Egypt. J. Chem. **Vol. 68**, No. 3 pp. 161 - 167 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Synthesis and Characterization of Levofloxacin-Conjugated Amphiphilic Peptides

Rakesh K. Tiwari[1,2], Keykavous Parang[1], Shaban A. Darwish[3*]

[1]*Department of Biomedical and Pharmaceutical Sciences, Center for Targeted Drug Delivery, Chapman University School of Pharmacy, Harry and Diane Rinker Health Science Campus, Irvine, CA 92866, USA.*
[2]*Department of Basic Medical Sciences, College of Osteopathic Medicine of the Pacific-Northwest, Western University of Health Sciences, Lebanon, OR 97355, USA*
[3]*Organometallic and Organometalloid Chemistry Department, National Research Centre, El Bohouth St., Dokki, Giza 12622, Egypt*

Abstract
The global challenge of antibacterial drug resistance has long impeded progress in the healthcare sector. To address this issue, an integrated approach involving antibacterial drugs and amphiphilic peptides has emerged as a promising strategy. This research aims to synthesize conjugates of levofloxacin (**LEV**) with rationally designed amphiphilic peptides (RFWRRIR, AKKWRKRW, and RWRIRWRKRA). Linear peptides, composed of a sequence of cationic and hydrophobic residues, were synthesized via solid-phase synthesis. Subsequently, **LEV**-containing amphiphilic peptides (**LEV**-RFWRRIR, **LEV**-AKKWRKRW, **LEV**-RWRIRWRKRA, and **LEV**-RWRIRWRK(**LEV**)RA) were synthesized under either solid-phase or solution-phase conditions, tailored to the specific levofloxacin-peptide conjugates needed. Four meticulously designed conjugates underwent purification through reverse-phase high-performance liquid chromatography (RP-HPLC) and characterization via matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS).

Keywords: Amphiphilic peptides; Levofloxacin; Levofloxacin-peptide conjugates; Solid-phase synthesis

1. Introduction

Antimicrobial resistance (AMR) in a wide range of infectious agents continues to be a serious threat to human health and the global economy. In 2019, bacterial AMR was directly responsible for 1.27 million global deaths and led to 4.95 million deaths [1]. Economically, the World Bank projects that AMR might drive up healthcare expenses by US$1 trillion by 2050 [2]. Given this menace, there is a need to leverage increased laboratory capabilities to ensure the continuous discovery of novel antimicrobial drugs, particularly during public health crises like the COVID-19 pandemic in 2020.

In this regard, the fluoroquinolone family (FQ) plays an important role as a synthetic antibiotic against both Gram-negative and Gram-positive pathogens [3-5]. FQ targets are intercellular, which means the drug needs to pass through the cell membrane [6]. However, the penetration process remains unclear and controversial [7]. Its mechanism of action depends heavily on the suppression of two main bacterial enzymes, type II topoisomerases, gyrase DNA, and topoisomerase IV [8], through a complexation approach [9], which leads to a blockade of DNA and, ultimately, inhibition of bacterial DNA replication and transcription [10, 11].

Levofloxacin, ((S)-9-floro-2,3-dihydro-3-methyl-l0-(4-methyl-lpiperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate) is among the second generation of the fluoroquinolone family, commonly used in the treatment of serious respiratory tract infections, skin infections, urinary tract infections, and even serious cases of sepsis [12]. Over time, similar to other antibacterial agents, bacteria have acquired resistance to it due to misuse and overuse, which are the main drivers in the development of drug-resistant pathogens. In this context, a combination of drugs with different molecules may be needed [13, 14], which could prevent the rapid development of bacterial resistance. Conjugates of amphiphilic peptides and levofloxacin may be an attractive option for innovative and effective AMR management.

Antimicrobial peptides (AMPs) are a unique class of antimicrobial agents [15, 16]. Generally, AMPs are small-molecule peptides with a balance between a net positive charge due to a large number of cationic residues (arginine and lysine) and hydrophobic amino acids [17]. These amphiphilic AMPs represent a promising pipeline to confront bacterial resistance

*Corresponding author e-mail: shaban_darwish@yahoo.com.sg.; (Shaban A. Darwish).
Receive Date: 14 June 2024, Revise Date: 29 July 2024, Accept Date: 29 July 2024
DOI: 10.21608/ejchem.2024.297620.9868

infections. Unlike conventional antibiotics that inhibit protein synthesis, the majority of amphiphilic AMPs exert their actions via the physical disruption of the cell membrane [18]. The physical nature of membrane disruption is less influenced by the mechanisms underlying bacterial antibiotic resistance, resulting in a lower likelihood of drug resistance development. Therefore, creating hybrid molecules of levofloxacin and amphiphilic peptides may be an efficient strategy to mitigate antibacterial drug resistance.

