Monatsh Chem (2009) 140:1221–1227
DOI 10.1007/s00706-009-0158-y

ORIGINAL PAPER

# Synthesis of [1-$^{15}$N,2-$^{13}$C]-labeled difloxacin

**Burkhard Schmidt · Ramona Schumacher-Buffel · Brigitte Thiede · Andreas Schäffer**

Received: 25 September 2008 / Accepted: 21 January 2009 / Published online: 29 May 2009


**Abstract** The synthesis of [1-$^{15}$N,2-$^{13}$C]-difloxacin, an arylfluoroquinolone antibacterial agent, is reported. As a crucial initial step, the starting materials ethyl 2,4,5-trifluorobenzoylacetate, [formyl-$^{13}$C]-triethyl orthoformate, and [$^{15}$N]-4-fluoroaniline were reacted to ethyl [$^{15}$N,3-$^{13}$C]-3-(4-fluoroanilino)-2-(2,4,5-trifluorobenzoyl)acrylate. After cyclization and ester cleavage, the resulting intermediate was reacted with 1-methylpiperazine to [1-$^{15}$N,2-$^{13}$C]-1-(4-fluorophenyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylate, i.e., [1-$^{15}$N,2-$^{13}$C]-difloxacin. The overall yield was 62% based on the non-labeled and 43% based on the labeled starting materials (both used in 1.4 molar excess). The product was identified by $^{1}$H-, $^{13}$C-, and $^{15}$N-NMR spectroscopy and by cochromatography (TLC, HPLC) with an authentic reference; its purity (HPLC) was above 98%. Prior to synthesis of [1-$^{15}$N,2-$^{13}$C]-difloxacin, non-labeled difloxacin was synthesized in order to optimize procedures and to identify and characterize all intermediates.

**Keywords** Isotopic labeling · Antibiotics · NMR spectroscopy · Difloxacin · Arylfluoroquinolone

## Introduction

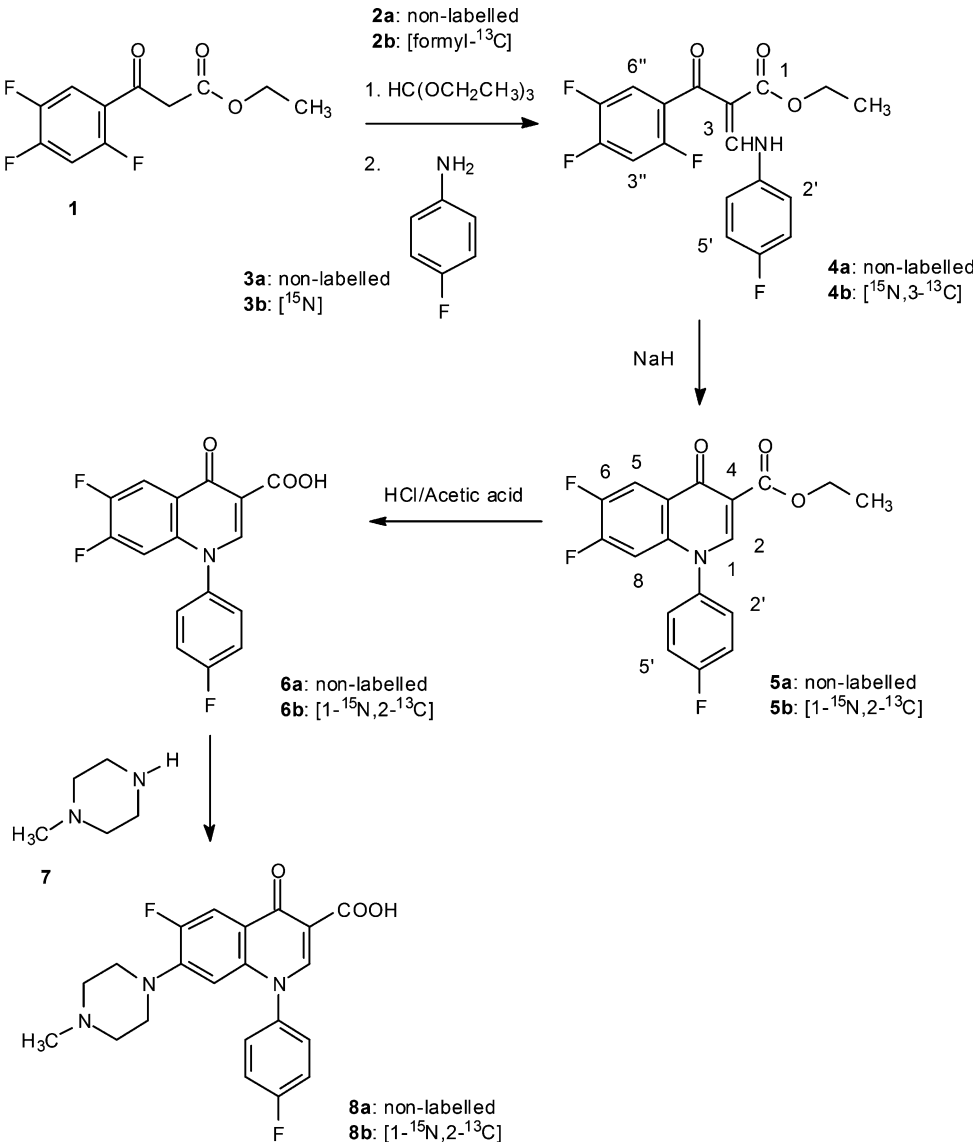
Fluoroquinolones have found wide application as antibacterial agents in human and veterinary medicine. Difloxacin (for the chemical structure, see Scheme 1) is effective against a wide range of gram-positive and gram-negative aerobes and anaerobes by acting as a DNA gyrase inhibitor. It has been developed and approved for veterinary use in pigs, cattle, sheeps, goats, poultry, and dogs [1–3]. In contrast to pesticides, which are used in agriculture by spraying considerably large areas, most veterinary pharmaceuticals are applied to individual organisms. Contamination of the environment during medication itself is consequently negligible. Nonetheless, compounds can be introduced into the environment by three main mechanisms. One route is via sewage, sewage treatment plants, and surface waters, which applies, e.g., for unused pharmaceuticals that are sometimes disposed of in the sewage system. Direct input into soils may occur via excretion of feces and urine by grazing animals. The main route, however, is via manure, which is widely used as an agricultural fertilizer. Thus, portions of antibiotics remaining non-metabolized or metabolites exhibiting residual biological activity are excreted by animals, end up in manure, and may subsequently contaminate agricultural soils [4–8].

