A Rapid and Versatile Method to Label Receptor Ligands Using "Click" Chemistry: Validation with the Muscarinic M1 Antagonist Pirenzepine

ARTICLE *in* BIOCONJUGATE CHEMISTRY · NOVEMBER 2006

Impact Factor: 4.51 · DOI: 10.1021/bc060140j · Source: PubMed

6 AUTHORS, INCLUDING:

Dominique Bonnet
University of Strasbourg
58 PUBLICATIONS **702** CITATIONS
SEE PROFILE

Jean-Luc Galzi
Centre National de Recherche Scientifique et…
100 PUBLICATIONS **5,226** CITATIONS
SEE PROFILE

Stéphanie Riché
University of Strasbourg
7 PUBLICATIONS **63** CITATIONS
SEE PROFILE

A Rapid and Versatile Method to Label Receptor Ligands Using "Click" Chemistry: Validation with the Muscarinic M1 Antagonist Pirenzepine

Dominique Bonnet,*[,†] Brigitte Ilien,*[,‡] Jean-Luc Galzi,[‡] Stéphanie Riché,[†] Cyril Antheaune,[§] and Marcel Hibert[†]

Département de Pharmacochimie de la Communication Cellulaire, Département Récepteurs et Protéines Membranaires, and IFR85, Service commun de RMN, Institut Gilbert Laustriat, UMR 7175-LC1 ULP/CNRS, Faculté de Pharmacie de Strasbourg, 74 Route du Rhin, 67401 Illkirch, France. Received May 29, 2006; Revised Manuscript Received July 28, 2006

Tagged biologically active molecules represent powerful pharmacological tools to study and characterize ligand−receptor interactions. However, the labeling of such molecules is not trivial, especially when poorly soluble tags have to be incorporated. The classical method of coupling usually necessitates a tedious final purification step to remove the excess of reagents and to isolate tagged molecules. To overcome this limitation, Cu(I)-catalyzed 1,3-dipolar cycloaddition, referred to as "click" chemistry, was evaluated as a tool to facilitate the access to labeled molecules. In order to validate the approach, we focused our attention on the incorporation of a fluorophore (Lissamine Rhodamine B), a nonfluorescent dye (Patent Blue VF), or biotin into a muscarinic antagonist scaffold derived from pirenzepine. The reaction performed in acetonitrile/water, in the presence of $CuSO_4$ and Cu wire, allowed us to obtain three novel pirenzepine derivatives with high purity and in good yield. No coupling reagents were needed, and the quasi-stoichiometric conditions of the reaction enabled the straightforward isolation of the final product by simple precipitation and its use in bioassays. The affinity of the compounds for the human M1 muscarinic receptor fused to EGFP was checked under classical radioligand and FRET binding conditions. The three pirenzepine constructs display a nanomolar affinity for the M1 receptor. In addition, both dye-labeled derivatives behave as potent acceptors of energy from excited EGFP with a very high quenching efficiency.

INTRODUCTION

Biomolecules bearing a biotin tag or dye labels represent useful pharmacological tools for studying receptors (*1*). Biotinylated compounds, together with their streptavidin partner, can be used in a wide variety of bioanalytical applications, such as affinity chromatography, affinity cytochemistry, and immunoassays (*2*), and also as biosensors for drug development (*3*) or for receptor purification (*4*). Fluorescent drug molecules allow the visualization of their receptor target at the single-cell level (*5*, *6*). More generally, these molecules are used as tracers in binding assays where they represent a safe and versatile alternative to radioligands, thus eliminating issues related to handling and disposal of radioactive materials (*7*). Recently, Ilien et al. reported on the synthesis of a series of fluorescent derivatives of the muscarinic M1 antagonist pirenzepine and on their binding properties at an enhanced green fluorescent protein (EGFP)−human M1 receptor chimera, examined through fluorescence resonance energy transfer (FRET) (*8*, *9*). Among these ligands, Lissamine Rhodamine B-labeled pirenzepine **1a** (Figure 1) displayed both a nanomolar affinity and the highest FRET efficiency. In a subsequent article, these authors provided evidence that pirenzepine derivatives labeled with a nonfluorescent dye, such as Patent Blue VF in compound **1b,** were useful tools to improve the reliability of FRET-based binding assays when performed at high probe concentrations (*10*).

Several methods are currently available to label biomolecules such as peptides or small organic molecules (*11*). However, most of these methods suffer from several limitations: (1) Activated tags are generally provided in small quantities suitable for biochemical applications, but amounts typically required for synthetic organic chemistry are prohibitively expensive. (2) To ensure good solubility of both partners, the coupling step has to be carried out in high boiling point solvents such as DMF or DMSO. This renders the final workup, i.e., the isolation of the labeled molecule, more difficult. (3) Excess of one of the partners is generally used to drive the reaction to completion. This makes final purification more tedious, as the excess of both reagents and coupling agents have to be removed. (4) Last but not least, the labeling is not chemoselective and other nucleophile groups have to be protected before performing the

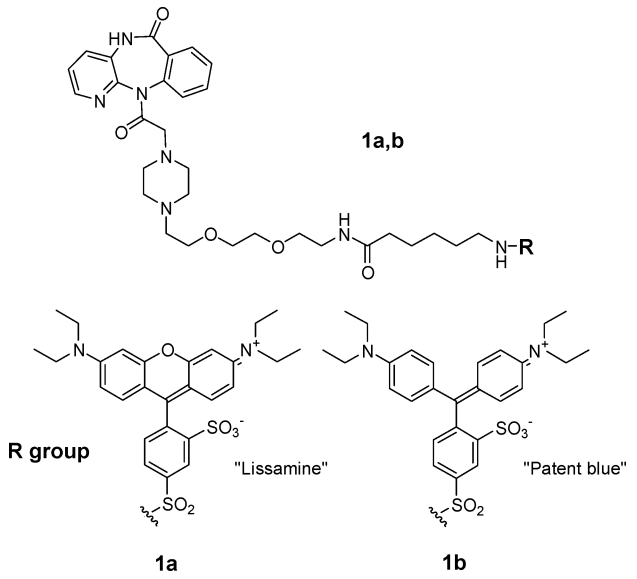


Figure 1. Chemical structures of fluorescent (Lissamine Rhodamine B-labeled; **1a**) and nonfluorescent (Patent Blue VF-labeled; **1b**) pirenzepine derivatives as described in refs 9 and 10.

