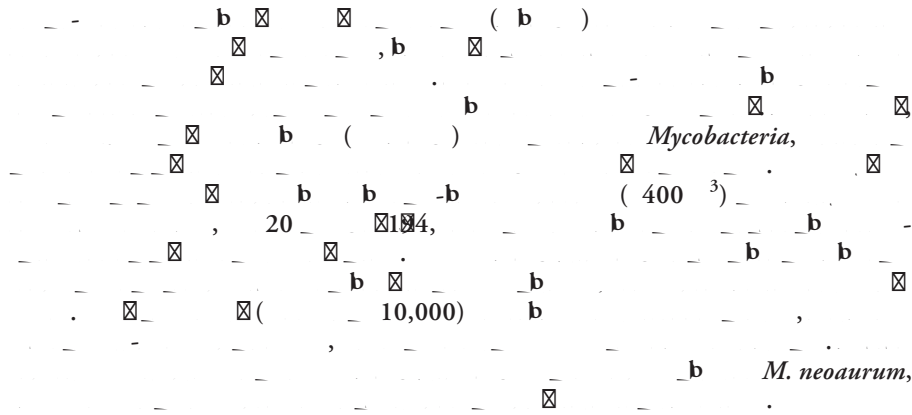




A large conserved family of small-molecule carboxyl methyltransferases identified from microorganisms

ab.1,2 a,1 c a d a ab.c.2



Significance

Small-molecule carboxyl methyltransferases (CbMTs) are critical for modulating biological processes and highly useful in industrial biotechnology; however, they were predominantly restricted to the SABATH family in plants. In this study, the discovery of the 3-OPC carboxyl methyltransferase (OPCMT), which have a distinct catalytic mechanism from SABATH MTs and are widely distributed in microorganisms, significantly broadens the knowledge and availability of small-molecule CbMTs. These methyltransferases (MTs) are indispensable for

whereas not found in human beings, suggesting that they could become ideal targets for antibiotics. In addition, their bulky substrate-binding pockets, differentiating them from other MTs, could facilitate the design of specific inhibitors. Finally, the broad substrate specificity and high catalytic efficiency of OPCMTs also provide a valuable tool for the sustainable production of methyl esters.

(IAA) (20), (21), (22), (23), (24-27) (Fig. S1, SI Appendix, Fig. S1). SABATH C MT (15). D
C MT (28), (26), (29), R, 3-, 4-, 20- (3-OPC) 3-, 4, 17-, 20- (3-OPDC) Mycolicibacterium neoaurum CCTCC AB2019054 (mJTU3) (30). C MT M. neoaurum. H, SABATH C MT (OPCMT MT) (<12%)

Author contributions: Z.L., Z.D., and X.Q. designed research; Z.L., Z.H., L.Z., and B.L. performed research; Z.L., Z.H., L.Z., B.L., X.H., and X.Q. analyzed data; and Z. L. and X.Q. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Copyright © 2023 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

¹Z.L. and Z.H. contributed equally to this work.

²To whom correspondence may be addressed. Email: linz@sjtu.edu.cn or quxd19@sjtu.edu.cn.

This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2301389120/-DCSupplemental>.

Published May 8, 2023.

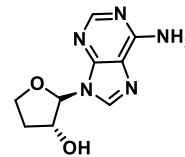
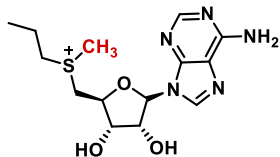
(F . 1). B
 OPCMT MT (10,000)
 M
 MT *M. neoaurum*, OPCMT

²155,
 3-OPC 3-OPDC, C MT
M. smegmatis T
 39 MT *M. neoaurum* MT *M. smegmatis*,
 16 (M, MT2-17). A
 M, MT, M, MT2, 3, 4, 5, 6 40%
 (F . 2A). 16M, MT
 I P (31),
 MT

