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Fluorescent-labeled ligands for the benzodiazepine receptor

Part 1: Synthesis and characterization of fluorescent-labeled benzodiazepines

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Because radioactive labeled ligands in receptor assays have several disadvantages, we synthesized a number of fluorescent-labeled benzodiazepines. Several fluorophores were attached at different positions of 1,4-benzodiazepine molecules in order to assess the impact of the fluorophores and their coupling position on the affinity for the benzodiazepine receptor. Besides the 1,4-benzodiazepines, the 1,2-annelated 1,4-benzodiazepines were also used for labeling. A metabolite of flumazenil (**18**), desethylflumazenil (Ro15-3890, **19**), was labeled with the fluorophore 4-bromomethyl-7-methoxycoumarin, with and without the incorporation of a spacer chain, yielding the methyl-methoxycoumarin (Mmc) derivatives Mmc-Ro15-3890 (**20a**) and Mmc−O−CO−$(CH_2)_3$−Ro15-3890 (**20b**), respectively. After the synthesis, the fluorescent-labeled benzodiazepines were purified by HPLC, using an analytical RP-C_{18} column. For the purification of **20b**, the chromatographic system was optimized, using multi-criteria decision making (MCDM) techniques. The binding affinities for the benzodiazepine receptor and the fluorescence characteristics were determined for the resulting products.

1. Introduction

Up to now, most of the receptor binding studies have been performed with radioactive labeled ligands. This allows measurements with high sensitivity and since the use of the radioisotopes [^{3}H] and [^{14}C] does not change the structures of the molecules, these ligands have similar binding affinities towards the receptor as the corresponding unlabeled analogues. However, the use of radioactivity has disadvantages, such as potential health hazards, high costs, generation of radioactive waste and the requirement for special laboratory facilities. Therefore, several groups have attempted to synthesize non-radioactive ligands. For the benzodiazepine receptor, fluorescent-labeled ligands [1–6] and biotin-labeled ligands [7–9] have been used as non-radioactive labels.

However, coupling of a fluorophore to a benzodiazepine molecule most often reduces the binding affinity towards the benzodiazepine receptor. To minimize this undesired effect, we coupled the fluorophore at different positions of 1,4-benzodiazepine and 1,2-annelated 1,4-benzodiazepine molecules to gather insight in the positions of the benzodiazepine molecule that are essential for the binding towards the benzodiazepine receptor and to learn which positions can be used for labeling. We also examined the impact of the type of the fluorescent label by comparing different fluorophores.

In this paper we describe the synthesis of several fluorescent-labeled 1,4-benzodiazepines, their purification, their fluorescence properties and their affinity towards the benzodiazepine receptor.

In addition to the 1,4-benzodiazepines, other benzodiazepines are also available for labeling, such as desethylflumazenil **(19)**, an imidazo-benzodiazepine [2, 4], and 1012-S **(23)**, a triazolo-benzodiazepine [3, 6, 7].

Compound **19** is a metabolite of the benzodiazepine antagonist flumazenil **(18)** and has no affinity for the benzodiazepine receptor. Using a ligand without affinity for the receptor has the advantage that in the case of hydrolysis of the labeled ligand the resulting products do not interfere with the assay. Hydrolysis is the main disadvantage of using didesethylflurazepam as a ligand for labeling, since didesethylflurazepam itself also has high affinity for the benzodiazepine receptor. In this paper we also describe the fluorescent labeling of desethylflumazenil. The fluorophore 4-bromo-methyl-7-methoxycoumarin was coupled directly to **19** and via a spacer.

After the synthesis of **20b**, the product containing the spacer was collected by precipitation with hexane. However, the precipitate was found to contain two products, the expected **20b** as well as **20a**. These two compounds were difficult to separate by RP-HPLC. Therefore, the chromatographic system for the purification was optimized chemometrically, using multi-criteria decision making (MCDM) techniques [10]. The effect of the mobile phase composition, consisting of three components (water, methanol and acetonitrile), on the chromatographic resolution (R_s) and capacity factors (k_1 and k_2) was studied. Our aim was to select a mobile phase composition giving adequate resolution R_s between the two compounds with the lowest capacity factor possible for the second eluting compound, **20b** (k_2).

The further procedure to purify the fluorescent-labeled **19** derivatives was identical to the purification procedure of the fluorescent-labeled 1,4-benzodiazepines.

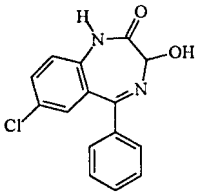
The choice of the optimal fluorescent-labeled ligand for benzodiazepine receptor assays, based on the most suitable K_1-value and fluorescence characteristics, is described in a second article [11].

2. Investigations, results and discussion

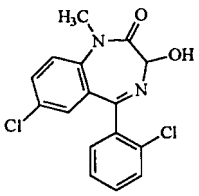
2.1. Synthesis of the fluorescent-labeled benzodiazepines

The fluorescent-labeled benzodiazepines were purified by reversed-phase HPLC with UV detection and their structures were verified by reversed phase HPLC-MS. The identities were not further confirmed by NMR, since the labels could only be coupled to one position of the benzodiazepine molecule. The verification of the molecular mass by MS was considered sufficient for identification. The m/z ratios of the fluorescent-labeled benzodiazepines are reported in Table 1.

