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Isolation and Identification of Plant Growth Substances in Immature Corn Seeds*, **

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Summary

The ethyl acetate-acetonitrile-soluble extract from fresh immature sweet corn seeds was fractionated by a series of chromatographies monitoring with the rice lamina inclination assay to give a strong active substance which was identified as castasterone by GC-EI-MS analysis of its bismethaneboronate derivative. An IAA methyl ester and a plant growth inhibitor were also found in the other extracts from the immature seeds.

Key words : Growth promoter, Castasterone, Growth inhibitor, Hydroxycinnamoyl glycerol, Immature corn seed.

Earlier investigation of plant growth substances in immature corn seeds revealed the occurrence of auxins[1], cytokinins[2] and gibberellins[3] as growth-promoting hormones. Although a number of studies on the physiological roles of these plant hormones in corn seeds[4, 5] have been reported, a problem still remains unclear about whether other biologically active substances may take part in regulating the development of corn seeds. In this regard, one of authors has previously reported that a non-indolic auxin-like substance, designated as the corn factor,[6] was obtained from corn germ using the rice lamina inclination (RLI) assay, an auxin bioassay[7]. The chemistry of the corn factor, however, was not established at that time because of the extreme difficulties in obtaining the necessary amount of the pure sample for structure analysis. Ever since brassinolide[8], the first steroidal plant hormone, was isolated as an active constituent of brassins[9] from rape pollen by the USDA group in 1979, more than twenty kinds of brassinolide analogs (brassinosteroids) have been successively isolated from various higher and lower plants[10]. During these isolation procedures, the RLI assay was effectively employed for monitoring the active substance in the extracts. This bioassay was very sensitive to brassinosteroids ; the difference in activity between brassinosteroids and auxins was more than ten thousand folds higher in brassinolide than IAA[11]. Our earlier investigation has shown that the isolated corn factor was highly sensitive to RLI assay and gave a specific coloration with sulfuric acid under UV light on silica gel TLC[6]. These data suggest that the corn factor might be a kind of brassinosteroid. Therefore, we reinvestigated, as described in this report, the purification and identification of the corn factor from sweet corn seeds with a giant germ. We also examined the presence of other plant growth substances in the same immature seeds. A fractionation of the active substances from the corn seeds extract was also monitored using the wheat leaf-unrolling (WLU) assays, according to the procedure reported by the late Dr. Kojiro

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Wada et al[11]. This bioassay was characterized as a method for the selective qantification of brassinosteroids co-existing with auxins in moderately purified fractions. As a result of the present study, we have identified castasterone (one of brassino-steroids) and IAA methyl ester (IAA Me) as the growth-promoting substances and a glycerol ester, (*trans-p*-hydroxy-cinnamoyl)-glycerol[12], as a plant growth inhibitor from immature sweet corn seeds.

The fresh immature seeds of sweet corn (*Zea Mays* L., cv. Honey bantam 9) were selected as the material for extraction, based on ; (1) the germ in a sweet corn seed was about three times larger than those of other corn seed cultivators, and (2) an acetonitrile extract of the immature seeds of sweet corn was more active than the mature and germinated seeds extracts in the RLI assay, as shown in Table 1. The fresh immature seeds (22.5 Kg) were extracted with methanol. The methanol extract, however, showed no promoting but inhibitory activity only, as judged from the slight change in bend angles between the lamina and sheath of the original excised segments. The methanol extract was concentrated, and the aqueous residue was successively extracted with *n*-hexane and ethyl acetate. Both extracts showed promoting activity ; among them the ethyl acetate extract showed the stronger activity (Table 2). The ethyl acetate was then extracted with acetonitrile, and the acetonitrile extract was subjected to silica gel column chromatography. The column was eluted with a mixture of chloroform and methanol. The 9th - 11th fractions eluted with 5% and 7% MeOH-$CHCl_3$ showed distinguishable activity from the others (Fig 1). The active fractions (513.7 mg) were combined and passed two times through a Sephadex LH-20 column using methanol as the solvent. The active elute (8.7 mg) was further purified using a preparative TLC

Table 1 Activities of EtOAc-MeCN-soluble fractions from different growth stages of sweet corn seeds at the concentrations of 0.5mg/ml.

