Latifolians A and B, Novel JNK3 Kinase Inhibitors from the Papua New Guinean Plant *Gnetum latifolium*

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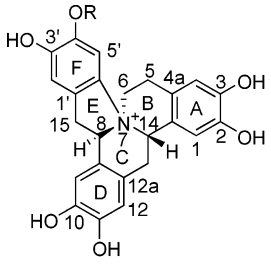
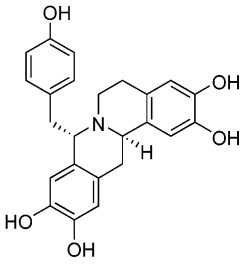
As part of our search for natural products active against the JNK3 kinase, two novel, charged benzylisoquinolines, latifolian A (**1**) and latifolian B (**2**), were isolated from the stem bark of the Papua New Guinean vine *Gnetum latifolium*. The planar structures were determined through detailed 2D NMR analysis. The relative configurations were assigned after examination of the ROESY data and through detailed molecular modeling studies.

The mitogen-activated protein kinase family of ser/thr kinases contains three classes of related kinases: the ERKs (or MAPKs); the p38 kinases; and the JNKs. JNK3 is thought to be neurone specific, as it is selectively expressed in the brain. It is thought to play a role in apoptosis and glu- or kainate-mediated neurotoxity. It is implicated in a number of neurodegenerative and immunoinflammatory diseases.[1] Inhibitors of the JNK3 kinase may therefore be expected to have therapeutic value for the treatment of these neurodegenerative and immunoinflammatory diseases. Toward this end approximately 100 000 natural extracts were screened against JNK3 kinase. An extract of the vine *Gnetum latifolium* Blume (Gnetaceae) proved to be active.

The dried and ground stem bark was extracted with a continuous flow gradient of H_2O to MeOH (0.1% TFA in both solvents). The eluent from the extraction was immediately fractionated on PAG/C18 in a continuous flow process. The selection of fractions for further purification was determined using a JNK-3 bioassay. The active material was further purified on C18 HPLC to yield pure latifolian A (**1**) in good quantity. This HPLC step also provided later eluting fractions that demonstrated inhibition of JNK-3. These fractions were combined and further purified to yield a methylated analogue of **1**, latifolian B (**2**). Both compounds were isolated as their TFA salts.



1 R = H
2 R = CH_3



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High-resolution MS analysis of **1** revealed a molecular ion (*m/z* 420.1438, M^+, Δ −0.4 mmu) consistent with a formula of $C_{24}H_{22}NO_6^+$. The ^1H NMR data (Table 1) revealed the presence of six singlet aromatic methines between δ 6.56 and 6.88. gHSQC analysis showed that these protons were attached to relatively shielded aromatic carbons (106.4−115.6 ppm). Further inspection of the NMR data revealed six exchangeable protons resonating between

