Mohammad, H. H., Connolly, J. A., Song, Z., Hothersall, J., Race, P. R., Willis, C. L., Simpson, T. J., Winn, P. J., & Thomas, C. M. (2018). Fine Tuning of Antibiotic Activity by a Tailoring Hydroxylase in a Trans-AT Polyketide Synthase Pathway. *ChemBioChem*, 19(8), 836-841. <https://doi.org/10.1002/cbic.201800036>

Peer reviewed version

Link to published version (if available):
[10.1002/cbic.201800036](https://doi.org/10.1002/cbic.201800036)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Wiley-VCH at <http://onlinelibrary.wiley.com/doi/10.1002/cbic.201800036/abstract> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Fine Tuning of Antibiotic Activity by a Tailoring Hydroxylase in a Trans-AT Polyketide Synthase Pathway

Authors: Hadi H Mohammad, Jack A Connolly, Zhongshu Song, Joanne Hothersall, Paul R Race, Christine L Willis, Thomas J Simpson, and Christopher Morton Thomas

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *ChemBioChem* 10.1002/cbic.201800036

Link to VoR: <http://dx.doi.org/10.1002/cbic.201800036>

WILEY-VCH

www.chembiochem.org

A Journal of

Fine Tuning of Antibiotic Activity by a Tailoring Hydroxylase in a Trans-AT Polyketide Synthase Pathway

Hadi H. Mohammad^[a,d], Jack A Connolly^[a], Zhongshu Song^[b], Joanne Hothersall^[a], Paul R Race^[c], Christine L Willis^[b], Thomas J Simpson^[b], Peter J Winn^[a] and Christopher M Thomas^{*[a]}

Abstract: Addition or removal of hydroxyl groups modulates the activity of many pharmacologically active biomolecules. It can be integral to the basic biosynthetic factory or result from associated tailoring steps. For the anti-MRSA antibiotic mupirocin, removal of a C8-hydroxy group late in the biosynthetic pathway gives the active pseudomonic acid A. An extra hydroxylation, at C4, occurs in the related but more potent antibiotic thiomarinol A. We report here *in vivo* and *in vitro* studies that show putative non-heme-iron(II)/ α -ketoglutarate-dependent dioxygenase TmuB, from the thiomarinol cluster, 4-hydroxylates various pseudomonic acids while C8-OH, and other substituents around the pyran ring, block enzyme action but not substrate binding. Molecular modelling suggested a basis for selectivity but mutational studies showed limited ability to rationally modify TmuB substrate specificity. 4-hydroxylation had opposite effects on the potency of mupirocin and thiomarinol. Thus TmuB can be added to the toolbox of polyketide tailoring technologies for *in vivo* generation of new antibiotics in the future.

Polyketide synthases (PKSs) build carbon backbones of complex molecules from simple building blocks, such as acetate and malonate, using enzymes similar to those found in fatty acid biosynthesis.^[1] Further "tailoring" of these backbones (e.g. via hydroxylation) to produce the final product can be critical and understanding the specificity of such enzymes is essential for assembly of new pathways to novel compounds. For example, cyclosporin loses its immunosuppressive activity after regio-specific hydroxylation at the 4th N-methyl leucine but retains its side effects on hair growth.^[2]

Hydroxyl groups in polyketides generally occur either by ketoreduction of β -keto thiol esters, or on α -carbons of thiol esters by a tailoring step. β -hydroxy groups can be predicted from the *pks* gene sequences while α -hydroxylation is less predictable. PKS pathways use a number of enzyme families for hydroxylation of α -carbons: cytochrome P450s,^[3] FAD-binding monooxygenases^[4] and non-hemeFe(II)/ α -ketoglutarate dependent dioxygenases.^[5]

