

Isolation and structure identification of new cytokinins from *Gleditsia caspia*

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Abstract

Phytochemical investigation of the *n*-BuOH- soluble fraction of the alcoholic extract of the fruits of *Gleditsia caspia* (Leguminosae) resulted in the isolation and identification of three new cytokinin derivatives identified as 6-*N*-(3-methylbut-2-enylamino)-2-hydroxy-9- β -D-glucopyranosyl purine, 6-*N*-(*cis*-4-hydroxy-3-methylbut-2-enylamino)-2-hydroxy-9- β -D-glucopyranosyl purine, and 6-*N*-(3-methylbut-2-enylamino)-2-hydroxy-9-[β -D-apiofuranosyl-(1" \rightarrow 6')- β -D-glucopyranosyl] purine. The structures of the isolated compounds were established by 1D- (^1H -, ^{13}C - and DEPT) and 2D- (COSY, HMQC and HMBC) NMR analysis.

Keywords: *N*-6-Substituted adenine derivatives, cytokinins, NMR, *Gleditsia caspia*

Introduction

Cytokinins are plant hormones which regulate plant cell proliferation.¹ Most natural cytokinins are *N*-6- substituted adenine derivatives generated as degradation products during depurination of DNA.² Since their discovery, a substantial number of biochemical and genetic studies have focused on elucidating the diverse roles of cytokinins in plant growth and, in particular, water-transport and water- stress conditions.³ Cytokinins are also involved in other essential processes such as the sink/source relationship, vascular development, chloroplast differentiation, apical dominance, and senescence. It seems that these effects result from molecular interactions with other plant hormones and environmental signals.⁴ Studies using structure-activity relationships have revealed the importance of the isoprenoid side chain for cytokinin activity.⁵ Although the effects of cytokinins in plants are well known, the mechanism of their action is still not fully understood.^{6,7} Moreover, *N*-6- substituted adenine derivatives show pharmacological activity in humans, have found application in molecular medicine.² *Gleditsia caspia* (Leguminosae) is a perennial shrub that is found in temperate and sub-tropical North and South America, tropical Africa, and Central and Eastern Asia. In traditional Chinese medicine the genus *Gleditsia* is used

in the treatment of apoplexy, as an expectorant, and for a pesticide.⁸ Our literature survey indicated that triterpenoidal saponins, steroids, alkaloids, and flavonoids have been isolated from the genus *Gleditsia*.⁹⁻¹¹ We report here the isolation and structural characterization of three new cytokinins isolated for the first time from the natural source.

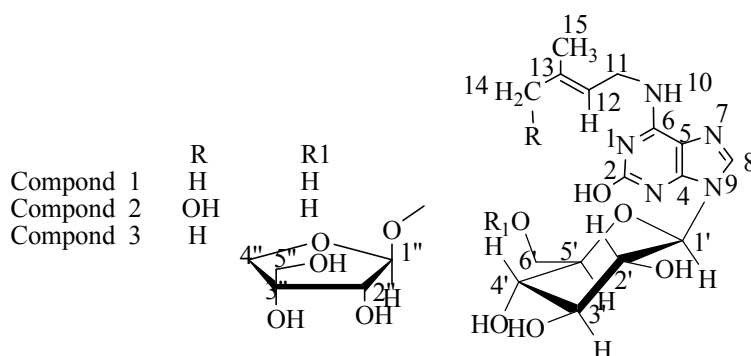


Figure 1. Structures of compounds **1**, **2** and **3**.

Results and Discussion

The fruits of *Gleditsia caspia* were extracted with ethanol and then the dried ethanolic extract was suspended in water and partitioned successively with petroleum, EtOAc, and *n*-BuOH. The *n*-BuOH fraction was passed through a polyamide column. The water eluate was chromatographed over reversed-phase silica gel to yield four fractions [A–D]. Fraction A was repeatedly chromatographed over Sephadex LH-20 and silica gel to yield three cytokinins **1–3**.