Table 1. NMR Data (DMSO, ^1H, 2DNMR, 600 MHz; ^{13}C, DEPT, 125 MHz) for **1**

position	^1H δ (m, *J* Hz)	^{13}C (ppm)	gHMBC	ROESY
1	6.60 (s)	114.2	C-3, C-4a, C-14	H-14, H_2-13
2		145.5		
3		146.3		
4	6.64 (s)	115.8	C-2, C-5	H_2-5
4a		122.2		
4b		121.4		
5a	3.09 (m)	24.5		H-5′, H-4, H_b-5, H_b-6
5b	3.37 (m)		C-4a, C-6	H-4, H_a-5, H_b-6
6a	3.90 (m)	58.9	C-4a, C-5, C-6′, C-14	H-5′, H-8, H_a-5, H_b-5
6b	3.98 (m)			H-5′, H-8, H-14, H_a-5
8	5.41 (brt, 6.8)	72.4	C-6, C-8a, C-9, C-12a, C-15, C-6′, C-1′	H_a-6, H_b-6, H-9, H_b-5, H_b-15
8a		122.8		
9	6.62 (s)	113.7	C-8	H-8, $H_{a/b}$-15
10		145.1		
11		146.1		
12	6.56 (s)	115.6	C-10, C-13	$H_{a/b}$-13
12a		120.9		
13a	2.98 (dd, 4.5, 17.7)	30.7	C-8a, C-12, C-12a, C-14	H-12
13b	3.19 (dd, 6.8, 17.7)			H-12
14	5.23 (br t, 4.9)	65.1	C-4b	H-1, H-13, H-15a, H-5′
15a	3.10 (m)	36.8		H-9, H_b-15, H-2′
15b	3.70 (dd, 7.4, 16.2)		C-8a, C-1′, C-2′, C-6′,	H-8, H-9, H_a-15, H-2′
1′		124.3		
2′	6.80 (s)	112.3	C-1′ (w), C-3′, C-4′, C-6′	$H_{a/b}$-15
3′		148.6		
4′		146.3		
5′	6.88 (s)	106.9	C-1′, C-3′, C-4′, C-6′	H_a-5, H_b-6, H-14
6′		137.3		
2-OH	9.14 (s)		C-2, C-1	
3-OH	9.86 (s)[a]		C-3/C-11, C-4/C-12	
10-OH	8.95 (s)		C-9, C-10	
11-OH	9.22 (s)[a]		C-3/C-11, C-4/C-12	
3′-OH	9.07 (s)		C-3′, C-2′	
4′-OH	9.46 (s)		C-4′, C-5′	

[a] May be interchanged within a column.

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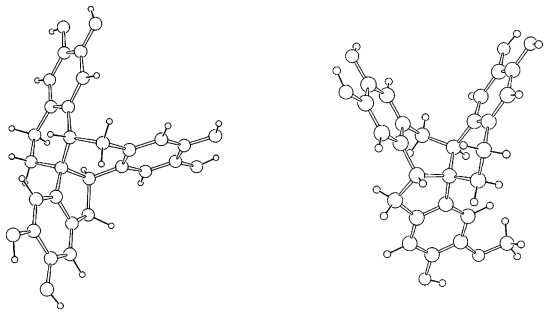

Figure 1. Energy-minimized 3D structures of latifolins A (**1**) and B (**2**) using Macromodel. For **1** ROESY correlations are explained by three minimized structures including the above, the lowest energy conformer. The energy differences between the minimized structures are small (<50 kJ/mol), i.e., all exist at room temperature (**1**: E_{min} = 269 kJ/mol, E_{27} = 295 kJ/mol, E_{502} = 305 kJ/mol). For **2** ROESY correlations are explained by two minimized structures including the minimum structure shown above (**2**: E_{min} = 337 kJ/mol, E_3 = 334 kJ/mol).

δ 8.9 and 10.0, indicative of six phenolic protons. The chemical shift of the phenolic carbons (between 145.0 and 149.0 ppm) suggested catechol-like moieties with the hydroxy groups *ortho* to each other. These ^{13}C and ^{1}H NMR data were consistent with the presence of three 1,2,4,5-tetrasubstituted benzene moieties, and this was confirmed by gHMBC analysis (Table 1). Also observed were two deshielded aliphatic methines (δ 5.23; 65.1 ppm and 5.41; 72.4 ppm) and four methylenes, one of which was relatively deshielded (δ 3.98, 3.90; 58.9 ppm). The deshielded nature of the methines and the 6-CH_2 methylene was partially explained by their proximity to the heteroatom in the benzylisoquinoline subunits. 2D NMR data then readily allowed the assembly of the A, B, C, D, and F ring systems (Table 1). gHMBC correlations from the H-8 methine were particularly important, since correlations to C-6, C-8a/C-9/C-12a, C-15, and C-6′/C-1′ established links to the B, D, E, and F rings, respectively. The final connection point from C-6′ to the nitrogen atom was deduced after consideration of the molecular formula and gHMBC data. The formation of this E ring meant the nitrogen was positively charged, which also explained the greater deshielding of C-6, C-8, and C-14 relative to molecules with similar structures (e.g., **3**).[2] This connectivity was also supported by a gHMBC correlation observed from 6-CH_2 (B ring) to C-6′ (F ring).

