Highly efficient on-DNA amide couplings promoted by micelle forming surfactants for the synthesis of DNA encoded libraries

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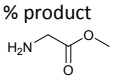
Supplementary information

Table S1 – Base screen. Conditions: 0.5M amine, 0.5M HATU, 1.5M base, **2** (10 nmol), 0.5M HATU, 30 µL reaction volume, 40 °C.

Base	% product	
		
DIPEA	90	10
Lutidine	97	42
N-methylmorpholine	72	0
Triethylamine	73	6
2,6-ditertbutyl-4-methylpiperidine	93 (6)	2
N-methylimidazole	87 (4)	27 (32)
Pyridine	95 (4)	21
DMAP	17	0
4-methoxypyridine	93 (4)	27
Quinoline	95 (3)	4
DABCO	64 (5)	0
4-Cyanopyridine	48	0

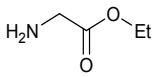
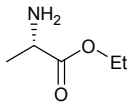
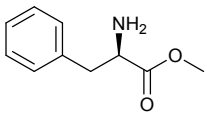
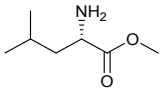
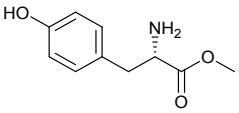
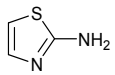
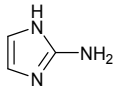
Side product amount shown in parentheses where applicable, remainder is unreacted **2**.

Table S2 – Optimisation of HATU coupling by Factorial Experimental Design. Conditions: **2** (5 nmol), amine (0.5M), 2,6-lutidine, HATU (0.5M), TPGS-750-M, 30 µL total volume, 16 h.

Run	Amine	Temp / °C	TPGS %	[base] / M	% Product	% Starting material	% Side product
1	2-Aminoimidazole	40	2	1	43	53	4
1	Glycine methyl ester	40	3.5	1.5	92	3	5
1	2-Aminoimidazole	40	5	1.5	48	40	12
1	2-Aminoimidazole	40	3.5	0.5	70	30	0
1	Glycine methyl ester	40	2	0.5	92	3	5
1	Glycine methyl ester	40	5	0.5	93	2	5
1	2-Aminoimidazole	50	3.5	1	59	31	10
1	Glycine methyl ester	50	3.5	0.5	92	4	4
1	2-Aminoimidazole	50	2	1.5	63	25	12
1	Glycine methyl ester	50	3.5	1	94	3	3
1	Glycine methyl ester	50	2	1.5	94	3	3
1	2-Aminoimidazole	50	5	0.5	38	59	3
1	Glycine methyl ester	50	5	1	94	3	3
1	2-Aminoimidazole	50	3.5	1	63	27	10

1	2-Aminoimidazole	60	5	1	50	32	18
1	Glycine methyl ester	60	5	0.5	2	1	97
1	2-Aminoimidazole	60	3.5	1.5	61	28	11
1	2-Aminoimidazole	60	2	0.5	2	2	96
1	Glycine methyl ester	60	2	1	19	2	79
1	Glycine methyl ester	60	5	1.5	69	8	23
2	2-Aminoimidazole	45	2	1.5	66	24	10
2	Glycine methyl ester	55	5	2.5	85	6	9
2	2-Aminoimidazole	45	5	2.5	41	56	3
2	Glycine methyl ester	55	3.5	1.5	71	3	26
2	Glycine methyl ester	55	2	2.5	87	4	9
2	2-Aminoimidazole	55	2	2.5	53	26	21
2	Glycine methyl ester	45	2	2.5	93	3	4
2	2-Aminoimidazole	55	5	1.5	63	27	10

Table S3 - Scope of the initial coupling conditions optimised by factorial design. Conditions: **2** (5 nmol), amine (0.5M), 2,6-lutidine (2M), HATU (0.5M), 3.5% TPGS, 30 µL total volume, 45 °C, 16 h.

Amine	% Conversion	% Product
	100	100
	100	100
	100	100
	100	100
	96	100
	94	100
	47	53
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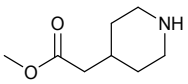
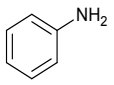
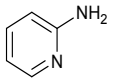
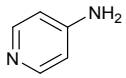
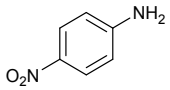
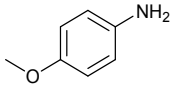
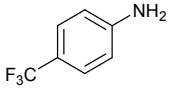
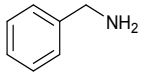
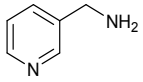
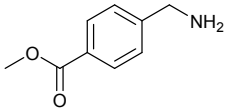
	100	100

	93	7
	50	50
	14	86
	17	1
	76	24
	64	100
	45	54
	90	10
	100	100
	90	10

Side product is the dimethylamide.

Table S4 – Coupling of NH_2-PEG_4-hexylamido-DNA with a diverse set of acids. Conditions: NH_2-PEG_4-hexylamido-DNA (5 nmol), acid (0.5M), 2,6-lutidine (2M), HATU (0.5M), 3.5% TPGS, 30 µL total volume, 45 °C, 16 hr.

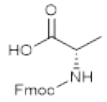
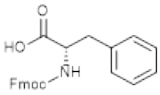
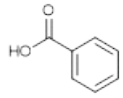
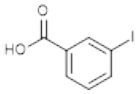
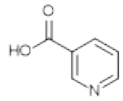
Amine	% Conversion	% Product
	86%	86%
	67%	67%
	100%	86%
	100%	100%
	100%	97%

Table S5 - Results of coupling agent screen; Conditions: **2** (5 nmol), amine (0.5M), 2,6-lutidine (2M), HATU (0.5M), 3.5% TPGS, 30 µL total volume, 45 °C, 16 hr.

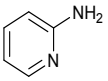
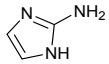
					
	% product	% starting material	% side product	% product	% starting material	% side product
COMU	0	54	46	0	42	37
BOP	0	7	93	0	36	64
PBOP	0	100	0	0	100	0
DCC, HOAT	57	43	0	23	77	0
EDC, HOAT	0	100	0	0	100	0
DIC, HOAT	74	25	1	64	36	0

Table S6. Optimisation of DIC mediated coupling by factorial design. Conditions: **2** (10 nmol), amine (0.5M), 2,6-lutidine, DIC, TPGS-750-M, 30 µL total volume, 3 h.

Amine	Temp / °C	TPGS %	[Base] / M	% Product	% Starting material	% Side product
2-Aminoimidazole	40	2	1	43	54	4
Glycine ethyl ester	40	3.5	1.5	92	3	5
2-Aminoimidazole	40	5	1.5	48	40	12
2-Aminoimidazole	40	3.5	0.5	70	30	0
Glycine ethyl ester	40	2	0.5	92	3	5
Glycine ethyl ester	40	5	0.5	93	2	5
2-Aminoimidazole	50	3.5	1	59	30	10
Glycine ethyl ester	50	3.5	0.5	92	4	4
2-Aminoimidazole	50	2	1.5	63	24	12
Glycine ethyl ester	50	3.5	1	94	3	3
Glycine ethyl ester	50	2	1.5	94	3	3
2-Aminoimidazole	50	5	0.5	38	59	3
Glycine ethyl ester	50	5	1	94	3	3
2-Aminoimidazole	50	3.5	1	63	26	10
2-Aminoimidazole	60	5	1	50	32	18
Glycine ethyl ester	60	5	0.5	2	1	97
2-Aminoimidazole	60	3.5	1.5	61	28	11
2-Aminoimidazole	60	2	0.5	2	4	96
Glycine ethyl ester	60	2	1	19	2	79
Glycine ethyl ester	60	5	1.5	69	8	13

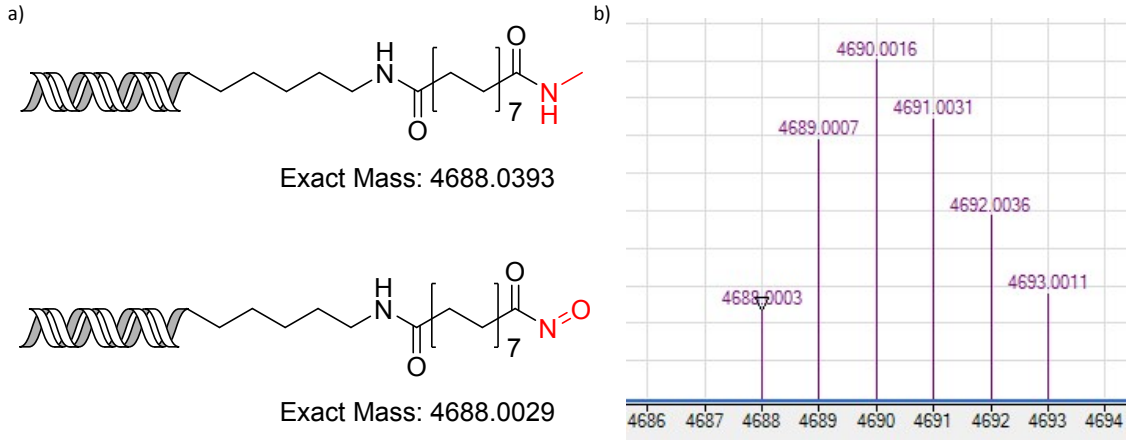
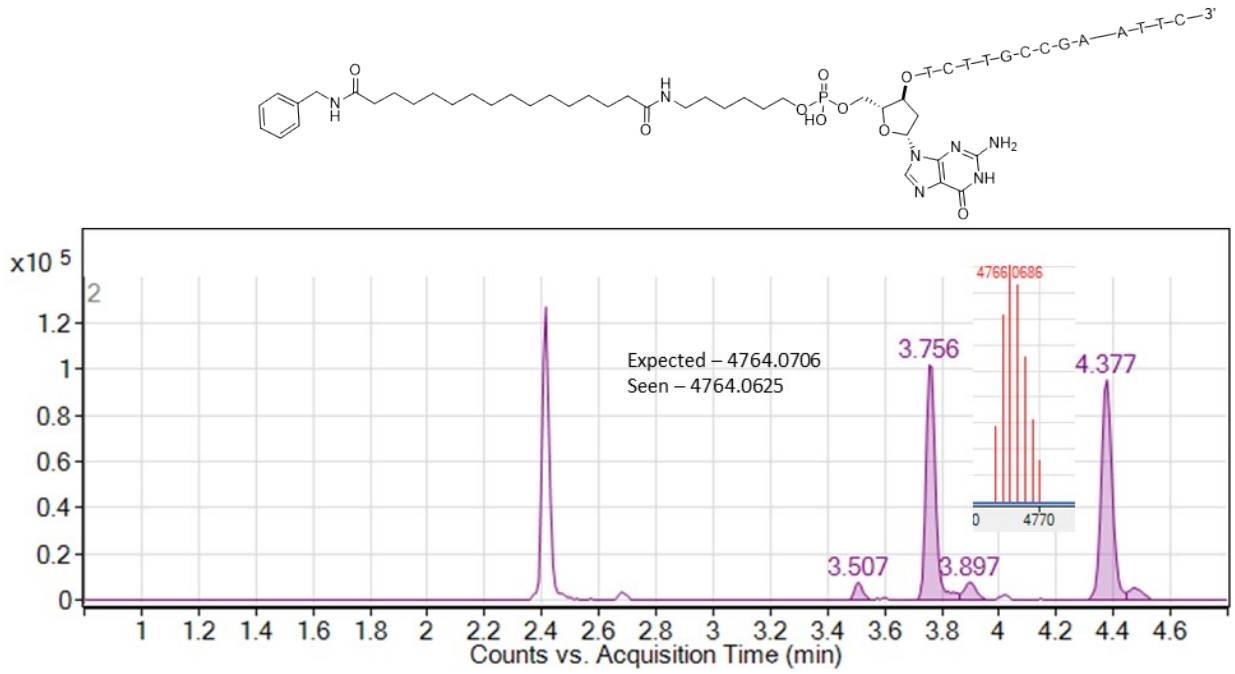


Figure S1 – a) Possible side products formed in reverse amide bond formation including predicted mass; b) observed molecular weight of major side product adduct formed in reverse amide reaction.



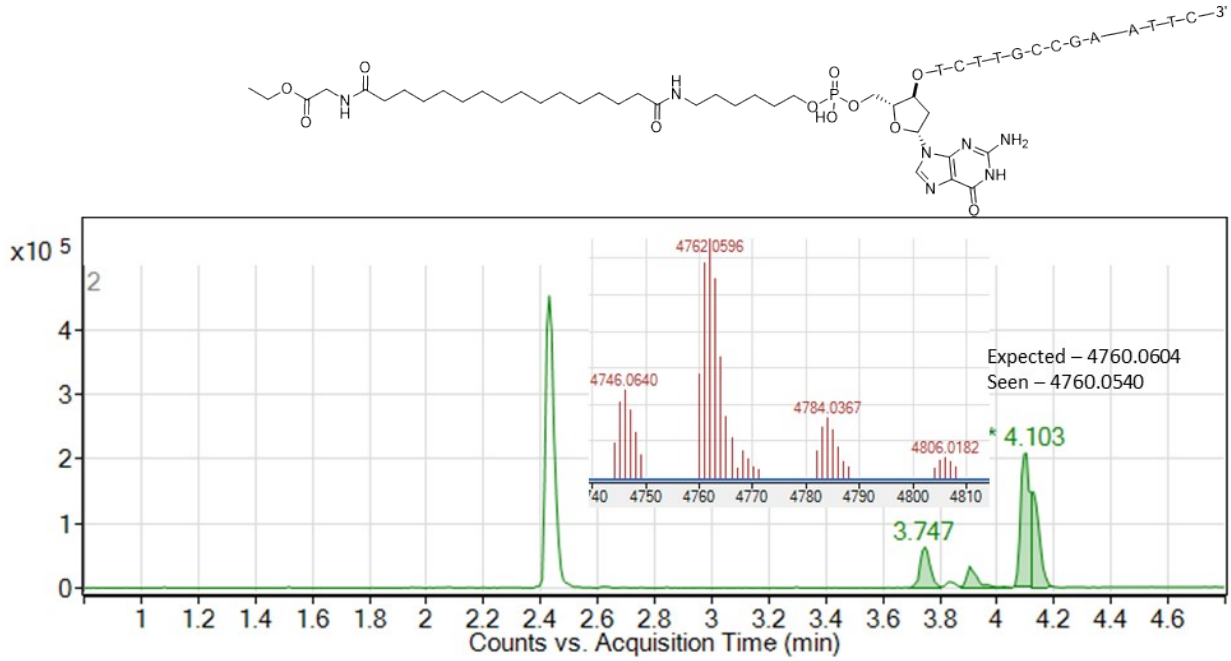


Figure S2 – Results with optimised conditions in the absence of micelles. Conditions: i) benzylamine or glycine ethyl ester solution (60 µl, 0.25 M in NMP) and HOAT (20 µl, 10 mg per 100 µl in NMP) heated at 55°C in Genevac (60 mins) to remove the NMP; ii) **2** (30 µl, 5 nmol in water), 2,6-lutidine (5.2 µl, 0.045 mmol) and DIC (2.2 µl, 0.015 mmol), 45°C, 5 h.

Experimental

The amines were used in the following salt forms

Glycine ethyl ester	Hydrochloride
Alanine ethyl ester	Hydrochloride
D-Phenylalanine methyl ester	Hydrochloride
L-Leucine methyl ester	Hydrochloride
L-Tyrosine methyl ester	Hydrochloride
Azetidine	Free base
Morpholine	Free base
4-Piperidine acetic acid methyl ester	Free base
Benzylamine	Free base
Methyl 4-(aminomethyl)benzoate	Hydrochloride
3-Picolylamine	Free base
Aniline	Free base
4-Nitroaniline	Free base
p-Anisidine	Free base

4-Trifluoromethylaniline	Free base
2-Aminopyridine	Free base
4-Aminopyridine	Free base
2-Aminothiazole	Free base
2-Aminoimidazole	Hemisulphate
Asparigine methyl ester	Hydrochloride
Cyclohexanemethylamine	Free base
Piperidine	Free base
1-(Cyclopropylcarbonyl)piperazine	Free base
o-Anisidine	Free base
3-Chloroaniline	Free base
4-Chlorobenzylamine	Free base
4-Fluoroaniline	Free base
Methyl 5-aminopyridine-3-carboxylate	Hydrochloride
Methyl 6-aminopyridazine-3-carboxylate	Free base

Head piece synthesis

MMT deprotection

The average loading of single stranded DNA attached to solid support was found by cleavage from the solid support using the below method and repeating 3 times. Nanodrop concentration of cleaved DNA showed that 103 mg gave 2 µmol of DNA.

The single stranded DNA used was a 14mer (GTCTTGCCGAATTC) with a 5' MMT amino C6 linker bound to solid support at the 3' end. The solid supported DNA (103 mg, ca. 2 µmol) was washed with 5% trichloroacetic acid in DCM (6 x 500 µl). A yellow colour indicates that the deprotection is in progress. Once this colour subsides the solid supported DNA was washed with DCM (3 x 500 µl) and left to air dry for 20 mins before coupling to the headpiece.

Acid headpieces 1 and 2

In a 1.5 ml micro centrifuge tube was added HATU (17 mg, 44 µmol), DIPEA (17 µl, 100 µmol) and DMF (1 ml). To this was added either hexadecanedioic acid (17 mg, 40 µmol) or 3,6,9,12,15-pentaoxaheptadecanedioic acid (18 mg, 40 µmol) and the mixture shaken for 20 mins at room temperature. The deprotected solid supported DNA (ca. 2 µmol) was added and the mixture shaken at room temperature overnight. The mixture was then filtered and washed with DMF (3 x 500 µl), MeCN (3 x 500 µl), MeOH (3 x 500 µl) and DCM (3 x 500 µl) and allowed to air dry for 20 mins. Water (1.5 ml) was added and the mixture shaken for 1 hour at room temperature, before filtration and washing with water (3 x 500 µl). 40% methylamine in water (500 µl) and 33% ammonia in water (500 µl) were mixed in a 1.5 ml centrifuge tube. The solid supported DNA was then added and the mixture shaken for 1 hour at room temperature. The mixture was then filtered and washed with water (3 x 500 µl) and concentrated to ~0.5 ml using a Genevac at 40°C. The crude product was then purified by HPLC, fractions concentrated using a Genevac at 40°C and dissolved in water (1 ml). The concentration of the samples was then quantified by UV using a NanoDrop1 by Thermofisher. The usual amount was ca. 0.5-1 µmol of DNA after HPLC purification. The exact amount of the complimentary 14mer

(GAATTCGGCAAGAC) was then added in water, and the mixture heated to 80°C for 1 hour, allowing to cool slowly. The double stranded DNA was then concentrated using a Genevac at 40°C until dry and dissolved in either water or 2% TPGS-750-M in water to form a 1 mM solution of the acid. The solution was then plated into wells of 20 µl with 20 nmol of DNA per well and frozen at -20°C. The samples were defrosted at room temperature for 30 mins prior to use in reactions.

Amine headpiece 3

In a 1.5 ml micro centrifuge tube was added HATU (17 mg, 44 µmol), DIPEA (17 µl, 100 µmol) and DMF (1 ml). To this was added 12-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)dodecanoic acid (26 mg, 40 µmol) and the mixture shaken for 20 mins at room temperature. The deprotected solid supported DNA (ca. 2 µmol) was added and the mixture shaken at room temperature overnight. The mixture was then filtered and washed with DMF (3 x 500 µl), MeCN (3 x 500 µl), MeOH (3 x 500 µl) and DCM (3 x 500 µl) and allowed to air dry for 20 mins. 40% methylamine in water (500 µl) and 33% ammonia in water (500 µl) were mixed in a 1.5 ml centrifuge tube. The solid supported DNA was then added and the mixture shaken for 1 hour at room temperature. The mixture was then filtered and washed with water (3 x 500 µl) and concentrated to ~0.5 ml using a Genevac at 40°C. The crude product was then purified by HPLC, fractions concentrated using a Genevac at 40°C and dissolved in water (1 ml). The concentration of the samples was then quantified by UV using a NanoDrop1 by Thermofisher. The usual amount was ca. 0.5-1 µmol of DNA after HPLC purification. The exact amount of the complimentary 14mer (GAATTCGGCAAGAC) was then added in water, and the mixture heated to 80°C for 1 hour, allowing to cool slowly. The double stranded DNA was then concentrated using a Genevac at 40°C until dry and dissolved in either water or 2% TPGS-750-M in water to form a 1 mM solution of the product. The solution was then plated into wells of 20 µl with 20 nmol of DNA per well and frozen at -20°C. The samples were defrosted at room temperature for 30 mins prior to use in reactions.

Coupling amines to acid headpiece

An aliquot of amine solution (60 µl, 0.25 M in NMP) and HOAT (20 µl, 10 mg per 100 µl in NMP) were added to a 50 µl glass insert for a Para-dox™ 96-well micro photoredox/optimisation Plate. The NMP was then removed at 55°C in a Genevac for 60 mins. To this solution was added the acid headpiece (30 µl, 5 nmol in 4.5% TPGS-750-M in water), 2,6-lutidine (5.2 µl, 0.045 mmol) and DIC (2.2 µl, 0.015 mmol). The vials were vortexed for 30 seconds each to enhance mixing. The samples were then heated in a Para-dox™ 96-well micro photoredox/optimisation plate at 45°C for 5 hours. Mass spectrometry was used to analyse reactions. Samples prepared by adding reaction mixture (1 µl) to water (20 µl) and filtered through a hydrophilic PTFE filter. To purify each sample they were diluted with water (50 µl), DCM (2 x 100 µl) was added to each and the vial vortexed. If an emulsion remained, the sample was centrifuged to aid separation. The organics were removed, and aqueous washed with ethyl acetate (2 x 100 µl). Aqueous sodium chloride (8 µl, 4M) and ethanol (264 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in water to give a 1mM solution.

