A CHIROPTICAL STUDY OF PENICILLINS

L. A. MITSCHER, PETER W. HOWISON* and THEODORE D. SOKOLOSKI

Division of Natural Products Chemistry
The Ohio State University, Columbus, Ohio 43210, U.S.A.

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ORD and CD spectra are reported for a series of penicillin antibiotics and related degradation products. The results, when compared with published X-ray data, suggest that some conformational changes can apparently go undetected by chiroptical techniques in this series and that the side chain substituent at C_6 plays a relatively minor role in determining the sign and intensity of the COTTON effect unless its stereochemistry is inverted. The rather large spectroscopic effect following C_6-epimerization has potential for development as an assay for the percentage of 6-epihetacillin in a given mixture. Some preliminary data are presented showing the utility of this method for investigating the kinetics of C_6-epimerization. Semiempirical predictive rules developed for simple amides are inadequate for rationalizing the penicillin spectra. The origin of the COTTON effects is briefly discussed and contrasted with the cephalosporin antibiotics.

Interest in the physical, chemical and spectroscopic properties of the β-lactam antibiotics remains high because of their outstanding clinical utility and a desire to rationalize their mode of action on a molecular level. A recent review volume has collected much of this scattered information[1]. One of the techniques which has found wide application for the study of the stereo-chemistry and solution conformation of antibiotics is optical rotatory dispersion and circular dichroism spectroscopy[2]. Although CD measurements of the cephalosporins have been discussed,[3,4] no corresponding study of the chiroptical properties of the penicillins has yet appeared except in brief extract[5]. Recently, RASMUSSEN and HIGUCHI have developed a sensitive assay for penicillins based on the ORD changes they undergo when hydrolyzed by penicillinase[11]. We would like to record here some of our findings with this drug class.

Materials and Methods

Antibiotics and Degradation Products: Dicloxacillin sodium, methicillin sodium, phenethicillin, cloxacillin sodium, oxacillin, hetacillin and epihetacillin are gifts of The Bristol Laboratories. Penicillin V potassium, ampicillin, penicillin G potassium and nafcillin sodium are gifts of Wyeth Laboratories. 6-Aminopenicillanic acid was purchased from The Aldrich Chemical Company. Benzylpenicilloic acid sodium salt, benzylpenicilloic acid and cephalothin sodium were gifts of M. SCHWARTZ of The State University of New York at Buffalo.

Circular Dichroism and Optical Rotatory Dispersion Measurements: ORD-CD measurements were carried out at ambient temperature (cell compartment approximately 29°C), with the optical train under constant N_2 flush, using a Durrum-Jasco ORD/UV/CD-5 instrument. The solvents used were triple distilled water, spectroscopic grade methanol or 0.1 M citrate buffers and the values cited in the tables are the average of at least two separate determinations.

Preliminary Kinetic Experiments with Hetacillin and Epihetacillin: In studying the kinetics of the alterations of hetacillin and epihetacillin in alkaline solutions, solutions of these compounds were prepared in glycine buffer (0.2 M, pH 11). Studies were made in duplicate using concentra-

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tions of hetacillin of 0.0243 % and 0.0195 % and of epihetacillin of 0.0257 % and 0.0203 %. A thermostated 1-cm pathlength cell was used and the temperature was maintained at 24.8±0.2°C. The rotation for each sample was measured as a function of time at the wavelength of maximum rotation for each species (240 nm for hetacillin and 238 nm for epihetacillin). Baseline blanks were determined using buffer alone. Whole spectrum scans were made at periodic intervals.

