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Thiotemplated Biosynthesis of Bacterial Polyyne Fatty Acids by a Designated Desaturase Triad

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Various bacterial species are capable of producing highly modified fatty acid derivatives with conjugated triple bonds, which play important ecological roles as antifungals and toxins in mutualistic and pathogenic interactions. Furthermore, the terminal polyyne moiety is of interest as pharmacophore and as tag in bioorthogonal chemistry and live imaging. To gain insight into the assembly of these highly reactive natural products, we investigated tetrayne (caryoynencin and protegencin) biosynthesis genes (*cay* and *pgn*) from *Trinickia caryophylli* and *Pseudomonas protegens*. Pathway dissection and reconstitution in the heterologous host *Burkholderia graminis* revealed the genes minimally required for polyyne formation. Mutational analyses and biochemical assays demonstrated that polyyne biosynthesis is thiotemplated, involving a fatty acyl-AMP ligase, a designated acyl carrier protein, and a thioesterase. Heterologous expression of point-mutated desaturase genes showed that three desaturases work synergistically to introduce four triple bonds. These findings point to an intricate desaturase complex and provide important information for future bioengineering experiments.

Introduction

Bacterial polyynes comprise a structurally intriguing family of highly modified fatty acid metabolites. Owing to the conjugated and terminal triple bonds, these compounds are highly reactive, and thus extremely unstable. Cepacin A (**1**) from the human pathogen *Burkholderia cepacia* and the biopesticidal bacterium *Burkholderia ambifaria*,[1] the antifungal antibiotic Sch 31828 (**2**) from *Microbispora* sp. SCC 1438,[2] and the antifungal collimonins (**3**–**6**) from the fungus-feeding bacterium *Collimonas fungivorans*,[3] are examples of hexadecanoids with two or three triple bonds. Octadecanoids with four triple bonds include the phytotoxic and antifungal caryoynencin (**8**) from the plant and mushrooms pathogens *T. caryophylli* and *B. gladioli*,[4] and the recently identified protegencin (**7**, syn. protegenin[5]) from *Pseudomonas protegens*[6] (Figure 1A). Their versatile ecological functions in protective symbioses[1b,4b] and their involvement in plant/algal,[6b] and mushroom[7] diseases have prompted the analyses of bacterial polyyne biosynthetic gene clusters (BGCs).[6a] These BGCs harbor conserved core genes coding for a fatty acyl-AMP ligase, three desaturases, an acyl carrier protein (ACP), a thioesterase, and an electron transport component

