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STRUCTURE–ACTIVITY RELATIONSHIPS OF CHEMICAL INDUCERS OF CAROTENOID BIOSYNTHESIS*

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Key Word Index—*Citrus paradisi*; Rutaceae; Marsh seedless grapefruit; carotenoid biosynthesis; lycopene; tertiary amines; bioregulators; partition coefficient.

Abstract—Fifteen amines having a profound effect on carotenogenesis in Marsh seedless grapefruit are reported. The compounds fall into three series: $Et_2N(CH_2)_nMe$ (n = 4–8), $Et_2N(CH_2)_nPh$ (n = 1–5), and $Et_2NCH_2CH_2OC_6H_4R$ (R=H, *p*-Me, *p*-Et, *p-iso*-Pr, *p-tert*-Bu). There was up to an 11-fold increase in the total carotene content. Lycopene, not normally accumulated, became a major pigment. The inducing ability of the amines on carotenoid biosynthesis is correlated with the octanol–water partition coefficient. The mode of action appears to be similar to that of 2-(4-chlorophenylthio)triethylamine hydrochloride.

INTRODUCTION

The effect of 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA) in a variety of citrus [1–3], carotenogenic mould [4, 5] and bacteria [6] has been reported. In citrus, CPTA caused a large increase in the total carotene content with lycopene (ψ,ψ-carotene), which is not normally accumulated, becoming a major pigment. The effect is believed to be caused by derepression of a gene regulating the synthesis of a specific enzyme(s) and inhibition of the cyclase(s) in the biosynthetic pathway of the carotenoids [4]. Several other derivatives of triethylamine have an effect similar to CPTA on citrus [7] and moulds [8].

This study was undertaken with the two-fold purpose of elucidating the essential structural characteristics of carotenogenesis inducing compounds and to find inducers that do not block the biosynthetic pathway at the lycopene level. The compounds studied fall into three series: $Et_2N(CH_2)_nMe$, n = 4 (**1**), n = 5 (**2**), n = 6 (**3**), n = 7 (**4**) and n = 8 (**5**); $Et_2N(CH_2)_nPh$, n = 1 (**6**), n = 2 (**7**), n = 3 (**8**), n = 4 (**9**) and n = 5 (**10**); and $Et_2NCH_2CH_2OC_6H_4R$, R=H (**11**), R=*p*-Me (**12**), R=*p*-Et (**13**), R=*p-iso*-Pr (**14**) and R=*p-tert*-Bu (**15**). All the compounds caused lycopene accumulation in Marsh seedless grapefruit. The biological activity was also correlated with the logarithm of the octanol–water partition coefficient (log *P*).

RESULTS AND DISCUSSION

Compounds **1**–**10** were applied as free amines in *iso*-PrOH. Compounds **11**–**15** and CPTA were applied as the hydrochlorides in *iso*-PrOH. This gave a more uniform response than the application of the hydrochloride in an aqueous solution. The untreated fruit had the normal light yellow colour. After treatment, the colour of the flavedo ranged from light orange to an intense red. The peel remained healthy on all the fruit except those treated with **5**, **10** and **15**. The damaged peel area was about 20, 40 and 20%, respectively. Only the undamaged peel was analysed. The response pattern within the grapefruit is determined essentially by the depth of

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penetration of the test compound. The endocarp showed no colour enhancement except with **5** which caused the undamaged tissues of the endocarp below the areas of damaged peel to develop the pink coloration typical of lycopene accumulation. In this paper, only the results from the flavedo are reported. The flavedo of all treated fruit showed lycopene accumulation (Tables 1, 2 and 3). Lycopene was not detected in the untreated fruits and it is not normally detected in mature grapefruit [9].

Treatment with **1**–**5** gave a fairly consistent response pattern as the length of the alkyl group was increased. The amount of any given carotene remained about the same or increased slightly with **1** and **2**, while **3** caused a larger response, and **4** and **5** caused very large increases. Lycopene accounted for most of the increase in the total carotene content but the intermediates, ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene) and neurosporene (7,8-dihydro-ψ,ψ-carotene), also increased appreciably. Of special significance are the 3·5

Table 1. Effect of **1**, **2**, **3**, **4** and **5** at 0·2 M on carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	Treatment **1**	**2**	**3**	**4**	**5**
Phytofluene	37·3	38·6	29·0	28·3	27·8	39·5
ζ-Carotene	2·25	2·47	3·13	4·32	14·6	17·8
Neurosporene	1·72	1·28	1·37	1·38	2·94	5·16
Lycopene		1·01	6·99	59·0	143	115
γ-Carotene	0·37	0·30	0·96	1·17	2·59	3·21
α-Carotene	0·54	1·04	1·02	1·26	2·07	2·11
β-Carotene	1·72	1·41	1·20	1·35	4·73	6·75
Total	43·9	46·1	43·7	96·8	197·7	189·5
Log *P*		2·94	3·44	3·94	4·44	4·94