Test solution	Activity
	θ ± S. E.
Control	47.3 ± 6.2
IAA 50μg/ml	125.5 ± 11.5
Mature stage	67.5 ± 9.4
Immature stage	118.3 ± 5.2
Germinated stage	89.1 ± 9.8

θ : 180° - the internal angle(°) between lamina and sheeth.
S. E. : Standard error.

Table 2 Activities of MeCN-soluble fractions and MeOH-soluble fraction from immature corn seeds in RLI and WLU assays.

Test solution	Activity	
	θ ±S. E.	mm±S. E.
Control	55.0±10.1	1.8±0.15
IAA, 50μg/ml	131.5± 9.5	1.8±0.16
Brassinolide, 0.05μg/ml	——	3.4±0.20
MeCN fr. from hexane ext., 50μg/ml	93.4±12.5	1.9±0.15
MeCN fr. from EtOAc ext., 20μg/ml	165.9± 5.5	2.8±0.15
MeOH-soluble fr., 500μg/ml	34.0± 4.1	1.8±0.16

θ : 180° - the internal angle (°) between lamina and sheeth in RLI assay.
mm: Leaf segment width in WLU assay.
S. E. : Standard error.

Table 3 Distribution of the activity in RLI assay after p-TLC of the active fraction obtained from the 2nd Sephadex LH-20 chromatography.

	Rf									
	0	0.15	0.25	0.35	0.45	0.55	0.65	0.75	0.85	1
θ	48.2	45.5	123.5	94.4	49.0	42.3	48.5	45.3	49.1	
±S. E.	9.2	11.3	6.3	9.2	13.2	10.5	8.5	11.0	9.2	

The assay was carried out at the concentration of a handred dilution of the extract from each zones.
θ : 180° - the internal angle (°)between lamina and sheeth.
Control showed the θ value at 49.5° ± 14.0.
Standard (IAA,50μg/ml) showed the θ value at 130.1° ± 7.5.
S. E. : Standard error.

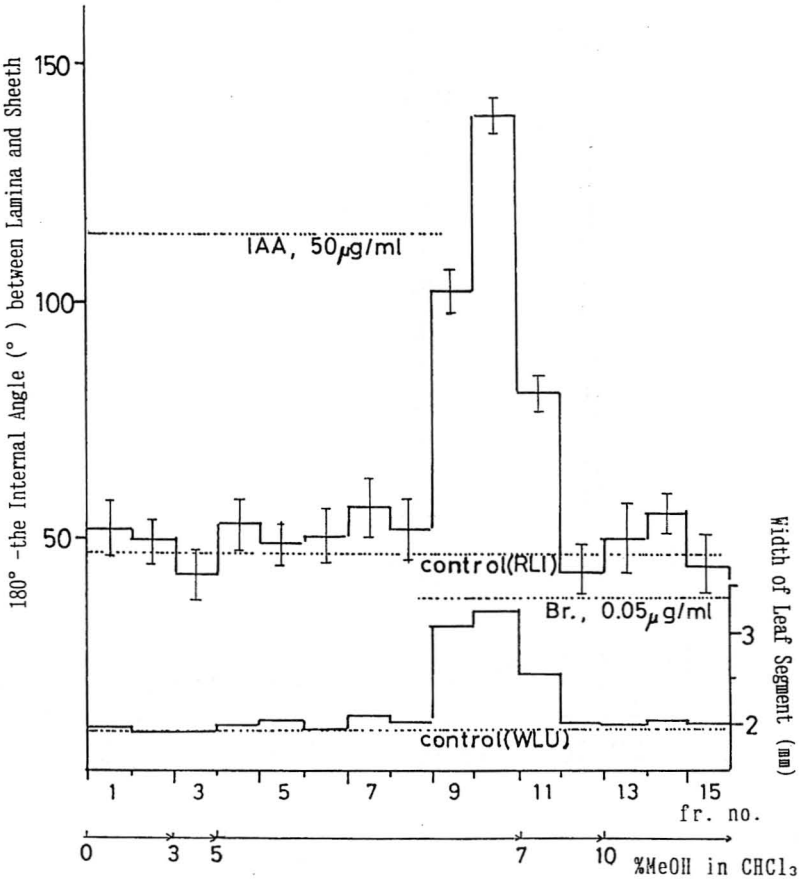

Fig. 1. Activities of the eluates from silica gel chromatography of the EtOAc-MeCN-soluble fraction in the RLI and WLU assays.

