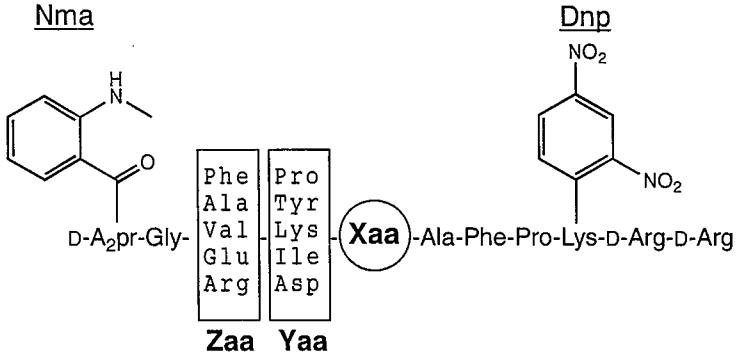
FRETS-25Xaa Series

* 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 3716-v

FRETS-25Thr Component-masses

FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GEDTA	710.69	710.2871	A2pr(Nma)GVYTAF	875.97	875.4178
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVIT	607.70	607.3330	A2pr(Nma)GFPTA	710.78	710.3388	A2pr(Nma)GAPTAFP	878.97	878.4287
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVDT	609.63	609.2758	A2pr(Nma)GRYT	714.77	714.3449	A2pr(Nma)GRITAF	883.01	882.4712
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GRPTA	719.79	719.3715	A2pr(Nma)GRDTAF	884.94	884.4141
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GEPT	621.64	621.2758	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GFKTAF	889.01	888.4494
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GVKT	622.71	622.3439	A2pr(Nma)GEKTA	723.77	723.3552	A2pr(Nma)GAITAFP	895.01	894.4600
A2pr(Nma)GAP	462.50	462.2227	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GFITA	726.82	726.3701	A2pr(Nma)GADTAFP	896.94	896.4028
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYT	629.66	629.2809	A2pr(Nma)GFDTA	728.75	728.3130	A2pr(Nma)GRKTAF	898.02	897.4821
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPTA	634.68	634.3075	A2pr(Nma)GVYTA	728.79	728.3493	A2pr(Nma)GEYTAF	905.95	905.3919
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GEIT	637.68	637.3071	A2pr(Nma)GRITA	735.83	735.4028	A2pr(Nma)GVPTAFP	907.02	906.4600
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDT	639.61	639.2500	A2pr(Nma)GRDTA	737.76	737.3457	A2pr(Nma)GAKTAFP	910.03	909.4709
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPT	639.70	639.3017	A2pr(Nma)GFKTA	741.83	741.3810	A2pr(Nma)GVITAFP	923.07	922.4913
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPT	648.71	648.3344	A2pr(Nma)GRKTA	750.85	750.4137	A2pr(Nma)GFYTAF	924.01	923.4178
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAITA	650.72	650.3388	A2pr(Nma)GEYTA	758.78	758.3235	A2pr(Nma)GVDTAFP	925.00	924.4341
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADTA	652.65	652.2817	A2pr(Nma)GFYTA	776.84	776.3493	A2pr(Nma)GRYTAF	933.02	932.4505
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKT	652.70	652.3180	A2pr(Nma)GAPTAF	781.86	781.3759	A2pr(Nma)GEPTAFP	937.01	936.4341
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GFIT	655.74	655.3330	A2pr(Nma)GRYTA	785.85	785.3820	A2pr(Nma)GVKTAFP	938.08	937.5022
