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IDENTIFICATION OF 3-HYDROXY-3-METHYLGLUTARIC ACID (HMG) AS A HYPOGLYCEMIC PRINCIPLE OF SPANISH MOSS (*TILLANDSIA USNEOIDES*)

K.M. WITHERUP, J.L. MCLAUGHLIN,*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907-1333

R.L. JUDD, M.H. ZIEGLER, P.J. MEDON, and W.J. KELLER

School of Pharmacy, Northeast Louisiana University, Monroe, Louisiana 71209

ABSTRACT.—Bioactivity-directed fractionation, using brine shrimp lethality and murine hypoglycemia, of an ethanol extract prepared from *Tillandsia usneoides*, led to the isolation of four apparently bioactive compounds from the water-soluble fraction. The compounds were identified as citric acid, succinic acid, 3-hydroxy-3-methylglutaric acid (HMG), and 3,6,3′,5′-tetramethoxy-5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside. The brine shrimp lethality of the acids was simply due to acidity; however, HMG elicited significant hypoglycemic responses in fasting normal mice. Ethyl and methyl esters of citric acid were prepared and tested in the murine hypoglycemic assay. Five of the predominant sugars were identified by tlc. Free thymidine was also isolated. Further evaluation of HMG and other potential inhibitors of HMG CoA lyase, in the treatment of symptoms of diabetes mellitus, is suggested.

A tea made from *Tillandsia usneolides* L. (Bromeliaceae) (Spanish moss) has been used in southern Louisiana to allay the symptoms of diabetes mellitus (1). In rats, H_2O and EtOH extracts (500 mg/kg, ip) produced significant hypoglycemia (11.1% and 9.4%, respectively) within 24 h; the H_2O extract produced a maximal hypoglycemia after 4 and 7 days of chronic exposure and blocked the development of alloxan diabetes; and plasma insulin levels were not affected by administration of the H_2O extract (2). *T. usneolides* has also been shown to possess antibiotic (3,4) and estrogenic activities (5), but none of these activities has been explained chemically.

This plant species has been the subject of several previous chemical investigations. Early investigators were mainly interested in inorganic analyses (6–13). Previously identified organic constituents include sugars (14), ascorbic acid and carotene (15), cyclopropane ring-containing triterpenes and straight-chain hydrocarbons (16,17), free and esterified sterols (18), and flavonoid glycosides (19,20). Our research was concerned with the activity-directed isolation and chemical identification of the compound(s) responsible for the hypoglycemic action.

Brine shrimp lethality (BST) is a convenient indicator of diverse bioactive materials (21) and was chosen to guide the early steps of this work. Hypoglycemia in fasting mice was used to confirm the bioactivities and to guide the latter stages of the fractionation when the acidity of organic acids precluded the effective use of the BST. Partitioning ($H_2O/CHCl_3$) of the EtOH extract (F001) located the activities in the H_2O (F002) residue. This extract (F002) was resolved using polyamide medium-pressure liquid chromatography (mplc), Sephadex G-10 gel filtration, high-speed centrifugal countercurrent chromatography (hsccc), and cation-exchange chromatography. The BST and/or murine hypoglycemia served as fractionation guides throughout the separation.

The first bioactive compound isolated and characterized (uv, ir, ^{1}H nmr, cims, eims, fabms) was 3,6,3′,5′-tetramethoxy-5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside, a compound previously reported from this species (20). Although this flavone was toxic to the brine shrimp,

TABLE 1. Murine Hypoglycemia (MH)[a] and Brine Shrimp Lethality (BST) Bioassay Results for the Acidic Pool and Isolated Compounds.

Sample	% Change in Plasma Glucose (MH)	Significance (p)	BST LC_{50} (ppm)	BST 95% Conf. Int. (ppm)
Acidic Pool	−23.7	<0.01	148	106–197
Flavone[b]	−1.0	N.S.	420	272–650
Citric Acid[c]	+47.3	<0.05	50	44–56
Succinic Acid	+9.9	N.S.	40	35–45
Thymidine[c]	+25.5	<0.05	>1000	—
HMG[d]	−41.7	<0.01	228	172–297

[a]n=7; mice fasted 14 h; blood samples collected 4 h after sample administration (250 mg/kg, ip); N.S.=not significant.

