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Novel human lens metabolites from normal and cataractous human lenses

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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

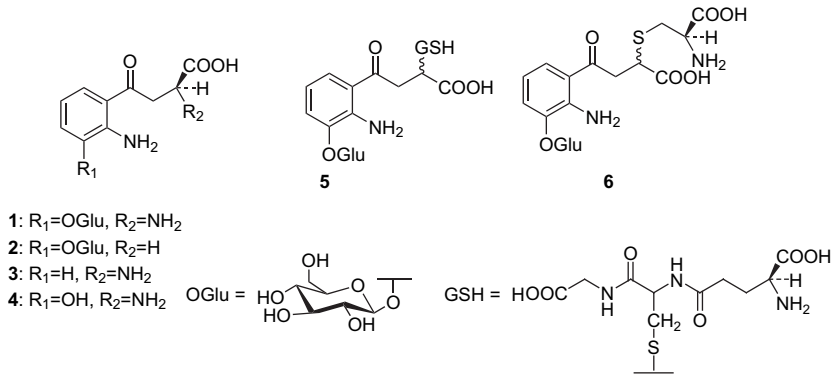

Figure 1. Major human lens UV filter compounds.

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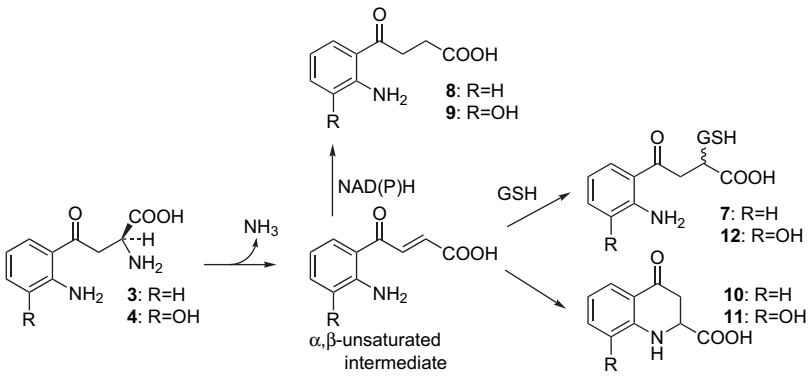


Scheme 1. Schematic diagram illustrating the degradation pathways of the human lens UV filter compounds **3** and **4**.

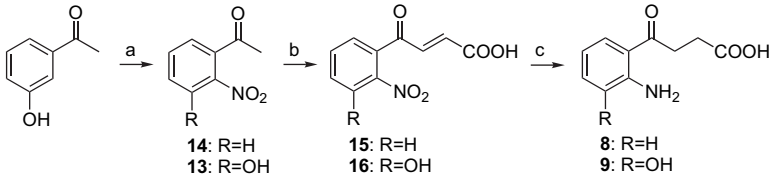
on human lens proteins,[9–11,7] and have also identified glutathionyl-3-hydroxykynurenine *O*-β-D-glucoside (**5**)[12] and cysteine-3-hydroxykynurenine *O*-β-D-glucoside (**6**)[13] as novel human lens UV filter compounds (Fig. 1). In addition, deaminated **3** readily reacts in vitro with glutathione (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.

