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Conversion of Steroids and Triterpenes by Mycobacteria: Stereospecific Hydrolysis of Steroidal Spiro-3ξ-oxiranes by *Mycobacterium aurum*[1]

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The irreversible inhibitor of bacterial Δ5-3-ketosteroid isomerase, (3*S*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol **1** and its epimer (3*R*)-spiro[5α-androstane-3,2′-oxirane]-17β-ol **2** are rapidly metabolized by strain A$^+$ *Mycobacterium aurum* in essentially quantitative yield to the same compound: 3β-(hydroxymethyl)-3α-hydroxy-5α-androstane-17-one **4**. Study of the metabolism of substrates **1** and **2** labeled with oxygen 18 on the epoxide ring showed that the formation of the same stereoisomer from the two different substrates was due to a highly regiospecific incorporation of hydroxide from water at carbon C2′ for **2** and carbon 3 for **1**. Kinetic studies of a mixture of **2** and **1** demonstrated a much faster rate of hydrolysis of the epoxide ring for the 3*R*-isomer **2** than for the 3*S*-isomer **1**. These findings effectively ruled out a unique mechanism of hydrolysis via formation of an intermediate carbocation after protonation of the epoxide ring. Activation of a water molecule by a basic group on the enzyme during hydrolysis of **2** but not during ring opening in **1** was thought to occur.

INTRODUCTION

In a previous publication (*1*), it was shown that strain A$^+$ of *Mycobacterium aurum* was able to degrade the 7-keto and 7-hydroxycholesterols to 3-oxygenated androstanes. It was suggested that elimination of the oxygen at position 7 occurred during an isomerization of the double bond from positions Δ5 to Δ4 by a simultaneous dehydration and isomerization. In order to test this hypothesis, we attempted to inhibit specifically the isomerization enzyme, Δ5-3-ketosteroid isomerase. An enzyme with similar properties in *Pseudomonas testosteroni* has been found to be inhibited by steroidal spiro-3-oxiranes (*2–4*). These compounds are irreversible inhibitors, and appear to be directed against the active site (*2*). However, we found that these compounds did not interfere with the metabolism of 7-keto-cholesterol by *M. aurum*, due to the fact that this bacterium rapidly degrades them. We report here our results on the metabolism of various steroidal spiro-3-oxiranes by *M. aurum*.

[1] This is Part III of a series; preceding papers are Refs. (*24*) and (*1*).
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MATERIALS AND METHODS

17β-Hydroxy-5α-androstane-3-one (98% grade), 5α-androstane-3,17-dione (98% grade) and 1-butyl boronic acid were from Sigma (St. Louis, MO); trimethylsufoxonium iodide (98% grade), sodium borohydride (99% grade) and sodium periodate (99% grade) were from Aldrich-Chimie (Strasbourg, France); methoxylamine hydrochloride was from Interchim (Montlucon, France); trimethylchlorosilane and bis(trimethylsilyl)trifluoroacetamide were from Pierce Chemical Co. (Rockford, IL). Water enriched in ^{18}O (98.13%) was obtained from the Centre d'Etudes Atomiques (Gif sur Yvette, France). Culture media were obtained from Difco (Detroit, MI) and silicic acid (Silicar CC7) was from Mallinckrodt (St. Louis, MO). Optical rotation was measured in chloroform, and the IR spectra were recorded on films between two NaCl slides.

Culture and Fermentation

Strain A^+ of *M. aurum* was isolated from a patient's sputum, and was supplied by Prof. H. David of the Institut Pasteur (Paris, France). This strain was innoculated (10% after 48 h preculture) into a medium containing 1% glucose, 0.5% casitone, 0.47% Middelbrook 7H9 medium (Difco) and 100 ml of distilled water. The culture was incubated at 37°C for 60 h with rotary stirring (120 rpm). 10 mg of substrate in 2 ml of dimethylformamide were introduced into each flask, and the media were stirred at 37°C for a further 24 h. The culture media were collected and extracted with dichloromethane. After several washes of the organic phase with distilled water, the solvents were evaporated under reduced pressure.

