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Fluorescent-labeled ligands for the benzodiazepine receptor

Part 2: The choice of an optimal fluorescent-labeled ligand for benzodiazepine receptor assays

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In this article, the binding affinities of the fluorescent-labeled benzodiazepines described in Part 1 are compared to assess the influence of the labeling position and the choice of fluorophore on the binding affinity. This comparison was extended by taking into account the data of other fluorescent-labeled benzodiazepines in the literature. The differences in the binding affinities observed could partly be explained by structure-activity relationships (SAR). On the basis of this comparison, fluorescent-labeled desethylflumazenil (Ro15-3890, **19)** derivatives were selected as the most suitable labeled ligands in fluorescent receptor assays. A methyl-methoxycoumarin derivative (Mmc−O−CO−$(CH_2)_3$−Ro15-3890) **(20b)** had a K_i-value of 6.5 nM, and a 7-nitrobenz-2-oxa-1,3diazole derivative (NBD−NH−$(CH_2)_3$−Ro15-3890, **21**) had a K_i-value of 5.7 nM. In order to yield sufficient sensitivity in the final receptor assay, a suitable fluoresent labeled ligand should have a K_i < 10 nM. A further advantage of the above two ligands is that the benzodiazepine moiety has no receptor affinity of its own. Thus, if some hydrolysis of the labeled ligand were to occur, the resulting Ro15-3890 **(18)** would hardly affect the outcome of the assay. In the second part of this paper the prerequisites of the fluorophore are being examined. In this regard, **20b** is preferred, because the coumarin derivative has higher fluorescence intensities in aqueous media than the NBD-derivative. Therefore, **20b** was selected as a fluorescent-labeled ligand in the development of a non-radioactive receptor assay for benzodiazepines.

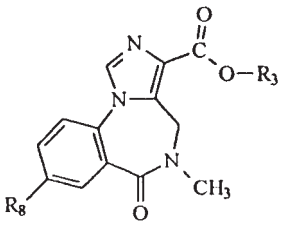
1. Introduction

In this paper, the binding affinities of fluorescent-labeled benzodiazepines are being evaluated in order to select an appropriate ligand for a benzodiazepine fluorescence receptor assay. This is being done in the light of structure activity relationships derived from the literature.

There are several criteria to be considered, such as the position of the label in the molecule, the type of fluorophore and the choice of the benzodiazepine for labeling. Besides the binding affinities of the ligands, the fluorescence characteristics of the latter also play an important role in the selection of the best ligand.

In our work, we used calf brains for the determination of the affinity constants of the different benzodiazepine derivatives. Yet, the inhibition constants of the fluorescent-labeled benzodiazepines described in the literature were sometimes determined with rat or calf brains. Figure 1 shows the correlation between calf [1] and rat [2] benzodiazepine receptors for the K_i-values of seven benzodiazepines. The species difference in the receptor binding of benzodiazepines appears to be small, which has also been observed in the comparison between receptors from human and rat brains [3]. Therefore, a comparison between our results with K_i-values determined with rat brain material seems warranted.

Table: Affinity of imidazobenzodiazepine-3-carboxylic acid derivatives for the benzodiazepine receptor [20]



Compd.	R_3	R_8	K_i (nM)
Ro 15-1788	CH_2CH_3	F	0.8
	CH_3	Cl	29.8
Ro 15-1310	CH_2CH_3	Cl	5.4
	$CH_2CH_2CH_3$	Cl	24.8
	$CH(CH_3)_2$	Cl	10.5
	cyclopropylmethyl	Cl	9.7
	$C(CH_3)_3$	Cl	4.0
	$CH(CH_2CH_3)_2$	Cl	26.9
	$CH_2C(CH_3)_3$	Cl	499.3
	$CH_2CH_2C(CH_3)_3$	Cl	184

