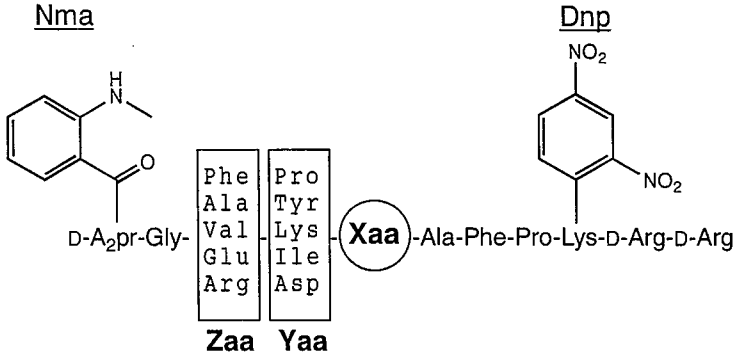
FRETS-25Xaa Series

* 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 3719-v

FRETS-25Val Component-masses

FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEDVA	708.72	708.3079	A2pr(Nma)GVYVAF	873.99	873.4385
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVIV	605.73	605.3537	A2pr(Nma)GFPVA	708.80	708.3595	A2pr(Nma)GAPVAFP	877.00	876.4494
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVDV	607.66	607.2966	A2pr(Nma)GRYV	712.80	712.3657	A2pr(Nma)GRIVAF	881.03	880.4919
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GRPVA	717.82	717.3922	A2pr(Nma)GRDVAF	882.96	882.4348
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GEPV	619.67	619.2966	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GFKVAF	887.04	886.4701
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GVKV	620.74	620.3646	A2pr(Nma)GEKVA	721.80	721.3759	A2pr(Nma)GAIVAFP	893.04	892.4807
A2pr(Nma)GAP	462.50	462.2227	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GFIVA	724.85	724.3908	A2pr(Nma)GADVAFP	894.97	894.4236
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYV	627.69	627.3017	A2pr(Nma)GFDVA	726.78	726.3337	A2pr(Nma)GRKVAF	896.05	895.5028
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPVA	632.71	632.3282	A2pr(Nma)GVYVA	726.82	726.3701	A2pr(Nma)GEYVAF	903.98	903.4127
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GEIV	635.71	635.3279	A2pr(Nma)GRIVA	733.86	733.4235	A2pr(Nma)GVPVAFP	905.05	904.4807
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDV	637.64	637.2708	A2pr(Nma)GRDVA	735.79	735.3664	A2pr(Nma)GAKVAFP	908.05	907.4916
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPV	637.73	637.3224	A2pr(Nma)GFKVA	739.86	739.4017	A2pr(Nma)GVIVAFP	921.09	920.5120
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPV	646.74	646.3551	A2pr(Nma)GRKVA	748.87	748.4344	A2pr(Nma)GFYVAF	922.04	921.4385
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAIVA	648.75	648.3595	A2pr(Nma)GEYVA	756.80	756.3443	A2pr(Nma)GVDVAFP	923.02	922.4549
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADVA	650.68	650.3024	A2pr(Nma)GFYVA	774.86	774.3701	A2pr(Nma)GRYVAF	931.05	930.4712
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKV	650.72	650.3388	A2pr(Nma)GAPVAF	779.88	779.3966	A2pr(Nma)GEPVAFP	935.03	934.4549
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GFIV	653.77	653.3537	A2pr(Nma)GRYVA	783.87	783.4028	A2pr(Nma)GVKVAFP	936.11	935.5229
