Bioorganic & Medicinal Chemistry Letters 30 (2020) 127107

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis, biological evaluation and structure-activity relationships of 5-arylidene tetramic acids with antibacterial activity against methicillin-resistant *Staphylococcus aureus*

Dimitris Matiadis[a,*], Dimitrios Tsironis[a], Valentina Stefanou[a], Spyridon Boussias[a], Angeliki Panagiotopoulou[b], Vickie McKee[c,d], Olga Igglessi-Markopoulou[a], John Markopoulos[e,*]

[a] National Technical University of Athens, School of Chemical Engineering, Laboratory of Organic Chemistry, Zografou Campus, Athens 15773, Greece
[b] Institute of Biosciences and Applications, NCSR "Demokritos", Ag. Paraskevi, 15310 Attiki, Greece
[c] Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark
[d] School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, Ireland
[e] Laboratory of Inorganic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, 15771 Athens, Greece

ARTICLE INFO

InChIKeys:
ITINXNLUXHSDPH-JYRVWZFOSA-N
VTIKXFQFIUTPJB-JYRVWZFOSA-N
XCWVVYBLKVYDOB-GHXNOFRVSA-N
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QIASDTRYOAULGK-UVTDQMKNSA-N
IMRAGIHYGYLUQR-VXYIRXSZSA-N

Keywords:
Pyrrolidine-2-dione
Tetramic acid
Lactam
X-ray crystallography
MRSA
Antibacterial activity

ABSTRACT

The steady rise of the antimicrobial resistance is a major global threat to human health that requires the urgent need for novel antibiotics. In this work we report the synthesis of a small library of 3-subsituted-5-arylidene tetramic acids in order to investigate the scope of our previously established methodology via an intermediate oxazolone and their antimicrobial activity. From this series of 14 tetramic acids, 11 derivatives are novel and one of them is a Schiff base, which was structurally characterized with single-crystal X-ray analysis and NMR spectroscopy. The compounds incorporating a lipophilic acyl group at carbon-3 of the ring showed moderate to high activity with minimum inhibitory activity of 4–32 μg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA), accompanied by no human cell toxicity and hemolytic activity within the tested concentration range. The substituent at *para* position of the aryl ring seemed to have no or little effect on the antimicrobial activity of these compounds.

The antimicrobial resistance (AMR) or multidrug resistance (MDR) is one of the greatest threats for humankind.[1] Undoubtedly, a large public health burden, increasing over time, is associated with infections caused by antibiotic-resistant bacteria.[2] Especially, the so-called ESKAPE pathogens[3] (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) represent the majority of nosocomial infections and characteristic models of pathogenesis, transmission and resistance.[4]

Specifically, studies indicate the rapidly increasing rates of infections associated with methicillin-resistant *S. aureus* (MRSA).[5] Clinical isolates of community-associated and healthcare-associated MRSA have developed resistance to many classes of antibiotics, such as β-lactams,[6] macrolides,[7] tetracyclines,[8] quinolones[9] and even last-resort agents, such as vancomycin,[10] daptomycin[11] and linezolid.[12] As a result, the development of novel antibiotics having new mechanisms of action is urgently needed to address the challenge of treating multidrug resistant

* Corresponding authors.
E-mail addresses: dmatiadis@gmail.com (D. Matiadis), jmmarko@chem.uoa.gr (J. Markopoulos).

https://doi.org/10.1016/j.bmcl.2020.127107
Received 2 February 2020; Received in revised form 9 March 2020; Accepted 10 March 2020
Available online 13 March 2020
0960-894X/

bacterial infections.

3-Acyltetramic acids present a great variety of biological activities, including antibacterial[13] and antifungal[14] properties. A few naturally occurring and synthetic tetramic acid-containing antibacterials have been found to inhibit effectively the methicillin-resistant or methicillin-sensitive *S. aureus*[15] (Fig. 1). The fungal toxin equisetin,[15a] as well as its analogue trichosetin,[15b] are 3-acyltetramic acids bearing an aliphatic bicyclic ring and show potent antibacterial activity against Gram-positive bacteria. Reutericyclin[15c] and magnesidin,[15d] effective inhibitors of *S. aureus*, are natural products incorporating a lipophilic alkanoyl group at nitrogen and at the carbon-3 of the ring respectively. Synthetic tetramic acids with novel structures [15e–g] or analogues of the above-mentioned natural products [15h,i] have been identified as potent inhibitors of *S. aureus*. The common point seems to be the presence of a long lipophilic group attached at the nitrogen or at the C-3 of the ring. Yet, there are only a few reports investigating the structure activity relationships of these compounds regarding their antibacterial properties.

Due to their aforementioned biological activities and the challenging structures of natural products bearing the tetramic acid moiety[16] many protocols for their synthesis have been developed, both conventional[17] and catalytic.[18] Tetramic acid metal coordination compounds[19] and derivatives such as amides,[20] peptide analogues,[21] macrocycles[22] and hybrids with commercial antibiotics[23] have been examined in order to further expand the potential of this valuable skeleton. However, only a few studies in the literature have reported the synthesis of 3-acyl or 3-phenyl 5-arylidenetetramic acids so far.[24]