RLI and WLU assays were carried out at concentrations of 1μg/ml and 10μg/ml for each test fractions.

plate on silica gel developed with ethyl acetate-ethanol (22:3). The active zone of Rf 0.25 - 0.45 was scraped out and extracted with methanol (Table 3). The active extract was finally purified by HPLC on Develosil ODS-5, which was developed with acetonitrile-H_2O (1 : 1) to give a semi-solid matter (160 μg). This matter showed sharp biological responses in the RLI assay at 0.0001 μg/ml and WLU assay at 0.01 μg/ml, and gave a single spot at Rf 0.31 on a silica gel plate (Merk Art. 5554, ethyl acetate-ethanol ; 22 : 3) which was colored in light blue under UV (365 nm) irradiation when sprayed with 50% sulfuric acid followed by heating at 110℃ for 5min. These chromatographic behaviors of the active semi-solid matter was close to that of castasterone, a ketonic brassino-steroid first isolated from chestnut insect galls[13]. The final identification was carried out by GC-EI-MS on its methaneboronate[14] ester derivative. The natural semi-solid matter was reacted with methaneboronic acid in pyridine, and the reaction mixture with the authentic methaneboronate ester of 2, 3-dihydroxystigmast-22-ene-6-one[15] as an internal standard was subjected to GC-EI-MS. The castasterone bismethaneboronate was also prepared and similarly analyzed. The relative retention time (1.175) of the natural derivative was found to coincide well with that (1.174) of the castasterone derivative, as shown in Fig. 2. The former's mass spectrum (Fig. 3) was identical to that of the castasterone derivative, thus, the active substance isolated from sweet corn seeds was concluded to be castasterone.

