Chapter 1

Introduction

1.1 General Introduction

Blindness affects many people worldwide. Currently it is estimated that 37 million people in the world are blind, and a further 124 million people suffer from vision impairment. Of these, only 75% can be treated for their blindness.[1] Vision 2020 reports that a further 1 to 2 million people a year go blind.[1] Unfortunately, a large proportion (90%) of the blind live in poor or under developed countries where resources are not readily available. For example, Africa has 6.8 million blind people, and the South East Asian region and the Western Pacific Region have at least 11.6 million blind people.[1]

Numerous diseases affect vision, including glaucoma, retinopathy, macula degeneration and cataract. Each of these diseases requires further medical research to understand their etiology, and to assist future generations with the development of more cost effective cures and treatments.

Cataract is the most common cause of blindness worldwide.[2,3] An enormous amount of research has been undertaken on this disease, but there are still many unanswered questions. Cataract is defined as the opacification or cloudiness of the lens.[4] Age-related nuclear (ARN) cataract is the most common type of cataract,[5] and is responsible for 48% of world blindness.[6] Today, the aging population is increasing and therefore the prevalence of cataract will continue to increase in the coming decades.[7]

This thesis will look at one UV filter, 3OHKyn, and examine its role in the human lens. The work presented in this thesis will add to our understanding of the changes that occur during nuclear cataract formation.

1.2 The Human Eye

The eye is spherical, enclosed by three layers and filled with fluid. The outermost layers of the eye include the sclera and the cornea, followed by the choroid, ciliary body and iris, and the innermost layer of the eye is the retina (Figure 1.1).[8] The human adult eye is approximately 24 mm in diameter.[9] The sclera is dense, white and an opaque coating of the eye and is not directly involved in the visual process. The cornea is a transparent tissue located anterior to the globe of the eye, and is covered by a thin tear film (~7-8 μm). Posterior to the cornea is the aqueous humor, which is secreted by the ciliary epithelium. Posterior to the aqueous humor is a transparent tissue, the lens, whose primary function is to focus and filter images onto the retina. The lens is suspended in place by zonular ligaments. The vitreous humor is a gel substance that occupies approximately 90% of the total volume of the eye, and provides structural support for the adjacent ocular tissues. Finally, detection of light occurs at the retina. Light first passes through the tear film followed by the cornea, aqueous humor, the lens, the vitreous humor and finally the light is absorbed by the photoreceptors on the retina, where an electrical signal is produced and that signal is transmitted through the ganglion cell layer to the brain.[8,9]

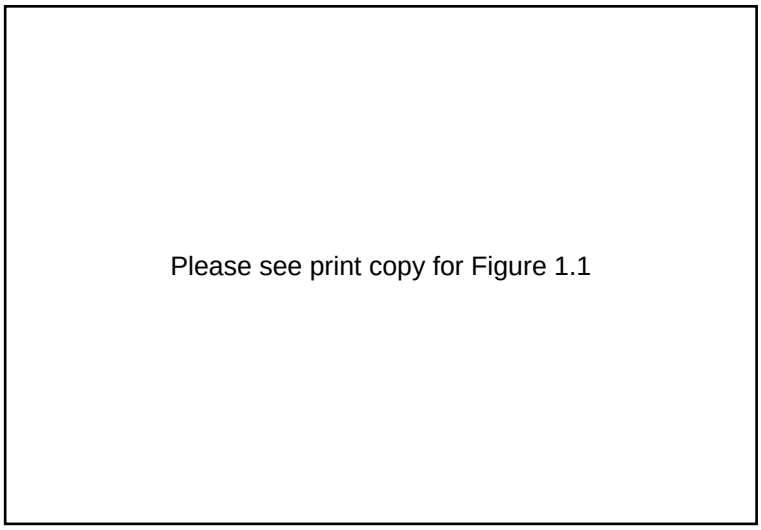


Figure 1.1 Schematic diagram of the cross section of the human eye.[10]

1.3 The Lens

The lens has a high concentration of protein and a unique arrangement of fibre cells, which together provide the high refractive index essential for focusing images onto the retina.[9,11,12] It is a transparent tissue surrounded by an elastic collagenous capsule that allows small molecules into and out of the lens from the surrounding structures, for example, nutrients from the aqueous humor.[13,14] The parts of the lens include the nucleus, cortex, epithelial cells, lens fiber cells and sutures (Figure 1.2).

The lens of an embryo consists only of the nucleus, and throughout life the lens continuously grows and develops. The epithelial cells have a high metabolic rate, and are located on the anterior surface underlying the capsule. These cells undergo mitosis at the equator and differentiate into elongated fibre cells. New fibre cells are laid down as concentric layers on previously formed embryonic fibres.[9] The fibre cells occupy the remaining lens, and metabolic activity occurs in this region of the lens *i.e.* the cortex.[15] Development of the fibre cells results in the accumulation of soluble crystallins, water channel membranes, lipids and numerous other components that insert into the fibre cell plasma membrane.[9]

As fibre cells mature they elongate and extend from the anterior surface of the lens and curve around to the back of the lens to meet in a region referred to as lens sutures[14] (Figure 1.2), and, there is a concomitant loss of the nuclei and other intracellular organelles such as the mitochondria, Golgi bodies and rough and smooth endoplasmic reticulum.[9] The lens nucleus contains the oldest cells and there is no protein turnover, since fibre cells do not contain DNA and RNA. Protein synthesis only occurs in the epithelium and the developing fibre cells of the lens. Amino acids are transported into the lens via the aqueous humor.[9,14]

