Engineering Microbiology 2 (2022) 100007

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Short Communication

In vitro characterization of a nitro-forming oxygenase involved in 3-(*trans*-2'-aminocyclopropyl)alanine biosynthesis☆

Linlin Pang[a], Weijing Niu[a], Yuwei Duan[a], Liujie Huo[a], Aiying Li[a], Jiequn Wu[c], Youming Zhang[a], Xiaoying Bian[a,*], Guannan Zhong[a,b,*]

[a] *Helmholtz International Lab for AntiInfectives, Shandong University-Helmholtz Institute of Biotechnology, State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China*

[b] *Suzhou Institute of Shandong University, Suzhou 215123, China*

[c] *Collaborative Innovation Center of Yangtze River Delta Region Green Pharmaceuticals, College of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou 310014, China*

ARTICLE INFO

Keywords:
Nitro-forming oxygenase
HDO superfamily protein
Belactosin A
Hormaomycin
Cyclopropyl ring

ABSTRACT

In vitro characterization experiments revealed the formations of 3-(*trans*-2'-aminocyclopropyl)alanine ((3-Acp)Ala) and 3-(*trans*-2'-nitrocyclopropyl)alanine ((3-Ncp)Ala) are originated via two homologous proteins, BelK and HrmI, which regioselectively catalyze the Nε-oxygenation of L-lysine. The two enzymes belong to the emerging heme-oxygenase-like diiron oxidase and oxygenase (HDO) superfamily and the catalytic center of BelK is validated by homology modeling and site-directed mutations. Based on the *in vitro* characterization, the biosynthetic pathways of (3-Acp)Ala and (3-Ncp)Ala are proposed.

The cyclopropane moiety has been found in several natural products of secondary metabolism from plants, fungi, and microorganisms (Wessjohann et al., 2003). The cyclopropyl skeleton lacks the ability to freely rotate and serves as a rigid structural element. This along with a C-C-C bond angle that significantly differs from the ideal bond angle of 109.5°, results in ring strain of up to 27.6 kcal/mol (Stability of Cycloalkanes 2021). The cyclopropyl ring is a versatile player that frequently appears in preclinical and clinical drug molecules as it enhances potency, reduces off-target effects, increases metabolic stability and brain permeability, decreases plasma clearance, alters the pKa of drugs to reduce its P-glycoprotein efflux ratio, and more. (Talele, 2016). Owing to its unique structure and excellent contributions to drug molecules, the cyclopropyl ring has led to advances in elegant synthetic methods (Chen et al., 2012).

Belactosin A (**1**, Fig. 1), a natural product isolated from *Streptomyces* sp. UCK 14 in 2000, has been shown to bind covalently to the proteasome β_5 subunit and prevent cell cycle division in tumor cells at the G2/M stage, thus presenting a novel lead in the development of potent anticancer agents (Asai et al., 2000, Asai et al., 2004). The most intriguing structural feature of belactosin A is the 3-(*trans*-2'-aminocyclopropyl)alanine ((3-Acp)Ala) (**3**) moiety. A similar chemical structure, 3-(*trans*-2'-nitrocyclopropyl)alanine ((3-Ncp)Ala) (**4**) with the opposite cyclopropyl stereoconfigurations is found in the cyclooctapeptide hormaomycin (**2**) (also known as takaokamycin, Fig. 1) produced by *Streptomyces griseoflavus* W-384 and functions as a bacterial signaling metabolite and a narrow spectrum antibiotic (Andres et al., 1989, Omura et al., 1984). Isotope labeling studies established that (3-Ncp)Ala was derived from L-lysine (**5**) (Brandl et al., 2005), yet the exact biosynthetic mechanism for this cyclopropanation remained elusive. Herein, we demonstrate that the iron-containing redox enzymes, BelK and HrmI, act as novel N-oxygenases, regioselectively oxygenating the ε-amino of L-lysine group to a nitro group thus initiating the biosyntheses of cyclopropyl rings.

