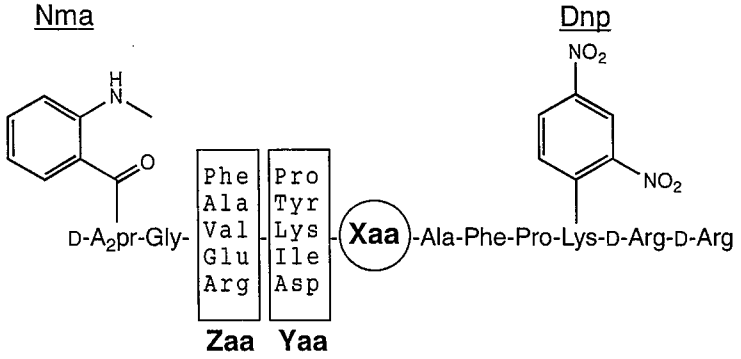
FRETS-25Xaa Series

* FRETS = Fluorescence Resonance Energy Transfer Substrates

Design of FRETS-25Xaa



Each substrate (#3701-v - #3719-v) in the FRETS-25Xaa series contains a highly fluorescent 2-(N-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal D-A2pr residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys. Xaa represents a fixed position of each of the 19 natural amino acids excluding Cys (*noted in product name #3701-v - #3719-v*). A mixture of 5 amino acid residues (P, Y, K, I, and D) is at the Yaa position along with a mixture of 5 amino acid residues (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combinations of each Xaa series resulting in a combinatorial library totaling 475 peptide substrates. Both Nma and Dnp groups are linked to the side chain of the individual residues, allowing for the determination of the cleavage site by a specific enzyme through mass spectrometric analysis and Edman degradation as well.

Principle

When an enzyme of interest cleaves any peptide bond between D-A2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at λex = 340 nm and λem = 440 nm increases in proportion to the release of the Nma fluorophore from the internal Dnp quencher.

Reagents

1) Each substrate stock solutions: each FRETS-25Xaa (#3701-v - #3719-v) in 1.0 ml of DMSO (1 mM, total of peptides)
2) Reference compounds stock solution: a 1:1 mixture of two solutions of #3720-v and #3721-v, each of which is reconstituted by dissolving peptides in 0.5 ml of DMSO at the concentration of 2 mM (1 mM, each reference compound)
3) Enzyme solution: an enzyme of interest in an appropriate buffer
4) Buffer

Procedure for the deduction of the substrate specificity of an enzyme with unidentified cleavage specificity

Choose the proper conditions for the measurement, such as substrate concentration and sensitivity setting, depending on the purpose of the experiment and the instrument available. Described here is one of the recommended procedures for determining the enzymatic cleavage site by the combination of the fluorometric analysis and liquid chromatography-mass spectrometry (LC-MS) analysis.

i) Primary screening: selection of the favored Xaa

·Substrate solution for primary screening (PS solution): Dilute 20 μl of each of the above substrate stock solution with 1980 μl of an appropriate buffer (10 μM)

·Reference compounds solution for primary screening (PR solution): Dilute 20 μl of the above reference compounds stock solution with 1980 μl of an appropriate buffer (10 μM)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix one of the PS solution and the PR solution in ratios of 10/0, 9/1, 8/2, 5/5 and 0/10

3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl each of all PS solutions into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor (leupeptin, E-64, pepstatin, EDTA and so on) or changing the pH of the reaction medium (using TCA, AcOH, NaOH and so on)
9) Choose the best Xaa-containing substrate for secondary screening

ii) Secondary screening: identification of the specificity of the enzyme (I)
·Substrate solution for secondary screening (SS solution): Dilute 200 μl of the stock solution of the best Xaa-containing substrate chosen by the above primary screening with 1800 μl of an appropriate buffer (100 μM)
·Reference compounds solution for secondary screening (SR solution): Dilute 200 μl of the above reference compounds stock solution with 1800 μl of an appropriate buffer (100 μM)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix the SS solution and the SR solution in ratios of 100/0, 95/5, 90/10, 80/20, 50/50 and 0/100
3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl of the SS solution into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor or changing the pH of the reaction medium upon completion of the reaction at the points of 0%, 5%, 10% and 20% of the total
9) Subject 100 μl aliquots to LC-MS

iii) LC-MS: identification of the specificity of the enzyme (II)
·Analytical conditions
column: ODS
eluant: A) H_2O containing 0.05% TFA, B) CH_3CN containing 0.05% TFA
gradient: 10% to 40% B) in A) over 50 min
detection: UV at 220 nm and 400 nm or fluorescence

