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An improved process for the preparation of (*S,S*)-2, 8-diazabicyclo [4,3,0] nonane, a key intermediate of moxifloxacin hydrochloride

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ABSTRACT

This contribution highlights the improved process for the preparation of high pure (*S, S*)-2, 8-diazabicyclo [4,3,0] nonane, a valuable intermediate of Moxifloxacin hydrochloride. A robust process was described for resolution step using water as solvent and systematically studied the impact of factors such as water quantity, temperature and mole ratio of resolving agent that influence the resolution. The racemisation process of the undesired isomer using acid is provided. Purification conditions were optimized for the amide reduction step by screening the effect of solvent washings at different pH values. The final debenzylation conditions were altered to reduce the time cycle and reaction temperature which minimize the impurity formation.

KEYWORDS

Resolution;
Racemisation;
Optical rotation;
Reduction;
Debenzylation.

INTRODUCTION

The fluoroquinolones have become widely used antibacterial agents in the treatment of ocular infections, with topical, intravitreal and systemic routes of administration being used. In general, fluoroquinolones have good activity against gram-negative and gram-positive bacteria[1]. Moxifloxacin hydrochloride (Avelox, Figure 1) is a recently-developed fluoroquinolone that has a broad spectrum of antimicrobial activity, including typical respiratory pathogens, atypical and intracellular respiratory pathogens, gram-negative pathogens and many anaerobes. This spectrum of activity makes moxifloxacin particularly suitable for the therapy of community-acquired respiratory tract infections. It also has enhanced activity against specific bacteria, such as Mycobacteria spp. and Legionella[2]. Moxifloxacin terminates bacterial growth by binding to DNA gyrase (topoisomerase **(2)**) and topoisomerase **(4)**, essential bacterial enzymes involved in the replication, translation, repair and recombination of deoxyribonucleic acid. Affinity for both enzymes improves potency and reduces the probability of selecting resistant bacterial subpopulations[3]. Moxifloxacin, a new antibiotic designed to treat community-acquired respiratory tract infections[4]. Moxifloxacin showed better efficacy than ciprofloxacin against *S. maltophilia* under conditions simulating human pharmacokinetics of the two compounds[5].

(*S, S*)-2, 8-diazabicyclo [4,3,0] nonane **(1)** is the key intermediate for the preparation of Moxifloxacin

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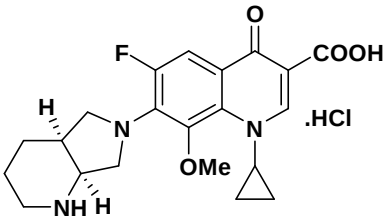


Figure 1

hydrochloride. The discovery synthesis[6] of 2,8-diazabicyclo [4,3,0] nonane in the literature (Scheme 1) involves the reduction of 6-benzyl-pyrrolo [3,4-b] pyridine-5,7-dione **(2)** using H_2/Ru-C or Pd/C to give cis-8-benzyl-7,9-dioxo-2,8-diazabicyclo[4,3,0]nonane **(3)** followed by reduction with $LiAlH_4$ or $NaBH_4$/$BF_3.(C_2H_5)_2O$ to afford 6-benzyl-octahydro-pyrrolo[4,3,0] pyridine **(4)**. The debenzylation of compound **(4)** using H_2/Pd provided 2, 8-diazabic [4,3,0] nonane **(1)** in racemic form.

There are two synthetic pathways reported previously[7] for the preparation of compound **(1)** as single enantiomer as shown in Scheme 2 and 3. The resolution was performed on compound **(3)** in Scheme 2 where as the resolution was done on compound **(4)** in Scheme 3. As it is advantageous to resolve a compound into single enatiomer during the initial stages of the process to obtain high throughput in the synthesis, we have chosen the Scheme 2 for the optimization studies.

The resolution step reported in the precedent literature was performed using D (-)-tartaric acid as resolving agent in mixture of ethanol and acetonitrile to obtain impure diasteriomeric tartrate salt. This was further resolved using D (-)-tartaric acid followed by purification in ethanol and ethylene glycol monomethyl ether mixture to afford pure diasteriomeric tartrate salt. It was treated with sodium bicarbonate in water and extracted into dichloromethane followed by evaporation to afford free amine compound **(5)** having the enatiomeric purity of 96.6%. The yield for the resolution step was 22% and the overall yield for the preparation of compound **(1)** from compound **(3)** was 19% only.

