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Supporting Information

Expanding the Substrate Scope of *N*- and *O*-Methyltransferases from Plants for Chemoselective Alkylation

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Experimental section

Materials

All substrates and reference compounds were purchased from Sigma-Aldrich (ATP, SAM, SAH, L-methionine, L-methionine-(methyl-¹³C), L-ethionine, 2-amino-4-bromo phenol, 2-amino-5-bromo phenol, 2-amino-4-chloro phenol, 2-amino-5-chloro phenol, 2-amino-4-nitro phenol, 2-amino-5-nitro phenol, 4-chloro-2-methoxyaniline, 5-chloro-2-methoxyaniline, 2-methoxy-4-nitro aniline, 2-methoxy-5-nitro aniline, caffeic acid, ferulic acid, isoferulic acid, 3,4-dihydroxybenzoic acid), Alfa Aesar (*N*-methylantranilic acid), and Fluka (anthranilic acid) in the highest purity available. Buffer ingredients, as well as cultivation media were obtained by Carl Roth.

Table S 1 – Used substrates and products with abbreviations and retention times. A, B, and C refers to the different analytical methods.

Substrate	Abbreviation / #	Retention time [min]		
		A	B	C
L-methionine	L-met	-	-	-
L-ethionine	L-eth	-	-	-
S-allyl-L-homocysteine	L-all	-	-	-
adenosine triphosphate	ATP	1.9	1.1	-
S-adenosyl-L-methionine	SAM	1.7	1.3	-
S-adenosyl-L-homocysteine	SAH	8.1	1.2	-
adenine	ade	9.3	1.6	-
S-adenosyl-L-ethionine	SAE	-	1.3	-
adenosyl-S-allyl-L-homocysteine	SAA	-	1.4	-
anthranilic acid	1	3.6	-	-
<i>N</i> -methyl anthranilic acid	1a	6.8	-	-
caffeic acid	2	3.2	-	-
3-methoxy-4-hydroxycinnamic acid	2b	6.4	-	-
3-hydroxy-4-methoxycinnamic acid	2b''	7.8	-	-
2-amino-4-nitrophenol	3	-	7.8	5.0
2-(methylamino)-4-nitrophenol	3a	-	8.6	-
2-methoxy-5-nitroanilin	3b	-	8.7	6.3
2-methoxy- <i>N</i> -methyl-5-nitroaniline	3ab	-	9.2	-
2-(ethylamino)-4-nitrophenol	3c	-	8.8	-
2-ethoxy-5-nitroaniline	3d	-	9.0	-
2-(allylamino)-4-nitrophenol	3e	-	8.9	-
2-allyloxy-5-nitroaniline	3f	-	9.1	-
2-amino-5-nitrophenol	4	-	8.2	6.0
2-(methylamino)-5-nitrophenol	4a	-	8.7	-
2-methoxy-4-nitroanilin	4b	-	8.8	6.7
2-methoxy- <i>N</i> -methyl-4-nitroaniline	4ab	-	9.1	-
2-(ethylamino)-5-nitrophenol	4c	-	8.9	-
2-ethoxy-4-nitroanilin	4d	-	9.0	-
2-(allylamino)-5-nitrophenol	4e	-	8.9	-
2-allyloxy-4-nitroanilin	4f	-	9.1	-
2-amino-4-bromophenol	5	-	7.9	-
4-bromo-2-(methylamino)phenol	5a	-	8.7	-
5-bromo-2-methoxyaniline	5b	-	9.0	-

Substrate	Abbreviation / #	Retention time [min]		
		A	B	C
2-amino-5-bromophenol	6	-	7.1	-
5-bromo-2-(methylamino)phenol	6a	-	7.9	-
4-bromo-2-methoxyaniline	6b	-	8.8	-
2-amino-4-chlorophenol	7	-	7.3	-
4-chloro-2-(methylamino)phenol	7a	-	8.5	-
5-bromo-2-methoxyaniline	7b	-	8.9	-
2-amino-5-chlorophenol	8	-	6.0	-
5-chloro-2-(methylamino)phenol	8a	-	7.4	-
4-bromo-2-methoxyaniline	8b	-	8.5	-

Methods

Cloning

Table S 2 – Primers for gene amplification and linearisation of pET28a(+).

Primer	Sequence 5'-3'
KAH9787224.1 (<i>Citrus sinensis</i>)_fw	CGCGCGGCAGCC <u>CATATG</u> GGTAGCCTGAGCGAATATCA
KAH9787224.1 (<i>Citrus sinensis</i>)_rev	GTGCGGCCGCA <u>AAGCTT</u> ATTTGAAAAATTCCATGATATACA
KDO86634.1 (<i>Citrus sinensis</i>)_fw	CGCGCGGCAGCC <u>CATATG</u> GGTAGCCTGAGCGAATA
KDO86634.1 (<i>Citrus sinensis</i>)_rev	GTGCGGCCGCAAGCT <u>AAGCTT</u> ATTTGAAAAATTCCATGAT
XP_007218135.1 (<i>Prunus persica</i>)_fw	CGCGCGGCAGCC <u>CATATG</u> GCAAGCAGCCTGGAAC
XP_007218135.1 (<i>Prunus persica</i>)_rev	GTGCGGCCGCAAGCT <u>AAGCTT</u> ATTTGAAAAATTCCATCAC
XP_006494578.1 (<i>Citrus sinensis</i>)_fw	CGCGCGGCAGCC <u>CATATG</u> GATAGCATTGTTGATGGTG
XP_006494578.1 (<i>Citrus sinensis</i>)_rev	GTGCGGCCGCAAGCT <u>AAGCTT</u> ATTTGTAAAATTCCATAAC
P28002.1 (<i>Medicago sativa</i>) fw	CGCGCGGCAGCC <u>CATATG</u> GGTAGCACCGGTGAAAC
P28002.1 (<i>Medicago sativa</i>) rev	GTGCGGCCGCAAGCT <u>AAGCTT</u> ACACTTTTTTAAGGAATTC
pET28a_Ndel	TATGGCTGCCGCGCGGCACC
pET28a_HindIII	AGCTTGCGGCCGCACTCGAG

*Restriction sites are underlined.

Table S 3 – 3-step PCR protocol.

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	98	30	1
Denaturation	98	10	30
Annealing	55	30	
Elongation	72	90	
Final elongation	72	240	1
Storage	8	∞	

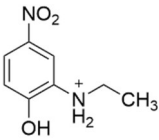
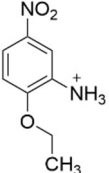
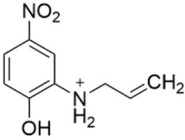
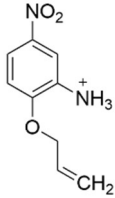
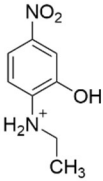
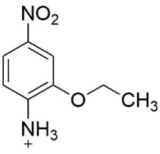
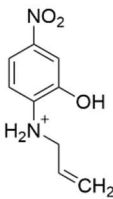
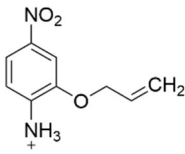
HPLC analysis

Table S 4 – Methods used for upscale reaction analysis and purification

	Method C	Method D
Assay	Analytical method of Up scale reaction	Purification method of Up scale reaction
HPLC system	Agilent 1260 infinity II	Agilent 1260 InfinityTM
Column	ACE 5-C18 300 column (4 µm, 4.6 mm × 150 mm)	SupelcoTM Discovery BIO wide pore (C18, 10 µm, 2.12 cm x 25 cm)
Mobile phase A	0.1% TFA	0.1% TFA
Mobile phase B	acetonitrile	acetonitrile with 0.1% TFA
Detection wavelenght	280 nm	280 nm
Flow rate	1 mL · min ⁻¹	8 mL · min ⁻¹
Injection volume	10 µL	500 – 900 µL
Gradient	1 min 90% A/ 10% B 6 min to 15% A/ 85% B 0.1 min to 0% A/ 100% B 1.9 min 0% A/ 100% B 0.1 min to 90% A/ 10% B 2.4 min 90% A/ 10%B	3 min 90% A/ 10% B 7 min to 50% A/ 50% B 15 min to 0% A/ 100% B 5 min 0% A/ 100% B 1 min 90% A/ 10% B 4 min 90% A/ 10% B

LC-MS analysis

Table S 5 – Investigated mother fragments in LC-MS analysis for confirmation of ethylation and allylation.

			
3c	3d	3e	3f
			
4c	4d	4e	4f

*m/z of [M+H]⁺ of products **3c/d** and **4c/d**: 183.18 Da and of products **3e/f** and **4e/f**: 195.19 Da

NMR analysis

Table S 5 – ¹³C-NMR signals for compounds used in the methylation assays.

	δ [ppm]
Methionine (S- <u>C</u> H ₃)	13.8
SAM (S- <u>C</u> H ₃)	23.5
Methionine (H ₂ N- <u>C</u> H)	59.4
Tris (HO- <u>C</u> H ₂)	61.6
Glycerol (<u>C</u> H ₂)	62.7
Glycerol (<u>C</u> H)	72.1

Protein sequences

The following protein sequences and enzyme sizes include the His₆-tag (marked in bold).

>*Ec*MAT (44.1 kDa)

MGSS**HHHHHH**SSGLVPRGSHMAKHLFTSESVSEGHDPDKIADQISDAVLDAILEQDPKARVACETYVKT
GMVLVGGEITTSAWVDIEEITRNTVREIGYVHSDMGFDANSCAVLSAIGKQSPDINQGVDRADPLEQG
AGDQGLMFGYATNETDVLMPAPITYAHLRVQRQAEVRKNGTLPWLRPDAKSQVTFQYDDGKIVGIDAV
VLSTQHSEEIDQKSLQEAVMEEI IKPILPAEWLTSATKFFINPTGRFVIGGPMGDCGLTGRKII VDTY
GGMARHGGGAFSGKDPSKVDRSAAAYAARYVAKNIVAAGLADRCEIQVSYAIGVAEPTSIMVETFGTEK
VPSEQLTLLVREFFDLRPYGLIQMLDLLHPIYKETAAYGHFGREHFPWEKTDKAQLLRDAAGLK

>*Tk*MAT (46.7 kDa)

MGSS**HHHHHH**SSGLVPRGSHMAGKVRNIVVEELVRTPVEMQKVELVERKGIGHPDSIADGIAEAVSRA
LSREYVKRYGII LHHNTDQVEVVGGRAYPQFGGGEVIKPIYI LLSGRAVEMVDREFFPVHEIALKAAK
DYLKRAVRHLDLEHHV IIDSRIQGGSVDLVGVFNKAKKNPIPLANDTSFGVGYAPLSETEKIVLETEK
YLN SDEFKKYPAVGEDIKVMGLRKGD EIDL TIAAAIVDSEVDNPDDYMAVKEAI YEAAKGIVESHTE
RPTNIYVNTADDPKEGIYYITVTGTSAEAGDDGSVGRGNRVNGLITPNRHMSMEAAAGKNPVSHVGKI
YNILSMLIANDIAEQVEGVVEEVYVRILSQIGKPIDEPLVASVQIIPKKGYSIDVLQKPAYEIADEWLA
NITKI QKMILEDKVN VF

>*Ec*MTAN (26.5 kDa)

MGSS**HHHHHH**SSGLVPRGSHMKIGIIGAMEEEVTLLRDKIENRQTISLGGCEIYTGQLNGTEVALLKS
GIGKVAALGATLLLEHCKPDVIINTGSAGGLAPTLKVGDIVVSDEARYHDADVTAFGYEYGLPGCF
AGFKADDKLIAAAEACIAELNLNAVRGLIVSGDAFINGSVGLAKIRHNFPQAI AVEMEATAIAHVCHN
FNVFPVVRAISDVADQQSHLSFDEFLAVAAKQSSLMVESLVQKLAHG

>*Pp*CaOMT (43.7 kDa)

MGSS**HHHHHH**SSGLVPRGSHMASSLERKSHPKINHAEPEDEITKEEEDSF CYAMQLVGSSVLMSLQ
SAIKLGIFDIIARKGPGAKLSSEIATKIGTENPEAPVMVDRI LRLLTSHSVLNC SAVAANGGSD FQR
VYSLGPVSKYFVNDEEGGSLGPLLTLIQDRVFLESWSQLKDAVVEGGIPFNRVHGMHAFEY PGLDPRF
NQVFNTAMFNHTTIVIKKLLHIYK GLEDKNLTQLVDVGGGLGVTNLITSR YQH IKGINF DLPHV VNH
APSYPGVEHVGGDMFASVPSGDAIFMKWILHDWSEHCLKLLKN CYKAIPDNGKVIVVEALLPAMPET
STATKTTSQLDVLMMTQNPGGKERSEQEFMALATGAGFSGIRYECFVCNFWVMEFFK

