Electronic Supplementary Material (ESI) for RSC Chemical Biology.

SUPPORTING INFORMATION

Squaric acid as a new chemoselective moiety for mass spectrometry-based metabolomics analysis of amines

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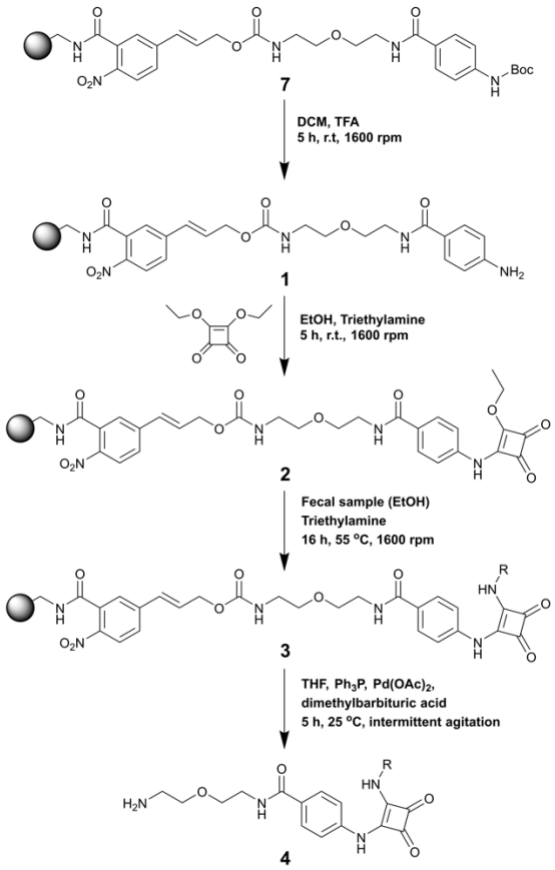
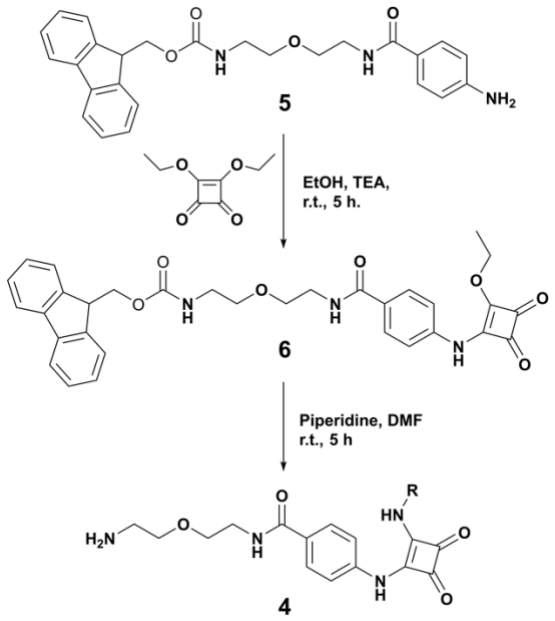


Scheme S1: Preparation of chemical probe activated for amine conjugation.



Scheme S2: Preparation of simplified chemical probe activated for amine conjugation.

2. Supporting figures

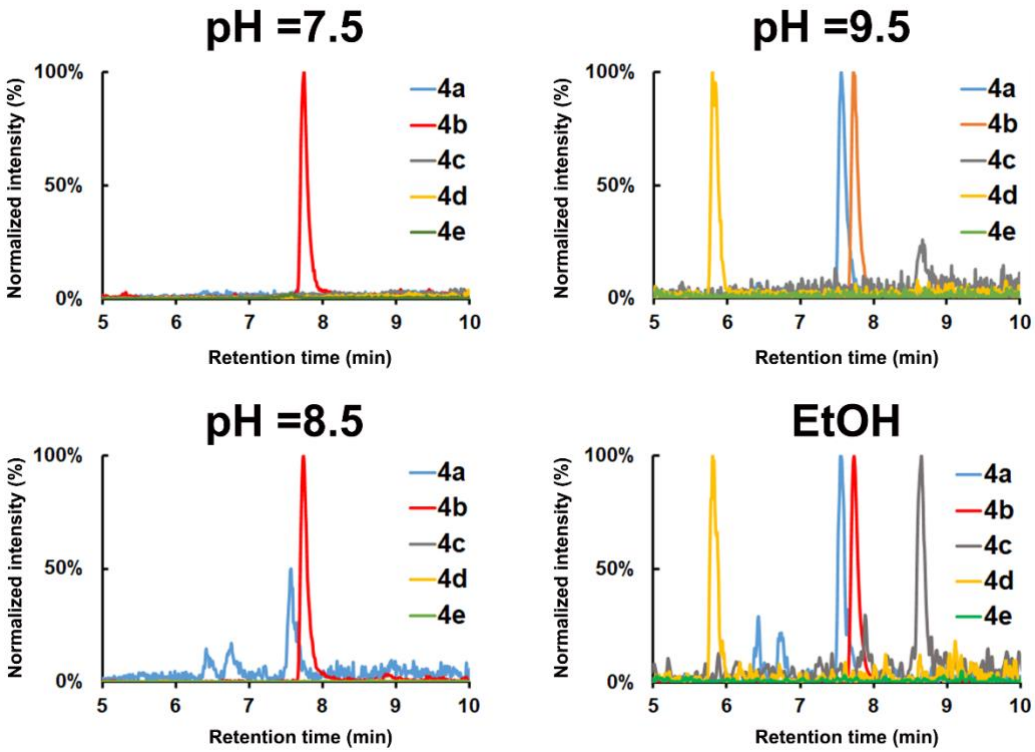


Figure S1: Extracted ion chromatograms resulting from reactivity experiments in different condition. Conjugated 1-aminopropane (**4a**), piperidine (**4b**), aniline (**4c**), L-serine (**4d**), *N*-acetyl-L-cysteine (**4e**)

