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Proanthocyanidins: Oligomeric Structures with Unique Biochemical Properties and Great Therapeutic Promise

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Proanthocyanidins represent a unique class of oligomeric and polymeric secondary metabolites found ubiquitously and in considerable amounts in plants and some algae. These substances exhibit a range of rather surprising physical and chemical properties which, once applied to living organisms, are translated into a multitude of biological activities. The latter include antioxidant properties, cancer chemoprevention, anti-inflammatory and anti-diabetic effects as well as some exceptional, yet highly interesting activities, such as anti-nutritional and antimicrobial activity. Despite the wide range of activities and possible medical/agricultural applications of proanthocyanidins, many questions still remain, including issues related to bioavailability, metabolism and the precise biochemical, extra- and intracellular targets and mode(s) of action of these highly potent materials. Among the various physical and chemical interactions of such substances, strong binding to proteins appears to form the basis of many of their biological activities. Once easy-to-use synthetic methods to produce appropriate quantities of pure proanthocyanidins are available, it will be possible to identify the prime biological targets of these oligomers, study oligomer-protein interactions in more detail and develop possible practical applications in medicine and agriculture.

Keywords: Antimicrobial activity, Antinutritional effects, Antioxidants, Enzyme inhibition, Metal chelation, Proanthocyanidins.

Proanthocyanidins form a unique class of high-molecular weight oligomeric and polymeric secondary metabolites which are present in many fruits, nuts and berries, and also found in cocoa, tea, wine and beer. Despite their limited bioavailability these macromolecular substances exhibit a wide range of sometimes stunning biological activities in the human body, including chemopreventive, vasorelaxing [1], antioxidative [2-4], antiproliferative/anticancer [5], antiadhesive [5], antimicrobial and even anti-nutritional effects (Figure 1).

Not surprisingly, proanthocyanidins have recently experienced a certain renaissance in nutritional and pharmaceutical research. During the last decade, a number of research articles have addressed the biosynthesis, chemistry, biochemistry and biological activities of these exceptional substances, and there are several excellent reviews now available on this matter [6-8]. Nonetheless, many chemical, biochemical and biological aspects surrounding proanthocyanidins are still unknown. The precise biosynthetic pathway leading to these oligomeric agents is still not fully understood [9], an effective and easy-to-conduct chemical synthesis of proanthocyanidins is just a wishful dream [10-15], and there are still several fundamental questions regarding the bioavailability, metabolic transformations, intra- and extracellular targets and modes of biological action of these agents [16-17].

From a chemical point of view, one particular property of the proanthocyanidins stands out, namely their ability to bind rather strongly to proteins. These interactions, which are primarily due to hydrogen bonding, van der Waals and electrostatic interactions, but may also include covalent bond formation, influence the function of the proteins and activity of the enzymes affected [18]. Indeed, it is likely that complex formation between proanthocyanidins and proteins is responsible for most of the biological activities associated with these bio-oligomers. Notably, this property of the proanthocyanidins is only emerging when the oligomeric state encompasses 4 or 5 condensed monomers, and often 'disappears' again at chain lengths above 10 or 11, because most of such polymeric materials are no longer soluble in aqueous media [19].

Before turning our attention to the specific nature and details of these oligomer-protein interactions, we will first consider the general structure and properties of the proanthoyanidins: Basically, proanthocyanidins are oligomeric or even polymeric natural substances of a molecular weight ranging from around 500 to over 3000, which are formally based on simple flavan or flavanol monomers, such as (+)catechin or (-)epicatechin. The name 'proanthocyanidin' reflects the fact that these oligomeric structures can be *broken down* chemically to form intensively colored anthocyanidins, *i.e.* proanthocyanidins may be considered as precursors of anthocyanidins (note: the proanthocyanidin oligomers are *not* based on anthocyanidin monomers).

The precise biosynthetic pathway of the proanthocyanidins is still shrouded in mystery: The crucial condensation step involving cross-linking of monomers and formation of new carbon-carbon bonds is often described as a black box – possibly involving flavanols and leucoanthocyanidins [6]. At the same time, the *chemical* synthesis of these oligomeric structures is extremely difficult and complicated further by the fact that oligomer formation involves the creation of additional chiral centers (see later on). Indeed, most studies to date have focused on dimers, trimers and tetramers, with hardly any easy-to-conduct synthetic procedures for petameric or even higher forms reported in the literature. Most of the synthetic procedures available to date are cumbersome and employ protected and activated flavanols which are coupled to flavan-3,4-diols (leucoanthocyanidins). The lack of straightforward synthetic methods for higher oligomeric agents is extremely unfortunate, however, since the ability of proanthocyanidins to form complexes with proteins and hence exert biological activity in most cases only begins at the tetrameric or pentameric state.