Chromatographic Analysis of Extracts

The extracts were analyzed without further purification by TLC, or by GLC after derivatization. TLC was carried out on 0.25-mm-thick Silicagel 60 F254 (Merck), and the plates were eluted with methyl ethyl ketone. The spots were visualized by heating the plates at 100°C for 5 min after spraying with 25% sulfuric acid in ethanol. GLC used a fused silica column (30 m × 0.45 mm) filled with OV-1 phase (Spiral, Dijon). Helium was used as carrier gas, and the oven temperature was programmed to rise linearly from 200 to 290°C at 2°C/min. The values of methylene index (MI)[3] were determined by coinjection of linear saturated hydrocarbons as standards. The extracts were derivatized as trimethylsilyl ethers, *O*-methyl oximes (*5*) or cyclic boronic diesters using 1-butyl boronic acid (*6*). GLC/MS was carried out under the same conditions as for the GLC. The mass spectrometer was a ZAB-2F instrument (V.G. Ltd., Manchester, UK) equipped with a mixed ionization source (electron impact or chemical ionization). Interface temperature was 200°C. The spectra were recorded on a DS-2050 data system at a 1 s/decade scan rate.

[3] Abbreviations used: MI, methylene index; TMS, trimethylsilyl.

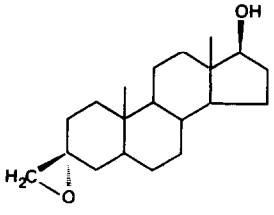
Isolation of Biotransformation Products

The extracts obtained from the fermentation were put onto a column containing 1 g of neutralized silica (Silicar CC7), and then eluted with a mixture of diethyl ether/hexane of increasing polarity (2-ml fractions). The fractions containing the mixture of **3** and **4** as detected by TLC were collected. The two stereoisomers were separated by HPLC (Si-60-5 Chrompack column, 4.6 × 250 mm; eluant ethyl acetate/hexane, 70/30) (k' = 6.42 for **3** and 5.67 for **4**).

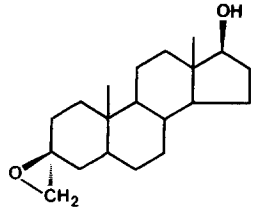
Chemical Syntheses

[*3-*^{18}*O*]*17-β-Hydroxy-5α-androstane-3-one.* Enrichment of 17-β-hydroxy-5α-androstane-3-one with ^{18}O was carried out using the method described by Bevins *et al.* (*3*). Mass spectrometry demonstrated that incorporation of ^{18}O into the ketone group was above 85%.

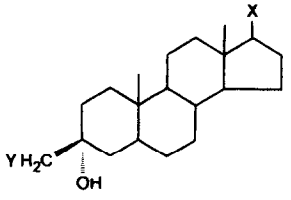
(*3R*)-*Spiro*[*5α-androstane-3,2'oxirane*]-*17β-ol* **2.** The action of dimethylsulfoxonium methylide $(CH_3)_2S(O)CH_2^-$ on the 3-ketosteroids is highly stereoselective, and only leads to the 3*R*-oxiranes (*7*). Thus compound **2** is obtained from 17-β-hydroxy-5α-androstane-3-one using the general method described by Corey and Chaykovsky (*8*). Purification on a neutralized silica column (Silicar CC7, eluant: diethyl ether/hexane 2/8) produced compound **2** in a 75% yield. Analysis by HPLC (Si-60-S Chrompack column 4.6 × 250 mm, eluant: dichloromethane/ethyl acetate, 9/1) showed the purity of the fraction (>99%), and the absence of the 3*S*-isomer **1.** mp 173–174°C (lit. 173–175°C (*9*)). $[\alpha]_D$ = +6° ($CHCl_3$, C = 1) (lit. + 3° ($CHCl_3$, C = 1, (*9*)). Mass spectra: *m/z* 304 (100%; $M^{+\cdot}$), *m/z* 289 (7%; M–CH_3),

