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Graphical Abstract

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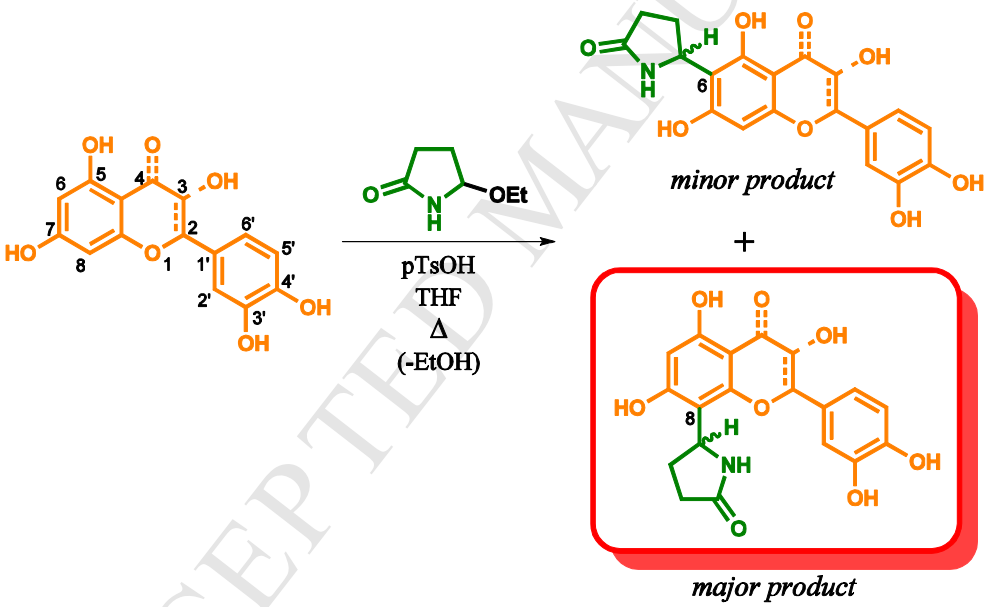

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ABSTRACT

We hereby report the biomimetic synthesis of three flavonoid alkaloids, namely 8-(2”-pyrrolidinon-5”-yl)quercetin, 6-(2”-pyrrolidinon-5”-yl)-(–)-epicatechin and 8-(2”-pyrrolidinon-5”-yl)-(–)-epicatechin. These known natural products were prepared *via* an acid-catalysed regioselective phenolic Mannich reaction involving the electrophilic attack of an *N*-acyliminium ion on the corresponding flavonoidal precursors. The products were purified by preparative HPLC. The reactions showed high C8-regioselectivity. The major isomers of the synthesized flavonoid alkaloids were further characterized in terms of their medicinal-chemical properties.

1. Introduction

Flavonoid alkaloids constitute a unique group of natural products. They contain – in almost all cases – a five- or six-membered nitrogen heterocycle which is connected to the C6 or C8 position of the A-ring of the flavonoid skeleton. Almost all molecules in this group are found in plants which are used in folk medicine as herbal remedy for the treatment of a wide range of conditions. Some compounds were reported to possess valuable pharmacological activity themselves.[1] Research into the field of flavonoid alkaloids gained even more attention when a synthetic flavonoid alkaloid derivative, namely flavopiridol[2] (brand name: Alvocidib, by Tolero Pharmaceuticals®) performed promisingly in phase II clinical trials and was granted the orphan drug designation by the FDA for the treatment of acute myeloid leukaemia in the United States in 2014.[3] Based on the still unexploited potential that lies in this family of natural products in terms of bioactivity and structural diversity, it can be rightfully stated that the isolation and synthesis of flavonoid alkaloids and their derivatives is a highly important field of research today.

The flavonoid alkaloid 8-(2”-pyrrolidinone-5”-yl)quercetin (**1**, **Fig. 1**) was isolated as a racemate from *Senecio argunensis* by N. Li and co-workers in 2008.[4] This molecule has a 2-pyrrolidone moiety attached to the C8 position of the A-ring of quercetin (**4**, **Fig. 1**). *Senecio argunensis* is a perennial herb distributed in northeast and northwest China. It is used in traditional Chinese medicine as a remedy for the treatment of, among other conditions, sore throat, dysentery and snake bite.

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† We regret to report that our co-author prof. György Kalaus died in 2014.

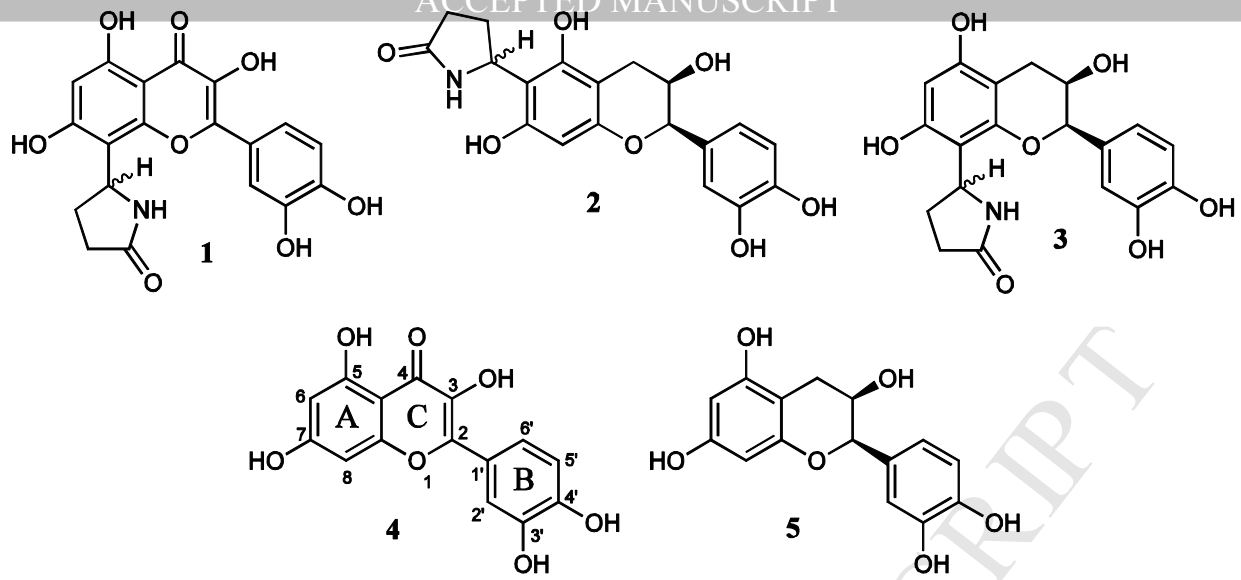
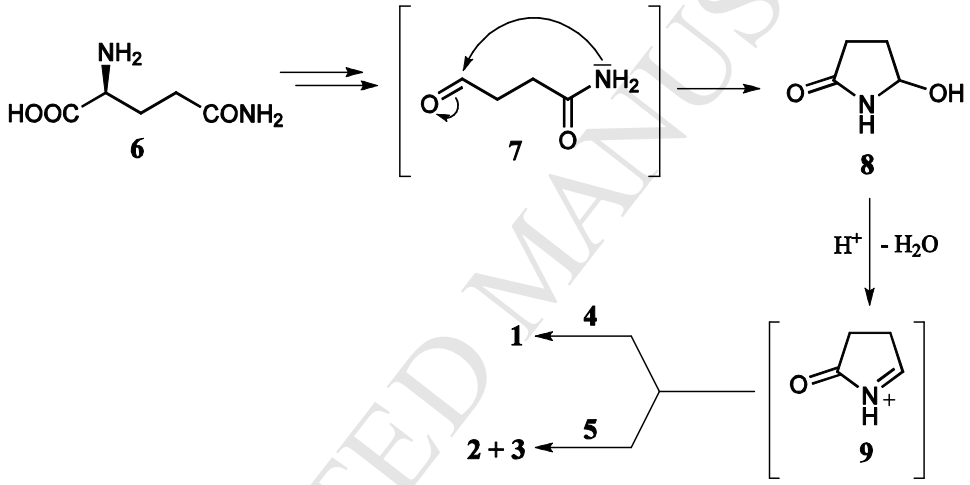


