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Discovery and biosynthesis of guanipiperazine from a NRPS-like pathway[†]

Jing Shi,^{‡a} Xiang Xu,^{‡a} Pei Yi Liu,^a Yi Ling Hu,^a Bo Zhang,^a Rui Hua Jiao,^a Ghader Bashiri,^b Ren Xiang Tan^{*a} and Hui Ming Ge^{ID *a}

Nonribosomal peptide synthetases (NRPSs) are modular enzymes that use a thiotemplate mechanism to assemble the peptide backbones of structurally diverse and biologically active natural products in bacteria and fungi. Unlike these canonical multi-modular NRPSs, single-module NRPS-like enzymes, which lack the key condensation (C) domain, are rare in bacteria, and have been largely unexplored to date. Here, we report the discovery of a gene cluster (*gup*) encoding a NRPS-like megasynthetase through genome mining. Heterologous expression of the *gup* cluster led to the production of two unprecedented alkaloids, guanipiperazines A and B. The NRPS-like enzyme activates two L-tyrosine molecules, reduces them to the corresponding amino aldehydes, and forms an unstable imine product. The subsequent enzymatic reduction affords piperazine, which can be morphed by a P450 monooxygenase into a highly strained compound through C–O bond formation. Further intermolecular oxidative coupling forming the C–C or C–O bond is catalyzed by another P450 enzyme. This work reveals the huge potential of NRPS-like biosynthetic gene clusters in the discovery of novel natural products.

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Introduction

Nonribosomal peptide synthetases (NRPSs) are modular meg-enzymes consisting of multiple covalently-linked domains. These megaenzymes catalyze the synthesis of important peptide natural products, including vancomycin, daptomycin, and cyclosporine A.^{1–3} The biosynthesis of such nonribosomal peptides depends on a minimal domain set for adenylation (A), thiolation (T), and condensation (C).^{1,4,5} The A domain selects and activates a carboxylic acid substrate through adenylation at the expense of ATP, then transfers the acyl group to the phosphopantetheinyl arm attached to the T domain to form a thioester linkage. The C domain mediates peptide bond formation in two adjacent modules. In comparison, single-module NRPS-like enzymes do not contain a C domain and lack this extension function. These enzymes are functionally diverse in primary and secondary metabolism. Depending the type of downstream releasing domains, NRPS-like enzymes catalyse a broad range of

reactions, including but not limited to Dieckmann cyclization,^{6,7} reduction,^{8,9} and Claisen condensation.¹⁰

The carboxylic acid reductases (CARs) belong to the single-module NRPS-like enzymes, and comprise an N-terminal adenylation domain, which is fused to a C-terminal reductase (R) domain *via* a T domain.¹¹ Mechanistically, the T domain delivers the acyl thioester to the R domain for reduction by NADPH to give an aldehyde product. For example, Lys2 can reduce α -amino adipate at C-6 to the semialdehyde in the biosynthesis of lysine in *Saccharomyces cerevisiae*,¹² CmlP is involved in the biosynthesis of the antibiotic chloramphenicol (Scheme 1) in *Streptomyces*,¹³ PvfC is responsible for the synthesis of a family of pyrazine N-oxides in *Pseudomonas virulence*,^{14,15} and HamD is related to valdiazin biosynthesis in *Burkholderia cenocepacia* (Scheme 1).¹⁶ CARs have also recently attracted interest as green biocatalysts due to their abilities to convert a broad range of carboxylic acid substrates to the corresponding aldehydes.^{17,18} CARs alone or combined with other enzymes have been applied to generate pharmaceuticals, biofuels, and other valuable products.^{17,19,20}

Recent advances of microbial genomics have led to the rapid accumulation of uncharacterized biosynthetic gene clusters (BGCs) in genome database. The diverse functions of CARs and their biocatalytic potential encouraged us to identify new members through genome mining. Here, we report the discovery of a unique four-gene operon encoding an F₄₂₀H₂-dependent reductase, a CAR, and two P450 proteins in *Streptomyces*. We reconstituted four enzymes that catalyse the

