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Synthesis and molecular docking studies of xanthone attached amino acids as potential antimicrobial and anti-inflammatory agents†

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A series of novel xanthone conjugated amino acids were synthesised and characterised by analytical and spectroscopic methods. All the synthesized analogues (**2**–**23**) were screened for their *in vitro* antimicrobial and anti-inflammatory activities. Compounds **7**, **8**, **9**, **12**, **18**, **19**, **20**, **21** and **23** showed excellent antimicrobial activities compared to antibacterial and antifungal reference drugs gentamicin and bavistin, respectively. Compounds **7**–**12** and **18**–**23** showed good anti-inflammatory activity compared to a standard drug, indomethacin. The preliminary structure–activity relationship revealed that tryptophan, tyrosine, phenylalanine, proline and cysteine conjugated compounds showed excellent antimicrobial and anti-inflammatory activities. This may be explained by the contribution of aromaticity and hydrophobicity of amino acids. Molecular docking studies were performed for all the synthesised compounds, among which compounds **20**, **21** and **23** showed the highest docking scores for antimicrobial activity while compounds **9**, **20** and **22** showed the highest docking scores for anti-inflammatory activity. Different amino acids conjugated xanthone derivatives were synthesized and evaluated for their *in vitro* biological activities. The conjugation was found to play a major role in improving the biological activities of those compounds.

1. Introduction

Infectious diseases are one of the major causes of death in the world and the development of novel antimicrobial agents without resistance is crucial. The increase of infectious diseases is a problem to the global population.[1] Bacterial infections, especially today with the emergence of multidrug-resistant bacteria caused by the misuse of antibiotics, are becoming a serious problem. Moreover, traditional drugs used in clinics are exhibiting less effectiveness in the treatment of infections.[2] Various cationic antimicrobial peptides, which are effectors of natural resistance, should act at the cytoplasmic film prompting permeabilization and in the long run layer disturbance. However, there are several limitations for utilizing naturally-derived antimicrobial peptides (AMPs), particularly for the treatment of invasive infections. These limitations include host toxicity, degradation by proteases, extensive serum binding, loss of antimicrobial activity in the presence of a physiological concentration of salts, and a high cost of production due to their complex design. Therefore, new avenues need to be pursued in order to transform AMPs into novel therapeutic agents capable of being used clinically.[3] Accordingly, interaction of antimicrobial peptides with anionic phospholipids is considered as one of the upcoming existing gap present in the bacteriostatic therapy to killing of microbes.[4] Therefore, it is necessary to develop new synthetic alternative compounds with greater biocidal efficacy with a clear detailed mechanism to decrease the problem of microbial resistance.

Xanthones are a class of heterocyclic compounds bearing oxygen and widely distributed in nature.[5] They occur in two main plant families, *Guttiferae* and *Gentianaceae*, and are also found in families of fungi and lichens.[6,7] Their structural scaffold and pharmacological properties have encouraged researchers to isolate these compounds from natural products or synthesise them as novel drug candidates. Xanthones have fascinated the field of medicinal chemistry in the last two decades and it is well evident in the literature. The recent literature reveals that xanthone is the parental moiety in naturally occurring and synthetic xanthone derivatives, which exhibits

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several pharmacological activities.[8–10] Xanthone is a key intermediate owing to its various biological activities, used primarily as an anticancer,[11] antimalarial,[12] antimicrobial,[13] anti-HIV,[14] anticonvulsant,[15] anticholinesterase,[16] antioxidant,[17] and anti-inflammatory[18] agent and as an inhibitor of several enzymes like α-glycosidase,[19] topoisomerase,[20] protein kinase,[21] aromatase[22] and so on.

The therapeutic applications of amino acids have received remarkable attention in renal failure, respiratory, cardiology, physiology, and neurological disorders and congenital defects. In recent years, various drug–amino acid conjugates were reported.[23] In addition to this, amino acid based drugs have adequate bioavailability, low toxicity, good permeability and pharmacokinetic properties.[24]

Our previous work aimed towards the development of new heterocycles as therapeutic agents;[25–30] herein we reported the synthesis of xanthone conjugated amino acids and their antimicrobial and anti-inflammatory activities. In addition, in this work, we have also conducted molecular docking studies of the compounds to correlate them with their antimicrobial and anti-inflammatory activities.

2. Results and discussion

2.1 Chemistry

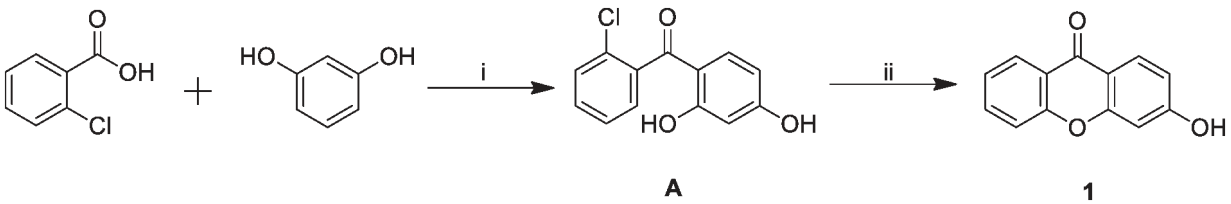
The desired compounds were synthesised according to a modified method[31] and the synthetic steps are illustrated in Schemes 1 and 2. 2-Chlorobenzoic acid and resorcinol were treated with anhydrous zinc chloride at 120 °C to give 2-chlorophenyl-(2,4-dihydroxyphenyl) methanone and cyclized with DMSO and NaOH at 120 °C to give the scaffold 3-hydroxy xanthone. This 3-hydroxy xanthone was conjugated with different Boc protected amino acids using HBTu as a coupling agent and TEA as a base. The Boc group was further cleaved by using HCl dioxan to give xanthone conjugated amino acids. All the derivatives were obtained in high yield. The structures of all the newly synthesised compounds and their intermediates were confirmed by ^{1}HNMR, ^{13}CNMR and mass spectral analysis. The formation of 2-chlorophenyl-(2,4-dihydroxyphenyl)methanone was confirmed by the absence of a singlet at 12.34 δ for –COOH and the presence of 2 hydroxy proton peaks at 12.07 δ and 10.9 δ in the ^{1}HNMR spectrum. In the IR spectra, the bands at 3510 and 3568 cm^{-1} for OH groups indicated the conversion of 2-chlorobenzoic acid into 2-chlorophenyl-(2,4-dihydroxyphenyl)methanone. The formation of 3-hydroxy xanthone was confirmed by the absence of one OH proton at 12.17 δ in the ^{1}H NMR spectrum and 3510 cm^{-1} in IR spectra. Further, the amino acids conjugates were confirmed by the absence of an –OH group at 10.9 δ in the ^{1}H NMR spectrum and de-Boc products were confirmed by the absence of tertiary butyl at 1.35–1.37 δ in the ^{1}H NMR spectrum. In the IR spectrum, most of our starting materials also possess one to three carbonyl groups; thus IR analysis was performed both before and after Boc protection for sharp comparison. In the IR spectra analysis, the presence of the Boc carbonyl group showed an additional absorbance peak in the range of 1660–1743 cm^{-1}. After the deprotection of the Boc protecting group, one of the carbonyl group signals that appeared in the range of 1660–1743 cm^{-1} disappeared. All the chemical structures were confirmed by ^{1}H, ^{13}C NMR and mass spectral analysis (see the ESI†).

2.2 Biology

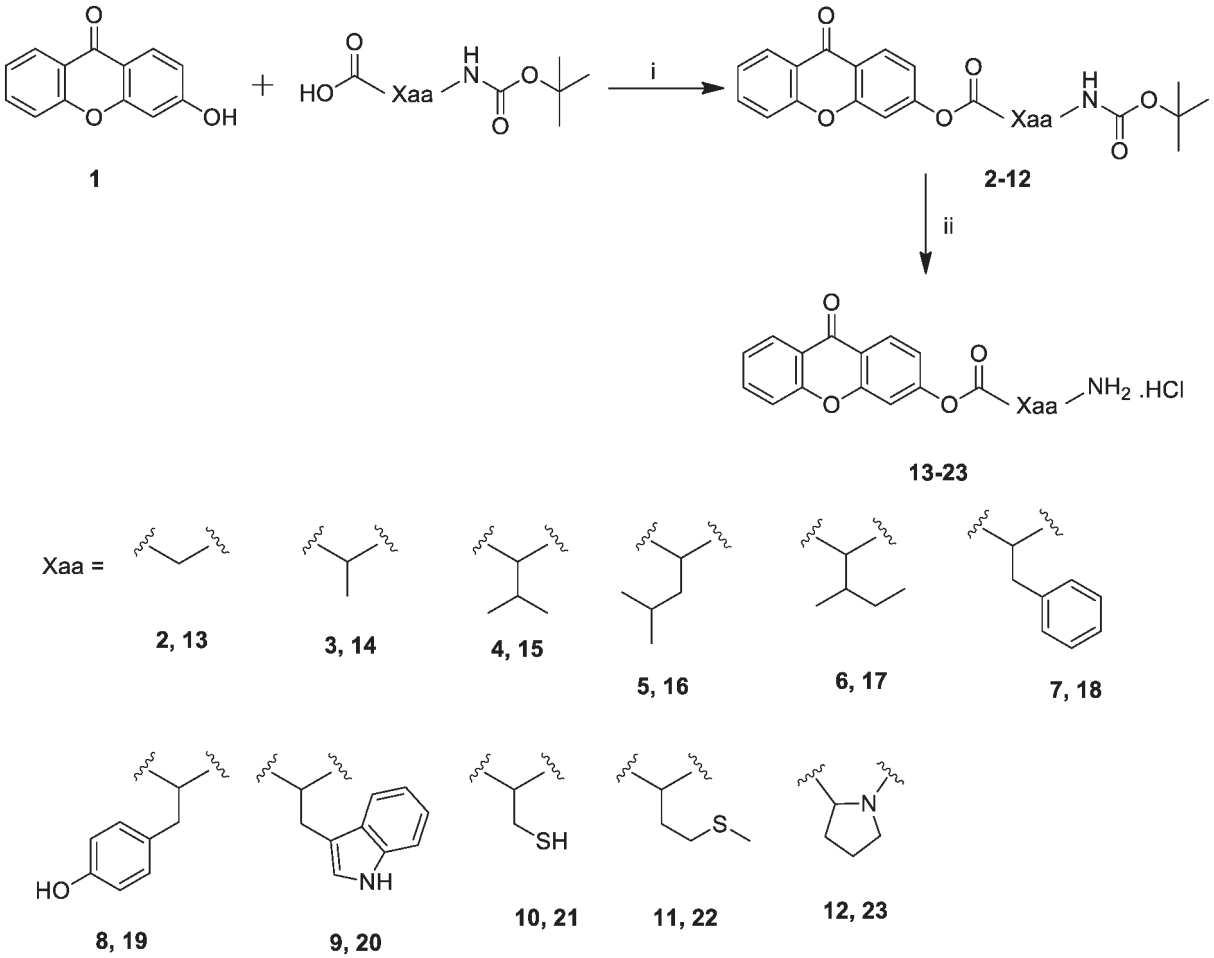
2.2.1 Antimicrobial activity. The synthesized xanthone conjugated amino acids (2–23) were evaluated for their *in vitro* antibacterial activities against two strains of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus substilis*) and two strains of Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) and their *in vitro* antifungal activities against *Aspergillus niger*, *Candida albicans* and *Fusarium oxysporum*, following the agar well diffusion method and a microdilution method.[32–35] The results of the antibacterial screening are summarized in Tables 1 and 3 and those of the antifungal screening are tabulated in Tables 2 and 3. All assays were performed in triplicate and the results were expressed as the mean of the diameter of the inhibition zone in millimeter (mm). Gentamicin and bavistin were used as standard drugs for antibacterial and antifungal activities, respectively.

