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Chemical Inducers of Carotenogenesis

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Fourteen triethylamine derivatives including the 2-(4-chlorophenylthio)triethylamine (CPTA) were all found to be effective in inducing the formation of lycopene in Marsh white seedless grapefruit (*Citrus paradisi*, Rutaceae); however, they differed in the degree of effectiveness. The structural feature necessary for inducer activity appeared to be the tertiary alkylamine. Compounds with an S atom joining the amine portion and the benzene moiety seem to be more effective than their oxy analogs. Benzene derivatives are more effective than alkyl derivatives. Log partition coefficient of the compounds can only be used as a general guide in designing the new chemical inducers; however, the effectiveness of the compounds depends not only on the lipo–hydro characteristics of the molecule but also on the electronic and steric states of the molecule.

The compound 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA) was found to have a profound effect on carotenogenesis in a wide array of carotenogenic tissues including the Marsh white seedless grapefruit (Coggins et al., 1970; Yokoyama et al., 1971, 1972; Hsu et al., 1972). In all cases, CPTA induces the formation of the C-40 acyclic carotenoid, lycopene. Chalcone derivatives of triethylamine, 2-(*p*-diethylaminoethoxybenzal)acetone (Yokoyama, 1972; Yokoyama et al., 1974), and other triethylamine derivatives (Poling et al., 1973) also had the same effect on carotenogenesis and their mode of action appeared to be similar to that of CPTA. They act as derepressors of a gene regulating the synthesis of a specific enzyme(s) in the lycopene pathway, and additionally they act as inhibitors of the cyclase(s) and cause lycopene to accumulate (Hsu et al., 1972). The present investigation aimed to determine the structural features necessary for the inducer activity and to see how these features influence the effectiveness of the inducers on carotenogenesis. The following triethylamine derivatives were prepared and their inducer activities were tested on Marsh white seedless grapefruit: 2-(4-chlorophenoxy)triethylamine (**1**), 2-(3-chlorophenoxy)triethylamine (**2**), 2-(2-chlorophenoxy)triethylamine (**3**), 4-[β-(diethylamino)ethoxy]benzaldehyde (**4**), 4′-[β-(diethylamino)ethoxy]-acetophenone (**5**), 4-[β-(diethylamino)ethoxy]benzophenone (**6**), *N*,*N*-dimethylaminopropoxybenzene (**7**), 2-phenylthiotriethylamine (**8**), 2-phenoxytriethylamine (**9**), 2-ethanethiotriethylamine (**10**), 2-diethylaminoethanethiol (**11**), 2-(4-nitrophenoxy)triethylamine (**12**), 2-(4-aminophenoxy)triethylamine (**13**), and CPTA.

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EXPERIMENTAL SECTION

Fruit Samples. Marsh white seedless grapefruits (*Citrus paradisi*, Rutaceae) were harvested at the fully mature stage. Two lots of fruit picked at different locations at different times were used for two separate experiments. Each sample consisted of six fruits.

Postharvest Treatment of Fruit. The test compounds were applied in their free amine forms to the fruit as 2-propanol solutions. For the control, pure 2-propanol was used. The solution was poured over the fruit in such a manner as to cover the entire surface for 5 min. The fruits were then air-dried and stored at room temperature (about 21°) for 1 and 2 weeks in polyethylene bags.

Extraction of Lipid and Preparation of Unsaponified Matter. Flavedo tissues of grapefruit were homogenized and extracted with acetone and methanol, followed, in some cases, by chloroform extraction in order to extract all the lycopene from the tissue. The lipid was saponified and the unsaponifiable material extracted by standard procedures (Davies, 1965).

Separation and Identification of Pigments. The unsaponified matter was partitioned between the light Petrol (30–60°) (PE) and 95% MeOH. Only the carotene fraction

(PE layer) was analyzed. The pigment was chromatographed on MgO–Hyflo-Supercel (1:1, w/w), and the various fractions were eluted with PE containing increasing amounts of acetone. The lycopene zone was eluted with acetone, then with EtOH and $CHCl_3$. The pigments were identified by their uv and visible spectra and adsorption characteristics relative to known compounds.

Quantitative Determination. The method used has been described by Davies (1965).

