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Journal of Fluorine Chemistry 126 (2005) 739–743
JOURNAL OF FLUORINE CHEMISTRY
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Synthesis and characterization of photolabeling probes of miltefosine

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Received 23 September 2004; received in revised form 26 January 2005; accepted 11 February 2005
Available online 7 April 2005

Abstract

Miltefosine is an ether-phospholipid analogue showing remarkable anti-cancer and anti-leishmanial activity thanks to its cell membrane-targeting properties. In order to study the mechanisms responsible for the biological effects of miltefosine using a photolabeling approach, we designed, synthesized and characterized photolabeling probes for studying the effects of miltefosine. In these probes, the photoactivatable tetrafluorophenylazido group is incorporated either at the polar head or in the alkyl chain of miltefosine. The probes showed fast, clear-cut photochemical reactions, which suggests that they are promising tools for use in photolabeling studies.

Keywords: Miltefosine; Photolabeling; Photolabeling probes; Tetrafluorophenylazides; Alkylphosphocholine

1. Introduction

Milltefosine (hexadecylphosphocholine, Scheme 1) is an alkyl phosphocholine showing remarkable anit-neoplastic, anti-tumor and anti-leishmanial activities [1,2]. It is one of the ether-phospholipid analogues and is generally thought to target the cell membranes. Miltefosine was originally developed as an anti-cancer drug [3], based on the seminal studies by Munder et al. [4], describing the immunomodulatory activities of lysophosphatidylcholines (Scheme 1). Lysophosphatidylcholines are a special kind of phospholipids, which are generated by the hydrolysis of a fatty acid present in the *sn*-2 position of various phospholipids. Since lysophosphatidylcholines can be easily hydrolyzed by several phospholipases and esterases in biological systems, more stable lysophosphatidylcholine analogues such as ether-phospholipids have been synthesized and characterized with a view to developing potential anti-tumor candidates [5]. Studies on the minimum structural features required for ether-phospholipids to be capable of anti-neoplastic activity led to the elimination of the glycerol backbone, leaving the alkylphosphocholines as the most likely candidates [6]. Among the various alkylphosphocholines, miltefosine was the first to be approved in 1992 as an anti-cancer drug for the topical treatment of certain forms of cutaneous cancer. Recently, miltefosine has attracted increasing attention because of its potential as an anti-leishmanial drug [7], and it has by now been registered for the oral treatment of visceral leishmaniasis in India, where it has achieved a success rate of up to 97% [8]. It has also proved to be an efficient means of treating immunodeficient patients with visceral leishmaniasis [9] and patients with cutanous leishmaniasis [10].

Although miltefosine has achieved considerable clinical success, little is known about its mechanism of action on either tumor cell lines or parasites. Due to its amphiphilic properties, miltefosine interacts mainly with the cell membranes of cancer cells as well as those of protozoa [1,2]. Many hypotheses have been proposed to explain the anti-cancer activity of miltefosine. The possible modes of action proposed so far include modulating membrane

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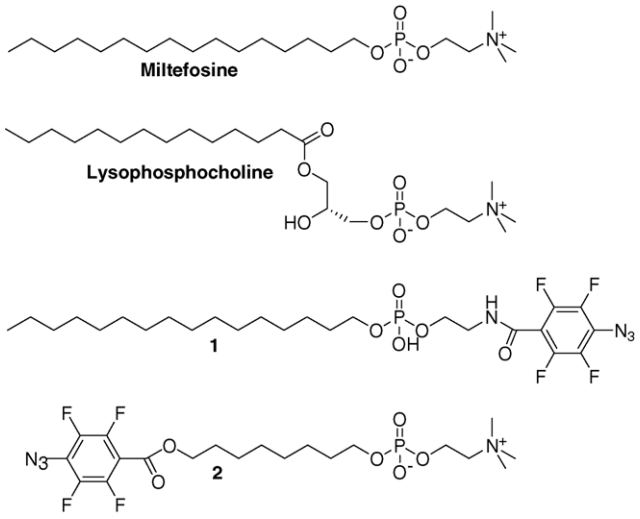
doi:10.1016/j.jfluchem.2005.02.019



Scheme 1. Miltefosine, lysophosphocholine and the designed photolabeling probes **1** and **2**.

permeability and membrane lipid composition, regulating phospholipid metabolism, alternating proliferation signal transduction pathways and inducing apoptosis [1]. Few studies have been carried out so far on the anti-leishmanial activity of miltefosine [2,11]. Since miltefosine heads for more than one cellular target, its action may involve complex mechanisms possibly affecting its overall biological properties. In order to study the molecular mechanisms involved, it was proposed first of all to identify the biological macromolecules interacting with miltefosine. For this purpose, we propose to use a photoaffinity labeling approach.

