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Multiplexed Quantification of Metabolites with MISSILE

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Abstract

We tested a strategy for multiplexed (4-plex) quantification of metabolites using the MISSILE identification method with liquid chromatography coupled to tandem mass spectrometry. We applied this methodology to study the metabolic effect of the proteasome inhibitor and chemotherapeutic drug Bortezomib in yeast cells. Using JUMPm software version 1.1 we simultaneously identified and quantified 95 metabolites across four experimental conditions and found that Bortezomib increased the accumulation of dipeptides but decreased the levels of specific lipid molecules (e.g. phosphtidylethanolamines) in a dose-dependent manner. This method combines metabolite identification and quantification, making untargeted metabolomics experiments more informative.

Keywords: Metabolomics; Metabolome; Mass spectrometry; Stable isotope labeling; Liquid chromatography; Metabolite quantification; Multiplex; Bortezomib; Proteasome inhibition; MISSILE

Abbreviations: MISSILE: Metabolome Identification by Systematic Stable Isotope Labeling Experiments; nUPLC-HRMS: Nanoscale Ultra-Performance Liquid Chromatography–High Resolution Mass Spectrometry

Introduction

We recently described a formula-centric strategy for the identification of metabolites in untargeted metabolomics experiments [1,2]. The method uses stable isotope labeling [3-5] to identify the formula and structure of metabolites in an automated fashion with support for false discovery rate estimation [6,7]. Here we have expanded on this strategy by adding a fourth isotope labeling condition to enable 4-plex relative quantification of metabolites in LC-MS experiments. Each label represents an independent experimental condition so that we can compare the relative levels of metabolites from pairwise duplicates, time course, or dose-response experimental designs. Multi-plexed analyses offer many advantages over traditional label-free methods [8], but current strategies rely on specific functional group tags to chemically label metabolites [9] which limit the scope of quantified metabolites and introduce complexity to the sample preparation workflow. As a test of our methodology, we performed a dose-response analysis on the effect of the proteasome inhibitor Bortezomib [10] in yeast cells. This drug is an effective therapy for multiple-myeloma, but drug-resistance invariably develops due to incompletely characterized mechanisms [11,12]. The effect of proteasome inhibition on protein [13] and gene regulation [14] has been studied in a variety of contexts, but much less is known about the role of metabolism in this process. We report our preliminary investigation and demonstrate the analytical tools for carrying out multiplexed metabolomics analyses.

Methods

Materials–LC-MS grade acetonitrile (ACN), water, and formic acid (Sigma), glass beads (Next Advance), Difco™ yeast nitrogen base, DMEM (BD Biosciences), nanoLC column (Waters), ^{13}C-6 glucose, ^{15}N-2 ammonium sulfate, (Cambridge Isotope Laboratories).

MISSILE protocol

For *Saccharomyces cerevisiae* (Fleischmann) labeling, cells were grown in four different minimal media conditions. A control media consisted of natural isotopic abundance components; Difco™ yeast nitrogen base without amino acids and ammonium sulfate (BD Biosciences), with 5 g/L ammonium sulfate (Sigma), and 20 g/L glucose (Sigma). For carbon-13 labeling, the media remained the same except that ^{13}C-6 glucose (Cambridge Isotope Laboratories) was used in place of standard glucose. Similarly for nitrogen-15 labeling, ^{15}N-2 ammonium sulfate was substituted into the media. Each culture was maintained for ~30 generations in the labeled media before drug treatment and metabolite extraction. For Bortezomib treatment, cultures were seeded to an OD_{600} of 0.1 and allowed to grow to 1 (~6 generations).

Bortezomib treatment

Bortezomib was solubilized in 10 mM, 1 mM, and 0.1 mM stock solutions (water) so that equal volumes could be spiked in for each treatment. For each 25 mL yeast culture, the OD_{600} was monitored and recorded every hour. Bortezomib was added to each culture at OD_{600}=0.5 absorbance units.

Sample preparation

Control and drug treatment yeast cultures were harvested and their metabolites were extracted. The liquid cultures were transferred to 15 mL conical vials and centrifuged at 1,000 g for 3 min to obtain

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a cell pellet. The supernatant was discarded and 1 mL of freezing 80% acetonitrile was added. Each vial was subjected to 3 min of vortexing at 3,000 rpm in a 1 on/1 off pattern to maintain sample temperature [2]. The lysate was transferred to a fresh vial to exclude the glass beads, and then centrifuged at 21,000 g for 5 min to clarify the liquid phase.

Spectrophotometric determination of multiplex mixing ratio

The absorption (300 nm) of each supernatant was measured in a 1 mL cuvette and used to calculate the mixing ratio of the 4 individual cultures. For each culture we multiplied the absorbance reading by the volume of supernatant recovered (900 µL) as an estimate of the total amount of metabolites present. We then calculated the amount of each sample needed to equal 90% of the lowest measured label. Using these values we mixed the supernatants of the 4 labeled samples into a single tube (~3 mL), vortexed, and aliquoted the mixture into 4 separate tubes.

Sample reconstitution

The mixed or individual aliquots were dried under centrifugal vacuum and resolubilized to 75 µL in buffer A and transferred to inserts for LC-MS analysis.

LC-MS analysis and parameters

Bortezomib treated yeast samples were analyzed on an Orbitrap Elite (Thermo Scientific) coupled to an Easy nLC™ system as previously described [2]. In summary, we used a nano Acquity UPLC column (75 µm × 100 mm) packed with 1.7 µm BEH C18 beads with 0.2% formic acid in water (Mobile Phase A) or acetonitrile (Mobile Phase B). Sample injection volume was 2 µL. LC-MS analysis was performed in positive ion (3 kV) mode with a 15 µm, 5 cm PicoTip emitter (New Objective). A top 5 data-dependent method was used to target ions for fragmentation (MS/MS) for later structural identification.

Results and Discussion

Proteasome inhibition of yeast cells and LC-MS analysis

Yeast cells in a respiring liquid culture were subjected to proteasome inhibition by addition of Bortezomib. We treated cells with three concentrations of drug; at the reported IC_{50} [15], 10-fold below, and 10-fold above, with a no-drug control (Figure 1a). To distinguish these four experimental conditions, carbon (i.e., glucose) and nitrogen (ammonium sulfate) sources in the culture were exchanged for various heavy stable isotope labeled compounds (i.e., ^{13}C-6-glucose and ^{15}N-ammonium sulfate). There was a decrease in the yeast growth rate with increasing concentrations of Bortezomib (Figure 1b). We subjected each condition to LC-MS analysis alone (Figure 1c) or multiplexed (mixed together) and observed changes in the base-peak chromatogram due to drug-treatment.