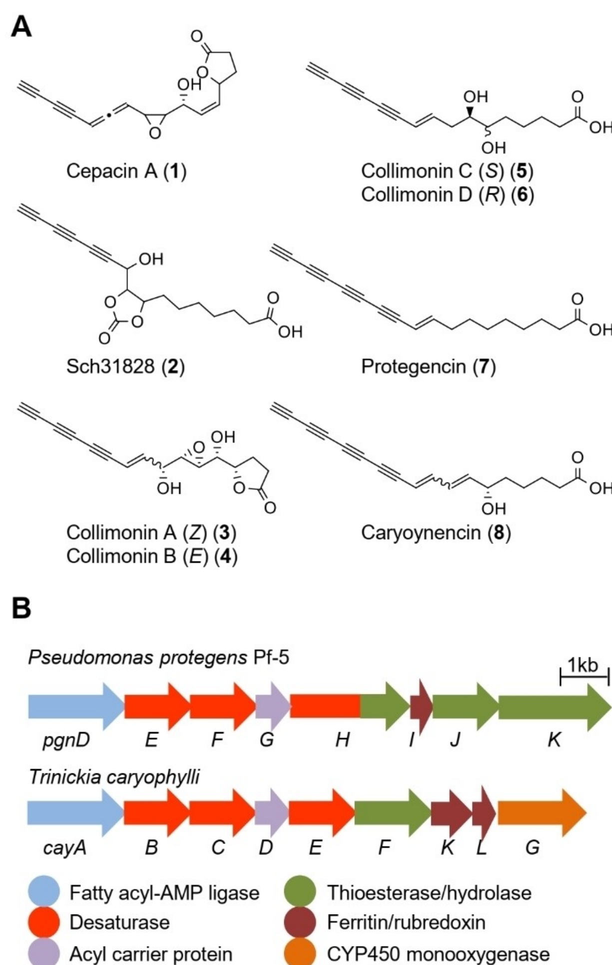

Figure 1. Examples of bacterial polyynes, and genetic basis for tetrayne biosynthesis. A) Structures of selected bacterial polyynes with terminal alkyne groups. B) Architectures of the protegencin (**7**) and caryoynencin (**8**) biosynthesis gene clusters.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200430

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such as rubredoxin (Figure 1B). In addition, the BGC may encode additional enzymes for modifications, such as the cytochrome P450 monooxygenase CayG, which has been shown to introduce an allylic alcohol moiety.[8]

Because of the high potential for bioprospecting,[9] as antifungals,[10] and for bio-orthogonal chemistry[11] and in vivo imaging[6b,7] there is high interest in understanding the biochemical basis for microbial alkyne and polyyne formation. Although various in silico and genetic analyses have been performed,[1b,3,5,6,8a,9] bacterial polyyne fatty acid biosynthesis is still little understood. Here, we provide insight into tetrayne octadecanoid biosynthesis by heterologous reconstitution, mutagenesis, and in vitro studies. We show the gene sets minimally required for polyyne biosynthesis, provide experimental evidence for a thiotemplated polyyne formation, and show that a desaturase triad works synergistically to produce four triple bonds.

Results and Discussion

To gain insight into the minimal set of genes required for polyyne fatty acid production, we cloned and heterologously expressed conserved caryoynencin and protegencin biosynthesis genes. Specifically, we created pRANGER-BTB3[12]-based expression plasmids containing genes *cayABCD*, coding for three desaturases and a fatty acyl-AMP ligase, *cayABCDE*, also containing a gene for an acyl carrier protein (ACP), and *cayABCDEF*, which contains an additional thioesterase gene, downstream of the pBAD promoter. We also generated constructs additionally containing *ferritin, cayK*, and/or *rubredoxin, cayL*, genes. The expression plasmids were introduced into *Burkholderia graminis*, which naturally does not produce polyyne fatty acids. The transformants were incubated in potato dextrose broth (PDB) medium at 30 °C for 2 days, gene expression was initiated by adding 1 mM arabinose, and metabolite production was monitored by HPLC with HRMS and UV detection (Figure 2A). Furthermore, we observed browning of bacterial colonies on agar plates, indicating the formation of unstable polyyne compounds (Figure 2B). When *cayABCD(E)* were heterologously expressed, no polyyne compounds could be detected (Figure 2A, traces b and g). When coexpressing the thioesterase gene, *cayF*, (*cayABCDEF*), a small peak corresponding to protegencin (**7**) was detected in the HPLC trace (Figure 2A, trace c). Coexpression of *ferritin, cayK*, and/or *rubredoxin, cayL*, genes markedly increased the production of protegencin (**7**) (Figure 2A, traces d–f), likely because the gene products are required for electron transfer. Alongside with

Figure 2. Heterologous reconstitution and mutational analysis of polyyne fatty acid biosynthesis. A) HPLC-DAD profiles (λ = 220–400 nm) of ethyl acetate extracts from heterologous expression of pRNAGER-BTB3 vector with a) empty (control), b) *cayABCDE*, c) *cayABCDEF*, d) *cayABCDEFK*, e) *cayABCDEFL*, f) *cayABCDEFKL*, g) *cayABCD*, h) *cayABCDK*, i) *cayABCDEK* and j) *cayADEFK* in *B. graminis* incubated in PDB medium at 30 °C for 2 days, k) and l) are standards of compound **9** and **7**, respectively. B) Incubation of above-mentioned strains in PDA plates under same condition. C) Model of the biosynthetic pathways to protegencin (**7**) and caryoynencin (**8**).

