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RESEARCH ARTICLE

Preliminary study to obtain some fluoroquinolone-tetracycline hybrids

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Objective: This paper aimed to synthesize hybrids of fluoroquinolones with tetracycline class representatives and conduct their preliminary characterization.

Methods: A reaction between tetracycline representatives (doxycycline, tetracycline), formaldehyde (acting as a molecular connector) and fluoroquinolone representatives (ciprofloxacin, moxifloxacin, and norfloxacin) was attempted through a classical reflux synthesis with an electrical heating source (heating mantles) and a microwave-assisted reflux synthesis. One synthesis group also used cupric chloride dihydrate as a catalyst. The samples were analyzed using Differential Scanning Calorimetry, Electrospray Ionization Mass Spectrometry, or High-Performance Liquid Chromatography.

Results: The results indicated the formation of a compound different from the parent components in the case of doxycycline-norfloxacin and possibly tetracycline-norfloxacin hybrids.

Conclusions: Both synthesis methods yielded similar results. The influence of the catalyst did not seem to have been significant. The synthesis method is simple and one-step, using non-toxic solvents. Future studies involving molecular docking and microbiology could be employed to further explore the mechanism of action and the microbiological effects of the hybrids.

Keywords: hybrid, fluoroquinolone, tetracycline, antibiotic

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Introduction

Since ancient times, bacterial infections have posed a significant challenge to humanity. Epidemics such as the bubonic plague caused by *Yersinia pestis* and cholera caused by *Vibrio cholerae* have resulted in thousands of deaths. Effective solutions, including vaccines and antibiotic treatments, have been developed to combat these diseases [1, 2]. Beyond these historical epidemics, bacterial infections continue to emerge and persist globally, whether in outbreaks or isolated cases. A significant concern in these situations is the growing resistance of bacteria and the diminishing effectiveness of existing antibiotics [3, 4].

Fluoroquinolones (FQNs) are a valuable class of antimicrobial agents derived from antibacterial quinolones, initially discovered while obtaining chloroquine, an antimalarial agent [5]. They are widely used to treat bacterial infections due to their bactericidal effect, which inhibits DNA replication and transcription by targeting DNA gyrase and topoisomerase IV [6–9]. The versatility of their molecular structure has allowed improvements in their pharmacokinetic and pharmacodynamic properties [10–13]. FQNs are prescribed for infections in various locations, including the urinary, respiratory, and gastrointestinal tracts, targeting Gram-negative and Gram-positive bacteria [14, 15]. However, their widespread use raises concerns about antibiotic resistance, underscoring the need for continuous discovery of new derivatives [16, 17]. Besides their antibacterial use, some FQN-derived compounds are utilized to treat conditions such as tuberculosis, malaria, viral and fungal infections, cancer, immunosuppression, and neurodegenerative diseases, maintaining high research interest due to their chemical properties [18].

Tetracyclines (TCs), discovered in the 1940s from Streptomyces species, are broad-spectrum antibacterial agents effective against a wide range of Gram-positive and Gram-negative bacteria, as well as pathogens like protozoan parasites, rickettsiae, mycoplasmas, and chlamydiae [19, 20]. Their primary mechanism of action is inhibiting protein synthesis by interacting with the 16S RNA of the 30S ribosomal subunit [21]. However, TCs also exhibit biological activities such as neuroprotective [22, 23], antiviral [24–26], anti-inflammatory [22, 27, 28], and antiapoptotic effects [22, 24], whose mechanisms are still being studied. They are widely used to treat various infections in

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humans and animals, including respiratory, urinary, genital, lymphatic, skin, and intestinal infections, and to prevent malaria caused by mefloquine-resistant *Plasmodium falciparum* [19, 29]. The extensive use of TCs has led to bacterial resistance, mainly through ribosomal protection proteins and efflux pumps in Gram-positive and Gram-negative bacteria. Despite this, TCs remain a significant class of antimicrobials, providing a valuable therapeutic option for various bacterial infections [30].

Although the discovery, synthesis, and therapeutic use of molecules within established classes of antibiotics have not fully addressed the problem of resistance, innovative methods for developing unique molecules with new targets and mechanisms of action offer hope for overcoming these challenges [31]. One such innovative method is hybridization. This approach involves creating new hybrid molecules from two substances with known actions. These hybrids are synthetic compounds incorporating two or more pharmacophores from known antimicrobial agents into their structure [32].

This paper aims to synthesize hybrids of FQNs with representatives of the tetracycline class and present their preliminary characterization. Obtaining these hybrids will contribute to developing compounds to overcome the current bacterial resistance.

