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- (17) For a similar use of **11** see: Heller, M. J.; Walder, J. A.; Klotz, I. M. *J. Am. Chem. Soc.* **1977**, *99*, 2780. See also ref 5b.
- (18) In several runs with $[16\text{-SH}] < 0.01$ M, the apparent infinity absorbance at 272 nm displayed slow upward drift, presumably owing to slow oxidation of product **13**. Indeed increasing absorption at 272 nm was observed when 8×10^{-5} M **13** and 5×10^{-3} M micellar CTACl in 0.04 M deoxygenated phosphate buffer were mixed with an equal volume of O_2 -saturated distilled water in a stopped-flow experiment. The apparent k_1 for this process was 0.045 s^{-1} , which is negligible compared to the rate constants of Table VII, provided that $[16\text{-SH}] > 0.01$ M. The increasing infinity titer problem was not encountered in (nonmicellar) reactions of thiocholine and iodoacetamide.
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- (24) Reference 2, p 220 ff.
- (25) The rate constant for this deacetylation is $1.5 \times 10^{-2} \text{ s}^{-1}$ when 16-Im-COCH₃ is formed from 5×10^{-3} M 16-Im and 2×10^{-4} M PNPA in 0.4 M phosphate buffer at pH 8, 25 °C: Moss, R. A.; Nahas, R. C.; Ramaswami, S. *J. Am. Chem. Soc.* **1977**, *99*, 627.
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Improved Acylation Rates within Cyclodextrin Complexes from Flexible Capping of the Cyclodextrin and from Adjustment of the Substrate Geometry

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Abstract: The acylation of β -cyclodextrin by bound substrates has been studied as a model for serine acylase enzymes such as chymotrypsin. Molecular model building suggested that previously examined substrates, which had given acylation rates only a few hundred times accelerated over the hydrolysis rates, were not optimal geometrically. In our work the geometry for such processes has been improved by fashioning an "intrusive floor" in the cyclodextrin cavity, leading to improved rates. Greater improvements have come from substrate modification, using substrates based on the cinnamic acid, adamantane, and ferrocene frameworks. The rates correlate well with the geometric predictions from molecular models. The best case leads to an acceleration of acylation, relative to hydrolysis, of 10^6 - 10^7 -fold, exceeding that for chymotrypsin with *p*-nitrophenyl acetate.

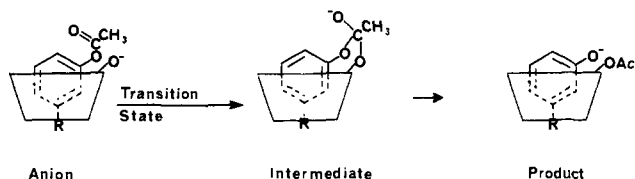
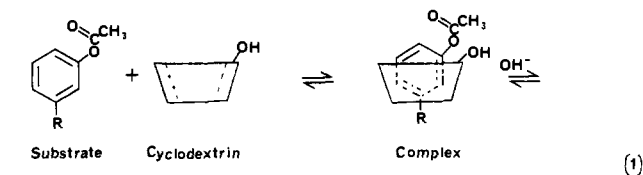
Introduction

The cyclodextrins (cycloamyloses) have attracted great attention as enzyme models. α -Cyclodextrin (cyclohexaamylose) and β -cyclodextrin (cycloheptaamylose), and to a lesser extent γ -cyclodextrin (cyclooctaamylose), have been studied because of their ability to bind large organic molecules into the cavity of the host cyclodextrin by the use of hydrophobic or generalized lyophobic¹ forces. Within the complex there may

be reaction *with* the hydroxyl groups of cyclodextrin^{2,3} or catalyzed by these hydroxyls.⁴ Derivatives of cyclodextrins have also been studied in which catalytic or reactive functional groups are present to attack the bound substrate.⁵⁻⁸

Although the ability of the cyclodextrins to bind substrates into a molecular cavity in solution made them interesting enzyme mimics, the accelerations resulting from such binding had been quite modest. For example, Bender et al.³ had studied

the acetylation of a cyclodextrin hydroxyl by bound phenyl acetate esters. Such a reaction may be considered to be a model for the first step in the action of esterase or protease enzymes in which the hydroxyl of a catalytic serine residue attacks the acyl group of a bound substrate. For the cyclodextrin case, Bender saw typical Michaelis-Menten kinetics and a maximum velocity for acetyl transfer in the complex which was dependent on hydroxide ion to the first order (eq 1).



Since the substrate can also be cleaved, in the absence of cyclodextrin, by direct nucleophilic attack of hydroxide ion, it is convenient to use such a process as the comparison with the cyclodextrin reaction:

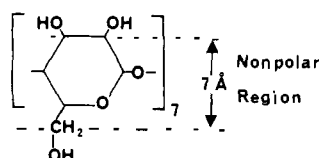
$$\text{rate of acetyl transfer in the cyclodextrin complex} = k_{\text{complex}}[\text{complex}][\text{OH}^-] \quad (2)$$

$$\text{rate of deacetylation without cyclodextrin} = k_{\text{un}}[\text{substrate}][\text{OH}^-] \quad (3)$$

Thus the ratio of $k_{\text{complex}}/k_{\text{un}}$ is used as a measure of the effectiveness of complexing in promoting deacetylation.

Such comparisons are often used in describing enzymatic catalysis. That is, the rate of the enzymatic step is compared with that for attack on the substrate by similar reagents or groups in free solution. By this criterion, cyclodextrins seemed to be poor enzyme models. Enzymes often achieve ratios for the catalyzed vs. uncatalyzed reaction of 10^5 – 10^{10} or greater. However, the best rate ratio found by Bender^{3,8} for acetyl transfer to β -cyclodextrin was only 250, the result for *m-tert*-butylphenyl acetate (eq 1). Thus there was concern that the potential for catalytic acceleration by cyclodextrin inclusion might be quite limited.⁹

To examine this problem, we have initiated a program of systematic variation of the geometry within the cyclodextrin complexes. Molecular model building¹⁰ suggested that the substrate of eq 1 can bind fully in the cavity in the complex, but that it is pulled up partly out of the cavity by formation of the tetrahedral intermediate. As a rough indicator of the information in such models, we have prepared Table I. This shows the percent occupancy of the nonpolar region of the cyclodextrin cavity by substrates and intermediates for all the systems considered in this paper. The numbers in Table I show



only the percentage of this vertical depth which is occupied by a nonpolar section of the substrate, and do not necessarily reflect the effectiveness with which that depth is filled or the energy associated with the binding.