[b]3,6,3′,5′-Tetramethoxy-5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside.

[c]Irritability may cause stress and subsequent hyperglycemic via epinephrine-induced hydrolysis of glycogen.

[d]3-Hydroxy-3-methylglutaric acid.

it did not significantly alter blood glucose levels in mice (Table 1).

In the early stages of chromatographic separation of the H_2O extract (F002), the most potent BST activity also coincided with hypoglycemic activity in the mice with 23.7% glucose reduction ($p<0.01$) for an acidic pool obtained via polyamide mplc. This acidic pool followed the initial column fractions, which contained a mixture of sugars. A Sephadex G-10 separation yielded succinic acid (mp, cims, ^{1}H nmr) and concentrated another acid, which was purified by hsccc and identified (mp, ir, cims, ^{1}H nmr, ^{13}C nmr) as citric acid. Although both of these acids were quite toxic to the brine shrimp, neither was hypoglycemic; indeed, citric acid was significantly hyperglycemic perhaps due to irritability (Table 1). Preparation of the brine with buffers subsequently demonstrated that the activity of these acids in the BST is attributable to their lowering of the pH of the brine (22).

Other acids from the analytical hsccc were toxic to the brine shrimp, and two of these gave cims evidence for a monoethyl ester (m/z 207) and a monomethyl ester (m/z 221) of citric acid. Whether present naturally or as artifacts, it was initially believed that these esters may have been responsible for the hypoglycemic activity. Thus, the monomethyl, dimethyl, trimethyl, and ethyl esters of citric acid were synthesized via controlled Fischer esterification, separated by hsccc, and tested in the mice. The dimethyl and trimethyl esters were significantly hyperglycemic and the diethyl ester was significantly hypoglycemic (Table 2). However, the latter was not detected (tlc) in the active acidic pool from the plant extract and, obviously, did not explain the hypoglycemic activity of the plant material.

Because the analytical hsccc system gave good resolution, prep. hsccc separation of the acidic pool from the polyamide column was performed. Three consecutive polyamide mplc runs yielded 6 g of the active acidic pool from a total of 50 g of F002. This acidic pool was then fractionated over five consecutive runs in the prep. hsccc. The fractions immediately subsequent to the elution of citric acid were combined and separated on another column of Sephadex G-10. Next an mplc column of cation-exchange resin was used to yield pure crystals of free thymidine (mp, ir, cims, ^{1}H nmr, ^{13}C nmr), which was, surprisingly, hyperglycemic (Table 1). Five sugars (arabinose, fructose, glucose, maltose, and sucrose) were identified (tlc) from the first eluents of the polyamide column.

TABLE 2. Murine Hypoglycemia[a] Bioassay Results for Citric Acid Esters.

Citric Acid Esters[b]	Mouse Assay	
	% Change in Plasma Glucose	Significance (p)
MME	−7.4	N.S.
DME	+35.8	<0.05
TME	+29.0	<0.05
MEE	−6.6	N.S.
DEE	−17.3	<0.05
TEE	−13.6	N.S.

[a] n=7; mice fasted 14 h; blood samples collected 4 h after sample administered (250 mg/kg, ip); N.S.=not significant.

[b] MME=monomethyl ester at 1-carboxyl (major) and 6-carboxyl (minor); DME=dimethyl ester; dimethyl ester at 1-,5-carboxyl (major) and 1-,6-carboxyl (minor); TME=trimethyl ester at 1-,5-,6-carboxyl; MEE=monoethyl ester at 1-carboxyl; DEE=diethyl ester at 1-,5-carboxyl; TEE=triethyl ester at 1-,5-,6-carboxyl.

An additional portion of the acidic pool of fresh *T. usneoides* was fractionated by the same hsccc system. A pool of active fractions was subjected to the same cation-exchange system as was used for the isolation of thymidine. This yielded pure 3-hydroxy-3-methylglutaric acid (HMG) (mp, ir, cims, fabms, ^{1}H nmr, and ^{13}C nmr), which was significantly hypoglycemic. HMG, which was then purchased commercially, showed a good dose-response relationship for hypoglycemia (Table 3) with a decrease in fasting blood glucose of 41.7% (p<0.01) at 250 mg/kg, ip. Thus, HMG is the active hyperglycemic principle of *T. usneoides*.