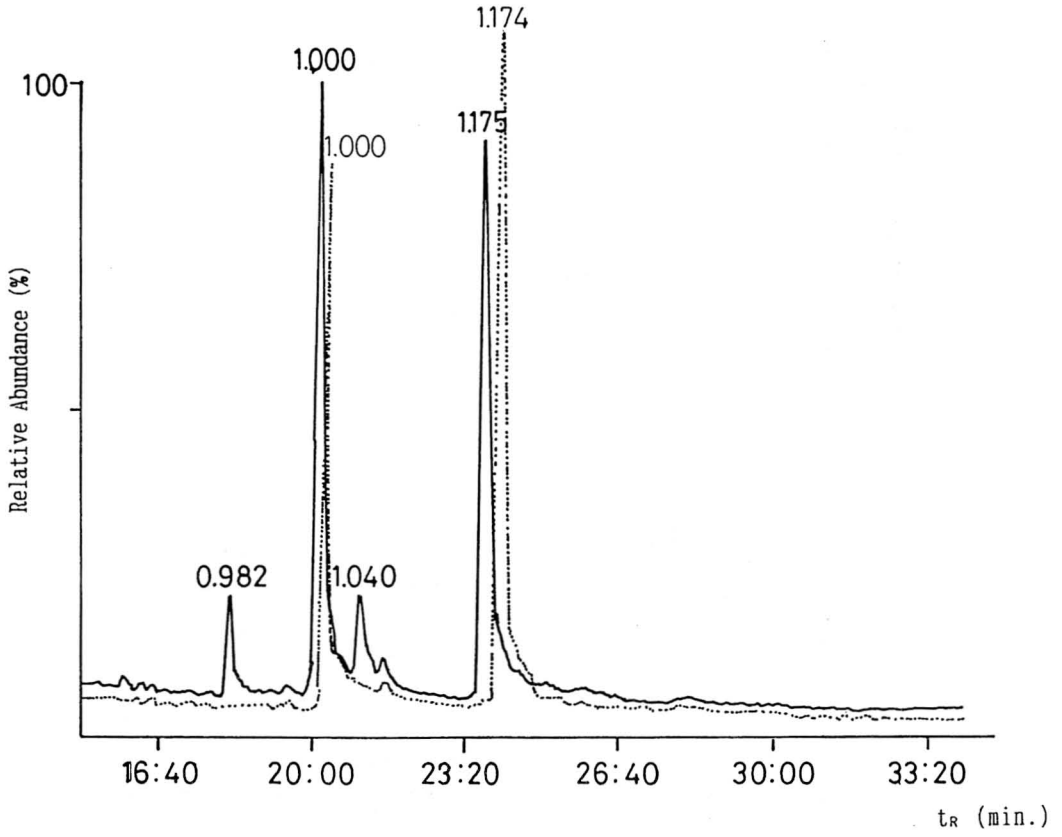

Fig. 2. Gas chromatograms obtained by GC-EI-MS of the reaction mixture of the semisolid matter with methaneboronic acid in pyridine and the authentic castasterone bismethaneboronate in the presence of the internal
standard, 2, 3-dihydroxystigmast-22-ene-6-one methaneboronate.

Solid line : semi-solid matter +internal standard.
Dashed line: authentic +internal standard.
Value on each peaks means the relative retention time with reference to the internal standard (1.000).

The content of castasterone in the seeds was calculated to be 6μg/kg based on their peak heights of total ion current during gas chromatography. The extra two peaks with relative retention times of 0.982 and 1.040 observed in Fig. 2 were too small to measure their mass spectra, hence no information could be obtained on their structures.

Next, the active *n*-hexane-soluble fraction was investigated. The *n*-hexane extract obtained as already described was extracted with acetonitrile. The acetonitrile-soluble fraction was fractionated by successively subjecting it to a silica gel column, Sephadex LH-20 column and HPLC to give the active principle. To guide the fractionation, an RLI assay was used. A light rose-colored oil (1.4mg) was obtained as a single spot on TLC which nearly possessed the same activity as that of IAA in the RLI assay, however, no activity at concentrations of 0.1 to 10μg/ml in the WLU assay. The EI-MS and ^{1}H-NMR spectra of the isolated compound were found to be identical with those of the authentic IAA methyl ester. The methanol-soluble extract with growth-inhibitory activity was purified through successive treatment with silica gel column chromatography, silica gel TLC and finally by recrystallization to give a pure active substance, (S)-(+)-1-(*trans*-*p*-hydroxycinnamoyl) glycerol, as reported in our previous paper. Interestingly, the substance could not be found in the mature seeds but only in the immature seeds just as reported in this study.

