γ-GLUTAMYL-PROPENYL-CYSTEINE SULFOXIDE (GPCS), A GAMMA-GLUTAMYL PEPTIDE FROM ONION (*ALLIUM CEPA* L.) - PHYTOCHEMISTRY AND PHARMACOLOGY

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1 Summary

Gamma-glutamyl peptides are widely found in plants. γ-Glutamyl-propenyl-cysteine sulfoxide (GPCS), a glutamyl peptide present in onion (*Allium cepa* L.), was identified by a bioassay-guided fractionation *in vitro* to be the compound responsible for the inhibition of bone resorption by onion in rats. Therefore, GPCS was selected for an extended investigation in our studies. The aim of the first part of this work was to examine the presence of GPCS in various plants, which were previously shown to inhibit bone resorption in rats. A fast and reliable method to determine GPCS in plant material was developed and validated. GPCS was found in onion at various concentrations, but no GPCS was detected in the material from other plants.

In the second part of the present work, the aim was to provide GPCS for further *in vitro* and *in vivo* studies. Small quantities (milligram-amounts) were obtained by isolation of GPCS from onion. As large-scale isolation (gram amounts) is not feasible under non-industrial conditions, and GPCS is not commercially available, an approach for synthesis was performed. The intermediate products S-(1-propenyl)-L-cysteine sulfoxide and Boc-L-glutamyl-α-t-butyl-N-oxo-succinimide ester were synthesised, but unfortunately, coupling of these two compounds failed and the final product could not be obtained.

The aim of the third part of this work was to examine pharmacodynamic and pharmacokinetic parameters of GPCS. *In vitro* experiments on the effects of GPCS on the activity, activation and recruitment of osteoclasts (OCs) were performed. The effect of GPCS on the activity of OCs was tested in the pit assay. GPCS, at 8 mM, significantly reduced the number of pits per OCs. The effect of GPCS on the activation of OCs was examined by determination of the OCs with actin ring(s). After incubation of the cells with 2 and 8 mM of GPCS, the percentage of OCs with actin rings did not change. The formation of OCs was inhibited at GPCS concentrations of 1 mM and higher in our experiments with CSF-1/RANKL primed bone marrow cells. A structure-function relationship was assessed by testing GPCS structurally related compounds in the same assay, namely γ-glutamyl-cysteine-ethylester, glycyl-cysteine, allyl-cysteine, cysteine, glutamyl-glycine and glycyl-valine. All test substances which contained a cysteine moiety inhibited osteoclastogenesis while the others did not show an effect. The pharmacokinetic properties of GPCS were studied *in vitro* and *in vivo*. Within 24 hours no change in the concentration of GPCS was observed when GPCS was incubated with simulated gastric acid. 24-hour incubation of GPCS with simulated intestinal fluid caused a 90 % decrease of the GPCS concentration, while GPCS concentration in onion incubated with simulated intestinal fluid decreased only by 30 %. In a pilot study with rats on the kinetics of GPCS *in vivo*, the bioavailability of orally administered GPCS was determined to be about 1 %. Finally, onion without GPCS and onion containing GPCS, as well as GPCS-

structure related compounds were tested in a rat model *in vivo* to analyse their effect on bone resorption. Allyl-cysteine and γ-glutamyl-cysteine-ethylester did not inhibit bone resorption while 3 g of onion without GPCS and 3 g of onion containing GPCS significantly inhibited bone resorption.

The *in vitro* results indicate that the effects of GPCS might be mediated by the cysteine moiety of the molecule. An *in vivo* oral application of pure GPCS in an aqueous solution is not suitable due to the low bioavailability of GPCS, however, application of GPCS embedded in onion might prevent cleavage of the compound and therefore increase the bioavailability. Because both onion without GPCS and onion containing GPCS inhibited bone resorption *in vivo*, we can postulate that the inhibition of bone resorption by onion is not exclusively mediated by GPCS. Future experiments using natural GPCS-free onions spiked with synthetic GPCS should clarify, to which extent GPCS contributes to the inhibition of bone resorption.

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4 Aims of the present work

Osteoporosis is widely recognised as a major public health problem. It is a systemic disease of the skeletal system characterised by reduced bone mass and microarchitectural deterioration of bone tissue which leads to an increased risk of fractures. Bone loss occurs because the equilibruim of bone formation and bone resorption is shifted to one side – to a decrease of bone formation and/or an increase of bone resorption. Dietary components and herbal products were reported to have positive influence on this process, particularly by inhibiting bone resorption [Putnam 2007], and thus may prevent osteoporosis and its consequences.

Several herbs and vegetables, among them onion (*Allium cepa* L.), were shown to inhibit bone resorption in rats [Muhlbauer 1999, Muhlbauer 2003a, Muhlbauer 2003b]. This effect was independent of their base-excess [Muhlbauer 2002] and an effect of flavonoids, especially rutin was unlikely to be the cause, as shown in previous experiments [Muhlbauer 2001]. To isolate and identify the substance in onion which might be responsible for the inhibition of bone resorption, a bioassay-guided fractionation was performed. γ-Glutamyl-propenyl-cysteine sulfoxide (GPCS[1]) was identified as the active compound *in vitro* which inhibits the resorption activity of osteoclasts (OCs) [Wetli 2005].

The aim of the first part of this PhD thesis was to examine the presence of GPCS in various plants, which were previously shown to inhibit bone resorption in rats. The requirement for this study was to develop a fast and reliable method to determine GPCS in plant material.

In the second part, the aim was to provide GPCS for further *in vitro* and *in vivo* studies. As large-scale isolation (gram amounts) is not feasible under non-industrial conditions, and GPCS is not commercially available, an approach for synthesis was performed.

The aim of the third part of this work was to examine whether GPCS does not only affect OC activity, but also its activation and recruitment. A structure-function relationship was assessed by testing structurally related compounds *in vitro*. The kinetic of GPCS was studied *in vitro* and *in vivo*. Finally, onion without GPCS and onion containing GPCS, as well as GPCS-structure related compounds were tested in a rat model *in vivo* to analyse their effect on bone resorption.

[1] The abbreviations used in this work are given in section 11.1 Appendix 1: List of Abbreviations.

5 Introduction

5.1 Allium cepa L.

5.1.1 Botanical systematics and history

Onion (*Allium cepa* L.) is a member of the Alliaceae family and belongs to the genus *Allium* which consists of about 450 species. Besides onion and garlic, also the other members of *Allium* like leek (*Allium porrum* L.), shallot (*Allium ascalonicum* L.), wild garlic (*Allium ursinum* L.) and many others are known for their unique and typical taste. *Allium* plants are widely distributed over Europe, Asia and America, and they have been used for millennia as spices, vegetables and for the treatment of diseases [Lanzotti 2006].

Onions probably originate from central Asia where they were produced since 3000 B.C.. They were brought to Europe about 2000 years ago. Onions and garlic were first cited in the Codex Ebers 1550 B.C., an Egyptian medical papyrus, and there recommended to cure heart problems, headache, bites, worms and tumours. In ancient times onions were consumed in large quantities because they were thought to be sacred food. The raw plant was given to asthmatics and to people suffering from pulmonary diseases. The Greeks and Romans cherished onions as curative agents [Block 1985, Corea 2005].

5.1.2 Bioactivity

Today onions are important phytonutrients, and a variety of studies suggests a positive effect of onions in the prevention and treatment of cancer, cardiovascular diseases, asthma, infections and hyperlipidemia [Griffiths 2002]. It was shown that food supplementation with onions significantly inhibits bone resorption in rats [Muhlbauer 1999, Muhlbauer 2002].

Onions, their extracts or isolated compounds, are able to modulate mammalian enzyme systems [Teyssier 2001], may cause apoptosis and show antiinflammatory, antioxidant [Yang 2004], antimicrobial, spasmolytic [Corea 2005], antifungal, antithrombotic, antilipidaemic [Ali 2000], antihyperglycemic [El-Demerdash 2005] and antiparasitic properties [Lanzotti 2006, Rose 2005]. In traditional medicine, fresh onion juice is used against common cold, cough, as well as local treatment of insect bites.

5.1.3 Chemistry

Bulbs of *Allium cepa* L. consist of water (~ 90 %), carbohydrates (~ 10 %), proteins (~ 1 %) and fat (~ 0.1 %). They are a rich source of bioactive compounds which are the thiosulfinates and structurally related substances, flavonoids, sapogenins and saponins. Their biosynthetic pathways and metabolisms within the plant were mainly studied by chemical analysis and radiotracer experiments [Jones 2004].

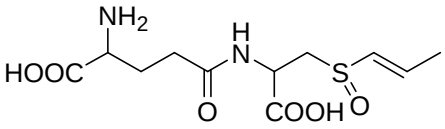
Organosulphur compounds

The typical odour of onion is developed as soon as its tissue is damaged because this damage leads to the release of volatile substances by enzymatic hydrolysis of non-volatile sulphur-containing storage compounds. These flavour precursors in onion are the odourless S-alk(en)yl-cysteine sulphoxides, which are typical for the *Allium* genus.

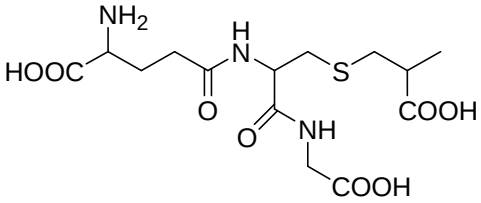
Sulphur is incorporated into the plant through the roots, reduced to sulphide, and primarily accumulated intracellularly through cysteine into the glutathione cycle leading to the non-volatile gamma-glutamyl peptides. Until now 24 sulphur-containing gamma-glutamyl peptides have been identified in *Allium* ssp. [Rose 2005]. They serve not only as sulphur and nitrogen storage compounds [Kasai 1980] but also as intermediates in the S-alk(en)yl-cysteine sulphoxide synthesis [Lancaster 1989, Lancaster 1991]. The major gamma-glutamyl peptides in onion are GPCS and S-2-carboxypropylglutathione [Shaw 1989] (Figure 1). According to Lancaster et al. [Lancaster 1991] they are not present in pre-bulbing onion, largely increased through bulbing and maintained at that level during bulb dormancy.

Figure 1: The two major gamma-glutamyl peptides in onion.

γ-Glutamyl-*trans*-S-1-propenyl-L-cysteine sulphoxide



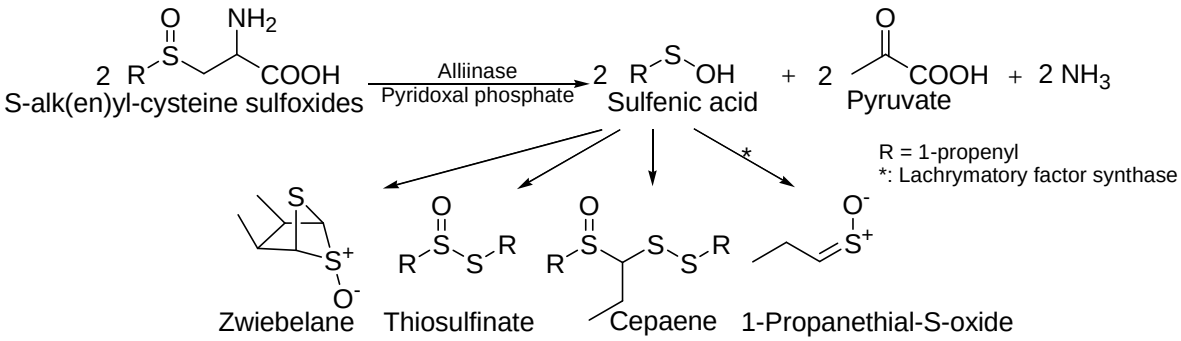
S-2-Carboxypropylglutathione



γ–Glutamyl (trans)peptidase, which is active in leaves, roots and bulbs of growing onion, transfers the γ–glutamyl group of the gamma-glutamyl peptides to amino acids or other peptides, leading to S-alk(en)yl-cysteine sulphoxides as product. After tissue disruption and mixture of the vacuole and cytoplasmic contents, enzymatic hydrolysis cleaves these compounds to pyruvate, ammonia and a thiosulfinate [Rose 2005]. Thiosulfinates are intermediates in the formation of sulphur volatiles [Freeman 1976] and thus responsible for the typical flavour [Block 1992]. They differ among the species according to their content of the enzyme alliinase and S-alk(en)yl-cysteine sulphoxide precursors [Lanzotti 2006].

The major and characteristic S-alk(en)yl-cysteine sulphoxide in intact onion is *trans*-1-propenyl-cysteine sulfoxide (isoalliin). By the influence of the enzymes alliinase and/or lachrymatory synthase it gives rise to sulfenic acids which react to thiosulfinates, cepaenes, zwiebelanes and the lachrymatory factor 1-propanethial S-oxide (Figure 2).

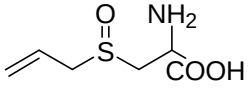
Figure 2: Formation of lachrymatory factor, zwiebelanes, cepaenes and thiosulfinates by alliinase and lachrymatory factor synthase



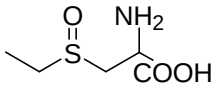
Further important S-alk(en)yl-cysteine sulphoxides found in onion and related species are S-propyl-cysteine sulfoxide (propiin), S-methyl-cysteine sulfoxide (methiin) as well as S-allyl-cysteine sulphoxide (alliin), a characteristic constituent of garlic [Jones 2004] (Figure 3).

Figure 3: Flavour precursors of the genus Allium

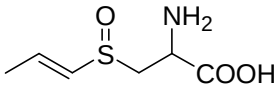
S-2-propenyl-cysteine sulfoxide (Alliin)



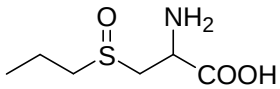
S-methyl-cysteine sulfoxide (Methiin)



trans-S-1-propenyl-cysteine sulfoxide (Isoalliin)



S-propyl-cysteine-sulfoxide (Propiin)



Flavour intensity depends on genetic factors and environmental conditions such as sulphur and nitrogen levels in the soil [Randle 1992, Randle 2000, Randle 1994], growing temperatures [Coolong 2003] and irrigation conditions [Freeman 1973].

Analysis of onion compounds has been reviewed in detail by Lanzotti [Lanzotti 2006]. It is performed using gas chromatography - mass spectrometry (GC-MS), liquid chromatography - mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR), supercritical fluid extraction – mass spectrometry, supercritical fluid extraction - gas chromatography - mass spectrometry, supercritical fluid extraction - liquid chromatography - mass spectrometry, gas chromatography - atomic emission spectroscopy, spectral photometric methods and liquid chromatography - diode array detection (LC-DAD). Problems, when using these methods, may occur because of the release and activity of the above mentioned enzymes during material preparation, because this might change the composition of the material to be analysed.

Saponins

Another class of bioactive compounds present in onion are saponins, namely sitosterol, gitogenin, oleanolic acid, amayrin, diosgenin, saponin beta-chlorogenin, cepagenin, alliospirosides A-D, alliofuroside A, tropeosides A1/A2 and tropeosides B1/B2, ascalocosides A1/A2 and ascalonicoside B [Lanzotti 2006].

Flavonoids

Onions are rich in flavonoids and phenolics. Major flavonoids in onion are quercetin and its glycosides, isorhamnetin and glycosides, rutin and kaempferol [Lanzotti 2006].

5.2 Bone

5.2.1 Function, structure and composition of bone

The skeleton, which consists of bone and cartilage, has several functions: it protects the internal organs such as heart, brain or the spinal cord and ensures the mechanical stability of the body. Bones are levers for muscles and thus support locomotion. They play an important role in the maintenance of mineral homeostasis as they serve as an ion reservoir, especially for calcium and phosphate. Bone has an influence on the acid-base-balance, because it absorbs or releases alkaline salts in cases of excessive blood pH changes.

The skeleton consists of two, anatomically different types of bone: cortical (or compact) bone and cancellous (or spongy or trabecular) bone. Cortical bone has mainly protective functions and is composed of 80 to 90 % of calcified tissue surrounding the cancellous bone as a thick, dense layer. Cancellous bone consists of a honeycomb-like network of thin trabeculae and is found in vertebrae and in the epiphysis of long bones. 15 to 25 % of cancellous bone is calcified and due to its structure, it provides stability to withstand mechanical load, but it also performs metabolic functions. Both cortical and trabecular bone surrounds the bone marrow stroma which contains precursor cells of bone-forming osteoblasts and bone-resorbing OCs.

Bone consists of water (5 - 10 %), organic matrix (20 – 40 %) and minerals (50 – 70 %), which surround the cells. The organic matrix of bone, mainly responsible for the elasticity and flexibility of bone, consists of collagen fibres, predominately type I, and non-collagenous proteins which make up to 15 % of the total protein content. Non-collagenous proteins are synthesised by bone forming cells and can be cleaved into proteogylcans, glycosylated proteins or gamma-carboxylated proteins. The role of these proteins is not yet well defined. Many of them seem to be multifunctional: they might be involved in the control of osteoclastic and osteoblastic metabolism and in the regulation of bone matrix mineralisation. The bone minerals are found on the collagen fibres in a form similar to the geological hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. This hydroxyapatite is less crystalline and contains some “defects”, for example missing hydroxide ions, or impurities like carbonate or magnesium. Thus, bone serves as an ideal reservoir for calcium, phosphate and magnesium ions. In addition, the minerals provide mechanical rigidity and load bearing strength to the bone.

The anatomy and function of bone has been reviewed in detail by Dempster et al. and Baron [Baron 1999, Dempster 2006].

5.2.2 Bone cells

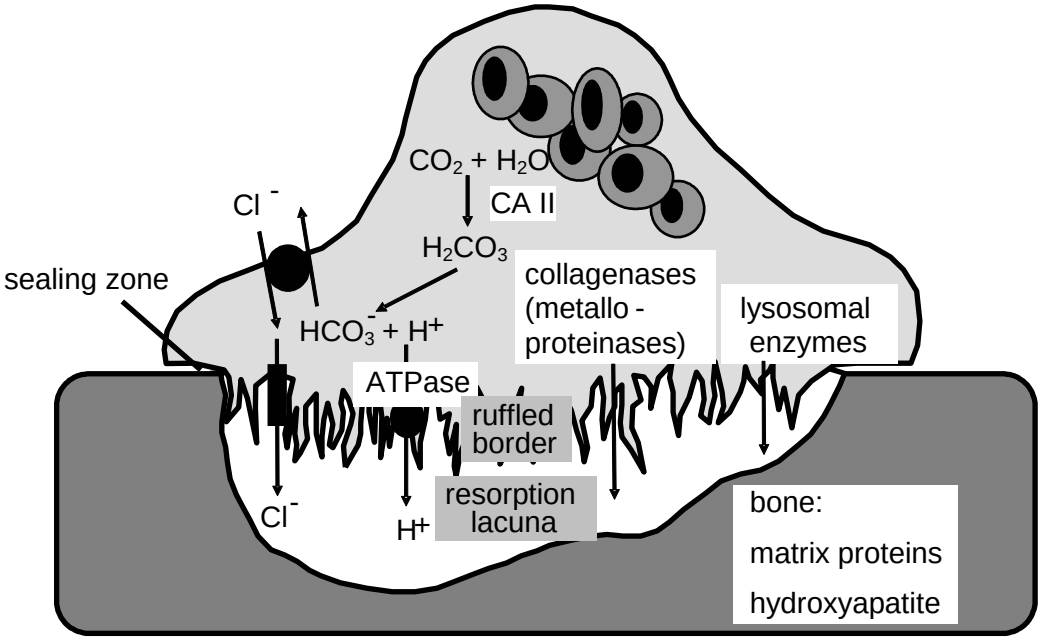
The principal cells of bone belong to the OC lineage or osteoblast lineage. OCs are derived from haematopoietic stem cells and they are the exclusive bone resorbing cells. The cells

that contribute to bone formation are derived from mesenchymal stem cells and are named osteoblasts, osteocytes and bone lining cells. Osteoblasts synthesise the bone matrix, osteocytes are embedded into the mineralised bone and bone lining cells cover the bone surface.

Osteoclasts (OCs)

OCs are multinucleated, bone resorpting giant cells which derive from cells of the monocyte/macrophage lineage under the influence of specific growth factors. They are in direct contact with the calcified bone surface via the OC's clear zone which surrounds the ruffled border. It is the location where bone resorption occurs (Figure 4).

Figure 4: Mechanisms of the resorption activity of an OC (courtesy of Prof. W. Hofstetter)



During the resorption process OC attach to bone. This cell-surface adhesion is mediated by integrin receptors [Nakamura 2007]. The OC becomes polarised; fibrillar actin in the cytoskeleton rearranges and forms a belt-like structure, the actin-ring, which is visible after specific staining. In addition, a tight junction between bone and osteoclastic membrane, the sealing zone, is formed which isolates the extracellular bone-resorbing compartment [Boyle 2003]. If the osteoclastic activity is disturbed, for example by inhibitors like calcitonin, structural anomalies of the actin ring may be observed [Suzuki 1996, Vaananen 2000]. The ruffled border, which is surrounded by the clear zone, includes infoldings of the membrane to increase the cell's surface area. Through this ruffled border, protons are excreted by the OC onto the bone surface. Protons, present in the cytosol of the OC, arise from molecules of carbon dioxide and water which react to carbonic acid under the catalysis of the enzyme carbonic anhydrase II. Proton transport to the bone matrix, mediated by a vacuolar

electrogenic proton pump (H^+-ATPase), and chloride ion transport, mediated by a chloride channel, form an acid environment of pH ~ 4.5 [Teitelbaum 2003]. Thus, the hydroxyapatite crystals in the bone are dissolved after digestion of their links to collagen which is degraded by lysosomal enzymes.

Lysosomal enzymes such as cathepsin K and tartrate resistant acid phosphatase, which are synthesised by OCs, are found in the endoplasmatic reticulum, golgi, and many vesicles. These acidified vesicles transport the enzymes to the apical pole of the OC where they fuse with the plasma membrane and thus form the ruffled border [Teitelbaum 1995]. Lysosomal enzymes are secreted through the ruffled border into the extra cellular bone-resorbing compartment by exocytosis. They degrade the bone matrix compounds and form lacunae because they are now at an optimal pH. Remaining residues of the resorption are either internalised by the OC, released from the clear zone in the extracellular bone-resorbing compartment or transported to the basolateral membrane and released. Intracellular hydrogen carbonate is exchanged through extracellular chloride through the basolateral membrane. These mechanisms avoid alkalinisation of the cell and provide efficient amounts of chloride for the ruffled-border chloride channels.

OCs are derived from haematopoietic precursor cells. The two essential growth factors in this process are receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [Pixley 2004, Yasuda 1998]. At an early stage of osteoclastogenesis, c-Fms, the receptor for M-CSF, is expressed by haematopoietic stem cells and M-CSF induces the proliferation and survival of monocytic cells and the expression of RANK, the receptor for RANKL [Arai 1999]. Later, M-CSF, RANKL and other downstream effectors of RANK signalling lead to the commitment and differentiation of pre-OCs which fuse to multinucleated cells. M-CSF is also involved in the cytoskeletal rearrangement of the OC for bone resorption [Ross 2006]. Both, RANKL and M-CSF are produced by marrow stromal cells and osteoblasts [Suda 1999].

Osteoblasts and osteocytes

The mature osteoblast is a cuboidal cell with a round nucleus at the base of the cell, huge Golgi-complex and well-developed rough endoplasmatic reticulum. Osteoblasts derive from mesenchymal stem cells (bone marrow stromal stem cells or connective tissue mesenchymal stem cells). The same cell line also gives rise to adipocytes, chondrocytes, fibroblasts and myoblasts. The mesenchymal stem cells which commit to the osteoblast lineage are called osteoprogenitors. Osteoprogenitors proliferate and differentiate to pre-osteoblasts and later to mature osteoblasts. Mature osteoblasts synthesise the bone matrix constituents including collagen, non-collagenous proteins, but also growth factors. At the end of this process, they become either osteocytes, lining cells or undergo apoptosis. Osteocytes are located in

lacunae of bone matrix. Originally they were osteoblasts which have become embedded by the bone matrix built by themselves. Osteocytes are connected with other osteocytes or lining cells via long cell processes in small channels called canaliculi and thus, form a network throughout the bone matrix. Bone lining cells are inactive osteoblasts covering all available bone surfaces. They provide a bone-blood barrier and regulate the flow of ions in and out of bone extracellular fluid.

The morphology, maturation and functional activities of osteoblast lineage cells have been reviewed by Dempster et al. and Aubin et al. [Aubin 2006, Dempster 2006].

5.2.3 Bone remodelling

Bone modelling is reshaping of bone characterised by an independent action of OCs and osteoblasts. This process affects size and shape of bone and occurs during growth for a period of about 18 years, or in adults during fracture healing for a period of about one year.

In contrast to bone modelling, bone remodelling affects bone density, mineralisation and microstructure and it continues throughout the life. Bone deposition and bone resorption occur at the same spot and the action of OCs and osteoblast are coupled processes. 2 – 3 % of cortical bone and 25 % of cancellous bone are remodelled per year. Ideally, bone remodelling maintains the current amount of bone, but at the age of about 35, the amount of bone resorption exceeds the amount of bone formation resulting in a decline in bone mass. Bone remodelling takes place continuously at the bone surface, mainly at the endosteum and in the Haversian system in response to mechanical load, calcium homeostasis or repair of microfractures. It is accomplished by teams of OCs and osteoblasts, called basic multicellular units (BMU), and it follows an activation-resorption-formation sequence consisting of four stages: (I) resorption, (II) reversal phase, (III) formation, and (IV) resting phase. It is hypothesised that pre-osteoclasts attach to the bone surface after they have been activated by factors released by lining cells, osteocytes or marrow cells. They fuse to form OCs, which start resorption and thereby dig out a tunnel to create a “cutting cone” into the cortical bone or lacunae into cancellous bone. Afterwards, the OCs detach, and during the reversal phase, some macrophage-like cells cover the surface and form a line that cements together old and new bone. In the next step, the formation period, preosteoblasts are activated, then differentiate and proliferate to osteoblasts, which follow along the surface and lay down initial matrix (osteoid) in parallel layers in the closing cone. After a certain time the initial matrix becomes mineralised and osteoblasts become lineage cells.

The process of bone remodelling has been reviewed by Baron and Dempster et al. [Baron 1999, Dempster 2006].

5.2.4 Osteoporosis

Osteoporosis is a systemic disease of the skeletal system characterised by reduced bone mass and microarchitectural deterioration of bone tissue. The WHO defines osteoporosis as a bone mineral density or a bone mineral content, that is -2.5 standard deviations below the young adult mean (20-year-old healthy female average) for the population, measured by techniques such as dual-energy X-ray absorptiometry [World Health Organisation 1994]. In a healthy person, bone mass increases until the age of approximately 30 and afterwards, it gradually decreases. A low bone mineral density in an elderly person implies a suboptimal bone mass in young adolescence or increased bone loss in later life, or both [Prentice 2004].
Osteoporosis results in an increased bone fragility and increased risk of fractures [Consensus development conference 1993, Prentice 2004]. The fractures occur throughout the skeleton, mostly at the vertebrae, wrist and hip, and lead to an increased morbidity, disability, and mortality in elderly people [Bonjour 1997]. With the increase in life expectancy, fracture prevalence will even rise. In addition to the individual burden, fractures impose enormous costs for health systems worldwide [Harvey 2006, Johnell 1997, Schwenkglenks 2004].
Most pharmacological treatments of osteoporosis decrease the risk of fractures by retarding the decrease in bone mineral density either by stimulating bone formation or by decreasing bone resorption [Boyle 2003]. One drug stimulating bone formation is teriparatide, a recombinant form of human parathyroid hormone 1-34. Bisphosphonates, estrogen and selective estrogen receptor modulators serve as antiresorptive treatment [Harada 2003], and calcium as well as vitamin D play an important role as a co-therapy [Dawson-Hughes 2006, Heaney 2006, Roux 2008].
However, due to the side effects and costs of these medications, prevention of osteoporosis and its consequences is preferable. A nutritional approach may thereby play a promising role. Various studies have demonstrated, that an increased intake of vegetables and fruits could positively influence the balance between bone formation and bone resorption [Putnam 2007], and that it is related to a higher bone mineral density [McGartland 2004, New 2000, Prynne 2006, Tucker 2002, Tucker 1999]. It was postulated that this effect is due to the food's base excess buffering metabolic acid [New 2000, Tucker 1999]. However, studies by Mühlbauer et al. have shown that the intake of several vegetables, fruits and herbs decreases bone resorption in rats [Muhlbauer 1999, Muhlbauer 2000, Muhlbauer 2003a, Muhlbauer 2003b], independently of their base excess, and the effect can be most likely attributed to pharmacological mechanisms [Muhlbauer 2002]. Onion was among the examined vegetables which were shown to inhibit bone resorption *in vivo*. Using a bioassay-guided fractionation, GPCS was identified by Wetli et al. as the active substance *in vitro* [Wetli 2005].

5.3 Analytical methods

Chromatography coupled to spectrometry was used to analyse GPCS in biological matrices (plant, rat plasma). Due to the high polar character of GPCS the method of first choice was reverse-phase high-performance liquid chromatography with diode-array or mass spectrometric detection (RP-HPLC-DAD or -MS). These methods, as well as sample preparation by solid phase extraction (SPE) are subsequently briefly described and referenced.

5.3.1 High-performance liquid chromatography (HPLC)

HPLC is the most frequently applied technique in pharmaceutical analytics. It is a column chromatography under high pressure used to separate and fractionate mixtures of substances or substances present in various biological matrices. For detection, it is usually combined with spectroscopic techniques.

A modern HPLC system is microprocessor-controlled, consists of a solvent reservoir, pumps, injection port, column with column oven, and detector. The solvent is pressed through the column by means of the pumps under high pressure. The sample mixture is dissolved in the mobile phase and each compound interacts with the stationary phase which is present in the column. The amount of interaction depends on the sorbent of the stationary phase and on the solvent of the mobile phase. Compounds with a high affinity to the sorbent remain longer on it and show a higher retention time than compounds with a low affinity. The retention time is the time when the compound is eluted from the column. Chemical and physical mechanisms, which retain the compounds on the sorbent, base upon adsorption, distribution, chiral interactions, ion-pair interactions, size-exclusion or affinity. Therefore numerous sorbents are available on the market, which are used to achieve optimal separation of complex mixtures. Several detection principles are used in HPLC: ultraviolet-visible (UV-VIS) spectroscopy, fluorescence, evaporative light scattering, electrochemistry, electrical conductivity, NMR, MS and refractive index.