Our comparative analysis of the available biosynthetic gene clusters of belactosin A (*bel*) (Wolf et al., 2017) and hormaomycin (*hrm*) (Höfer et al., 2011) (Fig. S1) revealed three highly conserved genes: *belK* and *hrmI* (encoding iron-containing redox enzymes), *belL* and *hrmJ* (encoding non-heme iron and α-ketoglutaric acid (α-KG) dependent oxygenases), and *belM* and *hrmT* (encoding diaminopimelate epimerases) (Table S1). Given the structural similarity between (3-Acp)Ala and (3-Ncp)Ala, the genes mentioned above were deduced to take part in the biosyntheses of cyclopropyl rings. It is important to note that the peptide backbone in hormaomycin is assembled by nonribosomal peptide synthetases (NRPSs), whereas in belactosin A is assembled by discrete amide ligases (Wolf et al., 2017, Höfer et al., 2011). Taking into account the wide differences in the biosyntheses and chemical struc-

☆ Given his role as Managing Editor, Dr. Xiaoying Bian, had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Shengying Li.

* Corresponding authors.
E-mail addresses: bianxiaoying@sdu.edu.cn (X. Bian), zhonggn@sdu.edu.cn (G. Zhong).

https://doi.org/10.1016/j.engmic.2021.100007
Received 23 October 2021; Received in revised form 17 November 2021; Accepted 19 November 2021
Available online 25 November 2021

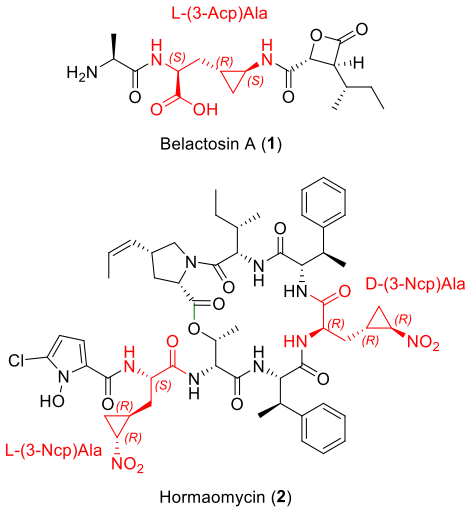

Fig. 1. Chemical structures of belactosin A (**1**) and hormaomycin (**2**). (3-Acp)Ala and (3-Ncp)Ala are highlighted in red.

tures between belactosin A and hormaomycin, it is very likely that (3-Acp)Ala and (3-Ncp)Ala are biosynthesized from separate molecules. Diaminopimelate epimerases catalyze the penultimate step during L-lysine biosynthesis in most plants, bacteria, and lower fungi (Xu et al., 2006). Despite the lack of experimental evidence, BelM and HrmT have been speculated to play a role in diverting lysine precursors from primary metabolism to secondary metabolism (Höfer et al., 2011). Therefore, BelK/HrmI and BelL/HrmJ are the most likely candidates for the biosyntheses of the cyclopropane moieties.

Next, we heterologously expressed and purified the non-heme iron and α-KG dependent oxygenase BelL (Fig. S2). However, *in vitro* characterization of BelL in the presence of L-lysine, α-KG, $FeSO_4 \cdot 7H_2O$, L-ascorbic acid and dithiothreitol (DTT) showed no potential product under various conditions, neither for the homologous protein HrmJ. This excluded the possibility of BelL or HrmJ being the first enzyme involved in the conversion of L-lysine to cyclopropyl rings, allowing us focus on the other oxygenases, BelK and HrmI.