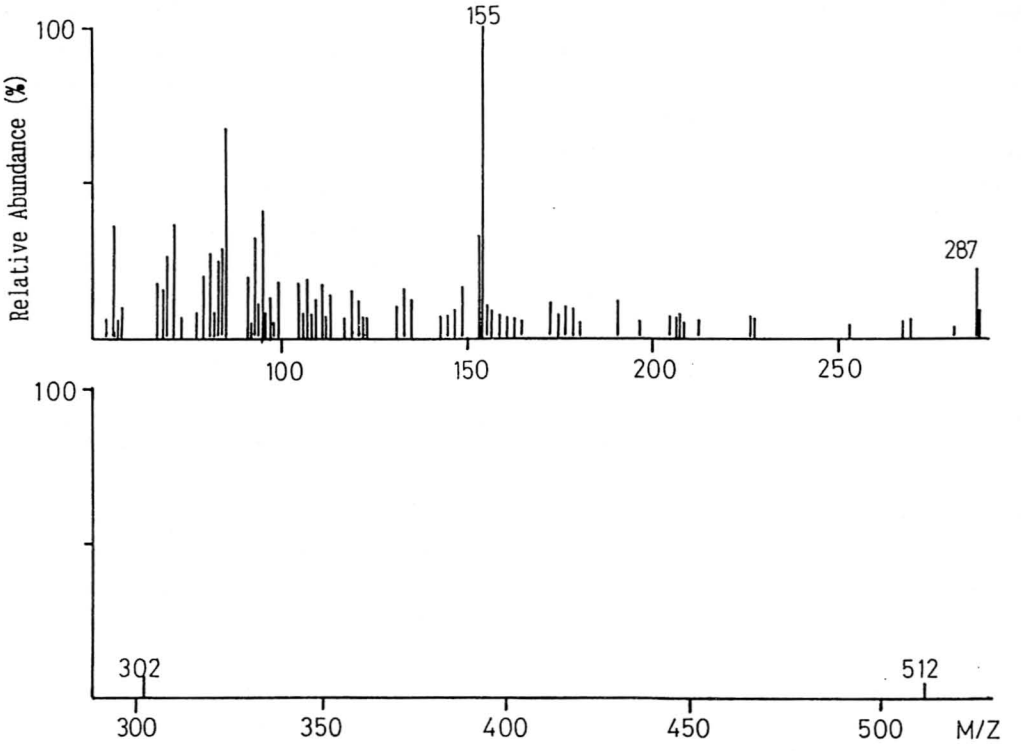

Fig. 3. Mass spectrum of the bismethaneboronate of a main peak (tr : 1.175) on GC-EI-MS for EtOAc-MeCN-soluble fraction.

Three biologically active substances, castasterone, IAA methyl ester and a glycerol *p*-hydroxycinnamate have been identified from immature sweet corn seeds. The corn factor that one of the authors has previously isolated from corn germ oil is most likely the castasterone identified in the present study based on the close similarity in both biological activity and chromatographical properties between the two compounds. Suzuki et al[16] investigated brassinosteroids in the pollen of *Zea Mays*, revealing that they contained mostly castasterone accompanied by other small amounts of its analogs. They also suggest that castasterone is the major brassinosteroid in the anther. Fujita[17] has shown that exogenous application of brassinolide to the ear silk of corn after the silking stage brings about normal growth of the recessive grains around the apex of the core. These observations suggest that brassinosteroids may play important roles in the regulation of not only the physiological phenomena of the pollen in tassels but also the growth of seed in the ear of corn.

The occurrence of IAA in the immature seeds[1] and its ethyl ester[18] in the mature sweet corn seeds have been reported. The IAA methyl ester in this study was most likely an artifact derived from endogenous IAA during the course of the methanol extraction. An inhibitor, (S)-(+)-1-(*trans*-*p*-hydroxycinnamoyl)-glycerol, entirely inhibits both the growth-promoting effects of IAA and brassinolide at a concentration of 100μg/ml in the RLI assay, and also inhibits the root elongation of Chinese cabbage seedlings at a concentration of 50μg/ml as described before. It is of interest in connection with the growth regulation of corn seeds that a large amount of the inhibitor in the immature seeds disappears in accordance with seed maturity.

EXPERIMENTAL

Melting points were uncorrected. Preparative TLC was performed on silica gel (Merk, Art. 7730, 0.5mm

thickness), and the silica gel for column chromatography was from the Kanto Chemical Co. Inc.; No.37047 unless otherwise stated. ^{1}H-NMR spectra were recorded with TMS as the internal standard at 360MHz on a Bruker AM-360WB spectrometer, or at 100MHz on a JEOL JNM-FX-100 spectrometer. Specific rotations were determined using a JASCO DIP-4 digital polarimeter.

MS and GC-MS Analyses. A Finnigan model 4000 mass spectrometer was used for the GC-EI-MS analyses of the biologically active substances obtained from the ethyl acetate extracts. The conditions were as follows. All of the runs were operated at 70 ev ionization voltage, 100 μA emmission current on the filament and an ion source temperature of 250℃. Column: Fused silica DB-5 (0.32 mm/60m, 0.25 μm film thickness, J & W Scientific Inc.) ; GC oven temp. : 125-300℃ (10℃/min ; inj. temp. : 120℃ (the sample was injected directly onto the column using the Hewlett-Packard injector) ; carrier gas: He, 40cc/sec, measured at GC oven temp. 150℃. Prior to gas chromatography, samples were converted into bismethaneboronates. A Hitachi M-80A mass spectrometer was used for the EI-MS measurements (70ev ionization voltage) of the IAA methyl ester obtained from the *n*-hexane extracts.

