Supplementary information

Scanning conformational space with a library of stereo- and regiochemically diverse aminoglycoside derivatives: The discovery of new ligands for RNA hairpin sequences

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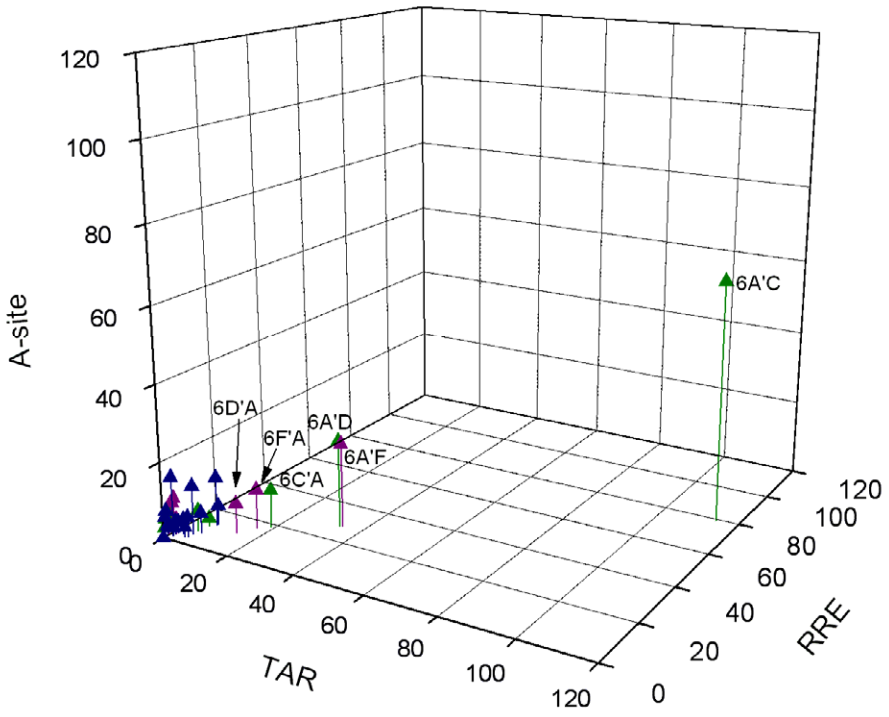
Table S1: Relative change in resonance unit (RU) observed in the association phase in the presence of 1 μM of each aminoglycoside derivative. The data have been normalised to account for differences in the immobilisation levels and relative molecular masses of the RNA molecules and the aminoglycosides. A response of 100% refers to an increase in RU of R_{max}, the maximum response observed when one binding site is occupied.

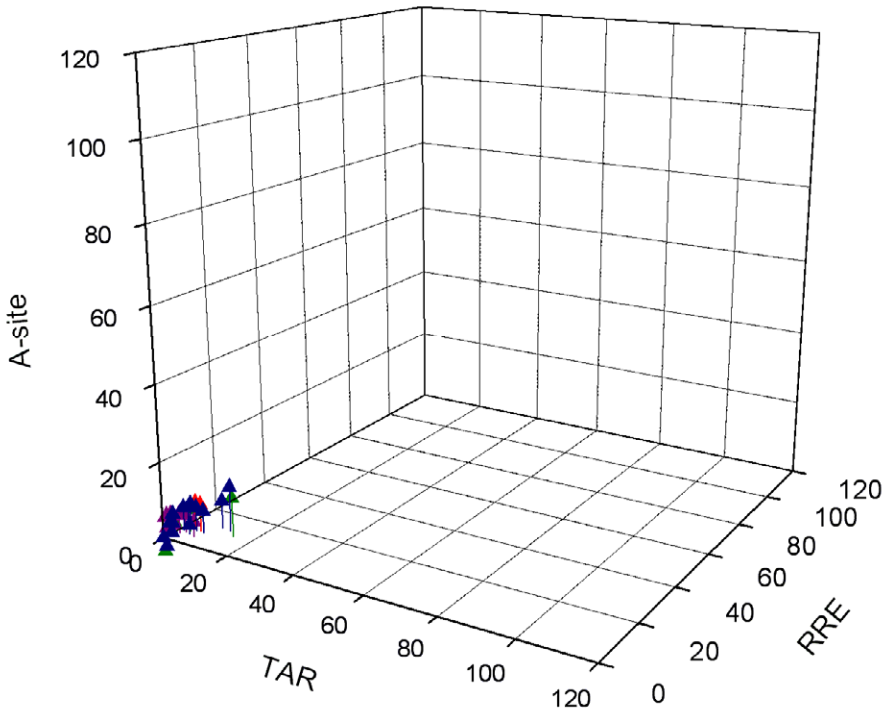
Compound screened	n[a]	TAR	RRE	A-site	Compound[b]	TAR[c]	RRE[c]	A-site[c]
6A	3	13	27	2	**6'A'**	8	18	2
6A'	3	16	23	1	**6'A**	11	17	3
6G	4	46	38	27	**6'G'**	30	27	18
9GH	4	12	14	12	**9'G'H'**	8	12	8
9GH'	4	9	12	9	**9'G'H**	7	13	6
9GF	5	7	10	9	**9'G'F'**	6	11	7
6AD	4	-2	3	3	**6D'A'**	-1	1	1
6AB	4	-4	3	4	**6B'A'**	-1	1	2
6AB'	4	-3	4	5	**6BA'**	-1	2	2
6AE	4	-3	7	7	**6E'A'**	1	4	3
6A'D	4	34	27	23	**6D'A**	14	11	8
6A'B	4	7	13	13	**6B'A**	5	7	6
6AF	4	-7	1	2	**6F'A'**	3	4	4
6A'B'	4	-2	3	4	**6BA**	0	2	2
6A'E	4	-2	5	7	**6E'A**	1	3	3
6A'F	4	34	28	22	**6F'A**	16	16	10
6AC	4	-1	4	5	**6C'A'**	1	3	4
6A'C	4	114	79	63	**6C'A**	19	18	10
6AA' [d]	4	7	13	5				

