# THE SYNTHESIS OF DEUTERIUM, TRITIUM AND CARBON-14 LABELLED NICOTINANILIDES

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## SUMMARY

The preparations of nicotin[2',4',6'-$^{2}H_3$]anilide, 4'-Cl-nicotin[2',6'-$^{2}H_2$]anilide, 4'-X-nicotin[ar-$^{3}$H]anilide, (X = H-, $CH_3$-, Cl- or $NO_2$-) and[6-$^{14}$C]nicotin[ar-$^{3}$H]anilide are described.

KEY WORDS: Labelled nicotinanilides

## INTRODUCTION

Nicotinanilides are potent molluscicides[1] which are non-toxic to fish[2]. This selective biological activity is potentially of great importance and, as part of a programme of research undertaken to investigate rate of uptake, tissue distribution, bioconcentration and metabolism, various labelled nicotinanilides and 4'-substituted nicotinanilides were prepared as described below.

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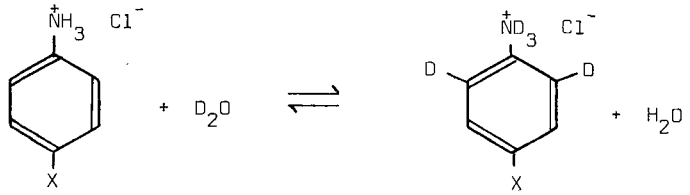
## RESULTS and DISCUSSION

Substituted aniline hydrochlorides were found to exchange with deuterium oxide according to the expected acid catalysed o,p- substitution pattern[3] :




X = H (D), Cl, $CH_3$, $NO_2$

The kinetics of these reactions were studied using an nmr technique[4] and this technique was used to determine practical equilibration times for the exchange reactions. Repeated exchanges yielded [2,4,6-$^2H_3$]aniline and 4-chloro[2,6-$^2H_2$]aniline with deuterium contents > 99% in the specified positions. Less than 5% deuterium exchange occurred in the 3,5- position.

The same conditions were used to prepare tritiated anilines in high yields from tritiated water of the required specific activity. The results are recorded in table 1.

Table 1

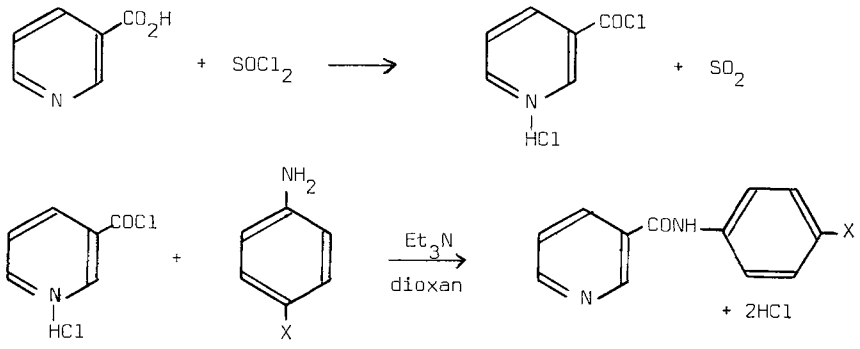
Specific Activities of 4-substituted Anilines after Exchange[a] in THO (1 $cm^3$) at 140°.

| Substituent | Quantity (mmol) | THO (Ci) | Time (hr) | Specific Activity (mCi $mmol^{-1}$) Observed | Calculated |
|---|---|---|---|---|---|
| H | 3.86 | 1 | 6 | 21.4 | 22.3 [b] |
| $NO_2$ | 2.87 | 1 | 5 | 12.3 | 15.9 [c] |
| $CH_3$ | 3.48 | 1 | 10 | 14.9 | 15.6 [c] |
| Cl [e] | 6.28 | ∿ 30 | 12 | 572 | ∿ 770 [d] |

a = exchanged as the hydrochloride salts
b = calculated for 3 exchangeable hydrogens atoms
c = calculated for 2 exchangeable hydrogens atoms
d = calculated for 4 exchangeable hydrogen
e = platinum catalyst added

In the case of the 4-chloroaniline we were attempting to prepare material of very high specific activity suitable for examination of biological specimens by high resolution electron microscope autoradiography. Consequently a platinum catalyst was added in the hope that it would promote additional exchange at the 3,5- positions but as table 1 shows this expectation was only partially realised. Experiments with deuterium oxide also showed that the 3,5- positions were reluctant to exchange even when platinum catalyst was present.

