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Isolation of 3′-*O*-Acetylchloramphenicol: A Possible Intermediate in Chloramphenicol Biosynthesis

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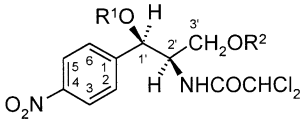
Abstract—3′-*O*-Acetylchloramphenicol, commonly formed from chloramphenicol by resistant bacteria, has been isolated from the antibiotic-producing organism. Biosynthetic experiments suggest that it is a protected intermediate in chloramphenicol biosynthesis, implicating acetylation as a self-resistance mechanism in the producing organism.

Acetylation, adenylation, and phosphorylation are common modifications conferring resistance to antibiotics in pathogenic microorganisms.[1,2] Many bacteria inactivate chloramphenicol (**1**) by acetylation,[3] which prevents the modified antibiotic from binding to prokaryotic ribosomes and inhibiting peptidyl transferase during protein biosynthesis.[4] Chloramphenicol acetyltransferase (CAT, EC 2.3.1.28),[3] catalyzes acetylation of the primary hydroxyl group by acetyl-CoA, yielding 3′-*O*-acetylchloramphenicol (**3**). In a slower, nonenzymatic reaction, the acetyl group migrates to the secondary hydroxyl group. 1′-*O*-Acetylchloramphenicol (**2**) is also a substrate for CAT, forming 1′,3′-*O*-diacetylchloramphenicol (**4**), although the reaction is slower than for chloramphenicol itself. None of the acetylated chloramphenicol derivatives exhibits antibiotic activity, and the action of CAT is the most common mechanism for acquiring resistance to chloramphenicol in bacteria.[3] Although detected in *Streptomyces* species, remarkably, however, CAT has never been reported in a strain producing the antibiotic.[1,4] The present report describes the isolation of 3′-*O*-acetylchloramphenicol from chloramphenicol-producing cultures of *Streptomyces venezuelae*, and experiments to determine the biosynthetic relationship between the antibiotic and its acetylated derivative.

Cultures of *S. venezuelae* strain 13s[5] were grown at 30 °C in glucose–isoleucine–salts solution[6] with aeration on a rotary shaker, and harvested after 7 days by filtration. The culture filtrate was extracted with three half-volumes of ethyl acetate, and the extract after evaporation in vacuo, was assayed by reversed-phase HPLC.[7] Chloramphenicol and an unknown metabolite with a longer retention time were detected. To obtain sufficient material for NMR analysis, the evaporated extract from 500 mL of culture filtrate was redissolved in water and fractionated on a silica C_{18} column (95×0.9 cm, Baker-bond, 40 μm) by gradient elution with methanol–water. The NMR spectra of the second metabolite were similar to those of chloramphenicol except for additional resonances at 172.5 and 20.6 ppm in the ^{13}C spectrum, and at 2.02 ppm (singlet, 3H) in the ^{1}H spectrum, which were consistent with an *O*-acetyl group.



1 $R^1 = H$, $R^2 = H$

2 $R^1 = COCH_3$, $R^2 = H$

3 $R^1 = H$, $R^2 = COCH_3$

4 $R^1 = COCH_3$, $R^2 = COCH_3$

To obtain a precise structural assignment, *O*-acetyl derivatives were prepared[8,9] from chloramphenicol (**1**)

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and acetic anhydride[10] and separated by preparative TLC (silica gel; $CHCl_3/CH_3OH$, 19:1).[9] Extraction of the separated, fluorescence-quenching bands into cold, dry CH_3CN[9] yielded chloramphenicol (**1**, R_f 0.15), 1′-*O*-acetylchloramphenicol (**2**, R_f 0.36), 3′-*O*-acetylchloramphenicol (**3**, R_f 0.45) and 1′,3′-di-*O*-acetylchloramphenicol (**4**, R_f 0.70); no isomerization of **2** or **3** in acetonitrile solution was apparent after several weeks storage at −20 °C. On the other hand, two TLC spots were detected, and by ^{1}H NMR spectroscopy 1:4 and 1:5 mixtures, close to the equilibrium ratio of **2** and **3**,[11] were observed when the separated bands of 1′- and 3′-*O*-acetylchloramphenicol were extracted from silica gel into ethyl acetate.

