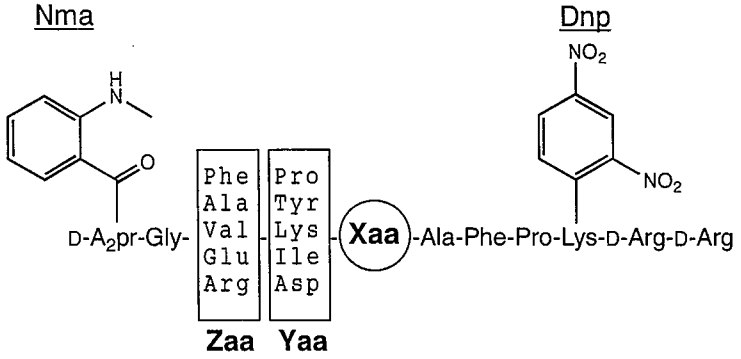
FRETS-25Xaa Series

* 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 3714-v

FRETS-25Pro Component-masses

FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GVIP	603.71	603.3380	A2pr(Nma)GEDPA	706.70	706.2922	A2pr(Nma)GVYPAF	871.98	871.4228
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GFPPA	706.79	706.3439	A2pr(Nma)GAPPAFP	874.98	874.4337
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GVDP	605.64	605.2809	A2pr(Nma)GRYP	710.78	710.3500	A2pr(Nma)GRIPAF	879.02	878.4763
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GRPPA	715.80	715.3766	A2pr(Nma)GRDPAF	880.95	880.4192
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GEPP	617.65	617.2809	A2pr(Nma)GEKPA	719.79	719.3602	A2pr(Nma)GFKPAF	885.02	884.4545
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GVKP	618.72	618.3489	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GAIPAFP	891.02	890.4650
A2pr(Nma)GAP	462.50	462.2227	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GFIPA	722.83	722.3752	A2pr(Nma)GADPAFP	892.95	892.4079
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAYP	625.67	625.2860	A2pr(Nma)GFDPA	724.76	724.3180	A2pr(Nma)GRKPAF	894.03	893.4872
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GAPPA	630.69	630.3126	A2pr(Nma)GVYPA	724.80	724.3544	A2pr(Nma)GEYPAF	901.96	901.3970
A2pr(Nma)GVP	490.55	490.2540	A2pr(Nma)GEIP	633.69	633.3122	A2pr(Nma)GRIPA	731.84	731.4079	A2pr(Nma)GVPPAFP	903.03	902.4650
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDP	635.62	635.2551	A2pr(Nma)GRDPA	733.77	733.3507	A2pr(Nma)GAKPAFP	906.04	905.4759
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPP	635.71	635.3067	A2pr(Nma)GFKPA	737.85	737.3861	A2pr(Nma)GVIPAFP	919.08	918.4963
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPP	644.72	644.3394	A2pr(Nma)GRKPA	746.86	746.4188	A2pr(Nma)GFYPAF	920.02	919.4228
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAIPA	646.74	646.3439	A2pr(Nma)GEYPA	754.79	754.3286	A2pr(Nma)GVDPAFP	921.01	920.4392
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADPA	648.66	648.2867	A2pr(Nma)GFYPA	772.85	772.3544	A2pr(Nma)GRYPAF	929.03	928.4555
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKP	648.71	648.3231	A2pr(Nma)GAPPAF	777.87	777.3810	A2pr(Nma)GEPPAFP	933.02	932.4392
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GFIP	651.75	651.3380	A2pr(Nma)GRYPA	781.86	781.3871	A2pr(Nma)GVKPAFP	934.09	933.5072
