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# Synthesis of N-(3-azido-4-chlorophenyl)-N'-[$^3$H-methyl] thiourea, an Efficient Photoaffinity Probe for the Urea Carrier.

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**Summary.**

Starting from commercial 4-chloro-3-nitroaniline, through a 5 step synthesis, was prepared 3-azido-4-chlorophenylisothiocyanate **5** which was reacted with [$^3$H]-methylamine. The latter was obtained by three methods :

*i-* [$^3$H]-$LiAlT_4$ reduction of benzylcarbamate gave rise to [$^3$H]-methylamine (S.A.: >70 Ci/mmol).

*ii-* Catalytic reduction of HCN with $^3H_2$ lead to [$^3$H]-$CH_3NH_2$ (S.A.: 0.7 Ci/mmol).

*iii-* Schmidt rearrangement of [$^3$H]-sodium acetate gave [$^3$H]-$CH_3NH_2$ (S.A.: 29 Ci/mmol).

Compound 7 at the highest specific activity had a self radiolysis rate precluding its practical use in biological studies whilst 29 Ci/mmol [$^3$H]-7 was satisfactory.

**Key Words** : Urea carrier, photoaffinity probe, [$^3$H]-methylamine.

## Introduction.

It is well known that urea crosses the plasma membrane of various kinds of cells such as mammalian red cells, mammalian kidney cells and amphibian urinary bladder epithelial cells, by a facilitated diffusion mechanism involving a membrane protein (1) which acts as a channel.

Certain inhibitors of the urea flux have been described in these tissues, among which are various low specific inhibitors, such as p-chloromercuribenzenesulfonic acid (2) and phloretin (3). Urea analogues have been shown to be much more specific in inhibiting the urea facilitated transport. They behave as competitive inhibitors (4).

No information is as yet available on the urea channel structure and on the molecular mechanism of the transport. Isolation and characterisation of this protein are required. One approach consists in isolating the carrier by the use of specific and covalent markers. In the absence of antibodies against the urea carrier, urea analogues would appear to be the most appropriate candidates for the specific recognition of the protein.We recently described the preparation of seven new photoactivable reagents as specific urea inhibitors (5). We demonstrated that at least two of these urea-modified analogues (compounds 6 and 7) can be used to label specifically and covalently the urea carrier of both red cells and urinary bladder epithelial cells.

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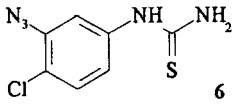
This paper describes the synthesis of the high specific activity tritiated compound 7.

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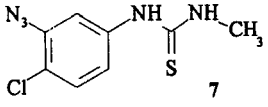


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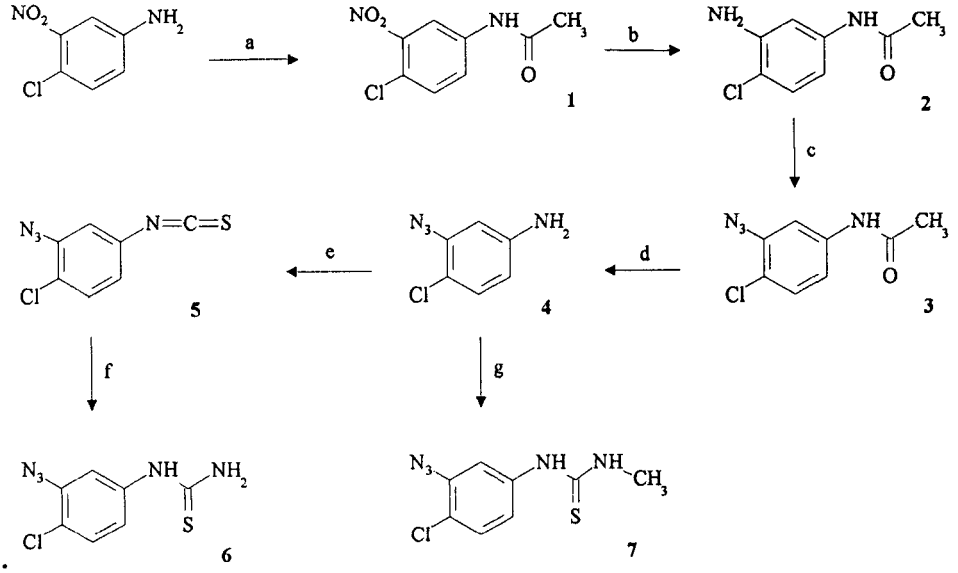
## Results.

