Hydrolysis of an Amide in a Carboxypeptidase Model Using Co(III) and Bifunctional Catalysts

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Abstract: The ability of a metal ion to cooperate with various inter- and intramolecular acids and bases and promote amide hydrolysis has been investigated. Phenolic and carboxylic functional groups were placed within reach of Co(III)-chelated amides in complexes that are models for zinc-containing peptidases such as carboxypeptidase A. The choice of substitutionally inert Co(III) as the metal ion makes mechanistic interpretation of the data less ambiguous, for it prevents equilibration of isomeric complexes. Co(III) was able to cooperate with a phenol group in a hydroxide-dependent process to enhance the rate of amide hydrolysis by 2 orders of magnitude above that due to metal alone. Under no conditions was there any intramolecular catalysis by the carboxyl group. $H_2PO_4^-/HPO_4^{2-}$ and $AcOH/AcO^-$ buffers were able to catalyze amide hydrolysis efficiently, and it is the basic portion of the buffer pair that was kinetically significant. The buffer-promoted intermolecular process competed with intramolecular catalysis by the phenol group, and imidazole, morpholine, and aniline were ineffective as buffer catalysts. These results are interpreted in terms of a bifunctional mechanism for tetrahedral intermediate breakdown by phenol, acetic acid, or $H_2PO_4^-$. Some discrimination between kinetically indistinguishable mechanisms is made. It is suggested that a similar bifunctional breakdown of the tetrahedral intermediate occurs in the enzyme-catalyzed hydrolysis of peptides.

The enzyme carboxypeptidase A[1] is one of a family of peptidases that utilizes zinc and a carboxylate ion as catalytic groups. Carboxypeptidase A itself has been extensively studied and characterized, both by X-ray[2] and by chemical methods.[3] Other related enzymes of interest include thermolysin and angiotensin converting enzyme. It seems likely that these enzymes all hydrolyze peptides in similar ways.[4]

Several different mechanisms have been considered for the action of carboxypeptidase A.[5] Most of them have built on the finding, in the X-ray studies of Lipscomb, that the substrate is bound to the enzyme with its carbonyl oxygen coordinated to the zinc of the enzyme.[6] Such coordination strongly suggests that zinc acts as a Lewis acid, so attention has focused on the possible roles of the other catalytic groups. Principal among these is the carboxylate group of Glu-270, but an important function was long ascribed to the phenolic hydroxyl of Tyr-248.[7] From the X-ray studies by Lipscomb the Glu-270 carboxylate is near the scissile-bound peptide group, while the phenolic hydroxyl of Tyr-248 is coordinated to the nitrogen atom of the scissile group (Figure 1). Thus the phenol was assigned a role as an acid group while the carboxylate was considered to be either a nucleophile or a general base.

Recently, site-directed mutagenesis has been used to replace tyrosine-248 with a phenylalanine group, and the enzyme retains its catalytic ability to hydrolyze peptides or related esters.[8] Thus any mechanisms that utilize the tyrosine hydroxyl group in the catalysis are invalid, at least in that respect. Furthermore, the mechanistic suggestion by Mock[9] that the Tyr-248 hydroxyl, as an anion, serves as a base group must also be incorrect.

It seemed to us that a study of a complete model for carboxypeptidase might help to elucidate several questions.[10] First of all, it would be of interest to see which mechanism is actually selected by a model system containing an amide substrate and all the catalytic groups of the enzyme. Secondly, it would be of interest to see how rapidly these catalytic groups could cleave the amide in such a model, in order to put the enzymatic rate in a chemical context. We wanted a model system that would be mechanistically relatively unambiguous, and for this reason we decided to use Co(III) as the metal. Since cobalt in this oxidation state is inert to substitution, we expected to be able to construct a complex with the amide substrate fixed in place.[11] This would avoid the possibility that the complex could equilibrate with isomeric structures, allowing mechanisms to operate in which the carbonyl oxygen is not coordinated to the metal ion. With labile metal ions such as Zn(II) these mechanistic ambiguities are common and difficult to solve.[12] Furthermore, it was known that the Co(III) complexes of various peptides are rather easily hydrolyzed by base.[13] We proposed to elaborate the sorts of systems that have been looked at previously to incorporate the other catalytic groups of the enzyme as well.

At the time we started this work it was not yet known that Tyr-248 does not play a catalytic role in carboxypeptidase A. Thus we explored compounds in which a peptide coordinated to Co(III)

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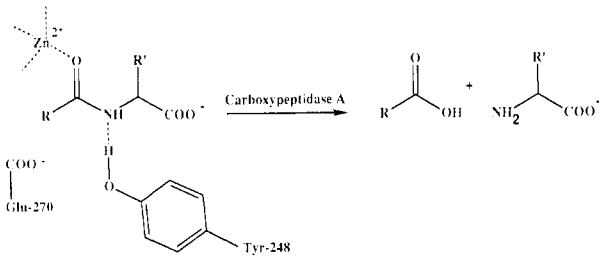
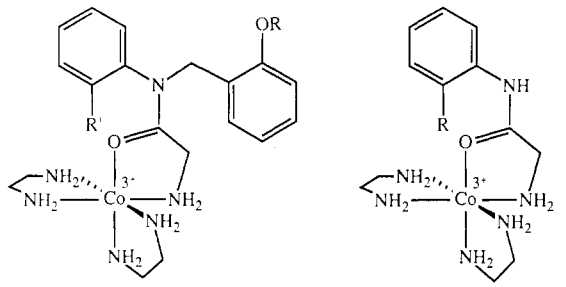


Figure 1. Schematic representation of CPA active site with bound glycyl-L-tyrosine.[6]



1, R = H, R' = CO_2H
2, R = H, R' = H
5, R = CH_3, R' = H

3, R = CO_2H
4, R = CO_2CH_3
6, R = H

Figure 2. Cobalt complexes **1–6** prepared as substrates for amide hydrolysis.

was also within reach of a carboxylate ion and of a phenol group, as in **1** (Figure 2). We also examined the ability of a metal ion to cooperate with either of these two additional functional groups in bifunctional models **2** and **3**. We found that indeed there was cooperative catalysis of the peptide cleavage by the metal and the phenol but that the carboxylate group does not contribute. Because Co(III) is substitutionally inert, it was possible to prepare these complexes with the substrate correctly coordinated to the cobalt but with the other two potential catalytic groups unable to coordinate to the metal themselves. If a catalytic acid and base group can mutually interact via a "short circuit", they will be less effective at interacting with the substrate. In our major study, we examined the catalytic effects of buffers, as unrestricted mimics of enzyme catalytic groups. We observed special buffer structural effects that imply a polyfunctional mechanism of direct relevance to the enzyme itself.

Results

The cobalt-coordinated amide complexes studied were prepared as shown in Scheme I. The molecules were characterized by ^1H NMR, UV–vis, and IR spectroscopies and elemental analysis. In addition, an X-ray structure of **4** was obtained (Figure 3), demonstrating the N,O coordination.[14] The UV–vis, ^1H NMR, and infrared data measured for **4** were compared with the data obtained for the complexes **1**, **2**, **3**, **5**, and **6** and confirmed that they also had the N,O-coordinated peptide structure.

Hydrolysis of amides **1–6** proceeded over a period of several hours to several weeks in buffered[15] solution (Scheme II). Because the organic hydrolysis products of **1**, **2**, and **5** are insoluble in pure water, we selected Me_2SO as a cosolvent that would allow sufficient solubilities of all the components in the reaction mixture. The hydrolysis reactions of all the amides in this study obeyed pseudo-first-order kinetics (reactions followed to at least 88% completion with correlation coefficients all > 0.99 and generally > 0.999). The buffers used in experiments designed to test the effects of neighboring groups were of the amino sulfonate type

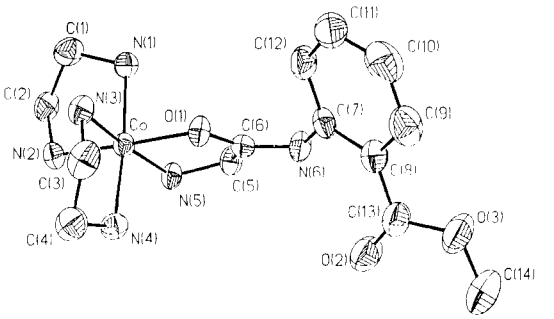


Figure 3. ORTEP diagram of the X-ray crystal structure of **4**. Ellipsoids represent 30% probability contours.

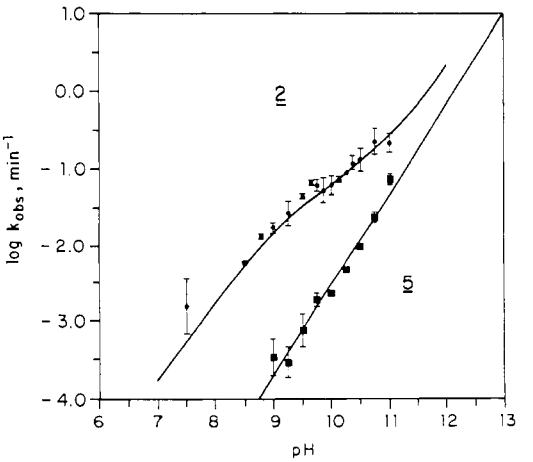


Figure 4. Plot of pH vs. log k_{obsd} for the hydrolysis of **2** (●) and **5** (■). The data points for **2** were fit to a theoretical curve defined by eq 3, using pK_a = 9.6, k_{OH^-} = 28.3 M^{-1} s^{-1}, and k'_{OH^-} = 3.30 M^{-1} s^{-1}. The data points for **5** fit linear eq 1, using k_{OH^-} = 0.48 M^{-1} s^{-1}.

("Good[15] buffers"), chosen because of their known lack of affinity for metal complexes.

Bifunctional Molecules: Effect of an Internal Phenol. The pH dependence of the hydrolyses of **2** and **5** in buffered 25% aqueous Me_2SO over the pH range 7.5–11.0 is plotted in Figure 4. In order to assess the effect of the neighboring phenol group, we compared the hydrolysis rates of **2** to those of compound **5**, in which the phenolic proton was masked with a methyl group. With the substrate at 0.50 mM, the hydrolysis rate of phenol amide **2** was independent of the buffer concentration over the range 0.10–0.50 M. The rate constants listed in Table I and Figure 4 were measured at a buffer concentration of 0.10 M. In contrast, the hydrolysis of methoxy amide **5** was slightly buffer catalyzed. The buffer-independent rate constants listed in Table I and Figure 4 were obtained by extrapolating linear plots of k_{obsd} vs. [buffer] to zero buffer concentration. The rate constants for **2** and **5** are juxtaposed in Figure 4 with theoretical curves calculated from eq 3 or 1, respectively (vide infra).

The hydrolysis rate for the methoxy amide **5** showed an apparent first-order dependence on the hydroxide ion concentration in the pH region studied (eq 1).[16] From kinetic data in the region between pH 9.0 and 11.0, k_{OH^-} is 0.480 M^{-1} s^{-1}.

$$v = k_{obsd}[\mathbf{5}] = k_{OH^-}[OH^-][\mathbf{5}] \quad (1)$$

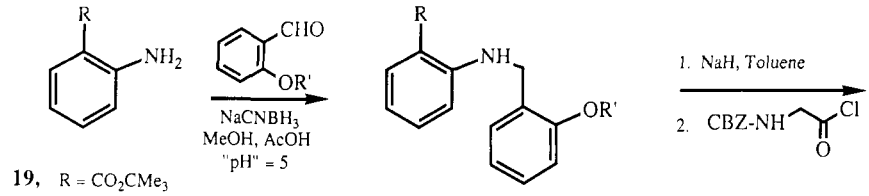
The pH–rate profile for **2** is more complicated. There is a pH 7.5–9.5 region in which the rate is first order in hydroxide ion; above this the phenol titrates and the data fall below the extrapolated first-order line. Since the rate continues to rise with pH, we have used eq 2, in which there are two rate constants for hydroxide reactions, and derived eq 3. In these equations, k_{OH^-} and k'_{OH^-} are the bimolecular rate constants for hydroxide-pro-

(14) Details of the crystal data collection and structure refinement are available as supplementary material (Table IV).

(15) Good, N. E.; Izawa, S. I. In *Methods in Enzymology*; San Pietro, A., Ed.; Academic: New York, 1972; Vol. 24, p 53. Although the addition of Me_2SO to the solution may change the solution "pH", and thus the absolute hydrolysis rates, this should not affect relative hydrolysis rates.

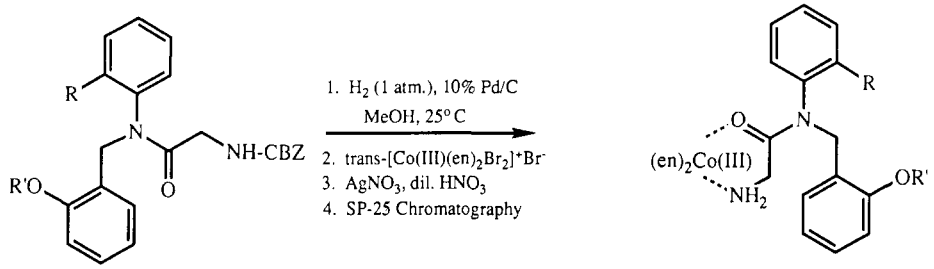
(16) Terms second order in hydroxide ion may become apparent at very high pH; see: Schowen, R. L.; Zuorick, G. W. *J. Am. Chem. Soc.* **1966**, *88*, 1223.