Table 2. Effect of **6**, **7**, **8**, **9** and **10** at 0·2 M on carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	Treatment **6**	**7**	**8**	**9**	**10**
Phytofluene	23·1	25·3	29·3	37·9	38·7	86·2
ζ-Carotene	1·13	1·38	3·87	6·77	27·5	53·8
Neurosporene	0·71	0·83	1·85	1·11	7·16	12·0
Lycopene		8·62	60·5	188	104	153
γ-Carotene		0·55	0·77	1·07	0·59	1·67
α-Carotene	0·57	0·77	0·84	0·61	2·19	0·76
β-Carotene	0·95	0·56	0·46	0·95	trace	0·68
Total	26·5	37·8	97·6	235·5	180·1	308·1
Log *P*		3·07	3·57	4·07	4·57	5·07

Table 3. Effect of **11**, **12**, **13**, **14**, **15** and CPTA at 0·1 M on carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	Treatment **11**	**12**	**13**	**14**	**15**	CPTA
Phytofluene	44·9	48·1	50·9	44·9	51·1	33·7	42·6
ζ-Carotene	2·74	5·45	19·0	13·7	20·6	4·57	10·1
Neurosporene	0·33	0·91	0·84	0·93	1·62	0·63	1·21
Lycopene		22·2	249	182	226	54·4	199
γ-Carotene	0·27	1·01	2·48	2·13	2·74	1·04	1·90
α-Carotene	0·35	0·57	0·41	0·38	0·40	0·63	0·23
β-Carotene	1·35	1·26	0·40	0·82	0·70	0·58	0·39
Total	49·9	79·5	323·0	244·9	303·2	95·6	255·4
Log *P*		3·05	3·55	4·05	4·35	4·65	4·25

and 4·6-fold increases in the cyclic carotenes, γ-, α- and β-carotene (i.e. β,ψ-, β,ε- and β,β-carotene), caused by **4** and **5**, respectively. This increase in cyclic carotenes is much larger than that caused by **6**–**15** or CPTA. Butyldiethylamine has also been observed to cause the development of a red colour in grapefruit but only with higher concentrations and longer treatment periods. The higher members of this series, decyldiethylamine, diethylundecylamine and diethyldodecylamine, caused increasing peel injury as the length of the alkyl chain increased. The last two damaged the peel wherever they were applied. The colour of the peel next to the damaged areas showed colour enhancement but to a lessening degree as the alkyl chain lengthened.

The response of fruit treated with **6**–**10** is similar to that of fruit treated with **1**–**5**. There was a much larger increase in ζ-carotene and neurosporene for **9** and **10** while the increase in cyclic carotenes was not very great. For series **1**–**5** and **11**–**15**, lycopene increased to a very high level and then dropped for the last member of the series. This drop in lycopene accumulation is observed with **9** but the amount of lycopene increased with **10** although remaining less than the maximum for the series. The very large increase in phytofluene (7,8,11,12,7′,8′-hexahydrolycopene), as well as ζ-carotene, caused **10** to give the greatest increase in the total carotene content. Whether this was caused in part by some side effect of the extensive peel damage or entirely by **10** is not certain. A similar effect has been previously observed [7] after treatment with [γ-(diethylamino)-propoxy]-benzene (**16**) and [δ-(diethylamino)-butoxy]-benzene (**17**). For **16** and **17**, the increases in phytofluene and ζ-carotene were also very large but no peel damage was observed. This effect may arise when the diethylamino and phenyl groups of the inducers are separated by a chain of four or five atoms.

Treatment with **11**–**15** gave a similar pattern. Neurosporene did not increase as much as previously although ζ-carotene did show a large increase. The cyclic carotenes, with the exception of γ-carotene, did not show a significant increase. There was a general decrease, not previously observed, of all the carotenes after treatment with **15**, as compared to **14**, instead of the steady increase of ζ-carotene and neurosporene observed in the other series. The greater biological activity of **11**–**15** and CPTA as compared to **1**–**10** is probably due to a higher degree of interaction between the compound and the active site.

Generally, the total carotene content increases through the first members of the series up to a maximum and then decreases from the maximum at the end of the series. This pattern was observed previously [7] for the series **11**, **16** and **17**. The total carotene content for fruit treated with **13** was lower than for **12** and **14**. Generally, the response increases with increasing concentration to a maximum value and further increases in the concentration cause no further increases in carotene content or become somewhat inhibitory to overall carotene synthesis. Doubling the concentration of **11** from 0·26 to 0·52 M reduced the observed response [7], while treatment with CPTA [3] at 0·018 M caused an accumulation of carotenes equal to that in Table 3. Treatment of Marsh seedless grapefruit, harvested at a different time and from a different location than those used in the other experiment, with 0·2 M solns of the hydrochlorides of **11**, **12**, **13** and CPTA in *iso*-PrOH gave total carotene contents of 180·3, 670·2, 233·3 and 369·1 μg/g dry wt, respectively, as compared to 29·1 μg/g dry wt for the control. Doubling the concentration caused large increases for **11** and **12**, while doubling **13** had almost no effect. That increasing CPTA concentrations begin to lose effectiveness in inducing greater carotene biosynthesis has also been observed in *Blakeslea trispora* [4].