The relative configuration was determined from extensive molecular modeling and analysis of the ROESY data. Molecular modeling was carried out using Macromodel (Schrödinger, L.L.C) to generate a series of energy-minimized structures for all the possible stereoisomers. The H-8 and H-14 methines showed quite different ROESY correlations and no correlation to each other. Consideration of the possible models indicated they were on opposite faces of the molecule. Only one relative configuration seemed to satisfy all the ROESY data, and it was also the lowest energy structure of the possible isomers. The structure with the 7*S**, 8*R**, 14*S** relative configuration satisfied the observed correlations. While the minimum energy structure accounted for most of the observed correlations in the ROESY experiment, two other structures generated (with the same configuration) were needed to account for the remaining correlations. The three structures were very close in energy (less than 25 kJ/mol difference between the three), and the major differences were in the orientation of the methylenes in the two six-membered rings (Figure 1). From this it was deduced that **1** is somewhat flexible and is moving through a series of low energy level conformers in solution at room temperature.

Latifolian B (**2**) was clearly very similar to **1**. The (+) ESIMS of **2** revealed a molecular ion at 434, a difference of just 14 mass units compared to **1**. Accurate mass measurement confirmed the molecular formula of $C_{25}H_{24}NO_6^+$ (Δ −0.2 mmu). The ^{1}H NMR spectrum immediately revealed the nature of the additional mass with an *O*-methyl singlet resonating at δ 3.71. Correspondingly, there were five rather than six phenolic resonances in the downfield region of the spectrum. The remainder of the spectrum was very similar to that of **1**. However, the H-5′ proton resonating at δ 6.88 in **1** was now resonating at δ 7.18 in **2**. This suggested that the methoxyl was substituted at C4′. This was confirmed by gHMBC correlations (Table 2) and the observation of a ROESY correlation from the *O*-methyl protons at δ 3.71 to H-5′ (δ 7.18). Molecular modeling and the ROESY data supported this compound as having the same relative configuration as **1**. In this case all the correlations observed in the ROESY experiment could be explained by two structures: the minimum energy structure (337 kJ/mol) and the third lowest conformer found (344 kJ/mol).

Latifolians A and B are new examples of the 8-benzylberberine alkaloid structure class. A large number of berberine type alkaloids have been reported, and they are distributed in many plant families. Berberine alkaloids that have a benzyl substituent at C8 are rare and have previously been reported only from *Aristolochia* gigantea,[3,4] *A. constricta*,[5] *Gnetium parvifolium*,[2] *Talinum paniculatum*,[6] and, surprisingly, the sponge *Theonella* sp.[7] Latifolians A and B were tested for inhibition activity against JNK3. Both compounds proved to be inhibitors of the kinase at low micromolar concentrations with IC_{50}'s of 13 and 10 μM, respectively.

Experimental Section

General Experimental Procedures. Water was Milli-Q filtered, while all other solvents were Omnisolv. C18 bonded silica columns (25 cm × 10 mm) were used for semipreparative HPLC. NMR spectra were recorded on a Varian Inova 600 or 500 MHz NMR spectrometer. Samples were dissolved in DMSO, and chemical shifts were calculated relative to the solvent peak (DMSO ^{1}H δ 2.51 and ^{13}C 39.51 ppm). Mass spectra were measured on a Fisons VG Platform II, using positive electrospray ionization mode. The elution solvent was a mixture of MeCN/H_2O 50% at 0.1 mL/min. HRESIMS was recorded on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. Samples were loaded through a rheodyne injector connected to a Waters 600 HPLC pump.

