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Isolation and characterization of regulatory peptides and bioactive compounds

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*To my family
Gun, Fredrik and Karin*

Abstract

Isolation of peptides and other bioactive compounds is an important and often necessary step to get the total information about their structures. This is demonstrated by a number of different characterizations in this thesis. Bioactive peptides and small organic molecules can act as signaling substances and messengers in multicellular organisms and are fundamental to higher forms of life. The following bioactive peptides and compounds were studied.

1) Different assays can be used for detection of novel peptides. Chemical assays are simple, but still robust, and have proven successful in several cases. In this thesis, an assay was developed to detect peptides that contain tryptophan. An N-terminal dipeptide Gly-Trp is characteristic for galanin, which was cleaved off, separated and visualized. In this manner a novel form of galanin from chicken was detected and isolated.

2) Antibacterial peptides were first found in insects and later in mammals. Many of these peptides are basic, some are Pro/Arg-rich (e.g. peptide PR-39). Investigation of basic peptides from pig spleen, resulted in the isolation of a new variant form of NK-lysin, previously only detected in a cDNA library from porcine bone marrow. PR-39 was identified by a method suitable for Pro/Arg-rich peptides using ladder sequence analysis with mass spectrometry.

3) Another biological assay, involving cAMP production in cell culture of SK-N-MC cells, was used to screen peptide fractions, and resulted in the isolation of a novel form of the hormone PYY, with a phosphate group attached to the Ser-13 residue. This finding demonstrates the benefit of peptide isolation to find posttranslational modifications not directly obvious from the corresponding DNA sequence. Using nano-electrospray tandem mass spectrometry, the phosphorylated form of porcine PYY was identified and the modification localized to Ser-13.

4) Specific antibodies raised against peptides are a valuable tool in peptide chemistry. We have isolated PEC-60 from pig and rat brains with a method that combines column purification procedures with the specificity of a radioimmunoassay (RIA) and the sensitivity of mass spectrometry to directly identify the peptide. The results show that PEC-60, like many other peptides, is localized in the gastrointestinal tract and also in the central nervous system. The specific regional brain distribution may imply a specific function.

5) Using RIA and HPLC purification, 4 novel forms of modified galanin were isolated and characterized by mass spectrometry. The modified forms contained β -aspartic shifts and oxidized tyrosine.

6) Hypoglycemic agents have been described in plants. *Gynostemma pentaphyllum* (Cucurbitaceae), an East-Asian herb, has been reported to have different activities, such as antitumor, cholesterol-lowering, immunopotentiating, hypoglycemic and antioxidant effects. We have isolated a novel insulin-releasing substance from this extract, determined the structure by NMR and mass spectrometry and characterized the effects on insulin release. We found that it is a novel saponin and we named it phanoside.

The results provide us with new knowledge about localization, structure, and processing of hormones and bioactive molecules.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. **Norberg Å**, Sillard R, Carlquist M, Jörnvall H, Mutt V. Chemical detection of natural peptides by specific structures. Isolation of chicken galanin by monitoring for its N-terminal dipeptide, and determination of the amino acid sequence. *FEBS Lett.* 288 (1991) 151-153.
- II. Bonetto V, Andersson A, Bergman T, Sillard R, **Norberg Å**, Mutt V, Jörnvall H. Spleen antibacterial peptides: high levels of PR-39 and presence of two forms of NK-lysin. *Cell. Mol. Life Sci.* 56 (1999) 174-178.
- III. Chen ZW, Eriste E, Jonsson AP, **Norberg Å**, Nepomuceno D, Lovenberg TW, Bergman T, Efendic S, Jörnvall H, Sillard R. Ser13-phosphorylated PYY from porcine intestine with a potent biological activity. *FEBS Lett.* 492 (2001) 119-122.
- IV. **Norberg Å**, Gruber S, Angelucci F, Renlund S, Wadensten H, Efendic S, Östenson CG, Jörnvall H, Sillard R, Mathé AA. Identification of the bioactive peptide PEC-60 in brain. *Cell. Mol. Life Sci.* 60 (2003) 378-381.
- V. **Norberg Å**, Griffiths WJ, Hjelmqvist L, Jörnvall H, Rökaeus Å. Identification of variant forms of the porcine neuroendocrine peptide galanin. *Manuscript*.
- VI. **Norberg Å**, Hoa NK, Liepinsh E, Phan DV, Thuan ND, Jörnvall H, Sillard R, Östenson CG. Isolation of a novel insulin-releasing substance from the plant *Gynostemma pentaphyllum*. *Manuscript*.

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ABBREVIATIONS

Three- and one-letter codes for the 20 genetically encoded amino acids

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CPP	carboxypeptidase P
CPY	carboxypeptidase Y
DMSO-d ₆	deuterated dimethylsulphoxide
ESI	electrospray ionization
G protein	guanine nucleotide-binding regulatory protein
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry (or mass spectrometer)
MS/MS	tandem mass spectrometry
NKL	NK-lysin
RP	reverse phase
PR-39	proline-arginine-rich peptide with 39 residues

1 INTRODUCTION

1.1 Messengers

All cells receive and respond to signals from their surroundings. Even the simplest bacteria detect and move in the direction of high concentrations of nutrients, such as glucose and amino acids. Unicellular eukaryotes also respond to signaling molecules secreted by other cells, allowing cell-to-cell communication. Mating between yeast cells is signaled by peptides that are secreted by one cell and bind to receptors on the surface of another [1]. In multicellular organisms, cell-to-cell communication reaches its highest level of sophistication. Whereas the cells of prokaryotes and unicellular eukaryotes are largely autonomous, the behavior of each cell in multicellular plants and animals must be carefully regulated to meet the needs of the organism as a whole. This is accomplished by signaling molecules that are secreted or expressed on the surface of one cell and bind to receptors expressed by other cells, thereby integrating and coordinating the functions of the individual cells that make up organisms as complex as human beings.

Bioactive compounds are involved in a broad array of functions where cells communicate in an endocrine manner on a distant target cell, in a paracrine manner to target cells close by or in an autocrine manner responding to their own signaling substances. Extracellular signaling can be described in 6 distinct steps 1. Synthesis of the signaling molecule by the signaling cell. 2. Release of the signaling molecule by the signaling cell. 3. Transport of the signal to the target cell. 4. Detection of the signal by the target cell receptor protein. 5. Change in cellular metabolism, function, or development triggered by the receptor-signal complex. 6. Removal of the signal, which terminates the cellular response.

The word “hormone” is derived from Greek and implying “arousal to activity” and constitutes a general term to indicate a secreted substance that exerts influence upon the metabolism of a target tissue or cell. Hormones coordinate growth, differentiation, and metabolic activities of cells, tissues, and organs in multicellular organisms. One large group of signaling molecules with great variation is peptides, ranging from only a few amino acid residues to more than a hundred in length.

