Monatshefte für Chemie 136, 159–168 (2005)
DOI 10.1007/s00706-004-0237-z

Monatshefte für Chemie
Chemical Monthly
Printed in Austria

Chemical and Chromatographic Behavior of Pseudohypericin and Isopseudohypericin, and the Occurrence of Isopseudohypericin in Saint John's Wort (*Hypericum perforatum*) Extracts

Youssef Naït-Si and **Jean-Dominique Fourneron***

Laboratoire des Systèmes Chimiques Complexes, CNRS [UMR 6171] Faculté des Sciences and Techniques de Saint-Jérôme, 13397 Marseille cedex 20, France

Received May 19, 2004; accepted (revised) June 21, 2004
Published online October 4, 2004 © Springer-Verlag 2004

Summary. Isopseudohypericin is formed from pseudohypericin in an alkaline, alcohol/water environment. The conditions for this conversion are specified, showing that these two compounds are in equilibrium at *pH* values of 5–10; pseudohypericin is stable below *pH* 5, and isopseudohypericin is stable above *pH* 10. It is also shown that the HPLC behavior of isopseudohypericin is dependent on the *pH* of the mobile phase: *pH* determines both the retention time and the UV-Vis spectrum characteristics of the corresponding peak analyzed using a diode array detector. Finally, different extraction conditions yield extracts containing different proportions of isopseudohypericin: it is suggested that this isopseudohypericin may be an artefact of extraction conditions and/or it may correspond to the compound cyclopseudohypericin, discussed by other experts but never formally characterized.

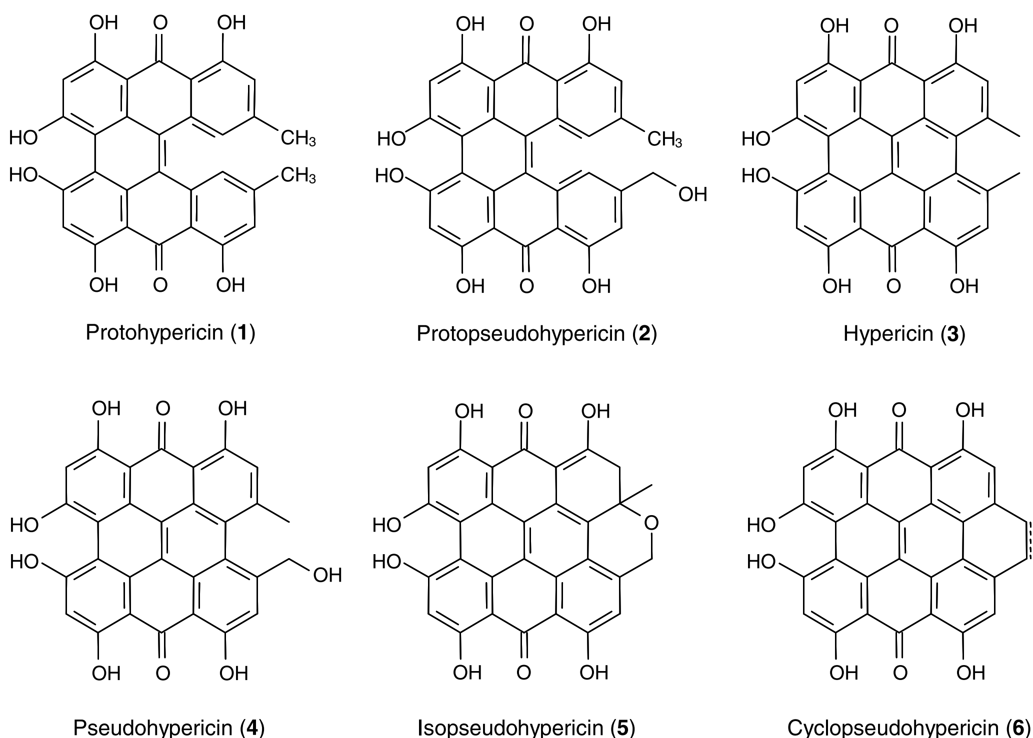
Keywords. Pseudohypericin; Isopseudohypericin; Cyclopseudohypericin; Equilibria; HPLC-DAD analysis.

Introduction

Given the many and various biological activities of St. John's Wort extracts [1, 2], it is important to understand the chemical reactivity and behaviour of the diverse compounds that mediate these activities. Some of the most physiologically active components in St. John's Wort extracts are phenanthroperylene quinones.

