Studies on natural and semisynthetic carotenoids

final research report

1. Introduction

In this project we wished to continue the studies on natural and semisynthetic carotenoids and expand the scope of our research to new carotenoid bioconjugates. In summary, isolation and characterization of natural carotenoids delivered a lot of interesting results, whereas in the semisynthetic we had our ups and downs, as expected.

2. Isolation

2.1. Tropical fruits

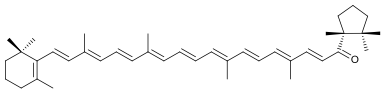
As a continuation of carotenoid isolation from natural sources, the complete carotenoid analysis of mamey (*Poutiera sapota*) was achieved by HPLC-DAD-MS, chemical tests, and co-chromatography with authentic samples. Altogether 47 components were detected and 34 identified from the total extract or after fractionation with column chromatography. The main carotenoids were cryptocapsin, sapotexanthin, and capsanthin 5,6-epoxide. Some further minor components containing the κ-end group with or without a hydroxy group and their 5,6-epoxy precursors were identified. Some comments were made about the biosynthesis of κ-carotenoids in red mamey [1].

From an extract of red mamey (*Pouteria sapota*), β-cryptoxanthin-5,6-epoxide, β-cryptoxanthin-5′,6′-epoxide, 3′-deoxycapsanthin, and cryptocapsin were isolated and characterized by UV−vis spectroscopy, electronic circular dichroism (ECD), nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS). Epoxidation of β-cryptoxanthin delivered the β-(5′*R*,6′*S*)- and (5′*S*,6′*R*)-cryptoxanthin-5′,6′-epoxides, which were identified by HPLC-ECD analysis [2-3]. These carotenoids among others are quite common in the fruits of Central America, and as they are natural provitamins A, they should play an important role in the diet of the mostly vitamin A deficient population of this region.

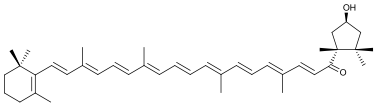
The fruit of red mamey (*Pouteria sapota*) contains a wide variety of carotenoids, generally in high concentration, which makes possible even the isolation of minor components in measurable amounts. Carotenoids were extracted from red mamey with acetone, subsequent saponification resulted a crude extract, which was submitted to column chromatography using aluminium oxide (Al_2O_3) as adsorbent. By using consecutive chromatographic steps and crystallization allene carotenoids, such as neoxanthin, (9'Z)-neoxanthin and capsoneoxanthin, were isolated from the most polar fractions in milligram amounts and in high purity. The amount of capsoneoxanthin was found sufficient for the complete analysis of this rare

carotenoid 15 years after its first isolation. The complete 1H- and 13C-NMR assignments of neoxanthin and (9'Z)-neoxanthin using 2D techniques were achieved for the first time, as well [4].

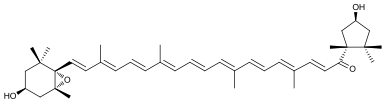
In cooperation with Prof. E. Murillo in Panamá a new compound, namely the sapotexanthin 5,6-epoxide was isolated from mamey and compared to their semisynthetic counterparts to achieve structure elucidation. There is as article in preparation on this topic, as well [5].



