Uncyclized xanthommatin is a key ommochrome intermediate in invertebrate coloration

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2   **Short title**: Uncyclized xanthommatin in ommochrome biosynthesis

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18   **Abbreviations**: CE, collision energy; CV, cone voltage; DAD, diode-array detector; ESI$^+$, positive-

19   mode electron spray ionization; LC, liquid chromatography; MeOH-HCl, acidified methanol with 0.5

20   % hydrochloric acid; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem

21   mass spectrometry; MW, molecular weight; *m/z*, mass-to-charge ratio; NMR, nuclear magnetic

22   resonance; RT, retention time; SD, standard deviation; SE, standard error; SIR, single ion recording;

23   UV,   ultraviolet.

24 **Abstract**

25 Ommochromes are widespread pigments that mediate multiple functions in invertebrates. The two

26 main families of ommochromes are ommatins and ommins, which both originate from the kynurenine

27 pathway but differ in their backbone, thereby in their coloration and function. Despite its broad

28 significance, how the structural diversity of ommochromes arises *in vivo* has remained an open

29 question since their first description. In this study, we combined organic synthesis, analytical

30 chemistry and organelle purification to address this issue. From a set of synthesized ommatins, we

31 derived a fragmentation pattern that helped elucidating the structure of new ommochromes. We

32 identified uncyclized xanthommatin as the elusive biological intermediate that links the kynurenine

33 pathway to the ommatin pathway within ommochromasomes, the ommochrome-producing organelles.

34 Due to its unique structure, we propose that uncyclized xanthommatin functions as a key branching

35 metabolite in the biosynthesis and structural diversification of ommatins and ommins, from insects to

36 cephalopods.

39 1. Introduction

40 Ommochromes are widespread phenoxazinone pigments of invertebrates. They act as light filters in

41 compound eyes and determine the integumental coloration of a large range of invertebrates (Figon and

42 Casas, 2019). Ommochromes are also of particular interest in applied sciences as their scaffold has

43 been used to design antitumor agents (Bolognese et al., 2002) and, very recently, to manufacture

44 biomimetic color changing electrochromic devices (Kumar et al., 2018). The two major families of

45 natural ommochromes are the yellow-to-red ommatins and the purple ommins that contain a

46 supplemental phenothiazine ring. Ommatins are currently the best described family of ommochromes

47 and occur throughout invertebrates. Ommins are much less characterized, although they are virtually

48 ubiquitous in insects and cephalopods (Needham, 1974; Riddiford and Ajami, 1971). After nearly 80

49   years and despite their broad significance, the structural and chemical relationships between these two

50   abundant families of ommochromes remain surprisingly mysterious (Figon and Casas, 2019).

51   The early steps of the biosynthesis of ommochromes in invertebrates cover the oxidation of

52   tryptophan into kynurenines (Fig 1B) (Figon and Casas, 2019), from insects (Linzen, 1974) and

53   spiders (Croucher et al., 2013) to planarians (Stubenhaus et al., 2016) and cephalopods (Williams et

54   al., 2019b). These oxidative steps are homologous to the kynurenine pathway of vertebrates in which

55   the last enzyme, the mitochondria-bound kynurenine 3-monooxygenase, catalyzes the formation of 3-

56   hydoxykynurenine. This *ortho*-aminophenolic amino acid is the currently accepted last common

57   precursor of all known ommochromes, from ommatins to ommins (Fig 1B) (Figon and Casas, 2019).

58   The catabolism of tryptophan then diverges from vertebrates because invertebrates lack the glutarate

59   pathway but possess the ommochrome pathway (Linzen, 1974).

60   Ommochromes   are   produced   within   specialized   intracellular   organelles,   called

61   ommochromasomes, most likely after the incorporation of 3-hydroxykynurenine (Figon and Casas,

62   2019; Mackenzie et al., 2000). Two hypotheses have been proposed to explain how ommatins

63   originate from there. (1) It has been suggested very early, but on weak evidence, that the oxidative

64   dimerization of 3-hydroxykynurenine into uncyclized xanthommatin and its subsequent intramolecular

65   cyclization account for the biosynthesis of ommochromes (Fig 1B) (Butenandt and Schäfer, 1962). (2)

66   The condensation of *ortho*-aminophenols with xanthurenic acid has been proposed to form directly the

67   oxo-pyrido[3,2-*a*]phenoxazinone chromophore of ommatins (Fig 1B) (Linzen, 1974; Panettieri et al.,

68   2018). Hypothesis 1 is currently more accepted because ommatins can be synthesized *in vitro* by the

69   oxidative condensation of 3-hydroxykynurenine, which is predicted to form an unstable intermediate,

70   the 3-hydroxykynurenine dimer called uncyclized xanthommatin (Butenandt and Schäfer, 1962; Figon

71   and Casas, 2019; Iwahashi and Ishii, 1997; Williams et al., 2019a; Zhuravlev et al., 2018).

72   Furthermore, uncyclized xanthommatin was speculated in biological extracts (Bolognese and

73   Scherillo, 1974), putatively identified in the *in vitro* oxidation of 3-hydroxykynurenine (Iwahashi and

74   Ishii, 1997) and its enzymatic formation predicted *in silico* by quantum calculations (Zhuravlev et al.,

75   2018). However, it has never been formerly extracted and characterized in biological samples.

76   Alternatively, hypothesis 2 does not involve the formation of any intermediate between the kynurenine

77   pathway and the ommatin pathway. Hence, to discriminate between the two hypotheses, one needs to

78   determine whether uncyclized xanthommatin is produced *in vivo* (Fig 1B). Finding this still-elusive

79   intermediate in biological extracts would therefore be a major step in characterizing the actual

80   biosynthetic pathway of ommochromes.

81   Deciphering the ommochrome pathway requires the characterization of metabolites in

82   biological extracts. However, biological ommochromes are remarkably refractory to NMR

83   spectroscopy, partly because of their poor solubility in most conventional solvents (Bolognese et al.,

84   1988b; Crescenzi et al., 2004; Parrilli and Bolognese, 1992). It was only very recently that the first [1]H-

85   NMR spectrum of xanthommatin, whose structure has been known for 60 years, was published after

86   extensive purification and optimization steps (Kumar et al., 2018). However, from a theoretical point

87   of view, mere ^{1}H-NMR data cannot provide enough information on the exact structure of

88   ommochromes. Indeed, they are rather poor in carbon-bonded hydrogens, which prevents access to all

89   positions in the structure. Furthermore, they are redox/pH sensitives and prone to tautomerization,

90   which complicate ^{1}H spectra greatly. Since our main target compound, uncyclized xanthommatin, is

91   unstable in solution (Bolognese et al., 1988a; Bolognese and Scherillo, 1974), it is highly improbable

92   that gold-standard techniques for structural elucidation, such as ^{13}C- and 2D-NMR, can be used

93   because they suffer from too low sensitivity. In order to elucidate the biological diversity of

94   ommochromes, one should therefore look for a combination of more sensitive analytical techniques

95   that provide orthogonal information, such as mass spectrometry and UV-Visible spectroscopy. For

96   nearly a decade, mass spectrometry (MS) has been used to elucidate the structure of both known and

97   unknown ommochromes from biological samples (Futahashi et al., 2012; Panettieri et al., 2018; Reiter

98   et al., 2018; Williams et al., 2016). Yet, evidence for common and compound-specific fragmentation

99   patterns of ommochromes are scarce [but see (Panettieri et al., 2018; Reiter et al., 2018)]. Together

100   with the seldom use of synthesized ommochromes, this lack of analytical data accounts for the very

101   little progress made since four decades to unravel the biological diversity of ommochromes (Figon and

102   Casas, 2019).

103   In this study, we synthesized xanthommatin and its decarboxylated form by the oxidative

104   dimerization of 3-hydroxykynurenine. Knowing that ommatins are methoxylated in acidified methanol

105   (MeOH-HCl), we incubated synthesized xanthommatin in MeOH-HCl to produce a range of ommatin-

106   derivatives. We constructed an analytical dataset of those ommatins by a combination of UV-Visible

107   spectroscopy, and (tandem) mass spectrometry after separation by liquid chromatography. From this

108   dataset, we derived a fragmentation pattern with valuable structural information, especially when

109   combined with UV-Visible spectra, to infer the structure of new ommochromes with strong

110   confidence. Hence, we could elucidate the structure of three methoxylated ommatins and, more

111   importantly, of uncyclized xanthommatin. Our experiments demonstrated that ommatins are easily and

112   rapidly methoxylated leading to artifacts in conditions matching standard extraction procedures from

113   biological samples. By combining our analytical tools with an artifact-free extraction protocol and a

114   subcellular fractionation of ommochromasomes, we reinvestigated the ommochromes of housefly

115   eyes. We could identify xanthommatin, decarboxylated xanthommatin and uncyclized xanthommatin

116   in ommochromasomes. Our results provide strong support to the hypothesis that ommatin biosynthesis

117   occurs in subcellular organelles through the dimerization of 3-hydroxykynurenine and its subsequent

118   intramolecular cyclization (hypothesis 1, Fig 1B). Furthermore, the unique structure of uncyclized

119   xanthommatin makes it a good candidate to link the biosynthetic pathways of ommatins and ommins,

120   which has important implications on how ommochromes have diversified in a wide range of

121   phylogenetically-distant species.

2. Material and Method

2.1 Insects

124   Houseflies (*Musca domestica*) were obtained at the pupal stage from Kreca. After hatching, houseflies

125   were either directly processed for ommochromasome purification or stored at -20 °C for ommochrome

126   extraction.

127   *2.2 Reagents*

128   Sodium dihydrogen phosphate, sodium hydrogen phosphate, L-kynurenine ($\geq$ 98 %), 3-hydroxy-D,L-

129   kynurenine, trifluoroacetic acid (TFA), Triton X-100, tris(hydroxymethyl)aminomethane (Tris),

130   potassium ferricyanide, magnesium chloride, potassium chloride, potassium pentoxide and

131   cinnabarinic acid ($\geq$ 98 %) were purchased from Sigma-Aldrich. Methanol, potassium chloride and

132   hydrochloric acid (37 %) were purchased from Carlo Erba reagents. Nycodenz® was purchased from

133   Axis Shield. L-tryptophan ($\geq$ 99 %) and xanthurenic acid ($\geq$ 96 %) were purchased from Acros

134   Organics. Sucrose (99 %) and sulfurous acid ($\geq$ 6 % SO_2) were purchased from Alfa Aesar. β-

135   Mercaptoethanol was purchased from BDH Chemicals. Acetonitrile and formic acid were purchased

136   from ThermoFischer Scientific.

137   **2.3 In vitro *synthesis of xanthommatin***

138   *2.3.1 Oxidative condensation of 3-hydroxykynurenine under anoxia*

139   A mixture of ommatins was synthesized by oxidizing 3-hydroxy-D,L-kynurenine with potassium

140   ferricyanide as previously described (Butenandt et al., 1954; Hori and Riddiford, 1981), with some

141   modifications. In a round bottom flask under argon, a solution of 44.6 mM of 3-hydroxy-D,L-

142   kynurenine was prepared by dissolving 455 μmol (102 mg) in 10.2 mL of 0.2 M phosphate buffer at

143   pH 7.1 (PB). In a second round bottom flask under argon, 174 mM of potassium ferricyanide (303 mg)

144   were dissolved in 5.3 mL of PB. Both solutions were purged with argon and protected from light. The

145   potassium ferricyanide solution was added slowly to the solution of 3-hydroxy-D,L-kynurenine. The

146   resulted reaction mixture was stirred at room temperature for 1 h 30 in darkness. Then, 10 mL of

147   sulfurous acid diluted four times in PB was added. The final solution was brought to 4 °C for 30 min

148   during which red flocculants formed. The suspension was then transferred into a 50 mL centrifuge

149   tube. The round bottom flask was rinsed with 8 mL of sulfurous acid previously diluted four times in

150   PB to ensure the complete reduction and flocculation of synthesized ommatins, as well as to remove

151   ferrocyanide. The final suspension was centrifuged for 10 min at 10 000 × g and at 4 °C. The solid

152   was desiccated overnight under vacuum over potassium hydroxide and phosphorus pentoxide. 104 mg

153   of a reddish brown powder was obtained and kept at 4 °C in darkness until further use.