Inspired by the above-mentioned literature and the promising biological results of our previously synthesized biofilm-inhibiting peptides [19], we expanded our work by combining these peptides with an antibacterial drug. The main objective of this work is to design, synthesize, and characterize different conjugates composed of amphiphilic linear peptides and levofloxacin.

2. Experimental section

Fmoc (9-fluorenylmethoxycarbonyl)-Rink amide resin, protected amino acid building blocks, and chemical reagents were purchased from AAPPTec, LLC. All solvents used for the synthesis and RP-HPLC were purchased from Sigma-Aldrich, USA, and used without further purification. The amphiphilic peptides and the levofloxacin-peptide conjugates were purified by RP-HPLC on a Shimadzu, C_{18} (19 x 250 mm) column, and the purity was confirmed by analytical RP-HPLC using a mobile phase composed of eluent A (water with 0.1% TFA) and eluent B (acetonitrile with 0.1% TFA). A Bruker Daltonics Autoflex MALDI-TOF was utilized to record mass spectra with α-cyano-4-hydroxycinnamic Acid (CHCA) as the matrix.

Experimental procedures

Synthesis the amphiphilic peptides

Arg-Phe-Trp-Arg-Arg-lle-Arg (3), Ala-Lys-Lys-Trp-Arg-Lys-Arg-Trp (5), and Arg-Trp-Arg-Ile-Arg-Trp-Arg-Lys-Arg-Ala (7).

The amphiphilic peptides (**3**, **5**, and **7**) were synthesized according to the literature [19] using Fmoc-Rink amide resin with HBTU as a coupling reagent. The molecular weight were confirmed by MALDI-TOF MS (m/z), compound **3** [$C_{50}H_{82}N_{21}O_7$]: Calcd: 1088.6701; Found, 1089.4437 $[M + H]^+$. Compound **5** [$C_{55}H_{90}N_{20}O_8$]: Calcd: 1158.7240; Found, 1158.9933 $[M + 2H]^+$. Compound **7** [$C_{67}H_{113}N_{29}O_{10}$]: Calcd: 1483.9214; Found, 1484.2317 $[M + 2H]^+$.

Synthesis of Levofloxacin-peptide conjugates

LEV-Arg-Phe-Trp-Arg-Arg-lle-Arg) (9)

PyBOP (10 mg, 0.02 mmol) and HOBt (5 mg, 0.04 mmol) were added to a solution of levofloxacin **8** (17 mg, 0.04 mmol) in DMF, followed by DIPEA (22 µl, 0.12 mmol). The reaction mixture was stirred at room temperature for 20 min. A solution of peptide **3** (50 mg, 0.04 mmol) in DMF was added dropwise under inert conditions. After completion of the reaction (monitored by MALDI-TOF), the reaction mixture was added dropwise to the cold ether. The crude conjugate **9** was precipitated and subjected to RP-HPLC for purification (Rt = 29 min), and the purity was confirmed by analytical HPLC. MALDI-TOF (m/z), [$C_{68}H_{99}FN_{24}O_{10}$] Calcd: 1430.7960, Found: 1431.4910 $[M]^+$.

LEV-Ala-Lys-Lys-Trp-Arg-Lys-Arg-Trp (11)

HBTU (136 mg, 0.36 mmol) was added to a solution of levofloxacin **8** (130 mg, 0.36 mmol) in DMF, followed by DIPEA (99 µl, 0.54 mmol). The reaction mixture was stirred at room temperature for 20 min, followed by the addition to peptidyl resin **4** (400 mg, 0.03 mmol, 0.09 mmol/g) in DMF. After completing the coupling, the levofloxacin-linear peptide conjugate was cleaved from the resins and the side chains under a freshly prepared cleavage cocktail of TFA/TIS/water (92.5/5/2.5, v/v/v) for 2 h. The mixture was filtered, and the conjugate was precipitated using cold diethyl ether. The crude conjugate was purified using RP-HPLC (Rt = 24 min), and the purity was confirmed by analytical HPLC and mass by MALDI-TOF (m/z), [$C_{73}H_{106}FN_{23}O_{11}$] Calcd: 1499.8426, Found: 1500.6279 $[M]^+$.

LEV-Arg-Trp-Arg-Ile-Arg-Trp-Arg-Lys-Arg-Ala (12) The same procedure as conjugate **11** was followed. The peptidyl resin **6** (300 mg, 0.02 mmol, 0.07 mmol/g), levofloxacin (101 mg, 0.28 mmol), HBTU (106 mg, 0.28 mmol), and DIPEA (77 µl, 0.42 mmol) were used. The crude conjugate was purified using RP-HPLC (Rt = 25 min), and the purity was confirmed by analytical HPLC. MALDI-TOF (m/z): [$C_{85}H_{130}FN_{32}O_{13}$] Calcd: 1826.0474, Found: 1826.8966 $[M + H]^+$.