Our group has contributed to the synthesis and study of many

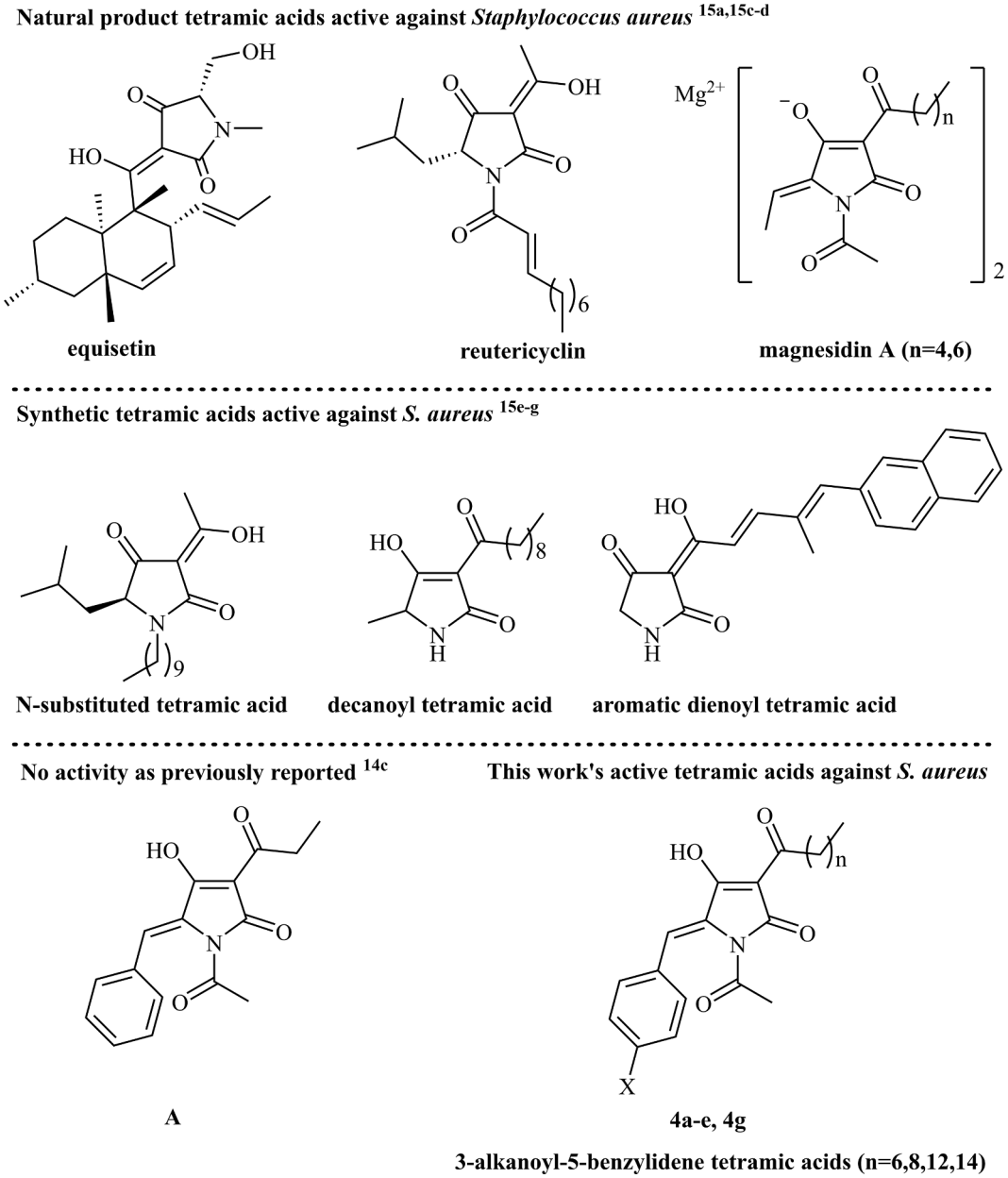
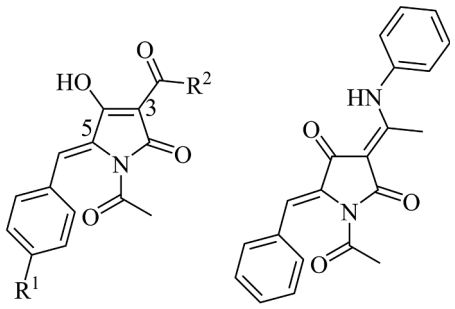


Fig. 1. Representative examples of the nature derived antibiotic tetramic acids equisetin, reutericyclin and magnesidin A, potent synthetic *S. aureus* inhibitors bearing lipophilic groups, our previous work model compound and this work's identified antimicrobial 3-alkanoyl-5-benzylidene tetramic acids. The tautomeric form of each structure is adopted from the cited literature.



3a-f, 4a-g **5**

3a-f:	**R^2=CH_3**	**4a-f:**	**R^1=H**
		4g:	**R^1=OCH_3**
3a:	**R^1=OCH_3**	**4a:**	**R^2=$(CH_2)_2CH_3$**
3b:	**R^1=CH_3**	**4b:**	**R^2=$(CH_2)_6CH_3$**
3c:	**R^1=Cl**	**4c:**	**R^2=$(CH_2)_8CH_3$**
3d:	**R^1=F**	**4d:**	**R^2=$(CH_2)_{12}CH_3$**
3e:	**R^1=CF_3**	**4e:**	**R^2=$(CH_2)_{14}CH_3$**
3f:	**R^1=NO_2**	**4f:**	**R^2=Ph**
		4g:	**R^2=$(CH_2)_2CH_3$**

Fig. 2. Structures of tetramic acids **3**–**5**.

tetramic acid derivatives,[24a-d,25] including their coordination compounds.[14c,24a,26] In our previous study, we published the first cadmium complex of N-acetyl-3-acetyl-5-benzylidene tetramic acid with promising antifungal activity against *Cryptococcus neoformans* with no human cell toxicity or hemolytic activity within the tested concentration range.[14c]

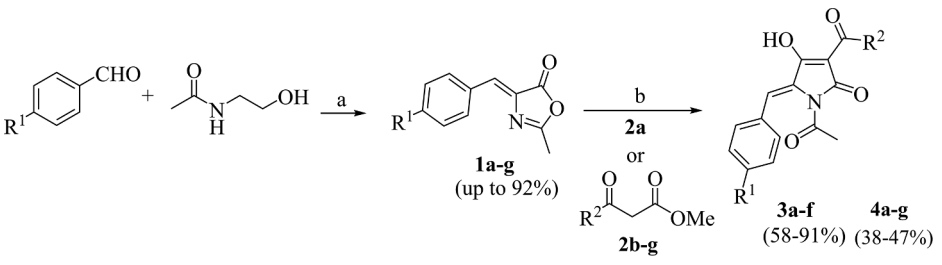
As a continuation of our efforts to expand the scope of our established methodology via an appropriate oxazolone intermediate[24a] and to further investigate the biological activities of this relatively underexplored class of antibiotics, we report herein the synthesis of a small library of *N*-acetyl-3-acyl-5-arylidene tetramic acids (**3**,**4**) and a novel tetramic acid Schiff base (**5**) (Fig. 2). In order to discuss the structure–activity relationships of their antimicrobial activities, appropriate modifications on two groups were made; the *para*-substituent on the 5-arylidene group (**3a-f**) and the 3-acyl group (**4a-g**). Moreover, the incorporation of a Schiff base component at C-3 was examined to investigate the influence of the carbonyl group, resulting in a novel structure (**5**) characterized with single-crystal X-ray analysis. All the synthesized compounds were primary-screened against 5 key ESKAPE pathogens (*E. coli*, methicillin-resistant *S. aureus* (MRSA), *K. pneumoniae, A. baumannii* and *P. aeruginosa*) and 2 fungi (*C. neoformans* and *Candida albicans*) at a concentration of 32 μg/mL and the active compounds were further screened in dose response antimicrobial assays to confirm their activity and determine their minimum inhibition concentration (MIC). The active compounds were tested for both human non-cancerous cell toxicity and hemolytic activity.