The structure of BelK was predicted online using the I-TASSER approach (Roy et al., 2010, Yang et al., 2015, Yang and Zhang, 2015), revealing high similarity to the crystal structure of SznF (PDB: 6M9R) from the streptozotocin biosynthetic gene cluster despite very low sequence homology (14.8%) (Fig. S3). SznF is also an iron-containing redox enzyme that participates in *N*-nitrosourea formation. The x-ray crystal structure shows that SznF contains an N-terminal dimerization domain, a central helical bundle domain and a C-terminal cupin domain (Ng et al., 2019, He et al., 2019). The latter domain is notably absent in the BelK predicted structure. *In vitro* characterization of BelK was performed using reduced nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) as the reductant and electron transfer (Fig. S4). After dansyl chloride (DNSC) derivatization and high-performance liquid chromatography electrospray ionization high resolution mass spectrometry (HPLC-ESI-HRMS) analysis, a new peak (**8**) with a longer retention time and 30 Da mass increase ($[M+H]^+$ *m/z*: calculated 410.1380 for $[C_{18}H_{24}N_3O_6S]^+$, found 410.1385) was detected in addition to DNS-Lys (**7**, $[M+H]^+$ *m/z*: calculated 380.1639 for $[C_{18}H_{26}N_3O_4S]^+$, found 380.1642) (Fig. 2B, S5). This compound disappeared if any one of the L-lysine, NADH, PMS or BelK was omitted, indicating that all of these materials were necessary for product formation. Interestingly, a similar result was found when HrmI was used instead of BelK, implying that the differences between (3-Acp)Ala and (3-Ncp)Ala are not determined by BelK or HrmI.

It is worth mentioning that **8** exhibited less polarity than 7, although **8** had two more oxygens and two less hydrogens. Thus, we hypothe-

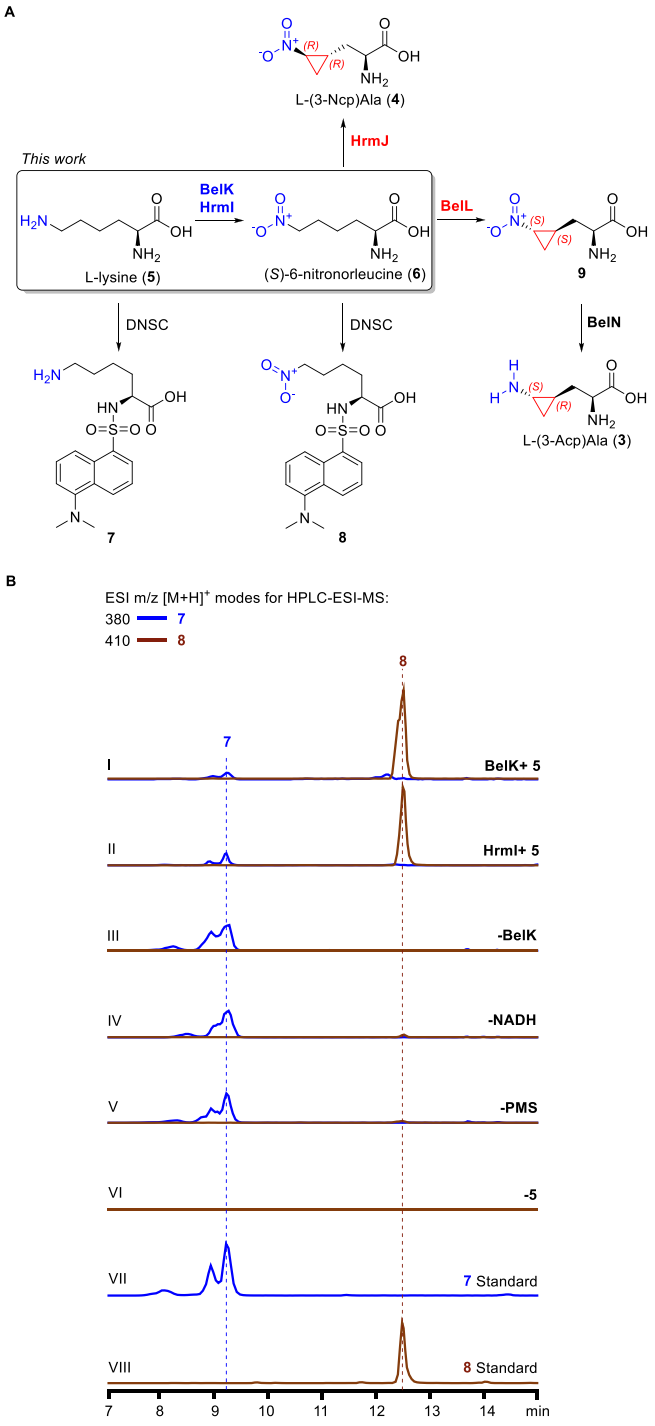

Fig. 2. *In vitro* characterization of BelK and HrmI. (A) Proposed biosynthetic pathways of (3-Acp)Ala and (3-Ncp)Ala. (B) Validation of the Nε-oxygenase activity of BelK and HrmI.

