Supplemental Information

Milk, Revealed "Silent" Chemistry: New Mode of Cycloretinal Synthesis

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INVENTORY OF SUPPLEMENTAL INFORMATION

Figure S1. BLG incubation optimization data used to determine the conditions employed in testing the aldehyde substrates listed in **Table 1**.

Figure S2. Expanded ^{1}H NMR spectra for BLG-mediated reactions highlighted in **Figure 3B**, and evidence of activity disruption by treatment with anionic detergent.

Figure S3. HPLC profile of extracts from BLG incubated with all-*trans*-retinal extends confirmation of ^{1}H NMR product evidence in **Figure 3**.

Figure S4. ^{1}H NMR analysis of RPE cell extract incubated with citral indicates lack of product formation assisted by proteins within the eye. A similar observation was made following incubation of citral with *E. coli* crude protein extracts depicted in **Figure 3C**.

Figure S5. ^{1}H NMR analysis of products formed during incubation of citral with desalted pasteurized milk shows that the condensation reaction can be attributed to BLG and is not dependent upon small molecules in milk. This is control data for the milk incubations shown in **Figure 4**.

Figure S6. ^{1}H NMR spectrum showing absence of cyclocitral formation under buffered/physiological conditions, a control experiment linked to **Figure 3**.

Figure S7. Synthesis of all-*trans*-retinal required for the rabbit study summarized in **Figure 5**.

Figure S8. ^{1}H NMR analysis of products formed during *in vitro* incubation of retinal with blood from a rabbit fed BLG in water demonstrates BLG survives uptake and retains ability to catalyze retinal condensation.

Supplemental Experimental Procedures. Details concerning BLG-promoted condensation reaction optimization, reaction profile HPLC, preparation of microbial and RPE cell extracts, BLG denaturation and assessment of unassisted aldehyde condensation under physiological buffered conditions.

SUPPLEMENTAL FIGURES

A

BLG:Substrate Equivalents	Volume 0.5 M citral (mL)	Yield (%)
1:1	0.54	10
1:2	1.09	31
1:3	1.63	58
1:5	2.72	58
1:10	5.43	58

B

pH	Yield (%)
1.0	0
3.0	9.8
5.0	13.6
7.0	50.7
9.0	27.9
11.0	0

C

Day	Yield (%)
1	12.3
2	22.4
3	36.2
4	58
5	58

D

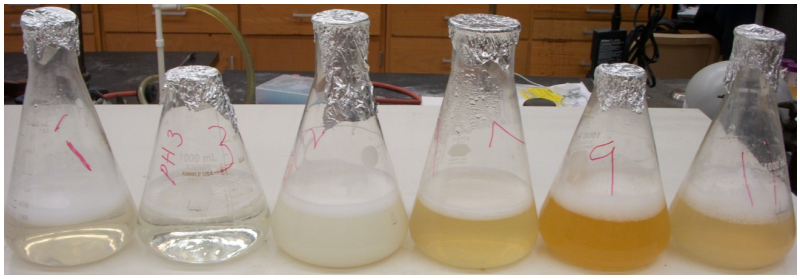

Figure S1. Percent yield for ranges of (A) protein to substrate ratio, (B) pH, and (C) reaction duration. (D) Color changes along with pH. From left to right: pH 1.0, 3.0, 5.0, 7.0, 9.0, and 11.0.

