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SYNTHESIS AND PURIFICATION OF HIGHLY TRITIATED ARYLDIAZONIUM PHOTOPROBES FOR CHOLINERGIC BINDING SITES

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SUMMARY

The synthesis and improved purification procedures of two photoactivatable ligands ^{3}H-A and ^{3}H-B (Figure 1) are presented. Particular attention was given to the use of resolutive reversed-phase HPLC conditions which were found to be essential for the characterization and the elimination of contaminating products and for the obtention of pure diazonium probes at high specific radioactivity (up to 50 Ci/mmol).

Key words : Aryldiazonium salts, photoaffinity, site-directed labelling, HPLC analysis, radiolabelled synthesis, cholinergic binding sites

INTRODUCTION

Structural information (constitutive analysis and topographical mapping) on receptor binding sites is now currently provided by a combination of chemical labelling, mutagenesis, physical, immunological and computer-modelling approaches. Photoaffinity labelling has proven to be an efficient site-directed labelling method which allows, when radioactive photoactivatable probes are used, to tag a receptor protein from the macromolecular to the binding site residues level (Review [1]).

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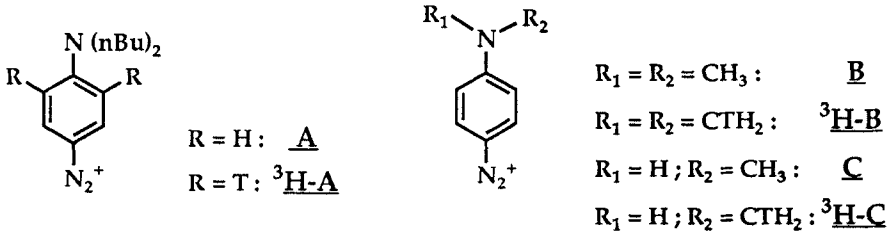
Aryldiazonium salts A and B (Fig. 1) display at once a high reactivity of the photogenerated aryl cation and an ideal substitution of the quaternary ammonium moiety of acetylcholine by the photoreactive charged diazonium group.

Figure 1 :



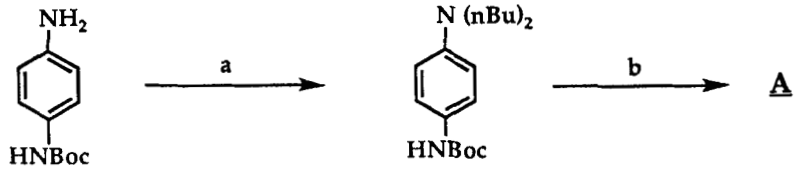
^{3}H-A and ^{3}H-B brought considerable structural information on the acetylcholine binding site of the nicotinic receptor[2-4] and on the active-site gorge of acetylcholinesterase[5-7] in alkylating several different amino acid residues.
In previous articles [8-10], we also described A and B as potent irreversible labels of the muscarinic acetylcholine receptors, provided energy-transfer photolabelling conditions were used.

All these findings prompted us to adapt the labelling strategy, developped in our laboratory for the nicotinic receptor and acetylcholinesterase, to the muscarinic receptor problematics. For example, the low natural abundance of these membrane-bound receptors precluded any fine structural analysis through site-directed labelling to be done without previous receptor purification steps and availability of probes at high specific radioactivity.
We describe here the synthesis and the purification of highly radiolabelled diazonium probes ^{3}H-A and ^{3}H-B (40-50 Ci/mmol.) together with optimal storage conditions.
A special attention was given to the characterization and elimination of contaminating products which needed optimal chromatographic conditions to be separated from the desired precursor and diazonium compounds. These analytical requirements were probably not necessary in the former studies [2-7] as the high isotopic dilution applied to the tritiated probes (0.1-5 Ci/mmol.) probably masked these contaminants.

RESULTS AND DISCUSSION

Unlabelled compounds were synthesized (Schemes 1 and 2) and purified in order to set up the diazotization reaction and the HPLC purification conditions for micromole quantities. Diazotization of precursors 2, 3 and 4 was reproducibly quantitative, giving the pure and stable corresponding diazonium derivatives A, B and C (Fig. 1) whose characteristics are listed in Table 1.

