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Design and Synthesis of Heterocyclic Conjugated Peptides as Novel Antimicrobial Agents

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Abstract Antimicrobial peptides have been recognized as a novel class of antibiotics and several candidates are currently in clinical trials. In the present study, new antimicrobial compounds were synthesized by coupling quinazolinone moiety with the fragments of elastin sequences VP, GVP, VGVP and GVGVP. They were evaluated for their antibacterial activity against both gram positive and gram negative bacterial strains. We are here reporting that heterocyclic conjugated tetra peptide and penta peptide showed enhanced antibacterial activity compare to the conventional antimicrobial drugs.

Keywords Antimicrobial drugs · Heterocyclic conjugated peptides · Synthesis

Introduction

A prolonged and extensive clinical use of classical antibiotics has led to widespread increase in resistant pathogenic bacteria (Nathan 2004; Wenzel 2004). Antibiotics interfere with the bacterial homeostasis. Hence, the bacteria are known to evolve resistance through mechanisms such as preventing the antibiotic from binding to or entering the organism, producing an enzyme that inactivates the antibiotic, and/or changing the internal binding site of the antibiotic (Projan 2003; Margareta et al. 2002). Therefore, it is of considerable interest to find antibiotic with a new mechanism of action, which can potentially evade the emergence of resistance. Hence the identification of new potential antimicrobial agents is significantly important in the present scenario.

Antimicrobial peptides are widespread in nature and constitute an effective component in natural immunity for host defense against microbial agents (Michael 1992). They provide immediate response to invading microbes and display broad spectrum of bactericidal and bacteriostatic actions. Various kinds of antimicrobial peptides have been identified from multicellular organisms, such as animals (Ganz and Lehrer 1997), plants (Broekaert et al. 1997) and insects (Lamberty et al. 2001). It has been suggested that the mode of action of these compounds on the membrane of microbes involves formation of ion-channel pores spanning the membranes without requiring a specific target receptor (Vaara and Porro 1996). Further studies have been devoted to improve activity, specificity and toxicity of antimicrobial peptides and to broaden clinical applications as next generation antimicrobials with high potential.

According to the Indian Ayurvedic System of Medicine, quinazolinone alkaloids are used as remedial practices to cure for cold, cough, bronchitis, rheumatism, phthisis and asthma (Jain et al. 1980). Recently, (−) vasicinone and its analogues were synthesized and characterized for antitumor, bronchodilating, hypotensive, anthelmintic and antianaphylactic activities (Santhosh and Narasimha 2001). Pegamine has been isolated from Peganum harmala, subsequently synthesized, exhibits cytotoxic activity. Luotonins isolated from *Peganum nigellastrum* shows antitumor activity (Santhosh and Narasimha 2001) and synthetic Luotonins series were analyzed for antimicrobial activity (Ross Kelly et al. 1999). The heterocyclic precursor **1** is the main structural component of these compounds which in presence of bulky group showed antimicrobial activity.

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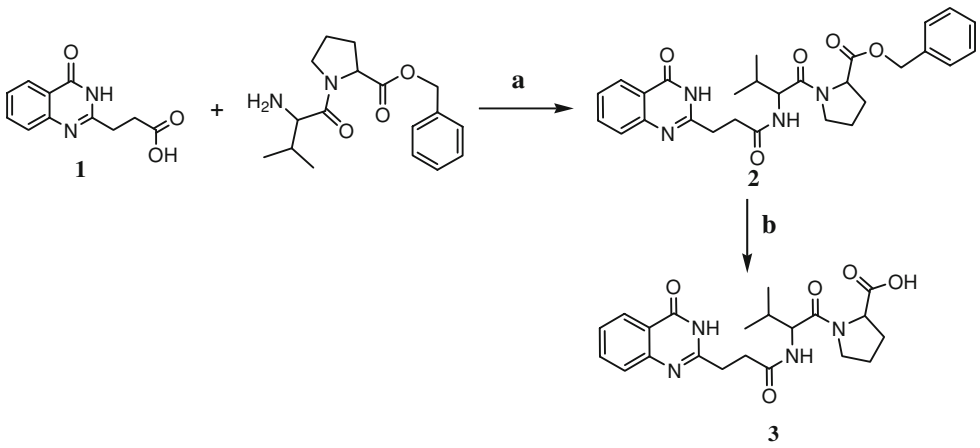
Scheme 1 Synthesis of heterocyclic conjugated peptides. **a** EDCI/HOBt and DIEA. **b** HCO_2NH_4/10% Pd-C



