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SYNTHESIS AND CHARACTERIZATION OF POTENTIAL PHOTOLABELING PROBES FOR STUDYING THE ANTIVIRAL MECHANISMS OF EICAR

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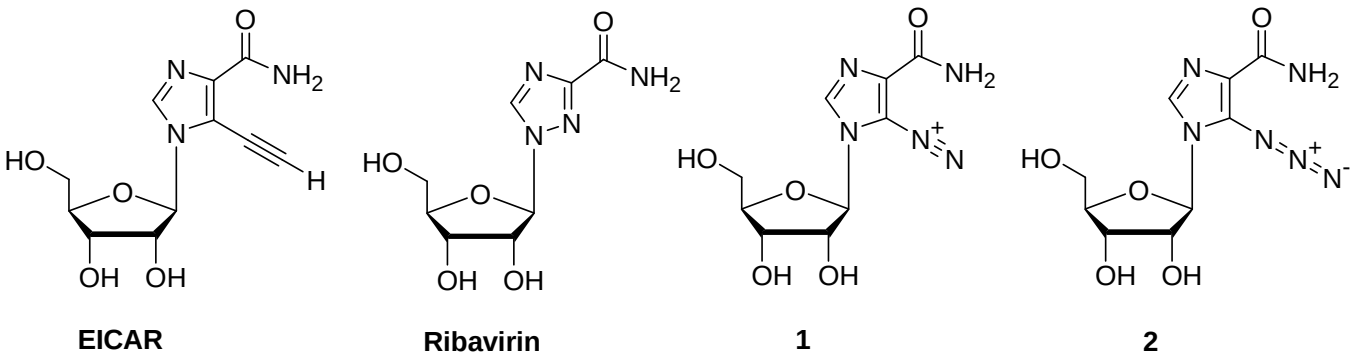
Abstract –The designed photolabeling probes: 5-diazonium-1-β-D-ribofurano-syl-1*H*-imidazole-4-carboxamide (**1**) and 5-azido-1-β-D-ribofuranosyl-1*H*-imida-zole-4-carboxamide (**2**) were synthesized *via* a short and convenient synthetic route, namely by diazotizing AICAR and subsequently performing substitution with NaN_3. Although **1** was not stable even at low temperatures, **2** showed a rapid and clean photochemical reaction, which suggests that it may be a valuable tool for use in photolabeling studies.

INTRODUCTION

Various antiviral derivatives of ribavirin have been synthesized since the discovery of ribavirin (Scheme 1), which was the first synthetic nucleoside showing a broad-spectrum of antiviral activity against many RNA and DNA viruses, as established over 30 years ago.[1] One of the most potent congeners of ribavirin is 5-ethynyl-1-β-D-ribofuranosyl-1*H*-imidazole-4-carboxamide (EICAR, Scheme 1).[2] The antiviral potency of EICAR is approximately 10 to 100 times greater than that of ribavirin.[3] In addition to its antiviral activity, EICAR exhibits antitumor activity and inhibits the growth of various tumor cells *in vitro*.[4] The mechanisms underlying the antiviral effects of EICAR have not yet been definitely elucidated, but they seem to be similar to those of ribavirin, which inhibits inosine 5'-monophosphate dehydrogenase *via* multiple modes of action.[3] Ribavirin was recently found to inhibit viral RNA polymerases and viral

capping enzymes, to induce mutagenic effects in viral RNA genomes, and to modulate the host immune responses.[5] The antiviral mechanisms of EICAR therefore seem to be complex and to involve multiple modes of action. In order to determine whether EICAR also targets viral RNA polymerases and viral capping enzymes as ribavirin, and to reach a better understanding of the complex mode of action of EICAR, we would like to propose a photolabeling study.[6]

In photolabeling studies, the photoprobe of a ligand, when subjected to photoirradiation, generates highly reactive species, leading to the covalent labeling of target molecules.[6] This enables us to identify the targets of the ligand, to map the binding site on the target, and to determine the ligand-target interactions. Aryl azides are by far the most widely used photolabeling probes at present, because they can be readily synthesized, show a high level of chemical stability in the dark and are highly reactive to photoirradiation.[6] Furthermore, aryl diazoniums are powerful photolabeling probes because of their extremely high reactivity when subjected to photoactivation. The latter compounds are, however, much less stable than aryl azides in the dark, which is a disadvantage as regards their use as photolabeling probes. It was therefore proposed here to use nucleosides (**1**) and (**2**) as probes (Scheme 1) to study the mechanisms underlying the antiviral effects of EICAR using a photolabeling approach. Like EICAR, these two probes are imidazole nucleosides. They have either a diazonium or an azido group in position 5. The ethynyl group in position 5 on the imidazole ring of EICAR is known to be vital to the biological activities of EICAR. The diazonium and azido groups both have similar geometry to the ethynyl group, and hence probes (**1**) and (**2**) presumably have similar biological activities, while being photochemically reactive. Here, we report on the synthesis and characterization of probes (**1**) and (**2**).

