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## Note

# Synthesis, antimicrobial and mosquito larvicidal activity of *N* -protected amino acid/peptide isoxazoles

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A series of *N*-protected amino acid/peptide isoxazoles **4-12** and **15-16** have been synthesized and the antimicrobial activity evaluated against three gram positive, three gram negative bacteria and five plant pathogenic fungi. Mosquito larvicidal activity of the newly synthesized compounds is also studied against fourth instar larve *Culex quinquefasciatus.*

**Keywords**: *N*-protected amino acid/peptide isoxazoles, antibacterial activity, antifungal activity, mosquito larvicidal test

The rapid emergence of multidrug resistant pathogenic bacteria has become a serious health threat worldwide[1]. It has been postulated that the development of resistance to known antibiotics could be overcome by identifying new drug targets *via* genomics, improving existing antibiotics and by identifying new antibacterial agents with novel structures and modes of action[2-4]. During the last four decades, these has been tremendous increase in the frequency of fungal infection[5]. Therefore, there is a need to screen for new antifungal therapeutics, which have high efficacy and low toxicity. The *Culex quinquefasciatus* mosquito is widespread and causes serious disease. Mosquito abatement primarily depends on continued application of organophosphates such as temephos[6]. Although effective, their repeated and excessive use has disrupted natural biological control systems. These problems have highlighted the needs for the development of new strategies for selective control of mosquito larvae. As part of a program aimed at identifying new antimicrobial agents and mosquito larvicidal testing, *N*-protected amino acid/peptide isoxazoles were identified as potential compounds. A survey of the existing literature indicated that there were no reports which described the use of heterocyclic coupled peptides as microbial and larvicidal agents. Therefore, a study was initiated to explore the activity of this class of compounds.

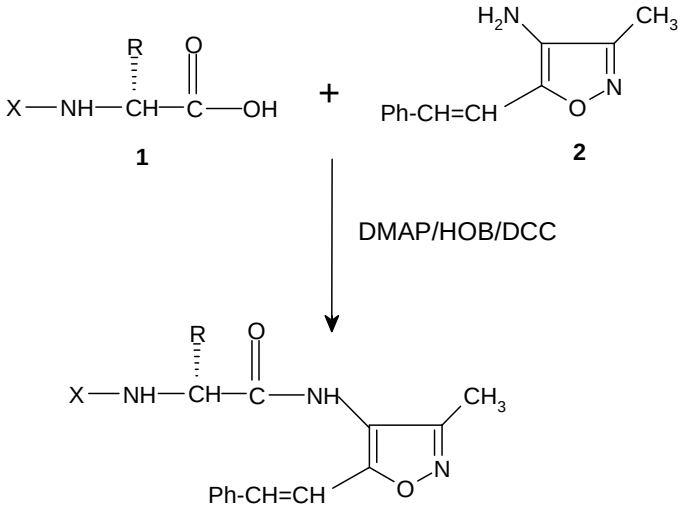
### Results and Discussion

Synthesis of *N*-protected aminoacid/peptide isoxazoles have been carried out by the reaction of 4-amino-3-methyl-5-styrylisoxazole **2** (Refs. 7,8) with *N*-protected amino acids **1** which are being blocked by employing *t*-butyloxycarbonyl (Boc), fluorenyl methoxy carbonyl (F-moc), benzyloxy carbonyl (z) and acetyl group (Ac) for $N^{\alpha}$-protection, *t*-butyl group for hydroxyl protection and trifluoro acetyl group ($F_3Ac$) for ε-amino protection. The coupling was accomplished with dimethylamino pyridine (DMAP), 1-hydroxybenzotriazole(HOBt) and dicyclohexyl carbodiimide(DCC) either in dichloromethane(DCM) or *N*,*N*-dimethyl formamide (DMF) as a solvent at RT. The coupling as monitored by TLC, was found to be complete in about 2-3 hr. After usual work-up, *N*-protected amino acid isoxazoles **4-12** and **15-16** were isolated as pure solids (**Schemes I** and **II**).

