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Identification of a major cytokinin in cocor' . milk

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Abstract. A major cytokinin found in coconut milk was isolated by using the tobacco callus growth-promoting assay as a guide during purification. The structure of the factor was determined to be *14*-O-{3-O-[β-D-galactopyranosyl-(1 → 2)-α-D-galactopyranosyl-(1 → 3)-α-L-arabinofuranosyl]-4-O-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-*trans*-zeatin riboside [G_3A_2-ZR] by various NMR techniques, including heteronuclear multiple bond connectivity by 2D multiple quantum NMR (HMBC), as well as mass spectroscopy and sugar analysis. The optimum concentration of G_3A_2-ZR for cytokinin activity in the tobacco callus assay was estimated to be 5×10^{-6} M, so that G_3A_2-ZR is one order of magnitude more potent than 1,3-diphenylurea and one order less potent than zeatin riboside. At least 20% of the cytokinin activity of coconut milk could be attributed to G_3A_2-ZR.
Key words. Coconut milk; cytokinin; zeatin; glycoside.

Cytokinins are plant hormones which regulate plant cell proliferation[1]. One of the classic sources of cytokinin activity is coconut milk, the fluid endosperm of the coconut (*Cocos nucifera L.*)[1,2]. A number of attempts to identify cytokinins in coconut milk have been reported. 1,3-Diphenylurea was isolated as a factor, though it has not yet been established whether the compound is really produced by the plant or is an artifact generated during the extraction procedure[3–5]. It has also been reported that coconut milk contains potent cytokinins such as zeatin, zeatin riboside (ZR), and O-glucosylzeatin[6–9]. However, the concentrations of these cytokinins, as well as the reported concentration of 1,3-diphenylurea, are quite low, and less than 1% of the cytokinin activity of coconut milk can be accounted for by these known cytokinins. In this paper, we report the isolation and structural identification of a major cytokinin which accounts for at least 20% of the cytokinin activity of coconut milk. The progress of the isolation was monitored by use of the tobacco callus growth-promoting assay[10].

Materials and methods

Coconut milk (67.45 l from 250 fruits), whose optimum concentration for cytokinin activity in the tobacco callus assay[10] was estimated to be 5% v/v (equivalent to 5×10^{-8} M zeatin, 3×10^{-7} M ZR, or 5×10^{-5} M 1,3-diphenylurea in the same assay), was subjected to polystyrene XAD-2 column chromatography (98ϕ × 530 mm). The major active fraction was eluted with MeOH (27 l, 29 g of residue after evaporation), and the residue was further separated on an LH-20 column (75ϕ × 450 mm, H_2O). The major activity was eluted at V_e/V_o of 1.6–2.6 and gave 18 g of residue after evaporation. Aliquots of the residue were subjected to HPLC on a Polygosil $_5C_{18}$ ODS column (8ϕ × 250 mm) eluted with 8% CH_3CN in H_2O. Several peaks were identified as active factors in the tobacco callus assay, with one major peak of activity giving 160 mg of residue after evaporation. We could not identify cytokinin activity in the fractions at retention times corresponding to those of authentic zeatin, ZR, or 1,3-diphenylurea, suggesting that the coconut milk used contained very small amounts, if any, of these known cytokinins[5]. The crude active residue obtained (160 mg) was further purified by the same HPLC to give 36 mg of chromatographically pure active factor (optimum concentration of 3 ppm), which was used for structural identification.

Results and discussion

The ^{1}H-NMR spectrum (49 protons bound to carbon), ^{13}C-NMR spectrum (43 carbons), and high resolution fast atom bombardment mass spectroscopy (HRFAB-MS) (M + H = 1102.4088), indicated that the molecular formula of the factor was $C_{43}H_{67}N_5O_{28}$ (calcd. M + H = 1102.4051). All the ^{1}H and ^{13}C signals could be assigned reasonably by H-H and C-H COSY techniques (table). Bonds were connected by HMBC and RCOSY techniques to give a planar structure of (hexose-hexose-pentose)-(pentose)-hexose-ZR (fig.). The sugar connections and branching were also supported by the MS/MS technique, which suggested a structure of (m/z 163-m/z 162-m/z 132) (m/z 133)-m/z 161-m/z 16-m/z 68-m/z 133

