A new endogenous natriuretic factor: LLU-α

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ABSTRACT   For over three decades, renal physiology has sought a putative natriuretic hormone (third factor) that might control the body's pool of extracellular fluid, an important determinant in hypertension, congestive heart failure, and cirrhosis. In our search for this hormone, we have isolated several pure natriuretic factors from human uremic urine that would appear, alone or in combination, to mark a cluster of phenomena previously presumed to be that of a single "natriuretic hormone." This paper reports the purification, chemical structure, and total synthesis of the first of these compounds, LLU-α, which proved to be 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman, presumably a metabolite of γ-tocopherol. Both natural LLU-α and synthetic material are identical (except for optical activity) with respect to structure and biological activity. It appears that the natriuretic activity of LLU-α is mediated by inhibition of the 70 pS K⁺ channel in the apical membrane of the thick ascending limb of the kidney.

As a result of salt-induced plasma-volume expansion in mammals, three concurrent events have been observed: sustained natriuresis, rising plasma concentration of a Na^+ transport inhibitor, and pressor activity. It has been presumed that these effects are due to elaboration of a low-molecular-weight "natriuretic hormone," the putative controller of extracellular fluid (1). Over 30 years of effort have failed to characterize the putative hormone (2, 3). Atrial natriuretic factor, when infused, produces a natriuresis and a decrease in blood pressure that are short-lived (4, 5) and whose physiological role is still undefined (6, 7).

The approach of essentially all other workers has been to investigate mammalian-derived isolates that inhibit the Na^+ pump (2, 8). From these studies, digoxin (9) and "iso-ouabain" (10–13) have been isolated; however, digoxin and ouabain lead to kaliuresis (4, 14–17). Therefore, we hypothesized that this search tool is an inadequate marker for natriuresis. Consequently, a natriuretic assay has been developed in which the *in vivo* physiological events, urine volume, K^+ and Na^+ concentrations, and mean arterial pressure are measured (18).

In this paper, we report the isolation of pure LLU-α, the determination of its structure by spectroscopy, its synthesis in racemic form, and its biological characterization. Given its structure, we infer that it is the product of *in vivo* oxidative metabolism of γ-tocopherol, a member of the vitamin E complex.

MATERIALS AND METHODS

Purification of LLU-α. Human uremic urine (800 liters) was collected, processed by ultrafiltration (3 kDa) and lyophilization, and then chromatographed on Sephadex G-25 to obtain the post-salt fraction as described (18). Freeze-dried material obtained therefrom was extracted by stirring with 9 volumes of isopropanol for 18 hr. The isopropanol extract was evaporated to dryness by use of a rotary evaporator. The resulting brown oil was dissolved in 20% methanol/water and subjected to chromatography on a C_{18} reversed-phase column. HPLC was performed employing a Beckman System Gold (model 126 pump and model 168 diode array detector) controlled by SYSTEM GOLD software (version 5.1). Chromatography involved Beckman Ultrasphere ODS (C_{18}) columns save for the final purification step, which employed an EM Science LiChrospher 100. All eluates were taken to dryness in a centrifugal vacuum concentrator (Jouan, Saint Herblain, France).

Step 1. Reversed-phase HPLC was performed by gradient elution with 0.2 M pyridinium acetate, pH 5.5, and methanol as described (4). Based on bioassay data, fractions 50–80 were combined for further purification.

Step 2. The next column (5 μm; 10 × 250 mm) was eluted at 2 ml/min with a gradient formed from 0.2 M acetic acid (A) and methanol (B) (60% A/40% B for 5 min, a linear gradient to 50% A/50% B over 5 min, and a linear gradient to 30% A/70% B over 55 min). The eluant for this and all succeeding chromatography was monitored with the diode array detector (202–390 nm). Sixty 1-min fractions were collected. The fractions designated α (4), as determined by bioassay and UV spectra, were pooled and dried.

Step 3. The solvents and column were the same as those in step 2; however, the gradient was changed (60% A/40% B for 5 min, a linear gradient to 40% A/60% B over 5 min, a linear gradient to 30% A/70% B over 28 min, and a linear gradient to 100% B over 2 min and 100% B for 3 min), and 40 1-min fractions were collected. The retention time for α, as identified by its UV spectrum, is 28 min. The α-containing fractions were pooled and dried.

Step 4. With use of the same size column, α was eluted isocratically at 2 ml/min with 45% 0.2 M acetic acid and 55% methanol for 37 min, collecting 70 half-minute fractions. α was identified by its UV spectrum at a retention time of 32.8 min.

