Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 119–123

TETRAHEDRON: *ASYMMETRY*

Enantioselective synthesis of a key intermediate of Levofloxacin using microbial resolution

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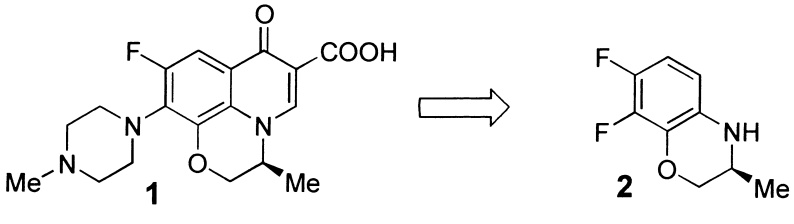
Received 28 October 1998; accepted 16 November 1998

Abstract

The important key intermediate in the synthesis of Levofloxacin, (*S*)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine, was prepared enantioselectively by microbial resolution. When lyophilized microorganism selected from soil was treated with the corresponding amide, the (*S*)-amine was obtained with high enantiomeric purity (99% *ee*).

1. Introduction

Levofloxacin **1** is an antibacterial agent with potent activity against Gram-positive and Gram-negative bacteria.[1] It has a tricyclic structure and possesses a methyl group at the C-3 position in the oxazine ring, and thus has an asymmetric center at this position. A number of chemical syntheses of the key intermediate, (*S*)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine **2**, have been reported (Scheme 1).[2–4] Although an enzymatic synthesis with asymmetric hydrolysis has also been reported,[5] the enantiomeric excess of **2** obtained with it was not particularly high. In contrast, only a few cases of the enantioselective hydrolysis of the amide have been reported.[6]



Scheme 1.

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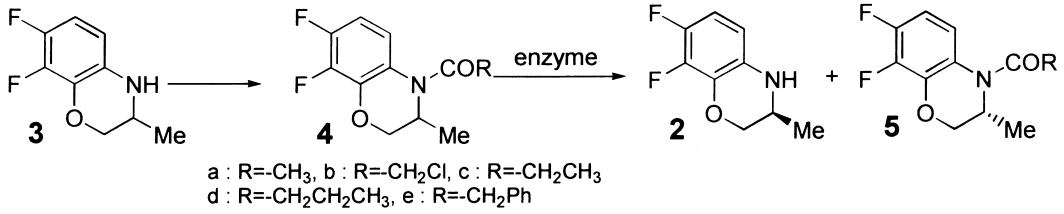
0957-4166/99/$ - see front matter
PII: S0957-4166(98)00475-3

In this paper, we describe, as shown in Scheme 2, an efficient and highly enantioselective synthesis of the key compound **2** using microbial resolution.



Scheme 2.

2. Results and discussion

We selected the racemic acetoamido **4a**, derived from **3** with acetic anhydride, as the substrate for enzymes and attempted to hydrolyze the C–N bond by microbial enantioselective hydrolysis. In our first trial of an enantioselective hydrolysis reaction, about 500 microorganisms were screened. A few strains, which belong to yeasts, were found to hydrolyze **4a**, however, they had poor activity, with chemical yields of **2** of 6–13% (runs 1–2).

Therefore, we attempted to screen for the microorganism with the highest specificity from soil (Table 1). One strain, which was selected from soil, did exhibit better activity, and the enantiomeric excess of **2** was particularly high (run 3). We found that the DSC 1012 strain yielded good to excellent *ee* for **2**.

The microbial reaction was applied to other aliphatic and aromatic amides **4b–e** to study the effect of chain length on reaction rate. The reaction was monitored by HPLC analysis and stopped when ca. 50% conversion had been achieved. As shown in Table 2, **4a** was the most reactive substrate for the DSC 1012 among the five substrates examined, and other compounds were not good substrates. In particular, for **4c–e**, the conversion rate was below 2% at 200 h and never reached 50%, even after a prolonged reaction time. The chain length of the alkyl group was thus found to have a great effect on reaction rate.

In a typical procedure for microbial resolution, a suspension of lyophilized cells (DSC-1012, 0.3 g) in a phosphate buffer 1500 ml (pH 7.0), was stirred for 15 min at 30°C. To the mixture, 1.5 g of amide **4a** was added. The whole mixture was stirred at 30°C. After 120 h of stirring, the amide **2**, which was

Table 1
Screening of enzymes catalyzing hydrolysis of racemate **4a**[7]

run[a]	strain[b]	conv.[c] (%)	amine **2**		amide **5a**		*E*[d] value
			ee(%)	config.	*ee*(%)	config.	
1	*Debaryomyces* sp. (IAM 1637)	12	97	*S*	15	*R*	24.3
2	*Debaryomyces* sp. (IFO 3330)	6	98	*S*	6	*R*	34.3
3	*Bacillus* sp. (DSC 1012)[e]	48	99	*S*	92	*R*	154

[a] 1mg **4a**, 2mg lyophilized cells, 0.1M phosphate buffer pH 7.0 (1ml) at 30℃-120hr.
[b] About 500 strains of type cultures (bacteria, yeasts, fungi) were tested in runs 1-2.
[c] Conversion ratio was determined from %*ee* of the amine **2** and the amide **5**.
[d] This enantiomeric ratio *E* was calculated from the equation : $E = \ln\{(1-c)[1-ee(s)]\}/\ln\{(1-c)[1+ee(s)]\}$.[7]
[e] DSC 1012 strain was isolated from soil, and was identified as *Bacillus* sp.