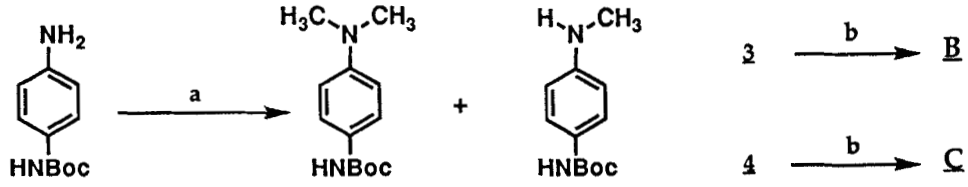
Scheme 1 :



1 2

Reagents and conditions : **a)** i : Butanal / AcOH ; ii : $NaBH_3CN$; **b)** i : CF_3COOH ; ii : $NaNO_2$

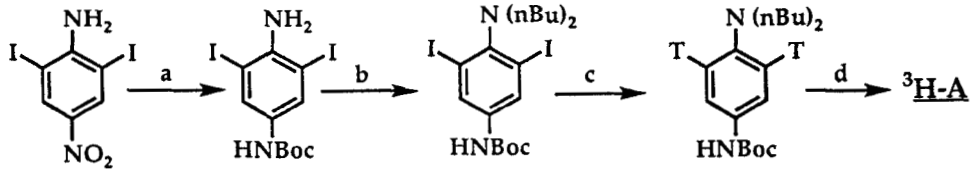
Scheme 2 :



1 3 4

Reagents and conditions : **a)** HCHO, H_2, Pd/C, EtOAc ; **b)** i : CF_3COOH ; ii : $NaNO_2$

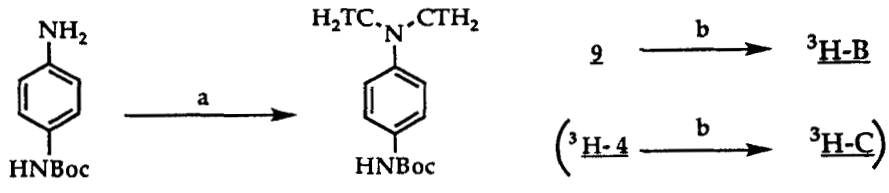
Scheme 3 :



5 6 7 8

Reagents and conditions : **a)** i : $NaBH_4$ /3S / THF ; ii : BocOBoc ; **b)** i : Butanal / AcOH ; ii : $NaBH_3CN$; **c)** TEA, T_2, Pd/C ; **d)** i : CF_3COOH ; ii : $NaNO_2$

Scheme 4 :



1 9

Reagents and conditions : **a)** HCHO, T_2, Pd/C, DMF ; **b)** i : CF_3COOH ; ii : $NaNO_2$

The synthesis of ^{3}H-A and ^{3}H-B (Schemes 3 and 4) followed a general three-step sequence : i- obtention of a precursor (1, 7) allowing the easy introduction of two tritium atoms per molecule, ii- tritiation at the CEA (Saclay) either by tritiolysis of carbon-iodine bonds (8) or by reductive amination (9) and finally, iii- conversion of the N-Boc-protected amino group into a diazonium moiety leading to the desired compounds ^{3}H-A and ^{3}H-B.

Following their obtention from the CEA, the radioactive batches containing the precursors 8 and 9 were stored in MeOH at -30°C until use. Reproducible losses in radioactivity (up to 40%) and in precursor absorbance (up to 20%) were detected after concentration of the samples. They indicated the occurence of radiolysis of the labelled products during the sample storage period and the evaporation step of the solvent.

Purity of the concentrated precursor samples was then checked by analytical injection on a reversed-phase HPLC column using the classically-described [5,11] gradient I as eluent.

Most of the time, substantial amounts of radioactivity and of $UV_{229\ nm}$ -absorbing material eluted besides the bisbutyle precursor 8 (not shown).

Purification of sample 8 was achieved on HPLC using the same gradient I and peak fractions (t_r= 15 min ; λ_{max}= 243 nm) were pooled. Their diazotization gave ^{3}H-A as the major product (80 to 100% yield) which was easily isolated and characterized (t_r = 26 min ; λ_{max}= 383 nm ; radioactive specific activity = 34-60 Ci/mmol.) after HPLC purification using the conditions of gradient II classically reported for aryldiazonium salts[5,11].

Surprisingly, relatively pure batches of sample 8 were not directly diazotizable under our conditions unless a preliminary purification step was done. This problem might be explained by the presence, in the starting material, of an unknown contaminant behaving as a potent diazotization inhibitor. Its removal was thus a prerequisite to an efficient diazotization of 8 at high specific radioactivity.