Until recently, little attention has been paid to the introduction of veterinary pharmaceuticals into the environment [8]. However, veterinary antibiotics have been detected in soils as the unintentional result of using manure [5–8]. There are only a few reports on groundwater contamination, although antibiotics have been detected in surface waters due to runoff from fields fertilized with manure [6, 9]. According to these findings, it is considered reasonable by regulatory authorities (legislation) to study both the effects and fate of veterinary antobiotics, such as difloxacin, in agricultural soils fertilized with manure from treated livestock [6, 8, 10, 11]. Until now, data on the latter issue have been scarce [5].

The present synthesis of $^{13}$C- and $^{15}$N-labeled difloxacin was performed in the context of a larger research unit

B. Schmidt (✉) · R. Schumacher-Buffel · B. Thiede · A. Schäffer
Institute of Biology V (Environmental Research), RWTH Aachen University, Worringerweg 1, 52056 Aachen, Germany
e-mail: burkhard.schmidt@post.rwth-aachen.de

**Scheme 1**




funded by the German Research Foundation, which deals with the effects and fate of the veterinary antibiotics sulfadiazin and difloxacin in soil after their introduction via manure of correspondingly medicated pigs. Results concerning the fate of sulfadiazin have already been published [12, 13]. Utilization of $^{13}$C-labeled sulfadiazin as an analytical reference was crucial for these experiments [13]. A similar reference labeled with stable isotopes was thus required for the study with difloxacin. In soil, sulfadiazin [12] and difloxacin [14] demonstrated a considerable tendency to rapidly form so-called non-extractable residues. These are thought to consist of covalent and non-covalent associations of the antibiotic or its metabolites with soil organic and inorganic matter that are not disintegrated by simple extraction procedures. In order to obtain information on the nature of these associations regarding their possible environmental impact, solid or liquid phase $^{13}$C NMR spectroscopy using a suitable $^{13}$C-labeled tracer was considered promising [15]. Thus, the projected difloxacin labeled with stable isotopes was also intended for field of application.

## Results and discussion

### *Synthesis planning*

According to the literature [16–20], a key step in the synthesis of arylfluoroquinolone antibacterial agents is treatment of an appropriately substituted (methyl or) ethyl benzoylacetate (**1**, in case of difloxacin) with triethyl orthoformate (**2a**) to give the one-carbon homologue enol ether. The resulting intermediate is allowed to react with aniline **3a** to yield the enamine keto ester **4a**, which

upon cyclization gives ethyl 1,4-dihydro-4-oxoquinoline-3-carboxylate **5a**. This product is then hydrolyzed to the carboxylic acid **6a** and reacted with an amine (1-methylpiperazine, **7**, in case of difloxacin) yielding arylfluoroquinolone (Scheme 1).

As outlined before, preparation of difloxacin labeled with stable isotopes was performed in order to obtain both an analytical reference (for mass spectrometry-based analysis, i.e., LC-MS) and a suitable tracer for $^{13}$C NMR studies on the nature of the so-called non-extractable residues emerging from the transformation of difloxacin in soil. Multiple labeling, such as a uniformly labeled aromatic ring, was preferable in the case of the analytical reference (with a distinctly different mass compared to the non-labeled molecule), while labeling of positions with high stability towards, e.g., microbial transformation was required for the tracer compound. The initial steps of difloxacin metabolism in humans [21, 22] and dogs [23] were shown to occur at the piperazine moiety, which subsequently can be removed completely. In contrast, the remaining carbon skeleton appears to be comparatively stable. Since a similar initial transformation pattern was suspected in soil, labeling was regarded to be the most favorable in the quinolone or 4-fluorophenyl moiety of difloxacin. This conclusion is supported by data published on the metabolism of the closely related fluoroquinolones ciprofloxacin and enrofloxacin by the brown rot fungus *Gloeophyllum striatum* [24, 25].

