DOCTORAL (PH.D.) THESIS

SYNTHESIS OF WATER-SOLUBLE CAROTENOIDS AND CAROTENOID DENDRIMERS

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I. Introduction

Carotenoids are naturally occurring tetraterpenes that deserved attention not only because of their flashy colors, but there are many other reasons why today they gained importance in analytical, biochemical and medical research. The beneficial effects of vegetables and fruits are mainly caused by carotenoids and vitamins. Today, nearly 700 kinds of carotenoids are isolated from natural sources. Among them, the β-carotene, lutein, zeaxanthin, and lycopene had been studied intensively.

Carotenoids have well-known biological effects. These natural pigments exhibit antioxidant activities, which are closely related properties of carbon chain which is containing conjugated double bonds, also called chromophore. Carotenoids react with reactive oxygen species (ROS) which are formed in the body and the antioxidant effect will develop by the oxidation or breaking up of polyene chain of carotenoids. In a chemical process, such as deactivation of the hydrogen peroxide, the carotenoids are converted to intermediates and completely decompose.

Carotenoids are applicable for preventing certain cancers, their antimutagenic effect is also known. Carotenoids reduce the risk of certain cardiovascular diseases. Zeaxanthin and lutein plays an important role in preventing of age-impaired vision (macular degeneration). Carotenoids with unsubstituted β-end group of the body of vitamin A precursors.

It can be seen that the body needs carotenoids, which can be fully covered by eating fruits and vegetables. Utilization and absorption of carotenoids in our body are determined by the properties of carotenoids: they are fat-soluble compounds.

Dendrimers have been studied intensively in the past 15 years. More than 50 dendrimer families are known, each has unique properties, surfaces, interiors and core structures which determine their specific use. There are many possible routes of application related to the unique molecular structure, multifunctional surface and the presence of cavities. These features make it possible to use them in the latest (nano) technologies. Extraordinary interest from the pharmaceutical area, but also a wide range of other industries can utilize these compounds.

Carotenoid dendrimers has not been known in the literature, thus they might be of interest biological assessments as well. This kind of dendrimers have a chance to change their physiological effects such as increased antioxidant effect of carotenoids, as observed with other biologically active compounds. The solubility and uptake could be more effective.

Humans being water based, can normally utilize much better compounds that are readily water-soluble. Carotenoids are hydrophobic compounds since the presence of long carbon chain and therefore their uptake is bound to fats. Better absorption could be achieved by enhancing water solubility. This may increase the antioxidant properties as well. Some hydrophilic carotenoid salts as cardioprotective drugs have also been introduced.

Nowadays it is not easy to find new carotenoids, the last century was the golden age in the structure determination and description of carotenoids. Today, the aims of experiments and research have nutritional and health implications. Only 3-4 research groups around the world are concentrating with more or less intensity on the introduction of novel reactions to carotenoid chemistry. One reason is the sensitive carotenoid compounds, air, light and easy to decompose traces of acid, working with them require a lot of care and precautions.

In my work I set the goal to develop new types of carotenoid derivatives, including to synthetize water-soluble compounds, as well as to introduce new types of reaction in carotenoid chemistry.

II. Aims of the study

1. Synthesis of carotenoid dimers and trimers in a simple, easy to follow reaction. In our experiments, we have planned to produce both hetero-and homodimers or trimers by using of simple cores which are easy to handle and can yield a stable final product.
2. Synthesis of water-soluble carotenoids. Synthesis of covalently bounded PEG-carotenoid conjugates and examination of the water solubility of the products.
3. The introduction of the click reaction to chemistry of carotenoids. The design of the end groups in the corresponding reaction partners. Development of optimal reaction conditions.

III. Experimental procedures

The NMR spectra of compounds were recorded on Varian Unity Inova (400/100 MHz $^{1}H/^{13}C$) spectrometer in deuterated chloroform ($CDCl_3$). The molecular weights were determined by Bruker Daltonics Autoflex MALDI II equipment.

The purity of the compounds was tested using HPLC apparatus. Measurements were performed by Dionex P580 HPLC gradient-type mixer pump, Dionex PDA-100 detector, Leon Chromeleon 6.70 software.

On the basis of the HPLC data's the synthesized materials are at least 95% pure, their UV spectrum is identical to the corresponding spectrum of carotenoids. We observed no measurable formation of cis isomers.

