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Original article

Synthesis of elastin based peptides conjugated to benzisoxazole as a new class of potent antimicrobials – A novel approach to enhance biocompatibility

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ABSTRACT

The peptides of elastin sequences chosen for the present study included tetrapeptides, pentapeptides and tricosapeptides (30 amino acids), synthesized by classical solution phase method and conjugated to [3-(4-piperidyl)-6-fluoro-1,2-benzisoxazole]. The structures of the compounds were confirmed by physical and spectroscopic techniques followed by the antimicrobial evaluation by both agar well diffusion and microdilution methods. Here we wish to report the effect of conjugation of these moieties which enabled us to identify a novel set of peptides conjugated to heterocycle which have exhibited more potent antimicrobial activity than the conventional drugs used. Further, conjugates of tricosamers **34** and **35** were able to inhibit the growth of fungal species at 3–5 μg/mL which is nearly 5 fold more potent than the reference drug.

1. Introduction

Antibiotics are among the most prescribed drugs in the world today, and since their development and commercialization, have saved countless millions of lives. The ideal antimicrobial agents are selective only in targeting the microorganism but not host cells. Resistance to antimicrobial agents is now recognized as a major global public health problem. Although there are new classes of compounds that are now frequently used to treat microbial infections, the frequency of deeply invasive microbial agents has increased 10 fold during the past decade. With the emergence of new microbial strain resistant to many currently available antibiotic treatments, there is increasing interest in the discovery of novel antimicrobial agents [1,2].

Many reports portray interesting biological activities of 1,2-benzisoxazoles and its derivatives. These have significant pharmacological and biological activities such as anticonvulsant [3], antipsychotic [4], anticancer [5] presented affinity for serotonergic and dopaminergic receptors [6]. In particular, 3-(*N*-benzylpiperidinylethyl)-1,2-benzisoxazoles inhibit acetyl cholinesterase, making them suitable candidates for the palliative treatment of Alzheimer's disease [7]. In view of the broad spectrum of the biological activity exhibited by these benzisoxazoles, the present work was aimed to incorporate [3-(4-piperidyl)-6-fluoro-1,2-benzisoxazole] which consists of benzisoxazole, piperidine and also fluoro group which would make them to be explored as better antimicrobials.

Elastin protein-based polymers have their origin in repeating sequences of the mammalian elastic protein, elastin [8,9]. The most prominent repeating sequence occurs in bovine elastin; it can be represented as $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ where n is eleven without a single substitution. Another repeat first found in porcine elastin is $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$ but this repeat has not been found to occur with n greater than 2 without substitution. The next most common recurring sequence in mammalian elastin is a polyhexapeptide $(Ala^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5\text{-}Val^6)_n$ where, with but a couple of isomorphous hydrophobic residue replacements such as Val by Ile or Leu, n is 8 in man [10]. The monomers, oligomers, and high polymers of these repeats have been synthesized and conformationally characterized [11]. These polymers have a number of medical and non-medical applications [12,13].

Earlier reports have shown that conjugation of different amino acids/peptides to various biologically active scaffolds has fetched the remarkable results which are very promising and even enthusiastic ([14–17] and several unpublished works). Moreover, the

Abbreviations: Boc, *t*-butoxycarbonyl; EDCI, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide HCl; HOBt, 1-hydroxybenzotriazole; IBCF, isobutyl chloroformate; NMM, *N*-methyl morpholine; TFA, trifluoroacetic acid.

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Table 1
^{1}H NMR data of the final protected peptides (**2**–**8**).

