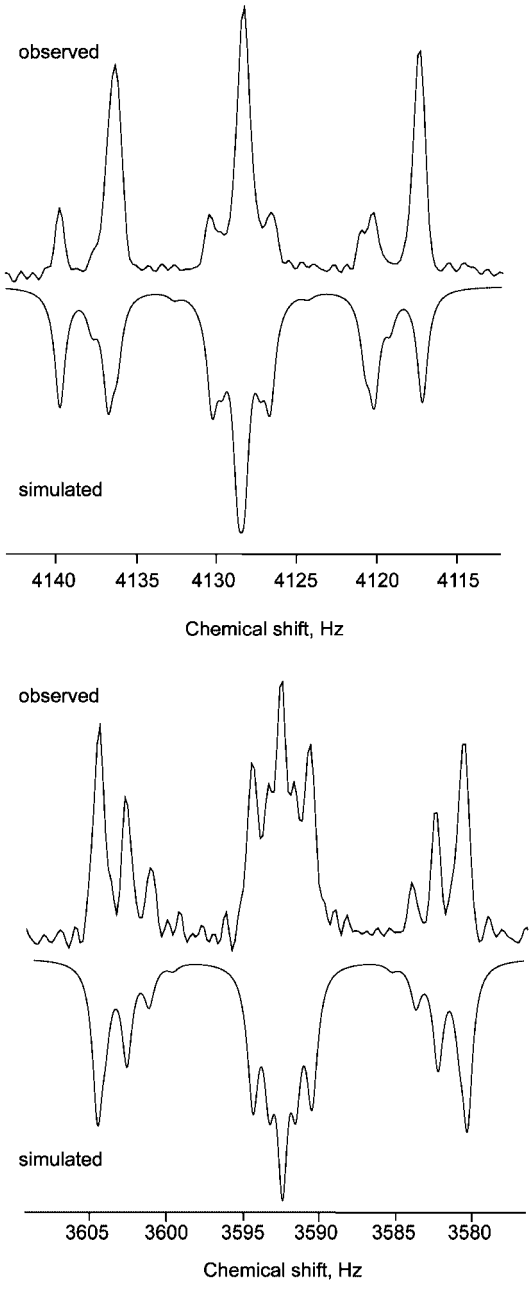


Figure 3. Observed and simulated ^{1}H NMR spectra of the 2,5-cyclohexadienone moiety of **2** (600 MHz spectra recorded in the HPLC–SPE–NMR mode with the crude extract of *H. ambavilla* and acetonitrile-d_3). The observed spectra were resolution-enhanced by Lorentz–Gauss transformation. The simulation parameters are reported in Table 1.

HPLC column, reversed-phase SPE fractionation of the crude extracts was performed using C_{18} SPE cartridges. The cartridges were eluted with a step gradient of acetonitrile in water. On the basis of analytical-scale HPLC monitoring, fractions containing constituents in the polarity region corresponding to peaks 5–9 of *H. ambavilla* and peaks E–J of *H. tomentosa* were obtained. The preparative SPE fractionation thus enabled a reduction of the extract amount by roughly 95%, essentially without the loss of **1**. The fractions enriched in **1** were finally purified in a two-step preparative HPLC procedure using C_{18} columns. The first step was an adaptation of the analytical separation (Figure 1) to preparative scale conditions. In the second step, a ternary solvent containing water, acetonitrile, and tetrahydrofuran (THF) was employed. However, while the HPLC separations required the presence of trifluoroacetic acid (TFA) in the mobile phase for satisfactory performance,

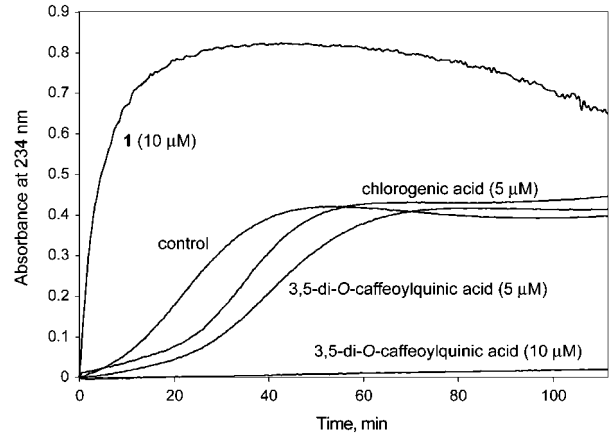


Figure 4. Effect of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and **1** on the Cu^{2+}-catalyzed oxidation of human LDL, expressed as different rates of formation of conjugated dienes.

