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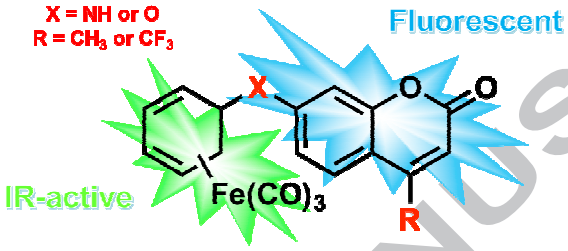

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for bimodal imaging/sensing applications !!!

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Synthesis and photophysical properties of iron-carbonyl complex-coumarin conjugates as potential bimodal IR-fluorescent probes

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ABSTRACT

An expedient synthesis of the first examples of iron-carbonyl complex-coumarin conjugates is reported. 7-Amino/7-hydroxycoumarin derivatives have been readily derivatized through an easily implemented single-step reaction involving the tricarbonyl(η^5-cyclohexadienyl)iron(1+) cation $[(C_6H_7)Fe(CO)_3]^+$. The scope and limitations of this *N*-/*O*-alkylation reaction were also investigated. The fluorescence properties of these novel metal-carbonyl complexes have been studied and support their further use as valuable building blocks in the design of bimodal contrast agents for combined vibrational and fluorescence imaging.

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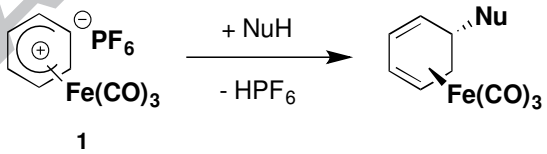
1. Introduction

A variety of techniques are currently used to elucidate basic cellular processes and many important biomolecular mechanisms associated with diseases and pathological states can be studied using such (bio)analytical tools. In this context, molecular imaging techniques play an important and growing role as they allow real-time visualization of a wide range of biomolecules or organelles, especially through the implementation of effective bio-labeling techniques and/or the use of rationally designed molecular bioprobes.[1] Since no single imaging technique exhibits the highest resolution and sensitivity, the deepest penetration, and the best observation timescale, it is often necessary to investigate a biological issue using different complementary set-ups.[2] Consequently, bimodal probes that can be defined as molecular structures exhibiting simultaneously two distinct spectroscopic properties such as fluorescence and magnetic properties[3], fluorescence and radioactive properties[4], or fluorescence and vibrational properties[5], are a clever means to combine the advantages of two complementary imaging techniques within the same molecule. They are well suited for bioimaging of living cells and small animals. Among the numerous bimodal imaging strategies currently explored, the one based on the use of luminescent and vibrational active metal-carbonyl complexes (also defined as Single Core Multimodal Probe for Imaging (SCoMPI)) has recently emerged.[5,6] Indeed, it seems to be a promising approach to perform correlative imaging without suffering the usual drawbacks of more conventional multicomponent conjugates used as bimodal radio/fluorescence or magnetic resonance imaging (MRI)/fluorescence probes. Indeed, these latter result from the chemical combination of two (or more) molecular fragments with complementary signaling units and physicochemical properties, and problems associated with their steric hindrance (that may negatively impact properties of tagged-(bio)molecules) and their premature cleavage *in cellulo* or *in vivo* (leading to a heterogeneous distribution/location of molecules related to each modality) are often encountered. Furthermore, mid-infrared (mid-IR) spectroscopy is a valuable technique for bio-imaging and bio-sensing, since the wavelength absorption is specific for a given chemical bond, and it allows chemical mapping of compounds with different functional groups (*e.g.*, amide[7], phosphate[7], or CO ligands of metal-carbonyl complexes[8,9]). We will focus on iron-carbonyl complexes which exhibit intense vibrational bands in the region (2200-1800 cm^{-1}) where most of the biological media are transparent (also called the mid-IR transparency window of cells).[8,10] Crucially, metal-carbonyl units can also be visualized through Raman spectroscopy (particularly beneficial for avoiding interferences from water) and more recently, surface-enhanced Raman spectroscopy (SERS)[8], and they have been already used in fluorescent cell imaging.[11] Multimodal detection involving both luminescence and infrared vibrational spectro- and microscopies of living cells has been successfully performed by the Policar group. They designed a SCoMPI through the chelation of an IR-active rhenium(I) tricarbonyl unit with a bidentate pyridyl-triazole ligand (*i.e.*, 4-(2-pyridyl)-1,2,3-triazolyl (pyta)) enabling radiative emission from ^{3}MLCT (metal-to-ligand charge transfer) excited state. The outcome of their research have shown that both techniques are consistent with one another; they pointed out the reliability of the present SCoMPI strategy for bimodal imaging and clearly demonstrated both integrity of the rhenium-carbonyl-pyta core inside cells and its accumulation in the Golgi apparatus of MDA-MB-231 breast cancer cells.[6a] Additionally, Mebi *et al.* reported the synthesis of new organometallic complex coupling photoactive 7-mercapto-4-methylcoumarin to a diironhexacarbonyl unit which was characterized by elemental and spectroscopic analyses.[12] The results of this study have shown that the new complex is electrochemically unstable and exhibits intramolecular photoinduced electron transfer (PeT) from coumarin to the iron-carbonyl unit leading to a severe quenching (quenching efficiency = 88%) of fluorescence of ligand. In view of these recent and important advances, there is a great need for alternative examples of bimodal IR/fluorescence-enabled molecules, and a clearer understanding of the influence on fluorescence properties when metal-carbonyl complexes are tethered to a coumarin fluorophore. Provided the fluorescence response persists in the presence of the metal complex, such bimodal molecules have potential to offer innovative chemical imaging tools by incorporating the unique information content of vibrationally-coupled IR stretching modes.[13]

