Inverse electron demand Diels-Alder bioconjugation reactions using 7-oxanorbornenes as dienophiles

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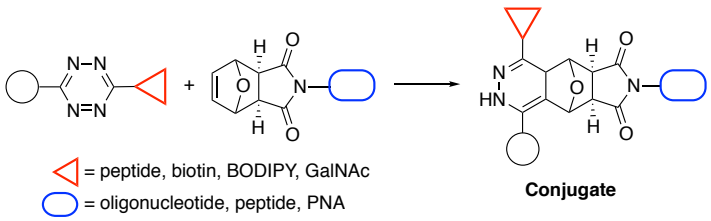


Abstract

Oligonucleotides, peptides and peptide nucleic acids incorporating 7-oxanorbornene as dienophile were reacted with tetrazines linked to either a peptide, D-biotin, BODIPY or *N*-acetyl-D-galactosamine. The inverse lectron demand Diels-Alder (IEDDA) cycloaddition, which was performed overnight at 37 ^{o}C, in all cases furnished the target conjugate in good yield. IEDDA reactions with 7-oxanorbornenes produce a lower number of stereoisomers than IEDDA cycloadditions with other dienophiles.

I

INTRODUCTION

Inverse electron demand Diels-Alder (IEDDA) cycloadditions are well established tools for bioconjugation. Most commonly the reaction involves 3,6-disubstituted-1,2,4,5-tetrazines and strained alkenes such as *n*-cyclooctenes, 1-methylcyclopropenes or norbornenes. The cycloaddition rate varies depending on the substituents of both the diene and the dienophile.[1] The choice of highly reactive dienophiles may be convenient for bioorthogonal experiments in which a high kinetic profile is desired.[2] However, when the aim is to prepare a bioconjugate employing a click-type reaction the use of stable reagents may be advantageous, since highly reactive reagents are more prone to decompose and generate more impurities.[3]

IEDDA cycloadditions do not generate a single compound (Scheme 1a). On the one hand, one stereocenter (an sp^3 carbon) is always present in the resulting 1,4-dihydropyridazine cycloadduct, which leads to mixtures of stereoisomers. On the other, the two possible diene-dienophile orientations give rise to different constitutional isomers. Since the two substituents of bioconjugation-convenient tetrazines are generally different, the final number of isomers can only be reduced by utilizing symmetrical dienophiles. For this purpose, we decided to explore the use of symmetrical 7-oxanorbornenes.

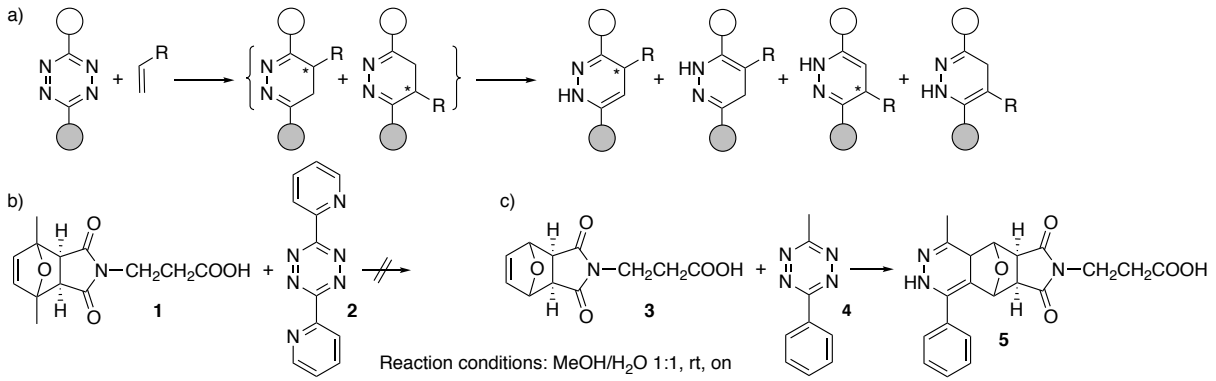
To our knowledge, 7-oxanorbornenes have not been used as dienophiles in IEDDA bioconjugation reactions, even though this possibility has been considered,[4] and 7-azanorbornenes only in one case.[5] Kinetic studies on the reactivity of several norbornenes[6] have shown that 7-oxanorbornenes are a bit less reactive than the corresponding norbornene: The reaction with 3,6-dipyridin-2-yl-1,2,4,5-tetrazine (**2**) was 1.5[6b] or three[6a] times slower depending on the structure of the bicyclic dienophile. Therefore, 7-oxanorbornenes may replace norbornenes and faster reacting dienophiles in applications not requiring quick bioorthogonal conjugation reactions.

We here describe that 7-oxanorbornenes can successfully replace norbornenes in IEDDA conjugations. Both polyamides and oligonucleotides have been derivatized with 7-oxanorbornnenes, and subsequently used

The 7-oxanorbornenes we have used can be obtained from Diels-Alder reactions between maleimides and furans. This reaction has been known since long ago,[7] and has been extensively used in the materials science field (references 8 are just some examples) and for a variety of synthetic purposes.[9]

To test the suitability of 7-oxanorbornenes as dienophiles, we first investigated the IEDDA reaction between the commercially available 3,6-dipyridine-2-yl-1,2,4,5-tetrazine (**2**) and 1,4-dimethylsubstituted 7-oxanorbornene **1**. In this case the dienophile was the *exo* adduct of a 2,5-dimethylfuran-protected maleimide, which we have used for the on-resin assembly of maleimido-oligonucleotides as well as for the preparation of a variety of conjugates.[10] No reaction was found to take place (Scheme 1b).

Observing that norbornenes typically used in IEDDA reactions do not have any substituent at either the 1 or 4 positions, we next investigated whether furan-protected maleimides such as **3** would be suitable dienophiles, and run the reaction shown in Scheme 1c. In this case, even though tetrazine **4** was less reactive than **2**, the reaction did take place, furnishing **5**.

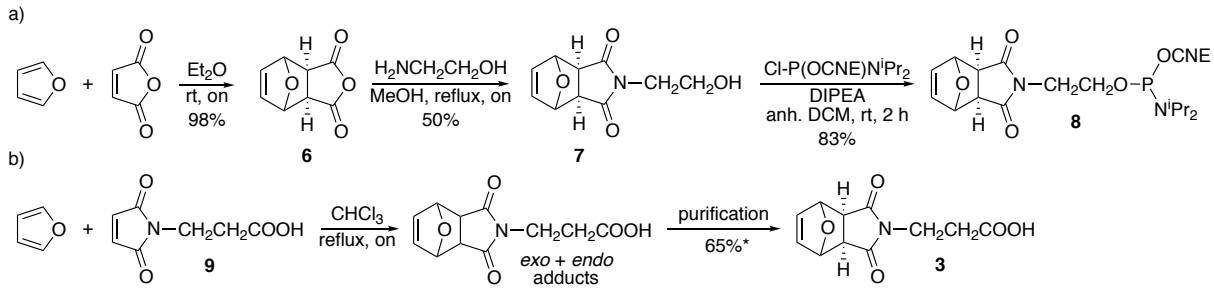


Scheme 1. Mixtures of compounds that result from IEDDA cycloadditions, where * depicts a stereocenter (a). Results of preliminary assays to assess the reactivity of 1,4-dimethyl-7-oxanorbornenes (b) and 7-oxanorbornenes (c) in IEDDA reactions.

Exo adducts are the thermodynamic products of maleimide (or maleic anhydride) Diels-Alder reactions with furans, and are known to be thermally more stable than the *endo* ones.[4,11] On this basis, we inferred that working with the 7-oxanorbornene *exo* adduct would be the best

choice in order to prevent its decomposition should IEDDA conjugation reactions require heating and/or long reaction times.

7-Oxanorbornene-containing compounds suitable for peptide and oligonucleotide derivatization were prepared as shown in Scheme 2. The Diels-Alder reaction between furan and maleic anhydride yields the *exo* isomer (**6**) only, which can then be transformed into the corresponding protected maleimide alcohol **7** and subsequently into the corresponding phosphoramidite **8**. Alternatively, commercially available (or prepared in the laboratory) 3-maleimidopropionic acid (**9**) can be reacted with furan, which gives a mixture of isomers that can be separated by precipitation.



Scheme 2. Synthesis of oxanorbornene-containing derivatives suitable for incorporation into oligonucleotides (a) and peptides (b). CNE=2-cyanoethyl; DIPEA=N,N-diisopropylethylamine. *Synthesis and purification yield.

Compound **8** was attached to the 5' end of resin-linked oligonucleotides, and **3** to the *N*-terminus of resin-linked peptides and peptide nucleic acids (PNAs), which allowed the corresponding 7-oxanorbornene-derivatized oligomers (**10-13**, Figure 1; see also Table 1 at the Experimental Section) to be obtained. As expected, 7-oxanorbornenes remained stable to the treatments that remove all permanent protecting groups from both oligonucleotides and polyamides after chain elongation, namely reaction with conc. aq. ammonia and with 95% trifluoroacetic acid (TFA) in the presence of scavengers, respectively.

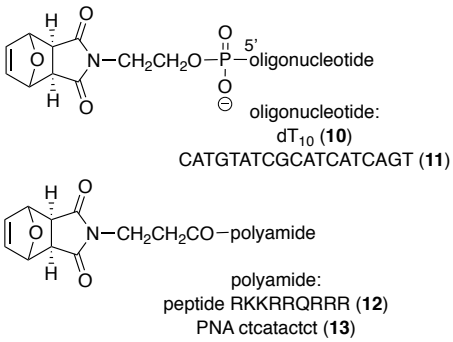


Figure 1. Oxanorbornene-derivatized oligonucleotides and polyamides prepared. 2'-Deoxyoligoribonucleotide sequences are depicted with upper case A, C, G and T; peptide sequences are also depicted with upper case letters, but can be recognized because they incorporate some of these as well as other letters; sequences depicted with lower case letters are those of PNAs.

Before embarking on the synthesis of more interesting tetrazines (whose synthesis is not straightforward[12]) and different conjugates, an additional proof-of-principle experiment was carried out to verify that the positive result shown in Scheme 1c could be reproduced. In this case the oxanorbornene-oligonucleotide **10** was used as dienophile, and reaction with tetrazine **2** gave the expected result (conjugate **pp2-10** was successfully isolated, see Table 2 at the Experimental Section).

