Synthesis of [3H]Pinoline, an Endogenous Tetrahydro-ß-carboline

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SUMMARY

Pinoline (6-methoxy-1,2,3,4-tetrahyro-9*H*-pyrido[3,4-*b*]indole) is an endogenous ß-carboline thought to be synthesized in the pineal gland from serotonin. It is a strong inhibitor of MAO-A and can displace [3H]citalopram from the 5-HT uptake site on human platelets in nM concentrations. The hydrochloride salt of 6-MeO-DHßC (6-methoxy-1,2-dihydro-9*H*-pyrido[3,4-*b*]indole), also a new compound, was synthesized as the immediate precursor to the title compound.

Key words: Tritium NMR, pinoline, ß-carboline, tryptamine, endogenous

INTRODUCTION

ß-Carbolines (ßCs) are of interest since they occur in many plants and animals (4). Synthetic analogues can precipitate reactions from anxiogenic to anxiolytic, depending on substitution, and interact with several neurotransmitter systems (10). They inhibit monoamine oxidase-A (MAO-A)

(5) and can interact with the benzodiazepine binding site as antagonists, agonists and inverse agonists (6). This certainly hints of some neuroregulatory role in stress modulation for endogenous ßCs. In animals they are thought to form as condensation products from tryptamines and aldehydes (8) and/or keto acids (7). Recent research has shown that ß-carbolines do not occur as artifacts in analysis or as dietary components (1). About half a dozen different ßCs have been identified in humans, though little else is known of their origin or function.

Condensation of endogenous tryptamines with formaldehyde, formed enzymatically from 5-methoxytetrahydrofolate (5-MTHF), result in ßCs unsubstituted at C-1 (Fig. 1). With acetaldehyde, from the metabolism of ethanol for example, products methylated at C-1 result. There is current speculation as to their role in alcoholism (2,11).
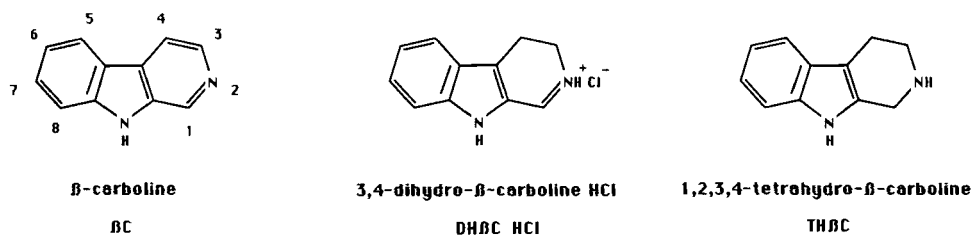

ß-carboline
ßC

3,4-dihydro-ß-carboline HCl
DHßC HCl

1,2,3,4-tetrahydro-ß-carboline
THßC

Figure 1

Tetrahydro-ß-carbolines (THßCs) can be produced synthetically by catalytic hydrogenation of dihydro-ß-carbolines (DHßCs). Essentially no work has been done on DHßCs which are unsubstituted at C-1, perhaps due to their lack of stability and subsequent availability. The trifluoroacetate of DHßC has recently been reported as a fine crystalline solid, produced in good yield (9), though these salts are not appropriate for receptor binding studies. Therefore we have developed a method for preparing the hydrochloride salt of dihydro-pinoline for biological investigations and as a precursor to [3H]pinoline (Fig. 2).
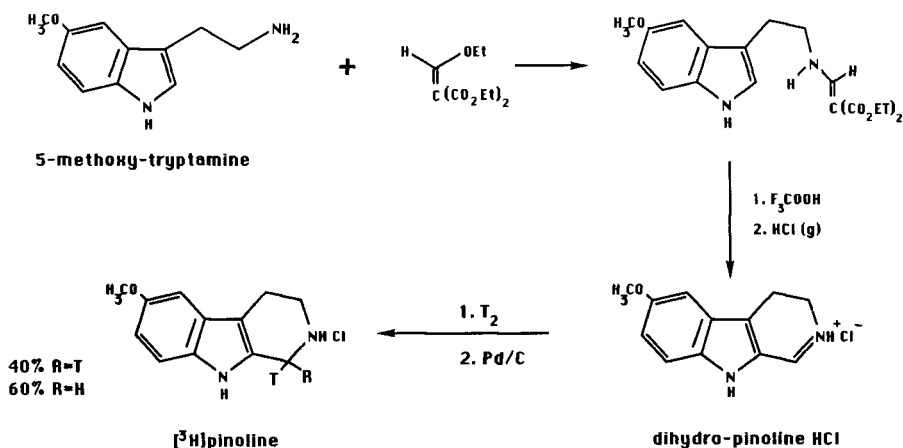
Figure 2

EXPERIMENTAL

Materials

Solvents were of analytical grade or better. Diethylethoxymethylene malonate and 5-methoxytryptamine were purchased from Sigma. Ethanol 200 proof and 10% Pd/C were purchased from Aldrich.