The NMR spectra[12] of chloramphenicol and 1′,3′-di-*O*-acetylchloramphenicol were consistent with published ^{1}H[13,14] and ^{13}C[15,16] NMR data. The monoacetyl isomers were distinguished in ^{1}H NMR spectra by chemical shift differences for H-2′ and H-3′ (i.e., resonances at 6.0 and 3.5 ppm for 1′-*O*-acetylchloramphenicol[13,17] and at 5.1 and 4.2 ppm for 3′-*O*-acetylchloramphenicol[13]) and by 1-2 ppm downfield shifts of the ^{13}C resonance of C-1′ or C-3′ upon acetylation (Table 1). The position of the acetyl group in **3** was further confirmed by an HMBC experiment that showed long range coupling between the carbon of the acetyl carbonyl and the H-2′,H-3′ spin system. Comparison of the ^{1}H and ^{13}C NMR data[18] with those of the synthetic monoacetylated samples revealed that the second metabolite had been isolated as a mixture of 1′- and 3′-*O*-acetylchloramphenicol. This is consistent with the relative ease with which mono-acetyl chloramphenicols interconvert,[11] but the approximately 1:6 and 1:9 ratios of **2**:**3** found in the product indicate that equilibrium was not established. Since the proportion of **3** in the isolated material exceeded that in the equilibrium mixture, 3′-*O*-acetylchloramphenicol is probably the initial acetylated chloramphenicol formed in the *S. venezuelae* culture, yielding 1′-*O*-acetylchloramphenicol by nonenzymatic isomerization.

To investigate the biosynthesis of the acetyl and dichloroacetyl groups, *S. venezuelae* cultures (500 mL) were supplemented at 72, 96 and 120 h after inoculation with sterile aqueous sodium [2-^{13}C]acetate (3×0.5 mol, 99.7% ^{13}C, MSD Isotopes) or ethyl [2,4-$^{13}C_2$]acetoacetate (3×0.25 mol, 99% ^{13}C, Cambridge Isotope Laboratories). The metabolites were isolated as described above and ^{13}C enrichments were determined by NMR spectroscopy[19] (Table 2). Label from [2-^{13}C]acetate was incorporated into the dichloroacetyl moieties of chloramphenicol and 3′-*O*-acetylchloramphenicol; the ^{13}C enrichment of the dichloromethine carbon was more than 5 times that of the amide carbonyl, supporting previous studies that showed labelling of both carbons from [2-^{14}C]acetate[20] and incorporation of [1,2-$^{13}C_2$]acetate with considerable bond scission.[16] Negligible enrichment of the acetyl group in 3′-*O*-acetylchloramphenicol was observed. Administration of ethyl [2,4-$^{13}C_2$]acetoacetate to *S. venezuelae* yielded complementary labelling results (Table 2); the acetyl group of 3′-*O*-acetylchloramphenicol was highly enriched, whereas the dichloroacetyl moieties of both metabolites were enriched to a much smaller extent.

While a common origin for the dichloroacetyl groups in chloramphenicol and 3′-*O*-acetylchloramphenicol is indicated by parallel labelling (in both experiments) of their dichloromethine and amide carbonyl carbon atoms, the different levels of ^{13}C incorporation into the dichloroacetyl and acetyl components of 3′-*O*-acetylchloramphenicol point to different routes of biosynthesis. Assuming the acetyl group is derived from acetyl–CoA, the labelling results imply that ethyl acetoacetate, which could generate acetyl–CoA from acetoacetate (or its ester) by a retro Claisen condensation without consumption of energy, is more efficient than acetate as a precursor of acetyl–CoA. They also suggest that acetate is not incorporated into the dichloroacetyl unit via acetyl–CoA.

