

NOTE

Synthesis and antimicrobial activity of ciprofloxacin and norfloxacin permanently bonded to polyethylene glycol by a thiourea linker

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The Journal of Antibiotics (2009) 62, 113–116; doi:10.1038/ja.2008.26; published online 16 January 2009

Keywords: antibacterial; fluoroquinolone; permanent bond; polyethylene glycol; telechelic

C₂-symmetric (divalent) biologically active molecules are an extensively studied field, as certain substances possessing a B-linker-B type structure (where B represents a bioactive molecule) have promising biological properties.¹ The synthesis and investigation of the dimers of the highly antibacterial quinolonecarboxylic acids were also carried out. However, the norfloxacin dimers linked with an alkyl chain, described by Coppel *et al.*,² were very slightly active. Later Kerns *et al.*^{3–5} synthesized the C₂-symmetric and mixed dimers of ciprofloxacin and norfloxacin using 1,4-phenylenebis-methylene and 2-butene-1,4-diyl linkers. These derivatives possessed comparable activity with that of the monomers and, in a few cases, higher MIC values were found against certain drug-resistant strains.

Polyethylene glycol (PEG), a cheap and nontoxic telechelic polymer diol of amphiphilic character is soluble both in water and organic solvents; therefore, it is often used as a carrier for drug molecules.

Bioactive molecules linked covalently to PEG possess favorable pharmacodynamic properties due to high water solubility and lipophilicity of the polymer, and PEGylation usually results in an enhanced biological stability and the products are less immunogenic. The PEGylated drug conjugates are primarily used in a 'releasable' form (that is, in a form linked by means of bondings unstable in a biological milieu).

In contrast, in the permanently bonded form, the drug molecule is attached to the polymer with covalent bondings stable in a biological milieu. The permanently bonded form is also advantageous, as numerous drug molecules introduced to therapy contains PEG in such a linkage. Related compounds are the PEGylated derivative of the bovine adenosyl deaminase enzyme ADAGEN,⁶ the PEGylated L-asparagine-containing ONCASPAR,⁷ PEG-INTRON,⁸ which con-

tains PEGylated interferon, and NEULASTA⁹ carrying a PEGylated granulocyte-colony stimulating factor. Incorporation of the mono-functional PEG chain(s) to these protein-type bioactive molecules resulted in decreasing immunogenicity of the parent compound, as well as in the increase of the circulation half-life time and the stability. There are few reports on permanently bonded drugs, but those compounds exhibit low biological activity.¹⁰

Ciprofloxacin was encapsulated in PEG-coated, long-circulating sustained-release liposomes.^{11,12} It is to be noted that Carraher *et al.*¹³ reported the synthesis and biological activity of ciprofloxacin covalently bonded to organotin polymers of **1** and **2**. Norfloxacin was also attached to methacrylate polymers.¹⁴

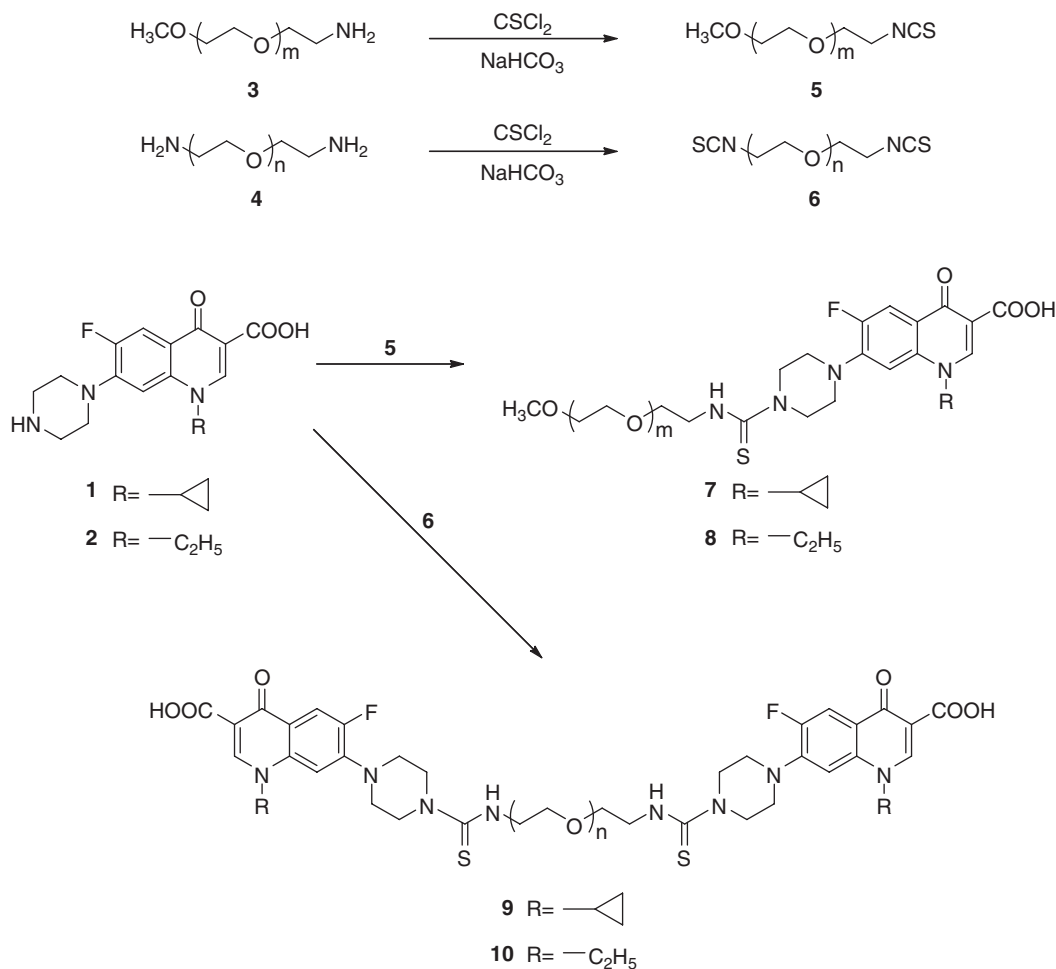
The goal of this work was to investigate the antimicrobial effect of the quinolonecarboxylic acid dimers prepared with a PEG linker, and to study the effect of PEGylation on the antibacterial activity.

