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CHEMICAL INDUCTION OF POLY-*CIS* CAROTENOID BIOSYNTHESIS*

STEPHEN M. POLING, WAN-JEAN HSU and HENRY YOKOYAMA

Fruit and Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, CA 91106, U.S.A.

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Key Word Index—*Citrus paradisi*; Rutaceae; Marsh white seedless grapefruit; carotenoid biosynthesis; bioregulators; poly-*cis* carotenoids; carotenoids; prolycopene.

Abstract—A new class of synthetic bioregulators is reported which cause the accumulation of poly-*cis* carotenoids in the flavedo of Marsh white seedless grapefruit. The compounds tested were all secondary amines: dibenzylamine, substituted dibenzylamines (4-F; 4-Cl; 4-Br; 2-, 3-and 4-Me; 4-NO_2; 4-CN; 4-Cl, 4'-Me; 4-Me, 4'-NO_2), *N*-benzyl phenethylamine and *N*-benzyl 2-naphthalenemethylamine. The most effective, 4-chlorodibenzylamine, caused the accumulation of 74 μg/g dry wt of poly-*cis* carotenoids. Prolycopene was the predominant pigment but substantial amounts of proneurosporene, poly-*cis*-γ-carotenes and other *cis* carotenes were also present. The mode of action of these new bioregulators is probably gene derepression, the same as that of the lycopene inducers. However, the secondary amines probably derepress a recessive gene governing the biosynthesis of poly-*cis* carotenoids; whereas, the lycopene inducers derepress the dominant gene that gives rise to the normal all-*trans* carotenoids. The new compounds did not seem to inhibit the cyclase(s), as the lycopene inducers do.

INTRODUCTION

The biosynthesis of carotenoids in citrus is affected by a variety of synthetic tertiary amines of the general formula RCH_2NEt_2 [1–8]. The carotene content greatly increases and lycopene (ψ,ψ-carotene) becomes the major pigment unless the tertiary amines are aliphatic esters of 2-diethylaminoethanol. If they are, β-carotene (β,β-carotene) is the predominant carotene [8]. All these compounds are postulated to act in the same way by derepressing a gene regulating the synthesis of a specific enzyme(s) in the carotenogenic pathway and inhibiting the cyclase(s) [3]. As compared with the other tertiary amines, aliphatic esters differ only in that they are rapidly hydrolysed and allow the accumulated lycopene to be converted into β-carotene.

All the previously reported compounds cause the accumulation of the normal, all-*trans* carotenoids. During our studies of the structure–activity relationships of compounds that affect carotenogenesis, we discovered a new class of bioregulators causing the accumulation of poly-*cis* carotenoids. The natural occurrence of *cis* and poly-*cis* carotenoids has been observed, but is not very common [9–14]. These new bioregulators are all secondary amines in contrast to the lycopene inducers, which were all tertiary amines. The compounds synthesized and tested on Marsh white seedless grapefruit were dibenzylamine (**1**); the following substituted dibenzylamines; 4-fluoro- (**2**), 4-chloro- (**3**), 4-bromo- (**4**), 2-methyl- (**5**), 3-methyl- (**6**), 4-methyl- (**7**), 4-nitro- (**8**), 4-cyano- (**9**), 4-chloro-, 4'-methyl- (**10**) and 4-methyl-, 4'-nitro- (**11**); *N*-benzyl phenethylamine (**12**) and *N*-benzyl 2-naphthalenemethylamine (**13**).

RESULTS AND DISCUSSION

Application of the free amines in *iso*-PrOH to the whole fruit stimulated carotenogenesis only in the flavedo; the endocarp was unaffected. Because the principal visible absorption bands are shifted to lower wavelengths for poly-*cis* carotenoids, as compared to the all-*trans* carotenoids [9], the more effective of these bioregulators caused the grapefruit to take on the bright orange colour normally seen in oranges rather than the red colour caused by the lycopene inducers.

The results obtained with these bioregulators are shown in Tables 1 and 2. The identities of the poly-*cis* carotenes were established by their absorption spectra [9] and iodine-catalysed photoisomerization [15]. Prolycopene and proneurosporene had spectra identical to their reported spectra, and after isomerization, spectra identical to those of the isomerized all-*trans* carotenes. The *cis*-lycopenes are the sum of two *cis*-carotenes that formed separate bands above the prolycopene band on the chromatographic column [5]. They had

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A laboratory of the California–Hawaii–Nevada Area, Western Region, Agricultural Research, SEA, USDA.