Unlabelled 7 was synthesized from 4-chloro-3-nitroaniline in 5 steps (scheme 1). The synthesis of the required 3-azido-4-chloroaniline **4** was previously reported by Boschetti et al (6). The nitroaniline **1** was acetylated under standard conditions. These authors used sodium dithionite to reduce the nitro group. We obtained better yields with Fe/HCl (7). Introduction of the azido group with sodium nitrite followed by sodium azide, and deacetylation of the acetanilide with potassium hydroxide were straightforward (6). The isothiocyanate **5** was obtained by action of thiophosgene in chloroform solution. The condensation with aqueous ammonia produced **6**. The analogue **7** was obtained by reaction of **4** with methyl isothiocyanate.

**Scheme 1 : Synthesis of 6 and 7**

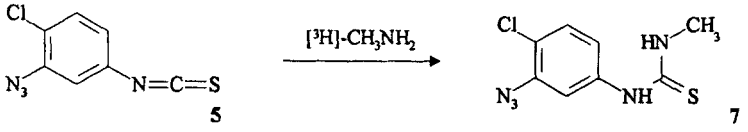



a) $Ac_2O$; b) Fe/HCl, EtOH/$H_2O$; c) $NaNO_2$, $NaN_3$, $H_2O$/HCl; d) KOH 30%; e) $Cl_2CS$, toluene/$NaHCO_3$ 5%; f) $NH_4OH$, $CHCl_3$; g) $CH_3NCS$, EtOH

Based on the results of the previously described biological tests , we selected 7 as a good candidate for the probe compound in the photoaffinity labelling experiment (8). We chose to put the label on the methyl group, which offered the most direct route to high specific activity tritium labelled 7.

A new synthesis of the compound 7 was devised starting from methylamine. (scheme 2). Reaction of methylamine with the previously synthesized isothiocyanate **5** gave the expected thiourea 7 in good yield. Then we focused our attention on the preparation of [³H]-methylamine.

**Scheme 2 : synthesis of N-(3-azido-4-chlorophenyl)-N'-[³H-methyl] thiourea.**

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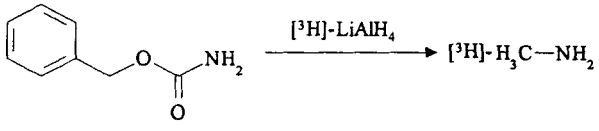
Two new methods were developed in order to obtain [³H]-$CH_3NH_2$ with different specific activities.

Firstly, high specific activity [³H]-$CH_3NH_2$ (>70 Ci/mmol) was prepared by [³H]-$LiAlH_4$ reduction of benzylcarbamate. The reaction conditions of deuterium experiments described by Kwant werre applied to the tritium labelling (9) (scheme 3). [³H]-$LiAlH_4$ was synthesized according to Burgos' procedure (10). The resulting tritiated compound 7 was successfully purified by HPLC but its fast self radiolysis precluded its use in biological tests.

**Scheme 3 : synthesis of high specific activity [³H]-$CH_3NH_2$.**



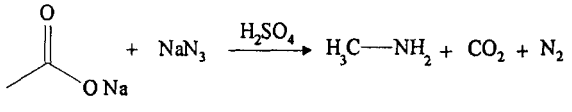

Secondly, low specific activity [³H]-methylamine (0.7 Ci/mmol) was obtained by the catalytic reduction of KCN with tritium in presence of $H_2SO_4$. The tritiated compound 7 obtained from this [³H]-methylamine, had a 2-month stability in ethanol at - 80 °C.

Two other batches of [³H]-methylamine were obtained by :

- The first method, where tritium was replaced by a mixture of hydrogen and tritium (10:1) gave a [³H]-methylamine (specific activity 7 Ci/mmol).

- In another preparation, [³H]-methylamine (S.A.: 29 Ci/mmol) was obtained using the Schmidt reaction on [³H]-sodium acetate (11) (scheme 4). [³H]-sodium acetate was obtained by the catalytic dehalogenation of bromoacetic acid with tritium.

**Scheme 4 : Schmidt reaction with sodium acetate.**




The compound 7 (specific activity: 29 Ci/mmol) has a 3-week stability in ethanol at - 80 °C. This product was suitable for a variety of biological assays, the results of wich will be reported later by Neau et al (12).

