- HUMAN LENS CHEMISTRY -

UV FILTERS AND AGE-RELATED NUCLEAR CATARACT

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JASMINKA MIZDRAK B.Sc (Hons)

Department of Chemistry and Biomolecular Sciences

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Scheme 6.4: Oxidation of Phe by HO$_2$ in the presence of O$_2$ (Path A) and absence of O$_2$ (Path B). The position of the hydroxyl group on Phe can be at o-, m- and p- position.$^{359}$

Scheme 7.1: The improved synthetic strategy towards 3OHKG. The preferred reaction conditions and product yields: i) Cu(NO$_3$)$_2$·2.5H$_2$O, AcO$_2$, AcOH, 10-15°C, 16 h, 25%; ii) HOCCOOH, MW, 110°C, 17 min, 60%; iii) MeOH, H$_2$SO$_4$, reflux, 6 h, 90%; iv) HOCCOOEt (50% sol. in toluene), PPA/SiO$_2$, MW, 110°C, 130 min, 58%; v) ABG, DCM, K$_2$CO$_3$, tetra-n-butyl ammonium bromide, 2.5 days, 70-78%; vi) EtOAc, aqueous NH$_3$, 3 h, not isolated; vii) EtOAc, H$_2$, Pd/C, 3 h, not isolated; viii) aqueous NaOH, NH$_3$, 8.5 h, 27% 3OHKG and 18-20% U-24; ix) H$_2$O, H$_2$, Pd/C, 10 min, 92%.

Scheme 7.2: The improved synthetic strategy towards 3OHKyn (A) and AHBG (B). The preferred reaction conditions and product yields: A, i) aqueous NH$_3$, 1.5 h, not isolated; ii) aqueous NH$_3$, H$_2$, Pd/C, 2 h, 72%; B, i) H$_2$, Pd/C, EtOAc/EtOH (4:1, v/v), 2 h, not isolated; ii) aqueous NaOH, 5 h, 41%.

Scheme 7.3: The synthetic strategy towards AHA and AHB. The preferred reaction conditions and product yields: i) HOCCOOH, MW, 110°C, 60 min, 54% (55) and 17 min, 60% (7); ii) H$_2$, Pd/C, EtOAc, AcOH (~0.2%), 22 h, 41% (AHA) and 3 h, 47% (AHB).

Scheme 7.4: The synthetic strategy towards GSH-Kyn, GSH-3OHKyn, Kyn yellow and 3OHKyn yellow from Kyn and 3OHKyn. The preferred reaction conditions and product yields: i) aqueous Na$_2$CO$_3$-NaHCO$_3$ (25 mM, pH 9.5), GSH, 37°C, 72 h, 51% (GSH-Kyn) and 44% (GSH-3OHKyn); ii) aqueous NaHCO$_3$ (0.49 M, pH ~9), reflux, 20 h, 26% (Kyn yellow) and 22% (3OHKyn yellow).

Scheme 7.5: The synthetic strategy towards Cys-3OHKG from 3OHKG. The preferred reaction conditions: aqueous Na$_2$CO$_3$-NaHCO$_3$ (25 mM, pH 9.2), Cys, 37°C, 72 h, 35%.
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The kynurenine-based UV filters are unstable under physiological conditions and undergo side chain deamination, resulting in α,β-unsaturated carbonyl compounds. These compounds can react with free or protein bound nucleophiles in the lens via Michael addition. The key sites of the UV filters kynurenine (Kyn) and 3-hydroxykynurenine (3OHKyn) modification in human lenses include cysteine (Cys), and to a lesser extent, lysine (Lys) and histidine (His) residues. Recent in vivo studies have revealed that 3-hydroxykynurenine-O-β-D-glucoside (3OHKG) binds to Cys residues of lens crystallins in older normal human lenses. As a result of this binding, human lens proteins become progressively modified by UV filters in an age-dependent manner, contributing to changes that occur with the development of age-related nuclear (ARN) cataract. Upon exposure to UV light, free UV filters are poor photosensitisers, however the role of protein-bound species is less clear. It has been recently demonstrated that Kyn, when bound to lens proteins, becomes more susceptible to photo-oxidation by UV light. Therefore, the investigation of 3OHKG binding to lens proteins, and the effect of UV light on proteins modified with 3OHKG and 3OHKyn, were major aims of this study. As a result of the role of these compounds as UV filters and their possible involvement in ARN cataract formation, it is crucial to understand the nature, concentration and modes of action of the UV filters and their metabolites present in the human lenses. Therefore, an additional aim was to investigate human lenses for the presence of novel kynurenine-based human lens metabolites and examine their reactivity.