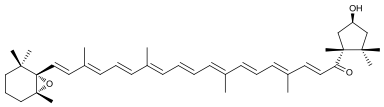
Sapotexanthin



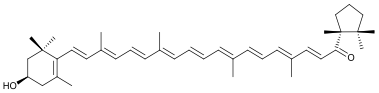
Cryptocapsin



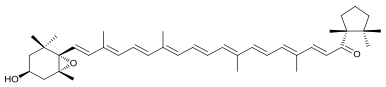
Capsanthin 5,6-epoxide



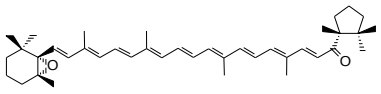
Cryptocapsin 5,6-epoxide



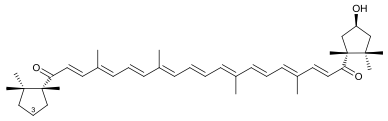
3'-Deoxycapsanthin



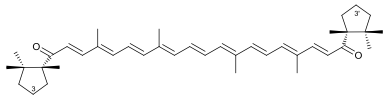
3'-Deoxycapsanthin 5,6-epoxide



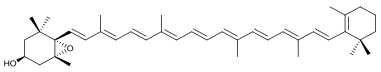
Sapotexanthin 5,6-epoxide



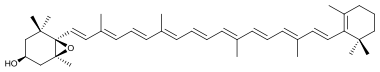
3-Deoxycapsorubin



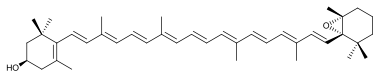
3,3'-Dideoxycapsorubin



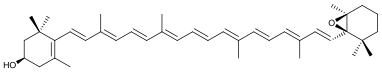
(3*S*,5*S*,6*R*)-β-Cryptoxanthin 5,6-epoxide



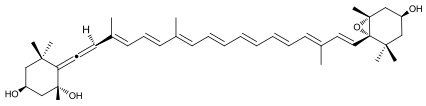
(3*S*,5*S*,6*R*)-β-Cryptoxanthin-5,6-epoxide



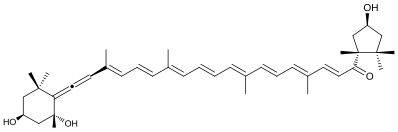
(3*R*,5'*R*,6'*S*)-β-Cryptoxanthin 5',6'-epoxide



(3*R*,5'*S*,6'*R*)-β-Cryptoxanthin-5',6'-epoxide



Neoxanthin



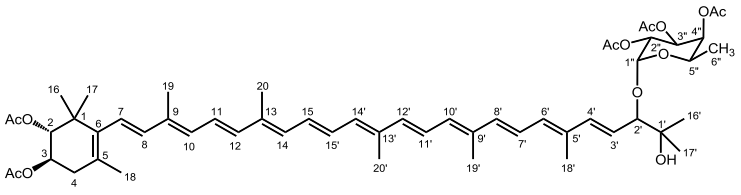
Capsoneoxanthin

To facilitate the isolation and analysis of carotenoids we provide a database which presents the separation characteristics of C18 and C30 stationary phases using carotenoids with diverse structures, including isomers, from the apolar carotenes to the very polar tetrahydroxy xantophylls. The retention behavior of more than 90 carotenoids on a C18 and C30 stationary phase were studied. The influence of structural differences of the carotenoids on the elution order for the two columns is discussed. Octadecyl bonded silica phase produces good separation for the whole polarity range of carotenoids. On this phase carotenoids are eluted in the order of their polarity. The examined polar compounds, including optical isomers, could be well separated on a C18 phase. However, the separation of regio- and geometrical isomers of carotenoids, as well as non-polar carotenes, is better achieved using a C30 phase. two HPLC methods, on C18 and C30 phases, were compared. These results were published in the form of a study review [6].

Some subtropical plants contain unusually high amounts of capsorubin. The complete and detailed carotenoid analysis of the fruits of Jipi-japa (*Carludovica palmata*) and the brown leaves of zamia (*Zamia dressleri*) was achieved using HPLC-DAD-MS technique, and co-chromatography with authentic samples. The main carotenoid capsorubin was isolated and characterized by UV-VIS, ^{1}H and ^{13}C NMR methods. A paper will be submitted soon including our findings [7].

2.2. Algal carotenoids

The freshwater cyanobacterium *Cylindrospermopsis raciborskii*, growing in the Fancsika lakes near Debrecen, was investigated for carotenoid composition. Besides β-carotene, echinenone and (9/9′*Z*)-echinenone a carotenoid glycoside was found to be the main component. This compound was isolated and subsequently acetylated for structural elucidation. The acetyl derivative was fully characterized by UV–vis, ECD, NMR and HRMS techniques. The detailed 1H and 13C NMR chemical shift assignment of the major carotenoid supported the unequivocal identification of (2′*S*)-2-hydroxymyxol-2′-α-L-fucoside [8].



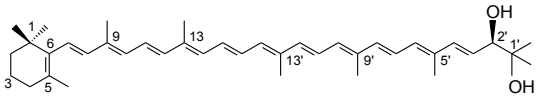
(2′*S*)-2-hydroxymyxol-2′-α-L-fucoside

A potentially new carotenoid glycoside was isolated from Nostoc algae as a minor pigment. Because of the low content of this carotenoid structure elucidation has not yet been completed. In cooperation with the Depatment of Botany in Debrecen more than 100 freeze-dried Nostoc samples will be evaluated in the near future to compare their carotenoid content with a special focus on the newly found glycoside.

