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A New Family of Highly Potent Inhibitors of Microbes: Synthesis and Conjugation of Elastin Based Peptides to Piperazine Derivative

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Abstract Elastin protein-based polymers have their origin in repeating sequences of the mammalian elastic protein, elastin. The sequences of elastin peptides chosen are tetrapeptides, pentapeptides and tricosamers (30 amino acids) and also aromatic amino acids. These have been conjugated to 1-(2,3-dichlorophenyl)piperazine to study the effect of conjugation on the activity. The conjugates so obtained were characterized by physical and analytical techniques followed by the antimicrobial evaluation. The study revealed that all the conjugates have exhibited enhanced activity than the conventional drugs. Further, the conjugates of tricosamers have shown extraordinary activity against the fungal species with MIC value of 3–5 μg/ml which is five fold more potent than the antibiotic used.

Keywords Peptides of elastin · Substituted piperazine · Conjugates · Effective antimicrobials · Hydrophobicity · Biocompatibility

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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Introduction

The increasing incidence of infection caused by the rapid development of bacterial resistance to most of the known antibiotics is a serious health problem (Chu et al. 1996). While many factors may be responsible for mutations in microbial genomes, it has been widely demonstrated that the incorrect use of antibiotics can greatly increase the development of resistant genotypes (Clark 1996). As multidrug-resistant bacterial strains proliferate, the necessity for effective therapy has stimulated research into the design and synthesis of novel antimicrobial molecules. Thus, the design and development of new agents that provide effective therapy for infections caused by organisms resistant to older agents is an urgent need (Emami et al. 2006).

The growing interest in heterocyclic compounds is basically because of their raised biological activity and also they make possible development of novel materials with unique properties. One very interesting and promising class of heterocycle is the series of piperazine and its derivatives. Piperazines have been widely used in biological screening resulting in numerous applications and constitute an attractive pharmacological scaffold present in several drugs (Foye et al. 1995). This small and rigid heterocyclic backbone could act on various pharmacological targets. Especially, piperazine nucleus could be found in a broad range of biologically active compounds displaying anticancer (Haga et al. 1985; Guo et al. 2003; Guo et al. 2004), calcium channel blockers (Shanklin et al. 1991; Nomura et al. 1995) and histamine antagonists (Hiristo et al. 1989; Gillard et al. 2002).

Protein-based polymers are polypeptides comprised of repeating sequences of amino acids, having their origin in a protein, elastin (Sandberg et al. 1985; Yeh et al. 1987). The

most striking repeating sequence $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ or $(VPGVG)_n$ is apparent in the bovine and porcine elastins and another repeat $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$, was first found in porcine elastin. The monomers, oligomers, and high polymers of these repeats have been synthesized and conformationally characterized (Gowda et al. 1994). Such protein-based polymers exhibit the same hierarchical structure and mimic the parent protein. These polymers have a number of medical and non-medical applications (Urry et al. 1993, 1996).

Extensive work has been reported on the conjugation of different amino acids/peptides to various biologically active moieties (Shivakumara et al. 2007; Suresha et al. 2009; Suhas et al. 2011; Suresha et al. 2011; Banoczi et al. 2008; Dutta et al. 2009) which reveals that conjugation plays a paramount role in exerting the activity. Also, involving amino acids/peptides in drugs makes them low toxic, ample bioavailability and permeability, modest potency and good metabolic and pharmacokinetic properties (Gadek and Nicholas 2003).

In a continuous effort to develop supplementary antimicrobial agents with improved results, the present work involves the conjugation of highly biocompatible elastin based peptides of varying hydrophobicity with 1-(2,3-dichlorophenyl)piperazine.

Materials and Methods

General

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). 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., (Bombay, 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 using a linear gradient of 0–100% acetonitrile/0.1% TFA (20 min). ^{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.