Tritium or deuterium labelled nicotinanilides were prepared by acylating the labelled anilines with nicotinyl chloride either in dioxan/triethylamine solution or in refluxing pyridine;




Specific activities, radiochemical yields and purities are recorded in table 2 :

Table 2

Preparation of Tritiated Nicotinanilides

| 4'-Substituent | Radiochemical Yield (%) | Radiochemical Purity (%) | |
|---|---|---|---|
| | | a | b |
| H | 45 | 97 | > 95 |
| $NO_2$ | 55 | 98 | > 95 |
| $CH_3$ | 68 | 98 | > 95 |
| Cl | 47 | 99 | > 95 |

a = reverse isotope dilution analysis (± 2%)

b = radiochromatograph (TLC) scanning (± 5%)

[6-$^{14}$C]Nicotinanilide was prepared from [6-$^{14}$C]nicotinic acid using the same synthetic pathway in apparatus modified for manipulation of labelled compounds on a semi-micro scale. The doubly labelled [6-$^{14}$C]nicotin[ar-$^{3}$H] anilide was prepared by dilution of [6-$^{14}$C]nicotinanilide with radiochemically

pure nicotin [ar-$^{3}$H]anilide followed by recrystallisation. The specific activities were respectively 98.2 μCi mmol$^{-1}$ for $^{14}$C and 411 μCi mmol$^{-1}$ for tritum giving an isotope ratio ($^{3}$H/$^{14}$C) of 4.6. The radiochemical purity was > 95% by TLC scanning.

## EXPERIMENTAL

### I. Materials

Tritiated water and [6-$^{14}$C]nicotinic acid were obtained from the Radiochemical Centre, Amersham, U.K. Deuterium oxide was purchased from Prochem (British Oxygen Co. Ltd., London U.K.). Other reagents and solvents were either 'AnalaR' grade or purified before use.

### II. Analytical Methods

(i) Melting points were measured using a Gallenkamp Melting Point Apparatus and were corrected from a calibration curve, prepared by use of micro-analytical reagents (BDH) as standard with melting points in the range 76° - 216°.

(ii) Microanalyses were performed on a Perkin Elmer Model 240 Elemental Analyser.

(iii) Spectroscopy: infrared (IR) spectra were recorded on a Perkin Elmer Model 325 Spectrometer and proton nuclear magnetic resonance (NMR) spectra were measured on a Perkin Elmer R12B 60 MHz Spectrometer at a probe temperature of 35°.

(iv) Thin layer chromatography (TLC): progress of reactions and product chemical purities were determined using analytical 5 x 20 cm plates. Plates were precoated with fluorescent indicator ($\lambda_{max}$ = 254 mm). Visualisation of material on the plates was by observation of the plates under UV light at 254 nm.

(v) Radiochromatogram: scans of thin layer chromatograms were carried out on either a Panax RTLS-1A Radiochromatogram Scanner or a Packard Model F201 Radiochromatogram Scanner.

(vi) Radiochemical assay: was performed on a Philips Liquid Scintillation Analyser or a Packard Tricarb Model 3314 Liquid Scintillation Counter. In some cases where clear homogeneous counting samples could not be obtained the samples were burned in a Packard Sample Oxidiser model 305 prior to counting. Counting samples were dissolved in toluene or blended with toluene using ethanol. The scintillator was Butyl-PBD (0.8%) in all cases. Observed count rates were corrected for background count and counting efficiency. For tritium samples counting efficiencies were determined by the samples channels ratio method. For carbon-14 samples, counting efficiencies were determined via an external standard channels ratio technique. Samples activities determined in this way were reproducible to ± 2% (single label) or ± 5% (double label).