Keeping all these rational points in mind, we have developed novel heterocyclic conjugated peptides by coupling peptides with heterocyclic precursor **1**. The resultant synthesized compounds are characterized and evaluated for antimicrobial activity against both gram positive and gram negative bacteria.

Materials and Methods

Reagents

All chemicals and reagents were obtained from Aldrich (USA), Spectrochem Pvt. Ltd. (India) and Rankem Pvt. Ltd. (India) and were used without further purification. The amino acids used were L-series unless mentioned. The ^{1}H NMR spectra were recorded on 300 MHz Bruker FT-NMR Spectrometer. The chemical shifts were reported as parts per million (δ ppm) using tetra methylsilane (TMS) as an internal standard. Mass spectra were obtained on LCMSD-Trap-XCT instrument. Agilent-1100 HPLC was used to find out the purity of the synthesized compounds. The progress of the reaction was monitored on pre coated silica gel plates (Merck) using chloroform/methanol/acetic acid (8.7:1:0.3) as a solvent system. The bacterial strains used were obtained from Department of Studies in Biotechnology, University of Mysore, India, namely, *Bacillus substilis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Xanthomonas campestris* pvs and *Xanthomonas oryzae*.

Synthesis

The 3-(4-oxo-3,4-dihydroquinazolin-2-yl)propanoic acid **1** was synthesized as previously reported using anthranilide and succinic anhydride (Santhosh and Narasimha 2001; Witt and Bergman 2000). The peptides were synthesized by stepwise solution phase method using Boc chemistry. The Boc group was used for temporary N^{α}-protection and its removal was achieved with TFA. The series of compounds **2**, **4**, **6**, **8** possess precursor **1** coupled with benzyl esters of VP, GVP, VGVP and GVGVP peptides, respectively and compounds **3**, **5**, **7**, **9** were their hydrolyzed product. The carboxyl group was protected by benzyl ester and its removal was effected by hydrogenolysis using HCO_2NH_4 as hydrogen donor and 10% Pd on carbon as catalyst (Anwer and Spatola 1980). The resulting peptides were coupled with **1** as shown in the Scheme 1.

General Procedure for the Coupling of Benzyl Esters of Peptides With 3-(4-Oxo-3,4-Dihydroquinazolin-2-yl)Propanoic Acid

To the stirred solution of 3-(4-oxo-3,4-dihydroquinazolin-2-yl)propanoic acid (0.20 g, 0.91 mmol) in DMF (5.0 ml) cooled to 0°C with crushed ice, added TFA.VP-OBzl (0.38 g, 0.91 mmol), DIEA (0.67 ml, 3.67 mmol), HOBt (0.30 g, 1.39 mmol), EDCI (0.42 g, 1.9 mmol) and THF (5 ml). The reaction mixture was stirred over night while slowly warming to room temperature. The reaction was quenched with H_2O (2 ml) and the solvent was removed. The residue was dissolved in EtOAc (30 ml) and the organic layer was washed with 1 N HCl (1 × 20 ml), saturated $NaHCO_3$ solution (1 × 20 ml), brine (1 × 20 ml).Then the organic layer was dried over $MgSO_4$ and concentrated at high vacuum. Chromatography (10% EtOAc-Hexane) over silica gel (100-200 mesh) provided compound **2** as white hygroscopic foam (Yield: 85%).