Compound **1**, was obtained as a white amorphous powder and gave on TLC yellowish-brown color with vanillin/H₂SO₄ reagent at *R_f* 0.78 with CHCl₃-MeOH-H₂O (61-32-7) as developing solvent. It displayed a [M+H]⁺ ion at *m/z* 382.1742 and a [M+Na]⁺ ion at *m/z* 404.1546 in the positive ion HR-FAB-MS spectrum, consistent with the molecular formula C₁₆H₂₃N₅O₆. Its UV spectrum showed absorption bands at 210 and 270 nm which are indicative of N-6- substituted adenine derivatives.¹² The IR spectrum showed absorption peaks at 3440, 1640 cm⁻¹, indicating the presence of hydroxyl and conjugated C=N groups, respectively. The presence of a hydroxy-adenine skeleton was indicated from the ¹H- NMR spectrum by the characteristic aromatic proton singlet at δ 8.03 and from the ¹³C- and DEPT- NMR by the presence of five sp² carbons with δ 102.90, 143.47, 153.58, 153.95 and 155.40, corresponding to C-4, -8, -6, -5 and -2, respectively, and from the presence of five nitrogen atoms as indicated from the FAB-MS. The isopentenyl side chain was indicated by an olefinic proton at δ 5.21 (1H, *br*, H-12), geminal methylene protons at δ 4.48 (2H, *br*, H₂-11) and two sharp singlets at δ 1.73 and 1.58 due to two methyl protons; H₃-14 and H₃-15, respectively, along with one methylene carbon at δ 39.95 (C-11), one methine carbon at δ 120.61 (C-12), one quaternary carbon at δ 135.34 (C-13) and two methyl carbons at 18.54 (C-14) and 25.94 (C-15), as shown in the ¹³C- and ¹³C- DEPT NMR

spectra (Table 1). The ^1H - NMR spectrum also showed broad signals at δ 4.82 (1H), 5.14 (1H), 5.41 (1H), 5.71 (2H) and 7.09 (1H) corresponding to $-\text{OH}$ and $-\text{NH}$ protons. For the assignment of the two methyl carbons in compound **1**, the more deshielded signal was assigned to the one which is *trans*- to the amino group.^{13,14}

Table 1. 1D- and 2D- NMR data (500 and 125.0 MHz in $\text{DMSO}-d_6$) for compound **1**

Position	δ_{C} , mult.	δ_{H} (J in Hz)	DQF-COSY	HMBC
2	155.40, q-C	-	-	-
4	153.95, q-C	-	-	-
5	102.90, q-C	-	-	-
6	153.58, q-C	-	-	-
8	143.47, CH	8.03, s	H-1'	C-1', 4, 5
11	39.95, CH_2	4.48, br	H-12	C-6, 12, 13, 15
12	120.61, CH	5.21, br	H-11, 14, 15	C-11, 13, 14, 15
13	135.34, q-C	-	-	-
14	18.54, CH_3	1.73, s	H-12	C-12, 13, 15
15	25.94, CH_3	1.58, s	H-12, 14	C-12, 13, 14
1'	86.85, CH	5.39, d (8.6)	H-8, 2'	C-4, 8, 2', 3', 5'
2'	73.05, CH	3.29, dd, 8.6, 9.0	H-1', 3'	C-1', 3', 4'
3'	76.69, CH	3.34*	H-2', 4'	C-1', 2', 4', 5'
4'	68.36, CH	3.46, t (9.0)	H-3', 5'	C-2', 3', 5', 6'
5'	79.94, CH	3.53, m	H-4', 6'a, 6'b	C-1', 3', 4', 6'a,b
6'a.	59.28, CH_2	3.69, dd (11.5, 2.8)	H-5, 6'b	C-4', 5'
6'b.	-	3.60, dd (11.5, 5.0)	H-5, 6'a	C-4', 5'

* The splitting pattern was masked by the water signal.