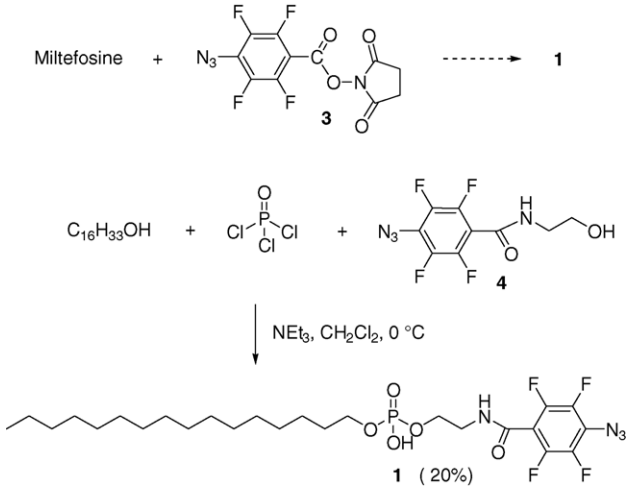
Photoaffinity labeling [12] is an efficient method for studying the interactions between drugs and their biological targets. This method involves the use of photoactivatable probes, which, upon being exposed to photoirradiation, generate highly reactive species, such as nitrene or carbene, which cross-link covalently with the targets at the binding sites. This process can be used to identify the biological targets that interact with the drugs and to map the binding sites on the targets, and thus to study the drug–target interactions. Aryl azides are frequently used as photoaffinity probes because they can be easily synthesized, as well as being chemically stable in the dark and highly reactive upon exposure to irradiation. Among the arylazides, fluorinated aryl azides [13] are exceptionally promising because they result in much more efficient photolabeling than non-fluorinated arylazides thanks to the retard of phenyl nitrene cyclization [14]. With a view to studying the mechanisms underlying the biological activity of miltefosine using photolabeling procedures, we designed two complementary photolabeling probes having fluorinated aryl azides (Scheme 1).

In probe **1**, the photoactivatable fluorinated arylazido group is introduced into the polar head. It is therefore proposed to probe the interactions between the probe and the lipid–water interface as well as to study the structure–function relationships occurring upon the modification of the polar head. In probe **2**, the photoactivatable group is located in the alkyl chain, which was designed to probe the interactions in which miltesoine is involved in the hydrophobic membrane core. The molecular design on which these probes were based was inspired by previous studies on photolabling probes designed for investigating the effects of phospholipids in biomemebranes and membrane proteins, where photoactivatable groups were introduced either into the polar head or into the fatty acid chain of the phospholipids [15,16]. Here, we report on the synthesis and characterization of the two photolabeling probes designed for studying the biological effects of miltefosine.

