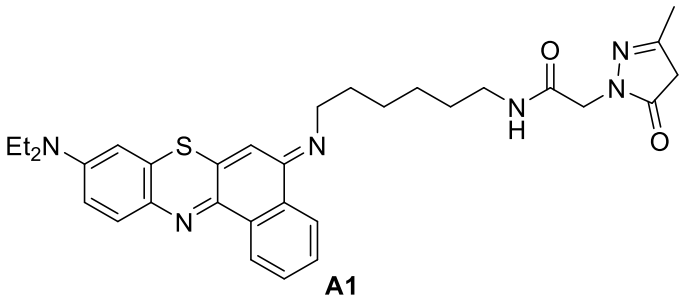
Problem Session

Nov. 2, 2024

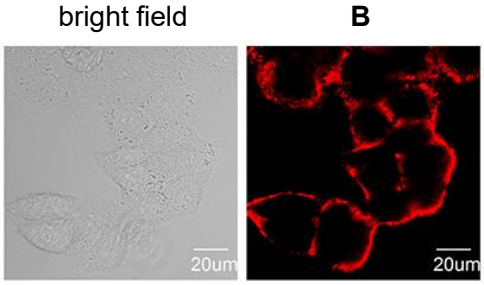
Hiroaki Itoh



A1

In the confocal fluorescent microscopy of cells incubated with compound **A1**, fluorescent image **B** was obtained.

conditions for fluorescent imaging: HepG2 cells (hepatoblastoma) were incubated with **A1** (0.2 μM) for 10 min. After washing the cells with phosphate buffered saline (PBS), the fluorescent image (excitation: 630 nm, emission: 650–750 nm) was obtained.



Based on the image **B** and the dataset **C** shown below, please propose a likely class of biomolecules that **A1** targets, and draw the most plausible interacting structure of **A1** with the target (or its substructure).

Dataset **C**

C1. Fluorescence tracking using cell fraction

procedure:

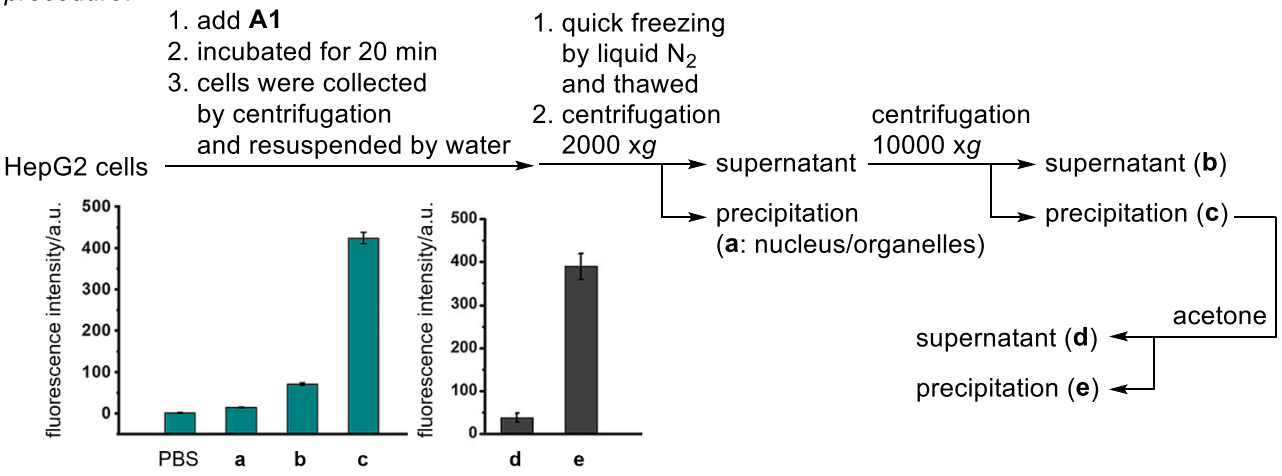
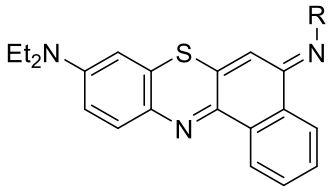
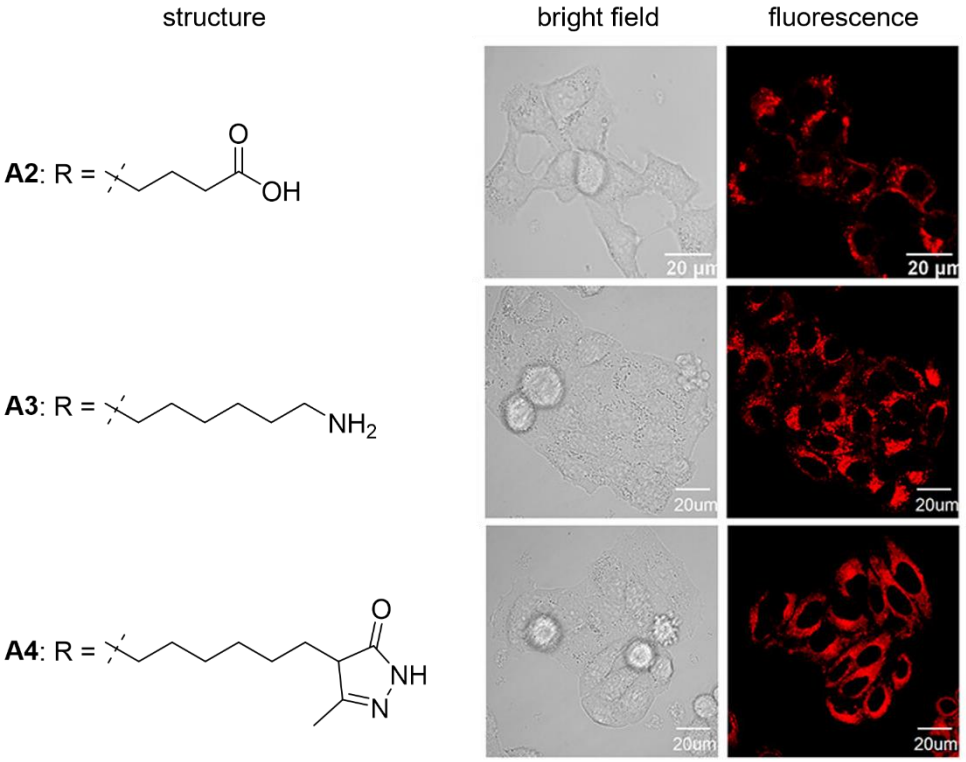


Figure 1. Fluorescence of cell fractions **a**–**e**. Each fraction was dissolved in the same volume of DMSO.