Step 5. Chromatography involved a C_{18} column (5 μm; 4.6 × 250 mm) eluted at 1 ml/min with a gradient of 50 mM acetic acid (A) and 45 mM acetic acid in acetonitrile (B) (85% A/15% B for 3 min and a linear gradient to 100% B over 42 min). Fifty half-minute fractions were collected starting at 10 min. LLU-α eluted at 24.8 min, as determined by its UV spectrum (Fig. 1) and bioactivity. The fractions containing LLU-α were pooled, dried, and subjected to one final chromatographic step. A LiChroCART LiChrospher 100 (C_{18} reversed-phase, 5 μm, 4 × 250 mm) (E. Merck, Darmstadt, Germany) column was eluted at 1 ml/min with water/acetonitrile/isopropanol/acetic acid (60:40:15:0.1). The major peak (R_t = 9.6 min) contained pure LLU-α.

In Vivo **Bioassay.** The assay for natriuresis in conscious rats has been described (18). Briefly, female Harlan Sprague Dawley rats (200–250 g) were cannulated in the femoral artery
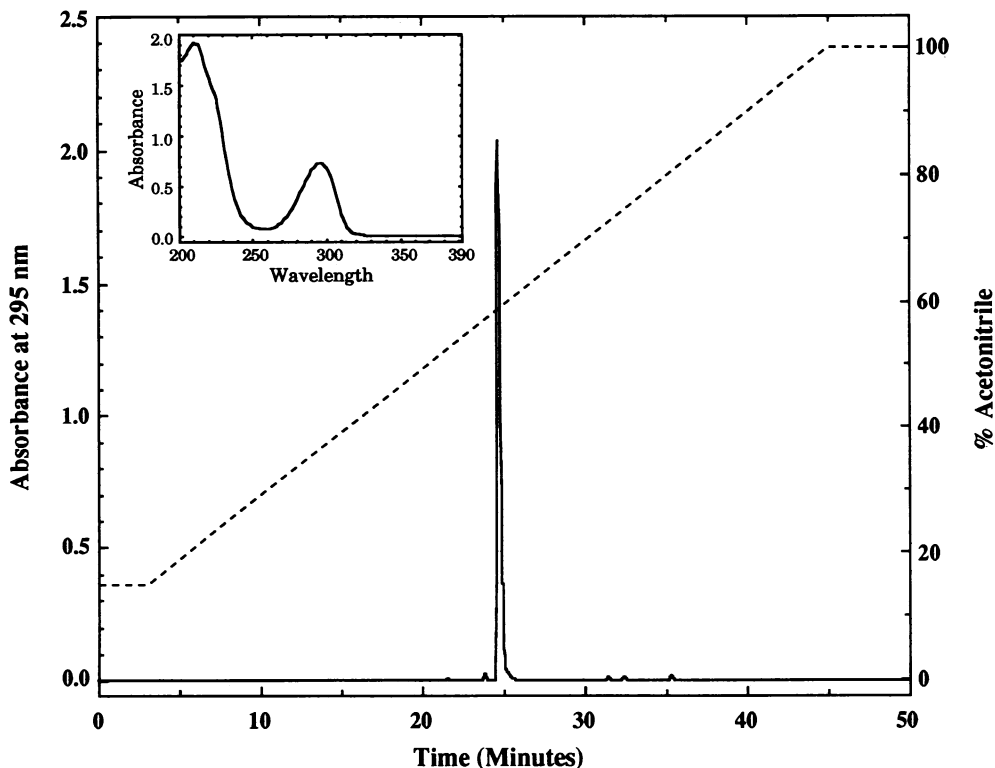

FIG. 1. Chromatogram of the acetic acid/acetonitrile purification step (step 5) for LLU-α. Absorbance at 295 nm of the eluant is shown as a solid line. The programmed acetonitrile gradient is plotted (dashed line). (*Inset*) UV spectrum, collected by diode array detector, of LLU-α eluting at retention time 24.8 min.

and vein for continuous monitoring of mean arterial pressure and infusion of saline and samples, respectively. The bladder was catheterized for collection of urine over 10-min periods. Furosemide (100 μg, $\approx$0.4 mg/kg body weight; 1 mg/ml in 0.17% saline) was injected as a positive control at the beginning of the sixth 10-min period. The sample was injected at the beginning of the 17th 10-min period. Urine was collected for another 150 min, and Na^+ and K^+ concentrations were determined with a Beckman E2A electrolyte analyzer. The volume of the urine was determined gravimetrically. From these data, the Na^+ excretion values (urine Na^+ concentration $\times$ urine volume per unit time, UNaV), as well as potassium excretion and urine volume, were calculated. The natriuretic response of a sample was normalized to the dose of furosemide infused. The net Na^+ excretion for the injection of furosemide or sample was calculated as follows. The median Na^+ excretion value (micromoles of Na^+ per 10-min period) for the five periods before injection of furosemide or sample was used to establish a baseline value for the calculation of ΔUNaV (micromoles of Na^+ per period $-$ baseline micromoles of Na^+) for the administration of either furosemide or sample, respectively. The sum of ΔUNaV for the four periods after injection of furosemide was the net Na^+ excreted for furosemide, defined as furosemide response (FR). The sum of ΔUNaV for the eight periods (150 min) after injection of the sample was the net Na^+ excreted for the sample, defined as sample response (SR). The natriuretic ratio (R) (or normalized natriuretic response) of a sample was calculated by dividing SR by FR (R = SR/FR). A sample was considered active if the R value for that sample was $\geq$0.55 (>99% confidence limits). For those experiments in which glomerular filtration rate (GFR) was determined, the assay was modified as follows. An additional cannula was implanted in the contralateral femoral vein for infusion of inulin-[^{14}C]carboxylic acid/saline solution. Before urine samples were collected, the animal was primed with 0.25 μCi (1 Ci = 37 GBq) inulin-[^{14}C]carboxylic acid in 1 ml of saline infused over 2 min. Then

