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Isotopically-labeled natural products. I. Biosynthesis and isolation of 1-menthol-^{14}C

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SUMMARY

The biosynthesis by and the isolation of 1-menthol-^{14}C from Mentha arvensis *grown in $^{14}CO_2$ are described. Crystals (18 mg; 3.2 mCi/g) were obtained after column chromatographic purification of the steam distillate of the leaves. Purity of greater than 99.5 % was established by gas-liquid radiochromatography.*

INTRODUCTION.

Menthol is used by the pharmaceutical, toiletry, and tobacco industries. However, labeled menthol has not become available commercially.

Radioactive mint oils have been produced by several groups [1, 2, 3] in biogenesis studies. Time-course experiments with $^{14}CO_2$ plus the incorporation of specific precursors have supported the biogenic scheme proposed by Reitsema [4]. Only Mitsuhashi and co-workers [5] isolated labeled menthol and, by chemical degradations, showed the incorporation of carboxyl-labeled acetate.

Two groups have produced radioactive material for tracer studies, but details have not been published. In one report, Stepka and Larson [6], described elaborate facilities and listed natural products isolated, but only mentioned growing mint. In the second report, Newell, Latimer, and Haefele [7] described the use of biosynthesized randomly-labeled menthol-^{14}C in cigarette smoke experiments.

Our specific interest in tobacco products, in which menthol is a frequent additive, created a need for high specific activity labeled menthol. We therefore grew *Mentha arvensis* in $^{14}CO_2$ and isolated the 1-menthol-^{14}C. The facility coupled with appropriate isolation procedures, should be useful for the production of a broad spectrum of labeled natural products.

PLANT CHAMBER.

The hermetically-sealed plant chamber, schematically presented in Figure 1, was based on that of Rapaport [8]. A 30 × 30 × 30 inch cube, constructed of UVT Plexiglas, was bolted to a 3/8 inch aluminium base. The edges were cemented and reinforced by Plexiglas strips (3/4 × 3/4 inch). Joints were sealed externally by General Electric SR-82 silicone resin and a 10 inch square door by Mortite caulking (Mortell Co.) external to a rubber gasket. An expansion volume (four 13 l respiratory bags) protected the sealed chamber from strain due to changes in temperature and atmospheric pressure. Four nutrient feed lines were connected to separatory funnels mounted above the chamber providing gravity feed. Air was circulated internally by a magnetically driven fan and externally by a diaphragm pump (Neptune Products Inc., Model 3). Externally, the air also passed through a brass heat exchanger plus a water removal trap. The latter consisted of a 3-liter separatory funnel with a side arm which was connected, in turn, to a 5-liter stainless steel beaker inside the chamber. The internal heat exchanger was a double-helix coil (50 ft. × 1/4 in. O.D. copper tubing) through which cold water (3-5° C) flowed. A temperature of 30.5 to 32° C with 50 to 55 % relative humidity was maintained with all lights on and the plant material in the chamber.

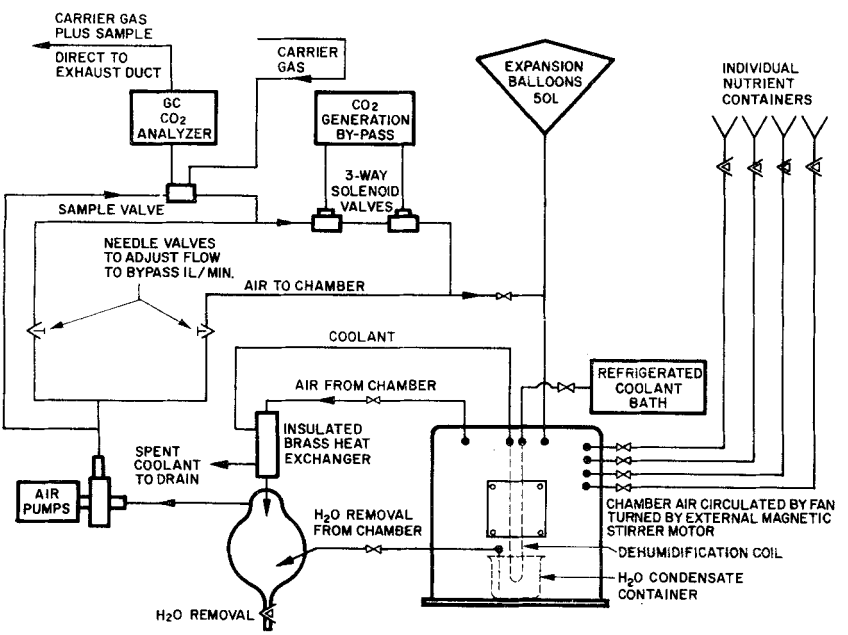

FIG. 1. Schematic diagram of plant chamber less lights.

Typical conditions were 24.5° C and 60 % R.H. with the lights off. Water, condensed within the chamber, was removed from the beaker at least once a day by the suction of the air pump when the mainstream air valve was closed.