To investigate the separate effect of the PEGylation and dimerization of **1** and **2** on the antimicrobial effect, both the mono- and bifunctional PEG derivatives of the antimicrobial agents were prepared.

Monomethoxy-PEG (MW_{average}=1132 Da) and PEG (MW_{average}=1382 Da) were converted¹⁵ into the monoamino (**3**) and diamino (**4**) derivatives, whose treatment with thiophosgene gave the corresponding mono- and diisothiocyanates **5** and **6**, respectively (Scheme 1). The reaction of **4** and **6** with ciprofloxacin and norfloxacin under mild conditions (Scheme 1) led to the PEG-conjugates (**7**, **8**, **9**, **10**), whose structures were proved by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and NMR spectroscopy. (Figure 1 represents the MALDI-time of flight (TOF) mass spectrum of **6** and **9**). Thiourea and its derivatives cannot be hydrolyzed by

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Received 20 October 2008; accepted 18 December 2008; published online 16 January 2009



Scheme 1 Formation of PEG-isothiocyanates and synthesis of PEGylated quinolonocarboxylic acid derivatives.

ureases of different origin^{16,17} or they are inhibitors of these enzymes.¹⁸ Therefore, the permanently bonded drug conjugates containing a thiourea-linking moiety are expected as stable molecules under biological conditions. Consequently, the prepared permanently bonded drug conjugates do not correspond to a delivery system, but represent original macromolecules carrying bioactive endgroups.

The data in Table 1 show the MIC values of our substances toward several bacterial strains. As PEGylation increases the molecular mass approximately 5–6 times, the change in the activity expressed in $\mu\text{mol l}^{-1}$ is more informative than the concentrations given in $\mu\text{g ml}^{-1}$.

The PEG conjugates possessed weak activity, or were inactive against the Gram-positive *Staphylococci*, with the exception of the dimeric ciprofloxacin **9** against strains **a** and **b** (in $7.5 \mu\text{mol l}^{-1}$). Nevertheless, **1** and **2** were also inefficient toward half of the Gram-positive strains investigated. At the same time, against *Bacillus subtilis*, the ciprofloxacin derivatives **7** and **9** have similar activity than the parent compounds (in 2.6 and $1.8 \mu\text{mol}$, respectively).

PEGylation emerged with more attractive results in the case of the Gram-negative bacterial strains. The PEG derivatives **7** and **9** had molar MIC values against *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* similar to those of ciprofloxacin and norfloxacin, and the norfloxacin compound **10** was also reasonably efficient against *E. coli* (**g**). PEGylation decreased the activity of norfloxacin more than that of ciprofloxacin. On the other hand, on

comparison of the microbial effect of **1** and **2** with that of the monovalent and dimeric derivatives (i.e., to **7** and **9**, and to **8** and **10**, respectively) against Gram-negative strains, no significant changes were observed. Consequently, we may conclude that (at least in the case of the MW=1500 PEG) dimerization of **1** and **2** does not significantly influence the biological activity against Gram negatives.