Synthesis

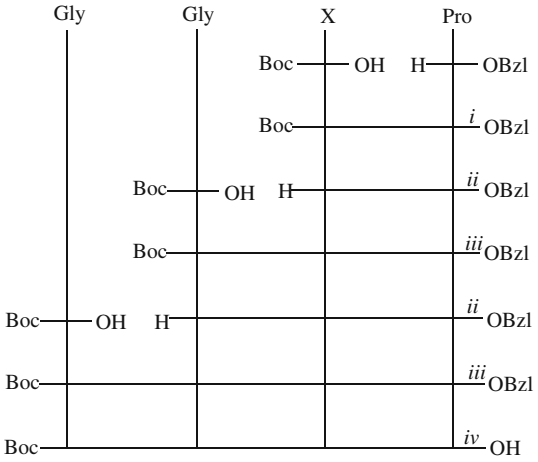
The 1-(2,3-dichlorophenyl)piperazine·HCl **1** was prepared as previously reported method (Oshira et al. 1991). The peptides were synthesized by classical solution phase method using Boc chemistry. The Boc group used for temporary N^{α} protection was removed with 4N HCl in dioxane or TFA. The C terminal 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 (Anwer and Spatola 1980) or hydrolysed using 1N 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 $HCOO^-NH_3^+$ and 10% Pd–C (Abiraj et al. 2005). 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, 2 and 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.

Synthesis of Tetrapeptides, Pentapeptides and Tricosamers

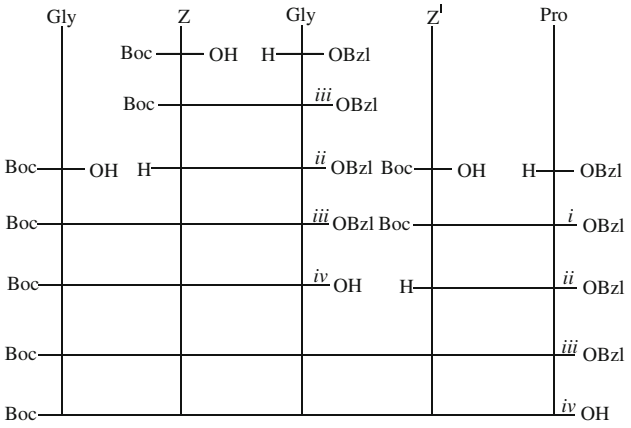
The peptides were synthesized as described in our earlier article (Suhas et al. 2011).

General Procedure for the Hydrogenolysis of Benzyl Esters of Tetra and Pentapeptides

Each peptide **2–6** (0.006 mol) was hydrogenolysed in methanol (10 ml/g of peptide) using ammonium formate (2.0 equi.) 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**).



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^{l}P-OH where Z = Z^{l} = Val for GVGVP; Z = Z^{l} = Phe for GFGFP; Z = Val and Z^{l} = Phe for GVGFP; Z = Glu(OcHx) and Z^{l} = 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 1N NaOH/MeOH

General Procedure for the Hydrolysis of Benzyl Esters of Tricosapeptides

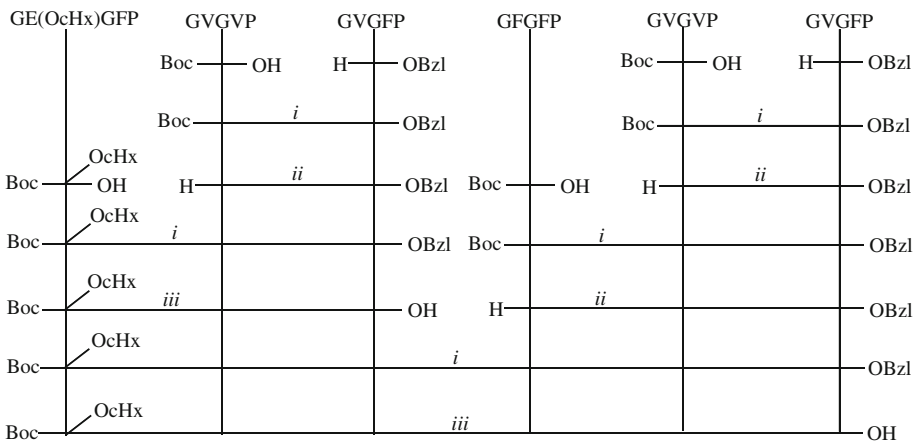
Each tricosamer **7**, **8** (0.006 mol) was hydrolysed in methanol (10 ml/g of peptide) using cold solution of 1 N NaOH (30 ml) for 2 h. The completion of the reaction was monitored by TLC, solvent was evaporated, cooled, neutralized with cold 1N HCl, extracted with $CHCl_3$, washed with 1N HCl followed 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) (Gowda et al. 2002) (**10**) R_f 0.54; M.P. 99 (Lit. 99) (Gowda et al. 2002) (**11**) R_f 0.44; M.P. 106–109 (Lit. 105) (Gowda et al. 2002) (**12**) R_f 0.45; M.P. 125 (Lit. 127) (Gowda et al. 2001) (**13**) R_f 0.46; M.P. 117 (Lit. 118) (Gowda et al. 2001) (**14**) R_f 0.48; M.P. 200–203 (**15**) R_f 0.46; M.P. 166–169.