The glucose moiety was identified from the one anomeric proton and carbon resonances at δ_{H} 5.39 (H-1'), δ_{C} 86.85 (C-1') of glucose. The identity of the sugar moiety was determined by a combination of COSY, HMQC and HMBC experiments (Table 1). The β -anomeric configuration of the glucose moiety was evident from the J - H1, H2 value of 8.6 Hz.¹⁵ The attachment of the glucose moiety to the N-6-position of the hydroxyl adenine skeleton was confirmed by the long-range correlations between H-1' (δ 5.39) of glucose and the methine carbon at δ 143.47 (C-8) and the quaternary carbon at δ 153.95 (C-4), in the HMBC spectrum of **1**. Therefore the structure of **1** was established to be 6-*N*-(3-methylbut-2-enylamino)-2-hydroxy-9- β -D-glucopyranosyl purine.

Compound **2**, was also obtained as a white amorphous powder, and gave on TLC yellowish-brown color with vanillin/ H_2SO_4 reagent at R_f 0.56 with CHCl_3 -MeOH- H_2O (61-32-7) as developing solvent. It has an $[\text{M}+\text{H}]^+$ ion at m/z = 398.1694 and $[\text{M}+\text{Na}]^+$ ion at m/z 420.1515 in the HR FAB-MS, 16 mass units more than that of **1**, suggesting the molecular formula of **2** to be

C₁₆H₂₃N₅O₇. The UV spectrum showed absorption bands at 215 and 266 nm, and the IR spectrum showed absorption bands for hydroxyl group at 3445 and for conjugated C=N groups at 1640 cm⁻¹.

Table 2. 1D- and 2D- NMR data (500 and 125.0 MHz, in DMSO-*d*₆) for compound **2**.

Position	δ _C , mult.	δ _H (<i>J</i> in Hz)	DQF-COSY	HMBC
2	154.03, q-C	-	-	-
4	153.44, q-C	-	-	-
5	103.01, q-C	-	-	-
6	153.16, q-C	-	-	-
8	143.65, CH	8.08, s	H-1'	C-1', 4, 5
11	39.92, CH ₂	4.55, d (7.1)	H-12	C-6, 12, 13, 15
12	121.35, CH	5.23, t (7.1)	H-11, 14, 15	C-11, 13, 14, 15
13	139.98, q-C	-	-	-
14	60.38, CH ₂	4.07, d (5.6)	H-12	C-12, 13, 15
15	21.88, CH ₃	1.64, s	H-12, 14	C-12, 13, 14
1'	86.92, CH	5.41, d (8.4)	H-8, 2'	C-4, 8, 2', 3', 5'
2'	73.14, CH	3.32*	H-1', 3'	C-1', 3', 4'
3'	76.58, CH	3.34*	H-2', 4'	C-1', 2', 4', 5'
4'	68.27, CH	3.46, t (9.0)	H-3', 5'	C-2', 3', 5', 6'
5'	80.02, CH	3.50, m	H-4', 6'a, 6'b	C-1', 3', 4', 6' a,b
6'a	59.50, CH ₂	3.75, dd (11.4, 2.8)	H-5, 6'b	C-4', 5'
6'b	-	3.65, dd (11.4, 5.0)	H-5, 6'a	C-4', 5'

* The splitting pattern was masked by the water signal.

The NMR data of **2** were superimposable with those of **1**, verifying a close structural relationship between **2** and **1**. The difference between **2** and **1** was the presence of hydroxyl *iso*-pentenyl side chain in **2** *versus* the *iso*-pentenyl side chain in **1**. This was established from the ¹H- NMR of **2** which indicates the presence of signals for two geminal methylene protons at δ4.55 (2H, br, H₂-11) and δ4.07 (2H, brs, H₂-14), a methine proton at δ5.23 (1H, br, H-12) and one methyl group, δ 1.64 (3H, s, H₃-15). ¹³C- and ¹³C- DEPT NMR spectra confirmed the hydroxy *iso*-pentenyl side chain by the presence of two methylene carbon signals at δ 60.38 (C-14) and 39.92 (C-11), one methine carbon signal at δ 121.35 (C-12), one methyl signal at δ 21.88 (C-15), and one quaternary carbon signal at δ 139.98 (C-13). Seven broad signals at δ 4.87 (1H), 5.35 (1H), 5.50 (1H), 5.83 (2H) and 7.40 (2H) corresponding to -OH and -NH protons, were also observed in the ¹H- NMR of **2**. Combined 2D- NMR (COSY, HMQC and HMBC) experiments determine the structure of the aglycon moiety of **2** as 2-hydroxyzeatin (Table 2).