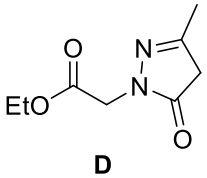
C2. Fluorescent images of the cells incubated with analogues of **A1** (**A2**, **A3**, **A4**)

*Conditions were same to the analysis using **A1**.





C3. Binding between compound **D** and proteinogenic amino acids



D

Table 1. ΔG in isothermal titration calorimetry (ITC) experiments with proteinogenic amino acids

amino acids	ΔG (Gibbs free energy change, kcal/mol)
Arg	-10.5
Lys	-2.9
His	-1.2
Asn	-0.1
Gln	-0.2
other proteinogenic amino acids	no detectable binding heat

Membrane protein labeling with noncovalent interaction

Question: Is there utility of membrane protein labeling through noncovalent interactions?

- Current major methods for cell membrane labeling
  (1) genetic engineering, (2) lipid bilayer insertion through hydrophobic interaction, (3) chemical modification of non-lipid membrane components (proteins, oligosaccharides) by covalent bond formation, this PS: specific example of chemical modification via noncovalent interactions with non-lipid components[1]

1. Fluorescent imaging using A1 and its analogue

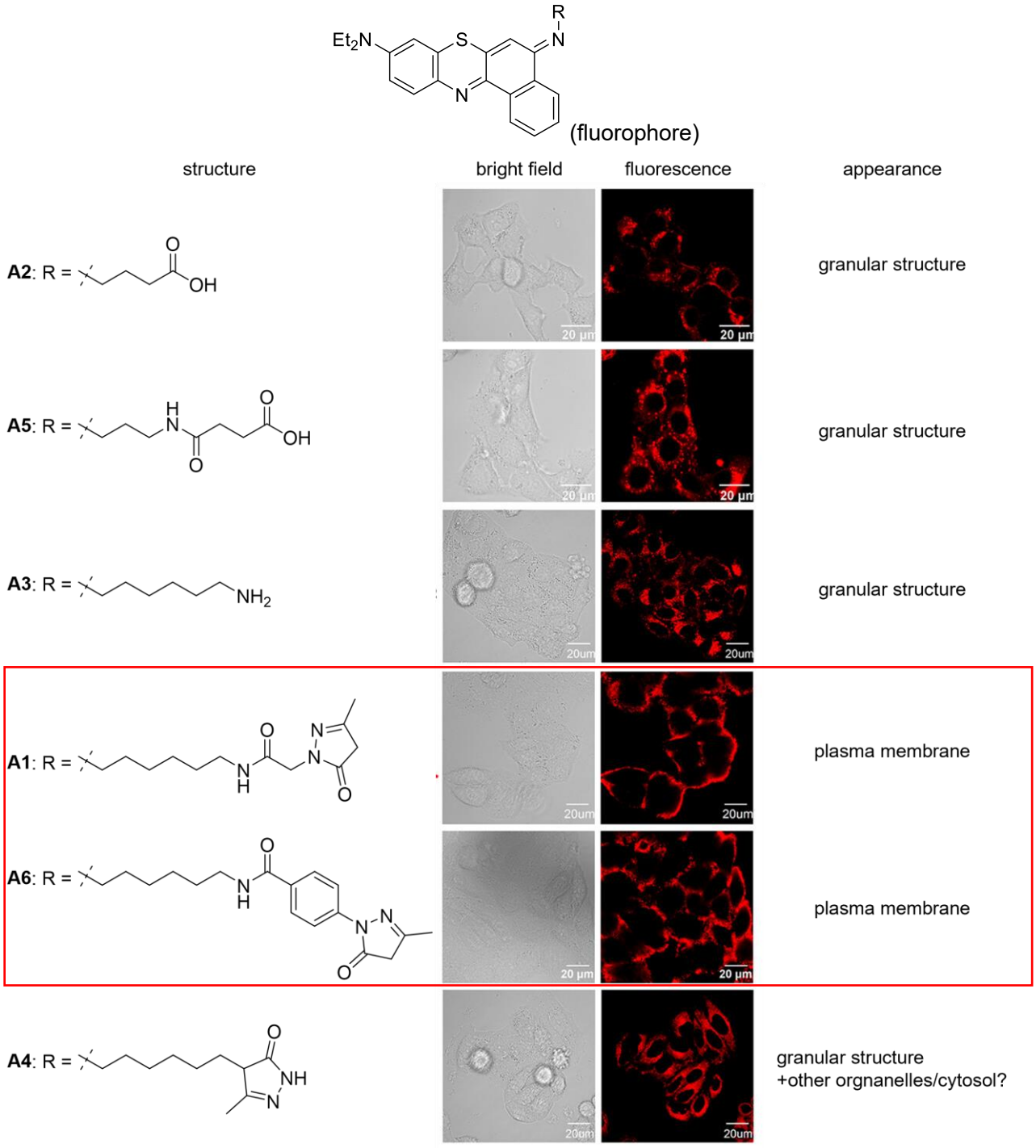
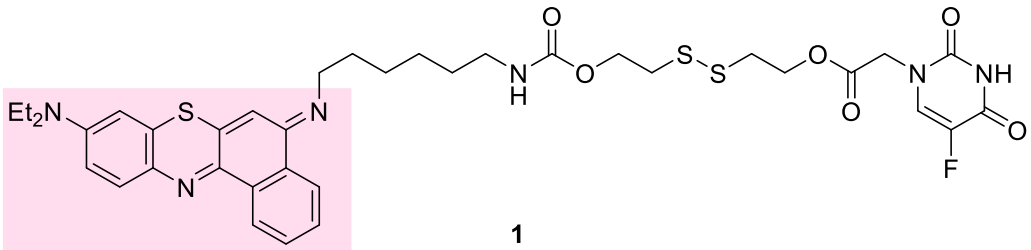


