if these angles are available, as in the case of the DNA double helix. Then, the angles θ_t found in this work can be related to the *rotational* angles of the transition moments around an axis perpendicular to the peptide plane.[54] We will correlate the angles θ_t with various helical conformations in future work.

Concluding Remarks. By the reversing-pulse electric birefringence method, the rotational relaxation time $\langle\tau\rangle_{EB}$, of the present $[Glu(OMe)]_n$ sample was evaluated from field-off decay curves in HFP under the same experimental conditions.[22] The axial translation per residue of the $[Glu(OMe)]_n$ helix backbone was then found to be 1.56 Å with the Broersma equation for the rodlike molecule.[22] On this basis, it was concluded that the tertiary winding of the helical chain was probably insignificant and that the conformation was α-helical. Hence, the observed polarization angles of peptide chromophores cannot be attributed to the overall molecular flexibility. The analysis of the $(\Delta\epsilon/\epsilon)$ spectrum of $[Glu(OMe)]_n$ clearly shows that the 190- and 205-nm bands are not polarized either perpendicular or parallel to the helix axis, as originally predicted by a simple exciton theory.[6,7] Furthermore, the intensity ratio of these two bands is about 3.3 (cf. Table I of the present work) or 4.9 (Table I of ref 16) in terms of the dipole strength, a marked contrast with theoretically calculated values of 1.66-1.38, as noted by Mandel and Holzwarth.[16] We ask what then are the alternative assignments of those component bands. The absorption spectra of small amide compounds are quite complex, and the number of transitions is still uncertain. Although the amide band at 180-210 nm has been assigned to a single $\pi-\pi^*$ transition, this view is by no means unanimous, as reviewed by Woody.[2] For instance, it has amply been demonstrated that an intermediate band is present for substituted amides between the strong 190-nm and weak 220-nm bands in the gas phase.[53,55-57] If such a band is also identified in the condensed phase, then the splitting of the peptide band in the helical conformation should be reappraised. Thus, before we reach any final conclusion on the peptide band splitting, the present line of ELD studies must be extended to other helical polypeptides on one hand, and film dichroism studies must also be carried out for oligomeric peptides and small amides on the other.

Registry No. Poly(γ-methyl L-glutamate) (homopolymer), 25086-16-2; poly(γ-methyl L-glutamate) (SRU), 25036-43-5.

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Biosynthesis of the Kinamycin Antibiotics by *Streptomyces murayamaensis*. Determination of the Origin of Carbon, Hydrogen, and Oxygen Atoms by ^{13}C NMR Spectroscopy†

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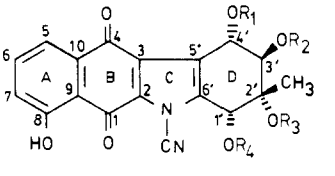
Abstract: Feeding of [1,2-$^{13}C_2$]acetate to cultures of *Streptomyces murayamaensis* gave kinamycin C and kinamycin D, each showing intact incorporation of acetate at carbons 1/2, 3/4, 10/5, 6/7, 8/9, 6′/1′, 2′/CH_3, and 4′/5′ and at the esters, as well as simple enrichment in C-3′, as shown by ^{13}C NMR analysis. The orientation of each acetate in kinamycin D was revealed by incorporation of [1-^{13}C,2,2,2-$^2H_3$]acetate. ^{13}C NMR analysis indicated enrichment at carbons 1, 3, 6, 8, 10, 2′, 4′, and 6′ and the ester carbonyls; isotope-shifted resonances were also observed for carbons 6, 10, and 2′ and the ester carbonyls, indicating retention of deuterium at the adjacent positions from the original acetate. Thus, the biosynthetic origin of all the carbons of kinamycin, except the cyano moiety, was revealed. When [1-^{13}C,1,1-$^{18}O_2$]acetate was fed, isotope-shifted peaks were observed for carbons 1, 8, and 4′ and the ester carbonyls of kinamycin C and of kinamycin D, indicating retention of ^{18}O at these positions, and when kinamycin D was produced in the presence of $^{18}O_2$, isotope-shifted peaks were observed for carbons 4, 1′, and 2′. These results are consistent with the kinamycin skeleton being derived from acetate via 1,3,8-trihydroxynaphthalene—subsequently oxidized either to 2-hydroxyjuglone or to juglone—and 4-amino-2-hydroxytoluic acid. The D ring is most reasonably generated by oxidation of tetracyclic intermediate **18b** to hydroquinone **19b** and then to epoxide **20b**, followed by rearrangement to epoxyquinol **21**, reduction, and epoxide opening with trans attack by water.

The kinamycins [A-D (**1-4**, respectively)] are a group of antibiotics produced by *Streptomyces murayamaensis* sp. nov. Hata and Ohtani that were isolated and characterized by Omura et al.[2,3] The structures were determined in part by chemical and spectroscopic means.[4] The complete structure of kinamycin C (**3**) was obtained from an X-ray crystallographic study,[5] and its relationship to the others was subsequently confirmed.[6] Thus, these metabolites contain highly novel features: a benzo[*b*]tetrahydrocarbazole skeleton and an *N*-cyano moiety. We recently published the complete assignments of the ^{13}C and ^1H NMR spectra of kinamycin D (**4**).[7]

The kinamycins are strongly active against Gram-positive bacteria, but less so against Gram-negative organisms.[3] The antimicrobial activity increases inversely with the number of acetates present. Periodate degradation of the D ring of the



