Identification of a small molecule inhibitor of importin beta mediated nuclear import by confocal on-bead screening of tagged one-bead one-compound libraries

Supporting Information

Martin Hintersteiner[1,3,‡], Géza Ambrus[2,‡], Janna Bednenko[2], Mario Schmied[3], Andrew J.S. Knox[1], Hubert Gstach[3], Jan-Marcus Seifert[3], Eric L. Singer[2], Larry Gerace[2]* and Manfred Auer [1,3]*

[1] The University of Edinburgh, School of Biological Sciences (CSE) and School of Biomedical Sciences (CMVM) , Michael Swann Building, 3.34, The King's Buildings, Mayfield Road, Edinburgh, EH9 3JR, UK

[2] Department of Cell Biology, The Scripps Research Institute, 10550 N Torrey Pines Road, La Jolla, Ca 92037, USA

[3] Affiliation when work was performed: Innovative Screening Technologies unit, Novartis Institutes for BioMedical Research (NIBR), Brunnerstrasse 59, A-1235 Vienna, Austria

*Corresponding authors. E-mail: manfred.auer@ed.ac.uk and lgerace@scripps.edu

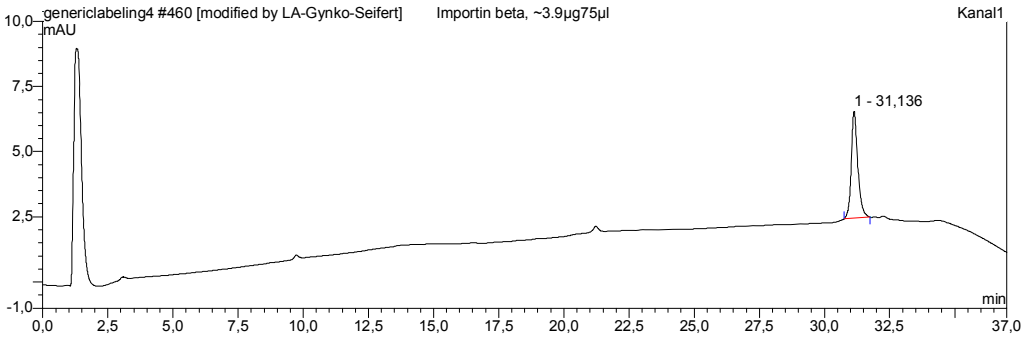
‡ These authors contributed equally to this work

Supporting Figures and Table

Figure S1 Characterization of purified importin β

A. RP-HPLC of unlabeled importin β at 215 nm detection wavelength. **B**. SDS-PAGE of importin β; NuPAGE 4-12% Bis-Tris Gel, 1 mm, reducing conditions, MES buffer, 200 V, with Mark 12 protein standard, silver staining

A



B

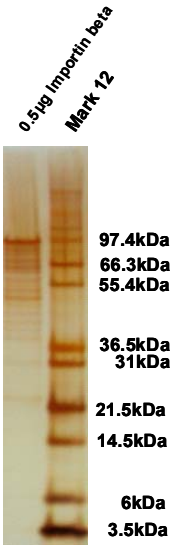
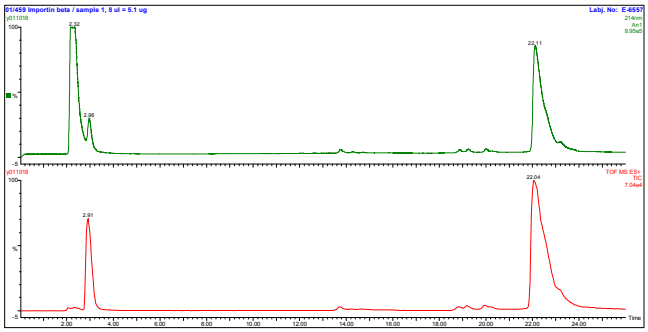


Figure S2 LC/ESI-MS analysis of recombinant importin β

LC/ESI-MS analysis of recombinant importin β. Briefly, 8 µl of a 6 µM solution of importin β were injected onto a Poros reversed phase column (1 R/H, 1x 50 mm) and eluted with a gradient of 25% to 70% B within 20 minutes at a constant flow of 0.06 ml min^{-1} (eluent A: 0.05% TFA in H_2O; eluent B: 90% CH3CN, 0.045% TFA in H_2O). The temperature of the column oven was set to 80° C. **A**. The chromatograms for 214 nm UV absorption and for total ion current detection (m/z = 500 – 2000). B. Expected and measured molecular weight for importin β. **C**. The deconvoluted MS spectrum (quadrupole MS with TOF detection) of the peak at a retention time of 14.8 minutes.

(A)



(B)

Expected:	**98242.3 Da**
Measured:	**A 98240.5 Da 60 %** **B 98375.5 Da 40 %**

(C)

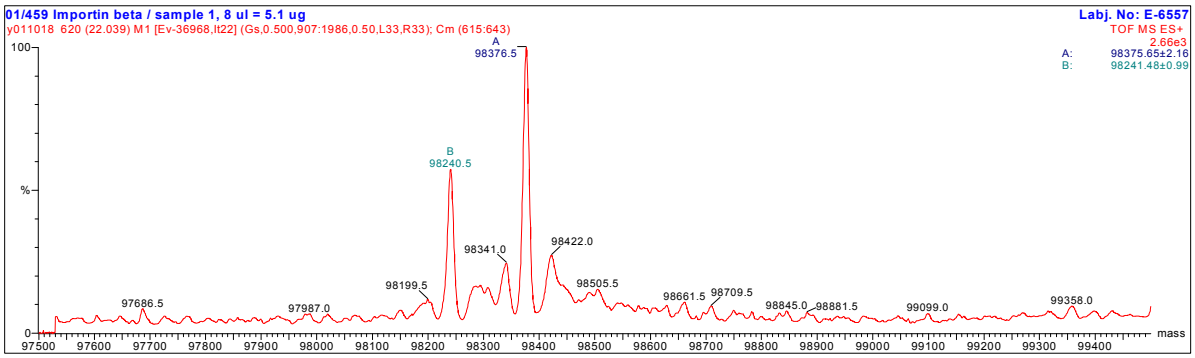


Figure S3 RP-HPLC analysis of Alexa488 labeled importin β

The upper panel shows the chromatogram detected by UV absorption at 280 nm. The lower panel shows the chromatogram recorded by fluorescence detection at 488 nm excitation / 520 nm emission. The labeled protein eluted at a retention time of 51.89 min (fwhm = 0.76 min), corresponding to 95% CH_3CN.

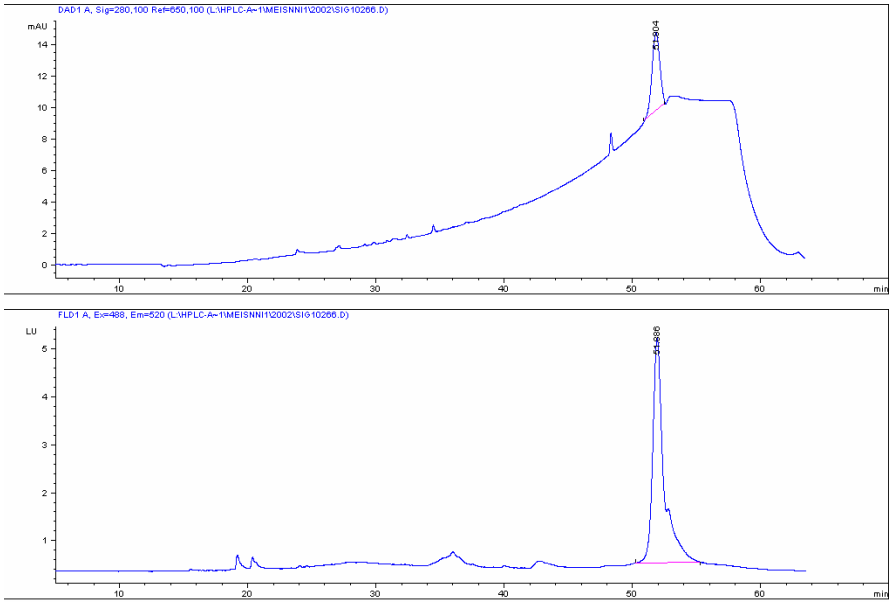


Figure S4 UV-VIS spectrum of Alexa488 labeled importin β

All spectra were taken in 20 mM HEPES pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate and 2 mM DTT, 0.01 mM CHAPS and referenced to the background absorption of the same buffer. A correction factor of 0.173 was determined for the Alexa488 contribution to the extinction at 280 nm based on the ratio between the dye absorption at 280 nm and 454 nm. Due to the Alexa488 absorption band around 300 nm, no correction for Raleigh scattered light was applied. **A**. The UV/VIS spectrum of recombinant importin β (black spectrum) and Alexa488 (red spectrum). **B**. The spectrum of Alexa488-labeled importin β.

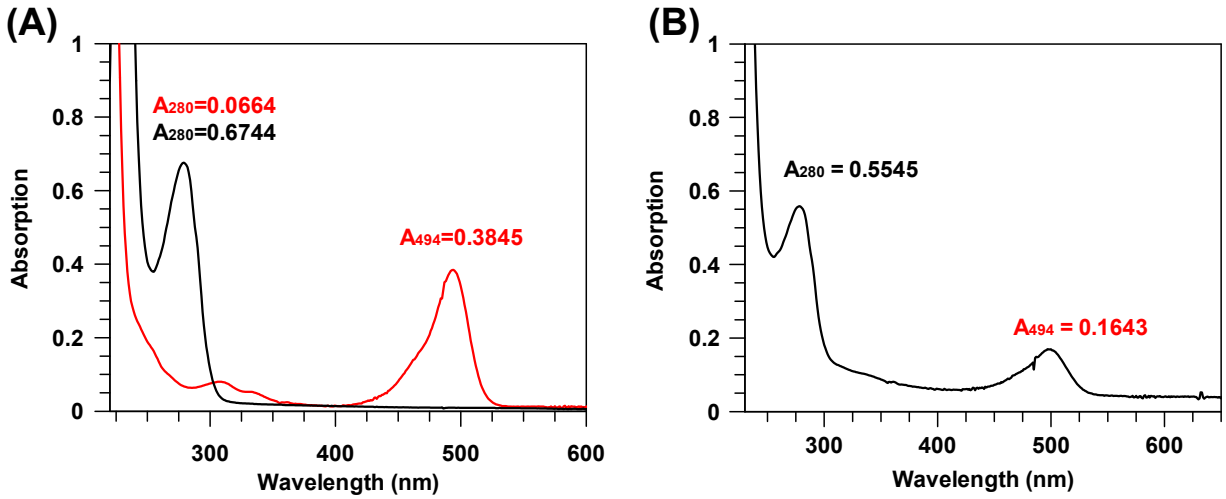


Figure S5. Combinatorial synthesis scheme for generating the bead-based libraries

Resin beads were distributed into different reaction vessels (small circles) and reacted with one building block per reaction vessel. After each combinatorial reaction step, the samples were pooled (large circles) mixed and re-distributed. The different resin batches were kept separated after the 3rd combinatorial reaction step, generating sublibraries with known substructures in combinatorial positions R3 and R4 and leaving two combinatorial positions for MS-decoding.

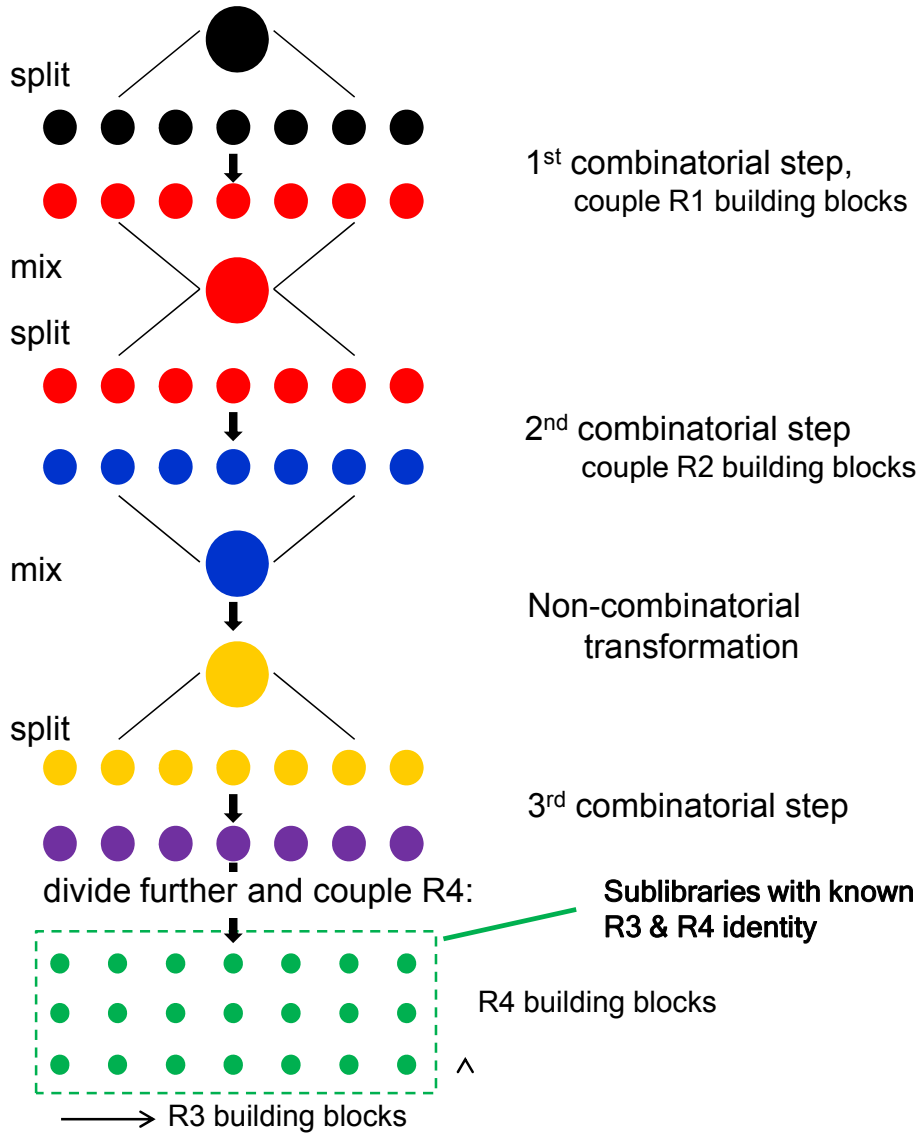
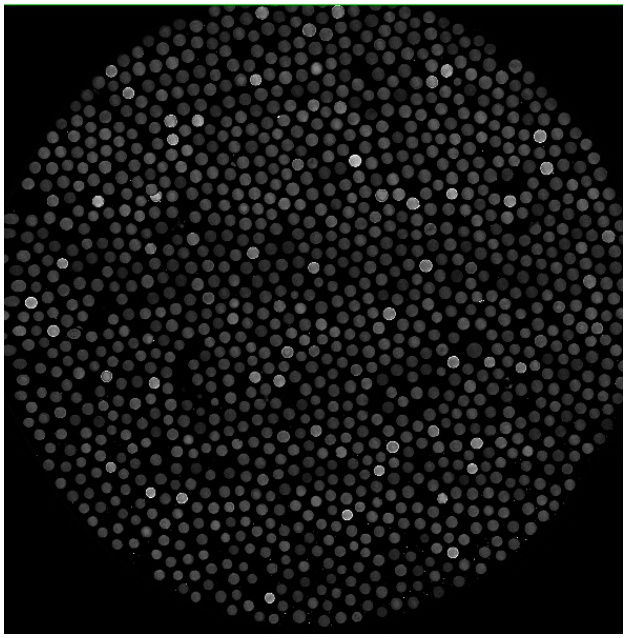


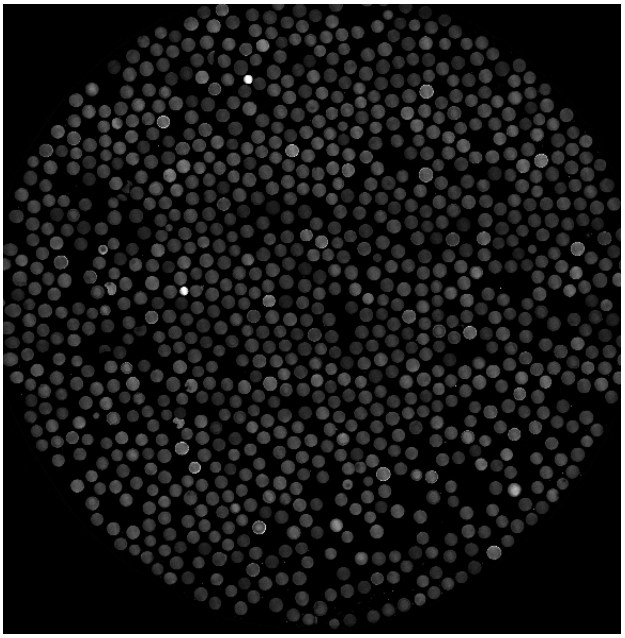
Figure S6. Representative Screening well images:

A. Scan image of sublibrary 1 (pyrrole scaffold). Sublibrary 1 contained 58 beads with fluorescent importin β bound to the ligands on the bead, 25 of which were picked. **B**. Scan image of sublibrary 2 (pyrrole scaffold). Sublibrary 2 contained 40 beads with ring formation, 25 of which were picked. **C**. Scan image of sublibrary 64 (4-amino-proline scaffold). Sublibrary 64 contained > 100 beads with ring formation, 20 of which were picked.