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFDT	657.67	657.2758	A2pr(Nma)GAITAF	797.90	797.4072	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GVYT	657.71	657.3122	A2pr(Nma)GADTAF	799.83	799.3501	A2pr(Nma)GAYTAFP	945.03	944.4392
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVPTA	662.73	662.3388	A2pr(Nma)GVPTAF	809.91	809.4072	A2pr(Nma)GEITAFP	953.05	952.4654
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GRIT	664.75	664.3657	A2pr(Nma)GAKTAF	812.91	812.4181	A2pr(Nma)GEDTAFP	954.98	954.4083
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GAKTA	665.74	665.3497	A2pr(Nma)GVITAF	825.95	825.4385	A2pr(Nma)GFPTAFP	955.07	954.4600
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GRDT	666.68	666.3085	A2pr(Nma)GVDTAF	827.88	827.3814	A2pr(Nma)GRPTAFP	964.08	963.4927
A2pr(Nma)GVY	556.61	556.2645	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEPTAF	839.89	839.3814	A2pr(Nma)GEKTAFP	968.06	967.4763
A2pr(Nma)GAPT	563.60	563.2704	A2pr(Nma)GFKT	670.76	670.3439	A2pr(Nma)GVKTAF	840.97	840.4494	A2pr(Nma)GFITAFP	971.11	970.4913
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GVITA	678.78	678.3701	A2pr(Nma)GAYTAF	847.91	847.3865	A2pr(Nma)GFDTAFP	973.04	972.4341
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GRKT	679.77	679.3766	A2pr(Nma)GEITAF	855.93	855.4127	A2pr(Nma)GVYTAFP	973.08	972.4705
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVDTA	680.71	680.3130	A2pr(Nma)GEDTAF	857.86	857.3555	A2pr(Nma)GRITAFP	980.12	979.5240
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GEYT	687.70	687.2864	A2pr(Nma)GFPTAF	857.95	857.4072	A2pr(Nma)GRDTAFP	982.05	981.4668
A2pr(Nma)GAIT	579.65	579.3017	A2pr(Nma)GEPTA	692.72	692.3130	A2pr(Nma)GRPTAF	866.96	866.4399	A2pr(Nma)GFKTAFP	986.12	985.5022
A2pr(Nma)GADT	581.58	581.2445	A2pr(Nma)GVKTA	693.79	693.3810	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GRKTAFP	995.14	994.5348
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GAYTA	700.74	700.3180	A2pr(Nma)GEKTAF	870.95	870.4236	A2pr(Nma)GEYTAFP	1003.06	1002.4447
A2pr(Nma)GVPT	591.66	591.3017	A2pr(Nma)GFYT	705.76	705.3122	A2pr(Nma)GFITAF	873.99	873.4385	A2pr(Nma)GFYTAFP	1021.12	1020.4705
A2pr(Nma)GAKT	594.66	594.3126	A2pr(Nma)GEITA	708.76	708.3443	A2pr(Nma)GFDTAF	875.92	875.3814	A2pr(Nma)GRYTAFP	1030.14	1029.5032

FRETS-25Thr Component-masses

FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic	FRETS-25Thr	Average	Monoisotopic
TAFPK(Dnp)rr	1041.12	1040.5152	A2pr(Nma)GRITAFPK(Dnp)	1274.38	1273.6204	A2pr(Nma)GAITAFPK(Dnp)r	1345.46	1344.6575	A2pr(Nma)GRKTAFPK(Dnp)r	1445.58	1444.7324