Guided by such models, we have modified β -cyclodextrin and selected new substrates so as to retain as much binding as

Table I. Percent Occupancy of the Cyclodextrin Cavity by Substrates and Tetrahedral Intermediates, as Judged from Molecular Models

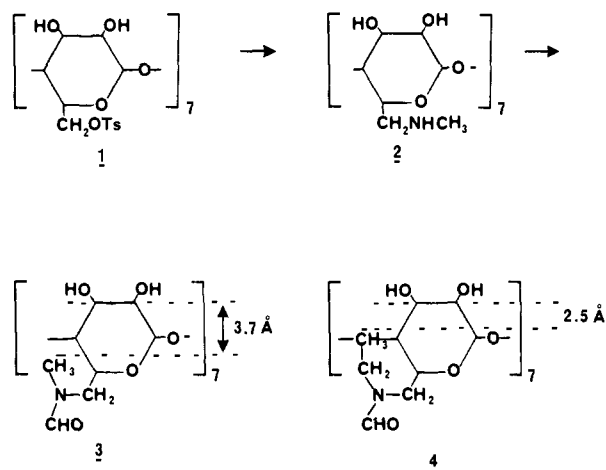
compd	β -cyclodextrin percent occupancy ^a	
	substrate	tetrahedral intermediate
<i>m-tert</i> -butylphenyl acetate (7)	100	25
2-methoxy-5- <i>tert</i> -butylcinnamate ester (8)	85	70
double acrylate ester (9)	85	70
adamantylpropionate ester (10)	65 (100) ^b	45
<i>tert</i> -butyladamantylpropionate ester (11)	100	80
ferrocenylpropionate ester (12)	80	75
ferrocenylacrylate ester (13)	80	75

^a Approximate vertical percentage penetration of the cyclodextrin cavity, excluding the hydroxyl groups, as judged from CPK models. See Discussion. ^b 65% refers to the reactive geometry, 100% to the dominant incorrect geometry.

possible on proceeding from bound substrate through bound transition state to bound tetrahedral intermediate (to product). Modifications which improved the situation by avoiding or diminishing the debinding at the transition state were, as expected, very effective at improving the rate of reaction within the complex. In fact, in our best cases we have brought the acceleration up to a level characteristic of that for a similar enzymatic reaction.

Results

The first approach was to modify β -cyclodextrin itself.¹¹ Cycloheptaamylose heptatosylate¹² (1) reacted with methylamine to produce the heptamethylamino derivative (2), and this polyelectrolyte was *N*-formylated to the hepta-*N*-methylformamide (3).



Molecular models suggested that in 3, and in the similarly prepared hepta-*N*-ethylformamide 4, the methyl (or ethyl) groups can cluster and enter the nonpolar cavity of cyclodextrin. The results is that the cavity should be capped, so as to become a pocket, and be shallower than the cavity of uncapped β -cyclodextrin. Several types of evidence confirm this picture.

First of all, we have examined the binding of adamantane-1-carboxylic acid (ACA, 5) to β -cyclodextrin³ and to 3 and 4. The binding of 5 to β -cyclodextrin should, from models, involve occlusion onto the face of the cavity since the diameter of 5 is slightly too large¹⁰ for complete inclusion.

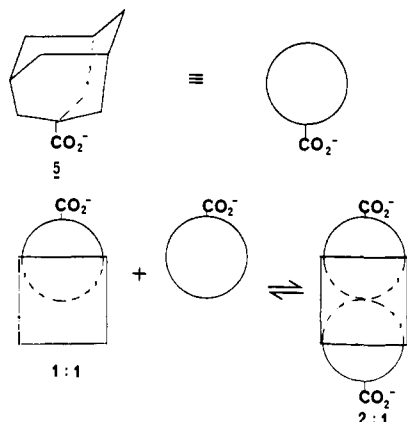
The binding of ACA (5) to cyclodextrin was determined by studying 5 as an inhibitor of the reaction of eq 1 with *m*-nitrophenyl acetate (6) as substrate. A standard treatment of this

Table II. Binding and Rate Constants for Deacylation of Substrate in a Cyclodextrin Complex and in Free Solution^a

substrate	host ^b	solvent	K_d , mM ^c	k_{complex}^d	k_{un}^e	$k_{\text{complex}}/k_{\text{un}}$
<i>m</i> -nitrophenyl acetate (6)	β CD	H ₂ O	5.3	0.012	1.9×10^{-4}	64
<i>m</i> -nitrophenyl acetate (6)	Me-capped (3)	H ₂ O	5.1	0.123	1.9×10^{-4}	660
<i>m</i> -nitrophenyl acetate (6)	Et-capped (4)	H ₂ O	25	0.210	1.9×10^{-4}	1140
<i>m-tert</i> -butylphenyl acetate (7)	β CD	H ₂ O	0.20	0.0041	1.1×10^{-5}	365
<i>m-tert</i> -butylphenyl acetate (7)	Me-capped (3)	H ₂ O	0.46	0.037	1.1×10^{-5}	3300
2-methoxy-5- <i>tert</i> -butylcinnamate ester (8)	β CD	Me ₂ SO/H ₂ O	10	0.0041	2.7×10^{-6}	1500
2-methoxy-5- <i>tert</i> -butylcinnamate ester (8)	Me-capped (3)	Me ₂ SO/H ₂ O	1.2	0.0011	2.7×10^{-6}	400
double acrylate ester (9)	β CD	Me ₂ SO/H ₂ O	15	0.017	3.5×10^{-6}	4900
adamantylpropionic ester (10)	β CD	Me ₂ SO/H ₂ O	0.3	0.084	3.9×10^{-5}	2150
adamantylpropionic ester (10)	Me-capped (3)	Me ₂ SO/H ₂ O	2.7	0.56	3.9×10^{-5}	14 000
<i>tert</i> -butyladamantylpropionic ester (11)	β CD	Me ₂ SO/H ₂ O	1.8	0.59	3.9×10^{-5}	15 000
<i>tert</i> -butyladamantylpropionic ester (11)	Me-capped (3)	Me ₂ SO/H ₂ O	(1.0 mM ²) ^f	0.37 ^f	3.9×10^{-5}	9500 ^f
ferrocenylpropionic ester (12)	β CD	Me ₂ SO/H ₂ O	5	0.40	2.8×10^{-6}	140 000
ferrocenylacrylic ester (13)	β CD	Me ₂ SO/H ₂ O	7	0.21	2.8×10^{-7}	750 000
ferrocenylacrylic ester (13)	Me-capped (3)	Me ₂ SO/H ₂ O	2	0.066	2.8×10^{-7}	235 000