6AA [e]	4	1	2	15				
6A'A [d]	4	5	3	5				
6A'A' [e]	4	6	7	5				
7AB	4	0	4	5	**7'A'B'**	3	6	4
7AB'	4	2	7	7	**7'A'B**	4	5	3
7AC	4	14	9	10	**7'A'C'**	9	12	7
7'AA	4	2	7	6	**7A'A'**	3	6	6
7AA	4	1	6	6	**7'A'A'**	4	7	6
7'AA'	4	12	11	12	**7AA'**	7	7	6
7'AB	4	1	1	1	**7A'B'**	0	2	3
7'AC	4	2	-2	-2	**7A'C'**	1	2	3
7'AB'	4	2	-1	-1	**7A'B**	1	3	4
8A	3	-3	6	7	**8'A'**	2	4	5
8'A	3	-3	5	6	**8A'**	0	3	4
8'G	4	1	8	8	**8G'**	3	6	6
8G	4	-1	8	9	**8'G'**	5	5	3
8'AC	4	4	8	7	**8A'C'**	6	7	7
8'AA'	4	-2	3	4	**8AA'**	3	5	5
8'AB	4	-4	2	3	**8A'B'**	0	2	3
8'AB'	4	-2	3	4	**8A'B**	2	2	3
8'AA	4	-1	5	5	**8A'A'**	0	3	3
8AA	4	-1	4	4	**8'A'A'**	0	1	2

