Synthesis and characterization of fluorescent probes for the development of a ceramide synthase FRET-based assay

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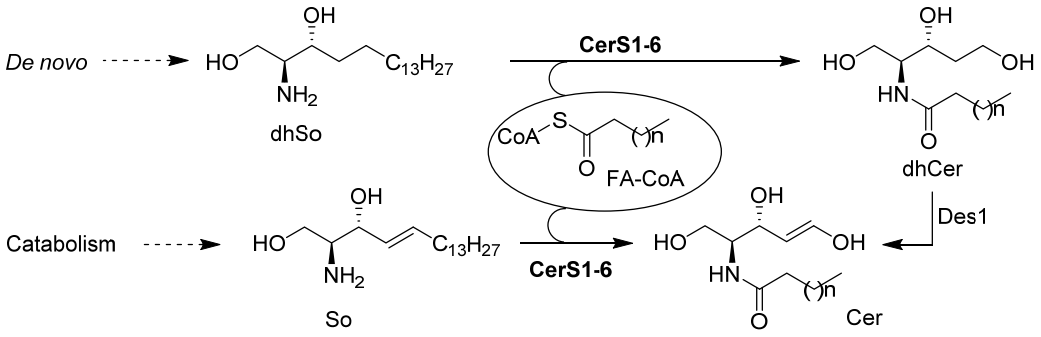
Abstract

Ceramides are recognized to occupy a pivotal position in sphingolipid metabolism, playing a fundamental role as membrane structural components, as well as second messengers. Ceramide synthases (CerS) are a family of enzymes that catalyze the *N*-acylation of sphingosine and dihydrosphingosine, thus controlling the levels of intracellular ceramides. In this work, we present the synthesis and the fluorescent properties of new probes for the development of a FRET-based assay for CerS activity. Based on our previous discovery of spisulosine as a suitable probe for CerS activity in cells, the use of a modified NBD-spisulosine with a clickable fatty acid as CerS substrates may allow the ultimate formation of a bichromophoric reporter for a FRET-based analysis of CerS activity in cells.

Introduction

Sphingolipids (SLs) are one of the major classes of lipids in eukaryotes. Canonical SLs derive from the sphingoid base dihydrosphingosine (dhSo) or (2*R*,3*S*) 2–amino–1,3–octadecanediol, which is metabolically modified to account for the different families of SLs known to date.[1] Among them, ceramides (Cer), arising from the formal *N*-acylation and 4,5-desaturation of dhSo, occupy a pivotal position of the metabolic pathways.[2] Apart from their fundamental structural role in cell membranes,[3] Cer are also important second messengers. In this regard, Cer have been reported to activate apoptosis in response to a variety of cell stress inducing agents.[4–6] Likewise, various types of cancer cells have been shown to reduce their Cer levels as a survival strategy through the overexpression of CDase enzymes.[7] Furthermore, Cer also participate in the regulation of autophagy, and stimulate cell cycle arrest, cell differentiation,[8] and senescence.[9]

The intracellular levels of Cer are the result of the catabolic processes from higher SLs (sphingomyelin, glycosphingolipids and ceramide-1-phosphate), as well from the biosynthesis *de novo* by the *N*-acylation of dhSo with a variety of fatty acids, prior to their desaturation by a specific desaturase (Des1), which introduces a C4(*E*) double bond into the sphingoid base. In this context, ceramide synthases (CerS) are a family of enzymes responsible for the *N*-acylation of dhSo (in the *de novo* pathway) or sphingosine (So) (in the catabolic pathway) to form dhCer and Cer, respectively (Scheme 1).[10]



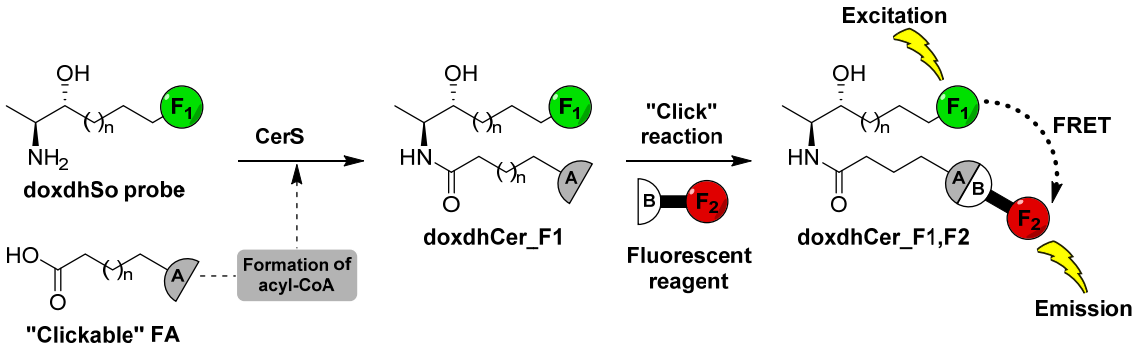
Scheme 1. Metabolic routes leading to Cer

Six isoforms of CerS have been identified in mammals, each one encoded by a unique gene (CerS1–6).[11] CerS enzymes are expressed differently in various tissues[12] and their levels of expression change during development, suggesting that populations of Cer with particular acyl chain lengths might be generated to meet the specific physiological needs of each tissue.[13] Moreover, the nature of the acyl chains is determinant for the biophysical properties of the

resulting Cer and also for the signalling pathways they participate.[14] The development of modern lipidomic techniques[15] has allowed to determine the relative abundance of the various Cer species in a range of biological contexts, and has provided some insight into the effect of the acyl chain composition on the physiological role of Cer.[16] Given the importance of CerS activity in cell fate, we became interested in the development of new chemical probes[17] towards this end. In a previous work, we reported on the use of 1-deoxydihydrosphingosine (doxdhSo, spisulosine or ES285) as a suitable probe for the profiling of CerS activity in intact cells.[18] On the basis that 1-deoxysphingolipids (doxSLs) can be virtually considered as "dead-end" metabolites due to the lack of the C1-OH group, we envisioned that a fluorescent probe derived from spisulosine, together with a suitable fatty acid analogue, could be used to develop a FRET-based assay to monitor CerS activity.

Objectives

Given the roles of CerS in cells, the discovery of new tools to monitor its activity becomes of interest to decipher the molecular mechanisms underlying these processes. For this reason, we are interested in the development of a FRET-based fluorescence assay to determine the activity of CerS, following the general experimental design depicted in Scheme 2.



Scheme 2. Schematic representation of the proposed FRET-based CerS activity assay.

A fluorescently labelled doxdhSo probe and a clickable FA analogue will be sequentially added to cells. Inside the cell, upon the endogenous activation of the FA analogue as a FA-CoA, the two compounds should be enzymatically conjugated by CerS to generate the corresponding doxdhCer_F1. Subsequently, it should be possible to fluorescently label the acyl chain of the doxdhCer molecule by means of a bioorthogonal reaction with a suitable fluorescent reagent. In the bichromophoric doxdhCer molecule, if the two fluorescent groups F1 and F2 are close

enough, upon excitation of the donor, fluorescence emission from the acceptor should be detected, due to FRET. Therefore, by measuring the differences in fluorescence emission arising from FRET we should be able to determine the activity of CerS. Groups A and B, in doxdhCer_F1 and in the fluorescent reagent, must be mutually reactive bioorthogonal groups. Thus, F1 and F2 must be two fluorophores with matching spectral properties, so they form a donor-acceptor FRET pair.

Based on this general scenario, we report on the synthesis and fluorescent properties of the NBD-labelled doxdhSo **RBM5-155** and a series of ω-functionalized fatty acids as potential CerS substrates. The resulting doxdhCer would be amenable to render bioorthogonal click reactions with suitable fluorescent reagents (see Figure 1). Thus, a small collection of differently functionalized fatty acids of diverse chain lengths, together with some suitable reactive partners for bioorthogonal copper-free click reactions (tetrazines for alkenes and bicyclononynes (BCN) for azides) have been synthesized. In addition, the expected CerS metabolites arising from condensation of the NBD probe **RBM5-155** with the ω-azido fatty acid **RBM5-065**, together with their corresponding click adducts with the BCN-derived fluorescent reagents **RBM5-142** and **RBM5-143** have also been synthesized as standards for the assay development that is currently underway in our laboratory.

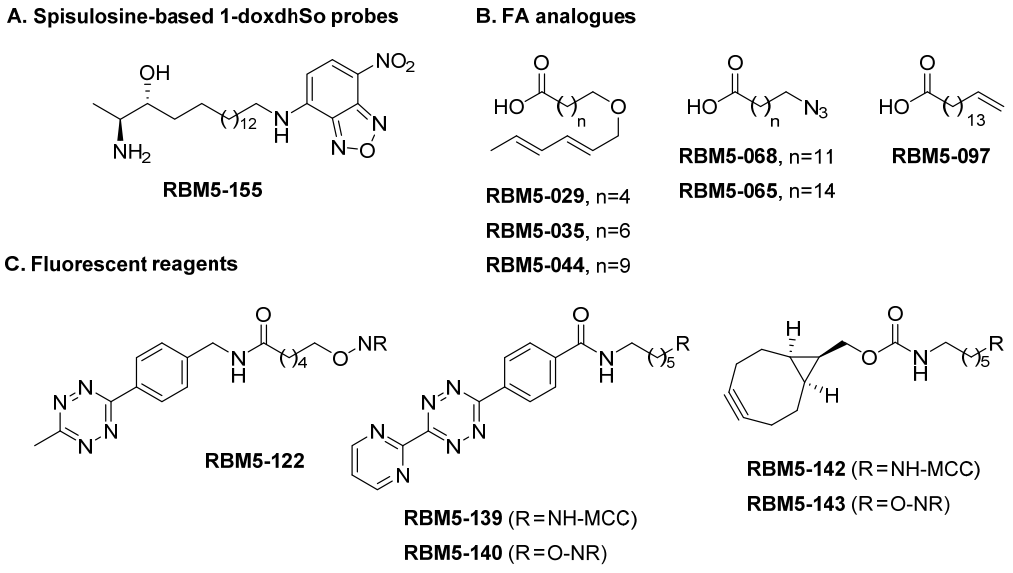


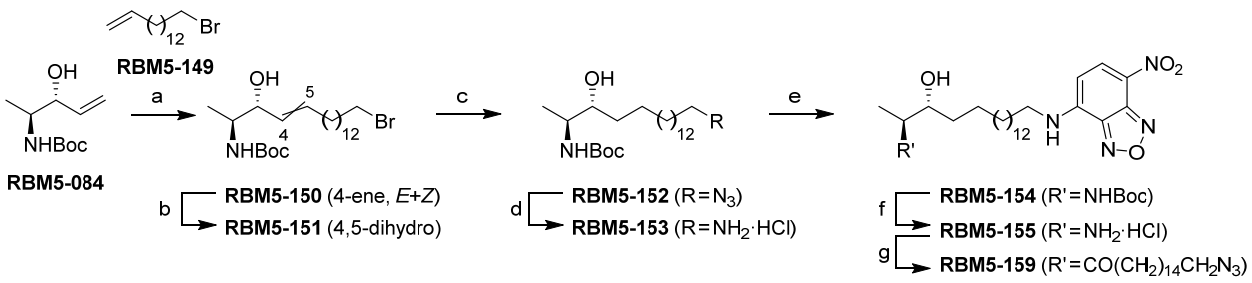
Figure 1. Overview of the different spisulosine-based doxdhSo probes, FA analogues and fluorescent reagents synthesized in this section. NR: Nile red; MCC: 7-methoxycoumarin-3-carboxylic acid

Results and Discussion.

Synthesis of the probes

a) Synthesis of the spisulosine-NBD RBM5-155

The synthesis of the NBD probe **RBM5-155** was carried out as depicted in Scheme 3. Starting from the *anti*-configured allylic alcohol **RBM5-084,**[19] an intermolecular Grubbs' second-generation catalyzed olefin metathesis with bromoalkene **RBM5-149**[20] afforded a highly *E*-enriched *E*:*Z* mixture (**RBM5-150**), in agreement with the literature.[19] This mixture was subjected to catalytic hydrogenation on Rh/Al_2O_3 to give the corresponding saturated ω-bromo intermediate **RBM5-151**. Subsequent nucleophilic displacement of the bromine atom with sodium azide furnished **RBM5-152**. Reduction of the azido group with the Pd/C–TES system[21], followed by reaction of the intermediate amine hydrochloride **RBM5-153** with NBD-Cl,[22] and *N*-Boc removal afforded probe **RBM5-155**, which was isolated and further manipulated as the corresponding hydrochloride, given its apparent sensitivity to the alkaline conditions required for the formation of the free amine.[23] Finally, *N*-acylation of **RBM5-155** with ω-azidopalmitic acid **RBM5-165** afforded the doxdhCer **RBM5-159**, which was required as standard for quantitative lipidomics (LC-MS) assays.