To provide new tools for engineering biosynthetic pathways we have investigated tailoring reactions in pathways of

two closely related antibiotics from the trans-AT family of modular polyketides,^[6] namely the thiomarinols and mupirocin. Thiomarinols (e.g. **1** and **2**) are produced by marine bacteria of the genus *Pseudoalteromonas* and are closely related to the commercially important mupirocin^[7] (a mixture of pseudomonic acids including A-C **3-5**, Fig. 1) produced by *Pseudomonas fluorescens* which is used topically against Methicillin Resistant *Staphylococcus aureus* (MRSA).^[8] The structures of the major metabolites, thiomarinol A **1** and pseudomonic acid A (PA-A, **3**), differ by the presence of a 10, 11-alkene in **1** but an epoxide in **3**, an 8-hydroxyoctanoic acid rather than 9-hydroxynonanoic acid side-chain and an additional pyrroline moiety. A further important difference for the current study, is that the more bioactive thiomarinol A **1** possesses a 4-hydroxyl group^[7]. Most genes in the mupirocin gene cluster have been allocated a role^[9] and sequence comparisons of the *mup* and *tml* gene clusters confirms the similarities but important differences in their biosynthetic pathways.^[10]

Figure 1. Structures of key compounds in this study. (A) Selected thiomarinols and pseudomonic acids; (B) Synthetic conversion of PA-A **3**; (C) Further 4-hydroxylated metabolites and mupirocin F; (D) Mupirocins not 4-hydroxylated by TmuB.

[a] Dr HH Mohammad, Mr JA Connolly, Dr J Hothersall, Dr PJ Winn and Prof CM Thomas*

School of Biosciences, University of Birmingham
Edgbaston, Birmingham B15 2TT, UK
E-mail: c.m.thomas@bham.ac.uk

[b] Dr Z Song, Prof CL Willis, Prof TJ Simpson
School of Chemistry, University of Bristol
Cantock's Close, Bristol BS8 1TS

[c] Dr PR Race
School of Biochemistry, University of Bristol
University Walk, Bristol BS8 1TD

[d] Dr HH Mohammad, College of Medicine, Kirkuk University, Kirkuk, Iraq

Supporting information for this article is given via a link at the end of the document. ((Please delete this text if not appropriate))

COMMUNICATION

WILEY-VCH

Understanding these differences might allow modification of mupirocin and other molecules to give new antibiotic leads. Feeding experiments with the PKS mutant of the thiocarinal producer *Pseudoalteromonas* spp SANK73390 showed that PA-A **1**, could be 4-hydroxylated and addition of the pyrroline occurred giving **6**, **7** and **8** (Figure 1B). In contrast, PA-B **2**, with the 8-OH, gained the pyrroline but no 4-hydroxylation occurred.^[11] Here we identify the 4-hydroxylase as TmuB and report its characterisation.

Results and Discussion

TmuB can 4-hydroxylate metabolites in P. fluorescens

Comparison of the mupirocin and thiocarinal gene clusters identified genes that lack paralogues in the mupirocin cluster and might encode the 4-hydroxylase. BLAST searches^[12] identified TmuB as related to phytanoyl-CoA dioxygenase, an enzyme of the non-heme-iron(II)/ α KG-dependent dioxygenase superfamily. We inserted the *tmuB* coding region into broad host range vector pJH10 under control of the *tac* promoter (pJH10-*tmuB*) and transferred it to WT *P. fluorescens* NCIMB 10586. HPLC analysis of culture supernatant after *tmuB* expression showed a new peak more polar than PA-A (Fig. 2). The new metabolite was isolated and confirmed by NMR spectroscopy to be 4-hydroxy PAA **6**.

Figure 2. HPLC analysis of culture supernatant from *P. fluorescens* strains showing that TmuB modifies PA-A **1** and PA-C **3** (produced by *mmpEΔOR*) but not PA-B **2** (produced by *ΔmupU*). Mupirocin F **4** is also 4-hydroxylated. Note also that *tmuB* expression increased overall yield of pseudomonic acid derivatives, so a new peak can appear even though the peak of the non-4-hydroxylated form is of a similar size to that observed without *tmuB*.

We expressed *tmuB* in *P. fluorescens* NCIMB10586 mutants that accumulate different intermediates to explore substrate specificity. As predicted from feeding PA-B **4** to the thiocarinal producer^[11], *tmuB* expressed in mutants *ΔmacpE*, *ΔmupO*, *ΔmupU* and *ΔmupV* that produce PA-B rather than PA-A^[15] did not change PA-B metabolite profile (Fig. 2), strengthening the conclusion that TmuB has specificity for PA-A **3** over PA-B **4**.