Ethyl 2,4,5-trifluorobenzoylacetate (**1**) and 4-fluoroaniline (**3**) are not commercially available as (uniformly) ring-$^{13}$C-labeled derivatives. The same holds for 2,4,5-trifluorobenzoic acid, which can be converted to **1** [16, 17, 20]. Uniformly $^{13}$C-labeled aniline is available; conversion of this precursor to **3**, however, was beyond the scope of the present study. Thus, labeling of difloxacin was carried out using [formyl-$^{13}$C]-triethyl orthoformate (**2b**). In order to increase the mass of the labeled compound, [$^{15}$N]-4-fluoroaniline (**3b**) was additionally utilized as an aniline component. This ultimately resulted in [1-$^{15}$,2-$^{13}$C]-difloxacin, which was considered suitable as a tracer and analytical reference.

### *Synthesis of non-labeled difloxacin and identification of intermediates*

For the optimization of methods and unequivocal identification of intermediates, non-labeled difloxacin was prepared prior to labeling with $^{13}$C and $^{15}$N (Scheme 1). A mixture of ethyl 2,4,5-trifluorobenzoylacetate (**1**), triethyl orthoformate (**2a**; molar excess: 1.4), and acetic anhydride was reacted as described in Refs. [16, 17]. After evaporation of the byproduct (ethyl acetate), remaining starting materials, **2a**, and acetic anhydride, the resulting intermediate was obtained in oily form. Removal of acetic anhydride was essential in order to avoid the side reaction to *N*-acetyl 4-fluoroaniline during the subsequent synthetic step. Under argon cover gas, the oily intermediate was dissolved in dichloromethane and converted to ethyl 3-(4-fluoroanilino)-2-(2,4,5-trifluorobenzoyl)acrylate (**4a**) by reaction with 4-fluoroaniline (**3a**; molar excess: 1.4 regarding **1**) [16, 17]. Crucial intermediate **4a** was purified by silica gel column chromatography, and characterized and identified by thin-layer chromatography (TLC) [26], $^{1}$H and $^{19}$F NMR, and GC-EIMS. Both NMR spectra demonstrated the presence of (*E*)- and (*Z*)-isomers of **4a** (with regard to the C2–C3 olefinic bond), the ratio of which varied among different experiments. Prominent $^{1}$H NMR signals were those of the NH and H-3 protons, respectively appearing as doublets at $\delta = 12.50/11.09$ and 8.50/8.41 ppm. Peaks at $\delta = 4.12/4.09$ (q) and 1.12/0.99 ppm (t) indicated the signals of the ethyl group, whereas those of all aromatic protons emerged as a multiplet with $\delta = 7.50$–6.80 ppm. The $^{1}$H NMR spectrum of **4a** correlated with published spectra of similar compounds [17, 19, 20]. In addition to (*E*)- and (*Z*)-isomers, the $^{19}$F NMR spectrum showed that four fluorine atoms were present in the molecule. These exhibited expected chemical shifts (main isomer given) at $\delta = -142.7$ (q), $-130.8$ (q), $-115.8$ (q), and $-115.3$ ppm (s), $^{19}$F–$^{19}$F spin–spin coupling, and corresponding constants. As examined by gas chromatography-mass spectroscopy with electron impact ionization (GC-EIMS), the mass spectrum of **4a** was similar to that of subsequent intermediate **5a** (including GC retention time) supposedly due to cyclization in the injector (250 °C) of the chromatographic system.

Cyclization of **4a** to ethyl 1-(4-fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate (**5a**) was executed using NaH in dry tetrahydrofurane under argon atmosphere in accordance with a published procedure [16, 17]. Intermediate product **5a** was examined without further purification by TLC [26] by $^{1}$H, $^{13}$C, and $^{19}$F NMR, as well as by GC-EIMS, and proved to be sufficiently pure for the subsequent synthetic reactions. The $^{1}$H and $^{19}$F NMR spectra unequivocally demonstrated the cyclization of **4a** to **5a**. The NH signal (of **4a**) was absent in the $^{1}$H NMR spectrum of intermediate **5a**, while only signals of three flourine atoms emerged in the $^{19}$F NMR spectrum of **5a** ($\delta = -138.3$, $-126.9$, and $-108.5$ ppm) as compared to **4a** (four signals). In the $^{1}$H NMR spectrum, aromatic protons H-5, H-8, H-2′/H-6′, and H-3′/H-5′ appeared with high resolution, and demonstrated expected $^{1}$H–$^{1}$H and $^{1}$H–$^{19}$F spin-spin coupling patterns and constants. The most noticeable feature of the $^{13}$C NMR spectrum of intermediate **5a** was the doublets of carbon atoms C4a, C8, C8a, C1′, C2′, C3′, C4′, C5′, and C6′ and the double doublets of carbon atoms of C5, C6, and C7, which were due to $^{13}$C–$^{19}$F spin–spin coupling. Coupling

constants were $^{13}C$–$^{19}F$: 252–256 Hz, $^{13}C$–C–$^{19}F$: 14–23 Hz, and $^{13}C$–C–C–$^{19}F$: 2–9 Hz. Unexpectedly, the $^{13}C$ signal of carbon atom C8 consisted only of a doublet—in contrast to C5, which gave rise to a double doublet caused by spin-spin coupling with both F-6 and F-7. This was probably due to insufficient resolution of the spectrum with regard to C8. At least, this assumption is supported by the observation that the peaks of the doublet of C8 were comparatively broad. Both the $^{1}H$ and $^{13}C$ NMR spectrum agreed with published spectra of fluoroquinolones (other than difloxacin) [17, 19, 20, 27, 28]. The EIMS of **5a** exhibited a molecular ion $M\cdot^{+}$ at $m/z = 347$ and a base peak at $m/z = 275$ derived from $M\cdot^{+}$–$COOC_2H_4$ fragmention. Fragment ion $m/z = 219$ was tentatively thought to result from fragmentation $M\cdot^{+}$–$COOC_2H_4$–$CH_2O$–CN. Other fragment ions were consistent with the structure of **5a**.

