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Fluorescent Probes

Rational Design of a Solvatochromic Fluorescent Uracil Analogue with a Dual-Band Ratiometric Response Based on 3-Hydroxychromone

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Dedicated to Professor J.-F. Biellmann

Abstract: Fluorescent nucleoside analogues with strong and informative responses to their local environment are in urgent need for DNA research. In this work, the design, synthesis and investigation of a new solvatochromic ratiometric fluorophore compiled from 3-hydroxychromones (3HCs) and uracil fragments are reported. 3HC dyes are a class of multiparametric, environment-sensitive fluorophores providing a ratiometric response due to the presence of two well-resolved bands in their emission spectra. The synthesized conjugate demonstrates not only the preservation but also the improvement of these properties. The absorption and fluorescence spectra are shifted to longer wavelengths together with an increase of brightness. Moreover, the two fluorescence bands are better resolved and provide ratiometric responses across a broader range of solvent polarities. To understand the photophysical properties of this new fluorophore, a series of model compounds were synthesized and comparatively investigated. The obtained data indicate that uracil and 3HC fragments of this derivative are coupled into an electronic conjugated system, which on excitation attains strong charge-transfer character. The developed fluorophore is a prospective label for nucleic acids.

Introduction

Fluorescent analogues of non-emissive natural nucleosides found broad applications in studying structure, dynamic properties and interactions of nucleic acids.[1] Artificial fluorescent nucleosides can be site-selectively incorporated into nucleic acid strands by an enzymatic polymerization reaction or by chemical synthesis. As a result, a well-defined location and geometry of the fluorophore within the nucleic acid structure can be achieved together with the ability to follow its functional transformations.[2] This opens many possibilities that cannot be realized with other types of covalent fluorescent labels. Advanced fluorescent nucleosides allow for the sensing of nucleic acid hybridization and conformational changes,[3] typing of single-nucleotide polymorphisms (SNPs),[4] and sensing the activity of DNA-binding proteins and enzymes.[5,6] These results stimulate new efforts to synthesize advanced emissive nucleosides based on new mechanisms of response. Fluorescence offers a limited number of parameters for quantitative detection. Typically, the events occurring in the microenvironment of fluorophores may be monitored by changes in: 1) fluorescence intensity; 2) fluorescence anisotropy; 3) excited-state lifetime; and 4) position of the emission band maximum (emission color).[7] Analytical assays based on fluorescent nucleosides commonly use one of these modalities. A classic example is 2-aminopurine (2AP), a constitutional isomer of adenine, changing emission intensity based on its local environment. 2AP fluoresces brightly in water ($\Phi = 0.68$), whereas stacking interactions with neighboring nucleobases within the nucleic acid strands strongly quenches 2AP fluorescence.[8] As a consequence, quantitative determination of fluorescence in-

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tensity may be problematic because it depends on the concentration and is sensitive to unaccountable quenching effects. Application of color-changing nucleosides can only partially resolve this problem; typically they are molecules that possess a highly dipolar excited state and exhibit polarity-dependent shifts in their emission together with the intensity changes, which can be rather strong. Both parameters can monitor changes occurring in their microenvironment.[9] Meanwhile, sensing based on fluorescent intensity or spectral shifts suffers from several limitations, related mainly to the problems of the calibration of the fluorescence response, which is commonly displayed in relative units. To overcome these limitations, an approach based on the λ-ratiometric sensing can be used.[10] Based on recording of the fluorescence signal as the ratio of intensities at two or more wavelengths, this sensing has undergone rapid development during recent years. Such simple two-channel probing makes possible to record an intrinsically calibrated analytical signal that does not depend on local probe concentration or instrumentation settings. Analytical assays for nucleic acids based on λ-ratiometry are known. The vast majority of them exploit double-dye systems, such as fluorescence resonance energy transfer (FRET) pairs and formation of excimers.[11] Nevertheless, this method requires double labeling to obtain informative signals. An attractive alternative using a single dye is still poorly explored because of the very limited number of dyes that can display this property and of the difficulties in their synthesis.[10,12] In this context, the focus of our research is the synthesis of fluorescent nucleosides containing single ratiometric emitters. With this aim, we concentrated our efforts on fluorescent nucleosides incorporating 3-hydroxychromone fluorophores as single-dye ratiometric reporters.

3-Hydroxychromones (3HCs) are a prospective class of fluorescent dyes offering exceptional solvatochromism together with dual-band fluorescence suitable for ratiometric detection. The two emission bands originate from a reaction occurring in the excited state: the excited-state intramolecular proton transfer (ESIPT, Figure 1).[13] Upon excitation, 3HC molecules attain the normal excited form (N*), which then undergoes an ESIPT reaction to produce the tautomer (T*) excited form. Both states are highly emissive, the T* band being typically 80–100 nm redshifted compared with the N* band (Figure 1).

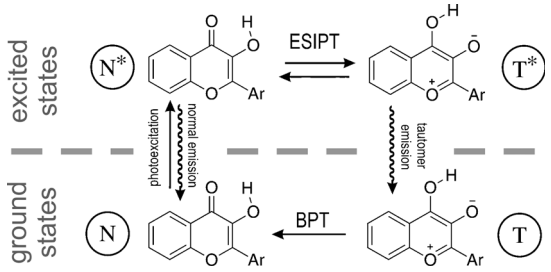


Figure 1. Origin of the dual emission of 3-hydroxychromones (ESIPT = excited state intramolecular proton transfer; BPT = ground state back-proton transfer; N and T denote the normal and tautomer forms, respectively).

The dual emission of 3HCs is highly sensitive to the polarity of the surrounding medium.[14] In particular, the ratio of the two emission bands changes with respect to the dielectric constant and H-bond donor ability of the environment.[15] High values of dielectric constant and strong H-bond acidity of the solvent stabilize the N* state and inhibit the ESIPT reaction, thus increasing the N*/T* intensity ratio. Therefore, the ratio of the two emission bands provides information about the microenvironment of the dye. In addition to the N*/T* ratio, information may be extracted from the relative positions of the two bands, so that 3HCs are multiparametric fluorescent probes.[15] Over the past two decades, 3HCs have found applications in sensing polarity of normal[16] and supercritical liquids,[17] probing lipid membranes[18] and proteins.[19] However, applications of 3HC-based probes to nucleic acid sensing remain meager.

The work presented herein expands our earlier efforts in the use of 3HC for nucleic acid probing. We showed that the H-bond-sensitive fluorophore 2-furyl-3-hydroxychromone conjugated to a polyamine chain can distinguish single- from double-stranded DNA through its two-band ratiometric response.[20] Subsequently, we synthesized a fluorescent nucleoside, which included the environment-sensitive 2-thienyl-3-hydroxychromone as a nucleobase mimic.[21] Incorporation of the nucleoside into oligonucleotides induced only a small destabilization of the duplex structure. Most importantly, the 3HC nucleoside showed significant changes in its ratiometric response upon binding to a complementary strand or as the result of the conformational changes caused by the chaperone viral nu-

Abstract in Ukrainian:

Флуоресцентні аналоги нуклеозидів, що володіють високою чутливістю до навколишнього оточення, є важливими для досліджень ДНК. В цій роботі ми представляємо дизайн, синтез та вивчення нового сольватохромного ратіометричного флуорофору, що складається з фрагментів 3-гідроксихромону (3ГХ) та урацилу. 3-Гідроксихромони є класом мультипараметричних чутливих до оточення флуорофорів, що дають ратіометричний сигнал через наявність двох добре розділених смуг в спектрах емісії. Сполуки, описані в цій роботі, показують не тільки збереження, а й покращення ратіометричних властивостей. Спектри поглинання та флуоресценції зміщені в червону область, яскравість флуоресценції збільшилась. Більш того, дві смуги флуоресценції виявились краще розділеними, а також дають ратіометричну відповідь у більш широкому інтервалі полярностей середовища. Для аналізу фотофізичних властивостей нового флуорофору, ми порівняли його з спеціально синтезованим рядом модельних сполук. Отримані дані свідчать про те, що фрагменти урацилу та 3ГХ у флуорофорі утворюють єдину спряжену електронну систему, що зазнає переносу заряду у збудженому стані. Розроблений нами флуорофор є перспективним для дослідження нуклеїнових кислот.

cleocapsid protein.[22] According to these data, 3HCs are prospective fluorophores for probing nucleic acids and their intermolecular interactions.

In this work, we developed a new 3HC-based ratiometric fluorophore with improved properties for the purpose of nucleic acid probing. We designed and synthesized a fluorophore composed of 3HC and uracil aromatic fragments assembled into a conjugated electronic system through an electron-conducting acetylenic linker and investigated its solvatochromic properties. The fluorophore showed improved fluorescence properties, including redshifted absorption and emission, higher brightness, and a new sensitivity profile to H-bond interactions. The dye constitutes a promising building block for incorporation into nucleic acids.