Methods

Chemicals and reagents

The solvent used was ethanol (Chimreactiv, Bucharest, Romania). The hybrid components (doxycycline monohydrate, tetracycline monohydrate, ciprofloxacin hydrochloride, moxifloxacin hydrochloride, norfloxacin) and the catalyst (cupric chloride dihydrate: $CuCl_2\ 2H_2O$) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The formaldehyde (a 37% solution) was purchased from Chemical Company (Iași, Romania).

The structures were sketched with Biovia DRAW 2021 [33].

Synthesis methods

The method introduced by Sriram et al. (2007) served as the starting point for this research. This method involves using microwave irradiation at 60% intensity for 3 minutes to facilitate the reaction between tetracycline representatives (tetracycline, oxytetracycline and minocycline), formaldehyde (acting as a molecular connector), and fluoroquinolone representatives (norfloxacin, lomefloxacin, ciprofloxacin and gatifloxacin) that contribute the secondary amino function (piperazine). In this experimental study, Mannich bases (beta-amino-ketones) of tetracyclines were synthesized, achieving yields between 41% and 78%, and the resulting products did not require any purification [34].

Our experiments were structured in three groups:

– S1: Classical reflux synthesis with an electrical heating source (heating mantles) using doxycycline and norfloxacin;
– S2: Microwave-assisted reflux synthesis, using doxycycline and ciprofloxacin, varying three parameters (the % of formaldehyde excess, the power, and the time);
– S3: Microwave-assisted reflux synthesis using doxycycline monohydrate, tetracycline monohydrate, ciprofloxacin hydrochloride, moxifloxacin hydrochloride, and norfloxacin, with and without $CuCl_2\ 2H_2O$ as catalyst.

The samples were assigned a code with the following format: [group][first two letters of the tetracycline component][first two letters of the fluoroquinolone component]-[energy source: "E" for electrical heating mantles, "M" for microwaves][number within the group - if the case][code for catalyst: "Cu" for $CuCl_2\ 2H_2O$ – if the case].

The details of the syntheses are presented in Table I.

For the S1 synthesis, the FQN and the TC components were put in a round bottom flask, successively adding the solvent and the solution of formaldehyde 37%. The condenser was mounted to the flask, and the flask-condenser installation was placed in a heating mantle (LabHEAT, ISOHEAT GmbH, Ubstadt-Weiher, Germany) placed over a magnetic stirrer (Heidolph, Schwabach, Germany) set to 500 rpm, and the synthesis time was 48 h.

For the S2 and S3 syntheses, the FQN and the TC components were put in the microwave's glass container, successively adding the solvent, the solution of formaldehyde 37%, and the catalyst (if applicable). The container was put in the ultrasonic bath (Analog Laboratory Ultrasonic Bath, AC-150H, MRC, Holon, Israel) for one minute and then placed in the microwave chamber (Ethos X – Advanced Microwave Extraction System, Milestone, Sorisole (BG), Italy), also coupling the condenser. The S3 parent compound solutions (S3Do, S3Te, S3Ci, S3Mo, S3No) were prepared similarly, except they were not microwaved. Figure 1 illustrates the schematic representation of the synthesis installations.

For the S1DoNo-E sample, we obtained two specimens, one by filtrating the suspension and air drying the precipitate (S1DoNo-E-PP) and one by evaporating the solvent from a portion of the supernatant (S1DoNo-E-SOL). The samples obtained from S1DoNo-E were analyzed using Differential Scanning Calorimetry (DSC) and Electrospray Ionization Mass Spectrometry (ESI-MS). The samples obtained from group S2 were analyzed using High-Performance Liquid Chromatography (HPLC), and the samples obtained from group 3 were analyzed by ESI-MS.

DSC analysis

The thermograms were registered using a DSC 60 apparatus with TA60-WS software (Shimadzu, Kyoto, Japan). The samples were obtained by pressing using Shimadzu 201-52943 lidded aluminum crimp cells. The DSC curves were recorded using 3 mg of sample in the 40-400°C temperature range, with an increase rate of 10°C/min.

