THE CHEMISTRY OF CEPHALOSPORIN P

By

TA-SEN CHOU

Taipei Institute of Technology

Taipei, Taiwan

The Republic of China

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THE CHEMISTRY OF CEPHALOSPORIN P

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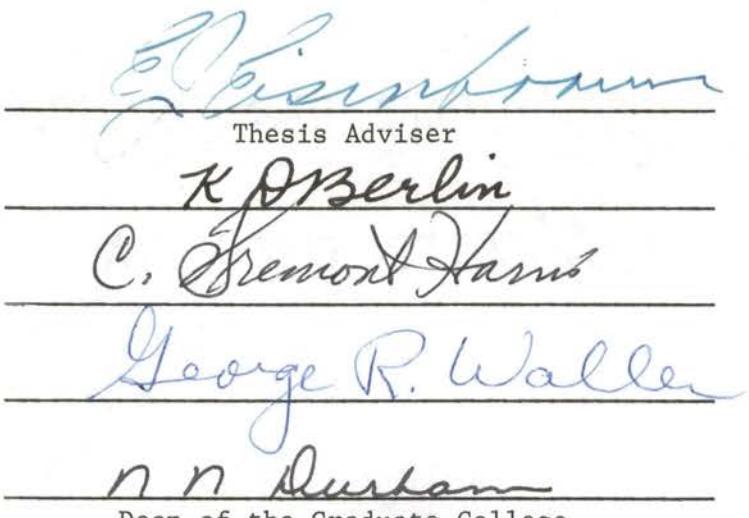

Thesis Adviser

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

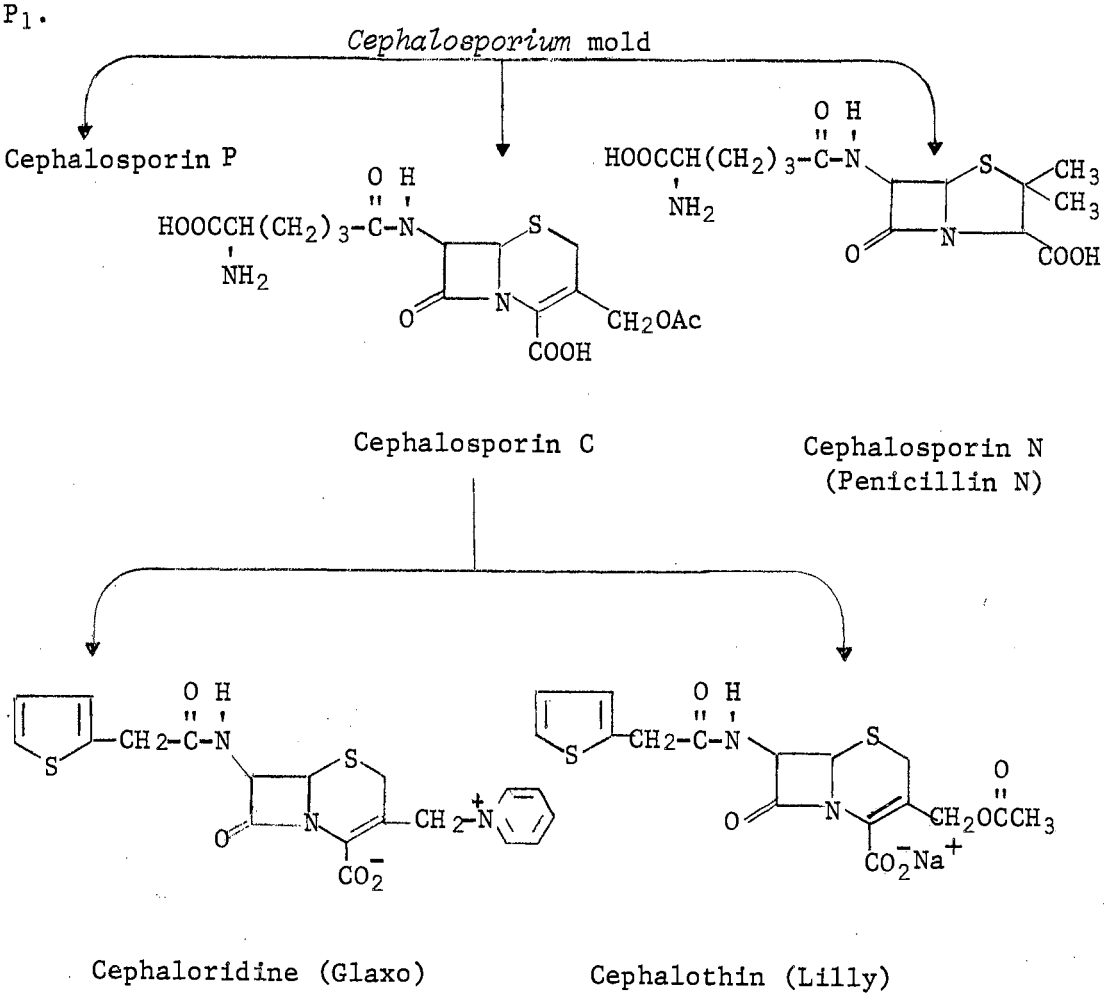
Steroid antibiotics are a group of acidic materials isolated from different fermentation sources. Three common ones in this category are fusidic acid from *Fusidium coccineum*, helvolic acid from *Cephalosporium caerulens*, and cephalosporin P_1 from *Cephalosporium acremonium*. These substances show relatively high activity against gram-positive microorganisms, and fusidic acid is marketed for such use in Europe. Chemically, they are characterized as polyisoprenoids and have an unique lanostane skeleton, differing from each other by placement of hydroxyl, acetoxy, or keto group. It is believed that these compounds may result from a new type of lanosterol biosynthesis pathway during fermentation.

Cephalosporin P_1 is the least known member of this group, even though it was isolated in 1951 along with the important cephalosporin C and cephalosporin N. Cephalosporin C has been converted to two useful drugs which are currently marketed as Cephalothin by Eli Lilly and Company and Cephaloridine by Glaxo Laboratories in Great Britain.

A partial structure for cephalosporin P_1 was reported in 1961 and was revised in 1963. In 1963, a small quantity of an organic extract from the fermentation broth of *Cephalosporium acremonium* was obtained from Eli Lilly and Company. Monodesacetylcephalosporin P_1 was first isolated, in our work, from the extract through a benzene

solvate procedure used in the isolation of fusidic acid from *Fusidium coccineum*. Later, cephalosporin P_1 and a small sample of isocephalosporin P_1 were also obtained from the same fermentation extract.

Structural studies were immediately initiated. In this thesis, a complete structure for cephalosporin P_1 is proposed through interpretation of the chemical and spectral data gathered from the structural investigations. While this work was in progress, two other research groups also obtained the same structure for cephalosporin P_1.



CHAPTER II

HISTORICAL

In 1945, Brotzu of the Institute of Hygiene, Cagliari University, Sardinia, isolated an antibiotic-producing microorganism from the sea near a sewer outlet off the coast of Sardinia. He cultured this microorganism on nutrient agar and found that fluid from the culture inhibited the growth of a number of gram-positive as well as a large number of gram-negative bacteria including *Staphyloccoccas aureus*, *Salmonella typhi*, *Vibris cholerae*, and *Bicillus authracis*. Brotzu later identified the microorganism as *Cephalosporium acremonium*, which has been shown to produce chemotherapeutic antibiotics.[1]

Although Waksman and Horning[2] had stated in 1943 that some members of the *Fusarium-Cephalosporium* group were decidedly antagonistic to the growth of bacteria on a solid medium, Brotzu[1] appeared to have made the first specific study of antibacterial substances from a species of *Cephalosporium*.

Gottshall[3] in 1951 reported the production of an antibiotic by a member of the genus *Tilachlidium* and by *Cephalosporium charticola*. It was subsequently shown that the *Tilachlidium* was a new species of *Cephalosporium*. This new species was named *Cephalosporium salmosynnematum*[4] and the antibiotic it produced was first named synnematin and later called synnematin B.[5] Synnematin B was shown to be identical with cephalosporin N, isolated from the culture fluid of *Cephalosporium*

acremonium.[6]

In 1951, Crawford and Heatley[7] cultured *Cephalosporium* (sp. C.M.I.49137) in a medium containing corn steep liquor and glucose using deep and strong aeration. The culture fluid was extracted with butyl acetate and an acidic antibiotic was found in the organic extract.

In the same year, Burton and Abraham[8] became convinced that at least two kinds of antibacterial substances were contained in the culture fluid, one of which was insoluble in common organic solvent but the other could be readily extracted into butyl acetate. The butyl acetate extract was then purified by counter-current distribution in a system composed of hexane, diisopropyl ether, acetone, and phosphate buffer at PH 6 followed by chromatography on Florisil. At least five acidic fractions were obtained in this manner which showed antibiotic activity. These were named cephalosporin P_1, P_2, P_3, P_4, and P_5 because of their range of activity against gram-positive bacteria with the former being the most effective. Cephalosporin P_1, P_2, and P_4 were obtained in crystalline forms, but only cephalosporin P_1 was obtained in sufficient quantity for further investigation.

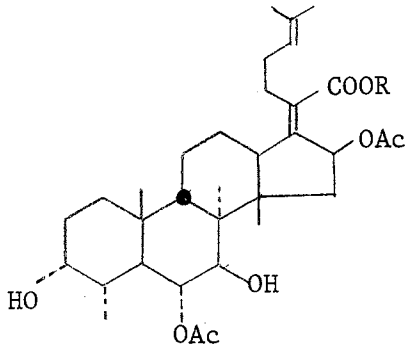
A second and extremely hydrophilic antibiotic was isolated from the water soluble portion of the culture fluid.[7,9] This new antibiotic was called cephalosporin N because of its pronounced activity against gram-negative bacteria. It was also found to be active against gram-positive bacteria.[10] Later, it was shown to be a true penicillin with a D-α-aminoadipic acid side chain which distinguishes it from penicillin. Consequently, it was renamed to penicillin N.[11]

Its isolation was troublesome but this was eventually overcome by Abraham, Newton, and Hale[12] who obtained it in nearly pure form.

Crude penicillin N was found to be contaminated with a chemically related antibiotic which had approximately the same antibiotic range.[13] This new antibiotic was named cephalosporin C by Newton and Abraham.[14] Neither penicillin N nor cephalosporin C were useful drugs. However, it was found that cephalosporin C could be modified to two useful antibiotics which are currently marketed as Cephalothin by Eli Lilly and Company and Cephaloridine by Glaxo Laboratories in Great Britain.

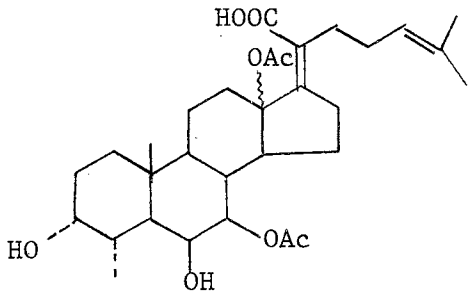
Since penicillin N and cephalosporin C are not structurally related to cephalosporin P_1, they will not be considered in this thesis.

The structure of cephalosporin P_1 is now depicted as 1,[15,16,17] which is in agreement with the chemical and spectral data of cephalosporin P_1 and its derivatives gathered to date.



1a R = H

1b R = CH_3



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A brief account of the chemical work which led to 1a for the structure of cephalosporin P_1 is summarized below.

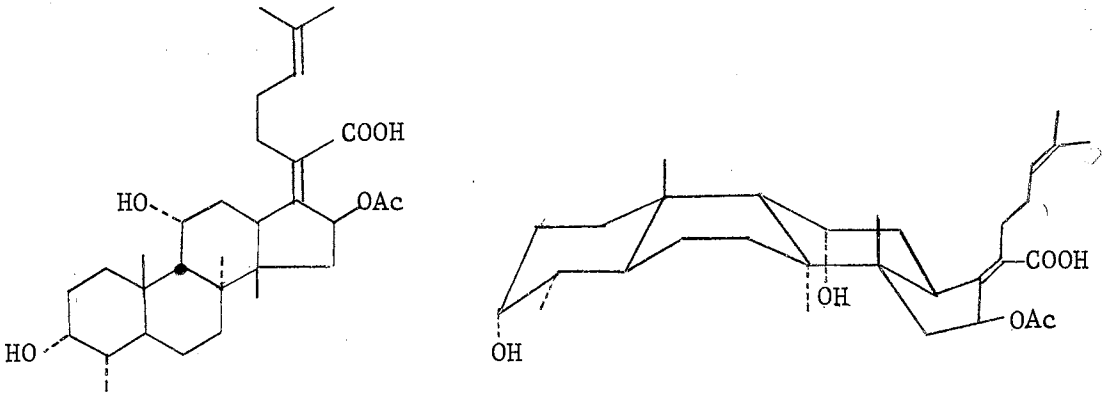
In their early work, Burton, Abraham, and Cardwell[18] proposed that cephalosporin P_1, with empirical formula $C_{32}H_{48}O_8$, was a tetracyclic monocarboxylic acid with a C_{28} skeleton. It contained two acetoxy groups, two hydroxyl groups, one easily reducible double bond and a tetrasubstituted double bond which was part of an α,β-unsaturated carboxylic acid moiety. One of the two acetoxy groups was easily hydrolyzed to give a monodesacetyl derivative of cephalosporin P_1 and hydrolysis of the second acetoxy group gave a didesacetyllactone. This work, therefore, lead to the conclusion that cephalosporin P_1 contained: $C_{27}H_{39}$-, $(-OCCH_3)_2$, $(-OH)_2$ and $-CO_2H$.

In 1961, Baird and Halsall[19] showed that cephalosporin P_1 is isoprenoid in origin by incorporating [2-C^{14}] mevalonic lactone into cephalosporin P_1 from a strain of *Cephalosporium*. At the same time, they proposed structure 2 for cephalosporin P_1 through interpretation of chemical and spectral data obtained from cephalosporin P_1 and its derivatives. It will be shown later that 2 is an incorrect assignment for cephalosporin P_1. Consequently, the proposed structures[19] for derivatives prepared from cephalosporin P_1 based on structure 2 are also invalid. The main reasons for the incorrect assignments stem from considering the chemistry of cephalosporin P_1 in terms of a molecular formula $C_{32}H_{48}O_8$ with a C_{28} nucleus and also a lack of knowledge about the conformation of this carbon skeleton.

In 1963, Halsall, Jones, and Lowe[20] found that the molecular formula of cephalosporin P_1 was $C_{33}H_{50}O_8$ instead of $C_{32}H_{48}O_8$, through

a mass spectrometric determination[21] of cephalosporin P_1 methyl ester and didesacetyl cephalosporin P_1 lactone. Using the C_{29} carbon skeleton of fusidic acid[22] as a model, a C_{29} carbon skeleton was suggested for cephalosporin P_1 and an additional methyl group was placed at position eight of 1.[22,23] After investigating the nuclear magnetic resonance (nmr) spectra of methyl ester derivatives of fusidic acid,[22] helvolic acid[24,25,26] and cephalosporin P_1, Melera[27] also confirmed the close relationship between these three steroidal antibiotics.

The complete structure of fusidic acid was achieved by Godtfredsen[28] who proposed structure 3. This structure was verified in 1965 by Cooper's[29] X-ray crystallographic studies on the 3-p-Bromobenzoate of fusidic acid methyl ester. Thus, the *trans-syn-trans* B-boat conformation of fusidic acid carbon skeleton is firmly established.



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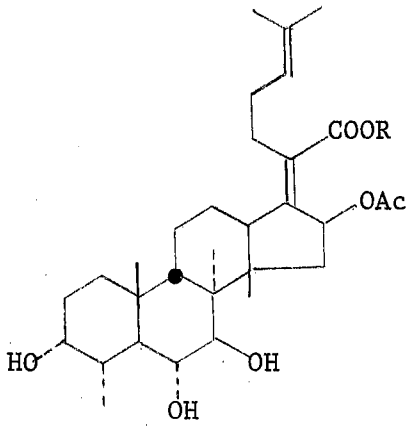
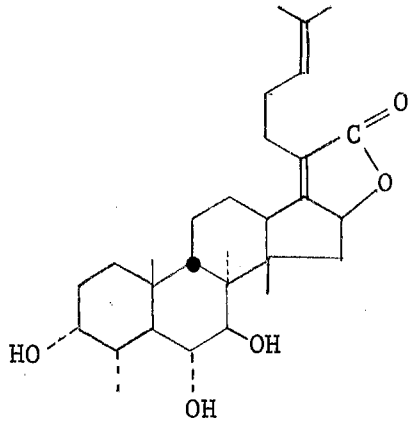
From the results of these mass and nmr spectra[20,27] and using the *trans-syn-trans* B-boat conformation of fusidic acid,[28] Halsall[15] in 1966 revised his structure 2 of cephalosporin P_1 to 1a. With these results, several previously prepared cephalosporin P_1

derivatives such as monodesacetylcephalosporin P_1,[18] didesacetylcephalosporin P_1 lactone,[18] the isopropylidene derivatives of monodesacetylcephalosporin P_1 methyl ester,[19] and the derivatives of tetrahydro monodesacetylcephalosporin P_1 methyl ester[19] can be represented by the structures 4a, 5, 6, and 7 respectively. The spectral



4a R = H

4b R = CH_3



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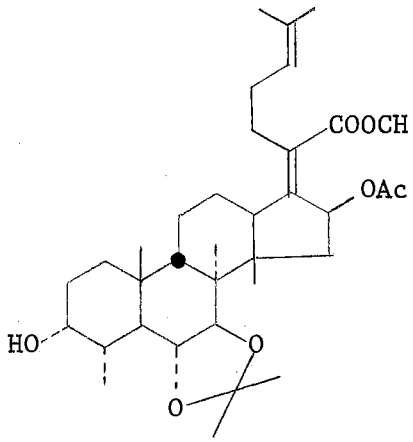

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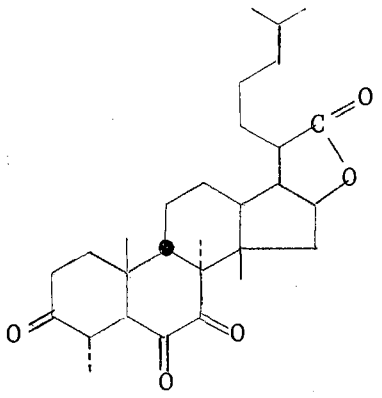

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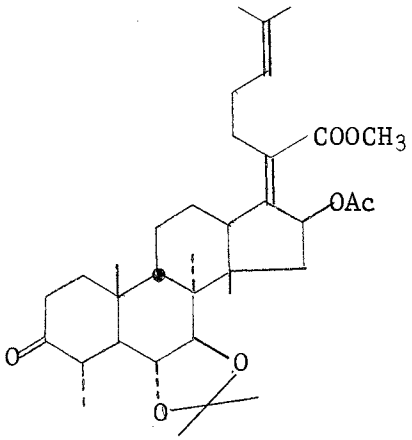
and chemical evidence which led Halsall to the revision of his earlier proposed structure 2 for cephalosporin P_1 to structure 1a were summarized. The C_{13} acetate of structure 2 was reassigned at C_{16} position in structure 1a based on the nmr spectrum of cephalosporin P_1 methyl ester (1b). The nmr spectrum of the methyl ester of 1a reveals that one of its secondary protons attached to the two acetoxy groups gives rise to a doublet with a coupling constant of 8.5 cps centered at δ 5.87. This is identical to the chemical shift of the secondary proton attached to the α C_{16} position in fusidic acid[27,28] and helvolic acid.[25,27] Therefore, the C_{13} acetoxy group in structure 2 was reassigned to C_{16} in structure 1. Thus, monodesacetylcephalosporin P_1 and didesacetylcephalosporin P_1 lactone first prepared by Burton[18] had the structures 4a and 5 respectively.