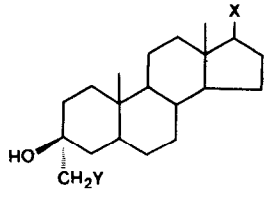


3R-isomer - 2



3S-isomer-1





4	X = =O ; Y = OH	**3**	X = =O ; Y = OH
6	X = —OH ; Y = OH	**5**	X = —OH ; Y = OH
8	X = —OH ; Y = H	**7**	X = —OH ; Y = H

m/z 275 (8%), *m/z* 245 (13%; breaks C14–C15 and C13–C17), *m/z* 220 (15%; breaks C1–C10 and C4–C5), *m/z* 85 (30%; M–220 + H^+).

(3S)-Spiro[5α-androstane-3,2'oxirane]-17β-ol ***1***. The action of dimethylsulfoxonium methylide $(CH_3)_2S^+CH_2^-$ on the 3-ketosteroids (*7*) gives a mixture of 40% of the 3*R*-isomer **2** and 60% of the 3*S*-isomer **1** with an overall yield of 95%. The two isomers **2** and **1** from 17-β-hydroxy-5α-androstane-3-one were purified and separated by HPLC under the conditions described above (k' = 2.64 for **1** and 3.26 for **2**). HPLC produced the 3*S* isomer **1** in a yield of more than 99% uncontaminated by **2**. mp 195–197°C (lit. 193–196°C (*7*)), $[\alpha]_D$ + 8° ($CHCl_3$, C = 1) (lit. + 6°, $CHCl_3$, C = 1). The mass spectrum was identical to that of **2**.

[3-^{18}O](3R)-Spiro[5α-androstane-3,2'-oxirane]-17-β-ol [^{18}O]-***2*** *and [3-^{18}O](3S)-spiro[5α-androstane-3,2'-oxirane]-17-β-ol* [^{18}O]-***1***. The synthesis of [^{18}O]-**2** and [^{18}O]-**1** were carried out from [3-^{18}O]17-β-hydroxy-5α-androstane-3-one using the method described above. Mass spectrometry demonstrated 95% incorporation of ^{18}O.

3β-(Hydroxymethyl)-5α-androstane-3β,17β-diol ***5*** *and 3α-(hydroxymethyl)-5α-androstane-3α,17β-diol* ***6***. These compounds were synthesized from **1** and **2** by alkaline hydrolysis according to the method described by Bevins *et al.* (*3*). The glycols obtained were purified by HPLC (Si-60-S Chrompack column 4.6 × 250 mm, eluant hexane/methyl ethyl ketone, 1/1) (k' = 6.18 for **6**, and 7.35 for **5**). The physicochemical parameters were in agreement with literature values (*3*). ***6*** mp 137–140°C (lit. 135–138°C), **5** mp 211–214°C (lit. 210–213°C). Mass spectra of **5** and **6** were identical: *m/z* 322 (0.4%, $M^{+\cdot}$), *m/z* 304 (5%, $M-H_2O$), *m/z* 291 (100%, $M-CH_2OH^{\cdot}$), *m/z* 273 (8%, $M-H_2O-CH_2OH^{\cdot}$), *m/z* 255 (23%, $M-2H_2O-CH_2OH^{\cdot}$).

Oxidation of the glycols ***3*** *and* ***4*** *by periodate*. An aqueous solution of sodium periodate (0.4 ml, 0.1 M) was mixed with 2 mg of **3** or **4** dissolved in 1 ml of methanol, and shaken in a closed tube for 20 h at room temperature. After extraction in hexane, the compound obtained ($M^{+\cdot}$ = 288 MS) was analyzed by GLC, along with an authentic sample of 5α-androstane-3,17-dione.

