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EVIDENCE FOR PLANT GROWTH PROMOTING BRASSINOSTEROIDS IN LEAVES OF *THEA SINENSIS*

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Key Word Index—*Thea sinensis*; Theaceae; plant growth regulator; brassinosteroids; brassinolide; castasterone.

Abstract—Steroids, which were active in inducing rice-lamina inclination, were separated from leaves of *Thea sinensis* and tentatively identified to be brassinolide and its 6-keto analogue castasterone, by mass fragmentographic analysis.

INTRODUCTION

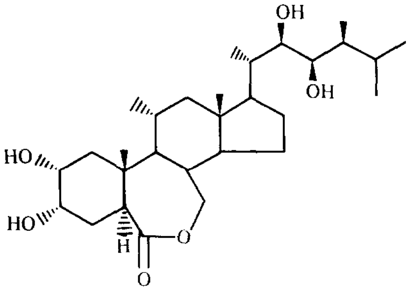
New plant growth-promoting steroids have been found in some higher plant tissues. These include brassinolide (**1**), from the pollen of rape (*Brassica napus*) [1], and its 6-keto analogue, castasterone (**2**), in the insect gall of chestnut (*Castanea crenata*) [2]. These naturally occurring compounds, which have been given the general term brassinosteroids [3], have proved to be a new class of plant growth regulator which are comparable to the known plant hormones in a number of bioassay systems [4, 5]. However, little is known of the physiological effects of brassinosteroids on plants or about their distribution in the plant kingdom.

Brassinosteroid-like active substances are found in a variety of higher plants. We have isolated three active substances, designated as *Distylium* factors A_1, A_2 and B, from fresh leaves of *Distylium racemosum* [6], using the rice-lamina inclination assay established by Maeda [7]. The very limited quantity of *Distylium* factors in the plant precluded their structural elucidation, but they seemed to be widespread in other higher plants surveyed. Furthermore, the similarity of responses induced by brassinolide and *Distylium* factors in several biological assays for auxin and cytokinin and their TLC behaviour suggest the ubiquitous presence of brassinosteroids in the plant kingdom.

Recent studies on the effect of brassinolide and its synthetic analogue, homobrassinolide, on lamina inclination showed that they dramatically promoted inclination at a very low concentration [8]. The rice-lamina inclination test is highly specific and sensitive and is one of the most useful assay methods in the screening of brassinosteroids in plants. We have re-examined the *Distylium* factors in the lamina inclination test and found them to produce the same result as the brassinosteroids. This report details the identification of brassinosteroids in fresh leaves of *Thea sinensis* (green tea).

RESULTS AND DISCUSSION

A methanol extract from fresh leaves of green tea was separated in the usual way to afford an ethyl acetate-soluble neutral fraction. This neutral fraction was then partitioned between acetonitrile and hexane and the acetonitrile fraction was purified by successive chromatographic separation [9], on alumina, Amberlite XAD-2, Sephadex LH-20, Si gel and by HPLC (see Experimental).



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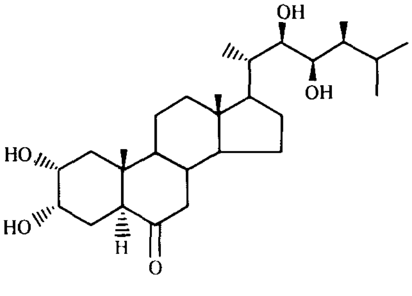

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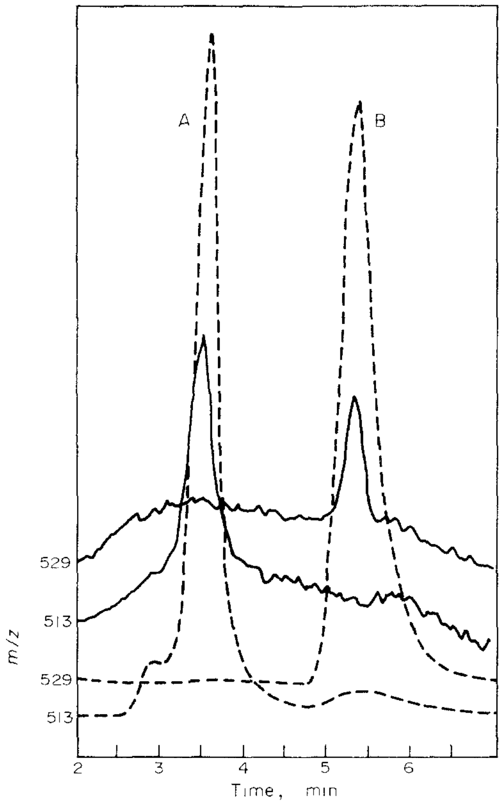

