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STRUCTURE–ACTIVITY RELATIONSHIP OF BRASSINOSTEROIDS*

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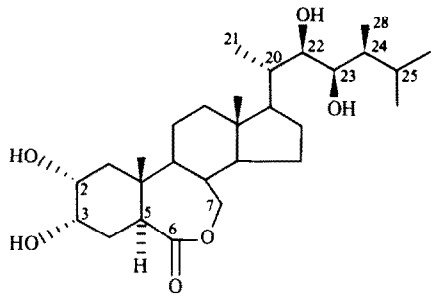
Key Word Index—Brassinolide; brassinosteroids; plant hormones; structure–activity relationship; *Raphanus* test; tomato test; rice-lamina inclination test.

Abstract—The plant growth-promoting activities of brassinolide and brassinosteroids with different side chains were investigated by means of the *Raphanus* and the tomato tests. In addition, the activities of brassinosteroids with a 6-ketone instead of a 7-oxalactone function in the B-ring were also determined. Brassinolide supplied via the root system remarkably promoted elongation of cotyledon petioles and hypocotyls of young radish and tomato plants at concentrations as low as 0.01 ppm, and also induced morphogenetic responses. Brassinolide was found to be the most active compound among the brassinosteroids tested in the two bioassays. The next were (22*R*,23*R*)-28-norbrassinolide and (22*R*,23*R*,24*R*)-24-epibrassinolide. (22*R*,23*R*,24*S*)-28-Homobrassinolide was much less active in these tests, although its activity was similar to that of brassinolide in the rice-lamina inclination test. The 6-oxobrassinosteroids were generally much less active than the brassinosteroids with a 7-oxalactone group. The structural requirements obtained by these two bioassays were as follows: (1) A,B-ring functions as in brassinolide, (2) (22*R*,23*R*)-configuration of 22,23-vicinal diol, and (3) methyl or no alkyl group at the C-24 position of the side chain. They are much more stringent than those obtained by the rice-lamina inclination test.

INTRODUCTION

In 1979, a new plant growth-promoting steroid named brassinolide (**1**) was isolated from the pollen of rape (*Brassica napus* L.) and its structure was determined as (22*R*, 23*R*, 24*S*)-2α, 3α, 22, 23-tetrahydroxy-24-methyl-*B*-homo-7-oxa-5α-cholestan-6-one (**1**) by spectroscopic data including X-ray crystallography [1]. In the bean-second internode bioassay, brassinolide promotes both cell elongation and cell division, resulting in curvature, swelling and, more dramatically, splitting of the internode at doses as low as 10 ng per plant [1]. Subsequent to the isolation of brassinolide, extensive and intensive investigations into the biological activities of brassinolide were carried out using a number of auxin, gibberellin and cytokinin bioassay systems in order to evaluate the plant growth-promoting activities of brassinolide. As a consequence, it was found that brassinolide possesses a broad spectrum of biological activities compared with the known plant hormones [2–4]. Applications of brassinolide analogues in agriculture were also examined in a preliminary form and promising results were reported [5].



1 Brassinolide

Although brassinolide was first isolated from the pollen of rape, the isolation of (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestan-6-one, castasterone, a possible biosynthetic precursor to brassinolide, from the insect galls of the chestnut tree (*Castanea* spp) [6] and the identification of brassinolide and its several analogues, 28-norbrassinolide (**8**), castasterone, brassinone (**20**) and 24-ethylbrassinone (**16**), in some higher plants [7], were recently reported. The presence of brassinolide-like substances was also suggested in a number of plants [8, 9]. Consequently, it has come to be recognized that brassinolide and its related compounds are widely distributed in the plant kingdom at the ppb level.

After the isolation of brassinolide, we [10] and Siddall *et al.* [11] succeeded in synthesizing this fascinating steroid. More recently, the synthesis was also reported by two other groups [12, 13]. Subsequently, we and Thompson *et al.* have independently focussed attention on the structure–activity relationship of brassinosteroids, and synthesized many brassinolide analogues [14–18]. The structural requirements for plant growth-promoting activity were investigated by us using the rice-lamina inclination test [16, 19] and also by Thompson *et al.* using

*Dedicated to Professor Edgar Lederer on the occasion of his 75th birthday.

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the bean internode bioassay [18]. These bioassays employ plant segments to evaluate the biological activity of brassinosteroids. However, the activity of these steroids should also be evaluated with intact plants. In this paper, we report the activities of brassinolide in the promotion of growth of intact radish and tomato plants as well as the results of an investigation on structure–activity relationships of brassinosteroids.