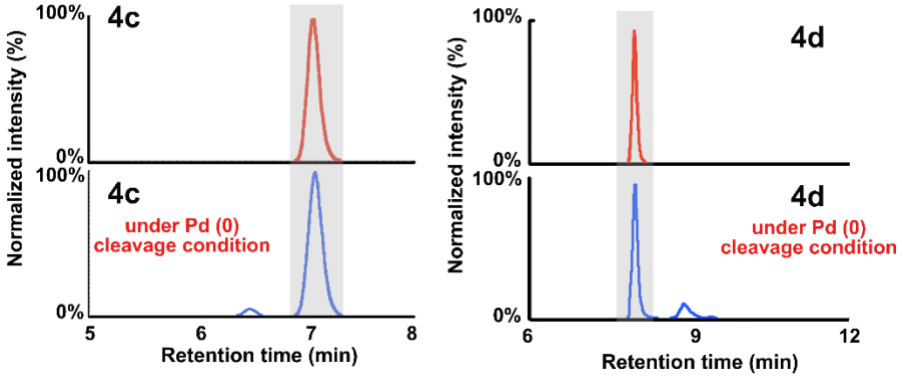


Figure S2: Extracted ion chromatograms resulting from stability experiments. **4c** and **4d** for treatment with $Pd(OAc)_2$, PPh_3, dimethylbarbituric acid in THF, 16 h, 25 °C. Intensities were normalized to 100% for each EIC separately.

Conjugates $[M+H]^+$	RT/min	6M 18M 21F	Annotation
319.1401	5.72		Ammonia
363.1658	5.94		Ethanolamine
373.1869	7.00		Pyrrolidine
377.1449	7.71/6.60		Glycine
387.2024	7.73		Piperidine
389.1456	10.02/10.95		2-Aminoacrylic acid
391.1608	6.79		Beta-Alanine; L-Alanine; Sarcosine; D-Alanine
392.1557	5.88		(Aminomethyl)carbamic acid
401.2180	7.79		Cyclohexylamine; 2-Methylpiperidine
403.1973	6.39		5-Aminopentanal
405.1396	8.76		3-Oxoalanine
407.1558	9.05/10.44/9.33		L-Serine; D-Serine; Protein serine
417.1764	6.70		L-Proline; D-Proline; 4-Amino-2-methylenebutanoic acid
418.2084	6.06		5-Aminopentanamide
419.1920	8.08		L-Valine; N-Methyl-a-aminoisobutyric acid; 5-Aminopentanoic acid
420.1876	7.43		2,4-Diaminobutyric acid; L-2,4-diaminobutyric acid
425.1810	8.99		p-Anisidine; 2-Propionylpyrrole; 4-Hydroxybenzylamine
427.1278	6.04		Taurine
428.1778	5.12		Melamine
431.1926	7.56		1-Piperidine carboxylic acid; Pipecolic acid; L-Pipecolic acid; D-Pipecolic acid;
432.2237	6.23/7.13		N-Acetylputrescine
433.1716	9.70		4-Hydroxyproline; 5-Aminolevulinic acid; L-Glutamic gamma-semialdehyde;
433.2073	8.86		L-Isoleucine; L-Alloisoleucine; L-Leucine; L-Norleucine; Aminocaproic acid;
435.1869	9.83		L-2-Amino-5-hydroxypentanoic acid; N-Lactoyl ethanolamine;
443.1931	1.59		L-Hypoglycin A
446.2394	7.21		N-Acetylcadaverine; 1-(3-Aminopropyl)-4-aminobutanal
448.1653	11.48		2-methyl-1,3-thiazolidine-2-carboxamide
448.2181	7.47/7.10/7.92/9.43		2,6-diaminohexanoic acid; L-Lysine; D-Lysine;
451.1420	9.92		3,5-dihydroxy-3,4-dihydro-1,4-benzothiazine
451.1823	9.21/10.86/10.18		6-Methyladenine; 1-Methyladenine; 3-Methyladenine; 7-Methyladenine
455.1773	8.94/6.60		FAPy-adenine
457.2821	6.34		Propylhexedrine
461.1794	11.16		L-Hexahydro-3-imino-1,2,4-oxadiazepine-3-carboxylic acid
461.2858	8.26		Aminopropylcadaverine; 4,4'-Diaminodibutylamine
462.1980	8.69		D-Alanyl-D-alanine; Alanyl-Alanine; 4-Acetamido-2-aminobutanoic acid
462.2334	5.23		N(6)-Methyllysine; Isoputreanine
463.1610	8.58		2-Indolecarboxylic acid; Indole-3-carboxylic acid; 3-Formyl-6-hydroxyindole
464.2127	8.06		5-Hydroxylysine
467.1591	8.37		Methionine sulfoxide; Ethiin
477.1737	5.74		Guanidinosuccinic acid
478.2072	5.07		Serotonin
479.1896	11.50		O-Ureidohomoserine
479.1920	11.84		5-Hydroxytryptophol; 2-Propenyl 2-aminobenzoate
480.1644	8.12		Melizame
481.2080	11.97		(R)-Salsolinol; alpha-Methylphenylalanine; N-methylphenylalanine; Salsolinol;
482.1697	4.96		Methionine sulfoximine
482.2020	6.65		5-Hydroxykynurenamine; Tyrosinamide; L-2-Amino-3-(4-aminophenyl)propanoic acid
483.1722	5.04		8-Hydroxy-7-methylguanine
487.1212	11.45		Phosphoserine; DL-O-Phosphoserine
493.1709	11.79		5-Hydroxyindoleacetic acid
501.1728	51.68		(S)-Isowillardiine
502.2647	10.22		Spermine dialdehyde
503.1482	4.92		Thiabendazole
507.1505	8.82		4,6-Dihydroxy-2-quinolinecarboxylic acid
513.2364	11.23		Methoxamine; Orciprenaline; Isoproterenol

Figure S3: The first 55 of the total 165 metabolites detected after analysis of fecal samples using the amine-specific chemical probe. Red indicates no detection; Green denotes detected metabolites, which are annotated on the basis of m/z value as an output from the XCMS analysis.