Table 1. Effect of compounds **1–4** and **7–9** at 0.1 M on the carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	**1**	**2**	**3**	**4**	**7**	**8**	**9**
Phytofluene	34.98	36.10	39.21	40.14	37.87	32.20	50.27	43.32
α-Carotene	0.14	0.11	0.10	0.16	0.12	0.11	0.14	0.09
β-Carotene	0.65	0.83	0.95	1.88	1.05	1.00	1.01	0.78
ζ-Carotene	4.37	10.12	12.64	21.16	18.64	14.03	18.71	12.45
Poly-*cis*-γ-carotene I*	0.31	0.70	1.35	3.18	3.84	2.50	1.49	0.78
Proneurosporene*		4.91	7.99	16.24	15.03	9.30	13.45	8.28
Prolycopene*	0.55	8.03	14.08	33.10	29.76	21.28	24.95	13.19
cis-Lycopenes*	1.21	5.12	7.58	9.69	9.31	9.45	10.10	6.00
Poly-*cis*-γ-carotene II*	0.87	2.35	5.48	11.94	6.81	5.55	5.91	4.50
Unknown 453	0.48	1.74	1.44	4.18	8.22	4.11	6.40	0.94
Total Carotenes	43.56	70.01	90.82	141.67	130.65	99.53	132.43	90.33
Total Xanthiphylls	25.48	28.76	30.04	39.36	36.58	26.08	39.74	28.47

* Tentative assignment of poly-*cis* carotenes in control.

the same spectrum, exhibiting λ_{max} at 419, 438 and 463 nm, and after isomerization, the same spectrum as isomerized lycopene. They probably contained fewer *cis* bonds than prolycopene. After isomerization, both poly-*cis*-γ-carotene I and II had a spectrum identical to that of isomerized γ-carotene (β,ψ-carotene); but before isomerization, neither was quite like the previously described poly-*cis*-γ-carotene [13] or pro-γ-carotene [14]. Poly-*cis*-γ-carotene I was pink and appeared just below proneurosporene on the chromatographic column like poly-*cis*-γ-carotene [13], but its spectrum, with λ_{max} at 403, 427 and sh 450 nm, was different. Poly-*cis*-γ-carotene II, on the other hand, had the same orange colour and spectrum as pro-γ-carotene [14], but appeared on the column above γ-carotene rather than below as would be expected [14, 15]. The structural difference between poly-*cis*-γ-carotene I and II remains to be determined. Unknown 453 was a red pigment remaining near the top of the column just above poly-*cis*-γ-carotene II and had λ_{max} at 433, 453 and sh 483 nm. When it was exposed to iodine, its spectrum did not change; so it was not likely a *cis*-carotene. Normally it does not occur in grapefruit in large quantities, if at all. Whether the amount of ζ-carotene measured was due to all-*trans* or a mixture of *trans* and poly-*cis*-ζ-carotene, as found in tangerine tomatoes [12], was not determined. The identification of poly-*cis* carotenes in both sets of controls (Tables 1 and 2) is only tentative because of the small amount of pigments collected.

Tables 1 and 2 show that all the secondary amines stimulated the production of poly-*cis* carotenes. In none of the samples was any trace of lycopene found. Compound **3** caused the greatest increase in pigments, but the other compounds caused similar although lesser responses. Prolycopene was the major pigment, but there were relatively large amounts of the other poly-*cis* carotenes. In marked contrast, the tertiary amine bioregulators primarily induced the synthesis of lycopene [2–7] or β-carotene [8]. The induction of poly-*cis* carotenes in plant tissues normally producing the all-*trans* carotenes has not been previously observed. It is quite possible that the increase in ζ-carotene we reported was due to the formation of