A. K. Sah *et al.*,[36] reported the synthesis of a class of amino acid derived *N*-glycoconjugates and screened these synthetic compounds for their antibacterial activity. In this paper, we also observed that molecules containing amino acids with aromatic and hydrophobic side chains like tryptophan displayed good antibacterial activity. R. Dahiya *et al.*,[37] also reported that amino acid conjugated iodoquinazolinones and nitroimidazoles were found to have potent antimicrobial activity. Based on the above interesting results, we were inspired to introduce conjugation with amino acids of different natures to xanthone analogues and thereafter to evaluate their *in vitro* antimicrobial activity.

The results revealed that most of the synthesised compounds displayed major effects on the growth of the tested



Scheme 1 Synthesis of xanthone. Reagents and conditions: i = anhydrous zinc chloride, 120 °C; ii = NaOH, DMSO, 80 °C.



Scheme 2 Synthesis of xanthone conjugated amino acids. Reagents and conditions: i = DMF, HBTu, TEA, rt; ii = HCl dioxan, 45 min, rt.

bacterial and fungal strains. The antimicrobial activity results clearly demonstrated that conjugation definitely improved the activities of the parent molecule (**1**) which could serve our purpose. The structure–antimicrobial activity relationship of the compounds revealed that xanthone precursor **1** conjugated with phenyl alanine (**7**), tyrosine (**8**), tryptophan (**9**), cysteine (**10**) and proline (**12**) showed excellent antibacterial and antifungal activities compared to glycine (**2**), alanine (**3**), valine (**4**), leucine (**5**) and isoleucine (**6**) conjugates and respective standard drugs. This could be explained by the presence of aromatic amino acids such as tryptophan, tyrosine and phenylalanine which were considered to play an important role in antimicrobial effects by anchoring amino acids/peptides to the bacterial membranes.[38] Among the phenylalanine, tyrosine and tryptophan conjugates, tryptophan conjugates exhibited the most potent activity due to the presence of high aromaticity, hydrophobicity, light stability and a stabilised amphiphilic structure necessary for antimicrobial activity.[39,40] Further deboc products of these compounds (**18**, **19**, **20**, **21** and **23**) showed superior activity to their counterparts (**7**, **8**, **9** **10** and **12**), respectively. This fact revealed that the increases in the polarity of these compounds[41] would enhance their permeability and thereby inactivate the microbes.[42] Overall the antimicrobial activity was in the order Trp > Tyr > Phe > Pro > Cys. Interestingly, we also screened free amino acids tyrosine and tryptophan (without conjugation) which showed the least or less antimicrobial activities. These results proved that conjugation plays a major role in the antimicrobial activities.

Structure–activity relationships (SAR) have become a useful tool to study the molecular determinants leading to the biological activity of synthesised analogues towards pathogens. The antimicrobial activity depends on the side chain functional groups of amino acids and the hydrophobicity, aromaticity and amphipathicity of molecules.[43] The more hydrophobic and aromatic amino acids present in the molecules improve the biological activity of the compounds. All the twenty amino acids have different natures, side chain functionalities, properties and also different hydrophobic natures. Some of the amino acids are very sensitive whose properties depend on the pH of the media. Therefore, only simple and stable amino acids were used for conjugation. The amino acid histidine is very sensitive and unstable. On the other hand, acidic amino acids like aspartic acid and glutamic acid are less hydrophobic with simple side chain functionalities. In addition, amino acids like arginine, asparagine and

Table 1 Antibacterial activity of the synthesized compounds (**1–23**)

	Zone of inhibition[a] (mm)															
	Gram-positive bacteria								Gram-negative bacteria							
	Staphylococcus aureus				*Bacillus substilis*				*Escherichia coli*				*Klebsiella pneumonia*			
Entry	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}
01	04 ± 1	07 ± 2	11 ± 2	14 ± 0	NA	NA	06 ± 0	10 ± 1	03 ± 1	07 ± 4	11 ± 1	13 ± 2	NA	NA	NA	NA
02	07 ± 2	12 ± 3	16 ± 3	22 ± 1	06 ± 1	12 ± 3	16 ± 1	20 ± 1	07 ± 1	03 ± 3	16 ± 1	21 ± 1	16 ± 1	12 ± 2	17 ± 1	23 ± 3
03	06 ± 2	10 ± 3	14 ± 3	19 ± 3	08 ± 1	14 ± 3	19 ± 3	22 ± 2	17 ± 2	14 ± 3	20 ± 4	23 ± 1	06 ± 1	13 ± 4	19 ± 1	22 ± 1
04	10 ± 1	13 ± 2	17 ± 3	22 ± 1	07 ± 2	13 ± 4	19 ± 1	22 ± 0	08 ± 2	13 ± 1	18 ± 1	20 ± 1	05 ± 1	11 ± 2	19 ± 3	24 ± 1
05	07 ± 3	13 ± 1	17 ± 3	22 ± 4	11 ± 2	16 ± 1	20 ± 1	24 ± 1	04 ± 2	10 ± 2	17 ± 2	21 ± 0	08 ± 2	11 ± 3	14 ± 3	20 ± 1
06	04 ± 2	10 ± 3	15 ± 0	19 ± 0	07 ± 1	11 ± 2	14 ± 3	19 ± 0	08 ± 1	12 ± 3	19 ± 1	22 ± 4	07 ± 1	14 ± 3	20 ± 1	25 ± 1
07	18 ± 1	25 ± 1	31 ± 0	34 ± 2	17 ± 2	26 ± 1	33 ± 1	36 ± 0	16 ± 2	25 ± 2	32 ± 1	34 ± 1	20 ± 1	27 ± 2	33 ± 1	37 ± 1
08	20 ± 2	26 ± 1	32 ± 1	36 ± 1	20 ± 1	28 ± 1	35 ± 2	37 ± 3	20 ± 1	27 ± 1	33 ± 0	36 ± 1	18 ± 1	24 ± 1	30 ± 0	34 ± 1
09	22 ± 1	29 ± 1	35 ± 0	40 ± 2	23 ± 1	29 ± 1	33 ± 2	39 ± 0	24 ± 1	30 ± 4	37 ± 0	41 ± 2	22 ± 1	29 ± 2	36 ± 0	41 ± 0
10	20 ± 1	28 ± 2	32 ± 1	34 ± 2	20 ± 1	26 ± 2	29 ± 0	34 ± 1	18 ± 1	26 ± 2	30 ± 0	34 ± 0	18 ± 0	24 ± 1	30 ± 1	34 ± 2
11	15 ± 1	17 ± 3	22 ± 0	27 ± 1	13 ± 1	18 ± 0	24 ± 1	29 ± 1	16 ± 1	19 ± 1	25 ± 1	30 ± 0	16 ± 1	22 ± 1	26 ± 3	31 ± 0
12	17 ± 1	24 ± 3	29 ± 1	33 ± 2	15 ± 1	24 ± 2	31 ± 0	35 ± 2	17 ± 2	26 ± 2	31 ± 0	36 ± 1	13 ± 1	21 ± 2	27 ± 3	32 ± 1
13	10 ± 2	15 ± 0	19 ± 2	23 ± 2	10 ± 2	15 ± 0	21 ± 2	26 ± 1	12 ± 2	17 ± 1	21 ± 3	24 ± 0	17 ± 0	22 ± 1	27 ± 2	30 ± 1
14	09 ± 1	13 ± 3	19 ± 2	22 ± 1	11 ± 2	16 ± 0	19 ± 1	23 ± 1	15 ± 1	19 ± 2	22 ± 1	26 ± 0	08 ± 2	16 ± 2	23 ± 1	28 ± 2
15	14 ± 0	17 ± 3	21 ± 2	24 ± 0	10 ± 1	15 ± 3	20 ± 2	24 ± 0	11 ± 0	15 ± 3	19 ± 0	23 ± 2	16 ± 2	12 ± 1	21 ± 0	27 ± 2
16	09 ± 1	12 ± 0	15 ± 0	21 ± 2	13 ± 1	19 ± 2	24 ± 3	29 ± 2	08 ± 2	14 ± 1	20 ± 1	24 ± 1	10 ± 1	16 ± 2	22 ± 0	26 ± 4
17	07 ± 2	12 ± 1	19 ± 1	23 ± 2	11 ± 2	17 ± 1	20 ± 2	24 ± 3	10 ± 1	13 ± 2	18 ± 2	22 ± 0	08 ± 2	13 ± 1	18 ± 2	24 ± 2
18	22 ± 2	27 ± 1	34 ± 3	38 ± 1	22 ± 0	27 ± 2	34 ± 2	38 ± 1	20 ± 1	27 ± 3	34 ± 2	37 ± 2	23 ± 2	29 ± 2	34 ± 2	40 ± 2
19	23 ± 2	27 ± 1	35 ± 2	39 ± 2	23 ± 1	26 ± 2	38 ± 2	40 ± 1	21 ± 1	26 ± 1	34 ± 3	39 ± 1	21 ± 1	26 ± 1	31 ± 0	37 ± 1
20	24 ± 1	31 ± 1	37 ± 0	42 ± 2	26 ± 1	32 ± 0	36 ± 1	43 ± 2	26 ± 1	31 ± 4	38 ± 1	43 ± 1	23 ± 0	30 ± 2	37 ± 2	44 ± 1
21	20 ± 2	29 ± 2	35 ± 2	39 ± 2	19 ± 1	26 ± 0	31 ± 1	34 ± 0	19 ± 0	28 ± 2	34 ± 2	39 ± 2	15 ± 1	23 ± 1	30 ± 1	37 ± 1
22	17 ± 1	19 ± 3	22 ± 1	28 ± 2	15 ± 2	20 ± 0	25 ± 1	31 ± 2	17 ± 1	21 ± 1	25 ± 2	29 ± 2	17 ± 2	24 ± 3	29 ± 1	33 ± 2
23	20 ± 1	27 ± 2	31 ± 2	35 ± 3	18 ± 2	26 ± 3	32 ± 0	37 ± 1	20 ± 2	25 ± 1	32 ± 1	39 ± 2	18 ± 2	28 ± 1	33 ± 3	38 ± 2
Tyr	04 ± 2	08 ± 2	12 ± 2	17 ± 2	NA	NA	09 ± 1	12 ± 0	04 ± 0	10 ± 1	12 ± 1	15 ± 1	04 ± 1	07 ± 1	11 ± 2	15 ± 2
Trp	06 ± 1	09 ± 3	13 ± 2	19 ± 0	04 ± 2	08 ± 1	12 ± 1	15 ± 1	06 ± 1	09 ± 1	14 ± 0	17 ± 0	06 ± 1	10 ± 2	13 ± 2	17 ± 2
Std	17 ± 1	21 ± 2	29 ± 1	32 ± 1	21 ± 0	24 ± 2	28 ± 1	33 ± 2	18 ± 0	24 ± 1	29 ± 2	33 ± 1	19 ± 1	24 ± 3	29 ± 1	34 ± 0
Control DMSO	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] Values are means of three determinations, the ranges of which are <5% of the mean in all cases. Std: gentamicin, NA: no activity, (±) standard deviation.

glutamine are sensitive and light unstable. Meanwhile, sulphur-containing amino acids like cysteine and methionine conjugated analogues have promising antimicrobial activity.

