Physicochemical Properties of Oosporein, the Major Secreted Metabolite of the Entomopathogenic Fungus *Beauveria brongniartii*

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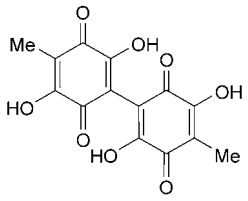
Oosporein (**1**) is the major secondary metabolite excreted by *Beauveria brongniartii* (SACC.) PETCH (Ascomycota: Clavicipitaceae), an entomopathogenic fungus infesting the larvae of *Melolontha melolontha* L. (Coleoptera: Scarabaeidae; common European cockchafer). Physicochemical parameters were established to allow a better understanding of the spatiotemporal distribution of this marker substance in the environment. *i*) The dependence of the oosporein solubility (C_s) from proton concentration and temperature was assessed. Oosporein is a rather strong organic acid. The basal solubility of oosporein (C_{s_0} in aqueous citrate/HCl buffer at pH 1.23) was found to be 24.8 ± 0.3 μM. The pK_a of the first deprotonation step was determined to be 2.42 ± 0.02. A nonlinear *van't Hoff* equation was established for the temperature dependency of the oosporein solubility ($\ln C_s = a + bT^{-1} + c\ln T$, with $a = -123.3$, $b = 2678.9$, and $c = 19.5$). *ii*) A second and third deprotonation step was found in *Britton–Robinson*-buffered aqueous solution. The associated pK_a values were determined as 6.79 ± 0.08 and 9.19 ± 0.03. *iii*) The stability of oosporein at different pH values and temperatures was addressed. Oosporein degraded quickly under moderate alkaline conditions and with increased temperatures. The half-life dropped below 20 h at pH ≥ 8.0 and $T \geq 43°$. The analysis of the *Arrhenius* plot allowed to calculate the activation energies (E_a) as 102 ± 17 (pH 6), 100 ± 5 (pH 8), and 91 ± 6 kJ/mol (pH 10). *iiii*) The octanol/water partition coefficient (P_O) and its pH dependence were also determined. The distribution coefficient (D_O) for oosporein at pH 1.2 was found to be 53.7 ± 4.1 ($\log D_O = 1.73 \pm 0.03$). The partition coefficient P_O for undissociated oosporein was calculated as 56.6 ($\log P_O = 1.75$). From the physicochemical properties, it can be concluded that oosporein can hardly be adsorbed by organisms.

Introduction. – Considerable progress has been made in the development of environmentally benign and effective new formulations of fungal biological control agents (BCAs) based on *Beauveria brongniartii* used for the control of *Melolontha melolontha* and *M. hippocastani* (Coleoptera: Scarabaeidae). Currently, four products, based on *Beauveria brongniartii*, are registered in three European countries (Austria, France, and Switzerland) for the control of the *Melolontha ssp.* [1–3]. To allow a responsible risk management of the applied BCAs, investigations on the significance of secondary metabolites released by entomopathogenic fungi and their environmental enrichment are needed as part of the preceding risk assessment [4]. Therefore, information on the spatiotemporal distribution of relevant metabolites in the final product as well as in the application environment has to be obtained. Oosporein (**1**) is the only known relevant secondary metabolite secreted by the commercially used *B.*

brongniartii production strains [5]. Therefore, an improved knowledge about the physicochemical properties of the questioned analyte can be the first step towards understanding the behavior of a chemical in the different environments. Bioavailability, toxicology, as well as stability of substances are mainly determined or influenced by the physical and chemical properties of a substance. Furthermore, factors like the degree of ionization, the aqueous and lipid solubility and the redox potential of involved compounds may have important effects on biochemical reactions. Until now, only few results have been published on the physicochemical characterization of oosporein (**1**) bio-synthesized by *B. brongniartii*. Most of the contributions focused on the pH dependence of UV- and VIS-light absorption resulting in solution colors ranging from light orange at acidic pH to violet at alkaline pH [6–9]. The solubility of **1**, its acidic character, its instability at pH > 5, its sensitivity to air, and its degradation on aluminium oxide have already been addressed by *Kögl* and *Van Wessem* [6] presenting **1** as new natural product. The same authors also did not fail to report shortly on the crystallization and melting behavior of **1**. Later contributions reproduced these findings, added new evidence as problems in defining the melting point [8][10][11], adsorptive behavior in chromatographic processes [10], or the degradation during workup [10]. This contribution will deal with the generation of further selected physicochemical parameters for oosporein (**1**) which have not been addressed yet.