IV. Results

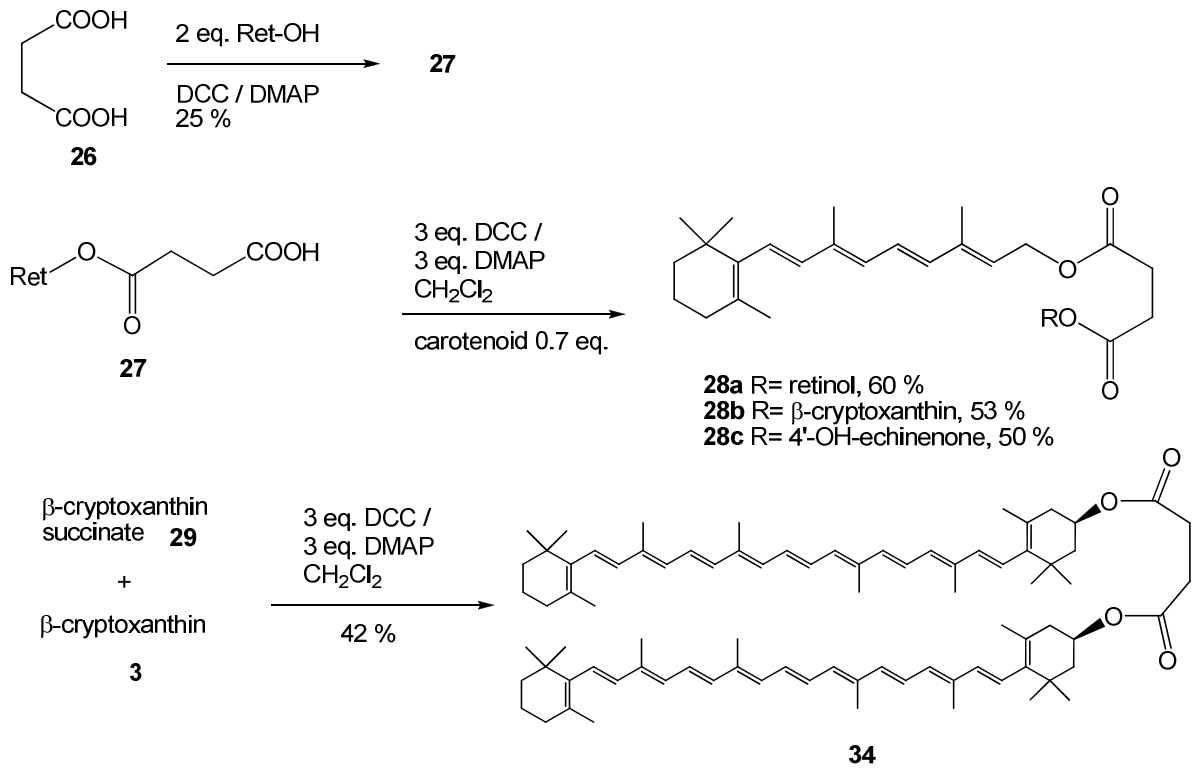
1. Synthesis of carotenoid dimers and trimmers

The experiments were started with synthesis of dimers with aliphatic carboxylic acids. First we reacted hydroxy-carotenoids with various acid anhydrides (Table 1.), and the obtained mono-esters (or monocarboxylic acids) have been esterified further with another hydroxy- carotenoid to obtain dimers.

Car-OH v. OH-Car-OH	2, or 4 eq. diacid anhydride → monoester or diester (CH_2Cl_2 / DMAP)		
	maleic anhydride	**succinic anhydride (26)**	**phthalic anhydride**
retinol (8)	*decomposed even on -70 ºC*	*85 %* (**27**, monoester)	*decomposed (even in piridine)*
izozeaxanthin (25)	*decomposed even on -70 ºC*	*instable product*	*decomposed (even in piridine)*
β-cryptoxanthin (3)	-----	*78 %* (**29**, monoester)	-----
8'-β-apocarotenol (26)	-----	*92 %* (**30**, monoester)	-----
4-hydroxy-echinenone (27)	-----	*72%* (**31**, monoester)	-----
zeaxanthin (4)	-----	*84%* (**32,** diester)	-----
lutein (1)	-----	*70%* (**33**, diester)	-----

Table 1. Esterification of carotenoids with diacid anhydrides

Different diesters were prepared by using Steglich esterification, the previously prepared carotenoid-succinates had been reacted with different hydroxyl carotenoids. In these reactions high yields could be achieved. The reaction was carried out not only with apocarotenoids (eg retinol (**8**)), but we also used carotenoids which containing 40 carbons (**28b, 28c**). An example of previously synthesized β-cryptoxanthin succinate (**29**) have been reacted to β-criptoxanthin (**3**) to form a homoester (**34**). The yields was 42%, which can be considered as good (Scheme 1.).