## III. Synthesis of Nicotinanilide and its Derivatives

1. 3-Pyridinecarboxylic acid chloride-hydrochloride (Nicotinyl chloride-hydrochloride. Thionyl chloride (10 $cm^3$) was added dropwise to nicotinic acid (3.1g; 0.025 mol) contained in a 100 $cm^3$ 2 necked round bottomed flask. The suspension was stirred and dimethylformamide (0.3 $cm^3$) was added. After 20 min. all the acid had dissolved and the solution was refluxed for a further 1 hour. Stirring was continued overnight at room temperature during which time white crystals precipitated. The flask was cooled in an ice bath for 1 hour and then the crystals were collected by force filtration through a sinter using nitrogen gas. The product was washed twice with cold benzene to remove the last traces of colour and then dried under vacuum. Yield 3.95g (88%); mp 157-160° (lit. mp 155.5 - 156.5)[5].

(Found: C,40.6; H,3.0; N,7.8%. Calc. for $C_6H_5Cl_2NO$ : C,40.4; H,2.8; N,7.9%).

2. Labelled anilines

(i) [2,4,6-$^2H_3$]Aniline

Aniline (1 $cm^3$; 11 mmol) was pipetted into a small reaction vessel. Deuterium oxide (2 $cm^3$, 99.8%) and concentrated deuterium chloride solution (10 mmol DCl/100 m mol $D_2O$) (1 $cm^3$) were then added. The vessel was frozen, evacuated, sealed and then immersed in an oil bath at 140°C for 5 hours. The vessel was opened, the deuterium oxide distilled off and a fresh 2.5 $cm^3$ of 99.8% deuterium oxide added followed by sealing and heating as before. After several exchanges the vessel was opened and the contents washed with water into a separating funnel containing aqueous potassium hydroxide solution (10% $^w/_v$) to give a precipitate of the free base at ∿ pH 10. The aniline was extracted into ether, then back extracted into dilute hydrochlorice acid, re-liberated by further addition of KOH, extracted into ether and dried over anhydrous sodium sulphate. (The double extraction procedure was adopted to ensure replacement of labile deuterium on the nitrogen atom with protium since the $-NH_2$ protons were used as internal standard for measurements of deuterium content by NMR). Filtration and removal of the ether yielded crude deuterated aniline which was not purified further. NMR and IR spectra were used to establish its purity.

(ii) [4-Chloro 2,6-$^2H_2$]aniline

Was prepared from 4-chloroaniline by similar exchange reactions.

(iii) [ar-$^3H$]Aniline

Aniline hydrochloride (500 mg; 3.86 mmol) was placed in a small reaction vessel[6] which was then attached to a vacuum manifold and evacuated.

Tritiated water (1 $cm^3$, 1 Ci) was distilled in, the tube sealed and immersed in an oil bath at 140° for 6 hours. The tritiated water was removed in vacuo via a break seal and the hydrochloride then washed with water into a separating funnel where it was washed successively with acid and base as before. Extraction with ether, drying and evaporation yielded slightly discoloured [ar-$^3$H]aniline (100 mg; 230 μCi $mg^{-1}$).

(iv) 4-Methyl[ar-$^3$H]aniline and 4-nitro[ar-$^3$H]aniline

Were prepared from 4-methyl- or 4-nitro-aniline exactly as described above. The 4-nitroaniline was insoluble at room temperature but completely soluble at 140°.

(v) 4-Chloro[ar-$^3$H]aniline

Platinum oxide (197 mg) was added to the standard reaction vessel, covered with ethanol (3 $cm^3$) and reduced by a stream of hydrogen until a fine black suspension was produced (approx. 40 min).

4-Chloroaniline hydrochloride (1.03g; 0.0062 mol) was added to the catalyst. The vessel was then evacuated, the ethanol distilled out, and tritiated water (about 30 Ci; 1 $cm^3$) stored under a mercury seal on the line was distilled in. After degassing of the reaction vessel (3 cycles of freezing with liquid nitrogen, evacuating and warming to room temperature) it was sealed and immersed in an oil bath at 140° for 12 hours.