It is well known that light induces the conversion of protopigments **1** and **2** into hypericin **3** and pseudohypericin **4** [3]. Hypericin **3** is stable in water/alcohol

* Corresponding author. E-mail: jean-dominique.fourneron@univ.u-3mrs.fr



Scheme 1

solution at both low and high *pH*. In contrast, we have shown that pseudohypericin **4** is rapidly converted into isopseudohypericin **5** [4, 5] in alkaline medium.

This article focuses first on the *pH*-dependent behavior of **4** and **5** in a water/alcohol environment; secondly, the behavior of these compounds in analytical HPLC is addressed; and thirdly, we sought to discover whether **5** is formed in the course of extraction of the plant into a diluted alcohol solution.

Results and Discussion

*Behavior of **4** and **5***

Recently we have reported that, in 80% ethanol (*Et*OH), **4** can be converted into **5** by the addition of sodium hydroxide. Broadly speaking, the rate of conversion is dependent on the concentration of NaOH.

To control the *pH* of the aqueous phase in these experiments, we used a series of buffer solutions covering *pH* values from 4 to 10. In addition, ''extreme'' conditions were tested with 0.1 *N* NaOH and HCl. Reactions were set up by adding a buffer solution (to a final concentration of 20% [v/v]) to an alcoholic solution of **4** (20 mg/dm^3 in anhydrous *Me*OH), and then recording the UV-Vis spectrum at regular intervals. The read-out of a typical experiment (at *pH* 7, in this case) is presented in Fig. 1.

The dynamics of this reaction can be followed using a previously described method [7]: in brief, the initial spectrum of **4** is attributed a coefficient, P (where $P<1$), and the resultant spectrum is subtracted from each of the spectra recorded