Plant Material. The stem bark of *Gnetum latifolium* (Gnetaceae) (BL 248) was collected in February 1998 near Veikabu bridge along the Hiritano highway 40 km from Port Moresby, Papua New Guinea, by one of the authors (T.R.) and identified by Paul Katik from Biodiversity Ltd.

Extraction and Isolation. The air-dried stem bark (20 g) was ground and extracted using an in-line chromatography system whereby the column containing plant material was connected to an MPLC column containing polyamide gel (PAG) that was, in turn, connected to a column containing C18. A gradient of H_2O to MeOH (0.1% TFA in both solvents) was passed through the linked columns and the eluent collected into test tubes. The majority of the activity existed in a band eluting in almost 100% MeOH. This material was further purified by reversed-phase HPLC on Hypersil 5 μm C18 (250 × 10 mm) with a linear gradient from 90% H_2O/10% MeOH to 60% H_2O/40% MeOH over 15 min followed by isocratic elution for 10 min. One hundred and twenty fractions were collected, and bioactivity was located in fractions 64−67, which was pure latifolian A (75 mg, 0.75%). A second band of activity was concentrated in fractions 84−89. This material was

Table 2. NMR Data (DMSO; ^{1}H, 2D NMR, 600 MHz; ^{13}C, DEPT, 125 MHz) for **2**

position	^{1}H δ (m, J Hz)	^{13}C (ppm)	gHMBC	ROESY
1	6.59 (m)	112.8	C-3, C-4a, C-14	H-14, H_b-13
2		144.7		
3		144.7		
4	6.59 (m)	114.8	C-2, C-5	H_2-5
4a		121.6		
4b		120.4		
5a	3.10 (brdd, 4.0, 17.8)	23.6	C-4a, C-4, C-6	H-5′, H-4, H_b-5, H_b-6
5b	3.36 (m)			H-4, H_a-5, H_b-6
6a	3.92 (m)	59.6	C-5, C-6′, C-8, C-14	H-8, H_a-5, H_b-13, H_b-15
6b	4.07 (m)			H-5′ (w), H-8, H-14, $H_{a/b}$-5
8	5.47 (brt, 6.5)	70.8	C-6, C-8a, C-9, C-12a, C-15, C-1′	H_a-6, H_b-6, H-9, H_a-5, H_b-15
8a		122.6		
9	6.58 (m)	112.8	C-8, C-11	H-8, $H_{a/b}$-15
10		144.5		
11		144.7		
12	6.51 (s)	115.0	C-8a, C-10, C-13	$H_{a/b}$-13
12a		120.2		
13a	2.97 (dd, 4.0, 17.4)	28.8	C-4b, C-8a, C-12, C-12a, C-14	H-1 (w), H-12, H_b-13, H-14
13b	3.21 (dd, 5.0, 17.4)			H-1 (w), H_a-6, H-12, H_a-13, H-14
14	5.42 (br t, 4.9)	64.9	C-4b	H-1, H_a-6, H_a-13
15a	3.04 (dd, 5.1,16.1)	36.8		H-9, H_b-15, H-2′
15b	3.74 (dd, 7.6, 16.1)		C-8, C-8a, C-1′, C-2′, C-6′	H_a-6, H-8, H-9, H_a-15, H-2′
1′		125.4		
2′	6.80 (s)	111.3	C-1′ (w), C-3′, C-4′, C-5′ (w), C-6′, C-15	$H_{a/b}$-15
3′		148.4		
4′		147.9		
5′	7.18 (s)	103.2	C-1′, C-3′, C-6′	H_b-6, CH_3O-4′
6′		136.4		
2-OH	9.01 (s)		C-2, C-1	
3-OH	9.17 (s)[a]		C-3/C-11, C-4/C-12	
10-OH	8.99 (s)		C-9, C-10	
11-OH	9.09 (s)[a]		C-3/C-11, C-4/C-12	
3′-OH	9.77 (s)		C-3′, C-2′	
4′-OCH_3	3.71 (s)	56.0	C-4′	H-5′

[a] May be interchanged within a column.

further purified by reversed-phase HPLC on Hypersil phenyl 5 μm 250 × 10 mm, with a gradient from 90% H_2O/10% MeOH to 65% H_2O/35% MeOH over 13 min to yield latifolian B (6.5 mg, 0.033%) as a white solid.