1) Inject 100 μl aliquots of each terminated solution at different stage of the reaction
2) Measure the MW of the cleaved product(s) in the peak(s) with the absorbance at 220 nm but not with 400 nm [identification of the N-terminal segment(s)]
3) Deduce their structure from the attached list of the theoretical MW for the cleaved products

* Comment 1: If the N-terminal segment has the identical retention time to the C-terminal segment or one of the starting uncleaved substrates, detection of the products by fluorescence is recommended.
* Comment 2: In the accidental case where the two products with the same MW (ex. Zaa-Yaa=Phe-Asp and Val-Tyr, Glu-Asp and Phe-Pro) are generated from one of the substrate, their analyses should be carried out by MS-MS sequencing and/or by Edman degradation.

Usefulness and limitation of FRETS-25Xaa series for screening of substrate specificities of proteases
We have confirmed that FRETS-25Xaa series are effectively used for the assay of numerous proteases such as trypsin, chymotyrpsin, elastase, thrombin, papain, calpain, pepsin and thermolysin. However, they did not work well for the assay of caspase-3 and furin, probably because they have only three changeable sites (Zaa-Yaa-Xaa) in each substrate (deficiency of P4 site). This fact implies that FRETS-25Xaa might not be applicable to the assay of an enzyme with wide range interacting sites with substrate.

Code 3703-v

FRETS-25Asn Component-masses

FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GAKN	607.66	607.3078	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GVYNAF	888.97	888.4130
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GEDNA	723.69	723.2824	A2pr(Nma)GAPNAFP	891.97	891.4239
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVIN	620.70	620.3282	A2pr(Nma)GFPNA	723.78	723.3340	A2pr(Nma)GRINAF	896.00	895.4664
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVDN	622.63	622.2711	A2pr(Nma)GRYN	727.77	727.3402	A2pr(Nma)GRDNAF	897.93	897.4093
A2pr(Nma)GF	441.48	441.2012	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GRPNA	732.79	732.3667	A2pr(Nma)GFKNAF	902.01	901.4446
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GEPN	634.64	634.2711	A2pr(Nma)GEKNA	736.77	736.3504	A2pr(Nma)GAINAFP	908.01	907.4552
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GVKN	635.71	635.3391	A2pr(Nma)GFINA	739.82	739.3653	A2pr(Nma)GADNAFP	909.94	909.3981
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYN	642.66	642.2762	A2pr(Nma)GFDNA	741.75	741.3082	A2pr(Nma)GRKNAF	911.02	910.4773
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPNA	647.68	647.3027	A2pr(Nma)GVYNA	741.79	741.3446	A2pr(Nma)GEYNAF	918.95	918.3872
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GEIN	650.68	650.3024	A2pr(Nma)GRINA	748.83	748.3980	A2pr(Nma)GVPNAFP	920.02	919.4552
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDN	652.61	652.2453	A2pr(Nma)GRDNA	750.76	750.3409	A2pr(Nma)GAKNAFP	923.03	922.4661
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPN	652.70	652.2969	A2pr(Nma)GFKNA	754.83	754.3762	A2pr(Nma)GVINAFP	936.06	935.4865