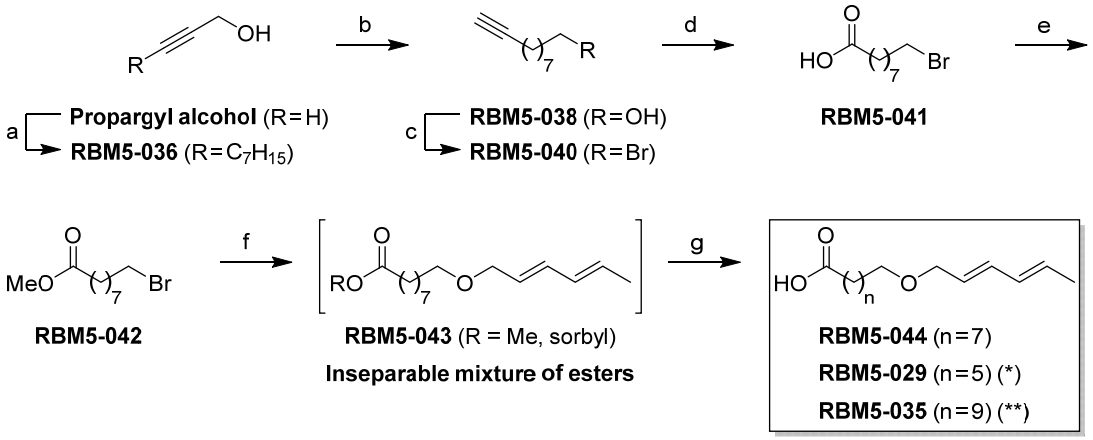
Scheme 3. Synthesis of doxSL probes. Reagents and conditions: (a) **RBM5-149**, Grubbs' 2nd gen. catalyst, CH_2Cl_2, reflux, 2 h, 44 %, *E*/*Z* = 95:5; (b) H_2, 5 wt. % Rh on Al_2O_3, MeOH, rt, 3 h, 86 %; (c) NaN_3, DMF, 80 ºC, 3 h, 95 %; (d) TES, Pd-C, MeOH : $CHCl_3$ (9:1), rt, 10 min, 85 %; (e) NBD-Cl, DIPEA, MeOH, 0 ºC to rt, overnight, 84 %; (f) AcCl, MeOH, 0 ºC to rt, overnight, 80 % (**RBM5-155**); (g) EDC, HOBt, Net_3, CH_2Cl_2, rt, 2 h, 74%

b) Synthesis of the fatty acid analogues

b1) Diene-tagged fatty acids

As shown in Scheme 4, the reaction between the *in situ* generated lithium acetylide of propargyl alcohol with 7-bromoheptane afforded the internal alkyne **RBM5-036**. Subsequent

isomerization of the triple bond with freshly prepared KAPA furnished the ω-alkynol **RBM5-038**.[24] The hydroxyl function was then replaced with a bromide through an Appel-type reaction, followed by the oxidative cleavage of the terminal alkyne with $KMnO_4$ to provide the intermediate **RBM5-041**. This carboxylic acid was refluxed in MeOH, in the presence of catalytic H_2SO_4, to yield the corresponding ω-bromo methyl ester **RBM5-042**, which was spectroscopically in agreement with the literature.[25]



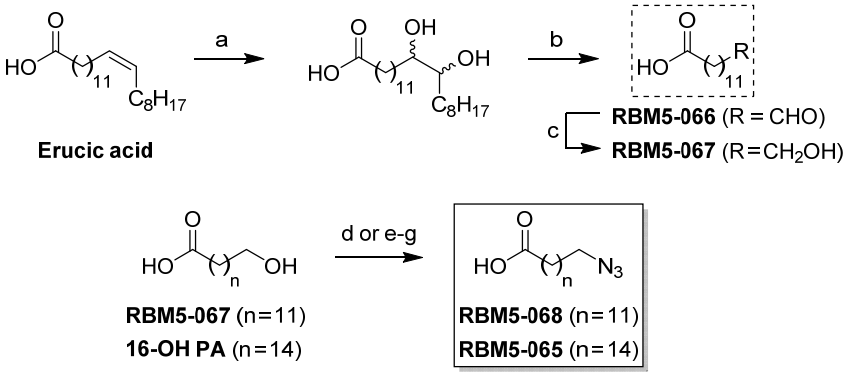
Scheme 4. Synthesis of the diene-tagged FAs **RBM5-029**, **RBM5-035** and **RBM5-44**. Reagents and conditions: (a) (i) *n*-BuLi, THF, HMPA, -78 ºC to -30 ºC, 30 min. (ii) 7-bromoheptane, -30 ºC to rt, overnight, 45 %; (b) Li, KO*t*Bu, 1,3-diaminopropane, 70 ºC to rt, 3 h, 72 %; (c) NBS, PPh_3, DMF, 0 ºC to rt, 1 h, 86 %; (d) $KMnO_4$, CTAB, H_2O/CH_2Cl_2/AcOH (10:4:1), 0 ºC to rt, overnight, 98 %; (e) H_2SO_4, MeOH, reflux, 4 h, 88-98 %; (f) Sorbyl alcohol, NaH, NaI, DMF, 0 ºC to rt, 3 h; (g) LiOH, THF/H_2O (3:1), 0 ºC, 2 h, 34 % (over two steps).

Several reaction conditions were assessed for the Williamson etherification reaction. However, even under the optimized conditions, the desired etherification was accompanied by a partial transesterification to an inseparable mixture of esters that uneventfully underwent alkaline hydrolysis to deliver the desired ω-dienic carboxylic acid **RBM5-044**. Similarly, the dienic acids **RBM5-029** and **RBM5-035** were synthesized from commercially available 7-bromoheptanoic acid and 11-dodecynol.

b2) Azide-tagged fatty acids

Even though many azido-functionalized lipids have already been described in the literature,[26–31] it is still unclear how the azido group emulates a carbon chain, and authors often differ in whether the nitrogen atoms of the azide should or should not be counted when designing an

ω-azido fatty acid analogue of a particular chain length. Therefore, both **RBM5-065** and **RBM5-068** were synthesized as potential palmitic acid surrogates, as shown in Scheme 5.



Scheme 5. Synthesis of the terminal azide-tagged FAs **RBM5-065** and **RBM5-68**. Reagents and conditions: (a) (i) H_2O_2, HCOOH, rt, overnight; (ii) KOH, H_2O, rt, overnight (b) $NaIO_4$, $CHCl_3$, H_2O, rt, overnight, 67 % (over three steps); (c) $NaBH_4$, EtOH, rt, 2 h, 85 %; (d) (i) NBS, PPh_3, DMF, 0 ºC to rt, 1 h, (ii) NaN_3, DMF, 80 ºC, 3 h, 59 % (over two steps) (**RBM-068**). For **RBM5-065**: (e) H_2SO_4, MeOH, reflux, 4 h, 78 %; (f) DPPA, DBU, DMF, rt, overnight, 81 %; (g) LiOH, THF : H_2O (3:1), 0 ºC, 2 h, 78 %

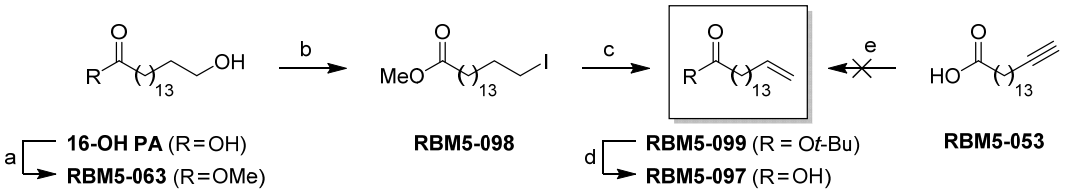
Starting from erucic acid (**¡Error! No se encuentra el origen de la referencia.**), epoxidation using hydrogen peroxide, followed by the alkaline ring opening,[32] gave a mixture of diastereomeric vicinal diols that was directly subjected to pinacol cleavage with $NaIO_4$ to furnish **RBM5-066** in 67 % yield over three steps. Subsequent hydride reduction of the aldehyde group gave the corresponding ω-hydroxyacid **RBM5-067**.[33] Finally, the primary alcohol was sequentially treated with NBS, PPh_3 and NaN_3 in DMF to form the ω-azido derivative **RBM5-068**.

On the other hand, commercial 16-hydroxypalmitic acid was converted into the corresponding methyl ester **RBM5-063** by means of standard protocols. Subsequently, the reaction with diphenylphosphoryl azide (DPPA) in the presence of DBU,[34] followed by the alkaline hydrolysis of the ester delivered the remaining azide-tagged fatty acid **RBM5-065**[30].

b3) Alkene-tagged fatty acids

Initially, we envisioned that the partial hydrogenation of the triple bond in **RBM5-053** (Scheme6, step e) using Lindlar's catalyst would be the most direct way to access the ω-alkene FA **RBM5-097**. However, as previously reported by Crombie *et al.*,[35] this methodology is far from optimal. First, reaction completion could not be achieved, even after long reaction times

and using high catalyst loadings, as determined by the observation in the ^{1}HNMR spectrum of a persistent triplet signal at 2.18 ppm, corresponding to the terminal alkyne CH. Moreover, the formation of a small amount of the fully saturated by-product was unavoidable, as evidenced by the appearance of a triplet at 0.88 ppm in the ^{1}H NMR spectrum, corresponding to the terminal CH_3. The reaction, thus, produced mixtures of **RBM5-097**, palmitic acid and unreacted **RBM5-053**, which were impossible to separate, even as the corresponding methyl esters, in different solvent systems. Taking all the above in consideration, we soon decided to change our strategy to rely on the base-promoted elimination of an appropriate terminal haloalkane, as reported by Hostetler *et al.*[20] Hereby, commercially available 16-hexadecanoic acid was converted into the corresponding methyl ester **RBM5-063** under typical Fischer esterification conditions. Subsequent iodination of the primary alcohol using NIS/PPh_3 gave the *ω*-iodoester **RBM5-098** in excellent yields. As expected, the treatment with an excess of KO*t*-Bu resulted both in the elimination of HI and the transesterification of the methyl ester to form **RBM5-099**. Finally, the acid-mediated removal of the *tert*-butyl ester group delivered the desired alkenyl FA **RBM5-097**.

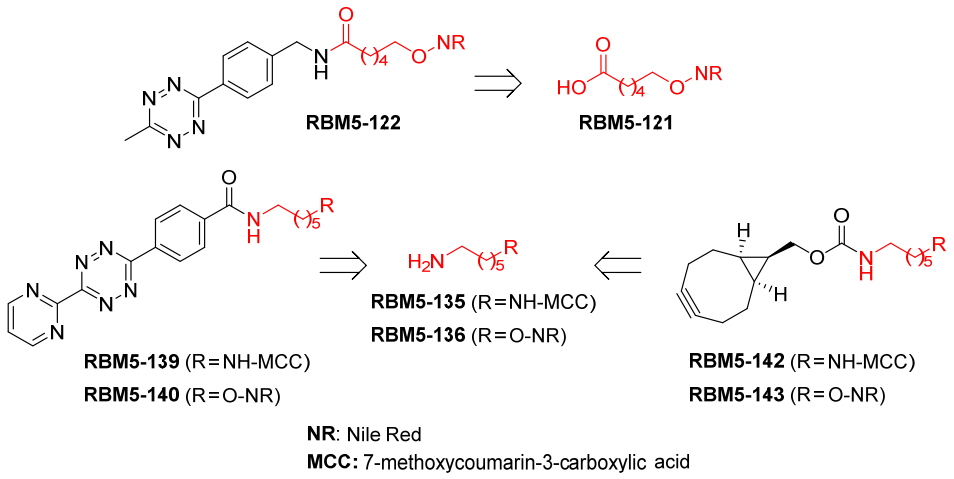


Scheme 6. Synthesis of the terminal alkene FA **RBM5-097**. Reagents and conditions: (a) H_2SO_4, MeOH, reflux, overnight, 93 %; (b) NIS, PPh_3, CH_2Cl_2, 0 ºC to rt, overnight, 98 %; (c) KO*t*Bu, THF, rt, 3 h, 78 %; (d) TFA : CH_2Cl_2 (1:1), 0 ºC to rt, 2 h, 84 %; (e) H_2, Pd-$CaCO_3$-quinoline, EtOAc, rt, 2 h.

c) Synthesis of tetrazine and BCN-derived fluorescent reagents

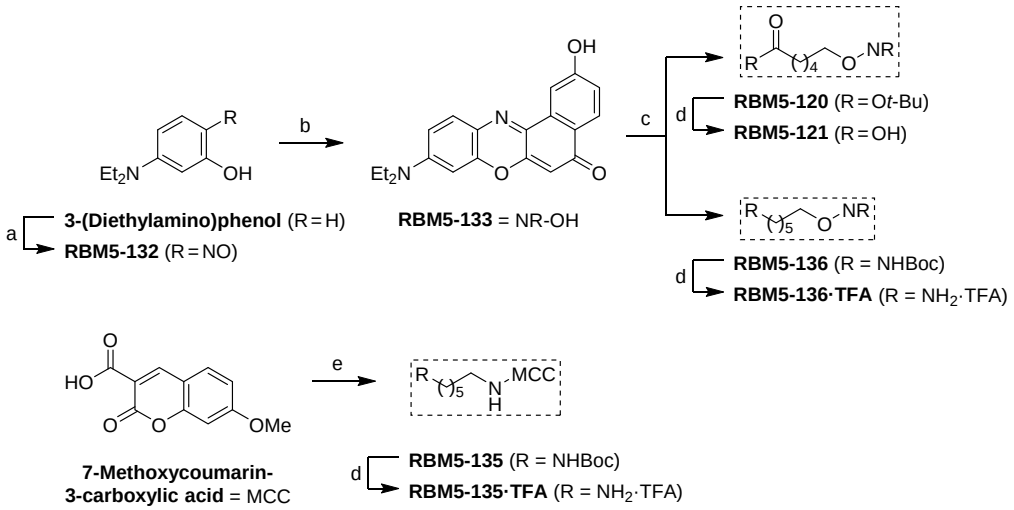
c1) Synthesis of the fluorescent moiety

As summarized in Scheme 7, the synthesis of the fluorescent reagents was envisioned by condensation of the fluorescent moieties **RBM5-121**, **RBM5-135** and **RBM5-136** with the appropriate tetrazine or bicyclononyne scaffolds.