For the synthesis of the different fluorescent-labeled substances, we selected the 3-hydroxybenzodiazepines **1** and **2**, the only active 1,4-benzodiazepines with a reactive



oxazepam (1)



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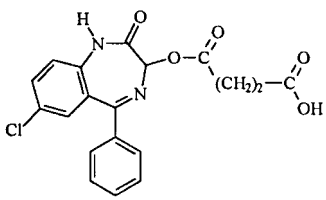
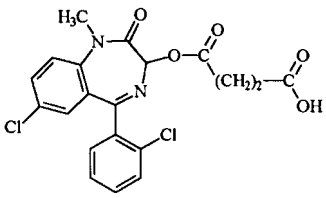


oxazepam hemisuccinate (3)



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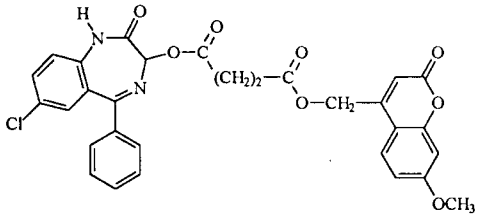
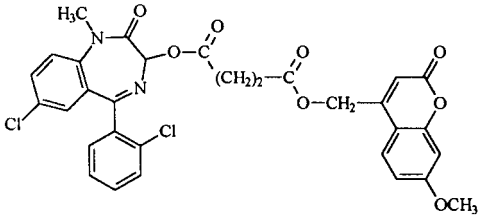


Mmc-O-CO-$(CH_2)_2$-CO-oxazepam (5)



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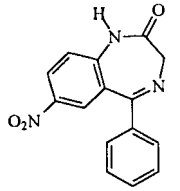
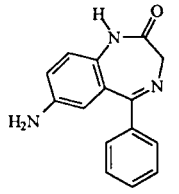
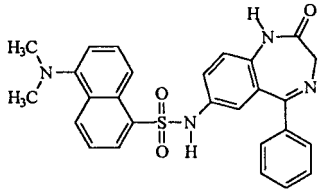


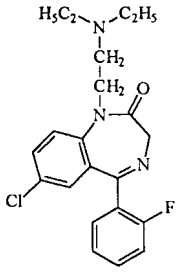
nitrazepam (7)



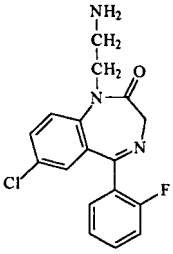
Ro5-3072 (7-aminonitrazepam) (8)



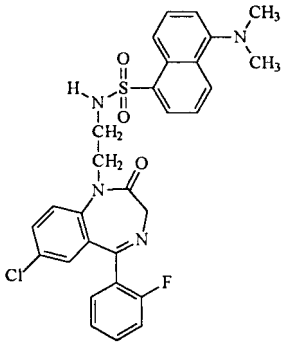
dansyl-Ro5-3072 (9)



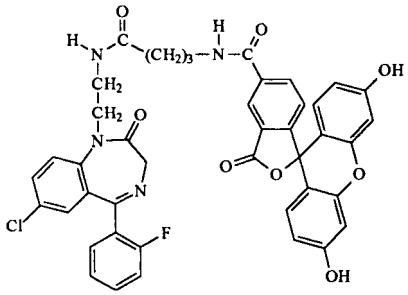
flurazepam (10)



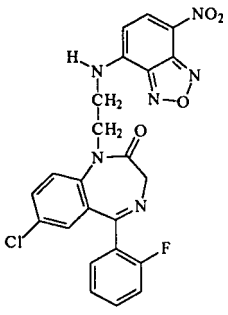
Ro-7-1986 (didesethylflurazepam) (11)



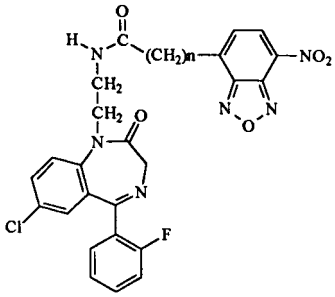
dansyl-Ro7-1986 (12)



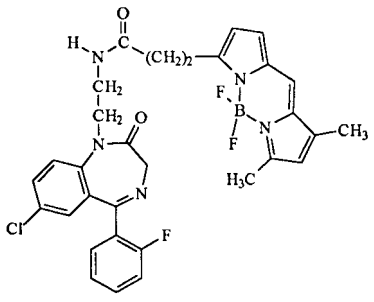
fluorescein-Ro7-1986 (13)



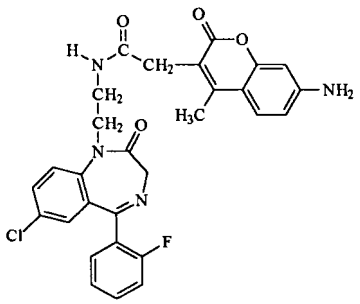
NBD-Ro7-1986 (14)



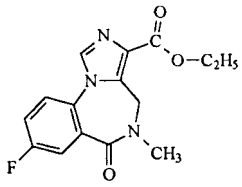
NBD-$(CH_2)_n$-CO-Ro7-1986 **(15a)**: n=2
(15b): n=5



