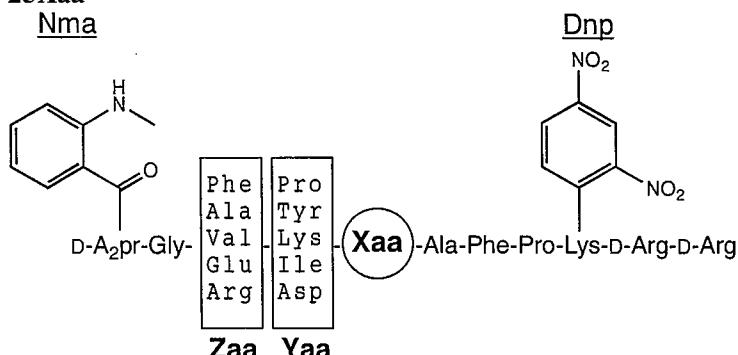# FRETS-25Xaa Series

* 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.

FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GEPG	577.59	577.2496	Ac-K(Dnp)rr	666.69	666.3126	A2pr(Nma)GAPGAFP	834.92	834.4024
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVKG	578.66	578.3176	A2pr(Nma)GFPGA	666.72	666.3198	A2pr(Nma)GRIGAF	838.95	838.4450
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GRYG	670.72	670.3187	A2pr(Nma)GRDGAF	840.88	840.3879
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GAYG	585.61	585.2547	A2pr(Nma)GRPGA	675.74	675.3453	A2pr(Nma)GFKGAF	844.96	844.4232
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GEKGA	679.72	679.3289	A2pr(Nma)GAIGAFP	850.96	850.4337
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GAPGA	590.63	590.2813	A2pr(Nma)GFIGA	682.77	682.3439	A2pr(Nma)GADGAFP	852.89	852.3766
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GEIG	593.63	593.2809	A2pr(Nma)GFDGA	684.70	684.2867	A2pr(Nma)GRKGAF	853.97	853.4559
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GEDG	595.56	595.2238	A2pr(Nma)GVYGA	684.74	684.3231	A2pr(Nma)GEYGAF	861.90	861.3657
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GFPG	595.65	595.2754	A2pr(Nma)GRIGA	691.78	691.3766	A2pr(Nma)GVPGAFP	862.97	862.4337
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GRDGA	693.71	693.3194	A2pr(Nma)GAKGAFP	865.97	865.4446
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GRPG	604.66	604.3081	A2pr(Nma)GFKGA	697.78	697.3548	FPK(Dnp)rr	868.94	868.4304
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GAIGA	606.67	606.3126	A2pr(Nma)GRKGA	706.79	706.3875	A2pr(Nma)GVIGAFP	879.01	878.4650
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GADGA	608.60	608.2554	A2pr(Nma)GEYGA	714.72	714.2973	A2pr(Nma)GFYGAF	879.96	879.3915
A2pr(Nma)GAPG	519.55	519.2441	A2pr(Nma)GEKG	608.64	608.2918	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GVDGAFP	880.94	880.4079
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GFIG	611.69	611.3067	A2pr(Nma)GFYGA	732.78	732.3231	A2pr(Nma)GRYGAF	888.97	888.4242
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GFDG	613.62	613.2496	A2pr(Nma)GAPGAF	737.80	737.3497	A2pr(Nma)GEPGAFP	892.95	892.4079
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GVYG	613.66	613.2860	A2pr(Nma)GRYGA	741.79	741.3558	A2pr(Nma)GVKGAFP	894.03	893.4759
A2pr(Nma)GAIG	535.59	535.2754	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GAIGAF	753.85	753.3810	A2pr(Nma)GAYGAFP	900.98	900.4130
