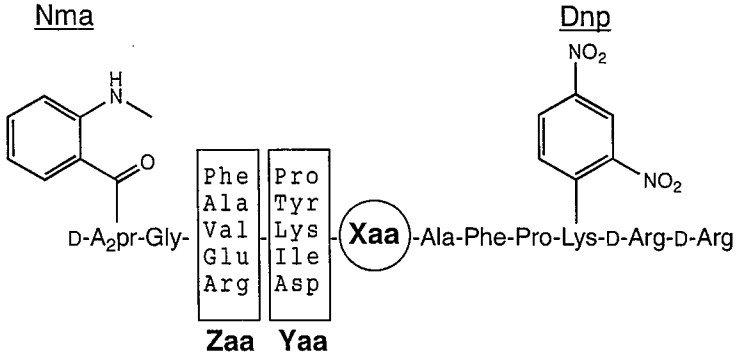
FRETS-25Xaa Series

* 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 3712-v

FRETS-25Met Component-masses

FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GVPM	621.75	621.2945	A2pr(Nma)GEIMA	738.85	738.3371	A2pr(Nma)GVYMAF	906.06	905.4106
A2pr(Nma)GA	365.38	365.1699	K(Dnp)rr	624.65	624.3054	A2pr(Nma)GEDMA	740.78	740.2799	A2pr(Nma)GAPMAFP	909.06	908.4215
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GAKM	624.75	624.3092	A2pr(Nma)GFPMA	740.87	740.3316	A2pr(Nma)GRIMAF	913.10	912.4640
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GVIM	637.79	637.3258	A2pr(Nma)GRYM	744.86	744.3377	A2pr(Nma)GRDMAF	915.03	914.4069
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVDM	639.72	639.2686	A2pr(Nma)GRPMA	749.88	749.3643	A2pr(Nma)GFKMAF	919.10	918.4422
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GEPM	651.73	651.2686	A2pr(Nma)GEKMA	753.87	753.3480	A2pr(Nma)GAIMAFP	925.10	924.4528
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GVKM	652.81	652.3367	A2pr(Nma)GFIMA	756.91	756.3629	A2pr(Nma)GADMAFP	927.03	926.3956
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYM	659.75	659.2737	A2pr(Nma)GFDMA	758.84	758.3058	A2pr(Nma)GRKMAF	928.11	927.4749
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPMA	664.77	664.3003	A2pr(Nma)GVYMA	758.88	758.3421	A2pr(Nma)GEYMAF	936.04	935.3847
A2pr(Nma)GVP	490.55	490.2540	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GRIMA	765.92	765.3956	A2pr(Nma)GVPMAFP	937.12	936.4528
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEIM	667.77	667.2999	A2pr(Nma)GRDMA	767.85	767.3385	AFPK(Dnp)rr	940.02	939.4637
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GEDM	669.70	669.2428	A2pr(Nma)GFKMA	771.93	771.3738	A2pr(Nma)GAKMAFP	940.12	939.4675
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GFPM	669.79	669.2945	A2pr(Nma)GRKMA	780.94	780.4065	A2pr(Nma)GVIMAFP	953.16	952.4841
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GRPM	678.80	678.3272	A2pr(Nma)GEYMA	788.87	788.3163	A2pr(Nma)GFYMAF	954.10	953.4106
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GAIMA	680.82	680.3316	A2pr(Nma)GFYMA	806.93	806.3421	A2pr(Nma)GVDMAFP	955.09	954.4269
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GADMA	682.75	682.2745	A2pr(Nma)GAPMAF	811.95	811.3687	A2pr(Nma)GRYMAF	963.11	962.4433
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GEKM	682.79	682.3108	A2pr(Nma)GRYMA	815.94	815.3748	A2pr(Nma)GEPMAFP	967.10	966.4269