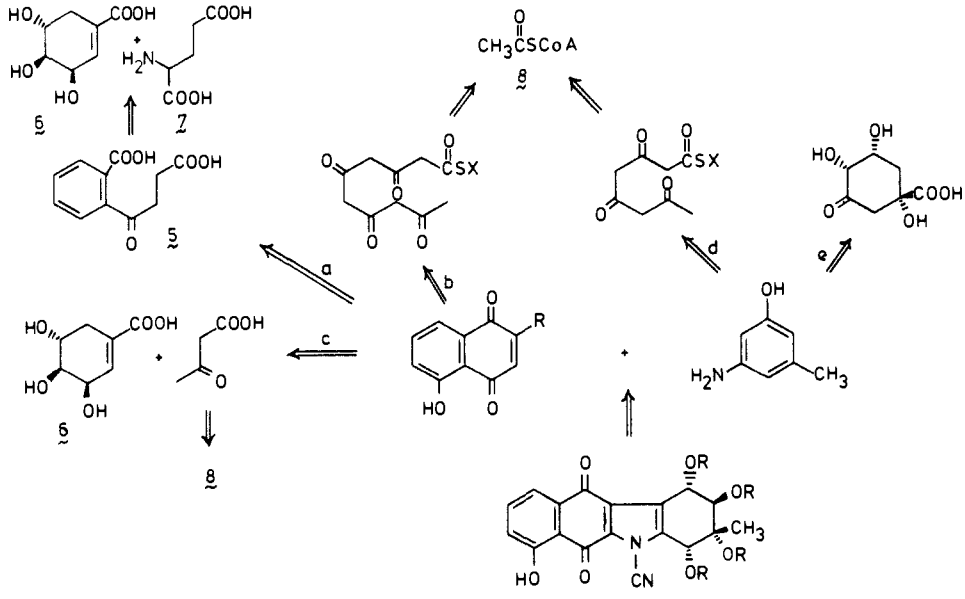
	R_1	R_2	R_3	R_4
1	H	Ac	Ac	Ac
2	H	H	Ac	H
3	Ac	Ac	H	Ac
4	H	Ac	H	Ac

† Dedicated to Professor George H. Büchi on the occasion of his 65th birthday.

‡ On leave from Tamagawa University, 6-1-1 Tamagawa Gakuen, Machida-shi, Tokyo 194, Japan.

0002-7863/86/1508-4625$01.50/0

Scheme I. Retrobiogenetic Analysis of Kinamycin Formation



kinamycins did not cause any significant loss of activity, indicating that the quinone portion of the antibiotics is probably involved in the mechanism of action, although this has not been established directly. Weak antitumor activity was exhibited by **3** against Ehrlich ascites carcinoma and against Sarcoma-180.[3]

Our interest has focused on the biosynthesis of the kinamycins.[8] A retrobiogenetic analysis suggested that the skeleton may have been derived by condensation of a naphthoquinone with 1 equiv of 3-amino-5-hydroxytoluene, as shown in Scheme I.

Naphthoquinones are known to be derived from a number of fundamentally different pathways. One (a) involves succinylbenzoic acid (**5**)—derived from shikimic acid (**6**)—and glutamic acid (**7**), as found in microbial (e.g., vitamins K[9,10]) and in plant (e.g., lawsone and juglone[10]) metabolism. In another microbial metabolism (b) naphthoquinones are derived from acetyl SCoA (**8**) (e.g., flaviolin[11] and skytalone[12]), and in a third pathway (c) they are derived from a combination of the shikimic acid pathway and acetate (e.g., rifamycin[13,14]). Examination of the putative D-ring precursor suggested a similarity (d) to 3,5-dihydroxytoluene (orcinol, derived from acetate via orsellinic acid[15]), as well as to the "C_7N" unit of the ansamycins[14] and mitomycins[16] that is known to be derived from a variation of the shikimate pathway (e).

Table I. Long-Range Couplings Observed by LR HETCOSY

proton	chem shift, δ	long-range coupling to carbon (chem shift, δ)
5	7.68	4 (180.8), 7 (124.3), 9 (115.6)
6	7.58	8 (162.4), 10 (133.8)
7	7.22	5 (120.3)
phenol	12.13	7 (124.3), 8 (162.4), 9 (115.6)
1′	5.48	2′ (73.7), 5′ (132.1), 6′ (127.8)
3′	5.59	2′ (73.7), 4′ (67.3), 2′-CH_3 (18.3)
4′	4.78	3′ (75.7), 5′ (132.1), 6′ (127.8)
1′-$OCOCH_3$	2.19	CH_3CO_2 (171.2)
2′-CH_3	1.22	1′ (71.3), 2′ (73.7), 3′ (75.7)
3′-$OCOCH_3$	2.26	CH_3CO_2 (172.3)

The permutations on these known possibilities as well as the potential that an entirely different pathway could be involved led us to explore the metabolism of this organism. In this paper we report results that clearly define the origin of the kinamycin skeleton from acetate, as well as the probable metabolic events that subsequently generate the unusual tetrahydroxycyclohexene D ring.

Results

NMR Spectroscopic Assignments. Prior to our own work, the ^{13}C NMR spectra of the kinamycins had not been assigned.[17] On the basis of a variety of spectroscopic methods, we have established unambiguous assignments for all carbons of the kinamycin skeleton.[7] Omura had previously assigned the ^1H NMR spectrum of **4**,[6] and we confirmed these assignments from a ^1H COSY spectrum. The results were also consistent with the published assignments for aclacinomycin.[19] A DEPT experiment indicated the proton multiplicity of each carbon, and a HETCOR[20] spectrum then allowed assignment of all carbon resonances except those for the six quaternary carbons at the ring junctions and the two

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(18) Except for the cyano carbon resonance, which has never been observed.
(19) Oki, T.; Kitamura, I.; Matsuzawa, Y.; Shibamoto, N.; Ogasawara, T.; Yoshimoto, A.; Inui, T.; Naganawa, H.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1979**, *32*, 801.
(20) $^1H/^{13}C$ heteronuclear chemical shift correlation.

Table II. ^{13}C NMR Spectrum of Kinamycin D and Incorporation of Labeled Precursors

		precursor[a]			
C	chem shift, δ	[1,2-$^{13}C_2$]-acetate,[b,f] J, Hz	[1-^{13}C,2,2,2-$^{2}H_3$]-acetate,[c] Δδ	[1-^{13}C,1,1-$^{18}O_2$]-acetate,[d] Δδ	[$^{18}O_2$],[e] Δδ
1	183.6	63.0		+0.03	
4	180.8	60.5			+0.04
1″	172.3	59.8		+0.03	
1″	171.3	59.1		+0.03	
8	162.4	63.7		+0.01	
6	136.2	58.5	+0.10		
10	133.8	62.0	+0.07		
2	132.8	63.0			
5′	132.1	47.4			
3	129.0	60.5			
6′	127.8	51.2			
7	124.3	58.5			
5	120.3	62.0			
9	115.6	63.7			
3′	75.7	s			
2′	73.7	41.6	+0.05, 0.10		+0.03
1′	71.3	51.2			+0.03
4′	67.3	47.4		+0.02	
2″	21.2	60.2			
2″	21.0	59.0			
Me	18.6	41.6			