The target compounds were synthesized bearing in mind the potential for antimicrobial activity and the scope of our previously published methodology for 3-acyl-5-benzylidene tetramic acids.[24a] The final products were prepared using a short synthetic route (Scheme 1) starting from *N*-acetylglycine [27]. *N*-Acetylglycine was condensed with the appropriate aromatic aldehyde in the presence of sodium acetate in acetic anhydride. The resulting azlactones **1a-g** proved to be ideal for the one-pot synthesis of 5-arylidenetetramic acids **3a-f** and **4 g**, since the time consuming and yield- or purity-decreasing steps of protection, deprotection and activation are eliminated. **1a-f** were subjected to nucleophilic attack from the anion of the β-ketoester **2a-g**, formed by non-nucleophilic base (NaH) deprotonation. Final products **3a-f** and **4 g** were precipitated in good purity after acidification with 10% HCl. Pure samples for analytical and biological tests were obtained after recrystallization from methanol or ethanol.

In a similar manner, tetramic acids **4a-f** were prepared from the oxazolone **1g** and the anion of ethyl benzoylacetate or the appropriate long hydrocarbon chain β-ketoester. The latter were prepared following a slightly modified literature method.[28] **4a-f** were purified by column chromatography. In this case, the extraction of the aqueous phase before acidification with petroleum ether instead of diethyl ether, increased the yield by 10–15% in comparison to our previously reported synthesis.[24a]

The results proved that the developed method via arylidene oxazolones allows wide variation of 3-acyl substituents and electron-withdrawing or donating groups at the *para* position of the 5-benzylidene group.

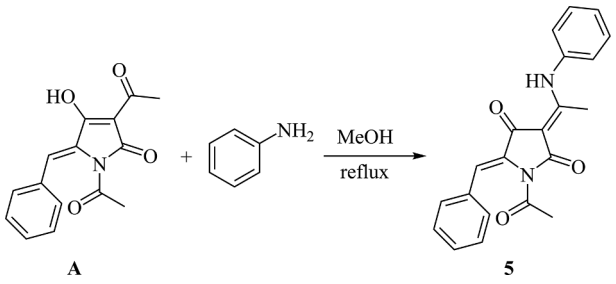
In order to investigate the structure-activity relation of the carbonyl oxygen at C-3 compared to a substituted imine analogue, we prepared the Schiff base derived from the condensation reaction between **A** and



1a:	**R^1=OCH_3**
1b:	**R^1=CH_3**
1c:	**R^1=Cl**
1d:	**R^1=F**
1e:	**R^1=CF_3**
1f:	**R^1=NO_2**
1g:	**R^1=H**

3a-f:	**R^2=CH_3**	**4a-f:**	**R^1=H**
		4g:	**R^1=OCH_3**
3a:	**R^1=OCH_3**	**4a:**	**R^2=$(CH_2)_2CH_3$**
3b:	**R^1=CH_3**	**4b:**	**R^2=$(CH_2)_6CH_3$**
3c:	**R^1=Cl**	**4c:**	**R^2=$(CH_2)_8CH_3$**
3d:	**R^1=F**	**4d:**	**R^2=$(CH_2)_{12}CH_3$**
3e:	**R^1=CF_3**	**4e:**	**R^2=$(CH_2)_{14}CH_3$**
3f:	**R^1=NO_2**	**4f:**	**R^2=Ph**
		4g:	**R^2=$(CH_2)_2CH_3$**

Scheme 1. Synthesis of tetramic acids **3a-f** and **4a-g**. Reagents and conditions: (a) acetic anhydride, CH_3COONa, 1 h, reflux; (b) NaH, dry THF, 0 °C to r.t., 10% HCl (**2a**: ethyl acetoacetate).



Scheme 2. Synthesis of the Schiff base **5**.

aniline. Compound **5** was prepared[29] in satisfactory yield and high purity by refluxing the starting materials in methanol for 2 h (Scheme 2).

The structures of the synthesized compounds were verified by ^{1}H, ^{13}C, and ^{2}D NMR experiments and elemental analyses and the structure of the Schiff base **5** was elucidated by X-ray diffraction studies (Table 1).

Broadly speaking, the structure of **5** is as expected (Fig. 3), although examination of the bond lengths (Table 2) suggests some delocalisation.

The molecules have an intramolecular hydrogen bond (N1⋯O1, 2.7253(11) Å) and the same nitrogen also makes an intermolecular hydrogen bond, linking pairs of molecules (N1⋯O1, 2.9980(12) Å under symmetry operation $-x + 1/2, y, -z + 1$). There is a reasonably convincing (aromatic)C—H⋯O hydrogen bond linking the molecules into chains (C2⋯O2, 3.5348(14) Å under symmetry operation $x-1/2, y + 1/2, z-1/2$). The result is a 2D hydrogen-bonded sheet lying parallel to the (1 0–1) plane; this appears to have large cavities (Fig. 4) but these "dimples" are filled by the next 2D layer (Table 3). There is no evidence of significant interaction between the sheets.

The ^{1}H, ^{13}C and 2D NMR spectra in DMSO-d_6 of the Schiff base **5** confirm the transfer of the hydrogen to the imine. The ^{1}H–^{1}H NOESY spectrum of the Schiff base **5** revealed the cross peaks between the *ortho* hydrogens of the phenyl group which appeared at 7.45 ppm with the hydrogen of the imine at 12.84 ppm and the CH_3 protons of the CH_3C = N group at 2.57 ppm.

Table 1
Crystal data and structure refinement for compound **5**.

Empirical formula	$C_{21}H_{18}N_2O_3$
Formula weight	346.37
Temperature/K	100(1)
Crystal system	monoclinic
Space group	I2/a
a/Å	15.08930(10)
b/Å	15.33770(10)
c/Å	14.55900(10)
α/°	90
β/°	92.2440(10)
γ/°	90
Volume/Å^3	3366.88(4)
Z	8
ρ_{calc} g/cm^3	1.367
μ/mm^{-1}	0.750
F(0 0 0)	1456.0
Crystal size/mm^3	0.165 × 0.055 × 0.04
Radiation	CuKα (λ = 1.54184)
2θ range for data collection/°	8.224–152.238
Index ranges	$-18 \le h \le 18, -19 \le k \le 19, -18 \le l \le 18$
Reflections collected	36,171
Independent reflections	3486 [R_{int} = 0.0177, R_{sigma} = 0.0073]
Data/restraints/parameters	3486/0/241
Goodness-of-fit on F^2	1.027
Final R indexes [I > =2σ (I)]	R1 = 0.0332, wR2 = 0.0846
Final R indexes [all data]	R1 = 0.0343, wR2 = 0.0855
Largest diff. peak/hole/e Å^{-3}	0.24/−0.22
CCDC	1975919

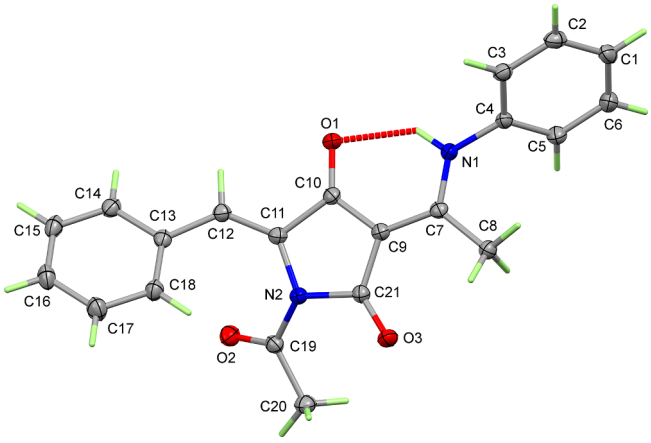


Fig. 3. Perspective view of **5** showing 50% probability ellipsoids for the non-hydrogen atoms. Hydrogen bond shown as a dashed red line.