Latifolian A (1): white solid (75 mg); $[\alpha]^{26}_D$ −33° (*c* 0.21, MeOH); UV (MeOH) λ_{max} (ϵ) 206 (ϵ 37 200), 290 (ϵ 6400) nm; IR (film) ν_{max} 3165, 1676, 1609, 1527, 1458, 1199, 1023 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz), see Table 1; ^{13}C NMR (DMSO, 125 MHz), see Table 1; ESI(+)MS *m/z* 420 $[M]^+$; HREIMS, *m/z* 420.1438 (M^+, $C_{24}H_{22}NO_6$, Δ −0.4 mmu).

Latifolian B (2): white solid (6.5 mg); $[\alpha]^{26}_D$ −12° (*c* 0.14, MeOH); UV (MeOH) λ_{max} 206 (ϵ 35 477), 289 (ϵ 5260) nm; IR (film) ν_{max} 3160, 1681, 1504, 1455, 1201, 1010 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz), see Table 2; ^{13}C NMR (DMSO, 125 MHz), see Table 2; ESI(+)MS *m/z* 434 $[M]^+$; HREIMS, *m/z* 434.1596 (M^+, $C_{25}H_{24}NO_6$, Δ −0.2 mmu).

Inhibition of c-Jun Amino-Terminal Kinase (JNK3) Phosphorylation of GST-ATF-2. A scintillation proximity assay (SPA) based on the inhibition of JNK-catalyzed transfer of the [γ]-phosphate group of [γ-^{33}P] ATP to biotinylated ATF-2 was set up to identify inhibitory compounds.

The assay was performed in a total volume of 27.5 μL containing 1.18 U/mL JNK3 (activated JNK3 N-terminal Hexa-His-tag) and 60 μg/mL biotinylated ATF-2 substrate in 50 mM 3-morpholinopropanesulfonic acid sodium salt (MOPS) (pH 7.2) with 150 mM NaCl, 0.1 mM EGTA, 10 mM dithiothreitol, 25 mM β-glycerolphosphate, 0.0009% Brij-35, 1.8 μM ATP, 0.18 mM $Mg(OAc)_2$, 0.88 mg/mL PVP, and 0.02 μCi [γ-^{33}P]-ATP.

Enzyme and substrate solutions were incubated separately for 30 min at room temperature and then for 1 h below 4 °C, before starting the reaction.

The enzyme solution contained 50 mM MOPS (pH 7.2) with 150 mM NaCl, 0.1 mM EGTA, 10 mM dithiothreitol, 25 mM β-glycerolphosphate, 0.0009% Brij-35, 20 μM ATP, 2.0 mM $Mg(OAc)_2$, and JNK3.

The substrate solution contained ATF-2 and [γ-^{33}P]-ATP in 50 mM MOPS (pH 7.2) with 150 mM NaCl, 0.1 mM EGTA, 10 mM dithiothreitol, 25 mM β-glycerolphosphate, 0.0009% Brij-35, 1.8 μM ATP, and 0.18 mM $Mg(OAc)_2$.

The reaction was then incubated for 20 min at room temperature and stopped by the addition of 50 μL of 0.25 mM EDTA (pH 7.6) containing 25 mg/mL streptavidin-coated SPA beads. Beads were allowed to settle for 6 h and radioactivity was counted on Microbeta Trilux.

Compounds were assayed for inhibition over 11 concentrations from 1.8 nM to 180 μM in duplicate. IC_{50} values were determined using GraphPad Prism Version 4.02.

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