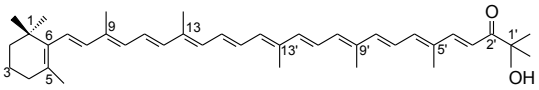
The effects of three non-steroidal anti-inflammatory drugs (NSAIDs: diclofenac, diflunisal and mefenamic acid) on growth, cyst formation and astaxanthin accumulation of the flagellated green alga *Haematococcus pluvialis* were investigated in the Department of Botany in Debrecen. Results of carotenoid (performed in our laboratory) and chlorophyll content analysis suggest more specific processes behind the observed phenomena than membrane damage [9].

2.3. Mushroom carotenoids

From the extract of the mushroom Scarlet elf cup (*Sarcoscypha coccinea*) (all-*E*,2R)-plectaniaxanthin, (all-E)-2'-dehydroplectaniaxanthin and a number of sterically unhindered (Z)-isomers of these carotenoids were isolated and partially characterized. The carotenoid composition of the Scarlet elf cup extract was determined by HPLC analysis. The $NaBH_4$-reduction of (all-*E*)-2'-dehydroplectaniaxanthin resulted in the racemic mixture of (*R*)- and (*S*)-plectaniaxanthin. The isolated (Z)-isomers were identified by their UV/Vis spectroscopic properties [10].



(all-*E*,2'*R*)-Plectaniaxanthin



(all-*E*)-2'-Dehydroplectaniaxanthin

3. Reactions of carotenoids with complex metal hydrides

Lutein oxidation was successfully reproduced with manganese dioxide to obtain oxolutein, which was crystallized and characterized. The reduction of 3'-oxo-lutein with different complex hydrides gave lutein and 3'-epilutein without the expected dihydro compounds. Reduction of capsanthin with DIBAH and L-Selectride produced 7',8'-dihydro-capsanthol epimers and 7',8'-dihydro-capsanthin, which were characterized by NMR methods. 3,3',6'-Triacetate and 3,3'-diacetate of capsanthol epimers were prepared for VCD (Vibration Circular Dichroism) investigations.

The reaction of 5,6-epoxy-carotenoids (violaxanthin, antheraxanthin) with L-Selectride and Super Hydride did not produce the expected compounds. The reaction of these carotenoids with DIBAH delivered a very complex mixture. The structure elucidation of the obtained compounds is in progress.

4. Carotenoid-biomolecule conjugates and their antioxidant activity

Our aim here was to continue our synthetic investigations on carotenoid-biomolecules conjugates with enhanced biological activity. As the azide-alkyne click reaction works for carotenoids pretty well, it was used for the synthesis of other bioconjugates.

4.1. Carotenoid-sugar conjugates

Carotenoid pentynoates were coupled to protected and deprotected monosaccharide azides via azide-alkyne click-reaction using bis-triphenylphosphano-copper(I)-butyrate ($C_3H_7COOCu(PPh_3)_2$) complex. Protected sugars delivered the conjugates with excellent yields, whereas with unprotected ones hydrophilic carotenoid sugar derivatives were obtained with good or moderate yields in one step [11].

4.2. Carotenoid-cyclodextrin conjugates

Carotenoid pentynoates were reacted with mono- and heptaazido-β-cyclodextrins and their acetylated derivatives under different conditions with various catalysts. Although reactions went with acceptable conversions, products could yet been isolated in pure form due to the amphipatic nature of the products. Nevertheless this project is worth pursuing in the future as the target molecules can be drug candidates (Figure 2.).