Results

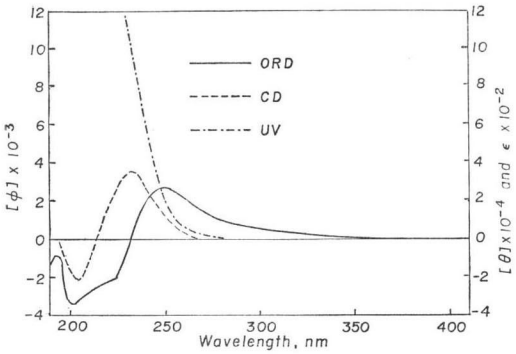
The extrema determined for the various penicillins are given in Table 1. In certain instances, high molecular absorptivity accompanied by low dichroic intensity prevented complete measurement of a peak. In most cases, however, a maximum could be measured satisfactorily even though end absorption often prevented measurement through a peak until baseline values were achieved. This was particularly true of measurements taken in buffer solutions and is more serious with the CD measurements than the ORD as, by their very nature, CD extrema occur at lower wavelengths than the first extremum of an ORD COTTON effect (see for example, Fig. 1*). In ORD measurements, it is common for the peak amplitudes to be superimposed upon a rising or falling background absorption which makes curve comparison less satisfactory than is the case with CD measurements. If the background rotations throughout a series are reasonably constant, then the amplitudes are at least roughly comparable. The CD measurements of these penicillin derivatives agree with the ORD measurements with regard to sign and relative intensity, so it is probable that such comparisons are valid in this case. One notes particularly the relative symmetry about the 0 axis of the first ORD extremum in Fig. 1 as being consistent with this view.

Table 1. ORD-CD of selected penicillins

Name	$[\phi]_{max}\times10^{-3}$ (nm)	$[\theta]_{max}\times10^{-4}$ (nm)	Solvent
6-Aminopenicillanic acid	2.87 (246)	4.22 (235)	0.1 M citrate buffer, pH 6.5
〃	—	5.87 (231)	HOH, pH 3.55
〃	—	5.11 (230)	HOH, pH 1.07
〃	—	5.44 (234)	HOH, pH 5.05
〃	—	5.30 (234)	HOH, pH 10.77
〃	—	1.50 (240)	Satd. soln. in MeOH
K Penicillin V	3.07 (244)	3.31 (230)	0.1 M citrate buffer, pH 6.5
Ampicillin	2.94 (248)	3.67 (232)	〃
Na Methicillin	2.69 (242)	2.51 (235)	〃
Hetacillin	3.40 (240)	4.55 (230)	〃
Epihetacillin	2.12 (238)	2.19 (230)	〃
Na Oxacillin	1.45 (245)	—	MeOH
Na Cloxacillin	2.05 (236)	—	〃
Na Dicloxacillin	1.37 (238)	—	〃
K Penicillin G	2.92 (243)	3.14 (234)	0.1 M citrate buffer, pH 6.5
Na Benzylpenilloic acid	—	—	〃
Benzylpenicilloic acid	0.58 (252)	—	〃

* This spectrum was run without buffer to increase the sensitivity of the measurements in the 180~220 nm range.

Fig. 1. ORD, CD and UV spectra of 6-aminopenicillanic acid in water at pH 6.5.



The simplest member of the penicillin series which retains at least some biological activity is 6-aminopenicillanic acid. In addition to being a β-lactam, this substance is also an amino acid. It is well known that amino acids give ORD-CD spectra of differing amplitude at different pH values[6]. Table 1 contains the data recorded in water over a pH range of 1.07~10.77 for 6-APA. The average CD molecular ellipticity for all pH values is $5.2\pm1.0\times10^{-4}$ $[\theta]$. This variation ($\pm19\%$) is larger than the normal operating precision of CD measurements ($\pm5\sim10\%$) indicating some pH effect so a common pH value of 6.5 was chosen for measurements throughout the series. At this pH, the penicillins are considered to have their greatest stability in aqueous solutions[7]. The oxacillin-cloxacillin-dicloxacillin family were not sufficiently soluble in buffer under these conditions, so their spectra were measured in methanol instead. Unfortunately, 6-aminopenicillanic acid was not soluble in methanol, so that a saturated solution was measured. Thus the low molecular rotation value listed for 6-APA in Table 1 is due to poor solubility, not to a large solvent effect.