General Procedure for the Coupling of Piperazine 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 1-(2,3-dichlorophenyl)piperazine·HCl (1.340 g, 0.005 mol) and NMM (0.55 ml, 0.005 mol) in DMF (13 ml) was added slowly. After 20 min, pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred over night at room temperature. DMF was removed under reduced pressure and the residue was poured into about 200 ml

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*) 1N NaOH/MeOH



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), 0.1N 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**). The schematic representation of the coupling is shown in Scheme 4.

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.

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 (Scheme 4). 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.

Biology

Antibacterial Activity

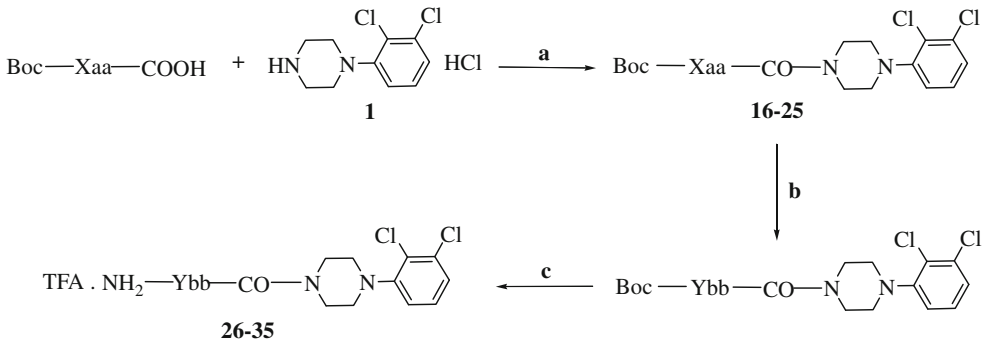
In vitro antibacterial activity was evaluated against human pathogens of both gram positive organisms namely *Coagulase positive staphylococcus* and *Klebsiella pneumoniae* and gram negative organisms namely *Xanthomonas oryzae* and *Escherichia coli* by agar well diffusion method (Fig. 1) as well as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

Agar Well Diffusion Method

The microorganisms were inoculated into 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 subcultured 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.

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}. MIC of the compounds was



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 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

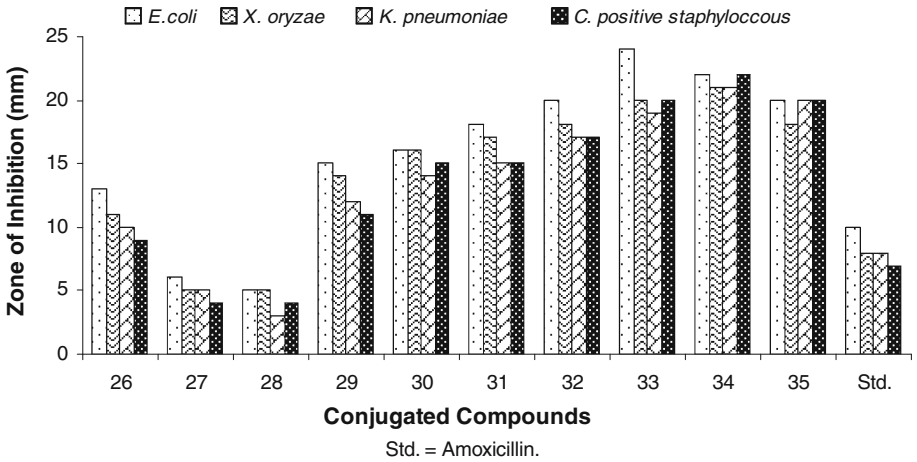


Fig. 1 Diagrammatic representation of antibacterial activity of the synthesized conjugates by agar well diffusion method