It is now over one hundred years since Bayliss and Starling demonstrated in 1902 that intravenous injection of an acid extract of canine upper intestine stimulated secretion of alkaline pancreatic juice, and the postulated blood-borne chemical messenger was named secretin [2]. The discovery of secretin established the concept of blood-borne endogenous messengers named hormones.

Despite the discovery of many new hormonal peptides and substances in the past four decades, such peptides and substances still remain to be discovered. Hormones have in general been detected by observation of their biological activity and isolation from tissue extracts utilizing bioassays as detection methods. Ancient Indians, Greeks, and Egyptians were aware that extracts of animal testes could promote virility, potency, and vigor in men. In modern times, castration and testis-transplantation experiments in roosters were conducted initially by John Hunter [3]. However, in 1849 Berthold [4] established the link between secretions of the testis and some of the sexually dimorphic features in the rooster. He drew the conclusion of a blood-borne messenger, in an experiment, where the testicles were removed from their original place and implanted in the abdominal cavity of the bird. In the early 20th

century many of the classical peptide hormones were discovered, in 1905 gastrin [5], in 1921-1922 insulin, and in 1928 CCK [6].

Isolation of peptides will continue to be important to achieve complete understanding concerning peptide hormones as processed mature peptides. For many peptides, posttranslational modifications are required for activity [7, 8]. While only 20 amino acids are specified in the genetic code and are involved as building blocks of the polypeptide chain, about 140 amino acids and amino acid derivatives have been identified as constituents of different proteins in organisms [9]. Medical chemistry, pharmacology, immunology and diabetes research, all search for novel biologically active peptides, such as “orphan” receptor ligands, peptides that affect insulin release in diabetes, or antibacterial peptides that are important in the innate immune system. Many assay systems can be used to detect new bioactive peptides [10-12].

Several genomic projects have revealed a number of genes encoding receptor-like sequences, with originally unknown functions. Many of the identified genes encode novel G protein-coupled receptors. The identity of the endogenous ligands for such receptors, the regulatory factors, hormones or neurotransmitters will provide information about cellular communication pathways and can help to develop candidates for new drugs and new strategies for treatment of diseases.

1.1.1 Biosynthesis

One striking difference between neuropeptides/peptide hormones and conventional neurotransmitters regards their biosynthesis. The active peptides derive from larger, inactive precursors that are generally at least 90 amino acid residues in length. Peptides are synthesized on the endoplasmatic reticulum by the ribosomes, and are produced as larger precursors, preprohormones. The signal peptide directs the product to the Golgi system where enzymatic steps lead to the bioactive peptide. A preprohormone is the primary translation of a hormone precursor mRNA, i.e. the precursor protein including the signal peptide. Cleavage sites are usually at basic residues. The endoproteases involved are prohormone convertases 1 and 2 (PC1 and PC2), which cleave at selected pairs of basic amino acids in the peptide precursors: Lys-Arg, Arg-Arg, Lys-Lys and Arg-Lys [13, 14].

1.1.2 Posttranslational modifications

Many bioactive peptides are subject to additional posttranslational modification. These modifications can include C-terminal amidation, N-terminal acetylation, sulphation of tyrosine residues, N-terminal pyroglutamate formation and phosphorylation of serine, threonine or tyrosine residues, the latter more common in cellular proteins.

Many of the posttranslational steps of neuropeptides are associated with movements of the maturing peptides down the axon towards the synapse in LDCVs (large dense core vesicles).

C-terminal amidation

C-terminal amidation by peptidylglycine α -hydroxylating mono-oxygenase (PHM) acts on peptide substrates after endoproteolytic cleavage and exopeptidase action, when a COOH-terminal Gly residue is exposed. The first step of the α -amidation reaction is performed by PHM, which is the NH₂-terminal portion of the bifunctional peptidylglycine α -amidating monooxygenase protein (PAM). PHM binds two Cu²⁺ atoms that participate in catalysis by

undergoing cycles of reduction and oxidation. PHM uses ascorbic acid as the reductant, with one atom of oxygen from O₂ incorporated into the peptide during the hydroxylation step. The second step of the α -amidation reaction is performed by a second enzymatic domain of PAM, peptidyl- α -hydroxyglycine α -amidating lyase (PAL) [15, 16].

Phosphorylation

Phosphorylation usually occurs as a means of signal mediation in a signal-transduction cascade. Secondary messengers carry the signal inside the cell and often use protein phosphorylation as a signaling device. In large receptors like the insulin receptor, the receptor is phosphorylated at several tyrosine residues upon binding of insulin and thereby mediates a signal [17]. In peptides, phosphorylation can be catalyzed by a protein kinase, like casein kinase [18], which recognizes the sequence Ser/Thr-X-Glu/Asp, where X is any amino acid. Phosphorylated peptides have only been found in few cases, such as ACTH, proenkephalin A, chromogranin A, and a few others [19-22]. Proenkephalin A-derived peptides have been shown to acquire antibacterial properties upon phosphorylation of specific serine residues [23].

β -aspartic shift

β -aspartic shifts can occur in many peptides [24-26] via a cyclic imide formation [27]. Both asparagine and aspartic acid residues can cyclize, with rates depending on both the identity of nearby residues, some of which can catalyze the deamidation reaction, and upon polypeptide conformation [28]. The cyclic imide can then open (Fig. 1) to form either a normal peptide or an isopeptide. Aspartyl and asparaginyl deamidation, isomerization, and racemization reactions have been studied in synthetic peptides to model these spontaneous processes. Studies indicate that both aspartic acid and asparagine residues may be subjected to nonenzymatic degradation of proteins, especially in cells such as erythrocytes and in those of the eye lens, where the macromolecules must function for long periods [29].

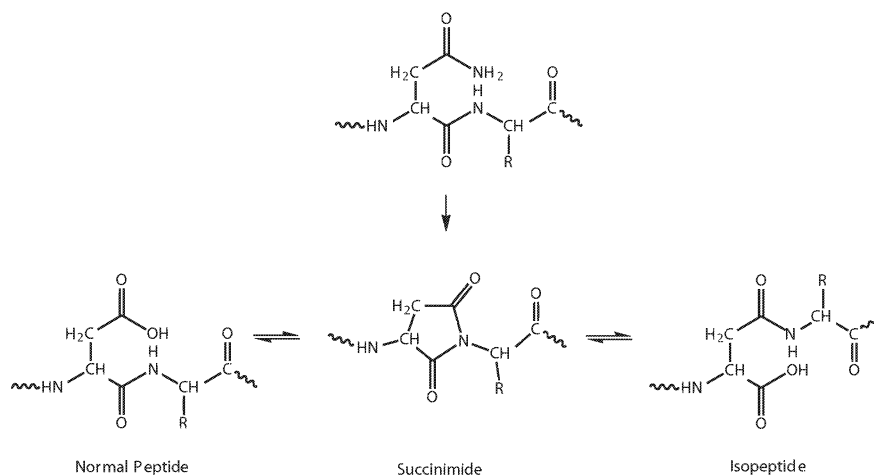


Figure 1. Aspartyl and asparaginyl deamidation, isomerization, and racemization reactions.