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Results and Discussion. – *Solubility of* **1** *as a Function of pH*. The solubility of oosporein (**1**) increased with increasing pH (*Fig. 1*). By fitting the experimental data points (citrate/HCl buffer system, 20°) to the *Henderson–Hasselbalch* equation based on the method of least squares, an ionization constant pK_a of 2.42 ± 0.02 and a basal solubility C_{s_0} of 24.8 ± 0.3 μM were calculated. As a consequence of these data, it can be stated that at a pH < 0.4, **1** is totally undissociated; at pH values above pH 3, the degree of dissociation as well as the solubility increases exponentially.

Solubility of **1** *as a Function of Temperature.* The solubility of **1** in aqueous, acidic solutions was weak and increased with increasing temperature (*Table 1*). Plotting the experimental data points according to *van't Hoff* (*Fig. 2*), resulted in a curve missing a clear linear relationship. Thus a nonlinear regression curve based on the method of least squares was calculated by *Eqn. 1*, where C_s is the solubility in mM, T the temperature in K, and a, b, and c are regression coefficients ($a = -123.3$, $b = 2678.9$, and $c = 19.5$). Based on these calculations, at 25° a heat-of-solution (ΔH_{sol}) value of 26.0 kJ/mol was obtained.

$$\ln C_s = a + bT^{-1} + c \ln T \quad (1)$$

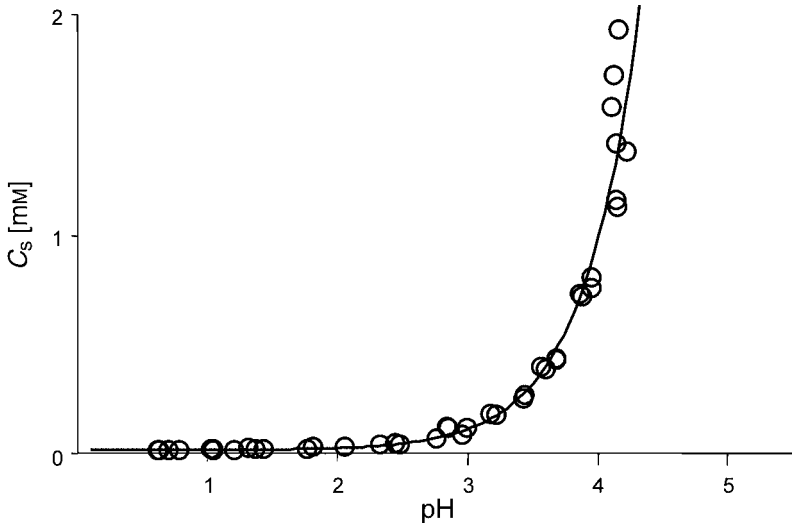

Fig. 1. *Solubility* (C_s) *of oosporein* (**1**) *in citrate/HCl buffer as a function of pH at 20°*. The data points represent the measured values; the plain curve is calculated from the *Henderson–Hasselbalch* equation.

Table 1. *Solubility of Oosporein* (**1**) *in Citrate/HCl Buffer* (pH 1.23) *at Different Temperatures*

Temp. [°]	C_s [μM]		D[a]) [%]	ΔH_{sol} [kJ/mol]
	exper.[b])	calc.[c])		
4.0	15.1 ± 0.5	16.0	− 1.6	22.6
12.0	20.9 ± 1.5	21.2	1.6	23.9
20.0	28.0 ± 0.5	28.1	1.9	25.2
29.0	37.7 ± 1.0	38.6	0.8	26.7
38.0	48.7 ± 0.1	52.9	− 5.5	28.1
47.0	72.5 ± 0.5	72.4	3.1	29.6

[a]) Relative difference of measured and calculated solubility values in %. [b]) Measured values (± s.d.). [c]) Calculated with *Eqn. 1*.

