3-Hydroxykynurenic acid: Physicochemical properties and fluorescence labeling

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ABSTRACT

Quinoline is one of the most important heterocyclic systems in life sciences. Some derivatives are normal metabolites, and others are used as antibacterial, antimalarial, and anticancer agents. In this work, we describe the synthesis, physicochemical properties, and fluorescence features of a new 4-quinolinone fluorophore, 3-hydroxykynurenic acid (3-HOKA). 3-HOKA was obtained by alkoxide-induced rearrangement of ethyl isatinacetate followed by acidification and then alkaline hydrolysis. The fluorescent compound was characterized by NMR, MS, IR, and UV-Vis spectra. 3-HOKA can exist under a keto-enol equilibrium, but the 4-quinolinone form is the predominant tautomer. In PBS (pH = 7.4), the anionic keto form of 3-HOKA showed a maximum absorption at 368 nm, a fluorescence peak at 474 nm, and a fluorescence quantum yield (Φ\textsubscript{F}): 0.73. 3-HOKA is photostable and a moderately weak oxygen generator. Viability assays on HeLa cells indicated that 3-HOKA did not induce significant cytotoxic effects. Under UV excitation, a bright blue fluorescence was selectively found in a singular body within the cytoplasm, a labeling pattern that suggests the possible localization of the probe in the centriole or related structures. Therefore, this novel fluorophore represents a promising prototype compound owing to its biocompatibility and potential biological applications.

1. Introduction

Fluorescent probes and among them quinoline-based structures, play a crucial role in bioimaging studies owing to their simple and rapid implementation, as well as their high sensitivity and high spatial resolution [1,2]. The use of fluorescent dyes easily functionalizable and compatible with biological experiments represents a distinctive feature for physiological research [3]. Several studies have used coumarin or quinoline scaffolds as central caged or fluorescent chemical cores to investigate small endogenous molecule functions [4]. These experiments remain limited and are highly dependent on both intrinsic biological activity and fluorescent properties of the probe (low cell and tissue penetration, biological side effects, decrease of natural function of studied molecules when labeled, among others). Moreover, the use of a small and biological inactive fluorophore coupled to the molecule of interest appears crucial to observe both its localization and its function in physiological conditions [5]. To be efficient, these fluorescent probes should be biocompatible (i.e., have low side effects at concentrations used for bioapplications) and have optimal fluorescent properties. In this context, quinolines appear quite attractive due to their relative synthetic versatility and subsequent tuning of molecular physicochemical properties [6]. For those reasons, quinoline is one of the most important heterocyclic systems, not only for its widespread inclusion in a variety of naturally occurring compounds [7], but also because many quinoline derivatives have found application in therapy, for example as antimalarial [8] and anticancer agents [9]. Quinoline-based frameworks also are key intermediates in assembling new materials with interesting electronic properties [10].

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Particularly, quinolin-4(1H)-one scaffold containing compounds represent extremely large group of organic substances that have been studied very extensively for many reasons [11]. Numerous dedicated articles can be found describing preparation and biological properties of variously substituted quinolin-4(1H)-ones. When the attention is focused on a variability in position 3 of the scaffold, most of them refer to the carboxy group bearing derivatives that became familiar particularly as very successful antimicrobial agents widely used in human therapy. On the other hand, substances bearing other functional groups (i.e., hydroxy or amino group) at the 3-position have been studied less frequently [12]. When the structure is further targeted to 3-hydroxyquinolin-4(1H)-one derivatives substituted with a carboxylate at position 2, the available information decreases rapidly [13]. Considering our experience in the synthesis and study of 3-hydroxyquinolin-4(1H)-one-2-carboxylic acid derivatives [14], a family of scarcely explored 4-quinolinones, we decided to study the physicochemical and biological properties of 3-hydroxy-4-oxo-1,4-di hy droquinoline-2-carboxylic acid (3-hydroxykynurenic acid, 3-HOKA), a new fluorophore.