^a Substrates 6 and 7 studied in H₂O at 25 °C at pH 9.0. All others in 60% (v/v) Me₂SO/H₂O at 30.0 °C with a "pH 6.8" buffer. ^b β CD is β -cyclodextrin, Me-capped is compound 3, Et-capped is compound 4. ^c The dissociation constant for the host-substrate complex determined by an Eadie plot of the kinetic data as a function of host concentration. The probable error is 10%. ^d The V_{max} for reaction of the substrate with the host at kinetic saturation. The V_{max} is first order in OH⁻, and shows no buffer catalysis at the concentrations (2-10 mM buffer) examined. ^e The rate constant for deacylation of substrate by OH⁻ in the absence of cyclodextrin. At 10 mM buffer there was generally a 50-75% contribution from buffer catalysis, and runs at several buffer concentrations were used to obtain the OH⁻ term which is listed. ^f A 2:1 complex is formed.

system gave a dissociation constant of 1.5 ± 0.1 mM for the complex of 5 with β -cyclodextrin (reported³ 0.7 mM) but at high concentrations of 5 (above 1.5 mM) our plots suggested that a second molecule of ACA (5) was binding. This was confirmed by a study¹³ of the optical rotation of a solution of β -cyclodextrin containing varying amounts of 5 in this higher concentration region. The data did not fit a plot for equilibration of β -cyclodextrin with a 1:1 complex (with an altered rotation), but did fit nicely a plot for formation of a 2:1 complex of 5 with β -cyclodextrin.

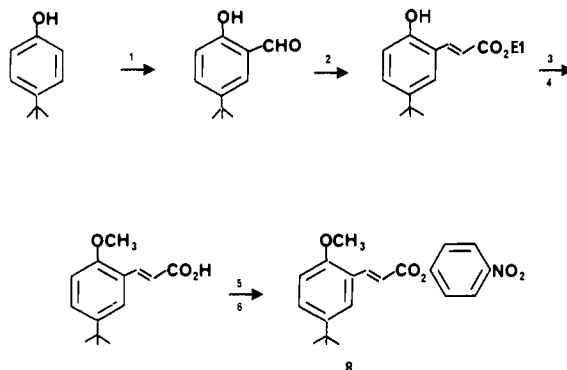


The results with the capped cyclodextrins 3 and 4 were very different. These clearly formed only 1:1 complexes with ACA (5), but with smaller dissociation constants (stronger binding). Thus the methyl-capped compound 3 had K_{diss} for ACA of 0.067 mM, while 4 had a K_{diss} for ACA of 0.075 mM. As will be discussed later, this shows that 3 and 4 indeed have floors which block one face of the β -cyclodextrin cavity. It also shows that these floors extend up into the cavity, so as to contact and help bind an ACA molecule occluded on the other face of the cavity.

The capped cyclodextrins 3 and 4 were examined in the reaction of eq 1 with *m*-nitrophenyl acetate (6) and *m-tert*-butylphenyl acetate (7) as substrates. The data, listed in Table



Scheme I



1, NaOH/CHCl₃; 2, Ph₃P=CHCO₂Et; 3, NaOH and Me₂SO₄; 4, KOH, then H⁺; 5, SOCl₂; 6, *p*-nitrophenol in pyridine.

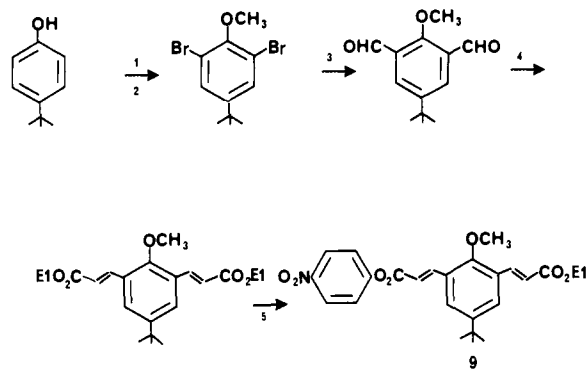
II, show that there is an improvement of $k_{\text{complex}}/k_{\text{un}}$ in 3, and even more in 4, compared with unmodified β -cyclodextrin. This is as expected if the shallower cavities bring the complex closer to the geometry of the transition state.

Our more general approach to improving the rates of β -cyclodextrin reactions was to find new substrates whose geometry was better suited to the reaction, so that the transition state could be as well bound as the substrate, or possibly better. This work will be described in order of increasing effectiveness, which is not the historical order.

A simple conceptual change in 7 is structure 8, the *p*-nitrophenyl ester of 2-methoxy-5-*tert*-butyl-*trans*-cinnamic acid. The compound was prepared by Scheme I. Compound 8 and indeed all the rest of the compounds in this study are not soluble in H₂O, but we had found¹ that cyclodextrin also binds substrates and reacts in other polar solvents. In fact, $k_{\text{complex}}/k_{\text{un}}$ for substrate 7 improves¹ from 365 in H₂O to 500 in 60% (v/v) Me₂SO/H₂O. Our data for k_{complex} , k_{un} , and K_d for compound 8 in Table II are thus obtained in 60% Me₂SO/H₂O at 30.0 °C with a buffer (sodium potassium phosphate) which has pH 6.8 in pure H₂O. The effective basicity is higher in our medium, by a little over 1 pH unit.¹

The increase in $k_{\text{complex}}/k_{\text{un}}$ for substrate 8, compared with 7, is quite modest. The data as a function of β -cyclodextrin concentration shows that only a 1:1 complex is formed, and the

