Inverse electron demand Diels-Alder pyridazine elimination: synthetic tools for chemical immunology

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An optimized synthesis of bifunctional cyclooctenes for click to release chemistry

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2.1 Introduction

Bioorthogonal chemistry is broadening in scope to include bond cleavage reactions alongside known ligation methods,[1–4] with many new reactions continuously emerging in the literature.[5–12] Amongst these, the inverse electron demand Diels-Alder (IEDDA) pyridazine elimination[13] comprises the first example of a bioorthogonal reaction that is today described as "click to release" (Figure 1 A). In the IEDDA decaging sequence, reaction of a tetrazine and a *trans*-cyclooctene (TCO) bearing a leaving group at the allylic position[14,15] results in a 4,5-dihydropyridazine intermediate. This adduct tautomerizes into 2,5- and 1,4-dihydropyridazines, the latter of which induces elimination of the allylic payload.[16,17] The biocompatibility of the tetrazine and TCO

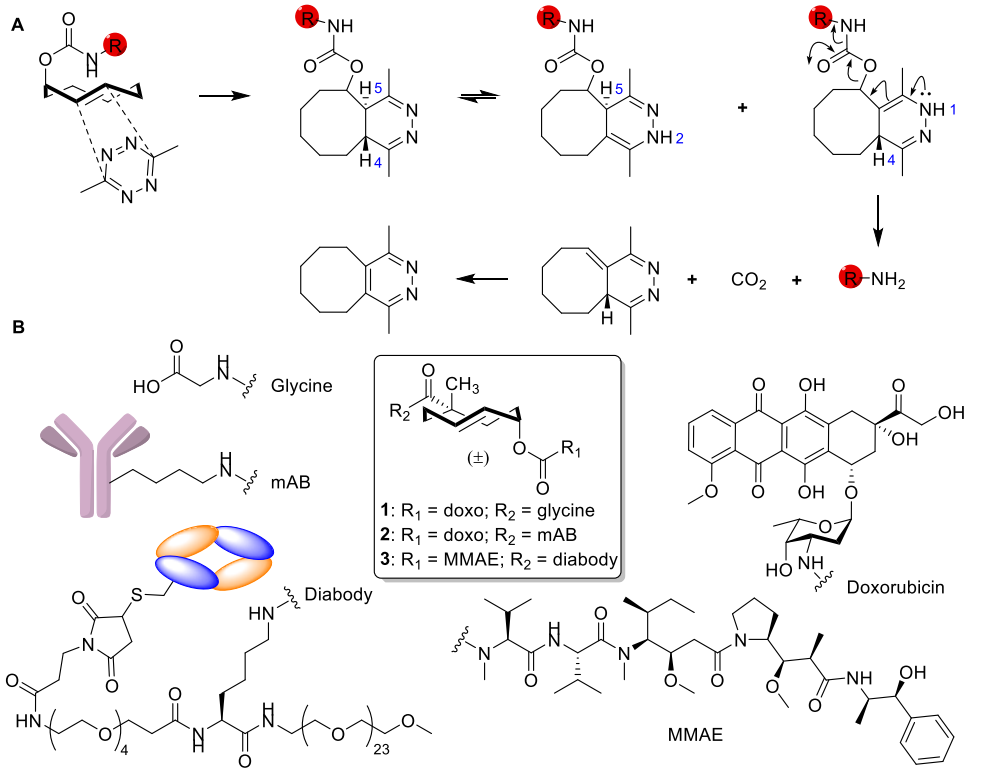
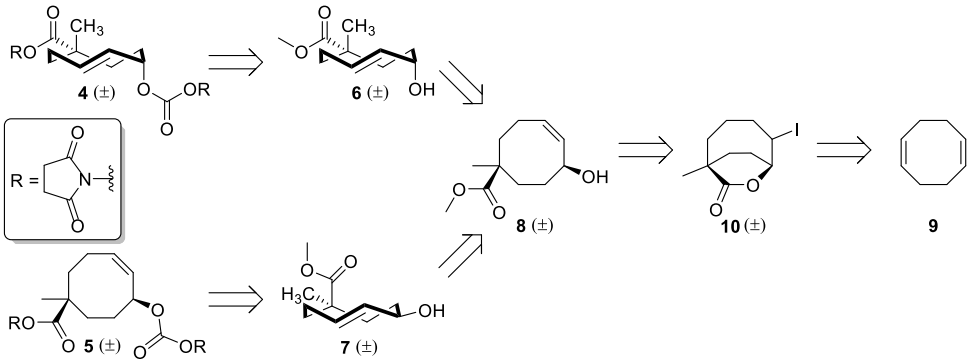


Figure 1 A) Overview of the inverse electron demand Diels-Alder (IEDDA) pyridazine elimination. B) Cytotoxic prodrugs **1-3** employed in *in vivo* experiments by Royzen and Oneto[22] and Robillard and co-workers.[18-19]

components, combined with their selectivity and overall deprotection rate have culminated in various applications, in which the area of cytotoxic (pro)drug activation[18–22] has received increasing interest.

While the tetrazine and TCO pair for this click reaction are inherently selective towards each other, the actual spatiotemporal control within biological systems is often achieved by employing another modality to direct localization of the reaction components.[18–22] Royzen and Oneto utilized tetrazine modified hydrogels in combination with TCO modified doxorubicin.[20–22] A phase 1 clinical trial towards the treatment of solid tumors is currently in progress for a tetrazine modified sodium hyaluronate and a TCO equipped with doxorubicin as payload and glycine to enhance aqueous solubility (**1**, Figure 1B).[22] This bifunctional TCO was initially reported by Robillard and co-workers to serve as cleavable linker for antibody-drug conjugates (ADCs), resulting in triggered release of doxorubicin *in vitro* and in tumor-bearing mice (**2**, Figure 1B).[18] This premise was refined with a diabody-based ADC (**3**, Figure 1B) to