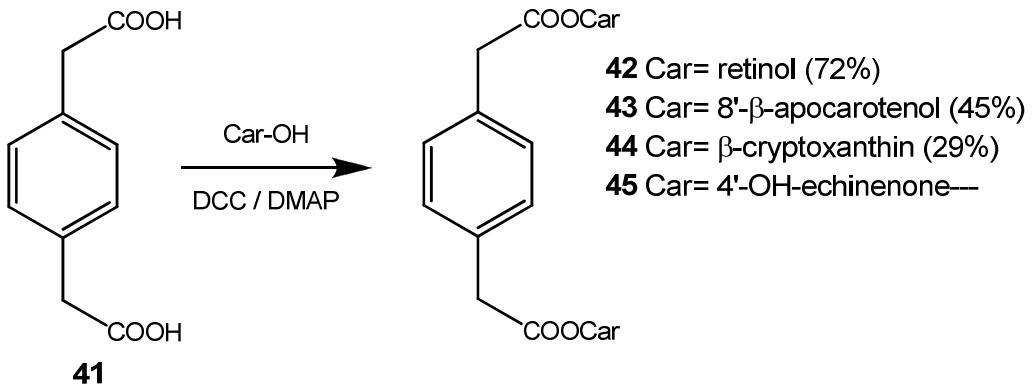
Scheme 1. Production of homo-, and heterodimers

If we applied excess of carotenoids using of DCC/DMAP/diacid reagent combination delivers the same homodimer. In our experiments retinol diester **(28a**) was prepared by using succinic acid (**26**). The yield was modest (25%) than in the two-step method (51%) and therefore not otherwise used in this procedure.

After synthesis of diesters, aromatic carboxylic acids and alcohols were reacted with carotenoids to produce dimers or trimers. These molecules can be considered as 1st generation dendrimers, their molecular weight is in the order of 1000-2000 Da.

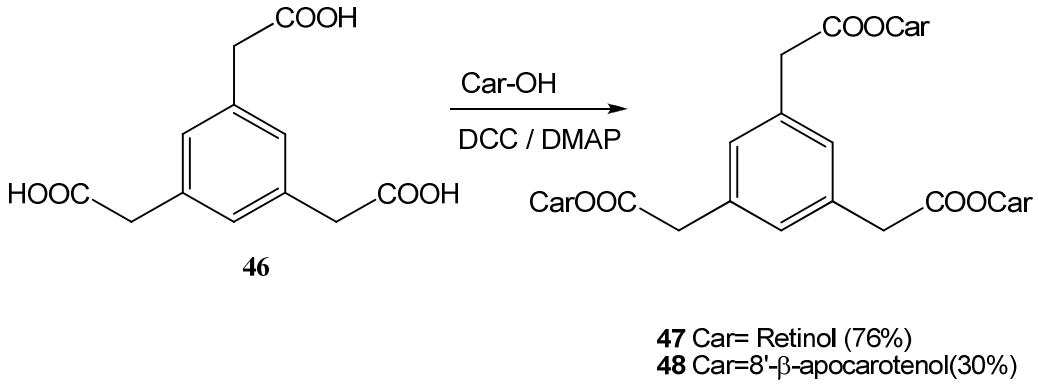
We had planned to use aromatic di-and tricarboxylic acids as cores, however, the experiments did not lead to successful reaction. This is probably due to the stereoelectronic and/or steric reasons.

Aromatic diacetic acid underwent the desired esterification (Scheme 2, **41**). In reaction with retinol (**8**) homo-diester were obtained with acceptable yields.