Results and Discussion

Design of the fluorophore

Design of new fluorophores with predefined photophysical characteristics is a great challenge. To reach this aim, the approach based on modification of known fluorescent cores appears particularly fruitful.[23] For designing a new fluorophore based on the 3-hydroxychromone core for nucleic acid labeling, several requirements need to be met: 1) the target molecule should contain a 3HC moiety with an unprotected hydroxyl group to use ESIPT as the principal sensing mechanism; 2) the fluorophore should be connected with one of the natural nucleobases to allow its incorporation into DNA as a corresponding nucleoside; 3) the 3HC and nucleobase parts should be connected through a short rigid linker to ensure a defined localization of the fluorophore with respect to the nucleic acid main axis. Based on these requirements, the following chemical structure of the fluorophore **1** (named **TCeU**) was proposed.

Fluorophore **1** is composed of two fragments, namely 2-thienyl-3-hydroxychromone, which was previously shown to be a good ratiometric reporter sensitive to H bonding[21] and uracil, as the simplest member of the genetic alphabet. 1,3-Dimethyl uracil was used to exclude any prototropic tautomerisation in the nucleobase part, which could trigger side reactions such as 5-*endo* cyclization during fragment assembly. An acetylenic linker was introduced between the 5′ position of the thienyl ring and the 5 position of uracil. Such a modification of uracil is commonly used in the synthesis of fluorescent nucleosides with new photophysical characteristics.[1b,9] Moreover, the selected configuration of the nucleobase and chromone moieties is expected to allow an intramolecular charge-transfer (ICT) between 4-carbonyl of chromone and N-1 atom of uracil (Figure 2). ICT was shown to play an important role in modulating the environment-sensing properties of 3HC dyes.[14a,24] Furthermore, it has been shown for 2-thienyl-3-hydroxychromones that their 5′-position is highly sensitive to electron-donating groups.[25]

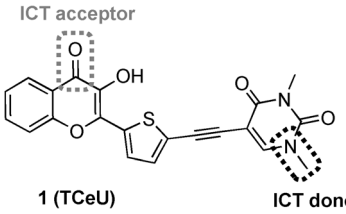


Figure 2. Proposed structure of the electronically connected 2-thienyl-3-hydroxychromone and uridine fragments.

To better understand the photophysics of this new compound, we compared it with two analogues, namely the parent 2-thienyl-3-hydroxychromone **2** (**TC**) and a TC bearing an acetylene moiety **3** (**TCe**; Figure 3).

In addition, we have synthesized and characterized for further comparison the methylated analogues of these three compounds **4**, **5** and **6** (named **MTC**, **MTCe**, and **MTCeU**, respectively) that are unable to undergo ESIPT.

Synthesis

Synthesis of the fluorophore **1 TCeU** and the model compounds is depicted in Scheme 1 and Scheme 2. Several synthetic strategies are reported to elaborate the 3HC scaffold. In our study, the Algar–Flynn–Oyamada (AFO) reaction was employed as the most facile and abundant reaction for constructing the 3-hydroxychromone core. Appreciable yields of the target chromones were achieved by using a "soft" one-pot protocol of the AFO reaction (Scheme 1).[26] An important feature of this protocol is the possibility to obtain pure product with minimal manipulation. According to this procedure, 2-hydroxyacetophonone **7** was treated with thiophene-aldehydes **8** and **9** in ethanol in the presence of sodium hydroxide with subsequent addition of hydrogen peroxide and acidic work-up to give 3-hydroxychromones **2 (TC)** and **10** in 50 and 48%

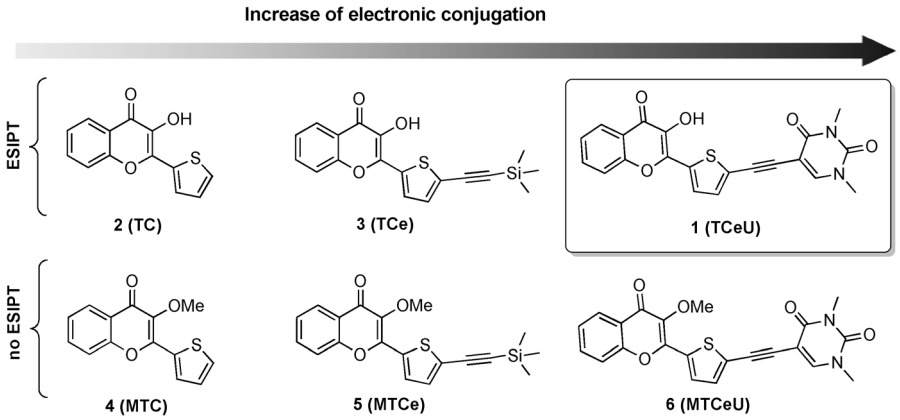
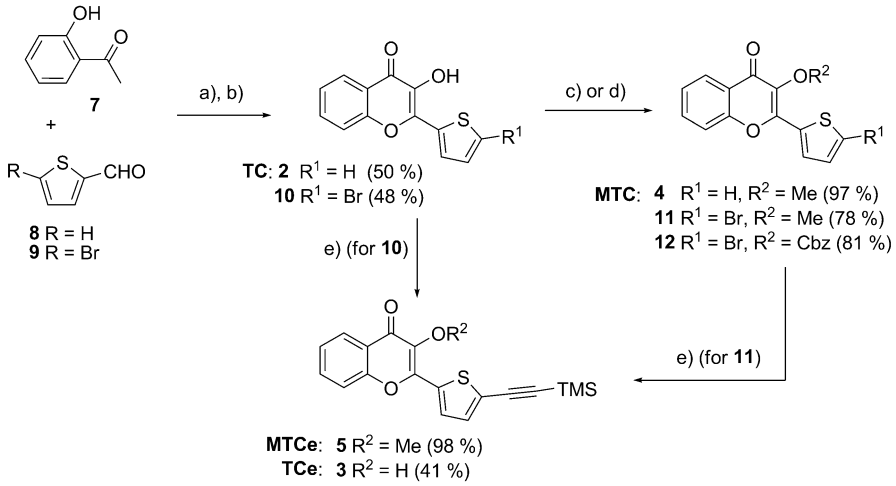
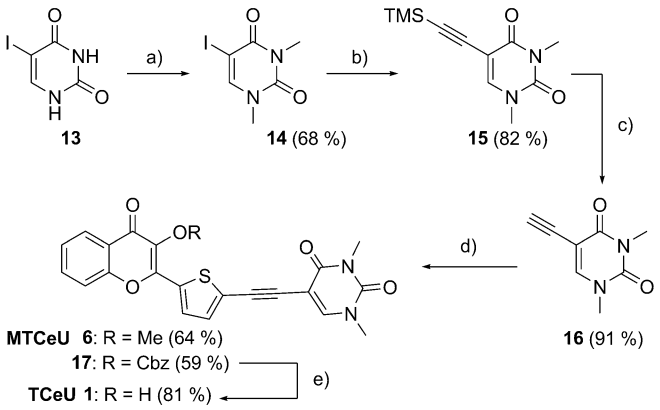


Figure 3. Structure of the designed fluorophore **TCeU** and model compounds.



Scheme 1. Synthesis of 2-thienyl-3-hydroxychromones. Reagents and conditions: a) NaOH, EtOH, RT, 48 h; b) H_2O_2 (30% aq.); c) Me_2SO_4, KOH, 18-crown-6, CH_2Cl_2/H_2O, RT, 1 h; d) Cbz-Cl, KOH, 18-crown-6, CH_2Cl_2/H_2O, RT, 1 h; e) $[PdCl_2(PPh_3)_2]$, CuI, NEt_3, THF, reflux.



Scheme 2. Modular synthesis of 3HC-uracil fluorophores. Reagents and conditions: a) Me_2SO_4, NaOH, H_2O, 100 °C, 2 h; b) TMS-acetylene, $[PdCl_2(PPh_3)_2]$, CuI, DIPEA, THF, 55 °C, 4 h; c) TBAF, THF, RT, 1 h; d) **11** or **12**, $[PdCl_2(PPh_3)_2]$, CuI, DIPEA, THF, 55 °C; e) NH_4OH, MeOH, RT, 1 h.

yields, respectively.[27] Derivative **4** (**MTC**) was obtained in 97% yield after methylation of **TC** by dimethyl sulfate under the phase-transfer catalysis (PTC) conditions reported previously.[28] Compounds **2** (**TC**) and **4** (**MTC**) were used for spectroscopic characterizations. Bromo-chromone **10** was converted under the same conditions into the methylated derivative **11** needed for the synthesis of the model compound **MTCe**.

The synthesis of the designed fluorophore **TCeU** requires an appropriate protection for the 3-OH group of the chromone. We selected a carbonate-type protecting group, the benzyloxycarbonyl group (known as Cbz or Z), which was successfully employed in our recent work for the synthesis of 3HC-containing nucleosides.[22] The Cbz group was introduced onto bromo-chromone **10** by treatment with Cbz-Cl under PTC conditions. Sonogashira coupling with TMS-acetylene and compounds **10** and **11** resulted in moderate to excellent yields of the model compounds **TCe** (**3**) and **MTCe** (**5**) required for the photophysical studies.

