

Synthesis of 3-Deoxyclarithromycin through Biotransformation of Clarithromycin by *Aspergillus niger* and its Antibacterial Activity

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Received on July 25, 2009

Accepted on March 18, 2010

Abstract

Biotransformation of clarithromycin (**1**), by the plant pathogen *Aspergillus niger* ATCC 10549, produced one main metabolite identified as 3-deoxyclarithromycin (**2**). Testing the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* showed that compound **2** has a moderate activity against the tested bacteria. This result provides further evidence that the presence of oxygen at position 3 in clarithromycin enhances the antibacterial activity. The structure of compound **2** was elucidated by different NMR techniques. 2D-NMR (COSY, HMBC and HMQC) experiments allowed full assignments of the proton and carbon chemical shifts.

Keywords: Clarithromycin; 3-Deoxyclarithromycin; Biotransformation; *Aspergillus niger*; Antibacterial activity.

Introduction

Clarithromycin (**1**), 6-O-methylerythromycin A, is a semi-synthetic derivative of the natural macrolide antibiotic erythromycin A. Methylation of the 6-hydroxy group of erythromycin A improved the antibacterial properties of the parent compound^[1-4] and markedly increased the acid stability, oral bioavailability and reduced gastrointestinal side effects of erythromycin^[5].

The development of bacterial resistance to existing antibacterial agents, has urged scientists to develop new antibacterial compounds. Many clarithromycin derivatives have been synthesized and the structure-activity relationships were described^[6, 7].

Microbial transformation of macrolides can be used to modify the structure of clarithromycin, and it may produce potent antibacterial compounds that are difficult to prepare otherwise. In addition, this method provides a low cost, one step way of preparing macrolide derivatives in a high stereo- and regio-selective approach and it may produce many derivatives that can be used to study the structural-activity relationships. In spite of these advantages, there is limited number of reports demonstrating the use of microorganisms to synthesize macrolides derivatives. Adachi *et al* used the plant pathogen fungi *Mucor circinelloides*. The study resulted in a selective mono-hydroxylation at C-14, C-15, and C-16^[8, 9]. N-demethylclarithromycin

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and 15-membered translactonization products were also isolated using the same fungi^[10].

In the present paper we describe the microbial transformation of clarithromycin (**1**) by the plant pathogen *Aspergillus niger*, aiming at exploring this technique as a one step method to prepare derivatives of clarithromycin (**1**). The antibacterial activity of the isolated metabolites was examined.

Experimental

General methods

Clarithromycin (**1**) was obtained from Hikma Pharmaceutical. Silica gel (70 - 230 mesh, Merck) was used for column chromatography (CC). Silica gel pre-coated plates (Merck, PF254; 20X20, 0.25mm and 0.50mm) were used for TLC. Spots were detected by spraying with anisaldehyde/ sulfuric acid spray reagent followed by heating to 140 °C. ¹H-, ¹³C- and 2D-NMR Spectra (CDCl₃ solutions) were recorded using Bruker Avance-300 or Bruker Avance-500 spectrophotometers. Chemical shifts (δ) are reported in ppm with TMS as an internal standard, and coupling constants (J) in Hz. EIMS were recorded on Jeol JMS-600H mass spectrometer. All reagents used were of analytical grade.

Incubation of clarithromycin (**1**)

Cultures of *A. niger* were obtained from the American Type Culture Collection (ATCC 10549). All cultures were grown on Sabouraud dextrose agar (SDA) and stored at 4 °C.

A. niger was grown in shake cultures at 30 °C in two conical flasks (250), each containing 100 mL of a sterile medium comprising of 2.25g MgSO₄.7H₂O, 2.25g KCl, 13.50g NaNO₃, 4.50g HK₂PO₄, 0.09g FeSO₄.7H₂O, 120.00g D-(+)-glucose in 4.5 liter distilled water at pH=6.50. The media was sterilized by autoclaving at 121 °C for 16 min. Incubations were initiated by suspending the surface growth from slants in sterile medium. After four days of incubation in the above described medium, clarithromycin (**1**) (0.05 g), dissolved in 2 mL of acetone, was equally distributed in the two conical flasks containing the media. Culture control blank contained sterile medium and microorganism without the substrate, and a substrate control blank consisted of sterile medium containing the same amount of substrate without the microorganism were incubated under the same conditions. The culture broth was sampled by chloroform extraction and examined by TLC every 24 hrs to monitor the progress of biotransformation. The transformation of clarithromycin (**1**) was almost complete after 4 days.