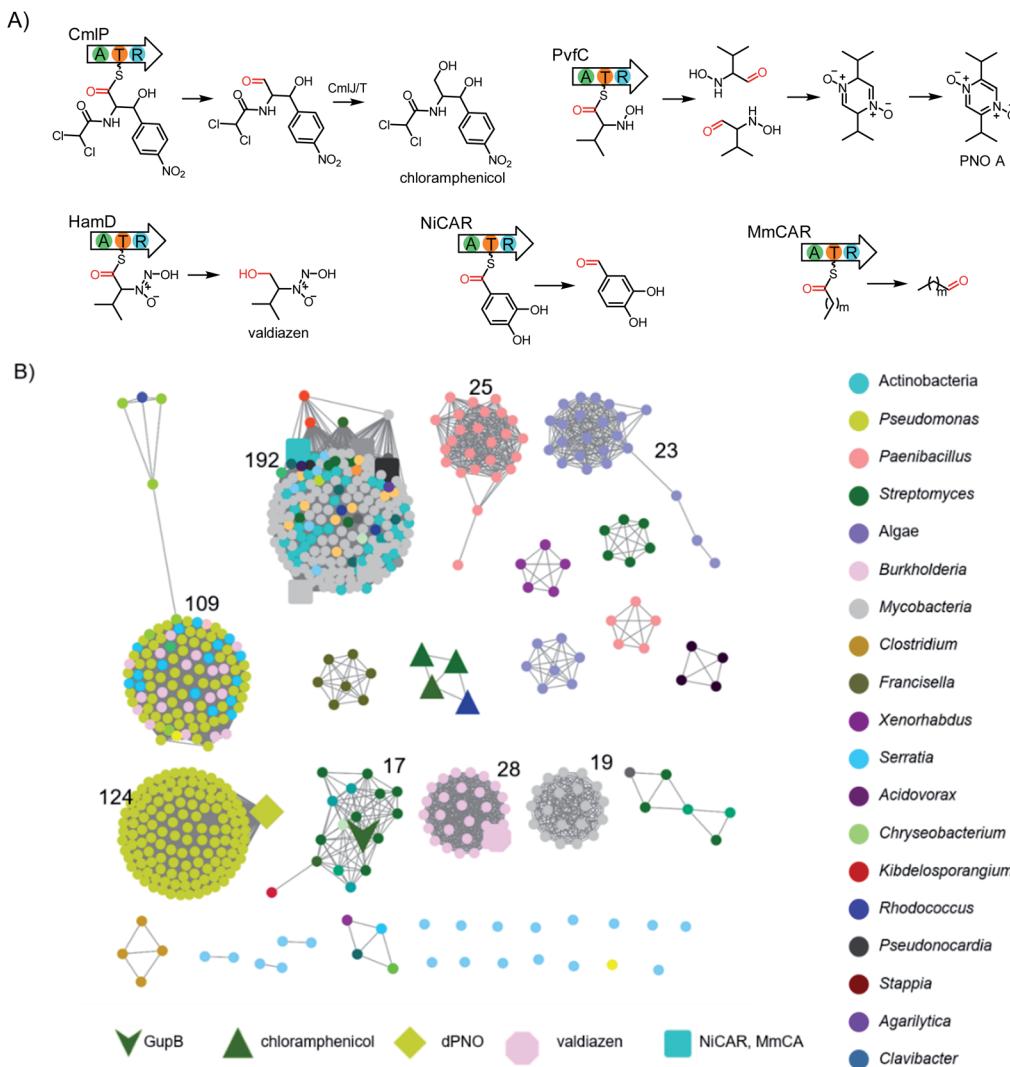
^aState Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Chemistry and Biomedicine Innovation Center (ChemBIC), Institute of Artificial Intelligence Biomedicine, Nanjing University, Nanjing 210023, China. E-mail: rxtan@nju.edu.cn; hmge@nju.edu.cn

^bLaboratory of Structural Biology, Maurice Wilkins Centre for Molecular Biodiscovery, School of Biological Sciences, University of Auckland, Auckland 1010, New Zealand

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‡ Contributed equally to this work.





Scheme 1 Genome mining of single-module CAR enzymes from bacteria. (A) The enzymatic reactions catalyzed by characterized bacterial CAR enzymes. (B) Sequence similarity network (SSN) analysis of single-module CAR enzymes with a sequence identity of >40% visualized by Cytoscape.

production of two unprecedented alkaloids (**1** and **2**). We demonstrated the detailed biosynthetic steps towards the formation of **1** and **2**.

