Supporting Information

Water-compatible Cycloadditions of Oligonucleotide-conjugated Strained Allenes for DNA-encoded Library Synthesis

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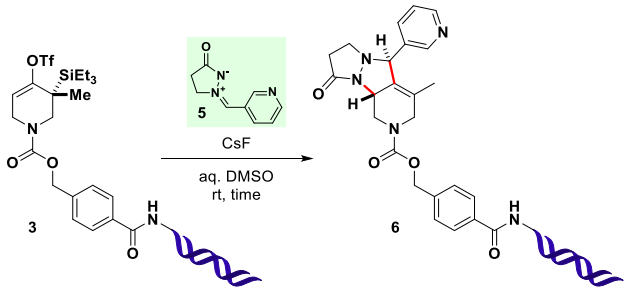
Contents

1. **Supplementary Tables**

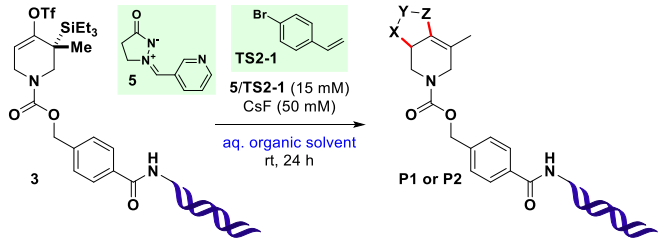
Table S1. Formation and trapping of a DNA-conjugated strained allenes.[a]



#	**5** [mM]	**CsF** [mM]	**%DMSO**	**t** [h]	**3** [%]	**6** [%]
1	100	8000	50	1	0	100
2	100	4000	50	1	0	100
3	100	2000	50	1	0	100
4	100	1000	50	1	2	98
5	100	500	50	1	28	72
6	100	250	50	1	64	40
7	100	3000	75	1	0	100
8	100	1500	75	1	0	100
9	100	750	75	1	1	99
10	100	375	75	1	28	72
11	100	~95	75	1	32	68
12	7.5	100	90	1	0	100
13	7.5	50	90	1	0	100
14	7.5	25	90	1	0	100
15	7.5	~13	90	1	0	100
16	7.5	~6	90	1	0	100
17	7.5	~3	90	1	27	73
18	7.5	~1.5	90	1	67	33
19	7.5	~0.8	90	1	96	4
20	8	50	90	12	0	100
21	4	50	90	12	0	100
22	2	50	90	12	0	100
23	1	50	90	12	0	95
24	0.5	50	90	12	0	84
25	0.25	50	90	12	0	56
26	0	50	90	12	0	0
27	15	50	25	24	100	0
28	15	50	50	24	97	3
29	15	50	75	24	0	100

[a] Reactions were run at room temperature (0.5 nmol scale, 10 µL total volume) and analyzed by UPLC-MS after ethanol precipitation. Product display (%AUC) was determined by UV (260 nm) considering DNA-species only. The relative configuration of the two newly formed stereogenic centers in **6** indicates the expected major product as observed in off-DNA precedence.

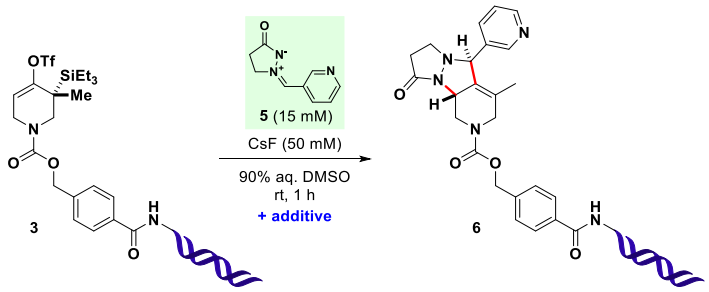
Table S2. Solvent screen for on-DNA strain-promoted cycloaddition reactions.[a]



BB	azomethine imine (5)						4-bromostyrene (TS2-1)					
%organic	25		50		75		25		50		75	
species	3	P1	3	P1	3	P1	3	P2	3	P2	3	P2
DMF	100	0	97	3	0	100	100	0	100	0	0	70
THF	100	0	100	0	100	0	100	0	100	0	97	3
MeCN	100	0	100	0	100	0	100	0	100	0	100	0
DMSO	100	0	97	3	0	100	100	0	100	0	0	89

[a] Reactions were run at room temperature (0.25 nmol scale, 10 µL total volume) for 24 h and analyzed by UPLC-MS after ethanol precipitation. %AUC of residual **3** or expected product **P** was determined by integration of UV signal (260 nm) considering DNA-species only. The results might be explained by the relatively high volatility of THF and MeCN: given the small reaction volume (10 µL), evaporation of organic solvent would quickly result in a highly aqueous medium slowing the consumption of **3** (also see **Table S1**).

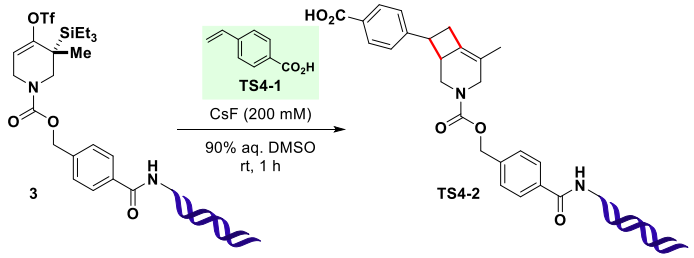
Table S3. Effect of excess aldehyde, triethylamine and hydrochloride salts on the conversion of **3**.[a]



#	additive/deviation	**3** [%]	**6** [%]	comment
1	none	0	100	efficient product formation
2	no **5**	0	0	several species form, masses inconclusive
3	benzaldehyde (28 mM)	0	100	no effect on desired transformation
4	benzaldehyde (112 mM)	0	100	no effect on desired transformation
5	NEt_3 (28 mM)	0	100	no effect on desired transformation
6	NEt_3 (112 mM)	0	100	no effect on desired transformation
7	MeNHOH·HCl (28 mM)	61	39	clean reaction profile, incomplete conversion of **3**
8	MeNHOH·HCl (112 mM)	100	0	no conversion of **3**
9	MeNHOH+$HNEt_3Cl$ (28 mM)	53	47	clean reaction profile, incomplete conversion of **3**
10	MeNHOH+$HNEt_3Cl$ (112 mM)	94	5	almost no conversion of **3**

[a] Reactions were run at room temperature (0.5 nmol **3**, 10 µL total volume) and analyzed by UPLC-MS after ethanol precipitation. Display (%AUC) of residual **3** and newly formed **6** was determined by UV (260 nm) considering DNA-species only. The relative configuration of the two newly formed stereogenic centers in **6** indicates the expected major product as observed in off-DNA precedence.

Table S4. Effect of acidic protons on consumption of **3**: titration of 4-vinylbenzoic acid.[a]



#	**TS4-1** [mM]	**CsF** [mM]	**ratio CsF/acid**	**t** [h]	**3** [%]	**TS4-2** [%]
1	180	200	1.1	1	43	46
2	90	200	2.2	1	0	71
3	45	200	4.4	1	3	77
4	~23	200	8.9	1	0	75
5	~11	200	17.8	1	0	89
6	~7	200	35.6	1	0	90

[a] Reactions were run at room temperature (0.25 nmol scale, 10 µL total volume) and analyzed by UPLC-MS after ethanol precipitation. Display (%AUC) of residual **3** (R_t = 3.3 min) and **TS4-2** (R_t = 2.5 min) were determined by UV (260 nm) considering DNA-species only.

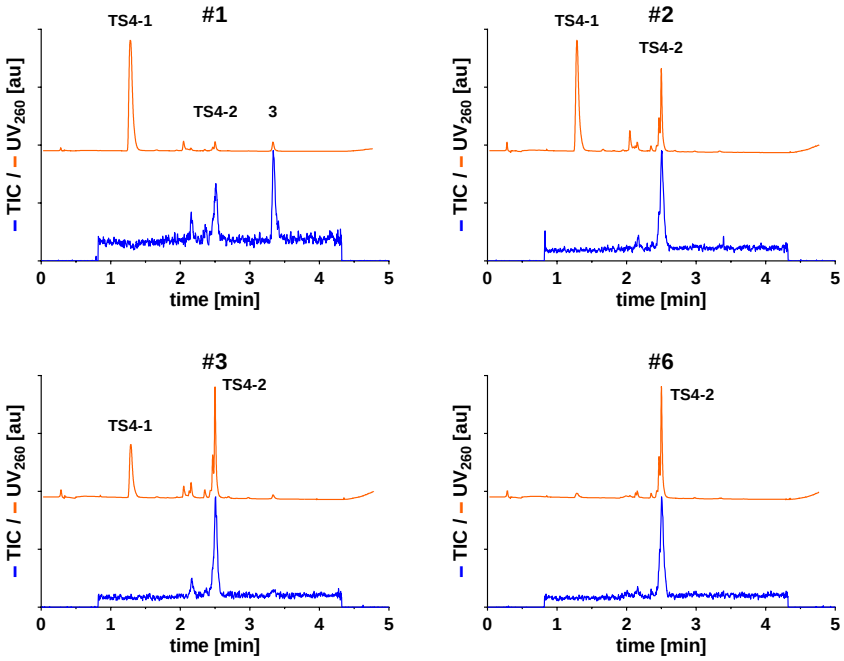
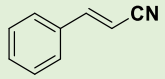
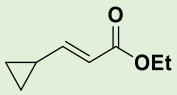
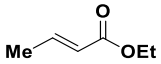
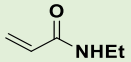
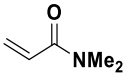
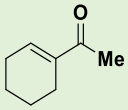
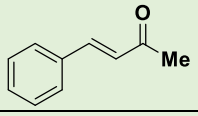
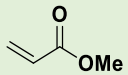
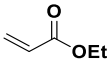
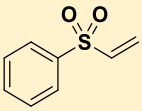
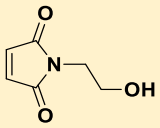
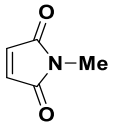
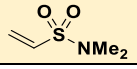
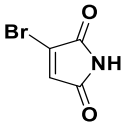
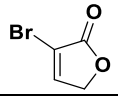


Table S5. Acceptor-substituted olefins in strain-promoted on-DNA [2+2] cycloadditions.[a]

structure	*E* in DMSO	*E* of similar compound in DMSO	time [h]	DNA alkylation observed?	comment
	-24.60		5.5	no	m/z agrees with expected product (up to 92% display)
	n. a.	 -23.59	1	no	m/z agrees with expected product (up to >69% display)
	n. a.	 -23.54	1	no	m/z agrees with expected product (up to >95% display)
	n. a.	< -23 (estimated)	1	no	m/z agrees with expected product (up to 75% display)
	-23.01		5.5	no	m/z agrees with expected product (up to >71% display)
	n. a.	 -19.07	1	no	efficient product formation, no additional alkylation observed
	-18.36		1	yes	product m/z agrees with [2+2] cycloaddition product and up to seven alkylation events (**see Figure S4**)
	n. a.	 -16.76	1	yes	product m/z agrees with [2+2] cycloaddition product and up to four alkylation events (concentration dependent)
	n. a.	 -14.07	1	yes	product m/z agrees with [2+2] cycloaddition product and up to 5 alkylation events (concentration dependent)
	n.a.	n.a.	2	yes	product m/z agrees with [2+2] cycloaddition product and up to 2 alkylation events (single experiment)
	n. a.		1		at high building block concentration: residual starting material and a very broad peak (polymerization?). At lower concentration, no defined DNA species detected.
	n. a.		1		at high building block concentration: residual starting material and a very broad peak (polymerization?). At lower concentration, no defined DNA species detected.

[a] Mayr electrophilicity parameters *E* (in DMSO) of acceptor-substituted olefins as published[1] or estimated by similarity. Conditions of on-DNA reactions: olefin (180 mM and 2-fold dilutions (5x down to ca. 6.6 mM)), strained-allene precursor **3** (0.25 nmol), CsF (200 mM), 90% aq. DMSO, rt, 1 or 5.5 h. Following EtOH precipitation, reaction outcomes were analyzed by UPLC-MS.

2. **Supplementary Figures**

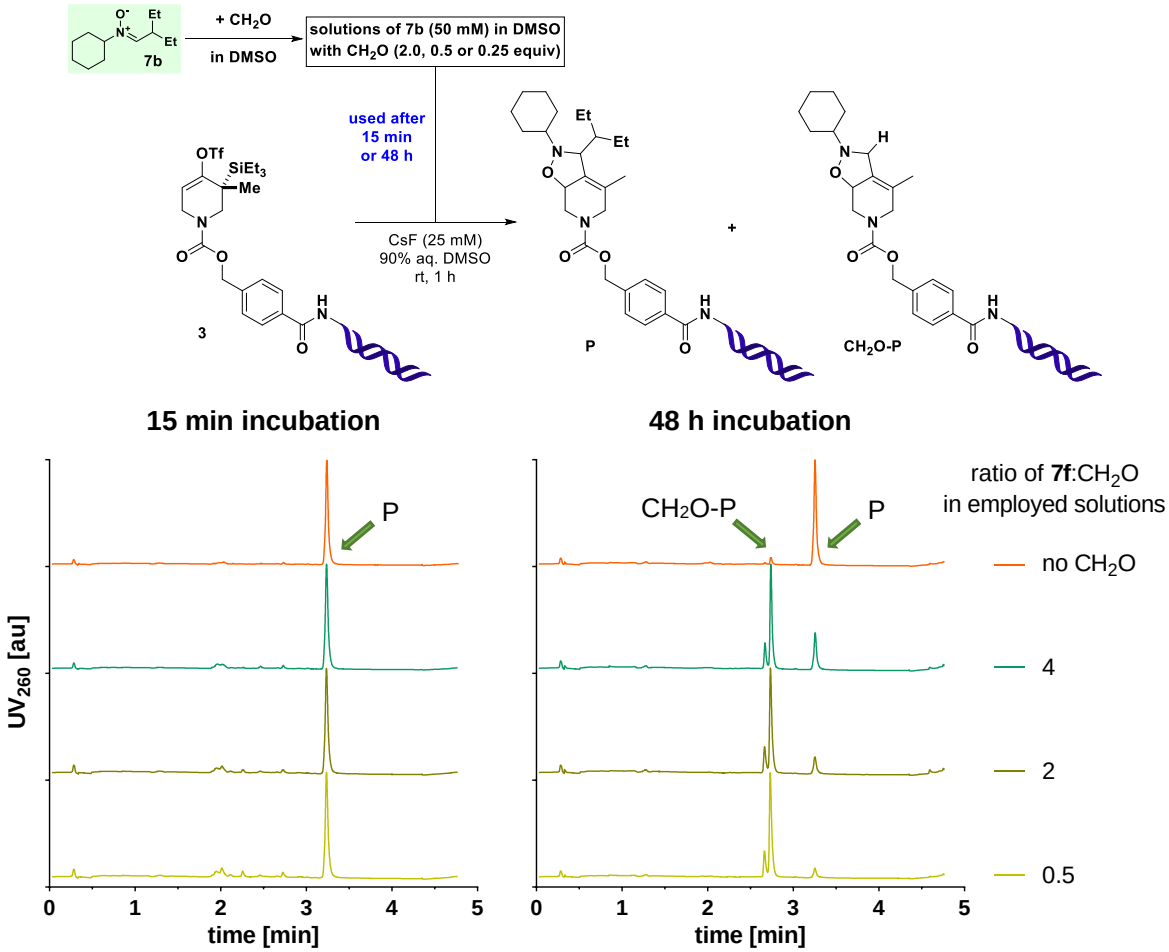


Figure S1. The effect of formaldehyde on strain-promoted [3+2] cycloadditions with nitrone **7b**. In the experiment, a solution of nitrone **7b** (100 mM in DMSO) was combined with the same volume of 37% aq. formaldehyde in DMSO (200 mM and 2-fold dilutions) to give stock solutions of varied ratios **7b**:CH_2O (all 50 mM in **7b**). After 15 min and 48 h incubation at room temperature, these stock solutions were employed in on-DNA strain-promoted cycloadditions. Reactions were performed with on-DNA allene precursor **3** (0.25 nmol), stock solutions **7b** (10 mM final concentration, 20 mM for no-formaldehyde control), and cesium fluoride (25 mM final concentration) in 90% aq. DMSO (room temperature, 1 h). Following ethanol precipitation, samples were analyzed by UPLC-MS. Reactions employing **7b**-formaldehyde mixtures (aged for 15 min), did not show significant formation of formaldehyde-derived product (Note: side reactions occur due to the low concentration of nitrone **7b**, see species eluting between 1.8 to 2.8 min). Employing the same stock solutions after 48 h resulted in significant formation of the corresponding formaldehyde-derived species (two peaks, presumably regioisomers). Importantly, small amounts of the same species were observed without added formaldehyde (orange top chromatogram), corroborating the presence low formaldehyde quantities in commercial DMSO.

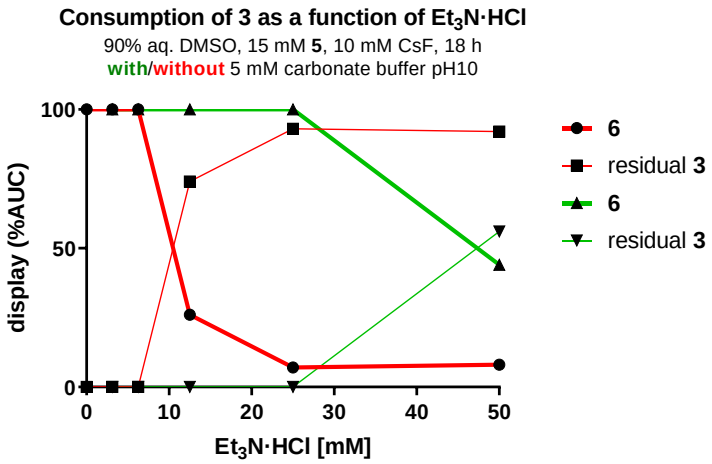


Figure S2. Effect of added bicarbonate buffer (pH10) on strained allene formation. on-DNA strained-allene precursor **3** (1 mM in water, 0.5 µL, 0.5 nmol) was combined with a solution of **5** in DMSO (9 µL, 15 mM final concentration). Solutions of CsF (200 mM) and varying concentrations of Et_3N·HCl (1000 mM and two-fold dilutions) in either carbonate buffer (100 mM, pH10) or water were added (0.5 µL to give final concentrations of 10 mM CsF, 5 or 0 mM carbonate buffer, and varied Et_3N·HCl content). Reactions were vortexed, spun down and incubated at room temperature for 18 h. Following ethanol precipitation, samples were analyzed by UPLC-MS and displays (%AUC) of residual **3** and product **6** were determined by integration of UV signals (UV 260 nm) considering DNA-species only. As the data shows, addition of carbonate buffer (pH10, 5 mM final concentration) was effective to overcome fluoride sequestration by added Et_3N·HCl up to a concentration of ca. 25 mM (18 h reaction time), whereas consumption of **3** dropped significantly around 10 mM Et_3N·HCl in the absence of buffer.

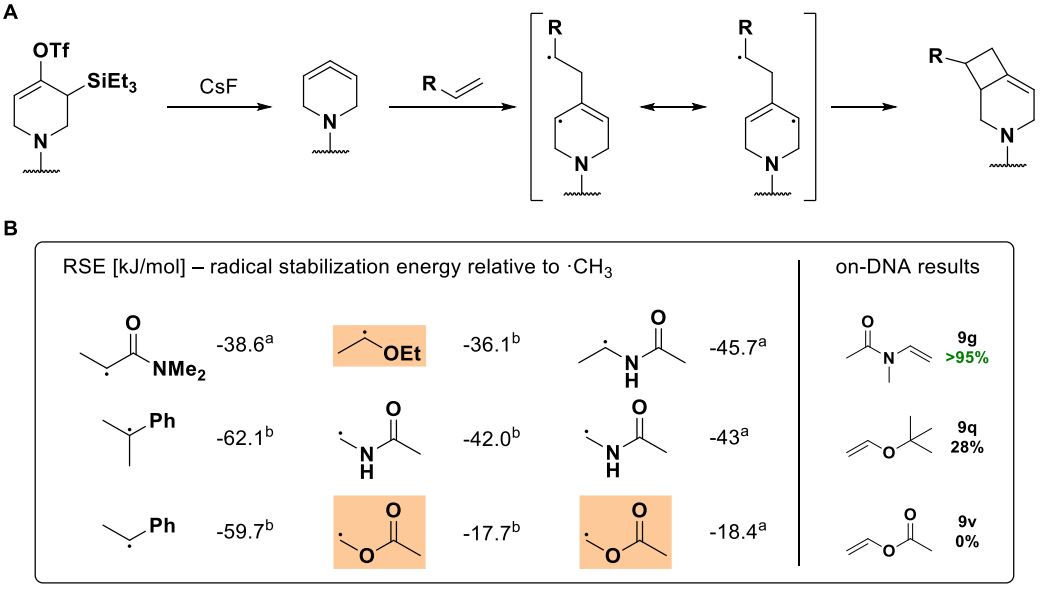


Figure S3. Radical stabilization energies in the context of strain-promoted on-DNA [2+2] cycloadditions. **A**: Mechanism of strain-promoted [2+2] cycloadditions with activated olefins as calculated by density functional theory.[2] **B**: Calculated radical stabilization energies (RSE, relative to $\cdot CH_3$) of radicals stabilized by functional groups relevant to this work (see **Figure 3** of the manuscript). [a]Energies calculated at the G3(MP2)-RAD level of theory.[3] [b]Energies calculates at the ROMP2 level of theory.[4] The highlighted species show relatively low RSE values, which might explain the unsuccessful [2+2] strain-promoted on-DNA cycloadditions of *O*-vinyl species. While vinyl ether **9q** afforded some expected product (28% display) along with numerous unidentified side products, application of vinyl acetate **9v** would not result in formation of distinct DNA species (suggesting polymerization).

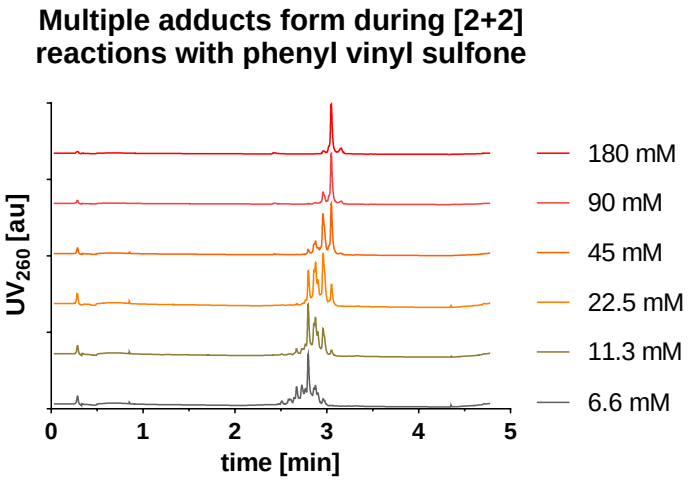


Figure S4. Reactions were performed with **3** (0.5 nmol), phenyl vinyl sulfone **9r** (final concentrations indicated) and cesium fluoride (200 mM final concentration) in 90% aq. DMSO (10 µL total volume, room temperature, 1 h). Following ethanol precipitation, reaction outcomes were analyzed by UPLC-MS. As show, various species form initially and converge at higher concentrations of phenyl vinyl sulfone. The main peak in the top chromatogram exhibits a deconvoluted mass of 6369 Da in agreement with the expected [2+2] addition product (5358 Da) and six additional alkylation events (5358 Da + 6x 168 Da = 6366 Da). The mass of a seventh adduct (6537 Da) was observed for the same peak.

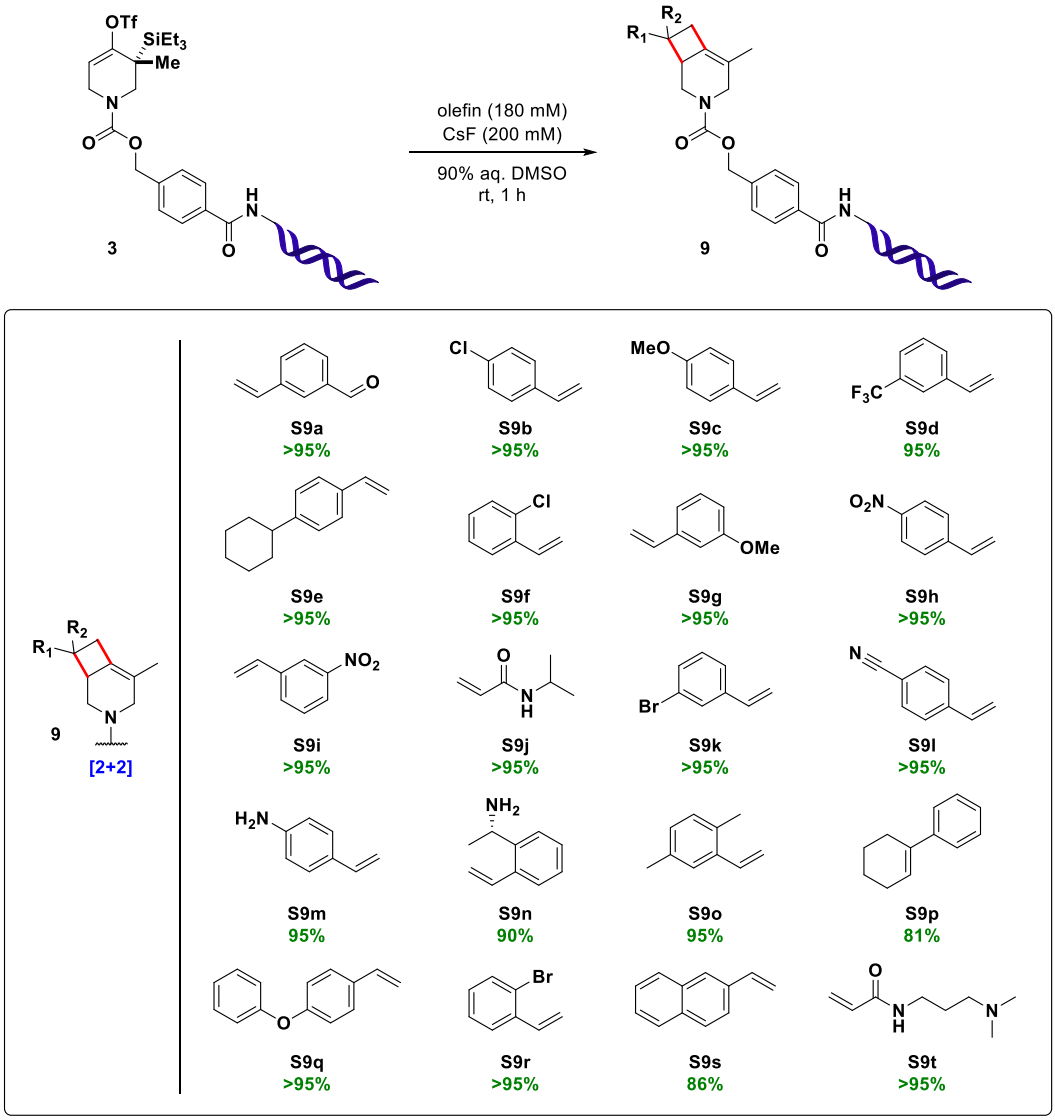


Figure S5. Additional examples of on-DNA strain-promoted [2+2] cycloadditions to afford compounds of type 9. Product display (%AUC) was determined by integration of UV signals (260 nm) considering DNA-species only.

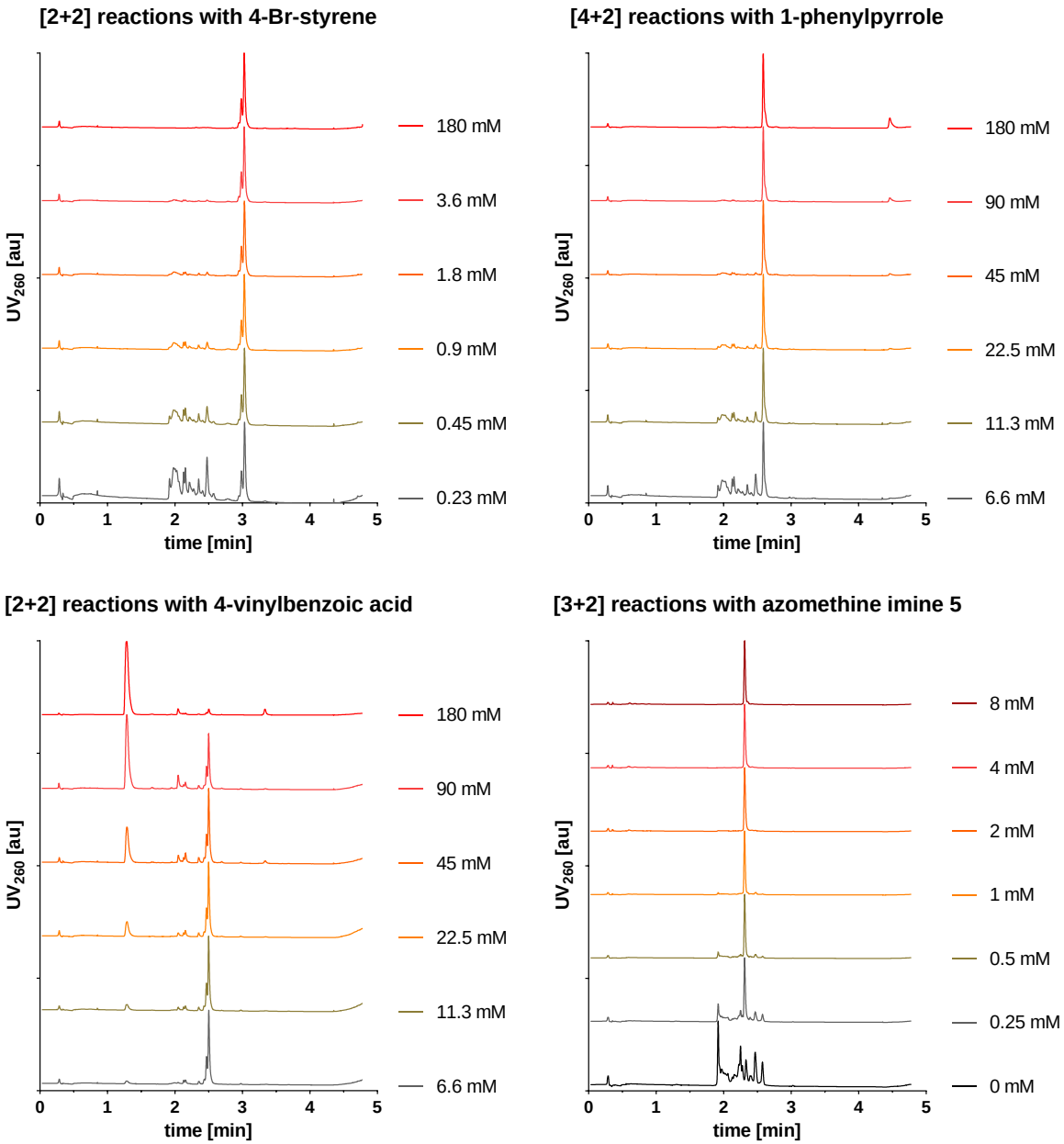


Figure S6. Titration of building blocks for each type of cycloaddition investigated in the present study (4-Br-styrene, 1-phenylpyrrole, 4-vinylbenzoic acid, and azomethine imine **5**). Reactions were performed with **3** (0.5 nmol) in 90% aq. DMSO (10 µL total volume, room temperature, 1 h) with the corresponding building block at a final concentration as indicated. Final concentration of cesium fluoride was 200 mM (50 mM for [3+2] reactions with azomethine imine **5**). Following ethanol precipitation, samples were analyzed by UPLC-MS.

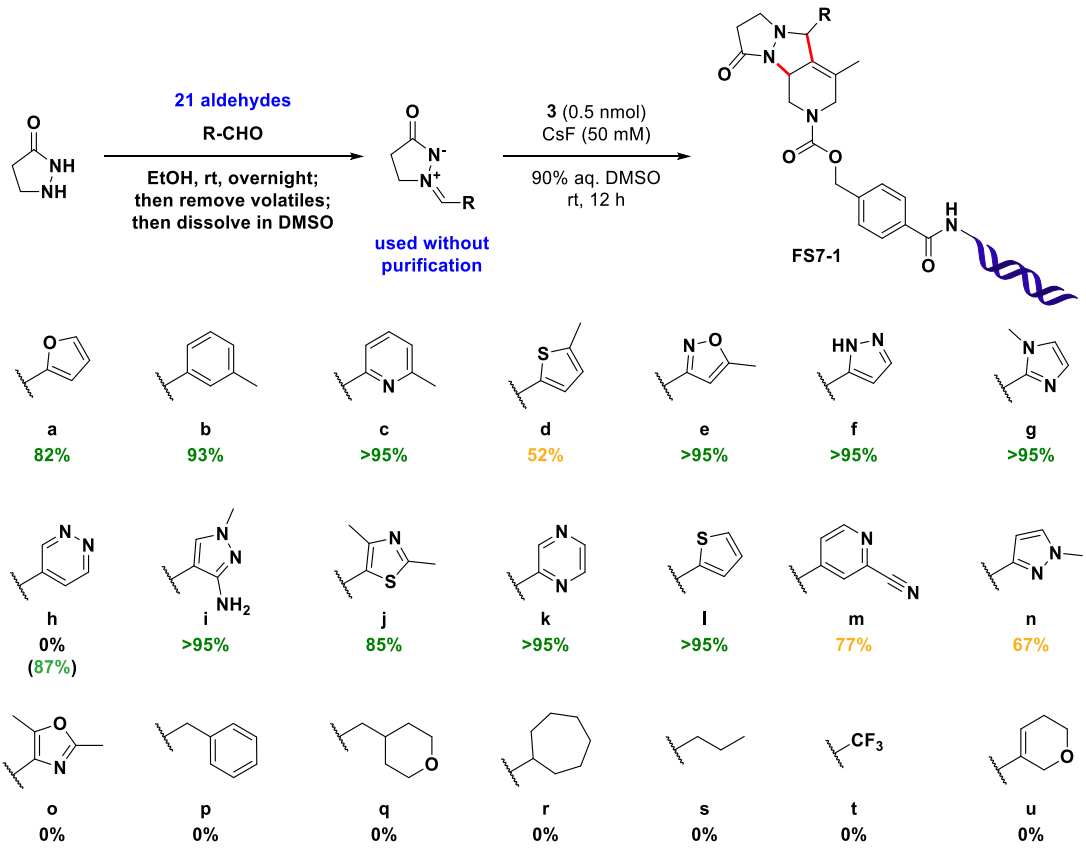


Figure S7. Purification-free preparation of azomethine imines and subsequent application in strain-promoted on-DNA cycloadditions. Value in parentheses corresponds to the outcome of an experiment employing the isolated and fully characterized azomethine imine. Reactions were performed with **3** (0.5 nmol) in 90% aq. DMSO (10 µL total volume) and analyzed by UPLC-MS following ethanol precipitation. Product display (%AUC) was determined by integration of UV chromatograms (260 nm) considering DNA-species only. For analytical data, see section 5 (p. S42).