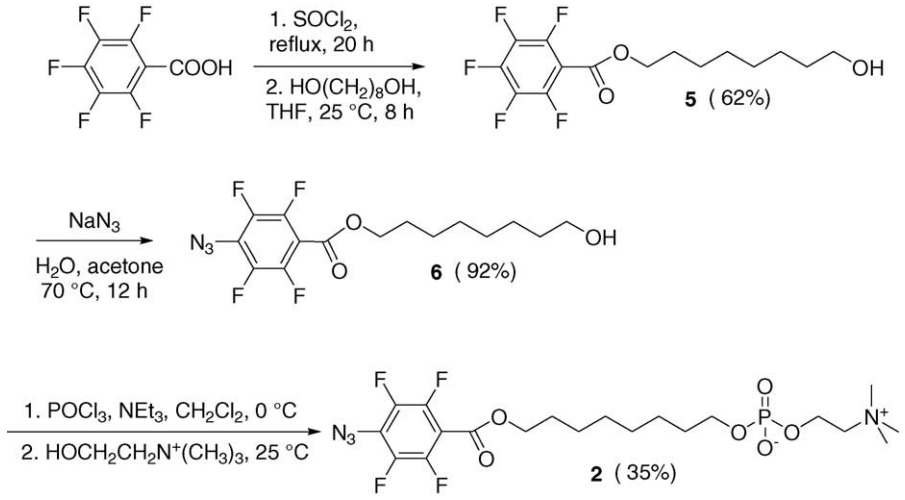
2. Results and discussion

We first attemped to synthesize **1** by directly coupling miltefosine to *N*-succinimidyl-4-azido-tetrafluorobenzoate **3** (Scheme 2) [13]. However, product **1** could not be obtained in pure form in this way because it was contaminated with the by-product 4-azido-tetrafluorobenzoic acid and could not be separated from this impurity using either a silica gel column or TLC seperation methods. We then performed the synthesis of **1** de novo by coupling $POCl_3$ to hexadecanol and then to *N*-(4-azido-tetrafluorobenzoyl)-ethanoamine **4** (Scheme 2), where **4** was obtained by treating ethanolamine with *N*-succinimidyl-4-azido-tetrafluorobenzoate **3**. After being extracted from the acidic aqueous solution (pH 1.0), **1** was obtained in pure form by performing column chromatography. Although TLC monitoring showed that a significant amount of **1** was formed during the reaction, the yield was rather low. One possible reason for the low yield here might be the poor solubility of **1**, which resulted in the loss of product during the work-up procedures.

The synthesis of **2** required the use of tetrafluorophenyl azide containing alcohol **6**, which was prepared by coupling the 1,8-octanediol to pentafluorobenzoic acid and subse-



Scheme 2. Synthesis of probe **1**.



Scheme 3. Synthesis of probe **2**.

quently introducing an azido group into the aromatic ring of **5** via nucleophilic substitution using NaN_3 (Scheme 3). This two-step procedure readily provided **6** with a good yield. By coupling $POCl_3$ successively to **6** and to choline tosylate (Scheme 3), **2** was obtained with a 35% yield without requiring any further optimization.

Both probes **1** and **2** were characterized by ^1H NMR, ^{13}C NMR, ^{19}F NMR, IR, UV and MS, giving satisfactory results. The maximum UV absorption wavelengths of these probes are 260 nm, and the molar absorption coefficients are around 5000 mol^{-1} L cm^{-1}, which are the values characteristic of aryl azides.

The probes **1** and **2** are stable in the dark. **1** is only marginally soluble in MeOH/CH_2Cl_2, while **2** is soluble in various solvents, such as MeOH, MeOH/CH_2Cl_2 and phosphate buffer. The poor solubility of probe **1** might be due to the introduction of the photoactivatable fluorinated arylazido group at the polar head. We tried to solubilize probe **1** in the phosphate buffer in the presence of the detergent sodium dodecylsulfate (SDS). The solubility of probe **1** in the buffer can in fact be significantly increased by adding SDS (Fig. 1). This result indicates that probe **1** still retains the amphiphilic nature of miltefosine. Similar findings has been reported on the modification of phospholipid probes designed to investigate the lipid–water interfaces of biomembranes [16].

A photochemical study was carried out with **1** in CH_2Cl_2/MeOH and **2** in phosphate buffer at pH 7.4. Fig. 2

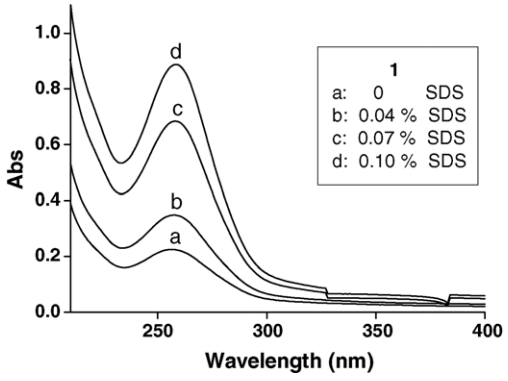


Fig. 1. UV spectral recording of the increase in the solubility of **1** in buffer solution (100 mM phosphate, pH 7.4 at 20 °C) observed upon adding the detergent, sodium dodecylsulfate (SDS).