A



B



C

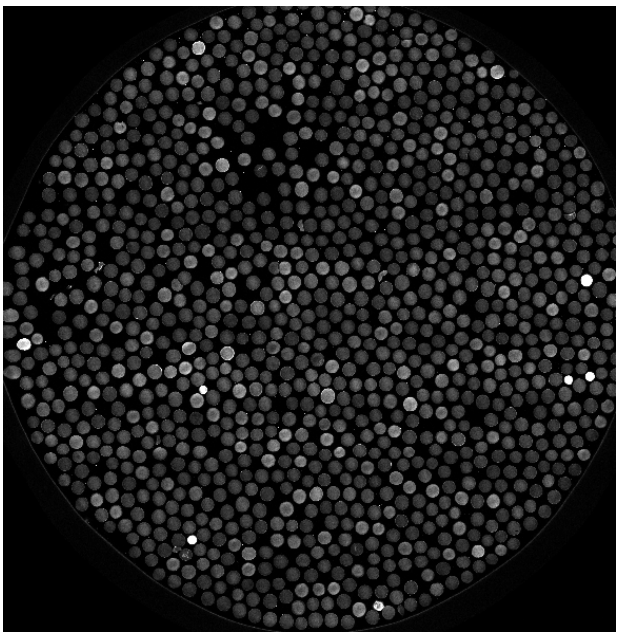


Figure S7. MS-based structure decoding of AIDA-tagged libraries

The built-in UV-dye AIDA contained in all library compounds facilitates MS-based structure decoding. **A.** After photolysis of individual hit beads the sample containing ~ 50 pmoles of cleaved compound is analyzed by HPLC-MS/MS^2. The UV-trace is used to detect the AIDA compound with high-sensitivity and the MS-spectra corresponding to this time-point of the gradient are examined for the any AIDA-specific fragments (marked A) and compound specific, *diagnostic fragments* (marked D) to assign the correct hit-structure. **B.** General fragmentation pattern of AIDA-tagged libraries: a series of compound-specific fragments, *C,* AIDA-containing fragments *A*, and fragments diagnostic for the building block used in the first combinatorial position, *D* can be assigned.

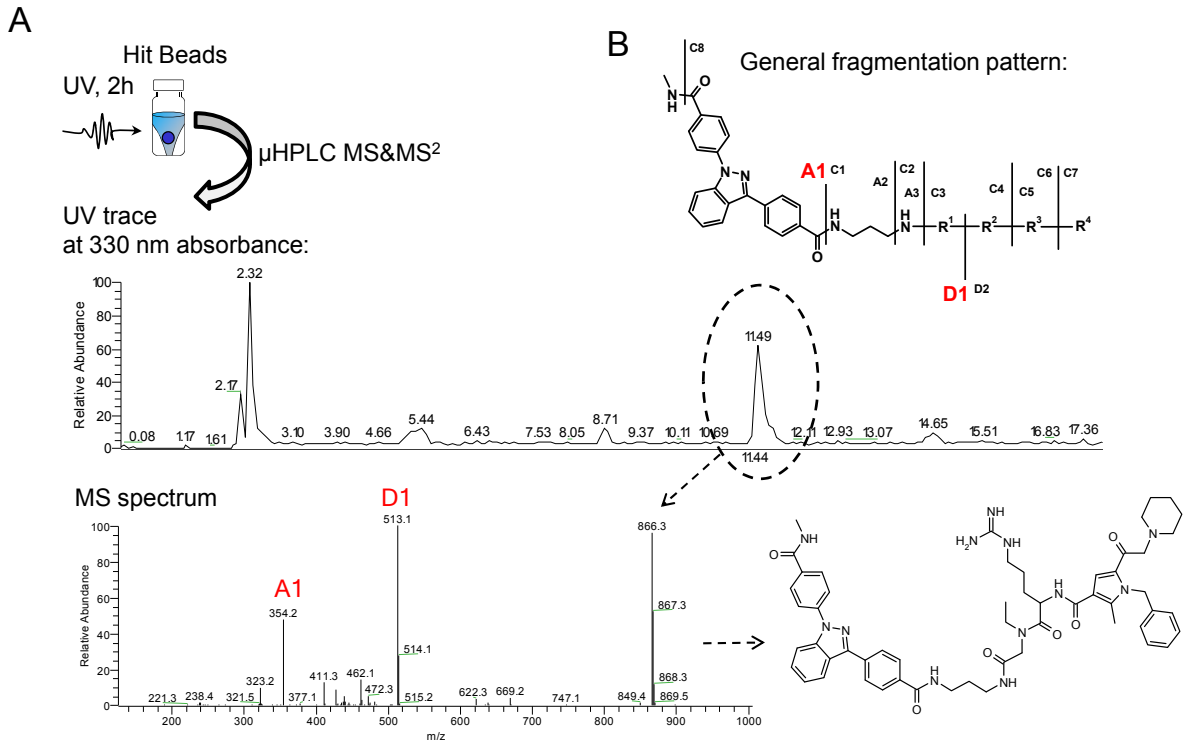


Figure S8. Repetitively identified hit structures (replicates)

Structures of compounds identified as hits more than once. The number of hit identifications are denoted for each structure.

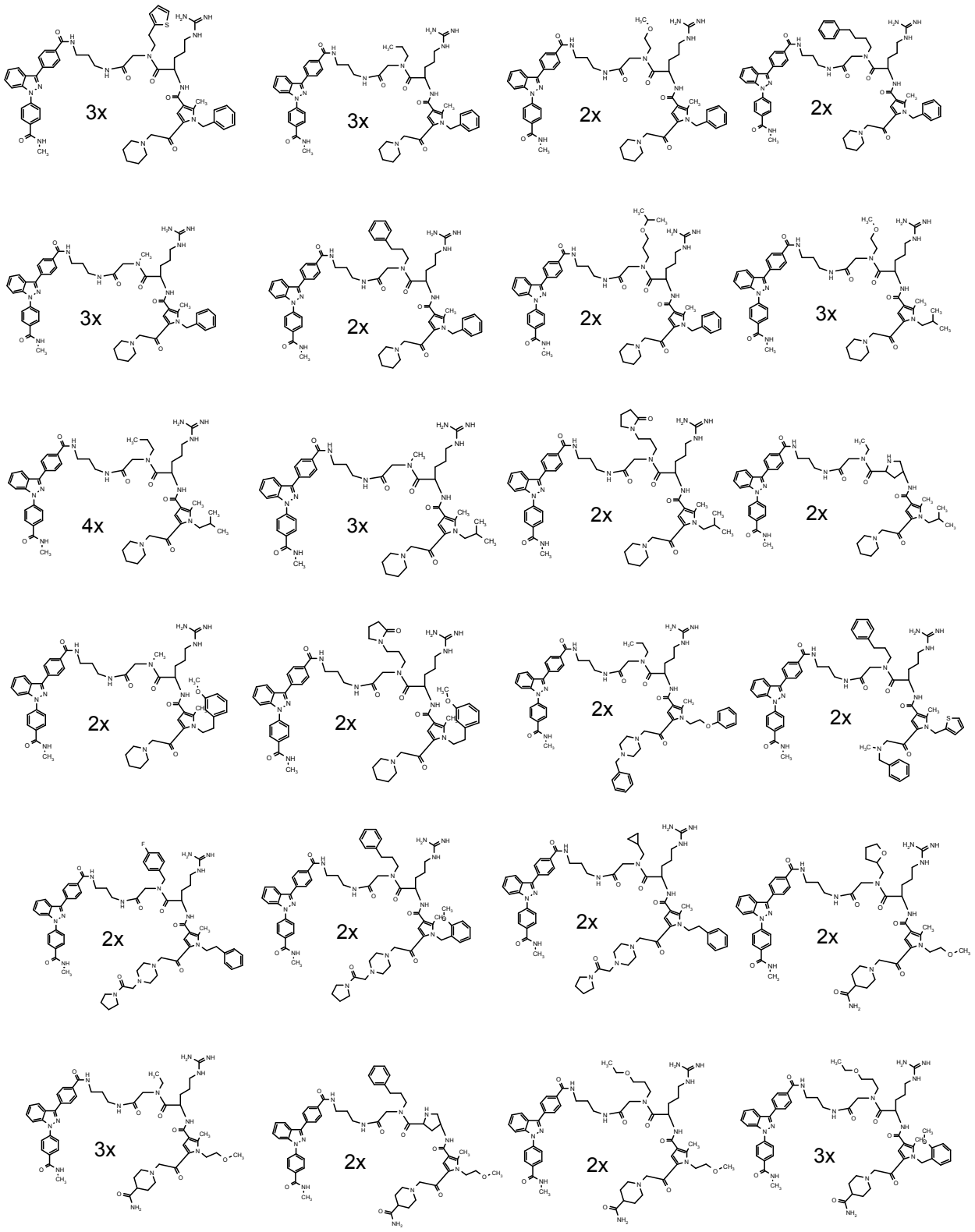


Figure S8. Repetitively identified hit structures (replicates) – continued

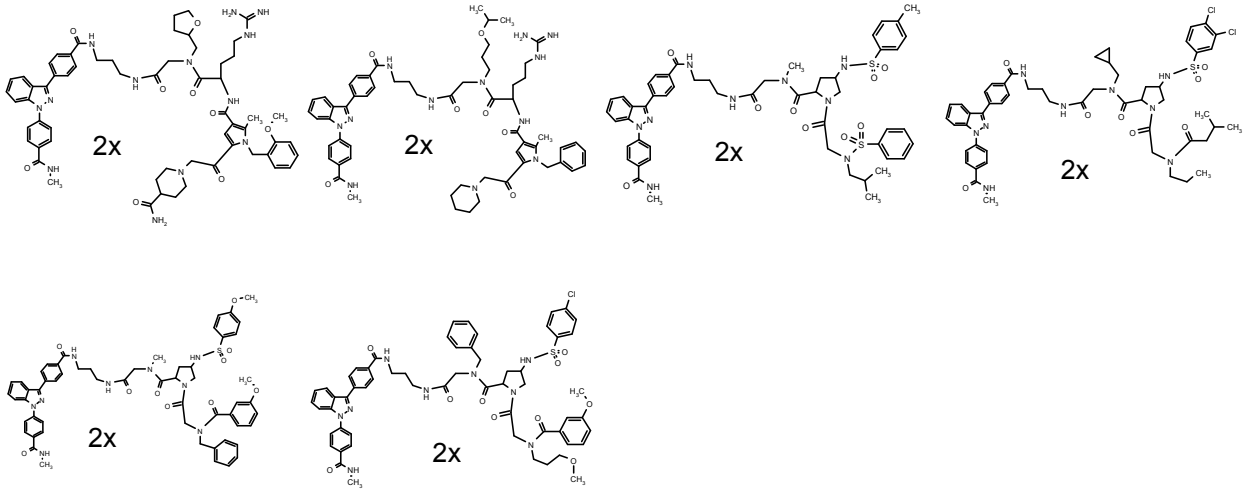


Figure S9. Building block frequency analysis

Building block frequency analysis of the analyzed hit-compounds for the combinatorial positions R1 and R2. Top-panel: pyrrole compounds, lower panel: Amino-proline compounds. The structures of the most frequent building blocks are drawn next to the respective bar.

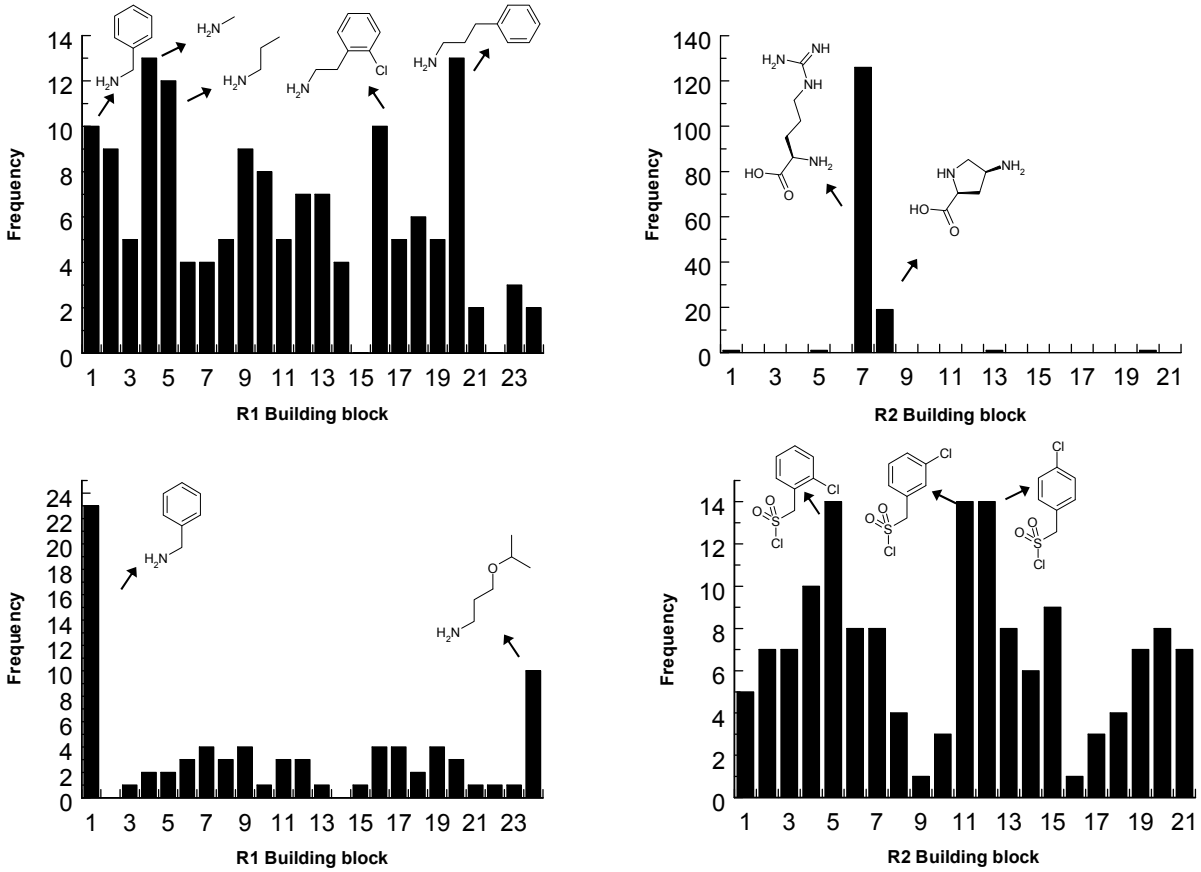
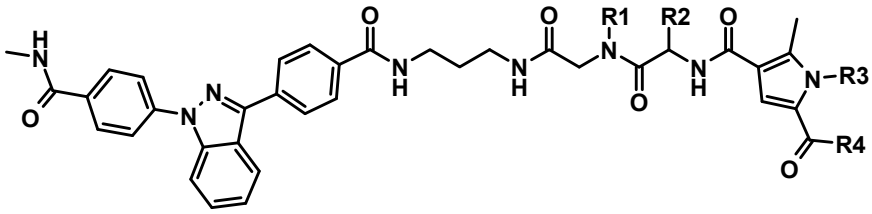


Figure S10. SAR analysis

Structures of compounds synthesized for SAR analysis and their respective inhibitory characteristics as measured by the in vitro nuclear import assay.



