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Emmanuelle Rémond, Jean-alain Fehrentz, Laure Liénart, Sébastien Clément, Jean-louis Banères, et al.. Fluorescent P-Hydroxyphosphole for Peptide Labeling through P-N Bond Formation. Chemistry - A European Journal, In press, 28 (47), 10.1002/chem.202201526 . hal-03736506

HAL Id: hal-03736506

<https://hal.umontpellier.fr/hal-03736506>

Submitted on 26 Jul 2022

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Fluorescent P-hydroxyphosphole for peptide labeling through P-N bond formation

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Dedication ((optional))

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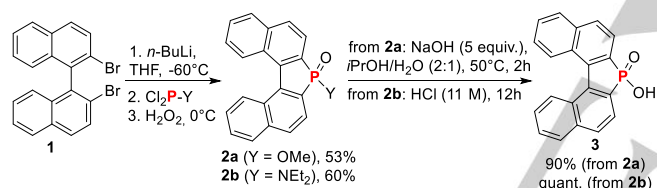
Abstract: Synthesis of fluorescent P-hydroxybinaphthylphosphole-oxide or -sulfide was achieved by trapping a binaphthyl dianion with methyl dichlorophosphite or P-(N,N-diethylamino)dichlorophosphine, followed by oxidation or sulfuration of the P-center. After saponification or acid hydrolysis, the P-hydroxyphospholes were coupled to peptides using the coupling agent BOP, under the conditions required for the synthesis in solution or on a solid support. This new method was illustrated by the labeling of the JMV2959, a potent antagonist of the Growth Hormone Secretagogue Receptor type 1a (GHS-R1a). The labeled conjugates were used to characterize GHSR ligands by competition assays, based on Fluorescence Resonance Energy Transfer (FRET). Such P-hydroxyphosphole-oxide or -sulfide constitutes a promising new class of compact fluorophores with large Stokes shift, for labeling biomolecules by grafting through the phosphorus atom.

Fluorescence is a powerful and versatile spectroscopic method for investigating molecular and biological interactions or cellular processes.¹ Today, the development of fluorescence techniques is continually renewed by advances in instrumentations,² the development of modular fluorophores and by methods for labeling biomolecules.^{3,4} In a recent past, much effort has also been devoted to the development of optoelectronic devices, optical imaging for diagnosis and guided-treatment exploiting a fluorescence signal, which should not interfere with autofluorescence of human tissue.⁵ Nowadays, the use of fluorophores depends on their physicochemical and optical properties, the nature of the targeted biomolecules and the type of imaging, *in vitro*, extra- or intra-cell.^{3,4} In this field, developing functionalized red-emitting small dyes with a large Stokes shift for convenient biomolecules labeling is a topical subject, either to minimize perturbations of the native biochemical processes, to avoid light reabsorption or to improve sensitivity.²⁻⁴ For this purpose, phospholes are attractive π -conjugated heterocycles, although they are mainly developed for optoelectronic devices, due to their unique electroluminescent properties.⁶ The phosphole core is a weakly aromatic five-membered heterocycle, having the characteristics of a dienic system bridged with a tetrahedral phosphorus atom. The specific photophysical properties of

phospholes are due to an unusual intramolecular charge transfer (ICT) of the excited state toward a hybrid LUMO, inducing a large Stokes shift, even in the case of emission of lower energies at high wavelengths.⁶ To date, the use of fluorescent phospholes in bioimaging is rare, in spite of their relatively low molecular weight, their absence of known toxicity and their high photochemical stability in regard to other classes of fluorophores.⁷⁻¹⁰ Recently, phosphole-based fluorophores bearing electron-withdrawing and -donating groups were reported to discriminate the cellular environment in adipocytes, according to the fluorescence emission color.⁷ The photostability of such scaffold was increased by developing structurally rigid phospholes having a diarylmethylene unit, bridged to the naphthophosphole-oxide (C-Naphox).⁸ The practical utility of a C-Naphox derivative functionalized by an N-hydroxysuccinimide (NHS) ester was reported for bioconjugation to antibodies for immunolabeling.⁹ In addition, non-fused pyridyl- and thienyl-substituted phospholes whose emission maxima depends on the polarity of the surrounding environment were also used to visualize lipid droplets in living cells.¹⁰ While most fluorophores are suitably functionalized to site-selective label biomolecules such as peptides and proteins, the use of fluorescent dyes bearing a phosphorus group for bioconjugation has been barely exemplified.¹¹ In pioneering work, Horner *et al.* reported an aminonaphthyl phosphinic fluoride reagent to label serine with emission up to 400 nm.¹² More recently, hydroxyamino acids like serine, threonine or tyrosine, have been labeled with a phosphoramidite linker bearing a (diethylamino)coumarine moiety, or with a phospholyl chloride by formation of P-O bonds.^{13,14} Finally, we and others have described fluorescent phospholes derived from homoalanine or phenylalanine by formation of P-C bonds, involving stannylphosphole reagents or phospholide anions obtained by cleavage with a metal of the P-phenyl bond starting from the corresponding phenylphospholes, respectively.^{14,15} As the above strategies and the functionalization of phospholes require severe conditions, this constitutes an unquestionable limitation for their design and for their use to label biomolecules.¹⁶ For these reasons, we decided to investigate the grafting of phosphole-based fluorophore through the P-center

