3-Hydroxy Retinal, a New Chromophore Identified in Insect Eyes: HPLC Separation and NMR Spectroscopic Identification of the Oxime Forms

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3-Hydroxy retinal acts as visual chromophore instead of retinal in the eyes of several insect orders. A HPLC separation system of the aldehyde and oxime isomers and their identification by 400 MHz ^{1}H NMR spectroscopy is described.

11-*cis* Retinal ((**1**) see Fig. 1) and its 3,4-dehydro derivative (11-*cis* A_2-retinal, (**2**)) were believed to be the sole chromophores in the visual pigments. Recently, another retinal compound – 3-hydroxy retinal (**3**) – was identified as the functional chromophore in the eyes of several related insect orders [1–3]. It was demonstrated that the adaptation of 3-hydroxy retinal as visual chromophore provides an advantage, since it allows – *e.g.* in the flies – the additional use of the alcohol compound, 3-hydroxy retinol (**4**), as antenna pigment [2].

The hydroxy group at position 3 of the cyclohexyl ring renders the molecule much more polar than retinal or a substitution at position 2 or 4. Such increased polarity requires long separation times and makes a complete separation of the isomers of 3-hydroxy retinal very difficult. The unambiguous identification of the chromophore isomers, however, is necessary for an exact correlation of the photochemistry to physiologically detected events. Only a few separation systems are reported so far which attempt to overcome the problem of increased polarity and still provide a sufficient separation [4–6].

In most cases of chromophore preparations from insect eyes, the free aldehyde can not be isolated in sufficient yield, possibly due to large amounts of phosphatidylethanolamines found in such eyes [1]. These compounds can react with free carbonyl groups and form highly polar and very stable Schiff bases. As a consequence of this side reaction, large amounts of the chromophore are withdrawn from an identification. Therefore, the chromophore isomers are usually converted into the oxime form. Such derivatization provides the additional advantage that due to their strong fluorescence even very small amounts of oximes can be detected. For an unambiguous assignment of the various oximes, we illuminated the all-*trans* form of the aldehyde, until a photostationary mixture was formed. From this mixture the oximes were prepared in the dark and characterized by NMR spectroscopy after separation on a preparative scale column (inset in Fig. 2). Fig. 2 shows an analytical scale separation of the oxime isomers, each of which present as a *syn*- and an *anti*-form.

A comparison with separations reported by other groups revealed a strong dependance of the relative elution order of some peaks from the type of silica gel used as stationary phase in the HPLC column. This result is most impressively demonstrated by comparing the positions of the all-*trans syn* and *anti* forms, which interchange when the column material

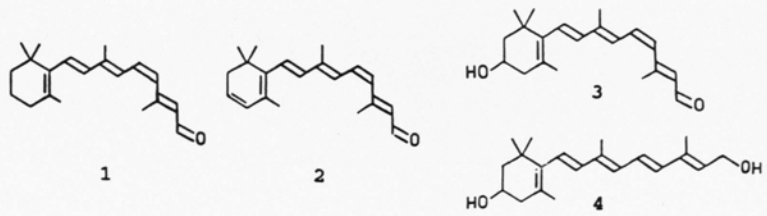

Fig. 1. Formulae of visual chromophores. **1**, A_1-retinal; **2**, A_2-retinal; **3**, 3-hydroxy retinal; **4**, 3-hydroxy retinol. The physiologically active isomeric forms are depicted (11-*cis* for **1**–**3** and all-*trans* for **4**).

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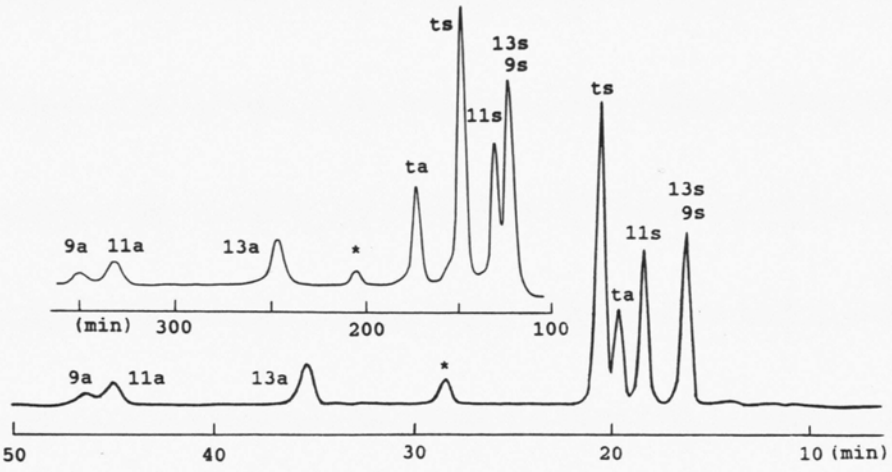