The protons of the phenyl group, *ortho* and *para* to electron-donating and electron-withdrawing R^1 substituents of compounds **3a-f** and **4g** show distinct upfield and downfield shifts in relation to the parent unsubstituted compound **A**. The chemical shift difference of the *ortho* resonance signals appears up to 0.90 ppm, while the *para* resonance signals chemical shift difference are up to 0.54 ppm, according to the substituent effect. Thus, the ^{1}H signals of the *ortho* and *para* positions when R^1 = CH_3 are found at 7.13 ppm and 7.11 ppm respectively whereas in the case of R^1 = NO_2 are found at 8.12 ppm and 7.42 ppm. Changes of ^{13}C NMR chemical shifts follow the same trend as the *ortho* ^{13}C signal is deshielded by 2.9 ppm (signal at 133.3 ppm) in the case of NO_2 substitution or shielded in the case of CH_3 substitution (signal at 128.2 ppm).

The infrared (FT-IR) spectrum of the Schiff base **5** exhibits two broad absorption bands at 3436 cm^{-1} and 3187 cm^{-1} that correspond to the proton of the enol and the nitrogen of the imine in accordance with the X-ray crystallographic data. The broadness of the absorptions indicate the presence of the intramolecular hydrogen bonding and the migration of the proton. The strong absorption band at 1574 cm^{-1} is attributed to the partial double bond character of the iminic C=N due to delocalization.

In order to investigate the potential anti-MRSA activity of the newly synthesized series of compounds **3a-f**, **4a-g** and the Schiff base analogue **5**, single point concentration (32 μg/mL; n = 2) broth antimicrobial screening was undertaken (Fig. 5). The preliminary results were evaluated in comparison to the *N*-acetyl-3-acetyl-5-benzylidene tetramic acid **A**, tested in our recently published work.[24a]

First, a series of tetramic acids incorporating the pharmacologically important F, OMe, Me, Cl, CF_3 and NO_2, keeping at the same time the 3-acetyl group of the initial compound A was evaluated. As a general trend, these compounds showed low activity against MRSA. Compound **3e** proved to be partially active at 32 μg/mL with mean %inhibition of 61.7, compared to growth and media controls. From the rest of the series only **3c** and **3f** were relatively more active than **A** (mean %inhibition 45.1 and 38.6 respectively in relation to 25.5 of A).

Next, a series of tetramic acids bearing a long aliphatic alkanoyl chain or a benzoyl group at C-3 of the ring was evaluated to test our hypothesis for enhancement of *S. aureus* inhibition with the

Table 2
Selected bond lengths (A) for **5**.

N1–C7	1.3265 (13)	C11–C12	1.3411 (15)
N1–C4	1.4301 (12)	O1–C10	1.2418 (12)
C7–C9	1.4040 (14)	O3–C21	1.2139 (13)
C9–C10	1.4287 (14)	O2–C19	1.2159 (13)
C10–C11	1.4917 (14)	N2–C11	1.4270 (13)

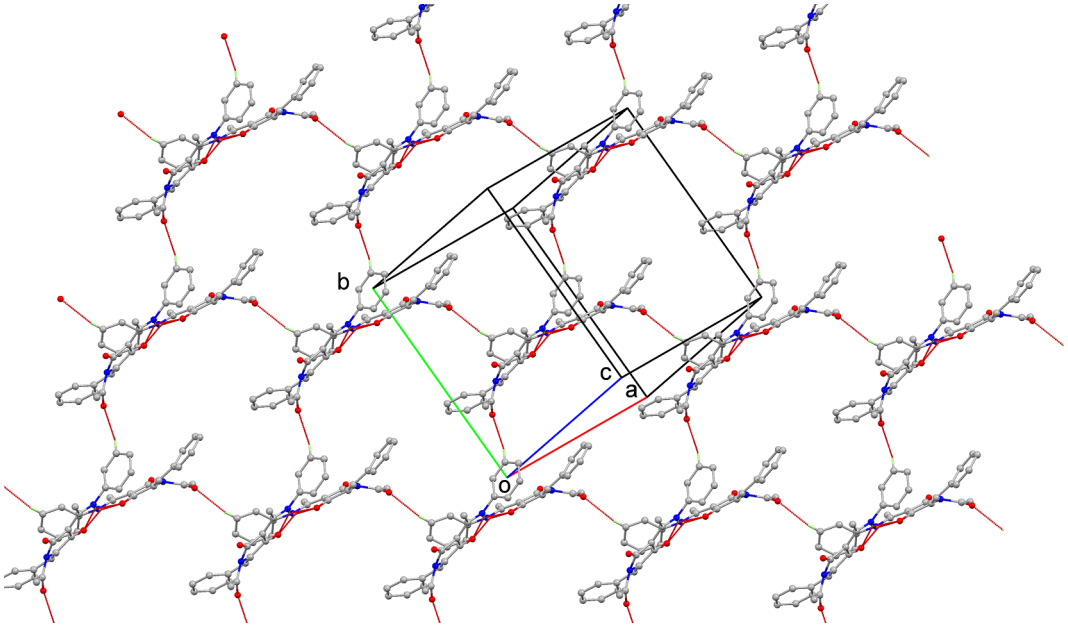


Fig. 4. H-bonded layer structure viewed perpendicular to the 1 0–1 plane.

Table 3
Hydrogen-bond geometry (Å, °) for **5**.