2. Experimental section

2.1. Materials and methods

Melting points were taken on a Büchi capillary apparatus and are uncorrected. TLC analyses were carried out on silica gel 60 F254 aluminum sheets. Preparative thin layer separations (PLC) were carried out by centrifugally accelerated radial chromatography using Chromatotron model 7924 T. The rotors were coated with Silica Gel 60 aluminum sheets. Preparative thin layer separations (PLC) were carried out by centrifugally accelerated radial chromatography using Chromatotron model 7924 T. The rotors were coated with Silica Gel 60 F254 and the layer thickness was 2 mm. Chloroform and chloroform with increasing percentages of methanol were used as eluents. Reagents, solvents and starting materials were purchased from standard sources and purified according to literature procedures. The microwave-assisted reactions were carried out in a Reactor Microwave Digestion System WX-4000, EU chemical instruments. 1H spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer. 1H and 13C NMR spectra were measured in DMSO-d6 solutions on a Bruker Avance III 600 MHz spectrometer at 25 °C. Standard concentration of the samples was 10 and 20 mg/ml., respectively. Chemical shifts are reported in ppm (δ) relative to TMS as an internal standard. Coupling constant (J) values are given in Hz. Splitting multiplicities are reported as singlet (s), broad signal (br s), broad doublet (br d), doublet (d), triplet (t), quartet (q), multiplet (m), doublet doublet (dd), doublet doublet (ddd).

High-resolution 13C and 15N solid-state spectra for the 3-HOKA sample were recorded using the ramp 1H,13C or 1H,15N CP-MAS sequence (cross-polarization and magic angle spinning) with proton decoupling during acquisition using the SPINAL64 sequence (small phase incremental alternation with 64 steps). All the ss-NMR experiments were performed at room temperature in a Bruker Avance II-300 spectrometer equipped with a 4-mm MAS probe. The operating frequency for protons, carbons and nitrogens was 300.13, 75.46 and 30.42 MHz, respectively. Ammonium chloride was used as an external reference for the 15N spectra and glycine was used as an external reference for the 13C spectra. The non-quaternary suppression (NQS) and 2D1H-13C HETCOR [15] experiments were recorded as in previous reports [16]. The contact time for the CP was 200 μs to avoid relayed homonuclear spin-diffusion-type processes in the 2D experiment. The spinning rate for all the samples was 10 kHz.

MS (EI) were recorded with a GC-MS Shimadzu QP-1000 spectrometer operating at 70 eV. High resolution mass spectra (HMR-ESI-MS), were acquired with a model GCT (Waters, Milford, MA, USA), operating at 8000 resolving power (50% valley definition) using heptacose (m/z 219) as the reference compound. The HRMS-ESI spectra were acquired using a Bruker microTOF-QII instrument (Positive Ion Polarization).

Electronic absorption spectra were determined with a Shimadzu UV3600 PC spectrophotometer. Fluorescence spectra were monitored with a QuantaMaster Model QM-4 PTI spectrofluorometer. The emission spectra of 3-HOKA was collected at the maxima band excitation wavelength and recorded up to 700 nm. Fluorescence quantum yields (ΦF) were determined by comparison with 1,3-diphenylisobenzofuran (ΦF = 0.74 in ethanol) [17] as a reference at λexc = λmax. Calculations were performed by Equation (1), where R and S superscripts refer to the reference and the sample respectively, I is the integrated area under the emission spectrum, A is the absorbance of solutions at the excitation wavelength and stands for the refractive index correction.

$$\Phi_F = \frac{S_I}{R_I} (1 - 10^{-\epsilon_d}) \left( \frac{n^2}{n_f^2} \right)^2 \left( \frac{1}{10^4 - 10^{-\epsilon_d}} \right)$$