When the isopropylidene derivative 6 was oxidized with chromic acid, the ketone 8 was formed. The optical rotatory dispersion (ORD) curve of ketone 8 showed a positive Cotton effect which did not depress in amplitude when the measurements were taken in acidic methanolic solution. These results indicate the A/B ring junction in cephalosporin P_1 (1a) is *trans* and C_4 methyl is α and equatorial. Reduction of 8 by sodium borohydride gives an epimeric alcohol; therefore, the C_3 hydroxyl group in cephalosporin P_1 (1a) must be α and axial. The triketone 7 was prepared from a didesacetylcephalosporin P_1 lactone derivative of cephalosporin P_1. The conversion of 7 to 9 provided evidence that C_5 is substituted with a proton and since the A/B rings are *trans* fused, this C-5 proton has an α and axial configuration.



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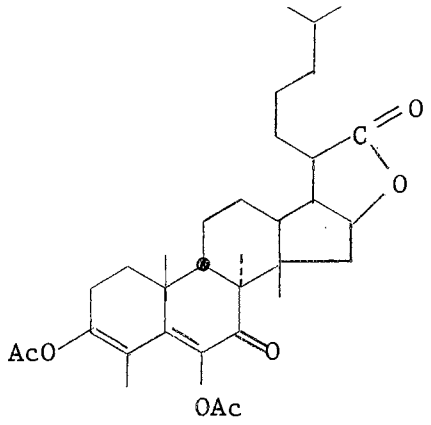

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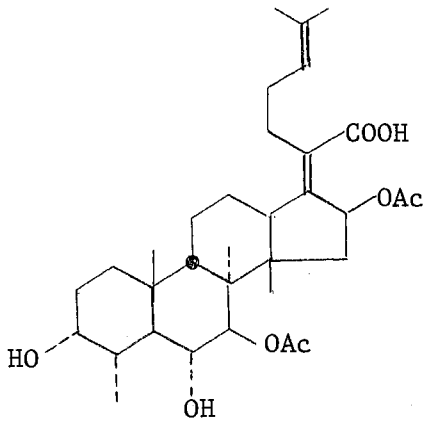
Using the B-boat conformation concept for fusidic acid, Halsall[15] reassigned the diol-monoacetate functions in ring B of cephalosporin P_1 from structure 2 to 1a through reinterpretation of the nmr spectrum of cephalosporin P_1 methyl ester. The hydroxyl group in cephalosporin P_1 methyl ester was first subjected to exchange with deuterium oxide. Its nmr spectrum then showed the following features: a doublet at δ 4.57 with a coupling constant of 10 cps, the half-height widths of the two doublet peaks were 5 and 5.5 cps respectively; a singlet at δ 3.50 with a half-height width of 3.5 cps. When the singlet peak at δ 3.50 was saturated by double resonance technique, the doublet with 10 cps at δ 4.57 remained but the half-height width of the two doublet peaks decrease to 3 and 3.5 cps respectively. Halsall interpreted these results to mean the signal at δ 4.57 was due to the secondary proton attached to the carbon holding an acetoxy group, and the peak at δ 3.50 was due to the secondary proton attached to the carbon bearing a hydroxyl group in the

diol-monoacetate functions in ring B of cephalosporin P_1. Since an axial and α proton was shown to be present at C_5 in cephalosporin P_1 (1a) and the C_8 position was fully substituted,[20,27] the doublet at δ 4.57 must arise from the coupling between the secondary proton attached to the carbon holding an acetoxy group at C_6 with the proton at C_5. The same reasoning may be used to show that the secondary proton (δ 3.50) must be at C_7 of 1a. The large coupling constant of 10 cps between the C_5 and C_6 protons indicate that they are diaxially coupled; thus, the C_6 proton must have a β and axial configuration. The decrease in half-height width of about 2 cps for the doublet peaks at δ 4.57 when the C_6 and C_7 protons were first decoupled indicate that the dihedral angle between C_6 and C_7 protons is about 110°. This is true only with a boat conformation for ring B. Therefore, the C_7 proton must have a β and axial configuration.

Oxley[16] in 1966 arrived at the same conclusion concerning the positions and configurations of the hydroxyl and acetoxy groups attached to ring B of cephalosporin P_1 (1a). He prepared an isomer of 1a which he believed to have the partial structure 10, by treating 1a with either cold, weak base or cold, strong acid. The nmr spectrum of the methyl ester of 1a and of the isomer 10 were taken.[16] The data obtained from the former is essentially the same as that obtained by Halsall[15] and Melera.[27] The nmr spectrum of the latter has signals at δ 4.63 (s) and δ 3.42 (J = 10 cps) which may be attributed to the partial structure 10.[16]



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The structure 1a for cephalosporin P_1 was also derived in our work.[17]

CHAPTER III

RESULTS AND DISCUSSION

In 1963, a small quantity of an organic extract from the fermentation broth of *Cephalosporium acremonium* was obtained from Eli Lilly and Company. From this crude extract, monodesacetylcephalosporin P_1 (4a) was isolated in about 18% yield. Later, cephalosporin P_1 (1a) was also obtained in sufficient quantity for detailed structural studies. At that time, the preliminary structure 2 for cephalosporin P_1 had been proposed by Halsall[19] and a close relationship between cephalosporin P_1, helvolic acid, and fusidic acid through mass[20] nuclear magnetic resonance[27] and infrared spectroscopic comparisons was established. In this thesis, evidence is now presented supporting structure 1a for cephalosporin P_1.

The organic extract from the fermentation broth of *Cephalosporium* was shown by paper chromatography and by bioassay to be free of cephalosporin C. The material migrating to the cephalosporin P area on the paper strip (70% propanol) showed antibiotic activity against *S. aureus* (10 γ), *B. subtilis* (50 γ), *S. faecolis* (25 γ), and *Trichophyton* (100 γ) but was not active against gram-negative organism.[34] These antibacterial activities were consistent with the data previously reported for cephalosporin P.[8]

Preliminary thin-layer chromatography studies of the organic

extract revealed that it contained a number of components which could only be separated with difficulty. Our attempts to repeat the separation and isolation of these components by column chromatography and counter-current distribution[8] proved to be fruitless. A benzene solvate isolation procedure[22] was adapted which provided crystalline material. This procedure was pointed toward isolation of an acidic component detected during the preliminary examination of the crude extract. A crystalline substance, mp 197-198°, $[\alpha]_D$ 37.6° (c 0.5, CH_3OH) was thus isolated through the benzene solvate procedure in about 18% yield. The elementary analyses and mass spectral measurements of this substance and a considerable number of its derivatives agreed with the formula $C_{31}H_{48}O_7$ for the unknown substance.

The physical constants of this substance were not in agreement with those of cephalosporin P_1 (1a). However, its molecular formula, $C_{31}H_{48}O_7$ and specific rotation, 37.6°, were the same as monodesacetylcephalosporin P_1 (4a), although its melting point, 197-198°, was not the same as previously reported.[8] Unfortunately, at this time a sample was not available for comparison.

The following evidence from chemical reactions and instrumental studies of reaction products was compiled to prove or deny that the crystalline material obtained from the benzene solvate isolation procedure is 4a and its methyl ester is 4b.

This crystalline substance was converted to its methyl ester with diazomethane. The melting point, 232-233°, and specific rotation, $[\alpha]_D$ + 29° (c 0.5, CH_3OH), of this methyl ester corresponded well with the data previously reported for 4b.[8] This new methyl ester showed a peak in the nuclear magnetic resonance spectrum (nmr)

at δ 1.92 (s) corresponding to the C_{16} acetoxy methyl protons of 1b. However, as expected, the second acetoxy methyl signal at δ 2.06 (s) due to 1b was missing.[27] The mass spectrum of 4b showed a parent ion peak at mass 546 ($C_{32}H_{50}O_7$) and a prominent M-60 peak at mass 486 indicating the presence of one easily removed acetate group. The M-120 peak, which is prominent in the mass spectrum of 1b, is a minor peak in the spectrum of this new substance.

In addition to these spectral data obtained from 4b, evidence gathered from some chemical reactions to be subsequently described also establish that this new crystalline compound is 4a. The carboxyl group is part of an α,β-unsaturated acid moiety since the ultraviolet spectrum showed absorption maximum at 220 mμ (ε 8000). The absorption due to vicinal hydroxyl groups was observed in the infrared spectrum of 4b at 3640 and 3580 cm^{-1}, which indicates intramolecular hydrogen bonding between two adjacent hydroxyl groups.[30]

The presence of vicinal hydroxyl groups was further confirmed through the formation of an acetonide derivative,[31] mp 158-159°. The proof of the structure 6 for this acetonide will be given in the latter part of this thesis. The infrared spectrum of this acetonide derivative still showed a band at 3640 cm^{-1} indicative of the presence of a third hydroxyl group in the new substance; thus the seven oxygen atoms of 4a ($C_{31}H_{48}O_7$) are accounted for. These seven oxygen atoms in 4a are assigned as two hydroxyl groups, one carboxyl group and one acetoxy group. This is also consistent with the functional group assignment of 4a.[8] As will be described later, a sample of 1a was obtained from the crude organic extract of *Cephalosporium acremonium*. The conversion of the pure cephalosporin P_1 (1a) to 4a

through the same benzene solvate isolation procedure completely confirmed that this new substance is 4a and its methyl ester is 4b.

After the isolation and identification of monodesacetylcephalosporin P_1 (4a) from the organic extract of the culture fluid of *Cephalosporium acremonium*, a question arose whether 4a was originally present in the crude organic extract or whether it was an artifact produced by alkaline hydrolysis of 1a since a 5% aqueous sodium hydroxide solution was employed during the benzene solvate isolation procedure. Further, an acetoxy group vicinal to a hydroxyl group is known to facilitate hydrolysis.[32]

In order to clarify this point, an effort was made to isolate 1a and 4a directly from the crude organic extract under neutral conditions so that interconversion between them was prohibited.

Cephalosporin P_1 (1a) was finally obtained from the crude extract in about 3-4% yield through column chromatography on neutral silica gel and repeated crystallization of various fractions from the column. The yield of crystalline material was greatly increased when the crude extract was methylated with diazomethane before column chromatography. This procedure, though tedious, gave 1b in about 20% yield.

The identity of 1b was made from elemental analysis and mass and nmr spectral studies. The elemental analysis showed that its molecular formula is $C_{34}H_{52}O_8$ corresponding to the parent ion peak at mass 588. The nmr spectrum agrees with that reported by Melera.[27]

An attempt to separate 4a from the crude extract by column chromatography in the same manner was not successful. However, certain fractions from the column were found to be enriched in 4a as

shown by a thin-layer chromatogram. These fractions were then methylated and subjected to preparative thin-layer chromatography on silica gel. The spot with R_f value corresponding to 4b was scraped from the plate and extracted to give about 3% yield of crystalline solid which was shown by X-ray crystallography to be identical with 4b obtained from the benzene solvate procedure.

These facts revealed that although 4a, obtained through the benzene solvate procedure from the crude extract, mainly originated from 1a, it is present as an original component of the extract.

The presence of 4a in the organic extract of *Cephalosporium acremonium* did not account for the entire antibacterial activity originally present in the extract. Some activity was found to remain in the butyl acetate layer from the benzene solvate process. These data indicated that the organic extract contained more than one active component[8] and that the activity of these components were not destroyed by the 5% sodium hydroxide solution used in the benzene solvate process. However, 4a does show antibacterial activity, although the activity was considerably reduced compared with that of 1a. In Table I, a comparison of antibacterial activity between fusidic acid (3), ramycin[33] and 4a in terms of minimum gamma per ml required to inhibit the microorganisms is shown. Ramycin was isolated by Dijck[33] from *Mucor ramannianus* and was structurally related to 1a from *Cephalosporium acremonium* and 3 from *Fusidium coccineum* by nmr, UV, and IR spectra.[34] Ramycin also gave an identical mass spectrum[34] with that of 3. Ramycin was later shown to be identical with 3.[35] The antibacterial activity presented in Table I confirmed this finding.

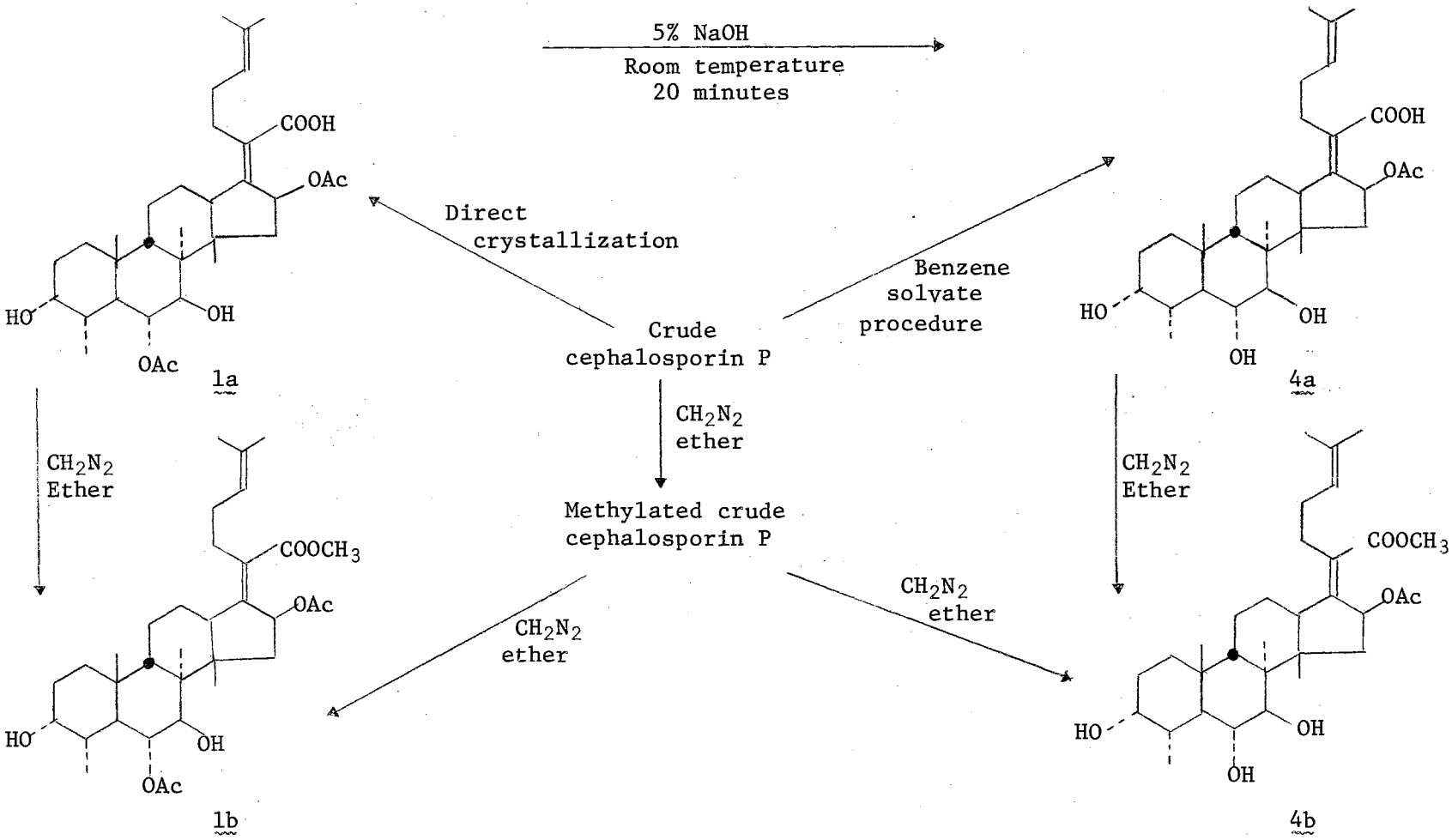


Figure 1. Isolation of Cephalosporin P_1 (1a) and Cephalosporin P_{da} (4a)

With the isolation of 1a and 4a in pure form and in sufficient quantities, it became possible to thoroughly investigate their structures.

TABLE I

ANTIBACTERIAL ACTIVITY OF RAMYCIN, FUSIDIC ACID (3) AND CEPHALOSPORIN P_{da} (4a)

	Ramycin	3	4a
S. aureus 3055	0.4	0.4	12.5
S. lutea	0.8	0.8	12.5
C. diphtheriae X-166	0.0125	0.0125	0.4
B. subtilis X-12	3.1	1.6	100
M. tuberculosis	25	25	100

At this time, the proposed structure 2 for cephalosporin P_1 was published.[19] Through mass and nmr spectral studies, a close relationship between cephalosporin P_1, helvolic acid, and fusidic acid also was established.[20,27] An additional tertiary methyl group which was not shown in structure 2 for cephalosporin P_1 was placed at C_8 position of fusidic acid (3).[23] However, the structure of cephalosporin P_1 as shown in structure 2 was incomplete. The placement and stereochemistry of various functional groups were uncertain and the conformation of the carbon skeleton also was not certain.

Certain experiments, to be described below, were devised to learn the placement and configuration of various functional groups

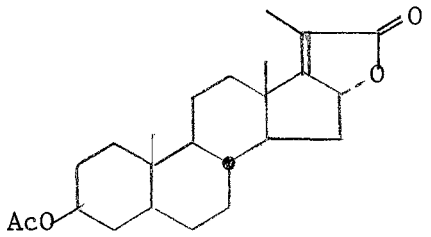
in 1a and 4a. Additional experiments were designed to test the results from mass, nmr, and infrared spectral determinations and whether all the data, as obtained, could be applied to the recently established *trans-syn-trans* B-boat conformation of fusidic acid (3).[28,29] The conformation of the cephalosporin P_1 molecule had to be established before the configuration assignment of its functional groups could have any meaning.

The relative placement of side chain carboxyl groups and the acetoxy group at C_{16} of 1a was established through several γ-lactone-forming reactions.

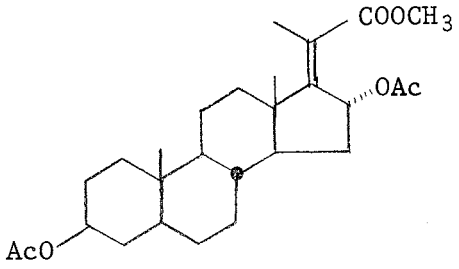
When 4a was pyrolyzed[36] at 240° under a nitrogen atmosphere, the α,β-unsaturated γ-lactone 5, mp 180-181°, was readily produced in about 35% yield. The structure of 5 was established by elemental analysis and instrumental data. The absorptions at 224.5 mμ (ε 13,500) of 5 and 1740 cm^{-1} are characteristic of an α,β-unsaturated lactone. The mass spectrum of 5 showed a parent ion peak at mass 472 ($C_{29}H_{42}O_5$). The M-60 peak which was prominent in the spectrum of 4b is no longer present as would be expected for the spectrum of 5. The nmr spectrum of 5 showed the chemical shift due to the secondary proton attached at C_{16} in 4b was no longer present.[27,28] Therefore, the lactone ring presumably formed by loss of acetoxy group from C_{16}.

In order to determine the configuration of the carbon oxygen bond at C_{16} of 5, optical rotatory dispersion (ORD) measurements on lactone 5 were performed. Saturated lactones have been shown to have Cotton effects with a first extremum at about 225 mμ.[37] The Cotton effect extremum of α,β-unsaturated carboxylic acids shift to 250 mμ.[38]

The use of the Cotton effect extremum in establishing the configuration of α,β-unsaturated γ-lactones was applied by Bucourt,[39] who found that steroidal α,β-unsaturated γ-lactones also exhibit a strong Cotton effect in the region of 250 mμ. Although no theoretical interpretation of the Cotton effects of α,β-unsaturated γ-lactones comparable to the octant rule[40] for ketones or the sector rule[37] for saturated lactones had been made, the ORD curve obtained for the α,β-unsaturated lactone 11, prepared by Mazur[41] from the α,β-unsaturated acid 12, could be used as a model. This lactone, 3β-acetoxy-16α-hydroxy-$\Delta^{17(20)}$-bisnor-5α-cholenic 22,16-lactone, has the partial structure at C_{16} as shown in 11, of which the stereochemistry is known through chemical correlations.



