Chapter 6:

SYNTHESIS OF TARGETED FLUORESCENT TRACERS FOR OPTICAL IMAGING

Optical fluorescence imaging is a valuable imaging technique due to its high resolution and sensitivity and its simple instrumentation. As penetration depth is limited, this technique is primarily used for the visualization of superficial structures, e.g. in intraoperative imaging, when targeted, exogenous contrast agents are employed to help the surgeon identify tissues that are otherwise barely distinguishable from the background. Moreover, this modality can be used for the imaging of organs accessible with endoscopy, such as the respiratory tract. In both cases, new fluorescent tracers that bind specifically to the respective tissue of interest are needed in order to broaden the applicability of this useful imaging technique. Hence, this chapter describes our efforts to develop new contrast agents for this purpose.

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INTRODUCTION

Molecular imaging plays a crucial role in modern medicine and several imaging methods are routinely applied in the clinic for diagnosis as well as monitoring of disease progression and treatment efficacy or guidance of surgical interventions. These methods include tomographic imaging, e.g. magnetic resonance imaging (MRI), positron emission tomography (PET) or computed tomography (CT), which offer the advantage of whole body imaging but are also limited by different factors, like poor temporal resolution due to subsequent image-reconstruction, requirement of hazardous radiation (PET), or limited choice of targeted contrast agents (CT, MRI).

Conversely, fluorescence optical imaging overcomes these drawbacks by enabling real-time, high-resolution visualization in the absence of damaging radiation, albeit at the cost of limited penetration depth. Moreover, it stands out due to its economical and straightforward usage.

Optical fluorescence imaging is based on the detection of the spatial distribution of fluorescent dyes that are administered to the patient as contrast agents. Such dyes can be excited with light of an appropriate wavelength and return to the ground state in a radiative fashion, i.e. by emitting light, which can be detected by a fluorescence camera. Fluorescein (Fig. 6.2) is one of such contrast agents, that emits light of $\lambda = 521$ nm after excitation with $\lambda = 494$ nm light. The use of this dye is well established in preclinical research and reactive derivatives are available to couple it to e.g. proteins in a straightforward manner. Conjugation of the dye affords targeted fluorescent agents, enabling the selective imaging of cellular structures of interest. Even though the clinical application of fluorescein is limited because the emission and excitation light is largely absorbed in biological tissue, it has been successfully used for e.g. intraoperative imaging. In that case, the limited penetration depth of the light is a negligible disadvantage.

In the following, the synthesis of different agents for optical fluorescence imaging based on the conjugation of fluorescein with a targeting moiety is described. The applied synthetic strategies rely on the introduction of the fluorescent moiety in the last step via a click reaction. The term click reaction was introduced by Sharpless et al. in 2001 and generally refers to a reaction that is highly selective towards two reacting moieties and hence proceeds also in the presence of many other species, especially biomolecules. A click reaction can generally be performed under simple reaction conditions in benign solvents, such as water, and is high-yielding, wide in scope and easy to purify. The copper(I)-catalyzed azide-alkyne Huisgen cycloaddition (CuAAC) is considered to be the first and most prominent example of this class of reactions. A key step in the further development of azide-alkyne cycloadditions, especially in terms of biocompatibility, was the introduction of a copper-free, strain-promoted version (SPAAC) using cyclooctynes.

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This chapter illustrates how CuAAC and SPAAC can be used for the synthesis of different examples of fluorescent tracers, designed for (i) the intraoperative visualization of parathyroid glands and (ii) the imaging of fungal infections.

DEVELOPMENT OF FLUORESCENT TRACERS FOR THE IMAGING OF PARATHYROID GLANDS

Intraoperative imaging is an emerging modality that helps clinicians to identify target tissue and distinguish it from healthy surrounding tissue during surgery.\textsuperscript{12,13} Oftentimes, making this distinction by the naked eye is very challenging, leading to post-operative complications due to incomplete removal of pathological or inadvertent dissection of healthy tissue. Therefore, clear visualization, achieved by employing targeted fluorescent tracers, is of tremendous help for clinicians and ultimately minimizes the burden for the patients.\textsuperscript{14}

One challenge that is encountered in clinical practice is the identification of parathyroid tissue during thyroid cancer surgery. Thyroid cancer affects mainly young females with an increasing incidence reaching approx. 8.7 cases/100 000 per year.\textsuperscript{15} The majority of the patients is cured by total thyroidectomy, and lymphadenectomy in the case of locoregional lymph node metastasis (affects up to 50% of all cases).\textsuperscript{16,17} During both

Fig. 6.1: First drawing of the human parathyroid glandular anatomy by Ivar Sandström (1852-1889). gl. pth.: parathyroid glands; gl. thyr.: thyroid gland; m. constr. ph. inf.: inferior pharyngeal constrictor muscle. Reproduced from ref. 19.
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surgical procedures, the risk of trauma of the parathyroid glands or their blood supply, resulting in permanent hypoparathyroidism, is very high. This common comorbidity is characterized by dysregulation of calcium homeostasis leading to e.g. tetany, bone pain and depression.\textsuperscript{18} Fig. 6.1 illustrates the anatomy of the parathyroid glands, being small organs of ca. 2-7 mm in length.\textsuperscript{19} Their identification during surgery is generally very difficult.\textsuperscript{20} Therefore, a fluorescent tracer, which selectively binds to those glands would be of immense help for the surgeon to avoid trauma of the respective tissue.