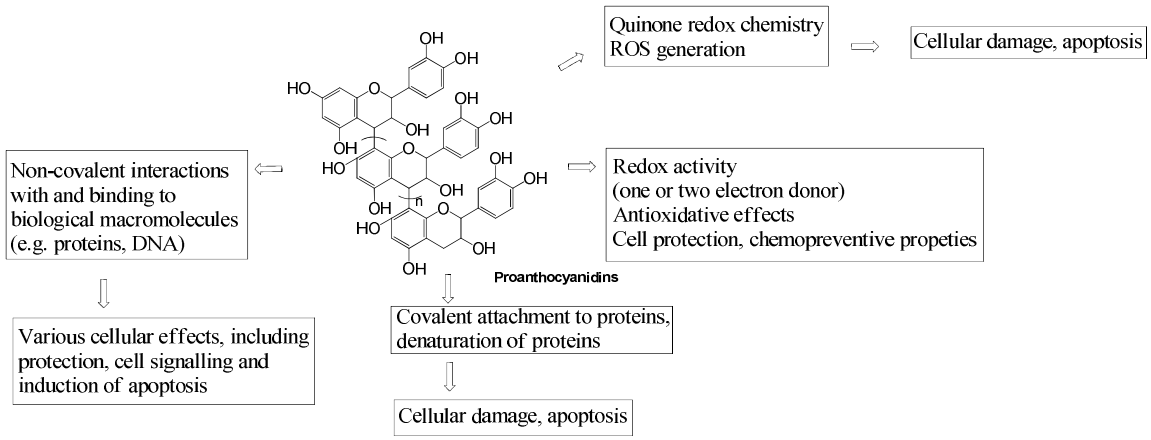

Figure 1: Basic chemical structure of proanthocyanins and several of the biological effects known to be associated with these substances.

Figure 1 illustrates the general molecular 'design' of some of the most commonly encountered proanthocyanidins. The flavanol monomers are linked via carbon-carbon bonds, primarily between the C4 and C6 positions or the C4 and C8 positions (B-type proanthocyanidins). Some plants contain proanthocyanidins whose monomers are linked further by an ether bond between C2 and C7. These A-type proanthocyanidins, which occur, for instance, in cranberries and peanut skins, exhibit particular biological activities which are not found in the B-type substances [20].

Regardless of the variation in monomers, proanthocyanidins exhibit common physical and chemical properties which are due to the extensive presence of hydroxyl-groups in these oligomeric structures: Here, each A-ring and B-ring (nomenclature of the monomeric unit) contributes two (or even three) OH-groups, which implies that a given octamer with a molecular weight of around 2300 contains at least 32 OH-groups. These groups are available for extensive electrostatic and hydrogen bonding. At the same time, the presence of several hydroquinone moieties (B-ring) enables proanthocyanidins to donate many electrons (at least 16 in the case of the octamer) and hence act as very potent antioxidants.[1]

The differences between the proanthocyanidin oligomers and the corresponding monomers, however, are not just 'additive'. While (epi-)catechin monomers can also donate electrons and act as antioxidants, they lack certain properties, such as strong binding to proteins, which only emerge at a certain oligomeric state (usually from the tetramer upward). Oligomers therefore are *qualitatively* different from the corresponding monomers - and also differ from the polymers with 11 or more units. One of these emergent properties associated solely with the oligomers, *i.e.* reduced bioavailability, actually has long counted against more extensive studies of this high molecular weight material in the context of human health. While the bioavailability of oligomers is obviously lower than the one of most monomers, recent studies, however, have confirmed that the oligomeric materials are still sufficiently water-soluble and also seem to cross from the gastrointestinal tract (GIT) into the blood [20]. This distinguishes such oligomeric substances from polymeric, water-insoluble and non-bioavailable forms, such as certain high-molecular weight *tannins*.[2] Indeed, analysis of proanthocyanidins in tissues (and blood) is difficult and may be hampered by the fact that such oligomeric substances associate easily with proteins and enzymes. It is therefore feasible (yet difficult to prove) that the proanthocyanidin content of such biological samples is frequently underestimated.

Importantly, research during the last five years has demonstrated that the classical kind of 'bioavailability', *i.e.* the transfer from the GIT into the blood stream, may not be essential for the biological activity of proanthocyanidins. We now know that (passage through) the GIT provides plenty of interesting biological targets for biologically active substances, without the need to enter the inner, sacred parts of the human body at all. Agents passing through the GIT may, for instance, affect the bacteria in the mouth or stomach, influence the composition of the colonic bacterial flora, affect adhesion of bacteria to the linings of the GIT, protect against toxins in the food (*e.g.* by chemically neutralizing carcinogens, such as azoxymethane or dimethylhydrazine [21]) and modulate the activity of enzymes involved in digestion (and uptake) of nutritional components. Furthermore, tumors present in the GIT, especially in the colon, can also be targeted by compounds passing though the GIT, yet without necessarily entering the bloodstream. Indeed, proanthocyanidins have shown antiproliferative, antiadhesive, antimetastasic and pro-apoptotic activities which may be of importance for cancer preventive and anticancer activity.