11



12

The ORD curve of lactone 5 showed $[\alpha]_{400}$ + 20°, $[\alpha]_{295}$ + 57°, $[\alpha]_{265}$ - 27°, $[\alpha]_{261}$ - 14°, (c 0.15, 1 cm, C_2H_5OH) as shown in Plate I. This curve is a mirror image of the ORD curve obtained from Mazur's lactone 11, $[\alpha]_{400}$ - 45°, $[\alpha]_{287}$ - 116°, $[\alpha]_{267}$ - 67°, and $[\alpha]_{263}$ - 76° (c, 0.165, 1 cm, C_2H_5OH). Since the lactone 11 has an α configuration at C_{16} and the Cotton effect for 5 was opposite in sign of the Cotton effect for 11, a β configuration was assigned to

the lactone 5 at C_{16}.

A similar conclusion about the assignment of configuration for 1a and 4a at C_{16} may be derived from the ORD data obtained from lactone 5. Arigoni[42] in 1964 showed that the lactone obtained from the 24, 25-dihydro derivative of fusidic acid (3) presented an intense negative circular dichroism maximum ($[\theta]_{250}$ = 29,700), which is comparable to the negative Cotton effect of the ORD curve of lactone 5. Since 3 was known to have a β configuration at C_{16},[28,29] therefore the C_{16} acetoxy groups in 1a and 4a are assumed to have the β configuration provided the lactone rings were closed in both cases without an inversion in configuration at C_{16}. This assumption seems reasonable since the same type of lactone ring formation was observed on heating the α,β-unsaturated ester 12 with potassium hydrogen sulfate at 170° to yield lactone 11.[41] This conversion shows the configuration of the double bond and C_{16} acetoxy group in the α,β-unsaturated ester 12 to be as indicated.[41] Moreover, the same transformation of 12 to 11 could also be accomplished through successive treatment with potassium hydroxide in boiling methanol, acidification, and reacetylation.[41] Application of these reactions to cephalosporin P_1 (1a) or 4a gave the lactone 5 in 75% yield. The identity of 5 from the two sources was confirmed since there was no depression in melting point of a mixture of the lactones. Other instrumental data from mass, nmr, IR, UV, and ORD spectroscopy also showed they were identical.

The acetoxy group at C_{16} of 1a was found to be more hindered and more resisted to hydrolysis than the acetoxy group attached to ring B. While the ring B acetoxy group, which is at the vicinal position of a

ORD CURVE OF LACTONES 5 AND 11

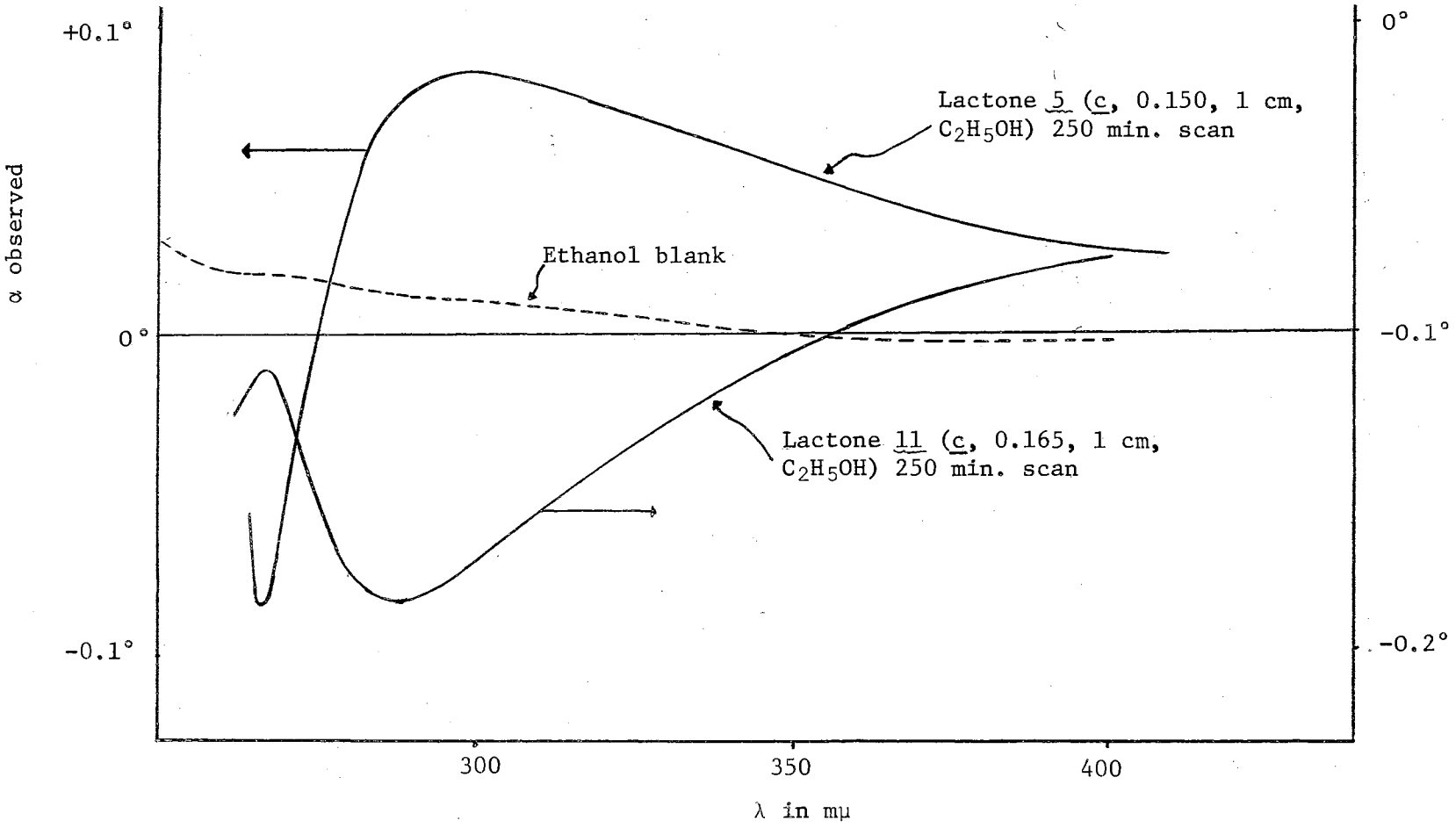


Plate I

hydroxyl group, is readily hydrolyzed[32] in a cold 5% sodium hydroxide solution after stirring for about twenty minutes, the C_{16} acetoxy group in 1a survives this treatment even after stirring for one hour. However, the C_{16} acetoxy group does hydrolyze after heating 1a and 4a in refluxing dilute alkali for four hours. The precipitate obtained from acidifying the aqueous solution with dilute hydrochloric acid was crystallized from methanol to yield the bisdesacetylcephalosporin P_1 (13), mp 125°, in 60% yield. Its structure was established by its infrared spectrum which showed the absorption due to a hydroxylgroup and the ultraviolet spectrum $\lambda_{max}^{C_2H_5OH}$ 226 mμ (ε 6,900) indicative of an α,β-unsaturated carboxylic acid group. The nmr spectrum no longer showed the signal corresponding to acetoxy methyl protons; therefore, the two acetoxy groups in 1a and one acetoxy group in 4a were completely removed.

That the configuration of the newly formed C_{16} hydroxyl group of 13 is β was shown by converting it into the α,β-unsaturated lactone 5. This was done by dissolving 13 in methanol and subjecting it to column chromatography on basic alumina. A crystalline solid, mp 180-181°, was obtained in 90% yield which was shown to be identical to lactone 5 by mixed melting point determination and comparison of IR, UV, nmr and mass spectra. The hydrolysis of the C_{16} acetoxy group in 1a with strong alkali without inversion of configuration is also consistent with the hydrolysis reaction observed in the fusidic acid (3) series.[22] The lactone ring formation of 5 presumably proceeds along the same reaction path as described in the previous paragraph.

To establish whether the alkaline conditions of the benzene

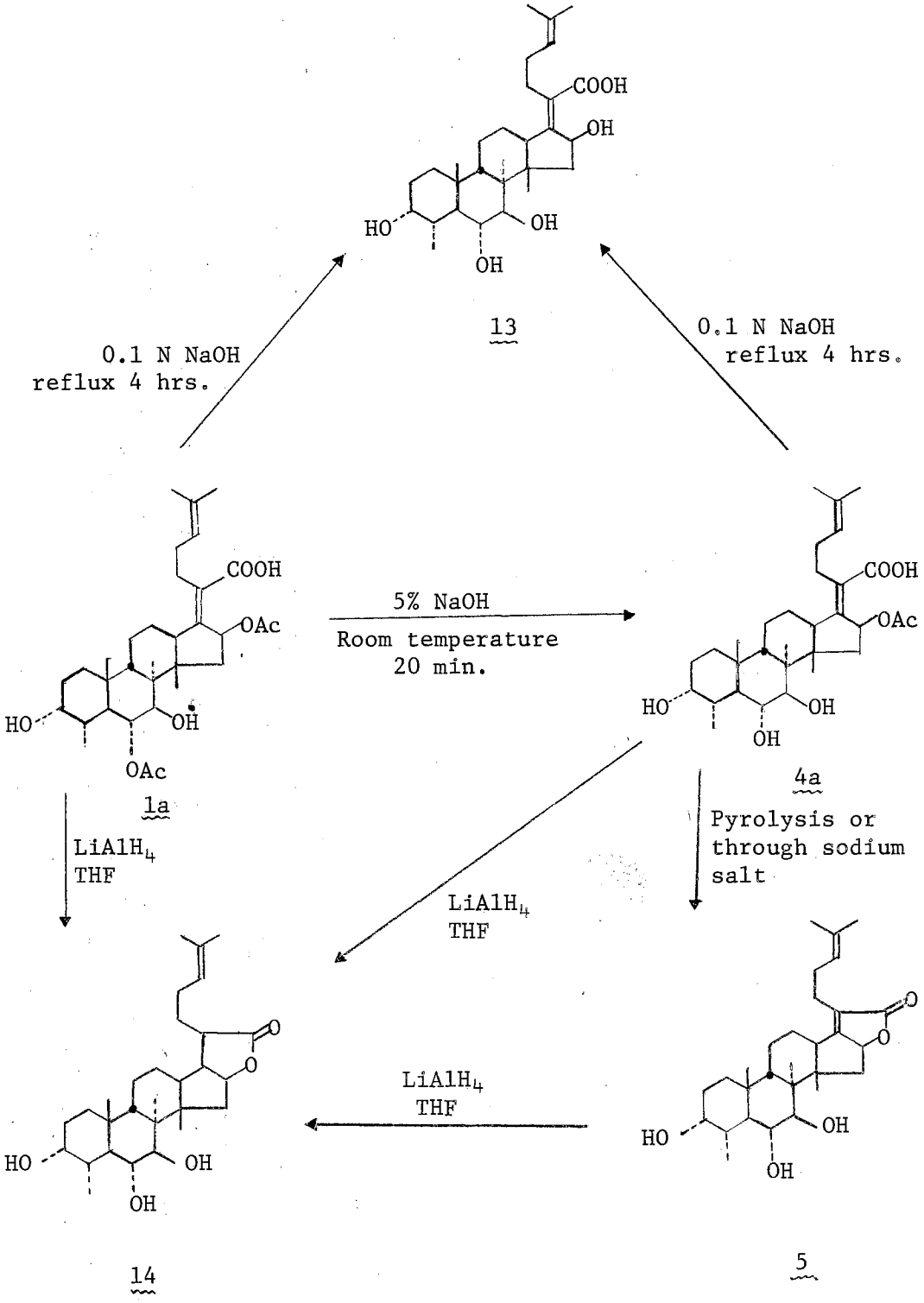
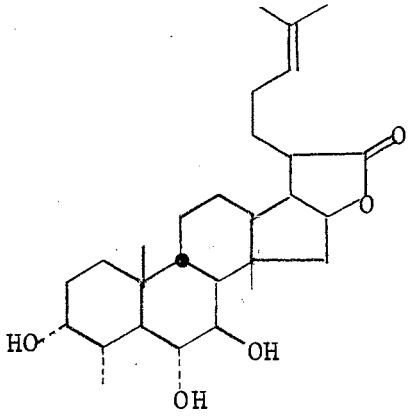


Figure 2. Stereochemical Studies Involving C_7 and C_{16} of Cephalosporin P_1 (1a) and Cephalosporin P_{da} (4a).

solvate procedure could have caused epimerization of monodesacetyl-cephalosporin P_1 (4a) during isolation, lithium aluminum hydride reductions[43] of cephalosporin P_1 methyl ester (1b) and monodesacetyl-cephalosporin P_1 (4b) were carried out. The reason for selecting this reaction is that the reduction of acetoxy groups with lithium aluminum hydride is believed not to alter the stereochemistry at an asymmetric center.[43] The reduction products from these esters (each gave two products) showed identical R_f values (0.35 and 0.61). The major products (R_f 0.61), mp 222-223°, from the two sources were proven to be identical by X-ray diffraction studies, indicating that the product from both reactions resulted from the same reaction path. The infrared spectrum showed a band at 1770 cm^{-1}, indicative of a saturated γ-lactone (14). The mass spectrum showed peaks at mass 474 and mass 392, which represent the parent ion of $C_{29}H_{46}O_5$ and side-chain elimination with hydrogen transfer through an expected McLafferty rearrangement.[44] In the mass spectrum of the unsaturated lactone 5, there is no peak corresponding to the elimination of side-chain.

The lack of absorption in the region of 225 mμ in the ultraviolet spectrum and absence of Cotton effect of the ORD curve in the vicinity of 266 mμ provided additional evidence that the unsaturated γ-lactone 5 had been reduced to the saturated γ-lactone 14 during lithium aluminum hydride reduction. Possibly the insolubility of the resulting salts prevent reduction by lithium aluminum hydride. The lactone 14 was found to be readily formed from the material with R_f 0.35 since, on treatment of the latter with dilute hydrochloric acid, a product is formed which has R_f 0.61 identical to the R_f value

of 14. Accordingly, the material with R_f 0.35 is probably the hydroxy acid. In a subsequent experiment, the unsaturated lactone 5 was reduced with lithium aluminum hydride to the saturated lactone 14 (R_f 0.61); therefore, lactone 5 is probably an intermediate in the conversion of 1b to 14. The hydride attack of the double bond at position 17 is assumed to occur from the less hindered α-side to give a *cis*-fused lactone ring. Thus, the saturated γ-lactone 14 probably has the β side-chain configuration at C_{17}.



14

These results indicate that no epimerization at C_6, C_7 or C_{16} had occurred during the benzene solvate isolation of 4a or during the preparation of 13. The reason for establishing that no epimerization had taken place is that acyl transfer and rearrangement through group participation during hydrolysis is known,[45] and in several examples, an inversion in configuration has been observed.[22,45]

The three hydroxyl groups in 4a were previously placed at C_3, C_6 and C_7.[19] The spectral and chemical data are now presented to confirm these assignments. As previously mentioned, the presence of vicinal hydroxyl groups in 4a was shown by the formation of the acetonide 6,

mp 158-159°, from the methyl ester 4b. The bands at 1380 and 1385 cm^{-1} in the infrared spectrum and the peak at δ 1.40 (s) in the nmr spectrum are consistent with the acetonide structure. The broad multiplet signal centered at δ 3.70 in the nmr spectrum of 6 is the same as the signal arising from the secondary proton attached to the carbon holding the C_3 hydroxyl group in the methyl ester of fusidic acid (3).[27] Therefore, it is reasonable to place the hydroxyl group in the hydroxy acetonide 6 at the C_3 position to be in accord with 3.[27,28] The assignment of this C_3 hydroxyl group in 6 is further established by oxidation to 8 with chromium trioxide in pyridine.[46] The nmr spectrum of 8 no longer showed the broad multiplet at δ 3.70. The infrared spectrum of 8 showed an additional band at 1708 cm^{-1} indicative of a six-membered ring ketone. This ketone carbonyl group is therefore located at C_3.

The location of the vicinal hydroxyl groups in 4a with respect to the C_3 hydroxyl group was established through a series of oxidations described below.

Monodesacetylcephalosporin P_1 (4a) was first hydrogenated in the presence of 2% palladium on carbon[31] to give a 90% yield of 24, 25-dihydromonodesacetylcephalosporin P_1 (15a), mp 201-202°. Treatment of 15a with diazomethane gave its methyl ester 15b, mp 262°. The reduction of the isopropylidine moiety in 15b was confirmed by the absence of acetone as an ozonization product of the methyl ester 15b. However, it was shown that an isopropylidine moiety was present in 4b since the 2,4-dinitrophenylhydrazone of acetone was obtained. In addition, the nmr spectrum of 15b no longer showed the vinylic proton signal at δ 5.13 (bt). This signal is characteristic of the C_{24}

vinylic proton at the side-chain of 1a and the methyl ester of fusidic acid (3)[27,28] as shown in Table II. Careful examination of the mass spectral data of 15b and 1b also reveal that some of the different fragmentations may be attributed to the difference in saturation at the C_{24} and C_{25} positions of the side-chain. This point will be discussed later.

The dihydro derivative 15b was subjected to chromic acid oxidation[46] which gave the yellow, crystalline triketone 16, mp 205-208°. The IR, UV, and nmr spectra and elemental analysis substantiate the structure. The ultraviolet spectrum of 16 is the most informative. It did not show absorption between 260 and 340 mμ; however, when the measurement was performed in acidic methanolic solution, two maxima at 219 mμ (ε 8,290) and 282 mμ (ε 4,500) appeared as shown in Plate II. This phenomenon was considered as strong evidence of a conjugated triketone system[19,47] involving carbonyl functions at C_3, C_6, and C_7 of the steroid skeleton of 1a and the possible placements of the vicinal hydroxyl groups at C_{11} and C_{12} positions are ruled out. The conjugated form of the triketone 16 in acidic or basic methanolic solution can be depicted in the partial structures 17a and 17b. This not only shows the relative positions of the three hydroxyl groups in 4a C_3, C_6, and C_7 but also demonstrates that a proton must be present at C_5 to make the conjugated forms 17a and 17b possible.