evaporation of the acidic HPLC fractions resulted in the degradation of **1**. Therefore, instead of the evaporation, the HPLC eluate was diluted with water and passed through C_{18} SPE cartridges. The cartridges were washed with water to remove all TFA present, and compound **1** was recovered by washing with neat acetonitrile. This resulted in an analytically pure sample of **1**. The isolated amount of **1** corresponded to under 0.2% in the defatted extracts of the two plants. Isolation of natural products from HPLC fractions by SPE rather than direct evaporation is thus a useful means of avoiding accumulation of unwanted (e.g., harmful or nonvolatile) mobile-phase additives.

The 4-hydroxycyclohexa-2,5-dien-1-one system present in **1** and **2** is rarely found in natural products. Previous examples of its occurrence include 4-(2-β-D-glucopyranosyloxyethyl)-4-hydroxycyclohexa-2,5-dien-1-one (cornoside) and its degradation products, as well as simple derivatives of (1-hydroxy-4-oxocyclohexa-2,5-dienyl)acetic acid, isolated from a number of species belonging to Asteraceae, Bignoniaceae, Cornaceae, Lamiaceae, Martyniaceae, Oleaceae, and Scrophulariaceae,[22–29] and also from *H. ambavilla*.[30] Brominated derivatives have been isolated from sponges.[31–33] Aculeatins A–D, isolated from *Amomum aculeatum* (Zingiberaceae), also contain a 4-oxygenated cyclohexa-2,5-dien-1-one moiety and display antibacterial and cytotoxic activities.[34]

Compound **1** was evaluated for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. However, **1** was inactive at concentrations up to 500 μg/mL. Methyl-1-hydroxy-4-oxocyclohexa-2,5-dienylacetate (jacaranone) has previously been reported to be a weak antioxidant.[35] Because caffeoyl derivatives of quinic acid possess antioxidant activity, both as radical scavengers and as inhibitors of Cu^{2+}-catalyzed oxidation of low-density lipoprotein (LDL),[21,36–41] initial evaluation of the activity of **1** using the latter method was performed. Solutions containing human LDL and Cu^{2+} were incubated with the test compound, continuously measuring changes of absorbance at 234 nm, which reflects the rate of formation of conjugated dienes in LDL. Chlorogenic acid and 3,5-di-*O*-caffeoylquinic acid were evaluated in parallel with **1**. The results are depicted in Figure 4. As expected, chlorogenic acid and 3,5-di-*O*-caffeoylquinic acid inhibited the oxidation of LDL in a dose-dependent manner. 3,5-Di-*O*-caffeoylquinic acid was more potent than chlorogenic acid because of the presence of an additional caffeoyl moiety.[42] Interestingly, **1** caused the instantaneous onset of oxidation and increased the rate as well as the extent of the oxidation (Figure 4), whereas the incubation of **1** with LDL alone (without Cu^{2+}) caused no time-dependent absorbance changes of the reaction mixture (data not shown). It is apparent that, while 10 μM 3,5-di-*O*-caffeoylquinic acid acted as an effective antioxidant, as expected, compound **1** was a strong pro-oxidant at the same concentration (the drop of

absorbance and increased noise toward the end of the incubation period are due to the precipitation of the oxidized lipoprotein). Although the mechanism of this pro-oxidant activity has yet to be investigated, it should be pointed out that the 4-hydroxycyclohexa-2,5-dien-1-one moiety of **1** is a potential precursor of a hydroxybenzene, and the energetic gain on account of aromatization of **1** to **3** may be of importance in free-radical reactions involving **1** and LDL.