The optical activity of all the synthetic *N*-protected amino acid/peptide isoxazoles has been determined. The $R_f$ values of all the products have been measured by the TLC analysis using silica gel G plates employing solvent systems ethyl acetate-*n*-hexane (7:3) and chloroform-methanol (9:1) (**Table I**). All the new isoxazole coupled peptides were purified by recrystallization from the appropriate solvents prior to biological testing and their structures established on the basis of spectral data (**Table II**).

### Antibacterial activity

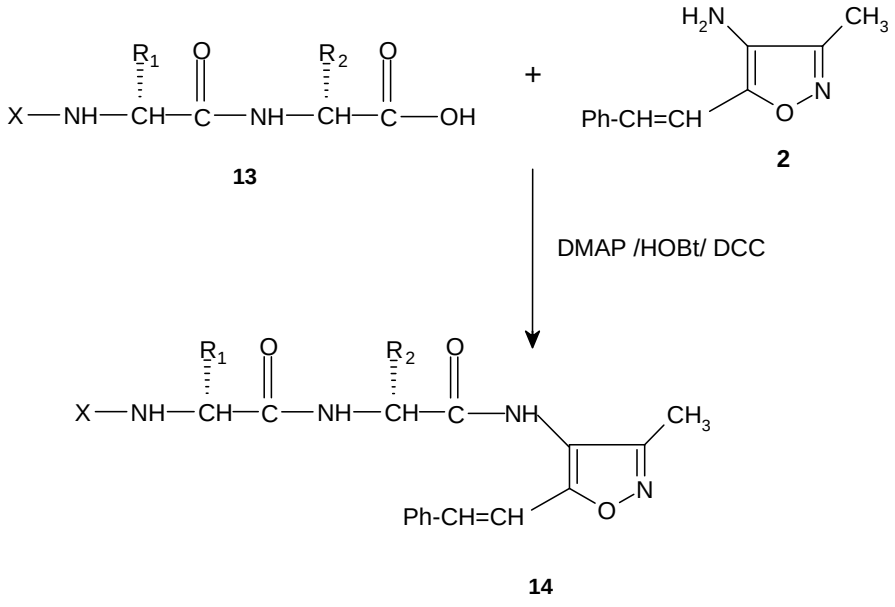
Six test organisms, *Bacillus subtilis* (MTCC 441), *Bacillus sphaericus* (MTCC 511), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella aerogenes* (MTCC 39) and *Chromobacterium violaceum* (MTCC 2656) were obtained from the Institute of Microbial Technology, Chandigarh. Cultures of test organisms were maintained on nutrient agar slants and were subcultured in Petri dishes prior to testing.




**1** **2** **3**

**4**, X = Boc, R = H (Gly)
**5**, X = Boc, R = $CH_3$ (D-Ala)
**6**, X = Boc, R = $CH_2CH(CH_3)_2$(D-Leu)
**7**, X = Fmoc, R = $CH_2OC(CH_3)_3$ (D-Ser(OBu$^t$))
**8**, X = Fmoc, R = $CH_2$-$C_6H_4$- $OC(CH_3)_3$ (D-Tyr(OBu$^t$))
**9**, X = Fmoc, R = $CH_2COOC(CH_3)_3$ (D-Glu(OBu$^t$))
**10**, X = Fmoc, R = $(CH_2)_4$-$NHCOCF_3$ (D-Lys($F_3$Ac))
**11**, X = Z, R = $CH(CH_3)CH_2CH_3$ (D-Ile)
**12**, X = Ac, R = $(CH_2)_3NH$ (C = NH)$NH_2$ (D-Arg)

**Scheme I**




**13** **2** **14**

**15**, X = Boc, $R_1 = CH_2C_6H_5$, $R_2 = CH_2CH(CH_3)_2$ (Boc -D-Phe-D-Leu)
**16**, X = Boc, $R_1 = CH_3$, $R_2$ = H (Boc-D-Ala-Gly)