2.3.2 Solubilization and analyses of synthesized ommatins

155   A solution of synthesized ommatins at 1 mg/mL of was made in methanol acidified with 0.5 % HCl

156   and pre-cooled at -20 °C (MeOH-HCl). The solution was mixed for 30 s and filtered on 0.45 μm

157   filters. All steps were performed, as much as possible, at 4 °C in darkness. The overall procedure took

158   less than two minutes. Immediately after filtration, the solution was subjected to absorption and mass

159   spectrometry analysis (see below). The filtered solution was then stored at 20 °C in darkness and

160   subjected to the same analysis 24 hours later.

2.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

162   One milligram of synthesized product was solubilized in 600 μL of d6-DMSO acidified with 25 μL of

163   TFA, as previously described (Kumar et al., 2018; Williams et al., 2019a). NMR spectra were

164   recorded on Bruker AVANCE AV 300 instruments and the NMR experiment was reported in units,

165   parts per million (ppm), using residual solvent peaks d6-DMSO (δ = 2.50 ppm) for ^{1}H NMR as

166   internal reference. Multiplicities are recorded as: s = singlet, d = doublet, t = triplet, dd = doublet of

167   doublets, m = multiplet, bs = broad singlet. Coupling constants (J) are reported in hertz (Hz).

168   ^{1}H NMR (300 MHz, d6-DMSO + 0.04 % TFA) δ 8.37 (bs, 3H, H_1), 8.21 (bs, 1H, NH_8), 8.04 (t, *J* =

169   4.5 Hz, 1H, H_5), 7.83 - 7.82 (m, 2H, H_4, H_6), 7.69 (s, 1H, H_7), 6.67 (s, 1H, H_9), 4.46 - 4.42 (m, 1H,

170   H_2), 3.89 (bs, 2H, H_3).

2.5 Ultra-Pressure Liquid Chromatography coupled to Diode-Array Detector and Electrospray Ionization Source-based Mass Spectrometer (UPLC-DAD-ESI-MS)

2.5.1 System

174   A reversed-phase ACQUITY UPLC® system coupled to a diode-array detector (DAD) and to a Xevo

175   TQD triple quadrupole mass spectrometer (MS) equipped with an electrospray ionization source (ESI)

176   was used (Waters, Milford, MA). Tandem mass spectrometry (MS/MS) was performed by collision-

177   induced dissociation with argon. Data were collected and processed using MassLynx software, version

178   4.1 (Waters, Milford, MA).

179   *2.5.2 Chromatographic conditions*

180   Analytes were separated on a CSH™ C18 column (2.1 x 150 mm, 1.7 μm) equipped with a CSH™

181   C18 VanGuard™ pre-column (2.1 x 5 mm). The column temperature was set at 45 °C and the flow

182   rate at 0.4 mL/min. The injection volume was 5 μL. The mobile phase consisted in a mixture of MilliQ

183   water (eluent A) and acetonitrile (eluent B), both prepared with 0.1 % formic acid. The linear gradient

184   was set from 2 % to 40 % B for 18 min.

185   *2.5.3 Spectroscopic conditions*

186   The MS continuously alternated between positive and negative modes every 20 ms. Capillary voltage,

187   sample cone voltage (CV) and collision energy (CE) were set at 2 000 V, 30 V and 3 eV, respectively,

188   for MS conditions. CE was set at 30 eV for tandem MS conditions. Cone and desolvation gas flow

189   rates were set at 30 and 1 000 L/h, respectively. Absorption spectra of analytes were continuously

190   recorded between 200 and 500 nm with a one-nm step. Analytes were annotated and identified

191   according to their retention times, absorbance spectra, mass spectra and tandem mass spectra (Table

192   1).

193   **2.6 Thermal reactivity of ommatins in acidified methanol in darkness**

194   *2.6.1 Conditions of solubilization and incubation*

195   Solutions (n = 5) of synthesized ommatins at 1 mg/mL were prepared in MeOH-HCl. The solutions

196   were mixed for 30 s and filtered on 0.45 μm filters. Aliquots of 50 μL were prepared for each sample

197   and stored at either 20 °C or -20 °C in darkness. All steps were performed, as much as possible, in

198   darkness and at 4 °C. The overall procedure for each sample took less than two minutes. During the

199   course of the experiment, each aliquot was analyzed only once by UPLC-ESI-MS/MS, representing a

200   single time point for each sample.

201   *2.6.2 Quantification of ommatins*

202   Unaltered (*i.e.* xanthommatin and its decarboxylated form) and their methoxylated forms were

203   detected and quantified by absorption and MS/MS (single reaction monitoring [MRM] mode)

204   spectrometry. MRM conditions were optimized for each ommatin based on the following parent-to-

205   product ion transitions: xanthommatin $[M+H]^+$ 424>361 *m/z* (CV 38 V, CE 25 eV), α^3-methoxy-

206   xanthommatin $[M+H]^+$ 438>375 *m/z* (CV 37 V, CE 23 eV), α^3,α^{11}-dimethoxy-xanthommatin $[M+H]^+$

207   452>375 *m/z* (CV 38 V, CE 25 eV), decarboxylated xanthommatin $[M+H]^+$ 380>317 *m/z* (CV 34 V,

208   CE 28 eV) and decarboxylated α^{11}-methoxy-xanthommatin $[M+H]^+$ 394>317 *m/z* (CV 34 V, CE 28

209   eV). See Fig S2 for detailed information on MS/MS optimization. Peak areas for both absorption and

210   MRM signals were calculated by integrating chromatographic peaks with a "Mean" smoothing method

211   (window size: ± 3 scans, number of smooths: 2). Absorbance values at 414 nm of unaltered ommatins

212   were summed and reported as a percentage of the total absorbance of unaltered and methoxylated

213   ommatins. The decay at -20 °C of uncyclized xanthommatin was followed by integrating both the

214   absorbance at 430 nm and the 443 *m/z* SIR signals associated to the chromatographic peak of

215   uncyclized xanthommatin at RT 6.7 min.

216   **2.7 Extraction and content analysis of housefly eyes**

217   *2.7.1 Biological extractions*

218   Five housefly (*M. domestica*) heads were pooled per sample (n = 5), weighted and homogenized in 1

219   mL MeOH-HCl with a tissue grinder (four metal balls, 300 strokes/min for 1 min). The obtained crude

220   extracts were centrifuged for 5 min at 10 000 × g and 4 °C. The supernatants were filtered on 0.45 μm

221   filters and immediately processed for absorption and MS analyses. All steps were performed, as much

222   as possible, in darkness at 4 °C. The overall extraction procedure took less than 20 min.

223   *2.7.2 Chromatographic profile*

224   The chromatographic profile of housefly eyes that includes L-tryptophan, xanthurenic acid, 3-D,L-

225   hydroxykynurenine, uncyclized xanthommatin, xanthommatin and decarboxylated xanthommatin is

226   reported based on their optimized MRM signals. L-Tryptophan and xanthurenic acid were quantified

227   based on their optimized MRM signals: $[M+H]^+$ 205>118 *m/z* (CV 26 V and CE 25 eV) and $[M+H]^+$

228   206>132 *m/z* (CV 36 V and CE 28 eV), respectively. See Fig S2 for detailed information on MS/MS

229   optimization. 3-D,L-Hydroxykynurenine and uncyclized xanthommatin were quantified based on their

230   absorption at 370 and 430 nm, respectively. L-Tryptophan, 3-D,L-hydroxykynurenine and xanthurenic

231   acid levels were converted to molar concentrations using calibrated curves of commercial standards.

232   Molar concentrations of uncyclized xanthommatin are reported as cinnabarinic equivalent, since both

233   metabolites possess the same chromophore and presented similar absorbance spectra (see Fig 5C).

234   **2.8 Purification and content analysis of ommochromasomes**

235   *2.8.1 Isolation protocol*

236   Ommochromasomes from housefly eyes were purified as previously described (Cölln et al., 1981),

237   with slight modifications. The isolation buffer was prepared with MgCl2 instead of CaCl2,

238   metrizamide was replaced by Nycodenz® (iohexol) and the final ultracentrifugation step was

239   performed on Nycodenz layers solely since Nycodenz mixes with sucrose layers. See Supplemental

240   File S1 for a more detailed protocol.

241   *2.8.2 Extraction of ommochrome-related metabolites from purified ommochromasomes*

242   Pellets (n = 5) were resuspended in 50 μL MeOH-HCl and directly subjected to UPLC-DAD-ESI-

243   MS/MS analysis. All steps were performed, as much as possible, in darkness and at 4 °C. The overall

244   extraction procedure took less than 2 min per sample.

245   *2.8.3 Metabolic analysis of purified ommochromasomes*

246   The chromatographic profile of purified ommochromasomes that includes L-tryptophan, xanthurenic

247   acid, 3-D,L-hydroxykynurenine, uncyclized xanthommatin, xanthommatin, decarboxylated

248   xanthommatin and β-mercaptoethanol-added ommatins is reported based on their optimized MRM

249   signals. L-Tryptophan, 3-D,L-hydroxykynurenine, xanthurenic acid and uncyclized xanthommatin

250   were quantified as described for crude extracts of housefly eyes.

251   *2.9 Statistical analysis*

252   Statistical analyses were performed using the R software, version 3.4.1 (www.r-project.org). Statistical

253   threshold was set to 0.05. For kinetic analyses, changes of metabolite quantities overtime were first

254   tested by correlation tests assuming monotonic variations (Sperman's rank correlation test).

255   Depending on the shape of the variation, either linear or logarithmic in this study, we performed linear

256   regressions on either unchanged or log-scaled time points, respectively. For comparisons of two

257   samples, we first assessed whether normality and homoscedasticity criteria were met with Shapiro-

258   Wilkinson normality tests and Fligner-Killeen tests of homogeneity of variances, respectively. When

259   data were heteroscedastic, we used Welch *t*-test rather than Student *t*-test. For multiple comparisons,

260   we also tested normality and heteroscedasticity. Since the normality criteria was not met in that case,

261   we performed a Kruskal-Wallis rank sum test followed by pairwise comparisons using a Wilcoxon

262   rank sum test with the Holm adjustment method. Detailed results of all statistical analyses are reported

263   in File S2.

264   3. Results

265   *3.1 UPLC-DAD-MS/MS structural elucidation of synthesized xanthommatin and its* in vitro

266   *derivatives*

267   Since xanthommatin is commercially unavailable, we achieved its *in vitro* synthesis by oxidative

268   condensation of 3-hydroxykynurenine under anoxia as previously reported (Butenandt et al., 1954).