The next step was the preparation of tetrazines conveniently derivatized for bioconjugation (Figure 2). Following described procedures that required some optimization (see Experimental Section), one tetrazine incorporating a carboxyl group (**14**) and one derivatized with an amine (**16**) were synthesized by reacting two nitrile-containing compounds and hydrazine hydrate, which was followed by oxidation with nitrous acid (Scheme 4, Experimental Section). **14** Was subsequently transformed into another amine-derivatized tetrazine (**15**) that included a spacer with two ethyleneglycol units, more reactive than **16** because of the presence of the 2-pyrimidinyl substituent instead of a methyl group (Scheme 4).

Thereafter, these compounds were used as starting materials to obtain the target tetrazines **18**, **19** and **20**), incorporating a biotin tether, a fluorophore (BODIPY) and *N*-acetyl-D-galactosamine, respectively (Figure 2; see also Schemes 5 and 6 at the Experimental Section). Additionally, tetrazine **14** was also attached to the *N*-terminus of a peptide to furnish tetrazine-peptide **17** (Figure 2, bottom). Other carboxyl-derivatized tetrazines have been incorporated to resin-linked peptides by reaction with DIPC (*N*,*N*-diisopropylcarbodiimide) in the presence of HOBt (1-hydroxybenzotriazole).[13] However, coupling of tetrazine **14** was deemed troublesome because of its high insolubility in virtually all organic solvents, including DMF. Tetrazine **14** could be satisfactorily coupled to the peptide-resin by reaction with HATU (1-[bis(dimethylamino)-methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium 3-oxide hexafluorophosphate) and *N*-ethyl-diisopropylamine using pyridine as solvent (2.5 h).

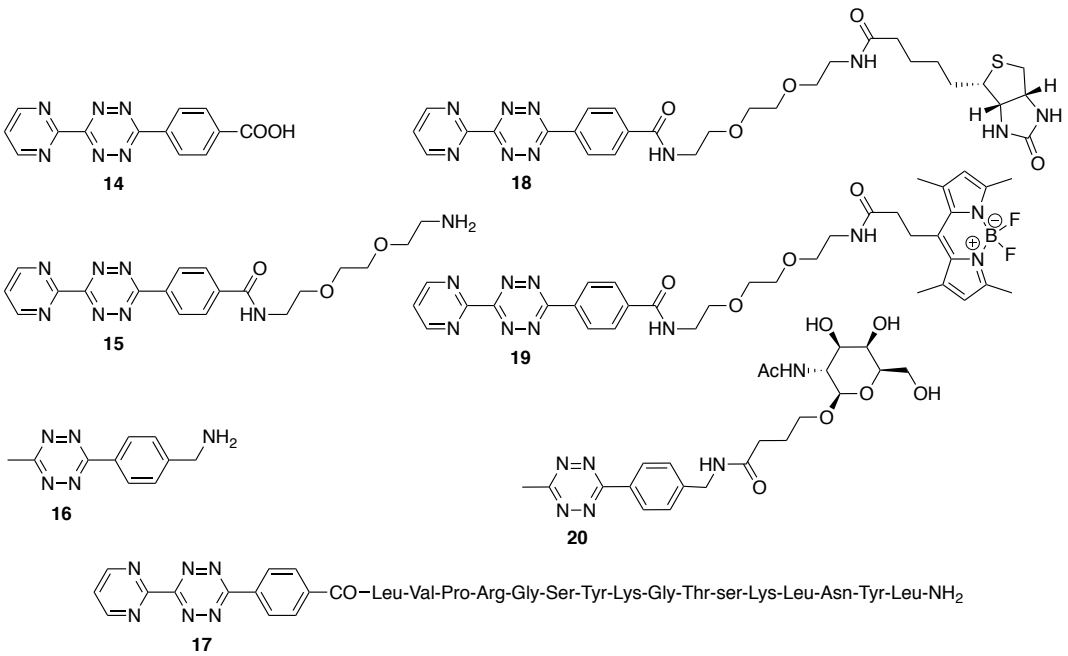


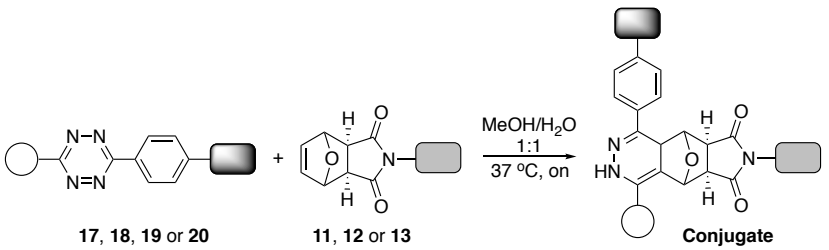
Figure 2. Carboxyl- and amine-derivatized tetrazines (**14-16**, left) used as starting materials for the preparation of tetrazines suitable for IEDDA conjugation (**17-20**, right and bottom).

In spite of its basic character, pyridine was a suitable, safe solvent, likely because of its moderate basicity. It is worth recalling that tetrazines have been described not to be stable to the piperidine solutions that remove Fmoc groups,[13a] and we have found them not to

withstand 1 M aq. NaOH and conc. aq. ammonia. Since the latter is the reagent required for the final deprotection of oligonucleotides prepared using standard methodology, tetrazine-derivatized oligonucleotides cannot be obtained by solid-phase synthesis. Tetrazine-containing nucleoside phosphoamidites have indeed been synthesized and utilized in solid-phase synthesis, but the tetrazine-oligonucleotide-resin was reacted with a cyclooctyne dienophile prior to the final ammonia-mediated deprotection.[14] Alternatively, tetrazine-containing oligonucleotides have been assembled making use of nucleoside triphosphates.[15]

In agreement with the literature,[13a] neither TFA nor the triisopropylsilane (TIS) scavenger present in the final deprotection treatment of the tetrazine-peptide caused any harm to the tetrazine core.

With all of the previously described compounds in hand a variety of oligonucleotide and polyamide conjugates were successfully prepared (Scheme 3). IEDDA Cycloadditions were carried out by reacting the oxanorbornene-containing compound with a 2-fold molar excess of the tetrazine derivative, overnight at 37 ^{o}C. Depending on the solubility of the reagents, the reactions were carried out either in water or in MeOH/H_2O mixtures (1:1 or 1:4, v/v). Progress of the reactions can be visually observed because the color of the mixture changes from purple (unreacted tetrazine) to yellowish. In all cases the target conjugate was isolated after reversed-phase HPLC purification, and characterized by MALDI-TOF MS (see Table 2 at the Experimental Section).



Conjugate	○	▬ *	▭ *	Yield (%)**
21	2-pyridiminyl	LVPRGSYKGTSKLNYL	CATGTATCGCATCATCAGT	25
22	Me	GalNAc	CATGTATCGCATCATCAGT	31
23	2-pyrimidinyl	biotin	CATGTATCGCATCATCAGT	34
24	2-pyrimidinyl	BODIPY	CATGTATCGCATCATCAGT	21
25	2-pyrimidinyl	LVPRGSYKGTSKLNYL	RKKRRQRRR	36
26	2-pyrimidinyl	biotin	RKKRRQRRR	45
27	2-pyrimidinyl	LVPRGSYKGTSKLNYL	ctcatactct	35
28	Me	GalNAc	ctcatactct	32
29	2-pyrimidinyl	BODIPY	ctcatactct	40

Scheme 3. Conjugates prepared. Full structures of starting materials are shown in Figures 1 and 2, and full structures of the conjugates are shown in Figure 3, Experimental Section). *Only the tag and the sequences of the oligomers are listed here, even though rectangles include one of the reagent's component (oligonucleotide, polyamide, tag, etc.) and the atoms that link it to the reactive part of the molecule (oxanorbornene, tetrazine). **Isolation/purification yield. 2'-Deoxyoligoribonucleotide sequences are depicted with upper case A, C, G and T; peptide sequences are also depicted with upper case letters, but can be identified because they incorporate some of these as well as other letters; sequences depicted with lower case letters are those of PNAs (PNA = peptide nucleic acid).

These results show that 7-oxanorbornenes are suitable dienophiles for use in IEDDA-involving bioconjugations. HPLC analysis of the crudes (see Supporting Information) shows that in most cases the reactions furnish fairly clean crudes, which points to low or very low undesired byproduct formation (some of the byproducts may derive from tetrazine decomposition, but we have no explanation for all byproducts formed). Isolation yields range between acceptable (21%) to highly satisfactory (45%), in any case within the standard range for purification of this type of molecules by reversed-phase HPLC.

In summary, the compounds that we have used in this work for polyamide and oligonucleotide derivatization (**3** and **8**, respectively) can be easily synthesized from inexpensive starting materials. Moreover, since their reactive 7-oxanorbornene moiety has a plane of symmetry, the number of isomers formed after the IEDDA cycloaddition is lower than when using non-symmetric dienophiles and therefore advantageous.

IEDDA cycloadditions involving 7-oxanorbornenes and tetrazines are a good alternative for the chemical preparation of bioconjugates. The procedure for the preparation of conjugates where one of the components is a carbohydrate, a peptide, an oligonucleotide or a PNA is straightforward, does not require a high excess of any of the two reagents, and takes place under mild conditions. The target compounds are isolated in fairly good yields, which may be

related to the fact that the 7-oxanorbornenes here described produce a lower number of isomers than other dienophiles.

EXPERIMENTAL SECTION

General information. 2,5-Dimethylfuran, furan, maleic anhydride, ethanolamine, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, 4-cyanobenzoic acid, sodium nitrite, Boc anhydride, anhyd pyridine, 4-aminobenzonitrile hydrochloride, γ-butyrolactone, benzyl bromide, 10% palladium on carbon, LiOH, 2,2′-(ethylenedioxy)bis(ethylamine) and DIPC were from Sigma-Aldrich. 4 M HCl in dioxane was from Fluka. EDC·HCl (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and HOBt·H_2O were from Iris Biotech. D-(+)-Galactosamine·HCl, D-biotin and 2,4-dimethylpyrrole were from Carbosynth. 2-Pyrimidine carbonitrile was from TCI. HATU was from Fluorochem. COMU ((1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate was from Acros. TMSOTf and 3,6-bis-(pyridine-2-yl)-1,2,4,5-tetrazine (**2**) were from Alfa Aesar. 32 % Concd aq ammonia was from Merck-Millipore. 3-Maleimidopropanoic acid (**9**) was from Bachem. Fmoc-amino acids (Ala, Arg(Pbf) (Pbf=2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl), Asn(Trt) (Trt=trityl), Cys(Trt), Gly, Leu, Lys(Boc), Ser(^{t}Bu), Tyr(^{t}Bu) and Val), and the Rink amide and NovaSyn TGR R resins were purchased from Novabiochem. Fmoc-PNA monomers (Bhoc-protected, Bhoc = benzhydryloxycarbonyl) were from Link technologies. TentaGel R RAM resin was from Rapp Polymere.

