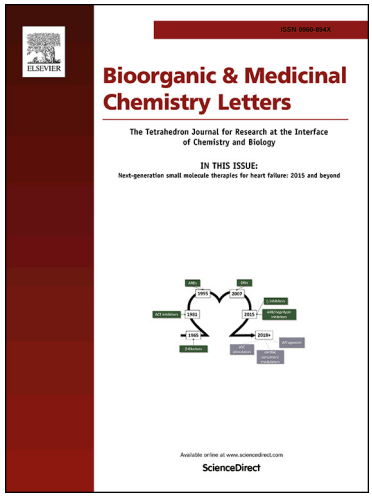
# Accepted Manuscript

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# Fluorescent-Fipronil: Design and Synthesis of a Stable Conjugate

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**Abstract**

Fipronil is a phenyl pyrazole molecule widely used across the world as both insecticide and veterinary drug. The main goal of this work was to synthesize a fluorescently labeled fipronil derivative for cellular imaging without affecting its intrinsic properties. We selected fluorescein as fluorescent probe and we investigated different strategies for stable chemical ligation between both entities, such as thiourea and direct peptide bond. While thiourea bond displayed low stability, direct peptide bond was difficult to achieve due to problems of steric hindrance. The best result was obtained by conjugation using click chemistry, which allowed to obtain fipronil stably labeled with the fluorescent probe.

**Keywords**

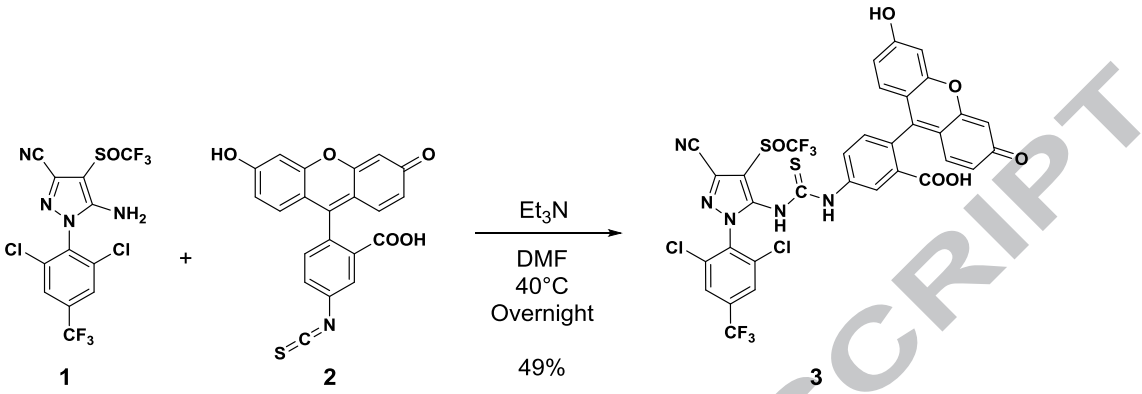
Fipronil; fluorescein; azide-alkyne cycloaddition; stability

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethylsufinyl)-1-*H*-pyrazole-3-carbonitrile) is a phenyl pyrazole insecticide used to protect crops with good selectivity between insects and mammals.[1] It became recently famous due to the egg scandal (millions of eggs have been contaminated with fipronil despite being banned by the European Union for use on animals intended for human consumption). Fipronil has been investigated for its insecticidal activity, toxicology, and impact on the environment.[2-7] In 2011 Vidau *et al.* showed that sublethal doses of fipronil highly increased mortality of honeybees previously infected by the microsporidian parasite *N. ceranae*.[2] However, uninfected honeybees exposed to fipronil were significantly less affected (mortality or behavior), meaning that uninfected honeybees would be able to respond to insecticides by enhancing detoxification process. In these studies, the activities of two enzymes implicated in insect detoxification, namely 7-ethoxycoumarine-*O*-deethylase (ECOD) and glutathione-S-transferase (GST) were assessed. While ECOD activity remained unchanged, GST activity was enhanced in the midgut and fat body, in contrast with the enhancement of infected honeybee susceptibility to fipronil. This means that GST would not be involved in detoxification process of fipronil. In 2013 the metabolic fate and the elimination of fipronil in rats were investigated using radiolabeled fipronil.[3] This study revealed that fipronil was slowly metabolized and excreted in these animals. Indeed, except in feces, no traces of parent compound were found in the analyzed samples. However, the highest level of radioactivity was found in adipose tissue and adrenals. To investigate further the toxic effect of fipronil and allow mechanistic studies, we wished to label fipronil with a fluorescent probe. For this purpose, we covalently functionalized fipronil with fluorescein using different chemical ligations and we investigated the stability of fluorescent fipronil (thermal and light sensitivity). For *in vivo* imaging, the conjugate has to display high stability to guarantee the colocalization of fipronil with the probe. We found that the strategy based on copper(I) catalyzed azide-alkyne cycloaddition (CuAAC) reaction was the most efficient to obtain a stably labeled fipronil derivative that will be used for cellular imaging.