Scheme I



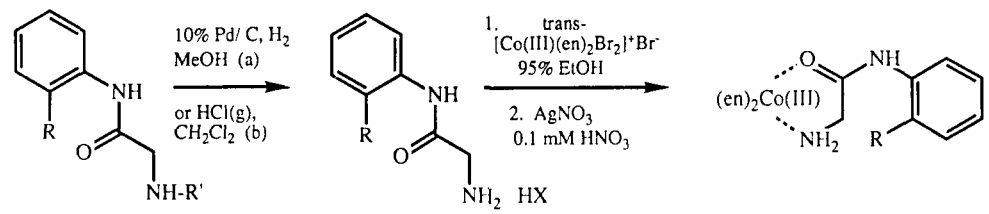
19, R = CO_2CMe_3

13, R = H, R' = $CH_2OCH_2CH_2OCH_3$
9, R = H, R' = CH_3
24, R = CO_2CMe_3, R' = $CH_2OCH_2CH_2OCH_3$
8, R = H, R' = H



14, R = H, R' = $CH_2OCH_2CH_2OCH_3$
16, R = H, R' = CH_3
25, R = CO_2CMe_3, R' = $CH_2OCH_2CH_2OCH_3$

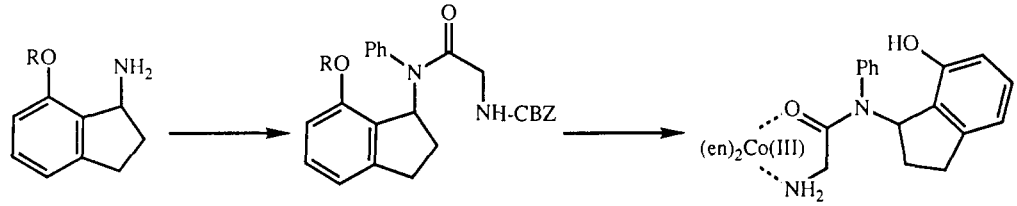
2, R = H, R' = H
5, R = H, R' = CH_3
1, R = CO_2H, R' = H



20, R = CO_2CMe_3, R' = CO_2Bn (a)
22, R = CO_2CH_3, R' = CO_2CMe_3 (b)
27, R = H, R' = CO_2CMe_3 (b)

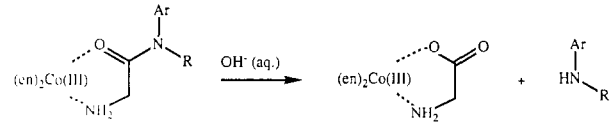
21, R = CO_2CMe_3, X = Cl
23, R = CO_2CH_3, X = Br
28, R = H, X = Br

3, R = CO_2H
4, R = CO_2CH_3
6, R = H

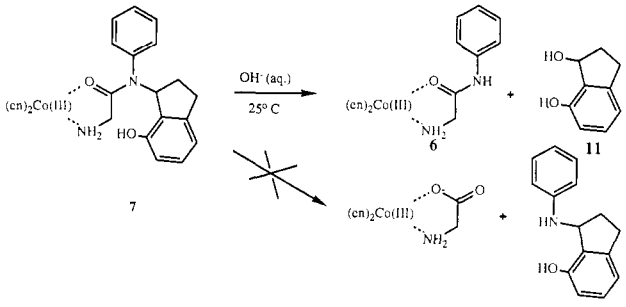


30, R = $CH_2OCH_2CH_2OCH_3$ **31**, R = $CH_2OCH_2CH_2OCH_3$ **7**

Scheme II



Scheme III



moted hydrolysis of **2** with the phenol unionized (**2**-OH) and ionized (**2**-O^-), and K_a is the equilibrium constant for this ionization.

$$v = k_{OH^-}[\mathbf{2}\text{-OH}]\,[OH^-] + k'_{OH^-}[\mathbf{2}\text{-}O^-]\,[OH^-] \quad (2)$$

$$[\mathbf{2}]_T = [\mathbf{2}\text{-OH}] + [\mathbf{2}\text{-}O^-]; \quad K_a = [\mathbf{2}\text{-}O^-]\,[H^+]/[\mathbf{2}\text{-OH}]$$

$$k_{obsd} = k_{OH^-}[OH^-]/\{1 + K_a/[H^+]\} + k'_{OH^-}[OH^-]/\{1 + [H^+]/K_a\} \quad (3)$$

The experimental data (Figure 4) fit eq 3 best when bimolecular rate constants k_{OH^-} and k'_{OH^-} have values of 28.3 and 3.30 M^{-1} s^{-1}, respectively, and the phenol pK_a is 9.6.[17]

A comparison of the bimolecular rate constants for **2** and **5** revealed that the phenol contributed significantly to the hydrolysis

(17) The pK_a of *o*-cresol is 10.3. The small difference between this and the pK_a of the phenol in **2** (9.6) may be due to the presence of the nearby Co^{3+} ion.[13g] It was not possible to measure the pK_a of the phenol in **2** directly because amide **2** hydrolyzes rapidly at elevated pH, $t_{1/2}$ = 15 min at pH 9.

Table I. Rates of Hydrolysis of Amides **1–5** at 25 °C

entry	complex	pH[a]	$10^3 k_{obsd}$, min^{-1} [b]
1	**2**[f]	7.50	(1.50 ± 1.10)
2	**2**	8.50	5.80 ± 0.28
3	**2**	8.80	(12.96 ± 1.14[c]
4	**2**	9.00	17.33 ± 2.10[d]
5	**2**	9.25	26.35 ± 8.40[c]
6	**2**	9.50	43.39 ± 2.28[d]
7	**2**	9.65	64.35 ± 2.90[c]
8	**2**	9.75	61.85 ± 1.18[c]
9	**2**	9.85	52.91 ± 18.58[c]
10	**2**	10.00	60.81 ± 15.24[c]
11	**2**	10.13	72.12 ± 4.53[c]
11	**2**	10.25	88.20 ± 2.21[c]
12	**2**	10.37	118.25 ± 23.89[c]
13	**2**	10.50	131.46 ± 16.67[c]
14	**2**	10.75	225.12 ± 84.15[c]
15	**2**	11.00	213.95 ± 57.71[c]
16	**5**[f]	9.00	0.36 ± 0.16
17	**5**	9.25	0.30 ± 0.13
18	**5**	9.50	0.80 ± 0.44[d]
19	**5**	9.75	1.79 ± 0.35
20	**5**	10.00	2.30 ± 0.05
21	**5**	10.25	4.61 ± 0.33
22	**5**	10.50	9.34 ± 0.08
23	**5**	10.75	23.38 ± 2.87
24	**5**	11.00	75.13 ± 10.51
25	**1**[f]	7.50	0.42 ± 0.28
26	**1**	8.00	0.93 ± 0.01
27	**1**	8.50	2.34 ± 0.16
28	**1**	9.00	7.01 ± 0.02
29	**1**	9.50	15.07 ± 0.87
30	**1**	10.00	22.66 ± 0.36
31	**3**	6.50	0.38 ± 0.05
32	**3**	7.50	2.55 ± 0.03
33	**3**	8.50	7.81 ± 2.64
34	**3**	9.50	9.25 ± 1.26
35	**3**	5.50	0.28 ± 0.01[e]
36	**3**	6.50	1.48 ± 0.02[e]
37	**4**	5.50	0.35 ± 0.01
38	**4**	6.50	0.79 ± 0.11
39	**4**	7.50	0.54 ± 0.19
40	**4**	8.50	1.37 ± 0.07
41	**4**	9.50	6.49 ± 0.35
42	**4**	5.50	1.73 ± 0.02[e]
43	**4**	6.50	4.94 ± 0.04[e]

[a] Buffers used in this study: HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid, pK_a = 7.5; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid, pK_a = 8.1; CHES, 2-[*N*-(cyclohexylamino]ethanesulfonic acid, pK_a = 9.3; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid, pK_a = 10.4; MES, 2-[*N*-morpholino]ethanesulfonic acid, pK_a = 6.1. The pseudo-first-order hydrolysis rates for **5** have been corrected for buffer catalysis is described in the text. [b] Pseudo-first-order rate constants for amide hydrolysis. Values reported for the average of at least two runs. Unless otherwise noted, observed rates were measured spectroscopically in 25% Me_2SO/75% aqueous buffer at 25 ± 0.1 °C at I = 0.35 M (NaCl), as described in the Experimental Section. [c] Reaction rate measured chromatographically. [d] Reaction rate measured both chromatographically and spectroscopically. [e] Reaction run in 80% CH_3CN/20% aqueous buffer at 50 °C. [f] I = 0.26 M (NaCl), after dilution with Me_2SO.

rate in the less alkaline pH-dependent region. Between pH 7.5 and 9.0, the presence of the phenol served to enhance the rate of amide hydrolysis by approximately 100-fold (k_{rel} at pH 9.0 = 104). At higher pHs, this rate enhancement dropped to a factor of 7.

In order to confirm that the process being observed spectroscopically was amide hydrolysis, the chemical yields of amines **8** or **9** produced at the kinetic end point of the reactions of **2** or **5**, respectively, were measured by using an external calibration method (described fully in the Experimental Section). The yields of **8** or **9** were found to be essentially quantitative (118 ± 18% and 106 ± 14%, respectively), confirming that side reactions were not important.

In amide **7**, the phenol is held more rigidly near the amide carbonyl group. However, incubation of this compound under hydrolysis conditions led to a fragmentation reaction. At pH 9.25 and 25 °C, amide hydrolysis did not occur; none of the expected amine product was formed. The products were aniline and indanyl alcohol **11**, and **11** appeared at a much faster rate than did aniline. Thus, compound **7** decomposed under these conditions by forming indanyl alcohol **11**[18] and complex **6** (which then hydrolyzed more slowly).[19] The rate of appearance of **11** was approximately equal to the rate of hydrolysis of **2**. If the phenol in **7** had been a significantly more effective "catalyst" than the phenol in **2**, hydrolysis, rather than decomposition, would thus have been observed.[20]

Effect of an Internal Carboxylate. Pseudo-first-order rate constants for the hydrolysis of **3** and **4** in aqueous solution at 25 °C are listed in Table I. In an effort to measure the effect of the neighboring carboxylate group in **3**, its pseudo-first-order hydrolysis rates were compared to those for **4**, where this group was masked as a methyl ester.

The data show that between pH 5.5 and 6.5 the rate constants for hydrolysis of **3** were very similar to those of **4**, while at higher pH the complex **3** hydrolyzed slightly faster. However, titration studies suggested that the faster hydrolysis rate of **3** was caused not by a neighboring group effect but rather by an ionization effect. The amido protons on **3** and **4** titrated at significantly different pHs, showing pK_a 7.2 for **4** but pK_a 9 for **3**.[21] The difference must be a consequence of hydrogen bonding.[22] This ionization effect implies that comparison of the hydrolysis rates of these two complexes provides no information about the extent of carboxylate catalytic participation, for they do not hydrolyze through analogous intermediates at pH values greater than 7.[23]

We also measured the relative hydrolysis rates of these two amide complexes in a partially nonaqueous solvent system where intramolecular catalysis might be kinetically more significant. In 80% aqueous CH_3CN at 50 °C no kinetic advantage of the carboxyl group was observed (Table I). The limited solubility of the cobalt complexes in organic solvents precluded the use of lower water concentrations.

Trifunctional Molecule: Effect of an Internal Phenol plus an Internal Carboxylate. Pseudo-first-order hydrolysis rates for trifunctional **1** in 25% aqueous Me_2SO as a function of pH are listed in Table I. We found that the rate constants for hydrolysis of **1** were actually lower than those of the bifunctional complex **2**.[24] Although this may be due in part to the differences between the two amine leaving groups, it seemed clear that the contribution of the proximal carboxyl group to the rate cannot be very significant.[25] Interestingly, catalysis by buffer was not observed in this system.

Effect of Added Buffers. Examination of the data in Table II shows that acetate buffer efficiently catalyzed the hydrolysis of both **2** and **5** in 25% aqueous Me_2SO at 50 °C. A linear increase in k_{obsd} was observed (Figure 9) as the $[buffer]_{total}$ was increased from 0.075 to 0.75 M. The pseudo-first-order rate constants measured at varying buffer ratios (but at a constant $[buffer]_{total}$) showed that acetate ion was the kinetically significant component of the buffer in both cases (Figure 5).[29] The bimolecular rate

(18) Prepared by treatment of 7-hydroxyindanone with lithium in liquid ammonia.

(19) A similar fragmentation of **2** does not occur. Amine **8** is retained for 6.2 min when eluted (reverse phase) with 75% aqueous MeOH, while aniline elutes with a retention time of 3.9 min in 70% aqueous MeOH.

(20) This compound was prepared, and studied, as a pair of diastereomers.

(21) The pK_a of the amido proton in $[Co(en)_2glyNH(CH_3)]^{3+}$ is reported in ref 12a to be 11.2 ± 0.2.

(22) For another example of this effect, compare the pK_a values for phenol (pK_a = 10) and salicylic acid (pK_{a2} = 12.4).

(23) Methyl anthranilate absorbs at 325 nm, while anthranilic acid absorbs at 308 nm. No anthranilic acid could be detected by TLC.

(24) Although the hydrolysis rate is still an order of magnitude faster than that of the methyl ether complex **5**.

(25) Although the geometry[26] in this system mimics that in aspirin, where general-base catalysis is observed,[27] the inability of a similarly placed carboxylate to enhance amide hydrolysis has been reported.[28]

(26) Gandour, R. D. *Bioorg. Chem.* **1981**, *10*, 169.

(27) Fersht, A. R.; Kirby, A. J. *J. Am. Chem. Soc.* **1967**, *89*, 4853.

(28) Bender, M. L.; Bergeron, R. J.; Komiyama, M. *The Bioorganic Chemistry of Enzymatic Catalysis*; Wiley: New York, 1984, p 223.

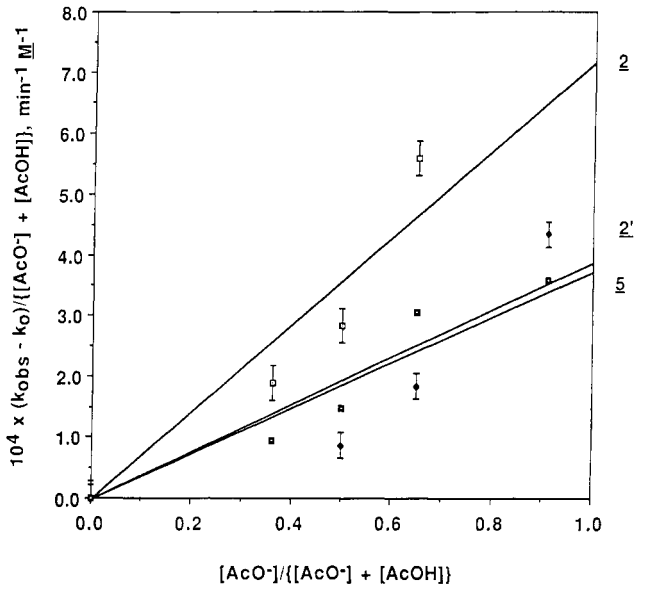


Figure 5. Dependence of the buffer-catalyzed hydrolysis rate of **2** (⊡, k_0 determined by extrapolation of k_0 obtained at higher pH values; □, k_0 determined by extrapolation of rates in acetate buffer to zero buffer concentration; see text and Table III) and **5** (♦; k_0 = 0) on the [AcO^-]/{[AcOH] + [AcO^-]} ratio. Lines drawn are computer-generated best-fit lines through the experimental points.

