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Mendeleev Commun., 2021, **31**, 615–617

Mendeleev Communications

Synthesis of modified conformationally fixed tricarbocyanine dyes for conjugation with therapeutic agents

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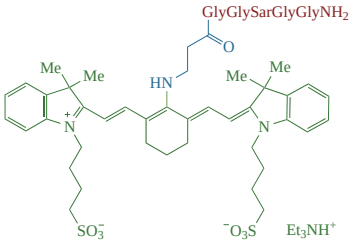
DOI: 10.1016/j.mencom.2021.09.008

New β-alanine modified tricarbocyanines containing various substituents at quaternized nitrogen atoms were obtained. The peptide synthesis method has been utilized for further conjugation thus giving promising tricarbocyanines containing two sulfonate groups and total negative charge of the fluorophore.



Keywords: fluorophores, conjugates, β-alanine, cell-penetrating peptides, tricarbocyanine dyes, fluorescent markers.

Dedicated to the memory of our head academician N. S. Zefirov

Recently, multifunctional structures with the fragments of different chemical origin have been investigated. The combination of individual moieties in one molecule allows several issues in theranostics to be resolved. Currently, the conjugates of fluorophores for biological purposes is actively developing.[1–5] Peptides of various amino acid content can serve as drug vectors that transport low molecular weight compounds across the membrane.[6,7] This property makes peptides prospective structural fragments in the conjugates. For example, the combination of a peptide with a fluorophore is expected to be promising for theranostics, particularly in oncology.

Cell penetrating peptides (CPP) have been developing since long as they penetrate into cells without the participation of membrane proteins.[8,9] Development of effective CPPs for selective drug internalization or imaging markers remains complicated.[10] One approach is the design of functional properties based on oligoarginines.[11] In the structural and functional studies of internalizable oligoarginines, these peptides are conjugated with hydrophobic[12] and hydrophilic fluorescent dyes.[13] However, when studying the internalization of fluorescent labels by peptides, it is important that the dye itself does not enter the cell. Consequently, testing the entry of dyes with different lipophilicities is topical. From this point of view, tricarbocyanine is a suitable scaffold for the dye design (*cf.* refs. 14–16). Structures with a conformationally fixed polymethine chain containing chlorine atom in the *meso*-position represent the most promising class due to their higher photostability and opportunities of modifications.[17,18]

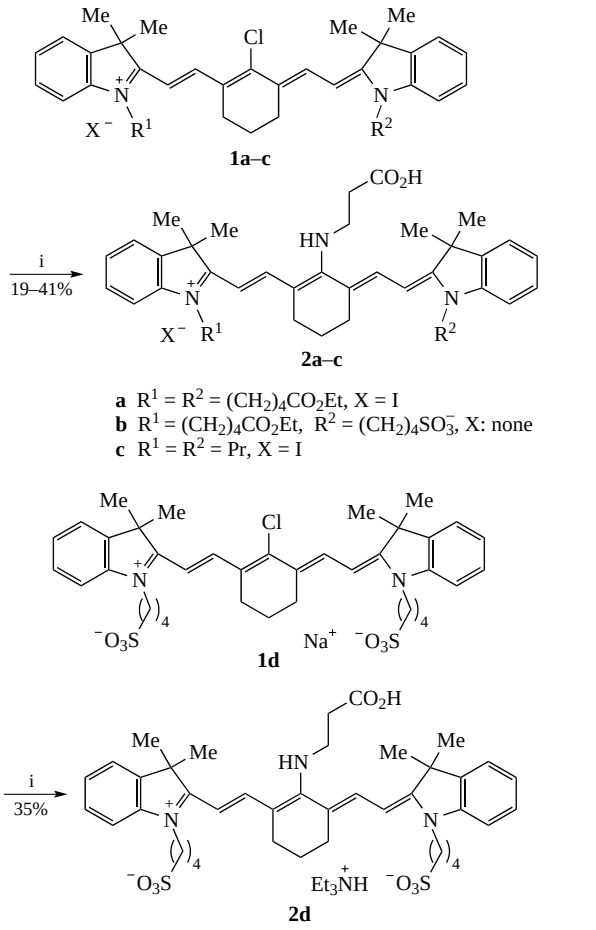
Binding of CPP directly to the fluorophore is not simple since the conditions for substitution in the *meso*-position are more stringent than those required for CPP stability.[19] ω-Amino acids,[20] *e.g.*, β-alanine, can act as linkers that ensure the binding of the fluorophore to CPP.

In this study, we synthesized a novel series of β-alanine modified tricarbocyanines **2a**–**c** and used the previously reported compound **2d**[21] to reveal the dependence of the intracellular entry on lipophilic/hydrophilic properties and the electric charge of the fluorophore (Scheme 1).

We tested the interaction of compounds **2a**–**d** with human colon cancer cell line the HCT116. The fluorescence intensity was determined in the dye-loaded cells before and after washing off the dye, and the medium. The lowest fluorescence intensity of cells after washing was in the case of **2d** bearing two negatively charged sulfonate groups. In contrast, the best cell permeable agent was **2c** containing alkyl substituents at quaternized N atoms (Table 1).