[a] Sites of enrichment indicated by J_{CC} coupling constants or by isotope-induced shifts of δ. [b] $CDCl_3$, 100 MHz; spectral width 25 000 Hz; 64K data points; quadrature detection; 35° pulse; aquisition time 1.31 s; line broadening 0.5 Hz; number of scans 26 326. [c] $CDCl_3$, 100 MHz; spectral width 20 000 Hz; 64K data points; quadrature detection; 35° pulse; acquisition time 1.64 s; number of scans 34 728. [d] $CDCl_3$, 100 MHz; spectral width 22 727 Hz; 64K data points; quadrature detection; 35° pulse; acquisition time 1.44 s; number of scans 31 999. [e] Same parameters as for (*c*) except for number of scans (32 001). [f] Average ^{13}C enrichment/kinamycin nucleus, 3.1%.

quinone carbonyl carbons. Six of these were assigned by application of a 2D NMR experiment that revealed the long-range $^{1}H/^{13}C$ couplings (^{3}J for aromatics; ^{3}J and ^{2}J for aliphatics).[7,21] These couplings are indicated in Table I.

Since H-1′ and H-4′ each showed long-range couplings to C-5′ and to C-6′, the latter two were the only carbon resonances that could not be assigned directly. An examination of their line widths failed to show a significant difference that could be attributed to quadrupolar broadening from the nitrogen atom. Although tentative assignments of these resonances were made by comparison with the spectra of tryptophan,[22] mitomycin C,[23] and the mitosenes,[24] these had to be reversed in light of the ^{13}C–^{13}C couplings to C-1′ and to C-4′ described below. The complete assignments are listed in Table II.

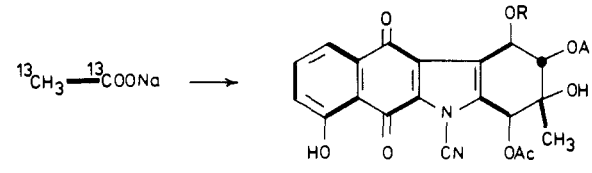
Biosynthetic Studies. The biosynthesis of the kinamycins, as represented primarily by **4**, was studied in shake cultures. *S. murayamaensis*[25] grown on an agar medium was used to inoculate a seed broth based on glucose, yeast and beef extracts and peptone. This was then used to inoculate production broths based on glycerol and soybean meal. Sterile addition of labeled precursors ca. 15 h after inoculation of the production broths and harvest ca. 33 h later were chosen as standard conditions for feeding experiments. After filtration, the kinamycins were extracted from both the broth and the mycelial mat with benzene until the extracts were colorless. After spectroscopic determination of the total amount of kinamycins produced (UV λ_{max} 448 nm), the mixture was separated by chromatography on silicic acid or on silica gel. Typically, only **4**, the major component, was of concern, and it was further purified by recrystallization from ethyl acetate. Occasionally, a significant amount of **3** was also produced, in which case it was also isolated and analyzed.

Table III. ^{13}C NMR Spectrum of Kinamycin C and Incorporation of Labeled Precursors

		precursor[a]	
C	chem shift, δ	[1,2-$^{13}C_2$]-acetate,[b,d] J, Hz	[1-^{13}C,1,1-$^{18}O_2$]-acetate,[c] Δδ
1	184.0	62.1	+0.03
4	178.0	61.1	
1″	172.0	59.8	+0.04
1″	171.1	59.2	+0.04
1″	170.2	60.6	+0.03
8	162.0	63.5	+0.01
6	136.2	58.6	
10	134.2	62.0	
2	132.5	62.0	
5′	130.1	51.0	
3	128.9	60.9	
6′	126.6	51.8	
7	123.7	58.5	
5	119.9	61.9	
9	115.5	63.4	
3′	75.4	s	
2′	73.5	40.3	
1′	70.9	51.2	
4′	68.1	51.6	+0.02
2″	21.1	59.8	
2″	21.0	59.2	
2″	20.9	62.0	
Me	18.6	41.1	

[a] Sites of enrichment indicated by J_{CC} coupling constants or by isotope-induced shifts of δ. [b] $CDCl_3$, 100 MHz; spectral width 25 000 Hz; 64K data points; quadrature detection; 35° pulse; acquisition time 1.31 s; line broadening 0.5 Hz; number of scans 36 596. [c] $CDCl_3$, 100 MHz; spectral width 20 000 Hz; 64K data points; quadrature detection; 35° pulse; acquisition time 1.64 s; number of scans 34 728. [d] Average ^{13}C enrichment/kinamycin nucleus, 3.1%.

Experiments were first carried out with sodium [1-^{14}C]acetate, and an incorporation of 1.8% was obtained; in this case authentic **4** was added as carrier at the start of the workup, and the final product was recrystallized to constant specific radioactivity. When a mixture of labeled ([1-^{14}C], 11.8×10^6 dpm) and unlabeled (4.8 mmol) sodium acetate was fed to two 200-mL fermentations, a 0.54% incorporation of ^{14}C was obtained. Sodium [1,2-$^{13}C_2$]-acetate (**9a**) was then fed to two 200-mL production broths. Workup yielded a nearly equal mixture of **3a** and **4a**, and these



9a **3a**, R = Ac; **4a**, R = H

were further separated by HPLC. In the 100-MHz ^{13}C NMR spectra of these metabolites all carbon resonances were enriched (average enrichment was 3.1% per position), and all ring carbon atoms except C-3′ showed coupling to one other carbon, as did the acetate carbon atoms.[26] The J_{CC}'s are listed in Table II for **4a** and in Table III for **3a**.

Having established that the entire carbon skeleton was derived from acetate, we then simultaneously confirmed the orientation

(21) This is an adaptation of a pulse sequence initially designed to detect the small heteronuclear couplings of $^{1}H/^{2}H$ geminal and vicinal interactions: Bleich, H.; Gould, S.; Pitner, P.; Wilde, J. *J. Magn. Reson.* **1984**, *56*, 515. Gould, S. J.; Palaniswamy, V. A.; Bleich, H.; Wilde, J. *J. Chem. Soc., Chem. Commun.* **1984**, 1075.

