Effect of Phenyl Substituents in Benzyl Quaternary Ammonium Derivatives of (+)-Limonene on Plant Growth-Retardant Activity

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The growth-retardant activity of 26 benzyl quaternary ammonium derivatives of (+)-limonene was evaluated using two bioassays, one based on the root growth of cucumber seed and the other on total fresh weight increase of alfalfa seeds exposed to dilute aqueous solutions of each compound. Inhibition of human blood serum cholinesterase was also determined manometrically. A highly significant correlation between serum cholinesterase inhibition and plant growth retardation found for 24 compounds on cucumber and 22 compounds on alfalfa suggests that these derivatives retard plant growth by inhibition of an enzyme system associated with a growth process. Electron withdrawing groups on the benzene ring, such as nitro and cyano, caused inactivation. A chloro group at 2 or 4 produced more activity than substitution at position 3. Monobromo and monomethyl derivatives substituted at 2 and 3 were equally active, while substitution at C-4 approximately doubled activity. The disubstituted chloro and methyl benzyl compounds were all highly active, regardless of the positions of these groupings.

The ability of certain quaternary ammonium compounds to reduce stem elongation of bean plants without causing abnormal, formative changes was first reported by Mitchell *et al.* (1949). One of the most active compounds was a quaternary ammonium carbamate, designated Amo-1618 (4-hydroxyl-5-isopropyl-2-methylphenyltrimethylammonium chloride, 1-piperidine carboxylate), prepared from the terpene thymol by R. L. Shriner at the University of Illinois. Much work has been done on the synthesis and testing of derivatives of Amo-1618 (Krewson *et al.*, 1959).

Knight *et al.* (1969) have studied the influence on plant growth-retardant activity of various substituents of aryl quaternary ammonium, phosfonium, and sulfonium compounds. 3-Chloro- and 4-chloro-benzyltributyl ammonium bromides were the most active compounds.

The plant growth-retardant activity of six benzyl quaternary ammonium derivatives synthesized from the terpene (+)-limonene, found abundantly in citrus peel oil, has been reported (Newhall and Pieringer, 1966). The effect of chain length in *n*-alkyl quaternary ammonium derivatives of (+)-limonene on plant growth has also been studied (Pieringer and Newhall, 1968). Two additional benzyl quaternary ammonium compounds, prepared from (+)-limonene, have been reported (Newhall and Pieringer, 1969), and their effect on the growth of citrus described (Pieringer and Newhall, 1970).

All of these limonene derivatives have somewhat different structural requirements for plant growth-retardant activity than other known growth regulants. A highly significant correlation between the plant growth-retardant activities of these compounds and their ability to function as inhibitors of human blood serum (pseudo) cholinesterase has been demonstrated (Newhall, 1969).

In the present study, 18 additional, new, substituted benzyl quaternary ammonium derivatives of (+)-limonene were synthesized both to determine the effect of various phenyl substituents on their plant growth-retardant and enzymic activities and to further study the correlation between these activities.

PROCEDURE

The benzyl quaternary ammonium compounds were prepared by quaternization of the mixed trans isomers of either 2-dimethylamino-Δ8(9)-*p*-menthen-1-ol (Newhall, 1964) or 2-dimethylamino-1-*p*-menthanol (Newhall, 1959) with the appropriate alkyl halide. As in the case of the previously reported benzyl quaternary ammonium derivatives, these products are also mixtures of the possible trans isomers having the general structure indicated in Table I. The presence of a single bond (sat.) or double bond (unsat.) at 8(9) in the isopropyl side chain is indicated, as well as the nature of the anion. Yield data and the results of elemental nitrogen analyses are included. Compounds are numbered consecutively for reference purposes. The only variation in synthesis was the substitution of dimethylformamide (DMF) as solvent for methylethylketone in quaternizations which were difficult, for steric or other reasons, and thus required a more polar solvent. These reactions, indicated in Table I by a superscript *c*, comprised warming 5 g of the amino alcohol with 10 g of the appropriate alkyl halide in 5 ml of DMF at 95° C for 16 hr. The DMF was removed azeotropically with *p*-xylene and the quaternary compound isolated (Newhall and