Antibacterial Assay

In vitro antibacterial assays were performed against *B. substilis*, *E. coli*, *P. fluorescens*, *X. campestris* pvs and *X. oryzae* by using the disc diffusion method (Lemriss et al. 2003). The bacterial strains were maintained on LB agar medium at 28°C. The bacteria were grown in LB broth and centrifuged at 10,000 rpm for 5 min. The pellet was

dissolved in double distilled water and used to inoculate the plates. The paper discs containing streptomycin and tetracyclin were used as positive control and DMSO as negative control. Each disc contained 10 μg of standard drugs and 10 μg synthesized compounds. The plates were maintained at 4°C for 2 h to allow the diffusion of drugs and then incubated at 28°C. All the compounds were tested in triplicate and inhibition zones were measured in mm after 24 h of incubation.

Analytical Data of Synthesized Compounds

Spectral data (NMR and mass spectra) confirmed the structures of the synthesized compounds (Table 1). The analytical data of the compounds are here presented.

The compound **2** was synthesized by following the general procedure using compound **1** (0.20 g, 0.91 mmol) with TFA.VP-OBzl (0.38 g, 0.91 mmol), DIEA (0.67 mL, 3.67 mmol), HOBt (0.30 g, 1.39 mmol) and EDCI (0.42 g, 1.9 mmol) in DMF (2 ml) and THF (2 ml). Yield: 85%., ^{1}H NMR (DMSO-D_6): δ 12.16 (s, 1H), 8.24–8.22 (d, 1H, $J = 6.6$ Hz), 8.08–8.05 (d, 1H, $J = 8.40$ Hz), 7.79–7.74 (t, 1H, $J = 7.5$ Hz), 7.58–7.55 (d, 1H, $J = 8.1$ Hz), 7.48–7.43 (t, 1H, $J = 7.8$ Hz), 7.34 (s, 5H), 5.1 (s, 2H), 4.35–4.31 (m, 2H), 3.52 (m, 1H), 3.75 (m, 1H), 2.83–2.82 (m, 2H), 2.76–2.72 (m, 2H), 2.15 (m, 1H), 1.88–1.82 (m, 4H), 0.87–0.83 (d, 6H, $J = 12.9$).

The compound **3** was synthesized by hydrolyzing compound **2** (0.15 g) using ammonium formate (2 eq) and 10% Pd/C (10% w/w) in methanol. Yield: 89%., ^{1}H NMR (DMSO-D_6): δ 12.16 (s, 1H), 8.24–8.22 (d, 1H, $J = 6.6$ Hz), 8.08–8.05 (d, 1H, $J = 8.50$ Hz), 7.79–7.74 (t, 1H, $J = 7.5$ Hz), 7.58–7.55 (d, 1H, $J = 8.1$ Hz), 7.48–7.42 (t, 1H, $J = 7.75$ Hz), 4.35–4.31 (m, 2H), 3.52 (m, 1H), 3.75 (m, 1H), 2.83–2.81 (m, 2H), 2.75–2.72 (m, 2H), 2.15 (m, 1H), 1.86–1.82 (m, 4H), 0.87–0.83 (d, 6H, $J = 12.9$).

The compound **4** was synthesized by following the general procedure using compound **1** (0.20 g, 0.91 mmol) and TFA.GVP-OBzl (0.43 g, 0.91 mmol), DIEA (0.67 ml, 3.67 mmol), HOBt (0.30 g, 1.39 mmol), EDCI (0.42 g, 1.9 mmol) in DMF (2 ml) and THF (2 ml). Yield: 84%., ^{1}H NMR (DMSO-D_6): δ 12.17 (s, 1H), 8.17 (s, 1H), 8.07–8.04 (m, 2H), 7.88–7.82 (t, 1H, $J = 7.8$ Hz), 7.58–7.55 (d, 1H, $J = 8.1$ Hz), 7.48–7.43 (t, 1H, $J = 7.6$ Hz), 7.37 (s, 5H), 5.09 (s, 2H), 4.37–4.31 (m, 2H), 3.74–3.72 (m, 3H), 3.51 (m, 1H), 2.86–2.82 (t, 2H, $J = 6.6$ Hz), 2.76–2.71 (t, 2H,$J = 6.6$ Hz), 2.13 (m, 1H), 1.90–1.83 (m, 4H), 0.85–0.81 (d, 6H, $J = 12$ Hz).