Again the vessel was evacuated, the break seal opened with a magnetic breaker and the tritiated water distilled out as before. Ethanol (3 x 3 ml) was distilled in and then out to remove exchangeable tritium. On the third washing it was equilibrated at room temperature overnight before being distilled out. The vessel was then opened and product and catalyst washed with water into a sinter beaker from which the product was filtered. The filtrate was treated dropwise with a sodium carbonate solution until the pH was alkaline (phenolphthalein indicator solution). The product was extracted into ether which was dried over sodium sulphate, filtered and

evaporated to dryness to yield crude 4-chloro[ar-$^{3}$H]aniline (650 mg; 81%). Purification was effected by sublimation. (Specific activity 10.50 mCi mg$^{-1}$). Of this, 468 mg was diluted with carrier (609 mg) and dissolved in ether. A stream of hydrogen chloride under nitrogen was bubbled in until the pH was acidic (methyl orange indicator). The precipitate was extracted with water. After an hour the free base was recovered as before. (Specific activity 2.04 mCi mg$^{-1}$). This washing procedure was repeated (specific activity 1.87 mCi mg$^{-1}$). A fourth washing gave a final specific activity of 1.95 mCi mg$^{-1}$. This material was not further purified.

## 3. Labelled Nicotinanilides

Two modifications of the same reaction scheme were used as described in the examples given below:

### Procedure 1

### (i) 4'-Chloronicotin[ar-$^{3}$H]anilide

To a solution of freshly prepared nicotinyl chloride-hydrochloride (1.56g; 0.09 mol) in 40 cm$^{3}$ of dry dioxan under an atmosphere of nitrogen was added dropwise a solution of 4-chloro[ar-$^{3}$H]aniline (429 mg; 1.95 mCi mg$^{-1}$), inactive 4-chloroaniline (300 mg) and triethylamine (1.76g; 0.017 mol) in 15 cm$^{3}$ of dry dioxan. The mixture was stirred for 2 hours at room temperature. Thin layer chromatography of the reaction mixture at this time indicated the presence of some unreacted 4-chloroaniline and so a further 0.5g of nicotinyl chloride-hydrochloride was added together with 0.4 cm$^{3}$ of triethylamine. After a further 20 minutes stirring, 4-chloroaniline could not be detected by TLC. The excess chloride and triethylamine hydrochloride were filtered off and the filtrate concentrated by rotary evaporation.

A column was dry packed with silica gel G60 (150g) and the filtrate preabsorbed onto it. Elution solvent was initially in the ratio

diisopropyl ether-ethyl acetate(15:5) and finally (during product elution) in the proportion 15:16. Eluted fractions (30 $cm^3$) were analysed using TLC and a radio elution curve obtained by liquid scintillation counting. Fractions 21-30 were combined, concentrated, dried to constant weight and assayed for tritium. Three recrystallisations from ethanol-water gave fine white needles of constant specific activity. Yield 694 mg (47%). Specific activity $6.36 \times 10^2$ µCi $mg^{-1}$.

Chemical purity of the product was determined by thin layer chromatography using the following systems

(a) silica gel GF : benzene-ethanol (85:15)

(b) silica gel GF : acetone-dioxan (50:50)

The spots were visualised by observation of the plate in UV light of wavelength 254 nm.

Radiochemical purity was determined by

(i) Radioscanning of the above TLC plates,

(ii) Reverse isotope dilution analysis : 4'-Chloronicotin[ar-$^3$H]anilide (1.555 mg; 989 µCi) was mixed in ethereal solution with carrier (230 mg) and dried to constant weight. The specific activity of a sample was determined (1017 dpm $mg^{-1}$). Recrystallisations from ethanol-water to constant specific activity gave a value of 1027 dpm $mg^{-1}$. The radiochemical purity was thus 99.0%.

(ii) Nicotin[ar-$^3$H]anilide and 4'-methylnicotin[ar-$^3$H]anilide

Were prepared by procedure 1.

