JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2002; **45**: 943–953.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.614

Research Article

New and convenient synthesis of a tritiated photoactivatable nicotinic agonist: [^{3}H]–AC5

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Summary

A new synthesis of a tritiated photosensitive nicotinic agonist ([^{3}H]−**AC5**), which uses [$^3H_2$] as the source of radioactivity, is described. Several improvements have been introduced to establish a reliable five-step synthesis displaying satisfactory overall yields (45%) and requiring a single purification (HPLC) in the final step. This photosensitive radiolabelled probe will enable us to undertake a time-resolved photolabelling study on the nicotinic acetylcholine receptor in order to analyze at the molecular level the allosteric transitions occurring after agonist stimulation. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: dynamic photoaffinity labelling; aryldiazonium salts; nicotinic acetylcholine receptor; radiolabelled synthesis

Introduction

*n*AChRs are pentameric allosteric transmembrane proteins that belong to the superfamily of the ligand-gated ion channels[1] (LGIC). Exposure

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Contract/grant sponsor: Ministere de l'Enseignement Supérieur, Naturalia
Contract/grant sponsor: CNRS

Received 16 April 2002
Accepted 13 May 2002

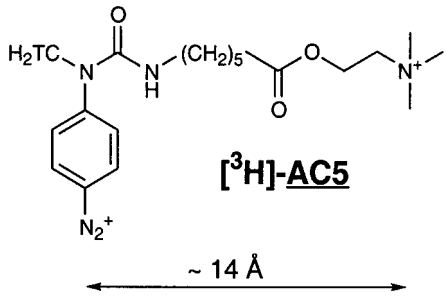


Figure 1. Structure of photoactivatable agonist [^{3}H]-AC5.

to agonists such as acetylcholine lead to at least three transitory states: a rapid (ms) opening of the channel, causing the depolarization of the membrane, followed by subsequent fast (ms–s) and slow (s–min) processes of desensitization.[2] This molecular phenomenon plays a key role in the regulation of the synaptic transmission.

These conformational transitions can be probed at the molecular level by using photoactivatable radiolabelled cholinergic ligands. The photolabelling experiments require the combination of rapid mixing procedures to flash photolysis. The experiments are analyzed at different mixing times, by quantification of the radioactivity incorporation into the receptor subunits and subsequently into the labelled amino acids after microsequencing (for review, see ref.[3]). Recently, the photosensitive partial agonist [^{3}H]-DCTA[4] was used in our laboratory to probe the conformational reorganization occurring within the ACh binding site after rapid mixing with the *Torpedo* receptor. It allowed to analyze, in particular, the slow desensitization process.[5]

The recent description of the crystal structure at 2.7 Å resolution of a water-soluble protein (AChBP) that has significant structural homology with the extracellular part of the *n*AChR,[6] prompted us to reinvestigate the time-resolved photolabelling experiments using a formerly described photosensitive nicotinic agonist AC5.[7] This molecule possesses outstanding binding and photochemical properties. Due to its flexible chemical structure and length (14 Å in the extended conformation, Figure 1) and referring to AChBP structure,[6] it should permit the mapping of inter-subunit areas in the neighbourhood of the ACh binding site, during these conformational transitions.[8] Indeed, AC5 diazonium moiety is probably located in a region of *n*AChR where significant topological changes occur during state transitions, as shown by fluorescence experiments with Dns-C_6-Choline.[9] The described

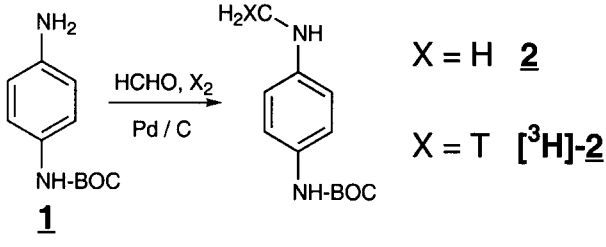
synthesis of the tritiated AC5 molecule[7] used [^{3}H]–CH_3I as the radioactive source which allowed a low yield synthesis of the [^{3}H]-**AC5** molecule with high specific radioactivity. The actual non-availability of the tritiated methyl iodide precursor compelled us to define a new synthetic strategy to proceed with this work.

The present article describes a five-step synthesis of [^{3}H]-**AC5** using [$3H_2$] as the radiolabel source. This synthesis is fully reproducible with high overall radiochemical yields (about 45%) and requires only a single HPLC purification in the final step.