>*Rg*ANMT (42.2 kDa)

MGSS**HHHHHH**SSGLVPRGSHMGSLSESHTQYKHGVEVEEDEEESYSRAMQLSMAIVLPMATQSAIQLG
VFEIIAKAPGGRLSASEIATILQAQNP KAPVMLDRMLRLLVSHRVLD CSVSGPAGERLYGLTSVSKYF
VPDQDGASLGNFMALPLDKVFME SWMGVKGAVMEGGIPFNRVHGMHIFEYASSNSKFS DTYHRAMFNH
STIALKRILEHYKGFENVTKLVDVGGGLGVTLSMIASKYPHIQAINFDLPHV VQDAASYPGVEHVGGN
MFESVPEGDAILMKWILHCWDDEQCLRILKN CYKATPENGKVIVMNSVVPETPEVSSSARETSLLDVL
LMTRDGGGRERTQKEFTELAIGAGFKGINFACCV CNLHIMEFFK

>CsANMT (41.9 kDa)

MGSS**HHHHHH**SSGLVPRGSHMGSLSLEYQKLAQKKHEEEEEESYSHAMQLAMGVVLPMATQAAIQLGVF
EIIAKAGELSAPEIAAQLQAQNVKAPMMLDRMLRLLVSHRVLECSVSGGERLYALNPVSKYFVSNKDG
ASLGHFMALPLDKVFMESWLGLKDAVMEGGIPFNRVHGMHIFEYASGNPRFNETYHEAMFNHSTIAME
RILEHYEGFQNVRLVDVGGGFGVTLSMITSKYPQIKAVNFDLPHVVQDAPSYAGVEHVGGNMFESVP
EGDAILMKWILHCWDDDHCLRILKNCYKAVPGNGKVIVMNSIVPEIPEVSSAARETSLLDVLLMTRDG
GGRERTKKEYTELAIAAGFKGINFASCVCNLYIMEFFK

>CsCaOMT (41.2 kDa)

MGSS**HHHHHH**SSGLVPRGSHMDSIVDGERDQSFAYASQLVMGTVLPMAIQAVYELGIFEILDKVGPGA
KLCASDIAAQLLTKNKDAPMMLDRILRLLASYSVVECSLDASGARRLYSLNSVSKYYVPNKDGVLLGP
LLQMNQDKVLLESWSQLKDAILEGGIPFNRAHGTVHFEYAGLDPKFNKHFNTAMYNYSLVMSNILES
YKGFNDNIKQLVDVGGSLGITLQAITTKYPYIKGINFDQPHVIDHAPSHPRIEHVGGDMFQSVPKGDAI
IMKSVLHDWNDEHCLKLLKNCYKSIPEDGKVIVVESMLPEVPNTSIESKSNSHFDVLMMIQSPGGKER
TRHEFMTLATGAGFGGISCELAIGNLWVMEFYK

>MsCaOMT (42.1 kDa)

MGSS**HHHHHH**SSGLVPRGSHMGSTGETQITPTTHISDEEANLFAMQLASASVLP MILKSALELDLLEII
AKAGPGAQISPIEIASQLPTTNPDAVMLDRMLRLLACYIILTCVVRTQQDGKVQRLYGLATVAKYLV
KNEDGVSISALNLMNQDKVLMESWYHLKDAVLDGGIPFNKAYGMTAFEYHGTDPFNKVFNKGMSDHS
TITMKKILETYTGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGVEHVGGDM
FVSIPKADAVFMKWICHDSDEHCLKFLKNCYEALPDNGKVIVAECILPVAPDSSLATKGVVHIDVIM
LAHNPGGKERTQKEFEDLAKGAGFQGFKVHCNAFNITYIMEFLKKV

>KDO86634.1 (*Citrus sinensis*) (45.7 kDa)

MGSS**HHHHHH**SSGLVPRGSHMGSLSLEYQKLAQKKHEEEEEEEEEESYSHAMQLAMGVVLPMATQAAIQLG
VFEIIAKAGELSAPEIAAQLQAQNVKAPMMLDRMLRLLVSHRVLECSVSGGERLYALNPVSKYFVSNK
DGASLGHFMALPLDKVFMESWYIIILSFFFFPLSGQIYIVVNLSNFKNACRLGLKDAVMEGGIPFNRV
HGMHIFEYASGNPRFNETYHEAMFNHSTIAMERILEHYEGFQNVRLVDVGGGFGVTLSMITSKYPQI
KAVNFDLPHVVQDAPSYAGVEHVGGNMFESVPEGDAILMKWILHCWDDDHCLRILKNCYKAVPGNGKV
IVMNSIVPEIPEVSSAARETSLLDVLLMTRDGGGRERTKKEYTELAIAAGFKGINFASCVCNLYIMEF
FK

Supplementary Figures

SDS-PAGE analysis

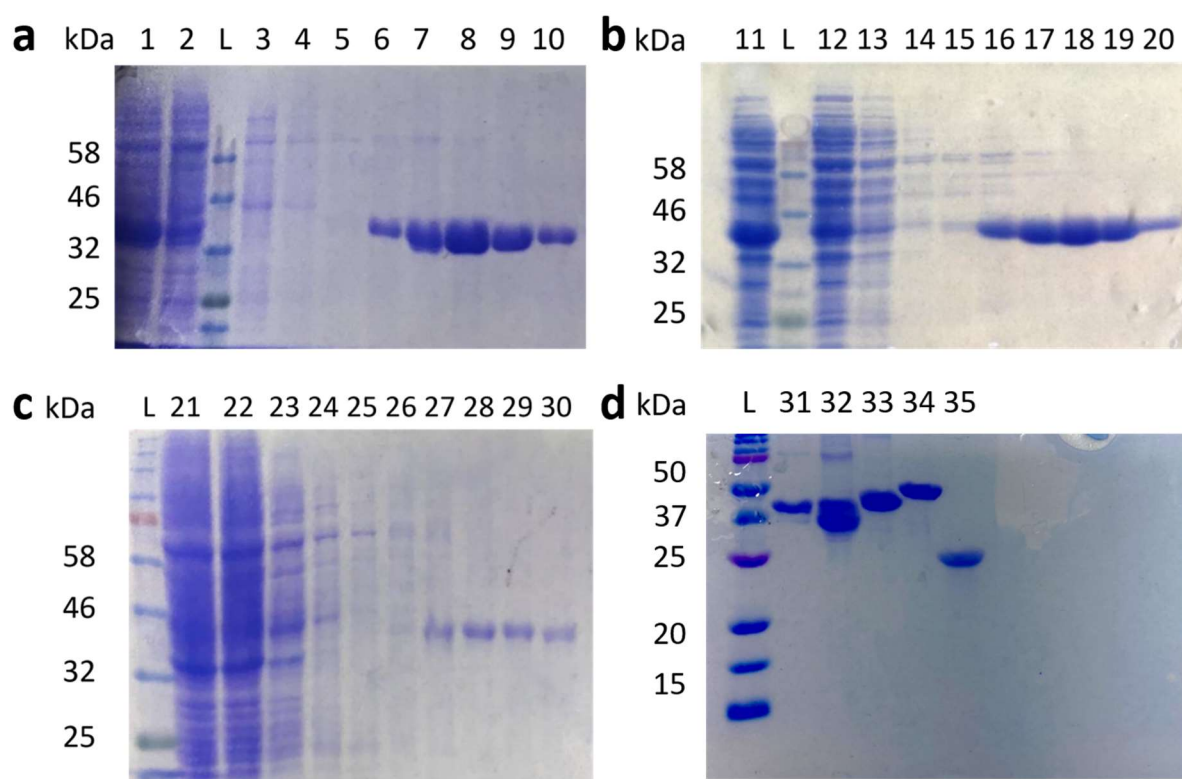


Figure S 1 – Visualisation of the enzymes used in this study. L=Ladder. **a:** SDS gel of CsANMT (41.9 kDa) fractions during purification. 1: crude lysate; 2: flow through; 3: 5 mM imidazole; 4: 10 mM imidazole; 5: 20 mM imidazole; 6: 50 mM imidazole; 7: 100 mM imidazole; 8: 150 mM imidazole; 9: 200 mM imidazole; 10: 300 mM imidazole. **b:** SDS gel of CsCaOMT (41.2 kDa) fractions during purification. 11: crude lysate; 12: flow through; 13: 5 mM imidazole; 14: 10 mM imidazole; 15: 20 mM imidazole; 16: 50 mM imidazole; 17: 100 mM imidazole; 18: 150 mM imidazole; 19: 200 mM imidazole; 20: 300 mM imidazole. **c:** SDS gel of MsCaOMT (42.1 kDa) fractions during purification. 21: crude lysate; 22: flow through; 23: 5 mM imidazole; 24: 10 mM imidazole; 25: 20 mM imidazole; 26: 50 mM imidazole; 27: 100 mM imidazole; 28: 150 mM imidazole; 29: 200 mM imidazole; 30: 300 mM imidazole. **d:** 31: RgANMT (42.2 kDa); 32: PpCaOMT (43.7 kDa); 33: EcMAT (44.1 kDa); TkMAT (46.7 kDa); EcMTAN (26.5 kDa).