In conclusion, the present work provided another example of achieving rigorous structure determination of new natural products in a crude extract by use of the HPLC–SPE–NMR technique. The results from HPLC–SPE–NMR were used to guide the targeted isolation of a selected compound, which greatly simplified the fractionation of the extract. This afforded accelerated access of the novel quinic acid derivative **1**. Thus, the HPLC–SPE–NMR technique is helpful in construction of natural-product libraries in a rational way. In HPLC–SPE–NMR, chromatographic separation of mixture components, separation of analytes from the HPLC mobile phase by SPE, and NMR data acquisition are performed as a fully automated, online series of events. Therefore, the technique provides a considerable speed advantage as compared to traditional microfractionations that involve evaporation of HPLC fractions.

Experimental Section

General Experimental Procedures. Optical rotation values were recorded using a Perkin-Elmer 241 polarimeter. Analytical-scale HPLC separations were performed on a Shimadzu HPLC system consisting of a SCL-10A system controller, SIL-10AD autoinjector, LC-10AT pump, and a SPD-M10A photo-diode array detector and operated with Shimadzu Class-VP version 6.10 software. Preparative-scale separations were performed using an Agilent 1100 LC system consisting of two preparative pumps, autosampler, sample collector, and a multiple-wavelength UV detector. The system was operated with ChemStation version B.01.01 software. The HPLC–SPE–NMR/HPLC–MS system consisted of a Bruker LC22 quaternary solvent delivery pump with a Degasys Populaire degasser, an Agilent 1100 autosampler, a Bruker photo-diode array detector, a Knauer K100 Wellchrom pump for postcolumn water delivery, a Spark Prospekt 2 solid-phase extraction device, and a Bruker Avance 600 spectrometer (hydrogen frequency of 600.13 MHz) equipped with a 30 μL ^{1}H{^{13}C} flow probe operating at 25 °C. A split led 5% of the HPLC eluate to an Esquire liquid chromatography (LC) ion-trap mass spectrometer, operated in the negative mode at default settings. Chromatography, peak trapping, and analyte transfer from the SPE unit to the NMR spectrometer were controlled with HyStar version 2.3 software, whereas the NMR experiments were conducted with Xwinnmr version 3.1 software (Bruker BioSpin). NMR spectra of isolated compounds were recorded at 25 °C, either with a Bruker Avance 600 spectrometer equipped with a 5 mm ^{1}H{^{13}C} probe or a Bruker Avance 400 spectrometer equipped with a 5 mm ^{13}C{^{1}H} probe (hydrogen frequencies of 600.13 and 400.13 MHz, respectively). NMR spectra simulations and iterations were performed with gNMR version 4.0 software (Adept Scientific). High-resolution mass-spectrometric measurements for exact mass determination were carried out in the positive-ion mode using a Bruker APEX Qe Fourier transform mass spectrometer equipped with a 7 tesla superconducting magnet and the external electrospray ion source (Apollo II source). The spectra were externally calibrated with a collision-induced dissociation spectrum of luteinizing hormone-releasing hormone (LHRH) free acid. The samples were introduced into the electrospray ion source at a rate of 2 μL/min. Water was purified by deionization and 0.22 μm membrane filtration (Millipore). HPLC-grade solvents from commercial suppliers were used for all operations. Chlorogenic acid was a commercial sample. 3,5-Di-*O*-caffeoylquinic acid was available from earlier studies at this laboratory.

Plant Material. Leaves and twigs of *Hubertia ambavilla* Bory variety *ambavilla* [*Senecio ambavilla* (Bory) Pers.] were collected at Plaine d'Affouche, and leaves of *Hubertia tomentosa* Bory variety *tomentosa* (*Senecio hubertia* variety *albicans* Cordem.) were collected at Maido, Réunion Island in July 1998. Voucher specimens (A. and H. Adsersen 5566 and A. and H. Adsersen 5559, respectively) were deposited in Herbarium C (Botanical Museum, University of Copenhagen, Denmark). The plant material was air-dried immediately after the collection and kept in paper bags until use.