* To whom correspondence should be addressed. Phone: +33(0)390244236. Fax: +33(0)390244310. E-mail: dominique.bonnet@pharma.u-strasbg.fr or ilien@esbs.u-strasbg.fr.

† Département de Pharmacochimie de la Communication Cellulaire.

‡ Département Récepteurs et Protéines Membranaires.

§ IFR85, Service commun de RMN.

10.1021/bc060140j CCC: $33.50
Published on Web 10/19/2006

coupling reaction. Therefore, the numerous difficulties associated with the preparation of such molecules prompted us to develop a novel method to ensure rapid, low-cost, and chemoselective labeling of biomolecules.

Herein, we describe a simple and efficient method, referred to as "click" chemistry, to label a small ligand with various tags (such as biotin, Lissamine Rhodamine B, or Patent Blue VF) using Cu(I)-catalyzed 1,3-dipolar cycloadditions. After optimization on a model reaction, the method was successfully applied to the synthesis of three novel pirenzepine (an M1 selective muscarinic antagonist) derivatives **14a**−**c**, with high yield and purity. An important issue was the impact of the triazole link on the biological activity of these ligands. Therefore, their binding properties at the muscarinic M1 receptor were examined through classical [^{3}H]-NMS competition experiments and through FRET-based saturation assays using the EGFP-(Δ17)hM1 chimera (*8*). Affinity parameters (K_i and K_d values) were determined and compared to those previously reported for the parent molecules **1a** and **1b**. Maximal amplitudes for EGFP fluorescence extinction, achieved at saturating concentrations of **14a** and **14b**, allowed the estimation of the efficiency of the energy transfer process promoted by their interaction with the receptor.

EXPERIMENTAL PROCEDURES

General. All chemicals were obtained from commercial suppliers and used without further purification. Lissamine Rhodamine B sulfonyl chloride and D(+)biotin were purchased from Acros and Avocado, respectively. 6-(Boc-amino)caproic acid *N*-succinimidyl ester **6** and 11-azido-3,6,9-trioxaundecan-1-amine were from Fluka and benzotriazole-1-yl-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) was supplied from Novabiochem. Compounds **9** and **13** were prepared as previously described in the literature (*9*, *10*). Silica gel 60F 254 plates for thin-layer chromatography (TLC) were purchased from Merck. Column chromatographic separations were carried out by flash chromatography using silica gel (Merck) with a particle size of 0.040−0.063 mm. ^{1}H NMR spectra were recorded at 200 or 300 MHz on a Bruker Advance spectrometer. Chemicals shifts are given in parts per million and coupling constants (*J*) in hertz. Analytical RP-HPLC separations were performed on a C18 Symmetry Shield column (4.6 × 150 mm) using a linear gradient of eluent B in eluent A (0−100% B in 30 min, flow rate 1 mL.min^{-1}, detection at 220 and 254 nm) and a linear gradient of eluent D in eluent C (0−100% D in 60 min, flow rate 0.8 mL.min^{-1}, detection at 220 and 254 nm). The following buffers were used: (eluent A) water containing 0.1% TFA; (eluent B) acetonitrile containing 0.1% TFA; (eluent C) water containing 0.1% HFBA; (eluent D) MeOH containing 0.1% HFBA. ESI-TOF (electrospray ionization time-of-flight) spectra were recorded on a Perseptive Biosystem Mariner 5155 spectrometer. The *m*/*z* range 200−2100 was scanned using an ion-spray voltage of 4500 V. The nozzle was ranged between 30 and 60 V.

***N*-(2-{2-[2-(2-Azidoethoxy)-ethoxy]-ethoxy}-ethyl)-4-nitrobenzamide 2.** 11-azido-3,6,9-trioxaundecan-1-amine (200 μL, 1 mmol) was dissolved in CH_2Cl_2 and Et_3N (210 μL, 1.5 mmol). 4-Nitrobenzoyl chloride (226 mg, 1.2 mmol) dissolved in CH_2Cl_2 was added to the solution. The resulting mixture was stirred for 4 h at room temperature. The crude reaction was then diluted with CH_2Cl_2 and washed with 10% citric acid solution and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Compound **2** was isolated by column chromatography (CH_2Cl_2/CH_3OH 98/2) to afford a yellow oil (281 mg, 77%); R_f = 0.35 (CH_2Cl_2/MeOH 98/2); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.28 (d, *J* = 8.4 Hz, 2H), 7.98 (d, *J* = 7.1 Hz, 2H), 3.95−3.35 (m, 14H), 3.35 (t, 4.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.7, 149.8, 140.5, 128.6, 123.9, 70.9, 70.7, 70.5, 70.2, 69.8, 50.9, 40.3; ES-MS MW calcd 368.14 *m*/*z*, found 390.17 [M + Na]$^+$; RP-HPLC purity >90%.

