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Bleaching Activity of 4-Phenyl-3-(substituted benzylthio)-4*H*-1,2,4-triazoles

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A variety of 4-aryl- and 4-alkyl-3-(substituted benzylthio)-4*H*-1,2,4-triazoles were prepared and evaluated for their bleaching activity by the lettuce seedling test. Among the series of tested compounds, 4-(3-fluorophenyl)-3-(4-trifluoromethylbenzylthio)-4*H*-1,2,4-triazole (39) exhibited the highest bleaching activity, causing complete bleaching symptoms at 10 μM. In the dark condition, compound 39 inhibited the formation of such carotenoids as β-carotene, violaxanthin, neoxanthin and lutein, resulting in the formation of ζ-carotene, phytoene, phytofluene and β-zeacarotene, which were not detected in the untreated control. Treatment by compound 39 at 50 μM resulted in the amount of accumulated ζ-carotene being seven-fold higher than that of phytoene, phytofluene and β-zeacarotene. These results suggest that compound 39 might have interfered with desaturation, especially ζ-carotene desaturation, during carotenoid biosynthesis.

Key words: bleaching; ζ-carotene; β-carotene; triazole

Bleaching herbicides interfere with the formation of colored carotenoids that play an essential role by photoprotecting chlorophyll against photooxidative destruction by singlet oxygen. A target of the many bleaching herbicides is phytoene desaturase on the carotenoid biosynthetic pathway (Fig. 1).[1] The inhibitors of phytoene desaturase, such as fluridone and flurtamone, cause an accumulation of phytoene.[2,3]

We have previously reported that 4-(4-chlorophenyl)-3-propargylthio-4*H*-1,2,4-triazole **(1)** showed bleaching activity against lettuce seedlings, and that 3-benzylthio-4-phenyl-4*H*-1,2,4-triazole **(2)** caused some bleaching symptoms. It was clear that a treatment by compound **1** at 50 μM resulted in a greatly reduced β-carotene content in radish seedlings with the accumulation of a large amount of phytoene.[4] We have recently found that compound **2** decreased the β-carotene content with the accumulation of both phytoene and ζ-carotene in lettuce seedlings. In light-grown seedlings treated with 250 μM of compound **2**, the accumulation of ζ-carotene was 2-fold higher than that of phytoene. It is known that some dihydropyrone[5] and 6-methylpyrimidine[6] derivatives inhibit ζ-carotene desaturase, resulting in the accumulation of ζ-carotene in treated plants. There have been few reports on the ζ-carotene desaturase inhibition of 1,2,4-triazole derivatives, except for amitorole which affects the lipid environment of desaturase in the thylakoid membrane resulting in the accumulation of phytoene and ζ-carotene.[7–9] In the present paper, we describe details of the structure-bleaching activity relationship of 4-aryl- and 4-alkyl-3-(substituted benzylthio)-4*H*-1,2,4-triazoles, and the effect of these compounds on the colored carotenoid content of lettuce seedlings.

Materials and Methods

Chemicals. All the 4-aryl- and 4-alkyl-3-(substituted benzylthio)-4*H*-1,2,4-triazoles used in this study were synthesized according to the same method reported previously.[4] The general procedure involved cyclization of 4-aryl- or 4-alkyl-1-formyl-3-thiosemicarbazides that has been prepared by treating formylhydrazine with aryl- or alkyl isothiocyanates. The resulting 4-aryl- and 4-alkyl-3-mercapto-4*H*-1,2,4-triazole was alkylated by an appropriate benzyl halide to afford to 4-aryl- or 4-alkyl-3-benzylthio-4*H*-1,2,4-triazole. The following procedure for the preparation of 4-(3-fluorophenyl)-3-(4-trifluoromethylbenzylthio)-4*H*-1,2,4-triazole **(39)** is typical. To a solution of 2.35 g of formylhydrazine in 20 ml of dry tetrahydrofuran was added 5.0 g of 3-trifluorophenyl isothiocyanate. After refluxing for 6 h, the insoluble product [4-(3-fluorophenyl)-1-formyl-3-thiosemicarbazide] was collected by filtration. A solution of the product and 2.58 g of potassium hydroxide in 50 ml of water was stirred for 2 h at 70°C. After cooling, the solution was acidified with a 2 N HCl solution. The resulting precipitate was collected by filtration, washed with water and recrystallized from hexane and ethyl acetate to afford 3.80 g (60%) of 4-(3-fluorophenyl)-3-mercapto-4*H*-1,2,4-triazole. To a mixture of 0.5 g of this 4-(3-fluorophenyl)-3-mercapto-4*H*-1,2,4-triazole and 2.0 g of potassium carbonate in 20 ml of acetone was added 0.67 g of 4-trifluoromethylbenzylbromide. After stir-