Entry	^{1}H NMR data ($CDCl_3$, δ ppm)
Boc-$G^1G^2A^3P^4$-OBzl (**2**)	Boc = 1.40 (9H, s); −NH = 8.01–8.15 (3H, m); Gly^1 = 3.87 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.14 (2H, s, $-^{\alpha}CH$); Ala^3 = 1.34 (1H, d, $-^{\beta}CH_3$), 4.62 (1H, m, $-^{\alpha}CH$); Pro^4 = 1.97–2.10 (2H, m, $-^{\gamma}CH_2$), 2.01–2.18 (2H, m, $-^{\beta}CH_2$), 3.64, 3.79 (2H, m, $-^{\delta}CH_2$), 4.60 (1H, m, $-^{\alpha}CH$); OBzl = 5.07 (2H, m, $-CH_2$), 7.10–7.34 (5H, m, Ar-H)
Boc-$G^1G^2I^3P^4$-OBzl (**3**)	Boc = 1.47 (9H, s); −NH = 8.14–8.26 (3H, m); Gly^1 = 3.91 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.62 (2H, s, $-^{\alpha}CH$); Ile^3 = 0.96–0.97 (6H, m, $-(CH_3)_2$), 1.43 (2H, m, $-^{\gamma}CH_2$), 2.18 (1H, m, $-^{\beta}CH$), 4.63 (1H, m, $-^{\alpha}CH$); Pro^4 = 1.97–2.18 (2H, m, $-^{\gamma}CH_2$), 2.01–2.17 (2H, m, $-^{\beta}CH_2$), 3.50, 3.91 (2H, m, $-^{\delta}CH_2$), 4.61 (1H, m, $-^{\alpha}CH$); OBzl = 5.10 (2H, m, $-CH_2$), 7.15–7.42 (5H, m, Ar-H)
Boc-$G^1G^2F^3P^4$-OBzl (**4**)	Boc = 1.41 (9H, s); −NH = 8.51–8.53 (3H, m); Gly^1 = 3.87 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.18 (2H, s, $-^{\alpha}CH$); Phe^3 = 3.67, 3.73 (2H, d, $-^{\beta}CH_2$), 4.80 (1H, t, $-^{\alpha}CH$); 7.21–7.32 (5H, m, ArH); Pro^4 = 2.00–2.05 (2H, m, $-^{\gamma}CH_2$), 2.14, 2.17 (2H, m, $-^{\beta}CH_2$), 3.70–3.82 (2H, m, $-^{\delta}CH_2$), 4.80 (1H, m, $-^{\alpha}CH$); OBzl = 5.12 (2H, m, $-CH_2$), 7.21–7.32 (5H, m, Ar-H)
Boc-$G^1V^2G^3V^4P^5$-OBzl (**5**)	Boc = 1.43 (9H, s); −NH = 8.34–8.42 (4H, m); Gly^1 = 3.83 (2H, s, $-^{\alpha}CH$); Val^2 = 0.98 (6H, d, $-(CH_3)_2$), 2.70 (1H, m, $-^{\beta}CH$), 4.75 (1H, s, $-^{\alpha}CH$); Gly^3 = 4.13 (2H, s, $-^{\alpha}CH$); Val^4 = 0.98 (6H, d, $-(CH_3)_2$), 2.69 (1H, m, $-^{\beta}CH$), 4.78 (1H, s, $-^{\alpha}CH$); Pro^5 = 2.09–3.64 (6H, m, $-CH_2$), 4.59 (1H, m, $-^{\alpha}CH$); OBzl = 5.05 (2H, m, $-CH_2$), 7.17–7.36 (5H, m, Ar-H)
Boc-$G^1F^2G^3F^4P^5$-OBzl (**6**)	Boc = 1.41 (9H, s); −NH = 8.04–8.12 (4H, m); Gly^1 = 3.87 (2H, s, $-^{\alpha}CH$); Phe^2 = 3.53, 3.57 (2H, m, $-^{\beta}CH_2$), 4.79 (1H, s, $-^{\alpha}CH$), 7.21–7.32 (5H, m, ArH); Gly^3 = 4.12 (2H, s, $-^{\alpha}CH$); Phe^4 = 3.47, 3.61 (2H, m, $-^{\beta}CH_2$), 4.81 (1H, s, $-^{\alpha}CH$), 7.21–7.32 (5H, m, ArH); Pro^5 = 1.80–3.64 (6H, m, $-CH_2$), 4.55 (1H, m, $-^{\alpha}CH$); OBzl = 5.08 (2H, m, $-CH_2$), 7.21–7.32 (5H, m, Ar-H)
Boc-GE(OcHx)GFP GVGVP GVGVP GVGVP GFGFP GFGFP-OBzl (**7**)	Boc = 1.42 (9H, s); −NH = 8.29–9.30 (24H, m); Gly = 4.09, 4.94 (24H, s, $-^{\alpha}CH$); Val = 0.98, 1.00 (36H, m, $(-CH_3)_2$), 2.46, 2.63 (6H, m, $-^{\beta}CH$), 4.44 (6H, m, $-^{\alpha}CH$); Glu = 1.11–1.24 (10H, m, $-CH_2$ of cyclohexyl ring), 2.05, 2.19 (4H, m, $-^{\beta\gamma}CH_2$), 3.69 (1H, m, −CH of cyclohexyl ring), 4.75 (1H, m, $-^{\alpha}CH$); Phe = 3.54, 3.70 (10H, m, $-^{\beta}CH_2$), 4.20, 4.65 (5H, m, $-^{\alpha}CH$), 7.00–7.63 (25H, m, ArH); Pro = 2.00, 3.52, 3.66 (36H, m, $-CH_2$), 4.76 (6H, m, $-^{\alpha}CH$); OBzl = 5.10 (2H, m, $-CH_2$), 7.00–7.63 (5H, m, Ar-H)
Boc-GE(OcHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OBzl (**8**)	Boc = 1.40 (9H, s); −NH = 8.38–9.34 (24H, m); Gly = 4.08, 4.90 (24H, s, $-^{\alpha}CH$); Val = 0.96, 1.02 (36H, m, $(-CH_3)_2$), 2.42, 2.60 (6H, m, $-^{\beta}CH$), 4.39 (6H, m, $-^{\alpha}CH$); Glu = 1.10–1.22 (10H, m, $-CH_2$ of cyclohexyl ring), 2.07, 2.14 (4H, m, $-^{\beta,\gamma}CH_2$), 3.65 (1H, m, −CH of cyclohexyl ring), 4.69 (1H, m, $-^{\alpha}CH$); Phe = 3.55, 3.74 (10H, m, $-^{\beta}CH_2$), 4.19, 4.57 (5H, m, $-^{\alpha}CH$), 7.19–7.69 (25H, m, ArH); Pro = 2.03, 3.54, 3.70 (36H, m, $-CH_2$), 4.72 (6H, m, $-^{\alpha}CH$); OBzl = 5.13 (2H, m, $-CH_2$), 7.19–7.69 (5H, m, Ar-H)

amino acid/peptide based drugs have low toxicity, ample bioavailability and permeability, modest potency and good metabolic and pharmacokinetic properties [18].

Armed with such valuable information and to shed some more light on the importance of conjugation, the overarching goal of this study is to get some insight into the hydrophobic elastin based peptide conjugates of benzisoxazoles with improved biocompatibility.

2. Results and discussion

Previous work has shown that it is possible to improve the activities and/or reduce the toxicities of naturally occurring peptide antibiotics by the modification of primary and/or secondary structures [19]. Using the new ideas as departure points, we have developed a new class of biocompatible elastin based peptides conjugated to biolabile benzisoxazole derivative. Hence, the present study included the provision for these two entities as a platform to investigate the effect of conjugation intricacies upon antimicrobial activity.

2.1. Chemistry

2.1.1. Peptide synthesis and characterization

Peptides were synthesized by classical solution phase method employing Boc chemistry. ^{1}H NMR data of the final protected peptides (**2**–**8**) are given in Table 1. The *C* terminal protected benzyl ester of these peptides was removed and then conjugated to benzisoxazole derivative **1** using EDCI/HOBt as coupling agent and NMM as base. The yields of the compounds obtained were found to be >84% and were characterized by TLC, M.P., ^{1}H NMR and mass spectroscopic analyses. The physical and analytical data of the conjugated compounds are provided in Table 2. The ^{1}H NMR and mass data were found to be in good agreement with the structures assigned.

2.2. Biological studies

The potentiality of the synthesized compounds as antimicrobials was appraised for their antibacterial studies against different strains of human pathogens of both gram positive bacteria namely *Klebsiella pneumoniae* and *Coagulase positive staphylococcus* and gram negative organisms like *Escherichia coli* and *Xanthomonas oryzae* and antifungal studies against *Aspergillus niger*, *Aspergillus flavus* and *Fusarium oxysporum*. The results obtained as zone of inhibition (mm) and minimum inhibitory concentration (μg/mL) are presented in Table 3 and Table 4 respectively. Amoxicillin and bavistin served as standard drugs for antibacterial and antifungal studies respectively.

It is unambiguous from the results obtained that all the heterocycle conjugated amino acids/peptides have shown enhanced activity than either heterocycle or free peptides which are inactive or weakly active (>50 μg/mL).

Interest was generated from the results of the earlier studies revealed by aromaticity and hydrophobicity [14] and hence initially simple aromatic amino acids such as Phe, Trp and Tyr were selected for conjugation. A more important gain would be that the Phe coupled heterocycle (**26**) has shown improved activity which is nearly one and half times more active than the standard drug used against all the species tested. But to our surprise other two amino acid conjugates **27** and **28** have shown moderate activity in spite of being aromatic and also having indole group in Trp and phenolic group in Tyr. Hence, the effect of substitution of Phe by other aromatic amino acids is not salutatory.

In view of this, developing novel antimicrobial agents became an area of great interest encompassing the concept of hydrophobicity and biocompatibility of elastin based peptide sequences with varied chain length. Next we focused our attention on tetrapeptide elastin sequences with varying hydrophobicity. Among the analogs tested GGAP (**29**), GGIP (**30**) and GGFP (**31**), the latter has exerted activity to its fullest i.e., GGFP which comprises of Phe has shown activity which is almost two times more active than the standard. But the remaining conjugates of tetrapeptide have exposed activity not as much as GGFP which could be attributed to the hydrophobicity dependency. Thus the order of activity among tetrapeptide conjugates is GGFP > GGIP > GGAP.