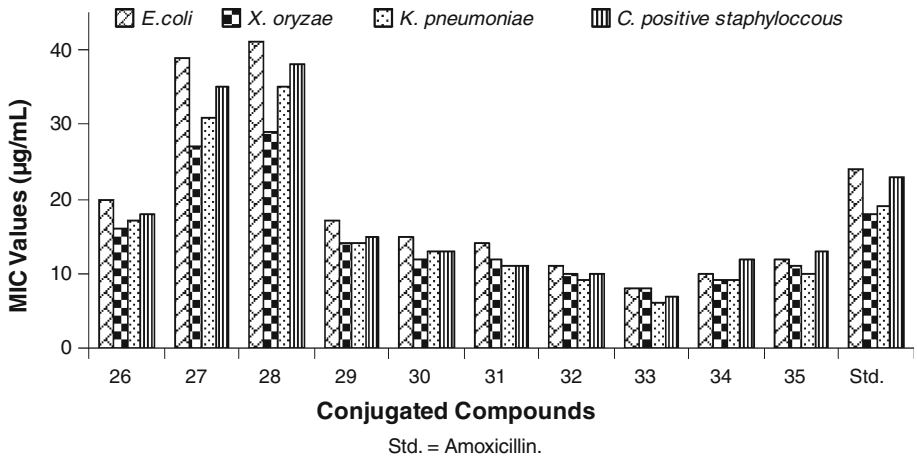


Fig. 2 Diagrammatic representation of antibacterial activity of the synthesized conjugates by microdilution method

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.

Antifungal Activity

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

Agar Well Diffusion Method

The fungal strains were subcultured separately into 25 ml of sterilized nutrient broth and incubated for 1 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.

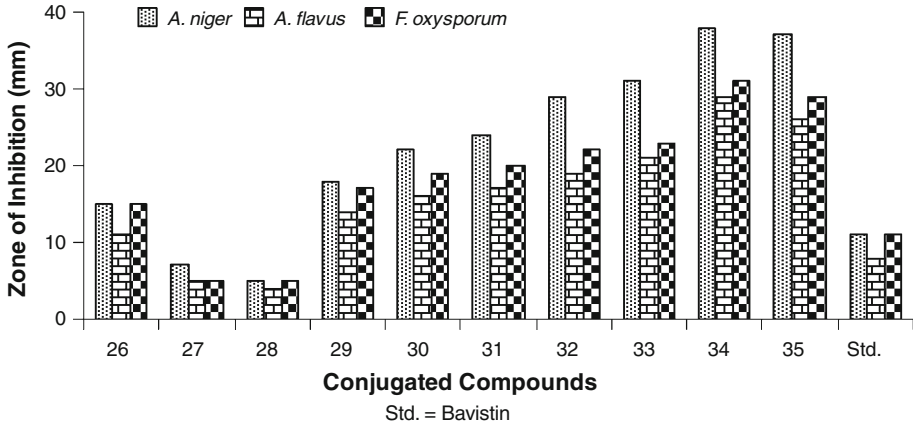


Fig. 3 Diagrammatic representation of antifungal activity of the synthesized conjugates by agar well diffusion method

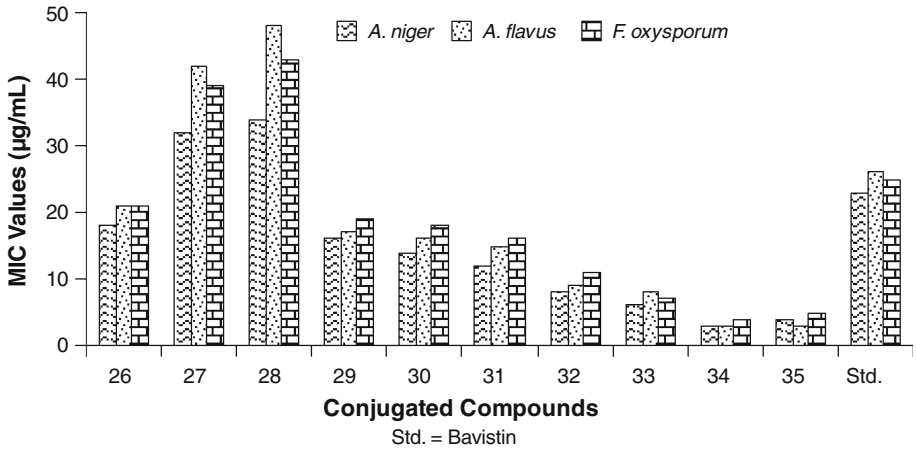


Fig. 4 Diagrammatic representation of antifungal activity of the synthesized conjugates by microdilution method

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.