Fig. 6.2 shows the different fluorescent tracers, namely indocyanine green (ICG), methylene blue (MB) and δ-aminolevulinic acid (ALA, which is converted into fluorescent protoporphyrin IX \textit{in vivo}), that have been suggested in literature reports for this purpose.\textsuperscript{21-23} However, their application is essentially limited by e.g. neurotoxicity (MB) or phototoxicity and unreliable imaging performance (ALA).\textsuperscript{24,25} ICG seemed to be a promising candidate, but the imaging is solely based on angiography making use of the high vascularization of the parathyroid glands. As a consequence, distinction of thyroid tissue or other well perfused organs remains problematic.

In order to tackle the problems described above, we aimed to develop a new fluorescent agent binding selectively to parathyroid glands. The design of the tracer was based on cinacalcet, a drug used for the treatment of secondary hyperparathyroidism. Cinacalcet binds selectively to the calcium sensing receptor (CasR) on parathyroid glands.\textsuperscript{26,27} Since CasR is a specific feature of parathyroids,\textsuperscript{28} we hypothesize that using this molecule as a targeting moiety would afford an agent that allows selective imaging of the tissue of interest without labeling adjacent tissue.
RESULTS AND DISCUSSION

Cinacalcet (Fig. 6.3) bears a secondary amine as functional group that would allow modification and attachment of a fluorescent moiety. However, studies on the structure-activity relationship have shown that this amine, in its protonated form, is essential for the binding affinity to CasR. For this reason, we decided to install an additional primary amine group on the molecule for labelling. Previous reports suggest that the naphthalene moiety of cinacalcet is buried in the binding pocket, whereas the other part of the molecule is oriented towards the outside. Based on these findings, the cinacalcet analogues depicted in Fig. 6.3 were designed. Since it is not completely clear, what effect modification of the aliphatic chain has on the binding affinity, we synthesized two analogues varying in the position of the amine substituent. This way we hoped to increase the chances to obtain a labelled agent with preserved affinity. For the ease of synthesis, the two molecules were each synthesized as a mixture of two diastereoisomers with the aim to first evaluate which of the two designs is better in terms of binding to CasR and then proceed with the synthesis of the pure epimers of the respective compound.

A commonly used practice for the conjugation of dyes is to use reactive N-hydroxysuccinimide (NHS) esters of the respective dyes to couple them to the molecule of interest (see chapter 7). However, previous attempts in our group to follow such a procedure (unpublished data) showed that the amide bond is formed with the secondary instead of the primary amine. Circumventing this by installation of a protecting group is not feasible due to difficulties in deprotecting the amine again after labelling without degradation of the often rather unstable dye itself. That is why we installed a handle bearing an azide functionality on the primary amine that allows attachment of the fluorescent moiety via a click reaction, assuring regioselectivity of the labelling.
The desired compounds were synthesized starting from the two isomers with N-Boc-protected primary amine, obtained earlier in our group. After installation of an Fmoc-protecting group on the secondary amine and subsequent deprotection of the primary one, a short alkyl chain with azide functionality was attached to the molecule. Final Fmoc deprotection yielded the two analogues of cinacalcet that could be labelled with fluorescein-alkyne in a CuAAC. Next, the respective conjugates of the two isomers were purified by semi-preparative HPLC and the purity and identity analyzed by UPLC-MS as shown in Fig. 6.5.
In order to evaluate the potential of the synthesized tracers for imaging of PG, their binding affinity towards CasR overexpressing cells was evaluated. To rule out unselective binding of the tested compounds to the cells, the same cell line but not expressing CasR was tested as a control. Initially, 1-FL seemed to selectively bind to the CasR as shown in Fig. 6.6. However, the results were not reproducible neither when the assay was repeated with higher concentrations of the tracers nor in a competitive binding assay with cinacalcet. Since the binding study was performed on genetically modified human embryonic kidney (HEK) cells and not parathyroid cells, we assume that the problem lies in the insufficient display of the CasR on the cell membrane of the cells.

For further investigation, we plan to assess the binding affinity of the synthesized conjugates on human tissue samples comprising parathyroid tissue. Such specimens are available from parathyroidectomy that is performed in cases of hyperparathyroidism. Undoubtedly, a reasonable concern is that the expression of CasR in pathological parathyroid tissue may differ from the one in healthy tissue. In fact, literature studies reveal downregulation of CasR. However, the receptor is still expressed and therefore we believe that this strategy may be used for identifying the conjugate with higher affinity. Subsequently, we aim to proceed with the synthesis of the single epimers of the respective compounds.
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Fig. 6.6: Binding studies of compounds 1-FL and 2-FL to HEK293 cells as control (circles) and engineered HEK293 cells expressing CasR (boxes). The fluorescein signal is depicted on the y-axis and concentration of the respective tracers on the x-axis. a) 1-FL tested in different concentration ranges; b) 2-FL tested in different concentration ranges. The results obtained for the different concentration ranges are not in agreement, pointing to reproducibility issues.