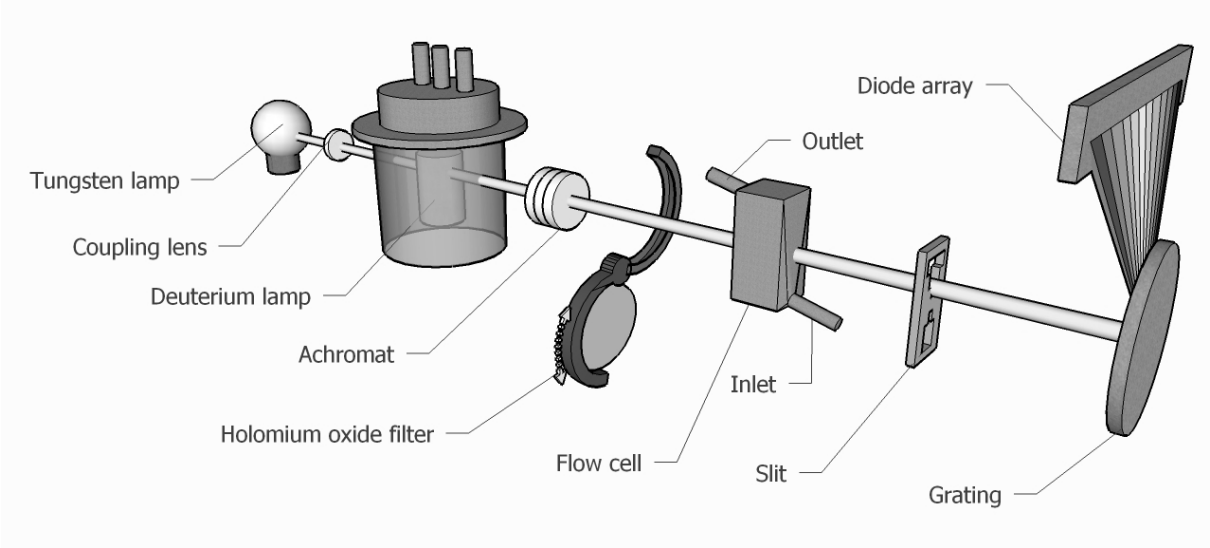
HPLC is reviewed in [Luque-Garcia 2007, Rücker 2001].

High-performance liquid chromatography - Diode array detection (HPLC-DAD)

Diode array detectors are widely used for the identification and quantitation of analytes. They allow simultaneous measurement of UV, VIS and near-infrared light absorption. The resulting spectra can then be compared to an online/offline database. As the absorption correlates with the concentration of the analyte, the signal can be used for quantitation.

A schematic view of an optical system of a diode array detector is given in Figure 5. In a diode array detector, a deuterium or holmium lamp emits light over the UV wavelength range, and a tungsten lamp emits light over the visible and near-infrared wavelength range. The light of the lamps is focussed by the achromat through the flow cell onto a holographic grating. The dispersed light from the grating falls onto a diode array which contains hundreds of individual photodiodes. The output of each diode is recorded by a computer. When the diode array detector is coupled with HPLC and a substance is eluted through the sensor cell, the information acquired by the diodes can either be used to obtain an absorption spectrum or, by selecting the appropriate diode, to obtain a chromatogram at a specific wavelength, usually at the maximum absorption of the analyte.
Sample analysis by HPLC-DAD is reviewed by Rücker et al. and Pragst et al. [Pragst 2004, Rücker 2001].

Figure 5: Schematic view of on optical system of a diode array detector (courtesy of D. Mabboux)



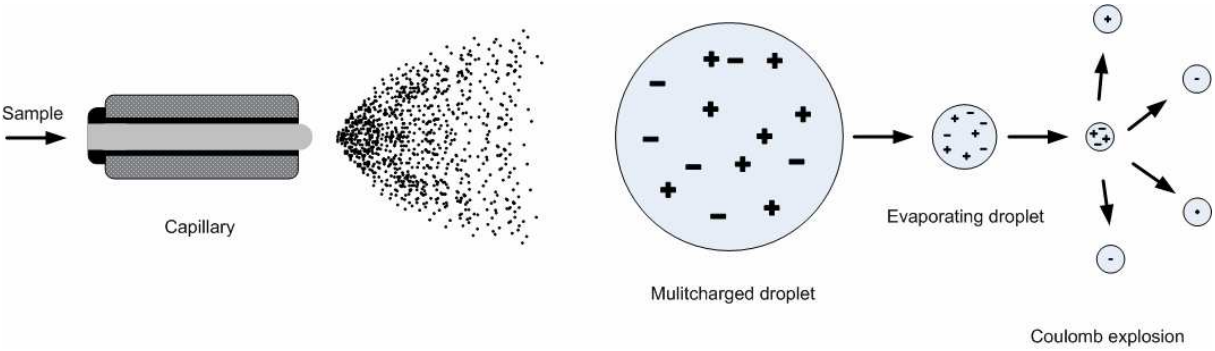
High-performance liquid chromatography - mass spectrometry (HPLC-MS)

Mass spectrometry analyses the mass to charge (m/z) ratio of ions. A typical mass spectrometry instrument consists of an ion source, a mass analyser and a detector. Two modes of sample introduction are used: samples are either infused directly into the source (method of choice to optimise the compound detection), or a HPLC system is attached to the source.
In our experiments, a HPLC system was coupled to a triple quadrupole mass spectrometer (QQQ) with an electrospray ionisation (ESI) source. The ESI source produces charged ions of the analytes in the liquid phase by pumping liquid through a small, charged metal capillary and producing an aerosol containing small sized droplets of 1-10 µm (Figure 6). Uncharged gases, like nitrogen, usually support the nebulising process, help to evaporate the solvent in

the droplets, and prevent the ions from being neutralised. The transition of the ions to the mass analyser is performed by a vacuum interface. A quadrupole mass analyser consists of four parallel metal rods which are arranged in a square. The opposing rod pairs are connected together electrically, and a direct current voltage is impressed to generate electrical fields. Ions travel down the rods and are filtered depending on particular magnitudes and frequencies in a way that only ions with a special characteristic m/z ratio reach the detector. Other ions oscillate and are neutralised by collision with the rods. Masses of all ions can be scanned by consistently altering the electric fields to give a mass spectrum. To improve sensitivity or to obtain structural information, mass analysers are used in series, like QQQ. The first and third quadrupole act as mass filters whereas the second is used as collision cell. Parent ions which were filtered or isolated from the first quadrupole are collisionally dissociated by the second quadrupole and its fragments are analysed by the third quadrupole. Finally, a detector counts the electrons generated from the ions and data are presented as a mass spectrum which contains a specific pattern of ion fragments of each analyte.

Mass spectrometry and its use in the quantification of peptides in biological systems is reviewed in [Bantscheff 2007, Price 1991, Rücker 2001, Squires 1998].

Figure 6: Schematic view of an electrospray ionisation process (courtesy of D. Mabboux)



5.3.2 Solid phase extraction (SPE)

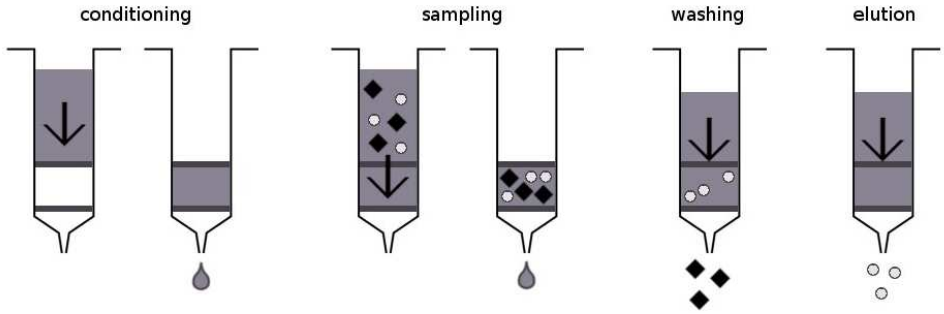
Sample preparation is a very important part of the analysis of substances in biological matrices. Its two main aims are the purification of the analyte from interfering matrix compounds (clean-up) and the concentration of the analyte.

A widely used, economic, fast and effective method for sample preparation is SPE. The separation principle is based on interactions between analyte, matrix and a sorbent. Common sample extraction products consist of a cartridge-like column or a 96-well-plate filled with a sorbent.

SPE usually consists of four steps. First, the sorbent is conditioned with a solvent to remove impurities of the sorbent and to increase separation reproducibility (“Conditioning”). In the second step the sample is loaded on the solid phase and the analyte is enriched on the

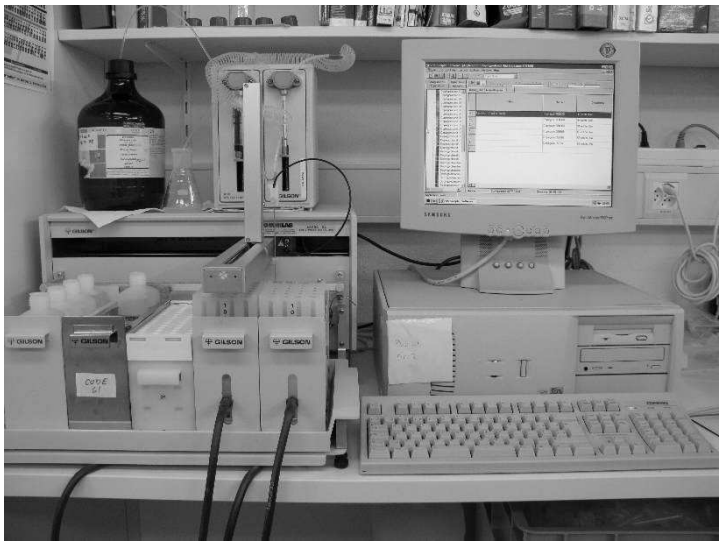
sorbent (“Sampling”). Afterwards, undesired matrix compounds are removed by washing the sorbent with a solvent which does not displace the analyte (“Washing”). Finally, the analyte is eluted (“Elution”) (Figure 7).

Figure 7: Schematic view of the several steps of SPE (courtesy of D. Mabboux)



The extraction process can be automated using robotic systems, like ASPEC™ (Figure 8). Even steps prior or following SPE, like serial dilution of standards, addition of internal standards, derivatisation or injection onto HPLC could be performed by these systems. Thus, throughput and reproducibility are increased, and the exposure of personal with hazardous materials is minimised. Several sorbents are available: they can be polymeric, silica-based, carbons or mixed and separate by non-polar, polar and/or ionic interactions with the analyte. Sample preparation by SPE is reviewed in [Brochure: Solid Phase Extraction 2006, Fontanals 2007, Hennion 1999, Poole 2000, Smith 2003].

Figure 8: SPE Robotic system



6 Part I: Determination of GPCS in various plants

6.1 Materials and methods

This section describes the materials and methods, which were used to determine GPCS in onions and various other plants.

6.1.1 Quantitation of GPCS using HPLC-DAD

Standards, solvents and chemicals

As GPCS was not commercially available it was isolated as described in chapter 7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS.
Bidistilled water was prepared in house. N-(L-α-Aspartyl)-L-phenylalanine methyl ester (aspartame), acetonitrile Chromasolv®, methanol Chromasolv® and ortho phosporic acid 85 % extra pur were purchased at Sigma-Aldrich[2] and Merck. Round filters were obtained from Schleicher & Schuell and 0.45-µm filters from Infochroma.

Plant material

Onions, garlic, leek, wood garlic, plum, red wine, crimp parsley, plain parsley, dill, sage, chinese cabbage, broccoli, rocket, smallage, common bean, red cabbage, cucumber, mushroom, tomato, lettuce, rosemary, thyme, boletus, fennel and orange were purchased on the local food market in Bern and either lyophilised or dried, subsequently pulverised, and stored at -20℃ until use.

Instrumentation

The HPLC system consisted of an Agilent 1100 Series degasser, capillary pump, diode array detector, thermostated column compartment, thermostated autosampler and an HP computer with ChemStation for LC 30 Rev. A. 09.03 [1417] (Agilent 1990-2002) software.

[2] A list of the addresses of suppliers is given in section 11.2 Appendix 2: List of suppliers.

Chromatographic conditions

The chromatographic conditions are shown in Table 1.

Table 1: Chromatographic conditions for quantitation of GPCS using HPLC-DAD

Stationary Phase:	125 x 4 mm i.d. column, packed with Spherisorb ODS-1, particle size 3.0 µm and a 8 x 4 mm i.d. precolumn, packed with the same column material (Machery-Nagel)
Mobile Phase:	Solvent A: Water containing 0.05 % phosphoric acid 85 % (v/v) Solvent B: Acetonitrile containing 0.05 % phosphoric acid 85 % (v/v) 75 % B, isocratic elution
Run time:	15 min
Flow:	0.7 ml/min
Temperature:	40℃
Detection:	Wavelength for the quantitation of GPCS with or without the internal standard method (aspartame as internal standard (I.S.)): UV 195 ± 4 nm, online recording of the UV spectra from 190 - 400 nm (DAD).

Validation

Peak identification (selectivity/specificity)

Chromatographic selectivity: The retention time of the analyte and the I.S. were assigned with the corresponding standards.
Spectroscopic selectivity: The DAD UV spectra in the range of 190 – 400 nm were compared to the spectra of the standards. The recorded spectra were also used for the peak purity check.

Calibration and linear range

The calibration of GPCS was performed using the I.S. and linear regression analysis over the concentration ranges of 10 µg/ml to 200 µg/ml with 36 µg/ml of I.S.. To obtain the calibration graph, the area ratio of GPCS to I.S. was plotted against the concentration of GPCS.
Five calibrator concentrations of 10, 50, 100, 150, and 200 µg/ml GPCS and 36 µg/ml I.S. were prepared in methanol/water/formic acid (50/50/0.05; v/v) from stock solutions of GPCS (1 mg/ml) and I.S.. Each sample was measured three times.

Intra- and interday precision and accuracy of the calibration

The intraday precision was determined measuring the samples for each concentration (30, 120, 180 µg/ml) five times within the same day.
The interday precision was established measuring the three control samples (30, 120, 180 µg/ml) nine times over a period of one month.

Mean, standard deviation (s.d.), relative standard deviation (r.s.d.) as well as accuracy were calculated. The control samples were tightly sealed and stored at –20℃ when not in use.

Limit of quantitation (LOQ)

The LOQ was defined at the concentration where the peaks could be clearly integrated with a signal to noise ratio of 10 to 1.

Determination of GPCS in plant material

Sample preparation

1.0 g of dry plant powder was accurately weighed. It was extracted with 50.0 ml of methanol/water/formic acid (50/50/0.05; v/v/v) with or without 36 µg/ml I.S. at room temperature using an ultrasonic bath for 5 min (modified method based on [Wetli 2005]). Then, the suspension was filtered through a 595 grade round filter and through a 0.45-µm filter. 7 or 10 µl were injected into the HPLC.

Intra- and interday precision and accuracy of the extraction

For the determination of the intraday precision, five extracts were prepared and analysed six times each using HPLC.

For the determination of the interday precision, fifteen extracts were prepared over a period of one month. Each sample was analysed six times using HPLC.

Mean, s.d. and r.s.d. were calculated. The control samples were tightly sealed and stored at –20℃ when not in use.

6.2 Results

In this chapter, the results of the determination of GPCS in various plant materials and of the validation of the HPLC method, are described.

6.2.1 Validation of HPLC-DAD method

Peak identification

The retention time for GPCS and I.S. was 5.8 min (range: 5.6 – 6.0 min) and 10.9 min (range: 10.7 - 11.1 min), respectively. The blank run showed no interferences with GPCS and I.S..

Calibration and linearity

The calibration was linear at the concentration range from 10 to 200 µg/ml. The linearity data are summarised in Table 2.

Table 2: Calibration data for the quantitation of GPCS obtained by performing triplicates for each of the 5 concentration levels (10, 50, 100, 150 and 200 µg/ml)

Analyte	Retention time [min]	m[1)]	b[1)]	Correlation coefficient r
GPCS	5.8	1.1222	– 0.0136	0.9993

[1)]y=mx + b; x: amount of GPCS; y: ratio of the areas under the peak GPCS:I.S.

Intra- and interday precision and accuracy of the calibration

The intraday precision at low, medium and high concentration levels was ≤ 0.2 %. The accuracy of the assay (± % of the target concentration added) ranged from -2.1 to +1.9 (Table 3).

Table 3: Intraday precision and accuracy for the quantitation of GPCS (control samples)

Added GPCS [µg/ml]	Found [Mean, µg/ml]	s.d. [µg/ml, n=5]	Precision [% r.s.d., n=5]	Accuracy [%]
30	30.6	0.36	0.1	-2.1
120	117.7	2.73	0.2	1.9
180	178.4	2.29	0.1	0.9

The interday precision at low, medium and high concentration levels was ≤ 0.9 % and the assay accuracy ranged from -1.8 to +2.8 % (Table 4).

Table 4: Interday precision and accuracy for the quantitation of GPCS (control samples)

Added GPCS [µg/ml]	Found [Mean µg/ml]	s.d. [µg/ml, n=9]	Precision [% r.s.d., n=9]	Accuracy [%]
30	30.9	0.26	0.9	2.8
120	117.8	0.61	0.5	-1.8
180	179.1	0.62	0.4	-0.5

Limit of quantitation (LOQ)

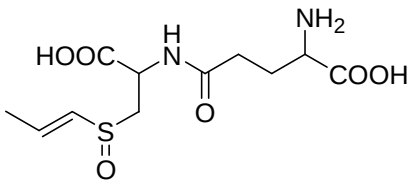
The LOQ corresponded to the lowest calibrator, i.e.10 µg/ml.

6.2.2 Determination of GPCS in plant material

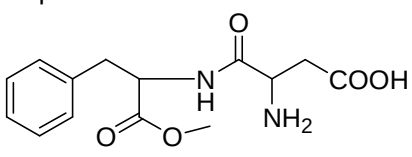
To examine the concentration of GPCS in onion, lyophilised onion powder was extracted with methanol-water-formic acid containing aspartame, which was used as I.S. (Figure 9).

Figure 9: Structures of GPCS and aspartame

GPCS



Aspartame



Validation of GPCS extraction from onion

Peak identification

The retention time for GPCS and I.S. was 5.9 min (range: 5.6 – 6.0 min) and 8.7 min (range: 8.2 - 9.1 min), respectively. The DAD UV spectra were identical to the corresponding standards and peak purity results confirmed that the GPCS and I.S. peaks were homogeneous and pure in the analysed samples.

Intra- and interday precision of the extraction of GPCS from onion

For the extraction of GPCS from dry onion powder the intraday precision was 2.38 % (Table 5).

Table 5: Intraday precision for the quantitation of GPCS in onion powder (extraction samples)

GPCS/onion powder [mg/g]	s.d. [mg/g, n=5]	Precision [% r.s.d., n=5]
4.27	0.10	2.38

The interday precision for the extraction of GPCS from dry onion powder was 3.64 % (Table 6).

Table 6: Interday precision for the quantitation of GPCS in onion powder (extraction samples)

GPCS/onion powder [mg/g]	s.d. [mg/g, n=15]	Precision [% r.s.d., n=15]
4.20	0.15	3.64

Screening of different varieties of onion according to the concentration of GPCS

Several varieties of onion were tested to determine their content of GPCS. Zero to 21 mg GPCS per g powder of onion were measured (Table 7).

Table 7: Content of GPCS in several varieties of onion

Variety	GPCS/onion powder [mg/g]
White onion	21
Red onion	0
Small shallot	1
Shallot sample I	7
Shallot sample II	1
Green onion	0

Screening of other plants concerning the presence of GPCS

As dry powders of garlic, leek, wild garlic, plum, red wine, crimp parsley, plain parsley, dill, sage, chinese cabbage, broccoli, arugula, celeriac, french bean, red cabbage, cucumber, field agaric mushroom, tomato, lettuce, rosemary, thyme, yellow boletus, fennel, and orange showed an inhibition of bone resorption in rats [Muhlbauer 1999, Muhlbauer 2003b] these plants were screened regarding the presence of GPCS. GPCS could not be found in any of these food or beverage samples.

6.3 Discussion

To screen onions and various other bone-resorption inhibiting plants regarding the presence of GPCS, a method for fast, routine, quantitative analysis of GPCS using HPLC-DAD was established. Gamma–glutamyl peptides have been examined previously using various methods using ^{35}S-sulphate, amino acid analyser, two-dimensional electrophoresis or chromatography [Breu 1996]. GPCS has first been detected by Virtanen et al. [Virtanen 1961a, Virtanen 1961b]. Methods to quantify GPCS in onions have been described by several authors [Coolong 2003, Kopsell 1999, Lancaster 1991, Lawson 1991, Matikkala 1967, Shaw 1989, Wetli 2005]. The method developed for our study is an improvement of the one described by Wetli et al. [Wetli 2005]. Less time is needed for sample preparation, because SPE and freeze-drying prior to analysis by HPLC-DAD could be omitted. The use of an I.S. results in higher accuracy: aspartame was found to be a suitable candidate. This dipeptide derivative is structurally similar to GPCS, and therefore, it showed similar extraction and elution properties. Furthermore, aspartame is not biogenic, of synthetic origin, and commercially easily available. An effective extraction was obtained by using a mixture of methanol/water/formic acid as extracting agent and sonication, avoiding the enzymatic cleavage of the target compound and thus stabilising the extract [Mütsch-Eckner 1992]. Chromatography was performed using a column filled with non end-capped octadecylsilyl material and a mobile phase consisting of (A) water containing 0.05 % phosphoric acid 85 % (v/v) and (B) acetonitrile containing 0.05 % phosphoric acid 85 % (v/v). Due to the lack of chromophores, detection had to be performed at 195 nm, where GPCS exhibits an UV absorption maximum which can be attributed to the $n \rightarrow \pi^*$ transition of the two carboxyl groups [Pretsch 2001]. The mobile phase used in the experiments shows a UV cutoff < 195 nm and therefore, detection was possible near the UV cutoff of the DAD. The developed extraction and chromatographic conditions were fully validated. The method was proved to be sufficiently reproducible with an intraday variability of 4.3 ± 2.4 % (mean ± r.s.d) and an interday variability of 4.2 ± 3.6 %. The linear regression of the calibration showed a good correlation coefficient (r = 0.9993) and the LOQ was set at 100 ng allowing reliable determination of GPCS in plant material (signal to noise ratio of 10 to 1).

After having validated the method, the plant samples were analysed. The content of GPCS in lyophilised onion powder ranged from 0 to 21 mg per g onion powder. These results correspond to the findings of others who measured GPCS in concentrations from 0 to 25 mg per g [Coolong 2003, Kopsell 1999, Lancaster 1991, Lawson 1991, Matikkala 1967, Shaw 1989]. In the onion, GPCS functions as sulphur and nitrogen storage [Kasai 1980] and as an intermediate in the biosynthetic pathway of flavour precursors [Lancaster 1989, Lancaster 1991]. Variation in the concentration of GPCS was shown to depend on selenium content of

the soil [Kopsell 1999] and growing temperature [Coolong 2003]. Other factors which most probably affect GPCS concentration in onions are sulphur and nitrogen content of the soil and irrigation conditions. These have been shown to influence the formation of onion flavour precursors [Block 1992, Freeman 1973, Randle 2000, Randle 1994].

Gamma-glutamyl peptides are widely distributed over the plant kingdom. However, a systematic division or classification of these compounds according to their occurrence seems to be impossible [Kasai 1980]. A number of γ-glutamyl derivatives have been found in the genus *Allium*, but neither garlic (*Allium sativum* L.), nor leek (*Allium ampeloprasum* var. *porrum* L.) and wild garlic (*Allium ursinum* L.) were reported to contain GPCS, which corresponds to the results obtained in our study. GPCS was also not detectable in other tested plants from different families, namely plum (*Prunus* L.), red wine, crimp parsley (*Petroselinum crispum* Mill.), plain parsley (*Petroselinum crispum var. neapolitanum* Mill.), dill (*Anethum graveolens* L.), sage (*Salvia* L.), chinese cabbage (*Brassica rapa ssp. Pekinensis)*, broccoli (*Brassica oleracea var. silvestris* L.), arugula (*Eruca sativa* Mill.), celeriac (*Apium* L.), french bean (*Diplotaxis tenuifolia* L.), red cabbage (*Brassica oleracea var. capitata f. rubra* L.), cucumber (*Cucumis sativus* L.), field agaric mushroom (*Agaricus hortensis)*, tomato (*Solanum lycopersicum* L.), lettuce (*Lactuca sativa* L.), rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus* L.), yellow boletus (*Boletus edulis)*, fennel (*Foeniculum vulgare* Mill.) and orange (*Citrus sinensis* L.). These findings are consistent with the literature: GPCS has not been reported to be present in any other plants than onion and sandal (*Santalum album* L.) [Kasai 1980, Kuttan 1974].

7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS

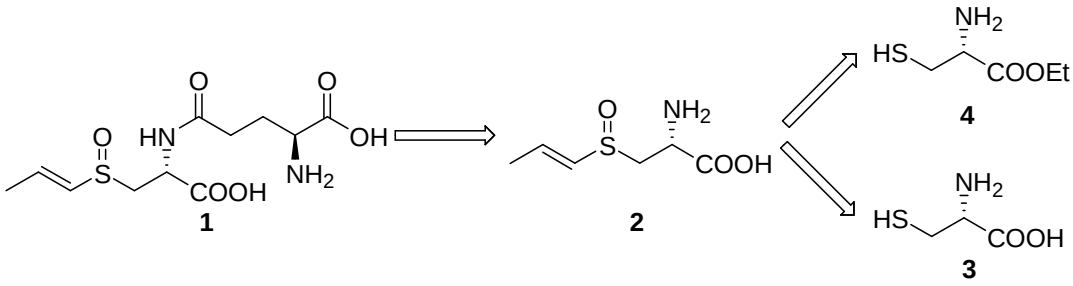
7.1 Retrosynthetic analysis of GPCS

We envisaged that GPCS (**1**) could be synthesised from *trans*-S-(1-propenyl)-L-cysteine sulfoxide (**2**) by coupling with Boc-L-glutamyl-α-t-butyl-N-oxo-succinimide ester. *Trans*-S-(1-propenyl)-L-cysteine sulfoxide (**2**) can be prepared by using two different methods.

The first method implies the synthesis of **2** starting from L-cysteine (**3**). Coupling with allylbromide [Lawson 1991], followed by isomerisation and oxidation leads to a *cis/trans* mixture of **2** [Carson 1966].

The second method starts from L-cysteine ethyl ester (**4**) using a Pd-catalysed C-S coupling as key step. The direct coupling with (*cis*)-1-bromoprop-1-ene circumvents the formation of the *trans*-isomer of **2** (Figure 10) [Namyslo 2006].

Figure 10: Retrosynthetic analysis of GPCS (**1**)



7.2 Materials and methods

7.2.1 Isolation of GPCS from *Allium cepa* L.

Plant material

Onions were purchased on the local food market in Bern and lyophilised, subsequently pulverised, and stored at -20℃ until use.

Chromatographic conditions

A method based on prior experiments of Wetli et al. [Wetli 2005] was used to isolate GPCS from onion.

200 g of lyophilised onion were extracted with 1 l of ethanol (15 %, v/v) under stirring at 60℃ for 1 h. The suspension was filtered through a towel and a 595 grade round filter. The residue was extracted again using the procedure mentioned above. The filtrates from both extractions were collected and after evaporation of the ethanol, the aqueous residue was lyophilised and stored at -20℃ until use.

About 12-g aliquots of the onion extract was dissolved in 100 ml of water and sonicated for 5 min. Separation was performed using cationic exchange column chromatography as

described in Table 8. The fractions were monitored by thin layer chromatography (TLC) on silica gel 60 F_{254} with n-butanol/ethanol/acetic acid/water (25/30/10/10, v/v/v/v) as mobile phase and ninhydrin reagent (1 g of ninhydrin dissolved in 475 ml of isopropanol and 25 ml of acetic acid) for detection. In addition, fractions were checked by HPLC-DAD using the method described in section 6.1.1 Quantitation of GPCS using HPLC-DAD.

Table 8: Conditions of cationic exchange chromatography

Stationary Phase:	43 cm x 30 mm i.d. column (Kuhn AG) packed with 75 ml of Dowex 50 WX8, 100 - 200 mesh (Sigma-Aldrich)
Resin regeneration:	750 ml hydrochloric acid 6 %
Resin wash:	1000 ml water
Mobile Phase:	1. 1000 ml water 2. 2 x 1000 ml 0.75 M ammonia
Flow:	~ 15 ml/min
Fraction collection:	250-ml cationic fractions were collected manually

Fractions containing GPCS were pooled, evaporated and lyophilised. 2 – 3-g aliquots of this material, dissolved in 20 ml of 0.05 M acetic acid, were used for further separation with anionic exchange column chromatography shown in Table 9. Fractions were monitored using TLC as described above; equal fractions were pooled and lyophilised prior to HPLC-DAD analysis with the procedure mentioned above.

Table 9: Conditions of anionic exchange chromatography

Stationary Phase:	40 cm x 26 mm i.d. column (Amersham Bioscience GmbH) packed with 50 ml of Dowex 1X8, 200 - 400 mesh (Sigma-Aldrich)
Resin regeneration:	500 ml NaOH 4 %
Resin activation:	2000 ml 2.5 M sodium acetate
Mobile Phase:	1. 250 ml 0.05 M acetic acid 2. 250 ml 0.5 M acetic acid 3. 250 ml 2 M acetic acid
Flow:	~ 2.5 ml/min
Fraction collection:	12.5 ml fractions were collected using a fraction collector 684 (Büchi)
Pump:	Minipuls 3 peristaltic pump (Gilson)

100-mg aliquots of the GPCS-containing material obtained by anionic exchange chromatography were separated by normal-phase medium pressure liquid chromatography (MPLC) as shown in Table 10. Fractions were monitored as described above and equal fractions were pooled. The ethanol was evaporated from fractions containing GPCS, and the aqueous residue was lyophilised prior to a final check using NMR.

Table 10: Conditions of normal-phase MPLC

Stationary Phase:	22 cm x 25 mm i.d. column (Büchi) packed with silica gel 60, 15 - 40 µm particle size (Merck) and a 13 cm x 10 mm i.d. pre-column filled with the same material
Mobile Phase:	1. 400 ml ethanol (75 %; v/v) 2. 200 ml ethanol (wash)
Flow:	1 - 2 ml/min
Fraction collection:	60 fractions of 5 ml each were collected using a fraction collector 684 (Büchi)
Pump:	chromatographic pump 681 (Büchi)

7.2.2 Synthesis of GPCS

Solvents and chemicals

Chemicals were purchased from Sigma-Aldrich or Merck and used without further purification. Solvents were of technical grade and distilled prior to use.

Instrumentation

Melting points (MP) were determined on a Büchi Melting point B-545 apparatus and were not corrected.

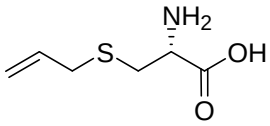
NMR spectra were measured on a Bruker Advance 300 spectrometer, which operated at 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei at 22°C. Chemical shift data are reported in units of δ (ppm) using as the internal standard either residual water (δ = 4.72 for ^{1}H NMR), DMSO (δ = 2.50 for ^{1}H NMR) or trichloromethane (δ = 7.27 for ^{1}H-NMR spectra and δ = 77.0 for ^{13}C NMR spectra). Multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br) for ^{1}H NMR spectra and quartenary (q), tertiary (t), secondary (s) and primary (p) for ^{13}C NMR spectra. Coupling constants (J) are reported in Hz.