Concerning the preparation of the nucleobase fragment, 5-iodouracil **13** was first bis-methylated on its N-1 and N-3 positions as reported.[29] A Sonogashira coupling in presence of TMS-acetylene provided the alkyne derivative **15** in good yields. Removal of TMS by treatment with tetrabutylammonium fluoride (TBAF) led to the required fragment for the final assembly. Sonogashira coupling between fluorescent moieties **11** and **12** and the nucleobase part **16** gave the desired dye **MTCeU** (**6**) and **17** in appreciable yields. The Cbz masking the 3-hydroxyl group was cleaved by a simple treatment with aqueous ammonia in methanol at room temperature to afford the target fluorophore **TCeU** (**1**).

Spectroscopic properties of the designed fluorophore

UV/Vis absorption and fluorescence properties of the designed fluorophore as well as of the model compounds were investigated in a set of eleven solvents varying in polarity (Table 1).

Table 1. Spectroscopic properties of **TCeU** in different solvents.

Solvent	$E_T(30)$[a]	$\Sigma\beta_2^H$[b]	λ_{abs}[c]	λ_{N^*}[d]	λ_{T^*}[e]	I_{N^*}/I_{T^*}[f]	Φ[g]
H_2O	63.1	0.35	401	500	–	–	0.08
MeOH	55.4	0.47	395	501	558	1.20	0.22
EtOH	51.9	0.48	397	488	574	0.63	0.18
BuOH	49.7	0.48	400	481	571	0.50	0.19
CH_3CN	45.6	0.32	392	472	576	0.54	0.15
DMSO	45.1	0.88	402	477	582	1.43	0.25
acetone	42.2	0.49	393	465	581	0.53	0.15
CH_2Cl_2	40.7	0.05	397	458	577	0.31	0.17
EtOAc	38.1	0.45	394	451	579	0.36	0.15
THF	37.4	0.48	396	448	585	0.73	0.18
$PhCH_3$	33.9	0.14	399	441	587	0.13	0.15

[a] Reichardt's empirical solvent polarity index.[30] [b] H-bond basicity.[15,31] [c] Position in nm of the absorption maximum. [d] Position in nm of the maximum of the normal emission band. [e] Position in nm of the maximum of the tautomer emission band. [f] Ratio of the intensities at the two emission maxima. [g] Fluorescence quantum yield measured using 3-hydroxyflavone in toluene ($\Phi = 0.29$) as a reference.[32]

Four protic solvents (water, methanol, ethanol, *n*-butanol) and seven aprotic solvents (acetonitrile, dimethyl sulfoxide, acetone, ethyl acetate, THF, dichloromethane, toluene) were selected. According to their H-bond basicity ($\Sigma\beta_2^H$) values, three

of the aprotic solvents may be classified as neutral (toluene, dichloromethane, acetonitrile), whereas the other aprotic solvents are rather basic.[15,31]

The compound **TCeU** exhibits a single absorption band centered at 392–401 nm. The positions of the maxima correlate poorly with the solvent polarity showing a little positive solvatochromism. In all solvents except for water, **TCeU** showed dual emission, in which the short-wavelength and long-wavelength bands can be attributed to the N* and T* states, respectively. The N*/T* ratio as well the position of the N* emission band were strongly solvent-dependent (Table 1, Figure 4).

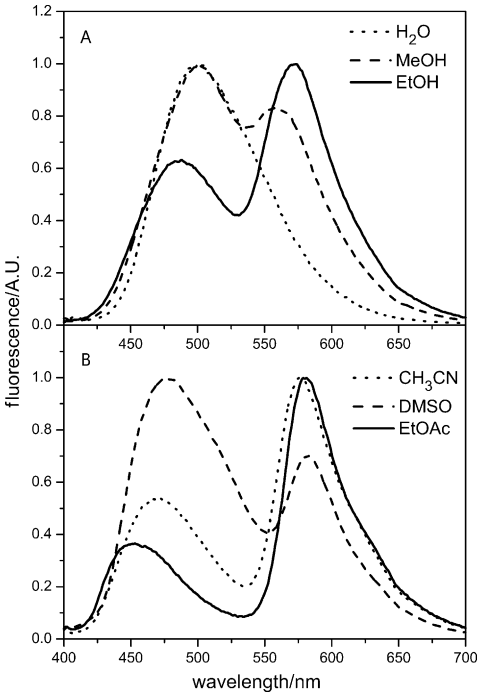


Figure 4. Fluorescence spectra of **TCeU** normalized at the maximal intensity in: A) protic, and B) aprotic solvents.

The N* band demonstrates positive solvatochromism, which is typical for this class of compounds.[33] The position of the T* band maximum is blueshifted in protic solvents, as compared with aprotic solvents, probably due to the formation of a H bond between the phenoxide oxygen of **TCeU** in the excited tautomer state and the solvent, as it was proposed for 2-furyl-3HC.[34] The Stokes shifts for the first and second emission bands are comprised between 2387–5356 and 7395–8027 cm^{-1}, respectively. The large Stokes shift obtained for the second emission band is characteristic for ESIPT probes. As a general trend, the N*/T* ratio increases with an increase in solvent polarity. Thus, the highest values of N*/T* ratio are observed for highly polar protic methanol and aprotic DMSO, whereas the lowest values are observed for the most apolar toluene. The fluorescence quantum yields are rather good in all solvents (0.15–0.25), except water (0.08).

Comparison of TCeU with model compounds

The absorption and fluorescence properties of **TC** and **TCe** were investigated and compared with those obtained for **TCeU** in the same set of solvents to get further insight regarding the effect of the electronic conjugation in the extended chromophore (Table 2). The absorption spectrum shifts to red

Table 2. Spectroscopic characteristics[a] of **TC**, **TCe**, and **TCeU** in different solvents.

Compound	Solvent	λ_{abs}	λ_{N^*}	λ_{T^*}	I_{N^*}/I_{T^*}	Φ
TC	MeOH	359	424	540	0.72	0.07
	EtOH	360	424	542	0.34	0.08
	CH_3CN	354	414	540	0.07	0.08
	EtOAc	354	412	543	0.05	0.12
	CH_2Cl_2	357	412	537	0.02	0.30
	$PhCH_3$	358	412	546	0.01	0.24
TCe	MeOH	378	442	561	0.43	0.13
	EtOH	379	439	563	0.22	0.14
	CH_3CN	373	431	563	0.10	0.14
	EtOAc	374	430	566	0.12	0.15
	CH_2Cl_2	379	432	561	0.05	0.19
	$PhCH_3$	379	431	572	0.03	0.20
TCeU	MeOH	395	501	558	1.20	0.23
	EtOH	397	488	574	0.63	0.18
	CH_3CN	392	472	576	0.54	0.15
	EtOAc	394	451	579	0.36	0.15
	CH_2Cl_2	397	458	577	0.31	0.17
	$PhCH_3$	399	441	587	0.13	0.15

[a] See the footnotes [c]–[g] in Table 1 for the definitions of these parameters.

in the following sequence: **TC**→**TCe**→**TCeU** (Figure 5). Extinction coefficients increase upon extending the π-electronic system of the fluorophore. Values are 23500, 30500, and 35000 $M^{-1}cm^{-1}$ in methanol for **TC**, **TCe**, and **TCeU**, respectively.

This sequential redshift corresponds well to the gradual increase in the conjugation length of the fluorophores. The data also suggest that the chromone and uridine parts in **TCeU** are fused into a single conjugated electronic system as evidenced by the disappearance of the characteristic absorption band of

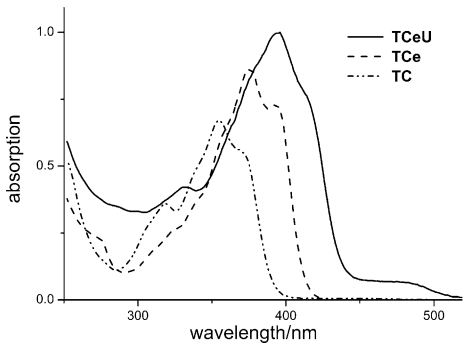


Figure 5. Absorption spectra of compounds **TC**, **TCe** and **TCeU** in ethyl acetate. Absorptions are proportional to their absorption coefficient.

uracil at about 260 nm (Figure 5). Interestingly, our designed fluorophore exhibits suitable absorption properties with an absorption band around 400 nm making possible its selective excitation in the presence of proteins and nucleic acids with conventional commercial lasers.

Similar to **TCeU**, the model compounds **TC** and **TCe** display dual emission, indicating that all three of them undergo ESIPT. The positions of the emission bands shift to the red with the increase in the fluorophore conjugation: **TC**→**TCe**→**TCeU**. For each solvent studied, the value of the N*/T* ratio of **TCeU** is much higher than those of **TC** and **TCe** (Table 2). Furthermore, the dual emission of these dyes depends differently on the solvent polarity.