SYNTHESIS OF A FLUORESCENT TRACER FOR THE IMAGING OF FUNGAL INFECTIONS

Mycosis, or fungal infection, is very common in the average population with a prevalence around 25% for mostly harmless, superficial infections. However, fungal pathogens can also cause much more severe invasive infections that go along with serious health conditions and present a big medical burden, especially to immunocompromised patients. Exact numbers are hard to estimate, since fungal infections are largely under-diagnosed. In general, diagnosis is done indirectly based on the interpretation of symptoms, imaging data (CT and X-ray scans), anamnesis or...
by direct detection of the pathogens in e.g. blood or sputum samples from patients. This analysis usually goes along with delayed identification of the infection and furthermore affords unreliable results in many cases. Overall, the available diagnostic means are immensely limited regarding sensitivity, selectivity and/or availability.

In the case of invasive pulmonary aspergillosis caused by *Aspergillus fumigatus*, one of the most common invasive fungal infections, incorrect or delayed diagnosis has lethal consequences in almost all cases. Hence, new bedside diagnostic tools are urgently needed. Recently, optical fluorescence imaging, making use of new endomicroscopy techniques, opened up new possibilities for the examination of the respiratory tract. The use of fluorescently labelled antibodies, binding selectively to *A. fumigatus*, has shown promising preclinical results for the imaging of invasive pulmonary aspergillosis. However, challenges regarding the use of antibodies lie in the relatively high instability and labor-intensive and thus costly production. For this reason, we aimed to develop a new fluorescent probe based on an easily-accessible, stable targeting moiety.

Amphotericin B was one of the first drugs approved for the treatment of invasive fungal infections in humans and for a long time remained the gold standard for antifungal therapy. It binds to the cell membrane of fungi and its antifungal activity is based on pore formation and binding to ergosterol (Fig. 6.7). Ergosterol is a sterol that is abundant in the cell membrane of fungi and protozoa assuming the same role as cholesterol in mammalian cells, rendering it a promising target for the selective imaging of these organisms. Therefore, we decided to base the design of a fluorescent tracer for the diagnosis of mycosis on amphotericin B as will be described in the following part of this chapter.

![Amphotericin B](image)

*Fig. 6.7: Representation of the binding mode of amphotericin B to ergosterol.*

Amphotericin B (Fig. 6.7) bears different functionalities, such as a carboxylic acid and various alcohol groups. However, there is only one amine functionality, making this site attractive for selective modification. As studies on structure-activity relationships suggest that this amine has to be protonated in order to bind to ergosterol, we decided to proceed with a reductive amination to install a linker for the attachment of the fluorescent label. By forming a secondary amine instead of an amide we did not significantly alter the $pK_a$ of the respective ammonium ion.
RESULTS AND DISCUSSION

A polyethylenglycol linker, bearing an azide functionality, was installed at the mentioned position allowing the attachment of a fluorophore to amphotericin B via a click reaction. This linker molecule was chosen due to its similar hydrophilicity to the amino sugar and its sterically undemanding nature resulting in minimal compromise of binding affinity. Accordingly, the respective aldehyde was reacted with amphotericin B in DMF in the presence of HCl using NaBH₃CN as a reducing agent (Fig. 6.8), following an adapted literature procedure. After purification of conjugate 6 by semi-preparative HPLC, a SPAAC was employed to attach the fluorophore. For this purpose, fluorescein...
isothiocyanate (FITC) was previously reacted with dibenzocyclooctyne-amine (DBCO-amine) to afford compound 7, enabling the coupling via a SPAAC. Finally, further purification by semi-preparative HPLC afforded final conjugate 8. Fig. 6.9 shows the chromatograms of the synthesized conjugates.

**Fig. 6.9: UPLC-MS analysis of purified compounds 6-8.** a) chromatograms recorded at λ = 400 nm, the additional peak in chromatogram of compound 7 at RT=9.8 min likely represents fluorescein bearing a free amine; b) mass spectra of the product peaks.

UV-Vis analysis of the substrate and product peaks on an HPLC system with a photodiode array (PDA) detector further confirmed the coupling of amphotericin B and fluorescein. Fig. 6.10 shows the UV-Vis absorbance spectra of compounds 6 (blue), 7 (yellow) and 8 (green). The spectrum of synthesized conjugate 8 is characterized by the distinctive absorption profile of amphotericin B between λ = 330 – 420 nm and of fluorescein with an absorption band with λ\text{max} = 496 nm. Notably, the amphotericin B
moiety seems to absorb more strongly than the fluorescein moiety. The reason for this is that the extinction coefficient of fluorescein depends on the pH: under basic conditions the equilibrium between the open and closed form is shifted towards the highly absorbing open form (see Fig. 6.2). Hence, the low absorption stems from the neutral conditions under which the measurement was performed (water/acetonitrile as eluents). The same effect was observed when a fraction containing product 8, isolated by semi-preparative HPLC, was analyzed by UV Vis spectrometry (Fig. 6.10 inset): Under the acidic purification conditions (water/acetonitrile with 0.1% formic acid) no absorbance above \( \lambda = 470 \) nm was observed, whereas after addition of dil. aq. NaOH, a clear absorption band with \( \lambda_{\text{max}} = 496 \) nm arose.