Table I. Substances and conditions used in the syntheses

Sample code	TC component		FQN component		Molecular connector: formaldehyde 37%	Solvent: ethanol 70% (mL)	Catalyst: $CuCl_2$ $2H_2O$		Power (W)	Time (min)
	mg	mmol	mg	mmol			mg	mmol		
S1DoNo-E	924	~2	640	~2	165 µL (~2 mmol, plus 10% excess)	200	-		N/A	48 h
S2DoCi-M1	57.8	~0.25	46.0	~0.25	39 µL (~0.25 mmol, plus 100% excess)	210	-		100	1
S2DoCi-M2	57.7		45.4						1000	1
S2DoCi-M3	58.2		46.2						100	10
S2DoCi-M4	57.3		46.0						1000	10
S2DoCi-M5	58.2		45.5		116 µL (~0.25 mmol, plus 500% excess)				100	1
S2DoCi-M6	57.8		45.9						1000	1
S2DoCi-M7	58.4		46.1						100	10
S2DoCi-M8	58.5		46.8						1000	10
S2DoCi-M9	58.4		46.1		77 µL (~0.25 mmol, plus 300% excess)				100	10
S2DoCi-M10	58.1		46.0						550	5.5
S2DoCi-M11	57.8		46.0						550	5.5
S2DoCi-M12	58.3		46.5						550	5.5
S2DoCi-M-control	57.8		46.4		-				1000	10
S3DoCi-M	29.0	~0.125	22.9	~0.125	58 µL (~0.125 mmol, plus 500% excess)	110	-		1800	20
S3DoCi-MCu	29.0		22.9				2.3	~0.012		
S3TeCi-M	28.9		22.9				-			
S3TeCi-MCu	28.9		22.9				3.0	~0.012		
S3DoMo-M	28.9		27.3				-			
S3DoMo-MCu	28.9		27.4				2.5	~0.012		
S3TeMo-M	29.0		27.4				-			
S3TeMo-MCu	29.1		27.4				2.7	~0.012		
S3DoNo-M	28.9		20.5			100	-			
S3DoNo-MCu	29.0		19.9				3.1	~0.012		
S3TeNo-M	28.9		20.1				-			
S3TeNo-MCu	29.1		20.0				2.6	~0.012		
S3Do	29.2	-	-	-	-	60	-		-	-
S3Te	29.1					60				
S3Ci	-	-	23.0	~0.125		50				
S3Mo			27.4			60				
S3No			20.3			40				

Do: doxycycline monohydrate, Te: tetracycline monohydrate, Ci: ciprofloxacin hydrochloride, Mo: moxifloxacin hydrochloride, No: norfloxacin, N/A: Not Applicable

HPLC analysis

A Merck Purosphere STAR RP-C18e, 125x4.6mm, 5µm chromatographic column was used. The mobile phase consisted of (A) 0.1% phosphoric acid and (B) acetonitrile, with a 5-70% gradient of B in 15 minutes, 1 mL/min debit, and 50°C column temperature. The injected volume was 15 µL, and the detection was done at 210 nm.

ESI-MS analysis

The AB Sciex 4600 QTOF mass spectrometer with Analyst 1.7 software and the Perkin Elmer FX10 HPLC with Chromera software were utilized for this determination.

The HPLC apparatus was coupled to provide a mobile phase flow of 0.2% formic acid solution and acetonitrile. The ionization parameters and the mobile phase compo-

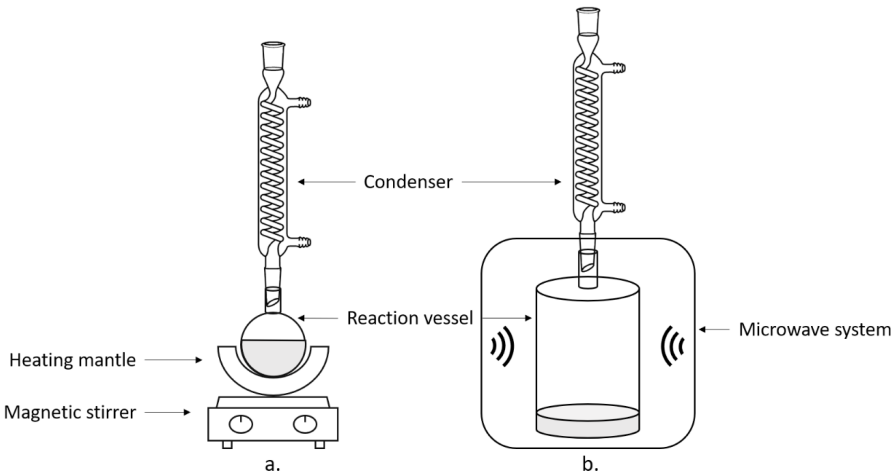


Fig. 1. Schematic representation of the synthesis methods: a. classical reflux synthesis, b. microwave-assisted reflux synthesis

sition were varied until the optimal conditions were obtained. The most advantageous mobile phase composition was 10% formic acid solution and 90% acetonitrile with a 0.2 mL/min flow rate. The optimal ionization parameters were: Ion Source – DuoSpray Ion Source, Ion Source Gas 1 – 15, Ion Source Gas 2 – 25, Curtain Gas – 10, Temperature – 450, IonSpray Voltage Floating – 4500, Scan type – TOF MS, Accumulation time – 0.249996 s, Period Duration – 10 min, Cycles – 2182, Delay Time – 0 s. Spectra were recorded for both negative and positive polarity. Furthermore, for the S1 group, fragmentation was performed at different collision energies for both negative and positive polarity to confirm that the hybrid was formed.

For the specimens derived from the S1DoNo-E sample (S1DoNo-E-PP and S1DoNo-E-SOL), dilute solutions in acetonitrile were obtained and centrifugated.