Di-levofloxacin-peptide conjugate

LEV-Arg-Trp-Arg-Ile-Arg-Trp-Arg-Lys(LEV)-Arg-Ala (13) The same procedure as conjugate **9** was followed. The peptide **7** (30 mg, 0.02 mmol), levofloxacin (14 mg, 0.04 mmol), PyBOP (10 mg, 0.02 mmol), HOBt (5 mg, 0.04 mmol), and DIPEA (11 µl, 0.06 mmol) were used. The crude conjugate **13** was purified using RP-HPLC (Rt = 25 min). The purity of the conjugate was confirmed by analytical HPLC. MALDI-TOF (m/z): [$C_{103}H_{148}F_2N_{35}O_{16}$], Calcd: 2169.1806, Found: 2169.9723 $[M + H]^+$.

Abbreviations

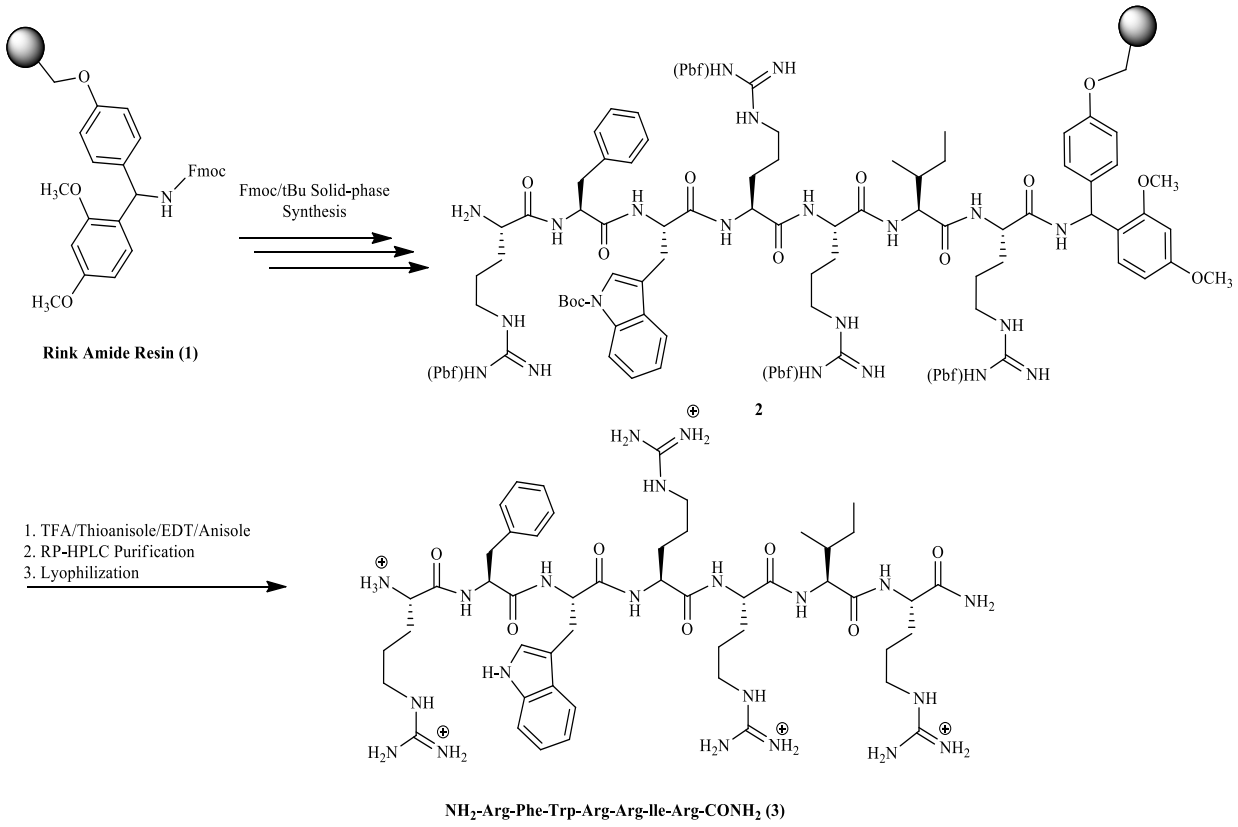
Ala, Alanine; Boc, Butyloxycarbonyl; F and Phe, Phenylalanine; I and Ile, Isoleucine; EDT, Ethane-1,2-dithiol; HOBt, 1-Hydroxybenzotriazole; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-Dimethylformamide; HBTU; 2-(1H-Benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate; Pbf, Pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; PyBOP, Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; R, Arginine; W; Tryptophan.

