

## A $\gamma$ -Glutamyl Peptide Isolated from Onion (*Allium cepa* L.) by Bioassay-Guided Fractionation Inhibits Resorption Activity of Osteoclasts

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One gram of onion added to the food of rats inhibits significantly ( $p < 0.05$ ) bone resorption as assessed by the urinary excretion of tritium released from bone of 9-week-old rats prelabeled with tritiated tetracycline from weeks 1 to 6. To isolate and identify the bone resorption inhibiting compound from onion, onion powder was extracted and the extract fractionated by column chromatography and medium-pressure liquid chromatography. A single active peak was finally obtained by semi-preparative high-performance liquid chromatography. The biological activity of the various fractions was tested in vitro on the activity of osteoclasts to form resorption pits on a mineralized substrate. Medium, containing the various fractions or the pure compound, was added to osteoclasts of newborn rats settled on ivory slices. After 24 h of incubation, the tartrate-resistant acid phosphatase positive multinucleated cells, that is, osteoclasts, were counted. Subsequently, the number of resorption pits was determined. Activity was calculated as the ratio of resorption pits/osteoclasts and was compared to a negative control, that is, medium containing 10% fetal bovine serum only and to calcitonin ( $10^{-12}$  M) as a positive control. Finally, a single peak inhibited osteoclast activity significantly ( $p < 0.05$ ). The structure of this compound was elucidated with high-performance liquid chromatography–electrospray ionization–mass spectrometry, time-of-flight electrospray ionization mass spectrometry, and nuclear magnetic resonance spectroscopy. The single peak was identified as  $\gamma$ -L-glutamyl-*trans*-S-1-propenyl-L-cysteine sulfoxide (GPCS). It has a molecular mass of 306 Da and inhibits dose-dependently the resorption activity of osteoclasts, the minimal effective dose being  $\sim 2$  mM. As no other peak displayed inhibitory activity, it likely is responsible for the effect of onion on bone resorption.

**KEYWORDS:** *Allium cepa* L.; Alliaceae; glutamyl peptide; bone resorption; osteoporosis; nutrition

### INTRODUCTION

Bone mass in adult humans decreases with age, leading to an increased risk of fractures. Osteoporotic fractures, besides causing suffering to the patient, are a major burden to health care, as the direct expenditure for osteoporosis and associated fractures is around U.S. \$17 billion/year in the United States (1). From a medical and economical view it would, therefore, be desirable if low bone mass could be prevented. A nutritional approach would be an inexpensive means to achieve this goal.

However, the effects of the nutritional strategies recommended today are rather modest. Indeed, even the effect of calcium in milk on the relative risk of hip fractures seems to be restricted to the 10% of the female population with the lowest intake of calcium (2). Thus, research into novel nutritional strategies preventing bone loss is needed.

The addition of 7% of dried onion bulbs to the diet of rats decreases bone resorption and increases bone mineral content in growing rats (3). This effect is independent of the base excess of onion (4), that is, independent of potassium citrate metabolically generating base, thought by some to protect bone by neutralizing noncarbonic metabolic acid, which would otherwise dissolve bone. Furthermore, an ethanolic extract from onion prevented bone loss in an osteoporosis model and inhibited the resorption activity of osteoclasts in vitro (5, 6). This suggested

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that the inhibitory activity of onion on bone resorption could be due to a pharmacologically active compound.

A first fractionation of the ethanolic extract showed no activity *in vivo* of the flavonoid-containing fraction, but instead the activity eluted with the more polar compounds. This rendered the easy approach of testing pure flavonoid standards abundant in onion inappropriate. Thus, an isolation strategy was necessary. Because *in vivo* models are not suitable for a bioassay-guided fractionation due to the large amounts of material requested, we have tested a well-established *in vitro* model: the osteoclast pit assay. As the polar material also inhibited the resorption activity of osteoclasts, this *in vitro* culture system could be used as a bioassay, prompting us to undertake the isolation and identification of the unknown compound(s) in onion inhibiting bone resorption.

## MATERIALS AND METHODS

**Plant Material.** Dried food grade flakes from white onion bulbs (*Allium cepa* L., Liliaceae) were purchased from Landolt and Hauser Inc., Näfels, Switzerland. The moisture was removed by adsorption over silica gel before grinding. Four hundred gram aliquots of onion powder were packed in polyethylene bags from which the air was evacuated before sealing. They were stored at 4 °C until use.

**Extraction.** Four hundred grams of onion powder was extracted twice with 2000 mL of aqueous ethanol (85% v/v) at 60 °C for 1 h and filtered. After evaporation of the ethanol, the aqueous residue was freeze-dried and stored at -20 °C.

**Bioassay-Guided Fractionation.** *Fractions A and B.* About 38 g of the onion extract was redissolved in 600 mL of aqueous ethanol 15% (v/v) and heated at 60 °C for 30 min under constant stirring. After cooling to room temperature, the mixture was centrifuged for 20 min at 7000 rpm and the supernatant subjected to fractionation by adsorption column chromatography (residue discarded). Fractions A and B resulted by using Amberlite XAD-4 (Fluka Chemie, Buchs, Switzerland) as stationary phase and eluting with (1) 1280 mL of aqueous ethanol 15%, (2) 1280 mL of water, and (3) 1400 mL of aqueous ethanol 85% at a flow of 10 mL/min. Thin-layer chromatography (TLC) on 10 × 10 cm silica gel 60 F<sub>254</sub> plates (Merck, Darmstadt, Germany) with chloroform/methanol/water (6.4:5:1, v/v) as mobile phase and detection with a 1% methanolic solution of diphenylboric acid/β-ethylaminoate, followed by a 5% ethanolic solution of polyethylene glycol 4000, showed the hydrophilic fraction A completely devoid of flavonoids, whereas the lipophilic fraction B contained flavonoids. Fractions A and B were then tested *in vivo* and *in vitro* by using the urinary [<sup>3</sup>H]tetracycline excretion rat model and the osteoclast pit assay, respectively. Fraction A showed bone resorption inhibiting effects, whereas fraction B was inactive and therefore discarded.

