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1. Introduction

Benzo[a]pyrene (B[a]P) is a known carcinogenic compound; when metabolized, it is changed into an epoxide, which can react with DNA; epoxides are hydrolyzed into monohydroxy-benzo[a]pyrene (OH-B[a]P).

Several experiments indicated that some of these B[a]P metabolites exhibit estrogenic activity. This property makes OH-B[a]Ps interesting compounds. One of the key points for understanding the induction of the estrogenic activity is to determine how the compounds interacts with the estrogen receptor. The most estrogenic active monohydroxy metabolites are 3- and 9OH-B[a]P (structures are shown in the appendix); this project is therefore focused on these two metabolites only.

Computational and toxicological studies of the 3- and 9OH-B[a]P/ER complex did not give conclusive results, as there are two possible orientations of the compound inside the ER pocket, which have theoretically the same probability to occur.

Thus, to determine the exact orientation of OH-B[a]Ps inside the ER, another approach is needed. The greatest problem is that it is difficult to determine which technique(s) can be used for this purpose. Fluorescence Line Narrowing Spectroscopy (FLNS) might be very useful.

2. Fluorescence Line Narrowing Spectroscopy

2.1. Broadening

In general, the bands of an absorption spectrum never are infinitely narrow, i.e. they are always broadened. This band broadening can be divided into two types: (a) the homogenous broadening and (b) the inhomogeneous broadening. As the inhomogeneous broadening is a result of the local environment, this broadening is not observed in the gas phase. The homogenous broadening is independent of the matrix of the molecule, and is therefore the same for all (chemically identical) molecules.

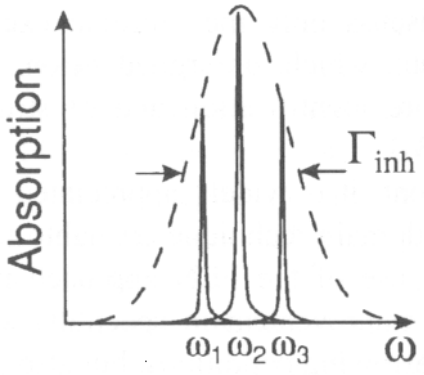

Figure 2.1.1
Band broadening in FLN

When identical molecules are present in an amorphous environment (i.e. a very disordered one), normally a large amount of band broadening is observed. However, this broadening mostly arises from the inhomogeneous broadening, i.e. it arises from the fact that molecules in a amorphous environment each have somewhat different energy levels. This results in a Gaussian distribution of individual bands, see figure 2.1.1. The solid lines have a Lorentzian shape due to homogenous band broadening. The numbers 1, 2 and 3 refer to three different analyte molecules having different environments, so slightly different energy levels are observed.

2.2. Principle of Fluorescence Line Narrowing Spectroscopy

FLNS has some unique properties. It offers the possibility to experimentally determine the vibrational levels of the excited state, as well as those of the ground state of a fluorescent molecule in its natural environment, something that no other technique offers. Also, FLNS give sharp peaks; differences in the spectra are therefore easy to detect.

FLNS is performed under cryogenic conditions (typically below 15K), so that their local environment does not change in time, at least not during the fluorescence process. The Gaussian distribution of energies gives the diagonal lines in figure 2.2.1. When a laser beam is used to excite the molecules (upwards pointing arrows on the left side in figure 2.2.1), only those molecules that have energy levels that correspond with the laser energy are excited (indicated by dotted horizontal line). These excited molecules will decay (without emitting radiation) until they reach the lowest stage of the S_1 (short downward pointing arrows in figure 2.2.1). After that, they will decay (by emitting a photon) to the ground state of the S_0 (long downward pointing arrows in figure 2.2.1). The result is that vibrational energies can be determined.