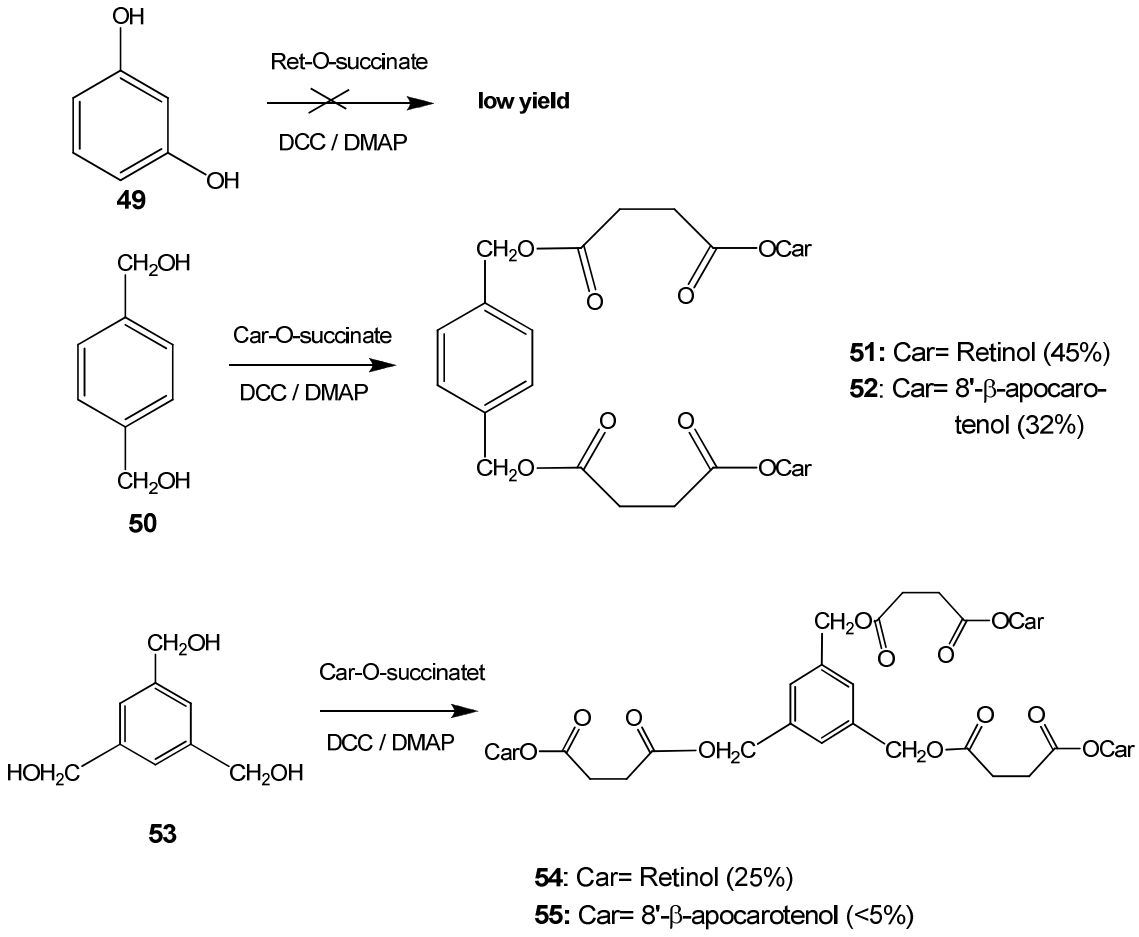
Scheme 2. Experiments with aromatic diacetic acid

Triester from retinol and 8'-β-apocarotenol had been prepared with the corresponding triacetic acid (**46)** (Scheme 3., **47**, **48**) were prepared in good yield.



Scheme 3. Experiments with aromatic triacetic acid

In the case of reaction of retinol succinate (**27**) with diphenol **49** (Scheme 4.) no product was observed. However, aromatic alcohols such as 1,4-diol (**50**) could be esterified with good yield with retinol succinate (**51**), and 8'-β-apocarotenol succinate (**52**). Using the 1,3,5-triol (**53**) the yields were much lower. Applying total (C40) carotenoids or their succinates in similar reactions no product formation was observed.



Scheme 4. Experiments with aromatic alcohols and phenols

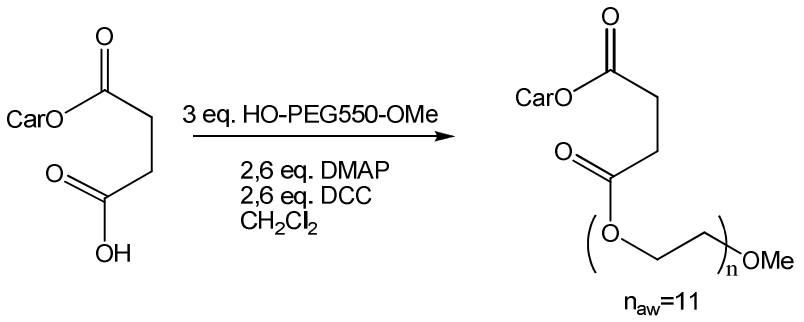
In the attempts to produce dendrimers the following results were obtained: 1st generation dendrimers had been succesfully produced from apocarotenols, but these cannot be further extended. The synthesis of trimers containing an outer free functionality (dihydroxy-carotenoids), did not work.

2. Synthesis of carotenoid-PEG conjugates

Many biomolecules (predominantly peptides) have been prepared as conjugates with polyethylene glycol (PEG) but covalently linked PEG conjugates of carotenoids has not previously been synthesized. The undoubted advantage PEG conjugates over ionic

compounds is that they do not alter the osmotic homeostasis, and the water solubility of PEG conjugates is not pH dependent.