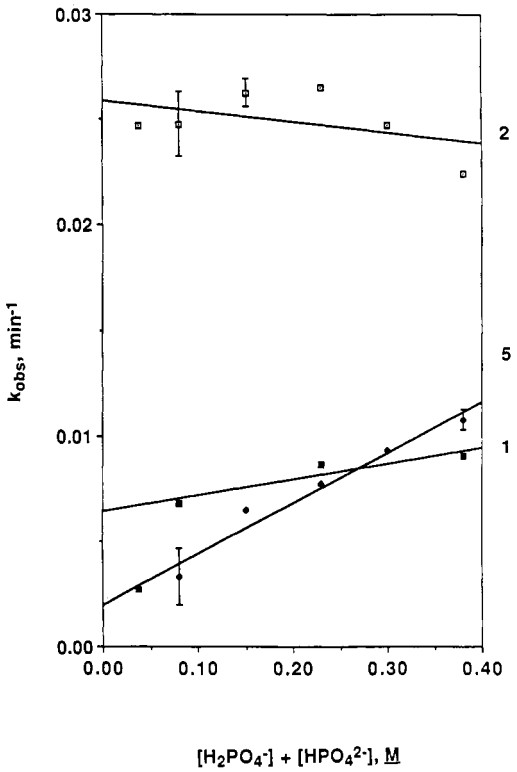


Figure 6. Plot of the observed pseudo-first-order rate constant for hydrolysis of **1** (■), **2** (⊡), and **5** (♦) vs. the concentration of phosphate buffer. Lines drawn are computer-generated best-fit lines through the experimental points.

constants calculated for acetate ion catalyzed hydrolysis of **2** and **5** are listed in Table III; it should be noted that their values do not differ by more than a factor of 2.

Other buffers were also tested for their ability to catalyze the hydrolysis of **2** and **5** (Table II). Neither imidazole nor morpholine buffers catalyzed the hydrolysis of **2**. In addition, aniline buffer did not catalyze the hydrolysis of **6**.[30] However, phosphate buffer

(29) Reference 10b.

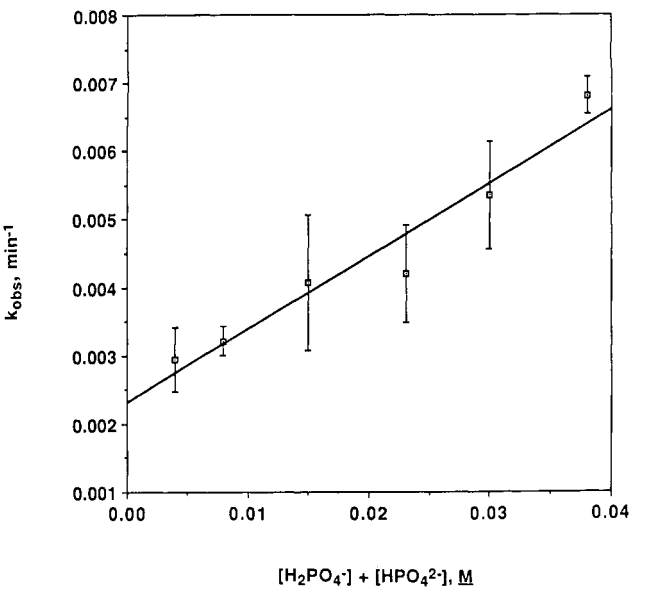


Figure 7. Plot of the observed pseudo-first-order rate constant for amide hydrolysis of **2** vs. the total concentration of phosphate buffer at low concentration, with additional 0.04 M HEPES buffer (in contrast to the conditions of Figure 6). Line drawn is the computer-generated best-fit line through the experimental points.

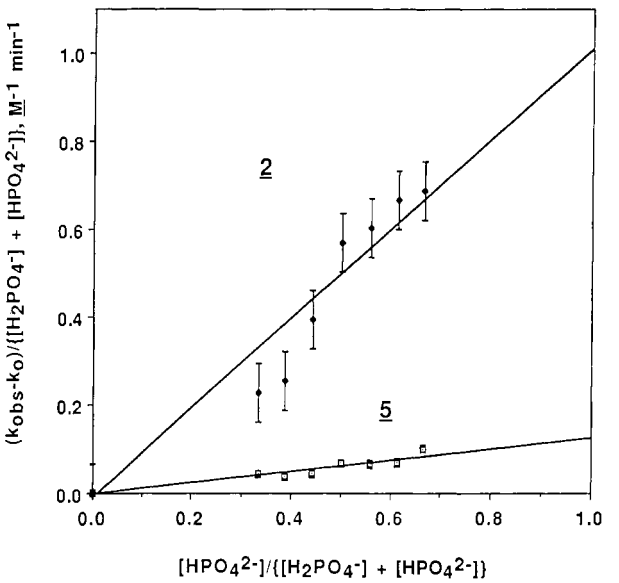


Figure 8. Dependence of the buffer-catalyzed hydrolysis rates of **2** (♦) and **5** (■) on the [HPO_4^{2-}]/{[HPO_4^{2-}] + [H_2PO^{4-}]} ratio. Lines drawn are computer-generated best-fit lines through the experimental points.

is an efficient catalyst for the hydrolysis of **1**, **2**, and **5** at neutral pH (see Figure 6). A linear increase in k_{obsd} as the [phosphate]$_{total}$ increased from 0.04 to 0.40 M was observed for **1** and **5** and between 0.004 and 0.04 M [phosphate]$_{total}$ for **2** (Figure 7). The ratio of $H_2PO_4^-$ to HPO_4^{2-} was varied at a constant [phosphate]$_{total}$ and showed HPO_4^{2-} to be the kinetically significant species for both **2** and **5** (Figure 8). Bimolecular rate constants are listed in Table III; it can be seen that $k_{HPO_4^{2-}}$ for **2** is only 8 times greater than $k_{HPO_4^{2-}}$ for **5**. Thus, much of the 10^2 effect of the phenol has been lost in the phosphate-catalyzed term, as was found for catalysis by acetic acid/acetate buffer (vide infra).

Much of this 10^2 effect is still present in the buffer-uncatalyzed term, k_{OH^-}, in this pH range. The value of k_{OH^-} for **2** was obtained

(30) Aniline should not be able to catalyze the hydrolysis of **6** by a nucleophilic mechanism because the "activated intermediate" would be identical with the starting material. This reasoning was used by Jencks and Carriuolo[31] to identify general-base catalysis of acetylimidazole by imidazole buffer.

(31) Jencks, W. P.; Carriuolo, J. *J. Biol. Chem.* **1959**, *234*, 1280.

Table II. Effect of Buffers on the Rate of Amide Hydrolysis of **2** and **5** at 50 °C in 25% Aqueous Me_2SO

pH[a]	[buffer], M	10^3k_{obsd},[b] min^{-1} 2	5	1
		Acetic Acid/Sodium Acetate Buffer		
4.50	0[d]	0.004 ± 0.001	0[f]	
	0[e]	0.08 ± 0.00$_3$		
	0.75	0.15 ± 0.00$_1$		
4.75	0[d]	0.008 ± 0.002	0[f]	
	0[e]	0.11 ± 0.01		
	0.75	0.22 ± 0.01	0.07 ± 0.00$_1$	
5.02	0[d]	0.015 ± 0.004		
	0[e]	0.20 ± 0.01		
	0.75	0.43 ± 0.02	0.14 ± 0.00$_1$	
5.75	0[d]	0.079 ± 0.019	0[f]	
	0[e]	0.92 ± 0.14		
	0.75	1.20 ± 0.17	0.33 ± 0.01	
4.75	0.15	0.14 ± 0.01		
4.75	0.30	0.15 ± 0.04		
4.75	0.45	0.16 ± 0.02		
4.75	0.60	0.20 ± 0.00$_2$		
4.75	0.75	0.22 ± 0.01		
		Na_2HPO_4/NaH_2PO_4 Buffer		
6.90	0[g]	1.26 ± 0.31	0[f]	
	0.04	10.35 ± 0.003	1.10 ± 0.01	
7.00	0[g]	1.37 ± 0.06	0[f]	
1	0.04	11.57 ± 1.14	1.07 ± 0.00$_4$	
7.10	0[g]	1.57 ± 0.12	0[f]	
	0.04	17.36 ± 0.79	1.74 ± 0.01	
7.20	0[g]	1.85 ± 0.004	0[f]	
	0.04	24.68 ± 0.09	2.73 ± 0.01	
7.30	0[g]	2.61 ± 0.78	0[f]	
	0.04	26.76 ± 1.87	2.55 ± 0.04	
7.40	0[g]	3.08 ± 0.32	0[f]	
	0.04	32.93 ± 8.16	1.72 ± 0.01	
7.50	0[g]	3.24 ± 0.01	0[f]	
	0.038	30.80 ± 0.42	1.56 ± 0.02	
7.20	0.004[c]	2.93 ± 0.47		
7.20	0.008[c]	3.21 ± 0.22		
7.20	0.015[c]	4.07 ± 1.00		
7.20	0.023[c]	4.20 ± 0.71		
7.20	0.030[c]	5.35 ± 0.79		
7.20	0.038[c]	6.80 ± 0.27		
7.20	0.038	24.68 ± 0.09	2.73 ± 0.09	
7.20	0.08	24.79 ± 1.55	3.32 ± 1.35	6.79 ± 0.01
7.20	0.15	26.28 ± 0.64	6.48 ± 0.01	
7.20	0.23	26.49 ± 0.11	7.71 ± 0.01	8.68 ± 0.04
7.20	0.30	24.78 ± 0.07	9.35 ± 0.02	
7.20	0.38	22.45 ± 0.12	10.81 ± 0.45	9.11 ± 0.03
		Imidazole Buffer		
6.90	0[g]	1.26 ± 0.31		
6.90	0.04	0.99 ± 0.03		
6.90 0.08	1.64 ± 0.09			
6.90	0.15	0.87 ± 0.05		
		Morpholine Buffer		
8.40	0.19	14.77 ± 0.03		
8.40	0.38	16.13 ± 0.03		
8.40	0.75	14.06 ± 0.04		

[a] See text for details. The pH reported is that of the aqueous buffer solution prior to dilution with organic solvent. [b] Observed pseudo-first-order rates for amide hydrolysis, in 25% Me_2SO/75% aqueous buffer solution at 50 °C. Ionic strength maintained with NaCl at 0.75 M (after dilution with Me_2SO). [c] Buffer solutions contained 0.04 M HEPES in addition to the buffer noted. [d] Value obtained by extrapolating data first order in hydroxide obtained at higher pH (6.9–7.5; this table) in HEPES buffer to this pH, assuming a first-order dependence on hydroxide concentration. [e] Value obtained by extrapolation of data obtained at this pH, but in the presence of acetae buffer to a zero concentration of acetate buffer. [f] Rate too slow to be measured under these conditions. [g] pH was maintained with 0.04 M HEPES buffer.

in two ways: the linear region of Figure 7 was extrapolated to zero phosphate concentration, and the pseudo-first-order rate constants were measured in the presence of a buffer known not to catalyze the reaction significantly (HEPES). The values obtained by these two methods agree to within 25% and are at least

Table III. Apparent Second-Order Rate Constants for Amide Hydrolysis in 25% Aqueous Me_2SO at 50 °C[a]

catalyst	pH	app k_2, M^{-1} s^{-1} 2	5
AcO^-	4.75	7.1×10^{-4}[b] (3.9×10^{-4})[c]	3.8×10^{-4}
HPO_4^{2-}	7.20	1.0	0.13

[a] Substrate concentration initially 0.50 mM. [b] Calculated as discussed in the text with a value of k_0 obtained by extrapolation of pseudo-first-order rate constants measured at pH 6.9–7.5 (0.04 M HEPES buffer to control pH) into this more acidic region. [c] Calculated as discussed in the text with a value of k_0 obtained by extrapolating pseudo-first-order rate constants in solutions containing 0.075–0.75 M acetate ion at pH 4.75.

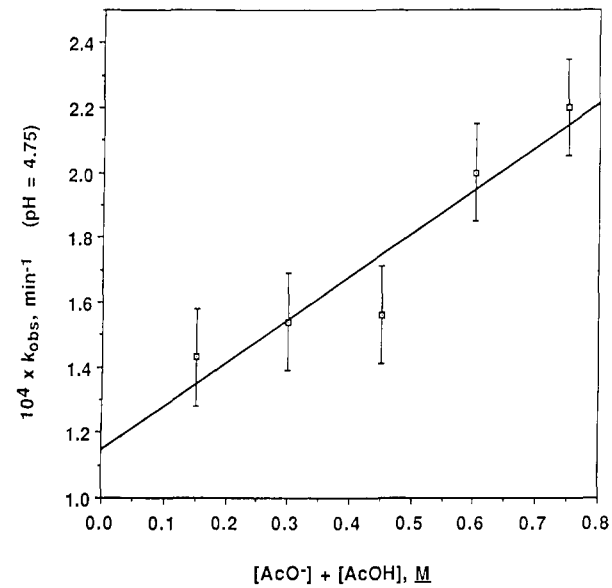
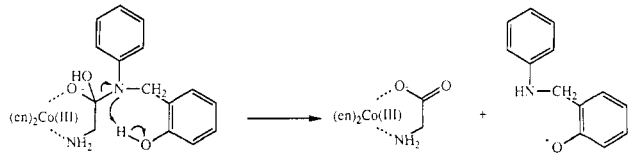


Figure 9. Plot of the observed pseudo-first-order rate constants for acetate-catalyzed hydrolysis of **2** vs. the concentration of acetate buffer in the medium. Line drawn is the computer-generated best-fit line through the experimental points.