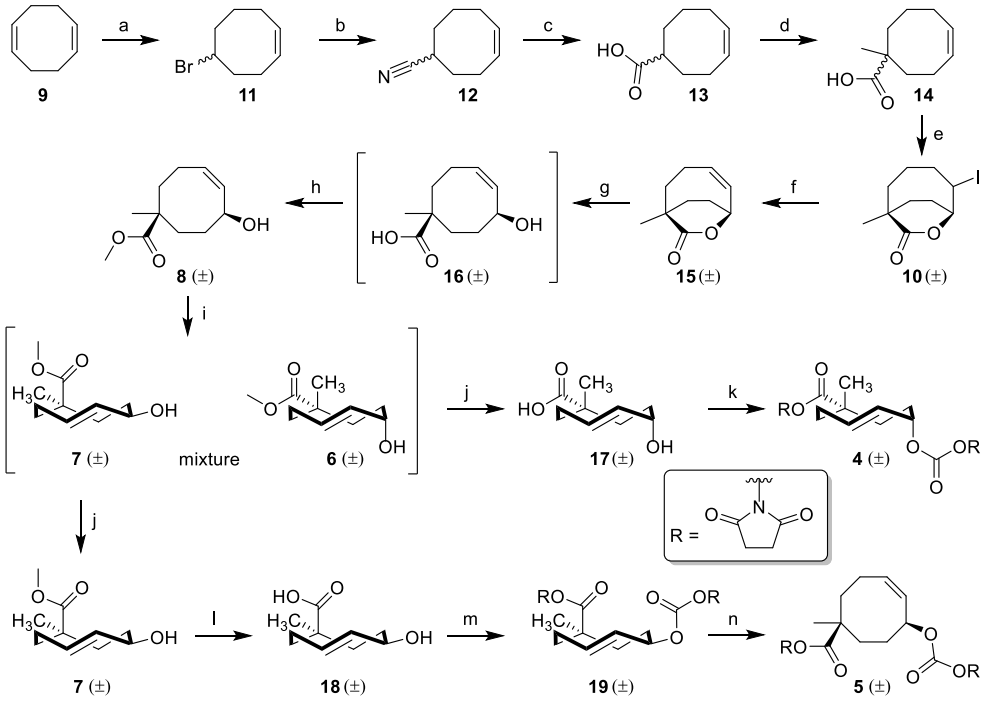
Scheme 1 Retrosynthetic route for bifunctional TCO reagent **4** and bifunctional CCO reagent **5** from a shared intermediate (**8**), which can be synthesized from **9** via **10**.

release monomethyl auristatin E (MMAE) from the same TCO motif, thereby outperforming the FDA approved, protease cleavable ADC Adcetris in two preclinical *in vivo* tumor models (LS174T and OVCAR-3, respectively).[19]

As the bifunctional TCO scaffold fulfills a central role towards *in vivo* utilization of the IEDDA pyridazine elimination, its synthetic accessibility is of importance to enable novel applications. This Chapter presents a simplified synthetic procedure towards bifunctional TCO reagent **4** based on the initially published route[18] (Scheme 1). A novel bifunctional *cis*-cyclooctene (CCO) reagent (**5**), which proved difficult to directly access from CCO intermediates, is additionally described. TCO reagent **4** and CCO reagent **5** were synthesized from TCOs **6** and **7**, respectively. Both reagents share a common intermediate (**8**), which was synthesized from 1,5-cyclooctadiene **9** via iodolactone **10**.

2.2 Results and discussion

Cyclooctadiene **9** was brominated in HBr/AcOH to obtain **11** in near-quantitative yield (Scheme 2). Nucleophilic substitution of **11** with NaCN in DMSO afforded nitrile **12** in 56% yield, which was subsequently hydrolyzed in the presence of KOH and H_2O_2 to obtain **13** in 74% yield. Reproducibility on large scale for this procedure, as described by Hartley[23] for a related system and adapted for **12** by Mitchell and co-workers,[24] was obtained by monitoring the consumption of nitrile **12** to the intermediate amide at 40°C, before refluxing overnight. Carboxylic acid **13** was α-methylated to obtain **14**, thereby preventing epimerization in subsequent steps and enabling regioselective conjugation chemistry.[18] These initial steps (bromination, cyanide substitution, oxidation, basic hydrolysis and α-methylation) were carried out using crude reaction mixtures obtained after aqueous workup. Iodolactonization of **14** afforded **10**, which was purified by crystallization from EtOH to result in a yield of 23% over five steps from



Scheme 2 Synthesis of cyclooctene reagents **4** and **5** from **9**. Reagents/conditions: (a) HBr, AcOH, 0°C to rt, 99%; (b) NaCN, DMSO, 100°C, 56%; (c) H_2O_2, KOH, H_2O, EtOH, 40 °C to reflux, 74%; (d) LDA, CH_3I, THF, -78°C to 40°C, 98%; (e) I_2, KI, $NaHCO_3$, DCM, H_2O, 0°C, 56%; (f) DBU, toluene, 70°C, 92%; (g) KOH, dioxane, H_2O, 0°C to rt; (h) CH_3I, DMF, 0°C to rt, 77% over 2 steps; (i) methyl benzoate, hν (254 nm), Et_2O/heptane, rt, 84%; (j) KOH, MeOH, H_2O, 4°C, 43% (**7**), 42% (**17**); (k) N,N'-disuccinimidyl carbonate, DIPEA, DMAP, MeCN, rt, 72%; (l) KOH, MeOH, H_2O, 50°C, 79%; (m) N,N'-disuccinimidyl carbonate, DIPEA, MeCN, rt, 66%; (n) hν (CFL), $CDCl_3$, rt, 74%.

9 at 500 mmol scale, without the need for the previously reported distillations.[18] Subsequent β-elimination in the presence of DBU afforded **15** in 92% yield.

The transesterification procedure starting from bicyclic lactone **15** (64 hours, 48% yield)[18] was replaced by a one-pot, two-step procedure to improve overall conversion and reaction time. Saponification afforded monocyclic carboxylic acid **16**, which was directly methylated to obtain methyl ester **8** in 77% yield over two steps. Photoisomerization[25] resulted in a 1 : 1.4 mixture of axial isomer **6** (axial hydroxyl, equatorial methyl ester) and equatorial isomer **7** (equatorial hydroxyl, axial methyl ester), respectively. The isomeric mixture was treated with potassium hydroxide at 4°C to selectively hydrolyze axial isomer **6**, followed by acid-base extraction to separately obtain carboxylic acid **17** and ester **7**. The bis-NHS functionalization of **17** into **4** (three days, 46% yield)[18] was accelerated using nucleophilic catalysis (DMAP). Hydrolysis of

