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Structural Identification of a Major Cytokinin in Coconut Milk as 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

Hisayoshi KOBAYASHI,[a] Naoko MORISAKI,[a] Yoshitaka TAGO,[a] Yuichi HASHIMOTO,*,[a] Shigeo IWASAKI,[a] Emiko KAWACHI,[b] Ryuji NAGATA,[b] and Koichi SHUDO[b]

Institute of Molecular and Cellular Biosciences,[a] University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113 and Faculty of Pharmaceutical Sciences,[b] University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.
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A cytokinin isolated from the fluid endosperm of *Cocos mucifera* L. (coconut milk), accounting for more than 20% of the total cytokinin activity, was structurally analyzed by NMR techniques, mass spectrometry, and sugar analysis by high performance liquid chromatography (HPLC). The planar structure of the cytokinin was deduced from its NMR and mass spectrometric data. The structure of the sugar moiety, including its absolute structure, was determined by HPLC analysis of alditol acetates and aldononitrile acetates derived from the cytokinin. The configuration of the sugar-sugar bonds was determined by NMR, and the structure was finally identified as 14-*O*-{3-*O*-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-*O*-(α-L-arabino-furanosyl)-β-D-galactopyranosyl}-*trans*-zeatin riboside.

Key words cytokinin; coconut milk; zeatin; glycoside; chiral HPLC

Cytokinins, like auxins and gibberellins, are important plant hormones which regulate many aspects of plant growth and development.[1,2] They play a decisive role in cell division and differentiation, while auxins and gibberellins control cell extension. In 1941, van Overbeek *et al.* found that coconut milk (the fluid endosperm of *Cocos nucifera* L.) possesses potent growth-promoting activity on plant tissue (callus) culture,[3,4] and thus coconut milk has been regarded as a classical source of cytokinin(s). Various studies on the purification and structural identification of the cytokinins in coconut milk have been reported. In 1952, Shantz and Steward isolated three factors with cytokinin activity,[5] and three years later, they determined the structure of the most potent factor among them to be 1,3-diphenylurea.[6] However, the cytokinin activity of this compound is very weak, accounting for less than 1% of the activity elicited by coconut milk. In addition, the presence of 1,3-diphenylurea as a natural product in plants has been disputed; it may be a contaminant.[7] Though a very potent cytokinin, kinetin, was found by Miller *et al.* in the same year, it is produced by pyrolysis of DNA, and is not a natural product.[8,9] Many researchers have tried to identify cytokinins in coconut milk, and various factors, including hexitols,[10] zeatin and its riboside (firstly isolated from *Zea mays* as a potent cytokinin),[11] have been reported. However, these known cytokinins[5-7,9,11] still account for less than 1% of the cytokinin activity of coconut milk.

Recently, we have reported the isolation of a major cytokinin in coconut milk.[7] The progress of the isolation was monitored by use of the tobacco callus growth-promoting assay.[12] On the basis of this assay method, the cytokinin we have isolated was estimated to represent at least 20% of the total cytokinin activity elicited by coconut milk.[7] We also reported the structure of the cytokinin as 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 in the short communication,[7] and named the compound "gazer". Here we present full details of the structural identification of gazer.

Results

NMR and Mass Spectrometric Study As already reported,[7] 160 mg of crude active compound was obtained from coconut milk (67.45 l from 250 fruits). It was further purified by HPLC to give 36 mg of chromatographically pure active factor. The isolated cytokinin, gazer, was analyzed by high-resolution fast atom bombardment mass spectrometry (HRFAB-MS) to give an $(M+H)^+$ ion peak of 1102.4088, suggesting the molecular formula of the factor to be $C_{43}H_{67}N_5O_{28}$ (calcd $M+H=1102.4051$).