A literature search showed that HMG was patented in 1971 as an anticholesterolemic agent (23). It is a competitive inhibitor of HMG-CoA reductase in bacterial systems (24), and the in vitro inhibition of microsomal HMG-CoA reductase from both liver and intestine has been shown (25). The enzymatic control of cholesterol biosynthesis is almost exclusively at the HMG-CoA reductase step at which HMG-CoA is converted to mevalonic acid (26). From this earlier interest in HMG as a potential hypocholesterolemic agent, the acute toxicity was studied in mice (27). The LD_{50} value was shown to be 7.33 g/kg, po and 3.23 g/kg, ip. Subtoxic and pharmacologic doses given to mice and cats during gestation gave no indication of tetratogenic effects. In a later study, human patients with familial hypercholesterolemia were given four different levels consisting of 750, 1500, 2250, and 3000 mg/day in three divided doses for a period of eight weeks (28). Each HMG dosage significantly decreased plasma cholesterol levels as compared to placebo after six weeks of treatment. The mean plasma cholesterol levels during the eight-week treatment period were 11% and 13% lower in the 2,250 mg and 3,000 mg HMG-treated groups (p<0.034 and <0.021, respectively). No toxicity or clinical side effects were noted throughout the study,

TABLE 3. Murine Hypoglycemic (MH) Dose/Response Test[a] for HMG.[b]

Dose (mg/kg, ip)	% Change in Plasma Glucose	Significance (p)
62.5	+7.9	N.S.
125	−21.0	<0.05
250	−41.7	<0.01

[a] n=7; mice fasted 14 h; blood samples collected 4 h after administration.

[b] 3-Hydroxy-3-methylglutaric acid.

and the report also mentioned that biochemical tests were performed to monitor certain enzymes and metabolites, including glucose. Unfortunately, the results of the glucose tests were not given, and any HMG-induced hypoglycemia was not noted.

By blocking HMG-CoA lyase, administration of HMG may produce a significant decrease of ketone bodies in the blood to the extent that the mouse brain is obligated to use serum glucose rather than acetoacetic acid (AcAc), as its major source of energy (29). In such a case, an increased amount of glucose would be metabolized by the brain to cause the hypoglycemic state. It is important to remember that the brain functions independently of insulin and glucagon.

HMG is a normal minor component of human urine and an abnormal major component in certain disorders of leucine catabolism; in the latter case, HMG aciduria is accompanied by protracted hypoglycemia (31), an observation that supports the HMG induction of hypoglycemia as we have discovered. Furthermore, an inborn human deficiency of HMG-CoA lyase is reported to have resulted in HMG aciduria with death at six months attributed to severe hypoglycemia; acetoacetic acidosis and ketosis were completely absent (32). These observations support our implication of HMG-CoA lyase as a probable site of hypoglycemic action of HMG. Thus, the effects of HMG (hypoglycemia, hypocholesterolemia, and, quite probably, decreased ketosis and decreased AcAc acidosis) would seem to make this simple compound, its analogues, and other HMGCoA lyase inhibitors worthy of further evaluation as drugs in the treatment of the deleterious consequences of diabetes mellitus.