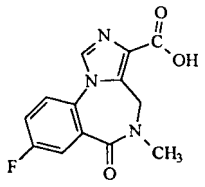
Bodipy®FL-Ro7-1986 (16)



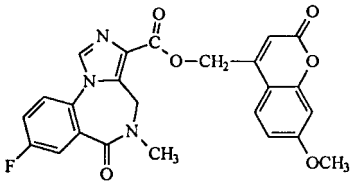
AMCA-Ro7-1986 (17)



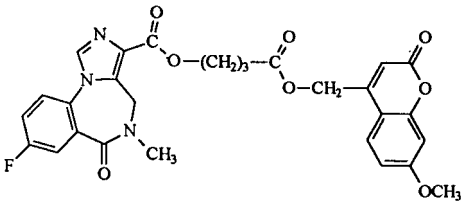
flumazenil (**18**)



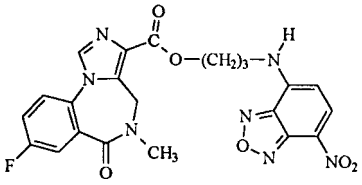
Ro15-3890 (desethylflumazenil) (**19**)



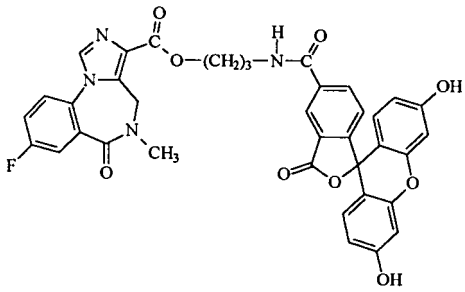
Mmc-Ro15-3890 (**20a**)



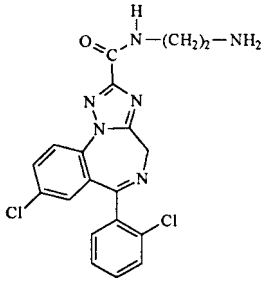
Mmc-O-CO-$(CH_2)_3$-Ro15-3890 (**20b**)



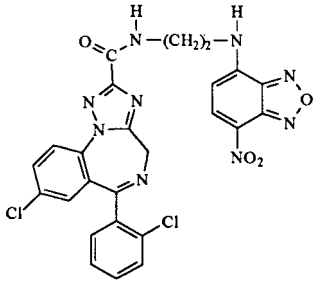
NBD-NH-$(CH_2)_3$-Ro15-3890 (**21**)



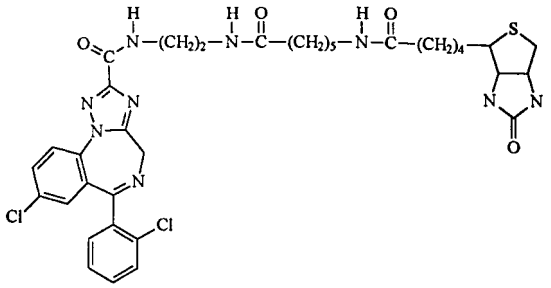
fluorescein-NH-$(CH_2)_3$-Ro15-3890 (**22**)



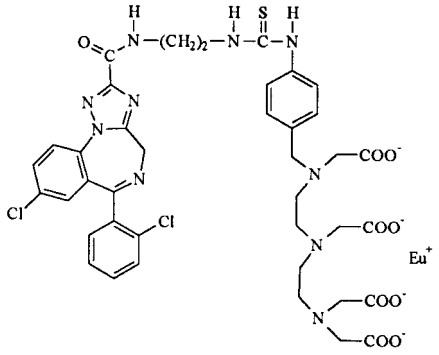
1012-S (**23**)



NBD-1012-S (**24**)



Biotin-1012-S (**25**)



Eu^+-1012-S (**26**)

Table 1: The m/z ratios of the synthesized fluorescent-labeled benzodiazepines and the mobile phase composition used for the purification of these compounds

	Compd.	m/z	Mobile phase composition H_2O/MeOH/ACN
Mmc−O−CO−$(CH_2)_2$−CO-oxazepam	**5**	575	70/30/0
Mmc−O−CO−$(CH_2)_2$−CO-lormetazepam	**6**	623	70/30/1
dansyl-Ro5-3072	**9**	485	40/60/0
dansyl-Ro7-1986	**12**	565	30/70/0
Mmc-Ro15-3890	**20a**	464	55/45/0
Mmc−O−CO−$(CH_2)_3$−Ro15-3890	**20b**	550	56/19/25

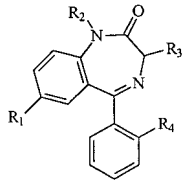
group. We also used two metabolites, **8**, with no affinity for the benzodiazepine receptor, and **11** for labeling.

The reactive groups of **8**, **11** and the 3-hydroxybenzodiazepines **1** and **2** are located at three different positions of the basic structure of the 1,4-benzodiazepine molecule, which is shown in Table 2.

This allowed us to examine which position of the benzodiazepine molecule can be labeled, so that the resulting product still exhibits sufficiently high binding affinity to the benzodiazepine receptor. The influence of the fluorophores used in this study on the affinity for the benzodiazepine receptor could also be examined because others have labeled **11** with different labels [1–3].