An alternate route was disclosed in the literature[8] in which 2,3-pyridine dicarboxylate was used as starting material. This route involves protection deprotection twice which increases the number of steps and affects the overall yield of the product. It also involves the use of $LiALH_4$ and NaH reagents which are hazardous and economically less viable on commercial scale.

An improved process has been reported recently[9] which comprises of the preparation of compound **(1)** starting from 2,3-dimethyl pyridine. This process involves the preparation of tosyl amide derivatives using alkoxide as base and the resolution using (*S*)-(+)-mandelic acid in ethanol medium. This route suffers from the draw backs like more number of steps, expensive reagents which makes the overall process less viable.

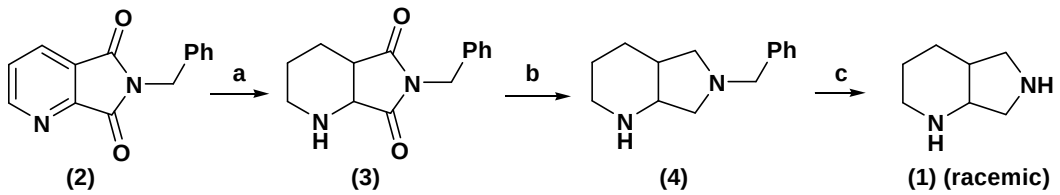
As per the another process disclosed in the patent[10], the resolution of compound **(3)** was done using (-)-2,3:4,6-di-O-isopropyledene-2-keto-L-gulonic acid in methyl ethyl ketone and another procedure described in the same patent uses (1*S*)-camphor sulphonic acid as resolving agent in acetone medium. These processes suffer from the disadvantages like high cost of resolving reagents, solvents and low yields.

There are alternate methods for the enrichment of the required enatiomer using simulated moving bed (SMB) column chromatography[11] and chiral HPLC[12] which are not preferable in commercial aspects.

Hence there is a continuous need for developing an improved, commercially viable and eco friendly process for the resolution step. We explored the use of D (-)-tartaric acid as resolving reagent using water as medium to make the process economic and less hazard to the environment.

Another objective of our process improvement is the racemisation of the undesired isomer which is obtained during the resolution step. The racemisation process reported in the literature[14] involves alkoxide as base in solvent mediums like tetrahydrofuran and toluene as solvents. We have developed an efficient racemisation process using sulfuric acid without using any solvent medium. The racemisation process has the advantage that it goes to completion and the formation of trans compound is virtually not observed. It can be carried out very effectively in technically simple manner and the racemate can be recycled in to the preparation of compound **(5)**. Moreover, the procedure according to the invention requires only simple chemicals and no particular precautions.

The reduction of the two amide bonds of compound **(3)** in Scheme 2 and 3 was affected by using $LiAlH_4$ or $NaBH_4/BF_3.(C_2H_5)_2O$ which is not economical and viable on commercial scale. As per recent method re-



Reagents and conditions: (a) H_2/Ru-C or Pd/C, (b) $LiAlH_4$, or $NaBH_4$ & $BF_3.(C_2H_5)_2O$, (c) H_2, Pd/C

Scheme 1

ported in the literature[13], sodium-bis-(2-methoxyethoxy) aluminum hydride in toluene which is commercially known as Vitride was employed as reducing agent for the amide bond reduction in Scheme 3. In this synthetic approach, as resolution was performed on compound **(4)** after the reduction with Vitride, the impurities carried over from the Vitride stage will be removed during the resolution with D(-)-tartaric acid and it does not require any further purification, but yield of the product will be effected.

We have employed Vitride as reducing agent in Scheme 4 in place of LAH or $NaBH_4/BF_3.(C_2H_5)_2O$ to make the process economic and feasible on commercial scale. We have developed the robust work up process for the efficient removal of impurities by giving solvent washings at different pH values to the product which is dissolved in basic aqueous medium.