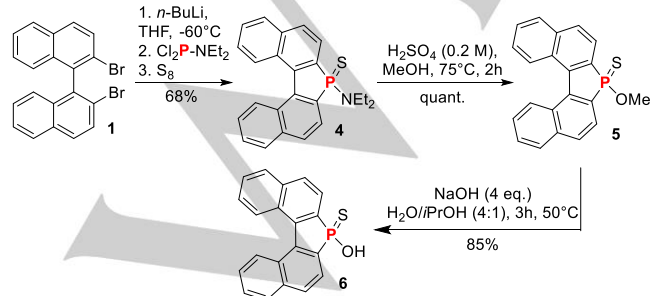
COMMUNICATION

under mild conditions. Herein, we report the synthesis of a new class of P-hydroxyphosphole-oxide fluorophores and their use in coupling to biomolecules by their amine functions. The coupling was achieved by activation of the P-hydroxyphosphole with the BOP as coupling agent, under required conditions for Solution Phase Synthesis (SPS) and Solid-Phase Peptide Synthesis (SPPS). This simple and new method was exemplified by the labeling of JMV2959, which is a potent antagonist of the Growth Hormone Secretagogue Receptor type 1a (GHS-R1a). Finally, binding assay of GHS-R1a based on Fluorescence Resonance Energy Transfer (FRET) with labeled ligands was demonstrated. The P-hydroxyphosphole-oxide **3** was synthesized in two steps starting from the commercially available 2,2'-dibromobinaphthyl precursor **1**, following a modified literature procedure.^{17,18} After metal-halide exchange using two equivalents of *n*-butyllithium (*n*-BuLi), the binaphthyl dianion was quenched with the methyl dichlorophosphite, followed by addition of hydrogen peroxide (H₂O₂) to afford a mixture of the P-methoxy- and P-hydroxyphosphole derivatives **2a** and **3**. The P-methoxybinaphthylphosphole-oxide **2a** was isolated in 53% yield, then saponified into the corresponding P-hydroxy derivative **3** in 90% yield. Alternatively, quenching the binaphthyl dianion with the (N,N-diethylamino)dichlorophosphine followed by oxidation with H₂O₂, led to the P-(N,N-diethylamino)binaphthylphosphole-oxide **2b** in 60% isolated yield. Finally, acid hydrolysis of **2b** using concentrated HCl allowed us to afford the P-hydroxybinaphthylphosphole-oxide **3** quantitatively (Scheme 1).



