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Design, synthesis, antibacterial and antitubercular activity of cationic antimicrobial peptide, ovine bactenecin5

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A series of shorter synthetic peptides analogues of ovine bactenecin 5 (OaBac5), Phe-Arg-Pro-Xaa (Xaa= Phe, Met, Tyr and Trp) are designed and synthesized. The tetra peptides synthesized are conjugated with the isonicotinic acid at the *N*-terminal of the tetra peptides to study the change in the biological activity. The shorter synthetic tetra peptide analogues of OaBac5 and isonicotinic acid conjugated tetra peptides are more active against Gram negative bacteria than Gram positive bacteria. For Gram negative bacteria, the overall order of antibacterial activity of the synthesized tetrapeptides is found to be Phe-Arg-Pro-Trp >Phe-Arg-Pro-Phe >Phe-Arg-Pro-Met ≥Phe-Arg-Pro-Tyr. The isonicotinic acid conjugated tetra peptides have showed reduced antibacterial activity when compared to the tetra peptides. All the peptide derivatives showed moderate antitubercular activity. None of the peptides exhibited prominent haemolytic activity.

Keywords: Antibacterial, antitubercular activity, cationic antimicrobial peptide, ovine bactenecin5

The past 50 years have been dubbed as the "antibiotic era" in which natural, semi synthetic, or synthetic antibacterial agents have been used with great success against the life threatening diseases. The rapid emergence of antibiotic resistant pathogens[1] is looked on as a major threat at the onset of the new millennium. While, microbial pathogens show a remarkable capacity to develop novel strategies to curb the effectiveness of conventional antibiotics, research on new antibiotics is somewhat lagging, with few antibacterial developed in recent years.

Researchers however are continuing their efforts to develop new classes of antimicrobials. One of these research lines aims to exploit the therapeutic potential of a variety of natural cationic antimicrobial peptides and their synthetic analogues, for the development of antimicrobials with a mechanism of action different from those of conventional antibiotics[2]. Most of these peptides are less than 10 KDa, have an overall net positive charge, they are hydrophobic and membrane active[3]. These peptides exhibit various biological activities and the fact that they have potential to overcome bacterial resistance makes them promising candidates for therapeutic drugs[4]. The number of discovered AMPs has now reached more than 800. A comprehensive searchable database can be found at http://www.bbcm.univ.trieste/~ tossi/pag2.htm. The killing mechanism of these peptides involves the interaction with and disruption of microbial membranes through a relatively nonspecific mechanism, largely based on differences in membrane lipid composition, the emergence of resistance may thus require multiple alterations in the bacterial surface and metabolism. Also the killing effects are very rapid, especially when compared with the conventional antibiotics. The antimicrobial peptides discovered till date have demonstrated their ability as antibacterial[5], antitubercular[6], antifungal[7], anti-malarial[8], antileishmanials[9] and for treating buco-dental infections[10], ocular infection[11] and sexually transmitted infections[12]. They have been reported to poses contraceptive potential[13], antitumoral[14] activity and antiviral[15] activity.

Many Ovis Aries bactenicins such as OaBac5, OaBac6, OaBac7.5, and OaBac11 were isolated[16] from sheep neutrophils. Since then OaBac5, as well as three variants of OaBac5 namely, OaBac5, OaBac5β, OaBac5 mini have also been isolated[17]. The antimicrobial properties of OaBac5 were investigated with an array of Gram positive and Gram negative microorganisms[18]. OaBac5 had potent activity against Gram negative bacteria (MICs, 0.125 to 8μg/mL) but weak activity against Gram positive bacteria and *Candida albicans* (MICs, 16 to 64μg/mL). OaBac5,

which is a 43 residue antimicrobial peptide (RFRPPIRRPPIR PPFRPPFRPPVRPPIRPPFRPPFR-PPIGPFP) and was characterized by high content of arginine and proline. It is made up of 6-residue *N*-terminus region followed by two copies of a 16-residue repeat and a 5-residue *C*-terminus region. The 6-resiue *N*-terminus region is highly cationic. This unique sequence with an Arg clustered region consists of five Phe-Arg-Pro triplets spaced by hydrophobic amino acid residue. Proline is known as a strong α-helix breaker, which can induce kinks in α-helix of proteins, suggesting that the repeating triplet may play an important role in antimicrobial activity.

