Development and perspectives of fluorescent receptor assays

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Chapter 3

Synthesis and characterization of fluorescent-labeled 1,2-annelated 1,4-benzodiazepines

3.1 Introduction

In Chapter 2, we described the synthesis of various fluorescent-labeled 1,4-benzodiazepines. Besides the 1,4-benzodiazepines, other classes of benzodiazepines are also available for labeling, such as desethylflumazenil, an imidazo-benzodiazepine [1,2], and 1012-S, a triazolo-benzodiazepine [3,4,5]. Desethylflumazenil (Ro15-3890) is a metabolite of the benzodiazepine antagonist flumazenil and has no affinity for the benzodiazepine receptor. Using a ligand without affinity for the receptor has the advantage that in case of hydrolysis the resulting products do not interfere in the assay. Hydrolysis is the main disadvantage of using didesethylflurazepam as ligand for labeling, since didesethylflurazepam itself also has high affinity for the benzodiazepine receptor [6]. In this paper we describe the fluorescent labeling of desethylflumazenil. The fluorophore 4-bromo-methyl-7-methoxycoumarin was coupled directly to desethylflumazenil and via a spacer.

After the synthesis of Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890, the latter was collected by precipitation with hexane. However, the precipitate contained two products, the expected Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890 but also Mmc-Ro15-3890. These two compounds were found difficult to separate by RP-HPLC. Therefore, the chromatographic system for the purification was optimized chemometrically, using multi-criteria decision making (MCDM) techniques [7]. The effect of the mobile phase composition, consisting of three components, water, methanol and acetonitrile, on the chromatographic parameters, 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, Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890 (k$_2$). Chromatograms were recorded with nine different mobile phase compositions, which were fixed in a factorial design.
The further procedure to purify the fluorescent-labeled desethylflumazenil derivatives was identical to the purification procedure of the fluorescent-labeled 1,4-benzodiazepines (see Chapter 2).

The most suitable fluorescent-labeled benzodiazepine for receptor binding studies was selected by comparing the $K_i$-values and fluorescence characteristics of the fluorescent-labeled ligands, which is described in Chapter 4.

### 3.2 Materials and Methods

#### Chemicals and apparatus

\([N\text{-}methyl-^3\text{H}]\text{flunitrazepam (82 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Desethylflumazenil (Ro15-3890) was a gift from Roche Nederland (Mijdrecht, The Netherlands). Quinine sulphate dihydrate (>99%), 4-hydroxybutyric acid (sodium salt), 4-bromo-methyl-7-methoxycoumarin and 18-crown-6 ether were purchased from Janssen Chimica (Beerse, Belgium). Methanol and acetonitrile, both hplc-grade, were supplied by LabScan (Dublin, Ireland). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Empore\textsuperscript{TM} Extraction Disks (C18, Ø47 mm) were obtained from Varian (Harbor City, CA, USA) and GF/B glass fibre filter discs (Ø25 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).
Synthesis of fluorescent-labeled 1,2-annelated 1,4-benzodiazepines

Synthesis

Synthesis of Mmc-Ro15-3890
Ten mg desethylflumazenil (Ro15-3890) were suspended in 2 ml acetonitrile. To this suspension 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml) and 10 mg potassium carbonate were added [8]. After derivatization for 1 hour at 60°C, the mixture was analyzed with HPLC-MS to identify the derivatization product (see below). The reminder was purified according the method described at pag 40.

Synthesis of Mmc-O-CO-(CH₂)₃-Ro15-3890
For the synthesis, 265 mg 4-hydroxybutyric acid (sodium salt) and 1.95 g potassium carbonate were suspended in 200 ml acetonitrile. To this suspension, 37.5 mg 18-crown-6 and 375 mg 4-bromo-methyl-7-methoxycoumarin were added and this mixture was incubated at 65°C for 1 hour. After the derivatization, the sediment formed was removed by filtration and the acetonitrile was evaporated under vacuum. The residue was dissolved in 50 ml chloroform. The chloroform was washed six times with 20 ml water, dried with anhydrous sodium sulphate and after evaporation under vacuum, 1-(4-hydroxybutyryl)-oxymethyl-7-methoxycoumarin was collected.

For the labeling of desethylflumazenil, 87.7 mg desethylflumazenil (Ro15-3890) were dissolved in 5 ml dry dichloromethane and 200 µl dry triethylamine were added. The reaction mixture was cooled on ice and 40 mg methanesulfonylchloride were added. After incubation at room temperature for 1 hour, the mixture was cooled on ice again, 100 mg 1-(4-hydroxybutyryl)-oxymethyl-7-methoxycoumarin were added and the reaction was continued at room temperature during the night. After the derivatization, the dichloromethane was evaporated and the residue was resuspended in 10 ml dry benzene. The precipitate was removed by filtration and the benzene fraction was evaporated under vacuum. The residue was dissolved in 50 ml dichloromethane. The dichloromethane was washed three times with 20 ml water, dried with anhydrous sodium sulphate and evaporated. The residue was dissolved in ethylacetate and recrystallized from hexane. After the recrystalliation, the product was collected and dissolved in methanol. The mixture was analyzed with HPLC-MS to identify the derivatization product (see below). The reminder was purified according the method described at pag 40.