2 (and 4)-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]-5 (or 3)-({[6-oxo-6-(prop-2-ynylamino)hexyl]amino}-sulfonyl)benzenesulfonate 3. To a solution of 6-(Boc-amino)-caproic acid *N*-succinimidyl ester (200 mg, 0.61 mmol) in a CH_2Cl_2/DMF (1/2) mixture were added propargylamine (62 μL, 0.91 mmol) and Et_3N (94 μL, 0.66 mmol). The resulting solution was stirred at room temperature overnight and then diluted with CH_2Cl_2 and washed with 10% citric acid solution, 10% aqueous Na_2CO_3, and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a white powder which was further purified through a short column of silica gel (CH_2Cl_2/CH_3OH 97/3) to afford compound **7** in 76% yield (125 mg). R_f = 0.38 (CH_2Cl_2/MeOH 97/3); ES-MS MW calcd 268.4, *m*/*z* found 291.2 [M + Na]$^+$. Compound **7** (100 mg, 0.37 mmol) was treated with a CH_2Cl_2/TFA (1/1) mixture for 20 min at room temperature. Following TFA removal under reduced pressure, the resulting deprotected compound **8** (yellow oil) was dissolved in CH_2Cl_2/DMF (5/1) mixture containing Et_3N (250 μL) and cooled at 0 °C. Lissamine Rhodamine B sulfonyl chloride (165 mg, 0.28 mmol) was added in portions over 20 min. The solution was allowed to warm up to room temperature and stirred overnight. The crude mixture was concentrated under reduced pressure. Compounds **3a** (para isomer) and **3b** (ortho isomer) were isolated by flash chromatography with a step gradient from 5% to 10% MeOH in CH_2Cl_2 and obtained as a red dark solid: **3a** (89 mg, 44%,); R_f = 0.37 (CH_2Cl_2/MeOH 93/7); ^{1}H NMR ($CDCl_3$/CD_3OD 9/1, 300 MHz) δ 8.57 (d, *J* = 1.8 Hz, 1H), 7.94 (dd, *J* = 1.8 and 8.1 Hz, 1H), 7.18 (d, *J* = 8.1 Hz, 1H), 7.04 (d, *J* = 9.6 Hz, 2H), 6.75 (dd, *J* = 2.1 and 9.6 Hz, 2H), 6.63 (d, *J* = 2.1 Hz, 2H), 3.80 (d, *J* = 2.5 Hz, 2H), 3.25 (bs, 1H), 2.95 (t, *J* = 6.6 Hz, 2H), 2.10−2.08 (m, 3H), 1.51−1.38 (m, 4H), 1.31−1.14 (m, 14H); ^{13}C NMR ($CDCl_3$/CD_3OD 9/1, 75 MHz) δ 175.7, 159.4, 158.5, 157.1, 147.8, 144.3, 135.2, 134.2, 132.0, 129.4, 128.2, 115.6, 115.2, 97.3, 72.4, 47.8, 47.4, 44.4, 37.1, 31.1, 30.6, 30.1, 27.4, 26.6, 13.9, 10.0; MW calcd 708.88, *m*/*z* found 731.36 [M + Na]$^+$; RP-HPLC purity >95%. **3b** (53 mg, 27%); R_f = 0.30 (CH_2Cl_2/MeOH 93/7); δ 8.44 (d, *J* = 1.7 Hz, 1H), 8.13 (dd, *J* = 1.7 and 7.8 Hz, 1H), 7.19 (d, *J* = 7.8 Hz, 1H), 7.01 (d, *J* = 9.4 Hz, 2H), 6.77 (dd, *J* = 2.4 and 9.4 Hz, 2H), 6.65 (d, *J* = 2.5 Hz, 2H), 3.82 (d, *J* = 2.5 Hz, 2H), 3.23 (bs, 1H), 3.03 (q, *J* = 7.5 Hz, 8H), 2.75 (t, *J* = 6.6 Hz, 2H), 2.11 (t, *J* = 2.5 Hz, 2H), 2.10−2.05 (m, 2H), 1.42−0.75 (m, 18H); ^{13}C NMR ($CDCl_3$/CD_3OD 9/1, 75 MHz) δ 175.7, 159.4, 157.3, 154.4, 150.7, 134.1, 133.5, 131.4, 127.6, 119.6, 115.6, 110.0, 99.0, 97.7, 72.7, 47.9, 47.6, 44.5, 37.2, 31.2, 30.2, 29.5, 27.5, 26.4, 14.0, 10.1; ES-MS MW calcd 708.88, *m*/*z* found 731.33 [M + Na]$^+$; RP-HPLC purity >95%.

2-{[4-(diethylamino)phenyl][4-(diethyliminio)cyclohexa-2,5-dien-1-ylidene] methyl}-5- ({[6-oxo-6-(prop-2-ynylamino)-hexyl]amino}sulfonyl)benzenesulfonate 4. A solution of aqueous lithium hydroxide hydrate 1 N (89 μL, 89.7 μmol) was added at room temperature to methyl 6-(4-[(4-diethylamino-phenyl)(4-diethylimino-2,5-cyclohexadien-1ylidene)methyl]-3-sulfo-1-phenylsulfonamido)hexanoate **9** (20 mg, 29.7 μmol) dissolved in dioxane. The mixture was stirred overnight at room temperature or 10 min at 80 °C using microwave irradiation. Dioxane was removed under reduced pressure, and water was added. The mixture was washed with CH_2Cl_2, acidified with a 1 M $KHSO_4$ solution, and extracted with CH_2Cl_2. Organic layers were combined and concentrated under pressure to give acid **10** which was used as crude material in the next step.

Crude acid **10** was dissolved in DMF with propargylamine (3 μL, 38.6 μmol). PyBOP (16 mg, 29.7 μmoles) was added, followed by DIEA (16 μL, 89.1 μmol). The resulting mixture was stirred at room temperature overnight. DMF solvent was removed under reduced pressure, and the crude material was purified by column chromatography (CH_2Cl_2/MeOH 95/5) to afford compound **4** as a blue powder (17.3 mg, 83%). R_f = 0.35 (CH_2Cl_2/MeOH 95/5); ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 300 MHz) δ 8.54 (d, *J* = 1.5 Hz, 1H), 7.88 (dd, *J* = 1.5 and 7.7 Hz, 1H), 7.33 (d, *J* = 9.5 Hz, 4H), 7.12 (d, *J* = 7.8 Hz, 1H), 6.72 (d, *J* = 9.5 Hz, 4H), 4.21 (d, *J* = 3.4 Hz, 1H), 3.84 (brd, *J* = 2.5 Hz, 2H), 3.58−3.48 (m, 8H), 2.98 (t, *J* = 6.5 Hz, 2H), 2.14−2.07 (m, 3H), 1.53−1.18 (m, 18H); ^{13}C NMR ($CDCl_3$/CD_3OD 9:1, 75 MHz) 174.1, 155.4, 147.0, 142.8, 140.9, 132.0, 131.2, 129.0, 128.4, 127.9, 127.4, 127.1, 126.8, 113.4, 46.1, 45.1, 43.0, 35.8, 29.8, 28.8, 26.0, 25.2, 12.8. ES-MS MW calcd 694.9, *m*/*z* found 717.4 $[M + Na]^+$; RP-HPLC purity >95%.

