Practical Course Instruction

Practical Course (MedPho – P1)
Physical Chemistry

M. Sc. Medical Photonics

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Experimental Instruction

1 Förster cycle — UV/vis absorption spectroscopy

1.1 Aim of experiment

Learn how to use a UV/Vis absorption spectrometer including evaluation and interpretation of the measured data and their application, in this particular case: determination of equilibrium constants and retrieval of thermodynamic data from spectroscopic measurements.

1.2 Preparations

Before starting the experiment, please make yourself familiar with the following topics and terms::

- Which transitions are observed in the UV/Vis area of the electromagnetic spectrum?
- What describe the terms: absorbance, absorption, transmission, absorption coefficient, Beer Lambert law?
- What is the general working principle of anUV/Vis spectrometer set up and components (also for particular working principles including one beam, two beams, simultan spectrophotometer)?
- What are possible field of applications of UV/Vis absorption spectroscopy?
- What describes the pK_a-value? How is the pH-value determined?
- What is dissociation enthalpy and dissociation entropy?
- How can UV/Vis absorption spectroscopy be used to determine the pK_a-value not only in the electronic ground state but also in the electronically excited state?

1.3 Tasks

1. Record the UV/Vis spectrum of bromthymolblue in aqueous solution as a function of pH-value .
2. Determine the equilibrium constants in the electronic ground state as well as in the electronically excited state.

3. Discuss your results.

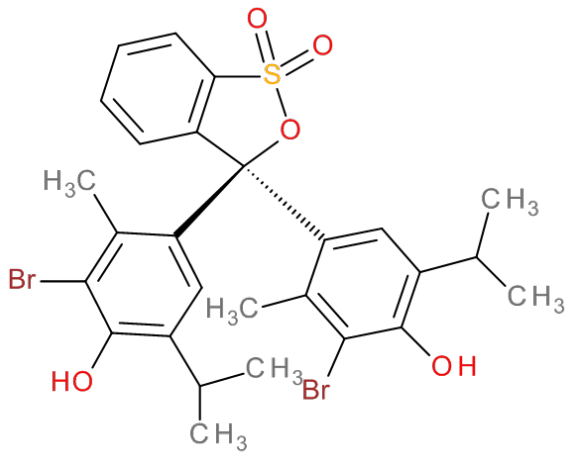

Figure 1.1: Bromthymolblue

1.4 Theoretical background

The pHdependency of absorption spectra results from structural changes due to protonation (e.g. of an amino group), deprotonation (e.g. of an OH group) or addition of OH^-. For benzopyrylium compounds the latter one can lead to subsequent reactions (see Fig. 1.2). However, those reactions are only observed at high pH-values and lead to the destruction of the chromophore system. At p$H < 12$ those reactions are usually slow, p$H > 12$ might prevent the correct evaluation of the spectra.

In general, the dissociation of an acid can be described as:

$$\mathrm{HA} \rightleftharpoons \mathrm{H^+} + \mathrm{A^-}.$$

After transformation of the resulting law of mass action one obtains the so-called Henderson-Hasselbalch equation:

$$\mathrm{p}K_\mathrm{a} = \mathrm{p}H - \lg \frac{[\mathrm{A^-}]}{[\mathrm{HA}]}. \tag{1.1}$$

With the assumption that the total concentration of the dye stays constant for each measurement, a relationship between pK_a and pH-value as well as the absorbance A can be obtained:

$$\lg \frac{A_\mathrm{HA} - A_\mathrm{M}}{A_\mathrm{M} - A_\mathrm{A^-}} = \mathrm{p}H - \mathrm{p}K_\mathrm{a}. \tag{1.2}$$

In this equation A_HA, A_M and $A_\mathrm{A^-}$ denote the absorbance of the pure, non-dissociated acid HA, the mixture M and the pure anion $\mathrm{A^-}$ under the assumption of constant total concentration. Plotting the logarithmic expression against pH yields a straight line which intersects with the pH-axis (x-axis) at pH = pK_a.

