The fluorescence of scorpions and cataractogenesis

Shawn J Stachel[1], Scott A Stockwell[2] and David L Van Vranken[1]

Background: Protein cross-linking and fluorescence are widely recognized markers of oxidative aging in human proteins. Oxidative protein aging is a combinatorial process in which diversity arises from the heterogeneity of the targets and is amplified by the nonselective nature of the reactants. The cross-links themselves defy analysis because they are generally embedded in a covalent matrix. Arthropods rely upon oxidative cross-linking in the hardening of the cuticle – a process known as sclerotization. Among arthropods, scorpions are noteworthy in that the process of sclerotization is accompanied by the buildup of strong visible fluorescence. To date, the nature of the fluorescent species has remained a mystery.

Results: We have identified one of the soluble fluorescent components of the scorpions *Centuroides vittatus* and *Pandinus imperator* as β-carboline – a tryptophan derivative that has previously been identified by hydrolysis and oxidation of lens protein. We have also shown that β-carboline-3-carboxylic acid is released from both scorpion exuvia (the shed cuticle) and human cataracts upon hydrolysis, suggesting that the protein-bound β-carboline and free β-carboline have common chemical origins.

Conclusions: Cataractogenesis and cuticular sclerotization are disparate oxidative processes – the former is collateral and the latter is constitutive. The common formation of β-carbolines shows that similar patterns of reactivity are operative. These fundamental mechanisms provide predictive insight into the consequences of human protein aging.

Addresses: [1]Department of Chemistry, University of California, Irvine, CA 92697-2025, USA. [2]US Army Medical Department Center and School, Medical Zoology Branch, Fort Sam Houston, San Antonio, TX 78234-6142, USA.

Correspondence: David L Van Vranken
E-mail: dlvanvra@uci.edu

Key words: β-carboline, cataract, fluorescence, scorpion, tryptophan

Received: **26 April 1999**
Accepted: **20 May 1999**

Published: **12 July 1999**

Chemistry & Biology August 1999, **6**:531–539
http://biomednet.com/elecref/1074552100600531

Introduction

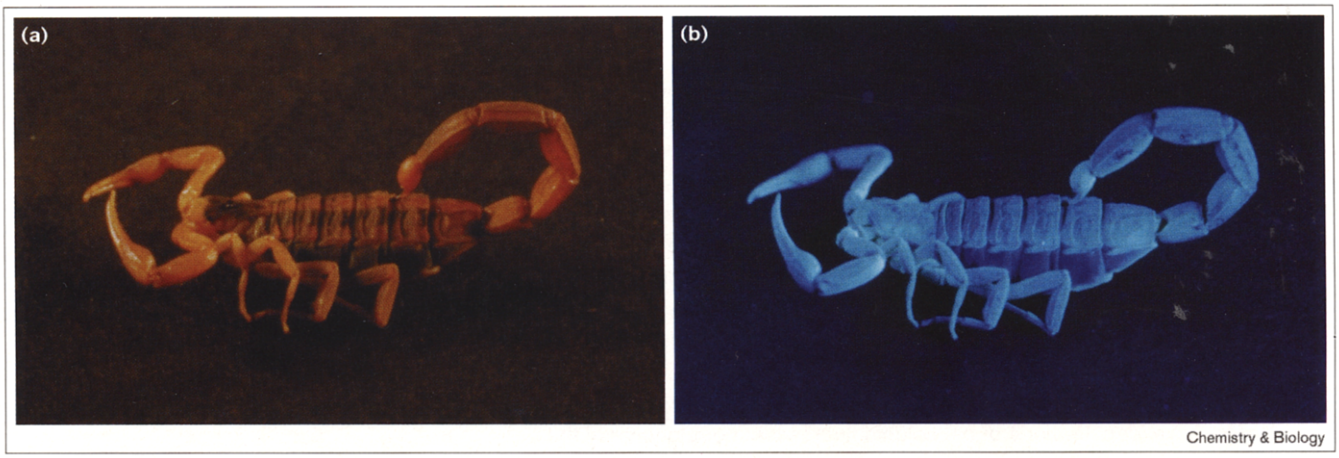
Scorpion fluorescence

Scorpions are among the oldest arachnids. Among the various orders of arachnids, scorpions are noteworthy for the general property of photoluminescence. The existence of fluorescence in scorpions under UV light was first reported in 1954 [1–5]. Several species of scorpion have been observed to fluoresce, but the intensity of the emission varies widely and is faint in some species [5–7]. Nevertheless, fluorescence still makes it easy to collect scorpions at night using a portable UV light (Figure 1). This enigmatic property of scorpions has been exploited to improve our knowledge of scorpion systematics, ecology and physiology, but to date, the identity or mechanism of formation of the molecular species responsible for the fluorescence has not been determined. Scorpions are primarily nocturnal and generally avoid sunlight, so the role of the fluorescence, if any, is currently unknown.

Examining sections of the cuticle of *Euscorpius italicus* with a light microscope, Pavan [2,3] demonstrated that the fluorescent substance is concentrated in the thin (about 4 μm thick) outermost layer of the cuticle known as the epicuticle. The emission intensity increases with the age and the hardness of the cuticle and is brightest in the hardest portions of the cuticle. Although fluorescence intensity varies among species, it is generally found that the soft cuticle of the first instar larva (i.e. the first molting stage) does not fluoresce [6] or fluoresces only weakly [8]. Second instar scorpions fluoresce faintly [6] or brightly [8], but the emission becomes quite strong in third and later instars. Immediately following ecdysis, the exuvium (i.e. the shed cuticle) retains the fluorescent quality, whereas the newly molted animal does not fluoresce. It has been reported that if a newly molted scorpion is immediately frozen and lyophilized the fluorescence never develops, suggesting that the constituents required for fluorogenesis are not present in the epicuticle immediately following ecdysis. To account for this observation, it has been suggested that the fluorescent substance is transported to the epicuticle of the scorpion through pore and wax canals during the cuticle tanning process [9]. Pavan [2] reported that the fluorescent substance in cuticular scrapings was insoluble in water below 100°C, and in a variety of other solvents including ethyl ether, chloroform, acetone, benzene, toluene, methanol and ethanol. In contrast, the alcohol in which scorpions are preserved begins to fluoresce, indicating that some part of the fluorescent substance is soluble in alcohol [5].