The main air stream was split into two parallel streams on the output side of the pump. One stream (6 l/min) went directly to the chamber, the other (1 l/min) passed through a carbon dioxide generation station. This consisted of a three-necked flask connected to 2 three-way solenoid valves with ball and socket joints.

For addition of $^{14}CO_2$, 2 g $Ba^{14}CO_3$ (41.1 mCi/g C) was placed in the three-necked flask which had a Kjeldahl safety bulb in the exit neck and a gas dispersion frit in the inlet. Lactic acid (13 ml, 50 % by volume), followed by 75 ml of water, was added from an additional funnel. Appropriate cycling of the solenoid valves allowed the $^{14}CO_2$ to be swept into the chamber by the circulating air. The same flask was used for an alkali scrub of $^{14}CO_2$ at the end of the biosynthesis; a solution of 10 g NaOH in 150 ml H_2O replaced the carbonate-lactate mixture. Carbon dioxide analyses were carried out gas chromatographically on 80-100 mesh Porapak Q (Waters Associates) at 37° C with helium carrier gas. Chamber air was sampled with a Cole-Parmer Masterflex (peristaltic) pump and a Loenco No. L206-6V gas sample valve. Calibrations of the Carle Model 100 Micro Detector were made by mass spectrometric analyses of samples collected in gas traps substituted for the flask at the generation station.

All flexible tubing was 3/8 or 1/4 inch O.D. unplasticized polyethylene, fittings were Swagelok and all connections passing through the chamber wall were made with bulkhead fittings sealed on each side of the "panel" with "O" rings. All air and nutrient lines were fitted with toggle or diaphragm valves. The valves allowed the isolation of the chamber with its expansion volume from the external systems for maintenance or repair of components.

Banks of lights were placed above and on two sides of the chamber. Two 4 inch electric fans circulated air over the top of the chamber below the lights. Illumination was controlled by a 24-hour timer. The distribution of lights, which gave an intensity of about 1.5 milliwatts/cm^2 (measured with a Yellow Springs Model 65 Radiometer), was as follows :

Overhead
7-40 watt (4 ft.) daylight fluorescent lamps (Sylvania F40D)
1-20 watt (2 ft.) daylight fluorescent lamp (Sylvania F20T12-D)
3-40 watt Plant-gro fluorescent lamps (Westinghouse F40/Gro)
3-40 watt incandescent lamps.

On Each Side
3-20 watt (2 ft.) daylight fluorescent lamps
5-20 watt (2 ft.) Plant-gro fluorescent lamps (Westinghouse F20T12/Gro)
2-40 watt incandescent lamps.

BIOSYNTHESIS.

Mint plants, *Mentha arvensis* L. var. *piperascens* or Japanese menthol mint, were grown from stolons supplied by the A. M. Todd Company. The stolons were planted in a sterilized mixture of three parts sand and one part vermiculite, with eight inch clay pots as containers. One stolon was planted per pot and each was grown to a large individual mint plant showing good color, growth, and size. The plants were grown in a controlled-environment room maintained at 30° C and 60 % R.H. with light supplied by fluorescent and incandescent lamps. Well-established plants were cut back in order to promote increased lateral stem growth with numerous actively growing young leaves. Each of the main stems produced several lateral stems which had three to four pairs of new leaves.

At this stage of growth, the mature leaves on each plant were removed. Four plants were placed in the chamber, and it was sealed. The plants were allowed to use the available CO_2 (0.12 % by volume of the chamber air) during a remaining light period of about 6 hours. On the following day three $^{14}CO_2$ (41.1 mCi/g C) additions, each equivalent to 0.05 % of the chamber volume, were made at 2 to 3 hour intervals. For the next 6 days, $^{14}CO_2$ was added three to four times daily, to give a total of 100 mCi.

Each plant was fed nutrient solution daily as needed. The lights were cycled with 16 hours of light and 8 hours of darkness per day. While in the chamber, all plants showed new growth and the appearance of the leaves did not indicate any physical abnormalities due to the exposure to the ^{14}C radiation. All leaves appeared to be normal in texture, color, and shape.

After the final $^{14}CO_2$ addition, the plants were allowed to metabolize for another 40 hours to convert precursors to l-menthol. Normal CO_2 was introduced four times during the light period of the interval. Respired $^{14}CO_2$ was removed by an alkali scrub during the second dark period. At the conclusion of the metabolic period, the air was monitored, then vented directly into an exhaust air duct, and the chamber was opened.

The plants were removed from the chamber, and the leaves were harvested in two groups. One sample consisted of the older leaves, the other of the younger leaves (terminal leaves and the next four pairs of opposite leaves). The leaves were submerged immediately in liquid nitrogen and were crushed manually with a pestle. Each sample of radioactive mint leaf powder was transferred to a distillation flask.

ISOLATION OF l-MENTHOL-^{14}C.