In this context, a series of tetra peptide analogues of OaBac5 had been synthesized by substituting different hydrophobic/cationic amino acid residues at the *C*-terminal of Phe-Arg-Pro triplet, to assess the effect of change of configuration on biological activity. In the view of optimizing the bioactivity, Isonicotinic acid was conjugated to the *N*-terminal of above shorter synthetic peptide analogues of OaBac5. The synthesized tetra peptides and isonicotinic acid conjugated tetra peptides were screened for their antibacterial and antitubercular activities.

Results and Discussion

Design of peptides and isonicotinic acid conjugated peptides

Previous work has shown that it is possible to improve the activities, or reduce the toxicities, of naturally occurring peptide antibiotics by producing synthetic analogs with modified primary and/or secondary structures[19]. This work was primarily aimed to synthesize some active shorter peptide analogues of OaBac5 to obtain some information about important structural features of the peptide. The repeating Phe-Arg-Pro triplet in OaBac5 was taken as the template to check the role of this triplet in the biological activity. The types of modifications included attachment hydrophobic/cationic amino acid residues at the *C*-terminal of Phe-Arg-Pro triplet and conjugating isonicotinic acid at the *N*-terminal of the resulted tetrapeptides to study the change in the biological activity.

Peptides synthesis, purification and characterization

The peptide synthesis was performed by solution phase method using Boc-chemistry. The synthesized tetra peptides were conjugated with isonicotinic acid. The protected peptides and the isonicotinic acid conjugated peptides were purified by SiO_2 column chromatography and at each step the peptides were characterized using TLC, melting points determination, elemental analysis, optical rotations and ^{1}H NMR spectroscopy. Purity of the products was confirmed by HPLC to be >98% gave satisfactory results. The yields, physical constants and analytical data of protected peptides and isonicotinic acid conjugated peptides were listed in **Table I**. The ^{1}H NMR data of the protected peptides and isonicotinic acid conjugated peptides were given in **Tables II** and **III**.

Antibacterial activity studies

The efficacy of shorter synthetic tetra peptide analogues of OaBac5 and isonicotinic acid conjugated tetra peptides were evaluated for their antibacterial activities against different Gram positive and Gram negative bacteria at various peptide concentrations. The MIC, minimal inhibitory concentration values of these tetra peptides and isonicotinic acid conjugated tetra peptides were given in **Tables IV** and **V**. All the shorter synthetic tetra peptide analogues of OaBac5 and isonicotinic acid conjugated tetra peptides were more active in arresting or killing the growth of Gram negative microorganisms when compared to Gram positive microorganisms at different peptide concentrations.

In vitro, at a concentration of 160-360 μg/mL, shorter synthetic tetrapeptide analogues of OaBac5 efficiently killed Gram negative bacteria. However, even at a concentration of 400 μg/mL of the shorter synthetic tetrapeptide analogues of OaBac5, unable to completely kill or suppress the growth of Gram positive bacteria. Among the four shorter synthetic tetrapeptide analogues of OaBac5, Phe-Arg-Pro-Trp was found to be the most potent antibacterial peptide, whereas Phe-Arg-Pro-Tyr was least potent antimicrobial peptide. The overall order of activity of the synthesized tetrapeptides is Phe-Arg-Pro-Trp >Phe-Arg-Pro-Phe >Phe-Arg-Pro-Met ≥Phe-Arg-Pro-Tyr.

The isonicotinic acid conjugated above tetra-peptides resulted in very little reduced antibacterial activity when compared to the designed tetrapeptides. *In vitro*, at a concentration of 160-360 μg/mL, the isonicotinic acid conjugated tetrapeptide analogues of OaBac5 efficiently killed Gram negative bacteria. However, even at a concentration of 400 μg/mL the isonicotinic acid conjugated tetrapeptide analogues of

Table I — Physical and analytical data of protected tetra peptides and isonicotinic acid conjugated peptides