**Scheme II**

**Table I** — Physical properties of *N*-protected amino acid/peptide isoxazoles*

| Compd | m.p. (°C) | Yield (%) | Mol. formula | $R_f$ Value | $[\alpha]_D^{20}$ ( c = 0.2,MeOH ) |
|---|---|---|---|---|---|
| Boc-Gly-isoxazole **4** | 136-40 | 70 | $C_{19}H_{23}N_3O_4$ | 0.58 | -1.9 |
| Boc-D-Ala-isoxazole **5** | 150-55 | 73 | $C_{20}H_{25}N_3O_4$ | 0.65 | -25.4 |
| Boc-D-Leu-isoxazole **6** | 92-95 | 70 | $C_{23}H_{31}N_3O_4$ | 0.73 | +12.2 |
| Fmoc-D-Ser (OBu$^t$)-isoxazole **7** | 125-28 | 71 | $C_{34}H_{35}N_3O_5$ | 0.82 | +16.7 |
| Fmoc-D-Tyr (OBu$^t$)-isoxazole **8** | 97-99 | 75 | $C_{36}H_{37}N_3O_6$ | 0.51 | +18.1 |
| Fmoc-D-Glu (OBu$^t$)-isoxazole **9** | 125-28 | 71 | $C_{36}H_{37}N_3O_6$ | 0.80 | +24.0 |
| Fmoc-D-Lys($F_3$Ac)-isoxazole **10** | 140-46 | 70 | $C_{35}H_{33}N_4O_5F_3$ | 0.68 | -1.1 |
| *Z*-D-Ile-Isoxazole **11** | 168-70 | 70 | $C_{26}H_{29}N_3O_4$ | 0.39 | -12.5 |
| *N*-Ac-D-Arg-isoxazole **12** | 208-10 | 72 | $C_{20}H_{27}N_6O_3$ | 0.44 | -10.2 |
| Boc-D-Phe-D-Leu-isoxazole **15** | 156-59 | 74 | $C_{27}H_{32}N_4O_3$ | 0.76 | -11.6 |
| Boc-D-Ala-Gly-isoxazole **16** | 121-23 | 69 | $C_{17}H_{20}N_4O_3$ | 0.88 | -12.0 |

* TLC analysis carried out using $CHCl_3$-MeOH (9:1) for compounds **4-10** and ethyl acetate-*n*-hexane (7:3) for compounds **11**,**12** and **15**,**16**.

