Preparation of 2-[^{125}I] Iodohistamine-Labelled Δ^8-Tetrahydrocannabinol-11-oic Acid for use in Cannabinoid Radioimmunoassay

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SUMMARY

A simple method is described for the preparation of 2-[^{125}I]iodohistamine-labelled Δ^8-tetrahydrocannabinol-11-oic acid with high specific activity for use in radioimmunoassay. This compound is produced in high yield and shows excellent radiochemical stability when stored at 4^{o}C. The radiolabelled cannabinoid has been shown to bind avidly to four different broadly specific cannabinoid antisera. It is the only [^{125}I] labelled cannabinoid suitable for use in radioimmunoassay with such antisera.

KEYWORDS: CANNABINOID RADIOIMMUNOASSAY RADIOTRACER IODOHISTAMINE

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INTRODUCTION

Although much effort has been directed towards the production of antisera for cannabinoid radioimmunoassay (RIA) (1-10) little success has been achieved in the production of suitable radiolabelled tracers. The only commercially available radiolabelled cannabinoid in the UK, suitable for RIA is the generally tritiated cannabinoid; Δ^9-[G-^{3}H]tetrahydrocannabinol (THC). This compound has relatively low specific activity (ca. 200GBq/mmol) which imposes limitation on its usefulness in RIA.

Pitt and his coworkers(11) have reported the preparation of a tritiated tracer, Δ^8-[4',5'-$^3H_2$]THC with high specific activity and more recently(12) an iodinated tracer 5'-[^{125}I] iodo-Δ^8-THC. The latter compound, had relatively low specific activity (3.7TBq/mmol) compared with other [^{125}I]tracers, required a lengthy preparation involving nucleophilic exchange and was prepared from a non-readily available cannabinoid derivative; 5'-iodo-Δ^8-THC.

The iodination method described here employs a more versatile approach to radiolabelling using the "iodo tag" 2-[^{125}I]iodohistamine, which is easily linked to the cannabinoid. This technique has the advantages that the labile cannabinoid is not subjected to harsh reaction conditions, high specific activities can be obtained (up to 81.4TBq/mmol) and the preparation of the radio-tracer is relatively quick and simple to perform. The starting compound, Δ^8-THC-11-oic acid (I, Figure 1), is also readily available.

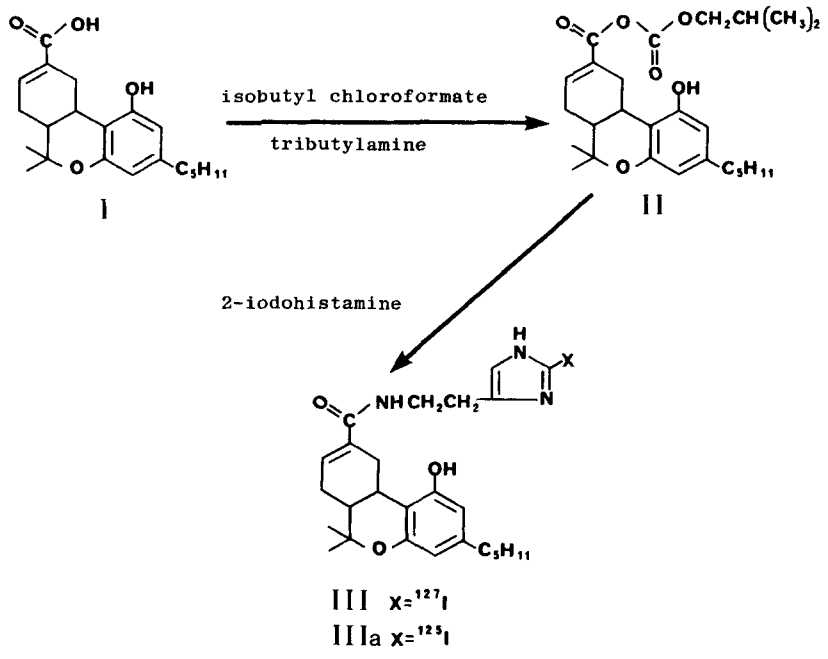


Figure 1 Structure of Δ^8-THC-11-oic acid (I), and synthesis of its isobutyric anhydride (II) and 2-iodohistamine derivatives (III, IIIa).

