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SYNTHESIS OF DEUTERIUM AND TRITIUM LABELLED m-AMINOLEVAMISOLE AND LEVAMISOLE

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SUMMARY

Levamisole (LEV) is a widely used anti-parasitic agent. In order to characterise the biochemical pharmacology of LEV in parasitic nematodes, [^{3}H]LEV and a more active analogue [^{3}H]*m*-aminolevamisole (MAL) have been prepared. Labelling was accomplished by tritiated water exchange of MAL under acid conditions. Multiple site labelling was achieved in the positions *ortho* and *para* to the amino group of MAL. Tritiation of MAL HCl in $[^3H]_2O$ was achieved at 103°C for 23 h. Crude [^{3}H]MAL was diazotised (hydrochloric acid and sodium nitrite) and deaminated (hypophosphorous acid) to effect the synthesis of [^{3}H]LEV. Products of both reactions were purified by preparative h.p.l.c. and characterised by h.p.l.c., t.l.c. and mass spectrometry. (Radiochemical yield was about 15% and purity >90%.) Specific activities of 39 Ci/mmol for [^{3}H]MAL and 37 Ci/mmol for [^{3}H]LEV were obtained.

Key Words: [^{3}H]meta-aminolevamisole, [^{3}H]levamisole, anthelmintic resistance, parasites

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INTRODUCTION

Levamisole (L[-]2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b] thiazole) (LEV; 4, Fig. 1) is an anthelmintic agent widely used in domestic animals (1). Interest in the mechanism of action of LEV has been stimulated by the emergence of LEV resistant strains of trichostrongylid nematode parasites of sheep. A more active analogue of LEV, *m*-aminolevamisole (MAL; 1, Fig. 1) has been tritiated and successfully used as a ligand in the characterisation of the LEV receptor site in the free-living nematode *Caenorhabditis elegans* (2,3). In this study [^{3}H]MAL (specific activity of 15-29 Ci/mmol) was prepared by catalytic (palladium/ carbon) deiodination under tritium gas (2). While useful in *C. elegans*, this isotope was of insufficient activity for detection of receptors in parasitic nematodes (N.C.Sangster, unpublished results). Tritium labelling of LEV has been subsequently achieved by debromination of a synthetic precursor and subsequent closure of two rings, however, this procedure yielded a lower specific activity (10 Ci/mmol)(4).

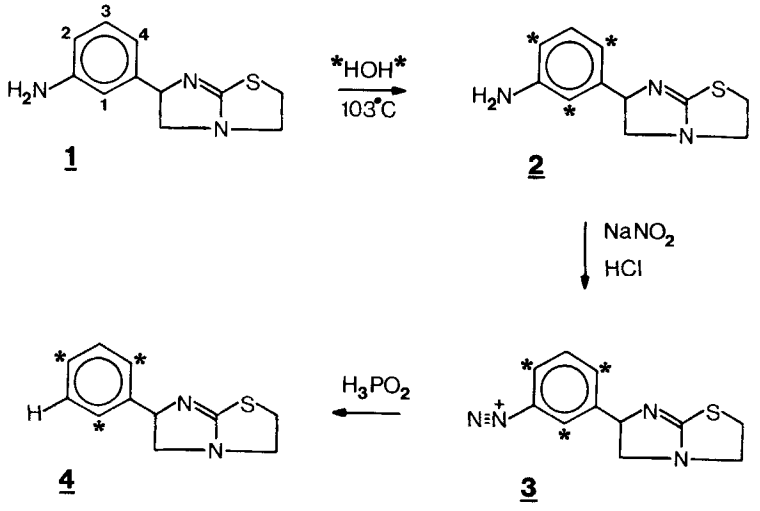

Figure 1 Synthetic route of [^{3}H]m-aminolevamisole (2) and [^{3}H]levamisole (4).
* denotes an isotope of H.

In order to develop radioisotope binding techniques for use with the receptors of parasitic nematodes we report the synthesis of [^{3}H]MAL and [^{3}H]LEV by tritium exchange labelling of MAL under acidic conditions with subsequent diazotisation and deamination to yield [^{3}H]LEV. Optimisation of the labelling procedure and determination of the position of label incorporation were accomplished by the use of deuterium labelled analogues.