Enzyme relationships

a

CLUSTAL O(1.2.4) multiple sequence alignment

MsCaOMT	--MGSTGETQITP-----THISDEEA---NLFAMQL-----ASASVLPMI	35
RgANMT	--MGSLSSES-----HTQYKHG-VEVEEDEEES---YSRAMQL-----SMAIVLPMA	40
CsANMT	--MGSLSSEY-----QKLAQKK-HEEE--EES---YSHAMQL-----AMGVVLPMA	38
KD086634.1 (Cs)	--MGSLSSEY-----QKLAQKK-HEEE-----YSHAMQL-----AMGVVLPMA	40
PpCaOMT	--MASSLERKSHPKINHAEPED-EITKEEEDS---FCYAMQL-----VGSSVLSMS	46
CsCaOMT	-----MD-SIVDGERDQS---FAYASQL-----VMGTVLPMA	28
RnCOMT	--MGDTKEQRILRYVQQNAKPGDPQSVLEAIDTYCTQKEWAMNVGDAKGQIMDAVIREYS	58
MxSafC	MIHHVELTQSVLQYIRDSSVRD-NDILRDLREETS KLPLRTMQIPPEQGQLSLVRLIG	59
	: : :	
MsCaOMT	LKSALELDLLEIIAKAGPGAQISPIEIASQLPTTNPDAVPMLDRMLRLLACYIILTCSVR	95
RgANMT	TQSAIQLGVFEIIAK-APGGRLSASEIATILQAQNPAPVMLDRMLRLLVSHRVLDSCSVS	99
CsANMT	TQAAIQLGVFEIIAK-A--GELSAPEIAAQQAQNVKAPMMLDRMLRLLVSHRVLECSVS	95
KD086634.1 (Cs)	TQAAIQLGVFEIIAK-A--GELSAPEIAAQQAQNVKAPMMLDRMLRLLVSHRVLECSVS	97
PpCaOMT	LQSAIKLGIFDIIARKGPGAKLSSEIATKIGTENPEAPVMVDRIILRLTSHSVLNC SAV	106
CsCaOMT	IQAVYELGIFIELDKVGPGAKLCSADIAAQLLTKNKDA PMMLDRILRLLASYSVVECSLD	88
RnCOMT	PSLVLELGAYCGYSA-----VRMA-----RLLQPGARLLTMEMN	92
MxSafC	ARKTLEVGVFTGYST-----LCAA-----LALPADGRVIACDLS	93
	. : . * * : .	
MsCaOMT	TQQDG-KVQRLYGLATVA-KYLVKNEDG-----VSISALNLMNQDKVLMSWY-----	141
RgANMT	G---PAGERLYGLTSVS-KYFVPDQDG-----ASLGNFMALPLDKVFMESWM-----	142
CsANMT	G-----GERLYALNPVS-KYFVSNKDG-----ASLGHFMALPLDKVFMESWL-----	136
KD086634.1 (Cs)	G-----GERLYALNPVS-KYFVSNKDG-----ASLGHFMALPLDKVFMESWYIIILSFF	145
PpCaOMT	AANGGSDFORVYSLGPVS-KYFVNDEEG-----GSLGPLLTLIQDRVFLESWS-----	153
CsCaOMT	A---SGARRLYSLNSVS-KYYVPNKDG-----VLLGPLLQMNQDKVLLESWS-----	131
RnCOMT	PDYAA-ITQQMLNFAGLQDKVTILNGASQDLIPQLKKKYD VDTLDMVFLDHWKDRYLPDT	151
MxSafC	EEWVS-IARRYWQRAGVADRIEVR LGDAHHSLEALVGSEHRTFDLAFIDADKESYDF--	150
	. : : : *	
MsCaOMT	-----HLKDAVLDDGGIPFNKAYGMTAFEYHGTDPRFNKVFNK	178
RgANMT	-----GVKGAVMEGGIPFNRVHGMHIF EYASSNSKFSPTYHR	179
CsANMT	-----GLKDAVMEGGIPFNRVHGMHIF EYASGNPRFNETYHE	173
KD086634.1 (Cs)	FFPLSGQIYIVNLSNFKNACRLGLKDAVMEGGIPFNRVHGMHIF EYASGNPRFNETYHE	205
PpCaOMT	-----QLKDAVVEGGIPFNRVHGMHAF EYPGLDPRFNQVFNT	190
CsCaOMT	-----QLKDAILEGGIPFNRAHGVHVF EYAGLDPKFNKHNT	168
RnCOMT	-----LLEKCGLLRKG-----	163
MxSafC	-----YYEHALRLVRPG-----	162
	.	
MsCaOMT	GMSDHSTITMKKILETYTGFE--GLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVI	236
RgANMT	AMFNHSTIALKRILEHYKGF E--NVTKLVDVGGGLGVTLSMIASKYPHIQAINFDLPHVV	237
CsANMT	AMFNHSTIAMERILEHYEGFQ--NVERLVDVGGGFGVTLSMITSKYPQIKAVNFDLPHVV	231
KD086634.1 (Cs)	AMFNHSTIAMERILEHYEGFQ--NVERLVDVGGGFGVTLSMITSKYPQIKAVNFDLPHVV	263
PpCaOMT	AMFNHTTIVIKLLHIYKGLDKNLTQLVDVGGGLGVTNLNITSRYQH IKGINFDLPHVV	250
CsCaOMT	AMYNYSLVMSNILES YKGF D--NIKQLVDVGGSLGITLQAITTKYPYIKGINFDQPHVI	226
RnCOMT	-----	163
MxSafC	-----	162
MsCaOMT	EDAPSYPGVEHVGGDMFVSIPKADAVFMKWICHDSDEHCLKFLKNCYEALPDNGKVIVA	296
RgANMT	QDAASYPGVEHVGGNMFE SVPEGDAILMKWILHCWDDDEQCLRI LKNCYKATPENGKVIVM	297
CsANMT	QDAPSYAGVEHVGGNMFE SVPEGDAILMKWILHCWDDDHCLRI LKNCYKAVPGNGKVIVM	291
KD086634.1 (Cs)	QDAPSYAGVEHVGGNMFE SVPEGDAILMKWILHCWDDDHCLRI LKNCYKAVPGNGKVIVM	323
PpCaOMT	NHAPSYPGVEHVGGDMFASVPSGDAIFMKWILHDWSDEHCLKLLKNCYKAIPDNGKVIVV	310
CsCaOMT	DHAPSHPRIEHVGGDMFQSVPKGDAIIMKSVLHDWNDEHCLKLLKNCYKSIPEDGKVIVV	286
RnCOMT	-----TVLLAD	169
MxSafC	-----GLIILD	168
	:	

MsCaOMT	ECILPVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGAGFQGFKVHCNAFNT	356
RgANMT	NSVVPETPEVSSSARETSLLDVLLMTRDGGGRERTQKEFTELAIGAGFKGINFACCVCNL	357
CsANMT	NSIVPEIPEVSSAARETSLLDVLLMTRDGGGRERTKKEYTELAIAAGFKGINFASCVCNL	351
KDO86634.1 (Cs)	NSIVPEIPEVSSAARETSLLDVLLMTRDGGGRERTKKEYTELAIAAGFKGINFASCVCNL	383
PpCaOMT	EALLPAMPETSTATKTTSQLDVLMMTQNPGGKERSEQEFMALATGAGFSGIRYECFVCNF	370
CsCaOMT	ESMLPEVPNTSIESKSNSHFDVLMMIQSPGGKERTRHEFMTLATGAGFGGISCELAIGNL	346
RnCOMT	NVIVPGTPDFLAYVRGSSS--F-----ECTHYSSYLEYMKVVDGLEKAIYQGPSSPDKS	221
MxSafC	NTLWSGKVADPSVVGDPETDSLRRINAKLLTDERVDLSMLPIADGLTLARKR-----	220
	: : . . : . .	

MsCaOMT	YIMEFLKKV	365
RgANMT	HIMEFFK--	364
CsANMT	YIMEFFK--	358
KDO86634.1 (Cs)	YIMEFFK--	390
PpCaOMT	WVMEFFK--	377
CsCaOMT	WVMEFYK--	353
RnCOMT	-----	221
MxSafC	-----	220

b

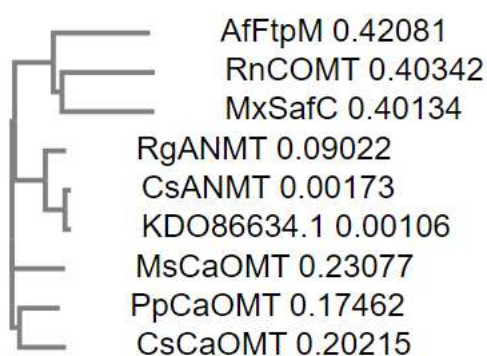
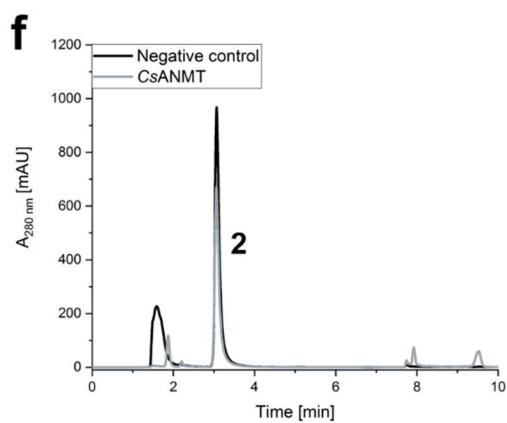
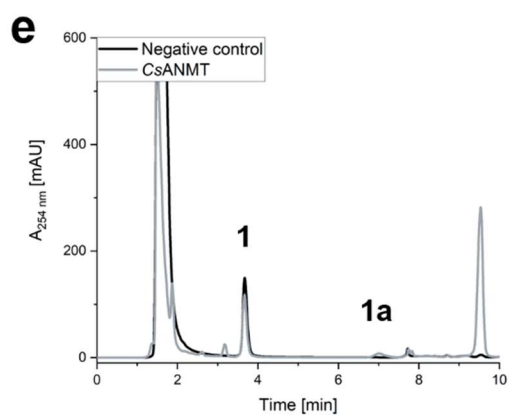
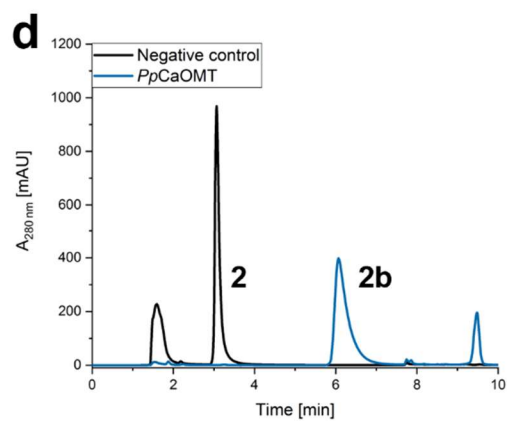
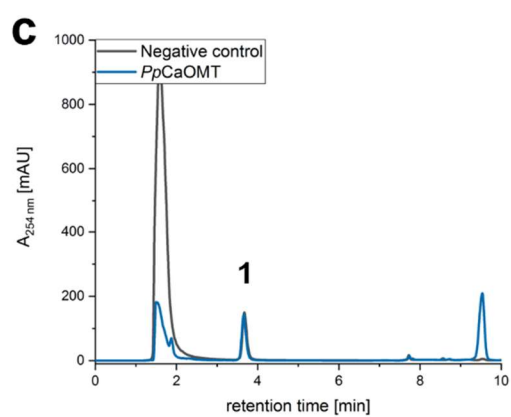
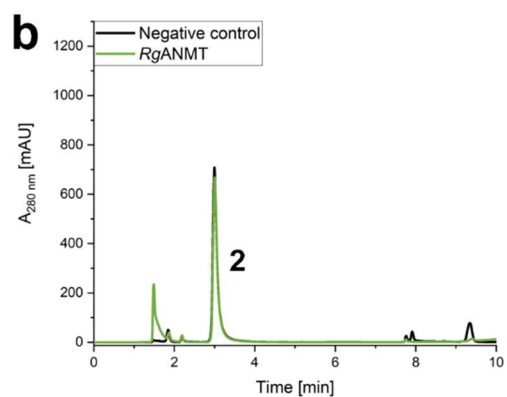
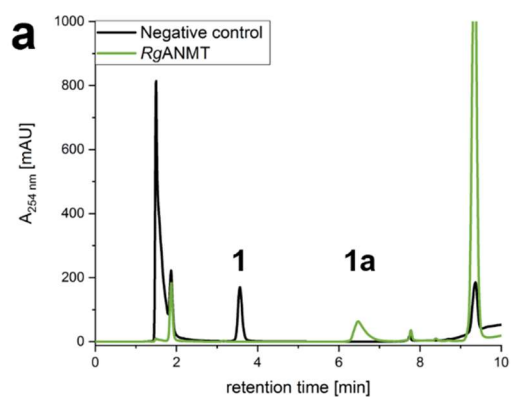


Figure S 2 – a: Amino acid alignment of all used MTs (*RgANMT*; *CsANMT*; *KDO86634.1 (Cs)*; *CsCaOMT*; *PpCaOMT*; *MsCaOMT*) and additionally *RnCOMT* and *MxSafC* as catechol O-MTs (class I enzymes according to *Joshi et al.*) for comparison.^[1] The alignment was created by Clustal Omega.^[2,3] **b:** Phylogenetic tree of the used MTs compared to *RnCOMT* and *MxSafC*, as well as a less related carboxyl methyltransferase from *Aspergillus fumigatus* (*AfFtpM*) that has been described recently.^[4,5]