Based on their promising antimicrobial activities, these synthetic compounds were further tested for their minimum inhibitory concentration (MIC). The results showed that compounds **7–9**, **12**, **18–20** and **23** exhibited excellent MBC and MFC activity against all the tested bacterial and fungal strains (MIC values were below the standard).

Mechanism of cell membrane damage. Bacterial cells are highly metabolically active and their cytoplasmic membranes are very delicate in nature. The present study suggests an effect of NPs on the cytoplasmic membrane of *S. aureus* which was subsequently stimulated to release its cellular materials; this phenomenon was further confirmed by SEM analysis, as mentioned in Fig. 1. Compounds **7**, **8**, **9**, **12**, **20** and **23** were evaluated, and they displayed time-dependent cell leakage. The effectiveness of compounds **7**, **8**, **12** and **23** was revealed by increasing the cellular contents in due time course. The results indicated that the hydrophobic effect of the molecule was efficiently involved in the cell membrane damage which in turn led to the cell content leakage, as presented in Fig. 2. This observation suggests that, the compounds acts as a biocidal agent and was confirmed in cellular content leakage at different time intervals against pathogens in the study. Thus, it is envisioned that any active molecule having potential interaction with the cytoplasmic membrane will lead to damage in membrane anatomical structure to release potassium ions, DNA, and other cellular materials. The report by Tyagi *et al.*[44] demonstrated that the effect of curcumin was associated with the membrane damage caused to *S. aureus* and *E. coli*, while the effectiveness of the hydrophobic nature of curcumin allows it to act as a biocidal molecule, which was determined by SEM and cellular leakage. These observations are closely correlated with the experimental results indicating that the synthesized hydrophobic molecules were also involved in the damage of the membrane anatomical structure and act as antimicrobial agents.

2.2.2 Anti-inflammatory activity. D. C. Gowda *et al.*[45] reported the synthesis and *in vitro* antimicrobial and anti-inflammatory activities of two series of novel quinazolinone (QZN 1 and QZN 2) conjugated amino acid analogues. Among these two class of conjugates, the phenylalanine (IC_{50} values are 52 and 44 μg mL^{-1}) and tryptophan (IC_{50} values are 40 and 38 μg mL^{-1}) conjugated quinazolinones displayed good anti-inflammatory activity. The same research group also[46] reported the synthesis and *in vivo* analgesic and anti-inflammatory activities of a class of novel amino acid or

Table 2 Antifungal activity of the synthesized compounds (**1–23**)

	Zone of inhibition[a] (mm)											
	Aspergillus niger				*Candida albicans*				*Fusarium oxysporum*			
Entry	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}	25 μg mL^{-1}	50 μg mL^{-1}	75 μg mL^{-1}	100 μg mL^{-1}
01	04 ± 1	07 ± 2	09 ± 2	11 ± 2	NA	NA	06 ± 1	10 ± 2	NA	NA	05 ± 1	09 ± 1
02	05 ± 1	09 ± 2	12 ± 1	15 ± 2	07 ± 0	10 ± 2	14 ± 2	16 ± 1	04 ± 1	09 ± 2	13 ± 1	15 ± 1
03	08 ± 1	12 ± 1	15 ± 2	18 ± 3	07 ± 1	12 ± 1	15 ± 1	17 ± 3	06 ± 1	10 ± 4	14 ± 2	18 ± 2
04	07 ± 1	13 ± 1	16 ± 2	20 ± 2	10 ± 2	13 ± 2	15 ± 3	21 ± 1	09 ± 0	14 ± 1	20 ± 2	23 ± 2
05	10 ± 2	15 ± 2	18 ± 1	22 ± 1	08 ± 1	11 ± 2	16 ± 1	20 ± 0	10 ± 1	12 ± 0	15 ± 1	19 ± 0
06	08 ± 1	12 ± 3	16 ± 1	20 ± 1	09 ± 2	13 ± 1	17 ± 2	22 ± 2	NA	NA	NA	NA
07	17 ± 1	28 ± 2	35 ± 1	40 ± 1	22 ± 1	27 ± 2	32 ± 1	38 ± 1	14 ± 1	22 ± 0	27 ± 1	32 ± 0
08	22 ± 1	30 ± 2	37 ± 2	41 ± 2	20 ± 2	29 ± 2	34 ± 1	37 ± 1	22 ± 1	29 ± 1	36 ± 2	42 ± 1
09	24 ± 1	31 ± 2	36 ± 1	44 ± 3	24 ± 2	31 ± 2	37 ± 2	42 ± 1	21 ± 1	30 ± 2	35 ± 2	40 ± 1
10	18 ± 1	26 ± 2	31 ± 1	34 ± 1	17 ± 1	22 ± 3	28 ± 0	34 ± 2	18 ± 1	24 ± 0	30 ± 1	35 ± 1
11	12 ± 1	15 ± 3	18 ± 1	22 ± 1	11 ± 0	16 ± 2	19 ± 1	21 ± 0	08 ± 2	14 ± 2	17 ± 2	23 ± 1
12	17 ± 1	24 ± 1	30 ± 2	33 ± 2	16 ± 1	22 ± 1	27 ± 0	34 ± 1	20 ± 1	24 ± 2	29 ± 2	35 ± 3
13	07 ± 1	10 ± 2	14 ± 1	17 ± 2	09 ± 2	11 ± 2	16 ± 2	20 ± 2	07 ± 2	10 ± 2	14 ± 0	17 ± 2
14	08 ± 1	12 ± 1	15 ± 2	18 ± 3	07 ± 1	12 ± 1	15 ± 1	17 ± 3	06 ± 1	10 ± 4	16 ± 2	18 ± 1
15	09 ± 1	12 ± 1	17 ± 2	21 ± 2	08 ± 1	11 ± 2	16 ± 3	19 ± 1	10 ± 0	15 ± 1	19 ± 2	21 ± 1
16	10 ± 2	14 ± 2	19 ± 1	22 ± 2	10 ± 0	15 ± 2	19 ± 2	21 ± 2	09 ± 1	13 ± 1	17 ± 1	19 ± 0
17	10 ± 1	15 ± 3	18 ± 1	23 ± 1	10 ± 2	14 ± 1	16 ± 2	20 ± 2	11 ± 0	15 ± 2	19 ± 2	21 ± 1
18	20 ± 1	30 ± 1	36 ± 2	41 ± 2	24 ± 2	28 ± 1	34 ± 0	40 ± 2	20 ± 1	25 ± 0	30 ± 1	36 ± 2
19	24 ± 2	31 ± 2	38 ± 2	40 ± 2	23 ± 1	30 ± 2	36 ± 1	39 ± 2	21 ± 1	28 ± 2	35 ± 1	41 ± 2
20	28 ± 1	31 ± 2	40 ± 1	46 ± 1	26 ± 1	33 ± 2	39 ± 2	44 ± 1	25 ± 1	32 ± 2	37 ± 2	42 ± 1
21	19 ± 1	27 ± 2	32 ± 1	37 ± 2	17 ± 1	26 ± 2	35 ± 1	40 ± 1	20 ± 1	28 ± 1	33 ± 1	39 ± 1
22	11 ± 1	18 ± 3	21 ± 1	24 ± 1	12 ± 0	17 ± 2	21 ± 2	23 ± 2	13 ± 2	17 ± 2	19 ± 2	22 ± 2
23	17 ± 1	24 ± 1	30 ± 2	33 ± 2	16 ± 1	22 ± 1	27 ± 0	34 ± 1	20 ± 1	24 ± 2	29 ± 2	35 ± 3
Tyr	NA	NA	07 ± 1	11 ± 2	04 ± 2	08 ± 1	13 ± 1	15 ± 0	03 ± 1	07 ± 2	12 ± 1	14 ± 0
Trp	04 ± 1	07 ± 2	11 ± 0	14 ± 2	05 ± 1	09 ± 2	13 ± 1	16 ± 1	04 ± 1	07 ± 2	09 ± 2	11 ± 1
Std	20 ± 1	27 ± 2	33 ± 1	36 ± 2	19 ± 1	24 ± 1	29 ± 1	32 ± 0	22 ± 1	27 ± 2	33 ± 1	36 ± 2
Control DMSO	—	—	—	—	—	—	—	—	—	—	—	—

[a] Values are means of three determinations, the ranges of which are <5% of the mean in all cases. Std: bavistin, NA: no activity, (±) standard deviation.

Table 3 Minimum inhibitory concentration (MIC) of the synthesized compounds

	MIC (μg mL^{-1}) values[a]						
	Antibacterial				Antifungal		
Entry	*S. aureus*	*B. substilis*	*E. coli*	*E. pneumoniae*	*A. niger*	*C. albicans*	*F. oxysporum*
7	25 ± 5	26 ± 1	22 ± 2	26 ± 2	28 ± 2	30 ± 2	27 ± 1
8	21 ± 2	20 ± 2	19 ± 6	24 ± 5	22 ± 5	24 ± 5	22 ± 2
9	18 ± 5	17 ± 3	16 ± 1	20 ± 5	20 ± 5	19 ± 5	21 ± 2
12	27 ± 3	26 ± 1	25 ± 2	24 ± 2	29 ± 3	24 ± 2	25 ± 5
18	22 ± 2	22 ± 0	22 ± 1	24 ± 2	26 ± 7	28 ± 5	26 ± 2
19	18 ± 5	19 ± 2	20 ± 2	17 ± 7	21 ± 7	23 ± 5	23 ± 1
20	16 ± 2	16 ± 2	15 ± 5	22 ± 5	21 ± 2	20 ± 2	20 ± 2
23	26 ± 5	25 ± 5	24 ± 1	26 ± 1	27 ± 3	28 ± 2	28 ± 5
Std (B)	26 ± 2	28 ± 4	26 ± 5	26 ± 2	—	—	—
Std (F)	—	—	—	—	27 ± 2	28 ± 2	27 ± 5

[a] Values are means of three determinations, the ranges of which are <5% of the mean in all cases. Std (B): gentamicin for antibacterial; Std (F): bavistin for antifungal.

peptide conjugated aurantiamide acetate analogues, and they demonstrated for the first time that the Boc group was essential for the pharmacological properties of those compounds. In addition, D. C. Gowda's study revealed that the presence of amino acids was necessary to exhibit enhanced activity. It was also noticed that compounds containing amino acid/peptide conjugates possessed highly potent activity compared to the reference standards. Hence, they concluded that those compounds could be developed as a class of new lead analgesic and anti-inflammatory agents. Herein, we report the design and synthesis of a class of xanthone conjugated amino acid analogues and the subsequently conducted *in vitro* anti-inflammatory activity studies of those conjugates.