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFIM	685.83	685.3258	A2pr(Nma)GAIMAF	827.99	827.4000	A2pr(Nma)GVKMAFP	968.17	967.4950
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GFDM	687.76	687.2686	A2pr(Nma)GADMAF	829.92	829.3429	A2pr(Nma)GAYMAFP	975.12	974.4320
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVYM	687.81	687.3050	A2pr(Nma)GVPMAF	840.00	839.4000	A2pr(Nma)GEIMAFP	983.14	982.4582
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GVPMA	692.83	692.3316	A2pr(Nma)GAKMAF	843.00	842.4109	A2pr(Nma)GEDMAFP	985.07	984.4011
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRIM	694.85	694.3585	A2pr(Nma)GVIMAF	856.04	855.4313	A2pr(Nma)GFPMAFP	985.16	984.4528
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GAKMA	695.83	695.3425	A2pr(Nma)GVDMAF	857.97	857.3742	A2pr(Nma)GRPMAFP	994.17	993.4855
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GRDM	696.78	696.3013	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GEKMAFP	998.16	997.4691
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GFKM	700.85	700.3367	A2pr(Nma)GEPMAF	869.98	869.3742	A2pr(Nma)GFIMAFP	1001.20	1000.4841
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GVIMA	708.87	708.3629	A2pr(Nma)GVKMAF	871.06	870.4422	A2pr(Nma)GFDMAFP	1003.13	1002.4269
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GRKM	709.86	709.3694	A2pr(Nma)GAYMAF	878.01	877.3793	A2pr(Nma)GVYMAFP	1003.17	1002.4633
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GVDMA	710.80	710.3058	A2pr(Nma)GEIMAF	886.03	885.4055	A2pr(Nma)GRIMAFP	1010.21	1009.5168
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GEYM	717.79	717.2792	A2pr(Nma)GEDMAF	887.96	887.3484	A2pr(Nma)GRDMAFP	1012.14	1011.4596
A2pr(Nma)GAPM	593.70	593.2632	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GFPMAF	888.04	887.4000	A2pr(Nma)GFKMAFP	1016.22	1015.4950
A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEPMA	722.81	722.3058	A2pr(Nma)GRPMAF	897.06	896.4327	A2pr(Nma)GRKMAFP	1025.23	1024.5277
A2pr(Nma)GAIM	609.74	609.2945	A2pr(Nma)GVKMA	723.88	723.3738	A2pr(Nma)GEKMAF	901.04	900.4164	A2pr(Nma)GEYMAFP	1033.16	1032.4375
A2pr(Nma)GADM	611.67	611.2373	A2pr(Nma)GAYMA	730.83	730.3108	A2pr(Nma)GFIMAF	904.09	903.4313	A2pr(Nma)GFYMAFP	1051.22	1050.4633
A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GFYM	735.85	735.3050	A2pr(Nma)GFDMAF	906.02	905.3742	A2pr(Nma)GRYMAFP	1060.23	1059.4960

FRETS-25Met Component-masses

FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic	FRETS-25Met	Average	Monoisotopic