3. Results and Discussion

Amphiphilic linear peptides with cationic and hydrophobic residues were synthesized utilizing the solid-phase peptide technique [19, 20]. The peptide sequences were designed based on a previously described computational model [21], primarily featuring arginine (R) and lysine (Lys) as a source of cationic component, and tryptophan (Trp) as the hydrophobic element. R and W residues play a vital role in designing AMPs, where electrostatic and hydrophobic interactions drive the peptide to penetrate the bacterial cell membrane [22].

The protected linear peptides were assembled on an acid-sensitive resin, Rink amide resin **1** [23]. Solid support **1** is crucial in designing peptides to create a C-terminal amide moiety, which reduces proteolytic degradation and enhances binding to negatively charged bacterial membranes compared to peptides with a C-terminal carboxylic moiety [24].

The first short amphiphilic peptide **3** was prepared as outlined in Scheme **1**, composed of seven amino acid residues (Arg-Phe-Trp-Arg-Arg-Ile-Arg) with R and W at different ratios of 1:4. The reaction sequence started with Fmoc-deprotection of the resin, followed by consecutive steps of coupling and deprotection to yield the targeted peptide **3** after removing the side chain-protecting groups and the resin from intermediate **2.**



Scheme 1. Representative synthetic scheme for peptides illustrated by **3**.

Other short amphiphilic peptides were synthesized via the peptidyl resin intermediates (**4**) and (**6**), composed of eight amino acids (Ala-Lys-Lys-Trp-Arg-Lys-Arg-Trp) (**5**) and ten amino acid residues (Arg-Trp-Arg-Ile-Arg-Trp-Arg-Lys-Arg-Ala) (**7**), containing W and R moieties at varying ratios of 2:2 and 2:5, respectively (Fig. **1).**

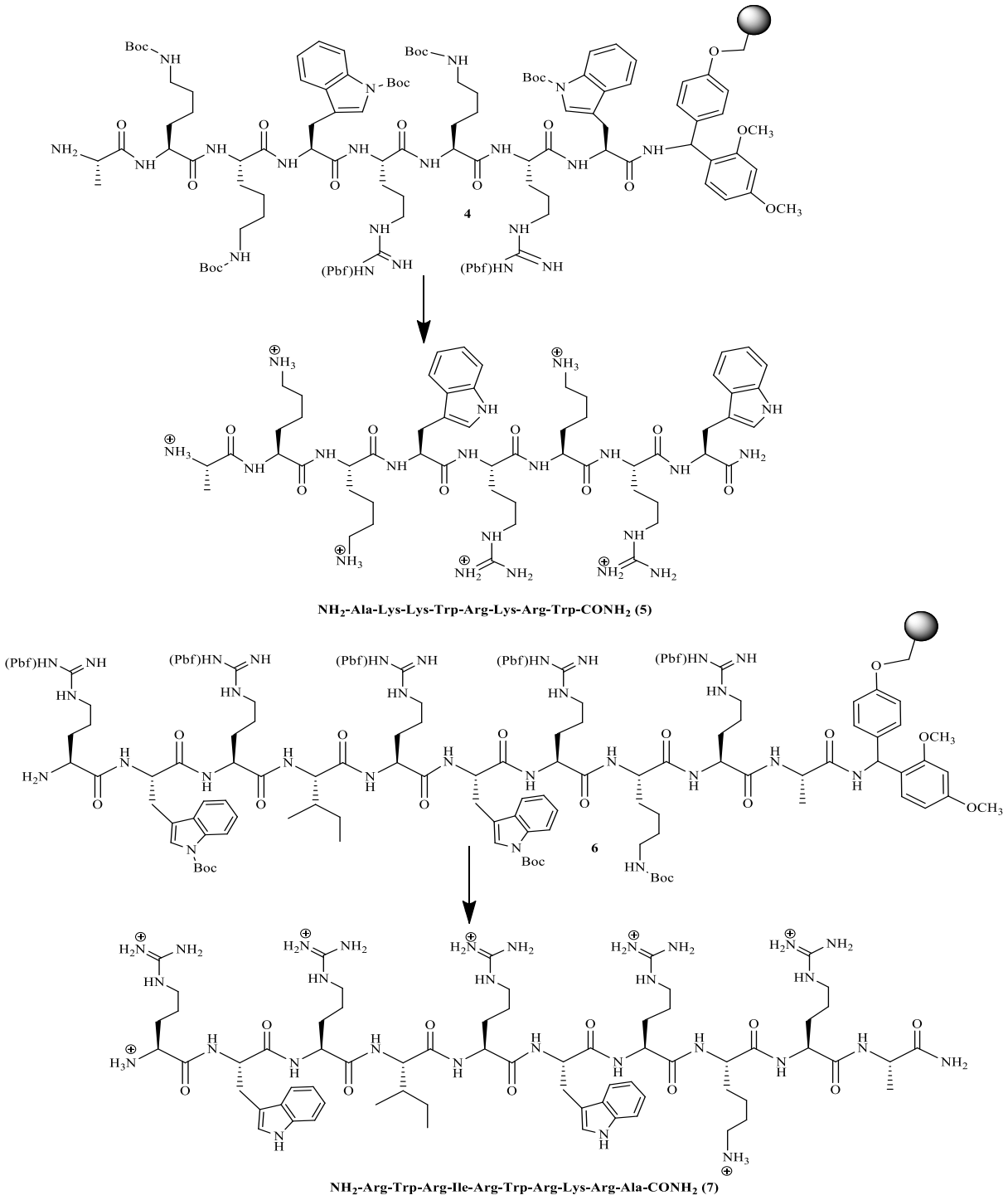
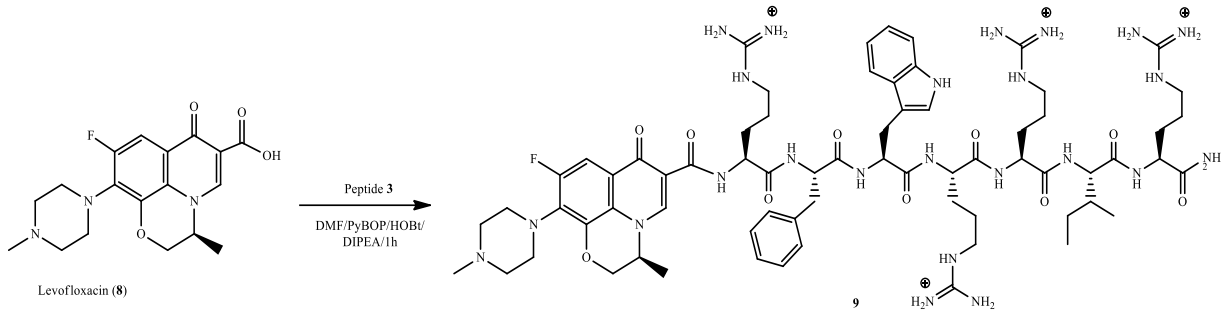