Standard chemical monitor bleaching rates were used to calculate the singlet oxygen (1O2) quantum yield (Φφ) [18]. For Φφ, studies, 3-HOKA was irradiated by light in the presence of imidazole and N,N-diethyl-4-nitrosoaniline (RNO) in 50 mM phosphate buffer saline pH = 7.4. 1O2 generated by photo-excitation of the sensitizer react with imidazole to form a transannular-type peroxide which bleaches RNO, and the bleaching can be monitored spectrophotometrically at 440 nm as a function of time. The reaction was carried out in 50 mM phosphate buffer saline solution (PBS), pH = 7.4 with imidazole (10 mM) and RNO (50 μM). The quantum yield for 1O2 generation was determined for 3-HOKA by measuring the relative rates of RNO bleaching under identical conditions after correcting for the incident light, using rose bengal as reference Φφ = 0.76 [19]. 3-HOKA and the reference were irradiated within the same wavelength interval λ1 - λ2 and Φφ was calculated according to Equation (2) [20], where r is the 1O2 photogeneration rate and the superscripts R and S stand for the sample and reference respectively, A is the absorbance at the irradiation wavelength and I0(λ) is the incident spectral photon flow (mol s⁻¹ nm⁻¹). When the irradiation wavelength range is narrow, the incident intensity varies smoothly with wavelength and the sample and reference have overlapping spectra, I, may be approximated by a constant value which may be drawn out of the integrals and cancelled. Experiments exciting 3-HOKA were performed in a polychromatic set-up using a projector lamp (Philips 7748SEHJ, 250 W).

$$\Phi_F = \frac{S_I}{R_I} (1 - 10^{-\epsilon_d}) \left( \frac{n^2}{n_f^2} \right)^2 \left( \frac{1}{10^4 - 10^{-\epsilon_d}} \right)$$

The photostability of 3-HOKA was analyzed in PBS by measuring the decrease in the intensity of the λmax over time irradiation with a visible light of 350–850 nm from a 250 W halogen lamp [21]. The fluence rate was adjusted to 20 mWcm⁻². Measurements were performed under air in PBS. Photodegradation rate constants k were calculated by Equation (3), where t, A0, A1 are their radiation time, absorbance at t = 0, absorbance at different times, respectively. 1,3-Diphenylisobenzofuran (DPBF), N,N-diethyl-4-nitrosoaniline (RNO), ethanol and PBS were available commercially and used without further purification.

$$\ln \frac{A_0}{A_1} = k t$$

2.2. Synthesis and physicochemical characterisation

2.2.1. Synthesis of 3-hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylic acid (3-HOKA) from kynurenic acid (KA)

a) Employing the methodology for the oxidation of phenols described by Elbs [22], 3-hydroxykynurenic acid (3-hydroxy-4-oxo-1,4-
2.2.2. Synthesis of 3-hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylic acid (3-HOKA) from ethyl isatinacetate (EIA)

Ethyl isatinacetate ([2,3-dioxo-2,3-dihydroindol-1-yl]acetic acid ethyl ester, EIA) was obtained following literature procedure [14c].

To a solution of sodium alkoxide prepared from sodium (350 mg, 15.2 mmol) in anhydrous ethanol (7.6 mL) under argon and heated in an oil bath (100−120 °C), EIA (3.8 mmol) was added as a powder in one portion. After 5–10 min the reaction mixture was poured into ice-HCl and extracted with CHCl₃ (3 × 10 mL). The organic layers were pooled, washed with water, dried with Na₂SO₄, filtered and evaporated in vacuo. Separation of the compounds obtained was achieved by centrifugal PLC. The first band eluted gave the ethyl 3-hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylate (65%). The slower moving band afforded traces of 3-HOKA.

A mixture of ethyl 3-hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylate (699 mg, 3 mmol) with 2N sodium hydroxide (3 mL) was heated on a water bath to 70 °C during 5 min. The solution was quickly cooled in an ice bath and by slow acidification with 10% HCl, 3-hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylic acid (3-HOKA) precipitates quantitatively.