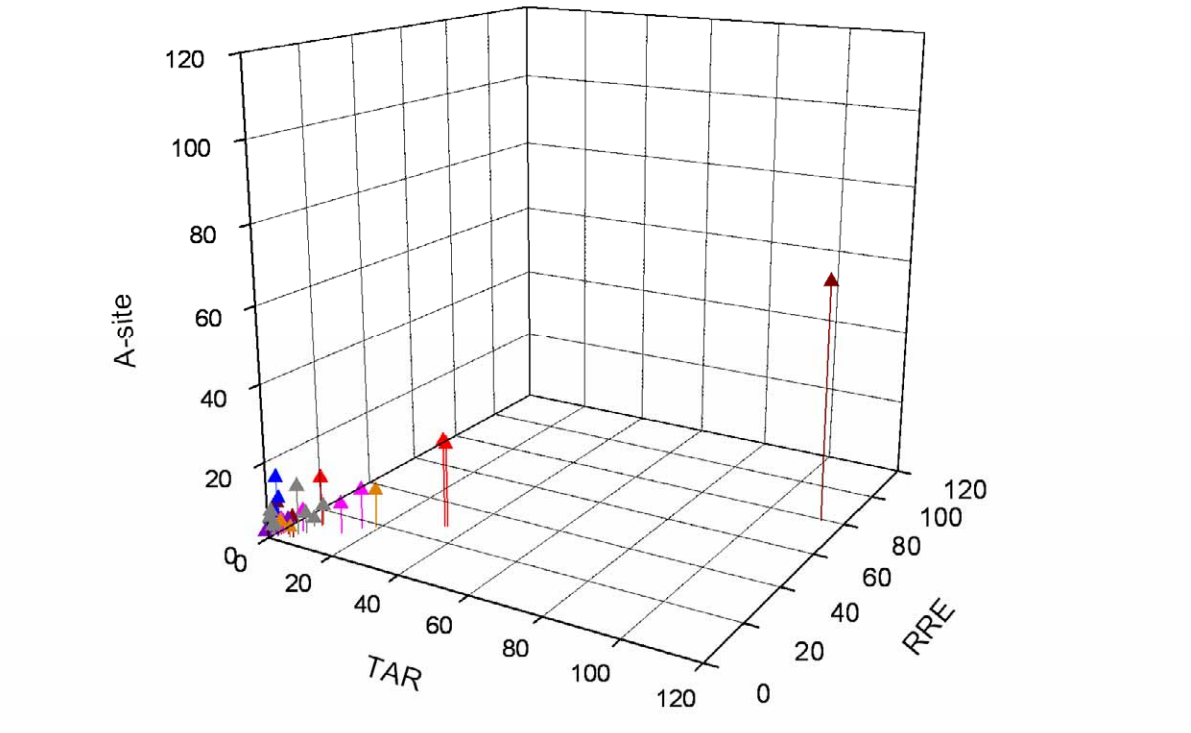
[a]Number of amino groups. [b]The compound was not prepared; its affinity for each RNA was determined indirectly by screening against enantiomeric RNA. [c]Enantiomeric RNA was used. [d]This achiral compound was not screened against enantiomeric RNA. [e]Both enantiomeric compound were prepared and screened against D-configured RNA.



Legend for Figure S1: Relationship between the relative change in resonance unit (RU) observed in the association phase in the presence of 1 μM of the aminoglycoside derivatives **6Sug$_1$Sug$_2$** (see Figure 4) and the regiochemistry of the aminoglycoside derivatives. Stereoisomeric derivatives with the same regiochemistry are shown in the same colour.



Legend for Figure S2: Relationship between the relative change in resonance unit (RU) observed in the association phase in the presence of 1 μM of the aminoglycoside derivatives **7Sug$_1$Sug$_2$** and **8Sug$_1$Sug$_2$** (see Figure 4) and the regiochemistry of the aminoglycoside derivatives. Stereoisomers with the same regiochemistry are shown in the same colour.



Legend for Figure S3: Relationship between the relative change in resonance unit (RU) observed in the association phase in the presence of 1 μM of the aminoglycoside derivatives **6Sug$_1$Sug$_2$** and **8Sug$_1$Sug$_2$** (see Figure 4) and the absolute configuration of the aminoglycoside derivatives. Regioisomeric derivatives with the same absolute configuation are shown in the same colour.

Table S2: Kinetic parameters and dissociation constants for the binding of aminoglycoside derivatives to RNA sequences. Experimental data was fitted to a three binding site model.

	$k_{a,1}$ / $M^{-1}s^{-1}$	$k_{d,1}$ / s^{-1}	$k_{a,2}$ / $M^{-1}s^{-1}$	$k_{d,2}$ / s^{-1}	$k_{a,3}$ / $M^{-1}s^{-1}$	$k_{d,3}$ / s^{-1}	$K_{D,1}$ / μM	$K_{D,2}$ / μM	$K_{D,3}$ / μM
A-site									
6A'B	140	4.1E-03	5500	0.25	57	0.029	29 ± 1	46	51
6A'C	180	4.3E-05	4400	0.065	470	5.1E-03	0.23 ± 0.01	14	11
6A'F	210	9.7E-04	1.9E+04	0.23	1200	0.010	4.7 ± 0.1	12	8.5
A-site, U1495A variant									
6A'B	85	5.1E-04	3600	0.13	650	0.011	6.0 ± 1.5	35	17
6A'F	260	7.1E-04	1.2E+04	0.14	1100	7.5E-03	2.8 ± 0.1	12	7.1
A-site, U1406A variant									
6A'B	0.011	2.7E-05	2000	0.078	290	3.9E-03	14 ± 1	2.3E4	39
6A'C	220	7.1E-05	3600	0.089	600	5.7E-03	0.33 ± 0.01	25	9.5
6A'F	300	1.2E-03	1.1E+04	0.17	820	6.8E-03	3.9 ± 0.1	15	8.4
TAR									
6A'C	350	9.4E-05	1900	0.024	360	1.3E-03	0.27 ± 0.03	13	3.6
6A'D	390	9.0E-05	2000	0.015	430	7.8E-04	0.26 ± 0.03	7.7	1.8
6A'F	390	1.0E-04	1900	0.21	1100	9.1E-03	0.26 ± 0.07	11	8.1
RRE									
6A'B	40	1.2E-03	2500	0.63	300	0.021	30 ± 13	250	69
6A'C	38	1.0E-04	55	0.82	56	5.5E-03	2.7 ± 0.5	1.4E4	98
6A'D	360	9.8E-05	1200	0.012	430	1.2E-03	0.27 ± 0.04	9.8	2.9