The compound **5** was synthesized by hydrolyzing compound **4** (0.15 g) using ammonium formate (2 eq) and 10% Pd/C (10% w/w) in methanol. Yield: 88%., ^{1}H NMR(DMSO-D_6): δ 12.19 (s, 1H), 8.18 (s, 1H), 8.08–8.04 (m, 2H), 7.78–7.73 (t, 1H, $J = 7.6$ Hz), 7.58–7.55 (d, 1H, $J = 8.1$ Hz), 7.48–7.43 (t, 1H, $J = 7.7$ Hz), 4.37–4.31 (m, 2H), 3.74–3.72 (m, 3H), 3.51 (m, 1H), 2.86–2.82 (t, 2H, $J = 6.9$ Hz), 2.75–2.71 (t, 2H, $J = 6.75$ Hz), 2.13 (m, 1H), 1.92–1.86 (m, 4H), 0.85–0.81 (d, 6H, $J = 12$ Hz).

The compound **6** was synthesized by following the general procedure using compound **1** (0.20 g, 0.91 mmol) and TFA.VGVP-OBzl (0.52 g, 0.91 mmol), DIEA (0.67 ml, 3.67 mmol), HOBt (0.3 g, 1.39 mmol), EDCI (0.42 g, 1.9 mmol) in DMF (2 ml) and THF (2 ml). Yield: 83%., ^{1}H NMR (DMSO-D_6): δ 12.18 (s, 1H), 8.17 (s, 1H), 8.07–8.05 (m, 2H), 8.04–8.01 (d, 1H, $J = 8.4$ Hz), 7.77–7.72 (t, 1H, $J = 7.65$ Hz), 7.57–7.54 (d, 1H, $J = 8.4$ Hz), 7.48–7.43 (t, 1H, $J = 7.6$ Hz), 7.35 (s, 5H), 5.11 (s, 2H), 4.42–4.31 (m, 3H), 3.78–3.72 (m, 3H), 3.52 (m, 1H), 2.85–2.84 (m, 2H), 2.76 (m, 1H), 2.70 (m, 1H), 1.95–1.91 (m, 5H), 1.84 (m, 1H), 0.87–0.81 (m, 12H).

The compound **7** was synthesized by hydrolyzing compound **6** (0.15 g) using ammonium formate (2 eq) and 10% Pd/C (10% w/w) in methanol. Yield: 87%., ^{1}H NMR (DMSO-D_6): δ 12.16 (s, 1H), 8.16 (s, 1H), 8.08–8.06 (m, 2H), 8.04–8.01 (d, 1H, $J = 8.4$ Hz), 7.79–7.74 (t, 1H, $J = 7.65$ Hz), 7.57–7.54 (d, 1H, $J = 8.1$ Hz), 7.48–7.43 (t, 1H, $J = 7.5$ Hz), 4.46–4.35 (m, 3H), 3.76–3.72 (m, 3H), 3.52 (m, 1H), 2.85–2.84 (m, 2H), 2.77 (m, 1H), 2.73 (m, 1H), 1.94–1.90 (m, 5H), 1.83 (m, 1H), 0.86–0.81 (m, 12H).

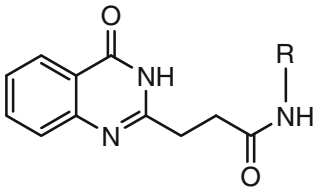
The compound **8** was synthesized by following the general procedure using the compound **1** (0.20 g, 0.91 mmol) and TFA.GVGVP-OBzl (0.57 g, 0.91 mmol), DIEA (0.67 mL, 3.67 mmol), HOBt (0.30 g, 1.39 mmol), EDCI (0.42 g, 1.9 mmol) in DMF (2 ml) and THF (2 ml). Yield: 85%., ^{1}H NMR (DMSO-D_6): δ 12.19 (s, 1H), 8.23–8.16 (m, 2H), 8.07–8.06 (m, 2H), 7.98–7.96 (d, 1H, $J = 8.4$ Hz), 7.82–7.75 (m, 1H), 7.58–7.56 (d, 1H, $J = 8.1$ Hz), 7.47–7.42 (t,1H, $J = 7.6$ Hz), 7.34 (s, 5H), 5.12 (s, 2H), 4.36–4.33 (m, 2H), 4.12(m, 1H), 3.76–3.74 (m, 5H), 3.56 (m, 1H), 2.83–2.81 (m, 2H), 2.67–2.64 (m, 2H), 2.13 (m, 1H), 1.92–1.87 (m, 5H), 0.86–0.78 (m, 12H).