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protegencin (**7**), we detected a congener (**9**) with a secondary alcohol group at C17 in lieu of the terminal alkyne, a shunt product previously observed in the wild type.[8a] Incomplete gene sets did not give rise to polyynes, irrespective of coexpression of *cayK* (*cayABCDK*/*cayABCDEK*/*cayADEFK*) (Figure 2A, traces h–j).

Analogous expression experiments with *pgn* genes gave the same results. The heterologous expression of either *pgnDEFGH* or *pgnDEFGHI* in *B. graminis* led to the production of protegencin (**7**) and compound **9** (Figure S1, traces b–c). In the absence of one or two desaturase and thioesterase genes (*pgnDEFG*, *pgnDGH*) and fatty acyl-AMP ligase/ACP (*pgnEFH*), no polyynes were formed (Figure S1, traces d–f). The presence of *rubredoxin* gene, *pgnI*, increased the amount of protegencin (**1**).

From these results we concluded that the biosynthesis of the polyynes is initiated by the ligase-catalyzed activation and loading of a fatty acid onto an ACP, followed by desaturation, which requires three desaturases and support by ferritin or rubredoxin. Finally, thioesterase releases the polyyne fatty acid from the ACP (Figure 2C).

To investigate the ACP (CayD/PgnG) – fatty acyl-AMP ligase (CayA/PgnD) system for fatty acid activation and loading, we performed *in vitro* assays. Therefore, we heterologously produced the *N*-His$_6$- variants in *Escherichia coli* BL21(DE3). Whereas *N*-His$_6$- or *C*-His$_6$-tagged CayA were always located in inclusion body as an insoluble form, we succeeded in isolating *N*-His$_6$-tagged CayD, *N*-His$_6$-tagged PgnD and *N*-His$_6$-tagged PgnG. The phosphopantetheinyl transferase Sfp (P39135) from *Bacillus subtilis*[13] was used to transform apo-CayD and apo-PgnD into the corresponding holo-forms (Figure 3A).

Using the in vitro system and MALDI-TOF analysis of the ACPs (CayD and PgnG) we tested a variety of fatty acids as substrates. We noted that fatty acyl-AMP ligase PgnD is capable of loading a range of unsaturated and saturated fatty acids, such myristic acid ($C_{14}H_{28}O_2$), palmitic acid ($C_{16}H_{32}O_2$), palmitoleic acid ($C_{16}H_{30}O_2$, *cis*-Δ^9), stearic acid ($C_{18}H_{36}O_2$), oleic acid ($C_{18}H_{34}O_2$, *cis*-Δ^9), elaidic acid ($C_{18}H_{34}O_2$, *trans*-Δ^9), vaccenic acid ($C_{18}H_{34}O_2$, *trans*-Δ^{11}), linoleic acid ($C_{18}H_{32}O_2$, *cis,cis*-Δ^9,Δ^{12}), and linoelaidic acid ($C_{18}H_{32}O_2$, *trans,trans*-Δ^9,Δ^{12})) onto the cognate ACPs (Figure 3A for PgnD, Figure S2 for CayD).

Since the C18 protegencin and caryoynencin are the major products in the cultivation of *P. protegens* and *T. caryophylli*, respectively, one would expect octadecanoid fatty acids to be the primary substrates for PgnG and CayD. Nonetheless, we also noted polyyne tetradecanoid and hexadecanoid fatty acids in *P. protegens* Pf-5, which suggested that either shorter chain lengths are accepted as substrates by the polyyne biosynthesis enzymes, or that the C18 products are shortened by β-oxidation.[8b] The ACP loading experiments demonstrate that there is some flexibility with regard to chain length in the ligase-ACP system, and further substrate control is likely during the downstream processes. The predicted tertiary structures of CayD and PgnG obtained by AlphaFold2[14] revealed that these proteins possess a typical ACP fold with three main α-helixes that run parallel to each other (Figure S3). Interestingly, the phylogenetic analysis of ACP family proteins from the ThYme[15] (Thioester-active enzYmes) database suggests that bacterial polyyne ACPs belong to a new clade. Their closest relatives are ACP domains (clade ACP6) in PKSs involved in mycobacterial lipids biosynthesis (Figure 3B, S4–6, Table S7).[16]