Peptide	Yield (%)	m.p. (˚C)	R_f^1	R_f^2	R_f^3	$[\alpha]_D^{25}$ (C,1) MeOH[a]	Mole. Formula	Found (Calculated) % C	H	N
Boc-Phe-Arg(NO_2)-Pro-OBzl	90	52-54	0.60	0.71	0.80	-49º	$C_{32}H_{46}O_8N_7$	58.50 (58.53	7.00 7.01	14.90 14.93)
Boc-Phe-Arg(NO_2)-Pro-Phe-OMe	85	56-58	0.71	0.78	0.96	-55º	$C_{35}H_{48}O_9N_8$	57.97 (58.01	6.60 6.62	15.41 15.46)
Boc-Phe-Arg(NO_2)-Pro-Met-OMe	78	62-64	0.80	0.85	-	-69º	$C_{30}H_{48}O_9N_8S_1$	51.70 (51.72	6.89 6.89	16.00 16.09)
Boc-Phe-Arg(NO_2)-Pro-Tyr-OMe	90	67-68	0.89	0.96	-	-62º	$C_{35}H_{47}O_{10}N_8$	56.80 (56.83	6.35 6.35	15.12 15.15)
Boc-Phe-Arg(NO_2)-Pro-Trp-OMe	93	56-57	0.79	0.86	0.91	-49º	$C_{37}H_{48}O_9N_9$	58.24 (58.26	6.24 6.29	16.50 16.53)
Nic-Phe-Arg(NO_2)-Pro-Phe-OMe	85	95-96	0.78	0.70	0.36	-90º	$C_{36}H_{43}O_8N_9$	59.21 (59.25	5.84 5.89	17.20 17.28)
Nic-Phe-Arg(NO_2)-Pro-Met-OMe	84	88-90	0.77	0.72	0.36	-88º	$C_{32}H_{43}O_8N_9S_1$	53.80 (53.85	6.00 6.03	17.60 17.67)
Nic-Phe-Arg(NO_2)-Pro-Tyr-OMe	90	91-93	0.74	0.70	0.21	-65º	$C_{36}H_{46}O_9N_9$	57.70 (57.75	6.10 6.14	16.81 16.84)
Nic-Phe-Arg(NO_2)-Pro-Trp-OMe	92	108-109	0.77	0.72	0.34	-93º	$C_{38}H_{44}O_8N_{10}$	59.30 (15.37	5.72 5.72	18.21 18.22)

[a]Specific rotation
R_f^1: $CHCl_3$:CH_3OH:CH_3COOH (95:05:3)
R_f^2: $CHCl_3$: CH_3OH:CH_3COOH (90:10:3)
R_f^3: $CHCl_3$: CH_3OH:CH_3COOH (85:15:3)

Table II — ^{1}H NMR data of protected tetra peptides in $CDCl_3$.

Peptide	Components	Chemical shift (δ)
Boc-Phe-Arg(NO_2)-Pro-Phe-OMe[a]	Boc[1]	1.27 (s, 9H, $(CH_3)_3$)
	Phe[2]	4.67 (d, 1H, $^{\alpha}$CH), 3.0 (m, 2H, $^{\beta}$CH), 8.4 (s, 1H, NH), 7.11-7.20 (m, 5H, Ar-H)
	Arg[3]	4.6 (1H, m, $^{\alpha}$CH), 1.85 (2H, m, $^{\beta}$CH), 1.48-1.54 (2H, m, $^{\gamma}$CH), 3.45 (2H, m, $^{\delta}$CH), 8.41 (1H, s, NH), 7.50 (1H, s, $^{\delta}$NH)
	Pro[4]	4.43 (1H, m, $^{\alpha}$CH), 1.90-2.0 (2H, m, $^{\beta}$CH), 1.98-2.04 (2H, m, $^{\gamma}$CH), 3.53 (2H, m, $^{\delta}$CH)
	Phe	5.15 (d, 1H, $^{\alpha}$CH), 2.88 (m, 2H, $^{\beta}$CH), 8.3(s, 1H, NH), 7.10-7.22(m, 5H, Ar-H)
	OMe[5]	3.63 (s, 3H, CH_3)
Boc-Phe-Arg(NO_2)-Pro-Met-OMe[b]	Met	4.45 (m, 1H, $^{\alpha}$CH), 2.9-3.0 (m, 2H, $^{\beta}$CH), 2.52 (m, 2H, $^{\gamma}$CH), 2.02 (s, 6H, CH_3), 8.4 (s, 1H, NH)
Boc-Phe-Arg(NO_2)-Pro-Tyr-OMe[c]	Tyr	4.62 (d, 1H, $^{\alpha}$CH), 2.8 (m, 2H, $^{\beta}$CH), 7.0-7.3 (m, 5H, Ar-H), 8.41 (s, 1H, NH)
Boc-Phe-Arg(NO_2)-Pro-Trp-OMe[d]	Trp	4.70-4.85 (d, 1H, $^{\alpha}$CH), 3.45 (m, 2H, $^{\beta}$CH), 8.49 (s, 1H, NH), 7.1-7.19 (m, 5H, Ar-H), 7.0 (m, 1H, indole-H), 9.18 (s, 1H, ring NH)