5-[(4*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-*N*-prop-2-ynylpentanamide 5. D(+)biotin (50 mg, 205 μmol) was dissolved in DMF in presence of DIEA (107 μL, 615 μmol). Propargylamine (28 μL, 410 μmol) was added, followed by PyBOP (106 mg, 205 μmol). After overnight stirring at room temperature, DMF solvent was removed under reduced pressure. The crude material was then purified by column chromatography on silica gel (CH_2Cl_2/MeOH 9/1) to get compound **5** as a white powder (35 mg, 60%). R_f = 0.24 (CH_2Cl_2/MeOH 9/1); ^{1}H NMR (CD_3OD, 200 MHz) δ 4.56−4.52 (m, 1H), 4.37−4.31 (m, 1H), 3.99 (d, *J* = 2.5 Hz, 2H), 3.32−3.22 (m, 1H), 2.97 (dd, *J* = 6.0 Hz, 12.0 Hz, 1H), 2.74 (d, *J* = 11.4 Hz, 1H), 2.61 (t, *J* = 2.5 Hz, 1H), 2.26 (*t*, *J* = 7.2 Hz, 2H), 1.84−1.40 (m, 6H); ^{13}C NMR (CD_3OD, 75 MHz) δ 176.4, 166.9, 81.5, 72.9, 64.2, 62.4, 57.8, 41.8, 37.3, 30.5, 30.3, 30.2, 27.5; ES-MS MW calcd 281.38, *m*/*z* found 304.15 $[M + Na]^+$.

General Procedure for the Preparation of Triazoles: **Synthesis of Compound 12.** Azido **2** (4.1 mg, 11.2 μmol) and alkyne **3a** (9.6 mg, 13.5 μmol) were dissolved in 9/1 acetonitrile/water mixture (600 μL) in presence of $CuSO_4$ (solution 26.7 μM, 40 μL, 1.11 μmol) and Cu wire (68 mg). The solution was stirred overnight at 30 °C, the solvent removed, and the crude material purified by column chromatography (CH_2Cl_2/MeOH 9/1). Compound **12** (9.0 mg, 75%) was obtained as a red solid. R_f = 0.28 (CH_2Cl_2/MeOH 9/1); ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 200 MHz) δ 8.62 (s, 1H), 8.13 (d, *J* = 8.5 Hz, 2H), 7.95 (m, 3H), 7.20 (d, *J* = 7.8 Hz, 1H), 7.07 (d, *J* = 9.3 Hz, 2H), 6.75 (d, *J* = 9.3 Hz, 2H), 6.59 (d, *J* = 1.9 Hz, 2H), 4.39−4.33 (m, 4H), 3.75 (t, *J* = 4.6 Hz, 2H), 3.60−3.33 (m, 18H), 2.95 (t, *J* = 4.6 Hz, 2H), 2.77 (bs, 1H), 2.35−1.93 (m, 4H), 1.6−1.0 (m, 18H); ^{13}C NMR (CD_3OD, 50 MHz) δ 158.1, 155.8, 133.8, 133.0, 130.5, 128.9, 128.0, 127.0, 123.8, 114.3, 114.0, 95.9, 70.6, 70.3, 69.7, 46.2, 43.1, 40.2, 12.7; ES-MS MW calcd 1075.4, *m*/*z* found 1076.6 $[M + H]^+$, 1098.6 $[M + Na]^+$; RP-HPLC purity >95%.

Compound 14a. The same procedure as that described above for compound **12** was used starting from azido **13** (4.7 mg, 9.5 μmol) and alkyne **3a** (8.0 mg, 11.3 μmol). After 4 h at 30 °C, triazole **14a** (8.9 mg, 78%) was isolated by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1/NH_4OH 1% in water) as a red solid. R_f = 0.21; RP-HPLC purity >95%; HRMS calcd for $C_{60}H_{75}N_{12}O_{11}S_2$ 1203.5114, found 1203.5156.

Compound 14b (5.0 mg, 70%, blue solid) was obtained by reacting azido **13** (3 mg, 6.0 μmol) and alkyne **4** (5.3 mg, 7.5 μmol) at 30 °C for 4 h and subsequent purification by column chromatography (CH_2Cl_2/MeOH 9:1/NH_4OH 1% in water). R_f = 0.27; RP-HPLC purity >95%; HRMS calcd for $C_{60}H_{77}N_{12}O_{10}S_2$ 1189.5322, found 1189.5298.

Compound 14c (5.9 mg, 92%, white solid) was obtained by reacting azido **13** (4.2 mg, 8.5 μmol) and alkyne **5** (3.0 mg, 10.6 μmol) following the same experimental conditions as for **14b**. R_f = 0.15 (CH_2Cl_2/MeOH 9:1/NH_4OH 1% in water); RP-HPLC purity >95%; HRMS calcd for $C_{37}H_{50}N_{11}O_6S$ 776.3661, found 776.3631.

Construction and Expression of the Chimeric hM1 Receptor. The human M1 muscarinic receptor with a truncated N-terminus fused to EGFP, referred to as EGFP-(Δ17)hM1, was obtained and stably expressed in HEK 293 cells as previously reported (*8*).

[^{3}H]-NMS Binding Assays. Competition experiments were performed using intact EGFP-(Δ17)hM1 expressing cells (60 000 cells) suspended in 1 mL HEPES/BSA buffer (10 mM HEPES, 137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, and 10 mM glucose, pH 7.4; supplemented with 1 mg.mL^{-1} bovine serum albumin). Incubation proceeded at 20 °C for 22 h in the presence of 100−150 pM [^{3}H]-NMS ([^{3}H]-*N*-methylscopolamine) of various concentrations of compounds to be tested or of 4 μM atropine for nonspecific binding measurement. K_i values of competitors were calculated from IC_{50} values, using the Cheng and Prusoff relationship (*12*) and taking a K_d value of 90 pM for the radioligand.