Scheme IV



50 times greater[32] than k_{OH^-} for **5** at this pH (k_0 for **5** in this pH range could not be measured accurately; only a lower limit could be assigned). Thus the phenol still functions to enhance the hydrolysis rate significantly in a buffer-independent process even at neutrality.

Interestingly, at high concentration of phosphate buffer, >0.04 M, the hydrolysis rate of **2** became independent of the buffer concentration; catalysis by HPO_4^{2-} showed *kinetic saturation*.[33] Similar effects were not observed with any other amide complex or any other buffer.

Discussion

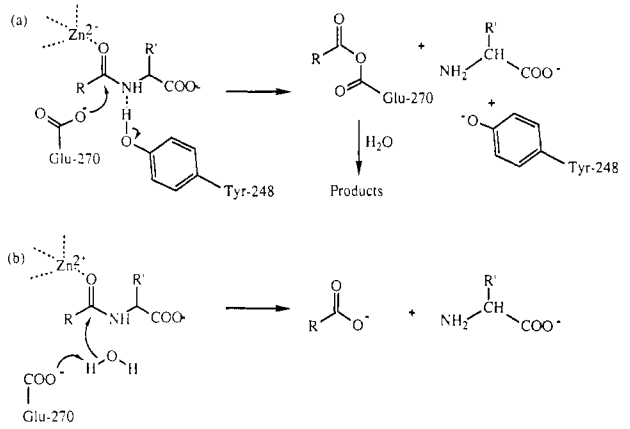
Model Systems. The phenol group cooperates in catalysis of the hydrolysis reaction performed by Co(III) and OH^-. In the lower pH region in which the phenol carries its proton it adds about a factor of 100 to the hydrolysis rate of the substrate. We observed about the same factor of 100 in a previous study[34] in which a neighboring phenolic group assisted the reaction of a carboxamide group with a carboxylate ion. In both cases this catalysis pre-

(32) The half-life for hydrolysis of **5** under these conditions is at least 200 h.

(33) Cunningham, B. A.; Schmir, G. L. *J. Am. Chem. Soc.* **1967**, *89*, 917.

(34) Chin, J.; Breslow, R. *Tetrahedron Lett.* **1982**, *23*, 4421.

Scheme V



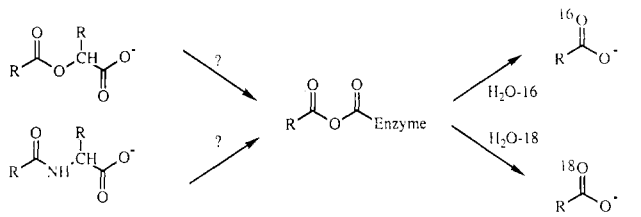
sumably reflects the protonation of the leaving nitrogen by the phenol group (Scheme IV, but see our discussion of kinetic equivalence later in this paper). If so, the addition of hydroxide ion to the carbonyl of the substrate in the cobalt complex is reversible; the rate-determining second step, in which the tetrahedral intermediate decomposes with loss of nitrogen, is assisted by the neighboring phenol. Of course it is well-known that the second step in amide hydrolysis is normally rate determining under basic conditions, so one would expect the phenol to be catalyzing this step by protonating the leaving group.[35] It is even more likely that the second step will be rate determining when Co(III) strongly facilitates the first reversible step.[36]

This is the sort of mechanism that was invoked for carboxypeptidase A itself when it was believed that Tyr-248 was a functioning catalytic group in the enzyme. Now that this catalytic group has been invalidated by the genetic engineering experiments of Rutter and Hilvert,[8] the relevance of our finding to the enzyme mechanism is less clear. Possibly another catalytic group plays this role in the enzyme. More likely, as we will discuss below, this function is no longer needed because the enzyme uses the Glu-270 in two sequential catalytic steps.

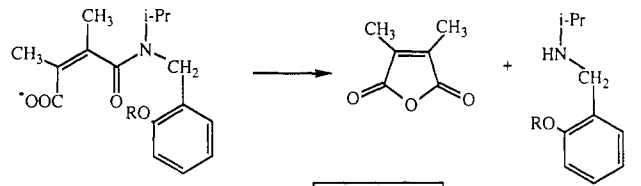
One of the major questions about the mechanism of action of carboxypeptidase A has concerned the function of this Glu-270 group. The two principal mechanisms have utilized it on the one hand as the nucleophile, on the way to the formation of an intermediate anhydride[37] (Scheme Va), and on the other hand as a general base[38] (Scheme Vb).

Evidence for a mechanism proceeding through an anhydride has included the apparent spectroscopic detection of an intermediate in an enzyme-catalyzed reaction.[39] However, it seems likely that this intermediate is simply one of the forms of the enzyme-substrate complex and not the postulated anhydride.[40] Disagreement on this point still exists. In addition there is a recent report that an anhydride intermediate can be trapped at low temperature by denaturing the protein and performing a hydride reduction.[41] However, the extent of reduction in this experiment is at a level only about double that of some of the control ex-

Scheme VI



Scheme VII



32

R	k(rel.)
H	10^2
CH_3	1

periments that could not have formed the postulated intermediate. There is also some question about whether the strong acid denaturation used in the workup might not induce chemical reactions between enzyme and substrate in its own right.[42] Thus, at the present time it is fair to say that there is no strong evidence supporting the intermediacy of an anhydride during carboxypeptidase A hydrolyses of peptides.

Support has also been advanced for the functioning of Glu-270 as a general base. We first reported that $H_2{}^{18}O$ exchange experiments could exclude an anhydride intermediate in these reactions except for the (very real) possibility that the enzyme traps a water molecule and prevents it from exchanging with solvent.[43] In a second approach to the problem, we examined the hydrolysis of a peptide substrate and of the analogous ester substrate by carboxypeptidase A.[44] If these two substrates hydrolyze through a common intermediate, that intermediate should partition identically on attack by two different nucleophiles regardless of how it was formed (Scheme VI).[45] Since carboxypeptidase is apparently unable to acylate any nucleophile except H_2O,[46] we studied this partitioning by using a mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$. We found that the isotope effect for attack by water was different in the hydrolysis of a peptide substrate and of a corresponding ester substrate with the same acyl group, thus excluding an identical intermediate for these two hydrolysis reactions. This is rather strong evidence against a common anhydride mechanism for the two processes, although as we pointed out there are some versions of such a mechanism that might still be permitted.

For these reasons it obviously was of interest to see whether in the model system a carboxylate ion could serve as an additional catalytic group and if so to establish the mechanism by which it operates.

As one approach to this question we examined the hydrolysis rate of compound **1**, a model that potentially contains all of the functionality of the enzyme–substrate compex. However, remarkably we found that **1** is less rapidly hydrolyzed than is **2**, which lacks the carboxyl group (Table I). Thus the principal established catalytic group of the enzyme, other than the metal ion, actually makes a *negative* contribution to the rate of our model system!

It seemed to us that there were two likely problems here. First, the carboxylate ion in compound **3** is a very weak base. Our

(35) (a) Bender, M. L.; Thomas, R. J. *J. Am. Chem. Soc.* **1961**, *83*, 4183. (b) Eriksson, S. O.; Bratt, L. *Acta Chem. Scand.* **1967**, *21*, 1812. (c) O'Connor, C. *Q. Rev. Chem. Soc.* **1970**, *24*, 553. (d) Pollack, R. M.; Bender, M. L. *J. Am. Chem. Soc.* **1970**, *92*, 7190. (e) Kershner, L. D.; Schowen, R. L. *J. Am. Chem. Soc.* **1971**, *93*, 2014.

(36) Sayre, L. M. *J. Am. Chem. Soc.* **1986**, *108*, 1633.

(37) Reeke, G. N.; Hartsuck, J. A.; Ludwig, M. L.; Quiocho, F. A.; Steitz, T. A.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 2220.

(38) Recently an interesting attempt to distinguish the anhydride and general-base pathways using solid-state NMR spectroscopy was reported: MacKenzie, N. E.; Fagerness, P. E.; Scott, A. I. *J. Chem. Soc., Chem. Commun.* **1985**, 635.

(39) (a) Makinen, M. W.; Kuo, L. C.; Dymowski, J. J.; Jaffer, S. *J. Biol. Chem.* **1976**, *254*, 356. (b) Makinen, M. W.; Yamamura, K.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3882. (c) Makinen, M. W.; Fukuyama, J. H.; Kuo, L. C. *J. Am. Chem. Soc.* **1982**, *104*, 2667.

(40) Hoffman, S. J.; Chu, S. S.-T.; Lee, H.; Kaiser, E. T.; Carey, P. R. *J. Am. Chem. Soc.* **1983**, *105*, 6981.

(41) Sander, M. E.; Witzel, H. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 681.

(42) March, J. *Advanced Organic Chemistry*, 2nd ed.; McGraw Hill: New York, 1977; p 369.

(43) Breslow, R.; Wernick, D. L. *J. Am. Chem. Soc.* **1976**, *98*, 259.

(44) Breslow, R.; Chin, J.; Hilvert, D.; Trainor, G. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4585.

(45) Breslow, R. *Organic Reaction Mechanisms*, 2nd ed.; Benjamin Cummings: New York, 1969.

(46) Breslow, R.; Wernick, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1303.

titration studies suggest that it has a pK_a a little above 2, much below the pK_a of a normal carboxylate, apparently as a result of the electrostatic effect of the Co(III) ion.[47] By contrast, the pK_a of Glu-270 in the enzyme is usually cited as 6.1, higher than normal.[1b] The second problem is probably related to the short circuit worries we mentioned earlier. The carboxylate may hydrogen bond to the phenol group in compound **1**; this could explain why the rate for **1** is less than that for **2**, with the carboxylate missing. Such a hydrogen bond would also make it even less likely that the carboxylate could act as a catalyst.

Carboxylate ions can nucleophilically attack amides in model systems.[48,34] We have described a model system (**32**) in which such attack cooperates with the catalytic action of a phenol group, which protonates a leaving amine nitrogen (Scheme VII).[34] The difference between that type of system and the current one has to do with the presence or absence of *fragmentation* as a result of such attack. In maleamic acid substrates such as **32**, attack by a carboxylate forms a maleic anhydride. The driving force is partly furnished by liberation of the amine particle and the resulting favorable entropy change associated with such fragmentation.[45] In compounds **1** or **3**, any nucleophilic attack by the carboxylate group would form an anhydride with the amine "leaving group" still attached; by far the most likely next step would be reattack by the amine to form the substrate again. One does not expect to see nucleophilic catalytic cleavage of an amide by a carboxylate ion when fragmentation is not part of the driving force. Indeed, no such reaction has ever been observed.

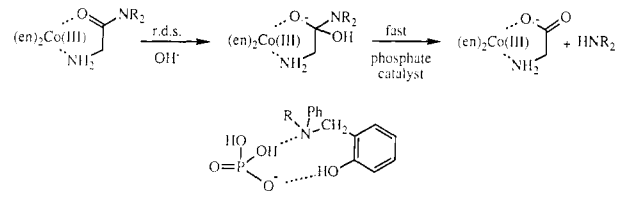
The carboxylate ion could still have served as a general-base catalyst, delivering a water molecule to the carbonyl group to form the tetrahedral intermediate. This is part of the mechanism we favor for the enzyme carboxypeptidase A itself. If the weak carboxylate ion of compound **3** were performing such a function in our model system, we would not see it. A catalyst that simply accelerated the reversible formation of a tetrahedral intermediate would not affect the rate if the second step is rate determining.

Another way to furnish a carboxylate ion as a mimic of Glu-270 was tried: the use of acetate buffer. Sodium acetate/acetic acid buffer was indeed a catalyst for the hydrolysis of compounds **2** and **5**, and by varying the buffer ratio at a constant buffer concentration it was easy to show (as the data in Table II and Figure 5 indicate) that the catalytic species was acetate ion, not acetic acid. That is, the kinetics showed a dependence on the concentration of acetate ion; the actual mechanism is another question, to be discussed below.

The bimolecular rate constant for acetate-catalyzed hydrolysis of compounds **2** and **5** is essentially identical for both compounds; the precise value of this rate constant depends on the value chosen for the buffer-independent term. It is difficult to measure this term accurately in this acidic pH region because the hydrolysis is quite slow, but an estimation of k_o was obtained both by extrapolation of observed hydrolysis rates in acetate buffer to an acetate concentration of zero and by extrapolation of the buffer-independent rates obtained at higher pH down into this more acidic pH range, assuming the hydrolysis remains first order in hydroxide. By either method, the bimolecular rate constants for **2** and **5** with acetate differ by no more than 3. Thus acetate catalysis is in direct competition with phenol + hydroxide ion catalysis. We have reached a similar conclusion for phosphate catalysis (vide infra) in which the data are even more extensive and precise.

In our earlier discussion we indicated that phenol was acting as a general acid to catalyze the departure of the nitrogen leaving group. We now must consider other possibilities, involving kinetically equivalent mechanisms. Thus in the cleavage of compound **2** we saw a kinetic term in OH^- and a kinetic term in the unionized phenol, although a kinetically equivalent possibility is that the catalyst group is the phenoxide ion (with one or more kinetically undetectable water molecules). If this were the case one could imagine that acetate ion and phenoxide ion would be competitors, both of them acting as general-base catalysts to deliver a water molecule to form the tetrahedral intermediate.[49] (It is hard to imagine that they could both be acting as nucleophiles, since the phenoxide as a nucleophile would not lead to the fragmentation necessary to permit this mechanism to operate, while acetate as a nucleophile would have only a neutral entropy driving force for converting a stable amide to an unstable anhydride. Forming such an anhydride would be particularly bad thermodynamically in the current case, because an anhydride is so much less basic than an amide that it would be a very poor ligand for Co(III).)

Scheme VIII



One argument against this possibility is that the second step, loss of the amine group from the tetrahedral intermediate, should be the slow step that could respond to catalysis.[35] Another argument is that there is no observed catalysis by several other buffers. In particular, imidazole is not a catalyst, although it is even more basic than acetate ion. Furthermore, aniline buffer shows no catalysis of the hydrolysis of **6**, although its basicity is comparable to that of acetate. If the buffer is simply acting as a general-base catalyst, it is not obvious why these other buffers are ineffective. As will be discussed below, there is a reasonable explanation if a different function is adduced for the acetate buffer.