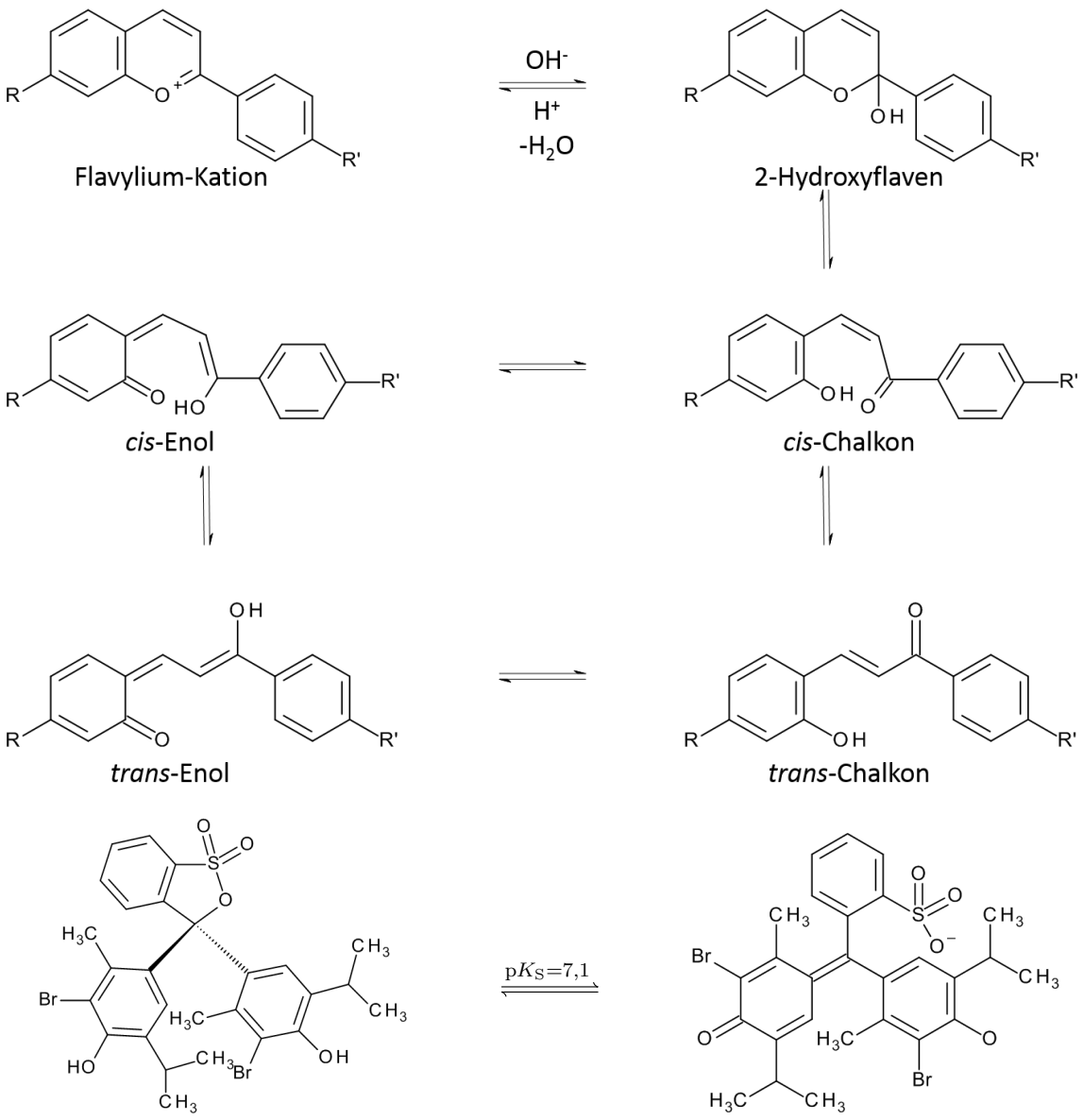

Figure 1.2: Possible consecutive reactions

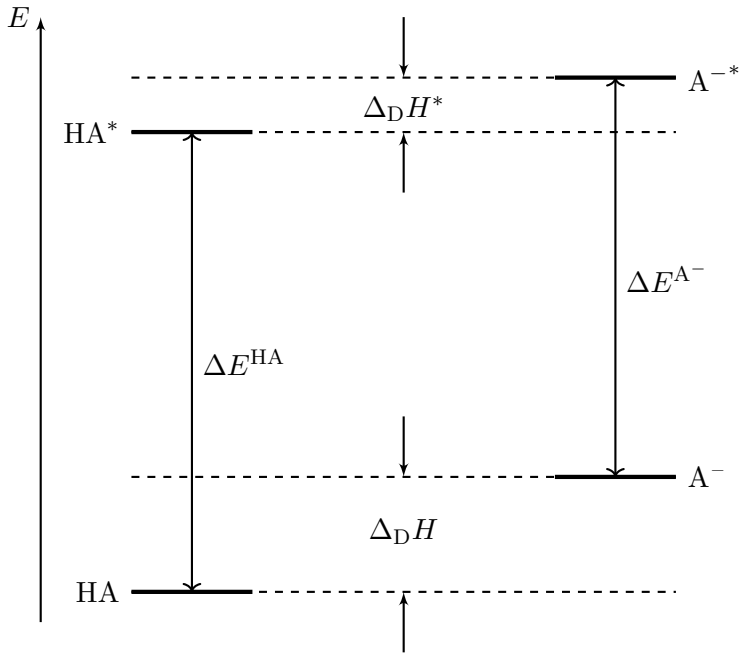

Figure 1.3: Förster cycle

The determination of the equilibrium constant for the reaction in the electronically excited state can be concluded from the energy level diagram depicted in Fig. 1.3 (Förster cycle):

From this diagram follows:

$$\Delta_{\mathrm{D}}H + \Delta E^{\mathrm{A}^-} = \Delta E^{\mathrm{HA}} + \Delta_{\mathrm{D}}H^*. \tag{1.3}$$

In this equation $\Delta_{\mathrm{D}}H$ and $\Delta_{\mathrm{D}}H^*$ denote the dissociation enthalpies in the ground and excited state, respectively. ΔE^{A^-} and ΔE^{HA} denote the energies for the electronic transitions at maximal absorbance of the base and the acid form, respectively. Assuming that the dissociation entropies of both states are the same, yields:

$$\ln \frac{K^*}{K} = \frac{\Delta_{\mathrm{D}}H - \Delta_{\mathrm{D}}H^*}{RT} = \frac{\Delta E^{\mathrm{HA}} - \Delta E^{\mathrm{A}^-}}{RT} = \frac{hc}{kT}\left(\tilde{\nu}_{\mathrm{HA}} - \tilde{\nu}_{\mathrm{A}^-}\right) \tag{1.4}$$

$$\mathrm{p}K^* = \mathrm{p}K - \frac{0,00625\ \mathrm{K\,m}}{T}\left(\tilde{\nu}_{\mathrm{HA}} - \tilde{\nu}_{\mathrm{A}^-}\right). \tag{1.5}$$

Equation (1.5) holds true for absorption as well as for emission. However, the results from both spectra only yield the same values in case where relaxation (geometry, solvatisation) is negligible in the excited state.

1.5 Experimental

Simultan-Spektralphotometer SPEKOL 1100 (online)

- switch on the device (rear)

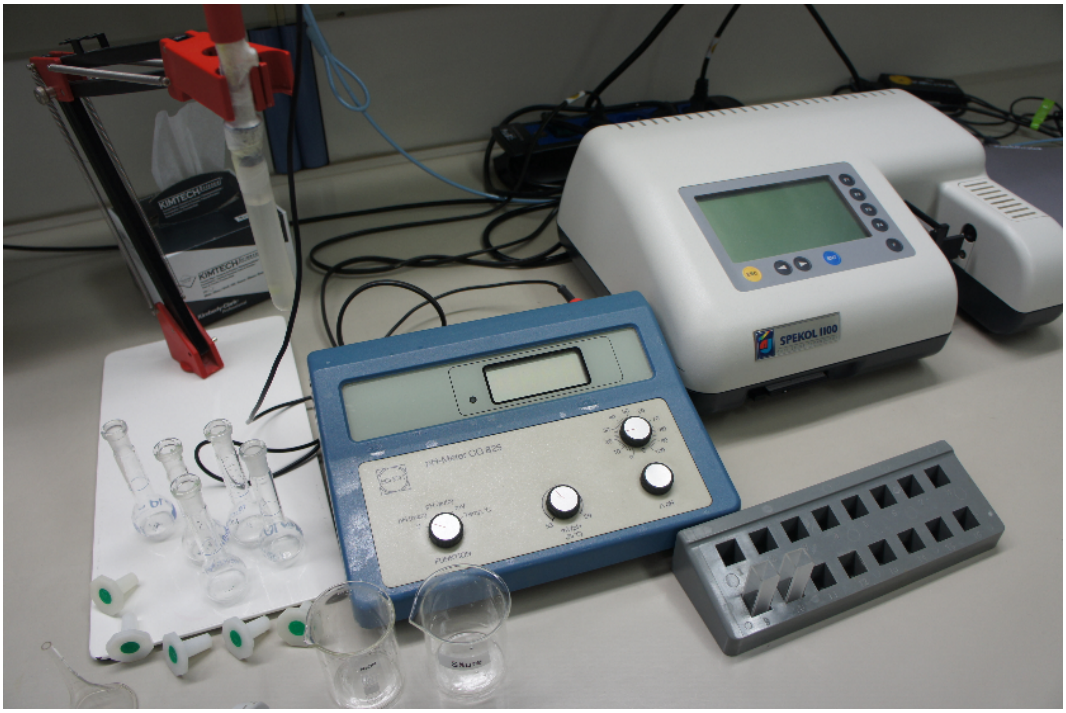

Figure 1.4: Experimental setup

- press any button after the self-test
- connect the device according to instructions at the workplace
- PC: Double-click on WinAspect icon
- Menu: "'Messung"' (measurement): device initialization, select parameter set ("default.par") or set parameter
- place cuvette with solvent (distilled water) in the sample cell holder
- reference measurement
- cuvette with solution → measurement
- save spectrum (menu "'Datei"' → Speichern unter (save as))
- Menu "'Datenbehandlung"' (data treatment) → Analyse, Digitalisieren (analysis, digitizing) → cursor: left mouse click: fixes cursor; right mouse click: opens the table with labeled wavelengths; clicking a wavelength and click on numerical value in "'Bearbeiten"'-Fenster (edit) allows changing the wavelength → please, record absorbance at the appropriate wavelength
- Menu "'Fenster"' (window): Neues Fenster (new window) → record new spectrum and continue as before

- Menu "'Fenster"' (window): Nebeneinander (side by side), displays all spectra next to each other; "'überlagern"' (overlay) enables display of overlayed spectra within one window. (the latter command is only applicable if at least two windows are opened (maximum 12 windows). The reading of the absorbance is in this display version not possible.-

1.5.1 Solutions

Please prepare the following solutions:

- Acid mixture (2,4 ml glacid acetic acid, 2,7 ml H_3PO_4 und 2,47 g H_3BO_3 in 1 l water)
- NaOH-solution ($0,1\ \frac{\text{mol}}{\text{l}}$)
- dye solution (solution with $A_{\max} \simeq 10$ in water or acetonitrile)

1.5.2 calibration of the pH-meter

1. Put function switch in position "'`pH (man)`"'
2. Set tuning knob "'° C"' on the temperature of the buffer solution
3. Dip electrode in the buffer solution with pH= 7.01 wait to reach equilibration, set the digital display on the value of the buffer solution using the tuning knob "'`ΔpH`"'.
4. Rinse electrode with distilled water and dip it in the second buffer solution (pH= 4.01). Adjust the digital display to the value of the second buffer solution using the tuning knob "'`mV/pH`"'.
5. Measurement of pH-value: keep function switch in position "'`pH (man)`"', dip electrode in measurement solution, wait to reach the equilibrium and read pH-value on the display.