Fig. 1. Mass fragmentographic detection of brassinolide and its 6-keto analogue, castasterone, in leaves of *Thea sinensis*. (A) Castasterone bismethaneboronate; (B) brassinolide bismethaneboronate; (———) natural; (– – – – –) authentic.

Two biologically active substances were obtained and the free compounds or their bismethaneboronate esters were detected by TLC as light-blue and purple spots under UV light at 365 nm after spraying with 0.5% vanillin–sulfuric acid and subsequent heating at 110° for 5 min. The polar substance, corresponding to the light-blue spot, appeared to be chromatographically similar to brassinolide while the less polar one (purple spot) corresponded to castasterone. The natural substances were compared with the synthetic standards using the microanalytical technique for brassinolide analogues [10]. The 2,3:22,23-bis(methaneboronate) derivatives of authentic brassinolide and castasterone afforded ions in CI–MS for the base peak corresponding to $[M+1]^+$ at *m/z* 529 and *m/z* 513, respectively. These ions were selectively monitored, by mass fragmentography during GC/MS analysis of samples, to exhibit sharp peaks at 3.5 min (peak A) and 5.3 min (peak B), respectively, as shown in Fig. 1. Under such conditions, the derivatives of the natural substances isolated from *T. sinensis* coincided in retention time with those of authentic brassinolide and castasterone. Both brassinosteroids contained in green tea occur in amounts of less than 1 µg/65 kg wet wt. This is much lower than their respective concentrations in rape pollen (4 mg/40 kg wet wt) and chestnut insect gall (95 µg/40 kg wet wt).

Thus, the brassinosteroids tentatively identified in green tea are brassinolide and its 6-keto analogue, castasterone, which is possibly a biosynthetic precursor of brassinolide. This is the first indication of brassinolides in plants other than *Brassica napus*.

EXPERIMENTAL

Plant materials and isolation procedure. Thea sinensis cv Sayamakaori leaves (65 kg) were collected in the suburbs of Sayama of Saitama Prefecture in Nov. 1980. The neutral fraction from the MeOH extract of the leaves was partitioned between MeCN and hexane and the MeCN fraction was chromatographed on alumina, from which the active fraction was eluted with 20–40% EtOH in EtOAc. The residue, evaporated to dryness, was dissolved in H_2O, passed through an Amberlite XAD-2 column and eluted with aq. EtOH. The activity appeared in fractions of 60–70% in H_2O. These fractions were successively purified by gel chromatography on Sephadex LH-20 (1% HOAc in EtOH), by TLC on Si gel (EtOAc; EtOAc–EtOH, 22:3) and by HPLC on Finepak SIL (*iso*-octane–EtOH, 70:30) and Finepak SIL C_{18} (MeCN–H_2O, 75:25).

Rice-lamina inclination test. The lamina joint sections, excised from the second leaves of etiolated seedlings (*Oryza sativa* cv Kinmaze) cultivated at 28° in darkness for 6 days, were used in all the expts. After having been floated in H_2O for 24 hr at 28° in the dark, they were transferred into 1 ml of test soln. The magnitude of the induced angle between lamina and sheath was measured after incubation for 48 hr under the same conditions.

Preparation of boronate esters. Methaneboronic acid (100 µg) was dissolved in 50 µl dry pyridine and 10 µl of this soln was added to each of the test samples. The mixture was heated at 60° for 30 min and 2 µl of the reaction mixture was used for GC analysis. Brassinolide and its 6-keto analogue, castasterone, as standard samples were synthesized by the method of ref. [11]. Castasterone was obtained by de-protection of the intermediate to brassinolide.