## Experimental.

**General.** $^1H$ NMR spectra were obtained on a 300 MHz Bruker instrument. They were recorded in $D_6$-DMSO unless otherwise noted. Chemical shifts are given in ppm relative to $Me_4Si$ (δ 0). The abbreviations s and d stand for singlet and doublet respectively. $^1H$ NMR coupling constants (J) are given in Hz. Mass spectra were obtained from a Finnigan mass spectrometer with the DCI/$NH_3$ ionisation method unless otherwise noted. IR spectra were recorded on a Beckman 4250 infrared spectrometer. The radioactivity of the tritiated samples was counted in a Wallac 1409 apparatus. $R_f$ values on TLC were recorded on Merck precoated (0.25 mm) silica gel 60 $F_{254}$ plates. The plates were revealed under UV at 254 nm. Melting points were measured using a Kofler block and are not corrected.

Elemental analyses were performed by Service de Microanalyses, C.N.R.S., Gif sur Yvette, France.

Methylisothiocyanate (Aldrich) was purified by distillation.

Abreviations :TFA : trifluoroacetic acid, THF : tetrahydrofuran, TMEDA : tetramethyl ethylene diamine.

**4-chloro-3-nitroacetanilide (1).** A solution of 4-chloro-3-nitro aniline (Aldrich) (40 g, 0.23 mol) in acetic anhydride (32 ml) with a drop of sulphuric acid, was heated at reflux for 2 hours. The mixture was cooled in an ice bath; the residue was filtered off and washed with water until the filtrate gave a pH of 7. The residue was dried under vacuum and recrystallized from toluene to give **1** (40.2 g, 81 %). TLC $R_f$ 0.6 (7:3 $CH_2Cl_2$/ethyl acetate); MS M+18=232 ($^{35}Cl$), 234 ($^{37}Cl$) (75% / 25%), M+35=249, 251 (75.8% / 24.2%); mp=147 °C; $^1H$ NMR δ 2.1 (s, $CH_3$), δ 7.65 (d, $C_5$-H, J=8Hz), δ 7.75 (dd, $C_6$-H, J=8Hz, J'=2Hz), δ 8.4 (d, $C_2$-H, J'=2Hz), δ 10.5 (s, NH); Anal. calcd for $C_8H_7ClN_2O_3$: C 44.86, H 3.30, N 13.09 found C 45.14, H 3.35, N 12.92.

**3-amino-4-chloroacetanilide (2).** In a 250 ml round-bottomed flask with mechanical stirring, **1** (5 g, 0.023 mol) was heated for 3 hours in a solution of ethanol (60 ml) containing water (5 ml), 37% hydrochloric acid (5 ml) and iron powder (5 g, 0.09 mol). The mixture was poured into water (300 ml). The aqueous phase was extracted three times with ether (100 ml). The combined organic extracts were dried over $MgSO_4$, filtered and evaporated. The residue was dissolved in ethyl acetate and extracted twice with 2N HCl (50 ml). The aqueous phase was washed with diethyl oxide (2x50 ml) and then neutralized by $Na_2CO_3$. Extraction with ether (2x50 ml) gave, after evaporation of this solvent, the compound **2** (3.2 g, 75%). TLC $R_f$ 0.3 (7:3 $CH_2Cl_2$/ethyl acetate); MS M+1=185 ($^{35}Cl$), 187 ($^{37}Cl$) (70% / 30%), M+18=202, 204 (74.5% / 25.5%), M+35=219, 221 (77% / 23%); mp=168 °C; $^1H$ NMR δ 2.1 (s, $CH_3$), δ 5.3 (s, $NH_2$), δ 6.7 (dd, $C_6$-H, J=10Hz, J'=3Hz), δ 7.05 (d, $C_5$-H, J=10Hz), δ 7.2 (d, $C_2$-H, J'=3Hz), δ 9.8 (s, NH); Anal. calcd for $C_8H_9ClN_2O$: C 52.16, H 4.93, N 15.22 found C 51.3, H 4.87, N 14.56.

