N-(3,4-Methylenedioxyphenyl)carbamates as Potent Flower-Inducing Compounds in *Asparagus* Seedlings as Well as Probes for Binding to Cytochrome P-450

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N-(3,4-Methylenedioxyphenyl)carbamates and related compounds were prepared to develop flower-inducing compounds in *Asparagus* seedlings and to assay binding to microsomal P-450 that has been postulated to be a possible triggering site for flowering. Some of the compounds had flower-inducing activity as effective as that of the previous potent compounds. The microsomal P-450 formed a type I spectrum with a representative of the present compounds as well as with previous, structurally different classes of flower-inducing compounds; which are carbamate and amidine derivatives and piperonyl butoxide, a methylenedioxy compound known as broad-spectrum P-450 inhibitor. Upon addition of NADPH, the spectra of these compounds changed to those with a peak centered at 460 nm, indicating that they all form a reactive, high affinity species to produce a complex with P-450. The target species of the compounds that induce flowering in asparagus seedlings was thought to be cytochrome P-450.

Introduction

We have developed structurally different classes of compounds that induce flowering in seedlings of *Asparagus officinalis* L. when applied during shoot apex differentiation, 4 to 10 days after sowing; they are *s*-triazines (Abe *et al.*, 1987), N-phenyl carbamates (Yanosaka *et al.*, 1989), anilide and benzamide compounds (Hara *et al.*, 1992), and alkanamidines (Kusukawa *et al.*, 1994). In search of the biochemical changes linked with the *in vivo* action of one representative carbamate compound, *n*-propyl N-(3,4-dichlorophenyl)carbamates (5091), we found that the concentrations of *p*-coumaric acid and later metabolites in the shikimic acid pathway decrease with increasing concentration of the chemical (Yanosaka *et al.*, 1991; Tanigaki *et al.*, 1993). The compound in fact inhibited *t*-cinnamic acid 4-hydroxylase (C4H), the gateway emzyme of phenylpropanoid metabolism, in the microsomes. Because C4H is known as a cytochrome P-450 monooxygenase (Durst, 1991), we have examined the flower-inducing activity of known cytochrome P-450 inhibitors, and found that piperonyl butoxide (PBO), a commercial P-450 inhibitor used as an insecticide synergist, causes flowering and inhibits C4H as well as the carbamate 5091.

However, the inhibition of phenylpropanoid metabolism as a cause of flower induction has been ruled out, because flowering does not occur even if the phenylpropanoid pathway is blocked by an inhibitor of phenylalanine ammonialyase, the enzyme involved in the step preceding that catalyzed by C4H (Tanigaki *et al.*, 1993). 5091 and PBO have been supposed to inhibit other cytochrome P-450 besides C4H. More recently, we have developed amidines as a potent series of flower-inducing compounds, and found that they inhibit C4H only weakly (Kusukawa *et al.*, 1994). However, whether or not the amidines inhibit P-450 other than C4H, as well as whether or not 5091 and PBO actually inhibit P-450 besides C4H, has remained to be proven. Incidentally, C4H is the only P-450 enzyme that has been identified in asparagus microsomes (Tanigaki *et al.*, 1993).

In this study, we prepared a series of N-(3,4-methylenedioxyphenyl)carbamates to develop flower-inducing compounds and probe binding to microsomal P-450; they have a structure that is a hybrid of previous carbamate series of compounds and PBO, a compound with a methylenedioxy group that is catalytically activated by P-450 to form a product complex with the enzyme (Ortiz de Montellano and Reich, 1986). Some of them

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had flower-inducing activity as effective as that of the potentest amidine compound, N-(4-chloro-2-trifluoromethylphenyl)-N′-*n*-propoxyacetamidine (AM12), and the C4H inhibitory activity of a representative one of the compounds, *n*-butyl N-(3,4-methylenedioxyphenyl)carbamate (CM15A), was again found to be very weak. The interaction of CM15A with P-450 in microsomes was then studied spectrophotometrically together with the previous 5091, PBO and AM12. All of these flower-inducing compounds showed the type I spectrum, which then underwent a change to the type III spectrum in the presence of NADPH. These findings are discussed.

Materials and Methods

The seeds of *Asparagus officinalis* L. cv. Mary Washington 500W were purchased from Kaneko Seed Company (Gunnma, Japan).

Glucose-6-phosphate and glucose-6-phosphate dehydrogenase (Type XV) (EC 1.1.1.49) were purchased from Sigma (St. Louis, U.S.A.).