Partition Coefficient for **1**. The partition coefficient (P) is a measure for the relative polarity and lipid solubility of organic molecules [12]. In ideal systems, where ionizations of the analyte can be excluded, the partition coefficient equals the quotient of the substance concentration in the nonaqueous phase (C_L) and the aqueous phase C_W) (*Eqns. 2* and *3*). The lipophilicity of a substance, often expressed as log$P$ (*Eqn. 3*), is additionally influenced by the pH of the aqueous buffer, whenever dissociation of the analyte can be expected. In these systems, the measured partition coefficient P is regarded as an apparent entity P_{app} or distribution coefficient D. For acidic analytes, D is related to the true partition coefficient P by *Eqn. 4* [13].

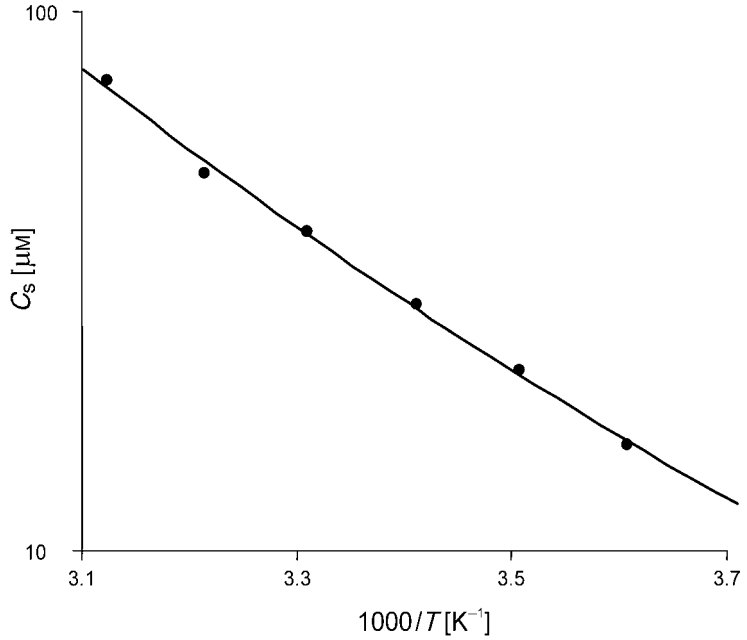

Fig. 2. Van't Hoff *plot of the equilibrium solubility data* (citrate/HCl buffer solution, pH 1.23) *of oosporein* (**1**). The data points represent the measured values, and the plain curve is calculated according to *Eqn. 1*.

$$P = C_L/C_W \tag{2}$$

$$\log P = \log C_L - \log C_W \tag{3}$$

$$\log P = \log D - \log 1/(1 + 10^{pH-pK_a}) \tag{4}$$

To mark that our values refer to octanol as lipophilic solvent, the subscript 'O' is used. In the case of oosporein (**1**), D_O was 53.7 ± 4.1 ($\log D_O = 1.73 \pm 0.03$; $n = 10$) at pH 1.2. Accordingly, the true partition coefficient for undissociated **1** was calculated to be 56.6 ($\log P_O = 1.75$). The pH dependence of the partition coefficient ($\log D_O$) is given in *Fig. 3*. The lipophilicity of **1** decreases rapidly with increasing pH, at neutral pH only *ca.* 0.15% of the total oosporein are present in the octanol phase.