Expression in NCIMB10586 *ΔmupC* producing mupirocin C **13**, and *ΔmupT* or *ΔmupW* producing mupirocin W **14** (structures shown in Fig. 1) gave no significant differences in metabolite profiles (data not shown). However, the *mmpEΔOR* strain producing PA-C **5**, gave a new metabolite (Fig. 2) confirmed as 4-hydroxy PA-C **9** following full characterisation by MS and NMR (Table 1S). Likewise *ΔmupF*, producing mupirocin F **10** gave the novel metabolite (Fig. 2) confirmed to be 4-hydroxy mupirocin F by LC-ESI-MS (MW=514) and NMR (Table 1S). Interestingly the mupirocin F peak at 22.3 min increased two-fold (Fig. 2), an example of increased overall production observed when *tmuB* is expressed. Thus the tetrahydropyran ring and its state of oxidation are critical for substrate modification by TmuB and it is not only an 8-OH group that can block the action of TmuB.

PA-B 4 is a competitive inhibitor of purified TmuB

WT *tmuB* was inserted into pET28a and expressed in *E. coli* BL21(DE3) with an N-terminal His-tag. As a control, *tmuB* from inactive mutant I109N, described below, was expressed in parallel. Purified WT and I109N TmuB protein were tested on purified PA-A **3**. Co-substrate (α -ketoglutaric acid) and co-factor (FeSO_4) were essential and WT TmuB generated a new peak at 18.2 min (Fig. 3A) confirmed to be 4-hydroxy PA-A **6** but TmuB I109N did not. Reactions at 23 °C, 25 °C, 30 °C and 37 °C for 180 min, showed maximum activity at 23 °C, the optimum growth temperature for the thiocarinal producer.

TmuB activity on PA-B was only observed after >6h incubation, generating a new metabolite (Figure 3A), confirmed as 4-hydroxy PA-B **12** (Fig. 1) by LCMS and NMR. Initial rates were determined at substrate concentrations from 3.75 to 240 μM to determine V_{max} and K_m (Fig. 3B, C, D). The catalytic efficiency (k_{cat}/K_m) for PA-A is three orders of magnitude higher than for PA-B, the major difference being turnover number rather than substrate binding (Fig. 3D).

Since the K_m for PA-B **4** is only 3-fold higher than for PA-A, while turnover number decreases 1000-fold, it may be a competitive inhibitor of TmuB. Reactions on PA-A **3** carried out with 30, 60 and 120 μM PA-B showed an increased K_m for PA-A while k_{cat} remained unchanged (Figure 1S). The apparent absence of hydroxylation of PA-B and other derivatives by TmuB *in vivo* may be due to this slow reaction rate: the product may be released from the cell before hydroxylation occurs or remain below the detection limit.

Mutational analysis of the TmuB substrate binding pocket

In the absence of TmuB crystallographic data, structural predictions were made from the coordinates of EasH^[13] (PDB 4NAO, 25% identity), plus AsqJ (PDB 5DAW, 21% identity)^[14] and FtmOx1 (PDB 4Y5T, 23% identity)^[15] because 4NAO lacks coordinates for the α -helix2/ β 3 loop which is resolved as a flap covering the active site entrance in the others (Fig. 2S). The TmuB homology model contains a double stranded β -helix (DSBH), or Jelly-roll fold, characteristic of the nonheme-Fe(II)/ α -ketoglutarate-dependent dioxygenase super-family (Fig. 3S)^[16]. PA-A **3** was docked with the best scoring models based on the criteria listed in Methods, with and without the α -helix2/ β 3 loop folded properly, to find dominant conformations/orientations consistent with the known biochemistry and identify residues interacting with the substrate. Without the α -helix2/ β 3 loop, the results show monic acid fitting the active site pocket and 9HN in the external groove (Fig. 4A). When the α -helix2/ β 3 loop is included it is located above the active site and interacts with the fatty acid chain (Fig. 4S). The tetrahydropyran ring was similarly

COMMUNICATION

WILEY-VCH

located in all models. Evolutionary Trace Analysis (ETA) identified about half the residues lining the pocket as likely to be functionally important (Fig. 5S) and this is supported by mutagenesis experiments in other proteins^[17]. For PA-A the target C-4 is 4.4 Å from the Fe cofactor which is close to the norm (4.5 Å) for these enzymes^[18]. The pyran ring sits near the pocket entrance which consists of residues R69, K105, I109 and M208 and interacts with R69, K105 and I109 plus L141 and A206 (Fig. 4B and C).