a continuous infusion of 0.4 μCi inulin-[^{14}C]carboxylic acid per milliliter per hour was begun. At the midpoint of each urine sample period, a 200-μl sample of blood was collected via the arterial cannula. The hematocrit was determined, and 5 μl of the plasma was counted in 1 ml of scintillation cocktail. Five microliters of the concurrent urine sample was also counted in 1 ml of scintillation cocktail.

Patch-Clamp Experiments. As described (19), pathogen-free Harlan Sprague Dawley rats (male or female; 80–120 g) were killed. Kidneys were then removed, and thin coronal sections were cut. The cortical and outer medulla thick ascending limb tubules were dissected and immobilized onto cover glasses (5 $\times$ 5 mm) coated with Cell-Tak (Biopolymers, Farmington, CT). The cover glass was then placed in a chamber mounted on an inverted microscope, and the tubules were superfused at 37°C with Hepes-buffered saline (135 mM NaCl/5 mM KCl/1.5 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM glucose/10 mM Na^+ Hepes, pH 7.4). The apical membrane was exposed by cutting the tubule with a sharpened micropipette. Single-channel currents were recorded in cell-attached and inside-out configuration with 140 mM KCl in the pipette with a resistance of $\approx$4–6 MΩ. The recordings were collected by use of an amplifier (Axon Instruments, Foster City, CA; model 200A), an eight-pole Bessel filter (Frequency Devices, Haverhill, MA; model 902LPF), a pulse code modulator (Sony, Tokyo; model PCM-501ES), and a videotape recorder. The channel currents were low-pass filtered at 1 kHz and converted to digitized signals at a sampling rate of 44 kHz. Analysis was done using PCLAMP version. 6.02 (Axon Instruments).

Na^+/K^+-ATPase Inhibition Assays. The assay for inhibition of Na^+/K^+-ATPase by measuring the reduction of $^{86}Rb^+$ uptake into Madin–Darby bovine kidney cells has been described in detail (18). Briefly, Madin–Darby bovine kidney cells (ATCC no. CCL22) were maintained in DMEM with 5% fetal bovine serum and 5% bovine calf serum in a 5% CO_2/95% humidified air atmosphere at 37°C and divided (1:2) weekly. The assay was performed by allowing the cells to
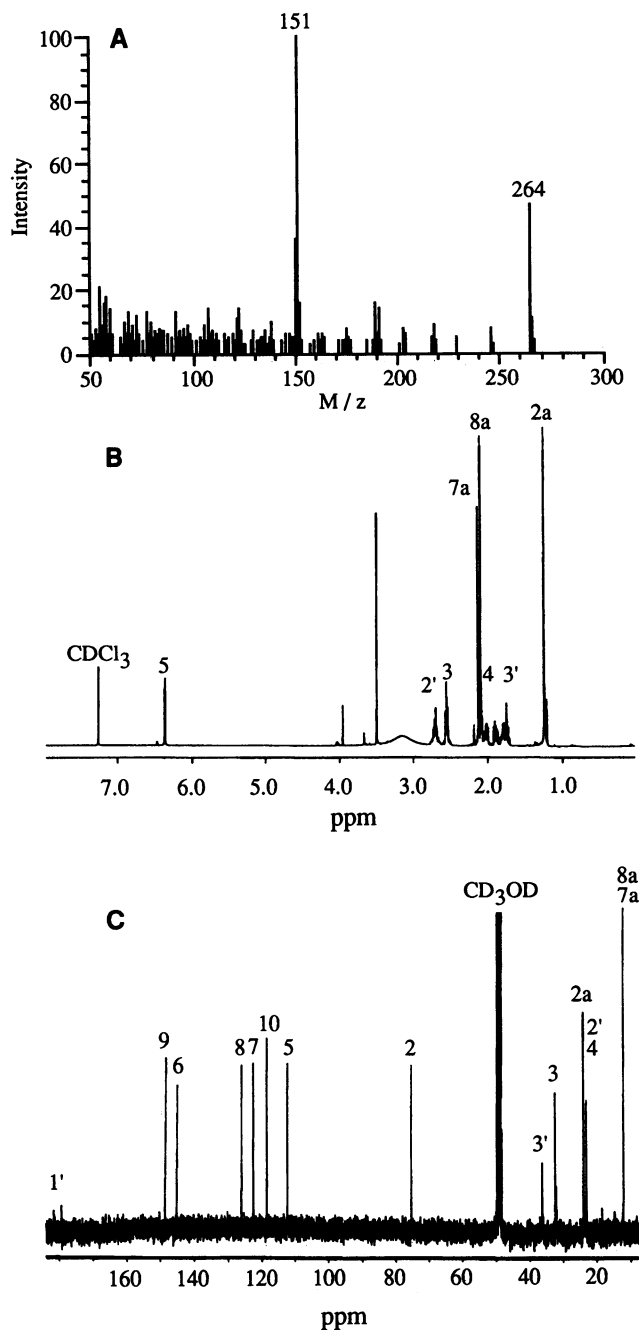
FIG. 2. Spectroscopic analysis of LLU-α. (*A*) EI-MS; the parent ion is m/z 264. (*B*) ¹H NMR; three methyl groups (2a, 7a, 8a) were readily identifiable. The multiplets for the two proton groups (2′, 3′, 3, 4) allowed for the assignment of two -CH₂ CH₂- moieties. The single proton at 6.3 ppm was initially identified as a vinyl (not aromatic) proton. (*C*) ¹³C NMR; a similarity of the chemical shifts to those of various tocopherol derivatives was noted.

