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Fast Assembly and High-Throughput Screening of Structure and Antioxidant Relationship of Carotenoids

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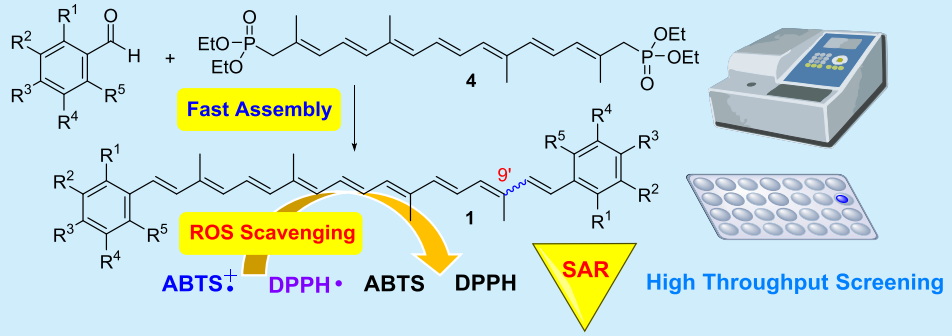
Supporting Information



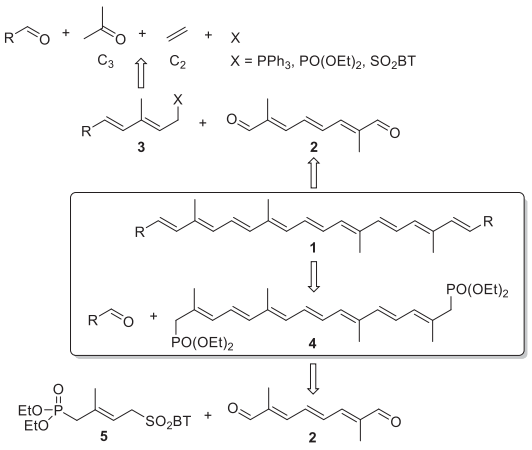
ABSTRACT: C_{20} heptaenyl diphosphonate **4** was prepared for one-pot synthesis of carotenoids **1**. Olefination with various aromatic aldehydes allowed fast assembly of the corresponding carotenoids. The SAR of carotenoids was investigated by high-throughput screening of ABTS and DPPH assays and their hierarchical clustering analysis. Antioxidant activity of carotenoids increased with the number of electron-donating substituents. Carotene **1a** with multiple electron-donating substituents was most proficient, which showed better radical scavenging activities than β-carotene and lycopene.

Carotenoids (**1**) are structurally unique natural products that generally contain nine conjugated C=C double bonds in the central chain with two terminal rings.[1] This structural motif is related with their energy-transferring activity from chlorophylls in photosynthesis.[2] Antioxidant activity by scavenging reactive oxygen species (ROS) is another important role of these red pigments.[3] Rapid destructive reactions with singlet oxygen or radical species explain the antioxidant mechanism of the conjugated polyene chain.[4] Given lipid peroxidation, DNA mutation, cancers, and other aging processes being induced by ROS,[5] it is necessary to develop powerful antioxidants to eliminate ROS generated during the respiration process.

It was envisioned that the structural modification of the terminal rings would significantly alter the effective conjugation and, thus, the reactivity of the conjugated polyene chain toward ROS.[6] An efficient synthetic method, as well as high-throughput screening (HTS), is an essential element for gaining information about the structure and activity relationship (SAR) to excavate lead compounds for new drugs.[7] Devising a highly efficient (robust and expeditious) synthetic method of carotenoids with various terminal rings would allow fast assessment of SAR of carotenoids for antioxidant activity.