1.5.3 Measurement procedure

1. Calibration of the pH-meter (see above)
2. Pipette 1 ml of the dye solution into a 10 ml measuring flask and fill it with the acid solution to 10 ml. Record the UV/Vis absorption spectrum in the spectral range 320–900 nm. Save the spectrum and measure afterwards the pH-value in the cuvette.
3. Repeat the procedure as described under 2, however, now using a buffer solution with pH-value 10-11 instead of the acid mixture (to prepare this, add a little bit of acid mixture to the 0.1 M NaOH solution and record the pH-value). Record the spectrum and save it and measure the pH-value of the final solution.
4. Repeat this procedure with different buffer solutions. Think of a suitable procedure to select the different pH-values in order to find the transition point. Perform a sufficient number of measurements with different pH-values around this transition point (at least 8 measurements with $\Delta\text{p}H \simeq 0,3$).

5. Select a suitable wavelength to follow the absorption of both, the base and the acid and enter those absorption values in a table following the example:

pH-value	absorbance at $\lambda_{\mathrm{HA}} = \ldots$ nm	absorbance at $\lambda_{\mathrm{A^-}} = \ldots$ nm

1.5.4 Data analysis / Evaluation

For a comfortable display and evaluation of the spectra you can export the spectra as ASCII file and import it for editing in a program like "Origin". Please, perform the following measurement and evaluation tasks and answer the questions:

1. Display all spectra in one graph using "ASPECT Plus" (select "'Spektren-Fenster"' (spectral window) and "'überlagern. . . "' (overlay), Origin or a comparable program. Please make sure to keep the right format of the exported files. Discuss the spectra cohort (maxima, isosbestic point, etc.).

2. Present a data table for the absorbances at the two chosen wavelengths λ_{HA} and $\lambda_{\mathrm{A^-}}$. Plot the absorbance as a function of pH-value ($A = f(\mathrm{p}H)$). What type of curve is obtained? How can you use this curve to determine the pK_{a}-value?

3. Derive equation (eq. (1.2)) from equation (eq. (1.1)) with the help of the molar fraction assuming from that the total concentration of dye ([HA] + [$\mathrm{A^-}$]) is constant for each measurement.

4. Calculate for each pH-value $\lg \frac{A_{\mathrm{HA}} - A_{\mathrm{M}}}{A_{\mathrm{M}} - A_{\mathrm{A^-}}}$ and display it in a table. Plot $\lg \frac{A_{\mathrm{HA}} - A_{\mathrm{M}}}{A_{\mathrm{M}} - A_{\mathrm{A^-}}} = f(\mathrm{p}H)$ and determine the pK_{a}-value by linear regression. Compare this value with the one obtained under point 2. If you observe significant deviations, decide which of the two values will be more reliable based on your measurement results. Give reasons for your opinion. Give the average of the two pKa values (or in case of major deviations, the selected value) as a result.

5. Explain why it is possible to mix the energies ($\Delta E^{\mathrm{A^-}}$, ΔE^{HA}) with the enthalpies ($\Delta_{\mathrm{D}}H$, $\Delta_{\mathrm{D}}H^*$) in equation (eq. (1.3)) resulting from the Förster cycle.

6. Determine the pK_{a}-value in the electronically excited state (photo dissociation) using equation (eq. (1.5)). Use the determined pK_{a}-value determined from your experiment (the average pK_{a}-value or the favored value). Compare the result with the one of the ground state reaction. Suppose, the acidic group is a phenolic OH-group. Which conclusions can you draw from the determined pK_{a}-values with regard to the electron distribution in the excited state compared to the ground state?

7. How would the method need to be adopted to determine the pK_{a}-value of a compound for which the absorbance of the acid $A_{\mathrm{A^-}}$ cannot be determined (e.g., due to decomposition, see above). Describe your suggestion in short words.

References

[1] Heinz G. Becker. *Einführung in die Photochemie: Mit 43 Tabellen*. Dt. Verl. d. Wiss, Berlin, 3., bearb. aufl. edition, 1991.

[2] Heinz-Helmut Perkampus. *UV-VIS Spectroscopy and Its Applications*. Springer Lab Manuals. Springer, Berlin, Heidelberg, 1992. URL: http://dx.doi.org/10.1007/978-3-642-77477-5.

[3] Werner Schmidt. *Optische Spektroskopie*. Wiley, Hoboken, 2nd ed. edition, 2014. URL: http://gbv.eblib.com/patron/FullRecord.aspx?p=1767446.

2 Vibronic Spectra and Fluorescence Spectroscopy

2.1 Aim of experiment

- Record UV/Vis spectra with vibrational progression
- Calculate molecular parameters from the spectroscopic data

A simple fluorescence apparatus is used to demonstrate:

- the measuring principle of a fluorescence spectrometer and the measurement of fluorescence and fluorescence excitation spectra,
- the relationship between absorption spectrum, fluorescence excitation spectrum and fluorescence spectrum
- the sensitivity of the method.

2.2 Preparations

1. Before starting the experiment, please make yourself familiar with the basics of vibronic spectroscopy, the vibronic spectrum of I_2 as well as inform yourself about the following terms:

   - Harmonic and anharmonic oscillator
   - Potential energy curve of two-atomic molecules in the electronic ground state and in the electronic excited states
   - Energy level diagram for a vibronic spectrum
   - Morse potential
   - Electronic transition moment
   - Franck-Condon principle and Franck-Condon factor
   - Vibrational progression
   - Boltzmann distribution of vibrational levels
   - Birge-Sponer diagramme

2. The dissociation energy of I_2 in the electronic ground state is $E_D = hc \cdot 12244\ \mathrm{cm}^{-1}$, the exponential factor $a = 1,87\ \text{Å}^{-1}$, the equilibrium distance $R_{eq} = 2,67\ \text{Å}$. Plot the resulting Morse potential. (For this, you can neglect in first approximation the difference between the measured dissociation energy and the quantitiy E_D in eq. (2.1).)

3. Use the instructions and the literature to find out about the basics and the essential of fluorescence spectroscopy.

2.3 Tasks

2.3.1 Vibronic Spectra

1. Record the absorption spectrum of I_2-vapor.
2. Determine the position of individual bands and give their respective wavelength.
3. Assign these bands with the help of the given table.
4. Calculate from the data of the gas spectra the following values:
   a) Eigenfrequency ν_0 of the electronic ground state and the corresponding wavenumber $\tilde{\nu}_0$,
   b) Eigenfrequency ν_0^* (and wavenumber $\tilde{\nu}_0^*$) of the electronically excited state,
   c) the dissociation energy of the electronic excited state E_D^*,
   d) the vibrational quantum number v^* of the electronically excited state at which dissociation occurs,
   e) the distance between the minima of the two potential energy curves E_P. Please, estimate the position of the 0-0-transitions with the help of the calculation transition energy.
5. Please, calculate the fraction of population in the vibrational states of the electronic ground state with $v = 0, 1$ and 2 at the temperature of measurement (Boltzmann distribution).
6. Please, plot the Morse potential of I_2 in the electronically excited state together with the ground state. The equilibrium distance in the excited state is $R^* = 2,97$ Å).