Scheme 7. General strategy for the synthesis of the fluorescent reagents

The synthesis of the precursors **RBM5-121** and **RBM5-136** (Scheme 8) began with the preparation of Nile red, as reported by Yang *et al.*[36] Next, the nucleophilic displacement of the bromide group in either *tert*-butyl 6-bromohexanoate or *N*-Boc-6-bromohexanamine by the potassium phenoxide of **RBM5-133** furnished the ethers **RBM5-120** and **RBM5-136**, respectively. Further acidic treatment delivered the corresponding unprotected precursors **RBM5-121** and **RBM5-136·TFA** in excellent yields (Scheme 8). As expected, the EDC-HOBt amide coupling between 7-methoxycoumarin-3-carboxylic acid and *N*-Boc-1,6-hexanediamine, followed by the TFA-mediated removal of the Boc amino protecting group gave the remaining amine precursor **RBM5-135·TFA.**

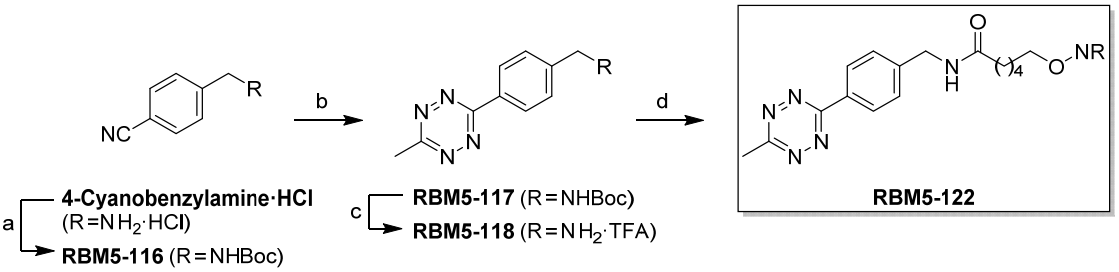


Scheme 8. Synthesis of the precursors **RBM5-121**, **RBM5-135** and **RBM5-136**. Reagents and conditions: (a) $NaNO_2$, HCl (aq.), 0 ºC, 5 h, 67 %; (b) naphthalene-1,6-diol, DMF, 160 ºC, 4 h, 19 %; (c) For **RBM5-136**, *N*-Boc-6-bromohexanamine, K_2CO_3, DMF, 85 ºC, overnight, 78 %; for **RBM5-120**,

tert-butyl 6-bromohexanoate, K_2CO_3, DMF, 85 ºC, overnight, 82 %; (d) TFA : CH_2Cl_2 (1:2), 0 ºC to rt, 1 h, quantitative; (e) *N*-Boc-1,6-hexanediamine, EDC, HOBt, NEt_3, CH_2Cl_2, rt, 2 h, 57 %

c2) Synthesis of the tetrazines RBM5-122, RBM5-139, and RBM5-140

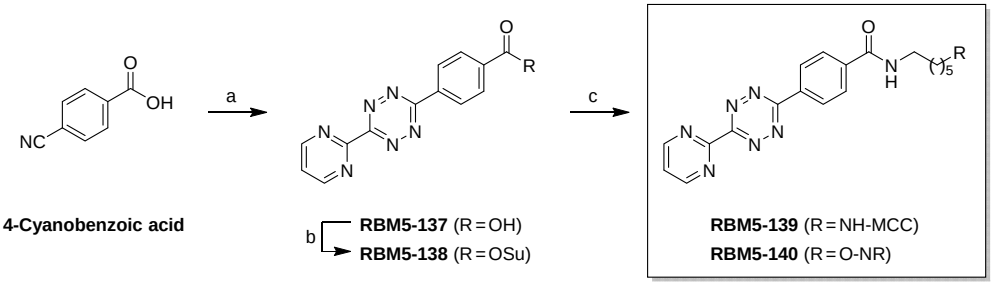
The synthesis of **RBM5-122** (Scheme 9) began with the treatment of 4-cyanobenzylamine hydrochloride with Boc anhydride in the presence of TEA to give the protected amine **RBM5-116**, which was reacted with acetonitrile and hydrazine hydrate under Ni catalysis to form the 1,2,4,5-tetrazine **RBM5-117**.[37,38] The formation of the unwanted symmetrical diaryl tetrazine was avoided by using an excess of acetonitrile. This resulted in the production of a substantial amount of the symmetrical dimethyl tetrazine, which could be easily removed taking advantage of its high volatility.[39] Subsequent removal of the *tert*-butyl carbamate moiety under acidic conditions, followed by an amide coupling with the carboxylic acid **RBM5-121** using EDC and HOBt as the coupling agents delivered the first tetrazine-based fluorescent reagent **RBM5-122**.



Scheme 9. Synthesis of the tetrazine-based fluorescent reagent **RBM5-122**. Reagents and conditions: (a) Boc_2O, NEt_3, CH_2Cl_2, 0 ºC to rt, overnight, 98 %; (b) (i) ACN, $NiCl_2$, hydrazine hydrate, 60 ºC, overnight, (ii) $NaNO_2$, HCl (aq.), 0 ºC, 2 h, 49 %; (c) TFA : CH_2Cl_2 (1:1), 0 ºC to rt, 2 h, 96 %; (d) **RBM5-121**, EDC, HOBt, NEt_3, CH_2Cl_2, rt, 2 h, 44 %.

The preparation of the remaining tetrazines was carried out as reported by Beckmann *et al* [40] (Scheme 10). Thereby, 4-cyanobenzoic acid, 2-cyanopyrimidine and hydrazine hydrate were refluxed in EtOH, without the use of any metal catalyst, to produce a mixture of tetrazines that, in this case, required a much more elaborated work-up to isolate the desired asymmetric tetrazine **RBM5-137**. Next, the carboxylic acid was converted into the corresponding activated *N*-hydroxysuccinimidyl ester **RBM5-138**, which readily underwent an addition-elimination reaction with the amines **RBM5-135·TFA** and **RBM5-136·TFA**, upon addition of triethylamine, to provide the desired dyes **RBM5-139** and **RBM5-140**. The low isolated yield

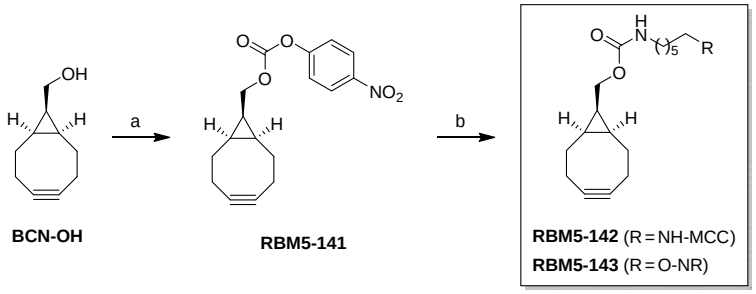
observed for **RBM5-139** was due to its poor solubility in a wide range of organic solvents, which caused a low recovery during the work-up and chromatographic purification steps.



Scheme 10. Synthesis of the tetrazine-based fluorescent dyes **RBM5-139** and **RBM5-140**. Reagents and conditions: (a) (i) 2-cyanopyrimidine, hydrazine hydrate, EtOH, reflux, overnight, (ii) $NaNO_2$, AcOH, 0 ºC, 2 h, 21 %; (b) HOSu, EDC, DMSO, pyridine, 40 ºC, 3 h, 80 %; (c) For **RBM5-139**: **RBM5-135·TFA**, NEt_3, CH_2Cl_2, rt, overnight, 25 %; **RBM5-140** was obtained from **RBM5-136·TFA** following the same procedure (95 %).

c3) Synthesis of the bicyclononynes RBM5-142 and RBM5-143

Both BCN-based fluorescent reagents **RBM5-142** and **RBM5-143** were obtained in excellent yields by reaction of the amine precursors **RBM5-135·TFA** and **RBM5-136·TFA** with the *p*-nitrophenyl carbonate mixed ester **RBM5-141**[41] (Scheme 11). These compounds were spectroscopically characterized in DMSO-d_6, since a substantial decomposition of the cycloalkyne moiety was noticed when using $CDCl_3$.

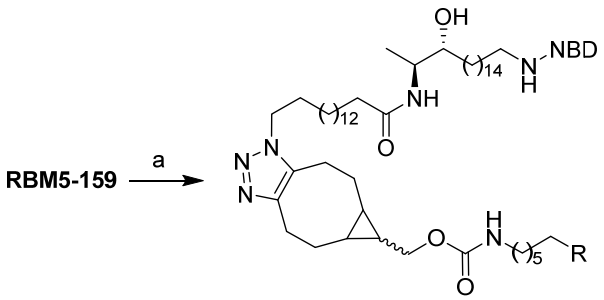


Scheme 11. Synthesis of the BCN-based fluorescent reagents **RBM5-142** and **RBM5-143**. Reagents and conditions: (a) 4-nitrophenyl chloroformate, pyridine, CH_2Cl_2, rt, 30 min, 84 %; (b) For **RBM5-142**: **RBM5-135·TFA**, NEt_3, CH_2Cl_2, rt, overnight, 89 %; **RBM5-143** was obtained from **RBM5-136·TFA** and **RBM5-141** following the same procedure (90 %).

d) Synthesis of doxdhCer by SPAAC reactions

The expected click adducts arising from the condensation of the NBDdoxCer **RBM5-159** with the fluorescent reagents **RBM5-142** and **RBM5-143** were also prepared for their complete

photochemical characterization and FRET studies, as well as standards for LC-MS quantification in the cell assay optimization. They were obtained by a strain-promoted alkyne-azide cycloaddition (SPAAC) reaction between NBDdoxdhCer **RBM5-159** and the fluorescent reagents **RBM5-142** (MCC) and **RBM5-143** (NR) (Scheme 12).



Scheme 12. Reagents and conditions: (a) **RBM5-142** (for **RBM5-160**) or **RBM5-143** (for **RBM5-161**), CH_2Cl_2, rt, overnight, 84-93 %.

Fluorescence properties and FRET efficiency of the bichromophoric probes RBM5-160 and RBM5-161

After confirming the suitability of the selected fluorescent partners for their use in FRET experiments by calculation of the spectral overlap integral and Förster radius (R_0) the normalised absorption and emission spectra of the compounds **RBM5-160** and **RBM5-161** in DMSO, EtOH and PBS buffer were determined (Figure 2). As expected, the absorption spectra of these compounds presented two bands owing to the presence of the two fluorescent labels. Compound **RBM5-160** present two maxima at around 350 nm (MCC moiety) and 470 nm (NBD moiety). For compound **RBM5-161**, the maxima were located around 485 nm (NBD moiety) and 550 nm (NR moiety).

The fluorescence intensity was strongly affected by the solvent, the highest fluorescence intensities being recorded in EtOH for both compounds. However, their emission in PBS buffer was significantly reduced as a result of their low aqueous solubility. The position and shape of the spectra in both compounds were slightly modified in the different solvents, due to solvatochromic effects. Furthermore, there were also considerable deviations in the absorption spectra of compounds **RBM5-160** and **RBM5-161**, when compared with the spectra of the related monochromophoric compounds, probably due to the intramolecular attractive interactions between the two fluorophores in each compound.[42].