Interestingly, most of the beneficial, chemopreventive and, in part, therapeutic properties associated with proanthocyanidins appear to be due to effects on proteins and enzymes, *i.e.* interactions which actually may be considered the physico-chemical hallmark of these oligomeric substances. The effects proanthocyanidins exert on proteins and enzymes in the GIT can even be experienced in daily life: Astringency is a property associated closely with proanthocyanidin oligomers and tannins, and which is a direct result of binding to - and subsequently denaturing – of proteins and

[1] Some authors refer to proanthocyanidins as 'polyphenols'. From a chemical, redox point of view, this name is slightly misleading: The key chemical moiety of the proanthocyanidins responsible for widespread antioxidant activity is a *hydroquinone* and not a phenol, *i.e.* ring B. While *ortho*- and *para*-hydroquinones are redox active and undergo reversible electron transfer reactions under physiological conditions (and hence may act as antioxidants), phenols generally do not undergo reversible electron transfer in aqueous media and are often rather poor antioxidants (*e.g.* tyrosine).

[2] As with referring to proanthocyanidins as 'polyphenols', assigning proanthocyanidins to the tannins is also somewhat unfortunate. Tannins include water-soluble and insoluble (highly polymeric) materials from various biological sources (berries, grape seeds, barks) and with a wide spectrum of biological functions and properties. Some tannins can be hydrolyzed (*e.g.* gallic acid esters), while others are 'condensed' (*e.g.* proanthocyanidins). The concept of 'tannins' clearly refers to a rather diffuse group of natural products; it lacks precision and embraces substances which often have very little in common.

enzymes in the mouth. [1] This property, which spices up our food and beverages with a distinct taste, is notably absent in the corresponding monomers.

Nonetheless, proanthocyanidins do not just bind to and subsequently denature a few proteins in the oral cavity. They also seem to attack bacteria rather effectively, and there is a rapidly growing number of reports describing a wide range of biological activities associated with these oligomeric substances, ranging from antimicrobial and antioxidant activities to chemopreventive, anticancer and antinutritional aspects. We will now take a closer look at these reported activities by following the proanthocyanidins on their passage through the human body. In doing so, we will once more underline the fact that proanthocyanidins do not have to enter the blood stream in order to exert effects which are highly beneficial to human health.

These beneficial effects begin already in the mouth. Besides a distinct effect on our taste buds, there are various reports describing antibacterial activities associated with different proanthocyanidins. Loehr *et al.* [22], for instance, have reported a rather interesting and thoroughly useful activity of a polyphenolic extract from the South African plant *Myrothamnus flabellifolia* Welw. against the bacterium *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium colonizing in the gingival sulcus, which contributes considerably to the progression of periodontitis [22]. The use of *M. flabellifolia* against periodontitis has an ethnopharmacological background and, as Loehr and colleagues could demonstrate, the plant extract (which is rich in dimeric and trimeric proanthocyanidins) at just 100 μg mL^{-1} inhibits adhesion/invasion of *P. gingivalis* by about 50%. Interestingly, the extract does not appear to kill the bacterial cells; it rather inhibits agglutination and gingipain enzymes and affects the gene expression of major adhesins. The extract also appears to exert some anti-inflammatory and cytoprotective effects on KB (Eagle) cells [23]. Together, these properties turn the *M. flabellifolia* extract into a rather interesting natural remedy against periodontal diseases. They also stimulate the search for similar, biologically active proanthocyanidin-rich preparations, perhaps from various seeds (*e.g.* grapes), hulls, barks or indeed from the skins of certain berries.

At the same time, the antibacterial activity of proanthocyanidins is not limited to *P. gingivalis*. There is evidence that such extracts especially, but not exclusively, also act against Gram-positive bacteria. In this context, Kylli *et al*. [24] have recently investigated proanthocyanidins from lingonberry (*Vaccinium vitis-idaea*) and the European cranberry (*Vaccinium microcarpon*). While dimeric and trimeric compounds showed little activity, oligomeric (medium degree of polymerization between 4 and 10) and polymeric (>10) materials exhibited a range of interesting biological activities, including antioxidant and anti-inflammatory effects. Notably, some of these substances (at 1 mg mL^{-1}) were also active against *Staphylococcus aureus*, confirming earlier reports of a general susceptibility of Gram-positive bacteria to proanthocyanidins. Mayer *et. al.* [25], for instance, have found that grape seed extract, which is an extraordinarily cheap and easy to obtain material rich in various flavonoids, oligo- and polymeric proanthocyanidins, is active against 10 different pathogens, including *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (VRE) (ATCC 19433), *Streptococcus pyogenes* (DSMZ 20565), *Haemophilus influenzae* (DSMZ 4690), *Enterococcus casilliflavus* (DSMZ 20680) and *Pneumococcus* (DSMZ 20566). The whole extent of antibacterial activity of the many different proanthoycanidins and proanthocyanidin-containing preparations is still unknown. At the same time, the (bio-) chemical causes of antibacterial activity are poorly understood, but may include iron depletion, inhibition of cell-associated proteolysis or inhibition of cell wall synthesis [24].