As 3OHKG is not commercially available, to conduct protein binding studies, an initial aim of this study was to synthesise 3OHKG (Chapter 2). Through the expansion and optimisation of a literature procedure, 3OHKG was successfully synthesised using commercially available and inexpensive reagents, and applying green chemistry principles, where toxic and corrosive reagents were replaced with benign reagents and solvent-free and microwave chemistry was used. A detailed investigation of different reaction conditions was also conducted, resulting in either the improvement of reaction yields or reaction time compared to the literature method. Applying the same synthetic strategy, and using key precursors from the synthesis of 3OHKG, the UV filters 3OHKyn and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid-O-β-D-glucoside (AHBG), were also successfully synthesised (Chapter 3).

Chapter 4 describes the investigation of both normal and cataractous human lenses in an attempt to identify novel human lens metabolites derived from deaminated Kyn and 3OHKyn
(Chapter 4, Part A). Initially, 4-(2-aminophenyl)-4-oxobutanoic acid (AHA), glutathionyl-kynurenine (GSH-Kyn), kynurenine yellow (Kyn yellow), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (AHB), glutathionyl-3-hydroxykynurenine (GSH-3OHKyn) and 3-hydroxykynurenine yellow (3OHKyn yellow) were synthesised and human lenses were examined for their presence. AHA and AHB were synthesised from similar precursors to those used in the synthesis of 3OHKG, while the GSH adducts and yellow compounds were synthesised from Kyn and 3OHKyn via base induced deamination. Following isolation and structural elucidation, AHA, AHB and GSH-Kyn were confirmed as novel human lens metabolites. They were quantified in low pmol/mg lens (dry mass) levels in normal and cataractous lenses of all ages, while GSH-3OHKyn, Kyn yellow and 3OHKyn yellow were not detected. In contrast to AHA, the lens metabolites AHB, GSH-Kyn and GSH-3OHKyn were found to be unstable at physiological pH. The spectral properties of these compounds suggest that they may act as UV filters.

Chapter 4 (Part B) also describes the identification and characterisation of a novel human lens UV filter, cysteinyl-3-hydroxykynurenine-O-β-D-glucoside (Cys-3OHKG). An authentic standard was synthesised via Michael addition of cysteine to deaminated 3OHKG. Cys-3OHKG was detected in low pmol/mg lens (dry mass) levels in normal lenses only after the 5th decade of life and was absent in cataractous lenses. Cys-3OHKG showed rapid decomposition at physiological pH.

Chapter 5 describes the identification and quantification of amino acids involved in covalent binding of 3OHKG to lens proteins. Model studies with bovine lens proteins and 3OHKG at pH 7.2 and 9.5 were undertaken. The amino acid adducts were identified via total synthesis and spectral analysis, and subsequently quantified upon acid hydrolysis of the modified lens proteins. Under both pH conditions, 3OHKG was found to react with lens proteins predominantly via Cys residues with low levels of binding also detected at Lys residues. Comparative studies with Kyn (pH 9.5) and 3OHKyn (pH 7.2 and 9.5) resulted in modified lens proteins at Cys residues, with only minor modification at Lys residues at pH 9.5. The extent of modification was found to be significantly higher at pH 9.5 in all cases. His adducts were not identified. 3OHKG-, Kyn- and 3OHKyn-modified lens proteins were found to be coloured and fluorescent, resembling those of aged and ARN cataractous lenses. In contrast, AHB and AHA, which can not form α,β-unsaturated carbonyl compounds, resulted in non-covalent modification of lens proteins. AHB may contribute to lens colouration and fluorescence as further reactions of this material yielded species that have similar
characteristics to those identified from 3OHKyn modification. These species are postulated to arise via auto-oxidation of the o-aminophenol moiety present in both 3OHKyn and AHB.