**Derivatization of fipronil with fluorescein through a thiourea bond**

As the 3-cyano and 4-trifluoromethylsulfinyl groups of phenyl pyrazole of fipronil are key pharmacophores, the main approaches reported for the derivatization of fipronil rely on the modification of the amino group on the pyrazole ring.[8] In this context, we initially relied on a previous work that investigated the derivatization of the free amine of fipronil **1** with fluorescein isothiocyanate (FITC) **2** through a thiourea bond (Scheme 1).[9] Fipronil reacted

with FITC in the presence of triethylamine at 40°C, giving the expected fluorescent conjugate **3** with 49% yield.




**Scheme 1.** Synthesis of fipronil functionalized with fluorescein through a thiourea bond.

The desired compound **3** was characterized by $^{1}H$ and $^{13}C$ NMR in different deuterated solvents and by LC-MS. First, NMR in DMSO-$D_6$ displayed unexpected signals in the aromatic area. This may be due to *cis-trans* equilibrium around the thiourea bond[10] and to the possible formation of the lactone on the fluorescein moiety. To avoid this equilibrium, NMR analysis was performed by increasing the temperature to 50°C (Figure S1). This analysis confirmed our hypothesis as less peaks were observed, but there were still undefined signals. However, when NMR was performed in deuterated methanol at 20°C, the peaks were better defined and their integration perfectly corresponded to the proton number of compound **3** (Figure S2). This result shows that the solvent certainly plays a crucial role in the conformation of the molecule. LC-MS analysis confirmed the synthesis of the desired compound ($m/z$ = 827.8 $[M+H]^+$).

For *in vivo* experiments, the compound has to be stable at physiological temperature for many hours or days. We performed HPLC analysis in order to study the stability of the conjugate (Figure 1). We observed that an aqueous solution of compound **3** stored at room temperature for 10 hours was unstable as four news peaks appeared. Compound **3** is probably photo- and temperature-sensitive. We also analyzed a solution stored at 4°C and we observed that it was slightly more stable, but still some degradation peaks appeared. Even when stored as a powder at 4°C, compound **3** displayed a certain level of degradation.

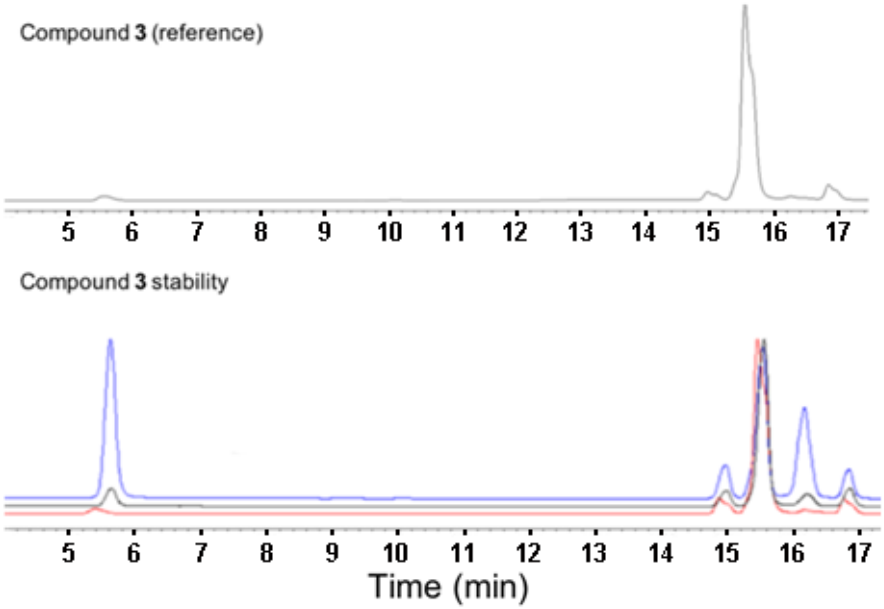



**Figure 1.** HPLC chromatograms of compound **3** (top) just after the synthesis and (bottom) stored in different conditions: in solution at rt (blue) and at 4°C (black), and as powder at 4°C (red).