Results

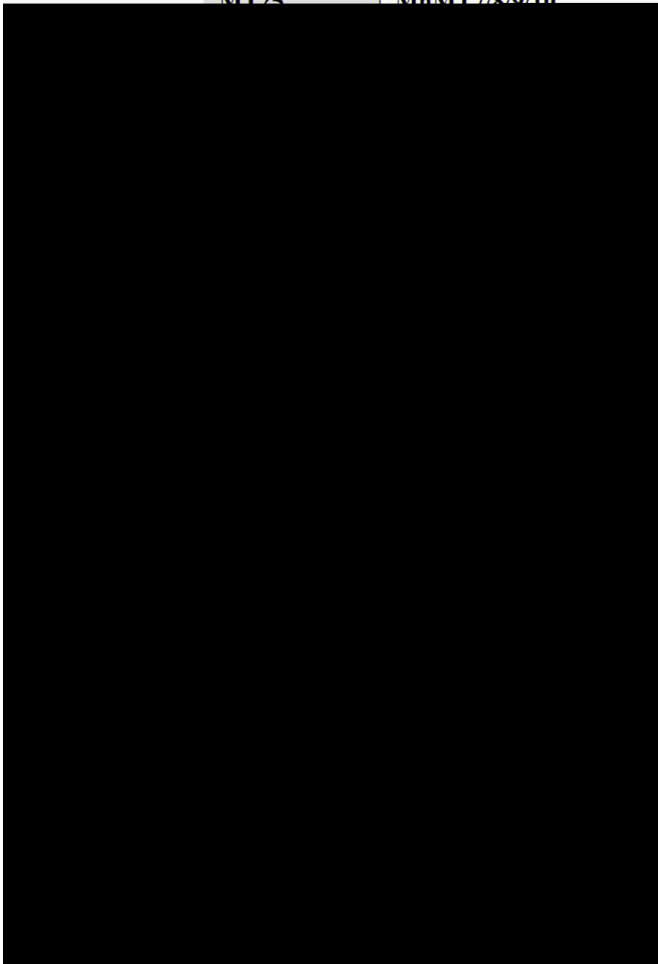
3-OPDC SABATH 3-OPC
 .A *M. neoaurum* CCTCC AB2019054
 67 MT 39 MT
 ; M, MT1, 27%
 SABATH C MT (1M6E 1) (17). T, M, MT1
Escherichia coli. B
 C MT 3-OPC 3-OPDC,
 I, SABATH
Mycolicibacterium smegmatis

T C MT, M, MT (M, MT4,
 7, 11, 14, 15, 17)
 T
 , M, MT4 3-OPC
 3-OPC SAM (F . 2B
SI Appendix, F . S2).
 M, MT4 SAM, M, MT4
 SAM- 3-OPC (OPCMT).
 C M, MT2, 3, 5, 6
 M, MT4, 3-OPC 3-OPC
 A 3-OPC 3-OPC
 12-



A

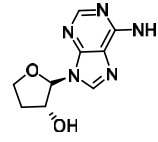
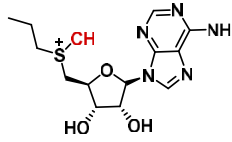
MT family	16 MnMTs
LCM	MnMT2/3/4/5/6
MT25	MnMT7/8/9/10



Characterization of MnMT4 as the MT responsible for 3-OPC methylation. () Grouping the 16 MTs of CCTCC AB2019054, all of which have homologous proteins in mc²155, in the InterPro database. OPCMT-like MTs are shown in red. () Biotransformation of 3-OPC to 3-OPCM using crude recombinant cell lysate. The ID of the six different MT families in InterPro: MT25 (IPRO41698); MT11 (IPRO13216); MT12 (IPRO13217); MT23 (PF13489); LCM (IPRO07213); THUMP-like (IPRO41497).