In contrast to **TCeU**, compounds **TC** and **TCe** show only limited positive solvatochromism of the N* band. Indeed, going from toluene to MeOH, the N* band shifts to the red by only 12 nm for **TC** and **TCe**, whereas for **TCeU** the redshift reaches 60 nm. The T* band of **TC** and **TCe** also does not reveal clear variations with solvent polarity. Remarkably, all three compounds showed an increase in the N*/T* ratio with increasing

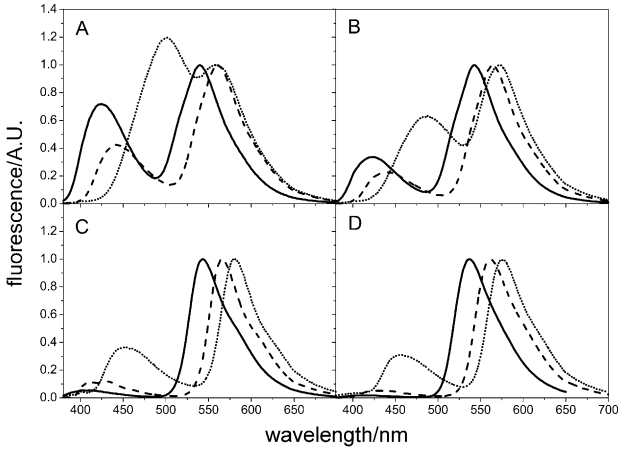


Figure 6. Normalized fluorescence spectra of **TC** (——), **TCe** (-----), and **TCeU** (•••••) in: A) methanol, B) ethanol, C) ethyl acetate, and D) dichloromethane.

solvent polarity (Figure 6). However, the N* band emission of **TCeU** is significant in aprotic solvents of different polarity, which is not the case of **TC** and **TCe**. Both compounds show only a marginal contribution of their N* form to the emission spectra in apolar solvents, such as ethyl acetate and dichloromethane (Figure 6C and D).

Both the strong solvatochromism of the N* band of **TCeU** and its high intensity in aprotic solvents are in line with a charge-transfer character of its N* state. A very similar charge-transfer character of the N* state was previously observed for 4′-(dialkylamino)-3-hydroxyflavone[14a] and its analogues having a push–pull structure.[35] It was hypothesized that in aprotic solvents, the strong interaction of the highly polar N* state with the environment may stabilize it dielectrically and thus shifts the ESIPT equilibrium N*⇄T* towards to the N* state.[36]

To further investigate the contribution of ICT to the photophysical behavior of the synthesized ESIPT dyes, their methylated analogues **MTC**, **MTCe**, and **MTCeU** were characterized in the same set of solvents (Supporting Information, Table S2). **MTC**, **MTCe**, and **MTCeU** showed comparable absorption coefficients to the parent non-methylated dyes and absorption bands centered at 337–341, 362–368, and 375–388 nm, respectively. When compared with the corresponding non-methylated chromones, the methylated analogues exhibit a significant blueshift of their absorption maximum. This significant blueshift may be explained by a possible distortion of the planar structure of the fluorophores due to an increased steric hindrance between the methoxyl group and the thienyl substituent in positions 3 and 2 of the chromone ring.[37]

As expected, no tautomer band in the emission spectra was observed due to blocked ESIPT. Notably, no significant blueshift in the emission maxima was observed, when compared with the N* band of non-methylated chromones. This indicates that the geometry of the relaxed excited state is close for both methylated and non-methylated compounds. The emission bands of the methylated 3HCs were centered at 426–450 nm for **MTC** and at 432–442 nm for **MTCe**, showing a poor dependence on solvent polarity. At the same time, compound **MTCeU** exhibited a strong solvent dependence of the position of its emission band, which ranges from 451 nm in ethyl acetate to 511 nm in water (Figure 7). Thus, the methylated compound **MTCeU**, similarly to its parent 3-hydroxychromone **TCeU**, shows a strong solvatochromism of its emission band, confirming our hypothesis on the charge-transfer character of the N* state. Our data confirm the presence of an ICT state due to the interaction of the chromone and uracil moieties.

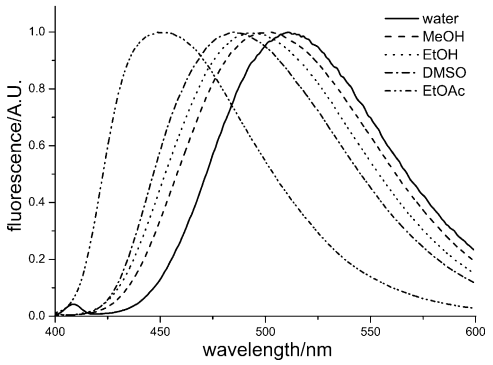
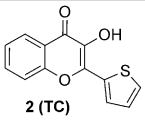
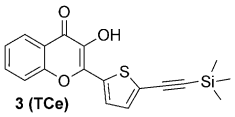
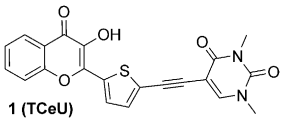


Figure 7. Normalized fluorescence spectra of **MTCeU** in representative solvents of different polarities.

A significant increase in the fluorescence quantum yield was observed for **MTCeU** (10–50%) as compared with **MTC** (0.5–2%) and **MTCe** (2–20%). The high quantum yields of **MTCeU** together with its solvatochromic properties compare favorably with other color-changing nucleoside analogues[38] and make this fluorophore a prospective candidate for incorporation in DNA and further investigations.

Table 3. Time-resolved decay parameters[a] for **TC**, **TCe**, and **TCeU** in polar solvents.

Compound	Solvent	$\tau_1^{N^*}$ [ns][a]	$\alpha_1^{N^*}$[b]	$\tau_2^{N^*}$ [ns][a]	$\alpha_2^{N^*}$[b]	$\tau_1^{T^*}$ [ns][a]	$\alpha_1^{T^*}$[b]	$\tau_2^{T^*}$ [ns][a]	$\alpha_2^{T^*}$[b]
 2 (TC)	EtOH	0.10 ± 0.01	1.00	–	–	0.08 ± 0.01	−0.45	1.2 ± 0.1	0.55
	MeOH	0.11 ± 0.01	1.00	–	–	0.10 ± 0.01	−0.47	0.76 ± 0.05	0.53
 3 (TCe)	EtOH	0.11 ± 0.01	1.00	–	–	0.09 ± 0.03	−0.53	2.6 ± 0.9	0.47
	MeOH	0.13 ± 0.01	1.00	–	–	0.13 ± 0.02	−0.15	2.00 ± 0.01	0.85
 1 (TCeU)	EtOAc	0.12 ± 0.01	1.00	–	–	0.10 ± 0.01	−0.47	2.4 ± 0.2	0.53
	EtOH	0.22 ± 0.01	0.54	0.50 ± 0.01	0.46	0.30 ± 0.13	−0.12	1.9 ± 0.1	0.88
	MeOH	0.43 ± 0.01	0.79	0.91 ± 0.02	0.21	0.35 ± 0.08	−0.25	1.55 ± 0.1	0.75

[a] The short- (τ_1) and long- (τ_2) lived decay times for the N* and T* emission bands, respectively. [b] The corresponding pre-exponential coefficients for the N* and T* emission bands, respectively.

Time-resolved studies of ESIPT

Time-resolved investigation of the ESIPT reaction in compounds **TC**, **TCe**, and **TCeU** was performed to understand the dramatic differences in the sensitivity of these compounds to solvent polarity. Time-resolved measurements were conducted in polar solvents in which both the normal and tautomer fluorescence bands were well separated (Table 3). Except for **TCeU** in the two protic solvents, the decay of the N* band of the three compounds was characterized by a single short-lived component ($\tau_1^{N^*}$), whereas the T* band decay was characterized by a fast rise component ($\tau_1^{T^*}$) with a lifetime value close to that of $\tau_1^{N^*}$, followed by a slower decay component ($\tau_2^{T^*}$). A similar behavior was already reported for (2-furyl)-3-hydroxychromone[39] and for 3-hydroxyquinolones,[40] the aza-analogues of 3HCs, strongly suggesting an irreversible ESIPT mechanism that is kinetically controlled. In this mechanism, the $\tau_1^{N^*}$ and $\tau_1^{T^*}$ values describe the kinetics of the proton transfer, whereas the long-lived decay component of the T* state ($\tau_2^{T^*}$) describes the lifetime of the T* form. For **TCe**, the ESIPT is slightly slower than in **TC**. For both compounds, the short-lived lifetimes are slightly higher in methanol, confirming that the ESIPT process is slower in this more H-bond-donating solvent.[39]

In the case of **TCeU**, the data indicate an apparent change in the ESIPT mechanism in polar protic solvents. Whereas the N* emission in ethyl acetate is characterized by monoexponential kinetics, the N* emission in EtOH and MeOH shows biexponential decay with commensurable amplitudes. The shorter component in these solvents still matches well with the corresponding rise component of the T* emission band, so it can be assigned to the ESIPT process. Interestingly, the value of this component drastically rises with the increase of the H-bond-donating ability of the solvent, indicating a slowing down of the ESIPT. The slowing down of ESIPT can be assigned to the formation of an intermolecular H bond between the 3-hydroxyl group of the dye with the solvent, which disrupts the intramolecular H bond with the carbonyl oxygen. Remarkably, the ESIPT process is slower in **TCeU** in comparison with **TC** and **TCe** in the same solvents. Indeed, an increase in the charge-transfer character of the N* state in **TCeU** due to the electron-donating uracil group may further strengthen the intermolecular solvent–solute H bond, thus additionally slowing down the ESIPT reaction.[39] The longer component in the N* emission, however, is not similar to the long-lived decay component in the T* emission, which one would expect in the case of reversible ESIPT.[36]

Thus, the proton transfer in **TCeU** in polar protic solvents likely remains irreversible, whereas the longer component of the N* emission decay may be assigned to the fluorescence of a different form, probably the intermolecular H-bonded species that is generally non-emissive in 3HCs, but was shown to emit in 3-hydroxyquinolones[40] and 4′-dialkylamino-3-hydroxyflavones.[41] The presence of this new emissive species is consistent with the higher quantum yield measured for **TCeU** in methanol and ethanol as compared with **TC** and **TCe**.