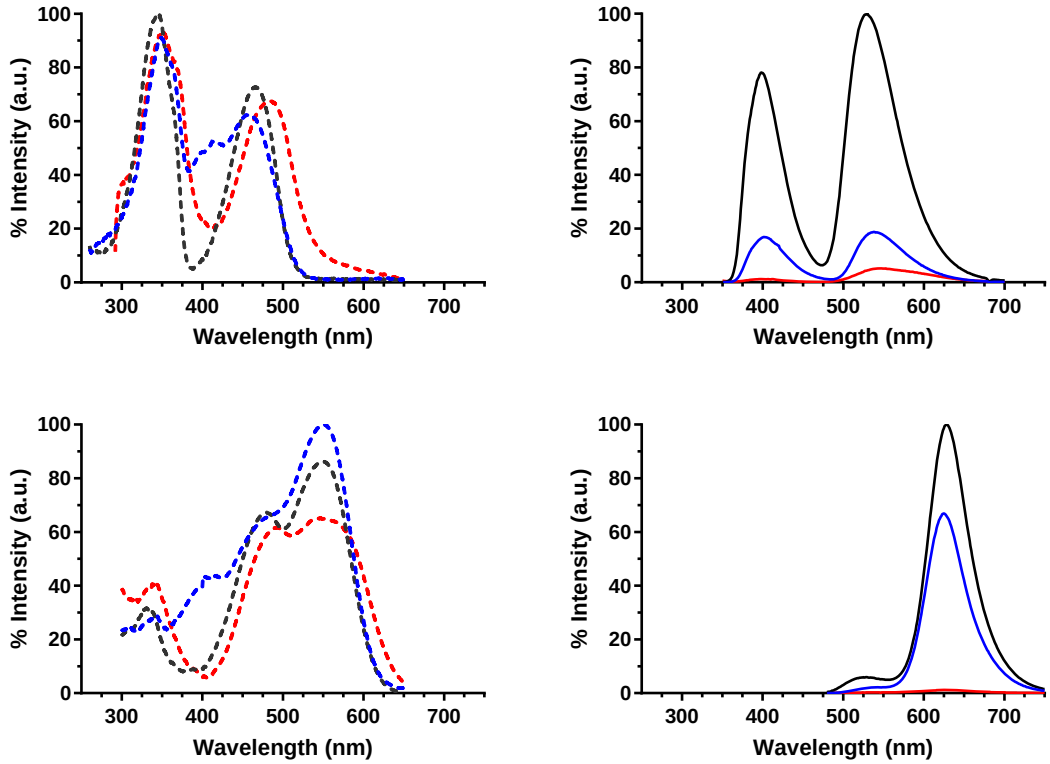


Figure 2. Normalised absorption (left panels) and emission (right panels) spectra for the bichromophoric compounds **RBM5-160** (top) and **RBM5-161** (bottom) at 5 µM in DMSO (blue), EtOH (black) and PBS (red). Excitation at 340 nm (MCC) and 455 nm (NBD), respectively. The absorption and emission spectra of **RBM5-160** in DMSO and PBS were normalized to those of the same compound in EtOH. The absorption spectra of **RBM5-161** in EtOH and PBS were normalized to those in DMSO. The emission spectra of **RBM5-161** in DMSO and PBS were normalized to those in EtOH.

The intramolecular FRET efficiencies of compounds **RBM5-160** and **RBM5-161** were estimated in DMSO and EtOH based on the loss of donor fluorescence in the presence of the acceptor. To this end, we compared the integrated fluorescence intensities (*I*), within the donor-specific wavelength interval, of the donor-alone (D) compounds (**RBM5-142** and **RBM5-154**) to those of the related donor+acceptor (DA) compounds (**RBM5-160** and **RBM5-161**, respectively). The pair **RBM5-142 / RBM5-160** was studied at one sole excitation wavelength (340 nm), whereas the pair **RBM5-154 / RBM5-161** was studied at two different excitation wavelengths (455 nm and 470 nm). The donor-specific emission wavelength intervals used for the former pair were [365,495] in DMSO and [360,490] in EtOH, whereas those used for the latter pair were [490,670] in DMSO and [480,660] in EtOH. A deconvolution process to correct for the overlapping of the emission bands of the donor and the acceptor components of **RBM5-160** and **RBM5-161** was required prior to their use for the calculation of the FRET efficiency

(see Supporting). As expected from the R_0 values, the calculated FRET efficiency of the NBD/NR pair ($E_{NBD/NR}$ = 0.88-0.96) was higher than that of the MCC/NBD pair ($E_{MCC/NBD}$ = 0.56-0.88). For both fluorophore pairs, the FRET process was more efficient in EtOH than in DMSO and, in the case of the NBD/NR pair, the two studied excitation wavelengths gave the same E value (Table 1).

Table 1. Study of the intramolecular FRET process of the bichromophoric compounds **RBM5-160** and **RBM5-161**. [a] FRET efficiencies (E) were calculated from the decrease of the donor emission; [b] Donor emission bleed-through is expressed as a percentage over the total integrated fluorescence intensity observed within the acceptor-specific emission wavelength interval. [c] Acceptor emission bleed-through is expressed as a percentage over the total integrated fluorescence intensity observed within the acceptor-specific emission wavelength interval. The values outside and inside the parentheses were calculated from the "ratio A" and "ratio B" coefficients, respectively.

Compound	Solvent	λ_{ex} (nm)	E[a]	DEB[b] (%)	AEB[c] (%)
RBM5-160	DMSO	340	0.56	0.73	44.17 (44.01)
	EtOH	340	0.86	0.65	28.42 (28.41)
RBM5-161	DMSO	470	0.90	1.74	46.14 (46.36)
		455	0.88	1.92	39.01 (39.03)
	EtOH	470	0.96	1.73	36.28 (36.33)
		455	0.96	1.93	24.44 (24.48)

Biomimetic SPAAC reaction between RBM5-159 and the fluorescent BCN reagents

The SPAAC reaction between the azide-tagged doxdhCer **RBM5-159** and the two BCN-tagged fluorescent dyes **RBM5-142** and **RBM5-143** (Scheme 12) was studied under similar dilution conditions to those applicable in the CerS assay. The first assays were carried out in DMSO at different reaction times and reagent ratios. The reaction progress was monitored by analyzing the changes in the fluorescence emission of the mixture upon irradiation at λex=455 (MCC excitation). In general terms, for **RBM5-160** (donor: MCC, acceptor: NBD). the fluorescence emission at λem=535 (NBD emission) at single concentrations of **RBM5-159** (5 and 20 μM) and increasing concentrations of the BCN-MCC reagent **RBM5-142** (from 5 to 50 μM) was

always lower than that of a standard of **RBM5-160** at 5 and 20 μM (results not shown). However, a similar experiment using the BCN-NR reagent **RBM5-143** (from 5 to 50 μM) led to fluorescence intensities at λem=625 (NR emission) comparable to those of a standard of **RBM5-161** using a 2.5 to 4 fold excess of the BCN reagent **RBM5-143** (Figure 3).

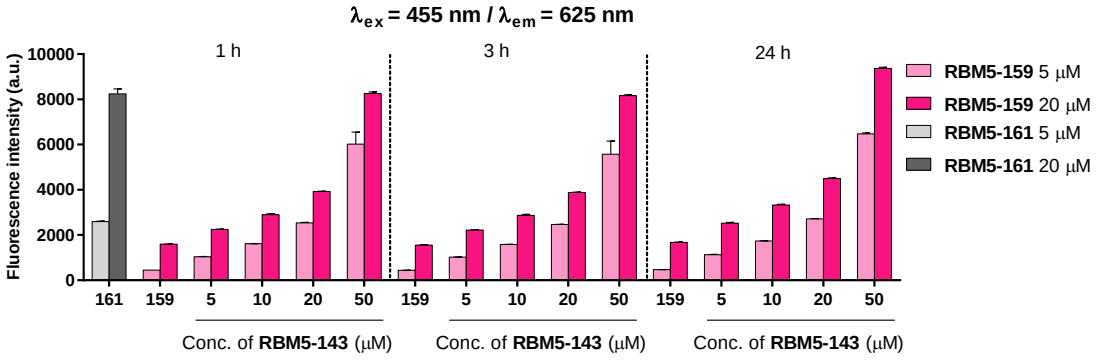


Figure 3. Bar diagram representing the changes in fluorescence emission at 625 nm (bottom), resulting from the excitation at 455 nm, of mixtures containing different ratios of compounds **RBM5-159** and **RBM5-143** in DMSO at various reaction times. Compound **RBM5-159** was used as the negative control, equivalent to 0 % conversion, whereas compound **RBM5-161** was used as the positive control, equivalent to 100 % conversion. The results correspond to the mean ± standard deviation of at least two independent experiments with triplicates.

Experiments in MeOH, EtOH and sodium acetate buffer (NaOAc 250 mM, NaCl 200 mM, 0.1 % Triton X-100) also shown a remarkable enhancement of the emission at 625 nm (λ_{exc} = 455 nm) in comparison with **RBM5-159**, together with an attenuation of the fluorescence emission at 535 nm (λ_{exc} = 455 nm). These results are in agreement with the formation of the desired cycloadduct **RBM5-161**, although a strong background emission for **RBM5-143**, as a result of the excitation cross-talk, was also evident (Figure 4).

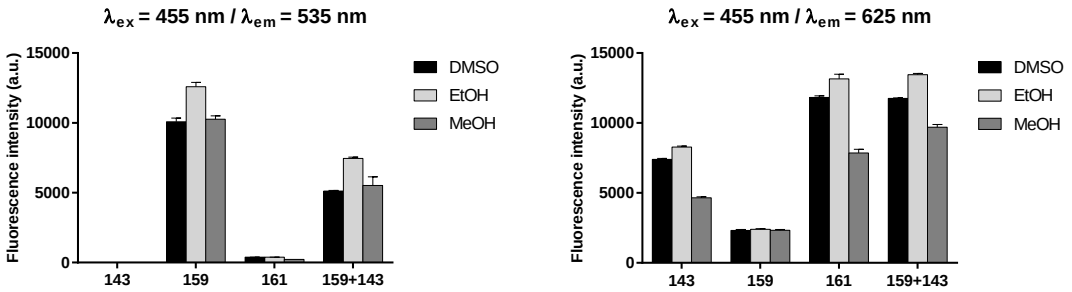


Figure 4. Bar diagram representing the fluorescence emission at 535 nm (left) and 625 nm (right), resulting from the excitation at 455 nm, of a mixture containing the compounds **RBM5-159** (25 μM) and **RBM5-143** (50 μM) after 1 h reaction at 37 ºC in DMSO (black), EtOH (light grey) and MeOH (dark grey). The corresponding starting materials (**RBM5-159** and **RBM5-143** at 25 μM) were used as the negative controls (0 % conversion), whereas the appropriate cycloadduct (**RBM5-161** at 25 μM) was used as the positive control (100 % conversion). The results correspond to the mean ± standard deviation of at least two independent experiments with triplicates.

CerS catalyzed acylation of RBM5-155 with ω-azidopalmitic acid RBM5-065 in cells

The suitability of probes **RBM5-155** and the ωN_3PA **RBM5-065** as CerS substrates was evaluated by examining the extent of their metabolic conversion into the *N*-acylated doxdhCer **RBM5-159**. Initial experiments were carried out in A549 and MEF cells. Even though the total amount of doxdhCers, arising from acylation of **RBM5-155** with endogenous fatty acids, was very similar in the two cell lines (~120-130 pmol equiv / 10^6 cells), the profile of the different doxdhCer species differed considerably. In A549 cells, there was a high proportion of $C18N_3$ and the elongated $C20N_3$ doxdhCer species (by the action of the endogenous elongases[43]), whereas the non-elongated doxdhCer **RBM5-159** accounted for less than a third of the total doxdhCers (~28 %). Conversely, in MEF cells, the non-elongated **RBM5-159** was the most abundant metabolite (85 % of the total doxdhCers), whereas the formation of elongated species was minimal. These results show that the elongation process is unpredictable and highly dependent on the cell line. Attempts to reduce or avoid the acyl chain elongation by using the fatty acid biosynthesis inhibitors TOFA or cerulenin were fruitless.[44–46] For practical purposes, overexpression of the CerS under study may improve the the signal-to-noise ratio of the assay. As a proof of concept, human embryonic kidney cells (HEK293T) were transfected with the human CerS5 gene using the cationic polymer polyethylenimine (PEI),[47] and were subsequently incubated with the doxdhSo probe **RBM5-155** and the *ω*-azidopalmitic acid. UPLC-TOF

analysis of the lipid extracts showed the higher proportion of C16-doxdhCer species in the transfected cells, in agreement with the expected preference of the CerS5 isoform for the C16 fatty acids.[48] (Figure 5)

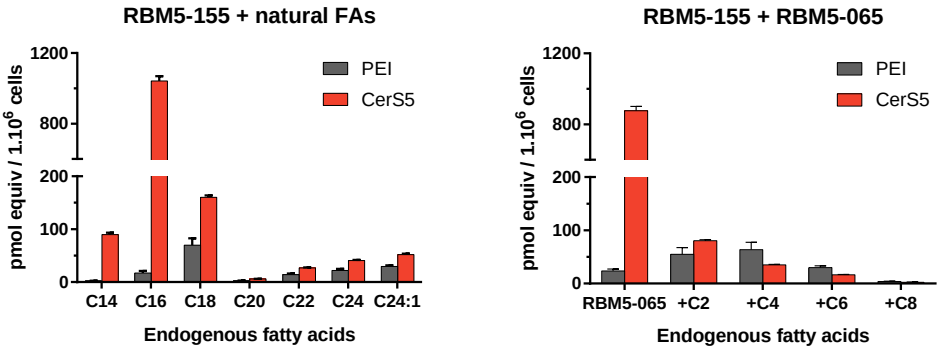


Figure 5. *N*-acylation of the doxdhSo probe **RBM5-155** with the endogenous natural fatty acids or the *ω*-azido fatty acid **RBM5-065**. HEK293T cells transfected with the plasmid containing human CerS5 (bars in orange) or simply treated with the transfection reagent PEI (bars in grey) were incubated for 90 min with **RBM5-155** (5 µM) in the absence (left) or the presence (right) of the ωN$_3$PA **RBM5-065** (0.5 mM) complexed in 0.5% acid-free BSA. After lipid extraction, the different doxdhCer species were quantified by UPLC-TOF. Left: Amount of doxdhCers containing endogenous natural fatty acids; Right: Amount of doxdhCers containing the administered **RBM5-065** and the corresponding elongated fatty acids. The results correspond to the mean ± standard deviation of at least two independent experiments with triplicates

Conclusions

In summary, the NBD probe **RBM5-155** and the modified fatty acid ωN$_3$PA **RBM5-065** are suitable CerS substrates in different cell lines. Despite the degree of incorporation and elongation of the fatty acid is strongly dependent on the particular cell line, studies carried out with the model doxdhCer **RBM5-159** indicated its suitability as a SPAAC reaction partner with the BCN modified Nile Red fluorophore **RBM5-143** to render the bichromophoric doxdhCer **RBM5-160**, exhibiting a high FRET efficiency in the solvent systems used. These results represent a promising starting point for the development of a FRET-based CerS activity assay, which should complement those currently available for this interesting enzyme. Efforts along this line are underway in our lab and will be reported in due course.