Figure 1. Structure of flavonoid alkaloids **1**-**3** and their flavonoid precursors (**4**, **5**)



Scheme 1. A plausible biosynthetic route to pyrrolidone-substituted flavonoids

6-(2”-Pyrrolidinone-5”-yl)-(–)-epicatechin (**2**, **Fig. 1**) and 8-(2”-pyrrolidinone-5”-yl)-(–)-epicatechin (**3**, **Fig. 1**) are both derivatives of the flavan-3-ol (–)-epicatechin (**5**, **Fig. 1**). They were isolated from the roots of *Actinidia arguta* by D. S. Jang and co-workers in 2009.[5] *Actinidia arguta* (or hardy kiwi) is a perennial vine native to northern China, Japan, Korea and Siberia. It produces an edible kiwi-like fruit used in traditional Chinese medicine to improve general health. In *in vitro* biological tests compounds **2** and **3** were both found to exhibit significant inhibitory effect against the formation of advanced glycation end products (AGEs), which play a key role in the pathogenesis of diabetic complications, cataracts, atherosclerosis and other neurodegenerative diseases.[5]

The biosynthesis of these flavonoid-pyrrolidone conjugates is hypothesized[6,7] to proceed *via* an aminocarbinol-derived iminium intermediate, which presumably arises through the *Strecker-degradation* of the corresponding amino acids present in the plants – in the case of molecules **1**-**3**, L-glutamine (**6**). The spontaneous cyclization of Strecker aldehyde **7** gives rise to 5-hydroxypyrrolidin-2-one (**8**), which is transformed into the *N*-acyliminium ion **9** upon protonation and subsequent loss of water. This *N*-acyliminium ion is a reactive electrophile, which attacks the activated A-ring of the flavonoids present in the plants to yield the C6- and C8-isomers of pyrrolidone-substituted flavonoids (**Scheme 1**). Plausible explanations of this biosynthetic route for similar cases are given by E. Leete[6] and by Tanaka *et al.*[7]

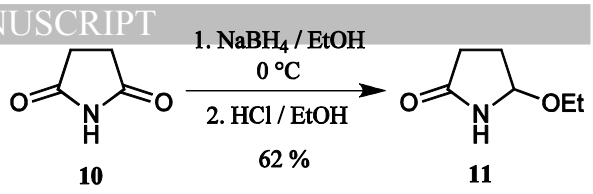
The aim of our work was to synthetically prepare flavonoid alkaloids **1**-**3** from their flavonoid precursors (**4**, **5**) in order to further investigate the desired products from a medicinal chemistry point of view.

2. Results and discussion

We have devised a feasible methodology for the synthesis of lactam-containing flavonoid alkaloids, utilizing a cyclic *N*-acylaminocarbinol reagent,[8] which can be easily prepared from succinimide in one step. This *N*-acylaminocarbinol is allowed to react with the appropriate flavonoid compound in a suitable solvent to yield the desired flavonoid alkaloids *via* an acid-catalysed regioselective phenolic *Mannich reaction*.[9] This type of transformation was applied in recent works by Tanaka *et al*. in the synthesis of a black tea polyphenol-type flavonoid alkaloid,[7] and also by Nguyen and co-workers in the selective synthesis of the C6- and C8-isomers of chrysin-derived flavonoid alkaloids and other pyrrolidine- and piperidine-substituted chrysin

derivatives.[10] Complementarily to their work, our biomimetic synthetic route provides a convenient way to pyrrolidone-substituted flavonoids and can be generally applied to various flavonoid substrates.

First we prepared the *N*-acylaminocarbinol reagent 5-ethoxypyrrolidin-2-one (**11**) by the partial reduction of succinimide (**10**) with sodium borohydride (**Scheme 2**), as described in the literature.[11]



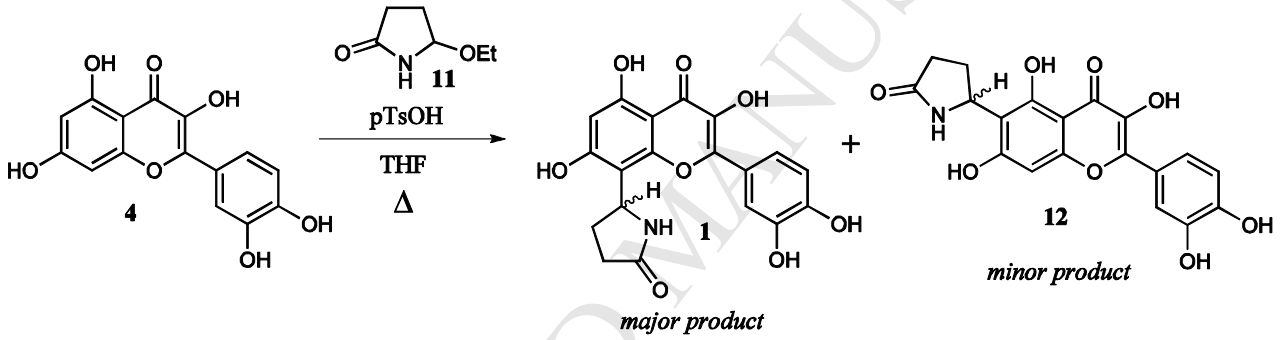
Scheme 2. Synthesis of 5-ethoxypyrrolidin-2-one (**11**)

Quercetin (**4**) was allowed to react with reagent **11** with acid catalysis in refluxing THF (**Scheme 3**). The crude product was subjected to preliminary purification in order to dispose of the remaining quercetin by refluxing its suspension in ethyl acetate (dissolving unreacted **4**), and filtering the purified product. The obtained solid was purified by preparative HPLC, after which the desired flavonoid alkaloid **1** was obtained together with its C6-isomer (**12**) in a ratio of 94:6, indicating an electrophilic attack predominantly directed towards C8 in the reaction. We note that the C8-substituted regioisomer was the only isomer of this natural product which was isolated from its natural source (*Senecio argunensis*).[4] the pertinent isomeric ratio of the natural forms of **2** and **3** isolated from *Actinidia arguta* was found to be **2** : **3** = 22:78.[5]

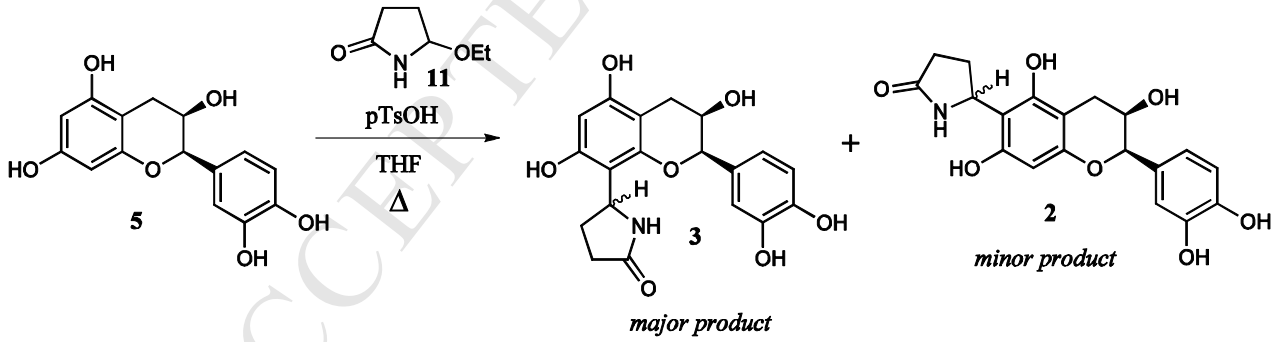
A plausible mechanism of these phenolic *Mannich reactions* (**Scheme 5**) is basically the same as that of the proposed biosynthetic route (**Scheme 1**). It involves the electrophilic attack of the *N*-acyliminium ion **9** on the aromatic A-ring of the flavonoid substrate at C6 or C8. The reactive iminium ion **9** arises from 5-ethoxypyrrolidin-2-one (**11**) after protonation and loss of ethanol.