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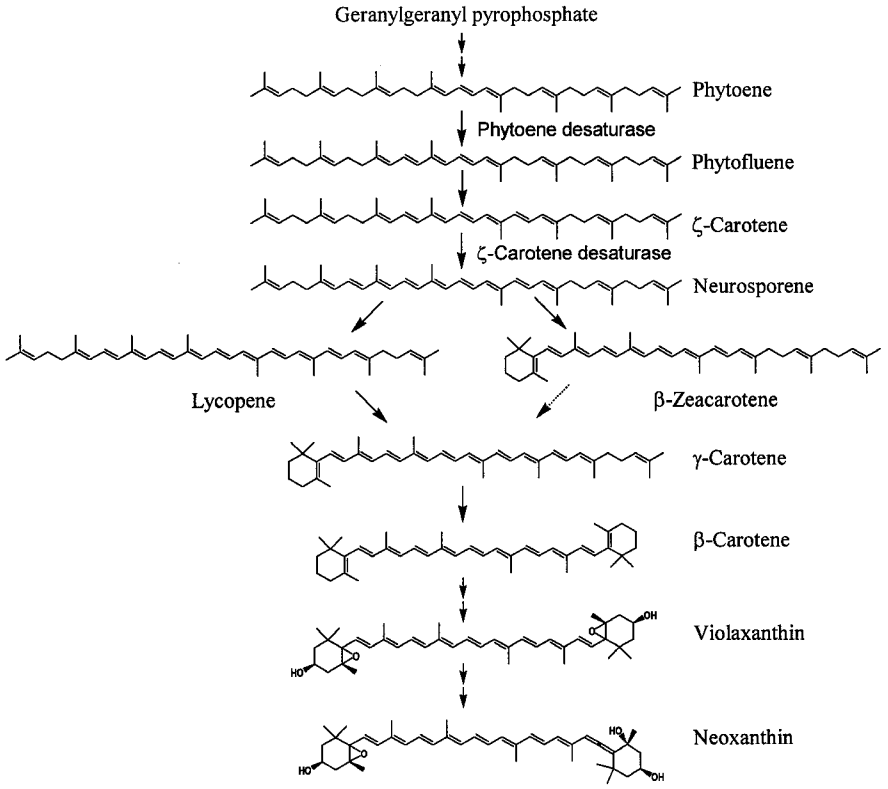


Fig. 1. Pathway for Carotenoid Biosynthesis.

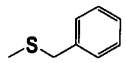
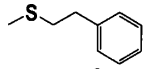
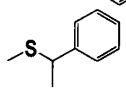
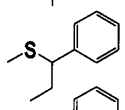
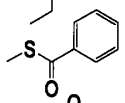
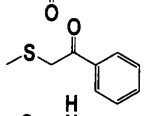
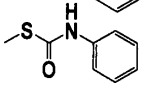
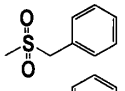
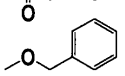
ring night over at room temperature, the solution was concentrated *in vacuo*. The residue was extracted with diethylether, successively washed with a 1 N NaOH solution, water and brine, and dried over Na_2SO_4. After evaporating the solution, the residue was recrystallized from hexane and ethyl acetate to yield 0.84 g of 4-(3-fluorophenyl)-3-(4-trifluoromethylbenzylthio)-4*H*-1,2,4-triazole (93%). Melting point: 94–95°C. ^{1}H-NMR (400 MHz, $CDCl_3$, TMS) δ: 4.52 (2H, s), 6.95–7.03 (2H, m), 7.18–7.23 (5H, m), 7.44–7.55 (4H, m), 8.28 (1H, s). *Anal.* Found: C, 54.61; H, 3.11; N, 12.01%. Calcd. for $C_{16}H_{11}F_4N_3S$: C, 54.39; H, 3.14; N, 11.89.