Procedure 2

(iii) Nicotin[2',4',6'-$^2H_3$]anilide

Aniline-$d_3$ (about 10 mmol) was stirred with dry pyridine (4.5 $cm^3$) and freshly prepared nicotinyl chloride-hydrochloride (2.62g; 14.7 mmol)

was added in portions. The mixture was refluxed for 1½ hours, cooled slowly and water (40 $cm^3$) added, resulting in a white precipitate which was stirred overnight. The product was filtered and dried to give a crude yield of 1.94g. Recrystallisations from ethanol-water gave fine white needles mp 119.5-121°. (Found : C,71.3; H,5.2; N,13.6. Calc. for $C_{12}H_7D_3N_2O$ : C,71.6; H,5.0; N,13.9%).

(iv) 4'-Chloronicotin[2',6'-$^2H_2$]anilide

Prepared from [2,6-$^2H_2$]aniline using procedure 2. Recrystallised from ethanol-water to give white needles mp 170-170.5°. (Found : C,61.8; H*,3.95; N,12.5%. Calc. for $C_{12}H_7D_2ClN_2O$ : C,61.4; H*,3.8; N,11.9%).

(v) 4'-Nitronicotin[ar-$^3$H]anilide

Was prepared by procedure 2.

(vi) [6-$^{14}$C]Nicotin[ar-$^3$H]anilide

Nicotinic acid (12.5 mg; 0.10 mmol) was dissolved in 1 $cm^3$ of water and added to an ampoule containing [6-$^{14}$C]nicotinic acid (50 µCi- 45 $mmol^{-1}$) and warmed to effect complete solution. This solution was transferred to a small reaction vessel which had a side arm in which a small sintered glass filter disc was fused.[7] The ampoule was washed out with a further 1 $cm^3$ of water and the reaction vessel connected to a vacuum line via the arm containing the sinter. The water was distilled out. Thionyl chloride (0.4 ml) was distilled in, the vessel removed from the vacuum line and refluxed for 1½ hours during which time complete solution of the nicotinic acid was obtained. The vessel was again connected to the vacuum line and excess thionyl chloride distilled out.

A small "spin-fin" stirrer was magnetically lowered into the vessel which was then clamped over a stirrer. A solution of aniline (0.109 mmol) and triethylamine (0.241 mmol) in dioxan (5 $cm^3$) was added dropwise from a syringe fitted with a long polythene needle. Addition was complete after 10 minutes and the suspension stirred for 1½ hours.

Insoluble salts were filtered off by inverting the vessel and applying slight nitrogen pressure. The filtrate was collected in a 10 ml pear shaped flask which was evacuated and the dioxan distilled out. This gave a crude yield of 20 mg which was dissolved in 1 $cm^3$ of acetone. A sample (2 µl) was chromatographed on silica gel:benzene-ethanol (5:1) and showed three radioactive peaks on scanning, corresponding to nicotinic acid, nicotinanilide and an unknown product of high $R_f$ in the ratio 5.5:23.5:1.

Nicotin[ar-$^3$H]anilide (120.6 µCi; 34 µCi $mg^{-1}$) was added to the crude product above, together with carrier (31.0 mg).

A small column was packed wet with silica gel - Merck 7734 (10g) and the acetone solution was slowly absorbed on it. The flask was washed with 1 $cm^3$ of eluent, diisopropyl ether-ethyl acetate (50:50). which was also absorbed. The column was then eluted and 36 x 6 $cm^3$ fractions collected. On the basis of results from TLC as well as count rates of fraction samples, numbers 15-35 were combined and solvent removed to yield slightly discoloured [6-$^{14}$C]nicotin[ar-$^3$H]anilide. A sample was dissolved in acetone and run on a silica gel plate in benzene-ethanol (5:1) and radioscanned. Only one peak could be detected either by visualisation or by radioactivity. A sample was counted under dual label conditions on the Philips Liquid Scintillation Analyser. This gave a specific activity for $^{14}$C as $1.00 \times 10^6$ dpm $mg^{-1}$ and a specific activity for $^3$H as $4.60 \times 10^5$ dpm $mg^{-1}$.

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