*2.1. Structure elucidation of flavonoid alkaloids **1**, **2**, **3** and **12***

Literature NMR and MS data[4,5] are available for compounds **1**, **2** and **3**. Li et al[4] elucidated structure **1** with high-resolution mass spectrometry (HRMS), infrared (IR) spectroscopy, and

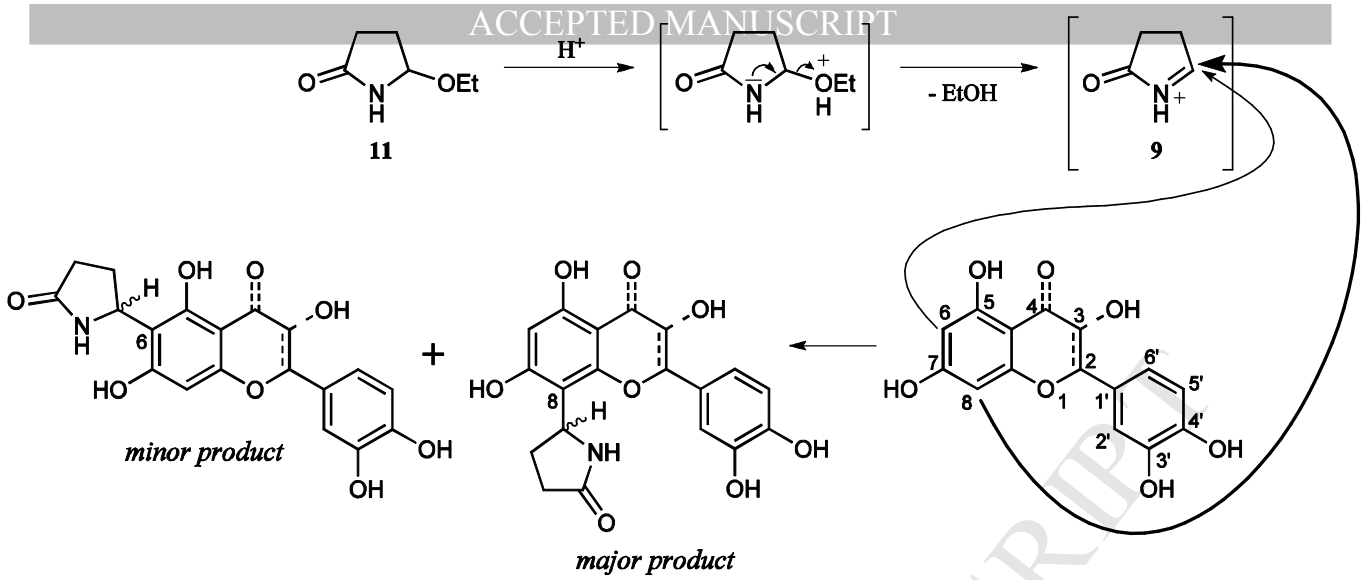


Scheme 3. Synthesis of 8-(2”-pyrrolidinone-5”-yl)quercetin (**1**) and the minor C6-isomer (**12**)



Scheme 4. Synthesis of 8-(2”-pyrrolidinone-5”-yl)-(–)-epicatechin (**3**) and 6-(2”-pyrrolidinone-5”-yl)-(–)-epicatechin (**2**)

In the next step, (–)-epicatechin (**5**) was allowed to react with 5-ethoxypyrrolidin-2-one (**11**) in the presence of an acid catalyst in refluxing THF (**Scheme 4**). A suspension of the crude product in ethyl acetate was refluxed (dissolving unreacted **5**) and filtered, in order to get rid of the remaining starting material contaminating the product. The obtained solid was purified by preparative HPLC, which gave the epimeric mixtures of the C8-(**3**) and the C6-substituted isomers (**2**) in a ratio of 87:13. The epimers of the regioisomers could not be separated by the applied method. The C8-regioselectivity observed in the reaction involving (–)-epicatechin is somewhat lower than in the case of quercetin, but nonetheless relatively high. One should note that various one-dimensional (1D) and two-dimensional (2D) NMR techniques, such as ^{1}H-^{1}H correlated spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC; one-bond ^{1}H-^{13}C connectivity data), and ^{1}H-^{13}C heteronuclear multiple bond connectivity (HMBC). Jang et al[5] elucidated structures **2** and **3** with the techniques mentioned above along with an additional NMR method, ^{1}H-^{1}H nuclear Overhauser effect spectroscopy (NOESY), and circular dichroism (CD) spectroscopy. They used CD to determine the absolute configuration at C2 and they analyzed the ^{1}H-^{1}H coupling constants to determine relative configuration between C2 and C3, concluding that the configuration of epicatechin derivatives **2** and **3** is (2*R*,3*R*).



Scheme 5. Proposed mechanism for the formation of flavonoid alkaloids (dashed lines indicate variations in the flavonoid core)