Figure 2. Fluorescent images of compounds **A1** and its analogues (excitation: 630 nm, emission: 650–750 nm). HepG2 cells (hepatoblastoma) were incubated with **A1** (0.2 μM) for 10 min. After washing the cells with phosphate buffered saline (PBS), the fluorescent image was obtained.

- The localization preference of the fluorophore (sulfur-substituted Nile blue) is likely irrelevant to the localization of **A1**. Colocalization of **1** with LysoTracker Green: *R* (Pearson's correlation coefficient) = 0.8[2]



sulfur-substituted Nile blue

1

- The observed plasma membrane localization does not depend on the fluorophore.

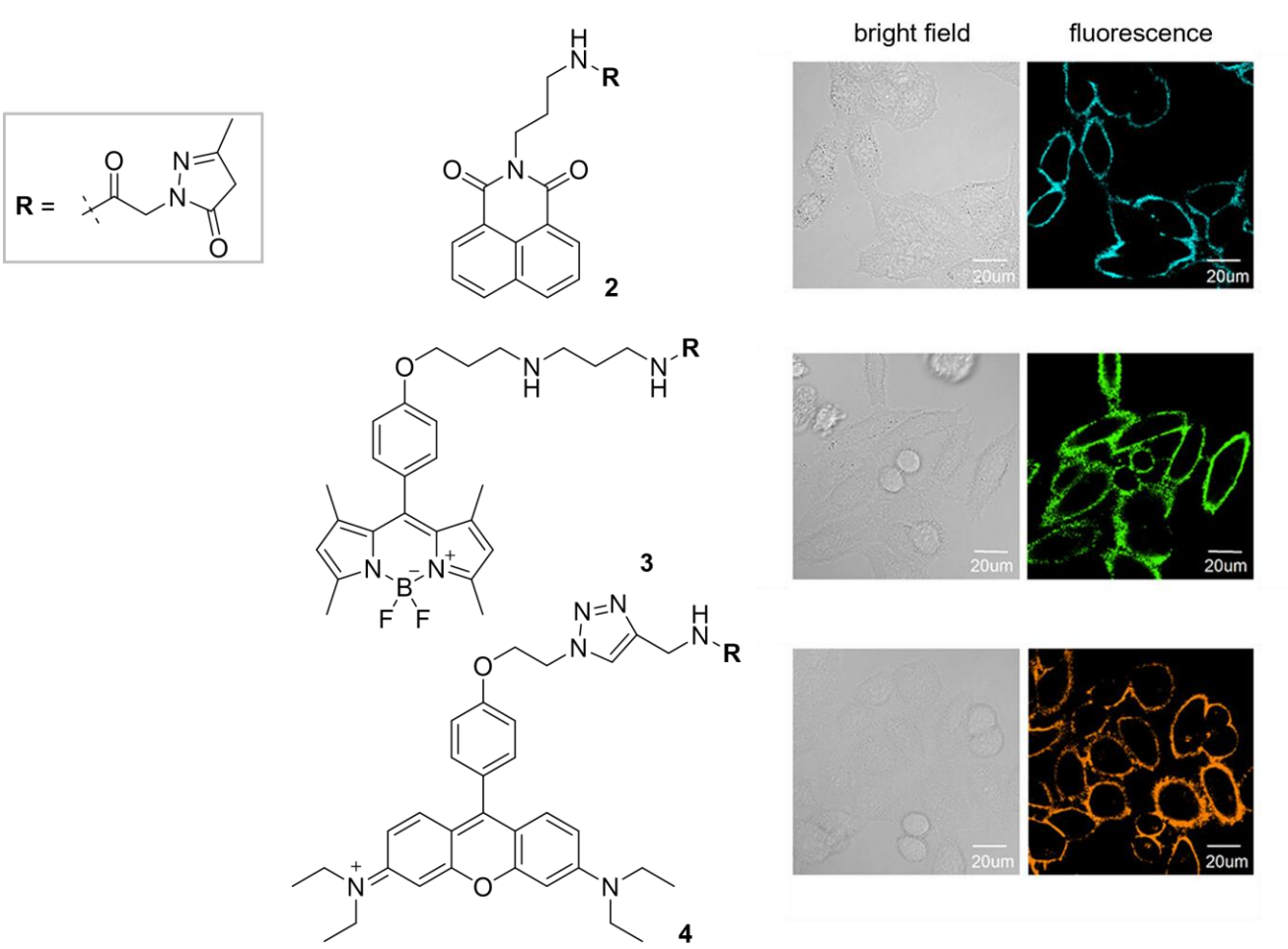


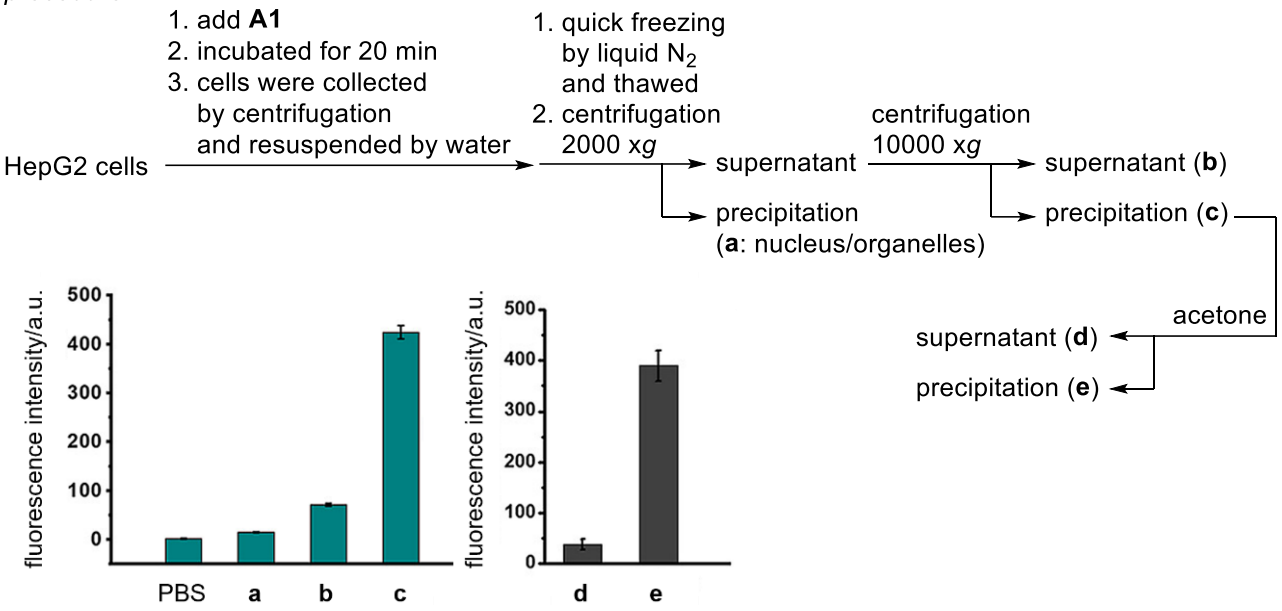
Figure 3. Fluorescent images of compounds **2** (excitation: 330 nm, emission: 380 nm), **3** (excitation: 496 nm, emission: 506 nm), and **4** (excitation: 554 nm, emission: 578 nm).

- Based on the above observations, membrane localization of **A1** is likely derived from a pyrazolone moiety.

2. Fluorescence tracking using cell fraction/ITC experiments

3. To determine the type of membrane components recognized by **A1**, fluorescence from the cell fractions was analyzed.