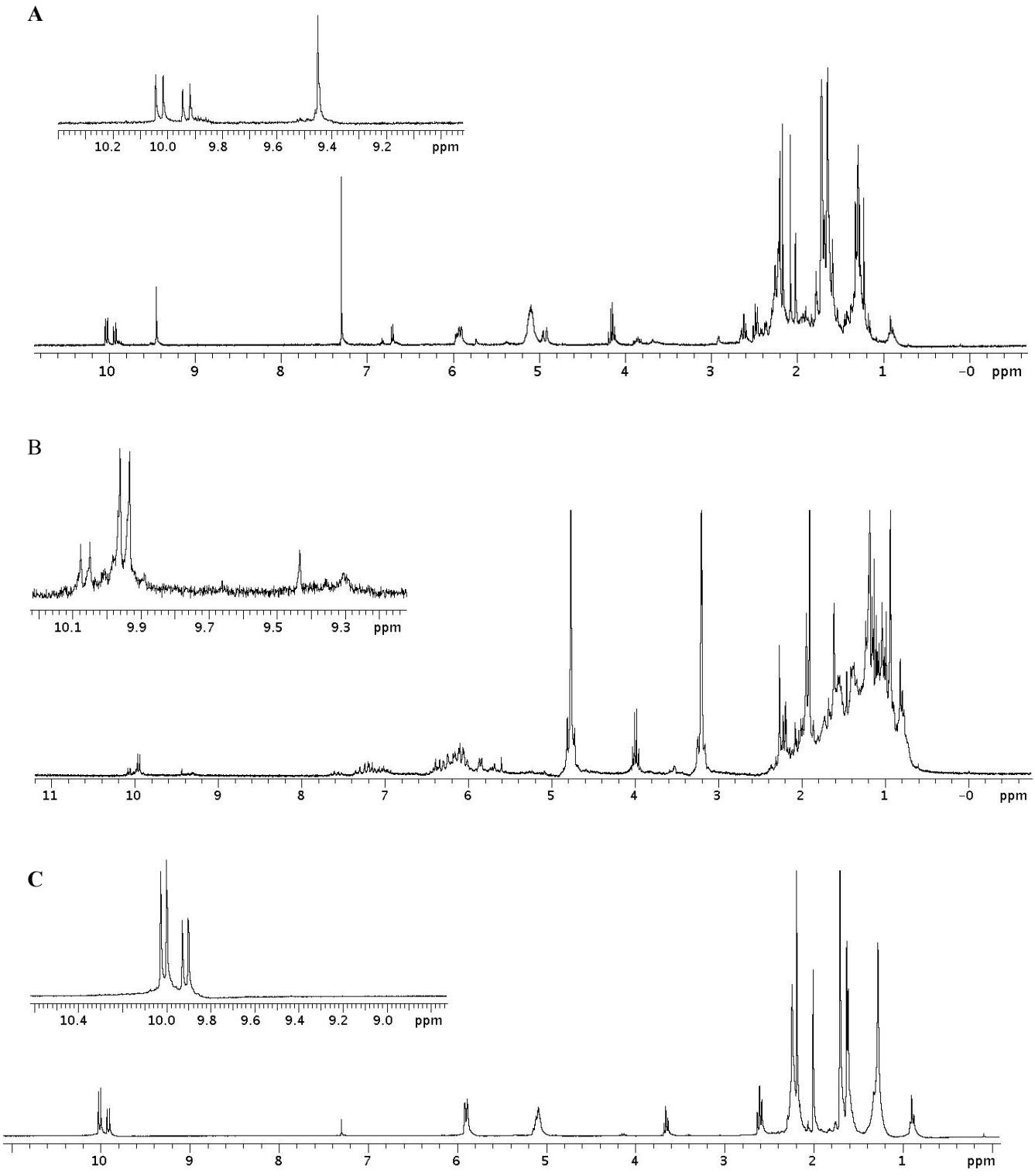

Figure S2. ^{1}H-NMR analysis of BLG-promoted cycloterpenal biosynthesis.
(A) Extract from 1% BLG incubated with 3 equivalents of citral.
(B) Extract from 1% BLG incubated with 3 equivalents of retinal.
(C) Extract from 10% SDS-denatured BLG incubated with 3 equivalents of citral.