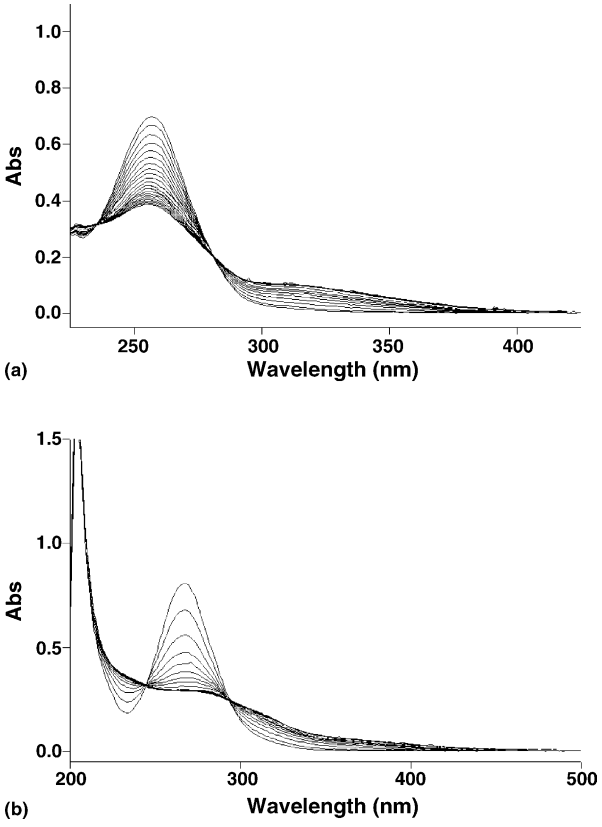


Fig. 2. Evolution of the UV absorption spectra when probes **1** and **2** were irradiated at a wavelength of 300 nm at 20 °C. The spectra were taken every 30 s. (a) **1** in MeOH/CH_2Cl_2 and (b) **2** in 100 mM phosphate, pH 7.4.

shows the photodecomposition of **1** and **2,** which occurred in response to irradiation at 300 nm. Both probes underwent a fast, clear-cut process of photodecomposition upon being irradiated at wavelength $\geq$ 300 nm. Irradiation of both **1** and **2** quickly led to the disappearance of the absorption band. The isobestic points observed indicated that the photochemical reaction was a single photo decomposition process. The fact that all these probes can be activated at wavelength $\geq$300 nm is an important point, since it means that the biological macromolecules will not be damaged by UV irradiation at this wavelength. These results indicate that both **1** and **2** retain the photochemical properties of fluorinated phenylazide and are therefore promising photolabeling probes for studying the molecular mechanisms underlying the biological activity of miltefosine.

3. Conclusion

Two photolabeling probes based on miltefosine, with the photoactivatable fluorinated arylazido group incorporated at the polar head (**1**) and in the alkyl chain (**2**), were synthesized with a view to studying the mechanisms responsible for the biological effects of miltefosine using a photolabeling approach. The process of photodecomposition of **1** and **2** was studied and found to involve a fast, clear-cut photochemical reaction. Probes **1** and **2** therefore both constitute promising tools for studying the mechanisms underlying the biological effects of miltefosine using a photolabeling approach. Further studies are now under way to investigate the interactions of these probes with biological membranes.

4. Experimental part

4.1. General

All reagents and solvents used for the synthesis of the ether-phospholipids were freshly purified or distilled before use. All the compounds were purified by flash chromatography on silica gel (200–300 mesh), which was purchased from Qingdao Ocean Chemical Plant. The ^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectra were recorded at 300, 564.6 and 150 MHz, respectively, on Varian Mercury-VX300 and Varian Inova-600 spectrometers. Chemical shifts were recorded in parts per million (ppm) with TMS as the internal references and CF_3COOH as the external reference. ESI mass spectra were determined using a Finnigan LCQ Advantage mass spectrometer. IR spectra were recorded using an Avatar 360 FT-IR spectrophotometer. UV absorption spectra were recorded using a Perkin-Elmer Lambda 35 UV–vis spectrophotometer. The *N*-succinimidyl-4-azido-tetrafluorobenzoate **3** was synthesized using a previously described procedure [13].

4: Ethanolamine (0.50 mL, 8.25 mmol) was added to a solution of **3** (280 mg, 0.84 mmol) in dry CH_2Cl_2 (10 mL). The reaction mixture was stirred at 20 °C for 2 h. The organic solvent was then removed. After extracting the product with CH_2Cl_2, the extract was dried over $MgSO_4$ and evaporated under reduced pressure. The residue obtained was purified by performing chromatography on silica gel with ethyl acetate/petroleum ether 1/1, yielding **4** (70%) as white solid. Mp: 139–140.5 °C. ^{1}H NMR (300 MHz, CD_3OD): δ = 3.69 (t, J = 5.7 Hz, 2H), 3.49 (t, J = 5.7 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$ + CD_3OD): δ = 163.4, 142.8 ($^{1}J_{CF}$ = 255 Hz), 140.2 ($^{1}J_{CF}$ = 240 Hz, $^{2}J_{CF}$ = 17.4 Hz), 118.1, 114.9, 59.4, 41.4. ^{19}F NMR (564.6 MHz, $CDCl_3$ + CD_3OD): δ = −139.2 (m, 2F), −151.3 (m, 2F). IR (KBr, cm^{-1}): 3279 (NH), 2925, 2849 (C–H), 2123 (N=N=N), 1661 (C=O). ESI-MS: m/z 278.4 $[M]^{+}$.