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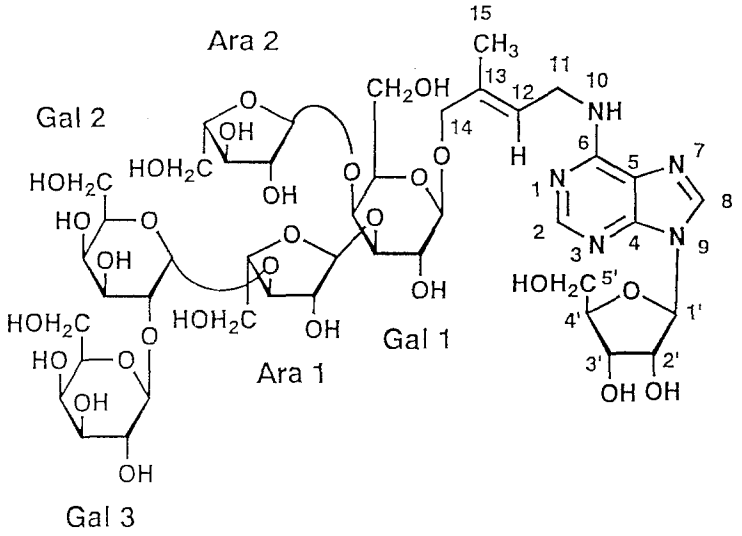
Table. NMR assignments of G_3A_2-ZR.

Position	^{13}C mult	^{1}H mult	J, Hz	HMBC (C no.)
Zeatin riboside				
2	153.5 d	8.14 s		4, 6
4	148.5 s			
5	120.4 s			
6	155.2 s			
8	141.0 d	8.21 s		4, 5
11	39.5 t	4.15 br		
12	125.9 d	5.69 br t	6.0	11, 14, 15
13	136.1 s			
14a	75.5 t	4.17 d	12.6	12, 15, Gal. 1 1
14b		4.28 d	12.6	12, 15, Gal. 1 1
15	14.6 q	1.79 s		12, 13, 14
1′	89.4 d	6.00 d	6.0	4, 8, 2′, 3′
2′	74.9 d	4.74 dd	5.0, 6.0	1′, 4′
3′	71.7 d	4.41 dd	3.2. 5.0	1′, 4′, 5′
4′	86.8 d	4.28 ddd	2.8, 3.8, 6.4	3′
5′a	62.6 t	3.83 dd	3.8, 12.8	3′
5′b		3.92 dd	2.8, 12.8	3′
Galactose 1				
1	102.0 d	4.43 d	8.0	14, Gal. 1 3
2	71.7 d	3.73 dd	8.0, 10.2	Gal. 1 1, Gal. 1 3
3	80.8 d	3.79 br d	10.2	Gal. 1 2, Ara. 1 1
4	75.1 d	4.13 br d	2.8	Gal. 1 3, Ara. 2 1
5	75.8 d	3.64 dr t		Gal. 1 1, Gal. 1 6
6	62.2 t	3.72 m		Gal. 1 4, Gal. 1 5
Arabinose 1				
1	110.3 d	5.27 d	2.0	Gal. 1 3, Ara. 1 4
2	81.1 d	4.39 dd	2.0, 3.6	Ara. 1 1, Ara. 1 3
3	86.0 d	3.96 dd	3.6, 7.4	Ara. 1 2, Gal. 2 1
4	83.4 d	4.31 m		Ara. 1 3
5a	62.5 t	3.77 m		Ara. 1 3, Ara. 1 4
5b		3.95 m		Ara. 1 3, Ara. 1 4
Galactose 2				
1	100.9 d	5.28 d	4.6	Ara. 1 3, Gal. 2 5
2	79.4 d	3.92 dd	4.6, 6.0	Gal. 2 3, Gal. 3 1
3	69.1 d	4.03 br		Gal. 2 2
4	70.3 d	4.04 br		Gal. 2 2, Gal. 2 3
5	72.1 d	4.09 m		Gal. 2 1, Gal. 2 6
6a	62.2 t	3.75 br		Gal. 2 4, Gal. 2 5
6b		3.77 br		Gal. 2 4, Gal. 2 5
Galactose 3				
1	106.0 d	4.53 d	7.8	Gal. 2 2, Gal. 3 5
2	72.1 d	3.60 dd	7.8, 10.0	Gal. 3 1, Gal. 3 3
3	73.7 d	3.67 dd	3.4, 10.0	Gal. 3 1, Gal. 3 2
4	69.6 d	3.94 br d	3.4	Gal. 3 2, Gal. 3 3
5	76.1 d	3.70 br		Gal. 3 4, Gal. 3 6
6a	62.0 t	3.79 br		Gal. 3 4, Gal. 3 5
6b		3.82 br		Gal. 3 4, Gal. 3 5
Arabinose 2				
1	109.4 d	5.40 d	1.2	Gal. 1 4, Ara. 2 4
2	82.4 d	4.18 d	1.2, 3.4	Ara. 2 3, Ara. 2 4
3	77.8 d	3.93 br		Ara. 2 2, Ara. 2 4
4	85.1 d	4.06 br		Ara. 2 3
5a	62.4 t	3.72 br		Ara. 2 3, Ara. 2 4
5b		3.83 br		Ara. 2 3, Ara. 2 4

(N_5-containing)-m/z 133. The ^{1}H-NMR signals at 1.79 (CH_3 group), 5.69 (olefinic proton), and two aromatic proton signals (8.14 and 8.21) characteristic of the adenine skeleton, as well as the corresponding ^{13}C-NMR signals, suggested the presence of a zeatin moiety.