Correlation of the spectroscopic properties of TCeU with solvent polarity

In the prospect of using **TCeU** as an environment-sensitive fluorophore for DNA probing, it is important to establish the relationships between the photophysical parameters of the fluorophore (position of absorption/emission bands, and N*/T* ratio) and quantitative parameters of the environment polarity. In this work, we used the Reichardt's polarity scale $E_T(30)$,[30] an empirical scale commonly used in the analysis of dye solvatochromism. It has also been established as a reliable scale for analyzing emission of 3HCs.[16,35]

We observe that the positions of the absorption band and the T* emission band of **TCeU** show a poor correlation with $E_T(30)$. In contrast, a strong correlation is observed for the position of the N* band, which undergoes a bathochromic shift with an increase in solvent polarity (Figure 8). Obviously, this solvatochromism is caused by the strong dipole moment of the N* state, which possesses an ICT character. The strong sol-

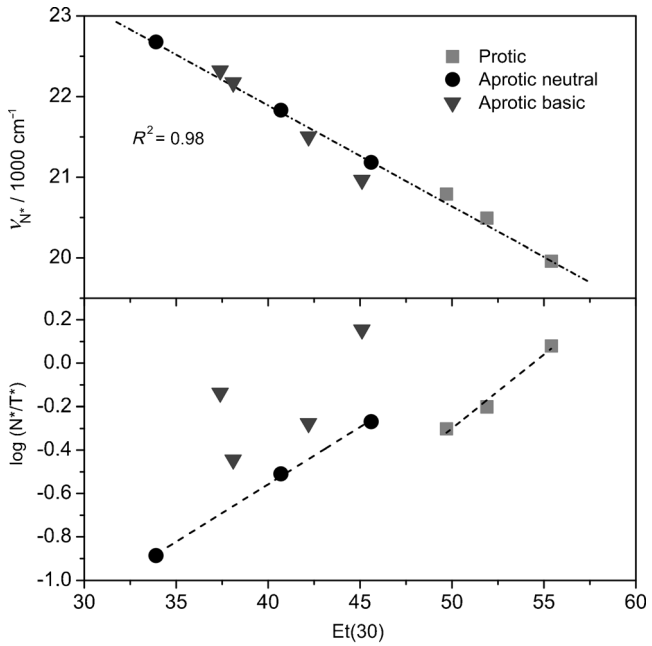


Figure 8. Dependence of the spectroscopic characteristics of **TCeU** on the solvent polarity index $E_T(30)$. Position of the N* band (top) and logarithm of the N*/T* ratio (bottom) as a function of $E_T(30)$. The light-gray squares, black circles, and dark-gray triangles represent protic (MeOH, EtOH, BuOH), aprotic neutral (CH_3CN, CH_2Cl_2, $PhCH_3$), and aprotic H-bond basic solvents (DMSO, acetone, EtOAc, THF), respectively.

vent-dependent variation in the N*/T* intensity ratio is the distinctive feature of 3HC fluorophores.[15,35] In the case of **TCeU**, the N*/T* ratio increases with the solvent polarity both in protic and neutral aprotic solvents, showing a linear correlation between log (I_{N^*}/I_{T^*}) and $E_T(30)$. Most importantly, the N*/T* ratio remains relatively high in aprotic media, so that the **TCeU** fluorophore is capable of distinguishing one apolar aprotic solvent from another; whereas, both the parent **TC** (Table 2, Figure 6) and its structural analogue 2-furyl-3-hydroxychromone[20] show a uniformly low N*/T* ratio in these solvents. By this feature, **TCeU** is comparable to 4′-dialkylamino-3-hydroxyflavones that also possess an ICT nature of their normal excited state. However, the dependence of the N*/T* ratio of **TCeU** versus $E_T(30)$ shows some upward deviation for aprotic solvents that present strong H-bond-acceptor ability (basicity). The upward influence of basic solvents on the relative intensity of N* band was observed even at trace concentration of a basic solvent originating from the stock solution; notably, this was observed only for fully conjugated **TCeU** (Supporting Information, Figure S1).

This deviation indicates that these aprotic solvents can hamper the ESIPT reaction in **TCeU**, probably by disrupting its intramolecular H bonding. This phenomenon is not usually observed for 3HC derivatives,[15] but it is a common property of 3-hydroxyquinolones.[40] A similar effect of basicity was also observed for several other 2-thienyl-3-hydroxychromones.[25] The effect of solvent basicity on **TCeU** emission confirms the fact that unlike other 3HCs, its complex with disrupted H bonding is probably emissive, in line with its relatively high quantum yields observed in H-bond-acceptor solvents (Table 1). Finally, the good correlation observed with all other solvents (Figure 8), indicates that the N*/T* ratio depends both on the polarity, H-bonding acidity and basicity of the solvent.

It should be noted that the strong variation of the dual emission of **TCeU** is observed in a broad range of solvent polarities, ranging from apolar toluene up to polar methanol. This is an important difference with its analogues **TC**, **TCe**, and the previously reported 2-furyl-3-hydroxychromone, which show strong variation of their dual emission mainly in polar protic solvents.[39] The present new feature, resulting from the charge-transfer character of the N* excited state, makes this new fluorophore prospective for incorporation into DNA, in which rather low polarities of internal regions can be found as well as the local environment of the major groove is expected to be relatively polar and comparable to methanol and therefore different to bulk water.[42] The incorporation of **TCeU** into DNA and its further application for monitoring DNA interactions is under way and will be reported in due course.

Conclusion

A new fluorescent dye based on the fusion of 3HC and uracil moieties has been designed, synthesized, and investigated. The dye undergoes an ESIPT reaction and exhibits a dual-band emission in a set of solvents of varying polarity. The ratio of its two emission bands changes as a function of the solvent polarity across a broad range. The position of the N* band correlates perfectly with the empirical solvent polarity scale $E_T(30)$. A strong correlation was found also between log(N*/T*) and $E_T(30)$ for protic and neutral aprotic solvents. Comparison with its structural analogues allowed for the demonstration of the electronic conjugation between 3HC and uracil heterocycles and the charge-transfer nature of its normal excited state that is responsible for the dependence of its N*/T* ratio on the solvent polarity. The fluorophore is a prospective candidate for incorporation into DNA as an environment-sensitive nucleobase mimic.

Experimental Section

General methods

All non-aqueous reactions involving water-sensitive reagents were performed in oven-dried glassware under argon using dry solvents. The synthetic intermediates were co-evaporated twice with toluene and dried in vacuo before use. All chemical reagents were obtained from commercial sources and were used as supplied. Anhydrous solvents were obtained according to standard procedures.[43] The reactions were monitored by thin-layer chromatography (TLC, Merck silica gel 60 F254 plates) and visualized both by UV radiation (254 and 365 nm) and by spraying with phosphomolybdic acid in ethanol followed by a subsequent warming with a heat gun. Flash column chromatography[44] was performed by using silica gel (40–63 μm). All NMR spectra (^1H, ^{13}C, ^2D) were recorded on 200 or 500 Bruker Advance Spectrometers (200 or 500 MHz). ^1H NMR (200 and 500 MHz), ^{13}C NMR (50 and 125 MHz, recorded with complete proton decoupling) spectra were obtained with samples dissolved in $CDCl_3$, CD_3OD, or [D_6]DMSO, with the solvent signal used as an

internal reference: $\delta = 7.26$ for $CHCl_3$, 3.31 for CD_2HOD, and 2.50 ppm for $(CD_3)(CD_2H)S(O)$ for ^1H NMR experiments, and $\delta = 77.0$ for $CDCl_3$, 49.0 for CD_3OD, and 39.4 ppm for $(CD_3)_2S(O)$ for ^{13}C NMR experiments.[45] Chemical shifts (δ) are given in ppm to the nearest 0.01 (^1H) or 0.1 ppm (^{13}C). The coupling constants (J) are given in Hertz (Hz). The signals are reported as follows: (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad). Assignments of ^1H and ^{13}C NMR signals were achieved with the help of D/H exchange, COSY, HMQC, HMBC experiments. Regular mass spectra (MS) were recorded on an Esquire 3000 Plus apparatus with ESI in both positive and negative mode. High-resolution mass spectrometry was conducted with a FINIGAN MAT 95 spectrometer with EI or ESI ionization techniques. Systematic flavone and nucleobase nomenclature is used below for the assignments of each spectrum. All solvents for absorption and fluorescence experiments were of spectroscopic grade. Absorption spectra were recorded on a Cary 4 spectrophotometer (Varian) using 1 cm quartz cells. Stock solutions were prepared using dimethyl sulfoxide, except for **TCeU** for which dioxane was used. The samples used for spectroscopic measurements contained approximately 0.1% v/v of the stock solvent. Fluorescence spectra were recorded on a FluoroLog 3.5 spectrofluorometer (Jobin Yvon, Horiba). Excitation wavelength was 360 nm. Time-resolved fluorescence measurements were performed with the time-correlated, single-photon counting technique using the excitation pulses at 315 nm provided by a pulse-picked frequency-tripled Ti:sapphire laser (Tsunami, Spectra Physics) pumped by a Millenia X laser (Spectra Physics). The fluorescence decays were collected at the magic angle (54.7°) of the emission polarizer to avoid any artifact due to vertically polarized excitation beam. The single-photon events were detected with a microchannel plate photomultiplier (Hamamatsu) coupled to a pulse preamplifier HFAC (Becker-Hickl) and recorded on a SPC-130 board (Becker-Hickl). Time-resolved data were analyzed by the maximum entropy method (Pulse 5 software).[46] In all cases, the χ^2 values were close to 1 and the weighted residuals as well as their autocorrelation were distributed randomly around 0, indicating an optimal fit.