Such a particular feature has not been reported in previous studies dealing with the synthesis of ^{3}H-A[5,7,11], either was this contaminant absent or its inhibitory effect masked by the concomitant high isotopic and volumetric dilutions applied to samples 8 before their diazotization.

Several other problems were encountered with the purification of the radioactive precursor 9 which was first undertaken by reversed-phase HPLC using gradient I as eluent.

Batch to batch variations were reflected in the recovery of 20 to 80% of the starting material (λ_{max}= 260 nm in MeOH ; radioactive specific activity= 48 Ci/mmol.) as an heterogeneous UV_{229nm} - absorbing peak, collected in a single 3ml-fraction whose elution time (t_r= 9 min) and maximal absorbance wavelength (λ_{max}= 240 nm in water) were however in agreement (Table 1) with the presence of precursor 9 (not shown).

TABLE 1

Spectral properties and retention times on HPLC of N-Boc-protected and diazonium compounds.

	SPECTRAL PROPERTIES		RETENTION TIME ON H.P.L.C.			
	Molar extinction coefficient	Maximal absorbance wavelength [b]	Gradient I	Gradient II	Gradient III	Gradient IV
	(M^{-1}/cm^{-1})	(λ_{max}, nm)	(min)	(min)	(min)	(min)
N-Boc-protected compounds [a]						
1	18000	238 (251)	8	16.5	21.5	-
2 (8)	21370	243 (272)	15	29	-	-
3 (9)	22400	241 (263)	9	19	29.5	-
4 (^{3}H-4)	16400	240 (258)	9	18.3	27	-
Aryldiazonium compounds [a]						
A (^{3}H-A)	28600	383	-	26	-	-
B (^{3}H-B)	37500	378	-	9	-	11.3
C (^{3}H-C)	40000	366	-	10.5	-	14.3

a) The corresponding tritiated compounds are indicated under parentheses.

b) λ_{max} of the compounds are in H_2O and in MeOH (values under parentheses).

Pure compounds (0.05 to 0.5 µmol) were analyzed by reversed-phase HPLC (see Experimental Section) under the following gradient conditions:

I : 100% A to 100% B in 30 min ;

II : 100% A to 100% B in 60 min ;

III : 100% A to 15% B in 15 min, isocratic 15% B (20 min), 15% B to 100% B in 10 min ;

IV : 100% A to 10% B in 15 min, isocratic 10% B (5 min), 10% B to 100% B in 10 min.

Under gradient II elution conditions (2- fold lowering of the gradient I slope), HPLC analysis of the content of batch 9 (Fig. 2a) allowed three poorly-resolved components to be seen (retention times ranging from 16 to 19 min) and representing about 50% of the injected radioactivity. The two corresponding fractions 9 (λ_{max}= 239 nm) and 10 (λ_{max}= 241 nm) were pooled, concentrated and diazotized. The measurement of a 370 nm -λ_{max} value for the diazotization reaction medium, uncompatible with the presence of pure diazonium ^{3}H-B (Table 1), precluded any accurate determination of diazotization yield and radioactive specific acitivity.

These results were in apparent contradiction with the straightforward reaction shown on Scheme 4 and with the clean diazotization reaction reproducibly obtained for the unlabelled analog 3.

