Flower Induction in Seedlings of *Asparagus officinalis* L. by N-Phenylcarbamates

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N-Phenylcarbamates, Flower Induction, *Asparagus officinalis*

A series of N-phenylcarbamates induced flowers in one-month-old seedlings of *Asparagus officinalis* L. Ninety to 100% of the plants flowered when the seeds were germinated in the presence of the most potent members of this class. The flowering occurred only once at the top of the seedlings, which then continued to grow normally. This made it possible to select the commercially preferred males of this dioecious plant at the seedling stage. Both male and female flowers were fertile, so cross-breeding was possible between flowering seedlings as well as between flowering seedlings and adults that had grown normally. Activity of flowering induction was not related with inhibition of photosystem II activity.

Introduction

Asparagus (*Asparagus officinalis* L.) is a dioecious species, and male plants are preferred for commercial production because of their greater yield, vigor, and longevity. It is impossible to distinguish the sex until flowering, which normally starts in the second or third year. A series of N^4,N^6-disubstituted 2-chloro-4,6-diamino-*s*-triazines induces flowering in one-month-old seedlings of asparagus when the seeds are germinated in their presence [1, 2]. The percentage of flowering plants is 40–45% with the most active derivative of this class.

s-Triazines are bioisosteric to carbamate compounds in their inhibition of photosystem II electron transport in spinach chloroplasts [3, 4]. The recent development of *s*-triazine anticytokinins [5] has led us to develop a series of carbamate anticytokinins [6] because the two structures are bioisosteric. Here, we explored flower induction in carbamate compounds, and found derivatives that cause a 90–100% level of flowering.

Materials and Methods

Synthesis

^{1}H NMR spectra were recorded on a JEOL PMX-60 spectrometer in $CDCl_3$ or Me_2SO-d_6 with tetramethylsilane as the internal reference. IR spectra were recorded on a Shimadzu IR-27G spectrometer. All melting points are uncorrected.

N-(3,4-Dichlorophenyl)carbamates **5–7**, **11–19**, **21**, **23**, **25–27**, *and* **29–36**

An appropriate alcohol (11.4 mmol) was added dropwise to anhydrous benzene containing 3,4-dichlorophenylisocyanate (2.0 g, 10.6 mmol) and two drops of triethylamine. The mixture was stirred for 1 h at room temperature, and then diluted with ether. The organic layer was washed with 1 N HCl and water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure.

The crude solid **12** was purified by silica gel column chromatography first with chloroform and then with *n*-hexane–ethyl acetate (90:10, v/v); purification of **27** was done with chloroform and then with ethyl acetate, and that of **33** was done with benzene. Compounds **2**, **3**, **17**, **29** and **36** were purified by recrystallization from *n*-hexane, and compound **37** from *n*-hexane–ethyl acetate. The other compounds were purified by silica gel column chromatography with chloroform as solvent. *n*-Pentyl **5** (27%), m.p. 58 °C; *n*-hexyl **6** (23%), oil; allyl **7** (54%), m.p. 54 °C; *s*-butyl **11** (59%), m.p. 74 °C; (1-methyl)butyl **12** (82%), oil; (2-methyl)butyl **13** (8%), m.p. 64 °C; (3-methyl)butyl **14** (20%), m.p. 71 °C; (4-methyl)-pentyl **15** (25%), m.p. 70 °C; *t*-butyl **16** (43%), m.p. 116 °C; neopentyl **17** (70%), m.p. 135 °C; (1-ethyl)-propyl **18** (46%), m.p. 57 °C; cyclopropylmethyl **19** (69%), m.p. 94 °C; (1-cyclopropyl)ethyl **21** (20%), m.p. 69.5 °C; cyclobutyl **23** (68%), m.p. 112 °C;

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cyclohexyl **25** (22%), m.p. 117 °C; cyclohexylmethyl **26** (39%), m.p. 136 °C; methoxyethyl **27** (22%), oil; phenyl **28** (34%), m.p. 136 °C; 2-methylphenyl **29** (58%), m.p. 116 °C; 3-methylphenyl **30** (17%), m.p. 115 °C; 4-methylphenyl **31** (6%), m.p. 143 °C; 2-fluorophenyl **32** (49%), m.p. 130 °C; 3-fluorophenyl **33** (43%), m.p. 112 °C; 4-fluorophenyl **34** (50%), m.p. 128 °C; 4-chlorophenyl **35** (56%), m.p. 144 °C; 3,4-dichlorophenyl **36** (67%), m.p. 153 °C.