To explore specificity, PA-B **4** was docked with the TmuB model using the same parameters as for PA-A **3**. Similar orientations were obtained and although binding energy and inhibitory constants for both PA-A **3** and PA-B **4** were similar, the

Figure 3. *In vitro* activity of TmuB and kinetics using different substrates. A: Activity of purified TmuB on PA-A **3** gives complete 4-hydroxylation of PA-A **3** to **6** but only 43% 4-hydroxylation of PA-B **4** after overnight incubation; B: Michaelis-Menten analysis of TmuB hydroxylating PA-A **3** and TMC **2**; C: Analysis of TmuB hydroxylating PA-B; and D: The enzyme kinetic parameters of TmuB for each substrate.

Figure 4. A. Homology model of TmuB showing PA-A **3** docked in the active site. 9HN fits into the external groove while monic acid inserts into the pocket. B. PA-A (Pink stick) and proximal residues in the active site. The target site C-4 is 4.38 Å from the Fe II (Orange sphere). C. The active site residues surrounding the pyran ring and were selected for mutagenesis. D. The effect of mutations on TmuB activity against PA-A.

C4 to active site Fe²⁺ distance was 6.3 Å compared to 4.4 Å for PA-A **3**. Thus the C-8 OH must alter the active site fit and detailed analysis showed that R69, D104, K105, I109, Q126, L141 and M208 may be involved. All these residues were mutated to smaller, hydrophilic amino acids to accommodate the extra groups. Substitutions least likely to cause TmuB inactivation were identified by alignments with other superfamily members and *in silico* mutation. Single and double *tmuB* point mutants were created in pJH10-*tmuB* and tested for PA-B hydroxylation in *P. fluorescens*: HPLC analysis showed that none gave observable hydroxylation but some reduced activity on PA-A (Fig. 4D). That 4-OH PA-B **12** might be lethal is unlikely given the MIC data presented below (Fig. 5) and the possibility that it is trapped in the cell seems unlikely since hydroxylated metabolites were easily isolated from culture supernatants. Thus it may not be possible to manipulate TmuB to allow *in vivo* hydroxylation of PA-B.

Effect of hydroxylation on antibacterial activity

Plate bioassay (plus 0.5 mM IPTG) showed that antibacterial activity of 4-hydroxylated mupirocin produced *in vivo* by NCIMB10586 (pJH10-*tmuB*) (mainly 4-hydroxy PA-A **6**) was reduced against *B. subtilis* 1064 (Fig. 5A) and MIC tests confirmed this (Fig. 5C). Lowered activity of 4-OH PA-B makes it unlikely that failure to convert *in vivo* is due to lethality. To explore explanations for the reduction, PA-A was docked to the crystal structure of the isoleucyl-tRNA synthetase (IleRS) *in silico*, yielding a conformation similar to the crystal complex of IleRS-PA-A (1FFY PDB)^[19]. The hydroxyl group in 4-OH PA-A **6** disrupted this conformation apparently because the 4-OH causes steric repulsion by H64 and D557 (Fig. 6S). To test the effect of thiocarbonyl lacking the 4-hydroxy group, a sample of thiocarbonyl **2** (Fig. 1) was prepared after the *tmuB* I109N

COMMUNICATION

WILEY-VCH

mutation was recombined into the thiomarinol producer. Bioassay and MIC tests with *B. subtilis*, *E. coli* and *S. aureus* showed that thiomarinol C (TMC) **2** was less potent than thiomarinol A **1** (Fig. 5). The different consequences of 4-hydroxylation on potency of PAs versus TMs may indicate that the length of hydroxy-acid moiety (9NA v 8HO) alters how these antibiotics bind their targets and emphasises the need for structures of more IleRS-antibiotic complexes.