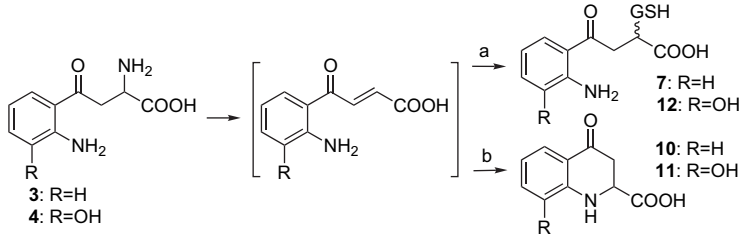
2. Results and discussion

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

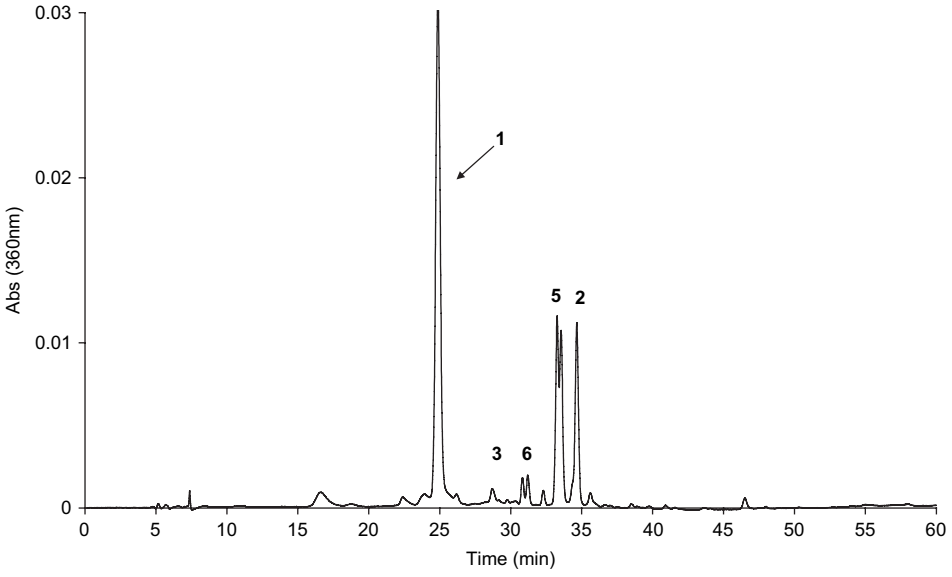


Figure 2. RP-HPLC profile of UV filter extract from a normal lens nucleus (88 years old) as described in Section 4.13. Detection was at 360 nm. 3-Hydroxykynurenine *O*-β-D-glucoside (**1**); 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (**2**); kynurenine (**3**); glutathionyl-3-hydroxykynurenine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

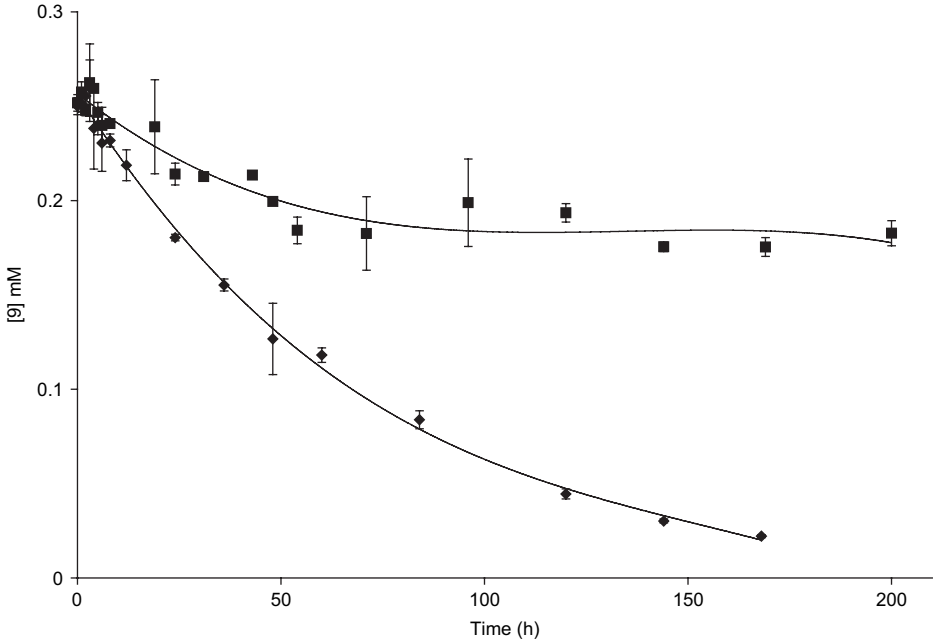


Figure 3. Compound **9** (0.25 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (2.0 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed 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