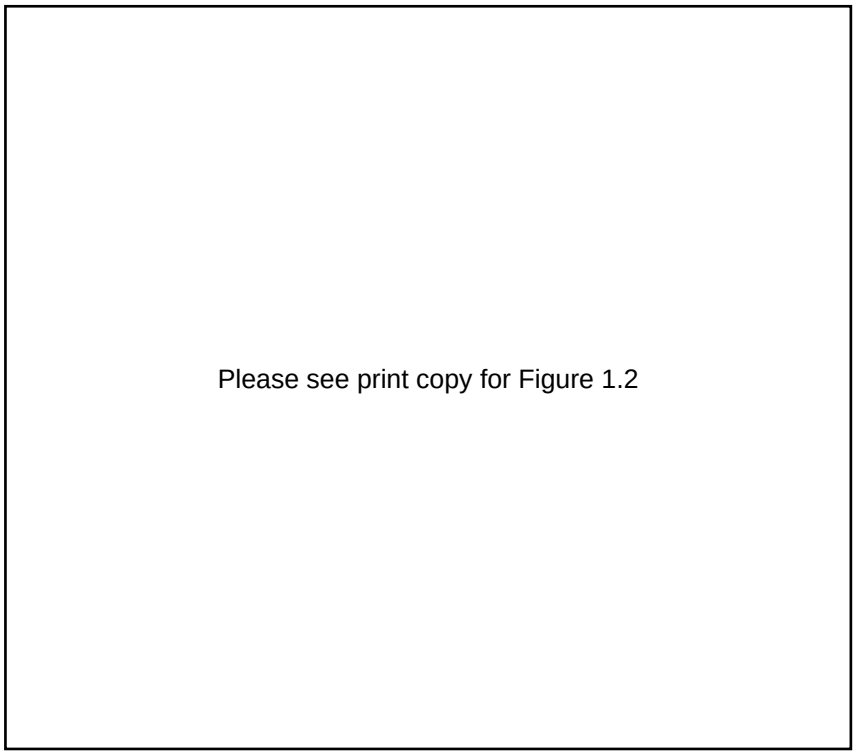


Figure 1.2 Cross-section of the human lens (adapted from J. Harding).[14]

1.4 Components of the Lens

Summary

The lens contains many chemical compounds. Table 1.1 lists the major components of the human lens. The lens contains amino acids of varying quantity, and many low molecular compounds including two antioxidants; glutathione and ascorbic acid. UV filters are also located in the lens and are low molecular weight compounds. In recent years the levels of 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (AHBG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-diglucoside (AHBDG) 3-hydroxykynurenine (3OHKyn), 3-hydroxykynurenine *O*-β-D-glucoside (3OHKynG), and kynurenine, (Kyn) have been quantified. In addition, oxygen has also been identified as a component of the human lens. Proteins, antioxidants and UV filters will be discussed in greater detail in the following section.

Table 1.1 Major components of the human lens.[9,16-19]

Please see print copy for Table 1.1

1.4.1 Proteins in the Lens

Proteins in the lens comprise approximately 38% of the wet mass.[20] The protein concentration in the centre of the human lens is approximately 450 mg/mL.[21] Crystallins are the major soluble proteins in the mammalian lens and make up over 90% of the lens proteins. The remaining 10% of the lens proteins are comprised of membrane and cytoskeleton proteins.[4] There is little or no protein turnover in the lens therefore the proteins are long-lived.[22] Crystallins are structural proteins responsible for the refractive properties and stability of the lens.[11,23-26] There are many classes of crystallins based on oligomeric size. Bovine and human lens proteins contain three classes of crystallins, the α-, β- and γ-crystallins. Other classes of crystallins, for example, the δ-crystallins are found in birds and reptiles[27], and ζ-crystallins are found in guinea pigs.[28]

α-Crystallin

α-Crystallin is a polydisperse, multimeric protein with a molecular weight distribution ranging from 300 kDa to over 1000 kDa.[29] It has two subunits, αA and αB, each with a monomeric mass of approximately 20 kDa. The amino acid sequence homology between αA- and αB-crystallin is approximately 60%.[25] α-Crystallin is a structural protein. It also functions as a molecular chaperone in the lens, by regulating protein folding during synthesis and protecting the lens proteins against misfolding and aggregation.[22,30-33] αA- and αB-crystallin are continuously synthesised during lens development,[34-37] and are found in the lens epithelial cells, but their synthesis is up regulated upon differentiation to the lens fibre cells.[38,39] αA-Crystallin is primarily found in the lens tissue, however αB-crystallin is ubiquitous, and is also found in non-lenticular tissues.[40-42] Increased amounts of αB-crystallin have been associated with various neurological diseases, for example, Alexander's disease,[43] Creutzfeldt-Jakob disease,[44,45] Alzheimer's disease[46] and Parkinson's disease.[44]

β- and γ-Crystallin

The β- and γ-crystallins are structural proteins in the lens. β-Crystallins are oligomers and γ-crystallins are monomers, both built up out of four Greek key motifs organised into two domains.[47] The β-crystallins have acidic (βA1 (23 kDa), βA2 (21 kDa), βA3

(25 kDa) and βA4 (22 kDa)) and basic (βB1 (27 kDa), βB2 (23 kDa) and βB3 (24 kDa)) polypeptides.[48-50] The β-crystallin genes are fibre cell specific, however the acidic β-crystallin genes have a wider expression pattern and their protein products are found in both the nucleus and the cortex of the lens.[36,37]

There are seven γ-crystallin genes in the mammalian genome. Six of the γ-crystallin genes (γA, γB, γC, γD, γE, γF) are closely linked in a repeated gene cluster and are similar in sequence, each with a mass of approximately 20 kDa. The seventh gene, γS-crystallin, is located on another chromosome and is more diverse in sequence.[51] The γ-crystallins are also fibre cell specific, and are the last crystallins to be synthesised during fibre cell differentiation, being preceded by the α- and then the β-crystallins.[38,39,52] γ-Crystallins are not found in the immature fibre cells in the cortical region. The γA-F-crystallin genes are expressed in early lens development and their products are mainly found in the lens nuclear region.[37,39]

1.4.2 Antioxidants

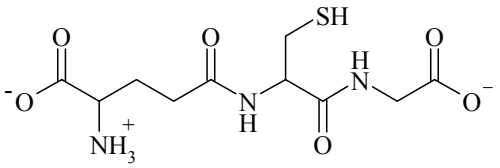
The lens, like many other tissues and cells in the body, is susceptible to oxidative damage. It is permeable and small molecules like hydrogen peroxide (H_2O_2), which is found in the aqueous humor, will diffuse into the lens and oxidise amino acid residues in proteins. Recently it has been shown that oxygen is present in the lens.[53,54] The lens requires antioxidants to offer protection, however with age, the level of antioxidants decreases.[19,55] The two antioxidants present in the lens include, ascorbic acid and reduced glutathione (GSH).