For the S3 group, 0.5 mL of sample was mixed with 0.5 mL hexane for 5 minutes at 3000 rpm. The mixture was then centrifugated for 5 minutes at 5500 rpm. 300 µL of supernatant were transferred in a tube and were evaporated under vacuum at room temperature. The sample was then reconstituted in 0.5 mL mobile phase.

Results

The appearance of some solutions changed after the reaction time specified (Table I). The S1DoNo-E sample changed from a light yellow solution to a pale brown suspension. The S1DoNo-E-PP specimen appeared as a light brown precipitate, and the S1DoNo-E-SOL specimen appeared as an orange dry substance. All the samples from the S2 group had the same appearance before and after the reaction time, namely a clear, very pale yellow solution. At the beginning of the reaction, all the samples from the S3 group appeared as clear, pale solutions ranging from yellow to orange or brown. At the end of the reaction, only samples S3DoNo-MCu and S3TeNo-MCu had changed, the first from a clear pale brown solution to an orange suspension and the second from a clear pale orange solution to an orange suspension.

The samples were kept in the refrigerator, and the aspect did not change further until the analyses were performed.

The structures of the components, the hypothetical structures of the potential hybrids, and the molecular masses are presented in Figure 2.

DSC analysis

The results obtained from the DSC analysis of the S1DoNo-E-PP and S1DoNo-E-SOL samples, along with the thermograms of the parent compounds (norfloxacin and doxycycline monohydrate), are presented in Figure 3.

HPLC analysis

The chromatograms of all the samples from the S2 group were similar. Figure 4 shows the obtained chromatograms for samples S2DoCi-M1 and S2DoCi-M8. The blue line corresponds to S2DoCi-M-control, a control solution of Do and Ci in 70% ethanol without formaldehyde (details regarding the quantities are presented in Table I). The peaks of both parent compounds (Do and Ci) can be seen in all chromatograms at approximately four (for Ci) and six (for Do) minutes.

ESI-MS analysis

The mass spectra obtained for the S1 group are presented in Figure 5. The m/z ratios corresponding to the expected molecular mass of the hybrid were present in the spectra both for negative (at a m/z of approximately 774 Da) and positive (at a m/z of approximately 776 Da) polarity, as well as the m/z corresponding to the parent compounds at approximately 320 Da (for No) and 445 Da (for Do). For group S1, fragmentation was also performed at different collision energies for negative and positive polarity to confirm that the obtained hybrid originates from the parent compounds (Figure 5).

For the S3 group, the m/z ratios corresponding to the expected molecular mass of the hybrids were not identified in either spectrum, except for the S3DoNo-M, S3DoNo-MCu, S3TeNo-M, and S3TeNo-MCu samples. In negative polarity, the closest to the expected mass was an m/z of approximately 779 Da for the abovementioned samples. In positive polarity, an m/z of approximately 775 was present for the S3TeNo-MCu sample.

Discussions

DSC analysis

Thermal methods can be employed to determine the specific thermophysical properties of hybrids. These analyses yield information regarding the stability of the hybrids, including their decomposition temperature, decomposition kinetics, and water content. When hybridization occurs, the resulting compound will exhibit thermal curves that differ significantly from its components [35].

This analysis was used for the samples in the S1 group. The melting point of norfloxacin was recorded at 222.96°C, an endothermic phenomenon represented by a very sharp peak on the thermogram (Figure 3); this fits in the 219.1 – 224,2°C melting interval determined by Dorofeev et al. [36], when using a temperature increase rate of 10°C/min, as we have used in the present work. For doxycycline, the endothermic peak was registered at 155°C. The melting point of doxycycline monohydrate is 167-168°C [37]. However, the obtained value is close to the one in the literature, and the difference could be explained by the water molecules present within the substance.

Some differences between the curves obtained for the products from the synthesis and those of the parent compounds can be observed. The curves obtained for the S1DoNo-E-PP and the S1DoNo-E-SOL samples are similar (Figure 3), indicating a similar substance in the precipitate and the supernatant.