Kinetics of Hydrolysis of ***1*** *and* ***2***

The bioconversion was carried out as described above, time zero was taken as the time of introduction of substrate (10 mg of a 1:1 mixture of [^{18}O]-**1** and unlabeled **2**. At various times, 5 ml of the reaction mixture was removed and put into 5 ml of dichloromethane cooled to −10°C in order to stop the enzymatic reaction. The aqueous phase was extracted three times with dichloromethane. After several washes in distilled water, 20% of the organic phase was transferred into a reaction vial, and the solvents were evaporated under nitrogen. Lithium aluminium hydride (2 mg) and 1 ml of anhydrous ether were added. The hermetically sealed tube was heated at 60°C for 1 h. The excess hydride was destroyed by 1 ml of ethyl acetate. The organic phase was washed several times before drying under nitrogen. The extract was then treated with 1-butyl boronic acid and a silylizing mixture (bis(trimethylsilyl)trifluoroacetamide containing 75% trimethylchlorosilane) under conditions described in Refs. (*5*) and (*6*). An aliquot of this fraction was analyzed by GLC/MS fragmentometry, monitoring the ions *m/z* 450

TABLE 1

Conversion of the Spiro-3-oxiranes **2** and **1** by *Mycobacterium aurum:* Relative Abundance of Metabolites Identified by GLC

Substrate	Reagent (37°C, 60 h)	Products	Relative amount % (GLC)
2	[a]	**4**	99
		3	1
1	[a]	**4**	97
		3	3
2	[b]	**6**	2
		2	98
1	[b] or [c]	**1**	100
2	[c]	**2**	100

[a] Live cells.
[b] Cultures autoclaved at 110°C for 1 h.
[c] Supernatant obtained after centrifugation of a culture at 5000*g* for 15 min.

and 452 (molecular ions of bis-TMS **8** and O-labeled bis-TMS **7** respectively), *m/z* 460 and 462 (molecular ions of labeled and unlabeled, derivatized **6** ions as cyclic boronate-*O*-TMS ethers).

RESULTS

*1. Conversion of the Spiro-oxiranes **1** and **2***

Under the experimental conditions described under Methods (24 h at 37°C) the spiro-oxiranes **1** and **2** were completely metabolized by live cells of *M. aurum*. The compounds were not affected by the supernatant of the culture medium. Slight hydrolysis of **2** was observed on contact with autoclaved bacteria in the culture medium (Table 1). The transformation of **1** and **2** was thus assumed to be due to enzymatic reactions. The slight hydrolysis with the autoclaved cultures was probably due to acidification of the medium (the pH fell from 6.6 to 5.8).

The enzymatic transformation of the 3*S*-isomer **1** and the 3*R*-isomer **2** led to essentially the same product, **4**, with 99% yield for **2** and 97% for **1**. The other product was referred to as compound **3** (Fig. 1).

*2. Characterization of the Products of Bioconversion of **3** and **4***

The molecular mass of these two compounds was determined by mass spectrometry by electron impact ($M^{+\cdot}$ at *m/z* 320), or chemical ionization with ammonia ($M \cdot NH_4^+$ at *m/z* 338). The empirical formula of **4** was determined by high resolution mass spectrometry (measured = 320.2335; calculated for $C_{20}H_{32}O_3$ = 320.2343). Functional groups were identified by ir spectroscopy which demon-