NMR spectra of gazer were measured in deuterated water. The ^{13}C-NMR spectrum of gazer (Table 1) indicated the presence of six anomeric carbons (δ: 89.4, 100.9, 102.0, 106.0, 109.4, and 110.3). The ^{1}H-NMR spectrum (Table 1) correspondingly showed the signals of six anomeric protons [δ: 4.43 (d, $J=8.0$ Hz), 4.53 (d, $J=7.8$ Hz), 5.27 (d, $J=2.0$ Hz), 5.28 (d, $J=4.6$ Hz), 5.40 (d, $J=1.2$ Hz), and 6.00 (d, $J=6.0$ Hz), suggesting the presence of six sugar moieties. All protons and carbons of the six sugars were assigned by the ^{1}H–^{1}H correlation spectroscopy (COSY), ^{13}C–^{1}H COSY and heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments. The coupling constants of ^{1}H-NMR and the chemical shifts of ^{13}C-NMR suggested the presence of three galactosyl, two arabinosyl, and one ribosyl moieties.[13]

As aromatic protons, two singlets (δ: 8.14 and 8.21) were found. These two characteristic singlets, as well as the ^{13}C-NMR spectrum, which indicates the presence of five sp^2 carbons [δ: 120.4 (s), 141.0 (d), 148.5 (s), 153.5 (d), 155.2(s)], and the presence of five nitrogen atoms

* To whom correspondence should be addressed.

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Table 1. ^{13}C-NMR and ^{1}H-NMR Chemical Shifts [δ, ppm from 3-(Trimethylsilyl) Propanesulfonic 2,2,3,3-d_4 Acid, Na Salt], Multiplicities, Coupling Constants (J, Hz) and HMBC Correlations (Carbon Number) of Gazer in D_2O at 35 °C

Position	^{13}C-NMR[a]	^{1}H-NMR[a]	J	HMBC
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, 3.2	3′
5′a	62.6 t	3.83 dd	3.8, 12.8	3′
5′b		3.92 dd	2.8, 12.8	3′
Galactopyranosyl-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 br t		Gal.1 1, Gal.1 6
6	62.2 t	3.72 m		Gal.1 4, Gal.1 5
Arabinofuranosyl-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
Galactopyranosyl-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
Galactopyranosyl-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
Arabinofuranosyl-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

a) s, singlet; d, doublet; t, triplet; q, quarted; m, multiplet; br, broad.

from HRFAB-MS, suggested the presence of an adenine skeleton. An olefinic proton triplet (δ: 5.69, J=6.0 Hz), geminal methylene protons [δ: 4.17 (d, J=12.6 Hz), 4.28 (d, J=12.6 Hz)] and a sharp singlet due to methyl protons (δ: 1.79), and two sp^2 carbons [δ: 125.9 (d), 136.1 (s)], one methyl carbon (δ: 14.6) and two sp^3 carbons [δ: 39.6 (t), 75.5 (t)] suggested the presence of a zeatin moiety.

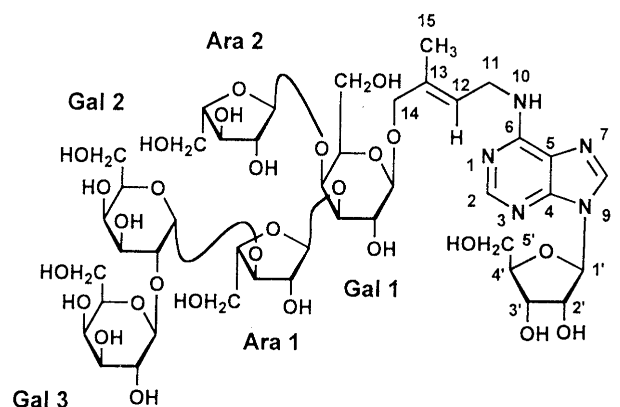

Fig. 1. Structure of Gazer

The HMBC experiments showed the bond connections of six sugar moieties and the zeatin moiety to give a planar structure of galactopyranosyl-(1→2)-galactopyranosyl-(1→3)-arabinofuranosyl-(1→3)-[arabinofuranosyl-(1→4)]-galactopyranosyl-zeatin riboside (Fig. 1). The configuration of the isopentenyl group of the zeatin moiety was determined to be *trans* by comparison of the chemical shifts of the ^{1}H- and ^{13}C-NMR signals with those of authentic *trans*- and *cis*-zeatin ribosides. This planar structure is consistent with the FAB mass spectral data. The protonated molecular ion peak at m/z 1102 (M+H) was analyzed by means of collisionally activated dissociation (CAD) technique (Fig. 2).[14] As shown in the figure, fragment ions were observed at m/z 970, 807, 675, 646, 512, 381 and 351. The pattern is consistent with the branching structure of gazer deduced from NMR experiments.