In this Letter, we report the synthesis of the first examples of iron-carbonyl complex-coumarin conjugates. Their spectral properties were evaluated to support their further use as valuable building blocks in the design of dual contrast agents for vibrational and fluorescence imaging.

2. Results and discussion

Tricarbonyl(η^5-cyclohexadienylium)iron(1+) complexes are powerful electrophiles with versatile applications as building blocks in synthetic organic chemistry.[14] Because of their positive charge, a large variety of nucleophiles readily react with the coordinated ligand (Scheme 1).[15]



1

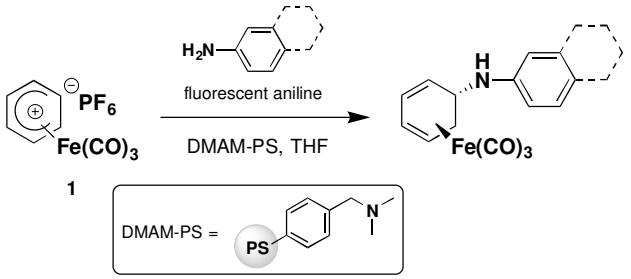
Scheme 1. The reaction of a tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation **1** (PF_6 salt) with nucleophiles (NuH).

The nucleophilic attack generally proceeds in high yields and takes place regioselectively at the terminus of the coordinated dienyl system (Davies-Green-Mingos rules[16]), and also stereoselectively *anti* to the tricarbonyliron fragment.[17]

Numerous synthetic methods are currently available for the reaction of highly electrophilic tricarbonyliron cations with different types of nucleophiles.[15,18] Additionally, these procedures have been used, for example, to prepare iron-carbonyl flavonoid derivatives for an IR-based study of *nod* gene regulation in *Rhizobium leguminosarum*,[19] and most of these methods should clearly be suitable for the introduction of charged tricarbonyliron cation into the heteroaryl moiety, and they should be easily transferable to more sensitive 7-amino and 7-hydroxycoumarin derivatives, scaffolds that possess good fluorescence properties, and a marked fluorogenic character when their aniline or phenol is substituted with an electron-withdrawing group (EWG) used for designing pro-fluorophores suitable for "turn-on" fluorescent detection of various (bio)analytes.[20,21]

Since our synthetic strategy is based on a nucleophilic substitution reaction between tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation and known substituted 7-amino (or 7-hydroxy)coumarin derivatives, our initial efforts were devoted to finding suitable conditions to readily perform such alkylation of weakly nucleophilic anilines.