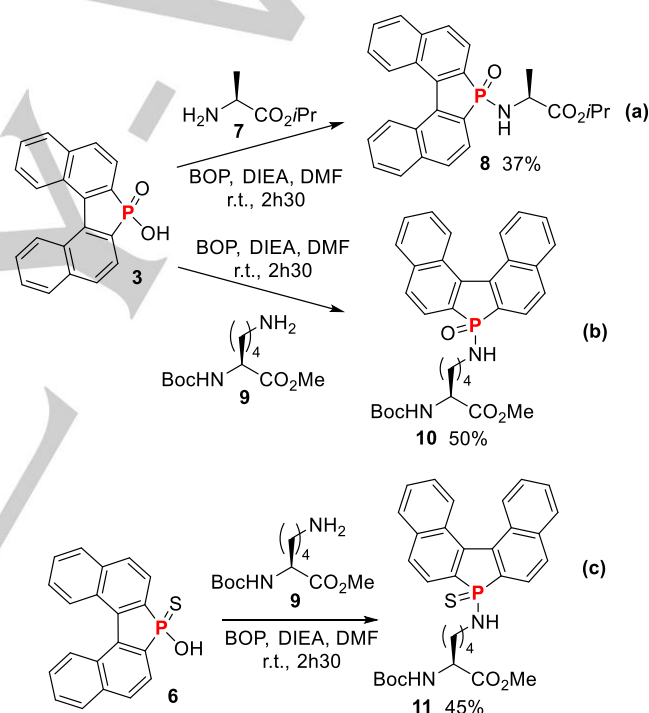
Scheme 1. Synthesis of P-hydroxybinaphthylphosphole-oxide **3**.

The P-hydroxybinaphthylphosphole-sulfide **6** was prepared in three steps starting from the 2,2'-dibromobinaphthyl **1** (Scheme 2). The binaphthyl dianion was firstly trapped with P-(N,N-diethylamino)dichlorophosphine, and sulfur was added to lead to the phosphoramido-sulfide **4** in 68% yield. After acid-catalyzed methanolysis, the corresponding P-methoxyphosphole-sulfide derivative **5** was quantitatively obtained. Finally, the P-hydroxybinaphthylphosphole sulfide **6** was prepared in 85% yield by saponification of **5** (Scheme 2).



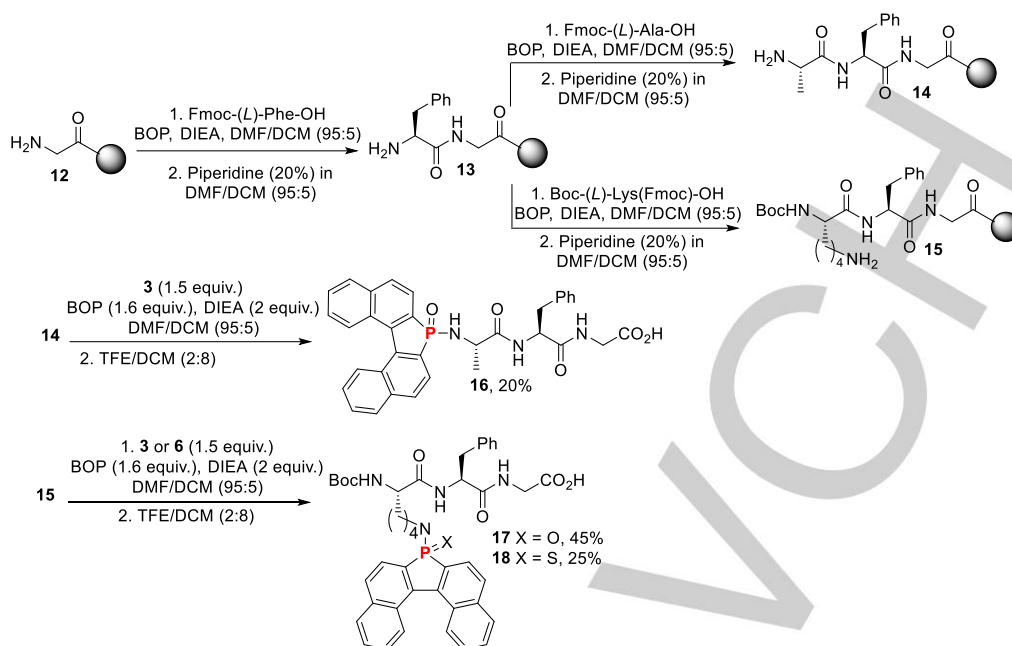
Scheme 2. Synthesis of P-hydroxybinaphthylphosphole-sulfide **6**.