Experimental

Solid-Phase RNA Synthesis

Automated solid-phase oligonucleotide synthesis was carried out using an Applied Biosystems 391 DNA synthesiser using standard ABI reagents and a standard coupling protocol for 5'-DMT phosphoramidite chemistry. Synthesis of the wild type sequences was carried out on a 1 μmol scale whilst all variant sequences were synthesised on a 0.2 μmol scale. All syntheses were performed using 500 Å DMT-*N*-*t*-butylbenzoyl-biotin succinate attached to lcaa CPG purchased from ChemGenes, Inc (USA). Standard 2'-*O*-*t*-butyldimethylsilyl-D-ribo-phosphoramidites, 2'-*O*-*t*-butyldimethylsilyl-L-ribo-cytidine-phosphoramidite and 5'-DMT-4'-thiocyanoethyl-2'-*O*-*t*-butyldimethylsilyl-D-uridine-phosphoramidite were purchased from ChemGenes, Inc (USA); 2'-*O*-*t*-butyldimethylsilyl-L-ribo-phosphoramidites were prepared in Leeds. The exocyclic amino groups of adenine and cytosine were benzoyl protected while guanine was isobutyryl-protected.

After synthesis, the CPG-bound oligonucleotides were cleaved off the resin, and the exocyclic amino and cyanoether protecting groups were removed using freshly prepared methanolic ammonia at 30 °C for 30 h. After evaporation to dryness, the TBDMS groups were removed at room temperature by dissolution in a 1:1 solution of DMSO and $NEt_3 \cdot 3$ HF (purchased from Aldrich) at room temperature overnight.

RNA Purification

All deprotected oligonucleotides were purified (and partially desalted and concentrated) by a two-step HPLC purification:

1. Anion exchange. Using a DNAPac™ PA-100 column and a Dionex DX 500 chromatography system, with H_2O and 1 M NH_4Cl eluent. An elution profile of 0→100% eluent over 30 min at 70 ºC was used.
2. Reverse phase. Using a Merck LiChrospher® 100 column and a Beckman System Gold chromatography system, with 100 mM NH_4OAc and 1:1 (v/v) 100 mM NH_4OAc/acetonitrile eluent. An elution profile of 0→100% eluent over 30 min at 60 ºC was used.

For analytical runs, the flow rate was 1 ml/min with UV detection set at a wavelength of 260 nm. For preparative runs the flow rate, was 2 ml/min with UV detection set at 290 nm.

Final desalting and concentration of the RNA samples was done using MF centrifugal concentrators with a 1 K cut off membrane. Samples were spun at 6,000 rpm for 2 days, salts were washed through the membrane by the addition of 1 ml portions of autoclaved milliQ water every 10 h.

The concentration of each of the RNA stock solutions (in water) was calculated from the measured absorbance at a wavelength of 260 nm and the μM extinction coefficient for each sequence. All absorbance measurements were made using a Perkin Elmer Lambda Bio 40 UV/Vis spectrophotometer. All extinction coefficients and expected molecular weights were derived using 'Expedite sequence editor' software.