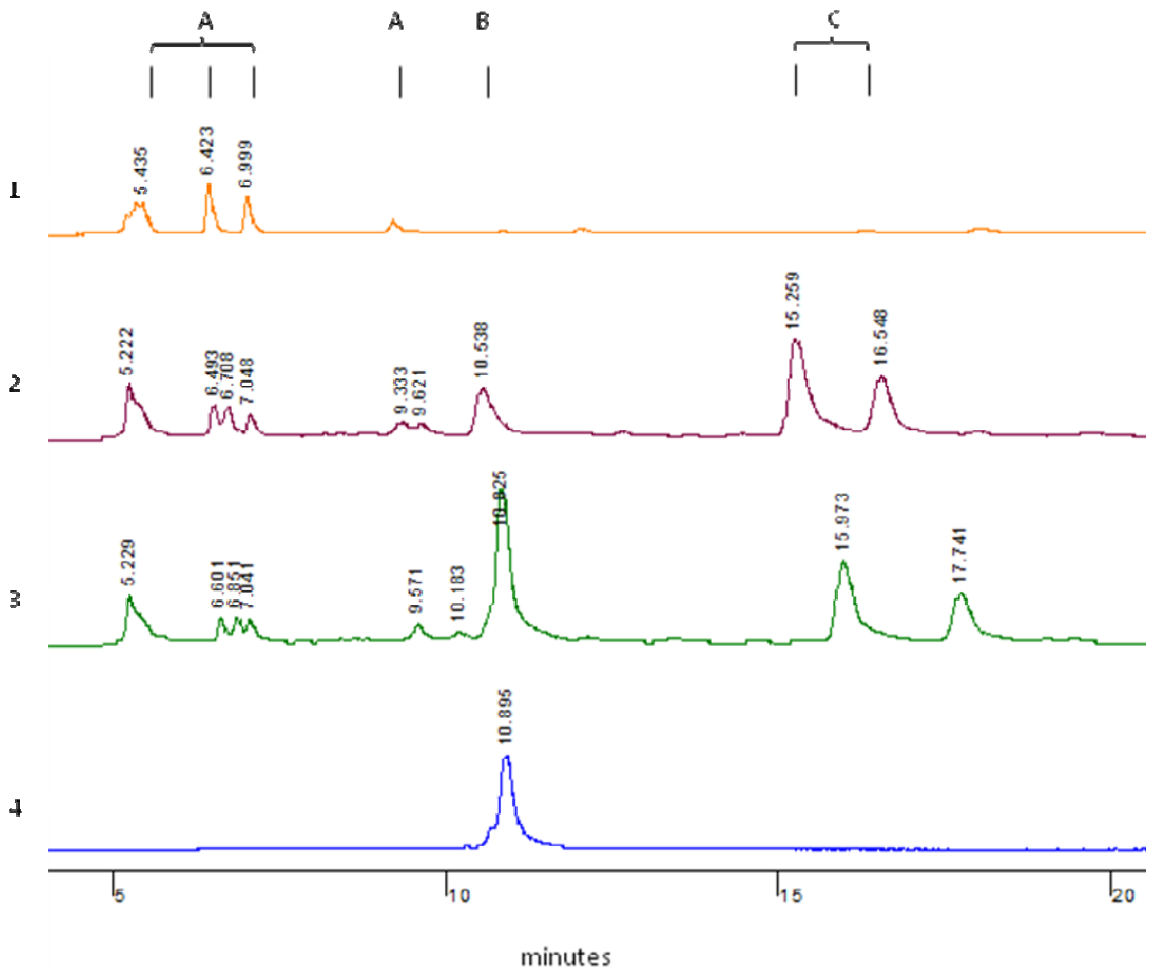

Figure S3. HPLC profile of BLG-promoted cycloretinal biosynthesis. [1] BLG extract, [2] BLG + retinal extract, [3] BLG + retinal extract spiked with synthetic cycloretinal (co-injection), and [4] synthetic cycloretinal. Peak identification is as follows: (A) BLG-bound metabolites, (B) cycloretinal, and (C) retinal-derived side products.