We have modified the debenzylation conditions by using Pd/C and formic acid as hydrogen source instead of previously reported conditions of H_2/Pd over carbon to reduce the cycle time of the reaction and to avoid the higher temperature for the completion of the reaction. The product **(1)** obtained in this process was having superior quality with respective to the GC purity as well as the enantiomeric purity.

EXPERIMENTAL

General methods

The ^{1}H-NMR spectral data was recorded at 300 MHz on Varian, the chemical shifts were reported in δ ppm relative to TMS as an internal standard. Optical rotations were recorded on Perkin-Elmer 341 model polarimeter. The mass analysis was performed on Agilent 1100 Q-trap LC-MS/MS mass spectrometer. Solvent removal was accomplished by rotary evaporator operating at house vacuum (40-50 Torr). The solvents and reagents were used without further purification.

Resolution of cis-8-benzyl-7,9-dioxo-2,8-diazabicyclo[4,3,0]nonane (3)

To a suspension of D(-)-tartaric acid (160 g, 1.066 moles) in water(3200 mL) was added cis-8-benzyl-7,9-dioxo-2,8-diazabicyclo[4,3,0]nonane **(3)** (400 g, 1.64 moles) at 25-30°C and slowly heated to 45°C. After attaining the temperature of 45°C, the reaction mixture was stirred for 30-45 minutes at same temperature. The contents were cooled to 30-35°C during 30 minutes followed by cooling to 20-25°C in 30 minutes. The reaction mixture is further cooled to 12-15°C in 45 minutes and maintained for 30 minutes at the same temperature. The compound was filtered, washed with water (160 mL) and dried at 60°C for 6 hours to afford tartrate salt of compound 3 as an off-white crystalline solid. Yield 258.5g (40%); $[\alpha]^{23}_D$ = -58.5 (c 0.5, 1N HCl).

The diasteriomeric tartrate salt 5 (250g, 0.634 moles) was suspended in water (250 mL) and toluene (250 mL) mixture and the pH of the reaction mass was adjusted to 8.0-8.5 using 25% aqueous sodium carbonate solution. The organic phase was separated and the aqueous layer was further extracted with toluene (3×200 mL). The combined organic layer was subjected to distillation to afford (*1S, 6R*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane **(5)** as light yellow liquid. Yield 151.7g (98%), purity by GC 99.5%, enantiomeric purity by GC 99.1% (the product ee was determined by GC after derivatisation with menthyl chloroformate); MS (DIP) 245 (M+1, 100%); ^{1}H NMR (300 MHz, DMSO) δ 7.22-7.35 (m, 4H), 4.55(s, 2H), 3.91 (d, 1H), 2.97 (dd, 1H), 2.53-2.66 (m, 2H), 1.78-1.68 (m, 2H), 1.42-1.28 (m, 2H).

Recovery of the undesired isomer, (*1R, 6S*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane (5)

The filtrate obtained from the above resolution step

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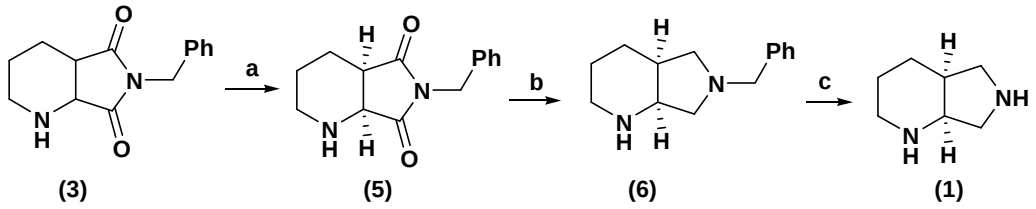
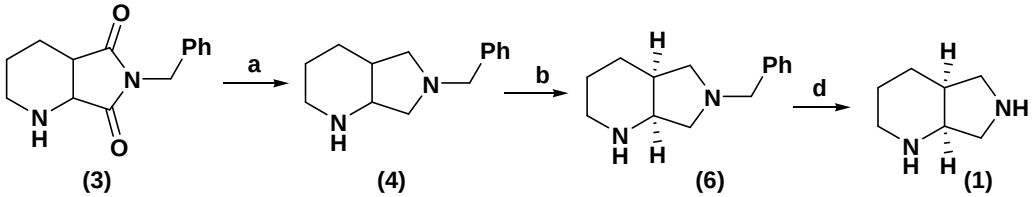