All RNA sequences were analysed in negative ion mode on a Platform II mass spectrometer (Waters UK Ltd., UK) with an ESI source coupled to a single quadrupole analyser. 50 μl samples at a concentration of 10 ρmol/μl were required for direct injection. Samples were sprayed in a solution of methanol (50%) water (50%) containing triethylamine (1%). Mass spectra for the sequences are also provided in the Supplementary Information. The capillary voltage was held at 2.30 kV, the counter electrode at 0.17 kV and the sampling cone voltage at 30 V. Mass calibration was performed on a separate injection using a 2 μg/μl aqueous sodium iodide clusters in negative ion mode. Neomycin sulfate was analysed on the Platform II in positive ion mode. The sample was prepared at a concentration of 10 ρmol/μl and sprayed in an aqueous solution of methanol/HCOOH (99:1). The capillary voltage was held at 3.5 kV, the counter electrode at 0.5 kV and the sampling cone voltage at 40 V. Mass calibration was performed on a separate injection using a 10 ρmol/μl horse heart myoglobin solution (methanol (50%) water (50%) with 0.5% formic acid) in positive ion mode.

Data processing was carried out with the MassLynx suite of programs supplied with the mass spectrometer: m/z spectra were smoothed or transposed onto a mass profile using Maximum Entropy techniques.

Surface Plasmon Resonance

General Procedures

All immobilisation and subsequent interaction studies were performed using a BIAcore 3000 instrument (Pharmacia Biosensor) set a temperature of 25 °C. Streptavidin-functionalised BIAcore sensorchips (Sensor chip SA) were purchased from Biacore AB. Standard desorb and sanitise routines were performed according to the BIAcore guidelines before docking new SA sensor chips. All buffers were autoclaved and filtered through sterile 0.2 μm nylon membranes (Nalgene) under vacuum. All RNA immobilisation and subsequent aminoglycoside binding experiments were performed according to the methods described by Wong and coworkers.[1]

Immobilisation of Biotinylated RNA

Before derivatisating the flow cells, the SA sensor chip was pre-conditioned using three successive 50 μl injections of chip preparation solution (50 mM NaOH, 1 M NaCl) at 50 μl/min, followed by a normalise routine using 70% glycerol BIAnormalising solution. Prior to immobilisation, solutions of biotinylated RNA (30 ρmol) in 240 μl of renaturing buffer (10 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, pH 6.8) were renatured by heating to 80 °C for 2 min followed by slow cooling to room temperature. Individual flow cells were functionalised by injecting 150 μl of RNA buffer using the INJECT command at a flow rate of 5 μl/min, followed by washing the Intergrated Fluidic Cartridge (IFC) and injection needle. Three flow cells were used to immobilise RNA while the fourth remained unmodified to serve as a blank control for matrix effects. Levels of RNA capture were calculated by subtracting response units after injection from response units before injection, for each flow cell.

General Procedures for Surface Plasmon Resonance Binding Studies

Aminoglycoside analyte samples were prepared by serial dilutions from stock solutions into Eppendorf tubes. All procedures for binding were automated as methods using

repetitive cycles of sample injection and regeneration. Typically, running buffer was injected in the first cycle to establish a stable baseline value. 200 µl samples were injected at a flowrate of 20 µl/min using the INJECT command. All aminoglycoside samples were injected from autoclaved 7 mm plastic vials (BIAcore) that were capped with pierceable plastic crimp caps to minimise carry-over and sample evaporation. Samples were injected in order of increasing concentration. Running buffer was injected for one cycle between each different concentration of the same analyte, and for two cycles between different analytes. The surface was regenerated using three pulses of 30 µL of 150 mM Na_2SO_4 solution.

Fitting of experimental data

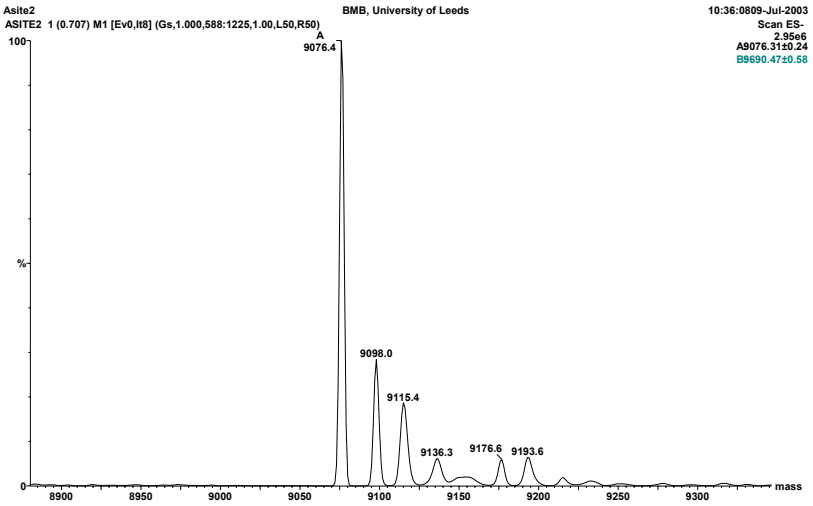
The sensorgrams generated were fitted using the BiaEvaluation software. Dissociation constants were determined by fitting the experimental data using the following formula:[2]