First, the coupling of P-hydroxybinaphthylphosphole-oxide **3** with the isopropyl alaninate **7** or with the N-Boc-lysine methyl ester **9** was investigated in solution in DMF using the BOP reagent in the presence of N,N-diisopropylethylamine (DIEA).¹⁹ When the isopropyl L-alaninate hydrochloride salts (H-Ala-OⁱPr.HCl) were used in coupling with **3**, only low conversion was observed. By contrast, when the reaction was performed with H-Ala-OⁱPr **7** bearing the free amino group in α -position, the corresponding phosphoramidate **8** was obtained in 37% yield (Scheme 3a). Under these conditions, the reaction of P-hydroxybinaphthylphosphole-oxide **3** with Boc-Lys-OMe **9** bearing a free amino group in terminal position on the lateral lysine chain led to the compound **10** in 50% yield (Scheme 3b). Likewise, coupling the P-hydroxybinaphthylphosphole-sulfide **6** in solution with the Boc-Lys-OMe **9** was investigated in the presence of BOP and DIEA (Scheme 3c). Under these conditions, it is worth noting that only the corresponding phosphole-sulfide lysine derivative **11** was observed and isolated in 45% yield, proving that the coupling was achieved by activation of the P-OH bond.



Scheme 3. Coupling the P-hydroxyphosphole-oxide **3** or -sulfide **6** to amino acids in solution.

The coupling to a model peptide grafted on solid support was envisaged in order to demonstrate the compatibility of the P-hydroxyphosphole in regard to solid phase peptide synthesis (SPPS) conditions. The model tripeptides **14** and **15** obtained by SPPS at a 0.376 mmol scale starting from the H-Gly preloaded 2-ClTrt resin **12**, were subjected to two cycles of coupling with a solution of P-hydroxybinaphthylphosphole-oxide **3** or P-hydroxybinaphthylphosphole-sulfide **6**, BOP reagent and DIEA (Scheme 4). Then, the cleavage of the supported tripeptides was performed in the presence of trifluoroethanol (TFE) as weak acid



Scheme 4. Coupling P-hydroxyphosphole-oxide **3** or P-hydroxyphosphole-sulfide **6** to the tripeptides **14** and **15** on solid support.

in dichloromethane in a ratio (2:8), to prevent cleavage of the P-N bond, since we observed degradation into the non-labeled tripeptide and P-hydroxyphosphole oxide **3** using strong acid conditions TFA/TIS (97:3). After precipitation, the labeled tripeptides **16**, **17** and **18** in the crude product, were characterized by LCMS and the yield of coupling was determined by measuring their UV-Visible absorption spectra. The tripeptides **16** and **17**, resulting from the coupling to alanine and lysine lateral chain were obtained in 20% and 45% yield respectively, which is consistent with the results obtained for the coupling in solution. Finally, the coupling of the P-hydroxyphosphole sulfide **6** to the tripeptide **15** on solid support was achieved in 25% yield (Scheme 4). The photophysical properties of the binaphthylphosphole derivatives **2-6** and the coupling phospholamides products **8**, **10** and **11** were then examined by UV-visible absorption and fluorescence spectroscopies (Figure 1). All photophysical data are summarized in Table 1. The maximum absorption wavelength (λ_{\max}) values of

phospholes **2-6** and phospholamides **8**, **10** and **11** in CH_2Cl_2 are rather similar, with λ_{\max} around 385-390 nm (Figure 1) and molar absorption coefficients (ϵ) ranging from 1400 to 5000 $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (Table 1). Their emission spectra in CH_2Cl_2 were recorded by excitation in their higher wavelength absorption band ($\lambda_{\text{ex}} = 385-390$ nm). Binaphthylphosphole compounds **2-4** and **8-11** exhibit a fluorescence emission around 465 nm (Table 1, entries 1-4 and 7-9). In contrast, a red shift was observed for the P-methoxy and the P-hydroxybinaphthylphospholes-sulfide **5** and **6** at $\lambda_{\text{em}} = 475$ and 485 nm, respectively (entries 5, 6). The quantum yields depend on the nature of the P-modification. Indeed, compared to the relatively high quantum yield of the binaphthylphosphole oxides **2a,b**, **3**, **8** and **10** ($\Phi_{\text{f}} = 0.13-0.15$; entries 1-3, 7 and 8), the Φ_{f} values for the sulfide derivatives **4-6** and **11** are lower ($\Phi_{\text{f}} = 0.01-0.02$) (entries 4-6, 11) probably due to the heavy atom effect arising from the sulfur atom.