Here, one particular mode of action, *i.e.* inhibition of adhesion to target cells, forms a recurrent theme in the literature. This is rather interesting, since proanthocyanidins, because of their ability to interact strongly with proteins, may be almost 'destined' to attach to (and possibly denature) proteins and enzymes at (cell) surfaces. Such a mode of action would also be possible for high molecular weight substances which otherwise may be unable to enter the cell in order to exert their activity within. In 2004, Schmidt *et al.* [5] extracted several C4-C8-linked oligomeric proanthocyanidins (average degree of polymerization from 3.25 to 5.65) from wild blueberry (*Vaccinium angustifolium* Ait.). These compounds, at a concentration of around 75 μg mL^{-1}, inhibited adhesion of uropathogenic *E. coli* cells (isolated from the urine of patients diagnosed with urinary tract infections) to human red blood cells (HRBC, A_1, Rh+) *in vitro*. How these oligomeric agents act on *E. coli*, and if they actually reach the urinary tract unchanged in order to act there against *E. coli* infections, is still not entirely clear. Interestingly, the blueberry-derived fraction with an average degree of polymerisation of 5.65 also showed considerable antiproliferative activity against cultured human prostate and mouse liver cancer cells at around 15 μg mL^{-1}.

Inhibition of attachment is also an important theme in the field of antiviral research. Here, effects on the *surface* of the viral particle indeed are most likely. In 2002, Shahat *et al.* [26] isolated and characterized various *O*-glycosidic flavonoids, procyanidins (oligomeric precursors of cyanidin) and C4-C8-linked tetrameric and pentameric proanthocyanidins from *Crataegus sinaica* (hawthorn). Most of these compounds exhibit antioxidant activity, but are also active against herpes simplex virus (HSV-1), probably by a - not specified - extracellular antiviral mechanism. More recently, Kuhn *et al.* [27] have reported that oligomeric proanthocyanidins extracted from sorrel (*Rumex acetosa* L). inhibit the attachment of HSV-1 to Vero cells with an IC_{50} of just 0.8 μg mL^{-1}. The authors were able to fractionate their proanthocyanidin extract and identify several flavan-3-ols, dimeric proanthocyanidins and gallic acids as the main constituents. Interestingly, substances with galloylation at position O3 are highly potent, and appear to block attachment of the virus to the cell surface, probably by directly interacting with and cross-linking of viral envelope proteins (*e.g.* glycoprotein D). From a chemical point of view, such *cross-linking of proteins* by galloylated proanthocyanidin dimers, trimers and oligomers is indeed possible, since the gallic acid residues are not only redox active, but are also able to participate in Michael addition reactions and imine formation with amine groups of proteins and enzymes.

Figure 2, albeit speculative at this time, illustrates such covalent interactions, which rely on (the oxidized form of) ring B or gallate groups attached to the proanthocyanidin 'core'. It should be noted that this type of cross-linking of proteins does not require large oligomeric proanthocyanidin chains. Furthermore, covalent cross-linking enables proanthocyanidins to interact with proteins without the need of hydrogen bonding, electrostatic or van der Waals interactions. Intriguingly, most of these interactions require prior

[1] Tannins are, of course, also used in the traditional method of tanning, *i.e.* processing of animal skins to turn them into leather by denaturing the proteins in the skin.

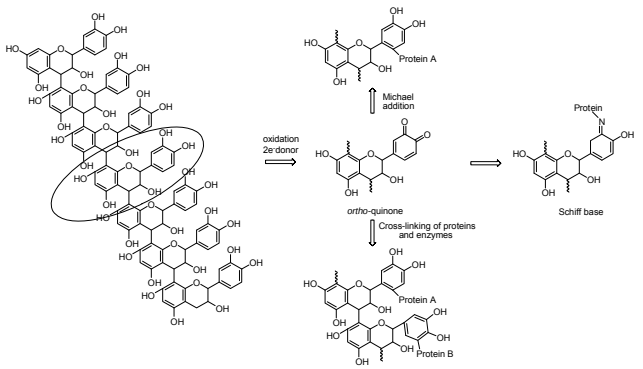

Figure 2: The chemistry behind covalent interactions of (oxidized) proanthocyanidins with proteins, ultimately resulting in protein-protein cross-linking. Such interactions include (several) Michael additions at various B-rings as well as Schiff base formation. Cysteine and lysine residues ((but also certain DNA bases) form the prime targets of this type of chemistry. Please note that most of these reactions only occur once the B-ring has been oxidized, *i.e.* after an initial oxidative activation of the proanthocyanidin oligomer.

oxidation of (some of) the B-rings, *i.e.* there may be a redox 'activation' step for this particular proanthocyanidin activity and some selectivity for oxidizing environments.