Conjugates $[M+H]^+$	RT/min	6M 18M 21F	Annotation
515.2499	11.24		2-nonenoylglycine
516.1543	11.79		Sulfacetamide
517.1675	9.00/9.14/9.52/8.51/8.82		Glycerylphosphorylethanolamine; sn-glycero-3-Phosphoethanolamine
518.1704	8.82/8.50/9.01		6-Aminopenicillanic acid
520.1863	9.07/9.94		Cysteinyl-Proline; Prolyl-Cysteine
520.2027	10.18		Glutamylalanine; gamma-Glutamylalanine; Hydroxyprolyl-Serine;
520.2402	11.72		Isoleucyl-Serine; Leucyl-Serine; Serylisoleucine; Serylleucine;
521.1627	9.53		L-Oxalylalbizziine
523.2663	5.12		Procarbazine
523.9941	16.86		Perchlorate
536.1195	10.18/10.31		Riluzole
536.1806	8.47		Cysteinyl-Hydroxyproline; Hydroxyprolyl-Cysteine
538.1558	9.93		Brassinin
545.1990	9.35/10.91/9.35		Cytidine; Cytarabine; gamma-Glutamyl-beta-cyanoalanine
545.2354	9.81		Glutaminylproline; Prolyl-Glutamine; Prolyl-Gamma-glutamate
547.1583	11.80		Lamivudine sulfoxide
547.2144	10.76/11.64/10.44		Asparaginyl-Hydroxyproline; Hydroxyprolyl-Asparagine
548.2179	10.45/10.46		Methionyl-Proline; Prolyl-Methionine
549.1575	10.64		Emtricitabine
551.1719	6.68/9.15/8.22		Sulfapyridine
552.1742	6.68/9.17		gamma-Glutamylcysteine; Glutamylcysteine
556.1838	9.86		Acetaminophen cystein
557.1289	10.91		Sulfathiazole
561.1922	6.67/9.68		Mizoribine
561.2261	11.09		Clobenzorex
561.2291	11.26		Glutaminylhydroxyproline; Hydroxyprolyl-Glutamine; Hydroxyprolyl-Gamma-glutamate
562.2495	6.51		Gamma-Glutamylisoleucine; Glutamylisoleucine; Glutamylleucine;
563.2101	9.03/8.89/9.39		Glutaminylaspartic acid; Glutamylasparagine; gamma-Glutamylasparagine;
564.0426	5.26		3-phosphonato-D-glyceroyl Phosphate(4-)
564.1929	12.08		Glutamylaspartic acid; L-beta-aspartyl-L-glutamic acid; gamma-Glutamylaspartic acid
564.2127	9.05/8.90/9.38		Hydroxyprolyl-Methionine; Methionyl-Hydroxyproline
565.2809	8.36		Protriptyline; Nortriptyline; demethylmaprotiline
566.1918	7.99		Aspartyl-Methionine; Methionyl-Aspartate; gamma-Glutamyl-S-methylcysteine
570.1991	10.43		Cysteinyl-Phenylalanine; Phenylalanyl-Cysteine
576.2520	12.23		Lysyl-Glutamate
580.2061	6.67/8.40		Glutamylmethionine; gamma-Glutamylmethionine
581.2336	8.56		Asparaginyl-Phenylalanine; Phenylalanyl-Asparagine
583.1356	12.84		Ajocysteine
586.1946	9.38		Cysteinyl-Tyrosine; Tyrosyl-Cysteine
587.1737	10.41		Cladribine
589.2218	10.92		Rutaecarpine
589.2232	10.79/10.40/10.67		N-Ribosylhistidine
590.2615	13.20		Imazamethabenz-methyl
591.2411	10.73/9.58		Ophthalmic acid;
592.1657	9.38		1-[4,9-Dihydro-2-(methylthio)-1,3-thiazino[6,5-b]indol-4-yl]-2-propanone
596.2171	10.90		Distichonic acid A; Distichonic acid B
601.1849	6.65		Fenbendazole
601.2470	5.97		Saxitoxin
605.2370	10.86		Indoleacetyl glutamine
605.2593	12.20		N5-Acetyl-N2-gamma-L-glutamyl-L-ornithine; Fenoterol; 6-Hydroxyetodolac;
605.2599	12.35		Fenoterol; 6-Hydroxyetodolac; 7-Hydroxyetodolac; alpha-noroxycodol; beta-noroxycodol
607.1528	11.79		Cytidine 2',3'-cyclic phosphate
611.1341	11.79		Lamivudine-monophosphate; 3-oxobrimonidine
613.1263	11.95		4'-Hydroxydiclofenac; 3'-Hydroxydiclofenac; 5-Hydroxydiclofenac
617.2589	12.24		2,5-dihydroxy-5-(methylamino)-3,4-diphenylpentanoic acid

Figure S4: The second 55 of the total 165 metabolites detected after analysis of fecal samples using the amine-specific chemical probe. Red indicates no detection; Green denotes detected metabolites, which are annotated on the basis of m/z value as an output from the XCMS analysis.