Enzyme screening



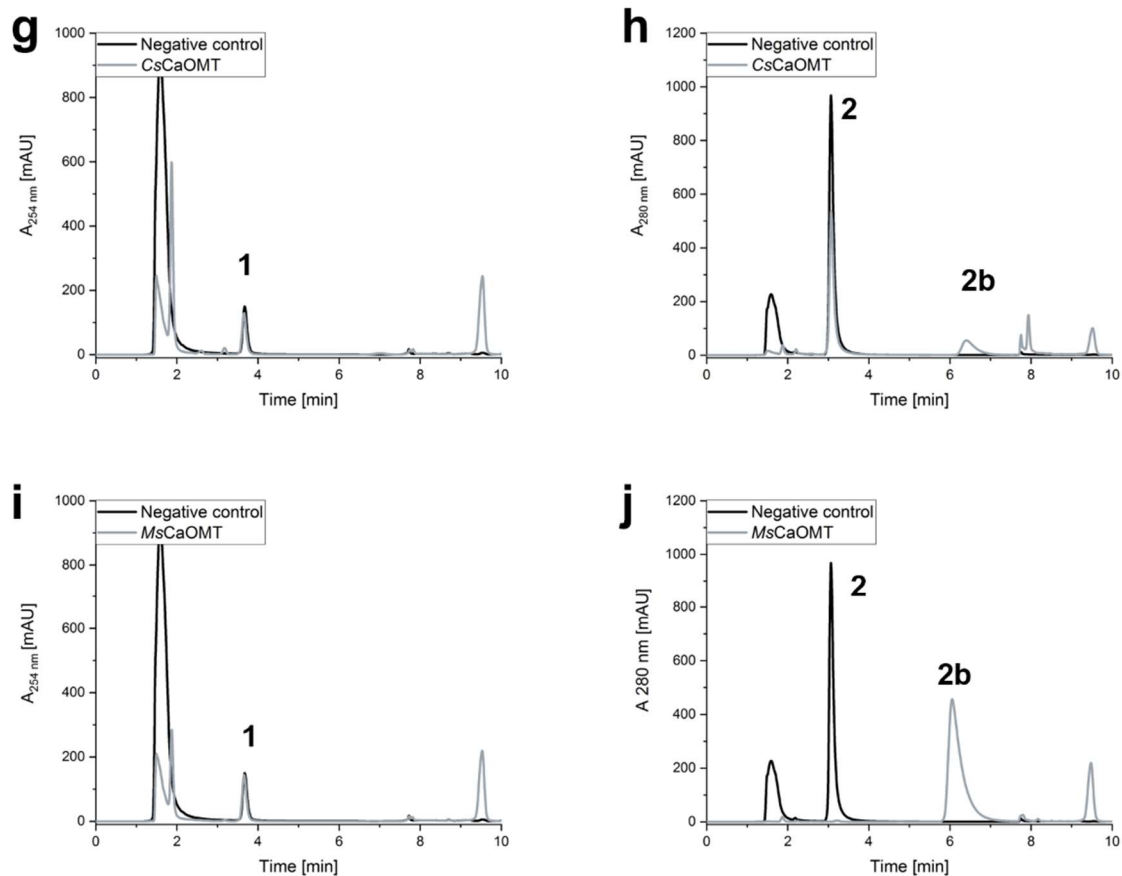
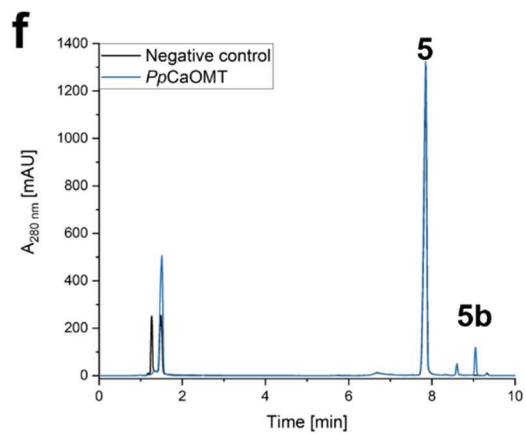
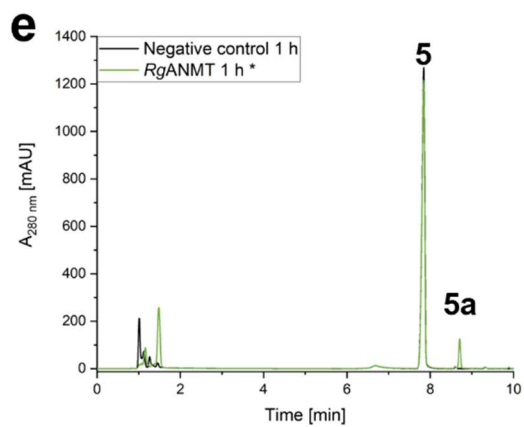
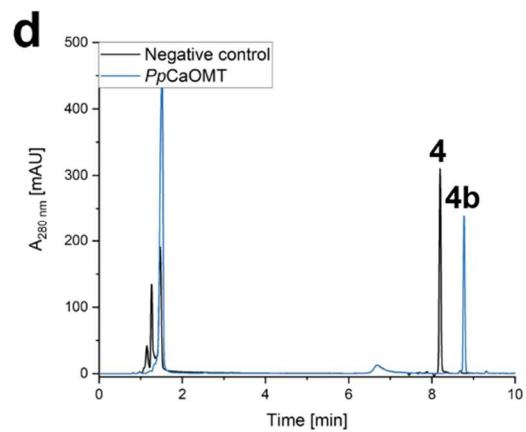
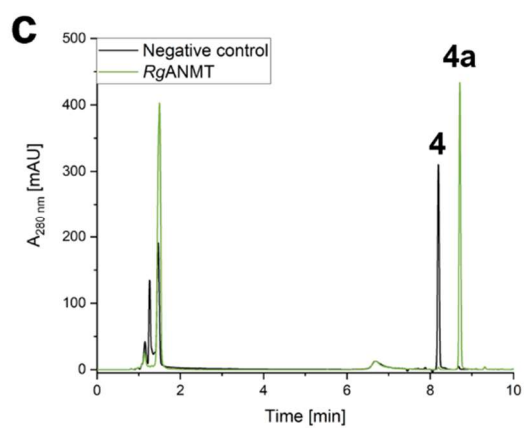
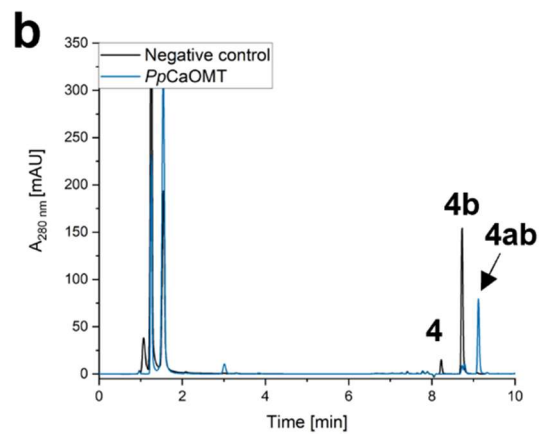
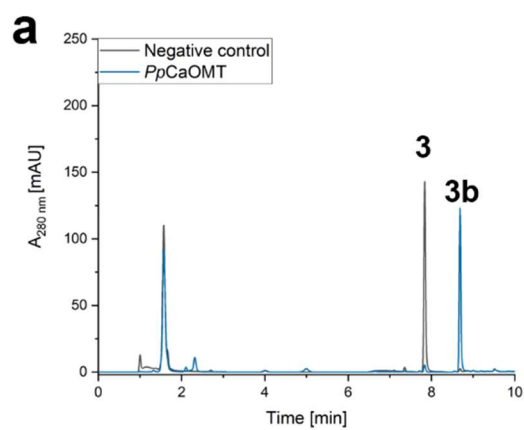


Figure S 3 – Enzyme screening analysed by HPLC Method A using the natural substrates **1** for the confirmation of *N*-methylation and **2** for *O*-methylation. The negative controls did not contain an MT but the MAT and MTAN enzymes (black). **a – b**: Reactions catalysed by *RgANMT*. **1** was fully converted to **1a** while **2** was not accepted as substrate. **c – d**: Reactions catalysed by *PpCaOMT*. **1** was not accepted while **2** was fully converted to **2b**. **e – f**: *CsANMT* accepted **1** as substrate and partly formed **1a**. **2** was not accepted as substrate. **g – h**: *CsCaOMT* only accepted **2** but not **1** as substrate and partly produced **2b**. **i – j**: *MsCaOMT* only accepted **2** as substrate leading to full conversion to product **2b**. This was already shown before.^[6]



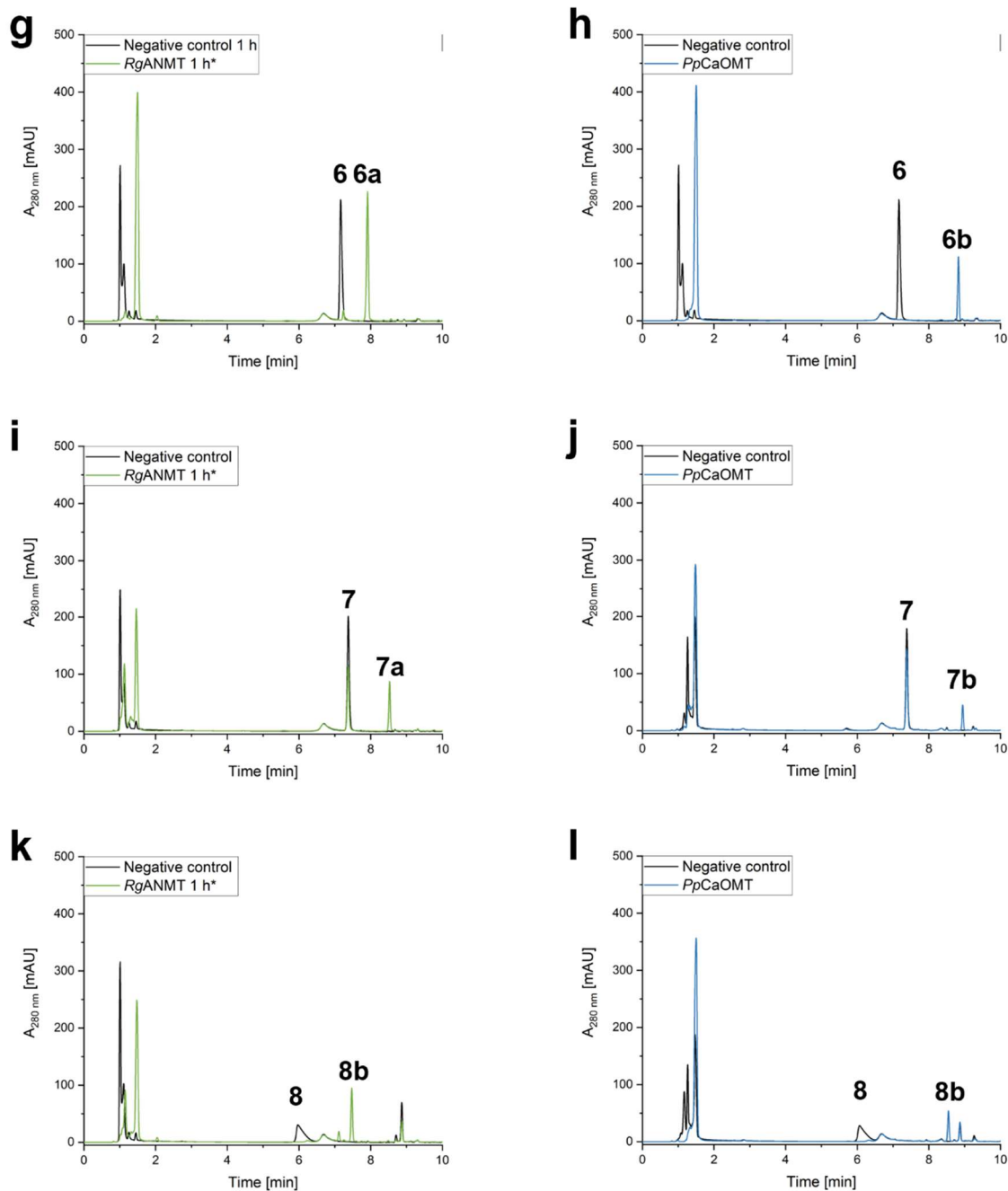
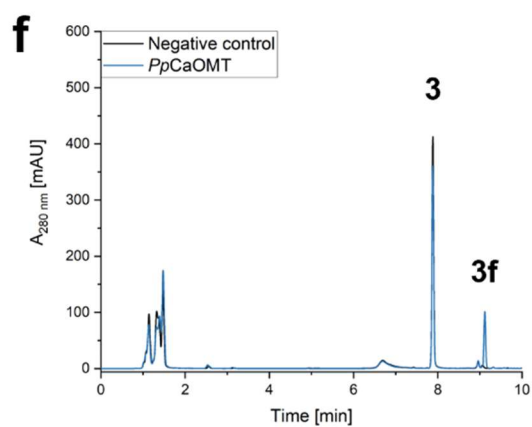
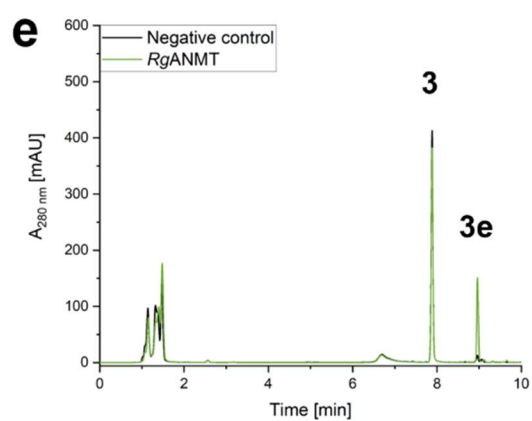
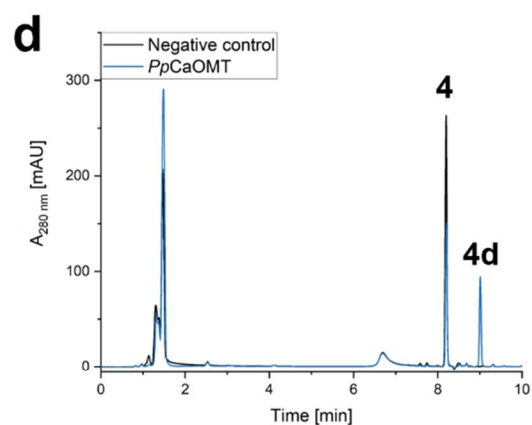
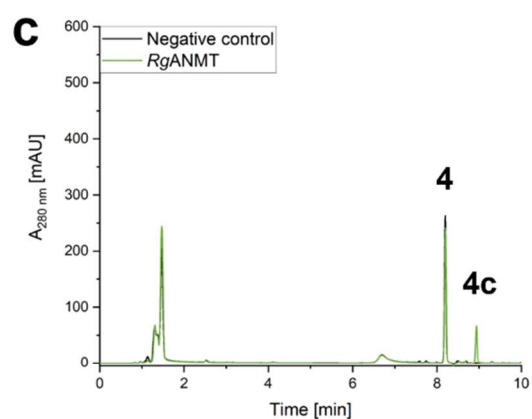
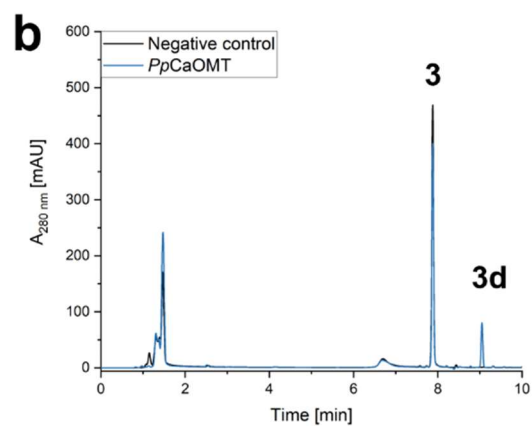
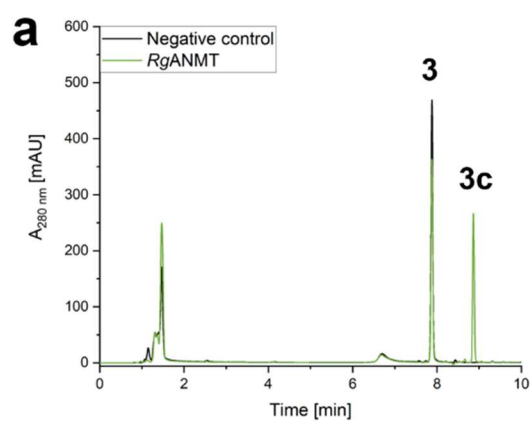