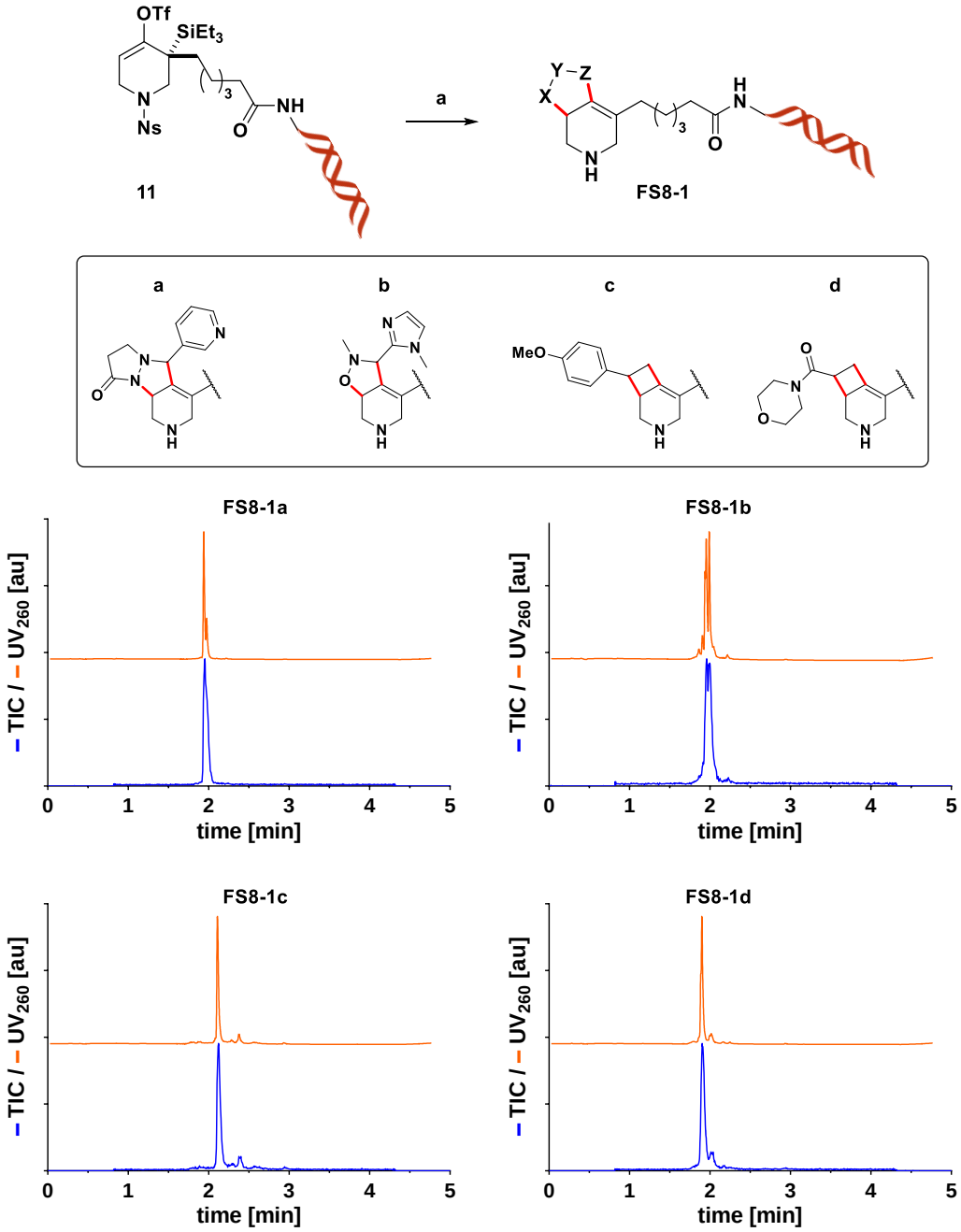


Figure S8. UPLC-MS traces of intermediate piperidines **FS8-1a–d** after strained-allene cycloaddition and Ns-deprotection.

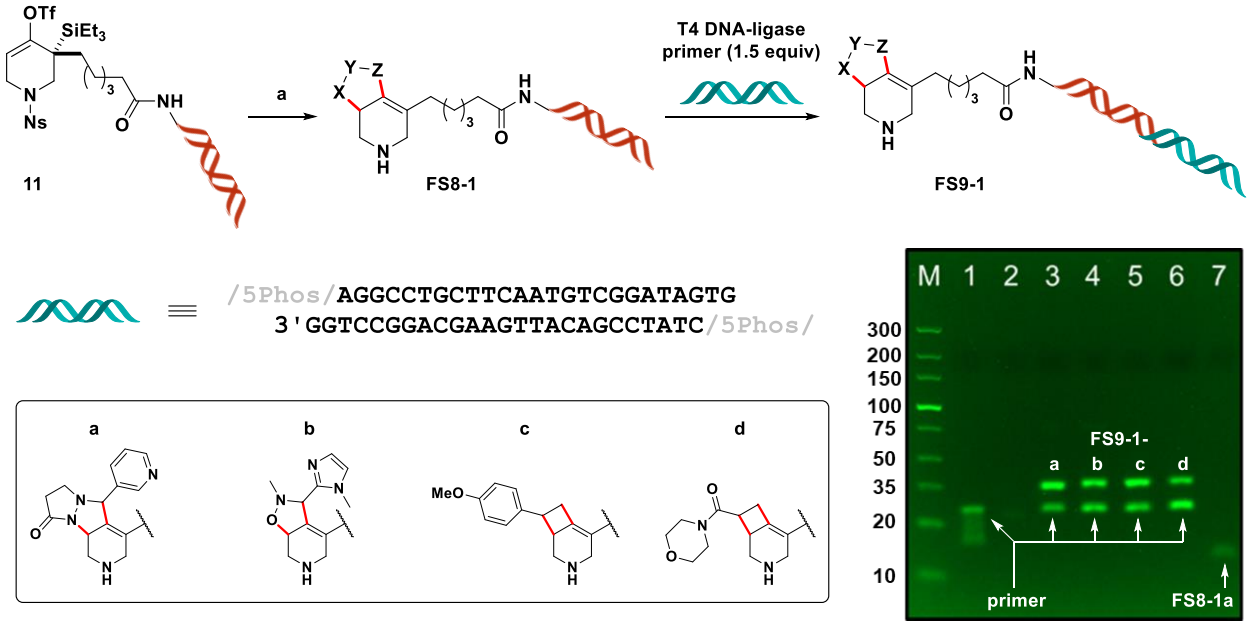


Figure S9. Ligation reactions of four on-DNA piperidines (**FS8-1a–d**), prepared by strain-promoted cycloaddition reactions (**Figure 4** in manuscript). Ligation conditions (4 reactions in parallel): **FS8-1a–d** (0.25 nmol per well), T4 DNA-ligase (2.5 U), 23-basepair primer (sequence shown, 1.5 equiv), ligation buffer (final concentrations: 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT), room temperature, overnight. Photograph shows gel electrophoretic analysis (E-Gel EX, 4% agarose, SYBR Gold II stain, software coloration "SYBR green") of reaction outcomes. Lanes show M: molecular ladder (number of basepairs indicated), 1: primer, 2: unrelated, 3–6: ligation reactions, 7: starting material **FS8-1a**. Lanes 3–6 indicate full consumption of starting materials **FS8-1a–d** (compare lane 7) with simultaneous formation of new species (**FS9-1a–d**) in agreement with the expected products (ca. 35 basepairs).

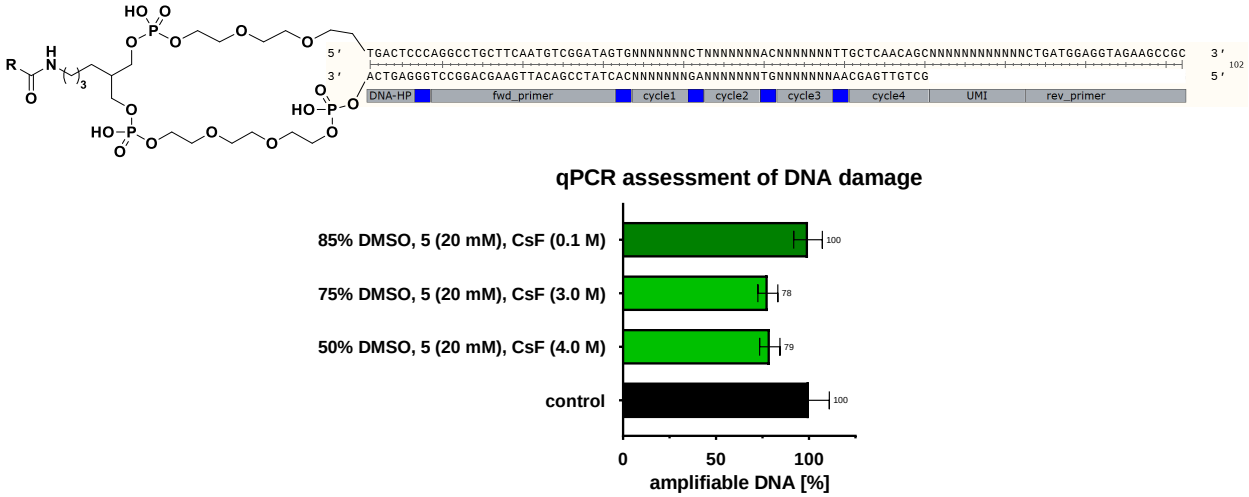


Figure S10. qPCR analysis of residual amplifiable material after 75 min incubation at room temperature. In a 96-well polypropylene plate, a solution of a full-length DNA-encoded library (construct shown, ca. 60 µM in water, 2 µL, ca. 0.12 nmol) was combined with water (control), DMSO solutions of azomethine imine **5** (20 mM final concentration) and aqueous cesium fluoride solutions to give final concentrations as indicated (20 µL total volume). The plate was sealed (adhesive aluminum foil), briefly vortexed, and incubated at room temperature for 75 min. All conditions were tested in triplicate (3x4 wells in total). Water (80 µL) was added and the resulting solutions were thoroughly mixed. Each sample was then diluted (two times 1:100, 2 µL sample in 198 µL water, dilution factor 1:10.000) and desalted by gel filtration (GE healthcare Illustra, G-25 microspin columns) as per the manufacturer's instruction. Amount of amplifiable material was determined by qPCR analysis in technical sextuplets (Applied Biosystems PowerUp SYBR Green Master Mix, catalog #A25742) relative to a 1:10 dilution series of the same template (DNA-encoded library).

Sample preparation: 2 µL template, 1 µL primers (5 µM each, 0.25 µM final concentration), 7 µL water, 10 µL master mix. qPCR parameters: 50 °C (60 s), 1.6 °C/s to 95 °C, then 40 cycles [90 °C (15 s), 1.0 °C/s to 60 °C (60 s), 1.6 °C/s to 90 °C].
fwd_primer 5' AATGATACGGCGACCACCGAGATCTACACCTCTCTATCTTAGCTCCCAGCGACCTGCTTCAATGTCGGATAGTG 3'
rev_primer 5' CAAGCAGAAGACGGCATACGAGATCAGCCTCGACCATGCTCACTGCGGCTTCTACCTCCATCAG 3'

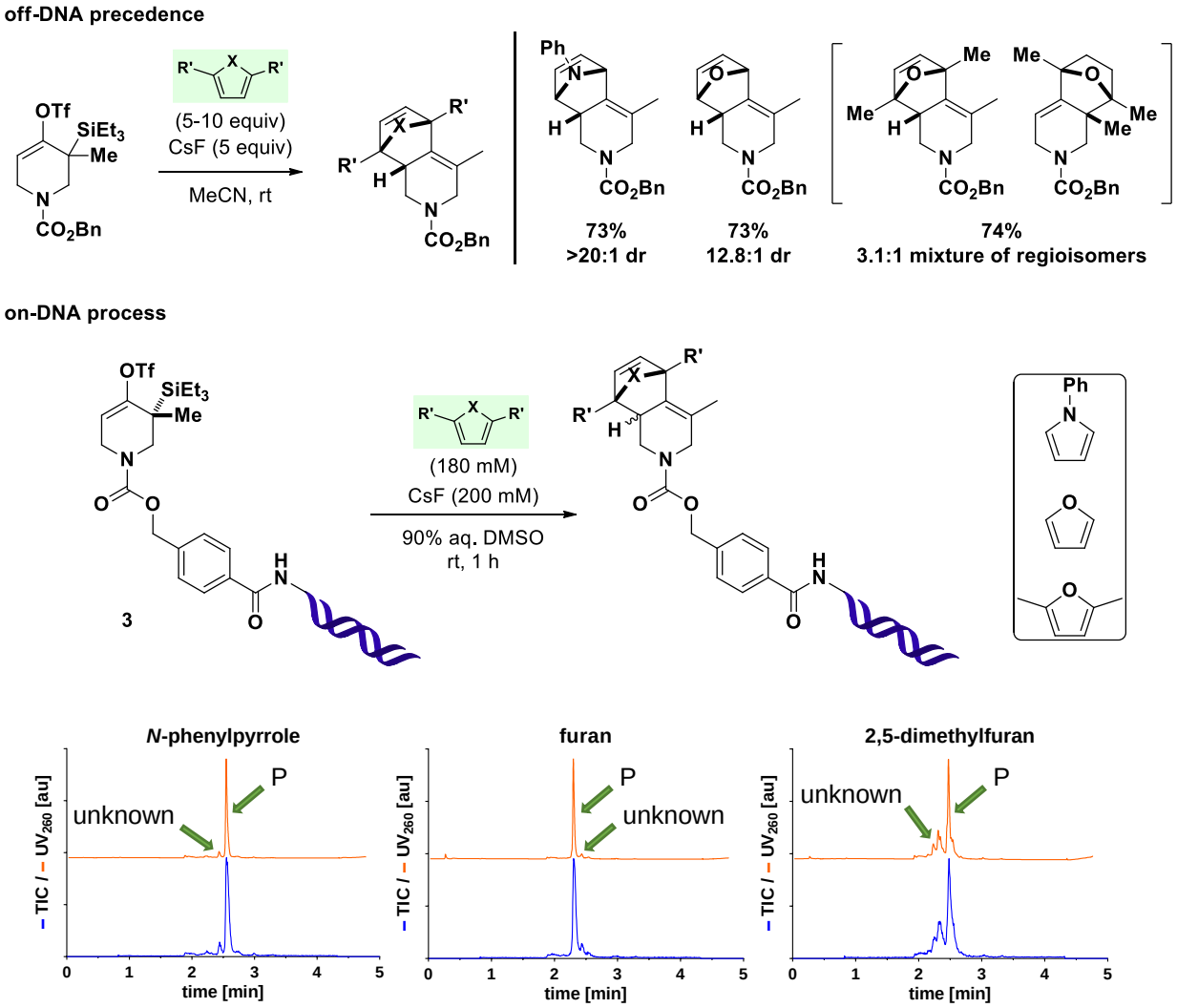


Figure S11. Top: off-DNA precedence of strain-promoted [4+2] cycloaddition with *N*-phenylpyrrole, furan and 2,5-dimethylfuran in MeCN. The products were formed as single diastereomer (*N*-phenylpyrrole), as diastereomerically enriched mixture (furan), and as a mixture of regioisomers (2,5-dimethylfuran), respectively. Relative configurations were assigned by 2D-NMR. **Bottom**: The corresponding on-DNA processes in 90% aq. DMSO. The reactions with *N*-phenylpyrrole and furan result in efficient formation of the expected products (mass of minor species inconclusive, possibly dimer of strained allene). The reaction with 2,5-dimethylfuran is sluggish, likely due to lower reactivity of the sterically congested building block.

3. General Information

a. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on Bruker AV-III HD 2-channel spectrometers operating at a frequency of 400.14 MHz (^{1}H) and 100.61 MHz (^{13}C) and equipped with either a 5 mm BBO-F conventional (room temperature) probehead or a 5 mm BBO-F cryoprobe. Unless indicated otherwise, data was acquired at 25 °C using the software ICON-NMR under TopSpin program control and processed with MestReNova (Version 12.0.2-20910). Spectra were referenced to residual solvent resonance according to literature.[5] For spectra recorded at elevated temperatures, the same literature values of residual solvent resonances (at ambient temperature) were used for referencing, neglecting temperature-dependent peak shifts. Resonance signals are reported as chemical shifts (δ) in ppm with multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal), couplings constant(s) in Hertz (Hz) and integral (not for ^{13}C). ^{13}C and ^{19}F NMR spectra were recorded with broadband ^{1}H decoupling.

b. Reaction monitoring by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

Samples were resolved on various Waters ACQUITY systems equipped with C18 columns (ACQUITY UPLC BEH C18 1.7 µm, 2.1x30 mm, ACQUITY UPLC BEH C18 1.7 µm, 2.1x50 mm, or ACQUITY UPLC CSH C18 1.7 µm, 2.1x50 mm) kept at 50 °C. UV signal was recorded with ACQUITY UPLC PDA detectors (210–400 nm). Light scattering signal was recorded with ACQUITY UPLC ELSD or SofTA 1100 ELSD detectors. Low-resolution mass spectra (LRMS) of eluting species were recorded with Waters SQD, SQD-2 or QDa detectors (electron spray ionization (positive and negative modes), scan time 0.3 sec, scanning range 120-1250 Da (QDa), 120-1600 Da (SQD) or 120-2850 Da (SQD-2)). High-resolution mass spectra (HRMS) of eluting species were recorded on systems equipped with Waters Xevo G2 Qtof or Waters Xevo G2-XS Tof detectors (electron spray ionization (positive mode), scan time 0.2 sec, scanning range 100-2050 Da).

Solvent A1	0.1% formic acid in water
Solvent B1	0.1% formic acid in acetonitrile
Solvent A2	5 mM ammonium hydroxide in water
Solvent B2	5 mM ammonium hydroxide in acetonitrile
Solvent A3	0.05% trifluoroacetic acid in water
Solvent B3	0.05% trifluoroacetic acid in acetonitrile

RXNMON-Acidic
flow: 1.0 mL/min, runtime: 2.0 min
column: ACQUITY UPLC BEH C18 1.7 µm, 2.1x30 mm

time	%A (solvent A1)	%B (solvent B1)
0.00	98	2
0.10	98	2
1.50	2	98
1.80	2	98
1.90	98	2
2.00	98	2

RXNMON-Basic
flow: 1.0 mL/min, runtime: 2.0 min
column: ACQUITY UPLC BEH C18 1.7 µm, 2.1x30 mm

time	%A (solvent A2)	%B (solvent B2)
0.00	98	2
0.10	98	2
1.50	2	98

1.80	2	98
1.90	98	2
2.00	98	2

ProductAnalysis-Acidic
flow: 1.0 mL/min, runtime: 5.2 min
column: ACQUITY UPLC BEH C18 1.7 µm, 2.1x50 mm

time	%A (solvent A1)	%B (solvent B1)
0.00	98	2
4.40	2	98
5.15	2	98
5.19	98	2

ProductAnalysis-Basic
flow: 1.0 mL/min, runtime: 5.2 min
column: ACQUITY UPLC BEH C18 1.7 µm, 2.1x50 mm

time	%A (solvent A2)	%B (solvent B2)
0.00	98	2
4.40	2	98
5.15	2	98
5.19	98	2

HRMS
flow: 1.0 mL/min, runtime: 2.2 min
column: ACQUITY UPLC CSH C18 1.7 µm, 2.1x50 mm

time	%A (solvent A3)	%B (solvent B3)
0.00	98	2
0.06	98	2
1.76	2	98
2.00	2	98
2.16	98	2

c. Ion-pair chromatographic analysis of DNA-conjugates by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

Samples were resolved on a Waters ACQUITY system equipped with a C18 column (ACQUITY Oligonucleotide BEH C18 1.7 µm, 2.1x50 mm, part #186003949) kept at 50 °C. UV signal was recorded with an ACQUITY TUV detector (260 nm, sampling rate 20 points/sec). Mass spectra of eluting species were recorded with a Waters SQ Detector 2 connected to a ZSpray™ source (negative ion mode, scan time 0.2 sec, scanning range 500-3000 Da, MaxEnt1 deconvolution, processing 2-20k Da).

Standard method (5-50% B)
flow: 0.5 mL/min, runtime: 6.0 min, injection volume: 10 µL, temp: 50 °C
column: ACQUITY Oligonucleotide BEH C18 1.7 µm, 2.1x50 mm
solvent A: 250 mM hexafluoroisopropanol and 8 mM triethylamine in water
solvent B: methanol

time	%A (solvent A)	%B (solvent B)
0.00	95	5
4.00	50	50
4.50	5	95
5.00	5	95
5.10	95	5
6.00	95	5

d. Preparative high performance liquid chromatography (prep-HPLC)

Purifications were performed on a Waters system equipped with Waters 515 pumps, a Waters 2545 binary gradient module, a Waters Acquity QDa detector, a Waters 2998 PDA detector and a Waters 2767 sample manager. The column (Waters XBridge Prep C18 OBD, 5 µm, 30 mm (inner diameter) x 50 mm) was kept at room temperature. Material was injected as solution in methanol/water mixtures (1.5 mL) and eluted with gradients of solvent A (water) and solvent B (acetonitrile), both modified with either 0.1% formic acid or 5 mM ammonium hydroxide (75 mL/min flowrate). Fraction collection was triggered by UV signal and mass (TIC) of eluting species. Predefined gradients were picked from a list of methods optimized for target retention time as observed during reaction monitoring by UPLC-MS.

e. Preparative high performance liquid chromatography of oligonucleotide-conjugates (microprep-HPLC)

Purifications were performed on an Agilent Infinity system equipped with an Infinity 1260 Bio Quat Pump (pump system, G5611A), an Infinity 1260 HiP Bio ALS (autosampler, G1330B), an Infinity 1290 TCC (thermostatted column compartment, G5667A), an Infinity 1260 DAD (diode array detector, G4212B), and an Infinity 1260 Bio FC-AS (fraction collector, G5664A) under Agilent OpenLab CDS (C.01.07 SR1) software control. The column (Waters XBridge BEH C18 OBD, 130 Å, 5 µm, 10x150 mm) was kept at 50 °C. Material was injected as aqueous solutions (up to 100 µL per injection) and eluted with gradients of solvent A (50 mM triethylammonium acetate in water) and solvent B (MeOH). Fraction collection was triggered by UV (260 nm).

10-70% method
flow: 5.0 mL/min, runtime: 20 min
column: Waters XBridge BEH C18 OBD, 130 Å, 5 µm, 10x150 mm

time	%A (solvent A)	%B (solvent B)
0.0	90	10
15.0	30	70
16.0	5	95
18.0	5	95
18.1	90	10
20.0	90	10

f. Other methods and equipment

Aqueous solutions of DNA were quantified photospectrometrically with a Thermo Scientific Nanodrop One. Electrophoresis was performed with an Invitrogen E-GEL Power Snap device using commercial gels (E-Gel EX, 4% agarose). Centrifugation was performed with a Beckman Coulter Allegra X-15R or Beckman Coulter Microfuge 18.

g. Flash chromatography

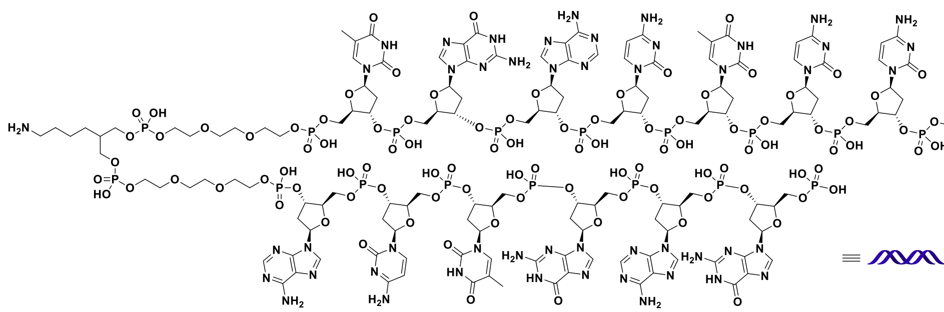
Automated flash chromatography was performed on Teledyne ISCO CombiFlash® systems equipped with TUV and ELSD detectors using prepacked columns (RediSep® Rf, prepacked with 4 g, 12 g, 24 g, 40 g, 80 g, 120 g, or 330 g silica, 35-70 µm). Crude material was usually adsorbed on diatomaceous earth (Biotage Isolute HM-N) and then subjected to chromatography (dry-loading) eluting with gradients of ethyl acetate in heptane, methanol in dichloromethane, or as specified. Fractions containing homogeneous material according to detection method (TUV/ELSD) and/or thin layer chromatography (TLC) were pooled and solvents were removed by rotary evaporation under reduced pressure at 40 or 45 °C to afford target compounds.

4. Chemical synthesis

a. General remarks

Anhydrous solvents (tetrahydrofuran (THF), acetonitrile (MeCN), dichloromethane (DCM), dimethylformamide (DMF), dimethylsulfoxide (DMSO), dimethylacetamide (DMA)) were purchased from Sigma-Aldrich and used as received. HPLC grade solvents (methanol (MeOH), ethyl acetate (EtOAc), heptane) were purchased from varying vendors and used as received. Solutions of potassium hexamethyldisilazide (KHMDS, 1 M in THF), *n*-butyl lithium (*n*-BuLi, 2.5 M in hexanes) and lithium tri-*sec*-butylborohydride (L-selectride, 1 M in THF) were purchased from Sigma-Aldrich. 2-[*N,N*-Bis(trifluoromethylsulfonyl)amino]-5-chloropyridine (Comins' reagent) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) were purchased from Oakwood Chemical. DNA-headpiece (DNA-HP) and AOP-headpiece (AOP-HP) were purchased from LGC Biosearch Technologies. Other chemicals were purchased from varying vendors and used as received, if not stated otherwise. Chemical reactions were routinely performed under nitrogen atmosphere. Reactions involving DNA-conjugates were performed in polypropylene vessels (usually Eppendorf Tubes®) or 96-well polypropylene PCR plates (Eppendorf twin.tec®) under ambient atmosphere.

Chemical structure of DNA-HP



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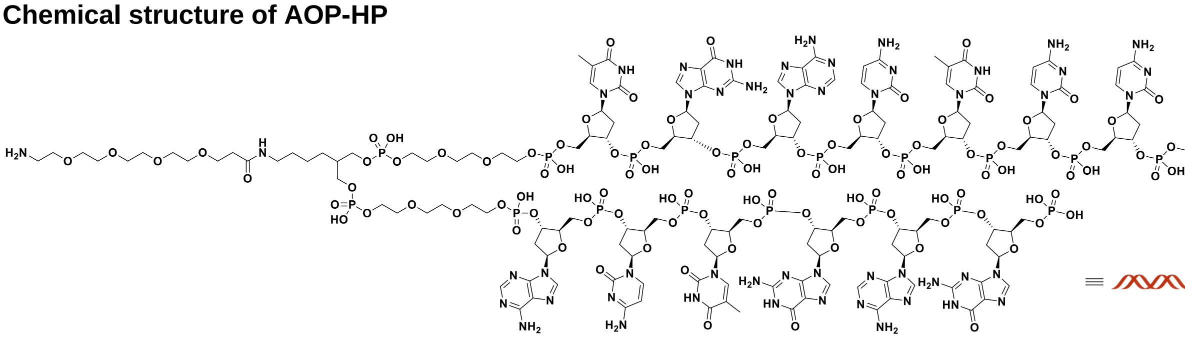
Chemical structure of AOP-HP



SMILES string:

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b. General procedures

GP1: Ethanol precipitation of 10 µL reactions in a 96-well PCR plate
Aq. NaCl (5 M, 1 µL) and EtOH (30 µL) were added. The plate was heat-sealed (Eppendorf heat-sealing foil), briefly vortexed and incubated on dry ice for 30 to 60 min. After centrifugation (5 min at 3700 RCF), the seal was taken off. The supernatant was removed by covering the plate with a paper towel and cautiously inverting. 80% aq. EtOH (40 µL) was added to the wells. The plate was heat-sealed and vortexed. After centrifugation (5 min at 3700 RCF), the supernatant was removed as before. EtOH (40 µL) was added and the procedure (sealing, vortexing, centrifugation, removal of supernatant) was repeated as before. Water (25 µL) was added and the plate was sealed (heat-sealing or using an adhesive foil). Samples were then analyzed by UPLC-MS.

GP2: Ethanol precipitation of larger scale reactions
To a reaction of a given volume (100% v/v), aq. NaCl (5 M, 10% v/v) and EtOH (300% v/v) were added. The reaction vessel (usually an Eppendorf tube) was briefly vortexed and incubated on dry ice for 30 to 60 min. After centrifugation (5 min at 11000 RCF), the supernatant was removed by pipette. To wash the pellet, 80% aq. EtOH (100-200% v/v) was added. The mixture was vortexed (or briefly sonicated). After centrifugation (5 min at 11000 RCF), the supernatant was removed by pipette. The washing procedure was repeated with 100% EtOH (100-200% v/v) and the obtained pellet was dried under vacuum.

GP3: on-DNA strain-promoted cycloaddition reactions (10 µL total volume)
In a 96-well polypropylene PCR plate, an aq. solution of the corresponding DNA-conjugated strained-allene precursor (0.5 or 1 mM, 0.5 µL, 0.25 or 0.5 nmol) was combined with a solution of a cycloaddition partner (1,3-dipole, activated olefin, or *N*-substituted pyrrole) in DMSO (e.g. 200 mM, 9 µL, 180 mM final concentration) and the plate was spun down. An aq. solution of cesium fluoride (e.g. 4 M in 100 mM bicarbonate buffer pH10, 0.5 µL, 200 mM final concentration) was added. The plate was heat-sealed (Eppendorf heat-sealing foil) and allowed to stand for a few minutes to cool. The plate was briefly vortexed, spun down and incubated at room temperature for 1 h. Following ethanol precipitation according to **GP1**, samples were analyzed by UPLC-MS.

c. Synthesis of DNA-conjugate 3

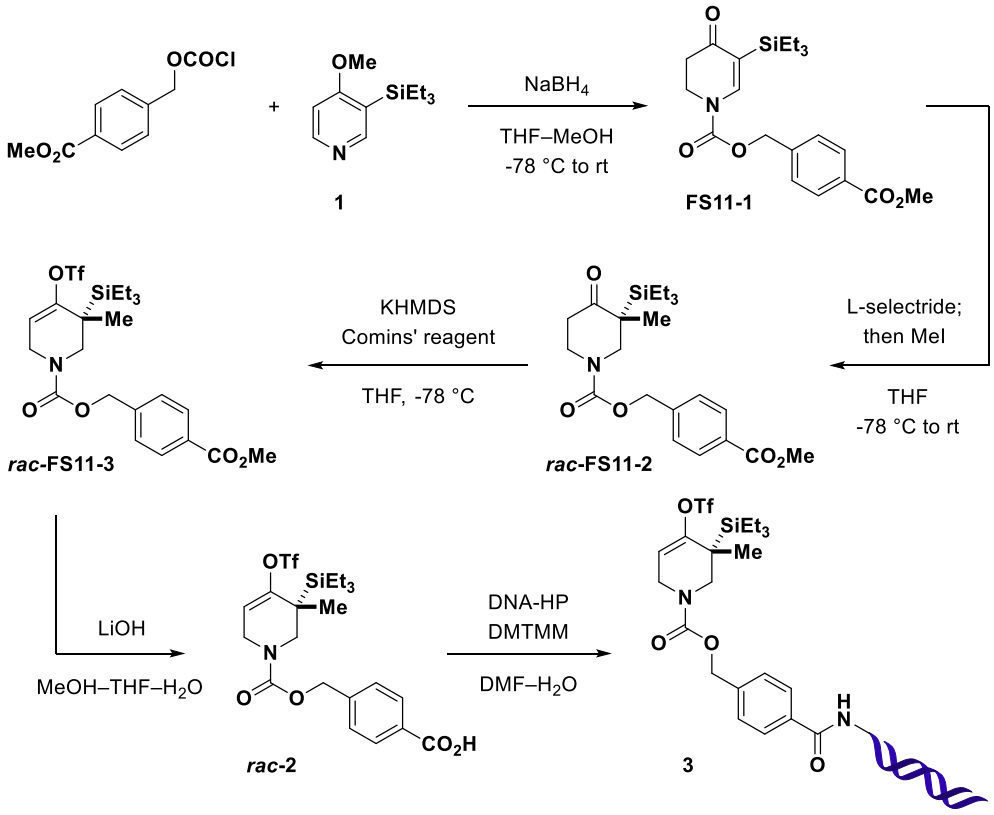
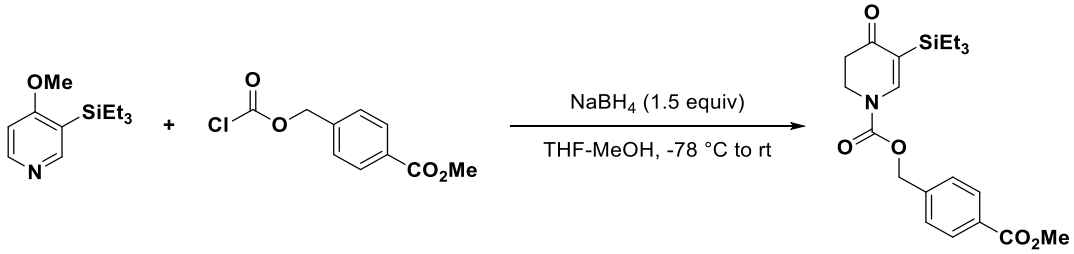


Figure S11 Synthetic route towards on-DNA strained allene precursor **3**.