The isotopic results do not define the order in which chloramphenicol and 3′-*O*-acetylchloramphenicol are

Table 1. ^{13}C and ^{1}H NMR data, including observed HMBC correlations, for 1′- and 3′-*O*-acetylchloramphenicol[a,b]

Position	1′-*O*-Acetylchloramphenicol (**2**)		3′-*O*-Acetylchloramphenicol (**3**)		
	δ^{13}C	δ^1H[c]	δ^{13}C	δ^1H	HMBC
1	148.8		150.2		H-3, H-5, H-1′
2	128.7	7.58 (2H)[d]	128.3	7.60 (2H)[d]	H-6, H-1′
3	124.5	8.18 (2H)[d]	124.2	8.16 (2H)[d]	H-2, H-5
4	146.5		148.5		H-2, H-3, H-5, H-6,
5	124.5	8.18 (2H)[d]	124.2	8.16 (2H)[d]	H-3, H-6
6	128.7	7.58 (2H)[d]	128.3	7.60 (2H)[d]	H-2, H-1′
1′	73.8	6.02 (d, J = 5 Hz, 1H)	71.2	5.07 (br s, 1H)	H-2, H-6, H-2′, H-3′
2′	56.5	4.16–4.31 (m, 1H)	55.2	4.15–4.35 (m, 3H)	H-3′
3′	61.4	3.43–3.63 (m, 2H)	64.0	4.15–4.35 (m, 3H)	H-1′
–$CHCl_2$	67.6	6.10 (s, 1H)	67.5	6.04 (s, 1H)	
–C=O (amide)	165.1		165.1		–$CHCl_2$
–CH_3	21.1	2.12 (s, 3H)	21.0	2.01 (s, 3H)	
–C=O (ester)	170.9		171.6		H-2′, H-3′, –CH_3
–NH		7.24 (br d, J = 9 Hz, 1H)		7.11 (br d, J = 8 Hz, 1H)	

[a]CD_3CN, Bruker AMX 400 spectrometer, chemical shifts refer to CHD_2CN (δ_H 1.94 ppm) and CD_3CN (δ_C 1.39 ppm).
[b]Assignments and one-bond C–H connections were determined in JMOD and HETCOR experiments.
[c]250.1 MHz.
[d]Apparent d, 9 Hz splitting.

Table 2. Incorporation of ^{13}C above natural abundance (%)[a]

Substrate	Chloramphenicol (**1**)		3′-*O*-Acetylchloramphenicol (**3**)			
	Cl_2CH–	–C=O	Cl_2CH–	–C=O (amide)	CH_3–	–C=O (ester)
Sodium [2-^{13}C]acetate	4.8 (5.2)[b]	0.6	4.9 (7.1)[b]	0.7	0.2 (0.9)[c]	−0.1
Ethyl [2,4-$^{13}C_2$]acetoacetate	0.5	0.2	0.5	—[d]	16.2 (15.0)[c]	—[d]

[a]The ^1H and ^{13}C NMR spectra of samples in CD_3OD were acquired on a Bruker AMX-400 spectrometer. A 3.7 μs 30° pulse with an acquisition time of 3.4 s and a 1-s delay were used to collect the ^1H NMR spectra. The ^{13}C NMR spectra (800 or 1000 scans) were acquired using 64 K data points in a 30 kHz spectral window, a 7.25 μs 90° pulse, a 1.08-s acquisition time, and a 4-s delay. A line broadening of 2 Hz was used to process the free-induction-decay data. Spectra for the natural-abundance reference and ^{13}C-enriched samples were acquired under identical conditions. The percentage ^{13}C enrichment was calculated[19] for individual carbons of the acetyl and dichloroacetyl units by normalizing peak intensities to the average peak intensity of the phenylpropanoid carbon atoms. Error limits are about ±0.2%. Numbers in parentheses are incorporations calculated by integrating the ^{13}C satellite peaks in the ^1H NMR spectrum.
[b]$^1J_{CH} = 180$ Hz.
[c]$^1J_{CH} = 130$ Hz.
[d]Signal not observed.

released by the biosynthetic process. To determine whether 3′-*O*-acetylchloramphenicol might be formed from chloramphenicol, cell-free extracts prepared by sonication of 7-day mycelium were incubated with chloramphenicol (1 mM) and acetyl–CoA (1 mM). No *O*-acetylchloramphenicol was detected by HPLC, and a colorimetric assay[21] showed no CAT activity. Mixing the *S. venezuelae* extract with an active extract from *Escherichia coli* cc118 did not diminish the *E. coli* CAT activity. The lack of CAT activity and the failure to detect genes homologous to known CAT genes in *S. venezuelae* (Wynands and van Pée, unpublished) suggest that 3′-*O*-acetylchloramphenicol is not formed from chloramphenicol in the producing organism.[1,4,22]