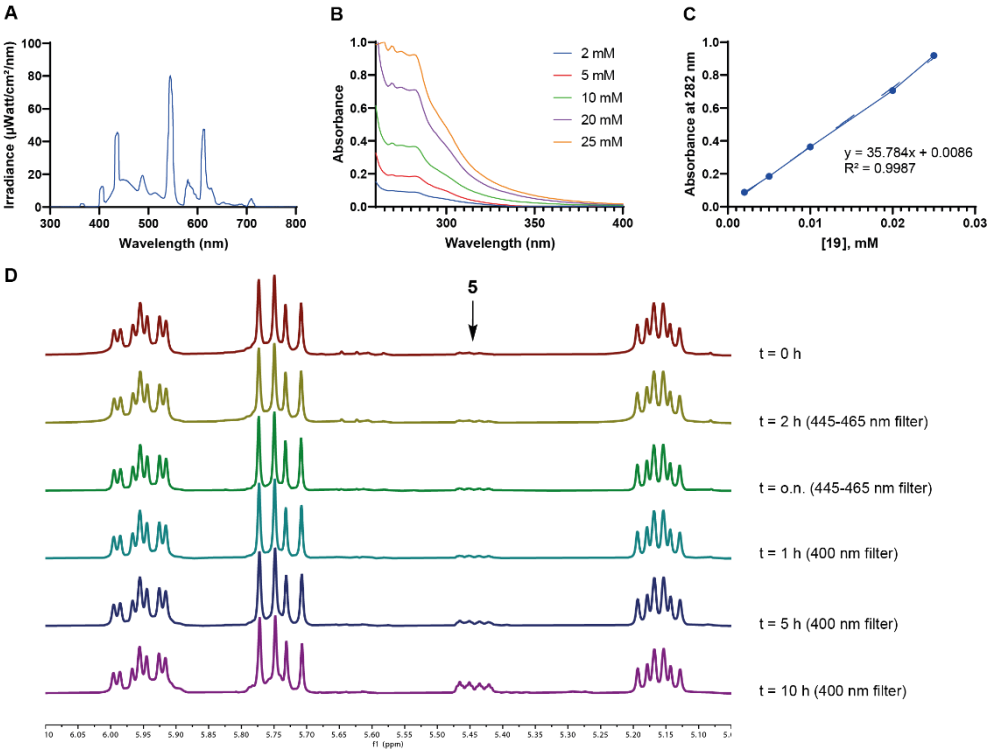


Figure 2 A) Emission spectrum of CFL lamp (Philips Tornado, 1450 lumen, 23 W, E27, ES 220-240V, 50/60 Hz, CDL 865, 170 mA, ≤ 2.5 mg Hg, 6500 K) used for photochemical conversion of **19** to **5**. B) UV-absorption of compound **19** (2, 5, 10, 20, 25 mM in $CHCl_3$). C) Linear fitting of the absorbance at 282 nm vs. the concentration of **19** yielded the extinction coefficient (ε) in L mol^{-1} cm^{-1} from the slope of the linear fit. D) ^1H NMR analyses of a white light irradiation experiment of **19**. Compound **19** (0.1 M in $CDCl_3$, 0.6 mL, NMR tube) was irradiated using a Xenon Arc (1000 W) at 20 cm distance. The temperature of sample was kept at 25°C using water cooling. The sample was measured before starting the experiment (top entry, red line), following irradiation with a filter which absorbs ≤ 445-465 nm ("445-465 filter"), following irradiation with a filter which absorbs ≤ 400 nm ("400 filter") at indicated times. Formation of **5** was followed by observing the characteristic signal marked at 5.45 ppm.

the product during chromatographic purification was prevented by employing neutralized silica gel, which resulted in a yield of 72%.

Synthesis of bifunctional CCO reagent **5** was envisioned from carboxylic acid **16** under similar conditions. However, attempts to directly functionalize **16** resulted in formation of bicyclic lactone **15**. Instead, equatorial isomer **7** was hydrolyzed (to afford **18**) and functionalized to obtain equatorial TCO reagent **19**, followed by *trans* to *cis* isomerization in the presence of visible light (26 W CFL bulb, Figure 2A) to obtain bifunctional CCO reagent **5**. Absorbance of **19** was characterized at 2 – 25 mM in $CHCl_3$ (Figure 2B) to reveal λ_{max} = 282 nm; absorbance at this wavelength was in agreement

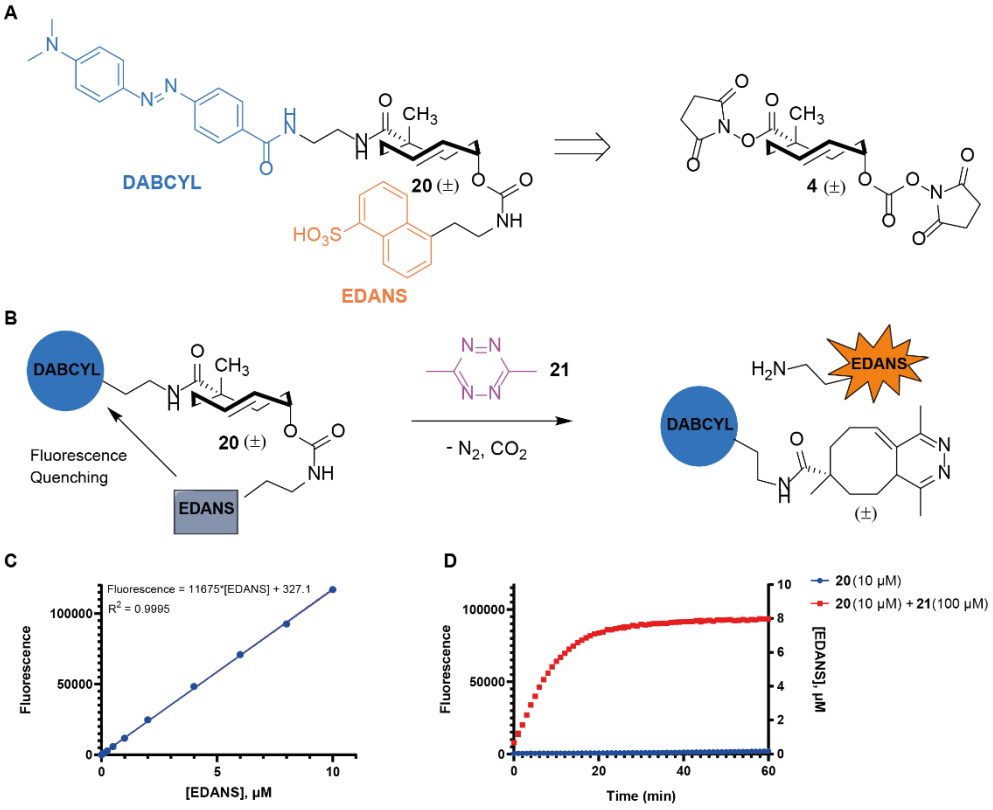


Figure 3 A) TCO **20** was projected to be synthesised from bifunctional TCO **4**. B) Schematic representation of the TCO-quenched fluorescence assay. The bifunctional TCO-reporter-quencher pair **20** does not display fluorescence in its native state due to fluorescence quenching between the EDANS fluorophore and DABCYL quencher. The EDANS fluorophore is released upon IEDDA pyridazine elimination, thereby disabling the fluorescence quenching and enabling a fluorescent readout for the elimination reaction. C/D) Quantification of fluorescence observed for TCO-reporter-quencher pair **20** (10 µM) using tetrazine **21** at 100 µM (n = 3). C) Linear regression of fluorescence observed for EDANS in µM. D) Fluororescence observed for TCO-reporter-quencher pair **20** (10 µM) upon treatment with tetrazine **21** (100 µM). The fluorescence observed was correlated to the linear regression for EDANS fluoresence, revealing an elimation efficiency of 80% after 60 minutes.

with Beer's law (Figure 2C). Xenon Arc (1000 W) irradiation of **19** with wavelength filters indicated light with a wavelength ≤ 445 nm is necessary to induce the photoisomerization of **19** to **5** (Figure 2D).