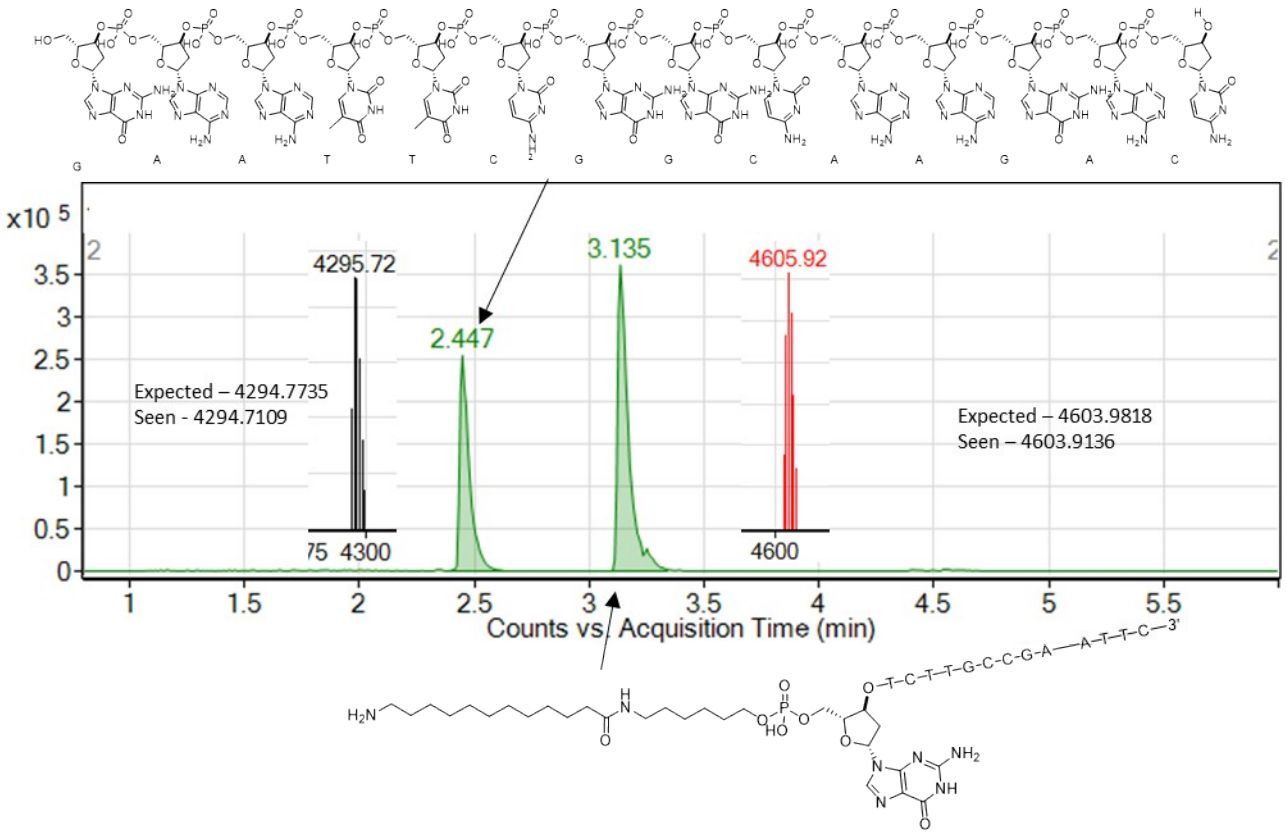
Coupling acids to amine headpiece

An aliquot of acid solution (60 µl, 0.25 M in NMP) was added to a 50 µl glass insert for a Para-dox™ 96-well micro photoredox/optimisation Plate. The NMP was then removed at 55°C in a Genevac for 60 mins. To this solution was added the amine headpiece (30 µl, 5 nmol in 3.5% TPGS-750-M in water), 2,6-lutidine (6.92 µl, 0.06 mmol) and HATU (5.7 mg, 0.015 mmol). The vials were vortexed for 30 seconds each to enhance mixing. The samples were then heated in a Para-dox™ 96-well micro photoredox/optimisation plate at 45°C overnight. Mass spectrometry was used to analyse reactions. Samples prepared

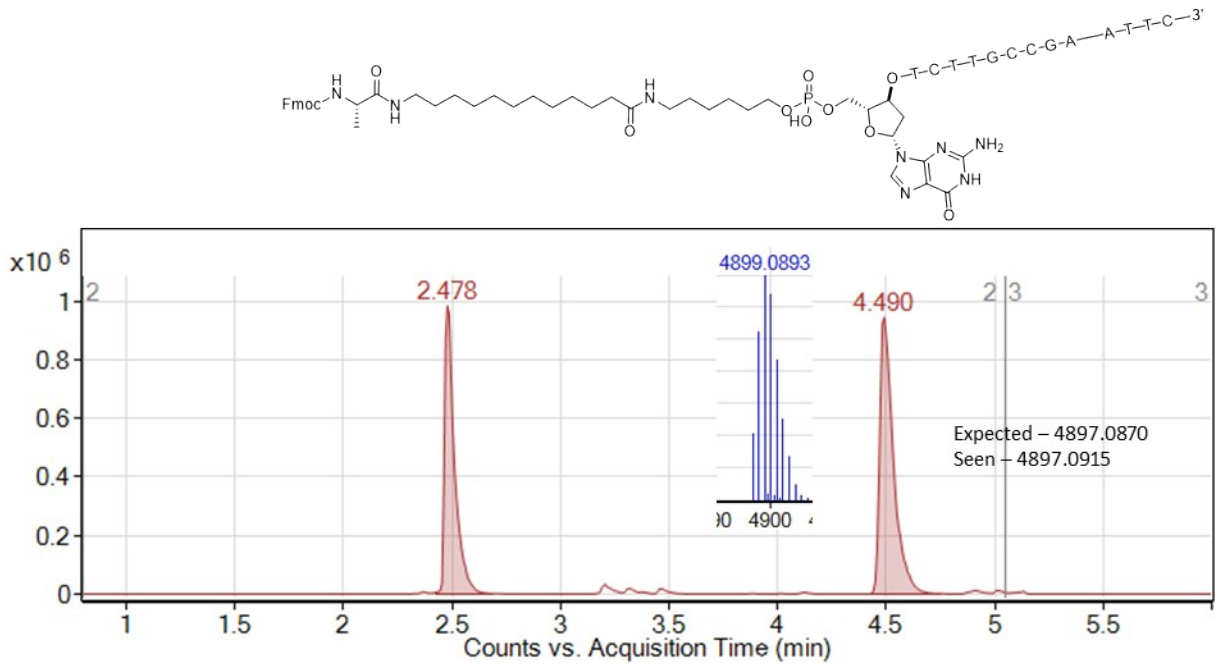
by adding reaction mixture (1 µl) to water (20 µl) and filtered through a hydrophilic PTFE filter. To purify each sample they were diluted with water (50 µl), DCM (2 x 100 µl) was added to each and the vial vortexed. If an emulsion remained, the sample was centrifuged to aid separation. The organics were removed, and aqueous washed with ethyl acetate (2 x 100 µl). Aqueous sodium chloride (8 µl, 4M) and ethanol (264 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in water to give a 1mM solution

Chromatograms and mass spectra

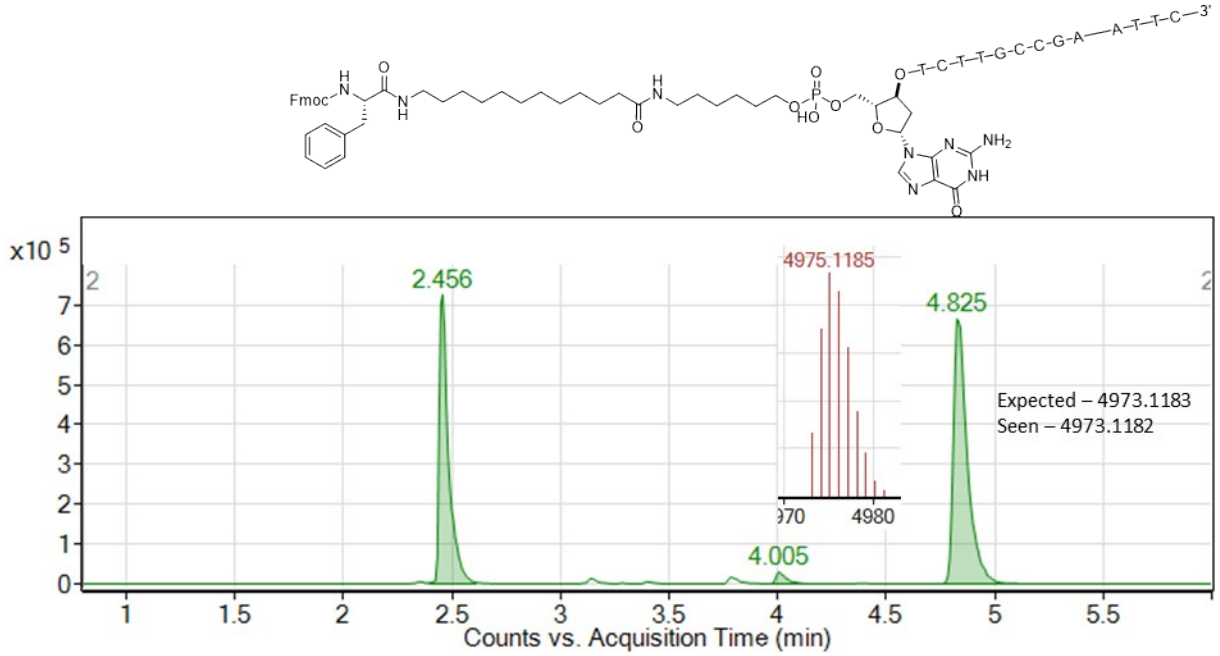
Amine headpiece **3**



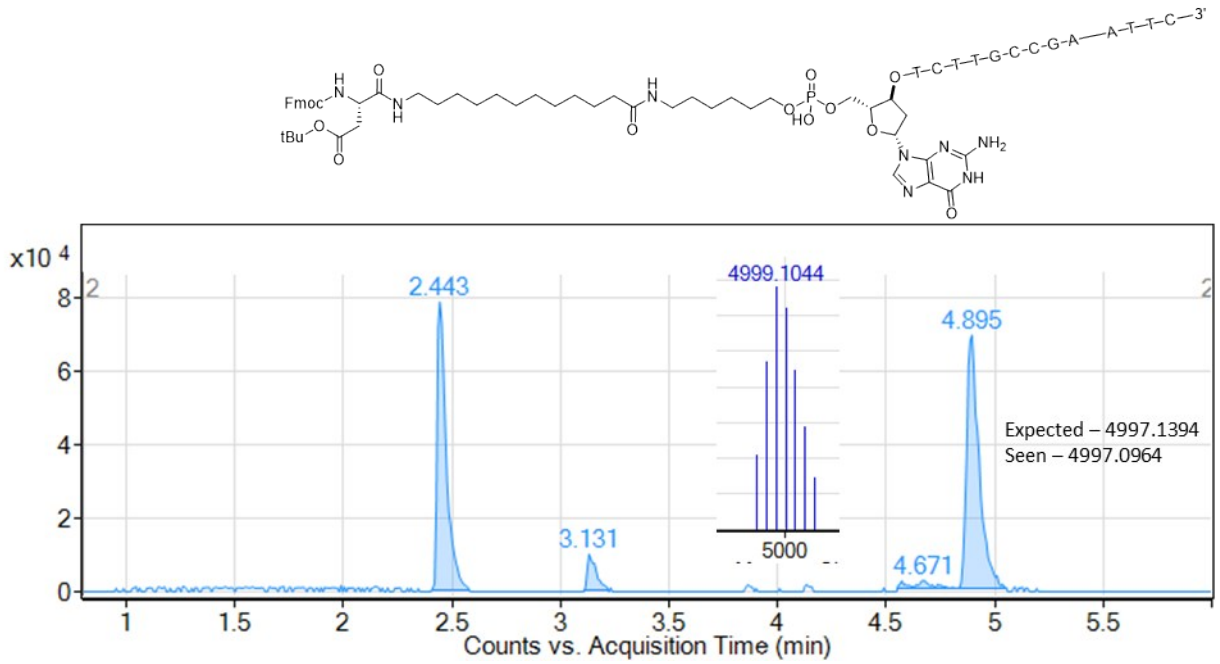
Amide coupling product, Fmoc alanine



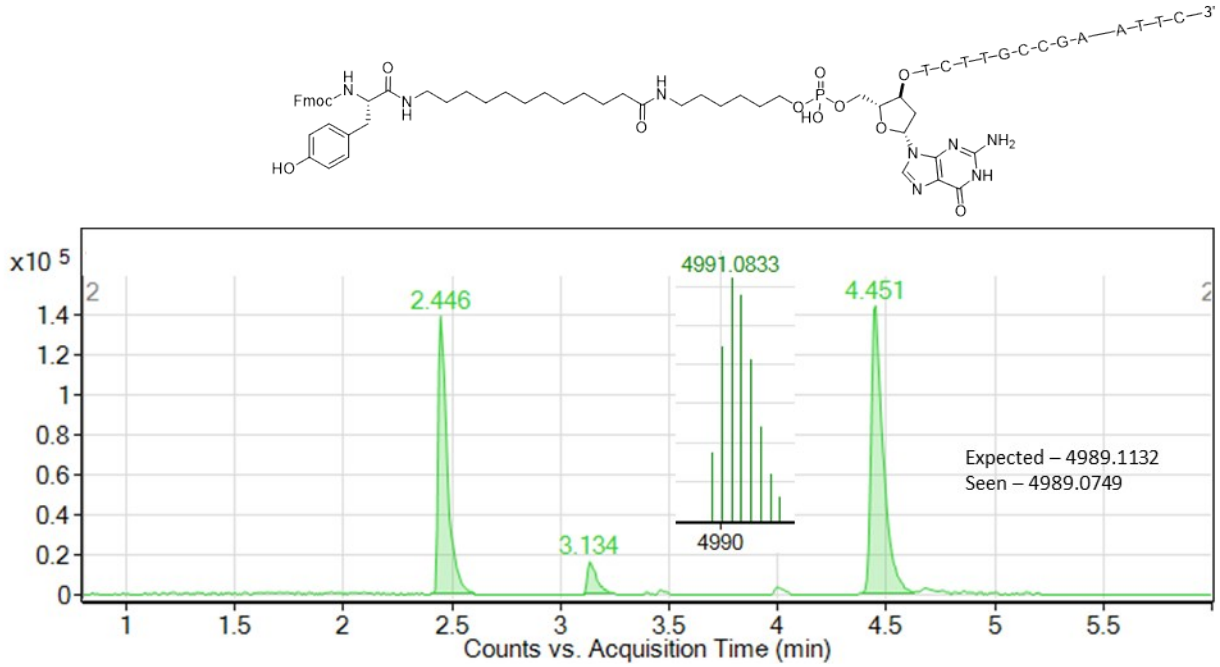
Amide coupling product, Fmoc phenylalanine



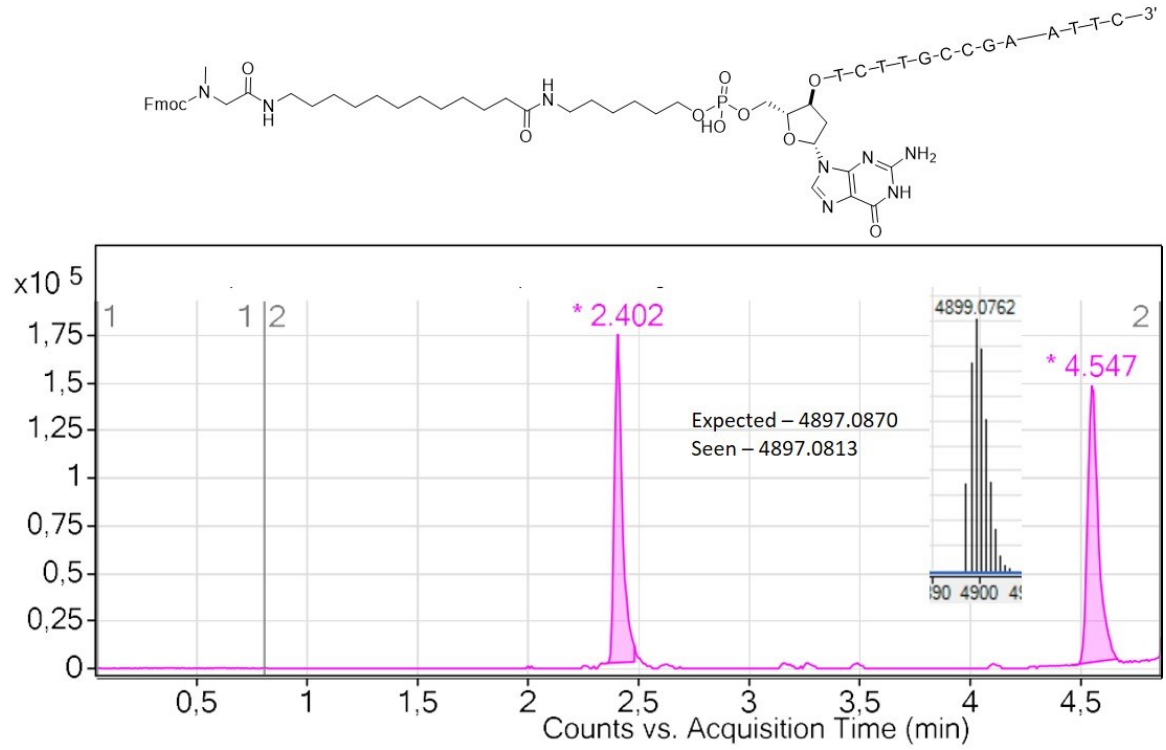
Amide coupling product, N-Fmoc O-tert-butyl aspartic acid