The only common structural characteristic of the compounds considered here and those previously reported that appears to be necessary to elicit the stimulation of carotenoid biosynthesis is the $-CH_2NEt_2$ group. Other structural factors only modify the response pattern. Compounds lacking an aromatic ring seem to cause less inhibition of the cyclase(s), while those with four or five atoms in the chain connecting the diethylamino group with the aromatic ring stimulate a greater increase in the lycopene precursors. The ability to interact with the active site and the value of log *P* seem only to affect the magnitude of the response and not the overall pattern.

The Hansch approach [10, 11] has been shown to have wide applicability toward correlating biological activities. The random walk process by

which the compound reaches the active site in the cellular phase from the extracellular phase by partitioning itself between the aqueous and lipid regions of the cell can be correlated with log *P*. The interaction of the compound with the active site from which the biological response eventually follows can be correlated with the Hammett constants. The excellent correlation obtained for plant growth regulators [12–14] lead us to consider possible correlations with carotenogenesis inducing activity. An advantage of this method is that the additive-constitutive nature of the partition coefficients [15] allows the calculation of log *P* without the necessity of making actual measurements.

The values of log *P* were calculated as follows. For **1–5**, 0·50 was added [11] to the experimentally determined value of log *P* of triethylamine [16] for each methylene group added to give the desired compound, i.e. for **5**, log *P* = 1·44 + 7 (0·50). For **6–10**, the log *P* of benzene [15] was added to the log *P* of triethylamine to obtain log *P* of **7**. The rest of the series was generated by adding or substracting 0·50 for each methylene group added or subtracted to give the corresponding compound. For **11–15**, the log *P* of methoxybenzene [17] was added to the log *P* of triethylamine to obtain the log *P* of **16**. The log *P* of **11** was obtained by subtracting 0·50 for the removal of one methylene group. For each non-branching methylene group added to give the desired compound, 0·50 was added to log *P* of **11**, while for each branching methylene group added, 0·30 was added to log *P* [18]. For CPTA, 0·70 was added for *p*-Cl [17] and 0·50 was added for replacement of oxygen by sulfur. The latter figure was arrived at by assuming the increase in the log *P* of phenoxyacetic acid [17] for replacing 3–MeO– with 3–MeS– can be applied to CPTA.

These values are fairly rough and ignore any complications such as the interaction of the amine group with the aromatic ring [19] or the presence of strong electron-withdrawing groups [15]. Their correlation with carotenogenic activity will serve as a crude but useful guide in designing more effective compounds. The calculated values are for the free amines. The log *P* of the acid salts will be reduced by at least 3 units. The formation of the hydrochloride of decylamine [15] from the free amine [20] reduced log *P* from 3·81 to 0·85. The strongly electron withdrawing nature of the quaternary amine may further reduce the hydrophobic nature of the two ethyl groups causing even greater reductions in the value of log *P*. There are enough organic acids present in the cells of the fruit, that whether the compound is applied as the hydrochloride or the free amine, it will probably exist as a salt in the cell. There may be some advantage for the free amines in passing through the layer of surface waxes because they will be more readily absorbed by the waxes than the amine salts.

There appears to be an upper limit for log *P* at which peel damage begins to occur. Compounds **5**, **10** and **15**, whose log *P* are greater than 4·6, all showed evidence of causing peel damage. It was noticed during the preparation of the alkyldiethylamines, that the hydrochlorides of the nonyl through dodecyl derivatives showed an increasing tendency to form an emulsion between ether and water. Those compounds with log $P \geq 4{\cdot}6$ probably cause peel damage by disrupting the lipid membranes of the cells. Those members of each series with log $P < 4{\cdot}6$ showed no evidence of peel damage.