Synthesis of FS11-1

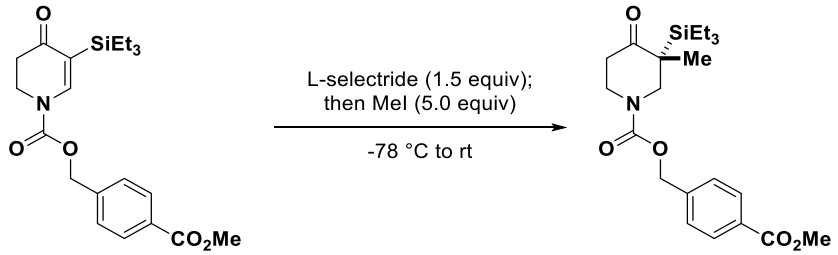


4-Methoxy-3-(triethylsilyl)pyridine (**1**) (4.47 g, 20.0 mmol, 1.0 equiv) was dissolved in MeOH (80 mL) and cooled to –78 °C. $NaBH_4$ (1.14 g, 30.0 mmol, 1.5 equiv) was added. A solution of 4-methoxycarbonylphenyl chloroformate (5.72 g, 25.0 mmol, 1.25 equiv) in THF (20 mL) was added via addition funnel over 10 min. Upon completion of the addition, the mixture had turned into a colorless suspension. The cooling bath was removed and the suspension was stirred for another 20 min. Aqueous HCl (10%, 50 mL) was added. After stirring for 5 min, the mixture was diluted with water (300 mL) and extracted with CH_2Cl_2 (1x 100 mL, 2x 50 mL). The combined organic extracts were washed with brine (100 mL), dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (10% EtOAc in heptane to 30%) afforded **FS11-1** (6.74 g, 16.7 mmol, 84% yield) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.09 - 8.03 (m, 2H), 7.80 (s, 1H), 7.49 - 7.42 (m, 2H), 5.31 (s, 2H), 4.05 - 3.98 (m, 2H), 3.92 (s, 3H), 2.59 - 2.50 (m, 2H), 0.90 (t, *J* = 7.8 Hz, 9H), 0.68 (q, *J* = 8.7, 8.1 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 196.6, 166.7, 152.8 (br), 147.9 (br), 140.2, 130.5, 130.1, 127.9, 113.8, 68.1, 52.4, 42.7, 36.2, 7.5, 3.0. **LRMS** (ESI+) req. for $C_{21}H_{30}NO_5Si$ $[M+H]^+$ 404.2, found 404.4.

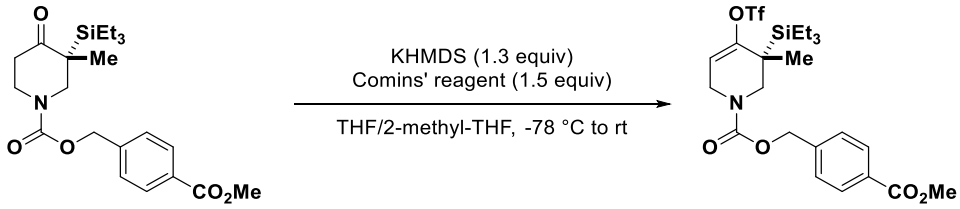
Synthesis of *rac*-FS11-2



FS11-1 (2.53 g, 6.27 mmol, 1.0 equiv) was dissolved in THF (31 mL) and cooled to –78 °C. A solution of L-selectride in THF (1 M, 9.4 mL, 9.4 mmol, 1.5 equiv) was added via syringe. The resulting yellow solution was stirred at –78 °C for 100 min. UPLC-MS analysis (RXNMON-Acidic) confirmed full consumption of starting material. Methyl iodide (2 mL, 31.3 mmol, 5 equiv) was added and the cooling bath was removed. The yellow solution was stirred under exclusion of light for 4.5 h. NEt_3 (3.5 mL, 25.1 mmol, 4 equiv) was then added to quench excess methyl iodide. The resulting suspension was stirred for 5 min, before it was diluted with water (300 mL). Organic material was extracted with CH_2Cl_2 (3x 50 mL). The combined organic extracts were washed with brine (100 mL), dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (5% EtOAc in heptane to 25%) afforded ***rac*-FS11-2** (1.62 g, 3.9 mmol, 62% yield) as colorless oil.

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ = 8.03 - 7.93 (m, 2H), 7.25 - 7.14 (m, 2H), 5.10 - 4.95 (m, 2H), 3.87 (dd, *J* = 14.0, 4.4 Hz, 1H), 3.73 - 3.64 (m, 1H), 3.60 - 3.51 (m, 3H), 3.27 - 3.09 (m, 2H), 2.30 - 2.17 (m, 1H), 2.16 - 2.05 (m, 1H), 1.12 - 1.05 (m, 3H), 0.88 (dd, *J* = 9.1, 6.6 Hz, 9H), 0.56 (qt, *J* = 11.6, 5.1 Hz, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C) δ = 209.7, 166.4, 155.2, 142.4, 137.7, 131.0, 130.2, 66.9, 51.6, 50.4, 45.5, 42.9, 39.5, 18.7, 7.9, 2.9. **HRMS** (ESI+) req. for $C_{22}H_{34}NO_5Si$ $[M+H]^+$ 420.2201, found 420.2219.

Synthesis of *rac*-FS11-3

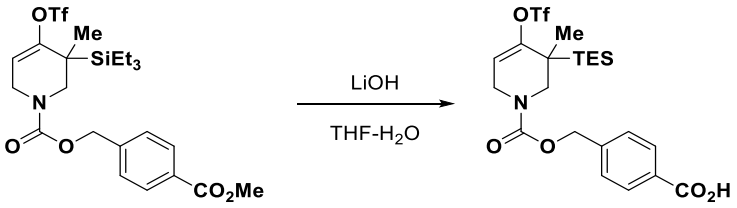


***rac*-FS11-2** (50 mg, 0.12 mmol, 1.0 equiv) was combined with THF (0.5 mL) and 2-[*N,N*-Bis(trifluoromethylsulfonyl)amino]-5-chloropyridine (Comins' reagent) (70 mg, 0.18 mmol, 1.5 equiv) was added. The mixture was cooled to –78 °C before a solution of KHMDS in THF (1 M, 0.16 mL, 0.16 mmol, 1.3 equiv) was added over 5 min. The reaction mixture was stirred for 45 min, then at room temperature for another 30 min. The mixture was diluted with EtOAc (150 mL), washed with half-saturated brine (3x 30 mL), sat. aq. sodium bicarbonate (30 mL) and brine (30 mL), dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 20%) afforded ***rac*-FS11-3** (25 mg, 0.045 mmol, 38% yield).

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ = 8.04 – 7.91 (m, 2H), 7.20 – 7.10 (m, 2H), 5.36 (t, *J* = 3.5 Hz, 1H), 5.08 – 4.87 (m, 2H), 3.78 – 3.55 (m, 3H), 3.54 (s, 3H), 3.14 (d, *J* = 13.2 Hz, 1H), 1.06 (s, 3H), 0.88 (t, *J* = 7.9 Hz, 9H), 0.63 – 0.53 (m, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C) δ = 166.3, 155.2, 154.9, 142.1, 137.7,

131.1, 130.3, 119.3 (q, *J* = 320.1 Hz), 110.2, 67.0, 51.5, 50.8, 42.8, 31.9, 18.7, 7.8, 3.0. **HRMS** (ESI+) req. for $C_{23}H_{33}F_3NO_7SSi$ $[M+H]^+$ 552.1694, found 552.1729.

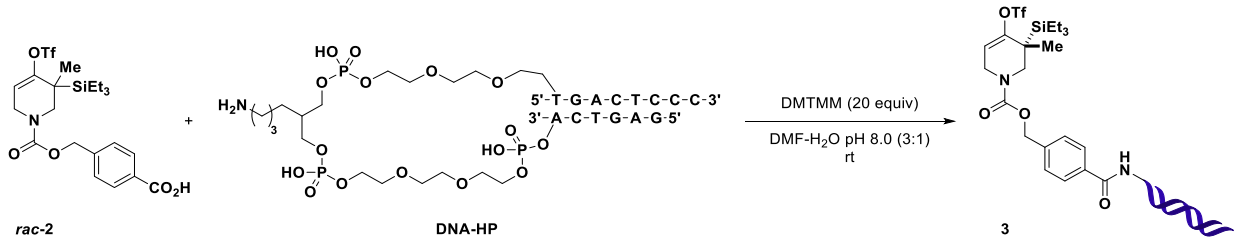
Synthesis of *rac*-2



***rac*-FS11-3** (79 mg, 0.143 mmol, 1.0 equiv) was dissolved in THF (1 mL). The solution was cooled to 0 °C and an aqueous solution of lithium hydroxide (1 M, 0.43 mL, 0.43 mmol, 3.0 equiv) was added. The resulting cloudy mixture was stirred overnight (thereby allowed to reach room temperature). The mixture was diluted with water (50 mL). Aqueous HCl (1 M, 5 mL) was added and the organic material was extracted with CH_2Cl_2 (3x 15 mL). The combined organic fractions were dried with $MgSO_4$, filtered and concentrated. The residue was dissolved in MeOH (0.5 mL) and filtered via syringe filter applying positive pressure (nitrogen) to force the solution through the filter. Material transfer was quantitated with additional MeOH (2x 0.5 mL). The filtrate was subjected to preparative HPLC (acidic, 55% to 80% MeCN in water) to afford ***rac*-2** (15 mg, 0.028 mmol, 19% yield).

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ = 8.05 – 7.97 (m, 2H), 7.13 (d, *J* = 8.0 Hz, 2H), 5.36 (t, *J* = 3.5 Hz, 1H), 5.04 – 4.87 (m, 2H), 3.80 – 3.51 (m, 3H), 3.15 (d, *J* = 13.3 Hz, 1H), 1.07 (s, 3H), 0.89 (t, *J* = 7.9 Hz, 9H), 0.58 (q, *J* = 7.6 Hz, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C) δ = 154.9, 143.0, 130.8, 110.1, 67.0, 50.9, 42.8, 31.9, 18.7, 7.8, 3.0. **^{19}F NMR** (377 MHz, *d8*-PhMe) δ = -75.1. **HRMS** (ESI+) req. for $C_{22}H_{31}F_3NO_7SSi$ $[M+H]^+$ 538.1537, found 538.1547.

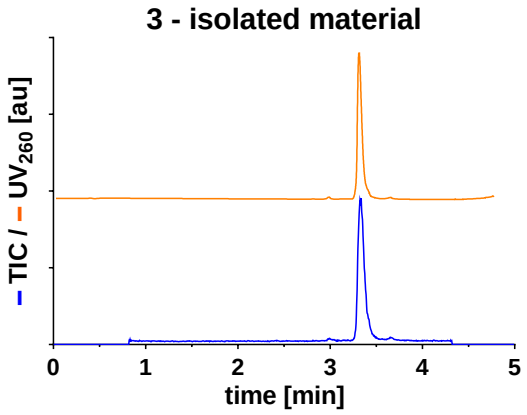
Synthesis of 3



***rac*-2** (3 mg, 5.6 µmol, ca. 28 equiv) was dissolved in DMF (0.6 mL) and added to a solution of DNA-HP (1 mM, 200 µL, 200 nmol) in phosphate buffer (100 mM, pH8.0). A freshly prepared solution of DMTMM in water (400 mM, 10 µL, 4 µmol, 20 equiv) was added. The resulting mixture was vortexed, spun down and incubated at room temperature for 3 h (UPLC-MS indicated full consumption of DNA-HP). The solution was transferred to a 20 mL centrifuge tube (Falcon®). Material transfer was quantitated with water (2x 0.1 mL, total volume now ca. 1 mL). Aq. NaCl (5 M, 0.1 mL) and EtOH (2.8 mL) were added. The mixture was vortexed and incubated on dry ice for 30 min. After centrifugation and removal of the supernatant, the pellet was washed with EtOH (5 mL, brief sonication, centrifugation, removal of supernatant), and then dried under high vacuum for 10 min. The pellet was dissolved in water (0.18 mL) and subjected to preparative HPLC (microprep-HPLC, 10-70% method). Fractions containing product were pooled and lyophilized. Material was transferred to an Eppendorf tube as solution in water (2x 0.5 mL, 1x 0.3 mL water). After lyophilization, the residue was dissolved in water (2x 50 µL) and desalted by spin-column chromatography (illustra MicroSpin G-25, GE healthcare) as per the manufacturer's instructions. DNA concentration of the

eluant was determined photospectrometrically (Nanodrop One): 4.988 g/L (1 mM, calculated with MW = 5000 Da, volume by pipette: V = 113 µL, 113 nmol, 57% yield).

MS (ESI-) expected for $C_{176}H_{243}F_3N_{53}O_{107}P_{17}SSi$ [M] 5454 Da, found 5455 Da.



d. Synthesis of DNA-conjugate 11

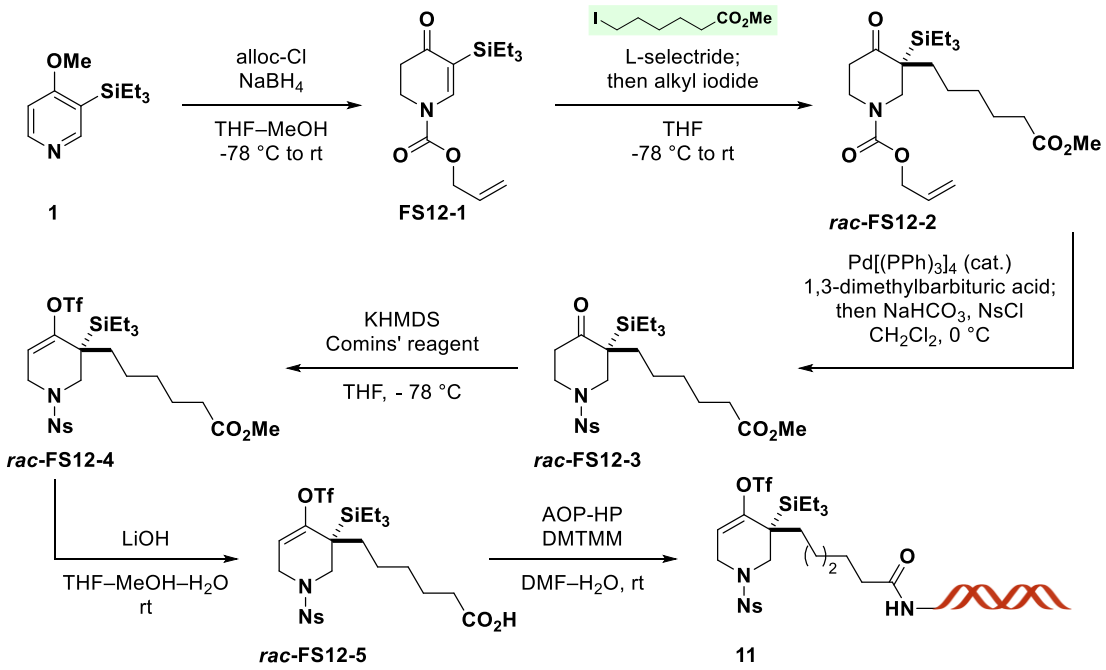
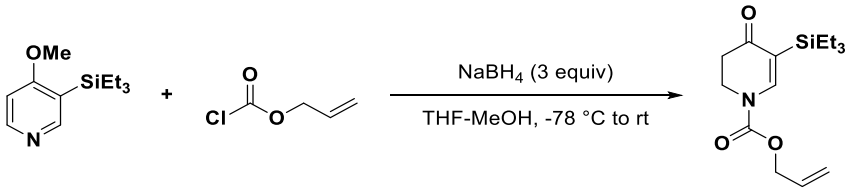


Figure S12 Synthetic route towards on-DNA strained allene precursor **11**.

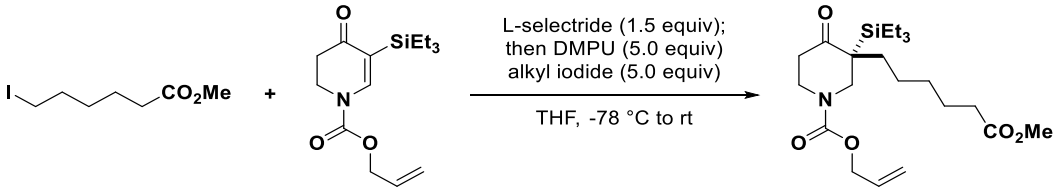
Synthesis of FS12-1



1 (5.1 g, 22.8 mmol, 1.0 equiv) was dissolved in MeOH (60 mL) and cooled to –78 °C. Sodium borohydride (2.59 g, 68.5 mmol) was added. A solution of allyl chloroformate (6.88 g, 57.1 mmol, 2.5 equiv) in THF (15 mL) was added via syringe over 10 min. Upon completion of the addition, the mixture had turned into a colorless suspension. The cooling bath was removed and it was stirred for another 20 min. Aq. HCl (10%, 50 mL) was added. After stirring for 5 min, the mixture was diluted with water (300 mL) and extracted with EtOAc (3x 50 mL). The combined organic extracts were washed with brine (100 mL), dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (5% EtOAc in heptane to 20%) afforded **FS12-1** (6.15 g, 20.8 mmol, 91% yield) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.77 (s, 1H), 5.95 (ddt, *J* = 16.5, 11.0, 5.7 Hz, 1H), 5.39 - 5.26 (m, 2H), 4.74 - 4.68 (m, 2H), 4.01 - 3.95 (m, 2H), 2.55 - 2.48 (m, 2H), 0.88 (t, *J* = 7.9 Hz, 9H), 0.67 (q, *J* = 8.1 Hz, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 196.7, 152.8 (br), 148.1 (br), 131.6, 119.1, 113.3, 67.8, 42.6, 36.2, 7.5, 3.0. **LRMS** (ESI+) req. for $C_{15}H_{26}NO_3Si$ $[M+H]^+$ 296.2, found 297.2.

Synthesis of *rac*-FS12-2

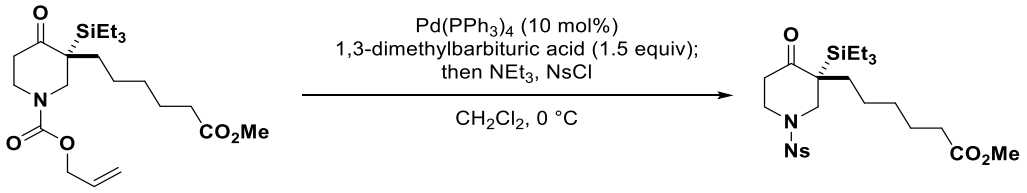


FS12-1 (1.0 g, 3.38 mmol, 1.0 equiv) was dissolved in THF (10 mL) and cooled to –78 °C. A solution of L-selectride in THF (1.0 M, 5.1 mL, 5.1 mmol, 1.5 equiv) was added dropwise over 5 min. The resulting slightly yellow solution was stirred for 1 h (UPLC-MS indicated full consumption of starting material). DMPU (2.0 mL, 16.9 mmol, 5.0 equiv) was added, followed by methyl 6-iodohexanoate (4.33 g, 16.9 mmol, 5.0 equiv). The resulting solution was stirred overnight under exclusion of light (thereby allowed to reach room temperature). The yellow solution was diluted with water (150 mL) and extracted with EtOAc (3x 30 mL). The combined organic extracts were washed with brine, dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 30%) afforded contaminated material. A second flash chromatography on silica (5% EtOAc in heptane to 30%) afforded ***rac*-FS12-2** (338 mg, 0.79 mmol, 23% yield) as colorless oil.

An analogous reaction employing **FS12-1** (2.6 g, 8.8 mmol, 1.0 equiv), L-selectride (1 M in THF, 13.2 mmol, 13.2 mmol, 1.5 equiv) and alkyl iodide (6.3 g, 24.6 mmol, 2.8 equiv) afforded ***rac*-FS12-2** (605 mg 1.42 mmol, 16% yield).

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ = 5.83 (ddt, *J* = 17.3, 10.5, 5.7 Hz, 1H), 5.15 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.03 (dq, *J* = 10.4, 1.4 Hz, 1H), 4.53 (dt, *J* = 5.7, 1.4 Hz, 2H), 3.77 (dd, *J* = 14.1, 0.9 Hz, 1H), 3.71 – 3.53 (m, 2H), 3.37 (s, 3H), 3.20 (ddd, *J* = 12.7, 10.5, 5.6 Hz, 1H), 2.24 (ddd, *J* = 15.5, 10.5, 7.3 Hz, 1H), 2.14 – 1.97 (m, 5H), 1.52 (p, *J* = 7.3 Hz, 2H), 1.43 – 1.30 (m, 1H), 1.30 – 1.09 (m, 5H), 0.92 (t, *J* = 7.9 Hz, 9H), 0.70 – 0.53 (m, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C) δ = 209.6, 173.1, 155.2, 134.1, 117.4, 66.3, 50.8, 50.5, 46.9, 42.3, 39.9, 34.2, 34.1, 30.5, 25.7, 25.2, 8.0, 3.2. **HRMS** (ESI+) req. for $C_{22}H_{40}NO_5Si$ $[M+H]^+$ 426.2670, found 426.2700; req. for $C_{22}H_{39}NNaO_5Si$ $[M+Na]^+$ 448.2490, found 448.2512.

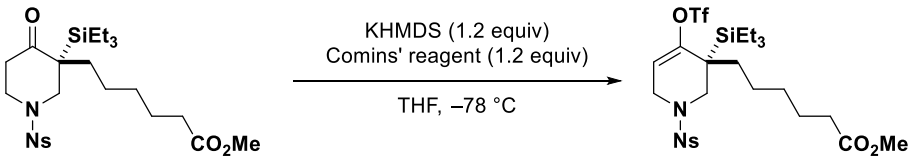
Synthesis of *rac*-FS12-3



***rac*-FS12-2** (421 mg, 0.99 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. 1,3-Dimethylbarbituric acid (232 mg, 1.48 mmol, 1.5 equiv) was added followed by $Pd(Ph_3P)_4$ (114 mg, 0.1 mmol, 10 mol%). After 40 min, nosyl chloride (438 mg, 1.98 mmol, 2.0 equiv) was added, immediately followed by triethylamine (414 µL, 2.97 mmol, 3 equiv). The resulting yellow solution was stirred for 15 min, before it was quenched by transferring the solution into a separation funnel filled with sat. aq. $NaHCO_3$ (50 mL), quantitating the transfer with additional CH_2Cl_2. Phases were separated and the aq. phase was extracted once with CH_2Cl_2 (20 mL). The combined organic extracts were dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (A: heptane/CH_2Cl_2 8:2, B: EtOAc, 5% B to 40%) afforded ***rac*-FS12-3** (260 mg, 0.49 mmol, 50% yield) as yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.94 (dd, *J* = 7.3, 2.1 Hz, 1H), 7.78 - 7.68 (m, 2H), 7.62 (dd, *J* = 7.2, 2.1 Hz, 1H), 3.87 (dd, *J* = 12.8, 1.8 Hz, 1H), 3.80 (dtd, *J* = 8.9, 4.9, 2.5 Hz, 1H), 3.63 (s, 3H), 3.35 (d, *J* = 12.9 Hz, 1H), 3.22 (td, *J* = 11.3, 5.1 Hz, 1H), 2.62 (ddd, *J* = 16.3, 10.8, 7.2 Hz, 1H), 2.46 (dt, *J* = 16.3, 4.7 Hz, 1H), 2.24 (t, *J* = 7.5 Hz, 2H), 2.10 - 1.98 (m, 1H), 1.54 (p, *J* = 7.3 Hz, 2H), 1.47 - 1.34 (m, 1H), 1.31 - 1.16 (m, 4H), 0.97 (t, *J* = 7.9 Hz, 9H), 0.79 - 0.68 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 208.7, 174.2, 148.8, 134.2, 131.7, 131.2, 130.5, 124.3, 51.5, 50.3, 49.7, 44.5, 39.8, 34.0, 32.7, 29.9, 25.2, 24.7, 8.0, 2.7. **HRMS** (ESI+) req. for $C_{24}H_{38}N_2O_7SSi$ $[M+H]^+$ 527.2242, found 527.2274.

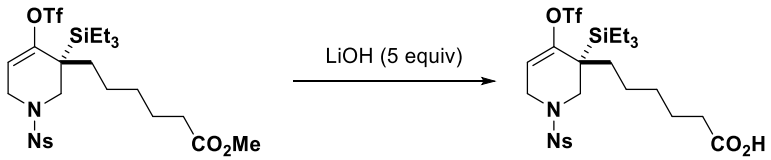
Synthesis of *rac*-FS12-4



***rac*-FS12-3** (83 mg, 0.16 mmol, 1.0 equiv) was dissolved in THF (1.5 mL) and cooled to –78 °C. A solution of KHMDS in THF (1 M, 0.19 mL, 0.19 mmol, 1.2 equiv) was added dropwise. 10 min after completion of the addition, a solution of Comins' reagent in THF (1 M, 0.19 mL, 0.19 mmol) was added dropwise. After 10 min, the reaction was quenched by the addition of sat. aq. NH_4Cl (10 mL). Extraction with EtOAc (3x 15 mL). The combined organic extracts were washed with brine, dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (20% EtOAc in heptane to 45%) afforded product contaminated with baseline material. A second chromatography on silica (solvent A: heptane/CH_2Cl_2 (8:2), solvent B: EtOAc, 5% B to 25%) afforded ***rac*-FS12-4** (40 mg, 0.061 mmol, 39% yield) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.95 (dd, *J* = 7.2, 2.0 Hz, 1H), 7.79 - 7.69 (m, 2H), 7.63 (dd, *J* = 7.3, 2.0 Hz, 1H), 5.71 (dd, *J* = 4.6, 2.7 Hz, 1H), 4.07 (dd, *J* = 15.9, 4.6 Hz, 1H), 3.72 - 3.61 (m, 2H), 3.66 (s, 3H), 3.27 (d, *J* = 12.7 Hz, 1H), 2.29 (t, *J* = 7.5 Hz, 2H), 1.74 - 1.56 (m, 4H), 1.50 - 1.34 (m, 2H), 1.34 - 1.21 (m, 2H), 1.01 (t, *J* = 8.0 Hz, 9H), 0.77 - 0.69 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 174.3, 153.5, 148.9, 134.3, 131.7, 131.3, 130.3, 124.4, 118.4 (q, *J* = 319.7 Hz), 108.6, 51.6, 50.3, 43.9, 35.9, 34.1, 32.8, 29.9, 24.8, 8.0, 2.9. **^{19}F NMR** (376 MHz, $CDCl_3$) δ = -74.6. **HRMS** (ESI+) req. for $C_{25}H_{38}F_3N_2O_9S_2Si$ $[M+H]^+$ 659.1735, found 659.1595.

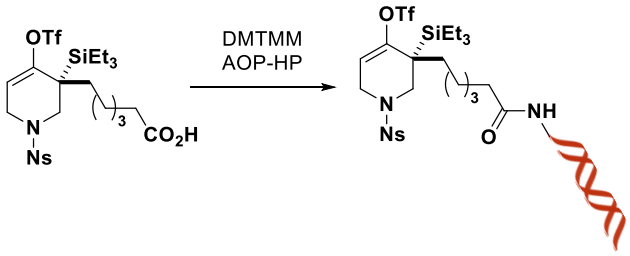
Synthesis of *rac*-FS12-5



***rac*-FS12-4** (10 mg, 0.015 mmol, 1.0 equiv) was dissolved in a mixture of MeOH (0.25 mL) and THF (0.10 mL). A solution of lithium hydroxide monohydrate in water (1 M) (0.076 mL, 0.076 mmol, 5.0 equiv) was added and the resulting mixture was stirred at room temperature for 90 min. Following dilution with EtOAc (50 mL), the mixture was washed with dilute aq. HCl (1 M, 10 mL) and brine (10 mL), dried with $MgSO_4$, filtered and concentrated. Flash chromatography on silica (non-automated, pipette column, heptane:CH_2Cl_2:EtOAc 5.5:2:2.5) afforded contaminated material. A second flash chromatography on silica (non-automated, pipette column, heptane:CH_2Cl_2:EtOAc 5.5:2.5:2 + 0.1% AcOH) afforded ***rac*-FS12-5** as colorless oil (1.7 mg, 0.00264 mmol, 17% yield).

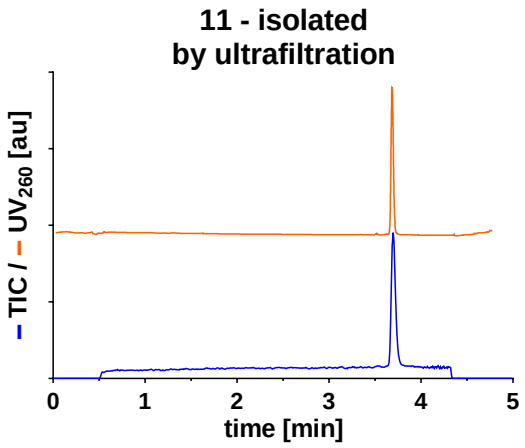
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.95 (dd, *J* = 7.2, 2.0 Hz, 1H), 7.82 - 7.67 (m, 2H), 7.63 (dd, *J* = 7.3, 2.0 Hz, 1H), 5.71 (dd, *J* = 4.6, 2.7 Hz, 1H), 4.08 (dd, *J* = 15.6, 4.9 Hz, 1H), 3.70 (d, *J* = 12.7 Hz, 1H), 3.64 (dd, *J* = 16.1, 2.8 Hz, 1H), 3.27 (d, *J* = 12.8 Hz, 1H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.71 - 1.58 (m, 4H), 1.49 - 1.36 (m, 2H), 1.35 - 1.25 (m, 2H), 1.01 (t, *J* = 8.0 Hz, 9H), 0.78 - 0.69 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 177.2, 153.5, 148.9, 134.3, 131.7, 131.3, 130.3, 124.4, 108.7, 50.3, 43.9, 35.9, 33.5, 32.8, 29.8, 24.8, 24.5, 8.0, 2.8. **^{19}F NMR** (376 MHz, $CDCl_3$) δ = -74.6. **HRMS** (ESI+) req. for $C_{24}H_{36}F_3N_2O_9S_2Si$ [M+H]+ 645.1578, found 645.1636.

Synthesis of 11

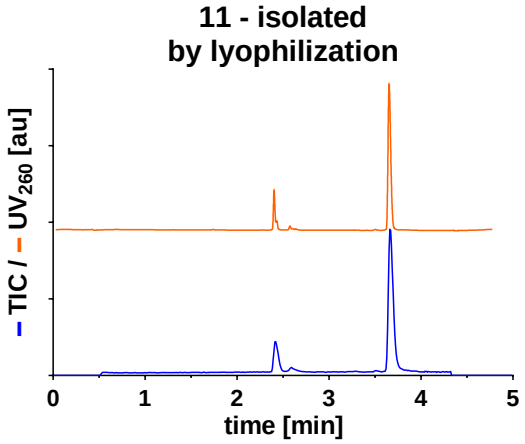


AOP-HP (2 mM in phosphate buffer pH8.0, 250 µL, 0.5 µmol) was combined with a solution of ***rac*-FS12-5** in DMF (10 mM, 300 µL, 3.00 µmol, 6 equiv). A solution of DMTMM in water (200 mM, 4.4 µL, 1.75 µmol, 3.5 equiv) was added. The mixture was briefly vortexed, spun down and incubated at room temperature for 3 h. UPLC-MS analysis indicated substantial amounts of residual AOP-HP. Another portion of DMTMM (4.5 µL, 1.8 µmol, 3.6 equiv) was added. The mixture was vortexed and incubated at for 4 h at room temperature. To remove potential DMT-adduct, 10% aq. piperidine (0.6 mL) was added. After 2 min, DNA was precipitated by the addition of aq. NaCl (5 M, 120 µL) and EtOH (4 mL). The cloudy mixture was vortexed and incubated on dry ice for 15 min. After centrifugation (3700 RCF, 5 min), the supernatant was decanted. The pellet was washed with additional EtOH (1 mL). The residue was dissolved in water (0.5 mL) and subjected to preparative HPLC (microprep-HPLC, 10-70% method, collecting into vials precharged with 100 mM phosphate buffer pH7). Fractions containing product were pooled and concentrated by ultrafiltration at 5 °C (Amicon® Ultra-4, 3000 Da nominal molecular weight limit, part #UFC800308 as per manufacturer's instruction). Upon completion, water (3.5 mL) was added and the filtration step was repeated to afford **11** as an aqueous solution, which was transferred to an Eppendorf tube. DNA concentration was determined photospectrometrically (Nanodrop One): 3.001 g/L (0.6 mM, calculated with MW = 5000 Da, volume by pipette: V = 200 µL, 120 nmol, 24% yield). The material (20 µL aliquots) was kept at -20 °C.

MS (ESI-) expected 5808 Da, found 5811 Da.

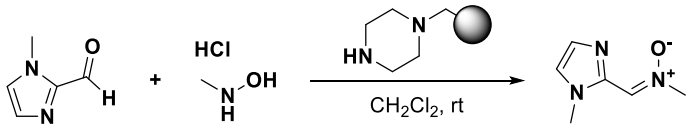


Note: Concentration of HPLC fractions by lyophilization would result in formation of impurities, amounting to 16% AUC in the example shown below (m/z of new species 5648-5650 Da).



e. Synthesis of 1,3-dipoles

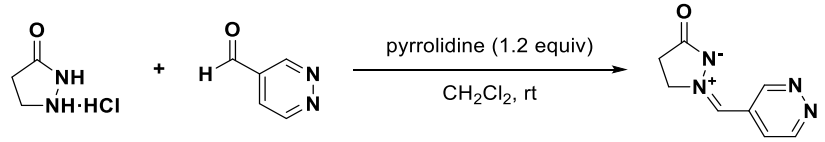
Synthesis of *N*-methyl-1-(1-methyl-1H-imidazol-2-yl)methanimine oxide



N-methylhydroxylamine hydrochloride (36 mg, 0.43 mmol, 1.0 equiv) was combined with 1-methyl-2-imidazolecarboxaldehyde (53 mg, 0.48 mmol, 1.1 equiv). CH_2Cl_2 (2.4 mL) and resin-bound piperazine (MP-piperazine from SupraSciences, loading 3 mmol/g, 160 mg) was added. The mixture was stirred overnight, filtered and concentrated. Flash chromatography on silica (5–25% MeOH in CH_2Cl_2, 0.1% NEt_3) afforded the product contaminated with triethylammonium hydrochloride. The material was partitioned between sat. aq. sodium bicarbonate and CH_2Cl_2. The phases were separated and the aq. phase was extracted with CH_2Cl_2:CF_3CH_2OH (4:1). The combined organic extracts were dried with $MgSO_4$, filtered and concentrated to afford the title compound (26 mg, 0.19 mmol, 43% yield) as a mixture of geometric isomers (ratio ca. 4:1).

Major isomer: **^{1}H NMR** (400 MHz, *d6*-DMSO) δ = 7.90 (s, 1H), 7.26 (s, 1H), 7.04 (s, 1H), 3.79 (s, 3H), 3.70 (s, 3H). **^{13}C NMR** (101 MHz, *d6*-DMSO) δ = 138.9, 128.9, 125.2, 123.1, 53.3. Minor isomer: **^{1}H NMR** (400 MHz, *d6*-DMSO) δ = 7.85 (s, 1H), 7.31 (s, 1H), 7.11 (s, 1H), 4.10 (s, 3H), 3.69 (s, 3H). **^{13}C NMR** (101 MHz, *d6*-DMSO) δ = 138.6, 129.0, 124.2, 123.5, 50.3, 32.6.