Scheme II

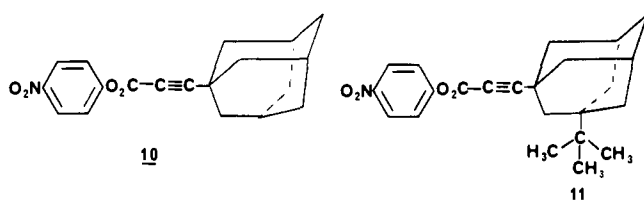


1, Br_2 and K_2CO_3 ; 2, NaOH and Me_2SO ; 3, BuLi , then DMF ; 4, $\text{Ph}_3\text{P}=\text{CHCO}_2\text{Et}$; 5, 1 equiv NaOH , then *p*-nitrophenol in pyridine

known binding constants for *tert*-butylphenyl and for nitrophenyl groups⁸ show that the binding must be to the *tert*-butylphenyl group. As Table I shows, there is still (from models) a significant lifting of the bound substrate partly out of the cavity as it goes to the tetrahedral intermediate, as in 7. However, our experimental data confirm that this is less in 8, since now the methyl-capped cyclodextrin 3 is less effective than is β -cyclodextrin, presumably because for 8 the cavity of 3 is now too shallow for the transition state.

We have synthesized a related compound 9 by Scheme II. This is simply substrate 8 with an extra projection. Models show (Table I) that the projection in 9 prevents the substrate from binding as deeply into the cavity but does not change the geometry of the tetrahedral intermediate. Since the geometry of the complex is thus closer to that for the transition state, $k_{\text{complex}}/k_{\text{un}}$ improves somewhat (Table II).

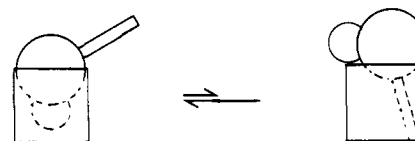
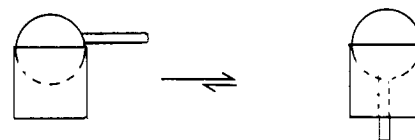
Molecular models, and our results with ACA (5), show that the adamantane nucleus binds strongly but shallowly to β -cyclodextrin; thus we have examined two substrates based on adamantane. One of them, the *p*-nitrophenyl ester of 1-adamantylpropionic acid¹⁴ (10), was simply prepared from adamantylacetylene while the corresponding compound (11)



based on *tert*-butyladamantane was prepared in a fashion similar to that of 10, but starting with 1-bromo-3-*tert*-butyladamantane.¹⁵

In the case of compound 11 models show that the β -cyclodextrin cavity is filled if the adamantane ring occludes onto the secondary face of the cyclodextrin, with the *tert*-butyl group extending into the rest of the cavity. This fixes the ester group near the secondary hydroxyls, so only a small geometric change (Table I) is needed for 11 to proceed to the tetrahedral intermediate, and on to product. The result is a large $k_{\text{complex}}/k_{\text{un}}$ (Table II), up to a value of 15 000.

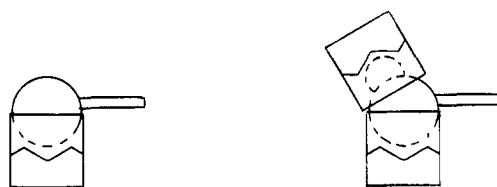
The situation with 10 is less obvious. In the absence of the *tert*-butyl group the orientation of the substrate is not locked with respect to β -cyclodextrin, and our data suggest that it binds chiefly in a nonreactive geometry. It has a $k_{\text{complex}}/k_{\text{un}}$ of only 2150, one-seventh of 11, and at the same time 10 is bound six times more strongly than is 11. This stronger binding only makes sense if the part of the cavity which is not occupied



by the adamantane nucleus is occupied by the acetylene chain.

Since this explanation and the observed stronger binding of 10 than of 11 imply that the acetylene unit binds better than does the *tert*-butyl unit, one should wonder why 11 did not bind in the same wrong way. Models show that, if the acetylene unit of 11 is put into the cavity, the *tert*-butyl unit prevents the adamantane nucleus from seating correctly.

To test these ideas, we have examined the binding and reaction of 10 and 11 with the methyl-capped cyclodextrin 3, in which the floor should block incorrect binding of both compounds. As the data in Table II show, this now raises the rate for 10 to that we had seen for 11 with simple β -cyclodextrin. The binding of 10 is weakened, now that the floor blocks its strong unreactive binding mode. Thus this directly confirms our picture. The results with 11 are striking. It retains essentially the same rate of reaction in the complex with 3, but now it binds *two* capped cyclodextrins (3) rather than one uncapped β -cyclodextrin. This is clearly shown by our plot of rate vs. cyclodextrin concentration. Since no such thing occurs with 10, the two molecules of 3 must both be binding to the *tert*-butyladamantane nucleus.



10 with 3

11 with two 3

This fits molecular models nicely. It can be described by saying that the floor in 3 would interfere with the *tert*-butyl group of 11, so this group rotates up out of the way and then binds a second 3 molecule. An alternate equivalent description reverses the conceptual roles of the two molecules of 3. In this description, the floor of 3 pushes the *tert*-butyl group up, forcing 11 partly out of the cavity. Now it is able to bind a second molecule of 3 to another face of the adamantane nucleus. The velocity of reaction within this ternary complex is, as expected from these ideas, similar to that of the binary complex of 11 with uncapped β -cyclodextrin, or of 10 with 3.

In considering how to design the optimal substrate for cyclodextrin acylation, we first built a molecular model of the tetrahedral intermediate for acylation by any *p*-nitrophenyl ester, and then asked what kind of an acyl group would fit it

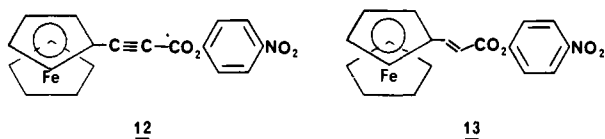
Table III. Activation Parameters for the Reactions of *p*-Nitrophenyl Ferrocenacrylate (**13**)^a

reaction	ΔH^\ddagger , kcal/mol	ΔS^\ddagger , eu
β -cyclodextrin acylation	5.7	-42.4
uncatalyzed hydrolysis	19.1	-23.6

^a All reactions in 60% Me₂SO/40% H₂O (v/v) at pH 6.8 with 10 mM phosphate buffer. Results are at 298 K.

best. The geometry required led us to derivatives of ferrocene.