(. ., 98%, 96%, 70%, 75%, 49% M. MT4, 2, 6, 5, 3,).
 I P (LCM) (IPRO07213).
 LCM (11). (R)
 LCM I ;
 (11, 12, 32, 33). C M. MT2-6
 MT LCM (<20%
), LCMT1 C-
 (11, 12) T P C-9
 E (33), SABATH C MT (<12%
),
 C MT, OPCMT MT (SI Appendix, F . S4).
 SAH SAM- MT . T

OPCMT MT SAH, M. MT4
 F SAM SAH, (K_D) SAH SAM (K_D = 2.91 10⁸ M SAH, K_D = 1.84 10⁵ M SAM), SAH M. MT4 (SI Appendix, F . S5). T SAH (EC 3.2.2.9) L S (EC 3.2.1.148) *E. coli*, SAH (34, 35). A SAH L S 96% (F . 3 4 SI Appendix, F . S7). K M. MT4 (k_{cat} = 765.3 97.40⁻¹, K_m = 489 103.6 M k_{cat}/K_m = 1.563 M⁻¹ s⁻¹) MT (F . 3 SI Appendix, F . S8), 3-OPC *M. neoaurum*. I SABATH MT, k_{cat} SA IAA 5.52 0.46⁻¹ 1.68 0.19⁻¹ (17). *M. smegmatis*²155 10 MT 30% M. MT4. A , M MT1 (67%) M. MT4. C *M. smegmatis* , M MT1 3-OPC, 100% OPC 12 (SI Appendix, F . S4). OPCMT MT *M. neoaurum* CCTCC AB2019054 *M. smegmatis*²155, M. MT4 PDB OPCMT MT , ML2640 (PDB ID: 2U Q) R 0731 (PDB ID: 6ID6), *Mycobacterium leprae* *Mycobacterium tuberculosis* 40% 39% M. MT4, I (36); *Mycobacteria*. T *E. coli*. B 3-OPC 3-OPC OPCMT C MT . N (17). C MT SABATH SAM ML2640 (F . 5A). S ML2640 R 0731 (α₁ α₄) α/β C- β- α- (F . 5B). N- MT . SAM- α/β C- MT . S ML2640 -SAM PPM1-SAM (PDB: 1RJD) (34) SAM α/β C- ML2640 (F . 5C SI Appendix, F . S9). A



SAM	ML2640		G G G	β_1	α_A	I
	A 164	L 165	MT (3). M	-OH		
NH,		SAM		A 135		
A_113	G 115	β_1	A 28, A 90,	G 189		
		α_5				

SAM (F . 5C_ [SI Appendix, F . S9](#)). S SAM-
 ML2640
 I MT (3, 37),
 SAM OPCMT MT
 ([SI Appendix, F . S10](#)).
 T OPCMT
 MT, 3-OPC SAM- ML2640 ,
 ([SI Appendix, F . S11](#)),
 SAM. 3-OPC
 3.2
 . M , 3-OPC
 (400 ³, [SI Appendix, F . S11](#))
 N- (α_1 α_4) C- 20,
 $\beta_4, \beta_5, \beta_7$
 T 194, T 229 (F . 5D).
 M 83 M 233 3-OPC
 3-OPC
 20
 T 194 (F . 5E). N , 20 T 194
 OPCMT MT ([SI Appendix, F . S10](#)).
 T , 20 T 194 ML2640

H (38). *M. tuberculosis*

(39, 40).

M. neoaurum CCTCC AB2019054 3-OPC (MIC)

3-OPC 3 M 0.6 M,

M. neoaurum.

T OPCMT MT

OPCMT

CCTCC AB2019054 CRISPR-C 9- (41)

F, M, MT4, 2, 6,

$\Delta 3mnm$ (SI Appendix, F . S13). H

MIC 3-OPC $\Delta 3mnm$

C MT, M, MT3 5,

M, MT3

$\Delta 3mnm$, $\Delta 4mnm$. A

MIC 3-OPC $\Delta 4mnm$

2 M 0.4 M,

M, MT5 $\Delta 4mnm$

(SI Appendix, F . S14).

A $\Delta 4mnm$

3-OPC

C (SI Appendix, F . S15),

M, MT5,

OPCMT C MT

OPCMT MT

T OPCMT

MT, ML2640

U P T

10,000 ML2640 (>30%) 300

(50

C MT SABATH), OPCMT

MT

ML2640

Mycobacterium (NCBI : 1,763, >5,000) *Streptomyces* (NCBI : 1,883, >2,000), *Nocardia* (NCBI : 1,817, >400), *Rhodococcus* (NCBI : 1,827, >200), *Amycolatopsis* (NCBI : 1,813, >150), *Chlamydomonas* (NCBI : 3,052, >50), *Kitasatospora* (NCBI : 2,063, >50). S

A

R 5,000 (>30%)

SAM (A 28, A 90, A 113, G 115, S 114, A 135, A 164, L 165, G 189, G 190) (F . 6 SI Appendix, F . S11 S16). O. P T 194,

ML2640 - 195F (F . 5F),

OPCMT MT

20 T /P 194

OPCMT MT,

(G . 25 T 115)

SABATH (17).