*Fractions A1 and A2.* One-gram aliquots of fraction A, dissolved in 5 mL of mobile phase, were then further separated by medium-pressure liquid chromatography (MPLC) using a model 681 pump and a 684 fraction collector (Büchi, Flawil, Switzerland) on a 46 × 2.5 cm, 15–25 μm Lichroprep RP-18 column (Merck) with a 13 × 1 cm precolumn. Elution was with (1) 340 mL of 5% aqueous methanol and (2) 300 mL of methanol, at a flow rate of 4 mL/min. Monitoring of the 60 4-mL fractions was performed by high-performance thin-layer chromatography (HPTLC) on 10 × 10 cm RP-18 F<sub>254</sub> plates (Merck) with water/methanol (19:1) as mobile phase and anisaldehyde reagent (5% acetic acid solution of anisaldehyde followed by heating at 120 °C) for detection. Fraction A1 contained mainly saccharides (glucose, fructose, and sucrose) and was active *in vitro*; fraction A2 was free of saccharides, almost inactive, and therefore discarded.

*Fractions A1-1, A1-2, A1-3, and A1-4.* Four hundred milligram aliquots of fraction A1 were further separated by MPLC on a 22 × 2.5 cm, 15–40 μm silica gel 60 column (Merck) with a 13 × 1 cm precolumn. Elution was first with 1208 mL of chloroform/methanol/water (6.4:5:1) followed by 300 mL of 70% methanol, at a flow rate of 4 mL/min. Monitoring of the 8-mL fractions (120 in total) was performed by TLC on 10 × 10 cm silica gel 60 F<sub>254</sub> plates (Merck) with *n*-butanol/*n*-propanol/acetic acid/water (3:1:1:1) as mobile phase

and anisaldehyde reagent for detection. The now saccharide-free fraction A1-4 showed a significant *in vitro* activity; the saccharide-containing fractions A1-1, A1-2, and A1-3 were not active in the osteoclast pit assay and therefore not further studied.

*Fractions A1-4A, A1-4B, A1-4C, and A1-4D.* Aliquots of 0.125 mg of fraction A1-4 were then finally purified by semipreparative, isocratic HPLC on an HP 1090 liquid chromatograph with diode array detection (DAD) (Hewlett-Packard, Waldbronn, Germany) using a 250 × 10 mm i.d., 5 μm Spherisorb ODS-1 column (filled in our laboratory by using a slurry technique) at 40 °C. The mobile phase was water/acetonitrile (1:1), containing 0.006% formic acid at a flow rate of 1.5 mL/min. Detection was at 195 nm. Equal fractions were pooled, evaporated, freeze-dried, and tested *in vitro*. Fraction A1-4C (yield = 15.2% w/w), corresponding to the major HPLC peak and chromatographically pure, inhibited osteoclast activity nearly as strongly as fraction A1-4; all other fractions were inactive.

**Scaled-up Isolation of A1-4C.** For the preparation of larger amounts of A1-4C (γ-L-glutamyl-*trans*-S-1-propenyl-L-cysteine sulfoxide, GPCS), 10.8-g aliquots of fraction A, dissolved in water, were first separated by cation exchange column chromatography on a 43 × 3 cm i.d. column filled with Dowex 50WX8 (Fluka Chemie). The noncationic fraction eluted with 975 mL of water and the cationic fraction with 1425 mL of 0.75 M ammonium hydroxide at a flow rate of 15–20 mL/min. TLC on silica gel 60 F<sub>254</sub> with *n*-butanol/*n*-propanol/acetic acid/water (3:1:1:1) as mobile phase and acetaldehyde and ninhydrin reagent for detection was used for the fraction monitoring. In addition, the fractions were checked by HPLC-DAD, using a 125 × 4 mm i.d., 3 μm Spherisorb ODS-1 column, water/acetonitrile (1:3), containing 0.05% phosphoric acid at a flow of 0.7 mL/min as mobile phase, and detection at 195 nm. The noncationic fraction contained mainly saccharides, whereas in the cationic fraction an enrichment of peptides and amino acids could be observed. Consequently, 700-mg aliquots of the freeze-dried cationic fractions were further separated by anion exchange column chromatography on a 400 × 26 mm i.d. column filled with Dowex 1X8 (Fluka Chemie). Elution was with (1) 237.5 mL of 0.1 M acetic acid, (2) 1250 mL of 0.5 M acetic acid, and (3) 1000 mL of 2 M acetic acid; the flow rate of 95 mL/h was maintained by a Minipuls 3 pericyclic pump (Gilson, Villiers-le-Bel, France), and the 12.5-mL fractions were collected and monitored by TLC. Identical fractions were pooled and freeze-dried prior to a final check by HPLC-ESI-MS and HPLC-DAD.

**Structure Elucidation of Compound A1-4C.** *HPLC–Electrospray Ionization–Tandem Mass Spectrometry (HPLC-ESI-MS-MS).* The instrumentation consisted of an HP 1100 liquid chromatograph with DAD (Hewlett-Packard), linked to an LQC ESI mass spectrometer (Finnigan, Bremen, Germany). Separation was performed isocratically at 40 °C on a 125 × 4 mm i.d., 3 μm Spherisorb ODS-1 column (Macherey-Nagel, Düren, Germany) with water/acetonitrile (1:4) containing 0.05% formic acid at a flow rate of 0.7 mL/min. The collision gas was helium, the energy 35%. Measurements were performed in both the positive and negative ionization modes.