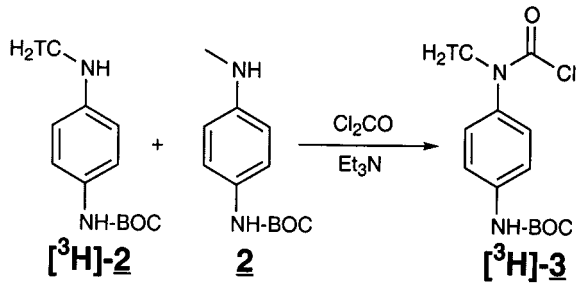
Results and discussion

Given the large amount of *n*AChR in the electropaque of *Torpedo marmorata* and the radiolytic sensitivity of chemical and biochemical materials with high specific radioactivity, we preferred not to exceed 1 Ci/mmol for this photoprobe. This specific radioactivity was sufficient to obtain good labelling patterns to identify the labelled amino acids. Accordingly, we synthesized the intermediate [^{3}H]-**2** using a classical reductive amination[10] of the phenylene diamine derivative **1**[11] (Scheme 1) to incorporate one tritium atom per molecule. This radioactive precursor was stored in MeOH at $-20°C$ and checked by HPLC for its purity before use. The *N*-methyl derivatives **2** and [^{3}H]-**3** were quantitatively transformed to the *N*-chlorocarbamoyl derivative [^{3}H]-**2** by treatment with phosgen[12] and this last was used directly without further purification (Scheme 2).

In our search for an efficient synthetic pathway, we transformed the brominated spacer **4** to its iodinated derivative **7** after subsequent protection and deprotection of the amine moiety (Scheme 3). This iodinated spacer **7** could be directly and efficiently coupled to the [^{3}H]-**3**



Scheme 1.



Scheme 2.

precursor forming compound [^{3}H]-**8**. The iodo-substituent was quantitatively converted by treatment with $N(Me)_3$ in anhydrous toluene to its quaternary ammonium salt [^{3}H]-**9** and isolated as a precipitate in the reaction mixture. This precursor [^{3}H]-**9** was taken up in water and lyophilized. The replacement of the bromo-substituent in **4** by the iodo derivative **7** allowed, therefore, interesting synthetic improvements: first, the coupling yields to form [^{3}H]-**8** were enhanced (98% instead of 50% maximum with the bromo compound); second, due to the precipitation of the iodide ammonium salt, the formation of [^{3}H]-**9** occurred with quantitative yields and allowed a convenient handling of the reaction product. This improved synthesis of [^{3}H]-**9** enabled us to use the crude reaction product for the final diazotization step without intermediate HPLC purification, preventing the degradation of moist-sensitive intermediates ([^{3}H]-**3**, **7** and [^{3}H]-**8**).