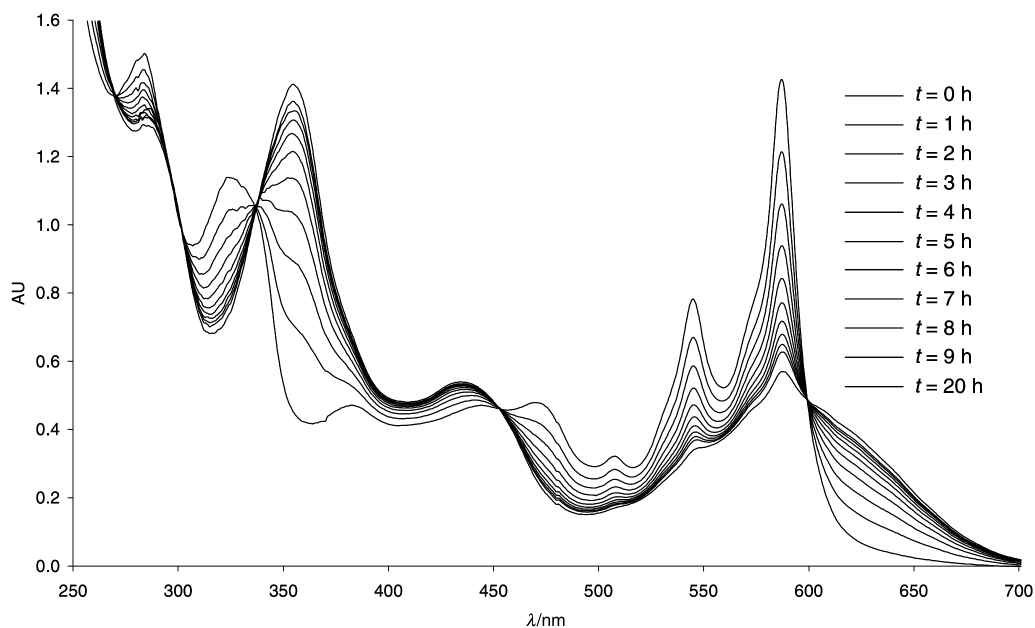


Fig. 1. Changes in the UV-Vis spectrum of **4** in methanol (*pH* 7) over time

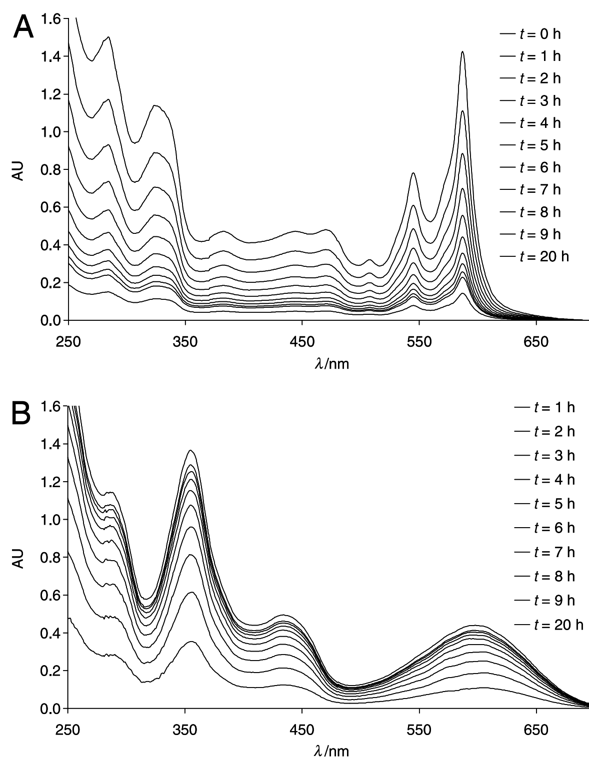


Fig. 2. (A) Spectra of the residual **4**; (B) spectra of the newly formed **5**

subsequently. This gives, for each time point, the spectrum of the residual **4** (which corresponds to *P* times the initial spectrum, Fig. 2A) plus the spectrum corresponding to the newly formed **5** (Fig. 2B).

Data from all the experiments at different *pH* values were treated in the same way and the amount of residual **4** at the various *pH* values was plotted against time (Fig. 3); the relationship is $P = \mathrm{f}(t)$.

The reaction is therefore characterized by its initial rate and the amounts of **4** (or **5**) at equilibrium. The percentage of residual **4** is plotted against *pH* in Fig. 4.

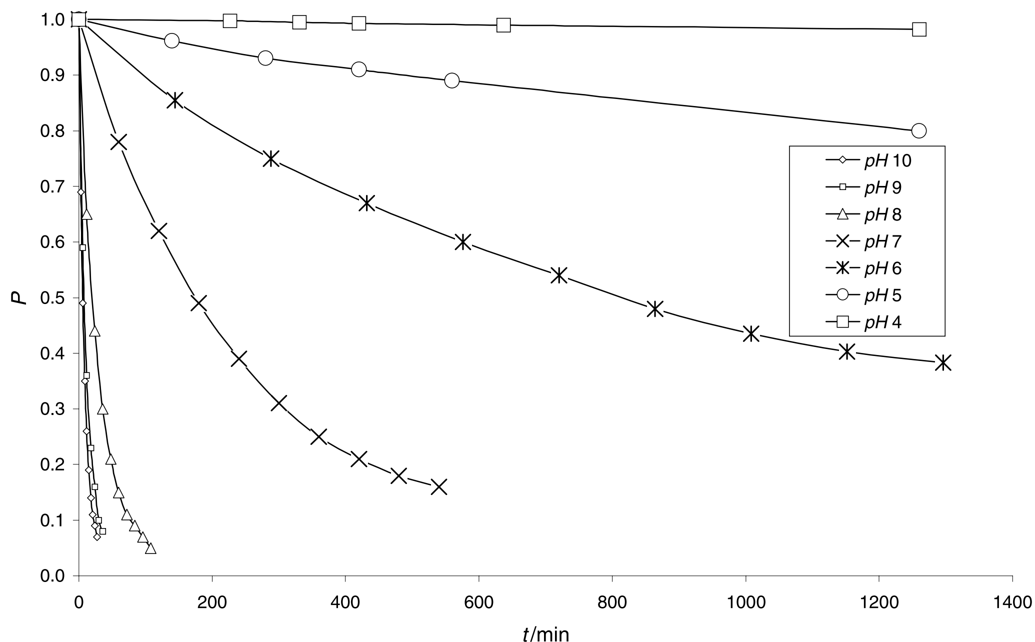


Fig. 3. *pH*-dependent changes in **4** (factor *P* *vs.* time)