The research summarized below was conducted in collaboration with Dr. Elmer Maurits (Leiden University), Alexi J.C. Sarris (Leiden University) and Dr. Thomas Hansen (Leiden University). Full details can be found in the published article.[26]

Implementation of the IEDDA pyridazine elimination in biological systems is reliant on the identification of suitable tetrazines in terms of reaction speed with respect to both initial Diels-Alder rate, rate of the ensuing retro-Diels-Alder and final elimination step. The nature of substituents on the two tetrazine carbons can exert drastic effects on both the rate and yield of the release obtained.[13,17,27,28] Fluorogenic reporters are important tools to characterize tetrazine release behavior, and these are often supported by NMR[13,16] or LC-MS[17,27,28] analyses. The profound effect of pH and buffer concentration on IEDDA pyridazine elimination complicates such studies.[16,17] Weissleder and co-workers[17] reported a quenched fluorescence assay, in which the tetrazine was modified with a quencher moiety to suppress fluorescence until alkylamine release occurred. This method, however, does not allow the characterization of unmodified tetrazines, which lack this quencher moiety, at low concentrations.

With these considerations in mind, bifunctional TCO **4** was used to design fluorogenic TCO **20**, which combines an EDANS fluorophore and a DABCYL quencher on the bifunctional TCO scaffold (Figure 3A). Condensation of **4** with the EDANS moiety occurred with complete regioselectivity towards the carbonate due to the steric hindrance induced by the methyl group. This was followed by functionalization with the complementary DABCYL quencher to obtain TCO-reporter-quencher pair **20**. Functionalization with EDANS and DABCYL occurred with the same degree of regioselectivity for CCO reagent **5**.

TCO **20** is fluorogenic by virtue of intramolecular fluorescence quenching until the EDANS fluorophore is released from the post-ligation construct, and could thus serve to determine overall properties of the IEDDA pyridazine elimination (Figure 3B). The reaction between **20** (10 μM) and 3,6-dimethyltetrazine **21** (100 μM) was characterized by quantifying the fluorescence emitted from the liberated EDANS fluorophore (Figure 3C/D). This experiment showed an EDANS release yield of 80% after 1 h, which corresponds with other reports.[13,16,17] Furthermore, a CCO analog of **20** was unresponsive towards treatment with tetrazine **21**, as no fluorescence was detected for this reaction pair.

Fluorogenic TCO **20** was subsequently employed to evaluate a panel of tetrazines selected from literature.[17,27,28] Pseudo-first-order rate constants (k_{obs}) and elimination yields after 4 h were determined based on a 96-well plate reader setup followed by data processing according to biphasic decay trend lines. Screening different concentrations of **20** with an excess of tetrazine enabled separation of the rate constants for the initial cycloaddition step and the subsequent tautomerization/elimination process, k_{IEDDA} and $k_{release}$, respectively.

2.3 Conclusions

In conclusion, a streamlined synthetic procedure is presented for the widely used bifunctional TCO reagent **4**. Crystallization of iodolactone **10** enables the initial steps of the route to be carried out using crude reaction mixtures whilst obtaining an intermediate product of high purity. Transesterification of **15** was replaced by a two-step saponification-methylation procedure, improving yield and decreasing reaction time. Bis-NHS functionalization of axial TCO **17** was accelerated by nucleophilic catalysis and the chromatographic purification of the final product **4** was optimized. A novel CCO reagent, **5**, was synthesized by *trans* to *cis* photoisomerization of equatorial TCO **19**.

TCO reagent **4** was employed for the design and synthesis of fluorogenic TCO **4**. This fluorogenic probe (**4**) combines the EDANS-DABCYL fluorophore-quencher pair with the bifunctional TCO scaffold, thereby linking the intramolecular fluorescence quenching to **4** and intermediates which occur after tetrazine ligation, but before elimination of the allylic payload. Kinetic analysis was conducted on a panel of tetrazines, thereby demonstrating the feasibility of this 96-well plate assay.

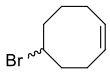
2.4 Experimental procedures

General methods: Commercially available reagents and solvents were used as received. Moisture and oxygen sensitive reactions were performed under an argon or N_2 atmosphere (balloon). MeCN, DMSO, DMF, DCM, toluene, THF, dioxane and Et_2O were stored over (flame-dried) 4 Å molecular sieves (8-12 mesh). DIPEA was stored over KOH pellets. TLC analysis was performed using aluminum sheets, pre-coated with silica gel (Merck, TLC Silica gel 60 F_{254}). Compounds were visualized by UV absorption (λ = 254 nm), by spraying with either a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in H_2O, a solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g/L) in 10% H_2SO_4, 20% H_2SO_4 in EtOH, or phosphomolybdic acid in EtOH (150 g/L), where appropriate, followed by charring at ca. 150°C. Column chromatography was performed on Screening Devices b.v. Silica Gel (particle size 40-63 μM, pore diameter 60 Å). ^1H, ^{13}C APT, ^1H COSY and HSQC spectra were recorded with a Bruker AV-400 (400/100 MHz) or AV-500 (500/125 MHz) spectrometer. Chemical shifts are reported as δ values (ppm) and were referenced to tetramethylsilane (δ = 0.00 ppm) or the residual solvent peak as internal standard. *J* couplings are reported in Hz. High resolution mass spectra were recorded by direct injection (2 μL of a 1 μM solution in H_2O/MeCN 1:1 and 0.1% formic acid) on a mass spectrometer (Q Exactive HF Hybrid Quadrupole-Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275°C) with resolution R = 240,000 at m/z 400 (mass range m/z = 160-2,000) and an external lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). The synthesis of tetrazine **21** is described in Chapter 4.[29]

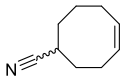
Preparation of neutralized silica gel: Unmodified silica gel (500 gram) was slowly dispersed into a 3 L round-bottom flask containing a stirring volume of H_2O (1.7 L). NH_4OH (28% w/w, 100 mL) was added and the alkaline suspension was stirred for 30 min. The suspension was filtered, washed with H_2O and the silica gel was dried on aluminium foil overnight at rt. The silica was transferred into a glass container and remaining traces of H_2O were removed by drying in an oven at 150°C overnight.

Photoisomerization methods: General guidelines were followed as described by Royzen *et al*.[25] Photochemical isomerization of **8** was performed using a Southern New England Ultraviolet Company Rayonet reactor (model RPR-100) equipped with 16 bulbs (part number RPR-2537A, λ = 254 nm). Photolysis was performed in a 1500 mL quartz flask (Southern New England Ultraviolet Company; part number RQV-323). A HPLC pump (Jasco; model PU-2088 Plus) was used to circulate solvent through the photolysis apparatus. An empty solid load cartridge with screw cap, frits, O-ring and end tips (40 g, iLOK, SD.0000.040, Screening Devices b.v.) was manually loaded with the specified silica gel to function as the stationary phase.