Results and Discussion

Peptide Synthesis and Characterization

Peptides were synthesized by classical solution phase method using Boc chemistry. These peptides were conjugated to piperazine derivative **1** using EDCI/HOBt as coupling agent and NMM as base. The yields of the compounds were found to be good and characterized by M.P., ^{1}H NMR and mass spectroscopic techniques. ^{1}H NMR data of the final protected peptides (**2**–**8**) are given in Table S1. The physical and analytical data of the conjugated compounds are provided in Table 1. The ^{1}H NMR and mass data were found to be in good agreement with the structures assigned.

Biological Evaluation

The efficacy of the synthesized compounds as antimicrobials were evaluated for their antibacterial studies against different strains of human pathogens of both gram positive bacteria namely *K. pneumoniae* and *C. positive staphylococcus* and gram negative organisms like *E. coli* and *X. oryzae* and antifungal studies against *A. niger*, *A. flavus* and *F. oxysporum*. The results obtained as zone of inhibition (mm) and minimum inhibitory concentration (µg/ml) are presented in Tables 2 and 3 respectively. Amoxicillin and bavistin were used as standard drugs for antibacterial and antifungal assays respectively.

Our earlier investigation (Suhas et al. 2011) revealed promising activity by the conjugation of benzisoxazole

Table 1 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	0.83	88.0	Gummy	478	478.4	Boc = 1.41 (9H, s); Phe = 3.20–3.51 (2H, d, $-^{\beta}CH_2$), 4.94 (1H, t, -$^{\alpha}$CH), 6.89–7.23 (5H, m, ArH), 8.10 (1H, s, –NH); Heterocycle = 3.51–3.74 (8H, m, $-CH_2$), 6.89–7.23 (3H, m, ArH)
17	0.81	90.0	129	517	517.4	Boc = 1.43 (9H, s); Trp = 3.14–3.34 (2H, d, $-^{\beta}CH_2$), 4.95 (1H, t, $-^{\alpha}$CH), 7.08 (1H, s, –CH of indole), 6.64–7.39 (4H, m, ArH), 10.21 (1H, s, -NH of indole), 8.08 (1H, s, –NH); Heterocycle = 3.54–3.71 (8H, m, $-CH_2$), 6.64–7.39 (3H, m, ArH)
18	0.84	96.0	79	653	653.4	Boc = 1.43 (9H, s); Tyr(2,6-Cl_2-Bzl) = 3.21-3.54 (2H, d, $-^{\beta}CH_2$), 4.93 (1H, t, $-^{\alpha}$CH), 5.27 (2H, s, $-CH_2$ of side chain protecting group), 6.72–7.49 (7H, m, ArH), 8.12 (1H, s, –NH); Heterocycle = 3.47–3.74 (8H, m, $-CH_2$), 6.72–7.49 (3H, m, ArH)
19	0.72	90.0	84–86	614	613.5	Boc = 1.40 (9H, s); Gly^1 = 3.87 (2H, s, $-^{\alpha}$CH); Gly^2 = 4.14 (2H, s, $-^{\alpha}$CH); Ala^3 = 1.30 (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 = 3.47–3.74 (8H, m, $-CH_2$), 6.72–7.49 (3H, m, ArH)
20	0.72	90.0	78–81	656	655.6	Boc = 1.42 (9H, s); Gly^1 = 3.81 (2H, s, $-^{\alpha}$CH); Gly^2 = 3.97 (2H, s, $-^{\alpha}$CH); Ile^3 = 1.04–1.06 (6H, d, $-(CH_3)_2$), 1.43 (2H, m, $-^{\gamma}CH_2$), 2.11 (1H, m, $-^{\beta}$CH), 4.60 (1H, m, $-^{\alpha}$CH); Pro^4 = 2.12–3.65 (6H, m, $-CH_2$), 4.57 (1H, m, $-^{\alpha}$CH); Heterocycle = 3.66–3.74 (8H, m, $-CH_2$), 6.90–7.26 (3H, m, ArH)