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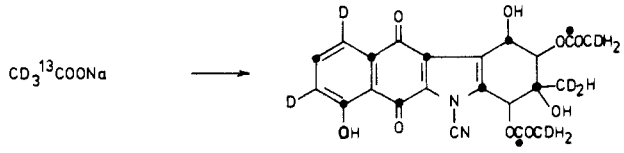
(24) Tomasz, M.; Lipman, R.; Verdine, G.; Nakanishi, K., unpublished results, private communication.

(25) The organism used was originally obtained from Professor S. Omura.

(26) Prior to our work, Professor S. Omura had obtained incorporations of [1-^{13}C]- and [2-^{13}C]acetate, but enrichments could not be detected in the ^{13}C NMR spectrum for all positions expected to be labeled on the basis of a polyketide origin, private communication.

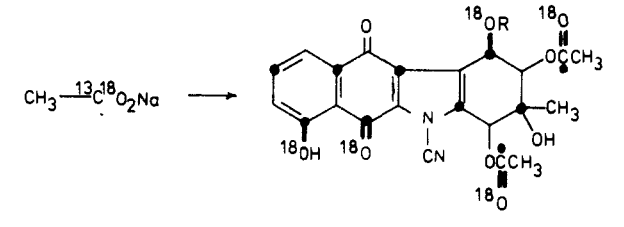
of the acetate units and determined which hydrogens were retained from the acetate methyl groups.[27] Thus, sodium [1-^{13}C,2,2,2-$^{2}H_3$]acetate (**9b**) was next fed and incorporated. Carbons 1, 3, 6, 8, 10, 2′, 4′, and 6′ as well as the two ester carbonyls of **4b** were now enriched. In addition, the resonances for C-6, C-10, and



9b **4b**

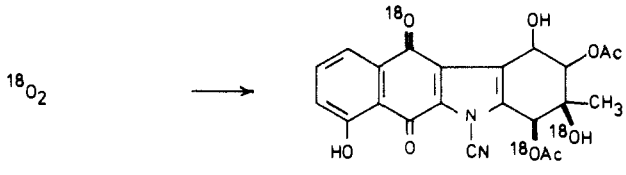
the ester carbonyl carbons each showed one deuterium isotope-shifted peak, while the resonance for C-2′ showed two additional peaks. It was particularly noteworthy that the resonance for C-6′ did not have an isotope-shifted peak. These data are also listed in Table II.[28]

We next investigated the origins of the oxygen atoms of **4** (and **3**). First, sodium [1-^{13}C,1,1-$^{18}O_2$]acetate (**9c**) was fed, and in the ^{13}C NMR spectra of the derived **4c** and **3c** five ^{18}O isotope-shifted resonances and six ^{18}O isotope-shifted resonances, respectively, were observed.[29,30] These corresponded to C-1, -8 and -4′ and the ester carbonyls (from the magnitude of the shifts, the labels were clearly at the doubly bonded ester oxygens[30]), as expected, confirming that three oxygens were retained from the polyketide intermediate. A fermentation was then carried out in the presence of $^{18}O_2$ on the apparatus described by Vederas.[31] The metabolite thus obtained, **4d**, yielded a ^{13}C NMR spectrum containing three isotope-shifted resonances: the expected peak for C-4, as well as peaks for C-1′ and C-2′. With only one oxygen unaccounted for and only one source of oxygen remaining, it was clear that the C-3′ oxygen was derived from water.



3c, R = $CH_3\overset{18}{C}O$

4c, R = H

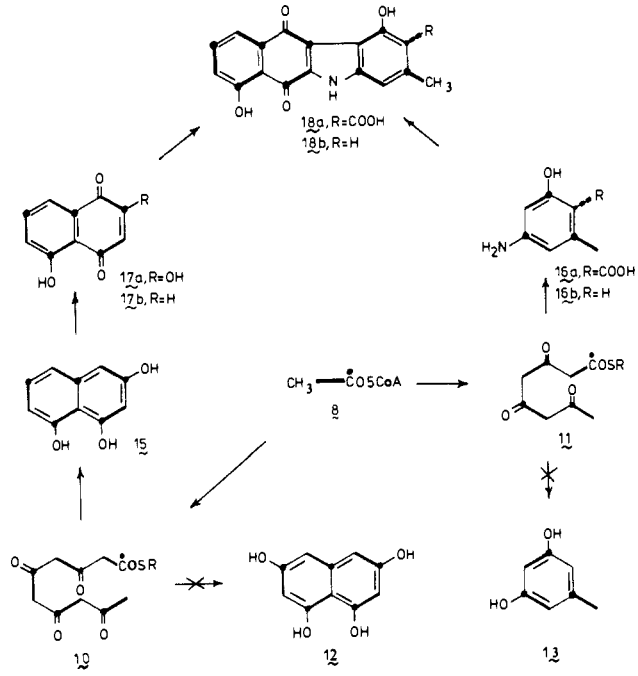


4d

Discussion

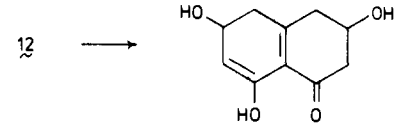
Results from the incorporation of **9a** established that the kinamycin skeleton is derived entirely from acetate and is consistent

Scheme II. Biosynthesis of the Kinamycin Skeleton from Acetyl-SCoA



with the intervention of two polyketide chains, **10** and **11**, one leading to a naphthoquinone and the other leading to a substituted toluic acid, respectively, as shown in Scheme II. The labeling pattern from incorporation of **9b** clearly identified the orientation of each precursor acetate unit. The most noteworthy feature of the early stages of the metabolism thus revealed is that no symmetrical intermediate is involved in either subpathway. Thus, 1,3,6,8-tetrahydroxynaphthalene (**12**), which would be produced by direct cyclization of **10**, was not involved. Similarly orcinol (**13**), which would be derived by cyclization of **11** to orsellinic acid[32] followed by decarboxylation,[15] could not have been involved.