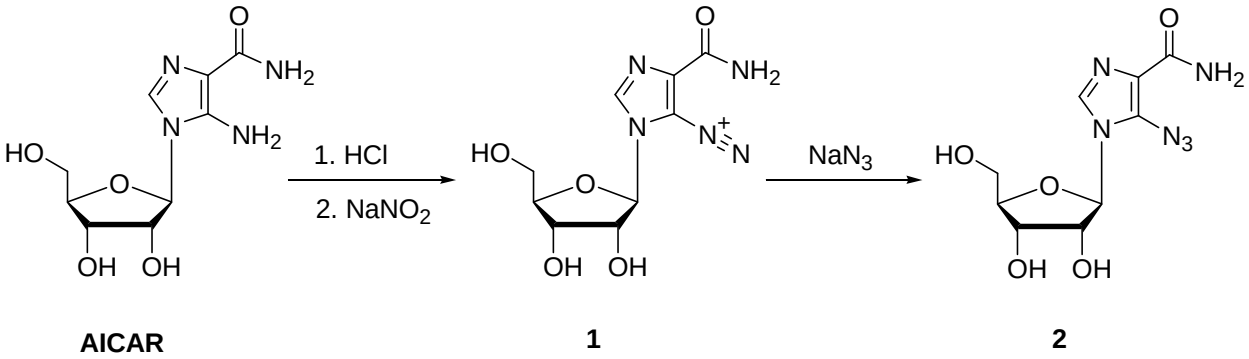


Scheme 1: Ribavirin, EICAR and the proposed photolabeling probes (**1**) and (**2**).

RESULTS AND DISCUSSION

The synthesis of **2** has been described by T. Fujii[7], starting with 5-amino-*N*'-methoxy-1-β-D-ribofurano-syl-1*H*-imidazole-4-carboxamidine, and giving an overall yield of 65% after a two-step process of

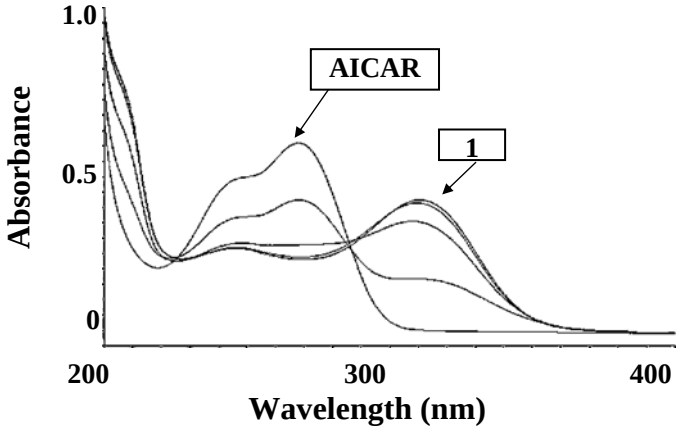
synthesis. However, the starting material is not commercially available and its synthesis from adenosine requires a three-step process, *via* oxidation, methylation and ring opening.[7] Consequently, the synthetic pathway previously described for **2** is lengthy and time-consuming.[7] Therefore, a short and convenient synthetic route, starting with 5-amino-1-β-D-ribofuranosyl-1*H*-imidazole-4-carboxamide (AICAR), followed by diazotization and subsequent substitution with NaN_3, should provide an efficient shortcut procedure for synthesizing **1** and **2** (Scheme 2).