Table 2. Effect of compounds **5–7** and **10–13** at 0.1 M on the carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	**5**	**6**	**7**	**10**	**11**	**12**	**13**
Phytofluene	38.81	48.84	41.48	40.70	33.06	43.14	30.37	53.89
α-Carotene	0.11	0.13	0.18	0.13	0.09	0.17	0.10	0.14
β-Carotene	0.62	0.84	1.07	1.44	1.08	1.08	0.84	1.08
ζ-Carotene	5.20	11.99	10.75	18.25	10.18	11.08	8.42	15.26
Poly-*cis*-γ-carotene I*	0.54	0.71	1.20	3.32	1.49	1.56	2.37	1.19
Preneurosporene*		6.55	5.44	11.67	5.13	5.51	4.42	8.74
Prolycopene*	1.31	7.72	8.90	25.23	9.86	10.50	13.80	9.83
cis-Lycopenes	1.45	5.57	5.57	13.99	7.68	7.75	8.44	8.31
Poly-*cis*-γ-carotene II*	0.80	2.45	4.12	8.37	3.67	2.42	3.71	5.05
Unknown 453		1.54	1.18	3.29	2.37	2.98	2.10	2.62
Total Carotenes	48.84	86.34	79.89	126.39	74.61	86.19	74.57	106.11
Total Xanthiphylls	24.64	34.77	35.51	40.01	31.92	37.18	26.94	46.21

* Tentative assignment of poly-*cis* carotenes in control.

poly-*cis*-ζ-carotene [12, 13]. Likewise, although not experimentally determined, the increase in the total xanthophylls observed in the treated fruit could have been due to the formation of *cis* and poly-*cis* xanthophylls. *cis*-Xanthophylls do occur naturally in some plants [10].

The data in Table 1 indicate that the ability to cause the synthesis of poly-*cis* carotenes increased with the electron-withdrawing ability of the substituent in the 4-position. Compound **7** showed increased activity even though the methyl group is not electron-withdrawing, probably because of the increased lipid solubility. These observations agree with our earlier findings about lycopene inducers [4–8]. The 2-, 3- and 4-methyl dibenzylamines (**5–7**) were all active, although **7** was the most active. In contrast, the 2-, 3- and 4-chloro [6] as well as the 2-, 3- and 4-methyl [7] derivatives of 2-phenoxytriethylamine differed greatly in their ability to increase lycopene synthesis. Of these lycopene inducers, the 4-substituted molecule was the most active, the 3-substituted molecule considerably less active, and the 2-substituted molecule almost completely inactive. The presence of substituents in both rings of the secondary amines did not increase activity; in fact, **10** and **11** were less active than either of their monosubstituted analogues. Compounds **12** and **13** show that both substituents on the amine need not be benzyl groups for activity. Further studies on the structure–activity relationship are underway.

The mode of action of the poly-*cis* inducers is probably gene derepression, the same as that of the lycopene inducers. However, the secondary amines probably derepress a recessive gene controlling the biosynthesis of the poly-*cis* carotenoids, whereas the lycopene inducers derepress the dominate gene giving rise to the normal all-*trans* carotenoids [4]. The existence of this recessive gene can only be postulated as present in grapefruit and other citrus. (We have also observed the induction of poly-*cis* carotenoids in treated Valencia and Washington Navel oranges and in Meyer lemons.) Important genetic studies have shown that such a gene exists in tomatoes [11, 16]. Tangerine tomatoes, which are homozygous for the recessive allele *t*, accumulate the orange pigment prolycopene at the expense of lycopene. Red fruit carry the dominant allele *t*+. The same situation could exist in other fruit and explain the action of the poly-*cis* inducers. This interpretation lends support to the postulated parallel pathways for all-*trans* and poly-*cis* carotenoids [12]. If this hypothesis is true, one would also expect to find poly-*cis*-ζ-carotene, phytofluene and phytoene present in the fruit. The poly-*cis* inducers appear to differ from the lycopene inducers in another respect. In addition to gene derepression, the lycopene inducers inhibit the cyclase(s), causing lycopene to accumulate at the expense of the cyclic carotenes [4]. The accumulation of significant amounts of poly-*cis*-γ-carotene I and II indicates that the cyclases are not inhibited by the poly-*cis* inducers and that their only apparent function is to derepress the recessive gene.

In summary, we have discovered a new class of bioregulators that stimulated the accumulation in grapefruit of poly-*cis* carotenoids in addition to the already existing normal, all-*trans* carotenoids. We postulate that these new bioregulators derepress a recessive gene controlling the poly-*cis* carotenoid pathway. These new bioregulators unlike the lycopene inducers did not inhibit the cyclase(s).

EXPERIMENTAL

Fruit samples. Marsh white seedless grapefruit were harvested from the same fields at the same time. All samples consisted of 8 fruit.

