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Synthesis, Azido–Tetrazole Equilibrium Studies and Biological Activity of 1-(2-Azido-6-Chloropyrid-4-yl)-3-Phenylurea, a Photoaffinity Labeling Reagent for Cytokinin-Binding Proteins

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Abstract—1-(2-Azido-6-chloropyrid-4-yl)-3-phenylurea was synthesized using known methods. Azido–tetrazole equilibrium for this compound was studied in various solvents, and the azide tautomer was found to be largely predominant. However, in water solution, it is suspected to exist in the tetrazole form in a significant amount. Like other 2,6-disubstituted pyridylurea analogs, it exhibits high cytokinin activity, and it is easily photolysable. Thus it appears to be a good candidate as a photoaffinity labeling reagent for cytokinin-binding proteins, receptors in particular. In the absence of the 6-chloro substituent, the tetrazole form was the only existing tautomer. The corresponding compound does not exhibit cytokinin activity and is not photolysable.

Introduction

In the course of our studies on the mode of action of cytokinins, we needed a photoaffinity labeling reagent as a probe for the hypothetical receptors of this important class of plant hormones. Azido derivatives of N^6-substituted adenines, analogs of natural cytokinins, have been previously synthesized as potential photoaffinity labeling reagents.[1] They exhibited high cytokinin activity and were easily photolysable. In fact, the 8-azido derivatives of zeatin and 6-(3-methylbut-2-enylamino)purine exhibited higher biological activity than the parent molecules. However, radiolabeling of these compounds has not been obtained, except for a ^{14}C-labeled 2-azido-6-benzylaminopurine[2] of low specific activity. This was successfully used to characterize a benzyladenine binding-site peptide[3] in CBF-1, a very abundant protein in wheat germ which binds lipophilic purine-type cytokinins.[4] More recently, a method was described for the synthesis of tritiated 2-azido-6-benzylaminopurine with high specific activity from 2-chloro-6-(3-tritiobenzylamino)purine prepared by iodine–tritium exchange in 2-chloro-6-(3-iodobenzylamino)purine.[5] However, this method has the inconvenience of introducing the tritium label at an early step of the synthesis.