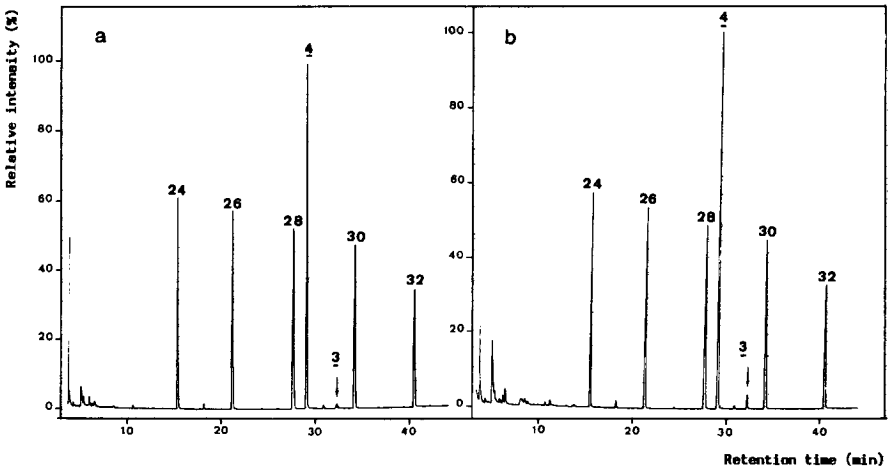


FIG. 1. GLC analysis of extracts of bioconversion of spiro-3-oxiranes by *Mycobacterium aurum* after derivatization with 1-butyl boronic acid: (a) bioconversion of compound **2**; (b) bioconversion of compound **1**. The peaks 24–32 correspond to the saturated linear hydrocarbons *n*-C24,*n*-C26,*n*-C28,*n*-C30,*n*-C32 (standards).

strated the presence of hydroxyl groups (3400 cm^{-1}) and carbonyl groups (1720 cm^{-1}). Mass spectrometry indicated the presence of a ketone group and two alcoholic groups, from the difference in mass between the trimethylsilyl-*O*-methyloxime derivatives and the nonderivatized compounds.

The action of sodium periodate on **4** led to 5α-androstane-3,17-dione, which indicated that it was one of the two epimers of 3ξ-(hydroxymethyl)-3ξ-hydroxy-5α-androstane-17-one.

The configuration of the carbon 3 of **4** was attributed after comparison with the reference compounds by chromatography (see Materials and Methods). Chemical hydrolysis in alkaline medium of **2** and **1** led, via opening of the oxirane with retention of configuration, to the reference triols **6** and **5**, respectively. Reduction by sodium borohydride of the 17-keto group was stereoselective leading to the 17β-hydroxylated compound. Compound **4** was thus identified as 3β-(hydroxymethyl)-3α-hydroxy-5α-androstane-17-one.

Reduction of **3** by sodium borohydride led to the triol **5** which indicated that **3** was 3α-(hydroxymethyl)-3β-hydroxy-5α-androstane-17-one.

3. Mechanism of the Enzymatic Opening of the 3S- and 3R-Epoxides 1 and 2 by M. aurum

The production of the same stereoisomer **4** from the two epimers **2** and **1** suggested that, during the enzyme catalyzed reaction, the configuration was retained for **2** and inverted for **1**.

Two possible mechanisms could be envisaged:

(i) acidic catalysis for the 3*S*-isomer **1** inverting the configuration of carbon 3, and alkaline catalysis for the 3*R*-isomer **2** with retention of the configuration.

(ii) a common mechanism leading to a carbenium ion in position 3, followed by nucleophilic attack from the least hindered side (α side).

In order to try and discriminate between these two mechanisms, the metabolism of compounds **2** and **1** labeled with ^{18}O on the epoxide ring was investigated.

An alkaline hydrolysis would involve nucleophilic attack on the least substituted carbon leading to a diol with the label on the tertiary hydroxyl group. An acid catalysis with formation or not of a carbenium ion at position 3 would give a diol with the label on the primary hydroxyl group (Diagram 1).

The localization of the isotope ^{18}O on the diols **4** obtained from the enzymatic transformation of **2** and **1** should thus enable the two mechanisms to be discriminated.

The position of this isotope is readily detected by mass spectrometry. A significant decomposition reaction of the $M^{+\cdot}$ ion of both compounds **3** and **4** leads to loss of the primary alcohol CH_2OH radical (Fig. 2a) (strong signal due to the ion M-31 at m/z 289). Note the weak signal for the M-33 ion (m/z 287) derived from loss of water and a methyl radical (signal approximately 1% of the M-31 ion).