(3) (5) (6) (1)

Reagents and conditions: (a) D (-)-tartaric acid, ethanol, acetonitrile, reflux, (b) $LiAlH_4$, THF, 10% aqueous NaOH solution, (c) H_2, Pd/C, methanol, 90°C

Scheme 2



(3) (4) (6) (1)

Reagents and conditions: (a) $LiAlH_4$, THF, 10% aqueous NaOH, (b) D (-)-tartaric acid, dimethylformamide, methoxy ethanol, 80°C, 45% aqueous NaOH, (c) H_2, Pd/C, methanol, 90°C

Scheme 3

(~3300 mL) was concentrated under vacuum to about 60% at below 55°C. The reaction mass was cooled to 25-30°C, the pH was adjusted to 4.0-4.5 with 10% aqueous NaOH solution and the contents were stirred for 2-3 hours at same temperature. The separated solid was filtered and washed with water (100 mL) and dried at 70°C to afford the (*1R, 6S*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane 8 as off-white solid. Yield 186g; enantiomeric purity (%) by GC 92.3: 7.7(*1R, 6S*: *1S, 6R*).

Racemisation of (*1R, 6S*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4, 3, 0] nonane (5)

A suspension (*1R, 6S*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane **(8)** (180 g, 0.737 moles) in sulfuric acid (90g, 0.918 moles) was heated to 85-90°C for 6-7 hours. The reaction mixture was cooled to 70°C and added toluene(756 mL) and it was further cooled to 25-30°C and adjusted the pH of the reaction mass to 8.0-8.5 using 25% sodium carbonate solution slowly. The contents were stirred for 10-15 minutes and organic phase was separated. The aqueous layer was again extracted with toluene (450 mL) and the combined organic layer was distilled off completely under reduced pressure to afford Cis-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane **(3)** as off-white solid. Yield 165.6g (92%), enatiomeric purity by GC (isomers ratio 50.01: 49.98).

Reduction of (*1S, 6R*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane (5)

To the stirred solution of Vitride (575 mL, 1.845 moles) in toluene (1200 mL) was added a solution (*1S, 6R*)-8-benzyl-7, 9-dioxo-2, 8-diazabicyclo [4,3,0] nonane **(5)** (150g, 0.615 moles) in toluene (300 mL) at 25-30°C during 2.5-3.0 hours under N_2 atmosphere. The reaction mixture was maintained for 5-6 hours at 25-30°C. Once the completion of the reaction was confirmed by monitoring Thin Layer Chromatography, the contents were cooled to 0.5°C. A solution of sodium potassium tartrate (150 g) in water (675 mL) was added slowly to the reaction mass at same temperature. The contents were allowed to reach 25-30°C and stirred for 15 minutes. The organic layer was separated and the aqueous layer was further extracted with toluene (3×200 mL). Water (525 mL) was added to the combined organic layer and adjusted pH to 2.0-2.5 with aqueous HCl (32%) and stirred for 15 minutes at 25-30°C. The organic phase was separated and discarded. The pH of the aqueous layer was adjusted to 5.0-5.5 using aqueous HCl (32%) and washed with toluene (2×200 mL). The aqueous layer was separated, the pH was adjusted to 12-13 using aqueous NaOH solution (40%) and extracted with toluene (3×225 mL). The combined organic phase was subjected to distillation under reduced pressure to afford compound **(6)** as brown liq-

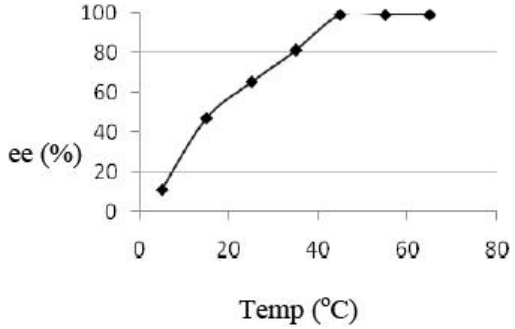


Figure 2 : Influence of temperature on resolution of compound (3)