Sugar Analysis The presence of three galactosyl, two arabinosyl, and one ribosyl moieties in gazer was deduced from NMR spectroscopic studies (*vide supra*). To confirm this, a small portion of gazer was hydrolyzed with 2 M trifluoroacetic acid followed by reduction with sodium borohydride to give a mixture of alditols. These alditols were acetylated by acetic anhydride to give a mixture of alditol acetates, which were analyzed by gas-liquid chromatography (GLC) on an OV-101 WCOT column. The chromatogram indicated the presence in the mother mixture of galactose, arabinose (or lyxose) and ribose with a molar ratio of 3.02 : 2.00 : 0.78 (roughly 3 : 2 : 1), based on a comparison with authentic sugars as standards. Though arabinose and lyxose cannot be distinguished by this analytical method because both sugars are led to the same alditol acetate in the reaction used,[15] the presence of lyxose was excluded by the ^{13}C-NMR spectrum of gazer.[16]

To determine the absolute configuration of the sugar moieties, a small portion of gazer was hydrolyzed with 2 M trifluoroacetic acid, then the products were oximated with hydroxylamine, dehydrated, and acetylated with acetic anhydride to give a mixture of aldononitrile acetates (Chart 1).[17] Authentic enantiomers of aldononitrile acetates were prepared similarly from standard samples of D- and L-isomers of galactose, arabinose, and ribose. The enantiomeric pairs of these authentic sugars could each be separated by HPLC on a Chiralcel OD-H column

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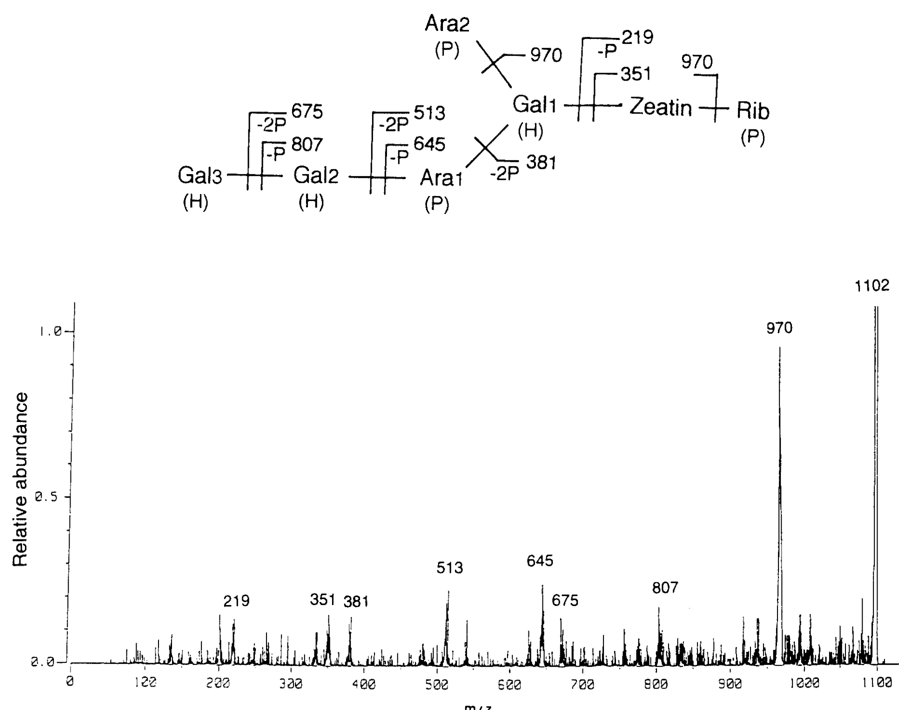

Fig. 2. Collisionally Activated Dissociation (CAD) Spectrum of the $(M+H)^+$ Ion, *m/z* 1102, of Gazer and Deduced Fragment Connections

H, hexose; P, pentose.

