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Isolation, structural elucidation and in vitro activity of 2-acetyl-2-decarboxamido-oxytetracycline against environmental relevant bacteria, including tetracycline-resistant bacteria

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Abstract

2-Acetyl-2-decarboxamido-oxytetracycline (ADOTC) is a major impurity of oxytetracycline (OTC) produced as a side product during fermentation. ADOTC was isolated from OTC and other impurities using preparative HPLC. The preparative column was an Xterra MS, C_{18} chromatographic column (100 mm × 19 mm i.d., 5 μm), and the mobile phase contained methanol–water (27:73 (v/v)) with 0.08 M formic acid added. The flow rate was 9.0 ml/min. It was possible to isolate few milligram ADOTC in a day. The compound was unambiguously identified using NMR and MS–MS. The anti-microbial activity against activated sludge bacteria was determined giving a potency of only 3% of that of OTC. With tetracycline-resistant bacteria, no anti-microbial activity was observed, indicating a mode of action similar to that of OTC.

Keywords: 2-Acetyl-2-decarboxamido-oxytetracycline; Preparative HPLC; NMR; Tandem mass spectrometry; Microbial testing; Resistance

1. Introduction

2-Acetyl-2-decarboxamido-oxytetracycline (ADOTC) is a side product of oxytetracycline (OTC) produced by *Streptomyces rimosus*. It is, therefore, found as an impurity in the raw material of OTC [1] and will be present in formulations of OTC including veterinary medicine. ADOTC is also expected to be present along with OTC in urine and faeces from treated patients and animals. ADOTC is mentioned as an impurity of OTC in the European Pharmacopoeia (Ph. Eur.) and the content of ADOTC has been limited to 2% of OTC. Other tetracyclines contain correspondingly 2-acetyl-2-decarboxamido-impurities as well [2]. The structures of OTC and ADOTC are shown in Fig. 1.

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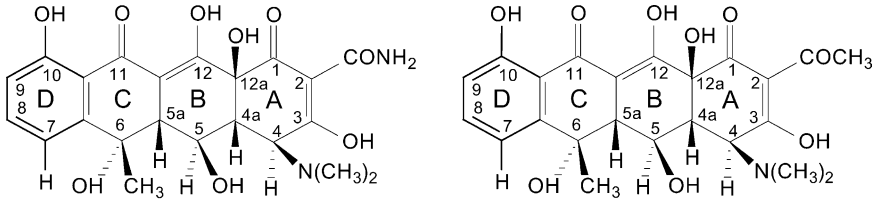

Fig. 1. Chemical structures of oxytetracycline and 2-acetyl-2-decarboxamido-oxytetracycline.

ADOTC has less anti-bacterial activity than OTC. Hochstein et al. reported an anti-bacterial activity of 10% of OTC [1] and Cakara an anti-bacterial activity of only 7% of OTC [3]. In this work, different in vitro bacterial assays were applied to determine the potency of ADOTC related to resistant and environmental relevant bacteria.

Some tetracycline analogues (e.g. anhydro-tetracycline, anhydro-chlortetracycline and the 4-epimers of these) are toxic to tetracycline-resistant bacteria. This toxicity is due to a bactericidal effect as opposed to the bacteriostatic effect of the parent compounds [4]. The literature does not reveal whether ADOTC belongs to these “atypical tetracyclines”.

ADOTC is not commercial available as a standard, and therefore, several methods have been developed to isolate or produce the compound. ADOTC has been isolated by Keiner et al. by preparative TLC [5]. Cakara isolated ADOTC from OTC by column chromatography and purified ADOTC by precipitation as the sulfosalicylic salt, which was finally converted into the hydrochloride of ADOTC [3]. Hochstein et al. produced ADOTC by means of a mutant strain of *S. rimosus*, which produced predominantly ADOTC [1]. In this work preparative HPLC was used to isolate ADOTC. The method was developed from an analytical HPLC method used to analyse OTC and degradation products in soil interstitial water, which gave a good separation of ADOTC from OTC and the other impurities [6].

The aim of this paper was to provide NMR and MS–MS data for the identification of ADOTC as well as to suggest a method for preparative HPLC to isolate ADOTC from OTC and other impurities. As OTC is heavily applied in agriculture and fish farming the anti-microbial potency of ADOTC was assessed on tetracycline resistant bacteria and activated sludge bacteria that provide a measure for a heterogeneous aerobic environmental relevant bacteria population.

