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APPENDIX A

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Identification of the new UV filter compound cysteine-L-3-hydroxykynurenine *O*-β-D-glucoside in human lenses

Peter G. Hains[a,b,1], Jasminka Mizdrak[c,1], Isla M. Streete[b], Joanne F. Jamie[c], Roger J.W. Truscott[a,b,*]

[a] *Save Sight Institute, University of Sydney, Sydney, NSW 2001, Australia*
[b] *Australian Cataract Research Foundation, Department of Chemistry, University of Wollongong, Wollongong, NSW 2522, Australia*
[c] *Department of Chemistry and Biomolecular Sciences, Division of Environmental and Life Sciences, Macquarie University, Sydney, NSW 2109, Australia*

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Abstract **UV filters protect the human lens and retina from UV light-induced damage. Here, we report the identification of a new UV filter, cysteine-L-3-hydroxykynurenine *O*-β-D-glucoside, which is present in older normal human lenses. Its structure was confirmed by independent synthesis. It is likely this novel UV filter is formed in the lens by nucleophilic attack of cysteine on the unsaturated ketone derived from deamination of 3-hydroxykynurenine *O*-β-D-glucoside. Quantitation studies revealed considerable variation in normal lens levels that may be traced to the marked instability of the cysteine adduct. The novel UV filter was not detected in advanced nuclear cataract lenses.**

Keywords: UV filters; Cysteine; Lens; Cataract

1. Introduction

The major UV filters found in human lenses are 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (AHBG), 3-hydroxykynurenine (3OHKyn) and kynurenine (Kyn) [1–5]. These UV filters function to protect the lens and retina from UV damage by absorbing light in the 295–400 nm range [6,7]. Work in our laboratory has shown these UV filters are unstable at physiological pH and undergo a range of reactions, including deamination to form α,β-unsaturated ketones [8], which are capable of reacting with nucleophilic amino acids including Cys, His and Lys [9,10]. The binding of UV filters, and other reactive small molecules [11–14] to crystallin proteins results in the normal human lens becoming more coloured with age [8,15–19].

The anti-oxidant glutathione (GSH) is present in the lens in high concentrations (~6 mM) [20], however, like the UV filters, its concentration has been shown to decrease with age [20,21]. GSH reacts with deaminated 3OHKG to form glutathione-3-hydroxykynurenine *O*-β-D-glucoside (GSH-3OHKG), a novel UV filter that, in contrast to other UV filters, increases in concentration with age [22]. In the lens, reaction of the α,β-unsaturated ketones with GSH may act to protect the crystallins from modification [23].

The decreasing concentration of UV filters may play an important role in the development of nuclear cataract [24] and as such, it is important to understand the changes that occur in normal and cataractous lenses. In this paper, we identify and quantify cysteine-3-hydroxykynurenine *O*-β-D-glucoside (Cys-3OHKG) in normal human lenses. It is likely that this novel human metabolite is formed in the lens by reaction of free cysteine with deaminated 3OHKG.

2. Materials and methods

2.1. Materials

All organic solvents and acids were of HPLC grade (Ajax, Auburn, NSW, Australia); trifluoroacetic acid (TFA) (>99%) and L-cysteine (free base) were from Sigma–Aldrich; chelex resin (100–200 mesh) was from BioRad. Milli-Q® water (purified to 18.2 MΩ cm^{-2}) was used in the preparation of all solutions. Dulbelco's phosphate-buffered saline (PBS), without calcium and magnesium, consisted of KCl 2.7 mM, KH_2PO_4 1.4 mM, NaCl 137 mM, Na_2HPO_4 7.68 mM, pH 7.0 [25]. The pre-washed chelex resin was added to the PBS buffer (~2 g/l) and left for 24 h prior to use. TLC plates were of normal phase 60 F_{254} (Merck, Germany) using a mobile phase of butanol/acetic acid/water (12:3:5, v/v/v). TLC plates were visualised under UV light (254 and 365 nm) and sprayed with ninhydrin.

2.2. Nuclear magnetic resonance (NMR) spectroscopy

^1H, ^{13}C, DEPT (45, 90, 135), COSY (^1H–^1H correlation spectroscopy), HSQC (^1H–^{13}C heteronuclear single quantum correlation) and HMBC (^1H–^{13}C heteronuclear multiple bond correlation) NMR experiments were acquired on a Bruker Avance 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz) at 25 °C. NMR spectra were run in D_2O and referenced to residual HDO. Resonances are quoted in ppm and coupling constants (*J*) are given in Hz.

*Corresponding author. Fax: +61 2 4221 4287.
E-mail address: rjwt@uow.edu.au (R.J.W. Truscott).

[1] P.G.H. and J.M. contributed equally to this manuscript.

Abbreviations: AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside; AHBdiG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-diglucoside; Cys-3OHKG, cysteine-3-hydroxykynurenine *O*-β-D-glucoside; ESI, electrospray ionisation; GSH-3OHKG, glutathione-3-hydroxykynurenine *O*-β-D-glucoside; Kyn, kynurenine; MS, mass spectrometry; MS/MS, tandem MS; 3OHKG, 3-hydroxykynurenine *O*-β-D-glucoside; 3OHKyn, 3-hydroxykynurenine; RP-HPLC, reversed phase-high performance liquid chromatography; TFA, trifluoroacetic acid

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2.3. Mass spectrometry (MS)

Peaks collected from RP-HPLC were lyophilised, resuspended in 50% (v/v) acetonitrile, 0.5% (v/v) formic acid and analysed by electrospray ionisation MS in positive ion mode using a Micromass Q-TOF2 equipped with a nanospray source. MS settings were as follows; cone voltage 25 V, LM/HM 12/12, MCP 2300 V, mass range 50–600 *m/z*. For tandem MS (MS/MS) analysis, ions were subjected to a range of collisions energy settings (typically between 10 and 25 eV). High resolution mass spectrometry was performed on a Q-TOF Ultima with lock spray. Leucine-encephalin (555.2692 Da) was used as the reference compound.

2.4. Fluorescence and UV–visible absorbance spectrometric measurements

UV–visible absorbance spectra were obtained using a Varian DMS 90 UV–visible spectrometer and Plastibrand® 0.5 ml disposable cuvettes (Germany). Fluorescence spectra were recorded on a Perkin–Elmer LS55 Luminescence spectrometer in two- and three-dimensional scan mode using Perkin–Elmer 10 × 10 mm Quartz SUPRASIL® 3.5 ml fluorescence cells. PBS was used as solvent for all measurements with Cys-3OHKG at a concentration of 5.5 mg/ml.

2.5. Cys-3OHKG synthesis

3OHKG [26] (20 mg, 0.064 mmoL) was dissolved in oxygen free Na_2CO_3–$NaHCO_3$ buffer (20 ml, 25 mM, pH 9.2). Free base L-cysteine (90 mg, 0.74 mmoL) was added and the pH was re-adjusted to pH 9.2 by the dropwise addition of 1 M NaOH. The light yellow solution was bubbled with argon for ~20 min, sealed with parafilm and incubated in the dark at 37 °C with shaking. The progress of the reaction was monitored by TLC ($R_{fCys\text{-}3OHKG}$ 0.26). After 24 and 48 h another portion of cysteine (90 mg, 0.74 mmoL) was added to the reaction mixture and the pH was readjusted to 9.2. After 72 h the light yellow reaction mixture was acidified to pH 6.5 by dropwise addition of 1 M acetic acid and lyophilised. Purification by preparative reversed phase-high performance liquid chromatography (RP-HPLC), as described below, afforded Cys-3OHKG (9 mg, 35.5%) as a light yellow solid.

Found: M, 490.1274 Da. Calculated for $C_{19}H_{26}N_2O_{11}S$: M, 490.1257 Da; δH 7.55 (1H, d, *J*, 8.3, H-6), 7.24 (1H, d, *J* 7.9, H-4), 6.71 (1H, dd, *J* 8.1, 8.3, H-5), 4.94 (1H, dd, *J* 1.5, 7.4, H-1′), 3.93 (1H, m, H-12), 3.75 (1H, m, H-9), 3.60 (~0.5H, m, H-8), 3.57 (~0.5H, m, H-8), 3.49 (~0.5H, m, H-8), 3.45 (~0.5H, m, H-8), 3.82 (1H, dd, *J* 1.1, 12.4, H-6′), 3.67 (1H, dd, *J* 5.26, 12.44, H-6′), 3.53 (1H, m, H-3′), 3.52 (1H, m, H-2′), 3.48 (1H, m, H-5′), 3.42 (1H, m, H-4′), 3.32 (~0.5H, dd, *J* 4.1, 14.7, H-11), 3.25 (~0.5H, dd, *J* 4.3, 14.8, H-11), 3.18 (~0.5H, m, H-11), 3.08 (~0.5H, m, H-11); δC 201.6 (CO-7), 201.4 (CO-7), 184.0 (CO-10), 172.5 (CO-13), 145.2 (C-3), 141.6 (C-2), 126.3 (C-6), 121.0 (C-4), 119.0 (C-1), 116.4 (C-5), 101.9 (C-1′), 76.5 (C-5′), 75.9 (C-2′), 73.2 (C-3′), 69.73 (C-4′), 60.8 (C-6′), 54.3 (C-12), 45.5 (C-9), 43.3 (C-9), 42.8 (C-8), 42.1 (C-8), 33.2 (C-11), 32.2 (C-11); electrospray ionisation (ESI)-MS/MS of *m/z* 491.1 (MH^+, 52%), 473.1 (100%), 370.1 (24%), 329.0 (13%), 311.1 (71%), 272.2 (11%), 208.2 (32%), 202.1 (63%), 110.0 (27%).

2.6. Stability studies

Cys-3OHKG was dissolved in chelex treated PBS (pH 7.0) that was degassed with argon for ~20 min to give a final concentration of 0.20 mM. 3 × 4 ml Cys-3OHKG solutions were sealed under argon and incubated in the dark at 37 °C with minimum shaking. Single aliquots (110 μl) were taken from each solution at 0, 1, 2, 3, 4, 6, 8, 11, 23, 32, 46, 57, 94, 120, 134, 168, 192, 216 and 240 h and analysed by RP-HPLC.

2.7. Purification of synthetic Cys-3OHKG

Reversed phased-high performance liquid chromatography (RP-HPLC) was performed on a Shimadzu HPLC equipped with LC-10ADvp pumps, a SIL-10Avp autoinjector, DGU-12A degasser and SPD-M10Avp diode array detector. Standard curves and stability sample analyses were performed on a Phenomenex (Luna, 100 Å, 5 μm, 250 mm × 4.6 mm, C18) column with the following mobile phase system: buffer A (water/0.05% TFA, v/v) and buffer B (80% acetonitrile/0.05% TFA, v/v). The flow rate of 1 ml/min was kept constant with the mobile phase gradient: 5% buffer B (4 min), 5–80% buffer B (12 min), hold at 80% buffer B (3 min), 80–5% buffer B (3 min) and hold at 5% buffer B (8 min). Detection was at 254 nm and 360 nm. Preparative separations were performed on the same instrument with the same mobile phase system as for the analytical separations using a Phenomenex (Luna, 100 Å, 10 μm, 250 mm × 15 mm, C18) column. The flow rate of 7 ml/min was kept constant with the mobile phase gradient: 5% buffer B (10 min), 5–70% buffer B (20 min), hold at 70% buffer B (5 min), 70–5% buffer B (5 min) and hold at 5% buffer B (10 min).

2.8. UV filter extraction and RP-HPLC

Normal human lenses were obtained from the Sydney Eye Bank, with ethical approval from the University of Wollongong Human Ethics Committee (HE99/001). Cataractous lenses were obtained from K.T. Seth Eye Hospital, Rajkot, Gujarat, India. UV filters were extracted from both nuclear and cortical lens sections as previously described [27]. A total of 8 normal lenses were analysed, 2 each from the 3rd, 5th, 7th and 9th decades. UV filters were also extracted from a set of 10 dark cataract lenses (assigned as Types III–IV according to the Pirie classification system [28], average age 66.6 years) and pooled. UV filters were additionally extracted from 15 individual cataract lenses (assigned as Types I–IV, inclusive). In all cases, lens extracts (individual and pooled) were lyophilised and resuspended in 100 μl of 0.1% (v/v) TFA, water prior to RP-HPLC analysis. Standard curve and lens separations were performed on a 250 mm × 4.6 mm 100 Å Varian Microsorb MV C18 column using an acetonitrile/H_2O gradient in 0.1% (v/v) TFA at a flow rate of 0.5 ml/min. The percentage of buffer B (0.08% (v/v) TFA, 80% [v/v] acetonitrile, water) in the gradient was 0% buffer B (5 min), 0–50% buffer B (50 min), and 50–0% buffer B (5 min). Eluant was monitored at 360 nm.

3. Results

A typical elution profile of UV filters extracted from a normal lens nucleus is shown in Fig. 1. An unknown compound eluted as a doublet at ~20% acetonitrile, 0.1% TFA. These peaks (Peak 3, Fig. 1) were further analysed by MS and MS/MS (Fig. 2A) with the identification of a major molecular ion at 491.13 $[M+H]^+$, correlating to a mass of 490.13 Da. The MS/MS data suggested the identity of the compound as Cys-3OHKG. This preliminary identification was from neutral losses of 162 Da (glucose), 109.05 Da (*ortho*-amino-phenol of 3OHKyn), 98 Da (deaminated side chain of 3OHKG), 121 Da (cysteine) and 18 Da (water), as indicated. All major ions observed in the spectra (Fig. 2A) can be accounted for by loss of various combinations of these compounds. The HPLC profile for both nuclear and cortical extracts were essentially identical (data not shown).

Cys-3OHKG was synthesised by incubating L-cysteine with 3OHKG at pH 9.2 and 37 °C. It has been previously demonstrated that the amino acid side chains of Kyn and 3OHKG deaminate readily under basic conditions [19,22,29,30] and that the α,β-unsaturated intermediate is readily attacked by cysteine via a Michael addition [31]. The pH of 9.2 was also beneficial in forming a higher concentration of thiolate anions, which is a stronger nucleophile than the thiol [31]. Due to rapid cysteine oxidation, it was necessary to add cysteine every 24 h to ensure a good yield of Cys-3OHKG. The purified material eluted as a diastereoisomeric doublet on RP-HPLC, with the same retention time as the compound purified from human lenses (data not shown). The MS/MS spectra were identical (Fig. 2B), clearly indicating the identity of the unknown as Cys-3OHKG. The structure of Cys-3OHKG is shown in Fig. 1 inset.