21	0.64	91.0	60–62	690	711.2 [M^++Na]	Boc = 1.44 (9H, s); Gly^1 = 3.83 (2H, s, $-^{\alpha}$CH); Gly^2 = 3.93 (2H, s, $-^{\alpha}$CH); Phe^3 = 3.18, 3.57 (2H, d, $-^{\beta}CH_2$), 4.93 (1H, t, $-^{\alpha}$CH); 6.99–7.26 (5H, m, ArH); Pro^4 = 1.99–2.00 (2H, m, $-^{\gamma}CH_2$), 2.05, 2.23 (2H, m, $-^{\beta}CH_2$), 3.57–3.62 (2H, m, $-^{\delta}CH_2$), 4.95 (1H, m, $-^{\alpha}$CH); Heterocycle = 2.23–2.94 (8H, m, $-CH_2$), 6.99–7.26 (3H, m, ArH)
22	0.62	94.0	116–118	741	740.7	Boc = 1.41 (9H, s); Gly^1 = 3.82 (2H, s, $-^{\alpha}$CH); Val^2 = 0.96 (6H, m, $(-CH_3)_2$), 2.58 (1H, m, $-^{\beta}$CH), 4.50 (1H, m, $-^{\alpha}$CH); Gly^3 = 4.13 (2H, s, $-^{\alpha}$CH); Val^4 = 0.96 (6H, m, $(-CH_3)_2$), 2.63 (1H, m, $-^{\beta}$CH), 4.49 (1H, m, $-^{\alpha}$CH); Pro^5 = 2.11–3.64 (6H, m, $-CH_2$), 4.52 (1H, m, $-^{\alpha}$CH); Heterocycle = 2.23–2.94 (8H, m, $-CH_2$), 6.99–7.26 (3H, m, ArH)
23	0.66	89.0	104–106	837	858.3 [M^++Na]	Boc = 1.42 (9H, s); Gly^1 = 3.82 (2H, s, $-^{\alpha}$CH); Phe^2 = 3.52, 3.58 (2H, m, $-^{\beta}CH_2$), 4.64 (1H, m, $-^{\alpha}$CH), 6.91–7.28 (5H, m, ArH); Gly^3 = 4.12 (2H, s, $-^{\alpha}$CH); Phe^4 = 3.47, 3.60 (2H, m, $-^{\beta}CH_2$), 4.66 (1H, m, $-^{\alpha}$CH), 6.91–7.28 (5H, m, ArH); Pro^5 = 2.11–3.64 (6H, m, $-CH_2$), 4.51 (1H, m, $-^{\alpha}$CH); Heterocycle = 3.40–3.73 (8H, m, $-CH_2$), 6.91–7.28 (3H, m, ArH)
24	0.73	88.0	167–170	3136	3142.2	Boc = 1.67 (9H, s); Gly = 4.09, 4.92 (24H, m, $-^{\alpha}$CH); Val = 0.98, 1.01 (36H, m, $(-CH_3)_2$), 2.45, 2.65 (6H, m, $-^{\beta}$CH), 4.41 (6H, m, $-^{\alpha}$CH); Glu = 1.15–1.25 (10H, m, $-CH_2$ of cyclohexyl ring), 2.07, 2.19 (4H, m, $-^{\beta,\gamma}CH_2$), 3.71 (1H, m, –CH of cyclohexyl ring), 4.74 (1H, m, $-^{\alpha}$CH); Phe = 3.52, 3.72 (10H, m, $-^{\beta}CH_2$), 4.21, 4.67 (5H, m, $-^{\alpha}$CH), 7.19–7.62 (25H, m, ArH); Pro = 2.02, 3.55, 3.69 (36H, m, $-CH_2$), 4.75 (6H, m, $^{\alpha}$–CH); Heterocycle = 3.40, 3.64 (8H, m, $-CH_2$), 7.19–7.62 (3H, m, ArH)
25	0.74	89.0	185–188	3136	3142.2	Boc = 1.62 (9H, s); Gly = 4.08, 4.90 (24H, m, $-^{\alpha}$CH); Val = 0.98, 1.00 (36H, m, $(-CH_3)_2$), 2.44, 2.62 (6H, m, $-^{\beta}$CH), 4.41 (6H, m, $-^{\alpha}$CH); Glu = 1.09–1.25 (10H, m, $-CH_2$ of cyclohexyl ring), 2.07, 2.17 (4H, m, $-^{\beta,\gamma}CH_2$), 3.68 (1H, m, –CH of cyclohexyl ring), 4.70 (1H, m, $-^{\alpha}$CH); Phe = 3.52, 3.70 (10H, m, $-^{\beta}CH_2$), 4.21, 4.59 (5H, m, $-^{\alpha}$CH), 7.20–7.62 (25H, m, ArH); Pro = 2.02, 3.55, 3.69 (36H, m, $-CH_2$), 4.75 (6H, m, $^{\alpha}$–CH); Heterocycle = 3.41, 3.64 (8H, m, $-CH_2$), 7.20–7.62 (3H, m, ArH)