**Table II** — $^1$H NMR and MS spectral data of *N*-protected amino acid/peptide isoxazoles

| Compd | $^1$H NMR (300 MHz, $CDCl_3$) | MS ($M^+$) |
|---|---|---|
| **4** | 1.5 (s, 9H, $3CH_3$), 2.3 (S, 3H, $CH_3$), 4.2 (d, 1H, $CH_2$), 5.3 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.5 (m, 5H, Ar-H and 1H, CH=CH), 8.1 (bs, 1H, NH) | 357 |
| **5** | 1.3 (d, 3H, $CH_3$), 1.4 (s, 9H, $3CH_3$), 2.2 (s, 3H, $CH_3$), 4.2 (q, 1H, CH), 5.1 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.5 (m, 5H, Ar-H and 1H, CH=CH), 8.0 (bs, 1H, NH) | 371 |
| **6** | 0.9 (d, 6H, $2CH_3$), 1.2 (m, 1H, CH), 1.4 (s, 9H, $3CH_3$), 1.8 (m, 2H, $CH_2$), 2.2 (s, 3H, $CH_3$), 4.2 (m, 1H, CH), 5.2 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.6 (m, 5H, Ar-H and 1H, CH=CH), 8.1 (bs, 1H, NH) | 413 |
| **7** | 1.2 (s, 9H, $3CH_3$), 2.2 (s, 3H, $CH_3$), 3.6 (d, 4H, $2CH_2$), 4.2 (m, 1H, CH), 4.4 (m, 1H, CH), 5.6 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.0-7.8 (m, 13H, Ar-H and 1H, CH=CH), 8.1 (bs, 1H, NH) | 565 |
| **8** | 1.4 (s, 9H, $3CH_3$), 2.2 (s, 3H, $CH_3$), 2.5 (d, 2H, $CH_2$), 3.6 (d, 2H, $CH_2$), 4.2 (m, 1H, CH), 4.4 (m, 1H, CH), 5.0 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.6 (m, 17H, Ar-H and 1H, CH=CH), 8.0 (bs, 1H, NH) | 641 |
| **9** | 1.4 (s, 9H, $3CH_3$), 2.2 (s, 3H, $CH_3$), 2.5 (m, 2H, $CH_2$), 3.6 (d, 2H, $CH_2$), 4.0 (m, 1H, CH), 4.2 (t, 1H, CH), 4.6 (m, 2H, $CH_2$), 6.0 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.0-7.8 (m, 13H, Ar-H and 1H, CH=CH), 8.1 (bs, 1H, NH) | 595 |
| **10** | 1.4-1.8 (m, 8H, $4CH_2$), 2.2 (s, 3H, $CH_3$), 3.5 (d, 2H, $CH_2$), 4.1 (m, 1H, CH), 4.2 (m, 1H, CH), 5.5 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-8.0 (m, 13H, Ar-H and 1H, CH=CH), 8.0 (bs, 1H, NH), 9.4 (bs, 1H, NH) | 646 |
| **11** | 1.0 (m, 6H, $2CH_3$) 1.6 (m, 2H, $CH_2$), 1.8 (m, 1H, CH), 2.2 (S, 3H, $CH_3$), 3.5 (s, 2H, $CH_2$), 4.2 (m, 1H, CH), 5.0 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.6 (m, 10H, Ar-H and 1H, CH=CH), 9.0 (bs, 1H, NH) | 447 |
| **12** | 1.2 (m, 4H, $2CH_2$) 1.6 (m, 2H, $CH_2$), 2.2 (S, 3H, $CH_3$), 3.1 (bs, 2H, NH), 3.6 (bs, 1H, NH), 3.9 (m, 1H, CH), 4.4 (bs, 1H, NH), 5.3 (bs, 1H, NH), 6.8 (d, 1H, CH=CH), 7.2-7.6 (m, 5H, Ar-H and 1H, CH=CH), 7.8 (bs, 1H, NH) | 399 |
| **15** | 1.0 (s, 9H, $3CH_3$) 1.2 (s, 6H, $2CH_3$), 1.3 (m, 1H, CH), 1.6 (m, 2H, $CH_2$), 2.0 (m, 2H, $CH_2$), 2.1 (s, 3H, $CH_3$), 3.6 (m, 2H, CH), 4.6 (bs, 1H, NH), 7.0-7.6 (m, 10H, Ar-H and 2H, CH=CH), 7.8 (bs, 2H, NH) | 460 |

The compounds were screened for their ability to inhibit bacterial growth against Gram negative bacteria *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella aerogenes* (MTCC 39), *Chormobacteium violaceum* (MTCC 2656) and Gram positive bacteria *Bacillus subtilis* (MTCC 441), *Bacillus sphaericus* (MTCC 511) and *Staphylococcus aureus* (MTCC 96) at 100 μg/mL concentration. The minimum inhibitory concentration (MIC) was done by broth dilution method[9]. Ciprofloxacin was used as standard for comparison.