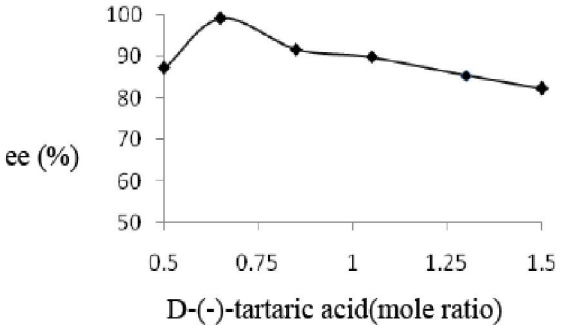


Figure 3 : Influence of D (-)-tartaric acid mole ratio on resolution of compound (3)

uid. Yield 115.6g (87.1%); Purity by GC 98.5%.

Debenzylation of (*S, S*)-8-benzyl-2, 8-diazabicyclo [4,3,0] nonane (6)

To a solution of compound 6 (110 g) in methanol (605 mL) was added 5% palladium over carbon (33g) and stirred for 10-15 minutes. To the reaction mass, formic acid was added slowly during 30-45 minutes at 35°C. The contents were slowly heated to 45°C and stirred for 3-4 hours at same temperature. The completion of the reaction can be monitored by Thin Layer Chromatography. The reaction mass was cooled to 10-15°C and the pH was adjusted to 7.5-8.0 using methanolic ammonia (~17%). The solvent was distilled off completely under reduced process followed by the fractional distillation to afford (S, S)-2, 8-Diazabicyclo [4,3,0] nonane **(1)** as color less liquid. Yield 60.6g (94.5%); Purity by GC 99.5%; enantiomeric purity by GC 99.6% (the product ee was checked by GC after derivatisation with Mosher's reagent); $[\alpha]^{23}_{D}$ = -2.5(undiluted); MS (DIP) 127 (M+1, 100%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.13 (1H,t), 2.93 (4H, m), 2.74 (1H, d), 2.57 (1H, td), 2.49 (1H, m), 2.21 (2H, br), 1.62 (2H, m), 1.51 (1H,m), 1.40 (1H, m).

RESULTS AND DISCUSSIONS

Resolution step

We explored the possibility of resolving the compound **(3)** using the D (-)-tartaric acid in water medium. We have systematically investigated on key parameters that influence the resolution of compound **(3)**. We have examined the impact of the parameters such as water quantity, temperature of the resolution, the mole ratio of the D (-)-tartaric acid and the product filtration temperature on the enatiomeric purity and yield of the product.

A systematic investigation on the influence of water quantity in the resolution of compound **(3)** demonstrated that the best results can be obtained with respect to yield and enatiomeric purity when 8.0 volumes of water were used (TABLE 1).

The reaction temperature also played a pivotal role in the chiral discrimination (Figure 2). The best selectivity (>99%) was obtained at 45°C. Interestingly, an increase in the temperature above 45°C did not affect the enantiomeric excess of the product.

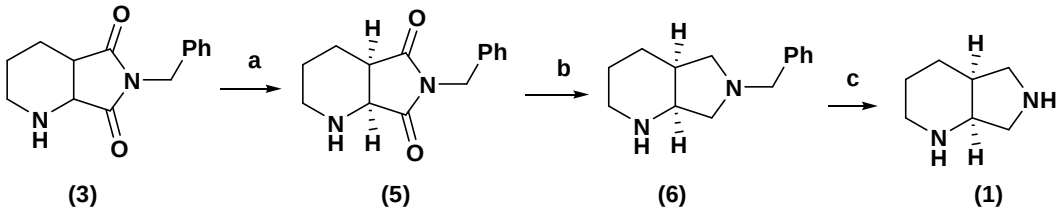
We have also extended our studies towards the effect of mole ratio of the resolving agent, D (-)-tartaric acid. The graphical diagram figure 3 illustrates how the resolution of compound **(3)** was influenced by altering the mole ratio of D (-)-tartaric acid. The optimal mole ratio of D (-)-tartaric acid was found to be 0.65.