The mass spectrum of ^{18}O-labeled **4** formed metabolically from [^{18}O]-**2** (Fig. 2b) exhibited a doublet at m/z 322 (isotopically labeled) and 320 (unlabeled). The ratio of the intensities of these signals corresponded to the degree of labeling of the precursor **2**. The ion fragments from elimination of CH_2OH were observed at m/z

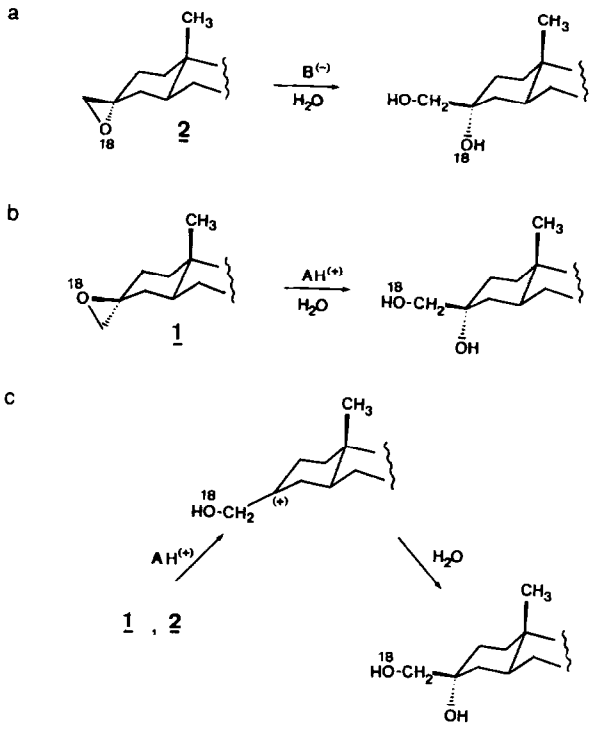


DIAGRAM 1. Different ways for the formation of the glycol **4** by ring opening of ^{18}O-labeled **1** and **2**: (a) base catalysis for **2**; (b) acid catalysis for **1**; (c) intermediate formation of a carbenium ion.