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GVPGA	618.68	618.3126	A2pr(Nma)GADGAF	755.77	755.3239	A2pr(Nma)GEIGAFP	909.00	908.4392
A2pr(Nma)GADG	537.52	537.2183	A2pr(Nma)GRIG	620.70	620.3394	A2pr(Nma)GVPGAF	765.86	765.3810	A2pr(Nma)GEDGAFP	910.93	910.3821
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GAKGA	621.69	621.3235	A2pr(Nma)GAKGAF	768.86	768.3919	A2pr(Nma)GFPGAFP	911.01	910.4337
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GRDG	622.63	622.2823	A2pr(Nma)GVIGAF	781.90	781.4123	A2pr(Nma)GRPGAFP	920.03	919.4664
A2pr(Nma)GVPG	547.60	547.2754	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GVDGAF	783.83	783.3552	A2pr(Nma)GEKGAFP	924.01	923.4501
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GFKG	626.70	626.3176	A2pr(Nma)GEPGAF	795.84	795.3552	A2pr(Nma)GFIGAFP	927.06	926.4650
A2pr(Nma)GAKG	550.61	550.2863	A2pr(Nma)GVIGA	634.72	634.3439	A2pr(Nma)GVKGAF	796.91	796.4232	A2pr(Nma)GFDGAFP	928.99	928.4079
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GRKG	635.72	635.3503	A2pr(Nma)GAYGAF	803.86	803.3602	A2pr(Nma)GVYGAFP	929.03	928.4443
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GVDGA	636.65	636.2867	A2pr(Nma)GEIGAF	811.88	811.3865	A2pr(Nma)GRIGAFP	936.07	935.4977
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GEYG	643.64	643.2602	A2pr(Nma)GEDGAF	813.81	813.3293	A2pr(Nma)GRDGAFP	938.00	937.4406
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GEPGA	648.66	648.2867	A2pr(Nma)GFPGAF	813.90	813.3810	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GVIG	563.65	563.3067	A2pr(Nma)GVKGA	649.74	649.3548	A2pr(Nma)GRPGAF	822.91	822.4137	A2pr(Nma)GFKGAFP	942.07	941.4759
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GAYGA	656.69	656.2918	A2pr(Nma)GEKGAF	826.90	826.3974	A2pr(Nma)GRKGAFP	951.08	950.5086
A2pr(Nma)GVDG	565.58	565.2496	A2pr(Nma)GFYG	661.70	661.2860	A2pr(Nma)GFIGAF	829.94	829.4123	A2pr(Nma)GEYGAFP	959.01	958.4185
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GEIGA	664.71	664.3180	A2pr(Nma)GFDGAF	831.87	831.3552	A2pr(Nma)GFYGAFP	977.07	976.4443
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GEDGA	666.64	666.2609	A2pr(Nma)GVYGAF	831.91	831.3915	A2pr(Nma)GRYGAFP	986.08	985.4770

FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic	FRETS-25Gly	Average	Monoisotopic
GAFPK(Dnp)rr	997.07	996.4890	A2pr(Nma)GRIGAFPK(Dnp)	1230.33	1229.5942	A2pr(Nma)GAIGAFPK(Dnp)r	1301.41	1300.6313	A2pr(Nma)GRKGAFPK(Dnp)r	1401.53	1400.7062