Synthesis of 6-methoxy-3,4-dihydro-ß-carboline hydrochloride

A 1.1 molar excess of diethylethoxymethylene malonate, 1.041 g (2.89 mM), was added to 500 mg (2.63 mM) of 5-methoxytryptamine and stirred for one hour at 95°C in a flask heated by a water bath. The reaction was cooled to room temperature and ethylacetate was added to give a total volume of 20 mL. This solution was gravity filtered, the solvent and excess malonate were removed *in vacuo*, leaving 950 mg of the crude enamine. Without further purification, the remaining oil was dissolved in as little trifluoroacetic acid as possible (1-2 mL to 1 g of crude enamine) while stirring in a flask on ice for 2 hours, forming the trifluoroacetate salt of 6-MeO-DHßC. Excess acid was partially removed *in vacuo* at room temperature.

The crude product was partitioned between 1 M HCl and ether. The aqueous phase was made basic by drop-wise addition of sat. aq. Na_2CO_3 and extracted with CH_2Cl_2. The organic phase was dried over anhyd. $MgSO_4$ for 1 hour. This solution was gravity filtered and the total volume was reduced *in vacuo* to 100 mL, the released vacuum was filled with nitrogen gas rather than ambient atmosphere to avoid decomposition of the free amine.

HCl gas was prepared by dropping conc. H_2SO_4 into a flask containing conc. HCl and a small amount of NaCl. The mixture was gently heated, and the generated gas passed through a refluxing column and a column of $CaCl_2$ before it was bubbled into the dry CH_2Cl_2/ 6-MeO-DHßC solution for about 30 seconds. The product was recovered by removal of the solvent *in vacuo*, and crystallized from dry ethylacetate ($MgSO_4$) while using a minimal amount of hexane to induce precipitation.

Synthesis of [³H]pinoline hydrochloride

20 mg (0.08 mM) of 6-MeO-DHßC hydrochloride was dissolved into 3 mL of 200 proof ethanol in a 15 mL reaction flask, giving a clear yellow solution, and frozen in a bath of liquid nitrogen under high vacuum (10^{-12} Torr). The reaction vessel was purged with nitrogen gas while the sample slowly thawed to room temperature. After the third evacuation, tritium (from a heated uranium hydride source), was introduced into the reaction vessel until the pressure reached 730 Torr, then 20 mg of 10% Pd/C was added to the solution from within the system. After one hour of stirring at room temperature the reaction solution appeared lighter in color. The reaction was terminated after a total time of 1.5 hours. The product was immediately converted to the hydrochloride salt by the addition of ethanolic HCl, thus avoiding possible loss of the product *via* sublimation. The solution was filtered to remove the catalyst; the filtrate was a pale-yellow solution. Ethanol, excess HCl and labile tritium were removed by lyophilization of the filtrate. A pale yellow solid remained which was dissolved in methanol and again lyophilized.

Characterization of the Products

High resolution mass spectra were recorded using a Trio-2-VG Masslab (Manchester U.K.) mass spectrometer. The electron energy was 70 eV, ion source temperature 200°C, and ionization current 200 μA. Samples were introduced by a glass holder with a direct insertion probe. Accurate mass measurements of molecular ions were carried out automatically with the data system. Perfluorokerosene was used as the reference compound.

NMR spectra were obtained from an IBM Instruments AF-300 spectrometer with an Oxford magnet of field strength 7.05 Tesla (70 K Gauss) interfaced with and Aspect 3000 computer with Digital Phase shifting (<1° resolution) using a 5 mm $^3H/^1H$ dual probe. All samples were dissolved in CD_3OD with TMS as the reference standard; SF= 300.135 MHz and SR= 4550.59 Hz for the proton spectrum; SF= 320.135 MHz and SR= 5438.10 Hz for the tritium spectra, which was also selectively decoupled at 5867.97 Hz.

6-MeO-DHßC hydrochloride. The yield was 512 mg (83 %), of a pale yellow crystalline powder which decomposed at 200°C. High resolution MS: m/z 201 (12 %), 200 (74 %), 199 (100 %), 184 (14 %), 172 (8%), 156 (11%), 130 (10%). Accurate mass (M+) 200.0941 (required for $C_{12}H_{12}N_2O$ (M+) 200.0946).