A conventional synthetic method of carotenoids relies on the olefination of C_{10} 2,7-dimethyl-2,4,6-octatrienedial (**2**) with C_{15} unit **3** (Scheme 1), which should be prepared in multiple steps generally from the corresponding aldehydes (RCHO) by sequential chain extension with C_3 (e.g., acetone) and C_2 (e.g., ethylene) units, followed by functionalization with the linchpin group X, which can be selected according to the olefination method among PPh_3 for Wittig, $P(O)OEt_2$ for Wadsworth-Emmons, and SO_2BT (benzothiazole) for Julia-Kocienski.[8] Novel C_{20} heptaene **4** containing phosphonate groups was devised to facilitate one-pot synthesis of various carotenoids **1**

Scheme 1. Comparison of the New One-Pot Synthesis of Carotenoids 1 with the Conventional Method



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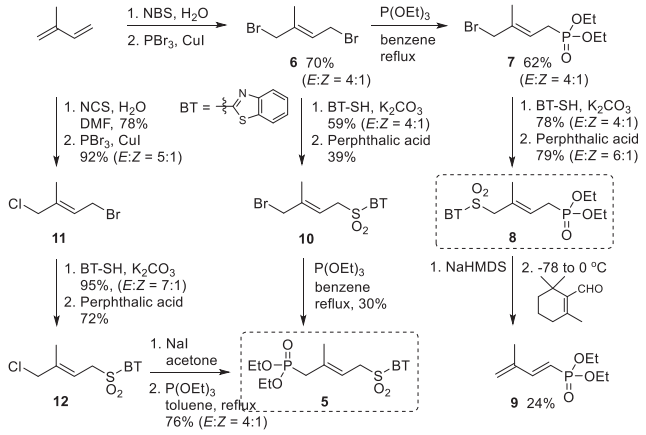
from diversely substituted aromatic aldehydes. New C_5 building block **5** with two linchpin groups of $P(O)OEt_2$ and SO_2BT was selected for sequential olefination,[9] and was selectively coupled at the SO_2BT site with C_{10} dial **2** to produce the key C_{20} diphosphonate **4**.

Preparation of C_5 units **5** and **8** were described in Scheme 2. Bromohydrin formation (NBS, H_2O) from isoprene, followed

Scheme 2. Preparation of C_5 Units 8 and 5 with Diethyl Phosphonate and Benzothiazolyl Sulfone Groups

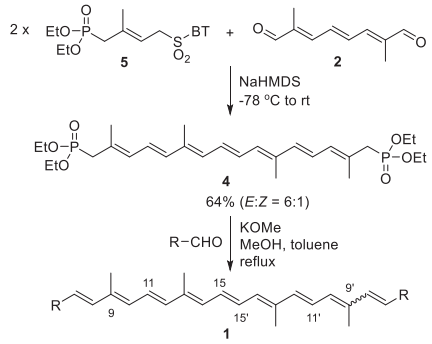


by allylic bromination (PBr_3, cat. CuI) produced 1,4-dibromide **6** in 70% yield with 4:1 *E*/*Z* selectivity. The order of phosphorylation and sulfonylation of dibromide **6** dictated the regiochemistry of bifunctional C_5 unit, in which the internal methyl group allowed facile allylation at the distal carbon by steric bias. C_5 Unit **8** was prepared first and tested for feasibility of sequential olefination. Arbuzov reaction with $P(OEt)_3$ gave allylic phosphonate **7** in 62% yield (4:1 *E*/*Z*). Sulfonylation by sulfide coupling (BT-SH, K_2CO_3) and monoperphthalic acid oxidation (urea–H_2O_2, phthalic anhydride) produced C_5 unit **8** in 62% yield (6:1 *E*/*Z*). The preliminary reaction of **8** with *β*-cyclocitral unfortunately gave no olefination at the phosphate site but elimination of BT-sulfone to produce dienyl phosphonate **9** in 24% yield. It seemed that superior reactivity of sterically less-crowded allylic position (by remote methyl group) was mismatched with less-reactive phosphonate group.