In the study of the changes hetacillin and epihetacillin undergo in aqueous solutions at pH 11, the rotation of each species was seen to decrease with time. Rotations were measured

Fig. 2. The relation between rotation and reaction time for hetacillin and epihetacillin at pH 11 and 23.8°C.

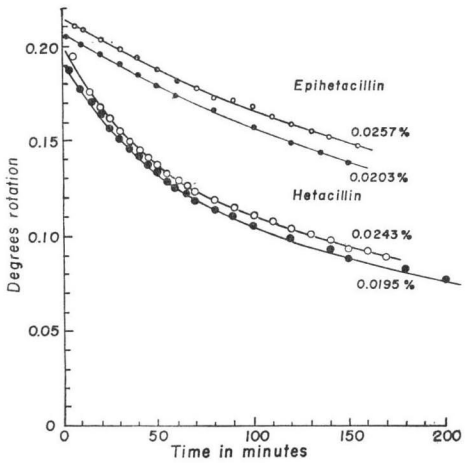


Fig. 3. The relation between log rotation and reaction time for hetacillin and epihetacillin at pH 11 and 23.8°C.

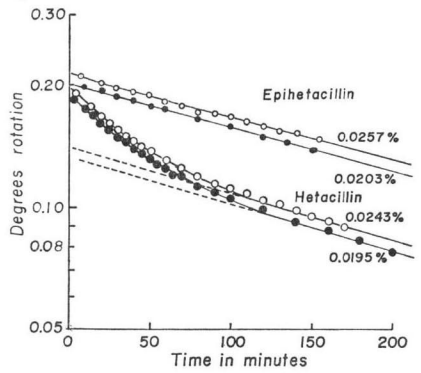
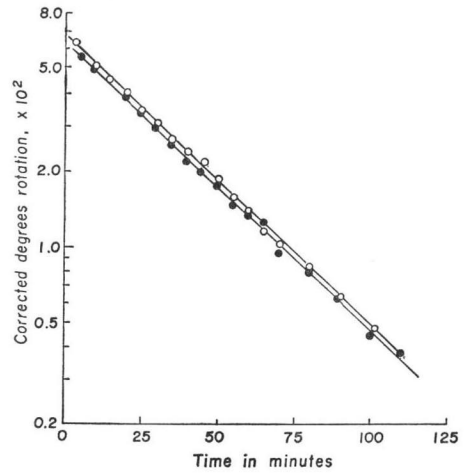


Fig. 4. The semilogarithmic relation between the rotation for hetacillin corrected for the second exponential epihetacillin process and reaction time.



relative to the base lines obtained. Fig. 2 shows the results obtained for hetacillin and epihetacillin in duplicate trials. A semilogarithmic plot of these data is shown in Fig. 3. The epihetacillin spectrum undergoes a linear change at both concentrations used over the time interval studied. As expected for a monoexponential process, the slopes of the lines at the concentrations used are essentially the same. The spectrum of hetacillin, at both concentrations used, undergoes a nonlinear semilogarithmic change with time. Assuming that such a nonlinear behavior might be biexponential in nature, where the limiting linear slope of the semilogarithmic rotation *vs* time plot is identical to that for epihetacillin, the initial rotation data obtained for hetacillin can be corrected for the second reaction. This correction was effected by extending the limiting linear portion of the semilogarithmic hetacillin *vs* time plot to zero time, as indicated in Fig. 3 and taking the difference between the rotation values thus drawn and the original experimentally determined values. These corrected rotation values were replotted on a semilogarithmic scale *vs* time. This resulted in an excellent linear relation (Fig. 4). The linearlity obtained (Fig. 4) confirms the assumption that the hetacillin spectral transformation as determined by rotation changes is indeed biexponential in nature.