Formula and structure identification of Bortezomib regulated metabolites

We used JUMPm software to globally analyze the metabolites from yeast cells treated with Bortezomib. As an example, we manually examined one of the down-regulated metabolites (a phosphatidylethanolamine). The MS1 scan shows four ions of varying mass, reflecting total incorporation of heavy stable isotopes into the chemical structure (Figure 1d). The mass shift of the four ions can also be used to determine the chemical formula of the metabolite (Figure 1e) as previously described. Each of the four parent ions for phosphatidylethanolamine was manually extracted to confirm their co-elution and identity as isotope labels (Figure 1f). The intensity of these peaks was detected by JUMPm and serves as the basis for the relative quantification. The structure identity was determined by JUMPm in a search of its associated MS/MS spectra (Figure 1g). These tandem mass spectra were collected in a Top 5 data-dependent fashion. For phosphatidylethanolamine, MS2 spectra were acquired from two of the four labeled parent ions, the ^{12}C parent (unlabeled control) and the double labeled $^{13}C^{15}N$ parent ion. Two structural fragments were observed for each parent with nearly identical relative intensities. Overall, we identified 181 metabolite formulas and quantified 95 metabolite structures among the four treatment conditions with a false discovery rate of less than 1% (Table 1).

Multiplexed quantification of Bortezomib regulated metabolites

We observed that higher Bortezomib concentrations had broader impacts on the yeast metabolic profile, with a greater proportion of metabolites showing a larger fold change in peak size relative to control (Figure 1i). The lowest concentration of drug had a relatively minor effect on metabolite levels, whereas the higher concentrations increasingly segregated metabolites into up- or down-regulated populations. This trend was also true for individual metabolites (Figure 1d). As expected we observed that Bortezomib treatment increased the levels of incomplete protein catabolites e.g., di-peptides (Figure 1j). Metabolites that were unaffected by drug-treatment included nucleo-bases (e.g. adenine) and other "housekeeping" metabolites such as glutathione. We also observed that membrane components and signaling lipids were strongly down-regulated with higher Bortezomib concentrations (Figure 1l and 1k; Table 1).

Advantages and limitations

The described multiplexed design has several advantages over classical unlabeled strategies for metabolite quantification. Peak alignment between analyses is a major confounding factor for unlabeled strategies, and much effort has been focused on resolving this issue. For multiplexed samples, peak alignment is unnecessary because the treatment conditions are analyzed simultaneously. Carbon and nitrogen stable isotope labeling does not affect the retention time of small molecules, so each labeled form co-elutes with the other treatment conditions. This co-elution is a major advantage because it controls for time and sample-dependent variations in retention time and ionization efficiency, a major source of technical error during metabolite quantification.

Using the current strategy we can expect to identify and quantify metabolites from four independent labeled conditions. The labels are comprised of light or heavy carbon and nitrogen atoms. Therefore we observe all four possible mass labels for a given metabolite if the chemical formula contains at least one nitrogen atom. We previously observed that ~50% of known metabolite formulas contain nitrogen while the rest do not. For metabolites without nitrogen, our strategy can only provide two independent labels. In that case, the two peaks represent the average of two cultures (i.e., cultures 1&2 or 3&4). Therefore the duplicate experimental design may be more suitable for studies focused on such metabolites.

We introduce a new capability to perform 4-plex relative quantification of metabolites in untargeted metabolomics experiments using the MISSILE strategy in combination with JUMPm software. Using this method, we can now analyze metabolites from two experimental conditions with duplicates, or with up to 4 independent experimental conditions (e.g. time series or dose response data). The

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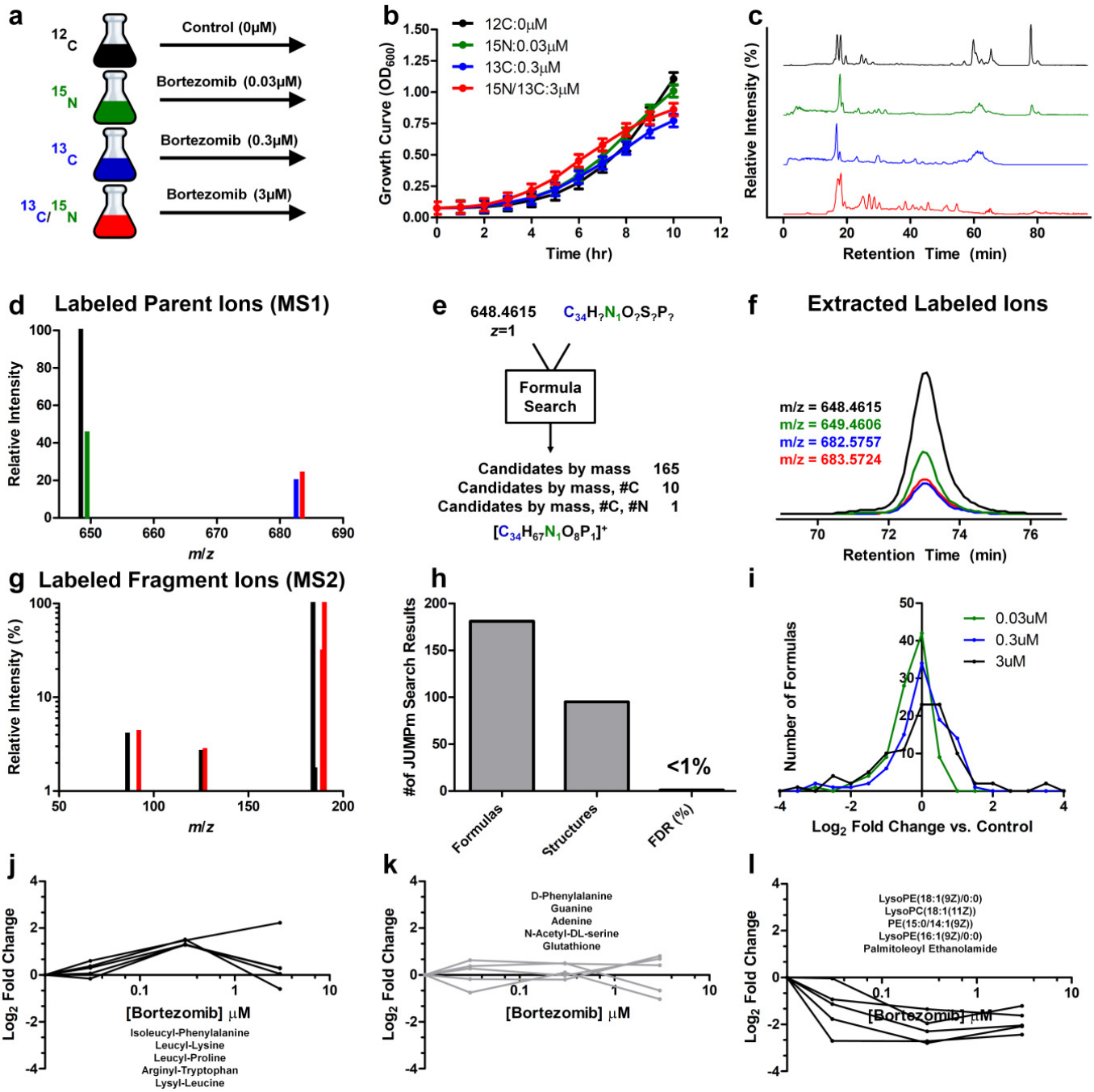