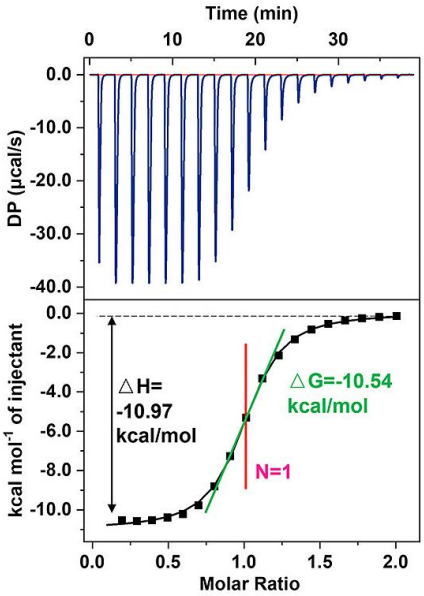
procedure:

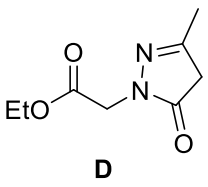


b: cytosol, **c**: membrane components, **d**: **lipid** (soluble in acetone), **e**: **membrane proteins and oligosaccharides** (c.f. acetone precipitation of proteins)

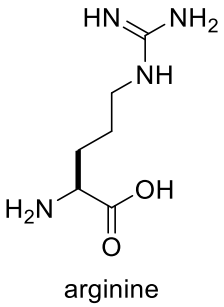
Based on the above fluorescence tracking analysis, **A1** likely recognizes non-lipid membrane components.

- The results of ITC experiments (dataset C3) indicated that **A1** likely recognizes **Arg residues of the membrane proteins** (there is only weak interaction with other basic amino acids).





D



arginine

Figure 4. Isothermal titration by Arg (30 mM) to compound **D** (3 mM) in buffer at 25 °C. Binding parameters of **A1**: Δ*H*: -10.97 kcal/mol, Δ*S*: -17.1 cal/mol/deg, Δ*G*: -10.54 kcal/mol, *N* = 1.

- The widely used plasma membrane labeling probe interacts with lipid components through hydrophobic interaction [e.g. DiO (3,3-dioctadecyloxacarbocyanine)]. Compound **A1** exhibited long retention in plasma membrane (24 h) while DiO was internalized within 2 h.