The arthropod cuticle is a remarkable composite material made up of proteins, chitin and lipids [10]. The structural

Figure 1



The common striped scorpion *Centruroides vittatus* under **(a)** normal illumination and **(b)** long-wave UV illumination (broadly centered around 350 nm).

rigidity of the cuticle is due primarily to a tanning process (sclerotization) that is driven by oxidation, but orchestrated by phenoloxidase and peroxidase enzymes. The generally accepted model for sclerotization involves the oxidative conversion of catecholic precursors (*N*-acetyl dopamine and *N*-β-alanyldopamine) into quinones or quinone methides. These electrophiles are attacked by the sidechains of proteins or the hydroxyl groups of chitin [11]. A second oxidation triggers secondary cross-link formation. Catechols therefore become the connectors that link proteins, either with other proteins or with chitin. Despite consensus on the general mechanisms for cuticular sclerotization, no quinone adducts have ever been isolated and characterized. However, dityrosines, which are well characterized, have been identified in arthropod cuticles [12,13]. Dityrosines are formed via oxidative phenolic coupling. Although dityrosines are fluorescent, they do not account for the wide range of fluorescence emission seen in scorpion cuticles because the fluorescence emission of dityrosines is around 400 nm [14]. There is little information about the amino acid sequence of proteins that make up the scorpion cuticle, but work on other insects (that are not highly fluorescent) points to the importance of tyrosine and histidine in sclerotization [15,16].

Lens proteins and fluorescence

The scorpion cuticle and the human lens undergo two similar time-dependent changes: hardening and evolution of strong visible fluorescence. These changes in physical properties reflect oxidative processes at the molecular level. Hardening may be essential for the scorpion cuticle, but it is an unwelcome consequence of oxidative aging in human lens proteins. Generally, cells resist oxidative modification to intracellular proteins through the process of renewal, but the lens is an exception [17]. Intracellular proteins in the central region of the human lens have virtually no turnover, so oxidative damage accumulates over many decades [18]. This oxidative damage is associated with fluorescence, molecular cross-linking and insolubility, and ultimately leads to opacification [19]. The resulting opacities, known as cataracts, occur in over 45% of people between the ages of 75 and 85.

Slow turnover is also a feature of extracellular structural proteins such as collagen, elastin and some proteoglycans. Fluorescence and cross-linking builds up in these long-lived structural proteins, much as they do in lens proteins, but the modified proteins are not visible to the naked eye [17].

The oxidation of proteins leads to increased coloration and fluorescence because of increased conjugation, but very little progress has been made in understanding the structures and mechanisms that underlie long-term oxidative changes. The attributes of proteins that are most prized by the analyst — homogeneity, solubility and quantity — are all lost in the aftermath of protein aging. Herculean efforts have revealed a number of fluorescent protein modifications, including kynurenines [20], dityrosine [14], pentosidine [21], and 3,4-dihydro-β-carboline derivatives [22]. This modest list is likely to grow as more is learned about the reactivity of amino acids in the context of proteins. The scorpion cuticle provides a unique opportunity to learn about the oxidative processes that lead to fluorogenesis in biological systems.

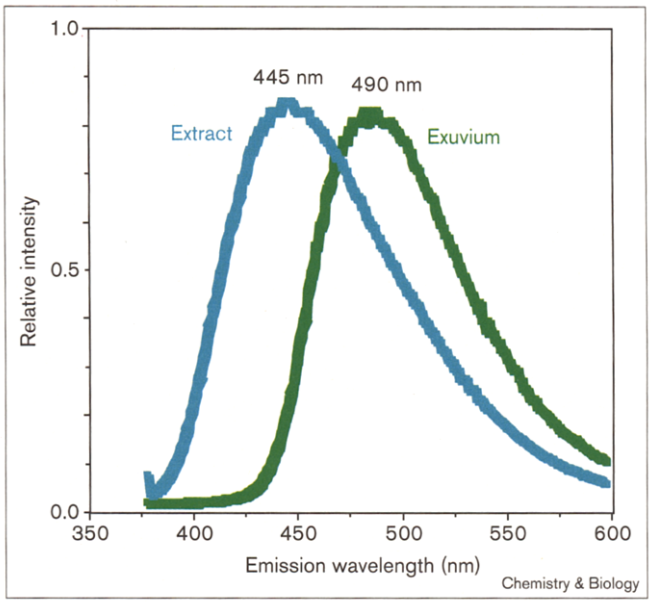
Results

Whole scorpions and crude ethanolic extract

Fluorescence

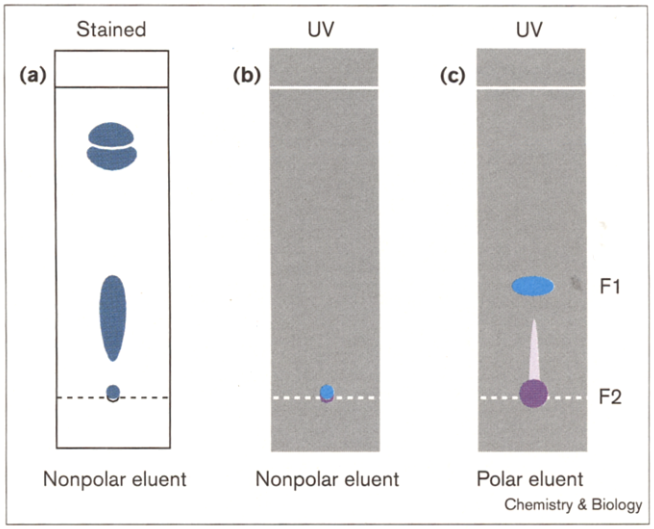
We first examined the extractable fluorescent species from whole specimens of the common striped scorpion *Centruroides vittatus*. *C. vittatus* is the most widely

Figure 2



Fluorescence emission spectra of *C. vittatus* extract (λ_{ex}=370 nm) and exuvium (λ_{ex}=360 nm).