2. Investigations, results and discussion

2.1. Binding affinities of 1,4-benzodiazepines

Table 1 lists the receptor affinity constants (K_i) of our newly synthesized fluorescent-labeled 1,4-benzodiazepines and of some ligands described in the literature. The basic

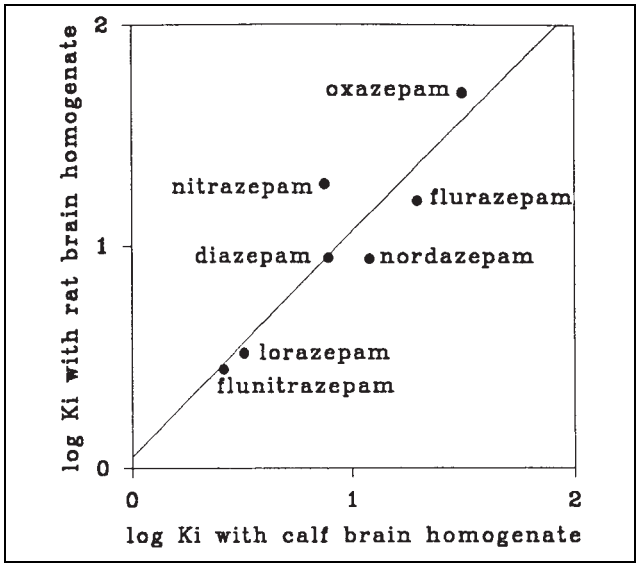


Fig. 1: Correlation between the log K_i values (in nM) of several benzodiazepines determined with calf brain homogenate (from ref. [1]) or with rat brain homogenate (from ref. [2]) (r = 0.90)

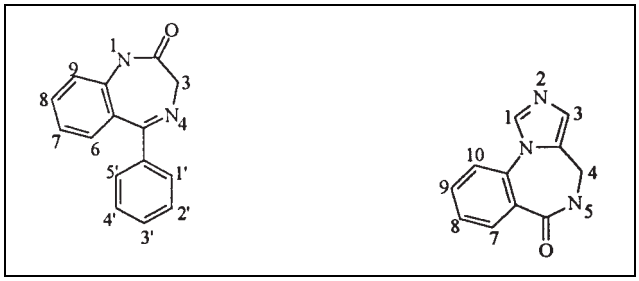


Fig. 2: Basic structures of 1,4-benzodiazepines and 1,2-annelated 1,4-benzodiazepines

structures of 1,4-benzodiazepines and 1,2-annelated 1,4-benzodiazepines are shown in Fig. 2. Detailed structures and numbers of the fluorescent-labeled benzodiazepines and their precursors can be found in part 1 of this article [4]. It should be observed that a suitable fluorescent-labeled ligand should have a $K_i < 10$ nM.

The 1,4-benzodiazepines **1** and **2** were labeled at the 3-position after coupling of succinic anhydride to the OH-groups, yielding **3** and **4**, respectively. The reactive carboxylgroup obtained in this way was then labeled with the fluorophore 4-bromomethyl-7-methoxycoumarin, which gave the fluorescent-labeled benzodiazepines **5** and **6**, respectively. The labeling in position 3 resulted in a 40–60 fold decrease in binding affinities towards the benzodiazepine receptor: For **1** from 16.7 nM to more than 1 μM and for **2** from 1.2 to 114 nM. A similar loss in affinity for labeling at the 3-position has also been described by others [5–7].