Similar triketone derivatives 18a, mp 172-177°, and 18b, mp 207-210°, were obtained from chromic acid oxidation[46] of 4a and 4b respectively. The ultraviolet spectra of 18a and 18b in acidic methanol solution also resembled that of 15. The triketone 16 melted 20 degrees above that reported by Okuda for the corresponding trione

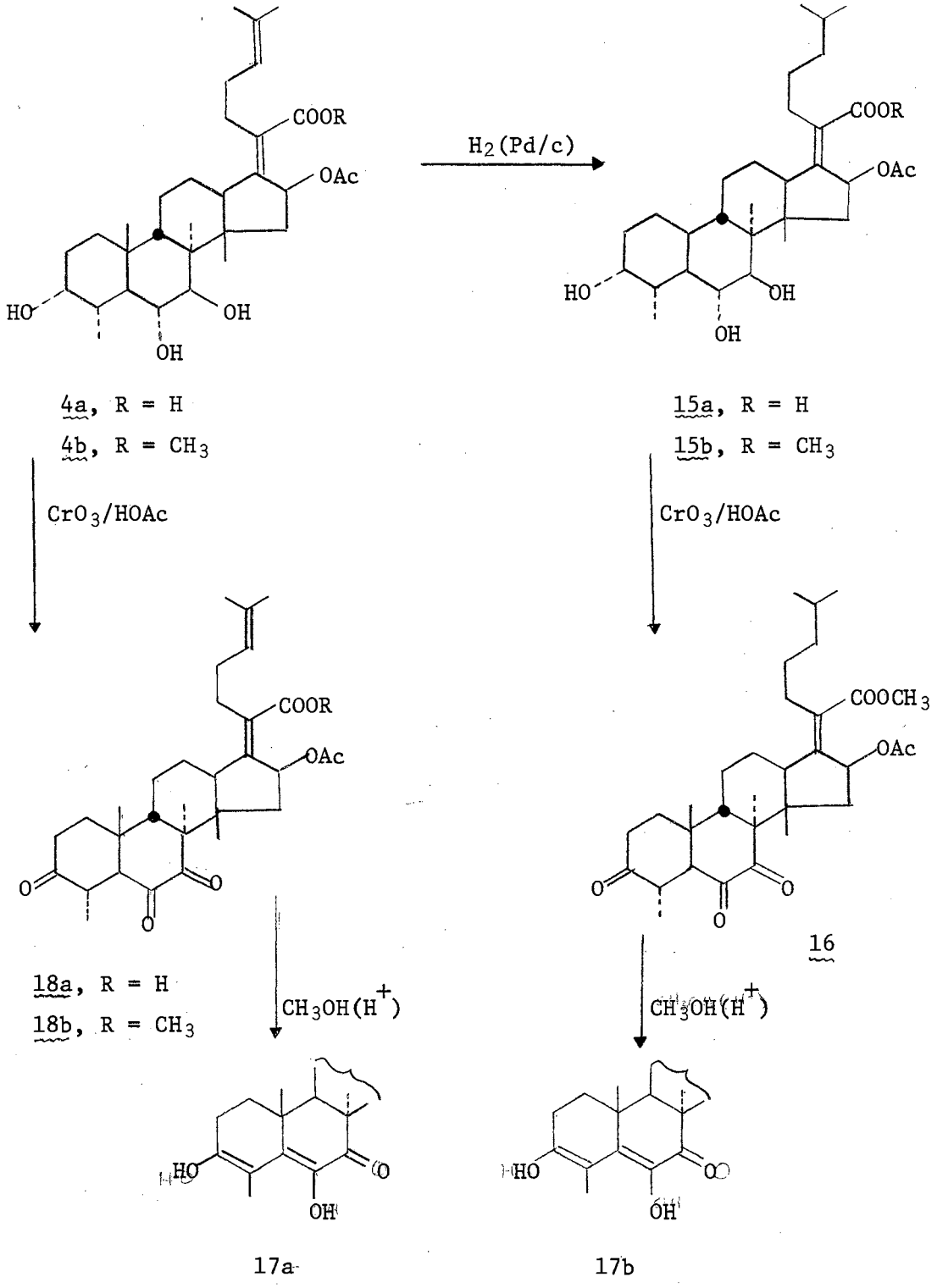


Figure 3. Proof of Placement of Hydroxyl Groups at C_3, C_6, and C_7 of 4a and 15a.

Ultraviolet Spectra of Triketones 16 and 18b

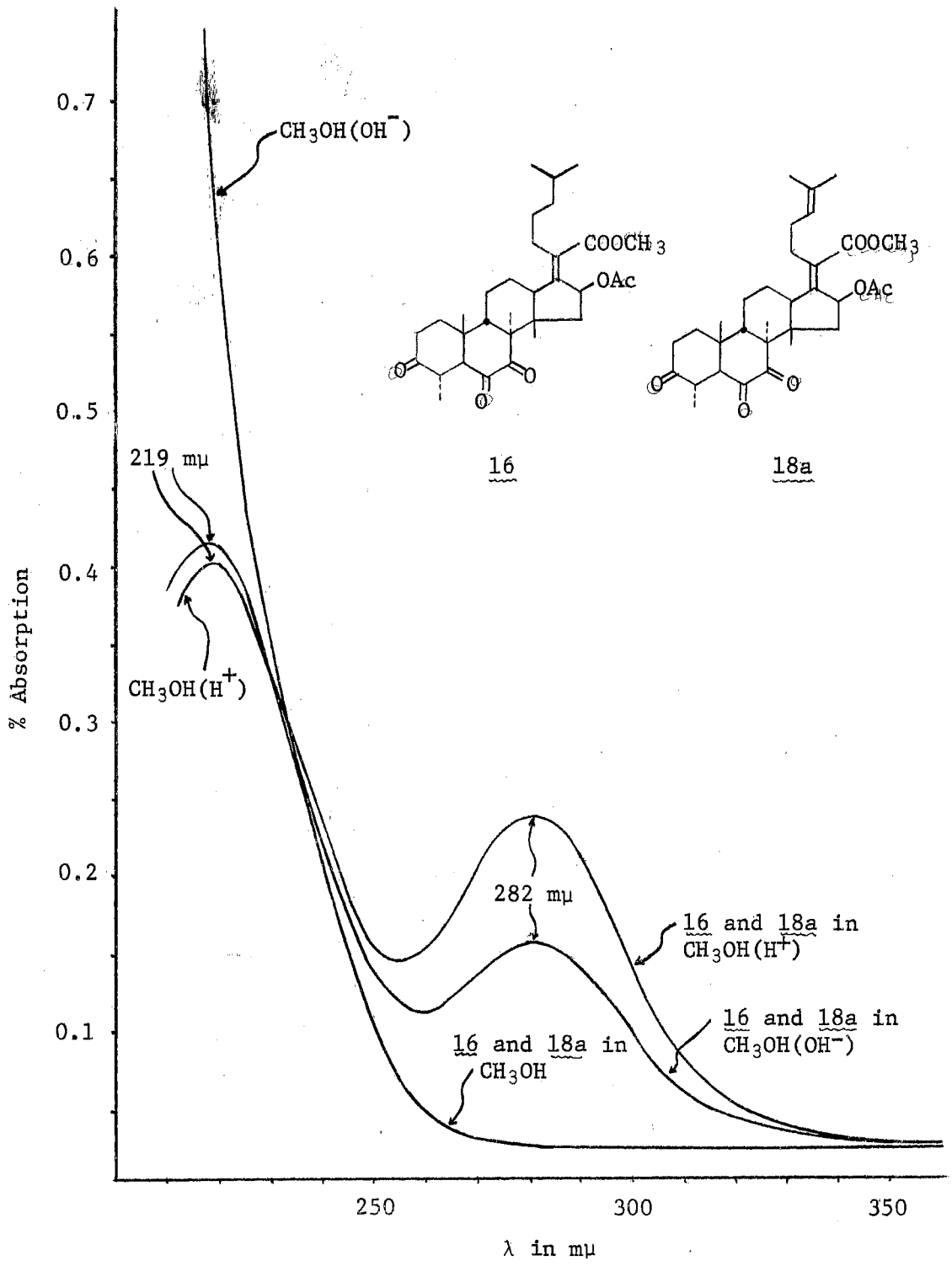


Plate II

from dihydrohelvolic acid methyl ester.[47] Since a comparison sample was not available, an effort was made to convert triketone 16 to its enol-acetate derivative 19[19] in the hope that 19 could be compared with a similar enol-acetate derivative prepared from helvolic acid by Okuda.[47] But because of the small quantity of triketone 15 available, the expected enol acetate 19 was not obtained in pure crystalline form and the attempt to link with Okuda's enol-acetate derivative of helvolic acid[47] through physical constants and spectral comparisons failed.

However, a direct comparison of methyl ester 4b with a sample of monodesacetylcephalosporin P_1 methyl ester prepared by Halsall was performed.[48] The melting point of the former was 233-236° and of the latter, 233-237°. The mixed melting point was 233-237°; thus, no depression had occurred. Additional comparison was made through infrared spectroscopy and thin-layer chromatography.

The enol-acetate 9, prepared by Halsall,[19] was later shown to be identical to an enol-acetate derivative prepared from helvolic acid.[48] Therefore, cephalosporin P_1 (1) must have the same type of carbon skeleton as that of helvolic acid.

After placement of the three hydroxyl groups in 4a and 4b at C_3, C_6, and C_7, attention was shifted to the determination of their relative configurations. The ready formation of acetonide 6 from 4b need not necessarily show the vicinal hydroxyl groups at C_6 and C_7 in 4 are *cis*, since *trans* diol systems have been shown to form acetonide derivatives quite easily. This has been observed for systems containing a flexible boat conformation.[49] According to Kuhn,[30] in certain cases, the relative configurations of adjacent hydroxyl groups can be

shown from the difference in hydroxyl stretching frequencies corresponding to an intramolecular hydrogen-bonded hydroxyl and a free hydroxyl group. Although the IR spectrum of 4b does show bands at 3580 and 3640 cm^{-1} indicative of hydrogen bonding between two adjacent hydroxyl groups, a definite decision about the configuration of the two hydroxyl groups could not be obtained.

An acetylation reaction was then used to test the reactivity of the three hydroxyl groups in 4b, hoping that some idea about their configurations could be gained.[50] Since 1b is a monoacetate of 4b, attempts were also made to quench the acetylation reaction of 4b at an intermediate stage in the hope that 1b could be isolated from the reaction mixture.

Anhydrous pyridine and acetic anhydride were used to acetylate 4b. The reaction mixture was tested on silica gel thin-layer plates. After four hours of reaction at room temperature, four pronounced spots with R_f values 0.75, 0.68, 0.62, and 0.48 respectively were shown on a thin-layer chromatogram. These spots were labeled A, B, C, and D. Spots A and C were the more pronounced of the four, with spot C more intense at the early stage of the reaction. The intensity of spot A gradually increased, and the spot due to starting material disappeared completely after two days. However, the four spots due to products persisted even after the reaction had been carried out for three days. Attempts were made to separate these four spots using preparative thin-layer chromatography, but because of a severe tailing effect, these efforts were not successful.

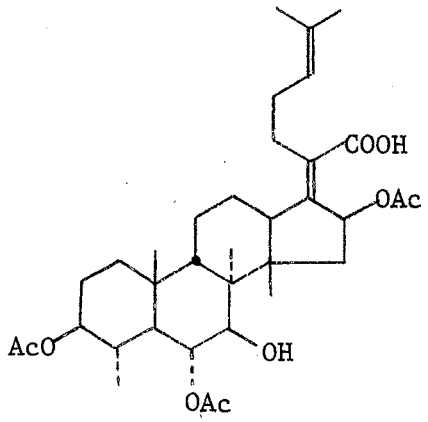
Following the isolation of 1a from organic extract of *Cephalosporium acremonium*, a sample of cephalosporin P_1 methyl ester (1b) was

thus available for a direct comparison of 1b with the acetylation products of 4b through R_f values. The R_f value of 1b was found to coincide with spot C of R_f 0.62. Thus, one of the acetylation products of 4b must be 1b.

This acetylation reaction was applied to 1b. The reaction was also carried out for three days at room temperature, and the reaction mixture was tested from time to time on silica gel tlc plates. Surprisingly, the starting material 1b completely disappeared after two days and a single product was detected, which showed an R_f value identical to spot A from 4b.

The reaction was repeated on larger scale, and again, a single product was observed. The product was isolated in 60% yield by extraction and purified with the aid of preparative thin-layer chromatography. The product was shown to be the amorphous[51] triacetate 20 by elemental analysis, and the IR spectrum which shows the presence of a hydroxyl stretching band. The broad multiplet centered at δ 3.70 corresponding to the secondary proton attached at C_3 of 1b was no longer present in the nmr spectrum, but the singlet at δ 3.50 remained. These data together with the upfield shift of the C_4 methyl signal to δ 0.81 (J 6.0 cps) indicated that the hydroxyl group at C_3 of 1b was acetylated. The new broad multiplet at δ 4.90 therefore arises from the secondary proton attached to the C_3 carbon holding the newly formed acetoxy group. The presence of three acetoxy groups in 20 was also indicated from its mass spectrum. In addition to the parent ion peak at mass 630, prominent peaks corresponding to M-60, M-120 and M-180 are also shown in Table III. The proof of location of the acetoxy group at C_6 instead of at C_7 of 1 and 20 was established by

nmr and ORD studies of 1a, 20, 21, and 22 will be described later.



20

Several attempts were made to acetylate the C_7 hydroxyl of 20 in order to obtain a peracetate derivative.[52] These experiments gave products which on thin-layer chromatography showed one spot when 1b or 20 was used and four spots when 4b was used.

From the above acetylation reactions, it is obvious that one of the two hydroxyl groups at C_6 and C_7 in 4a is very readily acetylated; thus, its orientation is probably equatorial.[50] The second reactive hydroxyl is at C_3 of 4a. This is also consistent with the previous axial assignment of the C_3 hydroxyl in structure 2.[19] However, the third hydroxyl group is so hindered that it cannot be acetylated under any conditions ordinarily used for acetylation of steroids;[52] and therefore, it must be in a 1,3-diaxial relationship[50] to one of the tertiary methyl groups at C_{10} or C_{14}[19,28] and undoubtedly located at C_6 or C_7.

Structures 1a and 20 have been used to illustrate the location and configuration of the functional groups in ring B. The correctness of the structural assignments in 1a and 20 was established by a

series of oxidation and reduction experiments applied to 21, 22, and 23 and instrumental studies of the products from these reactions.

The triacetoxy ketone 21 was prepared by chromic acid oxidation of the triacetoxy alcohol 20.[46] The IR spectrum of 21 showed the absence of hydroxyl stretching frequency and the presence of a band at 1710 cm^{-1} indicative of a six-membered ring ketone. In contrast to the difficulty in acetylating the C_7 hydroxyl group of 20, chromic acid oxidation of the C_7 hydroxyl group proceeded readily, which supports the axial assignment for this group.[50] Oxidation of 1b under the same conditions gave the diacetoxy diketone 22. As expected, the nmr spectrum of 22 showed a downfield shift of the C_4 methyl protons signal to δ 1.28 from its initial value of δ 0.90 in 1b. Other spectral data and elemental analysis also substantiated structure 22 for the diacetoxy diketone.

When the diacetoxy diketone 22 was subjected to Clemenson reduction with zinc and acetic acid, the monoacetoxy diketone 23 was formed.[53] The monoacetoxy diketone 23 was shown by tlc analysis to be contaminated with a small amount of its starting material, 22.

The nmr data for the nuclear protons of 1b, 20, 21, and 22 at 3, 6, and 7 are shown in Figure 5. Since an α and axial proton is known to be present at C_5 in 1b and the C_8 position is fully substituted,[20,27] the doublet at δ 4.57 in the spectra of 1b and 20 and at δ 5.30 in 21 and 22 are considered to be due to the signal from the secondary proton attached to the C_6 carbon which also holds an acetoxy group. Consequently, the signals δ 3.50 in the spectra of 1b and 20 are due to the secondary proton attached to C_7 carbon. As expected, the downfield shifts of the doublet signals from δ 4.57 in the spectra

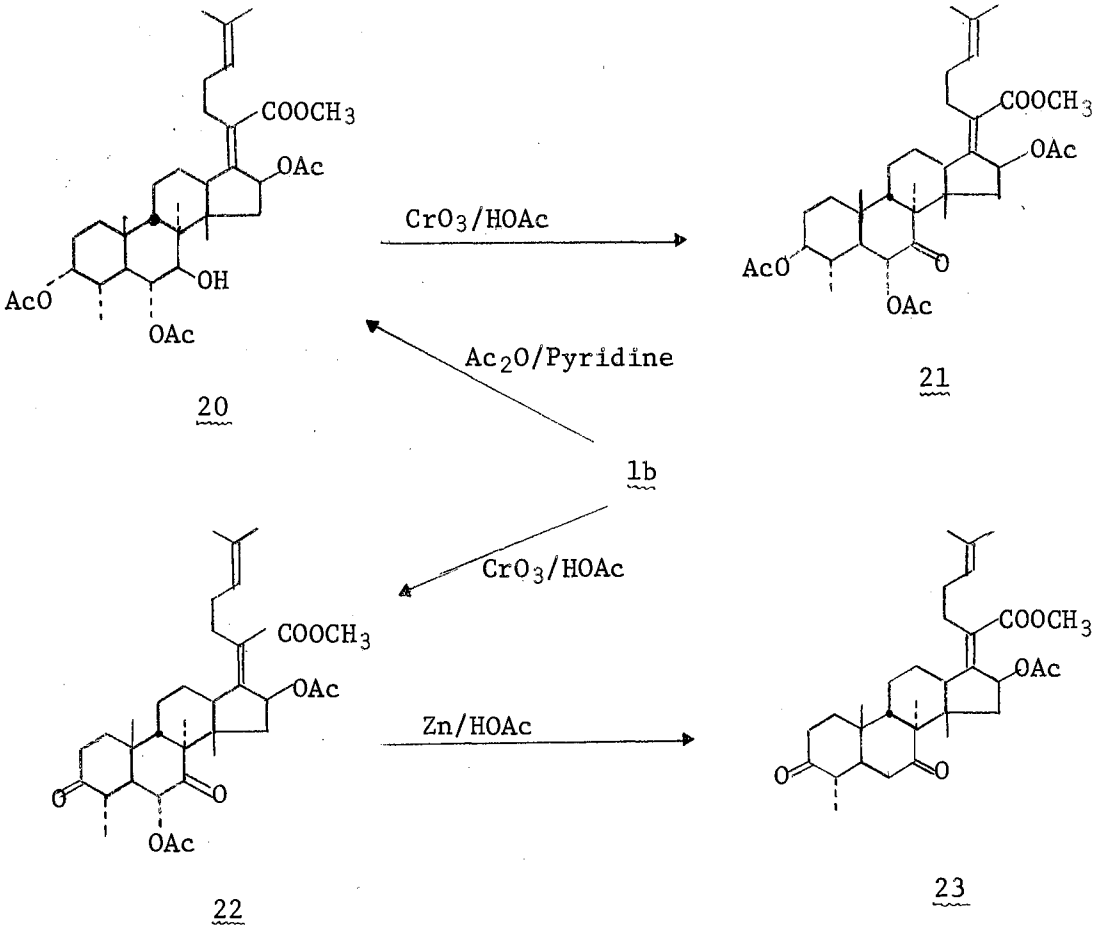
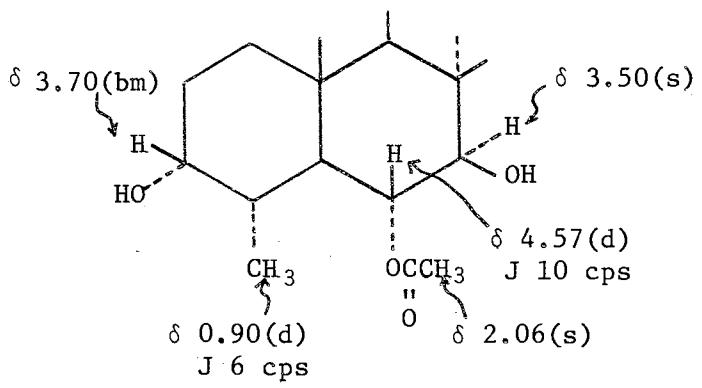


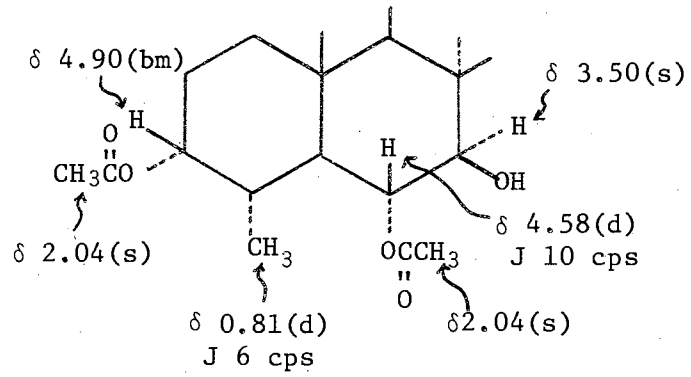
Figure 4. Preparation of Derivatives for ORD Studies

of 1b and 20 to δ 5.30-5.32 in those of 21 and 22 are due to the influence of a carbonyl group at C_7 of 21 and 22. The downfield shifts of the signals in the spectra of 21 and 22 corresponding to one of the acetoxy methyl protons at δ 2.06 for 1b and δ 2.04 for 20 to δ 2.18 for 21 and δ 2.20 for 22 are consistent with this observation. The large coupling constants between C_5 and C_6 protons in 1b, 20, 21, and 22 indicate that these are diaxially coupled; therefore, the C_6 proton must be β and axial. Thus, the C_6 acetoxy in 1b and in its derivatives, 20, 21, and 22, must be α and equatorial.