Figure 3



Thin-layer chromatograms of crude *C. vittatus* ethanol extract on silica gel. **(a,b)** Chromatograms run with a nonpolar eluent (1:4 ethyl acetate/hexanes); **(c)** Chromatogram run with a polar eluent (75:15:10 *n*-butanol/water/formic acid). (a) The chromatogram was stained with phosphomolybdic acid, revealing lipids and hydrocarbons; (b,c) chromatograms were illuminated with a long-wavelength UV lamp.

distributed of all American scorpions and is especially common in the Southwest and on the Gulf coast [23]. Ethanolic solutions (3:1 ethanol/water) in which scorpions have been stored slowly extract fluorescent species into solution. For our initial studies we used the ethanol solution from a collection of about 186 scorpions collected in Pecos County, Texas in 1974.

The purple–blue fluorescence of the ethanolic solution does not match the blue–green fluorescence of the whole scorpion or the exuvium. The emission maximum (λ_{ex}=380 nm) of the solution was 445 nm, consistent with the purple–blue fluorescence. The emission spectrum of the exuvium was shifted to 490 nm, consistent with the slightly greener hue (Figure 2) [24,25]. This difference suggests that the primary fluorophore in the alcohol extract is not the same as the primary fluorophore in the solid cuticle, but this effect may also be due to differences in the local environment of a similar fluorophore.

Thin-layer chromatography

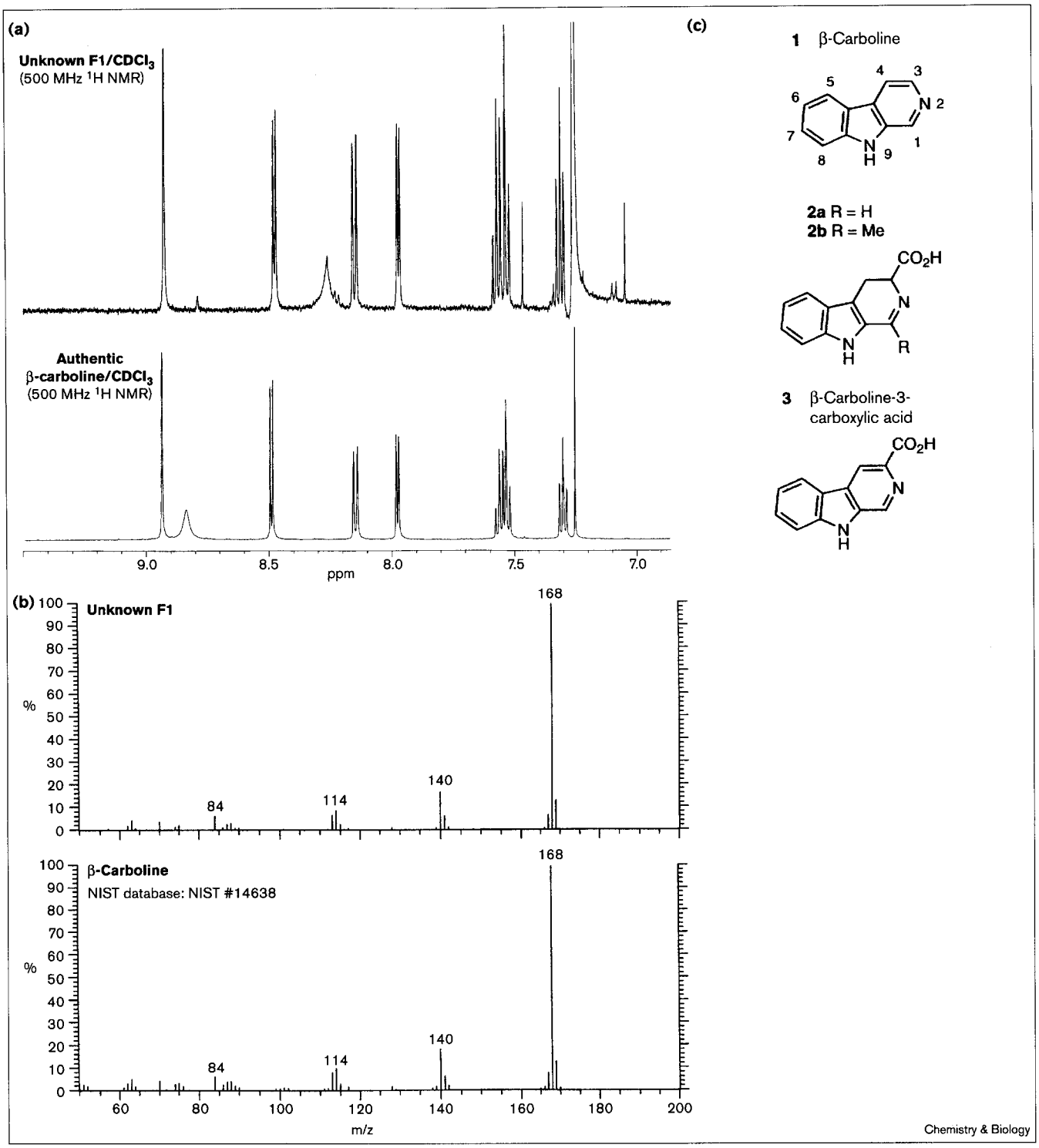
The ethanol extract from *C. vittatus* was analyzed using thin-layer chromatography (TLC; SiO_2) in three different eluent systems (Figure 3). Elution with 20% ethyl acetate/hexanes revealed three types of nonpolar components with R_f values of 0.30, 0.76 and 0.86. Immobile component(s) at the baseline were fluorescent under a 350 nm UV lamp. With a stronger eluent (75:15:10 butanol/formic acid/water; Figure 3) one of the fluorescent components (unknown **F1**) eluted as a homogeneous species with an R_f value of 0.34, whereas the remaining fluorescent material (unknown **F2**) was concentrated at the baseline, which is typical of high molecular weight species (e.g., hen egg lysozyme). The baseline material may have been heterogeneous.

The ethanolic extract (100 ml) was concentrated *in vacuo* to afford 0.72 g of a thick yellow oil. Analysis using ^{1}H nuclear magnetic resonance (NMR) in deuteriochloroform confirmed that this material was primarily composed of nonpolar compounds such as alkanes (branched and unbranched) and fatty acids (saturated and unsaturated), as expected for specimens of the genus *Centruroides* [26]. Singlets between 0.85 and 0.95 ppm suggested branched alkanes. The two triplets at 2.25 and 2.35 ppm correspond to the α-methylenes of fatty acids, whereas the signals between 5.3 and 5.4 ppm confirm unsaturation.