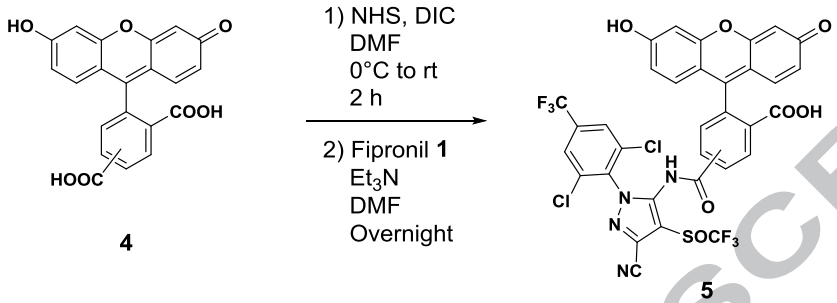
The main degradation product at 5.8 min was isolated by preparative HPLC and analyzed by LC-MS. This product corresponded to a mass of *m/z* = 348.2 identified as 5-aminofluorescein ($[M+H]^+$), confirming the low stability of the thiourea bond of compound **3** especially in solution. These results highlight the water sensitivity of thiourea bonds. Some studies have reported the instability of thiourea bonds in water, leading to ammonium and cyanate ions as hydrolysis products.[11-12]

Although this fluorescent compound was previously used in biological studies, we would like to underline that fipronil functionalized with FITC is not stable, even at low temperature and in powder form. Therefore, we can clearly conclude that this derivative is not suitable for biological experiments as only the fluorophore (not fipronil) would be detected. We recommend that careful stability studies are performed with fipronil derivatives before any *in vitro* and/or *in vivo* assay.

**Synthesis of fipronil-fluorescein *via* the formation of an amide bond**

Due to the low stability of the thiourea bond, we designed a new fipronil derivative based on the formation of an amide bond between the amino group of fipronil and the carboxylic function of 5(6)-carboxyfluorescein **4** (Scheme 2). The amide should be more stable compared to the thiourea bond. The reaction was performed by pre-activation of 5(6)-

carboxyfluorescein **4** with *N,N'*-diisopropylcarbodiimide (DIC) and *N*-hydroxysuccinimide (NHS). The activated ester reacted then with the amino group of fipronil in the presence of triethylamine to form desired compound **5**.




**Scheme 2.** Synthesis of fipronil functionalized with fluorescein through an amide bond.

The amidation reaction was monitored by HPLC, which confirmed the activation of both isomers of 5(6)-carboxyfluorescein as NHS esters (Figure 2). Indeed, after 2 h a peak shift of 2 min (from 6.9 and 7.1 to 8.9 and 9.1 min, respectively) was observed, confirming the formation of the activated 5(6)-carboxyfluorescein. However, after addition of fipronil and triethylamine, several peaks appeared at ~13.5 and 16 min but with very low intensity. After 3 h we could identify by LC-MS one peak with *m/z* = 794.8 corresponding to the desired product ($[M+H]^+$). However, even after heating at 40°C for 5 h, the reaction did not proceed further and the main peaks observed were still the activated fluorescein at 8.9 and 9.1 min and starting fipronil at 15.0 min. The crude mixture was purified by preparative HPLC. Nevertheless, we were not able to isolate a reasonable amount of compound **5**. This difficulty can be attributed to two factors: i) the low reactivity of the amino group of fipronil as the electron lone pair of the nitrogen is not highly nucleophilic due to the conjugation on the pyrazole ring; ii) steric hindrance due to the fact that all the aromatic groups considerably decrease the degree of freedom of the intermediates formed. As a consequence, we decided to try another approach to overcome these issues.