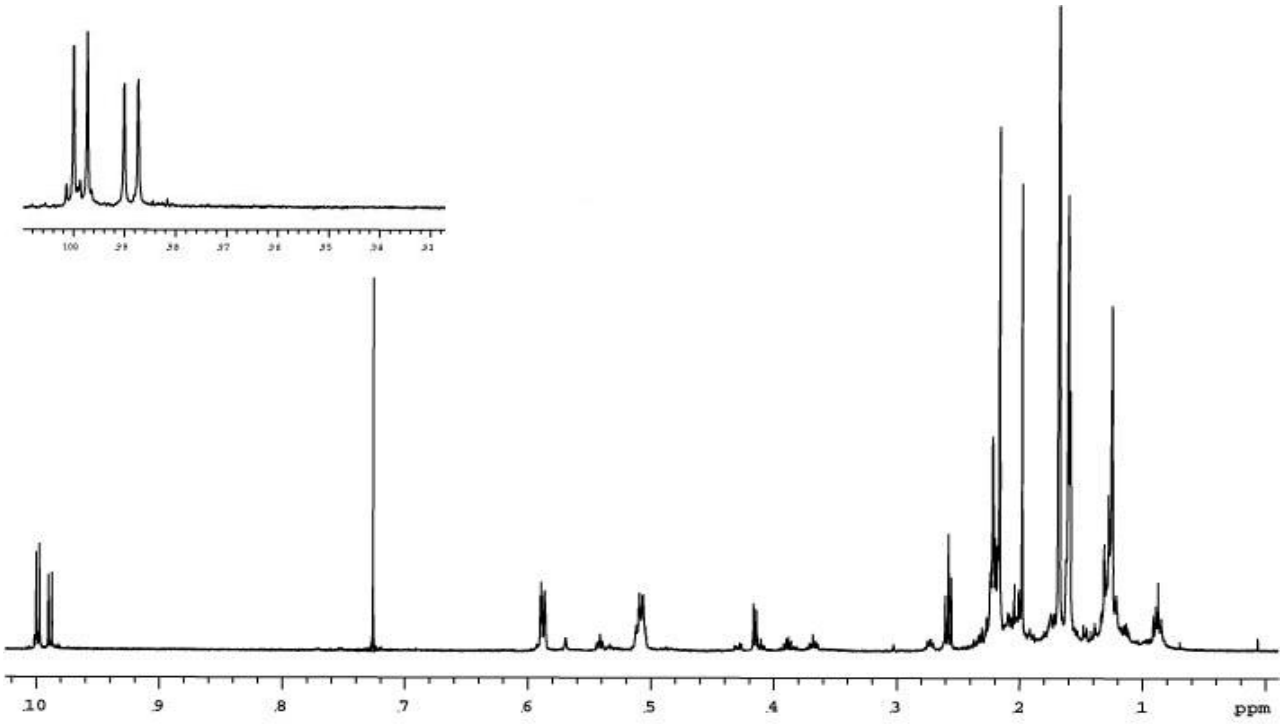

Figure S4. ^{1}H-NMR spectrum of extract from retinal pigment epithelial cell extract incubated with citral.