Besides the 1,4-benzodiazepines, we also selected another class, the 1,2-annelated 1,4-benzodiazepines, for labeling.

Table 2: Basic structure of 1,4-benzodiazepines. The positions R_1, R_2 and R_3 can be used for labeling



	Compd.	R_1	R_2	R_3	R_4
7-Aminonitrazepam	**8**	$-NH_2$	$-H$	$-H$	$-H$
Didesethylflurazepam	**11**	$-Cl$	$-CH_2-CH_2-NH_2$	$-H$	$-F$
Oxazepam	**1**	$-Cl$	$-H$	$-OH$	$-H$
Lormetazepam	**2**	$-Cl$	$-CH_3$	$-OH$	$-Cl$

Table 3: Capacity factors of the two compounds (k_1 and k_2) and the resolution (R_s) at different mobile phase compositions in the optimization experiment

% H_2O	% MeOH	% ACN	k_1 (min)	k_2 (min)	R_s
60	40	0	70.2	102	1.73
50	50	0	11.6	16.4	1.54
40	60	0	2.5	3.2	0.93
70	0	30	17.7	29.6	2.17
65	0	35	7.7	12.1	1.88
55	0	45	2.2	2.9	1.00
65	20	15	47.0	85.4	2.48
55	25	20	13.2	21.3	2.03
50	25	25	6.1	7.7	1.03

Compound **19**, a metabolite of **18**, was labeled with the fluorophore 4-bromomethyl-7-methoxycoumarin, with and without the incorporation of a spacer chain.

For the synthesis of **20b**, the fluorophore was first coupled to the carboxy-group of the spacer 4-hydroxybutyric acid, followed by the linkage of the hydroxy-group of the spacer to the ligand desethylflumazenil. This route was chosen to avoid cross-reactivity of the spacer molecules. McCabe et al. [2] and Havunjian et al. [4] first coupled the spacer to **19** in their procedure. To be successful, they had to protect the amino-group before coupling, which makes the synthesis more complex.

2.2. Purification of the fluorescent-labeled benzodiazepines

The synthesized fluorescent-labeled benzodiazepines were purified by reversed-phase HPLC. This was done with an analytical C_{18}-column, since a semi-preparative column did not provide adequate resolution. The use of an analytical column had the disadvantage that only small amounts of the fluorescent-labeled benzodiazepines could be purified. In one run, about 30 µg of the derivatization product could be brought onto the column. Applying more caused peak broadening, resulting in insufficient resolution. The fluorescent-labeled benzodiazepines were isolated from the mobile phase by solid phase extraction to obtain the product in a dry state. The amount of purified fluorescent-labeled benzodiazepine was established by weighing the glass test-tubes with and without the purified product. To minimize the error in weighing, the minimum amount of purified fluorescent-labeled benzodiazepine had to be at least 2 mg. The recoveries from this purification procedure was 50–70%. Residues were dissolved in some methanol and the purity was checked by reversed-phase HPLC, and was at least 95%. Takeuchi and Rechnitz [1] purified their fluorescent-labeled benzodiazepine **17** also by HPLC, but they did not isolate their product from the mobile phase. After the removal of the acetonitrile by evaporation, the concentration in their remaining eluent was determined from the molar extinction coefficient of the fluorophore, assuming that the molar extinction coefficient of the label had not been affected by coupling the benzodiazepine to the fluorophore. Because this assumption is questionable, we preferred to isolate the fluorescent-labeled benzodiazepine from the mobile phase.

For the purification of **20b**, a mobile phase consisting of methanol and water proved to be unsuitable because of insufficient resolution between **20b** and **20a**. Therefore, we switched to a ternary chromatographic system, in which the mobile phase of water/methanol/acetonitrile was optimized with regard to resolution and analysis time using MCDM [10]. The capacity factors of the two compounds (k_1 and k_2) and the corresponding resolutions for the different mobile phase compositions are represented in Table 3.

Fig. 1A shows a contour plot of the maximum capacity factor of compound 2 and Fig. 1B a contour plot of the minimum resolution. The two criteria for the mobile phase composition suitable for the purification are: R_s as large as