In Chapter 6, the potential roles of 3OHKG and 3OHKyn, and the related species AHA and AHB, in generating reactive oxygen species and protein damage following illumination with UV light was examined. The UV filter compounds were examined in both their free and protein-bound forms. Kyn-modified proteins were used as a positive control. Exposure of these compounds to UV light (λ 305-385 nm) has been shown to generate H₂O₂ and protein-bound peroxides in a time-dependent manner, with shorter wavelengths generating more peroxides. The yields of peroxides were observed to be highly dependent on the nature of the UV filter compound and whether these species were free or protein bound, with much higher levels being detected with the bound species. Thus, protein-bound 3OHKyn yielded higher levels of peroxide than 3OHKG, with these levels, in turn, higher than for the free UV filter compounds. AHB-treated lens proteins resulted in formation of low but statistically significant levels of peroxides, while AHA-treated lens proteins resulted in insignificant peroxide formation. The consequences of these photochemical reactions have been examined by quantifying protein-bound tyrosine oxidation products (3,4-dihydroxyphenylalanine [DOPA], di-tyrosine [di-Tyr]) and protein cross-linking. 3OHKG-modified proteins gave elevated levels of di-Tyr, but not DOPA, whereas 3OHKyn-modified protein gave the inverse. DOPA formation was observed to be independent of illumination and most likely arose via o-aminophenol auto-oxidation. AHB- and AHA-treated lens proteins resulted in statistically insignificant di-Tyr formation, while a light independent increase in DOPA was observed for both samples. Both reducible (disulfide) and non-reducible cross-links were detected in modified proteins following illumination. These linkages were present at lower levels in modified, but non-illuminated proteins, and absent from unmodified protein samples.

This work has provided an optimised synthetic procedure for 3OHKG and other lens metabolites (Chapters 2 and 3). Four novel lens metabolites have been identified and quantified in normal and cataractous human lenses (Chapter 4). Subsequent experiments, described in Chapter 5, identified the major covalent binding sites of 3OHKG to lens proteins, while AHA and AHB showed non-covalent binding. Further work described in Chapter 6 showed that protein-bound 3OHKG, Kyn and 3OHKyn were better photosensitisers of oxidative damage than in their unbound state. Together, this research has provided strong evidence that post-translational modifications of lens proteins by kynurenine-based metabolites and their interaction with UV light appear, at least in part, responsible for the age-
dependent colouration of human lenses and an elevated level of oxidative stress in older lenses. These processes may contribute to the progression of ARN cataract.
THESIS DECLARATION

This thesis contains no material that has been accepted for the award of any higher degree or diploma at any University or Institution, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text of the thesis.
The production of this thesis involved many people other than the author and I wish to gratefully acknowledge the contribution of the following people:

My supervisors, A/Prof Joanne F. Jamie (Macquarie University, Sydney, NSW), Prof Michael J. Davies (The Heart Research Institute, Sydney, NSW) and Prof Roger J. W. Truscott (Save Sight Institute, Sydney, NSW), for their hard work, patience and professional support throughout the years, and from whom I have learnt a great deal.

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My parents for their continuous love and support. Thank you mum Boja and dad Stevan, after all we have been through we are doing fine. Nobody can stop your dreams from coming true. Also, a big thanks to my brother Robert, sister-in-law Kristina and my lovely nephews Kristian (tetin miš) and Alek (teta buba/mačak) for support throughout the years. Finally I would like to thank my boyfriend Damir Jaksic for his unconditional love, endless support and for believing in me during these years. Your assistance in preparation of this document is invaluable. Thank you!
This thesis is dedicated to
my mum and dad
my brother, sister-in-law and nephews
my boyfriend

Love you all forever and thank you
Sections of the work described in this thesis have been reported or are in the preparation for the following publications:


Jasmina Mizdrak, Peter G. Hains, Roger J. W. Truscott, Joanne F. Jamie, Michael J. Davies, Tryptophan-derived UV filter compounds covalently bound to lens proteins are photosensitizers of oxidative damage. *Free Radicals in Biology & Medicine*, manuscript in preparation.