RESULTS AND DISCUSSION

After testing the sensitivity of a number of plants to brassinosteroids [20], we selected two highly sensitive plants, radish (*Raphanus sativus*) and tomato (*Lycopersicon esculentum*), which had previously been employed in the evaluation of plant growth regulators, including brassinosteroids [21–24]. The plant growth-promoting activities of brassinolide (**1**) bioassayed by the *Raphanus* test and the tomato test are summarized in Table 1. In the *Raphanus* test, brassinolide caused a significant elongation of the cotyledon petiole and hypocotyl of young radish at a concentration of 0.01 ppm. Morphogenetic changes (curvature) of the cotyledon petiole and the hypocotyl were also observed even at a concentration of 0.001 ppm. The morphogenesis of the hypocotyl induced by brassinolide, which is also induced by auxins, e.g. IAA at *ca* 10 ppm [21], can be ascribed to the cell division promoting activity of the steroid. In the tomato test, remarkable elongation of the hypocotyl and morphogenesis (curvature) of the cotyledon petiole and hypocotyl of young tomato plants were observed at a concentration of 0.01 ppm of brassinolide. Auxins and gibberellins are also active in these two bioassay systems but only at the 10 ppm level. Therefore, these tests are sensitive for brassinolide, and are useful for the evaluation of the plant growth-promoting activities of brassinolide and its analogues.

The results of such an evaluation are summarized in Table 2. In both bioassays, brassinolide (**1**) was the most active compound among the brassinosteroids tested. In the *Raphanus* test, the (22*S*,23*S*,24*R*)-isomer (**3**) of brassinolide (**1**) with the opposite configurations of the side chain functions at the C-22, C-23 and C-24 positions was as active as brassinolide (**1**) and more active than the corresponding (22*R*,23*R*,24*R*)-24-epibrassinolide (**2**). However, the relative activity was reversed in the tomato test. (22*R*,23*R*)-28-Norbrassinolide (**8**) which showed about 5% of the activity of brassinolide (**1**) in the rice-lamina inclination test [16], was also as active as brassinolide (**1**) in *Raphanus* test, and this steroid had about 10% of the activity of brassinolide (**1**) in the tomato test. However, (22*R*,23*R*,24*S*)- and (22*S*,23*S*,24*R*)-28-homobrassinolide (**4** and **5**) which possessed almost equal activity to brassinolide (**1**) in the rice-lamina inclination test [16], turned out to be much less active in these two bioassays; about 10–30 times less active in the *Raphanus* test and about 100 times less active in the tomato test. Among the 28-homobrassinolides (**4**–**7**), the steroids with the natural configuration at C-24 (**4** and **5**) were more active than those with the opposite configuration (**6** and **7**) in the *Raphanus* test. However, in the Tomato test these four 28-homosteroids showed almost the same activity. In addition, the 28-homobrassinolides were generally less active than the 24-methylbrassinolides (**1**–**3**) and the 28-norbrassinolides (**8** and **9**). This means that the C-24 methyl group of brassinolide (**1**) can be replaced by hydrogen, but not by an ethyl group. This is the most striking difference between the structural requirements obtained by these bioassays with intact plants and those with plant segments [16, 18], although the segments were from different plant species. With respect to the 22,23-vicinal diol, it is evident from Table 2 that the (22*R*,23*R*)-brassinosteroids were generally more active than the corresponding (22*S*,23*S*)-isomers except for the 24-epibrassinolides (**2** and **3**), and much more active than the (22*R*,23*S*)- and (22*S*,23*R*)-brassinosteroids as evidenced by the four isomers of 28-norbrassinolides (**8**–**11**). This importance of the stereochemical configuration of the