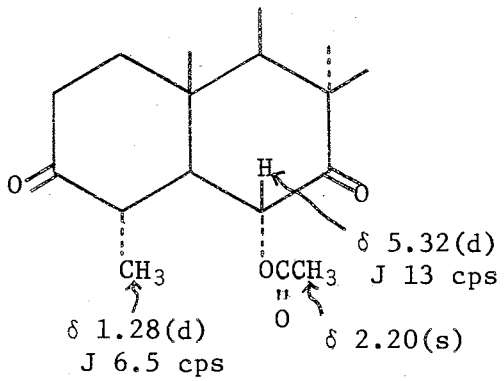
A distortion in boat conformation in ring B of 21 and 22 is



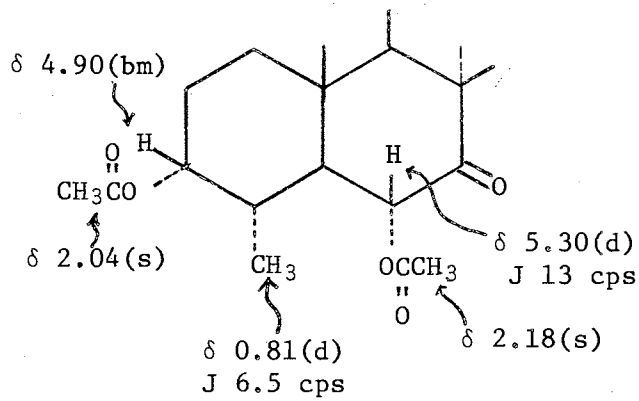
1b



20



22

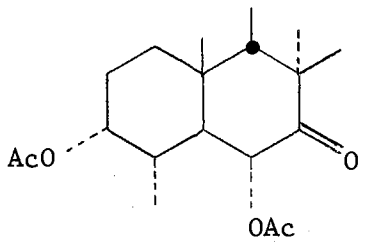


21

Figure 5. NMR Chemical Shifts of Nuclear Protons of 1b, 20, 21 and 22

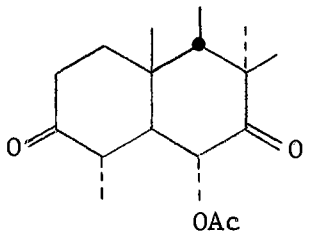
evident since the coupling constant between the C_5 and C_6 protons changed from 10 cps for 1b and 20 to 13 cps for 21 and 22.

Additional evidence that the stereochemical assignment of the 6α-acetoxy group for 1a, 20, 21, and 22 is correct was obtained from the ORD data of 21, 22, and 23, which showed negative Cotton effects with successive decrease in amplitude in their ORD curves. The ORD amplitude data are shown below:



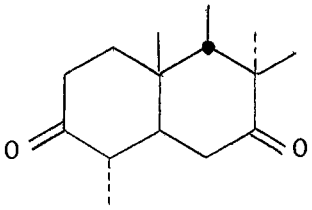
21

$[\alpha]_{317} = -1025°$



22

$[\alpha]_{320} = -516°$



23

$[\alpha]_{320} = -398°$

Examination of Dreiding models employing a ring B boat conformation for 21, 22, and 23 and application of the octant rule[40] shows the 6α-acetate is in a negative octant relative to the carbonyl at C_7, and thus a substantial negative contribution can be expected. A positive contribution to the Cotton effect could be expected from a 3-keto group which appears in a positive octant and therefore changes the negative value from -1025° for 21 to -516° for 22. The removal of the 6α-acetate which is in a negative octant relative to the C_7 carbonyl also changes the negative value from -516° for 22 to -398° for 23. On the other hand, these ORD data would have a different interpretation if the A/B ring junction were *cis*.

As previously mentioned during the discussion of the acetylation reactions of 4b, one of the vicinal hydroxyl groups in ring B was

probably 1,3-diaxial in its relationship to one of the tertiary methyl groups at C_{10} or C_{14}. Once the acetoxy group in ring B of 1b was fixed at the 6α position, it became possible to use this information in deciding the relationship between the axial C_7 hydroxyl and the axial C_{14} methyl of 1b and 4b.

The β and axial configuration of this C_7 hydroxyl group is evident in the nmr spectral data presented in Figure 6.

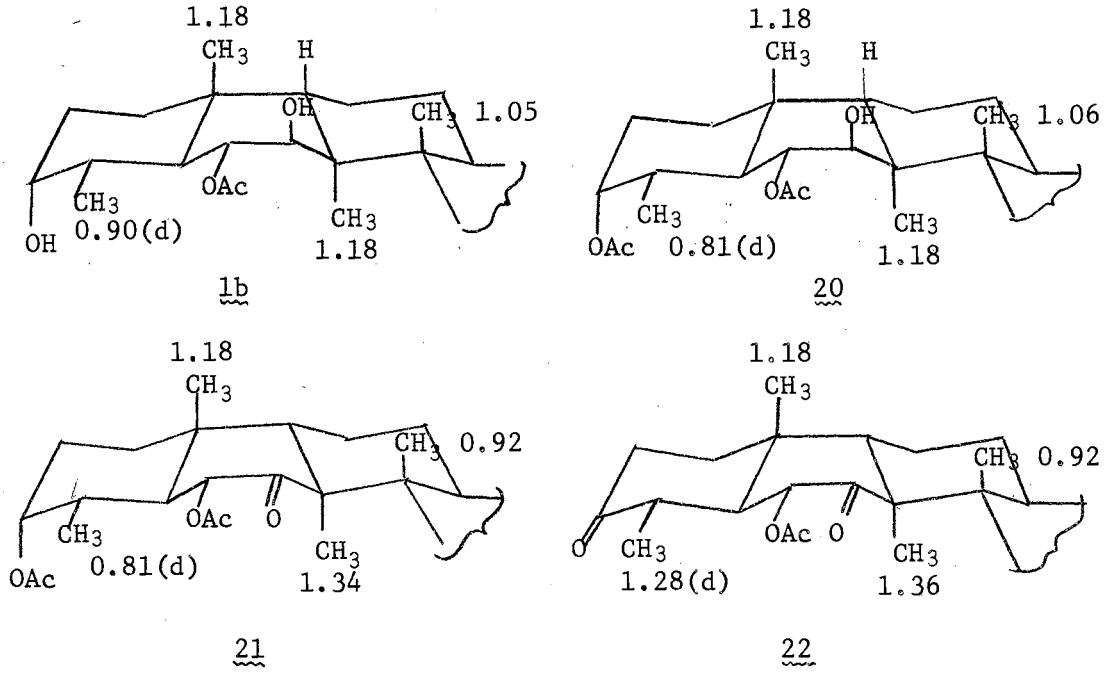


Figure 6. NMR Chemical Shifts of Methyl Protons in 1b, 20, 21 and 22.

The 1,3-diaxial interaction between the C_7 hydroxyl group and the C_{18} (methyl attached to C_{14}) methyl group in 1b and 20 are released when these hydroxyl groups are oxidized to sp^2 ketone carbonyls.[50] This is the reason for the upfield shifts to δ 0.92 for the signal corresponding to the C_{18} methyl protons of 21 and 22 compared with the position of the signal at δ 1.05 for the same methyl protons of 1a and 20. The presence of the C_7 keto group also causes the C_{32}

methyl (methyl attached to C_8) protons to shift downfield to δ 1.34 for 21 and δ 1.36 for 22. Should the C_7 hydroxyl group in 1b be α and equatorial oriented, the interaction between the C_7 hydroxyl and C^{18} methyl would not be so severe[50] and could not cause any pronounced upfield change in chemical shift for the C_{18} methyl protons. Therefore, the C_7 hydroxyl group must be β and axial in configuration.

It is known that when a hydroxyl group is acetylated, its bulkiness requires a more defined orientation away from an interacting group at 1,3-diaxial or 1,3-diequatorial positions than its parent hydroxyl group.[54] This fact is evident from the nmr data shown in Figure 6. The protons interacting with acetoxy groups experience a greater upfield shift than those interacting with hydroxyl groups.[54] Using this observation and careful examination of the nmr spectra obtained from cephalosporin P_1 derivatives, it is possible to differentiate the signals corresponding to the three tertiary methyl protons. These data are shown in Figure 6 and in Table II. The nmr data from a sample of fusidic acid methyl ester are also shown in Table II. It is evident from the data in this table that 1a and 3 are closely related.

A similar comparison was made through mass spectral measurements. These data are compiled in Table III. The molecular ion peaks of steroids containing functional groups could be obtained if a direct inlet technique were employed; therefore, a mass spectrometric comparison between fusidic acid and cephalosporin P_1 derivatives are informative.

It is known that at the inlet temperatures frequently employed in the determination of the mass spectra of steroidal alcohols and

TABLE II

NMR DATA FOR FUSIDIC ACID AND CEPHALOSPORIN P_1 DERIVATIVES

	1b	3	4b	10	20	21	22
CH_3 at C_4	0.90(d) J 6.0 cps	0.93(d) J 6.5 cps	0.93(d) J 6.0 cps	0.93(d) J 6.5 cps	0.81(d) J 6.0 cps	0.81(d) J 6.5 cps	1.28(d) J 6.5 cps
CH_3 at C_8	1.18(s)	1.39(s)	1.25(s)	1.20(s)	1.18(s)	1.34(s)	1.36(s)
CH_3 at C_{10}	1.18(s)	0.98(s)	1.15(s)	1.07(s)	1.18(s)	1.18(s)	1.18(s)
CH_3 at C_{14}	1.05(s)	0.91(s)	1.00(s)	1.00(s)	1.06(s)	0.92(s)	0.92(s)
CH_3 at $C_{26,27}$	1.60(d) J 1 cps	1.60(d) J 1 cps	1.60(d) J 1 cps	1.60(d) J 1 cps	1.60(d) J 1 cps	1.60(d) J 1 cps	1.61(d) J 1 cps
	1.68(d) J 1 cps	1.68(d) J 1 cps	1.68(d) J 1 cps	1.68(d) J 1 cps	1.68(d) J 1 cps	1.67(d) J 1 cps	1.69(d) J 1 cps
$CH_3\overset{O}{\overset{\|}{C}}-O$ at C_3	-	-	-	-	2.04(s)	2.04(s)	-
$CH_3\overset{O}{\overset{\|}{C}}-O$ at C_6	2.06(s)	-	-	-	2.04(s)	2.18(s)	2.20(s)
$CH_3\overset{O}{\overset{\|}{C}}-O$ at C_7	-	-	-	2.06(s)	-	-	-
$CH_3\overset{O}{\overset{\|}{C}}-O$ at C_{16}	1.97(s)	1.98(s)	1.92(s)	1.94(s)	1.98(s)	1.97(s)	1.97(s)
$2^{\circ}H$ at C_3	3.70(bm)	3.75(bm)	3.67(bm)	3.70(bm)	4.90(bm)	4.90(bm)	-

TABLE II (Continued)

	1b	3	4b	10	20	21	22
2°H at C_6	4.57(d) J 10 cps	-	3.40(d)	3.42(d) J 10 cps	4.58(d) J 10 cps	5.30(d) J 13 cps	5.32(d) J 13 cps
2°H at C_7	3.50(s)	-	3.40(s)	4.65(s)	3.50(s)	-	-
2°H at C_{11}	-	4.36(m)	-	-	-	-	-
2°H at C_{16}	5.87(d) J 8.5 cps	5.86(d) J 8.5 cps	5.83(d) J 8.5 cps	5.83(d) J 8.5 cps	5.85(d) J 8.5 cps	5.82(d) J 8.5 cps	5.85(d) J 8.5 cps
Vinylic H at C_{24}	5.13(bt)	5.12(bt)	5.13(bt)	5.12(bt)	5.12(bt)	5.13(bt)	5.13(bt)
$-COOCH_3$ at C_{21}	3.63(s)	3.63(s)	3.62(s)	3.62(s)	3.68(s)	3.62(s)	3.65(s)

acetates, thermally induced 1,2-elimination[55] of these functional groups may occur. For example, prominent M-60, M-78, and M-138 peaks corresponding to the loss of acetic acid, acetic acid plus water and two molecules of acetic acid plus water respectively are frequently observed in the spectra of cephalosporin P_1 derivatives. Actually in most cases, peaks at M-60 become the base peaks in the high mass range of these spectra. However, when care is taken to avoid thermal decomposition,[55] such as using the direct inlet, the molecular ions could be observed as shown in Table III. With the exception of lactones 5 and 14, all spectra were determined as the methyl ester or acetylated methyl ester derivatives of cephalosporin P_1. Although only those peaks in the high mass range are shown in Table III, the fragmentations associated with the low molecular weight peaks are similar to the spectra obtained from the bile acid methyl esters.[55,56]

Figures 7 and 8 depict the likely fragmentations which occur in this series of compounds under electron impact. Compounds 1b and 29b are used as models. The fragmentation patterns for the other cephalosporin P_1 derivatives in Table III can be explained in a similar manner. The choice and direction of elimination of acetate functions shown in Figures 7 and 8 are arbitrary when equally plausible alternatives are available. In the spectra of 1b, 3, 4b, and 20, peaks corresponding to M-60 and M-129 are prominent. These peaks are apparently due to the ready elimination of a molecule of acetic acid (mass 60) and an olefin species (C_5H_9) of mass 69 from the allylic cleavage of the side-chain. In the spectrum of the dihydro derivative 15b, the M-71 and M-131 peaks are intense since, in this case,

TABLE III

MASS SPECTRAL DATA FOR FUSIDIC ACID AND CEPHALOSPORIN P_1 DERIVATIVES

	1b		3		4b		5		14		15b		20		29b	
	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%
M	588	0	530	4	546	3	472	100	474	5	548	14	630	2	550	3
M-18							454	50								
M-36							436	80								
M-43															507	15
M-54							418	58								
M-60	528	26	470	33	486	27					488	63	570	42	490	100
M-69	519	20	461	62	477	26	403	64					561	21		
M-71											477	24				
M-83									392							
M-60-18			452	87	468	19					470	32			472	62
M-60-36			434	20	450	13					452	14			454	33
M-69-18							385									
M-69-36							367									

TABLE III (Continued)

	1b		3		4b		5		14		15b		20		29b	
	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%
M-60-32	496	29	438	18	454	37					456	88	538	28	458	33
M-60-32-18			420	40	436	27					438	41			440	43
M-60-32-36					418	22										
M-60-32-54					400	32										
M-60-69	459	26	401	28	417	50							501	30		
M-60-71											417	14				
M-60-69-18			383	100	399	100							483	10		
M-60-71-18											399	28				
M-60-69-36			365	95	381	69										
M-60-71-36											381	15				
M-60-69-32	427	27	369	19												
M-60-69-32-18			351	68												
M-60-69-32-36			333	26												
M-60-157															333	69

TABLE III (Continued)

	1b		3		4b		5		14		15b		20		29b	
	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%
M-60-157-15															318	31
M-60-157-18															315	87
M-60-157-36															297	47
M-60-158															332	22
M-60-158-15															317	55
M-60-158-18															314	23
M-120	468	36											510	62		
M-120-18	450	22											492	23		
M-120-32	436	35											478	34		
M-120-69	399	100											441	100		
M-120-69-18	381	69											423	26		
M-120-69-32	367	57														
M-120-69-36	363	28														
M-120-69-32-18	349	50														

TABLE III (Continued)

	1b		3		4b		5		14		15b		20		29b	
	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%
M-180													450	33		
M-180-32													418	32		
M-180-69													381	83		
M-180-69-18													363	60		

alkane (C_5H_{11}) species is eliminated from the side-chain of 15b. New peaks of M-157 ($C_9H_{17}O_2$) and M-158 ($C_9H_{18}O_2$) were observed in the spectrum of the tetrahydro derivative 29b. The latter peak probably corresponds to an elimination of the side-chain which may involve the McLafferty rearrangement.[44] From the fragmentations of these side-chains, it is evident that cephalosporin P_1 has a side-chain corresponding to a species of $C_9H_{13}O_2$. In Figure 9, partial fragmentation patterns of the unsaturated lactone 5 and the saturated lactone 14 are shown. The side-chain elimination through a McLafferty rearrangement is possible in lactone 14 since the C_{17}-C_{20} double bond is no longer present.