Compound **1**, which had been prepared as described in reference 10a, was available in the laboratory.

Acid-free DCM was obtained by filtration through basic alumina. DCM was dried using a dry-pack. Gold HPLC quality ACN (acetonitrile) was from Carlo Erba.

TLC was carried out on silica gel plates 60 F254 from Merck. Samples were lyophilized in a Christ or Labconco freeze-dryers. ^{1}H, ^{13}C, ^{31}P and ^{19}F NMR spectra were recorded on either a Varian Mercury 400 MHz spectrometer or a Brucker 400 MHz spectrometer. IR spectra were

recorded on a Nicolet 6700 FT-IR Thermo Scientific spectrometer, and UV spectra on a Jasco V-550 instrument. ESI (low and high resolution) mass spectra were obtained using an LC/MSD-TOF spectrometer from Agilent Technologies.

MALDI-TOF mass spectra were recorded on a 4800 *Plus* ABSciex instrument from Applied Biosystems (CCiT, UB). Samples were generally prepared by mixing 1 µL of an aqueous solution of the analyte and 1 µL of a mixture of an appropriate matrix. For the negative mode a 1:1 (v/v) mixture of ammonium citrate solution (50 mg/mL H_2O) and THAP·H_2O (10 mg/mL 1:1 ACN/H_2O). For the positive mode, a 1 µL of a solution of DHB (10 mg/mL 1:1 ACN/H_2O + 0.1% TFA) was used instead (DHB=2,5-dihydroxybenzoic acid; THAP=2',4',6'-trihydroxyacetophenone).

Peptide synthesis, purification and characterization. Peptide assembly was carried out manually in a polypropylene syringe fitted with a polyethylene disk, using the amino acid derivatives indicated above (general information). The solid matrix (Rink amide-derivatized resin) was first washed with DCM (3 ×), DMF (3 ×), MeOH (3 ×) and DCM (3 ×). After DCM washing, Fmoc removal was carried out by reaction with 20 % piperidine/DMF (1 × 3 min + 1 × 10 min), followed by washing with DMF (2 ×) and DCM (3 ×). Amino acid couplings were accomplished by using 3 equiv of Fmoc-amino acid, HOBt·H_2O and DIPC dissolved in the minimal amount of DCM, and a few drops of DMF, for 90 min at rt. This was followed by washing with DCM and DMF (3 × each). In case the Kaiser[16] test indicated that the coupling was not quantitative, it was repeated using 2 equiv of the same reagents for 30 min at rt. If necessary, unreacted amines were capped by reaction with Ac_2O/2,6-lutidine/DMF (5:6:89 mixture) for 15 min at rt, which was followed by washing with DMF (3 ×) and DCM (3 ×). 5-Fold molar excess of oxanorbornene-acid and DIPC was used for the incorporation of oxanorbornene-acid.

Deprotection and cleavage was carried out by reaction with a 95:2.5:2.5 TFA/H_2O/TIS mixture for 2.5 h at rt (or a 94:2.5:1:2.5 TFA/H_2O/TIS/EDT mixture for cysteine-containing peptides) (EDT=1,2-ethanedithiol). The filtrate was collected and the resin washed with TFA

(this filtrate was also collected). Filtrate and washings were concentrated under a N_2 stream, and diethyl ether was added to the resulting oil or semi-solid. After precipitating the peptide, the suspension was centrifuged (7800 rpm, 10 min, 5 ºC) and the diethyl ether supernatant discarded. This procedure was performed three times. The crude peptide was dissolved in water and lyophilized before analysis and purification by HPLC.

Pure peptides were quantified by UV/Vis spectroscopy on the basis of their absorbance at 280 nm (Tyr: ε_{280}=1490).

PNA synthesis, purification and characterization. PNAs were assembled on the TentaGel R RAM resin derivatized with a Rink amide linker using the building blocks indicated above (general information). Chain assembly was carried out as described above for peptides, with the only exception that couplings were effected by reaction with a 4 molar excess of PNA monomer and COMU in the presence of DIPEA. COMU and the PNA monomer were dissolved in the minimal amount of DCM and a few drops of DMF, and DIPEA (8 equiv.) was added to the resulting solution. After a 1-min preactivation, the mixture (then colored typically orange) was added to the solid matrix, and the suspension left to react for 90 min at rt. Oxanorbornene-containing acid (**3**) was coupled using 10 equiv. of both acid and DIPC. In all cases subsequent washings were the same as for peptide couplings. Cleavage and deprotection, and purification were performed the same way as for peptides.

PNAs were quantified by UV/Vis spectroscopy on the basis of the absorbance of the nucleobases at 260 nm (A , ε_{260}=13700; C, ε_{260}=6600; G, ε_{260}=11700; T, ε_{260}=8600). PNA monomers are depicted with small letters.

Oligonucleotide synthesis, purification and quantification. The synthesis of all 2'-deoxyribonucleic acids was performed in an automatic 3400 Applied Biosystems synthesized using the standard phophite triester methodology at the 1 μmol scale. Standard β-cyanoethyl phosphoramidite chemistry and ultramild nucleobase protecting scheme was employed (^{Pac}dA, $^{iPr\text{-}Pac}$dG, ^{Ac}dC, dT monomers purchased from Link Technologies; Pac = phenoxyacetyl, iPr-Pac = isopropylphenoxyacetyl). For the coupling of oxanorborne-

phosphoramidite **5** a BTT-mediated double coupling (0.1 M amidite solution in anhyd DCM, 0.3 M solution of BTT in anhyd ACN, 2× 10 min coupling time) was employed (BTT=benzylthiotetrazole). Anhyd ACN (DNA synthesis grade, from VWR) was used as received after addition of desiccant trap at least overnight before use. DCM (DNA synthesis grade, from Fisher) was used as received. Deblocking mixture (3% TCA in DCM), capping A (THF/Pac_2O/py 8:1:1 v/v/v), capping B (NMI/THF 1:9 v/v) and oxidizer (0.02 M I_2, H_2O/py/THF/I_2 90.54/9.05/0.41/0.43 v/v/v/w) were from Link Technologies and used as received (NMI=*N*-methylimidazole). As an activator a solution of BTT (crystalline, from Link Technologies) was weighed and diluted with anhyd ACN (0.3 M).

Deprotection and cleavage was carried out by reaction with concd aq ammonia (from Merck Millipore) for 2.5 h at rt. The filtrate was collected and the resin washed with MilliQ-H_2O. Ammonia from filtrates and washings was removed using a SpeedVac apparatus. The crude oligonucleotide was lyophilized before analysis and purification by HPLC.

Oligonucleotides were quantified by UV/Vis spectroscopy on the basis of the absorbance of the nucleobases at 260 nm (A, ε_{260}=13700; C, ε_{260}=6600; G, ε_{260}=11700; T, ε_{260}=8800).

Conditions for RP-HPLC analysis and purification. Reversed-phase HPLC analyses and purifications were performed in either Shimadzu systems (LC-10AS pumps and SPD-10A dual detector) or Alliance Waters 2695 separation module with a Waters 2996 Diode array for analysis and Alliance Waters 600 controller with a Waters 717 autosampler and Waters 2487 dual detector for purification. HPLC/MS analyses were recorded on an Alliance Waters 2690 separation module with a Waters micromass ZQ4000 MS detector.

Peptides and PNAs and polyamide conjugates. Analysis conditions: Jupiter Proteo column (4 µm, 90 Å, 250 × 4.6 mm) from Phenomenex. Linear gradients of 30 min were always used. Solvent A: 0.045% TFA in water, solvent B: 0.036% TFA in ACN, flow: 1 mL/min, detection wavelength: 280 and 320 nm (or 200 to 500 nm when using a diode array detector).

HPLC-MS analysis conditions: GraceSmart RP 18 (5 µm, 90 Å, 250 × 4.6 mm). Linear gradients of 30 min were always used. Solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in ACN. Detection wavelength (diode array detector): 200 to 500 nm.

Purification conditions: Jupiter Proteo column (10 µm, 90 Å, 250 × 10.0 mm) from Phenomenex. Linear gradients of 30 min were always used. Solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in ACN, flow: 3 mL/min, detection wavelength: 280 and 320 nm.

Oligonucleotides and oligonucleotide conjugates. Analysis conditions: Jupiter Proteo column (10 µm, 300 Å, 250 × 4.6 mm) from Phenomenex. Linear gradients of 30 min were always used. Solvent A: 100 mM TEAA (triethylammonium acetate) in water, solvent B: ACN, flow: 1 mL/min, detection wavelength: 260 nm (or 200 to 500 nm with a diode array detector).

Purification conditions: Jupiter Proteo column (10 µm, 300 Å, 250 × 10.0 mm) from Phenomenex. Linear gradients of 30 min were always used. Solvent A: 100 mM TEAA in water, solvent B: ACN, flow: 3 mL/min, detection wavelength: 254 and 280 nm.

Isolation and characterization of 7-oxanorbornene-polyamides and -oligonucleotides.

7-ONB-dT$_{10}$ (7-ONB oligonucleotide **10).** Purification gradient: 5-40% B, t_R = 12.3 min (same solvents as for analytical HPLC, see Table 1 below).

7-ONB-$^{5'}$dCATGTATCGCATCATCAGT$^{3'}$ (7-ONB oligonucleotide **11).** Purified using the analysis conditions (see Table 1 below).

7-ONB-RKKRRQRRR-NH$_2$ (7-ONB peptide **12).** Purification gradient: 10-50% B, t_R = 11.1 min (same solvents as for analytical HPLC, see Table 1 below).

7-ONB-ctcatactct-NH$_2$ (7-ONB PNA **13).** Purification gradient: 10-50% B (same solvents as for analytical HPLC but with 0.1% TFA in both A and B, see Table 1 below).