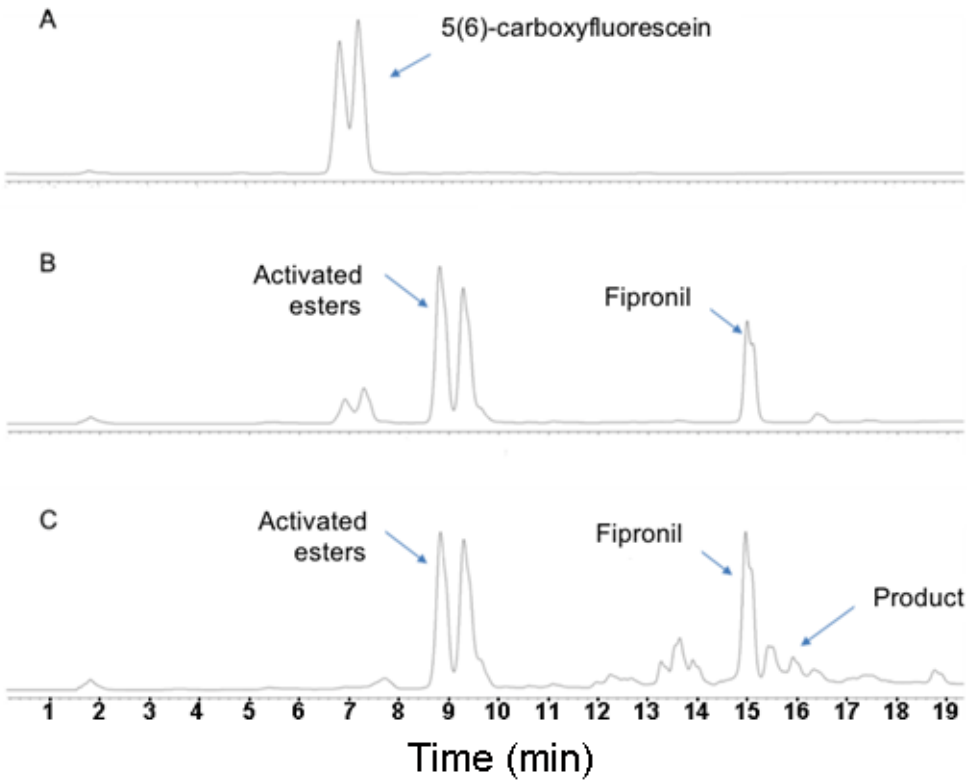
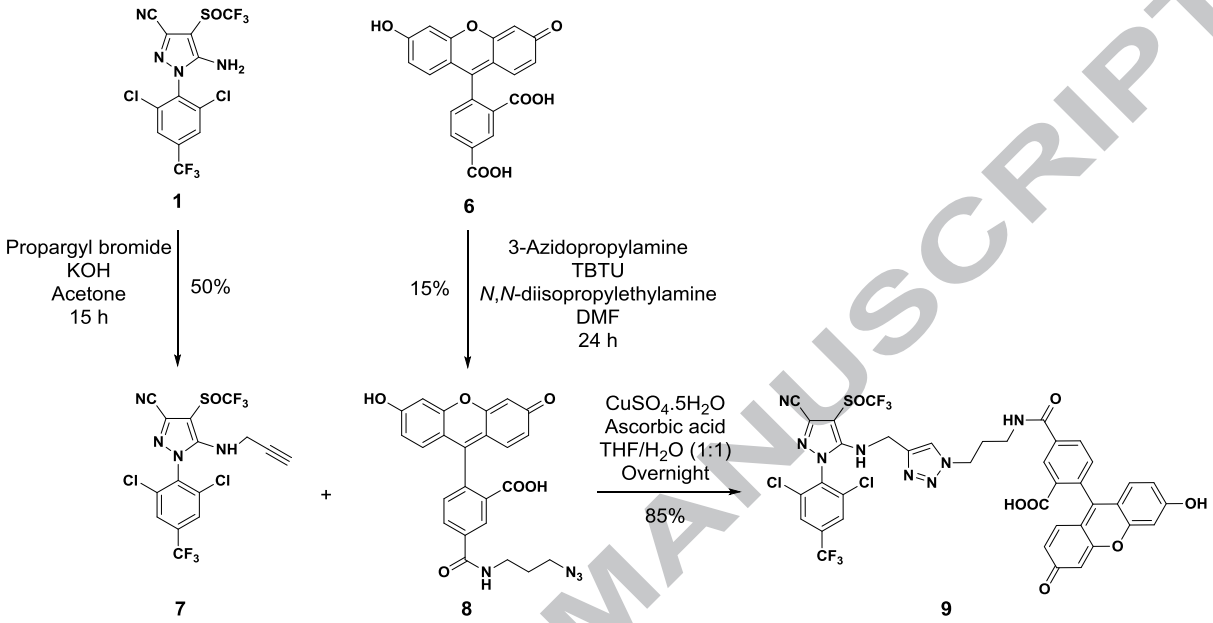