*ESI-MS-MS.* For confirmation of the results obtained by HPLC-ESI-MS-MS, A1-4C was further analyzed after acidic hydrolysis by 70% formic acid (100 °C for 22 h) by direct inlet ESI-MS-MS. The instrument was an Applied Biosystems/Sciex Qstar Pulsar mass spectrometer (Foster City, CA), which is a hybrid quadrupole time-of-flight (TOF) MS equipped with a nano-electrospray ion source.

*Nuclear Magnetic Resonance Spectroscopy (NMR).* NMR experiments were performed on a Bruker DRX500 instrument (Karlsruhe, Germany). Experiments were run at 500 MHz for <sup>1</sup>H and at 125 MHz for <sup>13</sup>C. D<sub>2</sub>O was purchased from Euriso-Top (Gif-sur-Yvette, France), and chemical shifts are reported relative to trimethylsilylpropionic acid (sodium salt) as an external standard (Wilmad Corp., Buena, NJ).

Solvents for all HPLC experiments were of Lichrosolv gradient grade quality; chemicals and solvents for MPLC and column chromatography as well as TLC detection reagents were of analytical quality from Merck. The pure GPCS standard was obtained from the New Zealand Institute for Crop and Food Research (Christchurch, New Zealand).

**Quantitative Determination of GPCS in Onion by HPLC.** GPCS in onion was quantified using an HP 1090 liquid chromatograph with DAD set at 195 nm (Hewlett-Packard). Analysis was performed

isocratically at 40 °C on a 125 × 4 mm i.d., 3 μm Spherisorb ODS-1 column (Macherey-Nagel) with water/acetonitrile (1:3, v/v) containing 0.05% phosphoric acid at a flow rate of 0.7 mL/min. The extract was prepared from ~1 g (accurately weighed) of dried, pulverized onion with 50 mL of methanol/water (50:50, v/v), containing 0.05% formic acid, by stirring at room temperature for 5 min according to the method of Mütsch-Eckner et al. (7). This method allowed an efficient extraction of polar compounds using hydrophilic solvents and at the same time inhibiting cleaving enzymes such as glutamyl peptidases and alliinases by the addition of methanol. The residues remaining after filtration were re-extracted twice to thoroughly extract GPCS. Finally, the methanol was removed from the filtrates in vacuo prior to freeze-drying. The residue was redissolved in 5.00 mL of HPLC mobile phase, and 10 μL was injected into the HPLC. GPCS calibration was performed using the external standard method and calculating the peak areas. The calibrator concentrations were 0.01, 0.05, 0.1, 1.5, and 0.2 mg/mL isolated GPCS. Each calibration point was measured three times. For the intra- and interday variability five onion extracts were prepared and analyzed two times within the same day and within 1 week, respectively. The limit of detection (LOD) was defined as the lowest detectable GPCS amount at a signal-to-noise ratio of 5:1.

**Biological Testing. Animals.** Wistar Hanlbm rats (RCC Ltd., Füllinsdorf, Switzerland) were reared and kept in standard animal facilities that comply with the Swiss and U.S. National Institutes of Health guidelines for the care and use of experimental animals. The experiment performed was approved by the State Committee for the Control of Animal Experimentation. At completion of the experiment the rats were killed with carbon dioxide.

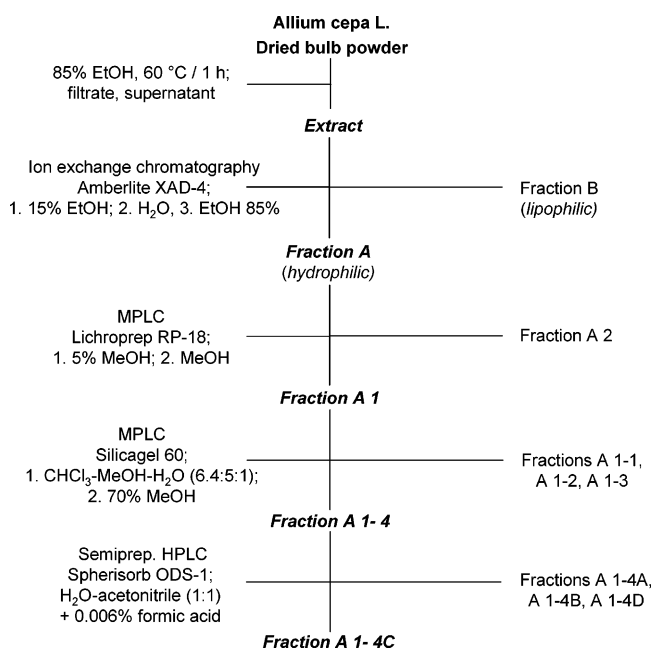
**In Vivo Assessment of Bone Resorption.** The urinary excretion of <sup>3</sup>H-labeled tetracycline (<sup>3</sup>H]Tc) from chronically prelabeled rats was used to assess bone resorption (3, 4, 8–11). This model has been validated previously by finding the expected effect of many inhibitors of bone resorption, such as bisphosphonates, calcitonin, and estrogen used clinically, or of stimulators of bone resorption, such as parathyroid hormone and dietary calcium restriction. For the present experiment 3 Wistar Hanlbm dams with 12 3-day-old male pups each were purchased. The 36 pups were injected from the first week of life twice a week for 6 weeks with increasing amounts of [<sup>3</sup>H]Tc (9). [<sup>3</sup>H]Tc is deposited into bone and is released when bone is resorbed (9). After discontinuation of labeling, the rats were transferred to metabolic cages. After 10 days of acclimatization, baseline bone resorption was monitored by measuring the daily urinary <sup>3</sup>H excretion. After 10 days of baseline measurement, the 10-day dietary intervention was started in rats, which were homogeneously assigned to the groups; that is, the baseline <sup>3</sup>H urinary excretion of all rats was ranked, and to each treatment group was assigned one animal with a similar rank until the number of animals per group was completed (*n* = 6 for the control group; *n* = 5 per treatment group). Using this protocol the mean <sup>3</sup>H excretion was similar for all groups at the start of the dietary intervention. <sup>3</sup>H in urine was determined by liquid scintillation counting. Aliquots of 1 mL of urine were counted in 10 mL of Irga-Safe Plus scintillator (Packard International, Zürich, Switzerland), and the result (dpm) was multiplied by the 24-h urine volume.