![Fig. 6.10: UV-Vis analysis of compound 6, 7 and 8. Overlay of the UV-Vis spectra of the corresponding peaks in the HPLC chromatogram. The spectra were normalized to the intensity of the highest absorption band. Inset: Absorption spectrum of an isolated fraction from semi-preparative HPLC purification of compound 8 (dark green) and after addition of dil. aq. NaOH to the same sample (light green).](image)

To date, it was unfortunately not possible to evaluate the potential of 8 for the selective imaging of fungal infections and imaging of invasive pulmonary aspergillosis in particular. The implementation of the experimental set up for assessing the selective binding of the conjugate to \( \text{A. fumiga tus} \) is still in progress and will be performed in the group of Prof. Dr. J.M. van Dijl at the department of Medical Microbiology (UMCG).

Additionally, further research focuses on the installation of alternative dyes to amphotericin B. Towards this end, we attached a DBCO moiety to NIR-fluorescent dye IRdye800CW for the coupling to amphotericin B analogue 6 (see experimental section). The advantages of NIR-fluorescence optical imaging and our further investigations to
synthesize suitable tracers for imaging of fungal and also bacterial infections will be discussed in the following chapter in more detail.

CONCLUSION

In conclusion, different potential tracers for optical fluorescence imaging of (i) parathyroid glands and (ii) fungal infections have been designed and successfully synthesized. The fluorescent moieties were introduced in the last synthetic step via a CuAAC or SPAAC respectively, in aqueous medium. The bio-orthogonality of this reaction, in particular the SPAAC used for the synthesis of the amphotericin B derivative, offers unique possibilities for the application also in biomedical research. Above that, fluorescein (or FITC) is widely available and affordable, expanding the applicability of this strategy. At this point, the purification, achieved by semi-preparative HPLC, presents the limiting factor in terms of scalability of synthesis and isolation of the conjugates.

Unfortunately, the evaluation of the synthesized tracers is still outstanding due to time constraints and diverse problems with the biological assays. In the future, we aim to revise the experimental setup of the assays in order to assess the binding affinity of the conjugates to their respective targets and to be able to critically evaluate and potentially optimize the molecular designs.

Generally, fluorescein is not considered optimal for in vivo imaging as the excitation and emission wavelength lie in the visible light range and it suffers from photobleaching. As indicated previously, new NIR dyes enable imaging of deeper lying structures and are therefore preferred. In view of these considerations, we proceeded with the synthesis of targeted NIR tracers, as described in the following chapter.

AUTHOR CONTRIBUTIONS

F. Reeßing: Synthesis, purification and analysis of compounds 1-FL, 2-FL, 1, 3, 4, 6, 7 and 8
Prof. Dr. W. Szymański: Synthesis, purification and analysis of compounds 2, 5 and S1-S4
D. Samplonius: Biological testing of 1-FL and 2-FL.

EXPERIMENTAL SECTION

GENERAL REMARKS

Starting materials, reagents and solvents were purchased from Sigma–Aldrich, Acros and Combi-Blocks and were used without any additional purification. Solvents for the reactions were purified by passage through solvent purification columns (MBraun SPS-800). The reaction progress was monitored by Thin Layer
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Chromatography (TLC). TLC analyses were performed on commercial Kieselgel 60, F_{254} silica gel plates with fluorescence-indicator UV254 (Merck, TLC silica gel 60 F_{254}). For detection of components, UV light at λ = 254 nm or λ = 365 nm was used. Flash column chromatography was performed with Silica gel, pore size 60 Å, 40-63 μm particle size.

Nuclear Magnetic Resonance spectra were measured with an Agilent Technologies 400-MR (400/54 Premium Shielded) spectrometer (400 MHz) or Bruker Avance 600 NMR Spectrometer (600MHz). All spectra were measured at room temperature (22–24 °C). The multiplicities of the signals are denoted by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). All \(^{13}\)C-NMR spectra are 1H-broadband decoupled. High-resolution mass spectrometric measurements were performed using a Thermo scientific LTQ OrbitrapXL spectrometer with ESI ionization. The ions are given in m/z-units.

Conjugates 1-FL and 2-FL were purified by reversed phase semi-preparative HPLC on a XTerra Prep MS C18 column (10 μm, 7.8 x 150 mm) with PDA detection. The eluents were acetonitrile and water, both with 0.1% trifluoroacetic acid. Conjugates of amphotericin B were purified by reversed phase semi-preparative HPLC on a Kinetex EVO C18 column (5 μm, 100 Å, 250 x 10.0 mm) with UV detection at 365 nm. The eluents were acetonitrile and water, both with 0.1% formic acid. The elution gradients were established as described below.

UPLC-MS analysis was performed on a Thermo Fisher Scientific Vanquish UPLC System on a reversed phase C18 column (Acquity UPLC HSS T3 1.8 μm, 2.1 x 150 mm), and with an LCQ Fleet mass spectrometer and UV-Vis detector at λ = 254 nm and 400 nm. The eluents were acetonitrile and water, both with 0.1% formic acid. The elution gradient was established from 10% to 90% organic phase.