Infrared spectra were recorded on a Jasco FT-IR-460 Plus spectrometer equipped with a Specac MKII Golden Gate Single Reflection Diamond ATR System in the 400-4000 cm^{-1} range and are reported in wave numbers (cm^{-1}).

Low and high-resolution mass spectra were recorded on a Waters Micromass Autospec Q mass spectrometer in electron impact mode at 70 eV.

Reactions were monitored by thin layer chromatography on plates coated with silica gel 60 with fluorescent indicator 254 nm (Merck). Substance spots were visualized by UV_{254} light and detected with ninhydrin solution. To prepare the ninhydrin solution, 1 g of ninhydrin was dissolved in 475 ml of isopropanol and 25 ml of acetic acid.

S-allyl-L-cysteine (5) [Lawson 1991]

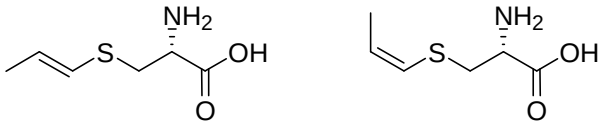


$C_6H_{11}NO_2S$
161.22

L-Cysteine hydrochloride (**3**) (1.57 g, 10.0 mmol) was dissolved in 30 ml of absolute ethanol and converted to the sodium salt with sodium (0.69 g, 30 mmol). Allyl bromide (1.2 g, 10.0 mmol) was added to the suspension and water was added dropwise until a clear solution was obtained. After 2 h of stirring, the solution was concentrated to 10 ml, acidified to pH 8.0 with 6 M hydrochloric acid and kept overnight at 5℃. The white precipitate was filtered, washed with absolute ethanol and dried under *vacuo*. The crude product was purified by recrystallisation from a mixture of ethanol and water to yield S-allyl-L-cysteine (**5**) (1.26 g, 78 %) as white needles.

MP:	208 – 210℃
IR (Film):	ν_{max} [cm^{-1}] = 3020-2870, 2590, 2120 (NH_3^+), 1576 (COO^-), 988 and 916 (allyl double bond)
^{1}H-NMR (300 MHz, D_2O):	δ [ppm] = 2.92 (dd, *J* = 14.9, 7.5 Hz, 1H, SC*H*), 3.05 (dd, *J* = 14.9, 4.3 Hz, 1H, SC*H*), 3.20 (d, *J* = 7.1 Hz, 2H, $CH_2CHC\mathit{H}_2$), 3.88 (dd, *J* = 7.4, 4.3 Hz, 1H, C*H*NH_2), 5.16 - 5.23 (m, 2H, $CHCH_2$), 5.75 – 5.89 (m, 1H, CH)
^{13}C-NMR (75 MHz, D_2O):	δ [ppm] = 30.8 ($CH_2CH\mathit{C}H_2$), 33.9 (SCH_2CH), 53.6 (CHN), 118.4 ($\mathit{C}H_2CH$), 133.6 ($CH_2\mathit{C}H$), 172.9 (OC=O).

Trans, cis-S-(1-propenyl)-L-cysteine (6) [Carson 1966]

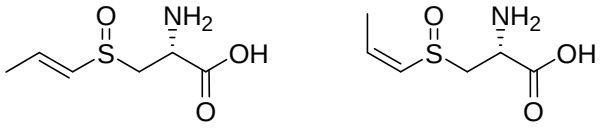


$C_6H_{11}NO_2S$
161.22

Potassium *tert*-butoxide (1.0 g, 8.9 mmol) was added to a suspension of S-allyl-L-cysteine (**5**) (1.0 g, 6.2 mmol) in 60 ml of dimethyl sulfoxide (DMSO) under external cooling. The pale amber solution was then stirred for 3 days at room temperature. 130 ml of ice-water and 3.5 ml of glacial acetic acid were added under cooling. The resulting solution was passed through a column filled with Amberlite IR-120 (H^+) and the amino acid was eluted with 130 ml of a 2 N ammonium hydroxide solution. Concentration of the eluate yielded a mixture of *trans*- and *cis*-S-(1-propenyl)-L-cysteine (**6**) as white crystals. After filtration, the solution was further concentrated, and thus a second crop was obtained. Recrystallisation of the material from aqueous ethanol yielded pure *trans, cis*-S-(1-propenyl)-L-cysteine (**6**) (600 mg, 60 %) in a ratio of 1 to 6 (*trans* to *cis*).

IR (Film):	ν_{max} [cm^{-1}] = 2950 (NH_3^+), 1580 (COO^-), no absorption at 920 (allyl double bond)
^{1}H-NMR (300 MHz, D_2O):	δ [ppm] = 1.68 (dd, *J* = 6.8, 1.5 Hz, 3H, CH_3 *cis*), 1.70 – 1.73 (m, 3H, CH_3 *trans*), 3.03 (dd, *J* = 14.9, 8.1 Hz, 1H, SCH_2 *trans*), 3.18 – 3.26 (m, 1H, SCH_2 *trans*), 3.16 (dd, *J* = 14.9, 7.4 Hz, 1H, SCH_2 *cis*), 3.27 (dd, J = 15.1, 4.3 Hz, 1H, SCH_2 *cis*), 3.90 (dd, *J* = 7.3, 4.1 Hz, 1H, CH_2CH *cis*), 3.86 – 3.91 (m, 1H, CH_2CH *trans*), 5.79 (dq, *J* = 9.0, 6.6 Hz, 1H, CH_3CH *cis*), 5.92 – 5.99 (m, 1H CH_3CH *trans*, 1 H SCH *trans*)
^{13}C-NMR (75 MHz, D_2O):	δ [ppm] = 13.9 (CH_3 *cis*), 17.7 (CH_3 *trans*), 33.6 (SCH_2 *trans*), 33.9 (SCH_2 *cis*), 53,7 (CHN *cis*), 54.2 (CHN *trans*), 120.0 (CH=CHS *trans*), 122.6 (CH=CHS *cis*), 127.9 (CH_3CH *cis*), 132.2 (CH_3CH *trans*), 172.5 (OC=O *cis*), 172.7 (OC=O *trans*)
MS:	$[M+1]^+$ m/z 162.2

Trans, cis-(R_S/S_S)-diastereomers of S-(1-propenyl)-L-cysteine sulfoxide (2) [Carson 1966]

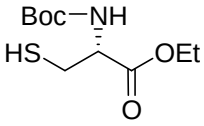


$C_6H_{11}NO_3S$
177.22

70 mg (0.4 mmol) of *trans, cis*-S-(1-propenyl)-L-cysteine (**6**) was suspended in 0.5 ml of a 3 % aqueous hydrogen peroxide solution and was stirred for 24 h. The solvent was carefully removed *in vacuo* at 28℃, affording the product (**2**) (60 mg, 85 %) as a complex mixture of diastereomers.

IR (Film):	ν_{max} [cm^{-1}] = 2980, 2921 (NH^{3+}), 1582 (COO^-), 1010 (S=O), no absorption at 920 cm^{-1} (allyl double bond)
MS:	$[M+1]^+$ m/z 178.4

N-Boc-L-cysteine ethyl ester (7) [Namyslo 2006]



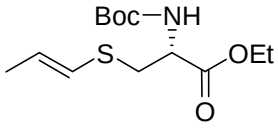
$C_{10}H_{19}NO_4S$
249.33

Triethylamine (6.11 ml, 22.0 mmol) was added to a suspension of L-cysteine ethyl ester hydrochloride (**4**) (3.71 g, 20.0 mmol) and anhydrous tetrahydrofuran (25 ml) at 0℃. A solution of di-*tert*-butyl dicarbonate (4.80 g, 22.0 mmol) in 25 ml of anhydrous tetrahydrofuran was added in portions and the mixture was stirred at room temperature for 2 h. 40 ml of water was added to the solution and the solution was extracted with ethyl acetate (3x50 ml). The combined organic phases were washed with brine, dried over potassium sulphate and the solvent was removed under reduced pressure. The product was purified by flash column chromatography on silica gel (cyclohexane/ethyl acetate, 7/1, v/v) to give *N*-Boc-L-cysteine ethyl ester (**7**) (4.83 g, 97 %) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.29 (t, *J* = 7.1 Hz, 3H, C*H*$_3$CH$_2$), 1.45 (s, 9H, *t*-C_4H_9), 2.97 (dd, *J* = 4.2, 4.2 Hz, 1 H, SCH_a), 2.98 (dd, *J* = 4.2, 4.2 Hz, 1 H, SCH_b), 4.23 (q, *J* = 7.1 Hz, 2H, CH_3CH_2), 4.57 (ddd, *J* = 7.8, 4.2, 4.2 Hz, 1 H, CHN), 5.42 (br d, *J* = 7.8 Hz, 1 H, NH)

^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.2 (CH_3CH_2), 27.4 (CH_2S), 28.3 ($OC(CH_3)_3$), 54.8 (CHN), 61.9 (CH_3CH_2), 80.2 ($OC(CH_3)_3$), 155.1 (NC=O), 170.3 (OC=O)

tert-Butyl (R)-2-[*trans*-prop-1-enylsulfanyl]-1-(ethoxycarbonyl) ethylcarbamate (8) [Namyslo 2006]



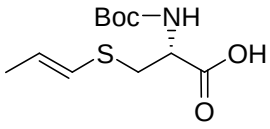
$C_{13}H_{23}NO_4S$
289.39

Tris(dibenzyideneacetone)-dipalladium(0)-chloroform ($Pd_2(dba)_3 \cdot CHCl_3$, 280 mg, 0.27 mmol), 1,1'-bis(diphenylphosphino) ferrocene (499 mg, 0.90 mmol), *trans*-1-bromo-prop-1-ene (0.85 ml, 9.90 mmol) and anhydrous triethylamine (2.50 ml, 18.9 mmol) were added to 50 ml of anhydrous tetrahydrofuran and stirred at room temperature for 20 min and at 40℃ for further 20 min. A solution of *N*-Boc-L-cysteine ethyl ester (**7**) (2.246 g, 9.01 mmol) in anhydrous tetrahydrofuran (2 ml) was added dropwise to the mixture and it was kept at 40℃ for 4 h. After the addition of 50 ml of water and extraction with trichloromethane (3x50 ml),

the combined organic phases were dried over potassium sulphate and the solvent was evaporated *in vacuo*. The product was purified by flash column chromatography on silica gel (pentane/EtOAc, 7/1, v/v) to give pure *tert*-butyl (R)-2-[*trans*-prop-1-enylsulfanyl]-1-(ethoxycarbonyl)ethylcarbamate (**8**) (2.0 g, 76 %) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.29 (t, *J* = 7.1 Hz, 3 H, C*H*$_3$CH$_2$), 1.45 (s, 9 H, *t*-C_4H_9), 1.73 (dd, *J* = 6.4, 1.2 Hz, 3 H, C*H*$_3$CH), 3.08 (d, *J* = 4.8 Hz, 2 H, CH_2S), 4.20 (q, *J* = 7.1 Hz, 2 H, CH_3CH_2), 4.54 (ddd, *J* = 7.8, 4.8, 4.4 Hz, 1 H, CHN), 5.36 (br d, *J* = 7.8 Hz, 1 H, NH), 5.77 (dq, *J* = 14.9, 6.4 Hz, 1 H, CH_3CH), 5.86 (dq, *J* = 14.9, 1.2 Hz, 1 H, CH=C*H*S)

(R)-2-*tert*-Butyloxycarbonylamino-3-[*trans*-prop-1-enylsulfanyl] propanoic acid (9) [Namyslo 2006]

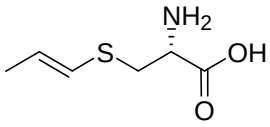


$C_{11}H_{19}NO_4S$
261.34

tert-Butyl (R)-2-[*trans*-prop-1-enylsulfanyl]-1-(ethoxycarbonyl)ethylcarbamate (**8**) (563 mg, 1.95 mmol) in anhydrous methanol (0.5 ml) was reacted with 1 M lithium hydroxide in methanol (3.89 ml) at 4℃ for 12 h. Subsequently, 7 ml of DOWEX 50WX8 (H^+) was added at the same temperature under stirring and filtered off. The filtrate was washed with ethyl acetate (3x50 ml). The aqueous layer was extracted with ethyl acetate (3x15 ml) and the combined organic layers were dried over potassium sulphate and evaporated *in vacuo* at 28℃ to yield (R)-2-*tert*-butyloxycarbonylamino-3-[*trans*-prop-1-enylsulfanyl]propanoic acid (**9**) (450 mg, 89 %)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.46 (s, 9 H, *t*-C_4H_9), 1.73 (dd, *J* = 6.4, 1.2 Hz, 3 H, C*H*$_3$CH), 3.11 (d, *J* = 4.9 Hz, 2 H, CH_2S), 4.59 (ddd, *J* = 7.7, 4.9, 4.0 Hz, 1 H, CHN), 5.38 (br d, *J* = 7.7 Hz, 1H, NH), 5.81 (dq, *J* = 14.8, 6.4 Hz, 1 H, CH_3CH), 5.90 (dq, *J* = 14.8, 1.2 Hz, 1 H, CH=C*H*S)

Trans-S-(1-propenyl)-L-cysteine (6) [Namyslo 2006]

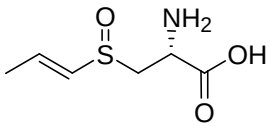


$C_6H_{11}NO_2S$
161.22

Trifluoroacetic acid (0.17 ml, 2.30 mmol) in anhydrous dichloromethane (0.17 ml) was added to (R)-2-*tert*-butyloxycarbonylamino-3-[*trans*-prop-1-enylsulfanyl]propanoic acid (**9**) (300 mg, 1.15 mmol) and stirred for 20 h. Afterwards, 30 ml of water and 15 ml of activated Amberlyst A-21 were added in portions and stirred for 10 min. The ion exchange resin was filtered off, washed with water (10x25 ml) and the collected aqueous solution was evaporated to dryness *in vacuo* at 28℃ to give *trans*-S-(1-propenyl)-L-cysteine (**6**) (130 mg, 70 %) as a white solid.

^{1}H NMR (300 MHz, D_2O): δ = 1.64 (br s, 3 H, C*H*$_3$CH), 2.96 (dd, *J* = 14.9, 7.9 Hz, 1 H, SCH_a), 3.15 (dd, *J* = 14.9, 3.0 Hz, 1 H, SCH_b), 3.82 (m, 1 H, CHN), 5.83–5.91 (m, 2 H, CH=CH)

Trans-S-(R_S/S_S)-diastereomers of S-(1-propenyl)-L-cysteine sulfoxide (2) [Namyslo 2006]



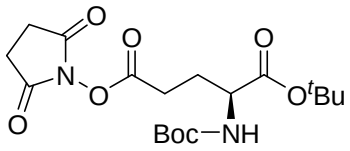
$C_6H_{11}NO_3S$
177.22

3 % of aqueous hydrogen peroxide (0.98 ml, 0.86 mmol) was reacted with *trans*-S-(1-propenyl)-L-cysteine (**6**) (132 mg, 0.82 mmol) overnight. Afterwards, the solvent was removed *in vacuo* at 28℃ and the *trans*-S-(R_S/S_S)-diastereomers of S-(1-propenyl)-L-cysteine sulfoxide (**2**) were obtained in quantitative yield as white solid.

^{1}H NMR (300 MHz, D_2O): δ (diastereomeric sulfoxide I) = 1.86 (d, *J* = 7.1 Hz, 3 H, C*H*$_3$CH), 3.17 (dd, *J* = 13.7, 8.2 Hz, 1 H, SCH_a), 3.56 (dd, *J* = 13.7, 5.6 Hz, 1 H, SCH_b), 4.03 (dd, *J* = 8.2, 5.6 Hz, 1 H, CHN), 6.42 (d, *J* = 14.3 Hz, 1 H, CH=C*H*S), 6.63 (dq, 1 H, *J* = 14.3, 7.1 Hz, CH_3C*H*)

δ (diastereomeric sulfoxide II) = 1.87 (d, 3 H, *J* = 7.1 Hz, C*H*$_3$CH), 3.34 (m, 2 H, SCH_2), 4.09 (dd, *J* = 8.0, 4.0 Hz, 1 H, CHN), 6.42 (d, *J* = 14.3 Hz, 1 H, CH=C*H*S), 6.58 (dq, *J* = 14.3, 7.1 Hz, 1 H, CH_3C*H*)

Boc-L-glutamyl-α-t-butyl-N-oxo-succinimide ester (10) [Lawson 1991]



$C_{18}H_{28}N_2O_8$
400.42

1,3-Dicyclohexylcarbodiimide (240 mg, 1.163 mmol) was added to a solution of Boc-L-glutamic acid-α-*t*-butyl ester (350 mg, 1.155 mmol) and N-hydroxy-succinimide (135 mg, 1.173 mmol) in 3 ml of tetrahydrofuran at -20°C. The mixture was allowed to warm to room temperature and stirring was continued for 12 h. 0.1 ml of glacial acetic acid was added and the mixture was stirred for another 1 h. The reaction mixture was diluted with 15 ml of ethyl acetate and filtered. The filtrate was washed with an ice-cold solution of 5 % sodium hydrogencarbonate in water (20 ml), water (10 ml) and brine (10 ml). Then it was dried using potassium sulphate and evaporated to give the Boc-L-glutamyl-α-*t*-butyl-N-oxo-succinimide ester (**10**) (380 mg, 83 %).

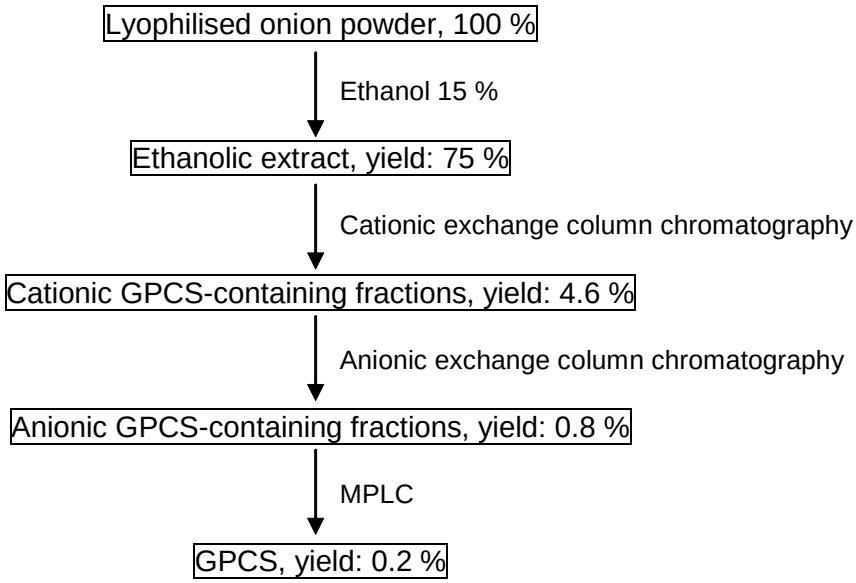
^{1}H-NMR (300 MHz, $CDCl_3$):	δ [ppm] = 1.45 (s, 9H, Bu^t), 1.48 (s, 9H, Bu^t), 1.96-2.08 (m, 2H, βCH$_2$ Glu), 2.19-2.37 (m, 2H, βCH$_2$ Glu), 2.47-2.78 (m, 3H, αCH$_3$Glu), 2.83 (s, 4H, CH$_2$NSu), 5.15 (d, J = 7.2 Hz, 1H, NH)
^{13}C-NMR (75 MHz, $CDCl_3$):	δ [ppm] = 24.6 (CH$_2$NSu), 27.2 (βCH$_2$ Glu), 27.8 (Bu^t), 28.1 (Bu^t), 52.9 (*C*HNH), 79.9 (*C*Bu^t), 82.4 (*C*Bu^t), 155.2 (C=O), 167.8 (C=O), 169.8 (2 C=O), 170.6 (C=O)
MS:	$[M+1]^+$ m/z 401.4

7.3 Results and discussion

7.3.1 Isolation of GPCS from *Allium cepa* L.

200 g of lyophilised onion powder was extracted with ethanol (15 %; v/v) and yielded in 150 g (75 %; w/w) of onion extract. A total of 10 cationic exchange column chromatographies were performed, each with 12 g of ethanolic extract. In sum, 9.13 g of GPCS-containing fractions were obtained corresponding to 4.6 % (w/w) of lyophilised onion powder. These fractions were used to perform anionic exchange column chromatographies which yielded in 1.58 g of GPCS-containing fractions (0.8 %; w/w). Finally, MPLC of 913 mg of these fractions yielded 313 mg of pure GPCS (0.2 %; w/w). This amount is lower than that obtained by Wetli et al. [Wetli 2005] (0.7 %; w/w) which might be attributed to different contents of GPCS in the starting material. An overview over the extraction is given in Figure 11.

Figure 11: Overview of the isolation of GPCS from onion



7.3.2 Synthesis of GPCS

The synthesis of GPCS (**1**) consisted of three main parts:

1. Synthesis of *trans*-S-1-propenyl-L-cysteine sulfoxide,
2. Synthesis of N-protected and carboxyl-activated L-glutamic acid and
3. Coupling of these two molecules.

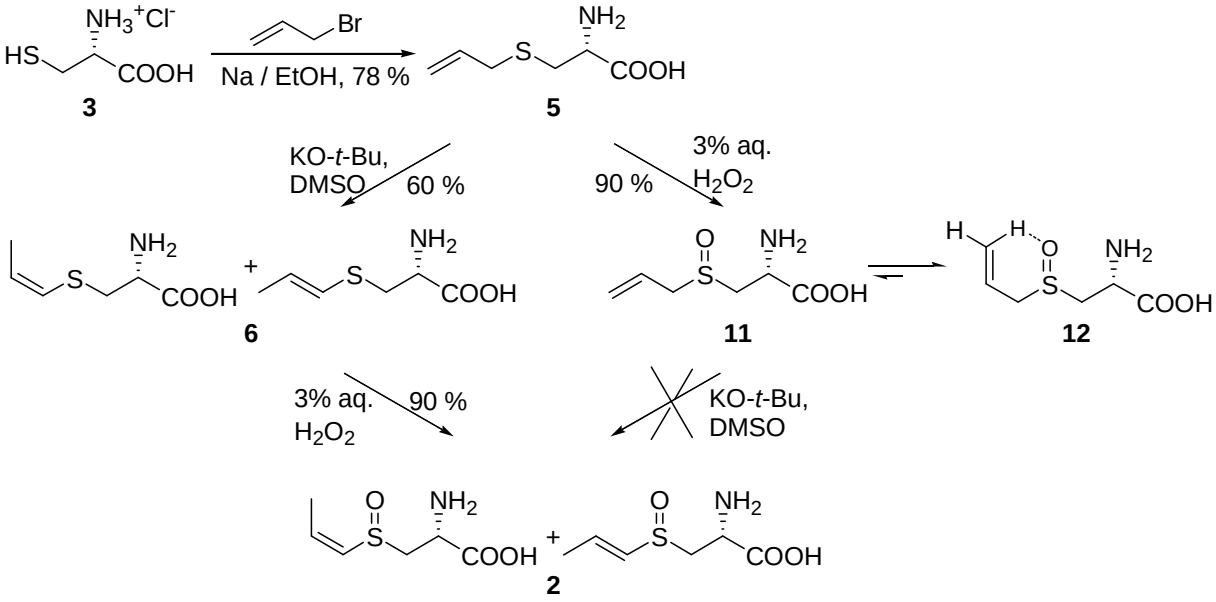
Synthesis of *trans*-S-1-propenyl-L-cysteine sulfoxide (2) [Namyslo 2006]

To synthesise *trans*-S-1-propenyl-L-cysteine sulfoxide (**2**) two different pathways were performed.

Pathway I

Attempts to obtain *trans*-S-1-propenyl-L-cysteine sulfoxide (**2**) by the first procedure led to a mixture of the *trans*- and *cis*-isomers of S-1-propenyl-L-cysteine sulfoxide (**2**) (Figure 12).

Figure 12: Synthesis of *trans, cis*-S-1-propenyl-L-cysteine sulfoxide



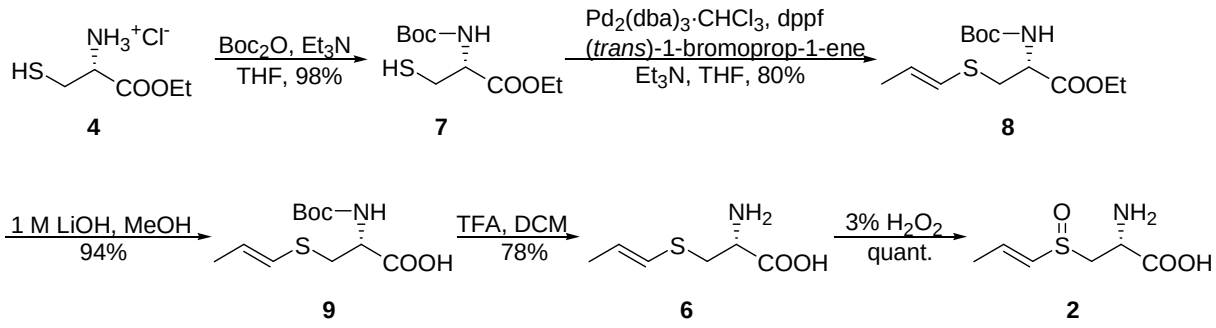
L-Cysteine hydrochloride (**3**) was alkenylated with allyl bromide [Lawson 1991]. It was performed in the presence of sodium in ethanol and yielded 78 % of S-(2-propenyl)-L-cysteine. In the next two steps, S-allyl-L-cysteine (**5**) was isomerised and oxidised to *trans, cis*-S-(1-propenyl)-L-cysteine sulfoxide (**2**). Two different options were pursued. S-Allyl-L-cysteine (**5**) was oxidised with hydrogen peroxide and yielded the sulfoxide as a mixture of diastereomers [Yu 1994]. Attempts to isomerise this product with potassium *tert*-butoxide in dimethyl sulfoxide according to Carson et al. [Carson 1966] failed. This might be explained by the configurations of S-(2-propenyl)-L-cysteine (**11**) possibly resulting in a 6-membered transition state (**12**).

Because this option was not successful, the order of isomerisation and oxidation was changed. Treatment of S-allyl-L-cysteine (**5**) with potassium *tert*-butoxide in dimethyl sulfoxide resulted in a mixture of *trans, cis*-S-(1-propenyl)-L-cysteine (**6**) [Carson 1966] with the *cis*- isomer as the major product (60 % yield). To improve the yield of the *trans* product longer reaction times (7 days) are necessary. Oxidation of *trans, cis*-S-(1-propenyl)-L-cysteine (**6**) was performed with an aqueous solution of 3 % aqueous hydrogen peroxide leading to *trans, cis*-diastereoisomers of S-(1-propenyl)-L-cysteine sulfoxide (**2**) (90 % yield, modification of the method described in [Yu 1994]).

Pathway II

Because the first pathway resulted in a mixture of *trans*- and *cis*-S-(1-propenyl)-L-cysteine sulfoxide (**2**), a second pathway was performed to obtain exclusively the *trans* product (Figure 13).

Figure 13: Synthesis of *trans*-S-1-propenyl-L-cysteine sulfoxide

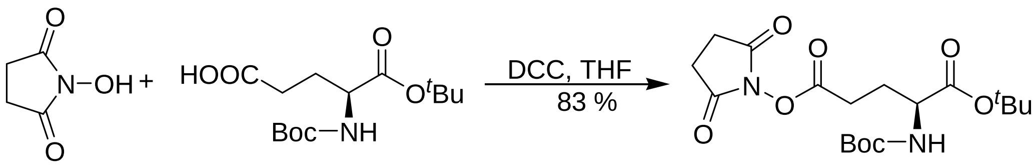


First, commercially available L-cysteine ethyl ester hydrochloride (**4**) was Boc-protected using di-*tert*-butyl dicarbonate and triethylamine in tetrahydrofuran, yielding N-Boc-L-cysteine ethyl ester (**7**) (98 %). Coupling with *trans*-1-bromoprop-1-ene in tetrahydrofuran under the catalysis of palladium(0) and purification by flash column chromatography, led to the *trans*-(prop-1-enyl) derivative (**8**) in 80 % yield from N-Boc-L-cysteine ethyl ester (**7**). The ethyl ester (**8**) was hydrolysed with lithium hydroxide giving the lithium carboxylate of **8**. To obtain the propanoic acid (**9**), a cationic exchange resin was used, yielding 94 % of the product. The N-Boc deprotection was performed with trifluoroacetic acid in dichloromethane and yielded the unprotected amino acid derivative (**6**) (78 %). A quantitative oxidation of *trans*-S-(1-propenyl)-L-cysteine (**6**) was achieved by using an aqueous solution of 3 % hydrogen peroxide.

Synthesis of N-protected and γ-carboxyl-activated L-glutamic acid

Boc-L-glutamyl-α-*t*-butyl-N-oxo-succinimide ester (**10**) was generated from N-hydroxy-succinimide (**13**), Boc-L-glutamic acid-α-*t*-butyl ester (**14**) and dicyclohexylcarbodiimide (DCC) in tetrahydrofuran and, yielding 83 % of the product [Lawson 1991] (Figure 14).

Figure 14: Synthesis of Boc-L-glutamyl-α-*t*-butyl-N-oxo-succinimide ester



13 **14** **10**

Coupling of *trans*-S-(1-propenyl)-L-cysteine sulfoxide (2) and N-protected and carboxyl-activated L-glutamic acid

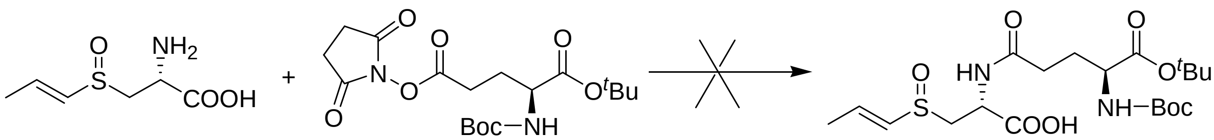
In order to obtain GPCS (**1**) the strategy was to couple *trans*-S-(1-propenyl)-L-cysteine sulfoxide (**2**) with N-protected and carboxyl-activated L-glutamic acid (**10**). This compound is known to react predominantly at the γ-function.

The Boc-group was selected for N-protection because it can easily be removed by trifluoroacetic acid in dichloromethane. In order to check the feasibility of amide coupling and to find optimal conditions for this reaction, different bases, solvents and reaction times for the amide coupling were tested (Table 11). Unfortunately, the product could not be formed.