*n-Propyl, (1-cyclopropyl)ethyl, and 4-chlorophenyl N-phenylcarbamates **38**, **40**, **42**, **43**, **55**, and **57–59***

Reaction was carried out between an appropriate alcohol (8.3 mmol) and a phenylisocyanate (6.5 mmol) by the same method as above.

Compounds **38**, **43**, and **57** were purified by silica gel column chromatography with chloroform as solvent; compound **42** was first purified with chloroform and then with benzene, and compound **58** was first purified with chloroform and then with *n*-hexane–ethyl acetate (90:10, v/v). Compounds **40** and **55** were recrystallized from *n*-hexane, and compound **59** was recrystallized from *n*-hexane–ethyl acetate. *n-Propyl carbamates:* 2-chlorophenyl **38** (15%), oil; 4-chlorophenyl **40** (74%), m.p. 64 °C; 2,5-dichlorophenyl **42** (71%), oil; 3,5-dichlorophenyl **43** (59%), m.p. 67 °C; 4-nitrophenyl **55** (43%), m.p. 118.5 °C. *(1-Cyclopropyl)ethyl carbamates:* 4-chlorophenyl **57** (30%), m.p. 77 °C; 3,5-dichlorophenyl **58** (63%), oil. *4-Chlorophenyl N-(4-chlorophenyl)carbamate* **59** (25%), m.p. 187 °C.

*n-Propyl-N-phenylcarbamates **37**, **39**, **41**, **44–54**, and **56***

n-Propyl chloroformate (1.00 g, 8.2 mmol) was added with stirring to anhydrous benzene containing 8.6 mmol each of an appropriate aniline and triethylamine. The mixture was stirred at room temperature for 20 min and extracted with ether. The organic layer was washed with 1 N HCl and water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure, giving a solid.

Compound **56** was recrystallized from *n*-hexane. Compounds **39** and **44** were purified by silica gel column chromatography first with chloroform and then with *n*-hexane–ethyl acetate (80:20, v/v); compound **41** was purified first with chloroform, second with *n*-hexane–ethyl acetate (80:20, v/v) and finally with benzene; compounds **49** and **53** were purified first with chloroform and then with benzene; and compounds **60** and **61** were purified with ethyl acetate. The other compounds were purified by silica gel column chromatography with chloroform as solvent. Unsubstituted phenyl **37** (24%), m.p. 50 °C; 3-chlorophenyl **39** (50%), oil; 2,4-dichlorophenyl **41** (8%), m.p. 62 °C; 3,4,5-trichlorophenyl **44** (28%), m.p. 116 °C; 4-fluorophenyl **45** (52%), m.p. 44.5 °C; 3,4-difluorophenyl **46** (86%), oil; 4-bromophenyl **47** (37%), m.p. 77 °C; 2-methylphenyl **48** (37%), oil; 3-methylphenyl **49** (25%), oil; 4-methylphenyl **50** (38%), oil; 3,4-dimethylphenyl **51** (79%), oil; 2-methoxyphenyl **52** (41%), oil; 3-methoxyphenyl **53** (26%), oil; 4-methoxyphenyl **54** (44%), m.p. 65.5 °C; 4-trifluoromethylphenyl **56** (22%), m.p. 105 °C.

*Cyclopropylmethyl and (1-cyclopropyl)ethyl N-(3,4-dichlorophenyl)-N-methylcarbamates **20** and **22***

To a solution of 1.2 mmol of cyclopropylmethyl or (1-cyclopropyl)ethyl-N-(3,4-dichlorophenyl)carbamate (**19** or **21**) in dimethyl sulfoxide were added 0.1 g (2.0 mmol) of sodium hydride (60% dispersion in oil) and excess methyl iodide. The mixture was stirred overnight at room temperature, diluted with small amounts of ether and ethyl acetate, washed with water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography with chloroform as the solvent. Cyclopropylmethyl **20** (95%), oil; (1-cyclopropyl)ethyl **22** (30%), oil.