Experimental

Chemistry

All chemicals were purchased from commercial sources and used as received unless otherwise noted. Dry solvents (THF, DMF and CH_2Cl_2), obtained from a PureSolv dispenser and subsequently degassed with inert gas, were used in most reactions. Synthesis grade (Hexane, EtOAc, Et_2O and CH_2Cl_2) or HPLC–grade (MeOH) solvents were used for extractions and purifications. Progression of the reactions was controlled by thin layer chromatography (TLC), using ALUGRAM® SIL G/UV254 (Macherey–Nagel) silica gel pre-coated aluminum sheets (Layer: 0.2 mm, silica gel 60). Compounds were detected by using UV light (λ=254 nm) and a stain solution of phosphomolibdic acid (5.7% in EtOH). Usual work up refers to washing of the combined organic layers with brine (2 x 25 mL), drying over anhydrous $MgSO_4$ and concentration to dryness. Compounds were purified by flash column chromatography, using silica gel (Chromatogel 60 Å, 35–75 μm) as stationary phase. Mobile phases and gradients are specified in each case. ^1H and ^{13}C Nuclear Magnetic Resonance spectra were recorded on a Varian – Mercury 400 (^1H NMR at 400 MHz and ^{13}C NMR at 100.6 MHz) spectrometer using $CDCl_3$ or CD_3OD as solvent. Chemical shifts of deuterated solvents were used as internal standards. Chemical shifts are given in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz). Splitting patterns have been described as singlet (s), broad singlet (br s), doublet (d), triplet (t), quartet (q), quintuplet (p), multiplet (m), apparent (app) or combinations of these descriptive names. Fourier transform infrared spectra were recorded on a FTIR Avatar 360 spectrophotometer. Samples were recorded neat using an attenuated total reflectance (ATR) accessory, or as thin films on NaCl plates (specified in each case in the following section). IR frequencies are reported in wave number (cm^{-1}). Specific optical rotations were recorded on a digital Perkin-Elmer 34 polarimeter at 25 ºC in a 1 dm, 1mL cell, using a sodium light lamp (λ=589 nm). Specific optical rotation values ($[\alpha]_D$) are expressed in $deg^{-1}\cdot cm^3\cdot g^{-1}$, and concentrations (c) are reported in g/100 mL of solvent. High–resolution mass spectra were recorded Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters,Milford, MA, USA) operated in positive electrospray ionisation mode. Samples were analyzed by FIA (Flow Injection Analysis), using ACN/water (70:30) as mobile phase. Samples were analyzed using a 10 μL volume injection. M/z ratios are reported as values in atomic mass units.

Absorption spectra were recorded on a Jasco V-730 UV-Vis spectrophotometer using a spectral bandwidth of 1 nm, a response time of 0.24 sec, a data interval of 1 nm (except for quinine sulphate and compound **RBM5-142**, in which case the data interval was of 0.2 nm) and a scan rate of 200 nm/min. Measurements were carried under inert atmosphere (continuous flow of nitrogen gas) at a constant temperature of 20 ºC. The temperature was maintained with a MultiTemp III Thermostatic Circulator from Pharmacia Biotech. Fluorescence emission spectra were recorded on a Photon Technology International (PTI) QuantaMaster fluorometer at room temperature. The excitation and emission monochromators were set at 0.5 mm, giving a spectral bandwidth of 2 nm (except for fluorescein and compounds **RBM5-155** and **RBM5-159**, in which case the monochromators were set at 0.35 mm, giving a spectral bandwidth of 1.4 nm). The data interval was 1 nm and the integration time was 1 sec. All measurements were carried using a Hellma 1.5 mL PTFE-stoppered fluorescence quartz cuvette (4 clear windows) with a 1 cm path length.

General procedures

General procedure 1: Acid-catalysed formation of methyl esters

A solution of the selected carboxylic acid (5 mmol) in MeOH (10 mL) was treated with a catalytic amount of concentrated H_2SO_4 at rt and the mixture was then refluxed for 4 h. The reaction was quenched by the addition of a few drops of saturated aqueous $NaHCO_3$ and the volatiles were removed under reduced pressure. The residue was taken up in water (50 mL), extracted with EtOAc (3 x 50 mL), and the combined extracts were washed with brine (2 x 25 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude mixture was purified by flash chromatography on a silica column to yield the corresponding methyl ester.

General procedure 2: Base-catalysed hydrolysis of esters

LiOH (3 equiv) was added in one portion to a stirred solution of the appropriate ester (0.8 mmol) in THF–H_2O (3:1) (80 mL) at 0 ºC. After stirring at the same temperature for 2 h, the reaction mixture was acidified with 1M aqueous HCl until pH 2 and extracted with EtOAc (3 x 20 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The crude mixture was purified by flash column chromatography to give the corresponding carboxylic acids.

General procedure 3: Base-promoted dehydrohalogenation of terminal haloalkanes

To a freshly prepared 1 M solution of KO*t*Bu (5 equiv) in dry THF (25 mL) was added a solution of the appropriate alkyl halide (5 mmol) in dry THF (5 mL), and the resulting suspension was stirred at rt under argon atmosphere. After 4 h, the reaction was quenched by the slow addition of 1 M aqueous HCl (30 mL) at 0 ºC, and the mixture was extracted with Et_2O (3 x 25 mL). The combined organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$, and concentrated to give a crude, which was purified as indicated for each compound.

General procedure 4: EDC/HOBt coupling for amide–bond formation (EIG290)

EDC·HCl (1.6 equiv) and HOBt (1.3 equiv) were sequentially added to an ice-cooled solution of the corresponding carboxylic acid (1.1 equiv) in anhydrous CH_2Cl_2 (25 mL), and the resulting mixture was vigorously stirred at rt under argon atmosphere. After 15 min, the previous mixture was added dropwise to a solution of the selected amine (5 mmol) and TEA (5 equiv) in anhydrous CH_2Cl_2 (25 mL), and the reaction was stirred at rt for 2 h. The mixture was next diluted with CH_2Cl_2 (50 mL) and washed with brine (2 x 25 mL). The organic layer was dried over $MgSO_4$, filtered, and the volatiles were removed under reduced pressure. Purification of the crude mixture by flash column chromatography afforded the corresponding amide.

Synthesis of the probes

a) Synthesis of NBD probes RBM5-155 and RBM5-159

15-Bromopentadec-1-ene (RBM5-149)

A stirred solution of **RBM5-148** [49] (2.77 g, 12.23 mmol), PPh_3 (3.53 g, 13.46 mmol) in anhydrous CH_2Cl_2 (100 mL) was cooled to 0 ºC, and NBS (2.61 g, 14.68 mmol) was added in small portions over 10 min. The resultant dark yellow solution was allowed to warm to rt and stirred for 1 h, after which the solvent was removed by vacuum evaporation. The residue was then diluted with water (50 mL), extracted with hexanes (3 x 50 mL) and worked-up as usual to give a crude, which was purified by flash chromatography on silica gel (isocratic 100 % hexanes) to afford **RBM5-149** as a colorless oil, (2.62 g, 74 %)

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H), 4.99 (dq, *J* = 17.1, 1.9 Hz, 1H), 4.93 (ddt, *J* = 10.2, 2.4, 1.4 Hz, 1H), 3.41 (t, *J* = 6.9 Hz, 2H), 2.08 – 2.00 (m, 2H), 1.85 (dt, *J* = 14.5, 7.0 Hz, 2H), 1.46 – 1.33 (m, 3H), 1.32 – 1.25 (m, 16H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 139.4, 114.2, 34.2, 34.0, 33.0, 29.8, 29.8, 29.7, 29.7, 29.6, 29.3, 29.1, 28.9, 28.3.

(2'*S*,3'*R*,4'*EZ*) *tert*-butyl (18-Bromo-3-hydroxyoctadec-4-en-2-yl)carbamate (RBM5-150)

To a stirred solution of **RBM5-084**[19,50] (480 mg, 2.38 mmol) and olefin **RBM5-149** (1.59 g, 5.49 mmol) in degassed CH_2Cl_2 (20 mL), 2nd generation Grubbs' catalyst (101 mg, 0.12 mmol) was added in one portion at rt. The resulting mixture was refluxed in the dark for 2 h, cooled to rt and concentrated *in vacuo* to afford a crude, which was purified by flash chromatography on silica gel (from 0 to 5 % MTBE in CH_2Cl_2). Compound **RBM5-150** was obtained as an inseparable mixture of *E*/*Z* isomers (colourless thick oil, 485 mg, 44 %).

^{1}H NMR (400 MHz, $CDCl_3$) (*E* isomer) δ 5.71 (dt, *J* = 14.6, 6.7 Hz, 1H), 5.42 (dt, *J* = 15.0, 7.5 Hz, 1H), 4.76 (br s, 1H), 4.11 (dd, *J* = 6.4, 3.0 Hz, 1H), 3.73 – 3.58 (m, 1H), 3.40 (t, *J* = 6.9 Hz, 2H), 2.09 – 1.96 (m, 2H), 1.85 (dt, *J* = 14.5, 6.9 Hz, 2H), 1.44 (s, 9H), 1.43 – 1.23 (m, 20H), 1.07 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) (mixture of *E*/*Z* isomers) δ 156.1, 155.7, 134.2, 133.5, 129.4, 128.7, 125.6, 124.9, 79.4, 79.2, 75.4, 73.5, 51.0, 50.0, 37.3, 33.9, 33.4, 32.8, 32.6, 32.3, 29.6, 29.5, 29.5, 29.4, 29.2, 28.7, 28.4, 28.1, 15.2, 14.3.

(2'*S*,3'*R*) *tert*-butyl (18-Bromo-3-hydroxyoctadecan-2-yl)carbamate (RBM5-151)

A solution of **RBM5-150** (450 mg, 0.97 mmol) in degassed MeOH (45 mL) was hydrogenated at 1 atm and rt in the presence of Rh/Al_2O_3 (60 mg, 15 % w/w). After stirring for 3 h, the catalyst was removed by filtration through a Celite® pad, and the solid was rinsed with MeOH (3 x 10 mL). The combined filtrates were concentrated *in vacuo*, and the residue was subjected to flash chromatography on silica gel (from 0 to 1 % MeOH in CH_2Cl_2).to yield **RBM5-151** as a white solid (395 mg, 87 %).

$[\alpha]^{20}_D$ = –3.65 (*c* 1, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.74 (br s, 1H), 3.70 – 3.67 (m, 1H), 3.64 (td, *J* = 8.0, 7.2, 2.7 Hz, 1H), 3.41 (t, *J* = 6.9 Hz, 2H), 1.85 (dt, *J* = 14.5, 7.0 Hz, 2H), 1.71 (br s, 1H), 1.44 (s, 9H), 1.43 – 1.35 (m, 4H), 1.34 – 1.23 (m, 22H), 1.08 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.0, 79.6, 74.6, 50.8, 34.2, 33.6, 33.0, 29.8, 29.7, 29.6, 28.9, 28.6, 28.3, 26.2, 14.5.

HRMS calcd. for $C_{23}H_{47}BrNO_3$ ($[M+H]^+$): 464.2734, 466.2713, found: 464.2729, 466.2718.

(2'*S*,3'*R*) *tert*-butyl (18-Azido-3-hydroxyoctadecan-2-yl)carbamate (RBM5-152)

To a stirred solution of **RBM5-151** (395 mg, 0.85 mmol) in anhydrous DMF (8 mL) was added NaN_3 (166 mg, 2.55 mmol). The mixture was heated to 80 ºC and stirred for 3 h under Ar. Water (20 mL) was next added, and the mixture was extracted with Et_2O (3 x 20 mL). Usual workup afforded a crude, which was purified by flash chromatography (from 0 to 1 % MeOH in CH_2Cl_2) to give **RBM5-152** (off-white wax, 346 mg, 95 %).

