Preparation of [22,23-$^3H_2$]-Dihydroavermectin B_1a of high specific activity

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SUMMARY

[22,23-$^3H_2$]-Dihydroavermectin B_1a of 2.13 TBq/mmol (57.7 Ci/mmol) specific activity was derived from avermectin B_1a by selective tritiation using Wilkinson's homogeneous catalyst. Data on binding affinity and nematotoxic activity of the title compound are reported.

Key words: [22,23-$^3H_2$]-Dihydroavermectin B_1a, [^3H]-Ivermectin, Wilkinson's catalyst, binding affinity, nematotoxic activity

INTRODUCTION

Avermectins a new family of natural products elaborated by Streptomyces avermectilis exhibit potent and specific activity against helminths (1-4), arachnids (5) and insects (6,7) apparently by interacting with γ-aminobutyric acid (GABA) mediated neurotransmission (8-11) through modulation of GABA/Benzodiazepine receptor binding (12,13). The

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Further study of the mode of action of these complex macrocyclic lactones required radiolabelled materials of high specific activity, and we wish to report the preparation of [22,23-$^{3}H_2$]-Dihydroavrmectin B_1a ([^{3}H]-Ivermectin) via selective tritiation of olefinic linkage between carbons 22 and 23 of avermectin B_1a (2aE,4E,5'S,6S,6'R,7S,8E,11R,13R,15S, 17aR,20R,20aR,20bS)6' [(R)-sec-butyl]-7-[[2,6-dideoxy-4-O-(2,6-dideoxy-3-O-methyl-α-L-arabino-hexopyranosyl)-3-O-methyl-α-L-arabino-hexopyranosyl] oxy]-5',6,6',7,10,11,14,15,17a,20,20a,20b-dodecahydro-20,20b-dihydroxy-5',6,8,19-tetramethyl-spiro-[11,15-methano-2H,13H,17H-furo-[4,3,2-pq] [2,6] benzodioxyacyclo-octadecin-13,2'-[2H]pyran]-17-one)),see Figure 1.

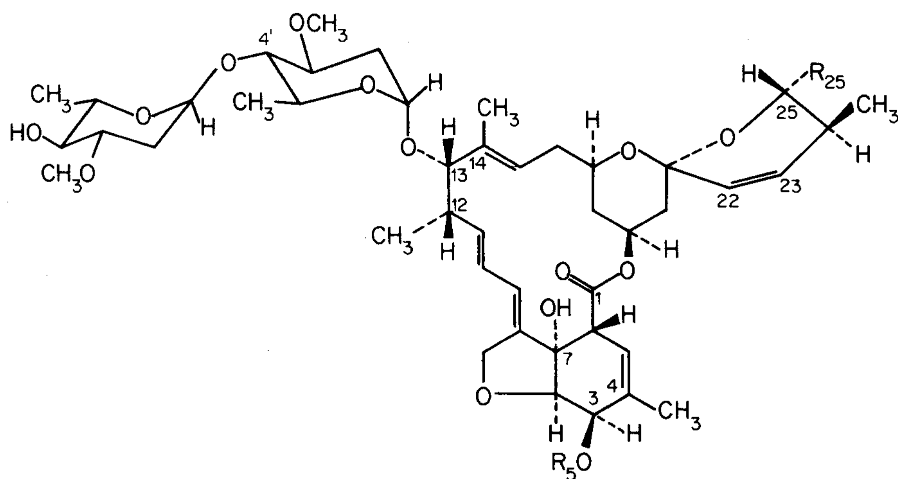

Figure 1. Avermectin B_1a, R_5=H, R_{25}=CH(CH_3)C_2H_5

RESULTS AND DISCUSSION

The desired compound is the [^{3}H]-Ivermectin and its preparation required the selective reduction of the olefinic linkage between carbon 22 and 23 as it causes only subtle changes in bioactivity (14). As avermectin B_1a containing the target olefin in cis substituted form was reduced selectively using Wilkinson's homogeneous hydrogenation catalyst (14) [^{3}H]-Ivermectin was derived from avermectin B_1a. Tritiation of avermectin B_1a for 19 hours using the catalyst in benzene at 25° C under 0.87 atm tritium provided [^{3}H]-Ivermectin in 80 % yield, together with 10 % of unreacted avermectin B_1a and some other impurities. Further purification by HPLC gave the final product (0.37 µmol) with 800 MBq radiactivity (2.13 TBq /mmol specific activity). The material can be stored in ethanolic solution (37 MBq /ml) at -70°C without considerable decomposition.

Binding of [^{3}H]-Ivermectin to synaptic membrane sites was tested using rat brain cortical synaptosomes. Data were analysed in terms of Scatchard analysis and gave K_d=5.7 nM and B_{max} of 275 pmol/mg protein. The K_d value is within the same order of magnitude with the value published for [^{3}H]-Avermectin B_1a binding to synaptic membranes (12), while the number of binding sites on synaptosomes are significantly enhanced (c.f. 7 pmol/mg protein, 12). Therefore the title compound is suitable for the further mechanistic studies.