The excess of monofunctional polyethylene glycol (PEG550-OMe or mPEG550) were used for esterification with previously synthesized carotenoid succinates (**27**, **29, 30**, **31**) at room temperature. (Scheme. 5).

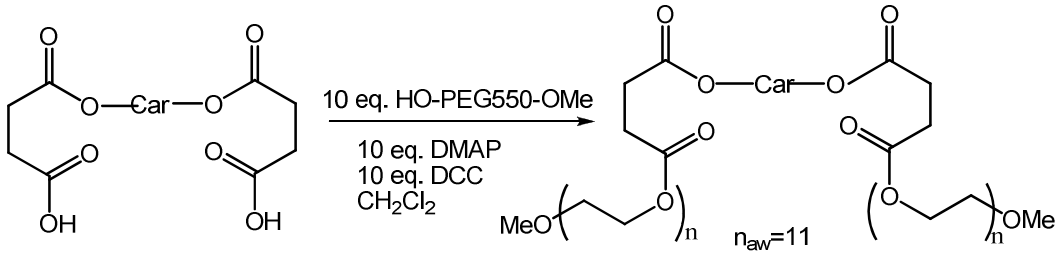


27 Car: retinol
29 Car: β-cryptoxanthin
30 Car: 8'-β-apocarotenol
31 Car: 4-OH-echinenone

60 Car: retinol (52%)
61 Car: β-cryptoxanthin (45%)
62 Car:8'-β-apocarotenol (37%)
63 Car:4-OH-echinenone (20%)

Scheme 5. Synthesis of PEG-carotenoid conjugates

The products were separated from the by-products on preparative thin layer chromatography. In the case of carotenoid disuccinates the same reaction conditions were applied and both ends were esterified with mPEG550 to yield the ester-linked conjugate (Scheme 6., **64, 65**).