$$R = R_{max} \{[c / (K_{D,1} + c)] + \{[c / (K_{D,2} + c)] + \{[c / (K_{D,2} + c)]\}$$

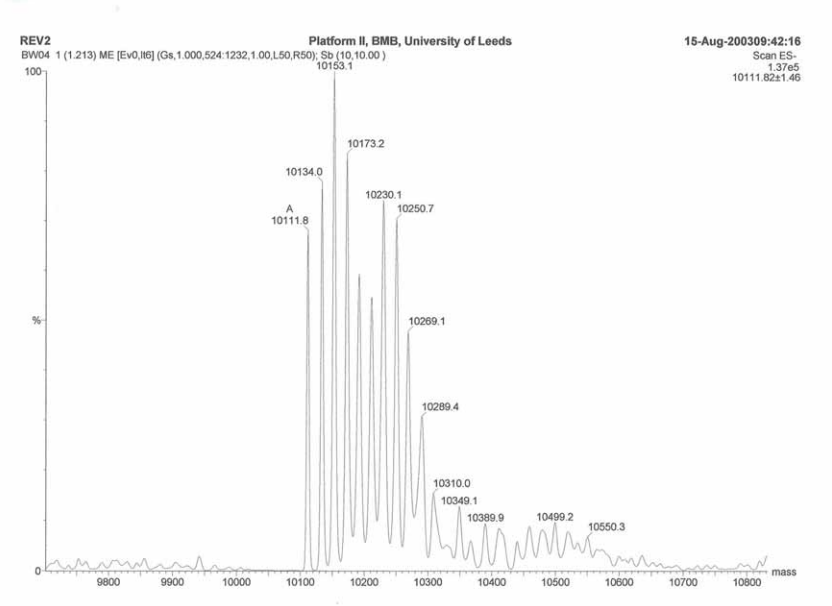
Where R = response, R_{max} = maximum response of one binding site occupied, c = concentration, $K_{D,1}$ = dissociation constant for specific binding, $K_{D,2}$ and $K_{D,3}$ = dissociation constants for lower affinity binding.