Mass fragmentography. A Shimadzu GC/MS 6020 with a CI ionization source was used. The column was packed with 2% OV-17 on Chromosorb W (80–100 mesh), 50 cm × 2 mm. The reactant gas was *iso*-butane; carrier gas (He) flow rate was 30 ml/min; electron energy 150 eV; box current 150 µA; accelerating high voltage 3.5 kV; ion source temp. 250°; separator temp. 310°.

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ISOLATION OF NORBERGENIN FROM *SAXIFRAGA STOLONIFERA**

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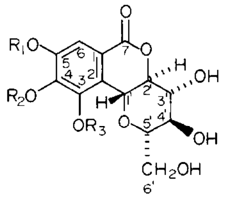
Key Word Index—*Saxifraga stolonifera*; Saxifragaceae; desmethylbergenin; norbergenin; bergenin; tri-*O*-methylnorbergenin.

Abstract—Norbergenin, a *C*-glucoside, was isolated from *Saxifraga stolonifera* and its structure assigned as 2β-D-glucopyranosylgallic acid δ-lactone.

Bergenin (**2**), one of the simplest *C*-glucosides, occurs in a number of plants [1–4]. In previous publications [5, 6], its distribution and biosynthesis have been reported. The results of incorporation of [^{14}C]glucose into **2** by leaves of *Saxifraga stolonifera* showed that gallic acid accepted a glucosyl moiety and was a precursor of **2**. During this study an unidentified phenolic, having high radioactivity, was isolated which was thought to be a demethylation product of **2**; it was named desmethylbergenin (**1**) [6]. While preparing a manuscript on its identification, Kalidhar *et al.* [7] reported the same compound in *Woodfordia fruticosa* (Lythraceae) and called it norbergenin. We now report the confirmation of its structure, as isolated from *S. stolonifera*.



1 R$_1$ = R$_2$ = R$_3$ = H
Norbergenin

2 R$_1$ = R$_3$ = H, R$_2$ = Me
Bergenin

3 R$_1$ = R$_2$ = R$_3$ = Me
Tri-*O*-methylnorbergenin

Our compound and its derivatives were identical in every way with norbergenin and its derivatives as described by Kalidhar *et al.* (see Experimental). In addition, the absolute configuration of **1** was determined by the aromatic chirality method [8, 9]. The three Cotton effects at $\Delta\varepsilon_{210} + 2.0$, $\Delta\varepsilon_{228} - 12.5$ and $\Delta\varepsilon_{290} + 0.8$ showed the chiral lactone ring to be a β-glucosyl moiety of **1**.

Table 1. ^{13}C NMR chemical shift of norbergenin (**1**), bergenin (**2**) and tri-*O*-methylnorbergenin (**3**)

	1		**2**	**3**
Carbon No.	CD_3OD–D_2O (2:1)	DMSO-d_6	DMSO-d_6	DMSO-d_6
1	117.49 *s*	116.08 *s*	118.03 *s*	126.45 *s*
2	114.08 *s*	112.76 *s*	115.93 *s*	119.05 *s*
3	143.31 *s*	142.43 *s*	148.04 *s*	150.80 *s*
4	140.93 *s*	139.61 *s*	140.59 *s*	147.70 *s*
5	146.58 *s*	145.94 *s*	150.92 *s*	152.82 *s*
6	111.25 *d*	109.30 *d*	109.50 *d*	109.00 *d*
7	166.65 *s*	163.73 *s*	163.34 *s*	163.54 *s*
1′	73.88 *d*	72.27 *d*	72.17 *d*	71.00 *d*
2′	75.14 *d*	73.78 *d*	73.73 *d*	73.97 *d*
3′	80.99 *d*	79.82 *d*	79.82 *d*	80.16 *d*
4′	71.44 *d*	70.90 *d*	70.71 *d*	70.41 *d*
5′	82.40 *d*	81.57 *d*	81.77 *d*	81.60 *d*
6′	62.33 *t*	61.25 *t*	61.16 *t*	61.11 *t*
OMe	—	—	—	61.10 *q*
	—	—	59.84 *q*	60.60 *q*
	—	—	—	56.00 *q*

21.15 MHz, TMS as int. standard.

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*Part 3 in the series "Studies on *C*-Glycosides in Higher Plants". For Part 2 see Taneyama, M. and Yoshida, S. (1979) *Bot. Mag.* **92**, 69.