PTAFPK(Dnp)rr	1138.24	1137.5679	AYTAFPK(Dnp)rr	1275.37	1274.6156	A2pr(Nma)GADTAFPK(Dnp)r	1347.39	1346.6004	A2pr(Nma)GEYTAFPK(Dnp)r	1453.51	1452.6422
ITAFPK(Dnp)rr	1154.28	1153.5992	A2pr(Nma)GRDTAFPK(Dnp)	1276.31	1275.5632	FYTAFPK(Dnp)rr	1351.47	1350.6469	A2pr(Nma)GFYTAFPK(Dnp)r	1471.57	1470.6680
DTAFPK(Dnp)rr	1156.21	1155.5421	A2pr(Nma)GFKTAFPK(Dnp)	1280.39	1279.5986	GRPTAFPK(Dnp)rr	1351.47	1350.6905	A2pr(Nma)GRYTAFPK(Dnp)r	1480.59	1479.7007
KTAFPK(Dnp)rr	1169.29	1168.6101	GAITAFPK(Dnp)rr	1282.41	1281.6578	GEKTAFPK(Dnp)rr	1355.46	1354.6742	A2pr(Nma)GAPTAFPK(Dnp)rr	1485.61	1484.7273
A2pr(Nma)GAPTAFPK(Dnp)	1173.23	1172.5251	EITAFPK(Dnp)rr	1283.39	1282.6418	A2pr(Nma)GVPTAFPK(Dnp)r	1357.47	1356.6575	A2pr(Nma)GAITAFPK(Dnp)rr	1501.65	1500.7586
A2pr(Nma)GAITAFPK(Dnp)	1189.28	1188.5564	GADTAFPK(Dnp)rr	1284.34	1283.6007	GFITAFPK(Dnp)rr	1358.50	1357.6891	A2pr(Nma)GADTAFPK(Dnp)rr	1503.58	1502.7015
A2pr(Nma)GADTAFPK(Dnp)	1191.21	1190.4993	EDTAFPK(Dnp)rr	1285.32	1284.5847	GFDTAFPK(Dnp)rr	1360.43	1359.6320	A2pr(Nma)GVPTAFPK(Dnp)rr	1513.66	1512.7586
A2pr(Nma)GVPTAFPK(Dnp)	1201.29	1200.5564	FPTAFPK(Dnp)rr	1285.41	1284.6364	A2pr(Nma)GAKTAFPK(Dnp)r	1360.48	1359.6684	A2pr(Nma)GAKTAFPK(Dnp)rr	1516.66	1515.7695
A2pr(Nma)GAKTAFPK(Dnp)	1204.29	1203.5673	A2pr(Nma)GRKTAFPK(Dnp)	1289.40	1288.6313	GVYTAFPK(Dnp)rr	1360.48	1359.6684	A2pr(Nma)GVITAFPK(Dnp)rr	1529.70	1528.7899
YTAFPK(Dnp)rr	1204.29	1203.5785	GVPTAFPK(Dnp)rr	1294.42	1293.6578	RYTAFPK(Dnp)rr	1360.48	1359.6796	A2pr(Nma)GVDTAFPK(Dnp)rr	1531.63	1530.7328
APTAFPK(Dnp)rr	1209.31	1208.6051	RPTAFPK(Dnp)rr	1294.42	1293.6691	GRITAFPK(Dnp)rr	1367.52	1366.7218	A2pr(Nma)GEPTAFPK(Dnp)rr	1543.64	1542.7328
A2pr(Nma)GVITAFPK(Dnp)	1217.33	1216.5877	A2pr(Nma)GEYTAFPK(Dnp)	1297.33	1296.5411	GRDTAFPK(Dnp)rr	1369.45	1368.6647	A2pr(Nma)GVKTAFPK(Dnp)rr	1544.72	1543.8008
A2pr(Nma)GVDTAFPK(Dnp)	1219.26	1218.5306	GAKTAFPK(Dnp)rr	1297.42	1296.6687	A2pr(Nma)GVITAFPK(Dnp)r	1373.51	1372.6888	A2pr(Nma)GAYTAFPK(Dnp)rr	1551.66	1550.7379
AITAFPK(Dnp)rr	1225.36	1224.6364	EKTAFPK(Dnp)rr	1298.41	1297.6527	GFKTAFPK(Dnp)rr	1373.52	1372.7000	A2pr(Nma)GEITAFPK(Dnp)rr	1559.68	1558.7641
ADTAFPK(Dnp)rr	1227.29	1226.5792	FITAFPK(Dnp)rr	1301.45	1300.6677	A2pr(Nma)GVDTAFPK(Dnp)r	1375.44	1374.6317	A2pr(Nma)GEDTAFPK(Dnp)rr	1561.61	1560.7070
A2pr(Nma)GEPTAFPK(Dnp)	1231.27	1230.5306	FDTAFPK(Dnp)rr	1303.38	1302.6105	GRKTAFPK(Dnp)rr	1382.53	1381.7327	A2pr(Nma)GFPTAFPK(Dnp)rr	1561.70	1560.7586