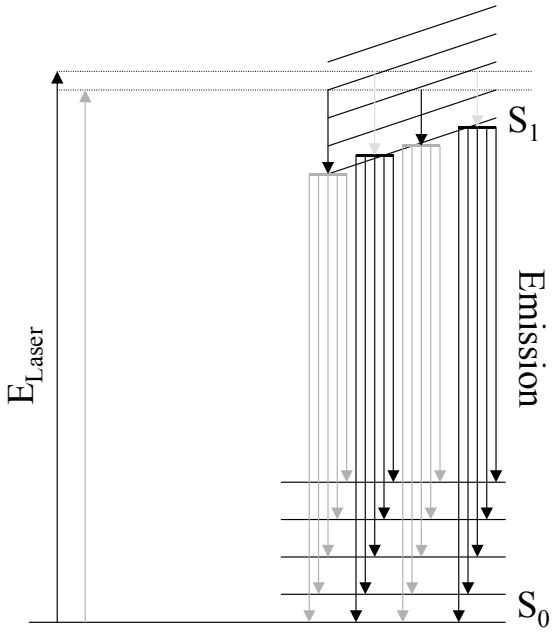

Figure 2.2.1
Excitation and emission in FLN

When the energy of the laser beam is changed, the energy of the emitted photon is changed by the same amount. When the energy of the emitted photon is subtracted from the energy of the laser beam, the resulting energy is that of the radiationless decay. However, not all vibrational levels can be determined at every excitation wavelength. This is because shorter excitation wavelengths (higher energies) result in other transitions in the S_1 state.
The measured radiationless transitions are only transitions between different vibrational levels of the excited state, and thus FLN can be used for determining these vibrational levels. The vibrational levels of the ground state can also be determined, but to do this, full emission has to be measured.

2.3 The advantage of FLN for determining environmental interactions

Why use FLN to determine the orientation of OH-B[a]Ps in the ER?

Figure 2.3.1 indicates the rigid matrix in which the analyte molecules are restrained. This figure makes clear that different molecules have different orientations inside the matrix and thus different interactions with the local environment. However, the orientations of the molecules present inside the matrix depend on the size and form of the matrix itself, too. It is clear that other matrix compositions lead to different possible orientations and different relative intensities of the fluorescence emissions. This is because the position and shape of the inhomogeneous band changes.

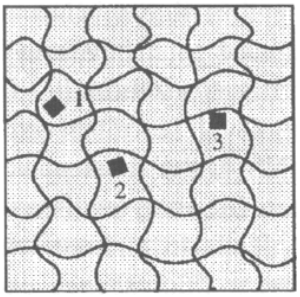

Figure 2.3.1
Matrix representation

Using this approach, information on the micro-environment of OH-B[a]Ps in the ER can be obtained. When the spectra obtained from OH-B[a]Ps in different solvents, without the ER, are compared with the spectra of the same OH-B[a]P fixed inside the ER, corresponding elements can be determined and related to interactions of the OH-B[a]P with the matrix. To give an example: if the spectra of the 9OH-B[a]P/ER-complex show energy levels that are also present when 9OH-B[a]P is restrained in a matrix of acetone or benzene only, but do not occur in the matrices of a (moderately) polar hydrogen bond-forming solvent such as ethanol or an extremely nonpolar solvent such as pentane or cyclohexane, the interactions of π-electrons will play an important role in fitting the 9OH-B[a]P in the ER.

To obtain fluorescence spectra of the compound, the samples will have to be excited. For this purpose, a laser is used, mainly due to its great monochromaticity.

3. Lasers

Here, an excimer laser pumped tunable dye laser setup was used.

3.1. Excimer Lasers

The lasing medium in excimer lasers is a diatomic species that forms weak bonds when in the excited state and is unstable in the ground state. *Excimer* refers to "*exci*ted state di*mers*". The laser used in this project was a XeCl excimer laser. The $XeCl^{\cdot}$ species is formed by an electrical discharge. The stable excited dimer lases at 308 nm and then decomposes in Xe and $Cl^{\cdot}$. The excimer laser produces 8 ns wide pulses of approximately 80 mJ each.

3.2. Dye Lasers

Dye Lasers are lasers that produce lasing photons due to fluorescence of dissolved organic molecules. For this, the molecules need to be pumped first. This can be done with a flash lamp, or another laser. For obtaining true laser light, a laser cavity is used. The wavelength of the dye laser can be tuned by using a grating. The range in which the light

can be tuned is very dependent on the type of dye used. The lifespan of the dye is variable; some dyes (e.g. stilbene) decompose rapidly. Dye lasers are very suitable for FLNS, because of their ease of tunability.

Concluding, FLNS, being a sensitive technique with high resolution, is a suitable technique for experimentally determining the vibrational energy levels of the excited state and for determining the interactions of fluorescent molecules with a certain environment.

Unfortunately, there are some disadvantages. The first is, that not all molecules are suitable for FLNS. One of the main demands is that the compound should be stable in the excited state. Another disadvantage is called hole-burning.

4. FLNS and hole-burning

Normally, when a set of molecules is frozen, they all have different orientations in the matrix. This results in a Gaussian shaped emission spectrum, as showed in figure 4.1. The Gaussian shape indicates the inhomogeneous broadening of the orientations of the compound in its environment.