1.1.3 Analytical methods

Mass spectrometry

In mass spectrometry (MS) ions are introduced into the vacuum, are accelerated through an electrical field and the mass/charge ratio (m/z) is measured. The sample is introduced into the mass spectrometer, which is generally kept under high vacuum ($<10^{-5}$ mBar). Compounds are converted into gas phase molecules either before or during the charging or ionisation process, which takes place in the ion source.

Electrospray ionization

In electrospray ionization MS (ESI MS), highly charged droplets dispersed from a capillary in an electric field are evaporated, and the resulting ions are drawn into an MS inlet. In ESI the analyte is introduced by a capillary, typically at 1-50 $\mu\text{l}/\text{min}$ flow rate. A potential up to 2-3 kV is applied between the capillary and the counter electrode [30, 31].

MALDI mass spectrometry

Matrix-assisted laser desorption ionization (MALDI) was developed by Karas and Hillenkamp [32]. The analyte is dissolved in a solution containing a weak aromatic acid (matrix) that is the UV absorbent, usually α -cyano-4-hydroxy-cinnamic acid. After UV irradiation, both analyte and matrix will desorb into the gas phase and thereafter are introduced into the mass analyser. Mass spectra of proteins with masses up to 300 kDa have been recorded [33].

MS/MS

Collision-induced dissociation (CID) of peptides is a widely used technique. The ion of interest is selected in the first mass analyzer, directed into a collision cell where an inert gas, usually argon causes fragmentation. A second mass analyser measures the masses of the fragment ions. Fragments will only be detected if they carry at least one charge. If this charge is retained on the N terminal fragment, the ion is classed as either a, b or c. If the charge is retained on the C terminal, the ion type is either x, y or z. A subscript indicates the number of residues in the fragment (Fig. 2). The accepted nomenclature for fragment ions was first proposed by Roepstorff and Fohlman [34]. Protonated peptide fragments in low energy CID fragment at the amide bond giving b-type and y type ions.

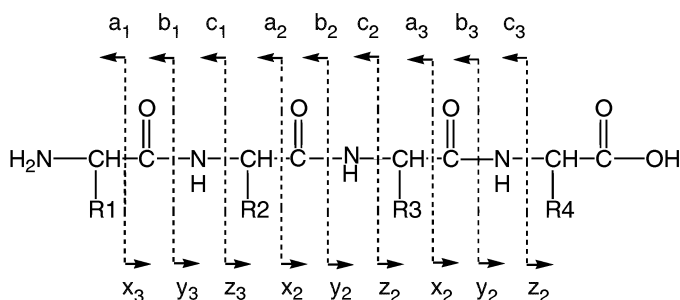


Figure 2. Nomenclature for peptide fragment ions.

NMR

Nuclear magnetic resonance (NMR) spectroscopy uses the fact that certain atomic nuclei are inherently magnetic. Only a restricted number of isotopes exhibit this property, called spin. Biologically important nuclei are ^1H , ^{13}C , ^{14}N and ^{31}P . The simplest is the hydrogen nucleus ^1H , a proton. The spinning of a proton generates a magnetic moment. This momentum can take two orientations, or spin states, α or β , in a magnetic field. The energy difference between these two states is proportional to the strength of the magnetic field. Absorption of electromagnetic radiation of appropriate frequency induces a transition from the lower to the upper level.

Nuclei of different location in a molecule absorb electromagnetic radiation at different frequencies. The different frequencies, termed chemical shifts are expressed in ppm relative to a standard such as water is measured. The spin of neighboring nuclei interact with each other in a manner that provides definitive structural information. Techniques like nuclear Overhauser effect (NOE) are used in two-dimensional spectroscopy to display pairs of nuclei in close proximity.

1.1.4 Peptides investigated

Galanin

Galanin was first isolated from pig intestine [35] using its C-terminal alanine amide to monitor the isolation with a chemical detection method [12] in which the C-terminal α -amide is cleaved off, isolated and detected. Galanin is a 29-residue bioactive neuroendocrine peptide (in human it has 30 residues). Galanin is widely distributed in endocrine tissues and in the central and peripheral nervous systems [36, 37]. Functionally, galanin modulates endocrine, neural and metabolic systems, and has a widespread cellular distribution [38]. However, the general role of galanin in physiology is difficult to determine because of variability between species. For example, in many mammalian species, galanin inhibits pancreatic insulin secretion, but not in the human [39]. Moreover, galanin has been suggested to be involved in other neuronal functions, such as learning and memory, epileptic activity, nociception, spinal reflexes and feeding [40]. A novel galanin-like peptide (GALP) has been reported isolated from porcine hypothalamus. The peptide has 60 amino acid residues and a non-amidated C terminus. The amino acid sequence of GALP-(9-21) is completely identical to that of galanin(1-13). In contrast to galanin, GALP shows highest affinity for the GALR2 receptor, while galanin is rather non-selective [41].

PEC-60

PEC-60, a peptide (P) with N-terminal glutamic acid (E), C-terminal cysteine (C), is a 60-residue peptide originally isolated from pig intestine. It is structurally related to the pancreatic secretory trypsin inhibitor with 41% residue identities, but PEC-60 does not inhibit trypsin. The disulfide arrangement of these two peptides is identical, showing related overall conformations [42]. PEC-60 inhibits glucose-induced insulin secretion from perfused pancreas in a hormonal manner, and has biological activity also in the immune system. PEC-60-like immunoreactive material has been reported in catecholamine neurons of the central and peripheral nervous systems, but the peptide has not been identified from that material [43]. The similarity to the Kazal type family of proteinase inhibitors earlier suggested based on amino acid homology was confirmed by the NMR structure [44].

NK-lysin

NK-lysin is a 78-residue antimicrobial, basic peptide, with three intrachain disulfide bonds. It was initially purified from pig small intestine, and has a high anti-bacterial activity against *Escherichia coli* and *Bacillus megaterium* and moderate activity against *Acinetobacter calcoaceticus* and *Streptococcus pyogenes* [45]. The peptide shows a marked lytic activity against an NK-sensitive mouse tumor cell line, YAC-1, but does not lyse red blood cells. NK-lysin belongs to the family of saposin-like proteins of pore-forming polypeptides [46]. The amino acid sequence of NK-lysin exhibits 33% identity with a putative human preproprotein, NKG5, derived from a cDNA clone of activated NK cells. NKG5 has been shown to stimulate mitogenicity of endothelial cells and may be involved in angiogenesis [47].