2. Materials and methods

2.1. Chemicals and reagents

Oxytetracycline hydrochloride purchased from Unikem (Copenhagen, Denmark) was used for isolation of ADOTC and as test substance in the growth inhibition test, 3,5-dichlorophenol purchased from Sigma Chemical Company (St Louis, MO) was used as reference substance in the growth inhibition test and deuterated methanol purchased from Lab Science (Copenhagen, Denmark) was used for NMR. All other chemicals were of reagent grade or HPLC quality.

2.2. Preparative HPLC-system

A LC-10AD liquid chromatograph (Shimadzu, Duisburg, Germany) was used for the preparative isolation of ADOTC. The detection was performed with an SPD-10A UV-detector (Shimadzu, Duisburg, Germany) at 254 nm. Peak areas were calculated with a DP700 integrator (CE Instruments, Milan, Italy). The preparative column was an Xterra MS (Waters Corporation, Milford, MO, USA) C_{18} chromatographic column (100 mm × 19 mm i.d., 5 μm). The mobile phase contained: methanol–water (27:73 (v/v)) with 0.08 M formic acid added. The operating flow-rate was 9.0 ml/min. The amount of OTC injected onto the HPLC-system was 12–20 mg dissolved in the mobile phase. The fraction corresponding to ADOTC was collected and diluted 1:3 with water giving a

final methanol concentration of 6–7%. Subsequently ADOTC was solid phase extracted through Oasis® HLB 1 cc Extraction Cartridges (Waters). The cartridges were conditioned with 2 ml methanol and 2 ml water, loaded with 500 ml sample, washed with 2 ml 5% methanol in water and, finally, eluted with 2 ml methanol. The methanol was then evaporated on a rotary evaporator.

The ADOTC was injected onto the HPLC, solid phase extracted and evaporated a second time to improve the purity.

3. MS analysis

The mass spectrometric measurements were performed on a Sciex API 3000™ (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionisation source. The instrument was operated in the positive mode and coupled to the outlet of the HPLC column via PEEK tubing. The chromatographic separation was performed as described by Halling-Sørensen et al. [6]. The temperature of the heated capillary was 500 °C and the source voltage set at 4.2 kV. Nebulizer gas, curtain gas, collision gas, declustering potential, focusing potential, entrance potential, collision energy, collision cell exit potential, were set at the following values: 8, 9, 4 psi and 41, 200, −10, 30 and 15 V, respectively. The fragmentation pattern of ADOTC and OTC was determined using product ion scan.

Collection and treatment of data was done using Analyst™ software (Applied Biosystems, Foster City, CA, USA) in Windows NT® platform based data processing.

3.1. NMR analysis

The NMR data of ADOTC was acquired using an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). ^{1}H NMR spectra were obtained at 400.13 MHz and ^{13}C NMR spectra were obtained at 100.6 MHz. All NMR spectra were recorded in CD_3OD. For the structural elucidation, the following spectra was obtained: ^{1}H NMR, ^{13}C NMR, COSY, NOESY, ^{1}H–^{13}C-COSY (HSQC) and HMBC.

Acquisition parameters are shown in Table 1.

3.2. Anti-microbial susceptibility testing to sensitive and resistant bacteria

Bacterial isolates were obtained from the continuous surveillance program for anti-microbial resistance in Denmark (DANMAP) as previously described [7]. Isolates were collected in 1997, 1998 and 1999. Twenty tetracycline resistant *E. coli* and 10 susceptible *E. coli* plus 18 tetracycline resistant and 10 susceptible *E. faecium* isolates from pigs and broilers, were tested for susceptibility to ADOTC. Susceptibilities to the compounds were determined by broth dilution susceptibility tests following NCCLS guidelines [8]. The tests were performed in Sensititre plates. All

Table 1
Acquisition parameters for the NMR analysis

	^{1}H-NMR	^{13}C-NMR	COSY	NOESY	^{1}H–^{13}C COSY	HMBC
TD (F1)	–	–	512	512	256	256
TD (F2)	32768	4096	1024	1024	4096	4096
SI (F1)	–	–	1024	1024	1024	512
SI (F2)	32768	65536	1024	1024	4096	4096
Pulses (°)	60	24	90	90	42	90
AQ	4.06	1.24	0.09	0.09	0.08	0.36
NS	137	227171	16	32	512	32
SWH (Hz)	4032	26315.8	5681.8	5681.8	26315.8	5681.8
LB	–	0.3	–[a]	–[b]	5	–
*D*1 (s)	1	1	1.5	2	1	1

TD: time domain data, SI: size of the real spectrum, AQ: acquisition time, NS: number of scans, SWH: spectral width, LB: line broadening prior to Fourier transformation, *D*1: relaxation delay between successive pulses.