MAFPK(Dnp)rr	1071.21	1070.5080	A2pr(Nma)GRIMAFPK(Dnp)	1304.48	1303.6132	A2pr(Nma)GAIMAFPK(Dnp)r	1375.55	1374.6503	A2pr(Nma)GRKMAFPK(Dnp)r	1475.68	1474.7252
PMAFPK(Dnp)rr	1168.33	1167.5607	AYMAFPK(Dnp)rr	1305.46	1304.6084	A2pr(Nma)GADMAFPK(Dnp)r	1377.48	1376.5932	A2pr(Nma)GEYMAFPK(Dnp)r	1483.61	1482.6350
IMAFPK(Dnp)rr	1184.37	1183.5920	A2pr(Nma)GRDMAFPK(Dnp)	1306.41	1305.5561	FYMAFPK(Dnp)rr	1381.56	1380.6397	A2pr(Nma)GFYMAFPK(Dnp)r	1501.67	1500.6609
DMAFPK(Dnp)rr	1186.30	1185.5349	A2pr(Nma)GFKMAFPK(Dnp)	1310.48	1309.5914	GRPMAFPK(Dnp)rr	1381.57	1380.6833	A2pr(Nma)GRYMAFPK(Dnp)r	1510.68	1509.6936
KMAFPK(Dnp)rr	1199.39	1198.6029	GAIMAFPK(Dnp)rr	1312.50	1311.6506	GEKMAFPK(Dnp)rr	1385.55	1384.6670	A2pr(Nma)GAPMAFPK(Dnp)rr	1515.70	1514.7201
A2pr(Nma)GAPMAFPK(Dnp)	1203.33	1202.5179	EIMAFPK(Dnp)rr	1313.48	1312.6346	A2pr(Nma)GVPMAFPK(Dnp)r	1387.56	1386.6503	A2pr(Nma)GAIMAFPK(Dnp)rr	1531.74	1530.7514
A2pr(Nma)GAIMAFPK(Dnp)	1219.37	1218.5492	GADMAFPK(Dnp)rr	1314.43	1313.5935	GFIMAFPK(Dnp)rr	1388.60	1387.6819	A2pr(Nma)GADMAFPK(Dnp)rr	1533.67	1532.6943
A2pr(Nma)GADMAFPK(Dnp)	1221.30	1220.4921	EDMAFPK(Dnp)rr	1315.41	1314.5775	GFDMAFPK(Dnp)rr	1390.53	1389.6248	A2pr(Nma)GVPMAFPK(Dnp)rr	1543.75	1542.7514
A2pr(Nma)GVPMAFPK(Dnp)	1231.38	1230.5492	FPMAFPK(Dnp)rr	1315.50	1314.6292	A2pr(Nma)GAKMAFPK(Dnp)r	1390.57	1389.6612	A2pr(Nma)GAKMAFPK(Dnp)rr	1546.75	1545.7623
A2pr(Nma)GAKMAFPK(Dnp)	1234.38	1233.5601	A2pr(Nma)GRKMAFPK(Dnp)	1319.49	1318.6241	GVYMAFPK(Dnp)rr	1390.57	1389.6612	A2pr(Nma)GVIMAFPK(Dnp)rr	1559.79	1558.7827
YMAFPK(Dnp)rr	1234.39	1233.5713	GVPMAFPK(Dnp)rr	1324.51	1323.6506	RYMAFPK(Dnp)rr	1390.57	1389.6724	A2pr(Nma)GVDMAFPK(Dnp)rr	1561.72	1560.7256
APMAFPK(Dnp)rr	1239.41	1238.5979	RPMAFPK(Dnp)rr	1324.51	1323.6619	GRIMAFPK(Dnp)rr	1397.61	1396.7146	A2pr(Nma)GEPMAFPK(Dnp)rr	1573.73	1572.7256
A2pr(Nma)GVIMAFPK(Dnp)	1247.42	1246.5805	A2pr(Nma)GEYMAFPK(Dnp)	1327.42	1326.5339	GRDMAFPK(Dnp)rr	1399.54	1398.6575	A2pr(Nma)GVKMAFPK(Dnp)rr	1574.81	1573.7936
A2pr(Nma)GVDMAFPK(Dnp)	1249.35	1248.5234	GAKMAFPK(Dnp)rr	1327.51	1326.6615	A2pr(Nma)GVIMAFPK(Dnp)r	1403.61	1402.6816	A2pr(Nma)GAYMAFPK(Dnp)rr	1581.76	1580.7307
AIMAFPK(Dnp)rr	1255.45	1254.6292	EKMAFPK(Dnp)rr	1328.50	1327.6455	GFKMAFPK(Dnp)rr	1403.61	1402.6928	A2pr(Nma)GEIMAFPK(Dnp)rr	1589.78	1588.7569
ADMAFPK(Dnp)rr	1257.38	1256.5720	FIMAFPK(Dnp)rr	1331.54	1330.6605	A2pr(Nma)GVDMAFPK(Dnp)r	1405.54	1404.6245	A2pr(Nma)GEDMAFPK(Dnp)rr	1591.71	1590.6998
A2pr(Nma)GEPMAFPK(Dnp)	1261.36	1260.5234	FDMAFPK(Dnp)rr	1333.47	1332.6033	GRKMAFPK(Dnp)rr	1412.62	1411.7255	A2pr(Nma)GFPMAFPK(Dnp)rr	1591.79	1590.7514