In order to confirm that a thioesterase catalyzes hydrolysis of thioester bond to release protegencin from ACP at the final step of protegencin biosynthetic pathway, *C*-His$_6$-tagged CayF was produced in *Escherichia coli* BL21(DE3) and tested with a coenzyme A surrogate,[17] the *N*-acetyl cysteamine (SNAC) thioester of protegencin (**10**).[8b] The *in vitro* assay of CayF with **10** in buffered solution (pH 8.0) showed that this enzyme readily hydrolyzes the thioester bond to produce protegencin (Figure 3C).[8b]

Gene cluster comparisons along with gene deletion and heterologous expression experiments suggested that bacterial polyyne fatty acid BGCs harbor three conserved genes for membrane-bound fatty acid desaturases (*cayB*, *cayC*, and *cayE*; *pgnE*, *pgnF*, and *pgnH*) (Table S3 and Figure S7–S10).[6a,8a] Bioinformatics indicated that desaturase genes *cayB*/*pgnE* and *cayC*/*pgnF* are related to *jamB*, which is involved in the formation of the terminal alkyne moiety of jamaicamide B in marine cyanobacteria.[18] Notably, JamB has been shown to act on a short-chain fatty acid bound to an ACP associated to a polyketide synthase.[18b] The gene products of CayE/PgnH are similar to delta 12 fatty acid desaturases.[8a] Like typical desaturases, the deduced products of the *cay/pgn* desaturase genes have conserved histidine regions (HXXHH/QXXHH) that coordinate di-iron centers at the active site.[19] From the expected topologies of the desaturases, the conserved histidine regions are located inside and outside the cytosol (Figure S7–S10). To confirm the proposed roles of the desaturases and to potentially detect biosynthetic intermediates, we individually mutated two histidine residues in two active centers into two leucine residues (HXXHH→HXXLL and QXXHH→QXXLL) in each desaturase (*cay* genes in Figure S11 and *pgn* genes in Figure S12A). Then, we individually replaced each point-mutated desaturase gene in the functional polyyne fatty acid biosynthesis gene cassettes (*cayABCDEFK*/*pgnDEFGH*) and expressed these in *B. graminis.* Metabolic profiling showed that single point mutations in the active centers in each desaturase fully abolished polyyne production (Figure 4, traces b–g for *cay* genes, and Figure S12B, traces b–g for *pgn* genes). These observations indicate that a set of three fully functional desaturases is required for polyyne formation and provides a rationale for the intriguing pattern of three desaturase genes in bacterial polyyne fatty acid BGCs.

Conclusion

The heterologous expression and functional analyses of polyyne biosynthesis genes from two different bacterial strains teach important lessons on the bacterial synthesis of highly unsaturated fatty acids. First, the expression and heterologous reconstitution studies identified the minimal set of genes that are required and sufficient for polyyne biosynthesis. These gene cassettes should minimally code for a fatty acyl-AMP ligase, an ACP, three desaturases, and a thioesterase. An additional