Confirmation of the structure of synthetic Cys-3OHKG was achieved by one-dimensional and two-dimensional NMR. ^1H NMR revealed three aromatic protons as well as protons from the glucose moiety consistent with those present in synthetic

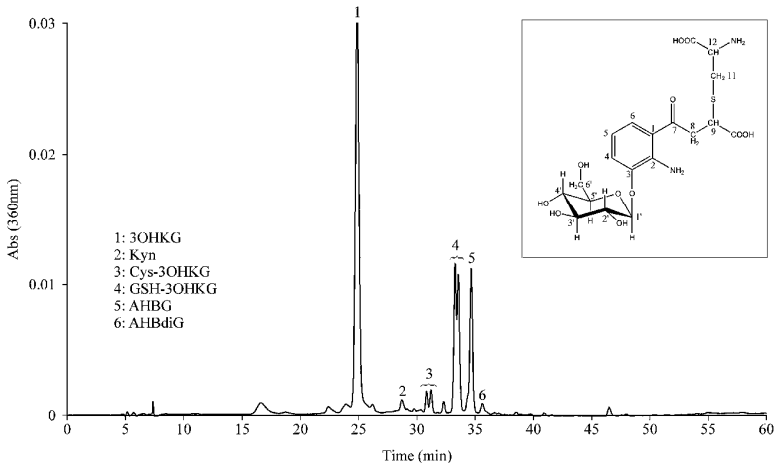

Fig. 1. RP-HPLC profile of UV filter extract from a normal lens nucleus (88 years old). The peaks are labelled 1–6 with the identifications as indicated in the figure. 3OHKG, 3-hydroxykynurenine *O*-β-D-glucoside; Kyn, kynurenine; Cys-3OHKG, cysteine-3-hydroxykynurenine *O*-β-D-glucoside; GSH-3OHKG, glutathione-3-hydroxykynurenine *O*-β-D-glucoside; AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside; AHBdiG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-diglucoside. Inset: structure of Cys-3OHKG.

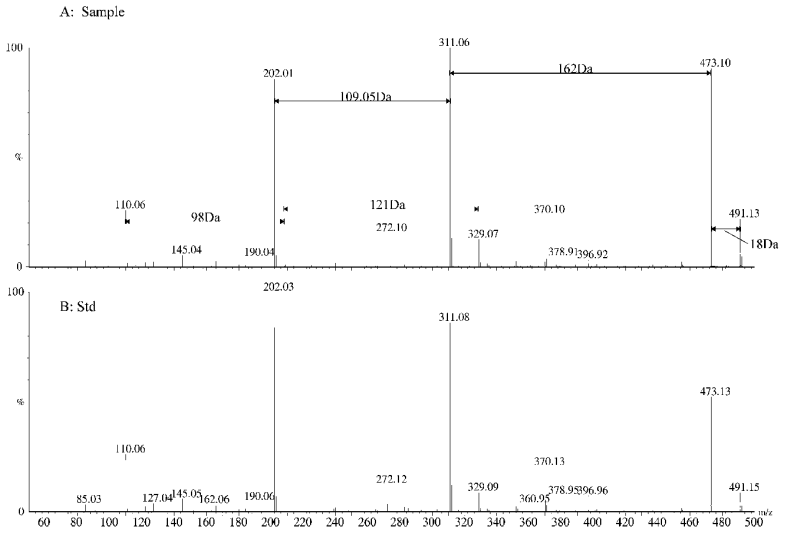

Fig. 2. ESI-MS/MS spectra. ESI-MS/MS of the unknown compound (A) Sample and authentic Cys-3OHKG (B) Std in positive ion mode. Mass differences between major ions are indicated in Daltons (Da).

3OHKG [26]. The side chain displayed a distinctive CH_2–CH moiety, assigned by the combination of DEPT (90 and 45) and HSQC. Chemical shifts at δ 3.60 and 3.57 ppm were assigned to the methylene protons at C-8 and at δ 3.75 ppm for the methine proton at C-9. The chemical shifts for the cysteine-methylene and methine protons were consistent with the literature [19].

A UV–visible absorbance spectrum of Cys-3OHKG in PBS at pH 7.0 showed major peaks at 262 nm and 365 nm. Cys-3OHKG was also found to be fluorescent under these conditions; the UV filter showing a maximum excitation wavelength at 337 nm and maximum emission at 438 nm (data not shown).

The stability of Cys-3OHKG was examined by incubating the compound in PBS (pH 7.0) at 37 °C. Cys-3OHKG was found to rapidly decompose, with the amount of Cys-3OHKG decreasing to ~30% after only 2 h (Fig. 3). This is more rapid than the decomposition of cysteine-kynurenine, which was shown to degrade to ~50% of the starting material after 24 h [19]. The major products formed from the decomposition of Cys-3OHKG, monitored by LC-MS, were deaminated 3OHKG and 3OHKG yellow [30]. After 240 h, 3OHKG yellow, formed by cyclisation of deaminated 3OHKG [30], represented approximately one third of the total deaminated 3OHKG.

UV filters from both the nuclear and cortical sections of individual normal human lenses, ranging in age from 24 to 88, were extracted and analysed by RP-HPLC. The amount of Cys-3OHKG was determined from the area under the curve at 360 nm, by comparison with a standard curve constructed using the synthetic compound. Cys-3OHKG was not detected in the

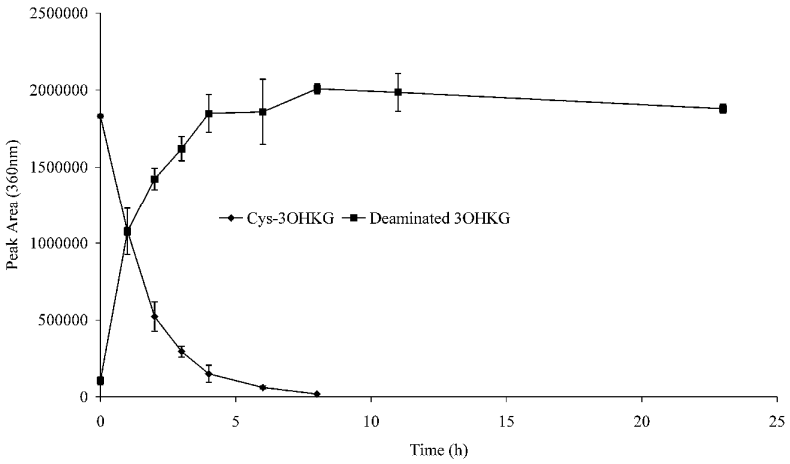

Fig. 3. Stability of Cys-3OHKG in chelex treated PBS at pH 7.0. The identity of deaminated 3OHKG was confirmed by LC-MS. Results are presented as average ± S.D.

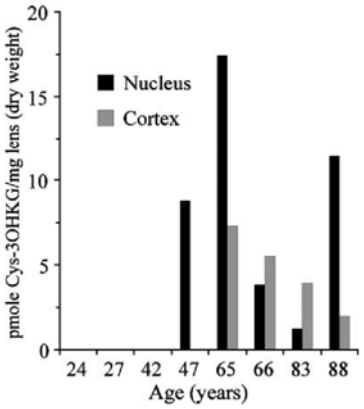

Fig. 4. Quantitation of Cys-3OHKG in normal human lenses. Cys-3OHKG was quantitated in the nucleus (black) and cortex (grey) of normal human lenses.

lens nucleus until the 5th decade and was absent in the cortex until the 7th decade (Fig. 4). The earlier detection of Cys-3OHKG in the nucleus is consistent with the onset of the barrier to diffusion that appears in human lenses at middle age [32]. This barrier between cortex and nucleus allows an increase in the average time that small molecules spend in the lens nucleus and thus more time for unstable molecules to decompose.

The concentration of nuclear Cys-3OHKG did not show any clear correlation with age, whereas, cortical Cys-3OHKG does appear to decrease with increasing age (Fig. 4). UV filters were additionally extracted from 15 individual cataract, and a pool of 10 dark cataract, lenses and analysed by RP-HPLC (data not shown). A number of fractions were collected around the known retention time of Cys-3OHKG and analysed by nano-spray MS. There was no evidence of Cys-3OHKG in any of the fractions collected from pooled or individual lenses.

4. Discussion

In this paper, we report the identification of a novel fluorophore, Cys-3OHKG, in normal human lenses. Its structure was confirmed by independent synthesis. It is very likely that Cys-3OHKG is formed in the lens by addition of free cysteine to the deamination product of 3OHKG in a manner analogous to that of the formation of GSH-3OHKG [22]. A pronounced increase in the covalent attachment of 3OHKG to lens proteins has also been noted after middle age [8] and it is probable that both cysteine and GSH compete with the nucleophilic residues on lens proteins for addition to the α,β-unsaturated ketone that is formed by spontaneous decomposition of 3OHKG. In agreement with this, a plot of Cys-3OHKG versus GSH-3OHKG in lenses showed a strong correlation (Fig. 5). Both of the free thiols may therefore be viewed as potential scavengers of this reactive UV filter molecule and, as a result, they can be considered to protect the long-lived proteins in the lens nucleus from post-translational modification.

Free cysteine is present in the cortex and nucleus of the human lens and it is involved in thiolation of lens proteins [33]. Dickerson and Lou [21] investigated the changes in free cysteine concentration in normal human lenses as a function of age. They found the concentration of cysteine decreased markedly after 40 years of age. This may be related to a decrease in the rate of cysteine uptake which diminished by 70% when cultured newborn lenses were compared to old (80–92 years) lenses [34]. Bova et al. found the concentration of 3OHKG, and other major UV filters, decreased linearly with age [20]. In our study, no clear age related correlation was found for the concentration of Cys-3OHKG in the nucleus, whereas, the concentration of cortical Cys-3OHKG did show signs of decreasing relative to age (Fig. 4). Cys-3OHKG was not detected in either the nucleus or cortex of normal lenses prior to the 5th decade, the age at which cysteine was found to decrease substantially [21]. Given the lack of a clear relationship between the nuclear levels of Cys-3OHKG and age, it appears the formation of Cys-3OHKG is not solely influenced by the concentration of free cysteine or 3OHKG. This is exemplified by a plot of Cys-3OHKG versus 3OHKG, where there is no correlation between the concentration of these compounds in the nucleus or cortex of human lenses (data not shown). By comparison, a plot of nuclear levels of Cys-3OHKG versus GSH-3OHKG, shows a strong correlation (R^2 = 0.9105, Fig. 5) indicating any deaminated 3OHKG

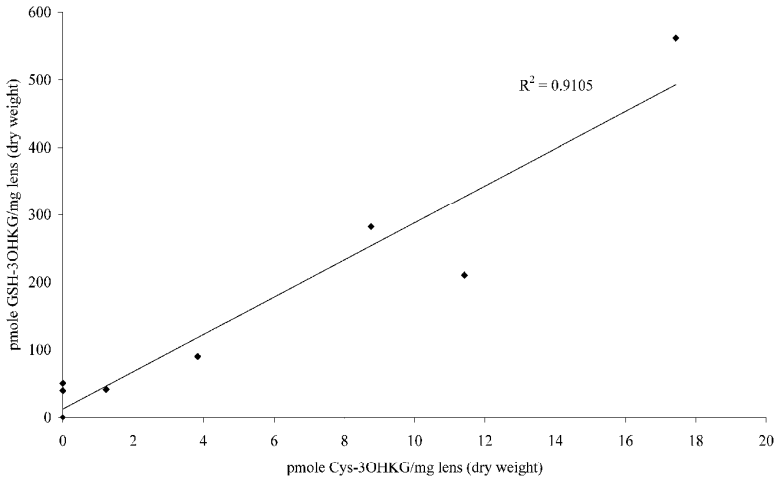

Fig. 5. Plot of the nuclear concentration of Cys-3OHKG versus GSH-3OHKG in normal human lenses. The quantity of each compound was determined by comparison to a standard curve. The R^2 value is shown on the graph.

is likely to react competitively with GSH or cysteine. This relationship also holds for cortical levels of each compound, albeit at a lower correlation ($R^2 = 0.7003$). One factor that may explain a lack of a clear correlation between Cys-3OHKG levels and age is the pronounced instability of Cys-3OHKG (Fig. 3). Cys-3OHKG undergoes elimination more rapidly than GSH-3OHKG. This may be related to greater accessibility by basic species (e.g. hydroxyl ion) to the H-8 of Cys-3OHKG, facilitating elimination of cysteine, or due to the free α-amino group of cysteine participating directly in deprotonation of H-8.

Like 3OHKG, Cys-3OHKG displays UV absorbance in the 300–400 nm band. This is relevant to its role as a lens UV filter compound, since wavelengths of light longer than 300 nm are transmitted by the cornea. Cys-3OHKG showed two absorbance maxima at 262 nm and 364 nm, very similar to GSH-3OHKG (260 nm and 365 nm) and the major UV filter, 3OHKG (264 nm and 365 nm). Cys-3OHKG was also found to be fluorescent, with an excitation and emission spectrum similar to that of 3OHKG [22].

RP-HPLC eluant was collected from individual and pooled cataract lenses around the known retention time of Cys-3OHKG and analysed by nanospray MS. No evidence of this compound could be found for 15 individual, or a pooled extract of 10 cataract lenses. This suggests either the concentration of free cysteine or 3OHKG had decreased to a level where Cys-3OHKG cannot form, or is formed in quantities below the detection limit of the instrumentation used in this study. This supposition is supported by recent work indicating the level of 3OHKG decreased ~5-fold in cataract lenses [24].

Additional reasons for the lack of Cys-3OHKG in cataract lenses may be due to the instability of Cys-3OHKG at physiological pH (Fig. 3), and the fact that it is very unlikely that free cysteine is available in the nuclei of such lenses. The nucleus of the advanced nuclear cataract lens is an oxidising environment in which GSH is very low or absent [35]. In conclusion, an unstable fluorescent and UV-absorbing molecule, Cys-3OHKG, has been identified in normal older human lenses but is absent from the lenses of patients with advanced nuclear cataract.