EXPERIMENTAL

Chemicals and Analytical Techniques

Unlabelled MAL was a gift of American Cyanamid (Princeton, NJ), LEV and BrLEV were from Sigma (St Louis, MO), [^{2}H]$_2$O from Pierce (Rockford,IL) and [^{3}H]$_2$O (2700 Ci/ml) from Amersham (Sydney, Australia). Other reagents were AR or h.p.l.c. grade. MAL and LEV were detected on Merck (Darmstadt, FRG) 5554 fluorescent t.l.c. plates developed in dichloromethane:methanol (50:10) and in addition, MAL was detected with iodine vapour on Gelman (Ann Arbor, MI) ITLC SG plates developed in dichloromethane:methanol (100:7) (2). For determination of radioactive purity 10, 1cm squares of t.l.c. plate were cut out and placed in scintillant (Omnifluor, Canberra Packard, Melbourne, Australia) and counted. The proportion of radioactivity which coeluted with unlabelled LEV or MAL was calculated as a percentage of the total counts recovered from the 10 squares. H.p.l.c. was performed with a Waters (Millipore/Waters, Sydney, Australia) 6UK injector, Spectraphysics (San Jose, CA) pump (SP8770) and a Waters 440 detector at 254nm. The mobile phase was 35% 0.01 M ammonium acetate buffer (pH 7.2) in methanol. Analytical runs, which were used to quantify the products, were performed on a Waters RCM Novapak C_{18} column (5 μm) with solvent delivered at 1 ml/min. Retention times for MAL and LEV were 115 and 175 s, respectively. Preparative runs were on an H.p.l.c. Technologies (Macclesfield, UK) Techsil 5 μm C_{18}6D column with the mobile phase delivered at 5 ml/min. MAL and LEV were extracted with dichloromethane from sodium bicarbonate-saturated solutions applied to a Chemelut column (Analytichem International, Harbor City, CA).

Mass spectra were obtained on a Finnigan/mat (San Jose, CA) 3200 GCMS (Quadrupole). Chemical ionisation was achieved using methane gas. N.m.r. spectra were performed

on a JEOL (Tokyo, Japan) FX90Q fourier transform n.m.r. spectrometer using tetramethylsilane as reference (δ = 0.00 ppm) in deuterated carbon tetrachloride.

Deuteration

a) $[^2H]_2O$ exchange

One mg of LEV HCl or MAL HCl was incubated with 50 μl $[^2H]_2O$ at 90°C for 4 h in a similar fashion to Hsi and Skaletzky (5). Conditions were optimised for temperature and time (Fig 2) and the extent of deuteration was determined by mass spectral analysis. The proportion of each deuterated species, was calculated by subtraction of the [^{13}C] and [^{34}S] contributions from the ion abundances of the D_0 to D_3 parent ions. The [^{1}H]n.m.r. spectrum of the 32 h sample (% deuteration shown in Fig. 2A) was recorded and compared with a spectrum for unlabelled MAL.

b) Deamination

Deamination was performed with unlabelled MAL and [^{2}H]MAL. Solutions of MAL (1 mg) in 27 μl of hydrochloric acid (1 M) and 0.33 mg sodium nitrite in 27 μl H_2O were held at 4°C and mixed to form the diazotised intermediate (3). After 30 min., 55 μl of hypophosphorous acid (50%) was added and the mixture allowed to warm to room temperature. After 16 h, the pH was adjusted to 10 with aqueous sodium hydroxide (I M) and the product extracted into chloroform on a Chemelut column. Only LEV was detected on t.l.c.

Tritiation of MAL

a) $[^3H]_2O$ exchange

MAL HCl (0.67 mg) in 250 μl of methanol was added to a thick walled glass tube and evaporated to dryness at room temperature under a stream of nitrogen. $[^3H]_2O$ (2 μl, 5 Ci) was transfered under vacuum into the tube, which was then sealed and heated at 103°C for 23 h. On cooling, the reaction was neutralised with saturated sodium bicarbonate (20 μl) and diluted with water (0.5 ml). The contents were transfered onto a Chemelut tube with two further 0.5 ml washes of water, were eluted with dichloromethane and evaporated to dryness under nitrogen. The residue was redissolved in 10 ml of methanol and dried under nitrogen to remove