The sugar components were analyzed by GLC after successive treatments of 116 µg of the isolated factor with trifluoroacetic acid (TFA) (hydrolysis), $NaBH_4$ (reduction to give alditols) and $(CH_3CO)_2O$ (acetylation)[11]. GLC analysis (OV-101 wall coated open tubular column (WCOT) column) of the reaction mixture indicated the presence of galactose, arabinose (or lyxose) and ribose with the molar ratio of 3.02:2.00:0.78 (roughly 3:2:1), based on a comparison with authentic sugars as standards. Though arabinose cannot be distinguished from lyxose by this GLC method, because both



Molecular formula	$C_{43}H_{67}N_5O_{28}$
HRFAB-MS (m/z)	
calcd:	1102.4051(M+H)
found:	1102.4088
$[\alpha]_D^{25}$	-5.17° (c=1, H_2O)
UV λ_{max}nm(ε):H_2O	210(16600) 267(18200)
IR ν_{max}(KBr)cm^{-1}	3400, 1628, 1080, 1045

Figure. Structure of G_3A_2-ZR.

of the sugars gave the same (or an enantiomeric pair of) alditol acetate product[11,12], the presence of lyxose was excluded by the ^{13}C-NMR spectrum[13]. Therefore, the structure of the active factor was determined to be Gal 1→2 Gal 1→3 Ara 1→3 (Ara 1→4) Gal→zeatin riboside (fig.).

The α/β-configuration of the sugar moieties was determined by measuring ^{1}H-^{1}H coupling constants. Z/E-Configuration of the isopentenyl moiety was determined by comparison of the ^{13}C-NMR spectrum with those of Z- and E-zeatin ribosides. For determination of the absolute configuration of each sugar component, 200 µg of the active factor was successively treated with TFA (hydrolysis), hydroxylamine (oximation) and $(CH_3CO)_2O$ (dehydration and acetylation) to give aldononitrile acetates[14]. The aldononitrile acetates were separated by silica gel and octadecylsilane (ODS) chromatography, and then analyzed by HPLC using Chiralcel OD-H (Daicel Chemical Ltd.). Comparison of the retention times of the aldononitrile acetates on HPLC with those of authentic aldononitrile acetates prepared from standard sugars revealed that the galactose and ribose had D-configuration, and arabinose, L-configuration. The final determined structure, 14-O-{3-O-[β-D-galactopyranosy l-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-O-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-*trans*-zeatin riboside (G_3A_2-ZR), is shown in the figure.

Thus, we isolated a major cytokinin in coconut milk and determined its structure to be G_3A_2-ZR. G_3A_2-ZR was estimated to have an optimum concentration of 5×10^{-6} M, being one order of magnitude more potent than 1,3-diphenylurea and one order less potent than ZR. Thus, the amount of G_3A_2-ZR (36 mg) isolated from 67.45 l of coconut milk corresponds to 5% of the total activity elicited by the coconut milk. Because HPLC analysis of partially purified coconut milk indicated that the amount of G_3A_2-ZR is more than 2.37 mg/l, at least 20% of the cytokinin activity of coconut milk can be attributed to this compound. Considering the losses during purification of the factors, we conclude that G_3A_2-ZR is a (or the) major cytokinin in coconut milk. Though we identified several other active factors in coconut milk, the cytokinin activity of each factor was less than 1% of the activity elicited. We could not unequivocally identify 1,3-diphenylurea, zeatin, or ZR[5].

A recent study of the molecular mechanism of cytokinin action suggests that the active form of ZR or other glycosylated zeatins is zeatin[15]. The cytokinin activity of G_3A_2-ZR might also be elicited after its hydrolysis to zeatin in the cells. G_3A_2-ZR is highly soluble in water while zeatin and zeatin riboside are lipophilic and rather insoluble in water. Production of a highly water-soluble cytokinin (or cytokinin precursor), i.e., G_3A_2-ZR, and its accumulation in coconut milk might be beneficial for nourishing the immature coconut embryo, which later produces a spongy mass of cotyledonary tissue that eventually fills the central cavity of the seed.

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