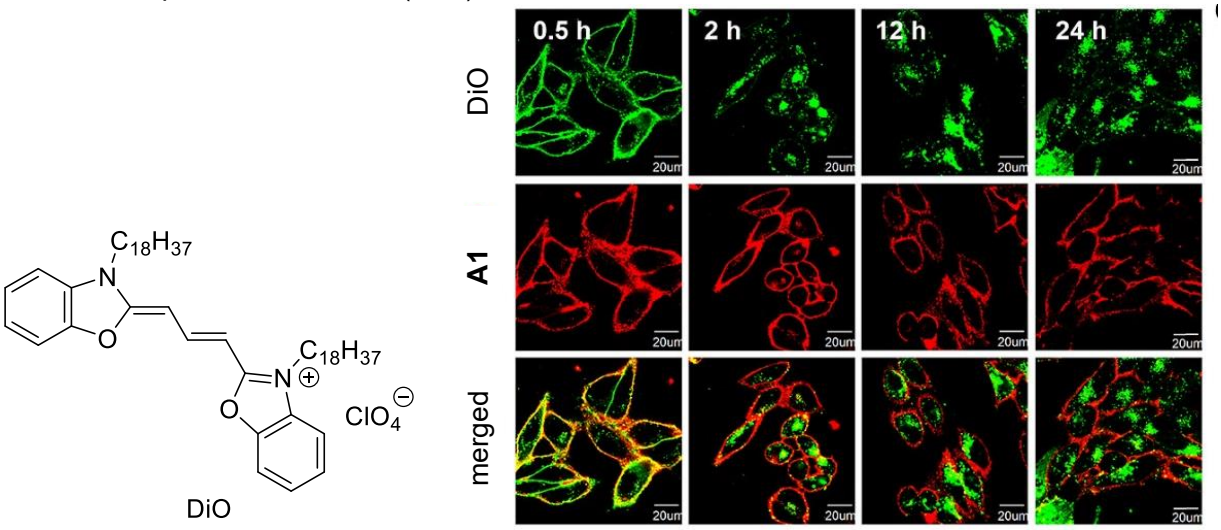


Figure 5. Structure of DiO (5 μM, excitation: 488 nm, emission: 500−540 nm) and colocalization with **A1** (0.2 μM, excitation: 630 nm, emission: 650−750 nm) in HepG2 cells.

4. Consideration of tautomerism and ionization of A1

- Tautomerism of the 1-substituted-5-pyrazolone (like **A1**) is known to depend on the polarity of media.[3] In non-polar solvents, compound **5** was reported to exist as $\mathbf{5_{CH}}$. In aqueous solvents, **5** was reported to exist in the form $\mathbf{5_{NH}}$ (large dipole moment) with 10% of $\mathbf{5_{OH}}$ (aromatization).
- 1(*H*)-5-Pyrazolone (like **A4**) was reported to predominantly exist as an enol form $\mathbf{6_{OH}}$, not as $\mathbf{6_{CH}}$.[4] In contrast to the 1-substituted-5-pyrazolone, the 1(*H*)-5-pyrazolone should be highly polar, and is known to have lack of solubility in non-polar solvents.

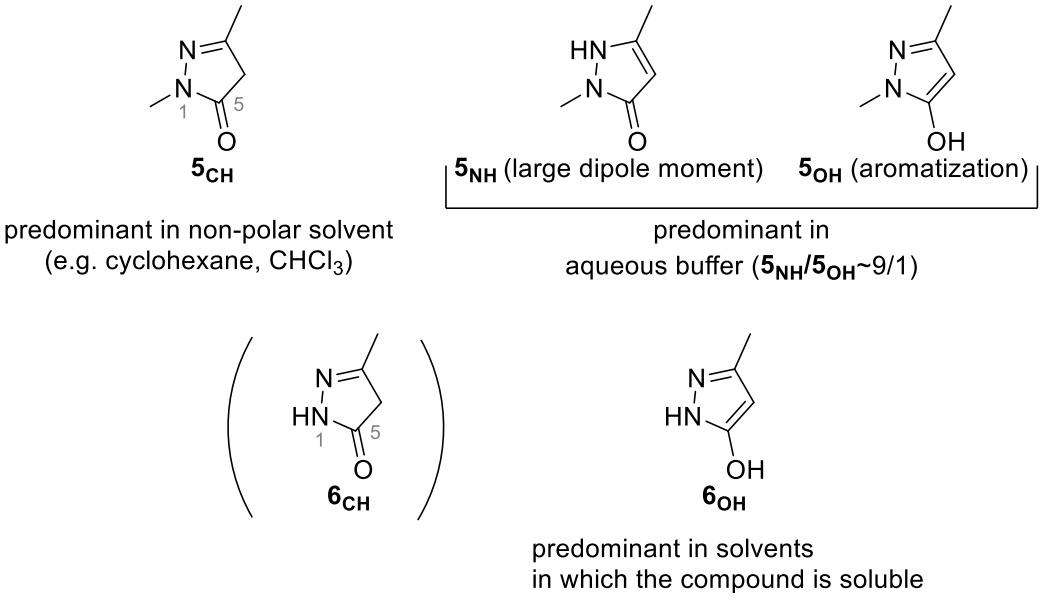
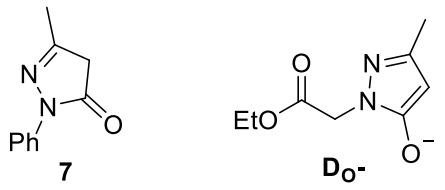


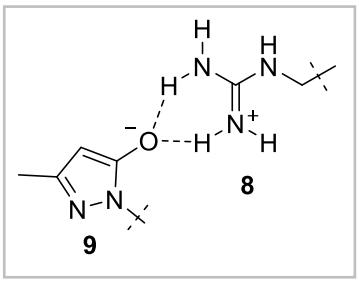
Figure 6. Tautomerism of 1-substituted-5-pyrazolone and 1(*H*)-5-pyrazolone.

- Neutralization titration indicated pK_a of compound **D** as 7.2 [c.f. pK_a of **7** (edaravone): 7.0[5]] Under

the physiological conditions, enolate form of $\mathbf{D_O}^-$ can exist due to the electronegativity of the oxygen atom and aromatization.



- Overall, interaction between guanidium cation **8** with anionic pyrazolone **9** can be considered. Considering the known anion-guanidium complex (see below), strong stabilization through bidentate hydrogen bonding can be proposed (note: In the original report, different interacting structure is proposed by calculation but diffuse functions are not considered).
- Compared to the carboxylates and the 1(*H*)-5-pyrazolones (e.g. compound **A4**), existence of the less polar CH form of 1-substituted-5-pyrazolones (which is soluble in non-polar solvents) can contribute to the preference to hydrophobic environment around the membrane proteins. The angle between the anionic oxygen and the N-alkyl chain could be also responsible for the properties of **A1**.