Discussion

The cephalosporins possess a much more complex chromophore than the penicillins because of the influence of the Δ_3-double bond system. The positive CD band of cephalosporins at 259 nm is attributed to a $\pi \rightarrow \pi^*$ transition of the double bond with vicinal contributions from the β-lactam, the thiazolidine sulfur and the carboxy group (Fig. 5). The negative transition at 228 nm is assigned to the overlap of the π electrons of the double bond and the p and π electrons of the β-lactam carbonyl group. The influence of these vicinal groups on the spectra was worked out by NAGARAJAN *et al.*[4] and BOYD[8]. With penicillin and its derivatives, the electronic situation is considerably simplified because the double bond is absent and, so, the penicillins belong in a very different spectroscopic class than the cephalosporins and their chiroptical properties and spectra are not directly comparable.

Fig. 5. Vicinal interactions on the $\pi \rightarrow \pi^*$ band of cephalosporins.

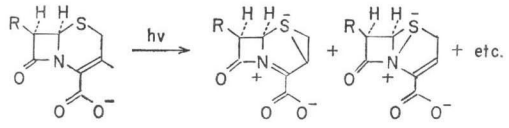
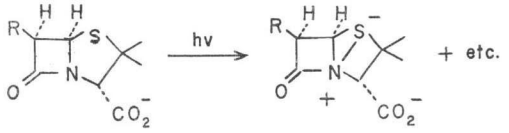


Fig. 6. Vicinal interactions on the $n \rightarrow \pi^*$ band of penicillins.



The penicillin chromophore consists primarily of the β-lactam grouping. The λ maximum is blue shifted compared to the average amide because of homoconjugative overlap with the thiazolidine sulfur atom (Fig. 6) and the nonplanarity of the β-lactam nitrogen impressed upon the system by the *cis* ring fusion. These inferences are supported by the theoretical analyses of the UV spectra of cepham and penam derivatives reported by BOYD[3]. In any case, 6-APA has two CD transitions in an instrumentally accessible spectroscopic region (see Fig. 1), and both of these are associated strongly with the β-lactam carbonyl group. The first COTTON effect is positive and occurs in the 230~240 nm region. This band occurs at an unusually high wavelength for a peptide $n \rightarrow \pi^*$ band and is probably associated with the vicinal effect of the thiazolidine S atom (Fig. 6) on the β-lactam group. NEELAKANTAN and URRY[5] and BOYD[8] attribute the ~210 nm COTTON effect to the peptide $n \rightarrow \pi^*$ band, and BOYD[8] predicts that the strongest vicinal interaction should be relatively weak and occur at approximately 245 nm. While this is true of the UV transition, the asymmetry of the fused system appears to be

sufficiently high to explain why the transition should be highly asymmetric and give rise to a strong CD signal.

The essential correctness of these inferences is supported by the ORD and CD spectra of sodium benzyl-penicilloic acid and benzyl-penicilloic acids in which the β-lactam bond has been opened by hydrolysis and no high intensity COTTON effect can be found in the 230 nm region.

When a second amide bond is introduced into the penicillins as, for example, with potassium benzylpenicillin, the position of the 245 nm band is not significantly changed and its intensity decreases somewhat (−25%). This result argues against a strong vicinal interaction of the original chromophore with the newly introduced amide and benzene moieties and the intensity change could be explained by either a small decrease in twisting or the presence of a new, bulkier, group in a + sign determining region. The benzylpenicilloic and benzylpenicilloic acid results also demonstrate that the newly formed peptide bond has a very small amplitude in the 230~250 nm region.

With ampicillin, an additional optically active center is present in the side chain. Still, the amplitude and band position are nearly the same as that of 6-APA. Phenoxymethyl penicillin and methicillin give closely similar spectra. This is further support for the rule that the nature of the side chain does not play a dominant ORD-CD role despite the nearness of these groups to the chromophore. This is of more than passing interest for X-ray studies have shown that the thiazolidine ring of ampicillin has a somewhat different solid state conformation than that of benzylpenicillin. If this difference is maintained in dilute aqueous solutions, then the CD spectra would not have reliably detected this conformational change. It is well known from many other chiroptical studies that only conformational shifts affecting the electrons of the chromophore itself will give strong spectroscopic changes. In particular, twisted chromophores (such as are present in α, β-unsaturated ketones, for example) are less sensitive to vicinal interactions than systems with undistorted geometry.