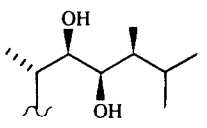
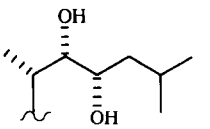
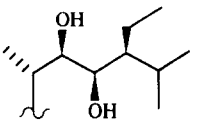
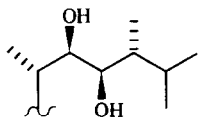
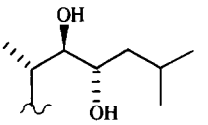
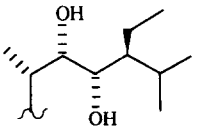
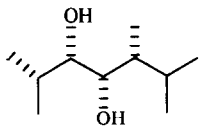
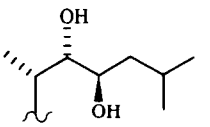
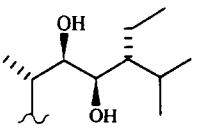
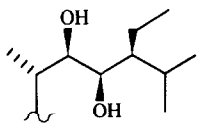
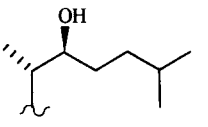
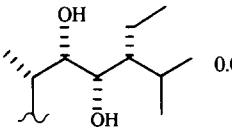
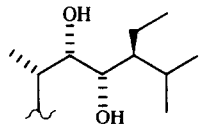
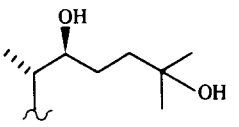
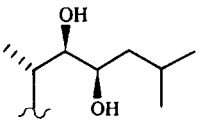
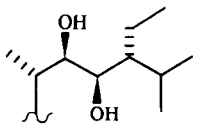
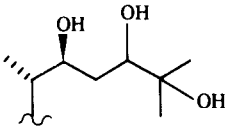
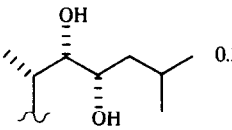
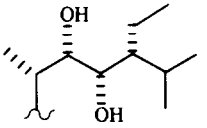
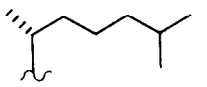
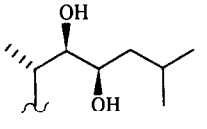
Table 1. Plant growth-promoting effect of brassinolide on young seedlings of radish and tomato

Concentration (ppm)	Elongation (%)*			
	Raphanus test			Tomato test
	Whole plant	Hypocotyl	Cotyledon petiole	Hypocotyl
Control	108.5 ± 5.3†	108.5 ± 6.2	108.6 ± 4.4	106.9 ± 7.2
30	138.8 ± 7.5	130.4 ± 8.7	163.1 ± 6.3	125.1 ± 10.1
10	137.9 ± 7.5	131.7 ± 8.7	156.5 ± 6.3	128.2 ± 10.1
3	138.3 ± 7.5	130.7 ± 8.7	158.6 ± 6.3	127.6 ± 10.1
1	132.0 ± 7.5	129.2 ± 8.7	140.9 ± 6.3	125.6 ± 10.1
0.3	127.0 ± 7.5	125.3 ± 8.7	132.0 ± 6.3	124.3 ± 10.1
0.1	126.3 ± 7.5	126.4 ± 8.7	126.1 ± 6.3	115.2 ± 10.1
0.03	121.7 ± 7.5	124.2 ± 8.7	115.4 ± 6.3	113.6 ± 10.1
0.01	118.3 ± 7.5	120.3 ± 8.7	113.8 ± 6.3	113.3 ± 10.1
0.003	112.8 ± 7.5	115.7 ± 8.7	106.0 ± 6.3	109.4 ± 10.1
0.001	110.0 ± 7.5	109.9 ± 8.7	110.1 ± 6.3	108.3 ± 10.1
0.0003	111.6 ± 7.5	112.1 ± 8.7	110.3 ± 6.3	106.7 ± 10.1
0.0001	112.0 ± 7.5	112.5 ± 8.7	110.8 ± 6.3	106.8 ± 10.1

*100% = initial length of the cotyledon petioles or the hypocotyls at the time of transfer into the test solution. Analysis of variance; 95% level.

†Incubated in water solution. This elongation (8.5%) was due to endogeneous plant hormones.

Table 2. Relative activity of brassinolide and its analogues bioassayed by *Raphanus* test and tomato test

	Lactone†	Relative activity* R	T		Lactone†	Relative activity* R	T		Ketones‡	Relative activity* R	T
1		100	100	**9**		10	1	**16**		1	0.3
2		10	10	**10**		1	0.1	**17**		0.01	0
3		100	3	**11**		1	0.5	**18**		0.1	0
4		10	1	**12**		1	0.3	**19**		0.03	0
5		3	1	**13**		0	0	**20**		3	1
6		1	1	**14**		0	0	**21**		0.3	0.3
7		0.5	1	**15**		0	0				
8		100	10								

*R *Raphanus* test; T tomato test.
†B-ring, 7-oxa-6-one function.
‡B-ring 6-ketone function.

22,23-vicinal diol function was also shown in the rice-lamina inclination test [16] and the bean internode bioassay [18].