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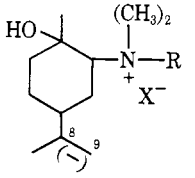
Table I. Enzyme and Plant Growth Inhibition of Quaternary Ammonium Derivatives of (+)-Limonene

General formula



Compound No.	R	8(9)	X	Yield %	N % (calcd.)	N[a] % (found)	Pseudo ChE inhibition ($I_{50} \times 10^5$) M	Cucumber growth inhibition (I_{50}) M	Alfalfa growth inhibition (I_{50}) M
1[b]	Benzyl	unsat.	Cl	...	...	...	20.0	1.0×10^{-2}	2.0×10^{-2}
2[b]	Benzyl	sat.	Cl	...	...	...	12.7	1.0×10^{-3}	3.1×10^{-2}
3[c]	2-Chlorobenzyl	sat.	Cl	96	3.88	3.90	2.0	1.9×10^{-4}	8.0×10^{-3}
4	3-Chlorobenzyl	sat.	Br	80	3.46	3.78	8.5	1.5×10^{-3}	2.8×10^{-2}
5[b]	4-Chlorobenzyl	sat.	Cl	...	...	...	6.3	2.2×10^{-4}	1.5×10^{-2}
6[b]	4-Chlorobenzyl	unsat.	Cl	...	...	...	11.4	6.0×11^{-4}	1.5×10^{-1}
7[c]	2-Bromobenzyl	sat.	Br	81	3.11	3.57	1.5	5.4×10^{-4}	1.0×10^{-2}
8[c]	3-Bromobenzyl	sat.	Br	91	3.11	3.17	8.5	5.7×10^{-4}	1.0×10^{-2}
9	4-Bromobenzyl	sat.	Br	78	3.11	3.53	1.3	2.6×10^{-4}	inactive
10	4-Fluorobenzyl	sat.	Cl	87	4.07	4.39	17.5	0.9×10^{-3}	inactive
11[c]	2-Methylbenzyl	sat.	Cl	91	4.12	4.24	5.5	5.3×10^{-4}	1.1×19^{-2}
12[c]	3-Methylbenzyl	sat.	Cl	100	4.12	4.27	4.0	5.5×10^{-4}	1.2×10^{-2}
13[b]	4-Methylbenzyl	sat.	Cl	...	...	...	4.5	2.2×10^{-4}	1.3×10^{-2}
14	4-Methylbenzyl	unsat.	Cl	79	4.14	4.34	8.0	2.8×10^{-4}	1.1×10^{-2}
15[c]	4-Ethylbenzyl	sat.	Cl	96	3.95	4.24	1.1	1.6×10^{-4}	4.2×10^{-3}
16[c]	4-Nitrobenzyl	sat.	Cl	99	7.55	7.69	7.5	inactive	inactive
17[c]	4-Cyanobenzyl	sat.	Br	85	7.08	7.02	7.0	inactive	inactive
18[b]	2,4-Dichlorobenzyl	unsat.	Cl	...	...	...	4.4	4.4×10^{-4}	0.9×10^{-2}
19[b]	2,4-Dichlorobenzyl	sat.	Cl	...	...	...	2.4	2.9×10^{-4}	1.7×10^{-2}
20[b]	3,4-Dichlorobenzyl	sat.	Cl	...	...	...	5.9	2.5×10^{-4}	1.3×10^{-2}
21[c]	3,4-Dimethylbenzyl	sat.	Cl	98	3.95	4.02	1.8	1.1×10^{-4}	1.8×10^{-2}
22	2,4-Dimethylbenzyl	sat.	Cl	72	3.95	3.47	3.7	1.3×10^{-4}	0.9×10^{-2}
23	2,4-Dimethylbenzyl	unsat.	Cl	71	3.98	3.84	7.2	3.6×10^{-4}	1.2×10^{-2}
24	2,5-Dimethylbenzyl	sat.	Cl	72	3.95	3.78	0.34	1.3×10^{-4}	0.7×10^{-2}
25	2,5-Dimethylbenzyl	unsat.	Cl	72	3.98	4.43	0.56	0.9×10^{-4}	0.5×10^{-2}
26	3,5-Dimethylbenzyl	sat.	Br	85	3.51	3.34	3.3	1.5×10^{-4}	0.6×10^{-2}