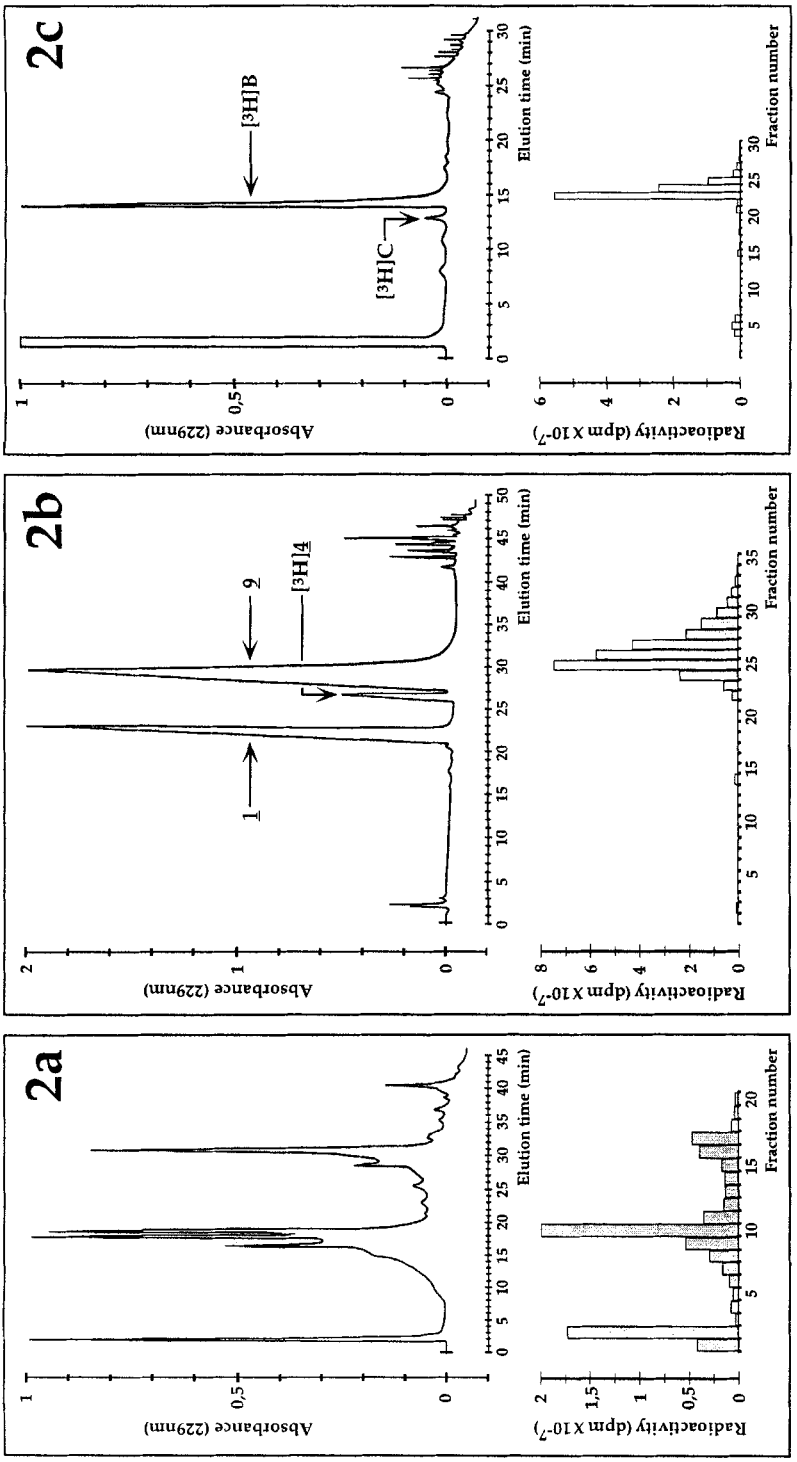


Figure 2: Reversed-phase HPLC analysis and purification of 9 and of its diazonium derivative ^{3}H-B.

Figure 2a: 0.15 µmoles of sample **9** are injected on the C18 column and eluted by gradient **II**. Fractions (3 ml) were collected and counted (10 µl aliquots) for radioactivity. **Figure 2b**: 0.5 µmoles of another sample of mixture **9** were HPLC-analysed under gradient conditions **III**. Fractions (1 ml) were collected and counted (5 µl aliquots) for radioactivity. **Figure 2c**: After their purification (fractions 25-30 pool as shown in Fig. 2b), 0.42 µmoles of **9** were diazotized and HPLC-analysed under gradient **IV** conditions. Fractions (1 ml) were collected and counted (3 µl aliquots) for radioactivity. Chromatographic conditions are as described under Experimental Section.

Carefull examination of the reductive amination of compound **1** (Scheme 2) demonstrated the presence of the mono-methylated derivative **4** in addition to the desired dimethylated compound **3**. This led us to suspect an identical evolution for the reductive amination of **1** under tritiation conditions.
Control experiments were then carried out, again under HPLC gradient conditions II, by injecting or co-injecting the three unlabelled Boc-protected compounds **1**, **3** and **4**.
All three retention time (t_r) values (Table 1) were in the 16-19 min time-range previously detected in Fig. 2a. It became clear also that the dimethyl-**3** and monomethyl-**4** derivatives, displaying almost identical λ_{max}- values in water, were poorly separated under these gradient II conditions (a fortiori under the classically-described I ones).