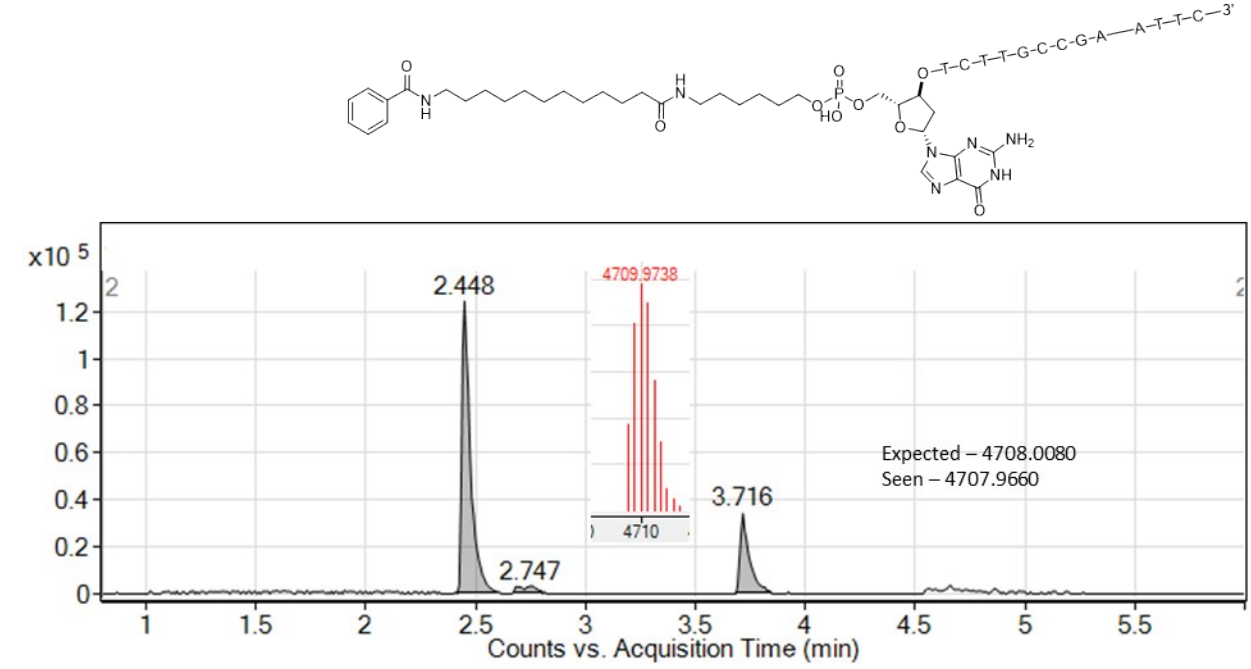
Amide coupling product, Fmoc tyrosine



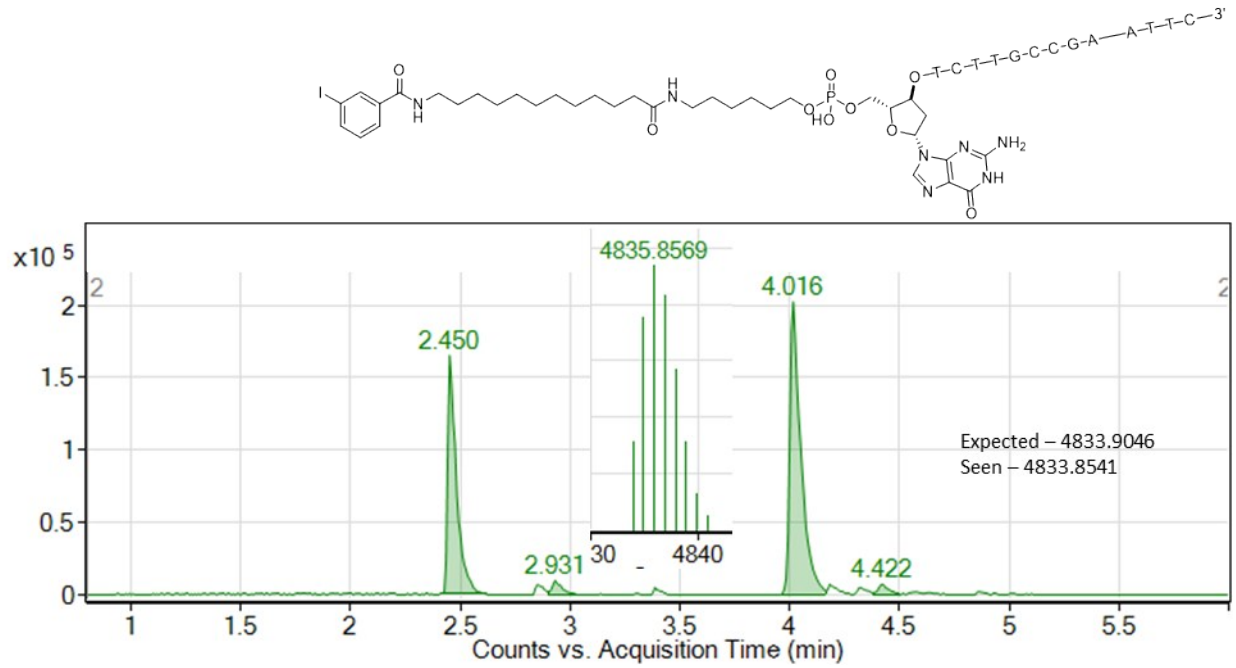
Amide coupling product, benzoic acid



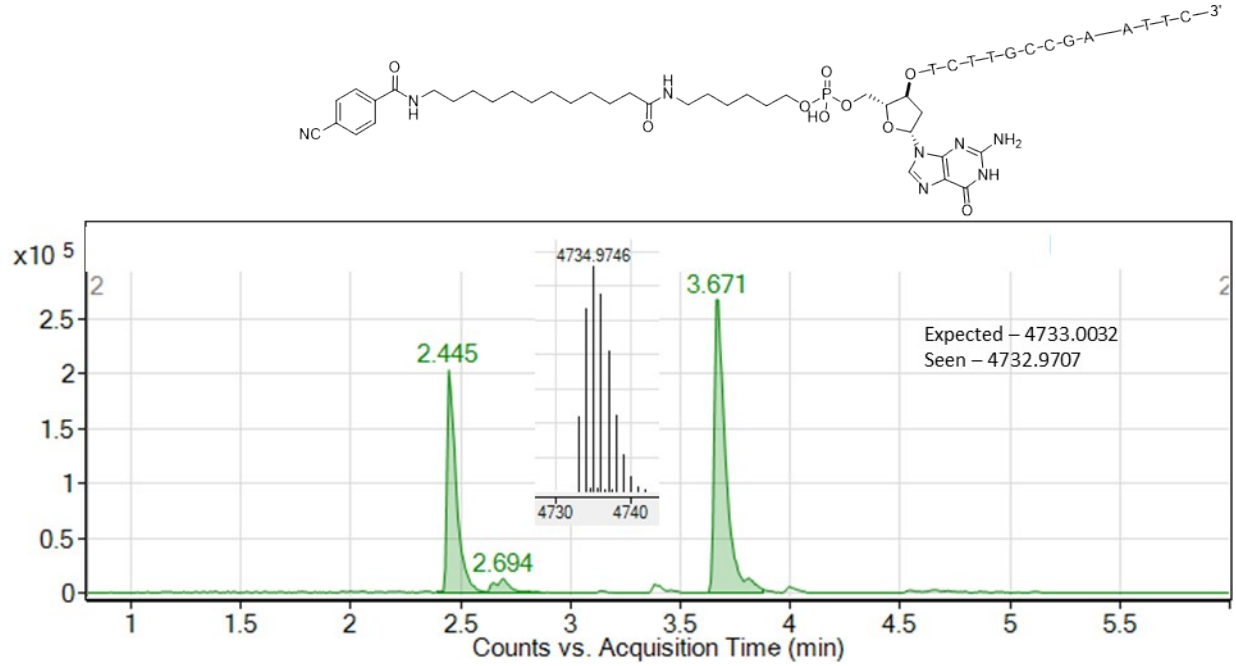
Amide coupling product, Fmoc sarcosine



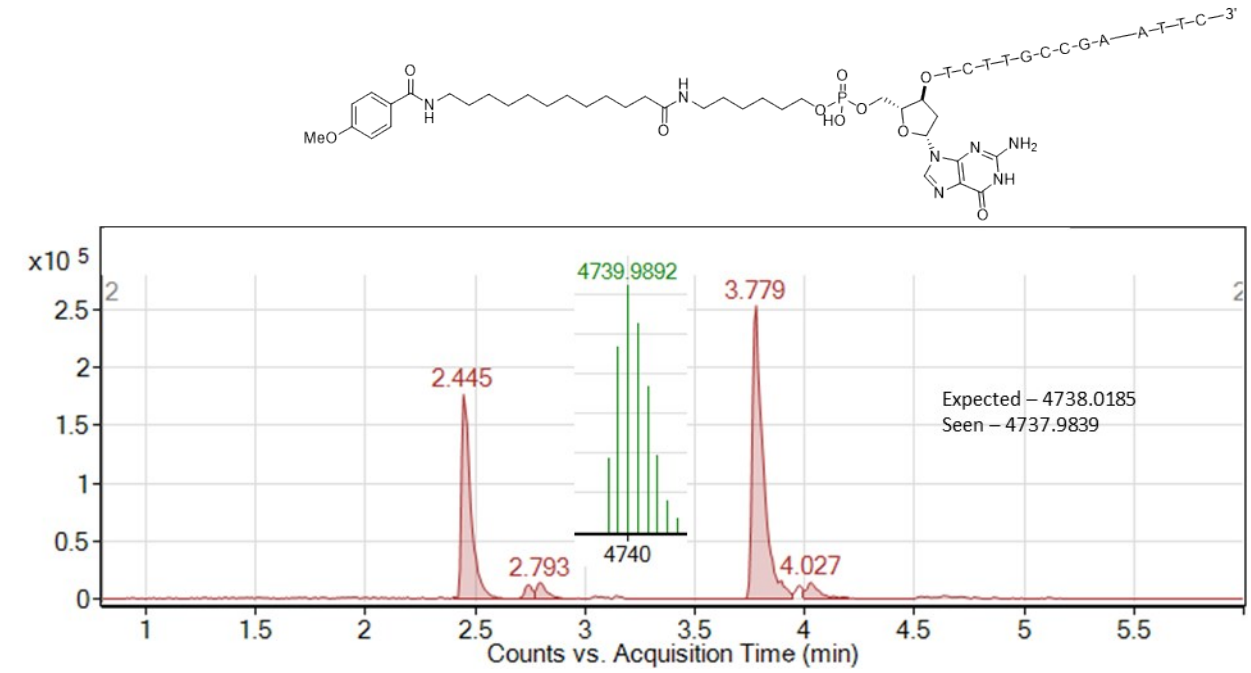
Amide coupling product, 3-iodobenzoic acid



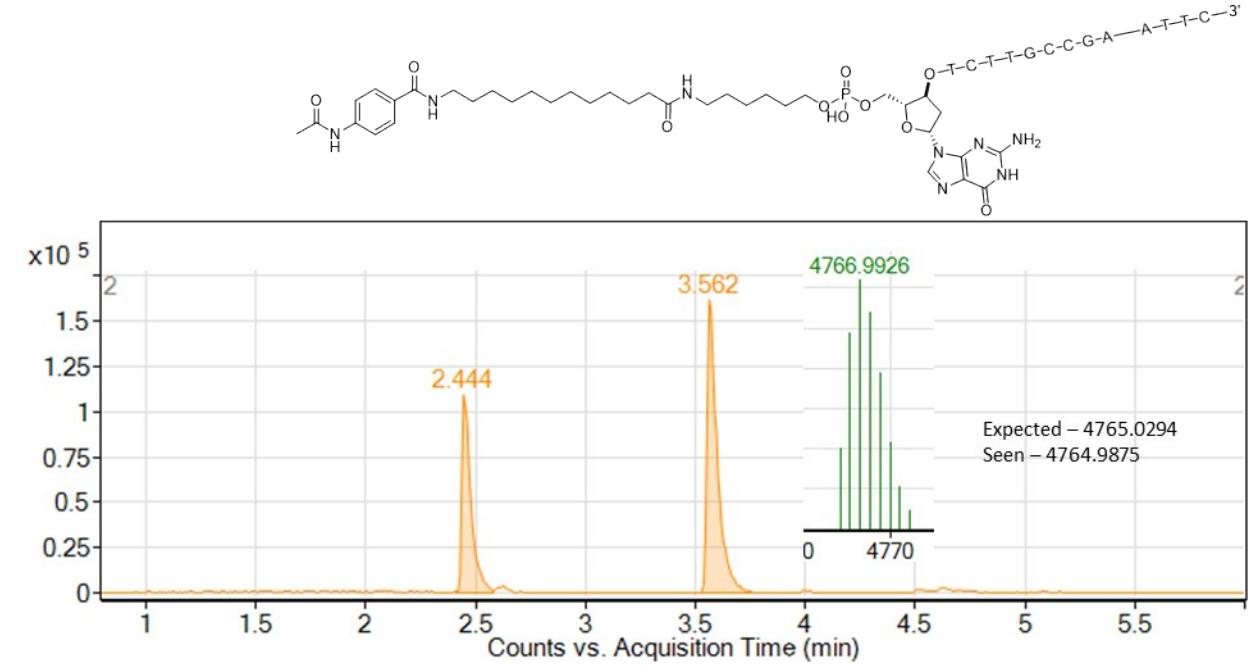
Amide coupling product, 4-cyanobenzoic acid



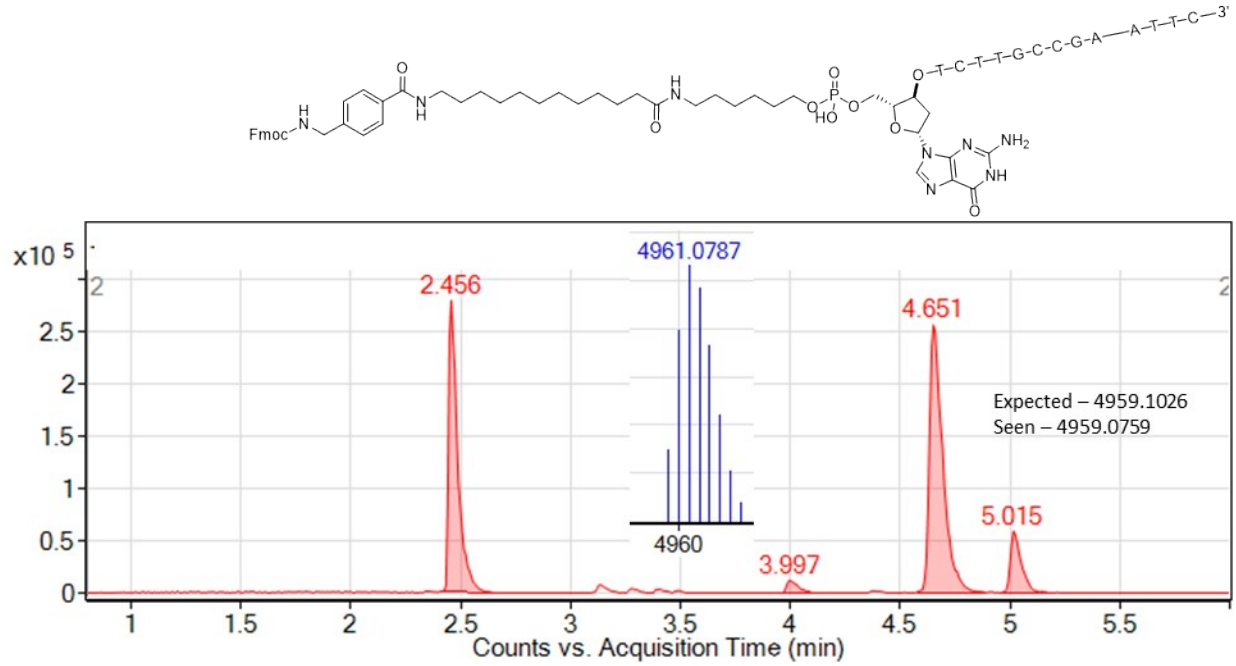
Amide coupling product, 4-methoxybenzoic acid



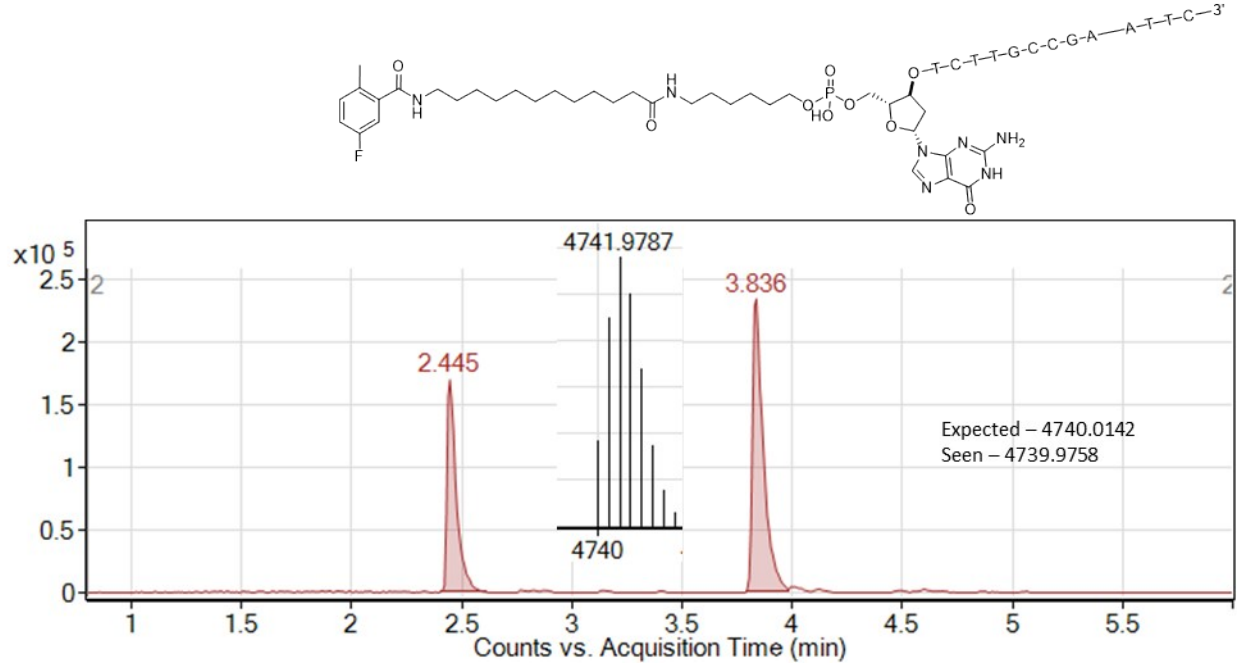
Amide coupling product, 4-carboxyaminomethyl benzoic acid



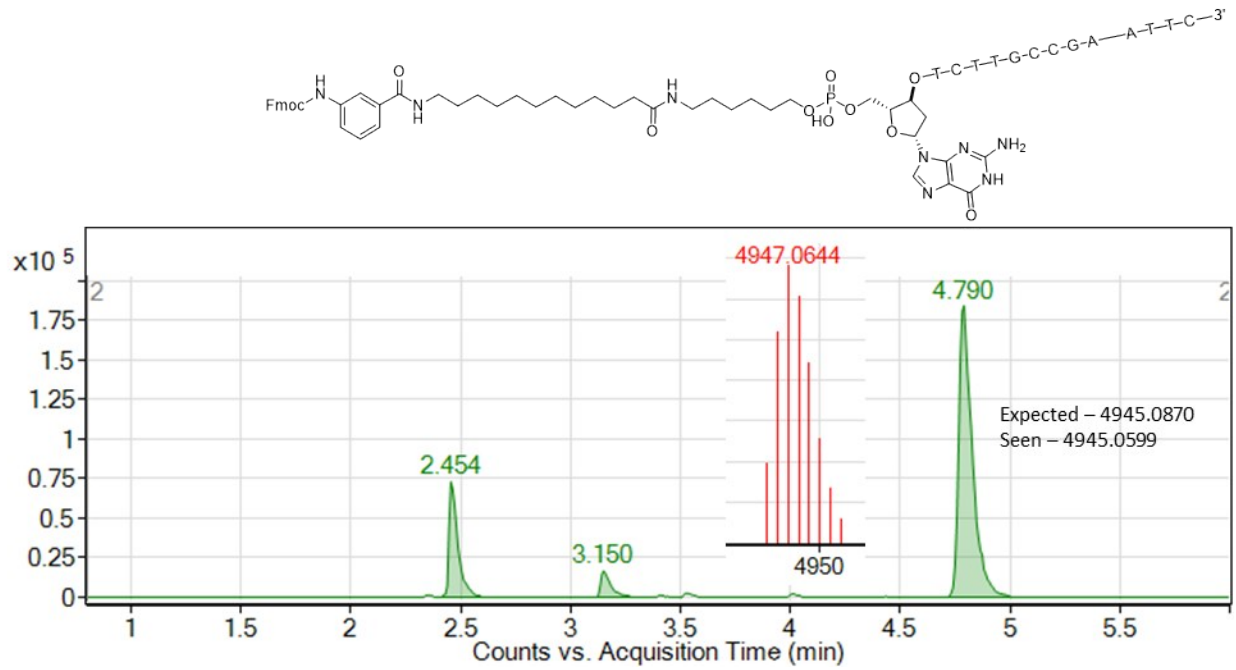
Amide coupling product, 4-Fmocaminomethylbenzoic acid



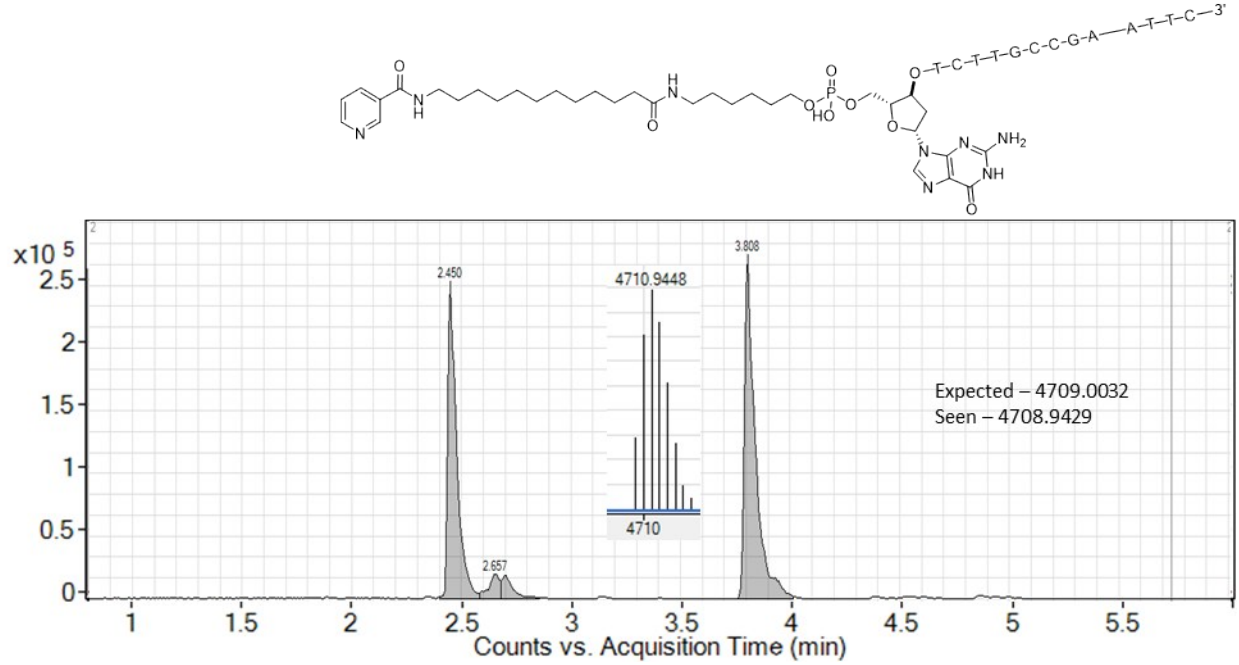
Amide coupling product, 2-methyl-5-fluorobenzoic acid