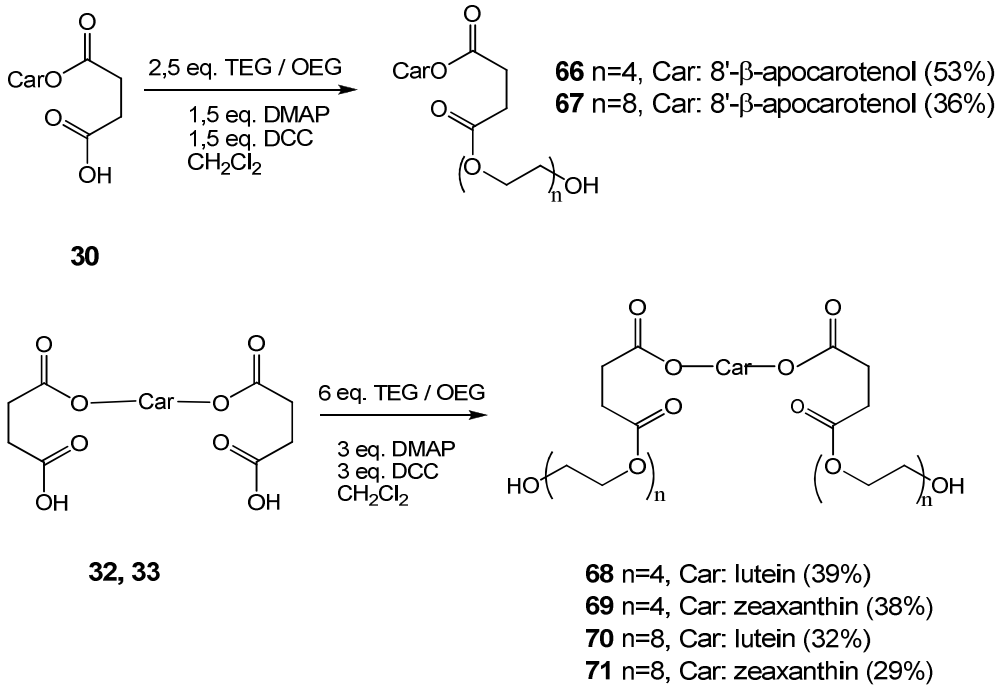


32 Car: zeaxanthin
33 Car: luthein

64 Car: zeaxanthin (56%)
65 Car: luthein (42%)

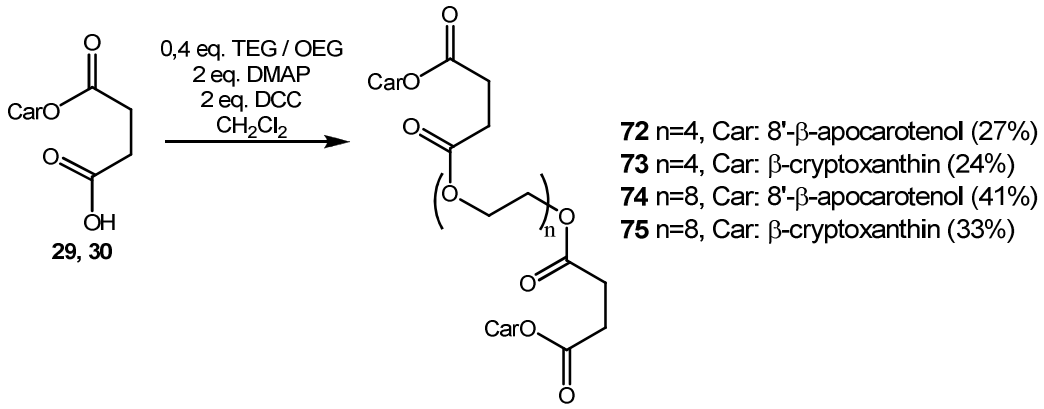
Scheme 6. Bifunctional carotenoids with mPEG-550

Similar carotinoid conjugates were prepared with octaethylene glycol (OEG) and tetraethylene glycol (TEG) by esterification (Scheme 7.). Carotenoid succinate (**30**), and disuccinates (**32**, **33**) were reacted with an the excess of TEG or OEG (**66**, **67**, **68, 69**, **70**, **71**). We observed that if the PEG chain length increased, the yield slightly decreased..



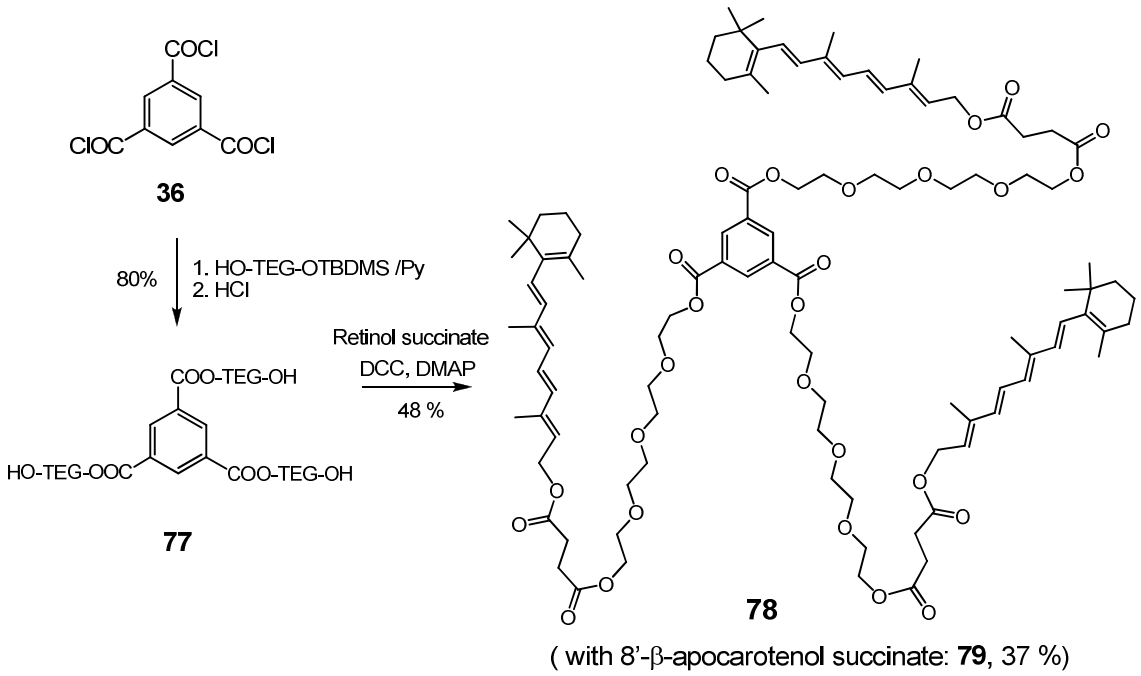
Scheme 7. Carotenoid conjugates with TEG and OEG

PEG chain linked homodimers have also been synthesized by the above method (Fig. 27) if the carotenoid monosuccinate was in excess (Scheme 8.). These compounds have a rather low content of PEG and a considerable water solubility cannot be expected from them.



Scheme 8. Carotenoid dimers with TEG and OEG

TEG linked carotenoid trimers were also prepared in a three step synthesis (Scheme 9., **78, 79**).