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFDV	655.70	655.2966	A2pr(Nma)GAIVAF	795.92	795.4279	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GVYV	655.74	655.3330	A2pr(Nma)GADVAF	797.85	797.3708	A2pr(Nma)GAYVAFP	943.06	942.4600
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVPVA	660.76	660.3595	A2pr(Nma)GVPVAF	807.94	807.4279	A2pr(Nma)GEIVAFP	951.08	950.4862
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GRIV	662.78	662.3864	A2pr(Nma)GAKVAF	810.94	810.4388	A2pr(Nma)GEDVAFP	953.01	952.4290
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GAKVA	663.77	663.3704	A2pr(Nma)GVIVAF	823.98	823.4592	A2pr(Nma)GFPVAFP	953.09	952.4807
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GRDV	664.71	664.3293	A2pr(Nma)GVDVAF	825.91	825.4021	A2pr(Nma)GRPVAFP	962.11	961.5134
A2pr(Nma)GVY	556.61	556.2645	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEPVAF	837.92	837.4021	A2pr(Nma)GEKVAFP	966.09	965.4971
A2pr(Nma)GAPV	561.63	561.2911	A2pr(Nma)GFKV	668.78	668.3646	A2pr(Nma)GVKVAF	838.99	838.4701	A2pr(Nma)GFIVAFP	969.14	968.5120
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GVIVA	676.80	676.3908	A2pr(Nma)GAYVAF	845.94	845.4072	A2pr(Nma)GFDVAFP	971.07	970.4549
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GRKV	677.80	677.3973	A2pr(Nma)GEIVAF	853.96	853.4334	A2pr(Nma)GVYVAFP	971.11	970.4913
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVDVA	678.73	678.3337	A2pr(Nma)GEDVAF	855.89	855.3763	A2pr(Nma)GRIVAFP	978.15	977.5447
A2pr(Nma)GAIV	577.67	577.3224	A2pr(Nma)GEYV	685.72	685.3071	A2pr(Nma)GFPVAF	855.98	855.4279	A2pr(Nma)GRDVAFP	980.08	979.4876
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GEPVA	690.74	690.3337	A2pr(Nma)GRPVAF	864.99	864.4606	A2pr(Nma)GFKVAFP	984.15	983.5229
A2pr(Nma)GADV	579.60	579.2653	A2pr(Nma)GVKVA	691.82	691.4017	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GRKVAFP	993.16	992.5556
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GAYVA	698.77	698.3388	A2pr(Nma)GEKVAF	868.98	868.4443	A2pr(Nma)GEYVAFP	1001.09	1000.4654
A2pr(Nma)GVPV	589.68	589.3224	A2pr(Nma)GFYV	703.78	703.3330	A2pr(Nma)GFIVAF	872.02	871.4592	A2pr(Nma)GFYVAFP	1019.15	1018.4913
A2pr(Nma)GAKV	592.69	592.3333	A2pr(Nma)GEIVA	706.79	706.3650	A2pr(Nma)GFDVAF	873.95	873.4021	A2pr(Nma)GRYVAFP	1028.16	1027.5240

FRETS-25Val Component-masses

FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic	FRETS-25Val	Average	Monoisotopic
VAFPK(Dnp)rr	1039.15	1038.5359	A2pr(Nma)GRIVAFPK(Dnp)	1272.41	1271.6411	A2pr(Nma)GAIVAFPK(Dnp)r	1343.49	1342.6782	A2pr(Nma)GRKVAFPK(Dnp)r	1443.61	1442.7531