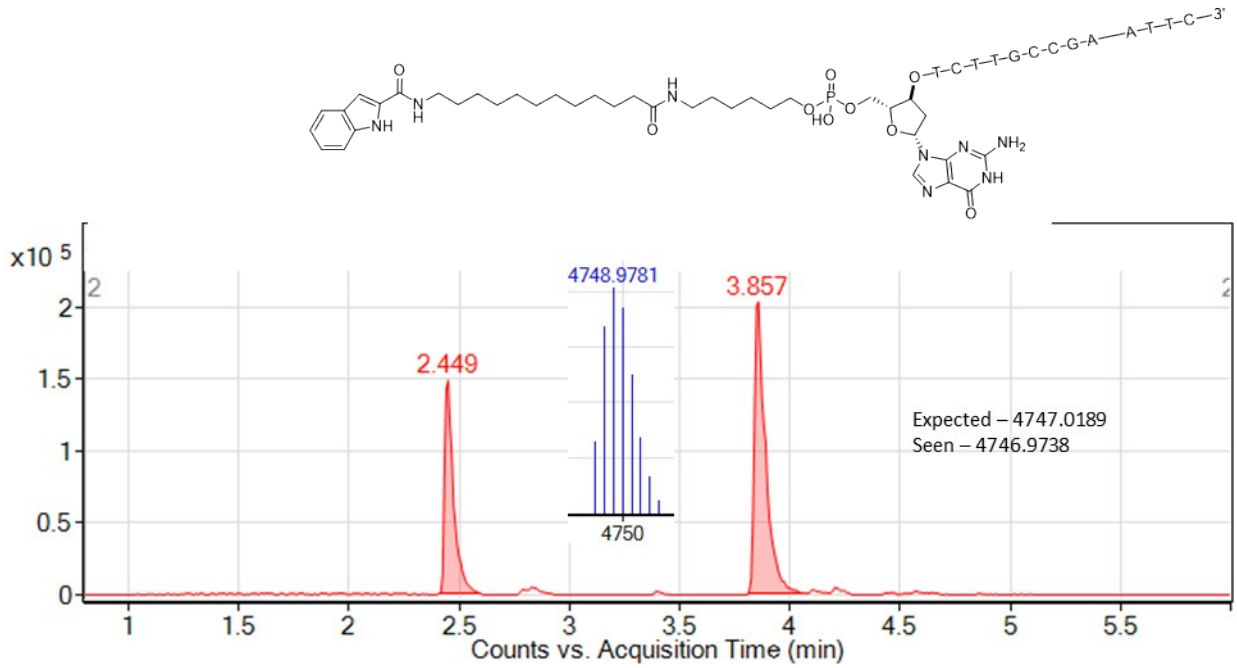
Amide coupling product, Fmoc-3-aminobenzoic acid



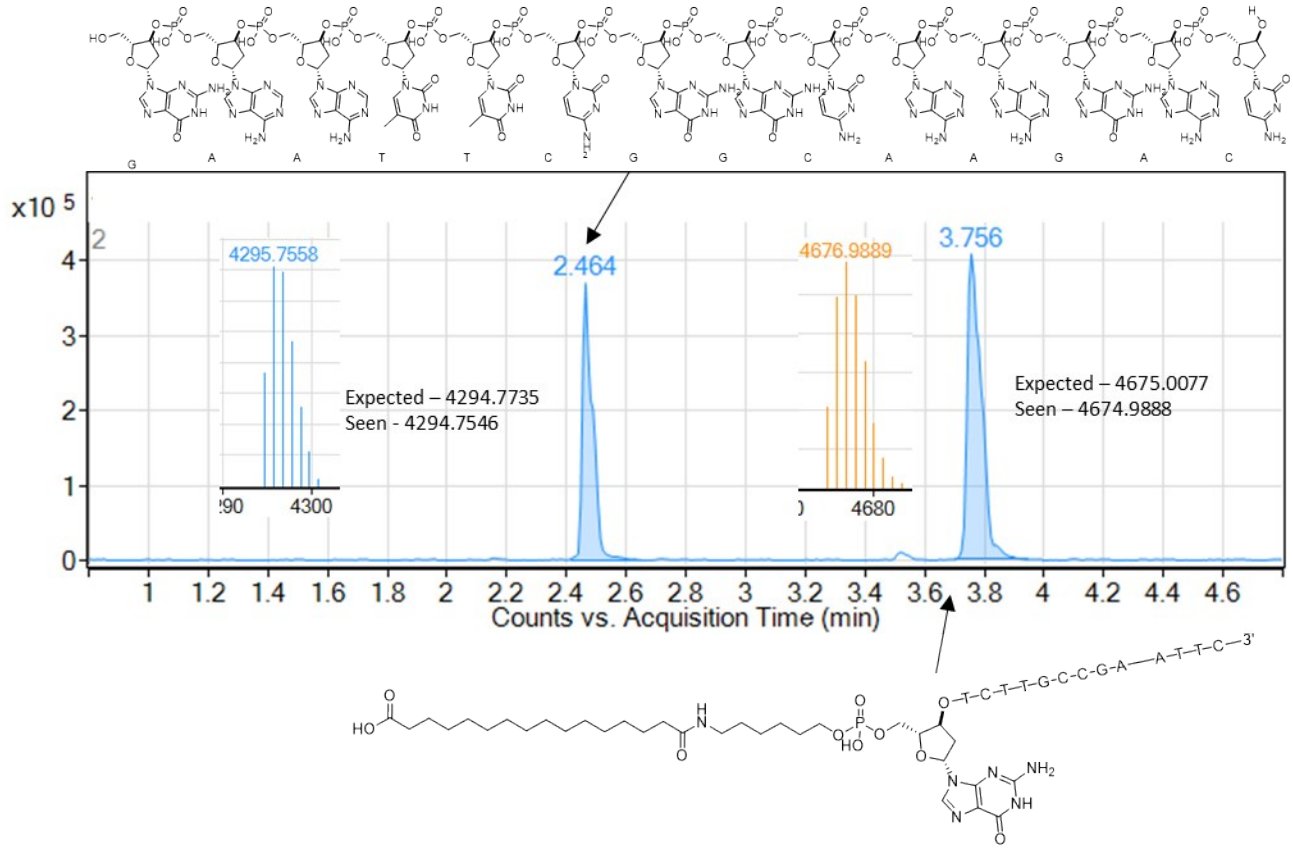
Amide coupling product, nicotinic acid



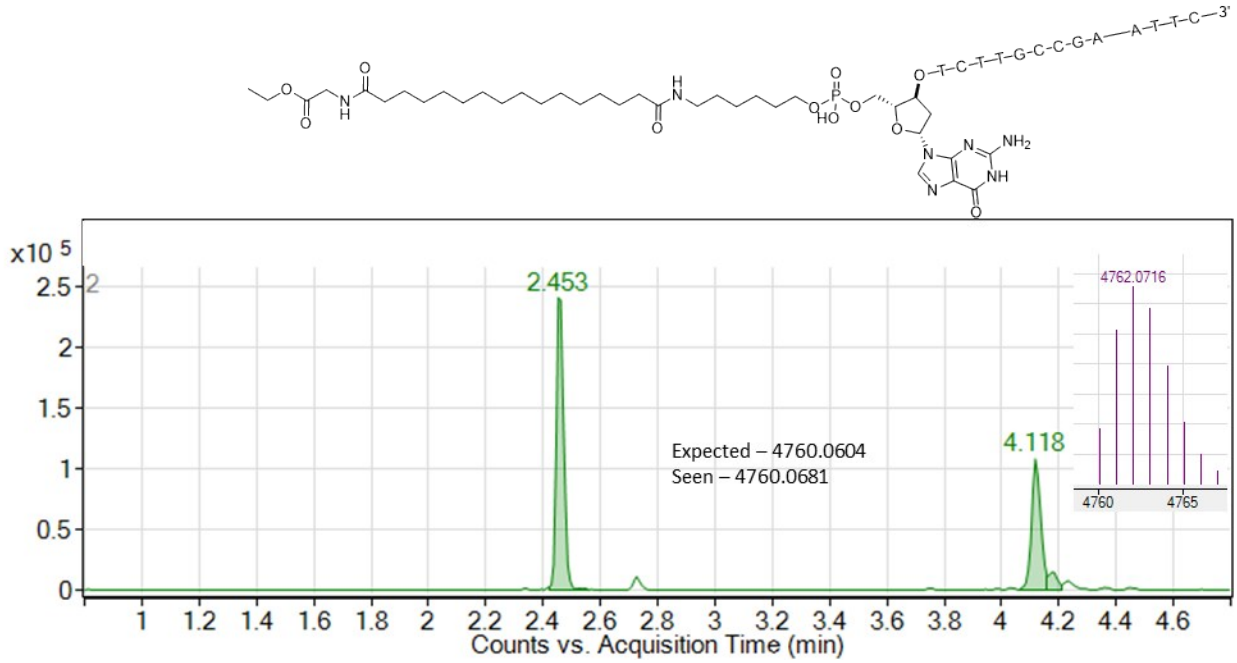
Amide coupling product, indole-2-carboxylic acid



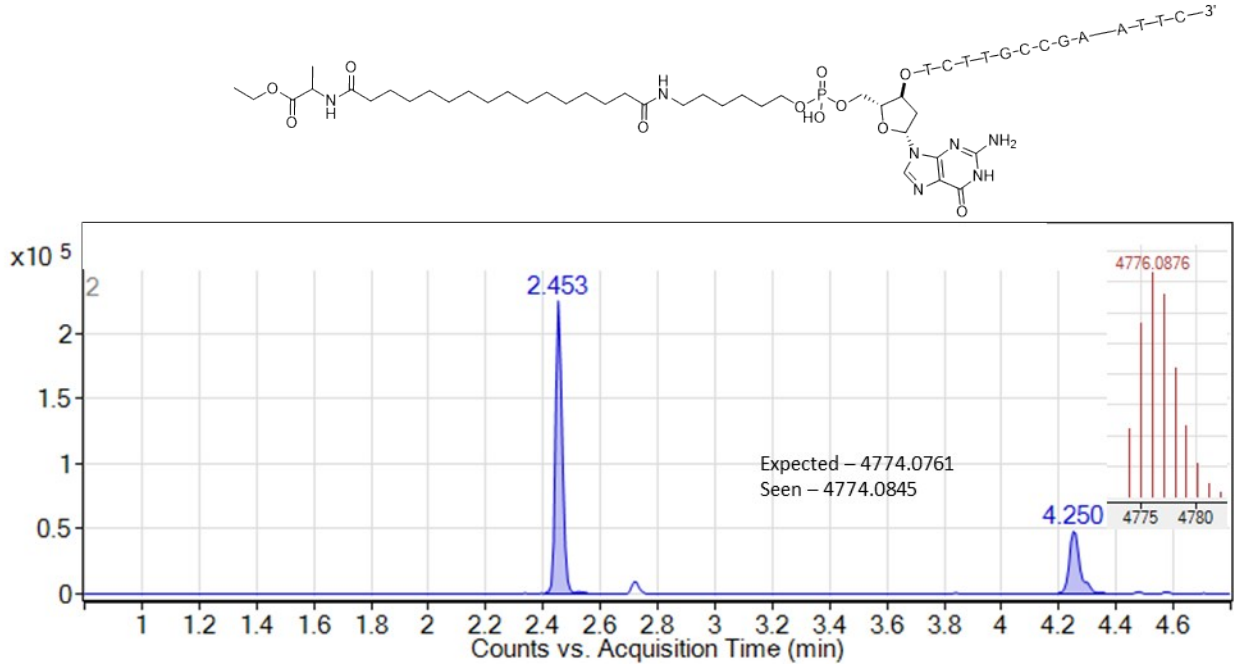
Acid headpiece **2**



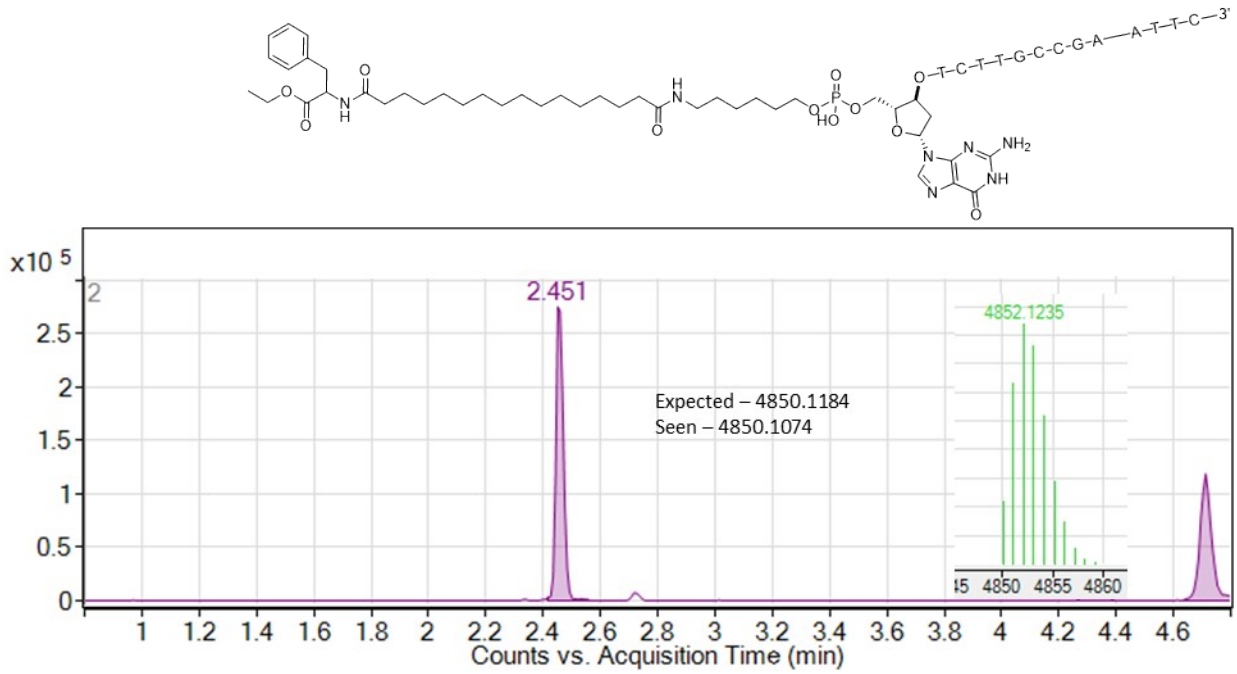
Amide coupling product, glycine ethyl ester



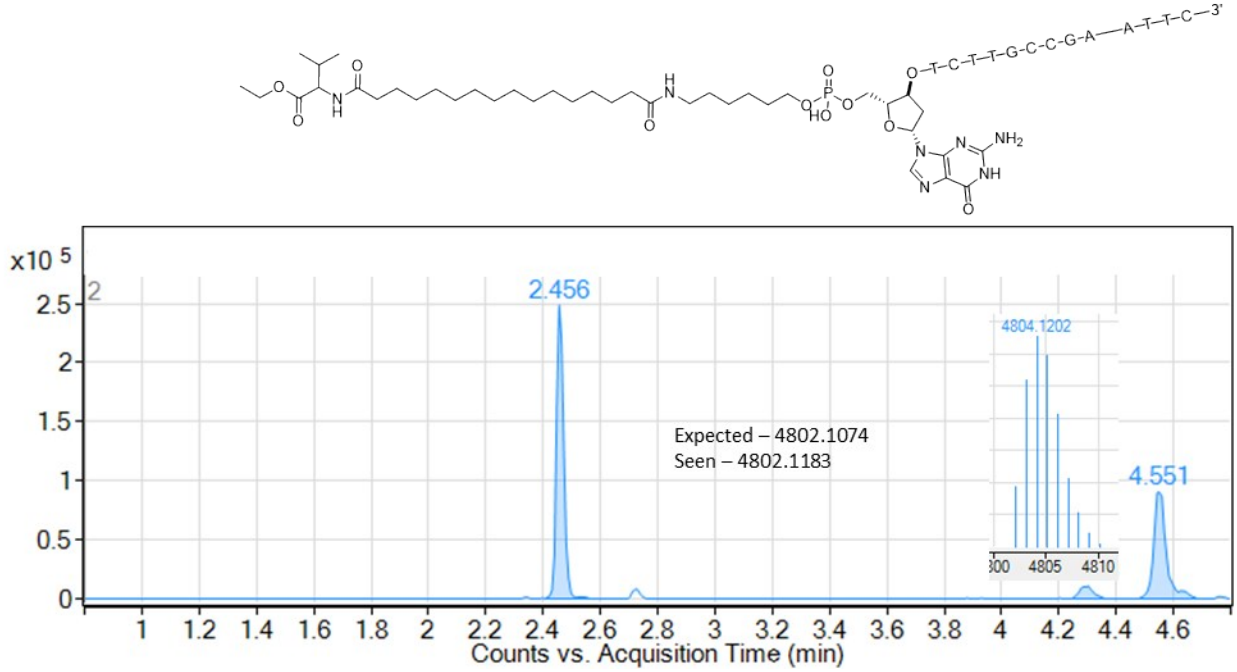
Amide coupling product, alanine ethyl ester



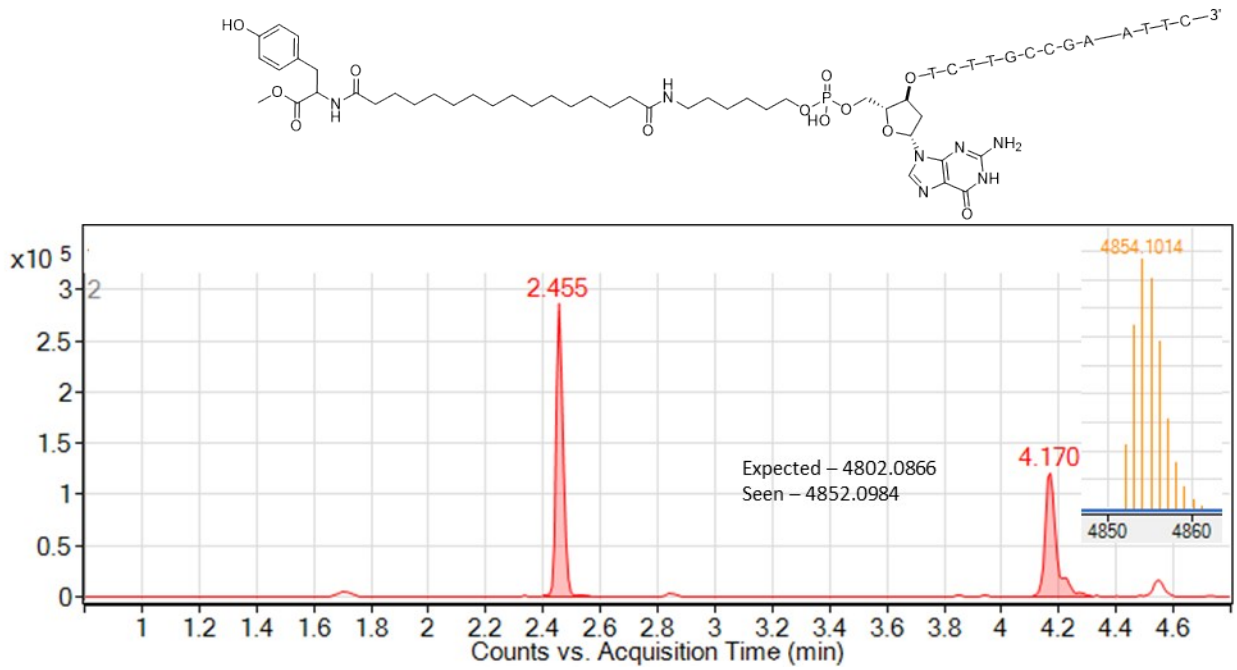
Amide coupling product, D-phenylalanine ethyl ester



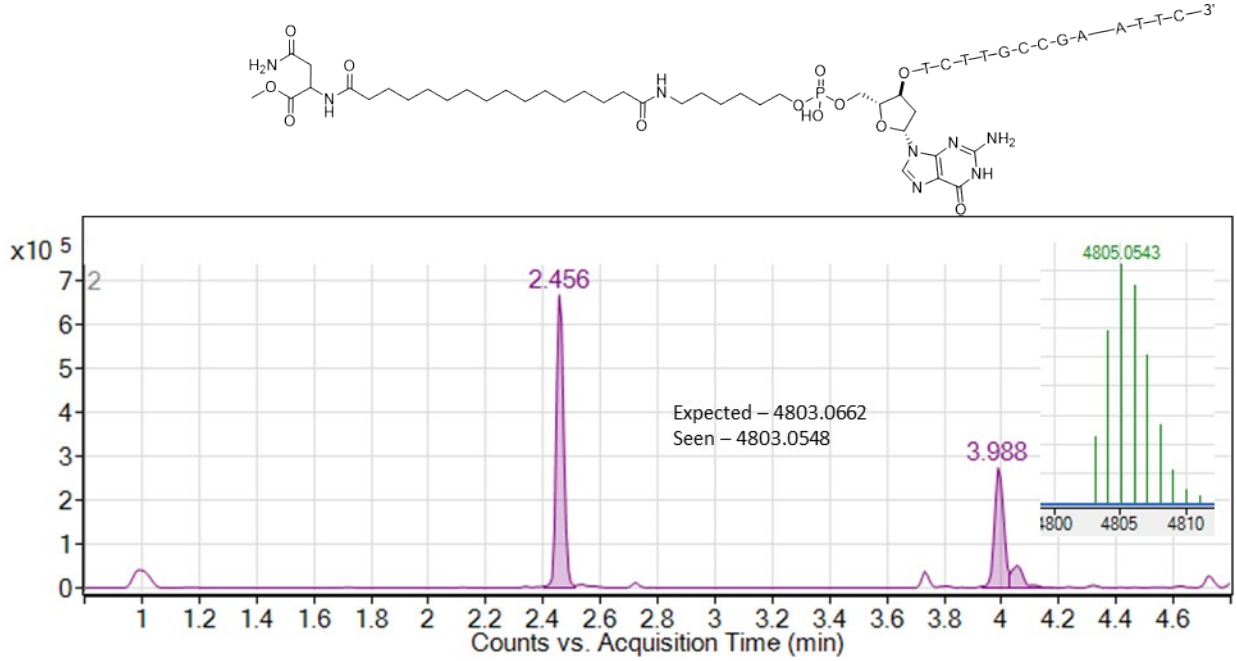
Amide coupling product, valine ethyl ester