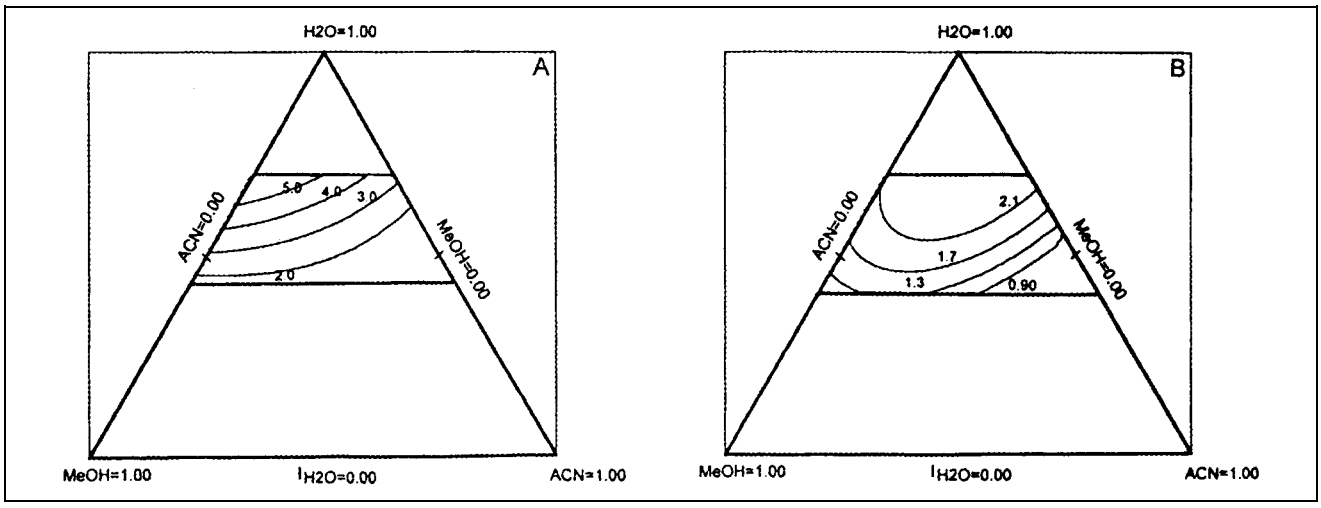


Fig. 1: (A): contour plot of the maximal capacity factor (ln values) of compound 2.
(B): contour plot of the minimal resolution

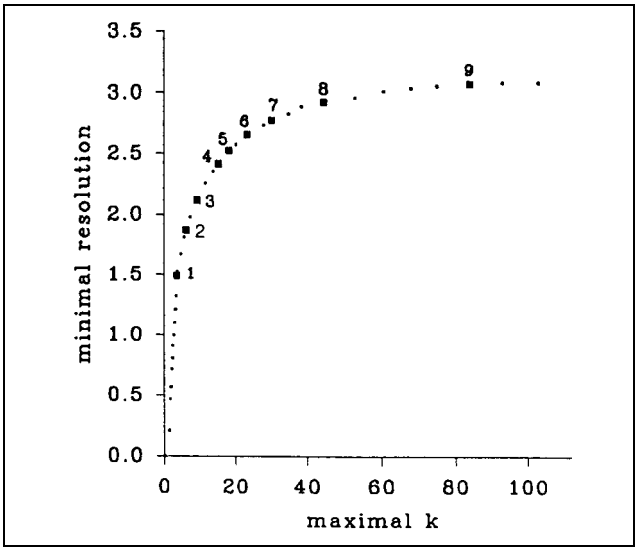


Fig. 2: Pareto-Optimal points for minimal resolution and maximal capacity factor of the second compound

Table 4: The variable settings of the Pareto-Optimal points

PO point number	% H_2O	% MeOH	% ACN	R_s	k_2
1	40	37	23	1.49	3.4
2	45	34	21	1.87	6.0
3	50	26	24	2.12	9.1
4	56	19	25	2.42	15.2
5	66	2	32	2.53	18.2
6	65	6	29	2.66	23.3
7	67	5	28	2.78	30.3
8	69	5	26	2.93	44.5
9	70	9	21	3.09	84.0

possible and k_2 as small as possible. From the MCDM-results, the Pareto-Optimal points were selected for the two factors R_s and k_2.

The Pareto-Optimal points are presented in Fig. 2 and show the two criteria R_s and k_2. Each point corresponds to a combination of the mobile phase composition. Nine combinations are also given in Table 4. From these results, the mobile phase composition water/methanol/acetonitrile 56/19/25 was selected as optimal for the purification.

Normally, a resolution of 1.5 is considered sufficient for a good separation between two compounds. However, for the purification of **20b**, almost saturated solutions of the derivatization mixture were applied onto the column, which caused peak broadening. Therefore, we preferred a higher resolution. A predicted resolution of 2.42 was considered acceptable, with predicted capacity factors for **20a** (k_1) and **20b** (k_2) of 9.4 and 15.2, respectively. Fig. 3 shows the actual chromatogram of the derivatization mixture for these optimal conditions. The retention times of **20a** and **20b** were 9.9 min and 14.4 min, respectively, corresponding with capacity factors of 8.9 and of 13.4, respectively.

Table 5: Fluorescence characteristics of fluorescent-labeled benzodiazepines in Tris-HCl buffer (pH 7.4; 50 mM)

	Compd.	relative fluorescence	λ_{ex} (nm)	λ_{em} (nm)
Mmc−O−CO−$(CH_2)_2$−CO-ox-azepam	**5**	59	331	402
Mmc−O−CO−$(CH_2)_2$−CO-lormetazepam	**6**	50	329	403
dansyl-Ro5-3072	**9**	4	326	494
dansyl-Ro7-1986	**12**	6	346	497
Bodipy FL-Ro7-1986	**16**	267	505	508
NBD−Ro7-1986	**14**	17	468	537
Mmc−Ro15-3890	**20a**	54	330	402
Mmc−O−CO−$(CH_2)_3$−Ro15-3890	**20b**	57	331	403

The molar fluorescence signals are expressed relative to the signal of 5 mM quinine sulphate in 1 N sulphuric acid (=100)