Intermediate **5a** was transformed to the free carboxylic acid **6a** by refluxing in a mixture of HCl, acetic acid, and water according to a recently published procedure [18]. Hydrolysis under basic conditions [16] was less effective, and isolation of the product was more complicated. 1-(4-Fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate (**6a**) was identified by TLC [26], $^{1}H$, and $^{19}F$ NMR spectroscopy. With the exception of the ethyl group ($^{1}H$ NMR), the spectra of ester **5a** and free acid **6a** were rather similar, agreeing with published data on fluoroquinolones [17, 19, 20, 27, 28]. Finally, intermediate **6a** was reacted with 1-methylpiperazine (**7**) using triethylamine as a base to give the projected non-labeled difloxacin (**8a**) according to a published method [18]. The product was identified (by comparison with an authetic reference) by TLC [26], HPLC, $^{1}H$, and $^{13}C$ NMR. $^{19}F$ NMR spectroscopy of **8a** was not possible due to its poor solubility in alternative solvents, and trifluoroacetic acid was required. The $^{1}H$ NMR of **8a** resembled that of **6a**. Main differences were the signals of protons H-5 and H-8, which were both doublets (spin–spin coupling with F-6) as compared to double doublets in case of **6a** (spin–spin coupling with F-6 and F-7). Additionally, the signals of the protons of the piperazinyl moiety emerged in the range of $\delta = 3.90$–$3.20$ ppm; the $^{1}H$ NMR pattern obtained was supported by literature data on such ring systems [29]. A similar trend was observed in the $^{13}C$ NMR spectrum of **8a**. The signals of carbon atoms C4a, C5, C6, and C7 appeared as doublets, which exhibited the expected $^{13}C$–$^{19}F$ spin-spin coupling constants (in case of C6, 250 Hz, and smaller constants with other carbon nuclei). In contrast to carbon C4a, a (broad) singlet, and not a doublet, was observed as a signal of carbon C8. A similar phenomenon was observed with intermediate **5a** (see discussion above). As expected, the signals of the piperazinyl part of the molecule were observed at $\delta = 56.5$, 48.9, and 46.0 ppm. According to HPLC analysis, the purity of the compound was above 98%.

*Synthesis of [1-$^{15}N$, 2-$^{13}C$]-labeled difloxacin*

After optimization of methods, the synthesis of [1-$^{15}N$,2-$^{13}C$]-difloxacin was executed as displayed in Scheme 1. Procedures and data given before correspond with maximum yields; these were used for the preparation of the labeled derivative. Intermediates **4b**, **5b**, and **6b** were identified by TLC, and **4b** and **5b** additionally by GC-EIMS. In order to minimize inevitable losses, only the projected [1-$^{15}N$,2-$^{13}C$]-difloxacin was thoroughly identified by $^{1}H$, $^{13}C$, and $^{15}N$ NMR spectroscopy and HPLC examination—in addition to TLC.

Ethyl [$^{15}N$, 3-$^{13}C$]-3-(4-fluoroanilino)-2-(2,4,5-trifluorobenzoyl)acrylate (**4b**) was prepared from **1**, [formyl-$^{13}C$]-triethyl orthoformate (**2b**), and [$^{15}N$]-4-fluoroaniline (**3b**). Based on compound **1**, the yield of **4b** was 99% after column chromatography. Cyclization gave ethyl [1-$^{15}N$, 2-$^{13}C$]-1-(4-fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate (**5b**; yield: 89.4% based on **1**). During TLC, intermediates **4b** and **5b** co-chromatographed with the corresponding non-labeled derivatives (**4a** and **5a**, respectively). Both **4b** and **5b** showed the same MS and retention time with GC-EIMS examination (see discussion on **4a** and **5a** before). Characteristic fragments emerged at $m/z = 277$ ($M\cdot^{+}$–$COOC_2H_4$; base peak) and $m/z = 219$ (277-$COOC_2H_4$–$CH_2O$–$^{13}C^{15}N$) besides $m/z = 349$, which corresponded with the molecular ion. The fact that both the non-labeled compound **5a** and labeled derivative **5b** exhibited the same fragment at $m/z = 219$ was regarded as proof for the suspected fragmentation pattern. Cleavage of ester **5b** gave [1-$^{15}N$, 2-$^{13}C$]-1-(4-fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate (**6b**); the yield was 62% based on ethyl benzoylacetate **1**.