The experiment was repeated under the same conditions using 40 conical flasks and 2.04 g clarithromycin (**1**) dissolved in 38 mL acetone. After 4 days, the fermentation products were filtered and extracted three times with chloroform. The residual mycelia were washed with methanol. The combined chloroform and methanol

solutions were dried over Na₂CO₃ and evaporated under vacuum to yield 3.4 g of a gummy mixture.

Isolation of metabolite (2)

The mixture was chromatographed on a silica gel column, packed in chloroform. The polarity of the eluent was increased gradually by increasing the percentage of methanol. 3-deoxyclarithromycin (**2**) was eluted from column by 85% CHCl₃/ MeOH and purified on TLC plates using 95% CHCl₃/ MeOH as mobile phase.

Identification of 3-deoxyclarithromycin (2)

The ¹H-NMR and ¹³C-NMR data of compounds **1** and **2** are summarized in table 1. 2D experiments (COSY, HMBC and HMQC) were used to assign the chemical shifts of compounds **1** and **2**.

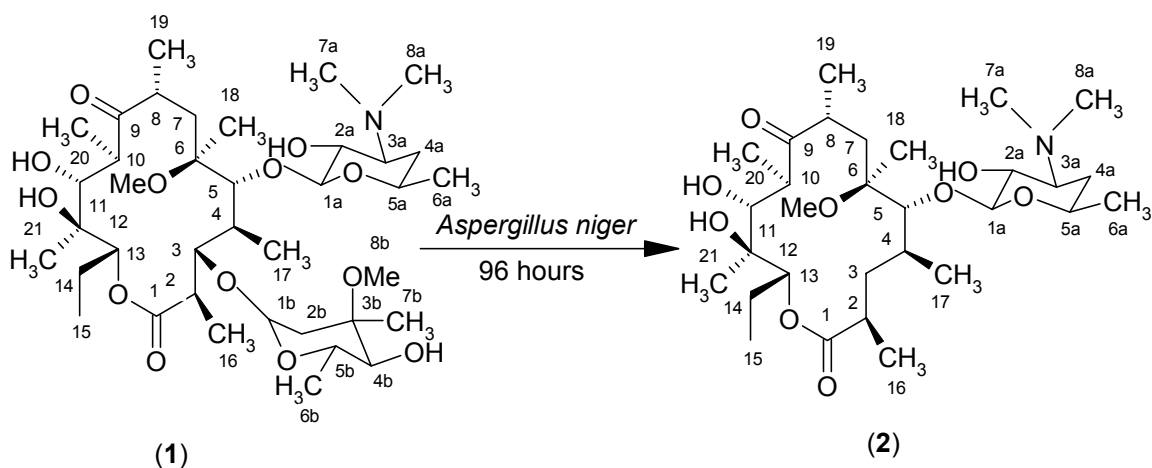
Antibacterial activity

The minimal inhibitory concentration (MIC) of 3-dexyclarithromycin (**2**) and clarithromycin (**1**) were determined according to NCCLS guidelines ^[11]. Two fold serial micro dilution method using Muller- Hinton broth was performed. The standard inocula of *Staphylococcus aureus* and *Escherichia coli* were prepared by direct suspension in and adjusted with sterile saline until the turbidity matched 0.5 McFarland standards. Each suspension was further diluted in sterile broth to obtain a final inoculum of approximately 5×10⁵ CFU/ mL. The exact inoculum size was determined via colony counts. MICs were read after 24 hrs incubation of 96-micoplate at 37 °C. The MIC was defined as the lowest concentration at which there was no visible growth

Results and discussion

Clarithromycin (**1**) is a wide spectrum macrolide antibiotic, obtained by introducing a hydroxy group at C-6 of erythromycin. This modification on the structure increased the stability and the potency of clarithromycin compared to erythromycin. In a search of new antibiotics with better pharmacokinetic properties and potency, different derivatives of compound **1** were prepared using different organic reagents. Only few reports employed microorganisms to prepare modified derivatives of calrithromycin (**1**), although this method proved to be highly regio- and stereoselective. As part of our ongoing research on discovering new reactions and new microorganisms, we have investigated biotransformation of clarithromycin (**1**) by using the plant pathogenic fungus *Aspergillus niger*. We have succeeded in preparing 3-deoxyclarithromycin **2** by a one step microbial transformation.