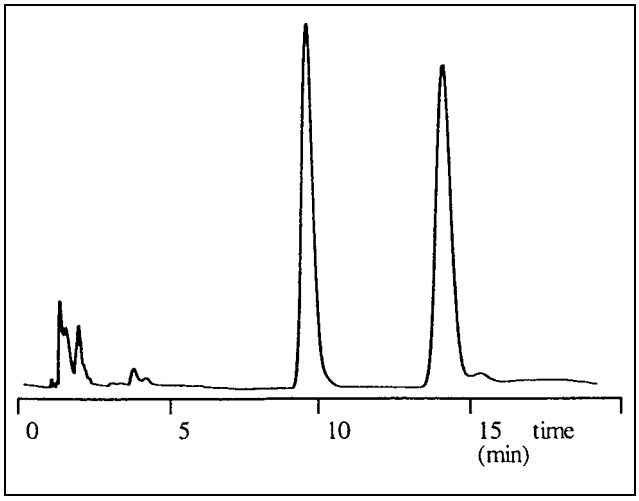


Fig. 3: Chromatogram of the derivatization mixture of **20b**. The mobile phase consisted of 56% H_2O, 19% MeOH and 25% ACN. The first peak at 9.9 min is **20a**; the second peak at 14.4 min is **20b**

Table 6: K_i-values of the fluorescent-labeled benzodiazepines and of their parent compounds

	Compd.	K_i
Oxazepam	**1**	16.7 nM
Mmc−O−CO−$(CH_2)_2$−CO-oxazepam	**5**	> 1 μM
lormetazepam	**2**	1.2 nM
Mmc−O−CO−$(CH_2)_2$−CO-lormetazepam	**6**	114 nM
Nitrazepam	**7**	7.4 nM
7-Aminonitrazepam (Ro5-3072)	**8**	470 nM
dansyl-Ro5-3072	**9**	>1 μM
Flurazepam	**10**	10.4 nM
Didesethylflurazepam (Ro7-1986)	**11**	4.9 nM
dansyl-Ro7-1986	**12**	167 nM
Bodipy FL-Ro7-1986	**16**	67 nM
NBD-Ro7-1986	**14**	51 nM
NBD−$(CH_2)_2$−CO−Ro7-1986 [3]	**15a**	132 nM
NBD−$(CH_2)_5$−CO−Ro7-1986 [3]	**15b**	163 nM
Fluorescein-Ro7-1986 [2]	**13**	74 nM
AMCA-Ro7-1986 [1]	**17**	8.6 nM
Flumazenil	**18**	0.6 nM
Desethylflumazenil (Ro15-3890)	**19**	>1 μM
Mmc-Ro15-3890	**20a**	121 nM
Mmc−O−CO−$(CH_2)_3$−Ro15-3890	**20b**	6.5 nM
Fluorescein−NH−$(CH_2)_3$−Ro15-3890 [2]	**22**	63 nM
NBD−NH−$(CH_2)_3$−Ro15-3890 [4]	**21**	5.7 nM
1012-S [7]	**23**	0.4 nM
NBD−1012-S [3]	**24**	85 nM

2.3. Fluorescence characteristics of the fluorescent-labeled benzodiazepines

The optimum excitation and emission wavelengths of the fluorescent-labeled benzodiazepines are reported in Table 5. The signals were measured as relative fluorescence values

with quinine sulphate as calibration sample. In this way, the fluorescence sensitivity of the different fluorophores can be compared easily to see which derivative has the most suitable fluorescence characteristics for use in a fluorescence receptor assay.

2.4. Binding affinities of the fluorescent-labeled benzodiazepines

The affinities of the fluorescent-labeled benzodiazepines were calculated from their inhibition curves. The latter were fitted with the program EBDA-Ligand, V4 (Biosoft, Cambridge, UK) [12] using a one-binding site model. The results are presented in Table 6.

3. Experimental

3.1. Chemicals and apparatus

[*N*-methyl-^{3}H]flunitrazepam (82 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Compound **2** was a gift from Wyeth Laboratoria (Hoofddorp, The Netherlands) and **7**, **8**, **11**, **18** and **19** were gifts from Roche Nederland (Mijdrecht, The Netherlands). Compound **1** was purchased from Genfarma (Maarssen, The Netherlands). The fluorescent-labeled benzodiazepines **14** and **16** were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Quinine sulphate dihydrate (>99%), succinic anhydride, 60% suspension of sodium hydride in mineral oil, 4-hydroxybutyric acid (sodium salt), 4-bromo-methyl-7-methoxycoumarin, 18-crown-6 ether and dansylchloride were purchased from Acros Organics (Geel, Belgium). Methanol and acetonitrile, both hplc-grade, were supplied by Lab-Scan (Dublin, Ireland). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Empore™ Extraction Disks (C18, Ø 47 mm) were obtained from Varian (Harbor City, CA, USA) and GF/B glass fibre filter discs (Ø 25 mm) were obtained from Whatman (Maidstone, UK). Rialuma, used as scintillation cocktail, was obtained from Lumac (Olen, Belgium). Demineralized water was further purified by an Elgastat Maxima instrument (Elga, High Wycombe, UK) before use in the buffers.