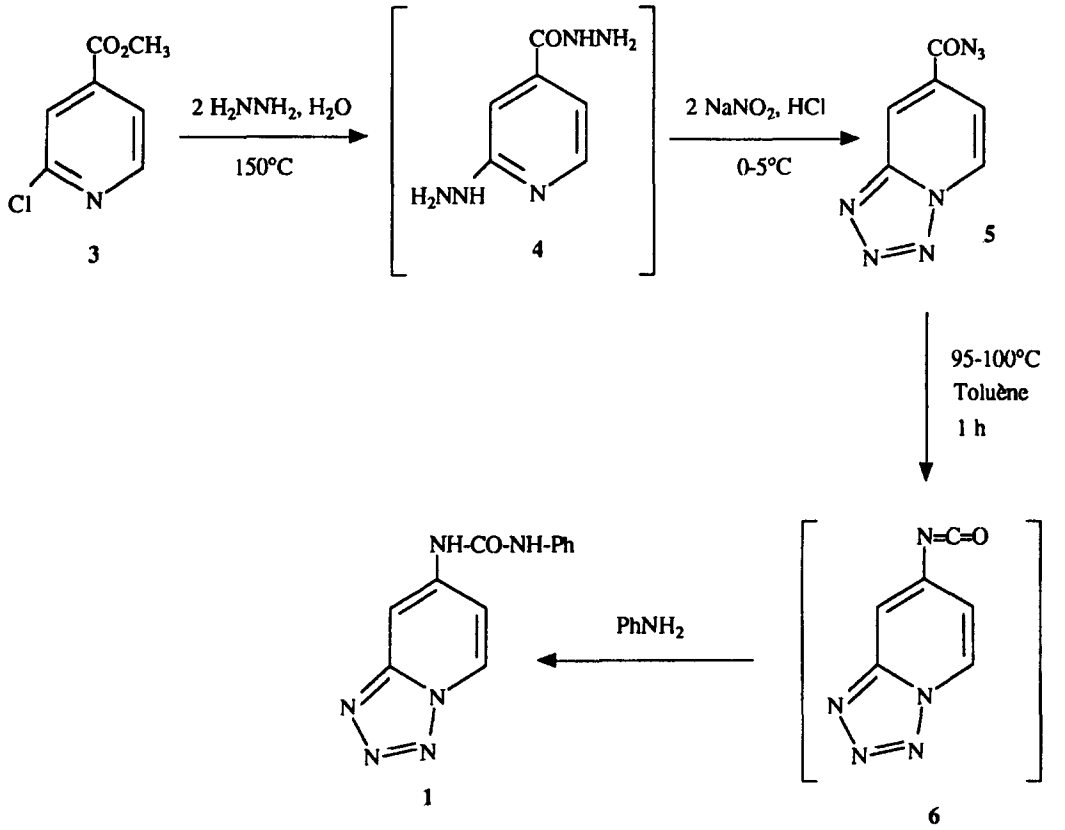
As an alternative, we sought to develop a photoaffinity probe derived from 3-phenyl-1-(pyrid-4-yl)ureas which compose a family of very potent cytokinins.[6] Optimum activity in this series requires electronegative substituents (halogens, CN, CF_3) in the 2- or 2,6-positions of the pyridine ring, and no substitution of the phenyl ring, except 3-fluoro. The azido group is considered to exert similar electronic effects to the bromo substituent.[7] Thus, it was expected that a 1-(2-azidopyrid-4-yl)-3-phenylurea derivative would be a biologically active and photoactivatable cytokinin, and might be easily tritiated in the phenyl ring, all favorable properties to obtain an efficient photoaffinity labeling reagent. However, while aryl azides have been widely used in photoaffinity labeling,[8] and while their photochemical properties have been extensively studied,[9] no use of the 2-azidopyridyl group has been described. Furthermore, the azido–tetrazole equilibrium which takes place in 2-azidopyridines[10] can influence both the biological activity and the photoreactivity.

We describe in this paper the synthesis of 1-phenyl-3-(tetrazolo[1,5-*a*]pyrid-7-yl)urea (**1**) and 1-(2-azido-6-chloropyrid-4-yl)-3-phenylurea (**2**). The azido–tetrazole equilibrium of these compounds, their biological activity as cytokinins and their photolysis have also been examined. The urea **2** appears to be a good candidate for the photoaffinity labeling of cytokinin-binding proteins, and a tritiated derivative is proposed for this purpose.

Results and Discussion

Syntheses

The urea **1** was prepared from methyl 2-chloroisonicotinate[11] (**3**) (Scheme 1). This ester was heated at 150 °C in a sealed tube with hydrazine hydrate to give the dihydrazino derivative **4**. Treatment of **4** with sodium nitrite in the presence of hydrochloric acid yielded a sole product, as revealed by ^{1}H NMR spectra recorded in DMSO-d_6 or chloroform-*d*. The IR spectrum of this compound showed only one azide absorption between 2130 and 2140 cm^{-1} attributed to



Scheme 1. Synthesis of 1-phenyl-3-(tetrazolo[1,5-*a*]pyrid-7-yl)urea (**1**).

the carbonylazide, since the azidopyridine would cause an absorption around 2160 cm^{-1}.[12] The tetrazolo[1,5-*a*]pyridylurea structure was then assigned to the obtained product **5**. The carbonyl azide **5** was heated in toluene at 90–100 °C for 1 h. It underwent the Curtius rearrangement to the isocyanate **6** which was further reacted *in situ* with aniline at room temperature to give the expected urea **1**. As in the carbonyl azide **5**, the existence of an azidopyridine form was ruled out by the absence of IR absorption around 2160 cm^{-1}.

The urea **2** was prepared through a similar way to that used for the synthesis of **1** (Scheme 2), starting from methyl 2,6-dichloroisonicotinate (**7**). The reaction conditions were different only in the first step, where the temperature of reaction was as low as 80 °C, to allow the selective substitution of one chloro group. The compounds **9** and **2** were obtained at least partially in their azido form, as revealed by their IR spectra.