As permanent covalent PEGylation does not affect the molar MIC values observed for certain microorganisms, it is believed that compounds **7–10** could be sufficient examples for the further investigation of the influence of PEGylation on antimicrobial activity.

In summary, it was observed that bonding of ciprofloxacin and norfloxacin to PEG with a permanent covalent linkage leads to an insignificant decrease in the molar activity of some of the products against certain Gram-negative bacteria. This fact opens the way to the synthesis of another stable polymer-antibiotic chimera, which may have better antimicrobial activity than that of the parent compounds.

EXPERIMENTAL SECTION

General methods

Unless otherwise stated, the starting materials and solvents were purchased from commercial sources (Sigma-Aldrich, St Louis, MO, USA or Fluka, Buchs, Switzerland) and used as received. All solvents were distilled before use. CH_2Cl_2 was distilled from P_2O_5 and stored over 4 Å molecular sieves. Triethylamine was distilled from KOH before use. ^1H NMR spectra were recorded at 360.13 MHz

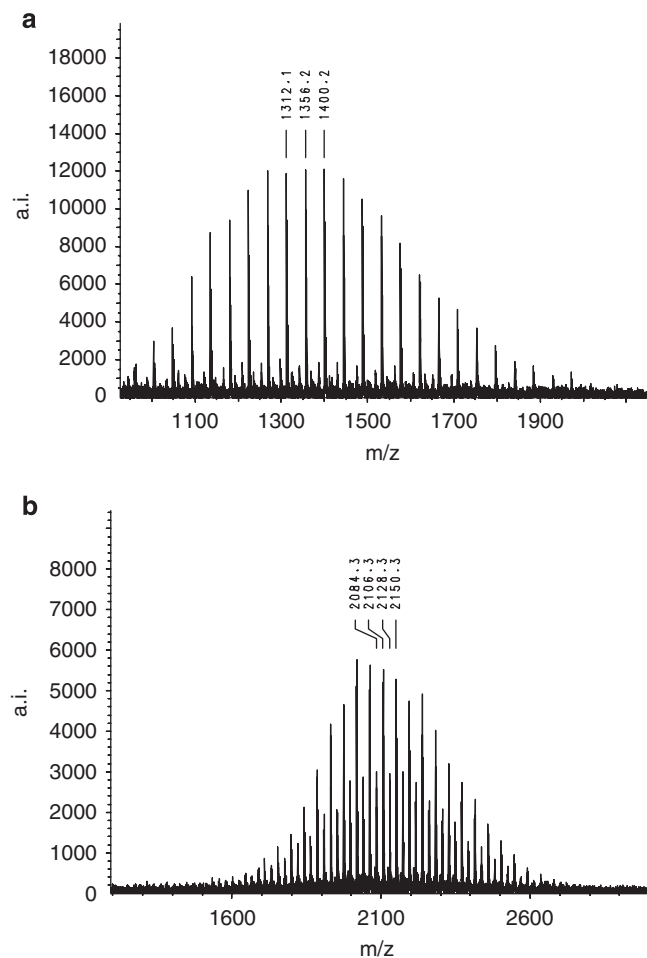


Figure 1 MALDI-TOF mass spectrum of **6** (spectrum a) and **9** (spectrum b).

with a Bruker WP-360 SY spectrometer, using DMSO-*d*₆ as solvent and tetramethylsilane as internal standard. Mass spectra were recorded with a Bruker Biflex-III MALDI-TOF mass spectrometer. For column chromatography, Merck silica gel (Kieselgel 60), 0.063–0.200 mm (70–230 mesh) was used. TLC was performed on Kieselgel 60 F₂₅₄ (Merck), using CH₂Cl₂–MeOH solvent system as eluent.

Spots were visualized by irradiation under UV lamp, and/or by spraying with an ammonium-molibdenate/sulfuric acid solution and heating. Evaporations were carried out under diminished pressure at 35–40 °C (bath temperature).

Monomethoxy-isothiocyanato-PEG (5)

To a solution of 60 ml of 0.54 M sodium hydrogencarbonate in water, a solution of thiophosgene (0.520 ml, 6.08 mmol) in dichloromethane (60 ml) was added. The mixture was stirred vigorously and a solution of **3** (3.0 g, 2.73 mmol) in dichloromethane (100 ml) was added dropwise for 1 h. After 15 min, the aqueous phase was extracted with CH₂Cl₂ (2 × 100 ml). Evaporation of the combined organic phases resulted in 2.87 g of **5** (93%). The structure and purity were proved by the MALDI spectrum.