sized that BelK/HrmI converted the ε-amino in L-lysine to a nitro group, as the α-amino of L-lysine was preserved in both (3-Acp)Ala and (3-Ncp)Ala. To validate this hypothesis, (*S*)-6-nitronorleucine (**6**) was synthesized according to a previously reported 4-step procedure (Fig. S6) (Zlatopolskiy et al., 2006). The DNSC derivatized **6** displayed an identical mass and retention time as **8** (Fig. 2B), confirming BelK and HrmI are N-oxygenases that oxidize the ε-amine of L-lysine to a nitro group (Fig. 2A). L-ornithine (**10**) was employed as an L-lysine analog to evaluate the substrate tolerance of BelK. However, no product was detected

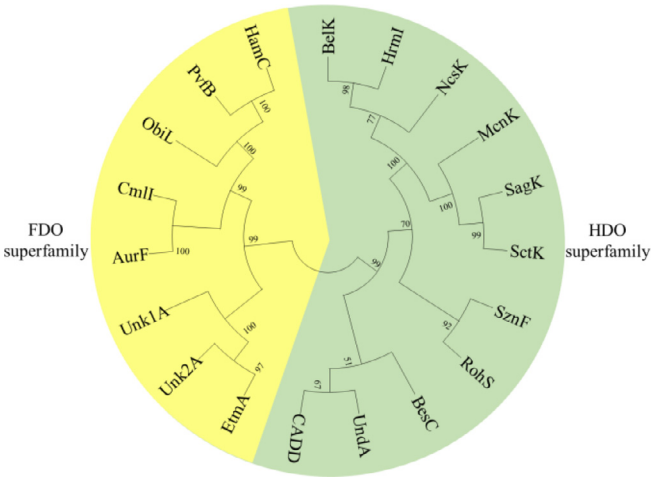

Fig. 3. Phylogenetic analysis of the FDO and HDO superfamilies. The tree was constructed using the neighbor-joining method and a bootstrap test with 2000 iterations.

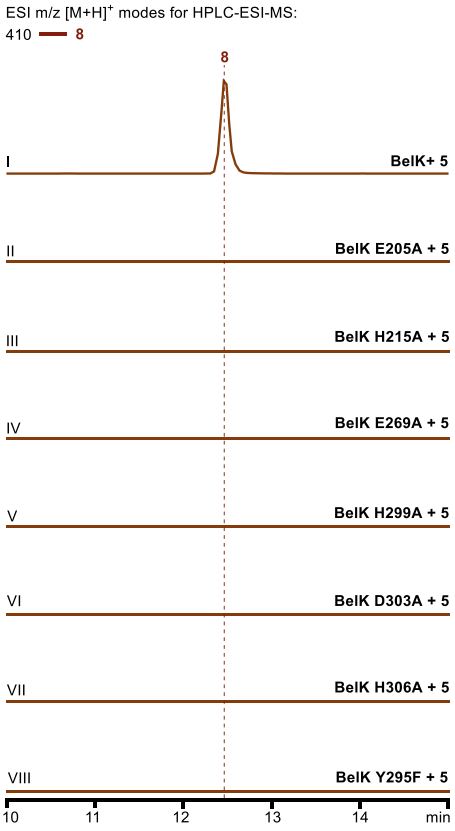

Fig. 4. Evaluation of the putative catalytic sites of BelK.

after DNSC derivatization (Fig. S7), indicating that BelK cannot catalyze the oxygenation of L-ornithine.