Figure 1: Metabolomics analysis of Bortezomib treated yeast cells (a) Experimental design of the 4-plex study. Labels indicate the stable isotope culture condition. Condition 4 (red) used both stable isotope labels simultaneously. (b) Growth curve for the four independent yeast cultures, monitored by OD_{600}. (c) Base-peak chromatograms for the four cultures analyzed independently prior to multiplex analysis. (d) An example MS1 spectrum for a metabolite peak after mixing the four yeast samples together. Colored peaks reflect the increased mass of the metabolite from stable isotope labelling and also the relative intensity changes due to Bortezomib treatment. This metabolite was identified as PE (15:0/14:1(9Z)). (e) Automated chemical formula-determination workflow for the example metabolite peaks shown in (d). Candidates represent chemical formulas within 4 ppm mass tolerance of the unlabelled peak. With increasingly strict search criteria the number of possible formulas dramatically decreases. The determined formula represents the charged parent ion. All formulas in Table 1 have been discharged. (f) Extracted ion chromatogram for each of the four labeled versions of the yeast metabolite shown in (d). Retention time and peak shape are not affected by isotope labelling, while the peak intensity reflects the changes due to Bortezomib treatment. (g) Tandem mass spectrum (MS/MS) of the labeled precursors from the example metabolite. (d-f) Each structure fragment shows two peaks, representing the two isotopes labeled parent ions which were fragmented in the Top 5 data-dependent method. (h) Fold change distribution of quantified metabolites compared to the no-drug control. Higher Bortezomib concentrations were associated with increased variation in metabolite levels. (i) Summary of JUMPm global metabolite search results. The formula false discovery rate (FDR) was less than 1% as determined by JUMPm based on the relative frequency of target and decoy formulas detected. (j-l) Up-regulated, unaffected, and down-regulated metabolites respectively as a function of Bortezomib concentration. Labels indicate identified metabolites using JUMPm software.