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doi.org/10.1002/cbic.202200430
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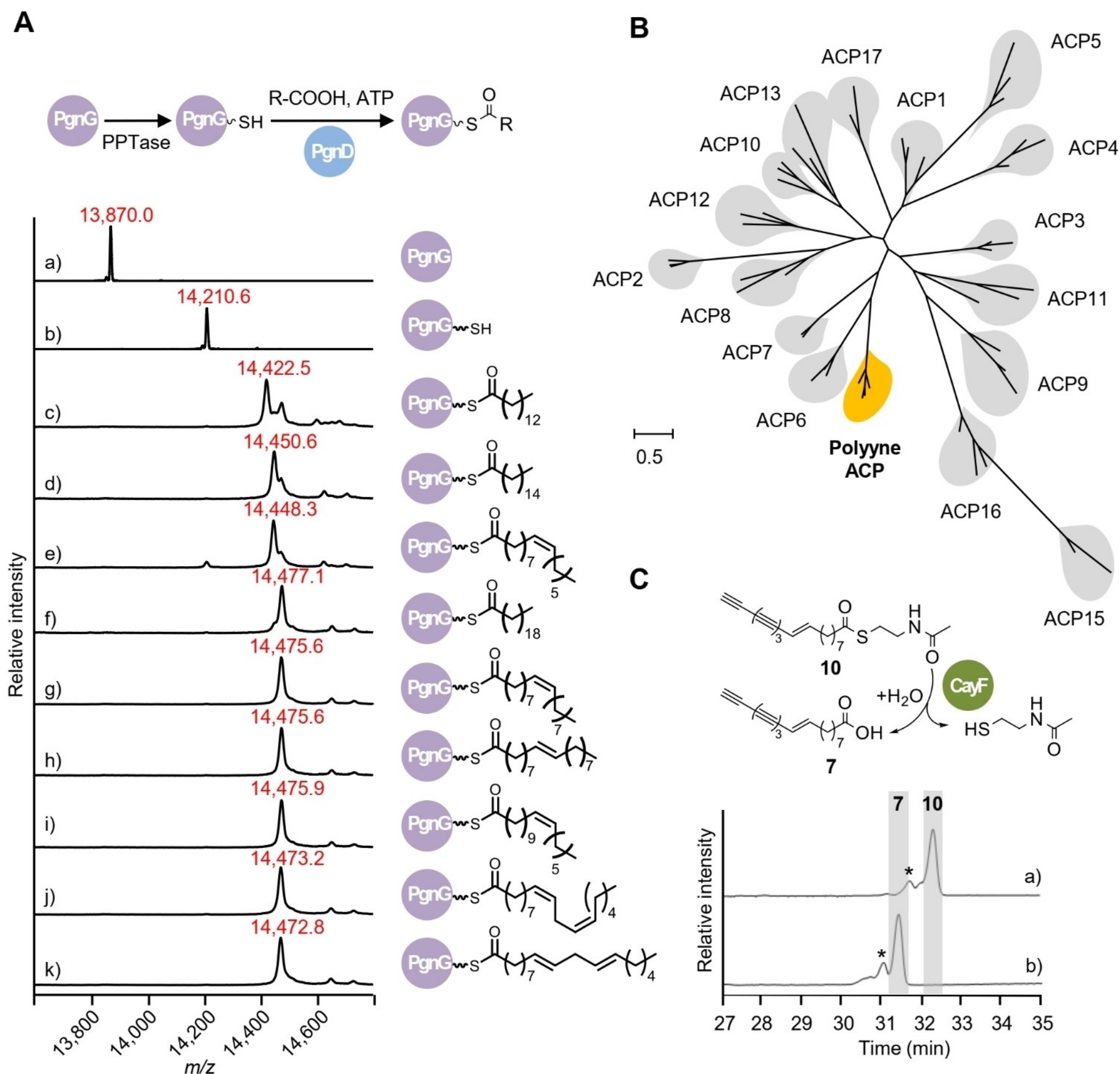