Nematotoxic activity of [^{3}H]-Ivermectin was tested in in vitro laboratory assays using dauerlarvae (15) and synchronised first stage larvae of the free living nematode Caenorhabditis briggsae (G-16 strain, see ref. 16) as well as the infective larvae of the entomophylic nematode Neoaplectana carpocapsae. We conclude that the tritiated ivermectin was at least as active as the non-tritiated one. Interestingly, neither ivermectin nor [^{3}H]-Ivermectin inhibited the nematode eggs from hatching, the newly born larvae, however, became paralized and died in concentrations over 0.1 µg/ml.

EXPERIMENTAL SECTION

Chemistry.A solution of avermectin B_1a (1.1 mg) and Wilkinson's catalyst (Merck,3.3 mg) in abs. benzene (1 ml) was stirred at 25°C under 0.87 atm $^3H_2$ gas (stored as uranium tritide,Technabexport, USSR) for 19 hours in a special glass vacuum-apparatus. After evaporation of benzene, additional solution-evaporation cycles were introduced (3 times) with 1:1 v/v methanol-water solution to remove the labile tritium. The product was checked by TLC (precoated silicagel plate,Merck) using 19:1 v/v chloroform-tetrahydrofuran solvent system. Two distinct radioactive spots, corresponding to the target material and the catalyst were observed (Radiochromatogram Scanner, Packard 7201). $[^3H]$-Ivermectin eluted from the plate by methanol had a radioactivity of 1.34 GBq (Liquid Scintillation Spectrometer, Searle Delta 500). The purity of the product was checked by HPLC (Waters Association Preparative LC System 500) and found that the crude product contained 80 % $[22,23-^3H_2]$-Dihydroavermectin B_1a, 10 % avermectin B_1a and some other impurities. The crude product was purified by HPLC using a Servachrom Silica Si10 polyol RP_{18}column (4.6 mm i.d. x 30 cm) eluted with 88.5:11.5 v/v methanol-water solution. The radioactivity of the final product was found to be 800 MBq (21.6 mCi) and corresponded to 0.37 µmoles as determined from its absorbance at 245 nM (UV-Vis Zeiss Spectrophotometer).

$[^3H]$-Ivermectin binding to synaptosomes. Synaptosomes prepared according to ref. (17) were suspended in bicarbonate-buffered physiological salt solution (Tyrode) at 4 mg protein/ml. 250 µl aliquot was incubated in the presence or absence of 3 µM ivermectin and varying concentrations of $[^3H]$-Ivermectin in tubes made from borosilicate glass at 22°C. After 30 min of incubation the samples were filtered (Whatman GF/B filters, binding of $[^3H]$-Ivermectin to filter was about 5-10 %) and washed with 4 x 2.5 ml of the above solution. The radioactivity on the filters was determined by standard liquid scintillation counting.

Nematotoxic activity. 10-25 dauerlarvae and 50-100 synchronized first stage (L1) larvae of the free living nematode Caenorhabditis briggsae (G-16 strain) were transferred to microtiter wells containing 50 µl of liquid worm medium and the respective $[^3H]$-Ivermectin concentrations.Half of the samples contained commercially available (Gibco) Caenorhabditis briggsae Maintenance medium supplemented with cholesterol of 50 µg/ml and myoglobin solution of 500 µg/ml in final concentration (18,19). The other half of the samples contained conventional S media (20) and 0.05 w/v E.coli OP 50 bacteria in final concentration. The applied doses of $[^3H]$-Ivermectin were 0, 0.05, 0.1, 0.2, 0.5 and 1 µg/ml.The compound was diluted in ethanol and then with 500 µg/ml myoglobin solution (in phosphate buffer, pH 6), so the final alcohol concentration was 1 %, which did not cause any

adverse effects on the worms in our experiments.The microtiter plate was checked 1, 3 and 7 days thereafter.It was found, that all the applied doses caused paralysis and later death for the worms, and the symptoms were the same as had been found earlier (11), while the animals in both media containing no [^{3}H]-Ivermectin but 1% of ethanol grow normally to fertile healthy adults. In concentrations below 0.2 μg/ml some of the dauers which did not moult to fourth stage (L4) larvae remained alive for a few days, but were paralized or slowly moving animals. The first stage larvae became paralized within 2 hours in each doses, but some of them managed to grow to L2 or L3 stages in concentrations below 0.2 μg/ml before died.After a week the effect proved irreversible in each doses containing 0.2 μg/ml [^{3}H]-Ivermectin or more, while some animal had been spent 7 days in 0.05 μg/ml dose recovered on normal agar (NGM) plate and were able to propagate.Infective larvae of the entomophylic nematode *Neoaplectana carpocapsae* were also treated and found that [^{3}H]-Ivermectin doses over 0.1 μg/ml paralized and killed them.

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