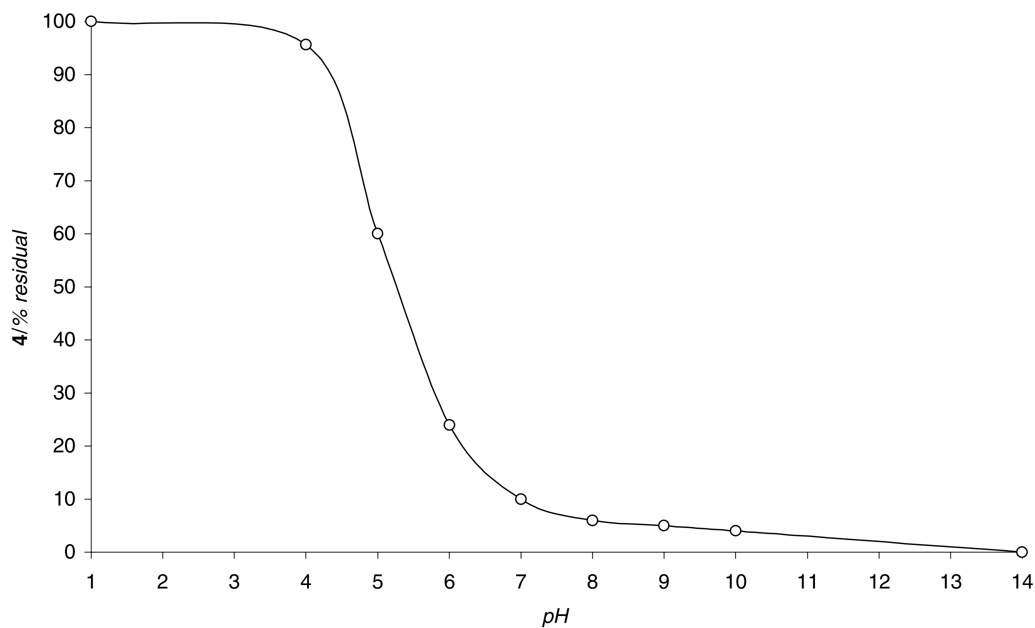


Fig. 4. Percentage of **4** present at equilibrium *vs.* *pH*

At high *pH* (over 10), **4** is very rapidly and completely converted into **5**. In contrast, **4** is stable at $pH < 4$, with no **5** detectable after thirty days. As the *pH* drops from 10 to 4, the rate of conversion drops regularly and the equilibrium position shifts towards **4**. Therefore similar series of experiments was carried out using **5** as the starting material. As expected, in a strongly alkaline medium ($pH > 10$), **5** is stable over a period of thirty days. As the *pH* is decreased from 10, **4** begins to form. At all *pH* values between 10 and 5, exactly the same UV-Vis spectrum is observed (*i.e.* the same equilibrium position is reached) whether the starting material is **4** or **5**. However at $pH < 4$, **5** is stable; the formation of **4** is not observed.

These results show that an equilibrium is established between **4** and **5** in an alcohol/water environment at *pH* values of 4–10. Above *pH* 10, **4** is completely converted into **5**, and below *pH* 4, both compounds are stable. How can these observations be explained? *Falk* has clearly shown that, in a polar environment, hypericin is a dissociated acid with a $pK_a \approx 2$ [8]. Underlying this strong acid character is the conformation of the molecule's *bay*-region coupled with the fact