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APPENDIX B

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Novel human lens metabolites from normal and cataractous human lenses

Jasminka Mizdrak,[a,†] Peter G. Hains,[b,†] Danuta Kalinowski,[a] Roger J. W. Truscott,[b] Michael J. Davies[c,d] and Joanne F. Jamie[a,*]

[a]*Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia*
[b]*The Save Sight Institute, Sydney, NSW 2001, Australia*
[c]*The Heart Research Institute, Sydney, NSW 2050, Australia*
[d]*Faculty of Medicine, University of Sydney, Sydney, NSW 2006, Australia*

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Abstract—4-(2-Aminophenyl)-4-oxobutanoic acid, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid and glutathionyl-kynurenine have been identified as novel metabolites in normal and cataractous human lenses following total synthesis and comparison with authentic human lens samples. Their structures are consistent with those derived from the major human lens UV filters kynurenine and 3-hydroxykynurenine, and it is proposed that these compounds also play a role as UV filters. These metabolites were isolated in pmol/mg levels (dry mass) in lenses. 4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid and glutathionyl-kynurenine were found to be unstable at physiological pH. Other potential metabolites, glutathionyl-3-hydroxykynurenine, kynurenine yellow and 3-hydroxykynurenine yellow, were not detected in either normal or cataractous lenses.

1. Introduction

The primate lens contains fluorescent tryptophan-derived compounds known as UV filters. These compounds absorb UV light (295–400 nm) and are believed to protect the lens and retina from UV-induced photo-damage.[1–4] Major UV filters detected to date in primate lenses, in decreasing order of abundance, include 3-hydroxykynurenine *O*-β-D-glucoside (**1**), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (**2**), kynurenine (**3**) and 3-hydroxykynurenine (**4**) (Fig. 1).[5]

Studies in our laboratory have shown that **1**, **3** and **4** undergo non-enzymatic deamination at physiological pH to form α,β-unsaturated carbonyl compounds (Scheme 1).[6–8] We have found that these unsaturated molecules undergo covalent binding (Michael addition) in vivo with amine and thiol nucleophiles including lysine, cysteine and histidine residues

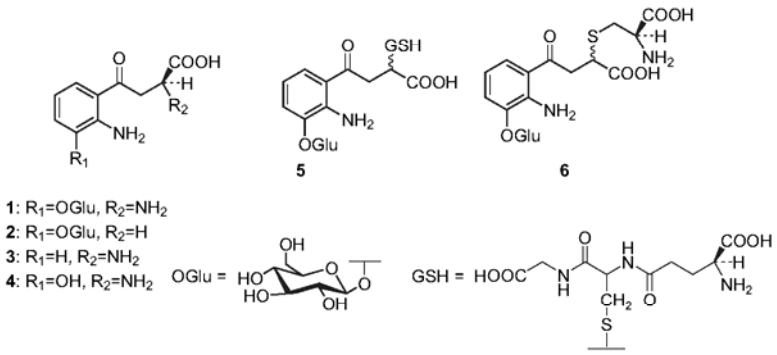

Figure 1. Major human lens UV filter compounds.

* Corresponding author. Tel.: +612 98508283; fax: +612 98508313; e-mail: joanne.jamie@mq.edu.au
† J.M. and P.G.H. contributed equally to this manuscript.

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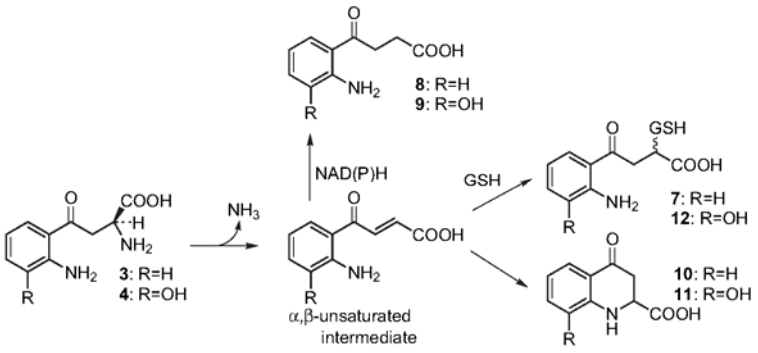


Scheme 1. Schematic diagram illustrating the degradation pathways of the human lens UV filter compounds **3** and **4**.

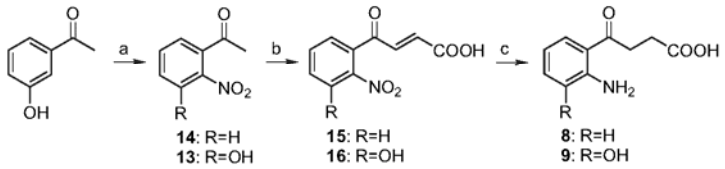
on human lens proteins,[9–11,7] and have also identified glutathionyl-3-hydroxykynurenine *O*-β-D-glucoside (**5**)[12] and cysteine-3-hydroxykynurenine *O*-β-D-glucoside (**6**)[13] as novel human lens UV filter compounds (Fig. 1). In addition, deaminated **3** readily reacts in vitro with glutathione (GSH) to give glutathionyl-kynurenine (**7**),[10] while deaminated **1**, **3** and **4** can be reduced by NAD(P)H to give **2**, 4-(2-aminophenyl)-4-oxobutanoic acid (**8**) and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (**9**), respectively.[8] The deamination and subsequent reduction of **1** is the likely metabolic process for formation of **2** in the lens.[6] In the absence of NAD(P)H, deaminated **1**, **3** and **4** undergo slow intramolecular Michael addition in vitro to give 3-hydroxykynurenine *O*-β-D-glucoside yellow, kynurenine yellow (**10**) and 3-hydroxykynurenine yellow (**11**), respectively (Scheme 1).[8]

Despite the similar structures and reactivities of **3** and **4** to **1**, no previous studies have examined human lenses for the presence of metabolites derived from the deamination of **3** or **4**. In this paper we report the synthesis of the reduced compounds **8** and **9**, the GSH adducts **7** and glutathionyl-3-hydroxykynurenine (**12**), the cyclised compounds **10** and **11** and the subsequent identification and quantification of **7**, **8** and **9** as novel human lens compounds in normal and cataractous lenses. In addition, the stability of **7**, **8**, **9** and **12** is described.

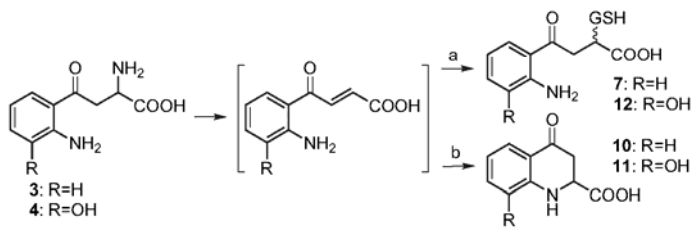
2. Results and discussion

2.1. Synthesis and spectral analysis of the proposed lens metabolites

In order to investigate if the proposed lens metabolites **7–12** were present in human lenses, authentic standards of each were synthesised (Schemes 2 and 3). 3-Hydroxyacetophenone was nitrated according to the method of Butenandt et al.[14] to afford 2-nitro-3-hydroxyacetophenone (**13**). 2-Nitroacetophenone (**14**) and **13** were separately condensed with glyoxylic acid according to a modified method of Bianchi et al.[15] to afford 4-(2-nitrophenyl)-4-oxo-2-butenoic acid (**15**) and 4-(3-hydroxy-2-nitrophenyl)-4-oxo-2-butenoic acid (**16**) in 45 and 55% yield, respectively. Hydrogenation of **15** and **16** at ca. 1 mg/mL in ethyl acetate with acetic acid (0.1–0.7%), catalysed by PtO_2, afforded **8** in 41% yield and **9** in 47% yield, respectively. Acetic acid was employed to assist protonation of the amino group produced and to prevent the formation of Michael adducts. Treatment of **3** and **4** with $NaHCO_3$ (ca. 0.5 M), following



Scheme 2. Synthesis of **8** and **9**. Conditions: (a) $Cu(NO_3)_2 \cdot 2.5H_2O$, AcOH, Ac_2O, ~16 h, 10–15 °C, 25% (**13**); (b) HCOCOOH, 24 h, 110 °C, 45% (**15**), 55% (**16**); (c) H_2/PtO_2, EtOAc, AcOH, rt, 41% (**8**), 47% (**9**).



Scheme 3. (a) Synthesis of **7** and **12**. Conditions: GSH, $Na_2CO_3/NaHCO_3$, pH 9.5, 48 h, 37 °C, 51% (**7**), 44% (**12**); (b) synthesis of **10** and **11**. Conditions: $NaHCO_3$, pH 9.5, reflux, 24 h, 26% (**10**), 22% (**11**).

the method of Tokuyama et al.,[16] promoted deamination and intramolecular Michael addition to produce **10** in 26% yield and **11** in 22% yield, respectively. In a similar manner to the synthesis of the GSH adduct **5**,[12] reaction of **3** and **4** in $Na_2CO_3/NaHCO_3$ buffer at pH 9.5 in the presence of GSH afforded diastereomeric mixtures (~1:1) of **7** in 51% yield and **12** in 44% yield, respectively.

Compounds **7**, **9** and **12** are novel compounds. The synthesis of **8** has been recently reported,[17] but no spectral data were provided. The mass spectrum (ES-MS/MS positive mode) of **8** showed a molecular ion at *m/z* 194 and major fragment ions at *m/z* 176 and 148. These are consistent with loss of water and formic acid, respectively. Using the same conditions, the mass spectrum of **9** revealed a molecular ion at *m/z* 210 with accompanying fragment ions at *m/z* 192 and 164 due to water and formic acid loss, respectively. Furthermore, the mass spectra of **7** and **12** showed the presence of a molecular ion at *m/z* 499 and 515, respectively, and fragment ions due to loss of glutamic acid and water. These were consistent with the mass spectra of **5**.[12]

The aromatic region of the ^{1}H NMR spectra of **7** and **8** revealed four adjacent aromatic resonances with chemical shifts and coupling patterns consistent with the aromatic ring of **3**.[11] Three aromatic protons were found for **9** and **12** with chemical shifts and coupling patterns characteristic of the aromatic ring of **4**.[18] Both **8** and **9** contained two isolated triplets at δ 2.65–3.26, consistent with the CH_2–CH_2 side chain.[19] The aliphatic side chains of **7** and **12** contained characteristic signals for a CH_2–CH moiety with diastereotopic methylene protons at δ ~3.3 and ~3.5 (2H, m) and a methine proton at δ ~3.8 (1H, m).[11] The distinctive deshielded diastereotopic protons are indicative of covalent attachment of the cysteine of GSH at C-2 of the **3** or **4** side chain.[11,12] Further analysis of **7** and **12** by COSY, HSQC and HMBC confirmed that the GSH moiety was intact and chemical shifts and coupling constants agreed with the literature.[12,20] The ^{1}H NMR spectra of **10** and **11** revealed the presence of four and three adjacent aromatic protons, respectively. Three additional protons from the methylene (δ 2.80–2.93) and methine (δ 4.20–4.35) groups were seen in both compounds. Their spectral data were in agreement with the literature.[21,16]

The absorbance and fluorescence spectral characteristics of **7**, **8**, **9** and **12** were similar to those observed by the major UV filters under the same conditions, i.e., **1**: λ_{max} at 263 and 365 nm and maximum fluorescence at λ_{ex} 360 nm/λ_{em} 500 nm; **2**: λ_{max} at 259 and 358 nm and maximum fluorescence at λ_{ex} 357 nm/λ_{em} 495 nm; **3**: λ_{max} at 257 and 359 nm and maximum fluorescence at λ_{ex} 355 nm/λ_{em} 485 nm; **4**: λ_{max} at 268 and 370 nm and maximum fluorescence at λ_{ex} 370 nm/λ_{em} 460 nm. Compounds **10** and **11** showed two fluorescence maxima at λ_{ex} 310/392 nm/λ_{em} 400/513 nm and λ_{ex} 370/392 nm/λ_{em} 457/547 nm, respectively. This was consistent with the literature.[12,19,16,22]

2.2. Identification and quantification of the proposed lens metabolites in human lenses

Eight normal human lenses ranging in age from 24 to 88 and two cataractous lenses of ages 60 and 70 were examined for the presence of the proposed lens metabolites. The nuclear and cortical regions were separately extracted with ethanol to allow isolation of kynurenine-based metabolites.[23] HPLC analysis was consistent with previous studies, showing the presence of the major UV filters **1**, **2**, **3** and **5** at 360 nm.[13] A typical lens profile is shown in Figure 2. Compound **4** was not detected in the investigated lenses. This may be due to the instability of **4**, which is an *ortho*-aminophenol and can readily oxidise.[24,25] Nuclear and cortical concentrations of **1** and **2** were found to be 0.1–3.6 nmol/mg lens (dry mass), while **3** and **5** were found in 0–580 pmol/mg lens (dry mass) (Tables 1 and 2). A marked decline in the levels of **1**, **2** and **3** was noted after the seventh decade of life, whilst the levels of **5** were generally higher after middle age. These findings are consistent with previous

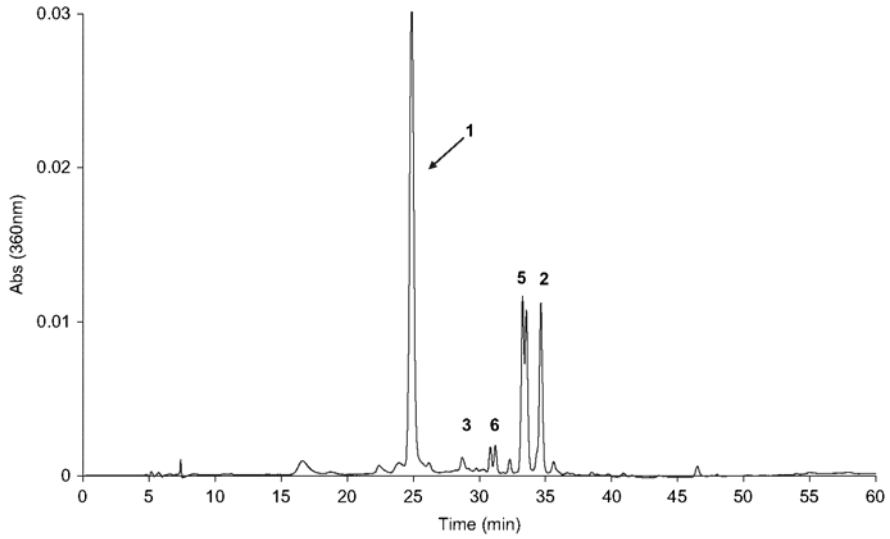


Figure 2. RP-HPLC profile of UV filter extract from a normal lens nucleus (88 years old) as described in Section 4.13. Detection was at 360 nm. 3-Hydroxykynurenine *O*-β-D-glucoside (**1**); 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (**2**); kynurenine (**3**); glutathionyl-3-hydroxykynurenine *O*-β-D-glucoside (**5**); cysteine-3-hydroxykynurenine *O*-β-D-glucoside (**6**).

studies.[5] The RP-HPLC absorbance traces of normal and cataractous lenses did not show the presence of the proposed lens metabolites, therefore, the eluent from the HPLC regions corresponding to the retention times of the synthetic compounds were collected, concentrated and investigated by ES-MS and MS/MS. MS analyses and spiking experiments with the authentic synthetic standards confirmed the presence of the reduced compounds **8** and **9** and the GSH adduct of **3** (**7**). The intramolecular Michael adducts **10** and **11** and the GSH adduct of **4** (**12**) were not detected in any of the lenses, suggesting that if present, they would be in very low levels (the estimated MS sensitivity was ~50 fmol).