Mass spectra of the RNA sequences

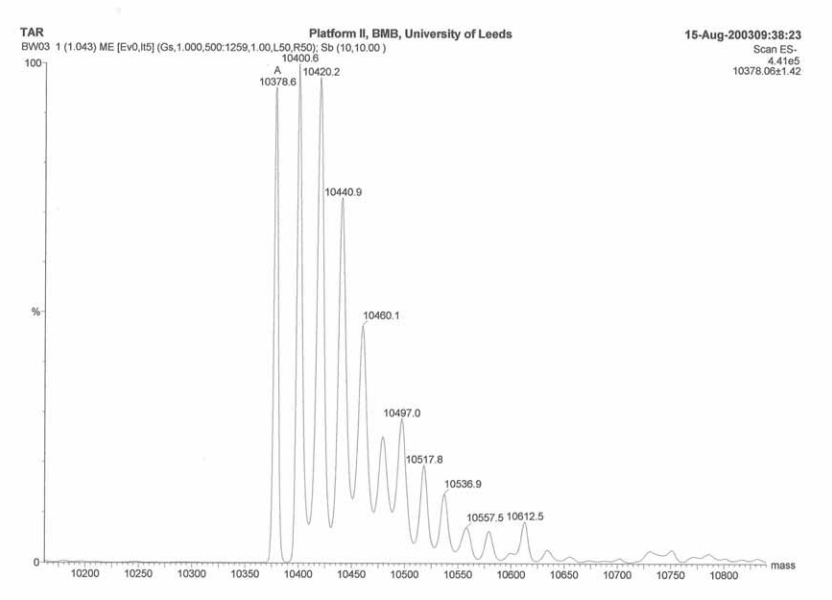
(a) A-site sequence



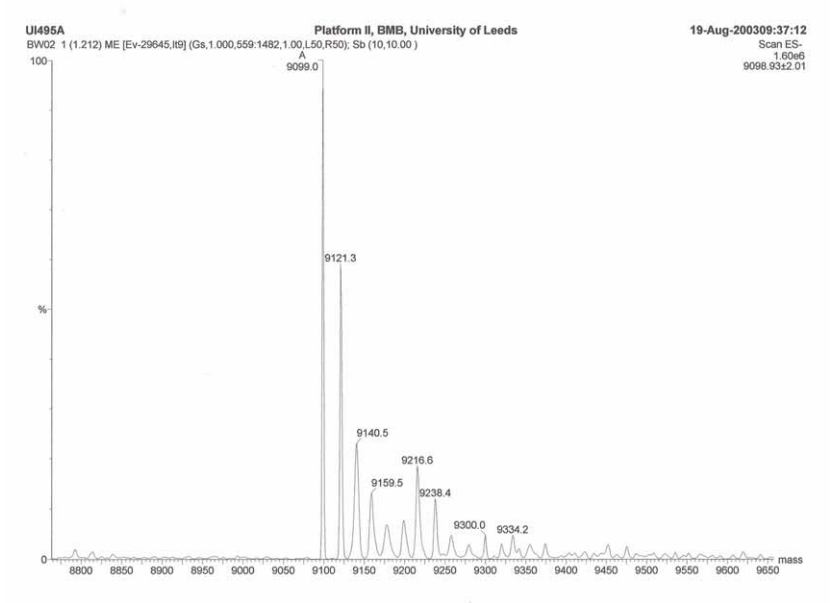
(b) RRE sequence



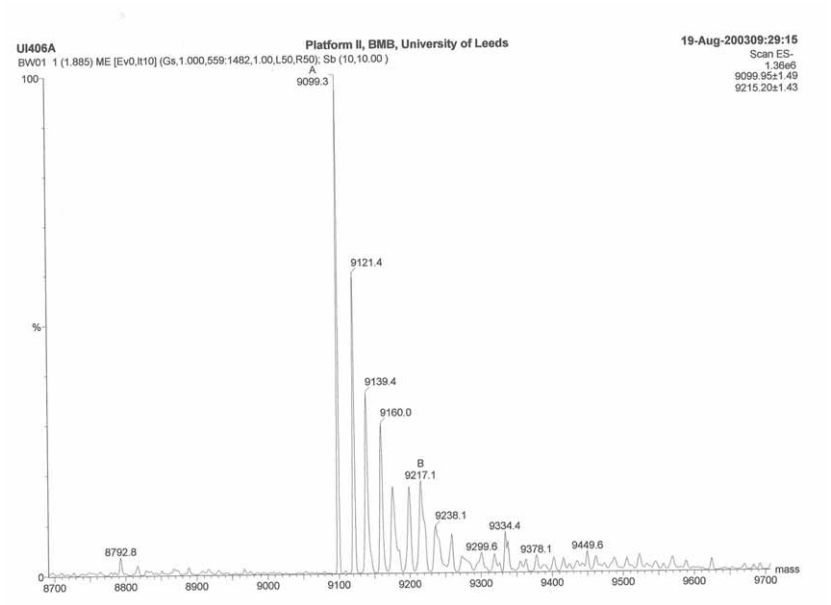
(c) TAR sequence



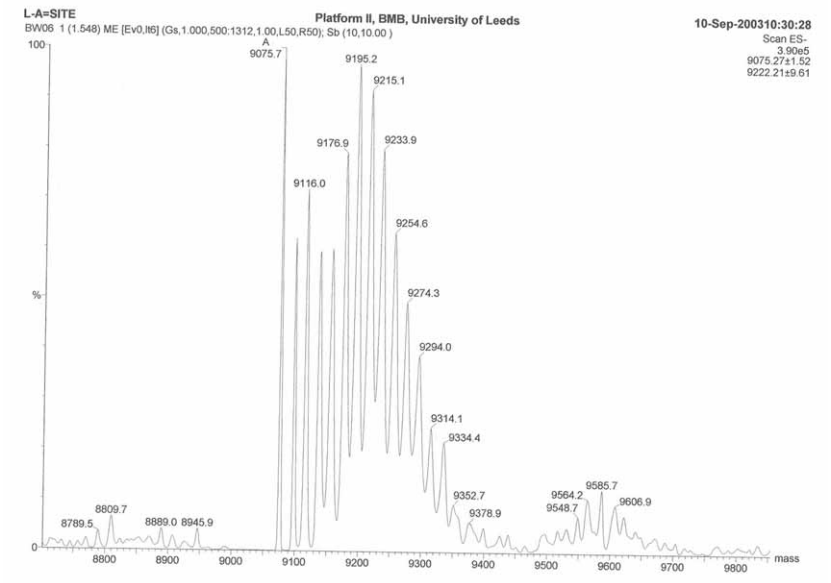
(d) U1495A variant of the A-site sequence