Table 1. Characterization data of 7-ONB-oligomer dienophiles and isolation yields

7-ONB[a] dienophile	anal. HPLC: t_R (min)[b]	MALDI-TOF MS[c]			Yield (%)
		m/z found	calcd mass	molec. formula	
10	17.2	3248.3	3248.5	$C_{110}H_{140}N_{21}O_{74}P_{10}$	14

11	12.8	6033.3	6031.0	$C_{195}H_{245}N_{68}O_{119}P_{19}$	15
12	18.7	1558.7	1556.9	$C_{64}H_{116}N_{32}O_{14}$	30
13	12.8	2786.2[d]	2855.1	$C_{117}H_{148}N_{52}O_{36}$	24

[a]ONB = 7-oxanorbornene. [b]HPLC Analysis gradients were 0-50% B for **10**, **12** and **13**, and 10-50% B for **11**; A = H_2O 100mM TEAA and B = ACN for **10** and **11**; A = H_2O + 0.045% TFA, and B = ACN + 0.036% TFA for **12** and **13**. [c]Negative mode, THAP/CA for **10** and **11**; positive mode, DHB for **12** and **13**. [d]*m/z* found 2786.2 $[M\text{-furan}+H]^+$, 2811.3 $[M\text{-furan}+Na]^+$ and 2827.2 $[M\text{-furan}+K]^+$.

7-Oxanorbornenes not linked to polyamide or oligonucleotide oligomers

3-((3a*R*,4*S*,7*R*,7a*S*)-1,3-Dioxo-1,3,3a,4,7,7a-hexahydro-2*H*-4,7-epoxyisoindol-2-yl)propanoic acid (3) Compound **3** was prepared following described procedures with minor modifications.[10a] Furan (861 µL, 11.84 mmol) was added to a suspension of 3-maleimidopropanoic acid (1.04 g, 6.12 mmol) in chloroform (10 mL), and heated in an aluminum reaction block under reflux overnight. Afterwards, the solvent was removed under reduced pressure and the residue was redissolved in a mixture of EtOAc and MeOH (5:1, 10 mL) and stirred for 15 minutes at 45 °C. Subsequently, the mixture was precipitated with hexanes (100 mL) yielding a white solid, which was filtered using a Büchner funnel and washed with additional EtOAc (3 x 15 mL). The filtering process was repeated to ensure that no product was left in the mother liquor to yield the final product as a white solid (939 mg, 65%). TLC (DCM/EtOAc/AcOH 30:68:2): R_f = 0.50. ^{1}H NMR (CD_3OD, 400 MHz): δ 6.54 (s, 2H), 5.15 (s, 2H), 3.71 (t, *J* = 7.2 Hz, 2H), 2.92 (s, 2H), 2.53 (t, *J* = 7.6 Hz) ppm. ^{13}C{H} NMR (CD_3OD, 101 MHz): δ 178.2, 174.5, 137.6, 82.2, 48.7, 35.6, 32.8 ppm. ESI-MS (negative mode) (*m/z*): $[M\text{-}H]^-$ calcd for $C_{11}H_{11}NO_5$ 236.05, found 236.20.

(3a*R*,4*S*,7*R*,7a*S*)-3a,4,7,7a-Tetrahydro-4,7-epoxyisobenzofuran-1,3-dione (6). To a suspension of maleic anhydride (5.00 g, 51 mmol) in Et_2O (10 mL) was added furan (20 mL, 275 mmol). The suspension was left stirring overnight at rt. Afterwards, the solvent was

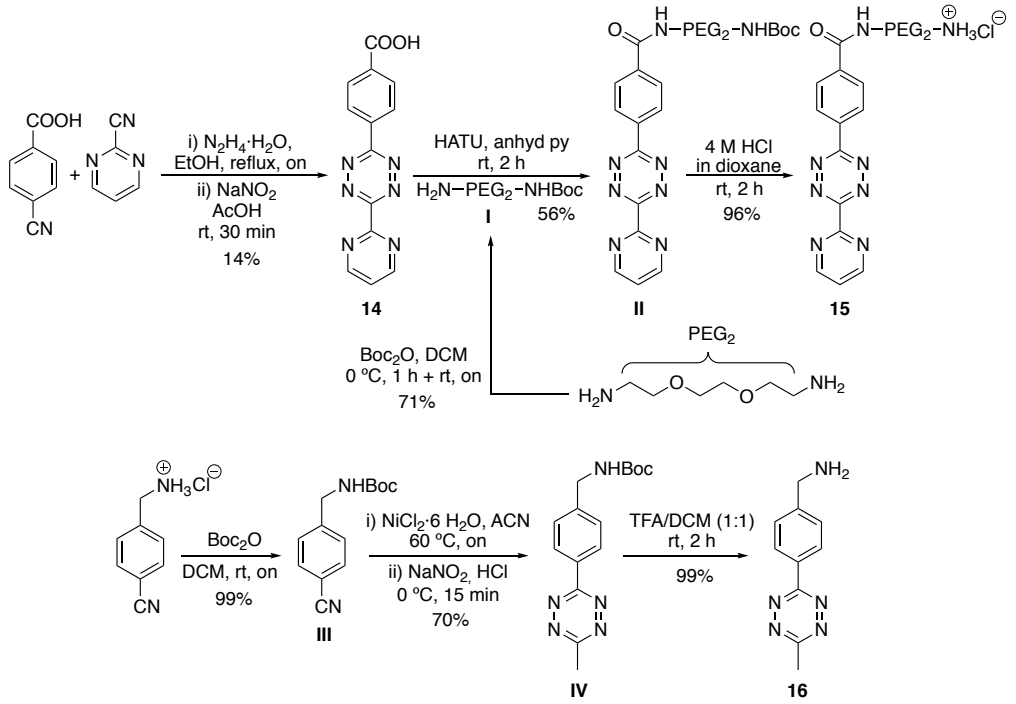
removed under reduced pressure to afford the title compound as a white solid (8.31 g, 98%). TLC (DCM/MeOH 95:5): R_f = 0.12. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.57 (s, 2H), 5.45 (s, 2H), 3.18 (s, 2H) ppm. ^{13}C{H} NMR (CD_3OD, 101 MHz): δ 172.5, 138.0, 85.5, 50.2 ppm. ESI-MS (positive mode) (*m/z*) *:* $[M+H]^+$ calcd for $C_8H_7O_4$ 167.03, found 167.47.

(3a*R*,4*S*,7*R*,7a*S*)-2-(2-Hydroxyethyl)-3a,4,7,7a-tetrahydro-1*H*-4,7-epoxyisoindole-1,3(2*H*)-dione (7). Compound **7** was prepared following the procedure described for an analog with two methyl groups with minor modifications.[10a] To a solution of **6** (1.00 g, 6.02 mmol) in MeOH (10 mL) was added ethanolamine (400 µL, 6.62 mmol) and the mixture heated in an aluminum reaction block and left refluxing overnight. Afterwards, the mixture was cooled in a freezer, and the precipitate filtered and washed with cold MeOH (3 × 5 mL). The title compound was obtained as a white solid (630 mg, 50%). TLC (DCM/MeOH 9:1): R_f = 0.20. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.54 (s, 2H), 5.12 (s, 2H), 3.45 – 3.38 (m, 4H), 2.92 (s, 2H) ppm. ^{13}C{H} NMR (DMSO-d_6, 101 MHz): δ 176.9, 136.9, 80.7, 57.7, 47.6, 41.1 ppm. ESI-MS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{10}H_{11}NO_4$ 210.07, found 210.57.

2-Cyanoethyl (2-((3a*R*,4*S*,7*R*,7a*S*)-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2*H*-4,7-epoxyiso-indol-2-yl)ethyl) diisopropylphosphoramidite (8). Compound **8** was prepared following procedures following the procedure described for an analog with two methyl groups with minor modifications.[10a] In an ice-cooled round-bottomed flask equipped with a magnet stirrer and an argon balloon was suspended **7** (332 mg, 1.58 mmol) in anhyd DCM (3 mL). Subsequently anhyd DIPEA (550 µL, 3.17 mmol) and a solution of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (250 mg, 1.05) in anhyd DCM (2 × 1 mL) was added, and the mixture was left reacting at low temperature for 20 min and at rt for an additional 2 hours. Afterwards, additional DCM (30 mL) was added and the solution transferred into a separatory funnel, washed with aq. $NaHCO_3$ 5% (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried over anhyd $MgSO_4$, filtered and the solvent removed under vacuum. The crude was further purified by silica gel column chromatography eluting with a 97.5:2.5 DCM/NEt_3 mixture (isocratic). The title compound was obtained as a pale yellow oil (360 mg, 83%).

TLC (DCM/NEt_3 98:2): R_f = 0.65. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.50 (s, 2H), 5.26 (s, 2H), 3.84 – 3.66 (m, 6H), 3.60 – 3.51 (m, 2H), 2.85 (t, *J* = 5.0 Hz, 2H), 2.63 – 2.59 (m, 2H), 1.15 (t, *J* = 7.0 Hz, 12H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 176.1, 136.5, 117.8, 80.8, 59.3, 58.6, 47.5, 43.1, 39.9, 24.6, 20.4 ppm. ^{31}P NMR ($CDCl_3$, 162 MHz): δ 147.93 ppm.

Tetrazines 14-16



Scheme 4. Synthesis of tetrazines **14**, **15** and **16**.

4-(6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (14). Compound **14** was prepared following described procedures with minor modifications.[17] To a solution of 2-pyrimidinecarbonitrile (935 mg, 8.90 mmol) in EtOH (30 mL), 4-cyanobenzoic acid (1.96 g, 13.33 mmol) and $N_2H_4 \cdot H_2O$ 65% (5.5 mL, 70 mmol) were added and the mixture was heated in an aluminum reaction block and refluxed overnight. Afterwards, the solution was cooled to room temperature and the precipitate washed with acetone (2 × 50 mL). To the remaining precipitate was added AcOH (10 mL) followed by aq. $NaNO_2$ (2.76 g, 40 mmol, 10 mL) and the mixture was left stirring at rt for 30 min. The pink precipitate was filtered and washed with H_2O (3 × 10 mL) and transferred into a round-bottomed flask containing boiling DMF

(20 mL). The DMF solution was kept at this temperature for 5 min and filtered while it was hot. The filtrate process was collected and dried under vacuum. This DMF treatment+filtration process was repeated 3 times. The title product was obtained as a purple solid (357.1 mg, 14%). TLC (DCM/MeOH/AcOH 78:20:2): R_f = 0.10. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.21 (d, *J* = 4.8 Hz, 2H), 8.68 (d, *J* = 8.4 Hz, 2H), 8.24 (d, *J* = 8.4 Hz, 2H), 7.84 (t, *J* = 4.9 Hz, 2H) ppm. ^{13}C{H} NMR (DMSO-d_6, 101 MHz): δ 172.5, 163.7, 163.3, 159.5, 159.0, 135.0, 130.7, 128.6, 123.5 ppm. ESI-HRMS (negative mode) (*m/z*): $[M-H]^-$ calcd for $C_{13}H_7N_6O_2$ 279.0636, found 279.0647.