[a] Nitrogen analyses were made using a semimicro-Kjeldahl-Gunning procedure. This was necessary because most compounds were amorphous, hygroscopic glasses which were difficult to purify, dry, and weigh. [b] Compounds reported previously. [c] Made using dimethylformamide as solvent.

Pieringer, 1966). All other quaternizations were done using methylethylketone and a reflux time of 16 hr (Newhall and Pieringer, 1966). The eight benzyl compounds described previously are indicated by a superscript *b*. Yields and elemental nitrogen analyses for these have been reported and are omitted in Table I. However, their molar I_{50} values for pseudocholinesterase and for growth retardation of cucumber and alfalfa have not been reported previously.

Infrared absorption curves were obtained for all new compounds (Table I) using thin films prepared by evaporating a few drops of a dilute methanol solution directly on a sodium chloride window. These spectra were consistent with the assigned structures (Table I). All derivatives exhibited strong absorption at 2.96 μ (OH) and 3.37 μ (C–H). The 8(9) unsaturated compounds had a sharp peak of medium intensity at 6.08 μ and a strong, broad peak at 11.2 to 11.3 μ, both of which were absent from the saturated derivatives.

Pseudocholinesterase inhibition was determined for all benzyl quaternary ammonium derivatives (Table I) using standard Warburg manometric techniques (Augustinsson, 1957). Human blood serum, diluted 1 in 10 with water, was used as the enzyme source with acetylcholine as substrate. The molar concentration giving 50% inhibition (I_{50}) was determined (Newhall, 1969) and is recorded as $I_{50} \times 10^5$ in Table I.

Two plant-growth tests were employed to determine the retardant activities of these compounds. A bioassay (Mitchell and Livingston, 1968) based on the root growth response of cucumber seed (*Cucumis sativus* L., 'Marketer') was found to be particularly sensitive to these compounds in the concentration range 0.0005 *M* to 0.00002 *M*. Three replicates of 10 seeds each were used for each of the five concentrations of test compound employed (0.0005, 0.0002, 0.0001, 0.00005, 0.00002 *M*). The molar concentration giving 50% growth retardation (I_{50}) was determined from a semilog plot of concentration against percent retardation, and is recorded in Table I.

A bioassay based on the growth response of alfalfa seed (*Medicago sativa* L., 'Hairy Peruvian') (Newhall, 1969) was used at three concentrations (0.005, 0.002, 0.001 *M*) of test compound. Three replicates of 100 seeds each were employed for each concentration, and molar I_{50} values determined (Table I) graphically as for cucumber seeds.

RESULTS AND DISCUSSION

Pseudocholinesterase inhibition ($I_{50} \times 10^5$) is plotted in Figure 1 on semilog paper against growth retardation (I_{50}) of cucumber. This shows a log-linear relationship between growth retardation and enzyme inhibition, respectively. Only compounds 16 and 17 (Table I), the 4-nitro- and 4-cyanobenzyl derivatives, respectively, are omitted. These two electron withdrawing substituents rendered both derivatives relatively inactive as growth retardants (alfalfa $I_{50} > 1$).