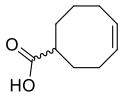
The emission spectrum of Figure 2A was recorded using an integrating sphere connected to an Avantes 2048L StarLine spectrometer detecting in the region of 300 – 1000 nm. The Avasoft 8.5 software from Avantes was used for recording the spectrum. Absorption spectra of compound **19** (Figure 2B) were recorded at room temperature with a Cary60 from Agilent and 1 cm x 1 cm quartz cuvettes from FireflySci.



(Z)-5-Bromocyclooct-1-ene (11): A 500 mL round-bottom flask charged with HBr (33 % w/w in AcOH, 85 mL, 490 mmol, 0.98 equiv) under N_2 was cooled to 0°C before adding (1Z,5Z)-cycloocta-1,5-diene (**9**, 61.3 ml, 500 mmol, 1.0 equiv) dropwise over 30 minutes. The reaction mixture was stirred for 72 h and allowed to warm to room temperature. The reaction mixture was diluted with H_2O (500 mL) and extracted with 10% Et_2O in pentane (3 x 750 mL). The combined organic layers were washed with H_2O (250 mL) and $NaHCO_3$ (satd., 250 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude bromide **11** (93.8 g, 496 mmol, 99%) was obtained as an oil and used in the next step without further purification: R_f = 0.7 (pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.70 – 5.54 (m, 2H), 4.40 – 4.21 (m, 1H), 2.47 – 2.37 (m, 1H), 2.34 – 2.19 (m, 3H), 2.19 – 1.99 (m, 4H), 1.79 – 1.67 (m, 1H), 1.60 – 1.48 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 129.7, 129.3, 55.7, 39.8, 37.2, 27.1, 25.4, 25.3. Spectroscopic data was in agreement with literature.[30]

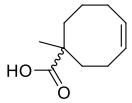


(Z)-Cyclooct-4-ene-1-carbonitrile (12): A 1 L round-bottom flask was charged with sodium cyanide (67.4 g, 1375 mmol, 2.78 equiv) and placed under N_2. Anhydrous DMSO (250 mL) was added, and the suspension was stirred at 100°C to dissolve the cyanide. The crude bromide **11** (93.7 g, 496 mmol, 1.0 equiv) was added to the reaction mixture over 30 minutes using a dropping funnel under N_2. The reaction mixture was stirred for 4 h at 100°C before cooling on ice and diluting with H_2O (2.5 L). The aqueous phase was extracted with pentane (5 x 1 L). Performing the extraction without cooling the reaction mixture often resulted in the formation of emulsions. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude nitrile **12** (37.8 g, 280 mmol, 56%) was obtained as an orange oil and used in the next step without further purification: R_f = 0.7 (10% Et_2O in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.76 – 5.50 (m, 2H), 2.85 – 2.73 (m, 1H), 2.51 – 2.35 (m, 1H), 2.33 – 2.19 (m, 1H), 2.19 – 2.06 (m, 2H), 2.06 – 1.92 (m, 1H), 1.92 – 1.74 (m, 4H), 1.55 – 1.37 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 130.8, 129.0, 123.4, 32.2, 29.5, 28.2, 27.1, 25.1, 23.4; HRMS: calculated for $C_9H_{14}N_1$ 136.11208 $[M+H]^+$; found 136.11210. Spectroscopic data was in agreement with literature.[24]

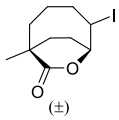


(Z)-Cyclooct-4-ene-1-carboxylic acid (13): The crude nitrile **12** (37.8 g, 280 mmol, 1.0 equiv) was dissolved in EtOH (140 mL) in a 3 L round-bottom flask. KOH (30% w/w, 420 mL) was added and the reaction mixture was stirred at 40°C. H_2O_2 (30% w/w, 250 mL, 2.45 mol, 8.75 equiv) was added to the reaction mixture over 2 h using a dropping funnel. After complete addition, the foamy reaction mixture was stirred for an additional 5 h at 40°C. TLC analysis (20% Et_2O in pentane) confirmed complete consumption of nitrile **12**. The temperature was gradually raised (increments of 20°C) to reflux. The reaction mixture was refluxed for 17 h before cooling down to room temperature. H_2O (1 L) was added and the mixture was washed with heptane (1 L). The organic layer was extracted with H_2O (2 x 750 mL). The combined aqueous layers were acidified with HCl (37% w/w) to pH 1. Then, the combined acidified aqueous layers were extracted with Et_2O (5 x 400 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude carboxylic acid **13** (31.8 g, 206 mmol, 74%) was obtained as a yellow oil and used in the next step without further purification: R_f = 0.5 (10% Et_2O, 1% AcOH in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.80 (br s, 1H), 6.09 – 5.26 (m, 2H), 2.56 – 2.45 (m, 1H), 2.45 – 2.32 (m, 1H), 2.21 – 2.02 (m, 4H), 1.97 – 1.84

(m, 1H), 1.79 – 1.52 (m, 3H), 1.47 – 1.33 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.4, 130.7, 129.6, 43.3, 31.5, 29.3, 27.9, 26.0, 24.1; HRMS: calculated for $C_9H_{15}O_2$ 155.10666 $[M+H]^+$; found 155.10705. Spectroscopic data was in agreement with literature.[24]

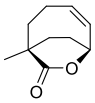


(Z)-1-Methylcyclooct-4-ene-1-carboxylic acid (14): Diisopropylamine (109 mL, 763 mmol, 3.7 equiv) was dissolved in anhydrous THF (250 mL) in a 2 L round-bottom flask under N_2. The solution was cooled to -50°C. *n*-Butyllithium (11 M in hexanes, 63.7 mL, 701 mmol, 3.4 eq) was added dropwise over 30 minutes whilst maintaining a temperature between -60°C and -40°C. The reaction mixture was stirred for 1 h at -50°C. Crude carboxylic acid **13** (31.8 g, 206 mmol, 1.0 equiv) was dissolved in anhydrous THF (100 mL) under N_2 and subsequently added to the reaction mixture using a double tipped needle under N_2 pressure. The reaction mixture was stirred for 1 h and allowed to warm to -20°C. Subsequently, the reaction mixture was stirred for 3 h at 50°C. Afterwards, the reaction mixture was cooled to -50°C before slowly adding CH_3I (51.6 mL, 825 mmol, 4.0 equiv) over 30 min. The reaction mixture was stirred for 17 h and allowed to warm to room temperature. Next, the reaction mixture was stirred for 3 h at 50°C. The reaction mixture was cooled on ice, quenched with NH_4Cl (satd., 50 mL) and concentrated *in vacuo*. The residue was diluted with HCl (2 M, 350 mL) and toluene (150 mL). The organic layer was washed with HCl (2 M, 100 mL). The combined aqueous layers were extracted with toluene (2 x 250 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude, methylated carboxylic acid **14** (34.15 g, 203 mmol, 98%) was obtained as a dark oil and used in the next step without further purification: R_f = 0.5 (10% Et_2O, 1% AcOH in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.65 (br s, 1H), 5.69 (dt, *J* = 11.1, 5.5 Hz, 1H), 5.53 – 5.42 (m, 1H), 2.42 – 2.05 (m, 5H), 1.90 – 1.82 (m, 1H), 1.79 – 1.50 (m, 4H), 1.25 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.0, 131.9, 126.5, 46.1, 35.2, 32.2, 27.0, 25.9, 24.7, 24.6; HRMS: calculated for $C_{10}H_{17}O_2$ 169.12231 $[M+H]^+$; found 169.12274. Spectroscopic data was in agreement with literature.[18]