**Feeding and Diet.** From the time when the rats were housed in the metabolic cages, they were given demineralized water to drink. The diets were given in a stainless steel crucible as wet food to minimize spillage in the cage; thus, deionized water was added to batches of food powder to give a doughlike consistency, which allowed food-balls to be formed. During the 10-day acclimatization period in the metabolic cages and during the 10-day baseline urine collection, the rats were fed a standardized "normal" diet 2134 (Kliba-Mühlen, Kaiseraugst, Switzerland) with similarly high Ca and P concentrations (1.1 g of Ca and 1.2 g of P per 100 g) as used in the "semipurified" diet described below. During the acclimatization period the rats were trained to consume 23 g of wet food/day (13.1 g of dry matter); rats that repeatedly did not eat the whole daily amount were eliminated during this period. For the dietary intervention the dry additives were mixed with a semipurified diet (see Table 1) (12). Thus, 1 g of dried onion or 639 g of dried ethanolic onion extract corresponding to 1 g of onion, or 595 mg of the dried hydrophilic fraction A corresponding to 1 g onion, or 7.1 mg of the dried lipophilic fraction B corresponding

**Table 1.** Composition of the Semipurified Diet to Which Onion, Onion Extract, or the Fractions Were Added

ingredient	g/kg of diet	ingredient	g/kg of diet
sodium caseinate	200.0	potassium carbonate	5.6
corn starch	521.0	magnesium oxide	2.0
dextrose	100.0	sodium chloride	1.1
pork fat	30.0	vitamin mix <sup>a</sup>	10.0
cellulose	40.0	trace element mix <sup>b</sup>	35.0
dicalcium phosphate	36.5	choline chloride (50%)	2.3
potassium phosphate	13.5	DL-methionine (25%)	3.0

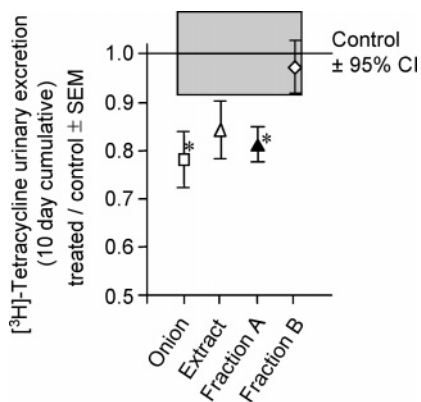
<sup>a</sup> Providing the following addition of vitamins per kg of diet: retinyl acetate, 1.34 mg; cholecalciferol, 0.025 mg; vitamin E, 100 mg; vitamin K<sub>3</sub>, 4 mg; vitamin B<sub>1</sub>, 6 mg; vitamin B<sub>2</sub>, 6 mg; nicotinic acid, 30 mg; pantothenic acid, 16 mg; folic acid, 2 mg; vitamin B<sub>6</sub>, 7 mg; vitamin B<sub>12</sub>, 0.05 mg; biotin, 0.2 mg; and choline, 1000 mg. <sup>b</sup> Providing the following additions of trace elements per kg of diet: copper, 8 mg; zinc, 38 mg; iron, 80 mg; iodine, 0.8 mg; manganese, 11 mg; and selenium, 0.18 mg.



**Figure 1.** Bioassay-guided fractionation of an ethanolic extract of *A. cepa* L. Fractions active in the osteoclast pit assay are shown in bold-italic (in the middle of the scheme); inactive fractions (on the right side of the scheme) were not further studied.

to 1 g of onion was given as daily treatment to each of the five rats per group. Appropriate amounts of the items to be investigated were added to batches of wet food sufficient for feeding five rats during 10 days. The additions were mixed with the diet according to the pharmaceutical art to achieve homogeneity. That is, if the addition was 31.5 g (50 + 3 portions of 595 mg) as for example in the case of fraction A (Figure 2), in a first step this material was carefully mixed with 31.5 g of diet. The resulting 63 g was then mixed with an additional 63 g of plain diet and so forth until the final 1219 g of diet including fraction A was obtained. These diets were then aliquoted into daily portions and kept frozen at -20 °C until use. The calcium and phosphate concentrations of the diets was verified in triplicate ashed samples dissolved in 1 mol/L HCl. Calcium was determined by atomic absorption spectrophotometry and phosphate by photometry (8, 13). The values given by the manufacturer were confirmed.

**Bioassay (in Vitro Assessment of Osteoclast Activity).** Osteoclasts were isolated from femora and tibiae of six 2-day-old rats and pooled, and aliquots of the cell suspension were settled for 40 min onto 4 × 4 mm and 0.6 mm thick ivory slices used as the mineralized substrate. The ivory was a gift from the zoo in Basel, Switzerland. After nonadherent cells had been washed off, individual slices were trans-



**Figure 2.** Effect on bone resorption in rats fed a purified diet containing (□) 1 g of dried onion, (△) 639 mg of dried ethanolic onion extract corresponding to 1 g of onion, (▲) 595 mg of the dried hydrophilic fraction A corresponding to 1 g of onion, and (◇) 7.1 mg of the dried lipophilic fraction B corresponding to 1 g of onion. The 95% confidence interval (CI;  $1.96 \times \text{SEM}$ ) of the untreated control group is given as a box with shaded background. Values are means  $\pm$  SEM.  $n = 6$  for control and  $n = 5$  for treated. \*,  $p < 0.05$ , indicates the significance of the difference of control versus treatments as assessed with Student's  $t$  test.