HMG was first isolated from two *Crotalaria* species (Leguminosae) (33). It is sometimes called dicrotalic acid. HMG was found conjugated to shikimic acid in the latex of *Euphorbia biglandulosa* (Euphorbiaceae) (34). In 1983, a quantitative gc analysis of the carboxylic acids found in wheat, rye, and barley seeds indicated the presence of HMG (35). Synthetic methods are previously described (36–38).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Polyamide (Aldrich) mplc was performed using a 5.4×44.4 cm Michel-Miller column with a H_2O to MeOH gradient. Gel filtration with H_2O eluent was carried out on a 2×185-cm column slurry-packed with 230 g Sephadex G-10 (Pharmacia). Analytical and prep. columns, using *n*-BuOH-*t*-BuOH-HOAc-H_2O (4:2:0.2:7) (aqueous mobile phase) and EtOAc-H_2O (1:1) (EtOAc mobile phase) as solvent systems, were utilized for the hsccc Ito coil apparatus (P.C., Inc.). Cation-exchange chromatography (cec) consisted of a 1.5×30 cm Michel-Miller column packed with Dowex 50×4-400 resin with 0.055 N HOAc as the eluent. Analytical tlc was conducted on Si gel 60 F254 (Merck) with EtOAc-MeOH-HOAc-H_2O (25:10:1.5:5) and RP-18 (Whatman) with MeCN-H_2O (9:1). Plates were sprayed with a vanillin/H_2SO_4 visualization spray. All aqueous samples were freeze-dried. Mps were obtained with a Mettler hot stage and are reported uncorrected. Ir and uv spectra were obtained with Beckman IR-33 and Beckman DU-7 spectrophotometers, respectively. Cims was performed on a Finnigan instrument using isobutane or NH_3. A Kratos MS-50 instrument was used for hrms. ^{1}H- (200 MHz) and ^{13}C-nmr (50 MHz) spectra were obtained using a Chemagnetics A-200 spectrometer. Spectral assignments for the isolated and synthesized compounds are given elsewhere (22).

PLANT MATERIAL.—A 7-kg quantity of dried *T. usneoides* was obtained from the Morris Kupchan plant collection as originally provided by the USDA (PR-6553, TK-8-68). Fresh moss (2.2 kg dry wt) was identified and collected in Monroe, Louisiana, by one of the authors (W.J.K.) in August of 1987.

BIOASSAYS.—The brine shrimp lethality bioassay (BST) (21) was used as a guide for early fractionations. A murine hypoglycemia (MH) assay was conducted as follows. Male Swiss-Webster mice (23–28 g each) were randomly assigned to each treatment group (7 mice), and these groups were placed in separate cages for a 3-day acclimation period. Mice were fasted 14 h prior to sample administration (usually at 250 mg/kg, ip) and for 4 additional h until blood was collected for analysis. Samples were prepared just prior to testing by dissolving in distilled H_2O. Control mice received

an equivalent volume of H_2O only. Samples were administered and blood collected at the same time each day to minimize diurnal variations. Blood was collected by decapitation and centrifuged at 5,000 rpm for 10 min; the plasma was then immediately analyzed using a glucose analyzer (YSI 23A). Hematocrit values were also recorded.

EXTRACTION AND ISOLATION.—Extraction of the Kupchan moss (7 kg) with 95% EtOH yielded 495.4 g of residue (F001) (BST LC_{50} >2000 ppm). This F001 residue was partitioned between $CHCl_3$-H_2O (1:1) to give 171.5 g of H_2O-soluble residue (F002) (BST LC_{50} 1625 ppm) and 223.5 g of $CHCl_3$-soluble residue (F003) (BST LC_{50} >2000 ppm) after solvent removal. The fresh dried moss (2.2 kg) was also extracted and partitioned as above to give F001 (165.4 g), F002 (46.1 g), and F003 (119.4 g).

Portions of F002 from the Kupchan moss were fractionated by polyamide mplc. Early fractions contained carbohydrate material (BST inactive, MH inactive) followed by the elution of acidic compounds (acidic pool) (BST LC_{50} 148 ppm; MH 23.7% plasma glucose reduction, $p<0.01$). Later fractions contained 3,6,3′,5′-tetramethoxy-5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside (BST LC_{50} 420 ppm; MH inactive). This compound was recrystallized from MeOH (mp 231–232°) and identified by a comparison of uv, ^{1}H-nmr, and ms data with literature values (20). The carbohydrate material was separated into two pools by Sephadex G-10 cc which contained mostly disaccharides and monosaccharides, respectively. Co-tlc with standard sugars identified arabinose, fructose, glucose, maltose, and sucrose. BST-monitored fractionation of the acidic pool on Sephadex G-10 yielded succinic acid (BST LC_{50} 40 ppm; MH inactive) and a mixture of compounds (BST LC_{50} 252 ppm) eluted in fractions just prior to the elution of succinic acid.