Chemicals. 4-[β-(Diethylamino)ethoxy]benzaldehyde (**4**), 2-diethylaminoethanethiol (**11**), and *N,N*-diethylaminoethyl chloride are available from Aldrich Chemical Co., Inc. The rest of the chemicals were chemically synthesized by published methods (Schuetz and Baldwin, 1958). With *N,N*-diethylaminoethyl chloride as the common starting reactant, *p*-chlorothiophenol, *p*-, *m*-, and *o*-chlorophenol, *p*-hydroxyacetophenone, *p*-hydroxybenzophenone, thiophenol, phenol, ethanethiol, *p*-nitrophenol, and *p*-aminophenol were used as the other reactant for CPTA and compounds **1, 2, 3, 5, 6, 8, 9, 10, 12,** and **13,** respectively. Compound **7** was prepared by allowing the *N,N*-dimethylaminopropyl chloride to react with phenol.

Calculation of Log Partition Coefficients. The calculations are based on the finding that the log partition coefficients (log *P*) of the organic compounds are additive and constitutive in nature (Leo et al., 1971) and under the assumption that no electronic effect was exerted by the groups introduced into the reference molecule. Since the values will be compared in a relative manner, no attempt was made to obtain the exact log *P* value for each compound tested in this study. The Δ log *P* (π) values for CH_2 and CH_3 groups are usually constant and an average value of 0.5 was assigned for each methylene group in the molecule (Fujita et al., 1964; Hansch and Anderson, 1967). For 2-phenoxytriethylamine (compound **9**) the log *P* value was obtained by subtracting 0.5 from the experimentally determined log *P* value of methoxybenzene (2.11) and then adding the value thus derived (1.61) to the log *P* value of triethylamine (1.44) (Lien et al., 1968); the value was 3.05. For a series of 2-phenoxytriethylamines, the Δ log *P* values (π) for each substituent group on phenoxyacetic acid were added to the log *P* value of 2-phenoxytriethylamine (Fujita et al., 1964). Thus, 0.7 for 4-Cl, 0.76 for 3-Cl, 0.59 for 2-Cl, −0.37 for 4-$COCH_3$, 0.24 for 4-NO_2, and −1.23 for 4-NH_2 were added to 3.05 and gave the log *P* values of 3.75, 3.81, 3.64, 2.68, 3.29, and 1.82 for compounds **1, 2, 3, 5, 12,** and **13,** respectively. For compound **4,** 0.1 was subtracted from the log *P* value of compound **5** and gave a value of 2.58 (Hansch et al., 1972). For compound **6,** 2.13 for the benzene ring constituent was added to the log *P* value of compound 4 to give 4.71. For the log *P* value for compound **7,** 0.5 was added for the increase of one CH_2 group between the N and O atoms and 1 was subtracted for the replacement of two ethyl groups by two methyl groups on the N atom from the log *P* of the reference molecule 2-phenoxytriethylamine (**9**); the value was 2.55. Diethyl sulfide has a log *P* value of 1.95 (Hansch and Anderson, 1967). The log *P* for compound **10** was calculated by subtracting 1 from the 1.95, for eliminating one of the ethyl group from the diethyl sulfide molecule, followed by adding 1.44, for the triethylamine; the value was 2.39. Subtracting 1 from the log *P* of compound **10** gave a value of 1.39 for compound **11.** Δπ, for the SCH_3 and OCH_3 substituents on the phenoxyacetic acid molecule, was 0.5 (Fujita et al., 1964). For compound **8,** 0.5 was thus added to the log *P* value of compound **9** for replacing the O atom with the S atom; the value was 3.55. For CPTA, 0.7 for 4-Cl was added to the log *P* of compound **8** to give 4.25.