Figure S 4 – HPLC chromatograms (Method B) of the substrate screening using *RgANMT* and *PpCaOMT*. Samples were taken after 20 h. The *N*-methylated products for substrate **5** – **8** were not stable under assay conditions and the product peaks were decreased after 20 h compared to the 1 h samples. The formation of **5a** – **8a** are shown after 1 h. **a**: Conversion of **3** to **3b** catalysed by *PpCaOMT*. **b**: Product **4a** was used as substrate for another methylation step catalysed by *PpCaOMT* to form product **4ab**. **4a** was synthesised in vivo. Small amounts of the origin substrate **4** were left in the stock solution leading to the formation of **4b** in small amounts as well. **c**: Conversion from **4** to **4a** catalysed by *RgANMT*. **d**: Conversion of **4** to **4b** catalysed by *PpCaOMT*. **e**: Formation of **5a** catalysed by *RgANMT* after 1 h. **f**: Formation of **5b** catalysed by *PpCaOMT*. **g**: Formation of **6a** catalysed by *RgANMT* after 1 h. **h**: Formation of **6b** catalysed by *PpCaOMT*. **i**: Formation of **7a** catalysed by *RgANMT* after 1 h. **j**: Formation of **7b** catalysed by *PpCaOMT*. **k**: Formation of **8a** catalysed by *RgANMT* after 1 h. **l**: Formation of **8b** catalysed by *PpCaOMT*.



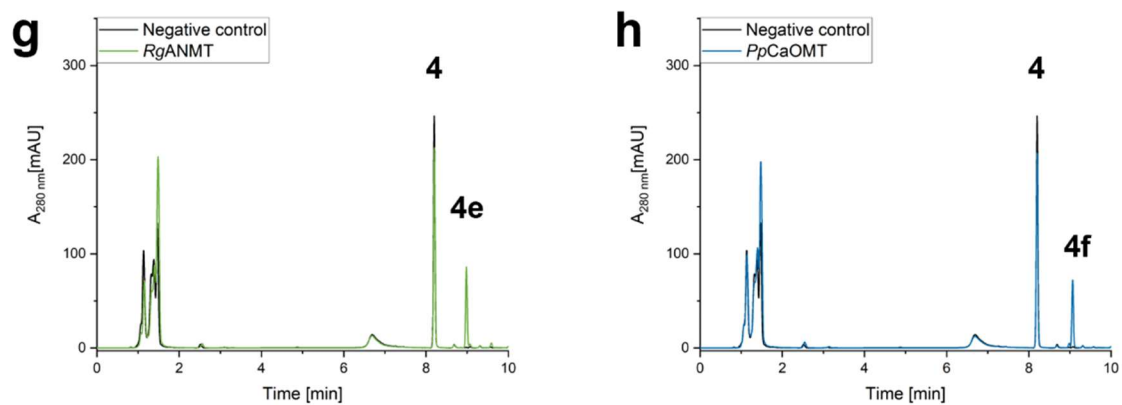


Figure S 5 – HPLC chromatograms (Method B) of the ethyl and allyl transfer assays using *RgANMT* and *PpCaOMT* as biocatalysts. The samples were taken after 20 h. **a:** Formation of **3c** catalysed by *RgANMT*. **b:** Formation of **3d** catalysed by *PpCaOMT*. **c:** Formation of **4c** catalysed by *RgANMT*. **d:** Formation of **4d** catalysed by *PpCaOMT*. **e:** Formation of **3e** catalysed by *RgANMT*. **f:** Formation of **3f** catalysed by *PpCaOMT*. **g:** Formation of **4e** catalysed by *RgANMT*. **h:** Formation of **4f** catalysed by *PpCaOMT*.

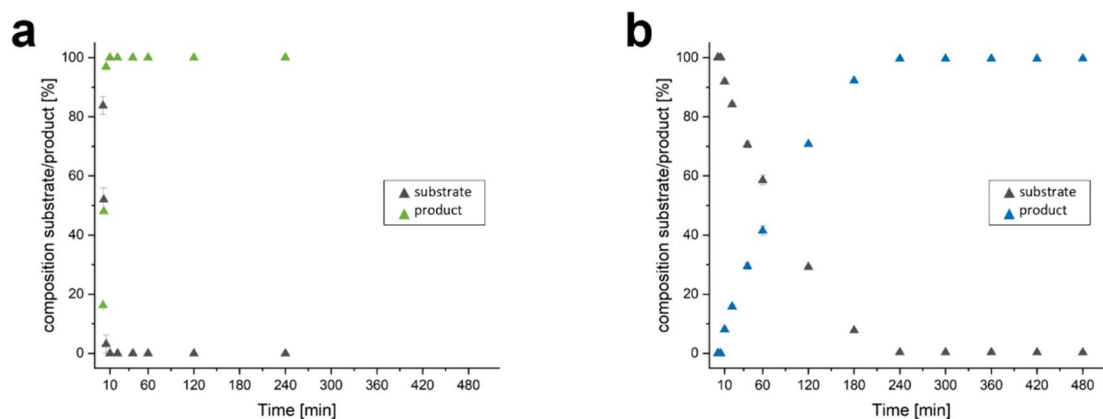


Figure S 6 – Time course experiments with *RgANMT* and *PpCaOMT* using substrate **4**. **a**: Reaction catalysed by *RgANMT*. After 5 min the reaction is completed and no substrate is left in the assay. **b**: The reaction catalysed by *PpCaOMT* with substrate **4** is completed after 240 min.

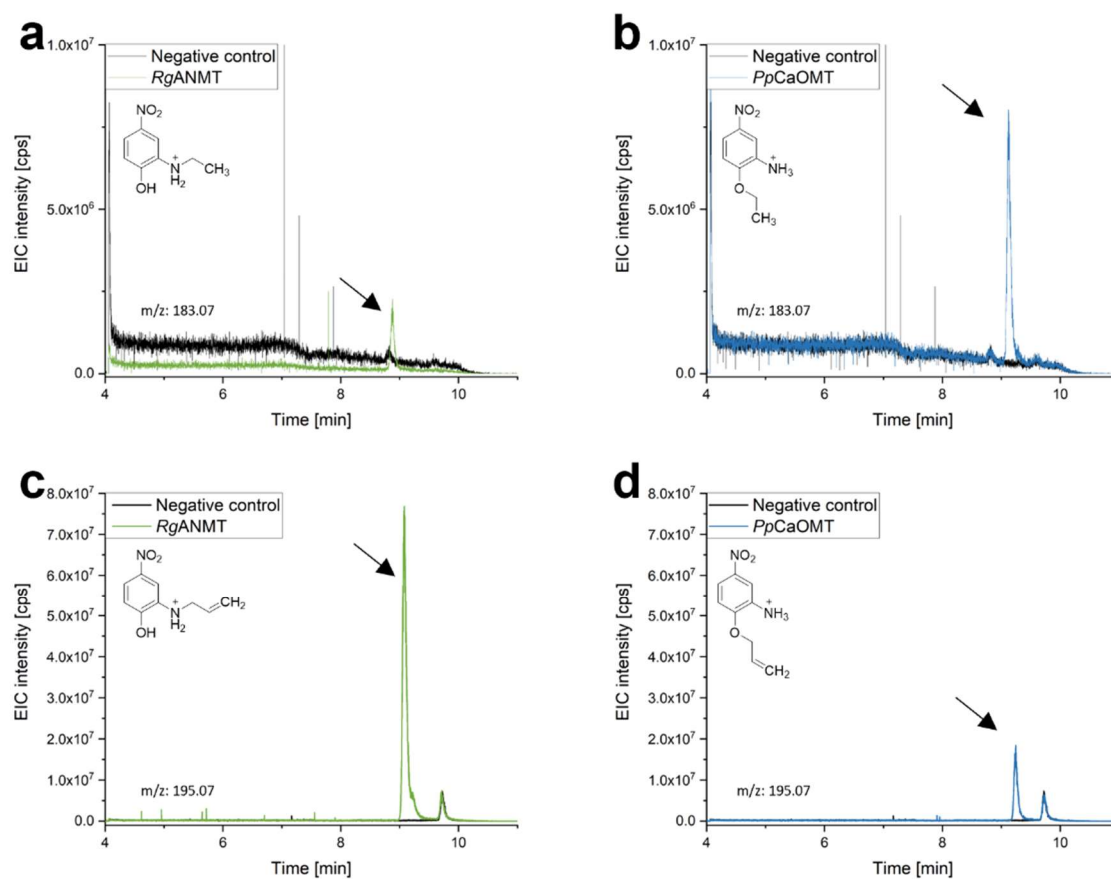


Figure S 7 – Extracted ion chromatograms for ethylation and allylation reactions catalysed by *RgANMT* and *PpCaOMT*. The product was found in the positive mode. **a**: Formation of **3c** catalysed by *RgANMT*. **b**: Formation of **3d** catalysed by *PpCaOMT*. **c**: Formation of **3e** catalysed by *RgANMT*. **d**: Formation of **3f** catalysed by *PpCaOMT*.