The compound **9** was synthesized by hydrolyzing compound **8** (0.15 g) using ammonium formate (2 eq) and 10% Pd/C (10% w/w) in methanol. Yield: 88%. ^{1}H NMR (DMSO-D_6): δ 12.19 (s, 1H), 8.22–8.16 (m, 2H), 8.06–8.05 (m, 2H), 7.99–7.96 (d, 1H, $J = 8.7$ Hz), 7.82–7.74 (m, 1H), 7.58–7.55 (d, 1H, $J = 8.4$ Hz), 7.49–7.44 (t, 1H, $J = 7.5$ Hz), 4.37–4.33 (m, 2H), 4.13 (m, 1H), 3.77–3.74 (m, 5H), 3.57 (m, 1H), 2.84–2.82 (m, 2H), 2.66–2.64 (m, 2H), 2.15 (m, 1H), 1.93–1.87 (m, 5H), 0.87–0.80 (m, 12H) (Table 1).

Results and Discussion

The biological activities of the synthesized heterocyclic conjugated peptides were tested against bacterial strains,

Table 1 Heterocyclic conjugated peptides with their analytical data



Compound no.	R[a]	Molecular formula	Theoretical mol. Wt	Actual MS values (M^+)	HPLC RT
2	VP-OBzl	$C_{28}H_{32}N_4O_5$	504.24	505	9.445
3	VP-OH	$C_{21}H_{26}N_4O_5$	414.19	415.3	6.147
4	GVP-OBzl	$C_{30}H_{35}N_5O_6$	561.26	562	9.485
5	GVP-OH	$C_{23}H_{29}N_5O_6$	471.21	472.1	6.272
6	VGVP-OBzl	$C_{35}H_{44}N_6O_7$	660.33	661.1	9.777
7	VGVP-OH	$C_{28}H_{38}N_6O_7$	570.28	571	7.786
8	GVGVP-OBzl	$C_{37}H_{47}N_7O_8$	717.35	717.8	9.878
9	GVGVP-OH	$C_{30}H_{41}N_7O_8$	627.3	628.4	7.889

[a] G = Glycine, P = Proline, V = Valine

Table 2 Inhibitory zone (diameter) mm of compounds against tested bacterial strains by disc diffusion method

Compounds	Inhibitory zone (diameter) mm[a]				
	Bacillus substilis	*Escherichia coli*	*Pseudomonas fluorescens*	*Xanthomonas campestris* pvs.	*Xanthomonas oryzae*
1	0	0	0	0	0
2	8	10	8	7	9
3	9	12	10	10	11
4	10	11	11	12	10
5	11	13	12	14	11
6	22	20	24	23	25
7	24	25	29	28	28
8	26	23	28	27	29
9	32	30	32	34	35
Streptomycin	17	21	23	–	–
Tetracyclin	–	–	–	19	18

Streptomycin sulphate (10 μg/disc); Tetracycline (10 μg/disc) were used as positive reference and Compounds (10 μg/disc)

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

B. substilis (gram positive) and *E. coli*, *P. fluorescens, X. campestris* pvs and *X. oryzae* (gram negative) and are given in Table 2. Each fragment of these compounds i.e., heterocyclic precursor **1** and VP, GVP, VGVP and GVGVP peptides were tested for antibacterial activity and found inactive (Urry et al. 1993).