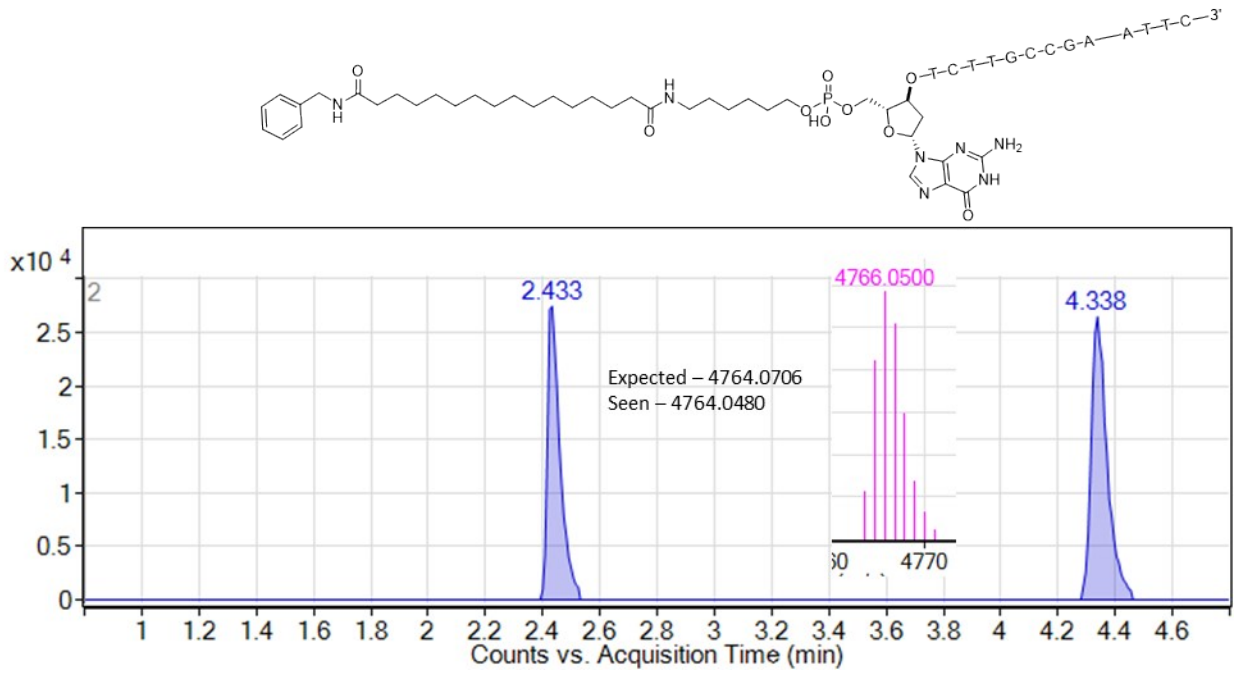
Amide coupling product, tyrosine methyl ester



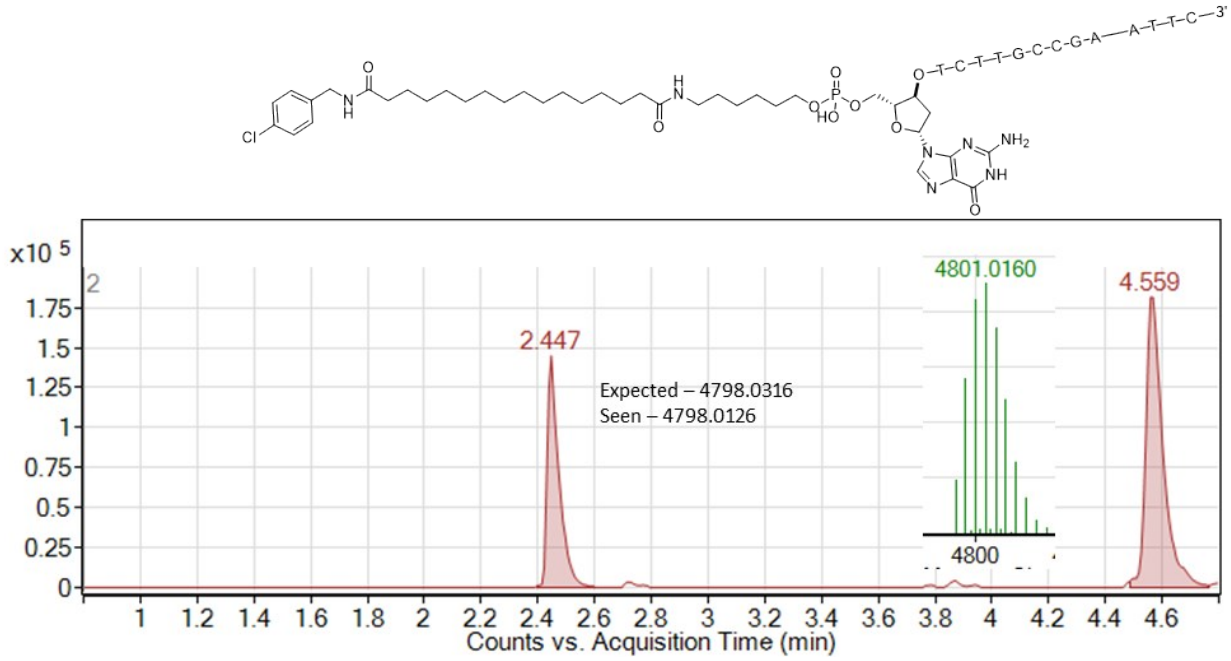
Amide coupling product, asparagine methyl ester



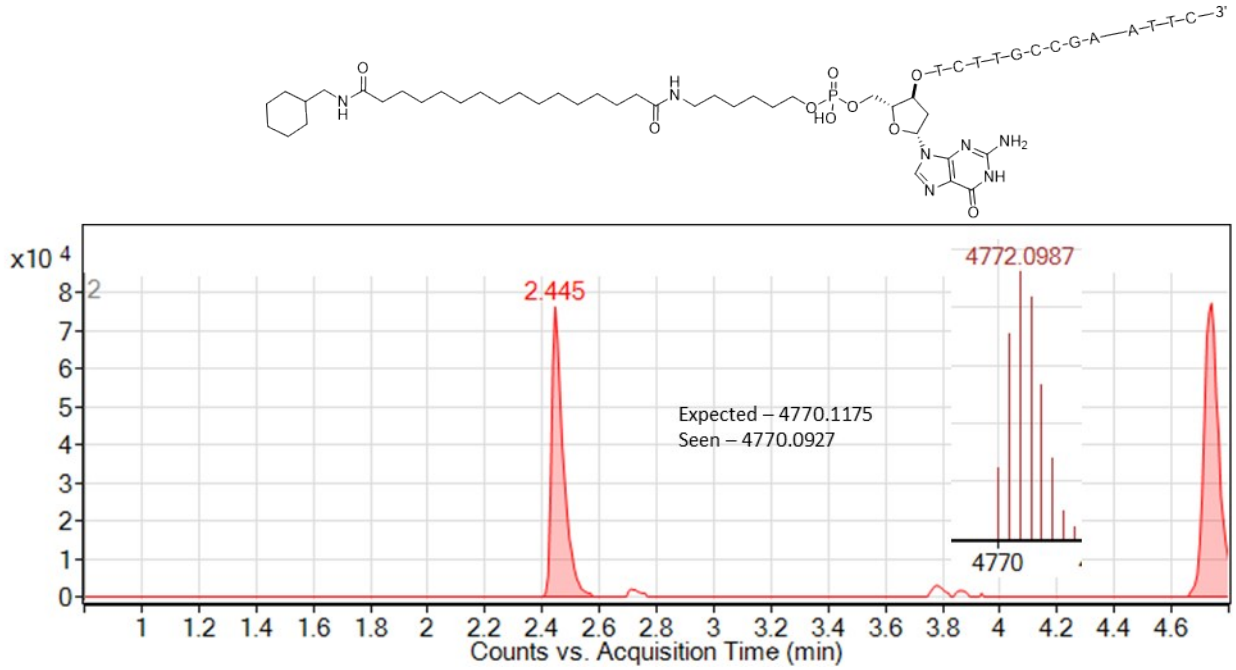
Amide coupling product, benzylamine



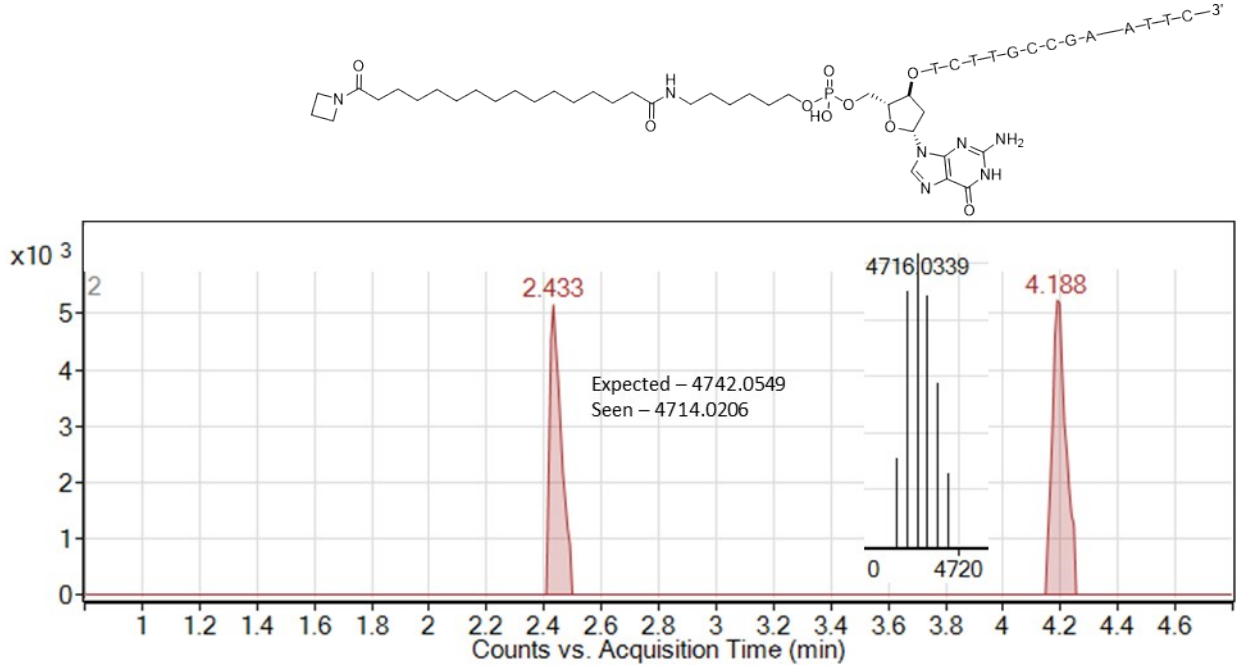
Amide coupling product, 4-chlorobenzylamine



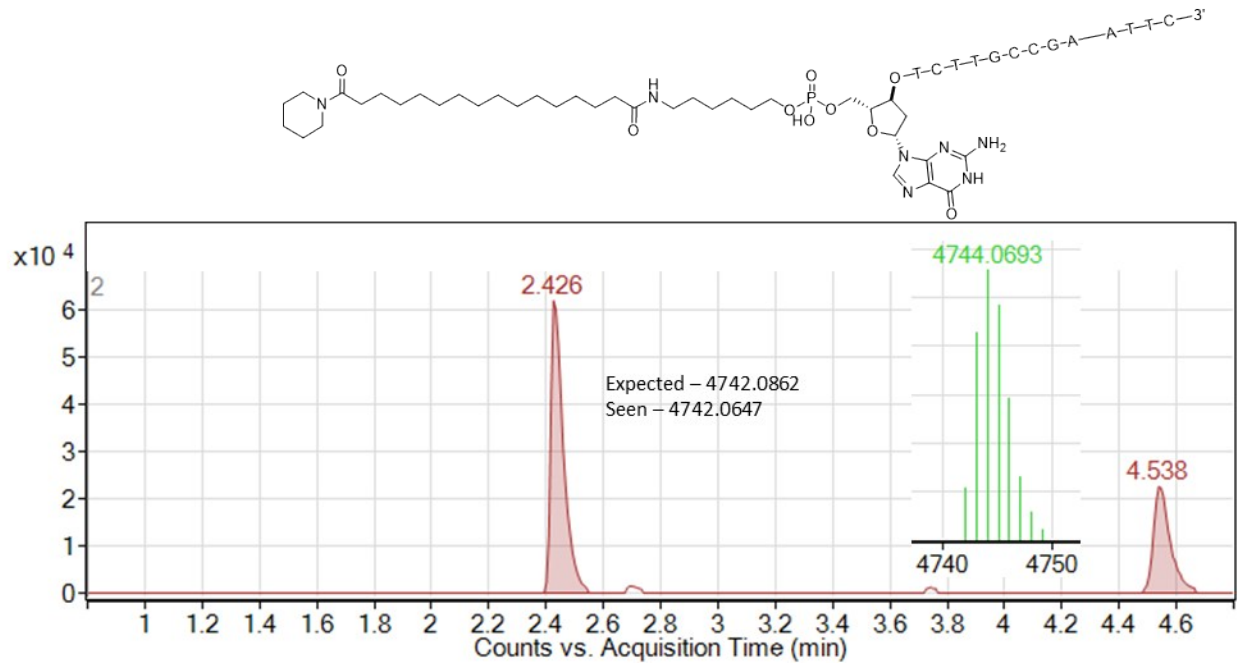
Amide coupling product, N-methylcyclohexylamine



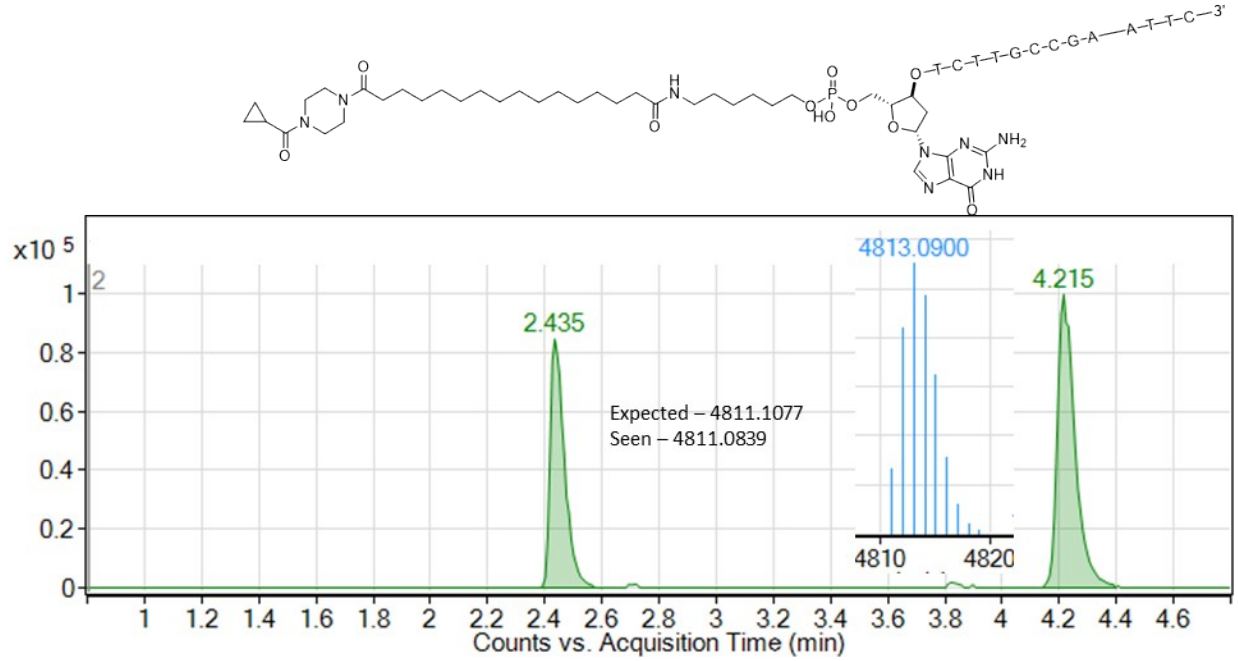
Amide coupling product, azetidine



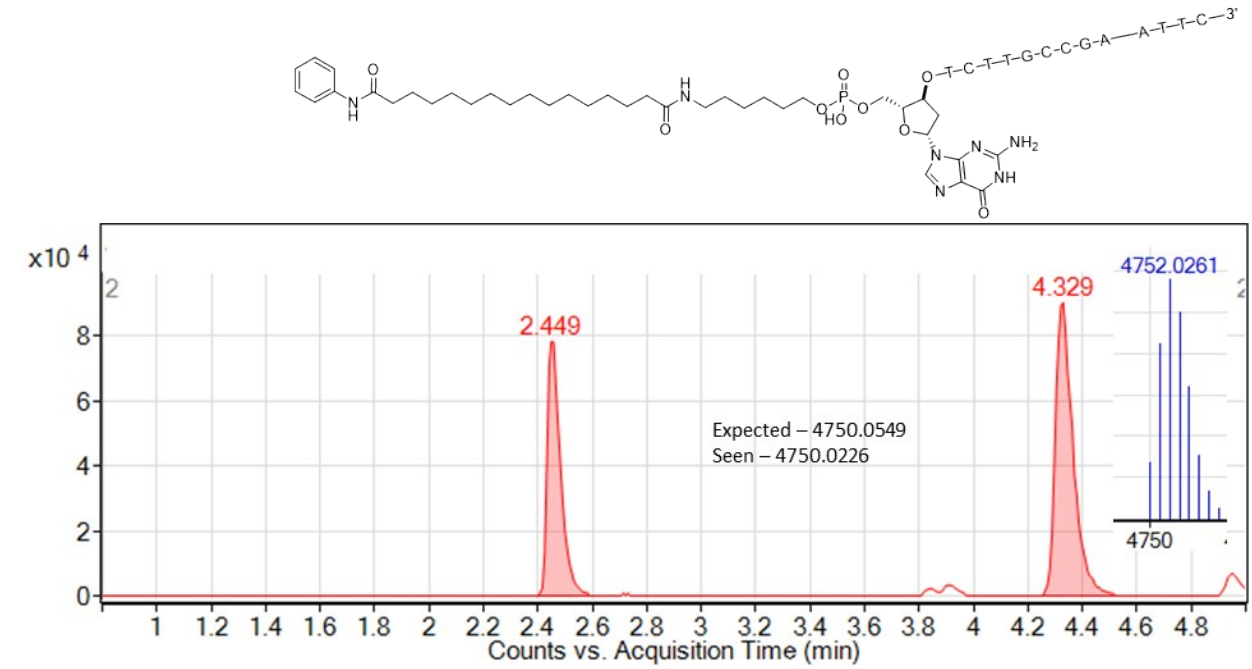
Amide coupling product, piperidine



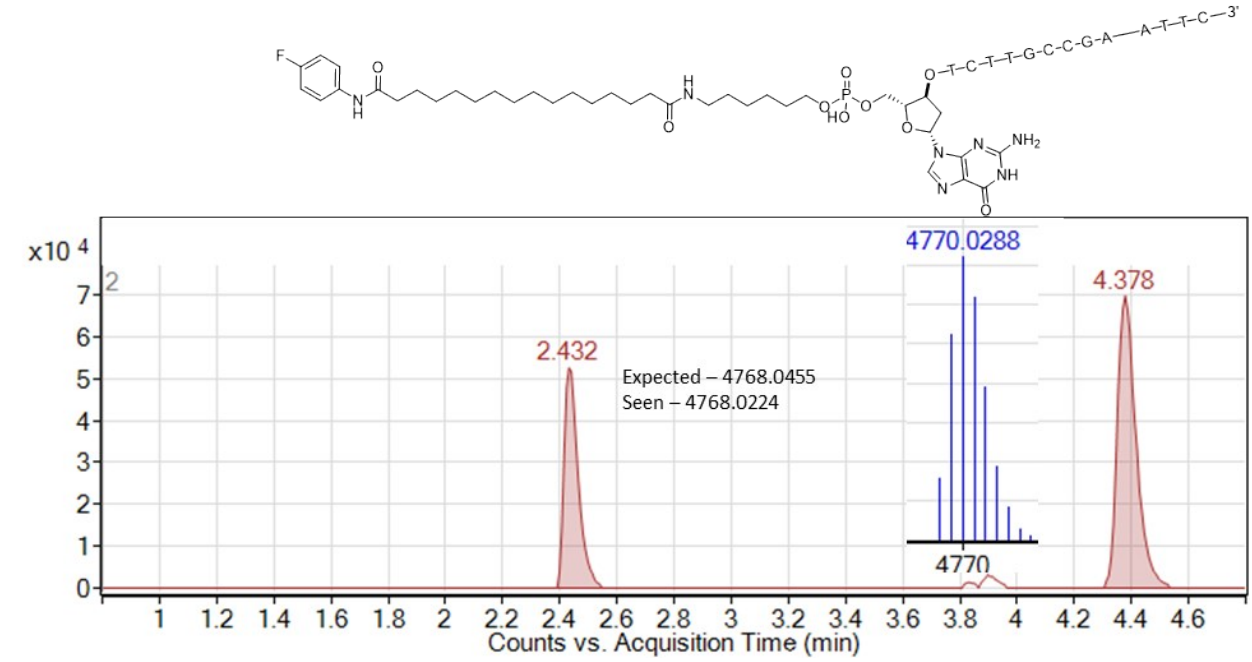
Amide coupling product, cyclopropylamidopiperazine