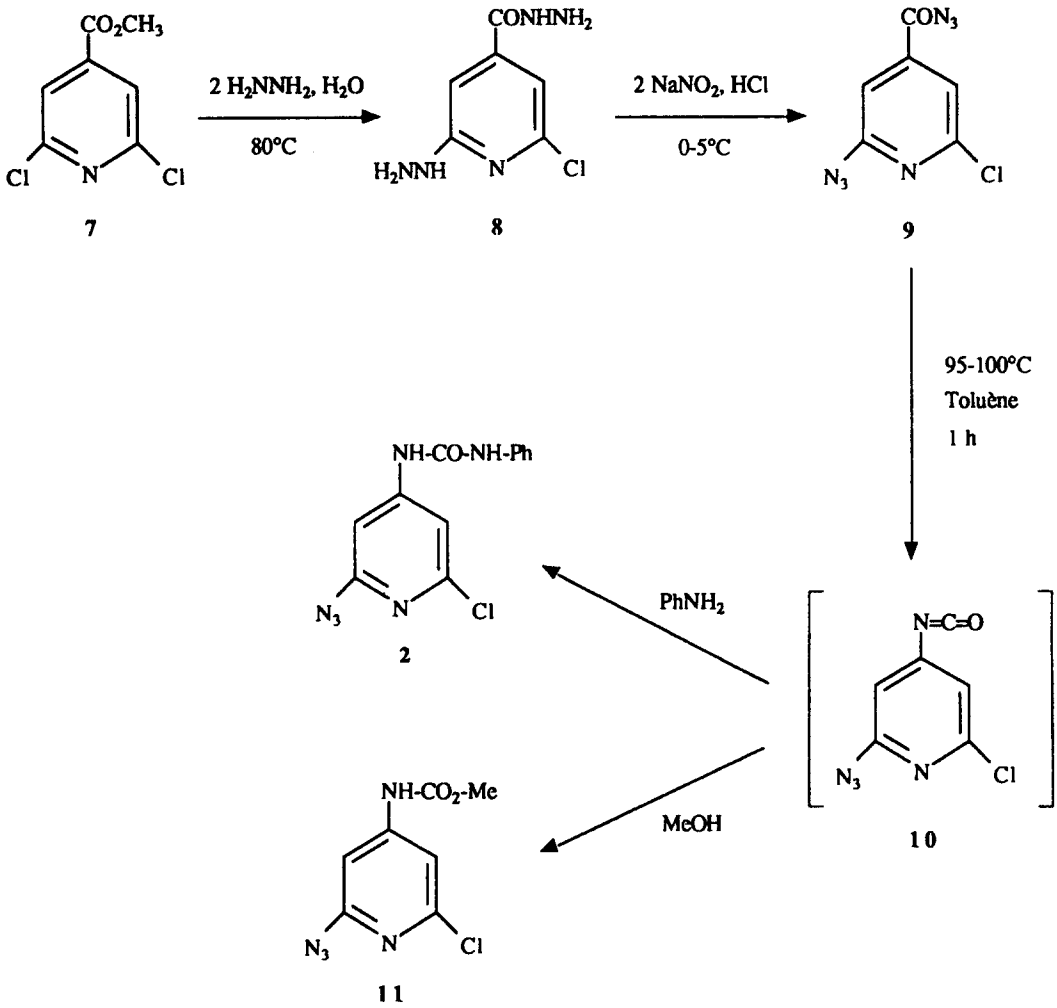
A radiolabeled derivative [^{3}H]**2** tritiated in the phenyl ring was prepared by reacting 4-[^{3}H]aniline in a toluene solution with a large excess of the isocyanate **10**. Unreacted **10** was quenched by methanol at the end of the reaction, leading to a mixture of [^{3}H]**2** and the carbamate **11**. This latter compound was prepared separately by the same reaction (Scheme 2).