Synthesis

3-Hydroxy-2-(thiophen-2-yl)-chromen-4-one (2): A 5 N NaOH solution (6 mL) was added drop-wise to a stirred solution of *o*-hydroxyacetophenone **7** (1.16 mL, 9.63 mmol) and 2-thiophenecarbaldehyde **8** (900 μL, 9.63 mmol) in ethanol (50 mL). The reaction mixture was stirred 48 h at RT before the drop-wise addition of 30% aq. hydrogen peroxide solution (2 mL). The resulting mixture was stirred 7 h at RT, then poured into cold water (300 mL) and neutralized with diluted acetic acid. The resulting precipitate was filtered and thoroughly washed with water and hexane to provide the target derivative **2** (1.17 g, 50%; $C_{13}H_8O_3S$ (244.27)) as a yellow solid. ^1H NMR (200 MHz, [D$_6$]DMSO): $\delta = 7.30$ (dd, $^3J = 5.0$, $^3J = 3.8$ Hz, 1H, Hβ-thioph.), 7.46 (td, $^3J = 8.0$, $^4J = 1.2$ Hz, 1H, H6), 7.72 (dd, $^3J = 8.4$, $^4J = 1.2$ Hz, 1H, H8), 7.73–7.84 (m, 1H, H7), 7.91 (dd, $^3J = 5.0$, $^4J = 1.2$ Hz, 1H, Hγ-thioph.), 7.97 (dd, $^3J = 3.8$, $^4J = 1.2$ Hz, 1H, Hα-thioph.), 8.10 (dd, $J = 8.0$, 1.2 Hz, 1H, H5), 10.28 ppm (s, 1H, OH); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 118.0$ (C8), 121.7 (C10), 124.5 (C6), 124.7 (C5), 127.7 (Cβ), 128.3 (Cα), 130.9 (Cγ), 132.3 (C2-*Th*), 133.6 (C7), 136.6 (C3), 143.1 (C2), 154.1 (C9), 171.9 ppm (C4); MS (ESI$^+$): *m/z*: 267.0 $[M+Na]^+$.

2-(5-Bromothiophen-2-yl)-3-hydroxy-chromen-4-one (10): A 5 N NaOH solution (6 mL) was added drop-wise to a stirred solution of *o*-hydroxyacetophenone **7** (1.16 mL, 9.63 mmol) and 5-bromo-2-thiophenecarbaldehyde **9** (1.14 mL, 9.63 mmol) in ethanol (50 mL) was. The reaction mixture was stirred 48 h at RT before a drop-wise addition of 30% aq. hydrogen peroxide solution (2 mL). The resulting mixture was stirred 7 h at RT, then poured into cold water (300 mL) and neutralized with diluted acetic acid. The resulting precipitate was filtered and thoroughly washed with water and hexane to provide as a yellow solid the target derivative **10** (1.49 g, 48%; $C_{13}H_7BrO_3S$ (323.16)). ^1H NMR (200 MHz, $CDCl_3$): $\delta = 7.19$ (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.41 (td, $^3J = 8.0$, $^4J = 0.8$ Hz, 1H, H6), 7.54 (d, $^3J = 8.4$ Hz, 1H, H8), 7.71 (ddd, $^3J = 8.4$, $^3J = 8.0$, $^4J = 1.4$ Hz, 1H, H7), 7.73 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.22 ppm (dd, $^3J = 8.0$, $^4J = 1.4$ Hz, 1H, H5); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 112.2$ (*Th*-Br), 117.8 (C8), 121.6 (Cα), 122.0 (C10), 122.9 (C6), 124.7 (C5), 129.3 (Cβ), 131.6 (C7), 136.1 (C2-*Th*), 141.6 (C3), 146.5 (C2), 153.3 (C9), 176.0 ppm (C4); MS (ESI$^+$): *m/z*: 344.9 $[M+Na]^+$.

3-Methoxy-2-(thiophen-2-yl)-chromen-4-one (4): A solution of 4.5 M KOH (2 mL), 18-crown-6 (5 mol%, 25 mg), and dimethyl sulfate (370 μL, 3.86 mmol) were added to a stirred suspension of **2** (472 mg, 1.93 mmol) in CH_2Cl_2 (8 mL). The reaction mixture became homogenous in 5 min and was stirred at RT for 1 h. After quenching by addition of H_2O (10 mL), the organic layer was extracted with CH_2Cl_2 (3×), dried over $MgSO_4$, filtered, and the volatiles were removed in vacuo. The residue was purified by flash chromatography on silica gel eluted with cyclohexane/EtOAc mixture (10:1→4:1, v/v) to provide the desired compound **4** as a yellow solid (483 mg, 97%; $C_{14}H_{10}O_3S$ (258.29)). ^1H NMR (200 MHz, $CDCl_3$): $\delta = 4.06$ (s, 3H, CH_3), 7.19 (dd, $^3J = 4.0$, 5.0 Hz, 1H, Hβ-thioph.), 7.36 (td, $^3J = 8.0$, $^4J = 1.0$ Hz, H6), 7.50 (dd, $^3J = 8.4$, $^4J = 1.0$ Hz, 1H, H8), 7.60 (dd, $^3J = 5.0$, $^4J = 1.2$ Hz, 1H, Hγ-thioph.), 7.65 (td, $^3J = 8.4$, $^4J = 1.6$ Hz, 1H, H7), 7.94 (dd, $^3J = 4.0$, $^4J = 1.2$ Hz, 1H, Hα-thioph.), 8.22 ppm (dd, $^3J = 8.0$, $^4J = 1.6$ Hz, 1H, H5); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 59.6$ (OMe), 117.7 (C8), 124.2 (C10), 124.6 (C6), 125.6 (C5), 127.4 (Cβ), 129.5 (Cα), 131.5 (Cγ), 131.7 (C2-*Th*), 133.3 (C7), 138.6 (C3), 151.5 (C2), 154.7 (C9), 174.0 ppm (C4); HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{11}O_3S$: 259.0423 $[M+H]^+$; found 259.0424.

3-Methoxy-2-(5-bromothiophen-2-yl)-chromen-4-one (11): A solution of 4.5 M aq. KOH (5 mL), 18-crown-6 (5 mol%, 65 mg), and dimethyl sulfate (930 μL, 9.84 mmol) were added to a stirred suspension of **10** (1.59 g, 4.92 mmol) in CH_2Cl_2 (35 mL). The reaction mixture became homogenous in 5 min and was stirred at RT for 1 h. After quenching by addition of H_2O (40 mL), the organic layer was extracted with CH_2Cl_2 (3×), dried over $MgSO_4$, filtered and the volatiles were removed in vacuo. The residue was purified by flash chromatography on silica gel eluted with cyclohexane/EtOAc mixture (10:1→4:1, v/v) to provide the desired compound **11** as a yellow solid (1.30 g, 78%; $C_{14}H_9BrO_3S$ (337.19)). ^1H NMR (200 MHz, $CDCl_3$): $\delta = 4.07$ (s, 3H, CH_3), 7.16 (d, $^3J = 3.8$ Hz, 1H, Hβ-thioph.), 7.38 (td, $^3J = 8.0$ Hz, $^4J = 1.0$ Hz, H6), 7.49 (dd, $^3J = 8.4$, $^4J = 1.0$ Hz, 1H, H8), 7.62–7.79 (m, 1H, H7), 7.66 (d, $^3J = 3.8$ Hz, 1H, Hα-thioph.), 8.22 ppm (dd, $^3J = 8.0$, $^4J = 1.6$ Hz, 1H, H5); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 59.7$ (OMe), 117.7 (C8), 119.8 (*Th*-Br), 124.2 (C10), 124.7 (C6), 125.7 (C5), 129.2 (Cα), 130.2 (Cβ), 132.7 (C2-*Th*), 133.5 (C7), 138.4 (C3), 150.2 (C2), 154.7 (C9), 173.8 ppm (C4); HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}BrO_3S$: 336.9529 $[M+H]^+$; found 336.9531.