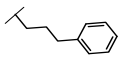
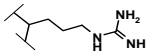
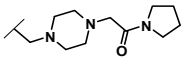
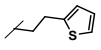
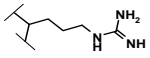
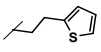
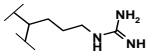
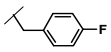
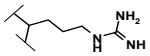
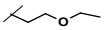
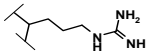
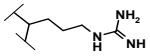
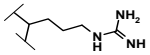
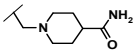
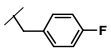
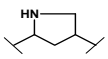
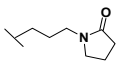
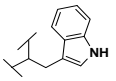
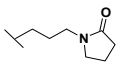
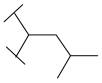
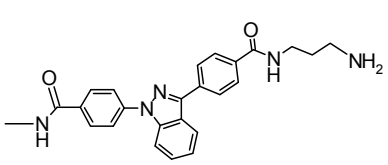
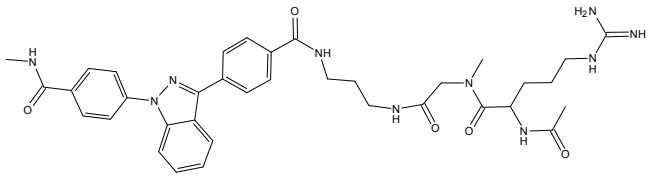
Compound	R1	R2	R3	R4	% Inhibition
Iß3A					78.8
Iß13A					61.5
Iß12A					85.1
Iß11A					74.9
Iß2A					68.3
Iß1A					65.6
Iß4A					35.8
Iß10A					78.2
Iß9A					31.6
Iß8A					37.6
Iß16A					inactive
Iß17A					14.8
Iß14A					15.6
Iß15A					77.0

Figure S11. Structure of unrelated control compounds, tested for binding to importin β

Only two measurements per compound (0 μM and 4 μM importin β) were performed, and both control compounds did not show binding (IßC1: r_{start} = 0.039, r_{end} = 0.075; IBC2: r_{start} = 0.053, r_{end} = 0.085) thus indicating that the binding of the importin β to hit compounds is specific.



IßC1



IßC2

Figure S12. Proteins used in the surface plasmon resonance measurements

Coomassie blue stained SDS-PAGE gel of recombinantly expressed and purified proteins used in the Biacore experiments. 2 µg of protein was loaded onto each lane.

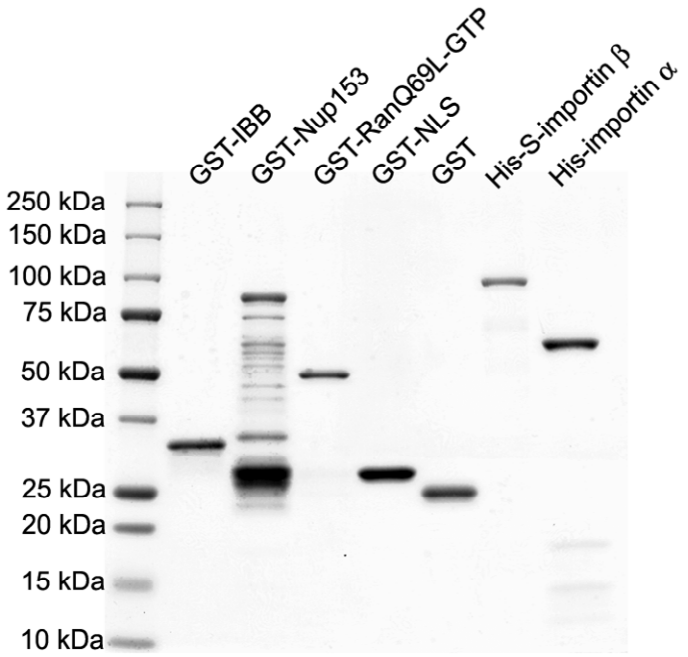


Figure S13. Surface plasmon resonance measurements of importin β interactions with GST-RanQLGTP, GST-IBB and GST-Nup153

Binding of importin β at various concentrations to GST-RanQ69LGTP (**A.**) GST-IBB (**B.**), or GST-Nup153 (**C.**) non-covalently immobilized to a GST antibody chip. Solid lines (__) represent actual data collected in duplicates and dotted lines (…) are theoretical simulations derived from global fit on the dataset. Global fits resulted in residuals less than 10% and no mass transport effect could be detected attesting to the goodness of the fit.

Binding of importin β at various concentrations in the presence of 5 μM *Iβ1A* to GST-Nup153 (**D.**) non-covalently immobilized to a GST antibody chip. Black lines represent actual data collected in duplicates and red lines are theoretical simulations derived from global fit on the dataset.

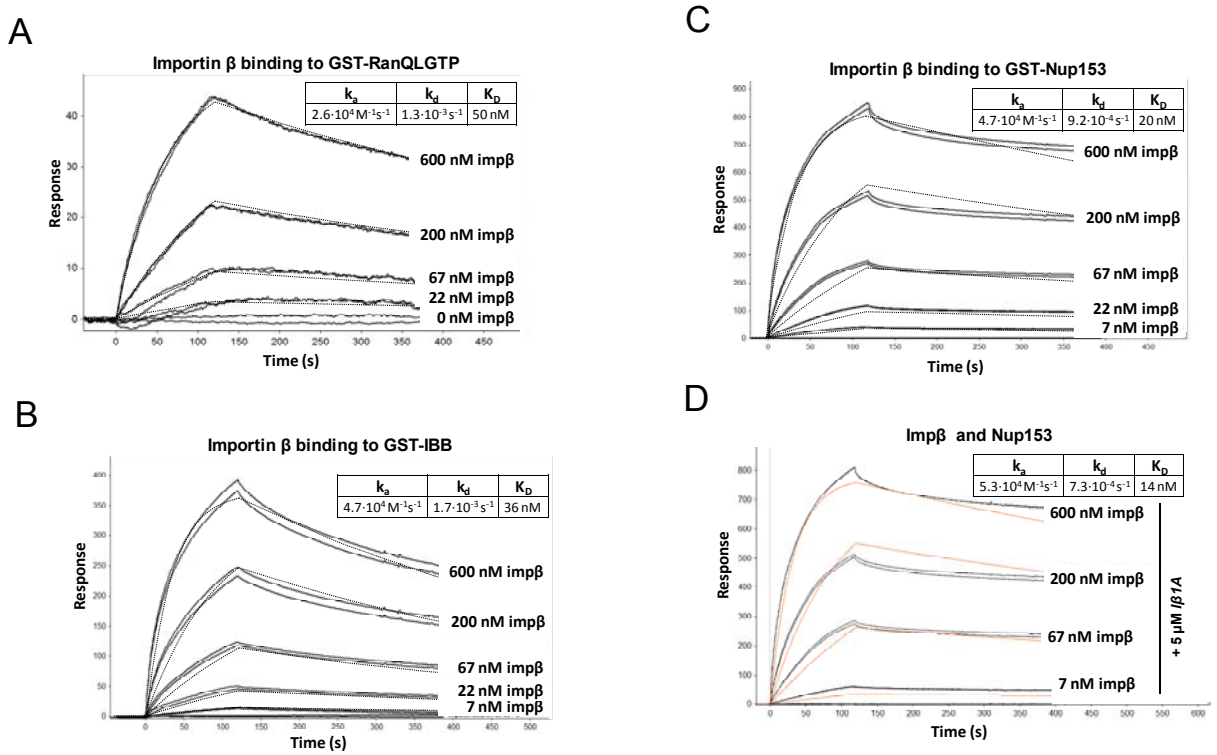


Table S1: List of ranked hit beads:

Rank	Pick label	Sublibrary #	RI [kHz]	SI [kHz]	CV (RI)
1	PS2/200x0x23/03	PYRROLE 01	374100	218831	13.1
2	PS2/200x0x23/47	PYRROLE 02	373784	250770	20.5
3	PS2/200x0x23/30	PYRROLE 02	372203	214404	23.4
4	PS2/200x0x23/27	PYRROLE 02	370622	218515	24.9
5	PS2/200x0x23/20	PYRROLE 01	369989	232429	20.2
6	PS2/200x0x23/48	PYRROLE 02	366511	217250	23.1
7	PS2/200x0x23/22	PYRROLE 01	361767	197644	15.6
8	PS2/200x0x23/04	PYRROLE 01	355759	201755	22.1
9	PS2/200x0x23/31	PYRROLE 02	351332	215985	20.4
10	PS2/200x0x23/02	PYRROLE 01	349434	200174	13.5
11	PS2/200x0x23/21	PYRROLE 01	348802	201755	19.7
12	PS2/200x0x23/49	PYRROLE 02	347537	221045	19.8
13	PS2/200x0x23/08	PYRROLE 01	344375	174243	16.2
14	PS2/200x0x23/05	PYRROLE 01	342477	217883	17.5
15	PS2/200x0x23/01	PYRROLE 01	331409	206814	22.8
16	PS2/200x0x23/29	PYRROLE 02	329828	214088	19.6
17	PS2/200x0x23/12	PYRROLE 01	327931	175824	17.1
18	PS2/200x0x23/09	PYRROLE 01	327614	205550	16.7
19	PS2/200x0x23/13	PYRROLE 01	327614	197328	14.8
20	PS2/200x0x23/39	PYRROLE 02	327614	220096	18.7
21	PS2/200x0x23/45	PYRROLE 02	321922	232429	20.2
22	PS2/200x0x23/18	PYRROLE 01	320341	203652	22.6
23	PS2/200x0x23/07	PYRROLE 01	317179	207447	19.8
24	PS2/200x0x23/16	PYRROLE 01	313068	178038	18.1
25	PS2/200x0x23/19	PYRROLE 01	309905	206498	12.9
26	PS2/200x0x23/06	PYRROLE 01	307692	211558	14.1
27	PS2/200x0x23/14	PYRROLE 01	298837	183730	15.9
28	PS2/200x0x24/26	PYRROLE 07	295991	246976	15.3
29	PS2/200x0x23/41	PYRROLE 02	294726	197644	16.9
30	PS2/200x0x23/36	PYRROLE 02	291880	200806	20.8
31	PS2/200x0x23/25	PYRROLE 01	290615	219464	15.2
32	PS2/200x0x23/44	PYRROLE 02	284291	191635	17.0
33	PS2/200x0x23/23	PYRROLE 01	282393	206814	21.3
34	PS2/200x0x23/10	PYRROLE 01	278282	197328	18.5
35	PS2/200x0x24/04	PYRROLE 03	275753	216301	15.7
36	PS2/200x0x23/42	PYRROLE 02	275120	203652	21.1
37	PS2/200x0x24/28	PYRROLE 07	270060	215985	15.7
38	PS2/200x0x23/15	PYRROLE 01	267214	172662	25.2
39	PS2/200x0x24/05	PYRROLE 03	266582	198593	17.1
40	PS2/200x0x23/37	PYRROLE 02	263420	162859	17.7
41	PS2/200x0x23/11	PYRROLE 01	263103	178038	18.7
42	PS2/200x0x24/68	PYRROLE 13	259625	215353	13.7
43	PS2/200x0x23/28	PYRROLE 02	258992	201439	18.8
44	PS2/200x0x23/17	PYRROLE 01	258044	173294	16.7
45	PS2/200x0x23/40	PYRROLE 02	255830	140722	23.7
46	PS2/200x0x24/16	PYRROLE 06	255514	191003	16.7
47	PS2/200x0x24/01	PYRROLE 03	253300	174243	19.1
48	PS2/200x0x24/54	PYRROLE 10	253300	197960	16.7
49	PS2/200x0x23/32	PYRROLE 02	252984	190371	18.1
50	PS2/200x0x24/32	PYRROLE 07	250454	184995	18.9
51	PS2/200x0x23/24	PYRROLE 01	250138	192584	19.4
52	PS2/200x0x24/31	PYRROLE 07	246027	186892	27.1
53	PS2/200x0x24/07	PYRROLE 03	244446	167602	20.9
54	PS2/200x0x24/02	PYRROLE 03	244130	191635	18.3
55	PS2/200x0x24/03	PYRROLE 03	243497	190687	17.1