Conjugates $[M+H]^+$	RT/min	6M 18M 21F	Annotation
619.2333	10.15		N-(1-Deoxy-1-fructosyl)histidine
620.2721	13.62		Fluvoxamine
624.2492	12.62		Avenic acid A; N2-Galacturonyl-L-lysine; N6-Galacturonyl-L-lysine
626.1385	11.88		Dorzolamide
631.1635	10.45		Cyclic AMP; Adenosine 2',3'-cyclic phosphate
631.2735	13.23		xi-Anomuricine
633.2501	9.16		Ciprofloxacin
644.2350	11.91		Clozapine N-oxide
650.2836	3.05		Enalaprilat
651.2245	8.62		Ampicillin; Cefradine
653.2501	13.27/13.22/12.59		Lomefloxacin
653.3329	8.82		Sphingosine 1-phosphate (d16:1-P)
656.2338	11.06/9.32/9.17		Allithiamine
665.1999	5.09		Cefadroxil
667.1700	9.17		Fludarabine; Metolazone; Indapamide
667.1715	9.17		Metolazone
669.2656	7.97		Tryptophyl-Tyrosine; Tyrosyl-Tryptophan
676.2266	9.14		Portulacaxanthin II
677.2745	8.10/9.74		Gatifloxacin
681.2700	7.61/8.05		2-(Arabinosylamino)-3-(glucosylamino)propanenitrile
682.1223	5.76		4,11-Dichloro-5,12-dihydroquino[2,3-b]acridine-7,14-dione
683.1920	11.78		Celecoxib
684.1215	7.86		Olsalazine-O-sulfate
685.2208	10.12/9.79		Succinyladenosine
700.2545	8.10		S-adenosyl-L-methioninate
707.2162	10.24		Cephaloglycin
710.2512	7.75		Wybutoxine
715.1277	11.77		Halofuginone
721.1469	5.76		S-(2,2-Dichloro-1-hydroxy)ethyl glutathione
725.1190	13.68		Gemcitabine diphosphate
725.1331	8.69		5-Amino-4-hydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid
726.1496	5.74		Thiamine(1+) Diphosphate(1-)
729.1330	7.36/6.78		Adenosine phosphosulfate
730.1244	7.78		IDP
741.2527	4.73		$C_{26}H_{31}N_3O_9S$
741.3840	9.16		LysoPE(0:0/15:0); LysoPE(15:0/0:0)
750.2259	8.85		Temocaprilat
773.2654	11.51		10-Formyldihydrofolate; (6S)-5-formyltetrahydrofolate
775.2574	12.05		$C_{19}H_{27}N_3O_9S$
777.1186	9.11		2',3'-Dideoxyadenosine-5-triphosphate; Dideoxyadenosine Triphosphate
781.3805	8.15		PC-M5'
782.2377	7.84		Cefepime
798.2439	6.77		1-Nitro-5-glutathionyl-6-hydroxy-5,6-dihydronaphthalene;
805.4193	7.96		LysoPE(0:0/20:3(11Z,14Z,17Z)); LysoPE(0:0/20:3(5Z,8Z,11Z));
825.1420	14.75/15.51/10.61		Ceftiofur
833.2655	11.47		Neoacrimarine F; Neoacrimarine I
861.1871	10.47		Adenosine diphosphate ribose
863.2811	11.46		$C_{22}H_{31}N_3O_{12}S$
867.3680	7.27		Hemorphin-4
889.1802	11.58		GDP-4-Dehydro-6-deoxy-D-mannose; GDP-4-Dehydro-6-L-deoxygalactose
895.2446	7.04		$C_{25}H_{27}N_3O_{12}S$
911.2816	11.54		$C_{26}H_{31}N_3O_{12}S$
914.2711	9.41		Oxidized glutathione
936.4078	7.26		Rescinnamine
953.3232	11.72		$C_{29}H_{37}N_3O_{12}S$

Figure S5: The third 55 of the total 165 metabolites detected after analysis of fecal samples using the amine-specific chemical probe. Red indicates no detection; Green denotes detected metabolites, which are annotated on the basis of m/z value as an output from the XCMS analysis.

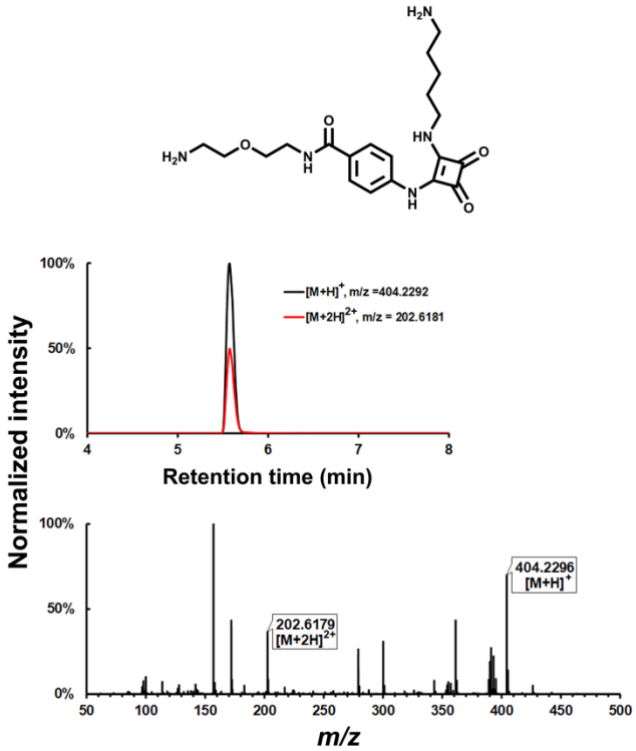


Figure S6: Representative EIC and mass spectrum for the conjugate of 1,5-diaminopentane in standard MS^E mode analysis. With a filter of m/z = 319 during our data analysis, we can exclude doubly annotated metabolites for charge states higher than 1.