PVAFPK(Dnp)rr	1136.26	1135.5887	AYVAFPK(Dnp)rr	1273.40	1272.6364	A2pr(Nma)GADVAFPK(Dnp)r	1345.42	1344.6211	A2pr(Nma)GEYVAFPK(Dnp)r	1451.54	1450.6630
IVAFPK(Dnp)rr	1152.31	1151.6200	A2pr(Nma)GRDVAFPK(Dnp)	1274.34	1273.5840	FYVAFPK(Dnp)rr	1349.50	1348.6677	A2pr(Nma)GFYVAFPK(Dnp)r	1469.60	1468.6888
DVAFPK(Dnp)rr	1154.24	1153.5629	A2pr(Nma)GFKVAFPK(Dnp)	1278.41	1277.6193	GRPVAFPK(Dnp)rr	1349.50	1348.7113	A2pr(Nma)GRYVAFPK(Dnp)r	1478.61	1477.7215
KVAFPK(Dnp)rr	1167.32	1166.6309	GAIVAFPK(Dnp)rr	1280.43	1279.6786	GEKVAFPK(Dnp)rr	1353.49	1352.6949	A2pr(Nma)GAPVAFPK(Dnp)rr	1483.63	1482.7480
A2pr(Nma)GAPVAFPK(Dnp)	1171.26	1170.5458	EIVAFPK(Dnp)rr	1281.42	1280.6626	A2pr(Nma)GVPVAFPK(Dnp)r	1355.50	1354.6782	A2pr(Nma)GAIVAFPK(Dnp)rr	1499.67	1498.7793
A2pr(Nma)GAIVAFPK(Dnp)	1187.30	1186.5771	GADVAFPK(Dnp)rr	1282.36	1281.6214	GFIVAFPK(Dnp)rr	1356.53	1355.7099	A2pr(Nma)GADVAFPK(Dnp)rr	1501.60	1500.7222
A2pr(Nma)GADVAFPK(Dnp)	1189.23	1188.5200	EDVAFPK(Dnp)rr	1283.35	1282.6054	GFDVAFPK(Dnp)rr	1358.46	1357.6527	A2pr(Nma)GVPVAFPK(Dnp)rr	1511.69	1510.7793
A2pr(Nma)GVPVAFPK(Dnp)	1199.31	1198.5771	FPVAFPK(Dnp)rr	1283.44	1282.6571	A2pr(Nma)GAKVAFPK(Dnp)r	1358.50	1357.6891	A2pr(Nma)GAKVAFPK(Dnp)rr	1514.69	1513.7902
A2pr(Nma)GAKVAFPK(Dnp)	1202.32	1201.5880	A2pr(Nma)GRKVAFPK(Dnp)	1287.43	1286.6520	GVYVAFPK(Dnp)rr	1358.50	1357.6891	A2pr(Nma)GVIVAFPK(Dnp)rr	1527.73	1526.8106
YVAFPK(Dnp)rr	1202.32	1201.5992	GVPVAFPK(Dnp)rr	1292.45	1291.6786	RYVAFPK(Dnp)rr	1358.51	1357.7004	A2pr(Nma)GVDVAFPK(Dnp)rr	1529.66	1528.7535
APVAFPK(Dnp)rr	1207.34	1206.6258	RPVAFPK(Dnp)rr	1292.45	1291.6898	GRIVAFPK(Dnp)rr	1365.54	1364.7426	A2pr(Nma)GEPVAFPK(Dnp)rr	1541.67	1540.7535
A2pr(Nma)GVIVAFPK(Dnp)	1215.36	1214.6084	A2pr(Nma)GEYVAFPK(Dnp)	1295.36	1294.5619	GRDVAFPK(Dnp)rr	1367.47	1366.6854	A2pr(Nma)GVKVAFPK(Dnp)rr	1542.74	1541.8215
A2pr(Nma)GVDVAFPK(Dnp)	1217.29	1216.5513	GAKVAFPK(Dnp)rr	1295.45	1294.6895	A2pr(Nma)GVIVAFPK(Dnp)r	1371.54	1370.7095	A2pr(Nma)GAYVAFPK(Dnp)rr	1549.69	1548.7586
AIVAFPK(Dnp)rr	1223.38	1222.6571	EKVAFPK(Dnp)rr	1296.43	1295.6735	GFKVAFPK(Dnp)rr	1371.55	1370.7208	A2pr(Nma)GEIVAFPK(Dnp)rr	1557.71	1556.7848
ADVAFPK(Dnp)rr	1225.31	1224.6000	FIVAFPK(Dnp)rr	1299.48	1298.6884	A2pr(Nma)GVDVAFPK(Dnp)r	1373.47	1372.6524	A2pr(Nma)GEDVAFPK(Dnp)rr	1559.64	1558.7277
A2pr(Nma)GEPVAFPK(Dnp)	1229.30	1228.5513	FDVAFPK(Dnp)rr	1301.41	1300.6313	GRKVAFPK(Dnp)rr	1380.56	1379.7534	A2pr(Nma)GFPVAFPK(Dnp)rr	1559.73	1558.7793