Ascorbic Acid

Ascorbic acid is present at high levels in the lens[56] and can function as an antioxidant. However, ascorbic acid displays prooxidant properties and in the presence of metal ions, free radical and other active oxygen species such as H_2O_2 are formed.[57] Ascorbic acid also induces the formation of non-disulfide bond crosslinks and insolubilisation of the lens proteins.[58-62]

GSH

GSH is a tripeptide, γ-glutamyl-cysteinyl-glycine, containing a thiol group (Figure 1.3).[63,64]



GSH

Figure 1.3 Structure of GSH.

GSH is found in millimolar levels in the lens.[65] The levels are highest in the epithelium[66] and are higher in the cortex than in the nucleus.[67] GSH is synthesised in the cortex[68,69] sequentially in two steps from L-glutamate, L-cysteine and glycine, according to the following reactions:

1. glutamate + cysteine + ATP ⟶ γ-glutamylcysteine + ADP + Pi

2. γ-glutamylcysteine + glycine + ATP ⟶ GSH + ADP + Pi

The first reaction is catalysed by γ-glutamylcysteine synthetase and the second reaction is catalysed by glutathione synthetase.[70,71] The functions of GSH in the lens include:[9,18,72]

- Maintaining protein sulfhydryl groups in a reduced form, and preventing the formation of high molecular weight aggregates;
- Detoxifying H_2O_2;
- Amino acid transportation as a γ-glutamyl donor to the α-amino groups of acceptor amino acids such as cysteine or glutamine;
- Ion transportation, especially Na^+ and K^+, by protecting the sulfhydryl groups of Na^+,K^+-ATPase from oxidation.

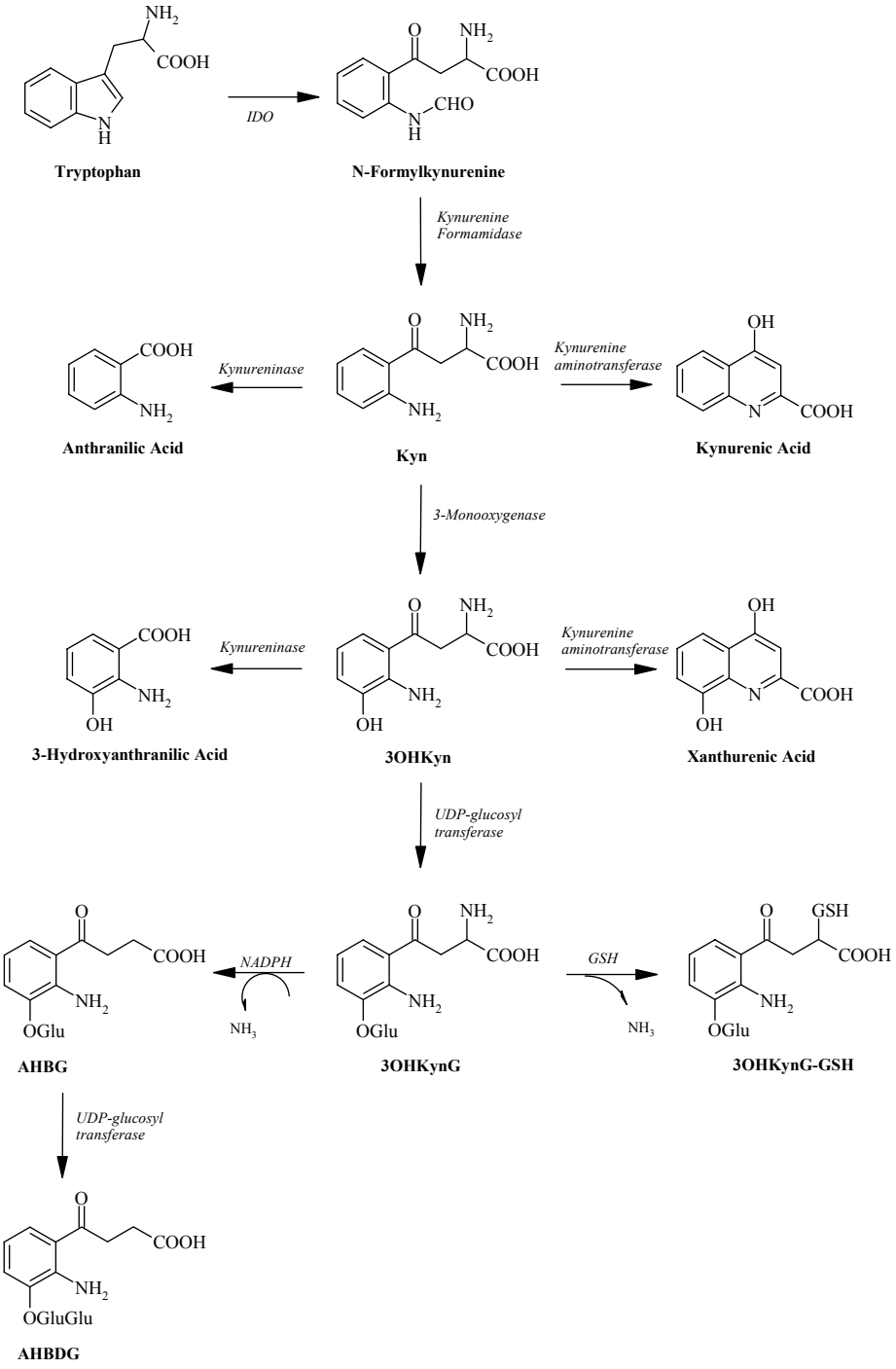
1.4.3 UV Filters

Low molecular weight fluorescent compounds called UV filters are also present in the human lens. Primate UV filters are synthesised from the metabolism of tryptophan

(Trp), and absorb UV light in the 300-400 nm region on the UV spectrum. UV filters have a peak absorption centred between 360 and 370 nm.[73] These compounds prevent UV-induced photodamage to the retina[74] and aid in visual acuity.[73] The most abundant UV filter in the lens is 3OHKynG,[75] followed by Kyn and 3OHKyn.[20,73,76-79] More recently, AHBDG,[80] AHBG,[81] and the glutathione adduct of 3OHKynG[82] have also been identified as fluorescent UV filter compounds in aged human lenses.