D–H···*A*	*D*–H	H···*A*	*D*···*A*	*D*–H···*A*
N1–H1···O1	0.906 (17)	1.966 (16)	2.7253 (11)	140.3 (14)
N1–H1···O1^{i}	0.906 (17)	2.368 (16)	2.9980 (12)	126.6 (13)
C2–H2···O2^{ii}	0.95	2.61	3.5348 (14)	164

Symmetry codes: (i) $-x + 1/2, y, -z + 1$; (ii) $x-1/2, y + 1/2, z-1/2$.

incorporation of a lipophilic hydrocarbon group. All of the compounds (**4a-g**) from this series were found active with 73.1–99.2% mean inhibition, excluding the benzoyl derivative which was found almost as active as **A** (22.9% mean inhibition).

Finally, with the aim of determining the importance of the carbonyl presence at the C-3 acyl group, a nitrogen analogue was prepared in the form of a Schiff base (**5**), derived from **A** and aniline. Analogue **5** was found totally inactive verifying our hypothesis based on the literature data[15] for the significance of a 3-acetyl or 3-acyl group on tetramic acid biological activities.

To confirm the activity of compounds **4a-e** and **4 g** an 8-point dose response (0.25 μg/mL – 32 μg/mL) antimicrobial activity assay was performed in duplicate, and activity expressed as minimum inhibitory concentrations (MIC). These assays were performed on both the target resulting from the primary screening results, MRSA ATCC 43300 as well as the full panel of bacteria/fungi for a uniform directly comparable data set for this lead candidate compound. Appropriate positive inhibitory antibiotic controls were included for each strain and data provided in the Supplementary information. The minimum inhibitory concentration (MIC) was determined following the CLSI guidelines, identifying the lowest concentration at which full inhibition of the bacteria or fungi was observed. Full inhibition of growth has been defined at ≤20% growth (or > 80% inhibition).

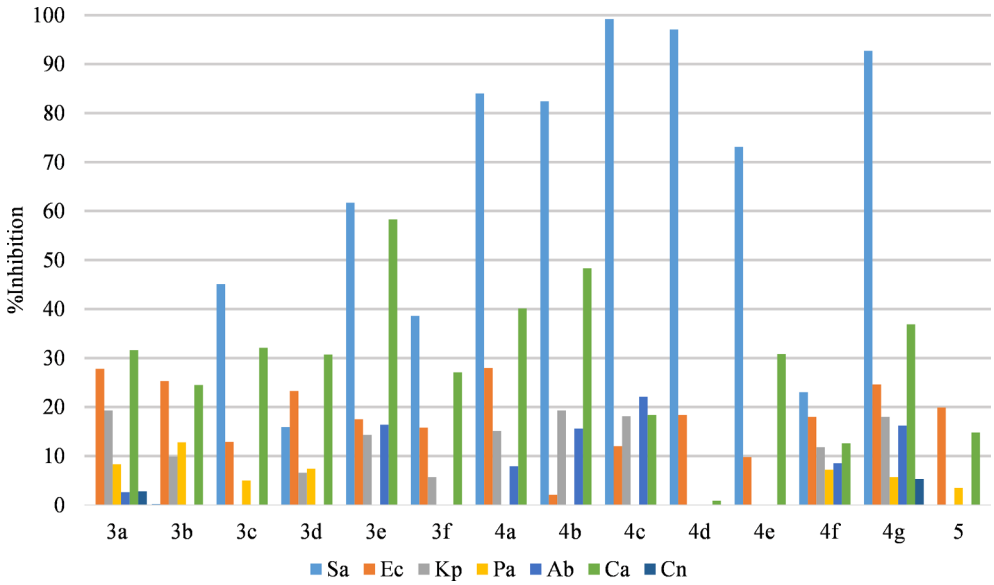


Fig. 5. Percentage inhibition at single concentration of 32 μg/mL of all preliminary screened compounds. Mean values. (Sa: *S. aureus*, Ec: *E. coli*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*, Ab: *A. baumanii*, Ca: *C. albicans*, Cn: *C. neoformans*).

Table 4
Antimicrobial activity against *S. aureus* (ATCC 43300, MRSA) as Minimum Inhibitory Concentration (MIC), cytotoxicity (CC_{50}) and hemolytic activity (HC_{10}) of compounds **4a-4e**, **4g** and **3e**. Log*P* values (miLog*P*) were calculated using Molinspiration platform (v2018.10).

Compound	MIC (μg/mL) MRSA	CC_{50} (μg/mL)	HC_{10} (μg/mL)	log*P*
4a (R^1 = H, R^2=$(CH_2)_2CH_3$)	32	> 32	> 32	2.94
4b (R^1 = H, R^2=$(CH_2)_6CH_3$)	4	> 32	> 32	4.96
4c (R^1 = H, R^2=$(CH_2)_8CH_3$)	4	> 32	20.4	5.97
4d (R^1 = H, R^2=$(CH_2)_{12}CH_3$)	4	> 32	> 32	7.99
4e (R^1 = H, R^2=$(CH_2)_{14}CH_3$)	4	> 32	> 32	8.69
4 g (R^1 = OMe, R^2=$(CH_2)_2CH_3$)	32	> 32	> 32	3.00
3e (R^1 = CF_3, R^2 = CH_3)	> 32	> 32	> 32	2.77
A (R^1 = H, R^2 = CH_3)[24a]	> 32	> 32	> 32	1.88

Compounds **4a** and **4g** demonstrated moderate inhibitory activity against MRSA (32 μg/mL) confirming thus the preliminary results that showed > 3-fold increase of the %inhibition at 32 μg/mL. In principle, these results indicate that irrespective of the substitution at the benzylidene group, the introduction of a more lipophilic acyl group at C-3 has substantial effect on MRSA inhibition.

Confirming our expectations, compounds **4b-e** exhibited excellent equal inhibitory activity against the target bacterium (4 μg/mL) (Table 4). The length of the hydrocarbon acyl chain plays a key role in the effectiveness of the anti-MRSA activity. The inhibition values at the MIC suggest the following order of activity **4b** > **4c** = **4d** < **4e** (86.6%, 94.4%, 94.4%, 81.7% mean values respectively). Therefore, for a further future optimization, compounds with 8 to 14 carbon chain (including the carbonyl carbon) should be employed.

Finally, compound **3e** was tested in the same way, but the MIC was determined > 32 μg/mL, only confirming the partial activity (60–80% inhibition value) at this concentration.

Higher log*P* values are related to better anti-MRSA activities (Table 4) in this series of tetramic acid compounds.

The aforementioned results came as no surprise to us, given the incorporation of a lipophilic group as we noticed in the – limited – relevant literature.[15] However, the determined MICs are impressive since the tested compounds bear a new skeleton and not an optimized known and studied one.

To investigate the specificity of compounds **4a-e, 4g** and **3e** as a potential antibacterial agents, they were assayed against a mammalian cell line and human red blood cells (RBCs) to determine general cell toxicity.