Rank	Pick label	Sublibrary #	RI [kHz]	SI [kHz]	CV (RI)
56	PS2/200x0x24/06	PYRROLE 03	238437	177405	21.3
57	PS2/200x0x24/52	PYRROLE 10	232113	177405	17.3
58	PS2/200x0x24/08	PYRROLE 03	231164	163175	21.4
59	PS2/200x0x24/48	PYRROLE 10	229899	189422	17.6
60	PS2/200x0x24/21	PYRROLE 06	228634	162859	18.1
61	PS2/200x0x24/51	PYRROLE 10	228318	172978	16.8
62	PS2/200x0x24/12	PYRROLE 03	227369	180884	19.4
63	PS2/200x0x24/29	PYRROLE 07	227369	198593	17.2
64	PS2/200x0x24/49	PYRROLE 10	227053	172662	14.3
65	PS2/200x0x24/11	PYRROLE 03	226737	178986	21.2
66	PS2/200x0x24/83	PYRROLE 14	225788	143252	24.7
67	PS2/200x0x24/72	PYRROLE 13	224840	192268	11.8
68	PS2/200x0x24/76	PYRROLE 14	224523	170448	15.1
69	PS2/200x0x24/10	PYRROLE 03	221677	175508	22.4
70	PS2/200x0x24/50	PYRROLE 10	221361	186576	15.4
71	PS2/200x0x24/70	PYRROLE 13	220412	159696	17.6
72	PS2/200x0x24/23	PYRROLE 07	219464	192900	12.7
73	PS2/200x0x24/43	PYRROLE 09	218515	154004	22.6
74	PS2/200x0x24/42	PYRROLE 09	217566	177405	16.1
75	PS2/200x0x23/33	PYRROLE 02	215036	165072	16.5
76	PS2/200x0x24/71	PYRROLE 13	213455	132817	24.2
77	PS2/200x0x24/14	PYRROLE 06	213139	168551	15.2
78	PS2/200x0x23/50	PYRROLE 02	211558	147679	17.9
79	PS2/200x0x24/27	PYRROLE 07	211242	176773	12.6
80	PS2/200x0x24/17	PYRROLE 06	210609	169183	16.4
81	PS2/200x0x24/46	PYRROLE 09	209661	188157	16.1
82	PS2/200x0x24/15	PYRROLE 06	209344	166021	15.6
83	PS2/200x0x24/53	PYRROLE 10	209028	190054	14.2
84	PS2/200x0x24/57	PYRROLE 10	207447	179935	17.4
85	PS2/200x0x24/58	PYRROLE 11	207447	135346	14.6
86	PS2/200x0x24/41	PYRROLE 09	206814	173927	14.1
87	PS2/200x0x24/24	PYRROLE 07	206498	169183	15.4
88	PS2/200x0x24/30	PYRROLE 07	206182	158431	19.5
89	PS2/200x0x24/09	PYRROLE 03	205866	156218	17.7
90	PS2/200x0x24/36	PYRROLE 08	205233	178986	15.9
91	PS2/200x0x24/25	PYRROLE 07	203968	170764	18.0
92	PS2/200x0x23/46	PYRROLE 02	203336	156850	19.0
93	PS2/200x0x24/89	PYRROLE 14	200806	136611	25.6
94	PS2/200x0x24/90	PYRROLE 15	200490	134082	20.5
95	PS2/200x0x24/59	PYRROLE 11	199857	151474	16.9
96	PS2/200x0x24/61	PYRROLE 11	198909	154953	17.7
97	PS2/200x0x24/40	PYRROLE 09	197011	162859	17.1
98	PS2/200x0x24/13	PYRROLE 06	196063	167918	18.7
99	PS2/200x0x24/69	PYRROLE 13	195746	147679	22.5
100	PS2/200x0x24/22	PYRROLE 06	195430	160329	12.9
101	PS2/200x0x24/73	PYRROLE 13	195430	165072	17.1
102	PS2/200x0x23/34	PYRROLE 02	192584	129654	21.7
103	PS2/200x0x24/88	PYRROLE 14	192268	136928	28.8
104	PS2/200x0x23/26	PYRROLE 02	189422	132184	21.3
105	PS2/200x0x24/37	PYRROLE 08	187841	178354	10.3
106	PS2/200x0x24/34	PYRROLE 08	186576	143252	14.5
107	PS2/200x0x24/56	PYRROLE 10	184995	158431	16.0
108	PS2/200x0x24/84	PYRROLE 14	184362	124595	23.8
109	PS2/200x0x24/74	PYRROLE 14	181832	116056	22.0
110	PS2/200x0x24/18	PYRROLE 06	180884	136928	14.8
111	PS2/200x0x24/45	PYRROLE 09	180884	153372	21.9
112	PS2/200x0x24/82	PYRROLE 14	180884	123962	24.1
113	PS2/200x0x24/60	PYRROLE 11	179302	142620	20.0
114	PS2/200x0x23/43	PYRROLE 02	178354	122065	18.6
115	PS2/200x0x24/19	PYRROLE 06	178354	146415	20.6

Rank	Pick label	Sublibrary #	RI [kHz]	SI [kHz]	CV (RI)
116	PS2/200x0x24/47	PYRROLE 09	176773	147996	17.7
117	PS2/200x0x24/44	PYRROLE 09	174875	148944	16.9
118	PS2/200x0x23/35	PYRROLE 02	173294	113527	19.2
119	PS2/200x0x24/64	PYRROLE 11	173294	139774	19.4
120	PS2/200x0x24/67	PYRROLE 11	172662	149577	16.7
121	PS2/200x0x23/38	PYRROLE 02	171713	116689	17.1
122	PS2/200x0x24/55	PYRROLE 10	170448	145782	15.2
123	PS2/200x0x27/83	PYRROLE 15	167602	141987	21.5
124	PS2/200x0x24/38	PYRROLE 09	166337	122697	18.2
125	PS2/200x0x24/62	PYRROLE 11	166021	137244	16.0
126	PS2/200x0x24/87	PYRROLE 14	164756	123330	25.8
127	PS2/200x0x27/76	PYRROLE 15	162859	148944	11.2
128	PS2/200x0x27/84	PYRROLE 15	161594	135663	18.4
129	PS2/200x0x24/65	PYRROLE 11	161277	137244	16.4
130	PS2/200x0x24/75	PYRROLE 14	156534	97399	20.0
131	PS2/200x0x24/81	PYRROLE 14	154320	127441	17.2
132	PS2/200x0x27/75	PYRROLE 15	154320	138509	16.2
133	PS2/200x0x24/33	PYRROLE 08	152423	121116	13.7
134	PS2/200x0x24/63	PYRROLE 11	152423	129338	17.9
135	PS2/200x0x24/79	PYRROLE 14	149577	117954	16.4
136	PS2/200x0x27/85	PYRROLE 15	148944	143252	14.9
137	PS2/200x0x24/35	PYRROLE 08	148312	124278	15.0
138	PS2/200x0x28/09	PYRROLE 08	147679	111945	11.5
139	PS2/200x0x24/86	PYRROLE 14	146415	106570	21.1
140	PS2/200x0x27/82	PYRROLE 15	146098	134714	12.9
141	PS2/200x0x28/07	AMINOPROLINE 52	145466	103723	23.9
142	PS2/200x0x24/66	PYRROLE 11	144201	117321	16.4
143	PS2/200x0x28/01	AMINOPROLINE 49	143568	115108	15.9
144	PS2/200x0x24/77	PYRROLE 14	143252	110997	15.4
145	PS2/200x0x27/79	PYRROLE 15	141039	120484	17.6
146	PS2/200x0x27/74	PYRROLE 15	140722	121432	15.4
147	PS2/200x0x27/78	PYRROLE 15	140406	125543	11.6
148	PS2/200x0x24/20	PYRROLE 06	139457	94237	16.2
149	PS2/200x0x24/85	PYRROLE 14	139457	92655	22.7
150	PS2/200x0x24/78	PYRROLE 14	139141	105621	22.2
151	PS2/200x0x27/73	PYRROLE 15	137560	120800	15.5
152	PS2/200x0x27/81	PYRROLE 15	134398	117954	13.0
153	PS2/200x0x27/80	PYRROLE 15	123646	105305	14.7
154	PS2/200x0x28/02	AMINOPROLINE 49	123330	81271	39.7
155	PS2/200x0x27/36	AMINOPROLINE 64	116373	75895	19.5
156	PS2/200x0x24/39	PYRROLE 09	115740	91707	14.7
157	PS2/200x0x27/49	AMINOPROLINE 64	113843	80639	18.9
158	PS2/200x0x24/80	PYRROLE 14	112262	86331	18.6
159	PS2/200x0x27/77	PYRROLE 15	111945	96134	20.1
160	PS2/200x0x27/29	AMINOPROLINE 63	100245	76528	18.2
161	PS2/200x0x27/14	AMINOPROLINE 62	93920	80006	8.4
162	PS2/200x0x27/34	AMINOPROLINE 64	82220	47751	34.3
163	PS2/200x0x27/38	AMINOPROLINE 64	75895	55340	19.4
164	PS2/200x0x28/03	AMINOPROLINE 49	75579	36366	35.2
165	PS2/200x0x27/01	AMINOPROLINE 62	73049	43324	35.2
166	PS2/200x0x27/02	AMINOPROLINE 62	68938	48067	20.1
167	PS2/200x0x27/30	AMINOPROLINE 63	68938	40477	37.5
168	PS2/200x0x27/61	AMINOPROLINE 66	68938	32572	36.1
169	PS2/200x0x28/15	AMINOPROLINE 57	67041	48067	21.8
170	PS2/200x0x27/67	AMINOPROLINE 66	66408	40794	31.5
171	PS2/200x0x28/14	AMINOPROLINE 57	65776	43007	27.8
172	PS2/200x0x27/09	AMINOPROLINE 62	65143	49016	20.7
173	PS2/200x0x27/04	AMINOPROLINE 62	63562	43956	42.0
174	PS2/200x0x27/69	AMINOPROLINE 66	63246	48699	23.6
175	PS2/200x0x27/71	AMINOPROLINE 66	62930	43640	53.1

Rank	Pick label	Sublibrary #	RI [kHz]	SI [kHz]	CV (RI)
176	PS2/200x0x27/56	AMINOPROLINE 65	62614	47435	46.2
177	PS2/200x0x27/27	AMINOPROLINE 63	62297	49964	21.5
178	PS2/200x0x27/64	AMINOPROLINE 66	62297	49648	15.5
179	PS2/200x0x27/70	AMINOPROLINE 66	61665	43007	26.8
180	PS2/200x0x27/06	AMINOPROLINE 62	61349	44588	30.8
181	PS2/200x0x27/05	AMINOPROLINE 62	60400	35734	28.9
182	PS2/200x0x27/13	AMINOPROLINE 62	59451	41426	27.5
183	PS2/200x0x27/11	AMINOPROLINE 62	58503	43956	56.0
184	PS2/200x0x27/12	AMINOPROLINE 62	58186	39213	32.8
185	PS2/200x0x27/03	AMINOPROLINE 62	57238	32572	57.3
186	PS2/200x0x27/28	AMINOPROLINE 63	56605	44588	21.2
187	PS2/200x0x27/15	AMINOPROLINE 62	56289	42375	26.8
188	PS2/200x0x27/45	AMINOPROLINE 64	55340	38896	24.6
189	PS2/200x0x27/16	AMINOPROLINE 63	54708	41110	28.9
190	PS2/200x0x27/21	AMINOPROLINE 63	54708	43640	16.1
191	PS2/200x0x27/07	AMINOPROLINE 62	54392	37948	25.5
192	PS2/200x0x27/20	AMINOPROLINE 63	54392	42375	24.8
193	PS2/200x0x27/57	AMINOPROLINE 65	54075	43007	18.8
194	PS2/200x0x27/53	AMINOPROLINE 65	53759	36683	25.0
195	PS2/200x0x27/39	AMINOPROLINE 64	53127	33204	29.4
196	PS2/200x0x27/18	AMINOPROLINE 63	52810	42691	21.0
197	PS2/200x0x27/19	AMINOPROLINE 63	52810	43324	18.4
198	PS2/200x0x27/10	AMINOPROLINE 62	52494	36683	26.2
199	PS2/200x0x27/25	AMINOPROLINE 63	52178	38264	34.7
200	PS2/200x0x27/08	AMINOPROLINE 62	51545	36366	34.9
201	PS2/200x0x27/65	AMINOPROLINE 66	51545	36366	31.0
202	PS2/200x0x27/44	AMINOPROLINE 64	51229	38264	32.0
203	PS2/200x0x27/47	AMINOPROLINE 64	51229	29726	34.9
204	PS2/200x0x27/62	AMINOPROLINE 66	51229	34153	76.7
205	PS2/200x0x27/17	AMINOPROLINE 63	50913	43007	19.0
206	PS2/200x0x27/40	AMINOPROLINE 64	49964	36366	26.3
207	PS2/200x0x27/42	AMINOPROLINE 64	49964	35102	28.4
208	PS2/200x0x27/52	AMINOPROLINE 65	49964	34153	30.2
209	PS2/200x0x28/05	AMINOPROLINE 51	49964	29726	42.7
210	PS2/200x0x27/46	AMINOPROLINE 64	49332	29409	28.7
211	PS2/200x0x27/51	AMINOPROLINE 65	49332	35734	22.4
212	PS2/200x0x27/22	AMINOPROLINE 63	49016	39213	42.3
213	PS2/200x0x27/66	AMINOPROLINE 66	49016	31623	31.2
214	PS2/200x0x27/59	AMINOPROLINE 65	48067	30358	26.1
215	PS2/200x0x27/32	AMINOPROLINE 64	47118	26880	32.1
216	PS2/200x0x27/23	AMINOPROLINE 63	46802	39213	22.0
217	PS2/200x0x27/68	AMINOPROLINE 66	46802	34153	20.4
218	PS2/200x0x27/24	AMINOPROLINE 63	46486	37948	28.7
219	PS2/200x0x27/33	AMINOPROLINE 64	46486	30042	27.8
220	PS2/200x0x27/37	AMINOPROLINE 64	46486	33520	35.6
221	PS2/200x0x27/63	AMINOPROLINE 66	45853	34153	26.8
222	PS2/200x0x27/35	AMINOPROLINE 64	45221	30674	27.1
223	PS2/200x0x27/54	AMINOPROLINE 65	45221	30674	33.7
224	PS2/200x0x27/55	AMINOPROLINE 65	45221	31623	26.4
225	PS2/200x0x27/48	AMINOPROLINE 64	44905	30991	34.8
226	PS2/200x0x27/26	AMINOPROLINE 63	44588	38264	18.2
227	PS2/200x0x27/50	AMINOPROLINE 64	44588	30042	31.7
228	PS2/200x0x27/60	AMINOPROLINE 65	43956	30991	25.2
229	PS2/200x0x27/72	AMINOPROLINE 66	43640	31307	25.6
230	PS2/200x0x27/58	AMINOPROLINE 65	43324	32572	24.4
231	PS2/200x0x28/06	AMINOPROLINE 51	42375	30042	26.5
232	PS2/200x0x27/31	AMINOPROLINE 64	42059	29093	27.5
233	PS2/200x0x28/04	AMINOPROLINE 51	41742	32255	30.3
234	PS2/200x0x27/43	AMINOPROLINE 64	40477	27196	30.6
235	PS2/200x0x28/13	AMINOPROLINE 57	40477	28144	33.9

On-bead screening and library setup

The screening process was typically performed in a 96-well microtiter plate. Each well was filled with ~ 2,000 beads of one individual sublibrary of the AIDA tagged one-bead one-compound library collection. All CONA screening libraries shared the same basic chemical setup consisting of a monodisperse batch of TentaGel-NH2 resin (90 µm diameter, Rapp Polymers, Germany) onto which 4-hydroxymethyl-3-nitro-benzoic acid was loaded as photolabile handle followed by the indazole dye AIDA. The indazole dye was thus incorporated as the first synthetic module. The fluorophore was separated from the combinatorial compounds by a diamino-propane spacer. In our synthesis scheme the sublibraries were obtained by keeping the resins separated after the third combinatorial synthesis step.

The assay layout was based on a fourfold redundancy. Therefore, in a sublibrary any one compound was present four times on average. Given the structural similarity of the compounds within the same sublibrary, any well with fewer than 8 hit beads has a high probability of containing only false positives. Such wells were excluded from further analysis. An initial qualitative inspection of the wells containing beads bearing pyrroles or wells containing aminoprolines revealed differences in the overall target binding. Hit beads containing compounds of the pyrrole scaffold had higher average fluorescence ring intensities than aminoproline containing hit beads. However, the number of hit beads in aminoproline sublibraries was significantly higher than the number of hit beads in pyrrole sublibraries.

Estimation of expected fluorescence anisotropy values, r for AIDA tagged hit compounds alone and in complex with a protein:

Theoretical anisotropies *r* can be calculated from the Perrin equation [E-1].

$$\frac{r_0}{r} = 1 + \frac{\tau}{\theta_R} = 1 + 6D\tau \qquad \text{[E-1]}$$

r_0: fundamental anisotropy of the fluorophore; Θ_R: rotational correlation time;
τ: fluorescence lifetime; D: rotational diffusion coefficient.