Azido–tetrazole equilibrium studies

^{1}H NMR spectrometry is a powerful technique for studying azido–tetrazole equilibrium.[10] In 2-azido and tetrazolopyridines, the pyridine ring H-signals appear downfield in the tetrazole tautomer when compared with those from the azido tautomer. Thus, all the signals can be easily assigned and the composition of mixtures of tautomers may be determined.

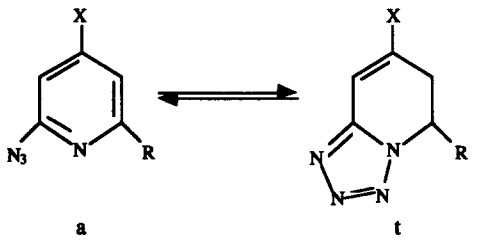
The carbonyl azide **9**, the urea **2** and the carbamate **11** were studied by ^{1}H NMR in DMSO-d_6. In this solvent, which is known to favor the existence of the tetrazole tautomer, the azide form was in fact predominant (Table 1). The amount of the azide represented more than 95% of the mixture of tautomers in the urea **2** and in the carbamate **11**, and 70% in the carbonyl azide **9**. When compared with the equilibrium observed in 2-azido-6-chloropyridine[10b] (Table 1), the effects of phenylureido or carbamoyl substituents clearly favor the azide form, while the azidocarbonyl substituent exerts a very weak effect on the equilibrium.

The ^{1}H NMR spectrum of the urea **2** recorded in other solvents like chloroform-*d* or deuterated methanol revealed the exclusive presence of the azide tautomer.



Scheme 2. Synthesis of 1-(2-azido-6-chloropyrid-4-yl)-3-phenylurea (**2**) and of the corresponding methyl carbamate **11**.

Table 1. Azido–tetrazole equilibrium in 2-azidopyridines studied by ^{1}H NMR in DMSO at 21 °C



Cpd N°	R	X	%a
2	Cl	NHCONHPh	> 95
9	Cl	CON_3	70
11	Cl	$NHCO_2Me$	> 95
	H	H	71.5*

*Value determined at 25 °C.[10b]

A ^{1}H NMR spectrum of **2** could not be obtained in water due to its low solubility. However, reversed phase HPLC of this compound using a water–methanol elution gradient gave rise to a dissymetric peak with a slow increase of the signal followed by a fast return to the baseline. This elution pattern likely indicates that a significant amount of the tetrazole isomer exists in a water-rich solvent, but that it is quickly transformed to the azide isomer when the solvent turns progressively to pure methanol.

Photoreactivity of the ureas 1 and 2

The urea **1** was found to be quite resistant to photolysis

in water, even when using a 400 W medium pressure mercury vapour lamp. This result confirms that **1** only exists in the tetrazole form. On the other hand, the urea **2** in dilute solution in water or alcohols was quickly photolysed, even when the UV light was filtered through a Pyrex glass ($\lambda > 290$ nm). These latter conditions led to a sole photolysis product in water. Without the Pyrex filter, a mixture of products was obtained.[13]

Biological activity studies

The ureas **1** and **2** were assayed using cytokinin auxotroph *Nicotiana tabacum* W 38 cell suspension.[14] No cytokinin activity was detected for the tetrazolopyridylurea (**1**). On the contrary, the urea **2** was found to be approximately as active as the common reference cytokinin 6-benzylaminopurine (BA). The concentrations allowing 50% of the maximum cell growth (A_{50}) were 15 nM for **1** and 10 nM for BA, respectively. The lack of activity of the tetrazolopyridylurea **1** suggested that the tetrazole tautomer in the urea **2** was also inactive, and that the cytokinin activity of this compound should be attributed exclusively to the azide form.

In conclusion, the urea **2** is an active cytokinin which can be easily photoactivated. As a tritiated derivative can be easily prepared, this compound appears to be a promising photoaffinity reagent for probing cytokinin-binding proteins, including receptors.