The HPLC-system used for the identification of the fluorescent-labeled benzodiazepine consisted of a Spectraflow 400 HPLC pump (ABI Analytical Kratos Division, Manchester, UK), a Spectraflow 757 variable wavelength UV detector (Kratos) and a modified Nermag R 3010 triple quadrupole mass spectrometer (Delsi-Nermag, Argenteuil, France), equipped with a custom-built prototype atmospheric pressure ionization (API) source. The spectra were recorded in the positive-ion mode. Injections were made using a Rheodyne 7125 injection valve, fitted with a 20 µl sample loop (Rheodyne, Cotati, CA USA).

The HPLC-system used for the purification of the fluorescent-labeled benzodiazepines consisted of a SP 8800 HPLC pump (Spectra Physics, San Jose, CA, USA), an autosampler model 460 fitted with a 20 µl loop (Kontron Instruments, Basle, Switzerland), a SPD-6A variable wavelength UV detector (Shimadzu, Tokyo, Japan) and a HeliFrac fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden).

3.2. Synthesis

3.2.1. Synthesis of Mmc-O-CO-$(CH_2)_2$-CO-oxazepam (**5**)

To a suspension of 0.5 g **1** in 35 ml dry tetrahydrofuran (1.74 mmol), 90 mg of a 60% suspension of sodium hydride in mineral oil (2.25 mmol) were added. After stirring for 0.5 hour at room temperature under a nitrogen atmosphere, succinic anhydride (0.27 g; 2.70 mmol) was added and stirring was continued at 40 °C. After 2 h, the tetrahydrofuran was evaporated under vacuum. The residue was suspended in 20 ml of water, transferred into a separation funnel, acidified with acetic acid, and extracted twice with 30 ml of dichloromethane. After drying the dichloromethane phase with anh. sodium sulphate, the dichloromethane was evaporated to half its volume and the addition of hexane afforded **3** [13].

For the labeling reaction, **3** (10 mg; 0.026 mmol) was suspended in 2 ml of acetonitrile. To this suspension, 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml; 0.074 mmol), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml; 0.009 mmol) and potassium carbonate (10 mg; 0.072 mmol) were added [14]. After derivatization for 1 h at 60 °C, the mixture was analyzed by HPLC-MS to identify the derivatization product (section 3.3.). The remainder was purified according the method described in section 3.4.

3.2.2. Synthesis of Mmc-O-CO-$(CH_2)_2$-CO-lormetazepam (**6**)

To a suspension of 0.2 g **2** in 10 ml dry tetrahydrofuran (0.60 mmol), 30 mg of a 60% suspension of sodium hydride in mineral oil (0.75 mmol) were added. After stirring for 0.5 h at room temperature under a nitrogen atmosphere, succinic anhydride (95 mg; 0.95 mmol) was added and stirring was continued at 40 °C. After 2 h the tetrahydrofuran was evaporated under vacuum. The residue was suspended in 20 ml of water, transferred into a separation funnel, acidified with acetic acid, and extracted twice with 30 ml of dichloromethane. After drying the dichloromethane phase with anhydrous sodium sulphate, the dichloromethane was evaporated to half its volume and the addition of hexane afforded **4** [13].

For the labeling reaction, **4** (10 mg; 0.023 mmol) was suspended in 2 ml of acetonitrile. To this suspension 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml; 0.074 mmol), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml; 0.009 mmol) and potassium carbonate (10 mg; 0.072 mmol) were added [14]. After derivatization for 1 h at 60 °C the mixture was analyzed by HPLC-MS to identify the derivatization product (section 3.3.). The remainder was purified according the method described in section 3.4.

3.2.3. Synthesis of dansyl-Ro5-3072 (**9**)

To a glass test-tube with dansyl chloride (100 mg; 0.37 mmol), 0.5 ml of 0.5 M sodium carbonate solution and 10 ml of a solution of **8** in acetone (1 mg/ml; 0.030 mmol) were added [15]. After incubation for 3 h at 45 °C, the clear solution was transferred into a second glass test-tube and the solvent was evaporated under a nitrogen atmosphere. The residue was dissolved in 10 ml of methanol.

*3.2.4. Synthesis of dansyl-Ro7-1986 (***12***)*

To a glass test-tube with dansyl chloride (100 mg; 0.37 mmol), 0.5 ml of 0.5 M sodium carbonate solution and 10 ml of a solution of **11** in acetone (1 mg/ml; 0.030 mmol) were added [15]. After incubation for 3 h at 45 °C, the clear solution was transferred into a second glass test-tube and the solvent was evaporated under a nitrogen atmosphere. The residue was dissolved in 10 ml of methanol.

*3.2.5. Synthesis of Mmc-Ro15-3890 (***20a***)*

Compound **19** (10 mg; 0.036 mmol) was suspended in 2 ml of acetonitrile. To this suspension 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml = 0.074 mmol), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml = 0.009 mmol) and potassium carbonate (10 mg; 0.072 mmol) were added [14]. After stirring for 1 h at 60 °C, the mixture was analyzed with HPLC-MS to identify the derivatization product (section 3.3). The remainder was purified according the method described in section 3.4.