Compounds **4-12** and **15,16** namely, *N*-protected amino acid/peptide isoxazoles utilized in the present investigation displayed high antibacterial activity and are more active than the standard drug ciprofloxacin (**Table III**). In this series the compounds **12** and **15**

consisting of dipeptide linkage possessing extra amino group in arginine and phenyl alanine moiety showed better activity. Similarly, the compounds **5** and **9** consisting of alanine and glutamic acid moieties imparted remarkable activity. This may be due to an extra carboxyl group. The compounds **4, 6, 10** and **11** have moderate antibacterial activity. The antibacterial activity of some of these *N*-protected amino acid isoxazoles compared to standard ciprofloxacin is promising and they can be exploited for formulation of bacteriocide after further study.

**Antifungal activity**

Five test organisms, *Aspergillus niger* (MTCC 282), *Chrysosporium tropicum* (MTCC 2821), *Rhizopus oryzae* (MTCC 262), *Fusarium moniliforme* (MTCC 1848), and *Curvularia lunata* (MTCC 2030) were obtained from the Institute of Microbial Technology, Chandigarh. Cultures of test organisms were maintained on potato dextrose agar slants and were subcultured in Petri dishes prior to testing.

The compounds were tested for their antifungal activity against five test organisms *Aspergillus niger* (MTCC 282), *Chrysosporium tropicum* (MTCC 2821), *Rhizopus oryzae* (MTCC 262), *Fusarium moniloformae* (MTCC 1848) and *Curvularia lunata* (MTCC 2030) using agar cup bioassay method[10] at 30 μg and 100 μg/mL concentration. Clotrimazole (fungicide) was used as the standard for comparison of the activity.

*N*-protected amino acid/peptide isoxazoles **4-12** and **15, 16** are significantly toxic towards all the five fungi and they are lethal at 100 μg/mL concentration (**Table IV**). Compounds **4** and **6** exhibited high antifungal activity which may be due to glycine and leucine moieties. Dipeptides **9** and **11** have moderate activity and they are highly toxic towards some fungi and moderate towards other fungi. Rest of the compounds **5, 7, 8, 10, 12** have moderate activity. The difference in toxicity of **4, 6, 9** and **11** towards five fungi can be attributed to the difference in their cell wall composition that affects the passage of the compound through the fungal cell wall. The antifungal activity of these compounds was compared with the standard drug clotrimazole and they have promising activity when compared with the standard drug.

**Mosquito larvicidal test**

*N*-protected amino acid/peptide isoxazoles **4-12** and **15, 16** were tested for toxicity against fourth instar mosquito larvae, *Culex quinquefasciatus*. Mosquito larval bioassay was performed according to standard methodology[11]. Toxicity and activity of the compounds were reported as $LC_{50}$ and $LC_{90}$ representing the concentration in ppm that killed 50% and 90% of larvae respectively. Two different concentrations **1, 2, 3, 4, 5** and **10, 15, 20, 25, 30** ppm were employed.

**Table III** — Antibacterial activity data MIC (in μg/mL) values of novel *N*-protected amino acid/peptide isoxazoles

| Compd | Microorganisms / conc (μg/mL) 100 | | | | | |
|---|---|---|---|---|---|---|
| | Gram positive | | | Gram negative | | |
| | *B. subtilis* | *B. sphaericus* | *S. aureus* | *P. aeruginosa* | *K. aerogenes* | *C. violaceum* |
| **4** | 16.5 | 15.0 | 15.5 | 20.0 | 15.5 | 13.0 |
| **5** | 8.0 | 10.5 | 11.5 | 8.0 | 12.5 | 15.0 |
| **6** | 16.5 | 15.0 | 18.0 | 18.0 | 15.0 | 15.5 |
| **7** | 18.5 | 18.0 | 16.5 | 15.5 | 20.0 | 15.5 |
| **8** | 20.0 | 15.5 | 20.0 | 16.5 | 18.5 | 15.0 |
| **9** | 10.0 | 8.0 | 12.0 | 8.0 | 12.5 | 10.0 |
| **10** | 15.0 | 15.0 | 15.0 | 20.5 | 15.0 | 16.5 |
| **11** | 15.0 | 15.5 | 12.5 | 20.0 | 15.5 | 20.0 |
| **12** | 8.5 | 10.0 | 10.5 | 12.5 | 10.5 | 12.0 |
| **15** | 10.0 | 12.5 | 8.5 | 10.0 | 10.0 | 13.5 |
| **16** | 18.5 | 16.5 | 16.0 | 20.0 | 16.5 | 15.5 |
| Ciprofloxacin | 20 | 20 | 25 | 30 | 25 | 25 |