Bioassay.

Lettuce seedling test. Lettuce (*Lactuca sativa L.* cv. Sacramento) seeding test was conducted by the method described previously.[10] After 4 days of cultivation, the bleaching activity was estimated on a scale of 0–4 according to the following ordinal categories: 0, no visual change compared with the control; 1, faint bleaching at the edges of the leaves; 2, intermediate between categories 1 and 3; 3, small green area remaining on the leaves; 4, complete bleaching. The bleaching activity from each treatment is indicated by the average result from 60 tested seedings.

Determination of pigments. Extraction of the chlorophyll and colored carotenoids from lettuce cotyledons was carried out by a modification of the method described previously.[4] On a filter paper in a Petri dish (15 cm in diameter) was poured 3 ml of an acetone solution of the test compound. After evaporating the solvent, 100 seeds were placed on the filter paper and cultivated in 15 ml of deionized water at 25°C under a 12-h photoperiod. After 4 days, the cotyledons were cut and stored at -40°C. A known amount of the cotyledons was ground with quartz in 2 ml of iced methanol in the dark. The extract was filtered, and the filtrate topped up to 10 ml with methanol. Two milliliter of the solution was taken out, and chlorophyll content was determined by its absobance at 665 and 650 nm, according to the method described by MacKinney. To extract the colored carotenoids, to the remaining methanol solution (8.0 ml), 3 ml of a methanol solution containing 22% KOH (the final KOH concentration was 6%) was added, and the mixture was incubated at 60°C for 20 min. The colored carotenoids were partitioned into 10% diethyl ether (v/v) in petroleum ether, and the ether solution was successively washed with water and brine, and dried (Na_2SO_4). After concentrating the ether solution, the residue was dissolved in 200 μl of methanol, and 20 μl of the solution was chlomatographed by HPLC on a 4.6×250 mm 5-μm Spherisorb ODS-II column. The pigments were separated by gradient elution chromatography according to a modification of Yi Li's method.[11] The elution program consisted of a linear gradient of water and a

Table 1. Bleaching Activity of 4-Phenyl-3-substituted 4*H*-1,2,4-triazoles against Lettuce Seedlings

No.	R	Bleaching activity Concentration (μM) 250	50
2		3	1
3		2	0
4		3	1
5		0	0
6		0	0
7		0	0
8		0	0
9		0	0
10		0	0

Bleaching was visually evaluated (Activity rating: 0, no bleaching; 1, faint bleaching; 2, intermediate between categories 1 and 3; 3, definite bleaching; 4, complete bleaching).

mixture of acetonitrile/methanol/2-propanol (7:2:1 (v/v/v)) from 88% to 100% of the organic mixture over 18 min at 1.5 ml/min. This system could separate very well the various xanthophylls and carotenes at the same time. The carotenoids were simultaneously detected by a diode array (Shimadzu CO., Japan) at five different wavelengths, and identified by their retention times and electron absorption spectra. Quantitative analyses of the carotenoids were conducted on the areas under selected peaks by integrating at each wavelength with the appropriate extinction coefficients, as described previously.[11-13] The determined extinction coefficients were as follows: phytoene, 863 at 290 nm; phytofluene, 1466 at 350 nm; ζ-carotene, 2331 at 425 nm; β-zeacarotene, 1940 at 427 nm; β-carotene, 2600, violaxanthin, 2500, neoxanthin, 2500 and luteine, 2600, at all 445 nm. Each determination is the mean of five independent measurements.

Results and Discussion

Bleaching activity

We have previously described the studies on the structure activity relationship between 3-alkylthio-4-(substituted phenyl)-4*H*-1,2,4-triazoles and the bleaching activity on 4-day-old lettuce seedlings. In the 4-phenyl-4*H*-1,2,4-triazole series, 3-propargylthio and 3-propylthio analogs showed the completed bleaching activity at 250 μM, and the activities were higher than that of other 3-alkylthio analogs. Since compound **2** caused some bleaching symptoms, several related 4-phenyl-4*H*-1,2,4-triazole analogs which have aryl group at 3-position were prepared and tested for their bleaching activity against lettuce seedlings (Table 1). 3-α-Methylbenzylthio analog **4** showed bleaching activity comparable to that of compound **2** at 250 μM, while 3-β-phenylethylthio analog **3** had low activity. No activity was apparent with 3-α-ethylbenzylthio **(5)**, 3-phenyl-thio ester **(6)**, 3-phenacylthio **(7)** and 3-phenyl-thiocarbamate **(8)** analogs, even at 250 μM. Oxidation of the sulfide group of compound **2** to a sulfone group (compound **9)** and conversion of the sulfur atom to an oxygen atom (compound **10)** completely eliminated the activity at 250 μM, indicating that the benzylthio group at the 3-position on the triazole ring was necessary for activity.