Synthesis

N-(3,4-Methylenedioxyphenyl)carbamates **1** *and* **5–7**: An appropriate alkyl chloroformate (11 mmol) was added dropwise to the solution of 3,4-methylenedioxyaniline (9 mmol) and triethylamine in dry benzene under stirring at 0 °C. The mixture was stirred for 2–3 hr at room temperature and extracted with ethyl ether. The ether layer was washed with 1 M HCl and water, dried over $MgSO_4$, and evaporated under reduced pressure to dryness. The residue was purified with silica gel column chromatography and recrystallization.

3,4-Methylenedioxyphenyl N-n-propylcarbamate **4**: The compounds were prepared as above from *n*-propyl isocyanate and 3,4-methylenedioxyphenol (sesamol).

N-(3,4-Methylenedioxyphenyl)-N′-n-propylurea **2**: The compounds were prepared as above from *n*-propyl isocyanate and 3,4-methylenedioxyaniline but without triethylamine.

N-(3,4-Methylenedioxyphenyl)butyramide **3**: The compounds were prepared as above from butyryl chloride and 3,4-methylenedioxyaniline.

N-(3,4-Methylenedioxyphenyl)carbamates **8–10**: To the mixture of triphosgene (18.4 mmol in 50 ml dry benzene) and triethylamine (73.6 mmol in 40 ml of dry benzene) was added 3,4-methylenedioxyaniline (36.8 mmol) in 50 ml of dry benzene. The mixture was stirred for 18 hr at room temperature and filtered. To $^1/_3$ of the filtrate was added an appropriate amount of alcohol (12 mmol) and triethylamine, and the mixture was stirred for 4 hr at room temperature. The mixture was extracted with ethyl ether, and the ether layer was washed with water, dried over $MgSO_4$, and evaporated to dryness. The residue was purified as above.

n-Butyl N-methyl-N-(3,4-methylenedioxyphenyl)-carbamate **11**: To the dimethylsulfoxide solution (20 ml) of **10** (2.4 mmol) was added 0.2 g of NaH (60% dispersion in oil) and an excess methyl iodide. The mixture was stirred at room temperature over night and extracted with ethyl ether. The ether layer was washed with water, dried over $MgSO_4$, and evaporated under reduced pressure to dryness. The residue was purified as above.

Elemental analyses of each compound were performed for C, H, and N within the error of ± 0.3%.

Flower induction

The chemicals were dissolved in dimethylsulfoxide, 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* were placed in a Petri dish (9 cm ∅ × 1.5 cm) that contained three layers of filter paper, four layers of tissue paper, and 20 ml of the test solution, and incubated at 25 °C for 10 days under darkness. The germination percentage was calculated at the end of the incubation period. Twenty germinated seeds were washed in running water for 10 min, placed in a Petri dish containing filter paper and tissue paper as above, and 20 ml of distilled water, and incubated for another 4 days at 25 °C with a 12 hr period of light from fluorescent lamps. The apices of the shoots were observed microscopically, and the number of plants with a flower bud was counted. The flowering rate (percent) was expressed as (no. of plants with flower buds/no. of plants that survived) ×100. The maximum range of the experimental error was ± 10%. Not all of the germinated seeds survived, so that the survival rate was also estimated. The range of experimental error was within ± 8% during germination and growth.

Isolation of microsomal fraction

The experiments were done as described elsewhere (Tanigaki *et al.*, 1983; Kojima and Takeuchi, 1989; Saunders *et al.*, 1977; Fujita and Asahi, 1985; Reichart *et al.*, 1982). Ten-day-old seedlings detached of seeds (20 g fr wt from about 2,000 seedlings), were homogenized with a homogenizer (Polytron PT 10 20 350D, Kinematica Co., Luzern, Switzerland) in four volumes (4 ml/g fr wt) of extraction buffer (50 mM tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5) containing 0.3 M *D*-sorbitol, 1 mM EDTA-2Na, 1% (wt/v) sodium isoascorbate, and Polyclar AT (0.05 g/g fr wt) at 2–4 °C. The homogenate was forced through four layers of cotton gauze, and the filtrate was centrifuged at 500×*g* for 10 min to remove debris and the Polyclar AT. The supernatant was centrifuged at 20,000×*g*, and the supernatant was centrifuged again at 100,000×*g* for 60 min. The precipitate was suspended in 1 ml of tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.5).