Negative control (acetone) – No activity
Values are indicated in μg/mL

**Table IV** — Antifungal screening results of *N*-protected amino acid/peptide isoxazoles

| Compd | Conc. (μg/mL) | Zone of inhibition in mm | | | | |
|---|---|---|---|---|---|---|
| | | *A. niger* | *C. tropicum* | *R. oryzae* | *F. moniliformae* | *C. lunata* |
| **4** | 30 | 40 | 45 | 40 | 40 | 45 |
| | 100 | 60 | 65 | 60 | 60 | 65 |
| **5** | 30 | 30 | 35 | 30 | 33 | 35 |
| | 100 | 45 | 45 | 50 | 45 | 50 |
| **6** | 30 | 40 | 40 | 40 | 35 | 45 |
| | 100 | 60 | 65 | 65 | 60 | 65 |
| **7** | 30 | 35 | 35 | 30 | 35 | 35 |
| | 100 | 50 | 45 | 45 | 40 | 45 |
| **8** | 30 | 30 | 30 | 35 | 35 | 30 |
| | 100 | 40 | 50 | 45 | 45 | 45 |
| **9** | 30 | 35 | 40 | 30 | 30 | 40 |
| | 100 | 45 | 65 | 40 | 45 | 65 |
| **10** | 30 | 40 | 35 | 35 | 40 | 30 |
| | 100 | 55 | 45 | 45 | 65 | 60 |
| **11** | 30 | 30 | 45 | 45 | 30 | 40 |
| | 100 | 45 | 65 | 65 | 45 | 60 |
| **12** | 30 | 25 | 30 | 35 | 30 | 35 |
| | 100 | 40 | 45 | 45 | 45 | 45 |
| **15** | 30 | 30 | 35 | 30 | 30 | 30 |
| | 100 | 35 | 45 | 50 | 40 | 40 |
| **16** | 30 | 30 | 30 | 35 | 30 | 35 |
| | 100 | 45 | 45 | 45 | 45 | 50 |
| Ciprofloxacin | 100 | 26 | 29 | 23 | 27 | 28 |

Negative control (acetone) – No activity

The toxicity of test compounds to fourth instar larvae of *C. quinquefasciatus* is reported in **Table V**. From the data it appears that the *N*-protected amino acid isoxazoles **4, 5** and **9** are most toxic to larvae at $LC_{50}$ value, followed by compounds **6** and **7**. *N*-protected amino acid/peptide isoxazoles **8, 10** and **15** are moderately toxic to larvae whereas compounds **11** and **12** are least toxic. The *N*-protected amino acid isoxazoles **4** and **9** are proved to be lethal for mosquito larvae, hence can be useful as more toxic substances.

## Experimental Section

D-Amino acids were purchased from Davas Pharmaceuticals, USA. TLC were run on Merck Silica Gel 60 $F_{254}$ coated aluminium plates and melting points were determined in open capillary tubes and are uncorrected. IR spectra in KBr pellets were recorded on Shimadzu FTIR spectrometer, $^1$H NMR spectra were recorded in $CDCl_3$ and in DMSO-$d_6$ on a Varian (300 MHz) spectrometer using TMS as internal standard and mass spectra were recorded on a VG-S.70 micro mass instrument operating at 70 eV. C, H and N analyses were carried out on Carlo Erba 106 and Perkin-Elmer analysers.