2.2.2.3. Analytical data of the synthesized compounds

2.2.2.3.1. 3-Hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylic acid (3-HOKA). Light brown powder, easily detected by TLC due to intense light blue fluorescence under UV irradiation (256 nm) when it is adsorbed on chromatographic supports. Mp: 260−262 °C (propanone) lit. 261−262 °C [23,24]; [α]D: 2.2 and 3.9; IR (KBr): 3425, 3004, 1721, 1676, 1631, 1483, 1026, 774 cm⁻¹ among others; ¹H NMR (DMSO-δ₆): δ = 8.16 (d, J = 8.3 Hz, 1H, H-5), 8.12 (d, J = 8.5 Hz, 1 H, H-8), 7.73 (s, J = 8.2 Hz, 1H, H-6), 7.60 (d, J = 8.1 Hz, 1H, H-7), 7.22 (t, J = 8.1 Hz, 1H, H-6); ¹³C NMR (DMSO-δ₆): δ = 162.0 (C-3), 139.6 (C-6), 130.6 (C-2), 128.1 (C-7), 123.6 (C-5), 122.5 (C-4a), 119.1 (C-4), 110.9 (C-14a), 110.2 (C-8), 104.6 (C-9), 104.1 (C-10), 102.6 (C-11), 56.4 (CH₂), 13.1 (CH₃); HRMS-EI: m/z calcd. for C₁₂H₁ₑNO₄: 206.04478 [M+H⁺] +, found: 206.04400. (See Supporting Information).

2.2.2.3.2. 3-Hydroxy-4-oxo-1,4-dihydroquinoline-2-carboxylic acid ethyl ester. White crystals, mp: 243−244 °C (methanol); IR (KBr): 3300, 2930, 2824, 2997, 1237, 1120, 750 cm⁻¹ among others; ¹H NMR (DMSO-δ₆): δ = 12.74 (br s, 1H, OH), 12.50 (br s, 1H, NH), 7.80 (d, J = 8.1 Hz, 1H, H-5), 7.55 (d, J = 8.1 Hz, 1H, H-8), 7.34 (t, J = 8.1 Hz, 1H, H-7), 7.22 (t, J = 8.1 Hz, 1H, H-6), 4.40 (q, J = 7.1 Hz, 2H, CH₂), 1.37 (br s, J = 7.1 Hz, 3H, CH₃); ¹³C NMR (DMSO-δ₆): δ = 161.5 (C-3), 162.4 (C-4), 135.7 (C-8a), 129.2 (C-2), 126.7 (C-4a), 125.4 (C-7), 122.5 (C-6), 112.3 (C-5), 113.2 (C-8), 111.1 (C-3), 62.2 (CH₂), 14.1 (CH₃); HRMS-EI: m/z calcd. for C₁₁H₁₂NO₄: 233.068807 [M⁺] ²; found: 233.068470.

2.2.3. 3-Bromo-4-oxo-1,4-dihydroquinoline-2-carboxylic acid. Off-white powder, mp: 289−290 °C (methanol) lit. 290 °C [24]; ¹H NMR (DMSO-δ₆): δ = 11.93 (br s, 1H, NH/OH), 11.90 (br s, 1H, NH/OH), 8.04 (dd, J = 7.9, 1.5 Hz, 1H, H-5), 7.66 (br d, J = 8.2 Hz, 1H, H-8), 7.58 (ddd, J = 8.2, 6.8, 1.5 Hz, 1H, H-7), 7.26 (ddd, J = 7.9, 6.8, 1.2 Hz, 1H, H-6).