PR-39

A proline arginine rich peptide (PR-39), isolated from pig intestine, is an antibacterial peptide with high activity against both *E. coli* and *Bacillus megaterium*. PR-39 has a very high content of proline (49 mol%) and arginine (26 mol%) [48]. PR-39 is suggested to kill bacteria by a mechanism that stops protein and DNA synthesis leading to degradation of these components [49]. PR-39, has several activities beyond its function of killing bacteria - it alters macrophage viability by inhibiting apoptosis, which was induced by nutrient depletion, LPS stimulation or camptothecin treatment. These findings suggest that PR-39, a porcine neutrophil-derived antimicrobial peptide, might function in the inflammatory milieu, not only to kill bacteria, but also to aid in modulating the viability of inflammatory cells by regulating apoptosis [50]. PR-39 is a member of the cathelicidin family of antimicrobial peptides characterized by conserved pro-peptide sequences that have been identified in several mammalian species [51].

PYY

Peptide YY (PYY) is a 36-residue peptide originally isolated from porcine upper small intestine using a chemical method to detect the C-terminal Tyr-amide structure [12]. PYY is related to the pancreatic polypeptide and to neurotensin, it exhibits biological activities and is present not only in the intestine, but also in the brain [12, 52]. PYY level is changed in several gastrointestinal disorders [53]. Endocrine cells that express PYY occur all along the gastrointestinal tract. Limited expression of PYY is found also in endocrine cells in the adrenal gland, respiratory tract and pituitary [54]. PYY released postprandially from the ileum and colon displays a potent inhibition of cephalic and gastric phases of gastric acid secretion through both central and peripheral mechanisms. In the gastric mucosa, PYY binds to Y1 receptors in the enterochromaffin-like cells to inhibit gastrin-stimulated histamine release and calcium signaling via a pertussis toxin-sensitive pathway [55]. PYY was early reported to stimulate eating behavior [56], as well as in later reviews, regarding neuropeptides having a role in anorexia and bulimia [57].

1.1.5 Saponins

Saponins are naturally occurring glycosides, mainly produced by plants, but also by lower marine animals and some bacteria [58]. Saponins have either a complex terpenoid or a steroid structure with sugars attached. Their structural diversity is very broad due to the large number of substituent functional groups and sugar moieties. Saponins that occur in plants are characterized by the property of producing a soapy lather. Moisture-absorbing amorphous saponin mixture can be used as a foaming and emulsifying agent and detergent. These

biochemicals have commercial applications such as separation in industrial and mining operations, and are useful in products such as photographic emulsions, cosmetics, and shampoos. When a saponin is digested, it yields sugars and a sapogenin aglycone. Many glucosides are toxic and can for example speed up hemoglobin degradation, but the saponin of ginseng is relatively safe.

Plant extracts have been used in traditional medicine since ancient times for treatment of various diseases including a disease similar to type 2 diabetes [59, 60]. In folklore, a variety of plant extracts have been used as oral hypoglycemic agents to treat diabetic patients [61]. Several biological effects have been ascribed to saponins such as anticarcinogenic, neurotrophic, immunostimulant, and hypoglycemic. They have also been found to considerably affect feed intake, reproduction and growth in animals [62]. Saponins have been found in wine, which potentially could provide an explanation for why wine drinking can benefit cardiovascular health. The compounds, are found in the waxy skins of grapes, but not in the juice [63]. Digoxin is a saponin derived from an extract of the common garden plant Foxglove [64]. A role of some saponins as protectants from phytopathogen infection and insect predation suggests that they may have fungicidal and insecticidal roles in nature [65].

Gynostemma pentaphyllum

G. pentaphyllum is a perennial liana that grows in south western part of mainland China, Taiwan, Korea, Vietnam and Indonesia. Saponins from *G. pentaphyllum* are called gypenosides. Extracts of *G. pentaphyllum* are used in treatment of inflammation, phlegm and cough, hypertension, liver diseases, headache, neuralgia, lower back pain, diabetes, kidney diseases, ulcer of stomach and duodenum, bronchitis, allergic dermatitis, insomnia, constipation, gall bladder stones, etc. Furthermore, water extracts of *G. pentaphyllum* and *G. lucidum* were found to possess significant anti-inflammatory activity against carrageenan induced edema [66]. Crude saponin fractions of *Panax ginseng* and *G. pentaphyllum* have similar degree of effects on hyperglycemia and hyperlipidemia [67]. *G. pentaphyllum* contains numerous saponins and 82 of them were identified in 1988 [68]. Some gypenosides are found in other plants such as ginseng [69].

2 AIMS OF THE PRESENT INVESTIGATION

The aim of this thesis is to isolate novel regulatory peptides, hormones, neurotransmitters and bioactive compounds from animal tissue and herbal extracts, to identify them, and to characterize their structures. This is achieved through development of different purification methods, guided by *in vitro* tests such as chemical assays for specific structures, insulin-release, cAMP production, antibacterial or radioimmunoassays and mass spectrometry.

3 METHODS

3.1 Extraction of animal tissues

Boiled, and then immediately frozen, pig upper intestine is obtained in deep-frozen state from slaughterhouses. The intestinal material is minced while still frozen, then extracted with

about two parts by weight of 0.5 M acetic acid, during constant stirring for about 20 h at a temperature not exceeding 10°C, and filtered. The pH of the solution is adjusted to 2.5-2.8 with 2M HCl, alginic acid is added to the filtrate, and the mixture is stirred for 30 min. The alginic acid carrying the adsorbed peptides is collected by suction filtration and washed on the filter with, in turn, 0.005 M HCl, ethanol (to remove fats), and again 0.005 M HCl. Elution is performed by repeatedly percolating small volumes of ice-cold 0.2 M HCl through the alginic acid pad on the filter. The pH of the eluate is adjusted to 3.0-3.5 by addition of solid sodium acetate. Finally, after, the peptides are precipitated from the eluate by addition of 320 g NaCl/l. The precipitate is collected on a suction filter. The yield of this salt cake, CTIP (Concentrate of Thermostable Intestinal Peptides) is about 1 g/kg boiled intestine.

Extraction with methanol (50 ml methanol/g peptide) is used in some cases. In paper I the methanol insoluble fraction is used, in paper V the methanol soluble fraction and in paper III the acid methanol soluble neutral insoluble fraction (AMESNI) is used.

Chicken intestine is extracted essentially in the same manner as pig intestine.