The succinic acid was recrystallized from MeOH (mp 181–183°), then identified by ^{1}H- and ^{13}C-nmr, ms, and co-tlc (glucose/aniline visualization spray) techniques. Analytical hsccc of the mixture of components using the *n*-BuOH-*t*-BuOH-HOAc-H_2O solvent system yielded citric acid (BST LC_{50} 50 ppm; MH 47.3% plasma glucose increase, $p<0.05$) in the early fractions. This was recrystallized from Me_2CO (mp 148–150°), and identified by ir, ^{1}H- and ^{13}C-nmr, ms, and co-tlc techniques. Fractionation of another portion of the acidic pool by preparative hsccc, using the above BuOH solvent system, concentrated murine hypoglycemic activity into several fractions just subsequent to the elution of citric acid.

The most active pool of these fractions (MH 43.4% plasma glucose reduction, $p<0.001$) was fractionated by Sephadex G-10 cc and then by hsccc to give free thymidine BST inactive; MH 25.5% plasma glucose increase, $p<0.05$). The thymidine was recrystallized from EtOH-H_2O (1:1) (mp 182–184°, 13 mg, 0.0006% yield) and identified by ir, ^{1}H-nmr, ^{13}C-nmr, hrms (*m/z* 243.0903 for $C_{10}H_{14}N_2O_5H^+$), and co-tlc data. A portion of the F002 residue derived from fresh moss was chromatographed by polyamide mplc to obtain the usual acidic pool. This pool was subjected to prep. hsccc using the same solvent system as before. Fractions after the elution of thymidine were pooled and then fractionated by additional hsccc to give pure 3-hydroxy-3-methylglutaric acid (HMG) (BST LC_{50} 228 ppm; MH 41.7% plasma glucose reduction, $p<0.01$). The HMG was recrystallized from Me_2CO (mp 105–107°, 30 mg, 0.3% yield from fresh dried moss) and identified by ir, ^{1}H-nmr, ^{13}C-nmr, cims, fabms, and co-tlc data.

SYNTHESIS OF ETHYL ESTERS OF CITRIC ACID.—A Fischer esterification reaction was used to prepare the ester products (22). A 3-g quantity of citric acid (Sigma) was placed in 100 ml of absolute EtOH to which 16 drops of concentrated H_2SO_4 were added. The reaction mixture was refluxed for 28 h to produce the liquid triethyl ester (2.17 g; 72.3% yield). The reaction was repeated as before but was stopped after 2 h when the mono- and diethyl esters were present (tlc) in a nearly 1:1 ratio. The workup procedure for all of the synthesized ester products was (a) neutralize reaction mixture with $Ba(OH)_2$ to pH 5-6, (b) evaporate solvent, (c) add Me_2CO to dissolve ester and gravity filter to remove insoluble $Ba(OH)_2$, (d) evaporate solvent, (e) add more Me_2CO and centrifuge 15 min to remove any remaining $Ba(OH)_2$, and (f) collect supernatant and evaporate solvent.

SYNTHESIS OF METHYL ESTERS OF CITRIC ACID.—An 80 ml volume of MeOH containing 3 g citric acid (Sigma) and 3 drops of concentrated H_2SO_4 was refluxed for 28 h to give 1.92 g of the semi-solid trimethyl ester (64.% yield) after workup (see above). The reaction was repeated but stopped after 2.5 h to give a mixture of the monoethyl and dimethyl esters.

SEPARATION AND CHARACTERIZATION OF THE ESTER MIXTURES.—The ethyl ester mixture (700 mg) was separated by analytical hsccc using an EtOAc-H_2O (1:1) solvent system with EtOAc as the mobile phase. The diethyl ester (367 mg; 52.4% yield) eluted first followed by the monoethyl ester (111 mg; 15.8% yield). The same method was used to separate 400 mg of the methyl esters to obtain 97 mg (24.3% yield) of the monomethyl ester and 124 mg (31.0% yield) of the dimethyl ester. The ester products were identified by cims and ^{1}H-nmr data. The spectral data indicated that the monomethyl ester was a mixture of 1-carboxymethyl (major) and 6-carboxymethyl (minor) ester products. The dimethyl ester consisted of the 1,5-carboxydimethyl (major) and 1,6-

carboxydimethyl (minor) ester products. The other esters were determined to be trimethyl (1,5,6-carboxytrimethyl), monoethyl (1-carboxymethyl), diethyl (1,5-carboxydimethyl), and triethyl (1,5,6-carboxytriethyl). The bioassay results for these citrate esters are summarized in Table 2.

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