OPCMT MT

F

1,500 OPCMT MT

Mycobacterium tuberculosis (NCBI : 77,643)

Mycobacterium avium (MAC) (NCBI : 120,793),

20 *M. avium* 104 (NCBI : 243,243), 27

Mycobacterium ulcerans (NCBI : 1,809). A

OPCMT

OPCMT

MT *M. neoaurum*,

OPCMT

A

Discussion

C

(15) D

C MT

P

SABATH

C MT ;

(16, 17). A

SABATH

(26, 28, 29). A

SABATH C MT

U

SABATH C MT, C MT

3-OPC M

2-

3-

10 100 (42). M

p-

(43),

2-

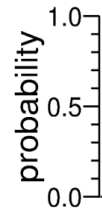
2-

P

C MT

F M,

C MT



(44). M. MT4
SABATH
F. M. I. SAM (45), OPCMT
B. OPCMT MT
N- (α_1 α_4) α/β -C- 300 A
OPCMT C MT SABATH C MT
(17). N- OPCMT MT
ML2640 -T20/ 194,
SAM, OPCMT MT
OPCMT MT.F
OPCMT MT
OPCMT MT
M. tuberculosis M. avium,
A.F
ML2640,
OPCMT MT

C MT,
ML2640
Materials and Methods
n SI Appendix, 2
n n n
DNA n n n -
n n E. coli w n n (46). DNA
(n A) n n nz w
B n (C., L. J n) n G n, (B n, C n).
DNA n n n n w n (n)
C n). n SI Appendix, 3. E n
M. neoaurum CC CC AB2019054 w n
n n w 2.5 /
, n 25 (47).
mnmt M. neoau-
rum
Gene deletion of mnmt4. mnmt4 n w
w - M. neoaurum CC CC AB2019054 n n.
A.1.78- - n n w n DNA
w w MnM 4- - / MnM 4- - , 1.92- - n
n w w w MnM 4- - / MnM 4-
- n n n w n n
Eco I/Hin III 18 B. n H 1w n
n M. neoaurum CC CC AB2019054 n.F w n n

mnm4 n Mko4 w
 C-Δ 4- (n SI Appendix, 3) n DNA n n .
Gene deletion of *mnm2*. *mnm2* n w
 n n mko4
 MnM 2- / MnM 2- . , 1.69- - n
 n w w w MnM 2- / MnM 2- .
Hin III 18 B. n H 2 w n
 n mko4 n.F w n n n
 w C , *mnm1*- n mko1 w n .l n
 n SI Appendix, 3) n DNA n n .
Gene deletion of *mnm6*. *mnm6* n w
 n n mko2
 MnM 6- / MnM 6- . , 1.93- - n
 n w w w MnM 6- / MnM 6- .
 n n n w n n Eco I/Hin III
 18 B. n H 3 w n n mko1
 n.F w n n n
 , *mnm6*- n mko6 (Δ*mnm6*) w n .l n
 w C n n C-Δ 6- / C-Δ 6- (n SI Appendix, 3) n DNA n n .
Gene deletion of *mnm3*. *mnm3* n w
 n n mko6
 MnM 3- / MnM 3- . , 1.78- - n
 n w w w MnM 3- / MnM 3- .
 n n n w n n Eco I/Hin III
 18 B. n H 5 w n n mko6
 n.F w n n n
 , *mnm3*-del n mko9 (Δ*mnm3*) w n .l n
 w C n n C-Δ 3- / C-Δ 3- (n SI Appendix, 3) n DNA n n .
Gene deletion of *mnm5*. *mnm5* n w
 w - *M. neoaurum* CC CCAB2019054 n -
 n.A1.60- - n n w n
 DNA w w MnM 5- / MnM 5- . , 1.65- -
 n n w w w MnM 5- /
 MnM 5- . n n n w n
 n Eco I/Hin III 18 B. n H 4 w
 n n *M. neoaurum* CC CCAB2019054 n.F w n
 n n n , *mnm5*- n
 mko5 w n .l n w C n n
 C-Δ 5- / C-Δ 5- (n SI Appendix, 3) n DNA
 n n .
 n , n n , n n ML2640 w
 n n n , n n n n , ML2640 -
 E 28 n w (194F-F / 194F- , 20 -
 F / 20 - , 229A-F / 229A-), w *DpnI* n , n
 n n n n n (194F-ML260 - E 28 , 20 -
 ML260 - E 28 , 229A-ML260 - E 28 . n 194F-ML260 -
 E 28 w 20 -F / 20 - , w *DpnI*
 n , n n n n n n 194F/
 20 -ML260 - E 28 . n n w n n E.
coli BL21 (DE3) n n .A n
 SI Appendix, 3
 nz n n w 200 μL BK
 (100 M, H 8.0). w 1 M 3-O C, 2 M AM,
 10 M AHE, 10 M L E, n 10 M n , n n 37 C
 10 n, 20 n, 0.5 , 1 , 4 , 8 n 12 , n w
 n n n 5 n n n n
 n n w LC-M n .