2.3.2 Fluorescence Spectra

1. Investigate the dependence of the fluorescence of a concentrated and a diluted solution of sodium fluorescein in $0,1$ n NaOH from the excitation wavelength. Describe and explain the observed effects.
2. Measure fluorescence spectra of the diluted solution of sodium fluorescein in $0,1$ n NaOH, excited at different excitation wavelengths. Check the validity of the rule of Kasha.
3. Measure fluorescence excitation spectra of the diluted sodium fluorescein solution at various emission wavelengths. What determines the shape of the fluorescence excitation spectrum? What information do fluorescence excitation spectra contain?
4. Determine the dependence of fluorescence intensity on absorbance or concentration of the sample. Estimate the limit of detection of the apparatus for sodium fluorescein.

2.4 Theoretic background

2.4.1 Vibronic Spectra

The potential energy curve of a two atomic atom can be in approximated with the Morse potential:

$$V(R) = E_{\mathrm{D}} \left(1 - \mathrm{e}^{-a(R-R_{\mathrm{eq}})}\right)^2 \tag{2.1}$$

mit

$$a = \sqrt{\frac{m_{\mathrm{red}}}{2E_{\mathrm{D}}}}\nu_0. \tag{2.2}$$

with:

E_{D} : difference between the minimum of potential energy and the energy that the resting atoms have after dissociation

R : bond distance

R_{eq} : bond distance in equilibrium

m_{red}: reduced mass

ν_0 : vibrational frequency of the harmonic oscillator

Solving the Schrödinger equation (with the expression from eq. (2.1) for the potential energy) gives for the energies E_v of an anharmonic oscillator in quantum states v the following expression:

$$E_v = h\nu_0 \left(v + \frac{1}{2}\right) - x_e h\nu_0 \left(v + \frac{1}{2}\right)^2 \tag{2.3}$$

$$= -x_e h\nu_0 v^2 + h\nu(1 - x_e)v + h\nu_0 \left(\frac{1}{2} - \frac{1}{4}x_e\right) \qquad v = 0, 1, 2, \ldots \tag{2.4}$$

mit

$$x_e = \frac{h\nu_0}{4E_{\mathrm{D}}} \tag{2.5}$$

Thus, a quadratic dependency of E_v as a function of v is obtained. A linear dependency of v is obtained if the first derivative if formed:

$$\frac{\mathrm{d}E_v}{\mathrm{d}v} = -2x_e h\nu_0 + h\nu_0(1 - x_e). \tag{2.6}$$

Substituting the differential quotient (i.e., the derivative) in approximation with a quotient of differences yields:

$$E_{v+1} - E_v \simeq (-2x_e h\nu_0 v + h\nu_0(1 - x_e)) \cdot ((v + 1) - v) \tag{2.7}$$

$$\simeq -2x_e h\nu_0 v + h\nu_0(1 - x_e), \tag{2.8}$$

$$\simeq -2x_e h\nu_0(v + 1) + h\nu_0 \tag{2.9}$$

Thus, a linear dependency from $v+1$ is obtained. From this dependency ν_0 and x_e can be determined and from those values E_D can be calculated. All what needs to be done is to find the energy differences, plot them against $v+1$ and perform a linear regression.

2.4.2 Fluorescence spectra

Types of luminescence

Fluorescence is the emission of light from atoms or molecules, in which they change from an high-energy electronic state to a low-energy electronic state (i. a. the ground state) of the same multiplicity. Radiative transitions between states of different multiplicity are called phosphorescence. Fluorescence and phosphorescence are combined as luminescence.

Luminescence phenomena can be classified according to the type of excitation energy supplied (see table 2.1). Luminescence investigations provide information about the properties of excited electronic states and can be used for qualitative and highly sensitive quantitative analysis.

Table 2.1: Types of luminescence

Luminescence type	type of excitation (example)
Photoluminescence	absorption of light
Chemiluminescence	chemical reaction
Radioluminescence	nuclear radiation
Electroluminescence	charge transport in a gas discharge (gas discharge tube) or semiconductor (light emitting diode)
Sonoluminescence	ultrasound
Thermoluminescence	thermally activated ion recombination

Fluorescence of organic compounds

With a large number of organic compounds, especially unsaturated hydrocarbons, an emission of light, the fluorescence, can be observed after irradiation in their absorption band. Fluorescence emission and radiationless deactivation processes of organic compounds can be described with the Jablonski diagram (Figure 2.1): At room temperature, most molecules are in the lowest vibrational state of the electronic ground state (S_0). Absorption of light leads in an electron-vibrational transition to an excited singlet state S_n with increased energy corresponding to that of the absorbed photon. Within 10^{-13} s to 10^{-11} s the molecule relaxes to the lowest excited electronic state S_1, which has a lifetime in the order of 10^{-9} s (nanoseconds). From this state the fluorescence takes place. Due to this fast relaxation, the fluorescence spectrum is independent of the excitation wavelength and bathochromically shifted from the long-wavelength absorption band. In many cases, the fluorescence spectrum appears mirror-inverted to the long-wave absorption band.

In competition to fluorescence, radiationless processes such as the return to the electronic ground state S_0 (IC = internal conversion), the transition to the triplet system (ISC =

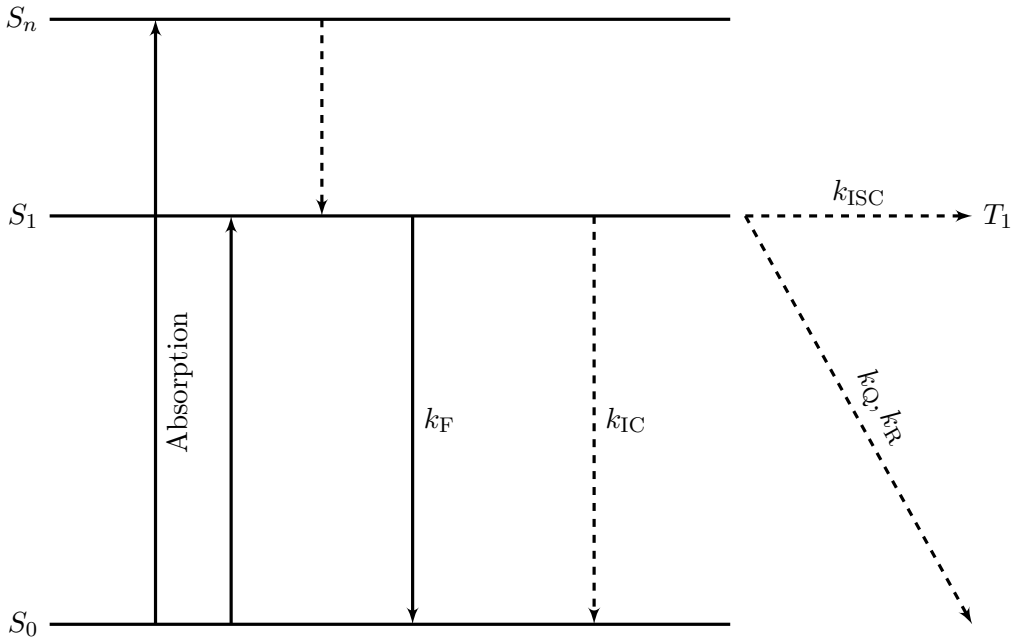

Figure 2.1: Jablonski-Diagramm

intersystem crossing), fluorescence quenching (Q) by electron, proton or energy transfer as well as photochemical reactions (R) take place. The fluorescence properties of molecules are thus determined both by the radiative process k_{F} and by the non-radiative processes $k_{\mathrm{IC}}, \ldots$, which in turn depend on the properties of the molecules in the S_1 state and their interaction with the environment (solvent, temperature, pH value, ...).