In vitro tests with purified TmuB showed thiomarinol C **2** is a good substrate for TmuB giving thiomarinol A **1** with similar catalytic activity to PA-A **3** ($K_m = 29.5 \mu\text{M}$ and $k_{cat} = 2212 \text{ s}^{-1}$) (Fig. 3B).

Figure 5. Antibacterial activity of the new derivatives. (A) Plate bioassays against *B. subtilis* from *P. fluorescens* strains expressing TmuB *in-trans*: *P. fluorescens* WT (producing 4-OH PA-A **6**), *P. fluorescens* mmpE Δ O (producing 4-OH PA-C **9**) and *P. fluorescens* Δ mupF (producing 4-OH mupirocin F **11**). (B) Plate bioassays on Thiomarinol A and C (**1** and **2**) extracted from WT and PM I109N SANK respectively. The discs were saturated with 100 μg of purified thiomarinol A and C and tested against *B. subtilis* 1064, *E. coli* DH5 α and *S. aureus* MRSA NCTC 12493. (C) Minimal inhibitory concentrations (MIC).

Conclusions

We have shown that TmuB 4-hydroxylates thiomarinol and its analogues including PA-A **3**, PA-C **5** and mupirocin F **10** in *P. fluorescens*. However, its activity is severely inhibited, not just by the 8-OH of PA-B **4**, but also by the further structural changes to the tetrahydropyran core (as in mupirocins C and W, **13** and **14**). It therefore seems likely that 4-hydroxylation occurs late in biosynthesis especially since an ACP-tethered substrate is not required, as TMC **2** and PA-C **5** are good substrates. We also conclude that the 9HN/8HO moiety must remain outside the active site pocket since its internal dimensions of 6.8 Å by 4.4 Å would be too small to accommodate the C1-3 of monic acid, 8HO and pyrroline in the pocket. 4-hydroxylation could therefore be one of the final steps in biosynthesis and *tmuB* could have been acquired relatively recently in thiomarinol cluster evolution, consistent with it being one of the genes (*tmly*, *tmuA* and *tmuB*) not present in the *mup* cluster^[10]. The fact that 4-hydroxylation increases thiomarinol potency could explain this acquisition but since *tmuB* expression also increased product levels in culture supernatants, enhancing passage across the membrane could be another explanation.

The detectable but low activity of TmuB on PA-B **5** suggested that TmuB might be modified to hydroxylate a broader range of substrates. Active site mutagenesis of enzymes of the nonheme-Fe(II)/ α KG-dependent dioxygenase superfamily has changed specificity and diverted catalytic function as reported recently with fumitremorgin B endoperoxidase from *Aspergillus fumigatus*^[15]. However, structure-guided site directed mutagenesis failed to improve the activity of our enzyme. This may be due to TmuB having flexible finger-like loops like those found in enzymes such as phytanoyl-CoA dioxygenase^[20] and prolyl hydroxylase^[21], which undergo conformational changes on substrate binding, making *in silico* substrate docking difficult. Therefore, determining accurate TmuB crystal structures with and without different substrates will be an important goal to increase its uses in polyketide tailoring technologies and facilitate the *in vivo* generation of new antibiotics in the future.

Acknowledgements

This work was funded by BBSRC grants BB/I014373/1 and BB/I014039/1 and through BrisSynBio, the Bristol Centre for Synthetic Biology (BB/L01386X/1). HHM was funded by an Iraqi Government Scholarship (HCED). JAC was funded by a BBSRC MIBTP (BB/J014532/1) studentship.

Conflict of interest

The authors declare no conflict of interest.