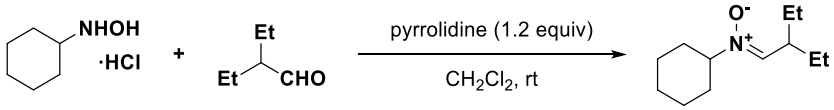
Synthesis of 5-oxo-2-(pyridazin-4-ylmethylene)pyrazolidin-2-ium-1-ide



According to a literature procedure,[6] 3-pyrazolidinone hydrochloride (22 mg, 0.18 mmol, 1.0 equiv) was combined with pyridazine-4-carbaldehyde (20 mg, 0.19 mmol, 1.05 equiv) and a solution of pyrrolidine in CH_2Cl_2 (0.24 M, 920 µL, ca. 1.2 equiv) was added. The cloudy mixture was centrifuged and the supernatant was removed. The pellet was washed with methanol (1 mL) and dried under high vacuum to afford the title compound as light-yellow solid (8 mg, 0.045 mmol, 25% yield) as a mixture of geometric isomers (ratio ca. 10:1).

^{1}H NMR (400 MHz, *d6*-DMSO) δ = 9.64 (t, *J* = 1.9 Hz, 1H), 9.41 (dd, *J* = 5.4, 1.3 Hz, 1H), 8.56 (dd, *J* = 5.6, 2.3 Hz, 1H), 7.69 (d, *J* = 1.9 Hz, 1H), 4.74 – 4.62 (m, 2H), 3.32 (s, 4H), 2.71 – 2.59 (m, 2H). **^{13}C NMR** (101 MHz, *d6*-DMSO) δ = 186.3, 153.1, 151.0, 128.5, 126.2, 125.5, 59.7, 29.8.

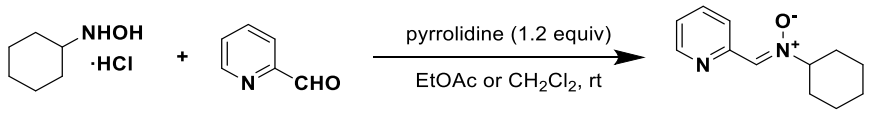
Synthesis of *N*-cyclohexyl-2-ethylbutan-1-imine oxide



According to a literature procedure,[6] *N*-cyclohexylhydroxylamine hydrochloride (100 mg, 0.66 mmol, 1.0 equiv) and 2-ethylbutanal (66 mg, 0.66 mmol, 1.0 equiv) were combined with CH_2Cl_2 (3.3 mL). Pyrrolidine (65 µL, 0.79 mmol, 1.2 equiv) was added and the resulting light yellow solution was stirred for 5 min at room temperature, before it was loaded onto a silica column preequilibrated with CH_2Cl_2. Elution (50% EtOAc in heptane to 100%), pooling of fractions containing a UV active species (R_f(EtOAc) = 0.24) and concentration afforded the title compound (114 mg, 0.58 mmol, 88% yield) as colorless solid.

^{1}H NMR (400 MHz, *d6*-DMSO) δ = 6.80 (d, *J* = 7.7 Hz, 1H), 3.83 – 3.72 (m, 1H), 2.71 (qt, *J* = 7.9, 5.5 Hz, 1H), 1.74 (ddt, *J* = 10.0, 7.2, 3.8 Hz, 6H), 1.59 (dt, *J* = 13.3, 3.6 Hz, 1H), 1.45 (dqd, *J* = 13.5, 7.7, 5.8 Hz, 2H), 1.39 – 1.20 (m, 4H), 1.10 (qt, *J* = 13.0, 3.3 Hz, 1H), 0.81 (t, *J* = 7.5 Hz, 6H). **^{13}C NMR** (101 MHz, *d6*-DMSO) δ = 138.8, 72.2, 38.1, 30.7, 24.7, 24.4, 24.0, 11.5.

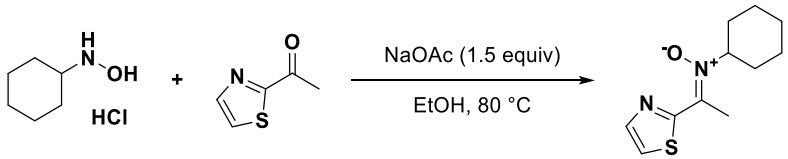
Synthesis of *N*-cyclohexyl-1-(pyridin-2-yl)methanimine oxide



According to a literature procedure,[6] *N*-cyclohexylhydroxylamine hydrochloride (152 mg, 1.0 mmol, 1.0 equiv) was suspended in CH_2Cl_2 (3 mL) and 2-pyridinecarboxaldehyde (96 µL, 1.0 mmol, 1.0 equiv) was added. Pyrrolidine (99 µL, 1.2 mmol, 1.2 equiv) was added (exotherm) and the resulting mixture was stirred at room temperature for 10 min. The solution was directly loaded onto a silica column (glass pipette). Material was eluted with EtOAc (2 mL fractions). Fractions 1-4 were pooled and concentrated to afford the title compound (202 mg, 0.99 mmol, 99% yield) as colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.15 (d, *J* = 8.3 Hz, 1H), 8.62 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.80 – 7.73 (m, 2H), 7.29 – 7.23 (m, 1H), 3.90 (tt, *J* = 11.5, 3.8 Hz, 1H), 2.17 – 2.07 (m, 2H), 2.01 – 1.88 (m, 4H), 1.75 – 1.67 (m, 1H), 1.39 (qt, *J* = 13.2, 3.4 Hz, 2H), 1.30 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 150.0, 149.6, 136.9, 133.8, 124.2, 123.9, 76.3, 31.3, 25.2, 25.1. **HRMS** (ESI+) req. for $C_{12}H_{17}N_2O$ $[M+H]^+$ 215.1335, found 205.1351.

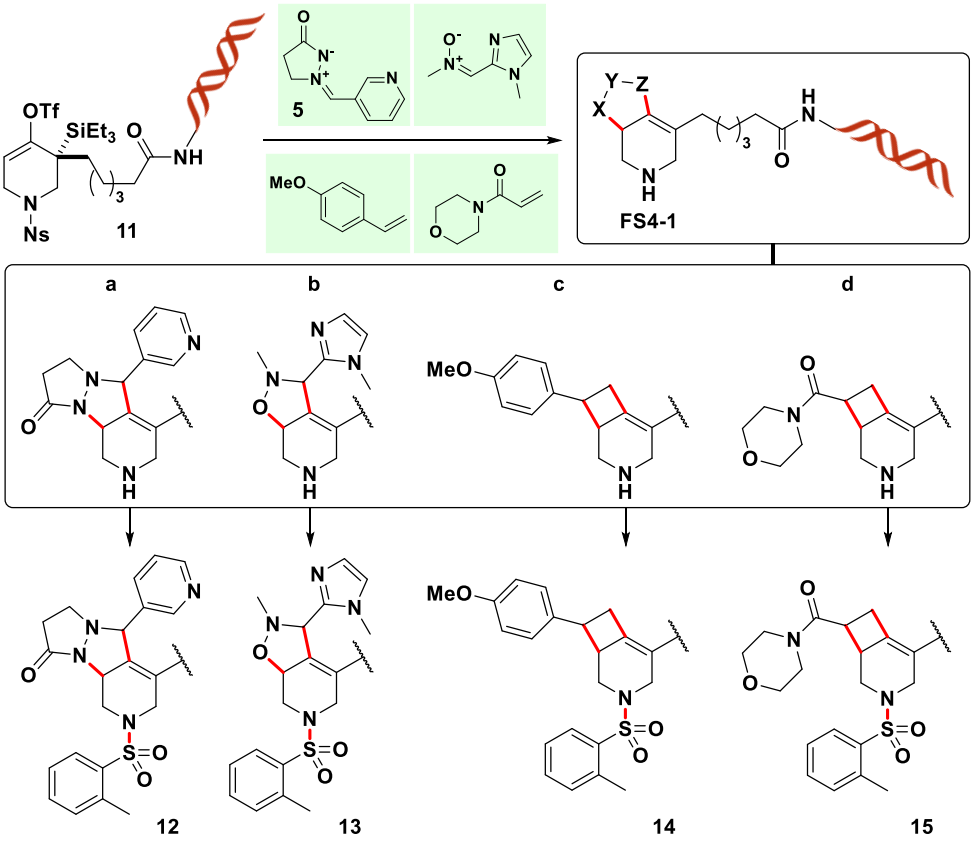
Synthesis of *N*-cyclohexyl-1-(thiazol-2-yl)ethan-1-imine oxide



N-cyclohexylhydroxylamine hydrochloride (114 mg, 0.75 mmol, 1.5 equiv) and sodium acetate (62 mg, 0.75 mmol, 1.5 equiv) were combined with a solution of 2-acetylthiazole in ethanol (0.2 M, 0.25 mL, 0.5 mmol, 1.0 equiv). The mixture was stirred at 80 °C for 38 h. After cooling to room temperature, the mixture was filtered (syringe filter, PTFE, 0.2 µm) and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 30%) afforded the title compound (31 mg, 0.14 mmol, 28% yield) as colorless oil. The *Z* geometry was established in a NOESY experiment, showing strong correlation between the CH and CH_3 groups.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.02 (d, *J* = 3.2 Hz, 1H), 7.42 (d, *J* = 3.1 Hz, 1H), 4.34 (tt, *J* = 11.3, 3.7 Hz, 1H), 2.78 (s, 3H), 2.15 - 2.02 (m, 2H), 1.98 - 1.85 (m, 4H), 1.76 - 1.67 (m, 1H), 1.45 - 1.30 (m, 2H), 1.30 - 1.20 (m, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 158.1, 143.1, 136.9, 121.5, 67.7, 30.1, 25.1, 25.1, 15.1. **LRMS** (ESI+) req. for $C_{11}H_{17}N_2OS$ $[M+H]^+$ 225.1, found 224.9.

f. Two-step diversification of 11



Step1: In four individual polypropylene tubes (Eppendorf), **11** (0.5 mM in water, 10 µL, 5 nmol) was combined with a solution of the corresponding cycloaddition partner (azomethine imine **5**, *N*-methyl-1-(1-methyl-1H-imidazol-2-yl)methanimine oxide (see above), 4-methoxystyrene, 4-acryloylmorpholine) in DMSO (200 mM, 11 µL, 2.2 µmol, 440 equiv). DMSO (66 µL) was added to adjust the final concentration of building blocks to 25 mM. The mixture was vortexed and spun down, before a solution of cesium fluoride (2 M in 100 mM bicarbonate buffer pH10, 1 µL, ca. 22 mM final concentration) was added. The solution was vortexed again and incubated at room temperature for 1 h. Bicarbonate buffer (100 mM, pH10, 50 µL) and 4-methoxythiophenol in DMSO (1250 mM, 10 µL) were added. The solution was vortexed, spun down and incubated at 80 °C for 70 min. DNA was then precipitated according to **GP2** to afford intermediate piperidines **FS8-1a–d** (also see **Figure S8**). Pellets were dissolved in water (10 µL) and DNA content was quantified photospectrometrically (Nanodrop One, calculated with MW = 5000 g/mol). Product display (%AUC) was determined by UPLC-MS (see **Figure S8**).

FS8-1a: >95%AUC (2.085 g/L, 0.42 mM, 4.2 nmol, 84% recovered DNA)
FS8-1b: 95%AUC (1.655 g/L, 0.33 mM, 3.3 nmol, 66% recovered DNA)
FS8-1c: 91%AUC (1.943 g/L, 0.39 mM, 3.9 nmol, 78% recovered DNA)
FS8-1d: 92%AUC (1.923 g/L, 0.38 mM, 3.8 nmol, 76% recovered DNA)

Step 2: In four individual wells (96-well polypropylene plate), solutions of **FS8-1a–d** (1 µL of on-DNA piperidines, 0.3–0.4 nmol) were combined with phosphate buffer (100 mM, pH8, 3 µL) and a solution of 2-methylbenzenesulfonyl chloride in acetonitrile (200 mM, 1 µL, 40 mM final concentration). The plate was sealed, vortexed, spun down and incubated overnight at room temperature. DNA was then precipitated according to **GP1** to afford final DNA-conjugates **12**–**15**. Pellets were dissolved in water (25 µL) and product display (%AUC) was determined by UPLC-MS (see **Figure 4, B**).

12: 96%AUC (MS req. m/z 5688 Da, found 5689 Da)
13: 92%AUC (MS req. m/z 5652 Da, found 5653 Da)
14: 79%AUC (MS req. m/z 5647 Da, found 5649 Da)
15: 88%AUC (MS req. m/z 5654 Da, found 5655 Da)

g. Preliminary results towards a pilot library

Fmoc-protected allene precursor (**FS13-3**) was synthesized and conjugated to DNA (AOP-HP) as depicted in Figure S13. The DNA-conjugate (**FS13-4**) allows for mild deprotection following strain-promoted cycloadditions. In preliminary studies with this substrate, the protecting group was partially or fully removed due to the basic conditions of the transformation (cesium fluoride). We found it convenient to add aq. piperidine at the end of the strain-promoted cycloaddition reaction to ensure quantitative Fmoc removal.

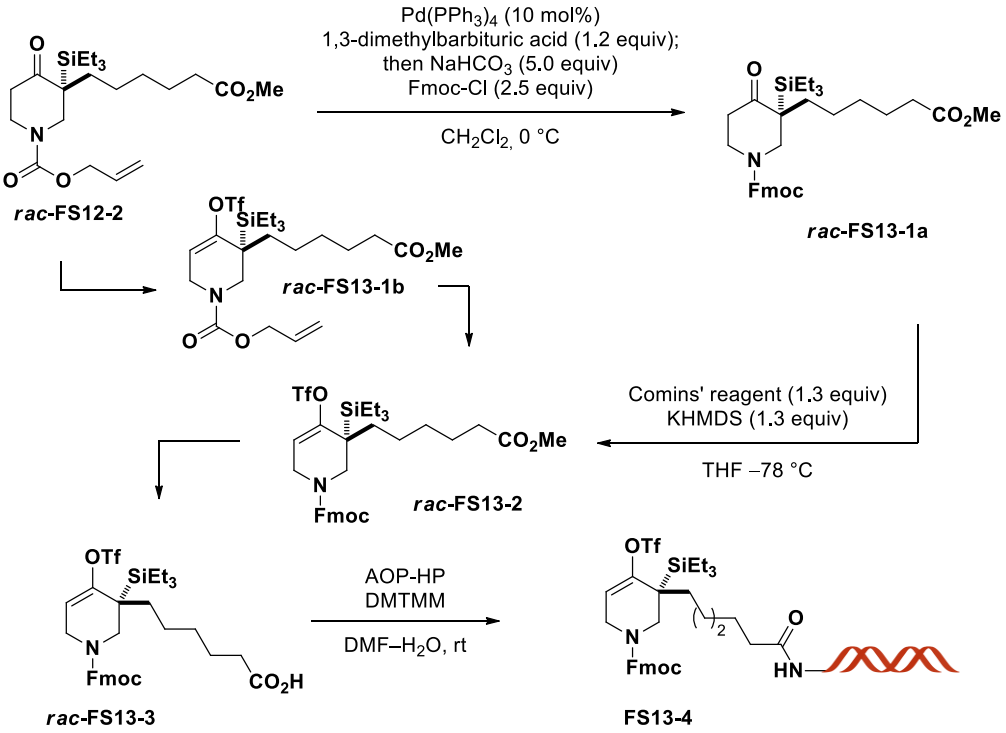
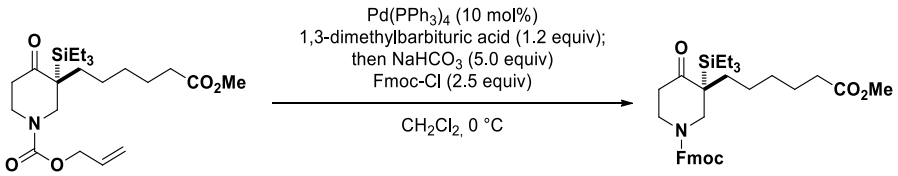


Figure S13 Synthetic route towards Fmoc-protected on-DNA strained allene precursor **FS13-4**.

Synthesis of *rac*-FS13-1a

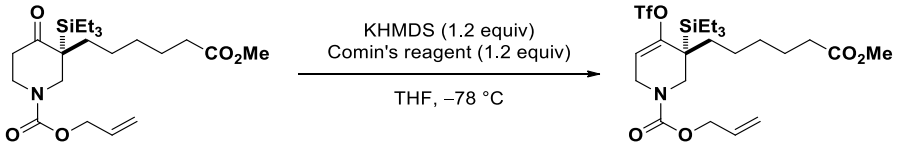


***rac*-FS12-2** (208 mg, 0.49 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (0.7 mL) and the resulting solution was cooled to 0 °C. A solution of 1,3-dimethylbarbituric acid (92 mg, 0.59 mmol, 1.2 equiv) and $Pd(PPh_3)_4$ (57 mg, 0.05 mmol, 0.1 equiv) in CH_2Cl_2 (0.7 mL) was added. The reaction mixture was stirred for 5 min before sodium bicarbonate (205 mg, 2.44 mmol, 5.0 equiv) was added, followed by Fmoc-Cl (316 mg, 1.22 mmol, 2.5 equiv). The suspension was stirred at 0 °C for 15 min. The reaction mixture was partitioned between a saturated aqueous solution of $NaHCO_3$ and CH_2Cl_2. The phases were separated and the organic

layer was washed with brine, dried over $MgSO_4$, filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 30%) afforded ***rac*-FS13-1a** (229 mg, 0.41 mmol, 83% yield) as colorless oil.

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ = 7.61 – 7.54 (m, 2H), 7.49 – 7.42 (m, 2H), 7.25 – 7.19 (m, 2H), 7.19 – 7.14 (m, 2H), 4.54 – 4.42 (m, 2H), 4.02 (t, *J* = 5.9 Hz, 1H), 3.68 – 3.45 (m, 3H), 3.38 (s, 3H), 3.18 (ddd, *J* = 12.6, 10.5, 5.6 Hz, 1H), 2.19 (ddd, *J* = 15.3, 10.6, 7.2 Hz, 1H), 2.12 – 1.92 (m, 3H), 1.51 (p, *J* = 7.4 Hz, 2H), 1.40 – 1.29 (m, 1H), 1.29 – 1.07 (m, 5H), 0.92 (t, *J* = 7.9 Hz, 9H), 0.68 – 0.50 (m, *J* = 7.4 Hz, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C, 2 rotamers) δ = 209.8, 173.3, 155.5, 145.2, 145.1, 142.3, 128.2, 127.6(3), 127.6(1), 125.4, 125.3, 120.5, 67.6, 51.0, 50.4, 48.5, 47.1, 42.5, 40.0, 34.4, 34.3, 30.6, 25.9, 25.3, 8.3, 3.3. **HRMS** (ESI+) req. for $C_{33}H_{46}NO_5Si$ $[M+H]^+$ 563.3067, found 564.3177; req. for $C_{33}H_{45}NNaO_5Si$ $[M+Na]^+$ 586.2965, found 586.2988.

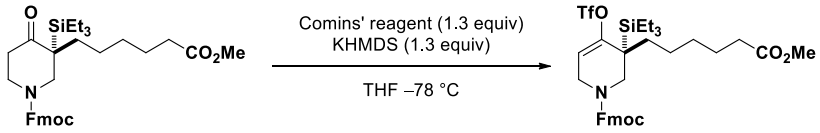
Synthesis of *rac*-FS13-1b



***rac*-FS12-2** (327 mg, 0.77 mmol, 1.0 equiv) was dissolved in THF (3 mL) and cooled to –78 °C. A solution of KHMDS in 2-methyltetrahydrofuran (1.0 M, 0.92 mL, 0.92 mmol, 1.2 equiv) was added dropwise over 5 min and the resulting yellow solution was stirred for another 15 min. A solution of Comins' reagent in THF (1 M, 0.92 mL, 0.92 mmol, 1.2 equiv) was added dropwise over 5 min and the resulting solution was stirred for another 15 min. The reaction was quenched by pouring it into a separation funnel preloaded with a saturated aqueous solution of NH_4Cl (100 mL). The organic material was extracted with CH_2Cl_2 (3 x 25 mL). The combined organic extracts were washed with brine, dried with $MgSO_4$, filtered and concentrated. Flash chromatorgraphy on silica (0% EtOAc in heptane to 25%) afforded ***rac*-FS13-1b** (322 mg, 0.58 mmol, 75% yield) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.92 (ddt, *J* = 16.3, 10.7, 5.6 Hz, 1H), 5.68 (s, 1H), 5.26 (dd, *J* = 29.6, 13.9 Hz, 2H), 4.60 (d, *J* = 5.5 Hz, 2H), 4.27 (dd, *J* = 18.0, 4.3 Hz, 1H), 3.95 – 3.73 (m, 2H), 3.65 (s, 3H), 3.49 – 3.30 (m, 1H), 2.27 (t, *J* = 7.6 Hz, 2H), 1.58 (qd, *J* = 7.7, 4.1 Hz, 4H), 1.35 – 1.21 (m, 4H), 0.99 (t, *J* = 7.9 Hz, 9H), 0.73 – 0.62 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 174.2, 154.9, 153.3, 153.0, 132.9, 118.4 (q, *J* = 319.5 Hz), 118.3, 117.9, 110.4, 110.2, 66.5, 51.5, 47.4, 42.4, 42.1, 35.4, 34.1, 32.7, 29.9, 24.8, 24.6, 24.4, 8.0, 2.8.

Synthesis of *rac*-FS13-2 (from *rac*-FS13-1a)

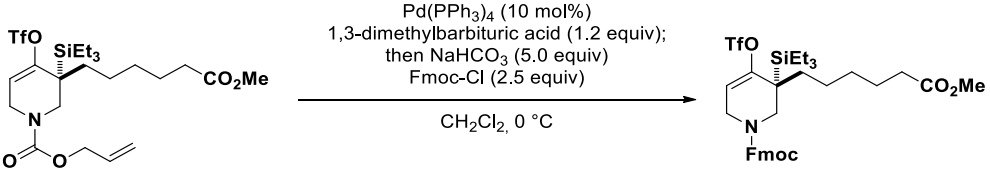


***rac*-FS13-1a** (30 mg, 0.05 mmol, 1.0 equiv) was dissolved in THF (0.5 mL) and the resulting solution was cooled to –78 °C. A solution of KHMDS (1 M in THF, 0.07 mL, 0.07 mmol, 1.3 equiv) was added over 1 min. After the addition, the reaction mixture was stirred at –78 °C for 5 min. A solution of Comins' reagent (25 mg, 0.06 mmol, 1.3 equiv) in THF (0.5 mL) was added and the reaction mixture was stirred at –78 °C for 1 h. A saturated aqueous solution of NH_4Cl was added and the mixture was extracted with EtOAc. The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 10%) afforded ***rac*-FS13-2** (10 mg, 0.01 mmol, 27% yield) as colorless oil.

^{1}H NMR (400 MHz, *d8*-PhMe, 80 °C) δ 7.58 (d, *J* = 7.4 Hz, 2H), 7.42 (d, *J* = 7.2 Hz, 2H), 7.26 – 7.14 (m, 4H), 5.44 (dd, *J* = 4.1, 2.9 Hz, 1H), 4.52 (dd, *J* = 10.8, 5.8 Hz, 1H), 4.42 (dd, *J* = 10.8, 5.9 Hz, 1H), 3.99 (t,

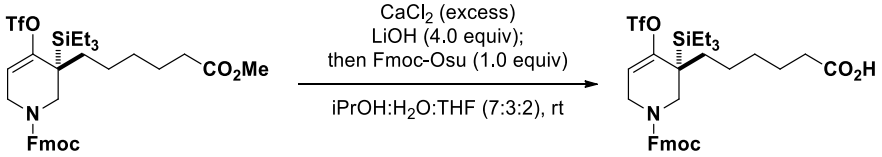
J = 5.9 Hz, 1H), 3.88 (dd, *J* = 18.2, 4.1 Hz, 1H), 3.58 (d, *J* = 13.5 Hz, 1H), 3.43 – 3.32 (m, 4H), 3.22 – 3.01 (m, 1H), 2.10 (t, *J* = 7.3 Hz, 2H), 1.69 – 1.40 (m, 4H), 1.40 – 1.13 (m, 4H), 0.94 (t, *J* = 7.9 Hz, 9H), 0.62 (q, *J* = 7.9 Hz, 6H). **^{13}C NMR** (101 MHz, *d8*-PhMe, 80 °C) δ = 173.3, 155.1, 153.9, 145.0, 142.3, 128.2, 127.7, 127.6, 125.4, 125.3, 120.6, 119.6 (q, *J* = 320.0 Hz), 110.9, 67.7, 51.0, 48.4, 48.2, 42.7, 36.3, 34.3, 33.4, 30.5, 25.4, 25.0, 8.2, 3.5. **HRMS** (ESI+) req. for $C_{34}H_{45}F_3NO_7SSi$ $[M+H]^+$ 696.2633, found 696.2692.

Synthesis of *rac*-FS13-2 (from *rac*-FS13-1b)



***rac*-FS13-1b** (200 mg, 0.36 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (0.9 mL) and cooled to 0 °C. A solution of $Pd(PPh_3)_4$ (41 mg, 0.04 mmol, 0.1 equiv) and 1,3-dimethylbarbituric acid (67 mg, 0.43 mmol, 1.2 equiv) in CH_2Cl_2 (0.9 mL) was then added and the resulting mixture was stirred for 3 min, before Fmoc-Cl (232 mg, 0.90 mmol, 2.5 equiv) and sodium bicarbonate (151 mg, 1.79 mmol, 5.0 equiv) were added. The heterogenous mixture was stirred for 20 min, then loaded directly onto a silica column (through a syringe filter, 0.2 µm), eluting with 0% EtOAc in heptane to 30%. Once the product was off the column (checked by TLC), the column was flushed with 10% MeOH in CH_2Cl_2. The flushing was combined with excess sodium bicarbonate and Fmoc-Cl, and stirred overnight. The mixture was filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 30%) afforded contaminated material, which was pooled with material containing the same contaminant from the first column. Another flash chromatography on silica (A: heptane:CH_2Cl_2 9:1, B: EtOAc, 0% B to 20%) afforded still contaminated material. Another flash chromatography on silica (A: heptane:CH_2Cl_2 1:1, B: heptane:CH_2Cl_2:EtOAc 1:1:0.05), 0% B to 100%) afforded ***rac*-FS13-2** (164 mg, 0.24 mmol, 66% yield). *Note*: the impurity, although more polar on TLC, eluted first under these conditions.

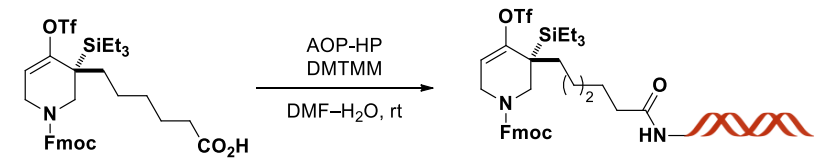
Synthesis of *rac*-FS13-3



***rac*-FS13-2** (120 mg, 0.17 mmol, 1.0 equiv) was dissolved in a freshly prepared solution of $CaCl_2$ (0.8 M in iPrOH:water, 7:3 *v*/*v*, 2.5 mL). THF (0.5 mL) was added, followed by LiOH (17 mg, 0.69 mmol, 4 equiv) and the resulting reaction mixture was stirred at room temperature for 2 d. UPLC-MS analysis showed that a fraction of the material was deprotected. Fmoc-Osu (58 mg, 0.17 mmol, 1.0 equiv) was added and the biphasic mixture was stirred at room temperature for 1 h. Water and a few drops of 5 M aqueous HCl were added. The suspension was extracted 3 times with EtOAc. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated. Flash chromatography on silica (0% EtOAc in heptane to 30%, +0.1% AcOH) afforded ***rac*-FS13-3** (34 mg, 0.05 mmol, 29% yield) as a colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.77 (d, *J* = 7.6 Hz, 2H), 7.56 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 5.73 – 5.61 (m, 1H), 4.73 – 4.35 (m, 2H), 4.35 – 4.12 (m, 2H), 3.94 – 3.77 (m, 1H), 3.71 – 2.92 (m, 2H), 2.29 (t, *J* = 7.6 Hz, 2H), 1.75 – 1.42 (m, 4H), 1.37 – 1.10 (m, 4H), 1.02 – 0.91 (m, 9H), 0.78 – 0.49 (m, 6H). **^{13}C HSQC** ($CDCl_3$) δ = 127.8, 127.2, 124.6, 120.1, 110.1, 65.7, 47.5, 47.4, 42.0, 33.8, 32.5, 29.5, 24.7, 7.8, 2.8. **HRMS** (ESI+) req. for $C_{33}H_{43}F_3NO_7SSi$ $[M+H]^+$ 682.2476, found 682.2518.

Synthesis of FS13-4



rac-FS13-3 FS13-4

FS13-4 was synthesized from ***rac*-FS13-3** as described for the preparation of **11** and isolated by preparative HPLC (74% yield by photospectrometry).

***rac*-FS13-3** (17 mM in DMF, 353 µL) was combined with DNA-AOP-HP (2.5 mM in phosphate buffer pH8.0, 80 µL, 200 nmol). DMTMM (400 mM in water, 10 µL, 4 µmol) was added. The mixture was vortexed, spun down and incubated on a shaker (30 °C, 1000 rpm) for 2 h. After EtOH precipitation (5 M NaCl, 44 µL, 1.4 mL EtOH, incubated on dry ice, removal of supernatant, pellet washed with 80% aq. EtOH (0.5 mL) and EtOH (0.5 mL), dried under vacuum) the pellet was transferred into an HPLC injection vial (3x 30 µL). Preparative HPLC (solvent A: trietylammonium acetate (50 mM) in water, B: MeOH, 5-95% B over 15 min, one injection) and concentration of the corresponding fractions by ultrafiltration (3k MW cutoff membrane) afforded **FS13-4** (6.316 g/L (photospectrometrically), 113 µL (by pipette), ca. 1.3 mM (calculated with MW = 5000 g/mol), 143 nmol, 74%). The solution was aliquoted (20 µL portions) and stored at -20 °C.

On-DNA cycloaddition reactions

Work towards a pilot library is currently in ongoing. This following section provides additional data obtained for a larger set of reagents. Cycloadditions were performed using **FS13-4** (3 M in water, 1 µL, 3 nmol) and commercially available building blocks as described above (90% aq. DMSO, 50 mM CsF, rt, 1 h). Usually, these conditions result in convenient loss of the protecting group. To ensure quantitative Fmoc-removal, reactions were treated with 10% aq. piperidine (1 volume) before ethanol-induced DNA-precipitation. Activated olefins and pyrroles were used as received and dissolved in DMSO (200 mM to give a final concentration of 180 mM). Dipoles were prepared in analogy to Figure S7 and used without purification (135 mM theoretical final concentration). The following table lists building blocks and dipoles that resulted in acceptable product formation (>75% AUC, corrected). Success rate: 56 out of 96 for commercial building blocks, 131 out of 384 for dipoles (preparation not optimized).