2.2.3.4. 3-Bromo-4-oxo-1,4-dihydroquinoline-2-carboxylic acid ethyl ester. White powder, mp: 251−252 °C (ethanol) lit. 250−251 °C [25]; ¹H NMR (DMSO-δ₆): δ = 12.77 (br s, 1H, NH), 8.13 (d, J = 8.2 Hz, 1H, H-5), 7.75−7.69 (m, 2H, H-7 and H-8), 7.40 (t, J = 8.2 Hz, 1H, H-6), 4.46 (q, J = 7.2 Hz, 2H, CH₂), 1.37 (br d, J = 7.2 Hz, 3H, CH₃); ¹³C NMR (DMSO-δ₆): δ = 164.2 and 161.9 (CO₂ and C-4), 140.8 and 138.5 (C-2 and C-8a), 132.9 (C-7), 125.3 (C-5), 124.9 (C-6), 123.4 (C-4a), 118.9 (C-8), 104.9 (C-3), 63.2 (CH₂), 13.8 (CH₃).

2.3. Biological tests

HeLa cells (human cervix adenocarcinoma ATCC®CCL-2™) were grown on 22 mm square coverslips placed into 35 mm culture dishes using Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum, 50 mg/mL streptomycin and 1% (v/v) 0.2 M L-glutamine (complete DMEM; all products from Gibco, Paisley, Scotland, UK). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂, and the culture medium was changed daily. Experiments were done during the exponential phase of cell growth.

A stock 1 mg/mL solution of 3-HOKA was prepared in PBS, filtered through a Millipore 0.22 μm filter (Merck SLG0P33R88), and then diluted with complete DMEM to reach the final concentrations: 10 μg mL⁻¹, 50 μg mL⁻¹, and 100 μg mL⁻¹. Cells were subjected to these 3-HOKA concentrations for 3 and 18 h in the dark, which were chosen as representative incubation times to analyze possible early and late effects. After 3-HOKA treatments and PBS washing, cells were again incubated for 24 h in the dark with complete culture medium, and then studies on the cell morphology and viability were performed. Cellular morphology was analyzed in living cells under phase contrast, differential interference contrast (DIC), or using bright-field microscopy after fixation with cold methanol (−20 °C) for 5 min [26], and with formaldehyde vapor in a moist chamber for different times [27]. Methanol-fixed cells were stained with 0.1% neutral red in distilled water for 2 min, washed, air-dried, and mounted in DePeX.

Cell viability was assessed by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [28]. Briefly, cell cultures were subjected to 0.05 mg/mL MTT in complete DMEM for 3 h, the medium was discarded, and the purple formazan product formed by reduction of MTT in viable cells was solubilized with pure DMSO. The formazan concentration was determined by absorption at 562 nm using a Synergy HT spectrophotometer (BioTek; Winooski, VT, USA). Cell viability was assessed by the ratio between the absorbance of treated cells and that of non-treated cells (control, 100% viability). Results were the mean values and standard deviation (SD) from at least three different experiments in triplicate. Statistical analysis was performed using one-way ANOVA with Tukey’s post-test.