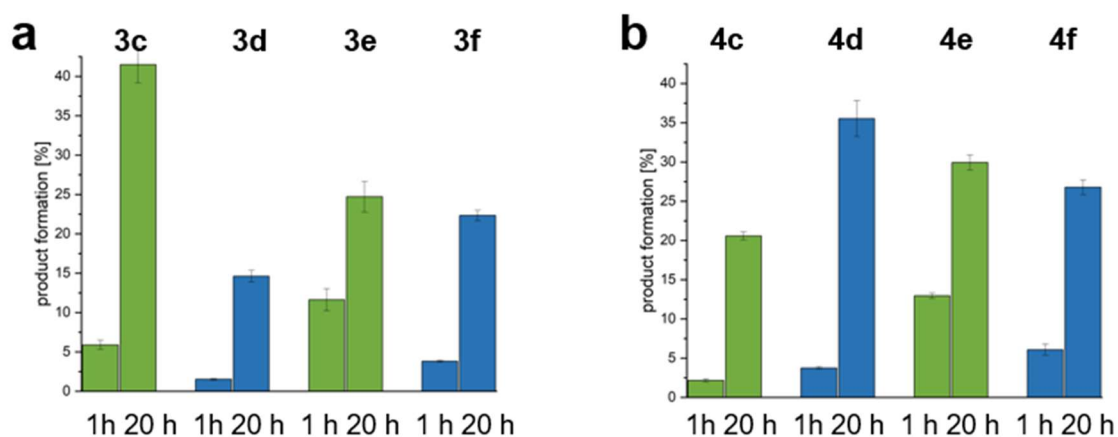


Figure S 8 – Conversion numbers for ethylation and allylation experiments catalysed by *RgANMT* (green) and *PpCaOMT* (blue) after 1 and 20 h. **a**: Conversion of ethylated and allylated products **3c-f** using substrate **3**. **b**: Conversion of ethylated and allylated products **4c-f** using substrate **4**.

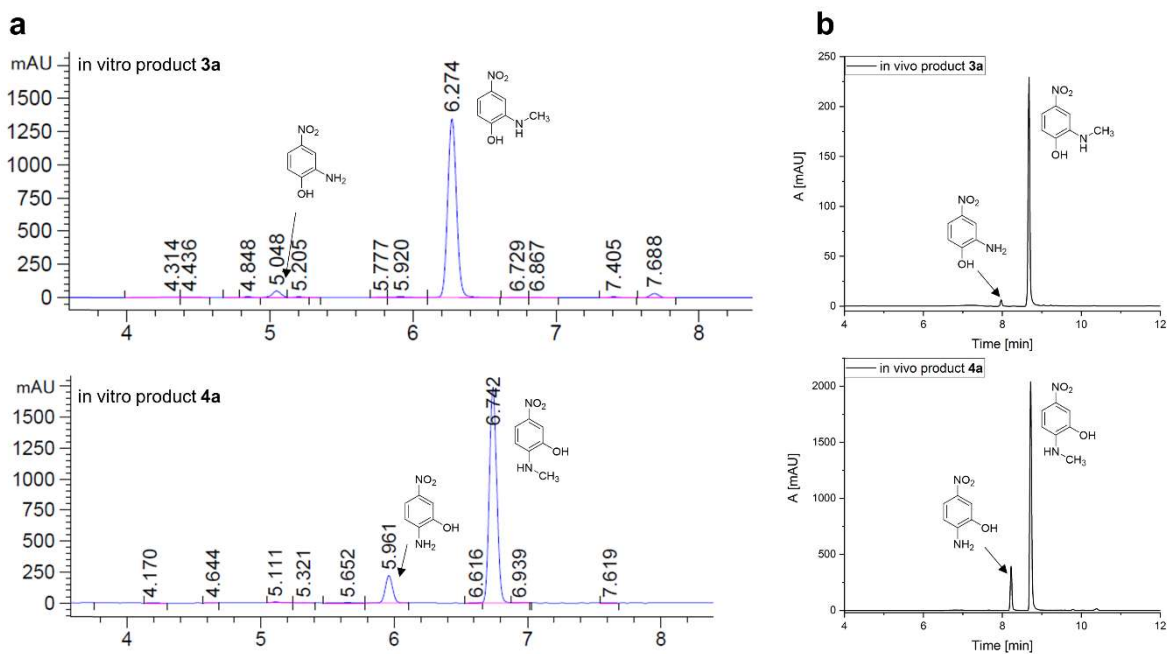


Figure S 9 – HPLC chromatograms of purified *N*-methylated products **3a** and **4a**. **a**: Products from upscale in vitro experiments catalysed by *RgANMT*. **b**: Products from upscale in vivo experiments catalysed by *RgANMT*.

NMR analysis

Confirmation of chemoselective reactions

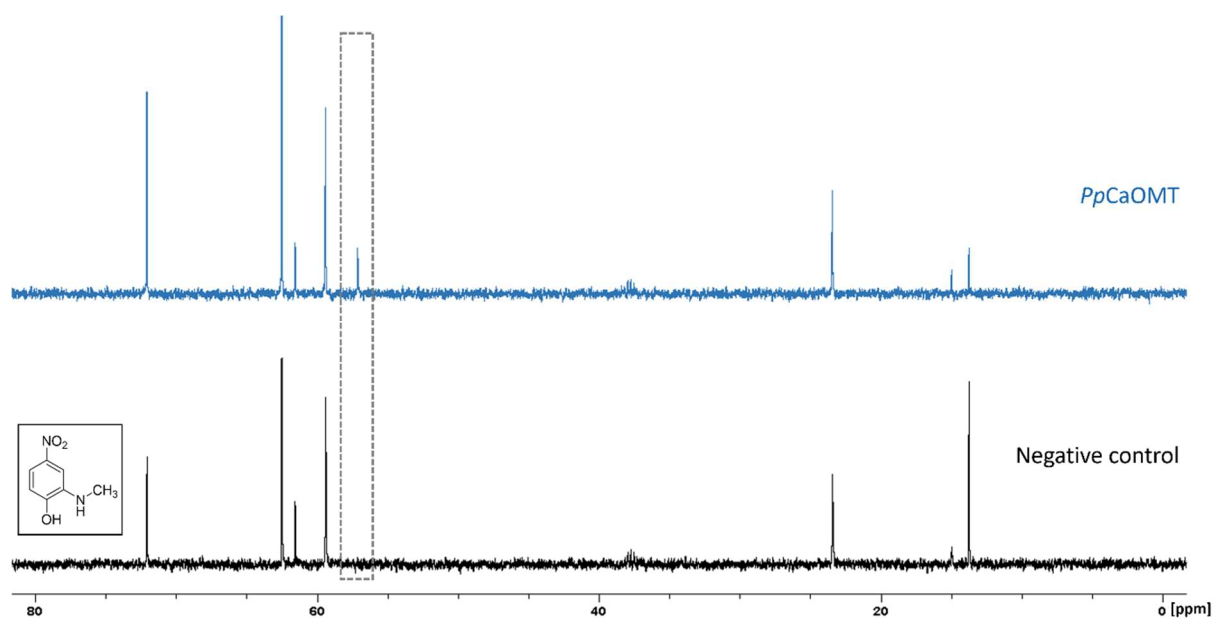


Figure S 10 – Double methylation using product **3a** as substrate. For the experiment, ^{13}C -labelled methionine was used. The concentration of **3a** was too low to visualize the carbon of the methyl group in *N*-position. The signal in the PpCaOMT reaction (blue) at 58 ppm belongs to the ^{13}C -labelled methyl group adjacent to the hydroxyl group. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

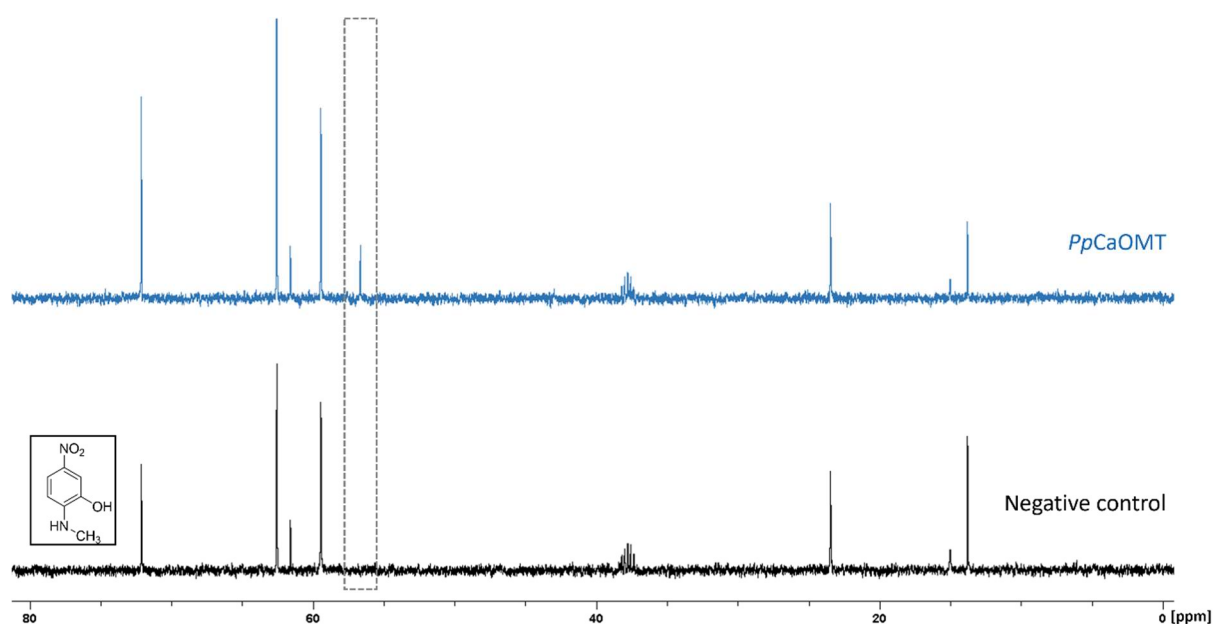


Figure S 11 – Double methylation using product **4a** as substrate. For the experiment, ^{13}C -labelled methionine was used. The concentration of **4a** was too low to visualize the carbon of the methyl group in *N*-position. The signal in the PpCaOMT reaction (blue) at 58 ppm belongs to the ^{13}C -labelled methyl group adjacent to the hydroxyl group. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

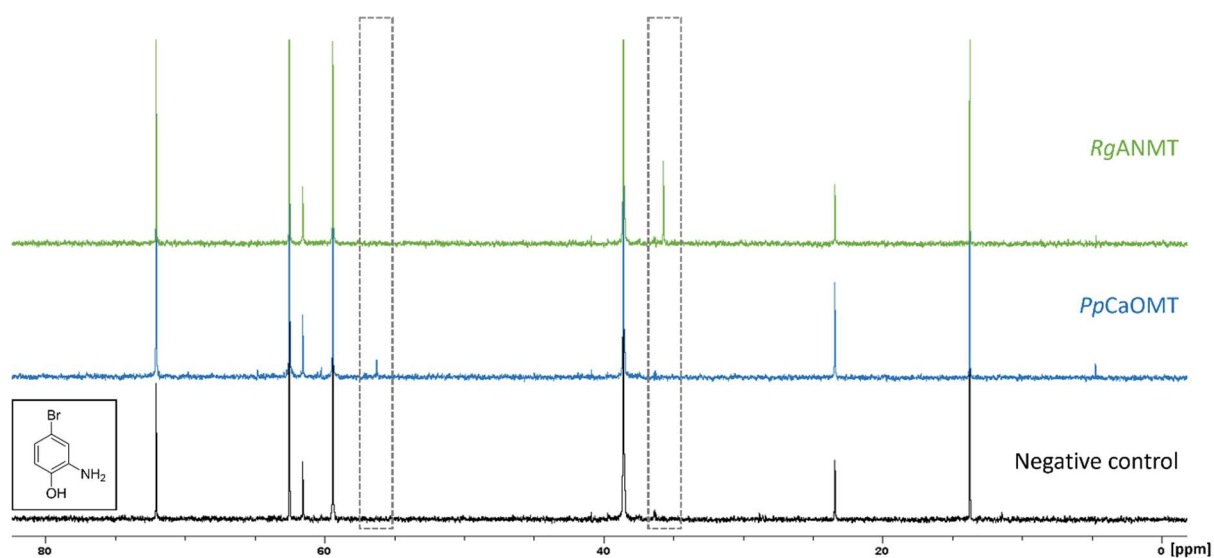


Figure S 12 – Methylation reaction catalysed by *RgANMT* (green) and *PpCaOMT* (blue) using substrate **5**. The ^{13}C -labelled methyl group was transferred onto the amino group (signal at 32 ppm) in the *N*-MT reaction and onto the hydroxyl group (signal at 56 ppm) in the *O*-MT reaction. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