Experimental

Chemistry

Melting points were taken on a Kofler bank or on a Reichert apparatus and are uncorrected. Elemental analyses were made by the Service Central d'Analyse du CNRS at Vernaison (France). ^{1}H NMR spectra were recorded with TMS as an internal standard, at 60 MHz on a Varian EM 360, or at 270 MHz on a Jeol GSX 270 WB spectrometer. IR and UV spectra were recorded on a Perkin-Elmer 841 and Lambda 2 spectrometers respectively. EI-MS were recorded on Varian MAT 112 or VG Autospec (HRMS) apparatus. TLC analyses were carried out on pre-coated plates of silica gel 60 F_{254} (Merck) and HPLC analyses were run on a Waters apparatus using Merck Lichrospher® RP 18 or Waters Novapak® C 18 columns and water–methanol mixtures as eluent. All operations involving azido compounds were driven in a dark room.

7-Azidocarbonyltetrazolo[1,5-a]pyridine (5). A mixture of methyl 2-chloroisonicotinate (**3**) (3.3 g, 19.2 mmol) and hydrazine hydrate (10 g, 0.2 mol) in ethanol (10 mL) was heated at 150 °C in a sealed tube for 6 h. The alcohol and the excess of hydrazine were evaporated under reduced pressure and the residue was triturated in water. The insoluble part was filtered off. The aqueous solution was evaporated and the resulting residue was then extracted several times with ethanol. The different fractions were collected and the solvent was removed by evaporation.

The crude 2-hydrazino-4-hydrazinocarbonylpyridine (**4**) obtained (0.417 g, 2.5 mmol), was dissolved in 10 M hydrochloric acid (100 mL). The mixture was cooled to 0 °C and a solution of sodium nitrite (0.69 g, 10 mmol) in water (10 mL) was then added dropwise with stirring, while the temperature was maintained below 5 °C. A brown precipitate appeared immediately. The mixture was extracted with diethyl ether, and the organic layers were washed with water and dried over Na_2SO_4. The solvent was then evaporated and the solid residue dried under reduced pressure over $CaCl_2$, giving product **5** (0.757 g, 21% based on **3**), which was not further purified; mp 90–93 °C; ^{1}H NMR 60 MHz (DMSO-d_6) 7.87 (1H, *dd*, 2 and 7.4 Hz, 6-H), 8.87 (1H, *d*, 2 Hz, 8-H), 9.57 (1H, *d*, 7.4 Hz, 5-H); IR (nujol) 2130–2140 cm^{-1} (ν_{N3}), 1690 cm^{-1} ($\nu_{C=O}$), 1630 cm^{-1} ($\nu_{C=N}$).

1-Phenyl-3-(tetrazolo[1,5-a]pyrid-7-yl)urea (1). 7-Azidocarbonyltetrazolo[1,5-*a*]pyridine (**5**) (0.757 g, 4 mmol) was heated at 90–100 °C in toluene (150 mL). After 1 h stirring, the mixture was cooled to room temperature and aniline (0.5 g, 5.37 mmol) was then added. The white precipitate obtained was filtered and dried under reduced pressure. Recrystallization from benzene–ethanol gave pure urea **1** (0.52 g, 51%) mp 261 °C; ^{1}H NMR 60 MHz (CF_3COOH) δ 7.4 (5H, *m*, phenyl Hs), 7.8 (1H, *d*, 7 Hz, 6-H), 8.76 (1H, *s*, 8-H), 9.0 (1H, *d*, 7 Hz, 5-H), 9.07 (1H, *s*, NH); ^{1}H NMR 270 MHz (DMSO-d_6) 7.07 (1H, *t*, 7.3 Hz, 4'-H phenyl), 7.33 (3H, *m*, 3' and 5'-H phenyl, and 6-H), 7.50 (2H, *dd*, 2.1 and 7.8 Hz, 2' and 6'-H phenyl), 8.22 (1H, *d*, 2.1 Hz, 8-H), 9.16 (1H, *d*, 7.5 Hz, 5-H), 9.06 and 9.59 (1H + 1H, 2*s*, 2 NH); EI-MS m/z 254 (19.7%, [M]$^+$), 226 (5, [M – N_2]), 199 (21.7, [M – N_2 – HCN]), 123, 119 (38.6, PhNCO), 93 (94, $PhNH_2$); UV $\lambda_{max}^{EtOH\ 95}$ 274 nm (ε 19,300); Anal. for $C_{12}H_{10}N_6O$: C, 56.69; H, 3.96; N, 33.05; Found: C, 56.23; H, 4.05; N, 32.61.