Spectroscopy. UV−visible absorbance spectroscopy was performed with a Cary 1E (Varian) spectrophotometer. Absorption spectra for compounds, diluted in MeOH, were recorded in the 450−750 nm wavelength range. Fluorescence measurements were made using a SPEX Fluorolog 2 (Jobin Yvon Horiba) spectrofluorimeter equipped with a 450 W Xe lamp. Data were stored using the DM3000 software provided with the spectrofluorimeter.

FRET-Based Binding Measurements. EGFP-(Δ17)hM1 cells (1×10^6 cells), suspended in 1 mL HEPES/BSA buffer, were incubated with increasing concentrations of fluorescent ligand, in the absence or presence of 10 μM atropine to assess the specificity of the signal. Equilibrium was allowed to proceed for 22 h at 20 °C. Thereafter, the incubation medium was transferred to a 1 mL thermostated (21 °C) quartz cuvette with magnetic stirring and recorded for fluorescence intensity at 510 nm (excitation set at 470 nm). Occupancy curves were generated by plotting amplitudes for fluorescence extinction as a function of ligand concentration as previously described (*10*). K_d values and maximal FRET amplitudes were from data fitting to the empirical Hill equation derived for saturation. Efficiency of fluorescence energy transfer (*E*) was defined as the fractional decrease in specific EGFP fluorescence due to ligand binding as previously reported (*8*).

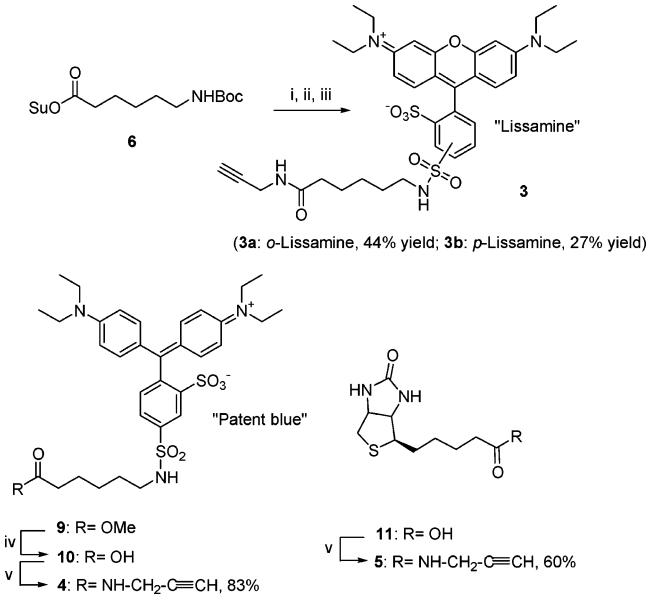
RESULTS AND DISCUSSION

Chemistry. The generation of (1,2,3)-triazoles through 1,3-dipolar cycloadditions of alkynes to azides, which belongs to a group of reactions referred to as "click chemistry" (*13*), proceeds at room temperature in the presence of copper(I) as a catalyst (*14*). Among various applications, this reaction has enabled the selective modification of virus particles (*15*), nucleic acids (*16*), and proteins (*17*) in biological environments.

In the first part of the study, the potential of this method for the labeling of bioactive molecules was examined using the azido model **2** and alkynes derived from Lissamine Rhodamine B **3**, Patent Blue VF **4**, and biotin **5** (Scheme 1).

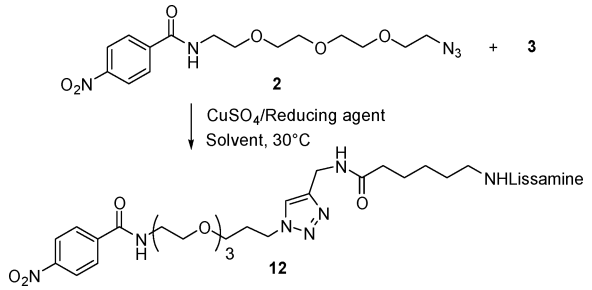
Alkyne **3** was obtained in three steps starting from commercially available 6-(Boc-amino)caproic acid *N*-succinimidyl ester **6**. Substitution of ester with propargylamine gave access to alkyne **7** that was subsequently deprotected in the presence of TFA. Resulting amine **8** was reacted with Lissamine Rhodamine B sulfonyl chloride (commercially available as a mixture of ortho and para isomers) in CH_2Cl_2/DMF mixture in the presence of Et_3N and 4-dimethylaminopyridine (DMAP) to afford **3** as a mixture of ortho **3a** and para **3b** isomers (1:1.6

Scheme 1. Routes for Preparation of Alkyne Derivatives 3−5[a]



[a] Legend: (i) propargylamine, CH_2Cl_2/DMF, Et_3N, rt, 16 h; (ii) TFA/CH_2Cl_2 (1:1), rt, 20 min; (iii) Lissamine sulfonyl chloride, CH_2Cl_2/DMF (5:1), Et_3N/DMAP, 0 °C, 15 min then rt, overnight; (iv) LiOH, dioxane/H_2O, 40 °C, 16 h or microwave irradiation (80 °C, 10 min); (v) propargylamine, PyBOP, DIEA, DMF, rt, overnight.

Scheme 2. Model Reaction to Optimize Cu(I)-Catalyzed 1,3-Dipolar Cycloaddition



ratio, respectively, as shown by ^{1}H NMR and RP-HPLC analysis (*18*)). Column chromatography on silica gel allowed the separation of both isomers **3a** and **3b** obtained in a 44% and 27% overall yield, respectively.