269   ^{1}H-NMR spectroscopy on the product validated that the main synthesized compound was

270   xanthommatin (see Material and Methods and Fig S1) (Williams et al., 2019a). The synthesized

271   product was then solubilized in methanol acidified with 0.5 % HCl (MeOH-HCl) and analyzed by

272   Liquid Chromatography (LC) coupled to Diode-Array Detection (DAD) and Mass Spectrometry (MS)

273   (Fig 2). The product was solubilized extemporaneously to avoid any chemical degradation before LC-

274   DAD-MS analyses (Fig 2A, B). Chromatograms showed two main peaks corresponding to

275   xanthommatin (retention time [RT]: 11.8 min, [M+H]$^+$ at *m/z* 424) and decarboxylated xanthommatin

276   (RT: 9.1 min, [M+H]$^+$ at *m/z* 380) (Table 1). Two co-eluting peaks were present in trace amounts at

277   RT 8.5 and 11.9 min and were associated to $[M+H]^+$ at *m/*z 460 and $[M+H]^+$ at *m/*z 504, respectively.

278   The detailed analysis of this sample enabled the detection of a small peak at RT 6.7 min ($[M+H]^+$ at

279   *m/*z 443) with its corresponding chromatogram at 430 nm (Fig 2A). A detailed analysis of this

280   particular compound is presented further in the text.

281   Table 1. **Analytical characteristics of ommatin-related compounds found *in vitro* and in biological extracts.**

Annotation (Formula, calculated MW)	RT (min)	Absorbance peaks	Monocharged ions (*m/z* loss)	Double-charged ions (*m/z* loss)	MS/MS fragments (*m/z* loss)
Detected in synthesized ommatins, crude extracts of housefly eyes and ommochromasome extracts					
3-Hydroxykynurenine ($C_{10}H_{12}N_2O_4$, 224.21)	1.6	231; 264; 376	224.9 $[M+H]^+$; 207.9 (-17); 161.9 (-63); 152.0 (-73)	Not detected	207.7 (-17); 161.9 (-63)
Uncyclized xanthommatin ($C_{20}H_{18}N_4O_8$, 442.38)	6.7	235; 420-450	442.9 $[M+H]^+$; 425.9 (-17); 408.8 (-34); 353.0 (-90)	213.6 $[M-17+2H]^{2+}$	425.9 (-17); 409.0 (-34); 390.9 (-52); 363.1 (-80); 353.0 (-90); 344.9 (-98); 335.1 (-108); 317.0 (-126); 307.0 (-136)
Xanthommatin ($C_{20}H_{13}N_3O_8$, 423.33)	11.8	234; 442	423.9 $[M+H]^+$; 406.8 (-17); 377.9 (-46); 350.9 (-73)	212.5 $[M+2H]^{2+}$; 189.5 (-23)	406.3 (-17; -18); 360.7 (-63); 350.8 (-73); 316.8 (-107); 304.8 (-119); 288.9 (-135)
Decarboxylated xanthommatin ($C_{19}H_{13}N_3O_6$, 379.32)	9.1	234; 442	379.9 $[M+H]^+$; 362.9 (-17); 333.9 (-46); 306.9 (-73)	190.5 $[M+2H]^{2+}$; 167.5 (-23)	362.7 (-17; -18); 333.7 (-46); 316.8 (-63); 306.8 (-73); 290.9 (-89)
Only detected in synthesized ommatins					
Xanthommatin sulphate/phosphate ester ($C_{20}H_{13}N_3O_{11}S$, 503.40; $C_{20}H_{14}N_3O_{11}P$, 503.31)	12.4	236; 445	503.9 $[M+H]^+$; 453.6 (-50); 430.5 (-73?); 379.8 (-124); 350.7 (-153)	252.5 $[M+2H]^{2+}$; 229.5 (-23)	487.0 (-17); 440.6 (-63); 430.8 (-73); 422.8 (-81); 396.5 (-107?); 384.9 (-119)
Decarboxylated xanthommatin sulphate/phosphate ester ($C_{19}H_{13}N_3O_9S$, 459.39; $C_{19}H_{14}N_3O_9P$, 459.30)	8.5	236; 443	459.8 $[M+H]^+$; 442.8 (-17); 413.8 (-46); 386.7 (-73)	230.4 $[M+2H]^{2+}$; 207.4 (-23)	442.8 (-17); 413.7 (-46); 396.8 (-63); 386.8 (-73)
Detected in synthesised ommatins incubated in MeOH-HCl in darkness					
α^3-Methoxy-xanthommatin ($C_{21}H_{15}N_3O_8$, 437.36)	12.6	217; 303; 452	437.9 $[M+H]^+$; 420.9 (-17); 391.9 (-46); 364.9 (-73)	219.4 $[M+2H]^{2+}$; 196.5 (-23)	420.7 (-17); 391.8 (-46); 374.8 (-63); 364.8 (-73); 314.8 (-123); 304.8 (-133)
α^3, α^{11}-Dimethoxy-xanthommatin ($C_{22}H_{17}N_3O_8$, 451.39)	13.0	217; 303; 452	451.9 $[M+H]^+$; 434.9 (-17); 391.9 (-60); 364.9 (-87)	226.5 $[M+2H]^{2+}$; 196.4 (-30)	434.9 (-17); 374.8 (-77); 364.9 (-87); 314.8 (-137); 304.8 (-147)
Decarboxylated α^{11}-methoxy-xanthommatin ($C_{20}H_{15}N_3O_6$, 393.35)	10.1	234; 442	393.9 $[M+H]^+$; 376.9 (-17); 333.9 (-60); 307.0 (-87)	197.6 $[M+2H]^{2+}$; 167.4 (-30)	376.7 (-17; -18); 333.8 (-60); 316.8 (-77); 306.9 (-87); 290.9 (-105)
Unknown altered xanthommatin (455)	14.4	242; 442	455.9 $[M+H]^+$	228.4 $[M+2H]^{2+}$; 205.4 (-23)	340.1 (-116); 324.6 (-131?); 295.0 (-161); 205.2 (-251)
Unknown altered methoxy-xanthommatin (469)	14.9	243; 390; 452	469.8 $[M+H]^+$	235.5 $[M+2H]^{2+}$; 212.4 (-23)	353.7 (-116); 338.8 (-131); 294.7 (-175); 204.8 (-265)
Unknown altered dimethoxy-xanthommatin (483)	15.0	242; 388; 450	484.0 $[M+H]^+$	242.5 $[M+2H]^{2+}$; 212.5 (-30)	353.9 (-130); 338.8 (-145); 294.8 (-189); 204.9 (-279)
Detected in synthesized ommatins incubated with β-mercaptoethanol in darkness and in ommochromasome extracts					
β-mercaptoethanol-added xanthommatin ($C_{22}H_{17}N_3O_9S$, 499.45)	11.7	238; 415	499.9 $[M+H]^+$; 426.8 (-73)	250.4 $[M+2H]^{2+}$; 227.5 (-23); 218.4 (-32)	482.7 (-17); 464.7 (-35)
β-mercaptoethanol-added decarboxylated xanthommatin ($C_{21}H_{17}N_3O_7S$, 455.44)	9.1	232; 412	455.9 $[M+H]^+$; 420.0 (-36); 382.8 (-73); 343.8 (-112)	228.4 $[M+2H]^{2+}$; 205.4 (-23); 196.5 (-32)	438.8 (-17); 421.0 (-35); 392.7 (-63)

282

283   With the aim to produce more ommatins and thus manipulate their molecular structure, we

284   incubated synthesized ommatins for 24 h in MeOH-HCl at 20 °C in darkness (Fig 2C, D). Based on

285   previous studies (Bolognese and Liberatore, 1988), we expected ommatins to get methoxylated,

286   mainly on their carboxylic acid functions. The comparative analysis of Fig 2A, B (2 minutes in

287   MeOH-HCl) and Fig 2C, D (24 h later) highlights different sets of peaks that appeared or disappeared

288   over the 24 h of incubation. Three major newly formed compounds were observed at RT 10.1, 12.6

289   and 13 min corresponding to [M+H]$^+$ at *m/z* 394, 438 and 452, respectively. We compared the UV and

290   MS characteristics of these compounds with those of xanthommatin and decarboxylated

291   xanthommatin. Absorbance spectra of the five compounds revealed strong similarities, particularly in

292   the visible region (> 400 nm, Fig 3A) suggesting that these three newly formed molecules shared their

293   chromophores with xanthommatin and decarboxylated xanthommatin. Mass spectra of the five

294   compounds also showed strong similarities (Fig 3B). They all experienced an in-source neutral loss of

295   -17 *m/z* (-NH_3) and formed a double-charged molecular ion [M+2H]$^{2+}$. 452 and 394 *m/z*-associated

296   compounds typically lost 14 units more than xanthommatin and decarboxylated xanthommatin,

297   respectively, during in-source fragmentation. Additionally, their double-charged fragmentations were

298   7 units higher than for xanthommatin and decarboxylated xanthommatin (= 14/2; Fig 3B). Overall,

299   gains of 14 *m/z* in the newly formed compounds compared to xanthommatin and decarboxylated

300   xanthommatin suggested methylation reactions occurring in acidic methanol.

301   Because we did not succeed in purifying each compound to analyze them separately by NMR

302   spectroscopy, we subjected the main [M+H]$^+$ to MS/MS fragmentation and compared it with

303   previously reported fragmentation patterns of kynurenine, 3-hydroxykynurenine, xanthommatin and

304   decarboxylated xanthommatin (Guijas et al., 2018; Panettieri et al., 2018; Reiter et al., 2018; Vazquez

305   et al., 2001; Williams et al., 2016) (http://metlin.scripps.edu, METLIN ID: 365). In these molecules,

306   the main ionization site is the amine function of the amino acid branch, which is also the most

307   susceptible to fragmentation. For both xanthommatin and decarboxylated xanthommatin, we observed

308   similar patterns of fragmentation of the amino acid branch with three neutral losses corresponding to -

309   NH_3 (-17 *m/z*), -CH_5O_2N (-63 *m/z*) and -$C_2H_3O_2N$ (-73 *m/z*) (Table 1). Those fragmentations have been

310   reported for these two ommatins (Panettieri et al., 2018; Reiter et al., 2018; Williams et al., 2016), as

311   well as for kynurenines (Vazquez et al., 2001), indicating that they are typical of compounds with a

312   kynurenine-like amino acid chain. Additionally, two neutral losses corresponding to -CO_2 (-44 *m/z*)

313   and -CH_2O_2 (-46 *m/z*) were observed only for xanthommatin (Fig 3C) due to the presence of the

314   carboxyl function on the pyridine ring (Fig 3D). We categorized those predictable MS fragments into

315   different successive fragmentation signatures called F_A to F_E (Table 2) and we used them to assign the

316   structure of unknown ommatins. The fragmentation of $[M+H]^+$ 394 *m/z* showed neutral losses

317   corresponding to -NH_3 (-17 *m/z* = F_A), -$C_2H_7O_2N$ (-77 = -63 -14 *m/z* = F_A + F_B + F_C - CH_2) and -

318    $C_3H_5O_2N$ (-87 = -73 -14 *m/z* = F_A + F_B + F_C + F_D - CH_2) on the amino acid branch. These results

319   strongly indicated that, in the 394 *m/z*-associated compound, the carboxyl function of the amino acid

320   branch was methoxylated (α^{11} position). Consequently, this compound was assigned to decarboxylated

321   α^{11}-methoxy-xanthommatin (Fig 3C). Those conclusions are in accordance with the similar absorbance

322   spectra of decarboxylated α^{11}-methoxy-xanthommatin and decarboxylated xanthommatin (Fig 3A), as

323   the amino acid branch is unlikely to act on near-UV and visible wavelength absorptions of the

324   chromophore. The fragmentation of $[M+H]^+$ 438 *m/z* showed the xanthommatin-like neutral losses, -

325   NH_3 (-17 *m/z* = F_A), -CH_5O_2N (-63 *m/z* = F_A + F_B + F_C) and -$C_2H_3O_2N$ (-73 *m/z* = F_A + F_B + F_C + F_D),

326   highlighting that this compound shared the same unaltered amino acid branch with xanthommatin.