The identification of the molecular formulas was made by elemental analysis for C, H, and N within the error of ±0.3%. The set of N-(3,4-dichlorophenyl)carbamates are listed in Table I and the others are listed in Table II. Compounds **1** [3], **2**, **3** [7], **4**, **8–10**, **24** [3], **28** [7], and **60–63** [6] were prepared and reported on previously.

Plant material

The seeds of *Asparagus officinalis* L. *cv.* Mary Washington 500W and Pole Tom were purchased from Takii Seed Company, Kyoto, Japan, and those of *cv.* Welcome were the product of Tane-no-Sakata Company, Yokohama, Japan. The seeds of *cv.* N-18 and NJ Green were kindly provided by the Nagaike Breeding Farm, Hokkaido, Japan, and those of *cv.* UC-157, Limbras 26, Franklim F1, and Hokkai 100 by the Hokkai Can Company, Tokyo, Japan.

Flower induction

The chemicals were dissolved in dimethyl sulfoxide, and the solution was diluted with distilled water to an appropriate concentration so that the final concentration of the organic solvent did not exceed 0.5% (v/v). Forty seeds of *Asparagus officinalis* L. were placed in a Petri dish (90 mm diameter, 15 mm high) that contained three layers of filter-paper and 20 ml of the test solution. The seeds were incubated at 25 °C for 12 days with a 12 h period of light from fluorescent lamps (National FL 40SS.D/37 and FL 200S.D, 80 W m^{-2}). The germination percentages were noted at the end of the incubation. The germinated seeds were throughly washed in running water, planted in Vermiculite, and grown for 13 days at 25 °C under the same light conditions as above. The flowering rate (%) was expressed by the (No. of plants with flowers/No. of plants that had emerged from the Vermiculite) $\times 100$. The maximum range of experimental error was $\pm 10\%$ through the repetitions and runs of the assay. Not all of the seeds that germinated emerged from the Vermiculite, so the emergence percentage was estimated by the (No. of plants that had emerged from Vermiculite/No. of seedlings planted) $\times 100$, the range of experimental error being within $\pm 8\%$.

The results are summarized in Tables I and II.

Inhibition of photosynthetic electron flow [3]

Chloroplasts isolated from 120 g of fresh, depetiolated spinach leaves were homogenized in an ice-cooled Waring blender with 200 ml of buffer consisting of tricine (50 mM), NaCl (10 mM), and sucrose (0.4 M) at pH 8.0. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at $2000 \times g$ for 10 min. The pellets were suspended in homogenizing medium and centrifuged again as above. The chloroplasts were suspended in 25 ml of the buffer, added to the same volume of ethylene glycol, mixed well, and stored at −20 °C until use. The amount of chloroplasts were measured by the method of MacKinney [8].

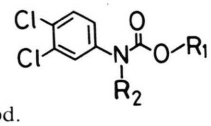
The reaction mixture consisted of 1.0 ml (15 μg) of chloroplasts suspension in a buffer (pH 7.8) of Tris (20 mM), NaCl (10 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), $CH_3NH_2 \cdot HCl$ (10 mM) and sucrose (0.4 M), 1.0 ml of (2,6-dichlorophenoxy)indophenol (DCIP) solution (40 μM) in a buffer (pH 7.2) of Tris-HCl (50 mM) and NaCl (10 mM), and 0.5 ml of a solution of the test compound. Test compounds were dissolved in water containing less than 2% (v/v) ethanol or methanol or less than 3% (v/v) Me_2SO, depending on their solubility. The reduction of DCIP was monitored at 600 nm with a Shimadzu UV-300 spectrophotometer modified for illumination with red light through a Toshiba R65 filter. The activity was expressed in terms of the logarithm of the reciprocal of the molar concentration at which 50% inhibition, pI_{50}, of the photosynthetic DCIP reduction is obtained; the range of the experimental error was within $\pm 0.08\%$.