n 3-O C w n
 (30).A 4-HBC(100 , 0.33)
 n (2.2 L), w z w C O₃(66.6 , 0.66)
 n w (0.2 L) n (1 L), n n
 6 . n, 5.4 L n w
 n n n 5 C.N , 5% N OH w
 H 11, n n w w CH₂C₂ n
 w w w w w . w n n w
 HC H = 2 n w w CH₂C₂ n w n ,
 w w n , n n N₂ O₄ w n n
 n n 3-O C(80.59 , 71%) w .
 n n n (K_D) MnM 4 w AM n AH w
 n n n n z F B
 O ED 96 (F B , A). A n n w
 37 C.N n n(N-N A) n w w MnM 4
 (40 M) n n n 20 MHE E , H 7.4, 300 MN C.A
 n n n n n n w
 w AM n AH, n n n w n
 n w O ED 96. In n n
 , AH(3, 15, 75 M) n AM(1, 5, 25 nM) w n w
 z MnM 4 n, n n n w
 n n n n , n w n z
 n w O ED 96. AM n AH w
 n n z w n n .

Construction of Protein Expression Plasmids

M, MT
 M. MT2/3/4/5/6-F M. MT2/3/4/5/6-R ,
 DNA *M. neoaurum* CCTCC AB2019054
NdeI
EcoRI ET28 (ET28 -
 M. MT2/3/4/5/6). SAH.
 L S S E-F / S_ E-R , L S-
 F / L S-R , DNA *E. coli* BL21 (DE3)
NcoI
 (ET28_S_ E_ ET28_L_ L S). A
 SI Appendix, T. S3.
E. coli BL21(DE3)
E. coli L
 B (LB) 50 μ L⁻¹ 37, C
 220 0.6 0.8 OD₆₀₀
 P (IPTG) 0.1 M,
 20 16, C.
 4,000 20 4, C
 (20 M HEPES, 300 M N_C ,
 10 M , H 7.5). A
 N -NTA
 (20 M HEPES, 300 M N_C , 50 M , H
 7.5), (20 M HEPES, 300 M
 N_C , 250 M , H 7.5).
 (500 μM 1 M) A U
 C F D. (M , MA;
 10 D) PD-10 D
 C (GE H , USA)
 (H 7.5) 10% 80, C 20 M HEPES
 N_ D 3000.

UPLC C18 (1.9 μm, 2.1 μm, 50 μm), SHIMADU, J. (A) (H₂O 0.1%, 0.1%) B (0.2 L⁻¹, 11, :0 1, 5 10% B; 1 8, 10 100% B; 8 9, 100% B; 9 10, 100 5% B; 10 11, 5% B. 254.