In the light of the above findings, our subsequent goal was to opt pentapeptide elastin sequences GVGVP and GFGFP. The conjugates of these two peptides have revealed the activity which is more than

Table 2
Physical and analytical data of the conjugated compounds (**16**–**25**).

Entry	R_f value	Yield (%)	M.P.°C	Theoretical mol. wt.	Actual MS values (M^+)	^{1}H NMR data ($CDCl_3$, δ ppm)
16	0.86	92.0	Gummy	468	467.5	Boc = 1.44 (9H, s); Phe = 3.64–3.73 (2H, d, $-^{\beta}CH_2$), 4.94 (1H, t, $-^{\alpha}CH$), 7.10–7.36 (5H, m, ArH), 8.11 (1H, s, −NH); Heterocycle = 1.89 (4H, m, $-CH_2$), 2.92 (1H, m, −CH), 3.60–3.61 (4H, m, $-CH_2$), 7.10–7.36 (3H, m, ArH)
17	0.72	96.0	108	507	506.5	Boc = 1.43 (9H, s); Trp = 3.58–3.66 (2H, d, $-^{\beta}CH_2$), 4.95 (1H, t, $-^{\alpha}CH$), 7.04 (1H, s, −CH of indole), 7.11–7.49 (4H, m, ArH), 10.32 (1H, s, −NH of indole), 8.08 (1H, s, −NH); Heterocycle = 1.91–1.93 (4H, m, $-CH_2$), 2.94 (1H, m, −CH), 3.66–3.67 (4H, m, $-CH_2$), 7.11–7.49 (3H, m, ArH)
18	0.71	90.9	112	643	642.5	Boc = 1.44 (9H, s); Tyr(2,6-Cl_2-Bzl) = 3.20–3.52 (2H, d, $-^{\beta}CH_2$), 4.88 (1H, t, $-^{\alpha}CH$), 5.24 (2H, s, $-CH_2$ of side chain protecting group), 6.94–7.66 (7H, m, ArH), 8.00 (1H, s, −NH); Heterocycle = 1.89 (4H, m, $-CH_2$), 2.98 (1H, m, −CH), 3.66 (4H, m, $-CH_2$), 6.94–7.66 (3H, m, ArH)
19	0.70	90.5	82–85	603	602.6	Boc = 1.40 (9H, s); −NH = 8.08–8.15 (3H, s); Gly^1 = 3.87 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.14 (2H, s, $-^{\alpha}CH$); Ala^3 = 1.34 (3H, d, $-^{\beta}CH_3$), 4.62 (1H, m, $-^{\alpha}CH$); Pro^4 = 1.97–2.10 (2H, m, $-^{\gamma}CH_2$), 2.11–2.18 (2H, m, $-^{\beta}CH_2$), 3.64,3.79 (2H, m, $-^{\delta}CH_2$), 4.60 (1H, m, $-^{\alpha}CH$); Heterocycle = 1.88,1.90 (4H, m, $-CH_2$), 2.88 (1H, m, −CH), 3.60 (4H, m, $-CH_2$), 6.98–7.49 (3H, m, ArH)
20	0.75	86.0	70–72	645	644.7	Boc = 1.40 (9H, s); −NH = 8.18–8.23 (3H, m); Gly^1 = 3.87 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.14 (2H, s, $-^{\alpha}CH$); Ile^3 = 0.84–1.06 (6H, m, $-(CH_3)_2$), 1.48 (2H, m, $-^{\gamma}CH_2$), 2.13 (1H, m, $-^{\beta}CH$), 4.62 (1H, m, $-^{\alpha}CH$); Pro^4 = 1.97–2.10 (2H, m, $-^{\gamma}CH_2$), 2.01–2.18 (2H, m, $-^{\beta}CH_2$), 3.64,3.79 (2H, m, $-^{\delta}CH_2$), 4.60 (1H, m, $-^{\alpha}CH$); Heterocycle = 1.88,1.90 (4H, m, $-CH_2$), 2.88 (1H, m, −CH), 3.60 (4H, m, $-CH_2$), 6.98–7.49 (3H, m, ArH)
21	0.74	88.1	80–82	679	678.7	Boc = 1.44 (9H, s); −NH = 8.51–8.53 (3H, s); Gly^1 = 3.84 (2H, s, $-^{\alpha}CH$); Gly^2 = 4.13 (2H, s, $-^{\alpha}CH$); Phe^3 = 3.62, 3.71 (2H, d, $-^{\beta}CH_2$), 4.96 (1H, t, $-^{\alpha}CH$); 6.99–7.26 (5H, m, ArH); Pro^4 = 2.00–2.05 (2H, m, $-^{\gamma}CH_2$), 2.14, 2.17 (2H, m, $-^{\beta}CH_2$), 3.70–3.82 (2H, m, $-^{\delta}CH_2$), 4.80 (1H, m, $-^{\alpha}CH$); Heterocycle = 1.90–1.97 (4H, m, $-CH_2$), 2.94 (1H, m, −CH), 3.57, 3.58 (4H, m, $-CH_2$), 6.99–7.26 (3H, m, ArH)
22	0.64	89.1	119–121	730	729.4	Boc = 1.41 (9H, s); −NH = 8.16–8.24 (4H, s); Gly^1 = 3.82 (2H, s, $-^{\alpha}CH$); Val^2 = 0.98 (6H, m, $-CH_3)_2$), 2.61 (1H, m, $-^{\beta}CH$), 4.71 (1H, s, $-^{\alpha}CH$); Gly^3 = 4.13 (2H, s, $-^{\alpha}CH$); Val^4 = 0.97 (6H, m, $-CH_3)_2$), 2.66 (1H, m, $-^{\beta}CH$), 4.92 (1H, s, $-^{\alpha}CH$); Pro^5 = 2.11–3.64 (6H, m, $-CH_2$), 4.52 (1H, m, $-^{\alpha}CH$); Heterocycle = 1.98–1.99 (4H, m, $-CH_2$), 2.89 (1H, m, −CH), 3.68, 3.70 (4H, m, $-CH_2$), 7.11–7.64 (3H, m, ArH)
23	0.60	88.5	125–127	826	825.8	Boc = 1.41 (9H, s); −NH = 8.38–8.49 (4H, s); Gly^1 = 3.82 (2H, s, $-^{\alpha}CH$); Phe^2 = 3.51, 3.60 (2H, m, $-^{\beta}CH_2$), 4.71 (1H, s, $-^{\alpha}CH$), 7.11–7.64 (5H, m, ArH); Gly^3 = 4.13 (2H, s, $-^{\alpha}CH$); Phe^4 = 3.44, 3.63 (2H, m, $-^{\beta}CH_2$), 4.92 (1H, s, $-^{\alpha}CH$), 7.11–7.64 (5H, m, ArH); Pro^5 = 2.11–3.64 (6H, m, $-CH_2$), 4.52 (1H, m, $-^{\alpha}CH$); Heterocycle = 1.98–1.99 (4H, m, $-CH_2$), 2.89 (1H, m, −CH), 3.68, 3.70 (4H, m, $-CH_2$), 7.11–7.64 (3H, m, ArH)
24	0.72	84.3	178–181	3125	3130.5	Boc = 1.45 (9H, s); −NH = 8.05–8.13 (24H, m); Gly = 4.04, 4.80 (24H, m, $-^{\alpha}CH$); Val = 1.06, 1.09 (36H, m, $(-CH_3)_2$), 2.45, 2.59 (6H, m, $-^{\beta}CH$), 4.40 (6H, m, $-^{\alpha}CH$); Glu = 1.17–1.30 (10H, m, $-CH_2$ of cyclohexyl ring), 2.09, 2.14 (4H, m, $-^{\beta,\gamma}CH_2$), 3.73 (1H, m, −CH of cyclohexyl ring), 4.71 (1H, m, $-^{\alpha}CH$); Phe = 3.51, 3.73 (10H, m, $-^{\beta}CH_2$), 4.19, 4.67 (5H, m, $-^{\alpha}CH$), 7.16–7.58 (25H, m, ArH); Pro = 2.04, 3.62, 3.68 (36H, m, $-CH_2$), 4.80 (6H, m, $-^{\alpha}CH$); Heterocycle = 1.93, 1.96 (4H, m, $-CH_2$), 2.98 (1H, m, −CH), 3.60, 3.67 (4H, m, $-CH_2$), 7.16–7.58 (3H, m, ArH)
25	0.70	84.6	150–153	3125	3130.5	Boc = 1.42 (9H, s); −NH = 8.01–8.11 (24H, m); Gly = 4.06, 4.85 (24H, m, $-^{\alpha}CH$); Val = 1.04, 1.07 (36H, m, $(-CH_3)_2$), 2.44, 2.60 (6H, m, $-^{\beta}CH$), 4.43 (6H, m, $-^{\alpha}CH$); Glu = 1.11–1.24 (10H, m, $-CH_2$ of cyclohexyl ring), 2.10, 2.15 (4H, m, $-^{\beta,\gamma}CH_2$), 3.70 (1H, m, −CH of cyclohexyl ring), 4.69 (1H, m, $-^{\alpha}CH$); Phe = 3.54, 3.75 (10H, m, $-^{\beta}CH_2$), 4.20, 4.61 (5H, m, $-^{\alpha}CH$), 7.19–7.69 (25H, m, ArH); Pro = 2.00, 3.58, 3.71 (36H, m, $-CH_2$), 4.78 (6H, m, $-^{\alpha}CH$); Heterocycle = 1.95, 1.97 (4H, m, $-CH_2$), 2.96 (1H, m, −CH), 3.62, 3.68 (4H, m, $-CH_2$), 7.19–7.69 (3H, m, ArH)