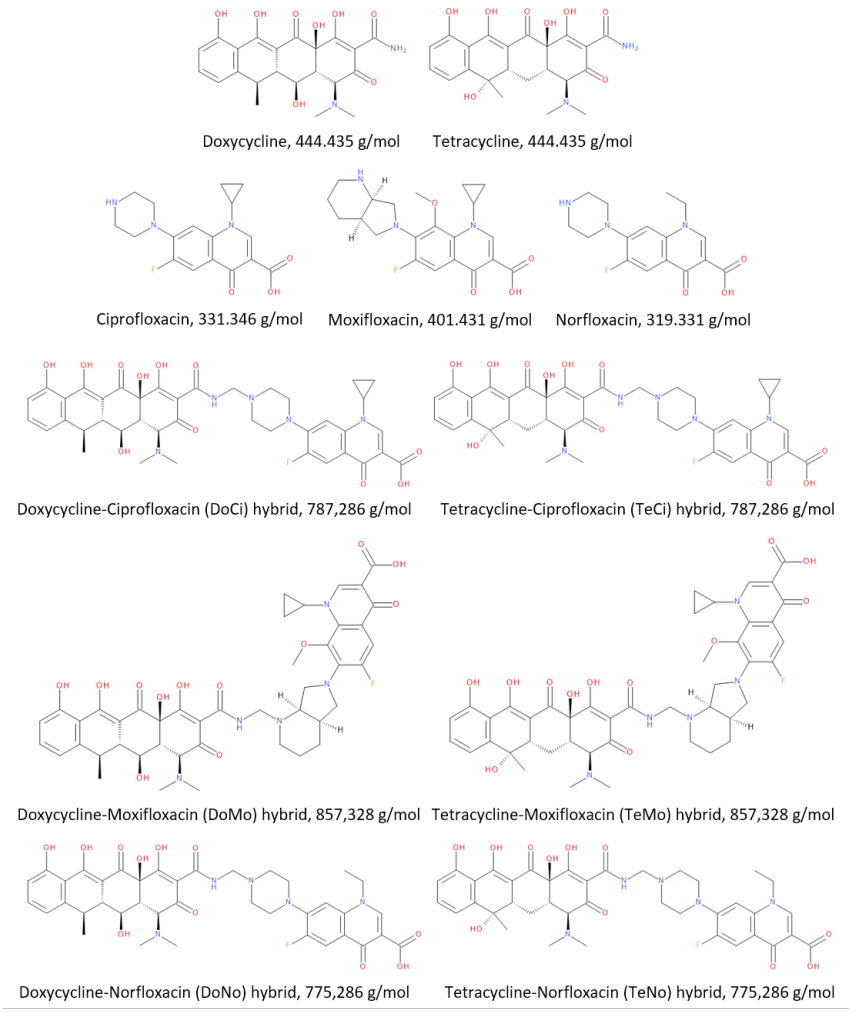


Fig. 2. Structure and molecular mass of the TC and FQN components and the hypothetical potential hybrids