1: A solution of hexadecanol (145 mg, 0.60 mmol) in dry THF (10 mL) was added dropwise to a mixture of freshly distilled phosphorus oxychloride (0.60 mL, 0.64 mmol) and triethylamine (1.0 mL, 7.2 mmol) in THF (5.0 mL), under nitrogen at 0 °C for 30 min. After 30 min at 0 °C, the mixture was left to warm up to 25 °C and then stirred for a further 2 h. A solution of **4** (165 mg, 0.59 mmol) in THF (10 mL) was then added dropwise to this mixture at 0 °C. The reaction mixture was stirred at 0 °C for another 30 min and then at 25 °C for 24 h before adding H_2O (2.0 mL), and stirred again at 25 °C for 1 h. The organic solvent was then removed. After extracting the solution (pH 1.0) with CH_2Cl_2, the extract was dried over $MgSO_4$ and evaporated under reduced pressure. The residue obtained was purified by chromatography on silica gel with MeOH/CH_2Cl_2 1/7, yielding **1** (20%) in the form of a white solid. Mp: 90 °C (decomposition) ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD): δ = 3.98 (m, 2H), 3.82 (m, 2H), 3.63 (m, 2H), 1.59 (m, 2H), 1.26 (m, 26H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$ + CD_3OD + CF_3COOH): δ = 158.4, 141.1, 137.7, 114.1 ($^{1}J_{CF}$ = 284 Hz), 113.5 ($^{1}J_{CF}$ = 282 Hz), 66.5, 64.0, 52.5, 31.0, 29.3, 28.7, 28.4, 28.3, 24.6, 21.7, 13.0. ^{19}F NMR (564.6 MHz, $CDCl_3$ + CD_3OD): δ = −138.3 (dd, J = 20.9, 9.60 Hz, 2F), −147.9 (dd, J = 20.9, 9.60 Hz, 2F). IR (KBr, cm^{-1}): 3347 (NH), 2919, 2851 (C–H), 2122 (N=N=N), 1666 (C=O). ESI-MS: m/z 581.0 $[M-H]^{-}$. UV: (MeOH/CH_2Cl_2) λ_{max} 257.8 nm (ε 4910 mol^{-1} L cm^{-1}).

5: Pentafluorobenzoic acid (424 mg, 2.0 mmol) was added to $SOCl_2$ (10 mL), and then refluxed for 20 h. After removing the $SOCl_2$, the residue was dissolved in THF (10 mL). The solution was added dropwise to a solution of 1,8-octanediol (584 mg, 4.0 mmol) in THF (10 mL) at 0 °C. After 30 min at 0 °C, the mixture was left to warm up to 25 °C and then stirred for another 8 h. The organic solvent was removed, and the residue obtained was purified by chromatography on silica gel with ethyl acetate/petroleum ether 1/5, yielding **5** (62%) in the form of a white solid. Mp: 34.5–35.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.36 (t, J = 6.6 Hz, 2H), 3.63 (t, J = 6.6 Hz, 2H), 1.70 (m, 2H), 1.55 (m, 2H), 1.34 (m, 8H). ^{13}C NMR (150 MHz, $CDCl_3$):

δ = 159.6, 145.9 ($^1J_{CF}$ = 255 Hz, $^2J_{CF}$ = 13.5 Hz), 141.2 ($^1J_{CF}$ = 240 Hz, $^2J_{CF}$ = 16.5 Hz), 123.3, 108.3 ($^2J_{CF}$ = 15 Hz), 66.9, 63.2, 32.9, 29.9, 29.3, 28.6, 25.9, 25.8. ^{19}F NMR (564.6 MHz, $CDCl_3$): δ = −138.8 (m, 2F), −149.3 (m, 1F), −160.8 (m, 2F). ESI-MS: *m/z* 341.0 $[M + H]^+$.