Table 3
Inhibitory zone (diameter) mm of the synthesized conjugates (**26**–**35**) against tested bacterial and fungal strains by agar well diffusion method.

Entry	Antibacterial activity				Antifungal activity		
	Zone of inhibition[a] (mm) ± SD (n = 3)						
	E. coli	*X. oryzae*	*K. pneumoniae*	*C. positive staphylococcus*	*A. niger*	*A. flavus*	*F. oxysporum*
26	14 ± 0.57	11 ± 1.00	10 ± 0.50	09 ± 0.76	16 ± 0.61	11 ± 0.35	14 ± 0.15
27	08 ± 0.40	06 ± 0.36	05 ± 0.55	04 ± 0.50	11 ± 0.70	04 ± 0.05	07 ± 0.47
28	07 ± 0.35	05 ± 0.12	04 ± 0.50	04 ± 0.25	10 ± 0.56	03 ± 0.55	06 ± 0.47
29	18 ± 0.50	13 ± 0.51	12 ± 0.60	11 ± 0.40	19 ± 0.45	13 ± 0.15	16 ± 0.24
30	20 ± 0.32	15 ± 0.43	14 ± 0.55	12 ± 0.04	22 ± 0.55	14 ± 0.15	18 ± 0.47
31	22 ± 0.36	16 ± 0.43	15 ± 0.41	14 ± 0.55	25 ± 0.40	16 ± 0.26	20 ± 0.60
32	23 ± 0.45	17 ± 0.21	17 ± 0.15	15 ± 0.40	27 ± 0.20	17 ± 0.32	21 ± 0.37
33	25 ± 0.28	20 ± 0.43	18 ± 0.25	18 ± 0.07	29 ± 0.17	21 ± 0.35	23 ± 0.45
34	15 ± 0.35	18 ± 0.25	17 ± 0.30	20 ± 0.61	36 ± 0.15	32 ± 0.51	31 ± 0.34
35	12 ± 0.36	14 ± 0.05	11 ± 0.20	16 ± 0.25	34 ± 0.20	28 ± 0.30	28 ± 0.25
Heterocycle	03 ± 0.20	04 ± 0.05	03 ± 0.10	03 ± 0.15	03 ± 0.20	02 ± 0.21	04 ± 0.13
Amoxicillin	11 ± 0.26	08 ± 0.11	08 ± 0.15	07 ± 0.47	–	–	–
Bavistin	–	–	–	–	12 ± 0.20	07 ± 0.15	10 ± 0.25

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

Table 4
Minimum inhibitory concentration (MIC) in μg/mL of the synthesized conjugates (**26**–**35**) against tested bacterial and fungal strains by microdilution method.

Entry	Antibacterial activity				Antifungal activity		
	Minimum inhibitory concentration (MIC) in μg/mL[a]						
	E. coli	*X. oryzae*	*K. pneumoniae*	*C. positive staphylococcus*	*A. niger*	*A. flavus*	*F. oxysporum*
26	20	15	16	18	18	22	21
27	38	26	31	36	32	41	39
28	40	29	34	39	34	43	41
29	17	13	14	16	15	18	19
30	15	12	12	14	13	16	17
31	14	11	11	13	11	15	16
32	11	10	08	10	08	09	10
33	08	09	06	08	06	08	08
34	10	09	09	11	03	03	04
35	12	10	10	13	04	04	05
Heterocycle	>50	>50	>50	>50	>50	>50	>50
Peptides	>50	>50	>50	>50	>50	>50	>50
Amoxicillin	24	18	20	23	–	–	–
Bavistin	–	–	–	–	22	26	25

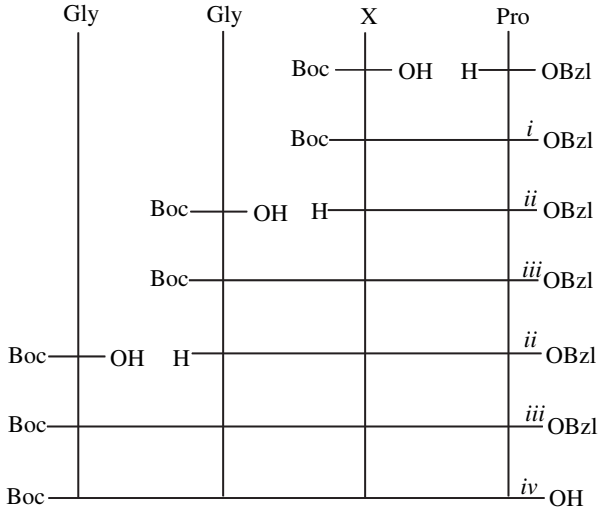
[a] Values are mean of three determinations, the ranges of which are <5% of the mean in all cases.

two fold the conventional drugs used. Inspection of the results further revealed that, irrespective of the same chain length of the peptides presence of two more hydrophobic Phe units in **33** has caused a slight increase in the activity compared to **32** which comprises of two Val moieties which is relatively less hydrophobic.