Sample Preparation. Ground plant material (25 g) of each plant was extracted with 3 × 400 mL of ethanol (18 h at room temperature). The extracts were filtered, pooled, concentrated in vacuo, and freeze-dried, to give 5.0 and 4.4 g of the crude extract of *H. ambavilla* and *H. tomentosa*, respectively.

HPLC Separations. Analytical-scale separations, including HPLC–SPE–
NMR experiments, were performed on a 150 × 4.6 mm i.d., Phenomenex Luna C_{18}(2) column (3 μm, 100 Å) with a guard column. A binary eluent consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) delivered at 0.8 mL/min was used. Gradient profile for *H. ambavilla* extract: 0 min, 10% B; 60 min, 18% B; 80 min, 44% B; 85 min, 90% B; 95 min, 90% B; 100 min, 10% B. Gradient profile for *H. tomentosa* extract: 0 min, 10% B; 60 min, 24% B; 80 min, 44% B; 85 min, 90% B; 95 min, 90% B; 100 min, 10% B. The chromatography was monitored at 254 and 320 nm. For monitoring the preparative-scale isolation of **1**, the following gradient profile was used for both extracts: 0 min, 5% B; 40 min, 32% B; 45 min, 95% B; 50 min, 95% B; 52 min, 5% B.

HPLC–SPE–NMR Experiments. The postcolumn eluate flow (0.8 mL/min) was diluted with water (flow rate of 2 mL/min) prior to analyte trapping on 2 × 10 mm SPE cartridges [GP resin, poly(divinylbenzene)]. Absorbance thresholds at 320 nm were used to trigger the trappings. A total of 5 (initial extract evaluation) or 8 (final structure elucidations by 2D NMR) cumulative trappings were performed for each peak selected for analysis. Acetonitrile-d_3 was used for elution and transfer of the analytes to the NMR flow probe. One-dimensional NMR spectra were acquired using the nuclear Overhauser effect spectrometry (NOESY) pulse sequence for dual presaturation of solvent resonances (H_2O and CD_2HCN) during the mixing time (100 ms) and relaxation delay (2.4 s). COSY and HSQC pulse sequences incorporated the water suppression enhanced through T_1 effects (WET) solvent suppression scheme.

Preparative Isolation of Compound 1. Ground plant material of *H. ambavilla* or *H. tomentosa* (500 g) was covered with 2.5 L of 99.9% ethanol at ambient temperature for 24 h. The solvent was removed by filtration, and the procedure was repeated twice. The combined filtrates were concentrated in vacuo below 40 °C, followed by removal of the residual solvent on a freeze dryer. The extracts were dispersed in 200 mL of MeOH/H_2O (1:9) and defatted with 3 × 200 mL of petroleum ether (bp 40–60 °C). The concentration of the methanol phases yielded 55 and 42 g of the extract of *H. ambavilla* and *H. tomentosa*, respectively. The defatted extracts were suspended in water to a concentration of 50 g/L, and 20 mL portions were applied to conditioned C_{18} SPE cartridges (Bakerbond SPE C-18, 10 g/70 mL). The cartridges were successively eluted with 2 × 100 mL of 10, 15, and 20% aqueous acetonitrile, followed by 2 × 100 mL of neat acetonitrile. Each 100 mL aliquot was collected individually and evaporated to dryness in vacuo, and the resulting remnant was dissolved in acetonitrile/water (1:9) to a concentration of 10 mg/mL and investigated using analytical-scale HPLC. The fractions eluted with 20% acetonitrile contained the majority of the target compound (**1**), with a small amount being eluted with 15% acetonitrile. Evaporation of fractions eluted with 20% acetonitrile yielded fractions enriched in **1** in an amount corresponding to 3% of the crude extract of the defatted extract of *H. ambavilla* and 4% of the defatted extract of *H. tomentosa*.