Interestingly, several of the studies cited above have also noted distinct 'anticancer' effects associated with the proanthocyanidin preparations. These 'anticancer' activities range from chemoprevention of cancer formation (*e.g.* by chemically neutralizing carcinogenic agents) to antiproliferative effects, cell cycle arrest and induction of apoptosis. Indeed, there are numerous reports of such activities in the literature, some dating back several decades [28-31]. We will focus here on a few selected examples, mostly from cancers of the GIT, *i.e.* tumors which at least in theory could get in contact with the proanthocyanidins during their passage through the human body. In 2008, Pierini *et al.* [29] reported significant reduction of the viability of esophageal adenocarcinoma cells by procyanidins derived from cider apples.[1] Importantly, the inhibitory effect, which is linked to cell cycle arrest in G_0/G_1 and involves caspase-mediated apoptosis, is critically dependent on the degree of polymerization: While flavan-3-ol monomers and dimers had no effect on both cell lines, significant activity was observed for oligomers and polymers at a concentration of 60 μg mL^{-1}.

Further down the GIT, oligomeric grape seed proanthocyanidins have been found to induce apoptosis in the human colorectal cancer cell line SNU-C4. At a concentration of 100 μg mL^{-1}, these extracts cause morphological changes typical of apoptosis, furthermore decreased Bcl-2 mRNA expression, increased levels of Bax and increased levels and activity of caspase-3 [30]. Similarly, procyanidins obtained from apples at 80 μg mL^{-1} are active against cultured human colon adenocarcinoma SW480 cells [16]. Similar to the grape seed proanthocyanidins, these compounds also appear to trigger caspase-mediated apoptosis, this time by activating the TRAIL-death receptors. While the exact 'chemistry' of how the procyanidins activate the death receptor is still unknown, it appears that the mechanism is independent of lipid raft formation (or alteration of DR4/DR5 expression, caveolin distribution).

Antiproliferative and proapoptotic effects have also been observed in cultured androgen-responsive prostate cancer cells (LNCaP) for oligomeric proanthocyanidins derived from grape seed and maritime pine bark. These effects include G1 growth arrest, inhibition of expression of cyclin-dependent kinases and cyclins, stimulation of tumor suppressors p21 and p27 and favorable changes in the Bcl-2/Bax ratio. In these particular cells, the activity of the proanthocyanidins also has been associated with a downregulation of the androgen receptor [31].

Besides affecting the AR-receptor or directly interacting with the TRAIL-receptor or other surface proteins, a recent study by Chung *et al.* [32] has suggested that proanthocyanidins may induce apoptosis in certain cells via an alternative mechanism involving increased levels of oxidative stress. Proanthocyanidins obtained from hop at concentrations of 50 to 100 μg mL^{-1} induce apoptosis, carbonylation and cytoskeleton disorganization in cultured HT-29 human colorectal cancer cells. These processes are associated with a sharp increase of intracellular levels of reactive oxygen species (ROS), which is accompanied by widespread oxidative modifications of proteins and enzymes, including inhibition of protein disulfide isomerase and destruction of the actin cytoskeleton.

The link between increased levels of ROS and apoptosis is not new; indeed, ROS-induced or mediated apoptosis has been observed on many occasions, leading to the notion of a critical 'redox threshold', which exists in cancer cells [33,34]. Once intracellular ROS levels reach this threshold, the cancer cell affected may undergo apoptosis. Nonetheless, it is somewhat surprising that proanthocyanidins, which are generally considered as 'antioxidants', should be involved in processes generating ROS. Early indications that flavonoids commonly considered as 'antioxidants' may, under certain conditions, also exhibit a more 'sinister', pro-oxidant behavior, which in turn may lead to experimental artifacts, have been reported by Long *et al.* [35] in 2000. Wang *et al.* [33] have recently confirmed the 'redox link' that exists between proanthocyanidins, ROS and cell death. In this study, highly oligomeric procyanidins from areca (betel) nut (degree of polymerization between 2 and 10) induced (caspase-mediated) apoptosis of lymphocytes via the depletion of intracellular thiols (apoptosis was attenuated upon addition of *N*-acetyl-L-cysteine). Interestingly, pro-apoptotic activity in this study depended critically on the degree of polymerization, with the pentamer representing the 'smallest' oligomeric form exhibiting reasonable activity. In line with this degree of polymerization-activity relationship, E_{50} values decreased successively from the pentameric (56.27 μg mL^{-1}) to the decameric form (41.80 μg mL^{-1}).

As already mentioned, the precise chemical and biochemical events underlying the antiadhesive, antibacterial, antiviral and antiproliferative (often cytotoxic) activities associated with many proanthocyanidin oligomers are still largely unknown. In any case, because of the wide range of activities reported to date, a 'one compound - one target' scenario explaining such activities is highly unlikely. A recent study by García-Conesa *et al.* [36] has considered the effects of oligomeric procyanidins from cider apple (*Malus domestica*, variety Antoinette) on the gene expression in human umbilical vascular endothelial cells. This study provides a first insight into the extremely complex mechanisms which - at least in part - may explain the activity of proanthocyanidins in living (human) cells. While (-)epicatechin and the procyanidin dimer B2 were inactive, the oligomeric procyanidins increased the expression of several genes that inhibit cell proliferation and migration. It appears that *regulation of gene expression* may indeed explain some of the antiproliferative effects observed for proanthocyanidins, as well as inhibition of cell migration and angiogenesis and possibly also cell cycle arrest (at the G_0/G_1 phase).