Brassinosteroids (**12** and **15**) lacking a 22,23-vicinal diol function, both of which showed about 1% of the activity of brassinolide (**1**) in the lamina test [25], exhibited weak or no activity, respectively, in these two bioassays. Interestingly, introduction of a hydroxyl group at C-25 completely suppressed the plant growth-promoting activities as in the case of compounds **13** and **14** although other biologically active steroids, e.g. 1,25-dihydroxyvitamin D_3, an active form of vitamin D_3, and ecdysteroids, insect moulting hormones, need a C-25 hydroxyl group for biological activity [26, 27]. However, one of the ecdysteroids, ponasterone A, which lacks the C-25 hydroxyl group, has been shown to be much more active than the native 20-hydroxyecdysone [28, 29].

In the case of (2α,3α,22,23)-tetrahydroxy-6-oxosteroids, the responses were generally much weaker than those obtained with the corresponding brassinosteroids with a

7-oxalactone group in the B-ring. The 22*R*,23*R*)-28-homo-6-oxosteroid (**16**), which had about 50% of the activity of brassinolide (**1**) in the rice-lamina test [16], showed only slight activity in the two bioassays. Among the 6-oxobrassinosteroids, the (22*R*,23*R*)-28-nor-6-oxosteroid (**20**) was the most active, giving about 10% of the response of the corresponding 28-norbrassinolide (**8**). The stereochemical requirements of the side chain obtained in brassinosteroids with 7-oxalactone also hold true for the corresponding 6-oxo analogues.

We conclude that the structural requirements of brassinosteroids for plant growth-promoting activities obtained by the *Raphanus* test and the tomato test are more stringent than those obtained by the rice-lamina inclination test [16]. Thus good responses are dependent on the following features: (1) 7-oxalactone, 2α,3α-vicinal diol and A,B-*trans* fused ring junction as in brassinolide (**1**); (2) a (22*R*,23*R*)-vicinal diol function at the C-22/C-23 position, and (3) a methyl or no alkyl group at C-24.

The clarification of the structural requirements of the brassinosteroids is important, as it will help (1) in the identification and investigation of the structure of the receptor site and (2) in the design of more active analogues of brassinolide (**1**). It can be assumed from these investigations into structure–activity relationships that brassinolide (**1**) should have two binding sites: a site specific for the 2α,3α-vicinal diol and a site recognizing the 22,23-vicinal diol. The affinity of the former site must be stronger than that of the latter one since only the brassinosteroids with a 2α,3α-vicinal diol group in the A-ring are active [30], while variations in the stereochemical configuration of the side chain part are more tolerated. However, it is of interest to note that the 6-oxobrassinosteroids are much less active than the corresponding 7-oxalactone steroids. The possibility that the 6-oxo group might be converted to the 7-oxalactone group in plants cannot be ruled out. These problems will be clarified using radioactive compounds.

EXPERIMENTAL

Brassinolide and its analogues. The following compounds were synthesized in our laboratory: brassinolide (**1**) [10], 24-epibrassinolides (**2** and **3**) [17], 28-homobrassinolides (**4**–**7**) [15, 16], 28-norbrassinolides (**8**–**11**) [14, 31], 23-deoxy- (**12**) and 22,23-bisdeoxybrassinosteroids (**15**) [32], 25-hydroxybrassinosteroids (**13** and **14**) [S. Takatsuto, N. Yazawa and N. Ikekawa, unpublished work], 28-homo-6-oxobrassinosteroids (**16**–**19**) [15, 16], and 28-nor-6-oxobrassinosteroids (**20** and **21**) [31].

Raphanus test and tomato test. Young radish (*Raphanus sativus* cv Tokinashi) seedlings and young tomato (*Lycopersicon esculentum* cv Giant cherry) seedlings were grown in wet sand in a greenhouse for 7 and 12 days, respectively. The plants were carefully transferred into 4 ml (radishes) and 2 ml (tomatoes) H_2O containing known amounts of brassinosteroids without damaging the roots. After incubation at 25° (radishes) and 30° (tomatoes) in the dark for 24 hr, the elongation percentages of the cotyledon petioles and the hypocotyls over the initials of those organs were measured. Curvatures of the cotyledon petioles and the hypocotyls induced by brassinosteroids were also observed. The minimum concns of the brassinosteroids which caused morphogenic changes were taken into account for the relative activity of the brassinosteroids as well as the minimum concn which caused about 110% elongation of the specific organs of these plants.

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