The effect of labeling at the 7-position was examined using **8** (Ro5-3072), a metabolite of **7**. This metabolite has less affinity towards the receptor, 470 nM as compared to 7.4 nM for nitrazepam itself. The 7-amino-group was labeled with dansyl chloride to form **9**. Yet, this labeling at the 7-position did not restore the binding affinity towards the benzodiazepine receptor. Actually, the binding affinity of >1 μM was even lower than of **8**. Haefely et al. [8] suggested that benzodiazepines need electronegative substituents at position 7, such as −Cl, −Br or $-NO_2$, in order to have suitable binding affinity. This has also been described by Sternbach et al. [6]. They also found that an increase in size of an alkyl substituent at position 7 caused lower binding and that substituents with a sulfone-group, which have strong electron-withdrawing properties, showed a decrease in affinity as well. These findings are in line with our observation that **9** has no affinity towards the benzodiazepine receptor, since labeling with dansyl chloride implies the introduction of a large substituent with a sulfone-group. The use of a fluorophore without a sulfone-group may limit this loss in binding affinity. Yet, since all fluorophores are relatively bulky substituents, high affinity towards the receptor is not expected after labeling at the 7-position. Therefore, we believe that labeling at either the 3- or the 7-position of 1,4-benzodiazepines will not result in a product with a sufficiently high affinity towards the benzodiazepine receptor, irrespective of the choice of the fluorophore.

Position 1 has also been studied by several researchers, e.g. in **11**, a metabolite of **10**, containing a primary amino-group [9–11]. Compound **11** itself has a relatively high affinity towards the benzodiazepine receptor ($K_i = 4.9$ nM), whereas the parent compound **10** has a K_i of 10.4 nM. Haefely et al. [8] described that relatively long side chains on the nitrogen 1-position induced only a moderate decrease in affinity. This seems to make **11** a suitable precursor for labeling. Compound **11** has also been used as ligand in affinity chromatography, by attaching it to agarose through a spacer chain [12]. Different fluorophores were coupled to the amino-group of **11**. Takeuchi and Rechnitz [9] used the coumarin 7-amino-4-methylcoumarin-3-acetic acid *N*-hydroxysuccinimide ester (NHS-AMCA) for labeling. The resulting product **17** had a K_i of 8.6 nM. Labeling **11** with 5-carboxylfluorescein N-hydroxysuccinimide ester, as described by McCabe et al. [10], resulted in **13** with a K_i of 63 nM. Velazquez et al. [11] used 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F), which resulted in **14** with a K_i-value of 79 nM. In our labeling experiments of **11** with various fluorophores, we found the following K_i-values: a similar K_i-value for **14**, namely 51 nM, a K_i-value of 67 nM for **16** and a K_i-value of 167 nM for **12**.

These results show that labeling of the 1-position of 1,4-benzodiazepines may be useful, depending on the choice of the fluorophore. Sternbach et al. [13] observed that precursors with a terminal dialkylamino-group on the side chain on the 1-position provided compounds with a higher affinity than compounds based on precursors with a terminal heterocyclic substituent. The lower affinity of the latter can be the result of steric hindrance of the heterocyclic substituents. This may be reduced by enlarging the distance between the amino-group and the substituents. Velazquez et al. [11] incorporated two spacers with different lengths between the parent benzodiazepine **11** and the fluorophore NBD. However, these spacers reduced the binding affinity, in that the incorporation of $-(CH_2)_2-CO-$ resulted in **15a** with a K_i of 132 nM and the incorporation of $-(CH_2)_5-CO-$ in **15b** with a K_i of 163 nM. This implies that the decrease in affinity by coupling a fluorophore to the benzodiazepine derivative **11** cannot simply be avoided by spacers.

2.2. Binding affinities of 1,2-annelated 1,4-benzodiazepines

Besides the 1,4-benzodiazepine derivative **11**, other classes, such as the 1,2-annelated benzodiazepines, have also been used for fluorescent labeling. The affinity constants of some fluorescent-labeled 1,2-annelated 1,4-benzodiazepines are reported in part 1 [4]. Takeuchi et al. successfully used **23**, a triazolobenzodiazepine, for labeling, either with biotin, resulting in **25** [14, 15] or with a europium chelating reagent, *N*-(*p*-isothiocyanato-benzyl)-diethylenetri-amine-*N*,*N*′,*N*″,*N*‴-tetraacetic acid [16, 17]. The latter label was measured by time resolved fluorescence. The affinity constants of these compounds were 0.31 and 1.2 nM, respectively, whereas the parent compound **23** had a K_i of 0.4 nM [18]. Compound **25** was used as ligand in a non-isotopic receptor assay for benzodiazepines. Velazquez et al. [11] labeled **23** with the fluorophore NBD-F. However, they observed an affinity constant for **24** of 85 nM, about 100-fold higher than the compounds of the group of Takeuchi.