SYNTHETIC PROCEDURES AND COMPOUND CHARACTERIZATION

\[ \text{S1: 3-Amino-3-(3-(trifluoromethyl)phenyl)propanoic acid.} \]

A solution of 3-trifluoromethyl-benzaldehyde (72.8 mmol, 12.7 g), malonic acid (72.8 mmol, 7.58 g) and ammonium acetate (45 mmol, 11.2 g) in EtOH (60 mL) was
heated at reflux overnight. The product precipitate was filtered off and washed with 
Et₂O, giving white powder (7.16 g, 40%). ¹H NMR (400 MHz, D₂O+K₂CO₃): δ 2.17 –
2.59 (m, 2H), 4.17 (t, J = 7.3 Hz, 1H), 7.30 – 7.42 (m, 1H), 7.42 – 7.50 (m, 2H), 7.54
(s, 1H); ¹⁹F NMR (376 MHz, D₂O+K₂CO₃): -62.3; HRMS (ESI-) calc. for [M-H]⁻
(C₁₀H₉F₃NO₂): 232.0580, found: 232.0594. The crude product was used in the next
step without further characterization.

S2: Methyl 3-((tert-butoxycarbonyl)amino)-3-(3-(trifluoromethyl)phenyl)propanoate.  
A mixture, containing compound 1 (2.00 mmol, 488 mg), Boc₂O (3.00 mmol, 655
mg), NaHCO₃ (3.00 mmol, 252 mg), H₂O (5 mL) and dioxane (5 mL), was stirred at
room temperature for 3 h. The volatiles were evaporated and the residual aqueous
solution was diluted with 0.1 N aq. HCl and extracted with AcOEt (2x). The combined
organic layers were dried (MgSO₄) and the solvent was evaporated, yielding crude
Boc-protected product. This compound was dissolved in DCM (20 mL) and MeOH
(0.40 mL) was added, followed by DMAP (25 mg) and EDC (2.80 mmol, 537 mg).
The resulting mixture was stirred at room temperature overnight. Next, the reaction
mixture was diluted with AcOEt and washed with 1 N aq. HCl (3x), sat. aq. NaHCO₃
(2x) and brine. The organic phase was dried (MgSO₄) and the volatiles were
 evaporated. Precipitation from Et₂O/pentane provided product as white powder
(558 mg, 80% yield over two steps). Mp. 68-69 °C; ¹H NMR (400 MHz, CDCl₃):
δ 1.41 (s, 9H), 2.64 – 3.08 (m, 2H), 3.62 (s, 3H), 5.13 (s, 1H), 5.63 (s, 1H), 7.33 – 7.64 (m, 4H);
¹³C NMR (100 MHz, CDCl₃): δ 28.2, 40.5, 50.8, 51.8, 80.0, 122.9 (m), 124.0 (q, J_C-F
= 272.3 Hz), 124.3 (q, J_C-F = 3.8 Hz), 129.1, 129.6, 130.9 (q, J_C-F = 32.3 Hz), 124.4, 155.0,
171.0; ¹⁹F NMR (376 MHz, CDCl₃): -62.7; HRMS (ESI+) calc. for [M+Na]⁺
(C₁₆H₁₂O₃F₃NO₄Na): 370.1236, found: 370.1236.

S3: tert-Butyl (3-oxo-1-(3-(trifluoromethyl)phenyl)propyl)carbamate.  
A solution of compound 2 (1.00 mmol, 347 mg) in dry DCM (5 mL) was stirred under
nitrogen atmosphere at -65 °C. A solution of DIBAL-H (1 M in DCM, 5 mL) was added
dropwise over 10 min. After 4 h stirring at -65 °C, the reaction was quenched by
addition of MeOH (3 mL) and H₂O (1 mL). The resulting solution was warmed up to
room temperature and diluted with AcOEt (100 mL). An aq. solution of Rochelle salt
(100 mL) was added and the biphasic mixture was stirred for 10 min. The phases
were separated and the organic phase was washed with 1 N aq. HCl (3x), sat. aq.
NaHCO₃ (2x) and brine. The organic phase was dried (MgSO₄) and the volatiles were
 evaporated, yielding crude product as a yellow oil (290 mg, 91%). ¹H NMR
(400 MHz, CDCl₃): δ 1.40 (s, 9H), 2.87 – 3.04 (m, 2H), 5.23 (s, 3H), 5.37 (s, 1H), 7.45
– 7.56 (m, 4H); ¹⁹F NMR (376 MHz, CDCl₃): -62.7.
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3: tert-Butyl (3-((((R)-1-(naphthalen-1-yl)ethyl)amino)-1-(3-(trifluoromethyl)phenyl)-propyl)carbamate.

A solution of compound 3 (0.91 mmol, 290 mg), (R)-1-(naphthalen-1-yl)ethan-1-amine (0.85 mmol, 146 mg) and sodium triacetoxyborohydride (1.20 mmol, 254 mg) in dry DCM (15 mL) was stirred at room temperature overnight. Sat. aq. NaHCO$_3$ (4 mL) was added; the phases were separated and the aqueous phase was extracted with DCM (15 mL). The combined organic phases were dried (MgSO$_4$) and the product was purified by flash chromatography (DCM/MeOH, 1:0 - 99:6 v/v) to give a clear oil (372 mg, 82%). The product was obtained as a roughly 1:1 mixture of diastereoisomers.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.44 (s, 9H), 1.51 (d, $J = 6.6$ Hz, 1.5H), 1.53 (d, $J = 6.6$ Hz, 1.5H), 1.55 – 1.87 (m, 2H), 1.90-2.05 (m, 2H), 2.52-2.70 (m, 2H), 4.51-4.62 (m, 1H), 4.87 (br s, 1H), 6.44 (br s, 1H), 6.62 (br s, 1H), 7.28-7.57 (m, 7H), 7.62 (t, $J = 7.1$ Hz, 1H), 7.76 (dd, $J = 8.0$, 5.5 Hz, 1H), 7.95 – 7.84 (m, 1H), 8.15 (t, $J = 8.5$ Hz, 1H); $^{19}$F NMR (376 MHz, CDCl$_3$): -62.6, -62.6. HRMS (ESI+) calc. for [M+H]$^+$ (C$_{27}$H$_{32}$F$_3$N$_2$O$_2$): 473.2410, found: 473.2403.