RESULTS AND DISCUSSIONS

(I) Essential Structural Features for Inducer Activity. Previous studies have shown that the net synthesis of carotenoid pigment in grapefruit ceases during maturation, and a large accumulation of phytoene is observed owing to the inhibition of the dehydrogenation steps in the sequential carotenogenic pathway (Yokoyama and White, 1967). The main carotenes of the control fruit sample were phytoene and phytofluene accompanied by small amounts of α-, β-, and ζ-carotene and neurosporene. No lycopene was detected. On treatment with CPTA (0.02 *M* or 0.5% levels) the amount of total polyene increased 13- to 14-fold. Massive amounts of lycopene accumulated and constituted about 87% of the total. The levels of γ-carotene and intermediates, phytoene, phytofluene, ζ-carotene, and neurosporene, also increased. In fruits treated with the oxy analog of CPTA, 2-(4-chlorophenoxy)triethylamine (compound 1), a similar carotenoid pattern was obtained. However, at a concentration of 0.02 *M* the inducer effect of the oxy analog was about half of that of CPTA. The effectiveness of the oxy analog was also found to be concentration dependent similar to that reported for CPTA (Coggins et al., 1970; Hsu et al., 1972). Inducer activity generally was higher with higher concentrations of the inducer. At too high a concentration, however, the effectiveness of the compound decreased. This drop in biological activity was probably due to the tissue damage caused by the high concentration of compound 1 (Supplementary Material I; see paragraph at end of paper).

Since 4-[β-(diethylamino)ethoxy]benzaldehyde (**4**) and its analogs (compounds **5** and **6**) and chalcone derivatives of triethylamine (Yokoyama, 1972) with different substituting groups on the benzene moiety of the oxy analog of CPTA can all induce lycopene formation (Supplementary Material II), the following question was raised. Is the substitution on the benzene moiety of the CPTA or its oxy analog necessary for the inducer activity? 2-Phenylthiotriethylamine (**8**) and its oxy analog, 2-phenoxytriethylamine (**9**), were synthesized and tested for inducer activity. Results show that substitution on the benzene moiety is not required for inducer activity; however, it seems to increase the effectiveness of the compounds (Supplementary Material III).

Despite the lower biological activities of compounds **10** and **11,** results indicate that the benzene moiety in the CPTA molecule also is not necessary for the inducer activity (Supplementary Material III). Further evidence supporting this view is the finding that certain *N,N*-diethylalkylamines synthesized in our laboratory are also biologically active on carotenogenesis in citrus fruits (Poling et al., 1975).

Previous studies have shown that the number of methylene groups between the N and the O atoms in the inducer molecules can be varied from 2 to 4 while the inducer activity still remained (Poling et al., 1973). Also, some *N,N*-diethylaminoalkylbenzenes were found to be biologically active (Poling et al., 1975) indicating that the thioether or ether linkage in the amine molecule is not essential for inducer activity.

Tributylamine was effective in the *Blakeslea trispora* system (Hsu et al., 1974) indicating that the ethyl groups on the amine part are not exclusively necessary for the regulatory activity of the amine compounds on carotenogenesis. To check whether this is also true in the citrus fruit system, *N,N*-dimethylpropoxybenzene (**7**) was synthesized. Test results show that when the two ethyl groups on the N atom were replaced with two methyl groups (**7**), the inducer activity of the molecule still remained. Thus, amines with other alkyl groups might have the same effect, but might differ in relative effectiveness.

The above results apparently eliminate the following structural features as essentials for the inducer activity of the amine compounds on carotenogenesis: the S atom, substitution on the benzene moiety, the benzene moiety, the thioether or ether linkage between the alkylamine and the

Table I. Correlation of Log Partition Coefficient (Log *P*) and Effectiveness of the Chemical Inducers

Chemical inducer	Log *P*	Experiment 1[a] Effectiveness[c]	Experiment 1[a] Total polyene[d]	Experiment 2[b] Effectiveness	Experiment 2[b] Total polyene
Control			53.7		21.3
$Et_2NCH_2CH_2OC_6H_4COPh(p)$ (6)	4.71	227.0	318.3		
$Et_2NCH_2CH_2SC_6H_4Cl(p)$ (CPTA)	4.25	605.0	689.8	256.7	297.4
$Et_2NCH_2CH_2OC_6H_4Cl(m)$ (2)	3.81			0.66	37.7
$Et_2NCH_2CH_2OC_6H_4Cl(p)$ (1)	3.75			130.4	176.4
$Et_2NCH_2CH_2OC_6H_4Cl(o)$ (3)	3.64				35.0
$Et_2NCH_2CH_2SPh$ (8)	3.55	7.2	49.2		
$Et_2NCH_2CH_2OC_6H_4NO_2(p)$ (12)	3.29			9.0	28.2
$Et_2NCH_2CH_2OPh$ (9)	3.05	6.2	52.8	0.8	36.1
$Et_2NCH_2CH_2OC_6H_4COCH_3(p)$ (5)	2.68	107.8	188.0		
$Et_2NCH_2CH_2OC_6H_4CHO(p)$ (4)	2.58	2.1	67.2		
$Me_2NCH_2CH_2CH_2OPh$ (7)	2.55	0.9	36.0		
$Et_2NCH_2CH_2SEt$ (10)	2.39	0.4	45.8		
$Et_2NCH_2CH_2OC_6H_4NH_2(p)$ (13)	1.82				29.4
$Et_2NCH_2CH_2SH$ (11)	1.39	0.9	6.0		