Microscopic observations and photography were performed using an inverted microscope Leica DM IL LED (Wetzlar, Germany), equipped with a digital camera DFC420, phase contrast, and DIC optics. An epifluorescence microscope Olympus BX61 and a digital camera Olympus DP50 (Center Valley, PA, USA) were used for fluorescence observations and photography under UV excitation (365−390 nm), generally with an exposure time of 3 s. Image processing and analysis (IPA) was performed using Adobe Photoshop CC2017, and the free FIJI software ([http://fiji.sc/Fiji]).
molecule, obtaining information in the solid-state when a monocristalline sample is difficult to be obtained [16a,29]. For the 3-HOKA sample, both $^{15}$N and $^{13}$C CP-MAS spectra show that only one molecule per unit cell is present since only one nitrogen resonance signal is present ($\Delta^{15}$N = −222.4 ppm), while the $^{13}$C CP-MAS spectrum shows nine signals when this molecule has ten carbons (Fig. 2, Table 1). Additionally, the $^{15}$N resonance signal of the 3-HOKA was quite similar to the reported value in ciprofloxacin derivatives where the 4-quinolone forms are the predominant tautomer [29b]. The assignment of the $^{13}$C CP-MAS spectrum was done according to the NQS and solution-state NMR experiments presented here. In the NQS experiment, the resonance signals of the quaternary carbons are only observed allowing the assignment of the carbons bounded to hydrogens (C-5-8) in the $^{13}$C CP-MAS spectrum, however, the unequivocal assignment of the NMR signals was done according to the 2D $^1$H-$^1$H HETCOR experiment (Fig. 3). In this 2D experiments, the different $^{13}$C nuclei correlated with the protons that were directly linked or nearby (inset in Fig. 3), doing possible to study the predominant tautomeric form and the connectivity and disposition of the different functional groups in the 3-HOKA molecule. The indirect $^1$H dimension shows two set of signals at 7–8 and 12–13 ppm corresponding to the aromatic hydrogens and hydrogens bounded to heteroatoms, respectively. Particularly, the long-range interactions B, C, D and K were essential to assume that 4-quinolone form is the predominant tautomeric of 3-HOKA either in the solid state or in solution (Fig. 3). Moreover, the unequivocal assignment of the C-4 and the carboxylic acid was done considering that only the C-4 was close and interacted with the aromatic proton H-5 (correlation B, Fig. 3). The carboxylic acid carbon (C-9) was only near to its own acidic proton at 12.6 ppm (correlation A, Fig. 3). Furthermore, three different protons corresponding to the -OH (δ$^1$H = 13.1), -NH (δ$^1$H = 12.3) and -CO$_2$H (δ$^1$H = 12.6 ppm) were well-resolved in the 2D spectrum. Interestingly, the H-8 presents a higher proton chemical shift (δ$^1$H = 8.4 ppm) than the rest of the aromatic protons (δ$^1$H = 7.6–7.7 ppm) being different to those observed in solution-state NMR, allowing the correct assignment of C-8a and NH due to the long-range interaction D and C, respectively (Fig. 2). It is important to mention, that the $^1$H chemical shift of H-5-8 is particularly affected depending on the deuterated solvents used in the solution-state NMR experiments.

The absorption spectra in different media are like other kynurenic acid derivatives previously reported [30]. Due to the presence of ionizable groups on the quinoline ring, the location and the intensity of the bands are strongly affected by pH changes. In PBS (pH = 7.4), the maximum absorption is centered at $\lambda$ = 368 nm, whereas in DMF it is positioned at $\lambda$ = 386 nm (Fig. 4). This red shift indicates that H-bonding occurs between the oxygen of the solvent and the hydrogen of the 3-HOKA carboxylic function which is probably not ionized in DMF [31]. Based on the previous literature, $\lambda_{\text{max}}$ absorption band in neutral aqueous media has been assigned to the anionic keto form, and in other organic solvents such as DMF, the main contribution of the absorption band arises from the neutral keto tautomer species [32].

A single fluorescence band is observed (DMF or PBS) when 3-HOKA is excited at $\lambda_{\text{max}}$ suggesting that 3-HOKA exists only in the keto form in both media (Fig. 4). The fluorescence $\lambda_{\text{max}}$ of 3-HOKA in DMF is shifted ~8 nm to the red in comparison to the $\lambda_{\text{max}}$ observed in PBS, which is consistent with other previously studied [32]. 3-HOKA presented a high value of fluorescence quantum yield in DMF ($\Phi_F = 0.77 \pm 0.02$) as well as in PBS ($\Phi_F = 0.73 \pm 0.03$) which indicates the great potential of 3-HOKA as a fluorescence labeling agent. Using the $N,N$-dimethyl-4-nitrosouanine (RNO) bleaching method, 3-HOKA showed low values of singlet oxygen quantum yield ($\Phi_\Delta$) in DMF ($\Phi_\Delta = 0.06 \pm 0.002$) and PBS ($\Phi_\Delta = 0.04 \pm 0.001$), suggesting that this is a moderately weak oxygen generator. The photostability was analyzed in PBS. The decay time of the $\lambda_{\text{max}}$ followed a first-order kinetics. 3-HOKA showed a low value of k (0.0007), which indicates that it is stable during the irradiation time of our experiments. 