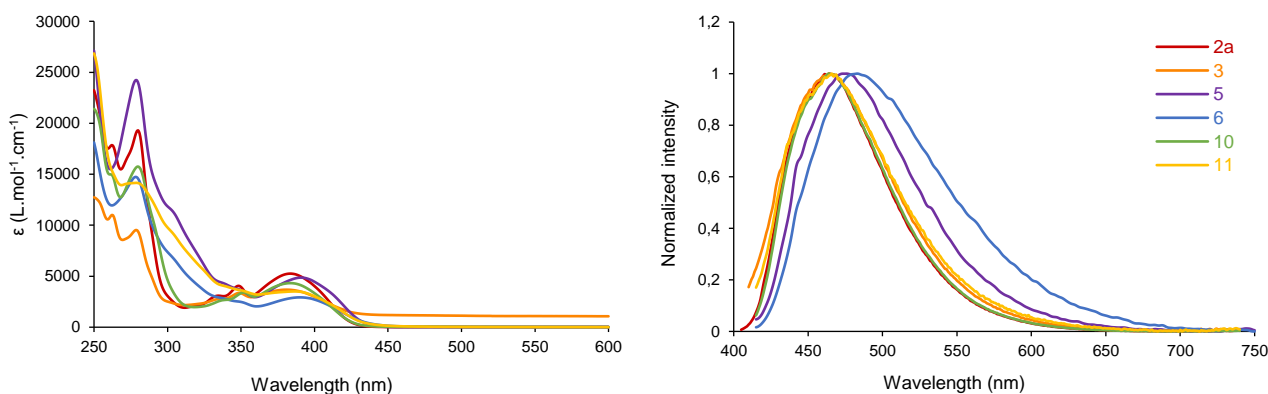


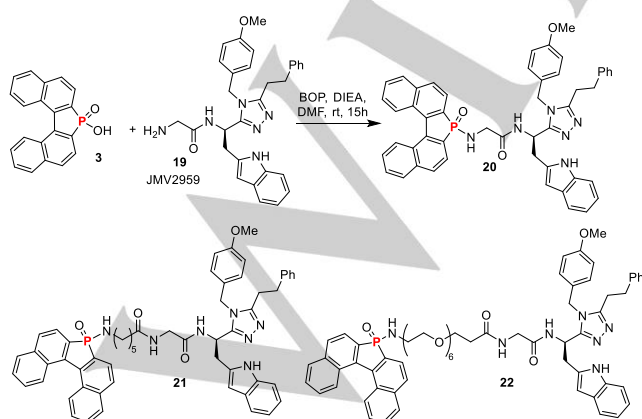
Figure 1. (A) UV-Visible Absorption and (B) normalized fluorescence spectra for selected compounds in CH_2Cl_2 (2×10^{-5} M).

entry	Compound	λ_{abs} (nm) (ϵ (L.mol ⁻¹ .cm ⁻¹))	λ_{ex} (nm)	λ_{em} (nm)	Stokes shift (nm)	Φ_{f} (%)
1	2a	280 (19000), 335 (sh) (2970), 348 (3870), 384 (4990)	385	465	81	0.15 (\pm 3%)
2	2b	280 (17300), 354 (sh) (3200), 385 (4540)	385	465	80	0.13 (\pm 4%)
3	3	278 (8060), 349 (3400), 380 (3750)	380	464	84	0.14 (\pm 4%)
4	4	274 (7850), 304 (5150), 390 (1410)	390	464	74	0.01 (\pm 15%)
5	5	279 (2490), 306 (sh) (10900), 391 (4890)	390	475	84	0.01 (\pm 15%)
6	6	278 (14400), 390 (2970)	390	485	95	0.02 (\pm 7%)
7	8	281 (16600), 352 (3270), 386 (4320)	385	468	82	0.15 (\pm 3%)
8	10	280 (14900), 352 (3410), 384 (4320)	385	464	80	0.15 (\pm 4%)
9	11	279 (13800), 387 (3470)	385	466	79	0.02 (\pm 3%)

Table 1. Photophysical data of binaphthylphospholes-oxides or sulfide **2a,b-6** and the coupling products **8, 10, 11**.