When a laser is used to excite the sample molecules, only those orientations that have energies that correspond with the laser energy are affected. When these molecules relax, they can redistribute over the total set of possible orientations thus creating a hole in the probability curve. This is shown in figure 4.2. The dotted curve represents the set of molecules that are redistributed; the normal curve shows the approximate distribution of the total set of molecules after hole-burning has occurred. The main disadvantage is, that this affects the time used to measure data. For example, if a molecule shows easy hole-burning is at 410.00 nm, and this wavelength is used for aligning and optimizing, this wavelength can not be used for data acquiring due to the low number of molecules left in the orientation corresponding to 410.00 nm. The only way to be able to use this wavelength again is to let the molecules redistribute over the total number of orientations again, as showed in figure 4. Warming the sample again until it is a liquid, and then freezing it again can do this. However, due to the time-

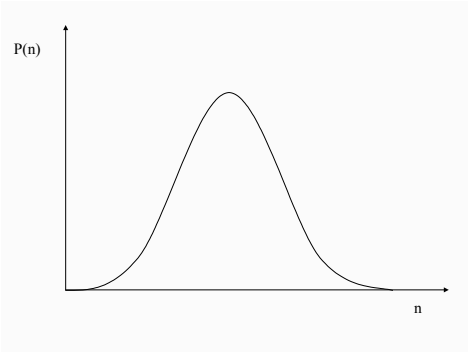

Figure 4.0.1
Normal distribution

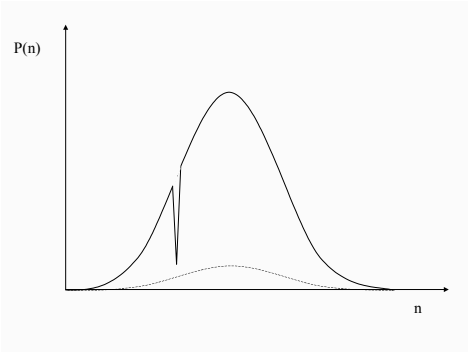

Figure 4.0.2
Distribution after hole-burning

consuming concept of warming and freezing, this is preferably avoided; this can be done by correctly using the excitation wavelengths. When the integer wavelengths (410.00, 411.00 etc) are used only for actual measuring, other wavelengths (410.25, 411.25 or 410.50, 411.50 etc) can be used for adjusting and testing the setup parameters. When hole-burning occurs at the non-integer wavelengths, it is no problem, because the integer ones, where there is no hole-burning yet, are used for actual measuring.

5. Experimental Setup

5.1. Chemicals and solutions

The OH-B[a]Ps used was from own stock; a $1.0 \cdot 10^{-3}$ M solution in ethanol (>99,8%, Sigma-Aldrich GmbH, Seelze, Germany) was made. This stock was diluted to $1.0 \cdot 10^{-5}$ M with (1) ethanol, (2) methylcyclohexane (MCH; 98%, J.T. Baker Chemicals NV, Deventer, The Netherlands), (3) acetone (>99.5%, Interchema, Rozenburg, The Netherlands) and (4) 50% v/v TRIS buffer (pH 7.3, from own stock) / 50% v/v glycerol (99% extra pure, Sigma-Aldrich GmbH, Seelze, Germany).

The dye solutions were all made in p-dioxane (>99.5%, Sigma-Aldrich GmbH, Seelze, Germany); concentrations 0.2 g/L (oscillating dye) and 0.07 g/L (amplifying dye). Please refer to the appendix for the structure of PBBO (the dye compound).