The ¹H- and ¹³C- NMR spectra of **2** revealed the presence of one β-D-glucopyranosyl moiety from the chemical shift and *J* value of one anomeric proton at δ 5.41 (d, *J* = 8.4 Hz, H-1') and the

chemical shift of one anomeric carbon at δ 86.92 (C-1'). The HMBC spectrum showed cross-peaks between the glucosyl anomeric proton (δ 5.41, H-1') and C-8 (δ 143.65) and C-4 (δ 153.44) and confirmed that the glucose moiety was attached to the N-6-position of the aglycon. The configuration of the *iso*-pentenyl group of the hydroxyzeatin was determined to be *cis*- by comparison of the chemical shifts of the ^1H - and ^{13}C - NMR signals with those of *trans*- zeatin riboside.^{13,16,17} Consequently, the structure of **2** was determined as 6-*N*-(*cis*-4-hydroxy-3-methylbut-2-enylamino)-2-hydroxy-9- β -D-glucopyranosyl purine **2**.

Compound **3**, was obtained as a white amorphous powder with molecular formula $\text{C}_{21}\text{H}_{31}\text{N}_5\text{O}_{10}$ as indicated from the HR-FAB-MS ions at m/z 514.2169 $[\text{M}+\text{H}]^+$ and 536.1988 $[\text{M}+\text{Na}]^+$. Combined with the evidence from monosaccharide composition analysis shown in the Experimental Section, this suggested that in compound **3** an apiose moiety (132 mass units) was present in addition to the skeleton present in **1**. It gave on TLC yellowish-brown color with vanillin/ H_2SO_4 reagent at R_f 0.31 with CHCl_3 -MeOH- H_2O (61-32-7) as developing solvent. Its UV and IR spectra showed absorption bands similar to **1** and **2**. The ^1H - NMR of **3** was very similar to that of **1**, but had extra signals for an additional sugar moiety indicated by the signals of two anomeric protons, the usual one at δ 5.42 (1H, d, $J = 8.4$ Hz, H-1' of glucose) and a second at δ 4.82 (1H, d, $J = 3.0$ Hz, H-1'' of apiose). The ^{13}C - NMR spectrum of **3** also showed a signal arising from an extra anomeric carbon at δ 109.65 (Table 3). The β -configuration on C-1 anomeric orientation of apiose was confirmed by comparing the ^1H - and ^{13}C - NMR of **3** with those of α -D- δ_{C} 104.5 and β -D- apiofuransides δ_{C} 111.5, respectively.^{18, 19} The signal of the glucosidic carbon, C-6' in **3** showed a typical glycosidation shift downfield by 6.6 ppm when compared to the unsubstituted C-6' of the glucose moiety in **1**, supporting the attachment of the apiose moiety to the glucosyl unit at C-6'. This was confirmed by HMBC cross-peaks between the anomeric proton of apiose and C-6' of glucose, and between H₂-6' of glucose and the anomeric carbon of apiose. Furthermore, the HMBC spectrum of **3** showed correlations between the anomeric proton of the glucose and C-4 and C-8 of the hydroxyadenine skeleton, confirming the attachment of the disaccharide moiety to the N-6- position of the aglycon.