derivative with elastin based peptides. Encouraged by this, in the present study it was felt worthy to link piperazine moiety to these peptides. It is evident from the results that all the heterocycle conjugated amino acids/peptides have exhibited enhanced activity compared to heterocycle or free peptides tested alone which are inactive or weakly active (>50 μg/ml).

Earlier studies report the significance of activity revealed by aromaticity and hydrophobicity (Shivakumara et al. 2007; Suresha et al. 2009, 2011; Suhas et al. 2011) and hence initially more hydrophobic and aromatic amino acids such as Phe, Trp and Tyr were selected for conjugation. The results revealed that the Phe coupled heterocycle (**26**) has shown improved activity. Surprisingly, other

Table 2 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	13 ± 0.26	11 ± 0.20	10 ± 0.30	09 ± 0.20	15 ± 0.36	11 ± 0.15	15 ± 0.10
27	06 ± 0.21	05 ± 0.10	05 ± 0.10	04 ± 0.11	07 ± 0.20	05 ± 0.05	05 ± 0.15
28	05 ± 0.26	05 ± 0.05	03 ± 0.30	04 ± 0.26	05 ± 0.10	04 ± 0.41	05 ± 0.47
29	15 ± 0.47	14 ± 0.30	12 ± 0.36	11 ± 0.47	18 ± 0.40	14 ± 0.25	17 ± 0.45
30	16 ± 0.25	16 ± 0.05	14 ± 0.30	15 ± 0.43	22 ± 0.41	16 ± 0.34	19 ± 0.30
31	18 ± 0.28	17 ± 0.25	15 ± 0.10	15 ± 0.20	24 ± 0.36	17 ± 0.25	20 ± 0.55
32	20 ± 0.30	18 ± 0.37	17 ± 0.40	17 ± 0.17	29 ± 0.52	19 ± 0.26	22 ± 0.25
33	24 ± 0.15	20 ± 0.36	19 ± 0.23	20 ± 0.30	31 ± 0.40	21 ± 0.32	23 ± 0.41
34	22 ± 0.20	21 ± 0.36	21 ± 0.26	22 ± 0.30	38 ± 0.43	29 ± 0.32	31 ± 0.30
35	20 ± 0.30	18 ± 0.32	20 ± 0.40	20 ± 0.05	37 ± 0.47	26 ± 0.26	29 ± 0.10
Heterocycle	03 ± 0.15	04 ± 0.25	02 ± 0.17	02 ± 0.32	04 ± 0.26	03 ± 0.20	03 ± 0.20
Amoxicillin	10 ± 0.20	08 ± 0.66	08 ± 0.11	07 ± 0.25	–	–	–
Bavistin	–	–	–	–	11 ± 0.25	08 ± 0.15	11 ± 0.17

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

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, it was found that substitution of Phe by other aromatic amino acids is not effective in exerting potent activity.

Based on this observation we were very much projected towards the conjugation of elastin based peptides with varying chain length and hydrophobicity. 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 exhibited more activity i.e., GGFP with Phe unit has exerted nearly two times more potent activity than the antibiotics used where as the other two tetrapeptides conjugated heterocycle have shown comparatively less activity than GGFP which could be due to less hydrophobicity of amino acids present at the second position. Thus the order of activity among tetrapeptide conjugates is GGFP > GGIP > GGAP.