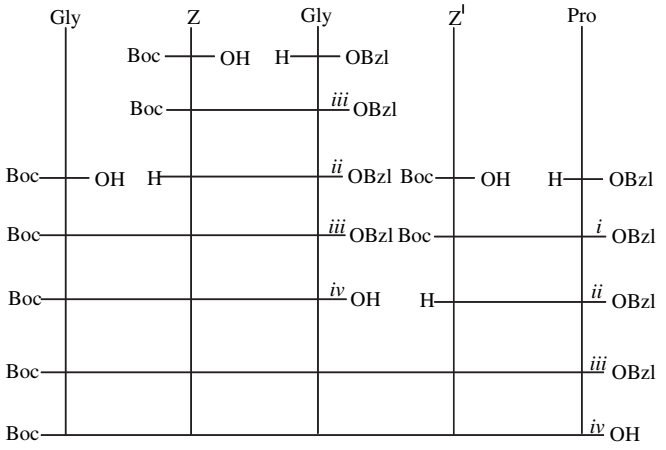
It is also attractive to speculate the observations that the presence of single Phe residue in **26** has shown enhanced activity (~1.5 times) while the retaining of only one Phe unit and increasing the peptide chain length has caused further increase in the activity (~two times) which is a case of GGFP (**31**). Also the other two analogs of tetrapeptide conjugates GGIP and GGAP have shown increased activity compared to Phe alone (**26**). On the other hand, when two Phe groups were introduced (greater hydrophobicity) as well as the peptide chain length increased, GFGFP (**33**) which resulted in the activity more than two times the standard drug used so also the case of GVGVP conjugate (**32**). Hence, it can be inferred that as the length of the peptide chain as well as the hydrophobicity increases the activity also increases. In short, based on the length of the peptide chain and hydrophobicity, the order of activity of the heterocyclic conjugated peptides is found to be GFGFP > GVGVP > GGFP > GGIP > GGAP > Phe>Trp > Tyr.

Upon getting the interesting results, our quest for a more reliable and suitable elastin based sequence for conjugation was portrayed by tricosamers **7** and **8** having improved hydrophobicity and also a polar species, Glu in the chain. Tricosamers prepared elsewhere in order to study the hydrophobicity-induced pKa shifts were used as such. The outcome of the conjugation of these tricosamers, **34** and **35** has yielded the antibacterial activity which is almost two times greater than the standard drug. Though it is twice active, the activity is less when compared to the GFGFP conjugate (**33**). This could be due to the long peptide chain length in spite of being a more hydrophobic entity and that would have resulted in the difficult passage of the molecule across the cell membranes of bacteria.

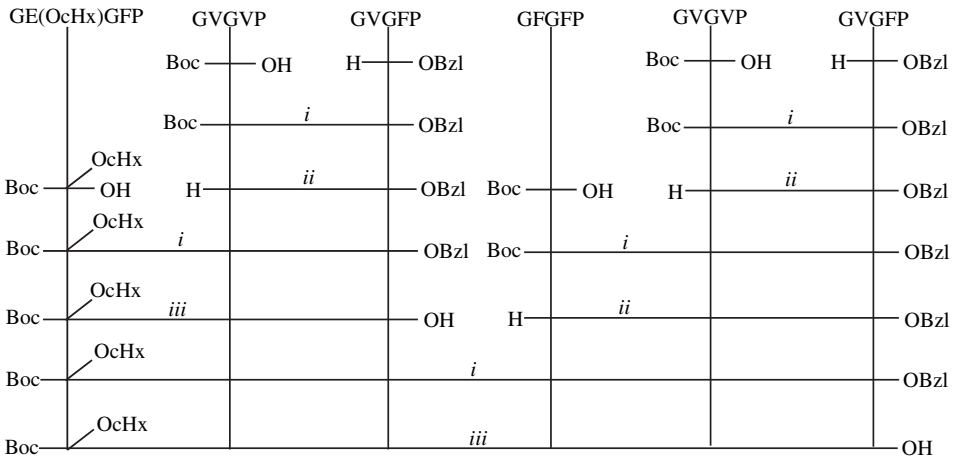
Contrary to conventional wisdom, antifungal activity of the tricosamers conjugated heterocycle has fetched a different set of results. Serendipitously, we succeeded in identifying highly potent antifungal agents of tricosamer conjugates which were able to inhibit the growth of fungal species at 3–5 μg/mL which is five times greater than the antibiotic used. Thus, both conjugates of tricosamers might better qualify as highly potent antifungal agents. Further insight into the results revealed that, among the two conjugates of tricosamers, the former **34** has put forth a slight enhancement over the latter (**35**). This may probably be due to variations of the positions of Phe and Val units in the tricosamers and also the distance as well as the charges present which would



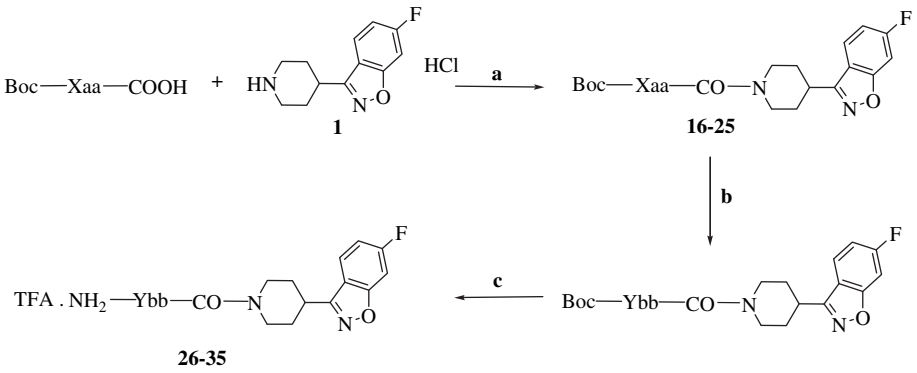
Scheme 1. Schematic representation of the synthesis of tetrapeptides Boc-GGXP-OH where X = Ala for GGAP; Ile for GGIP and Phe for GGFP by stepwise approach. i. IBCF/HOBt, NMM, ii. HCl/dioxane, iii. IBCF, NMM, iv. $HCOONH_4$/10%Pd-C



Scheme 2. Schematic representation of the synthesis of pentapeptides Boc-GZGZ′P-OH where Z = Z′ = Val for GVGVP; Z = Z′ = Phe for GFGFP; Z = Val and Z′ = Phe for GVGFP; Z = Glu(OcHx) and Z′ = Phe for GE(OcHx)GFP by (3 + 2) fragment coupling method. *i.* IBCF/HOBt, NMM, *ii.* HCl/dioxane, *iii.* IBCF, NMM, *iv.* $HCOONH_4$/10%Pd-C or 1*N* NaOH/MeOH.