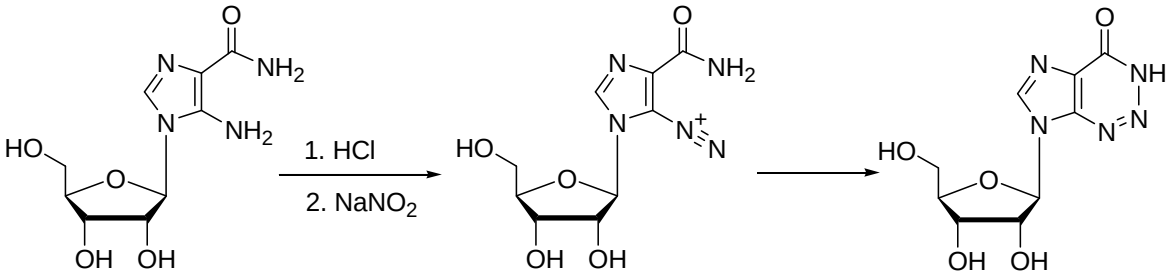
Scheme 2: Synthesis of **1** and **2** starting with AICAR, and performing diazotization and substitution.

The diazotization of AICAR was carried out with $NaNO_2$ in 6 N HCl at -20°C, and the reaction was monitored by UV spectrophotometer. When subjected to diazotization, **1** was formed, which has the UV absorption of around 310 nm (Figure 1) characteristic of aryldiazoniums.[6b] However, we were not able to isolate **1** because it was not stable even at 0°C under strongly acidic conditions. It has been suggested that some diazoniums might be a more stable form of tetrafluoroborate salts. We therefore carried out the diazotization of AICAR with $NaNO_2$ in HBF_4 or with isoamyl nitrite in HBF_4. However, our attempts to obtain **1** in the form of a pure tetrafluoroborate salt were not successful, since **1** decomposed rapidly.

Figure 1: UV spectral recording of the formation of **1** resulting from the diazotization of AICAR.



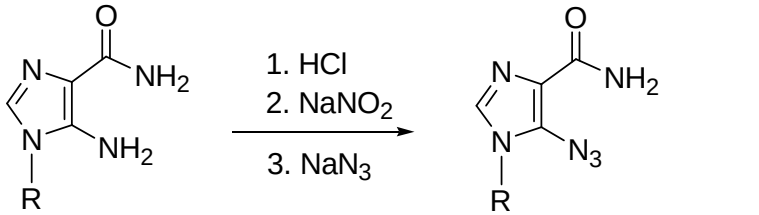
Although we were not able to isolate **1**, we did obtain **2** in moderate to high yields when we performed the substitution reaction on **1** with NaN_3 in situ at –20 °C. It was previously reported that the diazotization of AICAR with $NaNO_2$ in 6 N HCl at –25°C gave the ring closure product 2-azainosine as the main product[8] (Scheme 3), and the subsequent substitution resulted in a very poor yield.[9] However, we did not succeed in isolating the ring closure product, 2-azainosine, under the present experimental conditions.



AICAR **1**

Scheme 3: Diazotization of AICAR led to ring-closure product, 2-azainosine.[8]

We obtained **2** with a yield of around 40%, occasionally reaching 60%. In addition, we isolated 5-azido-1*H*-imidazole-4-carboxamide, the product of glycosyl hydrolysis of **2**, from the reaction mixture with a yield of around 10%. In addition, when we performed the diazotization of *N*-substituted 5-amino-1*H*-imidazole-4-carboxamide (**3a**) or (**3b**) with $NaNO_2$ in 6 N HCl at 0 °C, followed by substitution with NaN_3, we obtained the corresponding products (**4a**) or (**4b**) with a satisfactory, reproducible yield (Scheme 4). Performing diazotization and substitution on AICAR therefore turned out to constitute an efficient shortcut procedure for synthesizing **2** (Scheme 2). It is worth noting that it was crucial to control closely both the temperature and the pH during the reaction. Strongly acidic conditions were necessary to stabilize the diazonium intermediate formed, while suppressing the intra- and intermolecular diazo-coupling reaction.[10] However, the strongly acidic conditions favored the hydrolysis of the glycosyl linkage, which could be compensated for by lowering the reaction temperature.



3a: R = Me
3b: R = Et

4a: R = Me (71.4%)
4b: R = Et (73.4%)

Scheme 4: Diazotization-substitution reaction giving *N*-substituted 5-amino-1*H*-imidazole-4-carboxamide.