Post-harvest treatment of fruit. Solns of all test compounds were prepared at 0.1 M in *iso*-PrOH, and poured over the fruit to completely cover them. Control fruit were treated with *iso*-PrOH. The fruit were allowed to drain and were moved to a clean surface to air dry for several hr. Then they were placed in polyethylene bags and stored at room temp. (~21°). Some of the more lipid-soluble bioregulators caused some pitting of the peel and collapse of the albedo along wounds, but only **10** caused severe damage (~75% of peel area) at 0.1 M. Only the undamaged flavedo was removed and analysed 14 days after treatment.

Isolation, identification and quantification of the pigments. The pigments were isolated, and the all-*trans* carotenes were identified by published methods [5]. The carotenoids are listed in the tables in their order of elution. The poly-*cis* carotenes were identified on the basis of spectra [9] and iodine-catalysed photoisomerization [13]. The all-*trans* carotenes and prolycopene were quantified by use of standard $E^{1\%}_{1cm}$ [15]. $E^{1\%}_{1cm}$ for the poly-*cis* carotenes was estimated from the changes in absorption intensities from poly-*cis* to the equilibrium mixture and from the equilibrium mixture to the all-*trans* carotene. The values used were: poly-*cis*-γ-carotene I and II, $E^{1\%}_{1cm} = 2400$; proneurosporene, $E^{1\%}_{1cm} = 2200$; *cis*-lycopenes, $E^{1\%}_{1cm} = 1920$. $E^{1\%}_{1cm} = 2700$ was assumed for unknown 453 and "total xanthophylls". Compounds **2–13** were synthesized by reaction of the appropriate bromide (**4–11** and **13**) or chloride (**2**, **3** and **12**) with a 4-fold excess of benzylamine (**2–9**, **12** and **13**) or 4-methyl benzylamine (**10** and **11**). Compound **1** was commercially available. The bromides with electron-withdrawing groups were more reactive; the chlorides were less reactive; and the reaction times were varied accordingly. Synthesis of **4** is typical. Slowly, 25 g 4-bromobenzyl bromide was added to 54 g benzylamine stirred in a H_2O bath at room temp. After the addition was completed, the H_2O bath was heated to boiling for 1 hr; then the reaction mixture was removed from the bath and stirred at room temp. for 2 hr. The mixture was poured into 100 ml 20% NaOH and extracted 3× with 100 ml portions of Et_2O. The combined extracts were washed 8× with 200 ml portions of H_2O and dried (Na_2SO_4). The Et_2O was removed with a rotary evaporator. The residue was mixed with 100 ml EtOH and poured into 300 ml 10% HCl cooled in an ice bath. The ppt. was filtered, rinsed with 50 ml cold H_2O and dissolved in 1200 ml boiling H_2O. Any insoluble material that floated to the surface was removed, and the soln was cooled in an ice bath. The ppt. was filtered, rinsed with 100 ml cold H_2O and dried *in vacuo* at 65° for 2 hr. The ppt. was next dissolved in 1200 ml boiling EtOH and the soln was refrigerated overnight. The ppt. was filtered, rinsed with 100 ml cold EtOH and dried *in vacuo* at 65° for 2 hr. The hydrochloride was neutralized with 300 ml 15% NaOH, and the amine was extracted twice with 200 ml portions of Et_2O. From 15 to 20 min of shaking was required for the neutralization and extraction steps. The extract was washed 3× with 300 ml portions of H_2O, dried (Na_2SO_4) and stripped of Et_2O in a rotary evaporator. The yield of free amine was 22.5 g. The purity and identities of the amine

hydrochlorides were tested using HPLC and MS. HPLC was performed using a Whatman Magnum 9 ODS-2 reverse phase C_{18} column. The solvent was 45% MeOH in aq. 0.25 M H_3PO_4 adjusted to pH 3 with triethylamine. Absorbence was monitored at 206 and 254 nm. The retention times relative to **1** are: **2** (1.09), **3** (1.81), **4** (2.13), **5** (1.48), **6** (1.67), **7** (1.68), **8** (0.97), **4** (0.75), **10** (3.23), **11** (1.56), **12** (1.35) and **13** (3.23). MS were determined on a VG Micromass 7070F using the solid insertion probe. EI (70 eV) gave the following results: **1** 197 (M^+, 11%), **2** 215 (16), **3** 231 (23), **4** 275 (18), **5** 211 (27), **6** 211 (8), **7** 211 (20), **8** 242 (19), **9** 222 (20), **10** 245 (17), **11** 256 (21), **12** 211 (<0.05) and **13** 247 (5). $(M+1)^+$ was the base peak for all the compounds using CI (NH_3).

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