[a] Unshifted squared sine bell in both dimensions.

[b] Shifted squared sine bell in both dimensions.

plates were inoculated and incubated following NCCLS guidelines [9]. *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were included in each experiments as controls. The compounds were tested in two-fold dilutions from 0.25 to 32 μg/ml. The minimal inhibitory concentration (MIC) was read as the lowest concentration without visible growth. All MIC-values (mg/l) of the *E. coli* and *E. faecium* isolates were tested twice. The MIC_{50}-values (mg/l) were obtained at the concentrations where 50% of the isolates were inhibited (values from both tests were included).

3.3. Anti-microbial susceptibility testing to sludge bacteria

Activated sludge bacteria used in this investigation was obtained from the primary aeration tank of a pilot scale activated sludge waste water treatment plant located at the Institute of Environmental Science and Technology, The Technical University of Denmark, Lyngby, Denmark, receiving municipal wastewater from the Lyngby area. Within 1 h after collection, the sludge was pre-conditioned for 20–24 h at 20 °C.

Viable plate counting (pour plate method) of aerobic sludge bacteria was used in all the growth inhibition toxicity tests. The method was applied and validated exactly as described in detail by Halling-Sørensen et al. [10] and in ISO 15522 (1999) [11]. 3,5-Dichlorophenol was used as reference compound. In the application of this technique, bacteria were immobilized in the agar containing the relevant anti-bacterial agent. This resulted in the development of only well defined easily countable colonies. After 48 h, all plate colonies (activated sludge bacteria) were counted and the inhibition (*I*) (%) was calculated using:

$$I = \frac{D - E}{D} \times 100$$

where *D* is the mean number of counted colonies on the non-exposed agar plates at the end of the incubation period (48 h) and *E* the mean number of counted colonises on the anti-microbial treated agar plates at the end of the incubation period (48 h). From these data, EC_{50} (mg/l) values were calculated by weighted non-linear regression analysis using the Weibull equation to describe the concentration–response relationship. A computer program developed by Andersen et al. [12] was used to give the EC_{50}-values and corresponding 95%-confidence intervals.

4. Results and discussion

4.1. Isolation of ADOTC

The isolation of ADOTC was performed using isocratic preparative HPLC and the chromatogram is shown in Fig. 2. The retention times of ADOTC and OTC were ~12.5 and ~8.4 min, respectively. The method provided a good separation of ADOTC from OTC and the other impurities such as epi-oxytetracycline, tetracycline, α-apo-oxytetracycline and β-apo-oxytetracycline. The mobile phase was removed from the collected ADOTC using solid phase extraction, which is a gentle way to concentrate ADOTC. The compound was finally eluted from the extraction cartridge with pure methanol, which was then evaporated on a rotary evaporator. To obtain sufficient purity of ADOTC, the isolated substance was reinjected onto the chromatographic system and the recollected ADOTC was isolated from the mobile phase once more. Using this method, a few milligram of ADOTC could be isolated in a day.

Compared to earlier reported methods, the preparative HPLC method presented here is a method to isolate small quantities of ADOTC relatively fast. Another fast method, TLC, exists but it results in even smaller quantities [5]. Column chromatography [3] is more time-consuming but in return the

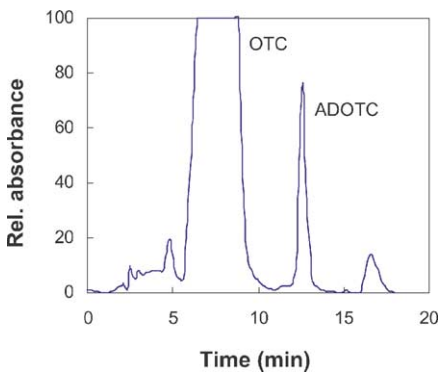

Fig. 2. Chromatogram of oxytetracycline (OTC) and the impurity 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC) under preparative isocratic HPLC conditions.