3. Supporting tables

Table S1: LOD experiment in standard amines.

Concentration	Signal/Noise				
	1-Aminopropane	Piperidine	Aniline	L-Serine	2-Amino-1-propanol
100 µM	16.73	9.75	6.41	ND	3.55
10 µM	0.78	1.41	3.14	ND	1.04
1.0 µM	ND	ND	ND	ND	ND
100 nM	ND	ND	ND	ND	ND

Table S2: A list of the standard library synthesized from commercial amines in this study.

Amine	Monoisotopic mass	Conjugates $[M+H]^+$	RT (min)
Ammonia	17.0265	319.1401	5.72
Methylamine	31.0422	333.1558	6.15
Dimethylamine	45.0578	347.1714	6.22
Ethanolamine	61.0527	363.1663	6.76
3-Aminopropanonitrile	70.0531	372.1667	5.21
1-Amino-propan-2-ol	75.0684	377.1820	6.40
2-(Methylamino) ethan-1-ol	75.0684	377.1820	6.00
3-Aminopropan-1-ol	75.0684	377.1820	6.34
Piperidine	85.0891	387.2027	7.73
1,4 Diaminobutane	88.1000	390.2136	5.22
Alanine	89.0476	391.1612	6.80
Aniline	93.0578	395.1714	9.84
1,5-Diaminopentane	102.1156	404.2292	5.58
2-Aminoisobutyric acid	103.0633	405.1769	5.09
L-α-aminobutyric acid	103.0633	405.1769	7.43
y-Aminobutyric acid	103.0633	405.1769	6.61

Diethanolamine	105.0789	407.1925	5.66
Hypotaurine	109.0197	411.1333	5.18
Histamine dihydrochloride	113.0953	415.2089	7.49
L-Valine	117.0789	419.1925	8.08
5-Aminovaleric acid	117.0789	419.1925	7.05
L-Norvaline	117.0789	419.1925	8.22
L-Homoserine	119.0582	421.1718	5.94
L-Threonine	119.0600	421.1736	6.38
α-Methylbenzylamine	121.0891	423.2027	9.13
2,6-Dimethylaniline	121.0891	423.2027	8.37
Phenethylamine	121.0891	423.2027	4.77
5-Methylcytosine	125.0589	427.1725	5.51
L-(-)-Pipecolic acid	129.0789	431.1925	7.56
L-Hydroxyproline	131.0582	433.1718	5.38
L-Isoleucine	131.0946	433.2082	8.82
L-Norleucine	131.0946	433.2082	5.10
6-Aminocaproic acid	131.0946	433.2082	7.64
Glycyl-glycine	132.0534	434.1670	5.37
D-Aspartic acid	133.0375	435.1511	6.29
DL-Homocysteine	135.0354	437.1490	7.21
Adenine	135.0544	437.1680	6.44
4-Aminobenzoic acid	137.0476	439.1612	11.05
O-Phosphoryl-ethanolamine	141.0190	443.1326	11.49
L-Histidinol	141.0902	443.2038	7.94
N-(5-Aminopentyl) acetamide	144.1262	446.2398	7.21
Spermidine	145.1578	447.2714	7.00
L-Lysine	146.1055	448.2191	6.88
L-Glutamine	146.1400	448.2536	7.50
L-Glutamic acid	147.0531	449.1667	6.51
Phenylglycine	151.0633	453.1769	7.34
N-Methyltyramine HCl	151.0997	453.2133	7.29
5-Aminosalicylic acid	153.0425	455.1561	5.18
DL-2-Aminocaprylic acid	159.1259	461.2395	10.33
Tryptamine	160.1000	462.2136	8.74
S-Allyl-L-cysteine	161.0511	463.1647	8.57?
N-Methyl-L-glutamic acid	161.0688	463.1824	5.94
Aminoadipic acid	161.0700	463.1836	9.97
DL-5-Hydroxylysine	162.1004	464.2140	9.80
7-Methylguanine	165.0650	467.1786	5.66
Pyridoxamine	168.0898	470.2034	8.12
L-Cysteic acid	169.0044	471.1180	10.78
3-Methyl-L-histidine	169.0851	471.1987	5.34
Glycylvaline	174.1004	476.2140	7.47
Glucosamine hydrochloride	180.0872	482.2008	6.74
N-α-Acetyl-L-lysine	188.1160	490.2296	10.24
Glycyl-L-leucine	188.1160	490.2296	8.24
N-Acetyl-L-Lysine	188.1161	490.2297	6.81
L-Homoarginine	188.1273	490.2409	8.19
L-Homocitrulline	189.1113	491.2249	10.99
2,6-Diaminopimelic Acid	190.0953	492.2089	9.82
Leucyl-Alanine	202.1317	504.2453	8.49
L-Tryptophan	204.0898	506.2034	8.49
D-(+)-Tryptophan	204.0898	506.2034	7.69
L-Kynurenine	208.0847	510.1983	9.82
L-Alany-L-glutamine	217.1063	519.2199	6.34
L-5-Hydroxytryptophan	220.0847	522.1983	9.87
L-Carnosine	226.1066	528.2202	8.65
L-Alanyl-L-phenylalanine	236.1161	538.2297	8.41
Glycyl-L-tyrosine	238.0953	540.2089	7.01
L-Cystine	240.0238	542.1374	8.69
Triamterene	253.1075	555.2211	11.35
5-Methylcytidine	257.1012	559.2148	8.49
Thiamine	265.1123	567.2259	9.37
Atenolol	266.1630	568.2766	8.50
2'-Deoxyguanosine	267.1000	569.2136	8.97
Guanosine	283.0916	585.2052	8.93
Hydrochlorothiazide	296.9644	599.078	7.49
Furosemide	330.0077	632.1213	6.65
Adenosine-5'-monophosphate	347.0630	649.1766	8.34
Folinic acid calcium salt hydrate	471.1503	773.2639	8.13
Cytidine 5'-triphosphate	482.9845	785.0981	9.90/10.22
Adenosine 5'-triphosphate	506.9957	809.1093	5.38
Guanosine 5′-triphosphate	522.9907	825.1043	7.16
NAD	664.1169	966.2305	8.44
S-(5'-Adenosyl)-L-methionine	399.1450	701.2586	7.48

Table S3: A list of validated metabolites and their associated diseases.