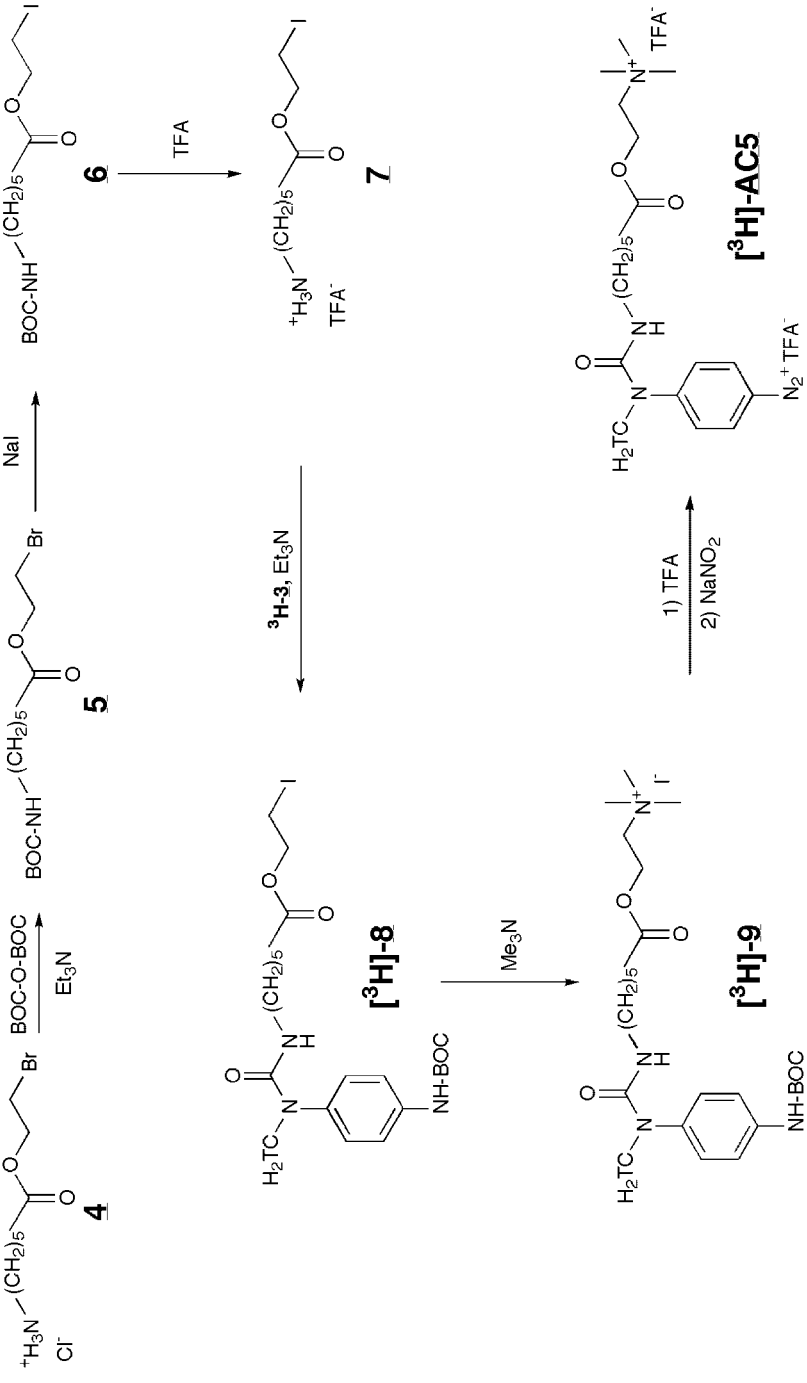
Diazotization must be operated in the dark, and gave [^{3}H]-**AC5** as the main product (60% yield, retention time 15 min with gradient I, Table 1) in the presence of a secondary compound that was probably the hydrolytic product of the ester (retention time 14 min with gradient I). To optimize this purification step, we used new eluting conditions (gradient II).

Characterization of [^{3}H]-**AC5** (Figure 2) was achieved by HPLC and UV analyses, and the product was stored as aliquots (50 µl) in aqueous solution (385 µM, 0.83 Ci/mmol) in the dark at −80°C.

Experimental section

General

Retention times on HPLC for the successive intermediate and the final compound are listed in Table 1. ^{1}H-NMR and ^{13}C-NMR spectra of



Scheme 3.

Table 1. Retention time (Rt) on reversed phase 10 μ-Hyperbond C18 for intermediates and final compounds in the synthesis of [^{3}H]-AC5 and of the non-labelled AC5. Compounds were analyzed under the following conditions: Gradient I = 0–60% B in 40 min; 60–100% B in 5 min. Gradient II = Isocratic 5% B. A = H_2O/TFA 0.1%; B = Acetonitrile

	Rt (min)	
Compounds	Gradient I	Gradient II
2	21	
3	35	
7 and [^{3}H]**7**	18	
8 and [^{3}H]**8**	39	
9 and [^{3}H]**9**	29	
AC5 and [^{3}H]**AC5**	15	21

unlabelled compounds were recorded in $CDCl_3$, CD_3OD or D_2O at 200 and 300 MHz, on Bruker VPC 200 and 300 instruments, δ are given in ppm. Mass spectra analysis of unlabelled compounds were performed on a Mariner ESI-Tof instrument from Applied Bio-System/Perkin-Elmer.

Analyses and purifications of both labelled and non-labelled compounds were made on a reversed phase 10 μ-Hyperbond C18 Bondex HPLC (Table 1) under the following gradient conditions: (I) 100% A to 60% B in 40 min, 60% B to 100% B in 5 min; (II) isocratic 5% B (A = H_2O/TFA 0.1%, B = acetonitrile).