*Table 11: Working conditions for the coupling of Boc-L-glutamyl-α-*t*-butyl-N-oxo-succinimide ester and trans-S-(1-propenyl)-L-cysteine sulfoxide (2)*

Base	Solvent	Time of reaction	Product
Triethylamine	Acetone	1 – 7 days	No reaction
Sodium hydrogencarbonate	Acetonitrile	1 – 7 days	No reaction
Triethylamine	Dimethylformamide	1 day	No reaction

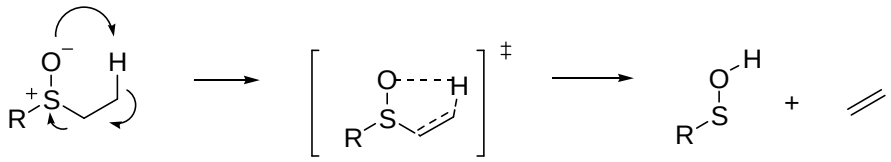
Figure 15: Amide coupling of *trans*-S-(1-propenyl)-L-cysteine sulfoxide (**2**) with N-protected and carboxyl-activated L-glutamic acid (**10**)



2 **10** **15**

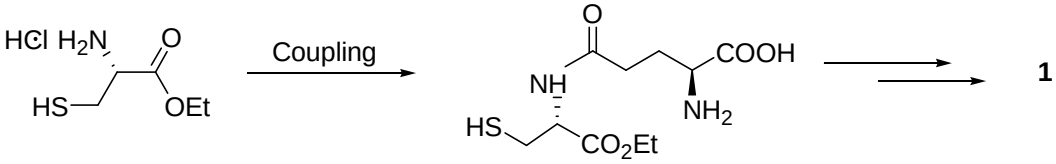
It is supposed that the coupling reaction with activated glutamic acid under standard conditions results in decomposition of S-(1-propenyl)-L-cysteine sulfoxide (**2**). The fact that the evaporation of **2** has to be performed at very mild conditions, at 28℃, and decomposition was observed when the temperature was increased, indicates instability of the substance. A second possibility is the internal elimination of sulfoxides to form olefins and sulfenic acids which may inhibit the coupling process (Figure 16) [Cubbage 2001].

Figure 16: Internal elimination of sulfoxides



Therefore, coupling at an earlier stage of the synthesis might lead to the desired product (Figure 17).

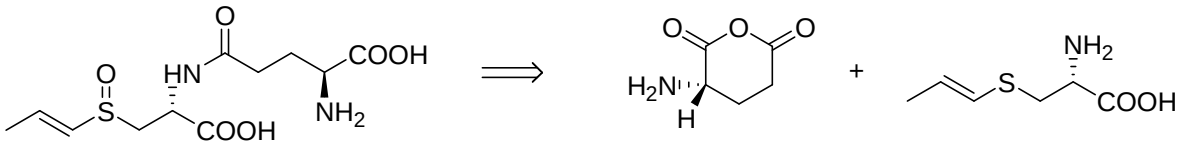
Figure 17: Alternative coupling strategy I



16 **17**

Furthermore, more efficient carboxyl activation groups for the coupling with the alpha amino acid could be tested. One possibility to couple the two amino acids might be the reaction of **6** with the N-protected L-glutamic anhydride (**18**), which is known to react predominantly at the gamma carbonyl function (Figure 18). This protocol has been used by Van den Broek et al. in the total synthesis of gamma-glutamylmarasmine [Van den Broek 1987].

Figure 18: Alternative coupling strategy II



1 **18** **6**

In addition, different protecting groups have to be taken into account. For N-protection the phthaloyl group, which can be removed by hydrazinolysis, as well as the Fmoc (9-Fluorenylmethyloxycarbonyl) could be investigated.

8 Part III: Pharmacodynamic and pharmacokinetic properties of GPCS *in vitro* and *in vivo*

8.1 Materials and methods

8.1.1 Effects of GPCS on the activity, activation and recruitment of osteoclasts *in vitro*

This section contains the description of materials and methods used to determine the effect of GPCS on the activity, activation and development of OCs.

Materials

Medium 199, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) solution (HEPES), Triton X-100, Hoechst H33342, calcitonin, paraformaldehyde (PFA), concentrated hydrochloric acid, L-cysteine, 1 M tartrate solution, 2.5 M acetate solution pH 5.2, napthol AS-BI phosphoric acid 12.5 mg/ml in N,N-dimethyl formamide, bovine serum albumin (BSA) and Fast Garnet GBC Salt were purchased at Sigma-Aldrich and Merck. Minimum essential medium with Earle's salts and without phenol red (MEM), alpha-minimum essential medium with Earle's salts (α-MEM), minimum essential medium with Hanks's salts (MEM Hank's), penicillin-streptomycin-solution (P/S; containing 10'000 units/ml penicillin G sodium and 10'000 µg/ml streptomycin sulphate in 0.85 % saline), fetal bovine serum (FBS), and glutamine were obtained from Gibco. Alexa Fluor 488 phalloidine was purchased from Molecular Probes, RANKL from Peprotech, IMMU-MOUNT from Thermo Shandon, and M-CSF was a gift from Chiron Corporation. Phosphate buffered saline (PBS) was prepared with 8.0 g/l of sodium chloride, 1.44 g/l of disodium hydrogen phosphate dihydrate, 0.2 g/l of potassium chloride and 0.2 g/l of potassium dihydrogen phosphate in bidistilled water. These chemicals were obtained from Sigma-Aldrich or Merck. γ-Glutamyl-L-cysteine-ethylester, glycyl-L-cysteine, glutamyl-glycine and glycyl-L-valine were purchased from Bachem. S-Allyl-L-cysteine was synthesised according to the procedure described in chapter 7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS. Bidistilled water was prepared in house.

Glass coverslips were purchased from Marienfeld GmbH & Co. KG. Dentin slices were in house custom made from ivory goods confiscated by customs. Sputter coating of dentin slices with gold was performed on a sputter machine SCD 004 from BALZERS UNION Limited.

All cell culture media were supplemented with 1 % P/S.

Study of osteoclast activity I: actin ring assay

Isolation of osteoclasts from neonatal rat bones

OCs were isolated from femora and tibiae of 2-day-old Wistar Hanlbm rats (bred in house at the Central Animal Facility of the Medical Faculty). The bones were cut into small pieces in Medium 199 containing 20 mM HEPES (pH 7.3). After repeated (three times) washing, the supernatants were pooled and centrifuged. The cell pellet was resuspended in Medium 199 containing 10 % heat-inactivated FBS (FBS Hia). 50 µl aliquots of the cell suspension were placed on ø 13 mm glass coverslips. Before use, these coverslips were coated overnight with a 0.9 % solution of sodium chloride containing 30 % FBS Hia, washed with water and dried. OCs isolated from femora and tibiae from eight rats were usually distributed onto 32 coverslips.

To allow adherence of OCs, the coverslips were incubated at 37℃ for 1 h. Non-adherent cells were removed by washing two times with Medium 199 containing 10 % FBS Hia. Thereafter, the coverslips were transferred into 24-well-plates containing Medium 199 supplemented with 10 % FBS Hia and were incubated at 37℃. After 3 h the medium was changed and the cells were again incubated for 15 min, if not stated otherwise. The medium was removed and Medium 199 containing 10 % FBS Hia, with or without test substances, was added.

Staining of actin and nuclei

After removal of the culture medium, the cells were fixed with 3 % PFA in PBS for 10 min and washed three times with PBS. The cells were permeabilised with 0.25 % Triton X-100 in PBS on ice for 5 min and then washed again two times with cooled PBS.

Subsequently, the cells were stained for actin by putting the coverslips upside down on drops of 30 µl Alexa Fluor 488 phalloidine (5 Us/ml, dissolved in PBS with 1 % BSA) in a box with a humid atmosphere for 40 min. After carefully dipping the coverslips two times in PBS, the nuclei were stained with the same method with Hoechst H33342 (5 µg/ml, dissolved in PBS) for 5 min. The coverslips were subsequently gently washed twice with demineralised water and dried at room temperature before they were embedded on microscope slides with IMMU-MOUNT.

The slides were blinded and the number of multinucleated OCs (nuclei > 2) and the number of OCs containing actin ring(s) were counted on a Nikon Eclipse E800 microscope (actin, excitation: 465 – 495 nm, fluorescence: 515 – 555 nm, beam splitter reflecting light < 505 nm; nuclei, excitation: 340 – 380 nm, fluorescence: 455 – 485 nm, beam splitter reflecting light < 400 nm). The number of OCs containing one or more actin rings was expressed as percentage of the total number of OCs and is a measure of activation of OCs.

Study of osteoclast activity II: pit assay

Isolation of osteoclasts from neonatal rat bones

OCs were isolated from bones of 2-day-old rats by the following procedure. A) Femora were dissected in MEM. After removal of soft tissues, the bones were finely chopped in MEM supplemented with 10 % FBS and acidified with 10 mmol/l hydrochloric acid. The supernatant was transferred to a 15-ml flask. B) The remaining bone pieces were washed twice with acidified MEM containing 10 % FBS. The supernatant was collected and pooled with the supernatant collected in step A). C) This cell suspension was vortexed for 20 sec and allowed to settle for 30 sec. The supernatant was transferred into a new 15-ml flask. D) 3 ml of acidified MEM containing 10 % FBS were added to the remaining bone pieces and the suspension was vortexed for 20 sec. After 30 sec the supernatant was combined with the supernatant collected in step C).

800 µl of the cell suspension was placed on 8 dentin slices which were placed in one well. After incubation for 45 min in 5 % carbon dioxide (CO_2)/95 % air at 37°C to allow cells to adhere, the dentin wafers were rinsed by dipping them carefully into sterile PBS. They were transferred into 48-well plates, which contained the pre-equilibrated test substances (dissolved in MEM with 10 % FBS) or MEM with 10 % FBS alone as control. The slices were incubated at 37°C in 5 % CO_2/95 % air for 24 h.

Quantitation of osteoclasts and resorption

To stop the resorption, the cells were fixed with 3 % PFA in PBS for 10 min and thereafter washed three times with demineralised water. To visualise the OCs, the cells were stained for tartrate resistant acid phosphatase (TRAP). For the preparation of the staining solution, 41 ml of demineralised water, 5 ml of a 1 M tartrate solution, 2 ml of a 2.5 M acetate solution pH 5.2 and 2 ml of Napthol AS-BI phosphoric acid 12.5 mg/ml in N,N-dimethyl formamide were mixed for 1 min with 10 – 15 mg Fast Garnet GBC Salt. The solution was filtered and used to stain the cells for 7 min. Finally, the dentin wavers were washed three times with demineralised water and air dried.

Multinucleated OCs (nuclei ≥ 2) were counted on a Nikon Optiphot microscope.

The cells were removed from the dentin wavers by gentle brushing with a toothbrush in 70 % isopropanol and afterwards in demineralised water. The absence of cells and debris was assessed with a microscope. The dentin discs were sputter-coated with gold and the resorption pits were counted blind on a Nikon Optiphot microscope.

Assessment of osteoclast formation *in vitro*

Culture of osteoclasts from bone marrow cells

Bone marrow cells, containing the precursor cells of OCs, were isolated from 6-weeks old mice (male, *ddy*) after euthanasia with CO_2. Femora were dissected, placed in MEM-Hank`s and soft tissue and muscles were removed. The articular ends were cut away and the bones were flushed with 5 ml MEM-Hank`s each. After centrifugation at 250 x *g* for 5 min the cell pellet was resuspended in 10 ml α-MEM containing 10 % FBS. Cells were counted in Turk-solution, which stains the nuclei of leucocytes, in a Neubauer chamber. Thereafter, the cell suspension was diluted with α-MEM containing 10 % FBS to 60'000 cells per ml. 50 µl of the cell suspension was distributed into each well of 96-well plates containing 50 µl of α-MEM supplemented with 10 % FBS, 60 ng/ml CSF and 10 ng/ml RANKL, with or without the substances to be tested, for a final concentration of 30 ng/ml CSF and 5 ng/ml of RANKL and the required concentrations of test substances. Media containing test substances were adjusted to the same pH as the control medium with 1 M sodium hydroxide solution and filtered through a 0.2-µm filter before use. The cells were incubated in 5 % CO_2/95 % air at 37℃. After three days, the medium was changed and cells were further incubated. Depending on the stage of maturation of the cells in the control group, the cultures were usually fixed on day 4 or 5 of the experiment.

Quantitation of osteoclast development

Cells were fixed with 4 % PFA in PBS for 10 min and washed three times with demineralised water before staining the cells for TRAP. The staining solution was prepared as described above. Cells were stained for 5 min and washed three times with demineralised water. Multinucleated, $TRAP^+$ cells were counted (nuclei ≥ 3) on a Wild-Leitz Diavert microscope.

Statistical analysis

The results were tested using analysis of variance (ANOVA, Bonferroni) using WinSTAT® version 2005.1 for Microsoft® Excel.

8.1.2 Studies to determine the stability of GPCS in simulated gastric acid and simulated intestinal fluid

In this section, materials and methods of studies on the stability of GPCS in simulated gastric acid and simulated intestinal fluid are shown.

Materials

As GPCS was not commercially available it was isolated as described in chapter 7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS.
Acetonitrile Chromasolv®, methanol Chromasolv®, pepsin and pancreatin were purchased from Sigma-Aldrich and ortho phosporic acid 85 %, sodium chloride, concentrated hydrochloric acid, potassium dihydrogen phosphate and sodium hydroxide were obtained from Merck. Onions were purchased on the local food market in Bern and lyophilised, subsequently pulverised, and stored at -20℃ until use.
Bidistilled water was prepared in house.

Instrumentation

See section 6.1.1 Quantitation of GPCS using HPLC-DAD.

Chromatographic conditions and method development

Optimisation of mobile phase

The mobile phase consisted of solvent A with bidistilled water containing 0.05 % phosphoric acid 85 % (v/v) and solvent B with acetonitrile containing 0.05 % phosphoric acid 85 % (v/v). To optimise the mobile phase, different water contents were tested isocratically: 25, 20, 15, 10, 7.5 and 5 % of solvent A. The flow was 0.7 ml/min.

Selection of stationary phase and column temperature

Two different stationary phases were tested to find optimal conditions for the determination of GPCS in simulated gastric acid and simulated intestinal fluid:
A) 125 x 4 mm I.D. column, packed with Spherisorb ODS-1, particle size 3.0 µm and a 8 x 4 mm I.D. precolumn, packed with the same column material (Machery-Nagel), and
B) 150 x 4.6 mm I.D. column, packed with Shodex ODP2 HP-4D, particle size 5 µm (Infochroma).
The temperature of the columns was 40℃.

Detection

Wavelength for the quantitation of GPCS was UV 195 ± 4 nm with online recording of the UV spectra from 190 - 400 nm (DAD).

Peak identification (selectivity/specificity)

Chromatographic selectivity: The retention time of the analyte was assigned with the corresponding standard.
Spectroscopic selectivity: The DAD UV spectra in the range of 190 – 400 nm were compared to the spectra of the standards. The recorded spectra were also used for the peak purity check. Blank samples of simulated gastric acid and simulated intestinal fluid were analysed to exclude interferences of the analytes with the matrices.

Calibration and linear range

Different stationary phases were used in this study. Thus, two calibrations of GPCS were performed by applying the external standard and linear regression analysis over the concentration ranges of 10 µg/ml to 200 µg/ml (Spherisorb ODS-1) and 5 µg/ml to 125 µg/ml (Shodex ODP2), respectively, for GPCS. To obtain the calibration graph for GPCS the area of GPCS was plotted against the concentration of GPCS.
Calibrator concentrations of 10, 50, 100, 150, 200 µg/ml GPCS (Spherisorb ODS-1) and 5, 50, 125 µg/ml GPCS (Shodex ODP2) were prepared in water/acetonitrile (1/3; v/v) from a stock solution of GPCS. Each sample was measured three times.

Stability of GPCS in simulated gastric acid

4.9 mg of GPCS were added to 5.0 ml of simulated gastric acid (preparation see below) and kept under stirring at 37°C. After 0.25 h, 50 µl of the reaction mixture was taken, diluted to 500 µl with water/acetonitrile (1/3; v/v) and analysed immediately using HPLC-DAD. The same procedure was repeated after 0.5, 1, 2, 4, 6 and 24 h of reaction.
Preparation of simulated gastric acid [Europäische Pharmakopöe 2005]: 200.0 mg of sodium chloride and 320.7 mg pepsin were dissolved in bidistilled water. After adding 8 ml of 1 N hydrochloric acid, the solution was diluted to 100.0 ml with bidistilled water.

Stability of GPCS in simulated intestinal fluid

2.3 mg of GPCS were added to 2.3 ml of simulated intestinal fluid (preparation see below) and kept under stirring at 37°C. After 0.25 h, 50 µl of reaction mixture was taken, diluted to 500 µl with water/acetonitrile (1/3; v/v) and analysed immediately using HPLC-DAD. The same procedure was repeated after 0.5, 1, 2, 4, 6 and 24 h.

Preparation of simulated intestinal fluid (SIF) [The United States Pharmacopoeia 2006]: 0.68 g of potassium dihydrogen phosphate was dissolved in 25 ml of bidistilled water. 7.7 ml of a 0.2 N solution of sodium hydroxide, 50 ml of bidistilled water and 1.0 g of pancreatin were added and mixed. The resulting suspension was adjusted to pH 6.8 by the addition of a 0.2 N solution of sodium hydroxide. It was diluted to 100.0 ml with bidistilled water and filtrated.

Stability of GPCS in water

2.0 mg GPCS were added to 2.0 ml bidistilled water and kept under stirring at 37°C. After 0.25 h, 50 µl of the reaction mixture was taken, diluted to 500 µl with water/acetonitrile (1/3; v/v) and analysed immediately using HPLC-DAD. The same procedure was repeated after 0.75, 1.25, 2, 4, 6 and 24 h.

Determination of GPCS in *Allium cepa* L. powder incubated in simulated intestinal fluid

500.5 mg lyophilised powder of *Allium cepa* L. were added to 10.0 ml of simulated intestinal fluid and kept under stirring at 37°C. After 0, 0.2 5, 0.75, 1.25, 2, 4, 6 and 24 h, 500 µl of the reaction mixture was taken and filtrated. 50 µl of the filtrate was diluted with 450 µl of acetonitrile/water (9/1; v/v) containing 0.05 % phosphoric acid 85 %, filtrated again, and analysed immediately using HPLC-DAD.

8.1.3 Pilot studies to determine the pharmacokinetic properties of GPCS in rats

The following experiments were performed to assess the pharmacokinetic properties of GPCS *in vivo*.

Animals

The pharmacokinetic properties of GPCS were studied in male Wistar Hanlbm rats. Rats were 8 – 10 weeks old and weighed about 250 g. They were kept in house in the Central Animal Facility of the Medical Faculty in compliance with the Swiss and US National Institute of Health guidelines for care and use of experimental animals. The State Committee for the Control of Animal Experimentation approved the performed experiments (authorisation number 48/05).

Experimental protocol

After a fasting period of 20 h, rats received 250 µl of a 10 mg/ml solution of GPCS (dissolved in 0.9 % sodium chloride) either by gavage or injection into the tail vein. Blood samples of maximum 150 µl were collected by stab puncture of the lateral tail vein into heparin coated Eppendorf tubes 5, 15, 30, 60 and 180 min after GPCS administration. A basal blood sample was taken one day before. After collection, blood was centrifuged immediately at 800 x *g*, at 4℃ for 10 min, and the plasma was stored at -70℃ until analysis. Samples were analysed using HPLC-DAD and HPLC-MS/MS after preparation with SPE.
Pharmacokinetic parameters were estimated using the area ratio of GPCS/I.S. versus time. Areas under the curve (AUC), the time points of the maximum area ratio GPCS/I.S. (t_{max}) and the oral bioavailabilities were calculated using PK functions (Add-In for Microsoft Excel).

Standards, solvents and chemicals

As GPCS was not commercially available it was isolated as described in chapter 7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS.
Bidistilled water was prepared in house. Acetonitrile Chromasolv®, methanol Chromasolv®, ortho phthaldialdehyde, ortho phosporic acid 85 %, concentrated hydrochloric acid, ammonium hydoxide solution 25 %, *tert*-butylmercaptan, sodium dihydrogen phosphate hydrate, disodium hydrogen phosphate dihydrate, sodium hydroxide, L-aspartyl-L-phenylalanine and sodium tetraborate decahydrate were purchased at Sigma-Aldrich and Merck. 0.45-µm filters were obtained from Infochroma.

To prepare the borate buffer pH 9.5, 4.77 mg of sodium tetraborate decahydrate was dissolved in 200 ml of water. The pH was adjusted to 9.5 with a solution of 10 M sodium hydroxide, and water was added to obtain a final volume of 250.0 ml.
The derivatisation reagent was prepared by dissolution of 140 mg of ortho phthaldialdehyde in 5 ml of methanol and addition of 200 µl of *tert*-butylmercaptan. The volume was adjusted with borate buffer pH 9.5 to 50.0 ml.
1.93 g sodium dihydrogen phosphate hydrate and 2.53 g disodium hydrogen phosphate dehydrate were dissolved in 1000.0 ml of water to obtain the phosphate buffer solution. The pH was adjusted with concentrated hydrochlorid acid to 6.5.

Instrumentation

HPLC-DAD

See section 6.1.1 Quantitation of GPCS using HPLC-DAD.

HPLC-MS/MS

The HPLC system consisted of an Agilent 1100 Series degasser, binary pump, thermostated column compartment (Agilent Technologies), MPS 3C autosampler with wash station (Gerstel AG). The HPLC system was coupled to an API 5000 triple quadrupole mass spectrometer with an electrospray Turbo V ionisation source (Applied Biosystems, MDS Sciex). All data were acquired and processed using Analyst 1.4.2 software.

Method development

SPE parameters

Four SPE columns with different column materials, Oasis MAX 3 cc SPE column, Oasis MAX 1 cc SPE column, Oasis MCX 1 cc SPE column and Oasis WAX 1 cc SPE column (Waters) were tested using various extracting solvents.
Prior to SPE, samples were prepared as follows. For the acidic and basic diluents, 20 µl of concentrated phosphoric acid (for Oasis MCX and Oasis WAX) and 20 µl of concentrated ammonium hydroxide (for Oasis MAX), respectively, were added to 500 µl of water. 500 µl of 0.11 mg/ml GPCS in water was diluted with 500 µl of the acidic or basic diluent. SPE was performed as described in Table 12 using a manual Adsorbex sample preparation unit (Merck).

Table 12: SPE method development for the determination of GPCS in plasma

SPE Column	Oasis MAX 3 cc	Oasis MAX 1 cc	Oasis MCX 1 cc	Oasis WAX 1 cc
Conditioning	2 ml Methanol	1 ml Methanol	1 ml Methanol	1 ml Methanol
	2 ml Water	1 ml Water	1 ml Water	1 ml Water
Loading	Diluted basic sample	Diluted basic sample	Diluted acidic sample	Diluted acidic sample
Washing	2 ml 5 % Ammonium hydroxide solution	1 ml 5 % Ammonium hydroxide solution	1 ml 2 % Formic acid in water	1 ml 5 % Ammonium hydroxide solution
	2 ml Methanol	1 ml Methanol	1 ml Methanol	1 ml Methanol
Elution	2 ml 2 % Formic acid in methanol/water 1/1 (v/v)	1 ml 2 % Formic acid in methanol/water 1/1 (v/v)	1 ml 2 % Ammonium hydroxide in methanol	1 ml 2 % Ammonium hydroxide in methanol

The eluates were evaporated to dryness under a stream of nitrogen at 40℃. 500 µl of acetonitrile/water (3/1; v/v) were added to the residue. The test tube containing the solution was closed, vortexed three times and sonicated for 3 min. The solution was filtered using a 0.45-µm filter and injected into the HPLC-DAD. To determine the most efficient extraction conditions, a solution of 0.11 mg/ml of GPCS in water was directly analysed using HPLC-DAD and the amount of GPCS found was directly related to the recoveries of GPCS in the different eluates.

Extraction procedure of plasma samples

The preparation of the plasma solution prior to the SPE was performed as follows. 100 µl of a 1.67 % solution of ammonium hydroxide was mixed with 50 µl plasma. 50 µl of internal standard (28 µl/ml of L-aspartyl-L-phenylalanine in water) and 800 µl of water were added, and the solution was vortexed three times. Plasma samples were purified with Oasis MAX 3 cc SPE columns (Waters) using an ASPEC robotic system (Automated Sample Preparation with Extraction Columns; Gilson; see Figure 8) or a manual Adsorbex sample preparation unit (Merck). The elution scheme is described in Table 13.

Table 13: Elution scheme: SPE of plasma for the determination of GPCS

Conditioning	Methanol	2 ml
	Water	2 ml
Loading	Sample prepared as described above	0.9 ml
Washing	5 % Ammonium hydroxide solution	2 ml
	Methanol	2 ml
Elution	2 % Formic acid in methanol/water 1/1 (v/v)	2 ml

The eluate was evaporated to dryness under a stream of nitrogen at 40℃. 100 µl of cetonitrile/water (3/1; v/v) were added to the residue. The test tube containing the solution was closed, vortexed three times and sonicated for 3 min. The solution was filtered using a 0.45-µm filter and analysed without further treatment, if not stated otherwise.

Chromatographic conditions and method development

System A: HPLC-DAD

Stationary phase and column temperature

As stationary phase a 125 x 4 mm i.d. column, packed with Spherisorb ODS-1, particle size 3.0 µm and a 8 x 4 mm i.d. precolumn, packed with the same material (Machery-Nagel) was used. The column temperature was 40℃.

Determination of GPCS without derivatisation

10 µl of the sample were injected into the HPLC.

Mobile phase

The mobile phase consisted of solvent A with water containing 0.05 % phosphoric acid (v/v) and solvent B with acetonitrile containing 0.05 % phosphoric acid (v/v). Separation was performed isocratically using 80 % solvent B for 30 min.

Detection

The wavelength for the quantitation of GPCS was 195 ± 4 nm with online recording of the UV spectra from 190 to 400 nm (DAD).

Determination of GPCS with derivatisation

Derivatisation of samples was performed by adding 200 µl of derivatisation reagent (preparation see above) to 50 µl of the sample. 10 µl was injected into the HPLC.

Optimisation of mobile phase

The mobile phase consisted of solvent A with phosphate buffer and solvent B with acetonitrile. To obtain optimal conditions of the mobile phase, different contents of phosphate buffer were tested isocratically: 5, 25, 50, 75 and 95 % of solvent A. The flow was 1.0 ml/min.

Detection

The wavelength for the quantitation of the GPCS derivative was 335 ± 4 nm with online recording of the UV spectra from 190 to 400 nm (DAD).

System B: HPLC-MS/MS

Samples were diluted 1:100 with water and 10 µl injected into the HPLC.

Mobile Phase

The mobile phase consisted of solvent A with acetonitrile containing 0.05 % formic acid (v/v) and solvent B with water containing 0.05 % formic acid (v/v). Separation was performed isocratically using 5 % of solvent A for 5 min. The column was washed with 95 % solvent B for 2 min and then reequilibrated to the initial conditions for 5 min. The flow was 0.2 ml/min.

Stationary phase and column temperature

A 150 x 2.1 mm i.d. column, packed with Synergi Fusion RP 80, particle size 4.0 µm (Phenomenex) was used. The column temperature was 23°C.

Optimisation of detection

Acquisition was performed in the Multi reaction monitoring mode (MRM) and the protonated (ESI^+) adduct ion was chosen as the precursor. Source conditions were established as follows: ion spray voltage 5 kV (ESI^+); collision gas, 5 psi; curtain gas, 30 psi; ion source gas 1 (nebuliser gas) 65 psi and 2 (turbo gas), 80 psi; ion source temperature, 500°C. High purity nitrogen (> 98 %) was used as desolvation, nebulisation and collision gas. MS/MS parameter optimisation was obtained by direct infusion at 10 µl/min of 50 ng/ml individual standard solutions in 50 % aqueous methanol. Declustering potential (DP), collision energy (CE) and cell exit potential (CXP) voltages were established for each analyte. The dwell time was set to 200 msec.

8.1.4 Experimental design to assess bone resorption *in vivo*

A previously developed and validated *in vivo* rat model to monitor acute changes in bone resorption [Antic 1996, Egger 1994, Muhlbauer 1990, Muhlbauer 1995, Muhlbauer 1999] was used to evaluate the effects of onion containing GPCS, onion without GPCS, allyl-cysteine, and γ-glutamyl-cysteine-ethylester on bone resorption. The following experiments were performed.

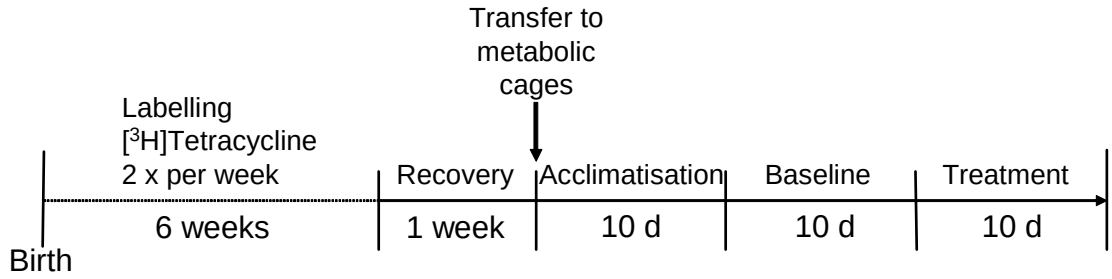
Animals

For each experiment, three female Wistar Hanlbm rats, with twelve 2 – 3 days old male pups each, were purchased from Charles River Wiga GmbH. The animals were kept in house in the Central Animal Facility of the Medical Faculty in compliance with the Swiss and US National Institute of Health guidelines for care and use of experimental animals. The State Committee for the Control of Animal Experimentation approved the performed experiments (authorisation number 48/05).

Experimental protocol

The skeleton of growing male rat pups was labelled by injecting [7-^{3}H(N)]-tetracycline for 6 weeks. After one week of recovery, the rats were weighed and transferred to metabolic cages where they were acclimatised for 10 days. During the following 10 days radioactivity was measured in the 24 h urine to determine base line excretion. Afterwards, the animals were weighed again and 24 animals were selected for the treatment phase. The animals were assigned to one of four groups (n = 6). The diet was changed to a semi-purified diet supplemented with different doses of onion, allyl-cysteine or γ-glutamyl-cysteine-ethylester. Semi-purified diet without supplements was used as the control. Under treatment, the measurement of the urinary secretion of [^{3}H] was continued for 10 days. At the end of the experiment the rats were euthanised with carbon dioxide. If not stated otherwise, the vertebrae were excised for microCT analysis (Micro-CT 40, Scanco Medical AG) and, as structural indices, the bone volume/total volume quotient (BV/TV) and the connection density were determined. A schematic overview of the experimental protocol is given in Figure 19.