We had shown earlier¹ that the ferrocene nucleus itself is strongly bound to β -cyclodextrin. Models suggested that the *p*-nitrophenyl esters of ferrocenepropionic acid (**12**) and of ferrocene-2-acrylic acid (**13**) should be excellent substrates. This proved to be the case.



Substrate **12** was prepared by carboxylating ferrocenylacetylene¹⁶ to the known acid¹⁷ and then forming the ester. As the data in Table II show, it has a normal binding constant but an outstanding reactivity, $k_{\text{complex}}/k_{\text{un}}$ being 140 000. This was expected from the geometric changes listed in Table I.

Substrate **13** was prepared by esterifying the known acid.¹⁸ It also had a normal binding constant to β -cyclodextrin, but showed an even larger rate acceleration, with $k_{\text{complex}}/k_{\text{un}}$ at 750 000. As with all our substrates, the product was *p*-nitrophenoxide ion and the β -cyclodextrin ester of the acid, in this case ferrocenacrylic acid. This ester could be hydrolyzed in base to liberate the β -cyclodextrin and the ferrocenacrylic acid.

Because the reaction of β -cyclodextrin with **13** was of special interest, we examined it further. Temperature studies yielded the activation parameters in Table III. These show that all of the acceleration comes from the remarkable decrease in *enthalpy* of activation for the reaction in the complex. This situation has been noted before for other cyclodextrin reactions.⁸ As with all reactions in solution, a detailed interpretation of the activation parameters is complicated by the fact that they include solvation changes. We have also looked at the reaction of **13** with our capped cyclodextrin **3**. The data in Table II show that it is slower, as expected since essentially the full cavity of β -cyclodextrin is needed to bind compound **13** and its transition state.

The products of all these reactions are esters of the cyclodextrins. Furthermore, they are certainly esters on the secondary face of cyclodextrin, as evidenced from the reactions of cyclodextrins such as **3** and **4** in which the primary hydroxyls are missing. This is also fully expected from molecular models. However, the models do not really distinguish between the hydroxyls at C-2 and those at C-3 as probable points of attack, since these two equatorial oxygens are very similarly disposed relative to the cavity.

We have built our models, and based Table I, on the assumption that the C-2 hydroxyl is the nucleophile, as it is in methylation, but Table I would be little changed by the use of the C-3 hydroxyl. Bender believes⁸ that the reaction of eq 1 involves the C-3 hydroxyl. Perhaps the most telling evidence is our observation that the cyclodextrin ester formed in the acylation by ferrocenacrylic *p*-nitrophenyl ester **13** is actually, by NMR, an almost equal mixture of *two* isomers. One shows a vinyl AB quartet with δ 6.05 and 7.40 and $J = 15$ Hz, while the other shows an AB quartet with δ 6.13 and 7.54 and $J =$

15 Hz. Thus it seems likely that either C-2 or C-3 can become esterified, as the model suggest.

The above NMR spectrum is most clearly resolved in the ferrocenacrylate ester(s) produced by reaction of β -cyclodextrin with ferrocenacryloyl imidazole, rather than the *p*-nitrophenyl ester (**13**). As we have reported previously,^{11b} this acyl imidazole also reacts rapidly with β -cyclodextrin, although $k_{\text{complex}}/k_{\text{un}}$ is less than for the *p*-nitrophenyl ester. Other amides of ferrocenacrylate, or the ethyl ester, are also much less reactive toward β -cyclodextrin. As expected, α -cyclodextrin (cyclohexaamylose) is approximately two orders of magnitude less reactive toward ester **13** than is β -cyclodextrin. The cavity in α -cyclodextrin is too small to bind ferrocene correctly.

Discussion

Molecular model have proven to be excellent predictive tools in this work. Thus the data in Table I, deduced from models, show good correlation with the rate data in Table II—slow substrates at the top of the tables lose a lot of binding on going to the tetrahedral intermediate, while fast substrates at the bottom of the tables lose very little. However, the data in Table I are only a very rough indication of what the models show.

The table was constructed by assuming that the hydrophobic cavity of β -cyclodextrin extends from carbons 2 and 3 on the secondary face all the way to the C-6 methylene group on the primary face. Substrates were placed as deeply into the cavity, from the secondary side, as they went without distorting, and similarly with tetrahedral intermediates formed using the C-2 cyclodextrin hydroxyl. Vertical distances were measured from the deepest point of penetration of a hydrocarbon portion of the substrate to the bottom of the cavity, and used to construct Table I. This procedure can of course be biased by error in the models or in our assumptions about geometries. Furthermore, these data do not show the *effectiveness* with which the cavity is filled. For instance, the ferrocenyl nucleus of substrates **12** and **13** is a plug filling the entire cavity into which it penetrates, while the phenyl rings of **7**, **8**, and **9** do not fill the cavity as well. Finally, the *energy* by which the substrates and intermediates are bound is yet another matter, since all segments of the cyclodextrin cavity may not contribute equally. Thus Table I is only a rough guide to expectations. Furthermore, the rate is affected by the energy of the *transition state*, not of the tetrahedral intermediate. We expect the transition state for this reaction to be similar to, but earlier than, the tetrahedral intermediate (Figure 1). Since *p*-nitrophenoxide ion is a better leaving group than is β -cyclodextrin anion, the tetrahedral intermediate should partition largely to product. This defines it as being past the transition state. Thus using the data of Table I for the intermediate may overestimate the geometric change at the transition state.

In Figure 1 we outline the *standard* free-energy relationships along the reaction path. When substrate binds to a cyclodextrin it rapidly and reversibly falls into a potential well from which it must climb in order to react. Thus the rate constant for reaction within the complex is diminished to the extent that substrate binds well and increased to the extent that the transition state is well bound. (Of course, if the concentrations are so low that substrate is not appreciably bound at equilibrium, then the bulk of the reactants are at point A, the rate constant is k_{complex}/K_d and is determined by the standard free-energy change $[D-A]$, and the energy at point C is irrelevant.)