[1] Note: Procyanidins form a specific group of proanthocyanidins.

Nonetheless, it remains unclear if such effects are due to direct interactions of proanthocyanidins with signaling cascades, regulator proteins (such as transcription factors) or possibly with DNA itself. The binding of such oligomeric natural products to DNA is a real possibility which has only insufficiently been studied in the past.[1] Interestingly, a report by Saito *et al.* [37] shows that synthetic, trimeric proanthocyanidins (but not their monomeric units, *i.e.* (-)epichatechin and (+)catechin) are effective inhibitors of mammalian DNA polymerase, with IC_{50} values around 100 nM. Topoisomerase enzymes represent another DNA-related target. Here, Fridrich *et al.* [38] have demonstrated that trimeric and tetrameric proanthocyanidins derived from grape seed extract at low micromolar concentrations are potent inhibitors of human topoisomerase I and II *in vitro*.

Besides proteins and enzymes involved in DNA replication and translation, there are, however, a range of other possible intracellular targets, including various kinases and NF-κB, which has led Nandakumar *et al.* [39] to review the "Multi-targeted prevention and therapy of cancer by proanthocyanidins". This overview of the many suspected intracellular targets underlines the complexity of proanthocyanidin activity, which ultimately turns research in this area into an almost frightening, endless endeavor. In order to disentangle these issues, we will therefore focus on targets which actually are likely to be reached by proanthocyanidins, and mechanisms which are specific and powerful enough to trigger *major* cellular responses. In doing so, we will encounter a few paradoxes and misconceptions, which need to be resolved along the way.

Redox activity, which is also associated with the flavonoid monomers, first comes to mind. Indeed, most authors consider proanthocyanidins as potent 'antioxidants', a view chemically supported - and, paradoxically, also challenged - by the presence of the *ortho*-hydroquinones (B rings) in these oligomers. Antioxidative properties ascribed to such substances have been considered in numerous assays, including radical-based redox assays. Here, many of the natural products discussed so far, such as grape seed extracts, wines and blueberry products, have been associated with significant 'oxygen radical absorbance capacity' (ORAC) [4]. The United States Department of Agriculture (USDA) has even compiled a report entitled "Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods" which is available from the department's website [40].

Not surprisingly, several studies have discussed the beneficial effects of proanthocyanidin antioxidant activity on various human disorders. In 1999, Rimbach *et al.* [41] reported that a French maritime pine bark extract rich in oligomeric procyanidins protects human endothelial cells against challenges by 3-morpholinosydnonimine and activated macrophages, and here especially against depletion of glutathione (GSH). Similar to the antiproliferative effects, antioxidant efficiency depends critically on the degree of polymerization, but also on galloylation and glycosylation. In a study by Plumb *et al.* [3], higher degrees of oligomerization (slightly) *decreased* antioxidant activity in the lipid phase of the test system, and, in the case of the tetramer, even in the aqueous phase. In 2008, Ortega *et al.* [1] reported the vasorelaxant activity of red wine, which is particularly pronounced for wines rich in catechins, oligomeric proanthocyanidins and anthocyanidin glycosides. This study supports the suspected cardioprotective properties of red wine (commonly known as the 'French paradox'), and confirms previous studies by Packer *et al.* [2], which have linked vasorelaxant activity to inhibition of angiotensin-converting enzyme (ACE) and modulation of nitric oxide metabolism.

When interpreting these studies, it is important that one bears in mind that a complex redox active system, such as an *ortho*-quinone-based oligomer, which in addition may also be (partially) galloylated, is not simply an electron donor (see above). It may undergo *redox cycling*, possibly reducing dioxygen to superoxide or hydrogen peroxide, which ultimately would result in a pro-oxidant, ROS generating activity. Furthermore, such quinones are also able to bind to metal ions rather strongly, hence indirect pro- or antioxidative effects via sequestration of adventitious metal ions or inhibition of metalloenzymes are also possible. In addition, hydroquinones are likely to interact with proteins and enzymes (*e.g.* via Michael addition, Figure 2), therefore possibly inhibiting enzymes involved in the generation of pro- or antioxidants. Not surprisingly, therefore, proanthocyanidins exhibit a highly complicated, sometimes paradox appearing behavior *in vitro* and *in vivo*, which may show facets of pro- and antioxidant activity, chemoprevention and induction of apoptosis as well as a range of other (activating or inhibiting) properties, depending on the location and cellular environment these compounds are ultimately 'placed in'.