Scheme 3. Schematic representation of the synthesis of tricosapeptides by [(5 + 5 + 5) + (5 + 5 + 5)] fragment coupling method. *i*. EDCI/HOBt, NMM, *ii*. TFA, *iii*. 1*N* NaOH/MeOH.



Scheme 4. Schematic representation of the coupling of amino acids/peptides to heterocycle **1**. Reagents and conditions: (a) EDCI/HOBt, NMM, 0 °C, overnight at rt (b) Polymer supported $HCOO^{-}NH^{+}{}_{3}$/10% Pd–C (c) TFA, 40 min,rt. Xaa = Phe, Trp, Tyr(2,6-Cl_2-Bzl), GGAP, GGIP, GGFP, GVGVP, GFGFP, GE(OcHx)GFP GVGVP GVGVP GVGVP GFGFP GFGFP, GE (OcHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP. Ybb = Phe, Trp, Tyr, GGAP, GGIP, GGFP, GVGVP, GFGFP, GEGFP GVGVP GVGVP GVGVP GFGFP GFGFP, GEGFP GVGVP GVGFP GFGFP GVGVP GVGFP.

facilitate to acquire better conformation of the molecule to trespass microbial environment.

Finally our stress on the existing study is to ponder over the consequences that may occur out of conjugation. In order to further refine this theme interest, we conducted an experiment in which equal amount of peptide and heterocycle were just mixed in required volume of DMSO to get the concentration of 50 μg/mL and performed the assay. The results revealed that there was only very less or no inhibition of the growth of microorganisms by this technique (>50 μg/mL). Our findings, thus, represent a break through in the longstanding impasse on the effect of conjugation. This observation provided the fact that the highly potent activity of these analogs is not due to the additive effect irrespective of the rank of hydrophobicity and length of the peptide chain and only it is because of conjugative effect which would provide additional interaction with the microbial cell membranes.

In summary, the present study involved the development of several conjugates of elastin peptides and benzisoxazole moiety

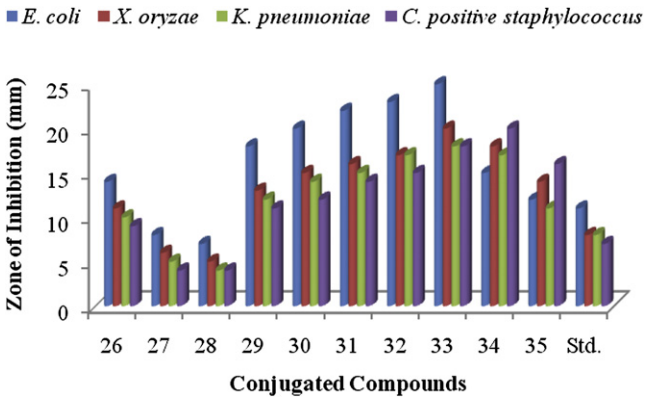


Fig. 1. Diagramatic representation of antibacterial activity of the synthesized conjugates by agar well diffusion method.

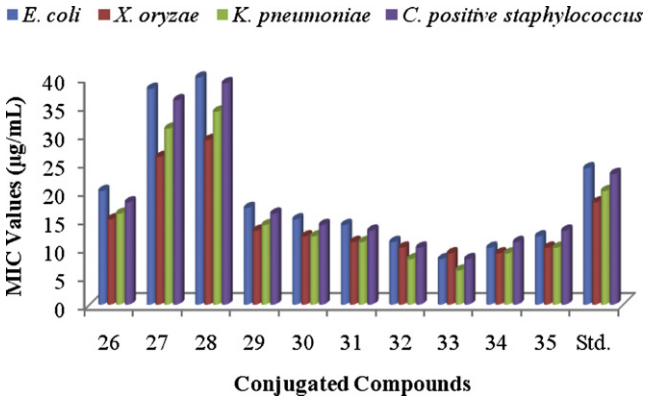


Fig. 2. Diagramatic representation of antibacterial activity of the synthesized conjugates by microdilution method.

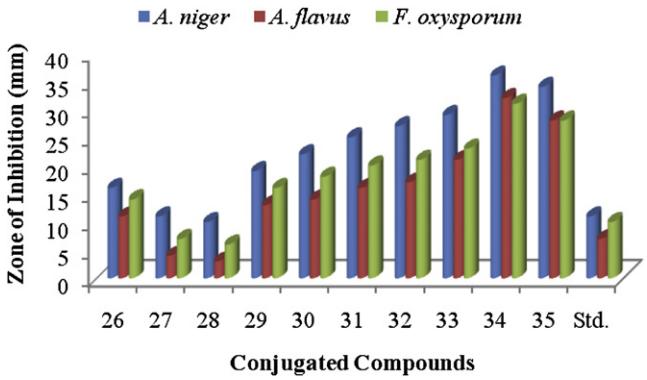


Fig. 3. Diagramatic representation of antifungal activity of the synthesized conjugates by agar well diffusion method.

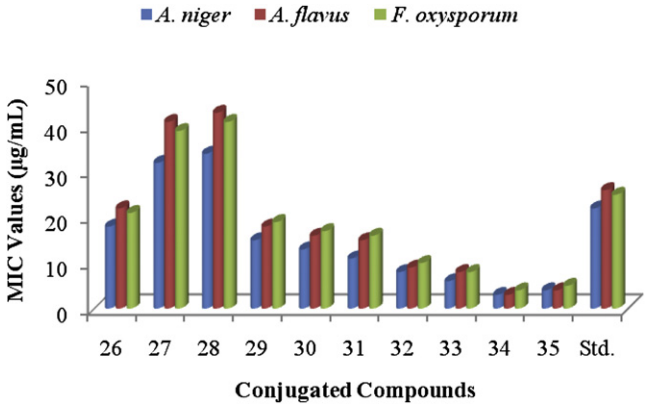


Fig. 4. Diagramatic representation of antifungal activity of the synthesized conjugates by microdilution method.

having high potency as antimicrobials. Results of variation of different amino acid residues in different positions and also the chain length will be the subject of future reports.