$[\alpha]^{20}_D$ = –4.13 (*c* 1, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.75 (s, 1H), 3.71 – 3.67 (m, 1H), 3.66 – 3.61 (m, 1H), 3.25 (t, *J* = 7.0 Hz, 2H), 1.75 (br s, 1H), 1.66 – 1.54 (m, 2H), 1.44 (s, 9H), 1.40 – 1.35 (m, 4H), 1.34 – 1.23 (m, 22H), 1.07 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.0, 79.6, 74.6, 51.6, 50.7, 33.6, 29.8, 29.8, 29.7, 29.7, 29.7, 29.7, 29.6, 29.3, 29.0, 28.5, 26.8, 26.2, 14.4.

HRMS calcd. for $C_{23}H_{47}N_4O_3$ ($[M+H]^+$): 427.3643, found: 427.3636.

(2'*S*,3'*R*) *tert*-butyl (18-Amino-3-hydroxyoctadecan-2-yl)carbamate (RBM5-153)

To a solution of **RBM5-152** (300 mg, 0.70 mmol) in degassed MeOH (16 mL) containing Pd–C (60 mg, 20 % w/w) neat TES (1.1 mL, 7.03 mmol) was added dropwise, and the resultant suspension was stirred at rt under an Ar-filled balloon. After stirring for 1 h, the reaction mixture was filtered through a pad of Celite® and the solid was rinsed with MeOH (3 x 5 mL). The combined filtrates were concentrated *in vacuo* and the residue was triturated with hexanes (4 x 2 mL) to give the desired amine hydrochloride, without the need of further purification.

^{1}H NMR (400 MHz, CD_3OD) δ 3.53 – 3.47 (m, 1H), 3.47 – 3.41 (m, 1H), 2.67 (t, *J* = 7.1 Hz, 2H), 1.53 – 1.46 (m, 4H), 1.44 (s, 9H), 1.30 (s, 24H), 1.08 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (101 MHz, CD_3OD) δ 157.8, 79.9, 75.3, 51.8, 42.3, 34.8, 33.0, 30.8, 30.7, 30.6, 28.8, 28.0, 27.1, 15.5.

HRMS calcd. for $C_{23}H_{49}N_2O_3$ ([M+H]$^+$): 401.3738, found: 401.3742.

(2'*S*,3'*R*) *tert*-butyl [3-Hydroxy-18-[(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino]octadecan-2-yl]carbamate (RBM5-154)

To a stirred solution of 4-chloro-7-nitrobenzo[*c*][1,2,5]oxadiazole (NBD-Cl) (132 mg, 0.66 mmol) in MeOH (15 mL) containing DIPEA (522 µL, 3.00 mmol) at 0 ºC was added dropwise a solution of **RBM5-153** (240 mg, 0.60 mmol) in MeOH (10 mL). After the addition was complete, the mixture was allowed to warm to rt and stirred overnight. Then, the volatiles were removed under reduced pressure and the residue was directly subjected to flash chromatography on silica gel (from 0 to 1 % MeOH in CH_2Cl_2) to provide **RBM5-154** as a shiny orange wax (278 mg, 82 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (d, *J* = 8.6 Hz, 1H), 6.31 (br s, 1H), 6.17 (d, *J* = 8.7 Hz, 1H), 4.75 (br s, 1H), 3.73 – 3.66 (m, 1H), 3.66 – 3.62 (m, 1H), 3.49 (ap q, *J* = 6.7 Hz, 2H), 1.86 (br s, 1H), 1.81 (dt, *J* = 14.9, 7.4 Hz, 2H), 1.53 – 1.44 (m, 2H), 1.44 (s, 9H), 1.41 – 1.35 (m, 4H), 1.33 – 1.23 (m, 20H), 1.07 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.0, 144.3, 144.2, 144.0, 136.7, 123.7, 98.6, 79.6, 74.6, 50.8, 44.2, 33.6, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.3, 28.6, 28.5, 27.0, 26.1, 14.5.

HRMS calcd. for $C_{29}H_{50}N_5O_6$ ([M+H]$^+$): 564.3756, found: 564.3761.

(2*S*,3*R*) 2-Amino-18-[(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino]octadecan-3-ol hydrochloride (RBM5-155)

An ice–cooled solution of **RBM5-154** (85 mg, 0.15 mmol) in MeOH (10 mL) was treated with neat AcCl (54 µL, 0.75 mmol) and the resulting mixture was allowed to warm to rt and stirred overnight in the dark. Then, the solvent was evaporated *in vacuo* and the residue was purified by flash chromatography on silica gel (from 0 to 20 % MeOH in CH_2Cl_2) to afford **RBM5-155** as a shiny orange wax (65 mg, 86 %).

^{1}H NMR (400 MHz, CD_3OD) δ 8.52 (d, *J* = 8.7 Hz, 1H), 6.35 (d, *J* = 8.9 Hz, 1H), 3.75 – 3.65 (m, 1H), 3.53 (br s, 2H), 3.31 – 3.22 (m, 1H), 1.77 (app p, *J* = 7.4 Hz, 2H), 1.55 – 1.37 (m, 8H), 1.36 – 1.25 (m, 18H), 1.22 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CD_3OD) δ 146.7, 145.8, 145.5, 138.6, 122.7, 99.6, 71.7, 52.6, 44.8, 34.0, 30.7, 30.6, 30.3, 29.2, 28.0, 27.0, 12.1.

HRMS calcd. for $C_{24}H_{42}N_5O_4$ ($[M+H]^+$): 464.3231, found: 464.3228.

(2'*S*,3'*R*)-16-Azido-*N*-[3-hydroxy-18-[(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino]octadecan-2-yl]hexadecanamide (RBM5-159)

EDC·HCl (12 mg, 64 µmol) and HOBt (7 mg, 52 µmol) were sequentially added to an ice-cooled solution of ω-azidopalmitic acid[30] (13 mg, 44 µmol) in anhydrous CH_2Cl_2 (6 mL). The resulting mixture was vigorously stirred at rt under Ar for 15 min, and next added dropwise to a solution of **RBM5-155** (20 mg, 40 µmol) and Et_3N (89 µL, 0.20 mmol) in anhydrous CH_2Cl_2 (6 mL), and the reaction was stirred at rt for additional 2 h. The mixture was next diluted with CH_2Cl_2 (50 mL), washed with brine (2 x 25 mL) and worked-up as usual to afford a crude, which was flash chromatographed (from 0 to 14 % EtOAc in CH_2Cl_2) to afford **RBM5-159** as a shiny orange solid (22 mg, 74 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (d, *J* = 8.6 Hz, 1H), 6.49 (br s, 1H), 6.17 (d, *J* = 8.7 Hz, 1H), 5.81 (d, *J* = 7.9 Hz, 1H), 4.05 – 3.96 (m, 1H), 3.66 – 3.59 (m, 1H), 3.53 – 3.45 (m, 2H), 3.24 (t, *J* = 7.0 Hz, 2H), 2.51 (br s, 1H), 2.17 (t, *J* = 7.6 Hz, 2H), 1.80 (app p, *J* = 7.4 Hz, 2H), 1.69 – 1.53 (m, 4H), 1.49 – 1.20 (m, 48H), 1.09 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4, 144.4, 144.1, 144.1, 136.7, 123.9, 98.6, 74.5, 51.6, 49.6, 44.2, 37.0, 33.7, 29.7, 29.7, 29.5, 29.5, 29.4, 29.3, 29.0, 28.6, 27.1, 26.8, 26.1, 25.9, 14.3.

b) Synthesis of the fatty acids

b1) Diene fatty acids

The synthesis of diene fatty acid **RBM5-029** is representative

Methyl 7-bromoheptanoate (RBM5-027)

Compound **RBM5-027** (light yellow oil, 3.13 g, 98 %) was obtained from 7-bromoheptanoic acid (3.00 g, 14.35 mmol) in refluxing MeOH (30 mL) containing a catalytic amount of H_2SO_4 (30 µL) according to the general procedure 1. Upon ^{1}H-NMR analysis, the crude of the reaction was deemed sufficiently pure for the next step.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66 (d, *J* = 1.7 Hz, 3H), 3.39 (t, *J* = 6.8 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.90 – 1.79 (m, 2H), 1.69 – 1.58 (m, 2H), 1.50 – 1.40 (m, 2H), 1.39 – 1.28 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.2, 51.6, 34.0, 33.9, 32.7, 28.4, 27.9, 24.8.

HRMS calcd. for $C_8H_{16}BrO_2$ ($[M+H]^+$): 223.0328, 225.0308, found: 223.0331, 225.0315.

Methyl 7-(((2*E*,4*E*)-hexa-2,4-dien-1-yl)oxy)heptanoate (RBM5-028a) and (2*E*,4*E*)-hexa-2,4-dien-1-yl 7-(((2*E*,4*E*)-hexa-2,4-dien-1-yl)oxy)heptanoate (RBM5-028b)

Sorbyl alcohol (660 mg, 6.72 mmol) was added carefully at 0 °C to a stirred suspension of NaH (60 % (w/w) in mineral oil, 296 mg, 7.40 mmol) in dry DMF (18 mL) and the mixture was allowed to warm to rt and stirred for additional 30 min. Then, the temperature was lowered again to 0 °C followed by the sequential addition of NaI (101 mg, 0.67 mmol) and **RBM5-027** (1.50 g, 6.72 mmol). After stirring at rt for 4 h, the reaction was quenched by the dropwise addition of ice-cold water (20 mL). When the evolution of gas ceased, the mixture was extracted with Et_2O (3 x 50 mL) and the combined organic extracts were washed with brine (3 x 20 mL), dried over anhydrous $MgSO_4$, filtered and evaporated under vacuum. The residue was subjected to flash column chromatography (from 0 to 4 % EtOAc in hexanes) to afford an inseparable mixture of esters **RBM5-028a** and **RBM5-028b** (colourless oil, 704 mg). First-eluting fractions (**RBM5-028b**) and last-eluting fractions (**RBM5-028a**) were independently collected to give a sample of the pure products, from which the following data were acquired.

RBM5-028a:

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.23 – 6.15 (m, 1H), 6.10 – 6.01 (m, 1H), 5.75 – 5.65 (m, 1H), 5.64 – 5.57 (m, 1H), 3.96 (d, *J* = 6.4 Hz, 2H), 3.66 (s, 3H), 3.40 (t, *J* = 6.6 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.75 (d, *J* = 6.2 Hz, 3H), 1.69 – 1.61 (m, 2H), 1.60 – 1.55 (m, 2H), 1.43 – 1.34 (m, 2H), 1.28 – 1.20 (m, 2H).

RBM5-028b:

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.28 – 6.12 (m, 2H), 6.10 – 5.98 (m, 2H), 5.79 – 5.67 (m, 2H), 5.66 – 5.56 (m, 2H), 4.56 (d, *J* = 6.6 Hz, 2H), 3.95 (d, *J* = 6.3 Hz, 2H), 3.39 (t, *J* = 6.5 Hz, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.78 – 1.71 (m, 6H), 1.68 – 1.61 (m, 2H), 1.60 – 1.54 (m, 2H), 1.42 – 1.33 (m, 2H), 1.28 – 1.20 (m, 2H).

HRMS calcd. for $C_{19}H_{31}O_3$ ($[M+H]^+$): 307.2268, found: 307.2268.

7-(((2*E*,4*E*)-Hexa-2,4-dien-1-yl)oxy)heptanoic acid (RBM5-029)

Compound **RBM5-029** (white solid, 466 mg, 22 % over two steps) was obtained from the previous mixture of esters **RBM5-028a** and **RBM5-028b** (704 mg) and LiOH (175 mg, 7.30

mmol) in THF-H_2O (3:1) (240 mL) according to the general procedure 2. The title compound was purified by flash column chromatography on silica gel (from 0 to 50 % EtOAc in hexanes).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.18 (dd, *J* = 15.1, 10.4 Hz, 1H), 6.05 (ddd, *J* = 14.8, 10.4, 1.8 Hz, 1H), 5.70 (dt, *J* = 14.8, 6.9 Hz, 1H), 5.62 (dt, J = 15.1, 6.2 Hz, 1H), 3.96 (d, J = 6.3 Hz, 2H), 3.40 (t, *J* = 6.6 Hz, 2H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.75 (dd, *J* = 6.7, 1.6 Hz, 3H), 1.68 – 1.61 (m, 2H), 1.60 – 1.53 (m, 2H), 1.39 – 1.33 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 180.0, 133.2, 131.0, 130.0, 127.0, 71.3, 70.2, 34.1, 29.7, 29.0, 26.0, 24.7, 18.2.