327   However, this compound experienced the neutral loss -$C_2H_4O_2$ (-60 = -46 -14 *m/z* = F_E - CH_2) on the

328   pyridine ring instead of -CH_2O_2 (-46 *m/z* = F_E) (Fig 3C), which strongly indicated a methoxylation on

329   the pyrido-carboxyl group (α^3 position). This is in accordance with the associated absorbance spectrum

330   being different from that of xanthommatin, which has a carboxylated chromophore (Fig 3A). Hence,

331   we proposed that this compound was α^3-methoxy-xanthommatin (Fig 3D). The 452 *m/z*-associated

332   compound showed neutral losses corresponding to -NH_3 (-17 *m/z* = FA), -$C_2H_7O_2N$ (-77 *m/z* = F_A + F_B

333   + F_C - CH_2) and -$C_3H_5O_2N$ (-87 *m/z* = F_A + F_B + F_C + F_D - CH_2) on the amino acid branch and -$C_2H_4O_2$

334   (-60 *m/z* = F_D - CH_2) on the pyridine ring, which strongly indicated methoxylations on both pyridine

335   ring and amino acid branch in positions α^3 and α^{11}, respectively. This is in accordance with the

336   associated absorbance spectrum being similar to that of α^3-methoxy-xanthommatin, which has a

337   methoxylated chromophore. Thus, we proposed this compound to be α^3,α^{11}-dimethoxy-xanthommatin

338   (Fig 3D). Overall, such positions of methoxylation agree well with the reactivity of ommatins in

339   MeOH-HCl (Bolognese and Liberatore, 1988).

340   Table 2. **Diagnostic neutral losses of ommatins**.

Annotation	Fragmented structure	Neutral loss	*m/z* loss
F_A	Amino acid	$-NH_3$	-17
F_B	Amino acid	$-H_2O$	-18
F_C	Amino acid	-CO	-28
F_D	Amino acid	-C+2H	-10
F_E	Pyridine ring	$-CO_2H$	-46

341

342   Using the same DAD-MS combination approaches, we annotated other ommatin-like

343   compounds produced during *in vitro* synthesis and after incubation in MeOH-HCl (Table 1). The 504

344   and 460 *m/z*-associated compounds differed from xanthommatin and decarboxylated xanthommatin by

345   80 Da, respectively. The associated double-charged ions $[M+2H]^{2+}$ and $[M-CH_2O_2+2H]^{2+}$ accordingly

346   differed by 40 *m/z* from those of xanthommatin and decarboxylated xanthommatin, respectively. Their

347   absorbance spectra were identical to the two ommatins. Their MS and MS/MS spectra revealed

348   identical losses to xanthommatin and decarboxylated xanthommatin: $-NH_3$ (-17 *m/z* = F_A), $-CH_2O_2$ (-

349   46 *m/z* = F_D), $-CH_5O_2N$ (-63 *m/z* = F_A + F_B + F_C) and $-C_2H_3O_2N$ (-73 *m/z* = F_A + F_B + F_C + F_D; see

350   Table 2). Furthermore, the $[M+H]^+$ 504 *m/z* experienced the same neutral losses $-C_2H_5O_4N$ (-107 m/z)

351   and $-C_3H_5O_4N$ (-119 *m/z*) than xanthommatin. Alternatively, the $[M+H]^+$ 504 *m/z* experienced a

352   unique in-source neutral loss of -153 *m/z* that could correspond to $-C_2H_3O_5NS$ or $-C_2H_4O_5NP$ (-73 -80

353   *m/z*). All those results suggested that the 504 and 460 *m/z*-associated compounds were sulphate or

354   phosphate esters of xanthommatin and decarboxylated xanthommatin, respectively. This in accordance

355   with the use of phosphate buffer for the *in vitro* synthesis and of sulfurous acid to precipitate

356   ommatins. To our knowledge, these two esters have never been described. Finally, during the

357   incubation in MeOH-HCl, minor ommatin-like compounds (classified as ommatins based on their

358   absorbance and the presence of double-charged ions) were formed and were associated to the 456, 470

359   and 484 *m/z* (Table 1). The differences of 14 units of their respective molecular ion *m/z*, as well as

360   their neutral losses in MS/MS being either similar or differing by 14 units, indicated that they were

361   methylated versions of each other. However, due to their very low amounts, we could not

362   unambiguously propose a structure.

363   These results showed that, in storage conditions mimicking extraction procedures with MeOH-

364   HCl, xanthommatin and its decarboxylated form are methoxylated, even in darkness. Those reactions

365   are likely to result from solvent additions with acidified MeOH-HCl (the most efficient solvent for

366   ommatin extraction). To further characterize the importance of those artifactitious reactions, we

367   followed the kinetic of the five ommatins described in Fig 3D in MeOH-HCl at 20 °C and in darkness.

368   *3.2 The ommatin profile is rapidly and readily modified overtime by artifactitious methoxylations*

369   *in acidified methanol*

370   Because absorbance spectra of all five considered compounds did not differ significantly and because

371   some of them were co-eluted (Fig 2), their detection and quantification were performed by MS/MS in

372   multiple reaction monitoring (MRM) mode. MRM conditions were independently optimized for each

373   compound based on the fragmentation of their amino acid branch (Fig S2).

374   The MRM signal of xanthommatin rapidly decreased overtime in a linear fashion, with a near

375   40 % reduction after only one day of incubation (Fig 4A). On the contrary, the MRM kinetics of α^3-

376   methoxy-xanthommatin had a logarithmic-shape, sharply increasing during the first day before

377   reaching a plateau during the two following days (Fig 4B). Both decarboxylated α^{11}-methoxy-

378   xanthommatin and α^3,α^{11}-dimethoxy-xanthommatin appeared after a few hours of incubation. Their

379   MRM signal then linearly increased overtime (Fig 4C, E). In parallel, the MRM signal of

380   decarboxylated xanthommatin stayed nearly constant, with only a small increase by 1.13 % over the

381   five first hours (Fig 4D). Those results further validate that xanthommatin was readily methoxylated in

382   darkness, primarily in position α^3. A slower methoxylation on the amino acid branch could account for

383   the delay in the appearance of the two other methoxylated forms. The levels of decarboxylated

384   xanthommatin did not vary much overtime although its methoxycarbonyl ester was produced (Fig 4D,

385   E). This result could be explained by the concomitant and competitive slow decarboxylation of

386   xanthommatin, a reaction that has already been described in MeOH-HCl upon light radiations

387   (Bolognese et al., 1988b).

388   Because we cannot compare MRM signal intensities of different molecules, we took benefit of

389   their similar absorption in the visible region, especially at 414 nm (Fig 3A, Fig S3), to quantify their

390   relative amounts. Although less sensitive and less specific than MRM-based detection, the absorbance

391   at 414 nm strongly indicated that, after only one day of incubation, one third of ommatins was

392   methoxylated (Fig 4F). Most of the methoxylated ommatins accumulated during the first 24 hours (Fig

393   4B). As expected, rates of methoxylation were significantly decreased by incubating synthesized

394   ommatins in MeOH-HCl at -20 °C (Fig S4A, B). After storage of a month at -20 °C, the methoxylated

395   ommatins represented nearly 1.2 % of ommatins (Fig S4C).

396   To conclude, our results showed that decarboxylated xanthommatin was mostly stable in

397   MeOH-HCl. By contrast, xanthommatin was rapidly converted into methoxylated derivatives. Since,

398   MeOH-HCl is the most efficient solvent for ommatin extraction, the conditions for extraction and

399   analysis of ommatins from biological samples should avoid wherever possible the formation of

400   artifactitious methoxylated ommatins.

401   *3.3 UPLC-DAD-MS/MS structural elucidation of uncyclized xanthommatin, the labile*

402   *intermediate in the synthesis of ommatins from 3-hydroxykynurenine*

403   The *in vitro* synthesis of xanthommatin by oxidizing 3-hydroxykynurenine additionally yielded a

404   minor compound at RT 6.7 min. It was characterized by a peak of absorbance at 430 nm and was

405   associated to the 443 *m/z* feature (Fig 2A, B). Upon solubilization in MeOH-HCl, the unidentified

406   compound was labile and disappeared after the 24h-incubation at 20 °C in darkness (Fig 2C, D). A

407   similar 443 *m/z* feature was described two decades ago during oxidations of 3-hydroxykynurenine in

408   various conditions (Iwahashi and Ishii, 1997). Based on its MS spectrum, it was assigned putatively to

409   the 3-hydroxykynurenine dimer called uncyclized xanthommatin. However, there was a lack of

410   analytical evidence to support its structural elucidation. No study has ever since reported the presence

411   of uncyclized xanthommatin, either *in vitro* or *in vivo*. Because this compound could be an important

412   biological intermediate in the formation of ommatins (Bolognese et al., 1988b; Iwahashi and Ishii,

413   1997), we further characterized its structure based on its chemical behavior, absorbance and

414   fragmentation pattern. We note that the apparent lability and the very low amounts of this unidentified

415   product precluded its characterization by NMR spectroscopy.

416   The absorbance and MS kinetics of the unidentified synthesized compound showed that it was

417   very labile (insets of Fig 5A, B). Indeed, we could not detect it after a week of storage at -20 °C

418   anymore. This behavior resembled that of a photosensitive ommatin-like isolated 40 years ago from

419   several invertebrates, which rapidly turned into xanthommatin after extraction (Bolognese and

420   Scherillo, 1974). The absorbance spectrum of this unidentified compound matched almost exactly the

421   UV-Visible spectrum of cinnabarinic acid measured in the same conditions (Fig 5C), and both are

422   similar to those reported for actinomycin D and 2-amino-phenoxazin-3-one (Nakazawa et al., 1981).

423   This result indicated that this compound contained the amino-phenoxazinone chromophore rather than

424   the pyrido[3,2-*a*]phenoxazinone scaffold of ommatins or the *ortho*-aminophenol core of 3-

425   hydroxykynurenine. Furthermore, its ionization pattern revealed striking similarities with ommatins.

426   Along with the molecular ion [M+H]$^+$ at 443 *m/z* (corresponding to MW 442 and to the formula

427    $C_{20}H_{18}N_4O_8$), we detected the double-charged ion [M-NH$_3$+2H]$^{2+}$ at 213.6 *m/z* (Fig 5D). We then

428   targeted the molecular ion for MS/MS to compare the obtained fragments with those reported above

429   (Table 2). If the compound was uncyclized xanthommatin, we predicted that F_A, F_B, F_C and F_D would

430   appear each twice, because uncyclized xanthommatin possesses two 3-hydroxykynurenine-like amino

431   acid chains. Only F_E should be absent, because no aromatic carboxylic acid exist in uncyclized

432   xanthommatin. Indeed, from two MS^2 spectra obtained at different collision energies, we could assign

433   each F_X twice and we did not find any fragmentation event corresponding to F_E. Hence, by starting

434   from the amino-phenoxazinone backbone suggested by the UV-Visible spectrum, the uncyclized form

435   of xanthommatin could be reconstructed after adding each F_{Xx} successively (Fig 5F).