Although no direct comparison between 1a and 3 has been reported, the nmr and mass spectral data previously compiled[20,27] and the data reported in Tables II and III strongly support a common carbon skeleton.[28,29]

This view is strengthened by the fact that bacteria which have acquired resistance to one of the antibiotics are also resistant to the other.[8,57] A comparison of the antibacterial activity between 1a and 3 was shown in Table I. The antibacterial activity of derivatives prepared from 1a and 4a are compiled in Table IV. Cephalosporin P_1 (1a) is shown to be the most active antibiotic of its group. It is also of interest that the derivatives of cephalosporin P_{da} are consistently less active than the corresponding derivatives of cephalosporin P_1. Fusidic and helvolic acid derivatives have been reported to be similar in their antibiotic activity.[58,59]

Godtfredsen suggested that fusidic acid (3), helvolic acid and cephalosporin P_1 (1a) are biogenetically related to the lanostane

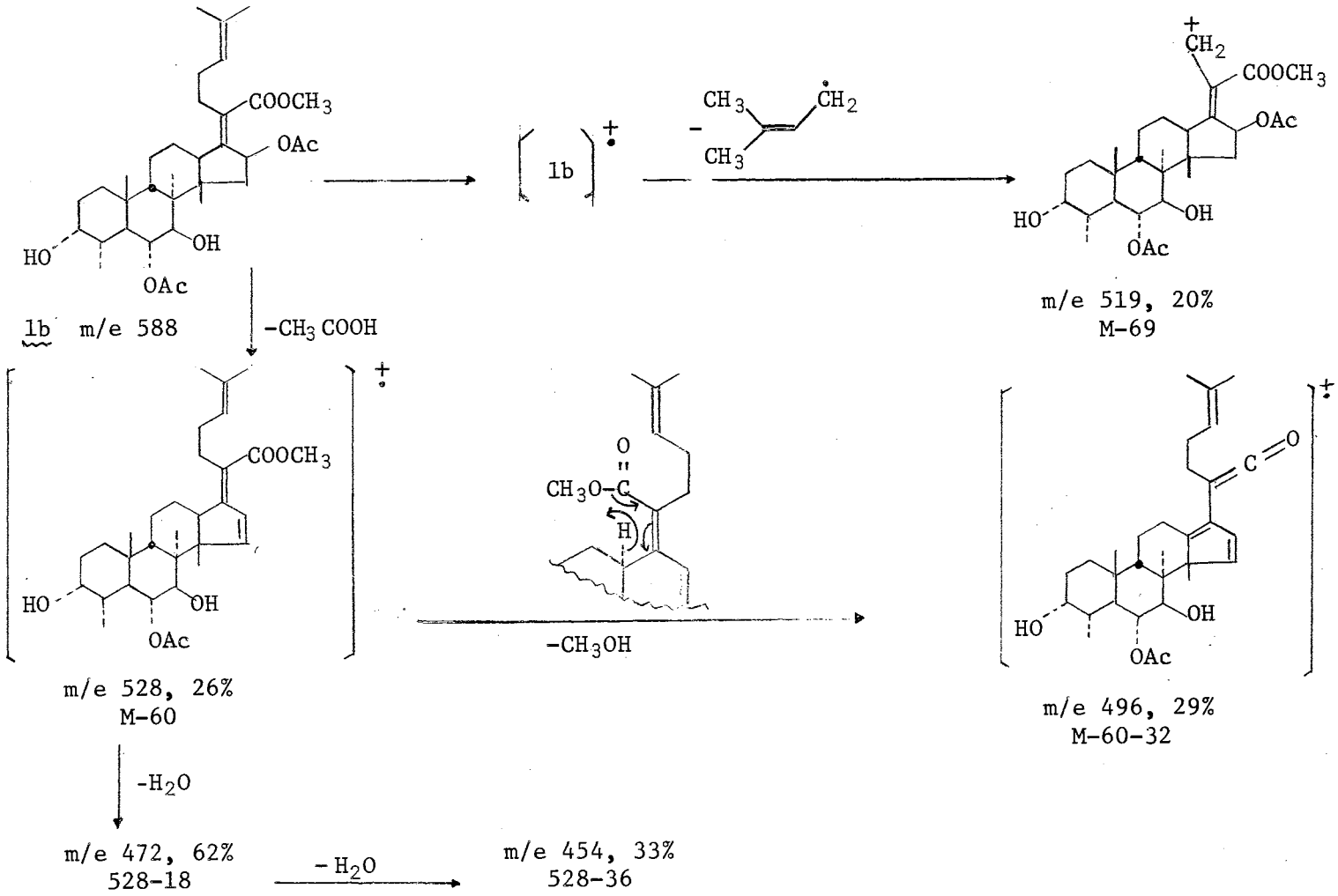


Figure 7. Postulated Partial Fragmentation Patterns for 1b

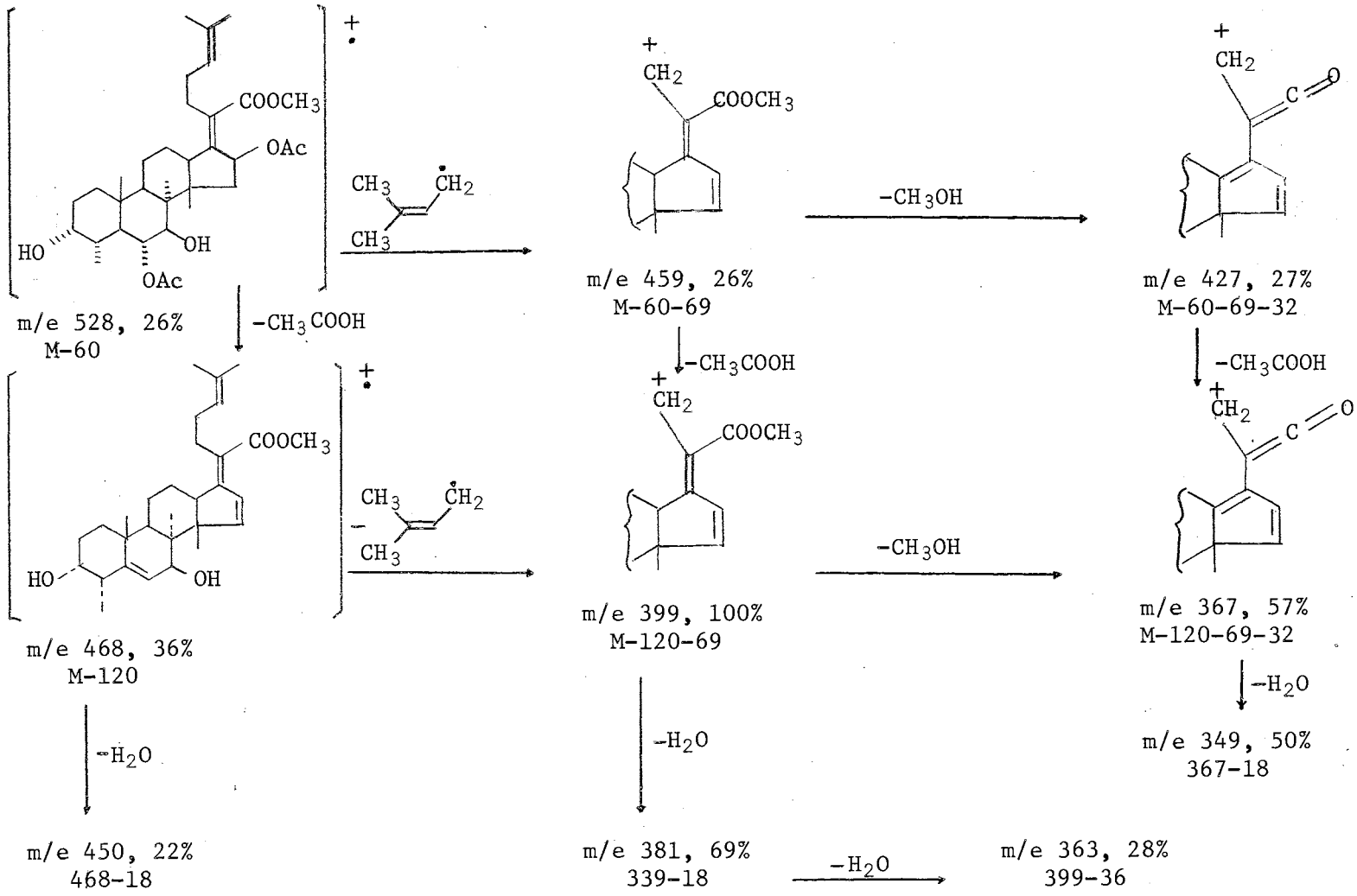


Figure 7 (Continued)

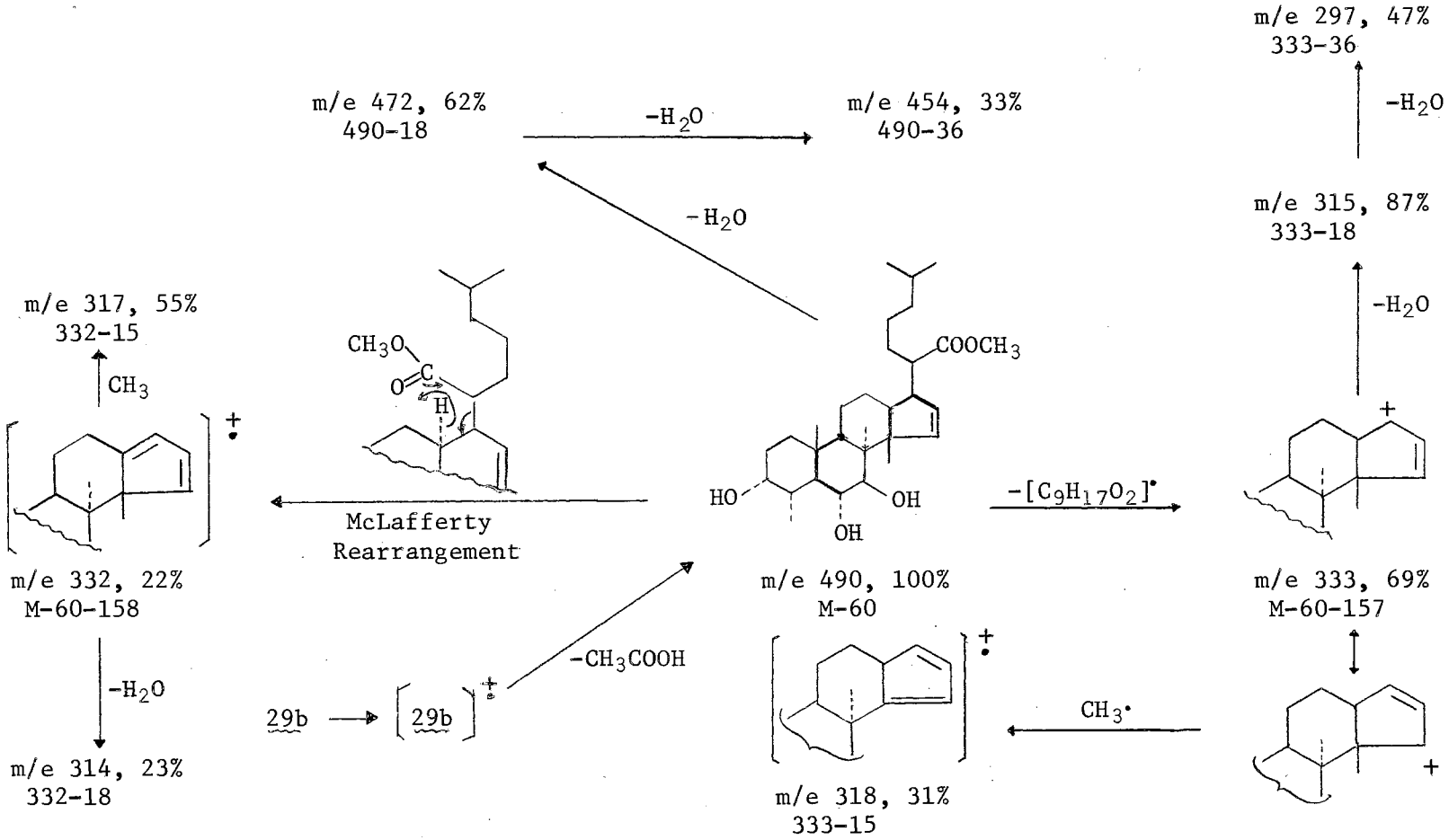


Figure 8. Postulated Partial Fragmentation Pattern for the Mass Spectrum of 29b

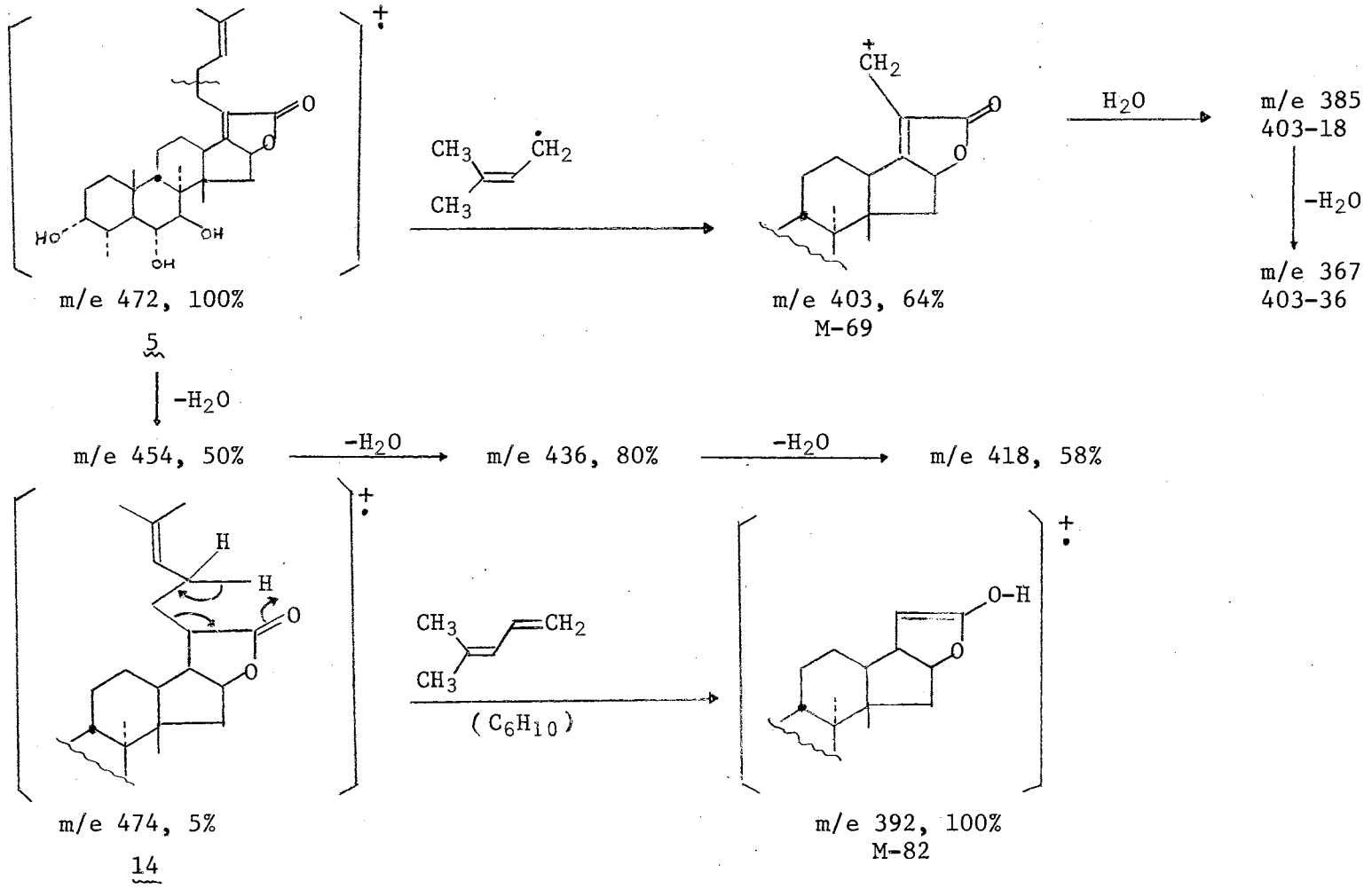


Figure 9. Postulated Partial Side-Chain Fragmentation Patterns for the Mass Spectra of 5 and 14

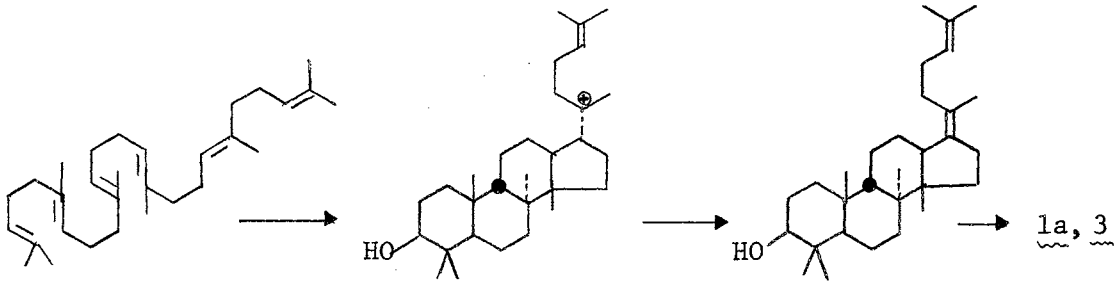
TABLE IV

ANTIBACTERIAL ACTIVITY[a]

	Corynebacterium diphtheriae	Sarcina lutea	Sarcina subflava	Staph. aureus 3055	Staph. aureus XI
Cephalosporin P_1	0.05	3.13	0.78	0.39	1.56
Cephalosporin P_1, dihydro -	0.10	6.25	3.13	0.39	0.78
Cephalosporin P_1, 3,7-dione -	6.25	100	100	50	100
Cephalosporin P_{da}	0.39	12.5	12.5	50	100
Cephalosporin P_{da}, 16-desacetoxy -	12.5	25	25	100	100
Cephalosporin P_{da}, dihydro -	0.39	6.25	12.5	6.25	25
Cephalosporin P_{da}, tetrahydro -	6.25	100	100	100	100
Cephalosporin P_{da}, 3,6,7-trione -	6.25	12.5	100	50	100
Cephalosporin P_{da} 16→20 lactone	6.25	25	6.25	100	100

[a]Minimum inhibitory concentration (mcg/ml)

group of tetracyclic triterpenes and therefore also related to the steroids.[28] The biosynthesis of 1a, 3 and helvolic acid can be postulated to proceed from squalene (24) through the tetracyclic carbonium ion 25 which is then stabilized by extrusion of a proton from C_{17}.[60] Whereas the formation of lanosterol from 25 involves a series of stereospecific 1,2-shifts of hydrogen atoms and methyl groups.[61] These rearrangements do not occur in the production of the hypothetic tetracyclic C_{30} precursor 26 of fusidic acid. The recent findings of Van Tamelen[62] and Corey[63] regarding introduction of oxygen at C_3 of the steroid nucleus probably apply as well.



24 25 26

The above physical and biogenetic evidence clearly establishes that 1a and 3 are related. The C_3 hydroxyl group in ring A of 1a was previously shown to be α and axial and the C_4 methyl group α and equatorial.[19] These conclusions are in accord with the configuration of C_3 hydroxyl group and C_4 methyl group for 3.

The side-chain structure proposed for cephalosporin P_1 in its early structure, 2,[19] is also in agreement with that for 3.[22] An identical side-chain structure is also derived from the di- and tetra-hydro derivatives of 1a and 4a as shown in Figure 10.

The easily reduced double bond ($\Delta^{24,25}$) in 1b and 4b was shown to

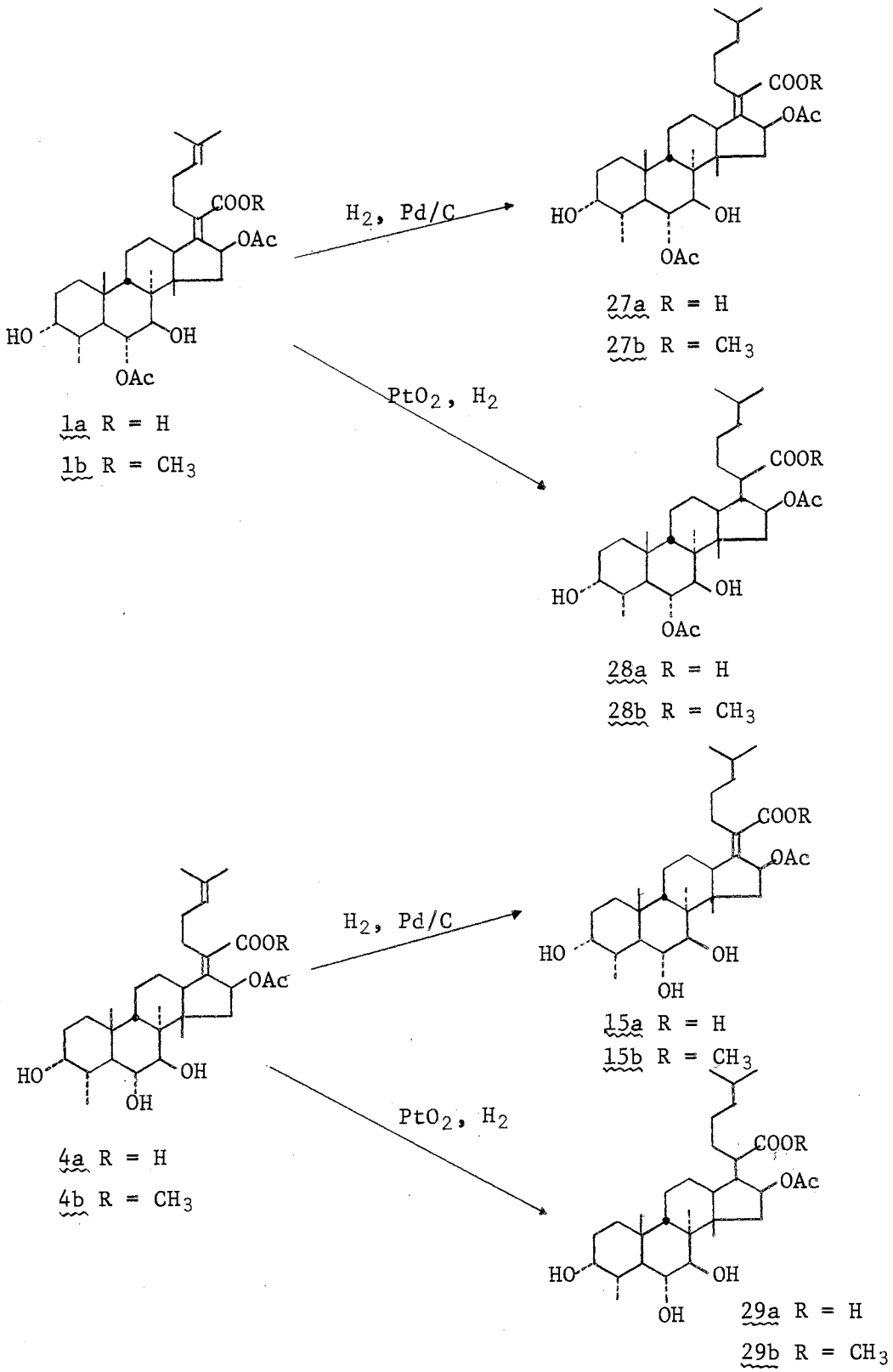


Figure 10. Preparation of Di- and Tetrahydro Derivatives of 1a, 1b, 4a, and 4b

be trisubstituted through observation of the vinylic proton signal at δ 5.13 (bm) as shown in Table III. This signal was no longer observed in the spectra of the dihydro derivatives 15b and 27b. The double bond ($\Delta^{24,25}$) is part of an isopropylidene moiety since acetone was obtained on ozonization of 4b. The tetrasubstituted double bond of 1a and 4a was shown to be $\Delta^{24,25}$ since absorption at 220 mμ was observed.