Table 2
Effect of chain length of alkyl group **4a–f**

substrate	reaction time	conv.[b]	amine **2**		amide **5a-e**	
			ee(%)	config.	*ee*(%)	config.
4a	120	48	99	*S*	94	*R*
4b	120	10	76	*S*	8	*R*
4c	120	1.6	-	-	-	-
4d	240	< 0.1	-	-	-	-
4e	240	< 0.1	-	-	-	-

[a] 100mM **4a-e**, 15mg lyophilized cells (DCS 1012), 0.1M phosphate buffer pH 7.0 (10ml) at 30°C.
[b] Conversion ratio was determined by HPLC.

hydrolyzed by enzyme, and the amide **5a** were separated by chromatography to obtain **2** (42%, 99% *ee*) and **5a** (46%, 92% *ee*).

3. Conclusion

We have demonstrated a highly enantioselective preparation of (*S*)-(−)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine **2**, which can easily be converted to Levofloxacin **1** in six steps,[2] using a microbial hydrolysis of the corresponding amide in high chemical yield and enantiomeric excess.

4. Experimental section

4.1. General procedures

Melting points were determined on a Yanagimoto apparatus and are uncorrected. Infrared spectra were recorded on an FT-720 spectrometer (Horiba). ^{1}H NMR spectra were recorded on a JEOL JNM-EX270 (270 MHz) instrument. Coupling constants are reported in hertz (Hz) and chemical shifts in ppm downfield from internal TMS. Mass spectra were recorded on a JEOL JMS-HX110 or JMS-AX505W mass spectrometer. Optical rotations were measured with a SEPA-300 polarimeter (Horiba). A Merck Kieselgel 60 Art7744 column was used for column chromatography. All chemicals were obtained from commercial sources and were used without further purification.

4.2. Microorganisms, media and culture conditions

Microorganisms were obtained from culture collections, except for the *Bacillus* sp. (DSC 1012), which was selected from soil, cultivating with 4-acetyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine **4a** as a sole carbon source at 30°C for 10 days. The identification of bacteria (DSC 1012) was based on Bergey's Manual of Determinative Bacteriology. Cultures were performed with a reciprocating shaker (150 rpm/min) on the following medium: peptone (10 g/l), meat extract (3 g/l), and NaCl (5 g/l) at pH 7.0 after sterilization (120°C, 15 min). At the late exponential growth phase, the cells were harvested by centrifugation (3000×g), resuspended in phosphate buffer (50 mM, pH 7.0), centrifuged again, and lyophilized. The cells were stored over several months at 5°C without significant loss of activity.

4.3. Measurement of enantiomeric purity

Enantiomeric excesses of **2**, **4a** and **4b** were analyzed by HPLC (Shimadzu LC-6A) on a CHIRALCEL OD column (4.6×250 mm, Daicel Chemical Co., Ltd) [eluent: *n*-hexane:ethanol, 20:1, flow rate: 1.0 ml/min, UV detection: 254 nm].

4.3.1. 4-Acetyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine ***4a***

7,8-Difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine **3** (5.2 g, 28.1 mmol) was dissolved in dichloromethane (50 ml); triethylamine (4.7 ml, 33.7 mmol) and acetic anhydride (3.1 ml, 33.0 mmol) were added to the solution at 0°C. The reaction mixture was left for 4 h at room temperature. The mixture was evaporated in vacuo. The residue was crystallized from ethanol to obtain **4a** as colorless crystals (5.8 g, 91.0%); mp 109–110°C; ^{1}H NMR ($CDCl_3$) δ: 1.20 (d, J=6.9 Hz, 3H, -C*H*$_3$), 2.30 (s, 3H, C*H*$_3$CO-), 4.18 (dd, 1H, J=2.6 Hz, 10.8 Hz, C_2-*H*), 4.32 (dd, 1H, J=1.3 Hz, 10.8 Hz, C_2-*H*), 4.7–5.2 (brs, 1H, C_3-*H*), 6.64–7.2 (m, 2H, C_5-*H* and C_6-*H*); m/z 228 (M^++1); IR (KBr) 1666, 1492, 1388 and 1151 cm^{-1}. Anal. calcd for $C_{11}H_{11}F_2NO_2$: C, 58.15; H, 4.88; F, 16.72; N, 6.16. Found: C, 58.33; H, 4.88; F, 16.82; N, 6.14.