Had **12** been produced, further enzymatic processing would have been expected to proceed from either end, leading to two subsets of metabolites distinguished by the nondegenerate labeling patterns from **9a**. In this case each carbon resonance of **4a** (or **3a**) would have appeared to be coupled to *each* of its neighbors, the spectrum being a composite of all species present. In contrast to kinamycin, the biosynthesis of skytalone (**14**)[12] from **9a** revealed



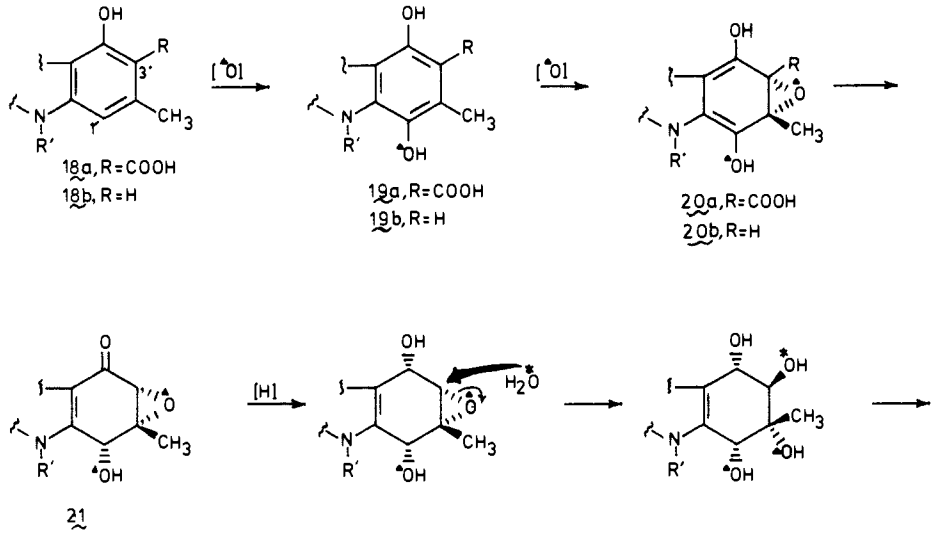
14

the intervention of the symmetrical tetrahydroxynaphthalene (**12**). It is, therefore, reasonable to assume that in the present case reduction and dehydration of **10** preceded aromatization, leading to the unsymmetrical 1,3,8-trihydroxynaphthalene (**15**).[33] This compound has been produced from **12** by melanin-negative mutants of *Verticillium dahliae*.[34]

In a similar vein it would be reasonable to anticipate that transamination of **11** occurs with subsequent cyclization to 4-amino-2-hydroxytoluic acid (**16a**). Coupling with naphthoquinone **17** derived from **15**, either before or after decarboxylation, would yield a benzo[*b*]carbazoloquinone (**18**). Unexceptional oxidations of **15** would yield 2-hydroxyjuglone (**17a**); however, 1,4-addition

(27) Abell, C.; Staunton, J. *J. Chem. Soc., Chem. Commun.* **1981**, 856.
(28) In principle, we could have fed [$^{2}H_3$]acetate and looked for retained deuterium directly by ^{2}H NMR. However, when the 61.4-MHz ^{2}H NMR spectrum of a sample of kinamycin D produced by fermentation in 5% $^{2}H_2O$ was obtained, it was found that the resonances for H-5, -6, and -7 were not resolved; neither were the resonances for H-1′ and H-3′.
(29) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252; *Ibid.* **1980**, *102*, 4609, 6699.
(30) Vederas, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 374. Diakur, J.; Nakashima, T. T.; Vederas, J. C. *Can. J. Chem.* **1980**, *58*, 1311. Vederas, J. C.; Diakur, J.; Nakashima, T. T. *J. Chem. Soc., Chem. Commun.* **1980**, 183.
(31) Moore, R. N.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694.
(32) Gaucher, G. M.; Shepherd, M. G. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 664.
(33) Bell, A. A.; Stipanovic, R. D.; Puhalla, J. E. *Tetrahedron* **1976**, *32*, 1353.
(34) Stipanovic, R. D.; Bell, A. A. *J. Org. Chem.* **1976**, *41*, 2468.

Scheme III. Development of the Kinamycin D-Ring Substitution Pattern

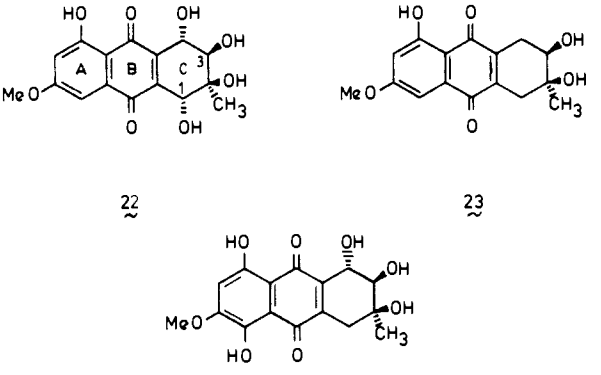


of aniline to 2-bromojuglone[35] takes place at C-2 while addition to the acetate of juglone takes place at C-3. Appropriately labeled **17a** and **17b** are being prepared and will be tested as intermediates to determine whether the enzyme has managed to overcome the inherent reactivity of **17a**.[36]

The deuterium labeling pattern obtained from **9b** is most significant in that no deuterium was found at C-1′ or at C-3′. Lack of deuterium at C-3′ would be consistent with cyclization of **11** to an aromatic structure followed by decarboxylation. Lack of deuterium at C-1′ would be consistent with aromatic hydroxylation of the putative tetracyclic intermediate (**18**), followed by rearomatization to hydroquinone (**19**) (Scheme III). This would necessitate loss of the presumptive deuterium label that would have been present at C-1′ in intermediate **18**. This explanation is also consistent with the incorporation of oxygen that was obtained with the fermentation in the presence of $^{18}O_2$.

From the incorporations of **9c** and $^{18}O_2$ the last key steps in the biosynthesis of the skeleton can be deduced, as shown in Scheme III. Incorporation of the former yielded the labeling pattern expected for normal polyketide metabolism. This also provided a control for the latter feeding that then focused on the origin of the remaining D ring oxygens. It is now logical to presume that the pathway continues from **19b** with a second oxidation to α-epoxide (**20b**). This may have then undergone stereospecific tautomerization to epoxyquinol (**21**). A stereospecific reduction followed by trans attack on the epoxide by water would have yielded the kinamycin skeleton with D ring possessing the four oxygens with the correct stereochemistry and with each derived from the correct source. An alternative pathway (**16a** → **18a** → **19a** → **20a** → 3′-OH-**19b**) via expoxidation and then decarboxylation of epoxy acid **20a**, analogous to the biosynthesis of multicolanic acid[37] is ruled out by the ^{18}O-labeling pattern.