For fast reaction within a complex it is clearly desirable to minimize substrate binding relative to binding of the activated complex at the transition state.^{19,20} In fact, one can argue for having *no* binding of the substrate, but such an argument ignores the need for prior complexing so that rates of collision in multicomponent reactions do not put an upper limit on the reaction velocity. In any case, our data are consistent with these

ideas. Thus the extra protrusion in substrate **9** relative to **8** prevents **9** from binding as deeply and increases K_d by a factor of 1.5. It also increases $k_{\text{complex}}/k_{\text{un}}$ by a factor of 3.3, consistent with the idea that **9** does not fall into as deep a potential well at point C of Figure 1.

A striking example is seen in the data for substrate **10**. Here the very strong binding of the substrate in an "incorrect" geometry leads to a big drop in the energy of point C relative to point D, but the blockage of this incorrect binding by the floor in cyclodextrin derivative **3** leads to a ninefold decrease in the binding constant for the substrate and a sevenfold increase in k_{complex} . The coincidence of these numbers is expected if the floor removes the special substrate stabilization resulting from incorrect binding.

It is interesting that the rate increase of **11** over **10** with β -cyclodextrin principally reflects the ability of the *tert*-butyl group in **11** to block incorrect through-the-cavity binding. One might have expected this projection on spherical adamantane to help orient the substrate so that the side chain is aimed correctly. However, rigid binding offers no rate advantage unless the aim is perfect.

At a certain point predictions become very difficult. We cannot easily distinguish between the situation, as seen in molecular models, for substrates **12** and **13**. Both of these ferrocene derivatives look very good, in the sense that the tetrahedral intermediates seem to retain almost all the binding of the substrate. Both are indeed excellent, but the acrylate ester **13** is five times better than the propiolate **12**. One might have worried that the acrylate ester **13** would have some single-bond flexibility in the side chain which must be frozen out during reaction, but the ferrocene nucleus is such an excellent electron donor²¹ that the acrylate side chain must be largely locked into conjugation.

It is possible that the very high rate of reaction of the ferrocenylacrylate **13** with cyclodextrin could reflect some factor other than just good geometry for transition-state binding. The ester is chemically quite unreactive, having the lowest k_{un} in Table II. This must reflect both the lesser electron-withdrawing effect of a vinyl group, relative to the acetylene units of some of the other substrates, and the deactivation of the ester group in **13** by electron donation from the ferrocene through the vinyl group. If binding of **13** blocked such conjugative stabilization, by putting the ferrocene unit into a less polar environment or by twisting the conjugated system, this could lead to a high value of $k_{\text{complex}}/k_{\text{un}}$. However, no such effects can operate in the adamantyl systems such as **11**, and the improvement over **11** in **12** and **13** is expected just on the basis of geometry, as Table I shows. Thus at the current time there seems to be no need to invoke other than geometric factors to explain the relative reactivities, as expressed by $k_{\text{complex}}/k_{\text{un}}$, in Table II.

The studies with cyclodextrins **3** and **4**, whose methyl or ethyl groups can intrude into the cavity, provide a consistent picture. Molecular models suggest that the original 7-Å depth of the hydrophobic pocket of β -cyclodextrin may shrink to 3.7 Å in **3** and 2.5 Å in **4**. Of course these intrusive floors are flexible, and binding of a substrate into the cavity could well change these dimensions even if they are the correct values for the modified cyclodextrins themselves.

The most striking confirmation of this picture of the structures of **3** and **4** is the finding that β -cyclodextrin binds *two* molecules of adamantanecarboxylate (**5**), while **3** and **4** bind only *one*. Our models show that an adamantane nucleus can occupy ca. 65% of the β -cyclodextrin cavity if it binds into the more open secondary face (cf. the value in Table I for binding of substrate **10** in the reactive geometry). This leaves ca. 35% of the bottom of the cavity plus the hydrophobic floor furnished by the bound adamantane for binding of a second molecule. However, in **3** the methyl groups fill the bottom of the cavity

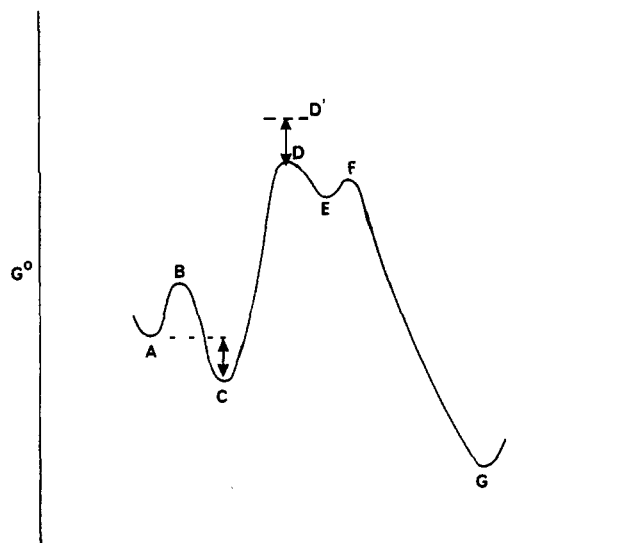


Figure 1. Uncomplexed substrate plus cyclodextrin (A) are in rapid equilibrium over a small barrier (B) with the complex (C). This proceeds through the transition state (D) to the tetrahedral intermediate (E), which then chiefly passes over the low barrier (F) to go to products (G). The binding energy of the substrate affects [A-C], and helps determine the dissociation constant of the complex K_d . The binding energy of the transition state relative to a hypothetical unbound transition state at D' helps to determine the rate constant, k_{complex} , for reaction in the complex.

so that only 50% of it is left, and binding of an adamantanecarboxylate into the top, with some downward displacement of the methyls, should occupy the entire cavity. Thus only a single adamantanecarboxylate is bound to **3**, and that one very strongly because of the extra hydrophobic interaction with the methyl floor. The binding must be into this cavity, and not by occlusion onto the bottom of **3**, since adamantanecarboxylate binding blocks the reaction of substrate **6** with the cyclodextrin **3**.