Each frozen sample was covered with water and steam distilled for one hour. The distillates (75 to 100 ml) were collected at 0° C; that from the younger leaves under toluene, that from the older leaves under benzene. Since the plant materials had to be processed quickly subsequent to harvesting,

fresh weights of the leaves were not obtainable. Instead, the leaf residues were freeze dried, and the weights, 6.9 and 4.2 g respectively for the younger and older leaves, were recorded.

Each steam distillate was saturated with sodium bicarbonate and the organic layer was removed. The oils were then concentrated by subambiant bulb-to-bulb vacuum distillation. All fractions were monitored and no appreciable radioactivity was found in the solvent traps. Gas-liquid radiochromatographic analyses showed that the menthol from the younger leaves had a higher specific as well as total activity than that from the older leaves. Purification was therefore carried out on this menthol-rich oil.

Gradient-elution adsorption chromatography on silica gel with hexane-benzene elution was used to purify the menthol. The column, 1/8 inch I.D. by 7 inch height, was prepared from silica gel (Brinkmann) activated overnight at 180° C and stored under hexane. The gradient, obtained by the addition of benzene at 0.3 ml/min to the reservoir containing 40 ml hexane with an equal flow to the column, was continued until the menthol had eluted. Menthol-rich fractions (numbers 7-13, 5 ml each) were combined and concentrated to less than 1 ml at 12-18° C *in vacuo*. Menthol (18 mg, m.p. 41.5° C uncorrected) crystallized on standing in the refrigerator (5° C).

GAS-LIQUID RADIOCHROMATOGRAPHY.

A Victoreen Model 4 000 Gas Chromatograph was employed for the menthol analyses. Mass data were obtained with a flame ionization detector and an Infotronics CRS-100 Digital Integrator. Radioactivities were determined with a 10 ml specially-constructed flow counter, connected to a Power Design 0-5 000 V power supply and a Nuclear-Chicago Model 8375 Digital Integrator.

The column (1/8 inch O.D. by 12 feet, stainless steel) contained 10 % Carbowax-20M on 60-80 mesh Chromosorb W-HMDS. The temperature was 175° C and helium flow 40 ml/min. A post-column splitter was fabricated from a 1/8 inch Swagelok stainless steel tee. Flow to the flame detector passed through a 22-gauge hypodermic needle silver soldered into 1/16 inch tubing. The split ratio (20 : 1), obtained by inserting the proper diameter stainless steel wire in the needle, was determined with toluene-^{14}C. Flow to the counter passed through a combustion tube (3/8 inch O.D. by 10 inch S.S., containing CuO maintained at 800° C) and a drying tube (1/4 inch O.D. by 12 inch copper, containing $MgClO_4$). The counting mixture was helium-propane (1 : 1) and the counter, shielded with 2 inches of lead, had a background of 13 c.p.m. Standardization and efficiency determinations were performed with toluene-^{14} C (u.l. ring label, New England Nuclear Corp.). Specific activities were determined in a Packard Tricarb Model 3003 liquid scintillation spectrometer. The scintillation solvent contained 4 g PPO and 100 mg POPOP per liter of toluene. An internal standard (toluene-^{14}C) was used for quenching correction.

Results and Discussion.

Commercially, l-menthol is crystallized by chilling the oil obtained by steam distillation of mint plants. This gives a 40 per cent yield [9]. Our desire for high yield as well as purity necessitated purification before crystallization. Fractions from gradient elution chromatography which contained 88-92 per cent menthol were combined. After solvent removal, 18 mg (57 μCi) of l-menthol-^{14}C was obtained. The radiochemical yield was 0.06 % based on $Ba^{14}CO_3$.

The specific activity of 3.2 mCi/g of menthol corresponds to 4.2 mCi/g of carbon. This is a 10-fold dilution from the 41.1 mCi/g carbon in the $^{14}CO_2$ fed. Initial analyses of the oil had shown the proportions of menthol to menthone to other constituents to be 7 to 2 to 1. The relatively high specific activity in the menthol, detected in the menthone as well, resulted from the extended feeding of $^{14}CO_2$ to young leaves, plus the added metabolic period. This schedule, based on the work of others with *M. piperita* [1, 3], permitted the accumulation of the desired labeled product without further optimization of conditions. The older leaves, although not mature, contained proportionally less ^{14}C than the younger leaves, as expected.

Gas-liquid radiochromatographic analyses established the radiochemical purity as greater than 99.5 per cent with a high degree of uniformity of labeling. The latter was indicated by equal ratios, within experimental error, of mass to radioactivity for degradation products. Detailed studies of the distribution of labeling will be reported separately.

A two-week long biosynthesis has been carried out with a total of 200 mCi $Ba^{14}CO_3$. The steam distilled oil was chromatographed and combined fractions contained 200 mg l-menthol-^{14}C with a single impurity, menthone (3.9 % by gas chromatography). The specific activity, 9.5 mCi/g menthol, corresponded to 12.5 mCi/g carbon or about a three-fold dilution.

The procedures employed have shown that l-menthol-^{14}C with high specific activity can be prepared biosynthetically and isolated in crystalline form with a high degree of chemical and radiochemical purity.

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