Results and Discussion

The compounds listed in Tables I and II were screened by assays that used *Asparagus officinalis* L. *cv.* Mary Washington 500W, a representative cultivar in world-wide use. First examined were the *n*-alkyl, alkenyl, and alkynyl N-(3,4-dichlorophenyl)-carbamates **1**–**8**. They were all active; the highest potency was observed with **3** at 200 μM, and with **4** and **7** at 400 μM. In the 3,4-dichlorophenyl series of compounds, those with a methyl or ethyl branch (**9**–**18**) in their alcohol moiety generally had poorer potency than that of the corresponding *n*-alkyl derivatives; those with a cyclopropyl or cyclobutyl structure gave higher rates of flower induction. Appropriate bulkiness and a conformational bend at the 1 or 2 position seemed to be preferable for activity, probably by best fitting the shape of the receptive site. The alcohol moiety of cyclopentyl **24**, cyclohexyl **25**, and cyclohexylmethyl **26**, which were not very active, may have been too bulky. The activity of phenyl derivative **28** was also poor, though it could be somewhat improved by introduction of a substituent on the benzene ring.

In the series of compounds where the alcohol moiety was fixed to *n*-propyl but the aromatic substituents were various (Table II), those with a group at the 3 or 4 position tended to be potent. Electron-withdrawing halo, nitro, and trifluoromethyl compounds and also electronically neutral unsubstituted and methyl derivatives had activity, a positional, steric effect appeared to be in operation rather than an electronic one. The poor effects of the methoxy compounds may have been due to their bulkiness. The potency of (1-cyclopropyl)ethyl **57** and **58** corresponded roughly with that of their *n*-propyl counterparts, as was so for the 3,4-dichlorophenyl series of

Table I. Effects of N-(3,4-dichlorophenyl)carbamates on germination, growth, and flowering of *Asparagus officinalis* L., and their activity against photosystem II electron transport in spinach chloroplasts.



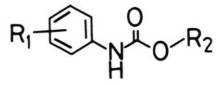
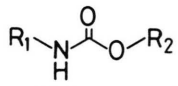
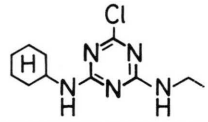
Compd. No.	R_1	R_2	*Asparagus officinalis* L. *cv.* Mary Washington 500 W Germination [%] 400 μM	Emergence [%] 400 μM	Flowering [%] μM 100	200	400	*Spinacia oleraceae* L. pI_{50}
1	Me	H	43	30	43	56	33	6.00
2	Et	H	46	56	33	57	63	4.92
3	*n*-Pr	H	57	75	54	84	67	5.11
4	*n*-Bu	H	70	55	42	61	82	5.74
5	*n*-Am	H	80	100	10	33	20	5.72
6	*n*-Hx	H	73	100	0	0	10	4.38
7	Allyl	H	55	45	39	78	89	5.50
8	Propargyl	H	46	81	57	44	35	4.82
9	*i*-Pr	H	53	70	47	39	64	5.12
10	*i*-Bu	H	60	90	27	29	53	4.97
11	*s*-Bu	H	53	45	14	67	33	4.85
12	1-Me-*n*-Bu	H	60	65	7	62	31	6.98
13	2-Me-*n*-Bu	H	73	85	6	31	18	5.13
14	*i*-Am	H	65	75	13	11	27	6.45
15	*i*-Hx	H	80	95	0	0	0	4.86
16	*t*-Bu	H	75	85	45	63	77	5.45
17	Neopentyl	H	63	80	0	20	19	5.11
18	1-Et-*n*-Pr	H	68	80	25	29	26	6.29
19	CH_2-*c*-Pr	H	60	65	62	69	92	5.39
20	CH_2-*c*-Pr	Me	80	75	53	88	100	
21	CH_2(Me)-*c*-Pr	H	58	85	33	59	82	
22	CH_2(Me)-*c*-Pr	Me	90	85	69	75	73	
23	*c*-Bu	H	70	50	43	62	90	5.24
24	*c*-Am	H	78	85	21	41	29	4.97
25	*c*-Hx	H	60	65	0	17	23	5.27
26	CH_2-*c*-Hx	H	70	85	0	0	0	4.70
27	CH_2CH_2OMe	H	53	55	27	54	36	6.51
28	C_6H_5	H	60	80	18	13	25	4.85
29	2-Me-C_6H_4	H	65	50	0	10	30	3.44
30	3-Me-C_6H_4	H	68	90	6	24	6	4.72
31	4-Me-C_6H_4	H	75	90	0	8	0	3.46
32	2-F-C_6H_4	H	60	85	0	0	6	na[a]
33	3-F-C_6H_4	H	65	75	0	7	12	na
34	4-F-C_6H_4	H	58	90	6	47	22	na
35	4-Cl-C_6H_4	H	90	70	0	15	0	
36	3,4-Cl_2-C_6H_3	H	93	70	0	0	7	
	control[b]		90	100	0	0	0	