The concentration of each lens metabolite was determined using a standard curve constructed with authentic synthetic standards. Compounds **8** and **9** were present in both the nucleus and cortex of all normal human lenses in 0–8.2 pmol/mg lens (dry mass) (Tables 1 and 2). Similar to the lenticular levels of **2**,[5] **8** and **9** did not show any clear age correlation in the first four decades, however, a steady decrease in **8** and **9** concentrations was observed after ca. 40 years of age in both the nucleus and cortex. This is in accordance with the decline of their metabolic precursors **3** (Tables 1 and 2) and **4**.[5] Interestingly, while **2** is typically present at 20–40% of the levels of **1** in normal human lenses,[5] **8** was only present at 0.5–2% of the levels of **3**. Levels of **9** could not be correlated to levels of its precursor since **4** was not detected in the investigated lenses.

Similar to **8** and **9**, the concentration of **7** was found to be low (0.3–28 pmol/mg lens (dry mass)) in both the nucleus and cortex. Even though no clear age-related correlation was seen for the concentrations of **7**, the normal nuclear concentration of **7** was generally higher than in the cortex of the same lens. This is consistent with **5**, which has been shown in this and related studies to generally be present in higher levels in the lens nucleus than in cortex.[5,26]

Table 1. Quantification of lens compounds in nuclei of normal and cataractous lenses

Nucleus age	**1**[b]	**2**[b]	**3**[b]	**5**[b]	**7**[a]	**8**[a]	**9**[a]
24	3600	692	586	0.00	1.80	4.00	2.67
27	3630	690	385	50.4	1.05	4.39	5.97
42	1840	408	135	39.5	0.63	0.86	1.14
47	2560	897	261	282	4.76	1.87	5.20
65	2220	527	188	562	28.0	1.83	2.40
66	658	268	73.1	89.9	3.09	1.37	0.69
83	426	72.0	61.4	41.1	2.00	0.70	0.00
88	452	127	14.9	210	7.60	0.32	0.63
60[c]	N/A	N/A	N/A	N/A	0.11	1.83	0.99
70[c]	N/A	N/A	N/A	N/A	0.10	1.21	1.31

pmol/mg lens (dry mass). N/A not determined.
[a] Determined by LC–MS.
[b] Determined by HPLC.
[c] Cataractous lenses.

Table 2. Quantification of lens compounds in cortices of normal lenses

Cortex age	**1**[b]	**2**[b]	**3**[b]	**5**[b]	**7**[a]	**8**[a]	**9**[a]
24	3100	558	317	0.00	0.34	2.24	1.76
27	2300	328	226	14.2	0.30	1.39	0.79
42	1500	365	114	27.5	2.21	8.20	6.04
47	3850	891	361	56.9	1.23	2.82	3.03
65	1900	407	177	173	18.4	1.40	2.79
66	1490	311	80.3	71.7	2.36	1.14	1.11
83	732	147	93.8	44.8	0.52	0.85	0.58
88	418	90.7	10.8	48.5	2.58	0.21	0.37

pmol/mg lens (dry mass).
[a] Determined by LC–MS.
[b] Determined by HPLC.

Due to the mode of lens removal in surgery, only the nuclear concentrations of the metabolites could be obtained for the cataractous lenses. The concentrations were found to be similar to those found in the normal nucleus (Table 1).

Taylor et al.[8] have investigated decomposition rates of the major UV filters at pH 7.0. They concluded that the deaminated UV filters cyclise very slowly to the yellow compounds and were more prone to react with lens components, such as GSH,[12] cysteine,[13] proteins[11,27,28,7] or NADPH[29] than to undergo intramolecular Michael addition. Therefore, the absence of **10** and **11** in the investigated lenses was not surprising.

2.3. Stability of the novel lens metabolites

The finding of the novel lens metabolites in only low pmol levels, particularly the reduced compound **8** and GSH adduct **7** (both derived from **3**), was surprising, especially given the much greater concentrations of **2** and **5** typically present in lenses relative to their precursor (**1**). Compound **1** has been shown to deaminate faster at pH 7.0 than **3**, but the difference is not significant enough to account for the lower levels of **7** and **8**.[8] The stabilities of **7**, **8** and **9** were therefore examined under extraction conditions (used to recover the UV filters from the human lenses) and HPLC conditions (used to analyse the extracts). They were also examined in phosphate-buffered saline (PBS, pH 7.0) under oxygen free conditions to mimic younger lenses, which have a relatively low oxygen environment and greater levels of antioxidants,[30,31] and in the presence of atmospheric oxygen to resemble older lenses, which are typically depleted of antioxidants.[31–33] In addition, the stability of **12** was investigated to confirm if the lack of its precursor (**4**) or stability of **12** may have contributed to its absence from the investigated lenses.

Known quantities of **7**, **8**, **9** and **12** in the presence of bovine lens tissue were separately extracted with 80% ethanol. Recovery was 57–79% (data not shown). This was comparable to the literature.[34] Additionally, **7**, **8**, **9** and **12** were stable over 24 h under extraction conditions and up to 5 h under HPLC conditions (data not shown). This confirms that the low concentrations measured for **7**, **8** and **9**, and the lack of detection of **12**, were not due to significant loss/breakdown during extraction and analysis of the lens extracts and are representative of the concentrations in the investigated lenses.

Compound **8** proved to be stable under physiological conditions in the presence of atmospheric oxygen for >200 h (data not shown). This suggests that formation of **8** may protect the lens from modification due to its greater stability compared to its precursor (**3**).[8] By contrast, **9** decomposed steadily under the same conditions (Fig. 3) resulting in its total disappearance after 7 days of incubation. The decomposition of **9** was accompanied by the generation of high molecular mass compounds (molecular masses >400) and predominantly less polar peaks, which were probably

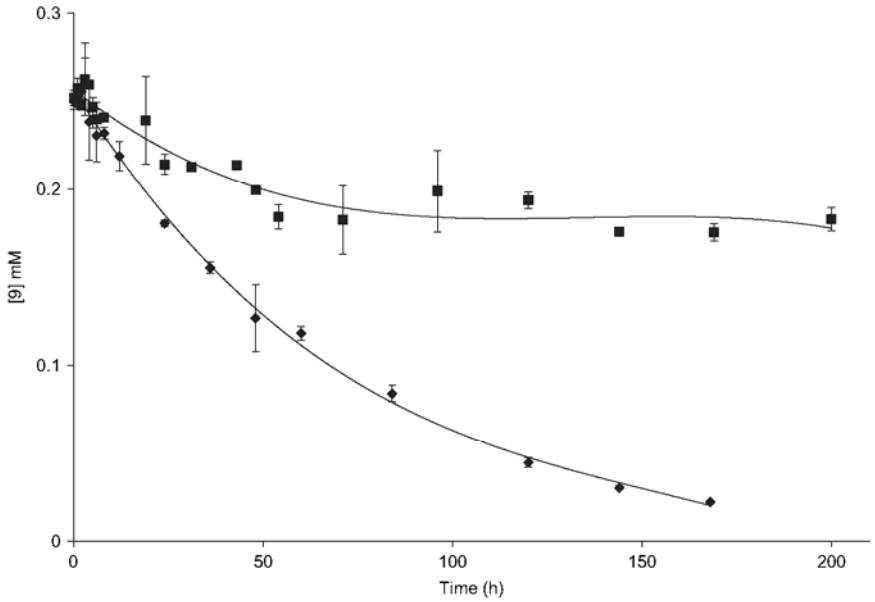


Figure 3. Compound **9** (0.25 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (2.0 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed by RP-HPLC as described in Section 4.2. Detection was at 254 nm. ◆—aerobic; ■—anaerobic.

dimeric aggregates arising from oxidation of this *ortho*-aminophenol.[35–37] The initially colourless incubation mixtures also became yellow/tanned over time, exhibiting λ_{max} at 240 and 420 nm, which are characteristic absorption peaks of xanthommatin based compounds.[38,39] A significantly greater stability of **9** was seen under anaerobic conditions in the presence of the potent radical scavengers, butylated hydroxytoluene and ascorbic acid, at pH 7.0. Only 27% of **9** was decomposed after 9 days of incubation in the presence of these agents (Fig. 3). Compound **9** is therefore expected to be unstable in the oxidising environment of cataractous lenses and may result in lens colouration and possibly contribute towards age-related nuclear cataract.[9,40]

Compound **7** decreased in concentration by 60–64% under physiological conditions, independent of the level of oxygen (Fig. 4). Similar to **7**, a significant decrease in the concentration of **12** was noted both in the aerobic (74% loss) and anaerobic conditions (80% loss) (Fig. 5). The breakdown products were identified by LC–MS and UV–vis as the

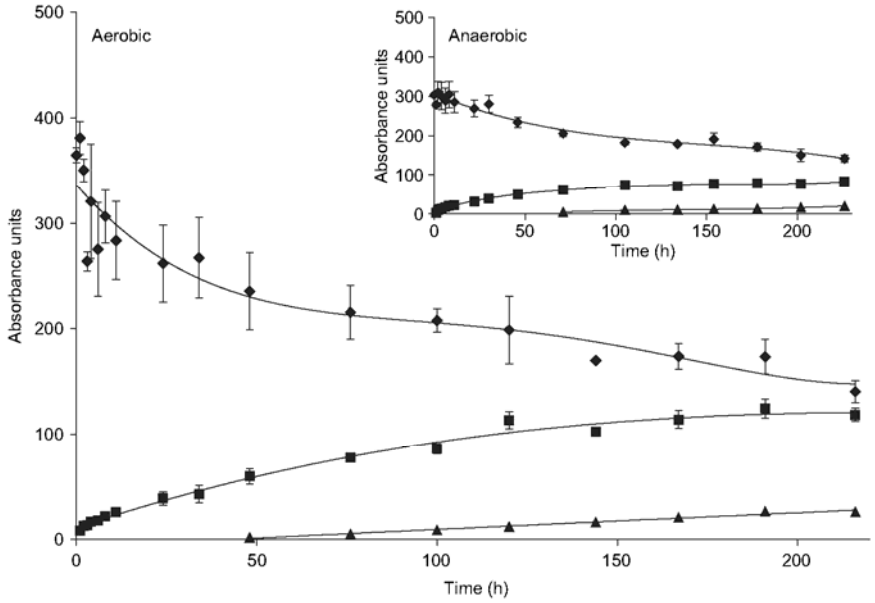


Figure 4. Compound **7** (0.4 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (0.2 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed by RP-HPLC as described in Section 4.2. Detection was at 254 nm. ◆—**7**; ■—deaminated **3**; ▲—**10**.

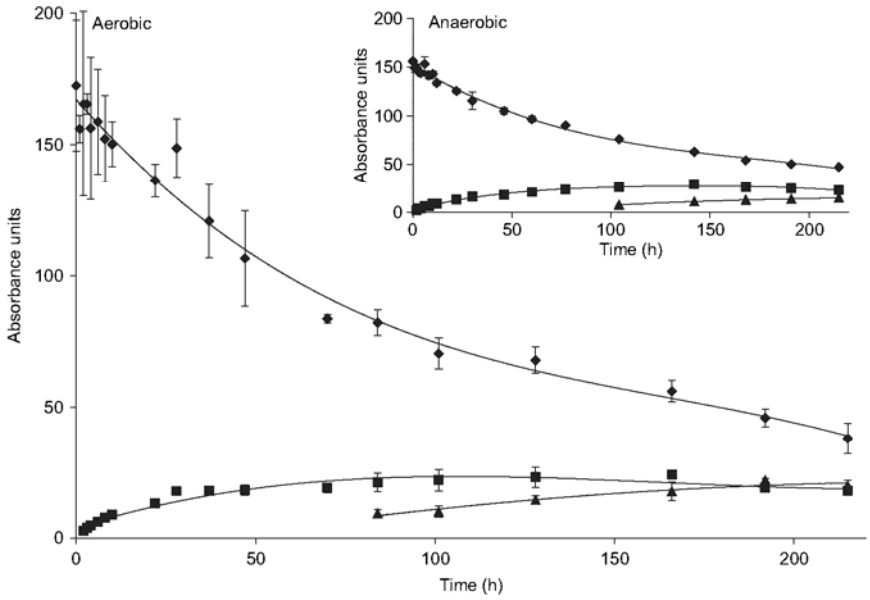


Figure 5. Compound **12** (0.1 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (2.0 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed by RP-HPLC as described in Section 4.2. Detection was at 254 nm. ◆—**12**; ■—deaminated **4**; ▲—**11**.

α,β-unsaturated ketones (via elimination of GSH) and yellow compounds, **10** and **11**. Similar instability of **3** and **4** was observed by Taylor et al.[8] The dynamic nature of GSH adduct formation and breakdown suggests that the GSH adducts may delay binding of deaminated **1**, **3** and **4** to lens proteins in younger lenses that have high levels of GSH (~4.5–6 mM),[5] but to a lesser degree in aged (GSH depleted) lenses.

3. Conclusion

This study describes the identification, via total synthesis and spectral analysis, and quantification of three novel human lens metabolites, 4-(2-aminophenyl)-4-oxobutanoic acid (**8**), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (**9**) and glutathionyl-kynurenine (**7**). These metabolites were isolated in pmol/mg levels (dry mass) in normal and cataractous lenses. Their spectral characteristics suggest that these compounds may have the capability to act as UV filters and protect the lens from UV-induced photodamage. Compound **8** was found to be very stable under conditions mimicking the lens environment. In contrast, both **7** and **9** were not stable. In particular, **9** was unstable under aerobic conditions mimicking older lenses and produced yellow-tanned products that may contribute to lens colouration observed with lens aging and age-related nuclear cataract. Compounds **7** and **12** showed similar decomposition rates to their precursors **3** and **4**, respectively.