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFDP	653.68	653.2809	A2pr(Nma)GAIPAF	793.91	793.4123	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GVYP	653.73	653.3173	A2pr(Nma)GADPAF	795.84	795.3552	A2pr(Nma)GAYPAFP	941.04	940.4443
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVPPA	658.75	658.3439	A2pr(Nma)GVPPAF	805.92	805.4123	A2pr(Nma)GEIPAFP	949.06	948.4705
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GRIP	660.76	660.3707	A2pr(Nma)GAKPAF	808.92	808.4232	A2pr(Nma)GEDPAFP	950.99	950.4134
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GAKPA	661.75	661.3548	A2pr(Nma)GVIPAF	821.96	821.4436	A2pr(Nma)GFPPAFP	951.08	950.4650
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GRDP	662.69	662.3136	A2pr(Nma)GVDPAF	823.89	823.3865	A2pr(Nma)GRPPAFP	960.09	959.4977
A2pr(Nma)GVY	556.61	556.2645	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEPPAF	835.90	835.3865	A2pr(Nma)GEKPAFP	964.07	963.4814
A2pr(Nma)GAPP	559.61	559.2754	A2pr(Nma)GFKP	666.77	666.3489	A2pr(Nma)GVKPAF	836.98	836.4545	A2pr(Nma)GFIPAFP	967.12	966.4963
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GVIPA	674.79	674.3752	A2pr(Nma)GAYPAF	843.92	843.3915	A2pr(Nma)GFDPAFP	969.05	968.4392
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GRKP	675.78	675.3816	A2pr(Nma)GEIPAF	851.94	851.4178	A2pr(Nma)GVYPAFP	969.09	968.4756
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GVDPA	676.72	676.3180	A2pr(Nma)GEDPAF	853.87	853.3606	A2pr(Nma)GRIPAFP	976.13	975.5290
A2pr(Nma)GAIP	575.66	575.3067	A2pr(Nma)GEYP	683.71	683.2915	A2pr(Nma)GFPPAF	853.96	853.4123	A2pr(Nma)GRDPAFP	978.06	977.4719
A2pr(Nma)GADP	577.59	577.2496	A2pr(Nma)GEPPA	688.73	688.3180	A2pr(Nma)GRPPAF	862.97	862.4450	A2pr(Nma)GFKPAFP	982.13	981.5072
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GVKPA	689.80	689.3861	A2pr(Nma)GEKPAF	866.96	866.4287	A2pr(Nma)GRKPAFP	991.15	990.5399
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GAYPA	696.75	696.3231	FPK(Dnp)rr	868.94	868.4304	A2pr(Nma)GEYPAFP	999.08	998.4498
A2pr(Nma)GVPP	587.67	587.3067	A2pr(Nma)GFYP	701.77	701.3173	A2pr(Nma)GFIPAF	870.00	869.4436	A2pr(Nma)GFYPAFP	1017.14	1016.4756
A2pr(Nma)GAKP	590.67	590.3176	A2pr(Nma)GEIPA	704.77	704.3493	A2pr(Nma)GFDPAF	871.93	871.3865	A2pr(Nma)GRYPAFP	1026.15	1025.5083

FRETS-25Pro Component-masses

FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic	FRETS-25Pro	Average	Monoisotopic
PAFPK(Dnp)rr	1037.13	1036.5203	A2pr(Nma)GRIPAFPK(Dnp)	1270.40	1269.6255	A2pr(Nma)GAIPAFPK(Dnp)r	1341.47	1340.6626	A2pr(Nma)GRKPAFPK(Dnp)r	1441.60	1440.7375