The cytotoxicity of all the aforementioned compounds as a growth inhibitor of human embryonic kidney cells (HEK293) was also determined by a dose response (0.25 μg/mL–32 μg/mL) cell viability assay, and found non-toxic up to the highest tested concentration of 32 μg/mL, i.e. CC_{50} deemed > 32 μg/mL.

None of the tested active compounds showed hemolytic activity at 32 μg/mL, except **4c** which exhibited HC_{10} (10% hemolytic concentration) of 20.4 μg/mL.

Therefore, compounds **4b**, **4d** and **4e** can be considered as the most promising compounds for further studies against MRSA and other Gram positive bacteria, given their high inhibitory activity and low toxicity (> 8-fold specificity of antibacterial activity over general cell toxicity). Taking into account the compliance with Lipinski's rule of five, among them, **4b** seems to be the best candidate, since it has molecular weight = 355.43 Da, log*P* = 4.96, number of hydrogen acceptors = 5 and number of hydrogen bond donors = 1. According to this rule,[30] most "drug-like" molecules have molecular weight ≤ 500 Da, log*P* ≤ 5, number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5. Molecules violating one or more of these rules may have a problem with bioavailability.

In conclusion, a series of 14 functionalized N-acetyl-5-arylidenetetramic acids were synthesized to investigate the scope of our methodology regarding substituents on the benzylidene group and on C-3 of the tetramic acid nucleus and their effects on antimicrobial activity. Among the compounds screened, compounds **4a-e** and **4g** showed interesting anti-MRSA activity. Three of the four most active compounds (**4b**, **4d**, **4e**) with MIC: 4 μg/mL against MRSA ATCC 43300 strain were found non-hemolytic and non-toxic against human embryonic kidney cells (HEK293) within the tested concentration range.

The structure-activity relationship (SAR) studies indicate that the compounds with 8–16 carbon-chained acyl groups at C-3 of the ring show remarkable activities over the less lipophilic groups. The variations of substituents at the *para* position of the benzylidene group at C-5 have no or little effect on the tested pathogens. The incorporation of an iminic group in the form of a Schiff base instead of the carbonyl group showed that the latter is essential for the anti-MRSA activity, or alternatively, that the existence of the imine hydrogen bond is canceling the above inhibition activity.

Compounds **4b**, **4d** and **4e** are the most promising ones for further modifications in order to develop an effective lead compound. The developed one-pot method via azlactones combined with the potential of tetramic acids for modifications encourage us to direct our efforts toward an expanded structure-activity relationship study.

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.

Acknowledgements

Antimicrobial and mammalian cell toxicity screening was performed by CO-ADD (The Community for Open Antimicrobial Drug Discovery),[31] funded by the Wellcome Trust (UK) and The University of Queensland (Australia).

DM would like to thank the Greek State Scholarship Foundation and SIEMENS for an IKY Fellowship of Excellence for Postdoctoral Studies in Greece – SIEMENS Program.

VMcK is grateful for funding from the Carlsberg Foundation (grant CF15-0675) for the X-ray diffractometer in SDU.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127107. These data include MOL files and InChiKeys of the most important compounds described in this article.