[³H]pinoline. Characterization was determined by NMR and comparison with cold pinoline produced under similar reaction conditions. Apparently all of the 6-MeO-DHßC was converted to product since none of the precursor could be detected by NMR. Specific radioactivity (S.A.) of the product, as determined by ¹H and ³H-NMR, was determined to be 35.9 ±1.8 Ci/mmol. The NMR for the product was found to be in agreement with the spectrum of unlabelled pinoline. The product was a pale-yellow powder.
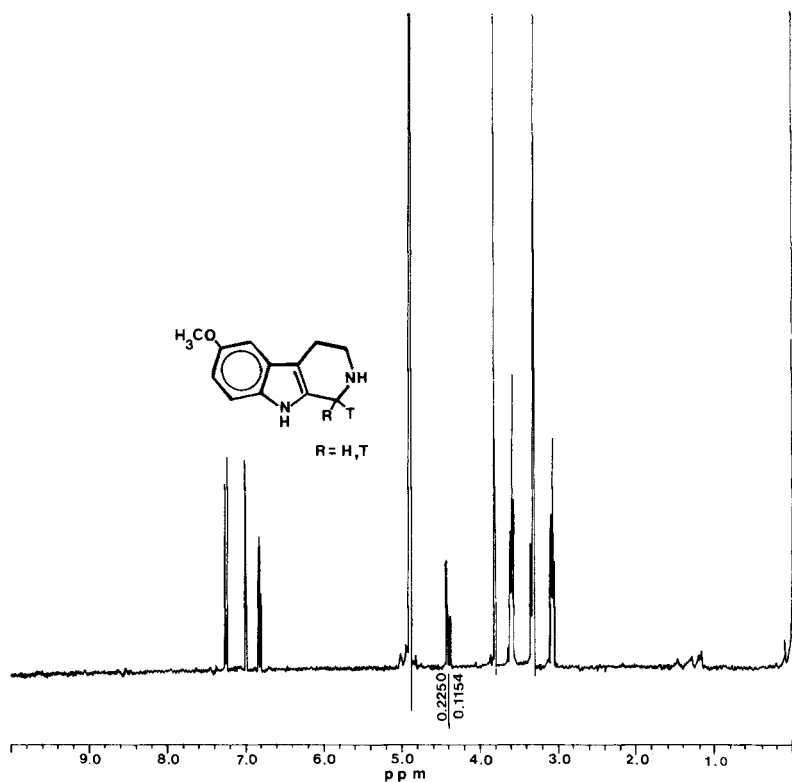
Figure 3. Proton spectrum of [³H]pinoline in CD₃OD; note the integral values for the doublet at 4.39 ppm resulting from tritium and hydrogen atoms attached to C-1.

Table 1.   ¹H-NMR of [³H]-Pinoline:

Atom	ppm H,	mult.
1	4.39,	d
3	3.05,	m
4	3.52,	m
5	6.97,	d
6-OCH₃	3.81,	s
7	6.80,	dd
8	7.23,	d

Specific radioactivity. Measurements are generally made by liquid scintillation counting of a known mass of compound, by mass spectrometry, or by consideration of the ^{3}H and ^{1}H NMR spectra (12). The NMR technique has been used to analyze compounds such as tritiated $NaBH_4$, where other methods are not facile or practical (13), and is most readily applied when the amount of ^{1}H replaced by ^{3}H at specific positions is high; *i.e.* 10-100% (14).

One NMR method for determining S.A. involves the integration of residual protons at the tritiated site relative to an unlabelled position with a known number of protons, and this requires only a proton spectrum of the product. Several issues need to be addressed when using this procedure: the substrate must be chemically and radiochemically pure; the residual protons are assumed to have the same T_1 as the integral reference protons; the proton spectra must be sufficiently well resolved to allow integration of the sites of interest; and the S.A. of a substrate containing one tritium atom per molecule is assumed to be 28.72 Ci/mmole. In general, we consider that a conservative (5-10%) error in NMR estimations of the S.A. should be assumed.