The UV filters are synthesised in the lens cortex. The first step of the Kyn pathway involves oxidative cleavage of the indole ring of Trp by the enzyme indoleamino 2,3-dioxygenase (IDO) to form *N*-formylkynurenine.[83] Kynurenine formamidase catalyses the formation of Kyn, 3-monooxygenase catalyses the formation of 3OHKyn, glycosylation via UDP-glucosyl tranferase yields 3OHKynG, deamination followed by Michael addition of GSH yields 3OHKyn-GSH, or deamination followed by reduction of the alkene yields AHBG, and further glycosylation via UDP-glucosyl tranferase yields AHBDG (see Scheme 1.1).[73,84,85]

The kynurenine pathway also occurs in other tissues, and Scheme 1.1 only shows some of the products of Trp metabolism. Interaction of Kyn and 3OHKyn with kynurenine aminotransferase yields kynurenic acid and xanthurenic acid respectively. Hydrolysis of Kyn and 3OHKyn with kynureninase yields anthranilic acid and 3-hydroxyanthranilic acid respectively, but they are not found in the lens. Xanthurenic acid was thought to be present in normal human lenses,[86] however a later study showed that it was absent.[87]



Scheme 1.1 Tryptophan metabolism: The Kynurenine pathway. Glu refers to glucose (β-linked), and GSH refers to glutathione.

1.5 Aging of the Human Lens

There are a number of structural and biochemical changes that occur in the human lens with age. It is unclear if the changes listed below are risk factors for the development of age-related cataract.

Barrier

A barrier to the diffusion of small molecules[88] including water,[89] into the lens nucleus begins at middle age. The barrier is located between the nucleus and cortex interface, however the biochemical basis of the barrier is unknown. As a result of the barrier, unstable compounds such as the kynurenines spend long periods of time in the centre of the lens and have more time to chemically react in this region. A barrier to the diffusion of GSH into the nucleus predisposes the lens center to oxidation.[90,91]

Antioxidants

The concentration of antioxidants in the lens decreases with age. The concentration of GSH in young human lens nucleus is ~4.5 mM and this decreases to ~1 mM with age. In the cortex, the concentration of GSH in the young lens is ~6 mM and this decreases to ~3 mM with age.[19,55] The barrier may contribute to the decrease in GSH concentration with age. In addition, there is an increase in the levels of 3OHKynG-GSH with age, and this compound forms when deaminated 3OHKynG binds covalently via a Michael addition to GSH.[19,82] GSH is an antioxidant, and it has an important role in protecting the lens from modifications. Once the level of GSH decreases, the lens crystallins then become prone to post-translational modifications.

Colouration and Fluorescence

A young human lens is colourless to a very pale yellow colour. With age the lens increases in colour and fluorescence.[92-95] In older lenses, absorption of light extends to approximately 500 nm.[95,96] Insoluble proteins in the lens nucleus appear to be responsible for the increase in non-tryptophan fluorescence, which is observed, with increasing age. A green fluorophore with maximum wavelength at Ex 440 nm/Em 520 nm, and a blue fluorophore with maximum wavelength at Ex 340 nm/Em 400 nm are observed.[97,98] The attachment of kynurenines and glycation products to the lens

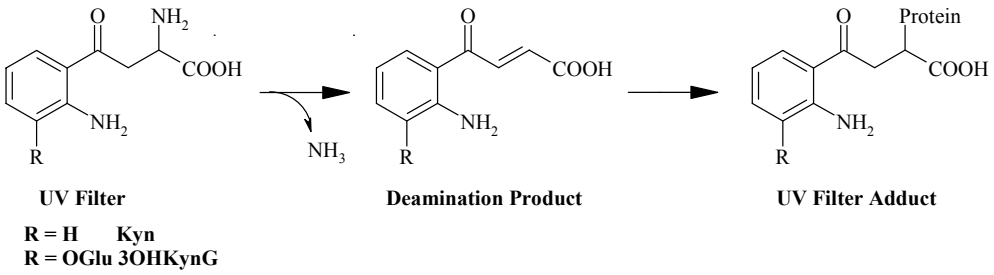
crystallins appear to be, at least in part, responsible for the age-dependent yellowing of the human lens.[76,99-107]

Presbyopia

Presbyopia is the loss of ability to focus properly on close objects or fine print. The ciliary muscle, (the muscle that surrounds the lens) expands and contracts, changing the curvature of the lens to accommodate for near and distant vision.[10] Presbyopia begins at approximately age 45 and gradually worsens over the coming decades. It begins when the aging lens thickens and becomes more rigid,[108] therefore the ciliary muscle is less efficient at changing the lens shape for near and distant focus.[10]

UV Filters

In the cortex and the nucleus, the concentration of free UV filters, 3OHKynG, Kyn and 3OHKyn, decreases with age at a rate of 12% per decade, from age 20 to 80 years old.[19] The UV filters, 3OHKynG, Kyn and 3OHKyn have been shown to be unstable at physiological pH, and deamination of the UV filters results in the formation of a reactive deamination product[109] (Scheme 1.2). The deamination product has been shown to undergo condensation reactions with reduced GSH in the lens,[82] and the nucleophilic amino acids of lens crystallins[110-112] (Scheme 1.2). Hood, *et al*. and Vazquez, *et al*. showed that 3OHKynG and Kyn are both bound to the human nuclear lens proteins, and that the amount bound increases with age.[101,103] The lens barrier[90] seems to be responsible for this phenomenon, since UV filters reside in the nucleus for longer periods of time, and there is a greater extent of deamination, which results in more UV filters bound to the highly nucleophilic amino acid residues on the crystallin proteins.



Scheme 1.2 Formation of protein UV filter adducts.