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 methanol. From these solutions, 20 µl were injected onto a reversed phase column (LiChrospher® 100 RP-18 (5 µm), 125 x 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.

Table 3.1  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.

<table>
<thead>
<tr>
<th>% H$_2$O</th>
<th>% MeOH</th>
<th>% ACN</th>
<th>$k_1$ (min)</th>
<th>$k_2$ (min)</th>
<th>$R_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>40</td>
<td>0</td>
<td>70.2</td>
<td>102</td>
<td>1.73</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>0</td>
<td>11.6</td>
<td>16.4</td>
<td>1.54</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>0</td>
<td>2.5</td>
<td>3.2</td>
<td>0.93</td>
</tr>
<tr>
<td>70</td>
<td>0</td>
<td>30</td>
<td>17.7</td>
<td>29.6</td>
<td>2.17</td>
</tr>
<tr>
<td>65</td>
<td>0</td>
<td>35</td>
<td>7.7</td>
<td>12.1</td>
<td>1.88</td>
</tr>
<tr>
<td>55</td>
<td>0</td>
<td>45</td>
<td>2.2</td>
<td>2.9</td>
<td>1.00</td>
</tr>
<tr>
<td>65</td>
<td>20</td>
<td>15</td>
<td>47.0</td>
<td>85.4</td>
<td>2.48</td>
</tr>
<tr>
<td>55</td>
<td>25</td>
<td>20</td>
<td>13.2</td>
<td>21.3</td>
<td>2.03</td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td>25</td>
<td>6.1</td>
<td>7.7</td>
<td>1.03</td>
</tr>
</tbody>
</table>

**Purification with HPLC**

Before purifying Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890, the mobile phase composition, consisting of the solvents water (H$_2$O), methanol (MeOH) and acetonitrile (ACN) was optimized using MCDM [7]. The chromatograms of the derivatization mixture of Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890 in methanol with nine different mobile phase compositions, which were fixed in 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 3.1.

For the purification of Mmc-Ro15-3890, 20 µl aliquots of the derivatization solutions were injected onto a reversed phase column (LiChrospher® 100 RP-18 (5 µm), 125 x 4 mm i.d., Merck, Darmstadt, Germany). Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890 was dissolved in methanol before injection into the HPLC-system. The mobile phases used are listed in Table 3.2. The eluent was monitored by UV detection at 254 nm. 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 Disks.
Synthesis of fluorescent-labeled 1,2-annelated 1,4-benzodiazepines

**Table 3.2** m/z ratios of the synthesized fluorescent-labeled Ro15-3890 derivatives and the mobile phase composition used for the purification of these compounds.

<table>
<thead>
<tr>
<th>m/z</th>
<th>mobile phase composition H₂O/MeOH/ACN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmc-Ro15-3890</td>
<td>464</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH₂)₃-Ro15-3890</td>
<td>550</td>
</tr>
</tbody>
</table>

After preconditioning of the disk with successively 5 ml methanol and 10 ml water, 100 ml of the water/methanol/acetonitrile solution 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 by 5 ml methanol. This extraction procedure was repeated until all water/methanol/acetonitrile 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 the 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 methanol and the purity was checked by HPLC (same conditions as were used for the purification). The solutions of the fluorescent-labeled benzodiazepines were stored at -20°C.

**Determination of the fluorescence characteristics of the fluorescent-labeled desethylflumazenil derivatives**

The stock solutions of the fluorescent-labeled desethylflumazenil 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 exitation 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) [9].

**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 x g in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands). The supernatant was centrifuged for 60 min at 100,000 x g. The resulting pellet (P₂) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged for 30 min at 100,000 x g. This washing
step was repeated once. All operations were performed at 4°C. The washed P₂-pellet was resuspended in five volumes (w/v) of phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkeroed, Denmark). The lyophilized P₂-pellet was stored at -20°C. For the receptor binding assays, the lyophilized P2-pellet was resuspended in Tris-HCl buffer (pH 7.4; 50 mM) with a glass-teflon Potter-Elvehjem homogenizer (2.5 mg/ml).