The regio-isomeric C_5 unit **5** was prepared from dibromide **6** by the above two-step sulfonylation to produce allylic BT-sulfone **10** (23% yield, *E*-form by recrystallization), followed by phosphorylation with $P(OEt)_3$ in 30% yield. This sequence of reactions suffered from low yields, and modification of reaction conditions was necessary to improve the yields of reactions. To further differentiate the reactivity of the two allylic positions, 4-bromo-1-chloro-2-methylbut-2-ene (**11**) was prepared in 72% overall yield (5:1 *E*/*Z*) from isoprene by chlorohydrin formation (NCS, H_2O, DMF) and allylic bromination (PBr_3, cat. CuI). The yield of the two-step sulfonylation to give BT-sulfone **12** was improved up to 68% (*E*-form by recrystallization). Arbuzov reaction was sluggish at the allylic chloride site of BT-sulfone **12**, and thus Finkelstein reaction (NaI, acetone) was preceded to produce C_5 unit **5** in 76% yield (4:1 *E*/*Z*). The *E*/*Z* ratio seemed to be deteriorated during the Finkelstein reaction. The pure *E*-isomer was easily purified by silica gel column chromatography and used for the next sequential olefination reactions.

A 2-fold Julia-Kocienski olefination of C_5 unit **5** with C_{10} dial **2** utilizing NaHMDS as a base smoothly progressed at −78 °C to room temperature (rt) to give C_{20} diphosphonate **4** (6:1 *E*/*Z*) in 64% yield (Scheme 3). Reactivity of the less-hindered

Scheme 3. Preparation of C_{20} Diphosphonate 4 and Expeditious Synthesis of Carotenoids 1



allylic carbon was reinforced by the more-reactive BT-sulfonyl group. Subsequent Wadsworth-Emmons olefination of the purified C_{20} all-(*E*)-diphosphonate **4** with various aromatic aldehydes with MeOK base in MeOH/toluene at reflux produced the corresponding carotenoids **1** containing aromatic rings in reasonable 23%–66% yields (recrystallized from THF/MeOH, see Table 1). The configuration of the polyene chain was carefully assigned to be either all-*E* or 9′-*Z*, or a mixture of both by comparing their ^1H NMR spectra with those of all-(*E*)-carotene **1b** and 9′-(*Z*)-carotene **1g** (Table 1).[10,11] The numbering sequence followed the carotenoid tradition, instead of the systematic IUPAC rule.[12]

It was found that the configuration of the polyene chain was controlled mostly by steric and electronic effects of the substituents in the terminal ring: *ortho*-methyl substitution preferentially produced all-*E* form; on the other hand, *para*-substituent favored 9′-*Z* form.[10] It was another noteworthy feature that furan gave all-*E* form, but thiophene produced 9′-*Z* form exclusively. Eighteen carotenoids were quickly assembled in this way, and HTS for their antioxidant activities were performed by measuring EC_{50} values (mol/L) for scavenging ABTS[13] [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH[14] (1,1-diphenyl-2-picrylhydrazyl) radicals. Antioxidant activities can be also evaluated by lipid peroxidation, DNA damage, OH radical and O_2 anionic radical scavenging, Cu(II)-chelating, Fe(III)-reducing assays.[13a] ABTS and DPPH assays were chosen for simple and fast HTS of the carotenoid antioxidant activities.

HTS is a process by which a large number of compounds can be tested in an automated fashion for biological activities.[7] The HTS assays for antioxidant with the microplate reader of 96-well proved to be robust and reproducible as well as time-saving. EC_{50} was defined as a measure of activity to be the concentration of antioxidant necessary to decrease the initial radical absorbance (A) to its 50% value. The measurements of EC_{50} (mol/L) for ABTS and DPPH assays were performed four times for each sample and the values of average and standard deviation were reported in Table 1. There are relatively good correlations between ABTS and DPPH assays in the order of carotenoids' radical scavenging activities. Hierarchical clustering analysis was performed for the EC_{50} values of carotenoids in ABTS and DPPH assays with Euclidian distance as a similarity measure and Ward's linkage

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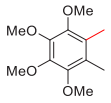
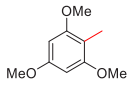
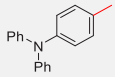
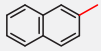
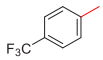
Org. Lett. XXXX, XXX, XXX–XXX