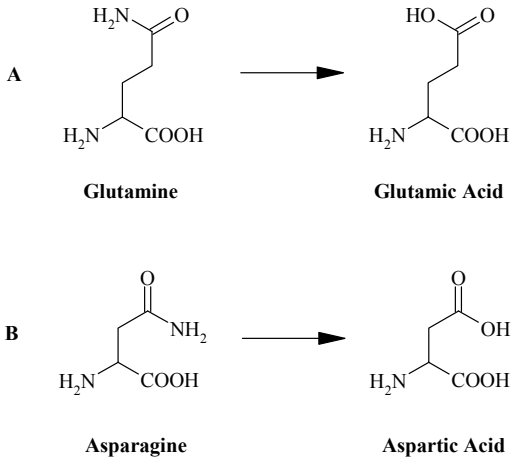
Post-Translational Modifications (PTMs) of the Crystallins

The crystallin proteins of the aging human lens undergo numerous PTMs. Most modifications increase with age. The modifications include, deamidation, glycosylation, phosphorylation, racemisation, and truncation, and will be discussed below.

Deamidation

Over time glutamine and asparagine residues undergo hydrolysis of a side chain amide to form glutamic acid and aspartic acid respectively (Scheme 1.3), and the molecular weight increases by 1 Da. Deamidation results in a more polar carboxylate anion, which may encourage ionic interactions that lead to conformational changes in the crystallins which could result in the formation of disulphide bonds within the cysteinyl crystallins although this is controversial.[91]

Deamidation has been identified in human aged γS-crystallin, at glutamine-92 and 170. Asparagine-24, 49, 118, 124 and 137 and glutamine-26, 47, 54, 66 and 67 are sites of deamidation in human aged γD-crystallin, and asparagine-24 and 137, and glutamine-26, 66, 67, 142 and 148 are deamidation sites in γC-crystallin.[113] Deamidation has also been observed at asparagine-146 in human aged αB-crystallin,[114] glutamine-50 in human αA-crystallin,[115] and asparagine-101 in the high molecular weight aggregate of αA-crystallin.[116]



Scheme 1.3 Deamidation of *A*, Glutamine; *B*, Asparagine.

Glycosylation

Nonenzymatic glycation of proteins via the Maillard reaction occurs by condensation of a sugar (e.g. glucose) molecule with protein amino groups (e.g. Lys) to form covalent adducts. This results in the colouration, fluorescence, crosslinking and sometimes insolubilisation of the protein.[117-122] Advanced glycation end (AGE) products form during the later stages of the Maillard reaction, and accumulate in long-lived proteins, like the lens crystallins,[104,122,123] but the exact mechanism of their formation is unknown. Dicarbonyls, glyoxal and methylglyoxal are often intermediates in the Maillard reaction.[124] Examples of AGE products identified in human lenses includes, pentosidine,[104] glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD),[125,126] N^{ε}-(carboxymethyl)lysine (CML),[127] N^{ε}-(1-(1-carboxy)ethyl)lysine (CEL),[128] and Vesperlysine A.[129] Methylglyoxal-derived hydroimidazolone AGE products have been recently discovered. They consist of three isomers but only two can be quantified in the lens since the third is highly unstable. The two isomers include N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornthine (MG-H1) and 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2).[130] 2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-Lysine) is also a newly identified AGE product in cataract and aged lens proteins.[106] The structures of the AGE products are shown in Figure 1.4.

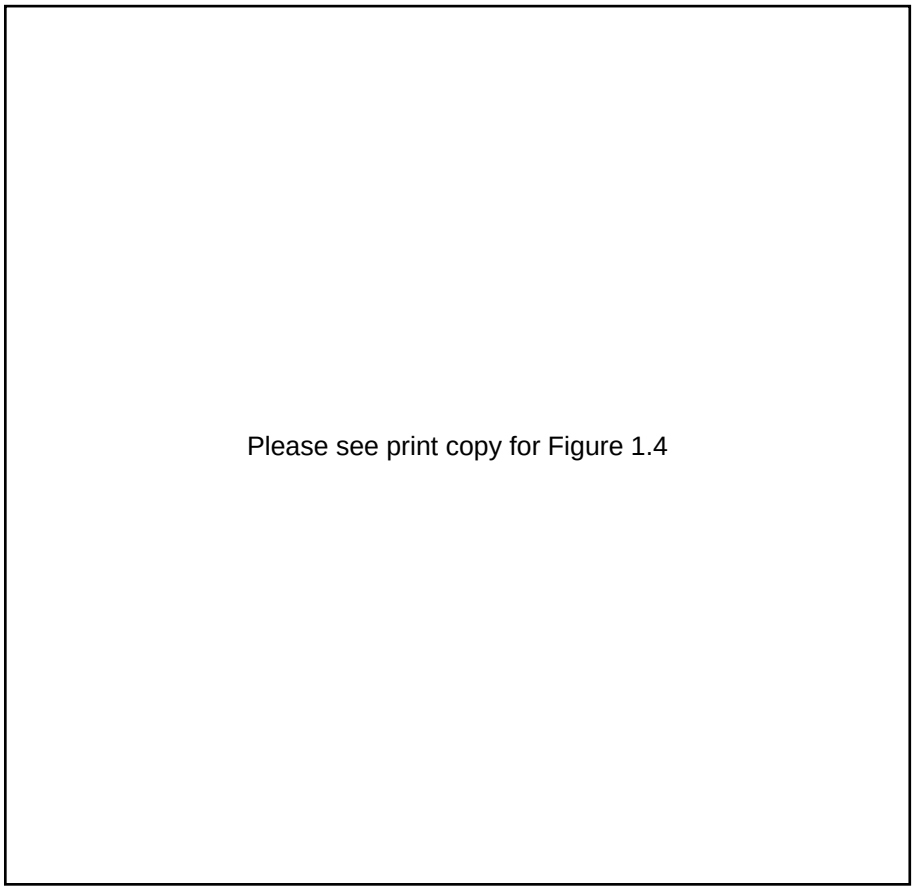


Figure 1.4 Structures of the AGE products found in human lenses.[105,106,130,131]

Phosphorylation

Aged α-crystalllin proteins are subject to phosphorylation of serine residues. In αB-crystallin serine-45[132] and 59[133] have both been identified as sites of phosphorylation.

Racemisation

In aged crystallin proteins from the human lens nucleus, aspartic acid undergoes racemisation. L-Aspartic acid residues are converted to D-Aspartic acid. D-Aspartic acid accumulates at a rate of 0.14% per year in the nuclear lens proteins.[134] D-Aspartic acid has been identified in high molecular weight aggregates and water-insoluble protein.[135] More specifically, D isomers have been identified at aspartic acid-58, 151,[136] 105 and 106[137] in αA-crystallin, and aspartic acid-36 and 62 in αB-crystallin.[138]

Truncation

Human aged crystallins undergo cleavages of the N- and the C-terminal polypeptides. Cleavages have been identified in α-, β- and γ-crystallins.[36,50,139-142]

Result of PTMs

Possibly as a result of these many PTMs, crystallins become progressively denatured over time and form large aggregates in the lens.