For this resolution step, the crystallization temperature also plays a significant role. The studies were conducted by crystallizing the product at different temperatures. TABLE 2 explains the influence of crystallization temperature on the yield and enatiomeric purity of the product during the resolution process. It indicates that the ideal temperature for the crystallization is 12-15°C.

Racemisation of undesired isomer (8)

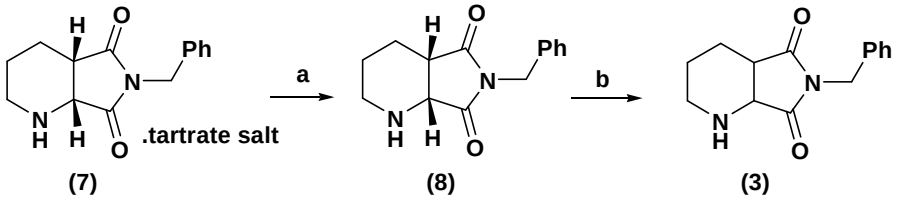
There is a synthetic approach documented in the literature[13] for the racemisation of the compound **(8)** which is recovered in the resolution step. The reported literature provides the racemisation process using the alkoxide base in solvent mediums like toluene and tetrahydrofuran which is less commercially viable. We have explored the possibility of using acid for racemisation of the undesired isomer **(8)**.

To isolate the (*R,S)*-isomer **(8)** (Scheme 5), typi-



Reagents and conditions: (a) D (-)-tartaric acid, water, 45°C, aqueous Na_2CO_3, (b) Vitride, toluene, 10% aqueous NaOH, (c) HCOOH, Pd/C, methanol, 35°C

Scheme 4



Reagents and conditions: (a) 10% aqueous NaOH, (b) H_2SO_4, toluene, 25% aqueous Na_2CO_3

Scheme 5

cally the mother liquors obtained in the resolution step were concentrated to upto 60% of the volume and neutralized with base to pH of 5-7 to liberate the tartaric acid. The isolated product contained approximately 5-10% of the (*S, R*)-isomer and 90-95% of (*R, S*)-isomer. This product was heated with sulphuric acid at 110°C for 16 h, cooled the reaction mass, diluted with water and neutralized with base. The subsequent extraction with toluene followed by distillation affords the racemic compound **(3)** in 92 % yield.

Before we arrived at sulfuric acid as preferred acid for racemisation, a number of acids were screened as shown in TABLE 3.

Amide bonds reduction

There are some procedures in the literature[14] for the reduction of amide bonds using Vitride/Red-Al, these were performed on compound **(3)** before resolution. There is no literature precedence for the reduction of resolved compound **(5)** using Vitride. During this transformation, impurities formation was observed. We studied the impact of solvent washings at different pH values to the crude product dissolved in aqueous medium and observed significant reduction in impurities by the washings at pH values 2.0-2.5 and 5.0-5.5 (TABLE 4). The yield loss of the product was more by washing at higher pH values. Based on results observed during this investigative study, the washings at pH 2.0-2.5 and 5.0-5.5 were incorporated in the process. The cumulative effect of these washings afforded the product with the purity of 98.5%.

Debenzylation step

The discovery route involved the debenzylation of compound **(6)** by hydrogenation using palladium over charcoal in alcohol medium. The reaction was taking several hours for completion of the transformation and high temperature was required in these conditions. We have optimized the process for debenzylation using formic acid as hydrogen source and methanol as medium in the presence 5% Pd/C. The reaction was completed within 4 hours by maintaining at 40-45°C. The catalyst was suctioned off and the filtrate was neutralized with triethylamine, evaporated by distillation followed by fractional distillation to afford high pure compound of **(1)**. This alteration in the debenzylation conditions has resulted in the reduction of the time cycle, temperature of the reaction and thus afforded product is having high purity by GC as well as enantiomeric purity.