The hetacillin-*epi*hetacillin spectra are particularly interesting. The relatively recent discovery that certain β-lactam antibiotics (generally those lacking an H atom on the 6-amino group) can be epimerized at C_6 at a rate competitive with, and sometimes exceeding that of, hydrolysis of the β-lactam bond and the finding that the equilibrium point is almost entirely on the side of the *epi*-configuration is strong evidence for the steric strain in the system[9]. Analytically, the *epi*-concentration in given samples is of some clinical significance as the *epi*-analogs have little or no antibacterial potency. Currently, an nmr technique is used, but the difference in coupling constants is small ($J_{cis\ 5,6}$=4.5Hz; $J_{trans\ 5,6}$=1.5Hz). A sharp drop in optical rotation is also observed (+343°→+232°). A microbiological assay can also be used. The difference in ORD maxima (Table 1) is quite large and should be sufficient to allow for convenient assay development, quite analogous to that recently reported for tetracyclines epimeric at C_4[10]. The method should also allow examination of the kinetics of this change in dilute equeous solutions. RASMUSSEN and HIGUCHI have already demonstrated that ORD-CD spectra of penicillins can be used to develop sensitive assays for these materials[11].

The preliminary results shown in Figs. 2, 3, and 4 indicate that the alkaline solution transformations of hetacillin and epihetacillin can indeed be studied using ORD. These studies show that epihetacillin decomposes by a monoexponential process with an apparent half-life of around 280 minutes (Fig. 3). Loss of the 238 nm ORD peak with time at pH 11 suggests β-lactam hydrolysis. By contrast, hetacillin solution kinetics apparently follow a biexponential process (Figs. 3 and 4) in accordance with the general equation

$$\text{Rotation} = Ae^{-\alpha t} + Be^{-\beta t}$$

where A, B, α, and β are constants. For hetacillin, the β constant is apparently the same as that describing the exponential loss of epihetacillin. The α phase constant has a value of 0.027 min^{-1} (Fig. 4). It is dangerous to extrapolate from this data to a speculation on the precise mechanism of hetacillin alteration in solution, but it appears that the α phase transition does involve the formation of epihetacillin and it is tempting to suppose that the results reflect epimerization followed by hydrolysis. This point appears worthy of further exploration.

The theoretical implications of the various findings with hetacillin-epihetacillin are also significant. Despite inversion of a bulky group α- to the chromophore, the spectrum is not inverted. A large decrease in positive rotation is seen and this would be consistent with either substantial relief of strain or inversion of one of the sign determining groups if at least one, stronger, determinant remained. Either explanation is plausible on inspection of molecular models. The latter explanation is acceptable if the thiazolidipe atoms 1 and 3 are especially important in determining sign and intensity as, indeed, theoretical calculations suggest[8] acid. Given these many evidences for strong, overriding, vicinal effects in the penicillin spectra, it is not surprising that lactam predictive rules developed for simpler systems lacking vicinal contributors should be inadequate for rationalizing the spectra. The strain in the penam system is also unprecedented among simple lactams and peptides. Given the absence of any closely related model system, it is perhaps not too surprising that empirical relationships derived for simpler molecules fit poorly. For precedent, one notes the frequent failure of the classic octant rule when dealing with non-chair or twist cyclohexanones. These considerations suggest that, for the present, empirical curve comparisons of a family of related antibiotics such as are made in this paper, and in NAJARAJAN's work[4], are the most fruitful approach until a more fundamental theoretical analysis can be elaborated. Apparent success with an empirical relationship which superficially appears to be applicable can seduce one into an unwarranted faith, for the nature of CD is such that only two signs are possible (+ or −) for a given spectrum and one will predict the correct sign roughly half the time by merely guessing.

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