- Examples of bidentate hydrogen bonding interaction between anionic species and guanidium are shown below.

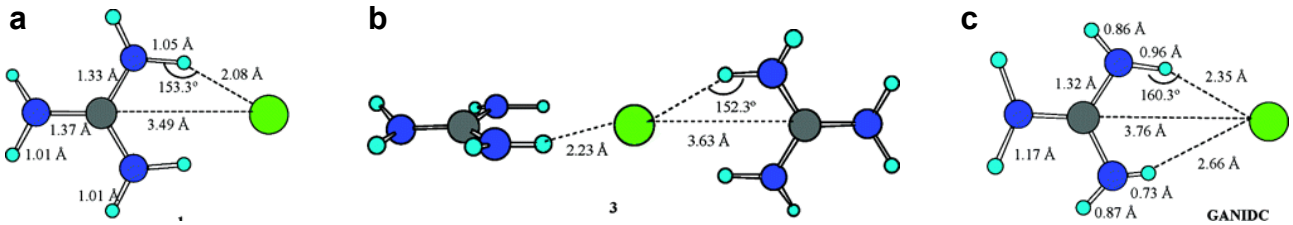


Figure 7. MP2/6-31+G* optimized structure of 1:1 (a) and 2:1 (b) guanidium-chloride complex and crystal structure (c) of guanidium chloride.[6]

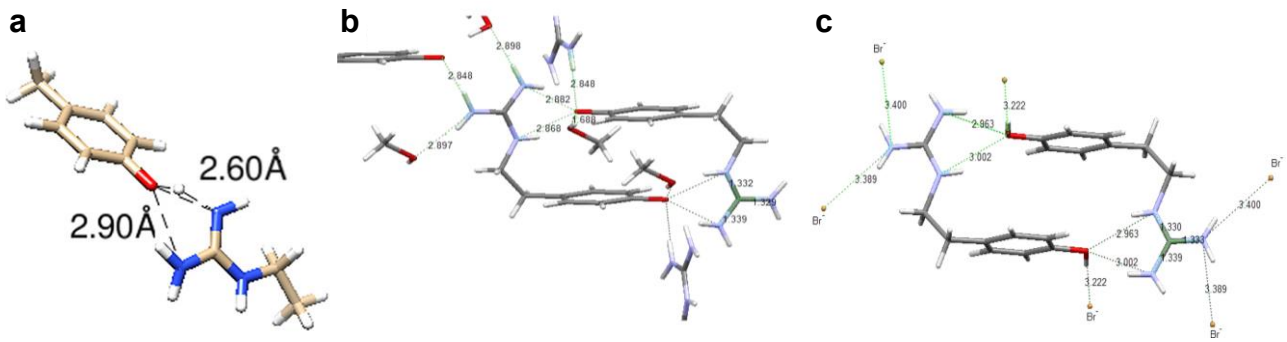


Figure 8. (a) Energy optimized structure of 1:1 complex between *p*-cresol and ethylguanidine (B3LYP/6-31++G**). The binding energy was calculated to be -17 kcal/mol.[7] (b) Crystal structure of (*p*-phenolyl)ethylguanidine. (c) Crystal structure of (*p*-phenolyl)ethylguanidium bromide.

5. The role of guanidium ion in anion recognition in biological context

- While interaction of guanidium ion with anionic species are widely utilized in catalyst design and supramolecular chemistry, here shows the importance of bidentate hydrogen bonding interaction in biological context, especially in the interaction with phosphates and carboxylates.

- Membrane permeability of polyarginine (based on HIV tat protein residues 49-57: RKKRRQRRR) is considered to be derived from the bidentate interaction with lipid polar head groups of phospholipids.[8] The high permeability is induced by guanidines, and not by primary amines (Figure 10). In the case of polyarginine, cancellation of repulsion between cationic sidechains through charge removal by anion binding is also driving force for the complexation.[9]

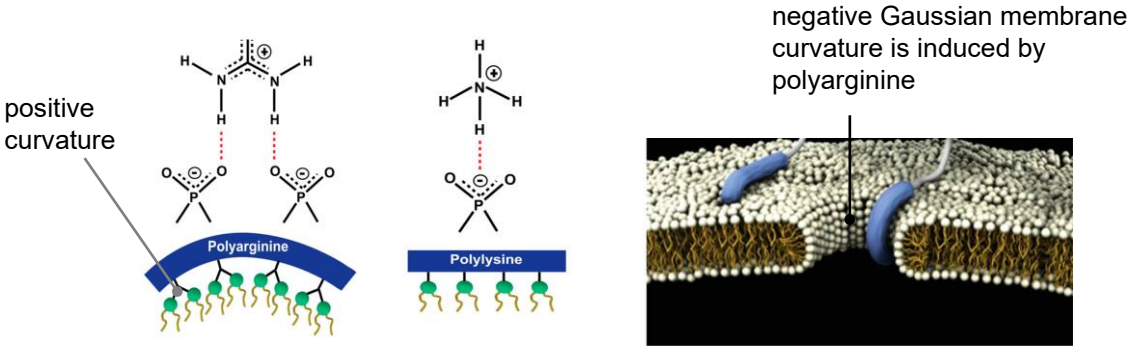


Figure 9. Proposed mechanisms of polyarginine-induced membrane permeabilization.[8]