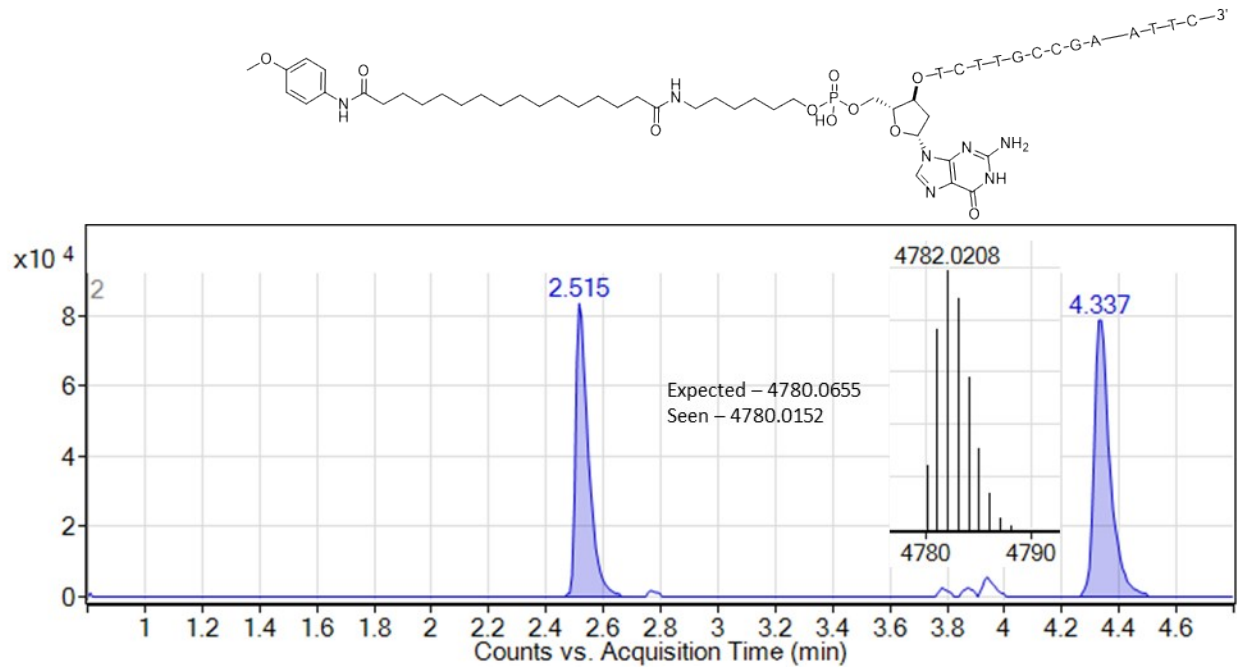
Amide coupling product, aniline



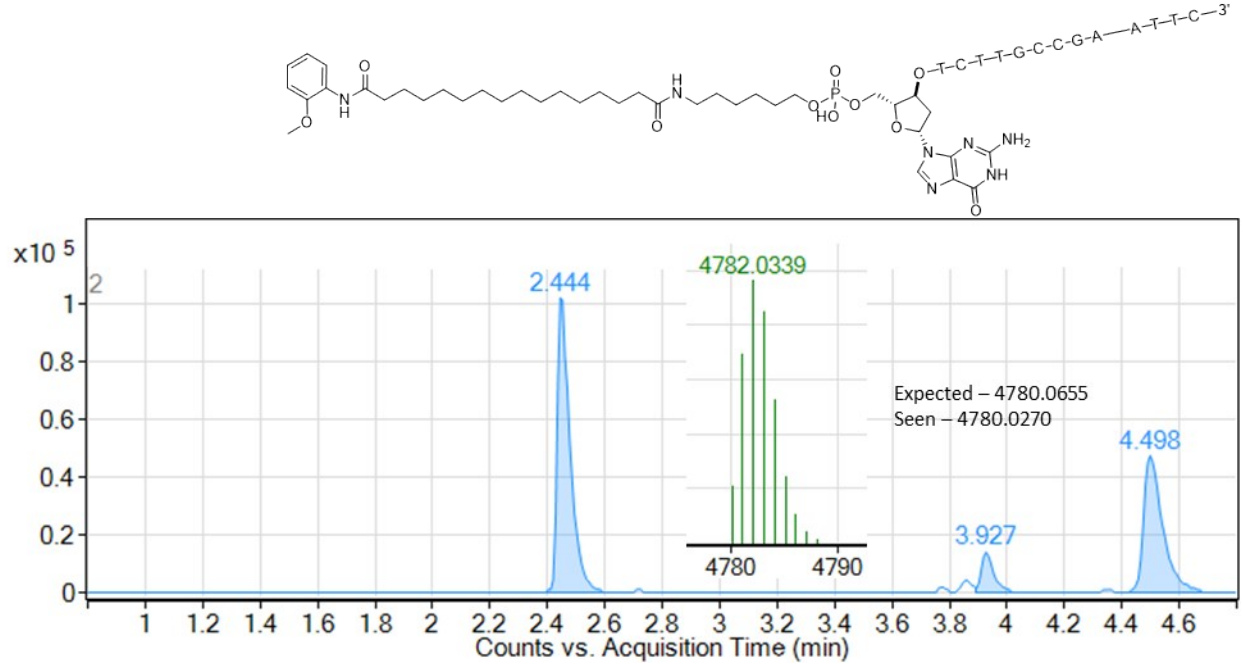
Amide coupling product, 4-fluoroaniline



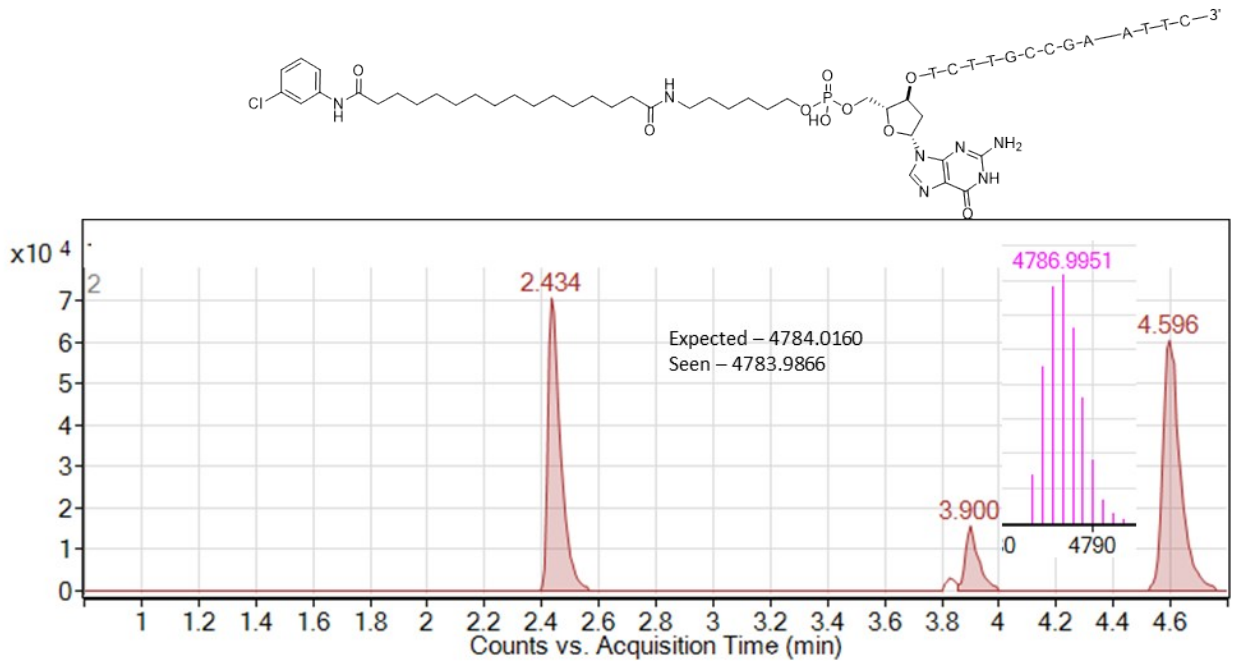
Amide coupling product, 4-methoxyaniline



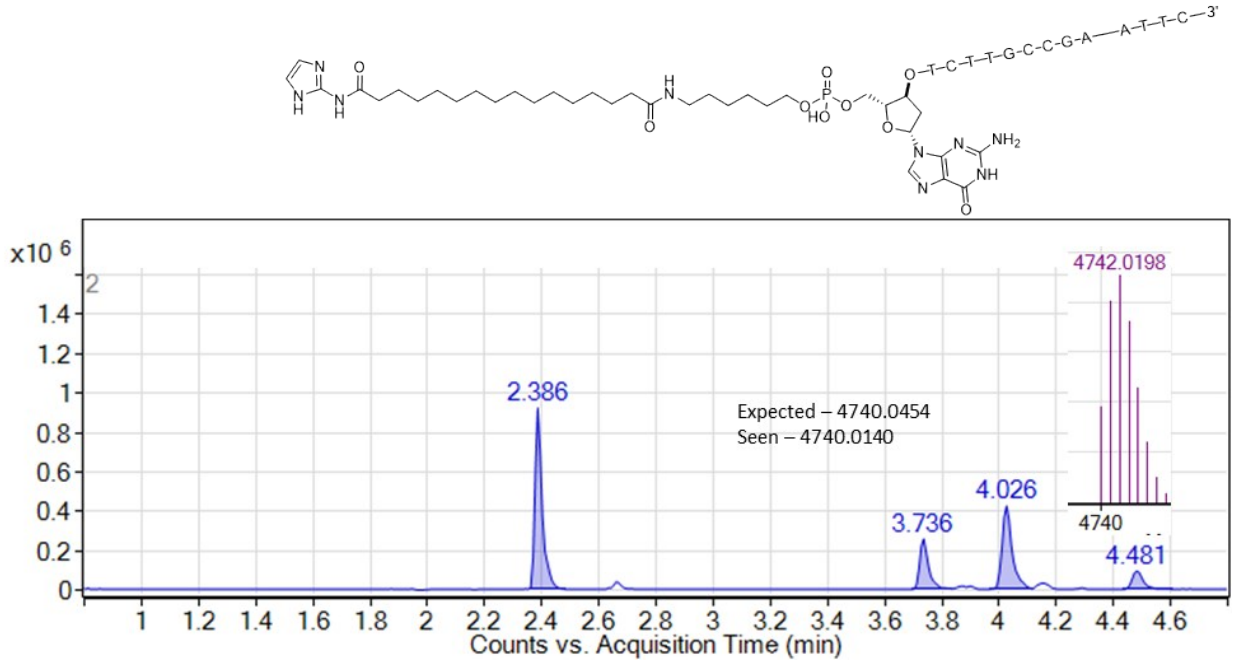
Amide coupling product, 2-methoxyaniline



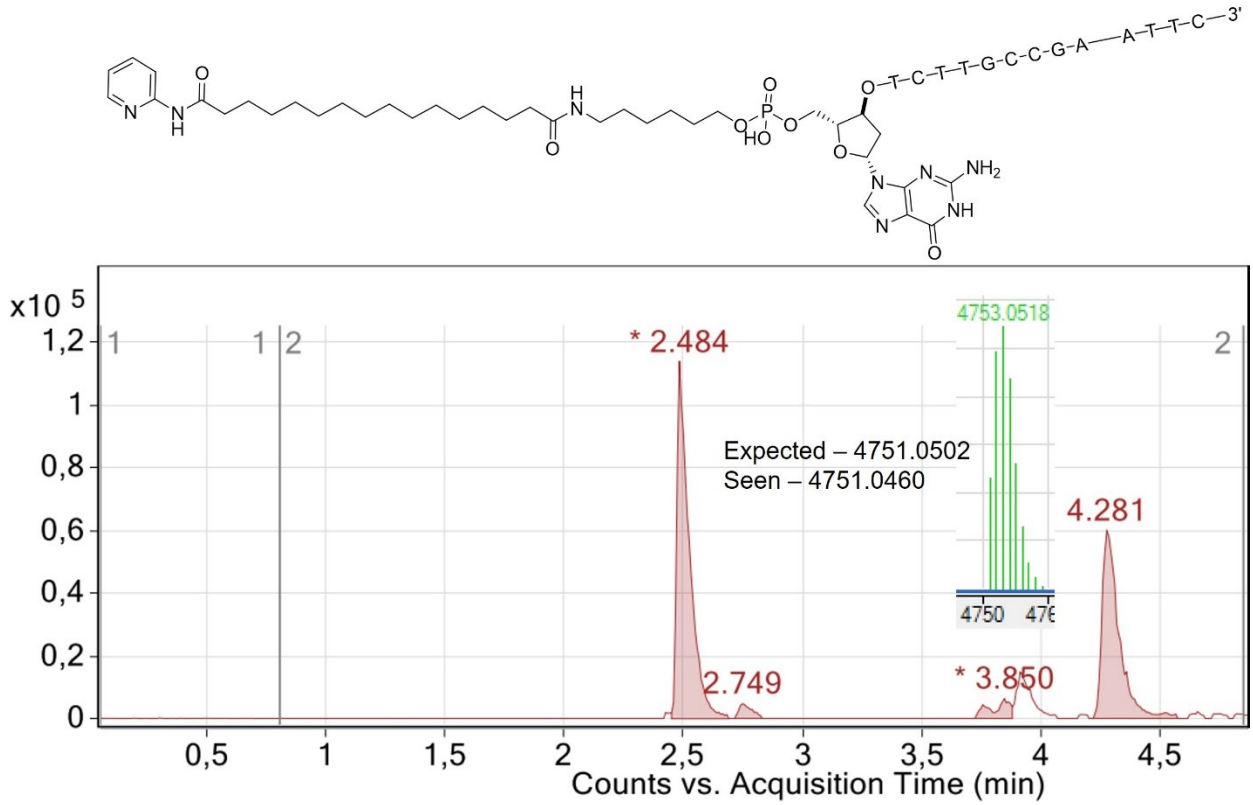
Amide coupling product, 3-chloroaniline



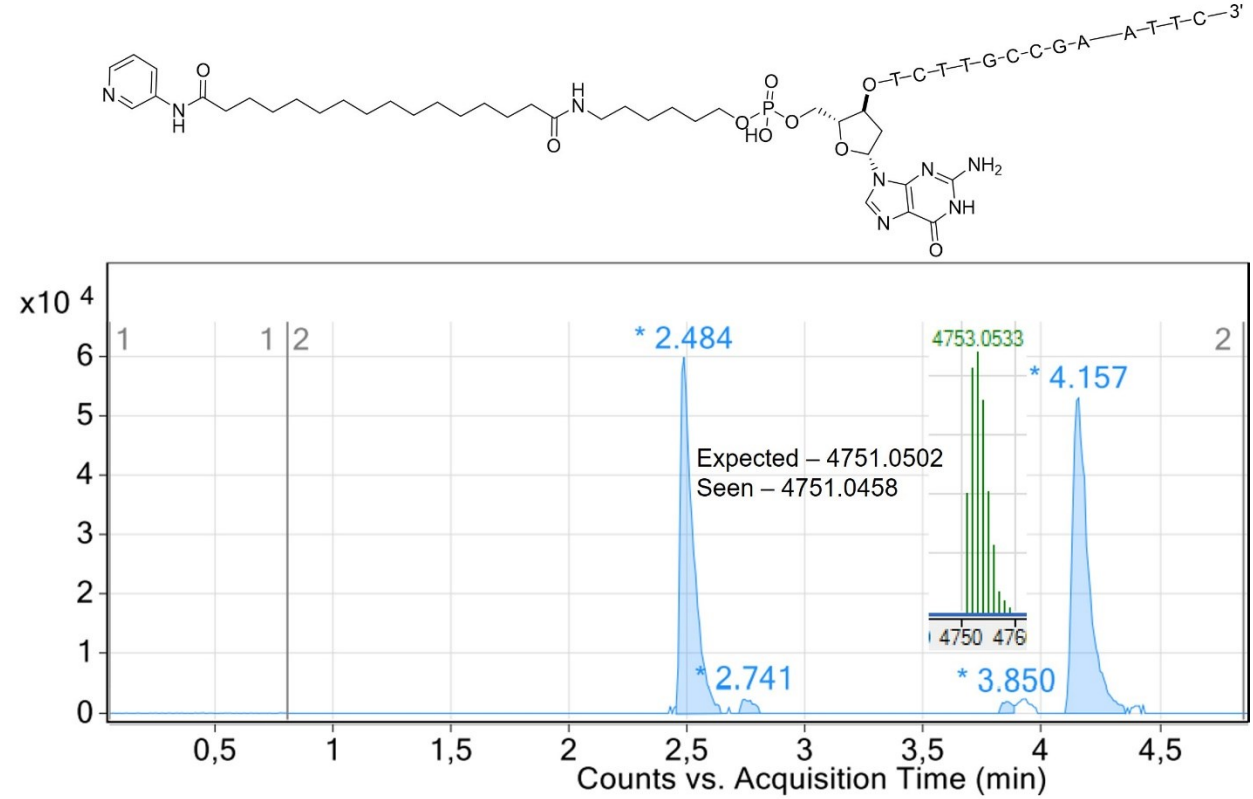
Amide coupling product, 2-aminoimidazole