Of course the principle of kinetic equivalence applies not only to catalysis by the phenol group. The apparent catalysis by acetate anion could instead be catalysis by OH^- and acetic acid; now the buffer would be acting as an acid, not a base. If the hydroxide ion reversibly adds to the carbonyl group of **2**, and the forward decomposition of the tetrahedral intermediate is catalyzed by acetic acid, it is clear why this would compete with catalysis by the phenol group. The phenol protonates the nitrogen atom leaving group, and acetic acid can competitively perform the same function.

This simple idea does not really explain all the observations, since it is not clear why other buffers would not be able to perform such acid catalysis. For instance, if the buffer is imidazole, then one would expect imidazolium ion to be able to protonate the nitrogen leaving group. A kinetic term that combines OH^- with a general acid is kinetically equivalent to a kinetic term in general base and responds in the same way to catalyst basicity. Thus the fact that imidazole is a significantly stronger base than acetate ion means that catalysis by hydroxide and imidazolium ion should also be more effective than catalysis by hydroxide and acetic acid (the higher concentration of OH^- more than makes up for the decreased HA acidity).

As the data in Table II shows, we do, however, see catalysis by HPO_4^{2-}. It is more basic than acetate, and the catalysis was very effective. Again, we believe that this catalysis involves the kinetically equivalent OH^- and $H_2PO_4^-$ pair. Over a phosphate buffer concentration ranging from 0.04 to 0.40 M, the rate of hydrolysis of compound **2** was unchanged but was *much faster* than the rate in the absence of this buffer and much faster than any other rate we have observed at pH 7.2, the operating pH in the phosphate buffer study. It is such an effective catalyst that it exhibits *kinetic saturation*. In the concentration range 0.0020–0.020 M the rate is linear in HPO_4^{2-} (Figure 7), but after 0.020 M phosphate the second step is now so effectively catalyzed by $H_2PO_4^-/OH^-$ that it is no longer rate determining (Figure 6).

(47) For another example, see: Broxton, T. J. *Aust. J. Chem.* **1983**, *36*, 1885.

(48) (a) Aldersley, M. F.; Kirby, A. J.; Lancaster, P. W. *J. Chem. Soc., Chem. Commun.* **1972**, 570. (b) Kirby, A. J.; McDonald, R. S.; Smith, C. R. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1495. (c) Aldersley, M. F.; Kirby, A. J.; Lancaster, P. W.; McDonald, R. S. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1487.

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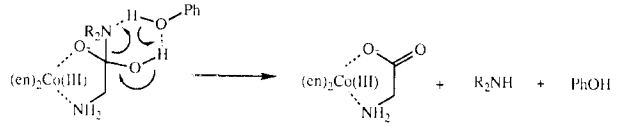
Scheme IX



We are observing a constant rate corresponding to the first OH^- addition step that forms the tetrahedral intermediate (Scheme VIII).

Phosphate catalysis is also observed with compound **5**, the methyl ether, but in this case the hydrolysis rate shows a normal kinetic increase as the buffer concentration is increased over the entire concentration range studied, and k_{cat} is approximately 8-fold smaller than for **2**. $H_2PO_4^-$ must, of course, hydrogen bond to the nitrogen atom of the leaving group during that second step, and this brings the negatively charged oxygens in an excellent position to hydrogen bond with the phenol group. The very high effectiveness of phosphate as a catalyst in the cleavage of **2** may well reflect such an additional binding interaction (Scheme VIII).

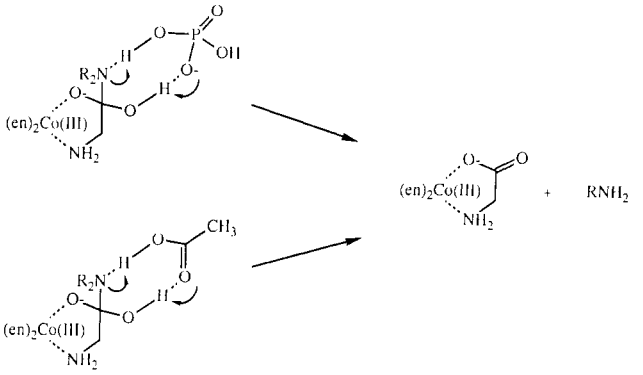
If the phenol could serve such a phosphate-binding interaction in the trifunctional molecule **1** and make addition of water to the amide carbonyl rate limiting, rate enhancements by all three of these functional groups might be observable. However, the rate of hydrolysis of **1** in phosphate buffer (Table II) is similar to that of **5** and is found to increase with buffer concentration, even to as high as 0.5 M. Thus, it appears that the carboxylate–phenol interaction has disrupted this phosphate binding, making the rate lower than that of **2** and eliminating the kinetic saturation.

A summary of the observations with various buffers reveals that the presence of catalysis is related not so much to the basicity of the buffer but rather to its actual structure. Catalytic groups containing an OH are effective, while those general acids derived from protonated amines are not. One might argue that basicities are perturbed by the presence of the charged cobalt ion, but it is hard to believe that the perturbation is so great as to drastically reverse the relative basicities of imidazole and acetate anion. (The pK_a of the phenol group in **2** is less than 1 unit lower than normal, while the pK_a of the carboxylate in **3** is only 2 units below normal although it is directly connected to the Co(III) through a conjugated system.) Instead, we believe that the presence or absence of catalysis reflects the need for *more* than simple protonation of the nitrogen leaving group during the second step of the reaction.

If a catalytic general acid protonated the nitrogen atom and the tetrahedral intermediate decomposed, the product would be a glycine coordinated to cobalt but still carrying its proton on the carboxy group. Any mechanism that can lead directly to the coordinated carboxylate *ion* would be preferred. We have pointed this situation out previously with respect to the enzyme carboxypeptidase A itself and have suggested that a general acid group in the enzyme must not only add a proton to the nitrogen leaving group but also remove a proton from the OH group of the tetrahedral intermediate.[12b] Indeed, we suggested that the phenolic hydroxyl group of Tyr-248 performed this function in the enzyme, although now this detailed proposal must be modified, as is discussed below. We also suggest this mechanism for the model system.

Examination of molecular models suggests that the phenolic hydroxyl group of **2** can act as a bridge between the nitrogen atom of the leaving group and the OH group of the tetrahedral intermediate. Thus it could be involved in a double proton transfer, one to the nitrogen and one from the tetrahedral OH group, so that the product is directly the stable complexed carboxylate ion (Scheme IX). Such a mechanism is also possible with acetic acid and with $H_2PO_4^-$, since again the OH group of the catalyst can act as a bridge to make a six-membered ring with two hydrogen bonds, within which two-proton transfers can occur. Larger rings are more likely; both acetic acid and $H_2PO_4^-$ could act as bifunctional proton-transfer catalysts with a different oxygen serving as the proton acceptor (Scheme X). Such bifunctional catalysis of proton transfers in the decomposition of tetrahedral intermediates is well established, especially with phosphate buffers.[50] In

Scheme X



fact, Buckingham et al.[51] observed such special effects of bifunctional buffers in a Co(III)–hydroxide cleavage of a singly coordinated peptide in work closely related to ours and interpreted it in a similar way. Note that this double proton transfer involves two *simultaneous* hydrogen bonds, in contrast to the sequential process suggested in Scheme IVb.

Such a double proton transfer mechanism is not available to any of the amine buffers that were in fact not catalysts. If an ammonium ion sets up a hydrogen bond to the leaving nitrogen atom there are no further orbitals available with unshared pairs that could be used to set up a second hydrogen bond and permit a double proton shift. Thus this mechanism seems to explain the otherwise unusual observations with respect to catalysis by buffers. As we will discuss, it is also strongly related to what we believe is the most probable mechanism for the enzyme itself.

Mechanism of Reactions Catalyzed by the Enzyme Carboxypeptidase A. With the finding that Tyr-248 is not a catalytic group in the cleavage of peptides and related esters by carboxypeptidase A, a serious problem has arisen with respect to the possible nucleophilic mechanism for peptide hydrolysis involving an anhydride intermediate. According to this mechanism (Scheme Va), the carboxylate ion of Glu-270 adds to the carbonyl group, and then the nitrogen departs to result in the formation of an acyl enzyme, which is an anhydride. However, there is now no identifiable acid group to protonate the nitrogen; unless such a catalytic group exists, this mechanism is out of the question for peptide substrates. (Some evidence supports anhydride intermediates in the hydrolysis of esters,[52] but we[44] and others[53] have evidence that esters and peptides use different hydrolysis mechanisms. No acid catalyst would be needed for conversion of an ester to an anhydride.) However, the general-base mechanism can function perfectly well for peptides without Tyr-248 as a catalytic group.

Matthews was the first to point out that a variant of the general-base mechanism could utilize Glu-270 for both steps.[54] In the first step the carboxylate ion acts as a general base, delivering a water molecule to the carbonyl group and forming a tetrahedral intermediate. This converts Glu-270 into a carboxylic acid; in the second step this carboxylic acid group could now put the proton on the nitrogen, permitting a catalyzed decomposition of the tetrahedral intermediate in the forward direction. The mechanism is directly analogous to that of chymotrypsin, in which an imidazole group of the enzyme first acts as a general base to deliver the hydroxyl of serine to the substrate carbonyl, and then the resulting

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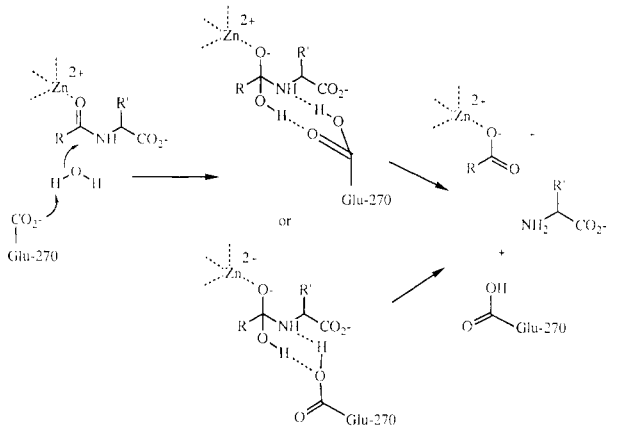
(54) Monzingo, A. F.; Matthews, B. W. *Biochemistry* **1984**, *23*, 5724.

Scheme XI



protonated imidazole acts as a general acid to assist the departure of the leaving nitrogen atom.[55]

We suggest a slight modification of this idea that seems more attractive for carboxypeptidase. Because after hydrolysis the carboxylic product is formed coordinated to the strongly electrophilic Zn(II), we believe it is very important that the product be formed as a carboxylate *anion*, not as a protonated carboxyl group. This means that the enzyme should do the same double proton transfer through a hydrogen-bonded circuit that we outlined above for our model system. In our proposed mechanism (Scheme XI) we show that the carboxyl group of Glu-270 can function to facilitate such a proton transfer, in direct analogy with the mechanism invoked (Scheme X) for acetic acid.

In our model systems we did not detect any behavior that could be clearly ascribed to catalysis of the first step, because in most of the cases the second step was rate limiting. Our fastest rate of reaction is approximately 10^4–10^6 slower than the enzymatic k_{cat}.[1b] However, the finding that phosphate buffer was able to accelerate the reaction so well that the first step in the model system was now rate limiting is intriguing. With the appropriate placement of catalytic groups of this sort, model systems might yet be devised that could approach the rate of carboxypeptidase A and related enzymes.

Conclusions

1. The hydrolysis of a Co(III)-coordinated glycine amide is catalyzed by an intramolecular phenol group but not a carboxyl group.

2. Bifunctional buffers are also catalysts of this hydrolysis by a two-proton transfer in the tetrahedral intermediate.

3. A similar two-proton transfer seems likely as part of the mechanism of peptide hydrolysis by carboxypeptidase A and related enzymes.

Experimental Section

Materials. All chemicals were of reagent grade and were used without further purification unless otherwise noted. Methylene chloride and benzene were distilled from calcium hydride, and THF was distilled from K°/benzophenone immediately before use. Solvents used in the kinetic studies were spectrophotometric grade or better. All reactions were carried out under an argon atmosphere unless otherwise indicated. Sodium hydride (50% oil dispersion) was washed with pentane and dried under vacuum before use. $MeOH_{NH_3}$ refers to methanol that has been saturated with NH_3 gas. ^1H NMR spectra were recorded on either a Varian XL-200 or a Bruker WM300 spectrometer. UV and visible spectra were recorded on Beckman DU8 or DU8B spectrophotometers equipped with themostated cell holders. Elemental analyses were obtained from Galbraith Laboratories Inc. or Schwarzkopf Microanalytical Laboratory. Mass spectra were obtained on either Finnegan 3300 or Ribomag 1010 instruments.

Kinetic Measurements. All hydrolysis reactions were run in buffered solutions. Ionic strength was maintained with NaCl. For reactions in partially nonaqueous solution, the pH values reported are those of the aqueous buffer solution prior to dilution with organic solvent. The pHs of the solutions in which the hydrolysis reactions were run were found not to differ by more than ±0.1 pH unit, at the end point, from the pH of the solutions that did not contain any cobalt complex. Isosbestic behavior in the ultraviolet region was observed during the hydrolysis of all cobalt complexes. The hydrolysis reactions were monitored at the wavelength where the largest absorbance change was observed upon hydrolysis. These wavelengths are **1**, 330 nm; **2**, 295 nm; **3**, 320 nm; **4** 325 nm; **5** 300 nm; and **6**, 300 nm. Typically, 1 mL of buffer solution in a semimicro (1.5-mL capacity) quartz cuvette was equilibrated to the required temperature in the cell holder of the Beckman spectrophotometer for at least 0.5 h before 3.33 μL of a standard 0.15 M solution of substrate (in Me_2SO, stored in a freezer until immediately before use) was added via micropipet. Absorbance data were acquired for at least three half-lives. Pseudo-first-order rate constants were calculated from a computer-assisted linear least-squares regression analysis of ln ($|A_t - A_0|$) vs. time.[56] Correlation coefficients were generally >0.999 and never <0.98. At least two runs were averaged for each data point. Agreement between runs was to within 20%. Some reactions of **2** and **5** were also measured by using high-pressure liquid chromatography.[57] Typically, 300 μL of the hydrolysis solution was equilibrated at 25 °C in a constant-temperature bath for at least 0.5 h before 1 μL of 0.15 M stock solution of **2** or **5** was added via micropipet to form a 0.50 mM solution. At appropriate times, 30 μL was removed via syringe, injected onto an analytical IBM C_{18} reverse-phase HPLC column, and eluted with 80% aqueous MeOH. The concentration of amine reaction products **8** or **9** was measured with an external calibration method. Plots of [**8**] vs. peak area were linear from 0.05 to 0.50 mM, while plots of [**9**] vs. peak area were linear from 0.05 to 0.45 mM. Reactions were followed to at least four half-lives, and pseudo-first-order rate constants were calculated as described above. Correlation coefficients were all >0.99. Good agreement between rate constants measured by both methods was found. Separate experiments confirmed that the cobalt complexes do not significantly hydrolyze in this eluent over the time course of the analysis.