4: (9H-Fluoren-9-yl)methyl (3-amino-3-(3-(trifluoromethyl)phenyl)propyl)((R)-1-(naphthalen-1-yl)ethyl)carbamate

A solution of compound 3 (2.33 mmol, 1.10 g), FmocCl (3.33 mmol, 862 mg) and DIPEA (6.66 mmol, 1.16 mL) in dry DCM (35 mL) was stirred under nitrogen atmosphere at room temperature overnight. The reaction mixture was diluted with AcOEt and washed with 1 N aq. HCl, sat. aq. NaHCO$_3$, and brine. The organic phase was dried (MgSO$_4$) and the product was purified by flash chromatography (pentane/Et$_2$O, 8:2 – 1:1, v/v) to obtain Fmoc-protected 3 (1.88 mmol, 1.31 g). The isolated product (0.94 mmol, 650 mg) was then dissolved in DCM (20 mL). Triisopropyl silane (1.18 mmol, 0.24 mL) and HCl in Et$_2$O (2 m, 20 mL) were added and the reaction mixture was stirred at room temperature overnight. The volatiles were evaporated and Et$_2$O and pentane were added to the residue. The mixture was stirred for 1 h and the precipitate was filtered off to afford compound 4 (464.5 mg, 64% calculated as monochloride salt). The $^1$H NMR spectrum is very complex due to large differences in the signals corresponding to the two different diastereoisomers, probably due to hindered rotation. The product was used in the next step without assignment of the signals. $^{19}$F NMR (376 MHz, CDCl$_3$): -62.1, -62.4; HRMS (ESI+) calc. for [M+H]$^+$ (C$_{37}$H$_{34}$F$_3$N$_2$O$_2$): 595.2567, found: 595.2566.
1: 4-Azido-\(N\)-(3-((R)-1-(naphthalen-1-yl)ethyl)amino)-1-(3-(trifluoromethyl)phenyl) propyl)butanamide

4-Azidobutanoic acid (0.67 mmol, 87.43 mg) was dissolved in 4.5 mL of DCM and oxalyl chloride (1.55 mmol, 0.13 mL) and two drops of DMF were added. The mixture was stirred at room temperature for 40 min. After evaporation of the volatiles under reduced pressure, DCM (4 mL) was added to the residue. The resulting solution was then added to a mixture of compound 4 (0.22 mmol, 131 mg) and DIPEA (0.67 mmol, 0.11 mL) in DCM (8 mL). The reaction mixture was stirred at room temperature for 2 h, after which the solvents were evaporated and the product purified by flash column chromatography (pentane/ACOEt, 4:1 – 1:1 v/v). The obtained product (105 mg) was dissolved in DMF (2 mL). Piperidine (4.6 mmol, 0.45 mL) was added and the mixture was stirred at room temperature for 20 min, after which the volatiles were evaporated. The product was dissolved in chloroform and washed with sat. aq. NaHCO\(_3\) solution to yield compound 1 as a roughly 1:1 mixture of diastereoisomers. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.55 (dd, 2H) 1.83 (q, 2H), 1.93-2.04 (m, 2H), 2.15 (m, 2H), 2.57-2.75 (m, 2H), 3.24-3.27 (m, 2H), 4.58-4.61 (m, 1H), 5.14 (m, 1H), 7.27-7.26 (m, ), 7.66 (d, 1H), 7.78 (t, 1H), 7.89 (t, 1H), 8.00 (s, 1H), 8.11-8.17 (m, 2H).
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(m, 1H); $^{19}$F NMR (376 MHz, CDCl$_3$): -62.5, -62.6; HRMS (ESI+) calc. for [M+H]$^+$ (C$_{26}$H$_{29}$F$_3$N$_5$O): 484.2319, found: 484.2311.

1-FL: 4-(4-(3-(3',6'-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-3-oxopropyl)-1H,1,2,3-triazol-1-yl)-N-(3-((R)-1-(naphthalen-1-yl)ethyl)amino)-1-(3-(trifluoromethyl)phenyl)propyl)butanamide

Copper(II) sulfate (0.02 mmol, 3.6 mg), and sodium ascorbate (0.04 mmol, 8 mg) were dissolved in H$_2$O (1 mL) and the mixture was stirred for 10 min in which the color of the mixture turned from blue to orange. A solution of sulfonated bathophenanthroline (0.01 mmol, 5.4 mL) in H$_2$O (1 mL) was added and the resulting mixture was then added to a solution of compound 1 (0.02 mmol, 10 mg) and fluorescein-alkyne (0.03 mmol, 13 mg) in DMSO (2 mL). The reaction mixture was stirred at room temperature overnight. Next, H$_2$O was added and the precipitate was filtered off. The collected product was then re-dissolved in a mixture of DMSO, H$_2$O and acetonitrile and purified by semi-preparative HPLC (gradient from 5% to 85% organic phase over 45 min).