The mean residence time (lifetime) of molecules in the S_1 state is determined by the rate constants of the involved deactivation processes according to equation (2.10).

$$\tau = \frac{1}{k_{\mathrm{F}} + k_{\mathrm{IC}} + k_{\mathrm{ISC}} + \ldots} \tag{2.10}$$

The fluorescence quantum yield Φ_{F} gives the ratio of the number of molecules that emitted fluorescence to the number of excited molecules (= number of emitted photons N_{F} / number of absorbed photons N_A).

$$\Phi_{\mathrm{F}} = \frac{N_{\mathrm{F}}}{N_A} \tag{2.11}$$

It can be expressed with the rate constants according to the equation (2.12):

$$\Phi_{\mathrm{F}} = \frac{k_{\mathrm{F}}}{k_{\mathrm{F}} + k_{\mathrm{IC}} + k_{\mathrm{ISC}} + \ldots} \tag{2.12}$$

From the experimentally determined values for Φ_{F} and τ the rate constant of the fluorescence k_{F} and the sum of the rate constants of the radiationless processes $k_{\mathrm{NR}} = k_{\mathrm{IC}} + k_{\mathrm{ISC}} + k_{textQ}[Q] + k_{\mathrm{R}}$

can be calculated:

$$k_{\mathrm{F}} = \frac{\Phi_{\mathrm{F}}}{\tau} \tag{2.13}$$

$$k_{\mathrm{NR}} = \frac{1 - \Phi_{\mathrm{F}}}{\tau} \tag{2.14}$$

Table 2.2 contains the values for the fluorescence quantum yields Φ_{F}, lifetimes τ and rate constants k_{F} and k_{NR} of some known compounds.

Table 2.2: Fluoreszenzrelevante Werte einiger Verbindungen

Compound/Solvent	Φ_{F}	τ/ns	$k_{\mathrm{F}}/10^6\ \mathrm{s}^{-1}$	$k_{\mathrm{NR}}/10^6\ \mathrm{s}^{-1}$
Benzene/Cyclohexane	0,058	29	2	32,5
Naphthalene/Cyclohexane	0,19	96	2	8,4
Anthracene/Cyclohexane	0,30	4,9	61	140
p-Terphenyl/Cyclohexane	0,77	0,95	810	240
quinine sulfate /0, 1 n H_2SO_4	0,55	20	27	22,5
Fluoresceine/0, 1 n NaOH	0,95	3,6	260	14

Fluorescence quantities

After excitation of a fluorescent sample with light of intensity $I_0(\lambda')$ or photon number $N_0(\lambda')$ of wavelength λ' part of the absorbed energy is emitted again in form of radiation of intensity I_{F} or photon number N_{F} at wavelength λ containing information about the properties of the sample. The measured quantities of fluorescence spectroscopy are as follows:

Fluorescence intensity In the literature the intensity (as radiant power per area F, unit $^{\mathrm{Watt}}/_{\mathrm{cm}^2}$) and the relative intensity, as well as the photon number or the photon current density ($^{N}/_{(t\Delta F)}$, also called photon intensity) are used as measures for the strength of fluorescence. The relationship between intensity and photon number is given by Planck's formula $E = h\nu = {}^{hc}/_{\lambda}$.

$$I = N\frac{h \cdot c}{\lambda} \cdot \frac{1}{t} \cdot \frac{1}{F}. \tag{2.15}$$

At a given wavelength, intensity and photon number can easily be converted into each other. In this manual *intensity* is always used as *photon intensity*.

The fluorescence intensity is essentially determined by the fluorescence quantum yield and the absorption coefficient (at λ') of the fluorescent substance, its concentration and the excitation intensity. With (2.11) and the Beer-Lambert law $A = \lg\left({}^{I_0}/_{I}\right) = \lg\left({}^{N_0}/_{N}\right)$, the relationship for the concentration dependence of the number of fluorescence photons is

$$N_{\mathrm{F}} = \Phi_{\mathrm{F}} N_A = \Phi_{\mathrm{F}}\left(N_0 - N\right) = \Phi_{\mathrm{F}}\left(N_0 - N_0 \cdot 10^{-\varepsilon c d}\right) = \Phi_{\mathrm{F}} N_0\left(1 - \cdot 10^{-\varepsilon c d}\right) \tag{2.16}$$

whose curve follows an exponential function, i.e. is nonlinear in principle (see figure 2.2).

It turns out that for the quantitative determination of concentration of the analyte by means of fluorescence, linear calibration curves can only be expected for small absorbances ($A \leq 0.05$) *for even if Beer-Lambert law is valid.*

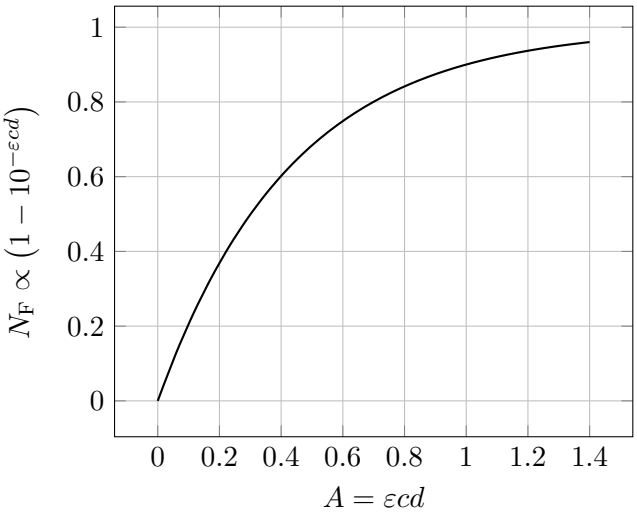

Figure 2.2: Dependence of the number of emitted photons of a sample on the absorbance

Fluorescence spectrum and fluorescence excitation spectra

Fluorescence spectra are represented by plotting the fluorescence intensity (or photon number) in relative units as a function of the emission wavelength (or wavenumber) (see Figure 2.3). The spectral shift in comparison to the absorption spectrum and the spectral shape are characteristic for the emitting system and its interaction with the environment (solvent).

Fluorescence excitation spectra are represented by applying the fluorescence intensity as a function of the excitation wavelength (or wavenumber) (see Figure 2.3). Spectrum shape and position correspond to the absorption spectrum if the sample is free of impurities (one-component system) and the absorbance is less than 0.05. The absorption spectrum is determined by the absorbance of the sample.

Fluorescence lifetime The determination of the fluorescence lifetime requires a technique to measure the time course of the fluorescence intensity after pulsed or modulated excitation. It provides an insight into the time scale of the processes in the excited state and determines the time course of the fluorescence intensity after pulsed excitation. In the simplest case (one-component system, no reactions or interactions in the S_1 state, avoidance of polarization effects), monoexponential decay of fluorescence occurs following:

$$I_{\mathrm{F}}(t) = I_{\mathrm{F}}(0)e^{-t/\tau}. \tag{2.17}$$

Fluorescence polarization The emitted fluorescence is more or less polarized. To determine the fluorescence polarization, the sample is excited with polarized light and the fluorescence is measured with a second polarizer (analyzer) in comparison to the excitation of parallel and perpendicular polarization direction. Fluorescence polarization contains information about rotational processes in the excited state or the microenvironment of the fluorescent molecule and enables the separation of transitions of different orientation in the absorption spectrum.