Dissociation of **1**. A bathochromic shift of the UV maxima with increasing pH was observed for oosporein (**1**) in *Britton–Robinson*-buffer systems. In contrast to a previously described solvent system [11], the absorption wavelength did not increase steadily with the pH but showed a distinct additional plateau at 318 nm, thus indicating the existence of at least three deprotonation levels. The absorption maxima of these levels were determined as 302 (pH 4), 318 (pH 7), and 325 nm (pH 10). Subsequently, absorption measurements at 318 and 325 nm between pH 5.9 and 10.7 (22 data points, two independent experiments) allowed us to calculate the pK_a values associated with the deprotonation of [oosporein]$^-$ to [oosporein]$^{2-}$ and further to [oosporein]$^{3-}$ as 6.79 ± 0.08 and 9.19 ± 0.03, respectively.

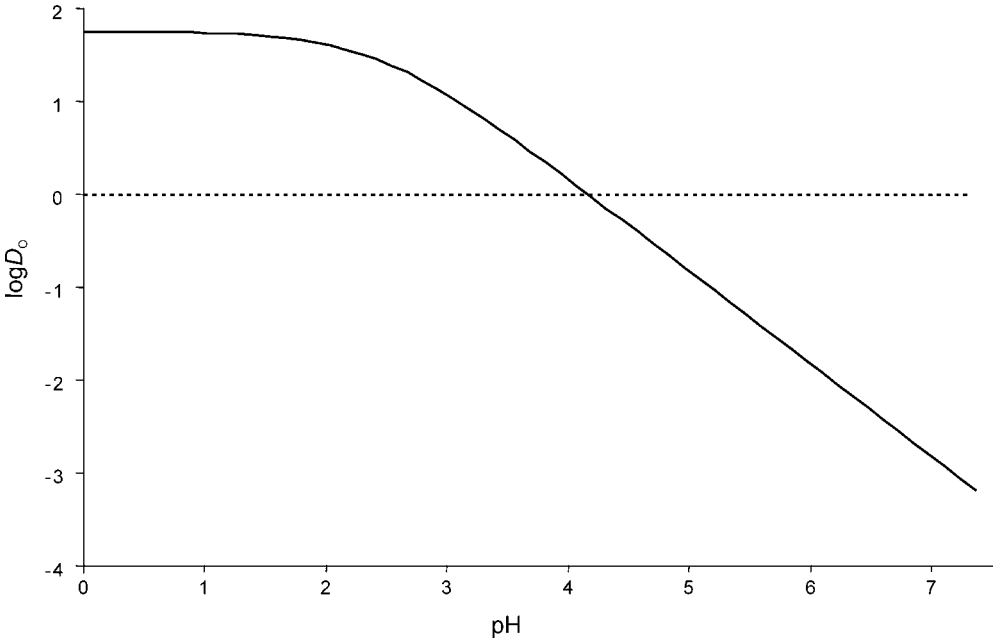

Fig. 3. *Change of log*D_O *of oosporein* (**1**) *with pH*. Curve calculated from the measured logP_O at pH 1.23 according to *Eqn. 4*.

Stability of **1** *as a Function of pH and Temperature.* In a first round of experiments at 53°, a pronounced pH dependency of the stability of oosporein (**1**) was observed. The pH dependency of the half-life was decreasing linearly between pH 2 and 6 from 37.9 days to 6.7 days, whereas an accelerated decomposition was observed at pH > 7 with half-lifes less than one day. Therefore, all further experiments at different incubation temperatures (23–53° in 10° steps) were limited to *Britton–Robinson* buffers with weakly acidic and alkaline pH (pH 6, 8, and 10). A correlation of the oosporein-breakdown rate with both temperature and pH was observed (*Table 2*). Half-lifes decreased with increasing temperature, whereas increasing the alkalinity of the solvent (pH 8 *vs.* pH 10) did not accelerate the oosporein breakdown. The slopes of the derived *Arrhenius* functions were independent of the pH value of the electrolyte used (*Fig. 4*). The corresponding activation energies (E_a) were calculated as 102 ± 17 (pH 6), 100 ± 5 (pH 8), and 91 ± 6 kJ/mol (pH 10). Therefore, the observed accelerated breakdown at alkaline pH (pH 8 and 10) does not depend on the kinetics of the initial reaction but on the occurrence of subsequent breakdown steps. Monitoring oosporein breakdown with HPLC-DAD (DAD = diode-array detector) allowed further investigation of the breakdown products. All observed degradation products had shorter retention times compared to oosporein (**1**). This indicates an increased polarity of the analyte, most likely caused by oxidative dimerization reactions involving lactone formation as already observed in model systems [14–17] and possible subsequent ring-opening reactions. Brownish precipitates, as observed previously [10]