adhere to a 96-well plate for 20–24 hr and then preincubating the compounds to be tested for 30 min with the cells before addition of ^{86}RbCl. As a positive control for inhibition of the Na$^+$ pump, ouabain was assayed concurrently over the range of 10^{-5}–10^{-8} M. All samples were assayed in quadruplicate and corrected for uptake inhibitable by ouabain.

Analytical Spectroscopy. The fractions containing pure LLU-α from the last purification step were combined, dried, and weighed. The dried sample (0.6 mg) was dissolved in 0.5 ml of CD₃OD (99.8%) and solvent was removed under a stream of argon. This step was repeated twice. In a dry argon atmosphere, the sample was then dissolved in 150 μl of

CD₃OD (¹²C 99.95%, D 99.5%) and transferred to a 2-mm NMR tube that was sealed. NMR spectroscopy of LLU-α was performed at the Department of Chemistry and Biochemistry at the University of California, Los Angeles. ¹³C (125.758 MHz) and ¹H (500.134 MHz) NMR and correlated spectroscopy spectra were recorded on a Bruker ARX 500 (Billerica, MA) using the UX NMR software. Electron-impact mass spectroscopy (EI-MS) were carried out at the University of California, Riverside with a resolution of 2000 Da and were recorded at an ionization voltage of 70 eV, at a source temperature of 220°C, and with introduction of sample by direct probe on a VG7070 EHF high resolution mass spectrometer. Fast atom bombardment MS was performed on a VG ZAB instrument (Manchester, U.K.).

General Synthetic Chemistry. Chemicals were obtained from Aldrich (Milwaukee, WI). All solvents were of analytical grade. Solvents were removed under reduced pressure at 40°C. The gravity and medium pressure chromatography was done on Silica gel (0.040–0.063 mm) (Bodmann ICN). NMR spectra for synthetic compounds were recorded on a General Electric GN-500 (¹H 500.135 MHz, ¹³C 125.768MHz) or a QE-300 (¹H 300.150 MHz, ¹³C 75.480 MHz) spectrometer at the Chemistry Department at the University of California, Riverside. Infrared spectra were acquired on Perkin–Elmer 1600 Series FTIR; data were processed on a personal computer with IR DATA MANAGER version 2.5 and Perkin–Elmer GEM version 3.02 software. EI-MS spectra were acquired on a gas chromatograph/mass spectrometer (Finnigan-MAT, San Jose, CA; model 4000), and data were processed on a personal computer with GALAXY 2000 (version 5.5; X-Spec Inc., Scott's Valley, CA). Elemental analyses were performed by Galbraith Laboratories Inc. (Knoxville, TN). Melting points were determined on a Fisher–Johns instrument and were uncorrected.

Synthesis of γ-Methyl-γ-Vinylbutyrolactone. Vinyl magnesium bromide (1 M in tetrahydrofuran, 400 ml, 0.44 mol) was added with vigorous stirring under N₂ at 0–5°C to a solution of ethyl levulinate (64 g, 0.40 mol) in tetrahydrofuran (60 ml) over 90 min. The reaction mixture was stirred for an additional 15 min then acidified with aqueous KHSO₄ solution (20%) to pH 4 and diluted with water (300 ml) to dissolve precipitated salts. This solution was extracted with ether (500 and 200 ml), and the combined etheral extracts were washed with water (150 ml), saturated NaHCO₃ solution (150 ml), and brine (150 ml) and then dried over Na₂SO₄. Ether was removed on a rotavapor to yield a yellow oily product that was distilled (20) under reduced pressure to yield γ-methyl-γ-vinylbutyrolactone (bp 110°C at 25 mm Hg) as a clear transparent liquid (27.8 g, 55%).