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27. Synthesis of tetramic acids and analytical data for novel compounds: The synthetic procedure was adapted from our previously published protocol with proper modifications.[24a] To a suspension of NaH (60% in mineral oil, 40 mg, 1.00 mmol) in dry THF at 0°C, the active methylene compound (β-ketoesters, 1.00 mmol) was added dropwise or portionwise over 10 minutes. After stirring for 1h, the appropriate oxazolone (0.50 mmol) was added at 0°C and the mixture was left under vigorous stirring at room temperature overnight. The resulting mixture was concentrated at < 40°C and the thick slurry was diluted with water (1.0 mL). The suspension was washed carefully with small quantity of Et_2O for **3a-f** and **4g** or petroleum ether for **4a-f** and acidified with 10% hydrochloric acid in an ice bath. The precipitated solids **3a-f** and **4g** were filtered, washed with water (x2) and petroleum ether (x2) and recrystallized from methanol or ethanol to yield off-white to bright yellow crystals. For **4a-f**, the aqueous phase was extracted with DCM (x3), dried with Na_2SO_4 and the organic solvent was evaporated to give the crude products, which were purified either by flash chromatography using gradient elution petroleum ether:ethyl acetate/dichloromethane:methanol or preparative TLC. The reaction completion was monitored by TLC using UV light and $FeCl_3$ stain. *N*-acetyl-3-acetyl-5-(4-methoxybenzylidene)-tetramic acid (**3a**): Bright yellow, Yield: 137mg (91%); Mp: 123-124°C (EtOH); R_f: 0.26 (Petroleum ether: Ethyl acetate 9:1); ^{1}H NMR (500MHz, DMSO-d_6) δ: 2.44 (s, 3H, CH_3CON), 2.51 (s, 3H, CH_3COC-3), 3.78 (s, 3H, CH_3OPh), 6.90 (d, 2H, J = 8.6 Hz, Ph), 6.93 (s, 1H, CH-Ph), 7.21 (d, 2H, J = 8.6 Hz, Ph); ^{13}C NMR (62.5 MHz, DMSO-d_6) δ: 26.1 (CH_3CON), 26.3 (CH_3COC-3), 55.2 (CH_3OPh), 101.5 (C-3), 113.3 (PhCH=), 118.0, 127.1, 127.4, 132.4, (Ph), 132.6 (C-5), 159.5 (C-2), 167.7 (CH_3CON), 179.5 (CH_3COC-3), 191.9 (C-4); Anal. Calcd for $C_{16}H_{15}NO_5$: C, 63.78, H, 5.02, N, 4.65. Found C, 63.99, H, 4.87, N, 4.58. N-acetyl-3-acetyl-5-(4-methylbenzylidene)-tetramic acid (3b): Yellow, Yield: 97mg (68%); Mp: 125-126°C; Rf: 0.42 (Petroleum ether: Ethyl acetate: Acetic acid 3:7:0.1); 1H NMR (500MHz, DMSO-d6) δ: 2.30 (s, 3H, CH_3Ph), 2.40 (s, 3H, CH_3CON), 2.48 (s, 3H, CH_3COC-3), 3.78 (s, 3H, CH_3OPh), 6.85 (s, 1H, CH-Ph), 7.11 (d, 2H, J = 7.1 Hz, Ph), 7.13 (d, 2H, J = 7.1 Hz, Ph); ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 20.9 (CH_3Ph), 26.3 (CH_3CON), 26.4 (CH_3COC-3), 101.1 (C-3), 116.7 (PhCH=), 128.2 (Ph), 128.3 (Ph), 130.2 (Ph), 130.4 (Ph), 131.5 (C-5), 167.7 (C-2), 168.2 (CH_3CON), 179.7 (CH_3COC-3), 191.3 (C-4); Anal. Calcd for C16H15NO4: C, 67.36, H, 5.30, N, 4.91. Found C, 67.52, H, 5.30, N, 4.77. N-acetyl-3-acetyl-5-(4-chlorobenzylidene)-tetramic acid (3c): Yellow, Yield: 135mg (88%); Mp: 116-117°C; Rf: 0.34 (Petroleum ether: Ethyl acetate: Acetic acid 3:7:0.1); 1H NMR (500MHz, DMSO-d6) δ: 2.42 (s, 3H, CH_3CON), 2.48 (s, 3H, CH_3COC-3), 6.87 (s, 1H, CH-Ph), 7.22 (d, 2H, J = 8.0 Hz, Ph), 7.36 (d, 2H, J = 8.0 Hz, Ph); 13C NMR (62.5 MHz, DMSO-d6) δ: 26.3 (CH_3CON), 26.4 (CH_3COC-3), 101.1 (C-3), 115.0 (PhCH=), 127.7, 130.5, 131.8, 132.4 (Ph), 134.2 (C-5), 167.8 (C-2), 168.2 (CH_3CON), 179.6 (CH_3COC-3), 191.1 (C-4); Anal. Calcd for $C_{15}H_{12}ClNO_4$: C, 58.93, H, 3.96, N, 4.58. Found C, 58.80, H, 5.69, N, 4.78. N-acetyl-3-acetyl-5-(4-fluorobenzylidene)-tetramic acid (3d): Off-white, Yield: 113 mg (78%); Mp: 125-126°C; Rf: 0.33 (Petroleum ether: Ethyl acetate: Acetic acid 3:7:0.1); 1H NMR (500 MHz, DMSO-d6) δ: 2.41 (s, 3H, CH_3CON), 2.48 (s, 3H, CH_3COC-3), 6.87 (s, 1H, CH-Ph), 7.14 (t, 2H, J = 7.2 Hz, Ph), 7.26 (t, 2H, J = 7.2 Hz, Ph); ^{13}C NMR (125 MHz, DMSO-d6) δ: 26.4 (CH_3CON), 26.6 (CH_3COC-3), 100.9 (C-3), 114.4 (d, Ph, J_{CF} = 21.6 Hz), 114.7 (d, Ph, J_{CF} = 21.6 Hz), 115.03 (PhCH=), 129.9 (Ph), 131.8 (d, Ph, J_{CF} = 3.1 Hz), 131.9 (d, Ph, J_{CF} = 3.1 Hz), 132.2 (d, Ph, J_{CF} = 8.3 Hz), 132.3 (d, Ph, J_{CF} = 8.3 Hz), 159.5 (C-5), 163.4 (C-2), 167.7 (CH_3CON), 168.4, 179.8 (CH_3COC-3), 191.1 (C-4).; Anal. Calcd for $C_{15}H_{12}FNO_4$: C, 62.28, H, 4.18, N, 4.84. Found C, 62.09, H, 4.12, N, 5.02. N-acetyl-3-acetyl-5-(4-trifluoromethylbenzylidene)-tetramic acid (3e): White, Yield: 112mg (66%); Mp: 110-111°C; Rf: 0.21 (Petroleum ether: Ethyl acetate: Acetic acid 3:7:0.1); 1H NMR (500 MHz, DMSO-d6) δ: 2.33 (s, 3H, CH_3CON), 2.46 (s, 3H, CH_3COC-3), 6.79 (s, 1H, CH-Ph), 7.35 (d, 2H, J = 8.4 Hz, Ph), 7.62 (d, 2H, J = 8.4 Hz, Ph); ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 27.75 (CH_3), 28.42 (CH^3), 100.18 (C-3), 111.99, 121.9, 131.5, 137.2 (q, CF_3, J_{CF}=272.9 Hz), 124.7 (q, Ph, J_{CF}= 3.4 Hz), 130.59 (q, C-CF_3, J_{CF} = 33.2 Hz), 131.04 (C-5), 140.9 (Ph), 168.3 (C=O), 170.88 (CH_3-C=O-N), 180.46 (C-OH), 190.80 (CH_3-C=O); Anal. Calcd for $C_{16}H_{12}F_3NO_4$: C, 56.64, H, 3.57, N, 4.13. Found C, 56.88, H, 3.66, N, 3.98. N-acetyl-3-acetyl-5-(4-nitrobenzylidene)-tetramic acid (3f): Dark orange, Yield: 95 mg (60%); Mp: 116-117°C; Rf: 0.36 (dichloromethane: methanol: Acetic acid 95:5:1); 1H NMR (500 MHz, DMSO-d6) δ: 2.38 (s, 3H, CH_3CON), 2.48 (s, 3H, CH_3COC-3), 6.87 (s, 1H, CH-Ph), 7.42 (t, 2H, J = 8.4 Hz, Ph), 8.12 (t, 2H, J = 8.4 Hz, Ph); ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 26.3 (CH_3CON), 26.9 (CH_3COC-3), 100.3 (C-3), 111.9 (PhCH=), 122.6, 130.6 (Ph), 133.4 (C-5), 143.1, 145.8 (Ph), 167.9 (C-2), 168.6 (CH_3CON), 179.6 (CH_3COC-3), 190.5 (C-4); Anal. Calcd for $C_{15}H_{12}N_2O_6$: C, 56.97, H, 3.82, N, 8.86. Found C, 56.60, H, 3.72, N, 9.04. N-acetyl-5-benzylidene-3-octanoyltetramic acid (4b): White solid, Yield: 99 mg (56%); Mp: 180 °C (dec.); Rf: 0.40 (petroleum ether/ethyl acetate 1:1); 1H NMR (500 MHz, DMSO-d6) δ: 0.84 (t, 3H, $CH_3CH_2CH_2$), 1.24 (br, 8H, $CH_3(CH_2)_4$), 1.47 (br, 2H CH_2CH_2COC-3), 2.45 (s, 3H, CH_3CON), 2.73 (br, 2H, CH_2CH_2COC-3), 6.68 (s, 1H, CH-Ph), 7.13-7.28 (br m, 5H, Ph); 13C NMR (62.5 MHz, DMSO-d6) δ: 13.9 [$CH_3(CH_2)_6$], 22.1, 24.7 [$(CH_2)_6$], 26.4 (CH_3CON), 27.8, 28.7, 29.0, 31.2 [$(CH_2)_6$], 98.1 (C-3), 118.9 (PhCH=), 127.0, 127.1, 127.3, 127.5, 129.7 (Ph), 130.4 (C-5), 167.8 (C-2), 170.8 (CH_3CON), 179.3 (CH_3COC-3), 195.8 (C-4); Anal. Calcd for $C_{21}H_{25}NO_4$: C, 70.96, H, 7.09, N, 3.94. Found C, 71.22, H, 6.88, N, 3.82. N-acetyl-5-benzylidene-3-decanoyltetramic acid (4c): Off-white solid, Yield: 105 mg (55%); Mp: 166 °C (dec.); Rf: 0.46 (petroleum ether/ethyl acetate 1:1); 1H NMR (500 MHz, DMSO-d6) δ: 0.84 (br s, 3H, $CH_3CH_2CH_2$), 1.22 (br, 12H, $CH_3(CH_2)_6$), 1.46 (br, 2H CH_2CH_2COC-3), 2.46 (s, 3H, CH_3CON), 2.72 (br, 2H CH_2CH_2COC-3), 6.67 (s, 1H, CH-Ph), 7.14-7.26 (br m, 5H, Ph), ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 13.9 [$CH_3(CH_2)_8$], 22.1, 24.5, 24.7 [$(CH_2)_8$], 26.7 (CH_3CON), 28.5, 28.7, 28.9, 29.0, 31.3, 33.7 [$(CH_2)_8$], 98.8 (C-3), 118.6 (PhCH=), 127.1, 127.4, 128.1, 128.4 (Ph), 129.7 (C-5), 167.8 (C-2), 170.8 (CH_3CON), 178.7 (CH_3COC-3), 194.1 (C-4); Anal. Calcd for $C_{23}H_{29}NO_4$: C, 72.04, H, 7.62, N, 3.65. Found C, 71.46, H, 7.42, N, 3.91. N-acetyl-5-benzylidene-3-tetradecanoyltetramic acid (4d): Off-white solid, Yield: 110 mg (50%); Mp: 134°C (dec.); Rf: 0.19 (petroleum ether/ethyl acetate 8:2); 1H NMR (500 MHz, DMSO-d6) δ: 0.84 (br t, 3H, $CH_3CH_2CH_2$), 1.23 (br s, 20H, CH3(CH2)10CH2CH2), 1.46 (br, 2H CH_2CH_2COC-3), 2.50 (s, 3H, CH_3CON), 2.73 (br, 2H CH_2CH_2COC-3), 6.65 (s, 1H, CH-Ph), 7.13-7.30 (br m, 5H, Ph); ^{13}C NMR (62.5 MHz, DMSO-d6) 13.9 [$CH_3(CH_2)_{12}$], 22.1, 24.4, 24.7[$(CH_2)_{12}$], 26.6 (CH_3CON), 28.7, 29.0, 29.1, 31.3 [$(CH_2)_{12}$], 98.3 (C-3), 115.7 (PhCH=), 127.1, 127.4, 127.6, 130.0 (Ph) 129.8 (C-5), 130.3 (Ph), 167.9 (C-2), 169.2 (CH_3CON), 182.9 (CH_3COC-3), 194.6 (C-4); Anal. Calcd for $C_{27}H_{37}NO_4$: C, 73.77, H, 8.48, N, 3.15. Found C, 74.16, H, 8.70, N, 2.97. N-acetyl-3-butyryl-5-(4-methoxybenzylidene)-tetramic acid (4g): Bright yellow, Yield: 109mg (66%); Mp: 89-91°C (EtOH); Rf: 0.42 (Petroleum ether: Ethyl acetate 9:1); 1H NMR (500 MHz, DMSO-d6) δ: 0.92 (t, 3H, J = 7.4 Hz, $CH_3CH_2CH_2$), 1.59 (sext, 2H, J = 7.4 Hz, $CH_3CH_2CH_2$), 2.83 (t, 2H, J = 7.4 Hz, $CH_3CH_2CH_2$), 2.50 (s, 3H, CH_3CON), 3.79 (s, 3H, CH_3OPh), 6.90 (d, 2H, J = 8.4 Hz, Ph), 6.94 (s, 1H, CH-Ph), 7.21 (d, 2H, J = 8.4 Hz, Ph); ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 13.8 ($CH_3CH_2CH_2$), 17.7 ($CH_3CH_2CH_2$), 26.3 (CH_3CON), 26.4 ($CH_3CH_2CH_2$), 55.2 (OCH_3), 101.0 (C-3), 113.3 (PhCH=), 117.9, 127.2, 127.4, 132.3 (Ph), 132.6 (C-5), 159.5 (C-2), 167.7 (CH_3CON), 179.2 (CH_3COC-3), 195.3 (C-4); Anal. Calcd for $C_{18}H_{19}NO_5$: C, 65.64, H, 5.82, N, 4.25. Found C, 65.45, H, 5.77, N, 4.39.