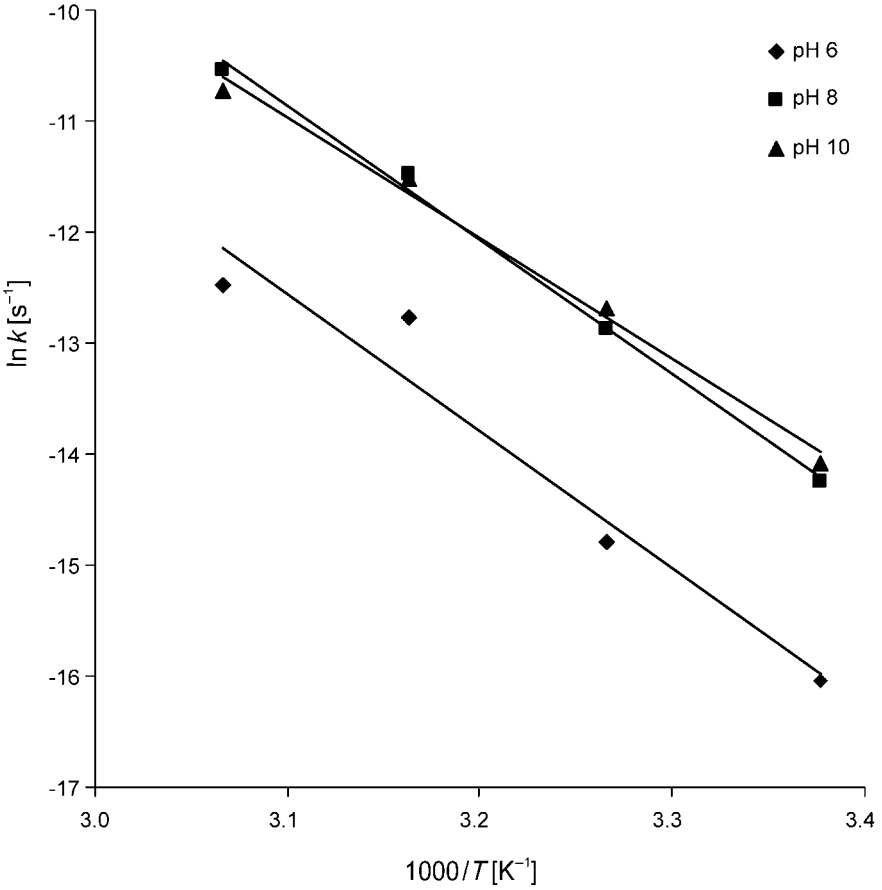

Fig. 4. Arrhenius *plot derived from the oosporein* (**1**) *breakdown experiments performed at pH 6, 8, and 10*

Table 2. *Oosporein Degradation at different pH Values and Temperatures: Summary of Averaged Rate Constants and Half-Lifes Including Their Relative Standard Deviation* (% r.s.d; ($n = 3$))

pH	Temp. [°]	Rate constant [10^{-3} h^{-1}]	Half-life [h]	% r.s.d.
6.0	23	0.3	1779.0	5.8
6.0	33	1.4	506.3	2.3
6.0	43	10.1	68.2	12.1
6.0	53	13.8	50.2	3.1
8.0	23	2.0	296.0	1.4
8.0	33	9.3	74.5	4.3
8.0	43	37.3	18.6	2.2
8.0	53	94.3	7.0	4.2
10.0	23	2.8	251.0	2.3
10.0	33	11.1	62.0	4.0
10.0	43	36.0	19.2	4.7
10.0	53	79.3	8.7	3.0

and in our oosporein purification protocols, were not formed in any of these experiments. Most of the reaction products still showed chromophores similar to that of the educt. The final qualitative and quantitative distribution of reaction products was pH dependent. Characterization of oosporein-breakdown products will be the focus of further research efforts involving HPLC-DAD-MS and HPLC-DAD-NMR hyphenation techniques.