Bioassay. The rice lamina inclination assay and the wheat leaf-unrolling assay were carried out using a rice cultivar "Sasanishiki" and a wheat cultivar "Norin No. 61", respectively.

Plant materials. Fresh mature seeds of corn (*Zea Mays* L., cv. honey bantam 9, sweet corn) were obtained from Sakata no Tane Co. Ltd.. The milk-stage of corn ears of honey bantam 9 were obtained from a farm in Fukushima prefecture, and the immature seeds were collected by plucking from the core of the ear by hand. The germinated seeds were prepared by incubation of the mature seeds described above on a wet saran mesh under fluorescent light at 26℃ for 4 days.

Preliminary experiment. The mature seeds were washed with water to remove the artificial colored material before use. The solvent extraction from 30 grains each of the mature, immature and germinated seeds was carried out as follows. Seeds were crushed and then extracted with 25ml of MeOH. The MeOH extract was partitioned against *n*-hexane. The MeOH layer was concentrated, and the residue was extracted twice with 10ml of EtOAc. The EtOAc-soluble fraction was treated with 5ml of MeCN to afford the MeCN-soluble fraction. The yields of the fraction were 18mg, 13mg and 21mg for the mature, immature and germinated seeds, respectively.

Large-scale extraction. Fresh immature seeds (22.5kg) of *Zea Mays* L. cv. Honeey bantam 9 were immersed in 40L of MeOH for a month at room temperature. The filtrate was reduced to the aq. solution and partitioned 3 times against 5L of *n*-hexane. The *n*-hexane solutions were combined and partitioned 3 times against 3L of MeCN. The MeCN extracts were concentrated to give an oil (24.7g) as the hexane-MeCN-soluble fraction. The aq. phase was partitioned 3 times against 5L of EtOAc. The EtOAc solutions described above were combined and concentrated under reduced pressure to afford a viscous oil. This was extracted several times with MeCN. The MeCN solutions were combined and concentrated under reduced pressure to give an oil (5.2g) as the EtOAc-MeCN-soluble fraction.

Purification procedure for EtOAc-MeCN-soluble fr. This fraction was dissolved in $CHCl_3$ and charged on a column (ϕ 5.2×40cm) of silica gel (650g). The column was eluted with $CHCl_3$-MeOH (500ml×2), and then with mixtures of $CHCl_3$-MeOH; 97 : 3 (v/v, 500ml), 95 : 5 (500ml×7), 93 : 7 (500ml×2) and 90 : 10 (500ml×3). The 8th and 9th fractions (5% MeOH) and the 10th fraction (7% MeOH) were combined, and then concentrated to give an active fraction (513.7mg). This was chromatographed on a Sephadex LH-20 column (ϕ 3.2×40cm) using MeOH. The eluate was collected in 10ml fractions with a flow rate of 15ml/hr. The active fractions between 150-170ml were combined. The fraction (72.8mg) was subjected to another Sephadex LH-20 column (ϕ 1.9×80cm; volume of each fraction, 5ml ; flow rate, 7ml/hr) eluting with MeOH to give the active fraction (elution vol., 135-145ml ;

8.7mg). This was chromatographed on preparative silica gel TLC plates using EtOAc-EtOH (v/v, 22 : 3) as the developing solvent. Silica gel being in a zone of Rf0.25-0.45 was collected and extracted with MeOH. The extract (1.7mg) was dissolved in MeCN and subjected to HPLC using a Develosil ODS-5 column (ϕ 0.46×25cm) with Develosil ODS-10 (ϕ 0.46×5cm) as a pre-column ; flow rate, 1ml/min; mobile phase, MeCN; collected fraction size, 1.0ml. The active fraction (Fr. Nos. 6 to 9 in each run) were collected by repetition of the HPLC and evaporated in vacuo to afford a colorless semi-solid (160μg).