The fractions were subjected to preparative-scale HPLC on a 250 × 20 mm C_{18} Phenomenex Luna column (5 μm), with a linear gradient of acetonitrile in water (20 mL/min, 0.1% TFA), rising from 20 to 30% over 20 min, followed by a column rinsing and equilibrating procedure; compound **1** eluted at 15.2 min. Final purification was achieved on a 250 × 10 mm Supelco Discovery C_{18} (5 μm) column using isocratic elution with a solvent mixture consisting of water/acetonitrile/THF (74.6:15.4:10) containing 0.1% TFA, with a flow of 4 mL/min; **1** eluted at 16.1 min. All separations were monitored at 320 nm. The total isolated amount of **1** was 116.2 mg.

3,5-Di-*O*-caffeoyl-4-*O*-[(1-hydroxy-4-oxocyclohexa-2,5-dienyl)-acetyl]quinic acid (1): amorphous white powder; $[\alpha]_D^{25}$ −151 (*c* 0.20, methanol). ^{1}H NMR (600 MHz, methanol-d_4) δ: 7.62 (1H, d, 15.9, H-β'), 7.57 (1H, d, 15.9, H-β'), 7.09 (1H, d, 2.15, H-2′), 7.07 (1H, d, 2.15, H-2′), 6.99 (4H, m, H-6′, H-6′, H-2″, H-6″), 6.80 (1H, d, 8.2, H-5′), 6.79 (1H, d, 8.2, H-5′), 6.31 (1H, d, 15.9, H-α′), 6.24 (1H, d,

15.9, H-α′), 6.06 (2H, m, H-3″, H-5″), 5.58 (2H, m, H-3, H-5), 5.25 (1H, dd, 8.5/3.6, H-4), 2.75 (1H, d, 14.4, H-α″B), 2.72 (1H, d, 14.4, H-α″A), 2.41 (1H, dd, 14.6/3.8, H-2_{ax}), 2.30 (1H, dd, 13.7/9.2, H-6_{ax}), 2.25 (1H, dd, 13.7/4.4, H-6_{eq}), 2.15 (1H, 14.6/5.6, H-2_{eq}). ^{13}C NMR (150 MHz, methanol-d_4) δ: 187.5 (C, C-4″), 177.4 (C, HOO*C*), 169.6 and 168.6 (each *C*O caffeoyl), 168.1 (–OO*C*–CH_2), 152.71 and 152.67 (each CH, C-2″ and C-6″), 149.9 and 149.8 (each C, C-4′), 147.9 and 147.7 (each C, C-3′), 147.0 (2 CH, C-β′), 128.6 and 128.5 (each CH, C-3″and C-5″), 127.9 and 127.8 (each C, C-1′), 123.4 and 123.3 (each CH, C-6′), 116.7 (2 CH, C-5′), 115.5 and 115.4 (each CH, C-2′), 115.3 and 114.9 (each CH, C-α′), 74.8 (C, C-1″), 73.4 (C, C-1), 69.9 (CH, C-4), 69.0 (CH, C-3), 68.0 (CH, C-5), 46.0 (CH_2, C- α″), 38.8 (CH_2, C-6), 36.7 (CH_2, C-2). HR ESI MS *m/z* 667.16559 $[MH]^+$. $C_{33}H_{31}O_{15}$ requires 667.16575 (ΔM 0.2 ppm).

Assay for Antimicrobial Activity.[43] The test organisms were *E. coli* (ATCC 11229), *S. aureus* (ATCC 6538), and *B. subtilis* (ATCC 6633). Compound **1** was tested in concentrations ranging from 0.031 to 500 μg/mL with streptomycin and buffer as the positive and negative references, respectively. The microtiter plates were incubated for 24 h at 37 °C before the addition 0.02% MTT solution for the reading of plates. Streptomycin had a minimal inhibitory concentration (MIC) of 12.5 μg/mL for all three test organisms. Compound **1** did not inhibit the growth of any of the tested organisms.

Assay for the Inhibition of Human LDL Oxidation.[44,45] Solutions containing 5 μM $CuSO_4$ and human LDL (0.05 mg of protein/mL) at pH 7.4 were incubated at 37 °C with varying concentrations of test compounds (**1**, chlorogenic acid, or 3,5-di-*O*-caffeoylquinic acid), continuously following changes in absorbance at 234 nm. Each assay was made in duplicate, and the average absorbance values were plotted as function of time.

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