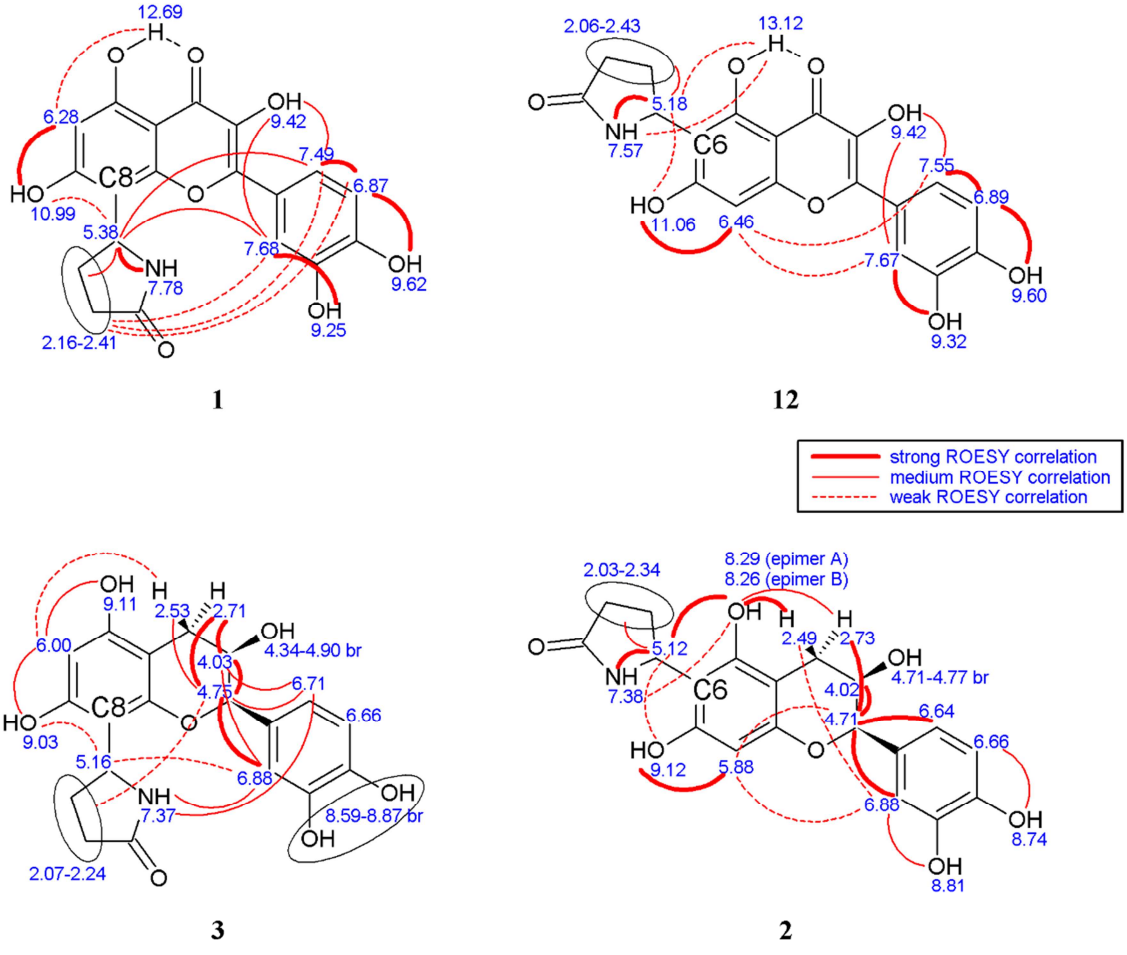


Figure 2. Qualitative representation of the observed 2D ROESY correlations of compounds **1**, **2**, **3** and **12**. For clarity, ^{1}H chemical shift values are given for only one of the two epimers of compound **3**. In the case of compound **2**, the ^{1}H NMR peaks of the two epimers overlap, except for the proton C(5)-O*H*, which facilitates the determination of the epimeric ratio of **2**.

Despite the detailed spectroscopic data available in the literature, a direct comparison of the NMR data of our synthetic samples with those reported in the literature[4,5] proved to be unsuitable for unambiguous structure identification. This is because the C6- and C8-substituted regioisomers have very similar ^{1}H and ^{13}C NMR spectra with only slight differences in the pertinent chemical shift values. The small experimental variations in these values with pH, temperature, concentration, etc., are in the same order of magnitude as the regioisomeric differences, making the comparison with the literature data

unreliable. The cases of epicatechin derivatives **2** and **3** are further complicated by the epimeric nature of these compounds, because the configuration of C5" is unspecified relative to C2 and C3, resulting in two ^{1}H NMR and ^{13}C NMR signal sets.

For the above reasons, we conducted an *ab initio* NMR-based structure determination for all synthetic samples, also with a view to giving full ^{1}H and ^{13}C NMR assignments where possible. This provided a solid basis for a *post factum* comparison of our assignments to those available in the literature (**1** in DMSO-d_6, **2** and **3** in CD_3OD). Note that for the purposes of a thorough and reliable spectral and structural assignment the choice of DMSO-d_6 as the solvent was critical in order to observe the OH and NH proton resonances, even though Jang et al assigned **2** and **3** in methanol-d_4. The application of ^{1}H NMR, ^{13}C NMR, COSY, HSQC (heteronuclear single quantum coherence; similarly to HMQC, this technique provides one-bond ^{1}H-^{13}C connectivity data, but with the advantage that it can differentiate methylene groups from CHs and CH_3s), and HMBC measurements was necessary for proving the structure of the flavon skeleton, the pyrrolidinone ring, and the connection between these moieties. Even so, the regioisomers could not be identified unambiguously from these experiments in the cases of the substituted epicatechin samples **2** and **3**. To determine whether a particular sample contained a C6- or a C8-substituted flavonoid, ^{1}H-^{1}H spatial proximity measurements (2D ROESY: two-dimensional rotating frame Overhauser effect spectroscopy) were therefore essential (**Fig. 2**).

In the cases of the C8-substituted flavonoids **1** and **3**, the regioisomers could be identified by the spatial proximity of the pyrrolidinone moiety and the catechol ring of the flavone skeleton (ring B). The C6-substituted flavonoids (**12** and **2**) give weak correlation peaks in their 2D ROESY spectra between the catechol ring and the C8-*H* proton. The application of 2D ROESY resulted in unambiguous structures for all synthetic samples. Subsequently we compared our NMR data with those of Li et al. and Jang et al. In order to facilitate that comparison, we also acquired ^{1}H NMR spectra for compounds **2** and **3** in CD_3OD, and a ^{13}C NMR spectrum in CD_3OD for the synthetic material before the separation of the regioisomers (this sample was available in a larger quantity, and contained **2** and **3** in ca. 1:6 ratio). We found a good agreement with the literature NMR data, except for a minor discrepancy in the ^{1}H NMR literature assignment of **1**[4]: the revised chemical shift values for C(3)-O*H* and C(4')-O*H* are 9.42 ppm and 9.60 ppm, which are proven with the corresponding three-bond *H*-O-C-*C* HMBC correlations.

The molar ratio of the two epimers of **2** can be estimated by integration of the singlets (linewidth at 50% peak height: 7 Hz) at 8.26 ppm and 8.29 ppm (C(5)-O*H*). This is the only pair of ^{1}H NMR peaks which appear separately for the two epimers at 800 MHz. The epimeric ratio for **2** is ca. 50%:50%. The C(5)-O*H* peaks for the epimers of **3** are broad and they overlap, thus the molar ratio of the two epimers of **3** is calculated by integration of the H-3 multiplets, because they separate well at 800 MHz (epimer A: ca. 58%, 4.02–4.04 ppm; epimer B: ca. 42%, 3.99–4.01 ppm). The estimated margin of measurement error is ca. 3%.

*2.2. Medicinal-chemical properties of compounds **1** and **3***

2.2.1. Physicochemical parameters

Typical predicted physicochemical parameters of compound **1** and the epimeric mixture of compound **3** are shown in **Table 1**.

Table 1. Predicted physicochemical parameters of compounds **1** and **3**

Cpnd.	clog*P*[#]	clog$D_{7.4}$/clog$D_{6.5}$[#]	TPSA[#] [Å^2]
1	1.93	0.11/0.87	159.38
3	1.48	0.79/1.01	156.55

[#] Physicochemical parameters were predicted for **1** and **3** with MarvinSketch v6.0.2 software.

The above data are in agreement with the results of the parallel artificial membrane permeability assay (PAMPA) measurements shown in **Table 2**. As a general rule of thumb, the total polar surface area (TPSA) cut-off values in the case of membrane permeability by passive diffusion are TPSA < 120 Å^2 for gastrointestinal absorption and TPSA < 70 Å^2 for BBB penetration.[12] Thus it comes as no surprise that none of the two compounds were permeable in the blood–brain barrier (BBB)- or gastrointestinal (GI)-PAMPA systems. However, the difference between the membrane retention values (MR%) showed a good correlation with the difference between the calculated log*P* (clog*P*) values, indicating that both tissue specific lipid membranes (GI and also BBB) bind compound **1** to a greater extent, which is slightly more lipophilic than the epimeric mixture of compound **3**.