A2pr(Nma)GVKMAFPK(Dnp)	1262.44	1261.5914	VYMAFPK(Dnp)rr	1333.52	1332.6397	A2pr(Nma)GEPMAFPK(Dnp)r	1417.55	1416.6245	A2pr(Nma)GRPMAFPK(Dnp)rr	1600.81	1599.7841
VPMAFPK(Dnp)rr	1267.46	1266.6292	GVIMAFPK(Dnp)rr	1340.55	1339.6819	A2pr(Nma)GVKMAFPK(Dnp)r	1418.62	1417.6925	A2pr(Nma)GEKMAFPK(Dnp)rr	1604.79	1603.7678
A2pr(Nma)GAYMAFPK(Dnp)	1269.38	1268.5284	RIMAFPK(Dnp)rr	1340.56	1339.6932	GEYMAFPK(Dnp)rr	1420.55	1419.6354	A2pr(Nma)GFIMAFPK(Dnp)rr	1607.84	1606.7827
AKMAFPK(Dnp)rr	1270.46	1269.6401	GVDMAFPK(Dnp)rr	1342.48	1341.6248	A2pr(Nma)GAYMAFPK(Dnp)r	1425.57	1424.6296	A2pr(Nma)GFDMAFPK(Dnp)rr	1609.77	1608.7256
A2pr(Nma)GEIMAFPK(Dnp)	1277.40	1276.5547	RDMAFPK(Dnp)rr	1342.49	1341.6360	A2pr(Nma)GEIMAFPK(Dnp)r	1433.59	1432.6558	A2pr(Nma)GVYMAFPK(Dnp)rr	1609.81	1608.7620
A2pr(Nma)GEDMAFPK(Dnp)	1279.33	1278.4975	A2pr(Nma)GFYMAFPK(Dnp)	1345.48	1344.5597	A2pr(Nma)GEDMAFPK(Dnp)r	1435.52	1434.5986	A2pr(Nma)GRIMAFPK(Dnp)rr	1616.85	1615.8154
A2pr(Nma)GFPMAFPK(Dnp)	1279.42	1278.5492	FKMAFPK(Dnp)rr	1346.56	1345.6714	A2pr(Nma)GFPMAFPK(Dnp)r	1435.61	1434.6503	A2pr(Nma)GRDMAFPK(Dnp)rr	1618.78	1617.7583
VIMAFPK(Dnp)rr	1283.50	1282.6605	A2pr(Nma)GRYMAFPK(Dnp)	1354.49	1353.5924	GFYMAFPK(Dnp)rr	1438.61	1437.6612	A2pr(Nma)GFKMAFPK(Dnp)rr	1622.85	1621.7936
VDMAFPK(Dnp)rr	1285.43	1284.6033	GEPMAFPK(Dnp)rr	1354.49	1353.6248	A2pr(Nma)GRPMAFPK(Dnp)r	1444.62	1443.6830	A2pr(Nma)GRKMAFPK(Dnp)rr	1631.86	1630.8263
A2pr(Nma)GRPMAFPK(Dnp)	1288.43	1287.5819	GVKMAFPK(Dnp)rr	1355.57	1354.6928	GRYMAFPK(Dnp)rr	1447.62	1446.6939	A2pr(Nma)GEYMAFPK(Dnp)rr	1639.79	1638.7361
A2pr(Nma)GEKMAFPK(Dnp)	1292.42	1291.5656	RKMAFPK(Dnp)rr	1355.57	1354.7041	A2pr(Nma)GEKMAFPK(Dnp)r	1448.60	1447.6667	A2pr(Nma)GFYMAFPK(Dnp)rr	1657.85	1656.7620
A2pr(Nma)GFIMAFPK(Dnp)	1295.46	1294.5805	A2pr(Nma)GAPMAFPK(Dnp)r	1359.51	1358.6190	A2pr(Nma)GFIMAFPK(Dnp)r	1451.65	1450.6816	A2pr(Nma)GRYMAFPK(Dnp)rr	1666.86	1665.7947
GAPMAFPK(Dnp)rr	1296.46	1295.6193	GAYMAFPK(Dnp)rr	1362.52	1361.6299	A2pr(Nma)GFDMAFPK(Dnp)r	1453.58	1452.6245			
A2pr(Nma)GFDMAFPK(Dnp)	1297.39	1296.5234	EYMAFPK(Dnp)rr	1363.50	1362.6139	A2pr(Nma)GVYMAFPK(Dnp)r	1453.62	1452.6609			
A2pr(Nma)GVYMAFPK(Dnp)	1297.44	1296.5597	GEIMAFPK(Dnp)rr	1370.54	1369.6561	A2pr(Nma)GRIMAFPK(Dnp)r	1460.66	1459.7143			
EPMAFPK(Dnp)rr	1297.44	1296.6033	GEDMAFPK(Dnp)rr	1372.47	1371.5990	A2pr(Nma)GRDMAFPK(Dnp)r	1462.59	1461.6572			
VKMAFPK(Dnp)rr	1298.52	1297.6714	GFPMAFPK(Dnp)rr	1372.55	1371.6506	A2pr(Nma)GFKMAFPK(Dnp)r	1466.66	1465.6925			