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MS1 scan	^{12}C *m/z*	^{15}N *m/z*	^{13}C *m/z*	$^{13}C/^{15}N$ *m/z*	^{12}C Intensity	^{15}N Intensity	^{13}C Intensity	$^{13}C/^{15}N$ Intensity	Formula	MS2 scan	Log_2 Fold Change (0.3µM/ Con)	Name	SMILES	InChIKey	Mscore	Precursor Label Type	MS1 S/N
7087	480.3097	481.3062	503.3870	504.3840	1721102	503870	247540	406383	$C_{23}H_{46}NO_7P$	7048	-2.80	LysoPE(18:1(9Z)/0:0)	[H][C@@](O)(COC(=O)CCCCCCC\C=C/CCCCCCCC)COP(O)(=O)OCCN	PYVRVRFVLRNJLY-MZMPXXGTSA-N	22.2	N15	29
7087	522.3567	523.3535	548.4441	549.4410	7158426	1098587	1090479	1319713	$C_{26}H_{50}NO_7P$	7116	-2.71	LysoPC(18:1(11Z))	CCCCCCC=CCCCCCCCCC(=O)OC[C@@H](O)COP([O-])(=O)OCC[N+](C)(C)C	PZRFVAHZNWPPAC-RUZDIDTESA-N	0.0	C13	95
7087	648.4615	649.4606	682.5757	683.5724	2451481	1121747	498976	595524	$C_{36}H_{68}NO_8P$	7095	-2.30	PE(15:0/14:1(9Z))	[H][C@@](COC(=O)CCCCCCCCCCCCCC)(COP(O)(=O)OCCN)OC(=O)CCCCCCC\C=C/CCCC	OIERHHCSSSRIJH-PGKKXZESSA-N	60.7	N15	43
5991	452.2785	453.2765	473.3489	474.3459	6712169	6533236	1728196	2895832	$C_{21}H_{42}NO_7P$	6021	-1.96	LysoPE(16:1(9Z)/0:0)	[H][C@@](O)(COC(=O)CCCCCCC\C=C/CCCCCC)COP(O)(=O)OCCN	DSOWUEHXZJUNID-WHXUGTBJSA-N	13.9	N15	1026
6194	298.2749	299.2719	316.3353	317.3323	529804	277625	207786	171710	$C_{18}H_{35}NO_2$	6190	-1.35	Palmitoleoyl Ethanolamide	CCCCCC\C=C/CCCCCCCC(=O)NCCO	WFRLANWAASSSFV-FPLPWBNLSA-N	0.0	C13	52
348	130.0503	131.0474	135.0672	136.0642	8262	6279	3432	10685	$C_5H_7NO_3$	329	-1.27	1-Pyrroline-4-hydroxy-2-carboxylate	OC1CN=C(C1)C(O)=O	AOMLMYXPXUTBQH-UHFFFAOYSA-N	8.5	C13	68
273	385.1306	391.1131	399.1777	405.1599	3266	1017	1842	2050	$C_{14}H_{24}N_6O_5S$	266	-0.83	S-Adenosylhomocysteine	N[C@@H](CCSC[C@H]1O[C@H]([C@H](O)[C@@H]1O)N1C=NC2=C(N)N=CN=C12)C(O)=O	ZJUKTBDSGOFHSH-WFMPWKQPSA-N	4.5	N15	12
5138	440.2786	441.2757	460.3458	461.3429	515593	650568	308222	319693	$C_{20}H_{42}NO_7P$	5093	-0.74	LysoPE(0:0/15:0)	[H][C@@](CO)(COP(O)(=O)OCCN)OC(=O)CCCCCCCCCCCCCC	GIUNBNKDUHWVHI-LJQANCHMSA-N	32.9	C12	80
651	104.1071	105.1042	109.1239	110.1210	122152	114269	75907	126557	$C_4H_{13}NO$	671	-0.69	Neurine	[OH-].C[N+](C)(C)C=C	NIPLJLVGZCKMP-UHFFFAOYSA-M	0.0	N15	23
256	166.0870	167.0840	175.1173	176.1142	1592	1494	1028	3138	$C_9H_{11}NO_2$	275	-0.63	Methyl N-methylanthranilate	CNC1=CC=CC=C1C(=O)OC	GVOWHGSUZUUUDR-UHFFFAOYSA-N	0.0	N15	22
259	148.0610	149.0581	153.0778	154.0748	5494	4859	3627	16905	$C_5H_9NO_4$	260	-0.60	DL-Glutamate	NC(CCC(O)=O)C(O)=O	WHUUTDBJXJRKMK-UHFFFAOYNA-N	0.0	C12	92
516	118.0865	119.0836	123.1034	124.1004	70584	66231	48595	90671	$C_5H_{11}NO_2$	483	-0.54	Norvaline	CCC[C@@H](N)C(O)=O	SNDPXSYFESPGGJ-SCSAIBSYSA-N	2.7	C13	29
348	159.0771	161.0712	165.0972	167.0913	9222	5738	7129	9637	$C_6H_{10}N_2O_3$	338	-0.37	1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	CC1(C)N(CO)C(=O)NC1=O	SIQZJFKTROUNPI-UHFFFAOYSA-N	2.6	C12	62
309	148.0610	149.0580	153.0778	154.0748	4897	5281	3834	8654	$C_5H_9NO_4$	260	-0.35	DL-Glutamate	NC(CCC(O)=O)C(O)=O	WHUUTDBJXJRKMK-UHFFFAOYNA-N	0.0	C12	56
7039	118.0864	119.0835	123.1032	124.1003	232026	227382	183446	304186	$C_5H_{11}NO_2$	6994	-0.34	Norvaline	CCC[C@@H](N)C(O)=O	SNDPXSYFESPGGJ-SCSAIBSYSA-N	0.0	C13	41