200 μL PBK (100 μM, H 8.0). 1 μM, 2 μM SAM, 10 μM SAHE, 10 μM L E, 10 μM OPCMT, 37, C 0.5, 1, 4, 8, 12, 50-L 250-L UPLC-MS

K, 3-OPC, 3-OPC, S UPLC 3-OPC (S, K, J). 3-OPC B, K M, M, G, P, 5.0, R, SEM (SI Appendix, F . S8.)

200 μL PBK (100 μM, H 8.0), 0.05 μM M, MT4, 1 μM SAHE, 1 μM L E, 0.5 μM SAM, M, MT2/3/5/6, 200 μL PBK (100 μM, H 8.0), 2 μM M, MT2/3/5/6, 10 μM SAHE, 10 μM L E, 2 μM SAM, 37, C 5 UPLC 254.

3-OPC SAM ACP PE(// 2, /) MD GROMACS 2018.5 (SI Appendix, F . S11.). A 14, 1 TIP3P 80, 80, 80, 10 (N⁺, C⁻);

- D.K.L., G., J., N., A. Nat. Prod. Rep. 2, 1238-1250 (2012).
- D.D. et al., N(1)- Nature. 530, 441-446 (2016).
- E.A. et al., M. ChemBioChem 23, 202200212 (2022).
- D.K.L., J.F., M., -N-, n opium poppy. J. Biol. Chem. 282, 14741-14751 (2007).
- C.C.H. et al., C. Mycobacterium tuberculosis. J. Biol. Chem. 277, 11559-11569 (2002).
- H.C. et al., M. Plant J. 4, 193-205 (2006).
- J., A.B.J., E.W., J.H.N., D.O'H., H. Arabidopsis thaliana. Angew. Chem. Int. Ed. 4, 3646-3648 (2010).

14 N⁺, A, 100, 300 K, 60, 40-300 K, A (NPT) 300 K, 2, 300 K, 1, 1-40.

P, R, E (PME), 14

M. neoaurum Δ4mmnt *M. neoaurum* CCTCC AB2019054 Δ4mmnt 5 L 30, C, 220, 400 L 1% 30, C, 220, A, 48, 60 L 100 M (H = 8.0). A, 2 L 300-L

200 μL 1 μM 3-OPC, 2 μM SAM, 10 μM L E, 10 μM SAHE, 37, C 0.5, 1, 4, 50-L 250-L UPLC-MS (SI Appendix, F . S15).

A, n, n n / SI Appendix. n M, G, n n L n n, D, L, n n n n 5000 O, CM, n', n, w, w, n, N, n K & D, C, n (2021 FC2100600, 2018 FA0900400, N, FC(31770063, n, 22107069, L.).

Author affiliations: ^aState Key Laboratory of Microbial Metabolism, School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China; ^bZhangjiang Institute for Advanced Study, Shanghai Jiao Tong University, Shanghai 200240, China; ^cKey Laboratory of Combinatorial Biosynthesis and Drug Discovery Ministry of Education & Abiochem Biotech Joint Center for Pharmaceutical Innovation, School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China; and ^dDepartment of Gastroenterology and Hepatology, Tongji Hospital affiliated to Huazhong University of Science and Technology, Wuhan 430071, China

- C.L. et al., M. Phytol. 1, 3, 665-672 (2012).
- J. et al., A. Proc. Natl. Acad. Sci. U.S.A. 103, 2075-2080 (2006).
- J., G., AM/ AH, AM- ACS Chem. Biol. 11, 583-597 (2016).
- J.L., J., 2A, J. Biol. Chem. 268, 19192-19195 (1993).
- et al., 2A, LCM-1. Mol. Cell. 41, 331-342 (2011).
- M., K, C, -O-, (COM): B, COM, Pharmacol. Rev. 51, 593-628 (1999).
- M., H.L., B., -O-, Br. J. Clin. Pharmacol. 77, 410-420 (2014).