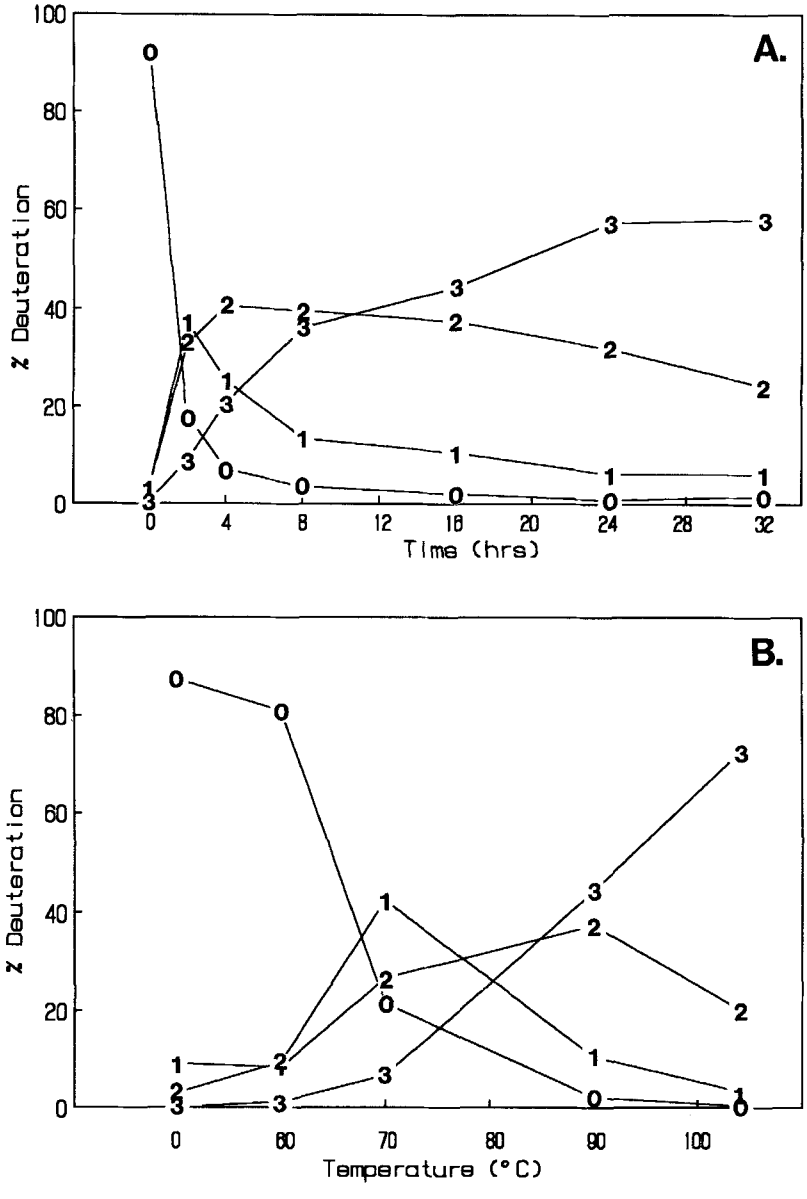

Figure 2 Optimisation of deuteration of *m*-aminolevamisole. Two mg of MAL was dissolved in 10 μl of $[^2H]_2O$ and incubated either at 90°C for varying times (A) or for 16 hrs at varying temperatures (B). 0, 1, 2 and 3 represent the abundances (corrected for carbon and sulphur, see text) of MAL species D_0, D_1, D_2 and D_3 labelled under each set of conditions, respectively.

exchangeable tritium to give crude [^{3}H]MAL (2). The crude [^{3}H] MAL was redissolved in methanol (4 ml) and divided into two 2 ml fractions for deamination to [^{3}H]LEV and [^{3}H]MAL purification, respectively.

b) Deamination

Crude [^{3}H]MAL in methanol (2 ml) was evaporated to dryness under N_2 and hydrochloric acid (8μl, 1 M) was added followed by an 8 μl aliquot of aqueous sodium nitrite (1 mg in 81 μl). All reactants were kept at 4°C. After 90 min, when diazotisation was complete hypophosphorous acid (14 μl) was added and the sample left at room temperature overnight. The product was neutralised with aqueous sodium hydroxide (2 M, 150 μl) and eluted on a Chemelut column to give crude [^{3}H]LEV, (4).

Purification

Both crude [^{3}H]MAL and [^{3}H]LEV were found by t.l.c. to be about 50% radiochemically pure. The remaining crude [^{3}H]MAL was injected into the preparative h.p.l.c. system and the peaks at 10, 14.5 and 19 min collected. By reinjection of each fraction under analytical conditions and by mass spectrometry the fraction eluted at 19 min was identified as [^{3}H]MAL. Purification of crude [^{3}H]LEV (4) also gave 4 fractions eluting at 18, 24, 27 and 30 min. Under analytical conditions and mass spectrometry [^{3}H]LEV was found to comprise the 27 min fraction. The appropriate preparative fractions containing [^{3}H]MAL and [^{3}H]LEV were evaporated to dryness under N_2 and redissolved in 5 ml of methanol. Forty μl samples of these solutions were injected into the analytical h.p.l.c. system and quantified by comparison with unlabelled standards. Purity and specific activity were determined by collection of 0.5 min fractions from the analytical h.p.l.c.