Receptor binding assay
For the binding assay, 50 µl [³H]flunitrazepam solution (0.5 nM final concentration) in Tris-HCl buffer (pH 7.4; 50 mM) was mixed in duplicate with 50 µl Tris-HCl buffer, containing the fluorescent labeled benzodiazepines (200 nM - 6 pM final concentration). 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 ice-cold Tris-HCl buffer and this mixture was filtered through pre-wetted GF/B filters. The tubes were rinsed twice with 4 ml 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 Results and discussion

Synthesis of the fluorescent-labeled benzodiazepines
The chemical structures of the starting products and the synthesized fluorescent-labeled benzodiazepines Ro15-3890 and 1021-S are shown in the Appendix. For the synthesis of Mmc-O-CO-(CH₂)₃-Ro15-3890, the fluorophore was first coupled to the carboxy-group of the spacer 4-hydroxybutyric acid and then the hydroxy-group of the spacer was linked to the ligand desethylflumazenil. This was done to avoid cross-reactivity of the spacer molecules. McCabe et al. [1] and Havunjian et al. [2] first coupled the spacer to desethylflumazenil. To be successful, they had to protect the amino-group before coupling, which makes the synthesis more complex. However, the latter approach is more practical when different fluorophores will be used for labeling.

The formation of Mmc-Ro15-3890 and Mmc-O-CO-(CH₂)₃-Ro15-3890 was verified by determination of their masses with MS. The m/z ratios are reported in Table 3.2.

Purification of the fluorescent-labeled desethylflumazenil derivatives
The synthesized fluorescent-labeled desethylflumazenil derivatives were purified by reversed-phase HPLC, comparable to the procedure for the fluorescent-labeled 1,4-benzodiazepines [6]. Mobile phases consisting of methanol and water were unsuitable for the purification of Mmc-
O-CO-(CH₂)₃-Ro15-3890 because of insufficient resolution between Mmc-O-CO-(CH₂)₃-Ro15-3890 and Mmc-Ro15-3890. Therefore we switched to a ternary chromatographic system, in which the mobile phase of water/methanol/acetonitrile was optimized chemometrically using MCDM. The capacity factors of the two compounds (k₁ and k₂) and the corresponding resolutions for the different mobile phase compositions are represented in Table 3.1. With these results, calculations were made using MCDM.

The effect of the mobile phase composition on k₁ and k₂ was best fitted according a quadratic model, with the equations

\[
\ln(k₁) = 10.65\%H₂O - 6.01\%MeOH + 3.17\%ACN + 0.91\%H₂O\%MeOH - 26.29\%H₂O\%ACN + 4.47\%MeOH\%ACN
\]

\[
(3.1)
\]

and

\[
\ln(k₂) = 11.28\%H₂O - 6.19\%MeOH + 1.18\%ACN + 1.35\%H₂O\%MeOH - 23.00\%H₂O\%ACN + 7.67\%MeOH\%ACN.
\]

\[
(3.2)
\]

The multiple correlation coefficients R² for k₁ and k₂ were 0.99632 and 0.99678, respectively. The resolutions Rₛ were calculated from the fitted k₁ and k₂ values and the effect of the mobile phase composition on Rₛ could be expressed by the equation

Figure 3.1  (A): contour plot of the maximal capacity factor (ln values) of compound 2.
(B): contour plot of the minimal resolution.
\[ R_s = 1.61 \times \% H_2O - 3.22 \times \% MeOH - 13.33 \times \% ACN + 9.41 \times \% H_2O \times \% MeOH + 24.71 \times \% H_2O \times \% ACN + 14.28 \times \% MeOH \times \% ACN. \]

(3.3)

Figure 3.1A contains a contour plot of the maximum capacity factor of compound 2 and Figure 3.1B contains 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 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 \( k_2 \).

![Figure 3.1A](image)

**Figure 3.2** The Pareto-Optimal points for minimal resolution and maximal capacity factor of the second compound.

A combination of \( R_s \) and \( k_2 \) is a Pareto-Optimal point if there exists no other combination which yields an improvement in one criterium without causing a degradation in the other criterion. The Pareto-Optimal points are presented in Figure 3.2 and show the relation between the \( R_s \) and \( k_2 \). Each point corresponds to a combination of the mobile phase composition and nine combinations are also presented in Table 3.3. 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 sufficient for a good separation between two compounds. However, for the purification of \( \text{MmC-O-CO-(CH}_2)_3\text{-Ro15-3890} \), almost saturated solutions of the derivatization mixture were applied onto the column, which caused peak broadening.

![Figure 3.2](image)
Table 3.3  The variable settings of the Pareto-Optimal points.