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPN	661.71	661.3296	A2pr(Nma)GRKNA	763.84	763.4089	A2pr(Nma)GFYNAF	937.01	936.4130
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAINA	663.72	663.3340	A2pr(Nma)GEYNA	771.77	771.3188	A2pr(Nma)GVDNAFP	937.99	937.4294
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADNA	665.65	665.2769	A2pr(Nma)GFYNA	789.83	789.3446	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKN	665.70	665.3133	A2pr(Nma)GAPNAF	794.85	794.3711	A2pr(Nma)GRYNAF	946.02	945.4457
A2pr(Nma)GEI	536.58	536.2595	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GRYNA	798.85	798.3773	A2pr(Nma)GEPNAFP	950.01	949.4294
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFIN	668.74	668.3282	A2pr(Nma)GAINAF	810.90	810.4024	A2pr(Nma)GVKNAFP	951.08	950.4974
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDN	670.67	670.2711	A2pr(Nma)GADNAF	812.83	812.3453	A2pr(Nma)GAYNAFP	958.03	957.4345
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYN	670.71	670.3075	A2pr(Nma)GVPNAF	822.91	822.4024	A2pr(Nma)GEINAFP	966.05	965.4607
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPNA	675.73	675.3340	A2pr(Nma)GAKNAF	825.91	825.4133	A2pr(Nma)GEDNAFP	967.98	967.4036
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRIN	677.75	677.3609	A2pr(Nma)GVINAF	838.95	838.4337	A2pr(Nma)GFPNAFP	968.07	967.4552
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKNA	678.74	678.3449	A2pr(Nma)GVDNAF	840.88	840.3766	A2pr(Nma)GRPNAFP	977.08	976.4879
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDN	679.68	679.3038	A2pr(Nma)GEPNAF	852.89	852.3766	A2pr(Nma)GEKNAFP	981.06	980.4716
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GFKN	683.76	683.3391	A2pr(Nma)GVKNAF	853.96	853.4446	A2pr(Nma)GFINAFP	984.11	983.4865
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GVINA	691.78	691.3653	A2pr(Nma)GAYNAF	860.91	860.3817	A2pr(Nma)GFDNAFP	986.04	985.4294
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GRKN	692.77	692.3718	A2pr(Nma)GEINAF	868.93	868.4079	A2pr(Nma)GVYNAFP	986.08	985.4658
A2pr(Nma)GAPN	576.60	576.2656	A2pr(Nma)GVDNA	693.71	693.3082	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GRINAFP	993.12	992.5192
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GEYN	700.70	700.2817	A2pr(Nma)GEDNAF	870.86	870.3508	A2pr(Nma)GRDNAFP	995.05	994.4621
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GEPNA	705.72	705.3082	A2pr(Nma)GFPNAF	870.95	870.4024	A2pr(Nma)GFKNAFP	999.12	998.4974
A2pr(Nma)GAIN	592.64	592.2969	A2pr(Nma)GVKNA	706.79	706.3762	A2pr(Nma)GRPNAF	879.96	879.4351	A2pr(Nma)GRKNAFP	1008.13	1007.5301
A2pr(Nma)GADN	594.57	594.2398	A2pr(Nma)GAYNA	713.74	713.3133	A2pr(Nma)GEKNAF	883.95	883.4188	A2pr(Nma)GEYNAFP	1016.06	1015.4399
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GFYN	718.76	718.3075	A2pr(Nma)GFINAF	886.99	886.4337	A2pr(Nma)GFYNAFP	1034.12	1033.4658
A2pr(Nma)GVPN	604.66	604.2969	A2pr(Nma)GEINA	721.76	721.3395	A2pr(Nma)GFDNAF	888.92	888.3766	A2pr(Nma)GRYNAFP	1043.13	1042.4985