Assay of C4H activity

The microsomal suspension (0.4 ml, 0.5 mg protein/ml) was mixed with 3 µl of dimethylsulfoxide containing a test compound and 0.4 ml of 50 mM tris(hydroxylmethyl)aminomethane-HCl buffer (pH 7.5) containing 3 mM NADP (sodium salt), 10 mM glucose-6-phosphate(disodium salt), 3 mM 2-mercaptoethanol, and glucose-6-phosphate dehydrogenase (4 units/ml), and incubated first at 30 °C for 10 min. To the mixture was added 0.4 ml of 2 mM *t*-cinnamic acid solution in the buffer, bringing the final concentration to 0.66 mM, and the mixture was incubated at 30 °C for 1 hr with vigorous shaking. The reaction was stopped by addition of 24 µl of acetic acid and boiling of the mixture for 1 min. The mixture was then cooled and centrifuged at 1,000×*g* for 10 min. The concentration of *p*-coumaric acid in the supernatant was assayed by a HPLC system equipped with a M & S ODS column (4.6 mm i.d.×15 cm), which was eluted with a gradient mode of MeOH: *n*-BuOH:acetic acid:H_2O = 1:1:2:96 (v/v) to 97:1:2:0 with monitoring at 280 nm. The concentration of *p*-coumaric acid after the reaction was subtracted from that before the reaction, and the amount of *p*-coumaric acid produced was calculated from the remainder in each experiment. The experiments were repeated three or four times, and Fig. 1 shows the average value with ± SE.

Binding asssay

The experiments were done with Shimadzu UV-3000 spectrophotometer. Test compounds were dissolved in dimethylsulfoxide, and a 3 µl aliquot was added to the sample cuvette containing 600 µl of microsomal fraction (1 mg protein/ml 50 mM K-Pi buffer (pH 7.5) containing 20% (w/w) glycerol), an identical volume of diemthylsulfoxide being added in the reference cuvette. The contents were mixed and the spectrum (type I) was recorded at 25 °C after 3 min. When a supply of NADPH was needed, the microsomal fraction was resuspended in 50 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.5) containing 2 mM glucose-6-phosphate, 1 mM 2-mercaptoethanol and 0.4 units/ml glucose-6-phosphate dehydrogenase. Test compounds were added as above, and the type I spectrum was recorded. Then NADP (sodium salt) was added to the sample cuvette so that the final concentration became 400 µM, and the spectrum (type III) was recorded after incubation for 10 min at 25 °C.

Protein determination

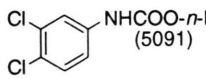
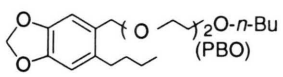
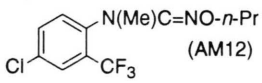
Protein was determined according to Bradford (1976).

Results

Flower inducing activity

The compounds **1–11** listed in Table I were prepared and screened by assays that used *Asparagus officinalis* L cv. Mary Washington 500W, a cultivar in world-wide use. First examined were carbamate, urea and amide compounds having a methylenedioxyphenyl function at one end of the molecule and *n*-propyl at another end (compounds **1–4**). The highest flower-inducing activity was observed with N-(3,4-methylenedioxyphenyl)carbamate (**1**), flowering rate being 100% at 200 µM. We then varied systematically the alkyl substituent of compound **1** to give compounds **5–10**. Ethyl derivative **5** had activity lower than **1**, and *s*-butyl, cyclopropylmethyl and *c*-pentyl **8**, **9** and **10** showed activity as high as or slightly higher than **1**. The potency of *n*-butyl **6** (CM15A) and *i*-butyl **7** was considerably

Table I. Effects of 3,4-methylenedioxyphenyl compounds on germination, growth, and flowering of *Asparagus officinalis* L. cv. Mary Washington 500W[a].

No.	X	[μM]	Germination [%]		Survival rate [%]		Flowering [%]			
			40	400	40	400	40	100	200	400
1	NHCOO–*n*-Pr		98	98	100	100	30	89	100	100
2	NHCONH–*n*-Pr		98	98	100	100	0	5	15	40
3	NHCO–*n*-Pr		100	100	90	100	0	10	55	65
4	OCONH–*n*-Pr		98	98	100	100	0	15	35	90
5	NHCOO–*n*-Et		98	100	100	100	5	65	95	100
6	NHCOO–*n*-Bu (CM15A)		95	98	100	95	90	95	100	100
7	NHCOO–*i*-Bu		100	95	100	95	90	100	100	100
8	NHCOO–*s*-Bu		93	95	90	80	61	80	88	100
9	NHCOO–CH_2-*c*-Pr		88	63	95	35	79	93	100	100
10	NHCOO–*c*-Pentyl		93	100	95	55	74	100	100	100
11	N(Me)COO–*n*-Bu		100	93	100	100	15	60	85	100
			–	57	–	75	–	54	84	67
			–	95	–	100	–	41	72	85
			95	100	100	100	95	100	100	100

[a] Data for 5091, PBO, and AM12 were cited from Yanosaka *et al.* (1989), Tanigaki *et al.* (1993), and Kusukawa *et al.* (1994), respectively.

high. There seemed to be a preferable steric condition for activity. N-Methylation of CM15A (**6**) caused lowering of activity (compound **11**). The biological data of 5091, PBO, and AM12 are also shown in Table I as reference.