The microbial transformation of a macrolide antibiotic, clarithromycin (**1**), was carried out using *A. niger*. The fungi catalyzed a specific hydrolysis of cladinose sugar of **1** to produce 3-deoxyclarithromycin (**2**) as the main metabolite (scheme 1).

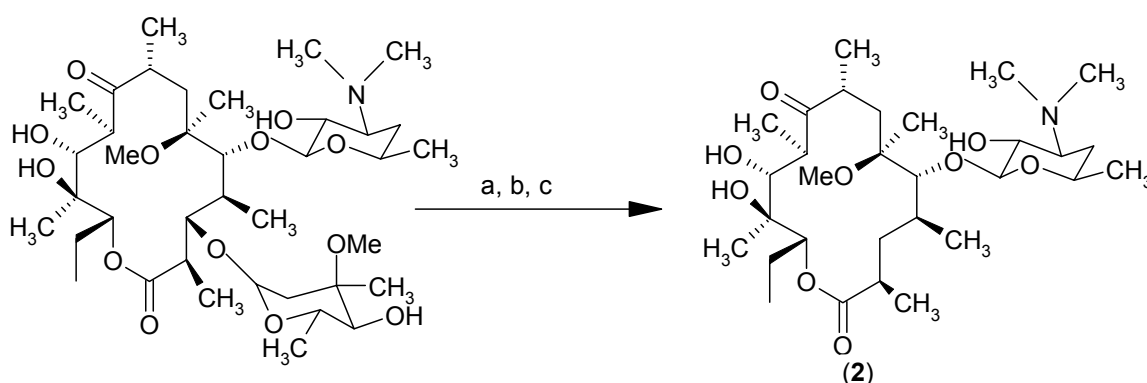


Scheme 1: Biotransformation of clarithromycin (**1**) by *A. niger*

Compound **2** was first detected by TLC plates in cultures broth incubated for 48 hrs. When fermentation period reached 96 hrs, the conversion was almost complete. Compound **2** was isolated by CC and purified on TLC to give 1.20 g of a white solid. The EIMS of **2** showed a molecular ion peak at m/z 621, the odd number indicates that the sugar moiety with the nitrogen is still there, the molar mass is 190 units less than that of clarithromycin (**1**), suggesting the loss of the cladinose unit. The ^1H -NMR spectrum of compound **2** revealed the presence of only one anomeric protons at δ 4.52 (1H, d, $J=7.2$ Hz), suggesting a hydrolysis of one of the clarithromycin sugars. The presence of only one methoxy group resonating at δ 2.98 (3H, s) and two N,N-dimethyl at δ 2.50 (6H, s) suggested that the cladinose sugar was removed. In addition the absence of the signal at δ 3.81 for the proton connected to the carbon at δ 77.9 suggested a complete reduction of C-3 to the corresponding methylene. A further evidence of the structure was obtained from comparing the carbon and DEPT spectra of compounds **1** and **2**. There were 30 carbon signals in **2**, which consisted of 4 quaternary carbons, 11 methines, 4 methylenes and 11 methyls, indicating that the number of carbon atoms is less by 8 than the parent clarithromycin (**1**). The entire signals corresponding to the cladinose unit were absent, whereas all the other signals corresponding to the 13 membered macrolide connected with desosaminyl sugar unit were present, with the exception of the disappearance of the methine signal at δ 77.9 and the appearance of a new methylene at δ 29.7 assigned to C-3. The presence of a methylene at C-3 was confirmed by 2D-NMR. The COSY experiment showed a correlation between the proton at δ 1.22 with the protons at δ 1.85, 2.60 and 2.15. HMQC showed that the two protons at δ 1.22 and 1.85 are attached to the carbon at δ 29.7 assigned to C-3, while the protons at δ 2.60 and 2.15 are attached to carbon atoms resonating at δ 45.6 and 37.6 corresponding to C-2 and C-4, respectively. An import long range correlation was obtained from HMBC between C-1 (δ 175.1) and the

proton at δ 1.85 providing a conclusive evidence for the structure of compound **2**. Full assignment of ^1H - and ^{13}C NMR signals are reported for the first time for compound **2** based on 2D COSY, HMQC and HMBC and they are summarized in table 1.

