Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **XLI**, 279-285 (1998)

SYNTHESIS OF [^{3}H]-ILLUDIN S, [^{3}H]-ACYLFULVENE, [^{3}H]&[^{14}C]-HYDROXYMETHYLACYLFULVENE (MGI 114)

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Summary

Tritiated derivatives of the toxic sesquiterpene illudin S (**1**) have been prepared by fermentation of *Omphalotus illudens* in the presence of [^{3}H]-sodium acetate. [^{3}H]-illudin S was converted to antitumor [^{3}H]-acylfulvene (**4**) by treatment with dilute sulfuric acid. Antitumor [^{14}C]-hydroxymethylacylfulvene (**5**) was best prepared by reacting acylfulvene with [^{14}C]-paraformaldehyde in dilute sulfuric acid.

Key Words: illudin S, acylfulvene, hydroxymethylacylfulvene, antitumor agents

Introduction

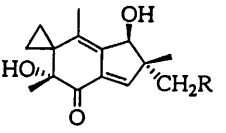
The sesquiterpene illudin S (**1**) is an extremely toxic substance found in the basidiomycete *Omphalotus illudens* (formerly *Clitocybe illudens*).[1] It is believed to be responsible for the poisoning that occurs when *Omphalotus* is mistaken for an edible mushroom.[2] The compound has antitumor activity but only a low therapeutic index when tested against a variety of rodent solid tumors and leukemias.[3] However, derivatives of illudins have been prepared which exhibit a greatly improved therapeutic index compared to the parent compounds illudin S and illudin M (**2**). They include dehydroilludin M (**3**),[4] acylfulvene (**4**),[5] and hydroxymethylacylfulvene (HMAF, also designated MGI 114, **5**).[6] HMAF in particular has been found to show outstanding activity against breast, lung, colon and skin cancer cell lines derived from human tumors.[7] The compound

CCC 0362–4803/98/040279–07$17.50

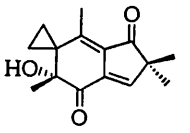
Received 3 October 1997
Revised 11 December 1997

is currently being evaluated in a human phase I clinical trial[8] and many phase II trials have been scheduled under the sponsorship of the National Cancer Institute.[9]

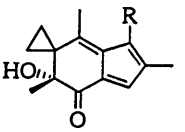
In order to investigate the mechanism of toxicity of these compounds and to carry out studies on pharmacokinetics and drug distribution, radiolabeled compounds were required. In this article we describe the preparation of [^{3}H]-illudin S, [^{3}H]-illudin M, [^{3}H]-acylfulvene, [^{3}H]-HMAF and [^{14}C]-HMAF.



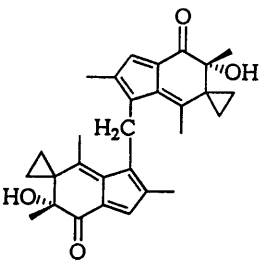
1. Illudin S, R = OH
2. Illudin M, R = H



3. Dehydroilludin M



4. Acylfulvene, R = H
5. HMAF, R = CH_2OH



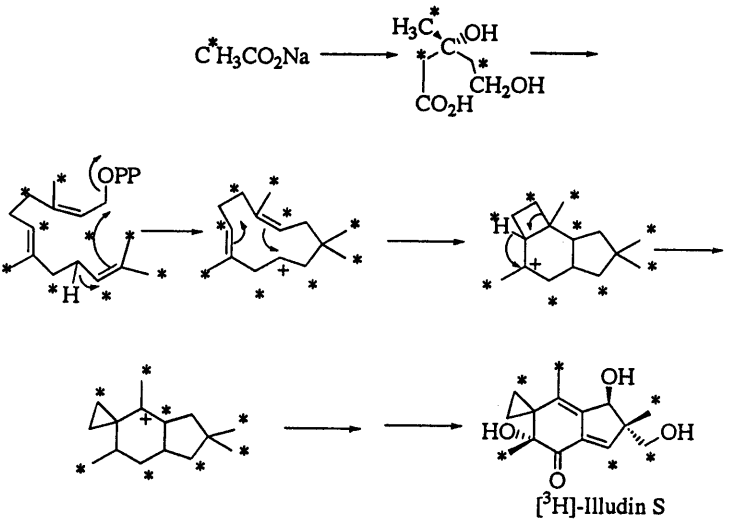
6. "Dimer"