HPLC chromatography

Reverse-phase high-performance liquid chromatography (HPLC; stationary phase: C_{18}, mobile phase: pH 6.0 50 mM $Et_3NH^+OAc^-$/acetonitrile) confirms the preliminary results of TLC. More than one fluorogenic species is present in the crude extract, but the major fluorophore **F1** has an excitation maximum around 350 nm and an emission maximum around 450 nm. The baseline material is at least four different highly polar components that elute rapidly from the C_{18} column. Qualitatively, these components showed similar fluorescence properties.

Figure 4



(a) ^{1}H NMR spectra and **(b)** mass spectra of purified **F1** (upper spectrum) and β-carboline (lower spectrum). **(c)** The structures of **1**, **2a**, **2b** and **3**.

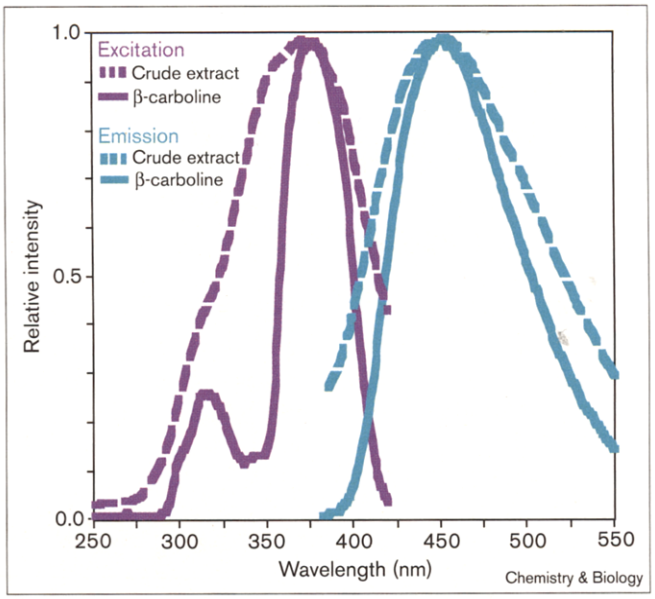
Isolation of β-carboline

We set out to isolate and characterize **F1**, the primary fluorescent species in the ethanol extract. Following removal of solvent from the crude extract, the residual viscous oil was chromatographed on silica gel using 5% methanol/chloroform. The initial fractions contained ~210 mg of nonpolar compounds. The high R_f fluorescent

product **F1** eluted next, followed by polar alkanes, probably fatty acids (320 mg). The remaining material did not elute even with 20% methanol/chloroform.

Further purification of the fluorescent fractions afforded ~0.1 mg of **F1**. The unknown **F1** was analyzed using fluorescence, ^{1}H NMR and mass spectrometry (Figure 4). Analysis of the high field ^{1}H NMR of purified **F1** was informative. The lack of peaks below 7 ppm confirmed that this compound was fully aromatic and therefore contained no peptide, sugar or lipid components. ^{1}H spin decoupling identified a four proton spin system typical of tryptophan derivatives. **F1** was then subjected to low- and high-resolution mass spectrometry. Mass spectrometry confirmed a molecular formula of $C_{11}H_8N_2$. Comparison of the electron ionization (EI) mass spectrum of compound **F1** with an authentic spectrum of β-carboline (norharman; **1**) from the NIST mass spectrometry database provided an exact match (Figure 4b). The ^{1}H NMR spectrum of authentic β-carboline was found to match **F1** (Figure 4a). Three other carbolines will be important for subsequent discussions: 3,4-dihydro-β-carboline-3-carboxylic acid (**2a**), 1-methyl-3,4-dihydro-β-carboline-3-carboxylic acid (**2b**) and β-carboline-3-carboxylic acid (**3**; Figure 4c).

Figure 5



Comparison of fluorescence excitation and emission spectra of β-carboline with the crude ethanolic extract from *C. vittatus*.

Having securely assigned **F1** as β-carboline (**1**) it was possible to assess its contribution to the fluorescence of the crude extract. It is clear from the fluorescence spectrum of the crude extract (Figure 5) and the HPLC data that the fluorescence properties of **F1** are similar to the fluorescence properties of the crude scorpion extract. The ethanol-extractable fluorescence of scorpions may therefore be attributed to β-carboline. As we were unable to separate and identify the low R_f fluorescent components **F2**, their contribution to the extractable fluorescence remains uncertain. Because the fluorescence of the ethanol extract closely matches **F1**, the **F2** components must be minor contributors to the extractable fluorescence, or else their fluorescence properties (excitation and emission maxima) are similar to those of β-carboline.

β-Carboline from fresh exuvia

Methanol extract of scorpion exuvia

It was necessary to confirm that formation of β-carboline is a normal part of scorpion cuticular biochemistry, rather than an artifact resulting from the extraction of noncuticle components or an artifact attributable to post-sclerotization aging of the preserved scorpions. The extreme sensitivity of fluorescence detection allowed us to quantify the amount of β-carboline in fresh exuvia. Exuvia from 12 freshly molted *C. vittatus* (0.164 g combined weight) were finely ground and then sonicated in methanol for 12 hours. The solution was then filtered and the solvent was removed *in vacuo*. The filtrate was analyzed using HPLC (equipped with a fluorescence detector) using harman (1-methyl-β-carboline) as an internal standard for fluorescence detection. The quantity of β-carboline (**F1**) in the fresh exuvial extract was estimated to be 0.9 μg (5×10^{-4}% w/w) on the basis of a calibration curve. A single exuvium from *Pandinus viatoris* (East Africa; 0.225 g) was also analyzed for β-carboline as an extractable component of the exoskeleton. The exuvium of *P. viatoris* was difficult to grind effectively so, not surprisingly, sonication in methanol extracted no detectable quantities of β-carboline.