Alkyne **4** was prepared starting from Patent Blue VF methyl ester **9** (*10*). In an attempt to obtain **4** in one step, aminolysis of **9** with an excess of propargylamine was tried and found to be inefficient, regardless of the solvent, the temperature, and the presence or absence of a catalytic amount of cyanide anion (*19*). Compound **4** was finally obtained in two steps. Ester **9** was first hydrolyzed in the presence of LiOH in a dioxane/water mixture at 40 °C overnight, leading quantitatively to acid **10**. Interestingly, hydrolysis was dramatically accelerated by using microwave irradiation (80 °C, 10 min). Crude acid **10** was then coupled with propargylamine following PyBOP in situ activation to provide **4** in 83% overall yield.

Finally, alkyne derived from biotine **5** was obtained by coupling propargylamine with D(+)biotin using PyBOP activation in DMF at 40 °C in 60% yield after purification on silica gel (Scheme 1).

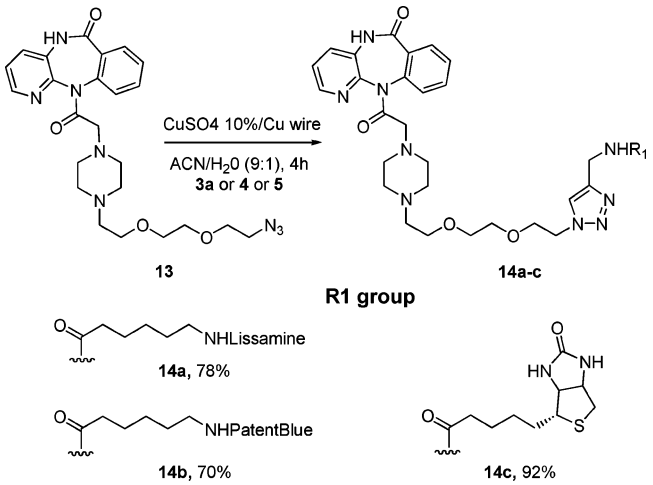
With alkynes **3**−**5** in hand, "click" reaction efficacy was evaluated with the model azido **2** and the alkyne derived from Lissamine **3**. The reaction was conducted in the presence of $CuSO_4$ and of a reducing agent to generate Cu(I) species in polar solvents at 30 °C (Scheme 2). The influence of key parameters such as the nature of the reducing agent and the solvent on the formation of the triazole derivative **12** was carefully investigated. RP-HPLC yields were determined following overnight stirring at 30 °C (Table 1).

Table 1. Optimization of the Synthesis of Triazole 12.

entry	solvent	reducing agent	RP-HPLC yield of **12**[a] (%)
1	EtOH/$H_2$0 (1:1)	TCEP	0
2	EtOH/$H_2$0 (1:1)	ascorbic acid	30
3	EtOH/$H_2$0 (1:1)	Cu wire	38
4	EtOH/$H_2$0 (9:1)	Cu wire	68
5	MeOH/$H_2$0 (9:1)	Cu wire	59
6	tBuOH/$H_2$0 (9:1)	Cu wire	65
7	ACN/$H_2$0 (9:1)	Cu wire	90
8	ACN/$H_2$0 (1:1)	Cu wire	74

[a] Yields were approximated by RP-HPLC and calculated using the ratio of surface area of reactant species to surface area of product species (see experimental procedures for RP-HPLC conditions).

Scheme 3. Novel Pirenzepine Derivatives Obtained Using "Click" Chemistry



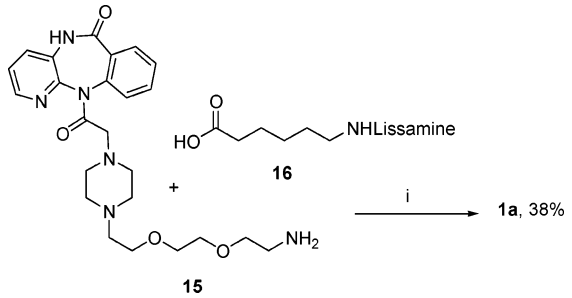
In our hands, the classical experimental conditions described in the literature (*14*), i.e., EtOH/H_2O (1:1) mixture in the presence of tris(carboxyethyl)phosphine (TCEP), failed (entry 1). Replacement of phosphine by ascorbic acid or Cu wire allowed an improvement of RP-HPLC yields from 30% to 38% (entries 2 and 3). The nature of the solvent and the ratio of water were found to be crucial for the reaction (entries 4−8), with the best result (90%) being obtained by using an acetronitrile (ACN)/water (9:1) mixture (entry 7). Using these conditions, compound **12** was isolated in 75% overall yield.

The scope and limitations of the method were further investigated by considering the incorporation of Lissamine Rhodamine B, but also of Patent Blue and of sparsely soluble biotin onto pirenzepine (Scheme 3). The azido pirenzepine derivative **13** was synthesized as previously described (*9*). 1,3-Dipolar cycloaddition was performed in the presence of 1.2 equiv of alkyne **3a** (para isomer), 0.1 equiv of $CuSO_4$, and Cu wire in an acetonitrile/water (9:1) mixture. Completion of the reaction was observed after only 4 h stirring at 30 °C. Thus, compound **14a** was isolated by column chromatography in 78% yield.

Similar results were obtained with alkynes derived from Patent Blue VF leading to the corresponding labeled pirenzepine **14b** in 70% isolated yield. Finally, the "click" reaction of alkyne **13** with azido-derived biotin **5** was found to be particularly clean and rapid, since conjugate **14c** was isolated in 92% yield following 4 h at 30 °C.

It is noteworthy that, owing to the excellent purity of the crude mixture, labeled compounds could be used directly for

Scheme 4. Synthesis of Fluorescent Labeled Pirenzepine 1a[a]



[a] Legend: (i) PyBOP, DIEA, DMF, 30 °C, 4 h.