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29. Synthesis and analytical data for 5: N-Acetyl-3-acetyl-5-benzylidene tetramic acid A 24a (136mg, 0.50mmol) was dissolved in 7mL of dry MeOH under reflux. Methanolic solution (1mL) of aniline (93mg, 1.00mmol) was added dropwise. The brown mixture was stirred under reflux for 2h when a light yellow solid precipitated. The solid was filtered and washed with 2 portions of ethanol (2mL each). The filtrate was evaporated under vacuum and the residual solid was washed well with ice-cold methanol. The yellow solid was recrystallized with ethanol forming light yellow needles. The completion of the reaction was monitored by TLC using permanganate stain. N-acetyl-3-(1-(phenylamino)ethylidene)-5-benzylidenetetramic acid (5): Light yellow needles. Yield: 86 mg (48%); Mp: 163-164 ˚C (EtOH) ; Rf: 0.65 (Petroleum ether: Ethyl acetate 1:1); nmax/cm-1 3435 (m), 3187 (m), 1613 (s), 1574 (s), 1625 (s), 1385 (m), 1240 (m); 1H NMR (500 MHz, DMSO-d6) δ: 2.55 (s, 3H, CH_3-C=N), 2.57 (s, 3H, CH_3CO), 6.94 (s, 1H, CH-Ph), 7.25 (d, 2H, J = 7.6 Hz, Ph), 7.29 (d, 1H, J = 7.4 Hz, Ph), 7.34 (t, 2H, J = 7.3 Hz, Ph), 7.45 (d, 2H, J = 7.7 Hz, Ph), 7.53 (t, 2H, J = 7.3 Hz, Ph) 12.85 (s, 1H, NH); ^{13}C NMR (62.5 MHz, DMSO-d6) δ: 16.0 (CH_3C=N), 26.5 (CH_3CO), 65.9 (C-3), 116.2 (PhCH=), 125.6, 127.7, 128.2, 129.5, 129.8, 130.0, 134.8 (Ph), 135.5 (C-5), 167.8 (CH_3CON), 169.8 (C-2), 184.0 (C-4); Anal. Calcd for C21H18N2O3: C, 72.82, H, 5.24, N, 8.09. Found C, 73.05, H, 5.49, N, 7.87.
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