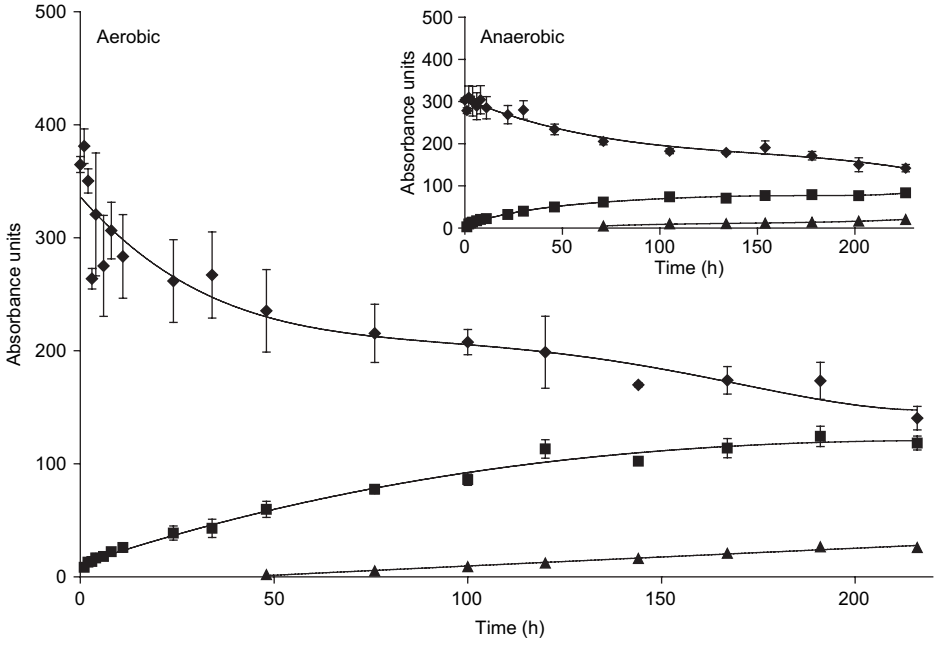


Figure 4. Compound **7** (0.4 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (0.2 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed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

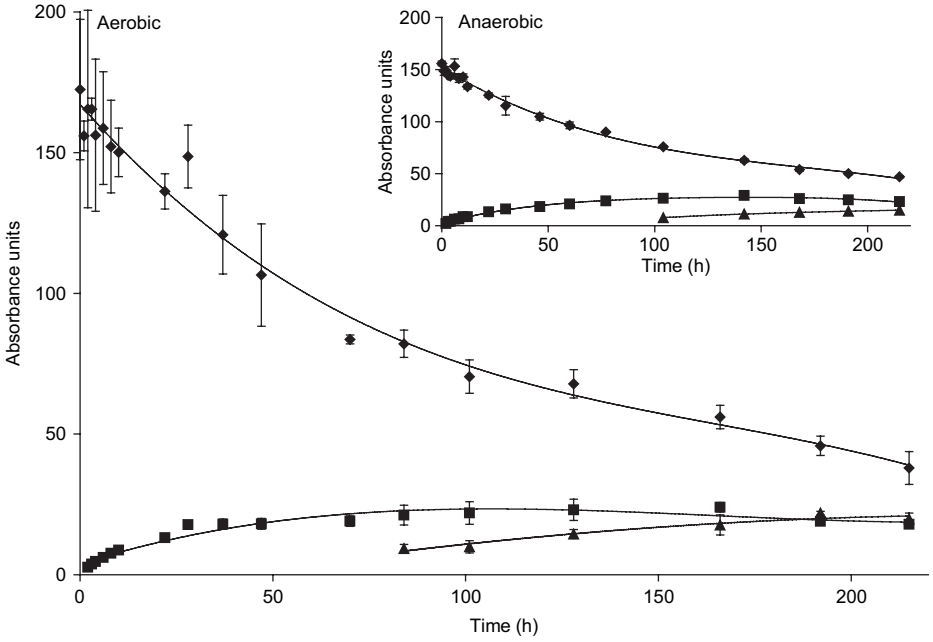


Figure 5. Compound **12** (0.1 mM) was incubated in PBS (pH 7.0) in the presence of atmospheric oxygen or in oxygen free PBS (pH 7.0) in the presence of ascorbic acid (2.0 mM) and butylated hydroxytoluene (100 μM). Aliquots of the reaction mixtures were taken at the indicated time points and analysed 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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