5.2. Experimental

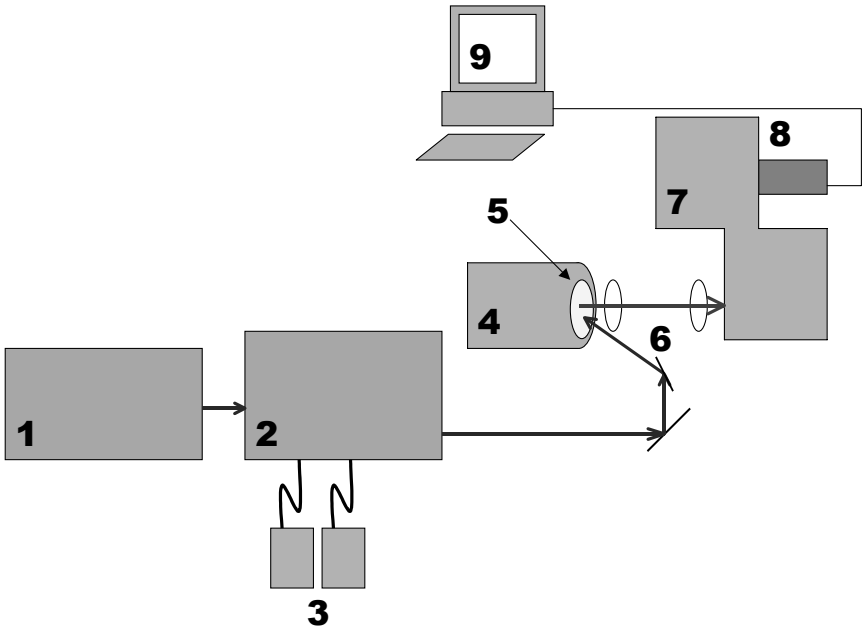

Figure 5.2.1
Schematic of experimental setup

Figure 5.2.1 shows the schematic of the experimental setup. Numbers are explained below.
The samples were introduced to a homemade sample holder, which was cooled by a Cryodyne model 21 closed-circuit helium refrigerator (4), obtained from CTI Cryogenics, Waltham, MA, USA. For exciting the sample, a LPD 3000 dye laser equipped with PBBO as dye (2) was used, pumped by a LPX 110i XeCl excimer laser (1) at 308 nm, both obtained from Lamda Physik, Göttingen, Germany. The PBBO dye was pumped through the dye laser using pumps (3) and gave tunability from approximately 396 to 415 nm. The sample was illuminated at an angle of 30° using prisms (6), and the emission was collected with a 3 cm F/1.2 quartz lens and focused on an 1877 0.6m Triple Monochromator from SPEX Industries, Edison, NJ, USA (7) using a 10 cm F/4 quartz lens (6). For detection, an Andor iStar DH-720 gateble CCD camera from Andor Technology, Belfast, Northern Ireland (8) was used; for data collection a personal computer equipped with software for the CCD camera was used (9).

The CCD camera was calibrated for wavelengths using an Argon calibration lamp (obtained from Oriel Optics Corporation, Stamford, CT, USA). For greatest resolution, the monochromator was used with the grating with 2400 lines/cm.

5.3. Several detection parameters

The CCD camera used for detection was gateble. This is necessary to be able to measure time-resolved. As the laser emission and the fluorescence emission are relatively close in wavelength, scattering may “pollute” the spectra. When gating is used, the camera can be set to start acquiring data when the laser scattering is over. This is possible, because the laser scattering occurs in another time scale than the fluorescence. Typical gate delay is 680 ns, while typical gate width is 50 ns.

The gating is triggered externally; this could be done because the laser offers its own trigger signal.

The camera is set to measure real-time when optimizing settings. However, when acquiring data thirty data accumulations were used to filter out the noise.

The gain of the camera (which sets the initial amplification of the signal) was typically set to 50.

6. Results and Discussion

To determine which interactions play an important role in the OH-B[a]P/ER complex, first, we must know what kind of interactions are of interest when measuring FLN spectra of mOH-B[a]Ps in different solvent matrices.

To make sure that different kinds of interactions could be distinguished, various solvents which all offer different kinds of interactions were chosen. As hydrogen bonding is a very typical interaction of a biological active compound with a receptor or enzyme, a

hydrogen bond-forming solvent is a first choice. Water was found unsuitable because of the formation of crystalline matrices when cooled; a glycerol/water mixture is amorphous. The receptor is in an aqueous solution, thus glycerol has to be added matrix. To be able to distinct peaks in the spectra coming from protein interaction and solvent interaction, a glycerol/water mixture was needed, too. The water part of this solvent was TRIS buffer at pH 7.3, to obtain conditions that are comparable to physiological ones.

Ethanol was chosen because of the known good FLN properties.

One solvent had to be chosen which could not form hydrogen bonds. For this purpose, methylcyclohexane is a good choice. MCH is known to give good amorphous matrices when frozen.

The last possible interaction that might be of interest is the interaction of the OH-B[a]Ps with π-electrons. For this, acetone is chosen, which has π-electrons. One great disadvantage of acetone is the formation of non-glassy matrices when cooled.