Figure 19: Time course of experiments to determine bone resorption in rats



Injection of [7-^{3}H(N)]-tetracycline into rats

From the first week on, male rat pups were injected subcutaneously with increasing doses of a [7-^{3}H(N)]-tetracycline solution (10 µCi/ml [7-^{3}H(N)]-tetracycline / 0.9 % sodium chloride solution) according to the scheme shown in Table 14.

Table 14: Scheme for injecting [7-3H(N)]-tetracycline solution into rats

Age of rats [week]	Injection volume [µl]
1	1 x 100
2	2 x 100
3	2 x 150
4	2 x 200
5	2 x 250
6	2 x 250

Preparation of diet

Standard diet was prepared by adding demineralised water to SODI 2134 diet powder (Kliba-Mühlen) in a ratio of 5/4 (powder/water, w/w) and by adding calcium gluconate, sodium dihydrogen phosphate and disodium hydrogen phosphate (Merck) to obtain a final concentration of 1.1 % calcium and 1.2 % phosphorus. To prevent interferences of natural components of a standard diet with bone resorption, rats received a semi-purified diet during the treatment phase [Brown 2001] which contained an animal protein source instead of a plant protein source. Semi-purified diet was prepared by adding demineralised water to SODI 2160 diet powder (Kliba-Mühlen) in a ratio of 6/5 (powder/water, w/w) to get a pastry consistence. Detailed compositions of the diets are given in section 11.3 Appendix 3: Composition of SODI 2134 and SODI 2160 diet powders.

Diets supplemented with onion were prepared by adding the appropriate amount of lyophilised onion powder to the semi-purified diet followed by thorough mixing. Usually, the diet for one experiment was prepared and stored in aliquots at -20℃ until use.

To prepare diets containing allyl-cysteine (synthesised as described in chapter 7 Part II: Isolation of GPCS from *Allium cepa* L. and synthesis of GPCS) or γ-glutamyl-cysteine-ethylester (Bachem), solutions of these substances in demineralised water were added to 30-g portions of the semi-purified diet and thoroughly mixed. Diets were fed to the rats immediately after preparation.

Feeding of animals

During the labelling and recovery phase, animals were kept in conventional cages and were allowed free access to food pellets (Kliba 3436, Kliba) and water. After transfer to the metabolic cages, during the acclimatisation and base line periods, the rats received demineralised water *ad libitum* and 30 g of standard diet every day at 11 am, if not stated otherwise. During the treatment phase rats obtained demineralised water *ad libitum* and 30 g of semi-purified diet (including the test compounds) every day at 11 am. Except for the Kliba 3436 diet, all diets were provided in stainless steel cups.

Determination of [^{3}H] excretion in urine

24-h urine samples were collected every day at 11 am. The volume of each urine sample was determined and aliquots of 1 ml, acidified with 0.1 M of hydrochloric acid, were mixed with 10 ml of Irga-Safe Plus scintillator (Perkin Elmer). Quantitation of the [^{3}H] content was performed by liquid scintillation counting using a TriCarb Liquid Scintillation Analyzer 2200CA (Packard), and the result was calculated for the volume of the collected urine.

Assignment of rats into groups

After 10 days of baseline measurement of the urinary [^{3}H] excretion, the rats were divided into 4 groups (n = 6) as follows:

In the first experiment the rats with high fluctuation and with very high or very low [^{3}H] excretions were excluded from the experiment. The 24 remaining rats were randomly assigned into the 4 groups by a computerised randomisation programme using the Web site Randomization.com <http://www.randomization.com>.

In the second and third experiment, during the 10 days of baseline measurement the following calculations were performed. The daily means of the [^{3}H] excretion from all rats ($\overline{X}$) and the absolute deviation of the individual rat's daily value from this mean ($|\overline{X} - X_i|$) were calculated. After the 10 days, for each rat the sum of their daily [^{3}H] excretion values ($\sum X_i$), the sum of their deviation from the daily means ($\sum \overline{X} - X_i$), and the sum of their absolute deviation value from the daily mean ($\sum|\overline{X} - X_i|$) were calculated.

These values were used to create a ranking, on top the rat with the least, and on the bottom the rat with the highest absolute deviation sum ($\sum|\overline{X} - X_i|$). The 12 rats with the highest

values were excluded from the experiment. The 24 remaining rats were divided into groups as follows. The first 12 rats from the ranking were set beforehand in the groups (3 rats per group). It was important that rats with negative and rats with positive deviation sums ($\sum \overline{X} - X_i$) were homogeneously distributed within the groups. To assign the 12 remaining rats, a computer programme was written [Mabboux 2007] enabling groups of 6 animals with equal means of their cumulative [^{3}H] excretions ($\sum X_i$).

Statistical analysis

The results were evaluated using analysis of variance (ANOVA, Bonferroni) using WinSTAT® version 2005.1 for Microsoft® Excel.

8.2 Results

8.2.1 Effects of GPCS on the activity, activation and recruitment of osteoclasts *in vitro*

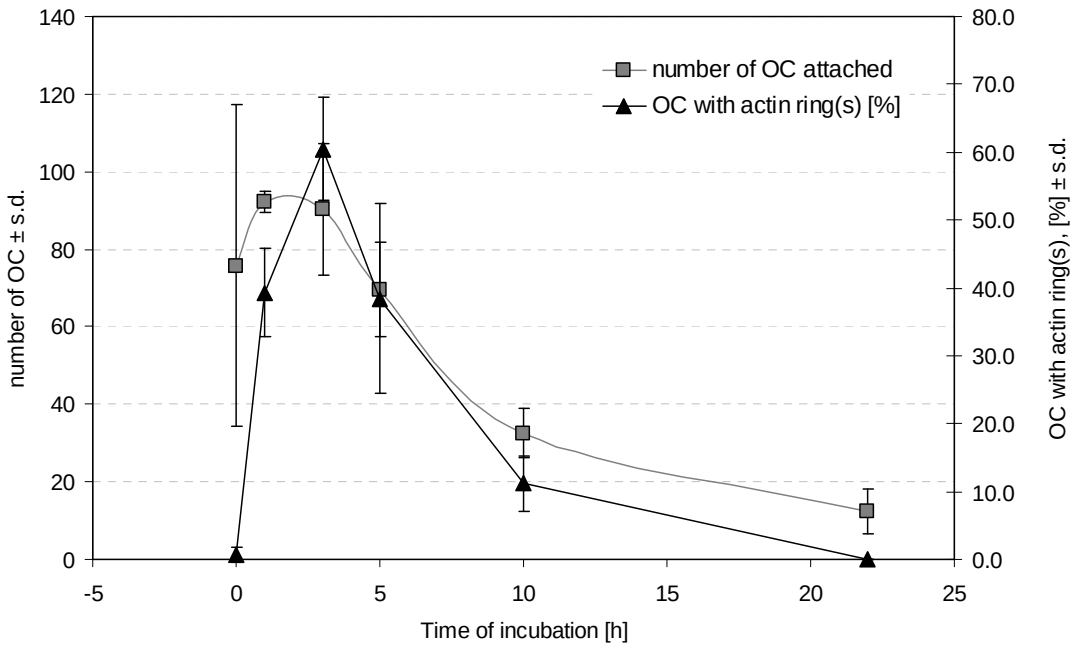
In this section the results of experiments which were performed to assess the effect of GPCS on OC activity and on OC recruitment *in vitro* are presented. In addition, the influence on OC development of substances, which are structurally related to GPCS, is shown.

Effects of GPCS on osteoclasts with actin ring(s)

GPCS was shown to inhibit the resorption activity of OCs *in vitro* [Wetli 2005]. To investigate effects on the activation of OCs, the cells were treated with GPCS and the number of OCs containing actin rings was determined.

To follow the development of actin rings in OCs over time and to establish optimal experimental conditions, the total number of OCs and the number of OCs with actin rings after incubation with Medium 199 containing 10 % FBS was determined over 21 h. The percentage of OCs with actin ring(s) increased up to 60 %, reaching a maximum within 3 h of incubation thereafter dropped to zero within the following 18 h. The number of OCs decreased after 5 h of incubation (Figure 20).

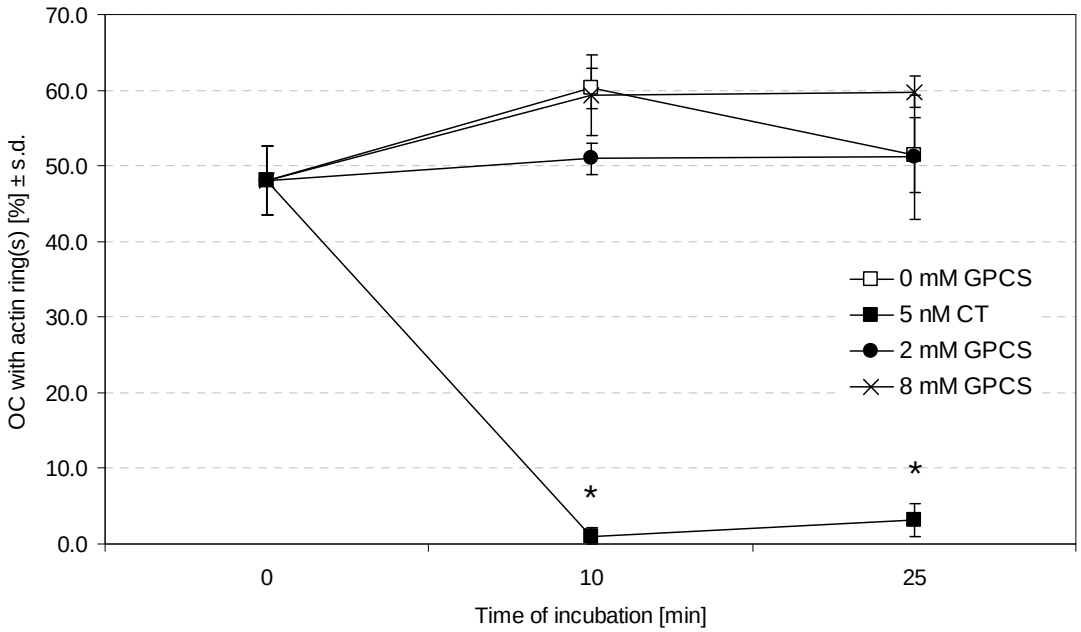
Figure 20: Time course to follow the development of actin rings in OCs after adherence on glass coverslips. OCs were incubated with medium for up to 21 h. The data represent the mean ± s.d. (n = 6) of one experiment.



In order to investigate the effect of GPCS on activated OCs, OCs were pre-incubated in medium for 3 h. A further increase of OCs with actin rings was induced if medium was

changed after these 3 h of preincubation and cells were incubated for another 15 min (data not shown). OCs were subsequently treated with Medium 199 containing 10 % FBS Hia and 0, 2 and 8 mM of GPCS. Calcitonin, a potent inhibitor of OC activity, was used as a positive control at a concentration of 5 nM. GPCS at 2 and 8 mM did not change the number of OC with actin rings (10 and 25 min of incubation), while calcitonin caused a complete disintegration of the actin ring(s) (Figure 21).

Figure 21: Effect of GPCS on the disintegration of actin rings. The cells were treated with 2 mM and 8 mM of GPCS and 5 nM calcitonin (CT) for 10 and 25 min. The data are presented as the mean ± s.d. (n = 6) of one experiment. * $p < 0.05$, significantly different from control.



Effects of GPCS on the resorption activity of osteoclasts

It was demonstrated by Wetli et al. [Wetli 2005] that GPCS inhibits the resorption activity of OCs *in vitro*. To confirm these results, pit assay experiments were performed. In two independent experiments, isolated rat OCs were cultured on dentin slices with 0, 2 and 8 mM of GPCS for 24 h. Thereafter, $TRAP^+$ multinucleated ($n \geq 2$) cells and resorption pits were counted. The number of OCs was similar in all groups (Figure 22). With increasing doses of GPCS, the number of pits decreased to 57 %, albeit not significantly (Figure 23). GPCS, at 8 mM, significantly decreased the number of pits per OC (Figure 24). 0.01 nM of calcitonin was used as a positive control. Compared to the control, calcitonin did not change the number of OCs, but prevented pit formation, resulting in a pits/OC ratio of zero.

Figure 22: Effect of GPCS on the number of OCs. Calcitonin was used as a positive control. Two experiments were performed, and the data are presented as percent of control (mean ± s.d., n = 16).

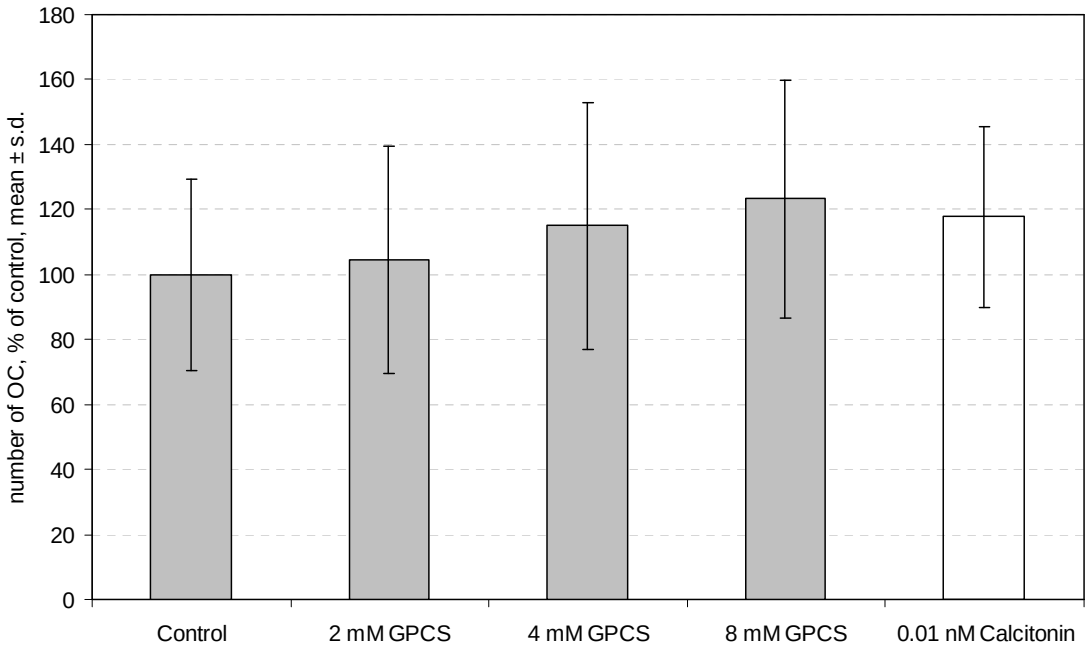


Figure 23: Effect of GPCS on osteoclastic resorption in the pit assay. Calcitonin was used as a positive control. Two experiments were performed, and the data are presented as percent of control (mean ± s.d., n = 16). * $p < 0.05$, significantly different from control.

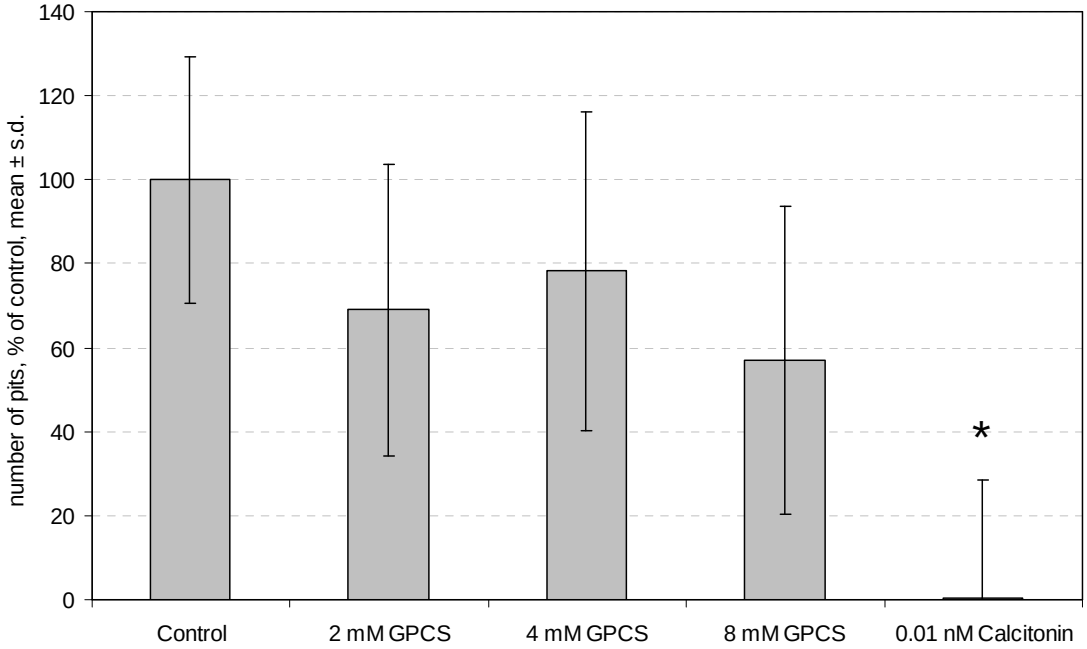
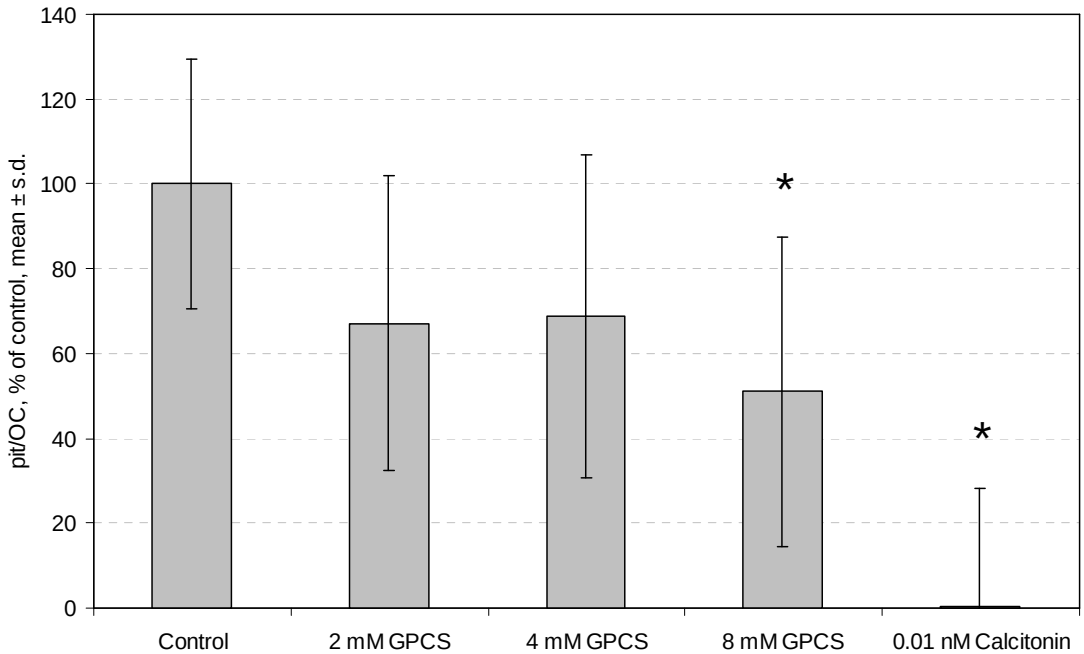


Figure 24: Effect of GPCS on the ratio of pits per OCs. Two experiments were performed, and the data are presented as percent of control (mean ± s.d., n = 16). * $p < 0.05$, significantly different from control.

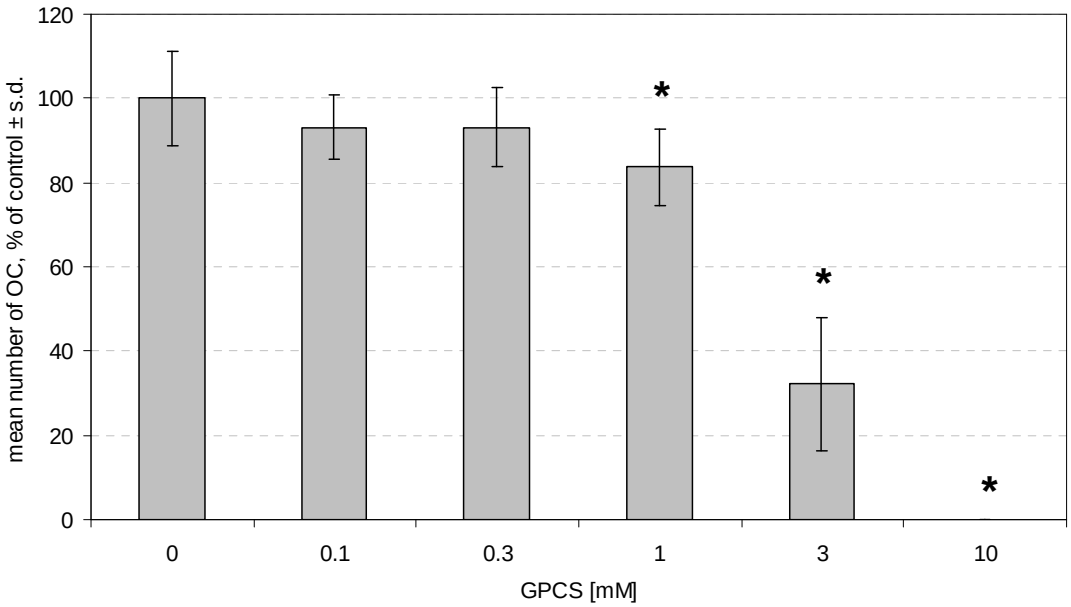


Effects of GPCS and other structurally related substances on the formation of osteoclasts

Effect of GPCS on the formation of osteoclasts

To investigate the effect of GPCS on the recruitment of OCs, bone marrow cells were incubated with M-CSF and RANKL and varying doses of GPCS for 4 - 5 days. Formed OCs, i.e. $TRAP^+$ multinucleated (n ≥ 3) cells were counted. GPCS significantly inhibited osteoclastogenesis at concentrations ≥ 1 mM (Figure 25).

Figure 25: GPCS in bone marrow cell cultures. Two experiments were performed. The data were combined and are presented as percent of control (mean ± s.d., n = 12). * $p < 0.05$, significantly different from control.



Effect of substances with structural similarities to GPCS on the formation of osteoclasts

To evaluate structure-function relationship and specificity of the inhibitory effect of GPCS on osteoclastogenesis *in vitro,* γ-glutamyl-cysteine-ethylester, glycyl-cysteine, allyl-cysteine, cysteine, glutamyl-glycine and glycyl-valine were tested in cultures of bone marrow cells. Substances containing a cysteine moiety inhibited the development of OCs in cultures of bone marrow cells with the following potencies: γ-glutamyl-cysteine-ethylester > glycyl-cysteine ≈ allyl-cysteine > cysteine (Figures 26-30).

Figure 26: Structures of γ-glutamyl-cysteine-ethylester, glycyl cysteine, allyl-cysteine and cysteine, ordered by their potency in inhibiting osteoclastogenesis.

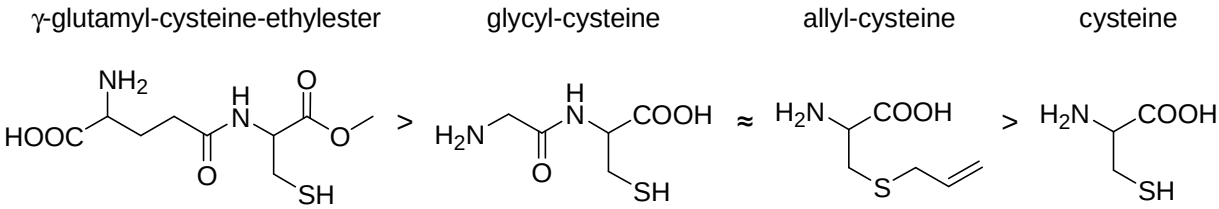


Figure 27: Effect of γ-glutamyl-cysteine-ethylester on the recruitment of OCs. Two independent experiments were performed and the results combined. The data are presented as percent of control (mean ± s.d., n = 12).
* $p < 0.05$, significantly different from control.

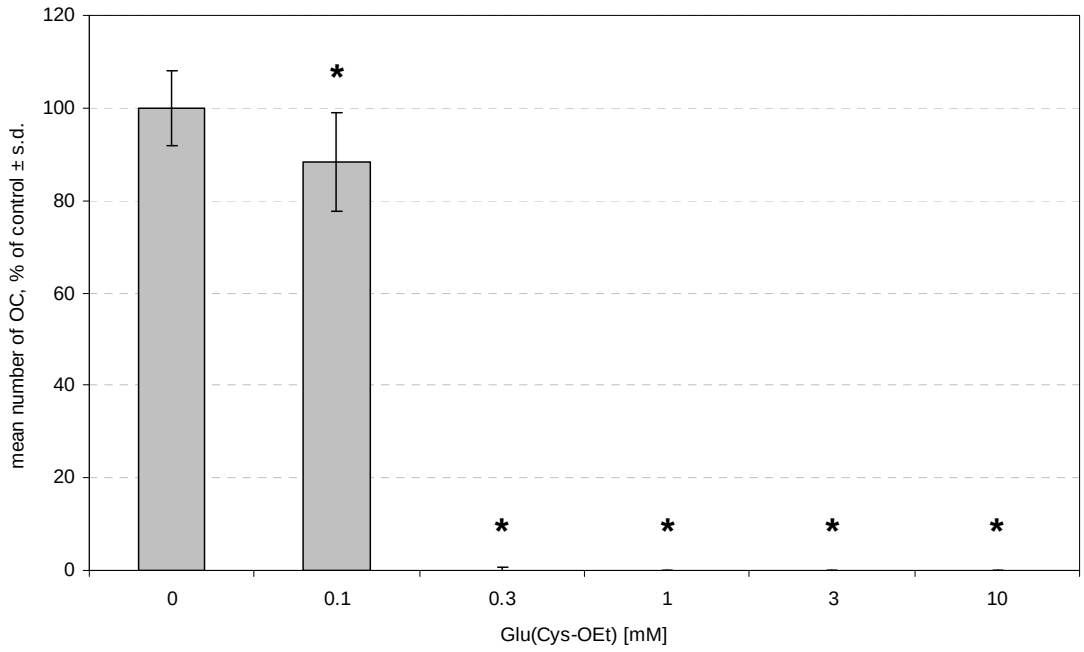


Figure 28: Effect of glycyl-cysteine on the recruitment of OCs. Two independent experiments were performed and the results combined. The data are presented as percent of control (mean ± s.d., n = 12).
* $p < 0.05$, significantly different from control.

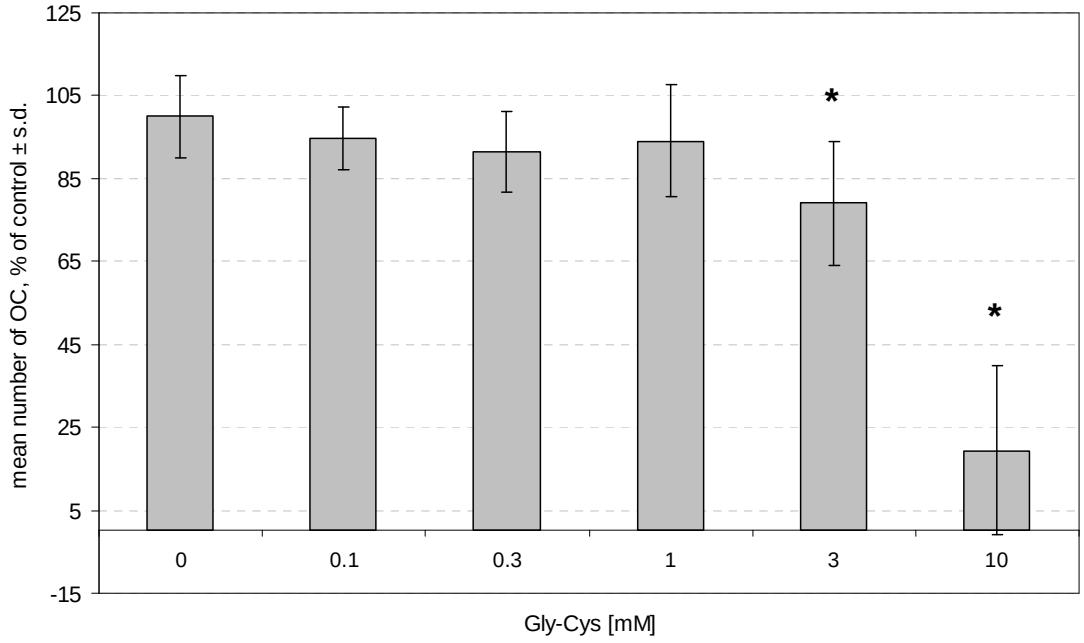


Figure 29: Effect of allyl-cysteine on the recruitment of OCs. Two independent experiments were performed and the results combined. The data are presented as percent of control (mean ± s.d., n = 12).
* $p < 0.05$, significantly different from control.