In the light of the above findings, our subsequent goal was to incorporate GVGVP and GFGFP pentapeptide elastin sequences for conjugation. The pentapeptide conjugates have shown more than two fold potent activity compared to reference drugs used. Among these, GFGFP conjugate (**33**) has shown slight enhancement in the activity over GVGVP conjugate (**32**) which may be due to the presence of two more hydrophobic Phe units in **33**.

Inspection of the results further revealed that the conjugate **26** having single Phe residue has shown enhanced activity. On the other hand, retaining of only one Phe unit and increasing the peptide chain length has caused further increase in the activity (~two times) which is evident from the conjugate GGFP (**31**). Similarly, the remaining two tetrapeptide conjugates GGIP and GGAP have shown increased activity compared to Phe alone (**26**). Also, when the length of the peptide chain increased as well as two Phe residues were introduced (greater hydrophobicity) as in GFGFP (**33**) has exhibited two times greater activity than the standard drugs. This trend was followed in GVGVP conjugate (**32**) also. Hence, it can be inferred that as the length of the peptide chain as well as the hydrophobicity increases the activity also increases (Suresha et al. 2009; Suhas et al. 2011). Thus, the order of activity of the heterocyclic conjugated peptides is found to be GFGFP > GVGVP > GGFP > GGIP > GGAP > Phe > Trp > Tyr.

In view of this, interest was generated to include peptides having improved hydrophobicity profile as well as a polar species, Glu in the chain which is exhibited by tricosamers **7** and **8**. Tricosamers prepared elsewhere in order to study the hydrophobicity-induced pKa shifts were used as such. Though the antibacterial activity of the conjugates of tricosamers **34** and **35** has shown almost two fold more potent activity than the reference drug it is less compared to pentapeptide conjugate **33**. The reason for this could be attributed to the long peptide chain length irrespective of being a more hydrophobic entity which would have resulted in the difficult passage of the molecule across the bacterial cell membrane.

On the contrary, tricosamers conjugated compounds **34** and **35** have exerted enhanced antifungal activity which

Table 3 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	16	17	18	18	21	21
27	39	27	31	35	32	42	39
28	41	29	35	38	34	48	43
29	17	14	14	15	16	17	19
30	15	12	13	13	14	16	18
31	14	12	11	11	12	15	16
32	11	10	09	10	08	09	11
33	08	08	06	07	06	08	07
34	10	09	09	12	03	03	04
35	12	11	10	13	04	03	05
Heterocycle	>50	>50	>50	>50	>50	>50	>50
Peptides	>50	>50	>50	>50	>50	>50	>50
Amoxicillin	24	18	19	23	–	–	–
Bavistin	–	–	–	–	23	26	25

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

were able to arrest the fungal growth at a concentration of 3–5 μg/ml which is five fold more potent than bavistin, the antibiotic used in the assay. Hence, the conjugates of tricosamers could be identified as highly potent antifungal agents.

The theme interest of the present investigation was to shed some light on the importance of the consequences of conjugation. Hence, we performed the antimicrobial assay in which equal amount of peptide and heterocycle were just mixed in required volume of DMSO to get the concentration of 50 μg/ml. The results revealed that there was only very less or no inhibition of the growth of microorganisms by this technique (>50 μg/ml). This observation led to the fact that the highly potent activity of these analogs (**26–35**) is because of conjugation which aids in providing additional interaction with the cell membrane there by arresting the growth of microbes.

These observations were noticed in our earlier report (Suhas et al. 2011) and hence it may be concluded that both benzisoxazole and piperazine conjugates exhibit similar type of activity. The present study thus provides a platform for the future reports which involves the variation of different amino acid residues at different positions as well as the chain length of the peptides.

Conclusion

This report highlights the importance of conjugation wherein all the peptides conjugated heterocycle have exhibited potent antimicrobial activity (6–19 μg/ml) than either heterocycle or peptides (>50 μg/ml) whereas the conjugates of amino acids displayed moderate activity. Of particular importance, the conjugates of tricosamers deserves a special mention as they have revealed extraordinary activity (3–5 μg/ml) in arresting the growth of all the fungal strains tested which is found to be five fold more potent than the antibiotic used. Hence, these could be developed as lead antimicrobial agents.

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