After acquiring sufficient data about the interaction of 9OH-B[a]P in several solvents, interaction in the ER was studied. This was done by incubating a 50 µL sample of ER, dissolved in ER buffer (phosphate buffer at pH 7.3 with 0.15M sodium chloride) in the presence of $5.0 \cdot 10^{-5}$ M of the 9OH-B[a]P. The incubation was performed at room temperature for approximately 5 minutes. Another possible incubation method would be incubating for 2 hours at approximately 277K. Other incubation conditions might give good results; however, it has to be taken into account that the human ER is only moderately stable at extreme conditions. 1-hour storage at 310K totally destroys the protein. Therefore, quick processing of the sample is needed. After incubation, 50 µL of glycerol was added. If conservation of the mixture is needed, storage below 200K is highly preferable due to the rapid decomposition of the ER.

6.1. 3OH-B[a]P

First measurements were carried out with $1.0 \cdot 10^{-5}$ M 3OH-B[a]P in ethanol, MCH, acetone and 50/50 v/v glycerol/buffer. After freezing to about 11K, the samples containing the 3OH-B[a]P were white grainy solids. Unfortunately, 3OH-B[a]P did not give good FLN spectra. It is thought that 3OH-B[a]P* has a very different electronic configuration than the ground state form, leading to band broadening. To rule out the possibility of faulty prepared solutions, measurements were carried out again with new solutions, but now containing a higher concentration. Because this did not give any improvement at all, it can be concluded that the problems are not the result of the concentration and that 3OH-B[a]P is not suitable for performing FLN.

6.2. 9OH-B[a]P

The 9OH-B[a]P metabolite did give better FLN spectra. First, FLN spectra were taken by exciting the samples at wavelengths starting at 397.00 nm further to 415.00 nm, 1.00 nm step size.

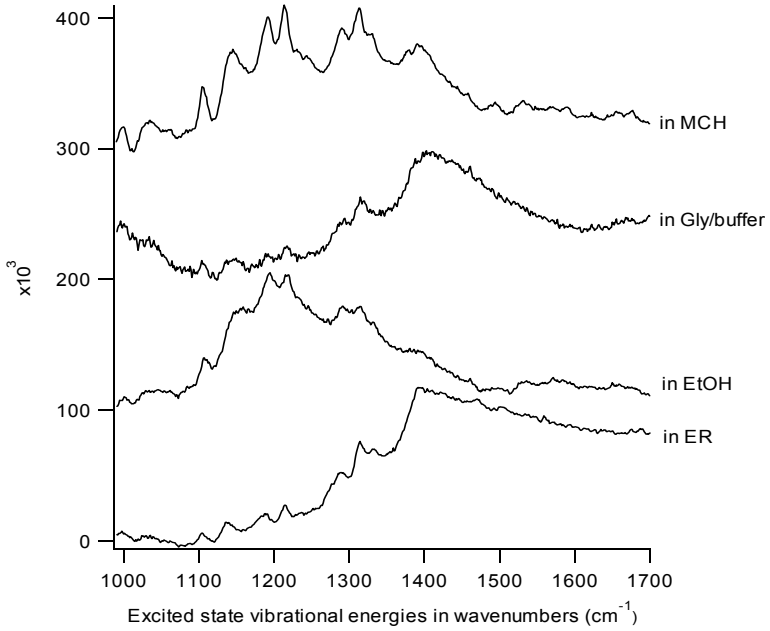

Figure 6.2.1
FLN spectra of 9OH-B[a]P in MCH, GlyBuf, EtOH and ER, respectively.
λ_{exc} = 401 nm

The wavelength in the thus acquired spectra were converted to wavenumbers, and corrected by subtracting the energy of the emission from the energy of the laser excitation. Figure 6.2.1 shows the raw spectra (acetone is not shown due to totally flat spectra); figure 6.2.2 shows the same data, with a subtracted background. This figure shows that subtracting the background from the data will give improvement when regarding the individual peaks, however, also makes it harder to distinguish between the different solvents.