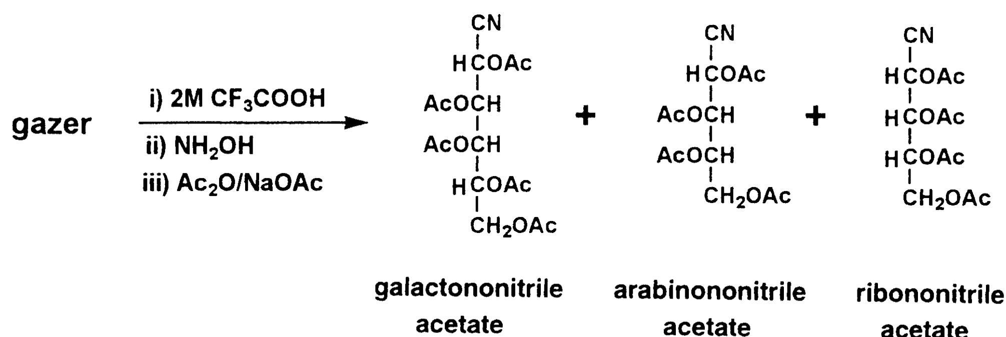

Chart 1. Derivatization to Aldononitrile Acetates

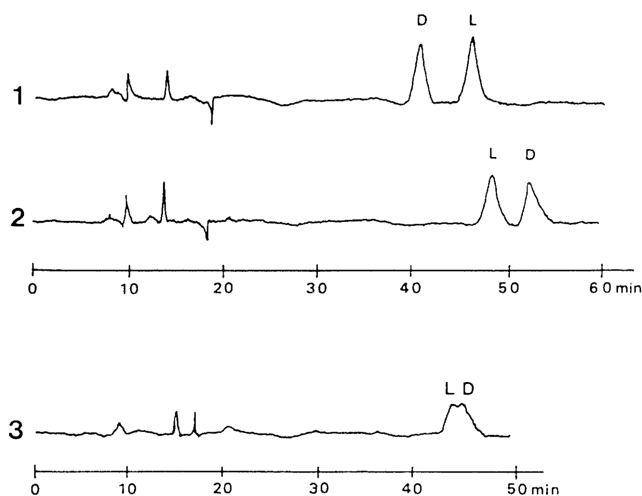

Fig. 3. Separation of Authentic Aldononitrile Acetate Enantiomers by Chiral HPLC

Aldononitrile acetates derived from D- and L-arabinose (1), galactose (2) and Ribose (3). Column, Chiralcel OD-H (4.6×250 mm); eluent, (panel 1 and 2) *n*-hexane : 2-propanol : CH_3CN = 97 : 2 : 1, (panel 3) *n*-hexane : 2-propanol = 97 : 3; flow rate, 0.5 ml/min; temperature, 23 °C; detection, UV 215 nm.

(Fig. 3). A mixture of aldononitrile acetates derived from gazer was separated by silica gel column chromatography and HPLC on an ODS column to give aldononitrile acetates derived from galactose, arabinose and ribose. Each fraction was analyzed by HPLC on a Chiralcel OD-H column (Fig. 4). Comparison of the retention times of the aldononitrile acetates with those of authentic enantiomers revealed that the galactose and ribose derived from gazer have D-configuration, and the arabinose has L-configuration.

Then, the α/β configuration of the sugar connections was determined by measuring the ^1H–^1H coupling constants and by comparing the ^{13}C-NMR chemical shifts with those of authentic sugars.[14] The structure of gazer 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 (Fig. 1).

Discussion

We have isolated a major cytokinin, gazer, in coconut milk and identified it as 14-*O*-{3-*O*-[β-D-galactopyranosyl-