Insolubilisation and Superaggregation

During normal aging there is an increase in the high molecular weight protein fraction and a conversion from water-soluble to a water-insoluble protein fraction. These changes do not compromise lens transparency. With age old lens proteins unfold and denature and these become prone to aggregation.[29,143,144] After age 40 water-soluble α-crystallin completely disappears from the human lens nucleus.[33,145]

1.6 Cataract

Cataract is the opacification of the lens.[4] Aging and the accompanying changes to the chemical composition of the lens components are the most likely reasons for cataract. Cataract can be classified by age of onset (e.g. congenital, juvenile or age-related), or by the location of opacity within the lens (e.g. cortical or nuclear).

Symptoms of Cataract

Typically age-related cataract develops slowly and initially affects vision only slightly. As opacification increases, vision becomes blurred, cloudy or dim. There is an increase in glare from bright lights and the sun. Vision may become more nearsighted, night vision may worsen and many colours will appear less vivid.[10]

Risk Factors

Aging is by far the major risk factor of cataract.[146,147] Other risk factors include diabetes, alcohol, smoking, diet, steroid use, UV light, heatstroke, hypertension and poverty.[2,146,148-154]

Treating Cataract

Currently there are no efficacious means available to prevent the formation of cataract. People suffering from cataract are treated with surgery. Previously surgeons used extracapsular (lens contents) or intracapsular (lens and capsule) surgery to treat cataract. Both procedures require large incisions to be made under the upper eyelid, and recovery takes many weeks. Today ultrasound phacoemulsification is the most common technique used by ophthalmologists. The technique involves a small incision, and an ultrasonic probe of high frequency sound waves is inserted, and used to break up the cataractous lens. It is then removed through a tiny needlelike tube. An artificial intraoculoar lens is then implanted. Patients generally recover well from this procedure.[10] Some surgeons choose to treat the opacities in the lens with an erbium:YAG laser (wavelength 2940 nm). The laser is inserted through a very small incision (0.8 mm compared to 2.5 mm for phacoemulsification), however one study showed that patients with highly dense nuclear opacities treated with laser, experienced increases in intraocular pressure, irreversible corneal edema and posterior capsule

rupture, compared to patients treated with ultrasound phacoemulsification,[155] whereas others report satisfactory outcomes from laser treatment.[156]

However, more research is needed to improve the current techniques in order to achieve greater efficacy and safety. A drug for prevention or therapy would be ideal since many patients suffering cataract are aged, surgical procedures can be traumatic for many elderly and the procedure is costly.

1.7 Age-Related Nuclear (ARN) Cataract

ARN cataract is the most common form of cataract, in which the proteins become coloured, oxidised, insoluble and crosslinked.[14,157-160] Pirie devised a classification system for ARN cataract. Type I cataract is cortical cataract, and Types II to V are nuclear cataracts in increasing order of severity (Figure 1.5). A normal lens nucleus is yellow, whereas a Type V cataract nucleus is brown/black.[161] Cortical cataract appears to be a result of an ionic imbalance.[162] Nuclear cataract lenses have a very low concentration of GSH in the centre of the lens.[163] This may be due to an increase in the barrier with cataract.

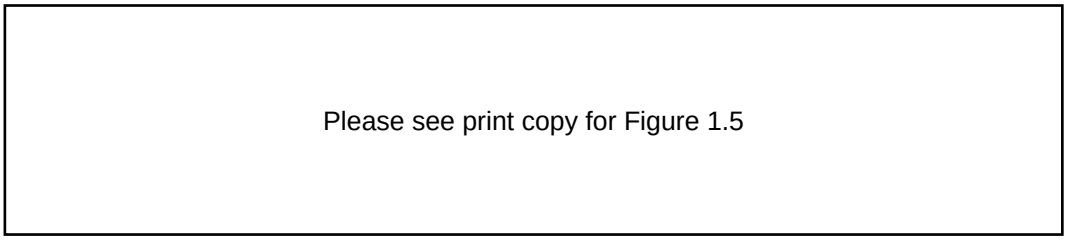


Figure 1.5 The Pirie classification of nuclear cataracts[161] using slit lamp photographs.[164] The colour of the nucleus increases with increasing severity of nuclear cataract.

Oxidation

During ARN cataract formation, oxidation of amino acids takes place. Approximately 90% of the Cys are oxidised to form disulfide bonds, and 45% of the methionines form methionine sulfoxide, in advanced nuclear cataract.[159] Hydroxyl radical oxidation of lens proteins is known to be associated with ARN cataract. Hydroxylation of protein-bound amino acids, such as, DOPA, *o*- and *m*-tyrosine, 3-hydroxyvaline, 5-hydroxyleucine and dityrosine, have all been observed.[165]

Insolubilisation

Proteins are usually solubilised in 8 M urea, however with the onset of ARN cataract, there is a major increase in a urea-insoluble fraction. This fraction can only be solubilised in the presence of a reducing agent such as, 2-mercaptoethanol or dithiothreitol.[158]

Crosslinking

Crosslinking is a characteristic feature of ARN cataract. Since the 1970s many crosslinked compounds have been identified in cataractous lens proteins. In 1972, Buckingham was first to document crosslinking in lens proteins.[166] Crosslinking refers to both disulfide bond and non-disulfide bond linkages. Buckingham demonstrated non-disulfide bond crosslinking in cataractous lens proteins. Normal and cataractous lens nuclear proteins were reduced and examined by gel filtration, showing the presence of high molecular weight proteins in the cataractous lens proteins.[166]

In 1973, Harding reduced and carboxymethylated human normal lens nuclear proteins, and Type II cataract lens nuclear proteins,[161] and reported that approximately 5% of the protein in the cataract lenses was composed of high molecular weight aggregates, as a result of protein-disulfide bond linkages, rather than protein-GSH bonds. However, disulfide bonds were not identified in normal lens nuclear and cortical proteins.[167]

Histidinoalanine (Figure 1.6) is an amino acid crosslink compound first identified in connective tissue,[168] and later identified in human lens nuclear proteins.[169] This compound is thought to be formed by the reaction between a histidine and a serine or a cysteine residue in close proximity.[170] Histidinoalanine has been quantified in normal human nuclear proteins, and similar levels were also identified in Type I and II nuclear cataract proteins. However the level of histidinoalanine in Type III and IV nuclear cataract proteins is seven and sixtyseven times higher than the normal human nuclear proteins, respectively.[169] Connective tissue, like lens tissue contains long-lived proteins. The level of histidinoalanine increases with age in connective tissue, and results in hardening of this tissue.[168,171] It is unclear if histidinoalanine is responsible for the hardening of the lens tissue.