Hydrolysates of scorpion exuvia

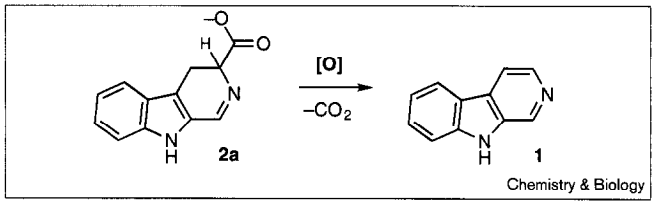
Scorpion exuvia have great resiliency and it was unclear whether β-carboline remained occluded in the intact material following extraction. The exuvia remaining from the methanol extraction were hydrolyzed in 4.2 N NaOH at 105°C for 12 hours. The solution was then adjusted to pH 9 with 4.2N HCl and extracted with ethyl acetate. The amount of β-carboline released (0.18 μg, 1×10^{-4}% w/w) was quantified by HPLC/fluorescence detection. A small amount of 1-methyl-β-carboline (harman) was detected in the hydrolysate (less than 5% of the amount of β-carboline detected in the hydrolysate). The exuvium of *P. viatoris* was also hydrolyzed under basic conditions. Analysis by reverse-phase HPLC showed the cuticle to contain 0.3 μg of β-carboline. The exuvia of the emperor scorpion (*Pandinus imperator*) was found to contain β-carboline, but the amount was not quantified.

β-carboline derivatives from protein hydrolysates

Dihydro-β-carboline-3-carboxylate derivatives

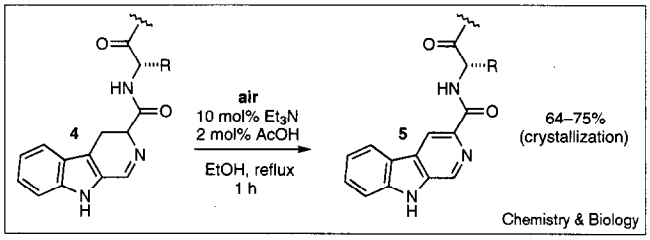
Dillon *et al.* [22] have demonstrated that the β-carboline precursors **2a** and **2b** are released from fluorescent, insoluble

Figure 6



Dihydro-β-carboline-3-carboxylic acids readily undergo oxidative decarboxylation.

Figure 7



Dihydro-β-carboline-3-carboxamides readily undergo oxidative dehydrogenation.

human cataract material by alkaline hydrolysis, suggesting that dihydro-β-carbolines are covalently bound to protein [27]. These dihydro-β-carboline 3-carboxylates are unstable, especially in basic media, and readily form β-carbolines by oxidative decarboxylation (Figure 6). Tryptophan therefore appears to have a similar fate in disparate sources: scorpions and humans. Neither protein-bound tryptophan nor free tryptophan (amino acid) has been implicated in the chemistry of sclerotization, but this may be due to the lack of reliable analytical techniques. Tryptophan is an enigmatic player in protein chemistry [28]. It is not reliably quantified by acidic protein hydrolysis, especially when carbohydrates are present [29]. More often than not, tryptophan is simply ignored [30]. Tryptophan provides a ready target for fluorogenesis and may be one of many hot spots for protein aging.

We next turned to the analysis of 3,4-dihydro-β-carboline-3-carboxylate derivatives in scorpion exuvia and cataractous lens protein (soluble and insoluble fractions). We were unable to isolate or identify the 1-methyl derivative of β-carboline (harman). As we were easily able to detect β-carboline, but not 1-methyl-β-carboline, in the exuvial hydrolysates we turned our attention to derivatives **2a** and **3** that lack the methyl group at the 1-position.

Currently, cataract surgery is the most successful surgery performed in the United States. The most common surgical procedure, phacoemulsification, involves sonication and removal of opacified lens material. The phacoemulsification effluent from a single eye operation was lyophilized and subjected to alkaline hydrolysis with 4N NaOH following the conditions of Dillon *et al.* [22]. The hydrolysate was brought to a pH of 8 and extracted with ethyl acetate. HPLC analysis (fluorescence detection) of the ethyl acetate extract failed to reveal the presence of β-carboline **1**. Dihydro-β-carboline-3-carboxylic acid **2a** was shown to be present, however, in the aqueous layer from the hydrolysate. The total amount of dihydro-β-carboline-3-carboxylic acid **2a** (or precursor) in a single lens was estimated to be 8 μg.

We returned to the scorpion cuticle. Surprisingly, when the exuvium of *C. vittatus* was subjected to alkaline hydrolysis and HPLC analysis, dihydro-β-carboline **2a** was not detected (the detection limit for our analysis was 0.1 μM).

β-carboline-3-carboxylate derivatives

The oxidative fate of dihydro-β-carboline-3-carboxylates is structure dependent. The corresponding carboxamides cannot decarboxylate as shown in Figure 6. Instead, such derivatives are prone to dehydrogenate under oxidative conditions as exemplified in Figure 7 [31]. It therefore seemed reasonable to analyze damaged proteins, not for the presence of dihydrocarboline **2a**, but for the presence of the fully aromatic carboline derivative **3**. The N-methyl amide of β-carboline-3-carboxylic acid emits at shorter wavelengths (emission maximum 400 nm at pH 5.5 and 460 nm at pH 1) than the parent heterocycle **1**.

Re-analysis of the alkaline hydrolysate from the cataract material confirmed the presence of β-carboline-3-carboxylate **3** corresponding to 4 μg/cataract lens. We next analyzed the alkaline hydrolysate of the *C. vittatus* exuvia (164 mg) for β-carboline-3-carboxylic acid **3** and found it to contain 9 μg of this material.

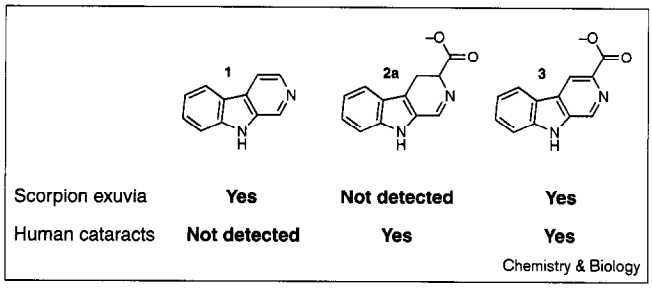
Tryptophan can readily form a β-carboline in the presence of formaldehyde and a one-electron oxidant if it is at the amino terminus of a peptide, but not if it is in the middle of a peptide. Identification of **3** in the alkaline hydrolysate suggests that it is covalently bound to and contributes to the fluorescence of the scorpion cuticle.