Another 1,2-annelated 1,4-benzodiazepine for labeling is the imidazobenzodiazepine **19**, a metabolite of the benzodiazepine antagonist **18**. Compound **19** itself has no affinity towards the benzodiazepine receptor ($K_i > 1$ μM), whereas **18** has a K_i-value of 0.6 nM. However, **19** contains a reactive carboxyl group. Direct labeling of this carboxyl group with 4-bromomethyl-7-methoxycoumarin resulted in **20a** with an affinity of 121 nM, which is unacceptable for use as a sensitive ligand in the benzodiazepine fluorescence receptor assay. Compound **18** contains

a terminal ethyl chain at the carboxyl group. Therefore, a spacer of three carbon atoms was introduced between **19** and the fluorophore. With this spacer, we could increase the affinity to 6.5 nM, with Mmc- as fluorophore [4]. McCabe et al. [10] and Havunjian et al. [19] also labeled desethylflumazenil via a spacer. The former used a different spacer than we did, with a terminal amino-group instead of our carboxyl-group, but the resulting alkyl chain was of the same length, namely three C-atoms. Havunjian et al. used NBD as fluorophore, resulting in **21**, and observed a K_i of 5.7 nM. However, McCabe et al. used fluorescein, resulting in **22**, and their ligand had a 10-fold lower affinity (K_i was 63 nM). This shows that the choice of the fluorophore can have a marked but rather unpredictable effect on the binding affinity.

Several structure-activity relationship studies have been performed to assess which positions of the imidazobenzodiazepines are involved in the binding to the benzodiazepine receptor. The Table shows the general structure and the affinity constants of some 3-substituted imidazobenzodiazepines [20]. These studies were done with a chlorine atom at the 8-position, and although **18** possesses a fluorine atom at this position to enhance the affinity, the influence of the substituents can still be examined. The length of the alkyl chain as well as the branching appear to play a role, in that the length has a negative effect on the affinity and branching a positive one. According to Fryer [21], the interaction of imidazobenzodiazepines also involves hydrogen bonding interactions between the imidazole nitrogen, π_1, as well the ester carbonyl oxygen, π_2, with the receptor site. Ananthan et al. [22] found that compounds possessing carbamate, acylamino, sulfonyl, oxazolinyl and benzoxazolyl functions have low affinities for the benzodiazepine receptors. This also indicates that favourable hydrogen bonding and steric interactions in regions surrounding the π_1 and π_2 proton-accepting sites are important for high affinity binding of the imidazobenzodiazepines. A model for the interaction of imidazobenzodiazepine-3-carboxylic esters was developed by Ananthan et al. [22] and is shown in Fig. 3.

The lack of affinity of **19** itself ($K_i > 1$ μM [4]) is probably due to the disturbed hydrogen bonding at the π_2 proton-accepting site of the carbonyl oxygen. Compound **20a** still has a low affinity for the benzodiazepine receptor (K_i = 121 nm), conceivably caused by steric hindrance.