7159	159.0768	161.0709	165.0970	167.0910	541456	352087	444023	532270	$C_6H_{10}N_2O_3$	7150	-0.29	1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	CC1(C)N(CO)C(=O)NC1=O	SIQZJFKTROUNPI-UHFFFAOYSA-N	0.0	C12	54
7153	176.1034	179.0946	182.1236	185.1147	1117889	694709	941790	1316654	$C_6H_{13}N_3O_3$	7142	-0.25	Citrulline	N[C@@H](CCCNC(N)=O)C(O)=O	RHGKLRLOHDJJDR-BYPYZUCNSA-N	0.0	C12	118
7911	148.0609	149.0580	153.0777	154.0747	294553	319728	252496	469843	$C_5H_9NO_4$	7931	-0.22	D-Glutamic acid	N[C@H](CCC(O)=O)C(O)=O	WHUUTDBJXJRKMK-GSVOUGTGSA-N	0.0	C12	782
720	176.1035	179.0948	182.1237	185.1151	742079	525381	641098	810360	$C_6H_{13}N_3O_3$	698	-0.21	Citrulline	N[C@@H](CCCNC(N)=O)C(O)=O	RHGKLRLOHDJJDR-BYPYZUCNSA-N	0.0	C12	180
1617	298.0975	303.0827	309.1344	314.1196	17453480	16726398	15118440	18394530	$C_{11}H_{14}N_5O_3S$	1618	-0.21	5'-Methylthioadenosine	CSC[C@H]1O[C@H]([C@H](O)[C@@H]1O)N1C=NC2=C1N=CN=C2N	WUUGFSXJNOTRMR-IOSLPCCCSA-N	5.1	C12	570
145	176.1038	179.0949	182.1239	185.1151	5172	2300	4496	4730	$C_6H_{13}N_3O_3$	141	-0.20	Argininic acid	NC(=N)NCCC[C@H](O)C(O)=O	BMFMQGXDDJALKQ-BYPYZUCNSA-N	11.2	C12	30
309	166.0869	167.0839	175.1172	176.1142	1076	953	938	1889	$C_9H_{11}NO_2$	275	-0.20	D-Phenylalanine	C1=CC=C(C=C1)CC(C(=O)[O-])[NH3+]	COLNVLDHVKWLRT-MRVPVSSYSA-N	0.0	N15	13
516	104.1072	105.1042	109.1240	110.1210	17570	14112	15487	10017	$C_4H_{13}NO$	507	-0.18	Neurine	[OH-].C[N+](C)(C)C=C	NIPLJLVGZCKMP-UHFFFAOYSA-M	0.0	C13	4
250	188.0712	189.0682	199.1082	200.1058	11300	10478	10192	32140	$C_{11}H_9NO_2$	251	-0.15	Indoleacrylic acid	OC(=O)\C=C\C1=CC2=CC(N1)C=CC=C2	SXOUIMVOMIGLHO-AATRIKPKSA-N	11.6	C12	194
268	263.1402	265.1344	277.1872	279.1814	2243	1315	2075	1785	$C_{14}H_{18}N_2O_3$	222	-0.11	L-phenylalanyl-L-proline	NC(CC1=CC=CC=C1)C(=O)N1CCCC1C(O)=O	WEQJQNWXCSUVMA-UHFFFAOYSA-N	46.0	C13	10
382	118.0866	119.0837	123.1034	124.1006	1712	2507	1679	8063	$C_5H_{11}NO_2$	401	-0.03	L-Valine	CC(C)[C@H](N)C(O)=O	KZSNJWFQEVHDMF-BYPYZUCNSA-N	3.1	C13	47
145	148.0611	149.0581	153.0779	154.0749	3782	4585	3738	6096	$C_5H_9NO_4$	164	-0.02	N-Acetyl-DL-serine	CC(=O)NC(CO)C(=O)O	JJIHLJJYMXLCOY-UHFFFAOYSA-N	7.2	C13	38
264	166.0869	167.0840	175.1173	176.1142	3501	3232	3601	3138	$C_9H_{11}NO_2$	275	0.04	Methyl N-methylanthranilate	CNC1=CC=CC=C1C(=O)OC	GVOWHGSUZUUUDR-UHFFFAOYSA-N	0.0	N15	22
273	152.0575	157.0426	157.0742	162.0593	5610	5698	5851	3301	$C_5H_5N_5O$	235	0.06	Guanine	NC1=NC(=O)C2=C(N1)N=CN2	UYTPUPDQBNUYGX-UHFFFAOYSA-N	4.0	C13	20
180	159.0771	161.0712	165.0973	167.0915	1844	771	1961	653	$C_6H_{10}N_2O_3$	223	0.09	1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	CC1(C)N(CO)C(=O)NC1=O	SIQZJFKTROUNPI-UHFFFAOYSA-N	0.0	N15	4
284	205.0980	207.0921	216.1350	218.1291	5375	6216	5723	2568	$C_{11}H_{12}N_2O_2$	265	0.09	(±)-Tryptophan	NC(CC1=CNC2=CC=CC=C12)C(O)=O	QIVBCDIJIAJPQS-UHFFFAOYNA-N	17.0	N15	17
504	188.0713	189.0683	199.1082	200.1053	292778	278202	313539	430796	$C_{11}H_9NO_2$	456	0.10	Indoleacrylic acid	OC(=O)\C=C\C1=CC2=C(N1)C=CC=C2	SXOUIMVOMIGLHO-AATRIKPKSA-N	0.0	C12	137
273	136.0624	141.0476	141.0792	146.0642	3002	1781	3233	1467	$C_5H_5N_5$	277	0.11	Adenine	NC1=NC=NC2=C1NC=N2	GFFGJBXGBJISGV-UHFFFAOYSA-N	0.0	C13	9
264	116.0710	117.0680	121.0878	122.0848	1509	1096	1632	557	$C_6H_9NO_2$	274	0.11	4-Amino-2-methylenebutanoic acid	NCCC(=C)C(O)=O	FTWHFXMUJQRNBK-UHFFFAOYSA-N	4.0	C13	4