Synthesis of Racemic 2,7,8-Trimethyl-2-(β-Carboxyethyl)-6-Hydroxychroman. LLU-α was prepared by modified methodology (21). γ-methyl-γ-vinylbutyrolactone (15 mmol) in dioxane (4.0 ml) was added, via syringe over 60 min at 120°C (oil bath, reflux) under nitrogen, to a solution of 2,3-dimethyl-1,4-hydroquinone (10 mmol) and boron trifluoride diethyl etherate (20 mmol) in dioxane (20 ml, dried over Na). The reaction mixture was cooled to room temperature and diluted with ether (300 ml) and then washed with water (three times, 100 ml) and dried (Na₂SO₄), and the ether was removed under vacuum. The brown oily residue was dissolved in methanol (30 ml), and then the methanol was removed under vacuum. The brown semisolid was suspended in methanol (30 ml) and refrigerated for 18 hr. The resulting white powder was collected on a sintered glass funnel and washed with methanol. The filtrate was concentrated under vacuum and the brown oil was purified on a flash silica-gel column eluted with ethylacetate/hexane/acetic acid (500:300:1). The residual yellow oil was crystallized from ether/hexane (1:1) at 5°C. The white crystals were recovered on a sintered glass funnel, washed with hexane, and dried first in air and then in a vacuum desiccator (1.37 g, 52%).
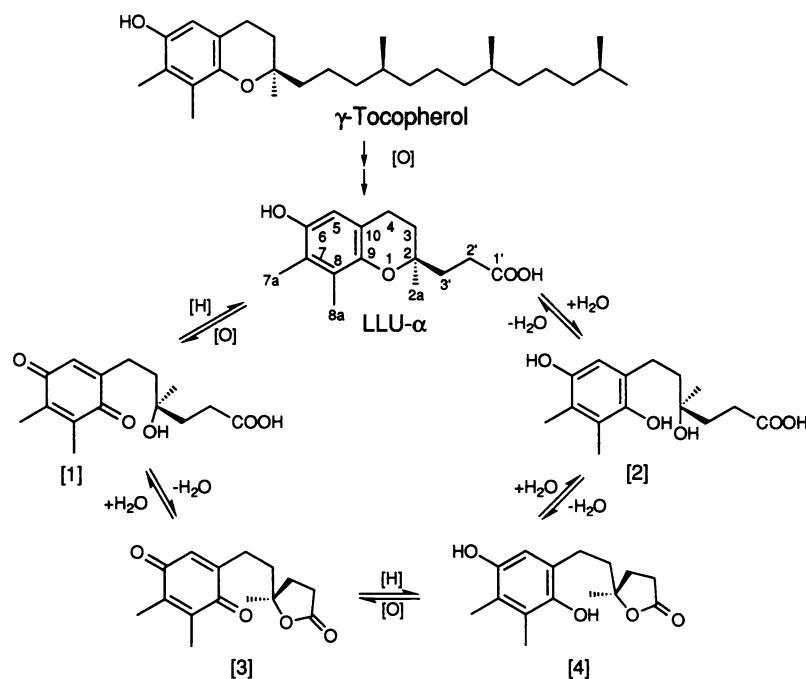
FIG. 3. Possible origin of LLU-α from γ-tocopherol and subsequent transformations. It is possible that the oxidation/reduction equilibrium and hydrolysis/dehydration equilibrium play a role in the modulation of natriuresis.

Racemic 2,7,8-Trimethyl-2-(β-Carboxyethyl)-6-Hydroxy-chroman (rac-LLU-α). Melting point 147–148°C; IR (KBr): 3700–2700, 1700, 1496, 1448, 1426, 1330, 1292, 1260, 1238, 1222, 1192 cm^{-1}; ^{1}H NMR, (CD$_3$OD) δ: 6.32 (s, 1H, CH), 2.69–2.65 (m, 2H, CH$_2$), 2.47–2.41 (m, 2H, CH$_2$), 2.06 (s, 3H, CH$_3$), 2.04 (s, 3H, CH$_3$), 1.98–1.92 (m, 1H, HCH), 1.88–1.80 (m, 1H, HCH), 1.80–1.70 (m, 2H, CH$_2$), 1.21 (s, 3H, CH$_3$); ^{13}C NMR (CD$_3$OD), (125 MHz) δ (ppm): 75.42 (2), 23.99 (2a), 36.39 (3), 23.50 (4), 112.43 (5), 145.36 (6), 122.69 (7), 12.22 (7a), 126.18 (8), 12.22 (8a), 148.79 (9), 118.65 (10), 179.48, 181.78 (1′), 23.14 (2′), 32.19, 32.52 (3′); fast atom bombardment MS: calculated: 264.1362, found: 264.1368 (C$_{15}$H$_{20}$O$_4$); UV (CH$_3$OH), λ (ε): 205.8 (32909), 223.0 (9311), 296.4 (4358) nm; EI-MS, m/z: 105, 106, 107, 111, 112, 113, 122, 123, 127, 129, 138, 149, 150, 151 (100%), 152, 185, 264 (11%, M$^+$) Da; Anal. (C$_{15}$H$_{20}$O$_4$) Calculated 68.16% C, 7.63% H, Found 68.08% C, 7.78% H.