EXPERIMENTAL

Materials

Unless otherwise stated, all reagents and solvents were of Analar grade obtained from BDH Chemicals Ltd Poole, Dorset, isobutylchloroformate from the same supplier was laboratory grade. Histamine dihydrochloride, and Tris base were obtained from Sigma Chemical Company Ltd., Poole, Dorset. Tributylamine (97%) was obtained from Aldrich Chemical Company Ltd., Gillingham, Dorset. Sodium [^{125}I]iodide (614MBq/µg) was obtained from Amersham International plc , Amersham, Bucks. Cannabinoids

were generously provided by the National Institute on Drug Abuse, Rockville, Maryland, USA. Ether was redistilled prior to use, and dioxane was passed through a column of aluminium oxide (basic, Brockmann grade 1 from BDH) to remove peroxides.

Cannabinoid antisera were purchased from Guildhay Antisera University of Surrey, Guildford (lot no. S133Y/22/5), and from Syva UK Ltd., Maidenhead, as part of an Emit kit for cannabinoid detection in urine. Antisera were also obtained as gifts from D.A. Verghise of Miles Laboratories Ltd., Slough, Berks, and Sanda Dani of The Isler Clinical Toxicology and Pharmacology Unit, The Sheba Medical Centre, Tel Aviv, Israel.

Equipment

High-performance liquid chromatography (HPLC) was carried out using a Waters 6000A HPLC pump, injection being made via a Rheodyne 7120 injection valve fitted with a 20µl or 2ml sample loop.

Gamma counting was carried out in a Nuclear Enterprises 1600 counter which had an efficiency of 61% for [^{129}I]iodine. Radioactive spots on paper chromatograms were located by cutting the paper into sections and counting these in a gamma counter and also with a Mini Instruments type 540 scintillation meter (Alrad Instruments Ltd., Newbury, Berks). To improve resolution of detection the scintillation probe was fitted with a lead shield, 3mm thick, in the centre of which was a small window ca. 2mm x 4mm.

Low resolution mass spectrometry (MS) was carried out using a VG Micromass 16F mass spectrometer fitted with a

single stage jet separator. The following conditions were used: emission, 100μA, electron energy, 70eV, source temperature 200°C (unless otherwise stated). Data were collected using a VG 2250 data system with the spectrometer scanning at 3s per decade.

High resolution mass measurements were obtained using a Kratos MS50 mass spectrometer and DS50 data system. Measurements were made under dynamic conditions scanning at 10s per decade and were averaged over 10 spectra. The following conditions were used: emission, 200μA; electron energy, 70eV; source temperature, 200°C.

Chromatography

Analytical HPLC was carried out with a stainless steel column (100mm x 5mm) packed with Hypersil 5-ODS (Shandon Southern Products Ltd., Runcorn, Cheshire). The solvent systems were:

A, methanol/water, (82.5/17.5) adjusted to pH6.1 with phosphoric acid solution (1M)

B, methanol/water, (77.5/22.5) adjusted to pH6.8 with phosphoric acid solution (1M)

Preparative HPLC was carried out with a stainless steel column (250mm x 8mm), packed with 10μ RP18 (Whatman, Maidstone, Kent).

Column eluate was monitored with a Cecil Instruments 212 ultraviolet monitor at 275nm, or in the case of [^{125}I] compounds, fractions were collected for γ-counting.

Chromatography papers were obtained from Whatman. Cannabinoid derivatives were chromatographed using the Bush B3 paper chromatography (PC) system(13). Petroleum ether (b.p.80-100^{o}C), toluene, methanol and water were mixed in the ratio 3.3/1.7/4/1 to give a biphasic mixture. The mixture was used to saturate the chromatography tank with vapour, and the upper phase was used as developing solvent. Papers, No 4 for analytical work and 17M for preparative work were run in the descending mode, at 20^{o}C without pre-equilibration.

Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254}, 0.25mm (Merk). Location was effected by UV light 254nm, iodine vapour, fast blue B salt (FBB, Sigma), ca. 1% aqueous solution oversprayed with sodium hydroxide solution (0.5M) and ninhydrin solution (Merk).

Methods

2-Iodohistamine - Histamine was iodinated according to the procedure of Tanchou and Slaunwhite(14).