In the present example a different approach was taken, utilizing both the tritium and proton spectra. The same care with assumptions should be applied as described in the first NMR method for S.A. assessment. In compounds where multiple tritium atoms may be incorporated on one carbon, the resonances of the isotopomers are resolved because of the primary isotope effect on the chemical shift. This is true for both proton and tritium spectra (Figures 3 and 4 respectively), and comparison of the two reveals the presence of the three possible species: R-CT$_2$, R-CTH, R-CH$_2$. Note that the R-CTH isotopomer has both tritium and proton spectra. This occurrence allows us to assess the mole fractions of the three species in the mixture, and thereby calculate a specific radioactivity for the product. Note also that the intensity (or integral) of each tritium signal is proportional to the number of tritium atoms observed, and not to the mole fraction of the isotopomer (*i.e.* 0.5 x R-CT$_2$ integral should be compared with R-CTH). Once

the mole fractions of the three isotopomers (M_{TT}, M_{TH} and M_{HH}) are known, the S.A. is readily calculated:

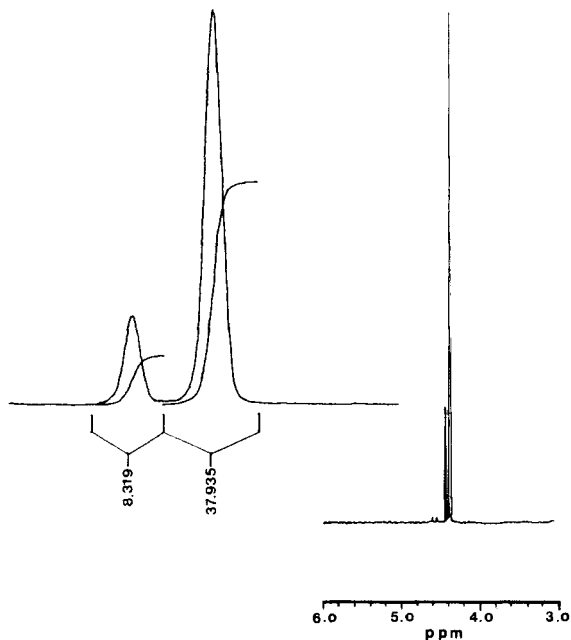$$e.g. \quad S.A. = (57.44 \times M_{TT}) + (28.72 \times M_{TH}) + (0 \times M_{HH}).$$

Figure 4. Proton-coupled tritium spectrum of [³H]pinoline; the larger peak represents R-CT₂ while the smaller partially represents R-CTH, the other half of the doublet is occluded by the larger peak.

In our example, integration of the proton-decoupled tritium spectrum (Fig. 5) easily yields the comparative proportions of the R-CT₂ and R-CTH species. In the proton spectrum there is overlap of the R-CH₂ singlet and one line of the R-CTH doublet, and this requires calculation of the R-CH₂ peak area (or accumulation of a tritium-decoupled proton spectrum). Combining these pieces of information gives $M_{TT} = 0.37$, $M_{TH} = 0.51$, and $M_{HH} = 0.12$. This yields a calculated S.A. of 35.9±1.8 Ci/mmole for the product.
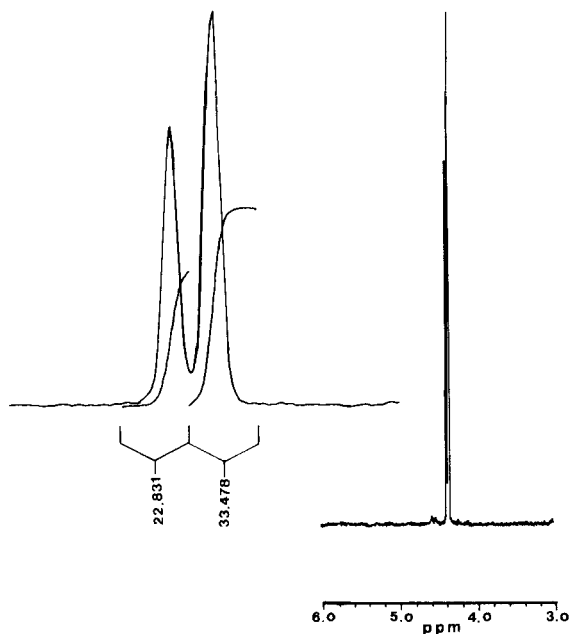
Figure 5. Proton-decoupled tritium spectrum; selectively decoupled at the signal resulting from the proton on C-1.

RESULTS AND DISCUSSION

[³H]pinoline has been synthesized with high specific activity by reducing the hydrochloride salt of 6-MeO-DHßC with tritium in the presence of a catalyst. The tritium form of this endogenous compound was prepared as a chemical probe for binding sites associated with the serotonergic system. Pinoline has been shown to displace specific ligands of the 5-HT transporter complex in nM concentrations (3). We hope to learn more about its endogenous role and putative binding site(s) by using [³H]pinoline as a radioligand. In addition, with the development of a method for producing stable hydrochloride salts of DHßCs which are unsubstituted at C-1, we can expand our investigations into this new group of ßCs.

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