To be a successful photolabeling probe, it is necessary for the probe to be stable in the dark and to be highly reactive to photoactivation for covalent labeling to occur. The diazonium probe (**1**) was not stable in the dark even at low temperatures, while azides (**2**) and (**4a-b**) were much more stable. We therefore undertook the photochemical studies only with **2** and **4a-b**. Both **2** and **4a-b** underwent rapid decomposition upon being exposed to photoirradiation. Since **2** was not readily soluble in organic solvents, the photochemical study was then undertaken with **4b** in various organic solvents such as $HNEt_2$, CH_2Cl_2 and 2,3-dimethyl-2-butene. HPLC analysis showed that the photodecomposition of **4b** occurred very rapidly (Figure 2). Photoirradiation of **4b** in $HNEt_2$ gave one main product along with other minor by-products (Figure 2a). Irradiation of **4b** in CH_2Cl_2 led to almost only one main product (Figure 2b), which was identified as **3b** by comparison with the authentic sample of **3b**. Photoirradiation of **4b** in 2,3-dimethyl-2-butene gave two main products (Figure 2c): the one was the amine (**3b**), and the other was the double bond insertion product, which was confirmed by MS/MS. Photoirradiation of **2** in phosphate buffer resulted in rapid photodecomposition (Figure 3). These results agree with the mechanism suggested in the case of aryl nitrenes, which undergo insertion, rearrangement and H-abstraction etc.[11] It has been reported that imidazoyl nitrene might undergo a ring opening reaction.[12] However, we did not observe this reaction under our experimental conditions. The results of our photochemical studies suggested that **2** might be a promising probe for use in photolabeling studies on the molecular mechanisms responsible for the action of EICAR.

Figure 2: HPLC analysis of photoirradiation of **4b** in different organic solvents: (a) **4b** in Et_2NH (b) **4b** in CH_2Cl_2 (c) **4b** in 2,3-dimethyl-2-butene.

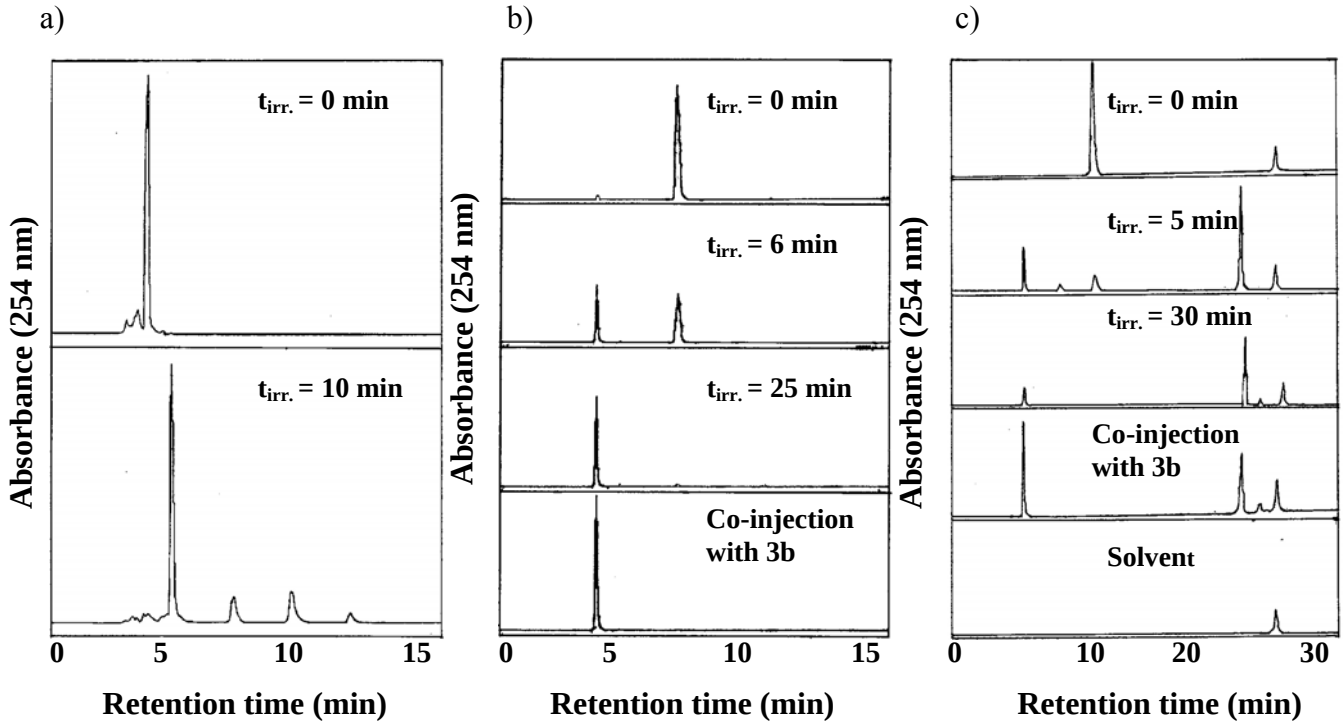
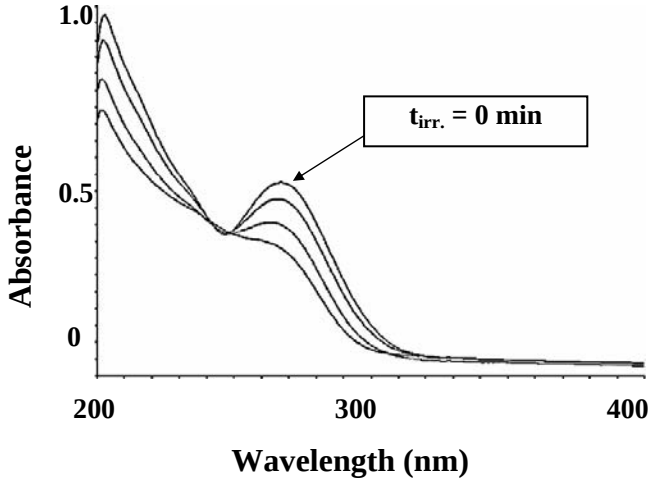