Effect on the activity of microsomal C4H

The 100,000×*g* microsomal fraction was prepared as previously reported (Tanigaki *et al.*, 1993) from 10-day-old seedlings detached of seeds. The optimum pH of C4H activity was 7.5 (data not shown). The conversion of *t*-cinnamic acid to *p*-coumaric acid was at a linear rate for at least 2 hr of incubation under the present experimental condition (Tanigaki *et al.*, 1993), and thus the reaction of 1 hr was studied in the experiments in which the effects of the chemicals were examined.

When the microsomal fraction was incubated with compound **6** (CM15A) in the presence of *t*-cinnamic acid, inhibition of *p*-coumaric acid production was very much less than that caused by the application of 5091 (Fig. 1).

Interaction with microsomal P-450

The interaction of the flower inducing compounds with microsomal P-450 was monitored using UV difference spectroscopy. The compounds selected for the examination were the previously developed 5091, PBO, AM12, and the present CM15A (**6**). All of these compounds were found to induce a type I difference spectrum, with an absorbance peak centered at 380–390 nm and a trough at 420–430 nm (Fig. 2). When the concentration of CM15A was stepwisely decreased, the peak lowered and the trough shallowed but no qualitative changes were observed (data not shown). *t*-Cinnamic acid (200 μM), the substrate of C4H, gave the spectrum only scarcely, and *n*-pro-

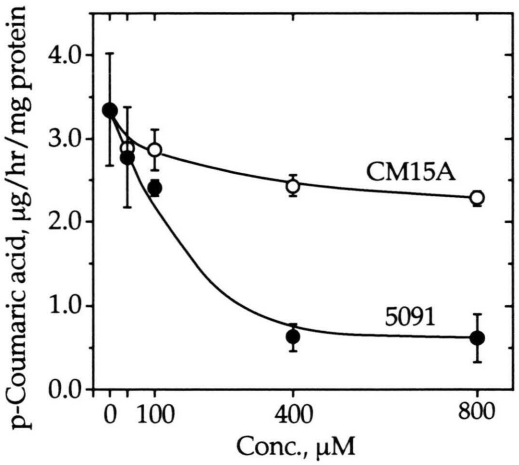


Fig. 1. Effects of CM15A and 5091 on C4H activity in the microsome preparation. Medium containing 0.66 mM *t*-cinnamic acid was incubated for 1 hr at 30 °C and centrifuged, and the concentration of *p*-coumaric acid in the supernatant was assayed by a HPLC system.

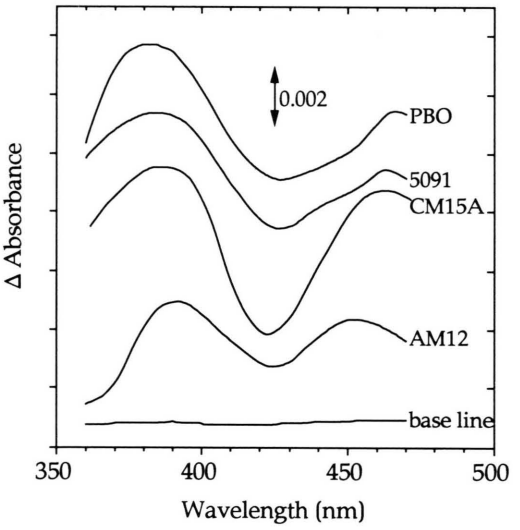


Fig. 2. Difference spectra resulting from addition of PBO (200 μM), 5091 (100 μM), CM15A (100 μM) and AM12 (100 μM) to the microsome preparation. Each test compound dissolved in dimethylsulfoxide was added to the sample cuvette, with the addition of an identical volume of dimethylsulfoxide to the reference cuvette, and the spectrum was recorded at 25 °C after 3 min.

pyl N-(4-methoxyphenyl)carbamate, an impotent carbamate for flower induction (Yanosaka *et al.*, 1989), did not give the spectrum (data not shown).