1,8-Dioxaphenanthrene. For structure of this compound [5], see Fig. 4. By-product of synthesis of 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman; m.p. 225–227°C (crystallized from methanol); IR (KBr): 3800–2700, 1698, 1442, 1412, 1382, 1352, 1320, 1300, 1260, 1222, 1196, 1178, 1138, 1118, 1104 cm^{-1}; ^{1}H NMR, (CD$_3$OD) δ: 2.58–2.50 (m, 4H, 2xCH$_2$), 2.50–2.40 (m, 4H, 2xCH$_2$), 2.04 (s, 6H, 2xCH$_3$), 2.0–1.75 (m, 8H, 4xCH$_2$), 1.21 (s, 6H, 2xCH$_3$); UV (CH$_3$OH), λ (ε): 207 (55219), 225 (15693), 299 (5365) nm; EI-MS, m/z: 101, 105, 107, 109, 110, 111, 113, 115, 119, 121, 127, 129, 135, 149, 151,

163, 175, 203, 277, 390 (7%, M$^+$) Da; Anal. (C$_{22}$H$_{30}$O$_6$) Calculated 67.67% C, 7.74% H, Found 67.15% C, 7.87% H.

RESULTS

Purification of LLU-α from ≈800 liters of human uremic urine afforded ≈0.6 mg of pure material that was stable in the crystalline state. This material was subjected to spectroscopic analysis for structure elucidation. Fig. 2A shows the electron-impact mass spectrum of LLU-α whose exact molecular weight of 264.1368 (fast atom bombardment MS) best fit the formula C$_{15}$H$_{20}$O$_4$. The UV spectrum (λ$_{max}$ 205 and 294 nm) suggested an extended conjugated system or an aromatic ring. ^{1}H NMR (Fig. 2B), ^{13}C NMR (Fig. 2C), and MS (Fig. 2A) established the presence of the various functional groups and allowed for the generation of partial structures. The major fragmentation in the MS (m/z 151) along with the similarity of the ^{13}C chemical shifts to those in various tocopherol derivatives led to the proposed structure of LLU-α (Fig. 3).

This structure was confirmed by the unambiguous synthesis of racemic LLU-α from 2,3-dimethyl-1,4-hydroquinone and γ-methyl-γ-vinylbutyrolactone catalyzed by BF$_3$·Et$_2$O (Fig. 4). The synthesis was based on the known synthesis of 2,5,7,8-tetramethyl-2-(β-carboxyethyl)-6-hydroxychroman (5-Me LLU-α; 21) from the condensation of 2,3,5-trimethyl-1,4-hydroquinone and the same lactone. For the current synthesis, in which 2,3-dimethyl-1,4-hydroquinone is used, a by-product, 1,8-dioxa-2,7,9,10-tetramethyl-2,7-di-(3′-propionic acid)-3H,4H,5H,6H-phenanthrene [5] (Fig. 4), resulted and was characterized (see *Materials and Methods*). All spectroscopic data and HPLC retention times for urine-derived LLU-α and synthetic rac-LLU-α were identical (Fig. 2; data in *Materials and Methods*).

Bioassays of isolated LLU-α exhibited a parabolic dose response curve in our *in vivo* natriuresis/diuresis/kaliuresis/blood pressure assay (18) over the range of 4–8 μg/kg when given i.v. In this dose range, we observed significant natriuresis and mild diuresis with no evidence of kaliuresis nor any effect on continuously monitored mean arterial pressure. The high-purity synthetic racemic LLU-α exhibited the same response at 4–12 μg/kg. Fig. 5 is a representative bioassay of the synthetic
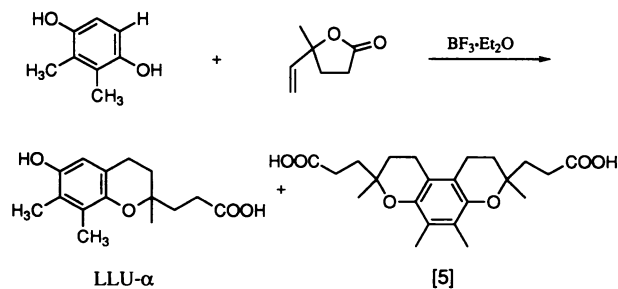

FIG. 4. Synthesis of rac-LLU-α.