***tert*-Butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (I).** Compound **I** was prepared following procedures described for an analog with minor modifications.[18] To an ice-cooled solution of 2,2′-(ethylenedioxy)bis(ethylamine) (4.00 mL, 27.49 mmol) in DCM (20 mL) a solution of Boc_2O (2.00 g, 9.16 mmol) in DCM (50 mL) was added dropwise over the course of 1 h. Afterwards, the mixture was allowed to warm to rt and left reacting overnight. Subsequently, the crude was transferred into a separatory funnel with additional DCM (50 mL) and washed with distilled H_2O (40 mL) and the aqueous phase extracted with DCM (2 x 20 mL). The combined organic layers were dried over anhyd $MgSO_4$, filtered and the solvent removed under reduced pressure to afford the title compound as a colorless oil (1.61 g, 71 % yield). TLC (Hexanes/EtOAc/NEt_3 48:50:2): R_f = 0.33. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.34 (br. s, 1H), 3.42 – 3.37 (m, 4H), 3.30 (t, 5.3 Hz, 4H), 3.10 – 3.03 (m, 2H), 2.63 (t, *J* = 5.2 Hz, 2H), 1.41 (br. s, 2H), 1.21 (s, 9H) ppm. ^{13}C{H} NMR ($CDCl_3$, 100 MHz): δ 155.8, 78.7, 73.2, 70.0, 41.5, 40.1, 28.2 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{25}N_2O_4$ 249.1809, found 249.1806.

***tert*-Butyl (2-(2-(2-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamido)ethoxy)ethoxy)-ethyl)carbamate (II).** To a 25 mL round-bottomed flask, **14** (400.0 mg, 1.43 mmol) and HATU (651.4 mg, 1.71 mmol) were added and dissolved in anhyd py (5 mL). The mixture was left stirring for 5 min and a solution of **I** (425.3 mg, 1.71 mmol) in anhyd py (2 mL) was added. The mixture was left stirring at rt for 2 h. Afterwards, the solvent was removed under

vacuum and to the crude was added DCM (20 mL) and washed with aq. HCl 10% (2 × 20 mL). The aqueous phase was extracted with additional DCM (2 × 20 mL). The combined organic layers were dried over anhyd $MgSO_4$, filtered and the solvent removed *in vacuo.* The extract was further purified by silica gel flash column chromatography eluting with DCM/MeOH mixtures, from 100:0 up to 97:3. The title compound was obtained as a pink solid (405.1 mg, 56%). TLC (DCM/MeOH 90:10): R_f = 0.50. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.15 (d, *J* = 4.9 Hz, 2H), 8.82 (d, *J* = 8.0 Hz, 2H), 8.07 (d, *J* = 7.7 Hz, 2H), 7.60 (t, *J* = 4.9 Hz, 1H), 6.93 (br. s, 1H), 4.98 (br. s, 1H), 3.73 (s, 4H), 3.70 (s, 4H), 3.63 – 3.54 (m, 4H), 1.45 (s, 9H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 167.5, 165.4, 164.9, 162.9, 156.1, 155.9, 135.2, 134.2, 130.2, 127.6117.7, 79.5, 73.2, 70.1 41.2, 40.0, 28.4 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{24}H_{31}N_8O_5$ 511.2412, found 511.2409.

2-(2-(2-(4-(6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamido)ethoxy)ethoxy)ethan-1-aminium chloride (15). In a 25 mL round-bottomed flask Boc-protected tetrazine **II** (200.0 mg, 0.39 mmol) was dissolved in 4 N HCl in dioxane (5 mL) and the solution stirred for 2 h at rt. Afterwards, the mixture was dried under vacuum to yield the title compound as a pink solid (155.1 mg, 96%). TLC (DCM/MeOH 90:10): R_f = 0.18. ^{1}H NMR (CD_3OD, 400 MHz): δ 9.18 (d, *J* = 4.9 Hz, 2H), 8.81 (d, *J* = 8.7 Hz, 2H), 8.79 (br. s, 1H), 8.14 (d, *J* = 8.7 Hz, 2H), 7.82 (t, *J* = 4.9 Hz, 1H), 3.78 – 3.64 (m, 12H), 3.13 (t, *J* = 5.2 Hz, 2H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 167.9, 164.2, 162.6, 158.8, 158.3, 138.4, 134.4, 128.3, 127.9, 123.1, 70.0, 69.9, 69.2, 66.5, 39.5, 39.2 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ M calcd for $C_{19}H_{23}N_8O_3$ 411.1888, found 411.1886.

***tert*-Butyl (4-cyanobenzyl)carbamate (III).** To a solution of 4-(aminoethyl)benzonitrile hydrochloride (1.00 g, 5.92 mmol) in DCM (20 mL) was added NEt_3 (2.06 mL, 14.80 mmol) and Boc_2O (1.42 g, 6.51 mmol) and the mixture left at rt overnight. Subsequently, additional DCM (30 mL) and aq. 10% HCl (20 mL) were added to the mixture and the solution transferred into a separatory funnel. The aqueous layer was extracted with additional DCM (2 × 20 mL). The combined organic layers were dried over anhyd $MgSO_4$, filtered and the

solvent removed under vacuum. The title product was obtained as a white solid (1.37 g, 99%). TLC (Hexanes/EtOAc 80:20): R_f = 0.25. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.62 (d, *J* = 8.2 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H), 4.97 (br. s, 1H), 4.37 (d, *J* = 6.2 Hz, 2H), 1.46 (s, 9H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 155.8, 144.6, 132.4, 127.8, 118.7, 111.1, 80.1, 44.2, 28.3 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{13}H_{17}N_2O_2$ 233.1285, found 233.1280.

***tert*-Butyl (4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamate (IV).** Compound **IV** was prepared following described procedures with minor modifications.[12c] In a high-pressure tube equipped with a stirring bar, **III** (1.30 g, 5.60 mmol), $NiCl_2 \cdot 6H_2O$ (660.2 mg, 2.80 mmol), ACN (3.0 mL, 55.96 mmol) and $N_2H_4 \cdot H_2O$ (6.8 mL, 139.91 mmol) were introduced. The tube was sealed and heated at 60 ºC in an oil bath overnight. Afterwards, to the crude was added H_2O (30 mL), the mixture was transferred into an Erlenmeyer flask and a solution of $NaNO_2$ (7.72 g, 111.93 mmol) in H_2O (30 mL) was added, followed by dropwise addition of aq. 10% HCl at 0 ºC until pH ≈ 3. After 15 min, the mixture was transferred into a separatory funnel and the aqueous layer extracted with EtOAc (4 × 50 mL). The combined organic layers were pooled together and washed with brine (50 mL), dried over anhyd $MgSO_4$, filtered and the solvent removed under vacuum. The extract was further purified by silica gel column chromatography eluting with hexanes/EtOAc mixtures, from 100:0 up to 80:20. The title compound was obtained as a purple solid (1.68 g, 70%). TLC (Hexanes/EtOAc 80:20): R_f = 0.25. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.46 (d, *J* = 8.1 Hz, 2H), 7.43 (d, *J* = 8.0 Hz, 2H), 5.21 (br. s, 1H), 4.38 (d, *J* = 5.7 Hz, 2H), 3.03 (s, 3H), 1.43 (s, 9H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 167.1, 163.8, 156.0, 144.0, 130.6, 128.1, 128.0, 79.7, 44.3, 28.4, 21.1 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{20}N_5O_2$ 302.1612, found 302.1610.

(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine (16). In a 100 mL round-bottomed flask was dissolved **IV** (1.50 g, 4.98 mmol) in DCM (10 mL), was added a HCl solution (4 M in dioxane, 10 mL) and the mixture was left stirring at rt for 2 h. Afterwards, the solvent was removed under vacuum and the mixture dissolved in sat. aq. $NaHCO_3$ (30 mL). The solution

was transferred into a separatory funnel and the aqueous layer was extracted with DCM (4 × 30 mL). The combined organic layers were pooled together, dried over anhyd $MgSO_4$ and the solvent removed *in* vacuo. The crude was further purified by silica gel column chromatography eluting with mixtures EtOAc/MeOH, from 100:0 up to 85:15. The title compound was obtained as a purple solid (1.00 g, 99 %). TLC (EtOAc): R_f = 0.20. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.60 (d, *J* = 8.1 Hz, 2H), 7.44 (d, *J* = 8.1 Hz, 2H), 4.31 (s, 2H), 3.03 (s, 3H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 167.4, 165.6, 143.0, 129.5, 127.1, 126.0, 45.9, 20.1 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{10}H_{12}N_5$ 202.1087, found 202.1091.

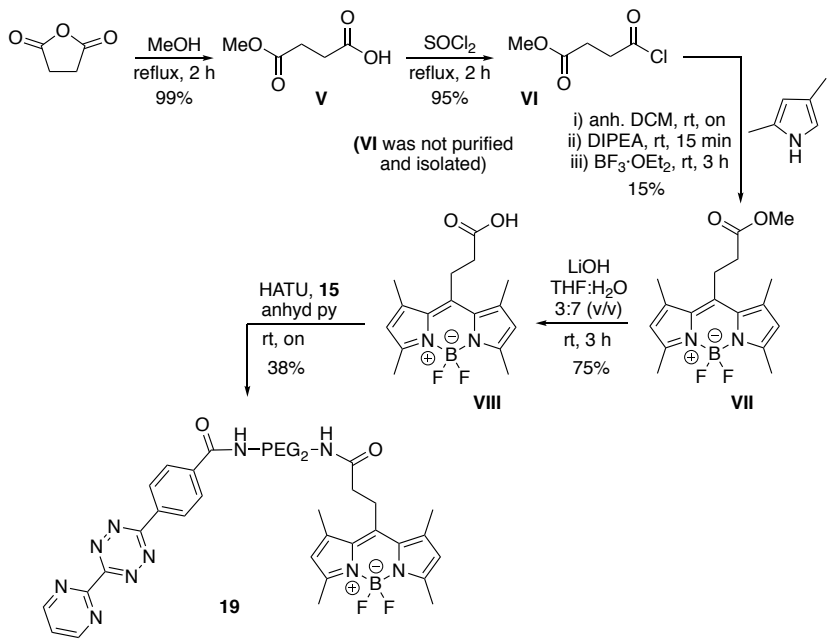
Tetrazines 17-20

Tetrazine-modified peptide (17). Elongation of the peptide sequence was carried out as described above. After Fmoc removal, tetrazine **14** was coupled to the resin-linked peptide by using 5 equiv of both **14** and HATU, anhyd pyridine as solvent and 150 min coupling time. Final deprotection and cleavage were performed as described above. Tetrazine-peptide **17** was purified by HPLC using a 0-40% gradient (same solvents as indicated above), t_R = 21.0 min. MALDI-TOF MS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{95}H_{141}N_{29}O_{23}$ 2056.1, found 2057.1.