The chemical shift values for the residues Boc[1], Phe[2], Arg[3], Pro[4] and OMe[5] of tetra peptides b, c and d were almost same as obtained for the tetra peptide a

OaBac5 unable to completely kill or suppress the growth of Gram positive bacteria.

Antitubercular activity studies

The efficacy of shorter synthetic tetrapeptide analogues of OaBac5 and isonicotinic acid conjugated tetrapeptides were evaluated for their antitubercular activity against *Mycobacterium tuberculosis* H_{37} Rv human strain at various peptide concentrations and results were summarized in **Table VI**.

Both the shorter synthetic tetrapeptide analogues of OaBac5 and the isonicotinic acid conjugated tetrapeptide analogues of OaBac5 showed considerably moderate antitubercular activity against *Myco*

Table III — ^{1}H NMR data of isonicotinic acid conjugated tetra peptides in $CDCl_3$.

Peptide	Components	Chemical shift (δ)
Nic-Phe-Arg(NO_2)-Pro-Phe-OMe[a]	Nic[1]	7.84 (5H, m, Ar-H)
	Phe[2]	4.85 (d, 1H, $^{\alpha}$CH), 3.05 (m, 2H, $^{\beta}$CH), 8.43 (s, 1H, NH), 7.1-7.3 (m, 5H, Ar-H)
	Arg[3]	4.55 (1H, m, $^{\alpha}$CH), 1.80 (2H, m, $^{\beta}$CH), 1.60(2H, m, $^{\gamma}$CH), 3.25 (2H, m, $^{\delta}$CH), 8.43 (1H, s, NH), 7.86 (1H, s, $^{\delta}$NH)
	Pro[4]	4.35 (1H, m, $^{\alpha}$CH), 2.0-2.1(2H, m, $^{\beta}$CH), 1.92 (2H, m, $^{\gamma}$CH), 3.08 (2H, m, $^{\delta}$CH)
	Phe	5.4 (d, 1H, $^{\alpha}$CH), 3.1 (m, 2H, $^{\beta}$CH), 8.0 (s, 1H, NH), 7.15-7.30 (m, 5H, Ar-H)
	OMe[5]	3.65 (s, 3H, CH_3)
Nic-Phe-Arg(NO_2)-Pro-Met-OMe[b]	Met	5.5 (m, 1H, $^{\alpha}$CH), 2.9 (m, 2H, $^{\beta}$CH), 2..0 (m, 2H, $^{\gamma}$CH), 1.8 (s, 6H, CH_3), 8.45 (s, 1H, NH)
Nic-Phe-Arg(NO_2)-Pro-Tyr-OMe[c]	Tyr	4.78 (d, 1H, $^{\alpha}$CH), 3.10 (m, 2H, $^{\beta}$CH), 7.0-7.28 (m, 5H, Ar-H), 8.84 (s, 1H, NH)
Nic-Phe-Arg(NO_2)-Pro-Trp-OMe[d]	Trp	4.6-4.7 (d, 1H, $^{\alpha}$CH), 3.0 (m, 2H, $^{\beta}$CH), 8.3 (s, 1H, NH), 6.8-7.2 (m, 5H, Ar-H), 6.90(m, 1H, indole-H), 9.1 (s, 1H, ring NH)

The chemical shift values for the residues Nic[1], Phe[2], Arg[3], Pro[4] and OMe[5] of isonicotinic acid conjugated tetra peptides b, c and d were almost same as obtained for the tetra peptide a.