Histamine dihydrochloride (4g, 21.7mmol) was dissolved in water (5ml) and the pH was adjusted to 7.5 with sodium hydroxide solution (40%). Addition of iodine (0.267g, 2.17mmol) in ethanol (21ml) was carried out, dropwise, over 2hrs with constant stirring. After a further hour, the solvent was evaporated to dryness under vacuum. The residue was then dissolved in water (ca. 5ml), the solution saturated with ammonium sulphate and, after adjusting the pH to 10 with sodium hydroxide solution, extracted twice with chloroform/propan-2-ol (3/1, 25ml). The combined extracts

were concentrated under vacuum, and then applied as a band to 3MM paper which was chromatographed in butanol/acetic acid/water (120/30/50). The reaction mixture gave two bands (location with ninhydrin), the lower band matching histamine in Rf. The upper band was removed, eluted with methanol and re-chromatographed. The upper band was again eluted, and 2-iodohistamine (49mg) was obtained after evaporation. This was shown to be >99% pure by TLC, using ethanol/ammonia (25%), (80/20) and location by UV_{254}, iodine and ninhydrin. Low resolution MS gave a molecular ion at m/z 237.

Δ^8-THC-11-oic mixed anhydride (II) The carboxyl function of I was activated using a modified Erlanger procedure(15). I(8.2mg, 23.8µmol) was dissolved in peroxide-free dioxane (0.5ml) and to this was added tri-n-butylamine (5.8µl, 24.4µmol) followed by isobutylchloroformate (3.15µl, 24.0µmol). HPLC using solvent system A indicated complete conversion of I (k' = 3.4) to the mixed anhydride II (k' = 12.3) after 75min. This solution was stored at -18^{o}C and used without purification.

Δ^8-THC-11-oic acid-2-iodohistamide (III) To Δ^8-THC-11-oic acid mixed anhydride (II) (equivalent to 8.2mg of acid I, 23.8µmol) in dioxane (0.5ml) was added 2-iodohistamine (8.5mg, 35.9µmol) in dimethylformamide (0.5ml). Analysis by HPLC using solvent B indicated complete reaction of the mixed anhydride after 10min, with the formation of Δ^8-THC-11-oic acid-2-iodohistamide (III) and some regeneration of I.

The solvent was removed under vacuum to give a brown oil which was partitioned between diethyl ether (5ml) and phosphate

buffer (5ml, 0.025M, pH5.1). The aqueous phase was washed twice with ether (5ml) and the combined organic extracts evaporated under vacuum (<50°C). The residue was dissolved in ether and applied as a band to 17M chromatography paper and run in the Bush B3 PC system. The band at Rf 0.47 was eluted with methanol. Final purification was carried out using preparative HPLC with methanol/water (80/20) as solvent. The methanolic extract was injected on to the column in 10 aliquots (ca. 0.2ml) and the peak corresponding to III was collected at k' = 6.51.

The HPLC solvent was removed under vacuum (<30°C) to give a white crystalline solid (2.35mg, 19% yield). Low resolution MS (source temperature 190°C) gave a molecular ion at m/z 563.

2-[^{125}I] Iodohistamine - The iodination procedure was a modification of that described by Hunter et al. (16). All reagents were freshly prepared. To histamine dihydrochloride (0.83µg, 4.5nmol) in phosphate buffer (10µl, 0.1M, pH8) was added sodium [^{125}I]iodide (37MBq, 0.45nmol) in sodium hydroxide solution (10µl, pH7-11) followed by chloramine T trihydrate (60µg, 0.213µmol) in phosphate buffer (10µl, 0.1M, pH8). The reactants were mixed by vortexing and after 2min the reaction was stopped by addition of sodium metabisulphite (200µg, 1.05mol) in borate buffer (20µl, 0.25M, pH8.6). The crude preparation was suitable for use without further purification.

Δ^8-THC-11-oic acid 2-[^{125}I]iodohistamide (IIIa) To the 2-[^{125}I]iodohistamine solution was added dioxane (40µl) followed by II (equivalent to 7µg of acid, 20.3nmol) in dioxane (10µl). The reaction mixture was maintained at room temperature for 60min then diluted with tris base solution (0.4ml, 0.25M) before it was extracted with ether (5ml) by vortexing for 90s. The ether extract was reduced in volume in a stream of nitrogen (oxygen-free) (<37^{o}C), in a well ventilated fume hood.