BUILDING BLOCK	ALDEHYDE FOR DIPOLE FORMATION (WHERE APPLICABLE)
DIETHYL VINYLPHOSPHONATE	
3-VINYLANISOLE	
2,5-DIMETHYLSTYRENE	
2-BROMOSTYRENE	
4-CHLOROSTYRENE	
METHYL-P-VINYLBENZOATE	
4-VINYL-IMIDAZOLE	
4-VINYLPHENOL	
4-VINYLBENZYL ACETATE	
(4-VINYLBENZYL)TRIMETHYLAMMONIUM CHLORIDE	
N,N-DIMETHYL-4-VINYLBENZYLAMINE	
4-AMINOSTYRENE	
3-NITROSTYRENE	
DIMETHYL VINYLPHOSPHONATE	
4-CYANOSTYRENE	
4-METHOXYSTYRENE	
2-VINYL-4,6-DIAMINO-1,3,5-TRIAZINE	
3-(TRIFLUOROMETHYL)STYRENE	
2-BROMO-6-(1H-PYRROL-1-YL)PYRIDINE	
P-SULFONAMIDO STYRENE	
3-BROMOSTYRENE	
3-(1H-PYRROL-1-YL)BENZALDEHYDE	
2-ISOPROPENYLANILINE	
1-PHENYLPYRROLE	
3-VINYLBENZALDEHYDE	
1-(2-AMINOPHENYL)PYRROLE	
4-PHENOXYSTYRENE	

4-METHYL-5-VINYLTHIAZOLE	
N-METHYLPYRROLE	
KETENE DIMETHYL ACETAL	
N-[TRIS(HYDROXYMETHYL)METHYL]ACRYLAMIDE	
2-HYDROXYETHL METHACRYLATE	
ALPHA-METHYLENE-GAMMA-BUTYROLACTONE	
TERT-BUTYL METHACRYLATE	
2(5H)-FURANONE	
METHACRYLAMIDE	
N-ISOPROPYLACRYLAMIDE	
N-[3-(DIMETHYLAMINO)PROPYL]ACRYLAMIDE	
N-BENZYLACRYLAMIDE	
2-ACETAMIDOACRYLIC ACID METHYL ESTER	
2-(DIMETHYLAMINO)ETHYL METHACRYLATE	
3-(1H-PYRROL-1-YL)ANILINE	
3-(DIMETHYLAMINO)PROPYL ACRYLATE	
2-BROMO-6-(1H-PYRROL-1-YL)PYRIDINE	
3-(1H-PYRROL-1-YL)BENZALDEHYDE	
N-ISOPROPYLACRYLAMIDE	
1-(4-NITROPHENYL)-1H-PYRROLE	
1-PHENYLPYRROLE	
4-BROMOSTYRENE	
(Z)-N-METHYL-1-(1-METHYL-1H-IMIDAZOL-2-YL)METHANIMINE OXIDE	
2-CHLOROSTYRENE	
4-NITROSTYRENE	
2-VINYLNAPHTHALENE	
3,5-DIMETHYL-4-VINYL-1H-PYRAZOLE	
N-VINYLACETAMIDE	
4-METHYLSTYRENE	
(Z)-N-CYCLOHEXYL-1-(PYRIDIN-2-YL)METHANIMINE OXIDE	
ALPHA-METHYLSTYRENE	
(Z)-5-OXO-2-(PYRIDIN-3-YLMETHYLENE)PYRAZOLIDIN-2-IUM-1-IDE	
1-VINYLIMIDAZOLE	
N-ETHYL ACRYLAMIDE	
4-ACRYLOYLMORPHOLINE	
N-ETHYLMETHACRYLAMIDE	
4-(1H-PYRROL-1-YL)ANILINE	
N-METHYLHYDROXYLAMINE	4-AMINOPYRIDINE-3-CARBALDEHYDE
N-ETHYLHYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-BENZYLHYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-METHYLHYDROXYLAMINE	3-METHYL-1H-PYRAZOLE-4-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	3-METHYL-1H-PYRAZOLE-4-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	4-METHYL-5-FORMYLTHIAZOLE
N-ETHYLHYDROXYLAMINE	4-METHYL-5-FORMYLTHIAZOLE
N-METHYLHYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
N-ETHYLHYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
N-BENZYLHYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	2-PYRIDINECARBOXALDEHYDE
N-METHYLHYDROXYLAMINE	1-METHYL-1H-IMIDAZOLE-4-CARBALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	1-METHYL-1H-IMIDAZOLE-4-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	1-METHYL-1H-IMIDAZOLE-4-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	1-METHYL-1H-IMIDAZOLE-4-CARBALDEHYDE
N-ETHYLHYDROXYLAMINE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-ETHYLHYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-BENZYLHYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
N-ETHYLHYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE

N-BENZYLHYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	OXAZOLE-2-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
N-ETHYLHYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
CYCLOHEXYLHYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
N-BENZYLHYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	3-CHLORO-2-FORMYLPYRIDINE
N-METHYLHYDROXYLAMINE	1H-PYRAZOLE-4-CARBOXALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	1H-PYRAZOLE-4-CARBOXALDEHYDE
N-METHYLHYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-ETHYLHYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-BENZYLHYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-ETHYLHYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-BENZYLHYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-ETHYLHYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-BENZYLHYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-METHYLHYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-ETHYLHYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-CYCLOPENTYLHYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-BENZYLHYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	PYRIDAZINE-3-CARBALDEHYDE
N-METHYLHYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-ETHYLHYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
CYCLOHEXYLHYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-BENZYLHYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	3-FORMYL-5-METHOXYPYRIDINE
N-METHYLHYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
N-ETHYLHYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
N-(TERT-BUTYL)HYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
CYCLOHEXYLHYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
N-BENZYLHYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
3-(HYDROXYAMINO)TETRAHYDROTHIOPHENE 1,1-DIOXIDE	PYRIMIDINE-5-CARBOXALDEHYDE
N-(PYRIDIN-2-YLMETHYL)HYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	3,5-DIMETHYLISOXAZOLE-4-CARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	3-METHYL-1H-PYRAZOLE-4-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	4-METHYL-5-FORMYLTHIAZOLE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	2-PYRIDINECARBOXALDEHYDE
3-PYRAZOLIDINONE	2-PYRIDINECARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	2-PYRIDINECARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	1-METHYL-1H-IMIDAZOLE-4-CARBALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	1-METHYL-1H-PYRAZOLE-3-CARBALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	4-METHYL-4H-1,2,4-TRIAZOLE-3-CARBALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	OXAZOLE-2-CARBALDEHYDE
3-PYRAZOLIDINONE	OXAZOLE-2-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	OXAZOLE-2-CARBALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	3-CHLORO-2-FORMYLPYRIDINE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	3-CHLORO-2-FORMYLPYRIDINE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	1H-PYRAZOLE-4-CARBOXALDEHYDE

N-(PYRIDIN-2-YLMETHYL)HYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	5-METHYLISOXAZOLE-3-CARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	2,5-DIMETHYLOXAZOLE-4-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	5-METHYLIMIDAZOLE-4-CARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
3-PYRAZOLIDINONE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	1-METHYL-2-IMIDAZOLECARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	PYRIDAZINE-3-CARBALDEHYDE
N-(PYRIDIN-2-YLMETHYL)HYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-(2-METHOXYBENZYL)HYDROXYLAMINE	3-FORMYL-5-METHOXYPYRIDINE
N-(PYRIDIN-2-YLMETHYL)HYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
N-[2-CHLOROPHENYLMETHYL]HYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
N-(4-CHLOROBENZYL)HYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE
(4-((HYDROXYAMINO)METHYL)PHENYL)METHANOL	PYRIMIDINE-5-CARBOXALDEHYDE
N-(2,4-DICHLOROBENZYL)HYDROXYLAMINE	PYRIMIDINE-5-CARBOXALDEHYDE

Validation of *N*-capping reactions

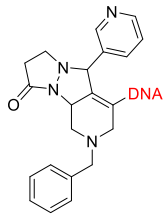
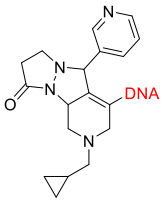
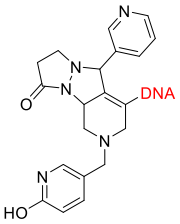
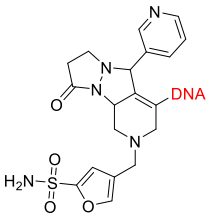
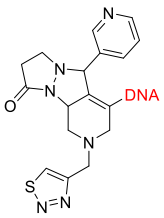
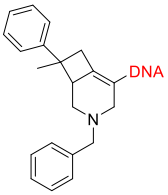
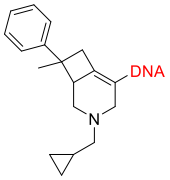
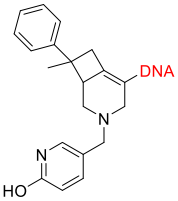
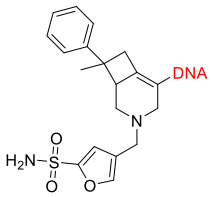
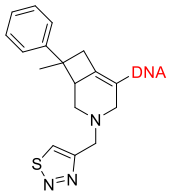
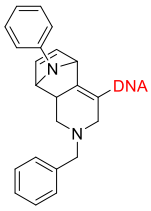
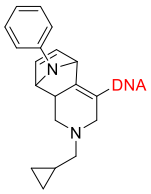
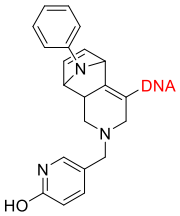
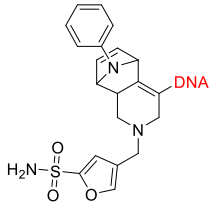
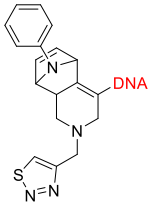
Reductive amination conditions:
On-DNA substrate (1 µL, 0.26–0.38 mM in water, 0.25–0.50 nmol) was combined with acetate buffer (1 M, pH 5, 2–4 µL). The aldehyde was added (200 mM in MeCN or DMF, 1–2 µL). After 10 min, NaH_3BCN (200 mM in water, 0.75–1.5 µL) was added and the resulting mixture was incubated at rt for 18 h. Reactions were subjected to ethanol-induced precipitation. The pellets were washed with EtOH:water (5:1) and analyzed by UPLC-MS.

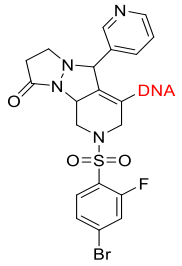
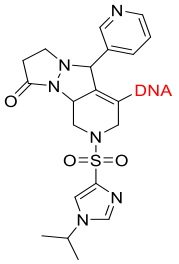
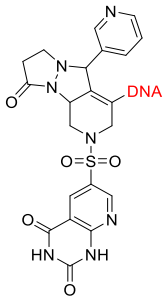
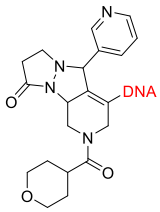
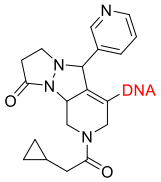
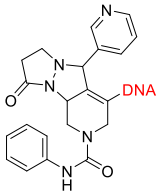
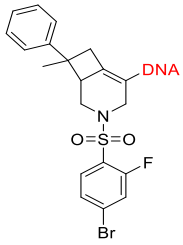
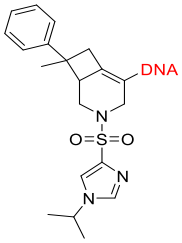
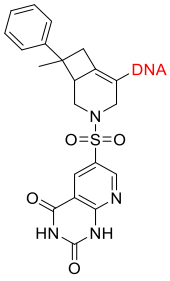
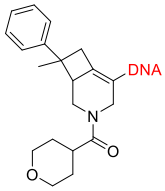
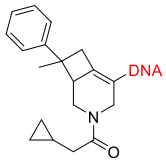
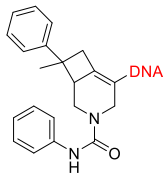
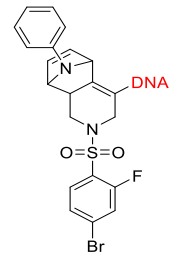
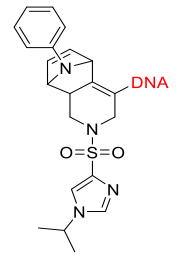
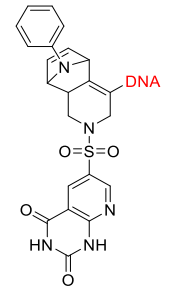
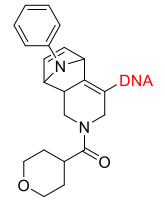
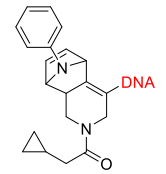
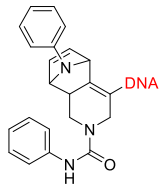
Sulfonylation conditions:
On-DNA substrate (1 µL, 0.26–0.38 mM in water, 0.25–0.50 nmol) was combined with phosphate buffer (0.1 M, pH 8, 0.75–1.5 µL). The sulfonyl chloride (200 mM in MeCN, 0.5–1 µL) was added and the resulting mixture was incubated at rt for 18 h. Reactions were subjected to ethanol-induced precipitation. The pellets were washed with EtOH:water (5:1) and analyzed by UPLC-MS.

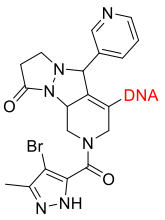
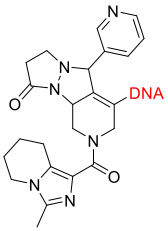
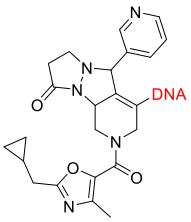
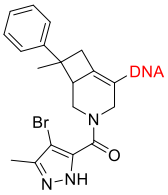
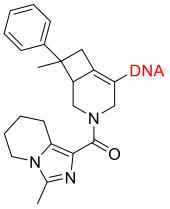
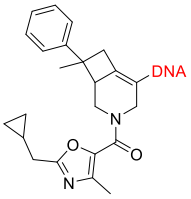
Acylation conditions:
On-DNA substrate (1 µL, 0.26–0.38 mM in water, 0.25–0.50 nmol) was combined with phosphate buffer (0.1 M, pH 8, 0.75–1.5 µL). The NHS-ester (200 mM in DMF, 0.5–1 µL) was added and the resulting mixture was incubated at rt for 18 h. Reactions were subjected to ethanol-induced precipitation. The pellets were washed with EtOH:water (5:1) and analyzed by UPLC-MS.

Carbamoylation conditions:
On-DNA substrate (1 µL, 0.26–0.38 mM in water, 0.25–0.50 nmol) was combined with phosphate buffer (0.1 M, pH 8, 0.75–1.5 µL). The isocyanate (200 mM in MeCN, 0.5–1 µL) was added and the resulting mixture was incubated at rt for 18 h. Reactions were subjected to ethanol-induced precipitation. The pellets were washed with EtOH:water (5:1) and analyzed by UPLC-MS.

The following charts show the chemical structures of expected products and the %AUC of observed DNA-conjugates as sum of individual species (e.g. stereoisomeric products) after performing the reaction sequence (strain-promoted cycloaddition/Fmoc-deprotection/*N*-capping).

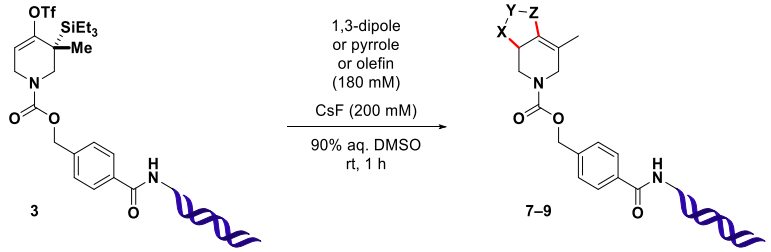
Reductive aminations				
 Exact Mass: 5624.2614	 Exact Mass: 5588.2614	 Exact Mass: 5641.2516	 Exact Mass: 5693.2135	 Exact Mass: 5632.2083
product: 98% unknown: 2%	**product: 90%** unknown: 10%	**product: 99%** unknown: 1%	**product: 98%** unknown: 2%	**product: 99%** unknown: 1%
 Exact Mass: 5567.2651	 Exact Mass: 5531.2651	 Exact Mass: 5584.2553	 Exact Mass: 5636.2172	 Exact Mass: 5575.2120
SM: 52% **product: 43%** unknown: 6%	SM: 9% **product: 82%** unknown: 9%	SM: 70% **product: 27%** unknown: 3%	SM: 12% **product: 57%** unknown: 32%	SM: 13% **product: 79%** unknown: 8%
 Exact Mass: 5592.2603	 Exact Mass: 5556.2603	 Exact Mass: 5609.2505	 Exact Mass: 5661.2124	 Exact Mass: 5600.2073
SM: 37% **product: 20%** unknown: 43%	SM: 1% **product: 48%** unknown: 51%	SM: 12% **product: 71%** N-methyl: 17%	SM: 1% **product: 25%** unknown: 72%	**product: 100%** (overlap with impurity)

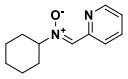
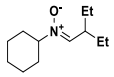
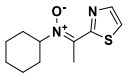
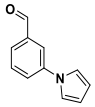
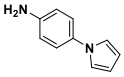
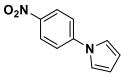
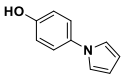
Sulfonylation			Acylation		Carbamoylation
					
Exact Mass: 5770.1087	Exact Mass: 5706.2451	Exact Mass: 5759.1989	Exact Mass: 5646.2669	Exact Mass: 5616.2563	Exact Mass: 5653.2516
product: 94% unknown: 6%	**product: 94%** unknown: 6%	**product: 100%**	SM: 10% **product: 53%** unknown: 38%	**product: 96%** unknown: 4%	**product: 91%** unknown: 9%
					
Exact Mass: 5713.1124	Exact Mass: 5649.2488	Exact Mass: 5702.2026	Exact Mass: 5589.2706	Exact Mass: 5559.2600	Exact Mass: 5596.2553
SM: 6% **product: 86%** unknown: 8%	SM: 57% **product: 32%** unknown: 11%	SM: 6% **product: 86%** unknown: 8%	**product: 92%** unknown: 8%	**product: 100%**	**product: 92%** unknown: 8%
					
Exact Mass: 5738.1077	Exact Mass: 5674.2440	Exact Mass: 5727.1978	Exact Mass: 5614.2658	Exact Mass: 5584.2553	Exact Mass: 5621.2505
product: 50% unknown: 50%	SM: 13% **product: 41%** unknown: 47%	**product: 84%** unknown: 16%	**product: 43%** unknown: 57%	**product: 52%** unknown: 48%	**product: 61%** unknown: 39%

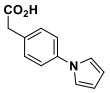
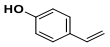
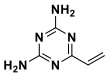
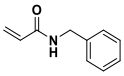
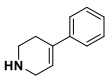
 Exact Mass: 5720.1573	 Exact Mass: 5696.2938	 Exact Mass: 5697.2778
product: 94% unknown: 6%	SM: 14% **product: 86%**	SM: 1% **product: 98%** unknown: 1%
 Exact Mass: 5663.1610	 Exact Mass: 5639.2975	 Exact Mass: 5640.2815
product: 93% unknown: 7%	SM: 30% **product: 69%**	**product: 94%** unknown: 6%

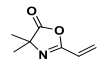
5. Analytics of DNA-conjugates

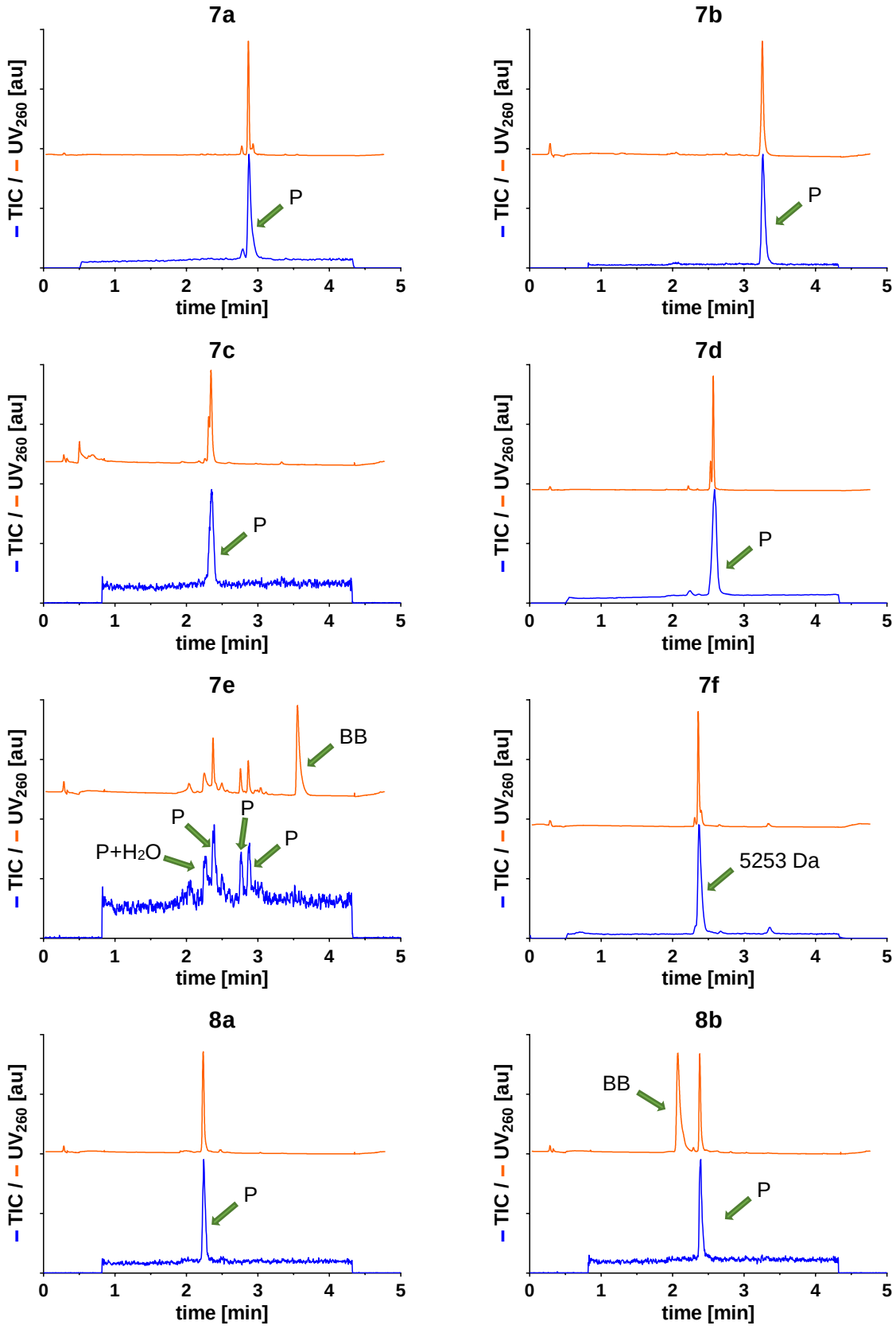
Table S6. Analytical data obtained for DNA-conjugates described in Figure 3.

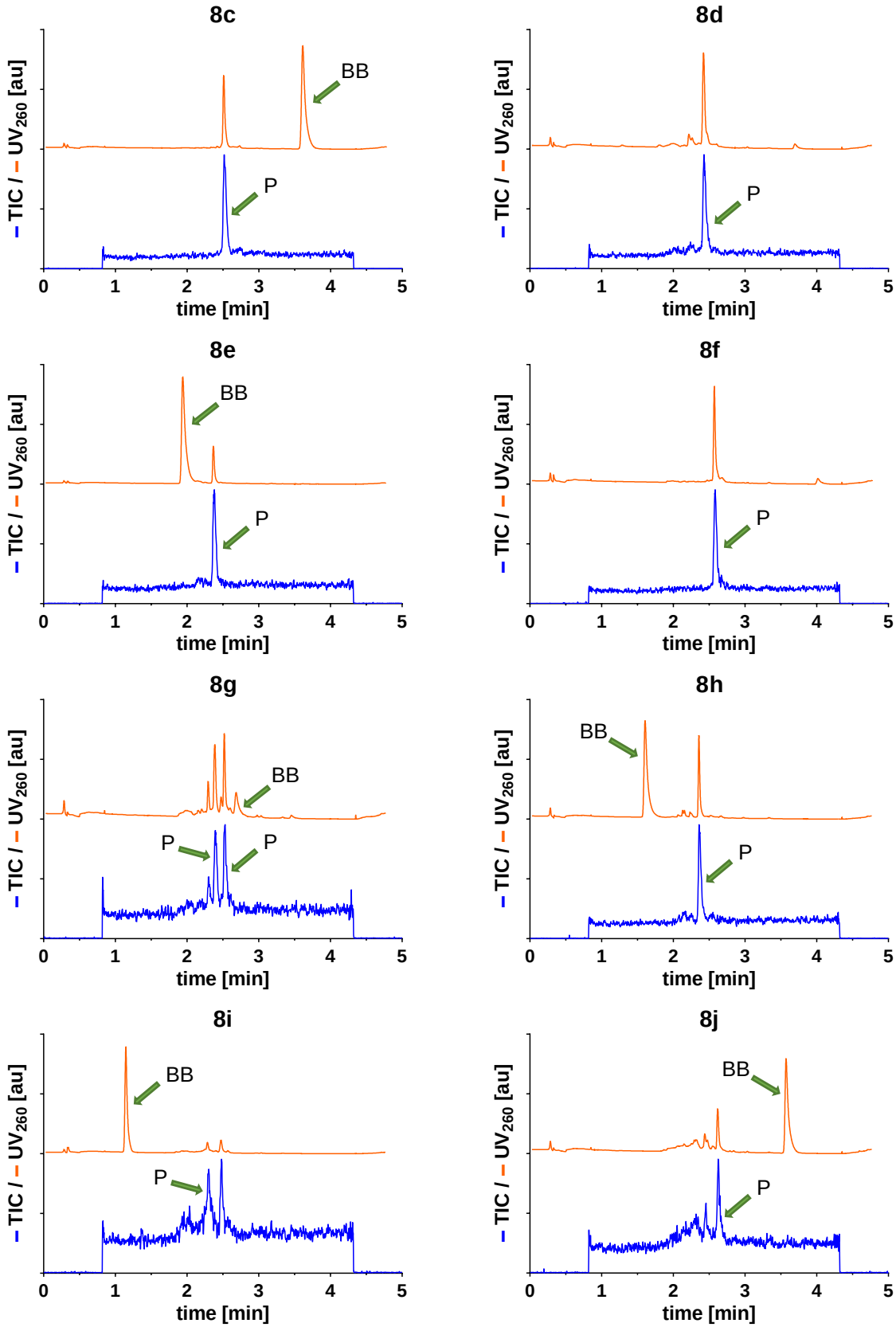


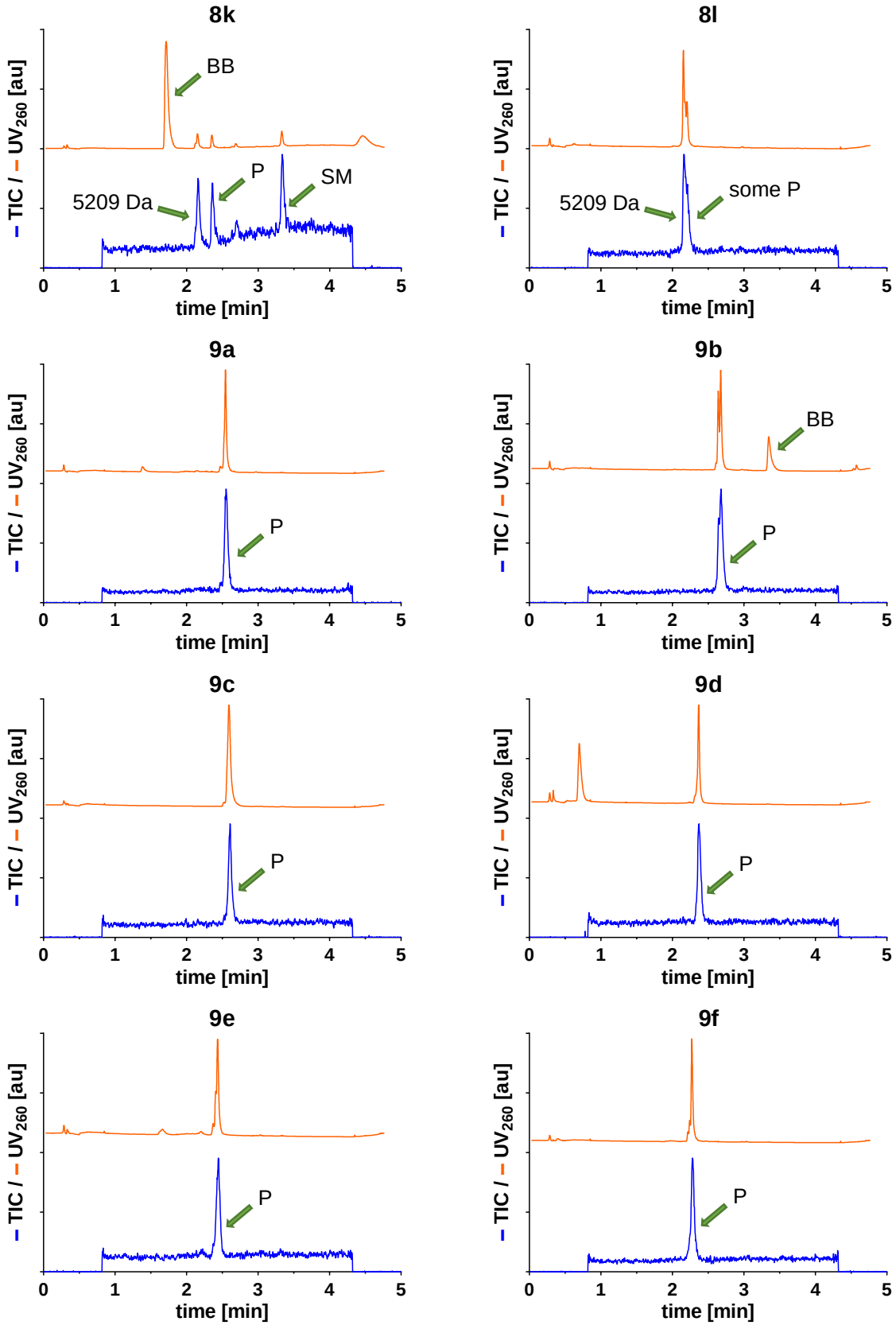
BB in Figure 3	Structure	product [%AUC]	calculated mass [Da]	found (MaxEnt1) [Da]
7a		92	5394	5398
7b		>95	5387	5388
7c		>95	5329	5332
7d		>95	5303	5306
7e		24% (two peaks) 27% (more polar, [2+2] N-oxide?)	5414	5415
7f		0	5305	5253 (-C_4H_8)
8a		>95	5271	5273
8b		>95	5348	5350
8c		>95	5361	5363
8d		>95	5343	5346
8e		>95	5348	5350
8f		>95	5378	5380
8g		84	5348	5350
8h		81	5349	5350
8i		57	5299	5301
8j		48	5378	5380

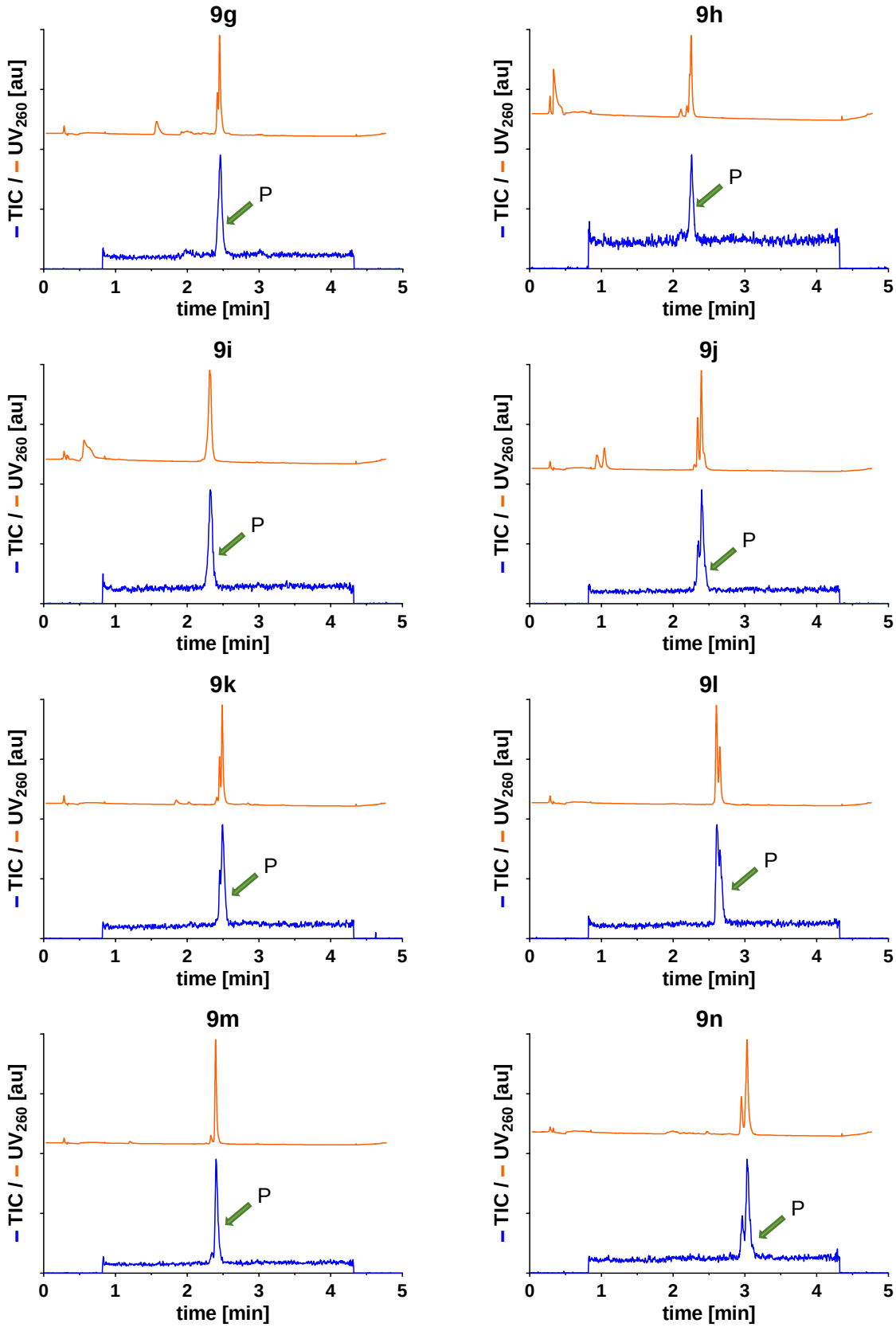
8k		25	5391	5391
8l		46	5314	5316
9a		>95	5310	5312
9b		>95	5315	5317
9c		>95	5312	5314
9d		>95	5284	5286
9e		93	5284	5287
9f		95	5275	5277
9g		>95	5289	5291
9h		91	5327	5328
9i		>95	5289	5290
9j		>95	5331	5322
9k		>95	5351	5353
9l		>95	5354	5357
9m		>95	5303	5305
9n		>95	5322	5324
9o		>95	5323	5325
9p		>95	5308	5308
9q		28	5290	5290
9r		0	5358	-
9s		0	5342	-
9t		0	5325	5455 (P+BB)
9u		0	5298	-
9v		0	5276	-
9w		0	5349	-

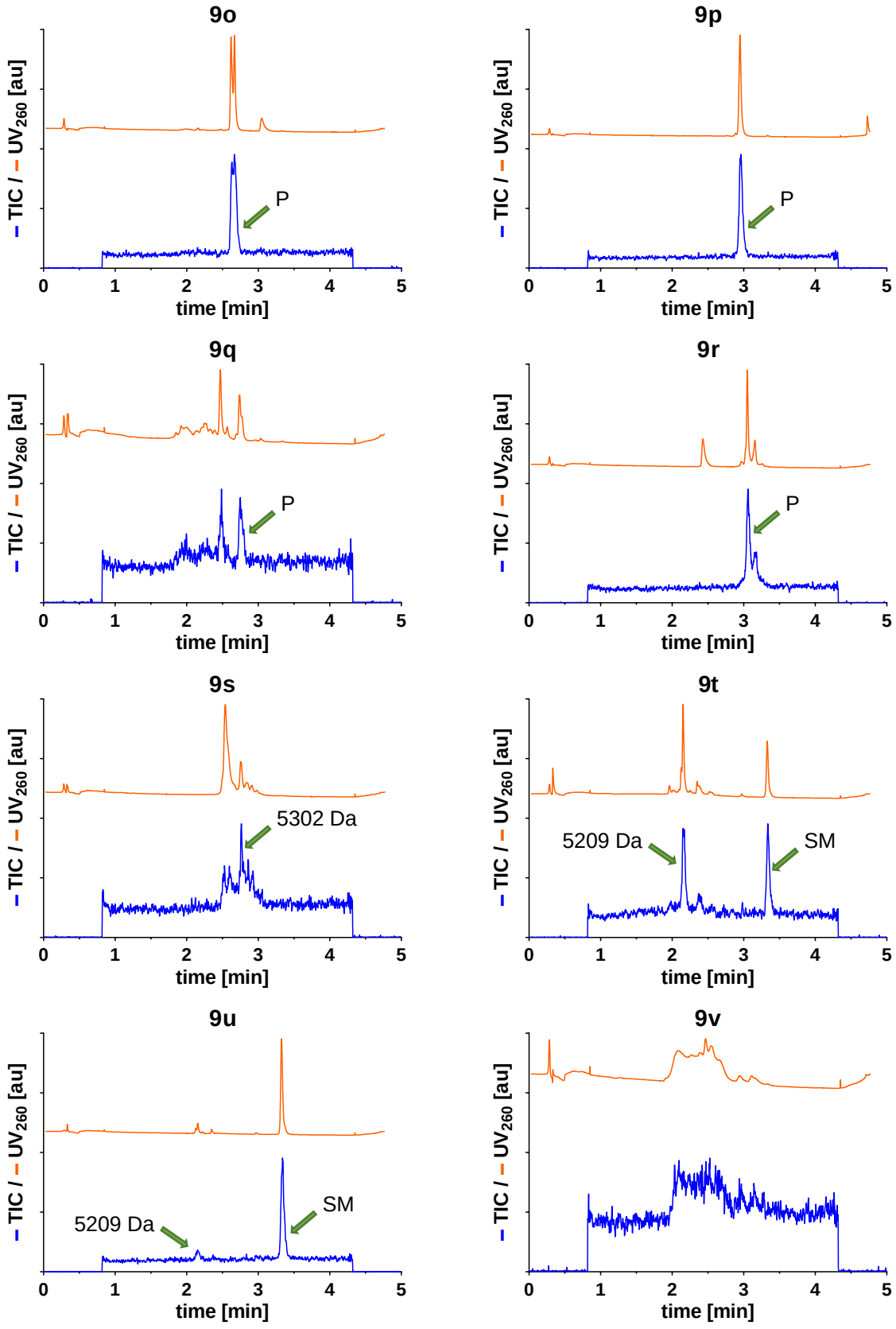
9x		0	5329	5480 (P+H_2O)

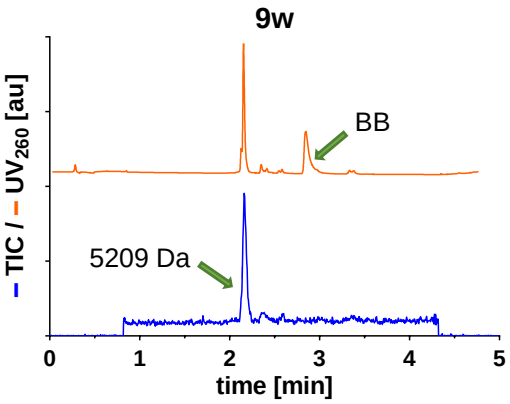

7a
7b
7c
7d
7e
7f
8a
8b
TIC / UV260 [au]
time [min]
P
BB
P+H2O
5253 Da


8c
8d
8e
8f
8g
8h
8i
8j
TIC / UV260 [au]
time [min]
BB
P
0
1
2
3
4
5


8k
BB
P
SM
5209 Da
8l
5209 Da
some P
9a
P
9b
BB
P
9c
P
9d
P
9e
P
9f
P
TIC / – UV260 [au]
time [min]
0
1
2
3
4
5


9g
9h
9i
9j
9k
9l
9m
9n
TIC / UV260 [au]
time [min]
P
0
1
2
3
4
5


9o
9p
9q
9r
9s
9t
9u
9v
TIC / UV260 [au]
time [min]
P
5302 Da
5209 Da
SM


9w
TIC / UV260 [au]
BB
5209 Da
0
1
2
3
4
5
time [min]

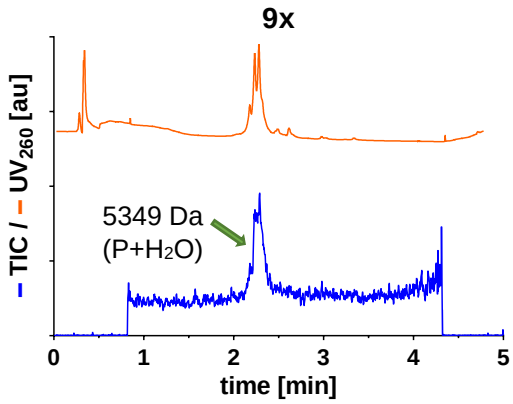
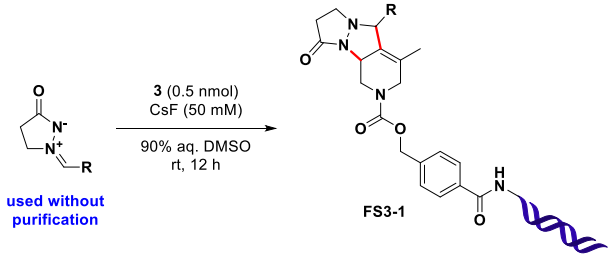
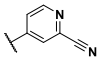
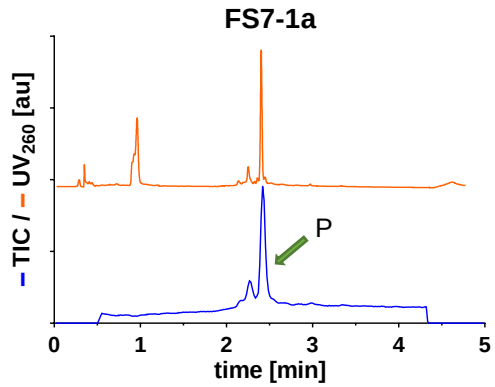

9x
TIC / UV260 [au]
5349 Da
(P+H2O)
0
1
2
3
4
5
time [min]

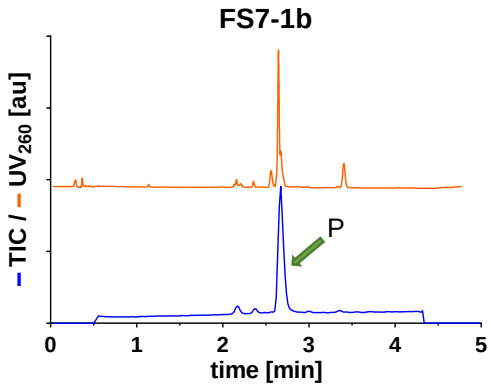
Table S7. Analytical data obtained for DNA-conjugates described in Figure S7.