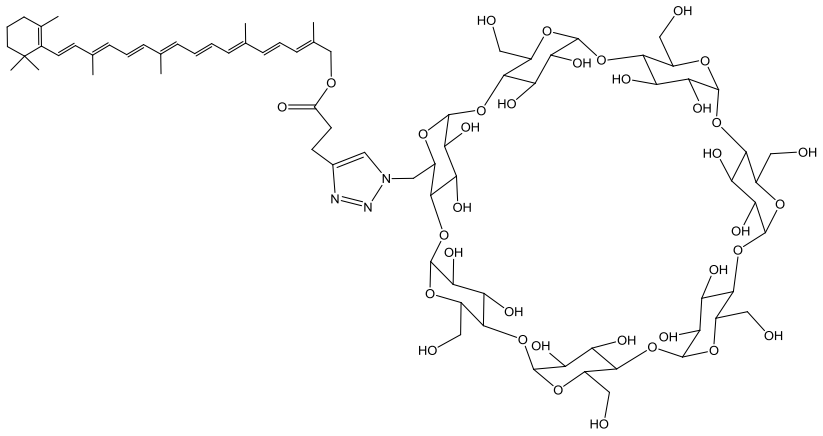
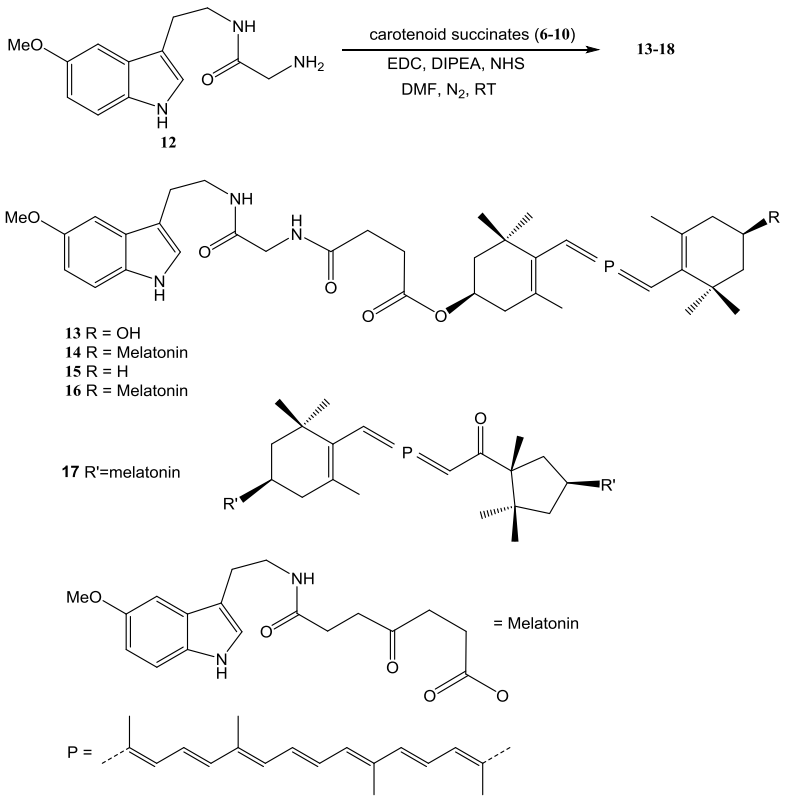


Figure 2. A carotenoid-CD conjugate

4.3. Carotenoid-melatonin conjugates

In a cooperation with the New York Eye Research Centre we were asked to synthetize zeaxanthin-melatonin conjugates for *in vivo* tests for macula degeneration. Unfortunately the conjugate could not be used in the assays due to poor water solubility.



entry	carotenol succinate	products (yields)
1	zeaxantin mono (**11**) and disuccinate (**7**)	**13** monomelatonin (73%) **14** dimelatonin (72%)
2	β-cryptoxanthin succinate (**8**)	**15** monomelatonin (51%)
3	capsanthin disuccinate (**9**)	**17** dimelatonin (47%)
4	lutein disuccinate (**10**)	**16** dimelatonin (40%)
5	8'-apocarotenol succinate (**6**)	**18** monomelatonin (42%)

Figure 3. Synthesis of melatonin conjugates

However, conjugation of carotenoids and melatonin could enchance the antioxidant activity, because the conjugate would counteract oxidative stress directly (carotenoid) and indirectly (melatonin). Hence, we decided to prepare such conjugates via the coupling of carotenoid succinates and the commercially available amino derivative of melatonin **12** (2-Amino-N-[2-(methoxy-1H-indol-3-yl]ethyl)-acetamide hydrochloride). These results together with antioxidant studies will be sent for publication this year (Figure 3.) [12].

4.4. Carotenoid-glycopeptide conjugates

Azide derivatives of glycopeptide antibiotic aglycons (teicoplanin, ristocetin) were attempted to be coupled with carotenoid pentynoates with click-reaction, because it was demonstrated, that if a lipophylic moiety (eg. n-decyl) is attached to these antibiotics their activity becomes higher and their spectrum wider. In our case the lipophylic (and also antioxidant) part would be a carotenoid derivative. Unfortunately, these reactions resulted in complex mixtures from which no products could be isolated.