Pseudo-first-order rate constants for hydrolysis of **6** in aniline buffer were also measured chromatographically. The concentration of $[Co(en)_2glyO]^{2+}$ could be measured as a function of time with an external calibration method.[58] Plots of $[Co(en)_2glyO]^{2+}$ concentration vs. peak area were linear from 0.05 to 0.50 mM. This compound eluted at 19–20 min with 30 mM *n*-hexanesulfonic acid, pH 3.5 (adjusted with polyphosphoric acid), in 40% aqueous MeOH on a C_{18} analytical reverse-phase HPLC column. Aniline eluted at 5–15 min under these conditions. Although the concentrations of aniline used were quite high, the two peaks were base line separated. Rates measured chromatographically for at least two runs agreed to within 15%.

Titrations. Measurements of pH were made with a Radiometer/Copenhagen Model PHM63 digital pH meter and a Cole Parmer combination Ag/AgCl electrode in aqueous solution at 25 °C. Ionic strength was not controlled.

***o*-[(2-Methoxyethoxy)methoxy]benzaldehyde (12).** To a THF suspension (100 mL) of sodium hydride (4.0 g, 82 mmol) was added salicylaldehyde (4.4 mL, 40.9 mmol) dropwise via syringe.[59] After dilution with an additional 100 mL of THF, MEMCl (21 mL, 200 mmol) was added while stirring. The cooling bath was removed and the reaction mixture allowed to warm to room temperature. After 20 min the reaction mixture was poured carefully into 200 mL of chilled pH 7.00 buffer, the excess THF was removed from the neutral solution under reduced pressure, and the product was extracted into ethyl acetate (200 mL + (3 × 50 mL)). The combined organic extracts were washed with 200 mL of saturated NaCl solution and dried ($MgSO_4$). Removal of the solvent under reduced pressure provided approximately 8 mL of a thick yellow oil which was flash chromatographed[60] on silica gel (40% ether/petroleum ether as eluent) to provide 6.8 g (79%) of **12** as a clear colorless oil: ^1H NMR (200 MHZ, $CDCl_3$) δ 3.38 (s, 2 H), 3.57 (m, 2 H), 3.88 (m, 3 H), 5.41 (s, 2 H), 7.13 (t, J = 7.2 Hz, 1 H), 7.28 (d, J = 8.4 Hz, 1 H), 7.55 (dt, J = 8.4, 1.9 Hz, 1 H), 7.85 (dd, J = 7.6, 1.8 Hz, 1 H), 10.50 (s, 1 H); IR (thin film, NaCl) 1690, 1215, 1100, 757 cm^{-1}; MS (CI/NH_3) calcd for $C_{11}H_{14}O_4$ m/e 210, found 211 (M + 1), 228 (M + 1 + 17); TLC (30% ether/petroleum ether) R_f 0.13. **7-[(2-Methoxyethoxy)methoxy]-1-indanone (29)**: 85% yield;[61] ^1H NMR ($CDCl_3$, 200

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MHz) δ 2.63–2.69 (m, 2 H), 3.05–3.11 (m, 2 H), 3.35 (s, 3 H), 3.51–3.56 (m, 2 H), 3.86–3.90 (m, 2 H), 5.43 (s, 2 H), 7.06 (d, J = 7.6 Hz, 1 H), 7.09 (d, J = 8.3 Hz, 1 H), 7.49 (t, J = 7.9 Hz, 1 H); TLC (1:1 ethyl acetate/petroleum ether) R_f 0.29.

***N*-[*o*-[(2-Methoxyethoxy)methoxy]benzyl]phenylamine (13).** To a solution fo aniline (0.87 mL, 9.6 mmol) and acetic acid (0.28 mL, 4.8 mmol) in 10 mL of methanol was added **12** (1.0 g, 4.8 mmol), followed by sodium cyanoborohydride (308 mg, 4.8 mmol) in one portion through the flask neck.[62] The clear colorless solution was stirred at room temperature for 36 h by which time TLC showed complete reaction. Concentrated HCl (~2 mL, to pH 2) was added, and the solution was basified to pH 12 (solid NaOH) after gas evolution stopped. Methanol was removed under reduced pressure, the basic aqueous layer was extracted with ether (2 × 50 mL), and the organic layers were washed with saturated NaCl solution (1 × 50 mL) and dried (Na_2SO_4). After removal of the solvent under reduced pressure, flash chromatography on silica gel (8:1 CH_2Cl_2/petroleum ether as eluent) provided 1.31 g (96%) of **13** as a colorless oil which turned brown upon exposure to air: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 3.38 (s, 3 H), 3.56 (m, 2 H), 3.84 (m, 2 H), ~4 (vbr s, 1 H), 4.36 (s, 2 H), 5.34 (s, 2 H), 6.60–6.8 (m, 2 H), 6.98 (dt, J = 7.0, 1.0 Hz, 1 H), 7.1–7.4 (m, 5 H); IR (thin film, NaCl) 3420, 1609, 1260, 757 cm^{-1}; MS (CI/NH_3) calcd for $C_{17}H_{21}NO_3$, m/e 287, found 288 (M + 1); TLC (9:1 CH_2Cl_2/petroleum ether) R_f 0.5. Prepared similarly was ***N*-[*o*-hydroxybenzyl]phenylamine (8)**: 74% yield; mp 100–102 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.43 (s, 2 H), 6.82–6.90 (m, 5 H), 7.15–7.34 (m, 4 H); IR (KBr pellet) 3400, 3271, 1255, 765 cm^{-1}. Anal. Calcd for $C_{13}H_{13}NO$ (found): C, 78.36 (77.93); H, 6.58 (6.87); N, 7.03 (6.75). ***N*-[*o*-Methoxybenzyl]phenylamine (9)**: 90% yield; mp 90–92 °C [lit.[63] mp 90–93 °C]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.1 (br s, 1 H + H_2O), 3.94 (s, 3 H), 4.41 (s, 2 H), 6.77 (m, 2 H), 7.00 (t, J = 7 Hz, 2 H), 7.2–7.45 (m, 4 H); IR (KBr pellet 3409, 1241, 1092 cm^{-1}; MS (CI/NH_3) calcd for $C_{14}H_{15}NO$, m/e 213, found 214 (M + 1); TLC (50% ether/petroleum ether) R_f 0.8. Anal. Calcd for $C_{14}H_{15}NO$ (found): C, 78.84 (78.92); H, 7.09 (7.25); N, 6.57 (6.28). ***tert*-Butyl *N*-[*o*-[(2-methoxyethoxy)methoxy]benzyl]anthranilate (24)**: 82% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.57 (s, 9 H), 3.38 (s, 3 H), 3.56 (m, 2 H), 3.68 (m, 2 H), 4.45 (d, J = 5.7 Hz, 2 H), 5.36 (s, 2 H), 6.56 (t, J = 7 Hz, 1 H), 6.64 (d, J = 8 Hz, 1 H), 6.95 (t, J = 7 Hz, 1 H), 7.15–7.4 (m, 4 H), 7.88 (d, J = 8 Hz, 1 H), 8.22 (br t, 1 H); IR (thin film, NaCl) 3375 1672, 1601, 1577, 1226, 744 cm^{-1}; MS (CI/NH_3) calcd for $C_{22}H_{29}NO_5$, m/e 387, found 388 (M + 1); TLC (30% ether/petroleum ether) R_f 0.58. **(±)-7-[(2-Methoxyethoxy)methoxy]-1-aminoindan (30)**: 79% yield; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.10–2.22 (m, 1 H), 2.30–2.42 (m, 1 H), 2.80–2.92 (m, 1 H), 3.05–3.18 (m, 1 H), 3.31 (s, 3 H), 3.45–3.54 (m, 2 H), 3.70–3.78 (m, 2 H), 4.00 (vbr s, 1 H), 5.00 (dd, J = 6.8, 2.5 Hz, 1 H), 5.24 (center of AB quartet, 2 H), 6.66 (d, J = 7.8 Hz, 2 H), 6.70 (t, J = 7.2 Hz, 1 H), 6.9–7.0 (m, 2 H), 7.15–7.25 (m, 3 H), IR (thin film, NaCl) 3400, 1603, 1241 cm^{-1}; MS (CI/NH_3) calcd for $C_{19}H_{23}NO_3$, m/e 313, found 314 (M + 1); TLC (30% ether/petroleum ether) R_f 0.46.

Benzyl [[[*o*-[(2-Methoxyethoxy)methoxy]benzyl]phenylcarbamoyl]methyl]carbamate (14). To a toluene suspension (100 mL) of carbobenzyloxyglycine (5.6 g, 27 mmol) was added DMF (1 mL) and then oxalyl chloride (2.7 mL, 30 mmol) dropwise at room temperature. Within 10 min the solution appeared clear. Toluene, excess oxalyl chloride, and HCl were removed under reduced pressure, leaving 5 mL of a yellow oil which was dissolved in 50 mL of toluene, and the distillation was repeated. To NaH (1.6 g, 33 mmol) suspended in 90 mL of toluene was added **13** (1.08 g, 3.8 mmol) via syringe as a concentrated toluene solution. After 10 min at room temperature, the acid chloride prepared above (in 30 mL of toluene) was added in one portion via syringe. TLC 2 min after addition showed complete reaction. The reaction mixture was poured carefully into 200 mL of chilled pH 7.00 buffer while stirring and the organic layer diluted to a total volume of 300 mL with ethyl acetate. After separation of the layers, the neutral aqueous layer was washed with an additional 3 × 30 mL of ethyl acetate, and the combined organic extracts were washed with a saturated NaCl solution (200 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure and flash chromatography of the resulting oil on silica gel (80% ether/petroleum ether eluent) provided 1.71 g (94%) of **14** as a clear colorless glass: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.38 (s, 3 H), 3.48 (m, 2 H), 3.58 (m, 2 H), 3.70 (d, J = 6 Hz, 2 H), 5.01 (s, 4 H), 5.10 (s, 2 H), 5.78 (br t, J = 6 Hz, 1 H), 6.9–7.4 (m, 14 H); IR (thin film, NaCl) 3350, 1730, 1675, 1240, 760 cm^{-1}; MS (CI/NH_3) calcd for $C_{27}H_{30}N_2O_6$, m/e 478, found 479 (M + 1), 496 (M + 1 + 17); TLC (ether) R_f 0.5. Prepared similarly was **benzyl [[[(*o*-methoxy)benzyl]phenylcarbamoyl]methyl]carbamate (16)**: 83% yield; mp 85–86.5 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.57 (s, 3 H), 3.71 (d, J = 4 Hz, 2 H), 4.95 (s, 2 H), 5.07 (s, 2 H), 5.78 (br, 1 H), 6.75 (t, J = 7 Hz, 1 H), 6.88 (t, J = 7 Hz, 1 H), 6.95–7.10 (m, 2 H), 7.15–7.45 (m, 10 H); IR (KBr pellet) 3225, 1720, 1654, 1249 cm^{-1}; MS (CI/NH_3) calcd for $C_{24}H_{24}N_2O_4$ m/e 404, found 405 (M + 1); TLC (70% ether/petroleum ether) R_f 0.43. **Benzyl [[[*o*-[(2-methoxyethoxy)methoxy]benzyl]((*tert*-butoxycarbonyl)phenyl)carbamoyl]methyl]carbamate (25)**: 97% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.58 (s, 9 H), 3.34 (s, 3 H), 3.4–3.8 (m, 6 H), 4.25 (d, J = 14 Hz, 1 H), 4.91 (center of AB quartet, 2 H), 5.06 (s, 2 H), 5.51 (d, J = 14 Hz, 1 H), 5.79 (br s, 1 H), 6.75 (d, J = 7 Hz, 1 H), 6.90 (t, J = 7 Hz, 1 H), 7.00 (d, J = 8 Hz, 1 H), 7.1–7.4 (m, 9 H + $CHCl_3$), 7.88 (dd, J = 7, 1 Hz, 1 H); IR (thin film, NaCl) 3370, 1725, 1715, 1670, 1451, 755 cm^{-1}; MS (CI/NH_3) calcd for $C_{32}H_{38}N_2O_8$, m/e, found 579 (M + 1), 596 (M + 1 + 17); TLC (ether) R_f 0.54. **(±)-Benzyl [[[7-[(2-methoxyethoxy)methoxy]-1-indanyl]phenylcarbamoyl]methyl]carbamate (31)**: 95% yield; mp 78–80 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.1 (m, 1 H), 2.4 (m, 2 H), 2.6 (m, 1 H), 3.34 (s, 3 H), 3.53 (t, J = 5 Hz, 2 H), 3.55 (center of doublet of AB quartet, 2 H), 3.82 (m, 2 H), 5.05 (s, 2 H), 5.31 (s, 2 H), 5.78 (br t, 4.5 Hz, 1 H), 6.42 (dd, J = 10, 2 Hz, 1 H), 6.7–7.4 (m, 13 H); IR (KBr) 3288, 1726, 1654 cm^{-1}; MS (CI/NH_3) calcd for $C_{29}H_{32}N_2O_6$, m/e 504, found 505 (M + 1), 522 (M + 1 + 17), 314 (M − 190).