Discussion

Let us compare and contrast our results for scorpion cuticles and human cataracts. In the broadest sense we know that alkaline hydrolysis releases β-carboline derivative **3** from scorpion cuticles and from human cataracts. Two important pieces of evidence suggest that these β-carbolines are not an artifact of the alkaline protein hydrolysis process. First, alkaline hydrolysis of tryptophan-containing proteins (such as albumin) does not lead to the formation of fluorescent components such as β-carbolines [22]. Second, free β-carboline **1** can be extracted from scorpion exuvia (shed cuticles) without basic hydrolysis.

On the basis of fluorescence spectra of lens homogenates, Chakrabarti and coworkers [32] have suggested that β-carboline might be present in the human lens. We were able

Figure 8



Comparison of β-carbolines detected in scorpion exuvia and human cataracts.

to detect β-carboline **1** in the scorpion cuticle but not in the cataractous lens material (Figure 8). If it is formed in the lens, then it must diffuse out such that the steady-state concentration is below our limit of fluorescence detection. Conversely, we were able to detect 3,4-dihydro-β-carboline-3-carboxylic acid **2a** in cataracts but not in the exuvial hydrolysates. Our failure to detect the dihydro-β-carboline **2a** in the scorpion exuvia might be attributable to the highly oxidative conditions involved in sclerotization (tanning). Under such conditions, one might expect that any 3,4-dihydro-β-carbolines attached to protein would fully oxidize to β-carbolines as shown in Figure 7.

β-carbolines seem to be a common marker for oxidative protein chemistry [33,34]. Clearly, carbolines are common, but the β-carboline 'profiles' of cataracts and scorpion cuticles are different. Although the indole moiety is probably derived from tryptophan via a Pictet–Spengler reaction, the source of the extra one-carbon (or two-carbon) unit that ultimately becomes the 1-position of the β-carboline remains a mystery. The presence of β-carbolines **1** and **3** in scorpion cuticles is also a mystery and it suggests a soluble precursor such as free tryptophan or tryptamine.

Despite the mystery, we can roughly estimate a lower limit for the rate of Pictet–Spengler chemistry in the human lens. On the basis of the cataractous lens material that was analyzed, we estimate that over 10 ng of β-carbolines were present. If averaged out over 60 years, this value corresponds to the formation of about 1,000,000,000,000 β-carboline derivatives per day in each eye.

Significance

There are numerous parallels between the sclerotization of scorpion cuticles and senescent cataract formation. Hardening and fluorescence are macroscopic physical properties that are common to both processes. At the molecular level, they involve macromolecular cross-linking, dityrosine formation and the formation of β-carboline derivatives. The normal cross-linking and fluorogenesis of lens proteins occur over many decades and are difficult to follow at the molecular level, whereas in scorpion cuticles these processes occur over several weeks and are more amenable to study. Also, in fluorogenic protein aging, the majority of the fluorescence is covalently bound to the insoluble cross-linked fraction, and this makes the characterization of the fluorescent species difficult.

Here we have shown that one of the soluble fluorescent components of scorpion cuticle is the tryptophan derivative β-carboline. We have also shown that β-carboline-3-carboxylic acid is released form both shed scorpion cuticles and human cataracts upon hydrolysis. Neither dityrosine nor β-carboline can account for the majority of the fluorescence in the human lens or the scorpion cuticle. However, β-carboline-3-carboxamide, attached to protein, could contribute to the fluorescence.

There is general agreement that β-carbolines found in human cataracts are derived from either free or protein-bound tryptophan, but the origin of the one-carbon fragment (at the 1-position) in these β-carbolines remains a mystery. The formation of β-carbolines has no clear functional role. It seems reasonable that, in scorpions, carboline formation is a form of collateral damage that accompanies the necessary process of oxidative cross-linking, whereas in the human lens it is one of many uncontrolled chemical processes that leads to senescence. As β-carbolines are potent activators of the benzodiazepine receptor [35], the structural and mechanistic origin of these species merits further study.

Materials and methods

Norharman and harman were purchased from Aldrich chemical co. Authentic β-Carboline-3-carboxylic acid was prepared by the method of Müller and coworkers [36]. 3,4-Dihydro-β-carboline-3-carboxylic acid was prepared by the method of Previero *et al.* [31].

Analytical TLC was performed using 0.25 mm commercial silica gel plates (EM science, silica gel 60 F_{254}) and visualized by short wave (256 nm) UV light or long wave (350 nm) UV light. Reverse-phase HPLC employed a Rainin C_{18} Microsorb stationary phase at a flow rate of 1 ml/min (analytical) or 15 ml/min (preparative). Peak detection was performed using either a Dynamax UV-1 detector (UV) or a Shimadzu RF-535 fluorescence detector at the wavelengths indicated. Fluorescence spectra were recorded on a SPEX Fluoromax-2 under magic angle conditions using Glans–Thompson polarizers. ^1H spectra were recorded using a Bruker Avance DRX 500 (500 MHz) in with a digital resolution of 0.15 ppm.

Phacoemulsification involves sonication and complete removal of the lens structure. Residual lens material is removed using a phosphate buffered solution containing hyaluronic acid (a high viscosity mucopolysaccharide).

Isolation of β-carboline from ethanolic extract

Scorpions (*C. vittatus*) were stored in 75% aqueous ethanol from the time of collection (1974). The alcohol slowly extracts a fluorescent component from the whole scorpions. A sample of the ethanolic extract (100 ml) was concentrated *in vacuo* to afford 0.72 g of a thick yellow oil. The residue was then adsorbed onto silica gel (6.5 g) and eluted with 5% methanol/chloroform. The fractions containing the fluorescent

compound **F1** were combined to give a sticky yellow solid (0.21 g). This residue was then purified further by preparative reverse-phase HPLC (mobile phase: 60% methanol/50 mM ammonium acetate buffer pH 8, isocratic; $\lambda_{ex} = 340$ nm/$\lambda_{em} = 380$ nm) to afford pure **F1** (0.1 mg) as a white solid. **F1** was shown to be identical in all respects with β-carboline.

Analysis of C. vittatus *exuvial extract for β-carboline*
The exuvia from 12 scorpions (*C. vittatus*, 0.164 g) were finely ground with and then sonicated in methanol (50 ml) for 12 h at room temperature. The mixture was filtered to remove the exuvial solids and the filtrate was concentrated *in vacuo*. The residue was dissolved in methanol (1 ml) and analyzed by HPLC (60% methanol/50 mM ammonium acetate buffer pH 8, isocratic; $\lambda_{ex} = 340$ nm/$\lambda_{em} = 380$ nm), using harman (1-methyl-β-carboline) as an internal standard for fluorescence detection. The quantity of β-carboline in the fresh exuvial extracts was estimated to be 0.9 μg (5×10^{-4}% w/w) on the basis of a calibration curve.