MS (MALDI-TOF) MW_{average}=1153.2 Da (M+Na)⁺. Calculated for C₅₀H₉₉O₂₄NS 1129.6.

Diisothiocyanato-PEG (6)

It was obtained from the diamino-PEG with the method applied for **5** using 2 equivalents of CSCL₂ and NaHCO₃. Yield: 92%.

MS (MALDI-TOF) MW_{average}=1356.2 Da (M+Na)⁺. Calculated for C₅₈H₁₁₂O₂₇N₂S₂ 1332.7.

Preparation of thiocarbamoyl-PEG derivatives of **1** and **2** (7–10)

Typical procedure (for **9**)

1 HCl (0.154 g, 0.42 mmol) and triethylamine (0.116 ml, 0.84 mmol) were dissolved in dry CH₂Cl₂ (35 ml), then 0.201 g (0.14 mmol) of **6** was added and the mixture was allowed to react for 3 days at room temperature. The product was purified by column chromatography on silica gel using an 85:15 CH₂Cl₂–MeOH solvent system as eluent. Yield: 67 mg (23%).

MS (MALDI-TOF) MW_{average}=2106.3 Da (M+Na)⁺, 2128.3 Da (M-H+2Na)⁺. Calculated for C₉₆H₁₅₆O₃₅N₈S₂F₂ 2083.0.

¹H NMR (360 MHz, DMSO-*d*₆) δ 8.67 (2H, s, quinolone CH), 7.92 (2H, d, J=13.3, aromatic), 7.55 (2H, d, J=7.4, aromatic), 4.02–4.04 (4H, m, –CH₂–NH–C=S), 3.80–3.86 (2H, m, cyclopropyl CH), 3.34–3.71 (m, polymer –CH₂–CH₂–O–, piperazine), 1.22–1.37 (8H, m, cyclopropyl methylene).

Compound **7**

1 HCl (0.089 g, 0.24 mmol) and triethylamine (66 μl, 0.84 mmol) were dissolved in dry CH₂Cl₂ (35 ml), then 0.19 g (0.16 mmol) of **5** was added and the procedure described for **9** was used. Yield: 55 mg (23%).

MS (MALDI-TOF) MW_{average}=1527.6 Da (M+Na)⁺, 1549.6 Da (M-H+2Na)⁺. Calculated for C₆₉H₁₂₁O₂₈N₄SF 1504.8.

¹H NMR (360 MHz, DMSO-*d*₆) δ 8.66 (1H, s, quinolone CH), 7.91 (1H, d, J=13.3, aromatic), 7.55 (1H, d, J=7.4, aromatic), 4.02–4.06 (2H, m, –CH₂–NH–C=S), 3.78–3.88 (1H, m, cyclopropyl CH), 3.34–3.71 (m, polymer –CH₂–CH₂–O–, piperazine), 3.24 (3H, s, H₃C–O–CH₂–CH₂–O–), 1.22–1.35 (4H, m, cyclopropyl methylene).

Compound **8**

5 (0.196 g, 0.17 mmol) was dissolved in dry CH₂Cl₂ (50 ml) and **2** (0.081 g, 0.25 mmol) was added. Using the general method, 48 mg of **8** was resulted (20%).

MS (MALDI-TOF) MW_{average}=1428.1 Da (M+Na)⁺, 1450.1 Da (M-H+2Na)⁺. Calculated for C₆₄H₁₁₃O₂₆N₄SF 1404.7.

¹H NMR (360 MHz, DMSO-*d*₆) δ 8.95 (1H, s, quinolone CH), 7.94 (1H, d, J=13.3, aromatic), 7.18 (1H, d, J=7.4, aromatic), 4.59 (2H, q, J=7.1, H₃C–CH₂–N), 4.00–4.06 (2H, m, –CH₂–NH–C=S), 3.27–3.71 (m, polymer –CH₂–CH₂–O–, piperazine), 3.24 (3H, s, H₃C–O–CH₂–CH₂–O–), 1.42 (3H, t, CH₃–CH₂–N).

Compound **10**

6 (0.196 g, 0.13 mmol) was dissolved in dry CH₂Cl₂ (50 ml) and **2** (0.118 g, 0.37 mmol) was added, and the reaction mixture was stirred for 3 days at room temperature. The product was purified by column chromatography on silica gel using a 85:15 CH₂Cl₂–MeOH solvent system as eluent. Yield: 48 mg (18%).

MS (MALDI-TOF) MW_{average}=2082.5 Da (M+Na)⁺, 2104.5 Da (M-H+2Na)⁺. Calculated for C₉₄H₁₅₆O₃₅N₈S₂F₂ 2059.0.