Recently we found that the amino derivatives of said antibiotics can be successfully coupled to active esters of acidic carotenoids (bixin, norbixin, crocetin etc.) so similar conjugates can and will be prepared in the future in coorporation with the Department of Pharmeceutical Chemistry at the Uninversity of Debrecen.

4.5. Carotenoid-ascorbic acid conjugates

Our aim was to couple carotenoids, by click reaction, to one or two molecules of ascorbic acid in order to improve water solubility and antioxidant potency.

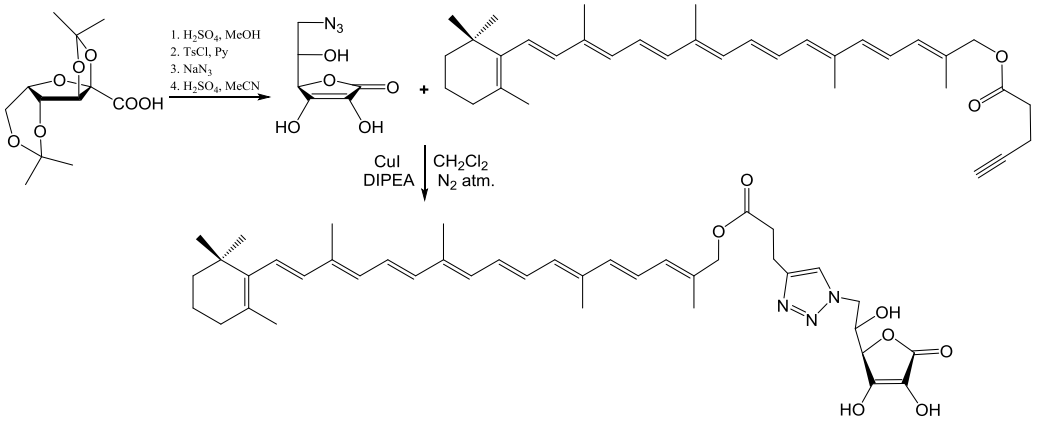


Figure 4. Preparation of 6-deoxy-6-azido ascorbic acid

The production of the 6-deoxy-6-L-azido ascorbic acid was achieved on gram scale, and its behaviour under the conditions of copper(I) catalyzed click reaction was studied. During several experiments with 3,5-dimethoxy-phenylacetylene as model compound we have finally found the appropriate conditions (CuI, DIPEA, CH_2Cl_2), and succeeded to couple azido ascorbic acid with 8'-apo-β-carotenol pentynoate (Figure 4.). The click reaction of the 6-deoxy-6-azido-L-ascorbic acid with other carotenoid pentynoates is under

investigation. These findings have been described in a diploma work (by Miki Hanaura) and a book chapter which we were invited to write [13].

4.6. Carotenoid-curcumin conjugates

Curcumin posesses a wide variety of interesting biological properties, among others antioxidant, anti-inflammatory, anti-cancer effects have been assigned to it. Coupling curcumin or fragments of it to carotenoids as another group of natural coumpounds showing excellent antioxidant effect might give rise to synergistic interactions in the antioxidant efficiency.