Results and Discussion

[^{14}C]-Illudin S and [^{14}C]-illudin M were prepared many years ago by fermentation of cultures of *Omphalotus illudens* in the presence of mevalonic acid 2- ^{14}C.[10] Labeled acetate (1,2- $^{13}C_2$ and 2- $^2H_3$) has also been used as substrate to produce labeled illudins.[11] We required radiolabeled compound with specific radioactivity of more than 100 mCi/mmol. Therefore we investigated the use of [2- $^3H_3$]- acetate as substrate. According to the established biosynthetic pathway, illudins are expected to contain tritium labels as indicated in Scheme 1.

In the first experiment [^{3}H]-CH_3COONa dissolved in 1 mL EtOH (25 mCi, 22 Ci/mmol, ICN, Pharmaceuticals Inc. Costa Mesa, California) was added to a culture (~ 100 mL) of *O. illudens* 4435 (ATCC) which had been growing for 5 weeks. After a further 3 weeks the culture liquid was separated and extracted with ethyl acetate. Chromatography of the extract yielded illudin S (30 mg) and illudin M (11 mg) both with specific activity of ~ 1 mCi/mmol.

Scheme 1



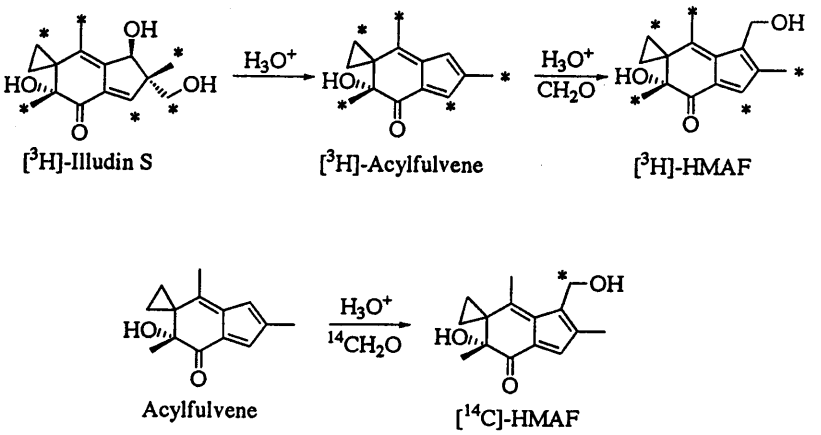
[^{3}H]-Illudin S

The experiment was repeated with a culture of *O. illudens* 4499 which produces mainly illudin S and little illudin M. It was grown on a smaller scale but with more [^{3}H]-CH_3COONa. Fermentation yielded 1.1 mg pure [^{3}H]-illudin S, specific activity 260 mCi/mmol. This material proved to be satisfactory for studies on the uptake of illudin S by relatively sensitive human myeloid leukemia (HL 60) cells and resistant human 8392 B cells. A sample of [^{3}H]-illudin S was converted to [^{3}H]-acylfulvene **4** with specific radioactivity 132 mCi/mmol and this material was also used successfully to measure uptake of the compound by various cells.[12]