This steric hindrance could be reduced by introducing an alkyl chain between the imidazobenzodiazepine moiety

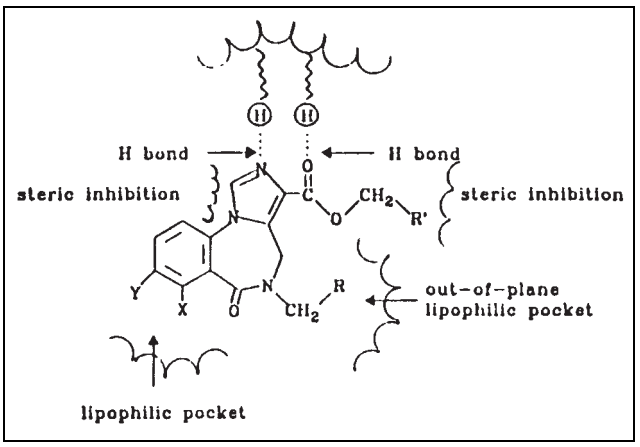


Fig. 3: Model for the interaction of imidazobenzodiazepine-3-carboxylic esters at the benzodiazepine receptor site, according to Ananthan et al. (reprinted with permission from ref. [22], copyright (1993) American Chemical Society)

19 and the fluorophore: **19** has been labeled in this way with three different fluorophores, the coumarin derivative Mmc **(20b)** (K_i = 6.5 nM [4]), fluorescein **(22)** (K_i = 63 nM [10]) and NBD **(21)** (K_i = 5.7 nM [19]), respectively. Two spacers were used; both had the same alkyl length, namely propyl, but the terminal groups were different. In our work, a terminal carboxyl-group was used for the labeling of Mmc, whereas McCabe et al. [10] and Havunjian et al. [19] used a terminal amino-group for the labeling with fluorescein and NBD, respectively.

It should be observed that the choice of the fluorophore may also affect the affinity. The fluorescein derivatives usually have a ten-fold lower affinity for the benzodiazepine receptor than the corresponding Mmc- and NBD-derivatives. This is probably caused by steric hindrance, due to the bulkiness of fluorescein.

Furthermore, our results with **20b** and **21** indicate that benzodiazepine compounds without affinity for the receptor can be successfully used as precursors for labeling and that it is not necessary to start with a precursor with a high affinity. In fact, the use of a precursor without affinity for the benzodiazepine receptor even represents a distinct advantage: In the event that hydrolysis occurs of the fluorescent labeled **19** derivatives, the resulting **19** has no affinity for the benzodiazepine receptor. However, an eventual hydrolysis of fluorescent-labeled **11** derivatives, such as **17**, will result in a compound with an even higher affinity for the benzodiazepine receptor than the labeled derivatives, thus causing substantial interference in the receptor assay. Therefore, we strongly prefer **19** as the benzodiazepine precursor for the synthesis of fluorescent-labeled ligands.

2.3. Fluorescence characteristics

A wide variety of fluorophores is available for labeling of the benzodiazepines, particularly for benzodiazepines containing a reactive amino-group. The selection of the most suitable fluorophore is not only dependent on the affinity of the fluorescent-labeled ligand for the benzodiazepine receptor. First, the fluorescent-labeled ligand must be stable for longer periods of time under various conditions. The fluorophore *o*-phthaladehyde (OPA), reactive to primary amines, is often used as post-column reagent in chromatographic assays [23, 24], but the resulting coupling products are unstable. Yet, the fluorophores used in part 1 [4], NBD-F, dansyl chloride, fluorescein, Bodipy FL and the coumarines, all yield stable products. Second, the fluorescence properties of the labeled ligand should allow sensitive and selective detection. The excitation wavelength should preferably exceed 500 nm to minimize autofluorescence of matrix components, such as proteins, but suitable substances are difficult to find. Fluorescein (λ_{ex} = 492 nm), rhodamine B (λ_{ex} = 550 nm) and Bodipy FL (λ_{ex} = 505 nm) fulfil this requirement, but the molecular sizes of these fluorophores are also larger. This makes them less suitable as labels in fluorescent receptor assays, since the bulkiness of these fluorophores may result in lower affinities for the benzodiazepine receptor due to steric hindrance.

Phycobiliproteins are highly soluble fluorescent proteins which are excitated at higher wavelengths (λ_{ex} ranging from 545 to 650 nm) [25]. These conjugates belong to the most sensitive fluorescent probes available and B- and R-phycoerythrin have been used successfully in immunoassays [26, 27]. However, since they have a molecular

weight of 240,000, they are not suitable for the direct labeling of ligands in fluorescent receptor assays.