Table IV — MIC[b] values of synthesized tetra peptides and isonicotinic acid conjugated peptides (μg/mL)[a] for Gram positive bacteria

Peptide	*Staphylococcous aureus*	*Bacillus Cereus*	*Strep. pneumoniae*	*Strep. pyogenes*	*Bacillus subtilis*
Phe-Arg-Pro-Phe	> 400	> 400	> 400	> 400	> 400
Phe-Arg-Pro-Met	> 400	> 400	> 400	> 400	> 400
Phe-Arg-Pro-Tyr	> 400	> 400	> 400	> 400	> 400
Phe-Arg-Pro-Trp	> 400	> 400	> 400	> 400	> 400
Nic-Phe-Arg-Pro-Phe	> 400	> 400	> 400	> 400	> 400
Nic-Phe-Arg-Pro-Met	> 400	> 400	> 400	> 400	> 400
Nic-Phe-Arg-Pro-Tyr	> 400	> 400	> 400	> 400	> 400
Nic-Phe-Arg-Pro-Trp	> 400	> 400	> 400	> 400	> 400
Ref. Drug[c]	120	80	120	80	80

[a]Values were expressed as mean values based on three separate experiments.
[b]MIC means minimal inhibitory concentration (μg/mL) required to for preventing bacterial growth.
[c]Separate reference drugs were used for separate bacteria.

Table V — MIC[b] values of synthesized tetra peptides and isonicotinic acid conjugated peptides (μg/mL)[a] for Gram negative bacteria

Peptide	*Escherichia coli*	*Pseudomonas aeruginosa*	*Shigella*	*K. pneumoniae*
Phe-Arg-Pro-Phe	240	200	240	240
Phe-Arg-Pro-Met	320	280	320	320
Phe-Arg-Pro-Tyr	240	280	360	320
Phe-Arg-Pro-Trp	160	160	240	160
Nic-Phe-Arg-Pro-Phe	240	200	240	240
Nic-Phe-Arg-Pro-Met	320	280	320	320
Nic-Phe-Arg-Pro-Tyr	280	280	360	320
Nic-Phe-Arg-Pro-Trp	160	160	240	160
Ref. Drug[c]	120	120	120	80

[a]Values were expressed as mean values based on three separate experiments.
[b]MIC means minimal inhibitory concentration (μg/mL) required to for preventing bacterial growth.
[c]Separate reference drugs were used for separate bacteria.

bacterium tuberculosis H_{37} Rv human strain. *In vitro*, at a concentration of 200 µg/mL, the tetrapeptide Phe-Arg-Pro-Trp and the isonicotinic acid conjugate of the same, Nic-Phe-Arg-Pro-Trp inhibited the growth of *Mycobacterium tuberculosis* H_{37} Rv human strain, less than 20 colonies and shows complete growth inhibition at 400 µg/mL. The tetrapeptide Phe-Arg-Pro-Phe and its isonicotinic acid conjugate, Nic-Phe-Arg-Pro-Phe also inhibited the growth of bacteria, less than 20 colonies at a concentration of 400 µg/mL.

Haemolytic activity studies

The short synthetic tetrapeptide analogues and the isonicotinic acid conjugated tetrapeptides synthesized in this study were essentially non-haemolytic, i.e. showed less than 30% of haemolysis even at 200µg/mL concentration. More than 20% haemolysis was observed for the tetrapeptide, Phe-Arg-Pro-Trp and the isonicotinic acid conjugate of the same, Nic-Phe-Arg-Pro-Trp. Only the peptides containing Trp are little haemolytic but not toxic. The results were summarized in **Table VII**.

Our results indicate that, the increase in the antibacterial activity is due to the increase in the hydrophobicity of the substituted amino acid at the *C*-terminal of the tetrapeptides and the isonicotinic acid conjugated peptides. The highly hydrophobic amino acid residues Trp, and Phe, containing peptides were shown to be promising antibacterial[20, 21]. The order of activity shown above also supports the structure-activity relationship. The selective Gram negative antibacterial activity of the above peptides can be attributed to the presence of the Arg- residue which ensures a strong electrostatic interaction between the peptides and the negatively charged bacterial surface. The large size of the Trp- residue ensured an efficient disruption of the membrane integrity, supporting the higher activity of the Trp- containing peptides.