PGAFPK(Dnp)rr	1094.18	1093.5417	AYGAFPK(Dnp)rr	1231.32	1230.5894	A2pr(Nma)GADGAFPK(Dnp)r	1303.34	1302.5741	A2pr(Nma)GEYGAFPK(Dnp)r	1409.46	1408.6160
IGAFPK(Dnp)rr	1110.23	1109.5730	A2pr(Nma)GRDGAFPK(Dnp)	1232.26	1231.5370	FYGAFPK(Dnp)rr	1307.42	1306.6207	A2pr(Nma)GFYGAFPK(Dnp)r	1427.52	1426.6418
DGAFPK(Dnp)rr	1112.16	1111.5159	A2pr(Nma)GFKGAFPK(Dnp)	1236.33	1235.5724	GRPGAFPK(Dnp)rr	1307.42	1306.6643	A2pr(Nma)GRYGAFPK(Dnp)r	1436.53	1435.6745
KGAFPK(Dnp)rr	1125.24	1124.5839	GAIGAFPK(Dnp)rr	1238.36	1237.6316	GEKGAFPK(Dnp)rr	1311.41	1310.6480	A2pr(Nma)GAPGAFPK(Dnp)rr	1441.55	1440.7011
A2pr(Nma)GAPGAFPK(Dnp)	1129.18	1128.4989	EIGAFPK(Dnp)rr	1239.34	1238.6156	A2pr(Nma)GVPGAFPK(Dnp)r	1313.42	1312.6313	A2pr(Nma)GAIGAFPK(Dnp)rr	1457.59	1456.7324
A2pr(Nma)GAIGAFPK(Dnp)	1145.22	1144.5302	GADGAFPK(Dnp)rr	1240.28	1239.5745	GFIGAFPK(Dnp)rr	1314.45	1313.6629	A2pr(Nma)GADGAFPK(Dnp)rr	1459.52	1458.6753
A2pr(Nma)GADGAFPK(Dnp)	1147.15	1146.4730	EDGAFPK(Dnp)rr	1241.27	1240.5585	GFDGAFPK(Dnp)rr	1316.38	1315.6058	A2pr(Nma)GVPGAFPK(Dnp)rr	1469.61	1468.7324
A2pr(Nma)GVPGAFPK(Dnp)	1157.23	1156.5302	FPGAFPK(Dnp)rr	1241.36	1240.6101	GVYGAFPK(Dnp)rr	1316.42	1315.6422	A2pr(Nma)GAKGAFPK(Dnp)rr	1472.61	1471.7433
A2pr(Nma)GAKGAFPK(Dnp)	1160.24	1159.5411	A2pr(Nma)GRKGAFPK(Dnp)	1245.35	1244.6051	A2pr(Nma)GAKGAFPK(Dnp)r	1316.42	1315.6422	A2pr(Nma)GVIGAFPK(Dnp)rr	1485.65	1484.7637
YGAFPK(Dnp)rr	1160.24	1159.5523	GVPGAFPK(Dnp)rr	1250.37	1249.6316	RYGAFPK(Dnp)rr	1316.43	1315.6534	A2pr(Nma)GVDGAFPK(Dnp)rr	1487.58	1486.7066
APGAFPK(Dnp)rr	1165.26	1164.5788	RPGAFPK(Dnp)rr	1250.37	1249.6428	GRIGAFPK(Dnp)rr	1323.46	1322.6956	A2pr(Nma)GEPGAFPK(Dnp)rr	1499.59	1498.7066
A2pr(Nma)GVIGAFPK(Dnp)	1173.28	1172.5615	A2pr(Nma)GEYGAFPK(Dnp)	1253.28	1252.5149	GRDGAFPK(Dnp)rr	1325.39	1324.6385	A2pr(Nma)GVKGAFPK(Dnp)rr	1500.66	1499.7746
A2pr(Nma)GVDGAFPK(Dnp)	1175.21	1174.5043	GAKGAFPK(Dnp)rr	1253.37	1252.6425	A2pr(Nma)GVIGAFPK(Dnp)r	1329.46	1328.6626	A2pr(Nma)GAYGAFPK(Dnp)rr	1507.61	1506.7116
AIGAFPK(Dnp)rr	1181.30	1180.6101	EKGAFPK(Dnp)rr	1254.35	1253.6265	GFKGAFPK(Dnp)rr	1329.47	1328.6738	A2pr(Nma)GEIGAFPK(Dnp)rr	1515.63	1514.7379
ADGAFPK(Dnp)rr	1183.23	1182.5530	FIGAFPK(Dnp)rr	1257.40	1256.6414	A2pr(Nma)GVDGAFPK(Dnp)r	1331.39	1330.6054	A2pr(Nma)GEDGAFPK(Dnp)rr	1517.56	1516.6807
A2pr(Nma)GEPGAFPK(Dnp)	1187.22	1186.5043	FDGAFPK(Dnp)rr	1259.33	1258.5843	GRKGAFPK(Dnp)rr	1338.48	1337.7065	A2pr(Nma)GFPGAFPK(Dnp)rr	1517.65	1516.7324