436   All these analytical characteristics strongly supported that this labile compound was the

437   phenoxazinone dimer of 3-hydroxykynurenine (Fig 5G), called uncyclized xanthommatin (Figon and

438   Casas, 2019). This structural assignment was further supported by the two following chemical

439   behaviors. First, the oxidation of an *ortho*-aminophenol (here 3-hydroxykynurenine) by potassium

440   ferricyanide is known to induce its dimerization through the loss of six electrons and protons (here 2x

441    $MW_{\text{3-hydroxykynurenine}}$ [224 Da] - 6 Da = MW_{dimer} [442 Da] = $MW_{\text{Uncyclized xanthommatin}}$). Second, the

442   spontaneous intramolecular cyclization involving the amine functions of the amino-phenoxazinone

443   core and the closest amino acid branch could explain the lability of uncyclized xanthommatin in cold

444   MeOH-HCl (insets of Fig 5A, B) (Williams et al., 2019a), as well as the formation of a major double-

445   charged ion corresponding to that of the reduced form of xanthommatin (dihydroxanthommatin; Fig

446   5G and Fig S5). The lability of uncyclized xanthommatin questions how we could detect it. We

447   believe there are three main reasons. First, our UHPLC-DAD-MS approach use some of the most

448   sensitive analytical techniques so far. Second, we used rather concentrated samples that allowed us to

449   inject it in sufficient amounts for chromatography, despite the steep decrease in uncyclized

450   xanthommatin during the first hours after extraction. Third, abiotic and biotic factors likely stabilize

451   uncyclized xanthommatin before and during extraction. In particular, we kept all extraction steps at 4

452   °C and in the darkness as much as possible to avoid increased rates of cyclization (Bolognese et al.,

453   1988a; Bolognese and Scherillo, 1974). Furthermore, ommochromes are known to bind to specific

454   proteins, such as ommochrome binding protein and reflectins, which protect them from degradation

455   (Williams et al., 2019b); it is therefore possible that uncyclized xanthommatin is stabilized in vivo by

456   binding to proteins. In conclusion, we have now the tools to identify uncyclized xanthommatin in

457   other samples, particularly biological materials.

458   *3.4 Biological localization of the metabolites from the tryptophan →ommochrome pathway*

459   Using our chemical and analytical knowledge of synthesized ommatins, we reinvestigated the content

460   of housefly eyes in ommochromes and their related metabolites. We chose this species because it is

461   known to accumulate xanthommatin and some metabolites of the kynurenine pathway in its eyes

462   (Linzen, 1974). However, nothing is known about decarboxylated xanthommatin and uncyclized

463   xanthommatin because they were recently described [(Figon and Casas, 2019) and this study], even

464   though the presence of uncyclized xanthommatin has been suspected (Bolognese and Scherillo, 1974).

465   Furthermore, a protocol to extract and purify ommochromasomes from housefly eyes is available

466   (Cölln et al., 1981), thus we can address the question of the localization of the metabolites from the

467   tryptophan→ommochrome pathway. Finally, we designed an extraction protocol in which all steps

468   were performed in darkness, at low temperature and in less than half an hour. In those conditions, we

469   were confident that artifactitious methoxylated ommatins would represent less than one percent of all

470   ommatins and that we could still detect uncyclized xanthommatin.

471   Based on MRM signals, we detected in methanolic extractions of housefly eyes (called crude

472   extracts; Fig 6A) the following metabolites of the tryptophan→ommochrome pathway: tryptophan, 3-

473   hydroxykynurenine, xanthurenic acid, xanthommatin, decarboxylated xanthommatin and uncyclized

474   xanthommatin (Fig 6B, Table 1). We ascertained the identification of uncyclized xanthommatin by

475   acquiring its absorbance, MS and MS/MS spectra in biological samples. They showed the same

476   features as synthesized uncyclized xanthommatin (Fig S6).

477   We then purified ommochromasomes from housefly eyes by a combination of differential

478   centrifugation and ultracentrifugation, and we compared the extracted compounds with those of crude

479   extracts (Fig 6A). The main metabolites of ommochromasomes were xanthommatin and its

480   decarboxylated form (Fig 6D), in accordance to the function of ommochromasomes as ommochrome

481   factories (Figon and Casas, 2019). Based on the absorbance at 414 nm, decarboxylated xanthommatin

482   represented 5.3 ± 0.1 % (mean $\pm$ SD, n = 5) of all ommatins detected in extracts of

483   ommochromasomes. In comparison, decarboxylated ommatins represented 21.5 ± 0.2 % (mean $\pm$ SD,

484   n = 10) of all ommatins synthesized *in vitro*, a percentage four times higher than in methanolic extracts

485   of purified ommochromasomes (Welch two sample *t*-test, t = 219.99, df = 12.499, p-value < $2.2e^{-16}$).

486   Regarding the precursors of ommochromes in housefly eyes, we could only detect tryptophan and 3-

487   hydroxykynurenine but not the intermediary kynurenine. Tryptophan remained undetectable in

488   extracts of ommochromasomes (Fig 6D). Xanthurenic acid, the product of 3-hydroxykynurenine

489   transamination, was particularly present in crude extracts of housefly eyes but much less in

490   ommochromasomes relatively to 3-hydroxykynurenine (Fig 6C vs. Fig 6E). We also detected the

491   uncyclized form of xanthommatin in ommochromasomes (Fig 6D). We calculated an enrichment ratio

492   of 3-hydroxykynurenine compared to either xanthurenic acid or uncyclized xanthommatin in both

493   crude and ommochromasome extracts, allowing a meaningful comparison between xanthurenic acid

494   and uncyclized xanthommatin in the two extracts. The lower this enrichment ratio is, the more

495   xanthurenic acid or uncyclized xanthommatin is present in the extract. We found that xanthurenic acid

496   was more enriched in crude extracts (mean enrichment of 2.1) than in ommochromasomes (mean

497   enrichment of 11; Student's *t*-test, t = -8.88, df = 8, p-value = $2.04e^{-5}$), while uncyclized xanthommatin

498   was more enriched in ommochromasomes (mean enrichment of 6.2) than in crude extracts (mean

499   enrichment of 21; Student's *t*-test, t = 5.71, df = 8, p-value = 0.00045).

500   We detected in ommochromasomes two minor ommatin-like compounds associated to the

501   molecular ions 500 and 456 *m/z* (Table 1), which co-eluted with xanthommatin and its decarboxylated

502   form, respectively (Fig 6D). Both unknown compounds were undetectable in crude extracts of

503   housefly eyes. Their associated *m/z* features differed from those of xanthommatin and decarboxylated

504   xanthommatin by 76 units, respectively (Table 1). Because the isolation buffer used for

505   ommochromasome purifications contained β-mercaptoethanol (MW 78 Da), we tested whether those

506   unknown ommatins could be produced by incubating synthesized ommatins with β-mercaptoethanol

507   in a water-based buffer. We did find that 456 and 500 *m/z*-associated compounds were rapidly formed

508   in those *in vitro* conditions and that their retention times matched those detected in ommochromasome

509   extracts (Fig S7). Therefore, the 456 and 500 *m/z*-associated ommatins detected in ommochromasome

510   extracts were likely artifacts arising from the purification procedure via the addition of β-

511   mercaptoethanol. These results further demonstrate that ommochromes are likely to be altered during

512   extraction and purification procedures, a chemical behavior that should be controlled by using

513   synthesized ommochromes incubated in similar conditions than biological samples.

514   ## 4. Discussion

515   *4.1 UPLC-DAD-MS/MS structural elucidation of new ommatins*

516   Biological ommochromes are difficult compounds to analyze by NMR spectroscopy

517   (Bolognese et al., 1988b). Only xanthommatin, the most common and studied ommochrome, has been

518   successfully subjected to ^{1}H-NMR (although no ^{13}C- and 2D-NMR data exist to date, to the best of our

519   knowledge) (Kumar et al., 2018; Williams et al., 2019a). The NMR-assisted structural elucidation of

520   unknown ommochromes remains therefore extremely challenging and deceptively difficult. To tackle

521   this structural problem, we used a combination of absorption and mass spectroscopies, which offer

522   high sensitivities and orthogonal information, after separation by liquid chromatography. We report

523   here the most comprehensive analytical dataset to date of ommatins, based on their absorbance, mass

524   and tandem mass spectra (Table 1). This dataset allowed us to elucidate with strong confidence the

525   structure of four new ommochromes, including three methoxylated forms and one labile biological

526   intermediate, and to propose structures for eight new other ommatins.

527   Studies reporting the MS/MS spectra of ommatins demonstrated that they primarily fragment

528   on their amino acid chain (Williams et al., 2016) and then on the pyrido-carboxylic acid, if present

529   (Panettieri et al., 2018). We confirmed those results for other ommatins, which indicates that

530   ommatins fragment in a predictable way despite being highly aromatic compounds. We took a step

531   further by positioning methoxylations based on the differences in fragmentation between

532   methoxylated ommatins and (decarboxylated) xanthommatin. Besides, xanthommatin not only formed

533   a 307 *m/z* fragment but also a major 305 *m/z* fragment (Fig 3C; Table 1). This sole fragment was used

534   in a previous study to annotate a putative new ommochrome called (iso-)elymniommatin, an isomer of

535   xanthommatin (Panettieri et al., 2018). Our results prove that MS/MS spectra cannot distinguish (iso-

536   )elymniommatin and xanthommatin unambiguously. The use of synthesized ommatins and further

537   experiments are thus needed to verify the existence of (iso-)elymniommatin in butterfly wings.

538   Overall, this analytical dataset will help future studies to identify known biosynthesized and

539   artifactitious ommatins in biological samples, as well as to elucidate the structure of unknown

540 ommatins by analyzing their absorbance and mass spectra in the absence of NMR data. Furthermore, it

541 is now possible to look for uncyclized xanthommatin in a wide variety of species.

4.2 Biological extracts are prone to yield artifactitious ommatins

543 It has long been reported that ommatins are photosensitive compounds that react with acidified

544 methanol (MeOH-HCl) upon light radiation, leading to their reduction, methylation, methoxylation,

545 decarboxylation and deamination (Bolognese et al., 1988c, 1988d; Bolognese and Liberatore, 1988;

546 Figon and Casas, 2019). Nevertheless, incubating tissues in MeOH-HCl for several hours at room

547 temperature has been commonly used to extract ommatins from biological samples efficiently

548 (Bolognese et al., 1988a; Riou and Christidès, 2010; Zhang et al., 2017). Our results demonstrate that,

549 even in the absence of light radiation, ommatins are readily and rapidly methoxylated by thermal

550 additions of methanol, primarily on the carboxylic acid function of the pyridine ring and secondarily

551 on the amino acid chain (Fig 7). The $[M+H]^+$ 438 *m/z* of α^3-methoxy-xanthommatin identified in our

552 study could correspond to the same $[M+H]^+$ previously reported in extracts of butterfly wings

553 (Panettieri et al., 2018). Hence, artifactitious methoxylations during extraction should first be ruled out

554 before assigning methoxylated ommatins to a new biosynthetic pathway. We also show that ommatins

555 react with other extraction buffers since we detected β-mercaptoethanol-added ommatins when

556 synthesized ommatins were incubated in a phosphate buffer containing that reducing agent. Overall,

557 these results emphasize the need to control for potential artifactitious reactions when performing any

558 extraction or purification protocol of biological ommochromes.

4.3 The metabolites of the tryptophan➔ommochrome pathway in ommochromasomes

560 It has long been hypothesized that precursors of ommochromes are translocated within

561 ommochromasomes by the transmembrane ABC transporters White and Scarlet (Ewart et al., 1994;

562 Mackenzie et al., 2000). Here, we clearly demonstrate that 3-hydroxykynurenine, but not tryptophan,

563 occurs in ommochromasome fractions of housefly eyes, confirming that 3-hydroxykynurenine is the

564 precursor imported into ommochromasomes by White and Scarlet transporters (Fig 8).