The tables show that although these compounds have a noticeable effect for log *P* of 3, i.e. inducing lycopene formation, any larger increase in the carotene content does not occur until log $P \geq 3{\cdot}5$. This is affected by the concentration used for treatment and by the degree of interaction that may be expected between the compound and the active site(s). Compounds **11–15** and CPTA, which probably interact more strongly at the active site(s), produce a noticeable effect at lower concentrations and for smaller values of log *P* than compounds **1–10**. The optimum value of log *P* seems to be in the range of 3·5–4·5. This trend was also observed [7] for the series **11**, **16** (log *P* = 3·55) and **17** (log *P* = 4·05). If a compound must travel through successive lipid and aqueous layers, the mobility will be greatest for equipartition between the two phases, i.e. log *P* = 0. If the formation of the salt from the free amine lowers log *P* by about 4 and the compound has to pass through several layers of membranes to reach the active site, then this optimum of log *P* would be explained. The greater interaction of **11–15** with the active site

may shift the optimum to a lower value, while the lack of an aromatic ring may require the alkyldiethylamines to have a greater hydrophobic nature to interact effectively with the active site even if it reduces the concentration at the active site.

The results from these and previous data can be summed up as follows. Chemical inducers of carotenoid biosynthesis have the general formula RCH_2NEt_2. The magnitude of the stimulation, but not the general pattern of response, depends on R. R should be such as to cause the log *P* of RCH_2NEt_2 to fall in the range 3·5–4·5 for citrus and probably for other higher plant tissues. Within this range, the highest concentration of the compound will reach the active site(s). The relationship between log *P* and carotenogenesis has not yet been established for microorganisms but a similar relationship probably exists. R also affects the magnitude of the response by the degree to which it affects the interaction with the active site(s). The nature of R can cause minor modifications in the overall pattern. When a five atom chain connects the amine group to the aromatic ring, there seems to be a greater accumulation of lycopene precursors, while when R lacks an aromatic ring, there seems to be less inhibition of the cyclase(s). The mode of action of all these compounds appears to be similar to that of CPTA.

EXPERIMENTAL

Fruit samples. Marsh seedless grapefruit were harvested at the fully mature stage. Fruit for testing **1–5** and **11–15** were harvested from a different location and at a different time than those used to test **6–10**. Each sample for the tests of **1–5** and **11–15** consisted of 6 fruits. The samples for testing **6–10** consisted of 5 fruits.

Post-harvest treatment of fruit. Test compounds were applied to fruit as 0·1 or 0·2 M solns in *iso*-PrOH. For control, pure *iso*-PrOH was used. **1–10** were applied as free amines while **11–15** were used as the hydrochlorides. The soln was poured over the fruit in such a manner as to cover the entire surface. Fruit were allowed to drain and then moved to a clean surface to air dry for several hr. Fruit were then stored at room temp. (~21°) for 2 weeks in polyethylene bags.

Extraction, isolation and quantitative determination of the pigments. Carotenoids were isolated and separated as described previously [9]. The method of Davies [21] was used for quantitative determinations. A portion of the ground flavedo was dried *in vacuo* to obtain the dry wt.

Identification of the pigments. The pigments were identified by their visible and UV spectra and adsorption behaviour relative to known compounds.

Compounds **1–10**. The bromide corresponding to the desired diethylamino derivative was added to a 2-fold excess of diethylamine and stirred and refluxed for 6 hr. The higher alkylbromides require longer to go to completion. Benzylbromide reacted vigorously and required cooling during the initial addition. The soln was cooled, neutralized with 15% NaOH and extracted with Et_2O. The ethereal soln was washed repeatedly with H_2O to remove the excess diethylamine and then extracted with 10% HCl. The HCl soln was washed with Et_2O, neutralized with 20% NaOH and extracted with Et_2O. The Et_2O soln was washed with H_2O, dried over Na_2SO_4 and the Et_2O evaporated to give the free amine which was used without further purification.

4-Phenylbutylbromide and 5-phenylpentylbromide. Corresponding acids were reduced with $LiAlH_4$ by the method of Nystrom and Brown [22]. After the addition of 10% H_2SO_4, the separated Et_2O layer was washed with 5% K_2CO_3 and then H_2O. After drying over anhydrous Na_2SO_4 and evaporation of the Et_2O, the alcohol was brominated by the method of Bachmann and Thomas [23]. After drying over anhydrous Na_2SO_4, the C_6H_6 was removed and the bromide used without further purification.

Compounds **11–15**. 0·5 mol of p-RC_6H_4OH was stirred with 40 g NaOH in 120 ml H_2O and heated to reflux. 0·5 mol $Et_2NCH_2CH_2Cl$–HCl in 100 ml H_2O was added dropwise over a 1 hr period. The soln was refluxed 5 hr more, cooled, the top layer separated and the aq. layer extracted with Et_2O. The top layer and Et_2O extract were combined and washed repeatedly with 5% NaOH and then H_2O. The Et_2O soln was extracted with 10% HCl. The HCl soln was reduced to dryness by warming in an evaporating dish under a stream of warm air. The HCl salt was recrystallized twice from *iso*-PrOH–Et_2O and dried *in vacuo* at 65°.

CPTA. The method of Schuetz and Baldwin [24] was used to prepare CPTA. The HCl salt was recrystallized ×2 from *iso*-PrOH and dried *in vacuo* at 65°.

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