Figure 3: UV-spectral recording of the photoirradiation of **2** in 50 mM phosphate buffer, pH 7.4.



In conclusion, we proposed here two photoprobes (**1**) and (**2**) for studying the mechanisms underlying the antiviral effects of EICAR by a photolabeling approach. These probes were synthesized by performing the diazotization and diazotization-substitution of AICAR, respectively. Probe (**1**) was not stable and decomposed rapidly, while probe (**2**) could be obtained readily with a yield of around 40%. The photodecomposition of **2** and **4b** was studied by UV spectral and HPLC analysis, and showed a rapid and clean photochemical reaction. Probe (**2**) therefore constitutes a potentially useful probe for studying the molecular mechanisms underlying the action of EICAR, using photolabeling approach. In addition, the imidazoyl azides are a promising new class of photoprobes, which provide a new range of solutions for developing new photolabeling reagents for use in photolabeling experiments. Since many bioactive compounds contain the imidazole ring, imidazoyl azides promise to be of a general interest because of the wide range of applications in which they can be used as photoprobes.

EXPERIMENTAL

General: The ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively, on a varian mercury-VX300 spectrometer and chemical shifts were recorded in parts per million (ppm) with TMS as the internal reference. MS spectra were determined using ZAB-HF-3F organic mass spectrometry. MS/MS spectral analysis was performed with an API III Plus Sciex Triple Quadripole spectrometer. IR spectra were recorded with an Avatar 360 FT-IR spectrophotometer. UV spectral analysis was performed with a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. HPLC were performed on a Waters^{TM} 600 pump with a Waters 600 controller and the components were detected using a Waters 2996 photodiode array detector. A C_{18} column (4.6 × 250 mm) was used for HPLC analysis. All the compounds were purified by performing flash chromatography on silica gel (200-300 mesh). Compounds (**3a**) and (**3b**)

were prepared as described in the literature.[13]

5-Diazonium-1-β-D-ribofuranosyl-1*H*-imidazole-4-carboxamide (**1**). 1 N $NaNO_2$ (0.15 mL, 0.150 mmol) was added in three parts to the solution of AICAR (33.5 mg, 0.129 mmol) in 6 N HCl (5 mL) at –20 °C. Upon each addition of $NaNO_2$ (50 μL), a sample was withdrawn and diluted 500-fold in distilled water, and measured with a UV spectrophotometer (Figure 1). **1** showed the UV absorption of around 310 nm characteristic of diazonium salt.