Using triethylamine as catalyst, intermediate **6b** was reacted with 1-methylpiperazine (**7**) to the projected [1-$^{15}N$,2-$^{13}C$]-difloxacin (**8b**). The yield of **8b** amounted to 62% based on **1** introduced into the reaction sequence, while its purity was above 98% as determined by HPLC analysis. Compound **8b** co-chromatographed (TLC, HPLC) with the non-labeled **8a**. Its $^{1}H$ and $^{13}C$ NMR spectra were similar to the corresponding spectra of **8a**; two exceptions however, were observed, which were due to the 1-$^{15}N$- and 2-$^{13}C$-labeling of **8b**. The signal of hydrogen atom H-2 emerged as a doublet at $\delta = 9.37$ ppm, resulting from $^{1}H$-$^{13}C$ spin-spin coupling ($J = 190.8$ Hz). Similarly, the signal of carbon atom C2a appeared as a large doublet ($\delta = 152$ ppm) in the $^{13}C$ NMR spectrum **8b**, which contrasted with both the size of remaining $^{13}C$ signals in this

spectrum and the singlet signal of carbon atom C2 in the spectrum of non-labeled **8a**. Both effects were brought about by $^{13}$C labeling of position C2 of the molecule and by spin-spin coupling ($J = 42$ Hz) between 2-$^{13}$C and 1-$^{15}$N. Additionally, a $^{15}$N NMR spectrum of compound **8b** was recorded, which demonstrated a doublet at $\delta = -215.7$ ppm and a $^{15}$N–$^{13}$C spin–spin coupling constant of $J = 38.4$ Hz.

## Conclusion

[1-$^{15}$N,2-$^{13}$C]-Difloxacin, i.e., [1-$^{15}$N,2-$^{13}$C]-1-(4-fluorophenyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylate, was synthesized from non-labeled ethyl 2,4,5-trifluorobenzoylacetate, and the labeled starting materials [formyl-$^{13}$C]-triethyl orthoformate and [$^{15}$N]-4-fluoroaniline. Overall yield was 62% based on the non-labeled and 42% based on the labeled starting materials (each introduced using a 1.4 molar excess). The isotope-labeled difloxacin is useful as an internal standard for mass spectrometry-based analytical procedures, which are required for quantification of the arylfluoroquinolone antibacterial agent in excreta of medicated livestock and in soils fertilized with manure from treated animals. The labeled compound can also be used as a tracer for $^{13}$C NMR studies on the nature of non-extractable residues arising from difloxacin in soil.

## Experimental

### *Materials*

Ethyl 2,4,5-trifluorobenzoylacetate (**1**) was supplied by ChemPur (Karlsruhe, Germany), difloxacin [1-(4-fluorophenyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylate, **8**] as hydrochloride by Fluka (Buchs, Switzerland; Vetranal®, analytical standard). Triethyl orthoformate (**2**) and 4-fluoroaniline (**3**) as well as 1-methylpiperazine were purchased from Riedel de-Haën (Selze, Germany) and Fluka (Buchs, Switzerland), respectively. [Formyl-$^{13}$C]-triethyl orthoformate (**2b**; $^{13}$C enrichment: 99.4 atom%, chemical purity: 98%) and [$^{15}$N]-4-fluoroaniline (**3b**; $^{15}$N enrichment: 95 atom%, chemical purity: 98%) were provided by Campro Scientific GmbH (Berlin, Germany).

### *Analytical methods*

Thin-layer chromatography (TLC) was executed on silica gel plates (SIL G-25 $UV_{254}$, thickness: 0.25 mm; Macherey-Nagel, Düren, Germany). The solvent systems used were system I: *n*-hexane:diethyl ether 1:1 (v/v) and system II: methanol:iso-propanol:$NH_3$ (conc.) 2.5:2.5:1 (v/v/v) [26]. Electron impact mass spectra (EIMS) were determined using a Hewlett–Packard 5890 Series II gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an FS-Supreme-5 column (25 m × 0.25 mm, 0.23 µm film thickness; CS, Langerwehe, Germany). The chromatograph was connected to a Hewlett-Packard 5971 A MSD (mass selective detector) operated in scan mode ($m/z = 50$–$500$) with 70 eV. Carrier gas was He and injection volume 1 mm$^3$. HPLC was performed on a Beckman System Gold personal chromatograph (Munich, Germany) with Programmable Solvent Module 126 and Diode Array Detector Modul 168. Analyses were performed at 20 °C on a reversed phase column (CC 250/4 Nucleodur Sphinx-RP 5 µm; Macherey-Nagel, Düren, Germany) Elution was carried out with solvents A (0.05 M $H_3PO_4$ in $H_2O$) and B ($CH_3CN$): A/B 95:5 (v/v) for 5 min, linear 20 min gradient to A/B 50:50, linear 10 min gradient to A/B 10:90, return to initial conditions in 5 min, and isocratic A/B 95:5 for 5 min. HPLC analyses were carried out with 100 mm$^3$ injection volume at a flow rate of 1 cm$^3$ min$^{-1}$ with detection of difloxacin at 260, 286, and 300 nm. $^1$H, $^{13}$C, $^{19}$F, and $^{15}$N NMR spectra were recorded using Bruker DPX300 and AV600 instruments; $\delta$ in ppm up- and downfield of internal standard $(CH_3)_4Si$.

### *Synthesis of non-labeled-difloxacin and [1-$^{15}$N,2-$^{13}$C]-labeled-difloxacin*

The procedures for the synthesis of non-labeled difloxacin were similar to those used for the synthesis of [1-$^{15}$N,2-$^{13}$C]-difloxacin (**8b**) (see below and Scheme 1). All intermediates (**4a**, **5a**, **6a**) and the final product (**8a**) were identified by NMR spectroscopy and occasionally by supplementary methods as follows.