The major advantages of our improved process (Scheme 4 and 5) are, i) the use of water as solvent for the resolution step which makes the process cost effective and environment friendly, ii) a simple and efficient racemisation process for the undesired isomer produced in the resolution step, iii) the reduction of amide using Vitride and insitu purification method to

TABLE 1 : Influence of water quantity on resolution of (3)

Volumes of the water	Yield (%)	Enatiomeric purity (ee %)
3.0	50	56
5.0	46	72%
6.5	43	89%
8.0	40	99%
9.5	33	99%
11.0	27	99%

TABLE 2 : Influence of crystallization temperature on resolution of compound (3)

Crystallization temperature(°C)	Yield(%)	Enatiomeric purity(ee %)
35-38	19	99
27-30	24	99
18-22	31	99
12-15	40	99
5-7	45	91

TABLE 3 : Racemisation of compound (8) using different acids

Acid used for racemisation	Chiral purity by GC S, R isomer: R,S isomer
Acetic acid	15.7 : 84.2
Trifluoroacetic acid	25.2 : 74.7
Sulphuric acid	50.01 : 49.99
Para toluene sulfonic acid*	37.57 : 62.42
Ortho phosphoric acid	45.21 : 54.78
Polyphosphoric acid	47.57 : 52.42
Methane sulphonic acid	41.9 : 58.09

***Toluene was used as solvent where as the remaining experiments were performed under neat conditions**

TABLE 4 : Purification of compound (6) by giving solvent washings at different pH values

pH	Purity by GC (%)
1.0-1.5	85.7
2.0-2.5	96.5
3.0-3.5	88.2
4.0-4.5	92.3
5.0-5.5	97.8
6.0-6.5	93.4
7.0-7.5	90.1

afford the product with high purity, iv) an advantageous debenzylation process which makes the process ecofriendly, v) the overall yield of the process is 32% which makes the whole process superior over the previously reported process (Scheme 2, the overall yield was 19% only).

CONCLUSION

We have extensively examined the effects of various factors that influence the resolution of compound **(3)** and developed a cost effective process. We have developed a robust and superior method of racemization of compound **(8)** and optimized the purification conditions for the amide reduction step to afford high pure compound **(6)**. The debenzylation conditions were modified to furnish the compound **(1)** with high yield and quality. This protocol has made the process of manufacturing compound **(1)** more efficient.

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REFERENCES

[1] A.Smith, S.B.Kaye; Drugs, **61(6)**, 747 **(2001)**.
[2] Juan-Pablo, Caeiro, B.L.Paul; Expert Rev.Anti Infect.Ther., **1(3)**, 363 **(2003)**.
[3] M.Dalene; Clin.Ophthalmol., **2(1)**, 77 **(2008)**.
[4] Ch.Nightingale; Pharmacotherapy, **20(3)**, 245 **(2000)**.
[5] B.Boubakar, F.Hala, A.Corinne, S.Marie-Claude, Q.Claudine; Antimicrob Agents Chemother., **48(3)**, 946 **(2004)**.
[6] P.Uwe, S.Thomas, K.Andreas, G.Klaus, S.Michael, H.Ingo, G.M.Karl, E.Rainer, Z.Hans-Joachim; US 4990517, **(1991)**.
[7] P.Uwe, S.Wilfred, H.Dieter, K.Andreas, S.Thomas, P.Thomas, G.Klaus, E.Rainer, B.Klaus-Dieter, M.Karl-Georg; US 5480879, **(1996)**.
[8] K.Wan Joo, P.Tae Ho, K.Moon Hwan, K.Bong Jin; US 5770597, **(1998)**.
[9] W.Zheqing, F.Shushan, C.Yongzhi; US 2008/0221329 A1, **(2008)**.
[10] F.Peter; US 6566523 B1, **(2003)**.
[11] D.Herbert, K.Andreas, K.Elvira, L.Walter, P.Hanns-Ingolf, S.Dietrich, G.Rolf, R.Tobias; US 2007/0213526 A1, **(2007)**.
[12] T.Radhakrishna, D.Sreenivas Rao, K.Vyas, G.Om Reddy; Journal of Pharmaceutical and Biomedical Analysis, **22(4)**, 691 **(2000)**.
[13] D.Herbert, M.Georg, J.Wilfried, K.Andreas, W.Joachim; US 6392044 B1, **(2002)**.
[14] A.Ramakrishnan, S.Bhawsar, V.Narayana; WO 2009/125425 A2, **(2009)**.