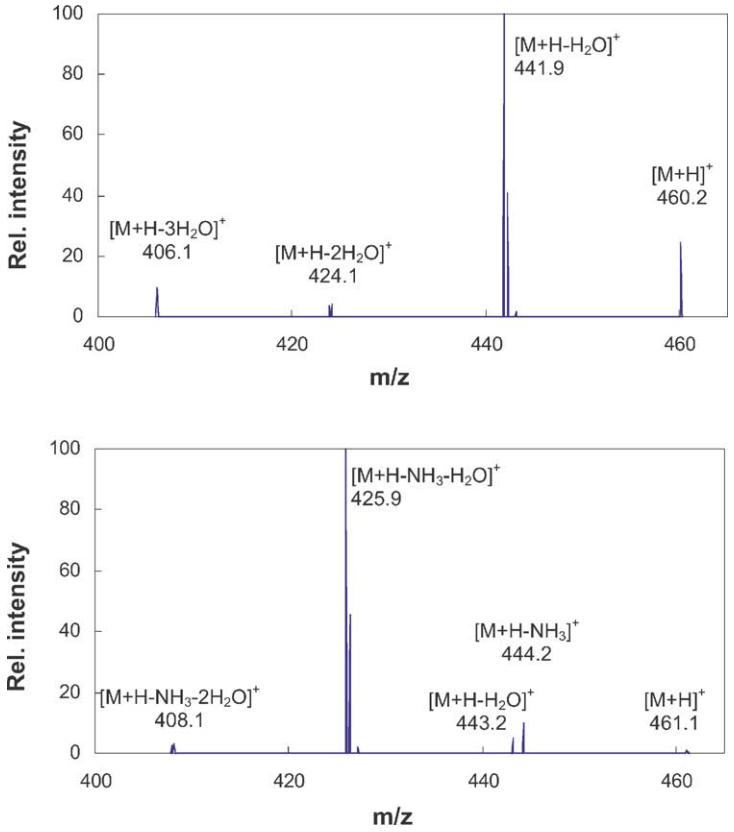

Fig. 3. Product ion scan of oxytetracycline (OTC) and 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC). Proposed structures of the product ions and *m*/*z* values are given.

yield of ADOTC is much larger. By the use of ADOTC-producing strains of *S. rimosus* [1] it is possible to produce large quantities, but this method is time consuming and it requires microbiological laboratory facilities.

4.2. Identification of ADOTC

Following isolation of ADOTC tandem mass spectrometry was used in the identification of the compound. The product ion scans of ADOTC and OTC are shown in Fig. 3. The protonated molecular ion of ADOTC has the mass 460, corresponding to a molecular weight of one less than the molecular ion of OTC at 461. The characteristic losses of OTC are: (1) the loss of water probably from the C-6 and C-5a position; and (2) the loss of NH_3 from the amide group at C-2. ADOTC lose water just as OTC, suggesting no differences at the C-6 and C-5a positions. There is no loss of NH_3, however, suggesting a difference at the C-2 position.

The ^{1}H NMR spectral data of ADOTC shows one more methyl-group (2.34 ppm) than OTC, which could correspond to a methyl group adjacent to a ketone or an aromatic group. The ^{13}C NMR spectral data of ADOTC is shown in Fig. 4. It shows one more peak than the ^{13}C NMR spectral data of OTC with a higher frequency than 190 ppm, corresponding to a carbonyl group. The ^{13}C NMR spectral data on the other hand does not contain a peak corresponding to the amide seen in OTC (177 ppm). This suggests that the compound contains a methyl ketone instead of the amide in OTC, and the compound is identified as 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC). This is in agreement with the product ion scan where no loss of NH_3 is seen.