**3-azido-4-chloroacetanilide (3).** To a solution of **2** (2 g, 0.011 mol) in water (24 ml) and 37% HCl (2.7 ml) at 0 °C, $NaNO_2$ (0.84 g, 0.012 mol) in water (8 ml) was added slowly with stirring, followed by $NaN_3$ (1.35 g, 0.02 mol) in water (8 ml). After 30 min at 0 °C and 30 min at room temperature, **3** (1.8 g, 78%) was recovered by filtration, washed with water and recrystallized

from ethanol/water. TLC $R_f$ 0.2 ($CH_2Cl_2$); MS M+1=211 ($^{35}Cl$), 213 ($^{37}Cl$) (76.7% / 23.3%), M+18=228, 230 (75.2% / 24.8%), M+35=245, 247 (75.8% / 24.2%); mp=153 °C; $^1H$ NMR δ 2.05 (s, $CH_3$), δ 7.3 (dd, $C_6$-H, J=10Hz, J'=3Hz), δ 7.4 (d, $C_5$-H, J=10Hz), δ 7.8 (d, $C_2$-H, J'=3Hz), δ 10.2 (s, NH); IR (KBr) azido 2120 $cm^{-1}$; Anal. calcd for $C_8H_7ClN_4O$: C 45.71, H 3.36, N 26.67 found C 45.88, H 3.41, N 26.38.

**3-azido-4-chloroaniline (4).** The compound **3** was hydrolyzed by boiling in 30% KOH solution for 1 hour. After cooling, the aniline **4** was extracted with $CH_2Cl_2$ (3x 20 ml) and recovered by evaporation under vacuum. The yield was 75%. TLC $R_f$ 0.8 ($CH_2Cl_2$); MS M+1=169 ($^{35}Cl$), 171 ($^{37}Cl$) (73.4% / 26.6%); mp=88 °C; $^1H$ NMR δ 5.5 (s, $NH_2$), δ 6.4 (dd, $C_6$-H, J=8Hz, J'=3Hz), δ 6.55 (d, $C_2$-H, J'=3Hz), δ 7 (d, $C_5$-H, J=8Hz); IR (KBr) azido 2120 $cm^{-1}$; Anal. calcd for $C_6H_5ClN_4$: C 42.85, H 3.00, N 33.34 found C 42.8, H 3.37, N 32.42.

**3-azido-4-chlorophenylisothiocyanate (5).** To thiophosgene (1.24 ml, 16 mmol) in toluene (5 ml), **4** (100 mg, 0.6 mmol) in 5% $NaHCO_3$ aqueous solution (5 ml) was added in a brown flask. The mixture was stirred for 4 hours at room temperature. The organic layer was separated, washed with a 1 M NaCl solution and evaporated. **5** (87.5 mg, 70%) was purified by column chromatography on silica gel from hexane. TLC $R_f$ 0.5 (Hexane); MS $M^+$=210 ($^{35}Cl$), 212 ($^{37}Cl$) (73.5% / 26.5%); $^1H$ NMR δ 7.25 (d, $C_6$-H, J=8Hz), δ 7.5 (d, $C_5$-H, J=8Hz), δ 7.6 (s, $C_2$-H); IR (KBr) azido 2120 $cm^{-1}$, isothiocyanate 2060 $cm^{-1}$; Anal. calcd for $C_7H_3ClN_4S$: C 39.91, H 1.44, N 26.60 found C 40.14, H 1.74, N 26.8.

**N-(3-azido-4-chlorophenyl-)-thiourea (6).** In a brown flask, $NH_4OH$ (1 ml, d=0.89) was added to **5** (30 mg, 0.14 mmol) in $CHCl_3$ (5 ml) at 0 °C. After stirring for 15 min at 0 °C, the mixture was allowed to warm to room temperature. After two hours, the organic layer was separated, washed with water and evaporated. The residue was chromatographed on silica gel using a 8:2 mixture of dichloromethane and ethyl acetate as eluent to give **6** (25 mg, 75%). TLC $R_f$ 0.5 (8:2 $CH_2Cl_2$/ethyl acetate); MS M+1=228 ($^{35}Cl$), 230 ($^{37}Cl$) (73.8% / 26.2%), M+18=245, 247 (73.5% / 26.5%), M+35=262, 264 (71.4% / 28.6%); mp=163 °C; $^1H$ NMR δ 7.2 (d, $C_6$-H, J=9Hz), δ 7.4 (d, $C_5$-H, J=9Hz), δ 7.75 (s, $C_2$-H), δ 9.9 (s, NH), the $NH_2$ signal was masked by the aromatic protons and disappeared on the addition of $D_2O$; $^{13}C$ NMR (300 MHz) δ 182 thiourea, δ 116, 122, 124, 132, 135, 139 aromatic carbons; IR (KBr) azido 2120 $cm^{-1}$; UV. (ethanol) $\lambda_{max}$=243 nm, ε=20580.