3-Benzyloxycarbonyloxy-2-(5-bromothiophen-2-yl)-chromen-4-one (12): A solution of 4.5 M aq. KOH (9 mL), 18-crown-6 (5 mol%, 43 mg), and benzyl chloroformate (700 μL, 4.88 mmol) were added to a stirred suspension of **10** (1.05 g, 3.25 mmol) in CH_2Cl_2 (13 mL). The reaction mixture became homogenous in 5 min and was stirred at RT for 1 h. After quenching by addition of H_2O (10 mL), the organic layer was extracted with CH_2Cl_2 (3×), dried over $MgSO_4$, filtered, and the volatiles were removed in vacuo. The residue was purified by flash chromatography on silica gel eluted with cyclohexane/EtOAc mixture (9:1→7:3, v/v) to provide the desired compound **12** as white crystals (1.20 g, 81%; $C_{21}H_{13}BrO_5S$ (457.29)).

$R_f = 0.53$ (cyclohexane/EtOAc = 7:3); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 5.37 (s, 2H, CH_2), 7.16 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.34–7.50 (m, 6H, H6 and Cbz), 7.52 (dd, $^3J = 8.4$, $^4J = 0.6$ Hz, 1H, H8), 7.65 (d, $^3J =$ 4.0 Hz, 1H, Hα-thioph.), 7.72 (td, $^3J = 8.4$, $^4J = 1.6$ Hz, 1H, H7), 8.25 ppm (dd, $^3J = 8.0$, $^4J = 1.6$ Hz, 1H, H5); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 71.3$ (OC(O)CH_2Ph), 117.8 (C8), 120.4 (*Th*-Br), 123.6 (C10), 125.4 (C6), 126.1 (C5), 128.4 (*o*-C_{Ph}), 128.7 (*m*-C_{Ph}), 128.8 (*p*-C_{Ph}), 131.0 (Cβ), 131.0 (Cα), 131.5 (C2-*Th*), 132.0 (C3), 134.1 (C7), 134.5 (*i*-C_{Ph}), 150.3 (C2), 151.8 (O*C*(O)CH_2Ph), 155.0 (C9), 171.3 ppm (C4); HRMS (ESI$^+$): *m/z* calcd for $C_{21}H_{14}BrO_5S$: 456.9740 $[M+H]^+$; found: 456.9732.

3-Hydroxy-2-(5-((trimethylsilyl)ethynyl)thiophen-2-yl)-chromen-4-one (3): $[PdCl_2(PPh_3)_2]$ (3 mol%, 20 mg) and CuI (3 mol%, 5 mg) were added to a stirred solution of aryl bromide **10** (323 mg, 1 mmol), trimethylsilylacetylene (210 μL, 1.5 mmol) and *N*,*N*-diisopropylethylamine (DIPEA; 280 μL, 2 mmol) in THF (6 mL) under argon. The mixture was heated at reflux for 1 h, then allowed to cool down to RT. The volatiles were removed in vacuo and the residue was purified by flash chromatography on silica gel eluted with CH_2Cl_2/MeOH (99:1→97:3, v/v) to provide the coupled derivative **3** as a yellow solid (142 mg, 41%; $C_{18}H_{16}O_3SSi$ (340.47)). ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 0.27$ (s, 9H, $SiMe_3$), 7.47 (dd, $^3J = 8.0$ Hz, $^3J = 7.5$ Hz, H6), 7.50 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.72 (d, $^3J =$ 8.5 Hz, 1H, H8), 7.81 (ddd, $^3J = 8.5$, $^3J = 7.5$, $^4J = 1.5$ Hz, 1H, H7), 7.85 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.10 (dd, $^3J = 8.0$, $^4J = 1.5$ Hz, 1H, H5); 10.59 ppm (br s, 1H, OH); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta =$ −0.4 ($SiMe_3$), 97.2 (-*C*≡C-TMS), 101.9 (-C≡*C*-TMS), 118.0 (C8), 121.7 (C10), 124.5 (C6), 124.8 (C5), 125.3 (C2-*Th*), 127.8 (Cα), 133.2 (Cβ), 133.5 (*Th*-*C*≡C), 133.7 (C7), 137.5 (C3), 141.9 (C2), 154.1 (C9), 172.0 ppm (C4); HRMS (ESI$^+$): *m/z* calcd for $C_{18}H_{17}O_3SSi$: 341.0662 $[M+H]^+$; found: 341.0657.

3-Methoxy-2-(5-((trimethylsilyl)ethynyl)thiophen-2-yl)-chromen-4-one (5): $[PdCl_2(PPh_3)_2]$ (4 mol%, 45 mg) and CuI (4 mol%, 12 mg) were added to a stirred solution of aryl bromide **11** (536 mg, 1.6 mmol), trimethylsilylacetylene (340 μL, 2.4 mmol), and DIPEA (430 μL, 3.2 mmol) in THF (10 mL) under argon. The mixture was heated at reflux for 2 h, then allowed to cool down to RT. The volatiles were removed in vacuo and the residue was purified by flash chromatography on silica gel eluted with cyclohexane/EtOAc mixture (9:1→5:1, v/v) to provide the coupled derivative **5** as yellow solid (554 mg, 98%; $C_{19}H_{18}O_3SSi$ (354.49)). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 0.28$ (s, 9H, $SiMe_3$), 4.09 (s, 3H, CH_3), 7.29 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.39 (dd, $^3J = 8.0$, $^3J = 7.0$ Hz, H6), 7.50 (d, $^3J =$ 8.0 Hz, 1H, H8), 7.67 (ddd, $^3J = 8.0$, $^3J = 7.0$, $^4J = 1.2$ Hz, 1H, H7), 7.79 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.24 ppm (dd, $^3J = 8.0$, $^4J = 1.2$ Hz, 1H, H5); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = -0.2$ ($SiMe_3$), 59.8 (OMe), 96.9 (-*C*≡C-TMS), 102.7 (-C≡*C*-TMS), 117.7 (C8), 124.3 (C10), 124.8 (C6), 125.8 (C5), 128.9 (C2-*Th*), 129.0 (Cα), 132.3 (*Th*-*C*≡C), 132.6 (Cβ), 133.5 (C7), 139.0 (C3), 150.5 (C2), 154.8 (C9), 173.9 ppm (C4); HRMS (ESI$^+$): *m/z* calcd for $C_{19}H_{19}O_3SSi$: 355.0819 $[M+H]^+$; found 355.0815.

5-Iodo-1,3-*N*,*N*-dimethyluracil (14): Dimethyl sulfate (870 μL, 9.2 mmol) was added drop-wise to a stirring slurry of 5-iodouracil **13** (1.00 g, 4.2 mmol) and NaOH (497 mg, 10.5 mmol) in H_2O (7 mL) at 0 °C. The mixture was heated under reflux for 2 h, cooled to RT, extracted with CH_2Cl_2 (3×), dried over $MgSO_4$, filtered, and reduced in vacuo. The resulting insoluble solid after the evaporation was washed several times with cyclohexane and subsequently with toluene, ether, EtOAc, and cold CH_2Cl_2. For each solvent, the solid particles were allowed to settle and the supernatant washings were taken out carefully with a syringe with a fine needle. The washings were collected and evaporated and the residual solid was washed as before. All the residues were combined and dried to obtain **14** (763 mg, 68%; $C_6H_7IN_2O_2$ (266.04)) as an amorphous white solid. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 3.41$ (s, 3H, N^3CH_3), 3.42 (s, 3H, N^1CH_3), 7.64 ppm (s, 1H, H6); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 29.4$ (N^3CH_3), 37.2 (N^1CH_3), 67.1 (C5), 147.3 (C6), 151.4 (C2), 160.4 ppm (C4); MS (ESI$^+$): *m/z*: 288.9 $[M+Na]^+$.

5-(Trimethylsilyl)ethynyl-1,3-*N*,*N*-dimethyluracil (15): $[PdCl_2(PPh_3)_2]$ (6 mol%, 89 mg) and CuI (6 mol%, 24 mg) were added to a stirred solution of alkenyl iodide **14** (544 mg, 2.0 mmol), trimethylsilylacetylene (441 μL, 3.0 mmol), and DIPEA (1.80 mL, 10.0 mmol) in a 2:1 THF/DMF mixture (10 mL) under argon. The reaction mixture was warmed to 55 °C for 4 h. Then, the volatiles were removed in vacuo and the residue was purified by flash chromatography on silica gel eluted with toluene/EtOAc (10:1→4:1, v/v) to provide the TMS-alkyne derivative **15** (390 mg, 82%; $C_{11}H_{16}N_2O_2Si$ (236.34)) as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.17$ (s, 9H, $SiMe_3$), 3.30 (s, 3H, N^3CH_3), 3.38 (s, 3H, N^1CH_3), 7.49 ppm (s, 1H, H6); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = -0.3$ ($SiMe_3$), 28.1 (N^3CH_3), 37.2 (N^1CH_3), 95.6 (-*C*≡C-TMS), 98.7 (-C≡*C*-TMS), 98.7 (C5), 146.2 (C6), 150.7 (C2), 161.5 ppm (C4); MS (ESI$^+$): *m/z*: 259.1 $[M+Na]^+$.