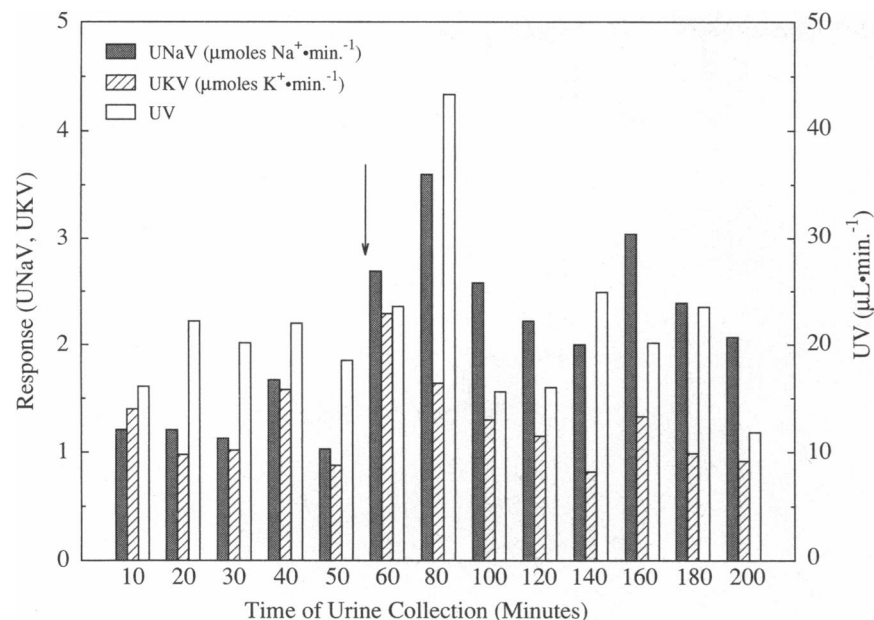FIG. 5. Natriuresis, kaliuresis, and diuresis resulting from the infusion of 1 μg (4 μg/kg) of synthetic, racemic LLU-α into a conscious, female Harlan Sprague Dawley rat. The arrow indicates the time of the infusion, immediately following the collection of the 10-min urine sample for the 50-min time point. The five 10-min periods before the infusion are the periods from which the median UNaV (in micromoles of Na$^+$ per minute) background value for calculation of the sample response is determined. For this assay, there is an increase in natriuresis and kaliuresis during the 10-min period after the injection. After that, the kaliuresis returns to background values, while the natriuresis remains elevated for the remainder of the experiment. Diuresis does not follow natriuresis. UKV, urine K$^+$ excretion volume; UV, urine volume.

racemic LLU-α that exhibits a prolonged natriuresis (as evaluated by the natriuretic ratio; $P = 0.026$ vs. vehicle controls; $n = 50$ for both experimental and control animals), mild diuresis, and absence of kaliuretic or blood pressure effects (unpublished data).

When GFR was evaluated over a range of doses (0.4–400 μg/kg) in this same animal preparation by radiolabeled inulin clearance, there was no apparent effect (Fig. 6). LLU-α also did not inhibit the Na$^+$/K$^+$-ATPase when evaluated over 1.9 $\times 10^{-5}$ to 1.9 $\times 10^{-10}$ M in Madin–Darby bovine kidney cells (unpublished data). Consequently, other biological systems are being examined to find a mechanism for the observed natriuresis. Fig. 7 represents a "patch clamp" experiment with thick ascending limb kidney cells that shows 86% reversible inhibition of 70 pS K$^+$ channel by 10 nM *rac*-LLU-α.

DISCUSSION

The natriuretic substance isolated in the present study, LLU-α, is quite likely a result of oxidative metabolism of γ-tocopherol. The known homolog, 5-Me LLU-α (a metabolite of α-tocoph-

erol), has been isolated from rabbit and human urine (22–25). However, this latter compound is not natriuretic in our assay (unpublished data), which suggests great specificity in the biology of LLU-α.

As with 5-Me LLU-α (22–25), mild oxidation provides the quinone [1] which is in equilibrium with the lactone [3] (Fig. 3). LLU-α is susceptible to hydrolysis [2] and cyclization to give the hydroquinone [4]. Following from the known chemistry of this system (22–25), LLU-α and compounds [1] through [4] probably result from the β-oxidation of (*R,R,R*)-γ-tocopherol. This reaction path would be expected to retain the spatial arrangement, which would be designated as (*S*) at C-2, assuming that γ-tocopherol is the source of LLU-α. LLU-α and its likely *in vivo* transformation products (Fig. 3) may be the source, all or in part, of helping modulate extracellular fluid volume in mammals.

LLU-α is not produced exclusively by human uremics. It has been isolated from normal human urine (4) along with other natriuretic factors as well as from urine from patients with congestive heart failure (unpublished data). The relative amounts of LLU-α detected in these urine samples are compared with that from uremic urine (Table 1). The amounts of LLU-α are rough estimates at this time. If the relative order of concentrations is maintained after further study, then the concentrations would be consistent with the concept of volume expansion resulting in the induction of a natriuretic substance or substances.