565   Our results confirm that xanthommatin is the main ommatin in ommochromasomes of

566   housefly eyes. We also showed that decarboxylated xanthommatin was present in significant amounts,

567   which could not be solely due to the slow decarboxylation of xanthommatin in MeOH-HCl. This result

568   indicates that both xanthommatin and its decarboxylated form are produced from 3-

569   hydroxykynurenine within ommochromasomes (Fig 8). We also detected xanthurenic acid, the

570   cyclized form of 3-hydroxykynurenine, in housefly eyes. Compared to 3-hydroxykynurenine,

571   xanthurenic acid was present in minute amounts within ommochromasomes. We hypothesize that

572   xanthurenic acid is produced within ommochromasomes via two non-exclusive pathways (Fig 8). The

573   first route is the *in situ* intramolecular cyclization of 3-hydroxykynurenine, which requires a cytosolic

574   transaminase activity (HKT; Fig 8) (Han et al., 2007). The second route is the degradation of

575   xanthommatin that would produce 3-hydroxykynurenine and xanthurenic acid. The phenoxazinone

576   structure of ommatins is indeed known to undergo ring-cleavage, particularly in slightly basic water-

577   based buffers (Butenandt and Schäfer, 1962). Hence, traces of xanthurenic acid might either come

578   from degradation during the purification protocol or from biological changes in ommochromasome

579   conditions (enzymatic activities or basification) leading to the cleavage of xanthommatin. To the best

580   of our knowledge, no biological pathways for the degradation of the pyrido-phenoxazinone structure

581   of ommatins have been described. The detection of xanthurenic acid in ommochromasomes might

582   therefore be the first step towards understanding the *in situ* catabolism of ommatins (Fig 8).

583   Experimental and computational chemists have long hypothesized that the pyrido[3,2-

584   *a*]phenoxazinone structure of ommatins should be synthesized *in vivo* by the dimerization of 3-

585   hydroxykynurenine and a subsequent spontaneous intramolecular cyclization (Butenandt, 1957;

586   Zhuravlev et al., 2018). However, the associated dimer of 3-hydroxykynurenine, called uncyclized

587   xanthommatin, proved to be difficult to characterize and to isolate from biological samples because of

588   its lability (Bolognese and Scherillo, 1974). In our study, we synthesized uncyclized xanthommatin by

589   the oxidative condensation of 3-hydroxykynurenine with potassium ferricyanide, an oxidant known to

590   form amino-phenoxazinones from *ortho*-aminophenols (Bolognese and Scherillo, 1974). We used a

591   combination of kinetics and analytical spectroscopy (DAD, MS and MS/MS) to confirm the *in vitro*

592 and biological occurrence of uncyclized xanthommatin. Because we detected xanthommatin, its

593 decarboxylated form, their precursor 3-hydroxykynurenine and the intermediary uncyclized

594 xanthommatin in both *in vitro* and biological samples, we argue that the *in vitro* synthesis and the

595 biosynthesis of ommatins proceed through a similar mechanism (compare Fig 7 and Fig 8). An

596 alternative biosynthetic pathway for ommatins has been proposed to occur through the condensation of

597 3-hydroxykynurenine with xanthurenic acid (hypothesis 2, Fig 1B) (Linzen, 1974; Panettieri et al.,

598 2018). Under the hypothesis that 3-hydroxykynurenine and xanthurenic acid condense into

599 xanthommatin, we would expect them to be in similar quantities within ommochromasomes (i.e.

600 enrichment ratio of 1), which was not the case since their concentrations differed by an order of

601 magnitude. On contrary, under the hypothesis that uncyclized xanthommatin is the intermediate form

602 between 3-hydroxykynurenine and xanthommatin, we would expect it to be enriched in

603 ommochromasomes compared to crude extracts, which was the case either. Furthermore, the fact that

604 the unstable uncyclized xanthommatin was more enriched in ommochromasomes than the stable

605 xanthurenic acid indicates that the flux of 3-hydroxykynurenine dimerization is high enough to

606 counterbalance the disappearance of uncyclized xanthommatin by intramolecular cyclization. Thus,

607 uncyclized xanthommatin is unlikely solely a by-product. At the very least, our results show that

608 xanthurenic acid is tightly linked to the ommochrome pathway and therefore cannot be considered as a

609 marker of a distinct biogenic pathway. Lastly, as far as we know, there has been no experimental

610 evidence for the formation of xanthommatin by condensing 3-hydroxykynurenine with xanthurenic

611 acid. In conclusion, the formation of uncyclized xanthommatin by the oxidative dimerization of 3-

612 hydroxykynurenine is likely to be the main biological route for the biosynthesis of ommatins within

613 ommochromasomes (Fig 8).

614   Whether the *in vivo* oxidative dimerization of 3-hydroxykynurenine is catalyzed enzymatically

615 remains a key question in the biogenesis of ommochromes (Figon and Casas, 2019). Theoretical

616 calculations suggested that both enzymatic and non-enzymatic oxidations of 3-hydroxykynurenine

617 would lead to the formation of the phenoxazinone uncyclized xanthommatin (Williams et al., 2019a;

618 Zhuravlev et al., 2018). There was some evidence of a phenoxazinone synthase (PHS) activity

26

619   associated to purified ommochromasomes of fruitflies (Yamamoto et al., 1976). However, no

620   corresponding PHS enzyme has ever been isolated nor identified in species producing ommochromes

621   (Figon and Casas, 2019). PHS is not the only enzyme capable of forming aminophenoxazinones from

622   *ortho*-aminophenols. Tyrosinase, laccase, peroxidase and catalase can also catalyze these reactions (Le

623   Roes-Hill et al., 2009). Particularly, peroxidase can produce xanthommatin and its decarboxylated

624   form from 3-hydroxykynurenine *in vitro* (Ishii et al., 1992; Iwahashi and Ishii, 1997; Vazquez et al.,

625   2000; Vogliardi et al., 2004), likely through the formation of uncyclized xanthommatin (Iwahashi and

626   Ishii, 1997). This result relates to the long-known fact that insects mutated for the heme peroxidase

627   Cardinal accumulate 3-hydroxykynurenine without forming ommochromes (Howells et al., 1977;

628   Osanai-Futahashi et al., 2016). Hence, our data support the hypothesis that the biosynthesis of

629   ommatins could be catalyzed by a relatively unspecific peroxidase such as Cardinal (Fig 8) (Liu et al.,

630   2017; Osanai-Futahashi et al., 2016), without the requirement of a specialized PHS.

631   *4.4 Uncyclized xanthommatin is a potential key branching point in the biogenesis of ommatins*

632   *and ommins*

633   The relatively recent description of decarboxylated xanthommatin in several species indicates that it is

634   a common biological ommatin (Figon and Casas, 2019). Yet, little is known about how

635   decarboxylation of ommatins proceeds *in vivo*. In this study, we show that decarboxylated

636   xanthommatin is unlikely to arise solely from the artifactitious decarboxylation of xanthommatin in

637   MeOH-HCl, and that the level of decarboxylated xanthommatin is lower in biological extracts (5.3 %)

638   than *in vitro* (21.5 %). Three biological mechanisms could account for the biosynthesis of

639   decarboxylated xanthommatin (Fig 8). (1) The decarboxylation of xanthommatin in water-based

640   environments and upon light radiations possibly accounted for the formation of decarboxylated

641   xanthommatin (Fig 8). Indeed, some aromatic compounds are known to be decarboxylated by the

642   action of water (Mundle and Kluger, 2009), and kynurenic acid, which is structurally related to

643   xanthommatin, is decarboxylated upon light radiations (Zelentsova et al., 2013). (2) Enzymatic and

644   non-enzymatic syntheses of ommatins might differ in their products and exact molecular steps (Ishii et

645   al., 1992; Zhuravlev et al., 2018). Thus, the proposed involvement of the heme peroxidase Cardinal in

646   the biosynthesis of ommatins (Howells et al., 1977) might lead to the natural formation of

647   xanthommatin over its decarboxylated form. (3) Bolognese and colleagues proposed that

648   decarboxylation happens by a rearrangement of protons, consecutively to the intramolecular

649   cyclization of uncyclized xanthommatin (Bolognese et al., 1988b). Such mechanism has been well

650   described for the biogenesis of eumelanin monomers, in which the non-decarboxylative rearrangement

651   of dopachrome is favored by the dopachrome tautomerase (Solano et al., 1996). Hence, this analogy

652   raises the intriguing possibility that a tautomerase might catalyze the formation of xanthommatin from

653   uncyclized xanthommatin (Fig 8), thereby controlling the relative content of decarboxylated

654   xanthommatin in ommochromasomes, which is known to vary among species, individuals and

655   chromatophores (Futahashi et al., 2012; Williams et al., 2016; Zhang et al., 2017). Why

656   decarboxylated xanthommatin levels depend on the biological context may rely on its biological

657   functions, which we now discuss.

658   Few studies have addressed the biological function of decarboxylated xanthommatin. Its ratio

659   to xanthommatin is known to vary among species and individuals (Futahashi et al., 2012; Williams et

660   al., 2016). In cephalopods, the ratio of xanthommatin to its decarboxylated form within a

661   chromatophore has been suggested to determine its color, ranging from yellow to purple (Williams et

662   al., 2016). However, our absorbance data do not support this hypothesis because the experimental

663   absorbance spectrum of decarboxylated xanthommatin was not different from that of xanthommatin in

664   the visible region. Moreover, purple colors are produced by chromophores that absorb wavelengths

665   around 520 nm, which has not been described for any ommatin in contrast to ommins (Figon and

666   Casas, 2019). Another study focusing on the quantum chemistry of pirenoxine, a xanthommatin-like

667   drug, proposed that the carboxylic acid function present on the pyrido[3,2-*a*]phenoxazinone of

668   pirenoxine could enhance its binding to divalent cations (Liao et al., 2011). This is coherent with the

669   fact that ommochromasomes accumulate and store cations, such as Ca^{2+} and Mg^{2+} (Gribakin et al.,

670   1987; Ukhanov, 1991). Thus, favoring xanthommatin over its decarboxylated form *in vivo* might

671   enhance the storage of metals in ommochromasomes, as proposed for eumelanins that contain high

672   proportions of the carboxylated monomers DHICA (Hong and Simon, 2007). To which extent the

673   binding of metals modifies the physical and chemical properties of ommatins, therefore their

674   biological roles, remains to be determined.

675   How ommatins and ommins, the two most abundant families of ommochromes, are

676   biochemically connected to each other is still a mystery (Figon and Casas, 2019). Purple ommins have

677   higher molecular weights than ommatins and derive from both 3-hydroxykynurenine and

678   cysteine/methionine, the latter providing sulfur to the phenothiazine ring of ommins (Linzen, 1974;

679   Needham, 1974). The best-known ommin is called ommin A, whose structure was proposed to be a

680   trimer of 3-hydroxykynurenine in which one of the phenoxazine ring is replaced by phenothiazine (Fig

681   8) (Needham, 1974). Since the pyrido[3,2-*a*]phenoxazinone cannot be reopen to an amino-

682   phenoxazinone in anyway (Bolognese and Liberatore, 1988), it is unlikely that the biosynthesis of

683   ommins is a side-branch of ommatins. Thus, the biochemical relationship between ommatins and

684   ommins should be found upstream in the biosynthetic pathway of ommochromes. Older genetic and

685   chemical studies demonstrated that ommins and ommatins share the kynurenine pathway and Linzen

686   proposed that the ratio of xanthommatin to ommins could depend on the level of methionine-derived

687   precursors (Linzen, 1974). The distinct structure of uncyclized xanthommatin raises the interesting

688   hypothesis that uncyclized xanthommatin is the elusive intermediate between the *ortho*-aminophenol

689   structure of 3-hydroxykynurenine, the pyrido-phenoxazinone chromophore of xanthommatin and the

690   phenoxazine-phenothiazine structure of ommins. We propose that the biosynthesis of ommins first

691   proceeds with the dimerization of 3-hydroxykynurenine into uncyclized xanthommatin, then with the

692   stabilization of its amino acid chain to avoid a spontaneous intramolecular cyclization, and finally with

693   the condensation with a sulfur-containing compound derived from methionine/cysteine (Fig 8).