The data also support the idea that the ethyl groups in flexibly capped cyclodextrin **4** protrude even further into the cavity. First of all, the binding of adamantanecarboxylate to **4** is weaker than it is to **3**, as expected if even more downward displacement of ethyls is required (or the adamantane nucleus does not seat fully into the cyclodextrin cavity). Secondly, the results of our kinetic studies with **3** and **4**, using *m*-nitrophenyl acetate (**6**), support this picture. The binding of substrate is unchanged comparing β -cyclodextrin with **3**, which must be a curious combination of decreased binding in the shallower cavity compensated by increased binding by the floor, but with **4** binding decreases (K_d increases). The interaction with the floor should be similar in **3** and **4**, so now the extra shallowness of the cavity is seen in K_d . Oddly, with *m-tert*-butylphenyl acetate (**7**) as substrate the extra shallowness of the cavity results in an increase in K_d even on comparing the β -cyclodextrin case with the methyl-capped **3**.

The data for k_{complex} with substrates **6** and **7** are quite sensible. Raising the substrate in the cavity by use of the methyl-capped **3** results in a nine- to tenfold acceleration for both **6** and **7**. The residual cavity in **3** is much closer to the 25% needed to bind the tetrahedral intermediate in its undistorted geometry. The transition state may well use most of the available cavity, both because it *precedes* the tetrahedral intermediate and because it may distort somewhat, the greater binding energy paying for the distortion (and because the estimates in Table I are only estimates). Thus with **3** we expect the increased rate because the net binding energy of substrate does not so much exceed that of the transition state as it does with uncapped cyclodextrin. An additional factor of 11 appears in the rate for substrate **6** with the ethyl-capped **4**, in which the

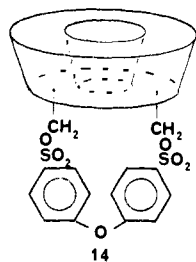


Figure 2. β -Cyclodextrin capped at positions⁶ 6A and 6D.

cavity is still shallower and thus closer to that needed to bind the undistorted transition state.

An indication that the transition states may be better bound by β -cyclodextrin than Table I indicates (assuming a resemblance to the tetrahedral intermediate) is the result of the study on the cinnamate ester **8** with capped and uncapped cyclodextrin. Of course the solvent is different from that for the studies with **6** and **7**, but we now see that the floor in methylcapped **3** binds the substrate to *more* than compensate for the decrease in cavity depth, K_d decreasing by eightfold. At the same time k_{complex} also decreases, in contrast to our results with **6** and **7**. The rate loss suggests that the cavity in **3** is now too *shallow* to bind the transition state for reaction of **8**, not quite consistent with a 53% residual cavity in **3** and a 45% requirement for the tetrahedral intermediate derived from **8**. The trend is in the right direction relative to **6** and **7**, but the results suggest that the transition state for **8** may need more than the cavity left in **3**. Of course, binding in a shallow cavity which holds the substrate too high to reach the reactive hydroxyls of cyclodextrin will have serious rate consequences.

The effects of methyl capping on the reactions of adamantane esters **10** and **11** are striking. The floor in **3** blocks incorrect binding of **10**, bringing its rate up to that of **11** with simple cyclodextrin. With **11** the "flexible methyl floor" is apparently strong enough to push the *tert*-butyl group out of the cavity, so that it binds a second molecule of **3**. Because of these complicated effects, it is not possible to say whether the floor also speeds up or slows down the reactions by the mechanisms we have discussed for the other substrates.

Finally the result with our best substrate, ferrocenylacrylic ester (**13**), is interesting. The floor in **3** increases substrate binding by 3.5-fold and decreases k_{complex} by 3.2-fold. It is as if the floor lowered the energy at point C of Figure 1, but had no effect on the energy of point D. The source of this differential binding effect is not obvious. It is the result of the intrusion of methyl groups into the cavity, not simply of capping, however. We have examined the reaction of substrate **13** with rigidly capped molecule **14** (Figure 2).²² Here we find that k_{complex} is slightly improved, so that $k_{\text{complex}}/k_{\text{un}}$ is now 1 000 000 and K_d is unchanged at 7.5 mM.

The very large acceleration we have achieved with the ferrocenylacrylate system **13** brings this acylation reaction into the range characteristic of a reaction of the enzyme α -chymotrypsin. The enzyme is acylated at neutrality by *p*-nitrophenyl acetate with a k_{complex} ²³ of 3.15 s⁻¹, which can be compared with the acylation rates of β -cyclodextrin of 0.21 s⁻¹ with a pH 6.8 buffer and 2.1 s⁻¹ at pH 7.8. Furthermore, the k_{un} for substrate **13** is 10² times less than is k_{un} for *p*-nitrophenyl acetate, because the ferrocenylacrylate system is electron donating relative to acetyl. Thus for the enzyme α -chymotrypsin $k_{\text{complex}}/k_{\text{un}}$ for *p*-nitrophenyl acetate is only ca. 10⁵, while for **13** with β -cyclodextrin $k_{\text{complex}}/k_{\text{un}}$ is almost 10⁶.

The comparison is even more striking when we include the solvent effects. Our Me₂SO/H₂O medium is meant to mimic the enzyme interior, so the k_{un} considered could be the un-

catalyzed rate constant in *water* solvent, as it is for the enzyme. We have found¹ that such an uncatalyzed reaction, with the same buffer, is ca. 25 times faster in 60% Me₂SO/H₂O than in H₂O alone. Thus $k_{\text{complex}}(\text{Me}_2\text{SO}/\text{H}_2\text{O})/k_{\text{un}}(\text{H}_2\text{O})$ for substrate **13** with β -cyclodextrin is ca. 1.8×10^7 , much greater than the enzymatic acceleration.

Such comparisons can be deceptive. α -Chymotrypsin is one of the slower enzymes, and *p*-nitrophenyl acetate is certainly not its optimal substrate. On the other hand, the enzyme has catalytic groups which are not present in simple β -cyclodextrin. Thus it seems fairest to say that geometry optimization for substrates of β -cyclodextrin has brought a great improvement in the acylation rate, up to the point at which it is comparable to the rate for one of the slower enzymatic processes. Further work with even better substrates and with β -cyclodextrin carrying other catalytic groups may add the additional rate factor of 10³ or so needed to bring these reactions up to the rates characteristic of really fast enzymatic reactions.