Altersolanol A[38] also contains a tetrahydroxymethylcyclohexene ring, although with a different stereochemistry. Its structure had originally been assigned on the basis of a correlation[39] of altersolanol B with bostrycin.[40] A revision of the stereochemistry and tautomerization of the latter compound, based on a total synthesis,[41] indicate that the structures of these three fungal metabolites should be **22**–**24**, respectively. Thus, the C ring of **22**



differs from the D ring of the kinamycins only in the stereochemistry at the tertiary alcohol. It has been reported[42] that incorporation of **9a** demonstrated that **22** is derived from a single polyketide chain, while incorporation of **9b** resulted in retention of deuterium at C-1 but not at C-3. The lack of deuterium at C-3 is consistent with our results and would be most easily explained by cyclization of the polyketide precursor to a fully aromatic intermediate. However, the retention of deuterium at C-1 is in contrast to our results and would suggest that hydroxylation in this pathway occurs after reduction of the ring (e.g., perhaps **23** is a precursor to **22**).[43]

From the ^{13}C NMR spectrum of **4b**, it was clear that the C-2′ methyl group had retained only two deuterium labels and the acetate methyl groups had each retained only one deuterium. Retention of only two deuterium labels in the starter unit of *O*-methylasparvenone[44] has previously been observed, and since in this metabolite the adjacent carbon is a methylene, it was proposed that one deuterium was lost as a consequence of a dehydration during the biosynthesis. The same result was obtained for a structurally related naphthoquinone.[45] In a few other cases,

(35) Thompson, R. H. *J. Org. Chem.* **1948**, *13*, 377, 870; *Ibid.* **1951**, *16*, 1082.
(36) Alternatively, the first step could be a Friedel–Crafts alkylation of **16**, analogous to known chemistry of orcinol: Kappe, T.; Soliman, F. S. G. *J. Heterocycl. Chem.* **1976**, *13*, 377. Crombie, L.; Crombie, W. M. L.; Forbes, R.; Witaker, A. *J. Chem. Res., Synop.* **1977**, 114.
(37) Grudgeon, J. A.; Holker, J. S. E.; Simpson, T. J.; Young, K. *Bioorg. Chem.* **1978**, *8*, 311. Holker, J. S. E.; O'Brien, E.; Moore, R. N.; Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1983**, 193.
(38) Stoessl, A. *Can. J. Chem.* **1969**, *47*, 767, 777.
(39) Gordon, M.; Stoessl, A.; Stothers, J. B. *Can. J. Chem.* **1972**, *50*, 122.
(40) The X-ray crystal structure of bostrycin *p*-bromobenzoate has been reported: Takenaka, A.; Furusaki, A.; Watanabe, T.; Noda, T.; Take, T.; Watanabe, T.; Abe, J.-n. *Tetrahedron. Lett.* **1968**, 6091.
(41) Kelly, R. T.; Saha, J. K.; Whittle, R. R. *J. Org. Chem.* **1985**, *50*, 3679.
(42) Stoessl, A.; Unwin, C. H.; Stothers, J. B. *Can. J. Chem.* **1983**, *61*, 372.
(43) It is claimed[40] that [^{13}C]-**22** was incorporated into the related anthraquinone macrosporin where the C ring has been aromatized with retention of hydroxyl only at C-3, but the mechanism for such a transformation is obscure.
(44) Simpson, T. J.; Stenzel, D. J. *J. Chem. Soc., Chem. Commun.* **1982**, 1074.
(45) Bentley, R.; Banach, W. J.; McInnis, A. G.; Walter, J. A. *Bioorg. Chem.* **1981**, *10*, 399.

such as rubrofusarin,[46] the isotope shifts have been sufficiently small such that an unresolved envelope of shifted peaks has been observed and it has been impossible to determine precisely how many deuterium labels were retained. However, the kinamycin results deserve some comment since the more typical finding for such incorporations into polyketides is retention of all three deuterium labels at starter units.[47] With regard to the starter methyl of the kinamycins, it is hard to imagine that formation of **16a** would require a dehydration involving the methyl group, and no clear explanation—other than coincidental exchange—for the absence of one deuterium is apparent. However, the loss of deuterium from the esters presents less of a problem. Thus, the monodeuterioacetate esters could have been formed from acetate that had already cycled through the citric acid cycle to malate. At this point only one deuterium would remain. Cleavage by malate synthase[48] would yield monodeuterioacetyl coenzyme A.

Conclusions

Although the precursor to the cyano moiety has not yet been unequivocally identified,[49] the rest of the kinamycin structure is clearly derived from acetate via two polyketide chains, one apparently leading to a naphthoquinone via 1,3,8-trihydroxynaphthalene and the other yielding 4-amino-2-hydroxytoluic acid. It is intriguing that juglone (**17a**), previously known only as a higher plant metabolite derived from shikimic acid and glutamate, may in the present case be derived from acetate and function as a key intermediate in kinamycin biosynthesis. Furthermore, the likely involvement of an epoxyquinol such as **21** reveals a similar metabolism to such diverse natural products as terremutin,[50] MM 14201,[51] and mycochrysone.[52] On the other hand, the different results obtained from the incorporation of **9b** into **4b** and into **22** indicate that the pathway to this latter compound may not be as closely related to kinamycin biosynthesis as a perusal of their structures would suggest. Oxygen-18-labeling studies for **22** would provide an interesting comparison to the results reported here. Tests of the putative intermediates revealed by our results, as well as examination of the intriguing enzymatic and genetic questions raised by these experiments, are being actively pursued.