As for other fluorophores, NBD-derivatives are excited at relatively high wavelengths ($\lambda_{ex} = 468$ nm), as compared to the coumarins (Mmc: $\lambda_{ex} = 315$ nm, AMCA: $\lambda_{ex} = 354$ nm) and dansyl chloride ($\lambda_{ex} = 340$ nm) derivatives.

Another requirement is that the fluorophore must have a high fluorescence intensity in aqueous media, since the sensitivity of the fluorescent receptor assay is determined by the possibility to quantitate either the bound or the free fraction of the fluorescent-labeled ligand, depending on the method. In order to assess the impact of detectability of the fluorescent ligand, a comparison has been made with a radioreceptor assay for benzodiazepines with radioactive-labeled flunitrazepam. [^{3}H]flunitrazepam had a specific activity of 3.2×10^{12} Bq/mmol and amounts of 0.7 fmol could be quantitated with an accuracy of 5% (with a counting time of 5 min). The detection limit of the fluorescent-labeled ligand is strongly dependent on the instrumentation used. Fluorescence detectors for chromatographic purposes are usually more sensitive than static detectors.

The sensitivity can be increased using laser-induced fluorescence (LIF), but a disadvantage of LIF is that a laser source can only emit light at a limited number of fixed wavelengths. The latter may not be compatible with the fluorophore of choice in fluorescent receptor assays.

Compound **16** has the highest fluorescence intensity [4], but it is not usable at the maximum excitation and emission wavelengths ($\lambda_{ex} = 505$ nm and $\lambda_{em} = 508$ nm), since the Stokes shift is only 3 nm. Rayleigh scattering precludes quantitation of Bodipy FL derivatives at these wavelengths. Alternatively, Bodipy FL derivatives can be quantitated at suboptimal wavelengths, yet at the expense of fluorescence intensity.

Fluorescein derivatives are also reported to have high fluorescence intensities in aqueous media at alkaline pH. However, the fluorescence intensities diminish with decreasing pH. Since we did not have **13** and **22** available, we were not able to measure their fluorescence intensities. However, since these fluorescent-labeled benzodiazepines had only moderate affinities for the benzodiazepine receptor (**13** had a K_i of 74 nM and **22** had a K_i of 63 nM), these compounds are not interesting as ligand in fluorescent receptor assays for benzodiazepines.

The Mmc-derivatives tested had fluorescences (relative to the fluorescence of 5 mM quinine sulphate in 1N H_2SO_4) ranging from 50 to 59 in Tris-HCl buffer, pH 7.4. These values are higher than those of **14** and the dansyl derivatives of **11** and **8**. Since we did not have access to the reference substance, we did not determine the relative fluorescence of **21**, but we assume that it is comparable to that of **14**. Ahnoff et al. [28] also described the low fluorescence intensity of NBD-hydroxyproline in water, whereas it had higher intensities in organic solvents. Although **21** has a higher excitation wavelength, we consider **20b** to be a more suitable fluorescent-ligand than **21**, since the fluorescence intensities of the Mmc-derivatives is three times higher than the fluorescence intensities of the NBD-derivatives.

2.4. Conclusions

It can be concluded that there are several limitations for a labeled ligand for use in the benzodiazepine receptor assay. To obtain affinity to the receptor, both the position of labeling, the molecular size (or bulkiness) of the fluorophore and the use of a spacer are important factors. In the end, a suitable fluorescent-labeled ligand for a benzodiazepine receptor assay should have a receptor affinity <10 nM.