Results and discussion

Genome mining uncovered the *gup* BGC

To identify single-module NRPS-like CAR encoding genes, we systematically investigated the genomic data in our own and the antiSMASH database,²¹ which totally contain over 150 000 bacterial BGCs. When the R domain was used as the search query, 1230 NRPS BGCs containing a terminal R domain were retrieved. After manually removing the multi-modular NRPSs, we were left with 608 single-module NRPS-like BGCs.

From these 608 hits, we generated a sequence similarity network (SSN) based on their corresponding R domain sequences.²² We showed that CARs from different organisms may evolve independently as many of them are related to their

taxonomies (Scheme 1).²³ Nonetheless, the known CAR enzymes with similar functions are clustered together. The largest clade of proteins contains homologs of NiCARs and MmCARs,²⁴ suggesting this clade is most likely involved in the reduction of aromatic or aliphatic carboxylic acids to their corresponding aldehydes. We also excluded homologous enzymes of PvfC, HamD, and CmlP from further investigation (Scheme 1).

We hypothesize that other tailoring enzymes in BGCs are important for the structural complexity of the corresponding natural products, as exemplified by the chloramphenicol BGC where a series of reductase, halogenase, amidase, and oxygenase encoding genes are present.¹³ Thus, we further submitted the CAR-gene containing loci to antiSMASH for gene cluster analysis.²¹ We identified an orphan BGC (*gup*) in *S. chrestomyceticus* consisting of a four-gene operon encoding a putative F₄₂₀H₂-dependent reductase, a CAR, and two P450s (Fig. 1). A subsequent BLAST homology search in the NCBI and JGI genome databases revealed that many other *Streptomyces*



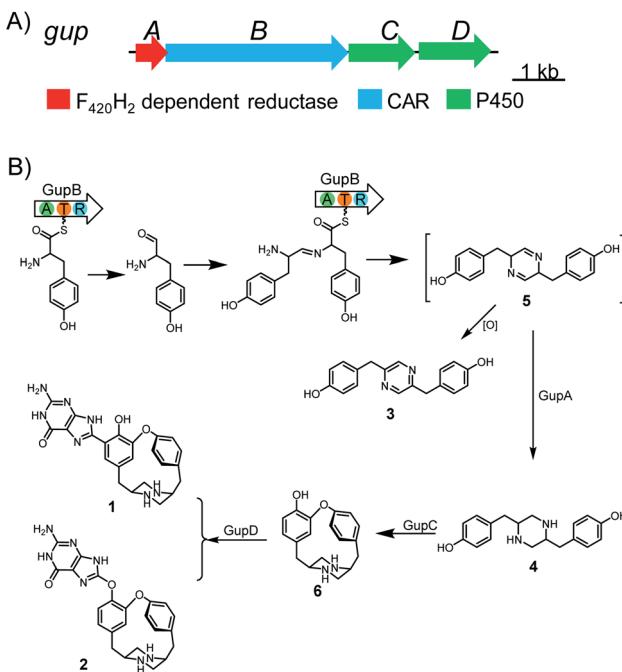


Fig. 1 Biosynthesis of guanipiperazines A and B (1 and 2). (A) Biosynthetic gene cluster of *gup*; (B) the proposed biosynthetic pathway of 1 and 2.

strains harbour this homologous, yet cryptic, gene cluster (Fig. S1†).

Heterologous production of guanipiperazines

Initial attempts to obtain the natural products encoded by the *gup* BGC in the *S. chrestomyceticus* wild-type strain by fermentation and media optimization failed. Therefore, we performed heterologous expression of the *gup* BGC in *S. lividans* TK24, a prolific host that is widely used for producing actinobacterial natural products.²⁵ We cloned the intact *gup* BGC into a *Streptomyces* integrative plasmid, pSET152, under the control of a strong promoter P_{kasO^*} ,²⁶ and introduced the plasmid into the *S. lividans* TK24 strain. After cultivating this strain in ISP4 medium for 10 days, two compounds, 1 and 2 were produced, both with m/z 446.2 [$M + H$]⁺. In contrast, these two compounds were not present in the control strain harbouring an empty pSET152 plasmid (Fig. 2). Subsequent large scale fermentation of the *S. lividans* TK24/*gupABCD* strain led to the isolation of sufficient amounts of 1 and 2. Their structures were characterized by analyses of the 1D and 2D NMR data, including ¹H, ¹³C, HSQC, HMBC, ¹H-¹H COSY, and NOESY (Fig. 1, Tables S4 and S5†). Both 1 and 2 showed antimicrobial activities against *Saccharomyces cerevisiae* with minimum inhibitory concentrations (MICs) of 4.5 and 5.6 μ g mL⁻¹, respectively.