4. Experimental

4.1. Materials

Acetonitrile (CH_3CN) was of HPLC grade. All other organic solvents were of AR grade and distilled prior to use. DL-Kynurenine sulfate salt (≥95%), 3-hydroxy-DL-kynurenine, GSH (99%), glyoxylic acid monohydrate (98%), Cu$(NO_3)_2 \cdot 2.5H_2O$ (99%), 2-nitroacetophenone (**14**) (95%) and trifluoroacetic acid (TFA) (>99%) were from Sigma–Aldrich. Glacial acetic acid (AcOH, >99.9%), acetic anhydride (99.0%) and ascorbic acid (99.7%) were purchased from BDH. Chelex resin (100–200 mesh) was purchased from BioRad and butylated hydroxytoluene from CalBiochem. CD_3OD (99.8%), CD_3COCD_3 (99.9%) and $CDCl_3$ (99.8%) were from Cambridge Isotope Laboratories. MilliQ® H_2O (purified to 18.2 MΩ/cm^2) was used in the preparation of all solutions. Dulbecco's phosphate-buffered saline (PBS), without calcium and magnesium, consisted of KCl 2.7 mM, KH_2PO_4 1.4 mM, NaCl 137 mM and Na_2HPO_4 7.68 mM.[41] The pH was adjusted to 7.0. Pre-washed chelex resin was added to the PBS buffer (~2 g/L) and left for 24 h prior to use. Normal human lenses were obtained postmortem from donor eyes at the Sydney Eye Bank (Sydney, Australia) and cataract lenses were obtained from K. T. Seth Eye Hospital (Rajkot, Gujarat, India) with ethical approval from the University of Wollongong Human Ethics Committee (HE99/001). After removal, lenses were immediately placed into sterile plastic screw-capped vials and kept at −80 °C until analysed. Thin-layer chromatography (TLC) plates were of normal phase $60F_{254}$ (Merck, Germany) and developed using a mobile phase of *n*-butanol/AcOH/H_2O (BAW, 12:3:5, v/v/v), unless otherwise stated, and reversed phase $18F_{254}$ using a mobile phase of 20% CH_3CN/H_2O (v/v). TLC plates were visualised under UV light (254 and 365 nm) and sprayed with ninhydrin. C18 reversed phase Sep-Pack® cartridges were purchased from Waters. Normal phase silica gel (230–400 mesh) was from Merck (Germany).

4.2. Instruments

Melting points were determined on a SMP 10 Stuart scientific (UK) apparatus and are uncorrected. Infrared spectra

were recorded on a Perkin–Elmer Paragon 1000 PC FTIR spectrometer.

^{1}H, ^{13}C, COSY (^{1}H–^{1}H correlation spectroscopy), HSQC (^{1}H–^{13}C heteronuclear single quantum correlation) and HMBC (^{1}H–^{13}C heteronuclear multiple bond correlation) NMR experiments were acquired on a Bruker Avance 400 spectrometer (^{1}H, 400 MHz; ^{13}C, 100 MHz) at 25 °C. NMR spectra were run in CD_3OD unless otherwise stated. The solvent signal was used as the internal reference. Resonances are quoted in parts per million and coupling constants (J) are given in Hertz.

Electrospray mass spectrometry (ES-MS) data were obtained in positive ion mode using a Micromass Q-TOF2 equipped with a nanospray source. MS settings were as follows: cone voltage 25 V, LM/HM 12/12, MCP 2300 V, mass range 50–600 m/z. For tandem MS (MS/MS) analysis, ions were subjected to a range of collisions energy settings (typically between 10–25 eV). High-resolution mass spectrometry (HRMS) was performed on a Q-TOF Ultima with lock spray. Leucine-encephalin (555.2692 Da) was used as the reference compound.

UV–vis absorbance spectra were obtained using a Varian DMS 90 UV–vis spectrometer. Fluorescence spectra were recorded on a Perkin–Elmer LS55 luminescence spectrometer. Slit widths were 10 nm for excitation and emission. PBS was used as solvent for all measurements.

Liquid chromatography–mass spectrometry (LC–MS) was run on a Shimadzu LC–MS-2010EV unit attached to an electrospray ionisation mass spectrometer (ES-MS). An Alltech (Prevail, 100 Å, 5 μm, 2.1×150 mm, C18) column was used at 27 °C. Mobile phase/gradient conditions consisted of buffer A (H_2O/0.1% formic acid v/v) and buffer B (CH_3CN/0.1% formic acid); 0–3 min (20% buffer B), 3–20 min (20–100% buffer B), 20–25 min (100% buffer B), 25–27 min (100–20% buffer B) and 27–35 min (20% buffer B). The flow rate was 0.2 mL/min and the source temperature was maintained at 170 °C. All spectra were acquired in continuum mode.

Reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Shimadzu HPLC. Standard curves and stability sample analyses were performed on a Phenomenex (Luna, 100 Å, 5 μm, 4.6×250 mm, C18) column with the following mobile phase system: buffer A (H_2O/0.05% TFA v/v) and buffer B (80% CH_3CN). The flow rate of 1 mL/min was kept constant with a mobile phase gradient as follows: 0–3 min (20% buffer B), 3–15 min (20–90% buffer B), 15–18 min (90% buffer B), 18–22 min (90–20% buffer B) and 22–28 min (20% buffer B). Detection was at 254 and 360 nm. Preparative separations were performed on the same instrument and using the same mobile phase system as for the analytical separations. Compounds **8** and **9** were purified using a Phenomenex (Luna, 100 Å, 10 μm, 15×250 mm, C18) column. The flow rate of 7 mL/min was kept constant with a mobile phase gradient as follows: 0–10 min (10% buffer B), 10–40 min (10–70% buffer B), 40–50 min (70% buffer B), 50–55 min (70–10% buffer B) and 55–65 min (10% buffer B). Compounds **7** and **12** were purified by the above preparative method with a mobile phase gradient as follows: 0–10 min (5% buffer B), 10–40 min (5–55% buffer B), 40–45 min (55% buffer B), 45–50 min (55–5% buffer B) and 50–60 min (5% buffer B). Compounds **10** and **11** were purified by the above preparative method while the mobile phase gradient was: 0–10 min (20% buffer B), 10–30 min (20–80% buffer B), 30–35 min (80% buffer B), 35–40 min (80–20% buffer B) and 40–50 min (20% buffer B).

4.3. Synthesis of 2-nitro-3-hydroxyacetophenone (13)

3-Hydroxyacetophenone (20.0 g, 0.15 mol) was dissolved in a mixture of glacial acetic acid (110 mL, 1.92 mol) and acetic anhydride (12.0 mL, 0.13 mol). This colourless solution was stirred and cooled down to 10–15 °C in a water/ice bath. Ground $Cu(NO_3)_2\cdot 2.5H_2O$ (40.0 g, 0.17 mol) was added to the reaction mixture over 3 h. The reaction mixture changed in colour to dark blue-green. The reaction was monitored by TLC (DCM, R_f 0.60) over a period of 8 h and left in the fridge O/N. The next day the reaction was continued at 10–15 °C for 7–8 h, with initial addition of ground $Cu(NO_3)_2\cdot 2.5H_2O$ (20.0 g, 0.08 mol) over a period of 1 h. Water (~200 mL) was added to the green solution and the reaction was stirred at 10–15 °C for ~1 h. The yellow precipitate was vacuum filtered, washed with cold water and air dried. The filtrate was extracted with DCM, washed with saturated brine, dried with $MgSO_4$ and decolourised with activated carbon. The yellow dried solid was purified by two sequential normal phase chromatography steps (DCM, followed by toluene/EtOAc, 5:1, v/v) to afford **13** (6.65 g, 25%, mp 134–136 °C, lit.[14] 131–132 °C) as a yellow solid. ν_{max} (KBr disc): 3100 (br), 1668, 1531, 1376, 1291, 798 cm^{-1}; δ_H ($CDCl_3$): 10.50 (1H, OH, s), 7.59 (1H, dd, J 7.3, 8.5, ArH-5), 7.23 (1H, dd, J 1.2, 8.5, ArH-6), 6.85 (1H, dd, J 1.2, 7.3, ArH-4), 2.50 (3H, s, CH_3); δ_C: 199.4 (CO-2), 155.0 (ArC-3), 140.6 (ArC-2, ArC-1), 136.9 (ArC-5), 121.0 (ArC-6), 118.1 (ArC-4), 30.3 (C-1).

4.4. Synthesis of 4-(2-nitrophenyl)-4-oxobut-2-enoic acid (15)

2-Nitroacetophenone (**14**) (0.50 mL, 3.03 mmol) was combined with melted glyoxylic acid monohydrate (2.78 g, 30.2 mmol) at 60 °C. The temperature was increased to 110 °C and the yellow reaction was left under vacuum. The reaction progress was monitored by TLC (EtOAc/1% AcOH, R_f 0.46). After 5 h another portion of glyoxylic acid monohydrate (0.20 g, 2.17 mmol) was added. After 14 h, the brown viscous reaction mixture was mixed with normal phase silica (~3 g) while still warm and set aside to cool down to rt. The mixture was purified by normal phase chromatography (DCM/1% AcOH) to yield **15** (0.3 g, 45%, mp 171–172 °C, lit.[15] 170–173 °C) as a white solid. ν_{max} (KBr disc): 3500–2300 (br), 1707, 1679, 1519, 1343, 1317, 1297, 740 cm^{-1}; δ_H (acetone-d_6): 8.25 (1H, d, J 8.0, ArH-3), 7.95 (1H, ddd, J 1.1, 7.5, 7.5, ArH-5), 7.85 (1H, ddd, J 1.3, 7.5, 8.0, ArH-4), 7.69 (1H, dd, J 1.3, 7.5, ArH-6), 7.26 (1H, d, J 16.1, H-3), 6.45 (1H, dd, J 16.1, H-2); δ_C: 192.8 (CO-4), 166.2 (CO-1), 147.6 (ArC-2), 139.9 (C-3), 135.8 (ArC-1), 135.5 (ArC-5), 134.4 (C-2), 132.5 (ArC-4), 129.8 (ArC-6), 125.4 (ArC-3); ES-MS m/z: 222 (MH^+, 89%).

4.5. Synthesis of 4-(3-hydroxy-2-nitrophenyl)-4-oxobut-2-enoic acid (16)

3-Hydroxy-2-nitroacetophenone (**13**) (0.4 g, 2.21 mmol) was combined with melted glyoxylic acid monohydrate (2.0 g, 21.7 mmol) at 60 °C. The yellow reaction mixture was further heated to 120 °C under vacuum for 24 h. The reaction progress was monitored by TLC (EtOAc/1% AcOH, R_f 0.48). After both 7 and 14 h, glyoxylic acid monohydrate (0.20 g, 2.17 mmol) was added. The brown viscous reaction mixture was mixed with normal phase silica (~3 g) while warm and set aside to cool down to rt. The mixture was purified by normal phase chromatography (DCM/1% AcOH) to yield **16** (0.28 mg, 55%, 158–159 °C, lit.[14] 158 °C) as a yellow solid. ν_{max} (KBr disc): 3500–2300 (br), 3300, 1704, 1676, 1601, 1518, 1431, 1273, 1168 cm^{-1}; δ_H ($CDCl_3$): 10.51 (1H, OH, s), 7.66 (1H, dd, *J* 7.3, 8.5, ArH-5), 7.31 (1H, dd, *J* 1.2, 8.5, ArH-6), 7.31 (1H, d, *J* 16.2, H-3), 6.90 (1H, dd, *J* 1.2, 7.3, ArH-4), 6.40 (1H, d, *J* 16.1, H-2); δ_C: 190.8 (CO-4), 166.3 (CO-1), 152.9 (ArC-3), 138.9 (C-3), 136.6 (ArC-2), 134.9 (ArC-1), 134.8 (ArC-5), 134.5 (C-2), 123.0 (ArC-6), 121.1 (ArC-4); ES-MS *m/z*: 238 (MH^+, 67%).

4.6. Synthesis of 4-(2-aminophenyl)-4-oxobutanoic acid (8)

A mixture of **15**[15] (255 mg, 1.15 mmol), EtOAc (280 mL), AcOH (450 μL) and PtO_2 (40 mg, 0.17 mM) was treated with H_2 gas at rt in the dark for 22 h. TLC analysis revealed multiple spots including **8** (normal phase, EtOAc/1% AcOH, R_f 0.88 and reversed phase, R_f 0.37) when visualised by UV light and sprayed with ninhydrin. The yellow reaction mixture was gravity filtered through a plug of Celite and the solvent was removed under vacuum. Crude **8** was dissolved in 10% CH_3CN/H_2O and loaded onto a preconditioned C18 reversed phase Sep-pack column and eluted with an increasing gradient of CH_3CN (10–40%). Fractions containing the product were pooled and lyophilised to yield **8** in ~80–85% purity. Further purification was achieved by preparative RP-HPLC as described above. The fraction containing **8** (t_R 31.3 min) was collected and lyophilised to yield an off-white solid (43 mg, 41%, mp 92–94 °C) in 99% purity. Found: M, 193.0745. Calculated for $C_{10}H_{11}NO_3$: M, 193.0739; ν_{max} (KBr disc): 3500–2300 (br), 3486, 3465, 3338, 1709, 1650, 763 cm^{-1}; δ_H: 7.82; (1H, dd, *J* 1.5, 8.2, ArH-6), 7.25 (1H, ddd, *J* 1.5, 7.0, 8.0, ArH-4), 6.75 (1H, dd, *J* 1.0, 8.0, ArH-3), 6.65 (1H, ddd, *J* 1.0, 7.0, 8.2, ArH-5), 3.25 (2H, t, *J* 6.4, H-2), 2.65 (2H, t, *J* 6.4, H-3); δ_C: 201.7 (CO-4), 177.0 (CO-1), 152.1 (ArC-2), 135.3 (ArC-4), 132.0 (ArC-6), 118.6 (ArC-1), 118.4 (ArC-3), 116.4 (ArC-5), 35.7 (C-2), 29.1 (C-3); ES-MS/MS of *m/z*: 194.08 (MH^+, 37%), 176.06 (100%), 148.03 (62%). λ_{max} 254 and 365 nm and maximum fluorescence at λ_{ex} 346.5 nm/λ_{em} 480.0 nm.