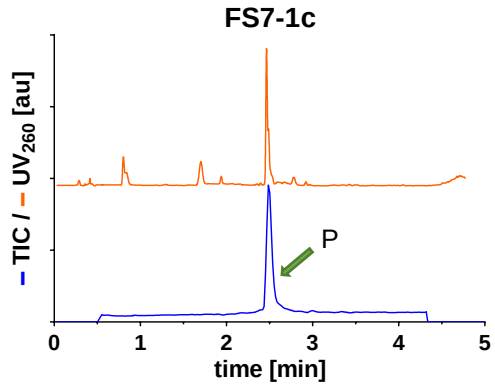


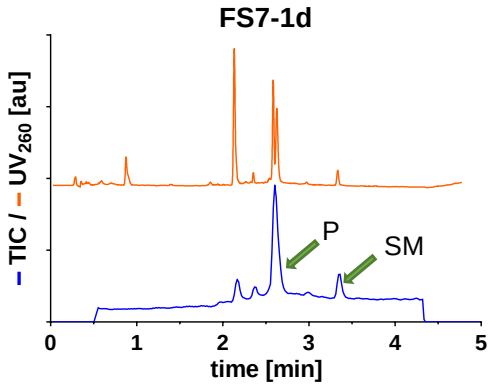
BB in Figure S7	R	product [%AUC]	calculated mass [Da]	found (MaxEnt1) [Da]
a		82	5354	5357
b		93	5378	5381
c		>95	5379	5382
d		52	5384	5388
e		>95	5369	5372
f		>95	5354	5357
g		>95	5368	5372
h		0 (87)[#]	5366	5370
i		>95	5383	5386
j		85	5399	5402
k		>95	5366	5369
l		>95	5370	5374
m		77	5390	5393
n		67	5368	5371
o		0	5383	5211/5193[$]
p		0	5378	5211/5193[$]
q		0	5386	5211/5193[$]
r		0	5384	5211/5193[$]
s		0	5330	5211/5193[$]
t		0	5356	5211/5193[$]
u		0	5370	5211/5193[$]

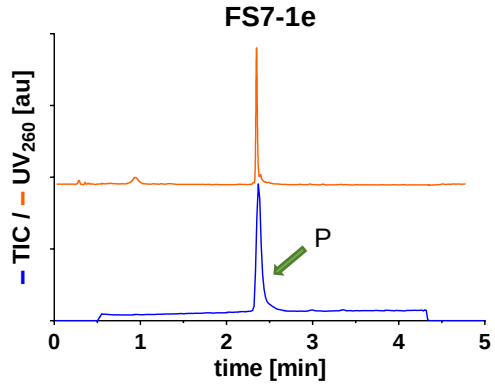
[#] Reaction performed with isolated azomethine imine. [$] Masses of observed main species in agreement with water addition to strained allene or ketone (from triflate hydrolysis/desilylation), req. 5208 Da, found 5211 Da), and diene (likely isomerized strained allene), req. 5190 Da, found 5193 Da.

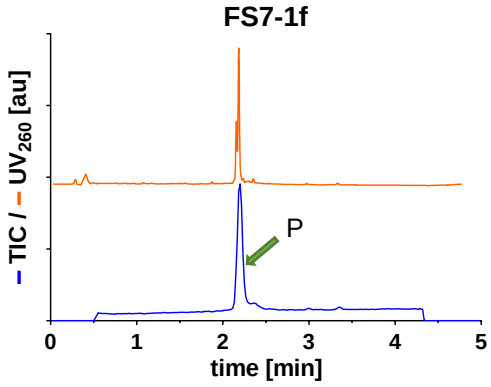

FS7-1a
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

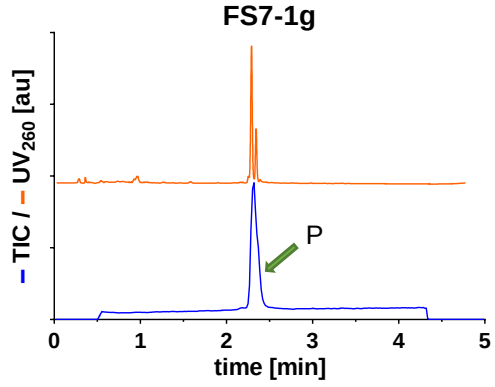

FS7-1b
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

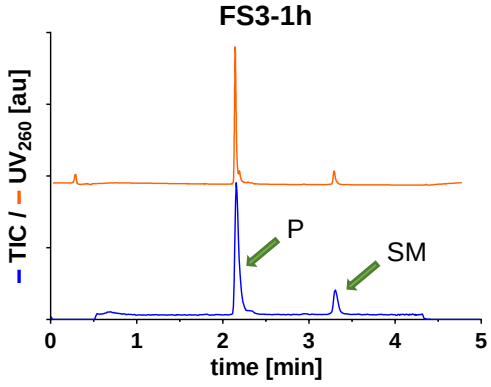

FS7-1c
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

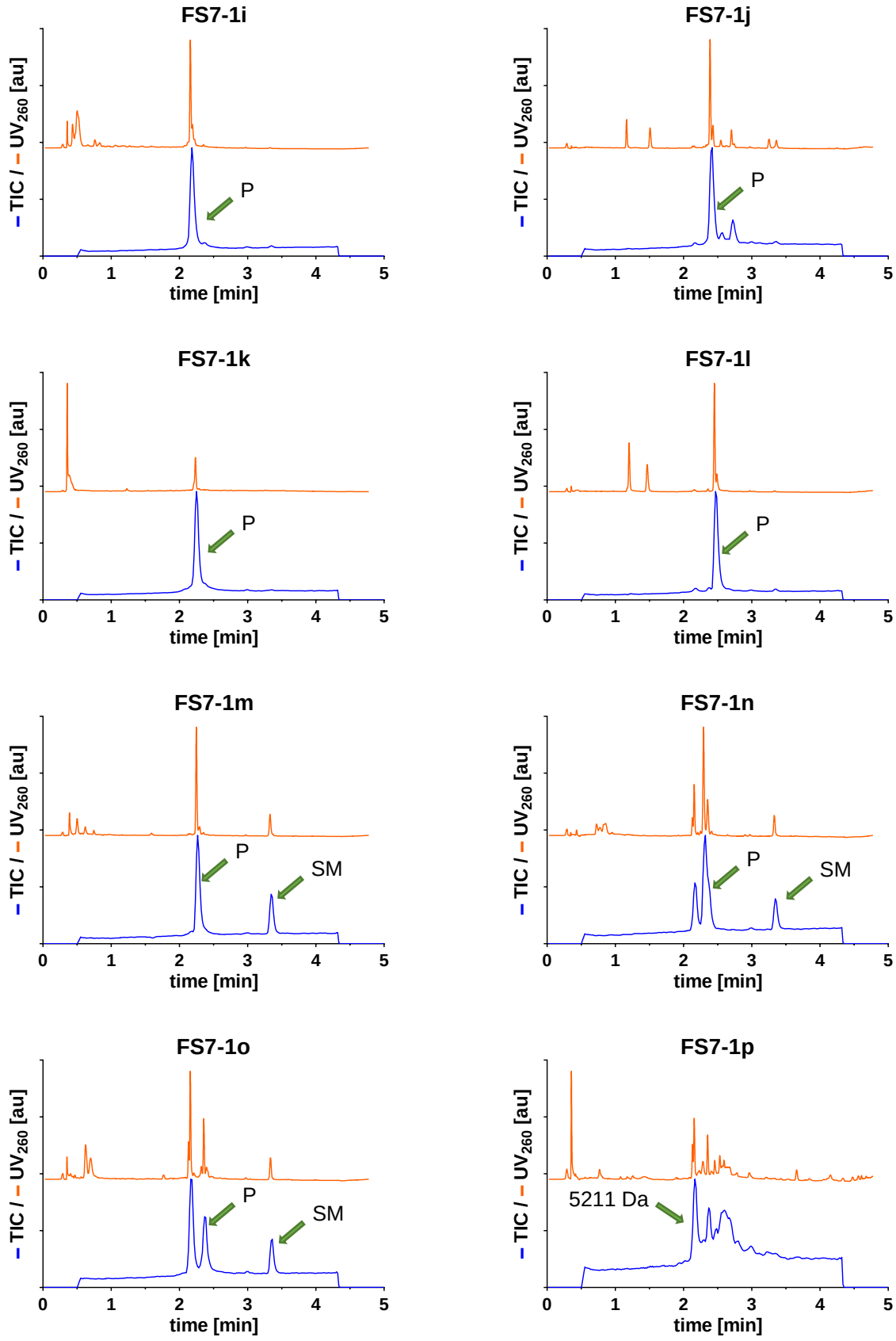

FS7-1d
TIC / UV260 [au]
P
SM
0
1
2
3
4
5
time [min]

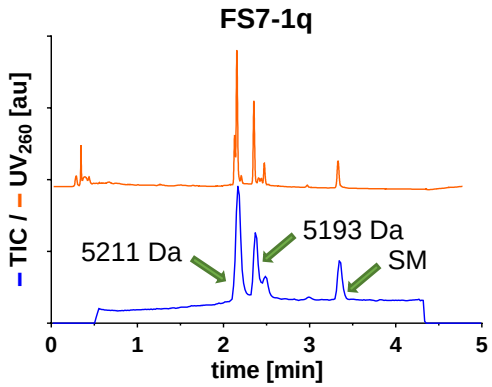

FS7-1e
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

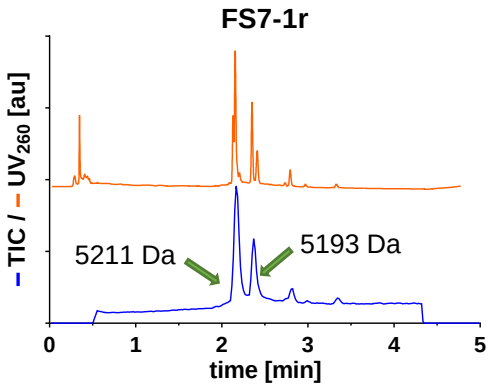

FS7-1f
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

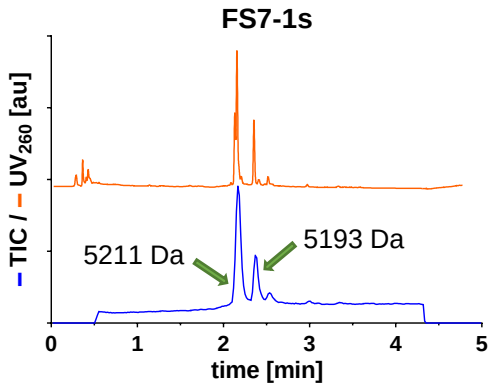

FS7-1g
TIC / UV260 [au]
P
0
1
2
3
4
5
time [min]

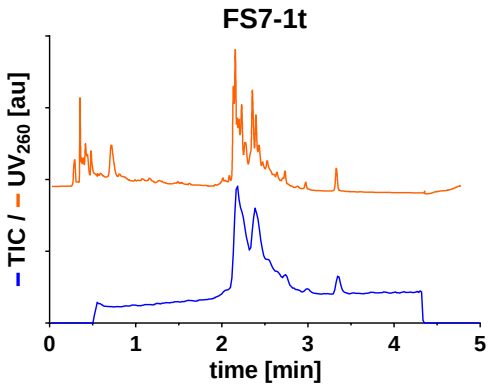

FS3-1h
TIC / UV260 [au]
P
SM
0
1
2
3
4
5
time [min]


FS7-1i
FS7-1j
FS7-1k
FS7-1l
FS7-1m
FS7-1n
FS7-1o
FS7-1p
– TIC / – UV260 [au]
time [min]
P
SM
5211 Da
0
1
2
3
4
5


FS7-1q
TIC / UV260 [au]
5211 Da
5193 Da
SM
0
1
2
3
4
5
time [min]


FS7-1r
TIC / UV260 [au]
5211 Da
5193 Da
0
1
2
3
4
5
time [min]


FS7-1s
TIC / UV260 [au]
5211 Da
5193 Da
0
1
2
3
4
5
time [min]


FS7-1t
TIC / UV260 [au]
0
1
2
3
4
5
time [min]

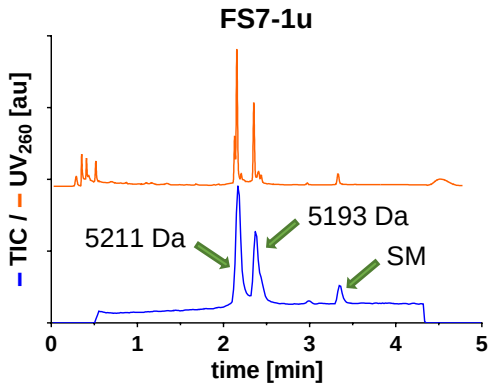
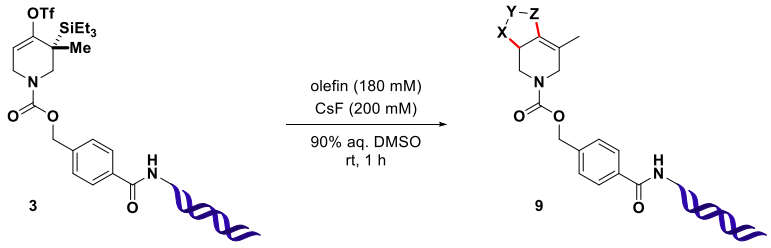
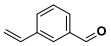
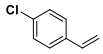
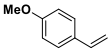
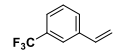
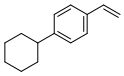
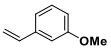
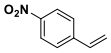
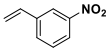
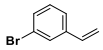
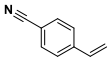
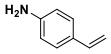
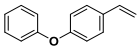
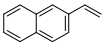
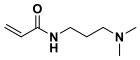
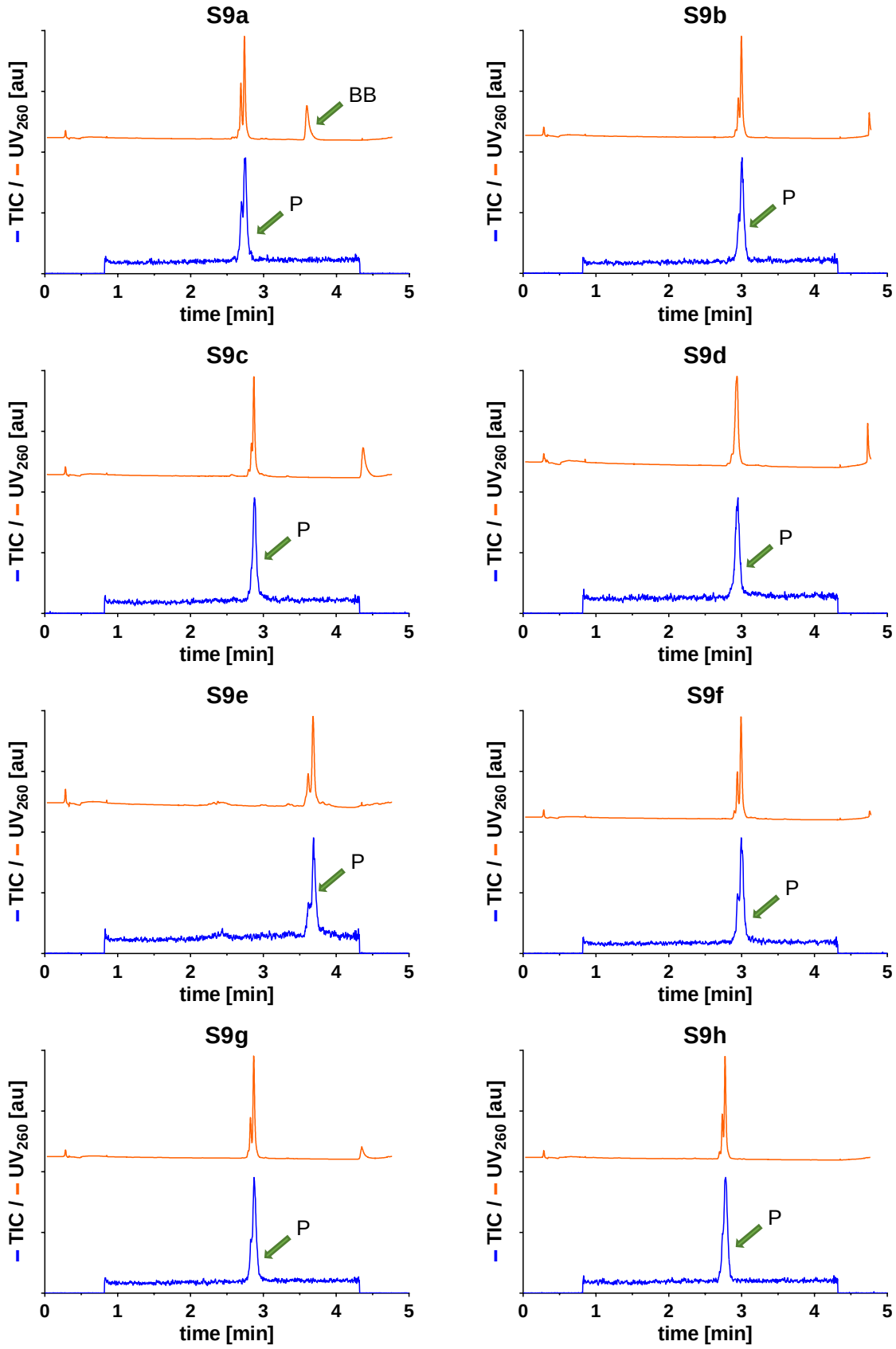

FS7-1u
TIC / UV260 [au]
5211 Da
5193 Da
SM
0
1
2
3
4
5
time [min]

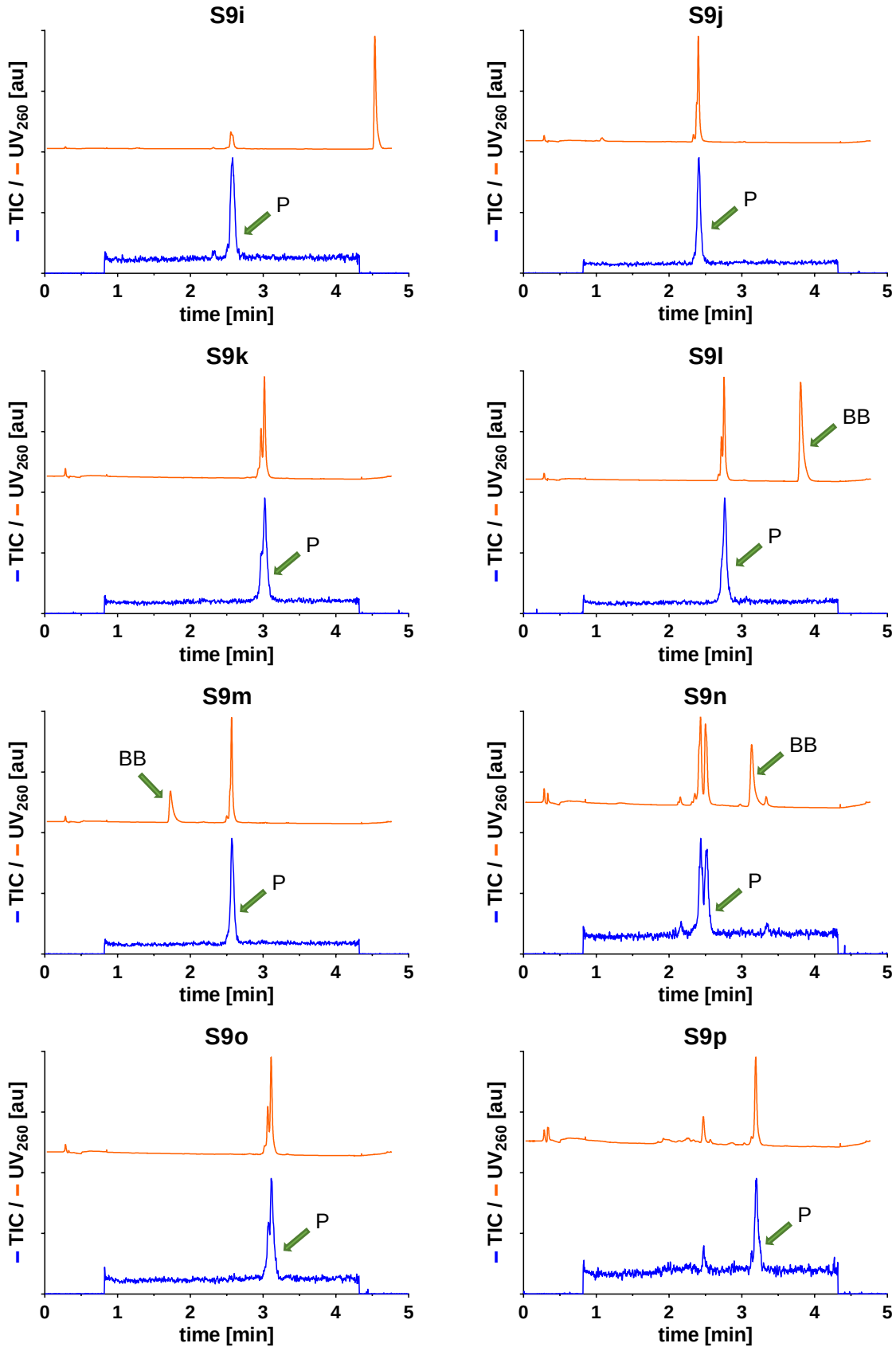
Table S8. Analytical data obtained for DNA-conjugates described in Figure S5.

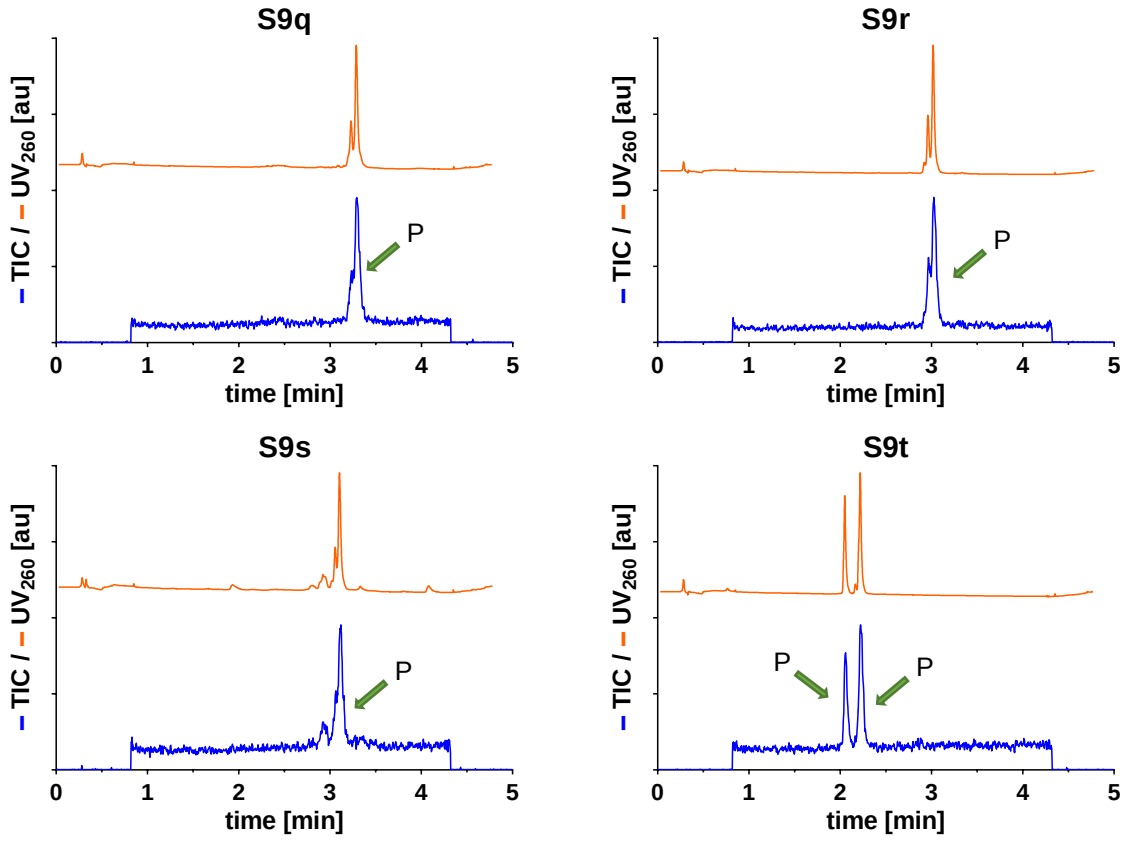


BB in Figure S5	R	product [%AUC]	calculated mass [Da]	found (MaxEnt1) [Da]
S9a		>95	5322	5323
S9b		>95	5328	5330
S9c		>95	5324	5326
S9d		95	5362	5364
S9e		>95	5376	5378
S9f		>95	5328	5329
S9g		>95	5324	5326
S9h		>95	5339	5341
S9i		>95	5351	5354
S9j		>95	5303	5305
S9k		>95	5372	5375
S9l		>95	5319	5320
S9m		95	5309	5310
S9n		90	5337	5339
S9o		95	5322	5324
S9p		81	5348	5350
S9q		>95	5386	5386
S9r		>95	5372	5375
S9s		86	5344	5346
S9t		>95	5346	5347

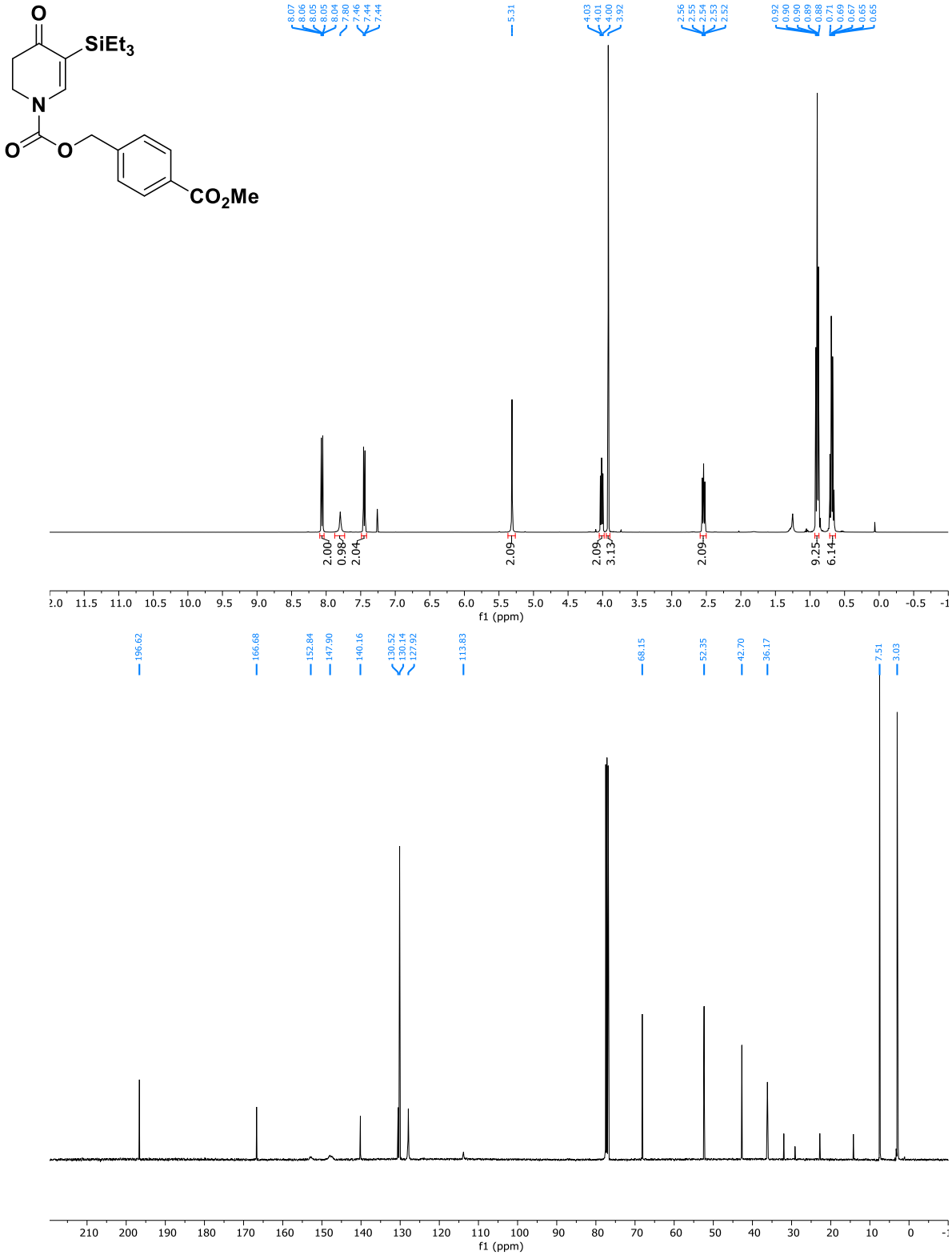
Reactions were performed with **3** (0.25 nmol) under the indicated conditions.