Distinct from the ferritin-like diiron oxidase and oxygenase (FDO) superfamily such as AurF, CmlI, ObiL and PvfB, HamC, EtmA, Unk1A, Unk2A that belong to the Pfam 11583 (Choi et al., 2008, Komor et al., 2016, Schaffer et al., 2017, Scott et al., 2017, He and Ryan, 2021, Morgan and Li, 2020), BelK and HrmI are placed in the Pfam 14518, the same as the non-heme diiron oxygenase SznF (Fig. S8) (McBride et al., 2020, McBride et al., 2021). Phylogenetically, BelK and HrmI fall into a clade close to SznF but far from the FDO superfamily (Fig. 3). Apart from SznF, other enzymes such as UndA (PDB: 4WWZ) involved in the biosynthesis of 1-undecene in *Pseudomonas aeruginosa* and *Chlamydia* protein associating with death domains (CADD) (PDB: 1RCW) implicated in the biosynthesis of *para*-aminobenzoic acid in *Chlamydia trachomatis* also contained a similar helical bundle topology as SznF (Rui et al., 2014, Manley et al., 2019, Zhang et al., 2019, Schwarzenbacher et al., 2004, Adams et al., 2014). These proteins together with the diiron N-oxygenase RohS, (involved in azomycin biosynthesis) and the olefin-forming desaturase BesC, (taking part in L-β-ethynylserine biogenesis) constituted the emerging heme-oxygenase-like diiron oxidase and oxygenase (HDO) superfamily (McBride et al., 2021, Hedges and Ryan, 2019, Marchand et al., 2019). As new members of the HDO superfamily, it is noteworthy that BelK and HrmI are the only members, aside from SznF, that contain an N-terminal dimerization domain. Attempts to remove the N-terminal domain of BelK resulted in no expression, demonstrating the importance of the N-terminal domain for BelK expression. Moreover, BelK and HrmI are different from other diiron nitro-forming enzymes uncovered to date. To the best of our knowledge, BelK and HrmI are the first diiron nitro-forming enzymes to act on the free amino acids, as most of the diiron nitro-forming enzymes act on either aryl amines (AurF, CmlI, ObiL and RohS) or carrier-bound amino acids (PvfB, HamC, EtmA and its homologues) (Fig. S9, S10).

Homology modeling of BelK performed by I-TASSER identified presumed diiron binding residues: E205, H215, E269, H299, D303 and H306 which are highly conserved in all BelK-like proteins (Fig. S3), such as HrmI, SagK from *S. agglomeratus* 6-3-2 (*sag* cluster), SctK from *S. chrestomyceticus* TBRC 1925 (*sct* cluster), McnK from *Micromonospora* sp. CNZ322 (*mcn* cluster) and NcsK from *Nocardia* sp. CS682 (*ncs* cluster) (Fig. S1, S11). Mutagenesis of any of the six predicted catalytic sites completely abolished the activity of BelK, validating the necessity of the presumed diiron binding residues (Fig. 4). In addition to these six sites, a tyrosine residue (Y295 in BelK) near the active sites is also conserved in all BelK-like proteins, SznF and CADD (Fig. S3, S11 and S12). Surprisingly, the mutation of Y295 to alanine resulted in the insolubility of BelK, indicating that Y295 plays a particularly prominent role in the proper structure of BelK. To further verify the function of this tyrosine residue, Y295F was constructed. In contrast to Y295A, Y295F was quite soluble but lost its catalytic activity (Fig. 4 and Fig. S4), suggesting that the hydroxyl group in Y295 is also vital for the correct function of BelK. Due to the instability of the diiron catalytic center of many HDO proteins, an extra coordinating side chain of E189 in SznF was recently identified (McBride et al., 2021), whereas the corresponding residue at this position is phenylalanine (F179 for BelK) in all BelK-like proteins, which is also noted in UndA and CADD (Fig. S3, S11 and S12). At present, it's difficult to identify the extra coordinating residue in BelK. Potentially, the carboxylate group of L-lysine may act as the extra coordination in all the BelK-like proteins, like with UndA (McBride et al., 2021).