Table VI — Antitubercular activity of the synthesized tetra peptides and isonicotinic acid conjugated peptides

Peptide	Peptide concentration (µg/mL)			
	50	100	200	400
Phe-Arg-Pro-Phe	+ +	+ +	+ +	+ -
Phe-Arg-Pro-Met	+ +	+ +	+ +	+ +
Phe-Arg-Pro-Tyr	+ +	+ +	+ +	+ +
Phe-Arg-Pro-Trp	+ +	+ +	+ -	- -
Nic-Phe-Arg-Pro-Phe	+ +	+ +	+ +	+ -
Nic-Phe-Arg-Pro-Met	+ +	+ +	+ +	+ +
Nic-Phe-Arg-Pro-Tyr	+ +	+ +	+ +	+ +
Nic-Phe-Arg-Pro-Trp	+ +	+ +	+ -	- -
Ref. Drug[a]	- -	- -	- -	- -

+ +: Denotes the growth of the bacterium
+ - : Denotes the growth less than 20 colonies of the bacterium
- - : Denotes no growth of the bacterium
[a]Streptomycin was used as the reference drug

Table VII — Haemolytic activity[a] of the synthesized tetra peptides and isonicotinic acid conjugated peptides

Peptide	Percentage of haemolysis at peptide concentration (µg/mL)					
	20	40	80	120	160	200
Phe-Arg-Pro-Phe	0.00	02.13	04.78	10.65	15.47	19.22
Phe-Arg-Pro-Met	0.00	0.00	0.00	02.43	04.63	07.36
Phe-Arg-Pro-Tyr	0.00	0.00	0.00	02.12	04.13	08.27
Phe-Arg-Pro-Trp	02.66	05.23	11.37	15.74	21.32	26.54
Nic-Phe-Arg-Pro-Phe	0.00	1.92	2.54	04.93	09.57	11.78
Nic-Phe-Arg-Pro-Met	0.00	0.00	0.00	01.64	03.96	06.27
Nic-Phe-Arg-Pro-Tyr	0.00	0.00	0.00	01.22	02.98	05.47
Nic-Phe-Arg-Pro-Trp	01.95	04.61	10.94	14.77	20.62	24.18

[a]Values were expressed as mean values based on three separate experiments

The isonicotinic acid attachment to the *N*-terminal of the tetrapeptides resulted in little reduced in antibacterial activity. This can be attributed to the decrease in the hydrophobicity of the peptide due to the conjugation with isonicotinic acid. Another reason is the non-availability of the phenylalanine amino acid residue of the Phe-Arg-Pro triplet, which is crucial for the bacterial membrane interactions thus reducing the activity to a little extent.

The same can be extrapolated to the antitubercular activity. The tetrapeptide, Phe-Arg-Pro-Trp and the isonicotinic acid conjugate of the same, Nic-Phe-Arg-Pro-Trp containing the most hydrophobic Trp- residue has shown considerably good activity. The low haemolytic activity of the present molecules was therefore an effect of their short size and relatively low hydrophobicity. Apparently, this rendered them highly selective for bacteria, which contain a higher proportion of anionic phospholipids compared with zwitterionic phospholipids in RBC [22].

Materials and Methods

All the amino acids used were of L-configuration unless otherwise specified. All tert-butyloxycarbonyl (Boc) amino acids, amino acid derivatives, HOBt and trifluoroacetic acid (TFA) were purchased from Advanced Chem. Tech., (Louisville, Kentucky, USA). Isonicotinic acid was purchased from Sigma Chemicals (St. Louis, USA). IBCF, NMM and EDCI were purchased from Sigma Chemicals (St. Louis, USA). All solvents and reagents were of analytical grade or were purified according to the standard procedure recommended for peptide synthesis. Silica gel (60-120 mesh) for column chromatography was purchased from Sisco Research Laboratories Pvt Ltd., (Mumbai, India). All the chemicals and reagents used for antibacterial and antifungal activities were of bacteriological grade unless otherwise indicated. Nutrient broth, Nutrient agar and L. J. Media were purchased from Hi-media chemicals (Mumbai, India).

The compounds on TLC plates were detected by UV light, by ninhydrin or chlorine/toluidine spray. The purity of the peptides was determined by HPLC analysis by using Thermo Electron with RP C18 column (5CN Cosmogel 215-4.6 mm) and UV detector-UV-VISL7400. 25µL of peptide sample in water injected for analysis. The analysis was carried out using appropriate 0-100% water/acetonitrile linear gradients in the presence of 0.1% TFA (Flow rate 1.0 mL/min).

The melting points of peptides were determined with Thomas-Hoover melting point apparatus and were uncorrected. The optical rotation was measured using Perkin-Elmer 243 digital polarimeter. Elemental analysis was carried out on VARIO EL III CHNOS Elementar. The compounds were dried over P_2O_5 under reduced pressure for 24 hr prior to the preparation of samples for all the analyses. ^{1}H NMR was recorded on a AMX-400 MHz spectrometer.