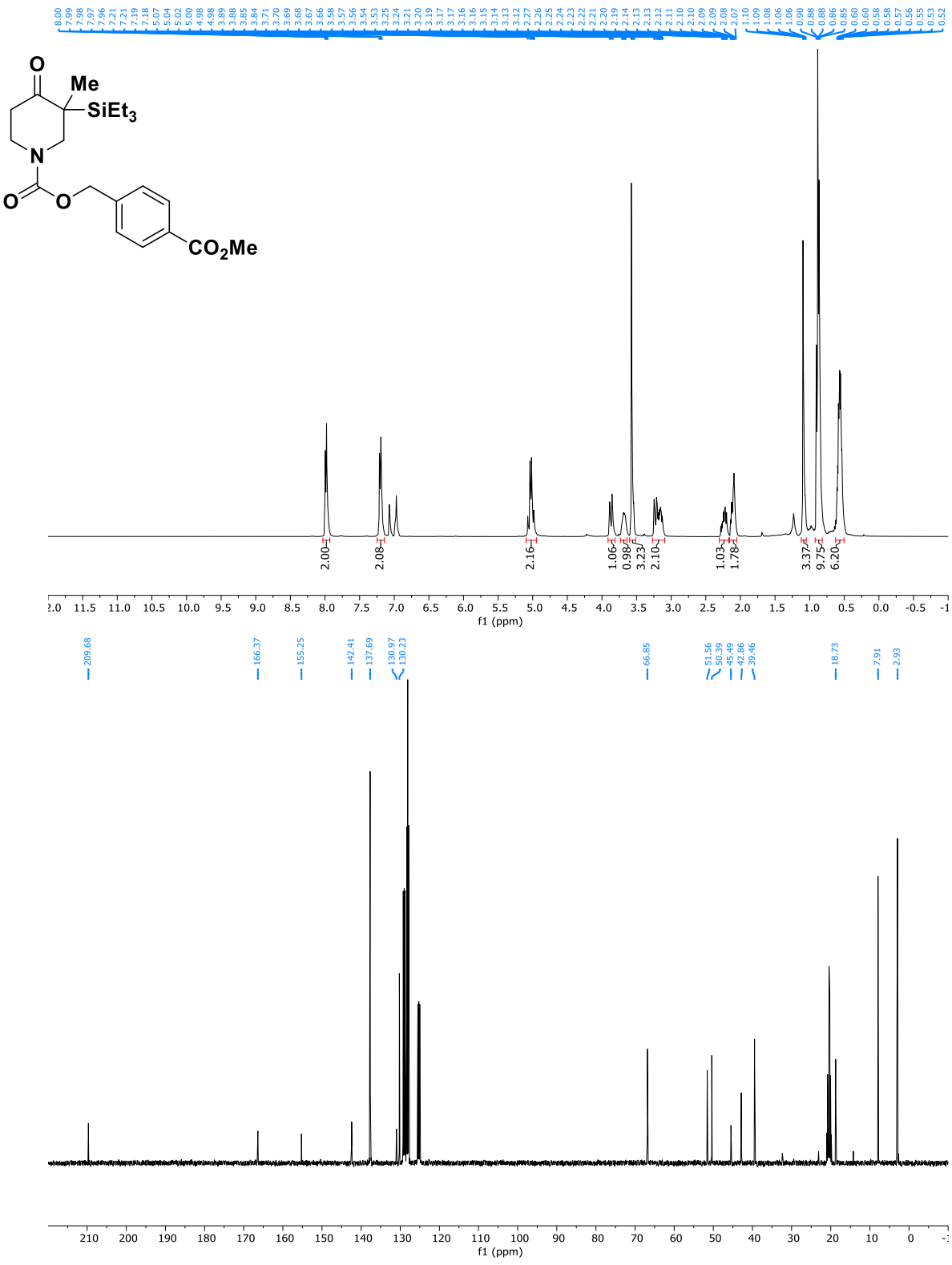

S9a
S9b
S9c
S9d
S9e
S9f
S9g
S9h
TIC / – UV260 [au]
time [min]
BB
P
0
1
2
3
4
5

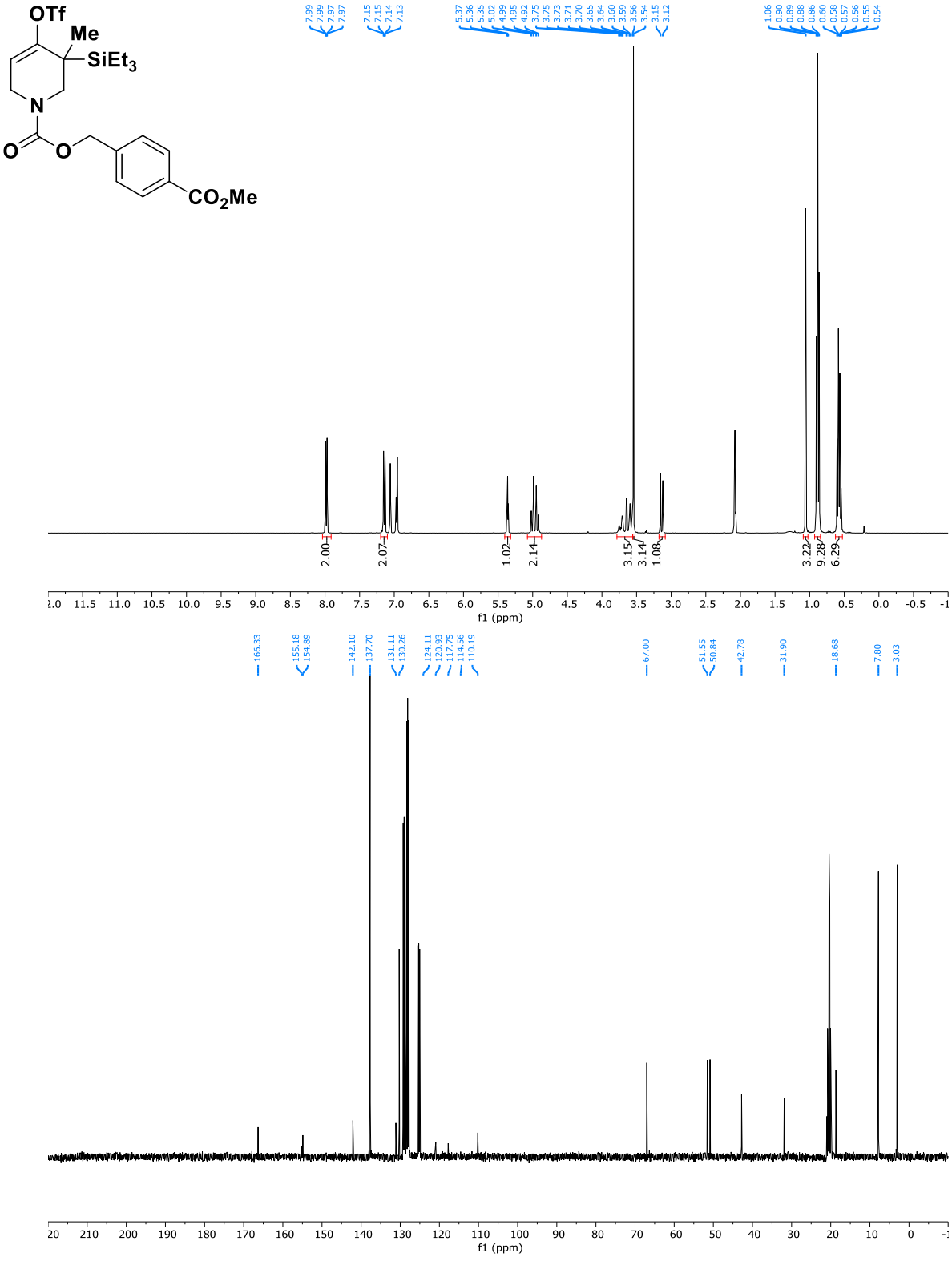

S9i
S9j
S9k
S9l
S9m
S9n
S9o
S9p
TIC / UV260 [au]
time [min]
P
BB
0
1
2
3
4
5

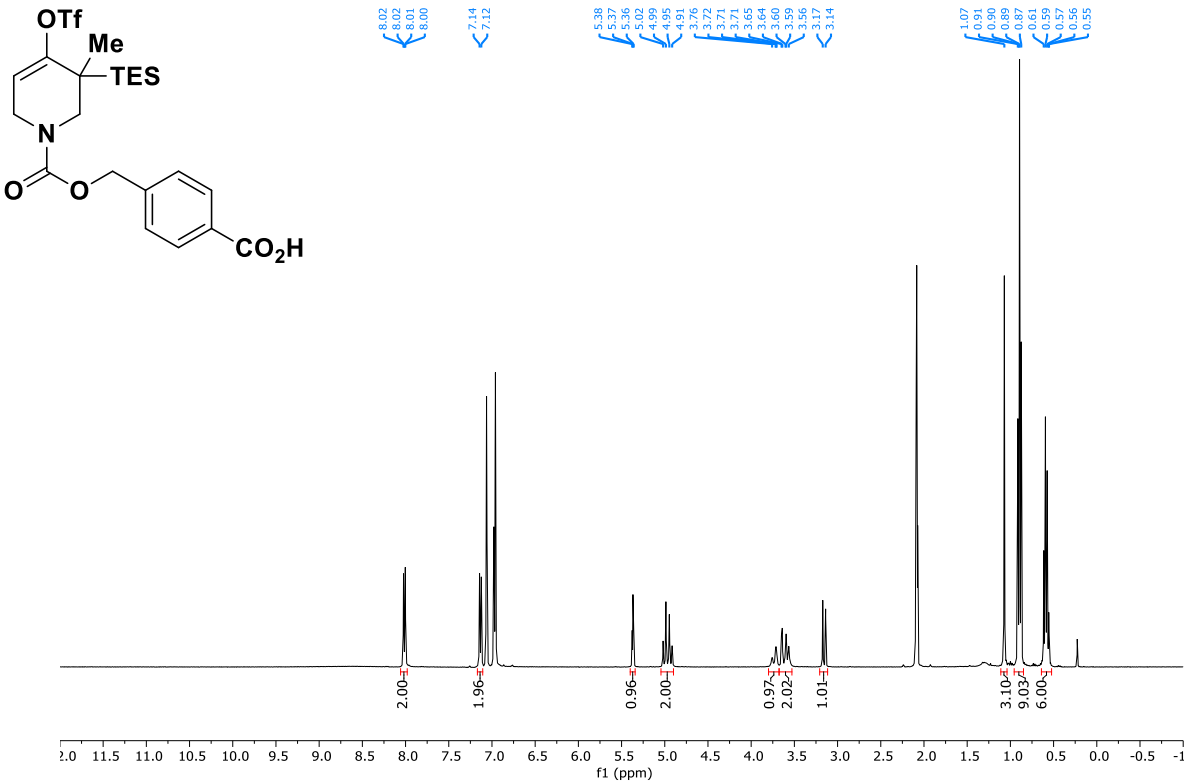

S9q
S9r
S9s
S9t
TIC / UV260 [au]
time [min]
P
0
1
2
3
4
5

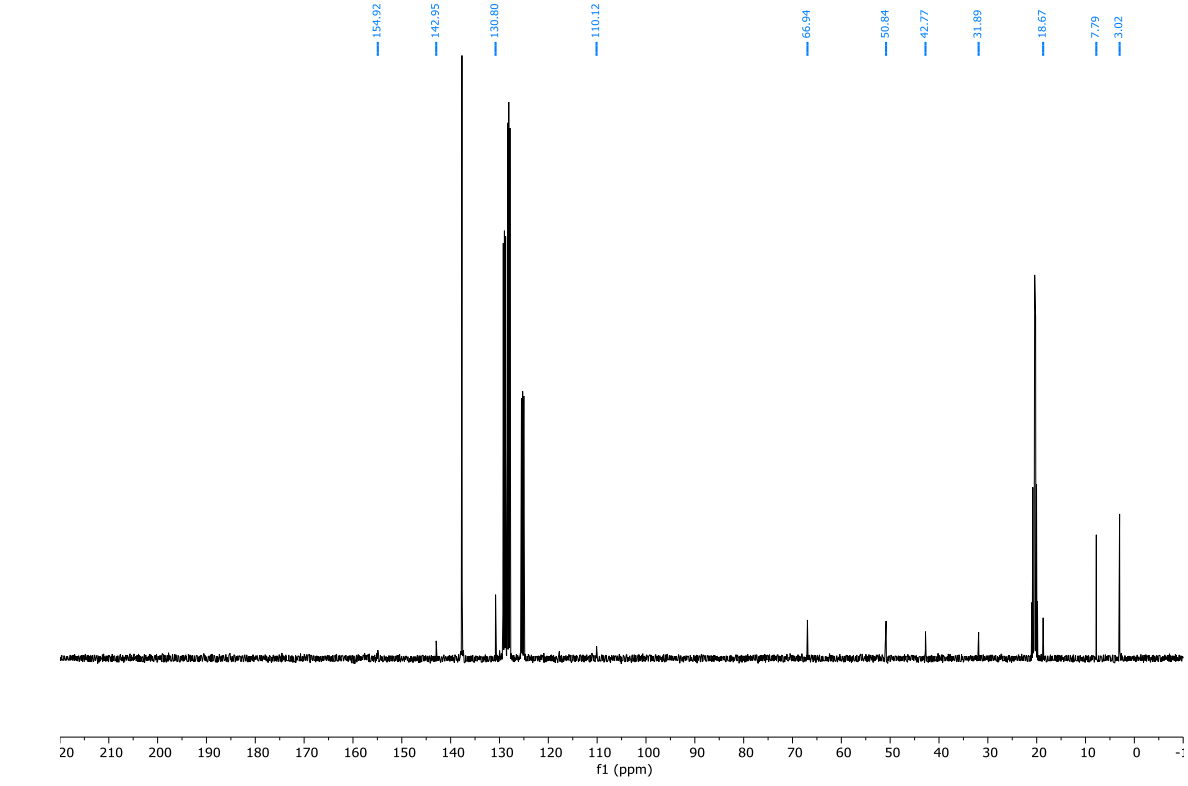
6. ^{1}H and ^{13}C NMR spectra

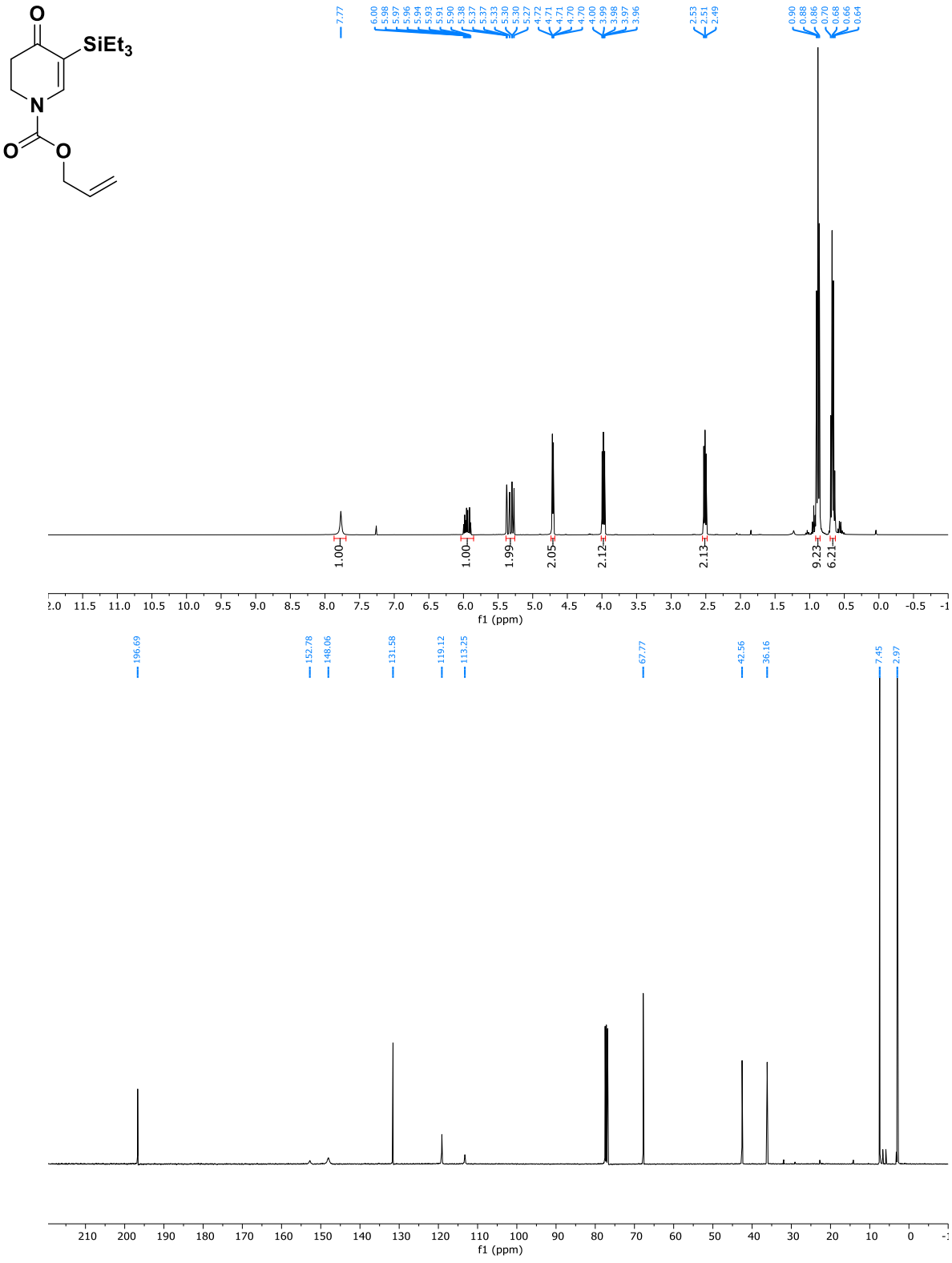


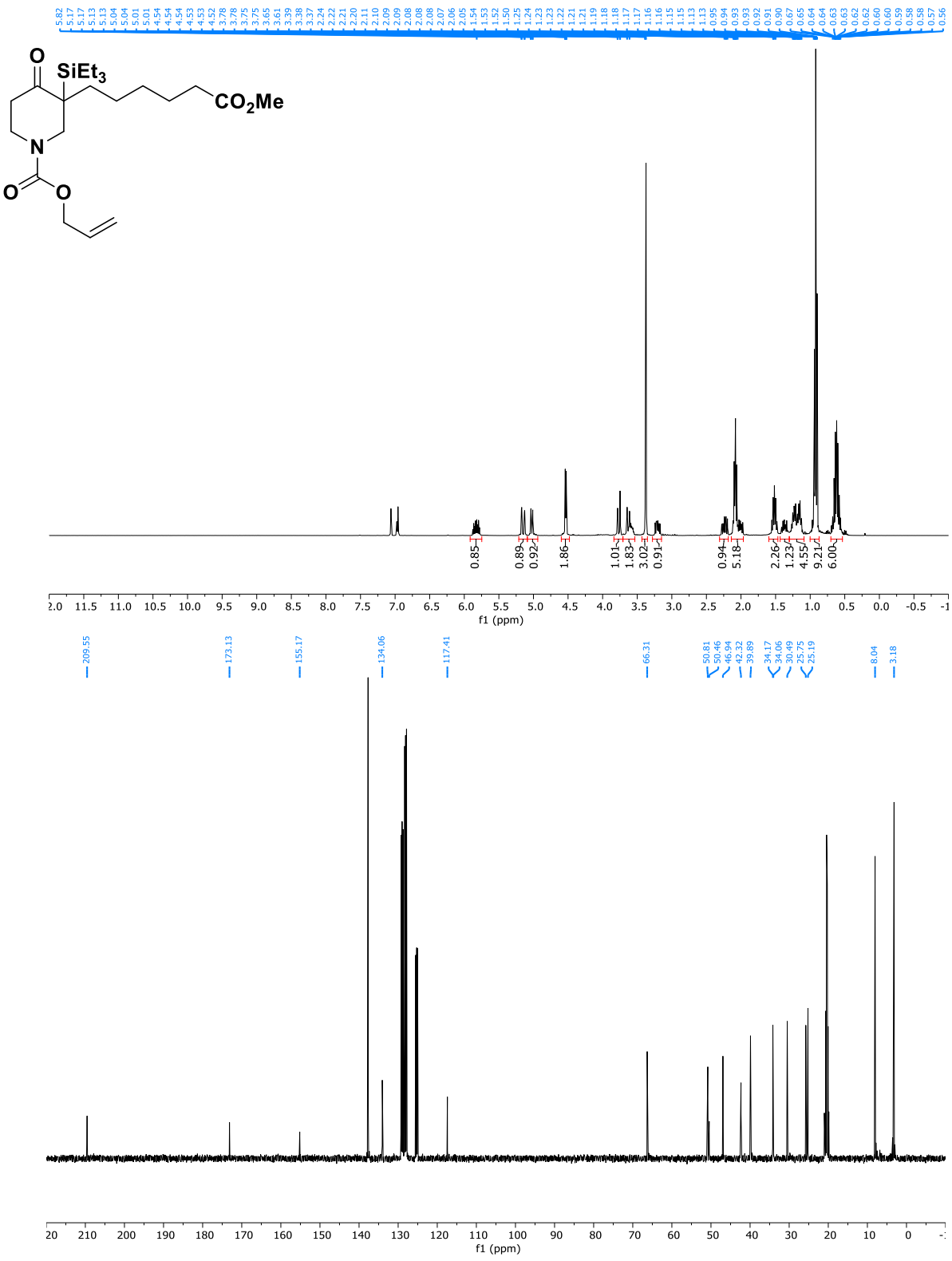

Me
SiEt3
N
O
O
O
CO2Me
f1 (ppm)
f1 (ppm)

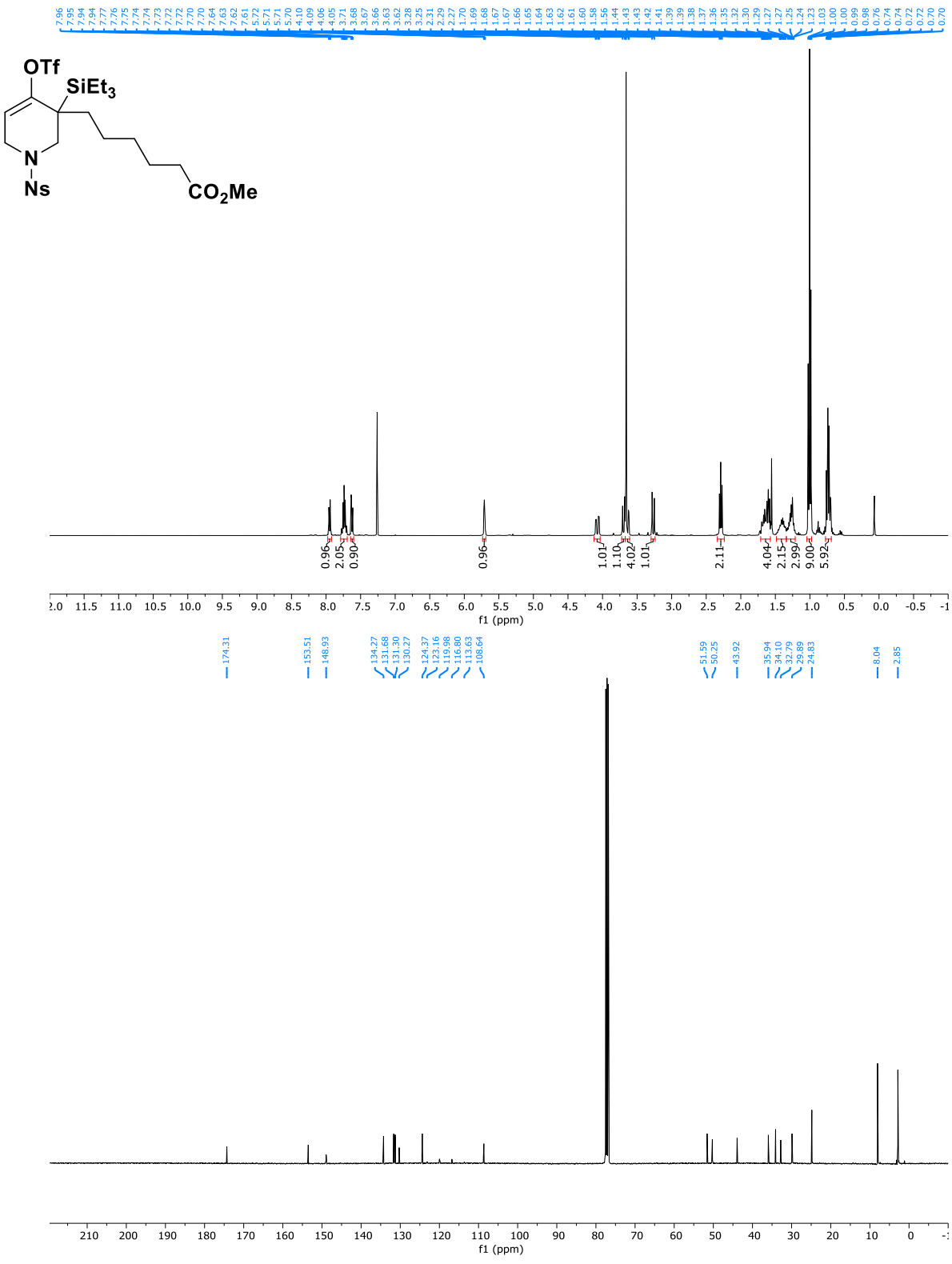

OTf
Me
SiEt3
N
O
O
CO2Me
f1 (ppm)
f1 (ppm)

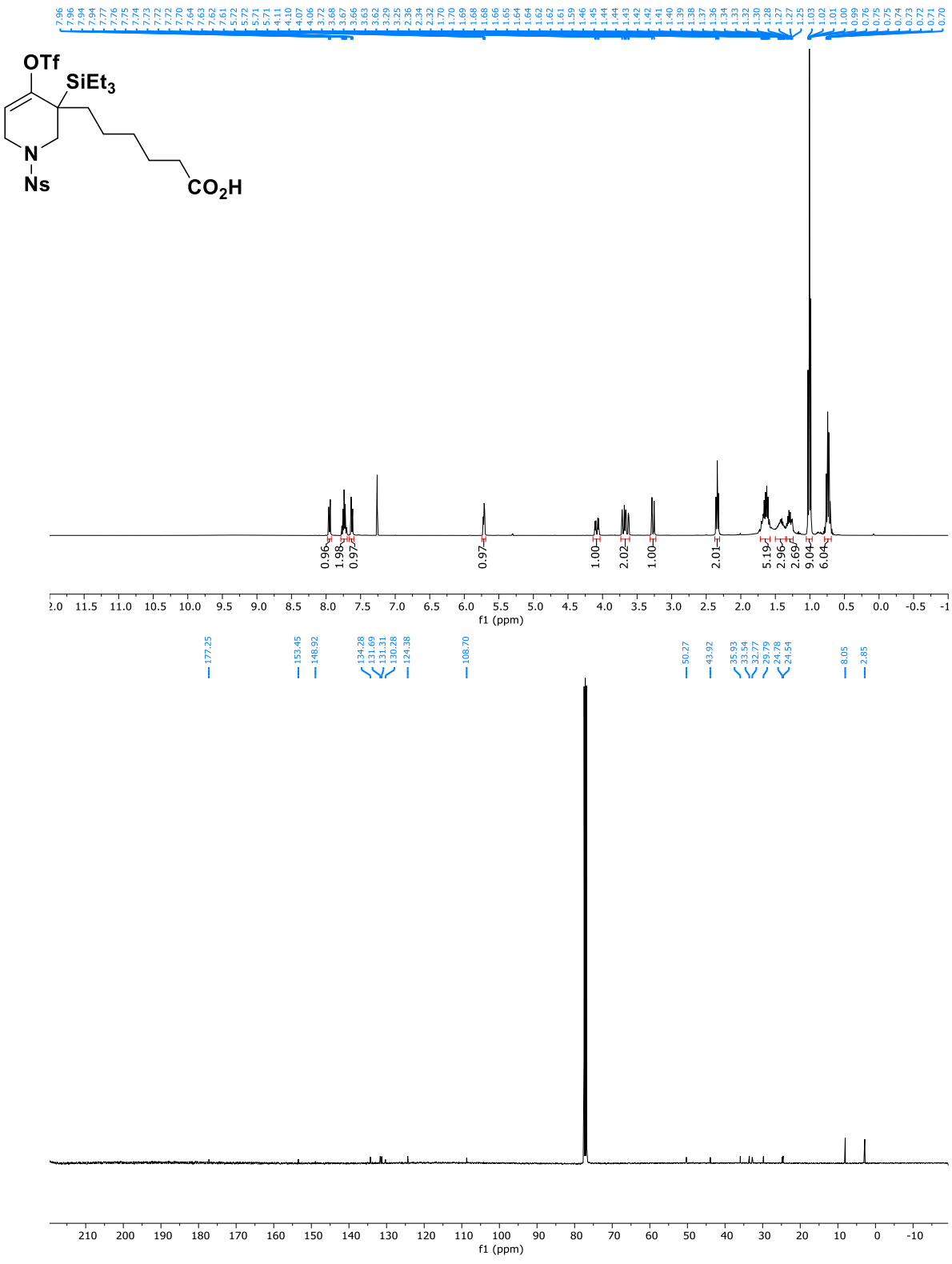

OTf
Me
TES
N
O
O
CO2H
8.02
8.02
8.01
8.00
7.14
7.12
5.38
5.37
5.36
5.02
4.99
4.95
4.91
3.76
3.72
3.71
3.71
3.65
3.64
3.60
3.59
3.56
3.17
3.14
1.07
0.91
0.90
0.89
0.87
0.61
0.59
0.57
0.56
0.55
2.00
1.96
0.96
2.00
0.97
2.02
1.01
3.10
9.03
6.00
f1 (ppm)

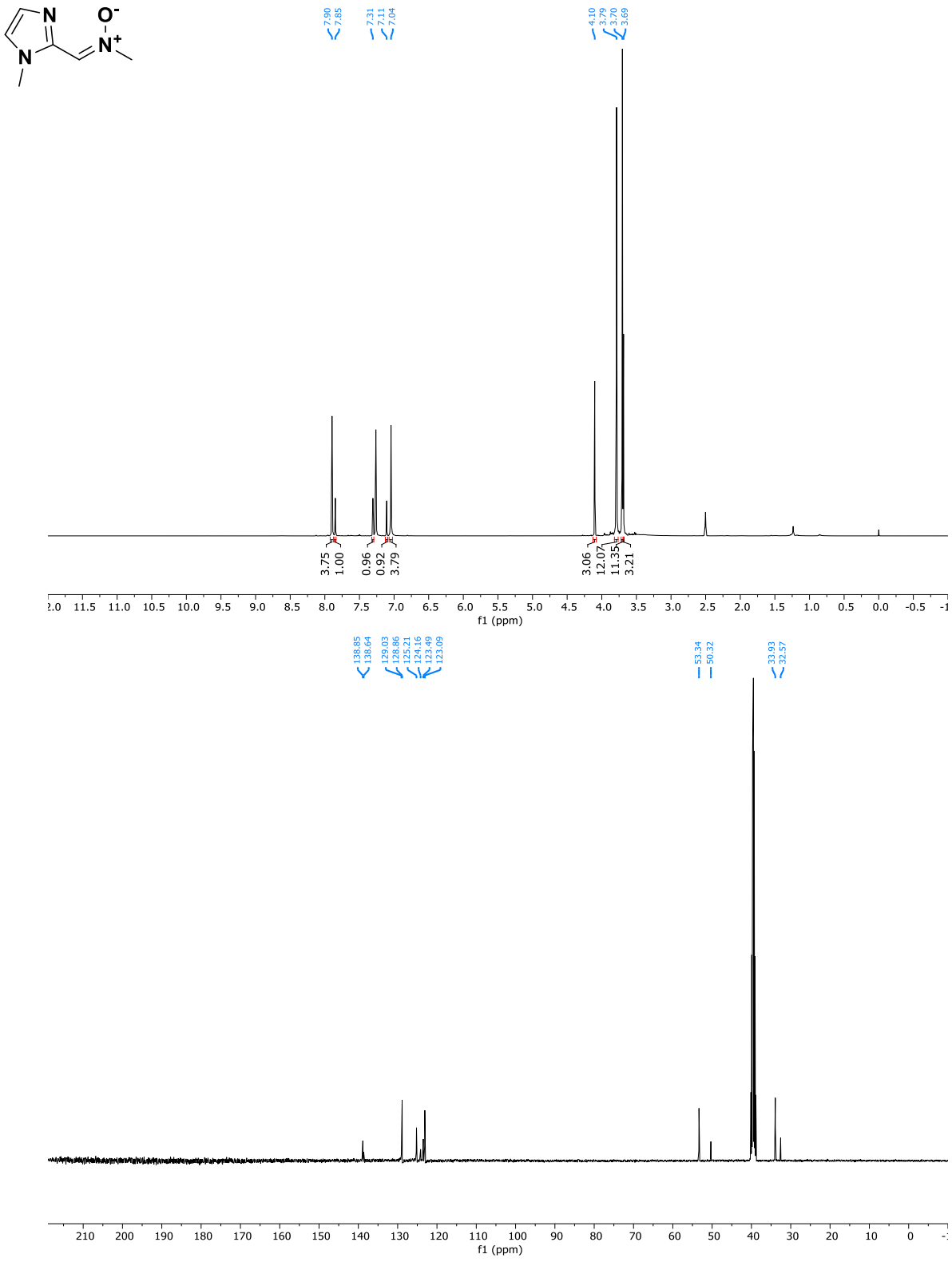

154.92
142.95
130.80
110.12
66.94
50.84
42.77
31.89
18.67
7.79
3.02
f1 (ppm)

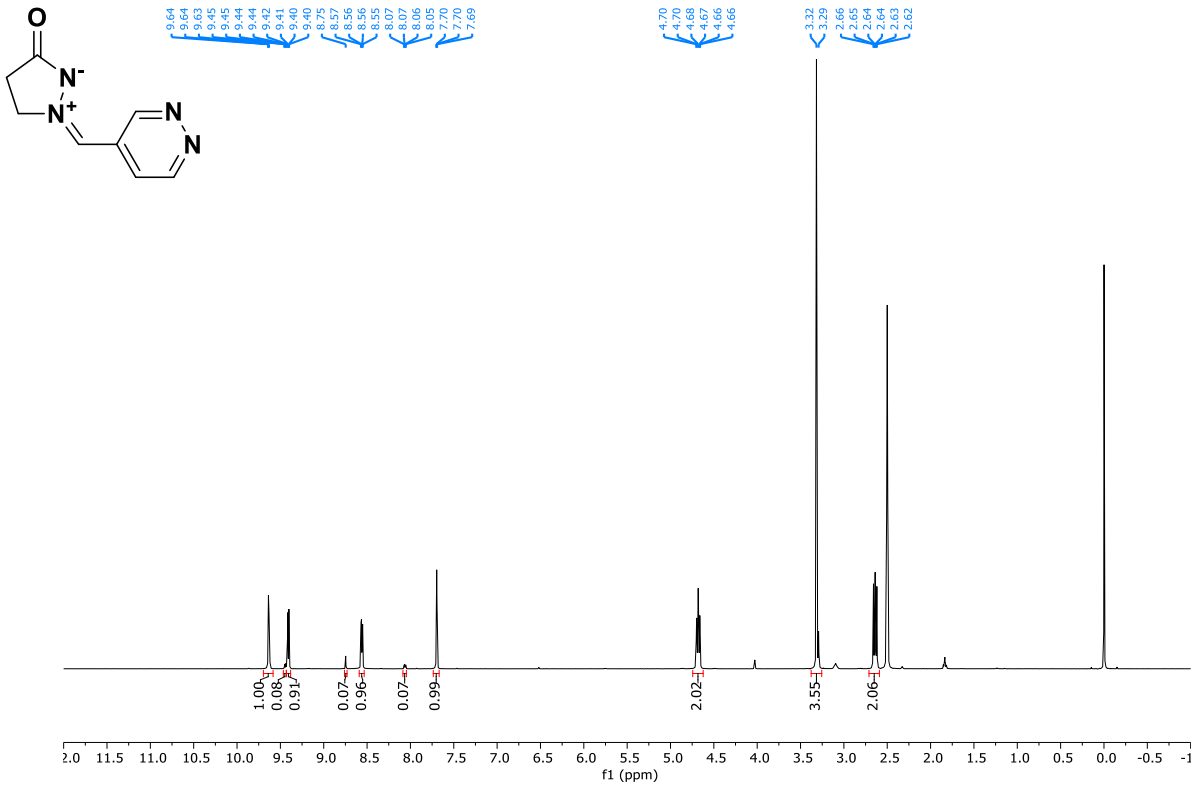

O
SiEt3
N
O
O
7.77
6.00
5.98
5.97
5.96
5.94
5.93
5.91
5.90
5.38
5.37
5.33
5.30
5.30
5.27
4.72
4.71
4.71
4.70
4.70
4.00
3.99
3.98
3.97
3.96
2.53
2.51
2.49
0.90
0.88
0.86
0.70
0.68
0.66
0.64
1.00
1.00
1.99
2.05
2.12
2.13
9.23
6.21
f1 (ppm)
196.69
152.78
148.06
131.58
119.12
113.25
67.77
42.56
36.16
7.45
2.97
f1 (ppm)