All the synthesised compounds were also evaluated for their *in vitro* anti-inflammatory activity using a known method reported in the literature.[47] A significant number of

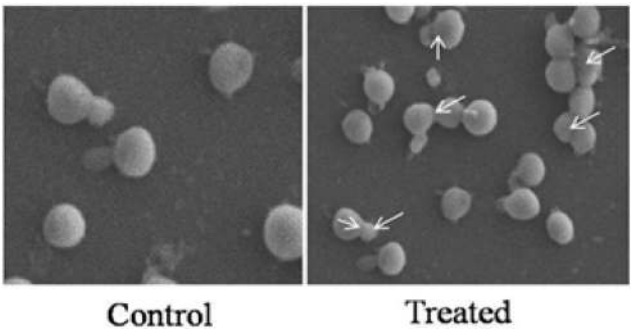


Fig. 1 The scanning electron microscopy analysis of *S. aureus* shows, the control cells having a regular and intact morphology whereas after treatment with compound **19** at MIC, the *S. aureus* cell morphology was altered due to the disruption of the cell membrane. This can be clearly visible as indicated by arrow marks at the site of damage.

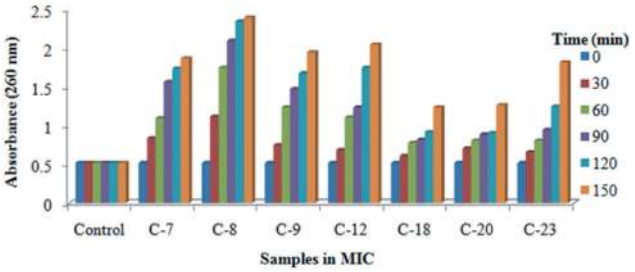


Fig. 2 The cellular content release: the treatment at MIC of *S. aureus* and incubation for different times indicating the release of cellular content due to cell membrane damage caused by the compounds.

Table 4 Anti-inflammatory activity of the synthesized amino acid conjugated xanthone derivatives (**1**–**23**)

Entry	Anti-inflammatory activity[a] IC_{50} ($\mu g\ mL^{-1}$)
1	90 ± 1.04
2	66 ± 1.24
3	70 ± 4.89
4	76 ± 1.16
5	64 ± 1.26
6	60 ± 1.24
7	38 ± 0.49
8	32 ± 1.19
9	24 ± 1.44
10	32 ± 1.24
11	36 ± 1.41
12	34 ± 0.81
13	70 ± 0.49
14	78 ± 1.36
15	82 ± 1.10
16	70 ± 1.10
17	68 ± 1.17
18	38 ± 0.19
19	32 ± 1.49
20	26 ± 1.42
21	30 ± 1.28
22	34 ± 1.87
23	36 ± 0.54
Indomethacin	40 ± 0.24

[a] Values are means of three determinations, the ranges of which are <5% of the mean in all cases.

compounds have exhibited excellent to moderate inhibitory activity compared to standard drug indomethacin. The IC_{50} of the compounds were determined and they displayed more than 50% inhibition (Table 4).

Compounds **7**, **8**, **9**, **10**, **11**, **12**, **18**, **19**, **20**, **21**, **22** and **23** showed good anti-inflammatory activity with IC_{50} values of 38, 32, 24, 32, 36, 34, 38, 32, 26, 30, 34 and 36 $\mu g\ mL^{-1}$, respectively, which were much better than that of the standard indomethacin (IC_{50} = 40 $\mu g\ mL^{-1}$). Aromatic hydrophobic (tryptophan, tyrosine and phenylalanine) and sulphur atom containing (cysteine and methionine) amino acids derived molecules showed excellent anti-inflammatory activity compared to other aliphatic or simple amino acids. The other compounds **2**–**6** and **13**–**17** showed moderate anti-inflammatory activity.

2.2.3 Molecular docking studies. In order to understand the structure-based correlation of synthesized compounds, we conducted molecular docking studies using the crystal structure of sPLA2 from humans and AmpC ß-lactamase from *E. coli*. The molecular docking results of the ligands (**20** and **21**) were similar to those of *in vitro* and *in silico* studies (Table 5) and the ADME drug-like criteria in Table 6. Inflammation is a complex immunological response of the body against tissue damage or microbial inflammation. Novel bioconjugated ligands showed promising anti-inflammatory activity against sPLA2 by our *in silico* molecular docking protocol. Gly29 and Asp48 are very crucial amino acids which reside near the catalytic site of sPLA2. Blocking these residues in-turn downregulates the enzyme activity thereby inhibiting the progression of inflammation. Ligand **22** formed π–π stacking with Gly29 and a hydrogen bond with Asp48, which is vital for a substrate to bind with sPLA2. Hence it clearly represents that ligand **22** could be a better bio-conjugate molecule against inflammation targeting sPLA2 (Fig. 3). Ligand **20** showed good interaction with His47 and His6 with a favorable energy pose (Fig. 4). Inflammation and microbial infection are very closely related; hence we checked the potency of the ligands for antibacterial activities. β-Lactamases are the most resistant to ß-lactam antibiotics and are an increasing menace to public health. Ligand **23** binds deeply into the active site of AmpC β-lactamase, suggesting very tight binding, thereby inhibiting the accessibility of the enzyme to act on a substrate (Fig. 5), indicating that it possesses both anti-inflammatory and antibacterial potency. QikProp was the prediction program used to calculate the ADME properties consisting of principal descriptors and physicochemical properties. Qikprop modules provide the ranges of molecular predicting properties for comparing the properties of a particular molecule with those of 95% of known drugs (Table 6).[48] The ligands obey Lipinski's rules: molecular weight below 500 Da, hydrogen bond donor (less than five) and acceptor (less than ten). QPlogPo/w (octanol/water partition coefficient) for the ligand is less than five.[47] The ligands satisfy the values of partition coefficient of octanol/gas

Table 5 Molecular docking scores of all the synthesized compounds against AmpC β-lactamase from *E. coli* and $sPLA_2$ from humans

	1KE4 (AmpC β-lactamase from *E. coli*)						5G3N ($sPLA_2$ from humans)					
Title	RMSD OPLS-2005	Docking score	Glide Evdw	Glide energy	Glide Emodel	Glide Lipo	RMSD OPLS-2005	Docking score	Glide Evdw	Glide energy	Glide Emodel	Glide Lipo
1	0.047	−4.55	−24.82	−26.34	−31.84	−2.90	0.047	−4.66	−34.26	−33.33	−38.12	−2.32
2	0.048	−2.72	−5.16	−6.45	−11.10	−3.11	0.048	−5.97	−46.76	−47.06	−60.13	−3.06
3	0.041	−4.18	−19.55	−20.95	−18.94	−3.19	0.041	−5.45	−40.01	−41.53	−52.89	−2.96
4	0.039	−4.41	−18.61	−18.38	−20.95	−3.43	0.039	−5.69	−44.01	−45.52	−53.03	−3.21
5	0.035	−5.05	−14.09	−16.73	−22.74	−4.35	0.035	−5.90	−44.44	−45.01	−55.87	−3.49
6	0.037	−5.33	−23.29	−26.89	−20.19	−4.57	0.037	−5.85	−31.96	−41.21	−50.97	−2.08
7	0.047	−2.89	−12.01	−12.62	−15.18	−3.88	0.047	−5.39	−44.08	−46.36	−56.90	−3.12
8	0.011	−3.88	−18.03	−18.31	−13.96	−2.54	0.011	−6.28	−44.19	−46.89	−57.27	−2.61
9	0.034	−3.9	−21.56	−22.67	−18.84	−2.97	0.023	−7.93	−48.60	−53.27	−70.41	−3.54
10	0.046	−3.35	−25.84	−28.43	−15.28	−4.42	0.046	−7.09	−35.62	−46.41	−62.06	−2.52
11	0.018	−4.93	−25.23	−29.18	−32.44	−3.32	0.018	−6.83	−45.12	−45.90	−54.70	−3.12
12	0.013	−4.40	−29.88	−36.94	−45.93	−3.44	0.009	−7.46	−40.37	−47.74	−67.49	−2.38
13	0.038	−0.35	−16.92	−18.81	−5.77	−3.47	0.006	−4.44	−43.36	−45.27	−56.34	−4.30
14	0.017	−3.01	−22.84	−35.06	−25.25	−4.51	0.048	−6.95	−36.61	−41.73	−59.29	−3.24
15	0.041	−3.12	−1.07	−4.77	1.74	−3.49	0.041	−6.47	−32.44	−40.55	−51.84	−2.14
16	0.023	−4.3	−4.30	−5.41	−2.10	−3.59	0.021	−7.64	−35.47	−45.22	−65.11	−2.42
17	0.012	−2.60	−0.85	−5.48	0.62	−3.04	0.012	−6.94	−36.94	−44.10	−55.27	−2.26
18	0.028	−5.40	−19.94	−20.64	−22.71	−3.93	0.028	−7.30	−50.43	−49.61	−65.25	−3.98
19	0.025	−5.68	−25.94	−37.28	−53.75	−3.30	0.018	−6.70	−35.81	−39.65	−53.63	−2.87
20	0.021	−8.31	−34.98	−37.60	−50.97	−3.55	0.007	−8.16	−32.55	−40.78	−54.6	−3.11
21	0.005	−8.74	−32.85	−38.52	−27.48	−3.41	0.004	−6.49	−38.28	−48.22	−63.14	−3.44
22	0.048	−2.63	−34.02	−37.24	−22.96	−4.39	0.048	−8.01	−36.61	−41.73	−59.29	−3.24
23	0.021	−8.90	−32.85	−32.52	−26.48	−3.41	0.004	−6.49	−30.28	−41.22	−53.14	−2.44
Gentamycin	0.019	−8.32	−18.81	−33.69	−49.91	−3.61	NA	NA	NA	NA	NA	NA
Indomethacin	NA	NA	NA	NA	NA	NA	0.001	−7.9	−27.66	−46.33	−66.71	−2.78

Table 6 ADME properties of the synthesized compounds

Ligands	QPlog HERG	QPPCaco	QPlogBB	QPPMDCK	QPlogKp	QPlogKhsa	% human oral absorption	Rule of five
1	−4.7	1084	−0.3	540	−2.2	−0.2	92	0
2	−6.5	442	−1.2	205	−2.7	0.3	93	0
3	−6.5	632	−1.1	301	−2.4	0.4	100	0
4	−6.6	744	−1.1	360	−2.2	0.7	100	0
5	−6.8	739	−1.2	357	−2.1	0.8	100	0
6	−6.8	749	−1.2	362	−2.1	0.8	100	0
7	−7.2	636	−1.2	303	−1.7	0.8	94	1
8	−7.0	194	−1.8	84	−2.7	0.7	94	0
9	−7.8	515	−1.4	242	−1.7	1.2	96	1
10	−6.6	623	−1.1	650	−2.3	0.6	100	0
11	−7.0	653	−1.2	539	−2.1	0.7	100	0
12	−5.7	1147	−0.6	574	−2.1	0.2	100	0
13	−6.0	92	−0.6	42	−5.0	−0.3	67	0
14	−6.2	125	−0.5	58	−4.8	−0.2	72	0
15	−6.3	163	−0.5	77	−4.5	0.0	78	0
16	−6.6	133	−0.7	62	−4.5	0.1	78	0
17	−6.4	149	−0.6	70	−4.5	0.1	79	0
18	−7.7	155	−0.6	73	−3.7	0.3	83	0
19	−7.5	44	−1.3	19	−4.9	0.1	69	0
20	−8.1	90	−1.0	41	−4.1	0.5	80	0
21	−6.3	127	−0.5	155	−4.6	−0.1	76	0
22	−6.8	130	−0.7	104	−4.5	0.0	78	0
23	−6.2	222	−0.2	108	−4.4	−0.1	79	0
Gentamycin	−8.0	1	−1.7	0	−8.7	−1.1	0	2
Range 95% of drugs	<−5	<poor, >500 great	−3.0 to 1.2	<25 poor, >500 great	−8.0 to −1.0	−1.5 to 1.5	>80% is high	0–4

(QPlogPoct), water/gas (QPlogPw) and brain/blood (QPlogBB), skin permeability (QPlogKp), and aqueous solubility (QPlogS), which is predicted for ligands within the permissible range.