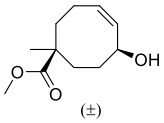
Bicyclic iodolactone 10: The crude, methylated carboxylic acid **14** (34.15 g, 203 mmol, 1.0 equiv) was dissolved in a mixture of DCM (400 mL) and H_2O (200 mL) in a 1 L round-bottom flask. $NaHCO_3$ (51.2 g, 609 mmol, 3.0 equiv) was added and the reaction mixture was stirred for 30 minutes before cooling to 0°C. I_2 (51.5 g, 203 mmol, 1.0 equiv) and KI (37.1 g, 223 mmol, 1.1 equiv) were added to a 500 mL Erlenmeyer flask and mixed with a spatula, forming a brown mixture. The iodine mixture was added portion wise to the cooled reaction mixture over 1 h. After complete addition, the reaction mixture was stirred for 4.5 h and allowed to reach room temperature. Subsequently, the reaction mixture was cooled on ice and quenched with $NaHSO_3$ (10% w/w) until discoloration was complete. Brine (200 mL) was added and the aqueous phase was extracted with DCM (2 x 250 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was obtained as a dark oil which was crystallized from EtOH to obtain iodolactone **10** (33.18 g, 113 mmol, 56%, 23% over 5 steps) as a solid: R_f = 0.35 (10% Et_2O, 1% AcOH in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.04 (dt, *J* = 8.1, 2.7 Hz, 1H), 4.66 – 4.50 (m, 1H), 2.59 – 2.39 (m, 2H), 2.37 – 2.24 (m, 1H), 2.22 – 1.99 (m, 2H), 1.98 – 1.75 (m, 3H), 1.71 – 1.61 (m, 1H), 1.56 – 1.42 (m, 1H), 1.29 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.5, 82.1, 43.8, 41.1, 37.0, 33.7, 32.5, 29.6, 26.8, 19.5;

HRMS: calculated for $C_{10}H_{16}IO_2$ 295.01895 $[M+H]^+$; found 295.01856. Spectroscopic data was in agreement with literature.[18]



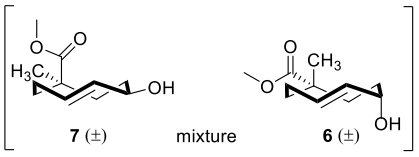
(±)

Bicyclic olefin 15: Iodolactone **10** (33.18 g, 113 mmol, 1.0 equiv) was co-evaporated with anhydrous toluene (2 x 50 mL) in a 1 L round-bottom flask, placed under N_2 and dissolved in anhydrous toluene (370 mL). DBU (22.1 mL, 147 mmol, 1.3 equiv) was added to the solution and the reaction mixture was stirred at 70°C under N_2. After 20 h, additional DBU (22.1 mL, 147 mmol, 1.3 equiv) was added and the reaction mixture was stirred for 72 h at 70°C under N_2. The reaction mixture was cooled to room temperature before washing with H_2O (2x 400 mL). The combined aqueous layers were extracted with toluene (400 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude bicyclic olefin **15** (17.24 g, 104 mmol, 92%) was obtained as an oil and used in the next step without further purification: R_f = 0.4 (20% Et_2O in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 5.95 (ddd, *J* = 11.9, 9.6, 5.4 Hz, 1H), 5.46 (ddd, *J* = 11.9, 4.8, 2.4 Hz, 1H), 5.10 (br s, 1H), 2.50 – 2.37 (m, 1H), 2.27 – 2.08 (m, 2H), 1.99 – 1.86 (m, 1H), 1.84 – 1.66 (m, 4H), 1.32 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.2, 129.2, 127.7, 79.2, 45.2, 43.0, 31.9, 29.4, 26.6, 24.0; HRMS: calculated for $C_{10}H_{15}O_2$ 167.10666 $[M+H]^+$; found 167.10643. Spectroscopic data was in agreement with literature.[18]

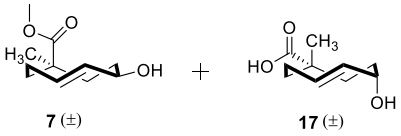


(±)

Cyclooctene methyl ester 8: The crude bicyclic olefin **15** (17.24 g, 104 mmol, 1.0 equiv) was dissolved in a mixture of dioxane (60 mL) and H_2O (60 mL) in a 1 L round-bottom flask. The mixture was cooled to 0°C before adding KOH (14.55 g, 259 mmol, 2.5 equiv) portion wise. The reaction mixture was stirred for 20 h and allowed to warm to room temperature. TLC analysis (50% EtOAc, 1% AcOH in pentane) confirmed conversion of the starting material into carboxylic acid intermediate **16**. Subsequently, the reaction mixture was concentrated *in vacuo* and co-evaporated with anhydrous toluene (3 x 60 mL) and anhydrous DMF (60 mL) to obtain the potassium salt of **16** as a crude, viscous oil: R_f = 0.5 (50% EtOAc, 1% AcOH in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 5.67 – 5.52 (m, 1H), 5.35 (ddt, *J* = 11.9, 5.6, 1.7 Hz, 1H), 5.03 – 4.88 (m, 1H), 2.36 – 2.25 (m, 1H), 2.25 – 2.13 (m, 1H), 2.13 – 2.01 (m, 3H), 1.78 – 1.65 (m, 2H), 1.65 – 1.53 (m, 1H), 1.27 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.2, 132.3, 129.6, 68.5, 45.9, 35.7, 33.7, 30.1, 26.8, 24.8. Compound **16** was placed under N_2, suspended in anhydrous DMF (175 mL) and cooled to 0°C. CH_3I (19.46 mL, 311 mmol, 3.0 equiv) was slowly added to the reaction mixture before stirring for 17 h and allowing the reaction mixture to warm to room temperature. H_2O (2 L) was added and the reaction mixture was extracted with Et_2O (3 x 1 L). The combined organic layers were washed with H_2O (500 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (10 → 20% EtOAc in pentane). Methyl ester **8** (15.78 g, 80 mmol, 77% over 2 steps) was obtained as a yellow oil: R_f = 0.4 (30% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 5.58 (dtd, *J* = 11.6, 5.6, 2.0 Hz, 1H), 5.41 – 5.28 (m, 1H), 5.00 – 4.86 (m, 1H), 3.66 (s, 3H), 2.36 – 2.22 (m, 1H), 2.19 – 2.00 (m, 4H), 1.77 (d, *J* = 4.7 Hz, OH), 1.74 – 1.63 (m, 2H), 1.60 – 1.52 (m, 1H), 1.22 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.8, 132.4, 129.3, 68.4, 51.9, 46.0, 35.9, 33.7, 30.4, 26.7, 24.9; HRMS: calculated for $C_{10}H_{15}O_2$ 167.10666 $[M-MeOH+H]^+$; found 167.10677. Spectroscopic data was in agreement with literature.[18]