Table 2. Binding Properties of Compounds 14a−c at the Muscarinic M1 Receptor.

		fluorescence resonance energy transfer[b]		
compound	[^{3}H]NMS binding[a] K_i (nM)	K_d (nM)	amplitude (%)	efficiency E
pirenzepine	7.1			
1a	46	18.7	48	0.68
1b	189	30	37	0.52
14a[c]	30	20	55	0.78
14b	16.3	11	46	0.65
14c	49			

[a] K_i values are from competition experiments performed in duplicate using [^{3}H]-NMS as the radioligand. The K_i value reported for compound **1b** (tested against [^{3}H]-QNB) is from ref 10. [b] Values listed for compounds **1a** and **1b** are, respectively, from refs 9 and 10. Other values are from a typical experiment performed in duplicate. [c] Only the para isomer was considered.

biological evaluation without any additional purification. Cu wire is simply picked up from the solution with tweezers, the crude product is diluted in water, and the resulting precipitate washed to remove $CuSO_4$. The use of metal resin scavengers, such as Chelex resin (Bio-Rad), may be regarded as an alternative process to get rid of Cu^{2+} (*20*).

To highlight the efficiency of the "click" reaction versus traditional coupling, pirenzepine amine **15** obtained by reduction of azido **13** (*10*) was reacted with 1.2 equiv of acid **16** (*21*) following PyBOP in situ activation in DMF (Scheme 4). After 4 h at 30 °C, this control experiment resulted in a low conversion of starting materials involving a tedious final purification by RP-HPLC to isolate Lissamine-labeled pirenzepine **1** in modest 38% yield (vs 78% for "click" reaction).

This first part of the study highlighted the efficiency of the "click" process to easily access the novel pirenzepine-labeled derivatives **14a−c**. The reaction is rapid, insensitive to water, and almost quantitative regardless of the nature and the solubility of tags incorporated. Interestingly, the absence of coupling reagents and quasi-stoichiometric conditions, combined with the use of an acetonitrile/water mixture, allow the isolation of the final labeled products by simple precipitation.

The main issue of the second part of the work was to check whether the triazole moiety was compatible with high-affinity binding of the pirenzepine derivatives to the muscarinic M1 receptor, a G protein-coupled receptor belonging to the bioamine group whose binding domain is deeply buried in the receptor protein.

Pharmacological Study of Novel Pirenzepine Derivatives 14a−c. Ligand binding affinity at equilibrium was evaluated through competition (conventional [^{3}H]NMS assay) and saturation (FRET-based assay) experiments, using the EGFP-(Δ17)hM1 chimera (*8*).

Binding parameters are listed in Table 2 and may be compared to those determined for pirenzepine and for the two parent ligands **1a** and **1b**, under similar experimental conditions.

The three novel pirenzepine derivatives retain nanomolar affinities for [^{3}H]-NMS binding sites. The Lissamine Rhodamine B conjugate **14a** exhibits a K_i value similar to that of its parent molecule **1a**, while the Patent Blue derivative **14b** displays a significantly higher affinity than **1b** (which has been tested against [^{3}H]-QNB; (*10*)). The introduction of the small biotine substituent in **14c**, as compared to the more voluminous dyes in **14a** and **14b**, does not lead to appreciable affinity changes. Thus, neither the incorporation of a triazole ring within the spacer nor the exchange of a large chromophore for a smaller moiety are detrimental to high-affinity binding of the compounds to the hM1 receptor.

For all tested molecules, similar affinity rank order and parameter values were determined from competition-type ([^{3}H]-NMS binding) or from saturation-type (FRET) equilibrium experiments. A subtle but constant increase in affinity is however noted for the compounds when tested for their ability to behave as energy acceptors from excited EGFP. Such a feature has been already reported, and was even exacerbated, when [^{3}H]-QNB- and FRET-derived binding affinity constants for a series of dye derivatives of pirenzepine were compared (*9*, *10*). Most probably, these variations stem from differences in the physicochemical properties of the ligands.

A surprising finding was the apparent increase in maximal extinction amplitude afforded at saturating micromolar concentrations of **14a** and **14b**, as compared to **1a** and **1b**, respectively. These changes cannot be attributed to modifications in EGFP-(Δ17)hM1-expressing cell properties, as a control Bodipy-pirenzepine derivative (Bo(10)PZ), taken at 200 nM, provided here a maximal extinction amplitude of 44.8%, in perfect agreement with previous determinations (*8*, *9*). Moreover, the muscarinic specificity of the FRET signal achieved at high probe concentrations was verified by the full protective (competitive) effect of 10 μM atropine. As far as identical dyes (Lissamine Rhodamine B for **1a** and **14a**; Patent Blue for **1b** and **14b**) and identical EGFP-fused receptors (FRET donor) are concerned, it is tempting to suggest that an increase in maximal extinction amplitude reflects an increase in FRET efficiency. Indeed, the efficiency of fluorescence energy transfer (E) is defined as the fractional decrease in EGFP fluorescence due to ligand binding and is expressed by $E = 1 - F_{DA}/F_D$, where F_{DA} and F_D are the specific donor fluorescence emission values in the presence or the absence of saturating concentrations of ligand, respectively (*8*). According to the Förster equation, $R = R_0(1/E - 1)^{1/6}$ (*21*, *22*), with a Förster radius R_0 value set as a constant, an increase in FRET efficiency is clearly linked to a shortening of the actual distance R which separates the EGFP donor from the acceptor dye. It is thus possible that the triazole ring allows a reorientation of the ligand chromophore within the receptor and its projection toward an external domain, closer to EGFP. Though such a possibility deserves further investigations, similar distance shortenings (close to 3.5 Å) could be estimated when using triazole-containing probes instead of their more flexible original analogues.

An alternate explanation is to consider that, owing to the presence of the triazole ring within the spacer, the ligand chromophore would no longer adopt a random orientation of its absorption transition dipoles relative to those of donor emission. Such a possibility is difficult to verify and would affect the estimation of the Förster radius value, R_0, that specifies the theoretical distance separating a given donor−acceptor pair for 50% energy transfer efficiency. Altogether, these results indicate that the substitution of an amide bond to a small and rigid 1,4-triazole ring within the spacer connecting pirenzepine to the label is not deleterious for binding. Furthermore, this may be considered an interesting way to shorten the separation between FRET partners, i.e., between the donor (EGFP) and the acceptor

(dye moiety of the ligand), and to further increase the sensitivity in detecting the formation of ligand−receptor complexes.