4.7. Synthesis of 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (9)

A mixture of **16**[14,15] (143 mg, 0.60 mmol), EtOAc (185 mL), AcOH (250 μL) and PtO_2 (35 mg, 0.15 mmol) was treated with H_2 gas at rt in the dark for 3 h. TLC analysis revealed **9** (normal phase, EtOAc/1% AcOH, R_f 0.65 and reversed phase, R_f 0.57) when visualised by UV light and sprayed with ninhydrin. The yellow reaction mixture was worked up similarly to **8** and further purification was achieved by using a C18 reversed phase Sep-pack column to yield **9** in ~80–85% purity. Subsequent purification by preparative RP-HPLC (t_R 23.8 min) yielded **9** as a light brown solid (59 mg, 47%, mp 134–135 °C) in 99% purity. Found: M, 209.0701. Calculated for $C_{10}H_{11}NO_4$: M, 209.0688; ν_{max} (KBr disc): 3500–2300 (br), 3370, 1709, 1681, 1652, 1195, 1149, 788, 719 cm^{-1}; δ_H: 7.82 (1H, dd, *J* 1.3, 8.3, ArH-6), 7.25 (1H, dd, *J* 1.3, 8.0, ArH-4), 6.57 (1H, dd, *J* 8.0, 8.3, ArH-5), 3.26 (2H, t, *J* 6.4, H-2), 2.66 (2H, t, *J* 6.4, H-3); δ_C: 202.0 (CO-4), 201.8 (CO-4), 176.9 (CO-1), 147.1 (ArC-3), 139.0 (ArC-2), 122.5 (ArC-6), 120.4 (ArC-1), 118.3 (ArC-4), 117.6 (ArC-5), 35.0 (C-2), 29.1 (C-3); ES-MS/MS of *m/z*: 210.08 (MH^+, 100%), 192.07 (28%), 164.07 (54%). λ_{max} at 267.4 and 369.4 nm and maximum fluorescence at λ_{ex} 346.5 nm/λ_{em} 435.0 nm.

4.8. Synthesis of glutathionyl-kynurenine (7)

DL-Kynurenine sulfate salt (100 mg, 0.32 mmol) was dissolved in argon-bubbled (~20 min) $Na_2CO_3/NaHCO_3$ buffer (20 mL, 25 mM, pH 9.5). GSH (500 mg, 1.62 mmol) was added and the light yellow solution was bubbled with argon for ~20 min, sealed with parafilm and incubated in the dark at 37 °C with shaking. The progress of the reaction was monitored by normal phase TLC (R_f 0.25) and reversed phase TLC (R_f 0.86). After 24 h, another portion of GSH (100 mg, 0.32 mmol) was added to the reaction mixture and the pH was readjusted to 9.5. After 72 h, the light yellow reaction mixture was acidified to pH 2 by dropwise addition of 25% HCl (v/v) and lyophilised. The crude solid was purified by preparative RP-HPLC (t_R 34.6 min), as described above, to obtain **7** (121 mg, 51%) as a ~1:1 mixture of diastereomers. Found: MH^+, 499.1534. Calculated for $C_{20}H_{26}N_4O_9S$: MH^+, 499.1499; ν_{max} (KBr disc): 3500–2300 (br), 1722, 1650 (br), 1545, 1200 (br) cm^{-1}; δ_H: 7.77 (1H, br d, *J* 8.2, ArH-6), 7.24 (1H, ddd, *J* 1.0, 7.6, 8.3, ArH-4), 6.73 (1H, d, *J* 8.3, ArH-3), 6.60 (1H, dd, *J* 7.6, 8.2, ArH-5), 4.74 (~0.5H, dd, *J* 4.8, 9.2, SCH_2CH), 4.68 (~0.5H, dd, *J* 5.7, 7.9, SCH_2CH), 4.00 (1H, t, *J* 6.2, $CH_2CHCOOH$), 3.94 (2H, s, CH_2COOH), 3.87 (1H, dd, *J* 3.8, 9.9, H-2), 3.62 (1H, m, H-3), 3.34 (1H, m, H-3), 3.31 (~0.5H, dd, *J* 4.8, 14.0, SCH_2), 3.22 (~0.5H, dd, *J* 5.7, 13.9, SCH_2), 3.07 (~0.5H, dd, *J* 7.9, 13.9, SCH_2), 2.92 (~0.5H, dd, *J* 9.2, 14.0, SCH_2), 2.58 (2H, t, *J* 6.9, $COCH_2CH_2$), 2.20 (2H, m, $COCH_2CH_2$); δ_C: 200.1 (CO-4), 200.0 (CO-4), 175.9 (CO-1), 175.8 (CO-1), 174.5 ($NHCOCH_2$), 174.4 ($NHCOCH_2$), 172.8 (CHCONH), 172.8 (CHCONH), 172.6 (CH_2COOH), 171.7 (CHCOOH), 152.6 (ArC-2), 135.6 (ArC-4), 132.0 (ArC-6), 118.3 (ArC-3), 117.9 (ArC-1), 116.2 (ArC-5), 54.5 (SCH_2CH), 53.8 (SCH_2CH), 53.7 ($CH_2CHCOOH$), 43.3 (C-2), 42.6 (C-2), 42.6 (C-3), 42.2 (C-3), 41.8 (CH_2COOH), 34.5 (SCH_2), 34.4 (SCH_2), 32.4 ($COCH_2CH_2$), 27.1 ($COCH_2CH_2$), 27.0 ($COCH_2CH_2$); ES-MS/MS of *m/z*: 499.0 (MH^+, 11%), 424.0 (30%), 370.0 (21%), 352.0 (62%), 259.0 (100%), 192.0 (21%), 179.0 (76%), 174.0 (30%). λ_{max} 256 and 356 nm and maximum fluorescence at λ_{ex} 350.5 nm/λ_{em} 475.0 nm.

4.9. Synthesis of glutathionyl-3-hydroxykynurenine (12)

3-Hydroxy-DL-kynurenine (100 mg, 0.44 mmol) was dissolved in argon-bubbled (~20 min) Na_2CO_3/$NaHCO_3$ buffer (100 mL, 25 mM, pH 9.5). GSH (500 mg, 1.62 mmol) was added to the light yellow solution and the solution was bubbled with argon (~20 min), sealed with parafilm and incubated in the dark at 37 °C with shaking. The progress of the reaction was monitored by normal phase TLC (R_f 0.28) and reversed phase TLC (R_f 0.87). Another portion of GSH (100 mg, 0.32 mmol) was added after 24 h and the pH was readjusted to 9.5. Similar to **7**, after 72 h the light yellow reaction mixture was acidified to pH 2 and purified by RP-HPLC (t_R 24.2 min), as described above, to obtain **12** (102 mg, 44%) as a ~1:1 mixture of diastereomers. Found: MH^+, 515.1520. Calculated for $C_{20}H_{26}N_4O_{10}S$: MH^+, 515.1448; ν_{max} (KBr disc): 3500–2300 (br), 1721, 1670 (br), 1545, 1200 (br) cm^{-1}; δ_H: 7.20 (~0.5H, dd, *J* 1.3, 8.4, ArH-6), 7.19 (~0.5H, dd, *J* 1.3, 8.4, ArH-6), 6.70 (1H, br d, *J* ~7.5, ArH-4), 6.40 (1H, br dd, *J* ~7.5, 8.4, ArH-5), 4.63 (~0.5H, dd, *J* 4.7, 9.2, SCH_2CH), 4.57 (~0.5H, dd, *J* 5.9, 8.0 SCH_2CH), 3.90 (1H, t, *J* 6.7, $CH_2CHCOOH$), 3.82 (1H, s, CH_2COOH), 3.81 (1H, s, CH_2COOH), 3.76 (1H, m, H-2), 3.50 (1H, m, H-3), 3.25 (1H, m, H-3), 3.20 (~0.5H, dd, *J* 4.7, 14.2, SCH_2), 3.10 (~0.5H, dd, *J* 5.9, 14.1, SCH_2), 2.97 (~0.5H, dd, *J* 8.0, 14.1, SCH_2), 2.80 (~0.5H, dd, *J* 9.2, 14.2, SCH_2), 2.47 (2H, t, *J* 7.0, $COCH_2CH_2$), 2.10 (2H, m, $COCH_2CH_2$); δ_C: 200.2 (CO-4), 200.1 (CO-4), 175.9 (CO-1), 175.9 (C-1), 174.5 ($NHCOCH_2$), 174.4 ($NHCOCH_2$), 172.9 ($CHCONH$), 172.8 ($CHCONH$), 172.6 (CH_2COOH), 171.7 ($CHCOOH$), 146.3 (ArC-3), 142.0 (ArC-2), 122.4 (ArC-6), 118.4 (ArC-1), 118.0 (ArC-4), 115.9 (ArC-5), 54.6 (SCH_2CH), 53.8 (SCH_2CH), 53.7 ($CH_2CHCOOH$), 43.3 (C-2), 42.8 (C-2), 42.6 (C-3), 42.4 (C-3), 41.8 (CH_2COOH), 34.5 (SCH_2), 34.5 (SCH_2), 32.4 ($COCH_2CH_2$), 27.1 ($COCH_2CH_2$), 27.0 ($COCH_2CH_2$); ES-MS/MS of *m/z*: 515.1 (MH^+, 32%), 440.1 (45%), 386.1 (32%), 368.1 (100%), 259.0 (97%), 208.1 (20%), 179.1 (41%). λ_{max} 256.5 and 356.5 nm and maximum fluorescence at λ_{ex} 350.5 nm/λ_{em} 475.0 nm.

4.10. Synthesis of kynurenine yellow (4-oxo-1,2,3,4-tetrahydroquinoline-2-carboxylic acid, 10)

Compound **10** was synthesised by the procedure of Tokuyama et al.[16] The progress of the reaction was monitored by normal phase TLC (R_f 0.85) and reversed phase TLC (R_f 0.61). Purification by preparative RP-HPLC (t_R 19.2 min), as described above, afforded **10** (26%) as a yellow-orange solid. The spectral data were in agreement with the literature.[16] Found: MH^+, 191.0571. Calculated for $C_{10}H_9NO_3$: MH^+, 191.0582; ν_{max} (KBr disc): 3500–2300 (br), 3341, 1695, 1655, 1618, 1283, 760 cm^{-1}; δ_H (acetone-d_6): 7.66 (1H, dd, *J* 1.5, 7.9, H-5), 7.30 (1H, ddd, *J* 1.5, 7.4, 8.0, H-7), 6.96 (1H, br d, *J* 8.0, H-8), 6.67 (1H, ddd, *J* 0.7, 7.4, 7.9, H-6), 6.20 (1H, s, N–H), 4.35 (1H, dd, *J* 5.5, 8.7, H-2), 2.90 (1H, dd, J 5.5, 16.2, H-3), 2.80 (1H, dd, *J* 8.7, 16.2, H-3), 1.40 (1H, s, COOH); δ_C: 191.6 (CO-4), 173.0 (COOH), 152.1 (C-8a), 135.8 (C-7), 127.4 (C-5), 119.5 (C-4a), 118.1 (C-6), 117.1 (C-8), 55.1 (C-2), 40.58 (C-3); ES-MS/MS of *m/z*: 192.2 (MH^+, 24%), 174.1 (32%), 146.1 (100%), 132.1 (14%). λ_{max} 260 and 378 nm and maximum fluorescence at λ_{ex} 310/392 nm and λ_{em} 400/513 nm.

4.11. Synthesis of 3-hydroxykynurenine yellow (8-hydroxy-4-oxo-1,2,3,4-tetrahydroquinoline-2-carboxylic acid, 11)

Compound **11** was synthesised by the procedure of Tokuyama et al.[16] The progress of the reaction was monitored by normal phase TLC (R_f 0.78). Purification by preparative RP-HPLC (t_R 15.5 min), as described above, afforded **11** (22%) as a yellow solid. The spectral data were in agreement with the literature.[16,21] Found: MH^+, 207.0386. Calculated for $C_{10}H_9NO_4$: MH^+, 207.0532; ν_{max} (KBr disc): 3500–2400 (br), 3400 (br), 1635, 1605, 1510, 1400, 1269, 1223, 787, 732 cm^{-1}; δ_H (acetone-d_6): 7.21 (1H, dd, *J* 1.3, 7.9, H-5), 6.82 (1H, dd, *J* 1.3, 7.9, H-7), 6.50 (1H, dd, *J* 7.9, 7.9, H-6), 4.20 (1H, dd, *J* 5.0, 10.5, H-2), 2.93 (1H, dd, *J* 5.0, 16.5, H-3), 2.80 (1H, dd, *J* 10.5, 16.5, H-3), δ_C: 193.4 (CO-4), 173.6 (COOH), 143.8 (C-8), 141.1 (C-8a), 117.3 (C-4a), 116.7 (C-7), 115.7 (C-5), 115.1 (C-6), 54.3 (C-2), 39.1 (C-3); ES-MS/MS of *m/z*: 208.0 (MH^+, 9%), 190.0 (27%), 162.0 (100%), 148.0 (5%), 120.0 (20%). λ_{max} 277 and 383 nm and maximum fluorescence at λ_{ex} 370/392 nm and λ_{em} 457/547 nm.

4.12. Stability studies

4.12.1. Stability under extraction, HPLC and pH 7.0 (aerobic) conditions. Compound **8** was dissolved in 80% ethanol/MilliQ® H_2O (v/v) to give a final concentration of 1.6 mM. The solutions (3×4 mL) were sealed and incubated in the dark at 37 °C with gentle shaking. Single aliquots were taken from each solution over 24 h and analysed by RP-HPLC. Separate incubations were repeated with **9** (0.2 mM), **7** (0.4 mM) and **12** (0.1 mM). Similar stability studies were carried out in MilliQ® H_2O/0.01% TFA (v/v) over 24 h and chelex treated PBS (pH 7.0) in the presence of atmospheric oxygen over 9 days.

4.12.2. Stability at pH 7.0 (anaerobic). Solutions of **9** (0.25 mM) with butylated hydroxytoluene (100 μM) and ascorbic acid (2.0 mM) in argon degassed chelex treated PBS (3×4 mL) were incubated in the dark at 37 °C with gentle shaking. Single aliquots were taken over 9 days from each solution and analysed by HPLC. Separate stability studies were repeated with **7** and **12** under similar conditions.

4.13. Lens preparation and RP-HPLC purification

Eight normal human lenses of ages 24, 27, 42, 47, 65, 66, 83 and 88 and two cataractous human lenses, assigned as Type III–IV according to the Pirie classification system,[42] of ages 60 and 70, were separated into cortex and nucleus using a 5 mm cork borer. The ends of each nuclear core were removed and added to the cortex. UV filters were extracted as described previously.[23] The average extraction recovery was measured by separately adding **7**, **8**, **9** and **12** in 500 pmol/mg lens protein. Subsequent extraction and HPLC analysis was as described. The retention times on RP-HPLC and ES-MS/MS spectra of synthetic **7–12** were determined. Lens extracts were separated by RP-HPLC using a Microsorb (MV, 100 Å, 4.6×250 mm, C18) column. The column was equilibrated in buffer A (H_2O/0.1% TFA (v/v)) at a flow rate of 0.5 mL/min. The following gradient was used: buffer B (80% CH_3CN/0.1% TFA, v/v), 0–5 min

(0–50% buffer B), 5–50 min (50% buffer B), 50–55 min, (50–0% buffer B) and 55–60 min (0% buffer B). The eluent was monitored at 360 nm. Fractions were collected at the known elution times of **8** (26.5% CH_3CN/0.1% TFA), **9** (20% CH_3CN/0.1% TFA), **7** (26.5% CH_3CN/0.1% TFA), **12** (20% CH_3CN/0.1% TFA), **10** (32.5% CH_3CN/0.1% TFA) and **11** (28% CH_3CN/0.1% TFA).