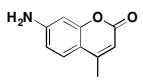
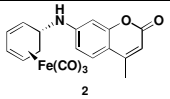
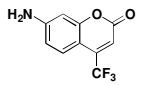
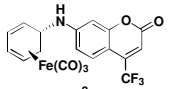
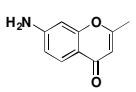
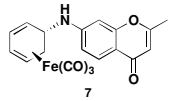
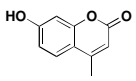
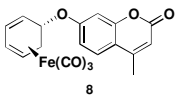
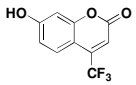
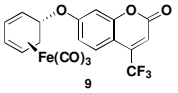
Synthesis of iron-carbonyl complex-aniline conjugates



Scheme 2. Reaction of tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation **1** (PF_6 salt) with fluorescent aniline derivatives (PS = polystyrene solid support).[27]

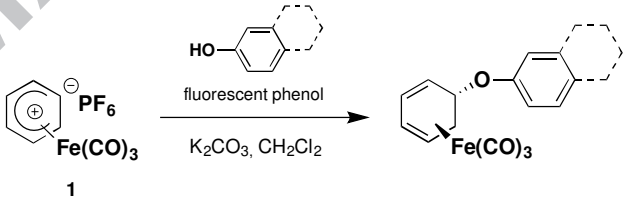
The one-step synthesis of iron carbonyl complexes from anilines including 7-aminocoumarin derivatives, 7-amino-2(1*H*)-quinolinones and 7-amino-2-methylchromone is shown in Scheme 2 (see also Table 1 and Figure 1 for targeted structures). The introduction of 7-aminocoumarin substituent onto the tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation is comparable to the facile nucleophile addition to cationic intermediates generated in S_N1 type reactions, and thus should be compatible with poorly nucleophilic aniline moieties. The introduction of commercially available 7-amino-4-methylcoumarin (7-AMC) was performed using a modified literature procedure[22] by reaction with **1** (PF_6 salt) in dry THF in the presence of polymer-supported base (dimethylamino)methyl-polystyrene (DMAM-PS) (see Table 1 and Supplementary data for synthetic details). The reaction mixture was stirred overnight before being quenched to give the product **2** in a satisfying 46% yield (Table 1, entry 1). We anticipated that this method could be used with various related readily available fluorescent anilines, where the substituents of the heterocyclic ring could be varied in order to assess possible effect on the fluorescence properties. In order to test this hypothesis, we used other commercially available 7-amino-substituted derivatives (Table 1 and Figure 1). Applying the optimized conditions previously found for the synthesis of 7-amino-4-methylcoumarin, we were pleased to observe that iron-carbonyl-7-aminocoumarin **3** and iron-carbonyl-7-aminochromone **7** could be isolated in good 74% and 86% yields respectively (Table 1, entries 2 and 3). Surprisingly, however, these reaction conditions did not work for all other fluorescent primary anilines tested since we did not manage to isolate either products **4**, **5** nor **6** (Figure 1), although their structures are closely related to **2** or **3**. The structures of the novel iron-carbonyl complex-aniline conjugates **2**, **3** and **7** were unambiguously confirmed by APCI-HRMS, NMR and IR spectroscopic analyses (see Supplementary data).

Table 1. Isolated yields for the reaction of tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation **1** (PF_6 salt) with fluorescent aniline/phenol derivatives.

Entry	NuH	Product	Isolated yield (%)
1		 **2**	46%
2		 **3**	74%
3		 **7**	86%
4		 **8**	81%
5		 **9**	78%

Synthesis of iron-carbonyl complex-phenol conjugates

With our new procedure involving the use of solid-supported DMAM-PS base in hand, we decided to expand the scope of this reaction to 7-hydroxycoumarin derivatives (see Table 1 and Figure 1 for targeted structures). However, we were unable to obtain the targeted products. During reaction of **1** with primary aniline derivatives (Scheme 2), the supported base (DMAM-PS) acts only as a trap for released HPF_6. In the case of reaction with 7-hydroxycoumarin derivatives, it is essential to deprotonate the phenol moiety to produce the reactive phenolate form, which is nucleophilic enough for the reaction to take place. To access the desired 7-hydroxycoumarin conjugates, the base and solvent were changed. The *O*-alkylation reaction was then readily achieved by reaction of 7-hydroxycoumarin derivatives with **1** (PF_6 salt) in dry CH_2Cl_2 and in the presence of K_2CO_3 as a base (see Scheme 3, Table 1 and Supplementary data for synthetic details).