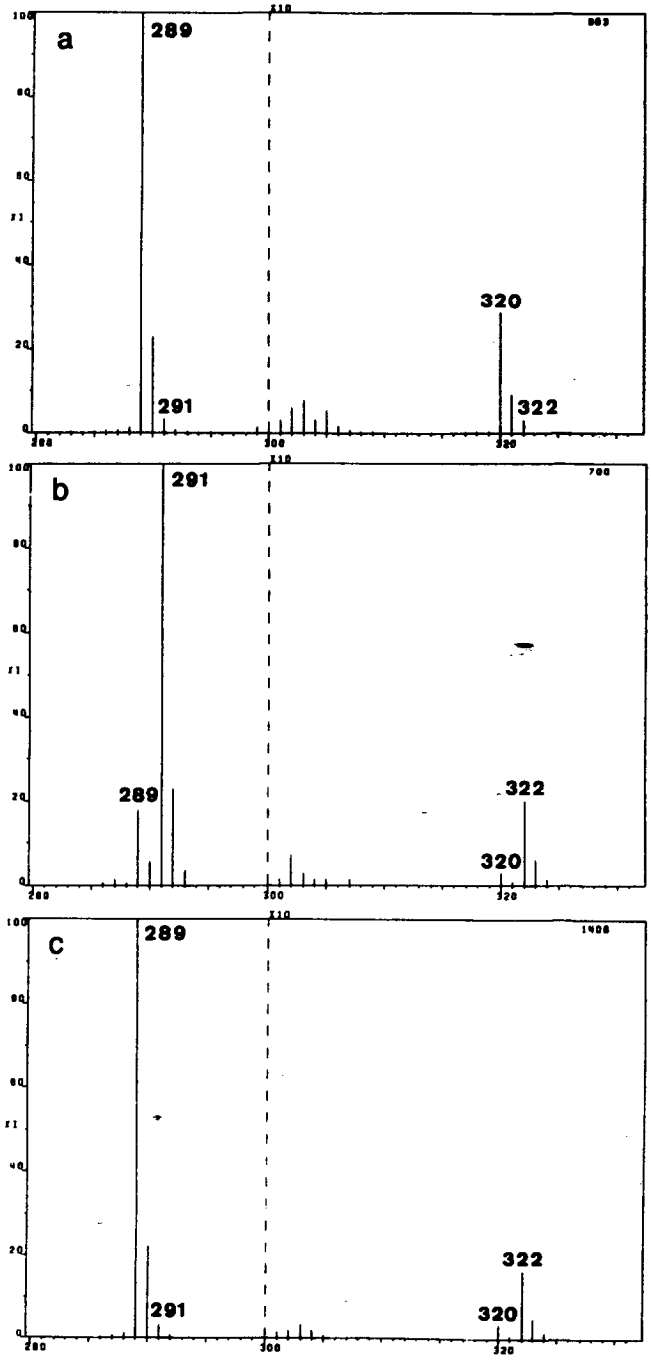


FIG. 2. Localization of ^{18}O in the products of bioconversion of [^{18}O]-**2** and [^{18}O]-**1** by *Mycobacterium aurum*. Partial electron impact mass spectrum of (a) unlabeled **4**, (b) [^{18}O]-**4** from [^{18}O]-**2**, (c) [^{18}O]-**4** from [^{18}O]-**1**.

291 and 289, respectively. The ratio of their intensities was comparable to that of the molecular ions indicating the loss of 31 mass units. This would localize the ^{18}O on the tertiary alcohol group.

On the other hand, in the mass spectrum of ^{18}O-labeled **4** produced by bioconversion of [^{18}O]-**1** (Fig. 2c) molecular ions were seen as a doublet at m/z 322 (labeled) and 320 (unlabeled). However, a unique ion fragment from loss of CH_2OH was observed at m/z 289. This ion did not contain the ^{18}O label, indicating that the label was situated on the primary alcohol group of **4**.

These results tend to support the first mechanism involving a difference in ring opening depending on the nature of the substrate.

4. Kinetics of Transformation of Compounds 1 and 2 by M. aurum

After 24 h of incubation with *M. aurum,* compounds **1** and **2** formed compound **4** in almost quantitative yield. Analysis of the medium at an intermediate time demonstrated the presence of compound **6**, formed by opening of the epoxide ring.

In order to measure the rates of hydrolysis of the steroidal spiro-oxiranes in relation to the stereochemistry of carbon 3, and independently of the reactions on carbon 17, we measured the rates of disappearance of the starting substrates and the rates of formation of the α-glycols (**4** and **6**).

In order to control for possible differences in action of the bacteria and extraction procedures, the experiment was carried out using an equimolecular mixture of **1** and **2**. Compound **2** was labeled with ^{18}O on the oxirane group. The kinetics of the reaction could thus be assessed from GLC/MS analysis of aliquots of the reaction mixture removed after various incubation times. The aliquots were reduced with lithium aluminium hydride and then derivatized with 1-butyl boronic acid. The hydroxyl groups were then transformed into the corresponding trimethylsilyl ethers. In this way, the 17-keto group was reduced to a 17β-hydroxyl (**4** → **6**), and the epoxide rings in the unreacted substrates **1** and **2** were hydrogenolyzed. This leads to the tertiary alcohols **7** and **8** with retention of the configuration on carbon 3. 1-Butyl boronic acid acts on the α-glycol to form the cyclic boronate (*16*), which is relatively resistant to electron impact ionization (as opposed to the noncyclized diols). The trimethylsilyl derivatives can be readily separated chromatographically. All products produced a strong signal from the molecular ion after electron impact ionization, and the derivatives of **6** exhibited an isotopic doublet (^{16}O and ^{18}O) depending on their origin. The mass fragmentometric analysis monitored the intensities of all the molecular ions throughout the GLC separation. Calculations taking account of the initial isotopic ratio $^{18}O/^{16}O$ of the labeled substrate **1** enabled the percentage transformation of each substrate to be determined. The results shown in Fig. 3 indicate that hydrolysis of **2** was much faster than that of **1**.