An attempt was also made to convert [^{3}H]-illudin S directly or via the acylfulvene into HMAF (Scheme 2). However, HMAF of satisfactory purity could not be obtained and the radiolabeled sample appeared to decompose quickly. We therefore investigated preparation of [^{14}C]-HMAF from acylfulvene and [^{14}C]-paraformaldehyde. The latter compound (5 mCi, specific radioactivity 1 mCi/mg, American Radiolabeled Chemicals Inc.) was contained in a vial to which was added acylfulvene (118 mg) dissolved in acetone (1 mL). Sulfuric acid (1 M, 0.25 mL) was added and the mixture was stirred at room temperature for 48 h. Work up and chromatography yielded unchanged acylfulvene, "dimer" **6**, and HMAF (7.94 mg, 21.4 MBq). The sample was analyzed and found to be 85% pure (radiochemical purity). The contaminant appears to be derived from the paraformaldehyde. It could be removed by adding unlabeled HMAF followed by recrystallization from ethanol-hexane. ^{14}C- HMAF was found to be more

stable in ethanol solution than in the solid state. It has been stored at -20 to -80°C (shielded from light) for more than two months without any decomposition. The [^{14}C]-HMAF has been used successfully for pharmacokinetics and toxicology studies. By means of this procedure more than 50 mCi of [^{14}C]-HMAF (some sample with specific radioactivity 56 mCi/mmol) has been prepared by Amersham, U.K.

Scheme 2



Experimental

Production of [^{3}H]-Illudin S and [^{3}H]-Illudin M

Liquid cornsteep medium (100 mL) was prepared from 4 g dextrose, 1 mL cornsteep liquor (Sigma) and mineral solution (2 mL, containing 0.3 g $NaNO_3$, 0.1 g KH_2PO_4, 0.05 g KCl, 0.04 g $MgSO_4 \cdot 7.H_20$) adjusted to pH 6. The medium was inoculated with *Omphalotus illudens* strain 4435, obtained from the New York Botanical Garden. After four weeks at room temperature during which the mycelial mat had covered the surface of the culture liquid, a solution of [^{3}H]-CH_3COONa in 1 mL EtOH (25 mCi, 22 Ci/mmol, ICN) was added to the culture with gentle shaking. It was set aside for three weeks then the brown culture liquid was extracted with ethyl acetate (3 X 100 mL), the extract was dried (Na_2SO_4) and the solvent removed in vacuo. Liquid chromatography on silica gel (230-425 mesh) of the residual brown gum with ethyl acetate:hexane

(1:1) gave pure illudin M 11 mg, λmax (EtOH) 235, 310 nm (ε 13900, 3000), sp. act. 0.8 mCi/mmol; pure illudin S 30 mg, λmax (EtOH) 235, 315 nm (ε 13200, 2800), sp. act. 0.6 mCi/mmol and dihydroilludin M (9 mg) λmax (EtOH) 253 nm (ε 22500), sp. act. 1.2 mCi/mmol.

The experiment was repeated with a solution of [^{3}H]-CH_3COONa in 22 mL water-ethanol (10:1), 100 mCi, 22 Ci/mmol which was added to mycelium with 10 mL culture liquid. After three weeks the culture liquid was poured off and extracted with ethyl acetate. This extract yielded illudin M (20 mg, sp. act. 18 mCi/mmol); dihydroilludin M (1 mg, sp. act. 92 mCi/mmol); illudin S (5 mg, sp. act. 33 mCi/mmol).

[^{3}H]-Acylfulvene

A new culture of *O. illudens* 4499 was obtained from the Center for Forest Mycological Research, Madison Wisconsin, courtesy of Dr. Tom Volk. This strain was selected because it produced mainly illudin S and in relatively good yield. The fungus was grown in a small vial as above. Three weeks after inoculation the culture liquid was carefully removed from the mycelial pad and extracted with ethyl acetate. The extract contained mainly illudin S and traces of illudin M and illudin S monoacetate. [^{3}H] CH_3COONa (2.18 Ci/mmol, 80.7 BGq/mmol, 22.6 Ci/mg, 100 mCi) was dissolved in water (2 mL) and the solution was added to the vial containing the mycelial pad. Fresh culture medium (3 mL) was also added and the vial was set aside for 17 days. Extraction of the culture medium with ethyl acetate followed by drying ($MgSO_4$) and removal of solvent gave a residue which on liquid chromatography yielded pure illudin S (1.1 mg, sp. act. 254 mCi/mmol).