Pig brains are obtained in deep-frozen state from slaughterhouses. Brains are minced and boiled for about 10 min, then cooled with ice and extracted in the same manner as the intestines, but when alginic acid is filtered off, the solution is collected. After filtration, 1 kg of Amberlite XAD 16 HP (Supelco) for each 40 l of liquid is added. The preparation is stirred for 45 min and allowed to sediment for 30 min. The liquid is decanted and Amberlite collected on a filter. Amberlite is washed with water (10 l) and the material adsorbed is eluted with 2 l of 45% isopropanol/water. After removal of the solvent, the yield from 50 kg of brains is 4.5 g crude peptide.

Spleens are washed and cleaned in saline, cut into small pieces and frozen. One kg of spleens from 20 pigs is boiled for 10 min, cooled with ice and extracted in 10 vol of 3% acetic acid overnight. The extract is filtered and peptides are captured with alginic acid from the filtrate [45]. One-third of the material is applied to DEAE-Sephadex, eluted with 0.2 M acetic acid, and lyophilized.

3.2 Extraction of plant tissues

The leaves and the stems of *G. pentaphyllum* are dried and pounded into powder. The powder is extracted with 70% ethanol in a Soxhlet extractor for 4 cycles. Ethanol is evaporated under reduced pressure to give a crude extract. 30 g of the crude extract is dissolved in 400 ml water and centrifuged for 20 min at 3000g. The supernatant is collected, diluted with 400 ml water, and extracted with 800 ml n-butanol, using a magnetic stirrer for 30 min. Water and n-butanol are allowed to separate for 30 min in a separating funnel. The butanol phase is collected and the solvent is evaporated until most of the butanol is removed. The residue from the butanol extraction is diluted with 2 l of water. Portions of 500 ml are mixed with 200 g Amberlite XAD 16 HP, and stirred for 30 min. The unabsorbed material is removed by filtration and the Amberlite is collected and washed with water. The adsorbed material is eluted from the Amberlite with aqueous acetonitrile at different concentrations (25%, 60% and 80% acetonitrile).

3.3 Chromatography

3.3.1 Animal extract

The salt cake is dissolved to a 10% solution in 0.2 M acetic acid and chromatographed in this solvent on a Sephadex G-25 column. The fractions are eluted with 0.2 M acetic acid and saturated with NaCl. The precipitate is collected by suction filtration and stored. Cation exchange chromatography is performed on CM-cellulose in paper I, II and V or on Resource S column in paper III and IV. The final steps are carried out on HPLC using cation exchange and reverse phase (RP) C18 columns.

3.3.2 Plant extract

The Ambertlite 60% acetonitrile fraction is chromatographed on a C18 RP column, the compounds eluted with a linear gradient of acetonitrile. Active fractions are rechromatographed on a polyaromatic column. The final purification is achieved on a high resolution RP column.

3.4 Insulin release assays

Male Wistar rats, weighing 250-280 g, were from a commercial breeder (B&K Universal, Sollentuna, Sweden). The animals are kept at 22°C with free access to food and water before being sacrificed for isolation of pancreatic islets. Islets are isolated by collagenase (Roche) digestion of the pancreas, followed by hand-picking [70]. The islets are then cultured for 24 h in RPMI 1640 medium (Flow Lab Ltd., Irvine, U.K.) containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin.

Batch incubations of islets are performed in Krebs-Ringer bicarbonate (KRB) buffer containing 118.4 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄ and 25 mM NaHCO₃ (equilibrated with 5% CO₂/95% O₂, pH 7.4) and 0.2% bovine serum albumin, 10 mM HEPES, and 3.3 or 16.7 mM glucose. Following pre-incubation for 30 min in KRB at 3.3 mM glucose, batches of 3 islets are incubated for 60 min in KRB with 3.3 or 16.7 mM glucose, and different concentrations of the crude ethanol extracts or the purified gypenoside compound.

After batch incubations, aliquots of the medium are analyzed for insulin content by a radioimmunoassay (RIA) [71].

3.5 C-terminal ladder sequence analysis

C-terminal sequence analysis of peptides and proteins using carboxypeptidases (CPs) in combination with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is convenient for protein and peptide characterization [72]. After a short digestion, a

sequence up to 20 residues can be identified, but the total number depends on the individual sequence. Carboxypeptidase P (CPP) and carboxypeptidase Y (CPY) can be used to complement each other in releasing all amino acid residues because of their relatively low specificities. The C-terminal ladder sequence analysis method is well suited for characterization of Pro/Arg-rich peptides like, PR-39 in paper II.

3.6 Antibacterial assay

Antibacterial activity is measured by an inhibition zone assay on thin agarose plates seeded with *Escherichia coli* strain D21 or *Salmonella typhimurium* LT-2 [10]. A value of 1000 units is defined as an activity against *E. coli* D21 equal to that of 1 µg of cecropin A. The chromatographic fractions are lyophilised, and samples of each fraction are redissolved at 5–50 µg/µl in 10–50 µl water, of which 3 µl is used for each determination.

3.7 Radioimmunoassays

3.7.1 Galanin-RIA

A RIA is performed essentially as described [37, 73]. Lyophilised chromatographic fractions or aliquots are preincubated with the antiserum (R2-8305) in 0.6 ml assay buffer for 24–48 h, after which ¹²⁵I-galanin in 0.4 ml is added and the mixture is incubated for 22 h. The sensitivity of the assay for porcine galanin is 4 fmol/assay tube, and the calculated IC₅₀ value for galanin is 9.0±0.5 fmol/ml (mean ± SEM, n=3). Since the K_d for the interaction of the antibody with unknown galanin-immunoreactive components, and the slope of the dilution curve for such components are unknown, conversion of bound over free ratio (B/F) into pmol galanin has been avoided, and the values are expressed as arbitrary units. A decrease in the B/F-ratio suggests that one or several galanin-immunoreactive compounds are present, reducing the binding of ¹²⁵I-galanin.

3.7.2 PEC-60 RIA

Lyophilized samples are reconstituted in the assay buffer. PEC-60-like immunoreactive material is quantified by RIA using PEC-60 standard, antiserum and ¹²⁵I-labeled PEC-60. The assay is run in duplicates as follows: 100-µl standards (prepared in the same buffers as samples) and samples are incubated at 4 °C for 48 h with 100 µl antibody to give approximately 30% “zero binding”. Following this incubation, 100 µl labeled PEC-60 is added and the solution is incubated for an additional 24 h. Free and antibody-bound PEC-60 are separated by 50 µl Sac-Cel (anti-rabbit solid-phase second antibody-coated cellulose suspension; IDS, Bolton, UK). Samples are left for 30 min at room temperature, the reaction is then blocked with 1 ml distilled H₂O. After centrifugation at 3000 g for 20 min at 4 °C, the supernatants are decanted and the pellets are counted in a gamma counter. The detection limit of the assay was 0.15 ng/ml and the intra-assay coefficient of variation was 3%. PEC-60

standard, antiserum and ^{125}I -labeled PEC-60 were prepared as described previously [74]. PEC-60 standard was purified from pig intestine as described previously [42].