In vivo characterization of GupA and GupB

Previous studies have reported the formation of a strained piperazine with a C-C cross-link between the phenol sides in fungus.^{8,27} To dissect the biosynthesis of 1 and 2, which feature

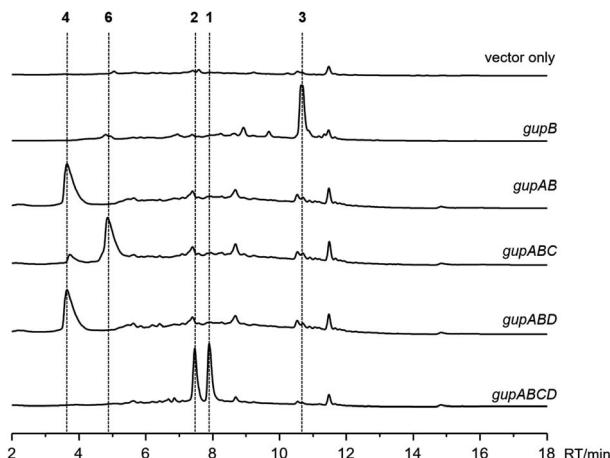


Fig. 2 HPLC analysis (monitored at 280 nm) of the heterologous expression of the *gup* biosynthetic gene cluster in *Streptomyces lividans* TK24.

a unique C–O cross-linked piperazine core and a guaninyl modification, we set out to reconstitute the biosynthetic pathway through a bottom-up strategy in *S. lividans* TK24. Expressing *gupB* alone in *S. lividans* TK24 led to the formation of a new product 3 (Fig. 1, Table S6†). Compound 3 has a pyrazine-type core, inconsistent with the structures of 1 and 2. To test if 3 is an on-pathway intermediate, we fed 3 to a *S. lividans* TK24/*gupACD* strain, in which the *gupB* gene was deleted from the *gup* BGC. The production of 1 or 2 could not be restored (Fig. S2†), indicating 3 could be a shunt metabolite.

GupA is annotated as an $F_{420}H_2$ -dependent reductase. Co-expression of the *gupB* and *gupA* genes yielded a piperazine-type compound 4 (Fig. 1, Table S7†). Feeding 4 to the *S. lividans* TK24/*gupACD* strain restored the production of 1 and 2 (Fig. S2†), indicating 4 is an on-pathway intermediate. Hence, the CAR GupB enzyme may successively activate two tyrosine molecules and reduce them to the corresponding amino aldehydes. Condensation between two amino aldehydes followed by dehydration led to the formation of a proposed intermediate 5. In the absence of GupA, 5 is spontaneously overoxidized by air to give 3; alternatively, 5 is reduced to form intermediate 4 in the presence of GupA.

In vitro characterization of GupA and GupB

To investigate the function of GupB *in vitro*, we overproduced the intact GupB protein in *Escherichia coli* BL21(DE3) cells and purified the *apo*-form to homogeneity via Ni-NTA affinity chromatography (Fig. S3†). An *in vitro* adenylation activity assay was used to monitor the pyrophosphate production in the presence of different amino acids.²⁸ L-Tyrosine is the preferred substrate for adenylation of the A domain of GupB, whereas D-tyrosine and other amino acids show relatively low activities (Fig. 3). Thus, we hypothesize that the chiral centers at C-3 and C-13 are retained in 1 and 2, as the downstream modifications would not affect their stereochemistry.