Conclusions. – The studies on the solubility of oosporein (**1**) as a function of temperature and pH showed that **1** is a rather strong organic acid ($pK_{a1} = 2.42$). The compound is slightly soluble in acidic aqueous solutions (basal solubility 24.8 μM), and the solubility rises with increasing pH. The solubility rises also with temperature, and a solution enthalpy of $\Delta H_{sol} = 26$ kJ mol^{-1} was derived. The degree of ionization increases with the pH ($pK_{a2} = 6.79$, second deprotonantion step; $pK_{a3} = 9.17$, third deprotonantion step). The degree of ionization of **1** under physiological conditions (pH 7) is high, and therefore, its lipophilicity is very low. At pH 7, only *ca.* 0.15% of **1** can be expected to be present in the lipophilic (octanol) phase. As a consequence, it cannot be expected that **1** can pass any type of biological membrane by the mechanism of passive diffusion under these conditions. Since it is also unlikely that an active-transport system exists for this molecule, it can be assumed that the rate of absorption of oosporein (**1**) in organisms is very low, unless the environment at the absorption site is very acidic. Furthermore, **1** degrades quickly under moderate alkaline conditions. The half-life drops sharply with increasing temperature and pH (12 days at 23° and pH 8, and 8 h at 53° and pH 8). Under moderate acidic conditions, the substance is more stable.

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Experimental Part

General. Oosporein (= 3,3′,6,6′-tetrahydroxy-4,4′-dimethyl-[bi(cyclohexa-1,4-dien-1-yl)]-2,2′,5,5′-tetrone; **1**) reference standard material (purity 98%) and culture filtrates were obtained from *Beauveria brongniartii* (strain BIPESCO 2; production strain of *Melocont*®-Pilzgerste; DSM 15205) submerse cultures as described [5][18]. MeCN, MeOH (both reagent grade), AcOH, boric acid, H_3PO_4, sodium hydroxide, and toluene (all anal. grade) were purchased from *Merck* (Darmstadt, Germany). Water for HPLC was produced by reverse osmosis followed by distillation. *Britton–Robinson* buffer was prepared from stock solns. according to the procedure given by *Mongay* and *Cerda* [19]. UV/VIS Spectra: *Shimadzu U-2000* UV/VIS photometer (*Shimadzu Europe*, Duisburg, Germany). pH measurements: *Beckman Φ^{TM}-71* pH meter (*Beckman Coulter*, Fullerton, USA), calibration with potassium tetraoxalate and potassium hydrogenphthalate at 20° according to DIN 19266. HPLC: *Hewlett-Packard 1090* instrument (*Agilent Technologies*, Heilbronn, Germany), equipped with a diode-array detector (DAD), an automatic injector, an auto sampler, and a column oven; *Phenomenex Synergi-Hydro-RP-80A* column (150 mm × 4.6 mm), particle size 4 μm (*Phenomenex*, Torrence, USA); solvent gradient of H_2O (solvent *A*) and MeCN (solvent *B*), both containing 0.1% (*v*/*v*) AcOH and 0.9% (*v*/*v*) HCOOH: 5 → 60% *B* in 6 min, then 60 → to 98% *B* in 2 min, and 98% *B* for a further 5 min; flow rate 1.0 ml/min; column temp. 23° (thermostated), re-equilibration of the column for 7 min between analyses; injection volume 10 μl; detection at 287 nm.