The anisotropy r is related to rotational volume V, and hence the molecular weight M of the analyte via the rotational correlation time Θ_R, which can be obtained by

$$\theta_R = \frac{\eta * V}{R * T} = \frac{\eta * M}{R * T}(\bar{V} + h) \qquad \text{[E-2]}$$

η: viscosity [cP]; V: volume of the rotating unit; $\bar{V}$ specific volume [ml g^{-1}];
h: hydration [g H_2O g^{-1}]

For the UV-dye AIDA the fundamental anisotropy r_0 has been determined to be 0.26, and the fluorescence lifetime τ=1 ns.

Structure-activity relationships

Based on the SAR results (Fig. 5 and Fig. S10), in the majority of instances stronger inhibition is correlated with a preference for arginine in position R2 and for N-ethylcyclohexylamine in position R4. When present, this combination yields the most active compounds (*Iβ1A*, *Iβ2A*, *Iβ11A* to *Iβ13A*). An equal potency is only obtained for compounds *Iβ10A*, *Iβ15A* and *Iβ3A*, with the first two having proline or serine, respectively, in R2 instead of arginine, while the latter has a 2-(4-ethyl-piperazin-1-yl)-pyrrolidin-1-yl-ethanone moiety in R4. The success of the arginine replacement by proline/serine in *Iβ10A* and *Iβ15A* is hard to explain with the available information. The 2-(4-ethyl-piperazin-1-yl)-pyrrolidin-1-yl-ethanone, however, may be considered as an expansion of the N-ethylcyclohexylamine moiety. Combinations with only one of the “preferred” structures in R2 or R4 result in only moderately active compounds (*Iβ4A*, *Iβ8A*, *Iβ9A*). The conclusion that the main criterion for recognition is having a “correct” R2 and R4 is supported by the fact that the three least active compounds, showing only weak or no inhibition of nuclear import (*Iβ14A, Iβ16A, Iβ17A*), all lack both an R2–arginine and an R4-N-ethyl-cyclohexylamine. Finally, a comparison of compounds *Iβ1A*

and *Iβ2A*, which only differ in R3 (*Iβ1A* = isopropyl, *Iβ2A* = phenyl) but have equal potencies, suggests no contribution of aromatic elements in R3. Overall, these SAR data confirmed the vital importance of the arginine residue in the second combinatorial position, consistent with the building block frequency analysis in the binding hits.

Statistical considerations for MS-decoding and repetitive identification of hit compounds:

We screened a diversity optimized selection of 96 sublibraries, distributed into the 96-wells of a 96-well microtiter plate. For 90µm TentaGel beads each well contains approximately 2000 beads. The total number of compounds screened is therefore ~ 190 000 compounds. Each of the sublibraries now contains approximately ~ 475 compounds (between 400 compounds and 500 compounds, depending on the exact sublibrary).

Each sublibrary was initially synthesized using 100 mg of resin – so the total reservoir of compound/beads is 200 000 beads and the redundancy is 421, i.e. each compound should be present on average 421 times in the reservoir.

The probability to have each compound contained k times in the 2000 beads screening sample is therefore given by

$$P_k(N,m,n) = \frac{\binom{m}{k} \times \binom{N-m}{n-k}}{\binom{N}{n}}$$

with P_k: probability to find a compound k-times within a sample, m: degeneracy i.e. average number of beads per peptide type, N: reservoir size, i.e. total number of beads per (sub)library, n: sample size to be screened for each (sub)library.

Using the numbers from above, the probability to find a compound e.g. four times is given by

$$P_k(N,m,n) = \frac{\binom{475}{4} \times \binom{199525}{1996}}{\binom{200000}{2000}}$$

For large numbers the Binomial distribution can be approximated by a Normal distribution, with average μ and standard deviation σ.

$$\mu = \frac{n \times m}{N} = 4.75 \qquad \sigma = \sqrt{\mu \times \left(1 - \frac{m}{N}\right)} = 2.18$$

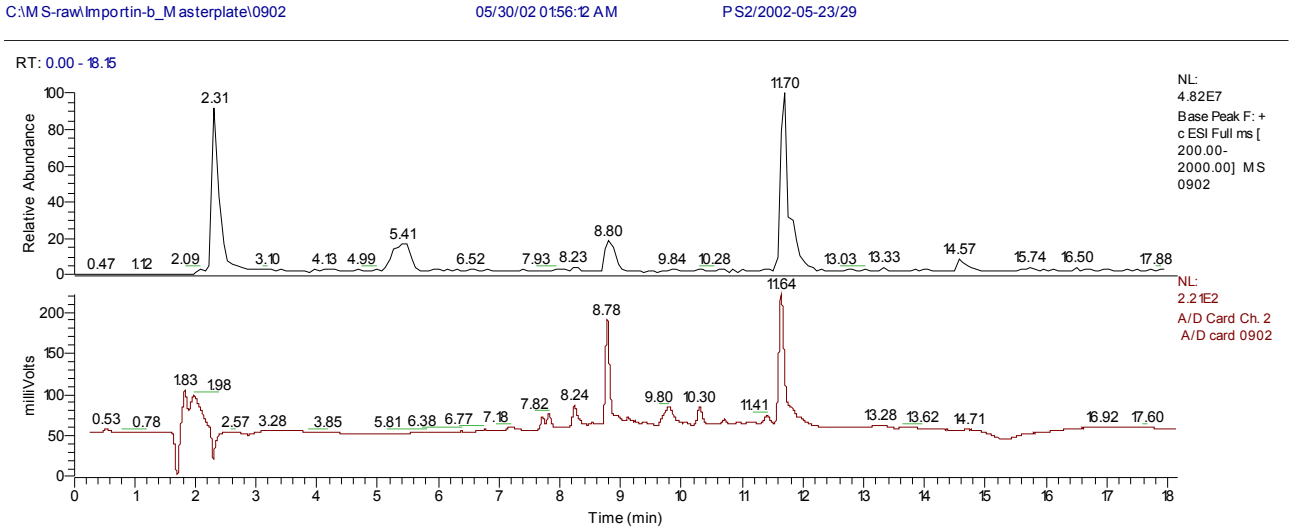
Using these parameters the probability to find a compound exactly four times in the library is 17.3 %. Furthermore, the probability to have a compound at least four times in the library is 71.6%.

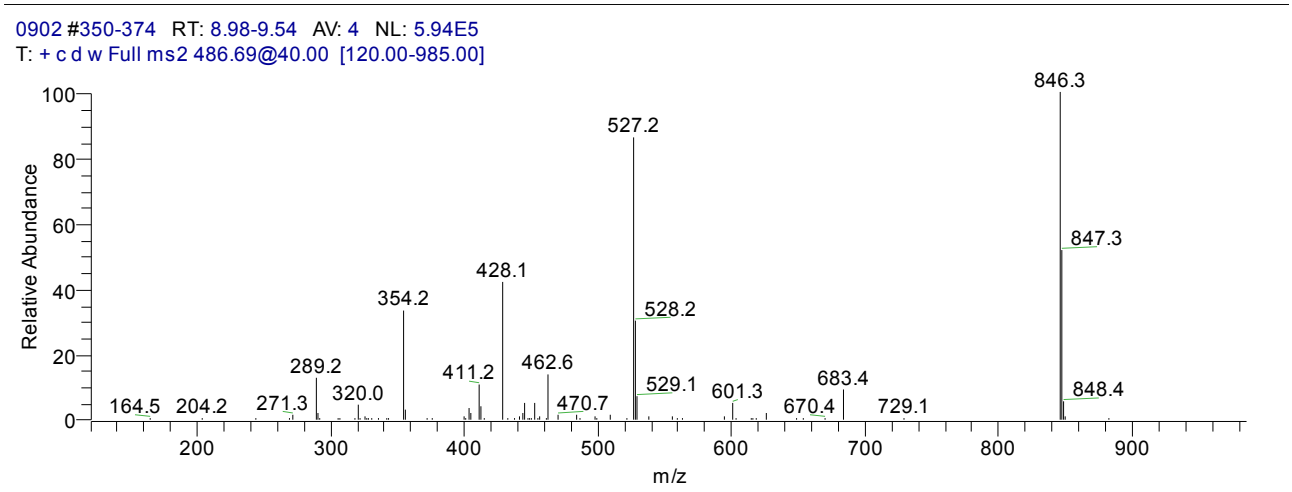
MS-decoding example:

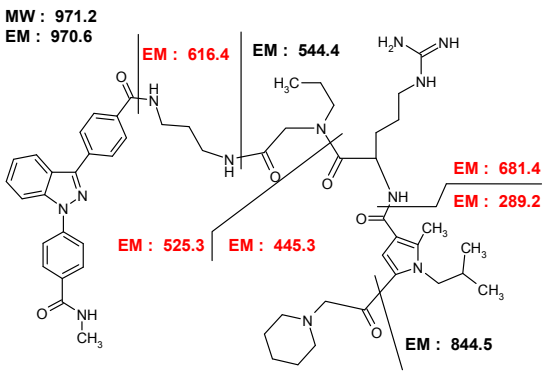
MS-decoding of compound structures was performed following this step-by-step procedure:
a) compound was cleaved from resin b) injected into a HPLC-MS instrument c) the UV-detector allows detection of the compound and assignment of retention time, based on the AIDA fluorescence. c) the MS-spectrum is used to detect molecular masses in the full compound range d) the MS-MS spectrum is used to see which fragments correspond to the selected mass peak in the MS-spectrum. e) the MS-MS spectrum is searched for typical AIDA fragment ions, (m/z 354, 411, 513) – if some of these are present, then the selected mass peak corresponds indeed to an AIDA compound, f) finally the MS-MS spectrum is searched for diagnostic fragment corresponding to a fragmentation between the first and the second combinatorial position.

To demonstrate the reliability of the MS-decoding procedure, the 4 different chromatograms and MS/MS² traces obtained from the four hit beads, leading to the four-fold decode result are shown:

Bead 1

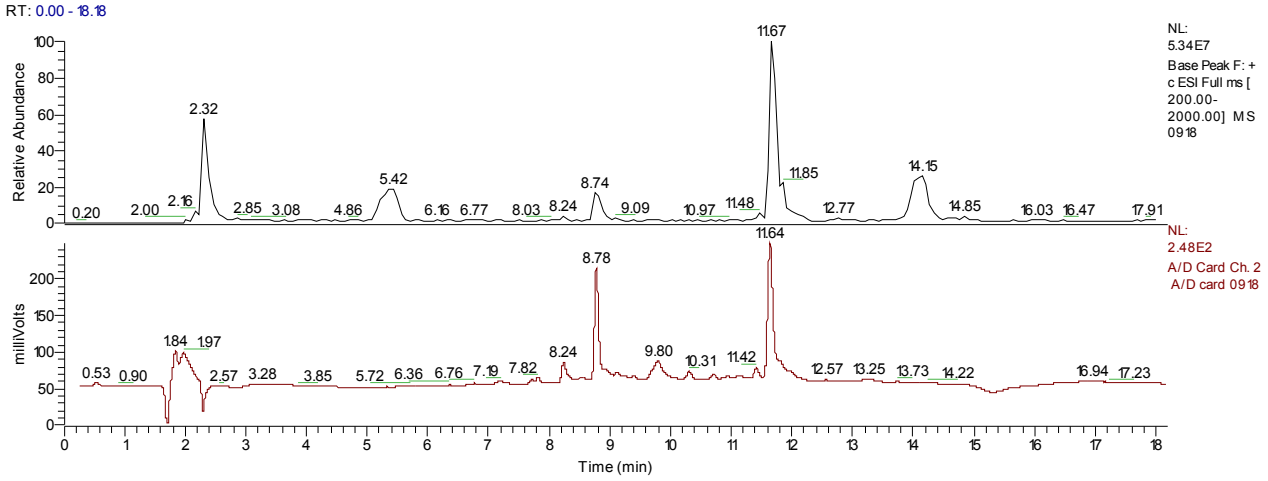


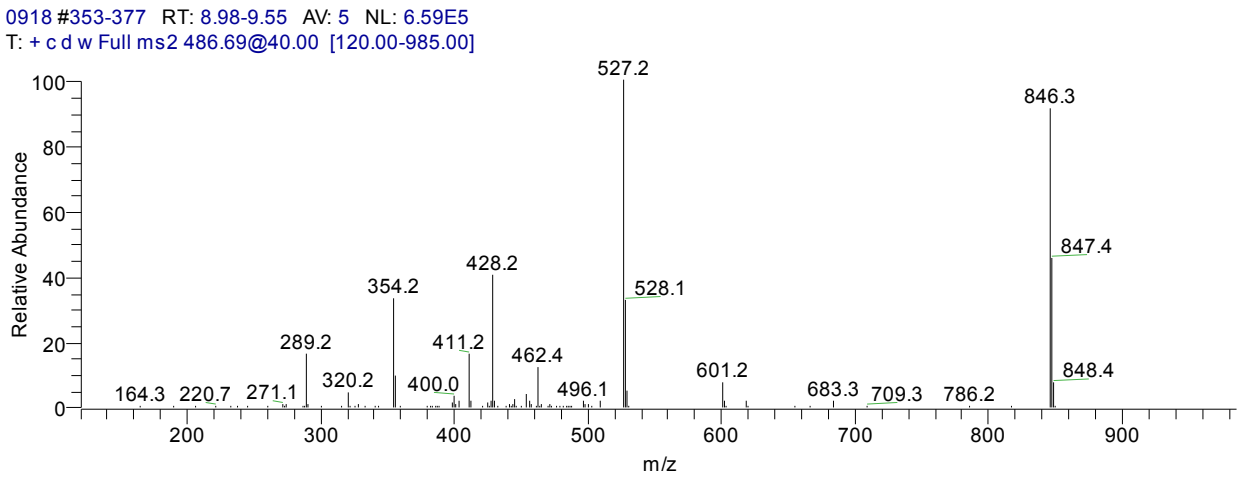


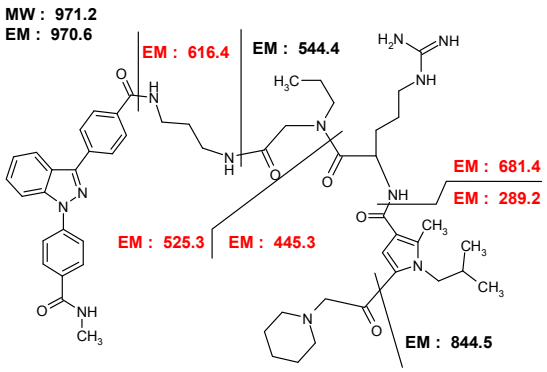


Bead 2

C:\MS-raw\Importin-b_Masterplate\0918 05/30/02 07:43:59 AM PS2/2002-05-23/45







Bead 3

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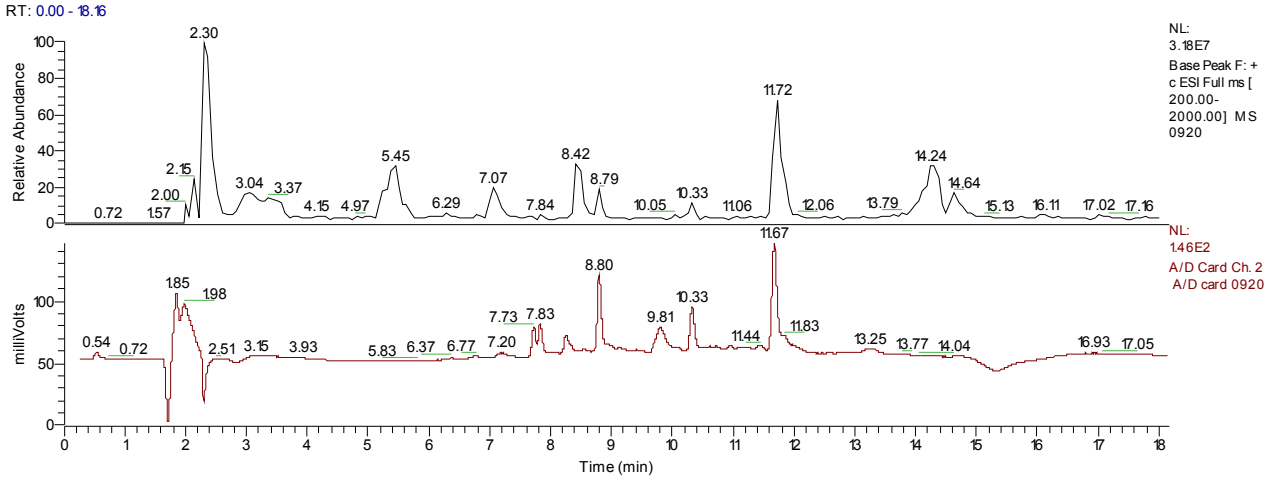
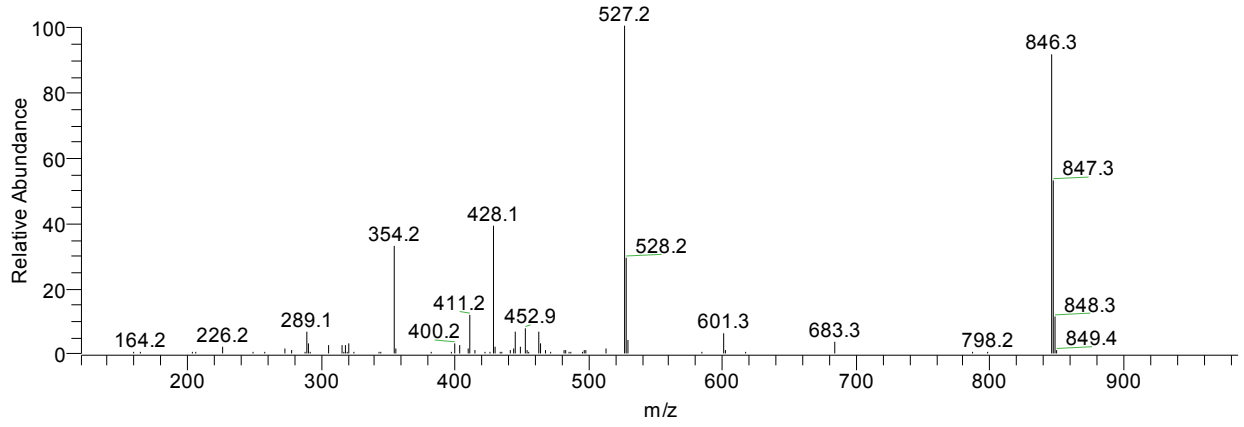
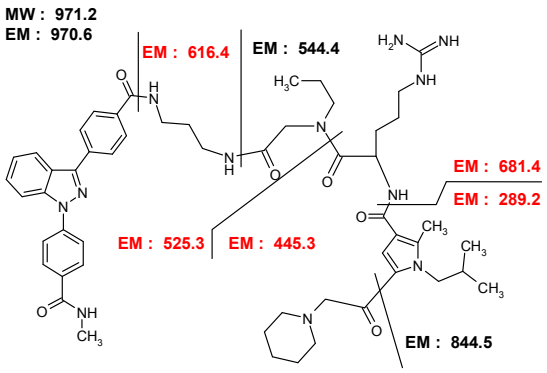


0920 #371-386 RT: 9.09-9.45 AV: 3 NL: 4.16E5
T: + c d w Full ms2 486.87@40.00 [120.00-985.00]





Bead 4

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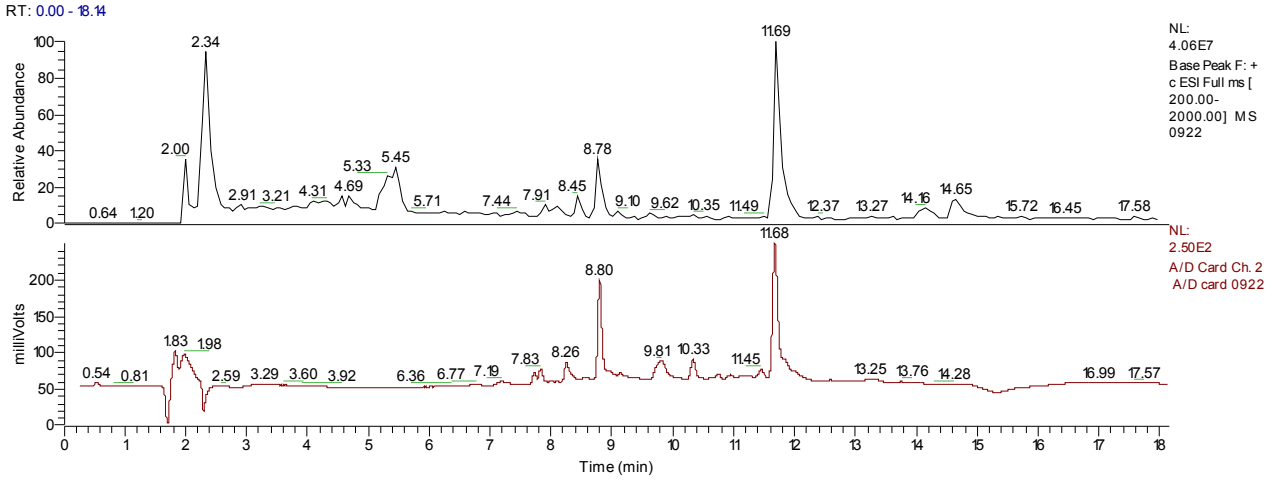

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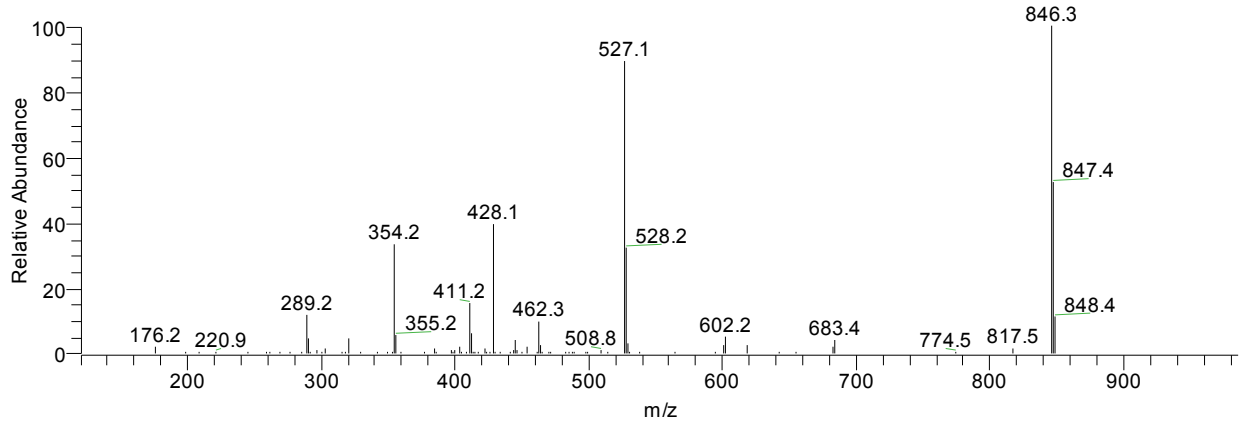
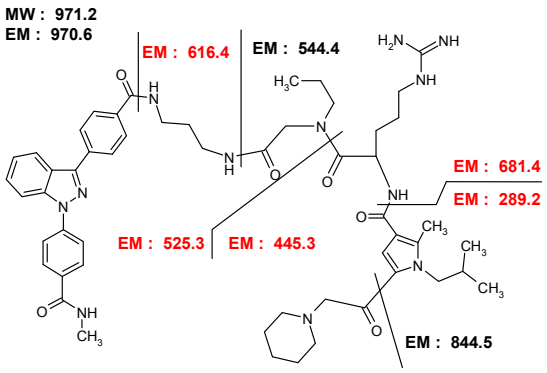




Materials and Methods

Cloning, expression and purification of importin β

Importin β was cloned in the IMPACT™ system (New England Biolabs) as a C-terminal fusion protein with a tag consisting of a self splicing intein domain and a chitin binding domain. Briefly, the protein was expressed in *E. coli* and purified from the soluble fraction by affinity chromatography on chitin agarose according to the manufacturer's protocol (New England Biolabs manual). Importin β was released from the resin-bound fusion tag by induction of intein-mediated self splicing with 30 mM DTT overnight at 4° C, and the native protein was recovered from the supernatant. The protein was finally obtained in a PBS buffer at pH 7.2, containing 200 mM NaCl , 0.01 mM CHAPS and 2 mM DTT. The final sequence of the recombinant protein with 10 additional amino acids at the C-terminus is the following:

```
  1 MELITILEKTVSPDRLELEAAQKFLERAAVENLPTFLVELSRVLANPGNSQVARVAAGLQ
 61 IKNSLTSKDPDIKAQYQQRWLAIDANARREVKNYVLQTLGTETYRPSSASQCVAGIACAE
121 IPVNQWPELIPQLVANVTNPNSTEHMKESTLEAIGYICQDIDPEQLQDKSNEILTAIIQG
181 MRKEEPSNNVKLAATNALLNSLEFTKANFDKESERHFIMQVVCEATQCPDTRVRVAALQN
241 LVKIMSLYYQYMETYMGPALFAITIEAMKSDIDEVALQGIEFWSNVCDEEMDLAIEASEA
301 AEQGRPPEHTSKFYAKGALQYLVPILTQTLTKQDENDDDDDWNPCKAAGVCLMLLATCCE
361 DDIVPHVLPFIKEHIKNPDWRYRDAAVMAFGCILEGPEPSQLKPLVIQAMPTLIELMKDP
421 SVVVRDTAAWTVGRICELLPEAAINDVYLAPLLQCLIEGLSAEPRVASNVCWAFSSLAEA
481 AYEAADVADDQEEPATYCLSSSFELIVQKLLETTDRPDGHQNNLRSSAYESLMEIVKNSA
541 KDCYPAVQKTTLVIMERLQQVLQMESHIQSTSDRIQFNDLQSLLCATLQNVLRKVQHQDA
601 LQISDVVMASLLRMFQSTAGSGGVQEDALMAVSTLVEVLGGEFLKYMEAFKPFLGIGLKN
661 YAEYQVCLAAVGLVGDLCRALQSNIIPFCDEVMQLLLENLGNENVHRSVKPQILSVFGDI
721 ALAIGGEFKKYLEVVLNTLQQASQAQVDKSDYDMVDYLNELRESCLEAYTGIVQGLKGDQ
781 ENVHPDVMLVQPRVEFILSFIDHIAGDEDHTDGVVACAAGLIGDLCTAFGKDVLKLVEAR
841 PMIHELLTEGRRSKTNKAKTLATWATKELRKLKNQAEGGREFLEPG
```

This recombinant importin β protein contains 886 amino acids (43 Lys, 23 Cys, 8 Trp), the molecular weight was 98242.3 (+ C-terminal MESNA 124, + C-terminal DTT 136), and the calculated pI is 4.67 (ExPASy, Swiss Institute for Bioinformatics). The theoretical extinction coefficients ε at 280 nm in 6.0 M guanidium hydrochloride 0.02 M phosphate buffer, pH 6.5, as calculated by the ProtParam software on the ExPASy server, based on the amino acid composition of the recombinant protein are are: 76280 [$M^{-1}cm^{-1}$] (all Cys as half cystines), and 74960 [$M^{-1}cm^{-1}$] (all Cys as cysteines).

Prior to fluorescence labeling the recombinant protein was analyzed by HPLC, UV spectroscopy, SDS-PAGE and μHPLC-MS. HPLC analysis was performed on a Poros R10 column (Perseptive Biosystems, custom filled by Dr. Maisch HPLC, Ammerbuch, Germany), 150 x 3 mm, 1 ml/min, 70° C, 5-60% B in 30 min, A: water/0.1% TFA, B: acetonitrile, 0.1% TFA, detection at 215 nm. SDS-PAGE was performed on Novex NuPAGE 4-12% Bis-Tris Gel (Invitrogen, # NP0321) under reducing conditions. Gels were stained with Silver Express staining kit (Invitrogen # LC6100). UV analysis was done on an Agilent 8453 UV-VIS single beam spectrophotometer in black 70 ml quartz microcuvettes. The purified protein was homogenous with a discrete main band at 97 kDa in the SDS-PAGE gel after silver staining and slightly faster moving bands at 50-97 kDa. The purity according to RP-HPLC analysis at 215 nm absorbance wavelengths was of > 95%. According to the deconvoluted mass spectrum shown in Figure S2, 60% of the protein was present with a free C-terminal carboxyl group (MW 98,242.3 $g.mol^{-1}$) and 40% had a C-terminal DTT thioester (+ 135 Da, MW 98,377.3 $g.mol^{-1}$).

Fluorescence labeling of importin β

Recombinant importin β was transferred into a buffer of 200 mM $NaHCO_3$ pH 8.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.01 mM CHAPS by dialysis for 2x10 hours at 4° C, using SLIDE-A-Lyzer dialysis cassettes (MWCO = 10,000 Da, Pierce). 1.56 μmol succinimidylester-activated Alexa488 (Molecular Probes) were dissolved in 100 μl DMF under argon and added to 2.6 ml of the 6.0 μM protein solution. The resulting dye: protein molar ratio was 100:1, the final DMF concentration was 3.7% (v/v). The reaction was incubated for 15 hours at 4° C under stirring and protected from light. Unreacted dye was removed by elution through a DG-10 gel filtration column with a buffer of 20 mM HEPES pH 7.4, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.01 mM CHAPS. The recovered protein solution of 4.5 ml was concentrated to a volume of 500 μl by centrifugation in a CENTRICON

ultrafiltration tube (MWCO = 10,000 Da, AMICON). The sample was divided into 30 µl aliquots, shock frozen in liquid nitrogen and stored at - 80° C.

Biochemical characterization

The concentration of labeled importin β was determined by UV absorption spectroscopy. Spectra were collected on an Agilent 8453 Spectrophotometer in the single beam mode, using 50 µl UV-silica cells (Ultra-Micro Cuvettes, Agilent) and the sample buffer as reference solution. The buffer subtracted spectra were corrected for Raleigh scattered light allowing for a contribution of Alexa488 to the absorption at 280 nm. The ratio between the absorption of the dye at 280 nm and at its absorption maximum at 494 nm was determined in a separate measurement with the free dye. Using this factor (A_{280} : A_{494} = 0.253), the dye contribution was subtracted from the measured extinction at 280 nm of the labeled protein. The protein concentration was calculated according to the Bouguer-Lambert-Beer law using the corrected protein absorption at 280 nm and the molar protein extinction coefficient of ε_{280} = 79,707.5 M^{-1} cm^{-1} (8 tryptophans, 23 tyrosines, see above). The labeling stoichiometry was determined by calculation of the ratio between the molar concentrations of importin β and the dye, using a molar extinction coefficient of Alexa488 in aqueous solutions of ε_{494} = 72,000 M^{-1} cm^{-1} (according to Molecular Probes).