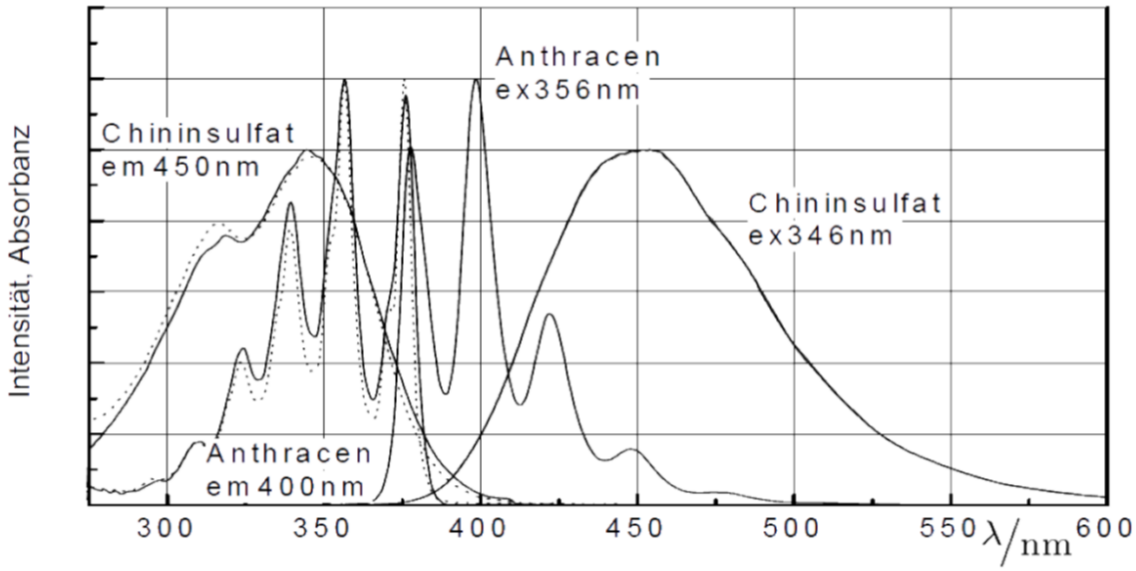

Figure 2.3: Normalized fluorescence spectra and fluorescence excitation spectra of dilute solutions (10^{-5} molar) of anthracene in cyclohexane and of quinine sulfate in 0.1 n H_2SO_4 and the corresponding absorption spectra (dotted)

Spectral fluorimeter setup

The parts of a spectral fluorimeter are shown schematically in Figure 2.4. It contains the following elements:

- a light source that emits in the UV-Vis range,
- monochromator for the selection of the excitation wavelength or for the analysis of the fluorescence light,
- the sample holder/chamber,
- optical elements for illuminating the sample and collecting the fluorescent light,

- photomultiplier,
- electronics and computing technology for signal processing, storage and display.

Mercury and xenon lamps are often used as *excitation light sources*. *mercury vapour lamps* emit a line spectrum with high intensities in the lines (at 254, 313, 365/66, 405, 436, 546, 577, 630 nm ...) over a low-intensity continuous background. *Xenon lamps* have a continuous emission spectrum whose intensity decreases in the UV range.

As detectors photomultipliers are used which, due to their high amplification (10^6 to 10^8), also allow the measurement of very low light intensities up to the detection of individual photons. Depending on the material of the photocathode, different types of photomultipliers differ in their absolute and spectral sensitivity. The amplification of the photomultiplier depends on the applied voltage.

Most fluorescence spectrometers measure fluorescence perpendicular to the direction of excitation (RA geometry). Fluorescent surfaces or samples of large absorbency (i.e. low penetration depth of the excitation light) are positioned in the sample space in such a way that the emitted fluorescence can be collected from the surface (frontal geometry).

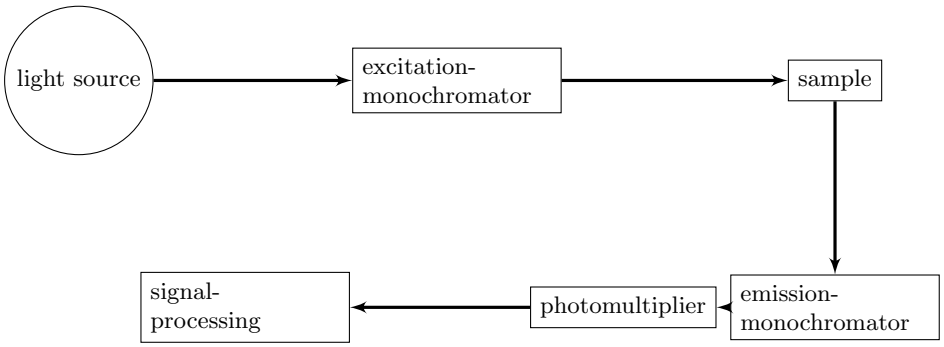

Figure 2.4: Setup of a spectral fluorimeter

Measurement of fluorescence spectra and fluorescence excitation spectra

Due to the limited aperture of the imaging optics or the emission monochromator, only a small part (0.1%) of the number of photons emitted by a sample into the entire solid angle can be measured with typical fluorescence spectrometers. Furthermore, the spectral sensitivity of the photomultiplier influences the shape of the measured spectrum and leads to deviations from the actual spectral shape. The measurement signal $M(\lambda, \lambda')$ of the apparatus, which is obtained by measuring the fluorescence $I_F(\lambda, \lambda')$ (λ': excitation wavelength, λ: emission wavelength) can be represented as follows:

$$M(\lambda, \lambda') = S(\lambda) \cdot I_F(\lambda, \lambda') \tag{2.18}$$

(2.19)

and with it (see (2.16))

$$I_{\mathrm{F}}(\lambda, \lambda') = \Phi_{\mathrm{F}} \cdot f(\lambda) \cdot I_{\mathrm{abs}}(\lambda') = \Phi_{\mathrm{F}} \cdot f(\lambda) \cdot I_0(\lambda') \cdot \left(1 - \cdot 10^{-A(\lambda')}\right) \tag{2.20}$$

In this means:

$S(\lambda)$: spectral sensitivity of the photomultiplier

Φ_{F} : fluorescence quantum yield

$f(\lambda)$: fluorescence spectrum (= corrected fluorescence spectrum = quantum spectrum)

$I_0(\lambda')$: spectral intensity distribution of excitation light

$I_{\mathrm{abs}}(\lambda')$: absorbed intensity

$A(\lambda')$: absorbance of sample at excitation wavelength

For the measured fluorescence spectrum (λ' and thus $I_{\mathrm{abs}}(\lambda') = \mathrm{const}$) applies:

$$M(\lambda) \approx f(\lambda) \cdot S(\lambda) \cdot \Phi_{\mathrm{F}}. \tag{2.21}$$

It is a spectrum that can be used for concentration determinations, fluorescence quenching studies, polarization, and more. For the determination of the fluorescence quantum yield or the quantitative comparison with literature data, the spectrum must be corrected with respect to the spectral sensitivity of the photomultiplier:

$$M_{\mathrm{korr}}(\lambda) \approx \frac{M(\lambda)}{S(\lambda)} \approx f(\lambda) \cdot \Phi_{\mathrm{F}}. \tag{2.22}$$

In addition, the measured intensity and the spectral shape can also be changed by absorption in the sample itself (filter effects). The choice of the measurement geometry (RA or frontal) has a considerable influence on the spectral shapes, especially the fluorescence excitation spectra, as well as on the concentration dependence of the fluorescence. Minimal falsifications are obtained when measuring on optically thin solutions ($A_{\max} \leq 0.05$). For small values of A the series development of equation (2.19) results in the relation

$$I_{\mathrm{abs}}(\lambda') = I_0(\lambda') \cdot \left(1 - \cdot 10^{-A(\lambda')}\right) \approx I_0(\lambda') \cdot \ln 10 \cdot A(\lambda') \tag{2.23}$$

which describes the linear relationship of the concentration dependence of the absorbed intensity and the absorbance and thus the fluorescence intensity (see Figure 2.2).