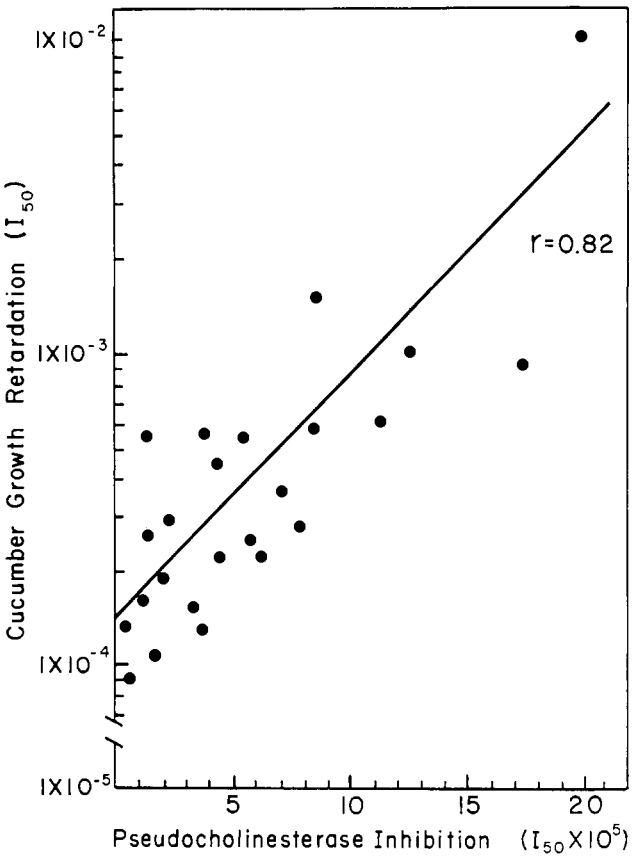


Figure 1. Pseudocholinesterase inhibition and growth retardation of cucumber by benzyl quaternary ammonium derivatives of (+)-limonene

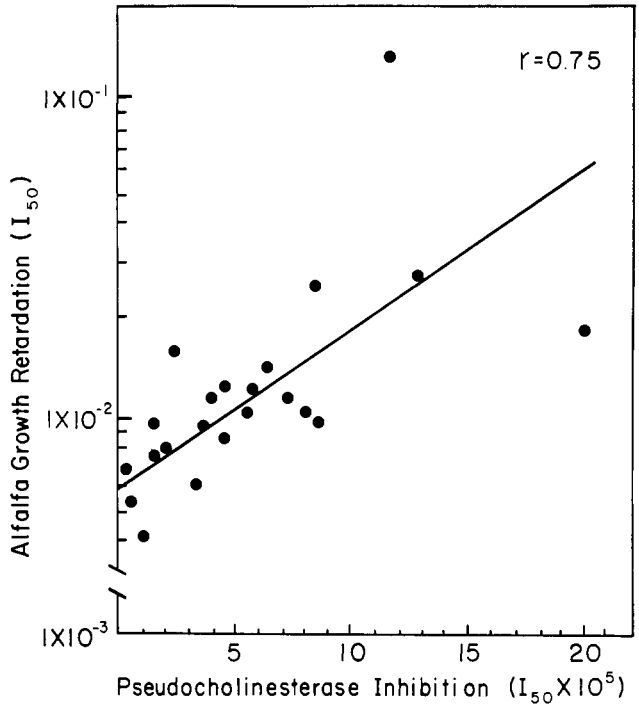


Figure 2. Pseudocholinesterase inhibition and growth retardation of alfalfa by benzyl quaternary ammonium derivatives of (+)-limonene

The correlation coefficient (r) calculated from the log of growth retardation and from enzyme inhibition by linear regression analysis for the other 24 compounds is 0.82. This shows a highly significant correlation between pseudocholinesterase inhibition and growth retardation of cucumber similar to that previously reported for a series of *n*-alkyl and benzyl quaternary ammonium derivatives of (+)-limonene (Newhall, 1969).