The illudin S was dissolved in water (0.75 mL) and 4 N H_2SO_4 (0.25 mL) added to the solution. It was kept overnight during which it became yellow. The solution was extracted with ethyl acetate and the extract was dried ($MgSO_4$). Removal of solvent and liquid chromatography (on silica gel) of the residue with ethyl acetate-hexane (1:8) as solvent gave pure yellow acylfulvene as a gum: UV λmax (EtOH) 235, 325 nm (ε 16600, 8300); 0.3 mg, sp. act. 132 mCi/mmol.

[^{3}H]-Hydroxymethylacylfulvene (HMAF)

Illudin S can be converted to HMAF directly or by conversion to acylfulvene followed by treatment of the latter with excess formaldehyde in dilute H_2SO_4. Model experiments run on a 5 mg scale gave better yields of HMAF when the two step method was used. Therefore a sample of

[^{3}H]-illudin S (1.7 mg, 254 mCi/mmol) was converted to acylfulvene as above. The crude acylfulvene was dissolved in tetrahydrofuran (0.4 mL) and formaldehyde solution (0.4 mL, 37% w/w) and 2 M H_2SO_4 (0.2 mL) were added. The solution was allowed to stand for 24 h then water was added and the solution was extracted with ethyl acetate. The extract was washed with $NaHCO_3$ solution, brine, and dried ($MgSO_4$). Removal of solvent and liquid chromatography (on silica gel) of the residue gave pure HMAF 0.2 mg, sp. act. 181 mCi/mmol. This compound decomposed quickly even though stored in the refrigerator during a period of two months.

[^{14}C]-HMAF

[^{14}C]-Paraformaldehyde (5 mCi, sp. act. 1 mCi/mg, American Radiolabeled Chemicals Inc., St Louis, MO) was obtained in a vial to which was added acylfulvene (118 mg) dissolved in acetone (1 mL). H_2SO_4 (1M, 0.25 mL) was added and the mixture was stirred at room temperature for 48 h. The yellow solution was extracted with ethyl acetate and the extract was washed with $NaHCO_3$ solution until neutral then dried ($MgSO_4$). Chromatography yielded unchanged acylfulvene, "dimer" 6, and HMAF (14.7 mg, 19 mCi/mmol).

The sample of HMAF was dissolved in ethanol (10 mL) and the solution was stored at -20 to -80°C. The solution was quite stable over a period of several months. The total radioactivity determined by liquid scintillation, was 1.07 mCi (39.6 MBq).

Analysis of the [^{14}C]-HMAF by TLC (Kieselgel 60 F254, Art 7734, Merck) using a linear analyzer showed one major radoiactive peak and one minor peak. The latter was less polar than HMAF and in certain solvent systems ($CHCl_3$:MeOH, 10:1; ethyl acetate:acetone:triethylamine, 40:20:2) it was slightly less polar than the "dimer" **6** as well. This minor peak has not been positively identified but it may have resulted from the paraformaldehyde used in the preparation of [^{14}C]-HMAF.

A sample of [^{14}C]-HMAF was diluted with non radioactive HMAF and recrystallized from ethanol:hexane (1:20) giving radiochemically pure [^{14}C]-HMAF (single radioactive peak on TLC). The total radioactivity of this sample was found to be 0.91 mCi. Hence the radiochemical purity of the original [^{14}C]-HMAF sample was 0.91 mCi/1.07 mCi (85% yield).

In order to improve the radiochemical purity of the sample of [^{14}C]-HMAF (21.4 MBq, 7.94 mg) it was rechromatographed on a Kieselgel 60 column (1.6 g, inside diameter 7 mm) with chloroform:ethanol:n-hexane (20:1:80) as eluent. Eight fractions were collected, four of which gave material with a radiochemical purity of > 98.6%. The compound was stored in ethanol

solution at -20 to -80°C and shielded from light. Under these conditions [^{14}C]-HMAF was stable over a period of several months. If stored at room temperature (10-25 °C), 10% of the compound decomposed after 2 months. The solid [^{14}C]-HMAF decomposed more rapidly at room temperature, only 48% remaining after 2 months.

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