Analysis of C. vittatus *exuvial hydrolysate for β-carboline*
The exuvial solids remaining from the methanolic extraction were hydrolyzed in 4.2 N NaOH (10 ml) at 105°C for 12 h. The solution was then adjusted to pH 9 with 4.2 N HCl and extracted with ethyl acetate (4×30 ml). The residue was dissolved in methanol (1 ml) and analyzed by HPLC (60% methanol/50 mM ammonium acetate pH 8, isocratic; $\lambda_{ex} = 340$ nm/$\lambda_{em} = 380$ nm). The quantity of β-carboline released from the exuvial hydrolysate was estimated to be 0.2 μg (1×10^{-4}% w/w) on the basis of a calibration curve.

Analysis of P. viatoris *(East Africa) for β-carboline*
The great resiliency of the *P. viatoris* exuvium made grinding ineffective. The whole exuvium (0.226 g) was sonicated in methanol (50 ml) for 12 h at room temperature. The solution was filtered and the solvent removed was removed in vacuo. The residue was dissolved in methanol (1 ml) and analyzed by HPLC. The extraction produced no detectable quantities of β-carboline (detection limit 1×10^{-7} M).

Analysis of hydrolyzed exuvia from P. viatoris *for β-carboline*
The extracted *P. viatoris* exuvium was hydrolyzed in 4.2 N NaOH (10 ml) at 100°C for 12 h. The mixture was then adjusted to pH 9 with 4.2 N HCl. The solids were removed by filtration and washed sequentially with H_2O (1×20 ml), ethyl acetate (2×20 ml), and acetone (2×20 ml). The organic solvents were removed *in vacuo* and the aqueous layer was extracted with ethyl acetate (4×30 ml). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. The residue was dissolved in methanol (1 ml) and analyzed by HPLC. The quantity of β-carboline released from the hydrolyzed exuvia was estimated to be 0.3 μg (1×10^{-4}% w/w) on the basis of a calibration curve.

Analysis of human cataracts for β-carboline
The phacoemulsification effluent (80 ml) from one elderly patient was lyophilized to provide a white powder (0.994 g). The residue was hydrolyzed with 4.2 N NaOH (10 ml) at 105°C for 12 h. The solution was then adjusted to pH 9 with 4.2 N HCl and extracted with ethyl acetate (4×30 ml). The aqueous layer was lyophilized for further studies (4.30 g obtained). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was dissolved in methanol (1 ml) and analyzed by analytical reverse-phase HPLC. The extraction produced no detectable quantities of β-carboline (detection limit 1×10^{-7} M).

*Analysis of exuvia (*C. vittatus*) for β-carboline-3-carboxylic acid*
The aqueous layer obtained from the base hydrolysis of 12 scorpion cuticles (see above) was lyophilized to provide 4.06 g of material. 0.146 g of this material was dissolved in 0.1% aqueous TFA (1 ml), filtered, and analyzed for β-carboline-3-carboxylic acid by analytical reverse-phase HPLC (mobile phase: A: 0.1% TFA, B: methanol; 35–45% B over 30 min, 45–100% B over 5 min; $\lambda_{ex} = 350/\lambda_{em} = 500$ nm). The quantity of β-carboline-3-carboxylic acid was estimated to be 9 μg on the basis of a calibration curve.

Analysis of human cataracts for β-carboline-3-carboxylic acid
A 114 mg sample of the lyophilization salts (4.30 g) obtained from the base hydrolysis of 0.994 g of the phacoemulsification solid (see above) was dissolved in 0.1N NaOH (1 ml). The solution was filtered and analyzed for β-carboline-3-carboxylic acid by analytical reverse-phase HPLC (mobile phase: A: 0.1% aq. TFA, B = methanol; 20–50% B over 20 min, 50% B for 10 min, then 50–100% B over 10 min; $\lambda_{ex} = 350$ nm/$\lambda_{em} = 500$ nm) using β-carboline-phenylalanine methyl ester as an internal standard. The quantity of β-carboline-3-carboxylic acid was estimated to be 4.0 μg on the basis of a calibration curve.

Analysis of human cataracts for 3,4-dihydro-β-carboline-3-carboxylic acid
A 100 mg sample of the lyophilization salts (4.30 g) obtained from the base hydrolysis of 0.994 g of the phacoemulsification solid (see above) was dissolved in 0.1% aqueous TFA (1 ml) and analyzed for 3,4-dihydro-β-carboline-3-carboxylic acid by analytical RP HPLC using UV detection. HPLC conditions (mobile phase: A: 0.1% aq. TFA, B: acetonitrile; 0–100% over 30 min; $\lambda_{ex} = 350/\lambda_{em} = 422$ nm). The quantity of 3,4-dihydro-β-carboline-3-carboxylic acid was estimated at 8 μg (calculated based on 4.3 g of salts) based on coinjection with a known quantity of authentic material.

Acknowledgements

This work is supported by the National Science Foundation (NSF CHE-9623903) and through the generosity of Glaxo-Wellcome, Eli Lilly, and the Camille and Henry Dreyfus Foundation. We are grateful for the assistance of Robert Jones, M.D., and Stephen Farley, M.D., for their assistance in obtaining cataract phacoemulsification effluent.