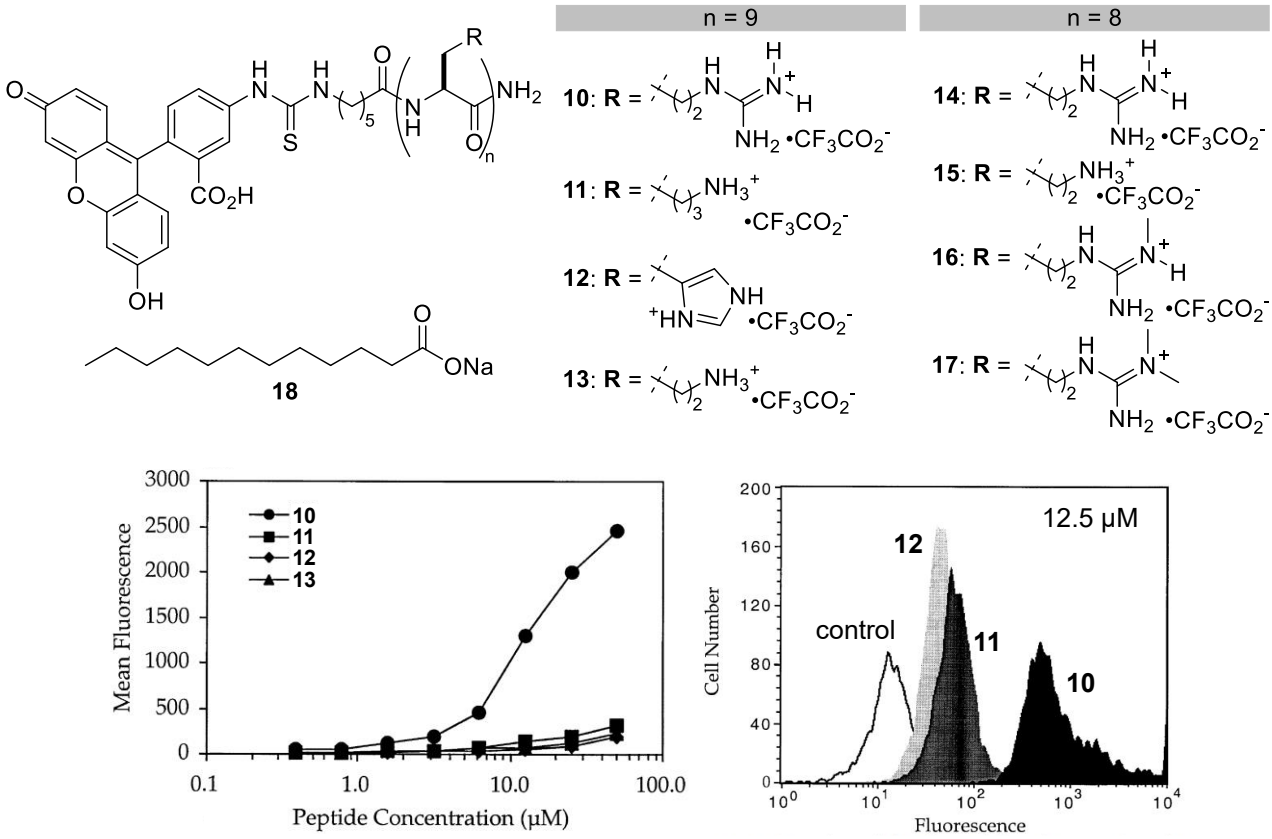


Figure 10. Flow cytometry-based analysis of the relative membrane permeability of peptides **10**-**13** using Jurkat cells (immortalized T lymphocyte).[10]

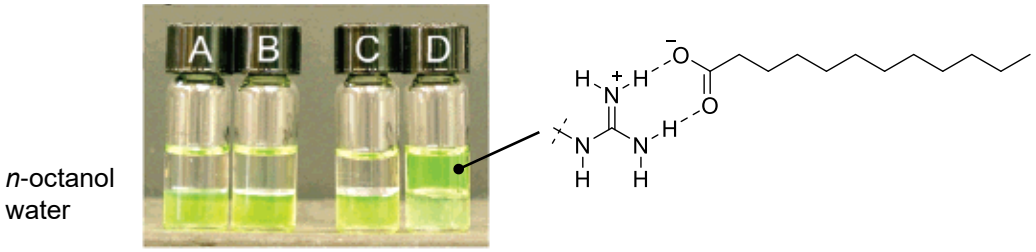


Figure 11. *n*-Octanol/water partition experiments. A: **15** (44 μM), B: **14** (44 μM), C: **15** (44 μM) + **18** (18 eq), D: **14** (44 μM) +**18** (18 eq).[11]

- Indication of importance of bidentate hydrogen bonding for carboxylate/phosphate recognition using methylated guanidines