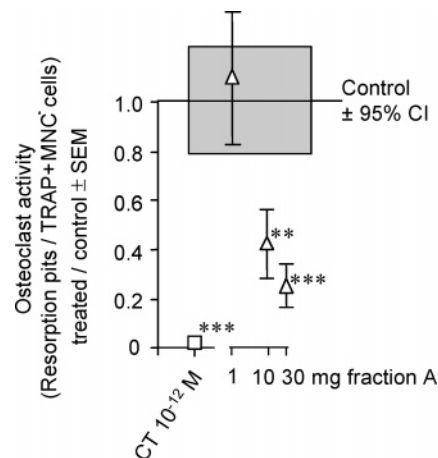
ferred to 48-well tissue culture plates and incubated for 24 h at 37 °C in a 5% CO<sub>2</sub>/air atmosphere (14, 15) in medium containing 10% fetal bovine serum (FBS) with or without the material to be tested. The concentration of bicarbonate in the MEM Earle's medium was reduced to 15 mM by the addition of 12 M HCl. In each experiment calcitonin (salmon calcitonin) (Novartis Pharma, Basel, Switzerland) was added as positive control at the concentration of  $10^{-11}$  or  $10^{-12}$  M. In one experiment parathyroid hormone (PTH bovine 1-34) (Bachem, Bubendorf, Switzerland) was added at the concentration of  $10^{-8}$  M, to stimulate bone resorption. For each material to be tested eight slices were used; for the untreated control  $2 \times 8$  slices were used. After fixation, osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) (Sigma, Buchs, Switzerland) and were counted blinded as TRAP positive (TRAP+) multinucleated (>2 nuclei) cells (MNC). After removal of the cells, the slices were sputter-coated with gold and the resorption pits counted blind (16). Osteoclastic resorption activity is calculated as the ratio of resorption pits to TRAP+MNC.

**Statistics.** The 95% confidence interval (95% CI) of the pertinent controls was calculated by multiplying the SEM by 1.96 and is given as a shaded box. Values of the treated groups are given as mean  $\pm$  SEM. The significance of differences was evaluated with Student's  $t$  test by using the GraphPad InStat statistical software (GraphPad Software, Inc., San Diego, CA), version 3.05.

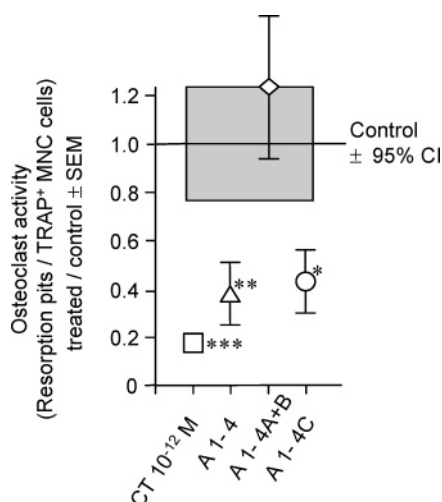
## RESULTS AND DISCUSSION

Consecutive bioassay-guided chromatographic fractionation (Figure 1) on nonionic-polymeric, reversed phase and normal phase columns of the active in vivo and in vitro ethanolic extract resulted in fractions A (51.5%, average yield corresponding to prior fraction), A1 (36.5%), and A1-4 (7.3%), respectively. Ten and a half milligrams of fraction A1-4 was purified by semipreparative RP-18 HPLC, leading to 1.6 mg (15.2%) of fraction A1-4C (GPCS).

The bone resorption inhibitory activity from onion was associated with the polar fraction A (Figure 2). On the contrary, fraction B containing flavonoids was devoid of activity when tested in vivo at a dose corresponding to 1 g of dry onion. This contrasts with the opinion of others who have proposed that rutin (17), a flavonoid abundant in onion, could be responsible for the observed effect of onion and other vegetables (3). In that study a single pharmacological dose of rutin inhibited bone loss in rats, a dose that was, however, much higher than that



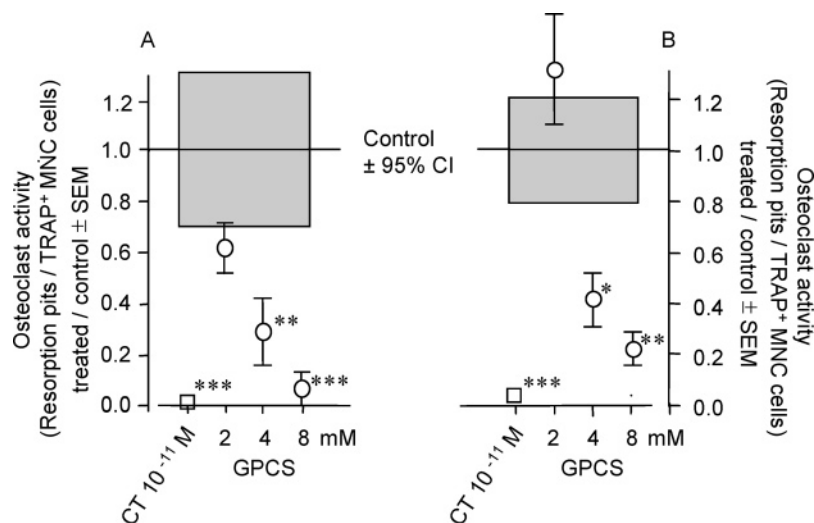
**Figure 3.** Dose-dependent effect of the hydrophilic fraction A and of calcitonin on in vitro resorption activity of osteoclasts. Fraction A was added to the medium at concentrations of 1, 10, and 30 mg/mL and calcitonin at  $10^{-12}$  M. Data presentation: the values of the treated groups ( $n = 8$ ) are given as the ratio treated/untreated  $\pm$  SEM. The 95% CI ( $1.96 \times \text{SEM}$ ) of the untreated group ( $n = 16$ ) is given as mean  $\pm$  SEM (box with shaded background). \*\*,  $p < 0.01$ , and \*\*\*,  $p < 0.001$ , indicate the significance of the difference of control versus treatments as assessed with Student's  $t$  test.