Indeed, some reports on anticancer activity of proanthocyanidins (*e.g.* cell cycle arrest, induction of cell death), such as the studies by Schmidt *et al.* [5], which describe the effects of blueberry proanthocyanidins on human prostate and mouse liver cancer cells, or the investigations of Kozikowski *et al.* [42], which have used synthetic pentameric proanthocyanidins to attack several breast cancer cell lines *in vitro*, including MCF-7, SKBR-3, MDA 435, and MDA MB 231, are certainly interesting, yet pose the question if such proanthocyanidins are really able to reach these 'targets' in the human body. We will, therefore, take a more realistic stand and return to 'targets' which can be reached easily whilst passing though the (human) body.

After passing through the esophagus (see above) and possibly acting as antibacterial agents against *Helicobacter pylori*, the proanthocyanidins reach the duodenum, where they may inhibit the pancreatic protease trypsin. This protease is essential for the processing of food and subsequent uptake of protein-derived material into the bloodstream. In a recent study by Gonçalves *et al.* [43] using proanthocyanidin fractions from grape seeds, a clear relationship between the degree of polymerization and trypsin inhibition has emerged, whereby inhibition increases with the degree of polymerization (the fractions containing proanthocyanidins with an average degree of polymerization of five and more were most active). Since inhibition of trypsin, which occurs at rather modest proanthocyanidin concentrations of around 200 μg mL^{-1}, prevents the absorption of certain food constituents, certain proanthocyanidins may possess rather interesting *antinutritional properties* (see below).

Indeed, antinutritional activity may be the most plausible and in many ways the most likely activity exerted by proanthocyanidins in the human body. Because enzymes involved in the processing/digestion of food are excreted into the GIT, they are also prime targets of proanthocyanidins passing (largely unchanged) through the GIT. Intriguingly, a range of digestive enzymes has been identified whose activity is affected by oligomeric proanthocyanidins, including α-amylase and α-glucosidase as well

[1] Such an interaction would assume, however, that the oligomeric proanthocyanidins are able to penetrate into the cell and also into the nucleus. Interactions with RNA in the cytosol are an additional, perhaps even more likely possibility.

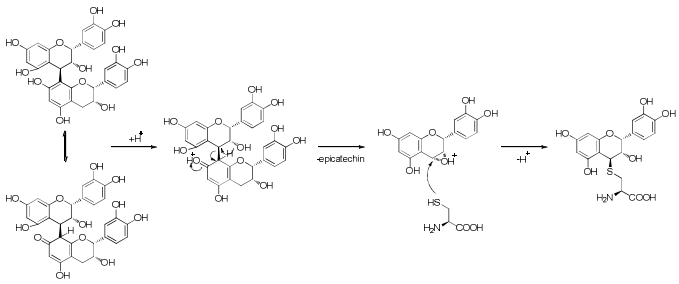

Figure 3: Oligomeric proanthocyanidins are sensitive toward cleavage by thiols. This specific chemistry is employed in thiolysis, an alternative to proanthocyanidin hydrolysis. Thiolysis is used, for instance, for the characterization of proanthocyanidins and also for the synthesis of semi-synthetic hybrid fragments.

as various proteases and lipases. The combined effect on all these enzymes may result in inhibition of starch, protein and lipid digestion, which in turn may be beneficial in the context of treating obesity and controlling type II diabetes [44,45]. There are also some reports that grape seed proanthocyanidins show certain antidiabetic effects, possibly via a specific insulinomimetic activity on insulin sensitive cells (and on glucose transporter 4 location) [46].

From a 'quasi-therapeutic' point of view, pancreatic lipase, an enzyme essential for triglyceride absorption, is of particular interest. This enzyme is secreted from the pancreas into the small intestine, where it hydrolyzes triglycerides into fatty acids and glycerol. Drugs like orlistat, which are used to treat obesity, inhibit lipase, prevent triglyceride uptake and ultimately cause weight loss. Oligomeric apple procyanidins seem to act in a similar way [47]. Here, inhibition of lipase depends critically on the degree of oligomerization. While (+)catechin and (-)epicatechin, as well as the dimer, show IC_{50} values above 125 μg mL^{-1}, lipase inhibition increases rapidly when moving to higher oligomers. From the pentamer onwards, IC_{50} values for the inhibition of lipase are around just 1 μg mL^{-1}, which is very competitive considering that these oligomeric substances occur in significant amounts of 20-30% in natural apple polyphenol extract. Indeed the apple extract itself inhibits lipase with an IC_{50} of 5.6 μg mL^{-1}, hence providing an interesting natural alternative to the prolonged use of orlistat and related drugs.

Binding of proanthocyanidins to pancreatic enzymes has recently been revisited by Brás *et al.* [18], who have investigated the binding of grape seed procyanidins to pancreatic elastase by experimental and computational methods. The results obtained as part of this study are highly revealing. They confirm the relationship between the (high) molecular weight of the proanthocyanidins, *i.e.* the degree of polymerization, and binding activity: Here, binding to elastase increases from the B3 dimer to the trimer, tetramer and ultimately the oligomeric fraction of the grape seed proanthocyanidins. Binding occurs by a static mechanism, and has some distinct effects on the structure and activity of elastase. The α-helix content, for instance, decreases upon binding, whilst the β-sheet content increases.