PPAFPK(Dnp)rr	1134.25	1133.5730	AYPAFPK(Dnp)rr	1271.38	1270.6207	A2pr(Nma)GADPAFPK(Dnp)r	1343.40	1342.6054	A2pr(Nma)GEYPAFPK(Dnp)r	1449.52	1448.6473
IPAFPK(Dnp)rr	1150.29	1149.6043	A2pr(Nma)GRDPAFPK(Dnp)	1272.32	1271.5683	FYPAFPK(Dnp)rr	1347.48	1346.6520	A2pr(Nma)GFYPAFPK(Dnp)r	1467.58	1466.6731
DPAFPK(Dnp)rr	1152.22	1151.5472	A2pr(Nma)GFKPAFPK(Dnp)	1276.40	1275.6037	GRPPAFPK(Dnp)rr	1347.48	1346.6956	A2pr(Nma)GRYPAFPK(Dnp)r	1476.60	1475.7058
KPAFPK(Dnp)rr	1165.30	1164.6152	GAIPAFPK(Dnp)rr	1278.42	1277.6629	GEKPAFPK(Dnp)rr	1351.47	1350.6793	A2pr(Nma)GAPPAFPK(Dnp)rr	1481.62	1480.7324
A2pr(Nma)GAPPAFPK(Dnp)	1169.24	1168.5302	EIPAFPK(Dnp)rr	1279.40	1278.6469	A2pr(Nma)GVPPAFPK(Dnp)r	1353.48	1352.6626	A2pr(Nma)GAIPAFPK(Dnp)rr	1497.66	1496.7637
A2pr(Nma)GAIPAFPK(Dnp)	1185.29	1184.5615	GADPAFPK(Dnp)rr	1280.35	1279.6058	GFIPAFPK(Dnp)rr	1354.51	1353.6942	A2pr(Nma)GADPAFPK(Dnp)rr	1499.59	1498.7066
A2pr(Nma)GADPAFPK(Dnp)	1187.22	1186.5043	EDPAFPK(Dnp)rr	1281.33	1280.5898	GFDPAFPK(Dnp)rr	1356.44	1355.6371	A2pr(Nma)GVPPAFPK(Dnp)rr	1509.67	1508.7637
A2pr(Nma)GVPPAFPK(Dnp)	1197.30	1196.5615	FPPAFPK(Dnp)rr	1281.42	1280.6414	A2pr(Nma)GAKPAFPK(Dnp)r	1356.49	1355.6735	A2pr(Nma)GAKPAFPK(Dnp)rr	1512.67	1511.7746
A2pr(Nma)GAKPAFPK(Dnp)	1200.30	1199.5724	A2pr(Nma)GRKPAFPK(Dnp)	1285.41	1284.6364	GVYPAFPK(Dnp)rr	1356.49	1355.6735	A2pr(Nma)GVIPAFPK(Dnp)rr	1525.71	1524.7950
YPAFPK(Dnp)rr	1200.31	1199.5836	GVPPAFPK(Dnp)rr	1290.43	1289.6629	RYPAFPK(Dnp)rr	1356.49	1355.6847	A2pr(Nma)GVDPAFPK(Dnp)rr	1527.64	1526.7379
APPAFPK(Dnp)rr	1205.33	1204.6101	RPPAFPK(Dnp)rr	1290.43	1289.6741	GRIPAFPK(Dnp)rr	1363.53	1362.7269	A2pr(Nma)GEPPAFPK(Dnp)rr	1539.65	1538.7379
A2pr(Nma)GVIPAFPK(Dnp)	1213.34	1212.5928	A2pr(Nma)GEYPAFPK(Dnp)	1293.34	1292.5462	GRDPAFPK(Dnp)rr	1365.46	1364.6698	A2pr(Nma)GVKPAFPK(Dnp)rr	1540.73	1539.8059
A2pr(Nma)GVDPAFPK(Dnp)	1215.27	1214.5356	GAKPAFPK(Dnp)rr	1293.43	1292.6738	A2pr(Nma)GVIPAFPK(Dnp)r	1369.53	1368.6939	A2pr(Nma)GAYPAFPK(Dnp)rr	1547.67	1546.7429
AIPAFPK(Dnp)rr	1221.37	1220.6414	EKPAFPK(Dnp)rr	1294.42	1293.6578	GFKPAFPK(Dnp)rr	1369.53	1368.7051	A2pr(Nma)GEIPAFPK(Dnp)rr	1555.69	1554.7692
ADPAFPK(Dnp)rr	1223.30	1222.5843	FIPAFPK(Dnp)rr	1297.46	1296.6727	A2pr(Nma)GVDPAFPK(Dnp)r	1371.46	1370.6367	A2pr(Nma)GEDPAFPK(Dnp)rr	1557.62	1556.7120
A2pr(Nma)GEPPAFPK(Dnp)	1227.28	1226.5356	FDPAFPK(Dnp)rr	1299.39	1298.6156	GRKPAFPK(Dnp)rr	1378.54	1377.7378	A2pr(Nma)GFPPAFPK(Dnp)rr	1557.71	1556.7637