<table>
<thead>
<tr>
<th>PO point number</th>
<th>% H₂O</th>
<th>% MeOH</th>
<th>% ACN</th>
<th>Rₛ</th>
<th>k₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.40</td>
<td>0.37</td>
<td>0.23</td>
<td>1.49</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>0.45</td>
<td>0.34</td>
<td>0.21</td>
<td>1.87</td>
<td>6.0</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>0.26</td>
<td>0.24</td>
<td>2.12</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>0.56</td>
<td>0.19</td>
<td>0.25</td>
<td>2.42</td>
<td>15.2</td>
</tr>
<tr>
<td>5</td>
<td>0.66</td>
<td>0.02</td>
<td>0.32</td>
<td>2.53</td>
<td>18.2</td>
</tr>
<tr>
<td>6</td>
<td>0.65</td>
<td>0.06</td>
<td>0.29</td>
<td>2.66</td>
<td>23.3</td>
</tr>
<tr>
<td>7</td>
<td>0.67</td>
<td>0.05</td>
<td>0.28</td>
<td>2.78</td>
<td>30.3</td>
</tr>
<tr>
<td>8</td>
<td>0.69</td>
<td>0.05</td>
<td>0.26</td>
<td>2.93</td>
<td>44.5</td>
</tr>
<tr>
<td>9</td>
<td>0.70</td>
<td>0.09</td>
<td>0.21</td>
<td>3.09</td>
<td>84.0</td>
</tr>
</tbody>
</table>

Figure 3.3  Chromatogram of derivatization mixture of Mmc-O-CO-(CH₂)₃-Ro15-3890, the mobile phase consisted of 56% H₂O, 19% MeOH and 25% ACN.

Therefore, we preferred a higher resolution. The predicted resolution was 2.42 and the predicted capacity factors for Mmc-Ro15-3890 (k₁) and Mmc-O-CO-(CH₂)₃-Ro15-3890 (k₂) were 9.4 and 15.2, respectively. Figure 3.3 shows a chromatogram of the derivatization mixture for these optimal conditions. The retention time of Mmc-Ro15-3890 was 9.9 min and of Mmc-O-CO-(CH₂)₃-Ro15-3890 was 14.4 min, which corresponds with the capacity factors of 8.9 and of 13.4, respectively.

**Fluorescence characteristics of the fluorescent-labeled desethylflumazenil derivatives**

The optimum excitation and emission wavelengths of the fluorescent-labeled desethylflumazenil derivatives in Tris-HCl buffer (pH 7.4; 50 mM) are reported in Table 3.4. A 5 mM solution of quinine sulphate in 1 N sulphuric acid was used as calibrator (= 100) and
fluorescence signals were measured as relative values compared to the calibrator and are reported in Table 3.4.

**Table 3.4** Fluorescence characteristics of fluorescent-labeled desethylflumazenil derivatives in Tris-HCl buffer (pH 7.4; 50 mM). The molar fluorescence signals of the fluorescent-labeled desethylflumazenil derivatives are expressed relative to the signal of 5 mM quinine sulphate in 1 N sulphuric acid (=100).

<table>
<thead>
<tr>
<th>Relative fluorescence</th>
<th>$\lambda_{ex}$ (nm)</th>
<th>$\lambda_{em}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmc-Ro15-3890</td>
<td>54</td>
<td>330</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890</td>
<td>57</td>
<td>331</td>
</tr>
</tbody>
</table>

**Table 3.5** $K_i$-values of the fluorescent-labeled benzodiazepines and of their parent compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>flumazenil</td>
<td>0.6 nM</td>
</tr>
<tr>
<td>desethylflumazenil (Ro15-3890)</td>
<td>&gt;1 µM</td>
</tr>
<tr>
<td>Mmc-Ro15-3890</td>
<td>121 nM</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890</td>
<td>6.5 nM</td>
</tr>
<tr>
<td>fluorescein-NH-(CH$_2$)$_3$-Ro15-3890 [1]</td>
<td>63 nM</td>
</tr>
<tr>
<td>NBD-NH-(CH$_2$)$_3$-Ro15-3890 [2]</td>
<td>5.7 nM</td>
</tr>
<tr>
<td>1012-S [5]</td>
<td>0.4 nM</td>
</tr>
<tr>
<td>NBD-1012-S [3]</td>
<td>85 nM</td>
</tr>
</tbody>
</table>

**Binding affinities of the fluorescent-labeled desethylflumazenil derivatives**

The affinities of the fluorescent-labeled desethylflumazenil derivatives were calculated from their inhibition curves. The inhibition curves were fitted with the program EBDA-Ligand, V4 (Biosoft, Cambridge, UK) [10] using a one-binding site model. The resulting $K_i$-values are presented in Table 3.5, together with some $K_i$-values from the literature, and the discussion about the most suitable position for labeling and best fluorophore to be used will be done in Chapter 4.

**Acknowledgement**

A.J.R.L. Hulst and E.J. de Vries (Department of Organic Chemistry, University of Groningen, The Netherlands) are thanked for their assistance with the synthesis of Mmc-O-CO-(CH$_2$)$_3$-Ro15-3890. M.M.W.B. Hendriks (Centre of Biometry, Wageningen, The Netherlands) is thanked for performing the chemometrical optimization of the chromatographic system.
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References