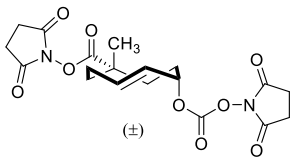


Mixture of axial TCO methyl ester 6 and equatorial TCO methyl ester 7: Methyl ester **8** (1.73 g, 8.73 mmol, 1.0 equiv) was irradiated (λ = 254 nm) for 24 h in the presence of methyl benzoate (3.30 mL, 26.2 mmol, 3.0 equiv) in a quartz flask containing a solution of Et_2O in heptane (1:3, 600 mL). During irradiation, the reaction mixture was continuously circulated over a silica column (40 g size, containing dry silica and 22 g of $AgNO_3$ impregnated silica[25] (10% wt, containing ca. 13 mmol $AgNO_3$, 1.5 equiv)) at a flowrate of 40 mL/min. The column was placed in the dark and shielded with aluminum foil during the irradiation. Absence of **8** from the reaction mixture was shown with ^1H NMR and the column was flushed with Et_2O/heptane (1:3, 500 mL) before drying over a stream of air. Next, the contents of the column were emptied into an Erlenmeyer flask containing NH_4OH (28% w/w, 200 mL) and DCM (200 mL). The biphasic mixture was stirred for ~ 1 h before filtration of the silica gel. The organic layer was separated and the aqueous layer was extracted with DCM (200 mL). The combined organic layers were washed with H_2O (100 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (33% EtOAc in pentane, isocratic) to obtain the isomeric mixture of axial isomer **6** and equatorial isomer **7** (1.46 g, 7.36 mmol, 84%, **6** : **7** in an approximate ratio of 1 : 1.4) as an oil: R_f = 0.7 (axial isomer **6**, 50% EtOAc in pentane), 0.6 (equatorial isomer **7**, 50% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 6.18 – 5.96 (m, 1H, **6**), 5.80 (ddd, *J* = 15.8, 11.6, 3.9 Hz, 1H, **7**), 5.63 (dd, *J* = 16.6, 2.4 Hz, 1H, **6**), 5.37 (dd, *J* = 16.2, 9.4 Hz, 1H, **7**), 4.48 (br s, 1H, **6**), 4.21 (td, *J* = 9.8, 5.7 Hz, 1H, **7**), 3.73 (s, 3H, **7**), 3.64 (s, 3H, **6**), 2.72 (qd, *J* = 11.9, 4.6 Hz, 1H, **7**), 2.38 – 2.03 (m, 3H, **6**, 3H, **7**), 2.01 – 1.60 (m, 5H, **6**, 2H, **7**, 2OH), 1.60 – 1.46 (m, 1H, **6**, 1H, **7**), 1.38 – 1.24 (m, 1H, **7**), 1.20 (s, 3H, **7**), 1.11 (s, 3H, **6**); ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.5, 177.4, 135.9, 135.1, 132.7, 130.5, 75.1, 69.8, 52.1, 51.4, 47.6, 46.0, 44.9, 44.7, 40.0, 38.9, 38.3, 34.8, 31.0, 30.9, 29.7, 18.2; HRMS: calculated for $C_{11}H_{18}O_3Na$ 221.11482 $[M+Na]^+$; found 221.11471. Spectroscopic data was in agreement with literature.[18]

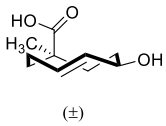


Equatorial TCO methyl ester 7, axial TCO carboxylic acid 17: The isomeric mixture of **6** and **7** (1.46 g, 7.36 mmol, 1.0 equiv) was placed under N_2, dissolved in MeOH (28 mL) and cooled on ice whilst stirring. A solution of KOH (4.54 g, 81 mmol, 11 equiv) in H_2O (14 mL) under N_2 was cooled on ice and subsequently added to the isomeric mixture. The reaction mixture was placed in the dark, shielded with aluminum foil and stirred for 68 h at 4°C. TLC (50% EtOAc in pentane) indicated complete hydrolysis of axial isomer **6**, whereas equatorial isomer **7** remained unaffected. The reaction mixture was diluted with H_2O (125 mL) and extracted with Et_2O (3 x 125 mL). The combined organic layers were washed with H_2O (25 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to obtain equatorial TCO methyl ester **7** (663 mg, 3.19 mmol, 43%) as a transparent oil: R_f = 0.6 (50% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 5.80 (ddd, *J* = 15.8, 11.6, 3.9 Hz, 1H), 5.37 (dd, *J* = 16.2, 9.4 Hz, 1H), 4.21 (td, *J* = 9.8, 5.7 Hz, 1H), 3.73 (s, 3H), 2.73 (qd, *J* = 11.9, 4.6 Hz, 1H), 2.30 – 2.20 (m, 1H), 2.17 – 2.05 (m, 2H), 1.93 (dddd, *J* = 14.0, 11.8, 10.2, 1.4 Hz, 1H), 1.83 – 1.71 (m, 1H + OH), 1.53 (ddd, *J* = 14.2, 12.6, 4.8 Hz, 1H), 1.31 (ddd, *J* = 15.4, 12.2, 1.4 Hz, 1H), 1.20 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.4, 135.9, 132.8, 75.1, 51.5, 47.6, 46.0, 40.0, 38.9, 34.8, 31.1; HRMS: calculated for $C_{11}H_{19}O_3$ 199.13287 $[M+H]^+$;

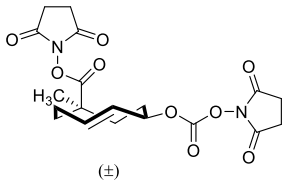
found 199.13290. The combined aqueous layers were acidified to pH 3 with AcOH (30 mL total), followed by extraction with Et_2O (3 x 125 mL). The combined organic layers were washed with H_2O (65 mL), dried over $MgSO_4$, filtered, partially concentrated *in vacuo* and co-evaporated with distilled toluene (3 x) to obtain axial TCO carboxylic acid **17** (569 mg, 3.09 mmol, 42%) as a yellow oil which was used in the next step without further purification: R_f = 0.3 (50% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.07 (ddd, *J* = 16.4, 10.8, 3.4 Hz, 1H), 5.64 (dd, *J* = 16.6, 2.5 Hz, 1H), 4.49 (br s, 1H), 2.39 – 2.08 (m, 3H), 2.06 – 1.77 (m, 4H), 1.65 (dd, *J* = 15.9, 6.1 Hz, 1H), 1.11 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.6, 135.1, 130.6, 69.8, 44.8, 44.5, 38.3, 30.9, 29.6, 18.0; HRMS: calculated for $C_{10}H_{17}O_3$ 185.11722 $[M+H]^+$; found 185.11728. Spectroscopic data was in agreement with literature.[18]