HMDB ID	Metabolites	Disease
HMDB0000051	Ammonia	Short bowel syndrome
HMDB0000164	Methylamine	Crohn's disease
HMDB0000087	Dimethylamine	Pancreatic cancer
HMDB0034301	Piperidine	Colorectal cancer
HMDB0001414	1,4 Diaminobutane	Alzheimer's disease
HMDB0000161	L-Alanine	Colorectal cancer
HMDB0001310	D-Alanine	Early preeclampsia
HMDB0002322	1,5-Diaminopentane	Colorectal cancer
HMDB0000452	L-α-aminobutyric acid	Alzheimer's disease
HMDB0000112	y-Aminobutyric acid	Alzheimer's disease
HMDB0000883	L-Valine	Schizophrenia
HMDB0003355	5-Aminovaleric acid	Irritable bowel syndrome
HMDB0000167	L-Threonine	Heart failure
HMDB0012275	2-Phenethylamine	Crohn's disease
HMDB0000070	Pipecolic acid	Colorectal cancer
HMDB0000725	L-Hydroxyproline	Alzheimer's disease
HMDB0000172	L-Isoleucine	Heart failure
HMDB0000742	DL-Homocysteine	Stroke
HMDB0001392	4-Aminobenzoic acid	Colorectal cancer
HMDB0000224	*O*-Phosphoryl-ethanolamine	Crohn's disease
HMDB0002284	*N*-(5-Aminopentyl) acetamide	Colorectal cancer
HMDB0000182	L-Lysine	Schizophrenia
HMDB0000641	L-Glutamine	Colorectal cancer
HMDB0034323	*S*-Allyl-L-cysteine	Alzheimer's disease
HMDB0000450	DL-5-Hydroxylysine	Colorectal cancer
HMDB0028854	Glycylvaline	Colorectal cancer
HMDB0000446	*N*-α-Acetyl-L-lysine	Colorectal cancer
HMDB0000472	L-5-Hydroxytryptophan	Ulcerative colitis
HMDB0000033	L-Carnosine	Alzheimer's disease
HMDB0000235	Thiamine	Hemodialysis
HMDB0000133	Guanosine	Colorectal cancer

4. General

All non-aqueous reactions were performed using flame- or oven dried glassware under an atmosphere of dry nitrogen. All reagents and solvents were purchased from Sigma-Aldrich or Fischer Scientific and were used without further purification. The in-house built metabolite library was obtained from MetaSci. Mass spectrometry grade solvents were used for UHPLC-ESI-MS analysis. Solutions were concentrated in vacuo on a Heidolph or a IKA rotary evaporator. Thin Layer Chromatography (TLC) was performed on silica gel 60 F-254 plates. Visualization of the developed chromatogram was performed using fluorescence quenching. Chromatographic purification of products was accomplished using flash column chromatography on Merck silica gel 60 (40−63 μm. All synthesized compounds were ⩾95% pure as determined by NMR. NMR spectra were recorded on Agilent 400 MHz spectrometer (1H NMR: 399.97 MHz, 13C NMR: 100.58 MHz). Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Glass vials used for handling magnetic beads were microwave vials from Biotage (0.2-0.5 mL or 0.5-2.0 mL). High-resolution mass spectra were acquired on a SYNAPT G2-S High-Definition Mass Spectrometer (HDMS) using an electrospray ionization (ESI) source with an AQCUITY UPLC I-class system and equipped with a Waters ACQUITY UPLC BEH C18 column (2.1 × 75 mm, 1.7 μm particle size) for chemoselective-probe based metabolomics analysis or Waters ACQUITY UPLC HSS T3 column (1.8 × 100 mm, 2.1 μm particle size) for compound characterization of compound **6**. The mobile phase consisted of a combination of 0.1% formic acid in MilliQ water (A) and 0.1% formic acid in LC-MS grade methanol (B). The column temperature was 40 °C and the mobile phase gradient applied was as follows: 0-2 min, 0% B; 2-15 min, 0-100% B; 15-18 min, 100% B; 18-20 min, 100-0% B; 20-25 min, 0% B, with a flow rate of 0.3 ml/min.

The samples were introduced into the q-TOF using positive electrospray ionization. The capillary voltage was set to -2.50 kV and the cone voltage was 40 V. The source temperature was 100 °C, the cone gas flow 50 L/min and the desolvation gas flow 600 L/h. The instrument was operated in MSE mode, the scan range was m/z = 50-1200, and the scan time was 0.3 s. A solution of sodium formate (0.5 mM in 2-propanol: water, 90:10, v/v) was used to calibrate the instrument and a solution of leucine-encephalin (2 ng/μl in acetonitrile: 0.1% formic acid in water, 50:50, v/v) was used for the lock mass correction at an injection rate of 30 s.

All chemical synthesis protocols and characterization data are available in the Supplementary Information.