Preparation of bismethaneboronate.[14] Methaneboronic acid (500μg) was dissolved in dry pyridine (500μl). Fifty μl of this solution was added to a mixture of the test sample (50μg) and a suitable amount of internal standard. The mixture was heated at 60℃ for 30min. Authentic castasterone (50μg) was also converted into the bismethaneboronate in the same way as stated above. 2μl of the reaction mixture was injected into the GC-EI-MS (Fig. 2).

Chemicals. 2, 3-dihydroxystigmast-22-ene-6-one prepared from stigmasterol[15] was used for the internal standard.

Purification procedure for n-hexane-MeCN-soluble fr. This fraction dissolved in 10% EtOAc-n-hexane was charged on a column of silica gel (500g; column size, ϕ 4.3×40cm) eluting with a mixtures of EtOAc-n-hexane. The EtOAc content was increased stepwise each 500ml of 10, 20, 30, 40 and 50% in *n*-hexane (collected fraction size, 100ml). The 18th to 20th fractions were combined and concentrated. The active fraction (2.2g) was purified twice with a Sephadex LH-20 column in the same manner as those of the EtOAc-MeCN-soluble fr. The active fraction (9.4mg) was subjected to HPLC in the same way as that of the EtOAc-MeCN-soluble fr. except the mobile phase used 50% water in MeCN The active fractions (Fr. Nos. 10 and 11) were combined and partitioned against EtOAc. The EtOAc phase was dried over anhydrous Na_2SO_4 and then concentrated to give a light rose-colored oil (1.4mg); EI-MS, m/z: 277(M^+), 198, 129, 94, 57 ; ^{1}H-NMR, δ ($CDCl_3$) : 3.78(3H, s), 3.78(2H, s), 7.89-7.29(3H, m), 7.36(1H, br.d, J=7.3Hz), 7.61(1H, br.d, J=7.3Hz), 8.05(1H, br.s).

Purification procedure for MeOH-soluble fr. This fraction was dissolved in MeOH, absorbed on celite, dried in vacuo, and loaded on a silica gel column (Bio-Rad Labs., Bio-Sil A; column size, ϕ 4×30cm). The MeOH content of the mobile phase was increased stepwise each 1L of 0, 3.3, 10 and 50% in EtOAc; collected fraction size, 100ml. The 18th to 21st fractions were combined and concentrated. The yellowish solid mass was crystallized from EtOAc to give colorless plates; mp 129-130℃ (1.01g). Spectral data of the inhibitor and its derivatives have been reported in our previous paper[12].

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トウモロコシ未熟種子に含まれる植物生長物質の単離と同定

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植物の未熟種子は完成された種子の形成に向けて活性状態にあると考えられる．したがって，そこに存在し，機能している生長調節物質は質的にも量的にも完熟種子とは異なることが期待される．本研究はトウモロコシの未熟種子を対象に生長調節物質の検索をおこなった結果を取りまとめたものである．

未熟トウモロコシ種子（*Zea Mays* L., cv. Honey bantam）の酢酸エチル可溶区中のアセトニトリル可溶区からライスラミナ屈曲試験に強い活性を示す画分を得た．この画分をメタンボロン酸で処理しGC−EI−MS法で分析した結果，その主成分はカスタステロンであることを確認した．また，ヘキサン可溶区からインドール酢酸メチルエステルを確認した．一方，メタノール可溶区からは生長阻害物質，(S)−(+)−1−(*trans-p*-hydroxycinnamoyl)−glycerol，の存在を明らかにした．カスタステロンは以前に報告したトウモロコシ胚芽油中に見いだされたコーンファクターの本体と考えられた．これらの生長調節物質は完熟種子にくらべ明らかに多く存在していた．特に，生長阻害物質のグリセロールエステルは完熟種子中からは検出できなかった．

キーワード：生長促進物質，カスタステロン，生長阻害物質，桂皮酸グリセライド，トウモロコシ未熟種子