Before and after washing the dyes, cells were examined on a Zeiss Axio Observer Z1 fluorescence microscope (magnification 25×). We found that the dyes containing less than two sulfonate groups **2a**–**c** penetrated into the cells (Figure 1).

The cytotoxicity testing using HCT116 cells and non-malignant fibroblasts showed that **2a** and **2c** (no sulfonate groups) caused cell death (see Table 1). These observations further confirmed that the nature of the substituents affects the ability of tricarbocyanines to penetrate cells. Thus, tricarbocyanines containing hydrophobic substituents at the quaternized nitrogen atom and having a total positive charge cannot serve as markers for the studies of the entry of conjugates into the cells. Only fluorophore **2d** can be used for conjugation of carbocyanines with peptides. Based on compound **2d**, conjugate **3** with the non-functional peptide spacer $GlyGlySarGlyGlyNH_2$ was prepared (Scheme 2).



Scheme 1 *Reagents and conditions*: i, β-alanine, DMF, argon, 85 °C, 3–22 h.

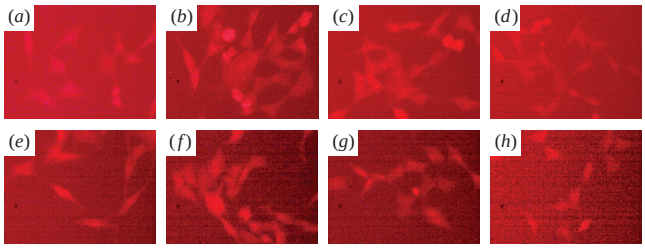
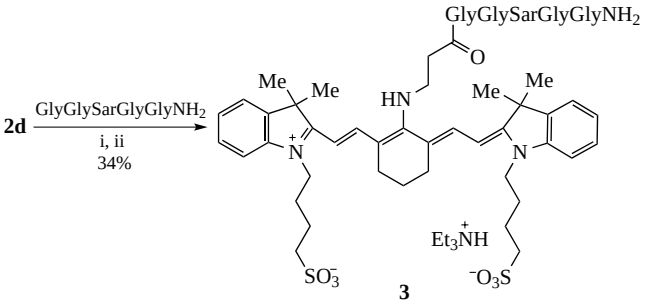


Figure 1 Fluorescent images of compounds **2a**–**d** at λ_{fl} = 630 nm: (*a*–*d*) before washing and (*e*–*h*) after washing (DMEM complete, containing 10% fetal bovine serum with glucose 4.5 g dm^{-3} with glutamine); (*a*, *e*) with **2a**, (*b*, *f*) with **2b**, (*c*, *g*) with **2c**, (*d*, *h*) with **2d**.

The fluorescence intensity studies with conjugates **3** and the original dye **2d** showed that an increase in the peptide length due to non-functional amino acids did not change the fluorophore–cell interaction (Figure 2).

In conclusion, the most promising fluorophores for conjugation with peptide vectors are tricarbocyanines containing at least two sulfonate groups and bearing a total negative charge



Scheme 2 *Reagents and conditions*: i, *N*-hydroxysuccinimide, DCC, DMF, 0–5 °C, 15 h; ii, GlyGlySarGlyGlyNH$_2$, Et$_3$N, room temperature, 5 days.

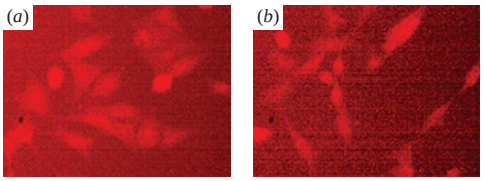


Figure 2 Fluorescent images of compound **3** at λ_{fl} = 630 nm: (*a*) before washing and (*b*) after washing (DMEM complete, containing 10% fetal bovine serum with glucose 4.5 g dm^{-3} with glutamine).

of the fluorophore. Penetration of spacer-linked dye-peptide conjugates is affected by the origin of substituents in the fluorophore, which makes it possible to consider conjugate **3** as a promising marker for assessment of the ability of the peptide as a cell permeability tool.

This work was supported by the State assignment no. 121021000105-7 and by the Russian Foundation for Basic Research (grant no. 19-53-26002).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2021.09.008.

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Table 1 Fluorescence intensity in HCT116 cells and cytotoxicity of compounds **2a**–**d**.[a]

Compound	λ_{fl}/nm	Fluorescence of cells before washing/×10^4	Fluorescence of cells after washing/×10^4	Fluorescence of the medium/×10^4	IC$_{50}$/μM HCT116	IC$_{50}$/μM fibroblasts
2a	750	56.7	6.8	101.0	2.3	4.6
2b	750	162.7	7.8	141.9	>50	>50
2c	760	128.9	10.3	101.0	2.7	3.4
2d	750	2.9	0.5	72.5	>50	>50

[a]The value represents mean ±SD of three independent experiments.

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Received: 30th April 2021; Com. 21/6551