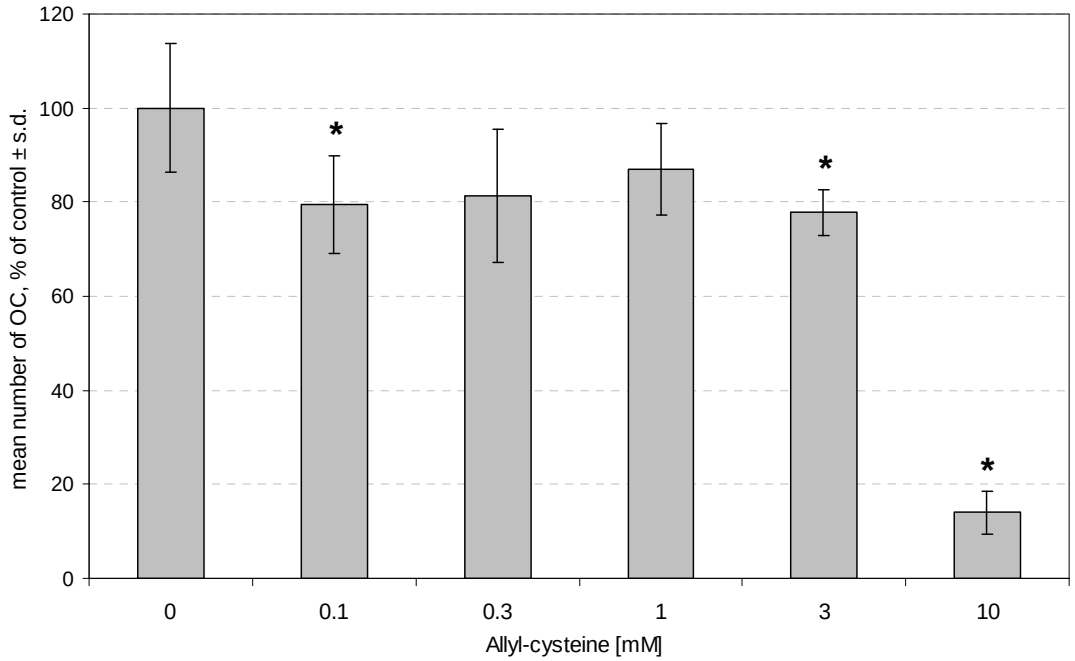
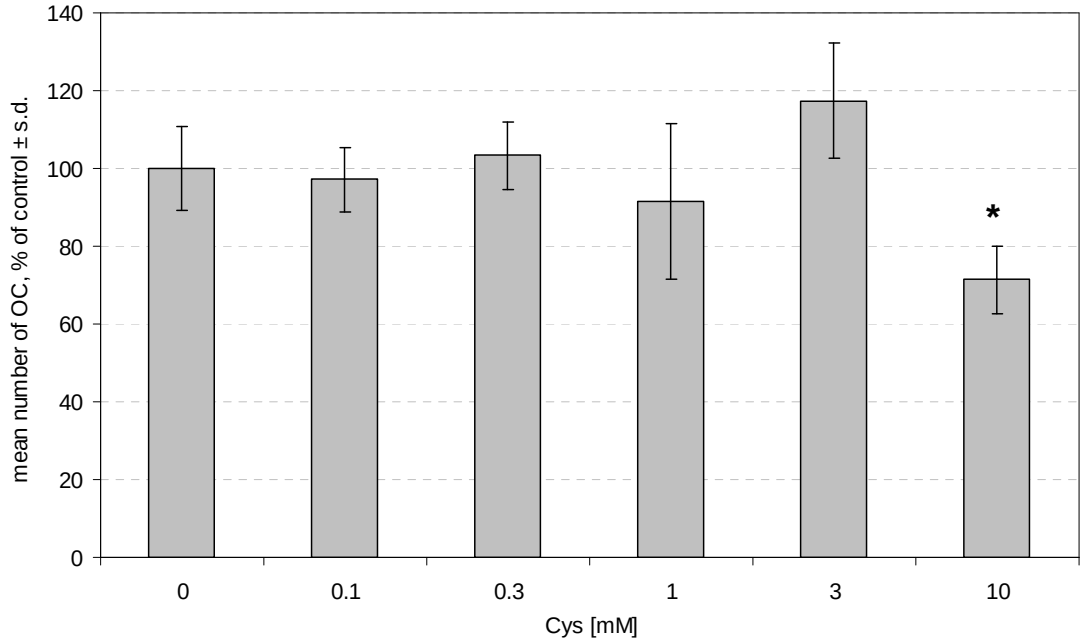


Figure 30: Effect of cysteine on the recruitment of OCs. One experiment was performed. The data are presented as percent of control (mean ± s.d., n = 6). * $p < 0.05$, significantly different from control.



Glutamyl-glycine and glycyl-valine, substances without a cysteine moiety, did not affect the development of OCs in the bone marrow cell culture (Figure 31-33).

Figure 31: Structures of glutamyl-glycine and glycyl-valine

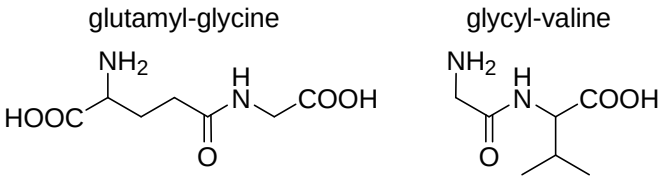


Figure 32: Effect of glutamyl-glycine on the recruitment of OCs. The data are presented as percent of control of two independent combined experiments (mean ± s.d., n = 12).

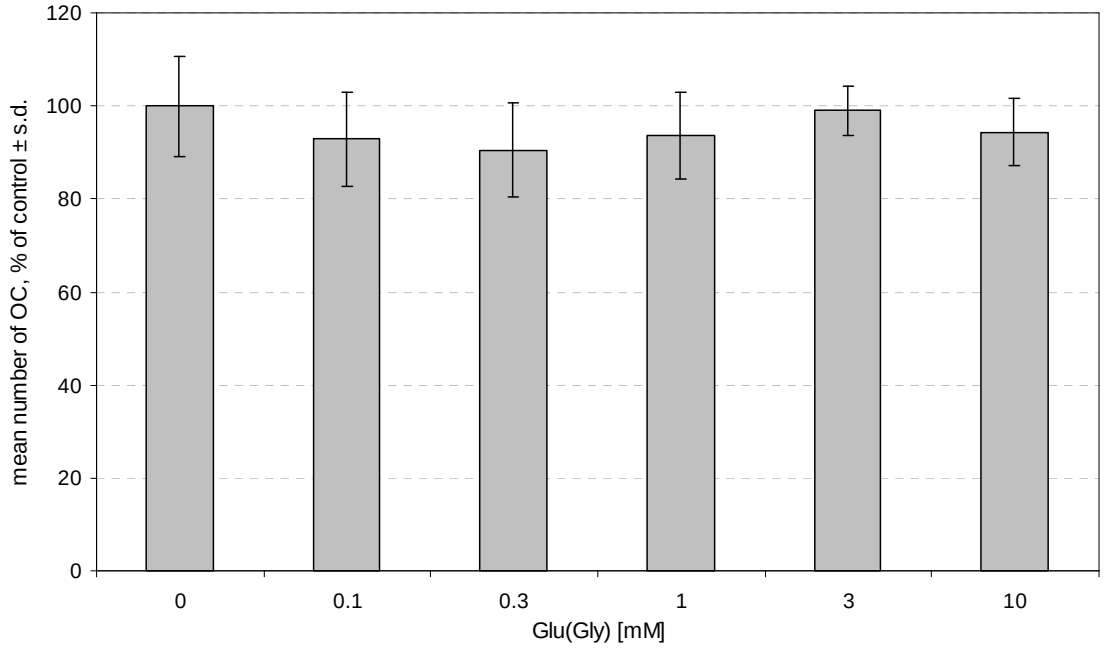
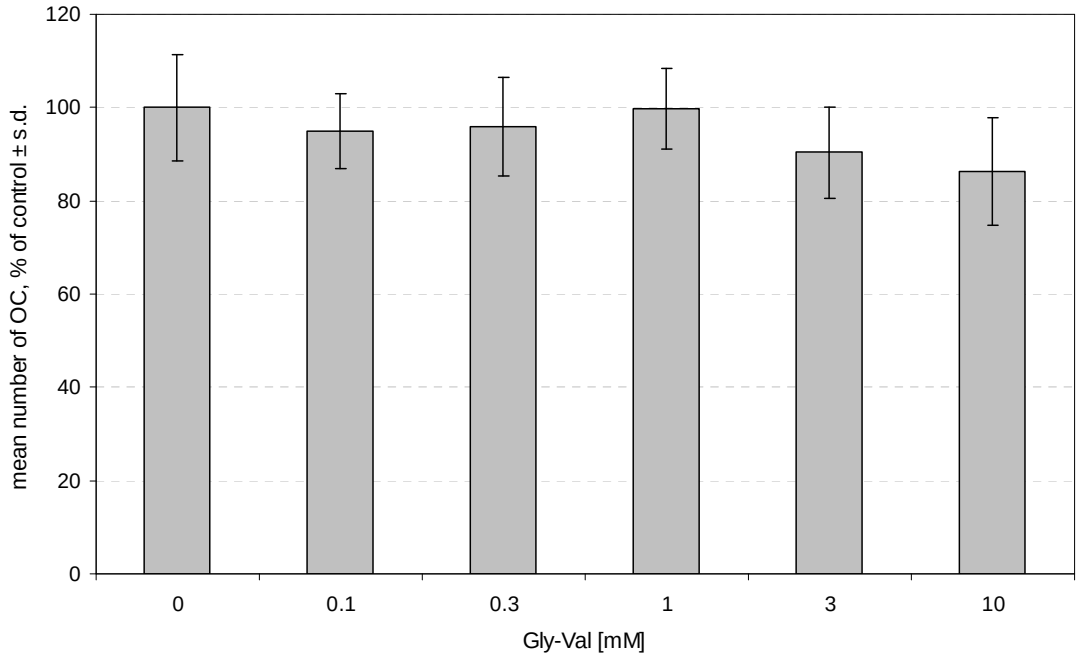


Figure 33: Effect glycyl-valine on the recruitment of OCs. The data are presented as percent of control of two independent experiments (mean ± s.d., n = 12).



8.2.2 Studies to determine the stability of GPCS in simulated gastric acid and simulated intestinal fluid

This section presents the results of the method development and of the stability study of GPCS incubated with simulated gastric acid and simulated intestinal fluid.

Method development

Determination of GPCS in simulated gastric acid

To determine GPCS in simulated gastric acid the mobile phase consisted of 25 % of solvent A and Spherisorb ODS-1 as stationary phase. A blank run of simulated gastric acid was compared with a GPCS spiked sample of simulated gastric acid and showed no interference between analyte and matrix.

Determination of GPCS in simulated intestinal fluid

In the first experiment to study the stability of GPCS in simulated intestinal fluid, 25 % of solvent A was used as mobile phase and Spherisorb ODS-1 as stationary phase. A comparison with the blank run of simulated intestinal fluid showed that GPCS interfered with the matrix. Thus, an optimisation of the mobile phase was necessary. It was performed by utilising 20, 15, 10 and 5 % of solvent A. However, no full baseline separation of the GPCS peak and the matrix peaks could be achieved. Hence, another stationary phase, the Shodex ODP2 HP-4D material, was tested. Again, different compositions of mobile phases (20, 15, 10, 7.5 and 5 % of solvent A) were tested. A complete separation of GPCS and matrix peaks was obtained at 10 % solvent A. There were no interferences between matrix and analyte.

To determine the content of GPCS in onion powder, which was incubated with simulated intestinal fluid, a mobile phase containing 7.5 % solvent A was most suitable to exclude interferences between matrix and analyte.

An overview of the final experimental conditions is given in Table 15.

Table 15: Final conditions for stationary and mobile phases to determine GPCS in various matrices

Matrix	Stationary phase	Mobile Phase
Gastric acid	Spherisorb ODS-1	25 % solvent A
Simulated intestinal fluid	Shodex ODP2 HP-4D	10 % solvent A
Simulated intestinal fluid + onion	Shodex ODP2 HP-4D	7.5 % solvent A

Peak identification

The retention time for GPCS was 5.8 min (range: 5.6 - 6.0 min) for the Spherisorb column and 13.1 min (range: 13.0 – 13.2 min) for the Shodex column. The blank run showed no interferences with the analyte.

Calibration and linearity

The calibration was linear in the concentration range of 10 to 200 µg/ml (Spherisorb ODS-1 column) and 5 to 125 µg/ml (Shodex ODP2 HP-4D column), respectively. The linearity data are summarised in Table 16.

Table 16: Calibration data for the quantitation of GPCS with the Spherisorb ODS-1 column obtained by performing triplicates for each of the 5 concentration levels (10, 50, 100, 150 and 200 µg/ml), and with the Shodex ODP2 HP-4D column obtained by performing triplicates for each of the 3 concentration levels (5, 50 and 125 µg/ml)

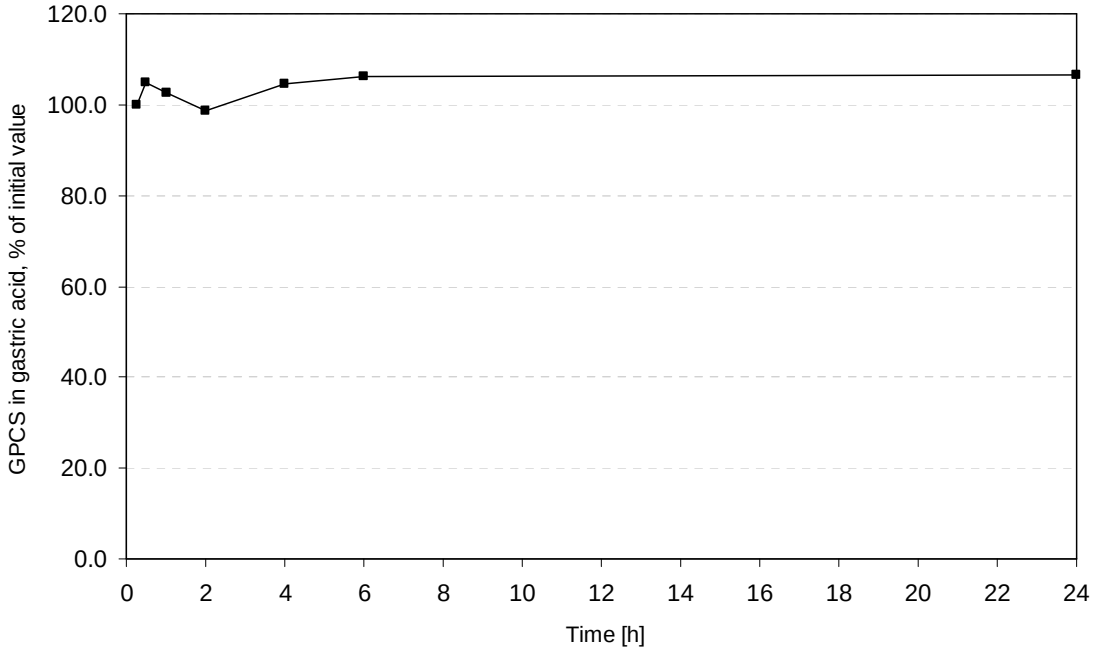
Column	Retention time [min]	m[1)]	b[1)]	Correlation coefficient r
Spherisorb ODS-1	5.8	3413	– 12.5	0.9998
Shodex ODP2 HP-4D	13.1	3632	– 12.9	0.9999

[1)] $y = mx + b$; x: amount of GPCS; y: area under the GPCS peak

Stability of GPCS in simulated gastric acid

GPCS was exposed *in vitro* to simulated gastric acid at 37℃. Within 24 h no change in the content of GPCS was observed (Figure 34).

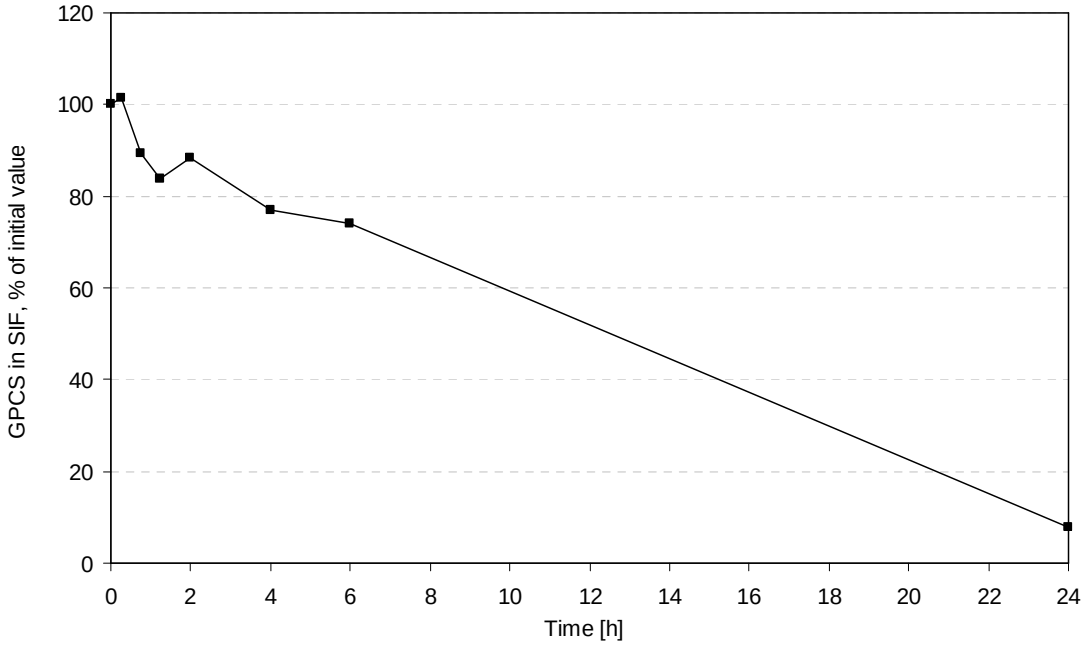
Figure 34: Stability of GPCS in simulated gastric acid. The results are expressed as percent of the baseline value.



Stability of GPCS in simulated intestinal fluid

The *in vitro* stability of GPCS in intestinal fluid was evaluated by incubation of GPCS in simulated intestinal fluid at 37℃ for 24 h. Within 2 h the content of GPCS decreased to less than 90 % of baseline. After 6 h, 75 % of GPCS were detectable and after 24 h of incubation only 10 % of GPCS were present (Figure 35).

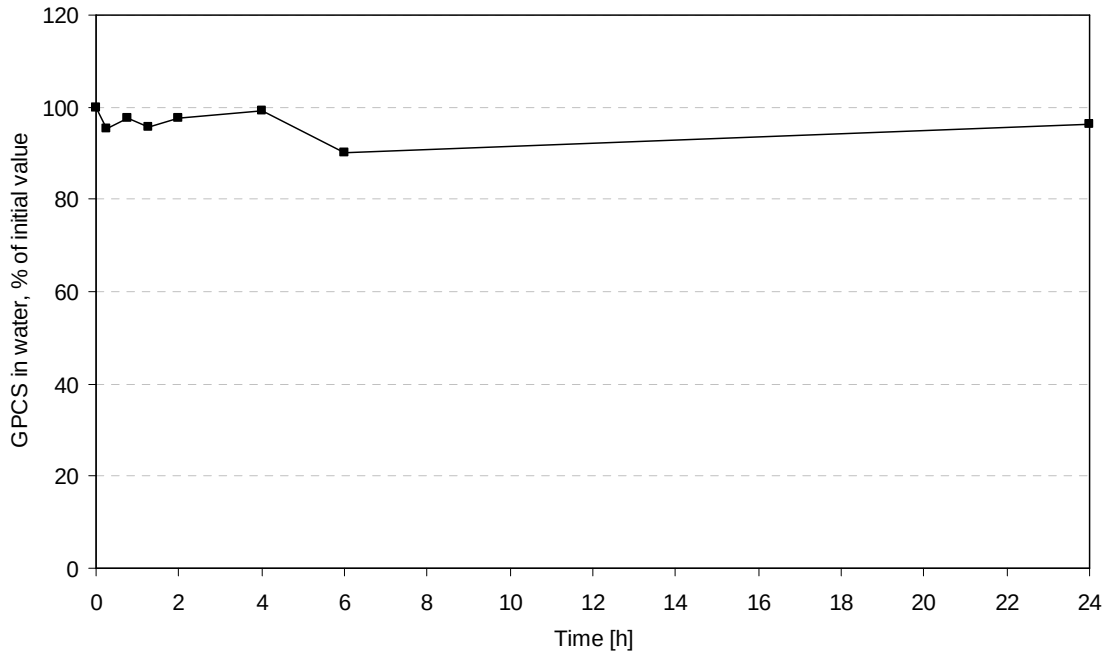
Figure 35: Stability of GPCS in simulated intestinal fluid (SIF). The results are expressed as percent of the baseline value.



Stability of GPCS in water

To check whether ingredients of intestinal enzymes are responsible for the decreasing content of GPCS in simulated intestinal fluid, GPCS was incubated in water at 37℃ for 24 h. GPCS remained stable over the whole incubation time (Figure 36).

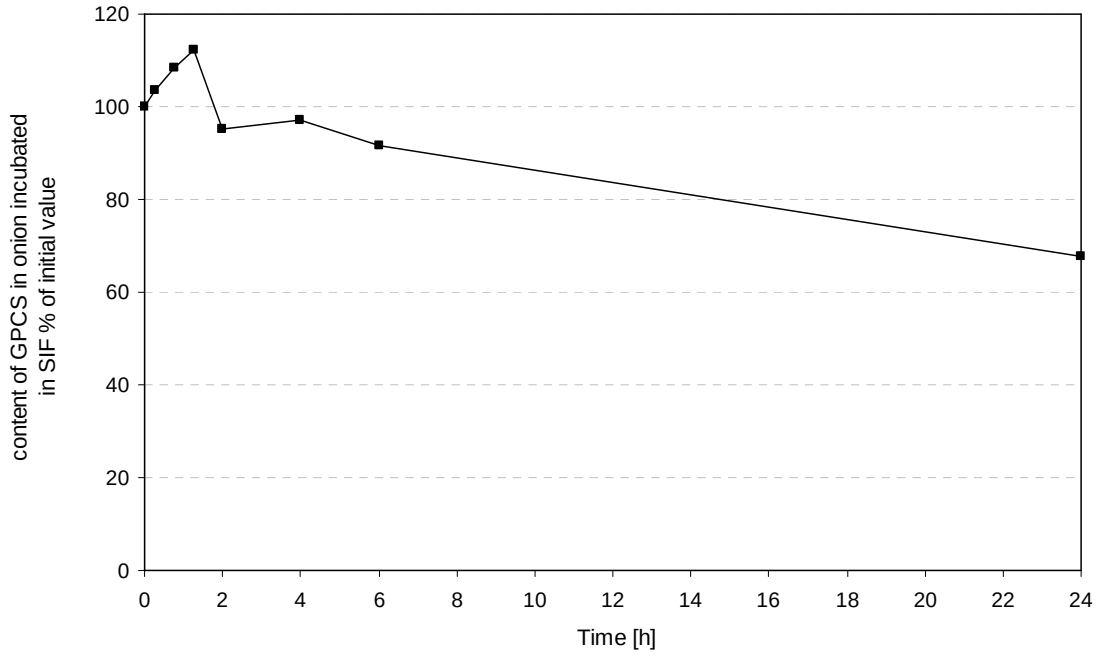
Figure 36: Stability of GPCS in water. The results are expressed as percent of the baseline value.



GPCS content of *Allium cepa* L. incubated in simulated intestinal fluid

To evaluate the effect of other onion constituents on the stability of GPCS in simulated intestinal fluid, lyophilised powder of onion was exposed to simulated intestinal fluid at 37℃ for 24 h, and the content of GPCS was determined. There was no change of GPCS content within 6 h of incubation. After 24 h a decrease of 30 % from the initial GPCS value was detectable (Figure 37).

Figure 37: GPCS content of onion incubated in simulated intestinal fluid. The results are expressed as percent of the baseline value.



8.2.3 Pilot studies to determine the pharmacokinetic properties of GPCS in rats

The results of the method development for the determination of GPCS in rat plasma and the results of a pharmacokinetic pilot study in rats are presented in this section.

Method development

SPE parameters

SPE was used to prepare the sample prior to analysis. To find suitable conditions for the SPE, various SPE columns with different elution procedures were tested. The recovery of GPCS in the eluates was determined using HPLC-DAD. The highest recovery of GPCS was found when OASIS MAX 3 cc (81 %) columns were used. No GPCS was detected when the extraction procedure was performed using Oasis WAX 1 cc columns (Table 17). Consequently, Oasis MAX 3 cc was chosen for plasma extraction.

Table 17: Recoveries of GPCS after SPE

SPE column	Recovery of GPCS [%]
Oasis MAX 3 cc	81
Oasis MAX 1 cc	30
Oasis MCX 1 cc	53
Oasis WAX 1 cc	0

Determination of GPCS

To study the pharmacokinetic properties of GPCS *in vivo*, 2 rats received 2.5 mg GPCS via gavage and 1 rat intravenously. At different time points after administration, blood was collected and centrifuged. The plasma was purified using SPE and the level of GPCS determined using HPLC-DAD and HPLC-MS/MS.

Determination of GPCS using HPLC-DAD

The limit of quantitation of GPCS in plasma using HPLC-DAD was in the µg-range. Therefore, no GPCS could be detected in the samples of the pharmacokinetic study. Thus, it was tried to improve sensitivity by adding a chromophor to the analyte, i.e. by derivatisation. GPCS was derivatised with ortho phthaldialdehyde in the presence of *tert*-butylmercaptan. However, this procedure did not sufficiently improve sensitivity (LOQ: µg-range) and was not further used. The next step was the analysis of the samples using HPLC-MS/MS.

Determination of GPCS using HPLC-MS/MS

Optimised parameters for the MRM acquisition mode

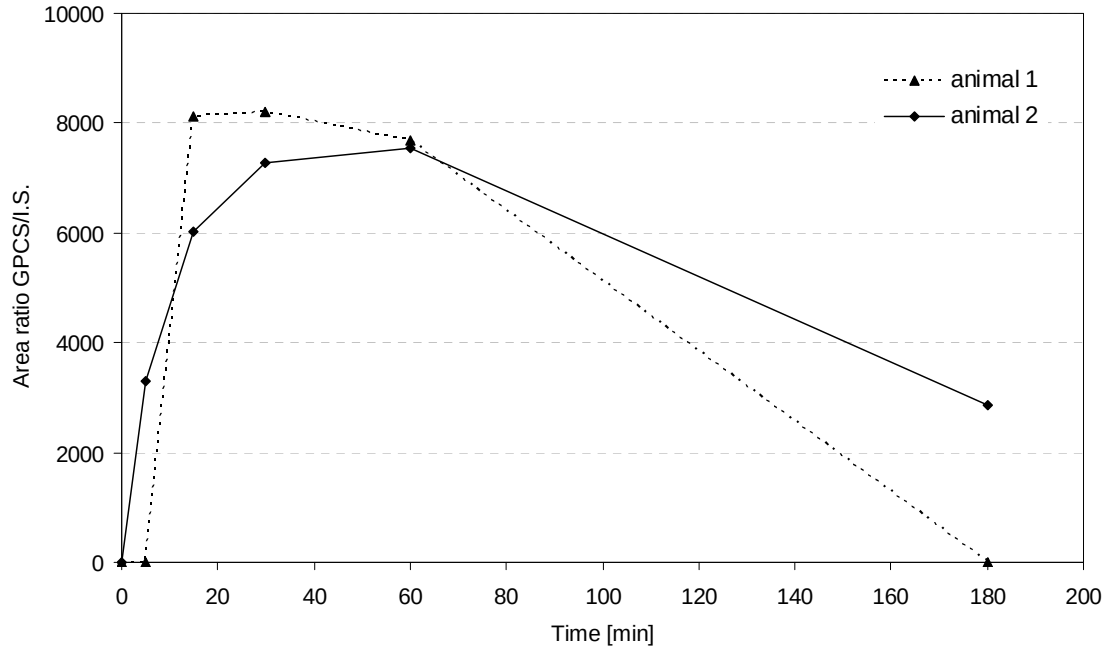
GPCS and I.S. (L-aspartyl-L-phenylalanine) were both eluted after 2.7 min. A mass spectrometric separation of the substances was obtained with MS/MS (MRM) in the electrospray positive mode. The optimal parameters to analyse GPCS (molecular weight: m/z 306) and I.S. (molecular weight: m/z 280) with HPLC-MS/MS in the MRM acquisition mode are given in Table 18.

Table 18: HPLC-MS/MS parameters optimised for the MRM acquisition mode (MRM transitions: 1.: first transition, 2.: second transition; Q , quantifier; q, qualifier; MRM ratio: area ratio of peak of the 1.transition/2.transition; CE, collision energy; CXP, cell exit potential)

Compound	Molecular weight	Precursor $[M+H]^+$	DP [V]	MRM transitions	CE [V]	CXP [V]	MRM ratio
GPCS	306	307	71	1. 307 → 217.20 (Q)	17	14	1.78
				2. 307 → 130.10 (q)	27	14	
I.S.	280	281	66	1. 281.2 → 235.2	21	12	1.32
				2. 281.2 → 166	19	14	

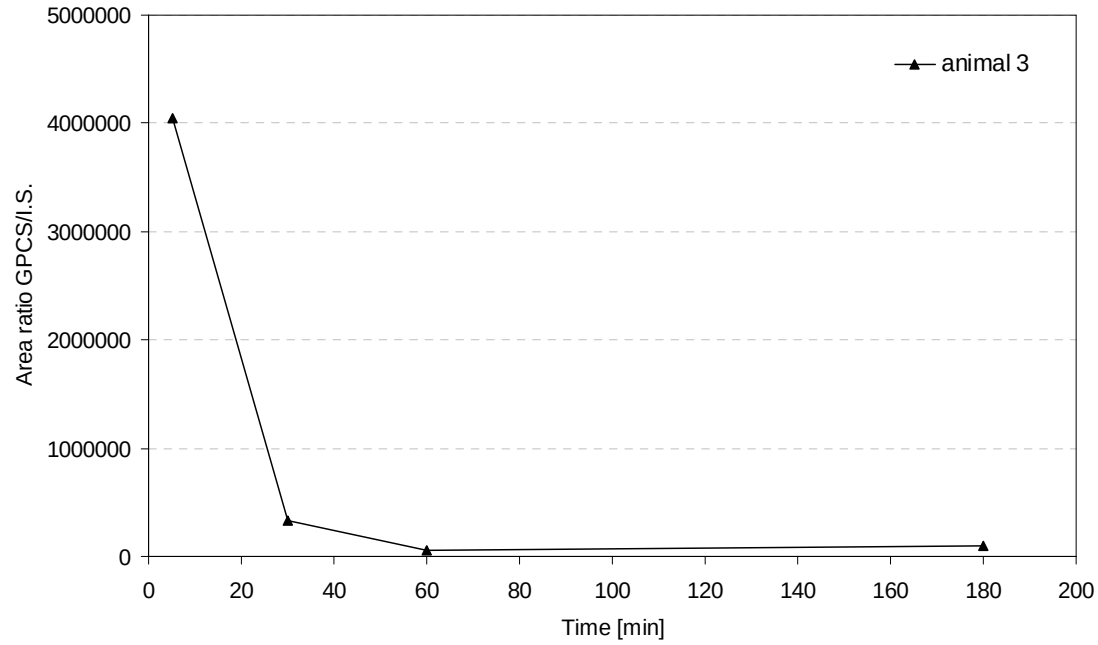
The GPCS plasma profiles of animal 1 and 2 after administration of GPCS via gavage are shown in Figure 38. Maximum GPCS levels (expressed as GPCS-I.S. area ratios) were obtained 30 min (animal 1) and 60 min (animal 2) after GPCS administration. Then, the GPCS levels dropped to baseline and about 50 % in animal 1 and 2, respectively (within 180 min post-dosing). Compared to the intravenous application, the oral bioavailability was determined to be 1 % for both animals.

Figure 38: GPCS rat plasma profiles after administration of GPCS via gavage



Animal 3 received GPCS intravenously. The highest level of GPCS was measured 5 min after GPCS administration dropping quickly almost to baseline within the observation period of 180 min (Figure 39).

Figure 39: GPCS rat plasma profiles after i.v. administration of GPCS.



8.2.4 Pharmacodynamics of substances structurally related to GPCS, of GPCS-containing, and of GPCS-free onion

In this section, the results of three experiments are presented, which were assessed to determine the effects of onion containing GPCS, onion without GPCS, allyl-cysteine and γ-glutamyl-cysteine-ethylester on bone resorption in rats.