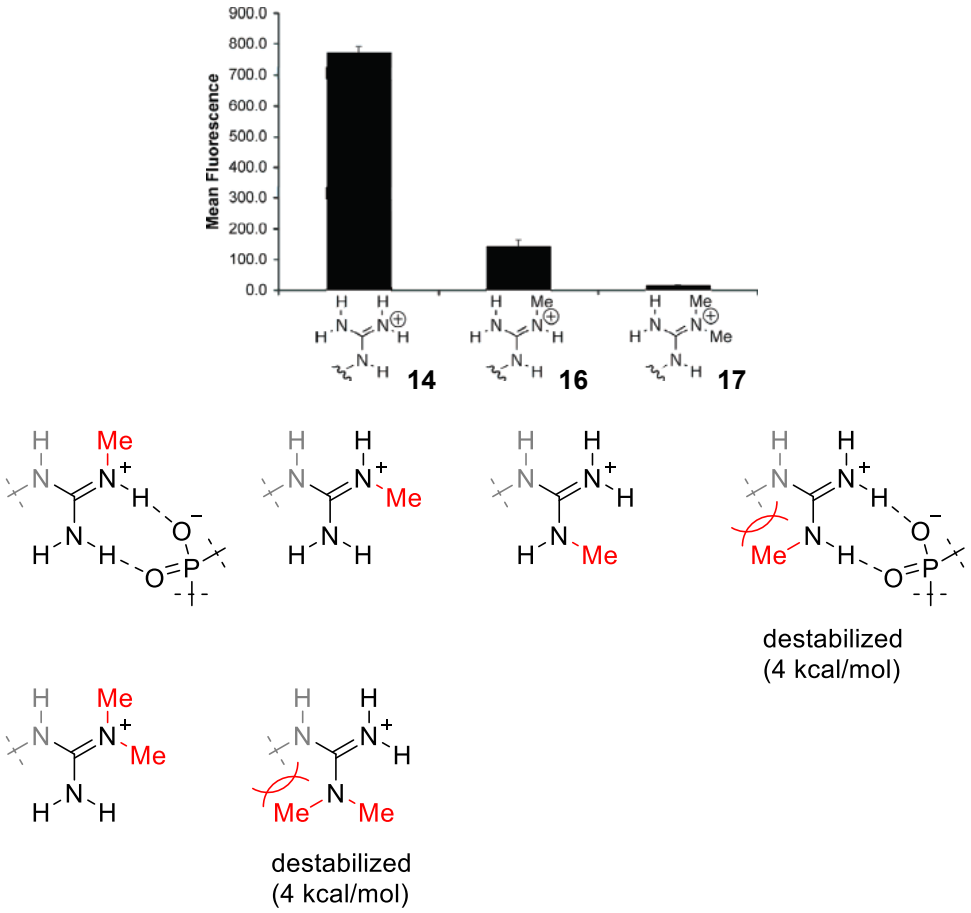


Figure 12. Uptake of **14**, **16**, and **17** (5 min, 50 mM each) by Jurkat cells.[11,12]

6. What was realized in the cell membrane labeling using 1-substitued-5-pyrazolone?

- Fast modification and long cell membrane retention in vitro (96 h, Figure 13a) and in vivo (>21 d) were reported. According to the results of calcein-AM/propidium iodide staining and MTT assay (up to 10 μM), detectable toxicity was not observed. Cell recognition was also demonstrated using biotin-conjugate **18** (Figure 13b).

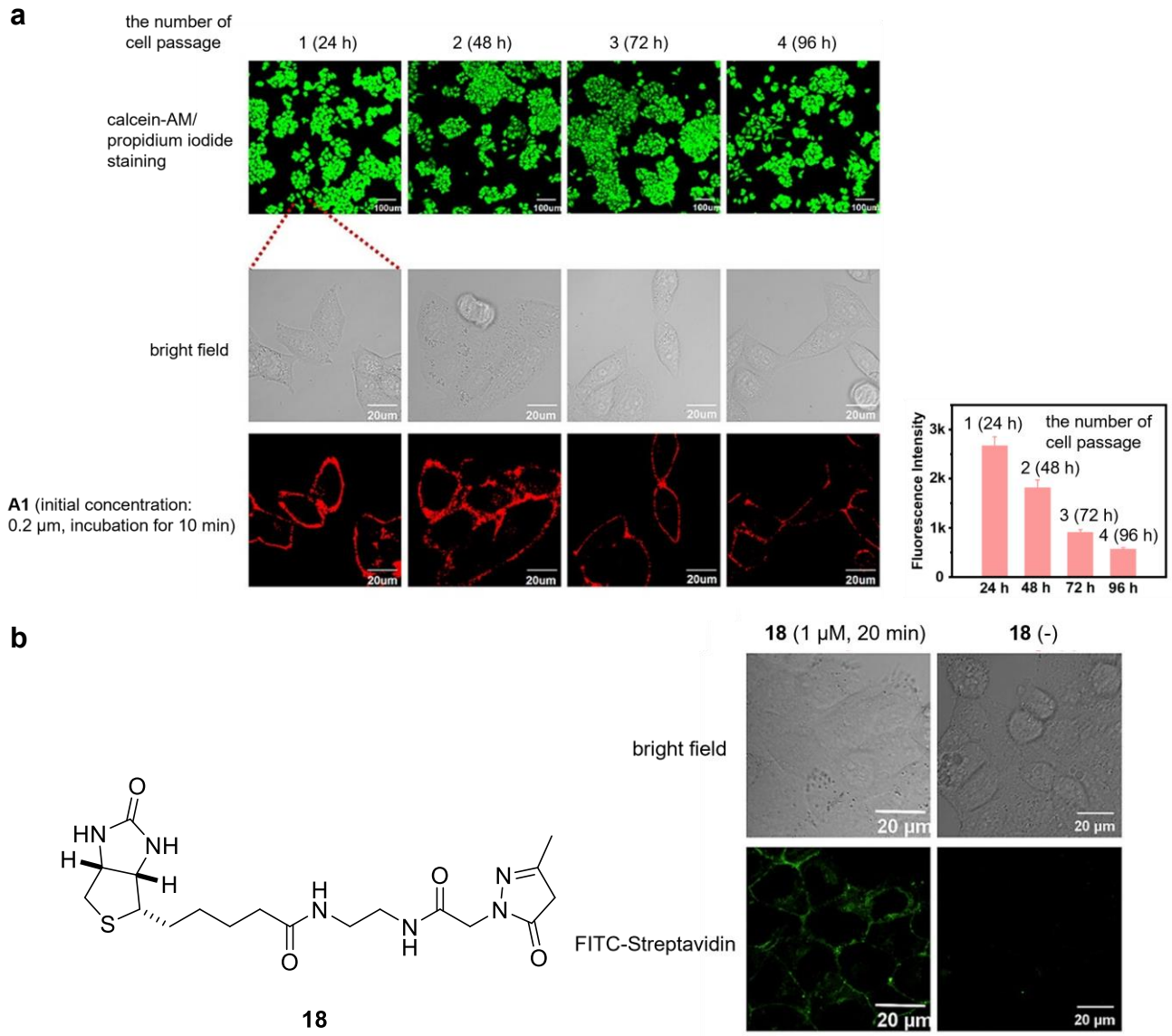


Figure 13. (a) Cell membrane retention of fluorescence of **A1** during cell passage. (b) FITC-Avidin-labeling of A431 cells (epidermoid carcinoma) using biotinylated pyrazolone **18**.

References

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