A *fluorescence excitation spectrum* is a spectrum in which the fluorescence intensity is measured at a constant emission wavelength as a function of the excitation wavelength. Using the relationships (2.18) and (2.19) (λ constant, λ' variable) gives:

$$M(\lambda') \approx I_0(\lambda') \cdot \left(1 - \cdot 10^{-A(\lambda')}\right) \tag{2.24}$$

For optically thin solutions ($A_{\max} \leq 0,05$) together with equation (2.23) yields:

$$M(\lambda') \approx \Phi_F \cdot f(\lambda) \cdot I_0(\lambda') \cdot A(\lambda'). \tag{2.25}$$

If the wavelength dependence of I_0 is taken into account, a corrected fluorescence excitation spectrum is obtained which corresponds in its form to the absorption spectrum of the fluorescent compound (cf. Figure 2.3).

$$\frac{M(\lambda')}{I_0(\lambda')} \approx \Phi_F \cdot f(\lambda) \cdot A(\lambda') \approx A(\lambda'). \tag{2.26}$$

The correspondence of the fluorescence excitation spectrum with the absorption spectrum is an important criterion for the purity of fluorescent substances.

Detection limit

The sensitivity of detection of the fluorescence method depends both on the apparatus used and on the substance or sample to be detected. Conditions for a high sensitivity of the apparatus are high excitation intensity, detector sensitivity and absence of scattered light of the monochromators. In addition to a high fluorescence quantum yield, the purity of the solvent is particularly important for the sample. Fluorescent compounds can be detected up to three orders of magnitude more sensitively by fluorescence spectroscopy than by absorption spectroscopy.

2.5 Experimental and data analysis

2.5.1 Vibronic spectra

Recording of the vapor phase absorption spectrum of I_2 with the absorption spectrometer Cary 5000.

A small amount of iodine is placed into a cuvette (path length 1 cm). This cuvette is placed into the spectrometer and allowed to equilibrate to 60 °C. At this temperature enough molecules have entered the gas phase so that the absorption can be easily recorded. Save the spectrum as `ASCII` file.

settings:

wavelength area (λ): 650...500 nm	**Data Interval:** 0,05 nm
Ave-Time: 0,4 s	**SBW (Splitbandwidth):** 0,25 nm

The recorded absorption spectrum of I_2 consists of several overlapping band systems (vibrational progression). In reference [4], detailed information can be found which are also depicted in table 2.3.

Table 2.3: Vibrational bands of I_2

v	v^*	λ/nm	v	v^*	λ/nm	v	v^*	λ/nm
0	27	541,2	1	18	571,6	2	13	595,7
0	28	539,0	1	19	568,6	2	14	592,0
0	29	536,9	1	20	565,6	2	15	588,5

Please, use the information from Table 2.3 to assign individual band systems, so that you obtain 3 sequences of transitions:

1. $v = 0 \rightarrow v^* = i, i+1, i+2, \ldots$
2. $v = 1 \rightarrow v^* = j, j+1, j+2, \ldots$
3. $v = 2 \rightarrow v^* = k, k+1, k+2, \ldots$

Calculate from these sequences the wavenumbers and vibrational energies. Plot these energies against v^* and determine from this ν_0^* and E_D^*. For data treatment, you can use the software "Origin".

Furthermore, please, plot the respective transition energies against v^*. How would such a representation look like for a harmonic oscillator? Use the above depiction to estimate the position of the 0-0-transition (non-linear regression, see eq. (2.3)). Please, discuss the result. Use the value for the 0-0-transition to estimate the distance between both potential energy curves of the task above.

2.5.2 Fluorescence Spectroscopy

Wavelength dependence

Prepare a diluted solution (absorbance approx. 0.05) from the existing solution (absorbance approx. 2). Insert the cuvettes with the sample solution into the sample holder of the sample chamber opened at the top and visually observe the effects occurring with both solutions when changing the excitation wavelength (excitation with tungsten lamp) in the range from 700 nm to 350 nm.

Note the following effects:

- intensity and color of fluorescence,
- spatial distribution of fluorescence in the sample,
- scattering effects,

- intensity of the excitation light before and after irradiation of the sample (observation on the screen with and without sample).

Fluorescence spectrum

Measure the fluorescence spectrum (excitation wavelength constant, emission wavelength variable) at (at least) two different excitation wavelengths (e.g. at the absorption maximum and at the short wavelength flank). The setting of optimal measuring conditions is achieved by

- adjusting the excitation monochromator to the desired excitation wavelength,
- setting the emission monochromator to a wavelength of 10 to 50 nm longer than the absorption maximum,
- selecting the measuring range on the recorder (e.g. 2) and the high voltage on the photomultiplier power supply (maximum 1.5 kV) so that a pointer deflection appears indicating fluorescent light (check by closing and opening the shutter on the excitation or emission monochromator),
- setting the emission monochromator to the fluorescence maximum,
- choosing measuring range and high voltage so that the pointer deflection lies in the last third of the scale.

The fluorescence spectrum is measured by determining the fluorescence intensity (in scale parts) as a function of the emission wavelength (measurement point by point, $\Delta\lambda = 5$ nm), starting at the excitation wavelength.

Fluorescence excitation spectrum

Please measure the fluorescence excitation spectra (emission wavelengths constant, excitation wavelength variable) at (at least) two different emission wavelengths (e.g. in the maximum of the fluorescence band and in the long-wavelength flank). The setting of the measurement conditions is analogous to that for the measurement of a fluorescence spectrum; instead of the emission monochromator, the excitation monochromator is now set to maximum intensity at a constant selected emission wavelength. The excitation spectrum is measured in the range of the absorption spectrum.

Concentration dependence and detection limit

Prepare about 5 solutions of the absorbance from 0.05 to 0.001 and measure the fluorescence intensity in the maximum of the fluorescence band with excitation in the absorption maximum. Plot the fluorescence intensity against the concentration of the solution ($c = {}^{A}/_{\varepsilon d}$) and discuss the curve. Estimate from the linear part of the curve and the signal of the solvent (blank value) which minimum concentration of sodium fluoresceine is still detectable with the given apparatus. Which factors determine the detection limit?

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3 Quantum chemical investigations on the inhibition process of cysteine proteases

3.1 Aim of experiment

- Introduction to modern computational quantum chemical methods and program package *Gaussian/GaussView*
- Calculation of partial charges and charge distributions of atoms inside a molecule
- Simulation of vibrational spectroscopy, i.e. IR and Raman

3.2 Preparations

Before starting the experiment, please make yourself familiar with the BASICS! of the following topics and terms:

- Schrödinger equation and Hamilton operator
- fundamentals of density functional theory (DFT)
- concept of local charges in molecules (i.e. electronegativity, inductive effects etc.)
- fundamentals of IR and Raman spectroscopy

3.3 Motivation

Proteases are a class of enzymes very often found in nature. Proteases selectively catalyze the hydrolysis of peptide bonds and are involved in numerous important physiological processes including digestion, wound healing, cell growth, cell signaling as well as immune responses. A dysregulation can lead to a wide range of diseases including emphysema, stroke, cancer, inflammation, viral infections and Alzheimer's. Protease inhibitors thus have considerable potential utility for therapeutic intervention.[1]

Epoxide based peptide inhibitors are known to selectively and irreversibly inhibit one class of protease enzymes, the cysteine protease. Here, the inhibition of the enzyme is realized by a nucleophilic attack of the thiol group (-SH) of a specific amino acid, cysteine, located in the enzyme's active center on one of the epoxide carbons of the inhibitor. The following ring

opening of the epoxide leads to a very stable compound, which thus deactivates the original function of the enzyme (Figure 3.1).