It was observed from the Table 2, the antimicrobial activity increases with increase in length of peptide chain. Although the peptides chosen were less hydrophobic and inactive against these strains, their coupling with heterocyclic precursor **1** showed activity for the mentioned bacterial strains. But heterocyclic precursor **1** conjugated with VGVP **6** and GVGVP **8** showed increased activity compare to conventional drugs. The antimicrobial effect of these compounds seems to be governed by the presence of ionic and increase in bulky structural motifs, which is in good agreement with the pharmacophore (Strom et al. 2003). Further debenzylated product of these compounds i.e. **7**, **9**, respectively showed enhanced activity than their counterparts. This may be attributed to the increase in polarity of the compounds.

Hydrophobicity of new chemical entities is supposed to be one of the most significant properties responsible for the observed increase in the antimicrobial activity. Hence the hydrophobicity was evaluated by measuring the retention time of each individual compound using RP-HPLC column surface (Table 1), which has affinity for hydrophobicity i.e., C18 (Krause et al. 1995). A plot of retention time of compounds against its antimicrobial activity gives the clear indication of correlation between the hydrophobicity and its activity for each compound.

As per the Fig. 1, it is clear that compounds containing more elongated peptides were found to be more hydrophobic which in turn correlated with higher antimicrobial

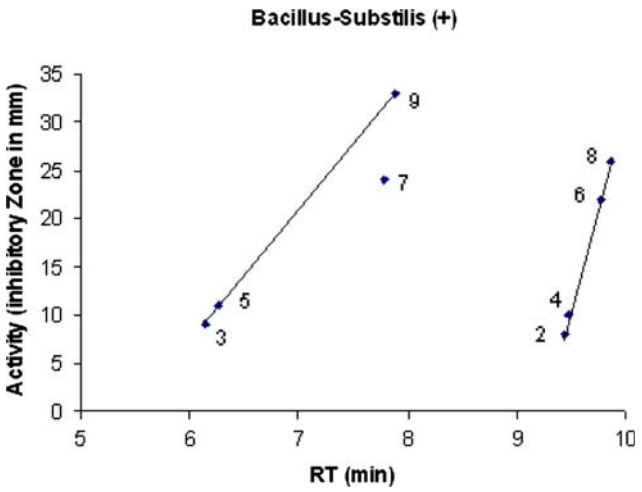


Fig. 1 Correlation between RT and antibacterial activity

activities. However, the respective hydrolyzed compounds are of less hydrophobic character compared to its counterparts, still they have shown more activity. This may be due to increase in polarity of these compounds. This indicates that increase in the polarity has an impact on the activity but given the small set of compounds tested, it is not possible to explain the observed enhanced activity. Expanding the set of compounds to overcome these limitations is in progress.

Further it is evident that activity towards gram −ve bacteria is more compared to the gram +ve. This may be due to the distance between the charged groups and the heterocyclic ring. Another widely postulated mechanism is that of the "self-promoted uptake" of the peptides across the outer membranes of gram −ve bacteria which consists lipopolysaccaride surface (Hancock 2001). This suggests that the peptides interact with the negatively charged outer membrane and subsequent channel formation in the cytoplasmic membrane via either "barrel-stave" or a "carpet" mechanism resulting in cell death (Bechinger 1999; Oren and Shai 1998; Shai 1999).

Conclusion

In the present work new antimicrobial compounds were synthesized by coupling quinazolinone precursor **1** with VP, GVP, VGVP and GVGVP peptides. Synthesized compounds were characterized for their antibacterial activity against bacterial strains *B. substilis* (gram positive) and *E. coli*, *P. fluorescens*, *X. campestris* pvs and *X. oryzae* (gram negative). Even though peptides and quinazolinone moiety which taken in isolation were inactive towards these bacterial strains, synthesized compounds showed activity. It was noticed that quinazolinone conjugated VGVP and GVGVP peptides showed increase in activity by nearly two fold compared to conventional antimicrobials. The increase in the activity may be attributed to the hydrophobicity, cationic nature of peptide and polarity of the molecules.

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