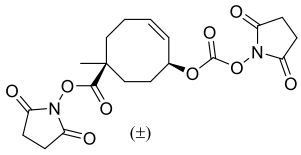
Axial TCO reagent 4: Axial TCO carboxylic acid **17** (515 mg, 2.80 mmol, 1.0 equiv) and *N,N′*-Disuccinimidyl carbonate (2.15 g, 8.39 mmol, 3.0 equiv) were combined in a 100 mL round-bottom flask. The mixture was co-evaporated with distilled toluene (3 x 5 mL), placed under N_2 and suspended in anhydrous MeCN (18 mL). DIPEA (1.95 mL, 11.2 mmol, 4.0 equiv) and DMAP (34 mg, 0.28 mmol, 0.1 equiv) were added and the resulting clear reaction mixture was placed in the dark, shielded with aluminum foil and stirred at room temperature for 17 h. The reaction mixture was concentrated *in vacuo*, co-evaporated with distilled toluene (3 x 5 mL) and subsequently purified by silica gel chromatography (neutralized silica, DCM → 2% acetone in DCM) to obtain the axial bifunctionalized product **4** (853 mg, 2.02 mmol, 72%) as a white, foamy solid: R_f = 0.3 (2% acetone in DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 – 5.95 (m, 1H), 5.62 (dd, *J* = 16.7, 2.1 Hz, 1H), 5.28 (br s, 1H), 2.84 (2 s, 8H), 2.53 – 2.19 (m, 4H), 2.19 – 1.91 (m, 4H), 1.28 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ174.2, 169.4, 168.7, 150.7, 133.1, 128.9, 78.9, 44.4, 44.4, 35.8, 30.9, 30.2, 25.8, 25.6, 18.1; HRMS: calculated for $C_{19}H_{22}N_2O_9Na$ 445.12175 $[M+Na]^+$; found 445.12150. Spectroscopic data was in agreement with literature.[18]



Equatorial TCO carboxylic acid 18: Equatorial TCO methyl ester **7** (350 mg, 1.77 mmol, 1.0 equiv) was placed under N_2 and dissolved in MeOH (10 mL). A solution of KOH (1.09 g, 19.4 mmol, 11 equiv) in H_2O (5 mL) under N_2 was cooled on ice and subsequently added to the methyl ester solution. The resulting reaction mixture was stirred for 6 d at 50°C, during which the reaction was kept in the dark and shielded with aluminium foil. The reaction mixture was diluted with H_2O (50 mL) and washed with Et_2O (2 x 100 mL). The combined organic layers were extracted with H_2O (50 mL). The combined aqueous layers were acidified to pH 3 with citric acid, followed by extraction with Et_2O (3 x 100 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to obtain equatorial TCO carboxylic acid **18** (256 mg, 1.39 mmol, 79%) as a white solid: R_f = 0.3 (50% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 (ddd, *J* = 15.8, 11.6, 3.9 Hz, 1H), 5.51 (dd, *J* = 16.2, 9.4 Hz, 1H), 4.25 (td, *J* = 9.6, 6.3 Hz, 1H), 2.77 (qd, *J* = 12.0, 4.6 Hz, 1H), 2.31 – 2.21 (m, 1H), 2.20 – 2.05 (m, 3H), 1.86 – 1.76 (m, 1H), 1.55 (ddd, *J* = 14.2, 12.6, 4.9 Hz, 1H), 1.35 (ddd, *J* = 15.2, 11.0, 2.6 Hz, 1H), 1.28 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.1, 135.8, 132.9, 75.2, 47.3, 45.8, 39.9, 38.9, 34.9, 30.9; HRMS: calculated for $C_{10}H_{16}O_3Na$ 207.09917 $[M+Na]^+$; found 207.09914.



Equatorial TCO reagent 19: Equatorial TCO carboxylic acid **18** (250 mg, 1.36 mmol, 1.0 equiv) and *N,N'*-Disuccinimidyl carbonate (1.39 g, 5.43 mmol, 4.0 equiv) were combined in a 50 mL round-bottom flask. The mixture was co-evaporated with distilled toluene (2 x 5 mL), placed under N_2 and suspended in anhydrous MeCN (7 mL). DIPEA (1.78 mL, 10.2 mmol, 7.5 equiv) was added and the resulting clear reaction mixture was placed in the dark, shielded with aluminum foil and stirred at room temperature for 36 h. The reaction mixture was impregnated with Celite, concentrated *in vacuo* and purified by silica gel chromatography (50% EtOAc in pentane, isocratic) to obtain the equatorial bifunctionalized product **19** (378 mg, 0.89 mmol, 66%) as a foamy white solid: R_f = 0.3 (50% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ5.95 (ddd, *J* = 15.9, 11.6, 4.0 Hz, 1H), 5.75 (dd, *J* = 16.4, 9.6 Hz, 1H), 5.16 (td, *J* = 10.1, 5.8 Hz, 1H), 2.92 – 2.77 (m, 8H), 2.73 – 2.59 (m, 1H), 2.42 (d, *J* = 14.2 Hz, 1H), 2.33 – 2.18 (m, 2H), 1.97 (dd, *J* = 15.6, 5.7 Hz, 1H), 1.70 (ddd, *J* = 14.3, 12.9, 4.7 Hz, 1H), 1.46 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5, 169.4, 168.9, 151.1, 135.0, 130.7, 83.5, 47.5, 45.2, 38.7, 35.1, 35.0, 30.9, 25.8, 25.6; HRMS: calculated for $C_{19}H_{22}N_2O_9Na$ 445.12175 $[M+Na]^+$; found 445.12164.



CCO reagent 5: Equatorial bifunctionalized TCO **19** (464 mg, 1.10 mmol, 1.0 equiv) was dissolved in $CDCl_3$ (22 mL) in a 100 mL round-bottom flask. The solution was purged with N_2, sealed and stirred whilst irradiating with a CFL (23 W, 1 cm distance to flask, see Figure 2) for 48 h. ^{1}H NMR analysis confirmed full conversion and the reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel chromatography (15% Et_2O in DCM, isocratic) to obtain the bifunctionalized *cis* product **5** (343 mg, 0.812 mmol, 74%) as an foamy white solid: R_f = 0.6 (30% Et_2O in DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.01 – 5.86 (m, 1H), 5.82 – 5.69 (m, 1H), 5.44 (ddt, *J* = 12.0, 5.8, 1.6 Hz, 1H), 2.92 – 2.76 (m, 8H), 2.47 – 2.15 (m, 5H), 2.00 – 1.79 (m, 3H), 1.46 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 169.2, 168.8, 151.3, 131.5, 125.7, 79.6, 45.8, 35.8, 29.6, 29.4, 26.5, 25.8, 25.6, 24.5; HRMS: calculated for $C_{19}H_{22}N_2O_9Na$ 445.12175 $[M+Na]^+$; found 445.12154.

2.5 References

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