Assessment of reaction yield using a scintillation meter indicated that >70% of the initial [^{125}I]iodine had been extracted into the organic phase.

The residue was redissolved in ether (ca. 10µl) and transferred, as a single spot under a stream of nitrogen (oxygen free) to No 4 paper. This was chromatographed in the Bush B3 PC system until the solvent front had moved 30cm (about 75min). The radioactive spot at Rf 0.47 was rapidly located using the modified scintillation meter, cut from the paper, and the radiolabelled cannabinoid eluted with methanol (2ml); extraction efficiency >99%. Radiochemical purity was found to be ca. 90% by HPLC, using solvent system B.

RESULTS AND DISCUSSION

The method described here for the activation of Δ^8-THC-11-oic acid (I) to give the mixed anhydride II is relatively quick and simple. The mixed anhydride is produced in high yield and when stored in dioxane at -18^{o}C is relatively stable with little decomposition (<5%) over 6 months, as determined by HPLC (System A).

Compound II reacted with 2-[^{125}I]iodohistamine to give two major radioactive compounds. It proved extremely difficult to obtain satisfactory or reproducible separation of these compounds using TLC, however excellent separation of all the radioactive and non-radioactive compounds was obtained using the Bush B3 PC system (Table 1). The two radioactive compounds were identified by comparison with their non radioactive analogues as Δ^8-THC-11-oic acid-2-iodohistamide (IIIa) and isobutyl-2-(2-iodoimidazo)-4-yl ethylcarbamate (IV). The latter compound was identified by high resolution MS and gave a molecular ion at m/z 337.0278 ($C_{10}H_{16}O_2N_3I$ requires 337.0267).

TABLE 1

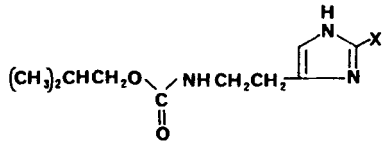
Rf of Compounds on Bush B3 System

Compound	Rf	Colour with FBB reagent
Δ^8-THC-11-oic acid mixed anhydride (II)	0.97	Pink
Δ^8-THC-11-oic acid (I)	0.66	Pink
Δ^8-THC-11-oic acid-2-iodohistamide (III and IIIa)	0.47	Pink
Isobutyl-2-(2-iodoimidazo)-4-yl ethylcarbamate (IV)	0.13	Mauve*
Δ^8-THC-11-oic acid histamide†	0.12	Pink
2-Iodohistamine	0	Red*
Histamine	0	Orange*
Na[^{125}I]	0	-

†This compound was prepared by the reaction of Δ^8-THC-11-oic acid mixed anhydride and histamine and was tentatively identified by its chromatographic properties and FBB colour reaction.

*Gave colour only after overspray with sodium hydroxide solution (0.5M).

This compound was formed through attack by-iodohistamine on the isobutoxy bearing carbonyl group of the mixed anhydride (II). This side reaction was also responsible for the regeneration of the starting compound I.



IV X=^{127}I, ^{125}I

Figure 2 isobutyl-2-(2-iodoimidazo)-4-yl ethylcarbamate

The method used initially for conjugation of 2-[^{125}I] iodohistamine to II was that of Hunter et al.(16), but the product ratio IIIa/IV was only ca. 0.4. This ratio was increased to >2 by reducing the pH of the conjugation medium to 4.2. Activation of 35µg of I was successful, but storage of the mixed anhydride II in bulk form was a more successful approach and was preferred as a routine procedure.

During the activation and conjugation steps, control of temperature (<10^{o}C) as originally described by Erlanger (15) and subsequently used by many other workers (e.g. 17,18) was found to be unnecessary.

It was noted that reducing the volume of dioxane in the conjugation reaction from 50µl to 10µl resulted in poor reaction efficiency and the formation of a large number of radioactive species.

The Bush B3 PC system proved extremely convenient for isolation of the separated IIIa, which could be eluted from the paper in >99% yield. This procedure was also preferable to purification by TLC since it avoided the potential risk of handling radioactive silica gel dust.

Decomposition of IIIa on PC (or TLC) media was reduced by spotting the chromatography paper (or plate) under a stream of nitrogen. Once extracted into methanol, however, the radiolabelled cannabinoid IIIa was found to be extremely stable, with no significant radioactive impurities being produced after storage at $4^{o}C$ for 3 months.