DISCUSSION

Although there have been a number of investigations on the hydrolysis of epoxides by enzymes from extracts of mammalian cells (*10–13*), there has been rela-

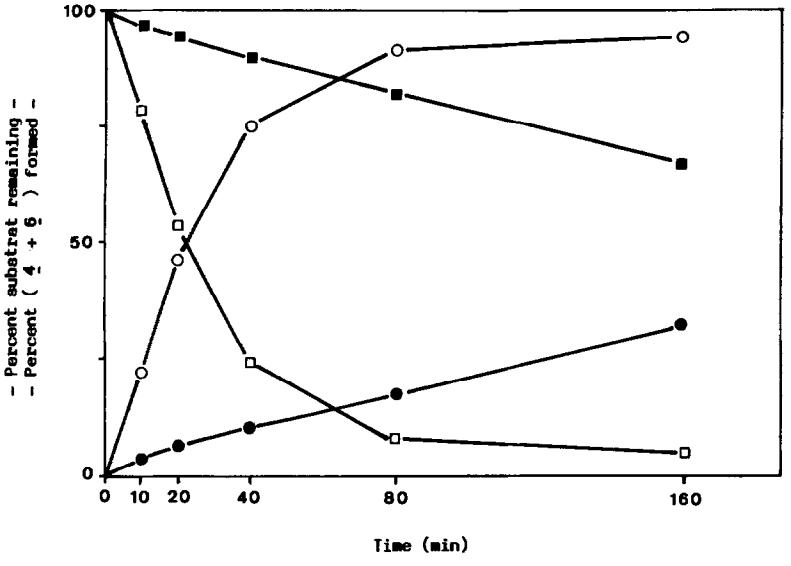


FIG. 3. Kinetics of hydrolysis of the spiro-3-oxiranes **2** and **1** by *Mycobacterium aurum*. □, Disappearance **2**; ■, disappearance **1**. ○, Appearance (**4** + **6**) formed from **2**; ●, appearance (**4** + **6**) formed from **1**.

tively little study of the opening of epoxides by bacterial enzymes (*14–16*). To our knowledge this is the first report on the hydrolysis of steroidal epoxides by microorganisms. The unusual feature of the system in strain A^+ of *M. aurum* is the difference in reaction mechanism depending on the stereochemistry of the epoxide: acid catalysis for the β-configuration of the epoxide ring of the (3*S*-isomer **1**) and alkaline catalysis for the α-configuration (3*R*-isomer **2**).

This result could be explained by either (i) the presence of several enzymes operating with different catalytic mechanisms, or (ii) different reaction mechanisms depending on the orientation of the substrate in the catalytic site of a single enzyme. For substrate **2**, the nucleophile (water) is activated by a basic group on the enzyme which orients the attack towards the least hindered site (equatorial methylene) (*17–20*). The rapid rate of hydrolysis of this compound, in contrast to the normal resistance of epoxides to nucleophilic attack without electrophilic assistance (*21*), would tend to indicate that protonation of oxygen is required (*3, 22*). In the compound with the opposite configuration **1**, the α-methylene in the ring is not in a favorable position with respect to the basic group on the enzyme. In this case, the reaction is driven by protonation of the oxygen in the ring (*23*).

The role of this hydrolytic potential in bacterial physiology remains to be determined. In this species which has high oxidative activity (*24–26*) it may serve as a protective process against endogenously formed, potentially toxic epoxides (*27*). The difference in enzymatic reaction mechanism depending on the configuration of the substrate is probably not limited to steroidal spiro-oxiranes. Further studies on other substrates are in progress.

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