Table 3. 1D- and 2D- NMR Spectroscopic Data (500 and 125.0 MHz in DMSO-*d*₆) for compound **3**

Position	δ_C , mult.	δ_H (<i>J</i> in Hz)	DQF-COSY	HMBC
2	155.50, q-C	-	-	-
4	153.87, q-C	-	-	-
5	102.91, q-C	-	-	-
6	153.71, q-C	-	-	-
8	143.75, CH	8.06, s	H-1'	C-1', 4, 5
11	39.84, CH ₂	4.48, d (6.8)	H-12	C-6, 12, 13
12	120.45, CH	5.20, t (6.8)	H-11, 14, 15	C-11, 13, 14, 15
13	135.51, q-C	-	-	-
14	18.52, CH ₃	1.72, s	H-12, 15	C-12, 13, 15
15	25.93, CH ₃	1.59, s	H-12, 14	C-12, 13, 14
1'	86.84, CH	5.42, d (8.4)	H-8, 2'	C-4, 8, 2', 3', 5'
2'	72.96, CH	3.34*	H-1', 3'	C-1', 3', 4'
3'	76.66, CH	3.30, t, 9.0	H-2', 4'	C-1', 2', 4', 5'
4'	68.58, CH	3.45, t (9.0)	H-3', 5'	C-2', 3', 5', 6'
5'	78.22, CH	3.65, m	H-4', 6'a, 6'b	C-1', 3', 4', 6'a,b
6'a	65.92, CH ₂	3.81, dd (11.5, 2.8)	H-5, 6'b	C-4', 5'
6'b	-	3.67, dd (11.5, 5.5)	H-5, 6'a	C-4', 5', 1''
1''	109.65, CH	4.82, d (3.0)	H-2''	C-6', 2'', 3'', 4''
2''	76.58, CH	3.83, d (3.1)	H-1''	C-1'', 3'', 4''
3''	79.22, q-C	-	-	-
4''a	73.72, CH ₂	3.85, d, 9.3	H-4''b	C-2'', 3'', 5''
4''b	-	3.59, d, 9.3	H-4''a	1''
5''a.	62.90, CH ₂	3.40, d (11.5)	H-5''b	C-2'', 3'', 4''
5''b.	-	3.31, d (11.5)	H-5''a	C-2'', 3'', 4''

* The splitting pattern was masked by the water signal.

The ¹H- NMR spectrum of **3** also showed eight broad signals for –OH and -NH protons at δ 4.08 (1H), 4.17 (1H), 4.63 (1H), 5.14 (1H), 5.51 (3H), and 7.19 (1H). Based on the above evidence, compound **3** was established to be 6-*N*-(3-methylbut-2-enylamino)-2-hydroxy-9- $[\beta$ -D-apiofuranosyl-(1''→6')- β -D-glucopyranosyl] purine.

Experimental Section

General. Melting points were obtained on a Stuart SMP3 apparatus. UV spectra were determined with a Hitachi 340 spectrophotometer, IR spectra from a Nicolet 205 FT-IR

spectrometer connected to a Hewlett-Packard Color Pro. Plotter. The ^1H - and ^{13}C - NMR measurements were obtained with a Jeol JNM ECA 500 spectrometer operating at 500 MHz (for ^1H -) and 125 MHz (for ^{13}C -) in $\text{DMSO}-d_6$ solution, and chemical shifts are expressed in δ (ppm) with reference to TMS, and coupling constants (J) in Hertz. The ^{13}C - multiplicities were determined by the DEPT pulse sequence (135°). COSY, HMBC and HMQC- NMR experiments were carried out using a Jeol JNM ECA-500 high field spectrometer. HR- FAB MS were taken on a VGZAB-HF reversed geometry mass spectrometer (BE configuration, where "B" is a magnetic sector and "E" is an electrostatic analyzer). Polyamide (ICN Biomedicals), and silica gel (Si gel 60, Merck), were used for open column chromatography. Flash column liquid chromatography was performed using J.T. Baker glassware with 40 μm Si gel (Baker) and Sepralyte C_{18} (40 μm) as stationary phases. TLC was performed on precoated silica gel 60 F_{254} (Merck) plates. Developed chromatograms were visualized by spraying with 1% vanillin- H_2SO_4 , followed by heating at 100 $^\circ\text{C}$ for 5 min or spraying the developed plates with 2% ninhydrin in acetone.