*Ethyl 3-(4-fluoroanilino)-2-(2,4,5-trifluorobenzoyl)-acrylate* (**4a**, $C_{18}H_{13}F_4NO_3$)

$^1$H NMR (300 MHz, $CDCl_3$), mixture of (*E*)- and (*Z*)-isomer (chemical shifts were not assigned to isomers): $\delta = 12.50/11.09$ (d, 1H, NH, $J = 13.5$ Hz), 8.50/8.41 (d, 1H, H-3, $J = 13.5$ Hz), 7.50–6.80 (m, 6 H, H-2′, H-3′, H-5′, H-6′, H-3′, H-6″), 4.12/4.09 (q, 2H, $CH_2CH_3$, $J = 7.0$ Hz), 1.12/0.99 (t, 3H, $CH_2CH_3$, $J = 7.0$ Hz) ppm; $^{19}$F NMR (282 MHz, $CDCl_3$), mixture of (*E*) and (Z) isomer (main isomer presented): $\delta = -142.7$ (dd, F-4″, $J = 22$ Hz, $J = 16$ Hz), −130.8 (dd, F-5″, $J = 22$ Hz, $J = 6$ Hz), -115. 8 (dd, F-2″, $J = 16$ Hz, $J = 6$ Hz), −115.3 (s, F-4′) ppm; EIMS: Same spectrum as compound **5a** (see below) due to cyclization of **4a** to **5a** in the injector of the GC system.

*Ethyl 1-(4-fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate* (**5a**, $C_{18}H_{12}F_3NO_3$)
$^1$H NMR (300 MHz, $CDCl_3$): $\delta = 8.40$ (s, 1H, H-2), 8.17 (dd, 1H, H-5, $J = 10.5$ Hz, $J = 8.7$ Hz), 7.51 (dd, 2 H, H-2′, H-6′, $J = 8.5$ Hz, $J = 4.6$ Hz), 7.34 (dd, 2H, H-3′, H-5′, $J = 8.5$ Hz, $J = 8.3$ Hz), 6.72 (dd, 1H, H-8, $J = 11.0$ Hz, $J = 6.2$ Hz), 4.31 (q, 2H, $CH_2CH_3$, $J = 7.1$ Hz), 1.34 (t, 3H, $CH_2CH_3$, $J = 7.1$ Hz) ppm; $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta = 172.6$ (C4), 165.0 (*C*OOEt), 163.3 (d, C4′, $J = 252$ Hz), 153.3 (dd, C6, $J = 256$ Hz, $J = 15$ Hz), 149.0 (C2), 149.0 (dd, C7, $J = 252$ Hz, $J = 14$ Hz), 137.7 (d, C8a, $J = 9$ Hz), 136.2 (d, C1′, $J = 3$ Hz), 129.5 (d, C2′, C6′, $J = 9$ Hz), 125.5 (d, C4a, $J = 5$ Hz), 118.0 (d, C3′, C5′, $J = 23$ Hz), 115.2 (dd, C5, $J = 19$ Hz, $J = 2$ Hz), 111.3 (C3), 106.5 (d, C8, $J = 23$ Hz), 61.1 ($OCH_2CH_3$), 14.3 ($OCH_2CH_3$) ppm; $^{19}$F NMR (282 MHz, $CDCl_3$): $\delta = -138.3$ (d, F-7, $J = 24$ Hz), -126.9 (d, F-6, $J = 24$ Hz), -108.5 (s, F-4′) ppm; EIMS: *m/z* (relative intensity) = 347 (7, $M^{\cdot+}$), 318 (1, $M^{\cdot+}$–$C_2H_5$), 302 (22, $M^{\cdot+}$–$OC_2H_5$), 275 (100, $M^{\cdot+}$–$COOC_2H_4$), 258 (12, 275-OH), 247 (9, 275-CO), 245 (10, 275-$CH_2O$), 226 (7, 245-F), 219 (18, 245-CN).

*1-(4-Fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate* (**6a**, $C_{18}H_8F_3NO_3$)
$^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 8.78$ (s, 1H, H-2), 8.34 (dd, 1H, H-5, $J = 10.4$ Hz, $J = 8.5$ Hz), 7.77 (dd, 2H, H-2′, H-6′, $J = 8.7$ Hz, $J = 4.9$ Hz), 7.54 (dd, 2 H, H-3′, H-5′, $J = 8.7$ Hz, $J = 8.7$ Hz), 7.26 (dd, 1H, H-8, $J = 11.4$ Hz, $J = 6.6$ Hz) ppm; $^{19}$F NMR (300 MHz, DMSO-$d_6$): $\delta = -137.4$ (d, F-7, $J = 24$ Hz), $-125.6$ (d, F-6, $J = 27$ Hz), $-110.3$ (s, F-4′) ppm.