694   Although this mechanism is hypothetical at this stage, it can explain two apparently unrelated

695   observations. First, it clarifies the reason why *cardinal* mutants of insects, which lack the heme

696   peroxidase Cardinal that possibly catalyzes the formation of uncyclized xanthommatin, lack both

697   ommatins and ommins, and accumulate 3-hydroxykynurenine (Howells et al., 1977; Osanai-Futahashi

698   et al., 2016). Second, it could explain how a single cephalopod chromatophore can change its color

699   from yellow (ommatins) to purple (ommins) across its lifetime (Reiter et al., 2018). The biochemical

700   mechanism might be analogous to the casing model of melanins (Ito and Wakamatsu, 2008), in which

701   pheomelanins and eumelanins (in this case ommins and ommatins, respectively) are produced

702   sequentially from the same precursors (uncyclized xanthommatin) through changes in sulfur

703   (methionine/cysteine) availability within melanosomes (ommochromasomes).

704   Overall, uncyclized xanthommatin appears as a key metabolite in the ommochrome pathway

705   by leading to either ommatins, decarboxylated ommatins or ommins (Fig 8). Therefore, the formation

706   of uncyclized xanthommatin might represent a key step in the divergence between the post-kynurenine

707   pathways of vertebrates and invertebrates, as well as in the structural diversification of ommochromes

708   in phylogenetically-distant invertebrates.

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889

890   Legends

891   Fig 1. **Current knowledge of the tryptophan→ommochrome pathway of invertebrates.** A) Main chemical structures and

892   chromophores of the tryptophan→ommochrome pathway. Numbering of ommatins used in this study is indicated on the

893   structure of xanthommatin. B) Kynurenine and ommochrome pathways form the early and late steps of the

894   tryptophan→ommochrome pathway, respectively. Ommatins are possibly biosynthesized via two routes: (1) the dimerization

895   of 3-hydroxykynurenine into the intermediary uncyclized xanthommatin, or (2) the direct condensation of 3-

896   hydroxykynurenine with its cyclized form, xanthurenic acid. Ommatin and ommin pathways share 3-hydroxykynurenine as a

897   precursor, but at which step they diverge is not known. Dashed arrows, steps for which we lack clear biological evidence.

898   HKT, 3-hydroxykynurenine transaminase. KFase, kynurenine formamidase. KMO, kynurenine 3-monooxygenase. Ox.,

899   oxidation. Red., reduction. TDO, tryptophan 2,3-dioxygenase.

900   Fig 2. **Chromatographic profiles of synthesized xanthommatin before and after storage in acidified methanol.**

901   Xanthommatin was synthesized by oxidizing 3-hydroxykynurenine with potassium ferricyanide. **(A-B)** The ommatin solution

902   was subjected to liquid chromatography (LC) two minutes after solubilization in methanol acidified with 0.5 % HCl (MeOH-

903   HCl). The eluted compounds were detected by their absorbance at 440 and 430 nm (A). The main molecular ions

904   (electrospray ionization in positive mode) associated to each peak were monitored by a triple quadrupole mass spectrometer

905   running in single ion reaction (SIR) mode (B). **(C-D)** The same ommatin solution was left for 24 hours at 20 °C in complete

906   darkness. Compounds were separated by LC and detected using the same absorbance (A) and MS modalities (B) as described

907   above.

908   Fig 3. **Absorbance- and mass spectrometry-assisted elucidation of the structure of the five major ommatins detected**

909   **after incubation in acidified methanol**. Ommatins incubated for 24 hours in acidified methanol were analysed by liquid

910   chromatography coupled to a photodiode-array detector and a triple quadrupole mass spectrometer. (A) Absorbance spectra.

911   For each metabolite, absorbance values were reported as percentages of the maximum absorbance value recorded in the range

912   of 200 to 500 nm. Major and minor absorbance peaks are indicated in black and grey fonts, respectively. (B) Mass spectra

913   showing molecular ions and in-source fragments. Black fonts, monocharged ions. Blue fonts, double-charged molecular ions.

914   (C) Tandem mass (MS/MS) spectra of molecular ions obtained by collision-induced dissociation with argon. Black

915   diamonds, [M+H]$^+$ *m/z*. Green fonts, fragmentations of the amino acid chain. Purple fonts, fragmentations of the pyridine

916   ring are indicated. (D) Elucidated structures of the five ommatins. MS/MS fragmentations are reported in green and purple

917   like in panel C. Black asterisk, main charged basic site. Blue asterisks, potential charged basic sites of the double-charged

918   molecular ions.

919   Fig 4. **Alterations of synthesized ommatins in acidified methanol at 20 °C in darkness.** Synthesized ommatins were

920   solubilized in methanol acidified with 0.5 % HCl (MeOH-HCl) and stored for up to three days at 20 °C and in complete

921   darkness. **(A-E)** Kinetics of alterations were followed by multiple reaction monitoring (MRM) mode of xanthommatin (A),

922   α^3-methoxy-xanthommatin (B), α^3,α^{11}-dimethoxy-xanthommatin (C), decarboxylated xanthommatin (D) and decarboxylated

923   α^{11}-xanthommatin (E). Values are mean ± SD of four to five samples. (A) Linear regression (R^2 = 0.96, F = 813.5, df = 1 and

924   32, p-value < 2.2e-16). (B) Linear regression with log-scaled time (R^2 = 0.83, F = 159.8, df = 1 and 32, p-value = 5.5e-14).

925   (C) Linear regression (R^2 = 0.98, F = 1665, df = 1 and 32, p-value < 2.2e-16). (D) Linear regression with log-scaled time (R^2

926   = 0.35, F = 17.16, df = 1 and 32, p-value = 0.00023). (E) Linear regression (R^2 = 0.95, F = 651.2, df = 1 and 32, p-value <

927   2.2e-16). **(F)** Relative quantifications of methoxylated ommatins compared to unaltered ones (i.e. xanthommatin and

928   decarboxylated xanthommatin) were performed by measuring the absorbance of ommatins at 414 nm for each time point.

929   Values are mean ± SD of five samples. Different letters indicate statistical differences (Kruskal-Wallis rank sum test: χ^2 =

930   17.857, df = 3, p-value = 0.00047; pairwise comparisons using Wilcoxon rank sum test and Holm adjustment: p-values <

931   0.05).

932   Fig 5. **Structural elucidation of uncyclized xanthommatin, the labile intermediate in the synthesis of xanthommatin.**

933   **(A-B)** Chromatographic peaks (absorbance at 430 nm [A] and [M+H]⁺ 443 m/z recorded in single ion reaction [SIR] mode

934   [B]) corresponding to the labile ommatin-like compound detected in in vitro synthesis of xanthommatin by the oxidation of

935   3-hydroxykynurenine with $Fe(CN)_6^{3-}$. Insets show the decay of chromatographic peaks during storage in methanol acidified

936   with 0.5 % HCl at -20 °C in darkness. Values are mean ± SD of five samples. (A) Linear regression with log-scaled time (R^2

937   = 0.75, F = 131.4, df = 1 and 43, p-value = 1.2e-14). (B) Linear regression with log-scaled time (R^2 = 0.72, F = 111.5, df = 1

938   and 43, p-value = 1.6e-13). **(C)** Absorbance spectra. Solid line, labile ommatin-like compound. Dashed line, the

939   aminophenoxazinone cinnabarinic acid. **(D)** Mass spectrum showing molecular ions and in-source fragments. Black fonts,

940   monocharged ions. Blue font, double-charged ion. **(E-F)** Tandem mass spectra of the molecular ion obtained by collision-

941   induced dissociation with argon at collision energies of 20 eV (E) and 30 eV (F). Fragmentations were classified in four types

942   (F_A to F_D) that occurred twice (F_{X1} and F_{X2}). Black diamonds, [M+H]⁺ *m/z*. **(G)** Evidence for the structural elucidation of

943   uncyclized xanthommatin. Colors of the MS/MS fragmentation pattern correspond to those in panels D-F. Black asterisks,

944   potential charged basic sites.

945   Fig 6. **Biological localization of uncyclized xanthommatin and tryptophan→ommochrome metabolites from housefly**

946   **eyes. (A)** Overview of the purification and extraction protocols of ommochromes from housefly eyes. **(B)** Chromatographic

947   profile in Multiple Reaction Monitoring (MRM) mode of the six main metabolites of the tryptophan→ommochrome pathway

948   detected in acidified methanol (MeOH-HCl) extracts of housefly eyes (crude extracts). DcXantho, decarboxylated

949   xanthommatin, 3-OHK, 3-hydroxykynurenine, Trp, tryptophan, UncXantho, uncyclized xanthommatin. Xantho,

950   xanthommatin. XA, xanthurenic acid. **(C)** Five μL of crude extract were injected in the chromatographic system and absolute

951   quantifications of tryptophan, xanthurenic acid, 3-hydroxykynurenine and uncyclized xanthommatin were performed based

952   on available standards (uncyclized xanthommatin levels are expressed as cinnabarinic acid equivalents). Open circles,
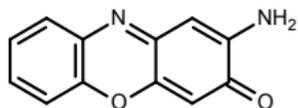
953   measures of five independent extracts. Filled circles and error bars, means ± SD of five samples. Ratios of 3-

954   hydroxykynurenine to xanthurenic acid and to uncyclized xanthommatin are shown (mean ± SD, N = 5). **(D-E)** Same as

955   panels B (D) and C (E) but for MeOH-HCl extracts of purified ommochromasomes from housefly eyes. The tryptophan

956   signal was below the signal-to-noise ratio. Statistical differences (p-value < 0.05) between ratios within panels (paired *t*-test)

957   and between panels (unpaired *t*-test) are indicated by different letters and capitals, respectively. N.D., not detected. See

958   Supplemental File S2 for information on statistical analyses. Photograph credits: (A) Sanjay Acharya (CC BY SA).

959   Fig 7. ***In vitro* formation and alteration of ommatins.** Oxidative condensation of the *ortho*-aminophenol 3-

960   hydroxykynurenine proceeds through the loss of six electrons, leading to the formation of the amino-phenoxazinone

961   uncyclized xanthommatin. Uncyclized xanthommatin then rapidly undergoes intramolecular cyclization and oxidation,

962   forming the two pyrido[3,2-*a*]phenoxazinone xanthommatin and decarboxylated xanthommatin. Decarboxylated

963   xanthommatin could also be produced from the direct decarboxylation of xanthommatin. In acidified conditions, ommatins

964   readily undergo thermal additions of methanol, which leads to their methoxylation. In solution, uncyclized xanthommatin

965   also decays by intramolecular cyclization and xanthommatin slowly decarboxylates. Relative sizes of arrows are indicative of

966   reaction rates.