A2pr(Nma)GVKGAFPK(Dnp)	1188.29	1187.5724	VYGAFPK(Dnp)rr	1259.37	1258.6207	A2pr(Nma)GEPGAFPK(Dnp)r	1343.40	1342.6054	A2pr(Nma)GRPGAFPK(Dnp)rr	1526.66	1525.7651
VPGAFPK(Dnp)rr	1193.31	1192.6101	GVIGAFPK(Dnp)rr	1266.41	1265.6629	A2pr(Nma)GVKGAFPK(Dnp)r	1344.48	1343.6735	A2pr(Nma)GEKGAFPK(Dnp)rr	1530.65	1529.7488
A2pr(Nma)GAYGAFPK(Dnp)	1195.24	1194.5094	RIGAFPK(Dnp)rr	1266.41	1265.6741	GEYGAFPK(Dnp)rr	1346.41	1345.6163	A2pr(Nma)GFIGAFPK(Dnp)rr	1533.69	1532.7637
AKGAFPK(Dnp)rr	1196.32	1195.6210	GVDGAFPK(Dnp)rr	1268.34	1267.6058	A2pr(Nma)GAYGAFPK(Dnp)r	1351.42	1350.6105	A2pr(Nma)GFDGAFPK(Dnp)rr	1535.62	1534.7066
A2pr(Nma)GEIGAFPK(Dnp)	1203.26	1202.5356	RDGAFPK(Dnp)rr	1268.34	1267.6170	A2pr(Nma)GEIGAFPK(Dnp)r	1359.45	1358.6367	A2pr(Nma)GVYGAFPK(Dnp)rr	1535.66	1534.7429
A2pr(Nma)GEDGAFPK(Dnp)	1205.19	1204.4785	A2pr(Nma)GFYGAFPK(Dnp)	1271.34	1270.5407	A2pr(Nma)GEDGAFPK(Dnp)r	1361.38	1360.5796	A2pr(Nma)GRIGAFPK(Dnp)rr	1542.70	1541.7964
A2pr(Nma)GFPGAFPK(Dnp)	1205.28	1204.5302	FKGAFPK(Dnp)rr	1272.41	1271.6523	A2pr(Nma)GFPGAFPK(Dnp)r	1361.46	1360.6313	A2pr(Nma)GRDGAFPK(Dnp)rr	1544.63	1543.7393
VIGAFPK(Dnp)rr	1209.36	1208.6414	A2pr(Nma)GRYGAFPK(Dnp)	1280.35	1279.5734	GFYGAFPK(Dnp)rr	1364.47	1363.6422	A2pr(Nma)GFKGAFPK(Dnp)rr	1548.71	1547.7746
VDGAFPK(Dnp)rr	1211.29	1210.5843	GEPGAFPK(Dnp)rr	1280.35	1279.6058	A2pr(Nma)GRPGAFPK(Dnp)r	1370.47	1369.6640	A2pr(Nma)GRKGAFPK(Dnp)rr	1557.72	1556.8073
A2pr(Nma)GRPGAFPK(Dnp)	1214.29	1213.5629	GVKGAFPK(Dnp)rr	1281.42	1280.6738	GRYGAFPK(Dnp)rr	1373.48	1372.6749	A2pr(Nma)GEYGAFPK(Dnp)rr	1565.65	1564.7171
A2pr(Nma)GEKGAFPK(Dnp)	1218.27	1217.5465	RKGAFPK(Dnp)rr	1281.43	1280.6850	A2pr(Nma)GEKGAFPK(Dnp)r	1374.46	1373.6476	A2pr(Nma)GFYGAFPK(Dnp)rr	1583.71	1582.7429
A2pr(Nma)GFIGAFPK(Dnp)	1221.32	1220.5615	A2pr(Nma)GAPGAFPK(Dnp)r	1285.37	1284.6000	A2pr(Nma)GFIGAFPK(Dnp)r	1377.51	1376.6626	A2pr(Nma)GRYGAFPK(Dnp)rr	1592.72	1591.7756
GAPGAFPK(Dnp)rr	1222.31	1221.6003	GAYGAFPK(Dnp)rr	1288.37	1287.6109	A2pr(Nma)GFDGAFPK(Dnp)r	1379.43	1378.6054			
A2pr(Nma)GFDGAFPK(Dnp)	1223.25	1222.5043	EYGAFPK(Dnp)rr	1289.36	1288.5949	A2pr(Nma)GVYGAFPK(Dnp)r	1379.48	1378.6418			
A2pr(Nma)GVYGAFPK(Dnp)	1223.29	1222.5407	GEIGAFPK(Dnp)rr	1296.39	1295.6371	A2pr(Nma)GRIGAFPK(Dnp)r	1386.52	1385.6953			
EPGAFPK(Dnp)rr	1223.30	1222.5843	GEDGAFPK(Dnp)rr	1298.32	1297.5800	A2pr(Nma)GRDGAFPK(Dnp)r	1388.45	1387.6381			
VKGAFPK(Dnp)rr	1224.37	1223.6523	GFPGAFPK(Dnp)rr	1298.41	1297.6316	A2pr(Nma)GFKGAFPK(Dnp)r	1392.52	1391.6735			