Bessems, *et al*. identified Lanthionine (Figure 1.6), a symmetric thioether formed by oxidative degradation of cystine, in human cataractous lenses.[172] Pentosidine (Figure 1.4), a non-disulfide bond crosslink product involving arginine and lysine linked in an imidazo (4,5,6) pyridinium ring formed by a 5-carbon sugar, has been identified in

human brunescent (high levels of pigmentation) cataract lens proteins.[104] Vesperlysine A (Figure 1.6), a lysine crosslink of the Maillard reaction, has been identified in normal and diabetic human aged lens proteins, and is identical to LM-1 (Figure 1.6).[105] Cheng, *et al*. isolated 1-(5-amino-5-carboxypently)-4-(5-amino-5-carboxypentlyamino)-3-hydroxy-2,3-dihydropyridinium (the authors abbreviated the name to, lysine-lysine pyridinium (K2P)) (Figure 1.6) from one hundred and fifty normal human lenses and three hundred and fifty Type I and II human cataract lenses. Quantitation in various lenses showed that the level of K2P was higher in cataract lens proteins.[107] OP-Lysine, MG-H1 and MG-H2 (Figure 1.4) are all AGE products, and non-disulfide bond crosslinks in cataract lens proteins.[106,130] The majority of non-disulfide crosslinks so far identified are linked via a Lys residue. The pKa value of Lys, His and Cys,[103] suggest that the Lys residues in proteins are the least ionized at the neutral pH and should react at a slower rate.[173]

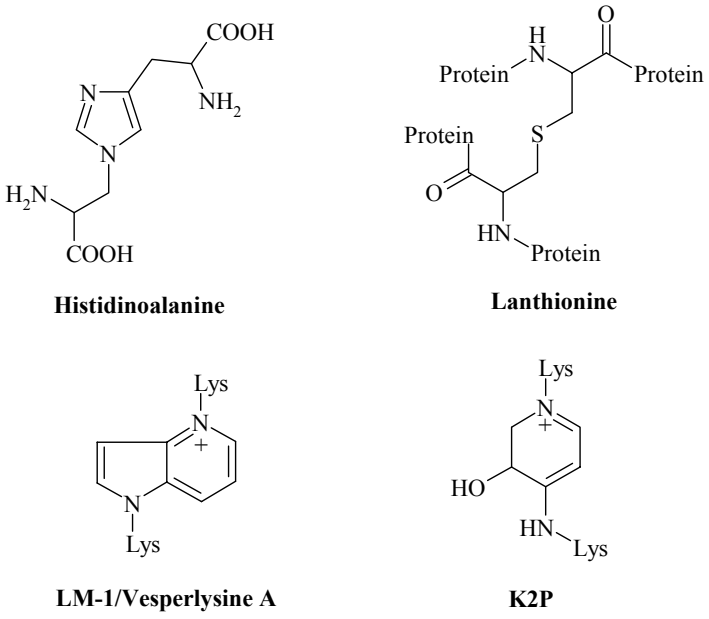


Figure 1.6 Structures of non-disulfide bond crosslink compounds identified in cataract lens proteins.

1.8 3OHKyn

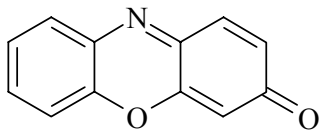
3OHKyn is an *o*-aminophenol. It is a UV filter in the lens,[19] however it is also found in other parts of the body, for example, the brain, where increased levels of 3OHKyn have been implicated in a number of disease states, such as, Huntington's disease and Parkinson's disease.[174-176] In neuronal cell cultures, 3OHKyn is cytotoxic at concentrations as low as 1 μM, causing oxidative stress through the formation of H_2O_2.[177,178] 3OHKyn also effectively chelates iron (III) and copper (II).[179] Oxidation of 3OHKyn is still observed even in Chelex treated phosphate buffers (phosphate buffers contain trace levels of copper and iron).[180]

Autoxidation of 3OHKyn results in the production of H_2O_2. The mechanism of formation of H_2O_2 via 3OHKyn autoxidation is shown in Scheme 1.4. It is believed that H_2O_2 is generated from reduction of oxygen by the phenoxide anion of 3OHKyn to generate $O_2^{\cdot-}$, and its dismutation to yield approximately 1 mole of H_2O_2 per mole of 3OHKyn.[181] The pK_a of the phenoxyl group of 3OHKyn is 9.6.[182] Thus a small fraction of 3OHKyn in the phenoxide state at neutral pH may be sufficient to allow autoxidation to proceed.[181]

Please see print copy for Scheme 1.4

Scheme 1.4 Mechanism of formation of H_2O_2, via 3OHKyn autoxidation.[181]

Ommochromes are natural pigments responsible for the colouration of eyes as well as certain other parts of the insect body. They are found primarily among arthropods. These pigments were first identified by Becker, and the name "ommochrome' was assigned as a result of the their location *i.e.* in the ommatidies of the insect eye.[183] Ommochromes are acidic pigments, insoluble in neutral solvents, however soluble in buffered solutions of pH 6. Ommochromes were initially divided into two groups; *Ommatins*, which exhibit a characteristic change in colour when oxidised or reduced; and *Ommins*, which are stable under alkali conditions and have molecular weights of approximately 650 Da.[183] Further investigations by Butenandt, *et al*. showed that the ommatins consisted of three pigments; xanthommatin, a yellow phenoxazone (Figure 1.7) pigment; rhodommatin, a red pigment; and ommin D, a red pigment.[184]