In Figure 2, pseudocholinesterase inhibition ($I_{50} \times 10^5$) is plotted on semilog paper against growth retardation (I_{50}) of alfalfa. The same log-linear relationship found for cucumber is evident. Compounds 16 and 17 are almost inactive on alfalfa, as are 9 and 10, the 4-bromo- and 4-fluorobenzyl derivatives, respectively ($I_{50} > 1$). The correlation coefficient (r) is 0.75 in this case for the 22 benzyl compounds plotted. This again shows a highly significant correlation between pseudocholinesterase inhibition and plant growth retardation.

Compounds (1, 2), (5, 6), (13, 14), (18, 19), (22, 23), and (24, 25) constitute six similar pairs of benzyl derivatives differing only in saturation or unsaturation at the 8(9) position (Table I). In each case, the saturated analog is approximately twice as active an inhibitor of pseudocholinesterase as the unsaturated. This observation was reported previously for other quaternary ammonium derivatives of (+)-limonene (Newhall, 1969). The molecular models of the saturated benzyl compounds show greater symmetry and are more planar than models of the 8(9) unsaturated analogs. If adsorption on an enzyme surface is critical to the plant growth retardant activity of these compounds, greater adsorption and attendant inhibition would be predicted from the molecular shape of the saturated derivatives. Greater growth retardation of cucumber by all 8(9) saturated compounds is also very pronounced. This effect is not shown, however, for alfalfa, which is a much less sensitive test plant for these compounds.

The data in Table I show that substitution of chlorine at positions 2 or 4 in the benzene ring produces derivatives more active in all tests than the 3-chloro compound. The 2 and 4 bromo derivatives are also more active as enzyme inhibitors than the 3-bromo derivative. There is no difference in the growth retardant activities of the 2 and 3-bromo compounds on cucumber. However, the 4-bromo derivative is about twice as active on cucumber, although it is inactive on alfalfa. The activity of the 4-fluoro compound (10) is about the same on pseudocholinesterase and cucumber as that of the unsubstituted benzyl compound (2). This may be because the fluoro group has about the same size as hydrogen. There is no difference between the activities of the monomethyl compounds substituted at either 2 or 3, but the 4-methyl compound is twice as active in the cucumber bioassay. The 4-ethyl derivative (15) is more active than the 4-methyl (13). The inactivity of compounds 16 and 17 as growth retardants has been mentioned. The dichloro derivatives (18, 19, 20) are highly active, with little difference between 2,4 or 3,4 substitution. The dimethyl compounds (21, 22, 23, 24, 25, 26) are also highly active, and there are only slight differences in their growth retardant activities. However, the 2,5-dimethyl compounds (24, 25) do show a marked increase in enzyme inhibition compared to either the 2,4, 3,4, or 3,5 analogs.

These results are similar in some respects to those obtained by Knight *et al.* (1969) on benzyltributyl quaternary ammonium salts. For these compounds, a *para*-chloro group was most important for high activity. Cathey (1964) has reported that for chlorobenzyltributylphosphonium salts the 4-chloro is more active than the 2-chloro compound. In Table I this order is reversed, and the 2-chlorobenzyl derivative (3) is shown to be more active in all tests than either the 3 or 4 substituted compounds. These facts indicate that the structural requirements for plant growth-retardant activity

are more similar in benzyl quaternary ammonium derivatives of (+)-limonene to those of known alkyl and aryl benzyl quaternary ammonium salts. Although derivatives of Amo-1618, prepared from the terpene carvacrol, might appear to be more similar to these limonene derivatives, their structural requirements for growth retardant activity are very different; *i.e.*, derivatives of Amo-1618 without a carbamate grouping are inactive.

These benzyl compounds and the previously reported (Newhall, 1969) *n*-alkyl quaternary ammonium derivatives of limonene thus constitute a unique group having particular structural requirements for activity. The contribution of the terpene moiety to this activity must be considerable, since all of these quaternary ammonium derivatives have biological activity. The striking correlation between pseudocholinesterase inhibition and plant growth retardation found for these compounds suggests that they inhibit a plant enzyme associated with a growth process.

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