6-Chloro-2-hydrazino-4-hydrazinocarbonylpyridine (8). Methyl 2,6-dichloroisonicotinate (**7**) (6.2 g, 30 mmol) was heated in ethanol (100 mL) at 80 °C in the presence of hydrazine hydrate (16 g, 320 mmol) for 3 h. The solvent and the excess of hydrazine were then evaporated under reduced pressure. The resulting residue was recrystallized from water and dried at 100 °C in vacuum, to yield **8** (6.31 g, 94%) mp 238 °C; ^{1}H NMR 60 MHz (DMSO-d_6) 4.3 (2H + 2H, l*s*, 2 NH_2), 8.0 (1H, *s*, NH-pyr.), 9.8 (1H, *s*, CO-NH); Anal. for $C_6H_8ClN_5O$: C, 35.75; H, 4.00; Cl, 17.58; N, 34.74; Found: C, 35.83; H, 3.95; Cl, 17.85; N, 34.49.

2-Azido-4-azidocarbonyl-6-chloropyridine (9) (9a, azide form, in equilibrium with 9t, tetrazole form). The hydrazide **8** (5.04 g, 25 mmol) was dissolved in a stirred solution of 10% hydrochloric acid (200 mL). The mixture was cooled in an ice-water bath and a solution of $NaNO_2$ (4.14 g, 60 mmol) in water (50 mL) was then added dropwise. A precipitate was immediately

observed. The mixture was extracted with diethyl ether (300 mL) and the organic layers were washed with water (2 x 100 mL) and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the yellow residue was purified by column chromatography (silica) using CH_2Cl_2 as eluent, giving pure **9** (3.46 g, 62%) mp 64 °C; ^{1}H NMR 270 MHz (DMSO-d_6) δ 7.3 and 7.6 (0.7H + 0.7H, 2*d*, 1.2 Hz, 3-H and 5-H pyr. of **9a**), 8.0 and 8.8 (0.3H + 0.3H, 2*d*, 1.2 Hz, 3-H and 5-H pyr. of **9t**); ^{1}H NMR 270 MHz ($CDCl_3$) 7.2 and 7.5 (2H, 2*d*, 1.2 Hz, 3-H and 5-H pyr.); Anal. for $C_6H_2ClN_7O$: C, 32.23; H, 0.99; Cl, 15.86; N, 43.85; Found: C, 32.50; H, 0.90; Cl, 15.67; N, 43.51.