3. Conclusion

Several amino acids/elastin based peptides were conjugated to benzisoxazole derivative and some representatives of this series were identified as novel and highly potent antimicrobial agents. Our studies revealed that conjugation plays a paramount role in inhibiting the growth of microorganisms tested. All the peptide conjugates have shown improved antimicrobial activity (6–19 μg/mL) compared to their precursors tested alone (>50 μg/mL) where as amino acids conjugates have shown moderate activity (15–43 μg/mL). Further, conjugates of tricosamers have revealed extraordinary activity (3–5 μg/mL) against all the fungal species tested which were found to be almost five times more potent than the reference drug used. Thus, these analogs could be regarded as most promising antimicrobial agents and developed as lead.

4. Experimental

4.1. Materials

All the amino acids used except glycine were of *L*-configuration unless otherwise mentioned. All Boc-amino acids, EDCI, HOBt and TFA were purchased from Advanced Chem. Tech. (Louisville, Kentucky, USA). IBCF and NMM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents and reagents used for the synthesis were of analytical grade. All the chemicals and reagents used for antimicrobial studies were of bacteriological grade unless otherwise indicated. Nutrient broth and nutrient agar were purchased from Hi-media chemicals (Mumbai, India). Silica gel (60–120 mesh) for column chromatography was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). The pathogens used for the microbial studies were obtained from a local hospital. The progress of the reaction was monitored by TLC using silica gel coated on glass plates with the solvent system comprising chloroform/methanol/acetic acid in the ratio 95:5:3 throughout the study and the compounds on TLC plates were detected by iodine vapors. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. All the HPLC analyses were performed with Lachrom-2000 Merck-Hitachi L7100 pump with RP18.250–4 mm column and UV detector-UV-VISL 7400. ^{1}H NMR spectra were obtained on VARIAN 400 MHz instrument using $CDCl_3$ and the chemical shifts are reported as parts per million (δ ppm) using TMS as an internal standard. Mass spectra were obtained on LCMSD-Trap-XCT instrument.

4.2. Synthesis

The [3-(4-piperidyl)-6-fluoro-1,2-benzisoxazole]HCl **1** was prepared as previously reported [20]. The peptides were synthesized by classical solution phase method using Boc chemistry. The Boc group used for temporary N^{α} protection was removed with 4 N HCl in dioxane or TFA. The *C* terminus carboxyl group was protected by the benzyl ester and its removal was effected by hydrogenolysis using $HCOONH_4$ as hydrogen donor and 10% Pd on carbon as catalyst [21] or hydrolysed using 1 N NaOH/MeOH. The hydroxyl group of Tyr was protected by 2,6-Cl_2-Bzl and γ-carboxyl group of Glu was protected by cyclohexyl ester and removed by treatment with polymer supported $HCOO^{-}NH_3^{+}$, 10% Pd–C [22]. All the coupling reactions for peptide synthesis were achieved with IBCF. The procedure followed for the synthesis of tetrapeptides, pentapeptides and tricosamers are outlined in the Scheme 1–3 respectively. The protected peptides were purified by column chromatography over silica gel and characterized by physical and analytical techniques. The purity of free peptides was checked by HPLC and found to have purity >95% in each case.

4.2.1. Synthesis of tetrapeptides

The tetrapeptide Boc-GGXP-OH [where X = Ala (**9**), Ile (**10**) and Phe (**11**)] was synthesized by a stepwise approach as shown in Scheme 1. In this stepwise approach, Boc-X-Pro-OBzl (X = Ala, Ile and Phe) was synthesized by the mixed anhydride method in the presence of HOBt, deblocked, and coupled with Boc-Gly-OH to obtain Boc-Gly-X-Pro-OBzl. This was further deblocked, and coupled with Boc-Gly-OH to obtain Boc-Gly-Gly-X-Pro-OBzl. This was then hydrogenolysed to the corresponding free acid using $HCOONH_4$/10% Pd–C.

4.2.2. Synthesis of pentapeptides

The pentapeptide Boc-GZGZ′P-OH [where Z = Z′ = Val for GVGVP; Z = Z′ = Phe for GFGFP; Z = Val and Z′ = Phe for GVGFP; Z = Glu(OcHx) and Z′ = Phe for GE(OcHx)GFP] was synthesized by (3 + 2) coupling strategy, the tripeptide Boc-Gly-Z-Gly-OH and the dipeptide HCl·NH_2-Z′-Pro-OBzl were synthesized separately and coupled to obtain the pentamer as shown in Scheme 2. In this approach, Boc-Z′P-OBzl was synthesized by the mixed anhydride method in the presence of HOBt, deblocked to obtain HCl·NH_2-Z′P-OBzl. On the other hand, Boc-Z-Gly-OBzl was synthesized by the

mixed anhydride method, deblocked and coupled to Boc-Gly-OH to obtain Boc-Gly-Z-Gly-OBzl, hydrogenolysed using $HCOONH_4$/10% Pd–C or hydrolysed using 1 N NaOH/MeOH to get Boc-Gly-Z-Gly-OH. Now the tripeptide and dipeptide fragments were coupled by the mixed anhydride method to obtain Boc-Gly-Z-Gly-Z′-Pro-OBzl. These were then treated with $HCOONH_4$/10% Pd–C or 1 N NaOH/MeOH to get corresponding free acid.

4.2.3. Synthesis of tricosamers

The tricosapeptides Boc-GE(OcHx)GFP GVGVP GVGVP GVGVP GFGFP GFGFP-OH (**14**) and Boc-GE(OcHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OH (**15**) were synthesized by the [(5 + 5 + 5) + (5 + 5 + 5)] fragment coupling strategy in solution phase as shown in Scheme 3. The pentamers required for this purpose were synthesized with appropriate side chain protection as described above. Among the tricosamers **14** and **15**, synthesis of **15** has been given below and the synthesis of **14** was followed in a similar way. In this approach, Boc-GVGVP-GVGFP-OBzl was synthesized using EDCI/HOBt, deblocked, and coupled to Boc-GFGFP-OH to obtain Boc-GFGFP-GVGVP-GVGFP-OBzl. This fragment was deblocked further to get TFA.GFGFP-GVGVP-GVGFP-OBzl. In an another appr oach, Boc-GVGVP-GVGFP-OBzl was synthesized using EDCI/HOBt, deblocked, and coupled to Boc-GE(OcHx)GFP-OH to get Boc-GE (OcHx)GFP-GVGVP-GVGFP-OBzl. This fragment was hydrolysed using 1 N NaOH/MeOH to get Boc-GE(OcHx)GFP-GVGVP-GVGFP-OH. Now the 15mer fragments were coupled using EDCI/HOBt to get Boc-GE(OcHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OBzl, hydrolysed using 1 N NaOH/MeOH to get Boc-GE(OcHx)GFP GVGVP GVGFP GFGFP GVGVP GVGFP-OH.

4.2.4. General procedure for the hydrogenolysis of benzyl esters of peptides

Each peptide **2**–**6** (0.006 mol) was hydrogenolysed in methanol (10 mL/g of peptide) using ammonium formate (2.0 equiv.) and 10% Pd–C (0.1 g/g of peptide) for 30 min at room temperature. The completion of the reaction was monitored by TLC. The catalyst was filtered and washed with methanol. The combined washings and filtrate were evaporated and the residue taken into $CHCl_3$, washed with water and dried over anhydrous Na_2SO_4. The solvent was removed under pressure and triturated with ether, filtered, washed with ether and dried to obtain debenzylated peptides (**9**–**13**).