Table 2. Bleaching Activity of 3-Benzylthio-4-substituted 4*H*-1,2,4-triazoles against Lettuce Seedlings

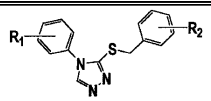
No.	R	Bleaching activity Concentration (μM) 250	50
11	allyl	0	0
12	*n*-butyl	0	0
13	*tert*-butyl	0	0
14	cyclohexyl	1	0
15	α-naphthyl	0	0
16	benzyl	1	0
17	β-phenylethyl	1	0

Bleaching was visually evaluated (Activity rating; 0, no bleaching; 1, faint bleaching; 2, intermediate between categories 1 and 3; 3, definite bleaching; 4, complete bleaching).

We also examined whether the phenyl group at the 4-position on the triazole ring in compound **2** could be replaced by other substituents (Table 2). The 4-allyl **(11)**, 4-*n*-butyl **(12)**, 4-*tert*-butyl **(13)** and 4-α-naphthyl **(15)** analogs were completely inactive. The 4-cyclohexyl **(14)**, 4-benzyl **(16)** and 4-β-phenylethyl **(17)** analogs had faint bleaching activity at 250 μM. This result indicates that the presence of the 4-phenyl group was significant for higher bleaching activity. Thus, among this series of 4-aryl- and 4-alkyl-3-substituted-4*H*-1,2,4-triazoles, 3-benzylthio-4-phenyl analog **2** exhibited the highest activity.

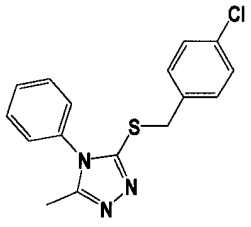
Further modification was made by introducing various substituents on the two benzene rings of compound **2** (Table 3). The introduction of some substituents on the benzene ring of the benzylthio moiety tended to increase the activity. Substitution at the

Table 3. Bleaching Activity of the 4-Phenyl-3-benzylthiol-4*H*-1,2,4-triazole Derivatives against Lettuce Seedlings



No.	R_1	R_2	Bleaching activity, Concentration (μM) 250	50	10	5	1
2	H	H	3	1	0		
18	H	2-Cl	3	1	0		
19	H	2-Me	4	3	0		
20	H	2-MeO	4	2	0		
21	H	3-Cl	4	3	0		
22	H	3-Me	3	3	0		
23	H	3-MeO	4	3	0		
24	H	4-Cl	4	4	2	1	0
25	H	4-Me	4	4	2	0	
26	H	4-Et	4	4	1	0	
27	H	4-MeO	4	4	2	0	
28	H	4-CF_3O	4	4	4	2	0
29	H	4-EtO	4	4	1	0	
30	H	4-F	4	2	0		
31	H	4-CF_3	4	4	4	3	1
32	H	3,4-diCl	4	3	2	0	
33	2-Cl	4-CF_3	0				
34	2-Me	4-CF_3	0				
35	2-MeO	4-CF_3	3	3	0		
36	3-Cl	4-CF_3	4	4	3	2	1
37	3-Me	4-CF_3	4	4	3	0	
38	3-MeO	4-CF_3	4	4	3	0	
39	3-F	4-CF_3	4	4	4	3	2
40	3-CF_3	4-CF_3	4	3	2	0	
41	4-Cl	4-CF_3	4	4	2	0	
42	4-Me	4-CF_3	3	2	0		
43	4-MeO	4-CF_3	4	1	0		
44	3,4-diCl	4-CF_3	2	1	0		

Bleaching was visually evaluated (Activity rating: 0, no bleaching; 1, faint bleaching; 2, intermediate between categories 1 and 3; 3, definite bleaching; 4, complete bleaching).