HRMS calcd. for $C_{13}H_{21}O_3$ ($[M–H]^-$): 225.1496, found: 225.1476.

b2) Alkene fatty acid RBM5-097

Methyl 16-iodohexadecanoate (RBM5-098)

NIS (2.54 g, 11.31 mmol) was added in small portions, over a period of 10 min, to a stirred solution of **RBM5-063** (2.70 g, 9.43 mmol) and PPh_3 (2.72 g, 10.37 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 ºC. After the addition was complete, the resultant dark brown solution was allowed to warm to rt. After stirring overnight, the solvent was evaporated *in vacuo*, and the residue was diluted with water (50 mL), extracted with hexanes (3 x 50 mL), and the combined organic layers were washed with brine (2 x 25 mL), dried over anhydrous $MgSO_4$ and concentrated to dryness. Purification of the crude product by flash column chromatography (from 0 to 10 % EtOAc in hexanes) yielded **RBM5-098** (3.68 g, 98 %) as a pale-yellow wax.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66 (s, 3H), 3.18 (t, *J* = 7.1 Hz, 2H), 2.29 (t, *J* = 7.6 Hz, 2H), 1.86 – 1.77 (m, 2H), 1.66 – 1.56 (m, 2H), 1.42 – 1.33 (m, 2H), 1.33 – 1.22 (m, 20H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 51.6, 34.3, 33.7, 30.7, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 28.7, 25.1, 7.5.

HRMS calcd. for $C_{17}H_{34}IO_2$ ($[M+H]^+$): 397.1598, found: 397.1605.

***tert*-Butyl 15-hexadecenoate (RBM5-099)**

Compound **RBM5-099** (colourless oil, 1.23 g, 79 %) was obtained from **RBM5-098** (2.00 g, 5.05 mmol), and KO*t*Bu (2.83 g, 25.23 mmol) in anhydrous THF (25 mL) according to the general procedure 3. The title compound was purified by flash chromatography on silica gel (from 0 to 1 % MTBE in hexanes).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1H), 4.99 (ddt, *J* = 17.1, 2.2, 1.6 Hz, 1H), 4.92 (ddt, *J* = 10.2, 2.3, 1.2 Hz, 1H), 2.20 (t, *J* = 7.5 Hz, 2H), 2.08 – 2.00 (m, 2H), 1.62 – 1.52 (m, 2H), 1.44 (s, 9H), 1.41 – 1.33 (m, 2H), 1.32 – 1.22 (m, 18H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 139.3, 114.0, 79.8, 35.6, 33.8, 29.6, 29.6, 29.5, 29.5, 29.3, 29.1, 29.1, 28.9, 28.1, 25.1.

15-Hexadecenoic acid (RBM5-097)

Neat TFA (5 mL) was added dropwise to an ice-cooled solution of *tert*-butyl ester **RBM5-099** (350 mg, 1.13 mmol) in dry CH_2Cl_2 (5 mL). After stirring at rt for 2 h, the reaction mixture was concentrated *in vacuo* to give a crude, which was subjected to flash column chromatography on silica gel (from 0 to 10 % EtOAc in hexanes) to afford **RBM5-097** (240 mg, 84 %) as a pale-yellow wax.

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.11 (br s, 1H), 5.81 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H), 4.99 (dd, *J* = 17.1, 1.7 Hz, 1H), 4.95 – 4.90 (m, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.04 (app q, *J* = 7.0 Hz, 2H), 1.63 (app p, *J* = 7.5 Hz, 2H), 1.41 – 1.20 (m, 20H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 180.2, 139.4, 114.2, 34.2, 34.0, 29.8, 29.7, 29.7, 29.6, 29.4, 29.3, 29.2, 29.1, 24.8.

HRMS calcd. for $C_{16}H_{29}O_2$ ($[M–H]^-$): 253.2173, found: 253.2169.

b3) Azido fatty acids

The synthesis of **RBM5-065** is representative

Methyl 16-hydroxyhexadecanoate (RBM5-063)

Compound **RBM5-063** (white solid, 980 mg, 93 %) was obtained from 16-hydroxyhexadecanoic acid (1.00 g, 3.67 mmol) in refluxing MeOH (20 mL) containing a catalytic amount of H_2SO_4 (20 µL) according to the general procedure 1. The title compound was purified by flash column chromatography on silica gel (from 0 to 20 % EtOAc in hexanes).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66 (s, 3H), 3.64 (t, *J* = 6.7 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.65 – 1.52 (m, 4H), 1.38 – 1.22 (m, 22H).

^{13}C NMR (101 MHz, cdcl$_3$) δ 174.5, 63.2, 51.6, 34.2, 32.9, 29.8, 29.7, 29.7, 29.7, 29.6, 29.4, 29.3, 25.9, 25.1.

Methyl 16-azidohexadecanoate (RBM5-064)

Method A: Compound **RBM5-064** (white solid, 460 mg, 85 %) was obtained from primary alcohol **RBM5-063** (500 mg, 1.75 mmol), NBS (373 mg, 2.09 mmol), PPh_3 (504 mg, 1.92 mmol) and NaN_3 (340 mg, 5.24 mmol) in dry DMF (18 mL) according to the procedure described for compound **RBM5-018**. The title compound was purified by flash column chromatography on silica gel (from 0 to 14 % Et_2O in hexanes).

Method B: DBU (339 µL, 2.27 mmol) and DPPA (489 µL, 2.27 mmol) were sequentially added to an ice–cooled solution of the primary alcohol **RBM5-063** (500 mg, 1.75 mmol) in dry DMF (3.5 mL) and the resulting mixture was allowed to warm to rt. After stirring overnight, water (10 mL) was added and the mixture was extracted with Et_2O (3 x 25 mL). The combined organic extracts were washed with brine (3 x 10 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography on silica gel (from 0 to 14 % Et_2O in hexanes) to afford **RBM5-064** (440 mg, 81 %) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66 (s, 3H), 3.25 (t, *J* = 7.0 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.66 – 1.53 (m, 4H), 1.40 – 1.22 (m, 22H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 51.6, 51.6, 34.3, 29.8, 29.8, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.0, 26.9, 25.1.

16-Azidohexadecanoic acid (RBM5-065)

Compound **RBM5-065** (off-white solid, 335 mg, 78 %) was obtained from methyl ester **RBM5-064** (450 mg, 1.44 mmol) and LiOH (104 mg, 4.33 mmol) in THF–H_2O (3:1) (160 mL) according to the general procedure 2. The title compound was purified by flash column chromatography on silica gel (from 100:0:1 to 80:20:1 hexanes/Et_2O/AcOH).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.34 (br s, *J* = 0.3 Hz, 1H), 3.25 (t, *J* = 7.0 Hz, 2H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.55 (m, 4H), 1.40 – 1.22 (m, 22H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 180.3, 51.6, 34.2, 29.8, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 29.0, 26.9, 24.8.

HRMS calcd. for $C_{16}H_{30}N_3O_2$ ($[M–H]^-$): 296.2344, found: 296.2337.

c) Synthesis of the fluorescent reagents

c1) Tetrazines

The synthesis of **RBM5-139** and **RBM5-140** are representative

***tert*-butyl (6-(7-Methoxy-2-oxo-2*H*-chromene-3-carboxamido)hexyl)carbamate (RBM5-135)**

Compound **RBM5-135** (white solid, 200 mg, 57 %) was obtained from *N*-Boc-1,6-hexanediamine (187 µL, 0.83 mmol), 7-methoxycoumarin-3-carboxylic acid (202 mg, 0.92 mmol), EDC·HCl (255 mg, 1.33 mmol), and HOBt (146 mg, 1.08 mmol) in anhydrous CH_2Cl_2 (40 mL) containing Et_3N (580 µL, 4.16 mmol) according to the general procedure 4. Purification of the crude material by flash column chromatography on silica gel (from 0 to 5 % MeOH in CH_2Cl_2) afforded the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.74 (br s, 1H), 7.56 (d, *J* = 8.6 Hz, 1H), 6.92 (d, *J* = 8.2 Hz, 1H), 6.85 (s, 1H), 4.55 (br s, 1H), 3.90 (s, 3H), 3.48 – 3.36 (m, 2H), 3.09 (m, 2H), 1.66 – 1.56 (m, 2H), 1.52 – 1.31 (m, 15H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 164.9, 162.1, 162.0, 156.7, 156.1, 148.3, 131.0, 115.0, 114.1, 112.6, 100.4, 79.1, 56.1, 40.6, 39.8, 30.1, 29.5, 28.5, 26.8, 26.5.

HRMS calcd. for $C_{22}H_{31}N_2O_6$ ($[M+H]^+$): 419.2177, found: 419.2181.

***tert*-butyl (6-((9-(Diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl)oxy)hexyl)carbamate (RBM5-136)**

Compound **RBM5-136** (red solid, 500 mg, 78 %) was obtained from **RBM5-133** (500 mg, 1.20 mmol), K_2CO_3 (397 mg, 2.87 mmol) and **RBM5-134** (369 mg, 1.32 mmol) in dry DMF (30 mL) according to the methodology described for **RBM5-120**. The title compound was obtained after flash column chromatography of the crude material (isocratic 6:4 hexane/EtOAc).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 8.7 Hz, 1H), 8.04 (d, *J* = 2.6 Hz, 1H), 7.61 (d, *J* = 9.1 Hz, 1H), 7.16 (dd, *J* = 8.7, 2.6 Hz, 1H), 6.66 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.47 (d, *J* = 2.7 Hz, 1H), 6.31 (s, 1H), 4.53 (br s, 1H), 4.17 (t, *J* = 6.4 Hz, 2H), 3.47 (q, *J* = 7.1 Hz, 4H), 3.19 – 3.09 (m, 2H), 1.91 – 1.82 (m, 2H), 1.58 – 1.51 (m, 4H), 1.44 (s, 11H), 1.26 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 183.4, 161.9, 156.1, 152.2, 150.8, 146.9, 140.2, 134.2, 131.2, 127.8, 125.7, 124.8, 118.4, 109.6, 106.7, 105.4, 96.4, 79.2, 68.3, 45.2, 40.7, 30.2, 29.3, 28.6, 26.7, 25.9, 12.8.

HRMS calcd. for $C_{31}H_{40}N_3O_5$ ([M+H]$^+$): 534.2962, found: 534.2965.

7-Methoxy-2-oxo-*N*-(6-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamido)hexyl)-2*H*-chromene-3-carboxamide (RBM5-139)

Neat TFA (1.5 mL) was added dropwise to an ice-cooled solution of *N*–Boc protected amine **RBM5-135** (100 mg, 0.24 mmol) in dry CH_2Cl_2 (3 mL). After stirring in the dark at rt for 2 h, the reaction mixture was concentrated to dryness to afford the corresponding crude amine trifluoroacetate (75 mg). This crude was taken up in CH_2Cl_2/Et_3N (10:1, 11 mL), followed by the addition of *N*-hydroxysuccinimide ester **RBM5-138** (90 mg, 0.24 mmol). After stirring overnight at rt in the dark, the reaction mixture was evaporated *in vacuo* and the residue was subjected to flash column chromatography (from 0 to 5 % MeOH in CH_2Cl_2) to give **RBM5-139** (35 mg, 25 %) as a pale pink solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (d, *J* = 4.8 Hz, 2H), 8.82 (d, *J* = 8.0 Hz, 2H), 8.79 (s, 1H), 8.08 (d, *J* = 8.3 Hz, 2H), 7.61 (s, 1H), 7.55 (d, *J* = 8.9 Hz, 1H), 7.52 (s, 1H), 6.92 (d, *J* = 9.3 Hz, 1H), 6.86 (s, 1H), 6.59 (br s, 1H), 3.91 (s, 3H), 3.57 – 3.46 (m, 4H), 1.70 – 1.28 (m, 8H).

HRMS calcd. for $C_{30}H_{29}N_8O_5$ ([M+H]$^+$): 581.2255, found: 581.2253.

***N*-(6-((9-(Diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl)oxy)hexyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (RBM5-140)**

Neat TFA (1.5 mL) was added dropwise to an ice-cooled solution of *N*–Boc protected amine **RBM5-136** (100 mg, 0.19 mmol) in dry CH_2Cl_2 (3 mL) After stirring at rt for 2 h in the dark, the reaction mixture was concentrated to dryness to afford the corresponding crude amine trifluoroacetate (82 mg). This crude was taken up in CH_2Cl_2/Et_3N (10:1, 11 mL), followed by the addition of *N*-hydroxysuccinimide ester **RBM5-138** (70 mg, 0.19 mmol). After stirring overnight at rt in the dark, the reaction mixture was evaporated *in vacuo* and the residue was subjected to flash column chromatography (from 0 to 5 % MeOH in CH_2Cl_2) to give **RBM5-140** (125 mg, 99 %) as a dark red solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (d, *J* = 4.8 Hz, 2H), 8.78 (t, *J* = 4.9 Hz, 2H), 8.20 (d, *J* = 8.7 Hz, 1H), 8.03 (d, *J* = 2.6 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 2H), 7.61 (t, *J* = 9.6 Hz, 1H), 7.59 (d, *J* = 9.5 Hz, 2H), 7.15 (dd, *J* = 8.7, 2.6 Hz, 1H), 6.66 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.45 (d, *J* = 2.7 Hz, 1H), 6.37 (t, *J* = 5.7 Hz, 1H), 6.32 (s, 1H), 4.19 (t, *J* = 6.3 Hz, 2H), 3.56 (q, *J* = 6.8 Hz,

2H), 3.46 (q, *J* = 7.1, 6.5 Hz, 4H), 1.95 – 1.86 (m, 2H), 1.79 – 1.49 (m, 6H), 1.26 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 183.7, 166.8, 162.0, 158.6, 152.3, 151.0, 139.9, 131.3, 129.1, 128.0, 128.0, 125.0, 122.8, 118.4, 111.8, 109.8, 106.8, 105.2, 96.4, 68.3, 45.2, 40.4, 29.7, 29.2, 25.9, 25.6, 12.8 (some of the carbon atoms with non bonded hydrogens were undetected due to the scarce solubility of the sample).