Table 1. Yield and Stereochemistry (All-*E*/9′-*Z*) of Carotenoids 1 Prepared from C_{20} Diphosphonate 4 and Their EC_{50} Values for ABTS and DPPH Assays

entry[a]	carotene **1**	R	yield (%)	ratio[b] (all-*E*/9′-*Z*)	ABTS EC_{50} (mol/L)	DPPH EC_{50} (mol/L)
1	**a**		24	3:1	0.0484±0.0169	0.4213±0.0054
2	**b**[10]		29	all-*E*	0.0807±0.0229	0.9865±0.0165
3	**c**		45	2:1	0.0982±0.0308	0.7541±0.0227
4	**d**		44	3:4	0.1075±0.0162	1.2756±0.0374
5	**e**		29	9′-*Z*	0.1102±0.0224	1.0799±0.0158
6	**f**[16]		40	all-*E*	0.1150±0.0308	1.4218±0.0611
7	**g**[10]		42	9′-*Z*	0.1180±0.0307	1.1375±0.0258
8	**h**		65	9′-*Z*	0.1288±0.0293	1.5052±0.0598
9	**i**		50	all-*E*	0.1316±0.0076	1.2391±0.0671
10	**j**		53	9′-*Z*	0.1512±0.0087	1.9174±0.1236
11	**k**[16]		66	9′-*Z*	0.1537±0.0201	1.5520±0.0890
12	**l**[17]		53	1:20	0.1776±0.0292	1.6906±0.2409
13	**m**[18a]		43	2:1	0.2184±0.0195	1.8279±0.0442
14	**n**[18]		39	9′-*Z*	0.3500±0.0138	3.2079±0.3821
15	**o**		42	1:5	0.3649±0.0168	2.3754±0.1256
16[c]	**p**		< 2	all-*E*	0.4270±0.0203	2.5043±0.7729
17	**q**		27	3:2	0.5709±0.0198	5.0130±0.9095
18	**r**		23	1:3.6	0.6531±0.0092	3.9859±0.3392

[a]The carotenoids were grouped (background shading) according to the hierarchical clustering analysis (see Figure S-1 in the Supporting Information). [b]The ratio of all-*E*/9′-*Z* configuration of carotenoids was calculated based on the integration of ^{1}H NMR peaks of the mixture (see page S84 in the Supporting Information as an example). [c]All-*E*-carotene **1p** was obtained in the synthesis of 9′-*Z*-carotene **1l** (see page S24 in the Supporting Information).

to extract unbiased information about the structural relevance to the carotenoids' antioxidant activity.[15] Eighteen carotenoids were grouped into five tribes in this way, which was denoted by background shading in Table 1 (also see the dendrogram of Figure S-1 in the Supporting Information).

The first group (Table 1, entries 1−3) demonstrated the strongest antioxidant activities, which showed the structural features of *ortho*- and multiple electron-donating substituents in the aromatic rings. It suggested that *ortho*-substitution induced more to the all-*E* configuration of the polyene chain.[10]

The second-best group in radical scavenging activity contained an electron-donating substituent at the *para*-position of the aromatic ring, which favored 9′-*Z* configuration of the carotenoid polyene chain (Table 1, entries 4, 5, 7, and 8). Two odd carotenes in this group are those with furan (Table 1, entry 6) and *meta*-dimethylbenzene (entry 9) as terminal rings, which exist as all-*E* form.

The aromatic rings of carotenoids in the third group possessed *para*-bromo or *ortho*-methyl substituents (Table 1, entries 10 and 13). 9′-(*Z*)-Carotene with benzene rings also

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Org. Lett. XXXX, XXX, XXX−XXX

belonged to this group (Table 1, entry 12). The correlation between *ortho*-substituent and all-*E* configuration was confirmed again (Table 1, entry 13). 9′-*Z* Configuration was the major for the other carotenoids, including the one with thiophene as the terminal rings (Table 1, entry 11). 9′-(*Z*)-Carotenoids with 2-naphthalene or *meta*-toluene rings, and all-(*E*)-carotene with benzene rings comprised the fourth group. The structural feature of the last (fifth) group of carotenoids was obvious that they contained electron-withdrawing *para*-substituted benzene rings, which exhibited the lowest radical scavenging activities (Table 1, entries 17 and 18).