497	210.0768	211.0739	220.1104	221.1074	31546	48871	34809	75814	$C_{10}H_{11}NO_4$	511	0.14	N-Formyl-L-tyrosine	C1=CC(=CC=C1CC(C(=O)O)NC=O)O	ROUWPHMRHBMAFE-VIFPVBQESA-N	0.0	C13	24
1929	457.1132	461.1014	474.1702	478.1584	777329	845863	865254	1128229	$C_{17}H_{21}N_4O_9P$	1891	0.15	Flavin Mononucleotide	CC1=CC2=C(C=C1C)N(C[C@H](O)[C@H](O)[C@H](O)COP(O)(O)=O)C1=NC(=O)NC(=O)C1=N2	FVTCRASFADXXNN-SCRDCRAPSA-N	0.0	C12	69
301	156.0774	159.0684	162.0975	165.0887	1322	1265	1485	2515	$C_6H_9N_3O_2$	338	0.17	L-2-Amino-3-(1-pyrazolyl)propanoic acid	NC(CN1C=CC=N1)C(O)=O	PIGOPELHGLPKLL-UHFFFAOYNA-N	5.0	N15	17
497	348.0715	353.0566	358.1052	363.0902	25113	32106	28228	56363	$C_{10}H_{14}N_5O_7P$	533	0.17	2-hydroxy-dAMP	[H][C@]1(O)C[C@@](O)([H])O[C@]1([H])COP(O)(O)=O)N1C=NC2=C(N)N=C(O)N=C12	GEQDRKVFKBSPSW-KVQBGUIXSA-N	11.2	N15	17
259	159.0771	161.0711	165.0972	167.0914	782	743	880	9293	$C_6H_{10}N_2O_3$	223	0.17	1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	CC1(C)N(CO)C(=O)NC1=O	SIQZJFKTROUNPI-UHFFFAOYSA-N	0.0	N15	56
273	188.0715	189.0685	199.1085	200.1054	20843	23582	23623	14358	$C_{11}H_9NO_2$	251	0.18	Indoleacrylic acid	OC(=O)\C=C\C1=CC2=C(N1)C=CC=C2	SXOUIMVOMIGLHO-AATRIKPKSA-N	11.6	C12	88
2929	318.1820	321.1732	335.2391	338.2303	712582	415598	812220	568953	$C_{17}H_{22}N_2O_3$	2883	0.19	Tryptophyl-Isoleucine	CCC(C)C(NC(=O)C(N)CC1=CNC2=C1C=CC=C2)C(O)=O	PITVQFJBUFDJDD-UHFFFAOYSA-N	0.0	C13	65
3421	175.1194	179.1076	181.1396	185.1277	953532	1225147	1091760	1934711	$C_6H_{14}N_4O_2$	3374	0.20	L-Arginine	N[C@@H](CCCN=C(N)N)C(O)=O	ODKSFYDXXFIFQN-BYPYZUCNSA-N	0.0	C13	252
497	188.0713	189.0683	199.1083	200.1053	185514	190547	212761	430796	$C_{11}H_9NO_2$	456	0.20	Indoleacrylic acid	OC(=O)\C=C\C1=CC2=C(N1)C=CC=C2	SXOUIMVOMIGLHO-AATRIKPKSA-N	0.0	C12	137
249	205.0979	207.0920	216.1349	218.1290	4919	4893	5842	6544	$C_{11}H_{12}N_2O_2$	265	0.25	(±)-Tryptophan	NC(CC1=CNC2=CC=CC=C12)C(O)=O	QIVBCDIJIAJPQS-UHFFFAOYNA-N	17.0	N15	36
7394	348.0712	353.0564	358.1048	363.0901	33807	41400	40979	61140	$C_{10}H_{14}N_5O_7P$	7351	0.28	3'-AMP	NC1=C2N=CN[C@@H]3O[C@H](CO)[C@@H](OP(O)(O)=O)[C@H]3O)C2=NC=N1	LNQVTSROQXJCDD-KQYNXXCUSA-N	8.3	C13	63
238	272.1733	277.1585	283.2103	288.1953	8982	11042	11020	11400	$C_{11}H_{21}N_5O_3$	222	0.30	Arginyl-Proline	NC(CCCNC(N)=N)C(=O)N1CCCC1C(O)=O	LQJAALCCPOTJGB-UHFFFAOYSA-N	62.8	N15	68
273	137.0464	141.0346	142.0632	146.0513	5164	5365	6561	480	$C_5H_4N_4O$	277	0.35	Hypoxanthine	OC1=NC=NC2=C1NC=N2	FDGQSTZJBFJUBT-UHFFFAOYSA-N	0.0	C13	3
300	116.0709	117.0679	121.0877	122.0848	929	529	1186	570	$C_6H_9NO_2$	274	0.35	4-Amino-2-methylenebutanoic acid	NCCC(=C)C(O)=O	FTWHFXMUJQRNBK-UHFFFAOYSA-N	4.0	C13	4
5192	583.2573	587.2454	616.3681	620.3563	223013	284064	285828	387537	$C_{33}H_{34}N_4O_6$	5199	0.36	Biliverdin	CC1=C(C=C2)C(NC1=O)=C\C1=C(C)C(CCC(O)=O)=C(N1)\C=C1/N\C(=C\C2=NC(=O)C(C=C)=C2C)C(C)=C1CCC(O)=O	RCNSAJSGRJSBKK-YKSNQIBWSA-N	0.0	C13	68
7213	348.0713	353.0565	358.1049	363.0901	196288	247135	260942	393713	$C_{10}H_{14}N_5O_6P$	7197	0.41	2'-Deoxyguanosine 5'-monophosphate	NC1=NC2=C(N=CN2[C@H]2C[C@H](O)[C@@H](COP(O)(O)=O)O2)C(=O)N1	LTFMZDNNPPEQNG-KVQBGUIXSA-N	29.5	N15	77