In addition, this is the first report of preparing 3-deoxyclarithromycin (**2**) by an enzymatic method. Compound (**2**) was first prepared from clarithromycin (**1**) in 1997 by Elliott *et al* [7]. The method included a selective transformation of C-3 hydroxy group of descladinosylclarithromycin to the C-3 xanthate followed by Bu_3SnH / AIBN mediated radical deoxygenation as shown in scheme 2. The procedure involved many steps and a lot of chemicals. Whereas the biotransformation method provides a one-step, environment friendly method at a lower cost.



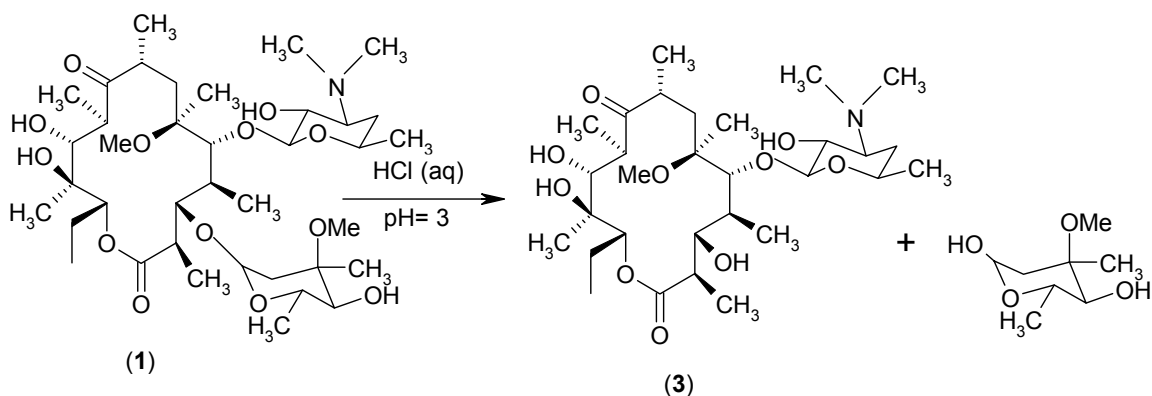
Scheme 2: Synthesis of 3-deoxyclarithromycin (**2**) from clarithromycin (**1**). (a) aq. HCl/ EtOH; (b) NaH (3.0 equiv)/ THF/ $-20\text{ }^\circ\text{C}$; then CS_2 (1 equiv); then MeI (1 equiv); (c) Bu_3SnH / AIBN/ C_6H_6 .

Table 1: ^1H - and ^{13}C -NMR of clarithromycin (**1**) and 3-deoxyclarithromycin (**2**)

No	Multiplicity	Clarithromycin (1)		3-Deoxyclarithromycin (2)	
		^{13}C NMR	^1H NMR (multiplicity, J in Hz)	^{13}C NMR	^1H NMR (multiplicity, J in Hz)
1	C	175.9	-	175.1	-
2	CH	45.2	2.90	45.6	2.60
3	CH	77.9	3.81	29.7 (CH_2)	1.22 , 1.85
4	CH	39.1	1.94	37.6	2.15
5	CH	78.4	3.69 (d, 10.4)	78.9	3.54 (d, J= 10.2)
6	C	78.5	-	75.2	-
7	CH_2	39.3	1.74, 1.86	38.8	1.45, 1.95
8	CH	45.0	2.62	44.5	2.67
9	C	221.1	-	220.8	-
10	CH	37.1	3.02	35.9	3.00
11	CH	70.8	3.77	70.1	3.55
12	C	74.9	-	74.6	-
13	CH	76.6	5.02 (11.0)	76.7	5.18 (d, J= 10.4)