Exploiting the experience of our Institute in the search of ghrelin receptor ligands for many years, we decided to use lead compound JM2959, a potent antagonist of the Growth Hormone Secretagogue Receptor type 1a (GHS-R1a) to demonstrate the interest of the P-hydroxyphosphole fluorophore and its new method of coupling. Ghrelin, the natural ligand of GHS-R1a, is an orexigenic hormone controlling, among others, growth hormone secretion, food intake and reward seeking behaviors.²⁰ It is a subject of great interest to develop agonists and antagonists of its receptor. Compound JM2959 **19** is a trisubstituted 1,2,4-triazole bearing a glycol group,²¹ that we labeled by coupling the P-hydroxybinaphthylphosphole-oxide **3** in SPS, using the BOP as coupling reagent in the presence of DIEA (Scheme 5). Under these conditions, the hydroxyphosphole-oxide **3** was successfully coupled to the JM2959 to afford the N-binaphthylphosphoryl-oxide conjugate **20** (Scheme 5). The conjugates **21** and **22** were obtained by coupling **3** to JM2959 **19** using the N-(6-amino)hexanoyl or the N-(2-aminoethoxy)pentaethoxypropanoyl spacers, respectively (Scheme 5). The labeled ligands **20-22** were purified by reverse-phase preparative HPLC using formic acid in the mobile phase.²² The purities assessed by analytical reverse C18 HPLC were found to be over 97% and their structures were confirmed by MS (electrospray).

The binding of the fluorescently-labeled antagonists **20-22** to the ghrelin receptor GHS-R1a was investigated by Förster Resonance Energy Transfer (FRET). Specifically, the fluorescent binaphthylphosphoryl-oxide moiety of the ligands **20-22** was used as a donor, whereas the AF488 dye attached to the N-terminus moiety of the recombinant GHSR assembled into lipid nanodiscs,²³ was used as an acceptor. To be noted, we used this fluorophore as an acceptor, because all the conditions for efficient labeling of the receptor were already set up for this probe.²⁴ Although not best adapted for an optimal FRET signal with the binaphthylphosphoryl-oxide moiety due to spectral overlap, the pair nevertheless provided a robust and reproducible signal that allowed unambiguously monitoring of the binding of the labeled antagonist and fully relevant and accurate quantitative parameters - K_d and K_i values - to be assessed. Under these conditions, a significant FRET signal was observed with conjugates **21** and **22**, *i.e.* when the binaphthylphosphoryl-oxide moiety was attached to the ligand through a spacer (Figure 2).



Scheme 5. Synthesis of the JM2959 conjugates **20-22**.

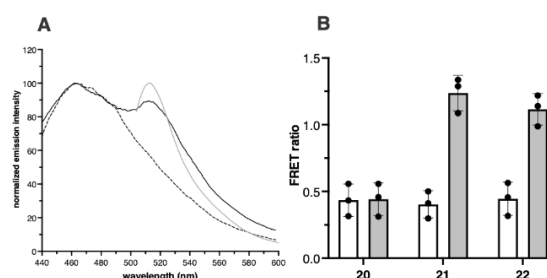


Figure 2. Binding of the conjugates to the ghrelin receptor. (A) normalized fluorescence emission spectra of conjugate **22** in the absence (dotted line) or in the presence (plain line) of the recombinant labeled with AF488 at its N-terminus. The emission spectrum in gray was obtained after direct excitation of AF488 at 500 nm. (B) FRET ratio between the labeled receptor and 10 μ M of the conjugates **20-22**. The FRET ratio was calculated as described in the Material and Methods section.

This signal reflected the proximity between the two fluorophores in the receptor:ligand complex, and therefore, strongly suggests that both compounds bind to the isolated receptor. In contrast, no significant FRET signal was obtained with the conjugate **20** whose binaphthylphosphole was directly bound to the glycol moiety of JMV2959. Whether the absence of FRET signal with this conjugate is due to the absence of linker being associated with an unfavorable geometry for FRET or to an impact of the fluorophore moiety on the binding properties of JMV2959 is an open question at this point. We further performed a FRET-monitored binding assay with conjugates **21** and **22**. The binding plots obtained (Figure 3A) indicate that compounds **21** and **22** specifically bind to the isolated receptor with an apparent affinity of 128.1 ± 21.1 and 317.5 ± 54.1 nM, respectively. These values are in the same range than those obtained for unlabeled JMV2959 ($K_i = 64$ nM; Figure 3B). Finally, to assess whether the labeled compounds could be used for evaluating the binding properties of GHSR ligands, we carried out a series of FRET-monitored competition experiments using the labeled conjugate **21** and unlabeled ligands of known pharmacological profile, *i.e.* the full agonist MK0677, the inverse agonist SPA and the neutral antagonist JMV3011.²⁵ As shown in Figure 2B, well-defined competition plots could be obtained under such conditions with K_i values in the same range than those previously assessed with a different fluorescent tracer,²⁵ suggesting that the labeled conjugate is fully adapted for binding assays aimed at characterizing GHSR ligands through a competition with an antagonist.