Keywords: antibiotics • hydroxylation • polyketides • mupirocin • thiomarinol

- [1] K. Weissman, *Nat. Prod. Reports* **2016**, 33, 203.
- [2] M. J. Lee, H. B. Kim, Y. J. Yoon, K. Han, E. S. Kim, *Appl. Env. Microbiol* **2013**, 79, 2253.
- [3] L. Song, L. Laureti, C. Corre, P. Leblond, B. Aigle, G. L. Challis, *J. Antibiot.* **2014**, 67, 71.
- [4] Y. Jiang, H. Wang, C. Lu, Y. Ding, Y. Li, Y. Shen, *ChemBiochem* **2013**, 14, 1468.
- [5] a) K. H. Almabruk, S. Asamizu, A. Chang, S. G. Varghese, T. Mahmud, *ChemBiochem* **2012**, 13, 2209. b) Y. Matsuda et al. *J. Am. Chem. Soc.* **2016**, 138, 12671.
- [6] Till, M. & Race, P.R. *Biotechnol. Lett.* **2014**, 36:877.
- [7] H. Shiozawa, T. Kagasaki, T. Kinoshita, H. Haruyama, H. Domon, Y. Utsui, K. Kodama, S. Takahashi. *J. Antibiot.* **1995**, 48, 907.
- [8] B. D. Cookson, R. W. Lacey, W. C. Noble, D. S. Reeves, R. Wise, R. J. Redhead, *Lancet* **1990**, 335, 1095.
- [9] a) C. M. Thomas, J. Hothersall, C. L. Willis, T. J. Simpson, *Nat. Rev. Microbiol.* **2010**, 8, 281. b) A. K. El-Sayed, J. Hothersall, S. M. Cooper, E. Stephens, T. J. Simpson, C. M. Thomas, *Chem. Biol.* **2003**, 10, 419.
- [10] a) J. Hothersall, et al., *J. Biol. Chem.* **2007**, 282, 15451. b) D. Fukuda, et al., *PLoS ONE* **2011**, 6, e18031.
- [11] a) A.C. Murphy, D. Fukuda, Z. Song, J. Hothersall, R. J. Cox, C. L. Willis, C. M. Thomas, T. J. Simpson *Angew. Chem. Int. Ed.* **2011**, 50, 3271. b) S. S. Gao, et al., *Angew. Chem. Int. Ed.* **2017**, 56, 3930.
- [12] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucl. Acids Res.* **1997**, 25, 3389.
- [13] J. Havemann, D. Vogel, B. Loll, U. Keller, *Chem. Biol.* **2014**, 21, 146.
- [14] A. Brauer, P. Beck, L. Hintermann, M. Groll, *Angew. Chem. Int. Ed.* **2016**, 55, 422.
- [15] W. Yan, et al., *Nature* **2015**, 527, 539.
- [16] R. P. Hausinger, *Crit. Rev. Biochem. Mol. Biol.* **2004**, 39, 21.
- [17] M. A. McDonough, K. L. Kavanagh, D. Butler, T. Searls, U. Oppermann, C. J. Schofield, *J. Biol. Chem.* **2005**, 280, 41101.

COMMUNICATION

WILEY-VCH

- [18] W. Aik, M. A. McDonough, A. Thalhammer, R. Chowdhury, C. J. Schofield, *Curr Opin Struct Biol* **2012**, 22, 691.
- [19] L. F. Silvan, J. Wang, T. A. Steitz, *Science* **1999**, 285, 1074.
- [20] a) I. J. Clifton, M. A. McDonough, D. Ehrismann, N. J. Kershaw, N. Granatino, C. J. Schofield, *J Inorg. Biochem.* **2006**, 100, 644. b) R. Chowdhury, M. A. McDonough, J. Mecinovic, C. Loenarz, E. Flashman, K. S. Hewitson, C. Domene, C. J. Schofield, *Structure* **2009**, 17, 981.
- [21] D. E. Lancaster, L. A. McNeill, M. A. McDonough, R. T. Aplin, K. S. Hewitson, C. W. Pugh, P. J. Ratcliffe, C. J. Schofield, *Biochem. J.* **2004**, 383, 429.

Accepted Manuscript

Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Text for Table of Contents

((Insert TOC Graphic here))

*Author(s), Corresponding Author(s)****Page No. – Page No.****Title**

Layout 2:

COMMUNICATION

((Insert TOC Graphic here))

*Author(s), Corresponding Author(s)****Page No. – Page No.****Title**

Text for Table of Contents