The focus of this exercise shall be the investigation of the regio-selectivity of the nucleophilic attack on a substituted epoxide by means of modern quantum chemical methods, whether this selectivity can be controlled by pH (protonation), and the identification of adducts depending on the attack site by means of computer-aided vibrational spectroscopy.

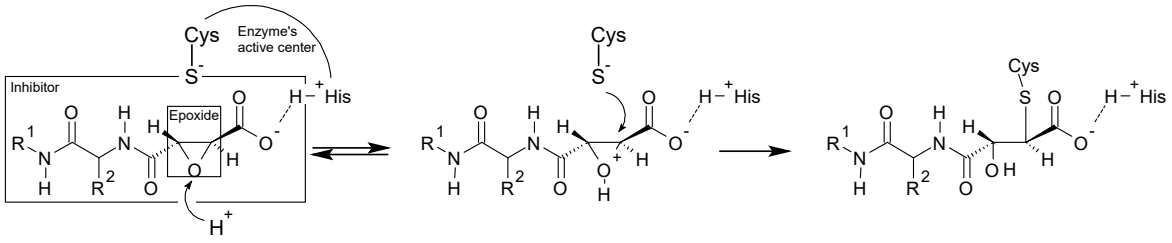


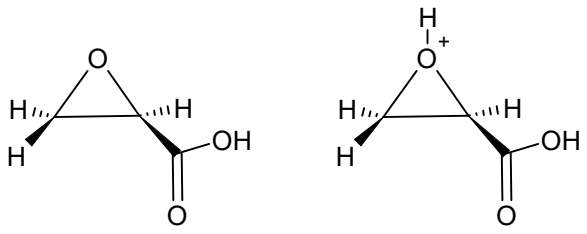
Figure 3.1: Proposed mechanism of inhibition of cysteine proteases by epoxysuccinates; adapted from [1]

3.4 Tasks

3.4.1 Partial charges, electron density and electrostatic potential of a substituted epoxide

Level of Theory: B3LYP / 6-31G(d)

In order to substantially reduce the computational efforts, we will crop the epoxysuccinate depicted in Figure 3.1 to a small substituted epoxide model system shown below. On that model, we now evaluate the possibility for a nucleophilic attack on each carbon atom of the epoxide. Which site is preferable? Do you expect the attack to be favorable in a neutral or acidic environment? Justify your decision! (*Hint:* A nucleophilic attack of a (partially) negatively charged particle preferably happens on the site with the most positive partial charge.)



Create the initial geometries shown above (epoxide substituted with a carboxyl group in neutral and protonated form) using *GaussView*, optimize these geometries and calculate the map of the electrostatic potential (ESP) on the surface of the total electron density using an

applicable iso-value. Visualize the EPSs together with the partial charges of each atom.

Setup and running the job

- build the carboxyl subsituted epoxide, starting from ethane and using the available tools in *GaussView*, e.g. "element fragment", "R-group fragment", "modify bond, angle, dihedral" and "add/delete valence" buttons
- once done building the molecule, open the calculation setup menu; a new window with several tabs will appear
- choose Job type: Optimization
- choose Method: ground state, DFT, B3LYP, basis set = 6-31G(d), charge = 0, spin = singlet
- in tab "General" choose "ignore symmetry" and "additional print"
- click on "submit" and save your input file under *<user>/Dokumente/*; the calculation will run in a separate window

  Hint: The geometry optimization procedure will look for a "nearby" **local** energy minimum point on the full 3N-6 dimensional potential ground-state energy surface of the molecule. The localization of such a point thus heavily depends on the input geometry you provide. Can you think of different stable isomers of our model system, e.g. depending on the relative orientation of the carboxyl group to the epoxide oxygen (e.g. by changing the dihedral angle O^{ep}-C-C=O)?
- repeat the above procedure for the protonated epoxide; carefully check the charge in the calculation setup as the protonated epoxide now is positively charged!

Analyzing and visualizing the results

- after the successful optimization open the resulting **.chk** file in *GaussView*
- visualizing the electron density:

  open the "Results" menu → Surfaces/Contours → in Cube Action choose New Cubes → type = total density → in Surface Action choose New Surface; (try different iso-values; for each iso-value you have to create a new surface; hide previous surfaces to see the effect)
- visualizing the ESP on the electron density:

  hide the density surface → in Surface Action choose New Mapped Surface → type = ESP; (repeat for different iso-values of the density)
- you can change the map to a transparent one in the "View" menu → Display Format → Surface tab → format transparent (uncheck "fade")
- showing charge distributions:

  open the "Results" menu → Charge Distribution → type = Mulliken; check Show Numbers

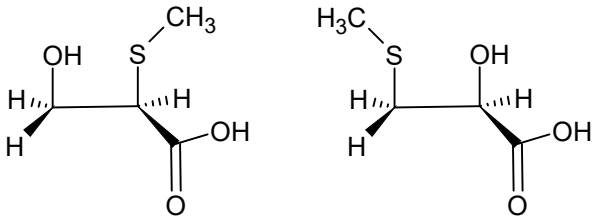
- you can save an image from the "File" menu → Save Image File

3.4.2 IR and Raman spectra for $H_3C\text{-}S^-$ addition products

Level of Theory: B3LYP / 6-31G(d)

We will now look at the adducts after the nucleophilic attack of a cysteine on the epoxide (cf. Figure 3.1). Again, in order to reduce calculation time, we use the model epoxide from **A** and we imitate the deprotonated cysteine by $^-S\text{-}CH_3$. Based on our epoxide model above, two attack sites are possible, leading to the two adducts shown below. With the help of IR and Raman spectra, is it possible to identify and differentiate the two different adducts? Which vibrational modes would be ideal so-called marker bands for such a differentiation with IR and Raman?

(*Hint:* bands/peaks that show pronounced alterations (frequency and/or intensity) between the spectra of two similar molecules (i.e. isomers), are typically chosen as marker bands, as they represent identifiable fingerprints for each isomer)



Build and optimize the structures of the adducts shown above according the procedure in **A**. In order to retrieve IR and Raman spectra, perform a frequency calculation for the optimized structures. Fortunately, the two steps (Optimization and Frequency calculation) can be neatly done in one single job by using Job Type = "Opt+Freq" in the calculation setup. Here, also choose Compute Raman = "Yes" for the Raman intensities to be calculated!

Once finished, load the **.log** file. Look at the IR and Raman results via the "Results" menu → Vibrations. You can look at and export the corresponding spectra, as well as animate and thus identify each vibrational mode of the molecule corresponding to the peaks in the spectrum. Look for substantial differences in the spectra for each adduct to identify suitable marker bands/peaks, e.g. the C-S stretching vibration etc.

(*Hint:* Maybe it is beneficial to show the spectra for both adducts in one plot (individually for IR and Raman) ; needs exported data (right click on spectrum → Save Data) and an external program (e.g. Excel, Origin, gnuplot etc).)

References

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