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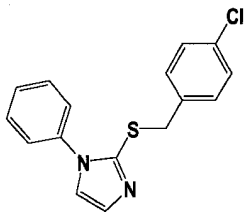

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Scheme

para position on the benzene ring exhibited relatively high activity. The 4-chloro (**24**), 4-methyl (**25**), 4-methoxy (**27**), and 4-ethoxy (**29**) analogs showed almost the same activity, causing complete bleaching symptoms at 50 μM. The additional introduction of a chlorine atom at the 3-position on the benzene ring of compound **24** slightly decreased the activity (compound **32**). The most favorable substituents were 4-trifluoromethoxy and 4-trifluoromethyl (compounds **28** and **31**, respectively), both compounds showing complete bleaching injury at 10 μM.

Based on the foregoing results, the 3-(4-trifluoromethyl)benzyl moiety of compound **31** was selected as the partial structure necessary for high activity, and some substituents were introduced to the phenyl group at the 4-position. The introduction of a chloro (compounds **33** and **41**), methyl (compounds **34** and **42**) or methoxy (compounds **35** and **43**) substituent to the phenyl group at the *ortho* or *para* position on the benzene ring of compound **31** caused a decrease in the bleaching activity, in particulars, the 2-chlorophenyl (**18**) and 2-methylphenyl (**19**) analogs were inactive at 250 μM. Only 3-fluorophenyl analog **39** showed slightly higher activity than that of compound **31**, whereas the other 3-substituted phenyl analogs, such as the 3-methylphenyl (**37**), 3-methoxyphenyl (**38**) and 3-trifuluoromethylphenyl (**40**) analogs, had lower activity and showed no bleaching symptoms at 5 μM. 3-Chlorophenyl analog **36** had slightly lower activity than that of compound **31**, and the additional introduction of a chlorine atom (compound **44**) led to greatly reduced activity. In conclusion, the 4-trifluoromethylbenzyl moiety and 3-fluorophenyl group on the triazole ring appeared to be essential for high bleaching activity.

It is noteworthy that the introduction of a methyl group into the 5-position of the triazole ring (compound **45**) in compound **24** induced a reduction in the potency, and 2-(4-chlorobenzylthio)-1-phenylimidazole (**46**) caused no visible bleaching symptoms at 250 μM (data are not shown). These results suggest that the 1,2,4-triazole ring of 4-phenyl-3-(substituted benzylthio)-4*H*-1,2,4-triazole played an important role in exhibiting the bleaching activity.

Effect on carotenoids content

Table 4 shows the influence of representative compounds **1** and **39** on the chlorophyll and carotenoid contents of lettuce seedlings cultivated under light or dark conditions. Under light conditions, both compounds **1** and **39** markedly reduced chlorophyll and such carotenoids as β-carotene, violaxanthin, neoxanthin and luteine. The amount of violaxanthin, neoxanthin and luteine diminished approximately in proportion to the decrease of β-carotene in the presence of compounds **1** and **39**. Compound **1** caused a large accumulation of phytone in lettuce seedlings, as has been observed for radish seedlings.[4]

On the other hand, in lettuce seedlings treated with 1 μM of compound **39** under light conditions, a relatively large amount of ζ-carotene was accumulated which was not detected in the untreated control. However, at higher concentrations, the accumulation of ζ-carotene decreased. The accumulation of phytoene caused by compound **39** was much less than that found with compound **1**.

In contrast to the results found under light conditions, treatment of compound **39** in darkness resulted

Table 4. Influence of 4-(4-Chlorophenyl)-3-propargylthio-4*H*-1,2,4-triazole (**1**) and 4-(3-Fluorophenyl)-3-(4-trifluoromethylbenzylthio)-4*H*-1,2,4-triazole (**39**) on the Chlorophyll and Carotenoid Content of 4-Day-old Light- or Dark-Grown Lettuse Leaves