[a] na = not active.
[b] Experiments with no chemicals added to the medium except for 0.5% (v/v) dimethyl sulfoxide.

compounds in Table I. The activity of pyridyl compounds **60–63** was not strong.

N-Phenylcarbamates are inhibitors of photosynthesis and thus herbicidal with certain aromatic and alcohol substituents. Compound **1** is the commercial herbicide swep; even in the presence of 400 μM, 30% of the seedlings grew normally. The asparagus plants seemed to be resistant to carbamate compounds when treated at the germination stage. Greening starts during the incubation with chemicals and their carryover to the next stage is possible, so we measured the photosystem II inhibitory activity of most of the compounds in Table I in spinach chloroplasts. No correlation was observed between the pI_{50} values

Table II. Effects of N-phenyl- and N-pyridylcarbamates on germination, growth, and flowering of *Asparagus officinalis* L. *cv.* Mary Washington 500W.

Compd. No.	R1	R2	Germination [%] 400 μM	Emergence [%] 400 μM	Flowering [%] μM 100	200	400
							
37	H	*n*-Pr	68	45	36	13	44
38	2-Cl	*n*-Pr	43	65	0	9	0
39	3-Cl	*n*-Pr	93	15	13	20	33
40	4-Cl	*n*-Pr	70	55	67	95	64
41	2,4-Cl_2	*n*-Pr	55	70	27	18	21
42	2,5-Cl_2	*n*-Pr	65	75	15	15	33
43	3,5-Cl_2	*n*-Pr	63	60	46	54	33
44	3,4,5-Cl_3	*n*-Pr	75	95	16	44	42
45	4-F	*n*-Pr	75	90	0	21	94
46	3,4-F	*n*-Pr	90	70	43	69	79
47	4-Br	*n*-Pr	68	85	28	55	94
48	2-Me	*n*-Pr	55	60	0	0	0
49	3-Me	*n*-Pr	88	35	16	53	57
50	4-Me	*n*-Pr	65	90	0	39	44
51	3,4-Me_2	*n*-Pr	68	75	5	63	87
52	2-OMe	*n*-Pr	40	65	0	0	0
53	3-OMe	*n*-Pr	15	67	0	0	0
54	4-OMe	*n*-Pr	18	86	0	7	0
55	4-NO_2	*n*-Pr	63	60	24	56	42
56	4-CF_3	*n*-Pr	88	70	75	81	93
57	4-Cl	CH_2(Me)-*c*-Pr	90	75	32	50	73
58	3,5-Cl_2	CH_2(Me)-*c*-Pr	85	90	18	67	56
59	4-Cl	4-ClC_6H_4	90	45	8	7	11
							
60	2-pyridyl	*n*-Pr	60	35	0	59	14
61	3-pyridyl	*n*-Pr	33	100	0	0	0
62	4-pyridyl	*n*-Pr	78	90	0	0	0
63	2-Cl-4-pyridyl	*n*-Pr	88	90	0	41	50
64			92	96	44	45	36

and toxicity; the emergence rate of the compounds with a pI_{50} value higher than 6 was rather low (**1**, **12**, **14**, and **27**; **18** was an exception) but even less growth occurred with many other compounds with lower pI_{50} values. Growth retardance by the latter compounds could be due to other causes. Neither direct nor inverse relationship was observed between photosystem II inhibition and the flowering rate.