RESULTS AND DISCUSSION

Prior to this study a number of methods of deuteration and tritiation were examined preliminary to this study. Attempted deuterium exchange by aluminium trichloride/$[^2H]_2O$ (6) and trifluoroacetic anhydride/$[^2H]_2O$ (7) were unsuccessful (unpublished results). Debromination of BrLEV by palladium/calcium carbonate

catalysis under tritium gas was also unsucccessful. In all cases no starting material or anticipated labelled products were observed by either t.l.c., h.p.l.c. or mass spectrometry. This observation is consistent with the results of Thijssen et al (4). Interestingly the deiodination of iodoMAL (2) suggests that, in contrast to debromination, the removal of iodine can be accomplished without complete degradation of the imidazothiazole system.

Of the techniques examined, only acid exchange of MAL in $[^2H]_2O$ at elevated temperatures gave deuterated MAL without decomposition. The level of deuterium incorporation was both time and temperature dependent. Mass spectral analysis of MAL at various times (up to 32 h) at 90°C showed the loss of the unlabelled MAL (D_o) molecular ion (M+1) at m/e 220 within 4 hrs and concomitant increase in the D_1 species up to 2–3 h followed by the D_2 species. The proportion of D_3 increases up to 16 h to plateau at 24 h. At 24 h the proportion of deuterium incorporation was estimated at 6% D_1, 32% D_2 and 57% D_3 (Fig 2A). The temperature dependence of deuterium incorporation at 16 h is presented in Fig 2B. At 60°C low proportions of D_1 and D_2 MAL species were observed. These two species reach maxima at 70 and 90°C, respectively. The proportion of D_3 MAL increases linearly from 70 to 103°C to finally account for 73% of the MAL species present.

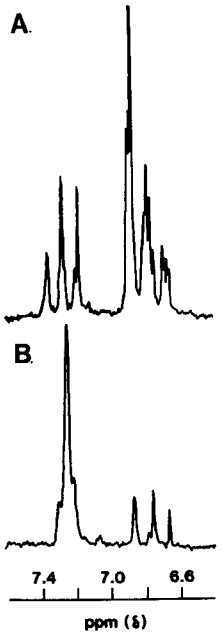

Figure 3 Aromatic region of [^1H]n.m.r. spectrum of MAL(A) and deuterated MAL (B).

The aromatic region (δ6.5– 7.5) of the n.m.r. spectra (Fig. 3A) of MAL consists of a high field multiplet (δ6.64– 6.96) comprising the three protons *ortho* and *para* to the amino group (positions 1,2 and 4 in Fig. 1) and a downfield single

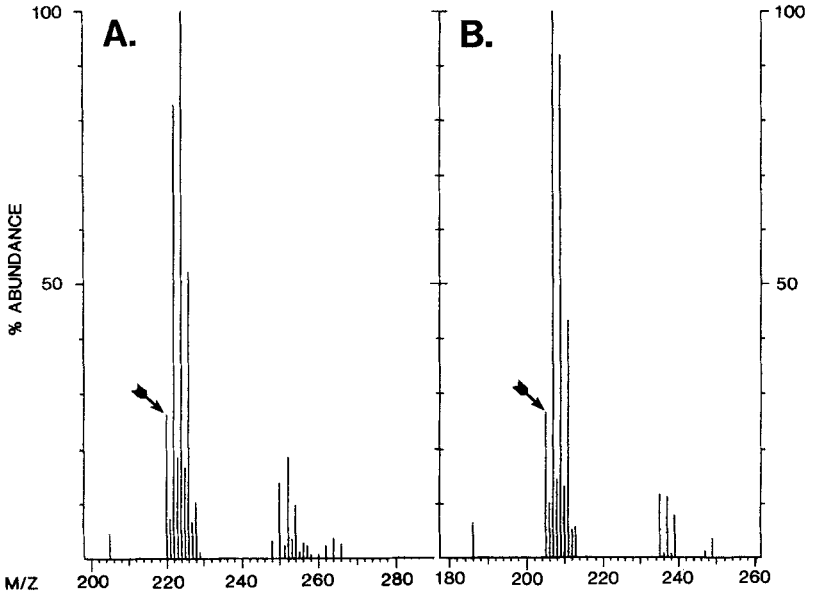

Figure 4 Mass spectra for [^{3}H]MAL (A) and [^{3}H]LEV (B). The parent ions of the species are indicated by arrows. Abundances are normalised to 100% for the most abundant species. Higher ions at 248 to 263 (A) and 235 to 249 (B) correspond to the expected M+29 and M+41 adduct ions observed with methane ionisation.