Feeding protocol

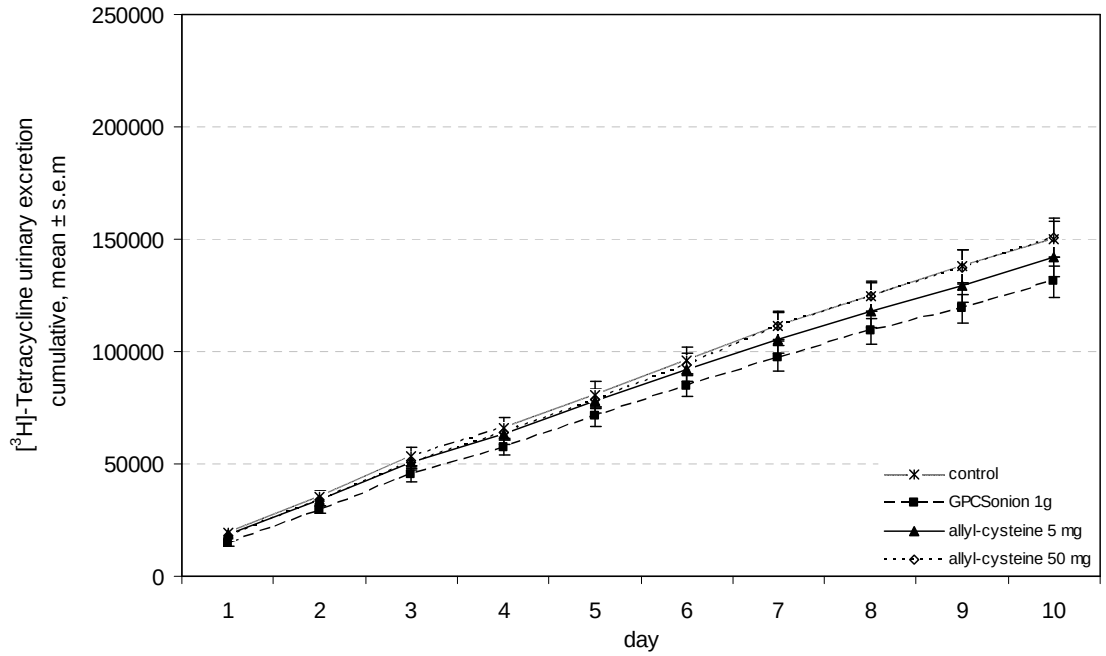
In the first experiment rats were fed with 23 g of standard diet after their transfer to the metabolic cages. They were weighed after 10 days and a loss of weight of about 10 % was observed. Thus, the amount of food was not adequate and subsequently was adapted to the needs of the rats by first providing them with 25 g food for the first 3 days of the baseline phase and afterwards with 30 g. The rats ingested their food within 1 h after it was placed in the cages. 20 days after the transfer to the metabolic cages, the rats which were assigned to the treatment were weighed again. An increase in weight was observed. In experiments 2 and 3, rats were fed as stated in section 8.1.4 Experimental design to assess bone resorption *in vivo*. Weighing the rats on days 10 and 20 after their transfer to the metabolic cages showed no loss of weight (section 11.4 Appendix 3: Assessment of bone resorption *in vivo:* experiment I to III, weight of rats on day 1, 10 and 20 in metabolic cages).

Assignment of rats to groups

After 10 days of [^{3}H] excretion baseline measurement, the rats were assigned to one of 4 groups with 6 animals each.

In the first experiment the assignment of rats to the groups was performed by a randomisation programme. Calculations which were performed post-experimentally showed that the mean cumulative [^{3}H] excretion in each group was not equal in all groups (Figure 40).

Figure 40: Experiment I, mean cumulative urinary [^{3}H] excretion during baseline measurement. Results are presented as mean ± s.e.m (n = 6).



In the second and third experiment, group assignment was performed as described in section 8.1.4 Experimental design to assess bone resorption *in vivo*. The mean cumulative urinary [^{3}H] excretion was equal for all groups at the beginning of the treatment phase (Figures 41-42).

Figure 41: Experiment II, mean cumulative urinary [^{3}H] excretion during baseline measurement. Results are presented as mean ± s.e.m (n = 6).

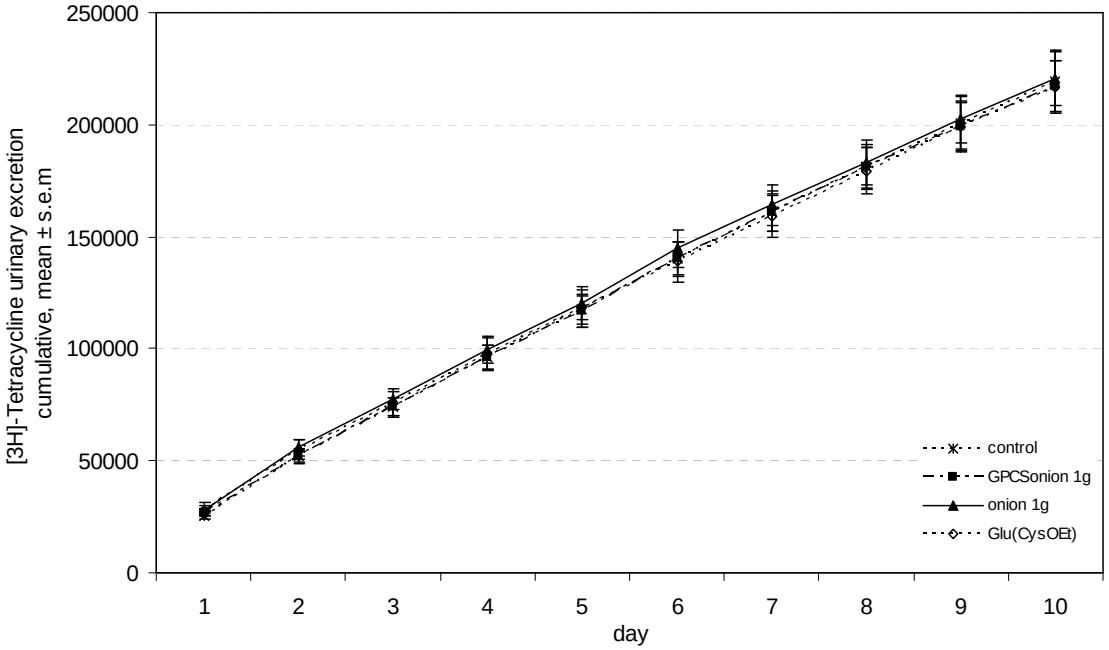
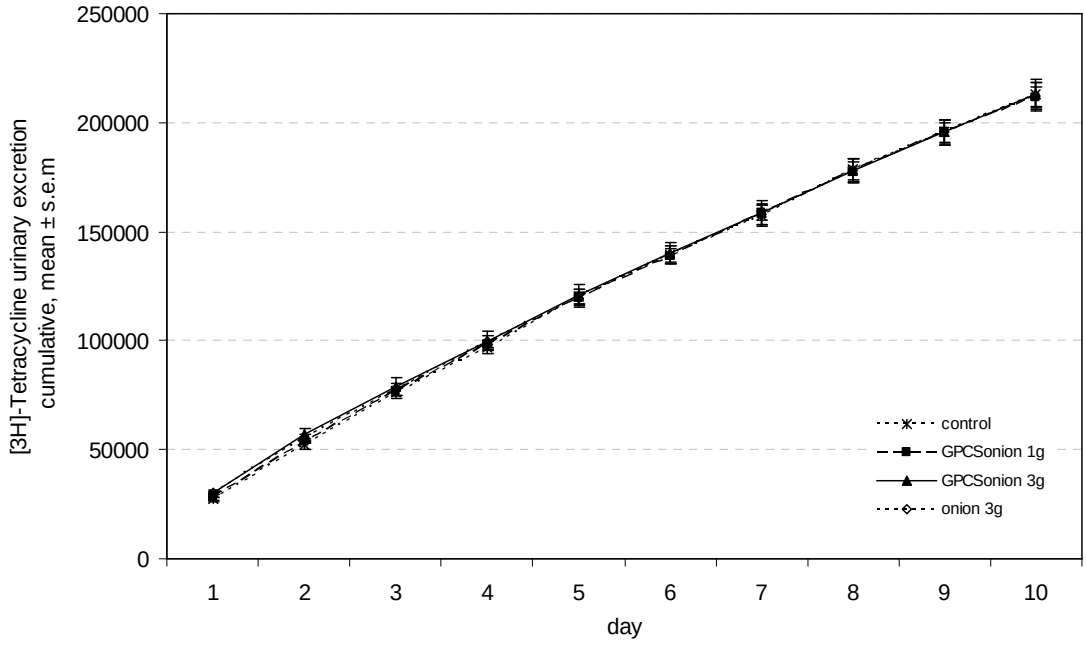


Figure 42: Experiment III, mean cumulative urinary [^{3}H] excretion of rats during baseline measurement. Results are presented as mean ± s.e.m (n = 6).

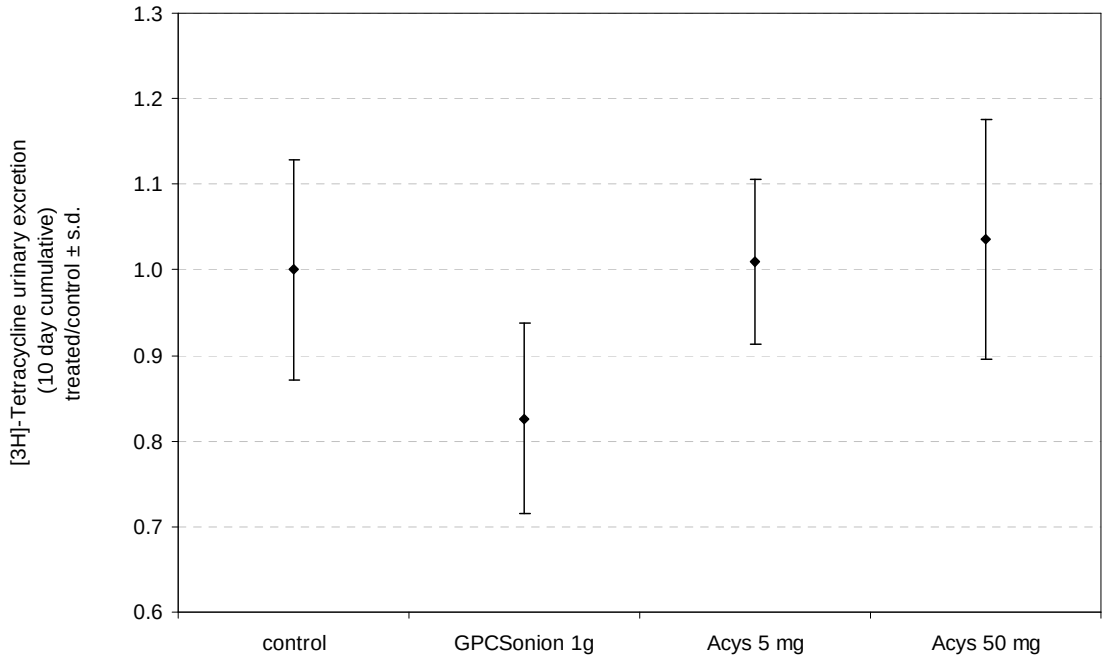


Effects of GPCS-containing and GPCS-free onion, allyl-cysteine and γ-glutamyl-cysteine-ethylester on bone resorption

In all experiment rats were fed standard diet during the baseline phase (day 1 – 10). During the treatment phase (day 11 – 20), they were fed a semi-purified diet containing the test compounds or onion powders, respectively.

In experiment 1, rats received daily semi-purified diet supplemented with 1 g of onion containing 20 mg GPCS. Furthermore, the effect of 5 mg and 50 mg allyl-cysteine was assessed because allyl-cysteine inhibited OC recruitment *in vitro*. At the end of the experiment, the cumulative urinary [^{3}H] excretion during the treatment phase was determined. Rats receiving onion containing GPCS showed a marginal, not significant, decrease in cumulative [^{3}H] excretion in comparison to control animals. Allyl-cysteine, at both concentrations, did not affect cumulative urinary [^{3}H] excretion (Figure 43).

Figure 43: Experiment I: 10 day cumulative urinary [^{3}H] excretion during treatment. Rats were fed a diet containing onion with GPCS (GPCSonion 1g), 5 mg allyl-cysteine (Acycs 5 mg), 50 mg allyl-cysteine (Acycs 50 mg) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6).



At the end of the experiment, rats were euthanised and vertebrae were excised to perform microCT analysis. No difference in the BV/TV and connection density between the groups was observed (Figures 44-45).

Figure 44: Experiment I: MicroCT analysis: BV/TV. Rats received a diet containing onion with GPCS (GPCSonion 1g), 5 mg allyl-cysteine (Acycs 5 mg), 50 mg allyl-cysteine (Acycs 50 mg) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6).

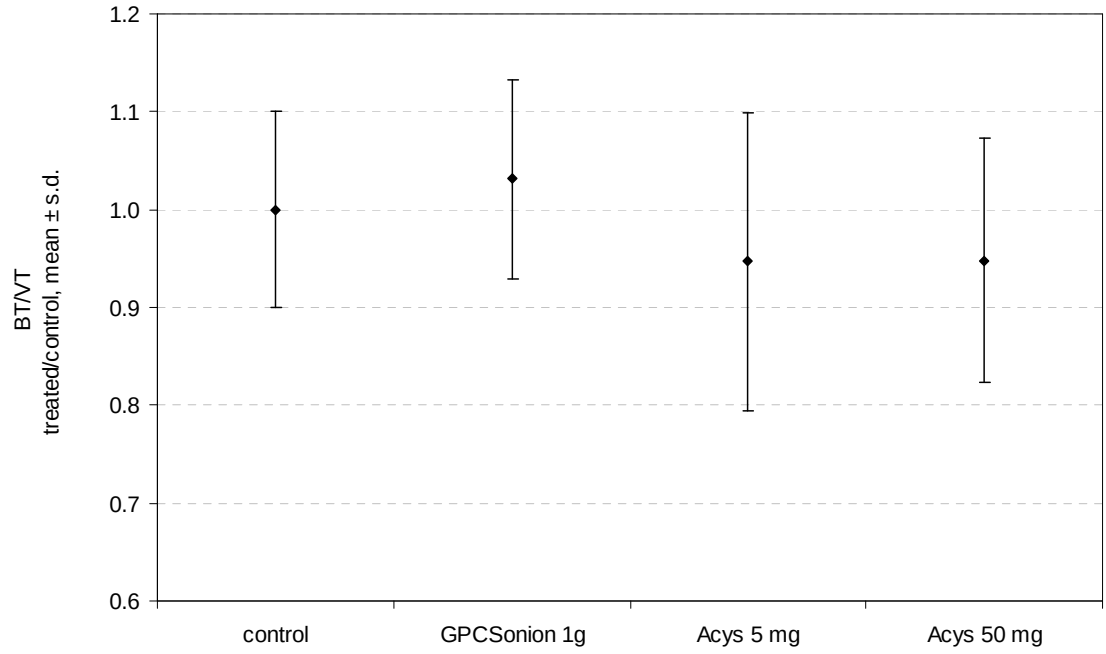
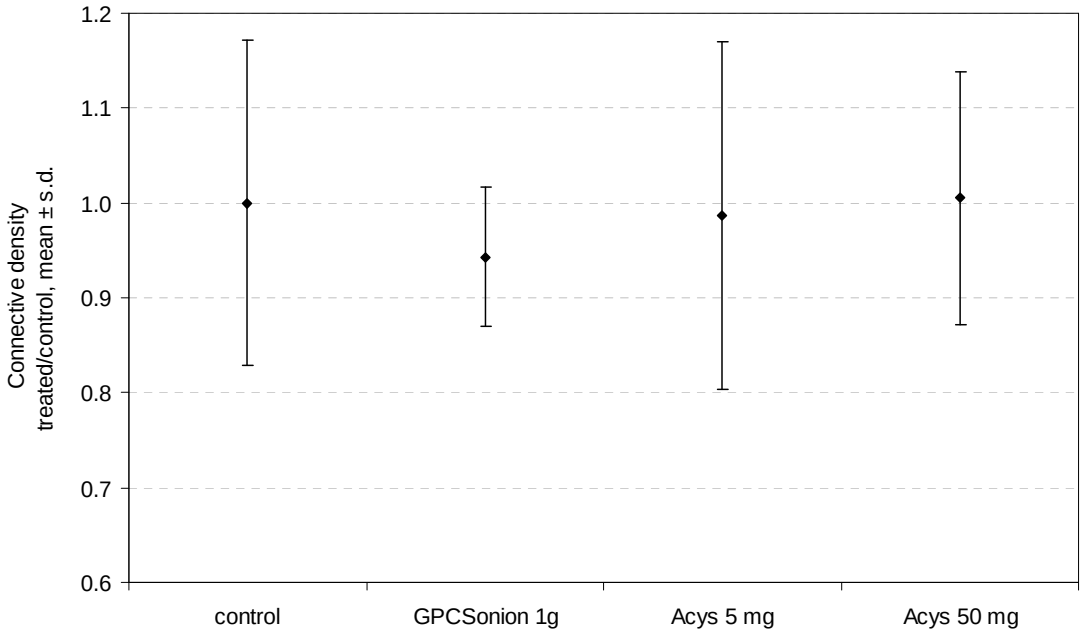


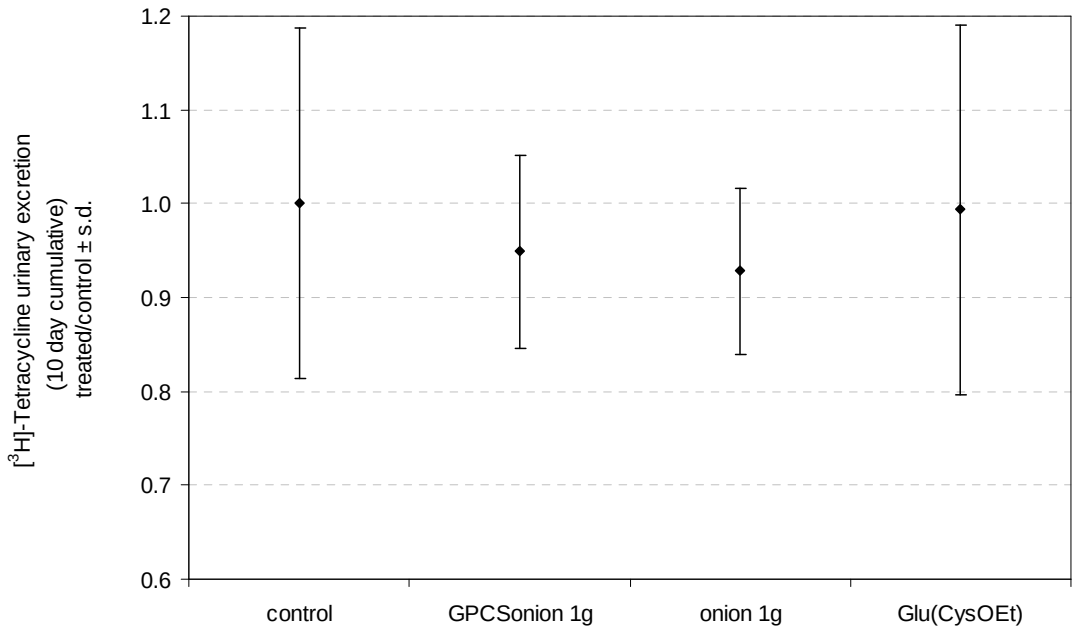
Figure 45: Experiment I: MicroCT analysis: connection density. Rats received a diet containing onion with GPCS (GPCSonion 1g), 5 mg allyl-cysteine (Acycs 5 mg), 50 mg allyl-cysteine (Acycs 50 mg) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6).



In the second experiment the effect of 1 g onion containing 20 mg GPCS was compared to that of 1 g of GPCS-free onion. In addition, γ-glutamyl-cysteine-ethylester was tested at 100 mg per day, because γ-glutamyl-cysteine-ethylester inhibited OC formation in cultures from bone marrow cells.

Cumulative urinary [^{3}H] excretion from the treatment phase showed no significant change in all treatment groups compared to the control. There was no difference in [^{3}H] excretion between rats receiving GPCS-containing onion and rats receiving GPCS–free onion (Figure 46).

Figure 46: Experiment II: 10 day cumulative urinary [^{3}H] excretion during treatment. Rats received 1 g onion with GPCS (GPCSonion 1g), 1 g onion without GPCS (onion 1 g), 100 mg γ-glutamyl-cysteine-ethylester (Glu(CysOEt)) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6).

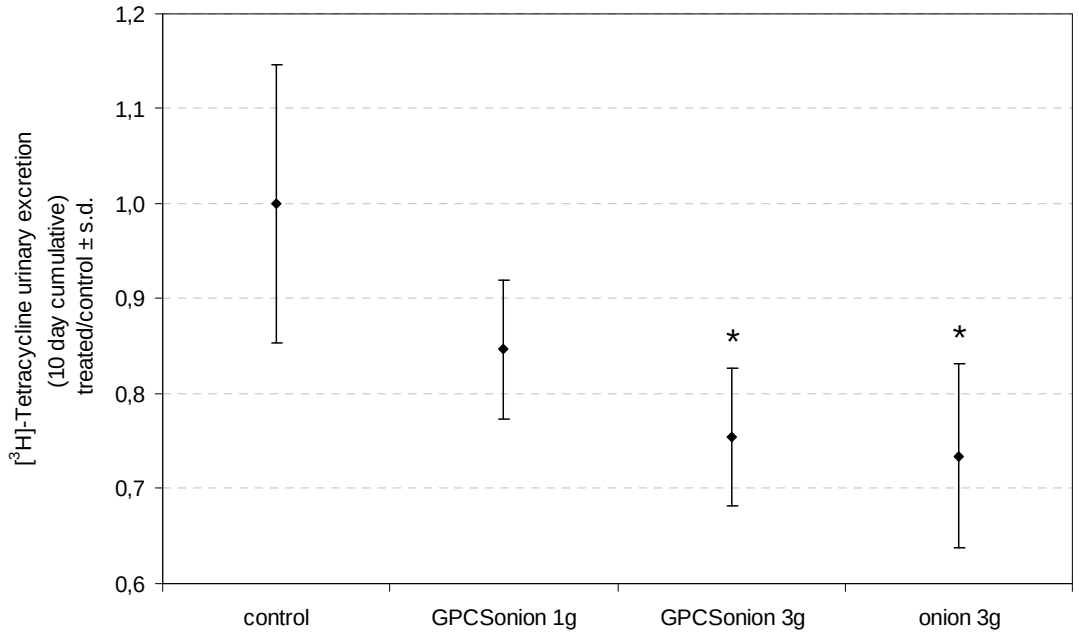


In the third experiment, the dose of onion was increased due to the lack of effects in experiments 1 and 2. First, an experiment was performed to determine an increased dose of onion to which the rats still respond to with a normal feeding and locomotory behaviour. Six 7-weeks old rats were transferred to metabolic cages and fed with SODI 2160 for 3 days. From the fourth to the seventh day, 2 rats were provided with 30 g SODI 2160 containing 3 g of onion, 2 rats received 30 g SODI 2160 containing 5 g of onion and 2 rats were fed SODI 2160 alone. The rats which were fed with 3 g of onion and those without onions showed normal behaviour and feeding patterns. The two rats which were provided with 5 g of onion refused to ingest their food within 1 h, showed an altered behaviour and their locomotory activity was severely reduced.

Based on these observations, in the third experiment, rats received semi-purified diet supplemented with 3 g onion containing 60 mg GPCS, 3 g onion without GPCS, 1 g onion containing 20 mg GPCS or semi-purified diet alone (control) over 10 days.

After the initial 10 days the cumulative values of urinary [^{3}H] excretion during the treatment phase were examined. Compared to the control, the cumulative urinary [^{3}H] excretion was decreased to 75 % in the rats fed 3 g GPCS-containing onion and 3 g GPCS-free onion, respectively. Rats which were fed GPCS-containing onion at 1 g showed a marginal, but not significant decrease in [^{3}H] excretion (Figure 47).

Figure 47: Experiment III: 10 day cumulative urinary [^{3}H] excretion during treatment. Rats received 1 g onion with GPCS (GPCSonion 1g), 3 g onion with GPCS (GPCSonion 3g), 3 g onion without GPCS (onion 3g) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6). * $p < 0.05$, significantly different from control.



At the end of the experiment, rats were euthanised and vertebrae were excised to perform micro-CT analysis. No difference in the BV/TV and connection density between the groups was observed (Figures 48-49).

Figure 48: Experiment III: MicroCT analysis: BV/TV. Rats received 1 g onion with GPCS (GPCSonion 1g), 3 g onion with GPCS (GPCSonion 3g), 3 g onion without GPCS (onion 3g) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6)

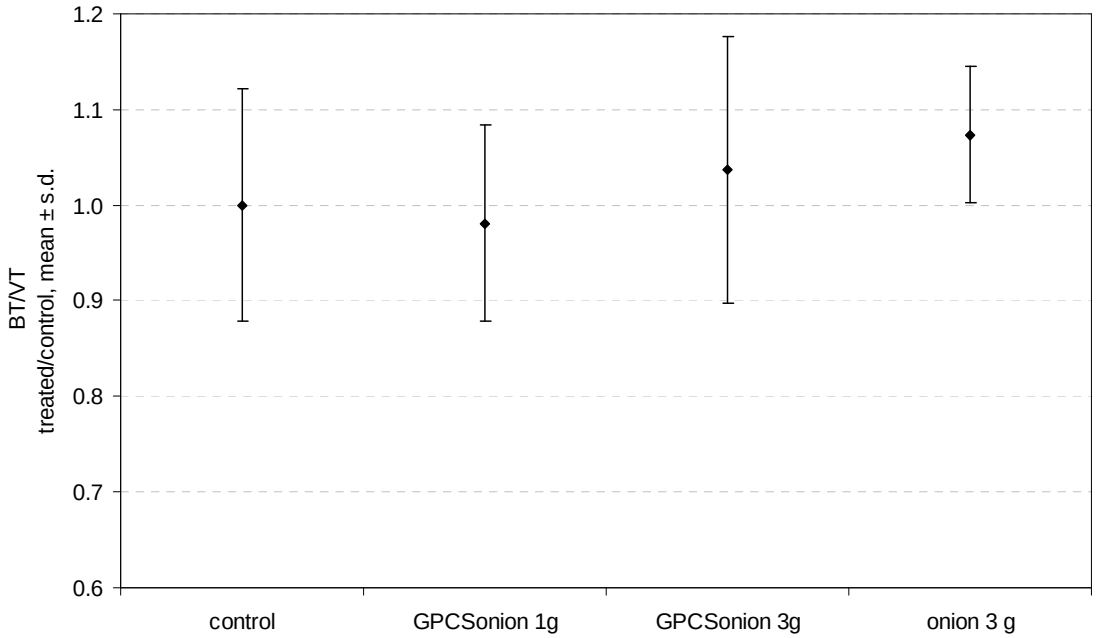
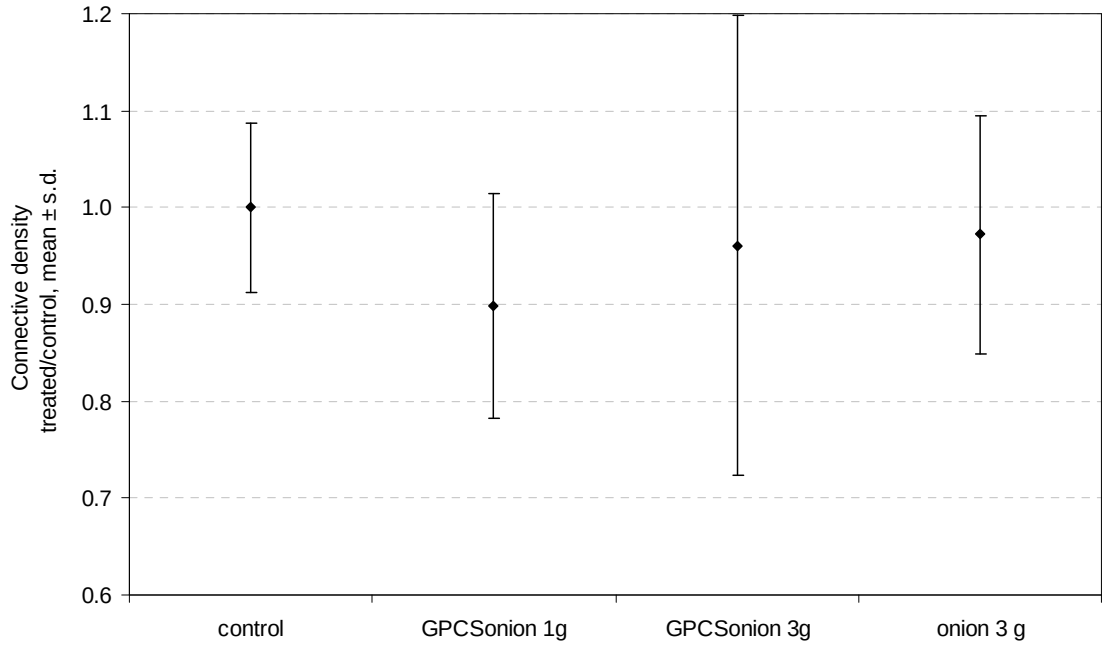


Figure 49: Experiment III: MicroCT analysis: connection density. Rats received 1 g onion with GPCS (GPCSonion 1g), 3 g onion with GPCS (GPCSonion 3g), 3 g onion without GPCS (onion 3g) or semi-purified diet alone (control) per day over 10 days. The data present treated/control mean values ± s.d. (n = 6)



In all three experiments, the course of urinary [^{3}H] excretion was monitored over 20 days of baseline and treatment phase. It showed a decrease in urinary [^{3}H] excretion in all groups from day 1 to day 20. After dietary change, there was an increase in [^{3}H] excretion from day 11 to day 12 (Figures 50-52).

Figure 50: Experiment I: Daily urinary [^{3}H] excretion of rats during baseline and treatment phase. From day 1 to day 10 rats received standard diet. From day 11 rats were administered semi-purified died supplemented with 1 g onion containing GPCS (onion 1g), 5 mg allyl-cysteine (Acys 5 mg), 50 mg allyl-cysteine (Acys 50 mg) or SODI 2160 diet alone (control) over 10 days. The data present mean (n = 6).