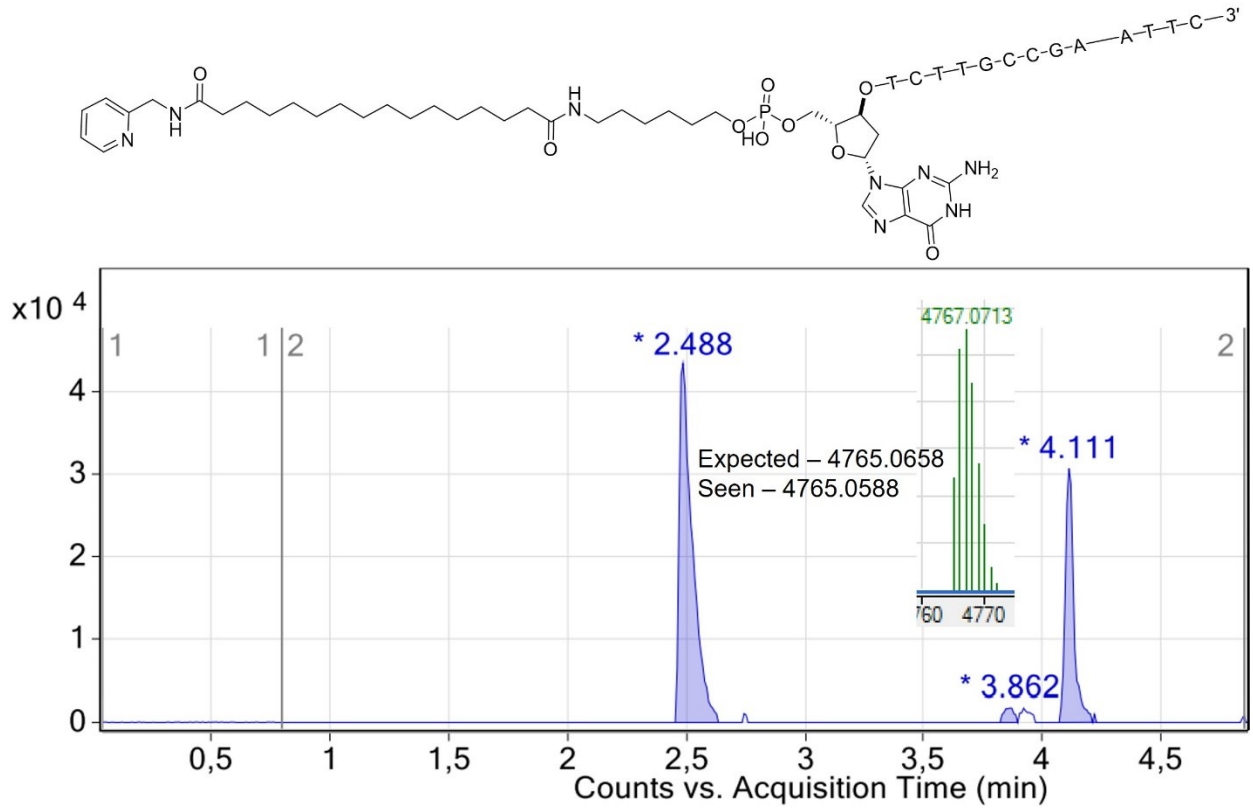
Amide coupling product, 2-aminopyridine



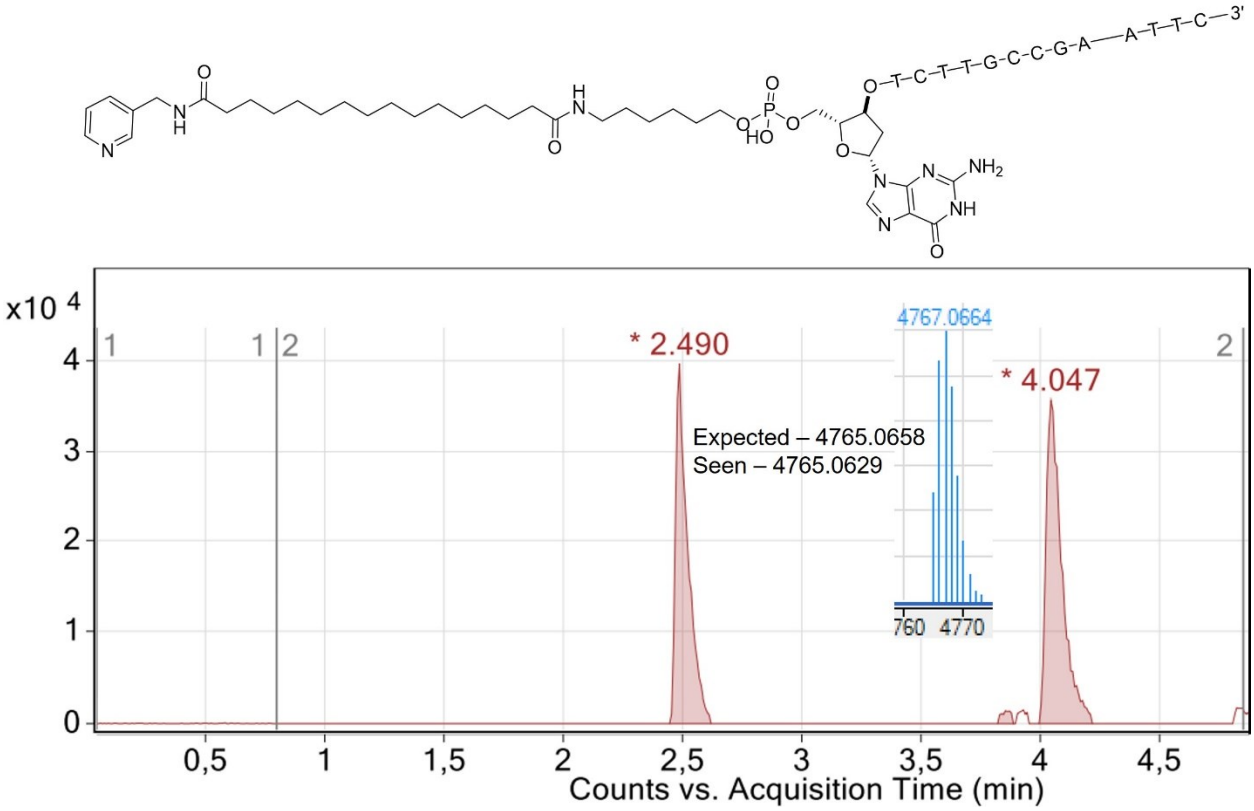
Amide coupling product, 3-aminopyridine



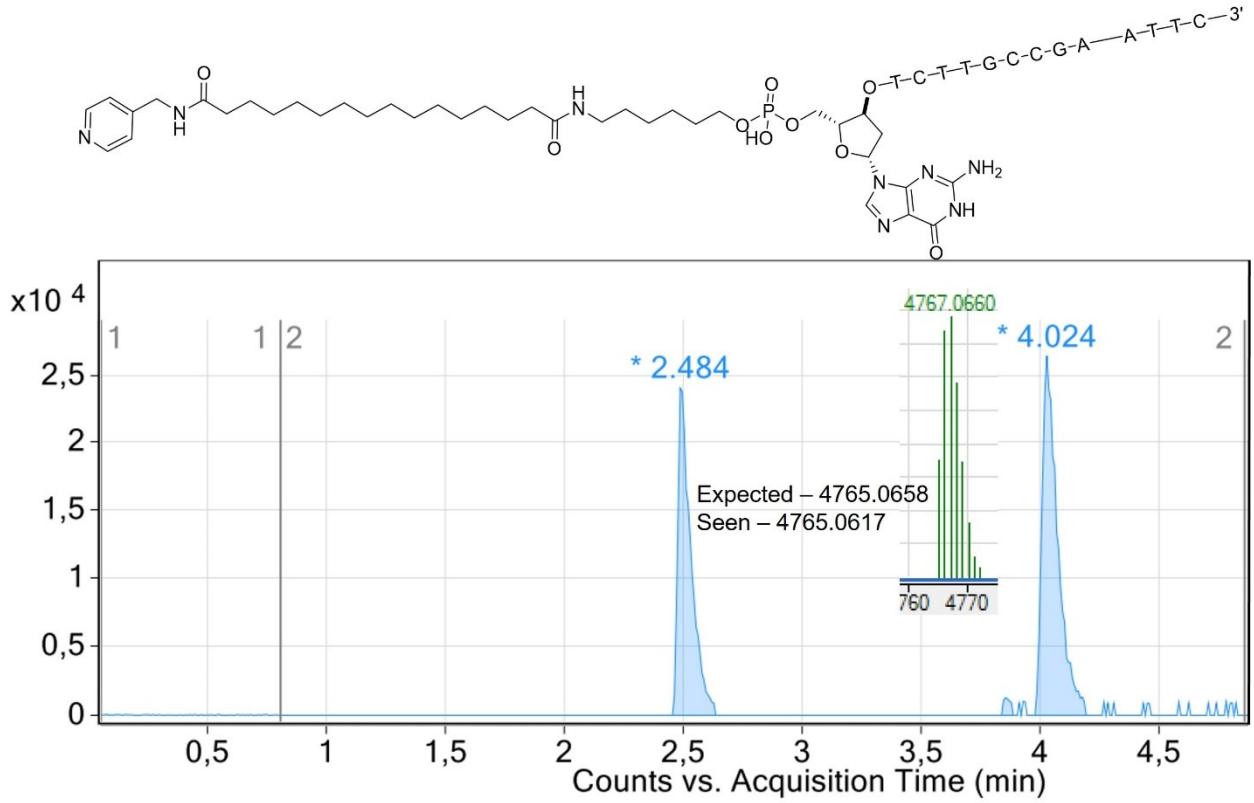
Amide coupling product, 2-aminomethylpyridine



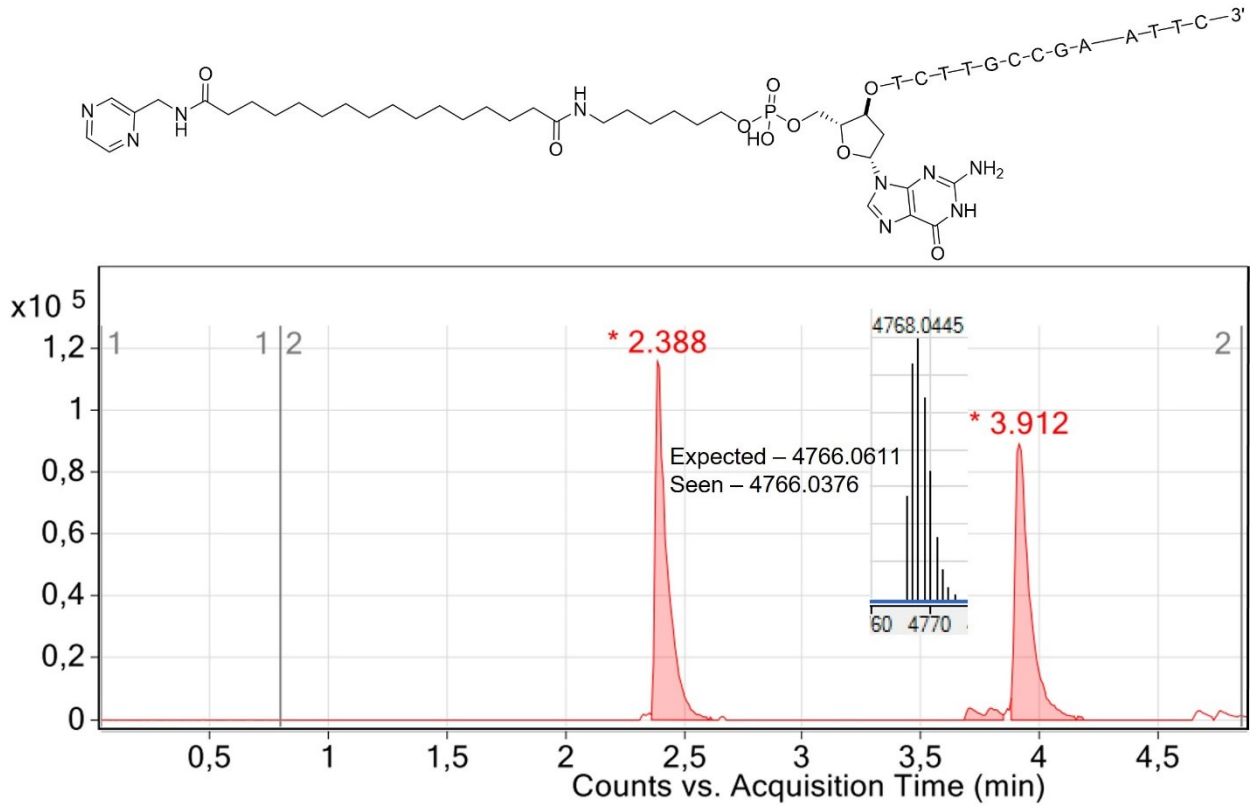
Amide coupling product, 3-aminomethylpyridine



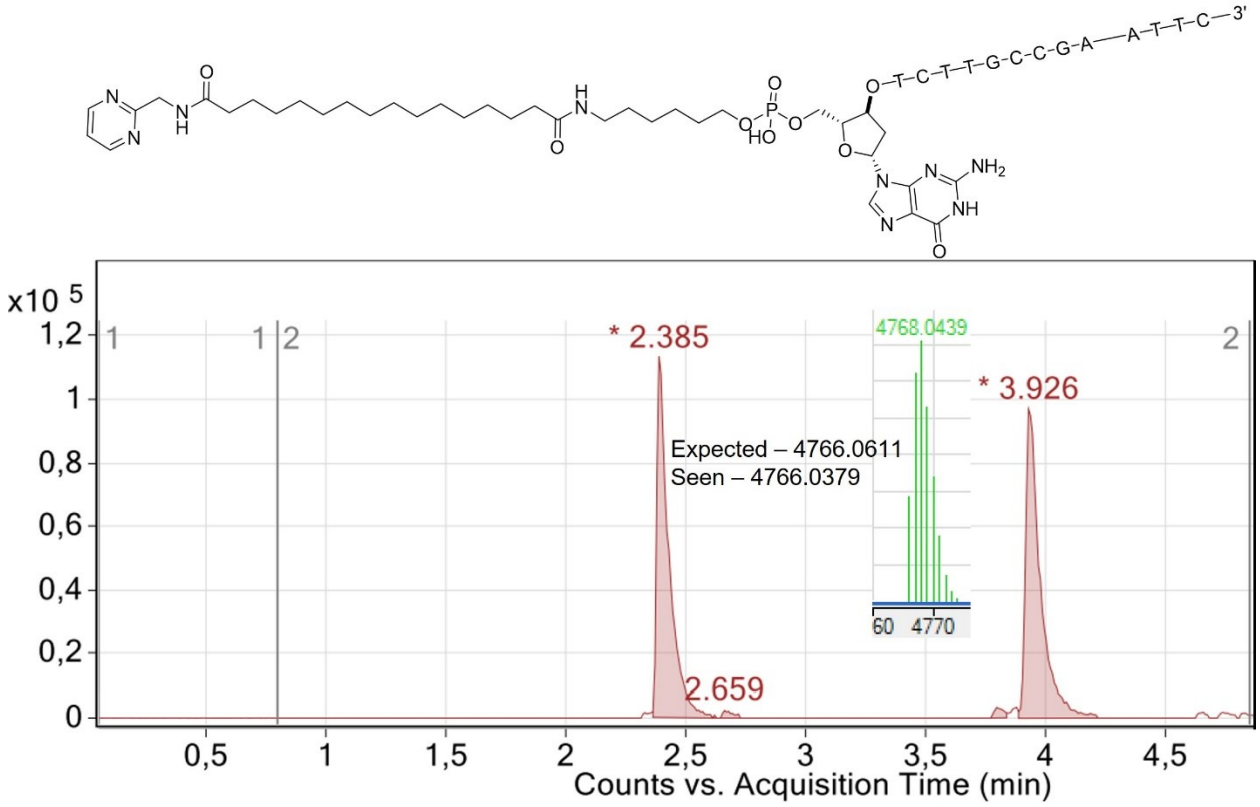
Amide coupling product, 3-aminomethylpyridine



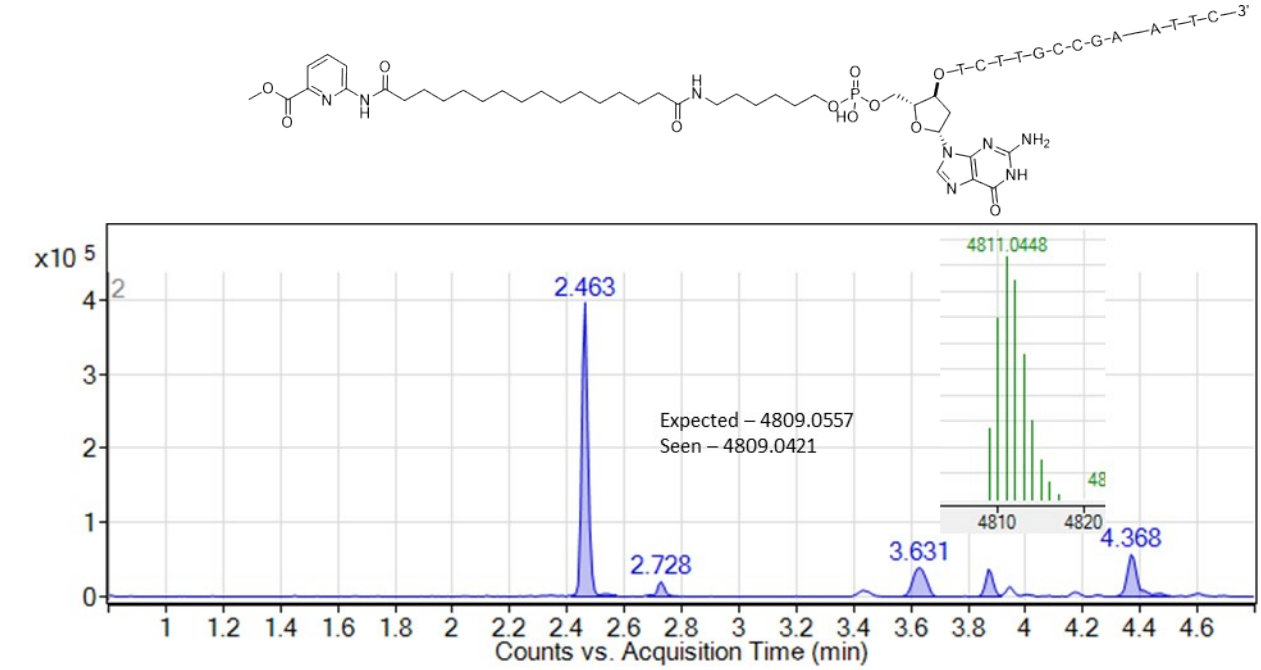
Amide coupling product, 2-aminomethylpyrazine



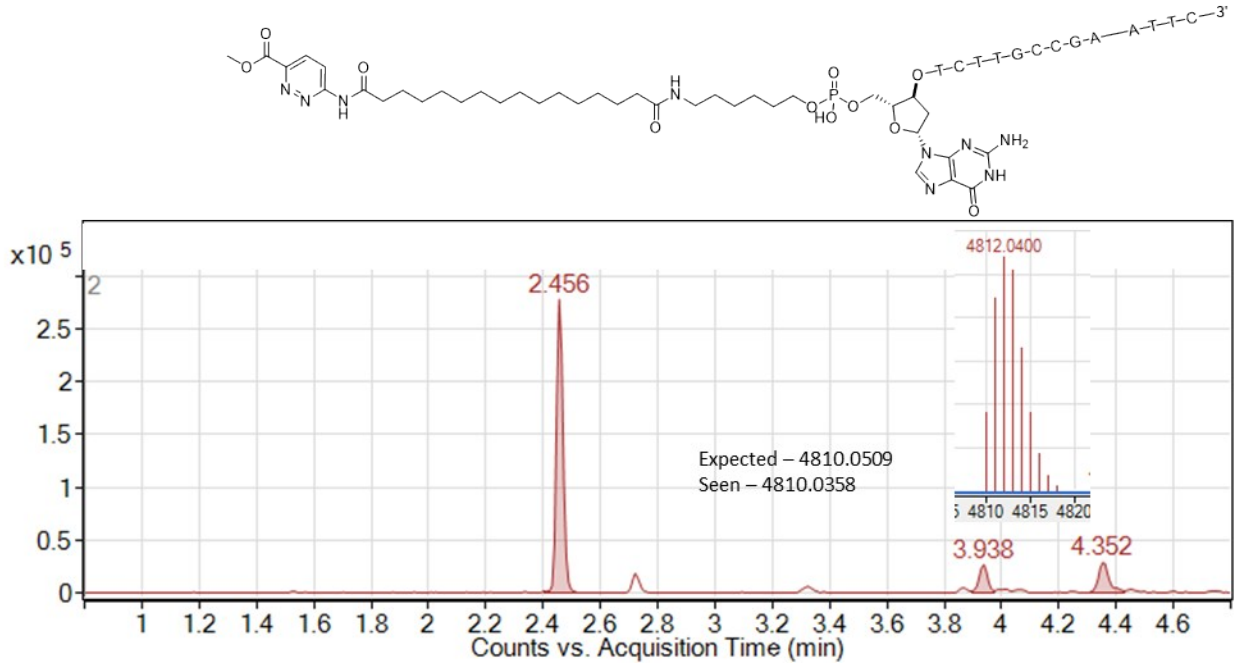
Amide coupling product, 2-aminomethylpyrimidine



Amide coupling product, methyl-5-aminonicotinate



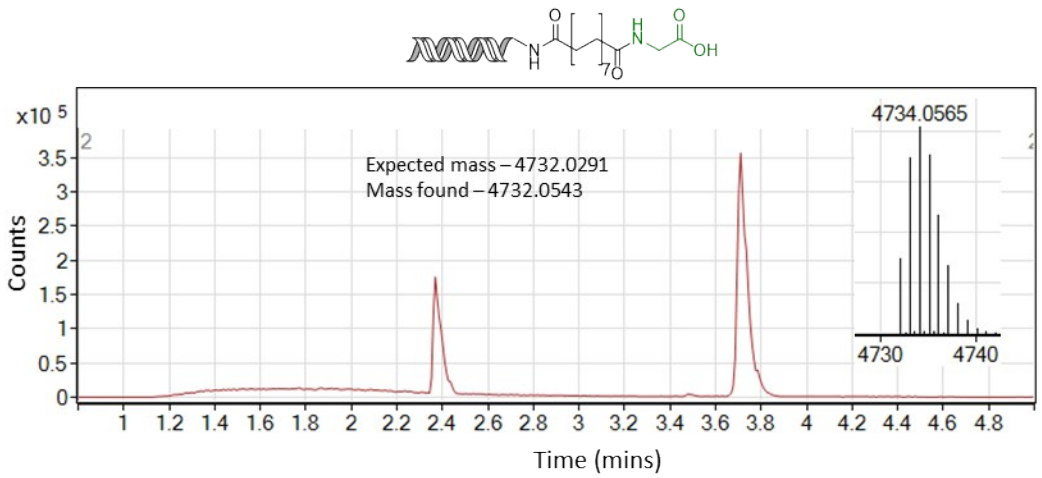
Amide coupling product, methyl-6-aminopyridazine-3-carboxylate

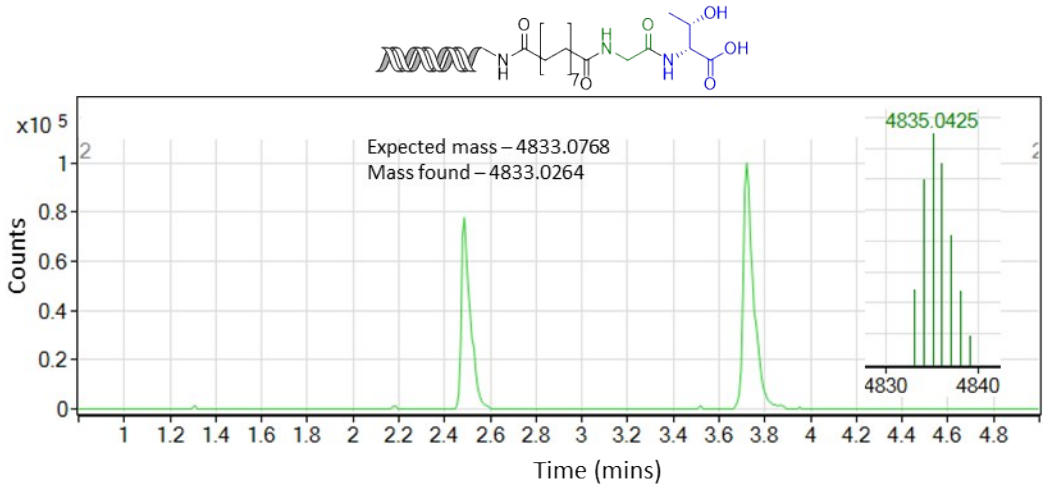


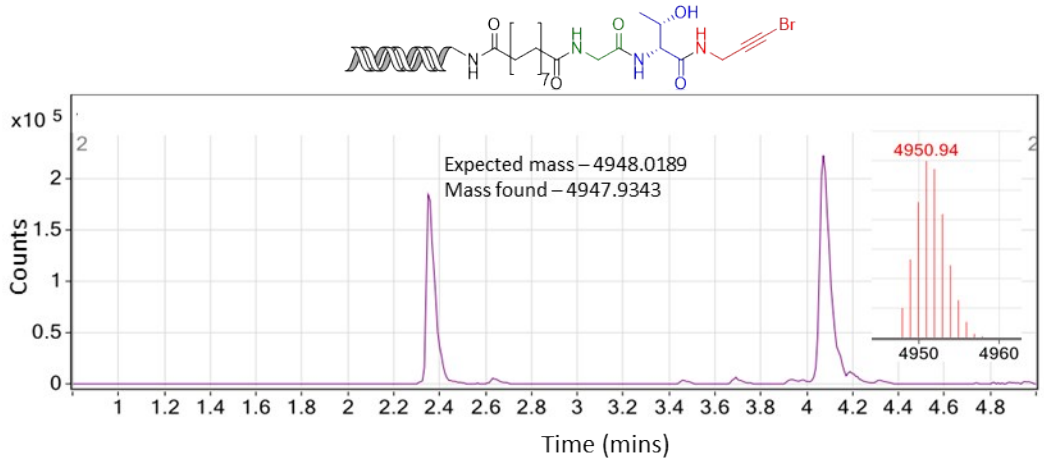
LCMS data for additional examples

Amine	RT / mins	Expected mass	Found mass
Cyclopropanemethylamine hydrochloride	4.191	4728.0706	4728.0269
4-Dimethylaminopiperidine dihydrochloride	4.277	4785.1284	4785.0835
6-Aminoindazole	4.191	4790.0611	4790.0217
3-Amino-1-N-methyl-azetidine dihydrochloride	3.953	4743.0815	4743.0423
2-aminoacetonitrile hydrochloride	3.856	4713.0345	4712.9694
3-Amino-1-methyl-1H-pyrazole	4.069	4754.0611	4754.0287
5-Amino-2-methoxypyridine	4.239	4781.0607	4781.0301

Three step amide synthesis using 14mer DNA.


x10 5
Counts
Expected mass – 4732.0291
Mass found – 4732.0543
4734.0565
4730
4740
Time (mins)


x10 5
Counts
Expected mass – 4833.0768
Mass found – 4833.0264
4835.0425
4830
4840
Time (mins)


x10 5
Counts
Expected mass – 4948.0189
Mass found – 4947.9343
4950.94
4950
4960
Time (mins)

Transmission electron microscopy

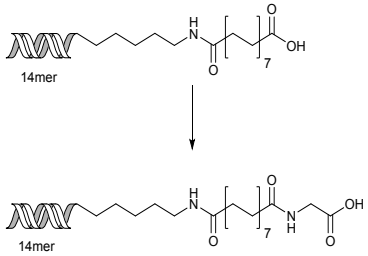
Sample A: To a 50 uL solution of TPGS-750-M 5% in water was added 50 uL of Invitrogen™ DEPC-treated water to obtain an overall concentration of 2.5% in water.