5-Azido-1-β-D-ribofuranosyl-1*H*-imidazole-4-carboxyamide (**2**). AICAR (96 mg, 0.37 mmol) was dissolved in 6 N HCl (3 mL) at –20 °C, and the cold solution was added dropwise (15 min) to the solution of 1 N $NaNO_2$ (0.39 mL, 3.9 mmol) at –20 °C. Then, NaN_3 (51 mg, 0.78 mmol) was added. The reaction mixture was kept at –20 °C for a further 2 h. The reaction solution was then brought to pH 8 by adding saturated sodium carbonate solution and extracted with ethyl acetate (10 × 10 mL). The combined organic phase was evaporated on a rotary evaporator and the residue was dissolved in methanol before being adsorbed on the silica gel for column chromatography with CH_2Cl_2/MeOH (10:1), giving **2** (40 mg, 38%) in the form of a white solid: mp 107 ˚C (decomp). ^{1}H-NMR (DMSO-d_6) δ 7.93 (s, 1H), 7.36 (br s, 1H), 7.18 (br s, 1H), 5.48-5.50 (m, 2H), 5.14 (d, 1H, J = 4.5 Hz), 5.01 (t, 1H, J = 5.0 Hz), 4.22-4.26 (m, 1H), 4.03-4.07 (m, 1H), 3.87-3.89 (m, 1H), 3.54-3.62 (m, 2H). ^{13}C-NMR (DMSO-d_6) δ 164.4, 132.5, 130.6, 124.6, 88.1, 85.9, 75.2, 70.5, 61.6. MS ($C_9H_{12}N_6O_5$, FAB) 281 $(M+Na-N_2+2H)^+$. IR (KBr, cm^{-1}) 2147. UV λ_{max} (EtOH) 267 nm (ε 5560).

5-Azido-1-methyl-1*H*-imidazole-4-carboxamide (4a). The solution consisting of **3a** (0.2 g, 1.43 mmol) in 6 N HCl (8 mL) was added to the pre-cooled aqueous solution of 1 N $NaNO_2$ (1.6 mL, 1.60 mmol). After 5 min, NaN_3 (0.18 g, 0.286 mmol) was added. The reaction was kept at 0°C for a further 4 h, and the pH of the solution was then adjusted to 8 by adding saturated sodium carbonate solution. The precipitate was collected by centrifugation, washed with water three times and this gave compound (**4a**). The combined solution obtained by centrifugation was extracted with dichloromethane (3 × 10 mL), and the extracted solution was concentrated and chromatographed on silica gel with CH_2Cl_2/MeOH (10:1) to obtain another part of **4a**. The total yield of **4a** (0.17 g) obtained was 71%. ^{1}H-NMR (DMSO-d_6) δ 7.54 (s, 1H), 7.24 (br s, 1H), 7.08 (br s, 1H), 3.44 (s, 3H). ^{13}C-NMR (DMSO-d_6) δ 164.6, 134.7, 131.2, 124.2, 31.3. MS ($C_5H_6N_6O$, FAB) 167 $(M+1)^+$. IR (KBr, cm^{-1}) 2150. UV λ_{max} (EtOH) 269 nm (ε 6872).

5-Azido-1-ethyl-1*H*-imidazole-4-carboxamide (4b). Compound (**4b**) was prepared from **3b** using the same procedure as that described in the case of **4a**: yield 73%. mp 94 ˚C (decomp). ^{1}H-NMR (DMSO-d_6) δ 7.61 (s, 1H), 7.28 (br s, 1H), 7.11 (br s, 1H), 3.85 (q, 2H, J = 6.6 Hz), 1.27 (t, 3H, J = 6.6 Hz). ^{13}C-NMR (DMSO-d_6) δ 164.6, 133.8, 130.5, 124.3, 19.8, 16.3. MS ($C_6H_8N_6O$,

FAB) 181 $(M+1)^+$. IR (KBr, cm^{-1}) 2146. UV λ_{max} (EtOH) 269 nm (ε 6686).

General Photolysis Procedure. Compound (**4b**) was dissolved, repectively, in Et_2NH, CH_2Cl_2 and 2,3-dimethyl-2-butene. Compound **(2)** was dissolved in 50 mM phosphate buffer at pH 7.4. The concentrations obtained in both cases were around 3.0×10^{-3} mol/L. The solutions were photolyzed under stirring using a 125 W Philipp mercury lamp for 0-30 min. at 20 °C. The irradiated sample was withdrawn for UV spectral measurement and HPLC analysis.

MS/MS analysis of photoproduct: After irradiation of **4b** in 2,3-dimethyl-2-butene, the double bond insertion product was isolated by HPLC and identified by MS for $C_{12}H_{20}N_4O$, with m/z 237 $[M + H]^+$. Further MS/MS spectral analysis of this ion gave fragment peaks of m/z 220 (loss of NH_3) and 192 (loss of $HCONH_2$). The ion measured at m/z 164 was found to be due to the consecutive decomposition of m/z 192 (loss of CH_2CH_2). Likewise, the ion of m/z 137 can be explained as the loss of a HCN molecule from the ion of m/z 164 . All these results confirm the structure of the double bond insertion product $C_{12}H_{20}N_4O$.

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