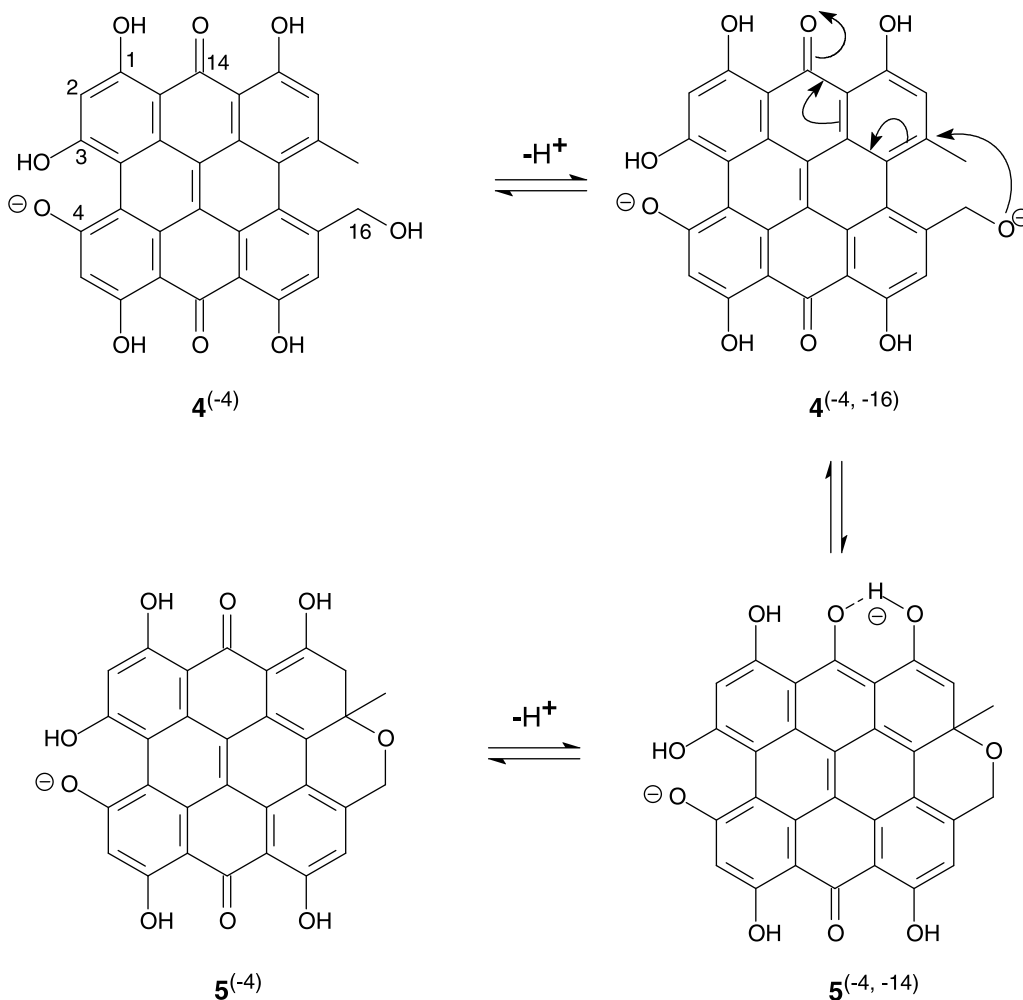


Fig. 5. Possible mechanism underlying the interconversion between **4** and **5**

that the negative charge can be delocalized at C7 and/or C14 where it can be stabilized by neighboring phenolic hydrogen atoms. It can be assumed that **4** and **5** behave in exactly the same way as hypericin when it comes to deprotonation of the *bay*-region so at *pH* 4, all the hydroxyl groups in that region have been stripped, *i.e.* **4** and **5** are in the forms $\mathbf{4}^{(-4)}$ and $\mathbf{5}^{(-4)}$ (Fig. 5) and are stable. If the ring closure, which converts **4** into **5** is to take place, there must be a negative charge on the primary hydroxyl group at C16. Mesomerism is not possible between $\mathbf{4}^{(-4)}$ and $\mathbf{4}^{(-16)}$ and the acidity at C16 (expected $pK_a = 16$) is insufficient for transfer of a proton between the *bay*-region and this site.

When the *pH* rises (although still in the acid range), additional deprotonation events occur. The first occur at the phenol groups ($pK_a = 12$) which do not induce conformational changes as in hypericin [5, 7]. On the other hand, deprotonation of the hydroxymethyl group ($\mathbf{4}^{(-4,-16)}$) very rapidly induces the formation of $\mathbf{5}^{(-4,-14)}$ with transfer of the negative charge onto the oxygen atom at C14 where it is effectively stabilized by the neighboring hydrogen atoms. Deprotonation of $\mathbf{5}^{(-4,-14)}$ gives the $\mathbf{5}^{(-4)}$ form which has been isolated and characterized [6].

Two other observations are worth mentioning. Firstly, the stock solution of **5** in anhydrous *Me*OH is stable for one year at −25°C, and for one month at room temperature. Secondly, at time point $t = 0$, three different UV-Vis spectra are observed for **5** at *pH* values of 4, 5, and 6 (Fig. 6); these are the same as spectra shown in a recent publication [6].

The spectrum recorded at *pH* 5 is the same as that observed in anhydrous *Me*OH [6]. It might correspond to the $\mathbf{5}^{(-4)}$ form. The spectrum at *pH* 4 could also correspond to the same form but carrying more protons. The spectrum at *pH* 6 might correspond to the $\mathbf{5}^{(-4,-14)}$ di-ionized structure. It should be noted that the same spectrum is also obtained at *pH* 7 (Fig. 2B) and *pH* 10.