The collected fractions were lyophilized several times with diluted aq. HCl to obtain the chloride salt of 1-FL.

S4: Methyl (S)-2-((tert-butoxycarbonyl)amino)-3-(3-(trifluoromethyl)phenyl)propanoate

N-Boc protected 3-trifluoromethyl-phenylalanine (1.50 mmol, 500 mg) was dissolved in DCM (15 mL) and MeOH (0.24 mL) was added, followed by DMAP (18 mg) and EDC (2.00 mmol, 384 mg). The resulting mixture was stirred at room temperature overnight. Afterwards, the reaction mixture was diluted with AcOEt and washed with 1 N aq. HCl (3x), sat. aq. NaHCO$_3$ (2x) and brine. The organic phase
was dried (MgSO₄) and the volatiles were evaporated to provide the product as a white powder (480 mg, 92%). Mp. 48-49 °C; ¹H NMR (400 MHz, CDCl₃): δ 1.40 (s, 9H), 3.08 (dd, J = 13.7, 6.0 Hz, 1H), 3.21 (dd, J = 13.7, 5.5 Hz, 1H), 3.71 (s, 3H), 4.55-4.63 (m, 1H), 5.05 (d, J = 7.5 Hz, 1H), 7.30-7.44 (m, 3H), 7.50 (d, J = 7.7 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ 28.2, 38.2, 52.3, 54.2, 80.1, 123.8 (q, J_C-F = 3.7 Hz), 124.0 (q, J_C-F = 273.5 Hz), 126.1, 128.9, 130.7 (q, J_C-F = 31.8 Hz), 132.7, 137.1, 154.9, 171.9; ¹⁹F NMR (376 MHz, CDCl₃): -62.7; HRMS (ESI+) calc. for [M+Na]+ (C₁₆H₂₀F₃NO₄Na): 370.1236, found: 370.1236.

5: tert-Butyl (1-(((R)-1-(naphthalen-1-yl)ethyl)amino)-3-(3-(trifluoromethyl)phenyl)-propan-2-yl)carbamate.

A solution of compound 6 (2.24 mmol, 780 mg) in dry DCM (11 mL) was stirred under nitrogen atmosphere at -65 °C. A solution of DIBAL-H (1 M in DCM, 12 mL) was added. After 4 h stirring at -65 °C, the reaction was quenched by addition of MeOH (6 mL) and H₂O (2 mL). The resulting solution was warmed up to room temperature and diluted with AcOEt (150 mL). Aqueous solution of Rochelle salt (100 mL) was added and the biphasic mixture was stirred for 10 min. The phases were separated and the organic phase was washed with 1 N aq. HCl, sat. aq. NaHCO₃ and brine. The organic phase was dried (MgSO₄) and the volatiles were evaporated. The product was purified by flash chromatography (pentane/Et₂O, 1:1 v/v) to give a white solid (300 mg). The aldehyde product was used in the next step without further purification. A solution of the aldehyde (253 mg), (R)-1-(naphthalen-1-yl)ethan-1-amine (0.78 mmol, 134 mg) and sodium triacetoxyborohydride (1.13 mmol, 240 mg) in dry DCM (17 mL) was stirred under dry nitrogen atmosphere at room temperature overnight. Sat. aq. NaHCO₃ was added, the phases were separated and the aqueous phase was extracted with DCM. The combined organic phases were dried (MgSO₄) and the product was purified by flash chromatography (DCM/MeOH, 1.0 - 98:2 v/v) to give a clear oil (330 mg, 38% overall yield). The product was obtained as a roughly 2:1 mixture of diastereoisomers due to the racemisation of the aldehyde obtained from compound S4. ¹H NMR (400 MHz, CDCl₃): δ 1.20-1.73 (m, 15H), 2.51 (dd, J = 12.4, 6.8 Hz, 1H), 2.57-2.99 (m, 3H), 3.85-3.94 (m, 1H), 4.50-4.56 (m, 1H), 4.71 (s, 1H), 7.24-7.65 (m, 8H), 7.75 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 8.20 (d, J = 7.8 Hz, 1H); ¹⁹F NMR (376 MHz, CDCl₃): -62.5.

Compound 5 was used to synthesize compound 2 in analogous way to the synthesis of compound 1 described above.
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2-FL: 4-(4-(3-(6-Dihydroxy-3-oxo-3H-spiro(isobenzofuran-1,9’-xanthen)-5-yl)amino)-3-oxopropyl)-1H-1,2,3-triazol-1-yl)-N-(1-((R)-1-(naphthalen-1-yl)ethyl)amino)-3-(3-(trifluoromethyl)phenyl)propan-2-yl)butanamide

Copper(II)sulfate (0.02 mmol, 3.6 mg), and sodium ascorbate (0.04 mmol, 8 mg) were dissolved in H$_2$O (1 mL) and stirred for 10 min in which the color of the mixture turned from blue to orange. A solution of sulfonated bathophenanthroline (0.01 mmol, 5.4 mg) in H$_2$O (1 mL) was added and the resulting mixture was then added to a solution of compound 2 (0.02 mmol, 10 mg) and fluorescein-alkyne (0.03 mmol, 12.8 mg) in DMSO (2 mL). The reaction mixture was stirred at room temperature overnight. Subsequently, H$_2$O was added and the precipitate was filtered off. The collected product was then re-dissolved in a mixture of DMSO, H$_2$O and acetonitrile and purified by semi-preparative HPLC (gradient from 5% to 85% organic phase over 45 min). The collected fractions were lyophilized several times with diluted aq. HCl to provide the chloride salt of 2-FL.