New eluting conditions (referred to as gradient III) and the collection of smaller fractions were selected for giving a much better separation (Table 1) of the different unlabelled species **1**, **3** and **4** and were similarily applied to HPLC analysis of the radioactive batch **9** (Fig. 2b).
Three peaks (monitored at 229 nm) eluted at retention times corresponding successively to those of compounds **1** (t_r= 21.5 min), **4** (t_r= 27 min) and **3** (t_r= 29.5 min).
Analysis of these peaks, collected as independent fractions, allowed their nature to be confirmed by UV spectroscopy (λ_{max}) and radioactivity counting and their relative proportions to be estimated (**1** : 238 nm, unlabelled, 23-27% ; tritiated ^{3}**H-4** : 240 nm, 20-22 Ci/ mmol., 6-16% ; **9** : 241 nm, 45-50 Ci/ mmol., 58-70%).

The findings of such an heterogeneous composition of the radioactive preparation **9** was unexpected since its synthesis (Scheme 4) was performed in DMF with an excess of reagent (HCHO) and catalyst (Pd/C), all conditions working together to accelerate and displace the reaction equilibrium (as compared to the experimental conditions reported in Scheme 2 for unlabelled compounds) towards the obtention of the dimethylated Boc-derivative **9** as a pure final product.
Precipitation of HCHO as a polymer and/or partial degradation of the catalyst are possible explanations for lot to lot variations in the purity of this tritiated precursor due to an uncomplete reductive amination.
This problem, probably masked in former studies [2,5-7,11] by the high isotopic dilution (10 to 500 fold) applied to the radioactive mixture **9**, represented a serious drawback in our experiments dealing with the synthesis of ^{3}**H-B** at high specific radioactivity.
Nevertheless, our new purification conditions allowed precursor **9** to be separated from contaminating compounds and especially from its mono-methylated congener and to be quantitatively diazotized.

Final purification of diazonium ^{3}**H-B** was achieved through the use of gradient IV-HPLC conditions which allowed, as shown in Fig. 2c, to separate ^{3}**H-B** (t_r= 14.3 min; λ_{max}= 378 nm ; specific radioactivity= 42-48 Ci/mmol.) from small amounts of ^{3}**H-C** (t_r= 11.3 min ; λ_{max}= 376 nm) resulting from the diazotization of residual ^{3}**H-4**.

Finally, the relative unstability of the purified aryldiazonium probes ^{3}**H-A** and ^{3}**H-B**, when stored in concentrated form and at the original high specific radioactivity, led us

to define an optimal compromise between concentration (up to 3 10^{-5}M in water), radioactive specific activity (up to 20 Ci/mmol.) and temperature (aliquots frozen at -80°C) to get an excellent stability of the compounds for several weeks.

CONCLUSION

In the present work, we have described the synthetis and new purification conditions allowing to obtain, with good yields, chemically pure and highly radiolabelled photoactivatable probes ^{3}H-A and ^{3}H-B.

The presence, in the initial radioactive precursor samples of unknown impurities behaving as quenchers of the diazotization reaction and of contaminating synthetic intermediates, hardly distinguishable from the desired Boc-protected radioactive compounds under the initially-described conditions [5,11], made it essential to design a more resolutive HPLC purification procedure.

Its application might be considered as a safe alternative to previously described purification conditions especially when aryldiazonium probes ^{3}H-A and ^{3}H-B, at the highest specific radioactivity available (40-50 Ci/mmol.), are needed.

EXPERIMENTAL SECTION

General

^{1}H-NMR spectra were recorded at 200 MHz, on a Bruker WP-200SY instrument, δ are given in ppm.

Mass spectra were obtained with a Finnigan spectrometer.

Melting points were taken on a Kofler block and are uncorrected.

N-terbutoxycarbonyl-para-phenylenediamine 1.

Its synthesis was as previously reported [12].

N,N-dibutyl-N'-terbutoxycarbonyl-para-phenylenediamine 2.

The reductive alkylation of 1, similar to that described for 6, is detailed in [13].

mp of 2 : 37-38°C

N,N -dimethyl-N'-terbutoxycarbonyl-para-phenylenediamine 3 and N-methyl-N'-terbutoxycarbonyl-paraphenylenediamine 4

The reaction scheme followed previously described procedures[14,15].