Figure 3. In vitro analysis of the thiotemplate system. A) Ligase-mediated loading of fatty acids onto ACP. General scheme and MALDI-TOF mass spectrometry spectra of ACP-bound substrates. a) PgnG alone, b) PgnG and SFP, c) PgnG, SFP and PgnD with myristic acid, d) same with palmitic acid, e) palmitoleic acid, f) stearic acid, g) oleic acid, h) elaidic acid, i) vaccenic acid, j) linoleic acid and k) linoelaidic acid. B) Phylogenetic tree of ACP family proteins. ACPs retrieved from the ThYme database are highlighted as gray clades. The clade of CayD homologues is highlighted in orange. The scale bar indicates amino acid substitutions per site. C) Scheme of thioesterase (CayF)-mediated hydrolysis of thioester surrogate **10**. HPLC-DAD profiles ($\lambda = 200$–400 nm) of enzyme reaction a) at $T = 0$ min, b) at $T = 30$ min. Asterisks indicate peaks of *Z*-isomers.

component for electron transport markedly increases polyyne production. Second, in contrast to the plant-derived alkyne fatty acids, which derive from free fatty acids, the formation of bacterial polyyne fatty acids is thiotemplated. It is a prerequisite for bacterial polyyne formation that the fatty acid is loaded onto a designated ACP, and eventually the furnished product is released by a thioesterase. Finally, the most remarkable finding is that three desaturases are capable of producing four triple bonds, and that it is essential for alkyne formation that this enzyme triad is intact. This finding contrasts with the individually acting acetylenases that introduce triple bonds into fatty acids in specialized plant and basidiomycete metabolism.[20] Insight into this peculiar bacterial polyyne assembly line provides important information for future bioengineering experiments. Beyond the heterologous production of highly instable, hard-to-synthesize polyyne compounds, this work may also set the basis for future analyses of an intricate desaturase complex.

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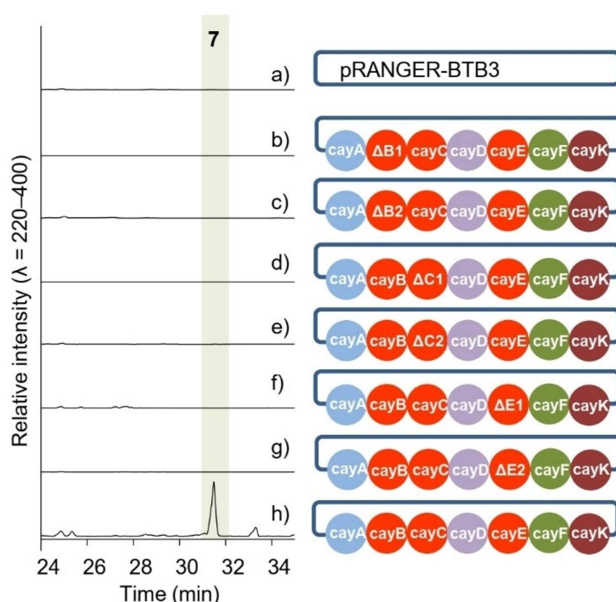

Figure 4. Functional analysis of the desaturase triad responsible for tetrayne formation. Point mutation of desaturases; sequences of point mutated a) *cayB1*, b) *cayB2*, c) *cayC1*, d) *cayC2*, e) *cayE*1 and f) *cayE*2. B) HPLC-DAD profiles (λ = 220–400 nm) of ethyl acetate extracts from heterologous expression of *cay* genes with point-mutated desaturase genes (*cayB*, *cayC* and *cayE*) in pRANGER-BTB3 in *B. graminis* incubated in PDB medium at 30 °C for 2 days.

Experimental Section

For experimental details, see the Supporting Information.

Acknowledgements

We thank A. Perner for LC HR-MS measurements, H. Heinecke for NMR measurements, and T. Kindel and A. Komor for MALDI measurements. Financial support by the Pakt für Forschung und Innovation and InfectoOptics (iTag) is gratefully acknowledged. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: alkynes · biosynthesis · fatty acids · natural products · polyynes

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Manuscript received: July 28, 2022
Revised manuscript received: September 14, 2022
Accepted manuscript online: September 15, 2022
Version of record online: September 29, 2022

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