15. L.C. ,H. .M C ,A.J.C n ,C n :N n n n n .*ChemCatChem*. **13**, 121 128 (2020).
16. J.C.DA ,F.C n,E ,C n ABA H M n*Arabidopsis Thaliana* .*Recent Adv. Phytochem*. **3**, 253 283 (2003).
17. C. *et al.*, n n n .*Plant Cell*. **15**, 1704 1716 (2003).
18. L.M.M ,N.K ,C.J.M nn,N.D , n n z n S- n - L- n n : nz nz n w *Antirrhinum majus*.*Arch. Biochem. Biophys.* **3** **2**, 145 151 (2000).
19. J.K n *et al.*,E n nn / - n n n n .*Plant Cell*. **1** , 3212 3229 (2007).
20. N. *et al.*, , n n n n n n -3- ABA H .*Plant Physiol*. **14** , 455 467 (2008).
21. M. n *et al.*,M n n *Arabidopsis* GAM 1 n GAM 2.*Plant Cell*. **1** , 32 45 (2007).
22. H. . *et al.*,J n n :A nz n - n n .*Proc. Natl. Acad. Sci. U.S.A.* ,4788 4793 (2001).
23. J.M ,J. ,H.G n, .D L , C n z n.*Plant Cell*. **20**, 524 542 (2008).
24. . n *et al.*, An *Arabidopsis thaliana* n n n .*Arch. Biochem. Biophys.* **44** , 123 132 (2006).
25. .A ,J.H.L w, nz n n .*J. Biol. Chem.* **245**, 709 713 (1970).
26. .N w , .B ,N.K ,A.L ,En n n *Escherichia coli* z n .*Appl. Environ. Microbiol.* --, 8052 8061 (2011).
27. N. n , .K.N ,B n n :A nz n .*Chem. Biol.* **22**, 1480 1490 (2015).
28. N. n ,A.J.H z,M.A. , .K.N ,L n n :In n n .*ChemBioChem* **1** , 784 788 (2018).
29. E.J n *et al.*,N w n : n n *Salvia dorisiana* .*J. Exp. Bot.* --**1**, 3052 3065 (2020).
30. H. n *et al.*,A .*Angew. Chem. Int. Ed.* . **0**, 5414 5420 (2021).
31. . n-L *et al.*,In n 2022. *Nucleic Acids Res.* **51**, D418 D427 (2022), 10.1093/n / 993.
32. N.L *et al.*, n n n n n n 1(M1), n 2A .*J. Biol. Chem.* **2** , 8351 8358 (2004).
33. E. .J n n,C. .H n n,B. n, n n n n A2 n n n w O(2) n n H(2)O. *Org. Lett.* **2**, 3225 3227 (2000).
34. C.L.H n ,J. . ,E. ,J. .N , . . ,An nz - S- n n - n n n n .*Anal. Biochem.* **32** , 100 105 (2004).
35. K.A.C n , .E. w , .D.B ,M.K. ,C z n n *Escherichia coli* 5'- n n /S- n n n :An nz n .*Biochem. Biophys. Res. Commun.* **22** , 724 732 (1996).
36. M.G *et al.*, M. *leprae* ML2640 n n .*Protein Sci.* **1** , 1896 1904 (2007).
37. J.L.M n,F.M.M M n, AM(n n)IAM: S- n n n - n n n .*Curr. Opin. Struct. Biol.* **12**, 783 793 (2002).
38. L. J ,L.A. , .L , n n n n n n .*Front. Microbiol.* **4**, 272 (2013).
39. J.C.C n *et al.*,I n n *Mycobacterium tuberculosis* .*J. Bacteriol.* **1** **1**, 5232 5239 (2009).
40. E. .B n *et al.*,C n n n n n n nz n - w n *Mycobacterium* . n .*J. Steroid Biochem. Mol. Biol.* **13** , 41 53 (2013).
41. . n *et al.*,C l -C 9,C l n C l -BE - n n n n n .*Nat. Protoc.* **15**, 2470 2502 (2020).
42. .B *et al.*,F n w n n n .*Food Chem. Toxicol.* **45**, 113 119 (2007).
43. .J.K n *et al.*,D z n n n n n n w n - n .*Science* **35** , 314 319 (2018).
44. L.C. *et al.*,C n n :A n n .*Angew. Chem. Int. Ed.* . **1**, Aew. *Chem. Int. Ed.*