3.8 Enzymatic digestion

3.8.1 Chymotrypsin cleavage of galanin

Degradation with chymotrypsin is carried out for 2 h at 37°C in 1 % ammonium bicarbonate at a substrate to enzyme ratio of 25:1.

3.8.2 Tryptic cleavage and desalting

Trypsin cleavage of PYY is carried out in 0.1 M ammonium bicarbonate at 37°C using modified enzyme from Promega (0.2 µg). The peptides in the digest are either separated by RP-HPLC or desalted on a Poros R3 resin (Perseptive Biosystems), where the peptides are eluted from with 60% acetonitrile containing 1% acetic acid.

3.9 Mass spectrometry

3.9.1 PR-39

For C-terminal ladder sequence analysis, peptides are digested with a mixture of CPY and CPP and analyzed on a Finnigan Lasermat 2000 (Thermo Bioanalysis, UK) [72].

3.9.2 Phospho-PYY

Mass spectrometric data are acquired on a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (MS) (Micromass, Manchester, UK), a Voyager DE Pro (Perseptive Biosystems, Framingham, MA, USA), and a Finnigan Lasermat 2000 as described [75, 76].

3.9.3 PEC-60

PEC-60 is analysed on an Ettan ESI-ToF mass spectrometer (Amersham Biosciences) with an Ettan LC connected, using a reverse phase column (Xterra™ MS C18, 3.5µm, 3 x 50 mm) eluted with buffers A (0.1% acetic acid/water) and B (0.1% acetic acid/95% acetonitrile/water) in a gradient of 15-35% B in 12 column volumes at a flow rate of 0.6 ml/min. MS-spectra are collected at a rate of 1 spectrum/s in positive ion mode. Extracted ion chromatograms are created for 5+ (m/z 1368.36), 6+ (m/z 1140.47), 7+ (m/z 977.69) and 8+ (m/z 855.60) ions of PEC-60, the chromatograms are compared, and the patterns analyzed for peak distributions.

3.9.4 Galanin

Nano-ES spectra are recorded on a tandem mass spectrometer (Q-TOF-1, Micromass, Manchester, England) [77] fitted with an orthogonal-sampling nano-ES interface (Z-Spray, Micromass). Mass spectra are recorded at a resolution of 9000 (full width at half maximum height, FWHM, definition), which corresponds to approximately 3000 on the 10% valley scale. Tandem mass spectra are recorded by selecting the precursor isotopic envelope of interest with the quadrupole mass filter, focussing the selected ions into a hexapole collision cell containing argon gas and accelerating undissociated precursor and fragment ions into an orthogonally arranged TOF analyzer. The product ion resolution was 9000 (FWHM definition). The collision energy employed was 90–120 eV.

3.9.5 *Gynostemma pentaphyllum*

Positive-ion mass spectra are recorded on an Ettan ESI-ToF electrospray time-of-flight mass spectrometer (Amersham Biosciences, Uppsala, Sweden) using a capillary exit voltage of 150 V. Samples of chromatographic fractions are infused at 10–50 $\mu\text{l}/\text{min}$ via the ESI source using a syringe pump. For alkali metal-adduct measurements, a 50- μl aliquot of the chromatographic fraction is mixed with 2 μl of 5 μM LiCl, NaCl or KCl solution in water and sprayed into the mass spectrometer.

3.10 NMR

NMR spectra are recorded with a Bruker DMX-600 spectrometer equipped with a cryoprobe in DMSO- d_6 solution at 25°C. Chemical shifts are reported in ppm relative to residual solvent signal ($\delta(^1\text{H})$ 2.50 ppm, $\delta(^{13}\text{C})$ 39.5 ppm). Two-dimensional spectra recorded included DQF-COSY, ROESY, TOCSY, sensitivity-enhanced ^{13}C -HSQC, and ^{13}C - ^1H HMBC. Pulsed-field gradients are used for all ^{13}C correlation spectra. ^{13}C -HMBC spectra were recorded with coupling evolution delay for the generation of multiple-bond correlations set to 62.5 ms. All 2D spectra are run with a 4096x1024 points data matrix, giving $\tau_{2\text{max}}=250$ ms for the ^1H nucleus in the acquisition dimension and $\tau_{1\text{max}}=200$ ms for ^1H or $\tau_{1\text{max}}=50$ ms for ^{13}C for indirect dimension. Prior to Fourier transform the data matrix is zero-filled twice and a multiplication by shifted sine-bell window function is applied. For ^{13}C -HMBC, the magnitude spectra are calculated.

4 RESULTS AND DISCUSSION

4.1.1 Chicken galanin (Paper I)

All known mammalian galanins have a conserved N-terminal sequence with Gly-Trp constituting a characteristic part of the peptide. We therefore decided to screen for chicken galanin by using this dipeptide, easily released by chymotrypsin. Chicken galanin was isolated by monitoring for the N-terminal glycytryptophan, separated by thin-layer chromatography and visualized with Ehrlich reagent, p-dimethylamino-benzaldehyd. The starting material for the isolation was a methanol-insoluble side fraction from the isolation of

chicken secretin [78]. 300 mg starting material was chromatographed on a cation exchange column at pH 8.0, and finally on a C-18 HPLC column. Fractions from both steps were assayed for Gly-Trp, and a pure homogeneous fraction was found.

Determination of the amino acid sequence by Edman degradation showed that chicken galanin is not identical to any of the galanins known at that time. It has a unique residue at position 28, several variations in the C-terminal part, and a strictly conserved N-terminal part of probably functional significance. At that time only three other galanin structures were known, all from mammals. In 2003 thirteen structures were listed in Swiss-Prot (Figure 3). Recently, galanin from Japanese quail was also described and shown to be identical to chicken galanin [79].

		1	6	11	16	21	26
Pig		GWTLN	SAGYL	LGPHA	IDNHR	SFHDK	YGLA
Human					VG	S	N TS
Cow					L S	Q	H
Rat						S	H T
Mouse						S	H T
Sheep							H
Dog						E	P T
Chicken					V	N	H FT
Quail	<i>C. japonica</i>				V	N	H FT
Bowfin	<i>A. calva</i>				V	LN	H
Trout	<i>S. gairdneri</i>				G G	TLS	H
Frog	<i>R. ridibunda</i>					N	H
Alligator	<i>A. mississippiensis</i>					NE	H I

Figure 3. Primary structures of characterized galanins. Empty spaces indicate residue identities with the top row of porcine galanin sequence. All peptides except the human form are C-terminally amidated. Data from the Swiss-Prot data bank.