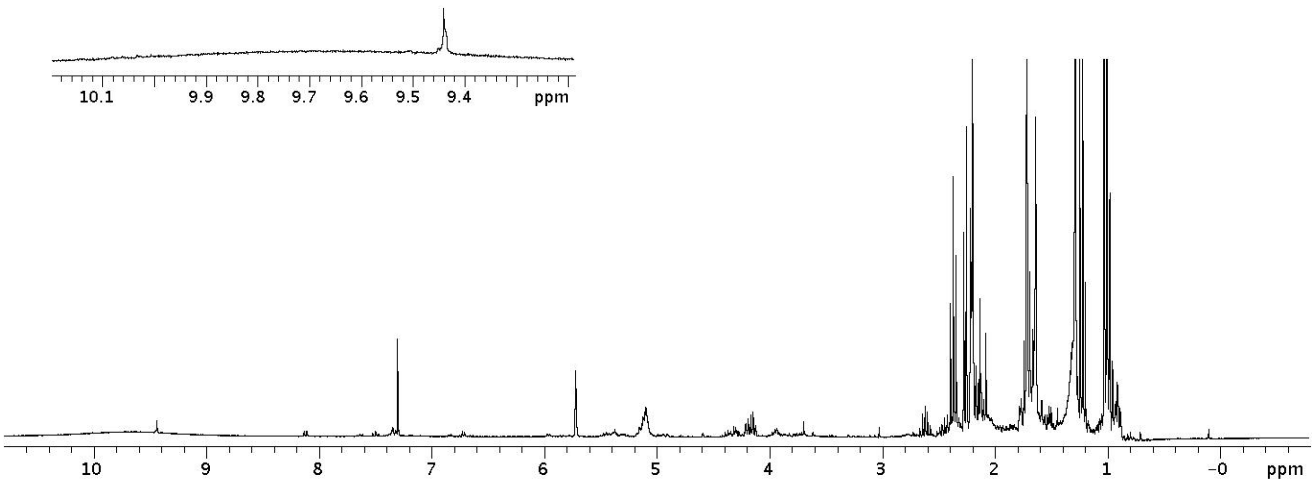

Figure S5. ^{1}H-NMR spectrum of extract from Sephadex G-25 filtered skim milk incubated with citral.

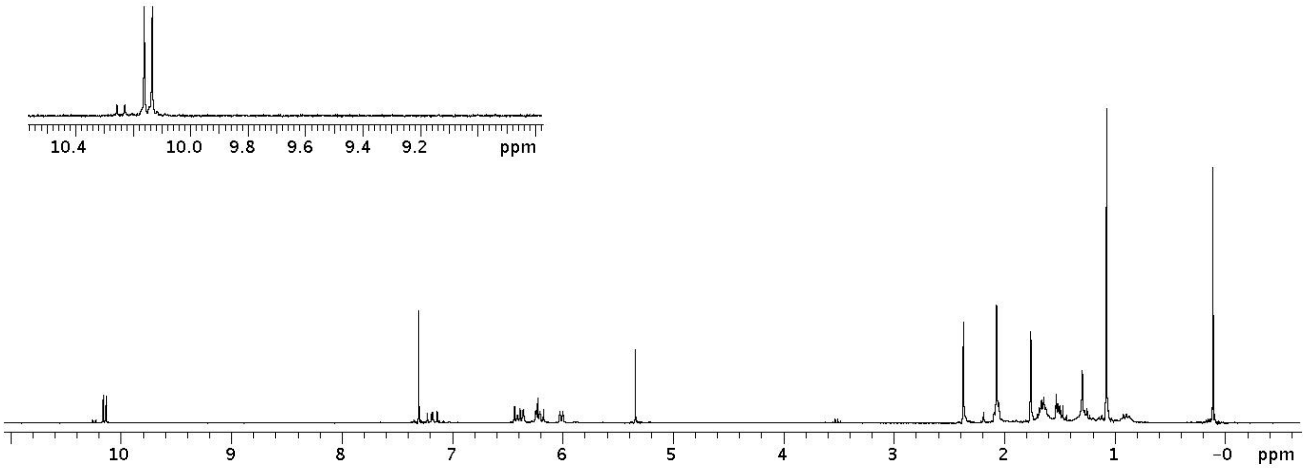

Figure S6. ^{1}H-NMR spectrum of extract from L-proline in PBS pH 7.4 incubated with retinal.