7255	175.1195	179.1076	181.1396	185.1277	1477916	1796139	1994965	3215890	$C_6H_{14}N_4O_2$	7208	0.43	D-Arginine	N[C@H](CCCNC(N)=N)C(O)=O	ODKSFYDXXFIFQN-SCSAIBSYSA-N	0.0	C12	1052
497	303.1462	307.1344	318.1967	322.1844	28925	30996	39127	43694	$C_{15}H_{18}N_6O_3$	480	0.44	Histidinyl-Phenylalanine	NC(CC1=CN=CN1)C(=O)NC(CC1=CC=CC=C1)C(O)=O	XMAUFHMAAVTODF-UHFFFAOYSA-N	20.1	C12	14
7159	308.0919	311.0830	318.1254	321.1165	408645	495074	557197	678460	$C_{10}H_{17}N_3O_6S$	7157	0.45	Glutathione	N[C@@H](CCC(=O)N[C@@H](CS)C(=O)NCC(O)=O)C(O)=O	RWSXRVCMGQZWBV-WDSKDSINSA-N	19.2	C12	68
378	308.0924	311.0835	318.1259	321.1170	8656	11535	11839	13974	$C_{10}H_{17}N_3O_6S$	344	0.45	Glutathione	N[C@@H](CCC(=O)N[C@@H](CS)C(=O)NCC(O)=O)C(O)=O	RWSXRVCMGQZWBV-WDSKDSINSA-N	0.0	C13	88
759	175.1195	179.1077	181.1396	185.1277	1707376	2330049	2339478	3978705	$C_6H_{14}N_4O_2$	732	0.45	D-Arginine	N[C@H](CCCNC(N)=N)C(O)=O	ODKSFYDXXFIFQN-SCSAIBSYSA-N	0.0	C13	1033
7255	147.1132	149.1073	153.1333	155.1274	47481	18895	65506	46668	$C_6H_{14}N_2O_2$	7220	0.46	D-Lysine	NCCCC[C@@H](N)C(O)=O	KDXKERNSBIXSRK-RXMQYKEDSA-N	0.0	C12	13
243	308.0927	311.0838	318.1263	321.1174	20323	27942	28097	32068	$C_{10}H_{17}N_3O_6S$	213	0.47	Glutathione	N[C@@H](CCC(=O)N[C@@H](CS)C(=O)NCC(O)=O)C(O)=O	RWSXRVCMGQZWBV-WDSKDSINSA-N	11.8	C12	193
378	303.1464	307.1346	318.1969	322.1850	1898	1715	2637	2006	$C_{15}H_{18}N_6O_3$	344	0.47	Histidinyl-Phenylalanine	NC(CC1=CN=CN1)C(=O)NC(CC1=CC=CC=C1)C(O)=O	XMAUFHMAAVTODF-UHFFFAOYSA-N	26.0	C13	13
1593	253.1301	257.1183	264.1669	268.1551	338926	369289	473371	305606	$C_{11}H_{16}N_6O_3$	1560	0.48	Histidinyl-Proline	NC(CC1=CN=CN1)C(=O)N1CCCC1C(O)=O	LNCFUHAPNTYMJB-UHFFFAOYSA-N	28.6	C13	9
180	308.0924	311.0835	318.1260	321.1173	4825	7479	6747	6442	$C_{10}H_{17}N_3O_6S$	213	0.48	Glutathione	N[C@@H](CCC(=O)N[C@@H](CS)C(=O)NCC(O)=O)C(O)=O	RWSXRVCMGQZWBV-WDSKDSINSA-N	11.8	C12	43
196	272.1731	277.1583	283.2100	288.1953	2973	4999	4193	4694	$C_{11}H_{21}N_5O_3$	222	0.50	Arginyl-Proline	NC(CCCNC(N)=N)C(=O)N1CCCC1C(O)=O	LQJAALCCPOTJGB-UHFFFAOYSA-N	62.8	N15	28
180	152.0574	157.0425	157.0742	162.0595	2590	3359	3670	1638	$C_5H_5N_5O$	143	0.50	Guanine	NC1=NC(=O)C2=C(N1)N=CN2	UYTPUPDQBNUYGX-UHFFFAOYSA-N	0.0	C12	11
273	272.1731	277.1582	283.2099	288.1950	9722	13926	13834	7080	$C_{11}H_{21}N_5O_3$	224	0.51	Prolyl-Arginine	NC(=N)NCCCC(NC(=O)C1CCCN1)C(O)=O	HMNSRTLZAJHSIK-UHFFFAOYSA-N	1.5	C12	43
180	175.1197	179.1079	181.1399	185.1281	12831	17475	18553	19686	$C_6H_{14}N_4O_2$	141	0.53	L-Arginine	N[C@@H](CCCN=C(N)N)C(O)=O	ODKSFYDXXFIFQN-BYPYZUCNSA-N	6.9	C12	131
1575	272.1724	277.1575	283.2092	288.1945	5083493	6237272	7491910	5875956	$C_{11}H_{21}N_5O_3$	1528	0.56	Arginyl-Proline	NC(CCCNC(N)=N)C(=O)N1CCCC1C(O)=O	LQJAALCCPOTJGB-UHFFFAOYSA-N	26.7	N15	157
3529	352.1666	355.1576	372.2338	375.2249	160772	145919	238006	177491	$C_{20}H_{21}N_3O_3$	3525	0.57	Phenylalanyl-Tryptophan	NC(CC1=CC=CC=C1)C(=O)NC(CC1=CNC2=CC=CC=C12)C(O)=O	JMCOUWKXLXDERB-UHFFFAOYSA-N	0.0	C13	23
259	308.0924	311.0836	318.1260	321.1174	1730	2750	2586	32068	$C_{10}H_{17}N_3O_6S$	213	0.58	Glutathione	N[C@@H](CCC(=O)N[C@@H](CS)C(=O)NCC(O)=O)C(O)=O	RWSXRVCMGQZWBV-WDSKDSINSA-N	11.8	C12	193
497	142.0980	145.0891	148.1181	151.1093	43998	67416	66564	95564	$C_6H_{11}N_3O$	517	0.60	L-Histidinol	N[C@H](CO)CC1=CN=CN1	ZQISRDCJNBUVMM-YFKPBYRVSA-N	0.0	C12	37
8784	137.0461	141.0343	142.0630	146.0512	23536	24881	37870	34265	$C_5H_4N_4O$	8761	0.69	Allopurinol	O=C1N=CN=C2NNC=C12	OFCNXPDARWKPPY-UHFFFAOYSA-N	0.0	C13	63
2290	302.1507	305.1419	318.2043	321.1956	488520	462842	787671	715698	$C_{16}H_{19}N_3O_3$	2319	0.69	Prolyl-Tryptophan	OC(=O)C(CC1=CNC2=CC=CC=C12)NC(=O)C1CCCN1	UEKYKRQIAQHOOZ-UHFFFAOYSA-N	0.0	C12	59
273	269.1621	273.1502	281.2023	285.1904	7461	7597	12484	1362	$C_{12}H_{20}N_4O_3$	224	0.74	Leucyl-Histidine	CC(C)CC(N)C(=O)NC(CC1=CN=CN1)C(O)=O	XWOBNBRUDDUEEY-UHFFFAOYSA-N	0.9	N15	9
759	229.1553	231.1494	240.1923	242.1864	322383	247587	543330	339793	$C_{11}H_{20}N_2O_3$	804	0.75	Leucyl-Proline	CC(C)CC(N)C(=O)N1CCCC1C(O)=O	VTJUNIYRYIAIHF-UHFFFAOYSA-N	0.0	C12	88
250	288.2044	293.1895	300.2447	305.2299	5799	4910	10001	5679	$C_{12}H_{25}N_5O_3$	221	0.79	Arginyl-Leucine	CC(C)CC(NC(=O)C(N)CCCNC(N)=N)C(O)=O	WYBVBIHNJWOLCJ-UHFFFAOYSA-N	2.2	C12	34
7153	258.1107	259.1078	266.1376	267.1346	76771	94433	133143	105987	$C_8H_{20}NO_4P$	7191	0.79	Glycerophosphocholine	C[N+](C)(C)CCOP([O-])(=O)OC[C@@H](O)CO	SUHOQUVVVLNYQR-QMMMGPOBSA-N	0.0	C12	9
180	269.1620	273.1502	281.2023	285.1906	1380	1911	2500	1267	$C_{12}H_{20}N_4O_3$	224	0.86	Leucyl-Histidine	CC(C)CC(N)C(=O)NC(CC1=CN=CN1)C(O)=O	XWOBNBRUDDUEEY-UHFFFAOYSA-N	0.9	N15	9
273	219.1350	221.1290	228.1652	230.1591	1090	876	1977	2337	$C_9H_{18}N_2O_4$	234	0.86	L-Lysopine	CC(NC(CCCCN)C(O)=O)C(O)=O	ZZYYVZYAZCMNPG-UHFFFAOYNA-N	6.0	C13	13