*3.2.6. Synthesis of Mmc-O-CO-$(CH_2)_3$-Ro15-3890 (***20b***)*

For the synthesis, 4-hydroxybutyric acid (sodium salt) (265 mg; 2.1 mmol) and potassium carbonate (1.95 g; 14 mmol) were suspended in 200 ml of acetonitrile. To this suspension, 18-crown-6 ether (37.5 mg; 0.14 mmol) and 4-bromo-methyl-7-methoxycoumarin (375 mg; 1.36 mmol) were added and this mixture was incubated at 65 °C for 1 h. After reaction, the sediment formed was removed by filtration and the acetonitrile was evaporated under vacuum. The residue was dissolved in 50 ml of chloroform. The chloroform was washed six times with 20 ml of water, dried with anh. sodium sulphate and after evaporation under vacuum, crude 1-(4-hydroxybutyryl)-oxymethyl-7-methoxycoumarin was collected.

For the labeling, 78.8 mg **19** (0.32 mmol) were dissolved in 5 ml of dry dichloromethane and 200 µl of dry triethylamine were added. The reaction mixture was cooled in an ice bath and methanesulfonylchloride (40 mg; 0.35 mmol) was added. After incubation at room temperature for 1 h, the mixture was cooled in an ice bath again, 1-(4-hydroxybutyryl)-oxymethyl-7-methoxycoumarin (100 mg; 0.34 mmol) was added and the reaction was continued at room temperature overnight. After reaction, the dichloromethane was evaporated and the residue was resuspended in 10 ml of dry benzene. The precipitate was removed by filtration and the benzene fraction was evaporated under vacuum. This residue was dissolved in 50 ml of dichloromethane. The dichloromethane was washed three times with 20 ml of water, dried with anh. sodium sulphate and evaporated. The residue was dissolved in ethyl acetate and recrystallized from hexane. After recrystallization, the product was collected and dissolved in methanol. The mixture was analyzed with HPLC-MS to identify the derivatization product (section 3.3). The remainder was purified according the method described in section 3.4.

3.3. Identification of the derivatization products by HPLC-MS

After the derivatization reactions, 100 µl of the solutions were evaporated and the residues were dissolved in 1 ml of methanol. From these solutions, 20 µl were injected onto a reversed phase column (LiChrospher® 100 RP-18 (5 µm), 125 × 4 mm i.d., Merck, Darmstadt, Germany) and eluted with a linear gradient water/methanol mobile phase, starting with 70% water and 30% methanol and finishing with 100% methanol after 10 min. The flow was 1 ml/min and the eluents were monitored by UV detection at 254 nm and mass spectrometric detection.

3.4. Purification with HPLC

For the purification of the fluorescent-labeled benzodiazepines, 20 µl aliquots of the derivatization solutions were injected onto a reversed phase column (LiChrospher® 100 RP-18 (5 µm), 125 × 4 mm i.d., Merck, Darmstadt, Germany). Compound **20b** was dissolved in methanol before injection into the HPLC-system. The mobile phases used are listed in Table 1.
The mobile phase composition for the purification of **20b**, consisting of the solvents water (H_2O), methanol (MeOH) and acetonitrile (ACN) was optimized using MCDM [10] in advance. The chromatograms of the derivatization mixture of **20b** in methanol with nine different mobile phase compositions, which were set up according a factorial design, were recorded and the capacity factors (k_1 and k_2) and the resolution (R_s) were calculated from the observed retention times. The mobile phase compositions are presented in Table 3. The other fluorescent-labeled benzodiazepines were purified using a mobile phase composition of only water and methanol.
During the chromatografic runs, the eluent was monitored by UV detection at 254 nm. For purfication, the fractions containing the fluorescent-labeled benzodiazepine were collected with the fraction collector. Water was added to reduce the organic modifier concentration to 10%. The fluorescent-labeled benzodiazepine was isolated from the water/methanol/acetonitrile solution with a C_{18} Empore™ Extraction Disk.
After preconditioning of the disk with successively 5 ml of methanol and 10 ml of water, 100 ml of the solution with the purified compound was applied onto the disk and the solution was pulled through by applying vacuum. The disk was kept under vacuum for 15 min after the solution passed the disk and the fluorescent-labeled benzodiazepine was eluted with 5 ml methanol. This extraction procedure was repeated until all solution had been treated. The methanol eluates were collected in a weighed glass test-tube and the methanol was evaporated under vacuum with an Univapo 150 H centrifuge (UniEquip, Martinsried, Germany). After evaporation of the methanol, the weight of the glass test-tube was determined again. The difference in weight was considered as the yield of the purified fluorescent-labeled benzodiazepine. The fluorescent-labeled benzodiazepines were dissolved in 10.0 ml of methanol and the purity was checked by HPLC (conditions as used for the purification). The solutions of the fluorescent-labeled benzodiazepines were stored at −20 °C.

3.5. Determination of the fluorescence characteristics of the fluorescent-labeled desethylflumazenil 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) [16].

3.6. Preparation of membrane-bound 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 × g in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands) [17]. The supernatant was centrifuged for 60 min at 100.000 × 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 × 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).

3.7. 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) [17]. To this mixture, 400 µl of the receptor suspension were added, vortexed and incubated for 45 min at 4 °C. The incubation was terminated by adding 4 ml of ice-cold Tris-HCl buffer and this mixture was filtered through pre-wetted GF/B filters. 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).

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