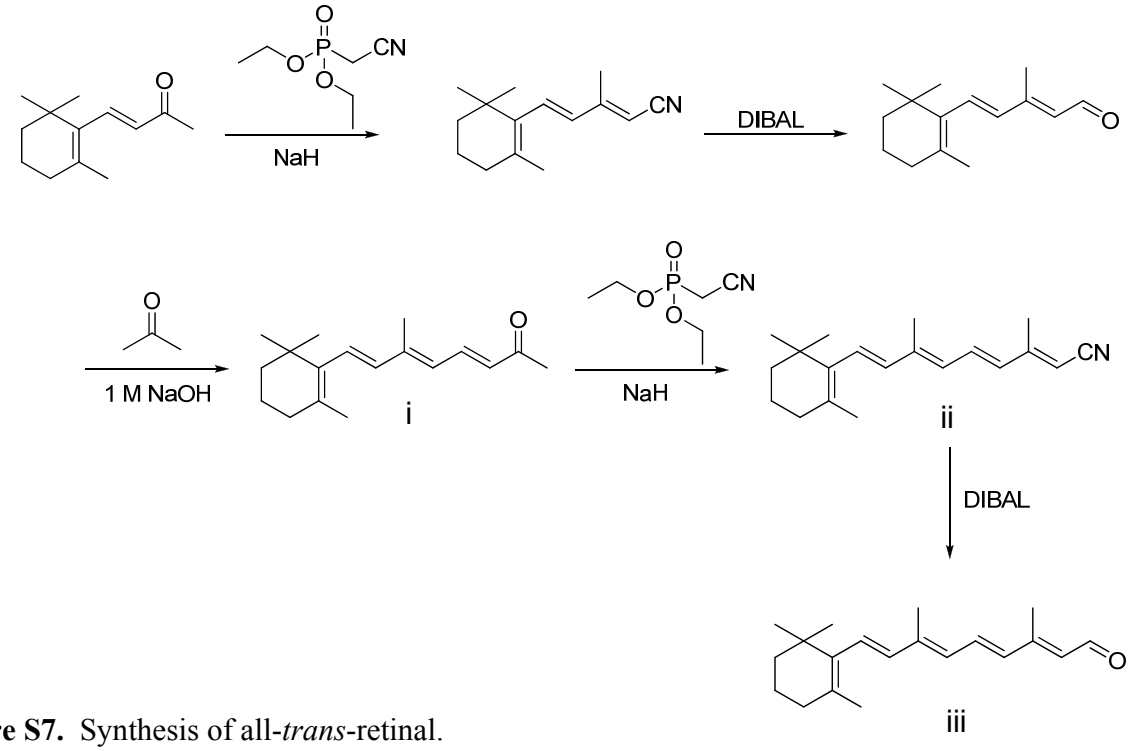

Figure S7. Synthesis of all-*trans*-retinal.