Tanchou and Slaunwhite(19) recently depreciated the mixed anhydride procedure(15) in favour of activation of carboxylic acids by N-hydroxysuccinimide ester formation when preparing radiotracers from 2-[^{125}I]iodohistamine. The mixed anhydride reaction as used here would appear to offer similar advantages to ester formation. Once prepared the mixed anhydride is stable and reacts to give a radiotracer in relatively high yield (about 50%). The preparation is simple and takes about 2.5hr including chromatographic purification and extraction.

The chromatographic purification results in a radiotracer of high specific activity which is avidly bound to cannabinoid antisera from a number of sources which have different specificities.

The iodinated cannabinoid derivative prepared by Pitt and his coworkers(12) was designed for use in an RIA specific

for THC. The structure of this derivative, namely an iodine atom at the 5'-position, would preclude its use with the more widely used, broad specificity cannabinoid antisera(3,9). The radio-tracer described here (IIIa) binds avidly to four such commercially available antisera, hence it will allow development of a broadly specific cannabinoid RIA based on commercially available reagents.

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REFERENCES

1. Grant, J.D., Gross, S.J., Lomax, P. and Wong, R.- Nature New Biol., 236:216 (1972).
2. Cais, M., Dani, S., Josephy, Y., Modiano, A., Gershon, H., and Mechoulam, R.-FEBS Lett., 55:257 (1975).
3. Teale, J.D., Forman, E.J., King, L.J., Piall, E.M., and Marks, V.J. - J. Pharm. Pharmac., 27:465 (1975).
4. Chase, A.R., Kelley, P.R., Taunton-Rigby, A., Jones, R.T., and Harwood, T.- Res. Monogr. Ser. Natl. Inst. Drug Abuse (USA), 7:1 (1976).
5. Cook, C.E., Howes, M.L., Amerson, E.W., Pitt, C.G., and Williams, D.- Res. Monogr. Ser. Natl. Inst. Drug Abuse (USA), 7:15 (1976).
6. Gross, S.J. and Soares, J.R.- Res. Monogr. Ser. Natl. Inst. Drug Abuse (USA), 7:10 (1976).

7. Rowley, G.L., Armstrong, T.A., Crowl, C.P., Eimstad, W.M., Hu, W.M., Kam, J.K., Rodgers, R., Ronald, R.C., Rubenstein, K.E., Sheldon, B.G., and Ullman, E.F. - Res. Monogr. Ser. Natl. Inst. Drug Abuse (USA), 7:28 (1976).

8. Soares, J.R., Gross, S.J. - Life Sci., 19:1711 (1976).

9. Rodgers, R., Crowl, C.P., Eimstad, W.M., Hu, M.W., Kam, J.K., Ronald, R.C., Rowley, G.L., and Ullman, E.F.- Clin. Chem., 24:95 (1978).

10. Tsui, P.T., Kelly, K.A., Ponpipom, M.M., Strahilevitz, M., and Sehon, A.H. - Can. J. Biochem., 52:252 (1974).

11. Pitt, C.G., Hobbs, D.T., Schran, H., Twine, C.E., Jr. and Williams, D.L. - J. Label Compds. 11:551 (1975).

12. Pitt, C.G., Seltzman, H.H., Setzer, S.R., and Williams, D.L. - J. Label. Compds., 17:681 (1980).

13. Bush, I.E.-Biochem. J. 50:370 (1952).

14. Tanchou, J.K., and Slaunwhite, W.R., Jr. - Prep. Biochem., 9:379 (1979).

15. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S. - J. Biol. Chem., 228:713 (1957).

16. Hunter, W.M., Nars, P.W. and Rutherford, F.J., 1975, in 5th Tenovus Workshop, Steroid Immunoassays (Eds. Cameron, E.H.D. and Griffiths, K.) pp 141. Alpha-Omega Press, Caerphilly, Wales.

17. Cook, C.E., Tallent, C.R., and Christensen, H.D. - Life Sci., 14:1075 (1974).

18. Stanczyk, F.Z. and Goebelsmann, U. - J. Steroid Biochem., 14:53 (1980).

19. Tanchou, J.K. and Slaunwhite, W.R., Jr. - J. Immunoassay, 1:129 (1980).