A2pr(Nma)GVKVAFPK(Dnp)	1230.37	1229.6193	VYVAFPK(Dnp)rr	1301.45	1300.6677	A2pr(Nma)GEPVAFPK(Dnp)r	1385.48	1384.6524	A2pr(Nma)GRPVAFPK(Dnp)rr	1568.74	1567.8120
VPVAFPK(Dnp)rr	1235.39	1234.6571	GVIVAFPK(Dnp)rr	1308.49	1307.7099	A2pr(Nma)GVKVAFPK(Dnp)r	1386.56	1385.7204	A2pr(Nma)GEKVAFPK(Dnp)rr	1572.73	1571.7957
A2pr(Nma)GAYVAFPK(Dnp)	1237.32	1236.5564	RIVAFPK(Dnp)rr	1308.49	1307.7211	GEYVAFPK(Dnp)rr	1388.49	1387.6633	A2pr(Nma)GFIVAFPK(Dnp)rr	1575.77	1574.8106
AKVAFPK(Dnp)rr	1238.40	1237.6680	GVDVAFPK(Dnp)rr	1310.42	1309.6527	A2pr(Nma)GAYVAFPK(Dnp)r	1393.50	1392.6575	A2pr(Nma)GFDVAFPK(Dnp)rr	1577.70	1576.7535
A2pr(Nma)GEIVAFPK(Dnp)	1245.34	1244.5826	RDVAFPK(Dnp)rr	1310.42	1309.6640	A2pr(Nma)GEIVAFPK(Dnp)r	1401.53	1400.6837	A2pr(Nma)GVYVAFPK(Dnp)rr	1577.74	1576.7899
A2pr(Nma)GEDVAFPK(Dnp)	1247.27	1246.5255	A2pr(Nma)GFYVAFPK(Dnp)	1313.41	1312.5877	A2pr(Nma)GEDVAFPK(Dnp)r	1403.45	1402.6266	A2pr(Nma)GRIVAFPK(Dnp)rr	1584.78	1583.8433
A2pr(Nma)GFPVAFPK(Dnp)	1247.36	1246.5771	FKVAFPK(Dnp)rr	1314.49	1313.6993	A2pr(Nma)GFPVAFPK(Dnp)r	1403.54	1402.6782	A2pr(Nma)GRDVAFPK(Dnp)rr	1586.71	1585.7862
VIVAFPK(Dnp)rr	1251.44	1250.6884	A2pr(Nma)GRYVAFPK(Dnp)	1322.43	1321.6204	GFYVAFPK(Dnp)rr	1406.55	1405.6891	A2pr(Nma)GFKVAFPK(Dnp)rr	1590.79	1589.8215
VDVAFPK(Dnp)rr	1253.37	1252.6313	GEPVAFPK(Dnp)rr	1322.43	1321.6527	A2pr(Nma)GRPVAFPK(Dnp)r	1412.55	1411.7109	A2pr(Nma)GRKVAFPK(Dnp)rr	1599.80	1598.8542
A2pr(Nma)GRPVAFPK(Dnp)	1256.37	1255.6098	GVKVAFPK(Dnp)rr	1323.50	1322.7208	GRYVAFPK(Dnp)rr	1415.56	1414.7218	A2pr(Nma)GEYVAFPK(Dnp)rr	1607.73	1606.7641
A2pr(Nma)GEKVAFPK(Dnp)	1260.35	1259.5935	RKVAFPK(Dnp)rr	1323.51	1322.7320	A2pr(Nma)GEKVAFPK(Dnp)r	1416.54	1415.6946	A2pr(Nma)GFYVAFPK(Dnp)rr	1625.79	1624.7899
A2pr(Nma)GFIVAFPK(Dnp)	1263.40	1262.6084	A2pr(Nma)GAPVAFPK(Dnp)r	1327.45	1326.6469	A2pr(Nma)GFIVAFPK(Dnp)r	1419.58	1418.7095	A2pr(Nma)GRYVAFPK(Dnp)rr	1634.80	1633.8226
GAPVAFPK(Dnp)rr	1264.39	1263.6473	GAYVAFPK(Dnp)rr	1330.45	1329.6578	A2pr(Nma)GFDVAFPK(Dnp)r	1421.51	1420.6524			
A2pr(Nma)GFDVAFPK(Dnp)	1265.33	1264.5513	EYVAFPK(Dnp)rr	1331.44	1330.6418	A2pr(Nma)GVYVAFPK(Dnp)r	1421.56	1420.6888			
A2pr(Nma)GVYVAFPK(Dnp)	1265.37	1264.5877	GEIVAFPK(Dnp)rr	1338.47	1337.6840	A2pr(Nma)GRIVAFPK(Dnp)r	1428.60	1427.7422			
EPVAFPK(Dnp)rr	1265.38	1264.6313	GEDVAFPK(Dnp)rr	1340.40	1339.6269	A2pr(Nma)GRDVAFPK(Dnp)r	1430.53	1429.6851			
VKVAFPK(Dnp)rr	1266.45	1265.6993	GFPVAFPK(Dnp)rr	1340.49	1339.6786	A2pr(Nma)GFKVAFPK(Dnp)r	1434.60	1433.7204			