Scheme 9. Synthesis of TEG containing trimers

Some PEG conjugates were tested for antioxidant activity and water solubility. The table below shows the results. The right column shows the maximum amount of dissolved material in 1 ml of 96% ethanol in mg. This alcoholic solution is miscible with water in any proportion without detection of cloudiness and precipitation. The best soluble compound in ethanolic water was the zeaxanthin disuccinate mPEG550-conjugate (**64**). As it was expected, if the length of the PEG chain decreases, so does the solubility.

Compound	mg/ml 96 % EtOH
61	37,5
63	3,34
64	66
66	2,7
71	2,67
72	0,375

Table 2. Maximum amount of dissolved compound in 96% ethanol

The antioxidant studies were done on human liver-cell cultures. Cells were treated with the hydrophilic carotenoids, and to induce oxidative stress hydrogen peroxide was

used. Survival rate of the cells was measured after 1 day. The results are shown in the following diagram (Figure. 1.): control (C) is cells without carotenoid added. The gray bars show survival of cells without oxidative stress, after treatment of hydrogen peroxide the black bars represent survival after oxidative stress.

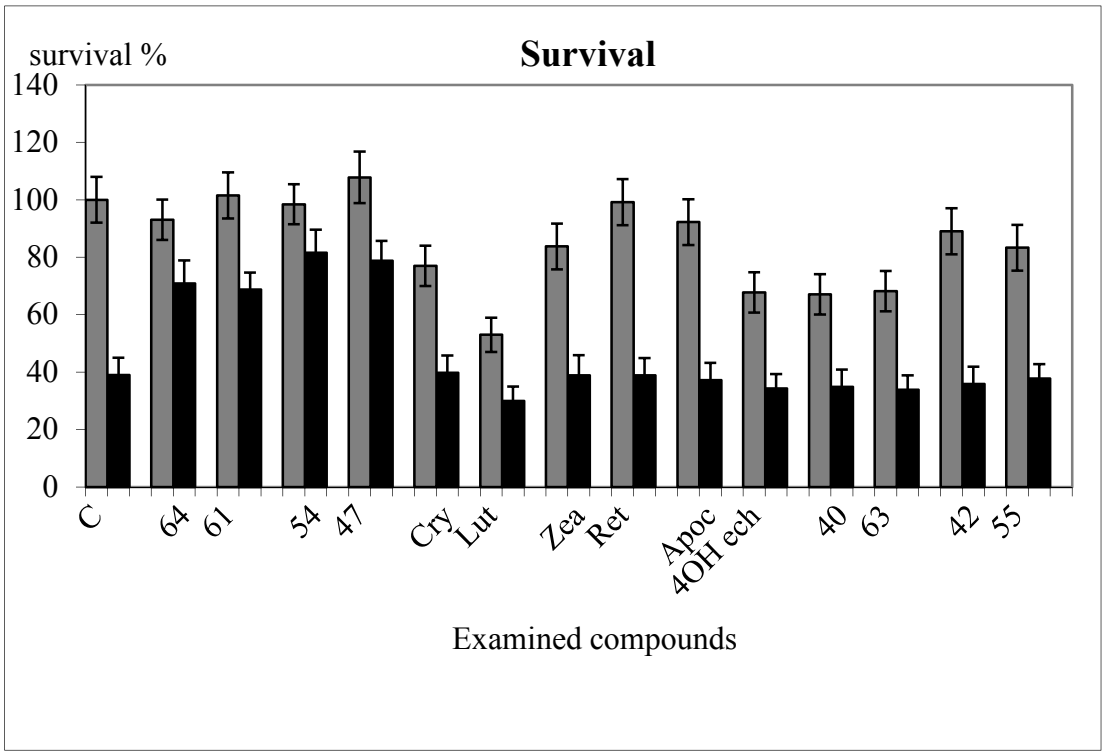


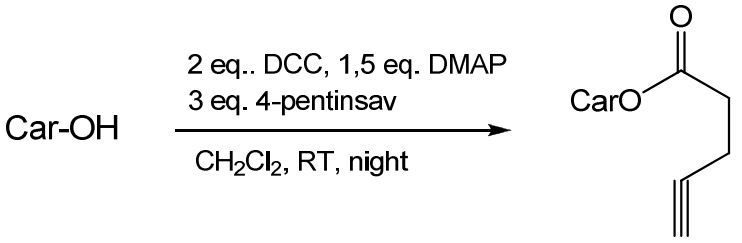
Figure 1. Survival rate of liver-cells after oxidative stress.