proton appearing as a multiplet at δ7.24 (position 3, Fig. 1). On deuteration (32 h, 90°C) the high field resonances centred on δ6.78 collapse to approximately 0.3 of a proton (Fig.3B), referenced to the triplet of the benzylic proton δ5.50 (which is not shown). The downfield resonance has collapsed to a broad singlet. On deuteration it appears that protons in positions 1, 2 and 4 have exchanged.

Tritiation of MAL was carried out at 103°C for 23 h in the presence of [^{3}H]$_2$O (5 Ci). After Chemelut solid phase extraction and evaporation the crude product was purified by preparative h.p.l.c. to yield [^{3}H]MAL (2). Ninety percent of the radioactivity comigrated with MAL on the two t.l.c. systems and on analytical h.p.l.c. Total yield of [^{3}H]MAL corresponded to about 72 μg or 14.4% of the starting material. (Note, half of the crude MAL was used for deamination.) Figure

4A shows the molecular ion region of the mass spectrum of [^{3}H]MAL. Analogous to the deuteration studies, replacement of up to three hydrogens of MAL by tritium had taken place. The specific activity calculated from the abundance of 1, 2 and 3 tritium labelled species was 39 Ci/mmol which compares favourably with the value of 42 Ci/mmol calculated from h.p.l.c. and dpm values.

Deamination of [^{3}H]MAL yielded [^{3}H]LEV (4) which after purification by preparative h.p.l.c. was 90% pure on t.l.c. Its mass spectrum (Fig. 4B) shows labelling corresponding to 37 Ci/mmol. This small decline in activity between [^{3}H]MAL and [^{3}H]LEV is seen most clearly as a decline in the abundance of more highly labelled species in Fig. 4. Some loss of tritium by self radiolysis of 3 or 4 may have occurred during the overnight reaction. Significantly, there was no loss of deuterium on deamination of [^{2}H]MAL, as determined by mass spectral analysis.

Under the same conditions the extent of labelling with tritium was lower than with deuterium. This could be due to a less efficient reaction but more probably accelerated degradation of the tritiated products. The low recovery of about 14% is consistent with radiolysis during the tritiation reaction. In addition, the reaction product was stored dried on glass at -20°C for 7 days before purification and quantitation. About half of the product may have degraded in this time (J.A.Lewis pers comm.) so that the initial yield from the labelling reaction may have been closer to 30%. Although this method of labelling lacks the specificity of dehalogenation if offers distinct advantages over the previous methods. 1) The reactions are simple and easy to perform. 2) The introduction of multiple tritium atoms yields a higher specific activity which is essential in the measurement of binding to the LEV receptor of parasitic nematodes. 3) The reactions provide both [^{3}H]MAL and [^{3}H]LEV; the latter may be a more stable ligand. 4) The procedure avoids the use of complex radioactive mixtures and handling of isotope through long and complex syntheses as proposed by Thijssen et al (4).

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REFERENCES

1. Janssen, P.A.J. - Prog. Drug Res. 20:347 (1976)
2. Lewis, J.A. and Paterson, I. - J. Labelled Compd. Radiopharm. 21:945 (1984)
3. Lewis, J.A., Fleming, J.T., McLafferty, S., Murphy, H. and Wu, C. - Mol. Pharmacol. 31:185 (1987)
4. Thijssen, J.B.A., Knaeps, A.G., Verluyten, W.L.M., Heykants, J.J.P. and Janssen, C.G.M. - J. Labelled Compd. Radiopharm. 24:779 (1987)
5. Hsi, R.S.P. and Skaletzky, L.L. - J. Labelled Compd. Radiopharm. 17:331 (1980)
6. Timmons, M.L., Pitt, C.G. and Wall, M.E. - Tetrahedron Letters 36:3129 (1969)
7. Vine, J.H., Dawson, M., McGee C.M., Lacey, E., Brooks, P.M. and Watson, T.R. - J. Chromatogr. (Biomed. Appl.) 420:129 (1987).