A2pr(Nma)GVKTAFPK(Dnp)	1232.34	1231.5986	VYTAFPK(Dnp)rr	1303.43	1302.6469	A2pr(Nma)GEPTAFPK(Dnp)r	1387.46	1386.6317	A2pr(Nma)GRPTAFPK(Dnp)rr	1570.71	1569.7913
VPTAFPK(Dnp)rr	1237.37	1236.6364	GVITAFPK(Dnp)rr	1310.46	1309.6891	A2pr(Nma)GVKTAFPK(Dnp)r	1388.53	1387.6997	A2pr(Nma)GEKTAFPK(Dnp)rr	1574.70	1573.7750
A2pr(Nma)GAYTAFPK(Dnp)	1239.29	1238.5356	RITAFPK(Dnp)rr	1310.46	1309.7004	GEYTAFPK(Dnp)rr	1390.46	1389.6426	A2pr(Nma)GFITAFPK(Dnp)rr	1577.74	1576.7899
AKTAFPK(Dnp)rr	1240.37	1239.6473	GVDTAFPK(Dnp)rr	1312.39	1311.6320	A2pr(Nma)GAYTAFPK(Dnp)r	1395.48	1394.6367	A2pr(Nma)GFDTAFPK(Dnp)rr	1579.67	1578.7328
A2pr(Nma)GEITAFPK(Dnp)	1247.31	1246.5619	RDTAFPK(Dnp)rr	1312.39	1311.6432	A2pr(Nma)GEITAFPK(Dnp)r	1403.50	1402.6630	A2pr(Nma)GVYTAFPK(Dnp)rr	1579.72	1578.7692
A2pr(Nma)GEDTAFPK(Dnp)	1249.24	1248.5047	A2pr(Nma)GFYTAFPK(Dnp)	1315.39	1314.5669	A2pr(Nma)GEDTAFPK(Dnp)r	1405.43	1404.6058	A2pr(Nma)GRITAFPK(Dnp)rr	1586.76	1585.8226
A2pr(Nma)GFPTAFPK(Dnp)	1249.33	1248.5564	FKTAFPK(Dnp)rr	1316.47	1315.6786	A2pr(Nma)GFPTAFPK(Dnp)r	1405.52	1404.6575	A2pr(Nma)GRDTAFPK(Dnp)rr	1588.69	1587.7655
VITAFPK(Dnp)rr	1253.41	1252.6677	A2pr(Nma)GRYTAFPK(Dnp)	1324.40	1323.5996	GFYTAFPK(Dnp)rr	1408.52	1407.6684	A2pr(Nma)GFKTAFPK(Dnp)rr	1592.76	1591.8008
VDTAFPK(Dnp)rr	1255.34	1254.6105	GEPTAFPK(Dnp)rr	1324.40	1323.6320	A2pr(Nma)GRPTAFPK(Dnp)r	1414.53	1413.6902	A2pr(Nma)GRKTAFPK(Dnp)rr	1601.77	1600.8335
A2pr(Nma)GRPTAFPK(Dnp)	1258.34	1257.5891	GVKTAFPK(Dnp)rr	1325.48	1324.7000	GRYTAFPK(Dnp)rr	1417.53	1416.7011	A2pr(Nma)GEYTAFPK(Dnp)rr	1609.70	1608.7433
A2pr(Nma)GEKTAFPK(Dnp)	1262.33	1261.5728	RKTAFPK(Dnp)rr	1325.48	1324.7113	A2pr(Nma)GEKTAFPK(Dnp)r	1418.51	1417.6739	A2pr(Nma)GFYTAFPK(Dnp)rr	1627.76	1626.7692
A2pr(Nma)GFITAFPK(Dnp)	1265.37	1264.5877	A2pr(Nma)GAPTAFPK(Dnp)r	1329.42	1328.6262	A2pr(Nma)GFITAFPK(Dnp)r	1421.56	1420.6888	A2pr(Nma)GRYTAFPK(Dnp)rr	1636.77	1635.8019
GAPTAFPK(Dnp)rr	1266.37	1265.6265	GAYTAFPK(Dnp)rr	1332.42	1331.6371	A2pr(Nma)GFDTAFPK(Dnp)r	1423.49	1422.6317			
A2pr(Nma)GFDTAFPK(Dnp)	1267.30	1266.5306	EYTAFPK(Dnp)rr	1333.41	1332.6211	A2pr(Nma)GVYTAFPK(Dnp)r	1423.53	1422.6680			
A2pr(Nma)GVYTAFPK(Dnp)	1267.34	1266.5669	GEITAFPK(Dnp)rr	1340.44	1339.6633	A2pr(Nma)GRITAFPK(Dnp)r	1430.57	1429.7215			
EPTAFPK(Dnp)rr	1267.35	1266.6105	GEDTAFPK(Dnp)rr	1342.37	1341.6062	A2pr(Nma)GRDTAFPK(Dnp)r	1432.50	1431.6644			
VKTAFPK(Dnp)rr	1268.42	1267.6786	GFPTAFPK(Dnp)rr	1342.46	1341.6578	A2pr(Nma)GFKTAFPK(Dnp)r	1436.57	1435.6997			