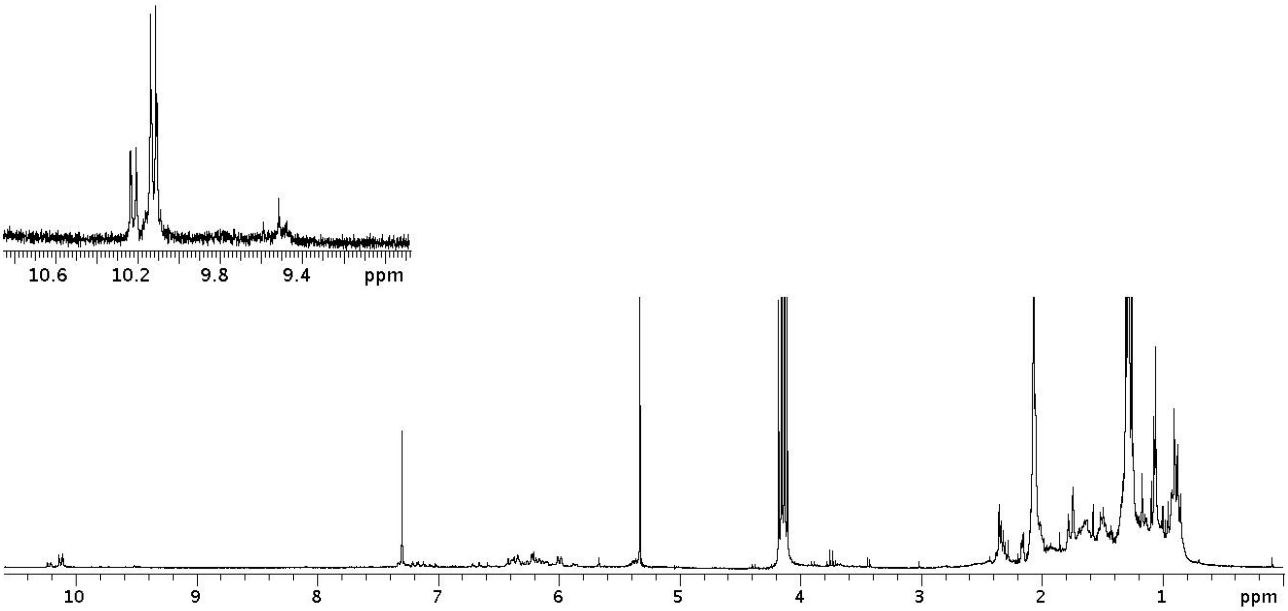

Figure S8. ^{1}H NMR spectrum of extract from *in vitro* incubation of retinal and blood from a rabbit fed BLG and water.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Determination of optimal protein to substrate ratio

To determine the optimal protein and substrate concentrations for dimer formation, a range of protein to substrate ratios from 1:1 to 1:10 were tested. A 1% (w/v) BLG solution was prepared in 500 mL phosphate buffered saline (PBS, 0.01 M phosphate, 0.0027 M KCl, 0.137 M NaCl, 500 mL, pH 7.0). An aliquot of 0.5 M citral in ethanol was added to the BLG solution to achieve the desired ratio, and the reaction was incubated at 37°C, 250 rpm for 4 days. The mixture was extracted, concentrated, and analyzed as outlined in the general procedure for BLG-promoted biosynthesis.

pH profile analysis

To examine the behavior of the BLG-promoted reaction as a function of pH, we evaluated the reaction at pH values of 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0. PBS (500 mL) was buffered with either 1.0 M HCl or 1.0 M NaOH to reach the desired pH using a Seven Easy pH meter (Mettler-Toledo, Switzerland, Product # Z654280). BLG (5 g) was subsequently dissolved in each buffered PBS solution to give a 1% solution and incubated with 3 equivalents of citral (0.5 M in ethanol, 1.630 mL) at 37 °C, 250 rpm for 4 days. The BLG mixture was extracted with 500 mL of ethyl acetate, concentrated *in vacuo* as detailed previously, and the metabolites analyzed by ^{1}H-NMR spectroscopy and mass spectrometry.

HPLC profile of BLG promoted biosynthesis of cycloterpenal homodimers

The products of a 500 mL BLG-retinal reaction were extracted as previously described. An aliquot of the extract (20%) was dissolved in 40 µL dichloromethane and analyzed by HPLC (Phenomenex Luna silica column, 5 µm particle size, 250 x 10.00 mm) with a dichloromethane mobile phase, 3 mL/min flow rate, and UV absorption monitoring at 254 nm. Additionally, an extract of 1% BLG in 500 mL PBS, synthetic (proline-catalyzed) cycloretinal, and an aliquot of the BLG-retinal reaction extract spiked with 0.25 mg synthetic cycloretinal were analyzed in the same manner (**Figure S3**).

General procedure for microbial protein crude extract controls

E. coli was cultured on agar plates using standard methods. A single colony was picked and placed in 3 mL of LB medium and allowed to grow overnight before being transferred to 500 mL of fresh medium. After 24 h, the culture was centrifuged and the cell pellet flash frozen in liquid nitrogen before being stored at -80 °C. Cells were lysed with a bead mill (BeadBeater, Biospec Products, Bartlesville, OK) equipped with 0.1 mm glass beads. Frozen cell pellets, PBS pH 7.0, and glass beads were added to the mill chamber on ice. Cells were lysed with ten pulses of 30 s each with 1 min intervals for cooling. The cell suspension was centrifuged and the resulting supernatant served as the crude protein extract. Protein concentration was measured by Bradford assay (Biorad, Hercules, CA) and diluted to a 1% (w/v) solution with PBS.