5-Ethynyl-1,3-*N*,*N*-dimethyluracil (16): TBAF (1 M in THF, 931 μL, 0.93 mmol) was added drop-wise to a stirred solution of **15** (200 mg, 0.85 mmol) in THF (150 mL). The reaction mixture was stirred at RT for 1 h. Then, the volatiles were removed in vacuo and the residue was purified by flash chromatography on silica gel eluted with toluene/EtOAc (10:1→3:1, v/v) to provide the terminal alkyne **16** (126 mg, 91%; $C_8H_8N_2O_2$ (164.16)) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.17$ (s, 1H, (-C≡CH), 3.37 (s, 3H, N^3CH_3), 3.43 (s, 3H, N^1CH_3), 7.50 ppm (s, 1H, H6); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 28.4$ (N^3CH_3), 37.4 (N^1CH_3), 74.9 (-*C*≡CH), 81.6 (-C≡*C*H), 98.0 (C5), 146.4 (C6), 150.9 (C2), 161.8 ppm (C4); MS (ESI$^+$): *m/z*: 187.0 $[M+Na]^+$.

5-[5-(3-Benzyloxycarbonyloxy-4-oxo-chromen-2-yl)-thiophen-2-yl-ethynyl]-1,3-*N*,*N*-dimethyluracil (17): $[PdCl_2(PPh_3)_2]$ (5 mol%, 19 mg) and CuI (5 mol%, 5 mg) were added to a stirred solution of aryl bromide **12** (236 mg, 0.50 mmol), **16** (92 mg, 0.55 mmol), and DIPEA (450 μL, 2.51 mmol) in THF (5 mL) under argon. The mixture was heated at reflux for 5 h, then allowed to cool down to RT. The volatiles were removed in vacuo and the residue was purified by flash chromatography on silica gel eluted with CH_2Cl_2/Et_2O (9:1→6:1, v/v) to provide the coupled derivative **17** as an orange solid (160 mg, 59%; $C_{29}H_{20}N_2O_7S$ (540.5)). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta =$ 3.39 (s, 3H, N^3CH_3), 3.47 (s, 3H, N^1CH_3), 5.38 (s, 2H, OC(O)O*C*H_2Ph), 7.27 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.37 (d, $^3J = 7.0$ Hz, 1H, *p*-H_{Ph}), 7.41 (t, $^3J = 7.0$ Hz, 2H, *m*-H_{Ph}), 7.42 (m, 1H, H6′), 7.48 (s, $^3J = 7.0$ Hz, 2H, *o*-H_{Ph}), 7.53 (d, $^3J = 8.0$ Hz, 1H, H8′), 7.57 (s, 1H, H6), 7.71 (ddd, $^3J = 8.0$ Hz, $^3J = 7.0$ Hz, $^4J = 1.8$ Hz, 1H, H7′), 7.76 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.23 ppm (dd, $^3J = 7.8$, $^4J = 1.8$ Hz, H5′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 28.4$ (N^3CH_3), 37.5 (N^1CH_3), 71.3 (OC(O)*C*H_2Ph), 85.6 (Th-*C*≡C-), 88.5 (Th-C≡*C*-), 98.3 (C5), 117.9 (C8′), 123.6 (C10′), 125.3 (C6′), 126.0 (C5′), 128.3 (*o*-C_{Ph}), 128.7 (*m*-C_{Ph}), 128.7 (*p*-C_{Ph}), 129.2 (C2′-*Th*-), 130.7 (Cα), 131.6 (*Th*-*C*≡C-), 131.9 (C3′), 132.8 (Cβ), 134.1 (C7′), 134.5 (*i*-C_{Ph}), 145.9 (C6), 150.6 (C2′), 150.7 (C2), 151.8 (OC(O)CH_2Ph), 155.0 (C9′), 161.1 (C4), 171.3 ppm (C4′); HRMS (ESI$^+$): *m/z* calcd for $C_{29}H_{20}N_2NaO_7S$: 563.0883 $[M+Na]^+$; found: 563.0872.

5-[5-(3-Hydroxy-4-oxo-chromen-2-yl)-thiophen-2-yl-ethynyl]-1,3-*N*,*N*-dimethyluracil (1): A solution of 33% aq. NH_4OH (870 μL, 22.2 mmol) was added drop-wise to a stirred solution of **17** (30 mg, 55.5 μmol) in CH_2Cl_2 (7 mL). The reaction mixture was stirred at room temperature for 1 h. The resulting insoluble solid after the evaporation was washed several times with cyclohexane and subsequently with toluene, ether, EtOAc, and CH_2Cl_2. For each solvent, the solution was centrifuged for settling solid particles and

the supernatant washings were carefully taken out with a syringe with a fine needle. The washings were collected and evaporated and the residual solid was washed as before. All the residues were combined and dried to obtain **1** (18 mg, 81%; $C_{21}H_{14}N_2O_5S$ (406.4)) as a bright-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.42$ (s, 3H, N^3CH_3), 3.48 (s, 3H, N^1CH_3), 6.94 (br s, 1H, OH), 7.36 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.42 (ddd, $^3J = 7.0$, $^3J = 8.0$, $^4J = 1.5$ Hz, 1H, H′6), 7.56 (d, $^3J = 8.0$ Hz, 1H, H8′), 7.57 (s, 1H, H6), 7.71 (ddd, $^3J = 7.0$, $^3J = 8.0$, $^4J = 1.5$ Hz, 1H, H7′), 7.88 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.23 ppm (dd, $^3J = 8.0$ Hz, $^4J = 1.5$ Hz, 1H, H5′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 28.5$ (N^3CH_3), 37.5 (N^1CH_3), 86.3 (Th-*C*≡C-), 87.5 (Th-C≡*C*-), 98.8 (C5), 118.1 (C8′), 121.0 (C10′), 124.7 (C6′), 125.5 (C5′), 126.8 (C2′-*Th*-), 129.1 (Cα), 133.1 (C7′), 133.8 (Cβ), 134.0 (C3′), 136.7 (*Th*-C≡C-), 141.6 (C2′), 145.5 (C6), 150.8 (C2), 155.1 (C9′), 161.3 (C4), 172.4 ppm (C4′); HRMS (ESI$^+$): *m/z* calcd for $C_{21}H_{15}N_2O_5S$: 407.0696 $[M+H]^+$; found: 407.0683.

5-[5-(3-Methoxy-4-oxo-chromen-2-yl)-thiophen-2-yl-ethynyl]-1,3-*N*,*N*-dimethyluracil (6): $[PdCl_2(PPh_3)_2]$ (6 mol%, 16 mg) and CuI (6 mol%, 4 mg) were added to a stirred solution of aryl bromide **11** (125 mg, 0.35 mmol), **16** (65 mg, 0.39 mmol), and DIPEA (415 μL, 1.76 mmol) in THF (4 mL) under argon. The mixture was heated at reflux for 5 h, then allowed to cool down to RT. The resulting insoluble solid after the evaporation was washed several times with cyclohexane and subsequently with toluene, ether, EtOAc, and CH_2Cl_2. For each solvent, the solution was centrifuged for settling solid particles and the supernatant washings were carefully taken out with a syringe with a fine needle. The washings were collected and evaporated and the residual solid was washed as before. All the residues were combined and dried to obtain **6** (95 mg, 64%; $C_{22}H_{16}N_2O_5S$ (420.4)) as a bright-yellow solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 3.21$ (s, 3H, N^3CH_3), 3.36 (s, 3H, N^1CH_3), 3.99 (s, 3H, OCH_3), 7.50 (d, $^3J = 4.0$ Hz, 1H, Hβ-thioph.), 7.50 (ddd, $^3J = 7.5$, $^3J = 8.0$, $^4J = 1.0$ Hz, 1H, H6′), 7.77 (d, $^3J = 7.5$ Hz, 1H, H8′), 7.84 (td, $^3J = 7.5$, $^4J = 1.5$ Hz, 1H, H7′), 7.98 (d, $^3J = 4.0$ Hz, 1H, Hα-thioph.), 8.09 (dd, $^3J = 8.0$, $^4J = 1.5$ Hz, 1H, H5′), 8.36 ppm (s, 1H, H6); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 27.8$ (N^3CH_3), 36.8 (N^1CH_3), 59.4 (OCH_3), 84.1 (Th-*C*≡C-), 90.0 (Th-C≡*C*-), 95.2 (C5), 118.2 (C8′), 123.6 (C10′), 124.8 (C6′), 125.1 (C5′), 130.0 (Cα), 131.3 (C2′-*Th*-), 132.1 (C7′), 134.1 (Cβ), 138.2 (C3′), 148.8 (C6), 149.5 (*Th*-C≡C-), 150.4 (C2′), 154.3 (C9′), 160.9 (C2), 172.7 (C4), 186.8 ppm (C4′); HRMS (ESI$^+$): *m/z* calcd for $C_{22}H_{16}N_2NaO_5S$: 443.0672 $[M+Na]^+$; found: 443.0670.

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