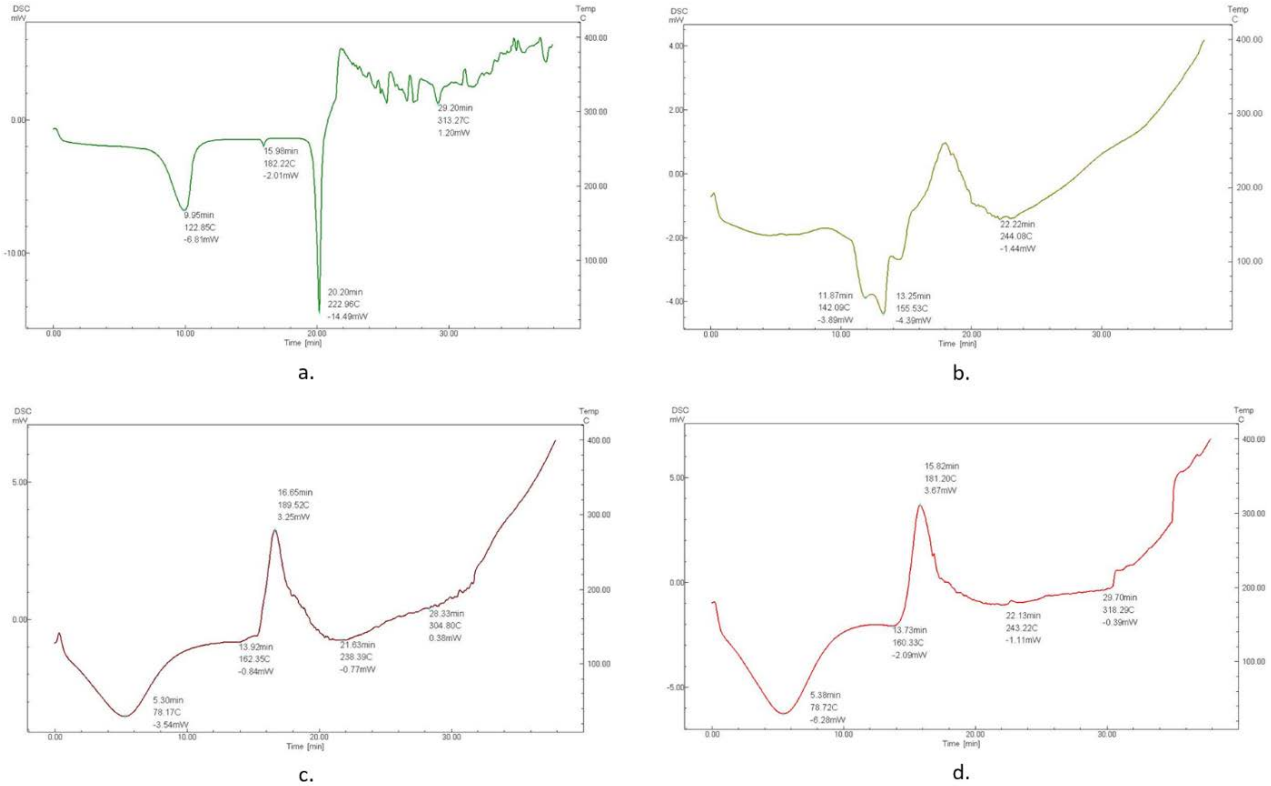


Fig. 3. DSC curves (power (mW), time (min), temperature (°C)) of a. norfloxacin, b. doxycycline monohydrate, c. S1DoNo-E-PP, and d. S1DoNo-E-SOL

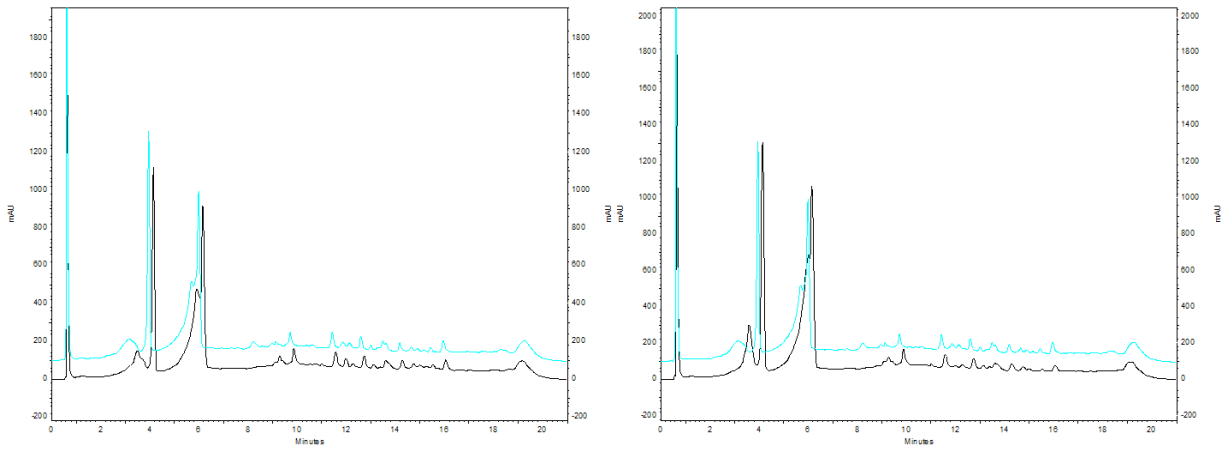
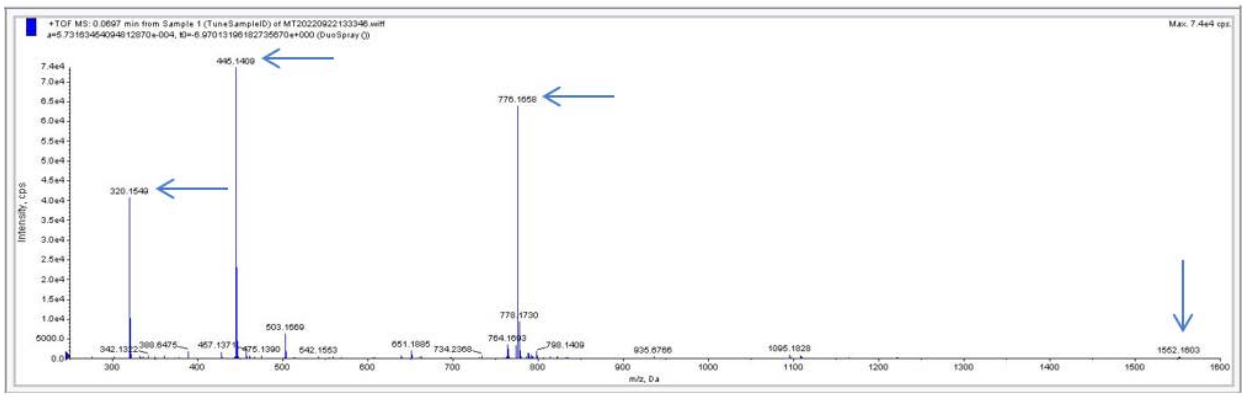
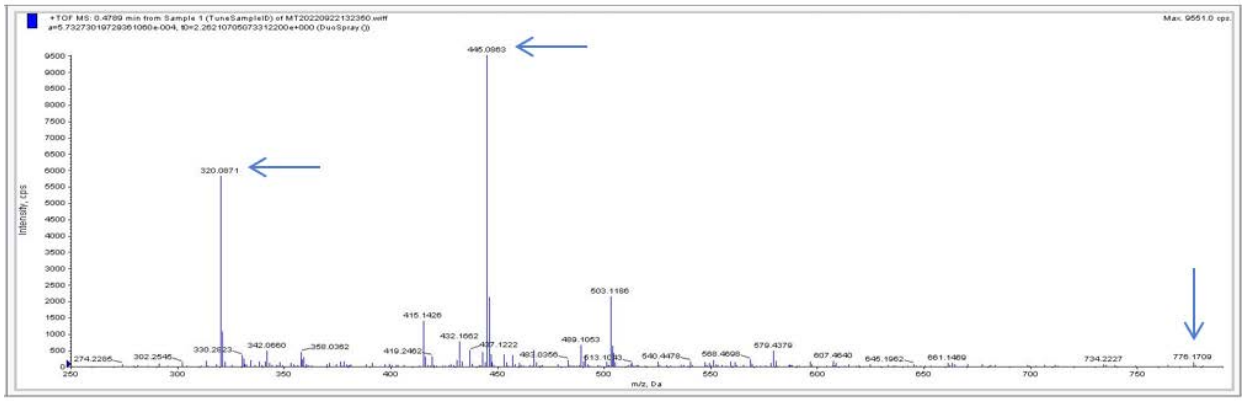