Biotin-tetrazine: *N*-(2-(2-(2-(5-((3a*S*,4*S*,6a*R*)-2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethoxy)ethoxy)ethyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (18). Into a 5 mL round-bottomed flask **15** (90.0 mg, 0.22 mmol), HATU (92.0 mg, 0.24 mmol) and D-Biotin (59.0 mg, 0.24 mmol) were dissolved in anhyd py (3 mL) and the solution stirred for 2 h at rt. Afterwards, the solvent was removed under reduced pressure and the crude was purified by silica gel column chromatography eluting with DCM:MeOH mixtures from 95:5 up to 85:15. The title compound was obtained as a pink powder (88.0 mg, 63%). TLC (DCM:MeOH 90:10): R_f = 0.45. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.21 (d, *J* = 5.2 Hz, 2H), 8.82 (t, *J* = 5.6 Hz, 1H), 8.68 (d, *J* = 8.3 Hz, 2H), 8.16 (d, *J* = 8.4 Hz, 1H), 7.87 –

7.80 (m, 2H), 6.40 (s, 1H), 6.34 (s, 1H), 4.29 (dd, J = 7.7, 5.0 Hz, 1H), 4.12 (ddd, J = 7.7, 4.4, 1.7 Hz, 1H), 3.62 – 3.46 (m, 8H), 3.40 (t, J = 5.9 Hz, 2H), 3.18 (q, J = 5.8 Hz, 2H), 3.08 (ddd, J = 8.5, 6.2, 4.4 Hz, 1H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 2.56 (d, J = 12.4 Hz, 1H), 2.06 (t, J = 7.4 Hz, 2H), 1.69 – 1.39 (m, 4H), 1.37 – 1.22 (m, 2H) ppm. ^{13}C{H} NMR (DMSO-d_6, 101 MHz): δ 172.6, 166.0, 163.6, 163.4, 163.1, 159.5, 159.0, 138.6, 134.3, 128.7, 128.6, 123.5, 70.0, 69.6, 69.3, 61.5, 59.6, 55.9, 38.9, 35.6, 28.6, 28.5, 25.7 ppm. Two signals (CH_2-NHCO) overlap with solvent. ESI-HRMS (positive mode) (m/z): $[M+H]^+$ calcd for $C_{29}H_{37}N_{10}O_5S$ 637.2664, found 637.2659.

BODIPY-tetrazine 19



Scheme 5. Synthesis of BODIPY-tetrazine **19**.

4-Methoxy-4-oxobutanoic acid (V). To a 25 mL round-bottomed flask was added succinic anhydride (2.00 g, 19.80 mmol) and MeOH (10 mL). Subsequently, the suspension was heated in an aluminum reaction block and refluxed for 2 h, cooled down and the solvent removed under reduced pressure to yield the title compound as a white solid (2.63 g, 99 %). TLC (DCM/MeOH 95:5): R_f = 0.50 ^{1}H NMR (CD_3OD, 400 MHz): δ 3.67 (s, 3H), 2.59 (s, 4H)

ppm. $^{13}C\{H\}$ NMR ($CDCl_3$, 101 MHz): δ 174.6, 173.3, 50.8, 28.4, 28.3 ppm. ESI-HRMS (negative mode) (*m/z*): $[M-H]^-$ calcd for $C_5H_7O_4$ 131.0350, 131.0347.

Methyl 4-chloro-4-oxobutanoate (VI). To a 25 mL round-bottomed flask was added **V** (2.00 g, 19.80 mmol) and thionyl chloride (10 mL) and the suspension was heated in an aluminum reaction block and refluxed for 2 h. Afterwards, the solvent was removed under reduced pressure to yield the title compound as a yellow oil which used without further purification. TLC (DCM/MeOH 95:5): R_f = 0.70. ^1H NMR (CD_3OD, 400 MHz): δ 3.61 (s, 3H), 2.50 (s, 4H) ppm. $^{13}C\{H\}$ NMR ($CDCl_3$, 101 MHz): δ 173.1, 172.6, 52.0, 42.2, 29.1 ppm.

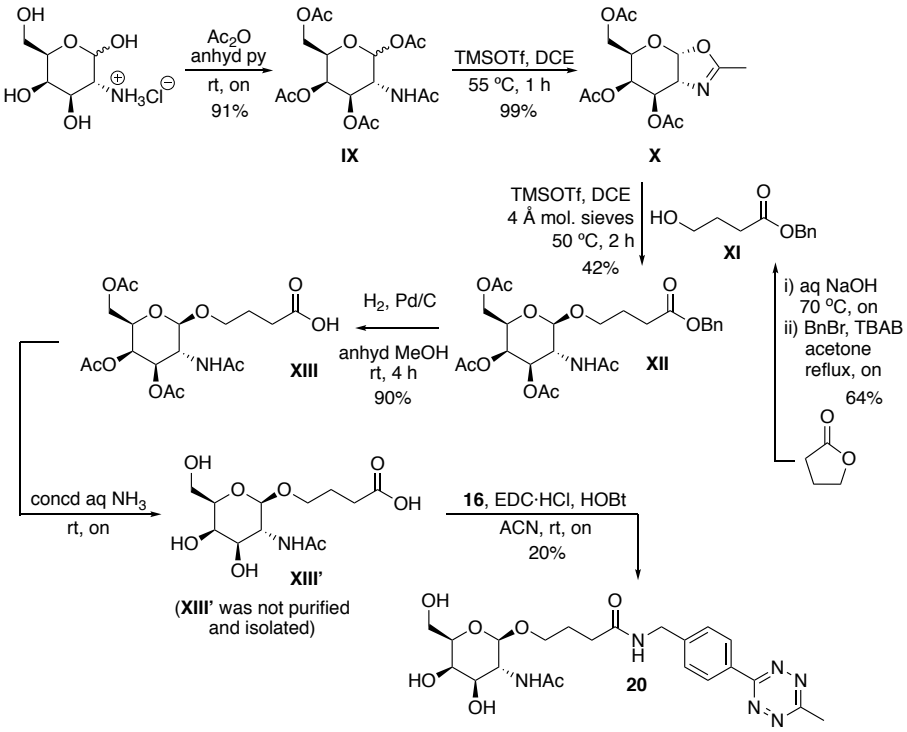
3-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)-propionic acid methyl ester (VII). Compound **VII** was prepared following described procedures with minor modifications.[19] To a 2,4-dimethylpyrrole (5.1 g, 54.0 mmol) solution in anhyd DCM (30 mL) was added **VI** (2.7 g, 18.0 mmol) dissolved in anhyd DCM (10 mL). The mixture was left stirring at rt overnight. Afterwards, DIPEA (15.6 mL, 90.0 mmol) was added and the mixture stirred for an additional 15 min. Then, $BF_3{\cdot}OEt_2$ (15 g, 108.0 mmol) was added and the mixture was stirred for 3 h at room temperature. Subsequently, the mixture was passed through a small silica plug and the solvent evaporated under reduced pressure. The resulting crude was purified by silica gel flash column chromatography eluting with hexanes:EtOAc mixtures, from 95:5 to 90:10. The title compound was obtained as a red solid (940 mg, 15%). TLC (Hexanes/EtOAc 8:2): R_f = 0.33. ^1H NMR ($CDCl_3$, 400 MHz): δ 6.06 (s, 2H), 3.74 (s, 3H), 3.34 – 3.28 (m, 2H), 2.64 – 2.57 (m, 2H), 2.51 (s, 6H), 2.43 (s, 6H) ppm. $^{13}C\{H\}$ NMR ($CDCl_3$, 101 MHz): δ 172.2, 154.8, 143.3, 140.6, 131.3, 122.1, 76.8, 52.2, 35.3, 23.7, 16.5, 14.6 ppm. ^{19}F NMR ($CDCl_3$, 376 MHz): δ -146.56 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{17}H_{22}BF_2N_2O_2$ 335.1737, found 334.1768 (^{10}B) and 335.1737.

3-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)-propionic acid (VIII). To a solution of **VII** (400.0 mg, 1.19 mmol) in THF (10 mL) was added a solution of LiOH (143.7 mg, 5.98 mmol) in H_2O (4 mL) and the mixture was left stirring for 3 h at rt. Afterwards, the crude was diluted with aq. HCl 10%, (10 mL) transferred into a separatory

funnel and extracted with DCM (3 × 30 mL). The combined organic layers were dried over anhyd $MgSO_4$, the solvent was removed under vacuum and the residue purified by silica gel flash column chromatography eluting with a hexanes/EtOAc/AcOH mixture (78:20:2). The title compound was obtained as an orange solid (285.0 mg, 75%). TLC (Hexanes/EtOAc/AcOH 78:20:2): R_f = 0.30. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.07 (s, 2H), 3.37 – 3.29 (m, 2H), 2.69 – 2.64 (m, 2H), 2.52 (s, 6H), 2.45 (s, 6H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 176.0, 154.9, 142.7, 140.3, 122.0, 110.0, 34.9, 23.4, 16.4, 14.5 ppm. ^{19}F NMR ($CDCl_3$, 376 MHz) δ -146.58 ppm. ESI-HRMS (negative mode) (*m/z*): $[M-H]^-$ calcd for $C_{16}H_{18}BF_2N_2O_2$ 319.1435, found 318.1476 (^{10}B) and 319.1439.