***N*-Glycyl-*N*-[*o*-[(2-methoxyethoxy)methoxy]benzyl]phenylamine (15).** To 150 mL of deoxygenated methanol (150 mL) was added 200 mg (10%) of palladium on carbon and then carbamate **14** (1.7 g, 3.6 mmol, in 20 mL of methanol). The black suspension was stirred under 1 atm of H_2 for 1.5 h, at which time TLC showed complete reaction.[64] The hydrogen atmosphere was replaced by argon, and the reaction mixture was filtered through silica gel (1-in. plug, 10% $MeOH_{NH_3}/CH_2Cl_2$) to remove the catalyst. After excess solvent was removed under reduced pressure, the yellow oil that remained was flash chromatographed on silica gel (3% $MeOH_{NH_3}/CH_2Cl_2$) to provide 868 mg of **15** (71%) as a slightly yellow glass: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.65 (br s, 2 H + H_2O), 3.13 (br s, 2 H), 3.35 (s, 3 H), 3.47 (m, 2 H), 3.59 (m, 2 H), 4.98 (s, 2 H) 5.00 (s, 2 H), 6.9–7.4 (m, 9 H + $CHCl_3$); IR (thin film, NaCl) 1655, 3376, 1102, 749 cm^{-1}; MS (CI/NH_3) calcd for $C_{19}H_{24}N_2O_4$ m/e 344, found 345 (M + 1); TLC (5% $MeOH_{NH_3}/CH_2Cl_2$) R_f 0.22. Prepared similarly was ***N*-glycyl-*N*-[(*o*-methoxy)benzyl]phenylamine (17)** in a quantitative yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.72 (br s, 2 H), 3.22 (br s, 2 H), 3.59 (s, 3 H), 4.97 (s, 2 H), 6.77 (d, J = 9 Hz, 1 H), 6.89–7.15 (m, 3 H), 7.15–7.4 (m, 5 H); IR (thin film, NaCl) 3375, 3313, 1663, 1246 cm^{-1}; MS (CI/NH_3) calcd for $C_{16}H_{18}N_2O_2$ m/e 270, found 271 (M + 1); TLC (5% $MeOH_{NH_3}/CH_2Cl_2$) R_f 0.2. ***tert*-Butyl *N*-glycyl-*N*-[*o*-[(2-methoxyethoxy)methoxy]benzyl]anthranilate (26)**: 87% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.56 (s, 9 H), 1.57 (br s, 2 H + HDO), 3.05 (br s, 2 H), 3.34 (s, 3 H), 3.4–3.8 (m, 4 H), 4.27 (d, J = 14.3 Hz, 1 H), 4.91 (center of AB quartet, 2 H), 5.36 (d, J = 14.3 Hz, 1 H); 6.75 (dd, J = 6.9, 1.5 Hz, 1 H), 6.93 (t, J = 7 Hz, 1 H), 7.01 (d, J = 8.2 Hz, 1 H), 7.1–7.4 (m, 4 H), 7.88 (dd, J = 6, 2.4 Hz, 1 H); IR (thin film, NaCl) 3378, 3320, 1711, 1663, 1304, 1128, 1100, 757 cm^{-1}; MS (CI/NH_3) calcd for $C_{24}H_{32}N_2O_6$, m/e 444, found 445 (M + 1); TLC (5% $MeOH_{NH_3}/CH_2Cl_2$) R_f 0.16. **(±)-*N*-Glycyl-*N*-[7-[(2-methoxyethoxy)methoxy]-1-indanyl]phenylamine**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.67 (br s, 2 H + HDO), 2.06–2.14 (m, 1 H), 2.32–2.45 (m, 2 H), 2.56–2.62 (m, 1 H), 2.97 (center of AB quartet, 2 H), 3.36 (s, 3 H), 3.55 (t, J = 4.5 Hz, 2 H), 3.80–3.89 (m, 2 H), 5.32 (center of AB quartet, 2 H), 6.44 (dd, J =6.1, 1.9 Hz, 1 H), 6.63 (d, J = 7.4 Hz, 1 H), 6.86 (d, J = 8.1 Hz, 1 H), 7.06–7.22 (m, 6 H); MS (CI/NH_3) calcd for $C_{21}H_{26}N_2O_4$, m/e 370, found 371 (M + 1); TLC (5% $MeOH_{NH_3}/CH_2Cl_2$) R_f 0.48.

(±)-*ab*-(2-Amino-*N*-salicylacetanilide-*O*,N^2)-*cf*,*de*-bis(ethylenediamine-*N*,*N'*)cobalt(3+) Trichloride (2). To amine **15** (120 mg, 0.35 mmol) dissolved in 570 μL of 95% ethanol was added *trans*-[Co(en)$_2$Br$_2$]$^+$Br$^-$ (160 mg, 0.38 mmol) to form a pale-green suspension.[65] After being stirred 2 h at room temperature, the reaction mixture was diluted with 3 mL of methanol, 5 mL of acetone, and 207 mg of a purple solid precipitated with diethyl ether. Without further purification, 150 mg of this solid was added to 168 mg (1 mmol) of silver nitrate dissolved in 2 mL of 10^{-4} M HNO_3.[13a] The solution was vortexed for 2 min, stirred at room temperature in the dark for 2 h, and centrifuged. Without any further manipulation, the supernatant was chromatographed on SP-25 Sephadex (20 g, 40-mm column diameter) using a gradient elution of 0.1 (500 mL)–1.0 N (500 mL) HCl to provide 75.6 mg (70%) of **2** as an

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orange solid: ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 2.3–3.0 (m, 8 H + Me_2SO), 3.68 (m, 1 H), 3.92 (m, 1 H), 4.82 (br s, 1 H), 4.88 (d, J = 12 Hz, 1 H), 5.06 (d, J = 12 Hz, 1 H), 5.22 (br s, 1 H), 5.66 (br s, 1 H), 5.80 (br s, 1 H), 5.9–6.25 (br m, 5 H), 6.46 (br s, 1 H), 6.7–6.85 (m, 2 H), 7.07 (t, J = 8 Hz, 1 H), 7.30 (d, J = 8 Hz, 1 H), 7.35–7.60 (m, 5 H), 9.60 (br s, 1 H); IR (KBr pellet) 1606 cm^{-1}; UV–vis (0.1 N HCl, 0.5 mM) 494 nm (100). Anal. Calcd for $CoC_{19}H_{32}N_6O_2{\cdot}3Cl^-{\cdot}3H_2O$ (found): C, 38.30 (39.02); H, 6.43 (6.32); N, 14.10 (13.78). Prepared similarly was **(±)-*ab*-(2-Amino-*N*-anisylacetanilide-*O*,*N*^{2})-*cf*,*de*-bis(ethylenediamine-*N*,*N*′)cobalt(3+) Trichloride (5)**: 7% yield; ^{1}H NMR δ 2.4–3.0 (m, 8 H), 3.36 (HDO), 3.60 (s, 3 H), 3.70 (br m, 1 H), 3.90 (br m, 1 H), 4.82 (br s, 1 H), 4.97 (center of AB quartet, 2 H), 5.16 (br s, 1 H), 5.69 (br s, 1 H), 5.75 (br s, 1 H), 6.00 (br s, 5 H), 6.46 (br s, 1 H), 6.85–6.95 (m, 2 H), 7.25 (t, J = 6 Hz, 1 H), 7.3–7.55 (m, 6 H); IR (KBr pellet 3443, 1607, 1583, 1249, 758 cm^{-1}; UV–vis (0.1 M HCl, 0.5 mM) 490 nm (~100). Anal. Calcd for $CoC_{20}H_{34}N_6O_2{\cdot}3Cl^-{\cdot}2HCl{\cdot}H_2O$ (found): Co, 9.11 (9.10); C, 37.14 (36.83); H, 5.92 (5.81); N, 12.99 (13.82). **(±)-*ab*,*cf*-Bis(ethylenediamine-*N*,*N*′)-*de*-(*N*-glycyl-*N*-salicylanthranilic acid)cobalt(3+) Trichloride (1)**: 43% yield; ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 2.20–3.00 (br m, 8 H + Me_2SO), 3.60 (br m, not integratable), 4.28 (d, J = 12 Hz, 1 H), 4.95 (br s, 1 H), 5.36 (d, J = 12 Hz, 1 H), 5.36 (br s, 1 H), 5.68 (br s, 1 H), 5.70–5.95 (br m, 3 H), 6.00 (br m, 2 H), 6.20 (br m, 2 H), 6.60–6.80 (m, 2 H), 7.00–7.30 (m, 3 H), 7.45–7.55 (m, 2 H), 8.05 (d, J = 9 Hz, 1 H), 9.60 (br s, 1 H); IR (KBr pellet) 1708, 1607, 1588 cm^{-1}; UV–vis (0.5 mM solution in 0.1 M HCl) 490 nm (100). Anal. Calcd for $CoC_{20}H_{32}N_6O_4{\cdot}2H_2O{\cdot}3Cl^-{\cdot}HCl$ (found): C, 36.69 (36.82); H, 5.70 (5.53); N, 12.84 (12.51). ***ab*-[2-Amino-*N*-(7-hydroxy-1-indanyl)acetanilide-*O*^{1},*N*^{2}]-*cf*,*de*-bis(ethylenediamine-*N*,*N*′)cobalt(3+) Triformate (7)**: 31% yield as a pair of diastereomers; ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 1.95–2.25 (m, 2 H), 2.25–2.9 (m, >22 H including Me_2SO), 3.35 (vbr s, HDO), 3.75 (m, 4 H), 4.84 (br s, 1 H), 5.04 (br s, 1 H), 5.2–5.55 (br m, 3 H), 5.58–6.00 (br m, 3 H), 6.02 (d, J = 6 Hz, 1 H), 6.22 (d, J = 6 Hz, 1 H), 6.48 (d, J = 9 Hz, 1 H), 6.60 (d, J = 9 Hz, 1 H), 6.70 (d, J = 9 Hz, 1 H), 6.2–6.8 (m, 8 H), 6.85–7.7 (m, 12 H), 9.90 (s, 1 H), 10.00 (s, 1 H); IR (KBr pellet) 3400, 1602 cm^{-1}; UV (0.5 mM solution in 0.1 M HCl) 490 (110), 340 (150) nm.

***N*-(Trifluoroacetyl)anthranilic acid (18)** was prepared in an 81% yield by reaction with trifluoroacetic anhydride:[66] mp 179–182 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.31 (t, J = 7.7 Hz, 1 H), 7.69 (t, J = 8.5 Hz, 1 H), 8.15 (d, J = 7.7 Hz, 1 H), 8.56 (d, J = 8.5 Hz, 1 H), 12.56 (br s, 1 H); IR (KBr pellet) 3260, 3000 (b), 2680 (b), 1735, 1678, 1420, 1270 cm^{-1}; TLC (50% ether/petroleum ether) R_f 0.5. Anal. Calcd for $C_9H_6NO_3F_3$ (found): C, 46.37 (46.29); H, 2.59 (2.91); N, 6.01 (6.03); F, 24.45 (24.17).

***tert*-Butyl Anthranilate (19).** A THF (25-mL) solution of *N*-(trifluoroacetyl)anthranilic acid (8.0 g, 34.3 mmol) and *N*,*N*′-carbonylbisimidazole[67] (8.3 g, 51.2 mmol) was stirred for 2 h at room temperature before 30 mL of sodium *tert*-butoxide in *tert*-butyl alcohol (1 M) was added via syringe.[68] After 36 h at room temperature, TLC showed that all starting material had disappeared. THF and *tert*-butyl alcohol were removed by evaporation under reduced pressure, and the oily residue which remained was dissolved in 200 mL of diethyl ether and extracted with H_2O (2 × 100 mL). After the combined aqueous layers were washed with diethyl ether (2 × 50 mL), the combined ethereal extracts were washed with saturated K_2CO_3 (100 mL) and NaCl solutions (100 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure provided 10.7 g of a white semisolid which appeared by ^{1}H NMR to be a mixture of the desired trifluoroacetamide ester and *tert*-butyl anthranilate. Without further purification, the mixture was dissolved in 250 mL of absolute ethanol and treated with sodium borohydride (6 g, 0.15 mol) at 0 °C for ~25 min, at which time TLC showed complete conversion to *tert*-butyl anthranilate. Excess hydride was quenched at 0 °C by slow addition of acetone (50 mL, then water (50 mL)). The volume was reduced to ~200 mL under reduced pressure and the cloudy white residue which remained extracted with ethyl acetate (150 mL + (2 × 50 mL)). The combined ethyl acetate extracts were washed with saturated NaCl solution (200 mL) and dried (Na_2SO_4) to provide 5.2 g (77%) of pure *tert*-butyl anthranilate **19** as a yellow oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.57 (s, 9 H), 5.70 (vbr s, 2 H), 6.65 (m, 2 H), 7.26 (dt, J = 8, 1 Hz, 1 H), 7.85 (dd, J = 8, 1 Hz, 1 H); IR (thin film, NaCl) 3500, 3392, 1690, 1250, 1120, 755 cm^{-1}; MS (CI/NH_3) calcd for $C_{11}H_{15}NO_2$, m/e 193, found 194 (M + 1); TLC (20% ether/petroleum ether) R_f 0.31.