273	229.1556	231.1497	240.1926	242.1865	10214	7017	18710	6745	$C_{11}H_{20}N_2O_3$	227	0.87	Leucyl-Proline	CC(C)CC(N)C(=O)N1CCCC1C(O)=O	VTJUNIYRYIAIHF-UHFFFAOYSA-N	11.6	C13	41
497	319.1411	323.1293	334.1915	338.1796	106384	119051	199921	231679	$C_{15}H_{18}N_4O_4$	469	0.91	Tyrosyl-Histidine	NC(CC1=CC=C(O)C=C1)C(=O)NC(CC1=CN=CN1)C(O)=O	ZQOOYCZQENFIMC-UHFFFAOYSA-N	37.3	C13	74
3439	361.1993	367.1814	378.2563	384.2386	972695	952115	1880096	1180116	$C_{17}H_{24}N_6O_3$	3394	0.95	Arginyl-Tryptophan	NC(CCCNC(N)=N)C(=O)NC(CC1=CNC2=CC=CC=C12)C(O)=O	QADCERNTBWTXFV-UHFFFAOYSA-N	0.0	N15	126
230	229.1558	231.1499	240.1928	242.1869	8972	6588	17757	11917	$C_{11}H_{20}N_2O_3$	227	0.98	Leucyl-Proline	CC(C)CC(N)C(=O)N1CCCC1C(O)=O	VTJUNIYRYIAIHF-UHFFFAOYSA-N	11.6	C13	72
230	260.1617	263.1530	271.1987	274.1898	1607	1416	3278	1953	$C_{11}H_{21}N_3O_4$	224	1.03	Hydroxyprolyl-Lysine	NCCCCC(NC(=O)C1CC(O)CN1)C(O)=O	BXAQOKHDAYJQPA-UHFFFAOYSA-N	2.4	C13	12
2503	253.1190	255.1131	265.1593	267.1533	299685	423382	632684	516946	$C_{12}H_{16}N_2O_4$	2541	1.08	Tyrosyl-Alanine	CC(NC(=O)C(N)CC1=CC=C(O)C=C1)C(O)=O	NLKUJNGEGZDXGO-UHFFFAOYSA-N	0.0	C13	51
273	260.1616	263.1529	271.1986	274.1895	1358	1509	2876	459	$C_{11}H_{21}N_3O_4$	224	1.08	Hydroxyprolyl-Lysine	NCCCCC(NC(=O)C1CC(O)CN1)C(O)=O	BXAQOKHDAYJQPA-UHFFFAOYSA-N	2.4	C13	3
2211	361.1993	367.1815	378.2565	384.2386	210915	396508	453475	363396	$C_{17}H_{24}N_6O_3$	2201	1.10	Tryptophyl-Arginine	NC(CC1=CNC2=C1C=CC=C2)C(=O)NC(CCCNC(N)=N)C(O)=O	LCPVBXOHXMBLFW-UHFFFAOYSA-N	0.0	C13	30
1575	288.2035	293.1889	300.2440	305.2292	1884540	2072277	4053500	2690484	$C_{12}H_{25}N_5O_3$	1564	1.10	Arginyl-Leucine	CC(C)CC(NC(=O)C(N)CCCNC(N)=N)C(O)=O	WYBVBIHNJWOLCJ-UHFFFAOYSA-N	0.0	C12	73
497	229.1555	231.1495	240.1924	242.1865	115962	119944	254926	153113	$C_{11}H_{20}N_2O_3$	457	1.14	Leucyl-Proline	CC(C)CC(N)C(=O)N1CCCC1C(O)=O	VTJUNIYRYIAIHF-UHFFFAOYSA-N	10.0	N15	59
180	288.2042	293.1895	300.2446	305.2299	1300	1485	2904	2439	$C_{12}H_{25}N_5O_3$	221	1.16	Arginyl-Leucine	CC(C)CC(NC(=O)C(N)CCCNC(N)=N)C(O)=O	WYBVBIHNJWOLCJ-UHFFFAOYSA-N	2.2	C12	15
2308	195.1134	197.1074	205.1469	207.1410	820378	984623	1877375	668150	$C_{10}H_{14}N_2O_2$	2335	1.19	Laccarin	CC1CCNC2=C(C(C)=O)C(=O)NC12	DAMBAJDWLIFTNW-UHFFFAOYSA-N	0.0	C12	49
273	260.1981	263.1892	272.2384	275.2293	4328	5171	9932	2789	$C_{12}H_{25}N_3O_3$	224	1.20	Lysyl-Leucine	CC(C)CC(NC(=O)C(N)CCCCN)C(O)=O	ATIPDCIQTUXABX-UHFFFAOYSA-N	2.2	C13	17
230	322.1890	327.1740	337.2392	342.2244	3944	4831	9093	6691	$C_{15}H_{23}N_5O_3$	278	1.21	Arginyl-Phenylalanine	NC(CCCNC(N)=N)C(=O)NC(CC1=CC=CC=C1)C(O)=O	PQBHGSGQZSOLIR-UHFFFAOYSA-N	28.4	N15	40
2563	279.1710	281.1652	294.2214	296.2155	288934	301991	703252	351849	$C_{15}H_{22}N_2O_3$	2534	1.28	Isoleucyl-Phenylalanine	CCC(C)C(NC(=O)NC(CC1=CC=CC=C1)C(O)=O	WMDZARSFSMZOQO-UHFFFAOYSA-N	0.0	N15	31
558	260.1976	263.1887	272.2379	275.2290	145455	179180	357184	179012	$C_{12}H_{25}N_3O_3$	587	1.30	Leucyl-Lysine	CC(C)CC(N)C(=O)NC(CCCCN)C(O)=O	OTXBNHIUIHNGAO-UHFFFAOYSA-N	0.0	C13	45
180	229.1557	231.1497	240.1927	242.1868	2654	2380	6625	2744	$C_{11}H_{20}N_2O_3$	227	1.32	Leucyl-Proline	CC(C)CC(N)C(=O)N1CCCC1C(O)=O	VTJUNIYRYIAIHF-UHFFFAOYSA-N	11.6	C13	18
3421	361.1993	367.1815	378.2563	384.2386	251942	381961	701564	1180116	$C_{17}H_{24}N_6O_3$	3394	1.48	Arginyl-Tryptophan	NC(CCCNC(N)=N)C(=O)NC(CC1=CNC2=CC=CC=C12)C(O)=O	QADCERNTBWTXFV-UHFFFAOYSA-N	0.0	N15	126
180	260.1980	263.1893	272.2384	275.2296	988	1283	2824	652	$C_{12}H_{25}N_3O_3$	224	1.52	Lysyl-Leucine	CC(C)CC(NC(=O)C(N)CCCCN)C(O)=O	ATIPDCIQTUXABX-UHFFFAOYSA-N	2.2	C13	4

Columns: [1]The MS1 scan number in the raw file in which the formula was detected. [2-5]The monoisotopic mass of the unlabelled (12C) and labeled (15N, 13C, 13C15N) peaks as detected. [6-9]The intensities of the detected peaks. [10]The chemical formula as determined by JUMPm. [11]The MS2 scan number in the raw file in which the structure was detected. [12]The Log2 ratio of the peak intensity for the 0.3uM Bortezomib treated sample divided by the control. [13]The best scoring structure candidate for the detected formula as determined by JUMPm. [14]The SMILES notaton for the annotated structure. [15]The InchiKey for the annotated structure. [16]The Mscore as determined by JUMPm, which scores the quality of the match between the observed MS2 fragment ions and the predicted MS2 fragment ions for the reported structure. [17]The type of label from which the MS2 scan was detected. [18]The signal to noise ratio for the detected peak in the MS1 scan.

Table 1: Metabolite formulas and structures identified from LC-MS analysis of Bortezomib treated yeast using MISSILE and JUMPm.

isotope labels are used by JUMPm software to determine chemical formulas and perform relative quantification and false discovery control.

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