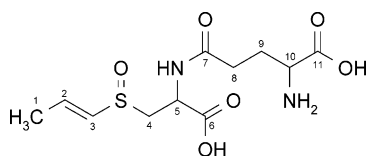
**Figure 4.** Effect of various onion fractions and calcitonin on in vitro resorption activity of osteoclasts. Fractions A1-4, A1-4B, and A1-4C were added to the medium at concentrations of 2.28, 0.43, and 0.53 mg/mL, respectively. Calcitonin was used at the dose of  $10^{-12}$  M. Data presentation is as described in Figure 2. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , and \*\*\*,  $p < 0.001$ , indicate the significance of the difference of control versus treatments as assessed with Student's  $t$  test.

contained in 1 g of onion (3). Therefore, it is not certain whether rutin contributes to the inhibition of bone resorption in vivo (18, 19).

Fraction A also inhibited the resorption activity of osteoclasts in vitro (Figure 3) when tested at doses corresponding to 17.4 and 52.2 mg/mL of dry onion equivalents. Therefore, this in vitro model could be used as a bioassay because it also requires only small amounts of material for activity testing. Fraction B was also investigated in this model (results not shown) at doses equivalent to 9, 17, and 26 mg/mL dry onion, that is, 0.06, 0.12, and 0.18 mg/mL medium. At the higher concentrations the lipophilic material was cytotoxic (very low number of surviving cells and many fragments of necrotic cells), whereas at 9 mg/mL onion equivalents, a dose with only a negligible effect on



**Figure 5.** Effect of GPCS and calcitonin on in vitro resorption activity of osteoclasts. GPCS was added to the medium at concentrations of 2, 4, and 8 mM. Calcitonin was used at the dose of  $10^{-11}$  M. Two separate experiments were performed: one without the addition of parathyroid hormone (**A**) and one in which to all cultures PTH ( $10^{-8}$  M) was added to stimulate bone resorption (**B**). Data presentation is as described in **Figure 3**. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , and \*\*\*,  $p < 0.001$ . indicate the significance of the difference of control versus treatments as assessed with Student's  $t$  test.



**Figure 6.** Structure of  $\gamma$ -L-glutamyl-*trans*-S-1-propenyl-L-cysteine sulfoxide (GPCS).

cell number, we could not detect an effect. Thus, fraction B containing the onion flavonoids was considered also as not inhibitory in vitro. This is in agreement with our additional evidence suggesting that rutin cannot explain the effect of vegetables on bone metabolism (18).

Further extensive isolation work by using semipreparative HPLC and activity screening allowed A1-4C to be identified as the only active fraction derived from the starting material A1-4 (**Figure 4**). Indeed, the pooled HPLC peaks eluting before A1-4C (fractions A1-4A and A1-4B) were not active. Furthermore, fraction A1-4D was also tested (2.53 mg/mL), but it was cytotoxic so that no conclusion could be drawn (results not shown). As HPLC showed that fraction A1-4C consisted of a single compound, its subsequent identification was attempted.

HPLC-ESI-MS-MS experiments with the compound **A1-4C** showed parent ions of  $m/z$  307 and 305 in the positive and negative ionization modes, respectively. Thus, the uncharged molecular ion of the compound in fraction A1-4C was 306 amu. A survey of the literature on onion compounds (20) revealed the compound to be  $\gamma$ -L-glutamyl-*trans*-S-1-propenyl-L-cysteine sulfoxide (GPCS) (**Figure 6**). Moreover, an observed ion of  $m/z$  130 confirmed the presence of a  $\gamma$ -glutamyl-type bond in the molecule as described by other investigators (21). In the ESI-MS-MS spectra after acid hydrolysis, fragments corresponding to glutamic acid, cysteine, and cystine could be observed, thus confirming the hypothesis.

$^1\text{H}$ ,  $^1\text{H}/^1\text{H}$  COSY, and  $^{13}\text{C}/^1\text{H}$  HSQC NMR measurements confirmed the presence of the two amino acids, glutamic acid (C7, C8, C9, C10, and C11) and cysteine (C4, C5, and C6) and an aliphatic C-chain with a double bond (C1, C2, and C3) in the molecule (**Figure 6**). The coupling constant of 15.26 Hz between H2 and H3 indicated a *trans* configuration (22). For chemical shifts and couplings see **Table 2**. Focusing on the  $^{13}\text{C}$ -carboxy region in the  $^{13}\text{C}/^1\text{H}$  HMBC spectrum revealed

**Table 2.** NMR Data for GPCS in  $\text{D}_2\text{O}$

position	$\delta_{\text{C}}$	$\delta_{\text{H}}$ multiplicity ( $J$ , Hz)
1	21.0	1.95 dd ( $J_{1,2}$ 6.87, $J_{1,3}$ 1.54)
2	147.0	6.65 dq ( $J_{2,1}$ 6.87, $J_{2,3}$ 15.26)
3	133.0	6.50 dq ( $J_{3,1}$ 1.54, $J_{3,2}$ 15.26)
4	57.0	3.30 dd ( $J_{5,4}$ 9.77, $J_{5,5}$ 13.28)
		3.45 dd ( $J_{5',4}$ 4.43, $J_{5',5}$ 13.28)
5	54.0	4.50 dd ( $J_{4,5}$ 4.43, $J_{4,5}$ 9.77)
6	178.5	
7	179.0	
8	35.0	2.50 t ( $J_{8,7}$ 7.78)
9	30.0	2.15 tt ( $J_{7,6}$ 6.41, $J_{7,8}$ 7.78)
10	57.0	3.80 t ( $J_{6,7}$ 6.41)
11	178.1	

couplings of the  $\gamma$ -C7 of the glutamic acid (174.00 ppm) with the  $\alpha$ -H5 of cysteine (4.50 ppm) and with the two  $\gamma$ -H8 of glutamic acid (2.50 ppm) and a coupling of the  $\alpha$ -C11 of the glutamic acid (173.10 ppm) only with the  $\alpha$ -H10 of glutamic acid (3.80 ppm). This proves clearly the presence of a  $\gamma$ -glutamyl-type bond in the molecule. These findings were confirmed by spectroscopic comparison with a pure reference sample of GPCS (23), which was analyzed under the same conditions.