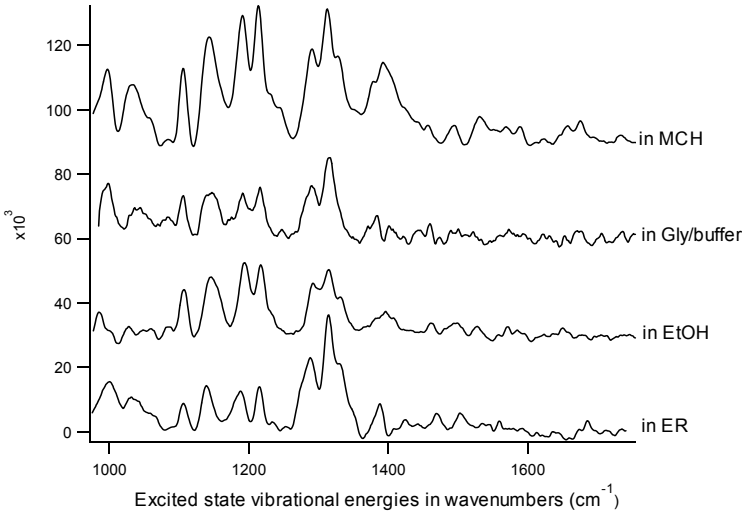

Figure 6.2.2
FLN spectra of 9OH-B[a]P in MCH, GlyBuf, EtOH and ER, respectively, after background subtraction;
λ_{exc} = 401 nm

Comparing the different FLN spectra of the various matrices, it proves to be difficult determining the differences. E.g., all solvents show approximately the same spectra when looking at wavenumbers 1250-1350. The main differences between the various spectra mainly lies in the intensity of the peaks; however, drawing conclusions from this data is doubtful.

The question arises how these data might be improved. For the ER, it might be useful to determine the yield of the incubation. As the spectrum of the 9OH-B[a]P/ER complex show great similarities with the spectrum of 9OH-B[a]P in GlyBuf, possibly incubation was unsuccessful, and 9OH-B[a]P in GlyBuf was measured instead of in ER. As the affinity of 9OH-B[a]P for the ER is much lower than of the natural occurring estrogens, the concentration ratio [ER] / [9OH-B[a]P] has to be much higher, compared with natural estrogen.

To improve the FLN results (e.g. narrower peaks, higher resolution), changing the conditions at which the FLN is performed is recommended. The greatest advantage can possibly be reached by achieving lower temperatures. The current data were all acquired at approximately 11K; lowering the temperature to, say, 4K might give significantly better results, due to smaller homogeneous broadening Also, even lower temperatures may result in less energy transfer from the xOH-B[a]P and the environment. Using cryogenic equipment of higher quality might assure better cooling.

7. Conclusion

FLNS is a technique that offers the possibility of sensitively and selectively determining the vibrational levels of the excited state of a fluorescent molecule. Because the broadening differs for several solvents, FLNS can be used for determining the orientation of a compound in a solvent and conclusively in a receptor, or an enzyme, too. Here, this property is used to determine the orientation of the estrogenic active monohydroxy benzo[a]pyrene derivatives 3- and 9OH-B[a]P in the human estrogen receptor.

Unfortunately, FLNS was not possible with 3OH-B[a]P; it is thought that this is because of changes in electronic configuration when exciting 3OH-B[a]P.
9OH-B[a]P gave good FLN, however, current results do not give enough unambiguous results. Implementing better incubation techniques and lowering the analyzing temperature might give better results.

8. References

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9. Acknowledgements

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10. Appendix

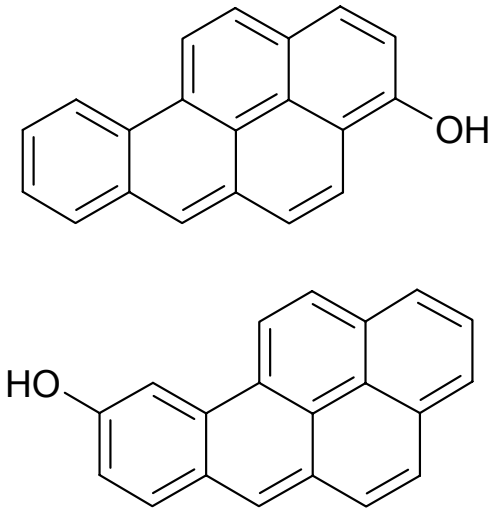

Figure 10.1
Structures of 3OH-B[a]P and 9OH-B[a]P, respectively

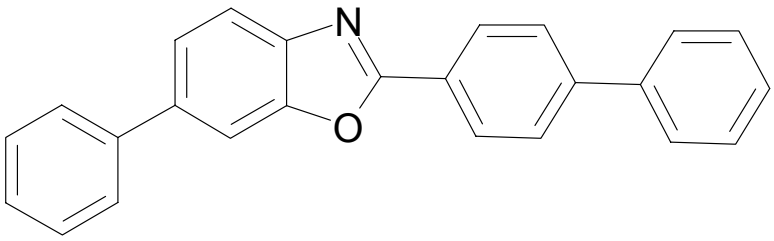

Figure 10.2
Structure of PBBO (Laser dye)