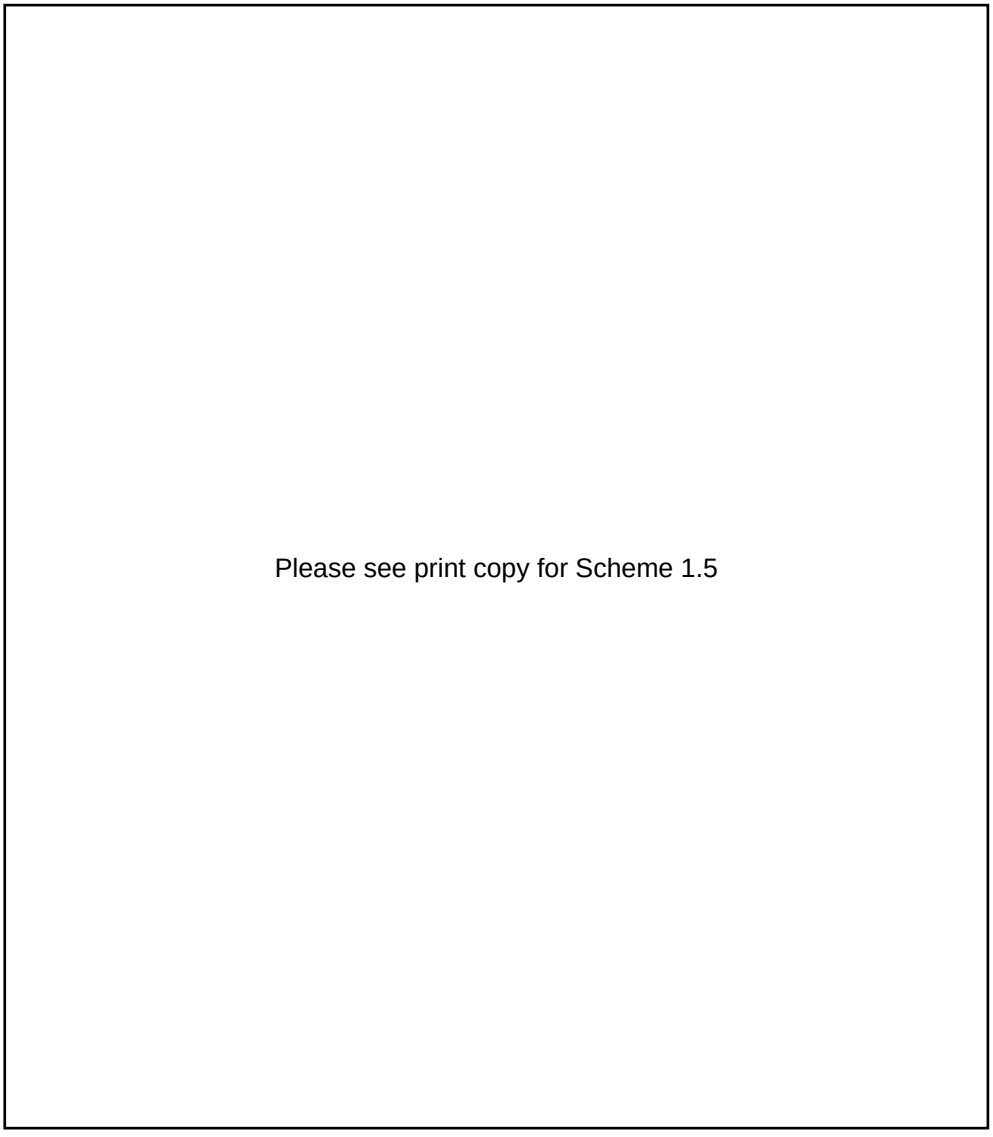


Phenoxazone

Figure 1.7 Structure of Phenoxazone.

Ommochromes are products of the metabolism of tryptophan. There are numerous pigments formed, since the final steps of the synthesis can diverge in such a way that a variety of pigments are produced. Xanthommatin is formed through the oxidative condensation of two molecules of 3OHKyn. The quinoline ring is formed by elimination of one mole of ammonia (Scheme 1.5). The synthesis of xanthommatin is in accordance with the general scheme for the synthesis of phenoxazones from *o*-aminophenols.[184] Phenoxazones exhibit a UV absorbance at 440 nm. Xanthommatin is reduced easily into dihydroxanthommatin by the uptake of two hydrogen atoms, and is reoxidised readily upon standing in air into xanthommatin, which is more stable. Ommatin D (Scheme 1.5) is stable towards oxidation, and is the sulphate ester of dihydroxanthommatin, that can be formed through a hydrolytic reaction. Rhodommatin (Scheme 1.5) is a derivative of dihydroxanthommatin and contains a glucose molecule, however the mechanism of its formation is unknown.[184]

Vazquez, *et al*. characterised major oxidations products from autoxidation of 3OHKyn under physiological conditions. The major autoxidation products were identified as xanthommatin, DHQCA and hydroxyxanthommatin.[181] Incubation of 3OHKyn with lens proteins, under oxidative conditions, results in tanned products that resemble cataractous material.[100,185,186]



Scheme 1.5 Formation of pigments in arthropods from oxidation of 3OHKyn.[184]

1.9 Aims of the Project

The aim of this study is to understand some of the roles of the UV filter 3OHKyn in the human lens. Based on previous studies it is known that UV filters, such as Kyn and 3OHKynG bind to lens protein in an age-dependant manner. But unlike Kyn and 3OHKynG, 3OHKyn is very unstable to oxidation and thus it is expected that 3OHKyn could play a role in the formation of ARN cataract, if it too were bound to proteins in the human lens.

Specific aims of this study are to:

- Synthesise the three 3OHKyn amino acid adducts expected in normal lenses, and characterise each adduct using mass spectrometry, NMR spectroscopy and UV-visible and 3-D fluorescence spectroscopy. In addition, to determine the stability properties of each adduct.
- Determine if 3OHKyn is attached to human lens proteins.
- Examine the potential role of 3OHKyn as a crosslinker of lens proteins.
- Identify novel compounds in the hydrolysates of human cataractous lens proteins.