¹H NMR (360 MHz, DMSO-*d*₆) δ 8.95 (1H, s, quinolone CH), 7.94 (1H, d, J=13.3, aromatic), 7.18 (1H, d, J=7.4, aromatic), 4.59 (4H, q, J=7.1, H₃C–CH₂–N), 4.02–4.06 (4H, m, –CH₂–NH–C=S), 3.27–3.71 (m, polymer –CH₂–CH₂–O–, piperazine), 1.42 (3H, t, CH₃–CH₂–N).

Biological assays

The efficacy of the prepared antimicrobials was determined with the broth microdilution method according to the NCCLS guideline.¹⁹ Bacterial strains were grown on 5% bovine blood agar plates at 35 °C overnight. Appropriate numbers of colonies were suspended in physiological saline to reach the density of 0.5 McFarland for inoculation.

Stock solutions of different concentrations of the substances were prepared in either distilled water or H₂O and methanol (1:1) or H₂O and DMSO (1:1), respectively, depending on the solubility of the given preparation. These were twofold serially diluted from 256 to 0.5 μg ml^{–1} in cation-adjusted Mueller–Hinton broth, then 100 μl of each dilution was transferred into microplate holes. Inoculation was carried out with 10 μl of each bacterial suspension.

Table 1 MICs of quinolonecarboxylic acid derivatives ($\mu\text{mol l}^{-1}$)

| Test microorganismus | MIC ($\mu\text{mol l}^{-1}$) | | | | | |
|---|--------------------------------|---------------|--------------------------|------------------------|-------------------------|------------------------|
| | Ciprofloxacin hydrochloride 1 | Norfloxacin 2 | Mono-ciprofloxacyl-PEG 7 | Mono-norfloxacyl-PEG 8 | Bis-ciprofloxacyl-PEG 9 | Bis-norfloxacyl-PEG 10 |
| <i>Staphylococcus aureus</i> ^a | 1.3 | 6.2 | 42.2 | >166.4 | 7.5 | >125.4 |
| <i>Staphylococcus aureus</i> ^b | 1.3 | 6.2 | 84.4 | >166.4 | 7.5 | >125.4 |
| <i>Staphylococcus aureus</i> ^c | >696.3 | >801.2 | >168.9 | >166.4 | >120.3 | >125.4 |
| <i>Staphylococcus haemolyticus</i> ^d | 43.5 | 400.6 | >168.9 | >166.4 | >120.3 | >125.4 |
| <i>Staphylococcus epidermidis</i> ^e | 1.3 | 3.1 | 84.4 | >166.4 | 15.0 | >125.4 |
| <i>Staphylococcus epidermidis</i> ^f | >696.3 | >801.2 | >168.9 | >166.4 | >120.3 | >125.4 |
| <i>Escherichia coli</i> ^g | 1.3 | 1.5 | 0.6 | 10.4 | 3.7 | 3.9 |
| <i>Pseudomonas aeruginosa</i> ^h | 1.3 | 1.5 | 10.5 | >166.4 | 3.7 | 62.7 |
| <i>Klebsiella</i> ⁱ | 174.0 | 801.2 | >168.9 | >166.4 | >120.3 | >125.4 |
| <i>Bacillus subtilis</i> ^j | 1.3 | 1.5 | 2.6 | 20.8 | 1.8 | 15.6 |
| <i>Enterobacter cloacae</i> ^k | 1.3 | 1.5 | 2.6 | 41.6 | 1.8 | 31.3 |
| <i>Acinetobacter baumannii</i> ^l | 174.0 | >801.2 | >168.9 | >166.4 | >120.3 | >125.4 |

^aATCC 29213 (methicillin susceptible).^bATCC 33591 (methicillin resistant).^c13797 (methicillin, ciprofloxacin resistant).^d13654 (ciprofloxacin resistant).^eATCC 25299 (methicillin susceptible).^f12333 (methicillin resistant, mecA gene positive).^gATCC 25992.^hNCTC 10006.ⁱ4660 (2005) (ESBL).^jATCC 6633.^k11213 (2005).^lATCC 6018.

Incubation was performed at 35 °C for 18 h and determination of the MIC was made with naked eyes on a mirror.

Solvents were also tested for inhibition of bacterial growth, and none of them exerted bacteriostatic effect at the concentration used.

ACKNOWLEDGEMENTS

This work was supported by the Hungarian Academy of Sciences and the National Scientific Research Found Grants no.: OTKA T042512, T046186, T046744 and RET-06/2004.

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