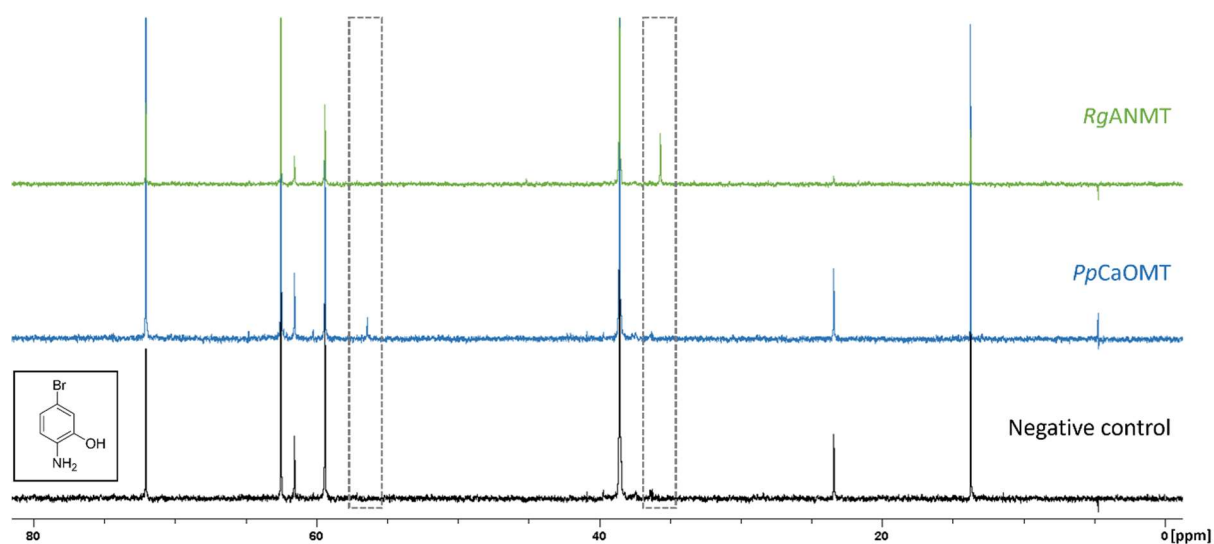


Figure S 13 – Methylation reaction catalysed by *RgANMT* (green) and *PpCaOMT* (blue) using substrate **6**. The ^{13}C -labelled methyl group was transferred onto the amino group (signal at 31 ppm) in the *N*-MT reaction and onto the hydroxyl group (signal at 56 ppm) in the *O*-MT reaction. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

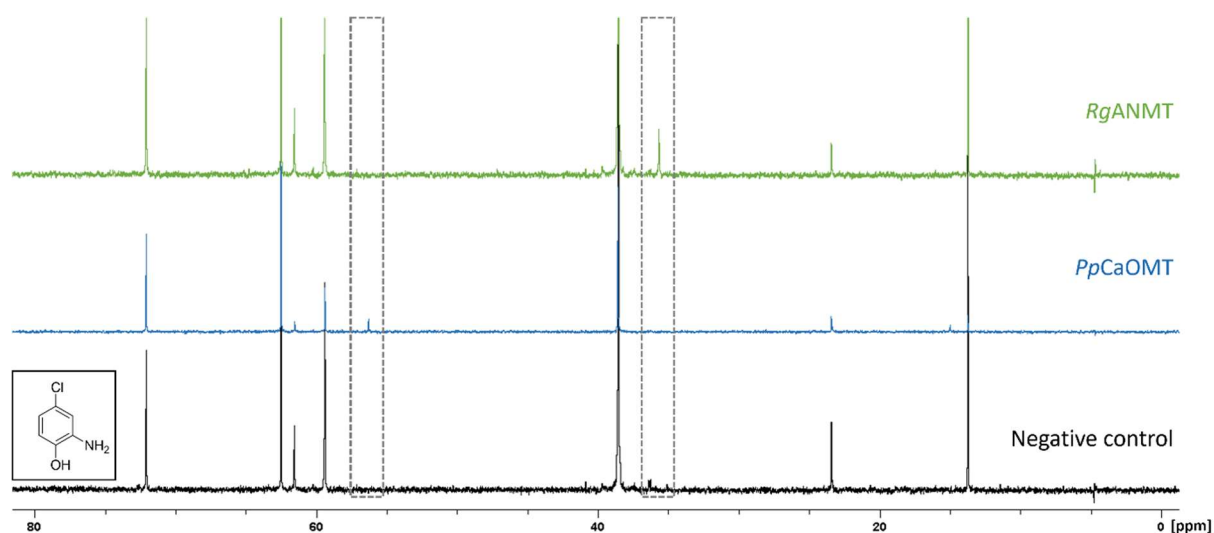


Figure S 14 – Methylation reaction catalysed by *RgANMT* (green) and *PpCaOMT* (blue) using substrate **7**. The ^{13}C -labelled methyl group was transferred onto the amino group (signal at 31 ppm) in the *N*-MT reaction and onto the hydroxyl group (signal at 57 ppm) in the *O*-MT reaction. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

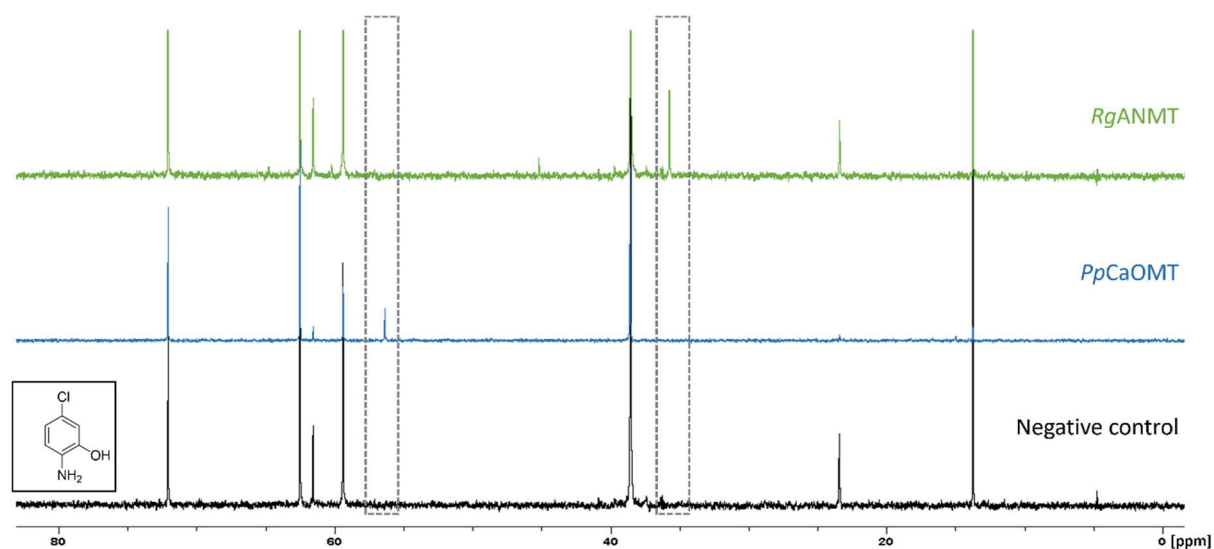


Figure S 15 – Methylation reaction catalysed by *RgANMT* (green) and *PpCaOMT* (blue) using substrate **8**. The ^{13}C -labelled methyl group was transferred onto the amino group (signal at 31 ppm) in the *N*-MT reaction and onto the hydroxyl group (signal at 56 ppm) in the *O*-MT reaction. ^{13}C -NMR (100.6 MHz, 5% D_2O); all other signals are assigned in **Table S 5** in the NMR chapter.

Upscale reactions NMR

in vivo

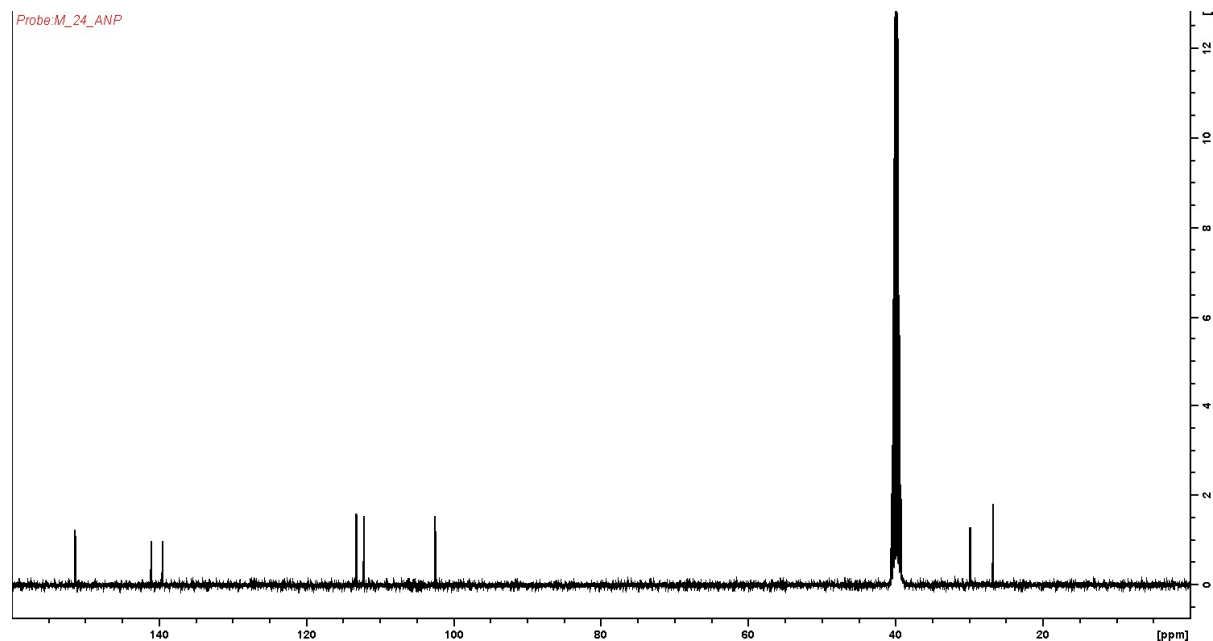


Figure S 16 – ¹³C-NMR for product **3a** from in vivo upscale experiments catalysed *Rg*ANMT after purification. ¹³C-NMR (100.6 MHz, D₆MSO) δ(ppm): 151.5, 141.1, 139.8, 113.3, 112.4, 102.8, 29.9, 27.0. The signal at 27.0 ppm is assigned to cyclohexane which was left from the purification method with the puriflash. The signal at 29.9 was assigned to the carbon of the methyl group adjacent to the amino group.