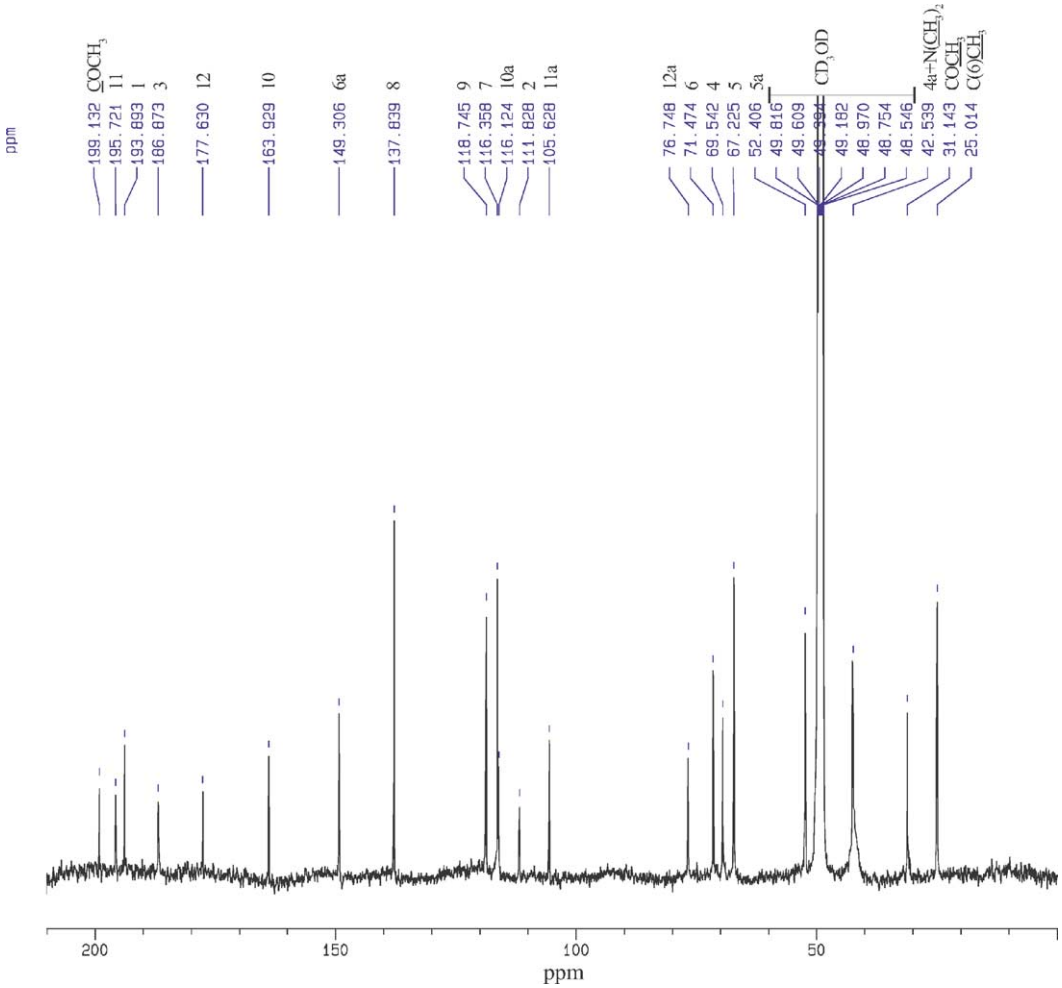

Fig. 4. ^{13}C NMR spectral data of 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC) measured in CD_3OD.

A full assignment of all ^{1}H and ^{13}C NMR resonances in ADOTC was obtained from ^{1}H NMR, ^{13}C NMR, COSY, NOESY, ^{1}H–^{13}C-COSY and HMBC experiments (see Table 2). ^{1}H and ^{13}C NMR resonances of OTC and ADOTC are compared in Table 3. The chemical shift values of OTC have been assigned by ^{1}H NMR, ^{13}C NMR, ^{1}H–^{13}C-COSY and HMBC experiments and by comparison with chemical shift values of chlortetracycline and tetracycline [13,14]. The chemical shift values of OTC and ADOTC are very similar and the only significant difference is near C-2, which carry the chemical difference of the two compounds. The carbonyl group in the methyl ketone group is 25 ppm more downfield than in the corresponding amide seen in OTC. The same is seen for C-2, which is 15 ppm more downfield in ADOTC than in OTC. There are only small differences for the rest of the groups in the two compounds.

4.3. Susceptibility of sensitive and resistant bacteria to ADOTC

The MIC_{50} values of OTC and ADOTC to sensitive and resistant bacteria are shown in Table 4.