Briefly, N-terbutoxycarbonyl-para-phenylenediamine 1 was dissolved in ethyl acetate (EtOAc) and 1.2 eqs. of formaldehyde (HCHO) in aqueous solution and Palladium on carbon (Pd/C 10%, 20 mg per mmol 1) were added. The mixture was hydrogenated at atmospheric pressure for 20-36 h, then filtered.

The solvent was evaporated and the residue was purified by chromatography on silica gel with EtOAc/hexane (3/7). The dimethyl derivative eluted first to give, after recrystallisation (EtOAc/hexane), compound 3 (30% yield) as a white solid.
The monomethyl derivative 4 (60% yield) slowly crystallised from an oil.
mp of 3 : 89-90°C
mp of 4 : 39-40°C
^{1}H-NMR ($CDCl_3$) of compound 3 : δ = 1.53 (s, 9H, terbutoxyl) ; 2.89 (s, 6H, $N(CH_3)_2$) ; 6.54 (broad s, 1H, NHBOC) ; 6.71 (d, 2H arom, J = 6.1 Hz) ; 7.23 (d, 2H arom, J = 5.8 Hz).
^{1}H-NMR ($CDCl_3$) of compound 4 : δ = 1.51 (s, 9H, terbutoxyl) ; 2.78 (s, 3H, NCH_3) ; 3.53 (broad s, 1H, $NHCH_3$) ; 6.51 (broad s, 1H, NHBOC) ; 6.54 (d, 2H arom, J = 8.8 Hz) ; 7.16 (d, 2H arom, J = 8.6 Hz).

2,6-diiodo-N-terbutoxycarbonyl-para-phenylenediamine 6.

To a solution of sodium borohydride sulfur (2.54 g, 19 mmol) in anhydrous tetrahydrofuran (THF, 20 ml) was added a mixture of 5 (3.9 g, 10 mmol) in dry THF (60 ml). The reaction mixture was heated at 60°C for 3 h and a solution of BocOBoc (2.83 g, 13 mmol) in dry THF (5 ml) was added. The mixture was stirred for an additional 24 h at 80°C and then allowed to cool down to room temperature. After evaporation of the solvent under vacuum, the residue was taken up in EtOAc. The organic layer was then washed with a saturated solution of NaCl, dried over Na_2SO_4 and concentrated under vacuum. The resulting solid was purified by flash chromatography on silica gel (hexane/EtOAc: 9/1) to afford 2.30 g of compound 6 (50 % yield) as a white solid.
Rf = 0.45 (hexane/EtOAc: 9/1)
^{1}H-NMR ($CDCl_3$) : δ = 1.50 (s, 9H, terbutoxyl) ; 6.20 (broad s, 1H, NHBoc) ; 7.70 (s, 2H, arom).

2,6-diiodo-N,N-dibutyl-N'-terbutoxycarbonyl-para-phenylenediamine 7.

To a solution of 6 (4 g, 8.7 mmol) in EtOH (300 ml) were added butyraldehyde (butanal, 62.2 ml, 0.69 mol), acetic acid (5%, 10 ml) and $NaBH_3CN$ (10.96 g, 0.17 mmol). The reaction mixture was stirred at room temperature for 24 h. After evaporation of the solvent under vacuum, the residue was resuspended in EtOAc. The organic layer was then washed with water, with a saturated solution of $NaHCO_3$ and with a saturated solution of NaCl, dried over $MgSO_4$ and concentrated under vacuum.
The resulting solid was purified by flash chromatography on silica gel (hexane/EtOAc : 95/5) to afford a yellow solid, which was recrystallized in hexane/diisopropyl ether (95/5) to afford 1.99 g of compound 7 (40 % yield) as a white solid.

Rf = 0.58 (hexane / EtOAc : 9/1)
mp of 7 : 110-111°C
^{1}H-NMR ($CDCl_3$) : δ = 0.89 (t, 6H, J = 7.2 Hz, 2 CH_3) ; 1.19 - 1.47 (m, 8H, CH_2) ; 1.51 (s, 9H, terbutoxyl) ; 3.09 (t, 4H, J = 7.5 Hz, CH_2NCH_2) ; 6.33 (broad s, 1H, NHBoc) ; 7.92 (s, 2H, arom).

2,6-(^{3}H)-N,N-dibutyl-N'-terbutoxycarbonyl-para-phenylenediamine 8.