A Packard Tri-carb 2100 TR liquid scintillation counter was used for quantification of radioactivity. Typically, 10 μl of sample were added to 1.5 ml of Packard emulsifier-safe and the solution was counted for 2 min. UV-visible spectra were recorded on a Biotek Uvikon XL. Toluene was distilled on sodium, CH_2Cl_2 was distilled on calcium hydride and acetone was distilled on calcium sulfate.

Analytical HPLC injections of the crude products [^{3}H]-**8**, [^{3}H]-**9** and [^{3}H]-**AC5** allowed us to characterize (UV spectra) and quantify (radioactivity counting) each product.

Syntheses

A general synthesis was developed for the unlabelled compound (**AC5**) to assess the synthetic methodology as well as the HPLC purification procedure.

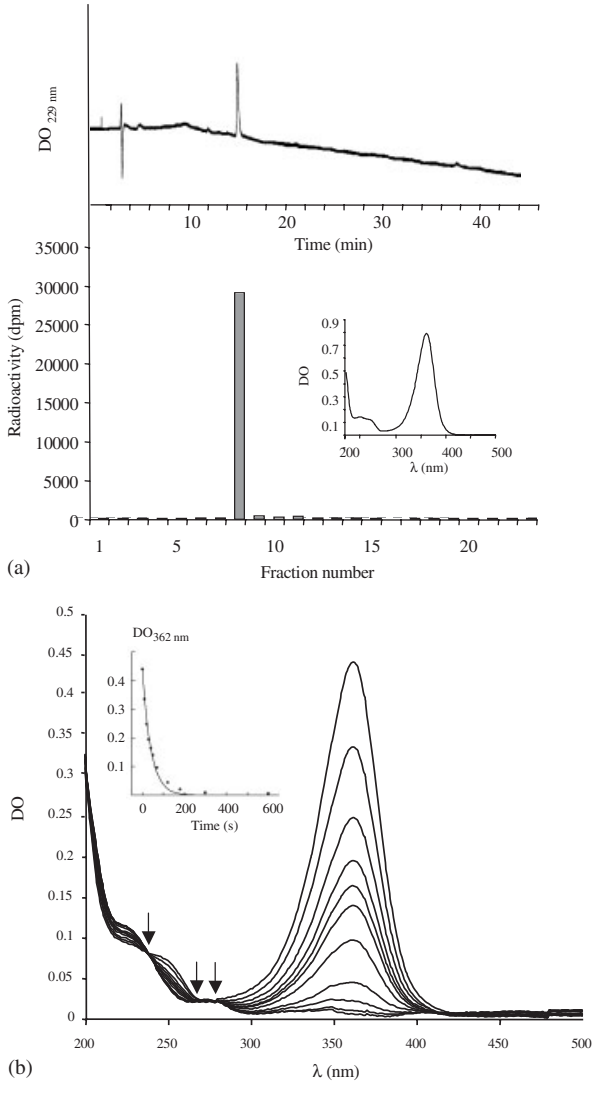


Figure 2. (a) reversed-phase HPLC analysis and purification of [^{3}H]-AC5. Upper part: 100 μl of [^{3}H]-AC5 (38 μM in water) were injected on the C18 column and eluted with gradient I (see Table 1). UV detection was monitored at 229 nm; lower part: fractions were collected and counted (10 μl aliquots) for radioactivity (UV spectral characteristics of fraction 8 are shown here). (b) Spectral changes associated with the photolysis of probe AC5 (15 μM in water, 10°C) irradiated at 364.6 nm (incident light energy: 70 μV). The initial absorbance at 362 nm decreases with a concomitant increase of new peaks (isobestic points (arrows) at 238, 270 and 277 nm). A half-time of 28 s was calculated according to a pseudo-first-order equation: $Do_t = Do_0 e(-k_{app}.x)$; $t_{1/2} = \ln 2/k_{app}$. AC5 is stable in the dark in water at 10°C ($t_{1/2} > 48$ h, data not shown)

The syntheses of *N*-terbutoxycarbonyl-para-phenylenediamine **1**[11], *N*-methyl-*N*′-terbutoxycarbonyl-para-phenylenediamine **2**[13], *N*-([^{3}H]-methyl)-*N*′-terbutoxycarbonyl-para-phenylenediamine [^{3}H]-**2**[10], *N*-([^{3}H]-methyl)-*N*-chloroformyl-*N*′-terbutoxy carbonyl-para-phenylenediamine [^{3}H]-**3**[12] and 6-amino-[(2-bromo)-ethyl)]-hexanoate chlorhydrate **4**[7] were previously reported.