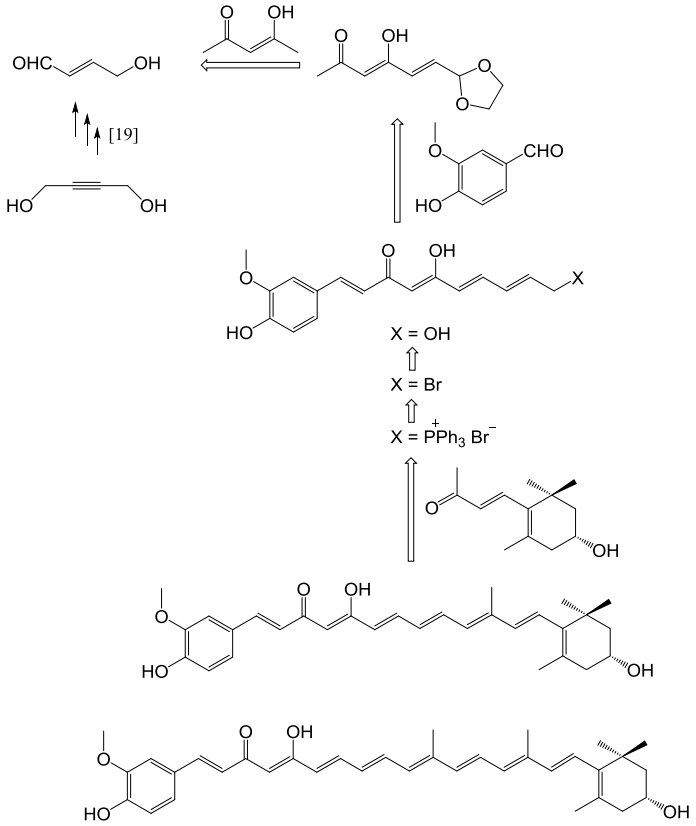
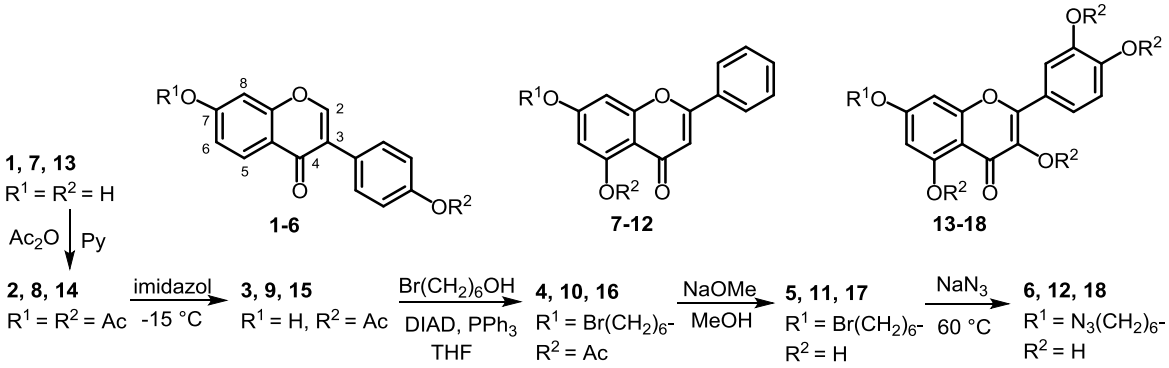


Figure 5. Synthetic plan for carotenoid-curcumin conjugates

Unfortunately the synthesis of curcumin hybrides according to the synthetic plan below could not be achieved due to unforeseen difficulties and so far only the „half hibride" was synthetized in low yields [14]. In the meantime the synthesis of curcumin esters of carotenoid succinates has been started.

4.7. Carotenoid-flavonoid conjugates

7-Azidohexyl ethers of daidzein (**1**), chrysin (**7**) and quercetin (**13**) were synthetised in five steps from the natural flavonoids. After selective deprotection of position 7 in peracetylated flavonoid, a bromohexyl moiety was coupled by Mitsonobu reaction, and after deacetylation, the product was substituted with azide. The resulting compounds were used in click-reaction (Fig.4.) with 8'-apo-β-carotenyl pentynoate, zeaxanthin-dipentynoate, and capsanthin dipentynoate. Bis-triphenylphosphano-copper(I)-butyrate complex ($C_3H_7COOCu(PPh_3)_2$) in dichloromethane was proven to be an efficient catalyst [13].



Preliminary studies for the Trolox-equivalent antioxidant capacity of zeaxanthin-daidzein conjugate against $ABTS^{\bullet+}$ radical were performed. Results showed that the conjugate is a ca. three times more efficient antioxidant that native daidzein.

As the prepared compounds have amphipatic character, their self-assembly in solution was also studied. Tetrahydrofuran solution of the conjugates were diluted with water and the solutions were examined by UV-vis and circular dichroism spectroscopy. The zeaxanthin conjugates gave chiral supramolecular structures, while capsanthin derivatives was not observed to be aggregated. The 8'-apocarotenoid conjugates also show some aggreg gation, however, it cannot be studied by circular dichroism spectroscopy. The atomic force microscopy investigation of the above conjugates on solid surface is in progress, as well as the light-scattering photometric study on the process of aggregation [15].

4.7. Antioxidant studies

The antioxidant properties of the newly synthetized derivativatives are being tested with the Trolox method and the FRAP method. The results will be published soon in the above mentioned articles.

References

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Q1 *if=3,154*

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