6:

Amphotericin B (15 mg, 16 µmol) was dissolved in DMF (1.5 mL). Solutions of 2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetaldehyde (4.2 mg, 19 µmol) in DMF (0.75 mL) and NaBH$_3$CN (1.2 mg, 19 µmol) in DMF (0.25 mL) were added. One drop of diluted aq. HCl (1:5 v/v with DMF) was added and the mixture was stirred at room temperature for 5 h. H$_2$O (1 mL) was added to quench the reaction. The mixture was
filtered (0.45 µm) and the product was purified by semi-preparative HPLC (gradient from 10% to 90% organic phase over 35 min).

**Fig. 6.12**: UPLC MS analysis of compound 6. *a*) chromatogram recorded at $\lambda = 400$ nm; *b*) mass spectrum of the product peak (peak 1).
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7:
Fluorescein isothiocyanate (0.40 mg, 1 µmol) and dibenzocyclooctyne-amine (0.28 mg, 1 µmol) were dissolved in DMSO (0.52 mL) and the solution was left at room temperature for 30 min to afford product 7 with complete conversion of both starting materials. $^1$H-NMR (400 MHz, DMSO-d$_6$): δ 1.95-1.99 (m, 1H), 2.68-2.70 (m, 1H), 3.5 (br s, 2H), 3.65 (d, 1H), 5.06 (d, 1H), 6.52-6.58 (m, 4H), 6.64 (s, 2H), 7.08 (d, 1H), 7.26-7.38 (m, 4H), 7.44-7.48 (m, 4H), 7.61-7.64 (m, 3H), 7.94 (s, 1H), 9.90 (s, 1H); $^{13}$C-NMR (151 MHz, DMSO-d$_6$): δ 34.0, 40.5, 55.5, 102.6, 102.7, 108.5, 110.2, 113.0, 114.7, 114.8, 116.8, 122.0, 123.0, 124.5, 125.7, 127.0, 127.3, 128.2, 128.6, 128.8, 129.5, 129.8, 130.1, 132.9, 141.7, 147.5, 148.9, 151.7, 152.3, 160.0, 169.0, 171.0, 180.6; HRMS (ESI+) calc. for [M+H]$^+$ (C$_{39}$H$_{28}$N$_3$O$_6$S): 666.1693, found: 666.1689.

8:
A solution of compound 6 (1.14 mg, 1.0 µmol) in DMSO (0.26 mL) was added to a solution of compound 7 (0.68 mg, 1.0 µmol) in DMSO (0.52 mL) and stirred at room temperature for 2 h. The product was subsequently purified by semi-preparative HPLC (gradient from 10% to 90% organic phase over 35 min).
Fig. 6.13: UPLC MS analysis of compound 8. a) chromatogram recorded at $\lambda = 400$ nm; b) mass spectrum of the product peak (peak 1).

A solution of DBCO-amine in DMSO (4.3 mM, 10 µL) and a solution of IRdye800CW-NHS ester in DMSO (4.3 mM, 10 µL) were mixed in DMSO and left at room temperature overnight. The next day the reaction mixture was analyzed by UPLC-MS (10 mM triethylamine acetate in water and acetonitrile as eluents) to confirm product formation and additional DBCO-amine was added if necessary.
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Fig. 6.14: UPLC MS analysis of compound 9. a) chromatogram recorded at \( \lambda = 680 \) nm; b) mass spectrum of the product peak (peak 1) recorded in negative mode. Peak 2 corresponds to the free acid of IRdye800CW.

PHOTOMETRIC QUANTIFICATION

The concentrations of conjugates 1-FL and 2-FL, purified by semi-preparative HPLC, lyophilized and redissolved in 10% DMSO in H\(_2\)O, were quantified by measuring the absorbance at \( \lambda = 480 \) nm. The concentrations of the taken samples were calculated using a calibration curve of fluorescein-alkyne, with the assumption that the modification outside the fluorescein moiety does not alter the extinction coefficient at that wavelength. The samples were diluted accordingly to afford the respective solutions for biological testing.

Fig. 6.15: Calibration curve using fluorescein alkyne as a reference for the quantification of 1-FL and 2-FL
**BIOLGICAL TESTING**

Wildtype HEK293 or CasR transfected HEK293 cells ($1.0 \times 10^5$) were incubated in PBS buffer (pH 7.4) with indicated concentrations of either 1-FL or 2-FL. After 30 min, the cells were washed twice with PBS buffer followed by resuspension in 200 µL PBS. Samples were immediately analyzed for fluorescence ($\lambda_{exc} = 488$ nm, detection filter 533/30 nm) on an Accuri C6 (BD Biosciences) flow cytometer.

**REFERENCES**

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