Table 2. Brain (BBB) and gastrointestinal (GI) specific permeability parameters of **1** and **3** (NP: non-permeable)

BBB-PAMPA			
Cpnd.	P_e (cm/s*10^{-6})	MR%	SD (MR%)
1	NP	45.3	3.5
3	NP	10	2.4
GI-PAMPA			
1	NP	20.4	3.3
3	NP	3.3	2.3

2.2.2. Antioxidant activity

1 and **3** had a strong antioxidant effect on the DPPH radical scavenging test (IC_{50}=12.1±1.8 and 7.5±1.1 µg/mL respectively), compared to the positive control quercetin (IC_{50}=9.4±0.7 µg/mL).

2.3. Anticancer activity and effect on the ABCB1 efflux transporter

In connection with the above studies on their BBB penetration, the cytotoxicity of the major isomers of the synthesized flavonoid alkaloid samples **1** and **3** was evaluated on the SH-SY5Y cancer cell line. None of the above compounds exerted cytotoxicity on the tested cell line (IC_{50}>150 µM for all the compounds). Compounds **1** and **3** were also evaluated for their potential to interfere with the ABCB1 transporter (commonly referred to as P-glycoprotein or P-gp), which plays a major role in the function of BBB through active transport.[13] Neither compound inhibited this efflux transporter as determined by measuring the intracellular accumulation of Rhodamin 123.

3. Conclusion

In the present work, we have developed a convenient way to synthesize pyrrolidone-containing flavonoid alkaloids, employing an easily accessible *N*-acylaminocarbinol reagent, and successfully synthesized 8-(2"-pyrrolidinone-5"-yl)quercetin (**1**), 6-(2"-pyrrolidinone-5"-yl)quercetin (**12**), 6-(2"-pyrrolidinone-5"-yl)-(–)-epicatechin (**2**) and 8-(2"-pyrrolidinone-5"-yl)-(–)-epicatechin (**3**) *via* an acid-catalysed regioselective phenolic *Mannich reaction*. The crude products were obtained as isomeric mixtures, which were separated by preparative HPLC. The pure

major isomers of the synthesized flavonoid alkaloids were further characterized in terms of their medicinal-chemical properties.

4. Experimental

4.1. General

Melting points were measured on a SANYO Gallenkamp apparatus and are uncorrected. IR spectra were recorded on a Bruker FT-IR instrument and a PerkinElmer Spectrum 100 FT-IR Spectrometer equipped with Universal ATR (diamond/ZnSe) accessory. NMR measurements (^{1}H, ^{13}C, gCOSY, 1D NOESY, 1D ROESY, 2D ROESY, gHSQCAD, gHMBCAD) were performed on Varian 400 MHz (equipped with 5 mm OneNMR ^{15}N-^{31}P/{^{1}H-^{19}F} PFG Probe), Varian 500 MHz (equipped with ^{1}H{^{13}C/^{15}N} 5 mm PFG Triple Resonance ^{13}C Enhanced Cold Probe) and Varian 800 MHz (equipped with ^{1}H{^{13}C/^{15}N} Triple Resonance ^{13}C Enhanced Salt Tolerant Cold Probe) spectrometers. ^{1}H chemical shifts are given on the delta scale as parts per million (ppm) with tetramethylsilane (TMS) as the internal standard (0.00 ppm). ^{13}C chemical shifts are given on the delta scale as parts per million (ppm) with tetramethylsilane (TMS) or dimethylsulfoxide-d_6 as the internal standard (0.0 ppm and 39.5 ppm, respectively). HRMS and MS analyses were performed on a Finnigan MAT 95 XP, and a Thermo LTQ FT Ultra as well as a Thermo LTQ XL (Thermo Fisher Scientific, Bremen, Germany) system. The ionization method was EI operated in positive ion mode on a Finnigan MAT 95 XP. Electron energy was 70 eV and the source temperature was set at 220°C. The ionization method was ESI operated in positive ion mode on the other two systems. For the CID experiment helium was used as the collision gas, and normalized collision energy (expressed in percentage), which is a measure of the amplitude of the resonance excitation RF voltage applied to the endcaps of the linear ion trap, was used to induce fragmentation. The protonated molecular ion peaks were fragmented by CID at a normalized collision energy of 35%. Data acquisition and analysis were accomplished with Xcalibur software version 2.0 (Thermo Fisher Scientific). TLC was carried out on TLC Silica gel 60 F_{254} on 20×20 cm aluminium sheets (Merck), and preparative TLC was carried out using Silica gel 60 $PF_{254+366}$ (Merck) coated glass plates. Column chromatography was performed using Silica gel 60 (0.063-0.200 mm) (Merck). Flavonoids (quercetin and (–)-epicatechin) were purchased from Sigma-Aldrich and used without further purification.

4.2. Preparative HPLC

4.2.1. Chemicals and reagents

The acetonitrile used in the preparative chromatographic separation was gradient grade LiChrosolv purchased from Merck. The trifluoroacetic acid (Uvasol) was obtained from Merck. Water was purified with a Milli-Q system. The sample solvent was spectroscopy grade dimethyl sulfoxide (Uvasol) purchased from Merck.

4.2.2. Apparatus

The separation of the isomeric mixtures of flavonoid alkaloids was performed with a Shimadzu chromatograph equipped with an LC-8A pump unit, SPD-M20A Photodiode detector and CBM-20A system controller. The samples were introduced via an SIL-10AP sample injector and the chromatograms were processed using the LCsolution software. The fractions were collected by an FRC10A fraction collector module. The temperature was controlled by a CTO-20AC prominence column oven.

4.2.3. Preparative separation procedure

The sample (500 µL/injection) was loaded to a Kinetex 5 µm Phenyl-Hexyl AXIA packed 21.2×150 mm column in the concentration of 20 mg/mL. The separation was achieved at 25 °C. Phase A was 0.1% TFA in water, phase B contained 0.1% TFA in acetonitrile. The developed linear gradient program increased the ratio of phase B from 5 to 30 percent by volume in 15 min. The flow rate was 21 mL/min. The fractions were collected based on UV absorption at 220 nm wavelength.

4.3. Medicinal-chemical properties

4.3.1. Antioxidant activity

The radical scavenging capacities of **1** and **3** were tested in the microplate format of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.[14] Quercetin was obtained from PhytoLab (Vestenbergsgreuth, Germany), and used as control.

4.3.2. Permeability measurements

The PAMPA-BBB method was previously published.[15] Briefly, a 96-well acceptor plate and a 96-well filter plate are assembled into a sandwich. The hydrophobic filter material of the 96 well filter plate is coated with 5 µL of a 2.6% (w/v) dodecane: hexane (25:75 v/v%) solution of porcine brain lipid (PBL). Subsequently, the acceptor wells at the bottom of the sandwich are filled with 300 µL of 10 mM PBS solution with 5% DMSO adjusted to pH 7.4. The donor wells at the top of the sandwich are hydrated with 150 µL of test compound solution. The test compound solution is prepared by diluting ×100 from a 10 mM stock solution in DMSO using PBS solution at pH 7.4 with 5% DMSO followed by filtration through a MultiScreen Solubility filter plate. The resulting sandwich is then incubated at 37 °C for 4 h. After incubation, the PAMPA sandwich plates are separated, and compound concentrations in donor and acceptor solutions are determined by HPLC-DAD.

A slightly modified version of PAMPA[16] was used to determine the gastrointestinal permeability (PAMPA-GI) as described previously.[17] Gradient-pH (donor: 6.5 → acceptor: 7.4) conditions were utilized. The hydrophobic filter material of the 96 well filter plate was coated with 5 µL of a 4% (w/v) dodecane solution of phosphatidylcholine:cholesterol (2:1).