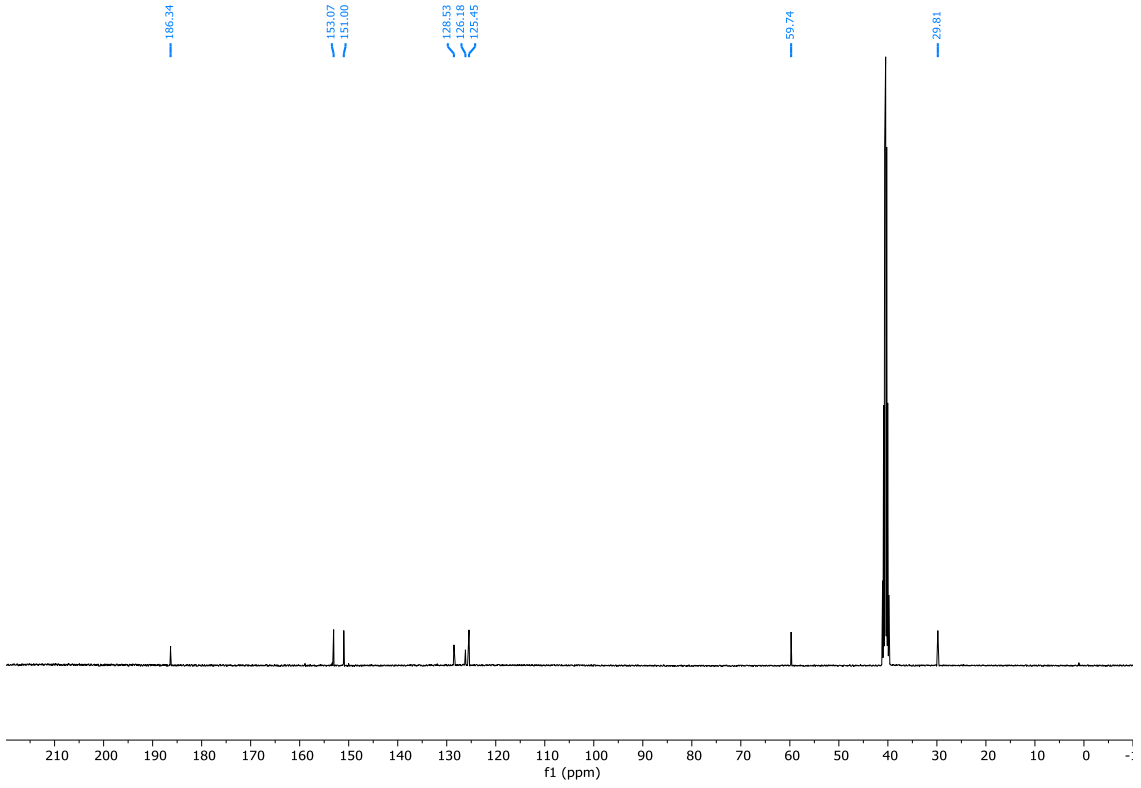

O
SiEt3
CO2Me
N
O
O
f1 (ppm)
f1 (ppm)

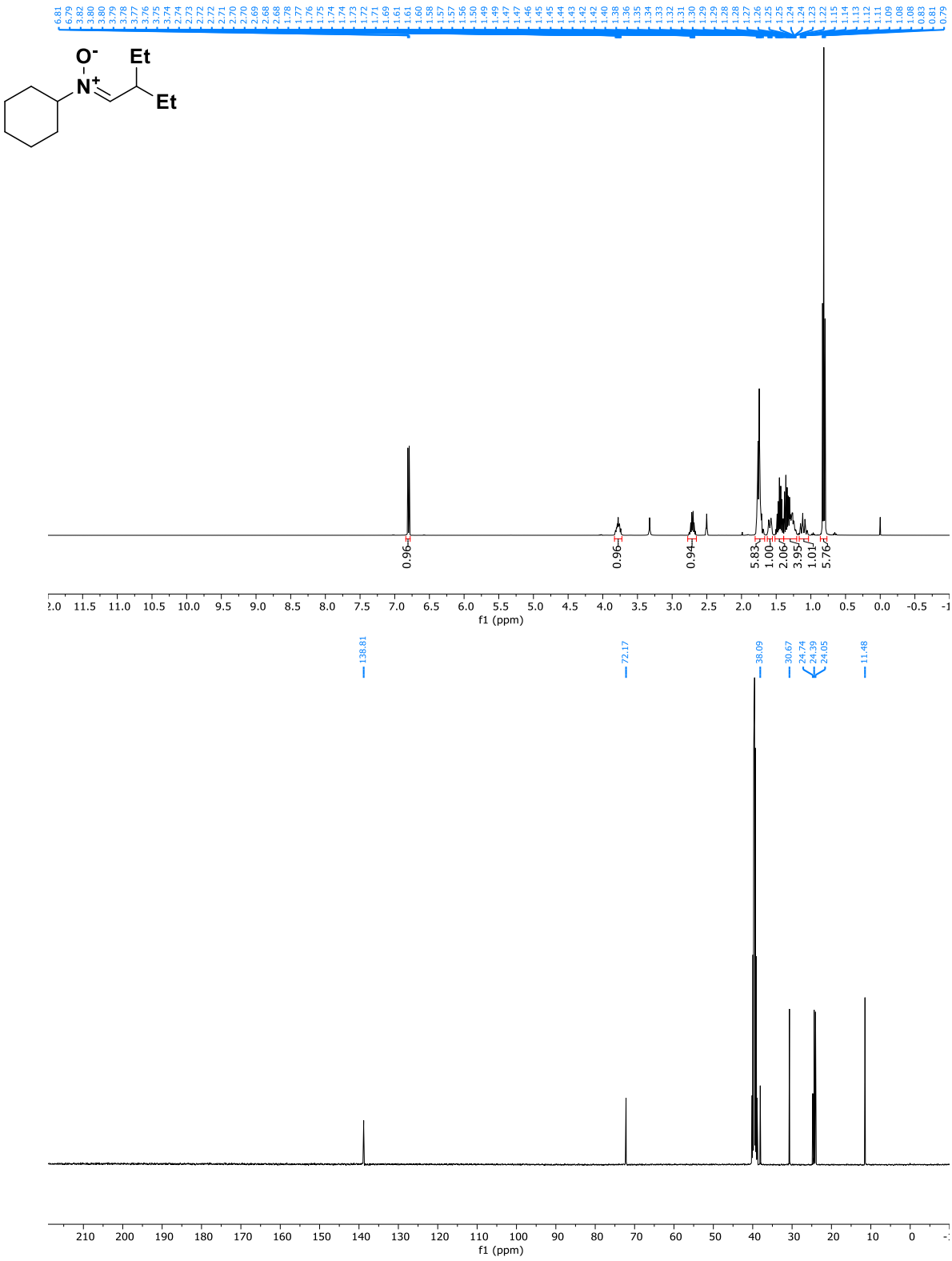

OTf
SiEt3
N
Ns
CO2Me
f1 (ppm)
f1 (ppm)

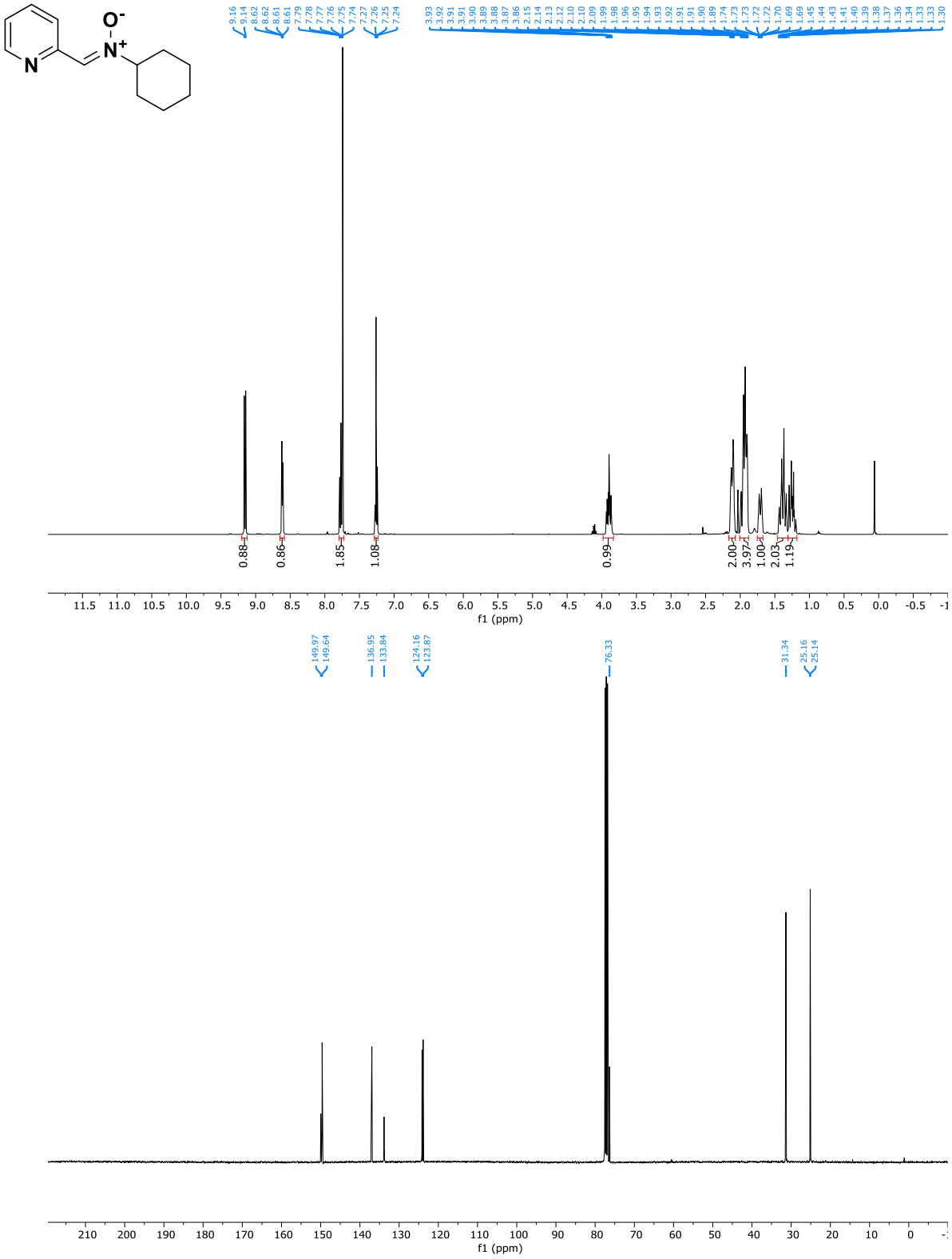

OTf
SiEt3
N
Ns
CO2H
f1 (ppm)
f1 (ppm)

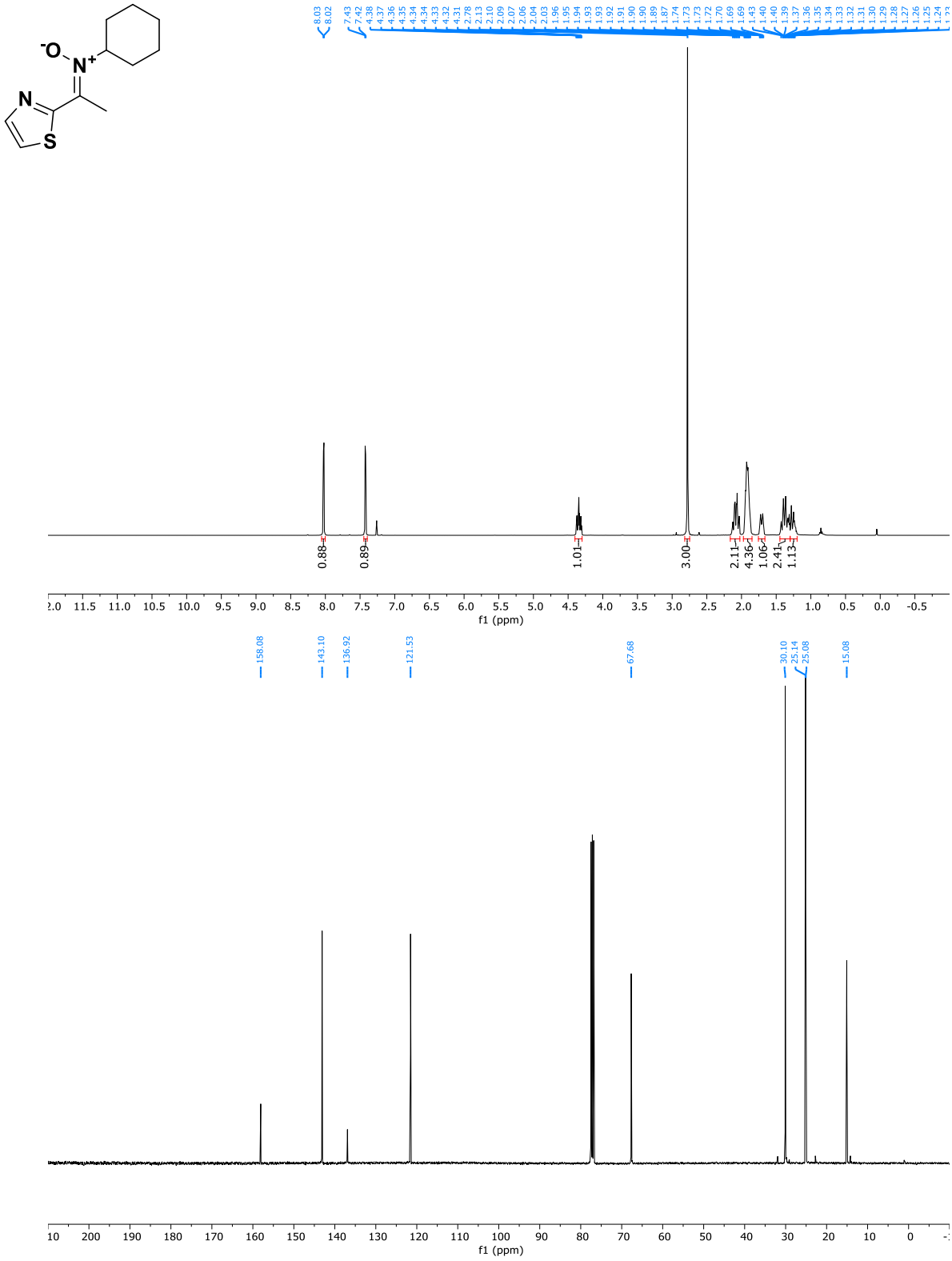

7.90
7.85
7.31
7.11
7.04
4.10
3.79
3.70
3.69
3.75
1.00
0.96
0.92
3.79
3.06
12.07
11.35
3.21
f1 (ppm)
138.85
138.64
129.03
128.86
125.21
124.16
123.49
123.09
53.34
50.32
33.93
32.57
f1 (ppm)

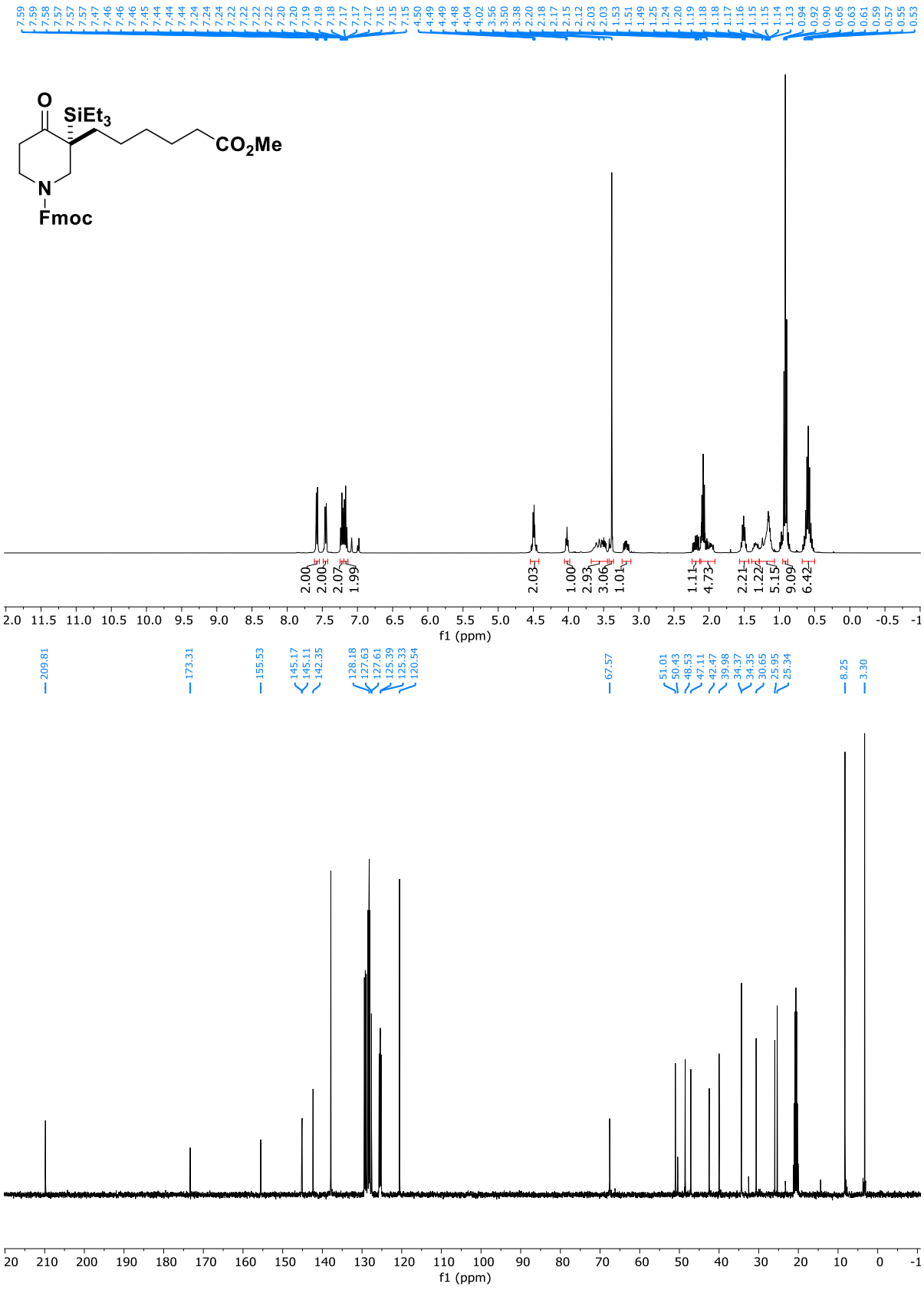

9.64
9.64
9.63
9.45
9.45
9.44
9.44
9.42
9.41
9.40
9.40
8.75
8.57
8.56
8.56
8.55
8.07
8.07
8.06
8.05
7.70
7.70
7.69
4.70
4.70
4.68
4.67
4.66
4.66
3.32
3.29
2.66
2.65
2.64
2.64
2.63
2.62
1.00
0.08
0.91
0.07
0.96
0.07
0.99
2.02
3.55
2.06
f1 (ppm)

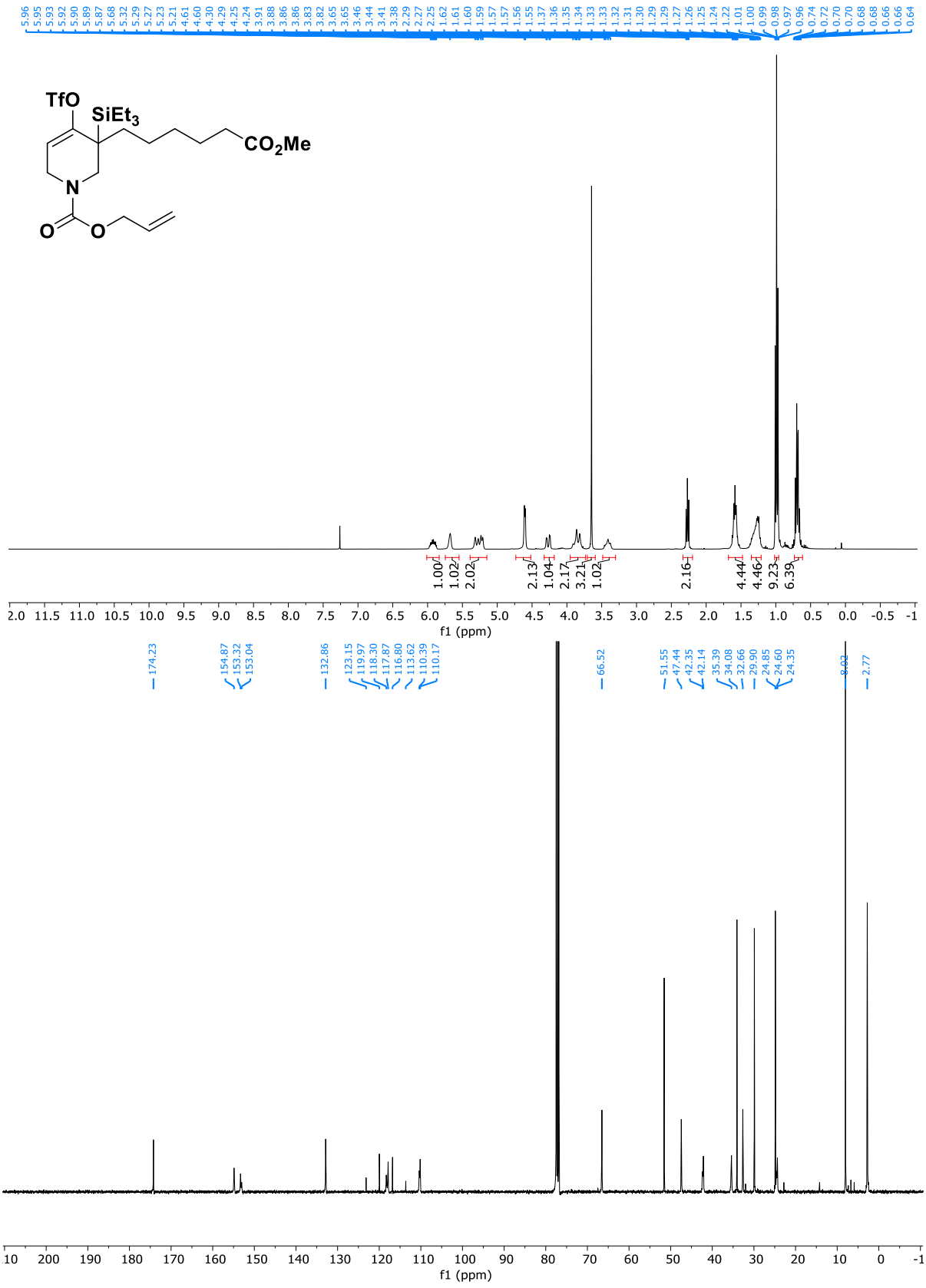

186.34
153.07
151.00
128.53
126.18
125.45
59.74
29.81
f1 (ppm)

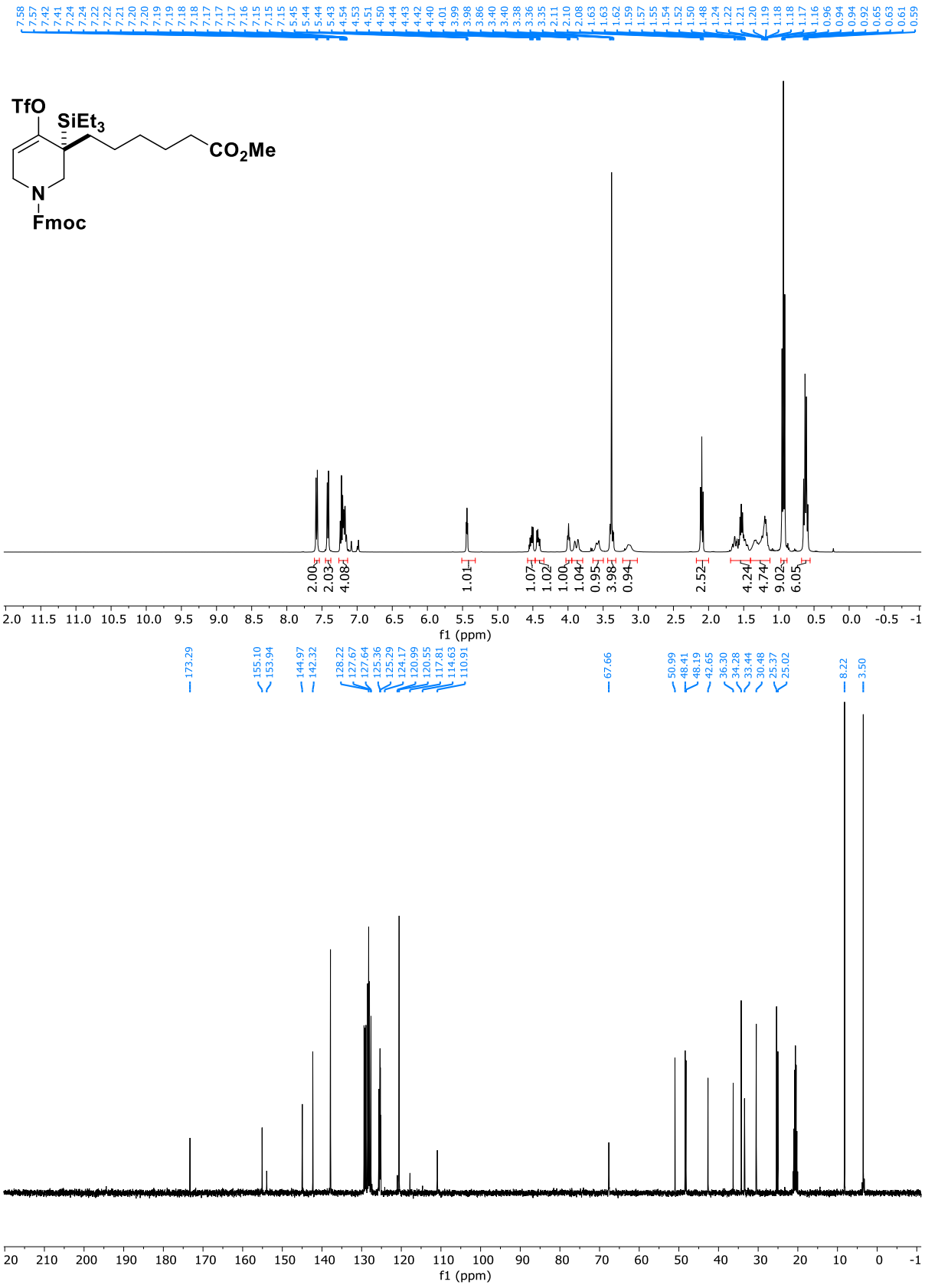

O⁻
N⁺
Et
Et
f1 (ppm)
f1 (ppm)

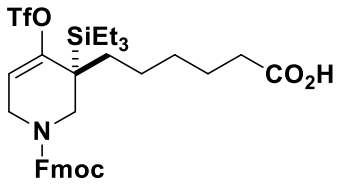

O-
N+
N
9.16 9.14 8.62 8.62 8.61 8.61 7.79 7.78 7.77 7.76 7.75 7.74 7.27 7.26 7.25 7.24
3.93 3.92 3.91 3.91 3.90 3.89 3.88 3.87 3.86 2.15 2.14 2.13 2.12 2.10 2.10 2.09 1.99 1.98 1.96 1.95 1.94 1.93 1.92 1.91 1.91 1.90 1.89 1.74 1.73 1.73 1.72 1.72 1.70 1.69 1.69 1.45 1.44 1.43 1.41 1.40 1.39 1.38 1.37 1.36 1.34 1.33 1.33 1.30
0.88 0.86 1.85 1.08 0.99 2.00 3.97 1.00 2.03 1.19
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)
149.97 149.64 136.95 133.84 124.16 123.87 76.33 31.34 25.16 25.14
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

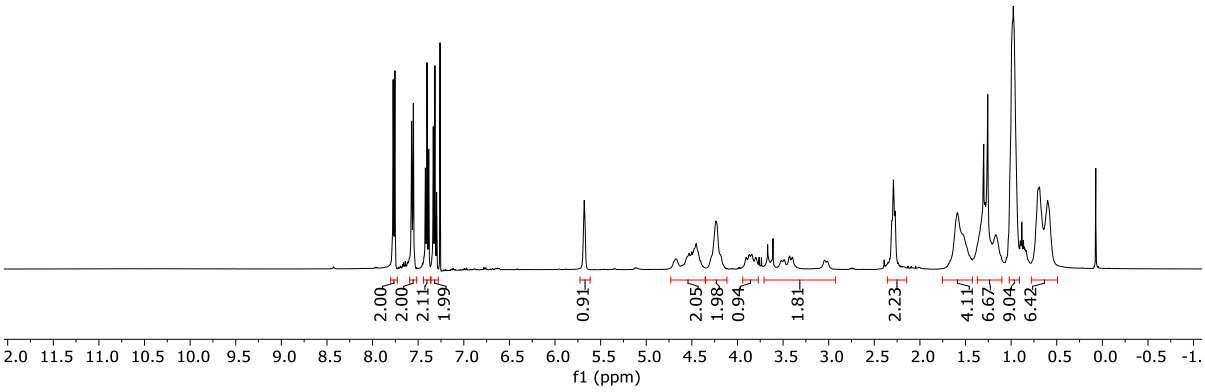

8.03
8.02
7.43
7.42
4.38
4.37
4.36
4.35
4.34
4.34
4.33
4.32
4.31
2.78
2.13
2.10
2.09
2.07
2.06
2.04
2.03
1.96
1.95
1.94
1.93
1.93
1.92
1.91
1.90
1.90
1.89
1.87
1.74
1.73
1.73
1.72
1.70
1.69
1.69
1.43
1.40
1.40
1.39
1.37
1.36
1.35
1.34
1.33
1.32
1.31
1.30
1.29
1.28
1.27
1.26
1.25
1.24
0.88
0.89
1.01
3.00
2.11
4.36
1.06
2.41
1.13
f1 (ppm)
158.08
143.10
136.92
121.53
67.68
30.10
25.14
25.08
15.08
f1 (ppm)

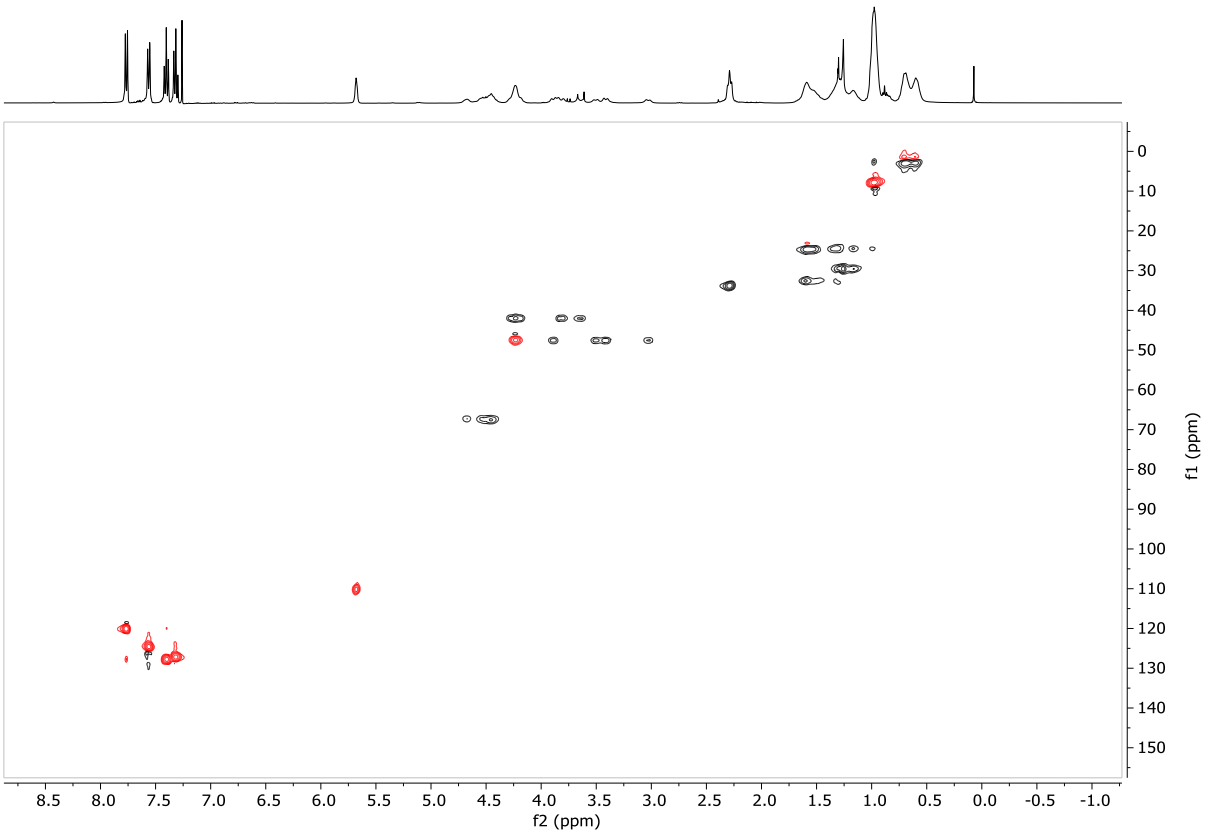

O
SiEt3
CO2Me
N
Fmoc
f1 (ppm)
f1 (ppm)


TfO
SiEt3
CO2Me
N
O
O
f1 (ppm)
f1 (ppm)


TfO
SiEt3
CO2Me
N
Fmoc
f1 (ppm)
f1 (ppm)


TfO
SiEt3
CO2H
N
Fmoc


2.00
2.00
2.11
1.99
0.91
2.05
1.98
0.94
1.81
2.23
4.11
6.67
9.04
6.42
f1 (ppm)


f2 (ppm)
f1 (ppm)

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