The following steps of the (3-Acp)Ala and (3-Ncp)Ala biosyntheses remains to be elucidated. It is likely that the non-heme iron and α-KG dependent oxygenases, BelL and HrmJ, are responsible for the formations of cyclopropyl rings with opposite stereo configurations (Fig. 2A). The Nε-nitro group is believed to be essential for cyclopropanation, as no products were detected in the BelL or HrmJ assays with L-lysine as the substrate. While this manuscript was under preparation, a non-heme iron and α-KG dependent oxygenase TqaL was reported to catalyze the aziridine biogenesis from L-valine, expanding the catalytic repertoire of the Fe(II)/α-KG oxygenases (Fig. S13) (Bunno et al., 2021). Considering that BelL, HrmJ and TqaL are members of the same Pfam (Pfam 10014), this published result of TqaL supports the hypothesis that BelL and HrmJ catalyze the generation of cyclopropyl rings.

It appears that a nitroreductase absent in the *hrm* cluster is required for the reduction of the nitro group in **9** to obtain (3-Acp)Ala (Fig. 2A). *In silico* analysis unveiled the genes *belN* and its homologues (encoding molybdopterin oxidoreductases) existing in the *sag, sct, mcn* and *ncs* biosynthetic gene clusters (Fig. S1). Aside from *belK/L/M/N*, no addi-

tional homologous genes were found in *bel* and the four clusters mentioned above. This surmises that the four genes are sufficient to generate (3-Acp)Ala and that the products of the *sag, sct, mcn* and *ncs* clusters might also contain the (3-Acp)Ala moiety. Further studies of BelL, HrmJ and BelN are currently underway in our laboratory.

In conclusion, BelK and HrmI, new members of the emerging HDO superfamily, have been biochemically characterized to regioselectively catalyze the unique Nε-oxygenation of L-lysine under reducing conditions. It has been reported that **6** inhibits human arginase I, resulting in higher cellular concentrations of L-arginine for NO biosynthesis. Therefore, this compound could be a candidate for the treatment of diseases in which upregulated arginase activity compromises NO-dependent physiological processes (Zakharian et al., 2008). Our results provide a new synthetic method for **6** with mild reaction conditions. Furthermore, this study highlights the versatile functions of the HDO superfamily, expands the existing knowledge for enzyme-mediated nitro group formation, and sheds light on the mechanism of (3-Acp)Ala and (3-Ncp)Ala biosynthesis. BelK and its homologues could serve as new genome mining probes for the discovery of novel bioactive natural products containing a nitro group or cyclopropyl skeleton.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Drs. Jingyao Qu, Zhifeng Li, Jing Zhu, Guannan Lin and Haiyan Sui (Core Facilities for Life and Environmental Sciences, State Key laboratory of Microbial Technology, Shandong University) for the detection and characterization by HPLC-ESI-HRMS and NMR, and Prof. Qingfei Zheng (Department of Radiation Oncology and Center for Cancer Metabolism, The Ohio State University) for proofreading the manuscript and helpful discussions. This work was supported by the National Key R&D Program of China (2019YFA0905700), the National Natural Science Foundation of China (21907057, 32070060), the Shandong Provincial Natural Science Foundation, China (ZR2019JQ11, ZR2019ZD18), the Natural Science Foundation of Jiangsu Province, China (BK20190201), the 111 project (B16030), the Youth Interdisciplinary Innovative Research Group (2020QNQT009), the Future Plan for Young Scholars, and the Fundamental Research Funds (2019GN032) of Shandong University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2021.100007.