Fig. 2. HPLC separation of *E*/*Z* isomers of 3-hydroxy retinal oximes (*syn* and *anti* forms, 13s, 13-*cis*, *syn*-form; ta, all-*trans*, *anti*-form). The HPLC system consisted of a Beckman pump type 114M, a variable wavelength detector (Beckman 163) and a separation column (25 × 0.4 cm), filled with Hypersil 3 μm as stationary phase. The chromatogram was run with a mixture of 19:1:80 (diethyl ether/ethanol/*n*-hexane, flow: 1 ml/min) as solvent and was detected at 360 nm. The small peak labelled by an asterisk appears during the oxime formation and could probably be the 7-*cis* isomer. Since only very small amounts of material could be collected, no identification was possible. Inset: Separation on a preparative-scale column (25 × 2.0 cm), filled with Lichrosorb SI 60, 5 μm. The compounds were eluted with a flow of 5 ml/min. Further conditions were as for the analytical separation. Note the different time scales of figure and inset.

is changed (see inset of Fig. 2. Here, a preparative scale column (25 × 2.0 cm), filled with SI 60, Lichrosorb 5 μm, was used at a flow of 5 ml/min). For a complete separation of the 9- and the 13-*cis* forms, which coelute in our system, the HPLC conditions described by Goldsmith *et al.* [4] were applied, which, however, in our hands for the rest of the isomer mixture gave no satisfying results. Especially the *syn* oximes of the two physiologically most important isomers, 11-*cis* and all-*trans*, elute very close to each other in our HPLC system and prevent a quantitative identification, when the solvent system described by Goldsmith *et al.* [4] was applied together with our HPLC column.

Until recently, only for the 11-*cis* isomer of the aldehyde the NMR data were known [5]. We present here the 400 MHz ^{1}H NMR data of the most relevant oxime isomers (*syn* and *anti*) and of the 11- and 13-*cis* and the all-*trans* isomers of the aldehyde. The compounds were separated with a preparative-scale HPLC column (for a representative chromatogram see inset of Fig. 2). The assignment of the NMR signals revealed that in general the olefinic hydrogens of (**3**) are found at very similar positions to those of retinal. The most important difference appears for the signal of hydrogen atom 7 which shows a strong upfield shift of 0.15 ppm for the oxime and also the aldehyde isomers [6]. The assignment was complicated by the fact that the NMR signals of some hydrogen atoms, which are closely located in the molecule, coincide in the spectrum. In some cases, even double resonance experiments gave no definite answer. Examples for this problem are the hydrogen atoms 7 and 8 of the *trans* isomer, and the hydrogen groups 7/8/10 and 11/12 of the 13-*cis* isomer (see Table I). For the all-*trans* (anti) isomer, a COSY experiment allowed a further assignment. For the 13-*cis* (anti) compound the positions of protons 10 and 12 were deduced from a comparison with the values of the corresponding retinal oxime [7].

Based on our identification of the isomers of 3-hydroxy retinal a further elucidation of the photochemical events also on a quantitative scale becomes now possible. Particularly, the function of the different isomers of the antenna pigment, 3-hydroxy retinol, can be examined in greater detail.

Table I: NMR data of 3-hydroxy retinal derivatives.

	Oxime derivatives, *E*/*Z* isomers						
	H_7	H_8	H_{10}	H_{11}	H_{12}	H_{14}	H_{15}
all-*trans*	6.12*	6.12*	6.12*	6.76	6.35	6.12*	8.15
(*syn*-form)	$J_{7/8}$: 16 Hz		$J_{10/11}$: 11.5 Hz			$J_{14/15}$: 10.5 Hz	
				$J_{11/12}$: 15 Hz			
(*anti*-form)	6.12*	6.12*	6.12*	6.82	6.38	6.66	7.49
9-*cis*	6.12*	6.62	6.04	6.84	6.28	6.13*	8.15
(*syn*-form)							
11-*cis*	6.17	6.11	6.54	6.44	5.93	6.17	8.12
(*syn*-form)				$J_{11/12}$: 11 Hz			
13-*cis*	6.15*	6.15*	6.15*	6.74*	6.74*	5.98	8.29
(*syn*-form)							
(*anti*-form)	6.14*	6.14*	6.14*	6.82*	6.82*	6.54	7.55

* Midpoint of signal group. Other signals: 1,1-dimethyl: 1.07; 5-methyl: 1.72; 9-methyl: 1.97/8 (*trans-anti:* 1.91, 11-*cis:* 1.93); 13-methyl *(trans-syn):* 2.0 (*trans-anti:* 2.02, 9-*cis,* 13-*cis syn:* 1.98, 13-*cis anti:* 2.05, 11-*cis:* 2.04). H−C_2: 1.47, 1.76; H−C_3: 4.0; H−C_4: 2.04, 2.38.

	3-Hydroxy retinal, *E*/*Z* isomers						
all-*trans*	6.27	6.15	6.19	7.11	6.38	5.97	10.09
11-*cis*	6.27	6.19	6.53	6.67	5.92	6.07	10.06
13-*cis*	6.27	6.16	6.19	7.01	7.27	5.85	10.18

Other signals: 1,1-dimethyl: 1.07; 5-methyl: 1.72; 9-methyl (13-*cis*, all-*trans*): 2.01 (11-*cis:* 1.98); 13-methyl *(trans):* 2.31 (11-*cis:* 2.33, 13-*cis:* 2.13); the cyclohexyl hydrogen atoms were found at the same positions as for the oxime derivatives.

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