4.3.2. 4-Chloroacetyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine ***4b***

Yield 92.9% (crystals); mp 106–107°C; ^{1}H NMR ($CDCl_3$) δ: 1.26 (d, J=6.9 Hz, 3H, -C*H*$_3$), 4.14–4.44 (m, 4H, ClC*H*$_2$CO- and C_2-*H*), 4.6–5.3 (brs, 1H, C_3-*H*), 6.69–7.5 (m, 2H, C_5-*H* and C_6-*H*); m/z 261 (M^+).

4.3.3. 4-Propanoyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine ***4c***

Yield 90.8% (oil); ^{1}H NMR ($CDCl_3$) δ: 1.19 (t, J=7.3 Hz, 3H, C*H*$_3$CH$_2$CO-), 1.20 (d, J=6.9 Hz, 3H, -C*H*$_3$), 2.46–2.64 (m, 2H, CH$_3$C*H*$_2$CO-), 4.16 (dd, 1H, J=3.0 Hz, 10.8 Hz, C_2-*H*), 4.32 (dd, 1H, J=1.6 Hz, 10.8 Hz, C_2-*H*), 4.7–5.1 (brs, 1H, C_3-*H*), 6.67–7.4 (m, 2H, C_5-*H* and C_6-*H*); m/z 242 (M^++1).

4.3.4. 4-Butanoyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine ***4d***

Yield 90.8% (oil); ^{1}H NMR ($CDCl_3$) δ: 0.95 (t, J=7.6 Hz, 3H, C*H*$_3$CH$_2$CH$_2$CO-), 1.19 (d, J=6.9 Hz, 3H, -C*H*$_3$), 1.62–1.81 (m, 2H, CH$_3$C*H*$_2$CH$_2$CO-), 2.39–2.65 (m, 2H, CH$_3$CH$_2$C*H*$_2$CO-), 4.15 (dd, 1H, J=2.6 Hz, 10.8 Hz, C_2-*H*), 4.32 (dd, 1H, J=1.5 Hz, 10.8 Hz, C_2-*H*), 4.7–5.2 (brs, 1H, C_3-*H*), 6.67–7.4 (m, 2H, C_5-*H* and C_6-*H*); m/z 255 (M^++1).

4.3.5. 4-Benzoyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine ***4e***

Yield 89.8% (crystals); mp 126–127; ^{1}H NMR ($CDCl_3$) δ: 1.32 (d, J=6.9 Hz, 3H, -C*H*$_3$), 4.28 (dd, 1H, J=2.6 Hz, 10.8 Hz, C_2-*H*), 4.34 (dd, 1H, J=1.6 Hz, 10.8 Hz, C_2-*H*), 4.69–4.84 (m, 1H, C_3-*H*), 6.64–6.72 (m, 2H, C_5-*H* and C_6-*H*), 7.31–7.52 (m, 5H, Ar-*H*); m/z 289 (M^+).

4.4. Microbial hydrolysis of ***4a***

Lyophilized microbial cells (DSC 1012, 0.3 g) were suspended in phosphate buffer (1500 ml, 0.1 M, pH 7.0) and stirred for 15 min at 30°C. 4-Acetyl-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine **4a** (1.5 g, 6.6 mmol) was then added and the whole mixture was stirred at 30°C, while the reaction was monitored by HPLC. After an appropriate degree of conversion (0.48) had been reached (120 h), dichloromethane was added (400 ml) and the cells were filtered through Celite. The aqueous layer of the filtrate was extracted with dichloromethane (400 ml×2). The combined organic layers were washed with water, dried, and evaporated in vacuo. The residue was chromatographed on silica. Elution with

toluene:ethyl acetate (20:1) gave (*S*)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-1,4-benzoxazine **2** as a pale brown oil (0.51 g, 42.0%, 99% *ee*). $[\alpha]_D^{25}$ −6.7 (*c*=0.51, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 1.20 (d, J=6.3 Hz, 3H, -C*H*$_3$), 3.42–3.53 (m, 1H, C_3-*H*), 3.79 (dd, 1H, J=8.3 Hz, 10.5 Hz, C_2-*H*), 4.28 (dd, 1H, J=2.6 Hz, 10.5 Hz, C_2-*H*), 6.18–6.62 (m, 2H, C_5-*H* and C_6-*H*); m/z 185 (M^+); IR (neat) 3386, 1612, 1513 and 1165 cm^{-1}. Elution with toluene:ethyl acetate (10:1) gave (*R*)-4-acetyl-7,8-difluoro-2,3-dihydro-3-methyl-1,4-benzoxazine **5a** (0.69 g, 46.0%, 92% *ee*). $[\alpha]_D^{25}$ −108.1 (*c*=0.59, $CHCl_3$). The ^{1}H NMR and IR spectra of compound **5a** obtained here were identical to those of compound **4a**.[7]

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