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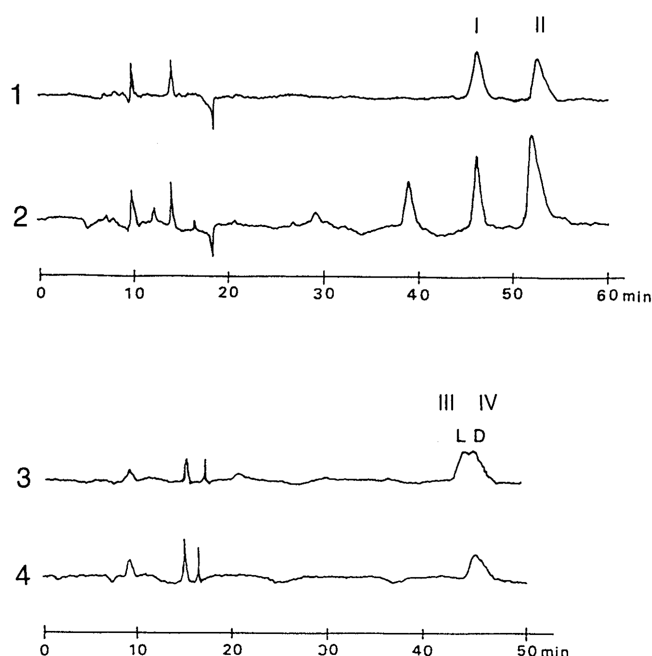

Fig. 4. Determination of the Absolute Stereochemistry of Aldononitrile Acetates Derived from Gazer by Chiral HPLC

The nitrile acetates derived from L-arabinose (peak I in panel 1), D-galactose (peak II in panel 1), L-ribose (peak III in panel 3), D-ribose (peak IV in panel 3), and gazer (panel 2 and 4). Panel 1 and 2: column, Chiralcel OD-H (4.6 × 250 mm); eluent, *n*-hexane : 2-propanol : $CH_3CN = 97:2:1$; flow rate, 0.5 ml/min; temperature, 23 °C; detection, UV 215 nm. panel 3 and 4: column, Chiralcel OD-H (4.6 × 250 mm); eluent, *n*-hexane : 2-propanol = 97 : 3; flow rate, 0.5 ml/min; temperature, 23 °C; detection, UV 215 nm.

(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-*O*-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-*trans*-zeatin riboside. The optimum concentration of gazer for tobacco callus growth-promoting activity is 5 μM,[7] so the compound is one order of magnitude more potent than 1,3-diphenylurea and one order less potent than zeatin riboside. As already reported, we could not detect 1,3-diphenylurea in coconut milk.[7] Though the role of 1,3-diphenylurea as a natural cytokinin remains controversial, the cytokinin activity of the compound is indisputable. Structural development of 1,3-diphenylurea has led to various very potent synthetic cytokinins with the pyridylurea skeleton.[1] The natural and synthetic cytokinins currently known can be divided structurally into two categories, *i.e.*, purine-type and urea-type cytokinins.[1] These two types of cytokinins are considered to be structurally related,[1] and we have reported the presence of a putative cytokinin receptor, CSBP (cytokinin-specific binding protein), which binds both purine-type and urea-type cytokinins in a mutually competitive manner.[18]

Gazer can be regarded as a glycosylated zeatin, and is classified structurally as a purine-type cytokinin. However, gazer does not bind CSBP,[7] suggesting that gazer elicits its cytokinin activity after hydrolysis to zeatin in plant cells. Therefore, gazer might be a storage form or precursor of zeatin. Gazer is highly soluble in water because of the presence of the six sugar moieties, while zeatin and zeatin riboside are lipophilic and hardly soluble in water. Production of highly water-soluble gazer 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. We noticed during the separation of gazer that coconut milk contains many substances with cytokinin activity, though their contents in coconut milk are low. Among these factors, several appear to be physicochemically very similar to gazer, *i.e.*, similar behavior in various kinds of chromatography, high hydrophilicity, and ultraviolet spectra almost indistinguishable from that of gazer (UV λ_{max} in water, 210 and 267 nm with ε of 16600 and 18200, respectively). These factors might be relatives of gazer which differ in their sugar components or linkages.

An analysis of these factors and an investigation of the relationship between their pattern and the growth-stage of the coconut fruit are planned.

Experimental

General Mass (FAB-MS, HRFAB-MS and CAD) spectra were recorded on a JEOL JMS-HX110 double-focusing mass spectrometer of E (electric field)-B (magnetic field)-E arrangement. NMR spectra were measured with a JEOL JNM A-500 (^1H: 500 MHz, ^{13}C: 125 MHz). IR spectrum was recorded on a JASCO A-102 infrared spectro-photometer. Optical rotation was measured on a JASCO DIP-100 polarimeter. UV spectrum was recorded on a Shimadzu UV-300 spectrophotometer. GLC was performed on a Shimadzu GC-7A apparatus, and peak areas were measured by use of a Shimadzu Chromatopac C-R2A. HPLC was performed on a Tosoh CCPM equipped with a Shimadzu photodiode array UV-VIS detector SPD-M6A. Chemical reactions of gazer and authentic sugar samples were performed in a Pierce Reacti-Vial (0.3 ml).