Scheme 3. Reaction of tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation **1** (PF_6 salt) with fluorescent 7-hydroxycoumarin derivatives.

These new conditions were applied to 7-hydroxycoumarin derivatives bearing different substituents in C-3/C-4 positions in order to assess possible effect on the fluorescence properties. We observe that compounds **8** and **9** can be isolated in good 81% and 78% yields respectively (Table 1, entries 4 and 5, for their analytical data, see Supplementary data). Unfortunately, under these conditions, we were unable to obtain compounds **10** and **11** (Figure 1). General outcome for all 7-NH_2/OH derivatives bearing substituent (EDG or EWG) in position C-3 failed to give the expected compounds and the starting substrates were recovered unmodified. This experimental observation can be attributed to the difference in electrons delocalization which lessens the *O* or *N* nucleophilicity.

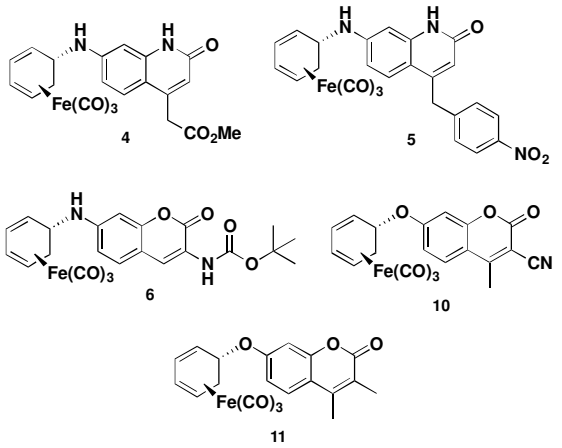


Figure 1. Structures of targeted iron-carbonyl complex-aniline/phenol conjugates not obtained using synthetic methodologies described in Schemes 2 and 3. For the synthesis of starting methyl 7-amino-2(1H)-quinolinone-4-acetate **S1** and 3-*N*-Boc-3,7-diaminocoumarin **S7** not yet reported in the literature, see Supplementary data.

Photophysical properties of iron-carbonyl complex-fluorophore conjugates

The photophysical properties of synthesized 7-*N*/*O*-substituted coumarins **2**, **3**, **8** and **9**, and 7-*N*-substituted 2-methylchromone derivative **7** were evaluated in DMSO (solvent in which these compounds are perfectly soluble), and in phosphate-buffered saline (PBS, pH 7.5), which mimics physiological conditions for three of them (**3**, **7** and **9**, due to their solubility in aq. buffers in the micromolar range). The results are compiled in Table 2 (see Figure 2 for absorption/fluorescence spectra of **9** and Supplementary data for the absorption/fluorescence spectra of other compounds). All compounds displayed a broad and intense absorption band with a maximum in the range 257-398 nm, mainly depending on the structure of the chromophore core (coumarin or chromone) and the nature of heterocyclic ring substituents, due to intramolecular charge transfer (ICT) from the *N*-/*O*-atom of amino/alkoxy group to ring carbonyl oxygen. Since the cyclohexadienyl sustituent is not directly conjugated with the *N*-/O-atom of coumarin/chromone scaffold, it is therefore logical to have a spectral behavior quite similar to that of parent free 7-amino/hydroxy coumarins/chromone. Upon excitation at 330 or 350 nm (depending on the compound studied), a significant blue or green fluorescence emission was observed, with a maximum ranging from 380 to 513 nm according to the solvent used and structural features of the coumarin/chromone scaffold. As usually observed with ICT molecules, large Stokes' shifts (58-185 nm) were obtained. Fluorescence quantum yields were lower than those of parent non-substituted 7-amino/hydroxy coumarins/chromone (see Table 2, entries 11-16 and fluorescence quantum yields reported in the literature for 7-hydroxy-4-trifluoromethylcoumarin (4-FMU), 7-amino-4-trifluoromethylcoumarin (C151) and 7-amino-2-methylchromone: 34% in phosphate buffer (pH 10)[23], 48% in DMSO[24] and 18% in water[25] respectively) but remain acceptable, especially under physiological conditions (11-24%). A number of hypotheses may be advanced as possible explanation for the partial quenching of fluorescence: (1) *N*- or *O*-substitution reduces the electron-donating ability of heteroatom and decreases the push-pull effect within the coumarin/chromone scaffold; (2) a possible intramolecular electron transfer (PeT process)[26] from the first excited singlet state of coumarin to iron-carbonyl unit[12,27]; (3) the formation of non-fluorescent *H*-aggregates[28] especially for compounds **3** and **7** in PBS, as supported by no perfect matching between absorption and excitation spectra (see Supplementary data). The behavior of compound **9** in DMSO is quite interesting (Table 2, entry 9 and Figure 2 for spectra). In addition to the main absorption band at 339 nm, a further red-shifted, flat and large absorption band in the range 435-450 nm was observed. Upon excitation at 448 nm, a strong fluorescence emission centered at 513 nm was obtained. However, it was not possible to determine the corresponding fluorescence quantum yield due to the lack of a linear relationship between fluorescence emission and absorption. A possible explanation may be the formation of *J*-aggregates[28] even if such self-assembly process of fluorophores is rarely observed in pure organic solvents.[29] Since compound **9** possesses the highest fluorescence quantum yield under physiological conditions with, in addition, both good absorption spectrum width (full-width half maximum, $\Delta\lambda_{1/2\ max}$) of 115 nm (Figure 2) and large Stokes' shift allowing excitation beyond 400 nm (thus avoiding damage of biomolecules and minimizing tissues' autofluorescence), it will make sense to consider the preparation of bimodal imaging agents through the conjugation of iron-carbonyl-7-hydroxycoumarin complexes to a biological vector (*e.g.*, peptides or antibodies). For example, by amidification of a carboxylic acid group (carboxymethyl substituent) pre-introduced onto the C-4 position of 7-hydroxycoumarin scaffold (Figure 3, top).[31]