Growth condition	Treatment		Chrorophyll (% of control)	Carotenoid content ($\mu g/g$ fresh weight)							
				Phytoene	Phytofluene	ζ-Carotene	β-Zeacarotene	β-Carotene	Violaxanthin	Neoxanthin	Luteine
light	Control		100	N.D.	N.D.	N.D.	N.D.	18.53 ± 4.45	4.17 ± 1.09	2.16 ± 0.67	19.55 ± 4.13
	Compound **1**	50 μM	54.5	4.98 ± 1.78	trace	0.17 ± 0.07	N.D.	3.81 ± 0.81	0.85 ± 0.24	0.28 ± 0.10	4.31 ± 1.52
		1 μM	40.2	0.50 ± 0.37	trace	1.36 ± 0.43	0.78 ± 0.41	1.56 ± 0.80	1.81 ± 0.99	0.65 ± 0.22	2.84 ± 1.36
	Compound **39**	5 μM	4.3	0.43 ± 0.14	N.D.	0.33 ± 0.11	0.17 ± 0.06	0.13 ± 0.03	0.15 ± 0.07	0.02 ± 0.008	0.49 ± 0.11
		50 μM	0.9	0.08 ± 0.002	N.D.	0.16 ± 0.04	0.023 ± 0.011	trace	trace	trace	0.031 ± 0.013
dark	Control		—[a]	N.D.	N.D.	N.D.	N.D.	0.29 ± 0.02	4.13 ± 0.37	0.44 ± 0.06	4.37 ± 0.47
	Compound **39**	1 μM	—	0.31 ± 0.10	trace	1.12 ± 0.48	1.63 ± 0.57	0.21 ± 0.08	3.91 ± 0.42	0.42 ± 0.08	3.51 ± 0.76
		5 μM	—	1.79 ± 0.31	1.21 ± 0.22	13.92 ± 2.99	3.08 ± 1.20	trace	0.24 ± 0.04	0.053 ± 0.010	0.26 ± 0.05
		50 μM	—	2.44 ± 0.08	1.46 ± 0.14	18.01 ± 6.74	0.29 ± 0.004	trace	0.085 ± 0.02	trace	0.14 ± 0.03

Each value is the average of five separate experiments. N.D.: not detected
[a] Chlorophyll was not found under dark conditions.

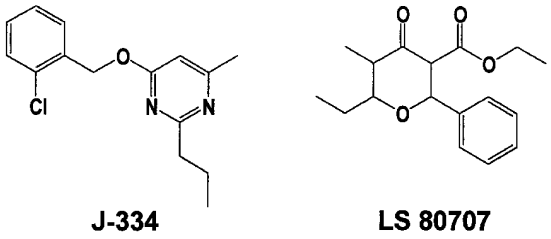

Fig. 2. Chemical Structures of ζ-Carotene Desaturase Inhibitors.

in a significant increase in the ζ-carotene content, particularly at higher concentrations. This result suggests that ζ-carotene was very sensitive to photodestruction in the light. Compound **39** caused the accumulation of phytoene as well, but its amount was much lower compared to ζ-carotene, suggesting that compound **39** predominantly inhibited ζ-carotene desaturation step rather than phytoene desaturation.

Neurosporene formed by the desaturation of ζ-carotene was not detected in the lettuce seedlings not treated and treated with compound **39**, while the accumulation of β-zeacarotene, which is a monocyclized product of neurosporene, was observed to some extent by treating with compound **39**. At lower concentrations revealing some bleaching injury, the β-zeacarotene content was higher than that of phytoene.

Although a number of bleaching herbicides have been found to inhibit the desaturation of phytoene, only a few compounds are known to interfere with the ζ-carotene desaturation reaction.[7] It has been reported that 4-(2-chlorobenzyloxy)-6-methyl-2-propylpyrimidine (J-334) caused an accumulation of ζ-carotene in *Dunaliella bardawil*.[14] However, low J-334 concentrations induced a large amount of β-zeacarotene, while at higher concentrations, it blocked the desaturation of phytoene as well as ζ-carotene. The herbicidal compound, ethyl-*cis*-5-methyl-6-ethyl-2-phenyl-5,6-dihydropyran-4-one-3-carboxylate (LS 80707) has been shown to interfere with ζ-carotene desaturase in the blue-green alga.[15] In this case, a large accumulation of phytoene was observed at the same time. We found that compound **39**, with increasing concentration, accumulated ζ-carotene in a much larger amount than those of phytoene, phytofluene and β-zeacarotene, indicating that the desaturation of ζ-carotene was preferentially inhibited. 4-Phenyl-3-(substituted benzylthio)-4*H*-1,2,4-triazoles in this article might be a reasonable lead for generating of a new bleaching herbicide as well as an effective tool to elucidate the structural and functional features of ζ-carotene desaturase.

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