It is also informative to compare the mass spectra obtained from 4b and their di- and tetrahydro derivatives. The side-chain structures can be derived from their fragmentation patterns as described in the previous paragraphs.

Following the determination of structures for 1a and 4a, attention was shifted to the isolation of new components from the crude organic extract of *Cephalosporium acremonium*. One new component has been isolated which gave R_f 0.44, which is between the R_f value of 1b (R_f 0.62) and 4b (R_f 0.21). The isolation was accomplished through preparative thin-layer chromatography on silica gel PF. A sample of the methyl ester derivative of this new component was obtained which gave a single spot on a tlc plate, although it was not obtained in crystalline form. The new component was shown to have structure 10 from the instrumental studies and elemental analysis, which gave a molecular formula of $C_{34}H_{52}O_8$. Its nmr spectrum showed signals corresponding to two acetoxy protons, and its mass spectrum showed a peak at mass 528, which apparently is the M-60 peak instead of parent ion peak. The general features of its nmr spectrum resembled that of 1b. However, there are minor differences since a singlet appeared at δ 4.65 and a doublet at δ 3.42 (J 10 cps) instead of a singlet at δ 3.50

and a doublet a δ 4.57 for 1a. This new compound is best described by adopting Oxley's isocephalosporin P_1 methyl ester structure 10[17] since the experimental data fit this structure.

Since a diol-monoacetate system was known to isomerize readily under influence of base or acid,[32] several experiments were carried out in the hope that 1b could be isomerized to this new component 10.

Cephalosporin P_1 methyl ester (1b) was found unchanged after exposure to dilute sodium carbonate (1-4 %) solution and to 1% sodium borohydride solution in isopropyl alcohol. But reaction did occur after stirring 1a with 5% sodium carbonate solution in isopropyl alcohol for three hours since two spots at R_f 0.62 and 0.44 were detected on tlc. The spot of R_f 0.62 is the same as the starting material 1b and the spot of R_f 0.44 proved to be the same as the new component 10 by the tlc and elemental analysis, as well as nmr and mass spectroscopy studies. Whether 10 was originally present in the crude organic extract of *Cephalosporium acremonium* or formed during the isolation process remains unknown.

As shown from the bioassay and thin-layer chromatography described above, the crude extract from *Cephalosporium acremonium* still contains additional antibacterial substances. It is hoped that these antibiotics may be isolated and their structures established.

CHAPTER IV

EXPERIMENTAL

Melting points were taken in capillary melting point tubes using a Thomas-Hoover apparatus and are uncorrected. The centigrade scale was used for all temperature measurements. The elemental analysis, optical rotatory dispersion and X-ray diffraction measurements were carried out in the Analytical Laboratory of Eli Lilly and Company. The major portion of the nmr spectral determinations using a Varian Association HR-60 spectrometer were also obtained from Eli Lilly and Company. Other instrumental determinations involving use of a Cary 14, Beckmann IR-5A, and Varian A-60 spectrometer were made at Oklahoma State University. A prototype LKB mass spectrometer-gas chromatograph was also used for mass spectrometry.

Isolation of Cephalosporin P_1 (1a)

The crude organic extract of *Cephalosporium acremonium* (3 g of Eli Lilly lot no. 316-440AD-166) was dissolved in 2 ml of acetone and eluted through a column packed with 50 g of Davidson silica gel (grade 62, mesh 60-200). The eluent (1 liter) was gradually changed from n-Hexane to 30% acetone in n-Hexane. The eluate was collected in 50 ml fractions. These fractions were concentrated in a vacuum evaporator and then tested on a thin-layer plate coated with 0.30 mm thickness of silica gel (Silica Gel $PF_{254+366}$, Brinkmann Instruments, Inc.). Three fractions eluted with 25% acetone in n-Hexane

were found to be enriched in 1a. These fractions were combined and again eluted with 20% acetone in n-Hexane (500 ml). The fractions enriched in 1a were chromatographed in the same manner once again. Finally, these light yellow fractions were decolorized by eluting through a column packed with 5 g of active carbon. The colorless eluate was evaporated to dryness and crystallized in methanol. A crystalline sample of 1a, mp 147-148°, weighing 100 mg, was obtained. The IR spectrum in chloroform showed bands at 2920, 2800-2550, 1720, 1460, and 1375 cm^{-1}. The identity of this sample with cephalosporin P_1 was made by conversion of this sample to its methyl ester derivative 1b. The identity of 1b with cephalosporin P_1 methyl ester is described in the next experimental section.

In a separate procedure, an additional 3 g of crude organic extract was dissolved in 40 ml of benzene and stirred at room temperature with 40 ml of water for four hours. A small quantity of benzene solvate of 1a formed, which weighed 80 mg when collected by filtration. The benzene solvate was washed with water and benzene, and then dissolved in methanol. Evaporation to dryness and two crystallizations from methanol gave 40 mg of 1a, mp 147-148°. The identity of this sample as 1a was established by mixed melting point test and comparison of infrared spectra with an authentic sample of 1a.

Preparation of Cephalosporin P_1 Methyl Ester (1b) from 1a

To a solution of 300 mg of cephalosporin P_1 (1a), mp 147-148°, in 200 ml ether was added an ethereal solution of freshly prepared diazomethane until a yellow color persisted. The solution was allowed to stand for 30 minutes at room temperature in a hood, and dilute acetic acid was then added to destroy the excess diazomethane. The

ether solution was washed with water, dilute sodium bicarbonate solution and again with water. The ether layer was evaporated to dryness in a vacuum evaporator and the residue was crystallized in ether. Recrystallization in methanol gave crystals of 1b, mp 196-197°, $[\alpha]_D$ + 28° (c 1.2, $CHCl_3$). The ultraviolet spectrum of 1b showed an absorption maximum at 220 mμ (ε 11,000). The infrared spectrum in chloroform has bands at 3500-3200, 2920, 2850, 1720, 1460, 1375, and 1255 cm^{-1}. The nmr and mass spectral data for 1b are shown in Tables II and III. These nmr data for 1b are the same as reported by Melera.[27] The X-ray diffraction pattern of 1b from this route and an authentic sample were found to be identical.[34]

Isolation of Cephalosporin P_1 (1a) in the Form of Methyl Ester 1b

A solution of 3 g of the organic extract of *Cephalosporium acremonium* (Eli Lilly lot no. 316-440AD-166) in 150 ml ether was methylated in the same manner as described for methylating 4a to 4b. The methylated crude cephalosporin P was evaporated to dryness, dissolved in 2 ml of acetone and eluted through a column packed with 50 g of Davidson silica gel (grade 62, mesh 60-200) in the same way as described for chromatographic isolation of 1a. A sample of 1b (600 mg) thus obtained melting at 196-197°.

Preparation of the Methyl Ester of Fusidic Acid (3)

A sample of 100 mg of sodium fusidate (Eli Lilly lot no. 290-522-152C) was dissolved in 5 ml of water and the aqueous solution was acidified by dropwise addition of 0.1N hydrochloric acid. The resulting precipitate was extracted with three portions of ether (total 30 ml) and the ether extract was dried over anhydrous magnesium sulfate. The ethereal solution of 3 was filtered and methylated with

freshly prepared diazomethane in the same manner as described for methylation of 4a to 4b. Fusidic acid methyl ester crystallized out when the ethereal solution was concentrated through evaporation. Recrystallization in methanol gave 96 mg of the methyl ester of 3, mp 153-154°. The nmr and mass spectral data of the methyl ester of 3 are shown in Table II and Table III.

Isolation of Cephalosporin P_{da} (4a) Through Benzene Solvate Procedure[22]

The crude organic extract of *Cephalosporium acremonium* (3 g of Eli Lilly lot no. 316-416AD-255, 316-415AD-FF, 316-440AD-166) was dissolved in 50 ml of n-butyl acetate and filtered. The butyl acetate solution was stirred at room temperature with 40 ml of 5% sodium hydroxide solution. The alkaline layer was separated, adjusted to pH 9 by addition of 1 M sodium dihydrogen phosphate (13 g per 100 ml), and was back washed with three 30-ml portions of petroleum ether. The aqueous layer was separated and benzene (50 ml) was added. Aqueous 10% hydrochloric acid solution was added dropwise to the solution with stirring until the pH was adjusted to 6. The benzene layer was separated, allowed to stand overnight, and filtered through a sintered glass funnel to collect the precipitated benzene solvate. The solvate was washed with water and benzene several times until the washings were colorless. The precipitate was then dissolved in methanol and evaporated to near dryness with a vacuum evaporator. The resulting colorless solid was recrystallized twice from aqueous methanol and once from isopropyl alcohol to yield 540 mg (18% yield) of colorless solid 4a, melting at 197-198.5°, $[\alpha]_D$ + 37.6° (c 0.5, CH_3OH). Thin-layer chromatography on silica gel showed one spot, R_f 0.58 (solvent system: petroleum ether/acetone/ethanol = 4/10/1,

thin-layer thickness 0.35 mm). A sample of crystalline 4a was assayed on paper chromatography using 70% propanol as eluting solvent. It was found to be active against a sensitive *Staph. aureus* 3055 as shown in Table I. Its ultraviolet spectrum in methanol has an absorption maximum at 220 mμ (ε 8,000). The infrared spectrum (potassium bromide pellet) shows bands at 3400, 2920, 1700, 1440, 1370, and 1260 cm^{-1}. A mass spectrum was obtained, but the molecular ion did not appear.

Anal. Calcd. for $C_{31}H_{48}O_7$: C, 69.89; H, 9.08. Found: C, 69.46; H, 9.26.

Preparation of Cephalosporin P_{da} Methyl Ester (4b) from 4a

To a solution of cephalosporin P_{da} (4a) (0.50 g, mp 197-198.5°) in 100 ml ether was added an ethereal solution of freshly prepared diazomethane until a yellow color persisted. The solution was allowed to stand for 30 minutes at room temperature in a hood; dilute acetic acid was then added to destroy the excess diazomethane. The solution was washed with water, dilute sodium bicarbonate solution and again with water. The ether layer was evaporated to dryness in a vacuum evaporator and the residue was crystallized in ether-hexane. Recrystallized once in methanol to yield 0.48 g of 4b, mp 232-233.5°, $[\alpha]_D$ + 29° (c 0.5, CH_3OH). The ultraviolet spectrum showed $\lambda_{max}^{C_2H_5OH}$ 220 mμ (ε 8,375). The infrared spectrum in chloroform (as saturated solution) has bands at 3640 (monomer OH), 3580 (dimer OH), 3500-3200 (intermolecular H-bonding OH), 3080, 2980, 2950, 1740 (acetate CO), 1718 (α,β-unsaturated ester CO) 1433, 1373, 1255 (ester), 1015, and 785 cm^{-1}. The mass spectrum showed a parent ion peak at mass 546 ($C_{32}H_{50}O_7$) (Table III). The nuclear magnetic resonance spectrum

(Table II) showed the presence of the following proton signals in ppm from TMS: δ 0.93(d), J 6.0 cps; δ 1.25(s); δ 1.15(s); δ 1.00(s); δ 1.60(d), J 1 cps; δ 1.68(d), J 1 cps; δ 1.92(s); δ 3.40(s); δ 3.40(d); δ 3.67(bm); δ 3.62(s); δ 5.83(d), J 8.5 cps; and δ 5.13(bt).

A direct comparison of 4b with the monodesacetylcephalosporin P_1 methyl ester prepared by T. G. Halsall[48] was made. It was found that an admixture did not have a depressed melting point and that identical R_f values were found during thin-layer chromatography. The infrared of 4b and the comparison sample was also found to be identical.

Anal. Calcd. for $C_{32}H_{50}O_7$: C, 70.30; H, 9.22. Found: C, 70.54; H, 9.22.

Isolation of Cephalosporin P_{da} Methyl Ester (4b) and Isocephalosporin P_1 Methyl Ester (10) by Preparative Thin-Layer Chromatography

The procedure used for isolation of 1a in the form of 1b was followed. A sample of 1 g of methylated crude cephalosporin P was eluted through a column packed with 25 g of Davidson silica gel in the same way as described for chromatographic isolation of 1a. The fractions (25-30% acetone in n-Hexane) were found to be enriched with 4b and 10. These fractions were combined and solvent removed to give about 80 mg of yellow residue. The residue was dissolved in 1 ml of methanol and applied to glass plates coated with 0.80 mm of silica gel. After developing in a chamber containing 1.5% methanol in chloroform, the spots were detected with a short wave (254 mμ) ultraviolet lamp. Two intense spots were scraped from the plates, and the silica gel was extracted with methanol and filtered. The more polar spot (R_f = 0.21) gave 27 mg of crystals, mp 224-228°. These crystals were identified as

4b through X-ray diffraction comparison with an authentic sample. The less polar spot gave 32 mg of 10 (R_f = 0.44) as non-crystalline material. The IR spectrum of 10 showed bands at 3500-3170, 2910, 1725, 1460, 1380, and 1255 cm^{-1}. The UV spectrum showed an absorption maximum at 220 mμ (ε 8,500). The nmr data for 10 are shown in Table II. The mass spectrum for 10 showed the following peaks at m/e = 528(M-60), 468(M-120), 519(M-69), 496(M-60-32), 459(M-60-69), 450(M-120-18), 436(M-120-32), 427(M-60-69-32), 399(M-120-69), 381(M-120-69-18), 367(M-120-69-32), 363(M-120-69-36), and 349(M-120-69-32-18).

Ozonolysis of Cephalosporin P_{da} Methyl Ester (4b)

Ozonized oxygen was bubbled through a solution of 4a (100 mg) in dry methylene chlorides (25 ml) containing dry pyridine (0.2 ml) at dry ice temperature for 10 minutes until the color of the methylene chloride solution turned blue. Zinc dust (1.25 g) and acetic acid (2.5 ml) were then added. After stirring at 0° for 20 minutes, the precipitate was filtered and washed with methylene chloride. The methylene chloride solution was steam distilled into a solution of 2,4-dinitrophenylhydrazine (80 mg) in a mixture of water (20 ml) and concentrated sulfuric acid (3 ml). The distillate containing 2,4-dinitrophenylhydrazone was separated into two layers (lower methylene chloride layer and lighter aqueous sulfuric acid layer). The two layers were separated and the aqueous sulfuric acid layer was washed with three 10 ml portions of methylene chloride until the yellow color of the 2,4-dinitrophenylhydrazone derivative no longer was apparent. The methylene chloride extracts were combined and evaporated to near dryness. Yellow crystals (mp 114-118°) formed after standing for a

short time. Recrystallization from aqueous ethanol raised the mp to 120-130°. (Acetone-dinitrophenylhydrazone melts at 123-124°.) The infrared spectrum of the isolated acetone-dinitrophenylhydrazone is identical with an authentic sample. Furthermore, the thin layer showed R_f 0.62, which is also identical with the authentic acetone-dinitrophenylhydrazone sample. From these data, it is clear that the yellow crystals are the 2,4-dinitrophenylhydrazone of acetone.

Sodium Carbonate-Catalyzed Isomerization of 1b to 10.

Cephalosporin P methyl ester (1a, 50 mg, mp 197-198°) was dissolved in 10 ml of isopropyl alcohol, and this solution was added to a solution of 5% sodium carbonate in 3 ml water and 97 ml isopropyl alcohol. After stirring at room temperature for 4 hours, the solution was acidified with diluted hydrochloric acid and poured into 150 ml water. The precipitate was extracted three times with 50 ml ether and after the ether extract was washed with 100 ml water, it was dried over magnesium sulfate and evaporated to dryness. The residue was spotted on a glass plate coated with a thin layer of silica gel and then developed in a solvent system of 2% methanol in chloroform. The spots were detected in a iodine vapor which showed two spots with R_f = 0.60 and 0.42 corresponding to the starting material 1b and the isomerized product 10 respectively. The identity of the spot having R_f = 0.42 with 10 was made through tlc and infrared comparisons.

Other isomerization experiments using 1%, 2%, and 3% sodium carbonate or 1% sodium borohydride in isopropyl solution failed to isomerize 1b after stirring at room temperature for four hours.

Pyrolysis of Cephalosporin P_{da} (4a) to Cephalosporin P_{da} Lactone (5)

Cephalosporin P_{da} (175 mg) was placed in a 10-ml, round-bottomed flask and pyrolyzed at 240° in a salt bath (8.5 g of KNO_2 + 10 g of KNO_3) for five minutes, when the evolution of gases almost ceased. During the pyrolysis, a stream of nitrogen was passed through the flask to maintain an inert atmosphere. After the reaction, the yellow amorphous solid weighed about 76% of original material. The amorphous solid was spotted on a glass plate coated with a thin layer of alumina and then developed in a petroleum ether/acetone = 1/3 solvent system. The spot were detected with a spray of concentrated sulfuric acid which showed four spots with R_f = 0.58 (trace), 0.47 (major, grey), 0.27 (green), and 0.04 (grey, brown). A stationary spot corresponding to 4a was observed. Cephalosporin P_{da} (4a) does not migrate on an alumina thin-layer plate. Column chromatography of 124 mg of the yellow amorphous solid on an alumina column (8 g alumina, grade V deactivated with 15% water) gave 10.7 mg white needle-like crystals, mp 180-181°, $[\alpha]_D$ + 76.2° (c 0.95, C_2H_5OH) on elution with 5% acetone in ether and 40 mg of a white solid when 10-20% acetone in ether was used. Rechromatography of the white solid on 3 g of grade V alumina gave 20 mg of white needles from the petroleum ether eluate. These white needles (mp 180-181°) were later identified as the same lactone 5 obtained from the first chromatographic separation. An ultraviolet spectrum showed a peak at $\lambda_{max}^{C_2H_5OH}$ 224.5 mμ (ε 13,500). An infrared spectrum in chloroform showed bands at 3460, 3050, 2910, 1740 (α,β-unsaturated γ-lactone), 1460, and 1380 cm^{-1}. The nmr spectrum showed that lactone 5 no longer contained an acetoxy group (Table II). The mass spectrum showed a parent ion peak

at mass 472 (Table III). The ORD curve of the lactone 5 showed $[\alpha]_{400}$ + 20°, $[\alpha]_{295}$ + 57°, $[\alpha]_{265}$ -27°, $[\alpha]_{261}$ -14° (c 0.15, 1 cm, C_2H_5OH). This curve is a mirror image of the ORD curve obtained for 3β-acetoxy-16α-hydro-$\Delta^{17(20)}$-bisnor-5α-cholenic-22,16-lactone (11), which showed $[\alpha]_{400}$ -45°, $[\alpha]_{287}$ -116°, $[\alpha]_{267}$ -67°, and $[\alpha]_{263}$ -76° (c 0.165, 1 cm, C_2H_5OH) (Plate I).