5. Description of procedures

5.1 Preparation of bead-bound, unactivated probe 1

MagnaBind Amine Derivatized Beads slurry (50 µL, 320 nmol, Thermo ScientificTM) was transferred into a 1.5 mL Eppendorf tube. Original solution from supplier was taken out by magnetic separation. The beads were washed with THF (2×150 µL) followed by phosphate buffer (2×150 µL, 25 nM, pH 7.5). DMF (150 µL) was added to the Eppendorf followed by 5 µL DIPEA and then vortexed for at least 30 s to yield the unprotonated amine. The beads were washed with DMF (150 µL) followed by DCM (150 µL). An amide coupling solution (4.5 mM PyBop, 3.3 mM HOBT, 1% DIPEA v/v in DCM) and probe solution (3 mM probe in DMF) were freshly prepared as reported previously in separate[1]. The probe solution (100 µL) and amide coupling solution (100 µL) were combined into the Eppendorf tube containing magnetic beads. The mixture was shaken and incubated using a Thermomixer (1,600 rpm, 25 °C, overnight.). The solution was removed and the beads consecutively washed with 2×150 µL THF and 2×150 µL DCM. After removal of all the solution, DCM (190 µL) and TFA (10 µL) were added in sequence to the Eppendorf for Boc deprotection. The mixture was shaken and incubated with a Thermomixer (1,500 rpm, 25 °C, 5 h). The reaction mixture was removed and followed by washing with THF (2×150 µL). DCM (150 µL) and DIPEA (10 µL) were added in sequence to the Eppendorf for amine deprotonation and TFA neutralization. The beads were washed with DMF (2×150 µL) and EtOH (2×150 µL). The beads were suspended in the EtOH (300 µL), ready to be used for chemoselective probe activation.

5.2 Activation of amine-specific chemoselective probe 2

The bead-bound, unactivated probe **1** (in 300 µL EtOH) were added with 3,4-Diethoxy-3-cyclobutene-1,2-dione (5 µL) and trimethylamine (3 µL). The suspension was agitated at 25 °C in a ThermoMixer (1,600 rpm) for 16 h. After the reaction was complete, the supernatant was removed and the beads were washed with THF (3 × 200 µL) followed by EtOH (2× 200 µL). The beads were suspended in the EtOH (300 µL), ready to be used for sample treatment.

5.3 Preparation of fecal metabolite extracts

A scalpel was used to collect approximately 30 mg of the frozen fecal sample from ten different patients (stored at 80 °C) in specialized tube D (MP Biomedicals). Ultrapure water (50 µL) and LCMS grade methanol (200 µL) were added into each tube. The mixture was vortexed and subsequently homogenized by a FastPrep 24 homogenizer (3 cycles, 6 m/s, 40 s, MP Biomedicals). The mixture was taken out from tube D into Eppendorf tubes and stored at -20 ℃ for at least 1 h for protein precipitation. The supernatant was collected after centrifugation (18,620 g, 5 min, 4 °C). The extracts were direct used in the bead treatment.

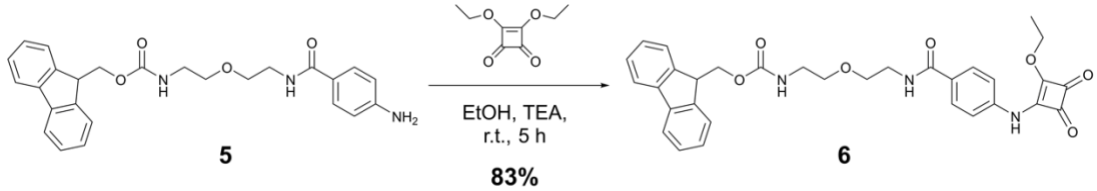
5.4 Treatment of fecal metabolite extracts

The activated beads **2** were used to treat the fecal extract in a solution of 1% *v/v* trimethylamine in ethanol. The mixture was shaken for 16 h at 1500 rpm and 55 °C. The fecal extract solution was removed from the beads and the beads were washed with THF (2 x 200 µL) before being resuspended in THF (300 µL).

5.5 Cleavage of the bead-bound chemical probe 3

The suspension of beads was transferred to a glass vial. Triphenylphosphine (97.0 µL, 12.9 mM in THF, 1.25 µmol) and dimethylbarbituric acid (90.0 µL, 30.7 mM in THF, 2.76 µmol) solutions were added to the vial, followed by palladium (II) acetate solution (84.0 µL, 6.53 mM in THF, 549 nmol). The vial was quickly sealed and a stream of nitrogen was passed through until approximately half the volume of the suspension remained. The vial was agitated at intervals on a vortexer and the reaction was allowed to continue 5 h. In parallel, a sample of unmodified beads was treated with the same cleavage conditions as the activated beads treated with fecal extract and used as control sample. The supernatant was removed from the beads using magnetic separation and the solvent removed using a vacuum centrifuge. The residues were redissolved in MeOH (30 µL each) and triphenylphosphine and triphenylphosphine oxide were precipitated through the addition of water (120 µL each). The suspension was centrifuged (benchtop centrifuge, 13,400 rpm, 5 min), the supernatant removed, and the solvent was again removed with the vacuum centrifuge. The residues were redissolved in water/acetonitrile solution (95:5 v/v) and submitted for LC-MS analysis.