FRETS-25Asn Component-masses

FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic	FRETS-25Asn	Average	Monoisotopic
NAFPK(Dnp)rr	1054.12	1053.5104	A2pr(Nma)GRINAFPK(Dnp)	1287.38	1286.6156	A2pr(Nma)GAINAFPK(Dnp)r	1358.46	1357.6527	A2pr(Nma)GRKNAFPK(Dnp)r	1458.58	1457.7276
PNAFPK(Dnp)rr	1151.23	1150.5632	AYNAFPK(Dnp)rr	1288.37	1287.6109	A2pr(Nma)GADNAFPK(Dnp)r	1360.39	1359.5956	A2pr(Nma)GEYNAFPK(Dnp)r	1466.51	1465.6375
INAFPK(Dnp)rr	1167.28	1166.5945	A2pr(Nma)GRDNAFPK(Dnp)	1289.31	1288.5585	FYNAFPK(Dnp)rr	1364.47	1363.6422	A2pr(Nma)GFYNAFPK(Dnp)r	1484.57	1483.6633
DNAFPK(Dnp)rr	1169.21	1168.5374	A2pr(Nma)GFKNAFPK(Dnp)	1293.39	1292.5938	GRPNAFPK(Dnp)rr	1364.47	1363.6858	A2pr(Nma)GRYNAFPK(Dnp)r	1493.58	1492.6960
KNAFPK(Dnp)rr	1182.29	1181.6054	GAINAFPK(Dnp)rr	1295.41	1294.6531	GEKNAFPK(Dnp)rr	1368.46	1367.6694	A2pr(Nma)GAPNAFPK(Dnp)rr	1498.60	1497.7225
A2pr(Nma)GAPNAFPK(Dnp)	1186.23	1185.5203	EINAFPK(Dnp)rr	1296.39	1295.6371	A2pr(Nma)GVPNAFPK(Dnp)r	1370.47	1369.6527	A2pr(Nma)GAINAFPK(Dnp)rr	1514.65	1513.7538
A2pr(Nma)GAINAFPK(Dnp)	1202.27	1201.5516	GADNAFPK(Dnp)rr	1297.34	1296.5959	GFINAFPK(Dnp)rr	1371.50	1370.6844	A2pr(Nma)GADNAFPK(Dnp)rr	1516.58	1515.6967
A2pr(Nma)GADNAFPK(Dnp)	1204.20	1203.4945	EDNAFPK(Dnp)rr	1298.32	1297.5800	GFDNAFPK(Dnp)rr	1373.43	1372.6272	A2pr(Nma)GVPNAFPK(Dnp)rr	1526.66	1525.7538
A2pr(Nma)GVPNAFPK(Dnp)	1214.29	1213.5516	FPNAFPK(Dnp)rr	1298.41	1297.6316	A2pr(Nma)GAKNAFPK(Dnp)r	1373.48	1372.6636	A2pr(Nma)GAKNAFPK(Dnp)rr	1529.66	1528.7647
A2pr(Nma)GAKNAFPK(Dnp)	1217.29	1216.5625	A2pr(Nma)GRKNAFPK(Dnp)	1302.40	1301.6265	GVYNAFPK(Dnp)rr	1373.48	1372.6636	A2pr(Nma)GVINAFPK(Dnp)rr	1542.70	1541.7851
YNAFPK(Dnp)rr	1217.29	1216.5738	GVPNAFPK(Dnp)rr	1307.42	1306.6531	RYNAFPK(Dnp)rr	1373.48	1372.6749	A2pr(Nma)GVDNAFPK(Dnp)rr	1544.63	1543.7280
APNAFPK(Dnp)rr	1222.31	1221.6003	RPNAFPK(Dnp)rr	1307.42	1306.6643	GRINAFPK(Dnp)rr	1380.51	1379.7171	A2pr(Nma)GEPNAFPK(Dnp)rr	1556.64	1555.7280
A2pr(Nma)GVINAFPK(Dnp)	1230.33	1229.5829	A2pr(Nma)GEYNAFPK(Dnp)	1310.33	1309.5364	GRDNAFPK(Dnp)rr	1382.44	1381.6599	A2pr(Nma)GVKNAFPK(Dnp)rr	1557.71	1556.7960
A2pr(Nma)GVDNAFPK(Dnp)	1232.26	1231.5258	GAKNAFPK(Dnp)rr	1310.42	1309.6640	A2pr(Nma)GVINAFPK(Dnp)r	1386.51	1385.6840	A2pr(Nma)GAYNAFPK(Dnp)rr	1564.66	1563.7331
AINAFPK(Dnp)rr	1238.36	1237.6316	EKNAFPK(Dnp)rr	1311.41	1310.6480	GFKNAFPK(Dnp)rr	1386.52	1385.6953	A2pr(Nma)GEINAFPK(Dnp)rr	1572.68	1571.7593
ADNAFPK(Dnp)rr	1240.28	1239.5745	FINAFPK(Dnp)rr	1314.45	1313.6629	A2pr(Nma)GVDNAFPK(Dnp)r	1388.44	1387.6269	A2pr(Nma)GEDNAFPK(Dnp)rr	1574.61	1573.7022