4.14. LC–MS quantification

Compounds **7–9** were analysed by LC–MS and detected in single-ion recording (SIR) mode on a Micromass Quattro micro in ESI positive mode.[43] Settings were as follows: LM1 16, HM1 15, cone 20 V, capillary 3.5 kV. Compounds were separated on a Phenomenex (Luna, 100 Å, 3 μm, 2.0×150 mm, C18(2)) column. The column was equilibrated in 95% buffer A (0.1% formic acid/H_2O, v/v) and 5% buffer B (80% CH_3CN/0.1% formic acid, v/v) at a flow rate of 0.1 mL/min. The following gradient was used: 0–5 min (5% buffer B), 5–50 min (5–50% buffer B), 50–55 min (50% buffer B), 55–60 min (50–5% buffer B) and 60–75 min (5% buffer B). The following masses were used for SIR data acquisition; 194.1 $[M+H]^+$ (**8**), 210.1 $[M+H]^+$ (**9**), 499.19 $[M+H]^+$ (**7**), 515.19 $[M+H]^+$ (**12**), 192.1 $[M+H]^+$ (**10**) and 208.1 $[M+H]^+$ (**11**). Lens compound was quantitated using standard curves of the synthetic samples.

4.15. HPLC Quantification

Compounds **1**, **2**, **3** and **5** were quantified using RP-HPLC at 360 nm. Compounds were separated on a Microsorb (MV, 100 Å, 4.6×250 mm, C18) column as described above. Standards were used as follows: synthetic **1** for the quantification of **1** and **5**, commercially available **3** for **3** and synthetic **2** for **2**.

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APPENDIX C

MEDIA COVERAGE

SCIENCE / TECHNOLOGY

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Chemistry Tour De Force In Hungary

Budapest congress provides European forum for the key challenges at the frontiers of chemistry today

Michael Freemantle

For centuries, chemists in Europe have been at the forefront of the molecular sciences, making discoveries that have changed the way we live and helping to tackle some of the most urgent problems facing the world today-for example, those relating to the environment, energy, health care, and medicine. Yet, despite the long history of chemistry in Europe, a congress on chemistry bringing together chemists from around Europe has never been held. That changed during the last week of August when the first-ever European Chemistry Congress took place at Eötvös Lorënd University, which is located on the west bank of the river Danube on the Buda side of Budapest, Hungary.

The meeting attracted 2,348 industrial, academic, and governmental chemists and molecular scientists from 57 countries. For the plenary sessions, the participants, just over 90% of whom were from Europe, gathered together in a white marquee, known as the plenary tent, on the university campus. The wind that blew along the Danube at times shook and rattled the tent but, apart from a few unsettling moments, failed to distract the audience or the plenary speakers from the chemistry that was presented.

The congress was organized by the European Association for Chemical & Molecular Sciences (EuCheMS) and cosponsored by the French Chemical Society, the German Chemical Society, and the U.K.'s Royal Society of Chemistry (RSC). EuCheMS was registered as a not-for-profit organization in Belgium earlier this year. Before 2004, it was known as the Federation of European Chemical Societies, which was founded in 1970. The association now has 50 member societies from 36 countries. The individual membership of the societies totals about 150,000.

INTERACTIVE PHOTO GALLERY

Michael Freemantle/C&EN

YOUNG CHEMIST Award finalist Mizdrak (right) poses with ACS President E. Ann Nalley.

LAUNCH INTERACTIVE PHOTO GALLERY

* Macromedia Flash Player 7 is required to view interactive feature.

The geographical diversity of Europe's chemical societies has led to a proliferation of national meetings and voices in European chemistry over the past century. EuCheMS aims to provide a powerful single voice for European chemists that addresses some of the major challenges faced today, both inside and outside the European Union.

"EuCheMS plans to establish European chemistry as a recognizable entity, comparable with the American Chemical Society in the U.S.," said W. James Feast, who became RSC president in July. Feast is a research professor at Durham University in England, and also works at Eindhoven University of Technology, in the Netherlands. "We have to put together the structures and mechanisms to support European chemistry for the sake of all our futures," he said. The Budapest congress was one of the initial steps in this mission.

Michael Freemantle/C&EN

OPEN AIR Plenary sessions at the congress in Budapest took place in a tent on the campus of Eötvös Loránd University.

"The growing need to enter into partnership with other science groups related to chemistry and the molecular sciences led European chemists to organize the congress," said congress Chairman Gábor Náray-Szabó, who is a past president of EuCheMS and the Hungarian Chemical Society and a chemistry professor at Eötvös Loránd University. "Our aim was to provide a showcase for chemical sciences in Europe," he told C&EN.

The meeting consisted of around 1,400 oral and poster presentations, including plenary lectures by five Nobel Laureates. A glance at the titles of the special topic symposia indicates the ambitious and wide-ranging nature of the congress. Symposia topics included theoretical and computational chemistry, spectroscopy, catalysis, medicinal chemistry, chemical imaging, nanomaterials, environmental chemistry, food and health, teaching, green and sustainable chemistry, polymer architecture, organic synthesis, coordination chemistry, biomolecules, and nuclear and radiochemistry. Some of the presentations are highlighted in meeting briefs that follow (see page 44).

Ahmed H. Zewail, professor of chemistry and physics at California Institute of Technology, was invited to present the opening plenary lecture. "Unlike the other plenary lecturers, I was not born in Europe, nor do I live in Europe, so I'm an honorary European today," he remarked. Zewail won the Nobel Prize in Chemistry in 1999 for his work on the development of femtochemistry, the study of fundamental chemical reactions on the femtosecond timescale using ultrashort laser flashes.

Zewail introduced some of the challenges facing chemists today by showing a letter, written in 2004 by a 10-year-old named Hani. "Although we can't see our future with a crystal ball, what we do today will affect our planet tomorrow," Hani wrote.

"Protecting our world now is an overriding challenge," Zewail said. "We can help to do this by making chemistry exciting for boys and girls. To do this, we need to get to the fundamentals of chemistry. And one of the fundamentals, which no other scientific discipline addresses, is the chemical bond."

The chemical bond is central to life, but it has been mainly studied in systems that are in equilibrium, he observed, adding that, in biological systems, structures are dynamic and often far from equilibrium.

"Nature employs huge and highly complex architectures to carry out specific and selective processes, for example, to move ions through channels in cells," he said. "One of the key challenges for chemists is understanding selectivity in biological systems. At present, we do not understand why there is a bias toward the formation of one complex structure rather than another for a specific function. And we know little about how dynamics influences and controls structure and function in biological systems."

Michael Freemantle/C&EN

PLENARY Feast (right) chaired the opening session in which Zewail (left) spoke about 4-D chemistry.

The development of instruments that can image atoms in real time and enable us to visualize structures as they pass through transition states will help us to understand the bonding in and dynamics of complex structures that are not at equilibrium, according to Zewail. He calls this four-dimensional visualization, the four dimensions being time and the three dimensions of space.

Zewail described a technique known as 4-D ultrafast electron microscopy (UEM) that is being developed by his group. UEM relies on the use of femtosecond laser pulses to free electron packets, some containing only one electron, from a photocathode. "Electrons scatter more efficiently than X-rays and therefore allow us to obtain diffraction patterns with atomic resolution," he explained. "We can use the 4-D imaging technique to visualize complex systems and observe how chemical bonds are forming, changing, and breaking with femtosecond time resolution and angstrom spatial resolution. Eventually, we hope to use UEM for biological imaging, for example, to study how cell membranes and ion channels function."

Kurt Wüthrich, professor of biophysics at **Swiss Federal Institute of Technology (ETH)**, Zurich, also described the use of instrumental techniques to explore the interface between chemistry and biology. Wüthrich, who is also the Cecil H. & Ida M. Green Professor of Structural Biology at **Scripps Research Institute**, won the **Nobel Prize in Chemistry in 2002** for his development of nuclear magnetic resonance spectroscopy for determining the 3-D structures of biological macromolecules in solution.

NMR spectroscopy, which was first reported in 1946, has played a pivotal role in modern biology and medicine, Wüthrich said in his plenary lecture. He focused his talk on structure determination of proteins such as prions.

"In my own field, the use of solution NMR techniques to study proteins has been pursued at an ever-increasing pace over the past 35 years," he noted. The Wüthrich group has employed NMR techniques to determine the structures of a variety of proteins including small membrane proteins reconstituted in water-soluble micelles.

In another plenary lecture, Jean-Marie Lehn, chemistry professor in the Collège de France at Louis Pasteur University in Strasbourg, discussed complexity and dynamics in chemical systems. Lehn won the **Nobel Prize in Chemistry in 1987** for his work on molecular recognition and the development of supramolecular chemistry.

"Chemistry started when, after the Big Bang, the universe became cool enough for molecules to form," he said. "The basic question is: How did matter become complex? The answer is self-organization."

Supramolecular chemistry explores systems undergoing self-organization. Lehn explained that it is intrinsically a dynamic chemistry because of the lability of the noncovalent interactions connecting the molecular components of a supramolecular entity and the resulting ability of the supramolecular species to exchange their constituents reversibly.

These characteristics of supramolecular chemistry are also observed in molecular chemistry when covalent bonds that break and re-form reversibly are introduced into molecules. Molecules with reversible covalent bonds can be used as building blocks to construct combinatorial libraries of molecules in dynamic equilibrium. When a template molecule is introduced to such a library, it selects the best-fitting molecule from the library and uses noncovalent interactions, such as hydrogen bonding, to form a host-guest complex.

Lehn has a name for the chemistry of systems that are dynamic at both the molecular and supramolecular levels. He calls it "constitutional dynamic chemistry," or CDC, and contrasts it with "constitutional static chemistry." The latter relies on explicit programming and self-organization by design to synthesize a desired molecular or supramolecular product. CDC, on the other hand, takes advantage of dynamic diversity to achieve variation and selection.

"The implementation of selection in chemistry introduces a fundamental change in outlook," Lehn observed. "CDC opens the way to adaptive and evolutive chemistry—a kind of Darwinian chemistry."

Mastering complexity is one of the key challenges for chemists, according to Igor Tkatchenko, a director of research at the University of Bourgogne in Dijon, France. He was vice chairman of the French Chemical Society until earlier this year and represented the society at the congress. "But the ultimate challenge lies in tackling problems relating to energy and the environment," he told C&EN.

Geoscientist **Paul Crutzen**, of Max Planck Institute for Chemistry in Mainz, Germany, and Scripps Institution of Oceanography, painted a frightening picture of the impact of human activities on Earth. "Human activities accelerated over the past few hundred years, creating a new geological era, the 'Anthropocene,' " said Crutzen, who shared the Nobel Prize in Chemistry in 1995 for his work on the formation and decomposition of ozone in the atmosphere.

Michael Freemantle/C&EN

CHALLENGES Crutzen (second from right) answers questions following his lecture on atmospheric chemistry and climate.

Over the past three centuries, he noted, the human population has increased to over 6 billion people; industrial output and fish catch have both increased 40-fold; and energy usage, 16-fold. Almost 50% of the land surface has been transformed by human activities. In particular, since World War II, there has been a "great acceleration" in population, water usage, transport, tourism, and many other human activities. "All this has played a major and increasing role in changing the basic properties of the atmosphere and Earth's surface," he observed.

"Human activities are affecting and, in many cases, out-competing natural processes, causing, for instance, the rise of greenhouse gases, which has had an impact on climate, urban and regional air pollution, and consequently on human and ecosystem health," he continued. "We have created chemical instability in the atmosphere. If we do not reduce rising temperatures, we may well cook."

Although tremendous progress has already been made, for example, in reducing emissions of chlorofluorocarbons and consequently ozone depletion, major questions remain and much research needs to be done, Crutzen said. Energy saving, the use of nuclear and renewable forms of energy, and carbon dioxide sequestration will help to reduce the emission of greenhouse gases into the atmosphere, he suggested. "But the tasks are enormous," he added.

One possibility Crutzen and others have proposed for consideration as a last resort, if we cannot reduce global warming by other means, is to fire rockets into the atmosphere to discharge hydrogen sulfide (C&EN, **Aug. 7, page 19**; **Sept. 18, page 13**). H_2S would form sulfate particles that could cool our planet in the same way sulfate particles produced by volcanic eruptions do.

George Olah, professor of chemistry and director of the Loker Hydrocarbon Research Institute at the University of Southern California, reminisced about Budapest at the beginning of his plenary lecture. "It is pleasure to be back in my native city, which I left 50 years ago," said Olah, who won the **1994 Nobel Prize in Chemistry** for his work on carbocation and hydrocarbon chemistry.

Michael Freemantle/C&EN

METHANOL MAVEN Olah (left) and *Angewandte Chemie* Editor Peter Gölitz greet congress participants at an event at which Olah signed copies of "Beyond Oil and Gas: The Methanol Economy," a book he coauthored (C&EN, **Oct. 2, page 51**)

Like Crutzen, his lecture focused on the environment. "Air belongs to everybody," he said in his plenary lecture. "It does not matter who releases CO_2 into the atmosphere. It's an international problem, and we need to find solutions.

"Despite the diminishing resources of nonrenewable fossil fuels and the effect of CO_2 formed from them on global warming, there is a continuing need for hydrocarbons and their derived products," he continued. "Nature gave us hydrocarbons, in the form of oil, coal, natural gas, and other resources, as a great present. But they are not renewable on a human timescale, and we are using them up at a frightening rate."

Olah outlined an approach to solving this problem that relies on methanol. This liquid, he said, can be used as a fuel for internal combustion engines and fuel cells, and it can also be converted to important synthetic hydrocarbons such as ethylene and propylene by catalytic dehydration. The methanol could be produced by direct oxidation of methane in existing supplies of natural gas without resorting to the production of synthesis gas (carbon monoxide and hydrogen) by Fischer-Tropsch chemistry, a process that consumes vast amounts of energy.