3. Conclusion

In the present work, a diversity of analogues with a conjugation of amino acids and biologically active xanthone

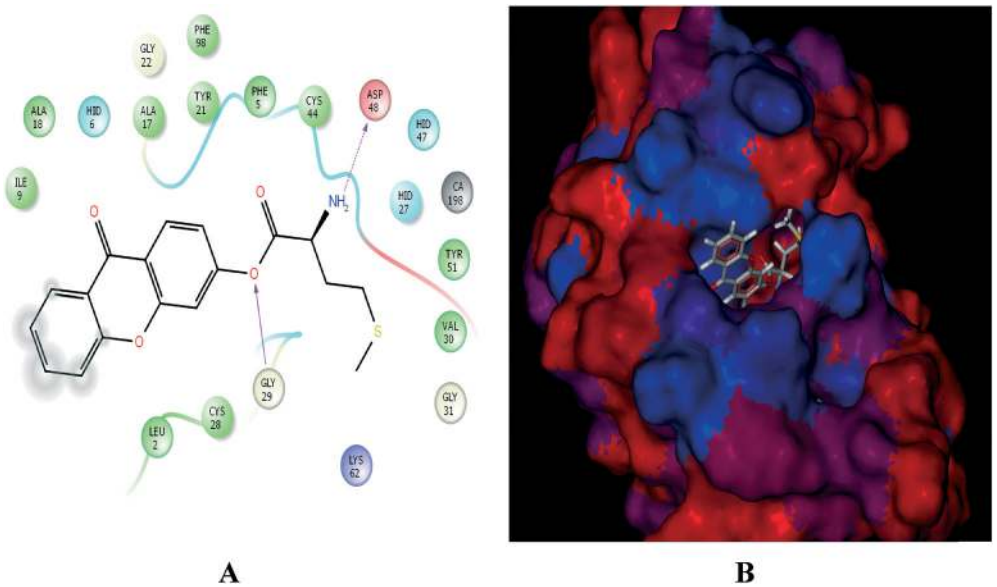


Fig. 3 (A) Molecular interaction of the 5G3N enzyme with ligand 22; (B) electrostatic surface representation of the protein depicting the best-docked pose for ligand 22.

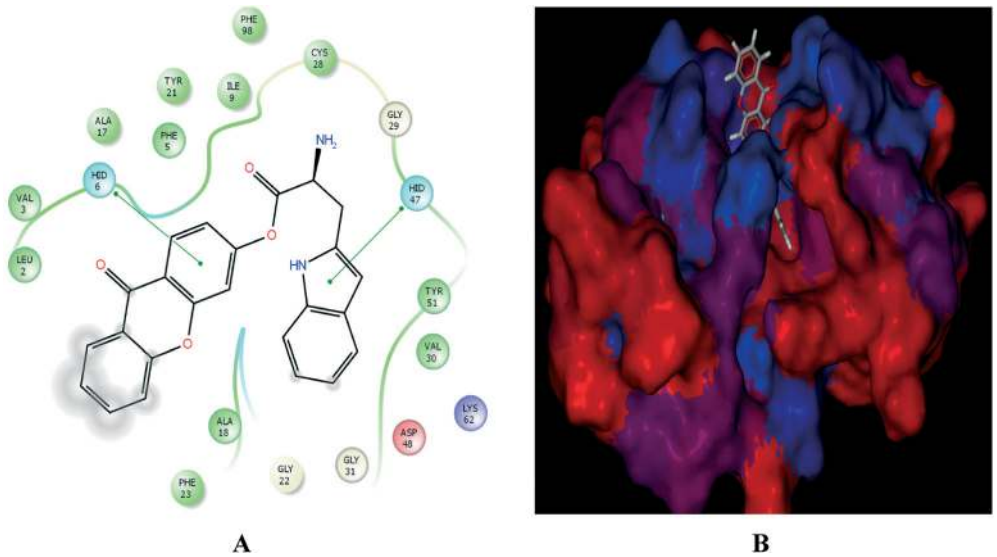


Fig. 4 (A) Molecular interaction of the 5G3N enzyme with ligand 20; (B) electrostatic surface representation of the protein depicting the best-docked pose for ligand 20.

heterocycles were synthesized and evaluated for their *in vitro* antimicrobial and anti-inflammatory activities by using agar well diffusion and human erythrocyte methods, respectively. Mainly, this study focuses on improving the biological activities and the development of new antimicrobial and anti-inflammatory therapeutic drugs. It is interesting to find that xanthone molecules alone are inactive when compared to the standard drugs. However, after conjugation with amino acids of different natures, the biological activities of those xanthone molecules increased remarkably. These results suggest that the conjugation played a significant role in improving the biological activities. The experimental results revealed that compounds **7**, **8**, **9**, **12**, **18**, **19**, **20**, **21** and **23** displayed excellent antibacterial and antifungal activities at the common antibiotic level. On the other hand, compounds **7–12** and **18–23** displayed good anti-inflammatory activity compared to the standard drug indomethacin. Further, SAR study analysis revealed that tryptophan, tyrosine and phenylalanine conjugated compounds possessed excellent antimicrobial activity, while tryptophan, tyrosine, phenylalanine, cysteine and methionine conjugates possessed good anti-inflammatory activity. We may conclude that the aromaticity and hydrophobicity of amino acids play a major role in biological activity. Molecular docking studies were performed for all the synthesised compounds, among which compounds **20**, **21** and **23** showed the highest docking scores for antimicrobial

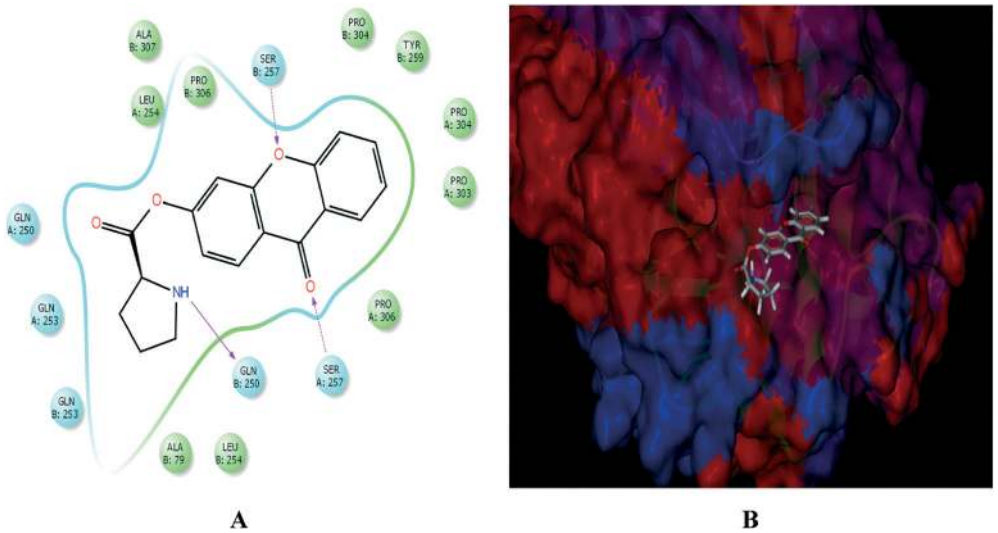


Fig. 5 (A) Molecular interaction of the 1KE4 enzyme with ligand 23; (B) electrostatic surface representation of the protein depicting the best-docked pose for ligand 23.

activity and compounds **9**, **20** and **22** showed the highest docking scores for anti-inflammatory activity.

4. Experimental section

4.1 General

All Boc-amino acids and HBTu used were in-house materials, and all the amino acids except glycine were of L-configuration unless otherwise mentioned. All the other reagents were obtained from Spectrochem Pvt. Ltd. (India) and Rankem Pvt. Ltd. (India) and used without further purification. The progress of the reaction was monitored by TLC using silica gel 60 F254, with the solvent system comprising hexane and ethyl acetate in the ratio of 03 : 01, and the compounds on the TLC plates were detected under UV light and iodine vapors. Melting points were determined using a Thermionic apparatus (India) and uncorrected. FT-IR spectroscopy was performed using a Perkin Elmer Spectrum Version 10.03.09 (Japan) using nujol media. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an Agilent Technologies (USA) spectrometer using DMSO (d_6)/$CDCl_3$ as solvent and the chemical shifts were reported as parts per million (δ ppm) using TMS as an internal standard. High resolution mass spectroscopic analysis was performed using a Bruker MicroTOF QII mass spectrometer in positive mode.

4.1.1 Synthesis of 2-chlorophenyl-(2,4-dihydroxyphenyl)-methanone (A). A mixture of resorcinol, 2-chlorobenzoic acid and anhydrous zinc chloride were heated to 120 °C. The temperature of the mixture was increased to 140–150 °C and maintained at that temperature for 2 h. After the reaction was completed, the reaction mass was then poured into sodium bicarbonate solution with vigorous stirring. The brown-coloured solid was filtered, washed repeatedly with water and dried to give the desired products.

Yield: 95.1%, R_f 0.60, m.p. 121–124 °C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.07 (s, 1H, OH), 10.9 (broad s, 1H, OH), 7.55 (m, 2H, ArH), 7.46 (s, 2H, ArH), 7.02 (d, 1H, ArH), 6.33 (s, 2H, ArH), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 103.4, 109.4, 112.9, 127.7, 129.3, 129.7, 130.1, 131.8, 135.6, 137.9, 165.3, 166.4, 197.6; HRMS (*m*/*z*): 249.1254 [M^+], 251.1254 [M + 2].

4.1.2 Synthesis of 3-hydroxy xanthone (1). 2-Chlorophenyl-(2,4-dihydroxyphenyl)methanone was dissolved in DMSO and heated to 80 °C. Sodium hydroxide flakes were added in lots over a 3 hour period and the process was found to be exothermic; the temperature of the reaction mixture was increased to 120 °C and maintained at that temperature for an hour. After the reaction was completed, the mass was cooled to room temperature and poured into ice sulphuric acid aqueous solution. The solid was filtered, repeatedly washed with water and recrystallized with ethyl acetate to give the desired product in 85% yield.

R_f 0.51, m.p. 230–232 °C, IR KBr (cm^{-1}): IR KBr (cm^{-1}): 3490 (OH), 1684 (CO); ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.95 (s, 1H, OH), 8.10 (d, 1H, J = 8.0 Hz, ArH), 7.99–8.01 (m, 1H, ArH), 7.76 (t, 1H, J = 7.2 Hz, ArH), 7.55 (d, 1H, J = 8.4 Hz, ArH), 7.39 (t, 1H, J = 7.6 Hz, ArH), 6.83–6.88 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 102.5, 114.4, 114.5, 118.2, 121.6, 124.5, 126.2, 128.4, 135.1, 155.9, 157.9, 164.4, 175.1; HRMS (*m*/*z*): 213.2145 [M + 1].