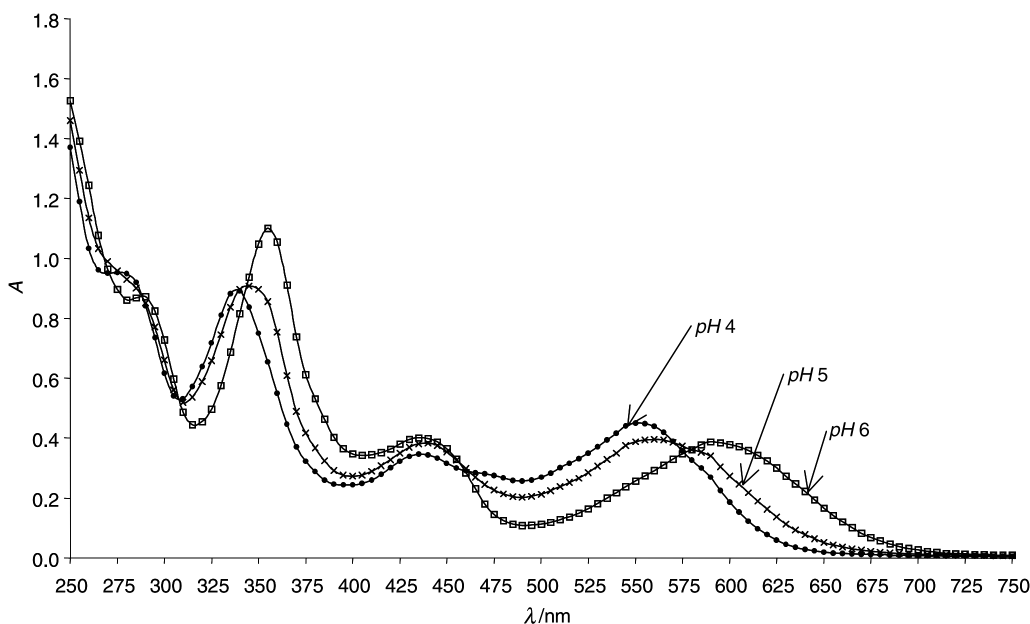


Fig. 6. UV-Vis spectra of **5** at *pH* 6, 5, and 4

HPLC Analysis

The *pH*-dependent sensitivity of isopseudohypericin in an alcohol/water environment complicates the question of HPLC analysis which uses this type of eluent. Data in literature suggest that the aqueous fraction of the mobile phase is often acidic (H_3PO_4) [9, 10]. As shown above, **5** gives different UV-Vis spectra at *pH* values of 4, 5, and 6 so eluent *pH* would be expected to have a significant impact on the analytical results obtained for this compound. We probed this question using a conventional C-18 column. The elution gradient was *Me*OH with one of three different aqueous phases: 0.1% H_3PO_4 adjusted *pH* 4, *pH* 5, and *pH* 7.5 using NaOH.

A solution of isopseudohypericin **5** (100 mg/dm^3) was made up in a mixture of *Me*OH and 0.1% H_3PO_4 (adjusted to *pH* 7.5). In parallel, a solution of pseudohypericin **4** was made up in a mixture of *Me*OH and 0.1% H_3PO_4 (*pH* 4). Each of these solutions was run with the three different eluents and retention time (*RT*) and area under the curve (*A*) at 590 nm were recorded (Table 1).

The retention times of **4** and **5** increase with *pH*, especially that of **5** which elutes in front of **4** at *pH* 7.5 but behind at *pH* 5 and 4. The decrease of the retention time with the increase of *pH* implies that **5** is under a form less and less lipophile what is compatible with a degree of deprotonation increasing with the *pH*. The area under the curve for **4** is unaffected by *pH* whereas that of **5** increases steadily between *pH* 4 and *pH* 7.5.

Finally, the UV-Vis spectra recorded using a DAD system similarly vary with the *pH* of the mobile phase: exactly the same spectra as those in Fig. 6 are observed for **5** at *pH* 4, 5, and 6 when a conventional spectrophotometer is used.

For quantitative determination, we calculated the response factor of **5** with respect to that of **4** in the system in which the mobile phase is at a *pH* 7.5. As might be expected from the UV-Vis spectra presented in Fig. 2, these response factors are identical at isosbestic point wavelengths, 340, 450, and 600 nm. Both the detection and quantitative determination of **5** by HPLC are therefore highly sensitive to the *pH* of the mobile phase. Retention time and the area under the curve all depend on *pH*, and this can give rise to confusion when it comes to interpreting chromatographic data.

Mixtures resulting from the interconversion of **4** and **5** were analyzed by HPLC and the estimated peak areas for residual **4** were found to be perfectly consistent with the UV-Vis results, *e.g.* at *pH* 7, results obtained with the reaction mixture give a curve which is perfectly superimposable on that shown in Fig. 3. The chromatogram recorded at 600 nm after 180 min is shown in Fig. 7.