GupA is a small protein (162 amino acids) that belongs to the RV1155- F_{420} superfamily, and contains a conserved F_{420} -

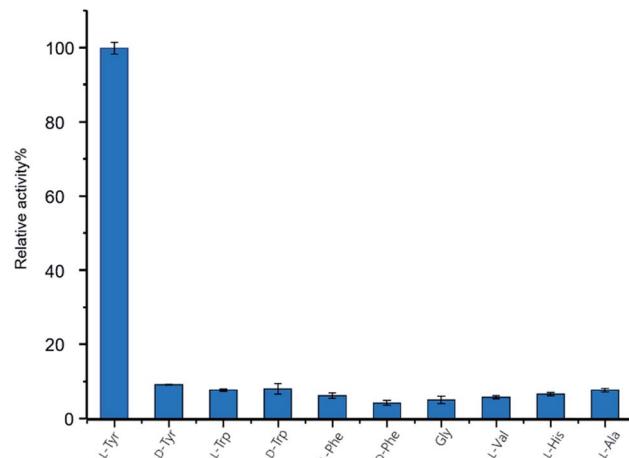


Fig. 3 The relative adenylation activities for GupB.

binding domain. However, the functions of members of this protein superfamily are unclear. We tested whether GupA can reduce the proposed intermediate 5. We purified the cofactor F₄₂₀ from a genetically-engineered strain of *Mycobacterium smegmatis*.²⁹ F₄₂₀-dependent glucose-6-phosphate dehydrogenase (FGD) from *M. tuberculosis* was used for *in situ* regeneration of the reduced form of F₄₂₀ (F₄₂₀H₂) (Fig. S5†).³⁰ We reconstituted the *holo*-GupB by incubating *apo*-GupB, CoA, and Sfp (a phosphopantetheinyl transferase in *Bacillus subtilis*).³¹ Further supplementation of ATP and L-tyrosine in the above reaction system led to the production of 3 (Fig. 4), indicating the reconstituted GupB protein is functional. When we incubated GupA, F₄₂₀, and the F₄₂₀H₂ regeneration system into the abovementioned enzymatic reaction, production of 4 was observed.

As cofactor F₄₂₀ is structurally similar to the flavins (FMN or FAD), we evaluated if GupA could utilize FMNH₂/FADH₂ as reducing agents. FMNH₂/FADH₂ can be regenerated by NADPH, FMN/FAD, and flavin reductase (Fre) from *E. coli*.³² However, the production of 4 was not detected upon the

introduction of FMNH₂/FADH₂ (Fig. S6†). These data reveal that GupB activates and reduces two L-tyrosine molecules and forms the condensed intermediate 5, while GupA is required for the formation of 4. To the best of our knowledge, GupA is the first member of the RV1155-F₄₂₀ superfamily to be functionally characterized.

Functional characterization of GupC and GupD

Next, we aimed to identify the enzymes responsible for the intramolecular diaryl ether (C–O bond) and intermolecular diaryl (C–C bond) and ether (C–O bond) couplings. The C–C and C–O aryl bonds have been observed in other peptides and alkaloids, and are often introduced by P450 enzymes; for example, OxyC, StaP, and CYP121 mediate the diaryl C–C bond formation in vancomycin, staurosporine, and mycocyclosin biosynthesis,^{33–35} respectively; and OxyA, OxyB and OxyE catalyse the diaryl C–O bond formation in vancomycin biosynthesis.³³ Therefore, we postulated the remaining two P450 encoding genes, *gupC* and *gupD*, which share 32% sequence identity, are involved in C–C and C–O bond formation.

The *gupC* and *gupD* genes were deleted individually from the *gup* BGC through in-frame deletion. LC-MS analysis showed that *S. lividans* TK24/*gupABC* did not produce 1 and 2, but accumulated 4 as the dominant product (Fig. 2). Meanwhile, the *S. lividans* TK24/*gupABC* strain generated a new compound 6 with *m/z* 297.2 [M + H]⁺ (Fig. 2), which is 2 Da less than that of 4. The structure of 6 is the expected strained piperazine compound (Fig. 1, Table S8†). Our *in vivo* heterologous expression data indicated that GupC catalyzes the intramolecular C–O bond formation, while GupD couples 6 and a guanine to form 1 and 2 through C–C and C–O linkage.