But the true methanol economy, as Olah calls it, will not depend on the methane oxidation route. It will rely on the use of hydrogen to reduce CO_2 sequestered from the atmosphere, he said.

Both the oxidation and reduction routes present major scientific and technological challenges. For example, current methods for both routes lead to the formation of formaldehyde and formic acid as by-products and low yields of methanol.

In addition, substantial amounts of energy would be needed to generate sufficient supplies of hydrogen from water to convert CO_2 into methanol. This energy, Olah suggested, could come from safe nuclear power plants as well as alternative energy sources such as sunlight and wind. The selective absorption of CO_2 from the atmosphere is also difficult, he added.

"The methanol economy can free mankind from dependence on diminishing supplies of natural fossil fuels," Olah said. "At the same time, recycling excess CO_2 from the atmosphere will mitigate the man-made effects of global warming."

The Budapest congress also tackled the challenge of enhancing the public's perception of chemistry.

"The image of chemistry is continually worsening all over the world," Náray-Szabó told C&EN. "One reason is the potential environmental threat by the uncontrolled production and use of chemicals."

Tkatchenko added that, although chemistry is a central science, it has poor visibility. "Chemistry should be regarded as a fundamental partner rather than just a service science in its interactions with physics, biology, and other sciences," he said. "Furthermore, we need to bring more youngsters to chemistry."

Michael Freemantle/C&EN

Tkatchenko (left) Nitschke (center) Náray-Szabó (right)

The Molecular Frontiers Institute, unveiled at the Budapest congress, attempts to address the image problem, particularly in regard to encouraging young people to take an interest in chemistry (C&EN, **Sept. 11, page 13**). Its activities will include the award of prizes to youngsters 12 to 18 years old who submit the best questions about molecular science.

Youth also featured in another event at the congress, the 2006 European Young Chemist Award. "The award was intended to showcase and recognize the excellent research being carried out by young scientists working in the chemical sciences," explained Bruno Pignataro, chemistry professor at the University of Palermo, Italy. He organized the competition, which was sponsored by the Italian Chemical Society in conjunction with other European chemical societies.

Almost 120 chemists, all of whom participated in the Budapest congress and were under 34 years old, entered the competition. The award was open not just to chemists working in Europe but also to European chemists working outside the European Union. For example, Jasminka Mizdrak, who was born in Croatia and was one of the 14 finalists, is carrying out Ph.D. research on the chemistry of human lens and cataracts at the University of Macquarie, in Sydney, Australia.

The 14 finalists made short presentations of their research during the congress. Jonathan Nitschke, an organic chemist at the University of Geneva, Switzerland, won first prize, which consisted of $2,280, a certificate, and a gold medal with the EuCheMS logo. His group is developing techniques based on the simultaneous formation of covalent and coordinative bonds during the course of a single overall self-assembly process.

"We are learning how to direct this process, enabling us to build structurally complex objects from simple subcomponents," Nitschke told C&EN. "We are also investigating new routes to metal-containing polymers and ways to use self-assembly to complement traditional techniques of organic synthesis."

Two second prizes, each consisting of $1,020, a certificate, and a silver medal with the EuCheMS logo, went to Lee Cronin, a chemistry professor at the University of Glasgow in Scotland, and Javier García-Martínez, who leads the molecular nanotechnology group at the University of Alicante in Spain. In his presentation at the congress, Cronin outlined his recent research on the supramolecular self-assembly of polyoxometalate clusters. García-MartÍnez described his work on hierarchical nanostructures prepared by combining biomolecules found in cell membranes and cationic surfactants.

The award jury also gave special mentions to two other finalists: Elisabete Carvalho, a Ph.D. student at the University of Porto in Portugal, and Iryna A. Koval, a recent Ph.D. graduate in chemistry at Leiden University in the Netherlands. Carvalho presented research on the interaction of salivary proteins with grape seed procyanidins. Koval talked about her work at Leiden on the use of synthetic model compounds to unravel the mechanism of the enzymatic cycle of catechol oxidase, an enzyme that is responsible for melanin formation in plant tissues.

Toward the end of the congress, Feast spoke about his vision of chemistry research in Europe. "As chemists, we live in interesting times and face a number of significant challenges that we have to solve if we are to pass the planet on to our grandchildren in a decent state," he said. "These challenges can also be regarded as opportunities for doing useful things for society and creating wealth.

"Society requires us to provide new pharmaceuticals and medical diagnoses, improved methods of food production, and new materials," he continued. "Chemists are also required to monitor and control the fate of chemicals, agrochemicals, and pharmaceuticals; develop renewable sources of energy; and remove and recycle greenhouse gases. In addition, there is a need for clean and energy-efficient methods for the production of chemicals, agrochemicals, and pharmaceuticals using renewable feedstocks."

The chemical sciences will thus play a key role in meeting the scientific, technological, economic, and human challenges of the 21st century, he added. Although these challenges are not unique to Europe, chemists there have the responsibility to help solve them, especially by building up influence and contacts among the public and decisionmakers, he said.

However, if European chemists want to use these congresses to showcase European chemistry and influence public opinion, they will need to get the press on board. Surprisingly, press were not encouraged to cover the congress. Accredited journalists who registered on the congress website received a press badge that entitled them to attend the congress on one day only.

The 2nd European Chemistry Congress will be coorganized by EuCheMS and the Italian Chemical Society. It will take place in Torino, Italy, on Sept. 16-20, 2008.

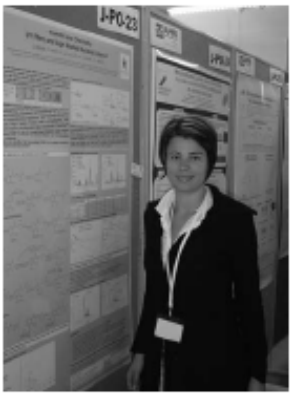
http://www.pubs.acs.org/

November / December 2006

MACQUARIE UNIVERSITY SYDNEY

MACQUARIE University News

Groundbreaking chemistry research into cataracts



PhD student Jasminka Mizdrak at the inaugural European Chemistry Congress.

Macquarie PhD student Jasminka Mizdrak's research caught the eye of the chemistry world when she gave a presentation at the first-ever European Chemistry Congress, held recently in Budapest, Hungary.

Croatian-born Mizdrak was selected from nearly 120 early-career chemists to be one of 14 finalists in the 2006 European Young Chemist Award. All finalists were asked to give a presentation at a special session of the Congress.

Under the joint supervision of Dr Joanne Jamie of Macquarie's Department of Chemistry and Biomolecular Sciences, the Save Sight Institute's Professor Roger Truscott and the Heart Research Institute's Professor Michael Davies, Mizdrak has been attempting to better understand how cataracts form in the human eye.

"There are about 38 million cases of blindness worldwide and around 40 per cent of these are due to cataract, a condition that results in opacification or clouding of the eye lens," she explains. "In Australia alone, 1.5 million Australians aged over 55 suffer from cataract, which is 31 per cent of this age group. Surgical removal and replacement of the lens – costing around $3000 per eye in Australia – is currently the only available treatment.

"The essential first step in developing preventative or therapeutic drugs is to fully understand how and why cataracts form. I have been focusing on the natural UV filters in the eye lens, which are implicated in age-related cataract. I have conducted model studies that have led to a greater understanding, at the molecular level, of the role of UV filters in cataract formation and have identified four novel UV filters in human lenses."

Macquarie University News, November/December 2006

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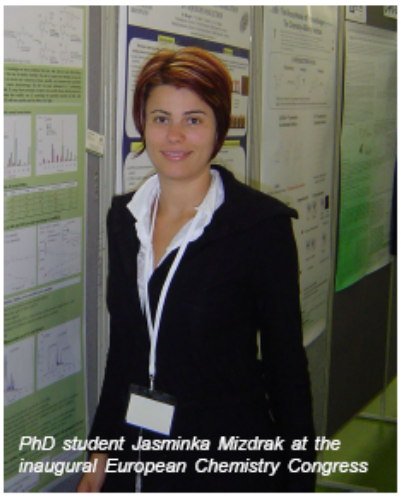
MACQUARIE UNIVERSITY ~ SYDNEY
November 2006

Cataract research catches the eye


PhD student Jasminka Mizdrak at the inaugural European Chemistry Congress

Croatian-born Macquarie PhD student Jasminka Mizdrak was selected from nearly 120 early-career chemists attending the first-ever European Chemistry Congress, held recently in Budapest, Hungary, to be one of 14 finalists in the 2006 European Young Chemist Award.

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Contact: Jasminka Mizdrak
(02) 9850 8275
jmizdrak@chem.mq.edu.au

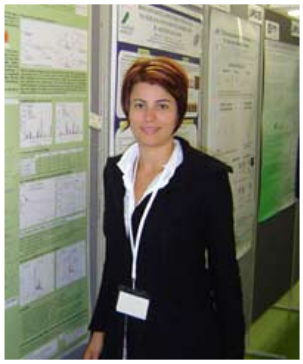
Research Active
Produced by the Public Relations and Marketing Unit
Macquarie University
North Ryde NSW 2109
Contact: Greg Welsh (Editor)
(02) 9850 7383
greg.welsh@mq.edu.au
www.researchactive.mq.edu.au

Postgrad and beyond

A Macquarie University newsletter for postgraduate study

health

Research to slow down cataracts



Jasminka Mizdrak

Slowing down the progression of age-related cataracts, and ultimately their prevention, are the primary aims of PhD research being undertaken at Macquarie University.

Under the joint supervision of Associate Professor Joanne Jamie from Macquarie's Department of Chemistry and Biomolecular Sciences, Save Sight Institute's Professor Roger Truscott and the Heart Research Institute's Professor Michael Davies, Jasminka Mizdrak has been attempting to better understand how cataracts form in the human eye.

"There are about 38 million cases of blindness worldwide and around 40 per cent of these are due to cataract, a condition that results in opacification or clouding of the eye lens," explains Mizdrak. "In Australia alone, 1.5 million Australians aged over 55 suffer from cataract, which is 31 per cent of this age group. Surgical removal and replacement of the lens - costing around $3000 per eye in Australia - is currently the only available treatment."

"The essential first step in developing preventative or therapeutic drugs is to understand how and why cataracts form. I have been focusing on the natural UV filters in the eye lens, which are implicated in age-related cataract, and have conducted model studies that have led to a greater understanding, at the molecular level, of the role of UV filters in cataract formation."

Research method

Mizdrak's work has involved extracting lenses from the eyeballs of cows. She has utilised cows as it has been shown that their proteins are very similar to human lens proteins and therefore are good models of the human proteins.

One strand of the project involved isolating the proteins from the lenses and irradiating them, in the presence of the UV filters, with UV light. "We use irradiation because of the fact that a lot of people believe that UV light can cause cataract and damage," says co-supervisor Dr Joanne Jamie. "Jasminka's work has been helping to show that when these small molecules bind with the lens proteins, the presence of UV light accentuates the damage."

Exciting findings

The changes that Mizdrak sees when the small molecules are bound to the proteins upon UV light radiation are quite distinctive of cataract according to Jamie.

"What is really exciting about this work is that Jasminka is helping to show the cause of the changes we see upon cataract formation," she says. "This is aiding our understanding of the mechanism of cataract formation and is really important because that can help us to prevent cataracts through different practices or drug design in the future."

International recognition

Mizdrak, who was born in Croatia, was selected from around 120 early-career chemists to present at the first-ever European Chemistry Congress, held in Budapest, Hungary last year. She was then selected to be one of 14 finalists in the 2006 European Young Chemist Award.

Mizdrak hopes to submit her thesis by April this year and in the long term is working towards a research position combining synthetic chemistry and biochemistry.

For further information contact Jasminka Mizdrak at jmizdrak@chem.mq.edu.au or Associate Professor Joanne Jamie joanne.jamie@mq.edu.au

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Jasminka's cataract research goal

Cataracts are one of the world's leading causes of blindness.

Croatian-born PhD student, Jasminka Mizdrak cataract research was recently acknowledged at the European Chemistry Congress in Budapest, Hungary.

One of 14 finalists of the European Young Chemists Award, Jasminka was the only finalist from the Southern Hemesphere.

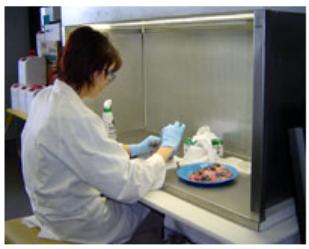
Jasminka's research is based on human age related nuclear (ARN) cataract. Currently, there is no prevention or cure for this condition.

The focus of her project is the identification and synthesis of human lens UV filters, investigation of their properties and responsiveness to UV light in either free or protein-bound state.

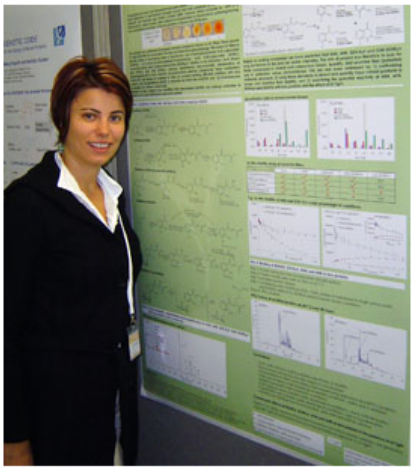
"I have conducted model studies that have led to a greater understanding, at the molecular level, of the role of UV filters in cataract formation and have identified four novel UV filters in human lenses," says Jasminka.

"This will aid understanding of the cause of cataract and assist in the development of either a preventative and/or therapeutic treatment for this disease."

The only treatment available at this point in time is surgery which costs approximately AU$3000 in Australia for each eye.



Jasminka in the lab extracting lenses for her experiments



Jasminka Mizdrak with a poster explaining her research discoveries so far

Jasminka started her science career at Macquarie with a Bachelor of Science(Biological Chemistry) with Honours.

She chose to commence her research at Macquarie because "the friendly and professional Department of Chemistry and Biomolecular Sciences attracted me to come back and start my PhD project. Great support and help is provided by all stuff members and students within."

Her project supervisors are Associate Professor Joanna Jamie (from the Department of Chemistry and Bimolecular Sciences at Macquarie), Professor Roger Truscott (from The Save Sight Institute) and Professor Michael Davies (from The Heart Research Institute).

"Since my project is very versatile I was lucky enough to explore collaborations between Macquarie and other universities and various institutes. This allowed me to explore different research fields and establish valuable contacts."

To learn more about her research, contact Jasminka Mizdrak. For more information about research opportunities at Macquarie University, visit the Higher Degree Research Unit website.

http://www.international.mq.edu.au/