As shown, the control cells and cells which treated with native hydrophobic carotenoids (*Lut **(1)**, Cry **(3)**, Zea **(2)**, Ret **(8)**, Apoc **(24)***), survived the oxidative stress approximately 40%. Application of PEG conjugates (**64, 61, 63**) gave a significant survival, 80%, 70%, and values around 50%. Trimers consisting of retinol (**54, 47**) have proven to give an excellent survival rate of 80%. The dimers' antioxidant effect **42**) was much weaker.

3. Click-reaction of carotenoids

Finally we intended to introduce azide-alkyne click chemistry to the field of carotenoids. To form the corresponding azide and alkyne functional groups two approaches are possible: the carotenoid includes the azide group and alkyne group is attached to the reactant or vice versa. Making alkyne derivatives of carotenoids is simpler, thus we mainly concentrated on this synthetic approach.

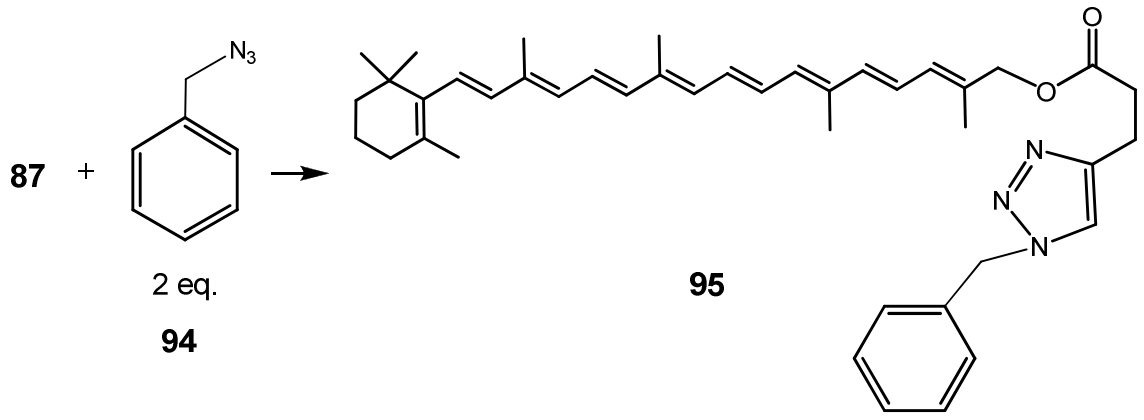
Carotenoid pentynoates have been prepared in good yield with 4-pentynoic acid. These compounds have the advantage that well crystallizable from toluene/methanol/water mixture (Scheme 10.).



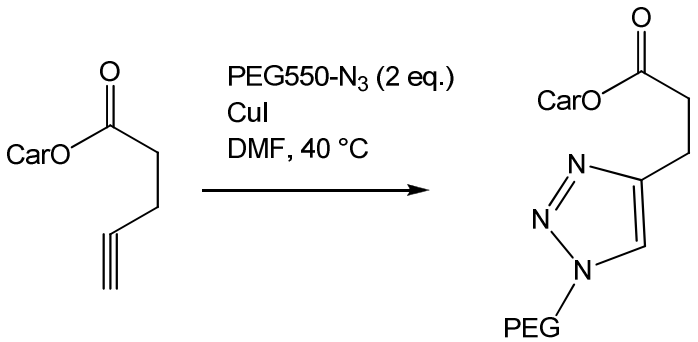
87 Car: 8'-β-apocarotenol (75%, with reduction)
88 Car: β-cryptoxanthin (90%)
89 Car: zeaxanthin (53%)
90 Car: izozeaxanthin (61%)
91 Car: lutein (47%)
92 Car: capsanthin (59%)
93 Car: 4-hidroxi-echinenone (52%)

Scheme 10. Synthesis of carotenoid pentynoates

To find the optimum reaction conditions the synthesized β-apocarotenol pentynoate (**87**) was reacted with benzyl azide (**94**) as model compound under different conditions. Based on the experiments, the best catalyst/solvent pairing was CuI/DMF. All subsequent reactions were performed with the use of CuI at 40 ° C in DMF.



These optimized conditions were applied for preparing of PEG-carotenoid conjugates (Scheme 11.).



96 Car: 8'-β-apocarotenol (52%)
97 Car: β-cryptoxanthin (47%)
98 Car: zeaxanthin (44%)
99Car: isozeaxanthin (33%)
100 Car: lutein (43%)
101 Car: capsanthin (33%)
102 Car: 4-hydroxy-echinenone (<3%)

Scheme 11. Reaction of carotenoid pentynoates with PEG-azides

The reactions delivered the adducts in good yields, which means that click-reaction most likely can be applicable among the carotenoids.

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