Scaled-up isolation of GPCS was carried out by subjecting 108.0 g of fraction A first to cation and then to anion exchange column chromatography, yielding 6.97 g (6.5%) of cationic fraction and 788.0 mg of GPCS (0.7%), respectively. Fractions devoid of GPCS were discarded. GPCS isolated by this methodology inhibited the resorption activity of osteoclasts dose-dependently, irrespective of whether the osteoclasts were stimulated with PTH (**Figure 5**). However, the magnitude of the inhibition was somewhat smaller when PTH was added to the cultures than when it was not. This seemed also to apply for the positive control calcitonin. Fraction A1-4C was active at the concentration of 0.53 mg/mL, that is, at 1.7 mM GPCS, whereas in the present experiment the inhibition was just not significant at 2 mM. Taken together, the minimal effective dose of GPCS in this model appears to be  $\sim 2$  mM in cultures not stimulated with PTH. In cultures stimulated with PTH this value falls between 2 and 4 mM; a graphical interpolation from **Figure 5** suggests that it may be  $\sim 3$  mM. As no other constituent of fraction A1-4C displayed inhibitory activity, GPCS appears to

be responsible for the effect of onion on bone resorption in vitro, a contention that should be confirmed in vivo as soon as sufficient compound is available. Future studies are also required to establish the doses necessary to inhibit bone loss in an osteoporosis model and to study its mechanism of action on bone cells.

According to the present literature, the compounds occurring in plant-derived foodstuffs that support bone health are limited to minerals (calcium, potassium, and magnesium), vitamins (K and C), phytoestrogens (coumestrol, zearalenol, isoflavones, and humulone), possibly other flavonoids (rutin and hesperidin), and monoterpenes (17, 24–32). Thus, compounds active on bone, and therefore candidates for a dietary approach to osteoporosis, are widely distributed in the plant kingdom. We found by HPLC 17.3 mg of GPCS in 1 g of dry onion, that is, 1.73%. The measured GPCS content is in agreement with the findings of others who measured GPCS concentrations in the range of 0.58–2.88% of dry weight (23, 33). Therefore, the rats treated with 1 g of onion received ~0.2 mM/kg of body weight of GPCS. Validation, using an onion batch with lower GPCS content, showed that the HPLC method is linear ( $r = 0.9998$ ), sufficiently reproducible (intraday variability, mean  $\pm$  RSD,  $0.05 \pm 8.2\%$ ,  $n = 5$ ; interday variability,  $0.1 \pm 10.8\%$ ,  $n = 5$ ), and sensitive (limit of detection = 30 ng of GPCS).

The identification of GPCS as a compound inhibiting the activity of bone-resorbing cells adds another compound, belonging to yet another class of molecules, to the list of natural compounds active on bone. Whether GPCS is a representative of a family of active compounds or an individual active compound is presently not known. To clarify this issue, it will be necessary to study its role in the activity of the other 25 active vegetable food items identified so far (12) and possibly identify other active members of this class of compounds.

#### ACKNOWLEDGMENT

We thank Martin Shaw from the New Zealand Institute for Crop and Food Research for sending us a reference sample of GPCS for the NMR experiments.