967   Fig 8. **Proposed biosynthetic pathway of ommochromes through the formation of uncyclized xanthommatin in**

968   **ommochromasomes.** See text for more details on each step**.** Relative sizes of arrows are indicative of reaction rates. TDO,

969   tryptophan 2,3-dioxygenase. KMO, kynurenine 3-monooxygenase. St, ABC transporter scarlet. W, ABC transporter white.

970   PHS, phenoxazinone synthase. HKT, 3-hydroxykynurenine transaminase.

A

ortho-aminophenol

Amino-phenoxazinone

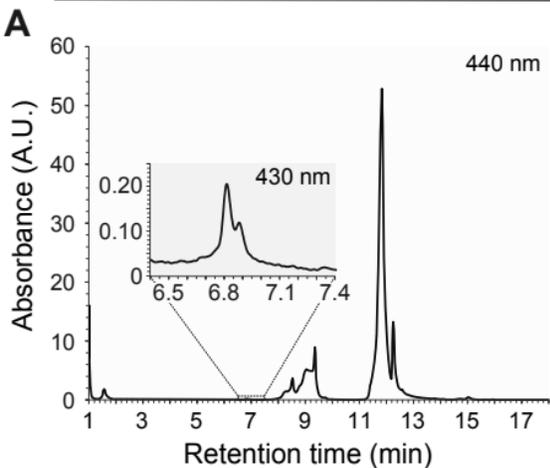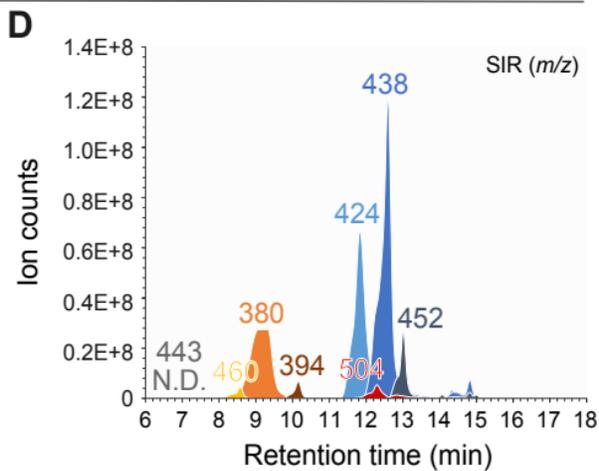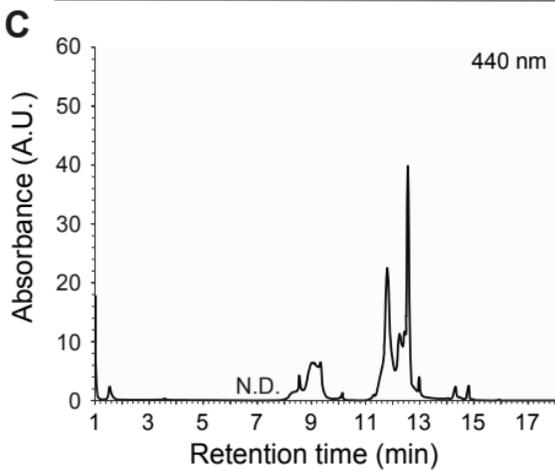Oxo-pyrido[3,2-*a*]-phenoxazinone

Phenoxazine

Phenothiazine

Xanthommatin

B

Kynurenine pathway (early steps)

Tryptophan
↓ TDO
N-Formylkynurenine
↓ KFase?
Kynurenine
↓ KMO
3-Hydroxykynurenine → HKT → Xanthurenic acid

Cysteine Methionine

① Uncyclized xanthommatin?

②

Xanthommatin

Red. / Ox.

Decarboxylated xanthommatin

Dihydro-xanthommatin

Methylated ommatins?

Ommatin D Rhodommatin

Ommin A
Ommins

Ommatins

Ommochrome pathway (late steps)

A 2 minutes in MeOH-HCl

440 nm

430 nm

B SIR (*m/z*)

443

424

380

460

504

C 24 hours in MeOH-HCl

440 nm

N.D.

D SIR (*m/z*)

438

424

452

380

394

443
N.D.

460

504

A

| RT: 10.7 min | RT: 8.4 min | RT: 11.8 min | RT: 12.4 min | RT: 9.5 min |

B

ESI$^+$-MS (423.9, 379.9, 437.9, 451.9, 393.9)

C

ESI$^+$-MS/MS 423.9, 379.9, 437.9, 451.9, 393.9

D

MW: 423; C$_{20}$H$_{13}$N$_3$O$_8$
Xanthommatin

MW: 379; C$_{19}$H$_{13}$N$_3$O$_6$
Decarboxylated xanthommatin

MW: 437; C$_{21}$H$_{15}$N$_3$O$_8$
α^3-Methoxy-xanthommatin

MW: 451; C$_{22}$H$_{17}$N$_3$O$_8$
α^3,α^{11}-Dimethoxy-xanthommatin

MW: 393; C$_{20}$H$_{15}$N$_3$O$_6$
Decarboxylated α^{11}-methoxy-xanthommatin

A Xanthommatin
Peak area vs Time in MeOH-HCl (hour)
MRM 424>361
RT: 11.8 min

B α³-Methoxy-xanthommatin
Peak area vs Time in MeOH-HCl (hour)
MRM 438>375
RT: 12.5 min

C α³,α¹¹-Dimethoxy-xanthommatin
Peak area vs Time in MeOH-HCl (hour)
MRM 452>375
RT: 13.0 min

D Decarboxylated xanthommatin
Peak area vs Time in MeOH-HCl (hour)
MRM 380>317
RT: 9.2 min

E Decarboxylated α¹¹-methoxy-xanthommatin
Peak area vs Time in MeOH-HCl (hour)
MRM 394>317
RT 10.1 min

F Proportion of unaltered ommatins (Relative absorbance at 414 nm, %) vs Time in MeOH-HCl

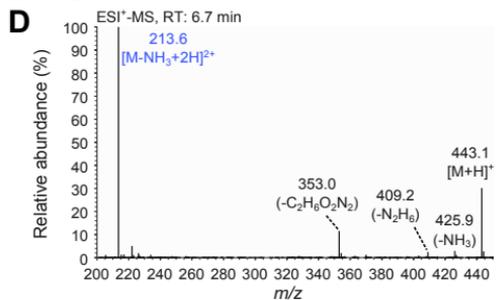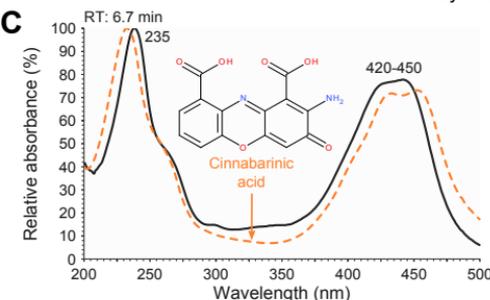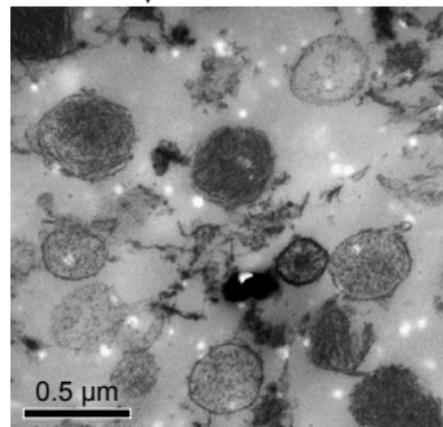**A** 430 nm — RT: 6.7 min — MeOH-HCl -20 °C

B SIR 443 *m/z* — RT: 6.7 min — MeOH-HCl -20 °C

--- Analytical characterization ---

C RT: 6.7 min — 235, 420-450, Cinnabarinic acid

D ESI⁺-MS, RT: 6.7 min
213.6 [M-NH₃+2H]²⁺
353.0 (-C₂H₆O₂N₂)
409.2 (-N₂H₆)
425.9 (-NH₃)
443.1 [M+H]⁺

E ESI⁺-MS/MS 443 *m/z*, RT: 6.7 min — CE 20 eV
F_{C2}: -CO 307.0
F_{B2}: -H₂O 335.1
F_{D1}: -C+2H
F_{C1}: -CO
F_{B1}: -H₂O 390.9
353.0
409.0
F_{A2}: -NH₃
F_{A1}: -NH₃ 425.9

F ESI⁺-MS/MS 443 *m/z*, RT: 6.7 min — CE 30 eV
307.0 317.0 F_{D1}
F_{C2}
F_{C2} 335.0 344.9 F_{B2}
353.1 F_{D1}
363.1 F_{C1}
391.0 F_{B1}
409.3 F_{A2} 425.7 F_{A1}
F_{A1}: -NH₃ F_{A2}: -NH₃
F_{B1}: -H₂O F_{B2}: -H₂O
F_{C1}: -CO F_{C2}: -CO
F_{D1}: -C+2H F_{D2}: -C+2H

G
F_{B2} F_{A2}
F_{C2} F_{C1}
F_{D2} F_{B1} F_{D1} F_{A1}

2x MW: 224, C₁₀H₁₂N₂O₄ 3-Hydroxykynurenine

Fe(CN)₆³⁻ -6H

MW: 442, C₂₀H₁₈N₄O₈ Uncyclized xanthommatin

In-source intramolecular cyclization

[M-NH₃+2H]²⁺

[Dihydroxanthommatin + 2H]²⁺

A

Purification of ommochromasomes

0.5 µm

B — Crude MeOH-HCl extract of housefly heads

3-OHK

UncXantho

Xantho

MRM

Trp

XA

DcXantho

Retention time (min)

Ion counts

C

x2.1 ±1.5   x21 ±5.6
a   b

Concentration (nmol/µL)

Trp   XA   3-OHK   Unc-Xantho

D — MeOH-HCl extract of purified ommochromasomes

MRM

Xantho

XA

3-OHK   Trp   Unc-Xantho

DcXantho

456   500

Retention time (min)

Ion counts

E

x11 ±1.7   x6.2 ±0.47
A   B

Concentration (nmol/µL)

N.D.

Trp   XA   3-OHK   Unc-Xantho

3-Hydroxykynurenine

Xanthommatin

α³-Methoxy-xanthommatin

α³,α¹¹-Dimethoxy-xanthommatin

$Fe(CN)_6^{3-}$ -6H

$-NH_3$ -2H

$+CH_3OH, -H_2O$ (CH_3OH, H^+)

$+CH_3OH, -H_2O$ (CH_3OH, H^+)

$-NH_3$ $-CO_2$ -2H

$-CO_2$

Uncyclized xanthommatin

Decarboxylated xanthommatin

$+CH_3OH, -H_2O$ (CH_3OH, H^+)

Decarboxylated α¹¹-methoxy-xanthommatin

Phosphate buffer 0.2 M, pH 7.0
Anaerobia, darkness

MeOH-HCl (0.5 %)
Darkness

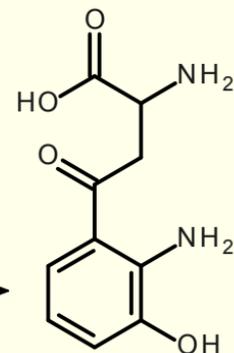------ Kynurenine pathway ------   ------------ Ommochrome pathway ------------

Xanthurenic acid

Xanthommatin

Tryptophan

3-hydroxy-kynurenine

Uncyclized xanthommatin

Decarboxylated xanthommatin

Ommin A

Ommochromasome

Methionine
Cysteine

TDO

KMO

HKT

St

W

x2
PHS?
Cardinal?

Catabolism?

HKT?

Tautomerase?

$-NH_3$
$-2H$

Light?

$-CO_2$

$-NH_3$
$-CO_2$
$-2H$