6: NaN_3 (80.7 mg, 1.24 mmol) was added to a solution of **5** (422 mg, 1.24 mmol) in H_2O (5.0 mL) and acetone (5.0 mL). The reaction mixture was stirred at 70 °C for 12 h. The organic solvent was then removed. After extracting the residual mixture with CH_2Cl_2, the extract was dried over $MgSO_4$ and evaporated under reduced pressure. The residue obtained was purified by chromatography on silica gel with ethyl acetate/petroleum ether 1/5, yielding **6** (92%) in the form of a white solid. Mp: 44.5–45.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.36 (t, *J* = 6.3 Hz, 2H), 3.62 (t, *J* = 6.6 Hz, 2H), 1.74 (m, 2H), 1.56 (m, 2H), 1.34 (m, 8H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.5, 145.3 ($^1J_{CF}$ = 255 Hz), 140.4 ($^1J_{CF}$ = 255 Hz, $^2J_{CF}$ = 15 Hz), 123.2, 108.0 ($^2J_{CF}$ = 15.3 Hz), 67.0, 63.0, 32.7, 29.7, 29.3, 28.4, 25.7. ^{19}F NMR (564.6 MHz, $CDCl_3$): δ = −139.2 (m, 2F), −151.4 (m, 2F). IR (KBr, cm^{-1}): 3369 (OH), 2933, 2859 (C–H), 2130 (N=N=N), 1737 (C=O). ESI-MS: *m/z* 362.3 $[M - H]^+$.

2: A solution of **6** in dry CH_2Cl_2 (10 mL) was added dropwise to a mixture of freshly distilled phosphorus oxychloride (1.2 mL, 1.3 mmol) and triethylamine (1.0 mL, 7.2 mmol) in THF (5.0 mL) at 0 °C under nitrogen. After 30 min at 0 °C, the mixture was left to warm up to 25 °C and then stirred for 1 h. A solution of choline tosylate (875 mg, 3.0 mmol) in freshly distilled pyridine (10 mL) was added to this mixture. The resulting mixture was stirred under nitrogen for 3 days at 25 °C. Water (1.0 mL) was added, and the solution obtained was stirred for 1 h. After being evaporated under reduced pressure, the residue was purified by chromatography on silica gel with H_2O/MeOH/CH_2Cl_2 4/35/65, yielding **2** (35%) in the form of a yellowish solid. Mp: > 70 °C (decomposition). ^{1}H NMR (300 MHz, D_2O): δ = 4.12 (m, 4H), 3.72 (m, 2H), 3.55 (m, 2H), 3.12 (s, 9H), 1.49 (m, 4H), 1.16 (m, 8H). ^{13}C NMR (150 MHz, D_2O): δ = 159.4, 145.3 ($^1J_{CF}$ = 255 Hz), 141.1 ($^1J_{CF}$ = 225 Hz, $^2J_{CF}$ = 15 Hz), 123.6, 107.1, 66.9, 66.2, 59.4, 54.2, 30.5, 29.1, 28.9, 28.2, 25.5. ^{19}F NMR (564.6 MHz, D_2O): δ = −141.3 (s, br, 2F), −153.4 (s, br, 2F). IR (KBr, cm^{-1}): 3390 (OH), 2933, 2858 (C–H), 2130 (N=N=N), 1733 (C=O). ESI-MS: *m/z* 529.2 $[M + H]^+$. UV: (H_2O) λ_{max} 267.1 nm (ε 6973 mol^{-1} L cm^{-1}).

4.2. General photolytic procedure

Compounds **1** and **2** were dissolved in MeOH/CH_2Cl_2 and in 100 mM phosphate buffer at pH 7.4, respectively, to a concentration of approximately 1.5×10^{-4} mol L^{-1}. The solutions (1.5 mL) were photolyzed under stirring using a 150 W USHIO Xenon Short Arc Lamp for 0–10 min at 20 °C. The absorption spectra of the irradiated samples were recorded using a CARY UV spectrophotometer.

Acknowledgements

We are grateful to Professor Andre Samat for giving us access to the irradiation equipment used here. We thank Dr. Jessica Blanc for revising the English manuscript. Financial support from the Ministry of Science and Technology in China (N° 2003CB114400, 2003AA2Z3506), Cheung Kong Scholar Foundation, Wuhan University and CNRS is gratefully acknowledged.

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