Ten tetracycline sensitive *E. coli* and 10 tetracycline sensitive *E. faecium* were tested for susceptibility to ADOTC. If a bacterial strain is not susceptible to a tetracycline concentration of 16 mg/l or more, the

Table 2
Connectivities observed in 1D and 2D NMR spectra of ADOTC measured in CD_3OD

	^1H	^{13}C	COSY	NOESY	HMBC[a]
1		193.893			
2		111.828			
3		186.873			
4	4.076 (s)	69.542	H-4a[b]	H-4a, $N(CH_3)_2$, H-5	
4a	2.570 (dd, $^2J_{4,4a} = 1.5$; $^2J_{4a,5} = 11.0$)	42.539	H-4[b], H-5	$N(CH_3)_2$, H-4, H-5, H-5a	C-1, C-12a, C-4, C-5
5	3.930 (dd, $^2J_{5,5a} = 8.3$; $^2J_{4a,5} = 10.8$)	67.225	H-4a, H-5a	$C(6)CH_3$, H-4a, H-5a, H-4	
5a	2.915 (d, $^2J_{5,5a} = 8.2$)	52.406	H-5	$C(6)CH_3$, H-4a, H-5a	C-11a, C-5
6		71.474			
6a		149.306			
7	7.162 (d, $^2J_{7,8} = 7.8$)	116.358	H-8	$C(6)CH_3$	C-9, C-10a, C-6
8	7.515 (t, $^2J_{7,8} = {}^2J_{8,9} = 7.8$)	137.839	H-7, H-9		C-6a, C-10
9	6.923 (d, $^2J_{8,9} = 7.8$)	118.745	H-8		C-7, C-10a
10		163.929			
10a		116.124			
11		195.721			
11a		105.628			
12		177.630			
12a		76.748			
CO**CH_3**	2.337 (s)	31.143			$COCH_3$
N**$(CH_3)_2$**	2.875 (s)	~42		H-4a, H-4	C-4
C(6)**CH_3**	1.802 (s)	25.013			
COCH_3		199.132		H-5a, H-5, H-7	

Coupling constants in Hz.
[a] Optimized for $J_{C,H} = 7$ Hz.
[b] The signal is very small.

strain is considered resistant [9]. Therefore, *E. coli* seems not to be susceptible to ADOTC ($MIC_{50} > 32$) as opposed to OTC ($MIC_{50} = 1$). The tetracycline sensitive *E. faecium* isolates showed susceptibility to ADOTC ($MIC_{50} = 8$). However, the compound was still much less toxic than OTC ($MIC_{50} = 0.5$).

Twenty tetracycline resistant *E. coli* and 18 tetracycline resistant *E. faecium* were screened for susceptibility to ADOTC. This was done to investigate whether ADOTC belongs to the atypical tetracyclines, which are toxic to tetracycline resistant bacteria. All MIC_{50} values of ADOTC as well as OTC to both the *E. coli* and the *E. faecium* tetracycline resistant isolates were >32. Thus, ADOTC did not demonstrate any significant anti-bacterial activity towards the tetracycline resistant isolates. Furthermore, the structure of ADOTC is different from the structure of the atypical tetracyclines due to the relative planarity of the B, C and D rings, which are predominantly lipophilic and non-ionized. Based on these results, ADOTC does not seem to belong to the atypical tetracyclines.

4.4. Growth inhibition of sludge bacteria

Table 5 shows the EC_{50}-values (mg/l) for the growth inhibition assay used for the aerobic activated sludge bacteria of the tetracyclines and the reference compound 3,5-dichlorophenol. The potency of the reference compound 3,5-dichlorophenol was found in accordance with previous obtained results with this testing method [10]. The impurity ADOTC was found less potent than the parent compound as the obtained 95% confidence limit of the EC_{50}-value did not overlap the one obtained for the parent compound. The potency of ADOTC was 3.2% of OTC (2.2–4.8% of OTC) on environmental relevant bacteria.

Hochstein et al. and Cakara reported a potency of 10 and 7% of OTC, respectively [1,3], which is more than was obtained in this work. This can be due to the use of different test organisms, however, Hochstein et al. and Cakara did not indicate which organisms were used in their studies.