A2pr(Nma)GEPNAFPK(Dnp)	1244.27	1243.5258	FDNAFPK(Dnp)rr	1316.38	1315.6058	GRKNAFPK(Dnp)rr	1395.53	1394.7280	A2pr(Nma)GFPNAFPK(Dnp)rr	1574.70	1573.7538
A2pr(Nma)GVKNAFPK(Dnp)	1245.34	1244.5938	VYNAFPK(Dnp)rr	1316.42	1315.6422	A2pr(Nma)GEPNAFPK(Dnp)r	1400.45	1399.6269	A2pr(Nma)GRPNAFPK(Dnp)rr	1583.71	1582.7865
VPNAFPK(Dnp)rr	1250.37	1249.6316	GVINAFPK(Dnp)rr	1323.46	1322.6844	A2pr(Nma)GVKNAFPK(Dnp)r	1401.53	1400.6949	A2pr(Nma)GEKNAFPK(Dnp)rr	1587.70	1586.7702
A2pr(Nma)GAYNAFPK(Dnp)	1252.29	1251.5309	RINAFPK(Dnp)rr	1323.46	1322.6956	GEYNAFPK(Dnp)rr	1403.46	1402.6378	A2pr(Nma)GFINAFPK(Dnp)rr	1590.74	1589.7851
AKNAFPK(Dnp)rr	1253.37	1252.6425	GVDNAFPK(Dnp)rr	1325.39	1324.6272	A2pr(Nma)GAYNAFPK(Dnp)r	1408.48	1407.6320	A2pr(Nma)GFDNAFPK(Dnp)rr	1592.67	1591.7280
A2pr(Nma)GEINAFPK(Dnp)	1260.31	1259.5571	RDNAFPK(Dnp)rr	1325.39	1324.6385	A2pr(Nma)GEINAFPK(Dnp)r	1416.50	1415.6582	A2pr(Nma)GVYNAFPK(Dnp)rr	1592.72	1591.7644
A2pr(Nma)GEDNAFPK(Dnp)	1262.24	1261.5000	A2pr(Nma)GFYNAFPK(Dnp)	1328.39	1327.5622	A2pr(Nma)GEDNAFPK(Dnp)r	1418.43	1417.6011	A2pr(Nma)GRINAFPK(Dnp)rr	1599.75	1598.8178
A2pr(Nma)GFPNAFPK(Dnp)	1262.33	1261.5516	FKNAFPK(Dnp)rr	1329.47	1328.6738	A2pr(Nma)GFPNAFPK(Dnp)r	1418.51	1417.6527	A2pr(Nma)GRDNAFPK(Dnp)rr	1601.68	1600.7607
VINAFPK(Dnp)rr	1266.41	1265.6629	A2pr(Nma)GRYNAFPK(Dnp)	1337.40	1336.5949	GFYNAFPK(Dnp)rr	1421.52	1420.6636	A2pr(Nma)GFKNAFPK(Dnp)rr	1605.76	1604.7960
VDNAFPK(Dnp)rr	1268.34	1267.6058	GEPNAFPK(Dnp)rr	1337.40	1336.6272	A2pr(Nma)GRPNAFPK(Dnp)r	1427.53	1426.6854	A2pr(Nma)GRKNAFPK(Dnp)rr	1614.77	1613.8287
A2pr(Nma)GRPNAFPK(Dnp)	1271.34	1270.5843	GVKNAFPK(Dnp)rr	1338.47	1337.6953	GRYNAFPK(Dnp)rr	1430.53	1429.6963	A2pr(Nma)GEYNAFPK(Dnp)rr	1622.70	1621.7386
A2pr(Nma)GEKNAFPK(Dnp)	1275.33	1274.5680	RKNAFPK(Dnp)rr	1338.48	1337.7065	A2pr(Nma)GEKNAFPK(Dnp)r	1431.51	1430.6691	A2pr(Nma)GFYNAFPK(Dnp)rr	1640.76	1639.7644
A2pr(Nma)GFINAFPK(Dnp)	1278.37	1277.5829	A2pr(Nma)GAPNAFPK(Dnp)r	1342.42	1341.6214	A2pr(Nma)GFINAFPK(Dnp)r	1434.56	1433.6840	A2pr(Nma)GRYNAFPK(Dnp)rr	1649.77	1648.7971
GAPNAFPK(Dnp)rr	1279.36	1278.6218	GAYNAFPK(Dnp)rr	1345.42	1344.6323	A2pr(Nma)GFDNAFPK(Dnp)r	1436.49	1435.6269			
A2pr(Nma)GFDNAFPK(Dnp)	1280.30	1279.5258	EYNAFPK(Dnp)rr	1346.41	1345.6163	A2pr(Nma)GVYNAFPK(Dnp)r	1436.53	1435.6633			
A2pr(Nma)GVYNAFPK(Dnp)	1280.34	1279.5622	GEINAFPK(Dnp)rr	1353.44	1352.6585	A2pr(Nma)GRINAFPK(Dnp)r	1443.57	1442.7167			
EPNAFPK(Dnp)rr	1280.35	1279.6058	GEDNAFPK(Dnp)rr	1355.37	1354.6014	A2pr(Nma)GRDNAFPK(Dnp)r	1445.50	1444.6596			
VKNAFPK(Dnp)rr	1281.42	1280.6738	GFPNAFPK(Dnp)rr	1355.46	1354.6531	A2pr(Nma)GFKNAFPK(Dnp)r	1449.57	1448.6949			