Fig. 1. Side chain protected peptidyl resins (**4** and **6**) and the corresponing linear peptides (**5** and **7**).

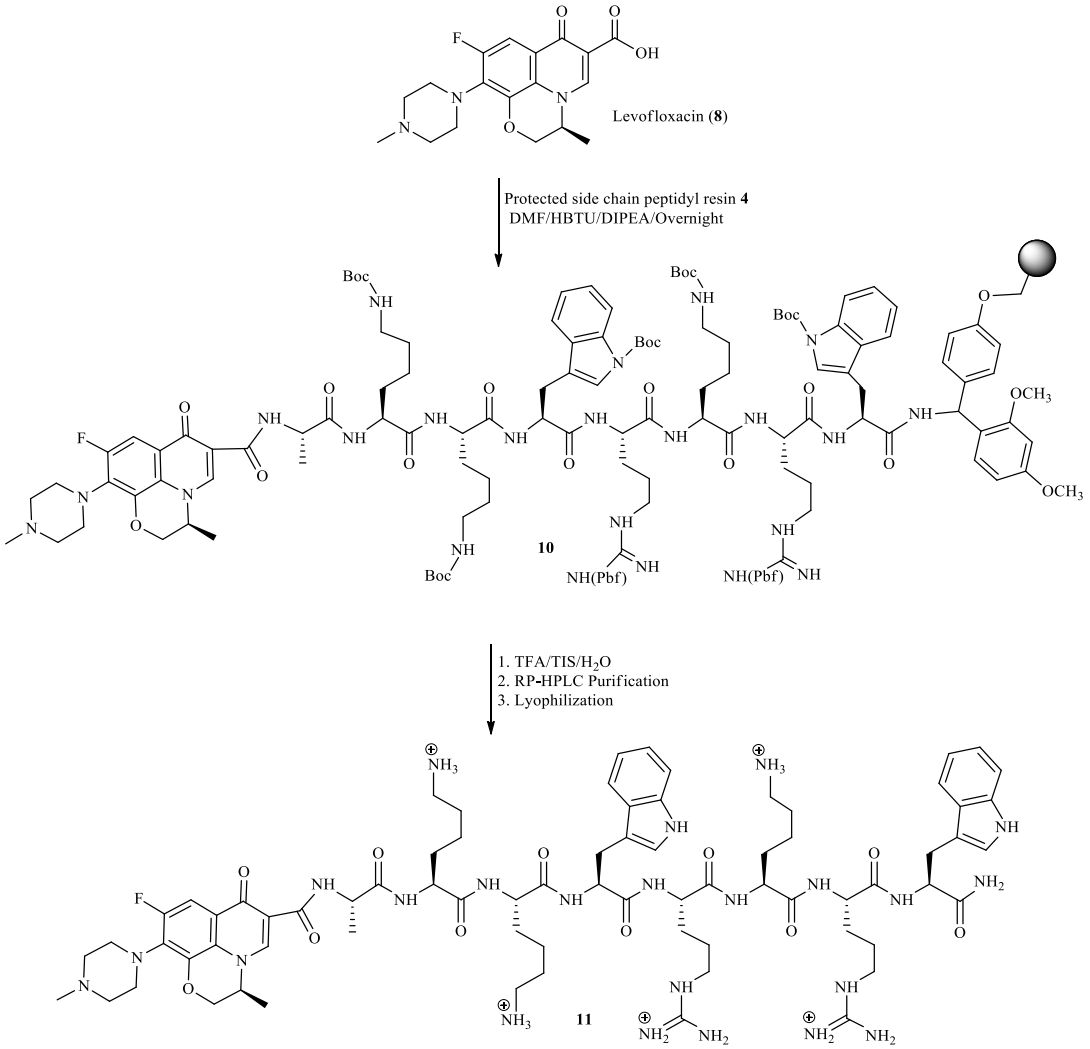
The characterization of the amphiphilic peptides was verified by MALDI-TOF MS, which closely matched the calculated masses.