4.1.3 General procedure for the synthesis of xanthone conjugated Boc protected amino acids. 3-Hydroxy xanthone (1 mmol) was dissolved in tetrahydrafuran (THF) and triethylamine (TEA) (1 mmol). HBTu (1 mmol) was added under stirring at room temperature. After 10 min Boc protected amino acids were added to the solution and the pH of the solution was adjusted to 8 by the addition of TEA and the reaction mixture was stirred for 5–6 hour. The solvent was removed under reduced pressure and the residue was poured into 100 mL cold 90% $KHCO_3$ solution and stirred for 30 min. The precipitated product was extracted with EtOAc and washed sequentially with 5% solution of $NaHCO_3$, water, and then with 0.1 N cold HCl solution and finally brine. The EtOAc

layer was dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. The obtained products were recrystallized from hexane to get crude products 2–12.

4.1.4 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)acetate (2). Yield 87.33%, R_f[a] = 0.48, m.p. 147–149 °C, IR KBr (cm^{-1}): 2839 (NH), 1681 (CO), 1667 (CO), 1610 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.41 (9H, s, $(CH_3)_3$), 3.34 (2H, s, CH_2), 4.05 (2H, d, J = 5.6 Hz, NH), 7.24 (1H, d, J = 8.5 Hz, ArH), 7.48 (1H, d, J = 8.8 Hz, ArH), 7.63 (2H, d, J = 8.1 Hz, ArH), 7.86 (1H, t, J = 15.3 Hz, ArH), 8.20 (2H, m, ArH); ^{13}C NMR ($CDCl_3$) δ: 28.5, 42.8, 79.0, 111.3, 118.5, 119.1, 119.5, 121.5, 125.0, 126.4, 128.1, 133.0, 155.7, 156.1, 156.3, 156.6, 169.3, 175.6; HRMS (m/z): 370.1829 [M + 1].

4.1.5 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)propanoate (3). Yield 89.41%, R_f[a] = 0.52, m.p. 171–172 °C, IR KBr (cm^{-1}): 2971 (NH), 1743 (CO), 1678 (CO), 1614 (CO); ^{1}H NMR (DMSO-d_6) δ: 1.18 (3H, d, J = 7.2 Hz, CH_3), 1.34 (9H, s, $(CH_3)_3$), 3.88 (1H, t, J = 7.2 Hz, CH), 6.85–6.90 (2H, m, ArH), 7.07 (1H, d, J = 7.6 Hz, ArH), 7.41 (1H, t, J = 14.0 Hz, ArH), 7.58 (1H, d, J = 8.0 Hz, ArH), 7.71–7.81 (1H, m, ArH), 8.02 (1H, d, J = 8.8 Hz, NH), 8.12 (1H, d, J = 6.0 Hz, ArH); ^{13}C NMR ($CDCl_3$) δ: 17.2, 28.4, 49.1, 79.0, 102.4, 114.2, 114.5, 118.1, 121.2, 124.6, 126.1, 128.4, 135.5, 155.8, 155.9, 157.9, 164.1, 175.3, 175.9; HRMS (m/z): 384.5412 [M + 1].

4.1.6 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-methylbutanoate (4). Yield 85.33%, R_f[a] = 0.49, m.p. 188–189 °C, IR KBr (cm^{-1}): 3284 (NH), 1743 (CO), 1695 (CO), 1648 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.04 (3H, d, J = 7.2 Hz, CH_3), 1.10 (3H, d, J = 7.2 Hz, CH_3), 1.42 (9H, s, $(CH_3)_3$), 2.33 (1H, t, J = 5.6 Hz, CH), 4.47 (1H, t, J = 5.2 Hz, CH), 5.09 (1H, d, J = 8.0 Hz, NH), 7.10–7.13 (1H, m, ArH), 7.30 (1H, d, J = 2.4 Hz, ArH), 7.37 (1H, t, J = 7.6 Hz, ArH), 7.46 (1H, d, J = 8.4 Hz, ArH), 7.68–7.73 (1H, m, ArH), 8.29–8.35 (2H, m, ArH); ^{13}C NMR ($CDCl_3$) δ: 17.7, 19.1, 28.2, 29.6, 31.1, 58.9, 80.2, 110.7, 117.8, 117.9, 119.8, 121.7, 124.1, 126.7, 128.3, 134.8, 155.2, 156.2, 156.7, 170.4, 176.2; HRMS (m/z): 412.5624 [M + 1].

4.1.7 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-methylpentanoate (5). Yield 82.14%, R_f[a] = 0.51, m.p. 180–181 °C, IR KBr (cm^{-1}): 3283 (NH), 1725 (CO), 1675 (CO), 1647 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.00 (6H, d, J = 6.0 Hz, $(CH_3)_2$), 1.44 (9H, s, $(CH_3)_3$), 1.75–1.84 (2H, m, CH_2), 4.51 (1H, d, J = 3.3 Hz, CH), 5.21 (1H, d, J = 1.9 Hz, NH), 7.09 (1H, d, J = 7.5 Hz, ArH), 7.26 (1H, d, J = 1.7 Hz, ArH), 7.32 (1H, t, J = 15.0 Hz, ArH), 7.40 (1H, d, J = 8.4 Hz, ArH), 7.65 (1H, t, J = 1.1 Hz, ArH), 8.25–8.31 (2H, m, ArH), ^{13}C NMR (DMSO-d_6) δ: 21.7, 22.8, 24.9, 28.3, 41.1, 52.5, 80.2, 110.7, 117.8, 117.9, 119.7, 121.6, 124.1, 126.6, 128.2, 134.9, 155.3, 155.6, 156.1, 156.6, 171.5, 176.3; HRMS (m/z): 426.1524 [M + 1].

4.1.8 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-methylpentanoate (6). Yield 87.10%, R_f[a] = 0.46, m.p. 175–176 °C, IR KBr (cm^{-1}): 3230 (NH), 1710 (CO), 1680 (CO), 1622 (CO); ^{1}H NMR ($CDCl_3$) δ: 0.87 (3H, t, J = 8.2 Hz, CH_3), 0.89 (3H, d, J = 7.2 Hz, CH_3), 0.95–0.99 (2H, m, CH_2), 1.33 (9H, s, $(CH_3)_3$), 2.40–4.42 (2H, m, CH), 4.36 (1H, m, CH), 5.20 (1H, d, J = 8.8 Hz, NH), 7.10 (1H, d, J = 8.8 Hz, ArH), 7.27 (1H, d, J = 8.4 Hz, ArH), 7.37 (1H, d, J = 8.0 Hz, ArH), 7.45 (1H, d, J = 8.4 Hz, ArH), 7.70 (1H, s, ArH), 8.33 (2H, d, J = 8.8 Hz, ArH), ^{13}C NMR (DMSO-d_6) δ: 11.5, 15.6, 25.1, 28.0, 37.9, 58.2, 81.5, 110.7, 117.8, 117.9, 119.8, 121.7, 124.1, 126.7, 128.3, 134.8, 155.2, 156.2, 156.7, 170.3, 176.2; HRMS (m/z): 426.0156 [M + 1].

4.1.9 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-phenylpropanoate (7). Yield 87.10%, R_f[a] = 0.46, m.p. 175–176 °C, IR KBr (cm^{-1}): 3310 (NH), 1710 (CO), 1680 (CO), 1622 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.47 (9H, s, $(CH_3)_3$), 3.27 (2H, d, J = 5.6 Hz, CH_2), 4.85 (1H, d, J = 6.4 Hz, CH), 6.13 (1H, s, NH), 6.80–6.81 (1H, m, ArH), 7.14–7.66 (8H, m, ArH), 7.80 (1H, d, J = 7.2 Hz, ArH), 8.13–8.14 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 37.6, 55.9, 110.9, 117.3, 117.9, 118.8, 120.7, 124.6, 126.8, 127.5, 128.9, 129.2, 129.9, 134.0, 135.8, 155.6, 156.1, 156.6, 170.5, 176.3; HRMS (m/z): 460.2641 [M + 1].

4.1.10 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-(4-hydroxyphenyl)propanoate (8). Yield 85.12%, R_f[a] = 0.40, m.p. 188–189 °C, IR KBr (cm^{-1}): 3281 (NH), 1721 (CO), 1674 (CO), 1618 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, $(CH_3)_3$), 3.21 (2H, t, J = 6.8 Hz, CH_2), 4.55–4.57 (1H, m, CH), 5.62 (1H, s, NH), 6.88–7.14 (6H, m, ArH), 7.29 (1H, t, J = 6.4 Hz, ArH), 7.45 (1H, d, J = 7.6 Hz, ArH), 7.68 (1H, d, J = 7.0 Hz, ArH), 7.75 (1H, t, J = 6.0 Hz, ArH), 8.12–8.13 (1H, m, ArH), 8.23 (1H, s, OH); ^{13}C NMR (DMSO-d_6) δ: 28.1, 36.9, 59.8, 80.6, 110.1, 115.4, 117.2, 118.1, 119.4, 121.6, 124.3, 125.6, 128.2, 129.4, 130.4, 133.1, 154.8, 155.4, 155.9, 156.8, 156.9, 169.3, 173.8; HRMS (m/z): 476.1476 [M + 1].

4.1.11 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-(1*H*-indole-2yl-)propanoate (9). Yield 81.77%, R_f[a] = 0.49, m.p. 190–191 °C, IR KBr (cm^{-1}): 3314 (NH), 2893 (NH), 1742 (CO), 1679 (CO), 1648 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, $(CH_3)_3$), 3.21 (2H, t, J = 6.0 Hz, CH_2), 4.55–4.57 (1H, m, CH), 5.62 (1H, s, NH), 6.88–7.14 (6H, m, ArH), 7.29 (1H, t, J = 8.8 Hz, ArH), 7.45 (1H, d, J = 8.2 Hz, ArH), 7.66 (1H, d, J = 5.6 Hz, ArH), 8.12–8.14 (2H, m, ArH), 10.2 (1H, s, NH); ^{13}C NMR (DMSO-d_6) δ: 28.3, 36.4, 59.1, 81.5, 99.5, 111.3, 111.8, 117.2, 117.6, 118.5, 119.4, 120.2, 120.8, 121.4, 125.0, 125.8, 127.5, 129.8, 132.4, 134.3, 136.5, 155.2, 156.1, 156.6, 169.4, 174.1; HRMS (m/z): 499.5412 [M + 1].

4.1.12 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-3-mercaptopropanoate (10). Yield 85.1%, R_f[a] = 0.52, m.p. 173–174 °C, IR KBr (cm^{-1}): 3310 (NH), 1712 (CO), 1681 (CO), 1621 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.41 (9H, s, $(CH_3)_3$), 1.52 (1H, s, SH), 3.24 (2H, t, J = 6.8 Hz, CH_2), 4.74–4.75 (1H, m, CH), 6.38 (1H, s, NH), 7.10–7.14 (2H, m, ArH), 7.21 (1H, d, J = 7.2 Hz, ArH), 7.49 (1H, d, J = 6.0 Hz, ArH), 7.68 (1H, d, J = 7.8 Hz, ArH), 8.10–8.11 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 26.4, 28.9, 60.8, 80.7, 110.9, 116.4, 117.4, 117.8, 120.4, 124.6, 125.3, 128.3, 135.4, 154.6, 155.8, 156.2, 160.8, 168.9, 174.8; HRMS (m/z): 416.2364 [M + 1].