Sample B: To a solution of 2.5 nmol of DNA conjugate **3** in 2.5 uL of Invitrogen™ DEPC-treated water was added 22.5 uL of Invitrogen™ DEPC-treated water to obtain a concentration of 0.1 mM.

Sample C: To a solution of 2.5 nmol of DNA conjugate **3** in 2.5 uL Invitrogen™ DEPC-treated water was added 5 uL of Invitrogen™ DEPC-treated water and 17.5 uL of TPGS-750-M 5% in water to obtain a concentration of 0.1 mM TPGS-750-M 3.5% in water.

Samples were vortexed and sonicated before grid preparation. 4 uL of the sample were loaded on a 300- mesh glow discharged carbon grid (Gilder Grids, carbon coated at Newcastle University) held in forceps. The sample was wicked away with filter paper (Whatman n°50). 10 uL of 2% aqueous uranyl acetate (Agar Scientific) was added to stain the sample for 30 seconds. The stain was blotted away, and the grid was air-dried. Grids were examined using a Hitachi HT7800 transmission electron microscope operated at 100 kV and equipped with an Emsis Xarosa camera with Radius software. The images collected were chosen on the 300- mesh grid as the most represented structures within the mesh.

Synthesis of 14mer DNA conjugate 75



Three cycle compound 6

Glycine conjugate 4

An aliquot of glycine ethyl ester hydrochloride (60 µl, 0.25 M in NMP) and HOAT (20 µl, 10 mg per 100 µl in NMP) were added to a 50 µl glass insert for a Para-dox™ 96-well micro photoredox/optimisation Plate. The NMP was then removed at 55°C in a Genevac for 60 mins. To this solution was added **2** (30 µl, 24 nmol in 4.5% TPGS-750-M in water), 2,6-lutidine (5.2 µl, 0.045 mmol) and DIC (2.2 µl, 0.015 mmol)**.** The vials were vortexed for 30 seconds each to enhance mixing. The samples were then heated in a Para-dox™ 96-well micro photoredox/optimisation plate at 45°C for 5 hours. Mass spectrometry was used to analyse reaction. Samples prepared by adding reaction mixture (1 µl) to water (20 µl) and filtered through a hydrophilic PTFE filter. HRMS (ESI): exact mass calcd : 4760.0604; found : 4760.0629.

The reaction was diluted with water (50 µl), DCM (2 x 100 µl) was added to each and the vial vortexed. If an emulsion remained, the sample was centrifuged to aid separation. The organics were removed, and aqueous washed with ethyl acetate (2 x 100 µl). Aqueous sodium chloride (8 µl, 4M) and ethanol (264 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in 0.25M LiOH (50 µl) and shacked for 1 hour. Aqueous sodium chloride (5 µl, 4M) and ethanol (165 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in water (100 µl) and purified by a 3000 Dalton molecular weight spin filter, washing with water (3 x 100 µl). The product was eluted and mass spectrometry was used to analyse reaction. HRMS (ESI): exact mass calcd : 4732.0291; found : 4732.0543.

Glycine, threonine conjugate 5

An aliquot of methyl L-threoninate hydrochloride (60 µl, 0.25 M in NMP) and HOAT (20 µl, 10 mg per 100 µl in NMP) were added to a 50 µl glass insert for a Para-dox™ 96-well micro photoredox/optimisation plate. The NMP was then removed at 55°C in a Genevac for 60 mins. To this solution was added **75** (30 µl, 15 nmol in 4.5% TPGS-750-M in water), 2,6-lutidine (5.2 µl, 0.045 mmol) and DIC (2.2 µl, 0.015 mmol)**.** The vials were vortexed for 30 seconds each to enhance mixing. The samples were then heated in a Para-dox™ 96-well micro photoredox/optimisation plate at 45°C for 5 hours. Mass spectrometry was used to analyse reaction. Samples prepared by adding reaction mixture (1 µl) to water (20 µl) and filtered through a hydrophilic PTFE filter. HRMS (ESI): exact mass calcd : 4847.0924; found : 4847.0302.

The reaction was diluted with water (50 µl), DCM (2 x 100 µl) was added to each and the vial vortexed. If an emulsion remained, the sample was centrifuged to aid separation. The organics were removed, and aqueous washed with ethyl acetate (2 x 100 µl). Aqueous sodium chloride (8 µl, 4M) and ethanol (264 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in 0.25M LiOH (50 µl) and shacked for 1 hour. Aqueous sodium chloride (5 µl, 4M) and ethanol (165 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in water (100 µl) and purified by a 3000 Dalton molecular weight spin filter, washing with water (3 x 100 µl). The product was eluted and mass spectrometry was used to analyse reaction. HRMS (ESI): exact mass calcd : 4833.0768; found : 4833.0264

Final 3-cycle compound 6

An aliquot of 3-bromoprop-2-yn-1-amine hydrochloride (60 µl, 0.25 M in NMP) and HOAT (20 µl, 10 mg per 100 µl in NMP) were added to a 50 µl glass insert for a Para-dox™ 96-well micro photoredox/optimisation Plate. The NMP was then removed at 55°C in a Genevac for 60 mins. To this solution was added **76** (30 µl, 4 nmol in 4.5% TPGS-750-M in water), 2,6-lutidine (5.2 µl, 0.045 mmol) and DIC (2.2 µl, 0.015 mmol)**.** The vials were vortexed for 30 seconds each to enhance mixing. The samples were then heated in a Para-dox™ 96-well micro photoredox/optimisation plate at 45°C for 5 hours. The reaction was diluted with water (50 µl), DCM (2 x 100 µl) was added to each and the vial vortexed. If an emulsion remained, the sample was centrifuged to aid separation. The organics were removed, and aqueous washed with ethyl acetate (2 x 100 µl). Aqueous sodium chloride (8 µl, 4M) and ethanol (264 µl) were added and the mixture incubated at -78°C for 1 hour. The mixture was then centrifuged and the ethanol layer removed. The pellet of DNA was then dissolved in water (100 µl) and purified by a 3000 Dalton molecular weight spin filter, washing with water (3 x 100 µl). The product was eluted and mass spectrometry was used to analyse reaction. HRMS (ESI): exact mass calcd : 4948.0189; found : 4947.9343.

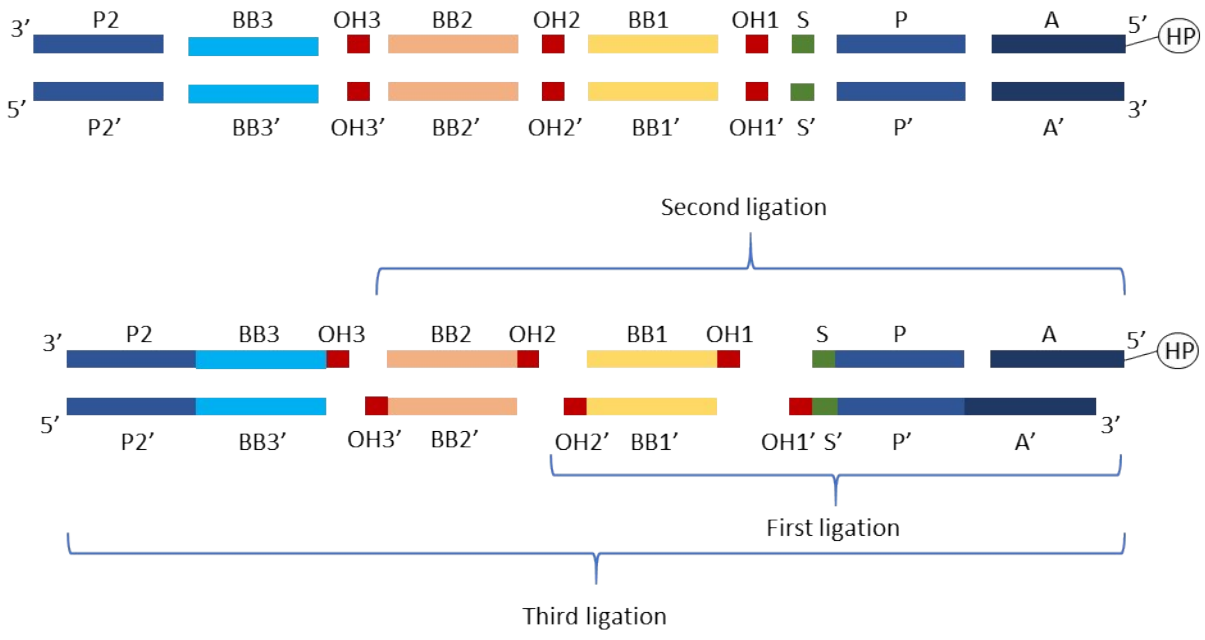
Encoded 3-cycle compound 10

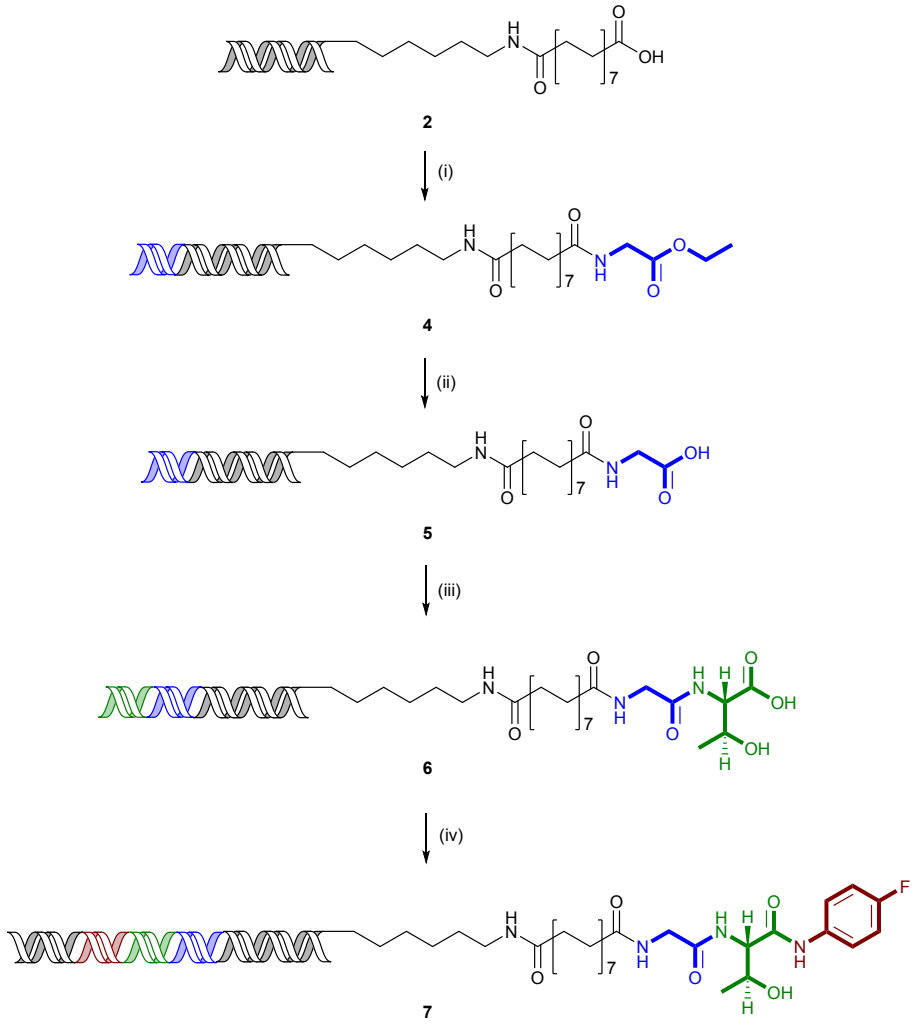
The following code abbreviations for each DNA section have been used:

Code	**Function**	**sequence 5'-3'**
A	Adapter – 5’ aminolinked head piece	GTCTTGCCGAATTC
A'	Complimentary adapter	GAATTCGGCAAGAC
P	Primer	AGGTCGGTGTGAACGGATTTG
P'	Complementary primer	CAAATCCGTTCACACCGACCT
S	Scaffold code	GCT

S'	Complementary S	AGC
OH1	Ligation overhang 1	GTAT
OH1'	Complementary OH1	ATAC
BB1	Building block 1	xxxxxxxx
BB1'	Complementary BB1	xxxxxxxx
OH2	Ligation overhang 2	CCTA
OH2'	Complementary OH2	TAGG
BB2	Building block 2	xxxxxxxx
BB2'	Complementary BB2	xxxxxxxx
OH3	Ligation overhang 3	TACG
OH3'	Complementary OH3	CGTA
BB3	Building block 3	xxxxxxxx
BB3'	Complementary BB3	xxxxxxxx
P2	Complementary to P2'	TGACCTCAACTACATGGTCTACA
P2'	Primer (reverse)	TGTAGACCATGTAGTTGAGGTCA

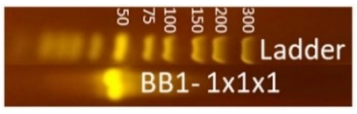
Coding strategy for encoded 3-cycle compound





Cycle 1

The first ligation included sequences containing adapter, primer, scaffold code, overhangs and building block 1 code. Prior to ligations, the 5' terminus of any DNA strands that required ligating was phosphorylated. In this case it included sequences with identifiers PS (AGGTCGGTGTGAACGGATTTGGCT), OH1BB1 (GTATGATCGACT) and A'P'S'OH1' (GAATTCGGCAAGACCAAATCCGTTCACACCGACCTAGCATAC), where the adapter sequence A (GTCTTGCCGAATTC) and BB1' sequence (TAGGAGTCGATC) did not require phosphorylation. The reactions were carried out at 450 µM for 1 hour. The ligations were then carried out in PCR tubes using 20 µl of each phosphorylation reaction mixture plus 9 µl of 1 mM solutions of non-phosphorylated A and OH2'BB1' (TAGGAGTCGATC). These reactions were made up to a total of 90 µl at 100 µM of DNA and left for 16 hours before denaturing for 10 mins at 75 °C. Gel electrophoresis showed a band at ca. 50 base pairs in length (expected 50 and 54 base pairs). It was purified by ethanol precipitation and the individual pellet was dissolved in 4.5% TPGS-750-M in water.



The first building block, glycine ethyl ester (60 µl, 0.25 M in NMP) of each was added to the reaction well, along with 20 µl HOAT at 1 mg per 10 µl and the sample reduced to dryness using a Genevac centrifugal evaporator. The DNA ligation product was added in 30 µl of 4.5% TPGS-750-M, along with 2,6-lutidine and DIC and the reaction heated to 40 °C for 3 hours in a sealed Paradox plate. After this time, the reaction was cooled to room temperature, diluted to 4 ml total volume and washed with DCM and ethyl acetate; centrifugation was required to remove the emulsion formed in this step. The resulting aqueous phase was then precipitated using ethanol. The pellet was dissolved in water and further purified using a 3000 Da spin filtration washing with water several times to remove any further organics and small ligation products. Nanodrop concentration indicated that the product **4** was 8.5 nmol (94%).

The compound was treated with 0.25 M lithium hydroxide for one hour, prior to ethanol precipitation giving 8.5 nmol of compound **5**.

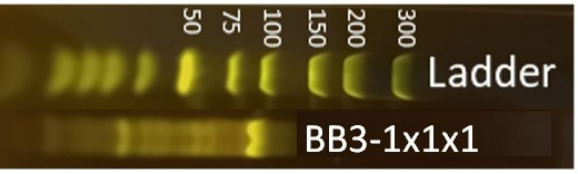
Cycle 2

To a solution of compound **5** (8.5 nmol, 1 mM) was phosphorylated as previously at a final concentration of 325 µM in an Eppendorf tube. The 5' terminus of the BB2OH2 sequence (CCTACGCGCACA) was phosphorylated in a PCR tube at 325 µM. The reactions were performed for 1 hour and denatured prior to the ligation reaction. The ligations were carried out in PCR tubes using 20 µl of each phosphorylation reaction mixture and 9 µl of a 1 mM solution of the non-phosphorylated OH3'BB2x' sequence (CGTATGTGCGCG). The overall DNA concentration as 100 µM, using 65 µl overall reaction media and was carried out for 16 hours and then denatured at 75 °C as previously. Gel electrophoresis analysis of the product showed that there was a significant band at approximately 65 base pairs in length with no visible starting material. The DNA was then precipitated using ethanol and dissolved in 4.5% TPGS-750-M ready for the second amide coupling building block addition.

The second building block, threonine methyl ester, solution in NMP was added to a reaction well along with HOAT and dried in a Genevac as previously. The solutions containing DNA were then added to the amino esters, along with DIC and 2,6-lutidine, sealed in a Paradox microplate and heated at 40 °C for 3 hours. The reaction was purified using the same procedures as the previous example, by washing with organic solvents, ethanol precipitation and molecular weight spin filtration. The 1x1x1 library was purified in the same manner. The two samples were dissolved in water and analysed by Nanodrop (5.6 nmol). The intermediate product was shaken with 0.25 M lithium hydroxide for 1 hour followed by an ethanol precipitation. The pellet was dissolved in water to provide a solution of **6** and analysed by Nanodrop (4.3 nmol, 51% overall).

Cycle 3

The **6** was dissolved in water to create a 1 mM solution. A longer DNA sequence was ligated incorporating a building block codon and the capping primer sequences (31 and 35 base pairs in length). The P2BB3xOH3 sequence (TACGACGGCACCTGACCTCAACTACATGGTCTACA) was phosphorylated at 165 µM total concentration of DNA. Once phosphorylation had taken place, the DNA was transferred to a PCR tube along with the P2'BB3 sequence (TGTAGACCATGTAGTTGAGGTCAGGTGCCGT) at a concentration of 55 µM and ligated for 16 hours using T4 ligase. Gel electrophoresis was used to analyse the product, a bright band was seen at ~100 BP (expected 97 BP). The DNA was precipitated using ethanol and dissolved in 4.5% TPGS-750-M ready for the final amide coupling.



A solution of the final amine building block, 4-fluoroaniline, (60 µl, 0.25 M in NMP), was added to a reaction tube along with HOAT, the solvent was removed and in the Genevac. The TPGS-750-M solution containing **6** was added along with DIC and 2,6-lutidine, sealed in a Paradox microplate and heated at 40 °C for 3 hours. The solution was washed with DCM and ethyl acetate, ethanol precipitated and redissolved in water. Further purification as carried out using a 10000 Da molecular weight spin filter. A final purification was carried out using preparative HPLC using buffers containing 16.3 mM TEA and 400 mM HFIP on a C18 column to reveal the 3 cycle product **7** (2.7 nmol, 63%).

PCR and Sequencing

PCR amplification was carried out using 1 µg (0.5 µl) of the DNA template and either 0.2 µM, 2 µM primers concentration along with a negative control with the primers omitted. 40 PCR cycles were carried out prior to analysis by gel electrophoresis on a 4% agarose gel. Analysis by agarose gel electrophoresis showed that there was a clear band around 140-150 base pairs in length. The expected length of the DNA strand post amplification using the 33-base pair NGS extensions was 148-base pairs. The PCR product was analysed by NGS (Genewiz, South Plainfield, NJ, USA), >80% of 77000 reads corresponded exactly to the expected sequence.