It can be concluded from the above hierarchical clustering analysis of HTS on the antioxidant activity of the carotenoids that *ortho*- and *para*-electron-donating aromatic rings increase the radical scavenging activity (groups 1 and 2), while electron-withdrawing ones decrease it (group 5). It is not easy to draw a general activity profile on the *E* vs *Z* configuration of carotenoids.[13b] Higher activities of the carotenoids in group 1, compared to those in group 2, can be ascribed not to the configuration of the polyene, but to the multiple electron-donating aromatic substituents. The *ortho*-substituents in group 1 induced the polyene chain more to all-*E* configuration by steric reason, whereas the *para*-substituents in group 2 favored 9′-*Z* configuration presumably by effective conjugation.[10] All-(*E*)-carotene with furan rings is more potent than 9′-(*Z*)-carotene with thiophene rings for the same rationale (Table 1, entries 6 and 11). The activity order for the methyl-substituent position in toluene rings was as follows: *para* > *ortho* > *meta* (Table 1, entries 8, 13, and 15). Higher radical scavenging activity was observed for 9′-(*Z*)-carotene containing benzene rings than its all-(*E*)-counterpart (Table 1, entries 12 and 16).

HTS of the radical scavenging activities was repeated for the representative carotenoids **1a**, **1d**, **1j**, **1n**, and **1q** in the above five groups, together with *β*-carotene and lycopene as positive controls. Measurement of EC_{50} values was perfromed in quadruplicate, and the average values were depicted as bar graphs in Figure 1. The same activity trend was confirmed for the above five groups. It was found that the natural carotenoids are very good radical scavenging antioxidants, but most of all, novel carotene **1a** exhibits slightly better antioxidant activity than those natural carotenoids do. It seems that electron-donating groups are more important than effective conjugation in stabilizing the carotenoid radical species in these radical scavenging assays.

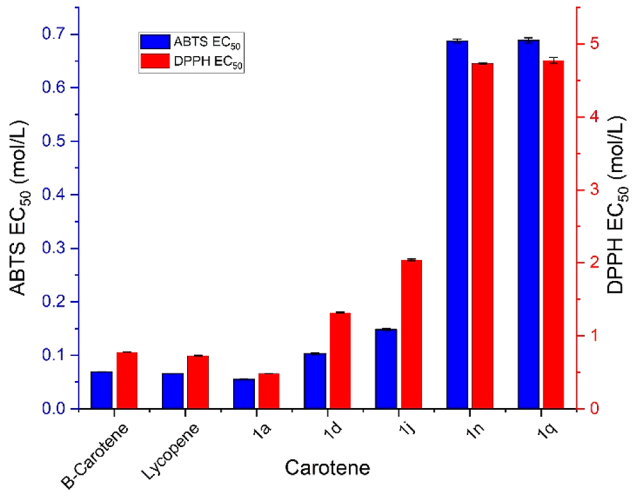

Figure 1. Comparison of antioxidant activities of the selected carotenoids in Table 1 with those of *β*-carotene and lycopene by EC_{50} (mol/L) in ABTS and DPPH assays (see Table S-3 in the Supporting Information).

In conclusion, versatile C_{20} heptaenyl diphosphonate **4** was developed for one-pot olefination of carotenoids **1** with various aromatic aldehydes, which diversified the terminal structures of the carotenoids. Fast assembly of diverse carotenoids, HTS for ABTS and DPPH radical scavenging activities, together with hierarchical clustering analysis provided structure and anti-oxidant-activity relationships for the carotenoids. Electron-rich aromatic rings increased the radical scavenging activity. The activity order of the methyl substituent in the toluene ring was as follows: *para* > *ortho* > *meta* position. Configurational effect of the polyene chain on the activity was found to be 9′-*Z* > all-*E* for the carotene with unsubstituted benzene rings. The strongest antioxidant activity was observed for **1a** with the aromatic rings of multiple electron-donating substituents, which exhibited stronger activity than natural *β*-carotene and lycopene. The SAR of carotenoids for antioxidant activity provides valuable designing principles for the carotenoid-based antioxidant drugs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03915.

Materials and methods for ABTS and DPPH assays, hierarchical clustering analysis, experimental procedures and analysis data for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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