The methylenedioxy function of PBO is known to be catalytically activated by P-450 to form a product complex with the enzyme (Ortiz de Montellano and Reich, 1986). To examine this possibility with asparagus microsomes, NADPH was generated in the sample cuvette after determinaton of the spectrum from oxidized P-450 (Fig. 3). Although the spectrum was contaminated with the peak of cytochrome b_5 centered at 430 nm, the peak centered at 460 ~ 465 nm appeared to indicate the formation of the so-called type III spectrum (Philpot and Hodgson, 1971); the results were the same with another methylenedioxy CM15A and non-methylenedioxy 5091 and AM12.

Discussion

Replacement of 3,4-dichloro function of the previous carbamate 5091 to 3,4-methylenedioxy gave a more potent flower inducing compound **1**, and the optimization of the structure produced compounds **6** (CM15A) and **7** the potency of which in flower induction was as high as that of a previous amidine compound AM12, the most potent compound so far developed (Kusukawa *et al.*, 1994).

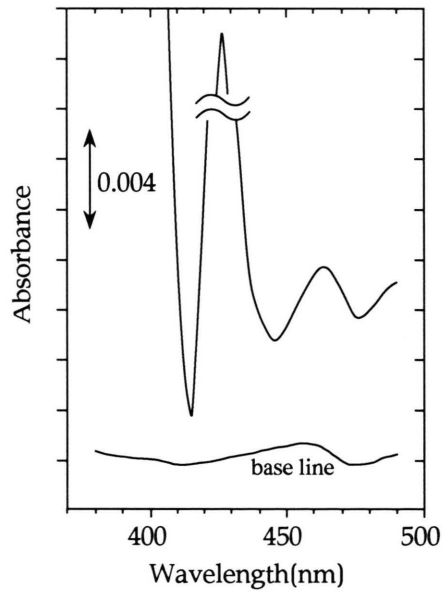


Fig. 3. PBO difference spectrum in the presence of NADPH. The test compound was added to the microsomes suspended in 50 mM tris(hydroxymethyl)aminomethane-HCl buffer containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase, and then NADP (sodium salt) was added to the sample cuvette. The spectrum (type III) was recorded after incubation for 10 min at 25 °C.

The present finding that the four structurally different classes of compounds 5091, PBO, AM12 and CM15A, formed type I spectra with asparagus microsomal P-450 suggests that binding with P-450 is a common feature of flower inducing compounds. The spectrum was not produced with a carbamate compound, *n*-propyl N-(4-methoxyphenyl)carbamate (Yanosaka *et al.*, 1989), that is structurally congeneric to 5091 but impotent for flower induction. The P-450 with which the flower inducing compounds commonly interact is considered to be not C4H, since poorly inhibitory CM15A formed the spectrum as well as strongly inhibitory 5091 and PBO. Moreover, the substrate of C4H, *t*-cinnamic acid, gave only a scarce spectral change with the microsomes from 10-day-old seedlings. The C4H content seemed to be relatively small; although data are not shown, *t*-cinnamic acid exhibited the type I spectrum with the microsomal fraction from 14-day-old seedlings, in which the phenylpropanoid metabolism has been reported to be accelerated much more than that in 10-day-old seedlings (Yanosaka *et al.*, 1989).

The spectral change at 450–460 nm observed when NADPH was generated in the mixtures of oxidized P-450 with 5091, PBO, AM12 and CM15A suggests that they form product complexes with P-450.

Methylenedioxy compounds like PBO are known as a representative class of inhibitors that produce this type (type III) of spectral change (Philpot and Hodgson, 1971), and thus the methylenedioxy CM15A is also thought to suffer a similar metabolic attack as PBO does. 5091 and AM12 do not have a methylenedioxy function, but are thought to give a product complex by a yet unknown mechanism. These findings show that another feature of the flower-inducing compounds is to suffer a metabolism to produce a reactive or high affinity species for reduced P-450.

The target species of the flower-inducing compounds is thought to be cytochrome P-450. However, what signal the cytochrome P-450 mediates for inducing flowering remains open. A deposit of a metabolite caused by blocking the P-450 enzyme may be a possibility in the day-neutral asparagus whose flowering is dependent on aging. One of the necessary steps to answer the above question is thought to apprehend the enzyme, and the set of the compounds would be also of use in pursuing the enzyme spectrophotometrically as the entity that interacts with them commonly strongly. They are also considered to be new classes of plant cytochrome P-450 inhibitors.

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