Fig 4. Chromatograms of a. S2DoCi-M1 and b. S2DoCi-M8





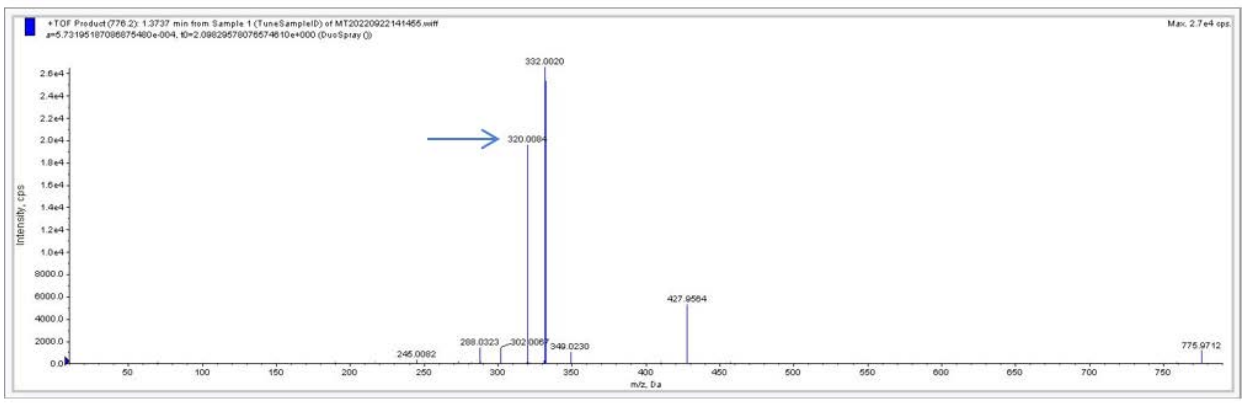


Fig. 5. Mass spectra of a. S1DoNo-E-PP (positive polarity, m/z ratio range 250-1600 Da), b. S1DoNo-E-SOL (positive polarity, m/z ratio range 250-800 Da), and c. fragmentation of the hybrid (positive polarity, collision energy 35 kV)

MS analysis

This analysis was used for the samples in the S1 and S3 groups. For the S1 group, the formation of the norfloxacin-doxycycline hybrid was indicated by the spectrum of the m/z ratio corresponding to the expected molecular mass (Figure 5). However, the signal corresponding to the expected mass is relatively weak, suggesting a minimal synthesis yield. Signals corresponding to dimers of the hybrid were also observed. Following fragmentation (Figure 5), it can be assumed that this occurred at the level of the molecular connector, with the formation of fragments corresponding to the parent compounds.

For the S3 group, only the spectra obtained for the S3DoNo-M, S3DoNo-MCu, S3TeNo-M, and S3TeNo-MCu samples seemed to indicate the formation of structures with a molecular mass close to the one expected for the hybrids. Even so, these signals were weak, implying a minimal synthesis yield.

HPLC analysis

Given that no precipitate formed in either of the solutions in group S2 (and a DSC analysis was impractical, given the lack of solid substance), we chose to analyze the samples by HPLC. The chromatograms indicated that no new structures were present. Only the peaks of the parent compounds were present (Figure 4).