The purity of Alexa488 labeled importin β was assessed by RP-HPLC analysis on an Agilent 1100 ChemStation instrument, equipped with a diode array UV-detector, serially arranged with a fluorescence detector. 1 to 10 µg protein were injected on a reversed phase column (VYDAC C4 protein&peptide, 4.6 mm x 150 mm; 5µm, 300 Å,VYDAC) and, after a 10 minute rinsing step, eluted with a solvent gradient at a constant flow of 0.5 ml min^{-1} and at ambient temperature (gradient: 10 minutes 100% A, to 100% B in 40 minutes, 100% B for 5 minutes, to 100% A in 5 minutes; solvent A: 5% (v/v) CH_3CN, 0.1% (v/v) TFA in H_2O; solvent B: 95% (v/v) CH_3CN, 0.1% TFA (v/v) in H_2O). UV absorption was detected at 215, 230 and 280 nm. Tryptophan fluorescence was detected at 280 nm excitation / 340 nm emission, Alexa488

fluorescence at 488 nm excitation / 520 nm emission. Data were processed with the Agilent ChemStation software.

The Alexa488 labeled importin β eluted in a single peak in RP-HPLC analysis (Figure S3). The labeling stoichiometry, determined by UV-VIS spectroscopy, was 2.8:1 (protein:dye), which corresponds to an average of 0.36 dye molecules per protein molecule (Figure S4). Even by increasing the concentration of reactive dye in the labeling reaction beyond a 100-fold molar excess, no higher labeling stoichiometry was achieved. The Alexa488 labeled importin β used for the screen showed nuclear import activity equivalent to the unmodified protein (data not shown).

Nuclear import assay using permeabilized cells

Nuclear import assays using permeabilized cells were carried out as previously described (*1*). Briefly, HeLa cells were grown in suspension, collected and washed with transport buffer (20 mM HEPES pH 7.4, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT and 0.1 µg/ml of each leupeptin, aprotinin, pepstatin). The cells were treated with 0.005% digitonin, which at this low concentration, permeates the plasma membrane, but does not disrupt the nuclear envelope. The cells were washed and incubated with the RanQ69L mutant and RanBP1 (Ran binding protein 1) for 15 min at 30° C to deplete endogenous transport factors (*2*). Each 40 µl import reaction was in transport buffer and contained 4,000 HeLa cells, an ATP regeneration system (0.5 mM ATP, 0.8 mg/ml creatine phosphate, 20 U/ml creatine phosphate kinase), 8 pmol cargo, involving either FITC labeled BSA conjugated SV large T antigen derived NLS (FITC-BSA-NLS) or the FITC labeled M9 NLS of hnRNP A1 fused to the core domain of nucleoplasmin (FITC-M9-nucleoplasmin) and either 2.5 mg/ml HeLa cell cytosolic extracts or recombinant import factors (10 pmol Ran, 25 pmol NTF2, and either 1.5-3 pmol transportin, or 2 pmol importin β and 15.6 pmol importin α). Control reactions either lacked importin β or transportin, contained 1 mg/ml wheat germ agglutinin (WGA) or contained 13.8 U/ml hexokinase, 25 µM glucose to deplete ATP, and 0.2 mM GMPPNP. The

digitonin permeabilized cells used in reactions containing cytosol were not preincubated with RanQ69L and RanBP1. Each CONA derived test compound was diluted from a 1 mM stock solution in DMSO to a final concentration of 10 µM (unless stated otherwise) in the recombinant factor mixture before addition to the HeLa cells. All import reactions were incubated for 30 minutes at 30° C and the level of nuclear import was quantified by flow cytometry (*2*).

Nuclear import assay in living cells using stably transfected HeLa cells:

The *in vivo* nuclear transport assay with GFP-NFAT stably transfected into HeLa cells have been essentially carried out as described before (*3-4*). In brief, GFP-NFAT expressing HeLa cells were grown in Dulbecco's Modified Eagle Medium complemented with 10% fetal bovine serum and 2 mM L-glutamine (cDMEM) and cells were seeded into poly-D-Lys coated clear bottom 96-well plates (Greiner) at 20,000 cells per well. 24 hours after seeding GFP-NFAT expression was induced by the addition of 1 µM trichostatin A. Nuclear transport reactions were carried out 24 hours later. To test the effect of *Iβ1A* on nuclear export and re-import processes, ionomycin at 1 µM was added to GFP-NFAT expressing cells. Half an hour later, cells were incubated with either 25 µM *Iβ1A* or 0.25% DMSO, each containing 1 µM ionomycin for 3 hours at 37ºC in a CO_2 incubator. Cells were washed 3x with, and left in cDMEM containing 25 µM *Iβ1A* or 0.25% DMSO but no ionomycin to induce export. Half an hour later ionomycin was added again at 1 µM and the re-import was left to proceed for half an hour. Throughout the procedure cells were kept at 37ºC in a CO_2 incubator except for when taking fluorescence microscopy images.

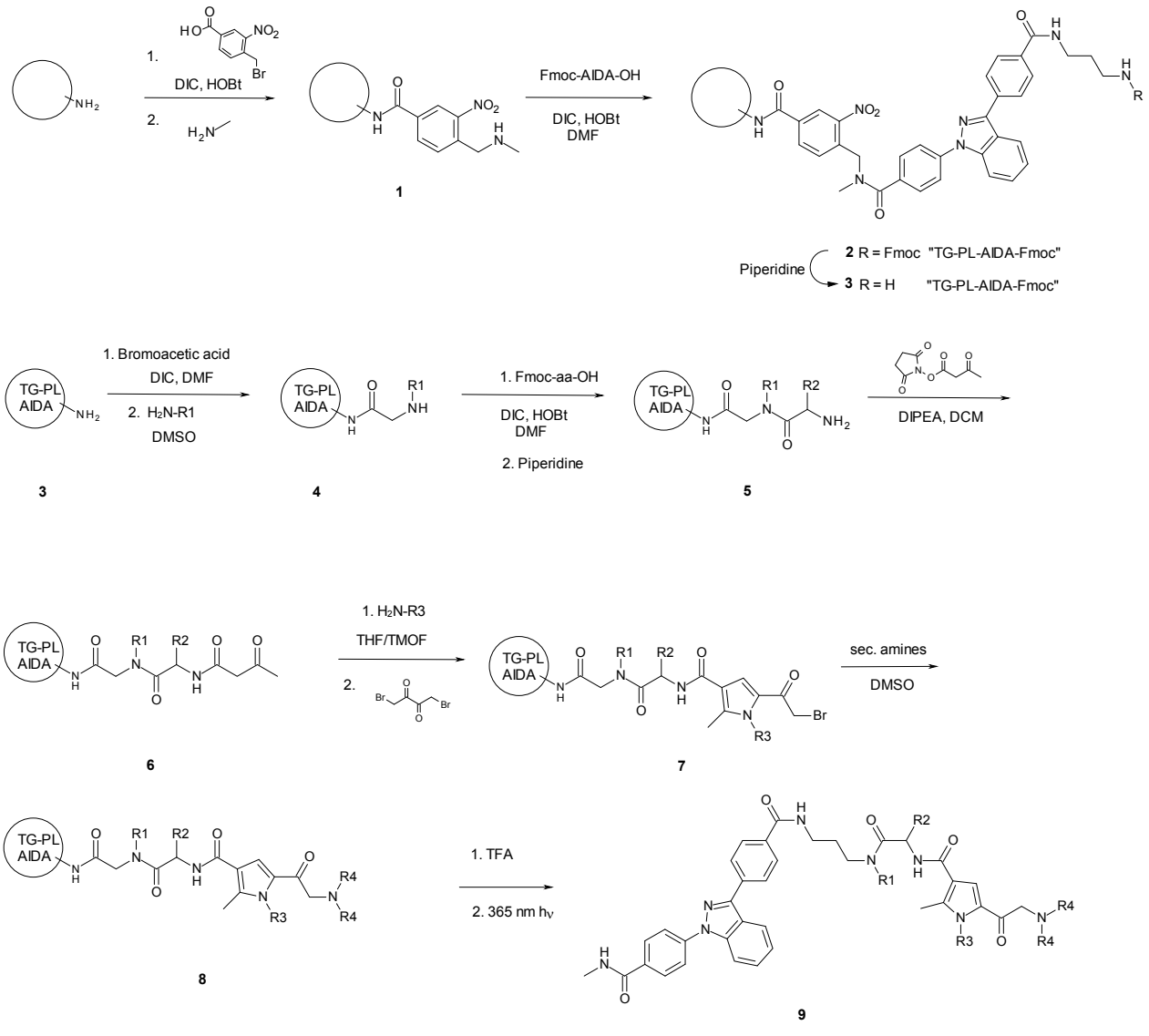
Surface plasmon resonance measurements:

CM5 sensor chips (Biacore) were used to covalently immobilize anti-GST antibodies at around 11,000 RU in both the test and reference flow cells. The recombinantly expressed and purified GST fused test proteins and unfused GST were injected into the test and reference flow cells, respectively, at 100 nM concentration in binding buffer (20 mM HEPES

pH 7.4, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 0.005% detergent P-20, 1 mM TCEP) and were left to stabilize for 5 min. Importin β was dissolved in binding buffer at the indicated concentrations and preincubated in the presence or absence of *Iβ1A* or DMSO at 0 ºC for 30 min before it was injected into the flow cells in duplicates. Flow cells were regenerated between run cycles with 10 mM pH2.2 glycine. Data was collected on a Biacore 2000 instrument and was analyzed using the Scrubber2 (Biologic Software Pty Ltd, Australia) with global fit and mass transport prediction functions.

Chemical Syntheses and compound Characterization

Synthesis of AIDA-tagged pyrrole compounds:



General procedure for resin loading (GP1):

TentaGel S NH2-resin (loading capacity=0.25mmol/g) was swollen in DCM and prewashed with DMF. 4-Bromomethyl-3-nitrobenzoic acid (3equ. relative to resin) and HOBt*H2O (3equ. relative to resin) were dissolved in a solution of 8% DMF in DCM (v/v). DIC (3equ. relative to resin) was added. After stirring at room temperature for 30min the solution was transferred to the

prewashed TentaGel S NH2-resin and the mixture was shaken at room temperature for 19h. The resin was filtered off, washed with DMF, DCM and MeOH and dried under reduced pressure. Completion of the reaction was detected by a negative Kaiser-ninhydrine test.

Methylamine substitution of photo-linker containing resin (GP2)

Dry DMSO was added to the resin followed by a solution of 8M methylamine in EtOH (20equ. relative to resin). After shaking for 3-3.5h at room temperature, the resin was filtered off and washed with DMSO, DMF, DCM, MeOH and DMF to obtain resin **1**. The chloranil test was positive.

Coupling of Fmoc-AIDA to the methylamine containing resins 1 (GP3)

Fmoc-AIDA-1 (3equ. relative to resin) and HOBt*H2O (3equ. relative to resin) were dissolved in DMF (80ml). DIC (6equ. relative to resin) was added. After shaking for 30min at room temperature the mixture was transferred to the resin **1** and shaken for 18h. The resin was filtered off and washed with DMF, DCM and MeOH to obtain resin **2**. The chloranil test was negative.

General protocol for Fmoc-deprotection (GP4)

A solution of piperidine in DMF (1:1, v/v) was added to the resin **2** and the mixture was shaken for 30min. The resin was filtered off, washed with DMF, DCM and MeOH and dried under reduced pressure. The Kaiser-ninhydrin test was positive.

Bromoacetic acid substitution of free amine containing resins (GP5)

Bromoacetic acid (5equ.) was dissolved in DMF and DIC (5equ.) was added. The solution was transferred to the resins **3** and shaken for 3-3.5h at room temperature.

The resin was filtered off, washed with DMF, DCM and MeOH and dried under reduced pressure. The Kaiser-ninhydrin test was negative.

Substitution of Bromoacetic acid containing resins by primary amines (GP6)

The bromoacetic acid coupled resin was swollen in DMSO. Then the appropriate primary amine (20equ.) was added to the resin and the mixture was shaken for 3h at room temperature. The resin was filtered off, washed with DMF, DCM and MeOH and dried under reduced pressure to give resin **4**. The chloranil test was positive.

Fmoc-amino acid coupling to resins containing secondary amines (GP7)

Fmoc-amino acids (3equ.) were dissolved in DMF and DIC (4.5equ.) and HOBt*H2O (3equ.) was added. The solution was shaken for 5 min. at room temperature and transferred to the resin. After shaking for 19h at room temperature the resin was filtered off washed with DMF, DCM and MeOH and dried under reduced pressure. The chloranil test was negative.

The N-terminal Fmoc group was then removed following GP4 to obtain resin **5**.

Acetoacetylation of resin 5

N-Hydroxysuccinimidyl acetoacetate (3equ.) was dissolved in DCM . DIPEA (4equ.) was added and the solution was transferred to the resin **5**. After shaking for 5.25h at room temperature the resin was filtered off washed with DMF, DCM and MeOH and dried under reduced pressure to give resin **6.** The Kaiser-ninhydrin test was negative.

Enaminone formation

A solution of THF and TMOF (1/1, v/v) was added to the resin **6**. The appropriate primary amine (10equ.) was added to the resin and the mixture was shaken for 18h at room temperature. The resin were filtered off, washed with THF/TMOF (1/1, v/v), DMF, DCM and MeOH and dried under reduced pressure.

Cyclisation of resin-bound enamineones with 1,4-dibromo-2,3-butanedione

2,6-di-tert.-Butylpyridine (2equ.) and 1,4-dibromo-2,3-butanedione (4equ.) were dissolved in dioxane. The resulting solution was added to the resin **7** and the mixture was shaken for 1.5h at room temperature. The resin was filtered off, washed with dioxane, DMF, THF and DCM and dried under reduced pressure to obtain resin **7**.

Substitution by secondary amines (GP8)

The appropriate secondary amine (10equ.) in DMSO was added to the resin **7** and the mixture was shaken for 15.5h at room temperature. The resin was filtered off, washed with DMSO, DMF, DCM and MeOH and dried under reduced pressure to obtain resin **8**.

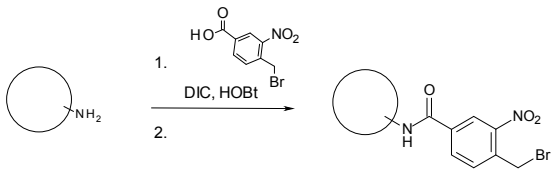
Cleavage of side chain protecting groups (GP9)

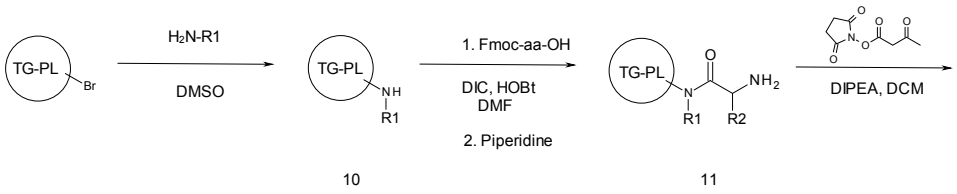
A solution of 20% TFA in DCM (v/v) was added to remove side chain protecting groups in the different combinatorial positions (R1-4). The mixture was shaken for 1h at room temperature. The resin was filtered off, washed with 20% TFA in DCM (v/v), DMF, DCM, EtOH and diethylether and dried under reduced pressure.