Extraction and Purification of Gazer Coconut milk (67.45 l) from 250 fruits of *Cocos nucifera* L. was used as the starting material for cytokinin separation.[7] The progress of the isolation was monitored by following the tobacco callus growth-promoting activity.[12] Briefly, the coconut milk was subjected to Amberlite XAD-2 column chromatography. Though cytokinin activity was contained in several fractions, the major active fraction eluted with methanol was collected, and further separated by Sephadex LH-20 column chromatography eluted with water. The active fractions (V_e/V_o 1.6—2.6) contained 18 g of residue after evaporation, and this was subjected to repeated semi-preparative HPLC on a Polygosil 5C18 ODS column eluted with 8% acetonitrile in water. Though several peaks were identified as active factors, the major peak of activity, giving 160 mg of residue after evaporation, was collected. The residue was further purified by the same HPLC to give 36 mg of chromatographically pure active factor, named gazer, which shows tabacco callus growth-promoting activity with an optimum concentration of 3 ppm.

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 (Gazer) $[\alpha]_D^{25}$ −5.17° ($c=1$, H_2O). IR (KBr) cm^{-1}: 3400, 1628, 1080, 1045. UV λ_{max} (H_2O) nm (ε): 210 (16600), 267 (18200). HRFAB-MS: Calcd for $C_{43}H_{68}N_5O_{28}$ [MH^+]: 1102.4051. Found: 1102.4088. ^1H- and ^{13}C-NMR: given in Table 1.

Component Sugar Analysis. (1) Derivatization to Alditol Acetates and Their Analysis Gazer (16 μg) was dissolved in 75 μl of 2 M trifluoroacetic acid in water, and heated at 121 °C in a Reacti-Vial for 1 h. Then the solution was evaporated to dryness, and the residue was redissolved in 50 μl of 1 M NH_4OH containing 0.5 mg $NaBH_4$. The mixture was allowed to stand at room temperature for 16 h, and evaporated to dryness. The residue was treated with acetic anhydride (50 μl) at 121 °C for 3 h. The mixture was evaporated to dryness and the resulting alditol acetates were extracted with $CHCl_3$. An aliquot of the alditol acetate extract was subjected to GLC on an OV-101 WCOT column [0.2 mm × 25 m, N_2 gas flow rate of 50 ml/min, 190 °C (4 min), heating rate of 1 °C/min]. Retention time (ratio); ribitol acetate: 13.2 min (0.78), arabinitol acetate or lyxitol acetate: 13.6 min (2.00), galactitol acetate: 24.6 min (3.02).

(2) Derivatization to Aldononitrile Acetates and Their Analysis Gazer (1 mg) was hydrolyzed with 2 M trifluoroacetic acid in water (200 μl) as described above, then, $NH_2OH \cdot HCl$ (1 mg), NaOAc (2.5 mg) and MeOH (200 μl), were added to the solution. The mixture was heated at 60 °C

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for 1 h, and then evaporated. The residue was treated with Ac_2O (200 μl) at 120 °C for 1 h. Then the solution was evaporated, and the aldononitrile acetates were extracted by addition of water (150 μl) and $CHCl_3$ (100 μl). The organic phase was washed with water three times, then fractionated by silica gel (0.4 ml) column chromatography (eluted with $CHCl_3$). Fractions containing aldononitrile acetates were evaporated and the residue was dissolved in 50 μl of CH_3CN. The solution was subjected to HPLC with an ODS column (eluted with 45% CH_3CN in water). The purified aldononitrile acetates thus obtained were analyzed by HPLC with a Chiralcel OD-H column (4.6 i.d. × 250 mm, eluted with iso-PrOH : CH_3CN : *n*-hexane = 2 : 1 : 97, 0.5 ml/min, 23 °C). For analysis of the aldononitrile acetates derived from ribose, the eluent was changed to 3% iso-PrOH in *n*-hexane.

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