4.4. Cytotoxicity on SH-SY5Y cancer cell line

4.4.1. Cell lines

SH-SY5Y neuroblastoma cells were cultured in EMEM media supplemented with non-essential amino acids, 1 mM Na-pyruvate and 10% inactivated fetal bovine serum, Nystatin, 2 mM L-glutamine, 100 U penicillin and 0.1 mg streptomycin, purchased from Sigma. Cells were cultured at 37 °C and 5% CO_2.

Two mouse lymphoma cell lines were used to test for the capacity of the compounds to inhibit the function of the ABCB1 transporter: a parental cell line, L5178 mouse T-cell lymphoma cells (ECACC catalog no. 87111908, U.S. FDA, Silver Spring, MD, U.S.), and a multi-drug resistant ($L5178_{MDR}$) cell line derived from L5178 by transfection with pHa MDR1/A retrovirus.[18] Cells were cultured in McCoy's 5A media supplemented inactivated horse serum and antibiotics as above. MDR cell line was selected by culturing the infected cells with 60 µg/L colchicine (Sigma).

4.4.2. Materials and methods

Compounds **1** and the epimeric mixture of **3** were dissolved in DMSO at a stock concentration of 10 mM.

4.4.2.1. Cytotoxicity on SH-S5Y5 cells

Ten thousand cells per well were seeded overnight. Serial dilutions of the compounds were prepared and added the following day to the plate. Cells were then incubated for 48 h, after which 10% MTT was added to each well. After 4 h, SDS was added to the medium and the results were read after o/n incubation. Fifty percent inhibitory concentrations (IC_{50}) were calculated using nonlinear regression curve fitting of log (inhibitor) versus normalized response and variable slope with a least squares (ordinary) fit of GraphPad Prism 5 software, for three independent samples.

4.4.2.2. Effect on Rhodamine 123 accumulation

Rhodamine 123 (Rd123) is a fluorescent dye and a substrate of the ABCB1 transporter whose overexpression prevents the intracellular accumulation of this dye.[19] Rd123 concentration inside the cells was determined by flow cytometry. Briefly, 2×10^6 cells/mL were treated with 2 and 20 μM of each compound and incubated for 10 min at RT. Rhodamine 123 (Sigma, Germany) was added to a final concentration of 5.2 μM. The samples were incubated for 20 min at 37 °C in water bath and then centrifuged (2000 rpm, 2 min). The pellet was resuspended in 0.5 mL of phosphate buffer saline (PBS) (Sigma, Germany). The washing step was repeated twice. The fluorescence of the samples was measured by flow cytometry (Becton Dickinson FACScan, BD, U.S.). Tariquidar at 2 μM was used as positive control. Fluorescence activity ratio measures the capacity of inhibition (accumulation of Rd123) and it is equal to the ratio between the FL-1 values of the $L5178_{MDR}$ cells treated and untreated.

4.5. Experimental procedures

*4.5.1. 5-Ethoxypyrrolidin-2-one (**11**)*

To a solution of succinimide (**10**) (7.157 g, 72.23 mmol) in ethanol (300 mL) sodium borohydride (4.00 g, 105.74 mmol) was added in one portion at 0 °C. The reaction mixture was stirred at 0 °C for 4 h, during which time five drops of 2 M ethanolic hydrogen chloride solution were added every 15 min. Then the reaction mixture was acidified to pH=3 with 2 M ethanolic hydrogen chloride solution over 30 min, after which it was stirred at 10 °C for 90 min. Then the reaction mixture was neutralised (pH = 7) with 5% ethanolic potassium hydroxide solution and evaporated in vacuo to give a syrupy solid, which was suspended in chloroform (80 mL), filtered, and the precipitate washed with chloroform (3×20 mL). The filtrate was evaporated in vacuo to give a colourless oil, which was dissolved in dichloromethane (80 mL) and washed with water (3×10 mL). The aqueous phase was extracted with dichloromethane (6×20 mL), then the organic phases were unified, dried ($MgSO_4$) and the solvent evaporated in vacuo to give the *title compound* **11** (5.788 g, 62%) as a colourless oil, which crystallized on standing, giving white crystals, mp 54–58 °C (lit.: 48–53 °C);[11] R_f (acetone) 0.72; ν_{max} (KBr) 3200, 2978, 1707, 1689, 1668, 1457, 1282, 1250, 1067, 986 cm^{-1}; δ_H (399.8 MHz, DMSO-d_6) 1.10 (3H, t, *J* 7.0 Hz, CH_3), 1.78–1.89 (1H, m, H_x-4), 1.95–2.05 (1H, m, H_x-3), 2.11–2.30 (2H, m, H_y-3, H_y-4), 3.27–3.35 (1H, m), 3.44–3.54 (1H, m): OCH_2, 4.83–4.89 (1H, m, H-5), 8.62 (1H, s, NH-1); δ_C (100.5 MHz, DMSO-d_6) 15.1 (CH_3), 27.7 (C-4), 28.1 (C-3), 61.6 (OCH_2), 85.0 (C-5), 177.4 (C-2); MS(ESI): M+H=130 ($C_6H_{12}NO_2$); ESI-MS-MS (cid=35) (rel. int. %): 84(100).

*4.5.2. 8-(2”-Pyrrolidinone-5”-yl)quercetin (**1**) and 6-(2”-pyrrolidinone-5”-yl)quercetin (**12**)*

To a mixture of quercetin (**4**) (2.459 g, 8.14 mmol) and 5-ethoxypyrrolidin-2-one (**11**) (1.261 g, 9.76 mmol) in THF (70 mL) 10 mol% p-toluenesulfonic acid monohydrate (155 mg, 0.814 mmol) was added and the mixture was refluxed for 4 h. The solvent was evaporated in vacuo and the solid residue was suspended in ethyl acetate, and refluxed for 5 min. Then the suspension was left to cool to room temperature and it was filtered. The filtrate was discarded and the filtered solid was subjected to the above procedure two more times to dispose of the quercetin remaining in the product. A crude product (1.922 g, 61%) was obtained, a sample of which (98 mg) was subjected to further purification by preparative HPLC, which gave *title compound* **1** (32.46 mg, 94.74% purity) as a yellow solid, mp 244–246 °C; R_f (17% CH_3OH/CH_2Cl_2) 0.46; ν_{max} (KBr) 3536, 3296, 3120, 3003, 2828, 2723, 1688, 1646, 1603, 1555, 1513, 1436, 1375, 1319, 1272, 1201, 1177, 1135, 1020, 1008 cm^{-1}; δ_H (799.7 MHz, DMSO-d_6) 2.16–2.21 (1H, m), 2.30–2.41 (3H, m): H_2-3”, H_2-4”, 5.37–5.40 (1H, m, H-5”), 6.28 (1H, s, H-6), 6.87 (1H, d, *J* 8.5 Hz, H-5’), 7.49 (1H, dd, *J* 8.5, 2.2 Hz, H-6’), 7.68 (1H, d, *J* 2.2 Hz, H-2’), 7.78 (1H, s, NH-1”), 9.25 (1H, s, C(3’)-O*H*), 9.42 (1H, s, C(3)-O*H*), 9.62 (1H, s, C(4’)-O*H*), 10.99 (1H, s, C(7)-O*H*), 12.69 C(5)-O*H*); δ_C (125.7 MHz, DMSO-d_6) 25.7 (C-4”), 30.8 (C-3”), 47.2 (C-5”), 98.1 (C-6), 102.9 (C-10), 106.2 (C-8), 115.1 (C-2’), 115.6 (C-5’), 119.9 (C-6’), 121.9 (C-1’), 135.4 (C-3), 144.9 (C-3’), 146.9 (C-2), 147.6 (C-4’), 153.7 (C-9), 159.4 (C-5), 162.0 (C-7), 176.0 (C-4), 176.8 (C-2”); HRMS (EI): M=385.07892 ($C_{19}H_{15}O_8N$; delta=-0.8 ppm) and *title compound* **12** (2.13 mg, 83.95% purity), also a yellow solid, δ_H (499.9 MHz, DMSO-d_6) 2.06–2.27 (2H, m, H_x-3”, H_x-4”), 2.31–2.43 (2H, m, H_y-3”, H_y-4”), 5.16–5.21 (1H, m, H-5”), 6.46 (1H, s, H-8), 6.89 (1H, d, *J* 8.5 Hz, H-5’), 7.55 (1H, dd, *J* 8.5, 2.2 Hz, H-6’), 7.57 (1H, br s, NH-1”), 7.67 (1H, d, *J* 2.2 Hz, H-2’), 9.32 (1H, s, C(3’)-O*H*), 9.42 (1H, s, C(3)-O*H*), 9.60 (1H, s, C(4’)-O*H*), 11.06 (1H, br s, C(7)-O*H*), 13.12 (1H, s, C(5)-O*H*); δ_C (125.7 MHz, DMSO-d_6) 25.3 (C-4”), 30.6 (C-3”), 46.5 (C-5”), 93.0 (C-8), 102.7 (C-10), 111.4 (C-6), 114.9 (C-2’), 115.6 (C-5’), 120.0 (C-6’), 121.8 (C-1’), 135.7 (C-3), 145.1 (C-3’), 146.8 (C-2), 147.7 (C-4’), 154.7 (C-9), 158.6 (C-5), 162.1 (C-7), 176.0 (C-4), 177.0 (C-2”); HRMS (EI): M=385.07907 ($C_{19}H_{15}O_8N$; delta=-0.4 ppm).