4.2.5. General procedure for the hydrolysis of benzyl esters of tricosapeptides

Each tricosamer **7**, **8** (0.006 mol) was hydrolysed in methanol (10 mL/g of peptide) using cold solution of 1 N NaOH (30 mL) for 2 h. The completion of the reaction was monitored by TLC, solvent was evaporated, cooled, neutralized with cold 1 N HCl, extracted with $CHCl_3$, washed with 1 N HCl followed by water and dried over anhydrous Na_2SO_4. The solvent was removed under pressure and triturated with ether, filtered, washed with ether and dried to obtain debenzylated peptides (**14**, **15**).

The *C* terminal free peptides so obtained were characterized by R_f values and M.P. (°C) and the data are as follows: (**9**) R_f 0.47; M.P. 67–69 (Lit. 66) [23] (**10**) R_f 0.54; M.P. 99 (Lit. 99) [23] (**11**) R_f 0.44; M.P. 106–109 (Lit. 105) [23] (**12**) R_f 0.45; M.P. 125 (Lit. 127) [24] (**13**) R_f 0.46; M.P. 117 (Lit. 118) [24] (**14**) R_f 0.48; M.P. 200–203 (**15**) R_f 0.46; M.P. 166–169.

*4.2.6. General procedure for the coupling of benzisoxazole derivative **1** with Boc-Xaa-OH where Xaa = Phe, Trp, Tyr(2,6-Cl_2-Bzl) and peptides **9**–**15***

To Boc-Xaa-OH (0.005 mol) and HOBt (0.765 g, 0.005 mol) dissolved in DMF (10 mL/g of peptide) and cooled to 0 °C was added NMM (0.55 mL, 0.005 mol). EDCI (0.956 g, 0.005 mol) was added under stirring while maintaining the temperature at 0 °C. The reaction mixture was stirred for an additional 10 min and pre-cooled solution of [3-(4-piperidinyl)-6-fluoro-1,2-benzisoxazole] HCl (1.285 g, 0.005 mol) and NMM (0.55 mL, 0.005 mol) in DMF (13 mL) was added slowly (Scheme 4). After 20 min, pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight at room temperature. DMF was removed under reduced pressure and the residue was poured into about 200 mL ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The precipitated product was taken into $CHCl_3$ and washed with 5% $NaHCO_3$ solution (2 × 20 mL), water (2 × 20 mL), 0.1 N cold HCl solution (2 × 20 mL) and finally brine solution (2 × 20 mL). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The products so obtained were recrystallized from ether/petroleum ether to get white colored desired conjugates (**16**–**25**).

*4.2.7. Deprotection of 2,6-Cl_2-Bzl of Tyr and OcHx of Glu of the conjugates **18**, **24** and **25***

To a solution of the conjugates **18**, **24** and **25** (0.001 mol) in methanol (10 mL/g of compound), 10% Pd–C (100 mg) and polymer supported formate (1 g) were added and the mixture was stirred at room temperature for 8 h. After completion of the reaction monitored by TLC, catalyst and the polymer were filtered, washed with methanol. The solvent was evaporated under reduced pressure and the product was taken into $CHCl_3$, washed with saturated NaCl and the solvent was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether and dried to get side chain deprotected conjugates.

4.2.8. General procedure for the deblocking of Boc-Ybb-Heterocycle

Each conjugate (**16**–**25**) (0.001 mol) was deblocked with TFA (10 mL/g of compound) for 40 min. The excess solvent was removed under reduced pressure, triturated with ether, filtered, washed with ether and dried under vacuum (yield 100%) to obtain TFA·NH_2-Ybb-Heterocycle (**26**–**35**) and these were used for the antibacterial and antifungal studies.

4.3. Biology

4.3.1. Antibacterial activity

In vitro antibacterial activity was evaluated against human pathogens of both gram positive organisms namely *C. positive staphylococcus* and *K. pneumoniae* and gram negative organisms namely *X. oryzae* and *E. coli* by agar well diffusion method (Fig. 1) as well as microdilution method (Fig. 2).

4.3.1.1. Agar well diffusion method. The microorganisms were inoculated in to the sterilized nutrient broth and maintained at 37 °C for 24 h. On the day of testing, bacteria were subcultured separately into 25 mL of sterilized nutrient broth. Inoculated sub-cultured broths were kept at room temperature for the growth of inoculums. Each test compounds (**26**–**35**) and standard drug (amoxicillin) of 10 mg was dissolved in 10 mL of DMSO to get a concentration of 1 mg/mL and further diluted to get a final concentration of 50 μg/mL. About 15–20 mL of molten nutrient agar was poured into each of the sterile plates. With the help of cork borer of 6 mm diameter, the cups were punched and scooped out of the set agar and the plates were inoculated with the suspension of particular organism by spread plate technique. The cups of inoculated plates were then filled with 0.1 mL of the test solution, amoxicillin solution and DMSO (negative control). The plates were allowed to stay for 24 h at 37 °C and zone of inhibition (mm) was then measured.

4.3.1.2. 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 O.D at A_{600}. MIC of the compounds was determined by agar dilution method. Suspension of each microorganism was prepared to contain approximately (1×10^4–2×10^4 CFU/mL) and applied to the plates with serially diluted compounds (dissolved in DMSO) to be tested and also reference drug 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. Zone of inhibition (mm) was measured after 24 h and MIC values were determined.

4.3.2. Antifungal activity

In vitro antifungal activity was evaluated against three fungal species namely *A. niger*, *A. flavus* and *F. oxysporum* by agar well diffusion method (Fig. 3) as well as microdilution method (Fig. 4).

4.3.2.1. Agar well diffusion method. The fungal strains were subcultured separately into 25 mL of sterilized nutrient broth and incubated for one day to obtain the inoculums. Each test compounds (**26**–**35**) and standard drug (bavistin) of 10 mg was dissolved in 10 mL of DMSO to get a concentration of 1 mg/mL and further diluted to get a final concentration of 50 μg/mL. Molten media of Sabouraud agar of 10–15 mL was poured into the petriplates and allowed to solidify. Fungal subculture was inoculated on the solidified media. With the help of 6 mm cork borer, the cups were punched and scooped out of the set agar. The cups of inoculated plates were then filled with 0.1 mL of the test solution, bavistin solution and DMSO (negative control). The plates were allowed to stay for 3 days at room temperature and zone of inhibition (mm) was then measured.

4.3.2.2. Microdilution method. Sabouraud agar was used for the preparation of plates. Suspension of each microorganism was prepared to contain 10^5 CFU/mL. The agar plates were inoculated with fungal strains and serially diluted test compounds and 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. Zone of inhibition (mm) was measured after 72 h and MIC values were determined.

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