Plant material

The fruits of *G. caspia* Desf. were collected from the Al-Orman garden, Giza, Egypt in April 2002. The plant material was identified by the late Professor Nabil El-Hadidy (Faculty of Science, Cairo University, Egypt). A voucher specimen {f202} has been deposited in the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Extraction and isolation. The powdered fruits (2.25 Kg) of *G. caspia* were subjected to exhaustive extraction with 95% EtOH (4 x 8L). The alcoholic extract was dried (205 g), and suspended in H_2O (400 ml), and then partitioned successively with light petroleum (10.0 g), EtOAc (8.2 g) and *n*-BuOH (34.0 g). The *n*-BuOH- soluble fraction was applied to a column of Polyamide and washed with H_2O and 25, 50, 75, and 100% MeOH. The water fraction was re-chromatographed over a Sepralyte C_{18} - flash column to give four fractions, of A, B, C and D. Fraction A (160 mg) was repeatedly applied to Sephadex LH 20 CC using CHCl_3 -MeOH (50:50) and MeOH, 100%, to gave two fractions A1 and A2. Fractions A1 and A2 were subjected separately to silica- gel CC, eluted with CHCl_3 -MeOH (90:10-80:20) and further purification with Sephadex LH 20 CC using MeOH to afford compound **2** (15 mg) from fraction A1 and compounds **1** (20 mg) and **3** (37 mg) from fraction A2.

Compound 1. White amorphous powder from MeOH, mp 177-179 $^\circ\text{C}$, R_f 0.78 (CHCl_3 -MeOH- H_2O , 61:32:7), UV (MeOH), λ_{max} : 210, 270nm; IR (KBr) 3440, 1640 cm^{-1} ; ^1H - and ^{13}C - NMR data are shown in Table 1; HR-FAB-MS m/z 382.1742 $[\text{M}+\text{H}]^+$ (Calcd for 382.1727), 404.1563 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{16}\text{H}_{23}\text{N}_5\text{O}_6$).

Compound 2. White amorphous powder from MeOH, mp 175-177 $^\circ\text{C}$, R_f 0.56 (CHCl_3 -MeOH- H_2O , 61:32:7), UV (MeOH), λ_{max} , 215, 266 nm, IR (KBr): 3445, 1640 cm^{-1} . ^1H - and ^{13}C - NMR data are shown in Table 2 HR-FAB-MS m/z 398.1694 $[\text{M}+\text{H}]^+$ (Calcd for 398.1676), 420.1515 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{16}\text{H}_{23}\text{N}_5\text{O}_7$).

Compound 3. White amorphous powder from MeOH, mp 191-193 °C, R_f 0.31 (CHCl₃-MeOH-H₂O, 61:32:7), UV (MeOH, λ_{\max}) 215, 270 nm. IR (KBr): 3445, 1640 cm⁻¹. ¹H- and ¹³C- NMR data are shown in Table 3 HR-FAB-MS m/z 514.2169 [M+H]⁺ (Calcd for 514.2149), 536.1988 [M+Na]⁺ (Calcd for C₂₁H₃₁N₅O₁₀).

Acid hydrolysis of compounds 1, 2 and 3.²⁰ Samples (5 mg) of 1, 2 and 3 were separately hydrolyzed with 0.1 N H₂SO₄ (1.0 ml) at 100 °C for 2 hours. After neutralization with NaHCO₃, the solvent was evaporated. The water-soluble part of the residue showed a spot at the same R_f as glucose for 1 and 2, and glucose and apiose for 3, on TLC (silica gel 14:6:1 CHCl₃-MeOH-H₂O).

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