It should be pointed out that inhibition of elastase is not only interesting from a nutritional point of view: Related proteolytic enzymes are involved in major disease processes, including bacterial colonization, inflammation, angiogenesis, tumor invasion and the formation of metastases. Because of similarities between elastase on the one hand and related proteases on the other, the effects that proanthocyanidins exert on pancreatic elastase therefore may be reflected for other hydrolytic enzymes. Since some of them are involved in pathological processes, proanthocayanidin-based inhibition of proteases could be of considerable therapeutic importance.

Before finally leaving the human body through the backdoor, the proanthocyanidins without any doubt will directly encounter one more potential target, *i.e.* the bacteria of the gut. We know today that the composition of these bacteria has a significant influence on human health and disease. Indeed, while some gut bacteria are useful in assisting the human body with the processing of food, others are less desirable - albeit not directly pathogenic. Such unwanted guests may, for instance, form and subsequent release irritants, toxins and even mutagenic substances into the gut. A recent landmark study by Dolara *et al.* [21] has shown that polyphenols derived from red wine not only possess antioxidant properties, neutralize carcinogenic compounds, alter gene expression profiles and inhibit colon carcinogenesis, but also strongly influence the colonic flora (as measured in the feces). The red wine polyphenols used in this study, which consist of 28% proanthocyanidins (with a mean degree of polymerization of 6.8), are able to alter the colonic flora of F344 rats from mostly *Bacteroides*, *Clostridium* and *Propionicbacterium* sp. in control animals to *Bacteroides*, *Lactobacillus* and *Bifidobacterium* sp. in the test animals. These changes are clearly beneficial to the host: Although the total bacterial count and the anaerobe/aerobe ratio of microorganisms remain mostly unaltered, the increased presence of Lactobacilli and Bifidobacteria improves intestinal function. In contrast, Clostridia have detrimental effects on the colonic mucosa. The alteration of the composition of the colonic flora therefore may provide protection against oxidative stress, inflammation and possibly also against exposure to carcinogens.

While our journey through the human body has revealed various potential and important targets for proanthocyanidins inside the GIT, limited bioavailability of the oligomeric material has quasi forced us to stay outside the true interior of the body. Not surprisingly, there are attempts to make proanthocyanidins more bioavailable. Apart from mixing proanthocyanidin extracts with oils and/or emulsifiers, some groups have also used the natural oligomeric and polymeric material as the starting point for the synthesis of 'designer' polyphenols. Here, we will briefly consider a recent publication by Fujii *et al.* [48], which describes the thiolysis of proanthocyanidins with the help of L-cysteine (Figure 3). Chemically, proanthocyanidins are rather stable compounds containing monomers linked with each other via carbon-carbon bonds. Nonetheless, good nucleophiles, such as thiols, are able to attack such carbon-carbon bonds and subsequently cause the breakdown of the oligomeric or polymeric materials into shorter fragments or even monomeric units. Importantly, the resulting fragments are not anthocyanidins (the products of acid hydrolysis), but thioethers.[1] In the case of L-cysteine, the products of thiolysis can be described as hybrid molecules consisting of the flavonoid (monomer or fragment) and L-cysteine. Although the data currently available for these 'semisynthetic' agents is limited and clearly of a preliminary nature, it is feasible that such 'processed' proanthocyanidins may be of interest in the context of chemoprevention and possibly even therapy in the future.

In conclusion, proanthocyanidins represent a vast and diverse class of oligomeric natural products with considerable chemopreventive

[1] Thiolysis of proanthocyanidins is also used in the context of analysis. Coupled with a chromatographic method, it allows the determination of the monomer composition of a given proanthocyanin and also provides some information regarding the mean degree of polymerization.

and even therapeutic potential. The question of bioavailability, which has long complicated research into these complex biological substances, is clearly not an issue when considering the many targets on the surface of the human body, be it the skin, respiratory system or the linings of the GIT. At the same time, certain proanthocyanidins may also be useful in the context of 'green pesticides'. It is now time to investigate the various proanthocyanidins and related compounds (*e.g.* tannins) with the scientific vigor and rigor these exciting macromolecules deserve. Here, synthetic chemistry urgently needs to provide effective and easy-to-perform methods for the synthesis of the biologically more interesting oligomers, *i.e.* compounds with degrees of polymerization between 4 and 10. At the same time, such pure materials need to be studied in different biochemical (activity) assays, using settings which are relevant *in vivo* (*e.g.* gut bacteria, skin cells, cells originating from the GIT, agricultural pests). Ultimately, the chemical properties and biochemical mode(s) of action need to be considered. Here, realistic protein and enzyme targets need to be identified, and interactions with other biopolymers, including membranes, proteins, DNA and RNA need to be studied.

In any case, proanthocyanidins provide plenty of opportunities for serious and fruitful research across the scientific disciplines in the near and medium future.

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