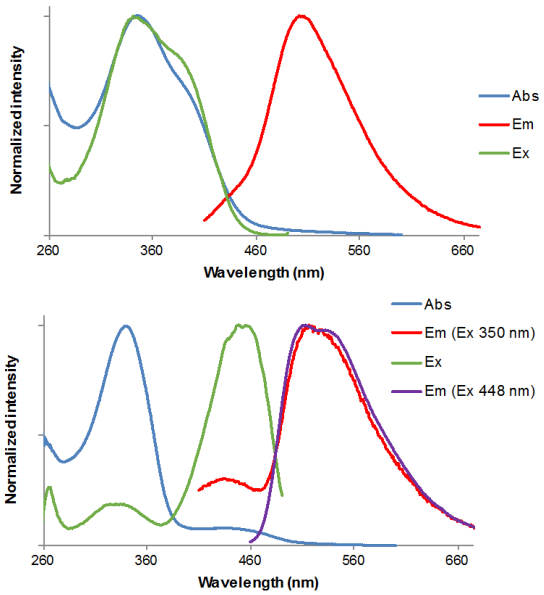


Figure 2. Normalized absorption (blue), excitation (Em. 500 nm, green) and emission (Ex. 350 nm, red and Ex. 448 nm, purple) spectra of compound **9** in PBS (top) and in DMSO (bottom) at 25 °C.

Table 2 Photophysical properties of iron-carbonyl complex-coumarin (chromone) conjugates at 25 °C.

Dye	Solvent[a]	Abs λ_{max} (nm)	Em λ_{max} (nm)	ε (M^{-1} cm^{-1})	Stokes' shift (nm/cm^{-1})	Φ_F (%)[b]

2	DMSO	360	446	-[c]	86/5 356	45
	PBS	-[c]	-[c]	-[c]	-[c]	-[c]
3	DMSO	398	495	11 390	97/4 923	13
	PBS	395	507	8 360	112/5 592	11
7	DMSO	259, 291, 328	415	11 080 5 150 5 510 10 570	156/14 513 124/10 268 87/6 391 185/16 286	10
	PBS	257, 300, 325	442	4 885 4 995	142/10 709 117/8 145	18
8	DMSO	322	380	-[c]	58/4 740	9
	PBS	-[c]	-[c]	-[c]	-[c]	-[c]
9	DMSO[d]	339	513	11 560	174/10 005	3
	PBS	343[e]	501	8 905	158/9 194	24
7-AMC[f]	DMSO	356	422	19 990	66/4 393	75
	PBS	343	444	16 520	101/6 632	83
C151[g]	DMSO	386	487	-	101/5 373	48
4-MU[f]	DMSO	324	385	14 380	61/4 890	17
	PBS	323	450	12 420	127/8 737	87
4-FMU[g]	PB[h]	385	501	16 300	116/6 014	34