On the other hand, it was observed by HPLC that *O*-acetylchloramphenicol added to washed mycelium prepared from 5- to 7-day (late growth phase), but not from 3- or 4-day (exponential phase) cultures, was largely hydrolyzed to chloramphenicol within 60 min. Since *O*-acetylchloramphenicol remained unaltered in culture fluid passed through a 0.2 μm filter, and in control incubations with boiled mycelium, the results implicate *O*-acetylchloramphenicol esterase activity in *S. venezuelae* mycelium. After sonication and centrifugation (10 min at 5000*g*) of 7-day mycelium, the esterase was located mainly in the insoluble pellet. Addition of the esterase inhibitors acetylsalicylic acid (12 mM)[22] and sodium fluoride (0.5 mM)[23] to *S. venezuelae* cultures during the antibiotic production phase increased accumulation of *O*-acetylchloramphenicol in the broth. An esterase hydrolyzing 3′-*O*-acetylchloramphenicol has been reported[22] to be present in cultures of most chloramphenicol-producing stretomycetes, but was located in the soluble fraction after sonication. It was not responsible for failure of the cultures to exhibit CAT activity.

The enzyme-catalyzed conversion of 3′-*O*-acetylchloramphenicol to chloramphenicol and the accumulation of acetylated antibiotic on addition of inhibitors implicate *O*-acetylation as a biosynthetic step en route to chloramphenicol, with the ester as a biosynthetic intermediate, and its hydrolysis as the last step in the pathway. Association of esterase activity in *S. venezuelae* with the late growth phase and with the particulate fraction of sonicated mycelium, together with evidence for export of chloramphenicol from *S. venezuelae* mycelium producing the antibiotic, suggest that hydrolysis of 3′-*O*-acetylchloramphenicol is linked to excretion of chloramphenicol. Metabolic shielding with acetylated intermediates to protect the producing organism is used during biosynthesis of the antibiotics bialaphos and kanamycin, and a series of phosphorylated compounds are intermediates in streptomycin biosynthesis.[1] Formation of 3′-*O*-acetylchloramphenicol and esterase-mediated generation of the metabolically toxic chloramphenicol during excretion may constitute a self-resistance mechanism in *S. venezuelae*.

Without coupling to chloramphenicol export, esterase catalyzed hydrolysis of *O*-acetylchloramphenicol in the last step of chloramphenicol biosynthesis would defeat acetylation of the antibiotic as a resistance mechanism in *S. venezuelae*. Not only would the active antibiotic be regenerated, but a futile cycle of acetylation/deacetylation would deplete the cell of acetyl–CoA and ultimately ATP. To evade chloramphenicol toxicity antibiotic-producing strains of *Streptomyces* have evolved alternative protective mechanisms. Under physiological conditions in which genes for biosynthesis of the antibiotic are not expressed, chloramphenicol can be phosphorylated[7] or degraded.[4] When the biosynthesis genes are expressed, export of chloramphenicol from the mycelium prevents access to susceptible target sites in the producing organism,[4] and a complementary self-resistance mechanism is provided by the use of acetylated intermediates in the biosynthetic pathway.

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18. ^{1}H (400.1 MHz) and ^{13}C (100.6 MHz) NMR data (CD_3OD). **2** (minor species): ^{1}H NMR δ 8.20 (apparent d), 7.60 (apparent d, partially obscured), 6.24 (s), 6.10 (d), H-2′ obscured, 3.45–3.65 (m), 2.14 (s). Only one enriched carbon of **2** at either 67.4 and 20.7 ppm was observed in the ^{13}C NMR spectra of *O*-acetylchoramphenicol isolated in the feeding experiments. **3** (major species): ^{1}H NMR δ 8.17 (apparent d, splitting of 9 Hz, 2H), 7.64 (apparent d, splitting of 8 Hz, 2H), 6.19 (s, 1H), 5.06 (d, $J=3$ Hz, 1H), 4.40–4.35 (m, 2H), 4.23–4.21 (m, 1H), 2.02 (s, 3H), ^{13}C NMR δ 172.5, 166.7, 150.8, 128.4, 124.2, 71.7, 67.3, 64.8, 55.7, 20.6.
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