5.6 Synthesis of Fmoc-protected simplified probe 6



5 **6**

Compound **5** (4.9 mg, 11.0 nmol), 3,4-diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester, 3.7 mg, 22.0 nmol) and triethylamine (3.3 mg, 33.0 nmol) were combined with ethanol (2.0 ml) in a microwave tube. The tube was sealed and stirred at 45 °C for 5 hours. After the complete consumption of the compound **5** by monitoring TLC, the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel using a gradient of 1-5% MeOH in DCM to yield compound **6** as white solid (5.2 mg, 83.0%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 21.5, 7.9 Hz, 4H, Fmoc *Ar*), 7.51 (d, *J* = 7.5 Hz, 2H, *Ph*), 7.38 (tt, *J* = 7.5, 0.9 Hz, 2H, *Ph*), 7.31 – 7.09 (m, 4H, Fmoc *Ar*), 6.74 (s, 1H, *amide*), 5.21 (s, 1H, *amide*), 4.78 (q, *J* = 7.1 Hz, 2H, OCH_2CH_3), 4.28 (d, *J* = 7.2 Hz, 2H, FmocCH_2), 4.13 (s, 1H, FmocC*H*), 3.70 – 3.62 (m, 4H, $ArCONHCH_2CH_2$, $FmocNHCH_2CH_2$), 3.61 – 3.55 (m, 2H, $ArCONHCH_2$), 3.44 – 3.40 (m, 2H, $FmocNHCH_2$), 2.80 (s, 1H, *aniline*), 1.44 (t, *J* = 7.1 Hz, 3H, OCH_2CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.4, 166.9, 156.9, 144.0, 141.4, 139.8, 130.5, 128.8, 127.9, 127.2, 125.2, 120.2, 118.6, 114.3, 70.7, 70.4, 69.5, 67.0, 47.3, 40.8, 40.1, 16.0. HRMS (ESI+) *m/z* $[M+H]^+$ calcd. for $C_{32}H_{32}O_7N_3^+$: 570.2235; found 570.2246.

5.7 Preparation of probe-conjugated standards for LOD measurement

A solution of Fmoc-protected probe **6** (10 µL, 10.0 mM in EtOH, 100 nmol, Scheme S2) was evaporated under reduced pressure. The residue was then combined with a solution of single amine standard (40 µL, 2 equiv., 5.0 mM in EtOH), 3 µL trimethylamine and 147 µL ethanol. The resulting solution was then shaken at 1600 rpm for 16 h at 55 °C. The solvents were then removed under reduced pressure, and the residues were treated with piperidine (80 µL) and shaken at 1600 rpm for 5 h at 25 °C. The piperidine was then removed under reduced pressure, and the residue was redissolved in EtOH (200 µL). The solution was diluted in a series of

concentration (100 µL, 10 µL, 1 µL, 100nM, 10 nM, 1 nM, 0.1 nM, 0.08 nM, 0.05nM and 0.01 nM) in a solution of water and acetonitrile (95:5 *v/v*) before being submitted for UPLC-MS analysis. In parallel, the amine standards have also been prepared in the concentration mentioned above for investigation of the improvement of the sensitivity.

5.8 Reactivity test experiment

For testing the reactivity of Fmoc-protected probe **6** for different amine metabolite classes. Herein, 1L of 4-methylmorpholine/acetic acid buffer solution was prepared in different pH (7.5, 8.5, and 9.5).

The Fmoc-protected probe **6** and amines conjugation was performed according to section 6.7 with minor modification. The probe 6 (50 µL, 10.0 mM in EtOH) was mixed with 1-aminopropane (primary amine, 8.33 µL, 10.0 mM in EtOH), piperidine (secondary amine, 8.33 µL, 10.0 mM in EtOH), aniline (aromatic amine, 8.33 µL, 10.0 mM in EtOH), L-serine (amino acid, 8.33 µL, 10.0 mM in EtOH), and N-acetyl-L-cysteine (thiol, 8.33 µL, 10.0 mM in EtOH) in 200 µL different buffer solution including pH 7.5, 8.5, 9.5 and ethanol with 1% trimethylamine for 16 hours at 55 °C. The solvents were then removed under reduced pressure, and the residues were treated with piperidine (80 µL) and shaken at 1600 rpm for 5 h at 25 °C. The piperidine was then removed under reduced pressure, and the residue was redissolved in MeOH (100 µL) followed by water (400 µL). The solution was diluted as necessary in a solution of water and acetonitrile (95:5 v/v) before being submitted for UPLC-MS analysis.

5.9 Stability test experiment.

The synthetic conjugated metabolites in section 6.8 were treated in the same condition as biorthogonal cleavage in section 6.5 for 16 hours.

5.10 Construction of amine-containing metabolite library

The library construction was performed by following the same procedure (section 6.4; 6.5) as human sample treatment replacing the human metabolite extracts into amine metabolite standards. Single injection was submitted to UPLC-MS.

5.11 LC-MS analysis

Four injections were performed for fecal extract-treated bead cleavage product and six injections for the control sample. For the first 90 s of the analysis, the output of the UHPLC system was diverted to waste and did not enter the mass spectrometer.

5.12 Data analysis

Data files from the LC-MS analysis were converted into the NetCDF file format using MassLynx 4.1 (Waters). The XCMS library was used to perform peak detection and align the chromatograms. The feature list was reduced by eliminating those features with an *m/z* value less than 319.1401 (the *m/z* value corresponding to the monoprotonated probe with ammonium conjugate). More abundant features in the control sample and less than five-fold higher abundance in the feces sample set were eliminated from the data analysis. The features that eluted earlier than 1.5 min were also removed. Mass values of each feature with 302.1136 Da subtracted (corresponding to the mass of the probe) were compared to the human metabolome

database in order to find plausible candidates for the parent metabolites. Commercial or synthetic standards (section 6.10) were then used to confirm the identity of the metabolites and identification of the correct regioisomers.

6. Reference

1. N. Garg, L. P. Conway, C. Ballet, M. S. P. Correia, F. K. S. Olsson, M. Vujasinovic, J. M. Lohr and D. Globisch, *Angew. Chem., Int. Ed.,* 2018, **57**, 13805-13809.

7. NMR Spectra