The tritiation step of compound 7 was performed at the CEA (Saclay) as follows. To a solution of 7 (10.9 mg, 0.019 mmol) in CH_3OH (2 ml) were successively added triethylamine (TEA, 9 µl) and Pd/C (10 %, 10.8 mg). The mixture was stirred under Tritium (20 Curies) at atmospheric pressure for 1 h at room temperature. The solution was then filtered over PTFE (0.5 µm) and CH_3OH (2 x 5 ml) was necessary to washout labile tritium atoms. After evaporation of the solvent under vacuum, the residue was resuspended in CH_3OH (50 ml) and compound 8 (1.07 Ci), initially obtained with a radiochemical purity superior to 94 % (TLC on silica gel) and a specific activity of 44 -60 Ci/mmol., was stored in CH_3OH at -30°C.
Rf = 0.50 (hexane / EtOAc: 9/1)

N,N-dimethyl(^{3}H)-N'-terbutoxycarbonyl-para-phenylenediamine 9.

The tritiation step of compound 1 was performed at the CEA (Saclay) as follows. To a solution of 1 (20.1 mg, 0.096 mmol) in freshly distilled dimethylformamide (DMF, 4 ml) were successively added HCHO (0.25 ml) and Pd/C (10 %, 150 mg). The mixture was stirred under Tritium (20 Curies) at atmospheric pressure for 4 h at room tempe- rature. Then, the solution was filtered over PTFE (0.45 µm) with DMF and over 0.2 µm filters with CH_3OH (2 x 10 ml). After evaporation of the solvent under vacuum, the residue was resuspended in CH_3OH (50 ml) and compound 9 (2.74 Ci), initially obtai- ned with a radiochemical purity superior to 99% (TLC on silica gel, hexane/EtOH : 9/1, MS, ^{3}H-NMR) and a specific activity of 47Ci/mmol., was stored in CH_3OH at -30°C.
MS (DCI/NH_3) : m/z 241 (M, 100%)
^{3}H-NMR : δ = 2.75 (s, CTH_2).

Diazotization

Unlabelled and tritiated precursors of diazonium A, B and C were submitted to an identical, micromolar-scaled, two-step procedure. Deprotection of the Boc-protected amino group of compounds 2, 3, 4, 8 and 9 (0.1 to 1 µmol) was done in trifluoroacetic acid (TFA, 200 to 500 µl) for 30 min at room temperature. The mixture was then cooled to -10°C, stirred vigourously and placed in the dark. $NaNO_2$ (1.2 eqs.) in aqueous solution was added portionwise over a period of 30 min and completion of the diazotization reaction was followed by UV spectroscopy. After dilution in H_2O to reach a final concentration close to 10^{-4}M, the purity of the diazonium compounds was checked by HPLC.
Special care was taken to avoid light exposure during the diazotization and subsequent manipulation steps.

Reversed-phase HPLC purification and storage conditions

Compounds (0.05 to 0.5 µmol) were injected on an analytical reversed-phase HPLC column (C_{18}; µ Bondapack Waters; 300x3.9 mm) and eluted (flow rate : 1.5 ml

per min) using solvents A (H_2O / 0.1% TFA) and B (CH_3CN) under the following gradient conditions:

I : 100% A to 100% B in 30 min ;

II : 100% A to 100% B in 60 min ;

III : 100% A to 15% B in 15 min, isocratic 15% B (20 min), 15% B to 100% B in 10 min ;

IV : 100% A to 10% B in 15 min, isocratic 10% B (5 min), 10% B to 100% B in 10 min.

Fractions of 3 ml (Gradients I and II) or of 1 ml (Gradients III and IV) were collected and characterized (UV spectroscopy, radioactivity).

Fractions containing pure diazonium compounds were pooled, submitted to repeated concentration-dilution (H_2O) steps performed under reduced pressure (0.1 mm Hg) using a dry ice/acetone cold trap. TFA and CH_3CN were rapidly eliminated and the samples were concentrated without damage. Aliquots were routinely stored at -30°C.

Optimal storage conditions of these aqueous solutions of diazonium probes greatly depended on their concentration and radioactive specific activity : unlabelled compounds were stable for years as crystals (B) or as concentrated aqueous solutions (up to 10^{-1}M) ; tritiated diazonium molecules at low specific radioactivity (up to 2 Ci/mmol) were stable for months while ^{3}H-A and ^{3}H-B, when prepared at a high specific radioactivity, remained stable for at least one month when isotopically (up to 20 Ci/mmol) and volumetrically (up to 3 10^{-5}M) diluted before storage as aliquots at -80°C.

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