[a] Chemicals were applied as 0.5% 2-propanol solutions except 11 (applied as 20% solution) to lot no. 1 fruits. Fruits were then kept at room temperature for 2 weeks after treatment. [b] Chemicals were applied as 0.02 *M* 2-propanol solutions to lot no. 2 fruits. Fruits were then kept at room temperature for 1 week after treatment. [c] Effectiveness is expressed as micrograms of lycopene induced/gram dry weight of flavedo. [d] Total polyene is expressed as micrograms of carotenes/gram dry weight of flavedo.

benzene moiety, and the ethyl groups of the amine part in CPTA and its oxy analog molecules. The structural feature necessary for inducer activity appeared to be the tertiary alkylamine. However, amines with a benzene moiety in the molecule and with a para chlorine substitution on the benzene ring and an S atom joining the amine portion and the benzene moiety seem to be most effective.

(II) Electronic Effects of the Substituents. Chlorine at the para position on the benzene ring with a σ value of +0.227 is considered to be an electron-withdrawing group. In order to investigate whether the higher biological activities of the chlorine-substituted compounds (CPTA and 1) are due to the electron-withdrawing ability of the chlorine, that is, whether the inducer activities of amine compounds are parallel to the electro characteristics of the substituting groups on the benzene moiety, 2-(4-nitrophenoxy)triethylamine (12) and 2-(4-aminophenoxy)triethylamine (13) were prepared and their effects on carotenogenesis were tested in comparison with that of 2-phenoxytriethylamine (9). It was shown that compound 9 applied to fruit at a concentration of 0.02 *M* induced formation of 0.75 μg of lycopene per g dry wt of flavedo. At 0.2 *M*, 65 μg of lycopene was obtained per g dry wt of flavedo. When a strong electron-releasing group, i.e. NH_2, with a σ value of −0.66 was introduced into the compound 9 molecule (compound 13), the biological activity of compound 9 was almost totally abolished; when a strong electron-withdrawing group, NO_2, with a σ value of +1.27 was introduced into the molecule (compound 12), activity increased (Supplementary Material IV). Among the chemical inducers tested, the higher biological activities of 1 over 12 and 5 (with a σ value of +0.874 for the p-$COCH_3$ group) over 4 (with a σ value of +1.126 for the p-CHO group) indicate that the effectiveness of the compounds does not depend solely upon the electron-withdrawing ability of the substituting groups in the benzene moiety. However, these data do not necessarily rule out the possible inductive effects produced by the substituting groups on the amine portion of the molecule.

(III) Steric Effects in Structure–Activity Correlations. In the oxy series of chemical inducers tested, 2-(4-chlorophenoxy)triethylamine (1) was the most effective in inducing the formation of lycopene. In order to investigate the steric effect of the substituents in the benzene moiety on the effectiveness of the inducers, meta and ortho isomers of compound 1 (compounds 2 and 3) were prepared, and their effectiveness toward carotenogenesis was determined. Results in Table I show that substitution of the chlorine atom at the meta position caused compound 2 to be less effective than the para isomer (1). The ortho isomer (3) was even less effective; at 0.2 *M*, it induced only 0.9 μg of lycopene per g dry wt of flavedo. The ortho isomer also was less effective than 2-(4-aminophenoxy)triethylamine (13) tested. In comparison with the reference compound, 2-phenoxytriethylamine (9), the para substitution of a chlorine atom enhanced the inducer activity of compound 9; however, substitutions at the meta and ortho positions decreased it drastically. Tributylamine was effective on carotenogenesis in the *Blakeslea trispora* system; however, due to the bulkiness of the butyl groups on the nitrogen atom, a much higher concentration of tributylamine is required in order to express its inducer activity (Hsu et al., 1974). The low activities observed for the meta- and ortho-substituted compounds in the present investigation might be due to the steric hindrance produced by the meta and ortho substitutions on the benzene ring, so that the molecules will no longer fit in the same active site of the enzyme, especially in the case of the ortho isomer compound.