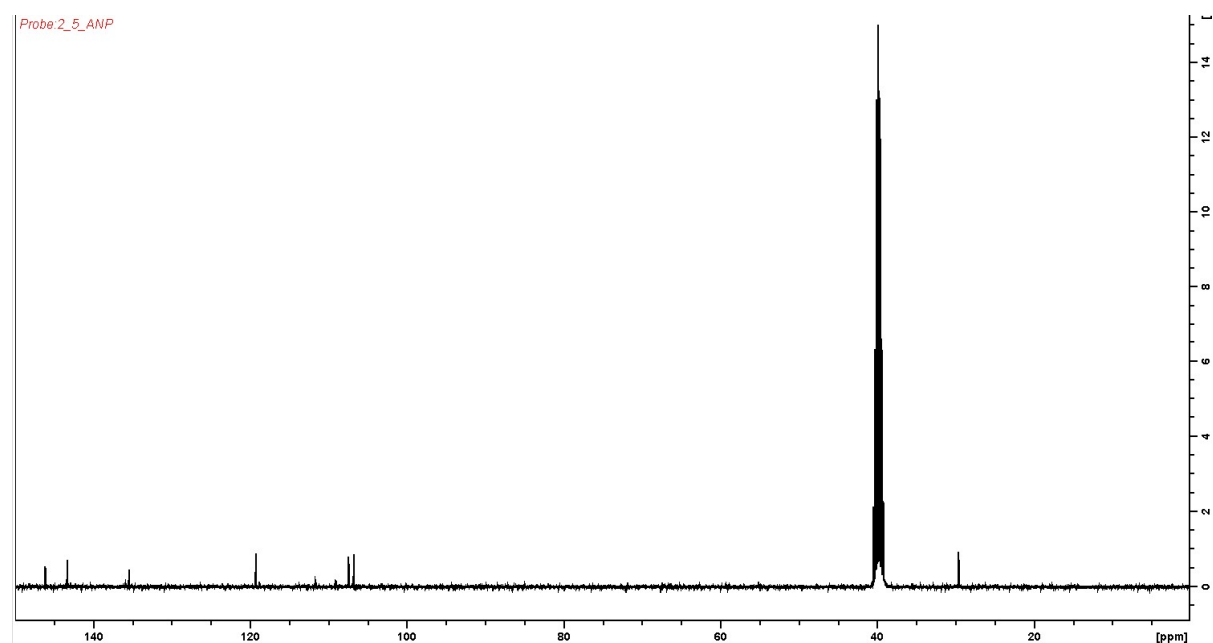


Figure S 17 – ¹³C-NMR for product **4a** from in vivo upscale experiments with *Rg*ANMT after purification. ¹³C-NMR (100.6 MHz, D₆MSO) δ(ppm): 146.1, 143.2, 135.5, 119.4, 107.4, 106.8, 29.6. The signal at 29.6 was assigned to the carbon of the methyl group adjacent to the amino group.

in vitro

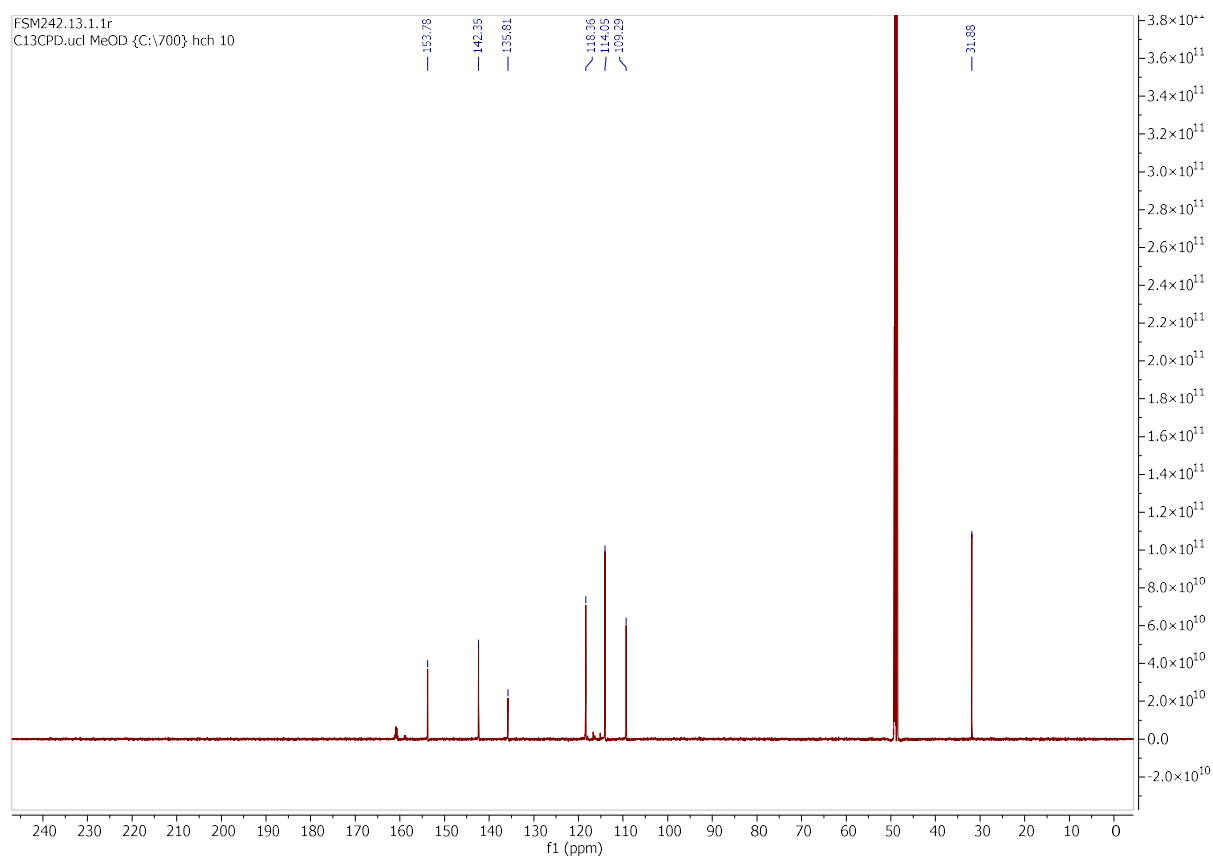


Figure S 18 – ^{13}C -NMR for product **3a** from in vitro upscale experiments with RgANMT after purification. ^{13}C -NMR (176 MHz, MeOD) δ (ppm): 153.8, 142.4, 135.8, 118.4, 114.1, 109.9, 31.9. HRMS (ES+) found $[\text{M}+\text{H}]^+$ 169.0605; $\text{C}_7\text{H}_9\text{N}_2\text{O}_3$ requires 169.0608.

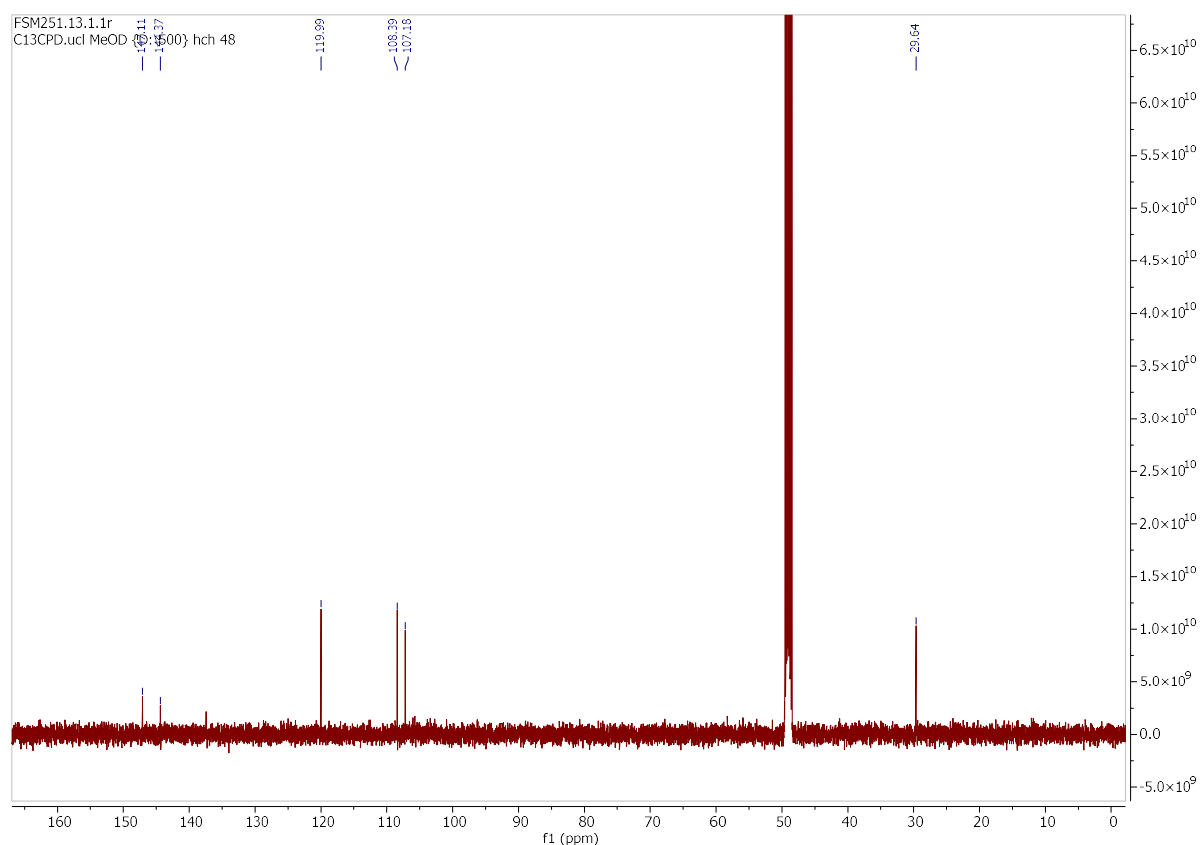


Figure S 19 – ^{13}C -NMR for product **4a** from in vitro upscale experiments catalysed by RgANMT after purification. ^{13}C -NMR (126 MHz, MeOD) $\delta(\text{ppm})$: 147.1, 144.4, 119.9, 108.4, 107.2, 29.6. HRMS (ES+) found $[\text{M}+\text{H}]^+$ 169.0607; $\text{C}_7\text{H}_9\text{N}_2\text{O}_3$ requires 169.06.

Synthesis S-allyl-L-homocysteine

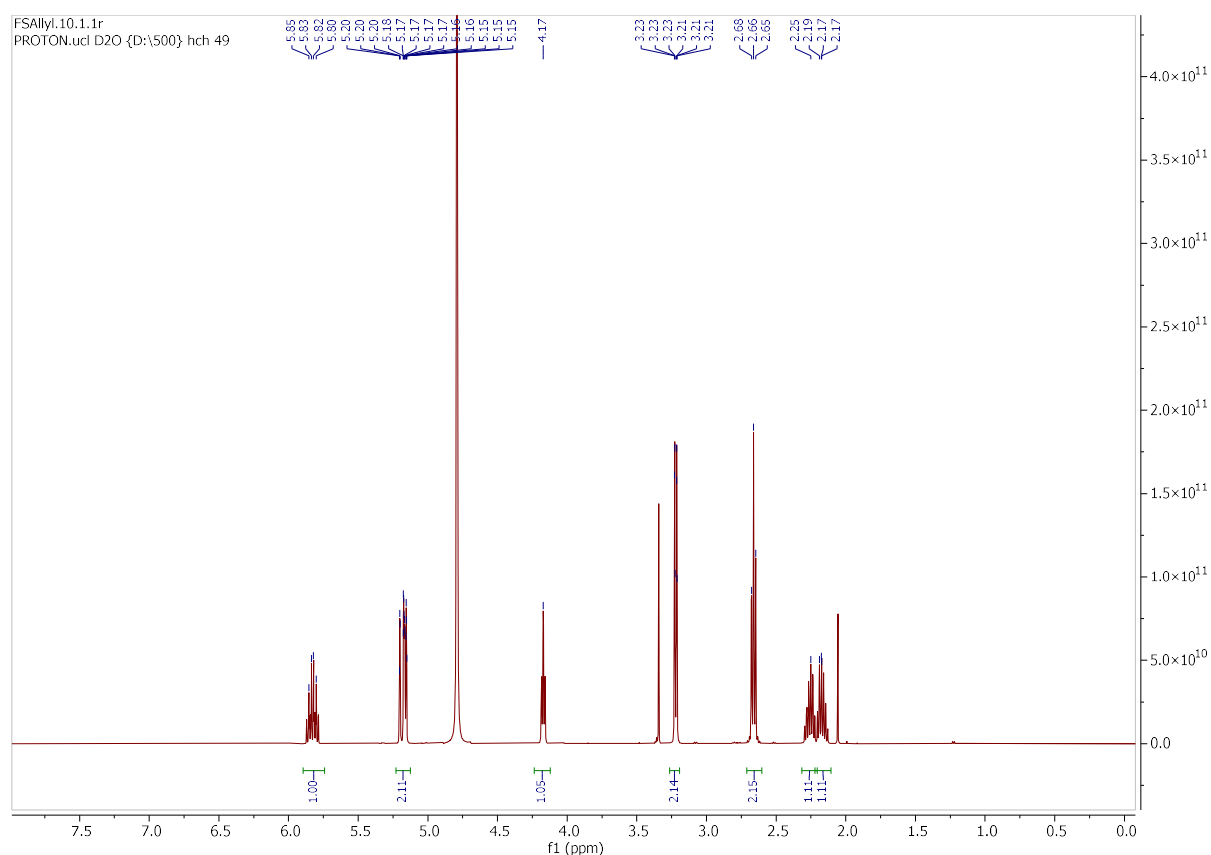


Figure S 20 – ¹H-NMR spectrum of chemical synthesis of S-allyl-L-homocysteine. ¹H-NMR (500 MHz; D₂O) δ (ppm) = 2.25–2.17 (m, 2H), 2.68–2.65 (m, 2H), 3.23–3.21 (m, 2H), 4.17 (m, 1H), 5.20–5.15 (m, 2H), 5.85–5.80 (m, 1H).

Supporting References

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