CONFERENCE PRESENTATIONS

The following oral or poster presentations have been made at conferences/symposia on research conducted during this PhD study:


v. The 1st European Chemistry Congress in Budapest (Hungary), 28-31 August 2006. Jasminka Mizdrak, Peter G. Hains, Roger J. W. Truscott, Michael J. Davies, Joanne F. Jamie. Human lens chemistry - UV filters and cataract. This international presentation resulted in media coverage, see Appendix C.

# LIST OF ABBREVIATIONS

The following abbreviations are used throughout the text:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABG</td>
<td>2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
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<td>AGE</td>
<td>Advanced glycation end</td>
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<td>Arginine</td>
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<td>Asparagine</td>
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<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<tr>
<td>AHA</td>
<td>4-(2-Aminophenyl)-4-oxobutanoic acid</td>
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<tr>
<td>AHB</td>
<td>4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid</td>
</tr>
<tr>
<td>AHBG</td>
<td>4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-O-β-D-glucoside</td>
</tr>
<tr>
<td>AHB DG</td>
<td>4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-O-diglucoside</td>
</tr>
<tr>
<td>ARN</td>
<td>Age-related nuclear</td>
</tr>
<tr>
<td>BAA</td>
<td>β-Benzoylacrylic acid</td>
</tr>
<tr>
<td>BLP</td>
<td>Bovine lens proteins</td>
</tr>
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<td>Boc</td>
<td>Butyloxycarbonyl</td>
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<td>CH₃CN</td>
<td>Acetonitrile</td>
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<td>Cys-BAA</td>
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<td>Cys-3OHKG</td>
<td>Cysteinyl-3-hydroxykynurenine-O-β-D-glucoside</td>
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<td>di-Tyr</td>
<td>Di-tyrosine</td>
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<td>DOPA</td>
<td>3,4-Dihydrophenyalanine</td>
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<td>D₂O</td>
<td>Deuterium oxide</td>
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<td>ES-MS</td>
<td>Electrospray mass spectrometry</td>
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<td>Em</td>
<td>Emission</td>
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<td>Ex</td>
<td>Excitation</td>
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<tr>
<td>FOX</td>
<td>Peroxide assay involving the oxidation of the Fe(II)-xylenol orange complex to the Fe(III) species</td>
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<tr>
<td>Gln</td>
<td>Glutamine</td>
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<td>Kynurenine</td>
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<tr>
<td>Kyn yellow</td>
<td>Kynurenine yellow</td>
</tr>
<tr>
<td>LC-MS</td>
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</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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</table>
MW Microwave
m/z Mass to charge ratio
NAD(P)H Reduced β-nicotinamide dinucleotide (phosphate)
NMR Nuclear magnetic resonance
δ Chemical shift
NaOH Sodium hydroxide
3OHKyn 3-Hydroxykynurenine
1O2 Singlet molecular oxygen in its 1Δg state
O2− Superoxide radical
3OHKG 3-Hydroxykynurenine-O-β-D-glucoside
3OHKyn yellow 3-Hydroxykynurenine yellow
3OHKG yellow 3-Hydroxykynurenine-O-β-D-glucoside yellow
pet. spirit Petroleum spirit
Phe Phenylalanine
Pro Proline
PSH Protein sulphydryl
PTM Post-translational modification
RP-HPLC Reversed phase-high performance liquid chromatography
RT Room temperature
Rt Retention time
SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser Serine
TFA Trifluoroacetic acid
Thr Threonine
TLC Thin layer chromatography
Trp Tryptophan
Tyr Tyrosine
UDP Uridine diphosphate
UV Ultraviolet