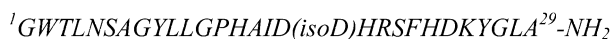
4.1.2 Galanin variant forms (Paper V)

We have isolated a series of variant forms of porcine galanin using chromatographic separations and radioimmunoassays. The modified variants include β -aspartyl shifted forms and oxidized forms containing Trp mono-oxide (Fig. 4). At least the β -aspartyl forms could be of native occurrence. The starting material for isolation of the galanin variants was a non-retained fraction from cation exchange obtained during purification of secretin. The non-retained material was used for further purification by HPLC. First, a reverse phase step on a C₁₈ column (Vydac, 22 mm I.D.) in 0.1% aqueous TFA was carried out with a gradient of acetonitrile. The active fraction was further purified on a cation exchange HPLC column in 0.2 M acetic acid, eluted with a gradient of 1.2 M ammonium acetate. The galanin immunoreactive material then obtained was finally chromatographed on a C₁₈ Vydac 4.6 mm I.D. column, either with 0.1% aqueous TFA, or 0.1% aqueous HFBA, and eluted with an acetonitrile gradient. By the use of tandem mass spectrometry the variants were identified as follows.

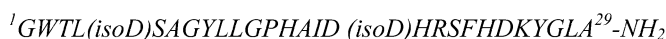
1) Galanin(isoD5), galanin with a β -aspartyl shift at position 5:



2) Galanin(isoD18), galanin with a β -aspartyl shift at position 18:



3) Galanin(isoD5, isoD18), galanin with two β -aspartyl shifts at positions 5 and 18:



4) Galanin(W2-Ox), galanin with oxidized Trp at position 2:



5) Galanin(3-29), galanin N-terminally truncated by two amino acid residues:

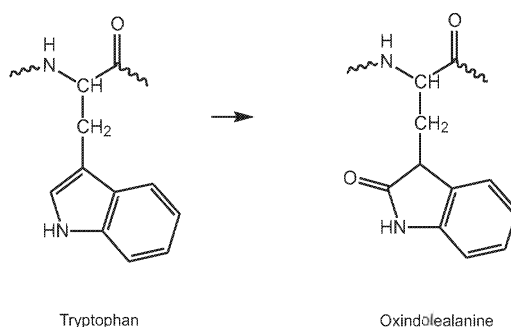


Figure 4. Tryptophan oxidation can lead to the formation of oxindolealanine [80].

4.1.3 NK-lysin and PR-39 (Paper II)

Normally, multicellular organisms live in harmony with microorganisms. Both animals and plants have potent, broad-spectrum antimicrobial peptides and many antibacterial peptides have been found in mammals [81]. α -defensin, β -defensin, PR-39, and NKL, are antimicrobial peptides and all have a net positive charge. In an attempt to characterize antibacterial peptides from pig spleen, an organ now shown to be rich in antibacterial peptides, we started with a heat-stable acid extract. Spleen is a secondary lymphoid organ and as such may contain a repertoire of antibacterial peptides from circulating cells. NKL_i (intestine), NKL_{bw} (bone marrow) (Fig. 5) and PR-39 were isolated by acetic acid extraction, ion exchange chromatography, and reverse-phase high-performance liquid chromatography.

By digesting with carboxypeptidases P and Y followed by C-terminal ladder sequence analysis with MALDI-MS, the structure of PR-39 was identified (Fig. 6).

NKL _i	<u>G</u> <u>Y</u> <u>F</u> CESCRKIIQKLEDMVGPQPNEDTVTQAAS <u>Q</u> VCD <u>K</u> LKILRG <u>L</u> CKKIMR <u>S</u> FLRRIS
NKL _{bw}	<u>G</u> <u>L</u> <u>I</u> CESCRKIIQKLEDMVGPQPNEDTVTQAAS <u>R</u> VCD <u>K</u> MKILRG <u>V</u> CKKIMR <u>T</u> FLRRIS
NKL _i	<u>W</u> DILTGKKPQAICVDIKICKE
NKL _{bw}	<u>K</u> DILTGKKPQAICVDIKICKE

Figure 5. Sequence alignment of NKL_i and NKL_{bw}. Nonidentical amino acids residues are bold and underlined.

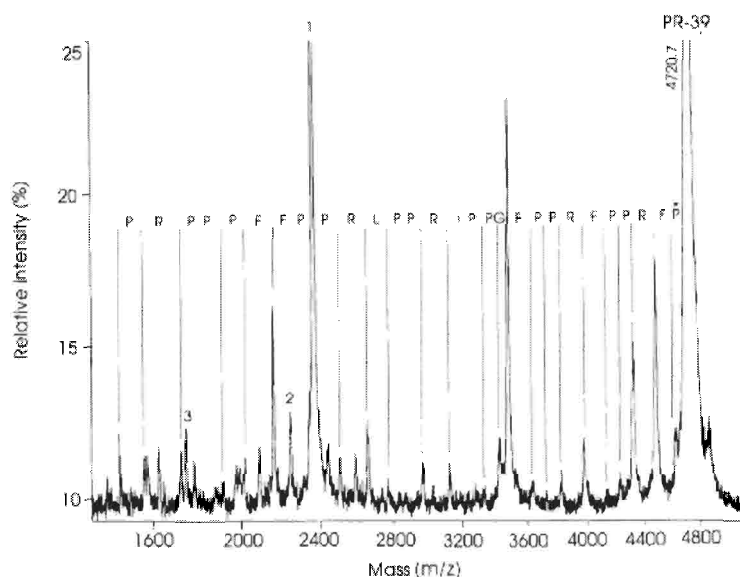


Figure 6. C-terminal ladder sequence analysis of PR-39 from porcine spleen. After 5 min incubation with CPY:CPP, the peptide digest was analyzed by MALDI-MS. P* indicates an amidated proline at the C-terminal position. 1, 2, and 3 are doubly-charged fragment ions.

4.1.4 Phospho-PYY (Paper III)

In this study, we have isolated a posttranslationally modified form of peptide YY (PYY) from pig intestine. The new form is phosphorylated at Ser-13, defining a class of gastrointestinal peptide hormones conjugated with a phosphate group. Phospho-PYY was isolated from pig intestine using several chromatographic steps including size-exclusion, reverse-phase and ion exchange chromatographies. The structure was determined after tryptic digestion by Edman degradation and ESI-MS/MS. Phosphorylation in PYY is probably catalyzed by a casein kinase, which recognizes the sequence -Ser/Thr-X-Glu/Asp-, where X is any amino acid. Comparison of PYY sequences from other species indicates that within PYY, the serine residue at position 13 is conserved throughout all characterized mammalian species (Fig. 7). This makes it possible that phosphorylation of Ser-13 also may occur in other species including humans.