The reduced activity of ADOTC compared to OTC is consistent with a set of rules of structural require-

Table 3
^{1}H NMR and ^{13}C NMR spectra of OTC and ADOTC measured in CD_3OD

	^{1}H		^{13}C	
	OTC	ADOTC	OTC	ADOTC
1			196.165	193.893
2			96.585	111.828
3			187.982	186.873
4	4.423 (d, $^{2}J_{4,4a} = 1.0$)	4.076 (s)	67.691	69.542
4a	2.914 (dd, $^{2}J_{4,4a} = 1.3$; $^{2}J_{4a,5} = 11.2$)	2.570 (dd)	43.573	42.539
5	3.881 (dd, $^{2}J_{5,5a} = 8.2$; $^{2}J_{4a,5} = 11.2$)	3.930 (dd)	66.372	67.225
5a	2.968 (d, $^{2}J_{5,5a} = 8.2$)	2.915 (d)	52.235	52.406
6			71.143	71.474
6a			148.993	149.306
7	7.166 (d, $^{2}J_{7,8} = 7.7$)	7.162 (d)	116.298	116.358
8	7.541 (*t*, $^{2}J_{7,8} = {}^{2}J_{8,9} = 8.1$)	7.515 (t)	137.948	137.839
9	6.947 (d, $^{2}J_{8,9} = 8.4$)	6.923 (d)	118.839	118.745
10			163.851	163.929
10a			116.045	116.124
11			195.003	195.721
11a			106.035	105.628
12			173.328	177.630
12a			74.308	76.748
CO**CH$_3$**		2.337 (s)		31.143
N**(CH$_3$)$_2$**	3.000 (s)	2.875 (s)	42.702	~42
C(6)**CH$_3$**	1.845 (s)	1.802 (s)	24.978	25.013
CONH$_2$/**C**OCH$_3$			174.411	199.132

Coupling constants in Hz.

Table 4
MIC_{50} values (mg/l) of OTC and ADOTC to sensitive and resistant strains of *E. coli* and *E. faecium*

	E. coli		*E. faecium*	
	Sensitive	Resistant	Sensitive	Resistant
ADOTC	>32 (32 to >32)	>32	8 (4–16)	>32
OTC	1 (0.5–2)	>32	0.5 (0.25–1)	>32 (16 to >32)

ments for tetracycline activity worked out by Mitscher [15]. Changes at position C-1, C-2, C-3, C-4, C-10, C-11, C-12 and C-12a will all reduce the potencies of tetracyclines [15]. ADOTC contain a methyl ketone group at C-2 instead of the amide group in OTC and this is believed to be the reason for the reduced activity. The amide is, therefore, believed to be essential for the activity of the tetracyclines.

Table 5
EC_{50}-values and 95% confidence limit of OTC, ADOTC and 3,5-dichlorophenol

Compound	EC_{50} (mg/l)	95% Confidence limit
OTC	0.17	0.14–0.21
ADOTC	5.3	4.4–6.4
3,5-Dichlorophenol	5.9	5.5–6.3

According to Mitscher, the nature of the substituent at position C-2 should have a pronounced effect on the water solubility [15]. ADOTC containing a methyl ketone at position C-2 is more lipophilic than OTC containing an amide. Estimations of $\log P$ of OTC and ADOTC using ACD ChemSketch [16] yields -1.22 ± 0.75 and -0.01 ± 0.75, respectively, indicating that ADOTC is 10 times more lipophilic than OTC. According to the literature, $\log P$ of OTC is -0.9, which is close to the estimated $\log P$ value [17]. Therefore, the estimated $\log P$ value of ADOTC is also expected to be close to the actual value. The difference in the lipophilicity can influence the inter-

action with the tetracycline-binding site in the 30S subunit of the bacterial ribosome. It is also expected to have an influence on the formation of a complex between the tetracycline and calcium and with that an altered transportation of the tetracycline into the bacterial cell by the active transport mechanism [18].

5. Conclusion

ADOTC, a major impurity of OTC was isolated from OTC using preparative HPLC, which gave a good separation of ADOTC from OTC and other impurities. The compound was unambiguously identified using MS–MS and NMR.

ADOTC is present in OTC formulations in concentrations of up to 2% of OTC and may, therefore, have impact on both resistance development and potency against pathogen and non-pathogen bacteria. As OTC is heavily applied in agriculture and fish farming ADOTC was applied on both activated sludge bacteria and environmental aerobic relevant OTC resistant bacteria. The results showed that ADOTC has a potency of only 3% of that of OTC on the activated sludge bacteria and did not exhibit potency towards the tetracycline resistant bacteria, whereas the compound was potent to the tetracycline sensitive pathogen bacteria.

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