Experimental Section

Peptide synthesis

The Tetra peptides, Phe-Arg-Pro-Xaa were synthesized by stepwise classical solution phase method[23]. The isonicotinic acid conjugated peptides were synthesized by following the procedure described in the last step. The Boc group was chosen for temporary N^{α}-protection and its removal was achieved with 4*N* HCl in dioxane or trifluoroacetic acid (TFA). The *C*-terminal carboxyl group was protected by benzyl or methyl ester, and its removal was effected by saponification with 1*N* NaOH. The N^{g}- of Arg was protected by nitro group and its removal was effected by hydrogenolysis using 10% Pd/C/ ammonium formate. All the coupling reactions were achieved with the standard coupling agents EDCI or isobutylchloroformate (IBCF). The protected peptides were purified by column chromatography over silica gel and characterized by physical and analytical techniques.

Boc-Phe-Arg(NO_2)-Pro-OBzl: Boc-Arg(NO_2)-Pro-OBzl (3.61g, 0.006 mole) which was synthesized, as described by Zhao *et. al*.,[24] was deblocked with 4.1*N* HCl/dioxane (36 mL) for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried (yield, 100%). The HCl.H-Arg(NO_2)-Pro-OBzl in DMF (36 mL) was neutralized with NMM (0.6mL, 0.006 mole) and coupled to Boc-Phe-OH (1.5 g, 0.006 mole) in acetonitrile (15 mL) and NMM (4.4 mL, 0.04 mole) using EDCI (1.21g, 0.006 mole). After 20 min, the *p*H of the solution was adjusted to 8 by the addition of NMM and the reaction mixture stirred overnight at room temperature. Acetonitrile was removed under reduced pressure and the residual DMF solution was poured into about 100 mL ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The peptide precipitated was extracted into $CHCl_3$. The organic layer was washed with water, 10% citric acid, water, 5% $NaHCO_3$ solution, water and dried

over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, the obtained peptide was triturated with dry ether, petroleum ether and dried under vacuum. The sample was recrystallized from ether/petroleum ether; yield 4.2 g (90%), m.p. 52-54ºC.

Boc-Phe-Arg(NO_2)-Pro-Xaa-OMe (Xaa= Phe, Met, Tyr, or Trp): Boc-Phe-Arg(NO_2)-Pro-OBzl (1.0 g, 0.0014 mole) was taken in 20 mL of methanol and saponified with 1*N* NaOH (2.88 mL, 0.0028 mole) to remove the bezyl ester group. The reaction was stirred up to the completion of the reaction as monitored by TLC, and the MeOH was evaporated, dissolved the product in 25 mL water, washed with $CHCl_3$, then neutralized with cold 1*N* HCl (5.0 mL). The precipitate was taken into $CHCl_3$ layer, washed with saturated NaCl and dried over sodium sulphate anhydrous. The Boc-Phe-Arg(NO_2)-Pro-OH was neutralized with NMM (0.11 mL, 0.0011 mole) and coupled with individual amino acids HCl. H_2N.Xaa-OMe (0.0011 mole) in DMF (10 mL/1 g of peptide) and NMM (0.11 mL, 0.0011 mole) using EDCI (0.23 g, 0.0011 mole) and worked up the same as Boc-Phe-Arg(NO_2)-Pro-Obzl synthesis to obtain the above compounds. The samples were purified over silica gel using chloroform-methanol as elutant. The yields and physical constants are given in **Table I**. The ^{1}H NMR spectral data of protected peptides is provided in **Table II**.

Nic-Phe-Arg(NO_2)-Pro-Xaa-OMe(Xaa= Phe, Met, Tyr, or Trp): 0.00025 mmole of Boc-Phe-Arg(NO_2)-Pro-Xaa-OMe (Xaa= Phe, Met, Tyr, or Trp) were deblocked individually with TFA (10 mL/1g for 40 min) for final Boc deprotection. The solvent was removed under reduced pressure, triturated with ether to obtain TFA salt of Phe-Arg(NO_2)-Pro-Xaa. The individual peptides (0.00025 mol) were coupled to isonicotinic acid in DMF (10 mL/g of peptide) and NMM (0.0025 mL, 0.00025 mol) using iso-butylchloroformate (0.034 mL, 0.00025 mmol) as described for the preparation of Boc-Arg(NO_2)-Pro-Obzl synthesis to obtain the corresponding iso-nicotinic acid conjugated tetra peptides**.** The yield and physical constants were given in **Table I**. The ^{1}H-NMR spectral data of isonicotinic acid conjugated peptides is provided in **Table III**.