Prior to carrying out the dimerization assay, a metabolite extract blank was generated. The protein extract (40 mL) was extracted with 200 mL of ethyl acetate, dried with anhydrous magnesium sulfate, and concentrated *in vacuo*. A ^{1}H-NMR spectrum was obtained to ensure that there were no metabolite peaks in the aldehydic region of interest. To establish whether crude protein extracts could support cycloterpenal formation, protein lysates were incubated with 3 equivalents of 0.5 M citral at 37 °C with shaking (250 rpm) for 4 days. As detailed previously, following incubation, the suspension was extracted and analyzed by ^{1}H-NMR spectroscopy and mass spectrometry.

Evaluation of RPE cell extract for cycloterpenal formation

RPE cells (ARPE-19, ATCC) were subcultured to give a total of 10 plates at 95% confluency. Cells were propagated in ATCC-formulated Dulbecco's modified Eagles medium DMEM:F12 Medium supplemented with 10% fetal bovine serum in a 5% carbon dioxide atmosphere at 37 °C. Cells were dislodged from the plate with 3 mL of a 0.05% (w/v) Trypsin-0.53 mM EDTA solution and transferred to a 50 mL conical tube. Cells were pelleted by centrifugation for 20 min. at 1,000 rpm. To remove traces of medium, the cells were washed twice with DPBS, 1X without calcium and magnesium, and subsequently resuspended in 10 mL of DPBS. The cell suspension was lysed with a 50 mL dounce homogenizer and the resulting lysate at 5,000 rpm (4 °C) for 30 min to remove cellular debris. Protein concentration was measured by Bradford assay and diluted to a 1% (w/v) solution with PBS.

A metabolite control was generated by extracting the lysate (5 mL) with ethyl acetate (50 mL), which was dried over anhydrous $MgSO_4$, concentrated *in vacuo*, and evaluated by ^{1}H-NMR spectroscopy. To establish whether the crude protein extract (10 mL) could support cycloterpenal formation, the lysate was incubated with 3 equivalents of 0.5 M citral at 37 °C with shaking (250 rpm) for 4 days. As detailed previously, following incubation, the suspension was extracted with 300 mL of ethyl acetate, centrifuged, dried over anhydrous $MgSO_4$, concentrated *in vacuo*, and analyzed by ^{1}H-NMR spectroscopy (**Figure S4**).

SDS denaturation of BLG

In a 1000 mL Erlenmeyer flask, 500 mL of a 1% BLG solution (PBS pH 7.0) was stirred with 10% (w/v) SDS for 2 h. Three equivalents of citral (0.5 M in absolute ethanol, 1.630 mL) was added to the SDS-protein solution. The mixture was placed in a 37 °C shaker and incubated at 250 rpm for 4 days. The BLG mixture was extracted and analyzed as detailed previously (**Figure S2C**).

Evaluation of cycloretinal formation under physiological conditions

L-proline (54.4 mg) was solubilized in 10 mL of buffer (0.01 M phosphate, 0.0027 M KCl, 0.137 M NaCl; pH 7.4) to which retinal was added dropwise (10 mg/1 mL ethanol). The reaction was incubated at room temperature (in the dark) for 11 h and subsequently extracted with 50% ethyl ether in hexane. The organics were dried over magnesium sulfate and concentrated *in vacuo* prior to NMR analysis (**Figure S6**).

Activity evaluation of BLG uptaken in rabbit blood

To determine the ability of BLG absorbed within blood to catalyze retinal condensation, 10 mL of blood each from the BLG/water control rabbit and the basic diet rabbit were incubated at 37°C with all-trans-retinal (400 mg in 1 mL absolute ethanol) for 4 days. The mixture was extracted with ethyl acetate (200 mL) by stirring for 1 h at room temperature and then centrifuged (30 min, 9000 rpm, 4°C) to separate the emulsion. The organic layer was removed, dried with anhydrous $MgSO_4$ and concentrated in vacuo prior to NMR analysis to detect cycloretinal formation (**Figure S8**).