For [^{3}H]-**3** synthesis, [^{3}H]-**2** (2.15 μmol, 14 mCi, 6.5 Ci/mmol) was isotopically diluted with **2** (12.9 μmol) to give a mixture [^{3}H]-**2**/**2** whose specific activity corresponds theorically to 0.93 Ci/mmol.

N-terbutoxycarbonyl-6-amino-[(2-bromo)-ethyl)]-hexanoate **5**

To a solution of **4** (2 g, 7.3 mmol) in 20 ml ethanol were added 2 ml (8.7 mmol, 1.2 equivalents) of di-*tert*-butyl-dicarbonate and 1.12 ml (8 mmol, 1 eq) of Et_3N. The reaction was stirred at room temperature for 5 h. After evaporation of the solvent, the residue was taken up in 150 ml CH_2Cl_2 and the organic layer was washed with water and dried (Na_2SO_4). The resulting oil was purified by chromatography on silica gel (pentane/ether: 8/2) to afford 2.2 g (6.6 mmol, 90% yield) of yellow oil.

^{1}H-NMR ($CDCl_3$):$\delta = 1.26-1.70$ (m, 15H, $3CH_2$, $3CH_3$); 2.27–2.35 (t, 2H, $J = 7.3$ Hz, CH_2); 3.01–3.12 (q, 2H, $J = 6.6$ Hz, CH_2); 3.43–3.50 (t, 2H, $J = 6.1$ Hz, CH_2); 4.30–4.37 (t, 2H, $J = 6.1$ Hz, CH_2); 4.60 (br s, 1H, NH).

N-terbutoxycarbonyl-6-amino-[(2-iodo)-ethyl)]-hexanoate **6**

To a solution of **5** (1.43 g, 4.2 mmol) in 20 ml of freshly distilled acetone was added 5.2 g (34.6 mmol, 8.2 eq) of anhydrous NaI and the mixture was heated under reflux for 45 h. After evaporation of the solvent, the residue was taken up in 150 ml CH_2Cl_2 and washed with 150 ml H_2O. The organic layer was dried with anhydrous Na_2SO_4. After evaporation of the solvent, the residue obtained was chromatographed on silica gel (heptane/ether: 7/3) to afford 1.51 g (3.9 mmol, 93% yield) of yellow oil.

^{1}H-NMR ($CDCl_3$):$\delta = 1.32-1.70$ (m, 15H, $3CH_2$, $3CH_3$); 2.31–2.36 (t, 2H, $J = 7.5$ Hz, CH_2); 3.07–3.14 (q, 2H, $J = 6.2$ Hz, CH_2); 3.26–3.31 (t, 2H, $J = 6.6$ Hz, CH_2); 4.29–4.35 (t, 2H, $J = 6.9$ Hz, CH_2); 4.55 (br s, 1H, NH).

6-amino-[(2-iodo)-ethyl)]-hexanoate **7**

1.51 g (3.9 mmol) of **6** was dissolved in 10 ml of freshly distilled CH_2Cl_2. 10 ml of anhydrous TFA were added drop-wise at 0°C to this solution. The reaction was stirred for 30 min at 0°C and then warmed up to 20°C for 45 min. The mixture was cooled to −78°C and lyophilized to avoid **7** from exposure to heat. The yellow oil was taken up twice in 10 ml of $CHCl_3$ and evaporated under vacuum to eliminate residual TFA. The residue was taken up in MeOH and recrystallized in isopropanol/hexane to afford 1.45 g of compound **7** (3.63 mmol, 93% yield) as a yellow-white solid.

^{1}H-NMR ($CDCl_3$):$\delta = 1.30-1.40$ (m, 2H, CH_2); 1.54–1.65 (m, 4H, $2CH_2$); 2.25–2.32 (t, 2H, $J = 7.2$ Hz, CH_2); 2.84–2.91 (t, 2H, $J = 7.1$ Hz, CH_2); 3.19–3.26 (t, 2H, $J = 6.7$ Hz, CH_2); 4.29–4.35 (t, 2H, $J = 6.7$ Hz, CH_2); 7.8 (br s, 2H, NH_2).

^{13}C-NMR ($CDCl_3$):$\delta = 1.48$, 24.6, 26.2, 31.2, 34.1, 40.4, 64.9 and 173.7.