**General procedure for the preparation of *N*-protected amino acid isoxazoles, 4-12**. To a solution of *N*-protected amino acid **1** (1 mmole) in dichloromethane (50 mL), DMAP (1 equivalent), HOBt (1 mmole) ad 4-amino-3-methyl-5-styryliso-xazole **2** (1 mmole) were added at 0°C with stirring. After a few min, DCC (2 mmoles) in $CH_2Cl_2$ was added to the reaction mixture during 25 min at 0°C with stirring. The stirring was continued for 2 hr at 0°C then at RT for another 24 hr (monitored by TLC). After the removal of *N*,*N*-dicyclohexyl urea by filtration it was washed with dichloromethane. The organic layer was concentrated under reduced pressure and washed with citric acid (2×10 mL), water (2×10 mL) and brine solution and dried over anhydrous $Na_2SO_4$. The organic layer was concentrated under reduced pressure and the crude product obtained was recrystallized from dichloromethane-*n*-hexane to afford the pure title compounds **4-12**.

**General procedure for the preparation of *N*-protected dipeptide isoxazoles, 15, 16**. To a cooled

**Table V** — Toxicity of *N*-protected amino acid/peptide isoxazoles against fourth instar larvae, *Culex quinquefasciatus*

| Compd | $LC_{50}$ | $LC_{90}$ | CHI square | Reg Coeff |
|---|---|---|---|---|
| **4** | 0.98 | 4.10 | 5.19 | 5.19 |
| **5** | 1.01 | 1.86 | 0.40 | 4.81 |
| **6** | 1.50 | 3.09 | 7.09 | 4.51 |
| **7** | 1.25 | 2.41 | 2.78 | 4.09 |
| **8** | 1.50 | 3.09 | 7.29 | 4.09 |
| **9** | 0.88 | 1.81 | 0.57 | 4.12 |
| **10** | 2.09 | 3.63 | 6.37 | 4.79 |
| **11** | 1.34 | 2.46 | 2.57 | 5.34 |
| **12** | 2.27 | 1.29 | 2.21 | 1.07 |
| **15** | 1.54 | 2.70 | 3.46 | 5.24 |

Negative control (acetone) – No mortality

solution of *N*-protected dipeptide **13** (1 mmole) in $CH_2Cl_2$ (50 mL), DMAP (1 equivalent), HOBt (1 mmol) and 4-amino-3-methyl-5-styrylisoxazole **2** (1 mmole) were added at 0°C with stirring. After a few minitues, DCC (2 mmol) was added to the reaction mixture during 30 min at 0°C with stirring. The stirring was continued at 0°C for 2 hr and then at RT for another 24 hr (monitored by TLC). The dicyclohexyl urea formed in the reaction was filtered out and washed with DCM. The organic layer was concentrated under reduced pressure and the residue dissolved in ethyl acetate and cooled to 5°C overnight. The excess urea derivative formed was filtered, then the solution washed with ethyl acetate, 1 *N* HCl, $KHCO_3$ and finally with water and brine solution and dried over anhyd. $MgSO_4$. The organic solvent was removed under reduced pressure and the resulting residue was recrystallized from dichloro-methane-*n*-hexane solvent to afford the pure title compounds **15** and **16**.

## Conclusion

This investigation happens to be the first of its kind wherein the coupling of isoxazole with *N*-protected amino acid/peptides has been performed. The dipeptides **5, 12** and **15** are found to inhibit the growth of bacteria at MIC 12.5 and 8.0 μg/mL whereas the dipeptides **5** and **12** are highly toxic towards the fungi used in the experiments. Compounds **4** and **5** are toxic to mosquito larvae. The results indicate that the dipeptides containing alanine and arginine moieties have significant ability to resist the bacterial and fungal infections and are also able to control mosquito related problems as they are toxic towards the mosquito larvae. This happens to be the first report to the best of our knowledge, that the peptides formed by coupling of a heterocycle with different amino acids could be useful as antibacterial and antifungal agents besides exhibiting toxicity towards the mosquito larvae.

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