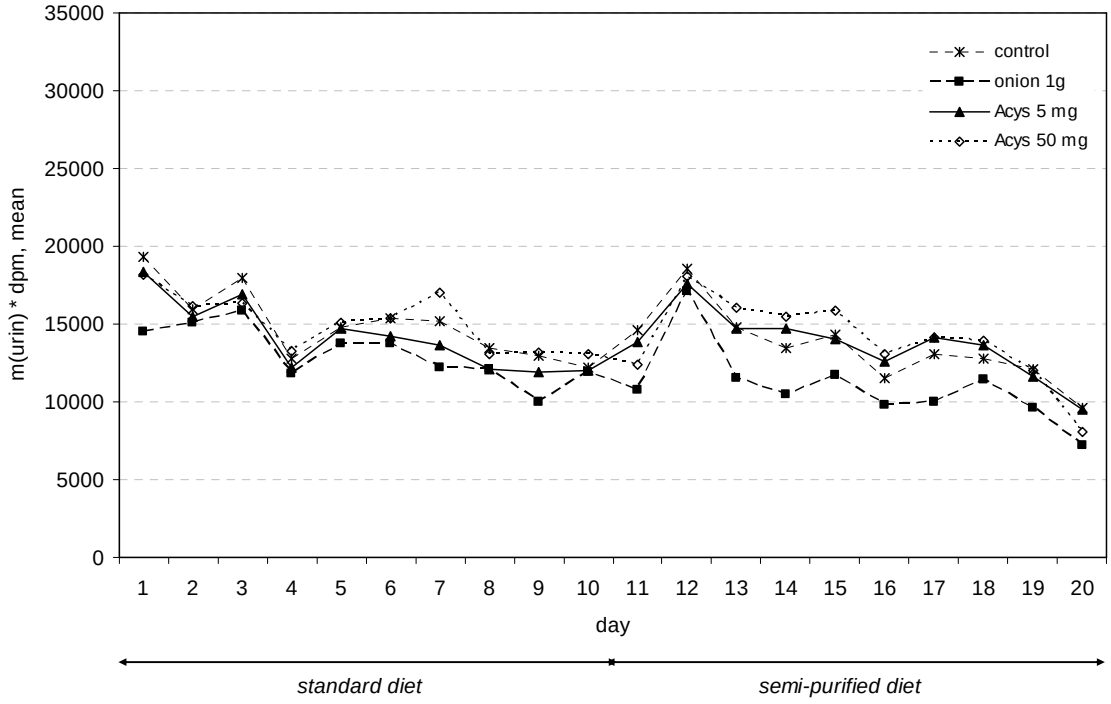


Figure 51: Experiment II: Daily urinary [^{3}H] excretion of rats during baseline and treatment phase. From day 1 to day 10 rats received standard diet. From day 11 rats were provided semi-purified died supplemented with 1 g onion containing GPCS (GPCSonion 1g), 1 g onion without GPCS (onion 1g), 100 mg γ-glutamyl-cysteine-ethylester (Glu(CysOEt)) or semi-purified diet alone (control) per day over 10 days. The data present mean (n = 6).

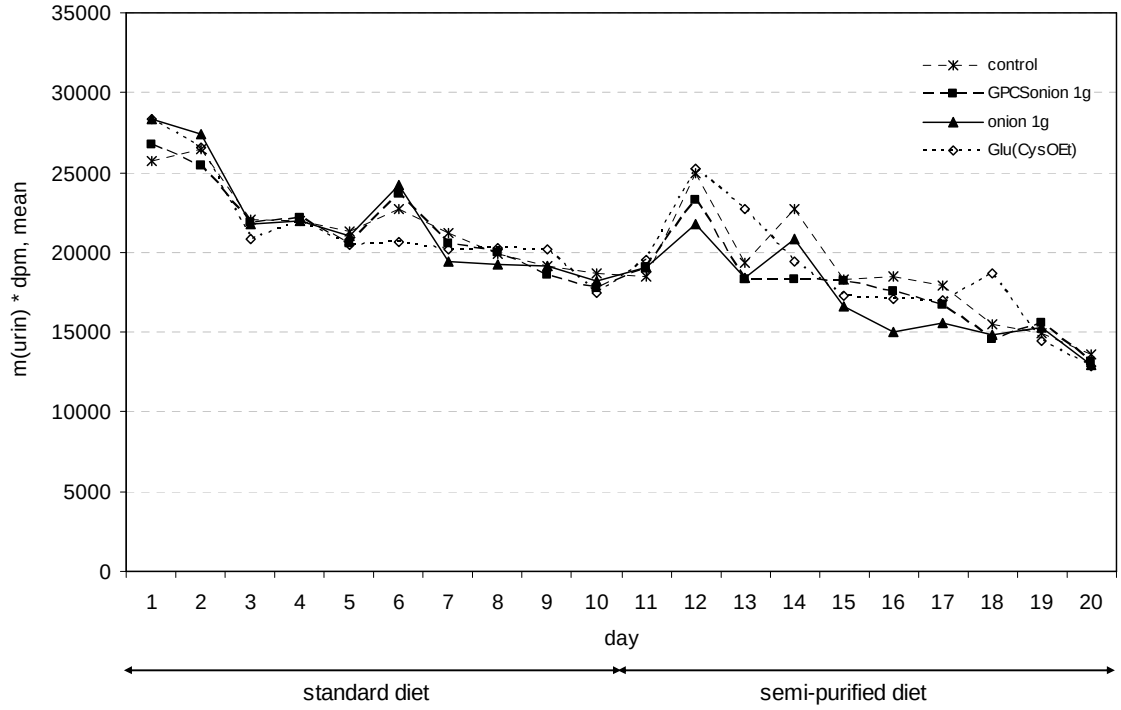
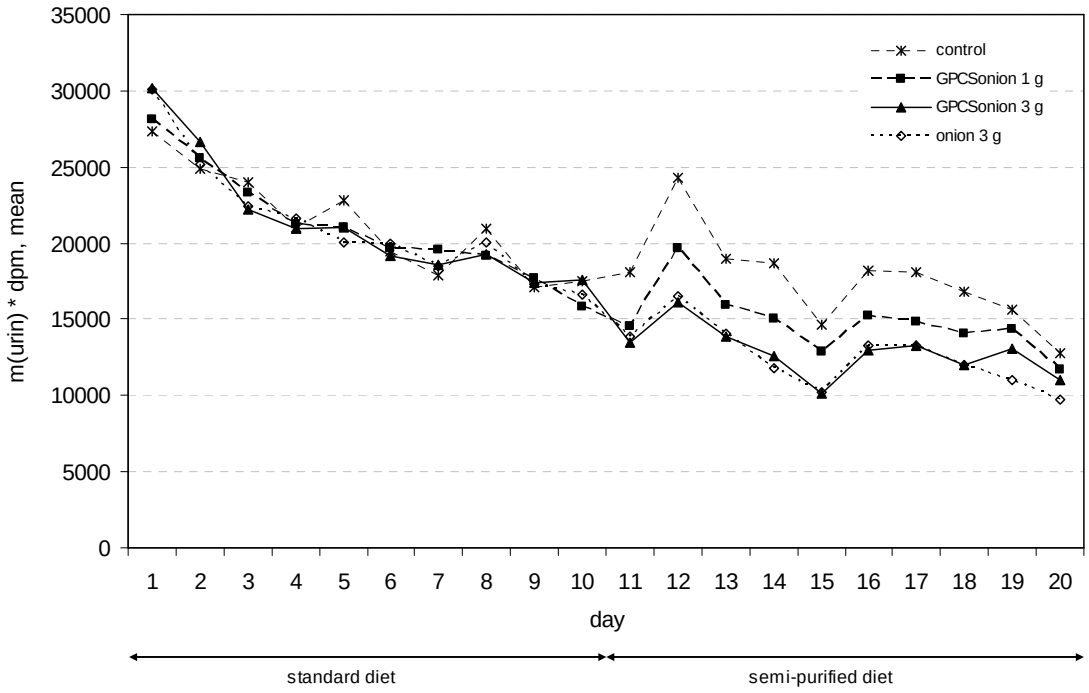


Figure 52: Experiment III: Daily urinary [^{3}H] excretion of rats during baseline and treatment phase. From day 1 to day 10 rats received standard diet. From day 11 rats were administered semi-purified died containing 1 g GPCS-containing onion (GPCSonion 1g), 3 g GPCS-containing onion (GPCSonion 3g) and 3 g GPCS-free onion (onion 3g) or semi-purified diet alone (control) per day over 10 days. The data present mean (n = 6).



8.3 Discussion

8.3.1 Effect of GPCS on the activity, activation and recruitment of osteoclasts *in vitro*

Previous studies demonstrated an inhibitory effect of onion on bone resorption [Muhlbauer 1999, Muhlbauer 2002, Muhlbauer 2003b, Wetli 2005]. GPCS was identified as the active compound *in vitro* showing an inhibition of the resorption activity of OCs [Wetli 2005]. Based on these results, further experiments were performed to elucidate the stage of osteoclastic resorption affected by the compound: development, activation or activity.

In our pit assay experiments, a reduced number of pits per OC was observed when cells were treated with 8 mM GPCS. Calcitonin, added at 0.01 nM, completely blocked pit formation. This concurs with data previously described by Wetli et al. [Wetli 2005]. The effect of GPCS on osteoclast activation was examined by determining the percentage of OCs with actin ring(s). GPCS did not change the percentage of OCs with actin ring(s). This might have either biological or/and technical reasons. On the one hand, GPCS did not exhibit an effect in the actin ring assay because it might not influence the activation of the OC, but has effects on other stages of the resorption process. On the other hand, the concentration of calcitonin which was required to reduce the percentage of OCs with actin ring(s) was 5 nM und thus 500 fold higher than in the pit assay. The actin ring assay might therefore be less sensitive than the pit assay. This may be due to different time of exposure to the substance under investigation. OCs in the pit assay were exposed to the test substances for 24 h immediately after their attachment to the dentin slices. In contrast, in the actin ring assay, OCs were allowed to attach to the glass coverslips for 3 h, when the percentage of OCs with actin rings reaches a maximum. Subsequently, the cells were exposed to the substances for 10 and 25 min.

GPCS inhibited OC formation at concentrations of 1 mM and higher in our experiments with CSF-1/RANKL primed bone marrow cells. This supports our hypothesis that onion may exert its inhibition of bone resorption not only by an inhibition of the activity of previously present OCs but also by a direct effect on the development of OC lineage cells affecting proliferation, differentiation, apoptosis or even cytotoxicity.

Specificity and structure-function-relationships of the inhibition of OC recruitment by GPCS were evaluated by testing peptides, peptide derivatives or dipeptides containing a cysteine moiety, a glutamyl moiety, both, or neither one in the molecule. Glutamyl-glycine and glycyl-valine did not change the number of OCs developing in the culture. All test substances which contained a cysteine moiety, γ-glutamyl-cysteine-ethylester, glycyl-cysteine, allyl-cysteine

and cysteine, inhibited osteoclastogenesis. These data suggest that the cysteine moiety of these molecules might be responsible for the inhibitory effect on the development of OCs.
Recently, a study has shown that aqueous solutions of onion powder inhibit osteoclastogenesis from co-cultures of bone marrow stromal cells and macrophage cells as well as RANKL induced ERK, p38 and NF-κB activation in macrophages [Tang 2009]. ERK and p38 are mitogen-activated protein kinases which are activated by RANK in OCs and OC precursors [Lee 2003]. NF-κB is a transcription factor which is present in the cytoplasm of most cell types [Gilmore 2006]. Many studies on bioactivity have been performed with allyl-cysteine. Allyl-cysteine, a compound derived from garlic (*Allium sativum* L.), and aged garlic extracts were shown to exhibit antioxidant [Amagase 2006], anticarcinogenic [Milner 2006, Nagini 2008], hepatoprotective [Gedik 2005] and immunostimulant [Lau 1991] activity. It was demonstrated by Geng et al. [Geng 1997] and Ide et al. [Ide 2001] that allyl-cysteine inhibits dose-dependently NF-κB activation induced by TNF-α or hydrogen peroxide in human T cells and endothelial cells. It was shown that osteoclastogenesis is inhibited in mice deficient of NF-κB proteins p 50 and p 52 [Abu-Amer 1997, Iotsova 1997]. NF-κB p 50 and p 52 control the differentiation of OC precursor cells induced by RANKL and TNF-α [Yamashita 2007]. The question whether the activity of allyl-cysteine, GPCS and the other compounds on OCs is related to an effect on NF-κB proteins, ERK or other mechanisms of osteoclastogenesis can not yet be answered.

8.3.2 Studies on the pharmacokinetic properties of GPCS *in vitro* and *in vivo*

GPCS was exposed to simulated digestive fluids *in vitro*. Simulated gastric acid contains pepsin, which is extracted from porcine gastric mucosa, and sodium chloride dissolved in a solution of hydrochloric acid and water. No change in the concentration of GPCS was observed when the compound was incubated under these conditions. Pepsin hydrolyses only peptide bonds, but preferentially cleaves hydrophobic, aromatic residues, which might be a reason why GPCS was not cleaved. Pepsin also cleaves at the carboxyl side of phenylalanine and leucine and to a lesser extent at the carboxyl side of glutamic acid residues [Enzyme explorer Pepsin 2008].
GPCS was also incubated with simulated intestinal fluid, a solution of pancreatin, sodium hydroxide and potassium dihydrogen phosphate in water (pH 6.8) [The United States Pharmacopoeia 2006]. Within 24 h, a 90 % decrease in the content of GPCS was observed. To test if intestinal enzymes are necessarily required for this effect, the content of GPCS was monitored in a solution of water over 24 h. GPCS remained stable over this period. Therefore, the presence of intestinal enzymes might be essential for degradation. Pancreatin, a product of porcine pancreas, contains many enzymes, including amylase,

trypsin, lipase, ribonuclease and protease [Data sheet Pancreatin 2008]. GPCS might have been cleaved most likely by proteases and probably by trypsin, both hydrolysing peptide bonds. Our experiment with onion powder incubated with simulated intestinal fluids over 24 h showed no change in GPCS content during the first 6 h. After 24 h, still 70 % of the initial GPCS concentration was found. *In vitro* studies on the influence of onions on digestive enzymes of rat pancreas were reported by Ramakrishna et al. [Ramakrishna Rao 2003]. In these experiments, pancreatic homogenates were incubated with onion powder and a reduced chymotrypsin activity was measured. This might be attributed to protease inhibitors present in onion. In a study of Deshimaru et al. [Deshimaru 2003] 3 protease inhibitors were purified from onion (OTI 1-3) and were reported to strongly inhibit trypsin, and to a weaker extent chymotrypsin activity. The reduced activity of chymotrypsin, trypsin and most likely other enzymes, may explain why the GPCS content is less decreased in simulated intestinal fluid when GPCS is present in onion matrix than as pure GPCS.

In a pilot study, we examined the pharmacokinetic properties of GPCS *in vivo*. The most powerful method for the sensitive detection of estimated (sub-)ng levels of GPCS in rat plasma was HPLC-MS/MS after sample purification using SPE. SPE was performed with Oasis MAX 3 cc columns, resulting in a recovery of about 80 % of GPCS. Oasis MAX is a strong mixed-mode anion-exchange polymeric sorbent designed to extract acidic compounds in complex matrices such as biological fluids [Brochure: Purity by SPE 2006]. L-Aspartyl-L-phenylalanine was used as I.S., because of the structural similarity to GPCS, and its stability under basic and acidic working conditions. This is very important during the extraction process, especially since elution is performed with solutions of ammonium hydroxide and formic acid. After SPE, samples were analysed with HPLC-DAD and HPLC-MS/MS. When using HPLC-DAD with or without derivatisation, no GPCS was found in the plasma extracts. GPCS is devoid of chromophors and thus, its UV absorption lies at a short wavelength at 195 nm which compromises the sensitivity when using UV detectors. Even derivatisation did not improve sensitivity. Consequently, an HPLC-MS/MS method was established which was sensitive enough to detect GPCS in the samples. Plasma profiles with typical t_{max} for substances given as single dose were obtained after oral and intravenous application [Bauer 2002]. The maximum area ratio of GPCS/I.S. was reached within 1 h for orally administered GPCS and the absolute bioavailability determined was about 1 %. This raises the question whether suitable concentrations of GPCS could be reached at the site of action indicating that either metabolites of GPCS might be responsible for an effect or GPCS is highly potent. The latter possibility is rather unlikely, because GPCS inhibited the activity and development of OCs *in vitro* at concentrations of 8 mM and from 1 mM and higher, respectively. The low bioavailability of the oral pure GPCS application might be due to pancreatic enzymes in the intestinal fluid, as shown by our *in vitro* experiments. But also other factors, such as

transporters, and physical conditions, for example tight intracellular conjunctions and the lipid characteristics of the cell membrane, might limit bioavailability [Pauletti 1997]. Due to the fact that the GPCS content is less decreased in simulated intestinal fluid when GPCS is present in onion, it is very likely that plasma levels of GPCS after oral onion administration would be higher than after administration of pure GPCS.

8.3.3 Studies on the pharmacodynamics of GPCS *in vivo*

When looking at the presence of GPCS in various plants (see chapter 6 Part I: Determination of GPCS in various plants), we found onions devoid of GPCS and onion containing high amounts of GPCS (20 mg per g dry onion powder). Each material was tested in pharmacodynamic studies to investigate, whether onion without GPCS exhibits other effects on bone resorption in rats than onion containing GPCS. In addition, allyl-cysteine and γ-glutamyl-cysteine-ethylester were tested as well, because these compounds showed a decrease of OC recruitment *in vitro*. To estimate the amount of bone resorption, we determined the urinary [^{3}H] excretion of rats chronically labelled with [7-^{3}H(N)]-tetracycline, a model, which was previously used and validated in several other studies [Antic 1996, Egger 1994, Muhlbauer 1990, Muhlbauer 1995, Muhlbauer 1999, Muhlbauer 2002, Muhlbauer 2003b, Wetli 2005]. [7-^{3}H(N)]-Tetracycline is incorporated into the bone and excreted into urine during bone resorption. In the first experiment, the feeding protocol was adopted from Wetli et al. [Wetli 2005]. Within the first 10 days of our experiment, the rats lost approximately 10 % of weight. Thus, lateron, the amount of food was increased and a gain in weight was observed. In experiment II and III, the daily food dose, which was found in experiment I to fit best to the need of the rats, was given from the start of the experiment, preventing loss of weight. After 10 days of [^{3}H] excretion baseline measurement, rats were assigned to different groups. In experiment I, rats were assigned randomly. Post-experimental analysis revealed, however, that the groups secreted unequal mean cumulative amounts of [7-^{3}H(N)]-tetracycline at the start of the treatment phase. Thus, not all groups had the same initial conditions, eventually giving rise to false results. Consequently, the mode of assignment was changed for the following experiments. A computer programme was designed which calculated an optimal assignment of the rats to the groups leading to an equal mean cumulative urinary [^{3}H] excretion at the beginning of the treatment phase.

In experiment I and II, a 1-g daily dose of onion containing GPCS was tested and exhibited no significant differences in the cumulative urinary [^{3}H] excretions, BV/TV, and connection density compared to control. In contrast to our results, in studies performed by Mühlbauer et al. and Wetli et al. [Muhlbauer 1999, Muhlbauer 2002, Muhlbauer 2003b, Wetli 2005], 1 g of onion significantly inhibited cumulative urinary [^{3}H] excretions. However, the content of GPCS present in the onions used by these authors is not known and might have been higher

than in our experiments. This might explain the lack of effect in our studies. As expected from the results of the [^{3}H]tetracycline assay and probably due to the short time of exposure to the substance, no differences between the groups in BV/TV and connection density of the cancellous bone were detected. The dose of onion was increased in experiment III. Daily doses of 3 g of onion, either containing or being devoid of GPCS, significantly inhibited cumulative urinary [^{3}H] excretion compared to control whereas 1 g of onion containing GPCS showed only marginal effects. There was no difference between the mean cumulative [^{3}H] excretion in both 3 g onion groups. This data suggests that, besides GPCS, other substances contribute to the inhibitory effects of onion on bone resorption *in vivo*. GPCS was previously found to be an active component following a bioassay based protocol [Wetli 2005], which, however, does not exclude the presence of other bone active components. It may also be possible that the contribution of GPCS to the inhibition of bone resorption by GPCS is insignificant, the observed *in vitro* effects at high concentrations being unspecific and other components of the onions mediating the biological response. While these possibilities warrant further investigations in the role of onions on bone resorption, it should be kept in mind that only one experiment supplementing the animals' daily diet with 3 g of onion was performed and the data needs to be reproduced. The previous validation and use of the [^{3}H]tetracycline model in several studies [Antic 1996, Egger 1994, Muhlbauer 1990, Muhlbauer 1995, Muhlbauer 1999], however, provide some strength to these results.

BV/TV and connection density were equal between all groups in experiment III which may be explained by the short exposure time (10 days) of the test substances. Long-term studies over 6 weeks on adult female rats have shown that onion-containing diet significantly decreases ovariectomy-induced bone loss and increases BV/TV and trabecular number and separation [Huang 2008].

In our experiments, allyl-cysteine and γ-glutamyl-cysteine-ethylester did not inhibit bone resorption *in vivo*, although they inhibited OC development *in vitro*. This may be due to an insufficient dose and/or to a low bioavailability of the two compounds *in vivo,* so that no adequate concentrations result at the site of action.

Monitoring the urinary [^{3}H] excretions over 20 days, we observed a decrease in the excretion from day 1 to day 20 which is attributed to the biological half-life of [7H-^{3}(N)]-tetracycline. An increase of urinary [^{3}H] excretions from day 11 to 12 was most probably induced by the change of diet on day 10 from standard to semi-purified diet without any plant derived inhibitors of bone resorption. This increase in the urinary [^{3}H] excretions was lower in groups with a significantly decreased mean cumulative [^{3}H] excretion. Since the food supplements and the semi-purified diet are given from the same time (day 10), the effect observed in this assay is an inhibition of the stimulation of bone resorption, rather than on the decrease of a normal physiological resorption. The data demonstrate that the food supplements can be

used to inhibit bone resorption. Therefore, this strategy might be a means for the prevention of bone loss.

9 Conclusions and outlook

The results of the present investigations show that GPCS does not only affect OC activity *in vitro*, but also OC recruitment, probably due to the cysteine moiety of the compound. As mentioned previously, studies by Tang et al. [Tang 2009] demonstrated that an inhibition of RANKL-induced osteoclastogenesis by onion might proceed via ERK, p38 and NF-κB pathways. Allyl-cysteine, a compound which was tested in our *in vitro* OC recruitment assay as well, inhibited osteoclastogenesis, and has previously been reported to inhibit NF-κB activation [Geng 1997, Ide 2001]. The question whether the *in vitro* activity of allyl-cysteine, GPCS, cysteine, γ-glutamyl-cysteine-ethylester and glycyl-cysteine on OCs is related to an effect on NF-κB, ERK or p38 could be answered by testing the compounds in the assays described by the authors previously mentioned. The results of our kinetic studies on GPCS and onion suggest that the oral application of GPCS in a solution of sodium chloride is unsuitable because of the low bioavailability, which might be attributed to pancreatic enzymes cleaving GPCS, especially proteases and trypsin. However, the presence of protease inhibitors in onions probably prevents the cleavage of GPCS and might therefore increase the bioavailability of the compound. Onion containing GPCS and onion without GPCS exhibited the same amount of inhibition of bone resorption *in vivo*. Due to the fact, that onion without GPCS exhibits an effect, we can postulate that either GPCS has no effect or is not the only substance from onion exerting an effect on bone resorption. Therefore, in further research, first priority should be given to the synthesis of GPCS to provide sufficient material for further *in vivo* studies. Future experiments using natural GPCS-free onions spiked with synthetic GPCS should clarify, to which extent GPCS contributes to the inhibition of bone resorption. If GPCS is involved in an inhibition of bone resorption, the exact mechanism should be elucidated and toxic effects should be excluded.

A variety of plants were tested according to the presence of GPCS. None of these plants contained GPCS and thus, the question which compounds are responsible for their *in vivo* bone resorption inhibiting effect remains unanswered. The active compounds might be isolated using a bioassay *in vitro* and the structure might be investigated using MS and NMR. Afterwards, the activity should be confirmed *in vivo*.

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11 Appendices

11.1 Appendix 1: List of Abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
b	Broad (NMR)
BSA	Bovine serum albumin
BV/TV	Bone volume/total volume quotient
CE	Collision energy
CXP	Cell exit potential
d	Douplet (NMR)
DAD	Diode array detection
DP	Declustering potential
ESI	Electrospray ionisation
FBS	Fetal bovine serum
FBS Hia	Heat-inactivated fetal bovine serum
GC-MS	Gas chromatography - mass spectrometry
GPCS	γ-Glutamyl-propenyl-cysteine sulfoxide
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) solution
HPLC	High performance liquid chromatography
i.d.	Inner diameter
I.S.	Internal standard
IS	Ion spray voltage
LOQ	Limit of quantitation
m	Multiplet (NMR)
M-CSF	Macrophage colony-stimulating factor
MEM	Minimum essential medium with Earle's salts and without phenol red
α-MEM	alpha-Minimum essential medium with Earle's salts
MEM Hank's	Minimum essential medium with Hanks's salts
MP	Melting point
MPLC	Medium pressure liquid chromatography
MRM	Multiple reaction monitoring mode
MS	Mass spectrometry
m/z	Mass to charge
NMR	Nuclear magnetic resonance spectroscopy
OC	Osteoclast
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
P/S	Penicillin-streptomycin-solution
q	Quartet (NMR)
QQQ	Triple quadrupole mass spectrometer

RANKL	Receptor activator of NF-κB ligand
RP	Reverse-phase
r.s.d.	Relative standard deviation
s	Singlet (NMR)
s.d.	Standard deviation
SIF	Simulated intestinal fluid
SPE	Solid phase extraction
TRAP	Tartrate resistant acid phosphatase
UV	Ultraviolet
VIS	Visible
v/v	Volume to volume ratio
w/w	Weight to weight ratio

11.2 Appendix 2: List of suppliers

Agilent Technologies, Wilmington, USA

Applied Biosystems, MDS Sciex, Concord, ON, Canada

Bachem, Burgdorf, Switzerland

BALZERS UNION Limited, Balzers, Liechtenstein

Charles River Wiga GmbH, Sulzfeld, Germany

Chiron Corporation, Emeryville, CA, USA

GERSTEL AG, Sursee, Switzerland

Gibco, Lubio Sciences GmbH, Lucerne, Switzerland

Gilson, Villiers La Bel, France

Infochroma, Zug, Switzerland

Kliba-Mühlen, Kaiseraugst, Switzerland

Machery-Nagel, Oensingen, Switzerland

Marienfeld GmbH & Co. KG Lauda-Königshofen, Germany

Merck, Dr. Grogg Chemie AG, Stettlen, Switzerland

Molecular Probes, Invitrogen, Lubio Sciences GmbH, Lucerne, Switzerland

Packard, Top Lab GmbH, Rickenbach, Switzerland

Peprotech London, UK

Perkin Elmer, Schwerzenbach, Switzerland

Phenomenex, Torrance, CA, USA

Scanco Medical AG, Basserdorf, Switzerland

Schleicher & Schuell, Sigma-Aldrich, Buchs, Switzerland

Sigma-Aldrich, Buchs, Switzerland

Thermo Shandon, Pittsburgh, USA

Waters, Baden-Dättwil, Switzerland

11.3 Appendix 3: Composition of SODI 2134 and SODI 2160 diet powders

11.3.1 Composition of SODI 2160 diet powder

Ingredient	Concentration in %
Corn starch	56
Casein	20
Dextrose	10
Cellulose	4
Pork fat	3
Arginine	0.71
Lysine	1.38
Methionine	0.56
Methionine, cystine	0.62
Tryptophan	0.25
Threonine	0.72
Calcium	1.1
Phosphorus	1.2
Magnesium	0.15
Sodium	0.30
Potassium	0.70
Chlorine	0.25

Ingredient	Concentration in mg/kg
Iron	80
Zinc	38
Copper	5
Iodine	0.8
Manganese	11
Selenium	0.2
Vitamin A	1.34
Vitamin D3	0.025
Vitamin E	100
Vitamin K3	4
Vitamin B1	6
Vitamin B2	6
Vitamin B6	7
Vitamin B12	0.05
Nicotinic acid	30
Pantothenic acid	16
Folic acid	2
Biotin	0.2
Choline	1000

11.3.2 Composition of SODI 2134 diet powder

Ingredient	Concentration in %
Corn starch	59
Soybean meal	20
Potato protein	9
Refined soybean oil	3.5
Oats	2.4
Dextrose	2
Casein	2
Arginine	1.06
Lysine	1.20
Methionine	0.40
Methionine, cystine	0.60
Tryptophan	0.24
Threonine	0.78
Calcium	0.17/1.1*
Phosphorus	0.20/1.2*
Magnesium	0.19
Sodium	0.19
Potassium	0.71
Chlorine	0.32

Ingredient	Concentration in mg/kg diet
Iron	78
Zinc	33
Copper	8
Iodine	1.3
Manganese	15
Selenium	0.2
Vitamin A	3.35
Vitamin D3	0.025
Vitamin E	100
Vitamin K3	4
Vitamin B1	6
Vitamin B2	6
Vitamin B6	7
Vitamin B12	0.05
Nicotinic acid	30
Pantothenic acid	16
Folic acid	2
Biotin	0.2
Choline	1000

* after preparation

11.4 Appendix 3: Assessment of bone resorption in vivo: experiment I to III, weight of rats on day 1, 10 and 20 in metabolic cages

	Experiment I			Experiment II			Experiment III		
Rat No.	Day 1	Day 10	Day 20	Day 1	Day 10	Day 20	Day 1	Day 10	Day 20
1	284	250	-	237	245	-	256	260	-
2	294	269	-	254	262	-	273	271	290
3	280	253	292	235	230	-	327	310	-
4	290	256	294	226	232	-	267	267	295
5	269	243	-	230	233	-	307	294	320
6	299	266	303	243	243	266	292	278	300
7	260	235	282	242	240	-	292	278	-
8	323	284	-	250	252	-	306	300	320
9	290	263	300	260	265	-	275	274	295
10	263	239	282	227	237	-	320	296	320
11	305	275	-	235	240	270	292	277	300
12	260	240	-	236	247	-	302	300	310
13	250	231	272	266	275	312	275	276	297
14	265	240	-	250	254	285	259	260	286
15	276	250	298	255	254	273	247	250	280
16	260	240	280	260	260	286	272	270	-
17	295	252	296	246	247	-	270	270	290
18	307	268	-	264	267	295	250	254	280
19	280	256	-	242	247	268	225	235	-
20	291	257	295	260	260	287	247	254	285
21	280	264	294	230	235	-	250	256	-
22	305	250	289	258	266	288	269	270	-
23	308	278	309	268	272	290	267	270	-
24	300	259	295	270	270	-	248	250	275
25	262	237	270	285	285	305	296	288	310
26	288	248	282	278	275	290	299	283	-
27	275	250	285	245	260	-	266	268	-
28	298	263	301	265	263	290	264	269	292
29	270	240	281	310	292	310	260	250	-
30	290	263	290	275	268	-	296	291	317
31	290	256	300	237	245	-	267	263	280
32	273	243	285	277	275	300	270	271	-
33	274	248	288	270	275	-	270	269	290
34	256	223	-	265	265	300	300	297	307
35	-	-	-	290	292	320	292	284	310
36	-	-	-	-	-	-	250	254	280
Mean	283	253	290	255	258	291	276	272	297
s.d.	18	14	10	20	17	15	23	17	14