Phe-Arg-Pro-Xaa-OH (Xaa= Phe, Met, Tyr, or Trp): The protected tetra peptides were hydro-genolyzed separately using ammonium formate (2 equivalents) and 10% Pd/C (1 equivalent) in methanol (10 mL/1 g) for 2 hr at room temperature. The catalyst was filtered and washed with methanol. The combined washings and filtrate were evaporated *in vacuo* and the residue taken into $CHCl_3$, washed with water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered, washed with ether and dried. The resulting peptides were saponified with *1N* NaOH as described in Boc-Phe-Arg(NO_2)-Pro-Xaa-OMe syntheis to remove the methyl ester group. The resulting peptides were treated individually with TFA (10 mL/1g for 40 min) for final Boc deprotection. The solvent was removed under reduced pressure, triturated with ether to obtain TFA salt of Phe-Arg-Pro-Xaa. The free peptides resulted were purified by gel filtration using Sephadex G-10 and checked the purity by HPLC, using a linear gradient of 0-100% acetonitrile/0.1% trifluoroacetic acid.

Nic-Phe-Arg-Pro-Xaa-OH (Xaa= Phe, Met, Tyr, or Trp): The protected isonicotinic acid conjugated tetra peptides were hydrogenolyzed separately using ammonium formate (2 equivalents) and Pd/C (1 equivalent) in methanol (10 mL/1 g) for 2 hr at room temperature. The catalyst was filtered and washed with methanol. The combined washings and filtrate were evaporated *in vacuo* and the residue taken into $CHCl_3$, washed with water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and triturated with ether, filtered, washed with ether and dried. The resulting peptides were saponified with 1*N* NaOH as described above to remove the methyl ester group. The resulted free peptides were purified by gel filtration using Sephadex G-10 and checked the purity by HPLC, using a linear gradient of 0-100% acetonitrile/0.1% trifluoroacetic acid.

The pure cultures of some of the Gram positive bacteria and Gram negative bacteria were obtained from IMTECH (Indian Institute of Microbial Technology) Chandigarh, India. Nutrient broth media was used for the antibacterial screening and antitubercular activity was carried out by Lowenstein-Jensen egg media (L. J. media). The reference drugs cloxacillin for *Staphylococcous aureus*, erythromycin for *Bacillus cereus*, ceftriaxone for *Strep. pneumoniae*, amoxicillin for *Strep. pyogenes*, penicillin G for *Bacillus subtillis*, ciprofloxacin for *Escherichia coli*, gentamycin sulphate for *Pseudomonas aeruginosa*, gentamycin sulphate for *Shigella* and *K. pneumoniae* were used. The antibacterial assay was carried out by following microbroth serial dilution method as described by Gennaro *et. al.*[25] with slight modifications. The

antitubercular activity of the synthesized compounds was carried out against *Mycobacterium tuberculosis* H_{37} Rv human strain as described by Watt *et. al*.[26] with slight modifications. Haemolytic activity was used as a coarse measurement of the toxicity of the peptides to normal human cells. Haemolytic activity was assayed by the method as described by Yoshida, *et. al*.[27]

Conclusion

The results indicate that cationic residues mainly participate in antibacterial activities, and the hydrophobic residues affect both antibacterial and haemolytic activities. A consideration of cationic residues and hydrophobic residues is required in the design of the desirable peptides with strong antibacterial activity but less or no haemolytic activity. Information from the present work would be useful for the investigations on the structure-activity relationship of the synthetic analogues of OaBac5. At the same time the incorporation of the isonicotinic acid to the peptides and the antitubercular activity of the compounds is also a novel approach.

In conclusion, the shorter tetrapeptides and isonicotinic acid conjugated tetrapeptides with small size, easy to synthesize, simple amino acid composition, high antimicrobial activity and low toxicity, makes them highly potent as novel templates for the design of pharmaceutical compounds and peptidomimetics for topical and systemic treatment of the antibiotic resistant strains of bacteria.

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