References

1. Pavan, M. (1954). Presenza e distribuzione di una sostanza fluorescente nel tegumento degli Scorpioni. *Boll. Soc. Ital. Biol. Sper.* **30**, 801-803.
2. Pavan, M. (1954). Primi dati per la caratterizzazione della sostanza fluorescente nel tegumento degli Scorpioni. *Boll. Soc. Ital. Biol. Sper.* **30**, 803-805.
3. Pavan, M. (1954). Studi sugli Scorpioni. I. Una nuova caratteristica tipica del tegumentodegli Scorpioni. *Ital. J. Zool.* **21**, 283-291.
4. Pavan, M. & Vachon, M. (1954). Sur l'existence d'une substance fluorescente dans les téguments des Scorpions (Arachnides). *C. R. Acad. Sciences Paris* **239**, 1700-1702.
5. Lawrence, R.F. (1954). Fluorescence in arthropoda. *J. Ent. Soc. South Africa* **17**, 167-170.
6. Stahnke, H.L. (1972). UV light, a useful field tool. *BioScience* **22**, 604-607.
7. Baggini, A. & Pavan, M. (1955). Studi sugli Scorpioni. III. Scorpioni ed altri Chelicerati esaminati alla luce de Wood per la fluorescenza dell'epicuticola. *Ital. J. Zool.* **22**, 329-340.
8. Polis, G. & Sissom, W.D. (1990). Life history. In *The Biology of Scorpions.* (Polis, G., ed.) Stanford University Press, Stanford.
9. Filshie, B.K. & Hadley, N.F. (1979). Fine structure of the cuticle of the desert scorpion, *Hadrurus arizonensis. Tissue & Cell* **11**, 249-262.
10. Neville, A.C. (1975). *Biology of the Arthropod Cuticle.* Springer-Verlag, New York.
11. Hopkins, T.L. & Kramer, K.J. (1992). Insect cuticle sclerotization. *Annu. Rev. Entomol.* **37**, 273-302.
12. Anderson, S.O. (1964). The cross-links in resilin identified as dityrosine and trityrosine. *Biochim. Biophys. Acta* **93**, 213-215.
13. Sugumaran, M., Henzel, W.J. & Lipke, H. (1982). Chitin-bound protein of sarcophagid larvae: metabolism of covalently linked aromatic constituents. *Biochemistry* **21**, 6509-6515.
14. Giulivi, C. & Davies, K.J.A. (1994). Dityrosine: a marker for oxidatively modified proteins and selective proteolysis. In *Methods in Enzymology, Vol. 233. Oxygen radicals in biological systems, Part C.* (Packer, L., ed.) Academic Press, Inc., San Diego, California, USA.
15. Kramer, K.J., Hopkins, T.L. & Schaefer, J. (1995). Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochem. Mol. Biol.* **25**, 1067-1080.
16. Anderson, S.O., Hojrup, P. & Roepstorff, P. (1995). Insect cuticular proteins. *Insect Biochem. Mol. Biol.* **25**, 153-176.
17. Sell, D.R. & Monnier, V.M. (1995). 10. Aging of long-lived proteins: extracellular matrix (collagens, elastins, proteoglycans) and lens crystallins In *Handbook of Physiology: Aging.* (Masoro, E.J., ed.) Oxford University Press, New York.

18. Young, R.W. (1991). 2. The structure of the lens and its defenses against aging. In *Age-Related Cataract.* Oxford University Press, New York.
19. Young, R.W. (1991). 3. Age-related deterioration of the lens. In *Age-Related Cataract.* Oxford University Press, New York.
20. Finley, E.L., Dillon, J., Crouch, R.K. & Schey, K.L. (1998). Identification of tryptophan oxidation products in bovine alpha-crystallin. *Protein Sci.* **7**, 2391-2397.
21. Sell, D.R. & Monnier, V.M. (1990). End-stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from aging human collagen. *J. Clin. Invest.* **85**, 380-384.
22. Dillon, J., Spector, A. & Nakanishi, K. (1976). Identification of beta carbolines isolated from fluorescent human lens proteins. *Nature* **259**, 422.
23. Keegan, H.L. (1980). *Scorpions of Medical Importance.* University Press of Mississippi, Jackson, MI.
24. Koehler, F.H., Jr. (1979). Physical characteristics of the fluorescence spectra of scorpions [Master's Thesis]. California State University.
25. Fasel, A., Muller, P.A., Suppan, P. & Vauthey, E. (1997). Photoluminescence of the African scorpion *Pandinus imperator.* *J. Photochem. Photobiol. B* **39**, 96-98.
26. Toolson, E.C. & Hadley, N.F. (1979). Seasonal effects on cuticular permeability and epicuticular lipid composition. In *Centruroides sculptatus* Ewing 1928 (Scorpiones: Buthidae). *J. Comp. Physiol. B* **129**, 319.
27. Manabe, S., Yuan, J., Takahashi, T. & Urban, R.C.J. (1996). Age-related accumulation of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid in human lens. *Exp. Eye Res.* **63**, 179-186.
28. Hunt, S. (1985). Degradation of amino acids accompanying *in vitro* protein hydrolysis. In *Chemistry and Biochemistry of the Amino Acids.* (Barrett, G.C., ed.) Chapman & Hall, New York.
29. Blackburn (1978). *Amino Acid Determination: Methods and Techniques.* (2nd ed.), Marcel Dekker, New York.
30. Okot-Kotber, B.M., Morgan, T.D., Hopkins, T.L. & Kramer, K.J. (1994). Characterization of two high molecular weight catechol-containing glycoproteins from pharate pupal cuticle of the tobacco hornworm, *Manduca sexta. Insect Biochem. Mol. Biol.* **24**, 787-802.
31. Previero, A., Barry, L.-G., Torreilles, J., Fleury, B., Letellier, S. & Maupas, B. (1984). Aromatization of 3,4-dihydro-β-carboline-3-carboxylic acid and its derivatives through a carbanion intermediate: mechanistic study and its use in chemical synthesis. *Tetrahedron* **40**, 221-234.
32. Sen, A.C., Ueno, N. & Chakrabarti, B. (1992). Studies on human lens. 1. Origin and development of fluorescent pigments. *Photochem. Photobiol.* **55**, 753-764.
33. Smith, G.J., Markham, K.R. & Mellhuish, W.H. (1994). The presence of photoreactive beta-carboline fluorophores in weathered wool keratin. *Photochem. Photobiol.* **60**, 196-198.
34. Rommelspacher, H., May, T. & Susilo, R. (1991). Beta-carbolines and tetrahydroisoquinolines: detection and function in mammals. *Planta Med.* **57**, S85-S92.
35. Braestrup, C., Nielsen, M. & Olsen, C.E. (1980). Urinary and brain beta-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. Natl Acad. Sci. USA* **77**, 2288-2292.
36 Lippke, K.P., Schunack, W.G., Wenning, W. & Müller, W.E. (1983). β-carbolines as benzodiazepine receptor ligands. 1. Synthesis and benzodiazapine receptor interaction of esters of β-carboline-3-carboxylic acid. *J. Med. Chem.* **26**, 499-503.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.