Table 1. HPLC characteristics of peaks for **4** and **5**; dependence on mobile phase's *pH*

pH	**4**		**5**	
	RT	*A* (590 nm)	*RT*	*A* (590 nm)
4	10.29	0.57	10.97	0.75
5	9.39	0.56	9.88	0.96
7.5	8.74	0.58	7.28	1.38

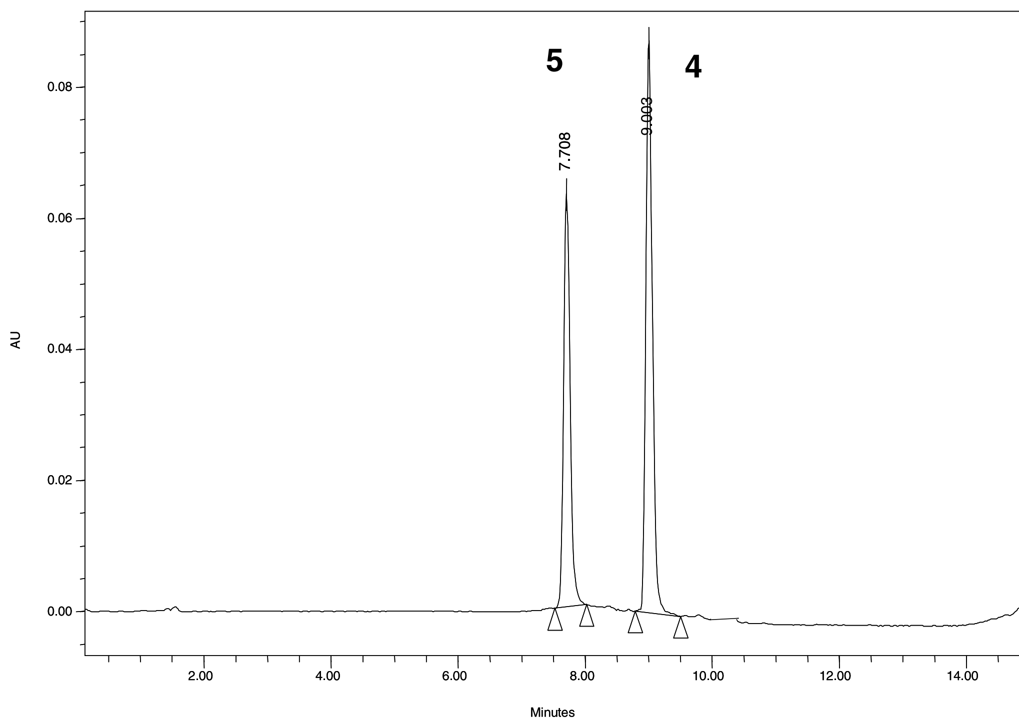


Fig. 7. Chromatogram (600 nm) of the reaction mixture after 180 min

Occurrence of **5** *in Plant Extract*

The instability of **4** in an alcohol/water environment coupled with the sensitivity of **5** to HPLC conditions leads us to believe that: i) **5** may be present in plant extracts prepared in the traditional way using aqueous ethanol, and ii) cyclopseudohypericin **6**, mentioned by various experts as a phenanthroperylene quinone of Saint John's Wort might in fact be isopseudohypericin **5**.

We prepared three different extracts of the plant. For the first two, we used a 60:40 mixture of *Et*OH and H_2O; in one case, tap water (TW, *pH* 7.4), and in the other, distilled water (DW, *pH* 5.1) was used. In the third extraction protocol, anhydrous *Me*OH was used. In all three procedures, a volume of 0.2 dm^3 of solvent was used to extract 20 g of plant material at 20°C with 24 hours of simple maceration and stirring from time to time.

At the beginning, the *pH* readings of the extraction mixtures were 8.3 with TW, and 7.1 with DW. After 24 hours of contact with the plant, this had dropped in both cases to *pH* 4.9. Supernatants were then analyzed by HPLC with the mobile phase at *pH* 7.5. Chromatograms were recorded at 600 nm, at which wavelength **4** and **5** have the same response factor. It can be seen that there is no difference between the TW and DW extracts which both contain the same percentages of **4** (97.8%) and **5** (2.2%). Extraction with anhydrous *Me*OH gives significantly different proportions, namely 99.2 and 0.88%. Thus, extraction into a hydrated medium substantially increases the concentration of **5** in the extract.

At the beginning of extraction, the *pH* is high enough for the conversion of **4** into **5**. Subsequently, the extraction medium becomes acidic, probably due to the

abundance of phenolic compounds (mainly flavones) in the plant material. This inhibits the conversion of **4** into **5**, as indicated by the fact that their relative concentrations subsequently remain stable over a period of 96 h.

If, after 24 hours of maceration in TW, the *pH* is adjusted to the initial level by the addition of dilute NaOH, within three hours the percentage of **5** has risen from 2.1 to 4.0; after 24 h, the percentage has further increased to 6.0%.