PYY	pig	YPAKPEAPGEDA <u>S</u> PEELSRYYASLRHYLNLVTRQRY
NPY	pig	YPSKPDNPGEDA <u>P</u> AEDLARYYSALRHYINLITRQRY
PP	pig	APLEPVYFGDDA <u>T</u> PEQMAQYAAELRRYINMLTRPRY
PYY	human	YPIKPEAPGEDA <u>S</u> PEELNRYYSALRHYLNLVTRQRY
PYY	rat	YPAKPEAPGEDA <u>S</u> PEELSRYYASLRHYLNLVTRQRY
PYY	bovine	YPAKPQAPGEHA <u>S</u> PDELNRYYSALRHYLNLVTRQRF
PYY	chicken	AYPPKPESPGDAA <u>S</u> PEEIAQYFSALRHYINLVTRQRY

Figure 7. Alignment of the amino acid sequences of PYY from different species with those of porcine NPY and PP. Position 13 is highlighted by bold type and underlined.

To identify the modification and its position, the purified peptide was digested with trypsin. Analysis of the resulting peptides by MALDI-TOF MS indicated that the modified residue is in the N-terminal tryptic fragment, PYY (residues 1-19; YPAKPEAPGEDASPEELSR). This fragment was investigated using ES-MS/MS. Series of y- and b-type ions were detected and the y-ions following y_7^+ (at m/z 897.40) were found to be shifted. Interpretation of the product ion spectrum showed that the 80 Da modification, -HPO₃ (79.9663 Da) or -SO₃ (79.9568 Da) was located to Ser-13. To further ascertain the identity of the modification the peptide was analyzed in the negative ion mode. The doubly deprotonated parent ion (m/z 1059.95) was subjected to CID. The product ion spectrum showed an intense peak at 78.9664, characteristic of PO₃⁻, indicating the presence of a phosphate group in the peptide. From these results we conclude that the native PYY is phosphorylated at Ser-13.

4.1.5 PEC-60 from brain tissue (Paper IV)

We have isolated PEC-60 from pig and rat brains with a method that combines column purification procedures with the specificity of a radioimmunoassay (RIA) and the sensitivity of mass spectrometry to directly identify the peptide. The results show that PEC-60, like

many other peptides, is expressed in the gastrointestinal tract and the central nervous system (CNS). Furthermore, the distribution in brain regions was found to be different from that of other neuropeptides (neurotensin, CGRP, galanin, neurokinin A, substance P, somatostatin and neuropeptide Y). All these peptides usually have their highest concentration in the hypothalamus and lowest in the frontal cortex. In contrast, the PEC-60 concentration was highest in the frontal cortex and low in hypothalamus. The specific regional brain distribution and interaction with classical neurotransmitters raise the possibility that PEC-60 may play a role in CNS disorders, including dopamine dysregulation.

4.1.6 Phanoside, a novel insulin releasing compound (Paper VI)

Gynostemma pentaphyllum Makino (Cucurbitaceae), an East-Asian herb, has been reported to have numerous different activities, such as antitumor, cholesterol-lowering, immunopotentiating, antidiabetic and antioxidant effects. We have observed that a *G. pentaphyllum* extract has a hypoglycemic effect in rats. In the present study, we demonstrate that ethanol extracts of *G. pentaphyllum* strongly stimulate insulin secretion from isolated rat pancreatic islets. We have isolated a novel insulin-releasing substance from this extract, determined the structure of several active isomers by NMR and mass spectrometry, and characterized the effects on insulin release. Purification and investigation of the structures of active substances were accomplished as follows. The plants were collected in the mountains in Vietnam, dried in sunlight before pounding into powder. The powder was extracted in 70% ethanol in a Soxhlet extractor for 4 cycles. Ethanol was evaporated under low pressure. The crude extract was dissolved in water, extracted with butanol, adsorbed to a reverse phase resin and eluted with acetonitrile/water. All steps were monitored by an insulin release assay. The activity was finally purified by three reverse phase HPLC steps. We isolated the active compound and demonstrated by NMR and mass spectrometry that it is a novel saponin with a molecular mass of 914.5 Da. We name it phanoside, (21-,23-epoxy-,3 β -,20-,21-trihydroxydammar-24-ene-3-O- $\{[\alpha$ -D-rhamnopyranosyl(1 \rightarrow 2)]- $[\beta$ -D-glycopyranosyl(1 \rightarrow 3)]- β -D-lyxopyranoside (Fig. 8). The compound has a strong insulin-releasing activity in isolated pancreatic islets.

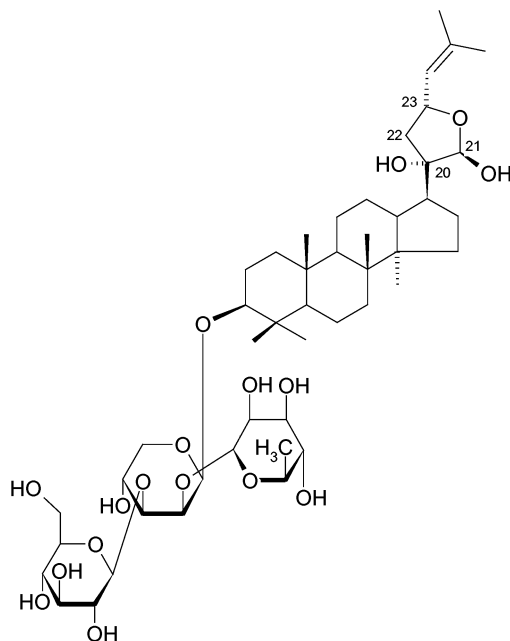


Figure 8. Structure of the novel saponin, phanoside.

5 CONCLUSIONS

Bioactive peptides have often been discovered by monitoring of their biological activity. Examples are classical peptide hormones, like insulin and secretin. A route to localize a novel peptide to cells or organs is antibodies. Immunohistochemical investigations with antibodies and RIA may indicate a new localization and function. In this thesis, the bioactive peptide, PEC-60, initially isolated from intestine is now identified in brain tissue from pig and rat.

New forms of the neuroendocrine peptide galanin were isolated and characterized, one from a new species (chicken), and five posttranslationally modified porcine forms.

A novel NK-lysin variant from porcine spleen was isolated using an antibacterial assay.

The finding of a phosphorylated PYY was unexpected, and clearly demonstrates the value of peptide isolation. Given the slightly lower receptor binding potency and weaker inhibitory activity of cAMP production of the phosphorylated PYY than of the non-phosphorylated PYY, phosphorylation may imply a fine regulation pathway of PYY activity.

Diabetes is a growing problem all over the world. Therefore new drugs and treatments for this disease are important. Control of diabetes is usually achieved by diet, physical exercise oral hypoglycemics and insulin. Isolation of the new saponin, phanoside, may lead to new possibilities how hyperglycemia can be regulated.

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