***N*-(2-(2-(2-(3-(5,5-Difluoro-1,3,7,9-tetramethyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-10-yl)propanamido)ethoxy)ethoxy)ethyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (19).** To a 10 mL round-bottomed flask was weighed **XIII** (78.9 mg, 0.25 mmol), **15** (100.0 mg, 0.22 mmol) and HATU (93.7 mg, 0.25 mmol), anhyd py (5 mL) was added and the mixture left stirring overnight at rt. Afterwards, the solvent was removed under vacuum and the crude was purified by silica gel flash column chromatography eluting with DCM:MeOH mixtures from 100:0 up to 95:5. The title compound was obtained as a bright orange solid (68.0 mg, 38 %). TLC (DCM:MeOH 95:5): R_f = 0.37. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.13 (d, *J* = 4.8 Hz, 2H), 8.74 (d, *J* = 8.5 Hz, 2H), 8.00 (d, *J* = 8.6 Hz, 2H), 7.60 (t, *J* = 4.9 Hz, 1H), 6.96 (br.s, 1H), 6.33 (t, *J* = 5.6 Hz, 1H), 6.00 (s, 2H), 3.74 – 3.59 (m, 8H), 3.53 (t, *J* = 5.1 Hz, 2H), 3.45 (t, *J* = 5.2 Hz, 2H), 3.34 – 3.24 (m, 2H), 2.47 (s, 6H), 2.46 – 2.42 (m, 2H), 2.40 (s, 6H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 170.8, 166.7, 164.0, 163.1, 159.4, 158.4, 154.4, 144.4, 140.6, 138.6, 133.9, 131.2, 128.9, 128.2, 128.0, 122.6, 121.8, 120.4, 70.4, 70.2, 69.8, 69.7, 40.0, 39.4, 37.3, 23.8, 16.5, 14.5 ppm. ^{19}F NMR ($CDCl_3$, 376 MHz): δ -145.89, 146.75 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+Na]^+$ calcd for $C_{35}H_{39}BF_2N_{10}NaO_4$ 736.3187, found 735.3111 (^{10}B) and 736.3143.

GalNAc-tetrazine 20



Scheme 6. Synthesis of GalNAc-tetrazine **20**.

(2*R*,3*R*,4*R*,5*R*,6*R*)-3-Acetamido-6-(acetoxymethyl)tetrahydro-2*H*-pyran-2,4,5-triyl triacetate (IX). Compound **IX** was prepared following described procedures with minor modifications.[20] In a 100 mL round-bottomed flask D-(+)-galactosamine hydrochloride (4.00 g, 18.60 mmol) was dissolved in anhyd py (20 mL). Subsequently, Ac_2O (17.6 mL, 186.00 mmol) was added and the reaction mixture was stirred at rt overnight. Afterwards, the mixture was cooled in an ice bath and ice-cold water (100 mL) was added. The white solid precipitate was collected by vacuum filtration washing it with additional ice-cold water (2 × 20 mL). The precipitate was co-evaporated with toluene (30 mL) to remove residual water. The title product was obtained as a white powder (6.60 g, 91 %). TLC (Hexanes/EtOAc 1:1): R_f = 0.55. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.70 (d, *J* = 8.8 Hz, 1H), 5.54 (d, *J* = 9.6 Hz, 1H), 5.37 (d, *J* = 3.2 Hz, 1H), 5.08 (dd, *J* = 11.3, 3.3 Hz, 1H), 4.44 (dt, *J* = 11.3, 9.2 Hz, 1H), 4.14 (qd, *J* = 11.3, 6.5 Hz, 1H), 4.02 (t, *J* = 6.5 Hz, 1H), 2.17 (s, 3H), 2.13 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.94 (s, 3H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 170.8, 170.4, 170.3, 170.2, 169.6,

93.0, 71.9, 70.3, 66.3, 61.3, 49.8, 23.3, 20.9, 20.7, 20.6 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{24}NO_{10}$ 390.1395, found 390.1374.

(3a*R*,5*R*,6*R*,7*R*,7a*R*)-5-(Acetoxymethyl)-2-methyl-3a,6,7,7a-tetrahydro-5*H*-pyrano[3,2-d]oxazole-6,7-diyl diacetate (X). Compound **X** was prepared following described procedures with minor modifications.[20] To a solution of **IX** (500.0 mg, 1.28 mmol) in DCE (30 mL) was added TMSOTf (245 µL, 1.41 mmol) and the mixture was heated at 55 ºC in an aluminum reaction block for 1 h. Afterwards, the crude was cooled down and treated with NEt_3 (212 µL, 1.92 mmol) for 10 min. Afterwards, the crude was trespassed into a separatory funnel and diluted with additional DCM (30 mL). The organic phase was washed with aq. sat. $NaHCO_3$, the solvent removed under vacuum and the residue was purified by silica gel flash column chromatography eluting with EtOAc/NEt_3 (99:1). The title compound was obtained as a pale-yellow oil (420.1 mg, 99 %). TLC (EtOAc/NEt_3 99:1): R_f = 0.35. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.95 (d, *J* = 6.8 Hz, 1H), 5.42 (t, *J* = 3.0 Hz, 1H), 4.87 (dd, *J* = 7.4, 3.4 Hz, 1H), 4.27 – 4.04 (m, 3H), 3.96 (td, *J* = 7.1, 1.4 Hz, 1H), 2.08 (s, 3H), 2.03 (s, 6H), 2.01 (d, *J* = 1.2 Hz, 3H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 170.4, 170.1, 169.7, 166.4, 101.4, 71.8, 69.5, 65.3, 63.5, 61.6, 20.7, 20.7, 20.5, 14.4 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{20}NO_8$ 330.1183, found 330.1180.

Benzyl 4-hydroxybutanoate (XI). Compound **XI** was prepared following described procedures with minor modifications.[21] To a solution of NaOH (1.40 g, 34.87 mmol) in H_2O (30 mL) was added γ-butyrolactone (2.67 mL, 34.87 mmol) and the mixture was heated in an aluminum reaction block at 70 ºC overnight. The crude was concentrated under vacuum, the solid suspended in acetone (30 mL) and TBAB (560 mg, 1.74 mmol), BnBr (5.0 mL, 41.84 mmol) were added and the mixture was left refluxing overnight. Subsequently, the crude was cooled and concentrated *in vacuo*, the residue redissolved in EtOAc (100 mL) and the solution transferred into a separatory funnel. The organic phase was washed with 1 N aqueous $NaHSO_4$ (30 mL), aq. sat. $NaHCO_3$ (30 mL) and brine (30 mL), dried over anhyd $MgSO_4$ and concentrated. The crude was further purified using silica gel flash column chromatography

eluting with hexanes:EtOAc mixtures, from 90:10 to 75:25, to afford the title compound (4.30 g, 64%) as a colorless oil. TLC (Hexanes/EtOAc 60:40): R_f = 0.60. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40 – 7.30 (m, 5H), 5.12 (s, 2H), 3.67 (t, *J* = 6.2 Hz, 2H), 2.49 (t, *J* = 7.2 Hz, 2H), 1.94 – 1.86 (m, 3H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 177.8, 140.9, 128.5, 127.6, 126.9, 68.6, 65.2, 31.0, 27.8 ppm. ESI-MS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{15}O_3$ 195.10, found 195.40.

(2*R*,3*R*,4*R*,5*R*,6*R*)-5-Acetamido-2-(acetoxymethyl)-6-(4-(benzylperoxy)-4-oxobutoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (XII). Compound **XII** was prepared following procedures described for an analog with minor modifications.[22] To a 100 mL round-bottomed flask, 4 Å molecular sieves (1 g), **X** (2.23 g, 6.78 mmol) and DCE (30 mL) were added, followed by a solution of **XI** (1.45 g, 7.46 mmol) in DCE (5 mL). The mixture was stirred for 30 min at 50 ºC in an aluminum reaction block. Afterwards, TMSOTf (612 µL, 3.39 mmol) was added dropwise and the solution was left reacting 2 h at 50 ºC. Subsequently, the solution was left to cool down and transferred into a separatory funnel, after filtering off the sieves, and DCM (30 mL) and aq. sat. $NaHCO_3$ (40 mL) were added. The aqueous phase was extracted with additional DCM until the organic layer was colorless (2 × 30 mL). The combined organic phases were pooled together, dried over anhyd $MgSO_4$, filtered and the solvent removed *in vacuo*. The extract was further purified by silica gel column chromatography eluting with DCM/MeOH mixtures, from 100:0 up to 95:5. The title compound was obtained as a pale yellow foam (1.48 g, 42 %). TLC (DCM/MeOH 95:5): R_f = 0.45. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.40 – 7.30 (m, 5H), 5.37 – 5.29 (m, 2H), 5.06 – 4.92 (m, 2H), 4.20 – 4.07 (m, 3H), 3.96 – 3.85 (m, 3H), 3.49 (d, *J* = 6.7 Hz, 2H), 2.14 (s, 3H), 2.06 (s, 3H), 2.00 (s, 3H), 1.94 (s, 3H), 1.62 – 1.37 (m, 4H) ppm. ^{13}C{H} NMR ($CDCl_3$, 101 MHz): δ 175.9, 170.1, 168.7, 158.6, 158.5, 147.5, 144.9, 139.6, 136.5, 136.1, 135.9, 135.7, 130.2, 130.1, 129.2, 128.2, 128.1, 127.9, 127.9, 127.9, 127.1, 126.8, 113.2, 113.2, 86.0, 80.9, 61.5, 55.3, 51.6, 49.0, 47.5, 45.9, 35.4, 35.1, 34.2, 33.8 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{25}H_{34}NO_{11}$ 524.2126, found 524.2134.

4-(((2*R*,3*R*,4*R*,5*R*,6*R*)-3-Acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)butanoic acid (XIII). To a 100 mL round-bottomed flask and under an argon atmosphere, **XII** (700.0 mg, 1.34 mmol) and 10% Pd/C (w/w) (70.0 mg, 0.06 mmol) were added. Subsequently, anhyd MeOH (15 mL) was added, through a septum, and the argon was purged by flushing with two balloons full of H_2 (directly bubbling it into the solvent) over the course of 1 h (rt). Afterwards, a new balloon with H_2 was placed and the mixture stirred at rt for an additional 3 h. Then, the mixture was filtered through a Celite plug washing with MeOH (2 × 10 mL). After solvent removal under vacuum, the title compound was obtained as a white foam (520.1 mg, 90 %). TLC (DCM/MeOH 9:1): R_f = 0.60. ^{1}H NMR (CD_3OD, 400 MHz): δ 4.61 (d, *J* = 12.2 Hz, 1H), 4.37 (d, *J* = 8.4 Hz, 1H), 4.38 (s, 1H), 4.02 – 3.69 (m, 4H), 3.60 – 3.45 (m, 2H), 3.34 (d, *J* = 0.5 Hz, 2H), 2.24 (td, *J* = 7.3, 2.5 Hz, 2H), 2.14 (s, 3H), 2.06 (s, 3H), 2.00 (s, 3H), 1.94 (s, 3H) ppm. ^{13}C{H} NMR (CD_3OD, 101 MHz): δ 178.2, 172.9, 137.6, 82.2, 61.9, 54.5, 36.4, 34.9 ppm. ESI-HRMS (negative mode) (*m/z*): $[M-H]^-$ calcd for $C_{18}H_{26}NO_{11}$ 432.1511, found 432.1510.