*4.5.3. 6-(2”-Pyrrolidinone-5”-yl)-(–)-epicatechin (**2**) and 8-(2”-pyrrolidinone-5”-yl)-(–)-epicatechin (**3**)*

To a mixture of (–)-epicatechin (**5**) (440 mg, 1.52 mmol) and 5-ethoxypyrrolidin-2-one (**11**) (235 mg, 1.82 mmol) in THF (30 mL) 10 mol% p-toluenesulfonic acid monohydrate (29 mg, 0.152 mmol) was added and the mixture was refluxed for 2 h. The solvent was evaporated in vacuo and the solid residue was suspended in ethyl acetate, and refluxed for 5 min. Then the suspension was left to cool to room temperature and it was filtered. The filtrate was discarded and the filtered solid was air-dried by suction. A crude product (765 mg) was obtained, a sample of which (103 mg) was subjected to further purification by preparative HPLC, which gave an epimeric mixture of *title compound* **2** (5.30 mg, 95.95% purity) as a white solid, mp 183–189 °C (lit.: 195–197 °C);[5] R_f (17% CH_3OH/CH_2Cl_2) 0.27; ν_{max} (diamond/ZnSe) 3272, 1652, 1620, 1516, 1451, 1344, 1282, 1199, 1159, 1118, 1058 cm^{-1}; δ_H for epimer A (799.7 MHz, DMSO-d_6) 2.03–2.08 (1H, m, H_x-4”), 2.13–2.18 (1H, m, H_x-3”), 2.22–2.27 (1H, m, H_y-4”), 2.30–2.34 (1H, m, H_y-3”), 2.48–2.50 (1H, m, H_x-4), 2.71–2.74 (1H, m, H_y-4), 4.01–4.03 (1H, m, H-3), 4.70–4.72 (1H, m, H-2), 4.71–4.77 (1H, br m, C(3)-O*H*), 5.12 (1H, dd, *J* 9.1, 5.3 Hz, H-5”), 5.88 (1H, s, H-8), 6.63–6.65 (1H, m, H-6’), 6.65–6.67 (1H, m, H-5’), 6.88–6.89 (1H, m, H-2’), 7.38* (1H, s, NH-1”), 8.29 (1H, s, C(5)-O*H*), 8.74 (1H, br s, C(4’)-O*H*), 8.81 (1H, br s, C(3’)-O*H*), 9.12 (1H, s, C(7)-O*H*); δ_C for epimer A (201.1 MHz, DMSO-d_6) 25.9 (C-4”), 28.6 (C-4), 30.9 (C-3”), 47.5 (C-5”), 64.6 (C-3), 77.6 (C-2), 95.0 (C-8), 99.5 (C-10), 109.0 (C-6), 114.6 (C-5’), 114.7 (C-2’), 117.8 (C-6’),