4.1.13 9-Oxo-9*H*-xanthen-3-yl-2-((*tert*-butoxycarbonyl)-amino)-4-methyl(thio)butanoate (11). Yield 82.3%, R_f[a] = 0.47, m.p. 182–183 °C, IR KBr (cm^{-1}): 3289 (NH), 1714 (CO), 1688 (CO), 1619 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s, $(CH_3)_3$), 1.99–2.01 (2H, m, CH_2), 2.17 (3H, s, CH_3), 2.29 (2H, t, J = 7.0 Hz, CH_2), 4.51–4.53 (1H, m, CH), 6.20 (1H, s, NH), 7.08–7.11 (2H,

m, ArH), 7.27 (1H, d, J = 5.8 Hz, ArH), 7.49 (1H, t, J = 6.6 Hz, ArH), 7.65 (1H, d, J = 7.4 Hz, ArH), 8.13–8.15 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 15.8, 28.1, 29.3, 30.4, 57.9, 80.1, 111.5, 117.1, 117.6, 118.3, 121.2, 124.9, 125.1, 127.6, 134.5, 155.5, 155.9, 156.3, 161.2, 167.5, 173.0; HRMS (m/z): 444.1265 [M + 1].

4.1.14 1-*tert*-Butyl-2-(9-oxo-9*H*-xanthen-3-yl)pyrrolidine-1,2-dicarboxylate (12). Yield 83.71%, R_f[a] = 0.55, m.p. 183–184 °C, IR KBr (cm^{-1}): 1710 (CO), 1682 (CO), 1616 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.39 (9H, s, $(CH_3)_3$), 2.23 (2H, m, CH_2), 2.37 (2H, t, J = 7.2 Hz, CH_2), 3.33–3.38 (2H, m, CH_2), 4.47 (1H, m, CH), 7.23 (1H, d, J = 7.2 Hz, ArH), 7.48 (2H, s, ArH), 7.64 (1H, d, J = 6.8 Hz, ArH), 7.85 (1H, d, J = 6.4 Hz, ArH), 8.17 (1H, d, J = 7.2 Hz, ArH), 8.25 (1H, t, J = 8.8 Hz, ArH), ^{13}C NMR (DMSO-d_6) δ: 23.6, 24.6, 28.4, 29.7, 30.7, 40.7, 59.0, 79.7, 111.2, 118.5, 119.0, 121.5, 125.0, 126.4, 126.2, 128.3, 136.0, 154.1, 155.5, 156.1, 156.5, 171.1, 175.6; HRMS (m/z): 410.9165 [M + 1].

4.1.15 Deblocking of Boc group (13–23). Het-Xaa-Boc (2–12) (0.002 mmol) was stirred with 2.0 mL of HCl-dioxan for 45 min at room temperature. After completion of the reaction, the reaction mixture was concentrated under high vacuum to obtain Het-Xaa-NH_2·HCl (13–23) which was then triturated with dry ether, filtered and dried.

4.1.16 9-Oxo-9*H*-xanthen-3-yl 2aminoacetate hydrochloride (13). Yield 91.24%, R_f[a] = 0.31, m.p. 155–156 °C, IR KBr (cm^{-1}): 2952 (NH), 1710 (CO), 1638 (CO); ^{1}H NMR ($CDCl_3$) δ: 4.35 (2H, s, CH_2), 5.17 (2H, s, NH_2), 7.07–7.15 (2H, m, ArH), 7.29 (1H, d, J = 7.8 Hz, ArH), 7.42 (1H, t, J = 5.8 Hz, ArH), 7.66 (1H, d, J = 7.0 Hz, ArH), 7.85 (1H, t, J = 6.2 Hz, ArH), 8.12–8.14 (2H, m, ArH); ^{13}C NMR ($CDCl_3$) δ: 41.4, 111.4, 117.2, 117.6, 118.0, 121.9, 124.8, 124.9, 128.6, 135.2, 155.6, 155.9, 156.4, 168.3, 173.1; HRMS (m/z): 306.4251 [M + 1].

4.1.17 9-Oxo-9*H*-xanthen-3-yl 2aminopropanoate hydrochloride (14). Yield 95.4%, R_f[a] = 0.42, m.p. 164–165 °C, IR KBr (cm^{-1}): 2984 (NH), 1741 (CO), 1668 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.20–1.23 (3H, d, J = 6.8 Hz, CH_3), 3.77–3.79 (1H, m, CH), 5.59 (2H, s, NH_2), 7.08–7.15 (2H, m, ArH), 7.26 (1H, d, J = 4.8 Hz, ArH), 7.51 (1H, m, ArH), 7.66 (1H, t, J = 8.2 Hz, ArH), 7.71 (1H, d, J = 5.6 Hz, ArH), 8.04 (1H, d, J = 7.8 Hz, ArH); ^{13}C NMR ($CDCl_3$) δ: 17.9, 50.1, 111.53, 116.9, 117.5, 117.9, 120.8, 124.9, 126.6, 127.3, 134.0, 154.8, 155.7, 156.8, 168.9, 174.4; HRMS (m/z): 320.4521 [M + 1].

4.1.18 9-Oxo-9*H*-xanthen-3-yl 2-amino-3-methylbutanoate hydrochloride (15). Yield 94.52%, R_f[a] = 0.38, m.p. 767–169 °C, IR KBr (cm^{-1}): 2970 (NH), 1738 (CO), 1650 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.04 (6H, d, J = 6.8 Hz, $(CH_3)_2$), 2.28 (1H, t, J = 6.2 Hz, CH), 4.55 (1H, m, CH), 5.82 (2H, s, NH_2), 7.13–7.14 (1H, m, ArH), 7.26 (1H, d, J = 4.4 Hz, ArH), 7.30 (1H, t, J = 8.4 Hz, ArH), 7.48 (1H, d, J = 7.2 Hz, ArH), 7.69–7.70 (1H, m, ArH), 8.10–8.12 (2H, m, ArH); ^{13}C NMR ($CDCl_3$) δ: 18.2, 18.4, 30.7, 59.8, 111.7, 117.3, 117.9, 119.1, 120.3, 123.9, 126.4, 128.6, 133.0, 155.3, 155.9156.1, 167.5, 173.5; HRMS (m/z): 348.2351 [M + 1].

4.1.19 9-Oxo-9*H*-xanthen-3-yl-2-amino-4-methylpentanoate hydrochloride (16). Yield 95.20%, R_f[a] = 0.34, m.p. 175–777 °C, IR KBr (cm^{-1}): 3230 (NH), 1682 (CO), 1614 (CO); ^{1}H NMR ($CDCl_3$) δ: 0.98 (6H, d, J = 5.8 Hz, $(CH_3)_2$), 1.70–1.72 (2H, m, CH_2), 4.58 (1H, d, J = 4.8 Hz, CH), 6.28 (2H, s, NH_2), 7.15 (1H, d, J = 8.2 Hz, ArH), 7.28 (1H, d, J = 5.8 Hz, ArH), 7.38 (1H, t, J = 12.4 Hz, ArH), 7.48 (1H, d, J = 7.2 Hz, ArH), 7.68 (1H, d, J = 2.8 Hz, ArH), 8.12–8.15 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 22.8, 25.6, 42.6, 50.4, 110.2, 117.4, 117.8, 119.6, 121.4, 124.5, 125.9, 127.8, 134.6, 155.1, 156.4, 156.9, 169.4, 175.1; HRMS (m/z): 362.4521 [M + 1].

4.1.20 9-Oxo-9*H*-xanthen-3-yl-2-amino-3-methylpentanoate hydrochloride (17). Yield 96.15%, R_f[a] = 0.36, m.p. 180–182 °C, IR KBr (cm^{-1}): 3286 (NH), 1678 (CO), 1614 (CO); ^{1}H NMR ($CDCl_3$) δ: 0.96 (3H, t, J = 6.8 Hz, CH_3), 1.16 (3H, d, J = 6.8 Hz, CH_3), 1.67–1.70 (2H, m, CH_2), 2.40–4.42 (2H, m, CH), 4.36 (1H, m, CH), 5.82 (2H, s, NH_2), 7.15 (1H, d, J = 6.8 Hz, ArH), 7.30 (1H, d, J = 7.2 Hz, ArH), 7.42 (1H, d, J = 5.8 Hz, ArH), 7.49 (1H, d, J = 8.0 Hz, ArH), 7.76 (1H, d, J = 3.8 Hz, ArH), 8.14 (2H, d, J = 8.8 Hz, ArH), ^{13}C NMR (DMSO-d_6) δ: 11.9, 15.8, 24.2, 36.5, 56.6, 111.1, 117.3, 117.7, 118.8, 120.6, 124.3, 125.3, 127.8, 135.3, 155.0, 156.12, 156.9, 168.5, 174.3; HRMS (m/z): 362.1235 [M + 1].

4.1.21 9-Oxo-9*H*-xanthen-3-yl-2-amino-3-phenylpropanoate hydrochloride (18). Yield 92.14%, R_f[a] = 0.30, m.p. 168–169 °C, IR KBr (cm^{-1}): 3181 (NH), 1674 (CO), 1612 (CO); ^{1}H NMR ($CDCl_3$) δ: 3.30 (2H, d, J = 6.8 Hz, CH_2), 4.76 (1H, d, J = 7.6 Hz, CH), 5.17 (2H, s, NH_2), 7.00–7.02 (1H, m, ArH), 7.21–7.51 (8H, m, ArH), 7.74 (1H, s, ArH), 8.32–8.36 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 28.2, 38.2, 54.8, 80.4, 110.7, 117.9, 119.8, 121.7, 124.2, 125.9, 126.7, 127.4, 128.3, 128.8, 129.3, 134.9, 135.4, 155.0, 156.2, 156.6, 176.3; HRMS (m/z): 395.3521 [M + 1].

4.1.22 9-Oxo-9*H*-xanthen-3-yl-2-amino-3-(4-hydroxyphenyl)-propanoate hydrochloride (19). Yield 90.18%, R_f[a] = 0.31, m.p. 170–172 °C, IR KBr (cm^{-1}): 3525 (OH), 3177 (NH), 1674 (CO), 1626 (CO); ^{1}H NMR ($CDCl_3$) δ: 3.17 (2H, t, J = 7.2 Hz, CH_2), 4.50–4.51 (1H, m, CH), 5.89 (2H, s, NH_2), 6.92–7.11 (6H, m, ArH), 7.31 (1H, t, J = 7.6 Hz, ArH), 7.49 (1H, d, J = 8.0 Hz, ArH), 7.69 (1H, d, J = 7.8 Hz, ArH), 7.80 (1H, t, J = 5.8 Hz, ArH), 8.15–8.17 (1H, m, ArH), 8.56 (1H, s, OH); ^{13}C NMR (DMSO-d_6) δ: 37.2, 55.3, 111.2, 116.5, 117.1, 117.8, 119.0, 120.4, 124.6, 125.3, 128.5, 129.5, 130.4, 133.2, 154.1, 155.3, 155.8, 156.6, 156.4, 170.3, 174.5; HRMS (m/z): 411.1236 [M + 1].