From a structural viewpoint, amphiphilic peptides represent particularly interesting entities for hybridization with pharmaceutical molecules. Therefore, four conjugates of levofloxacin and amphiphilic peptides were designed and synthesized using either solid-phase or solution-phase synthesis methods. Regarding conjugate **9, it** was synthesized via solution-phase synthesis through a condensation reaction. The free amino terminus of peptide **3 was** coupled with levofloxacin **8** in the presence of PyBOP and HOBt as coupling reagents under basic conditions to afford the levofloxacin-peptide conjugate **9** (Scheme **2**). MALDI spectra showed a distinct peak at 1431.4910 Da, corresponding to $[M + H]^+$.



Scheme 2. Synthesis of levofloxacin-peptide conjugate (**9**).

On the other hand, the synthesis of conjugate **11** was accomplished under solid-phase conditions. The side N-terminal alanine residue of the peptidyl resin **4 was** coupled with levofloxacin **8** during a conventional amidation reaction**,** utilizing the widely employed aminium-based coupling reagent HBTU as an activator under basic conditions to give **LEV**-peptidyl resin conjugate **10**. The conjugate was then subjected to cocktail cleavage with freshly prepared trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water, generating the **LEV**-peptide conjugate **11** free from the side chain-protecting group and the resin (Scheme **3**). The targeted hybrid was confirmed by MALDI, showing a distinct peak at 1500.6279 Da, corresponding to $[M + H]^+$.



Scheme 3. Synthesis of linear peptide-levofloxacin conjugate (**11**).

Under similar solid-phase synthesis conditions, the **LEV**-peptide conjugate **12** was constructed. Treatment of the side chain-protected peptidyl resin **6** with a levofloxacin-activated carboxylic group afforded the corresponding mono levofloxacin-

peptide conjugate **12,** after the final cleavage of the resin and removal of the side chain-protecting groups (Fig. **2**). The MALDI spectra showed a peak at 1826.8966 Da, corresponding to $[M + H]^+$.