MS (ESI, positive ion 130 eV): calculated for $(C_8H_{17}NO_2I)^+$:286.13; found: 286.03.

(2-iodo)-ethyl-6-N-[N′-([^{3}H]-methyl)-para-amino-terbutoxy-carbonyl-phenylurea]-hexanoate [^{3}**H**]-8

4 mg (14 μmol, 13.3 μmol, 13.3 mCi) of [^{3}**H**]-**3** and 7.2 mg (18 μmol, 1.3 equivalent) of **7** were dissolved in 300 μl of freshly distilled CH_2Cl_2 and 5 μl (36.1 μmol, 2.6 equivalents) of Et_3N were added. The reaction was stirred for 7 h at room temperature and monitored by HPLC (gradient I) until completion. 1 ml of CH_2Cl_2 was then added and the mixture was extracted with 1 ml H_2O. The aqueous layer was extracted again with 1 ml CH_2Cl_2 and the organic layers were pooled, dried over Na_2SO_4 and evaporated. The residue was taken up in 2 ml of anhydrous CH_2Cl_2 and analyzed with HPLC gradient I: the mixture contained 10.4 mCi of [^{3}**H**]-**8** (80% radiochemical yield).

(2-trimethylammonium)-ethyl-6-N-N′-([^{3}H]-methyl)-para-amino-terbutoxycarbonyl-phenylurea]-hexanoate iodide [^{3}**H**]-**9**

6 mg (11.2 μmol, 10.4 mCi) of [^{3}**H**]-**8** were dissolved in 300 μl of anhydrous toluene saturated with Me_3N and the reaction was stirred at room temperature for 24 h. The solvent was removed under vacuum and the residue was taken up in the 300 μl of toluene freshly saturated

with Me_3N and stirred for 70 h. The washing procedure was repeated twice and the solvent was then removed under reduced pressure. The residue was treated with 2 ml of toluene and extracted with 2 ml of water. The aqueous layer contained 10.2 mCi of [^{3}H]-**9** (98% radiochemical yield). The crude product was then lyophilized and taken up in 4 ml of MeOH (2.55 mCi/ml, 3 mM) and stored at −20°C.

(2-trimethylammonium)-ethyl-6-N-[N′-([^{3}H]-methyl)-para-diazonium-phenylurea]-hexanoate trifluoroacetate [^{3}H]-**AC5**

450 µl (1.3 µmol, 1.1 mCi) of the methanolic [^{3}H]-**9** solution was dried under vacuum and cooled to 0°C before dropwise addition of 300 µl of cold TFA. Stirring the mixture for 30 min at 15°C allowed full deprotection of the amino group. The mixture was cooled to −10°C and handled in the dark. $NaNO_2$ (1.2 eq) in aqueous solution (8.3 mg/ml) was added portionwise over a 30 min period. Completion of the diazotization was monitored by UV spectroscopy. The mixture was then lyophilized twice with 1 ml H_2O to remove excess of TFA before HPLC purification using gradient II. Purification step gave [^{3}H]-**AC5** with purity >95% (as confirmed by final HPLC and UV analyses, Figure 2a) with good radiochemical yield (60%).

[^{3}H]-**AC5** was then stored in aliquots of aqueous solutions (385 µM) in the dark at −80°C until its use.

Spectral properties of **AC5**: $\lambda_{max} = 362$ nm, $\varepsilon_{362} = 23400\ M^{-1}\,cm^{-1}$.

[^{3}H]-**AC5** specific activity measured: SA = 0.83 Ci/mmol.

Conclusion

The present article describes a reliable synthesis of the photosensitive nicotinic agonist [^{3}H]-**AC5**. Despite a five-step synthesis from the radioactive precursor [^{3}H]-**2**, [^{3}H]-**AC5** was obtained with good overall yield (44%). Its synthetic precursor [^{3}H]-**9**, is chemically and photochemically stable, offering a convenient way to store the compound for longer time-periods.

The photoactivatable agonist [^{3}H]-**AC5** has already proven its efficacy in labeling specifically the *n*AChR from *Torpedo marmorata*.[7] This photolabelling efficacy does ensure sufficient radioactivity incorporation into the *n*AChR to permit a molecular analysis of the labelling patterns during conformational transitions. This work is presently in progress.

Acknowledgements

The authors thank the Ministère de l'Enseignement Supérieur, Naturalia and the CNRS for financial support.

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