130.3 (C-1'), 144.3 (C-4'), 144.4 (C-3'), 154.0 (C-9), 154.4 (C-5), 154.6 (C-7), 176.8 (C-2"); δ_H for epimer B (799.7 MHz, DMSO-d_6) 2.03–2.08 (1H, m, H_x-4"), 2.13–2.18 (1H, m, H_x-3"), 2.22–2.27 (1H, m, H_y-4"), 2.30–2.34 (1H, m, H_y-3"), 2.50–2.52 (1H, m, H_x-4), 2.70–2.73 (1H, m, H_y-4), 4.01–4.03 (1H, m, H-3), 4.70–4.72 (1H, m, H-2), 4.71–4.77 (1H, br m, C(3)-O*H*), 5.12 (1H, dd, *J* 9.1, 5.3 Hz, H-5"), 5.88 (1H, s, H-8), 6.63–6.65 (1H, m, H-6'), 6.65–6.67 (1H, m, H-5'), 6.88–6.89 (1H, m, H-2'), 7.38* (1H, s, NH-1"), 8.26 (1H, s, C(5)-O*H*), 8.74 (1H, br s, C(4')-O*H*), 8.81 (1H, br s, C(3')-O*H*), 9.12 (1H, s, C(7)-O*H*), *: in the epimeric mixture of **2**, the NH-1" peaks of the two epimers are separated by 4 ppb; δ_C for epimerB (201.1 MHz, DMSO-d_6) 25.9 (C-4"), 28.6 (C-4), 30.9 (C-3"), 47.6 (C-5"), 64.6 (C-3), 77.6 (C-2), 95.0 (C-8), 99.4 (C-10), 109.0 (C-6), 114.6 (C-5'), 114.7 (C-2'), 117.8 (C-6'), 130.3 (C-1'), 144.3 (C-4'), 144.4 (C-3'), 154.0 (C-9), 154.3 (C-5), 154.5 (C-7), 176.7 (C-2"); HRMS (ESI+): M+H=374.12344 (delta=0.03 ppm; $C_{19}H_{20}O_7N$). HR-ESI-MS-MS (CID=35%; rel. int. %): 356(100), 339(9), 311(2), 246(27), 234(32), 222(20), 217(2), 206(2), 205(3); and an epimeric mixture of *title compound* **3** (37.02 mg, 97.89% purity), also a white solid, mp 178–186 °C (lit.: 195–197 °C);[5] R_f (17% CH_3OH/CH_2Cl_2) 0.27; ν_{max} (diamond/ZnSe) 3277, 1648, 1610, 1521, 1455, 1357, 1280, 1199, 1159, 1099, 1067 cm^{-1}; δ_H for epimer A (799.7 MHz, DMSO-d_6) 2.07–2.13 (2H, m, H_x-3", H_x-4"), 2.17–2.24 (2H, m, H_y-3", H_y-4"), 2.51–2.54 (1H, m, H_x-4), 2.69–2.73 (1H, m, H_y-4), 4.03–4.04 (1H, m, H-3), 4.32–4.90 (1H, br, C(3)-O*H*), 4.74–4.75 (1H, m, H-2), 5.14–5.18 (1H, m, H-5"), 6.00 (1H, s, H-6), 6.66 (1H, d, *J* 8.1 Hz, H-5'), 6.71 (1H, dd, *J* 8.1, 2.0 Hz, H-6'), 6.88 (1H, d, *J* 2.0 Hz, H-2'), 7.37 (1H, br s, NH-1"), 8.59–8.87 (2H, br, C(3')-O*H*, C(4')-O*H*), 9.03 (1H, br s, C(7)-O*H*), 9.11 (1H, br s, C(5)-O*H*); δ_C for epimer A (201.1 MHz, DMSO-d_6) 25.8 (C-4"), 28.5 (C-4), 30.8 (C-3"), 47.1 (C-5"), 64.5 (C-3), 77.9 (C-2), 95.2 (C-6), 98.4 (C-10), 106.6 (C-8), 114.4 (C-2'), 114.7 (C-5'), 117.5 (C-6'), 130.4 (C-1'), 144.1 (C-4'), 144.3 (C-3'), 153.7 (C-9), 154.5 (C-7), 155.1 (C-5), 176.5 (C-2"); δ_H for epimer B (799.7 MHz, DMSO-d_6) 2.03–2.07 (1H, m, H_x-3"), 2.15–2.20 (2H, m, H_2-4"), 2.17–2.21 (1H, m, H_y-3"), 2.47–2.50 (1H, m, H_x-4), 2.67–2.71 (1H, m, H_y-4), 3.99–4.01 (1H, m, H-3), 4.32–4.90 (1H, br, C(3)-O*H*), 4.76–4.77 (1H, m, H-2), 5.13–5.16 (1H, m, H-5"), 5.99 (1H, s, H-6), 6.66–6.68 (2H, m, H-5', H-6'), 6.87 (1H, m, H-2'), 7.30 (1H, br s, NH-1"), 8.59–8.87 (2H, br, C(3')-O*H*, C(4')-O*H*), 9.03 (1H, br s, C(7)-O*H*), 9.11 (1H, br s, C(5)-O*H*); δ_C for epimer B (201.1 MHz, DMSO-d_6) 25.6 (C-4"), 28.1 (C-4), 30.6 (C-3"), 47.2 (C-5"), 64.3 (C-3), 78.1 (C-2), 95.0 (C-6), 98.4 (C-10), 106.4 (C-8), 114.5 (C-2'), 114.7 (C-5'), 117.7 (C-6'), 130.4 (C-1'), 144.2 (C-4'), 144.3 (C-3'), 153.8 (C-9), 154.2 (C-7), 155.0 (C-5), 176.4 (C-2"); HRMS (ESI+): M+H=374.12337 (delta=-0.16 ppm; $C_{19}H_{20}O_7N$). HR-ESI-MS-MS (CID=35%; rel. int. %): 356(87), 339(1), 246(5), 234(13), 222(100), 206(1), 205(8).

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References and notes

1. Khadem, S.; Marles, R. J. *Molecules* **2012**, *17*, 191–206.
2. Jain, S. K.; Bharate, S. B.; Vishwakarma, R. A. *Mini-Rev. Med. Chem.* **2002**, *12*, 632–649.
3. *Oncology Times* **2014**, *36*(10), p. 91.
4. Li, N.; Shao, L.; Zhang, C.; Zhang, M. *J. Asian Nat. Prod. Res.* **2008**, *10*, 1143–1146.
5. Jang, D. S.; Lee, G. Y.; Lee, Y. M.; Kim, Y. S.; Sun, H.; Kim, D.; Kim, J. S. *Chem. Pharm. Bull.* **2009**, *57*, 397–400.
6. Leete, E. *J. Nat. Prod*. **1982**, *45*, 605–607.
7. (a) Tanaka, T.; Watarumi, S.; Fujieda, M.; Kouno, I. *Food Chem.* **2005**, *93*, 81–87.; (b) Tanaka, T.; Matsuo, Y.; Kouno, I. *Int. J. Mol. Sci.* **2010**, *11*, 14–40.
8. For reviews on the chemistry of *N*-acyliminium ions and the synthesis of their precursors see: (a) Speckamp, W. N.; Hiemstra, H. *Tetrahedron* **1985**, *41*, 4367–4416.; (b) Speckamp, W. N.; Moolenaar, M. J. *Tetrahedron* **2000**, *56*, 3817–3856.; (c) Marson, C. M. *ARKIVOC (Gainesville, FL, U.S.)* **2001**, 1–16.; (d) Maryanoff, B. E.; Zhang, H.; Cohen, J. H.; Turchi, I. J.; Maryanoff, C. A. *Chem. Rev. (Washington, DC, U.S.)* **2004**, *104*, 1431–1628.; (e) Royer, J.; Bonin, M.; Micouin, L. *Chem. Rev. (Washington, DC, U.S.)* **2004**, *104*, 2311–2352.; (f) Yazici, A.; Pyne, S. G. *Synthesis* **2009**, 339–368.; (g) Yazici, A.; Pyne, S. G. *Synthesis* **2009**, 513–541.
9. For relevant studies on regioselective phenolic *Mannich reactions* see: (a) Tramontini, M.; Angiolini, L. *Mannich Bases – Chemistry and Uses*, CRC Press: Boca Raton, 1994; pp 32–38.; (b) Omura, Y.; Taruno, Y.; Irisa, Y.; Morimoto, M.; Saimoto, H.; Shigemasa, Y. *Tetrahedron Lett.* **2001**, *42*, 7273–7275.
10. (a) Nguyen, T. B.; Wang, Q.; Guéritte, F. *Eur. J. Org. Chem.* **2011**, 7076–7079.; (b) Nguyen, T. B.; Lozach, O.; Surpateanu, G.; Wang, Q.; Retailleau, P.; Iorga, B. I.; Meijer, L.; Guéritte, F. *J. Med. Chem.* **2012**, *55*, 2811–2819.
11. Hubert, J. C.; Wijnberg, J. B. P. A.; Speckamp, W. N. *Tetrahedron* **1975**, *31*, 1437–1441.
12. Kelder, J.; Grootenhuis, P. D. J.; Bayada, D. M.; Delbressine, L. P. C.; Ploemen, J. P. *Pharm. Res.* **1999**, *16*, 1514–1519.
13. Schinkel, A. H. *Adv. Drug Deliv. Rev.* **1999**, *36*, 179–194.
14. Hu, C.; Kitts, D. D. *Phytomedicine* **2005**, *12*, 588–597.
15. Müller, J.; Esső, K.; Dargó, G.; Könczöl, Á.; Balogh, G. T. *Eur. J. Pharm. Sci.* **2015**, *79*, 53–60.
16. Kansy, M.; Senner, F.; Gubernator, K. *J. Med. Chem.* **1998**, *41*, 1007–1010.
17. Borbás, E.; Balogh, A.; Bocz, K.; Müller, J.; Kiserdei, É.; Vigh, T.; Sinkó, B.; Marosi, A.; Halász, A.; Dohányos, Z.; Szente, L.; Balogh, G. T.; Nagy, Z. K. *Int. J. Pharm.* **2015**, *419*, 180–189.
18. Pastan, I.; Gottesman, M. M.; Ueda, K.; Lovelace, E.; Rutherford, A. V.; Willingham, M. C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4486–4490.
19. Forster, S.; Thumser, A. E.; Hood, S. R.; Plant, N. *PLOS ONE* **2012**, *7*, e33253.