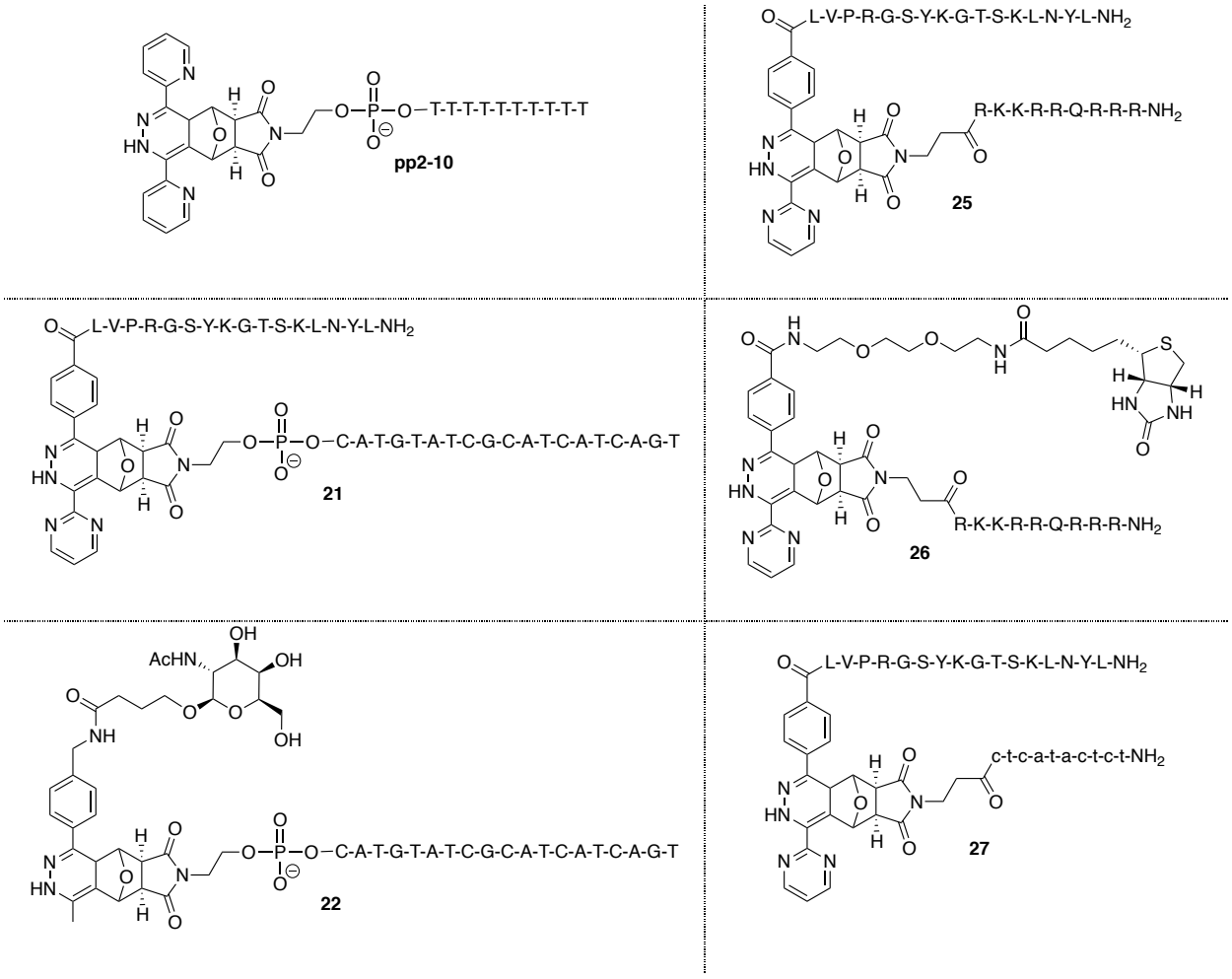
4-(((2*R*,3*R*,4*R*,5*R*,6*R*)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-*N*-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)butanamide (XIII' and **20).** Into a 100 mL round-bottomed flask, **XIII** (200 mg, 0.467 mmol) was weighed. Subsequently, 32% conc. aq. ammonia (20 mL) was added and the mixture was left stirring at rt overnight to yield **XIII'**. Afterwards, ammonia was removed *in vacuo*, and to the crude was added ACN (3 mL), EDC·HCl (133.4 mg, 0.70 mmol), HOBt (94.3 mg, 0.70 mmol) and **16** (274.9 mg, 0.634 mmol)**.** The mixture was left reacting for an additional 12 h (rt). The solvent was removed under vacuum and the crude was purified by silica gel flash column chromatography eluting with DCM/MeOH 100:0 up to 95:5 mixtures. The title compound was obtained as a pink solid (20.0 mg, 20%). TLC (DCM/MeOH 9:1): R_f = 0.41. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.61 (d, *J* = 8.3 Hz, 2H), 7.79 (d, *J* = 8.2 Hz, 2H), 4.49 (d, *J* = 4.5 Hz, 1H), 4.35 (d, *J* = 8.4 Hz, 1H), 4.26 (s, 2H), 3.98 – 3.43 (m, 10H), 3.04 (d, *J* = 9.7 Hz, 3H), 2.43 – 2.29 (m, 2H), 1.97 (s, 3H), 1.93 – 1.80 (m, 2H) ppm. ^{13}C{H} NMR (CD_3OD, 101

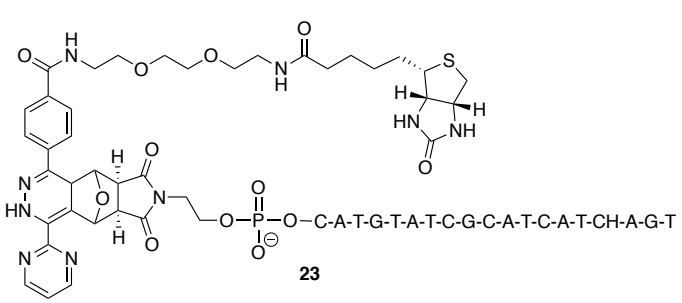
MHz): δ 174.6, 172.9, 167.4, 165.0, 138.0, 129.8, 127.5, 125.6, 108.7, 78.6, 71.3, 70.7, 62.2, 60.3, 43.6, 32.8, 25.7, 23.6, 20.0 ppm. ESI-HRMS (positive mode) (*m/z*): $[M+H]^+$ calcd for $C_{22}H_{31}N_6O_7$ 491.2249, found 491.2245.

Conjugates

Conjugation reactions. Unless otherwise stated, all conjugation reactions were carried out using 2 equiv of tetrazine derivative in regards to the oxanorbornene-containing moiety, in 1:1 MeOH/H_2O (v/v) solutions at 37 ºC (heated in an Eppendorf thermomixer) and overnight reaction times. In all cases, a 0.5 mM final concentration of the oxanorbornene-containing compound was employed. Monitoring and purification of the major compounds was achieved by RP-HPLC, and analysis characterization of the conjugate by MALDI-TOF MS.

Figure 3. Structures of the conjugates prepared





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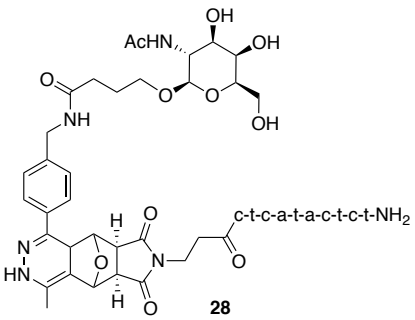

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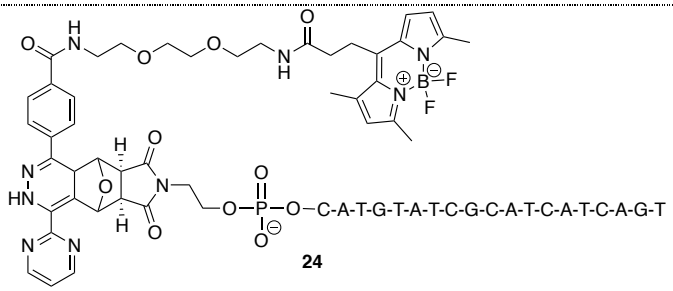

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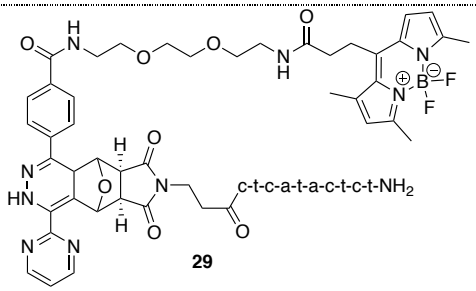

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Table 2. Characterization data of conjugates and isolation yields

Conjugate	anal. HPLC: t_R (min)[#]	MALDI-TOF MS			Yield (%)
		m/z found	calcd mass[&]	molec. formula	
pp2-10	16.8 & 18.0[a]	3456.4	3456.6	$C_{122}H_{148}N_{25}O_{74}P_{10}$	40
21	13.1[b]	8057.0	8058.1	$C_{290}H_{386}N_{95}O_{142}P_{19}$	25
22	9.0[b]	6490.2	6493.2	$C_{217}H_{275}N_{72}O_{126}P_{19}$	31
23	13.1[b]	6635.1	6639.2	$C_{224}H_{281}N_{76}O_{124}P_{19}S$	34
24	15.0[b]	6712.0	6715.3	$C_{230}H_{284}BF_2N_{76}O_{123}P_{19}$	21
25	25.1 & 25.4[c]	3585.0	3585.2	$C_{159}H_{257}N_{59}O_{37}$	35
26	23.7[c]	3569.6	3463.4	$C_{146}H_{182}N_{60}O_{37}$	45
27	15.6[d]	4869.9	4883.1	$C_{212}H_{287}N_{79}O_{59}$	36
28	14.5[e]	3318.5	3317.3	$C_{139}H_{176}N_{56}O_{43}$	32
29	19.7[c]	3539.0	3539.4	$C_{152}H_{185}BF_2N_{60}O_{40}$	40

[#]HPLC gradients were: a = 0-50% B (A =100 mM aq TEAA, B = ACN); b = 10-40% B (A = H_2O 100mM TEAA, B = ACN); c = 0-50% B (A = H_2O + 0.045% TFA, B = ACN + 0.036% TFA); d = 0-50% B (A = H_2O + 0.1% formic acid, B = ACN + 0.1% formic acid); e = 10-50% B (A = H_2O + 0.045% TFA, B = ACN + 0.036% TFA). [&]$[M+H]^+$.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. HPLC traces and spectra (pdf).

Acknowledgments

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