[a]PBS = 100 mM phosphate + 150 mM NaCl, pH 7.5.
[b]Determined using 7-hydroxycoumarin (Φ_F = 76% in sodium phosphate buffer, pH 7.5, λ_{ex} = 330 nm (**7** & **8**) & 350 nm (**2**, **3** & **9**)) as standard.[30]
[c] Preliminary photophysical characterization of **2** and **8** in DMSO was performed in School of Chemistry of University of East Anglia and School of Pharmaceutical Sciences of Ribeirão Preto and no longer available for the more comprehensive study (determination of both molar exctinction coefficient and quantum yield in DMSO and PBS) conducted at ICMUB.
[d] In DMSO, compound **9** exhibits a second, red-shifted and flat absorption band (in the range 435-450 nm) whose the intensity increases upon dilution (probably *J*-aggregates).
[e]Broad absorption band assigned to sum of absorption spectra of phenol and phenolate forms (pKa = 7.3).
[f]Photophysical properties determined by us using conditions given in footnote b. See Supplementary data for the corresponding absorption/fluorescence spectra.
[g]C151 = coumarin-151 = 7-amino-4-(trifluoromethyl)coumarin, see ref. 24. 4-FMU = 4-(trifluoromethyl)umbelliferone, see ref. 23.
[h]PB = 0.1 M phosphate buffer, pH 10.

Conclusion

We have developed a convenient synthesis of fluorescent IR-active iron complexes using a non-soluble base (supported tertiary amine or K_2CO_3) in an effective nucleophilic substitution reaction between fluorescent anilines/phenols and tricarbonyl(η^5-cyclohexadienylium)iron(1+) cation. Despite the substitution of fluorogenic center (7-NH_2 or 7-OH) of coumarin/chromone scaffold and a possible PeT process, these unusual iron-carbonyl complexes exhibit moderate to good blue-green fluorescence both in pure organic solvents and aq. buffers. This is a positive and promising first step for further use of these building blocks in the construction of bimodal IR-fluorescence bioimaging probes. In a more ambitious and prospective goal, further functionalization of the secondary aniline of **2**, **3** or **7** with an enzyme-triggerable self-immolative carbamate spacer[32] may be also considered to access ratiometric IR-fluorescent probes (bimodal "smart" probes), for which both IR and fluorescence signals could be dramatically modified by the action of the targeted bioanalyte (Figure 3, bottom).

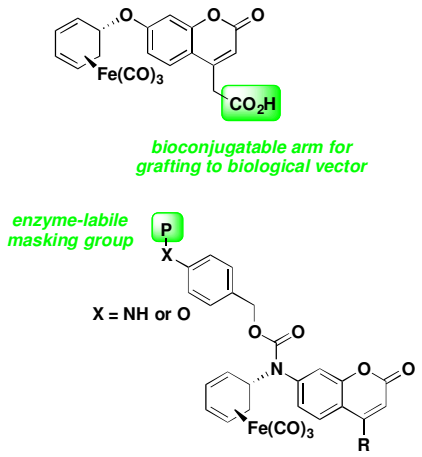


Figure 3. Possible ways of functionalization of iron-carbonyl complex-coumarin conjugates for their conversion into bimodal IR-fluorescence bioimaging probes (top) and ratiometric IR-fluorescent probes for enzyme sensing (bottom, R = H, alkyl or hydrophilic substituent).

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Supplementary data

Supplementary data (detailed synthetic procedures, spectroscopic characterizations and/or absorption/excitation/emission spectra of compounds **2**, **3**, **7-9**, **S2**, **S7**, **7-AMC** and **4-FMU**) associated with this article can be found, in the online version:

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Highlights:

Novel iron-carbonyl complex-coumarin conjugates have been synthesized.

Reaction of dienyl iron-tricarbonyl cation with fluorescent anilines and phenols.

Iron-carbonyl complexes are fairly fluorescent both in water and organic solvents.