1-(2-Azido-6-chloropyrid-4-yl)-3-phenylurea (2). To a stirred solution of the isocyanate **10**, prepared as described for the synthesis of the urea **1**, from the carbonylazide **9** (0.46 g, 2.06 mmol) in toluene (50 mL), was added aniline (0.3 g, 3.22 mmol). The mixture was left for 15 h at room temperature. The white precipitate was then filtered and air-dried. Recrystallization from benzene–ethyl acetate gave pure urea **2** (0.5 g, 84%) mp 188 °C; ^{1}H NMR 270 MHz (DMSO-d_6) azide form **2a**, δ 6.98 (1H, *d*, 1.4 Hz, 3-H pyr.), 7.03 (1H, *t*, 7.3 Hz, 4'-H phenyl), 7.30 (2H, *t*, 7.8 Hz, 3' and 5'-H phenyl), 7.36 (1H, *d*, 1.4 Hz, 5-H pyr.), 7.45 (2H, *d*, 7.8 Hz, 2' and 6'-H phenyl), 9.06 and 9.46 (2H, 2*s*, 2 NH); signals from trace (< 5%) of the tetrazole tautomer **2t** were seen at δ 7.65 and 8.15 (3-H and 5-H pyr.) and at δ 9.18 and 9.60 (2 NH); ^{1}H NMR 270 MHz (CD_3OD) azide form **2a**, δ 6.91 (1H, *d*, 1.4 Hz, 3-H pyr.), 7.02 (1H, *t*, 7.3 Hz, 4'-H phenyl), 7.25 (2H, *t*, 7.5 Hz, 3'-H and 5'-H phenyl), 7.27 (1H, *d*, 1.2 Hz, 5-H pyr.), 7.39 (2H, *d*, 7.3 Hz, 2'-H and 6'-H phenyl); no signals observed for **2t**; EI MS m/z 288–290 (19.4 and 6.4%, $[M]^+$), 225 (22.1, $[M - N_2 - Cl]^+$), 157–159 (79 and 25.6), 129–131 (58.7 and 18.6), 119 (39.5, PhNCO), 93 (100, $PhNH_2$), 77 (99.4, $[C_6H_5]^+$), 65 (48.2), 51 (42.3), 39 (32.2); IR (nujol) 2165 cm^{-1} (ν_{N3}); UV $\lambda_{max}^{EtOH\ 95\%}$ 264 nm (ε = 30,300), 250 nm (ε = 35,100); Anal. for $C_{12}H_9ClN_6O$: C, 49.93; H, 3.14; Cl, 12.28; N, 29.11; Found: C, 50.39; H, 3.22; Cl, 12.18; N, 28.92.

Methyl 2-azido-6-chloropyrid-4-ylcarbamate (11). This compound was prepared in a similar way to the urea **2**, from the carbonylazide **9** (0.223 g, 1 mmol) and methanol (1 mL) in toluene (50 mL). The mixture was concentrated to dryness and the resulting residue recrystallized from ethanol–water, giving pure carbamate **11** (0.14 g, 61.5%) mp 95–99 °C; ^{1}H NMR 270 MHz (DMSO-d_6) azide form **11a**, δ 3.72 (3H, *s*, CH_3O), 6.96 and 7.30 (2H, 2*d*, 1.7 Hz, 3-H and 5-H pyr.), 10.51 (1H, *s*, NH); signals from trace (< 5%) of tetrazole tautomer **11t** were seen at 3.77 (CH_3O), 7.50 and 8.15 (3-H and 5-H pyr.) and 10.70 (NH); EI MS m/z 227–229 (45 and 17%, $[M]^+$), 199–201 (63 and 28, $[M - N_2]^+$), 184–186 (40 and 14, $[M - N_2 - CH_3]^+$), 172–174 (15 and 5, $[M - N_2 - HCN]^+$), 164 (65, $[M - N_2 - Cl]^+$), 113 (100, $[M - N_2 - HCN - CH_3COO]^+$); HRMS Found 227.021002 $[M]^+$; $C_7H_6N_5O_2Cl$ requires 227.020245.

1-(2-Azido-6-chloropyrid-4-yl)-3-(4-[^{3}H]phenyl)urea [^{3}H]2. To a solution of the isocyanate **10** in toluene (3 mL, 13.5 μmol) prepared from a stock solution of the carbonyl azide **9** (1 mg mL^{-1}) was added a solution of 4-[^{3}H]aniline (obtained from CEA, France) (20 mL, 9.25 x 10^8 Bq). The mixture was heated at 60 °C for 24 h. The reaction was monitored by HPLC. The excess of isocyanate **10** was quenched by methanol (1 mL). The radiolabeled compound was purified by reversed phase HPLC (44% radiochemical yield).

Photolysis experiments. Solutions of the ureas **1** and **2** in water (15 μM) or in methanol (1 mM) were photolysed in a Pyrex reactor (400 mL) equipped with a medium pressure mercury lamp located in a quartz immersion apparatus cooled internally by running water. A Pyrex filter which cuts short wavelength light (< 290 nm) was used in some experiments. The reactions were monitored by UV spectroscopy or HPLC.

Biological assay. The cytokinin activity of the ureas **1** and **2** tested according to the assay was previously described,[15] except that inactinic glass test tubes were used.

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