3. Results and discussion

3.1. Synthesis

3-HOKA can be obtained from the preformed 4-quinolinoine nucleus, by C-3 hydroxylation of commercially available kynurenic (KA) acid. Oxidation using peroxydisulfate in basic media (Elbs’ reaction) [22] afforded poor 3-HOKA yields (28%). Alternatively, the halogenation-hydrolysis sequence [24] failed. On the other hand, by alkoxide-induced rearrangement of ethyl isatinacetate (EIA) with 4 equivalents of hydrolysis sequence [24] failed. On the other hand, by alkoxide-in-
3.3. Cell viability assay

No morphological changes were observed in living HeLa cells treated with different concentrations of 3-HOKA after short (3 h) or prolonged times (18 h). Cell cultures showed a completely normal morphology and the cell density was the same as that of untreated controls (Fig. 5). Mitotic cells were also clearly detected even at the highest 3-HOKA concentration and treatment time. Likewise, different 3-HOKA treatments do not produce significant cytotoxic effects as revealed by the MTT assay, and the cell viability appeared very well conserved (Fig. 6).

3.4. Application in cell imaging

In HeLa cells treated for 3 h with 3-HOKA at any concentration only a blue signal in granular components resembling lysosomes was sometimes observed, and likewise, poorly resolved mitochondrial structures were also detected, but in both cases the weak signal and diffuse cytoplasmic emission hindered their precise visualization. However, after 18 h treatment with 3-HOKA, a perinuclear peripheral body with high blue fluorescence could be very often visualized within the cytoplasm of living cells (Fig. 7). Granular, crystallloid, arrowhead, cross- or L-shaped forms were the most frequent aspect of this body. Because its appearance, this structure will be referred as the “singular body”. It was clearly observed using the three 3-HOKA concentrations, but in untreated controls or in cells treated for only 3 h this body was not found.

After treatments for 3 or 18 h, the 3-HOKA fluorescence showed considerable fading, and therefore prolonged observations under UV exciting light could be not performed. On the other hand, a fluorescent structure, very like the singular body, was often observed in the intercellular bridge connecting two daughter cells after mitosis (Fig. 8). It is known that this bridge contains the persisting midbody formed by remaining spindle microtubules, which appear interdigitated and tightly packed together with a dense matrix material [33]. Attempts made for fixing treated cells to preserve the fluorescent 3-HOKA signal were unsuccessful. The fixation with formaldehyde vapor for 1 min greatly reduced or abolished the 3-HOKA emission of the singular body, whereas fixing for 5 or 10 min generated too much formaldehyde-induced fluorescence that obliterated any other signal. However, because

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Table 1

$^{13}$C NMR chemical shifts of 3-HOKA observed in solid state and in solution.

<table>
<thead>
<tr>
<th>NMR experiments</th>
<th>Chemical shifts (ppm)</th>
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<tbody>
<tr>
<td></td>
<td>C-2</td>
</tr>
<tr>
<td>Solution</td>
<td>143.9</td>
</tr>
<tr>
<td>Solid-state</td>
<td>133.6*</td>
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Fig. 2. $^{13}$C CP-MAS (A), NQS (B) and $^{15}$N CP-MAS (C) spectra for the 3-HOKA.
Fig. 3. 2D $^1$H–$^{13}$C HETCOR spectrum for the 3-HOKA sample.

Fig. 4. Normalized UV–Vis absorption and emission spectra of 3-HOKA (12 μM) in DMF and PBS (pH = 7.4).
the low density of the cell periphery, the fluorescent body located in the connecting region between daughter cells could be sometimes recognized. After methanol fixation, no 3-HOKA signal was conserved within cells. The occurrence of a diffuse blue emission induced by 3-HOKA in the cytoplasm of living cells did not allow the precise recognition of lysosome-like granules and mitochondrial autofluorescence. In this context, it is also noteworthy that under UV excitation, the well-known blue nuclear emission of DAPI- and Hoechst 33342-labeled live cells also hindered the visualization of NADH-dependent mitochondrial autofluorescence [27,34].