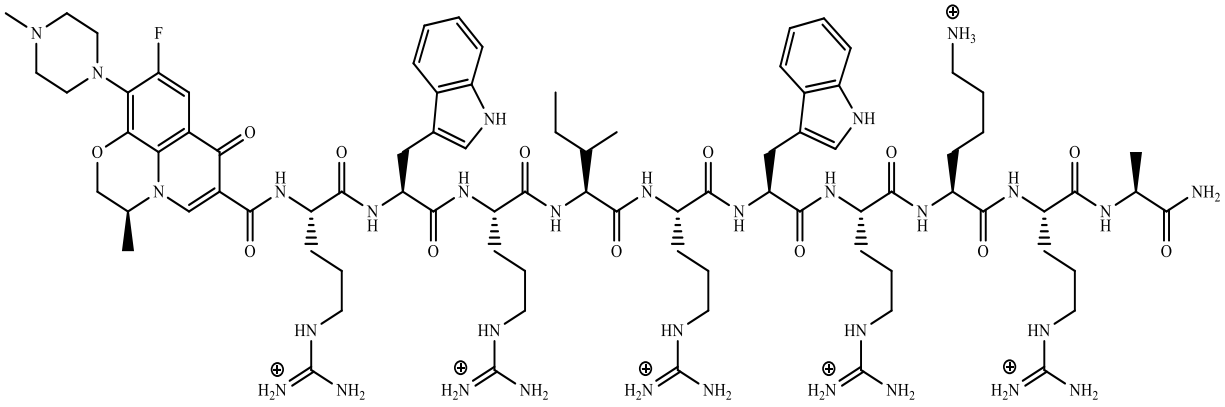
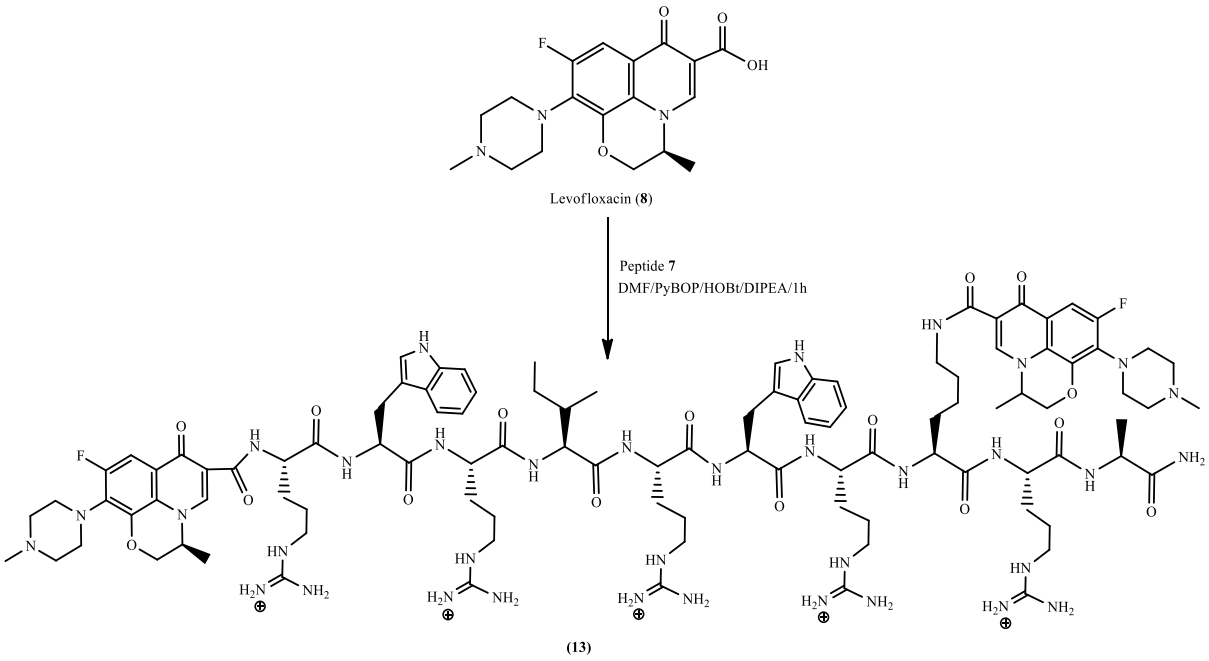


Fig. 2. Levofloxacin-linear peptide conjugate (**12**).

The conjugation of two molecules of an antibacterial drug with the amphiphilic peptides appears to be of considerable significance. As seen from peptide structure **7**, the two amino functional groups (*N*-terminal arginine and lysine side chain amino groups) can be leveraged for chemical conjugation. Under solution-phase conditions, two molecules of levofloxacin were coupled to the peptide through the lysine side chain and *N*-terminal under the standard coupling conditions (similar to conjugate **9**), generating an amphiphilic peptide-double levofloxacin motif as represented in Scheme **4**. Analysis of the product by MALDI spectra showed a peak at 2169.9723 Da, standing to $[M + H]^+$.



Scheme 4. Amphiphilic linear peptide attached two molecules of levofloxacin (**13**).

All the amphiphilic peptides and their conjugates with levofloxacin were purified through reverse-phase high-performance liquid chromatography (RP-HPLC), and the purification was confirmed by analytical HPLC (Supplementary material). It is worth mentioning that the research plan will be extended to synthesize more levofloxacin-amphiphilic peptide conjugates and evaluate their antibacterial efficacy against various strains *in vivo* animal studies.