These results clearly show that **5** is generated in the course of extraction of plant material into an alcohol/water solution. The small amounts of **5** measured in these extraction conditions are reminiscent of the chromatogram of *Gaedcke* [11] and the results of *Poutaraud* [9], although both of these authors discuss cyclopseudohypericin rather than isopseudohypericin.

On the basis of this work, it is not possible to say whether very small quantities of **5** are naturally present in the plant or not. However, the results unequivocally show that more **5** is obtained when the plant material is extracted using a mixture of *Et*OH and water, especially if the *pH* is kept around neutral. As a result, at least part of **5** is generated artificially.

Neither do these results make it possible to define whether cyclopseudohypericin and isopseudohypericin are structurally identical or not. Until the compound believed to be cyclopseudohypericin will be isolated and its structure elucidated, this question will remain unanswered. Our contribution to this issue is to make available to the scientific community (on the Internet at the address www.geocities.com/sjwsclub/) digitalized versions of the UV-Vis spectra of **5** (as shown in Fig. 5) which can be used to accurately identify minor peaks in chromatograms derived from extracts of Saint John's Wort.

Experimental

Chemicals

The two phenanthroperylenes **4** and **5** where prepared according to Ref. [4]; **4** fulfils pre-established purity criteria [7]. The buffer solutions used (*pH* 4, 7, and 10) are supplied as *pH* meter reference solutions by Fischer Bioblock Scientific (F 67403 Illkirch). Intermediate buffer solutions (*pH* 5, 6, 8, and 9) were generated by adding 1 *N* NaOH or HCl to the above. The methanol used for extraction of plant material and for HPLC analysis was from Riedel-de Häen.

UV-Vis Analysis

UV-Vis spectra were recorded on a Kontron 922 apparatus using 1 cm SiO_2 cuvettes. Resolution was 1 nm and the wavelength scanning speed was 100 nm/min. Spectral data were recovered in the form of an Excel® file.

HPLC Analysis

HPLC was performed on a Waters apparatus with an E600 pump and the diode array detector DAD 996. The unit was controlled using Millenium^{32} software from Waters. Solutions were injected *via* a Rheodyne valve with a 20 mm^3 injection loop. The column (CC125/4 Nucleosil 100-5 C18) was obtained from Macherey-Nagel. DAD settings were: wavelength from 220 to 740 nm; recording frequency: 1 spectrum per second with a resolution of 1.231 nm. Elution was performed at room

temperature (20–21°C) in the following steps: $t = 0$, *Me*OH/H_2O = 50/50; $t = 3$ min (linear gradient), *Me*OH; $t = 10$ min (isocratic phase), *Me*OH; $t = 10.1$ (return to initial conditions), MeOH/H_2O = 50/50; $t = 15$ min (stabilization), MeOH/H_2O = 50/50. The flow rate was 1 cm^3/min. The signals generated by the PDAD were processed by Millenium^{32} software. UV-Vis spectra were obtained by using the function "Spectrum Point" of Millenium^{32} in a compatible Excel® file.

Extraction of Plant Material

The material extracted consisted of the flowering tips of cultivated plants (PMA28, F-28140, Varize) harvested in 2003. Twenty grams of plant material were roughly cut up and extracted into 0.2 dm^3 of solvent (aqueous *Et*OH or anhydrous *Me*OH). HPLC analysis was conducted on the supernatant after membrane filtration (pore size = 0.45 μm).

References

[1] Hölzl J, Ostrowski E (1987) Dtsch Apoth Ztg **23**: 1227
[2] Nahrstedt A, Butterweck V (1999) Pharmacopsychiatry **30**: 129
[3] Poutaraud A, Di Gregorio F, Chan Fook Kin V, Girardin P (2001) Planta Medica **67**: 225
[4] Naït-Si Y (2002) Thesis. University of Aix-Marseille III
[5] Fourneron J-D, Herbette G, Caloprisco E (1999) C R Acad Sc Série II, 127
[6] Fourneron J-D, Naït-Si Y, Rosas R, Faure R, Viant P (2003) Tetrahedron Lett **44**: 6285
[7] Naït-Si Y, Fourneron J-D (2004) Monatsh Chem **135**: 1319
[8] Leonhartsberger JG, Falk H (2002) Monatsh Chem **133**: 167
[9] Poutaraud A, Lobstein A, Girardin P, Weniger B (2001) Phytochem Anal **12**: 355
[10] Kraemer W, Wiartalla R (1992) Pharm Ztg Wiss **5**: 202
[11] Gaedcke F (1997) Dtsch Apoth Ztg **137**: 117