Fluorescent vital probes are now increasingly used in microscopic cell and molecular biology studies in the field of biomedical sciences [1,35]. Cell organelles and structures such as lysosomes, mitochondria, Golgi apparatus, endoplasmic reticulum, plasma membrane, chromatin, nucleoli, lipid droplets, etc., are currently visualized in live cells using fluorescent probes belonging to different heterocyclic derivatives (i.e., coumarin, acridine, azine, xanthene, oxazine, carbocyanine, BODIPY, etc.) [35]. Therefore, the fluorescence of 3-HOKA was considered adequate to search for some possible localization within live cells. A bright blue emission in the structure named singular body was clearly visualized after 18 h treatment with 3-HOKA. Although morphological criteria are valuable to identify unambiguously cell structures, in the present case this assignment was a difficult task. It is worth to note the singular character of this body, which is in contrast with the occurrence of multiple cytoplasmic organelles such as lysosomes, mitochondria, peroxisomes, Golgi dictyosomes, lipid droplets, etc. As formaldehyde or methanol fixation prevents the 3-HOKA fluorescence, additional immunocytochemical methods to identify the singular body could be not applied.

The significance of this intriguing cell component is unknown and deserves further investigation, but it is tempting to speculate that because morphological features such as the number, position, size and shape, this peculiar structure could represent the pair of centrioles (∼0.7 μm in length and ∼0.2 μm in width). Some occasional images

![Fig. 5. DIC images of cells untreated (control) and treated with 3-HOKA at different concentrations and for different times. No morphological evidence of cell damage can be seen. Arrows indicate metaphase cells, showing the equatorial plate as a steep central bar.](image1)

![Fig. 6. Cell viability assessed by the MTT method after 3 h (A) and 18 h (B) treatment with 3-HOKA at different concentrations, showing the absence of significant cytotoxic effects.](image2)
showed an almost orthogonal configuration of 2 bars (Fig. 7A, inserts), which strongly suggests the typical geometry of the centriolar pair. Fluorescence fading, inadequate position, and/or out of focus images would result in a bad or no visualization of this body in some cells. Round halos or large granules could also correspond to the centrosome, which is formed by amorphous pericentriolar materials, and behaves as the microtubule-organizing center. Centrosomes have been visualized as round bodies by using antibodies to centrosomal proteins and immunofluorescence microscopy [36].

However, no reports have been found regarding the simple and direct fluorescent visualization of centrioles or centrosomes by small molecular vital probes. It is noteworthy that some morphological features of the centriole, such as its perinuclear position or location near the plasma membrane are shared by the singular body, which could suggest a correspondence between the two structures. Centrioles can move from sites near the nuclear envelope to the cell surface forming the basal bodies of cilia and flagella [37]. Interestingly, during the meiotic division in fruit fly spermatocytes, the distal part of the centriole is first surrounded by a membranous sheath and then protrudes from the cell surface covered by the plasma membrane to form the sperm flagellum [38]. Regarding 3-HOKA, our observations could be related with some effects of tryptophan metabolites along the kynurenine pathway. The treatment of cultured neurons with quinololnic acid, a compound generated from kynurenic acid, induced microtubular
damage [39]. Kynurenic acid also interferes with the organization and maintenance of the cytoskeletal intermediate filaments [40]. However, in our case no microtubules or other cytoskeletal components were labeled after 3-HOCA treatment, which indicates that this compound has different binding properties and effects compared with those tryptophan derivatives. Further investigations to identify precisely the cell structure labeled by 3-HOCA fluorescence are in course.

4. Conclusion

The 3-HOCA could be a new probe for the labeling of centrioles, with special application to the study of flagellated microorganisms, especially taking into account the null cytotoxicity of the compound. Therefore, this novel fluorophore represents a promising prototype compound owing to its biocompatibility and potential biological applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dyepig.2018.10.027.

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