The ratio of male and female flowers was about unity throughout the experiments. The growth indices and flowering rate changed little between 20–30 °C. The growth and concomitant flowering were much suppressed at lower and higher temperatures than these (data not shown). Flowering usually occurred only once at the top of the plants; the situation is shown in Fig. 1. This may suggest that the triggering occurred only at an early stage of germination.

The involvement of cytokinins with flower organ formation has been suggested [9], and the present

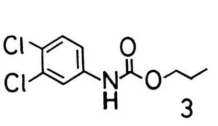
Fig. 1. Seedlings of *Asparagus officinalis* L. *cv.* Mary Washington 500W with chemically-induced flowers at their tops.

compounds were originally developed as anticytokinins [6], so they may interfere with cytokinins at the germination stage of asparagus to cause flowering. Germination experiments were therefore carried out in the presence of both a carbamate and a cytokinin benzyladenine before the seedlings were planted in Vermiculite. In this situation, however, antagonism by cytokinin could not be examined. Although with 1 and 4 μM of benzyladenine, plants grew and flowered normally, significant malformation occurred with 10 μM, making it impossible to find the flowering percentages and thus to observe antagonism; at 40 μM, the plants died (data not shown). The carbamates may disturb one of the many actions of cytokinins, leading to flowering.

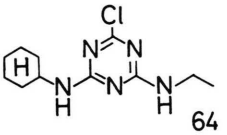
Flower induction for other cultivars than Mary Washington 500W was examined for practical application. Table III summarizes the results with compound **3**, a representative member of the present class, and, for reference, the results with 2-chloro-4-cyclohexylamino-6-ethylamino-s-triazin (**64**) [2], the most potent member in the earlier, flower-inducing s-triazines. Flower induction of the carbamate **3** was always greater than that of the triazine **64**. Male and female fertility was also examined. Fruits with viable seeds were produced by pollination between chemically induced flowers and also between chemically induced and naturally formed flowers. Table IV summarizes the results of reciprocal pollination, and Fig. 2 shows seedling bearing a fruit at its top.

This study showed that the carbamate compounds had a novel and strong flower-inducing activity for asparagus seedlings of a variety of cultivars, which

Table III. Effects of carbamate and s-triazine compounds on flowering in seedlings of asparagus cultivars[a].



3



64

Variety	Growth rate [%]	Flowering [%]	Growth rate [%]	Flowering [%]
Mary Washington 500W	95	84	75	45
Pole Tom	80	94	100	60
UC 157	75	93	95	68
N 18	50	60	50	10
NJ Green	90	78	60	8
Limbras 26	83	58	100	32
Franklim F1	79	69	45	33
Welcome	80	88	90	39
Hokkai 100	80	69	85	35

[a] Experiments were done with compounds **3** and **64** at 200 μM.

Table IV. Pollination experiments made between chemically induced and naturally formed flowers of *Asparagus officinalis* L.[a].

Male	Female Mary Washington 500W Naturally formed	Mary Washington 500W Chemically induced	Limbras 26 Chemically induced
Mary Washington 500W chemically induced	0	0	
naturally formed		0	0
Limbras 26 chemically induced	0		0
Franklim F1 chemically induced	0		

[a] Chemical induction of flowering was done with compound **3** at 200 μM. The pollination experiments were done between the indicated combinations, and viable seeds were obtained.

made it possible to select the commercially preferred males at an early stage. The compounds may be of use in increasing the efficiency of crossbreeding experiments, and in studying the reproductive differentiation of plants.

Fig. 2. Seedling of *Asparagus officinalis* L. *cv.* Mary Washington 500W with a fruit at its top. Pollination was done between chemically-induced flowers.

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