Benzyl [[[(*tert*-Butoxycarbonyl)phenyl]carbamoyl]methyl]carbamate (20). To 60 mL of a methylene chloride solution of carbobenzyloxyglycine (3.0 g, 14.3 mmol) were added *tert*-butyl anthranilate (2.77 g, 14.3 mmol) and *N*-ethyl-*N*′-((dimethylamino)propyl)carbodiimide hydrochloride[69] (4.12 g, 21.5 mmol) at 0 °C. After warming to room temperature, the reaction mixture was stirred for 7 days before being diluted with an additional 60 mL of methylene chloride, washed with H_2O, 1% $NaHCO_3$, 1% citric acid, and H_2O (all 100 mL), and then dried (Na_2SO_4). After removal of the solvent under reduced pressure, the oily solid that remained was recrystallized from ether/petroleum ether to provide 4.4 g (80%) of crystalline product: mp 88–90 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.58 (s, 9 H), 3.11 (d, J = 5 Hz, 2 H), 5.20 (s, 2 H), 5.58 (br s, 1 H), 7.09 (t, J = 7 Hz, 1 H), 7.23 (s, $CHCl_3$), 7.2–7.6 (m, 7 H), 7.99 (dd, J = 8, 1 Hz, 1 H), 8.69 (d, J = 8 Hz, 1 H); IR (KBr pellet) 3305, 3230, 1730, 1720, 1670, 1255, 1150, 760 cm^{-1}; MS (CI/NH_3) calcd for $C_{21}H_{24}N_2O_5$ m/e 384, found 385 (M + 1) 402 (M + 1 + 17); TLC (40% ether/petroleum ether R_f 0.21. **Methyl *N*-[*N*-[(*tert*-butyloxy)carbonyl]glycyl]anthranilate (22)** was prepared similarly: 83% yield; mp 117–119 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9 H), 3.85 (s, 3 H), 3.96 (d, J = 4.8 Hz, 2 H), 5.29 (br s, 1 H), 7.12 (t, J = 7.2 Hz, 1 H), 7.48 (t, J = 8.4 Hz, 1 H), 7.96 (d, J = 7.9 Hz, 1 H), 8.65 (d, J = 8.4 Hz, 1 H), 11.49 (br s, 1 H); IR (KBr) 3380, 3320, 1730, 1710, 1690, 1270, 765 cm^{-1}; MS (CI/NH_3) calcd for $C_{15}H_{20}N_2O_5$, m/e 308, found 309 (M + 1), 209 (M − (CO_2 + C_4H_8)); TLC (50% ether/petroleum ether) R_f 0.21. Anal. Calcd for $C_{15}H_{20}N_2O_5$ (found): C, 58.43 (58.19); H, 6.54 (6.58); N, 9.09 (9.19). ***N*-[*N*-[(*tert*-Butyloxy)carbonyl]glycyl]aniline (27)** (carbobenzyloxyglycine was replaced with ((*tert*-butyloxy)carbonyl)glycine): 95% yield; mp 142–144 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.45 (s, 9 H), 3.92 (d, J = 5.8 Hz, 2 H), 5.45 (br s, 1 H), 7.10 (t, J = 8 Hz, 1 H), 7.22–7.35 (m, 2 H), 7.50 (d, J = 8 Hz, 2 H), 8.38 (br s, 1 H); IR (KBr pellet) 3405, 3358, 1709, 1690, 1540, 1315 cm^{-1}; MS (CI/NH_3) calcd for $C_{13}H_{18}N_2O_3$, m/e 250, found 251 (M + 1), 268 (M + 1 + 17), 151 (M − (C_4H_8 + CO_2)); TLC (50% ether/petroleum ether) R_f 0.22. Anal. Calcd for $C_{13}H_{18}N_2O_3$ (found): C, 62.38 (62.40); H, 7.25 (7.33); N, 11.19 (11.19).

***tert*-Butyl *N*-Glycylanthranilate Hydrochloride (21).** To 20 mL of deoxygenated ethanol was added 800 mg of 10% Pd/C and then **20** (2.4 g, 6.1 mmol, in 10 mL of ethanol). The black suspension was stirred under 1 atm of H_2 at room temperature for 40 min, at which time approximately 1 mL of concentrated HCl was added and the catalyst removed by filtration through Celite (rinse with 5 × 15 mL of ethanol). The filtrate was evaporated under reduced pressure to yield a white solid which was recrystallized from methanol/diethyl ether to provide 1.6 g (92%) of the hydrochloride salt: mp 245–250 °C dec; ^{1}H NMR (CD_3OD, 200 MHz) δ 1.62 (s, 9 H), 4.00 (s, 2 H), 4.85 (br s, HDO), 7.18 (t, J = 8.5 Hz, 1 H), 7.50 (dt, J = 1.6, 8.0 Hz, 1 H), 8.00 (dd, J = 1.6, 8.0 Hz, 1 H), 8.49 (d, J = 8.5 Hz, 1 H); IR (KBr pellet) 3380, 3250, 1715, 1690, 1285, 1150, 770 cm^{-1}; MS (CI/NH_3) calcd for $C_{13}H_{18}N_2O_3$, m/e 250, found 251 (M + 1), 195 (M + 1 − C_4H_8); TLC (3% MeOH/$CHCl_3$) R_f 0.16. ***N*-Glycylaniline hydrobromide (28)**: 84% yield; mp 201–203 °C; ^{1}H NMR (CD_3OD, 200 MHz) δ 3.40 (s, CH_3OH), 3.93 (s, HDO), 4.89 (s, 2 H), 7.15 (t, J = 7 Hz, 1 H), 7.36 (t, J = 7 Hz, 2 H), 7.63 (d, J = 7 Hz, 2 H); IR (KBr pellet) 3440, 3300, 1670–, 1540 cm^{-1}. Anal. Calcd for $C_8H_{10}N_2O{\cdot}HBr$ (found): C, 41.58 (41.60); H, 4.80 (4.86); N, 12.12 (12.10); Br, 34.58 (34.05).

(±)-*ab*,*cf*-Bis(ethylenediamine-*N*,*N*′)-*de*-(*N*-glycylanthranilic acid)-cobalt(3+) Trichloride (3). To hydrochloride salt **21** (600 mg, 2.1 mmol) dissolved in 4.4 mL of 95% ethanol was added (800 mg, 1.9 mmol) *trans*-$[Co(en)_2Br_2]^+Br^-$ and then triethylamine (0.3 mL, 2.2 mmol) dropwise with stirring. After 14 h at room temperature, the reaction mixture had turned purple. After cooling (0 °C), 1.02 g of pink solid was isolated and suspended in 5.5 mL of 10^{-4} M HNO_3. To this was added silver nitrate (1.3 g, 7.8 mmol), and the reaction mixture was stirred overnight at room temperature in the dark and then centrifuged to remove silver salts. Evaporation of the solvent under reduced pressure provided a bright-orange solid (290 mg) which was dissolved in 30 mL of CH_2Cl_2. To this was added 6 mL of CF_3CO_2H, and the reaction mixture was stirred at room temperature for 24 h. Removal of the solvent under reduced pressure and chromatography on Sephadex SP-25 (25 g, 0.1–1.0 M HCl eluent) provided the desired product in 21% yield: ^{1}H NMR (Me_2SO-d_6, 250 MHz) δ 2.3–3.0 (m, 8 H + Me_2SO residue); 3.8 (vvbr s, HDO), 4.20 (m, 2 H), 4.94 (br s, 1 H), 5.36 (br s, 1 H), 5.66 (br s, 1 H), 5.8–6.35 (m, 7 H), 7.41 (t, J = 7.5 Hz, 1 H), 7.85 (t, J = 7.5 Hz, 1 H), 7.90–8.02 (m, 2 H), ~8.6 (br s, 1 H); IR (Me_2SO-d_6 solution) 3043, 1615, 765 cm^{-1}; UV (0.5 mM in 0.1 M HCl) 491 (100) nm. Anal. Calcd for $CoC_{13}H_{26}N_6O_3Cl_3{\cdot}3HCl$ (found): C, 26.51 (26.60); H, 4.96 (5.29); N, 14.27 (14.56).

Methyl *N*-Glycylanthranilate (23). Carbamate **22** (2.5 g, 8.11 mmol) was dissolved in 100 mL of methylene chloride and treated with gaseous HBr for 5 min, during which time a white solid precipitated. The solvent

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was removed under reduced pressure and the crude product recrystallized from methanol/diethyl ether to provide 2.16 g (92%) of the desired product as a white powder: mp 210-212 °C; ^{1}H NMR (CD_3OD, 200 MHz) δ 3.95 (s, 3 H), 3.97 (s, 2 H), 7.24 (dt, J = 7.8, 1.1 Hz, 1 H), 7.62 (dt, J = 8.0, 1.7 Hz, 1 H), 8.08 (dd, J = 8.0, 1.6 Hz, 1 H), 8.47 (dd, J = 7.8, 1.1 Hz, 1 H); IR (KBr pellet) 3200, 3140, 1720, 1700, 1250, 778 cm^{-1}; MS (CI/NH_3) calcd for $C_{10}H_{12}N_2O_3$, m/e 208, found 209 (M + 1).

(±)-*ab*,*cf*-Bis(ethylenediamine-*N*,*N'*)-*de*-(*N*-glycyl-2-(methoxycarbonyl)aniline)cobalt(3+) Trinitrate (4). To hydrobromide salt **23** (1.9 g, 6.57 mmol) dissolved in 10 mL of 95% ethanol was added *trans*-$[Co(en)_2Br_2]^+Br^-$ (2.15 g, 5.12 mmol) and then diethylamine (0.68 mL, 6.57 mmol) dropwise. The reaction mixture was stirred overnight at room temperature, chilled to precipitate a pink solid which was isolated on a medium frit, washed with ice-cold 96% ethanol, and dried in vacuo (yield 2.2 g). Silver nitrate (0.45 g, 2.6 mmol) was added to 500 mg of this solid dissolved in 3 mL of 10^{-4} M HNO_3, and the solution was vortexed and allowed to stand in the dark overnight before the silver salts were removed by filtration. Lithium nitrate (concentrated aqueous solution) was added dropwise at room temperature to the filtrate, which precipitated 78 mg (17%) of X-ray quality crystals: ^{1}H NMR (300 MHz, Me_2SO-d_6) δ 2.2-2.8 (m, 8 H + Me_2SO residue), 3.32 (HDO), 3.38 (s, 3 H), 4.07 (br s, 3 H), 4.48 (br s, 1 H), 5.13 (br s, 1 H), 5.34 (br s, 3 H), 5.65 (br s, 3 H), 5.80 (br s, 1 H), 7.42 (t, J = 7.3 Hz, 1 H), 7.6-7.8 (m, 2 H), 7.94 d, J = 7.7 Hz, 1 H), 9.06 (br s, 1 H); IR (Me_2SO-d_6 solution) 3500, 1720, 1616, 1261 cm^{-1}; UV-vis (0.5 mM solution in 0.1 M HCl) 487 (~100) nm. Anal. Calcd for $CoC_{14}H_{28}N_9O_{12}\cdot 2H_2O$ (found): Co, 9.67 (9.76); C, 27.59 (27.11); H, 5.29 (5.33); N, 20.69 (20.60). **(±)-*ab*-(2-Aminoacetanilide-*O*,*N*^{2})-*cf*,*de*-bis(ethylenediamine-*N*,*N'*)cobalt(3+) Trinitrate (6).** After centrifugation (discard pellet), the orange supernatant was chromatographed on G-10 Sephadex (0.5 M HCO_2H eluent) to remove excess soluble silver salts and then on Sephadex SP-25 (0.1-1.0 M gradient elution). The major 3$^+$ orange band was collected and evaporated to an orange glass which was recrystallized from dilute nitric acid to give a 42% yield of the desired product: ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 2.35-2.90 (br m, 8 H), 3.35 (s, HDO), 4.05 (br s, 3 H), 4.53 (br s, 1 H), 5.19 (br s, 1 H), 5.32 (br s, 2 H), 5.58 (br s, 3 H), 5.75 (br s, 2 H), 7.26 (t, J = 7 Hz, 1 H), 7.42 (t, J = 7 Hz, 2 H), 7.53 (d, J = 7 Hz, 2 H), 8.94 (br s, 1 H); IR (Me_2SO-d_6 solution) 3580, 1613, 1581, 1349, 829 cm^{-1}; UV-vis (0.5 mM solution in 0.1 M HCl) 490 (~100) nm. Anal. Calcd for $CoC_{12}H_{26}N_9O_{10}\cdot 1H_2O$ (found): Co, 11.05 (10.96); C, 27.02 (26.72); H, 5.29 (5.32); N, 23.64 (23.42).

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Supplementary Material Available: Table IV showing crystal data and structure analysis results (1 page). Ordering information is given on any current masthead page.

Metal Ion Catalyzed Reactions of Acrylonitrile, Acrylamide, and Ethyl Acrylate by way of Their Diels-Alder Cycloadducts

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Abstract: In this paper, we report our successful efforts in coupling a cycloaddition/cycloreversion sequence to the well-documented metal ion promotion of functional group conversion in the cases of acrylonitrile to acrylamide, acrylamide to ethyl acrylate, and ethyl acrylate to acrylic acid. With 9-(2-pyridyl)anthracene as the diene, cycloadducts were prepared that underwent metal-promoted nitrile hydration, ethyl ester hydrolysis, and amide alcoholysis reactions. In addition, we report the synthesis of 9,10-bis(((2-(dimethylamino)ethyl)methylamino)methyl)anthracene, a diene capable of chelating to metal ions, as demonstrated by the successful synthesis of (9,10-bis(((2-(dimethylamino)ethyl)methylamino)methyl)anthracene-*N*,*N'*,*N''*,*N'''*)dizinc tetrachloride.

"Molecular recognition" is an active area of chemical research that seeks to understand how to bind a "guest" molecule to a "host" molecule or material selectively, and its relevance to the rational design of catalysts is clear. The biomimetic origins of this field have, quite logically, focused attention on binding guests out of aqueous solution, and therefore enzyme-like binding mechanisms (e.g., hydrophobic binding, hydrogen bonding, ion pairing, and ion-dipole interactions) have been successfully exploited by the pioneering efforts of many groups.[1] Of course, not all guest molecules of importance are water soluble, and in these cases the general approach has been to couple a polar or intercalative binding mechanism onto a nonpolar, lyophilic backbone.[2] Occasionally, such complexations are also freely reversible, thereby meeting one of the least flexible tenets of true catalysis.

Many guest molecules do not possess the necessary "handle" with which to afford the transient associations available via precedented complexation mechanisms. Our work with acrylate-type substrates points this out clearly; few of the previously utilized methods of host-guest interaction seem practical in this case. We are, therefore, examining a mode of complexation that has not been utilized previously, *reversible cycloaddition*, that will effectively allow for the molecular recognition of dienophilic substrates. This idea is outlined in Figure 1 (cat. = catalytic group, f.g. = functional group), which is a highly generalized and versatile scheme for the formulation of Diels-Alder reaction based catalysts. Reversibility in the Diels-Alder reaction has substantial literature precedent,[3-5] and our own work learning to accelerate retro-Diels-Alder reaction rates is aimed at making such reversibility accessible at lower temperatures in the acrylate series.[6]

(1) The design of water-soluble compounds that catalyze reactions via mechanisms analogous to those of certain enzymes (often called "artificial enzymes", "synzymes", "synthetic enzymes", or "enzyme mimics") has been a fruitful area of research for about 25 years or so. The literature is by now so vast that it is not possible to list just a few key reviews that will provide lead references for most of what has been done.

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