#### LITERATURE CITED

- Melton, L. J.; Heaney, R. P. Too much medicine? Or too little? *Bone* **2003**, *32*, 327–331.
- Kanis, J. A. The use of calcium in the management of osteoporosis. *Bone* **1999**, *24*, 279–290.
- Mühlbauer, R. C.; Li, F. Effect of vegetables on bone metabolism. *Nature* **1999**, *401*, 343–344.
- Mühlbauer, R. C.; Lozano, A.; Reinli, A. Onion and a mixture of vegetables, salads and herbs affect bone resorption in the rat by a mechanism independent of their base excess. *J. Bone Miner. Res.* **2002**, *17*, 1230–1236.
- Ingold, P.; Kneissel, M.; Mühlbauer, R. C.; Gasser, J. A. Extracts from onion prevent tibial cortical and cancellous bone loss induced by a high phosphate/low protein diet in aged retired breeder rats. *Bone* **1998**, *23*, S387 (Abstr. W388).
- Mühlbauer, R. C.; Li, F.; Guenther, H. L. Common vegetables consumed by humans potentially modulate bone metabolism in vitro and in vivo. *Bone* **1998**, *23*, S387.
- Mütsch-Eckner, M.; Sticher, O.; Meier, B. Reversed-phase high-performance liquid chromatography of *S*-alk(en)yl-L-cysteine derivatives in *Allium sativum* including the determination of (+)-*S*-allyl-L-cysteine sulphoxide,  $\gamma$ -L-glutamyl-*S*-allyl-L-cysteine and  $\gamma$ -L-glutamyl-*S*-(*trans*-1-propenyl)-cysteine. *J. Chromatogr.* **1992**, *625*, 183–190.
- Mühlbauer, R. C.; Fleisch, H. The diurnal rhythm of bone resorption in the rat: effect of feeding habits and pharmacological inhibitors. *J. Clin. Invest.* **1995**, *95*, 1933–1940.
- Mühlbauer, R. C.; Fleisch, H. A method for continual monitoring of bone resorption in rats: evidence for a diurnal rhythm. *Am. J. Physiol.* **1990**, *259*, R679–R689.
- Egger, C. D.; Mühlbauer, R. C.; Felix, R.; Delmas, P. D.; Marks, S. C.; Fleisch, H. Evaluation of urinary pyridinium cross-link excretion as a marker of bone resorption in the rat. *J. Bone Miner. Res.* **1994**, *9*, 1211–1219.
- Antic, V. N.; Fleisch, H.; Mühlbauer, R. C. Effect of bisphosphonates on the increase in bone resorption induced by a low calcium diet. *Calcif. Tissue Int.* **1996**, *58*, 443–448.
- Mühlbauer, R. C.; Lozano, A.; Reinli, A.; Wetli, H. Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. *J. Nutr.* **2003**, *133*, 3592–3597.
- Chen, P. S., Jr.; Toribara, T. Y.; Warner, H. Microdetermination of phosphorus. *Anal. Chem.* **1956**, *28*, 1756–1758.
- Arnett, T. R.; Spowage, M. Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. *Bone* **1996**, *18*, 277–279.
- Jones, S. J.; Boyde, A.; Ali, N. N. The resorption of biological and non-biological substrates by cultured avian and mammalian osteoblasts. *Anat. Embryol.* **1984**, *170*, 247–256.
- Vitté, C.; Fleisch, H.; Guenther, H. L. Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption. *Endocrinology* **1996**, *137*, 2324–2333.
- Horcajada-Molteni, M. N.; Crespy, V.; Coxam, V.; Davicco, M. J.; Remesy, C.; Barlet, J. P. Rutin inhibits ovariectomy-induced osteopenia in rats. *J. Bone Miner. Res.* **2000**, *15*, 2251–2258.
- Mühlbauer, R. C. Rutin cannot explain the effect of vegetables on bone metabolism. *J. Bone Miner. Res.* **2001**, *16*, 970 (letter to the Editor).
- Barlet, J. P. A possible rut(in) the road. *J. Bone Miner. Res.* **2001**, *16*, 971 (reply).
- Breu, W. *Allium cepa* L. (onion) Part 1: Chemistry and analysis. *Phytomedicine* **1996**, *3*, 293–306.
- Isobe, M.; Uyakul, D.; Liu, K. L.; Goto, T. FAB-MS/MS spectrometry in determining the primary structure of  $\gamma$ -glutamyl-containing peptides. *Agric. Biol. Chem.* **1990**, *54*, 1651–1660.
- Kuttan, R.; Nair, N. G.; Radhakrishnan, A. N.; Spande, T. F.; Yeh, H. J.; Witkop, B. The isolation and characterization of  $\gamma$ -L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide from sandal (*Santalum album* L). An interesting occurrence of sulfoxide diastereoisomers in nature. *Biochemistry* **1974**, *13*, 4394–4400.
- Shaw, M. L.; Lancaster, J. E.; Lane, G. A. Quantitative analysis of the major  $\gamma$  glutamyl peptides in onion bulbs (*Allium cepa*). *J. Sci. Food Agric.* **1989**, *48*, 459–467.
- Tucker, K. L.; Hannan, M. T.; Chen, H.; Cupples, L. A.; Wilson, P. W.; Kiel, D. P. Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women. *Am. J. Clin. Nutr.* **1999**, *69*, 727–736.
- Feskanich, D.; Weber, P.; Willett, W. C.; Rockett, H.; Booth, S. L.; Colditz, G. A. Vitamin K intake and hip fractures in women: a prospective study. *Am. J. Clin. Nutr.* **1999**, *69*, 74–79.
- New, S. A.; Bolton-Smith, C.; Grubb, D. A.; Reid, D. M. Nutritional influences on bone mineral density: a cross-sectional study in premenopausal women. *Am. J. Clin. Nutr.* **1997**, *65*, 1831–1839.
- Draper, C. R.; Edell, M. J.; Dick, I. M.; Randall, A. G.; Martin, G. B.; Prince, R. L. Phytoestrogens reduce bone loss and bone resorption in oophorectomized rats. *J. Nutr.* **1997**, *127*, 1795–1799.
- Arjmandi, B. H.; Alekel, L.; Hollis, B. W.; Amin, D.; Stacewicz-Sapuntzakis, M.; Guo, P.; Kukreja, S. C. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *J. Nutr.* **1996**, *126*, 161–167.

- (29) Tobe, H.; Muraki, Y.; Kitamura, K.; Komiyama, O.; Sato, Y.; Sugioka, T.; Maruyama, H. B.; Matsuda, E.; Nagai, M. Bone resorption inhibitors from hop extract. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 158–159.
- (30) Chiba, H.; Uehara, M.; Wu, J.; Wang, X.; Masuyama, R.; Suzuki, K.; Kanazawa, K.; Ishimi, Y. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *J. Nutr.* **2003**, *133*, 1892–1897.
- (31) Mühlbauer, R. C.; Lozano, A.; Palacio, S.; Reinli, A.; Felix, R. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. *Bone* **2003**, *32*, 372–380.
- (32) Yamaguchi, K.; Shinohara, C.; Kojima, S.; Sodeoka, M.; Tsuji, T. (2E,6R)-8-hydroxy-2,6-dimethyl-2-octenoic acid, a novel anti-osteoporotic monoterpene, isolated from *Cistanche salsa*. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 731–735.
- (33) Kopsell, D. A.; Randle, W. M. Selenium affects the *S*-alk(en)yl cysteine sulfoxides among short-day onion cultivars. *J. Am. Soc. Hortic. Sci.* **1999**, *124*, 307–311.

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**Received for review November 12, 2004. Revised manuscript received February 22, 2005. Accepted February 23, 2005. Presented in part at the 10th Annual Meeting of the Swiss Bone and Mineral Society, Bern, Switzerland, May 6, 2004. This investigation was supported in part by the Swiss National Science Foundation (Grant 32-65329) and by Novartis Consumer Health (Nyon, Switzerland).**

JF0404571