Solubility Measurements of **1**. The solubility of **1** as a function of temp. and pH was performed in a citrate/HCl buffer (pH 1.23; ionic strength 0.069) with 0.03% (*w*/*v*) polysorbate *80*. The solns. (25 ml) containing **1** in excess were prepared in lockable, cylindrical glass vessels (Ø 4 cm) equipped with a magnetic stirrer

(500 r.p.m.). All experiments were performed in a thermostatted water bath (±0.05; pre-equilibration time 30 min). Samples were withdrawn by sucking the soln. through a 0.45-μm membrane filter (*HAWP, Millipore*, Billerica, USA) directly into a glass pipette [20]. Concentrations were determined spectrophotometrically at 287 nm. The temp. dependence of the solubility of **1** was performed at 4, 12, 20, 29, 38 and 47° (temp. of the water bath). Four samples from two parallel experiments were withdrawn at different times between 115 and 300 min. The pH dependence of the solubility was determined at 20°. Starting from a citrate/HCl buffer at pH 1.23 seventeen buffer solns. were created by adding 1N NaOH or 1N HCl. Oosporein (**1**) was added under stirring until saturation was reached. The pH of the sat. soln. was measured after 1 h. Then a sample was withdrawn and the concentration was determined spectrophotometrically. By adding 1N NaOH to the test suspensions, higher pH values were established. Thus, starting from 17 solns., the saturation concentration of **1** at a total of 38 pH values between pH 0.63 and 4.78 was measured. The pK_a value and the basal solubility C_{s_0} were determined fitting the *Henderson–Hasselbalch* equation ($pH - pK_a = \log (C_s - C_{s_0})/C_{s_0}$) to the exper. data (least square fit).

Partition Coefficient for **1**. The determination of the apparent partition coefficient followed the procedure by *Moll* and *Bender* [12]. Oosporein (**1**) solns. (20 ml; six different concentrations) and solvent blanks in citrate/HCl buffer (pH 1.23; ionic strength 0.069) were incubated in lockable, cylindrical glass vessels (Ø 4 cm) equipped with a magnetic stirrer (500 rpm). After equilibration of the solns. (30 min) to 20° in a water bath (±0.1°), octan-1-ol (two independent experiments with either 5 ml or 10 ml) was added, and stirring was prolonged for 1 h. Phase separation was achieved by centrifugation at 3000 r.p.m. for 5 min (*Megafuge 1.0*, *Heraeus Sepatech*, Germany). Samples were withdrawn from both phases and, after appropriate dilution, the concentrations were determined spectrophotometrically at 287 nm for the buffer phase and at 290 nm for the octanol phase.

Dissociation of **1**. The dissociation of **1** was monitored by UV absorption measurements performed in *Britton–Robinson* buffer systems. Buffer solns. were prepared in 0.5-pH-unit steps, and solns. in-between these steps were obtained by mixing appropriate aliquots of the parent solns. yielding a total of 22 buffered solns. in the pH range 5.6–11.2. All pH values of the obtained buffers were measured twice. Oosporein was diluted in one step from a 1 mg/ml stock soln. in MeOH to final concentrations of 5 and 10 ppm (16 and 32 μM), resp. The concentrations were checked on random samples by HPLC-DAD. The addition of **1** to the buffer solns. had no measurable effect on the pH value. The pK_a values were determined from two separate experiments measuring at two wavelengths (318 and 325 nm) in each experiment by appropriate curve-fitting procedures.

Stability of **1**. The experiments for the pH dependence of the stability of **1** were preformed in 25-ml volumetric flasks (heat-sterilized before usage, 0.2 mg NaN_3 added) at 50° in a dark and thermostatted incubation chamber. Aliquots of 1 ml were withdrawn at appropriate intervals and sealed in HPLC vials. All solns. were stored at 4° until analysis. Five *Britton–Robinson*-buffer systems covering a pH range from 2.04 to 9.80 with constant ion strength of 0.10M (0.11M in the case of the pH 9.80 soln.) were prepared accordingly to *Mongay* and *Cerda* [19]. The pH values (2.40, 4.20, 5.75, 7.80, and 9.80) were checked after the addition of **1** (**1** was added by 1:50 dilution from a MeOH stock soln. to a final concentration of *ca.* 0.1 mg/ml) and were found constant (within ±0.02 units). Oosporein (**1**) quantification was performed by HPLC measurements. All measurements were made in triplicate, and reference solns. were randomly added into the analysis process to detect system instabilities.

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