(IV) Correlation of Biological Activity and Log Partition Coefficient of the Chemical Inducers. It has been shown that the effectiveness of biologically active compounds such as plant growth regulators, bactericides, insecticides, local anesthetics, thyroxin analogs, and carcinogenic compounds depends largely upon the lipo–hydrophilic character of the molecule; that is effectiveness depends on the log function of the partition coefficient (log *P*) of the compound between the lipo–hydro phases (Hansch et al., 1962; 1963a,b; Hansch and Fujita, 1964; Fujita et al., 1964; Muir et al., 1967). The variable effectiveness on carotenogenesis among the triethylamine derivatives tested in this study suggests the possible existence of a penetration problem for getting the compounds into the fruit flavedo tissue. The Hansch approach was thus considered. The additive–constitutive nature of the partition coefficients of organic compounds between a water and an apolar phase enables

us to calculate the partition coefficients of compounds from suitable reference molecules without actually determining the partition coefficients for each compound (Leo et al., 1971). Table I shows the log P and the effectiveness of the compounds tested in the present investigation. In experiment 1, the relations between the effectiveness and the log P in the series of CPTA and compounds **8, 10,** and **11** seem to indicate that the higher the log P of the compound, the higher the biological activity will be. Activity was highest for CPTA which has the highest log P value in the series. In the series of 2-phenoxytriethylamines, the effectiveness of the compounds correlates fairly closely with the log P values except for compound **5.** The addition of the $COCH_3$ group to the benzene ring of the 2-phenoxytriethylamine (compound **9**) lowers its log P value (3.05) by 0.37; therefore, compound **5** acquires a log P value of 2.68. At a concentration of 0.5%, activity was much higher for compound **5** (107.8 μg of lycopene accumulated per g dry wt of flavedo) than for compound **9** (6.2 μg of lycopene per g dry wt of flavedo). In comparison of the effectiveness between the two series, compound **6** has a higher log P value (4.71) than CPTA (4.25), but is only one-third as effective as CPTA. Also, compound **8** has a higher log P value (3.55) than compound **5** (2.68), but it has a much lower activity than compound **5.** In experiment 2, the effectiveness of the compounds correlates quite well to their log P values. The log P values of the phenoxyacetic acids with 2-, 3-, or 4-chloro substitution on the benzene ring did not differ greatly (Fujita et al., 1964). The 2-(3-chlorophenoxy)- and 2-(2-chlorophenoxy)triethylamine (**2** and **3**) had log P values (3.81 and 3.64) similar to that of the para isomer (**1,** 3.75). However, they were much less effective. The above results indicate that the lipo–hydrophilic characteristics of the compounds can be used as a general guide; however, it does not provide complete correlation between the structure and the inducer activity of the compounds. Effectiveness was not compared between the compounds tested in the two separate experiments since two different lots of fruits were used in the tests.

CONCLUSION

Results in the present investigation indicate that none of the following structural alterations totally abolishes the inducer activity of the 14 triethylamine derivatives tested or changes the response pattern: (1) the replacement by the O atom of the S atom connecting the amine portion and the benzene moiety in the CPTA molecule; (2) elimination of the substitution on the benzene ring; (3) elimination of the benzene ring itself; (4) replacement of the ethyl groups of the amine portion by other alkyl groups. However, these alterations change the effectiveness of the compounds thus modified. The S compounds seem to be more effective than their O analogs; benzene derivatives are more effective than alkyl derivatives; and the substitutions of certain groups on the benzene ring at the para position seem to make the compound more effective. The log partition coefficient of the compounds can be used as a general guide in designing the structure of new chemical inducers. However, the possible electronic and steric effects exerted by the groups introduced into the molecule should also be considered.

Supplementary Material Available. A listing of specific effects of the 14 triethylamine derivatives on carotenogenesis in the grapefruit will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JAFC-75-831.

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