To verify functions of the two P450s *in vitro*, GupC and GupD were overexpressed in *E. coli* BL21(DE3) cells and purified to near homogeneity as (His)₆-fusion proteins. In addition, P450 enzyme activity requires the supply of electrons to the iron center *via* the electron transport chain. The bacterial P450 enzyme utilizes a two-component system consisting of ferredoxin (Fdx) and ferredoxin reductase (Fdr). Thus, we overexpressed and purified the native Fdx and Fdr enzymes from *S. chrestomyceticus* (Fig. S3†). Incubation of GupC and 4 in the presence of the electron transport system and NADPH led to the consumption of 4 with the concomitant appearance of 6. These findings indicate GupC catalyzes the intramolecular C–O bond formation.

When GupD was incubated with 6 and GTP in the presence of the electron transport system, we observed the formation of 1 and 2 (Fig. 5). This indicated that GupD acts as a guanyl transferase that catalyzes the attachment of a guanine moiety to the piperazine core *via* a C–C or C–O bond. This function of GupD is reminiscent of the P450 enzyme PcmD (with 37% identity to GupD),³⁶ which transfers a guanyl moiety to a cyclodipeptide in the biosynthesis of purineyclamide. PcmD uses a guanine as the substrate instead of GTP, whereas GupD only accepts GTP as the substrate, and is inactive to ATP, UTP, and guanine (Fig. S7†).

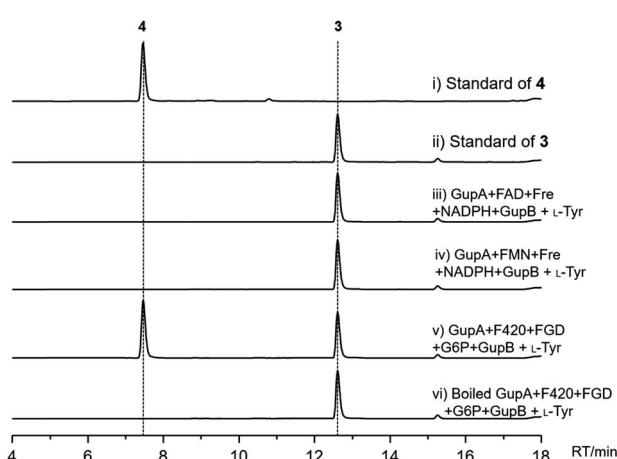


Fig. 4 HPLC analysis (monitored at 280 nm) of GupB and GupA enzymatic reactions *in vitro*.



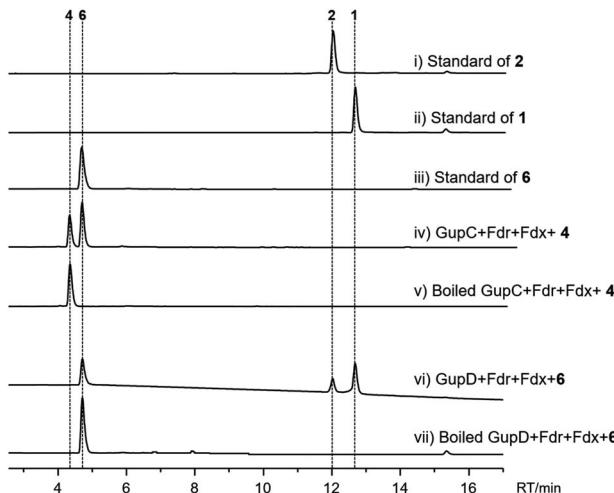


Fig. 5 HPLC analysis (monitored at 280 nm) of GupC and GupD enzymatic reactions *in vitro*.

Conclusions

In conclusion, by genome mining towards the single-module NRPS-like genes in bacteria we discovered a four-gene operon (*gup*) that is actually abundant and widespread in *Streptomyces* strains. Heterologous expression of the *gup* BGC led to the characterization of two novel alkaloids, guanipiperazines A and B, which feature a rare, highly strained piperazine core and a guaninyl modification. GupB is a new CAR enzyme in secondary metabolism that plays a key role in the construction of the piperazine core. An $F_{420}H_2$ -dependent reductase and two P450 enzymes subsequently mediate the remaining redox steps to form the guanipiperazines. Our work highlights the diverse functions of CAR enzymes, and paves the way for new natural products to be discovered from other clades of CAR enzymes in the future.

Conflicts of interest

The authors declare no conflict of interest.

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