The finding that LLU-α inhibits a K$^+$ channel in the thick ascending limb may explain its natriuretic effect. Inhibition of this channel could significantly block the recycling of K$^+$ and, thereby, inhibit the function of the Na$^+$/2 Cl$^-$/K$^+$ cotrans-
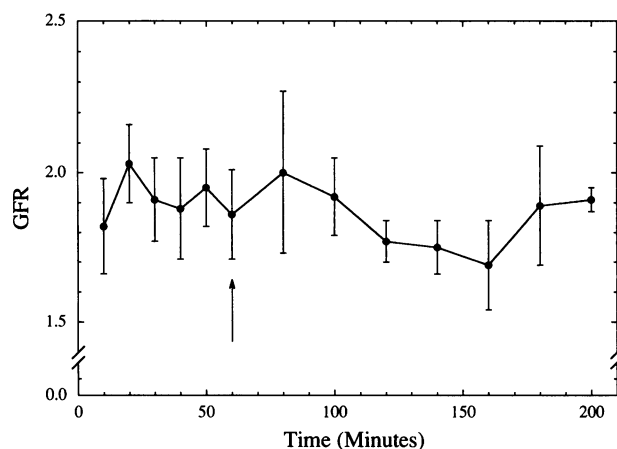

FIG. 6. Synthetic, racemic LLU-α does not affect GFR. GFR was measured for the 50-min baseline period before sample injection (indicated by the arrow) as well as for 150 min afterwards. The GFR was determined at 10-fold dose intervals over the range 0.4–400 μg/kg in a total of seven animals. (Bars = SE.) There was no significant change in GFR for any individual animal.

Table 1. Estimation of relative amounts of LLU-α present in human urine*

Normal	Uremics	CHF†
+/−	++	+
$n = 2/5$‡	$n = 10/10$	$n = 1/1$

*Because of the small volumes used ($\leq$3 liters) for some samples (especially samples from normal, healthy individuals), peak height or area of peak from step 5 chromatography was used instead of mass of material isolated.

†CHF, congestive heart failure.

‡The ratio given for n represents the number of individuals for which LLU-α was detected relative to the total number of individuals whose urine was examined.
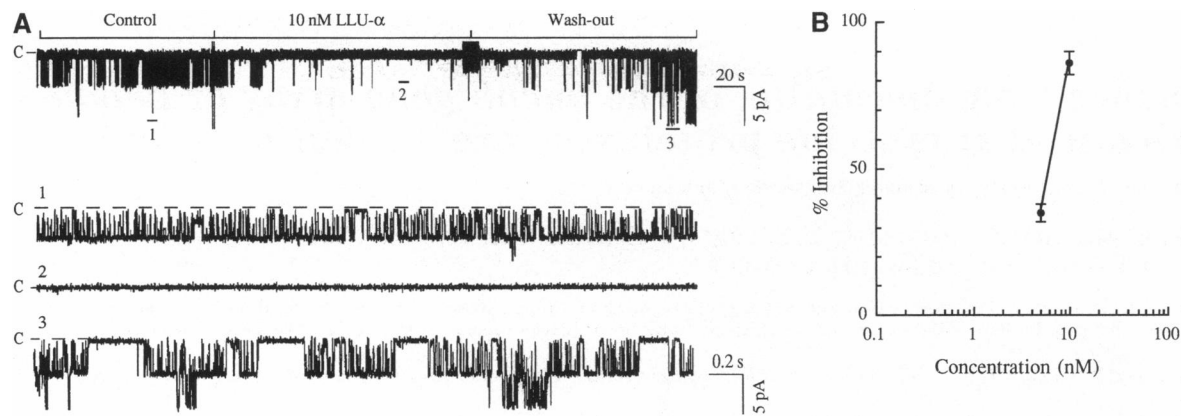
FIG. 7.   Effect of *rac*-LLU-α on the 70 pS K$^+$ channel. (*A*) A single channel recording made in an inside-out patch. The recording shows that 10 nM *rac*-LLU-α reversibly blocked the activity of the 70 pS K$^+$ channel in the apical membrane of the thick ascending limb of the rat kidney. The channel closed level is indicated by C. Three parts of the tracings indicated by a number are extended at fast time resolution at the bottom. (*B*) Inhibition of the 70 pS K$^+$ channel at two different concentrations of *rac*-LLU-α. The data represent the average of five determinations in as many patches. ID$_{50}$ = 5 nM.

porter, resulting in natriuresis (26–28). Other compounds that also ostensibly produce natriuresis via inhibition of K$^+$ channels have been described; however, LLU-α is the most potent inhibitor yet reported (29–31), with an ID$_{50}$ of about 5 nM.

The K$^+$ channel inhibition taken together with the lack of inhibition of the Na$^+$ pump and the absence of hemodynamic effects, as indicated by GFR, is consistent with our thesis concerning the putative natriuretic hormone (2, 18).

A large number of biological experiments remain to be carried out with LLU-α to understand its mechanism of action and its importance to the physiology of extracellular fluid volume control.

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