Regarding the position of labeling, there are two (or three when **23** is also taken into account) benzodiazepine compounds which can be used as precursors. For the 1,4-benzodiazepines, only labeling at position 1 will not disturb the binding affinity. The precursor of choice is **11**, which has a primary amino group. The size of the fluorophore has an enormous influence on the binding affinity, due to steric hindrance. Therefore, small fluorophores, such as coumarins or NBD, are to be used. The other suitable precursor is the imidazobenzodiazepine **19**, which has a free carboxyl group. A prerequisite to obtain high affinity was not only the use of a small fluorophore, but also a spacer to optimize the distance between the benzodiazepine moiety and the fluorophore.

Besides the size of the fluorophore, the fluorescent characteristics of the latter are also important. To minimize autofluorescence of matrix components, the excitation wavelength should preferably exceed 500 nm. However, the fluorophores that fulfill this requirement also have a large molecular size, which makes them too bulky to yield sufficient binding affinity. Second, the fluorescence properties of the labeled ligand should allow sensitive detection in aqueous media, since the receptor assay can only be executed in these media. For this reason, the coumarin fluorophores have the advantage over NBD, since the former have a three times higher fluorescence intensity than NBD in Tris-HCl buffer (pH 7.4).

Another important aspect of the fluorescent-labeled ligand is that after an eventual hydrolysis of the labeled ligand, the resulting products have no affinity for the receptor.

From all these points, it can be concluded that the coumarin derivative **20b** is the fluorescent-labeled ligand of first choice: It has a high affinity for the benzodiazepine receptor, it has a relative high fluorescence intensity in aqueous media and the resulting compounds after an eventual hydrolysis have no affinity for the benzodiazepine receptor.

3. Experimental

3.1. Preparation of membrane-bound benzodiazepine receptors

Calf brains, obtained from the local slaughterhouse and stored at −80 °C after discarding the cerebella, were homogenized in six volumes (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i. Breisgau, Germany) fitted with a Teflon pestle and centrifuged for 10 min at $1000 \times g$ in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands) [29]. The supernatant was centrifuged for 60 min at $100.000 \times$ g. The resulting pellet (P_2) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged for 30 min at $100.000 \times$ g. This washing step was repeated once. All operations were performed at 4 °C. The washed P_2-pellet was resuspended in five volumes (w/v) of phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkerød, Denmark). The lyophilized P_2-pellet was stored at −20°C. For the receptor binding assays, the lyophilized P_2-pellet was resuspended in Tris-HCl buffer (pH 7.4; 50 mM) with a glass-teflon Potter-Elvehjem homogenizer (2.5 mg/ml).

3.2. Benzodiazepine receptor binding assay

For the binding assay, 50 µl of a [^{3}H]flunitrazepam solution (0.5 nM final concentration) in Tris-HCl buffer (pH 7.4; 50 mM) were mixed in duplicate with 50 µl of a Tris-HCl buffer, containing the fluorescent labeled benzodiazepines (200 nM − 6 pM final concentration) [29]. To this mixture, 400 µl of the receptor suspension were added, vortexed and incubated for 45 min at 4 °C. The incubation was ended by adding 4 ml of ice-cold Tris-HCl buffer and this mixture was filtered through pre-wetted GF/B fil-

ters. The tubes were rinsed twice with 4 ml of ice-cold buffer, which was also filtered. The filters were transferred into 6 ml polyethylene counting vials and dispersed in 3.5 ml Rialuma. The vials were shaken for 2 h and counted for 5 min in a Tri-Carb 4000 Packard scintillation counter (Canberra Packard, Groningen, The Netherlands).

3.3. Determination of the fluorescence characteristics of fluorescent labeled derivatives

The stock solutions of the fluorescent-labeled **19** derivatives were diluted one hundred fold with Tris-HCl buffer (50 mM; pH 7.4) and the fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorometer (Zürich, Switzerland).
Since not all spectra were recorded on the same day, a calibrator, quinine sulphate, was also determined with an excitation wavelength of 351 nm and an emission wavelength of 448 nm. Quinine sulphate was dried till constant weight and dissolved in 1 N sulphuric acid (5 mM) [4].

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