Syntheses of hybrids

Doxycycline was chosen as this study's first TC component because no such hybrid is described in the literature and the availability of the substance [34]. The other components of the hybrids were selected based on several factors, such as availability, the presence of a nitrogen heterocycle in position 7 (for the FQN components), and approval for human use (we excluded FQNs that were withdrawn from the market, even if they had a nitrogen heterocycle in position 7).

We chose the classical reflux synthesis with an electrical heating source method as a first attempt (S1) because of its low cost, simplicity, and equipment availability. Reflux synthesis is a thermal method where the solvent vaporizes, condenses in a refrigerant, and returns to the flask, minimizing solvent loss.

Given that the classical reflux method has disadvantages, such as a prolonged synthesis time, which could also lead to the degradation of the compounds, we also used the microwave-assisted method, which minimizes the necessary reaction time. Consequently, we attempted a microwave-assisted reflux synthesis (the S2 group), using doxycycline monohydrate and ciprofloxacin hydrochloride, based on the synthesis method of Sriram et al. [34] to obtain better results than the classical approach.

Thus, the HPLC analysis revealed only the presence of the parent compounds. It was unexpected because the synthesis method was described as successful in the literature [34]. To get the desired results, we decided to try more combinations of parent compounds for group S3, including a $CuCl_2$ 2H2O-catalyzed version for each combination. We chose this catalyst because it seemed the best out of a series of catalysts tested for improving Mannich condensation reactions [38].

From the S3 group, close m/z ratios were observed only for the expected masses of the DoNo and TeNo hybrids, which is valuable information. It seemed that only the norfloxacin hybrids were forming (as the results from the samples from the S1 and S2 groups also pointed out). Since norfloxacin was the only FQN used as a base rather than hydrochloride, we considered this might have influenced the syntheses.

The classical method, as well as the microwave-assisted method, produced similar results. However, the classical approach produced a compound (assumingly the DoNo hybrid) with a molecular mass closest to the one expected. Nonetheless, this does not prove that the classical approach was better since there were differences in the amount of parent compounds used (in the classical approach, we could use larger quantities considering the prolonged heating time, which allowed a more effective dissolution of the compounds).

Regarding the use of the catalyst, it is unclear if it significantly influenced the reaction. When examining the results for the S3TeNo-MCu sample, it was observed that the m/z ratio was the closest to the expected mass of the hybrid compound. This aspect suggests that while the overall impact of the catalyst on the reaction efficiency remains uncertain, the specific conditions associated with the S3TeNo-MCu sample yielded a mass spectrometry result that aligned closely with theoretical predictions, indicating a potentially higher degree of accuracy in the formation of the desired hybrid molecule.

In the future, we plan to optimize the synthesis of the No-containing hybrids, using larger quantities of substances to allow us to perform further analyses of these hybrids (such as IR spectra and elemental analysis). The preliminary syntheses presented in this work were attempted with smaller quantities due to the poor solubility of both FQNs and TCs. Larger amounts of parent compounds over multiple synthesis attempts would have meant using significant solvent volumes. Also, we would like to try to obtain the other hybrids using the FQN bases rather than the hydrochlorides. Testing the hybrids' antimicrobial activity would be another valuable determination, as well as molecular docking studies, that could provide information regarding the possible mechanism of action of the hybrids.

Conclusion

This work focused on synthesizing hybrids between some FQN (ciprofloxacin, moxifloxacin, and norfloxacin) and TC (doxycycline, tetracycline) representatives, using a Mannich reaction with formaldehyde acting as a molecular connector. The preliminary attempt to synthesize the norfloxacin-doxycycline hybrid was successful. DSC and

MS analyses support the formation of a new compound distinct from the parent compounds.

Both synthesis methods (classical and microwave-assisted) produced similar results. It is not clear if the use of the catalyst improved the reaction. An indicator of a better result when using the catalyst could be that for the S3TeNoMCu sample, the m/z ratio was the closest to the expected mass of the hybrid.

The synthesis method is simple and one-step, using non-toxic solvents and a conventional heating mantle or microwaves as the energy source. The obtained results are encouraging and represent valuable information for optimizing these synthesis methods of the targeted hybrids.

Molecular docking and microbiological studies could be used to investigate the mechanism of action and microbiological effects of the hybrids further. This research can constitute a starting point for future studies by underlining the need for new antibacterial compounds, among which FQN-TC hybrids could be valuable representatives.

Authors' contribution

IAL (Conceptualization; Data curation; Funding acquisition; Investigation; Visualization; Writing – original draft, Writing – review & editing)
LF (Investigation; Resources; Writing – review & editing)
ZIS (Investigation; Resources; Writing – review & editing)
ȘAG (Conceptualization; Investigation; Resources)
OLM (Conceptualization; Visualization; Writing – review & editing)
AR (Conceptualization; Resources; Supervision; Writing – review & editing)

Conflict of interest

None to declare.

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