Anal. Calcd. for $C_{29}H_{44}O_5$: C, 73.69; H, 9.38. Found: C, 73.58; H, 9.42.

Alkaline Hydrolysis of Cephalosporin P_{da} (4a) Sodium Salt to Cephalosporin P_{da} Lactone (5)

Cephalosporin P_{da} (4a) (90 mg) was dissolved in a minimum amount of dry acetone (2 ml) and methanol (1 ml). Sodium hydroxide solution (33%) was added dropwise until moist pH paper showed a pH of 7.5-8.0. The solution was then poured into 50 ml of acetone. The sodium salt of 4a was separated by filtering through a sintered-glass funnel and washed with dry acetone. It was then dissolved in water and acidified by addition of 0.1 N hydrochloric acid solution to give a white precipitate. This precipitate was dissolved in ether, washed with water, dilute sodium hydrogen carbonate solution and water, and dried over anhydrous sodium carbonate. Chromatography on a Florisil column gave 48 mg of a white crystalline solid, melting at 179-180.5°, $[\alpha]_D$ + 75.5° (c 0.83, C_2H_5OH). This material was shown to be identical with lactone 5 prepared by pyrolysis of 4a since there was no depression in melting point on admixture and only one spot was obtained on silica gel thin-layer chromatography of a mixture of 5 from the two sources. The infrared, ultraviolet, and nuclear magnetic resonance spectra of 5 from the two sources were also identical, and

the ORD curve has a negative Cotton effect, identical to the ORD curve of lactone 5 obtained by pyrolysis of 4a.

Anal. Calcd. for $C_{29}H_{44}O_5$: C, 73.69; H, 9.38. Found: C, 73.59; H, 9.42.

Alkaline Hydrolysis of Cephalosporin P_{da} (4a) to Monodesacetylcephalosporin P_{da} (13)

Cephalosporin P_{da} (4a) (100 mg, mp 197-198.5°) was heated at reflux for four hours with 0.1 N hydrochloric acid solution (3.8 ml) and the resulting precipitate filtered, washed with water and crystallized from methanol and water. Recrystallization from the same solvent yielded 79 mg of crystals, mp 125-126.5°. The infrared spectrum (potassium bromide pellet) showed bands at 3400, 2800, 1710, and 1700 (α,β-unsaturated carboxylic acid) cm^{-1}. The acetate band at 1260 cm^{-1} was missing. The ultraviolet spectrum showed $\lambda_{max}^{C_2H_5OH}$ 226 mμ (ε 6,900). Base shifted the absorption to longer wavelength. The nmr spectrum no longer showed the peak due to acetate methyl protons.

In an attempt to purify an impure sample of 13 on a silica gel column (Davidson silica gel, grade 62, mesh 60-200), a new crystalline solid, mp 181°, was obtained. This new crystalline solid was found to be identical to lactone 5 since no depression in melting point of the admixture was observed and their X-ray diffraction patterns were also identical. Their IR, UV, nmr and mass spectra were also the same.

Since lactone 5 can be made in a better yield (90%) using this procedure than by pyrolysis of 4a, the pyrolysis procedure is no longer used to produce lactone 5.

Alkaline Hydrolysis of Cephalosporin P_1 (1a) to 13

The procedure described for hydrolysis of 4a to 13 was followed.

A starting material of 25 mg of 1a was hydrolyzed to yield 12 mg of crystalline compound 13, which was shown to be identical to an authentic sample by mixed melting point and IR spectrometric comparison.

Lithium Aluminum Hydride Reduction of 1b, 4b, and 5

Lithium aluminum hydride (1 g) was heated in 100 ml of refluxing digylme with good stirring for 10 min. Heating was stopped and a solution of 100 mg of 4b in 100 ml of diglyme was added quickly. The reaction was exothermic. After the reaction had slowed, heating was resumed to maintain reflux for about 30 min. Saturated sodium sulfate solution was added gradually to the stirred suspension through an addition funnel to destroy excess lithium aluminum hydride. After a small excess of sodium sulfate solution was added, a white precipitate formed and settled to the bottom of the flask. The suspension was filtered. The filtrate was dried to give 26.5 mg of solid, which was shown to contain two components having R_f = 0.35 and 0.61.

The same procedure was used to reduce 1b and 5, and the resulting solid also contained two components with the same R_f values. The major component 14 (R_f = 0.61) was obtained in crystalline form, mp 222-223°. The reduction product 14 from the three sources was shown to be identical by X-ray diffraction studies. The IR spectrum of 14 showed a band at 1770 cm^{-1}. The mass spectrum of 14 showed peaks at mass 474 and mass 392. The UV spectrum of 14 did not show absorption in the region of 225 mμ and the ORD curve did not have a Cotton effect in the vicinity of 266 mμ.

The material with R_f 0.35 was readily converted to 14, R_f 0.61, by obtaining a pure sample from a preparative thin-layer plate and treating it with methanol containing hydrochloric acid.

Preparation of Methyl Cephalosporin P_{da} Acetonide (6)

Cephalosporin P_{da} methyl ester (4b) (100 mg), mp 232-233.5°, was dissolved in 2 ml dry acetone, and to this solution was added 20 mg of p-toluenesulfonic acid (monohydrate) and a few drops of 2,2-dimethoxypropane to react with any water. The reaction mixture was brought to pH 7-8 with bicarbonate solution, water (3 ml) was added, and the acetone evaporated under reduced pressure. The resulting precipitate was dissolved in ether and the ether solution washed with water, bicarbonate solution, and then dried and filtered. A similar ether solution was obtained from the aqueous portion of the reaction mixture. These ether solutions of crude acetonide were spotted on a silica gel thin-layer plate and both showed the same two spots. One spot corresponded to the starting material and the other was presumed to be the desired acetonide. The ether solutions were combined and evaporated to give 90 mg of solid which was chromatographed on a basic alumina column (1 cm x 15 cm). The acetonide of 4b, mp 158-159.5°, 30 mg, was eluted with petroleum ether. The starting material, 4b (6 mg), was eluted with ether-methanol. The ultraviolet spectrum showed an absorption maximum in ethanol at 220 mμ (ε 5,650). The infrared spectrum has bands at 3640, 3020, 2900, 2800, 1740, 1730, 1465, 1385, 1380, 1236, and 1175 cm^{-1}. The nmr spectrum showed a singlet peak at δ 1.40 corresponding to the gem-dimethyl protons.

Anal. Calcd. for $C_{35}H_{54}O_7$: C, 71.64; H, 9.28. Found: C, 71.65; H, 9.25.

Oxidation of Acetonide 6 to 8

A slurry of chromium trioxide-pyridine complex[46] was prepared by adding chromium trioxide (6.2×10^{-3} mole) to 7 ml of vigorously

stirred cold pyridine over a period of 10 min. The acetonide 6 (20 mg) in 0.3 ml pyridine was added to one-tenth portion of the prepared slurry. The mixture was stirred for approximately 30 min, and then it was allowed to remain undisturbed at room temperature for 15 hours. The mixture was poured into 20 ml water and extracted with 20-ml portions of ether. The combined ether extracts were washed successively with 10 ml of 5% sodium carbonate solution and 20 ml water. The ether solution was evaporated to near dryness and purified by eluting through a column packed with 10 g of basic alumina. The amorphous solid 8 (8 mg) eluted in the 5% acetone in chloroform fraction did not crystallize but gave a single spot on a silica gel thin-layer plate. The IR spectrum of 8 showed bands at 2900, 1740, 1710, 1465, 1380, 1368, and 1240 cm^{-1}.

Preparation of Dihydrocephalosporin P_{da} (15a)

A solution of 4a (100 mg, mp 197-198.5°) in absolute ethanol (25 ml) was shaken at room temperature under a hydrogen atmosphere in the presence of 2% palladium on carbon (20 mg). When the reaction had proceeded for 30 minutes, the catalyst was removed by filtration. A precipitate appeared on adding water to the filtrate. Crystallization of the precipitate from methanol-water yielded 91 mg of 15a, mp 201-202°. The ultraviolet spectrum showed absorption maximum at 222 mμ (ε 8,000).

Anal. Calcd. for $C_{31}H_{50}O_7$: C, 69.63; H, 9.43. Found: C, 69.59; H, 9.39.

Preparation of Dihydrocephalosporin P_{da} Methyl Ester (15b)

The dihydrocephalosporin P_{da} methyl ester (15b) was prepared through methylation of 15a or through hydrogenation of 4b. The

procedure described for hydrogenation of 4a to 15b and for methylation of 4a to 4b were used. From both routes, the yield of 15b was 90%. The melting point of 15b after two crystallizations from methanol was 261.5-262.5°. The UV spectrum showed absorption at 220 mμ (ε 8,375) and its mass spectrum showed a parent ion peak at mass 548. The nmr spectrum no longer showed the peak due to the vinylic proton at C_{24} of 4b. The complete nmr and mass spectral data are shown in Tables II and III.

A sample of 15b (30 mg) in methylene chloride (20 ml) was ozonized in the same manner as for ozonization of 1b. No acetone dinitrophenylhydrazone was obtained in this case.

Anal. Calcd. for $C_{32}H_{52}O_7$: C, 70.04; H, 9.55. Found: C, 69.71; H, 9.55.

Preparation of the 3,6,7-Triketone 16

Dihydrocephalosporin P_{da} methyl ester (15b) (63 mg) was dissolved in 2 ml of acetic acid and a chromic acid solution (10% of chromium trioxide in 95% acetic acid) was added dropwise with vigorous shaking until the color of the solution became slightly green-brown (about 0.3 ml was used). The solution was poured into 50 ml of water and the resulting precipitate was extracted with ether. The ether solution was washed with water, dilute sodium bicarbonate solution and water, and dried over anhydrous magnesium sulfate. The ether solution was evaporated under vacuum to near dryness and crystallized from ethanol-water. The yellow crystals of 16 (10 mg) melted at 193-198°. Two crystallizations raised the melting point to 205-208°. The ultraviolet spectrum showed no absorption between 260 and 340 mμ. However, the addition of acid produced $\lambda_{max}^{CH_3OH,H^+}$ 219 and 282 mμ (ε 8,290 and

4,500). Similar absorptions were also observed in alkaline solution as shown in Plate II.

Anal. Calcd. for $C_{32}H_{46}O_7 \cdot H_2O$: C, 68.54; H, 8.63. Found: C 68.08; H, 8.33.

Preparation of the 3,6,7-Triketone 18a

A solution of 40 mg of 4a in glacial acetic acid was treated with 0.5 ml of solution of 5% of chromium trioxide in 95% acetic acid in the same manner as described for preparation of 16. The yellow crystals of 18a (8 mg) thus obtained melted at 172-177°.

Anal. Calcd. for $C_{31}H_{42}O_7 \cdot H_2O$: C, 68.34; H, 8.14. Found: C, 67.96; H, 8.36.

Preparation of the 3,6,7-Triketone 18b

A solution of 50 mg of 4b in glacial acetic acid was treated with 0.6 ml of 5% chromium trioxide in 95% acetic acid in the same manner as described for preparation of 16. Yellow crystals of 18b (15 mg) were obtained, mp 207-210°. The UV spectrum showed no absorption between 260 and 340 mμ. When the UV spectrum of 18b was taken in acidic methanol solution, two absorption maxima at 219 and 283 mμ were observed, as shown in Plate II.

Anal. Calcd. for $C_{32}H_{44}O_7 \cdot H_2O$: C, 68.79; H, 8.30. Found: C, 69.19; H, 8.38.

Preparation of 3-Acetoxycephalosporin P_1 Methyl Ester (20)

Cephalosporin P_1 methyl ester (1b) (180 mg, mp 232-233.5°) was dissolved in a mixture of acetic anhydride (1.5 ml) and pyridine (2.5 ml). The solution was allowed to stand for 40 hours at room temperature. Water was added to the solution and the amorphous precipitate of 20 was collected, washed with water, and dried at 100° (0.01 mm Hg)

to remove traces of pyridine. The amorphous precipitate failed to crystallize but showed a melting range of 101-103.5°. Thin-layer chromatography on silica gel showed one spot having R_f 0.75 compared with R_f 0.62 for 1b. The infrared spectrum of 20 in chloroform shows bands at 3500-3200 (interhydrogen bonding OH), 2880, 1725, 1430, 1365, 1235, and 1015 cm^{-1}. The mass spectrum showed peaks at mass 630, 570, and 441 corresponding to parent ion, M-69 and M-69-120 as shown in Table III. The nmr spectrum showed proton signals at δ 0.81(d), J 6 cps; δ 4.58(d), J 10 cps, and δ 4.90(bm) corresponding to the methyl protons at C_4 and the secondary protons at C_3 and C_6. The complete nmr and mass spectral data are shown in Tables II and III.

Anal. Calcd. for $C_{36}H_{54}O_9$: C, 68.54; H, 8.63. Found: C, 68.50; H, 8.84.

Acetylation of Cephalosporin P_{da} Methyl Ester (4b)

To a solution of 4b (100 mg) in 3 ml pyridine, 1 ml acetic anhydride was added and the reaction mixture was allowed to stand at room temperature for three days. The mixture was tested on silica gel thin-layer plates after four hours, and four pronounced spots with R_f values 0.75, 0.68, 0.62, and 0.48 were observed. These same four spots persisted even after the reaction had been carried out for three days. The reaction mixture was then poured into 100 ml of water and the resulting light-yellow precipitate was collected, washed with water and dried at 100° at a reduced pressure to remove traces of pyridine. An attempt to separate this mixture (38 mg) with a silica gel column was not successful. The mixture was tested again on a thin-layer plate and the spots having R_f 0.62 and 0.75

were identified as 1b and 20 respectively through the comparison with the R_f values of authentic samples. The other spots were not identified. Preparative thin-layer separation gave amorphous samples which gave IR spectra identical with those of 1a and 20 respectively.

Preparation of Monoketone 21 from 20

A solution of 100 mg of triacetate 20 (amorphous solid purified by preparative thin-layer plates) in 5 ml of acetic acid was oxidized with 5% chromium trioxide in 0.8 ml of 95% acetic acid in the same way as described for compound 16. The residue failed to crystallize but was purified by preparative thin-layer plates coated with silica gel to give an analytically pure sample of monoketone 21. The nmr data are shown in Table II. The IR spectrum has bands at 2910, 1740, 1710, 1455, 1380, and 1250 cm^{-1}. The ORD curve of 21 showed $[\alpha]_{300}$ = -853°, $[\alpha]_{317}$ + -1025°, $[\alpha]_{325}$ = -836°, and $[\alpha]_{340}$ = -456° (c 0.235, 1 cm, C_2H_5OH).

Anal. Calcd. for $C_{36}H_{52}O_9$: C, 68.76; H, 8.34. Found: C, 68.27; H, 8.55.

Preparation of Diketone 22 from 1b

A solution of 100 mg of 1b, mp 147-148°, in 5 ml acetic acid was oxidized with 5% chromium trioxide in 1.6 ml 95% acetic acid in the same manner as described for oxidizing compound 16. The diketone 22 was not obtained in crystal form but purified by silica gel thin-layer plates to give a pure sample of 22. The nmr data are shown in Table II. The IR spectrum has bands at 2910, 1740, 1725, 1710, 1450, 1380, and 1250 cm^{-1}. The ORD curve of 22 showed $[\alpha]_{310}$ = -333°, $[\alpha]_{320}$ = -516°, $[\alpha]_{325}$ = -442°, and $[\alpha]_{340}$ = -216° (c 0.120, 1 cm, C_2H_5OH).

Anal. Calcd. for $C_{34}H_{48}O_8.H_2O$: C, 67.75; H, 8.36. Found: C, 67.09; H, 8.10.

Reduction of 22 to 23

To a stirred solution of 22 (30 mg) in 1 ml 90% acetic acid, 70 mg zinc dust was added in small portions during 15 minutes. After stirring at reflux temperature for 60 min, the precipitate was filtered off and the filtrate poured into water. The product was extracted with methylene chloride and the extract was washed with water, dried, and evaporated to dryness *in vacuo*. The residue could not be crystallized. Testing on silica thin-layer plates showed it contained a small amount of the starting material 22. The ORD curve of 23 showed $[\alpha]_{310} = -186°$, $[\alpha]_{320} = -398°$, $[\alpha]_{325} = -336°$, and $[\alpha]_{340} = -142°$ (c 0.113, 1 cm. C_2H_5OH).

Preparation of Dihydrocephalosporin P_1 (27a)

The procedure described for preparation of 15a was used. The acid 27a was obtained in about 80% yield with a melting point of 153-154.5° after crystallization from methanol.

Preparation of Dihydrocephalosporin P_1 Methyl Ester (27b)

Cephalosporin P_1 methyl ester (1b) was hydrogenated with 2% palladium on carbon to 27b in 90% yield. The procedure described for hydrogenation of 15a was followed. The melting point of 27b after two crystallizations from methanol was 207-209°. The UV spectrum in ethanol showed absorption maximum at 222 mμ (ε 7,100).

Preparations of Tetrahydrocephalosporin P_1 (28a) and Tetrahydrocephalosporin P_1 Methyl Ester (28b)

A sample of 50 mg of 1a and 50 mg of 1b were hydrogenated separately in the same manner as described for hydrogenation of 29b. The

tetrahydrocephalosporin P_1 (28a) obtained from hydrogenation of 1a and the methyl ester 28b obtained from hydrogenation of 1b were shown by tlc to be contaminated with 29a and 29b respectively.

Preparation of Tetrahydrocephalosporin P_{da} (29a)

The procedure described for 29b was used. The acid 29a was obtained in about 80% yield with a melting point of 234-237° after crystallization from methanol.

Preparation of Tetrahydrocephalosporin P_{da} Methyl Ester (29b)

A solution of 4b (50 mg, mp 197-198.5°) in 20 ml absolute ethanol and 0.5 ml acetic acid was stirred at room temperature under a hydrogen atmosphere in the presence of 10 mg of Adam's catalyst. When the reaction had proceeded for one hour, the catalyst was removed by filtration. A precipitate appeared on pouring the filtrate into 100 ml of water. Crystallization of the precipitate from methanol yielded 43 mg of 29b, mp 170-171.5°. The ultraviolet spectrum showed no absorption in the region of 220 mμ and a test with tetranitromethane was negative. The IR spectrum showed bands at 3630, 3570, 3500-3200, 2900, 2840, 1725, 1460, 1375, and 1260 cm^{-1}. The nmr spectrum no longer showed the peak corresponding to the C_{24} proton of 1b. The mass spectral data are shown in Table III.

Anal. Calcd. for $C_{32}H_{54}O_7$: C, 69.78; H, 9.88. Found: C, 70.05; H, 9.92.

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VITA

Ta-Sen Chou

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE CHEMISTRY OF CEPHALOSPORIN P

Major Field: Chemistry

Biographical:

Personal Data: The author was born in China on August 19, 1938, the son of B. I. and L. C. Chou.

Education: Graduated from Cheng-kung High School, Taiwan, The Republic of China, in 1952, and from Taipei Institute of Technology in 1957.

Professional Experience: Chemical Engineer, China Fiber Company, Taiwan, China, 1957-1958; 2nd Lt., Chinese Navy, 1958-1960; Chemist, Joint Industrial Research Center of Ministry of Economics of The Republic of China, Taiwan, China, 1960-1962; Graduate Research Assistant, Oklahoma State University, Stillwater, Oklahoma, 1963-1967.

Membership in Professional Societies: Member of Phi Lambda Upsilon and American Chemical Society.