A2pr(Nma)GVKPAFPK(Dnp)	1228.36	1227.6037	VYPAFPK(Dnp)rr	1299.44	1298.6520	A2pr(Nma)GEPPAFPK(Dnp)r	1383.47	1382.6367	A2pr(Nma)GRPPAFPK(Dnp)rr	1566.72	1565.7964
VPPAFPK(Dnp)rr	1233.38	1232.6414	GVIPAFPK(Dnp)rr	1306.47	1305.6942	A2pr(Nma)GVKPAFPK(Dnp)r	1384.54	1383.7048	A2pr(Nma)GEKPAFPK(Dnp)rr	1570.71	1569.7801
A2pr(Nma)GAYPAFPK(Dnp)	1235.30	1234.5407	RIPAFPK(Dnp)rr	1306.48	1305.7054	GEYPAFPK(Dnp)rr	1386.47	1385.6476	A2pr(Nma)GFIPAFPK(Dnp)rr	1573.75	1572.7950
AKPAFPK(Dnp)rr	1236.38	1235.6523	GVDPAFPK(Dnp)rr	1308.40	1307.6371	A2pr(Nma)GAYPAFPK(Dnp)r	1391.49	1390.6418	A2pr(Nma)GFDPAFPK(Dnp)rr	1575.68	1574.7379
A2pr(Nma)GEIPAFPK(Dnp)	1243.32	1242.5669	RDPAFPK(Dnp)rr	1308.41	1307.6483	A2pr(Nma)GEIPAFPK(Dnp)r	1399.51	1398.6680	A2pr(Nma)GVYPAFPK(Dnp)rr	1575.73	1574.7742
A2pr(Nma)GEDPAFPK(Dnp)	1245.25	1244.5098	A2pr(Nma)GFYPAFPK(Dnp)	1311.40	1310.5720	A2pr(Nma)GEDPAFPK(Dnp)r	1401.44	1400.6109	A2pr(Nma)GRIPAFPK(Dnp)rr	1582.77	1581.8277
A2pr(Nma)GFPPAFPK(Dnp)	1245.34	1244.5615	FKPAFPK(Dnp)rr	1312.48	1311.6836	A2pr(Nma)GFPPAFPK(Dnp)r	1401.53	1400.6626	A2pr(Nma)GRDPAFPK(Dnp)rr	1584.70	1583.7706
VIPAFPK(Dnp)rr	1249.42	1248.6727	A2pr(Nma)GRYPAFPK(Dnp)	1320.41	1319.6047	GFYPAFPK(Dnp)rr	1404.53	1403.6735	A2pr(Nma)GFKPAFPK(Dnp)rr	1588.77	1587.8059
VDPAFPK(Dnp)rr	1251.35	1250.6156	GEPPAFPK(Dnp)rr	1320.41	1319.6371	A2pr(Nma)GRPPAFPK(Dnp)r	1410.54	1409.6953	A2pr(Nma)GRKPAFPK(Dnp)rr	1597.78	1596.8386
A2pr(Nma)GRPPAFPK(Dnp)	1254.35	1253.5942	GVKPAFPK(Dnp)rr	1321.49	1320.7051	GRYPAFPK(Dnp)rr	1413.54	1412.7062	A2pr(Nma)GEYPAFPK(Dnp)rr	1605.71	1604.7484
A2pr(Nma)GEKPAFPK(Dnp)	1258.34	1257.5778	RKPAFPK(Dnp)rr	1321.49	1320.7163	A2pr(Nma)GEKPAFPK(Dnp)r	1414.52	1413.6789	A2pr(Nma)GFYPAFPK(Dnp)rr	1623.77	1622.7742
A2pr(Nma)GFIPAFPK(Dnp)	1261.38	1260.5928	A2pr(Nma)GAPPAFPK(Dnp)r	1325.43	1324.6313	A2pr(Nma)GFIPAFPK(Dnp)r	1417.57	1416.6939	A2pr(Nma)GRYPAFPK(Dnp)rr	1632.78	1631.8069
GAPPAFPK(Dnp)rr	1262.38	1261.6316	GAYPAFPK(Dnp)rr	1328.43	1327.6422	A2pr(Nma)GFDPAFPK(Dnp)r	1419.50	1418.6367			
A2pr(Nma)GFDPAFPK(Dnp)	1263.31	1262.5356	EYPAFPK(Dnp)rr	1329.42	1328.6262	A2pr(Nma)GVYPAFPK(Dnp)r	1419.54	1418.6731			
A2pr(Nma)GVYPAFPK(Dnp)	1263.36	1262.5720	GEIPAFPK(Dnp)rr	1336.46	1335.6684	A2pr(Nma)GRIPAFPK(Dnp)r	1426.58	1425.7266			
EPPAFPK(Dnp)rr	1263.36	1262.6156	GEDPAFPK(Dnp)rr	1338.38	1337.6113	A2pr(Nma)GRDPAFPK(Dnp)r	1428.51	1427.6694			
VKPAFPK(Dnp)rr	1264.44	1263.6836	GFPPAFPK(Dnp)rr	1338.47	1337.6629	A2pr(Nma)GFKPAFPK(Dnp)r	1432.58	1431.7048			