CONCLUSION

In this paper, we describe a versatile and convenient method to facilitate the labeling of the small pirenzepine ligand with either a fluorescent probe, a nonfluorescent dye, or biotin. Cu(I)-catalyzed 1,3-dipolar cycloaddition, performed in partially aqueous media, is rapid, chemo- and regioselective, and leads to the formation of a stable triazole connection. The three new probes exhibit a nanomolar affinity for EGFP(Δ17)-hM1 receptors, indicating that the triazole link does not perturb binding to M1 receptors. It is also compatible with FRET-based binding assays. "Click" chemistry may thus be regarded as a convenient method to tag GPCR ligands with different probes useful for various applications. For instance, these three novel pharmacological tools will be involved in studies aimed at crystallizing muscarinic M1 receptor. Fluorescent and colored ligands will be used to detect and quantify correctly folded receptors. The biotinylated derivative will be used to prepare affinity chromatography columns necessary to the ultimate purification of functional M1 receptor.

ACKNOWLEDGMENT

This work was supported by the Centre National de la Recherche Scientifique, the Université Louis Pasteur, the European Community's Sixth Framework Program (grant LSHB-CT-2003-503337). We thank Pascale Buisine (IFR85) for ES-MS analyses, Aziz Chenaf for participating in the binding assay experiments, and Line Bourel for proofreading the manuscript.

LITERATURE CITED

(1) Baindur, N., and Triggle, D. (1994) Concepts and progress in the development and utilization of receptor-specific fluorescent ligands. *J. Med. Res. Rev. 14*, 591−664.

(2) Wilcheck, M., and Bayer, E. (1998) The avidin-biotin complex in bioanalytical applications. *Anal. Biochem. 171*, 1−32.

(3) Sakai, T., Naidenko, O., Iijima, H., Kronenberg, M., and Koezuka, Y. (1999) Syntheses of biotinylated alpha-galactosylceramides and their effects on the immune system and CD1 molecules. *J. Med. Chem. 42*, 1836−1841.

(4) Honda, T., Janosik, T., Honda, Y., Han, J., Liby, K. T., Williams, C. R., Couch, R. D., Anderson, A. C., Sporn, M. B., and Gribble, G. W. (2004) Design, synthesis, and biological evaluation of biotin conjugates of 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid for the isolation of the protein targets. *J. Med. Chem. 47*, 4923−4932.

(5) Hegener, O., Jordan, R., and Häberlein, H. (2004) Dye-labeled benzodiazepines: Development of small ligands for receptor binding studies using fluorescence correlation spectroscopy. *J. Med. Chem. 47*, 3600−3605.

(6) Hess, S. T., Huang, S., Heikal, A. A., and Webb, W. W. (2002) Biological and chemical applications of fluorescence correlation spectroscopy: a review. *Biochemistry 41*, 697−705.

(7) Middleton, R. J., and Kellam, B. (2005) Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol. 9*, 517−525.

(8) Ilien, B., Franchet, C., Bernard, P., Morisset, S., Weill, C. O., Bourguignon, J.-J., Hibert, M., and Galzi, J.-L. (2003) Fluorescence resonance energy transfer to probe human M1 muscarinic receptor structure and drug binding properties. *J. Neurochem. 85*, 768−778.

(9) Tahtaoui, C., Parrot, I., Klotz, P., Guillier, F., Galzi, J.- L., Hibert, M., and Ilien, B. (2004) Fluorescent pirenzepine derivatives as potential bitopic ligands of the human M1 muscarinic receptor. *J. Med. Chem. 47*, 4300−4315.

(10) Tahtaoui, C., Guillier, F., Klotz, P., Galzi, J.-L., Hibert, M., and Ilien, B. (2005) On the use of nonfluorescent dye labeled ligands in FRET-based receptor binding studies. *J. Med. Chem. 48*, 7847−7859.

(11) Montalbetti, C. A. G. N., and Falque, V. (2005) Amide bond formation and peptide coupling. *Tetrahedron 61*, 10827−10852.

(12) Cheng, Y.-C., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099−3108.

(13) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40*, 2004−2021.

(14) Rostovtsev, V. V., Green, L. G., Vokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.

(15) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc. 125*, 3192−3193.

(16) Seo, T. S., Li, Z., Ruparel, H., and Ju, J. (2003) Click chemistry to construct fluorescent oligonucleotides for DNA sequencing. *J. Org. Chem. 68*, 609−612.

(17) Link, A. J., and Tirrell, D. A. (2003) Cell surface labeling of *Escherichia coli* via copper(I)-catalyzed [3+2] cycloaddition. *J. Am. Chem. Soc. 125*, 11164−11165.

(18) Corrie, J. E. T., Davis, C. T., and Eccleston, J. F. (2001) Chemistry of sulforhodamine amine conjugates. *Bioconjugate Chem. 12*, 186−184.

(19) Hoegberg, T., and Ulff, B. (1984) Stereoconservative reductive methyl- and dimethylamination of isomeric 3,3-diarylpropenals. Synthetic and mechanistic studies on control of the stereochemistry. *J. Org. Chem. 49*, 4209−4214.

(20) Greenberg, R. R., and Kingston, H. M. (1983) Trace element analysis of natural water samples by neutron activation analysis with chelating resin. *Anal. Chem. 55*, 1160−1165.

(21) Förster, T. (1948) Zwischenmolekulare energiewanderung und fluoreszenz. *Ann. Phys. (Leipzig) 2*, 55−75.

(22) Lakowicz, J. R. (1999) *Principles of fluorescence spectroscopy.* Kluwer Academic/Plenum Press, New York.

(23) Lefevre, C., Chol Kang, H., Haugland, P. R., Malekzadeh, N., Arttamangkul, S., and Haugland, P. R. (1996) Texas Red-X and rhodamine Red-X, new derivatives of sulforhodamine 101 and lissamine rhodamine B with improved labeling and fluorescence properties. *Bioconjugate Chem. 7*, 482−489.

BC060140J