**N-(3-azido-4-chlorophenyl-)-N'-methyl-thiourea (7).** To **4** (150 mg, 0.89 mmol) in ethanol (6 ml), methylisothiocyanate (304 µl, 4.5 mmol) was added and the mixture was stirred at room temperature for 48 hours. After evaporation of solvent, the residue was dissolved in ethanol and crystallized by adding water. TLC $R_f$ 0.2 (8:2 hexane/ethyl acetate); MS (DEI) $M^+$=241 ($^{35}Cl$), 243 ($^{37}Cl$) (70% / 30%); $^1H$ NMR δ 3.1 (d, N-$CH_3$, J=5Hz), δ 6 (s, NH), δ 6.95 (d, $C_6$-H, J'=8Hz), δ 7.05 (s, $C_2$-H), δ 7.4 (d, $C_5$-H, J'=8Hz), δ 7.8 (s, NH); $^{13}C$ NMR (300 MHz) δ 182 thiourea, δ 116, 122, 124, 132, 135, 139 aromatic carbons; IR (KBr) azido 2120 $cm^{-1}$; UV. (ethanol) $\lambda_{max}$=247.5 nm, ε=25930; Anal. calcd for $C_8H_8ClN_5S$: C 39.75, H 3.34, N 28.98 found C 39.84, H 3.31, N 29.25.

**[$^3$H]-$CH_3NH_2$ (low specific activity).** In a septum flask, 20 mg of $PtO_2$ (80% Pt, Adams) were reduced under a tritium atmosphere (20 Ci). After 3 hours stirring, KCN (10 mg, 0.15 mmol) in water (180 µl) and 1 N $H_2SO_4$ (2.3 ml) were added. Tritium (20 Ci) was introduced in the flask. After stirring overnight, the catalyst was filtered off, washed with methanol. Solvents were removed under reduced pressure and 0.7 Ci/mmol methyl amine (230 mCi, 0.33 mmol) was recovered by transfer under vacuum.

TLC $R_f$ 0.46 (50:25:25 n-butanol/acetic acid/water).

**[$^3$H]-$CH_3NH_2$ (high specific activity).** In this one pot procedure, all reactions were carried out at 20 °C. A 1.6 M solution of n-butyllithium (0.86 mmol) in hexanes containing TMEDA (0.92 mmol) was stirred under a tritium gas atmosphere until no more gas absorption occured. The solvent and TMEDA were removed under reduced pressure to yield [$^3$H]-LiH (0.67 mmol). A 0.4 M solution of $AlCl_3$ (0.1675 mmol) in THF was added to [$^3$H]-LiH. After 15 min of stirring LiCl precipitated. Then, a 0.4 M solution of benzylcarbamate (0.1675 mmol) in THF was added. After overnight stirring, the mixture was cooled in a ice bath and hydrolysed with 15% aqueous KOH. The tritiated methylamine was purified by transfer under vacuum to yield 128 mCi of pure methylamine.

**[$^3$H]-$CH_3NH_2$ (specific activity 29 Ci/mmol).** Bromoacetic acid (80.3 mg, 0.58 mmol) was stirred with 10% Pd/C (101.7 mg) in a mixture of water (1 ml), 2N NaOH (2 ml) and methanol (2 ml) with 50 Ci tritium gas until no more gas absorption occured. Filtration of the catalyst and evaporation of the solvents gave [$^3$H]-sodium acetate (9.4 Ci). [$^3$H]-Sodium acetate (2.37 Ci, 0.08 mmol) and sodium azide (17 mg, 0.26 mmol) were stirred in $H_2SO_4$ 100% (0.2 ml) at 35 °C for 20 min and then at 70 °C for 1 hour at which time gas evolution ceased. After cooling in an ice bath, the mixture was diluted with water. The product (1.5 Ci, yield 63 %) was recovered by azeotropic distillation with water (70 ml). Specific activity : 29 Ci/mmol (measured by mass spectrometry).

**N-(3-azido-4-chlorophenyl)-N'-[$^3$H-methyl]-thiourea.** [$^3$H]-$CH_3NH_2$,HCl (156 mCi, S.A. 29 Ci/mmol, 0.005 mmol) was dissolved in 0.75 N NaOH (0.1 ml). An excess of **5** (4 mg, 0,019 mmol) in $CHCl_3$ (0.5 ml) was added and the mixture was stirred vigorously at room temperature for two hours. Then the aqueous layer was removed and the organic layer was washed with water (0.5 ml). After evaporation of the solvent, the [$^3$H]-7 was purified on a silica Zorbax column and eluted at a flow rate of 2 ml/min with a 7:3 mixture of hexane/ethyl acetate (Vr = 70 ml).

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