*Difloxacin [1-(4-fluorophenyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylate]* (**8a**, $C_{21}H_{19}F_2N_3O_3$)
$^1$H NMR (300 MHz, trifluoroacetic acid-$d_1$): $\delta = 9.11$ (s, 1H, H-2), 8.22 (d, 1H, H-5, $J = 12.2$ Hz), 7.52 (m, 2H, H-2′, H-6′), 7.36 (dd, 2H, H-3′, H-5′, $J = 7.6$ Hz, $J = 7.6$ Hz), 6.74 (d, 1H, H-8, $J = 6.4$ Hz), 3.9–3.6 (m, 4H, piperazinyl 2- and 6-$CH_2$), 3.5–3.2 (m, 4H, piperazinyl 3- and 5-$CH_2$), 3.00 (s, 3H, $H_3C$-N) ppm; $^{13}$C-NMR (151 MHz, trifluoroacetic acid-$d_1$): $\delta = 174.0$ (C4), 172.4 (COOH), 167.5 (d, C4′, $J = 255$ Hz), 157.8 (d, C6, $J = 258$ Hz), 152.2 (C2), 150.5 (d, C7, $J = 10$ Hz), 143.9 (C8a), 137.3 (C1′), 130.9 (d, C2′, C6′, $J = 7$ Hz), 120.9 (d, C3′, C5′, $J = 25$ Hz), 118.1 (d, C4a, $J = 12$ Hz), 114.2 (d, C5, $J = 25$ Hz), 109.2 (C8), 106.2 (C3), 56.5 (C3″, C5″), 48.9 (C2″, C6″), 46.0 (N–$CH_3$) ppm.

*Ethyl [$^{15}$N,3-$^{13}$C]-3-(4-fluoroanilino)-2-(2,4,5-trifluorobenzoyl)-acrylate* (**4b**, $C_{17}^{13}CH_{13}F_4^{15}NO_3$)
A mixture of 1.210 g ethyl 2,4,5-trifluorobenzoylacetate, (**1**; 4.917 mmol), 1.064 g [formyl-$^{13}$C]-triethyl orthoformate (**2b**; 7.179 mmol), and 2.259 g acetic anhydride (22.13 mmol) was heated to 130 °C for 120 min. During the reaction, the emerging ethyl acetate evaporated and was separated by a condenser. After cooling to 22 °C, remaining portions of acetic anhydride and **2b** were removed under high vacuum. The remaining oily orange product was put under an argon atmosphere and dissolved in 25 $cm^3$ of dry dichloromethane. To this solution, 0.798 g [$^{15}$N]-4-fluoroaniline (**3b**; 0.68 $cm^3$, 7.179 mmol) was added, and the resulting mixture was stirred for 60 min at 22 °C under argon. Remaining portions of **3b** and dichloromethane were removed under high vacuum. The resulting brown solid crude product was dissolved in chloroform and adsorbed onto 10 g of silica gel 60 (0.063–0.2 mm; Fluka). This adsorbate was laid on a 100-g column of silica gel 60 (3 × 34 cm, solvent: petrol ether:diethyl ether 95:5 v/v). The crude product was fractionated using a gradient of petrol ether:diethyl ether and fractions of 100 $cm^3$ as follows: 95:5 (v/v) 10 fractions, 90:10 (v/v) 10 fractions, 80:20 (v/v) 10 fractions. Fractions 16–27 were combined and dried under high vacuum. Yield of **4b** was 1.780 g (99% based on **1**); the intermediate was examined by TLC (solvent system I: $R_f = 0.38$) and GC-MS. EIMS: same spectrum as compound **5b** (see below) due to cyclization in the injector of the GC system.

*Ethyl [1-$^{15}$N,2-$^{13}$C]-1-(4-fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate* (**5b**, $C_{17}^{13}CH_{12}F_3^{15}NO_3$)
Intermediate **4b** was dissolved in 40 $cm^3$ of dry tetrahydrofurane under argon, and 234 mg of an oily suspension (60%) of NaH (5.847 mmol) was added. The mixture was heated to 50 °C for 240 min under argon and was then cooled to 0 °C. After addition of 0.8 $cm^3$ of acetic acid (13.644 mmol), the mixture was concentrated to about 2 $cm^3$ (rotary evaporator), when 100 $cm^3$ of water was added. The sedimented orange brown solid was separated by filtration and dried (high vacuum). Yield of **5b** was 1.535 g (89% based on **1**). The product was examined by TLC (solvent system I: $R_f = 0.22$) and GC-MS. EIMS: *m/z* (relative intensity) = 349 (9, $M^{\cdot+}$), 320 (1, $M^{\cdot+}$–$C_2H_5$), 304 (22, $M^{\cdot+}$–$OC_2H_5$), 277 (100, $M^{\cdot+}$–$COOC_2H_4$), 260 (12, 277-OH), 249 (10, 277-CO), 247 (12, 277-$CH_2O$), 228 (9, 247-F), 219 (17, 247-$^{13}C^{15}N$).

*[1-$^{15}$N,2-$^{13}$]-1-(4-Fluorophenyl)-6,7-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylate* (**6b**, $C_{15}^{13}CH_8F_3^{15}NO_3$)
Intermediate **5b** was dissolved in 10.5 $cm^3$ of a mixture of water, HCl (conc.), and acetic acid (conc.) 1:1:1 (v/v/v), and was refluxed for 180 min. With cooling to 22 °C, the product sedimented, and was separated by filtration and washed with ethanol. The light brown product was dried under high vacuum yielding 1.232 g **6b** (62% based on **1**).

The product was analyzed by TLC (solvent system I: $R_f = 0.19$; solvent system II: $R_f = 0.35$).