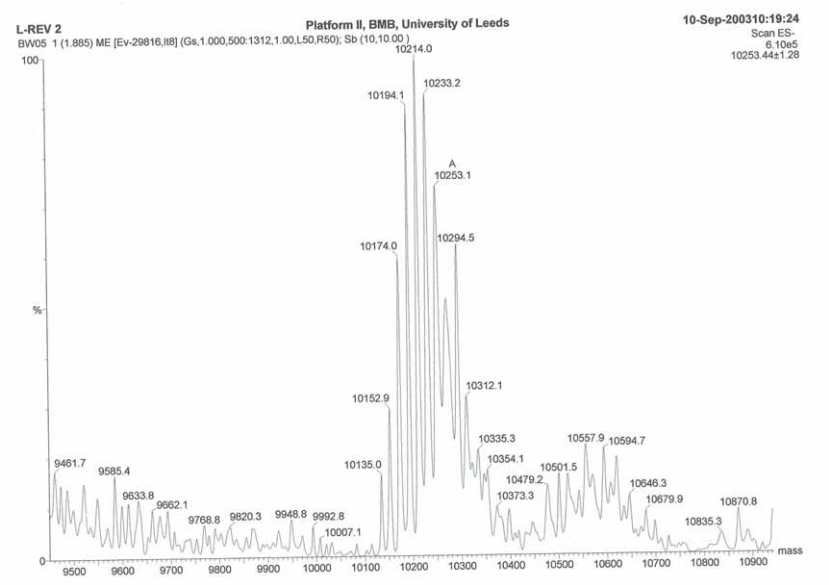
(e) U1406A variant of the A-site sequence



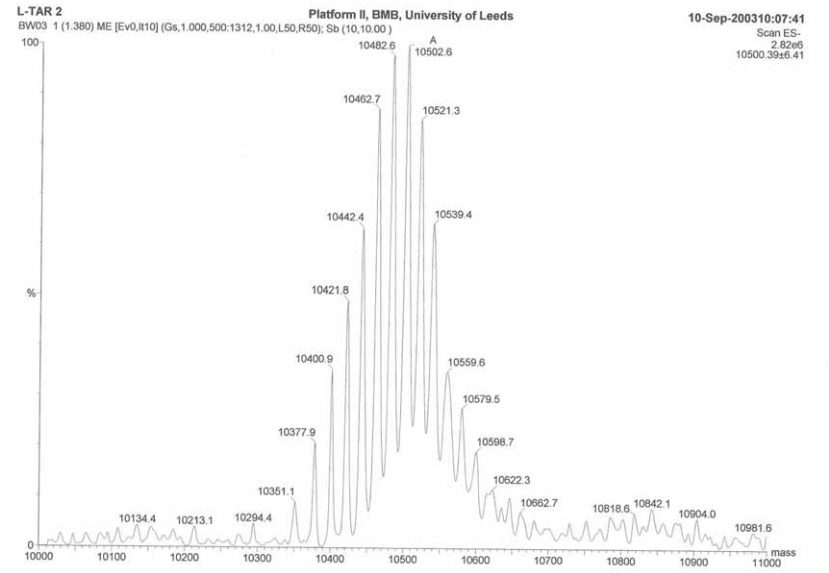
(f) Enantiomeric A-site sequence prepared from L-configured phosphoramidites



(g) Enantiomeric RRE sequence prepared from L-configured phosphoramidites



(h) Enantiomeric TAR sequence prepared from L-configured phosphoramidites



References:

1. M. Hendrix, E. S. Priestley, G. F. Joyce and C.-H. Wong, *J. Am. Chem. Soc.* 1997, **119**, 3641.
2. S. H. L. Verhelst, P. J. A. Michiels, G. A. van der Marel, C. A. A. van Boeckel and J. H. van Boom, *ChemBioChem* 2004, **5**, 937.