Experimental Section

General Procedures. Melting points were determined in a Hoover capillary melting point apparatus and are uncorrected. ^1H, ^2H, and ^{13}C NMR spectra were taken on a Bruker AM 400 spectrometer at 400.0, 61.4, and 100.6 MHz, respectively, using a 5-mm multinuclear probe. All ^{13}C and ^2H spectra were taken under Waltz proton-decoupling conditions. NMR spectra are reported as parts per million downfield from Me_4Si (δ 0.0). IR spectra were obtained with a Perkin-Elmer 727B spectrometer, and UV spectra were obtained on either a Cary 210 or an IBM 9420 instrument. Microsamples were weighed on a Cahn Model 29 electrobalance. All radioactive measurements were carried out in a Beckman LS 7800 liquid scintillation counter using the Beckman HP scintillation cocktail. All measurements were done in duplicate to a ±4% standard deviation. Counting efficiencies were determined automatically by using the Beckman DPM program and sealed [^{14}C]-quenched standards purchased from Beckman, and the standard curves were then tested for accuracy with standards prepared from kinamycin D and [^{14}C]-*n*-hexadecane. HPLC was done with a Waters U6K injector, 6000A pump, R401 refractive index detector, and two Partisil PXS 10/25 columns connected in series. Thin-layer (TLC) and preparative-layer (PLC) chromatographies were done on Merck precoated silica gel 60 PF-254 plates, 0.25 of 2.0 mm thick, respectively. Fermentations were carried out in a Lab-Line gyrotory incubator shaker. Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25–35 °C.

Sodium [1-^{14}C]acetate was purchased from ICN Pharmaceuticals. Sodium [1,2-$^{13}C_2$]acetate was purchased from Merck Sharp and Dohme, while sodium [1-^{13}C,2,2,2-$^2H_3$]acetate, sodium [1-^{13}C,1,1-$^{18}O_2$]acetate, and D_2O were purchased from Cambridge Isotope Laboratories. Strains of *S. murayamaensis* were gifts from Professors Omura and Hornemann; all incorporation experiments were done with the former strain. Reference samples of kinamycin D were provided by Professors Omura and Hornemann and of kinamycin C by Professor Hornemann. Silicar CC-4 brand of silicic acid (Mallinckrodt 7086) was used for column chromatography. All chemicals were of reagent grade, and all solvents were distilled prior to use.

Standard Culture Conditions. *S. murayamaensis* was maintained at 5 °C on Krainsky's agar slants composed of 1.0% glucose, 0.05% asparagine, 0.05% $K_2HPO_4 \cdot 3H_2O$, and 1.0% agar. Seed cultures were prepared by inoculating 100 mL of medium containing 2% glucose, 0.5% peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% NaCl, and 0.3% $CaCO_3$, with growth from an agar slant. The cultures, contained in 500-mL Erlenmeyer flasks, were incubated at 25–26 °C and 300 rpm for 48 h. Production broths (200 mL in 1-L Erlenmeyer flasks), consisting of 1.0% glycerol, 1.0% soybean meal, and 0.3% NaCl, were adjusted to pH 8.0 with 3 N NaOH prior to sterilization and were subsequently inoculated 5% v/v with vegetative inoculum from seed cultures. The production cultures were incubated for 48 h. For precursor feedings, the labeled acetate was added in a sterile manner through Millipore filters after ca. 15 h, corresponding to approximately the onset of detectable kinamycin production. For sodium [1,2-$^{13}C_2$]acetate two 200-mL broths were used, while for the other two acetate feedings five such broths were used.

Isolation. The cultures were filtered through cheesecloth, and the broth and mycelial mat were each extracted with benzene until the extracts were colorless. After they were dried over Na_2SO_4, the combined extracts were concentrated to dryness in vacuo. One liter of broth usually yielded 500–600 mg of crude material, which was taken up in a minimal volume of $CHCl_3$/EtOAc (=5/1) and chromatographed on Silicar CC-4 (55–60 g in a 32-mm-diameter column) prepared in chloroform. The column was eluted first with this solvent mixture and then with the same components in a ratio of 3/2. Crude kinamycin D, containing varying amounts of kinamycin C, was usually obtained from early fractions eluted with the latter solvent. This was either recrystallized directly from ethyl acetate or first purified further by PLC ($CHCl_3$/EtOAc = 3/2). The sample composed of **4a** and **3a** and the sample obtained from the H_2O/D_2O fermentation were finally separated by HPLC (isooctane/THF = 65/35, 3 mL/min, 1600 psi).

Incorporation of Sodium [1,2-$^{13}C_2$]Acetate. Sodium [1,2-$^{13}C_2$]acetate (0.197 g) was mixed with an equal amount of sodium acetate and with 2.57×10^7 dpm of sodium [1-^{14}C]acetate. Half the mixture was added sterilely to each of two 200-mL production broths 15 h after inoculation. After an additional 33 h, the fermentations were worked up to yield kinamycin C (**3a**) (1.9 mg) and kinamycin D (**4a**) (6.1 mg). Analysis of these samples in $CDCl_3$ by ^{13}C NMR indicated a ca. 3% incorporation at each labeled position.

Incorporation of Sodium [1-^{13}C,2,2,2-$^2H_3$]Acetate. Sodium [1-^{13}C,2,2,2-$^2H_3$]acetate (0.492 g) was mixed with an equal amount of sodium acetate and with 2.40×10^7 dpm of sodium [1-^{14}C]acetate. The mixture was divided into five portions, and each was added sterilely to a 200-mL production broth 13 h after inoculation. Workup 35 h later through the Silicar CC-4 chromatography yielded material that was recrystallized to afford **4b** (20 mg). The ^{13}C NMR spectrum of this material was obtained.

Incorporation of Sodium [1-^{13}C,1,1-$^{18}O_2$]Acetate. Sodium [1-^{13}C,1,1-$^{18}O_2$]acetate (0.492 g) was mixed with 817 mg of sodium acetate and with 4.40×10^7 dpm of sodium [1-^{14}C]acetate. The mixture was divided into five portions, and each was added sterilely to a 200-mL production broth 12 h after inoculation. Thirty-five hours later workup through the PLC step afforded 10.2 mg of a nearly equal mixture of **4c** and **3c**. This was analyzed by ^{13}C NMR spectroscopy.