No	Multiplicity	Clarithromycin (1)		3-Deoxyclarithromycin (2)	
		¹³ C NMR	¹ H NMR (multiplicity, J in Hz)	¹³ C NMR	¹ H NMR (multiplicity, J in Hz)
14	CH ₂	21.0	1.46, 1.96	21.5	1.48, 1.95
15	CH ₃	10.6	0.83 (t, 7.3)	10.5	0.80 (t, J= 7.2)
16	CH ₃	15.9	1.20 (d, J=6.0)	15.3	1.25 (d, J= 6.1)
17	CH ₃	9.1	1.08 (d, J=6.2)	8.4	1.12 (d, J= 6.0)
18	CH ₃	19.7	1.41 (s)	18.9	1.35 (s)
19	CH ₃	18.0	1.15	17.8	1.18
20	CH ₃	12.3	1.13	12.7	1.15
21	CH ₃	16.2	1.14 (s)	16.2	1.14 (s)
6-OCH ₃	CH ₃	50.6	3.05 (s)	50.5	2.98 (s)
1a	CH	102.7	4.45 (d, J=7.1)	105.7	4.52 (d, J= 7.2)
2a	CH	69.5	3.39	70.4	3.37
3a	CH	65.8	2.93	65.8	2.99
4a	CH ₂	29.2	1.23, 1.65	29.2	1.22, 1.80
5a	CH	65.5	3.44	69.8	3.60
6a	CH ₃	21.4	1.23	21.2	1.29
7a	CH ₃	40.3	2.52 (s)	40.2	2.50 (s)
8a	CH ₃	40.3	2.52 (s)	40.2	2.50 (s)
1b	CH	96.1	4.95		
2b	CH ₂	34.9	1.60, 2.38		
3b	C	74.2	-		
4b	CH	77.8	3.00		
5b	CH	66.0	4.02		
6b	CH ₃	18.7	1.32 (s)		
7b	CH ₃	21.4	1.25 (s)		
8b	OCH ₃	49.5	3.32 (s)		

It is noteworthy that *A. niger* is known to induce hydroxylation of methylene carbons. As far as we know, this is the first report of the ability of this fungus to reduce hydroxylated carbon into the corresponding methylene. We believe that the reaction may have occurred in many steps; starting by enzymatic acid catalyzed hydrolysis of the cladinose sugar, followed by dehydration and hydrogenation. It is well known that the acid stability of clarithromycin (1) is more than erythromycin A, nevertheless, clarithromycin (1) degrades below pH= 3 at 37 °C to 3-decladinosylclarithromycin (3) as shown in scheme 3^[12]. No degradation products were detected in the substrate control flask, indicating that the reaction is an enzymatic catalyzed reaction. We have tried to isolate compound 3 or any other intermediates by repeating the experiment for shorter periods of time, but we did not succeed to detect any of the expected intermediates.



Scheme 3: Acid hydrolysis of clarithromycin (1)

The *in vitro* antibacterial activity of 3-deoxyclarithromycin (2) and clarithromycin (1) were tested against a gram positive bacterium; *Staphylococcus aureus* and a gram negative *Escherichia coli*. The MICs were determined by serial dilution method and are summarized in table 2. The results showed that compound 2 has a moderate activity and it is less potent than compound 1. This result is in agreement with that reported by Elliott *et al*^[7].

Table 2: Minimum Inhibition Concentration (MIC) of Clarithromycin (1) and 3-Deoxyclarithromycin (2)

Organism	Minimum Inhibition Concentration (MIC) µg/ mL	
	Clarithromycin (1)	3-Deoxyclarithromycin (2)
<i>Staphylococcus aureus</i>	0.25	25
<i>Escherichia coli</i>	0.38	1.5

The result provides further evidence that the caldinose sugar plays an important role in the activity of macrolides antibiotics. Although Rousell-Uclaf demonstrated that 3-ketoclarithromycin analogues showed a potent antibacterial activity^[13, 14], the decrease of antibacterial activity upon removal of C-3 oxygen of clarithromycin was demonstrated by Elliott *et al*^[7].

In summary, biotransformation of clarithromycin (1) by the plant pathogen fungus *A. niger* provides a good method for selective one step 3-deoxygenation of compound 1. However the deoxy product 2 was found to be less potent than clarithromycin (1)

Acknowledgement

The author would like to thank the Deanship of Scientific Research at the University of Jordan for financial support. Thanks are also due to Dr. Mohammad Abu Shhail at Hikma Pharmaceuticals.

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