References

Wessjohann, L.A., Brandt, W., Thiemann, T., 2003. Chem. Rev. 103, 1625–1648.
Stability of Cycloalkanes, 2021. Ring Strain.. June 22 https://chem.libretexts.org/@go/page/31410 .
Talele, T.T., 2016. J. Med. Chem. 59, 8712–8756.
Chen, D.Y.K., Pouwer, R.H., Richard, J.-A., 2012. Chem. Soc. Rev. 41, 4631–4642.
Asai, A., Hasegawa, A., Ochiai, K., Yamashita, Y., Mizukami, T., 2000. J. Antibiot. 53, 81–83.
Asai, A., Tsujita, T., Sharma, S.V., et al., 2004. Biochem. Pharmacol. 67, 227–234.
Andres, N., Wolf, H., Zähner, H., et al., 1989. Helv. Chim. Acta 72, 426–437.
Omura, S., Mamada, H., Wang, N.J., et al., 1984. J. Antibiot. 37, 700–705.
Brandl, M., Kozhushkov, S.I., Zlatopolskiy, B.D., et al., 2005. Eur. J. Org. Chem. 123–135 2005.
Wolf, F., Bauer, J.S., Bendel, T.M., et al., 2017. Angew. Chem. Int. Ed. 56, 6665–6668.
Höfer, I., Crüsemann, M., Radzom, M., et al., 2011. Chem. Biol. 18, 381–391.
Xu, H., Andi, B., Qian, J., West, A.H., Cook, P.F., 2006. Cell Biochem. Biophys. 46, 43–64.
Roy, A., Kucukural, A., Zhang, Y., 2010. Nat. Protoc. 5, 725–738.
Yang, J., Yan, R., Roy, A., et al., 2015. Nat. Methods 12, 7–8.
Yang, J., Zhang, Y., 2015. Nucleic Acids Res 43, W174–W181.
Ng, T.L., Rohac, R., Mitchell, A.J., Boal, A.K., Balskus, E.P., 2019. Nature 566, 94–99.
He, H.-Y., Henderson, A.C., Du, Y.-L., Ryan, K.S., 2019. J. Am. Chem. Soc. 141, 4026–4033.
Zlatopolskiy, B.D., Radzom, M., Zeeck, A., de Meijere, A., 2006. Eur. J. Org. Chem. 1525–1534 2006.
Choi, Y.S., Zhang, H., Brunzelle, J.S., Nair, S.K., Zhao, H., 2008. Proc. Natl. Acad. Sci. USA 105 6858–6863.
Komor, A.J., Rivard, B.S., Fan, R., et al., 2016. J. Am. Chem. Soc. 138, 7411–7421.
Schaffer, J.E., Reck, M.R., Prasad, N.K., Wencewicz, T.A., 2017. Nat. Chem. Biol. 13, 737–744.
Scott, T.A., Heine, D., Qin, Z., Wilkinson, B., 2017. Nat. Commun. 8, 15935.
He, H.-Y., Ryan, K.S., 2021. Nat. Chem. 13, 599–606.
Morgan, G.L., Li, B., 2020. Angew. Chem. Int. Ed. 59, 21387–21391.
McBride, M.J., Sil, D., Ng, T.L., et al., 2020. J. Am. Chem. Soc. 142, 11818–11828.
McBride, M.J., Pope, S.R., Hu, K., et al., 2021. Proc. Natl. Acad. Sci. USA 118, e2015931118.
Rui, Z., Li, X., Zhu, X., et al., 2014. Proc. Natl. Acad. Sci. USA 111, 18237–18242.
Manley, O.M., Fan, R., Guo, Y., Makris, T.M., 2019. J. Am. Chem. Soc. 141, 8684–8688.
Zhang, B., Rajakovich, L.J., Van Cura, D., et al., 2019. J. Am. Chem. Soc. 141, 14510–14514.
Schwarzenbacher, R., Stenner-Liewen, F., Liewen, H., et al., 2004. J. Biol. Chem. 279, 29320–29324.
Adams, N.E., Thiaville, J.J., Proestos, J., et al., 2014. mBio 5 e01378-14.
Hedges, J.B., Ryan, K.S., 2019. Angew. Chem. Int. Ed. 58, 11647–11651.
Marchand, J.A., Neugebauer, M.E., Ing, M.C., et al., 2019. Nature 567, 420–424.
Bunno, R., Awakawa, T., Mori, T., Abe, I., 2021. Angew. Chem. Int. Ed. 60, 15827–15831.
Zakharian, T.Y., Di Costanzo, L., Christianson, D.W., 2008. J. Am. Chem. Soc. 130, 17254–17255.