Incubation of *S. murayamaensis* in the Presence of $^{18}O_2$. Fermentation in the presence of $^{18}O_2$ required the use of an alternate medium that did not foam excessively. A salt solution (1 L) composed of 0.1% asparagine, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.04% $MgSO_4 \cdot 7H_2O$, and 0.01% $FeSO_4 \cdot 7H_2O$ and a solution of glucose (25 g) in 25 mL of distilled water were sterilized separately. Fifty milliliters of a seed culture, the glucose solution, and

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(48) Smith, E. L.; Hill, R. L.; Lehman, I. R.; Lefkowitz, R. J.; Handler, P.; White, A. *Principles of Biochemistry*, 7th ed.; McGraw-Hill: New York, 1983; p 425.
(49) In an unusual paper it was claimed on the basis of infrared spectroscopic evidence that C-1 of acetate specifically labeled the cyano carbon: Ajisaka, K.; Takeshima, H.; Omura, S. *J. Chem. Soc., Chem. Commun.* **1976**, 571. However, the spectra provided could only be interpreted in this manner if the metabolite were nearly 100% enriched—which was not the case (see ref 26). We have never observed the ^{13}C resonance for the cyano carbon from any of our biosynthetic samples. This is probably due to quadrupolar relaxation by the two nitrogen substituents. Efforts to produce kinamycin in a medium containing inorganic ^{15}N are in progress. However, from feedings of [*guanido*-^{14}C]arginine, [2-^{14}C]glycine, and [3-^{14}C]serine, radioactive **4** was only obtained with the last of these, and this is currently being pursued further.
(50) Miller, M. W. *Tetrahedron* **1968**, *24*, 4839.
(51) Box, S. J.; Gilpin, M. L.; Gwynn, M.; Hanscomb, G.; Spear, S. R.; Brown, A. G. *J. Antibiot.* **1983**, *36*, 1631.
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950 mL of the salts solution were combined in the sterile fermentor apparatus described by Vederas.[31] The buret was charged with 50% $^{18}O_2$ and was replenished as needed. The experiment was carried out twice: the first time 1.2 L was consumed in 52 h, and the second time 1.2 L was consumed in 57 h. Each was worked up separately through the Silicar CC-4 chromatography, and the combined material was purified by PLC to yield 14.2 mg of **4d**, which was analyzed by ^{13}C NMR spectroscopy.

Incubation of *S. murayamaensis* in the Presence of D_2O. *S. murayamaensis* was grown in the standard production medium (two 200-mL broths) to which 10 mL of D_2O had been added sterilely to each flask. After 48 h, the fermentations were filtered and extracted with benzene, and—after they were dried and concentrated in vacuo—the extracts were chromatographed twice by PLC. This yielded 39 mg of kinamycin D that was recrystallized to afford 9.7 mg of pure metabolite: ^2H NMR (61.4 MHz, $CDCl_3$) δ 1.09 (b), 2.14 (b), 4.55 (vb), 5.40 (vb), and 7.58 (sharp peak overlapped by broad peak).

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Registry No. Kinamycin C, 35303-08-3; kinamycin D, 35303-14-1; acetate, 64-19-7.

Divalent Metal Ion Catalysis in Amide Hydrolysis. The Hydrolysis of *N*-Acylimidazoles

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Abstract: Rate constants have been determined for hydrolysis of *N*-picolinoylimidazole, *N*-picolinoylbenzimidazole, and *N*-(6-carboxypicolinoyl)benzimidazole in water at 30 °C. Hydroxide ion, hydronium ion, and water catalyzed reactions were observed. The second-order rate constants for OH^- catalysis, k_{OH}, are similar with *N*-picolinoylimidazole and *N*-picolinoylbenzimidazole. However, the pH-independent and apparent hydronium ion catalyzed reactions of the benzimidazole derivatives are slower than those of the *N*-acylimidazole. The latter reaction is retarded because of the relatively low pK_a of the *N*-acylbenzimidazole conjugate acid ($pK_{app} < 2$). Pronounced metal-ion catalysis occurs in the hydrolysis of these compounds in the presence of Cu^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}. With the picolinoyl derivatives the plots of k_{obsd} vs. metal ion concentration were linear even at metal ion concentrations as high as 0.01 M. At the nonsaturating metal ion concentration of 0.01 M (0.001 M with Cu^{2+}) the rate enhancements in the OH^--catalyzed reactions ranged from 4×10^2 with Co^{2+} to 4×10^5 with Cu^{2+}. A Ni^{2+}-promoted water-catalyzed reaction was also observed in the hydrolysis of *N*-picolinoylbenzimidazole. The hydrolysis of *N*-isonicotinoylbenzimidazole is not significantly catalyzed by divalent metal ions. Therefore, metal ion catalysis requires the pyridine nitrogen to be adjacent to the carbonyl. Saturation was observed at low metal ion concentrations in the hydrolysis of *N*-(6-carboxypicolinoyl)benzimidazole. Metal ion promoted OH^--catalyzed reactions then occur with large enhancements in k_{OH}, ranging from 10^5–10^6 with Zn^{2+}, Co^{2+}, and Ni^{2+} to 10^9 with Cu^{2+}. The value of k_{OH} in the presence of a saturating concentration of Cu^{2+} is 5×10^{11} M^{-1} s^{-1}. Thus, a bimolecular attack of external OH^- can be ruled out; the reaction must involve intramolecular attack of metal ion bound OH^-. At pH > 6 formation of the metal ion–*N*-(6-carboxypicolinoyl)-benzimidazole complex becomes the rate-limiting step in the Ni^{2+}-catalyzed reactions, and a pH-independent reaction is observed. Large metal ion catalytic effects are found in the OH^--catalyzed reactions of the *N*-acylbenzimidazoles because the C–N bond is easily broken.

A number of proteolytic enzymes require the presence of a metal ion for activity.[2,3] Carboxypeptidase A is a Zn(II) metalloenzyme that catalyzes the hydrolysis of peptides having a free terminal carboxyl group and O-acyl derivatives of α-hydroxy carboxylic acids.[2-4] Mechanisms have been suggested for the enzymatic reactions in which the Zn(II) ion is involved.[2-4] Divalent metal ion catalyzed ester hydrolysis reactions have been extensively studied.[5-13] Metal ion promoted OH^- catalysis occurs in these reactions, and enhancements in the second-order rate constant k_{OH} as large as 10^8 have been observed.[7,9] Metal ion promoted water reactions have also been detected in the hydrolysis of phenolic esters[13] and anhydrides of picolinic acid.[14] In contrast, there have been few reports of metal ion catalysis in the hydrolysis of amides,[15-17] and in those cases the rate enhancements have been

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