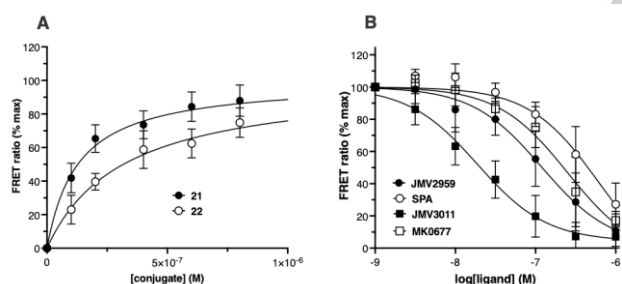


Figure 3. Binding properties of conjugates **21** and **22**. (A) FRET-monitored assay for binding of conjugates **21** and **22** to GHSR in nanodiscs labeled with AF488 at its N-terminus. (B) FRET-based competition assay between conjugate **21** and unlabeled ligands for binding GHSR. In all cases, data are mean \pm SD of three experiments.

In summary, the first examples of P-hydroxybinaphthylphosphole-oxide and -sulfide as new class of fluorophore ready to couple to amino acids or peptides by the phosphorus atom, are described. The synthesis was achieved by metal halide exchange from 2,2'-dibromobinaphthyl, addition of dichlorophosphinyl reagent, oxidation with H_2O_2 or sulfuration, followed by basic or acid hydrolysis. Very interestingly, the coupling of the P-hydroxybinaphthylphosphole-oxide with amino groups has been achieved in SPS and SPPS using the coupling agent BOP, to afford the corresponding N-binaphthylphospholyl-oxide amino acid or peptide derivatives by formation of a P-N bond. This new method of coupling phosphole-based fluorophores was applied to the labeling of JMV2959, an antagonist of the Growth Hormone Secretagogue Receptor type 1a (GHSR). The binaphthylphospholyl-oxide was successfully coupled to the glycol moiety of JMV2959 directly, or through spacers. In the present

case, FRET-based binding assays involving the resulting conjugates and recombinant GHSR demonstrate that the presence of a spacer is required to maintain high binding affinity. Besides, exploiting the chemistry of 2,2'-dihalogenobiaryl precursors to design P-hydroxyphospholes as compact fluorophores with large Stokes shift to provide a labeled antagonist tracer for binding assays with the ghrelin receptor, also opens an innovative breakthrough to label peptide and others biomolecules, by grafting through the phosphorus-atom.

Acknowledgements

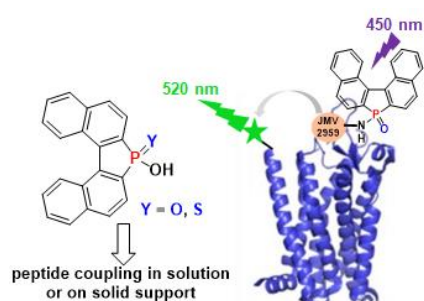
We acknowledge Agence Nationale de la Recherche (ANR, project ANR-18-CE07-0019-01) for the financial support. The LCMS and NMR analyses were performed at the Plateforme Technologique Laboratoire de Mesures Physiques and IBMM analytical facilities.

Keywords: Fluorescent P-hydroxyphospholes • phosphoramidate • peptide coupling • FRET • Fluorescent labelled GHSR ligands

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A straightforward synthesis of new P-hydroxyphosphole-oxide and -sulfide based fluorophores exhibiting large Stokes shifts, and their coupling to biomolecules through the formation of P-N bond is presented herein. The fluorescent P-hydroxybinaphthylphosphole-oxide was coupled to a ghrelin receptor (GHSR) antagonist and the binding of the conjugates was highlighted by Förster Resonance Energy Transfer (FRET).