4. Conclusions

The incorporation of antibacterial drugs into amphiphilic linear peptide scaffolds is an intriguing area of work in AMR management. Three linear amphiphilic peptides were synthesized using Fmoc solid-phase chemistry. The amphiphilic peptides were conjugated with a mono- or di-antibacterial drug, levofloxacin, under amidation reaction conditions, employing either solid-phase or solution-phase synthesis to generate four levofloxacin-peptide conjugates. The meticulously designed hybrid conjugates were characterized using MALDI-TOF MS, and their purity was confirmed by analytical HPLC.

5. Conflicts of interest

The authors declare that no reported conflicts.

Associated Content

MALDI mass spectrometry and Analytical HPLC of the targeted peptides and the corresponding conjugates

6. Acknowledgments

The authors thank the core labs at the Chapman University School of Pharmacy for access to all instrumentation.

7. References

[1] C.J.L. Murray, *et al.* The Lancet., 399 (2022) 629-655.
[2] O. Jonas, A. Irwin, J. Berthe, F. Le Gall, V. Marquez, Drug-resistant infections: a threat to our economic future (Vol. 2): final report (English). HNP/Agriculture Global Antimicrobial Resistance Initiative Washington, D.C.:World Bank Group. http://documents.worldbank.org/curated/en/323311493396993758/final-report
[3] T.D.M. Pham, Z. M. Ziora, M. A. T. Blaskovich, Med. Chem. Commun., 10 (2019) 1719-1739.
[4] X. Huang, Y. Bao, S. Zhu, X. Zhang, S. Lan, T. Wang, Bioorg. Med. Chem. Lett., 25 (2015) 3928-3932
[5] A. Foroumadi, S. Emami, S. Mansouri, A. Javidnia, N. Adeli, F. H. Shirazi, A. Shafiee, Eur. J. Med. Chem., 42 (2007) 985-992
[6] T.D. Gootz, K.E. Brighty, Med. Res. Rev., 16 (1996) 433-486.
[7] T. Mach, P. Neves, E. Spiga, H. Weingart, M. Winterhalter, P. Ruggerone, M. Ceccarelli, P. Gameiro, J. Am. Chem. Soc., 130 (2008) 13301-13309.
[8] C. Levine, H. Hiasa, K.J. Marians, Biochim. Biophys. Acta, Gene Struct. Expression, 1400 (1998) 29-43.
[9] A. Fabrega, S. Madurga, E. Giralt, J. Vila, Microb. Biotechnol., 2 (2009) 40-61.
[10] J.S. Wolfson, D.C. Hooper, Clin. Microbiol. Rev., 2 (1989) 378-424.
[11] D. N. Fish, A. T. Chow, Clin. Pharmacokinet., 32 (1997) 101-119.
[12] D.S. North, D.N. Fish, J.J. Redington, Pharmacotherapy, 18 (1998) 915-935.
[13] N. Riahifard, K. Tavakoli, J. Yamaki, K. Parang , R. Tiwari, Molecules, 22 (2017) 957.
[14] E.H.M. Mohammed, S. Lohan, T. Ghaffari, S. Gupta, R. Tiwari, K. Parang, J. Med. Chem., 65 (2022) 15819-15839.
[15] S. Ji, F. An, T. Zhang, M. Lou, J. Guo, K. Liu, Y. Zhu, J. Wu, R. Wu, Eur. J. Med. Chem., 265 (2024) 116072.
[16] K. Mangano, D. Klepacki, I. Ohanmu, C. Baliga, W. Huang, A. Brakel, A. Krizsan, Y. S. Polikanov, R. Hoffman, N. V. Laslop, A. S. Mankin, Nat. Chem. Biol., 19 (2023)1082–1090.
[17] L.T. Nguyen, E.F. Haney. H.J. Vogel, Trends Biotechnol., 29 (2011) 464-472.
[18] M.N. Melo, R. Ferre , M.A.R.B. Castanho, Nat. Rev. Microbiol., 7 (2009) 245-250.
[19] S. Darwish, A. Noreddin, R. Tiwari, W.F. Elkhatib, Int. J. Pept. Res. Ther., 25 (2019) 1075-1085.
[20] S. Darwish, K. Parang, J. Marshall, D. J. Goebel, R. Tiwari, Tetrahedron Lett., 58 (2017) 3053-3065.
[21] S. Gupta, A.K. Sharma, S.K. Jaiswal, V.K. Sharma, Front Microbiol., 7 (2016) 949-959.
[22] T. Anunthawan, C. Núñez, R.E. Hancock, S. Klaynongsruang, Biochim. Biophys. Acta., 1848 (2015) 1352-1358.
[23] H. Rink, Tetrahedron Lett., 28 (1987) 3787-3790.
[24] S. Zhu, W. Li, N. Simpson, F. Separovic, M.A. Sani, Biophys. J., 120 (2021) 769-777.