**Figure 2.** HPLC chromatograms at 254 nm of 5(6)-carboxyfluorescein (A), the reaction mixture just after the addition of fipronil t=0 (B), and the reaction mixture after stirring for 3 h at room temperature (C).

**Synthesis of fipronil-fluorescein conjugate *via* click chemistry**

In view of the difficulties to synthesize a stable fluorescently labeled fipronil derivative, we explored a third strategy using click chemistry, in particular the CuAAC reaction,[13,14] between fipronil and fluorescein derivatized with an acetylene and an azide linker, respectively. The concept of click chemistry was introduced by K. B. Sharpless in 2001 and it refers to reactions that are orthogonal, high yielding, and fast.[15] Moreover, in our case the presence of linkers allowed decreasing steric hindrance. For this purpose, fipronil initially reacted with a slight excess of propargyl bromide in the presence of potassium hydroxide (Scheme 3).[16] However, following a procedure reported in literature we isolated only the di-alkylated derivative **S1** (80% yield), as confirmed by $^{1}H$ and $^{13}C$ NMR and LC-MS (*m/z* = 513.0 $[M+H]^{+}$) (Figure S3). To solve this problem, we improved the published protocol. We increased the dilution to reach lower concentrations, reduced the amount of propargyl bromide to a sub-stoichiometric amount (0.9 eq.), and decreased temperature from rt to 0°C during the addition of propargyl bromide in order to avoid the formation of the di-alkylated

compound. Moreover, propargyl bromide was added dropwise as a solution in acetone for 15 min. Using these optimized conditions, we were able to obtain the desired mono-alkylated compound **7** in a reasonable yield (50%).




**Scheme 3.** Synthesis of fluorescein-fipronil derivative **9** through CuAAC.

In parallel, fluorescein was derivatized with an azide group. For this purpose, 5-carboxyfluorescein **6** was activated with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) and then reacted with 3-azidopropylamine to give the desired fluorescein-azide derivative **8** (Scheme 3).[17] Surprisingly, we discovered that the work-up reported in literature did not work in our case (addition of 10% aqueous NaOH followed by diethyl ether extraction). Therefore, we modified the work-up by acidification of the reaction mixture to reach pH = 2 using HCl 6N and extraction of the compound with ethyl acetate. The crude residue was purified by preparative HPLC to give the desired compound **8** in 15% yield. The low amount of isolated product can be explained by the difficulty to dilute the compound in a water miscible solvent that forced us to add DMF, resulting in an important loss of the compound in the HPLC injection peak.

The last step consisted in the CuAAC reaction between the two precursors previously synthesized. The reaction was performed in the presence of a catalytic amount of copper sulfate and ascorbic acid in 1:1 THF/water, leading to the formation of compound **9** in mild conditions and with 85% yield after purification by silica gel column (Scheme 3). Overall, the synthetic strategy based on CuAAC was longer (3 steps) compared to the other approaches (1

step) investigated in this work, but it resulted much more efficient. The stability of compound **9** was investigated by HPLC and we observed no degradation peak both in powder form and in solution over a period of 24 h. In addition, we stored the compound as a powder and in DMSO (1 mg/mL) at -20°C for more than six months without observing any degradation.

In conclusion we have investigated different chemical ligations to derivatize fipronil with fluorescein. The approaches relying on the formation of thiourea or amide bond between the two entities were not satisfying due to instability and low yield, respectively. The most efficient strategy was based on CuAAC reaction allowing to obtain a compound with high stability and not photosensitive. These properties ensure the relevance of *in vitro* and *in vivo* biological experiments. The fipronil-fluorescein conjugate will be used for cellular imaging for mechanistic investigation of fipronil activity. As we encountered many problems while trying to reproduce protocols reported in literature, we optimized conditions to solve these issues. Hence, we believe this work will be very helpful for the design of fipronil-based derivatives with high stability in biological assays.

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**Graphical Abstract**