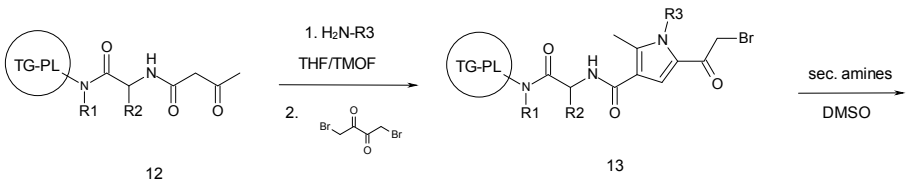
Cleavage of final compounds from resin (GP10)

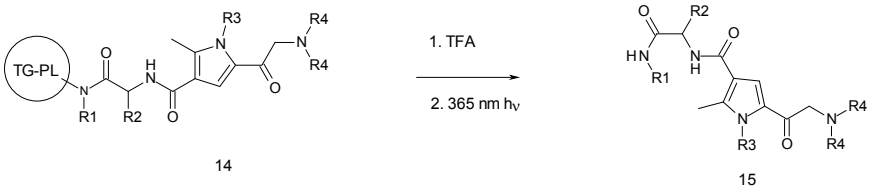
The resin containing the final product was suspended in a solution of TFA (1.5% v/v) in MeOH and irradiated with UV-light (366 nm) under stirring for 90 min. The resin was then drained and extensively washed with MeOH. The combined filtrates were concentrated *in vacuo* and the resulting residue was dissolved in DMF/MeOH (50% v/v). The solution was then purified by preparative HPLC and analyzed by analytical HPLC and ESI-MS.

Synthesis of Non-AIDA tagged pyrrole compounds:



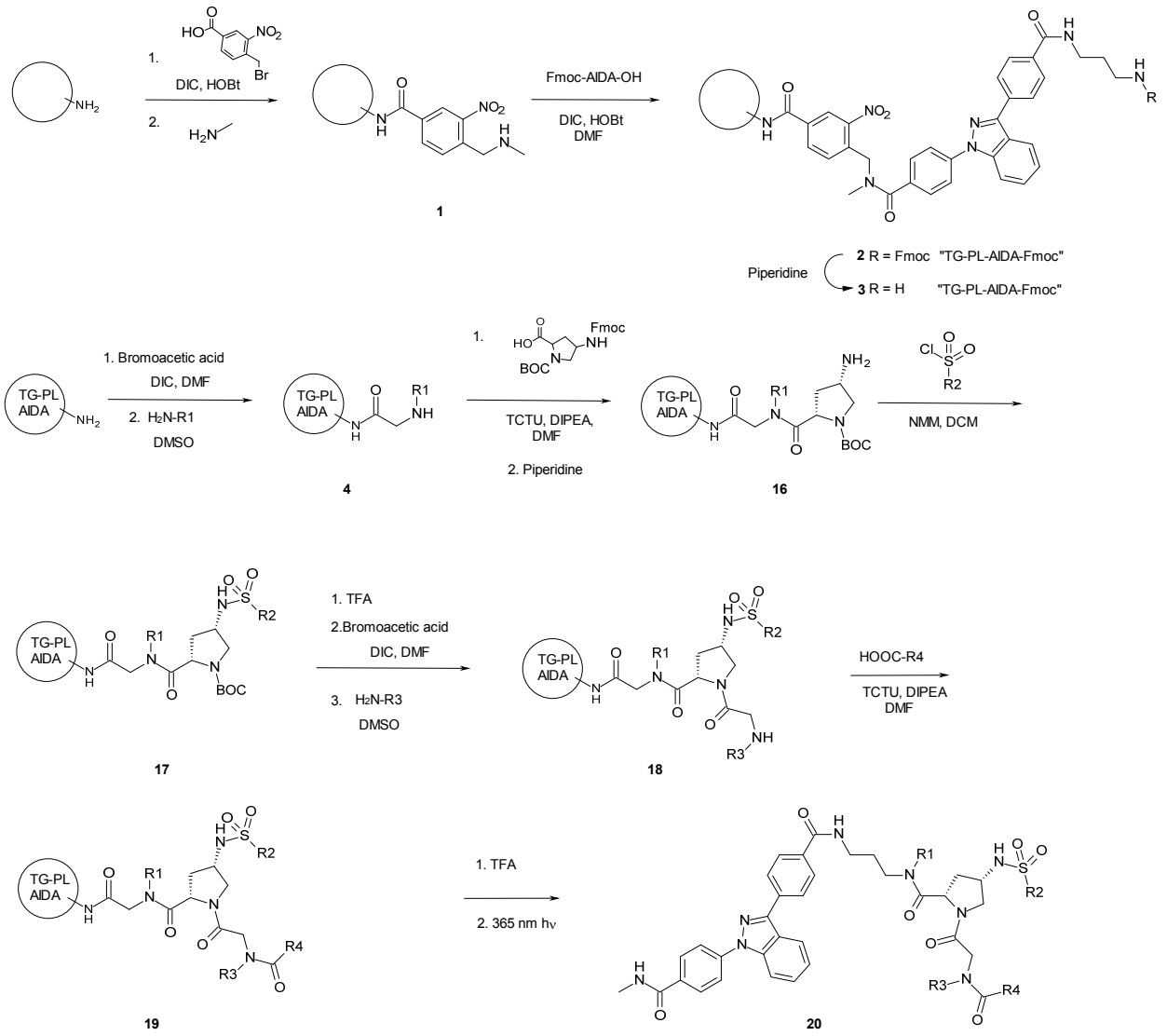






TentaGel NH2 (90 µm) was loaded with photolinker as described in GP1. Nucleophilic substitution in the bromobenzyl-position of the photolinker using the appropriate primary amines was carried out according to GP6 to obtain resin **10**. This was followed by coupling of the appropriate Fmoc-amino acids according to GP7 and Fmoc-deprotection according to GP4, to give resin **11**. Acetoacetation, enamineone formation with the appropriate primary amines and subsequent cyclization to the pyrrole structure was performed as described for the AIDA-tagged pyrrole compound syntheses above. Finally, nucleophilic substitution with the appropriate secondary amines in the fourth combinatorial position, TFA-cleavage of side chain protecting groups and final compound cleavage from resin was carried out according to standard protocols GP-8, GP9 and GP10, respectively giving the final compounds of general structure **15**.

Synthesis of 4-amino-proline compounds:



then coupled to resin **1** using GP3 and the Fmoc-group was removed with piperidine using GP4.

Next, bromoacetic acid was coupled to the TG-PL-AIDA resin **3** according to GP5. Nucleophilic substitution with the appropriate primary amines according to GP6 gave resin **4**.

Coupling of (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid to resin 4

(2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid (2 equ. was dissolved in DMF and TCTU (2.5 equ.) and DIPEA (5 equ.) were added. The solution transferred to resin **4** and shaken for 19 h at room temperature. Then the resin was filtered off and washed with DMF, DCM and MeOH and dried under reduced pressure. A chloranil test was negative.

The N-terminal Fmoc-protecting group was then removed according to GP4 to give resin **16**.

Reaction of resin 16 with sulfonyl chlorides

A solution of the appropriate sulfonyl chloride (3equ.) and NMM (10equ.) in DCM was added to resin **16**. After shaking for 18h at room temperature the resins were filtered off and washed with DCM, DMF, DCM and MeOH and dried under reduced pressure to give resin **17**. A Kaiser-ninhydrin tests was negative.

Boc-deprotection of resin 17

A solution of 20 % TFA in DCM (v/v) was added to resin x and the mixture was shaken for 60 min at room temperature. The resin was then filtered off, and treated a second time with 20 % TFA in DCM (v/v) for 60 min. Then the resin was washed with 20% TFA in DCM (v/v), DCM, DMF, and MeOH and dried under reduced pressure.

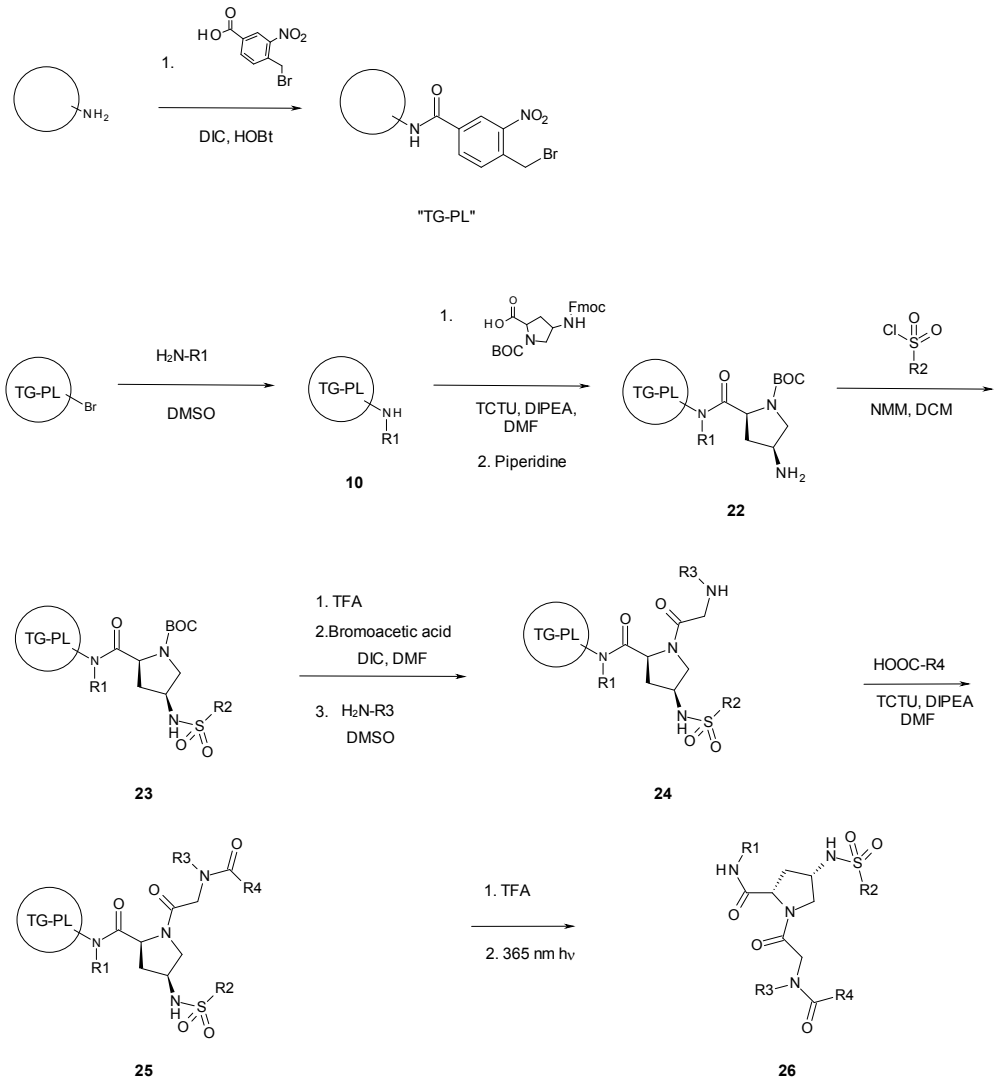
Bromoacetic acid was then coupled to the free amino-group of the resin as described in GP5, Nucleophilic substitution with the appropriate primary amines according to GP6 gave resin **18**.

Acetylation of resin 18 with carboxylic acids :

The appropriate carboxylic acid (2.5 equ.) and TCTU (2.5 equ.) were dissolved in DMF. Then DIEA (2.5 eq.) was added and solution was transferred to resin **18**. After shaking for 13 h at room temperature the resin was filtered off and washed with DMF, DCM and EtOH and dried under reduced pressure to give resin **19**. A Chloranil test was negative.

Side chain deprotection and final cleavage of compounds from the resin were carried out as described in standard protocols GP10 and GP9.

Synthesis of Non-AIDA tagged 4-aminoproline compounds:



TentaGel NH2 (90 µm) was loaded with photolinker as described in GP1. Nucleophilic substitution in the bromobenzyl-position of the photolinker using the appropriate primary amines was carried out according to GP6 to obtain resin **10**. This was followed by coupling of (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid to the resin **21** according to the procedure described above for the AIDA-tagged 4-amino-proline compounds.

The all subsequent reactions as the cleavage of the final compounds from resin was carried out using the identical procedures as described for the AIDA-tagged 4-amino-proline compounds (see above).

Re-synthesis of Selected hit compounds as AIDA and Non-AIDA conjugates

For the re-synthesis of selected hit compounds the same synthetic procedures were applied as originally used for the on-bead library synthesis. All compounds were synthesized on TentaGel beads (90 µm, Rapp Polymers, Tübingen, Germany), photocleaved from the solid support and purified by preparative RP-HPLC. Compound purity was then analyzed by RP-HPLC and compound identity confirmed by ES-MS.

Analytical HPLC: For analytical separations an Abimed (D-Langenfeld) HPLC system was used, consisting of two pump units 306, a dynamic mixing chamber module 811C, a manometric pressure module 805, an UV-Detector UV/VIS 155 and an autoinjector 234. The separation was performed on an analytical column GromSil150 ODS-5 ST (3 µm, 120 x 2 mm) manufactured by Grom (D-Herrenberg). A gradient of water and acetonitrile (0% to 100% over 15 min), both containing 0.1% TFA (v/v) with a flow rate of 0.4 ml/min was used. UV-traces were recorded at 214 nm and 305 nm. The purity of the products was assigned on the basis of the peak area as determined from the 214 nm trace.

ES-MS: ES-MS-analysis was performed on a Micromass (Altrinchan/UK) Quattro II triple quadrupole mass spectrometer with a waters (D-Eschborn) 515 make-up pump (isocratic flow of 60 µl/min acetonitrile/water 1:1, containing 0.1 % formic acid) and a Abimed (D-Langenfeld) Gilson 232X autosampler.

Individual compound analytics:

Compound name, number:	Retention time (min)	Purity % (Area% at 214 nm)	ES+-MS (Intensity)
Ib1A 1	8.09	90	971.6 (30), 486.4 (100).
Ib1N 2	6.80	94	618.4 (10), 504.4 (30), 273.3 (10), 252.8 (100), 223.3 (12).
Ib2A 3	8.07	94	1005.7 (25), 503.5 (100).
Ib2N 4	6.93	97	652.5 (12), 538.5 (32), 413.2 (12), 290.2 (10), 269.8 (100), 240.2 (13).
Ib3A 5	8.13	97	1223.7 (18), 612.5 (100).
Ib3N 6	7.55	94	756.6 (10), 378.9 (100), 329.3 (22).
Ib4A 7	7.42	97	1002.7 (60), 513.3 (18), 502.0 (100)
Ib4N 8	5.97	94	535.5 (36), 367.3 (10), 288.8 (12), 268.2 (100), 259.8 (41), 230.2 (15).
Ib5A 9	9.87	94	1042.5 (50), 675 (15), 379 (19), 338.3 (100),
Ib5N 10	9.50	99	1151.4 (15), 912.6 (10), 613.3 (10), 597.4 (20), 575.2 (100), 379.5 (15), 338.4 (95), 321.2 (10).
Ib6A 11	9.96	100	1044.5 (35), 675.8 (19), 379.5 (15), 338.3 (100), 321.2 (10).
Ib6N 12	9.65	99	1151.4 (20), 912 (10), 613 (10), 597.4 (25), 575.2 (100), 379.5 (10), 338.3 (90), 321.2 (10).
Ib7A 13	9.80	100	1042.4 (45), 675.8 (20), 379.5 (15), 338.3 (100), 321 (10).

Ib7N 14	9.42	99	1173.4 (20), 1151.4 (20), 613.2 (35), 597.2 (70), 575.3 (100), 379.4 (10), 338.4 (90), 321.4 (10).

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