*[1-$^{15}$N,2-$^{13}$C]-Difloxacin {[1-$^{15}$N,2-$^{13}$C]-1-(4-fluorophenyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylate}*
(**8b**, $C^{13}_{20}CH_{19}F_2N^{15}_2NO_3$)

Intermediate **6b** was put under argon and dissolved in 30 cm$^3$ of dry acetonitrile. After addition of 3.0 cm$^3$ of triethylamine and 1.537 g of 1-methylpiperazine (**7**; 1.7 cm$^3$, 15.345 mmol), the reaction mixture was refluxed for 300 min and cooled to 22 °C. The mixture was kept at 4 °C over night, and the pearly to light sandy product was subsequently separated by filtration, washed with acetonitrile, and dried under high vacuum. Yield of **8b** was 1.225 g (62% based on **1**). The product **8b** was examined by TLC (solvent system II: $R_f = 0.23$), HPLC, $^1$H, $^{13}$C, and $^{15}$N NMR. $^1$H NMR (600 MHz, trifluoroacetic acid-$d_1$): the spectrum was similar to that of the non-labeled compound, except the signal of hydrogen atom H-2, which appeared as a doublet ($J = 190.8$ Hz, $^1$H–$^{13}$C coupling) at $\delta = 9.37$ ppm; $^{13}$C NMR (151 MHz, trifluoroacetic acid-$d_1$): the spectrum was similar to that of the non-labeled compound, except the large signal of carbon atom C2, which appeared as a doublet ($J = 42$ Hz, $^{13}$C–$^{15}$N coupling) at $\delta = 152$ ppm; $^{15}$N NMR (61 MHz, trifluoroacetic acid-$d_1$): doublet ($J = 38.4$ Hz, $^{15}$N–$^{13}$C coupling) at $\delta = -215.7$ ppm.

**Acknowledgments** We are grateful to Prof. Dr. W. Leitner, Dr. J. Klankermayer, Dr. F. H. Kowaldt, T. Pullmann, and I. Bachmann-Remy (Institute for Technical and Macromolecular Chemistry, RWTH Aachen University) for support with synthesis and NMR spectra. The study was funded by the German Research Foundation, DFG (Research Unit FOR566 'Veterinary Medicines in Soils: Basic Research for Risk Analysis').

## References

1. Wolfson JS, Hooper DC (1989) Clin Microbiol Rev 2:378
2. Shryock TR (2001) J Clin Microbiol 39:1680
3. Brown SA (1996) J Vet Pharmacol Ther 19:1
4. Golet EM, Xifra L, Siegrist H, Alder AC, Giger W (2003) Environ Sci Technol 37:3243
5. Thiele-Bruhn S (2003) J Plant Nutr Soil Sci 166:145–167
6. Boxall ABA, Fogg LA, Blackwell PA, Kay P, Pemberton EJ, Croxford A (2004) Rev Environ Contam Toxicol 180:1
7. Kümmerer K (2003) J Antimicrob Chemother 52:5
8. Halling-Sørensen B, Nielsen SN, Lanzky PF, Ingersiev F, Holten Lützhoft HC, Jørgensen SE (1998) Chemosphere 36:357
9. Kreuzig R, Höltge S, Brunotte J, Berenzen N, Wogram J, Schulz R (2005) Environ Toxicol Chem 24:777
10. Kreuzig R (2002) Mitt Umweltchem Ökotox 8:9
11. Kreuzig R, Heise J, Höltge S (2006) Mitt Umweltchem Ökotox 12:39
12. Schmidt B, Ebert J, Lamshöft M, Thiede B, Schumacher-Buffel R, Ji R, Corvini PFX, Schäffer A (2008) J Environ Sci Health Part B 43:8
13. Lamshöft M, Sukul P, Zühlke S, Spiteller M (2007) Anal Bioanal Chem 388:1733
14. Schmidt B, Schäffer A, unpublished results
15. Dec J, Bollag JM (1997) Soil Sci 162:858
16. Chu DTW, Fernandes PB, Claiborne AK, Pihulaec E, Nordeen CW, Maleczka RE, Pernet AG (1985) J Med Chem 28:1558
17. Chu DTW, Fernandes PB, Maleczka RE, Nordeen CW, Pernet AG (1987) J Med Chem 30:504
18. Glushkov RG, Marchenko NB, Levshin IB (1997) Pharm Chem J 31:240
19. Baker WR, Cai S, Dimitroff M, Fang L, Huh KK, Ryckman DR, Shang X, Shawar RM, Therrien JH (2004) J Med Chem 47:4693
20. Grohe K, Heitzer H (1987) Liebigs Ann Chem 1987:29
21. Granneman GR, Snyder KM, Shu VS (1986) Antimicrob Agents Chemother 30:689
22. Garcia MA, Solans C, Aramayona JJ, Rueda S, Bregante MA (2000) Chromatographia 51:487
23. Freedom of Information Office, Center for Veterinary Medicine, FDA (1997) Freedom of information summary, NADA 141-096, difloxacin hydrochloride. http://www.fda.gov/cvm/FOI/893.htm. Last accessed 21 May 2009
24. Wetzstein HG, Schmeer N, Karl W (1997) Appl Environ Microbiol 63:4272
25. Wetzstein HG, Stadler M, Tichy HV, Dalhoff A, Karl W (1999) Appl Environ Microbiol 65:1556
26. Choma IM (2003) J Liq Chrom Rel Technol 26:2673
27. Wallis SC, Gahan LR, Charles BG, Hambley TW (1994) Aust J Chem 47:799
28. Holzgrabe U, Branch SK (1994) Magn Reson Chem 32:192
29. Spragg RA (1968) J Chem Soc B:1128