4.1.23 9-Oxo-9*H*-xanthen-3-yl-2-amino-3-(1*H*-indole-2yl-)-propanoate hydrochloride (20). Yield 90.95%, R_f[a] = 0.39, m.p. 180–181 °C, IR KBr (cm^{-1}): 3436 (NH), 2996 (NH), 1712 (CO), 1652 (CO); ^{1}H NMR ($CDCl_3$) δ: 3.18 (2H, t, J = 7.2 Hz, CH_2), 4.60–4.62 (1H, m, CH), 6.01 (2H, s, NH_2), 6.90–7.16 (6H, m, ArH), 7.32 (1H, t, J = 7.2 Hz, ArH), 7.48 (1H, d, J = 8.8 Hz, ArH), 7.69 (1H, d, J = 6.4 Hz, ArH), 8.09–8.10 (2H, m, ArH), 10.5 (1H, s, NH); ^{13}C NMR (DMSO-d_6) δ: 37.5, 56.8, 100.5, 110.5, 111.2, 117.2, 117.5, 118.9, 119.9, 120.6, 121.5, 121.5, 124.4, 125.5, 127.6, 128.2, 133.6, 135.6, 136.6, 155.9, 156.8, 156.9, 170.3, 174.6; HRMS (m/z): 435.1264 [M + 1].

4.1.24 9-Oxo-9*H*-xanthen-3-yl-2-amino-3-mercapto-propanoate hydrochloride (21). Yield 92.14%, R_f[a] = 0.40, m.p. 156–158 °C, IR KBr (cm^{-1}): 3207 (NH), 2651 (SH), 1682 (CO), 1602 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.46 (1H, s, SH), 3.19 (2H, t, J = 7.2 Hz, CH_2), 4.70–4.71 (1H, m, CH), 6.10 (2H, s, NH_2), 7.11–7.13 (2H, m, ArH), 7.28 (1H, d, J = 6.8 Hz, ArH), 7.52 (1H, d, J =

7.2 Hz, ArH), 7.69 (1H, d, J = 8.2 Hz, ArH), 8.14–8.16 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 26.3, 59.8, 111.1, 117.2, 117.6, 118.2, 121.5, 124.3, 125.3, 128.6, 134.3, 155.2, 155.7, 156.3, 1610.8, 171.5, 173.6; HRMS (m/z): 352.1864 [M + 1].

4.1.25 9-Oxo-9*H*-xanthen-3-yl-2-amino-4-methyl(thio)-butanoate hydrochloride (22). Yield 92.40%, R_f[a] = 0.37, m.p. 170–173 °C, IR KBr (cm^{-1}): 3207 (NH), 1682 (CO), 1602 (CO); ^{1}H NMR ($CDCl_3$) δ: 1.97–1.99 (2H, m, CH_2), 2.22 (3H, s, CH_3), 2.32 (2H, t, J = 7.8 Hz, CH_2), 4.56–4.57 (1H, m, CH), 6.10 (21H, s, NH_2), 7.09–7.10 (2H, m, ArH), 7.28 (1H, d, J = 6.2 Hz, ArH), 7.44 (1H, t, J = 8.0 Hz, ArH), 7.69 (1H, d, J = 6.8 Hz, ArH), 8.10–8.12 (2H, m, ArH); ^{13}C NMR (DMSO-d_6) δ: 15.4, 29.6, 31.5, 58.9, 110.3, 117.2, 117.8, 118.6, 120.4, 125.1, 126.4, 127.6, 133.5, 155.9, 156.1, 156.9, 170.6, 174.2; HRMS (m/z): 380.1265 [M + 1].

4.1.26 9-Oxo-9*H*-xanthen-3-yl-pyrrolidine-2-carboxylate hydrochloride (23). Yield 92.42%, R_f[a] = 0.38, m.p. 171–173 °C, IR KBr (cm^{-1}): 1657 (CO), 1623 (CO); ^{1}H NMR ($CDCl_3$) δ: 2.18 (2H, m, CH_2), 2.45 (2H, t, J = 6.8 Hz, CH_2), 3.41–3.42 (2H, m, CH_2), 4.52 (1H, m, CH), 6.81 (1H, s, NH), 7.14 (1H, d, J = 6.4 Hz, ArH), 7.35–7.45 (2H, m, ArH), 7.67 (1H, d, J = 7.6 Hz, ArH), 7.87 (1H, d, J = 5.6 Hz, ArH), 8.11 (1H, d, J = 8.0 Hz, ArH), 8.24 (1H, t, J = 8.2 Hz, ArH), ^{13}C NMR (DMSO-d_6) δ: 22.7, 24.6, 29.6, 31.8, 41.5, 57.8, 110.1, 117.3, 119.6, 120.6, 124.5, 125.1, 126.8, 127.4, 135.4, 155.2, 155.7, 156.1, 170.1, 174.5; HRMS (m/z): 346.1254 [M + 1].

5. Biological evaluation

5.1 Antibacterial activity

In vitro antibacterial activity was evaluated against human pathogens, namely, both Gram-positive organisms *S. aureus* and *B. substilis* and Gram-negative organisms *E. coli* and *K. pneumoniae* by using the agar well diffusion method[32] as well as a microdilution method[33] with slight modifications.

Agar well diffusion method. The microorganisms were inoculated into sterilized nutrient broth and maintained at 37 °C for 24 hours. On the day of testing, the bacteria were subcultured separately in 100 mL sterilized nutrient broth. The inoculated subcultured broths were kept at room temperature for the growth of inocula. Using a sterile cork borer, wells (6 mm) were made into each petriplate. The compounds were dissolved in DMSO as 5 mg mL^{-1} and from this 5, 10, 15 and 20 μL (25, 50, 75, 100 μg per well) were added into the wells by using sterile pipettes. The antibiotic standard gentamicin for antibacterial activity (as the positive control) was tested against the pathogens. The samples dissolved in DMSO which showed no zone of inhibition acted as negative controls. The plates were incubated at 37 °C for 24 h for bacteria. After appropriate incubation, the diameter of the zone of inhibition of each well was measured. Duplicates were maintained and the average values were calculated for eventual assessment of antimicrobial activity.

Microdilution method. All the microorganisms were grown in Muller-Hinton broth. After cultivation for 16–18 h at 37 °C, the bacteria were harvested and their density was determined by measuring OD at A_{600}. The MIC of the compounds was determined by the agar dilution method. A suspension of each microorganism was prepared to contain approximately 1 × 10^4 – 2 × 10^4 CFU mL^{-1}, applied to the plates with serially diluted compounds (both the tested compounds and the reference drug were dissolved in DMSO) and incubated at 37 °C overnight. The minimum inhibitory concentration was considered to be the lowest concentration that completely inhibited the growth of microorganisms on the plates. The diameter of the zone of inhibition (mm) was measured after 24 h and MIC values were determined.

5.2 Antifungal activity

In vitro antifungal activity was evaluated against human pathogens *A. niger*, *C. albicans* and *F. oxysporum* by the agar well diffusion method[34] as well as a microdilution method[35] with slight modifications.

Agar well diffusion method. The microorganisms were inoculated into sterilized nutrient broth and maintained at 37 °C for 24 hours. On the day of testing, the bacteria were subcultured separately into 100 mL sterilized nutrient broth. The inoculated subcultured broths were kept at room temperature for the growth of inocula. Using a sterile cork borer, wells (6 mm) were made into each petriplate. The compounds were dissolved in DMSO as 5 mg mL^{-1} and from this 5, 10, 15 and 20 μL (25, 50, 75, 100 μg per well) were added into the wells by using sterile pipettes. Simultaneously the antifungal standard bavistin for antifungal activity (as the positive control) was tested against the pathogens. The samples dissolved in DMSO which showed no zone of inhibition acted as negative controls. The plates were incubated at 28 °C for 48 h for fungi. After appropriate incubation, the diameter of the zone of inhibition of each well was measured. Duplicates were maintained and the average values were calculated for the final antimicrobial activity.

Microdilution method. Sabouraud agar was used for the preparation of plates. A suspension of each microorganism was prepared to contain 10^5 CFU mL^{-1}. The agar plates were inoculated with fungal strains and serially diluted test compounds and the reference drug dissolved in DMSO. The plates were incubated at 25 °C for 48–72 h. The minimum inhibitory concentration was considered to be the lowest concentration that completely inhibited the growth of microorganisms on the plates. The zone of inhibition (mm) was measured after 48 h and MIC values were determined.

5.3 Bacterial cell microscopy

Scanning electron microscopy (SEM) was carried out to study *S. aureus* membrane damage by, treating MIC of compounds by incubating for 2 h, and then the cells were pelleted by centrifugation (10 000 rpm for 5 min) at 4 °C. The cells were fixed by glutaraldehyde (2.5%) in PBS, pelleted and deposited on a glass slide, followed by stepwise drying treatment with 30% to 100% ethanol. After 2 days of

drying under room temperature, these were used for SEM analysis.[49,50]

5.4 Release of cellular material

The effects of the compounds were analyzed by measuring the cellular material (DNA) from *S. aureus* as the model organism according to the protocol of Chauhan and Kang.[51] The experiment was carried out by inoculating log phase cultures into 0.1% sterile peptone water and without samples as a control. After incubation at 37 °C (for 0, 30, 60, and 120 min), 1 mL of broth was transferred to an Eppendorf tube, centrifuged at 3500 rpm and the supernatant was measured at 260 nm using a spectrophotometer. Results were expressed in the form of optical density (OD) for the samples incubated at different intervals of time for samples. The assay was performed in triplicate and repeated thrice.

5.5 Anti-inflammatory activity[47]

Human erythrocyte suspension. The human blood was purchased from a public hospital in Mysore, India and collected in a heparinized vacutainer. The collected healthy human blood was washed with 0.9% saline and centrifuged for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and a 40% v/v suspension was prepared with isotonic phosphate buffer of 154 mM NaCl in 10 mM sodium phosphate buffer at pH 7.4 to be used as a stock erythrocyte or RBC suspension.

Hypotonic solution-induced haemolysis. The tested sample consisted of 0.5 mL stock erythrocyte (RBC) suspension, 5 mL hypotonic solution (50 mM NaCl in 10 mM sodium phosphate buffer saline at pH 7.4) and different concentrations of sample (20, 40, 60, 80 and 100 μg mL^{-1}). The blank control consisted of 0.5 mL RBC suspension and 5 mL hypotonic buffered solution alone. The mixtures were incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm and the supernatant was measured spectrophotometrically at 540 nm. The % inhibition of haemolysis was calculated according to the following formula:

$$\%\ \text{Inhibition of haemolysis} = \left[\frac{A_1 - A_2}{A_1}\right] \times 100$$

where:

A_1 = Absorbance of hypotonic buffered solution alone

A_2 = Absorbance of test/standard sample in hypotonic solution

5.6 Molecular docking and ADME predictions

The coordinates of 1KE4 and 5G3N were obtained from the Brookhaven Protein Data Bank.[52] Ligands were drawn using Maestro 2D sketcher and energy minimization was computed by OPLS 2005. Proteins were prepared using the Maestro 9.3 platform (Schrödinger, Inc.). Protein structures were corrected by using the Prime software module of Schrödinger to correct the missing loops in the protein. Water molecules were removed beyond 5 Å from the heteroatom. Water molecules which are important in aiding the interaction with the receptor were optimized using protein pepwizard. Automated, necessary bonds, bond orders, hybridization, explicit hydrogen atoms and charges were assigned. OPLS 2005 force field was applied to the protein to restrain minimization and RMSD of 0.30 Å was set to converge heavy atoms during the preprocessing of protein before starting the docking. Using extra-precision (XP) docking and scoring, each compound was docked into the receptor grid of radii 20 Å × 20 Å × 20 Å, and the docking calculations were judged based on the Glide score, ADME results and Glide energy. QikProp, the prediction program used to calculate the ADME properties of all the ligands and molecular visualization was conducted using Maestro 9.3.[52]

Conflict of interest

The authors declare no competing interests.

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