HRMS calcd. for $C_{39}H_{38}N_9O_4$ ($[M+H]^+$): 696.3041, found: 696.3043.

c2) Bicyclononynes

[(1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-yl]methyl [6-(7-methoxy-2-oxo-2*H*-chromene-3-carboxamido)hexyl]carbamate (RBM5-142)

To an ice-cooled solution of *N*–Boc protected amine **RBM5-135** (93 mg, 0.22 mmol) in dry CH_2Cl_2 (6 mL) was added dropwise neat TFA (1.5 mL). After stirring at rt for 2 h in the dark, the reaction mixture was concentrated to dryness to afford the corresponding crude amine trifluoroacetate (71 mg). This crude was taken up in CH_2Cl_2 (10 mL), followed by the sequential addition of Et_3N (108 µL, 0.78 mmol) and *p*-nitrophenol activated carbonate ester **RBM5-141**[41] (70 mg, 0.22 mmol). After stirring overnight at rt in the dark, the reaction mixture was evaporated *in vacuo* and the residue was flash chromatographed (from 0 to 20 % EtOAc in CH_2Cl_2) to give the desired carbamate **RBM5-142** (98 mg, 89 %) as an off-white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.76 (br s, 1H), 7.58 (d, *J* = 8.7 Hz, 1H), 6.93 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.86 (d, *J* = 2.4 Hz, 1H), 4.69 (br s, 1H), 4.13 (d, *J* = 8.1 Hz, 2H), 3.91 (s, 3H), 3.44 (td, *J* = 7.1, 5.8 Hz, 2H), 3.20 – 3.12 (m, 2H), 2.34 – 2.15 (m, 6H), 1.67 – 1.24 (m, 11H), 0.99 – 0.86 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 164.9, 162.1, 162.1, 156.9, 156.8, 148.3, 131.0, 115.0, 114.1, 112.6, 100.4, 99.0, 62.7, 56.2, 41.0, 39.7, 30.0, 29.5, 29.2, 26.7, 26.5, 21.6, 20.2, 18.0.

HRMS calcd. for $C_{28}H_{35}N_2O_6$ ($[M+H]^+$): 495.2490, found: 495.2496.

[(1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-yl]methyl [6-[[9-(diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl]oxy]hexyl]carbamate (RBM5-143)

To an ice-cooled solution of *N*–Boc protected amine **RBM5-136** (100 mg, 0.19 mmol) in dry CH_2Cl_2 (6 mL) was added dropwise neat TFA (1.5 mL). After stirring at rt for 2 h in the dark, the reaction mixture was concentrated to dryness to afford the corresponding crude amine trifluoroacetate (81 mg). This crude was taken up in CH_2Cl_2 (10 mL), followed by the sequential addition of Et_3N (93 µL, 0.67 mmol) and *p*-nitrophenol activated carbonate ester **RBM5-141**[41] (60 mg, 0.19 mmol). After stirring overnight at rt in the dark, the reaction mixture was evaporated *in vacuo* and the residue was flash chromatographed (from 0 to 20 % EtOAc in CH_2Cl_2) to give the desired carbamate **RBM5-143** (104 mg, 90 %) as a shiny dark-red solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 8.7 Hz, 1H), 8.03 (d, *J* = 2.6 Hz, 1H), 7.60 (d, *J* = 9.0 Hz, 1H), 7.15 (dd, *J* = 8.7, 2.6 Hz, 1H), 6.65 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.45 (d, *J* = 2.7 Hz, 1H), 6.29 (s, 1H), 4.70 (br s, 1H), 4.19 – 4.11 (m, 4H), 3.46 (q, *J* = 7.1 Hz, 5H), 3.25 – 3.15 (m, 2H), 2.34 – 2.16 (m, 6H), 1.90 – 1.82 (m, 2H), 1.67 – 1.30 (m, 10H), 1.26 (t, *J* = 7.0 Hz, 6H), 0.97 – 0.87 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 183.4, 161.9, 156.9, 152.2, 150.9, 147.0, 140.3, 134.2, 131.2, 127.9, 125.7, 124.8, 118.4, 109.6, 106.7, 105.5, 99.0, 96.5, 68.3, 62.8, 45.2, 41.1, 30.1, 29.3, 29.2, 26.6, 25.9, 21.6, 20.2, 17.9, 12.8.

HRMS calcd. for $C_{37}H_{44}N_3O_5$ ($[M+H]^+$): 610.3275, found: 610.3279.

d) Synthesis of doxdhCer by SPAAC reactions

Compound RBM5-160

Compound **RBM5-142** (7 mg, 15 µmol) was added to a stirred solution of **RBM5-159** (9 mg, 12 µmol) in CH_2Cl_2 (4 mL). After stirring overnight at rt in the dark, the reaction mixture was concentrated to dryness and the residue was flash chromatographed on silica gel (from 0 to 5 % MeOH in CH_2Cl_2) to afford the desired SPAAC reaction adduct **RBM5-160** (14 mg, 93 %, inseparable mixture of diastereomers) as a dark-orange solid.

^{1}H NMR (400 MHz, DMSO-d_6) (mixture of diastereomers) δ 9.54 (s, 1H), 8.81 (s, 1H), 8.63 (t, *J* = 5.7 Hz, 1H), 8.50 (d, *J* = 9.1 Hz, 1H), 7.90 (d, *J* = 8.7 Hz, 1H), 7.49 (d, *J* = 8.5 Hz, 1H), 7.14 – 7.04 (m, 2H), 7.04 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.41 (d, *J* = 9.3 Hz, 1H), 4.47 (d, *J* = 6.1 Hz, 1H), 4.18 (t, *J* = 7.0 Hz, 2H), 4.07 – 3.96 (m, 2H), 3.89 (s, 3H), 3.66 – 3.58 (m, 1H), 3.50 – 3.39 (m, 2H), 3.34 – 3.26 (m, 2H), 3.26 – 3.17 (m, 1H), 2.99 – 2.88 (m, 4H), 2.77 – 2.61 (m,

2H), 2.15 – 2.05 (m, 3H), 2.01 (t, *J* = 7.3 Hz, 2H), 1.73 – 1.60 (m, 4H), 1.56 – 1.05 (m, 60H), 0.97 (d, *J* = 6.7 Hz, 3H), 0.94 – 0.82 (m, 2H).

HRMS calcd. for $C_{68}H_{105}N_{10}O_{11}$ ($[M+H]^+$): 1237.7959, found: 1237.7983.

Compound RBM5-161

Compound **RBM5-143** (10 mg, 15 μmol) was added to a stirred solution of **RBM5-159** (10 mg, 13 μmol) in CH_2Cl_2 (4 mL) . After stirring overnight at rt in the dark, the reaction mixture was concentrated to dryness and the residue was flash chromatographed on silica gel (from 0 to 5 % MeOH in CH_2Cl_2) to afford the desired SPAAC reaction adduct **RBM5-161** (16 mg, 88 %, inseparable mixture of diastereomers) as a dark-red solid.

^{1}H NMR (400 MHz, DMSO-*d*$_6$) (mixture of diastereomers) δ 9.53 (br s, 1H), 8.48 (d, *J* = 8.9 Hz, 1H), 8.03 (d, *J* = 8.6 Hz, 1H), 7.94 (d, *J* = 2.5 Hz, 1H), 7.64 – 7.59 (m, 1H), 7.49 (d, *J* = 8.6 Hz, 1H), 7.25 (dd, *J* = 8.7, 2.5 Hz, 1H), 7.10 (t, *J* = 5.7 Hz, 1H), 6.81 (d, *J* = 9.2 Hz, 1H), 6.64 (d, *J* = 2.4 Hz, 1H), 6.38 (d, *J* = 9.0 Hz, 1H), 6.18 (s, 1H), 4.47 (d, *J* = 6.1 Hz, 1H), 4.21 – 4.10 (m, 4H), 4.02 (d, *J* = 7.8 Hz, 2H), 3.67 – 3.57 (m, 1H), 3.50 (q, *J* = 7.0 Hz, 4H), 3.46 – 3.39 (m, 2H), 3.25 – 3.18 (m, 1H), 3.15 – 3.04 (m, 4H), 3.05 – 2.95 (m, 2H), 2.94 – 2.86 (m, 2H), 2.78 – 2.59 (m, 2H), 2.13 – 1.90 (m, 7H), 1.83 – 1.73 (m, 2H), 1.70 – 1.59 (m, 4H), 1.54 – 1.10 (m, 58H), 0.96 (d, *J* = 6.7 Hz, 3H), 0.93 – 0.82 (m, 2H).

HRMS calcd. for $C_{77}H_{114}N_{11}O_{10}$ ($[M+H]^+$): 1352.8745, found: 1352.8760.

FRET efficiency

The FRET efficiencies of the donor-acceptor pairs were determined by comparing the integrated fluorescence intensities of the donor alone (D) and in the presence of the acceptor (DA). For this purpose, a series of solutions of the D compounds (**RBM5-142** and **RBM5-154**) and the DA compounds (**RBM5-160** and **RBM5-161**) was prepared such that the *Abs* value at the corresponding λ_{Ex} was approximately between 0.01 and 0.1, in order to avoid re-absorption effects. The absorption and emission spectra of each solution were recorded using a 1 cm path length quartz cuvette, as described above. Both D and DA compounds were analysed under the same conditions. The absorption and emission spectra of the DA compounds were subjected to deconvolution regression, as explained above, to isolate the spectra of the donor component (**RBM5-160_donor** and **RBM5-161_donor**).

The integrated fluorescence intensity was then plotted against the *Abs* value at the λ_{Ex} and adjusted to a linear regression function forced through the origin using GraphPad Prism version 7.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Then, the FRET efficiency was calculated using **Equation ¡**Error! No hay texto con el estilo especificado en el documento.**.1**, where $Grad_D$ and $Grad_{DA}$ are the slopes of the plots of the integrated fluorescence intensity *vs* absorbance at the λ_{Ex} of the donor alone and the donor component (upon spectral deconvolution) in the presence of the acceptor, respectively.

$$E = 1 - \frac{Grad_{DA}}{Grad_D}$$

Equation ¡Error! No hay texto con el estilo especificado en el documento.**.1**

Biology

CerS assay

To overexpress CerS5, 24 h before transfection, HEK293T cells were seeded in 6-well plates (2×10^5 cells per well). Then, cells were transfected with 2.5 μg/well of plasmid harbouring the human CerS5 gene using 0.01 mg/mL PEI in opti-MEM for 6 h. Complete DMEM medium supplemented with 10% FBS was added and cells were incubated for 48 h. After transfection, cells were treated with **RBM5-155** (10 μM final concentration) and ω-azidopalmitic acid (500 μM final concentration) for 2 h. The medium was remove and the cells were washed with 400 μL PBS. UPLC-MS analysis was performed as mentioned below

Lipid extraction

Cell pellets were suspended with 100 μL of H_2O and mixed with 750 μL of methanol: chloroform, 2:1. Samples were heated at 48°C overnight and next day, 75 μL of 1 M KOH in methanol were added, followed by 2 h incubation at 37°C. Afterwards, the saponification was neutralised with 75 μL of 1 M acetic acid and solvent was removed using a Speed Vac Savant SPD131DDA (Thermo Scientific).

UPLC-MS analysis

Lipid extracts, fortified with internal standards (*N*-dodecanoylsphingosine, *N*-dodecanoylglucosylsphingosine, *N*-dodecanoylsphingosylphosphorylcoline and C17-sphinganine 0.2 nmol each) were solubilised in 150 μL of methanol. Samples were then centrifuged at 9,300

g for 3 min and 130 µL of the supernatant were injected to a Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters, Milford, MA, USA) operated in positive electrospray ionisation mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points every 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mm x2.1 mm i.d., 1.7 µm C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: methanol/water/formic acid (74/25/1 v/v/v); phase B: methanol/formic acid (99/1 v/v), both also contained 5 mM ammonium formate. A linear gradient was programmed—0.0 min: 80 % B; 3 min: 90 % B; 6 min: 90 % B; 15 min: 99 % B; 18 min: 99 % B; 20 min: 80 % B. The flow rate was 0.3 mL min^{-1}.

Supporting Material

Representative NMR spectra and fluorescence studies

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