Conclusions

1. The rate of acylation of β -cyclodextrin by bound substrate responds in a reasonable way to improved geometry, as judged from models.
2. The geometry can be improved by the addition of intrusive floors to β -cyclodextrin, or by substrate optimization.
3. These changes bring the rate of reaction in the complex compared with the rate of uncatalyzed hydrolysis up from the best previously known values of ca. 10² to values of 10⁶ or better.
4. The result with our best substrates is an acceleration of acylation, by complexing, which is comparable to that in a simple enzymatic acylation reaction.
5. Even our best cases are still not fully optimal, so larger rate effects can be anticipated.

Experimental Section

All substrates were carefully purified, and were homogeneous by TLC criteria, as well as by NMR. Aqueous solutions were prepared from distilled or double-distilled water, and other solvents and chemicals were either spectro- or reagent grade. Reported melting points are uncorrected. A Radiometer PHM 63 pH meter was used to determine pH values. β -Cyclodextrin was supplied by CPC International and was generally used without purification.

Reaction Kinetics. Reaction rates for *p*-nitrophenyl esters were followed at 30.0 \pm 0.5 $^\circ\text{C}$ in thermostated cell holders, in either a Cary 118 or Gilford 2400-S spectrophotometer, by monitoring the increase in absorbance at 410 nm. Pseudo-first-order rate constants were calculated from time vs. absorbance curves using a standard linear least-squares application to the integrated first-order rate equation.

The reaction medium was dimethyl sulfoxide/water (60:40 v/v) in which the water phase was buffered to pH 6.8 with 10 mM KH₂PO₄/NaOH. Thus the final buffer concentration was 4 mM. Kinetics experiments were initiated by adding 10 μL of a stock substrate solution (in Me₂SO) to 1 mL of the thermally equilibrated reaction medium, to give a final substrate concentration of 1.0×10^{-4} M. In a few cases the final substrate concentration used was lower, when the substrate exhibited low solubility in the reaction medium. The absorbance vs. time curves were then generated graphically using the chart speed as the time base, or digitally, for very slow reactions using electric clock readings of time.

Values for the dissociation constants K_d , and maximal acylation rate constants k_{complex} , were determined kinetically according to the method of Eadie.²⁴ Thus typically five to ten concentrations of β -cyclodextrin were used to generate plots of $(k_{\text{obsd}} - k_{\text{un}})$ against $(k_{\text{obsd}} - k_{\text{un}})/[\beta\text{-cyclodextrin}]$. Application of the standard least-squares analysis yielded $-K_d$ as the slope and k_{complex} as the *Y* intercept of the straight line.

Values for k_{un} were calculated by extrapolation to zero buffer concentration. Typically reaction rates were measured at buffer concentrations of 4, 2, and 1 mM with ionic strength maintained with isotonic KCl solution and pH adjusted with 1 N NaOH. Plots of k_{obsd}

against [buffer] gave as the Y intercept k_{un} . Buffer catalysis was typically 50–75% of the observed rate at 4 mM.

Kinetics studies on substrates **6** and **7** were performed in H₂O at 390 and 280 nm, respectively, with pH 9.0 ($I = 0.2$) NaHCO₃/NaCO₃ buffer at 25 °C. The substrate was introduced into 2 mL of aqueous buffer in 5–15 μ L of CH₃CN.

***p*-Nitrophenyl 2-Ferrocenylacrylate (13)**. Ethyl 2-ferrocenylacrylate was prepared by the Wittig reaction of 2 equiv of carbethoxymethylenetriphenylphosphorane with 1 equiv of ferrocenecarboxaldehyde in methylene chloride at room temperature for 12 h. The product was isolated by silica gel chromatography, eluting with hexane/ether (9/1), to give an orange, crystalline solid (86%). The ethyl ester was saponified at room temperature with 10% KOH in ethanol and gave after acidification the deep red ferrocenacrylic acid¹⁸ in 92% yield. The acid was converted to the acid chloride by refluxing in benzene with phosphorus trichloride for 5 h, and upon evaporation of the solvent the residue was treated with *p*-nitrophenol and pyridine in dry THF. After removal of pyridine and starting material by extraction the *p*-nitrophenyl 2-ferrocenylacrylate was isolated as metallic red crystals: mp 158–159 °C; IR (CHCl₃) 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 4.22 (5, s), 4.55 (4, m), 6.16 (1, d, $J = 16$ Hz), 7.37 (2, d, $J = 9$ Hz), 7.81 (1, d, $J = 16$ Hz), 8.18 (2, d, $J = 9$ Hz); CIMS $M + 1 = 378$.

***p*-Nitrophenyl Ferrocenylpropiolate (12)**. Ferrocenylacetylene was synthesized according to the procedure of Schlögl and Egger,¹⁶ converted to the propiolic acid by carbonation of the anion as described by Benkeser and Fitzgerald,¹⁷ and finally esterified with *p*-nitrophenol as described above for the ferrocenylacrylic acid to form **12**: mp 165–167 °C dec; IR (CH₂Cl₂) 2295, 1745 cm⁻¹; ¹H NMR δ 4.32 (5, s), 4.58 (2, m), 4.80 (2, m), 7.41 (2, d), 8.32 (2, d).

***p*-Nitrophenyl 2-Methoxy-5-*tert*-butylcinnamate (8)**. 5-*tert*-Butylsalicylaldehyde was prepared from 4-*tert*-butylphenol using standard Reimer-Tiemann conditions;²⁵ it was purified by silica gel chromatography, eluting with 3% ether in hexane. The aldehyde was converted to the ethyl cinnamate by the Wittig procedure described for the ferrocenylacrylate. The phenol was methylated with dimethyl sulfate in the standard manner and then the ester was saponified. The acid was converted to the acid chloride with thionyl chloride and then to the *p*-nitrophenyl ester (**8**) as with the ferrocenylacrylate: mp 113–114 °C; IR (CH₂Cl₂) 1730 cm⁻¹; ¹H NMR (CDCl₃) δ 1.25 (9, s), 3.95 (3, s), 6.76, 6.93 (2, overlapping doublets, $J = 15, 8$ Hz), 7.39, 7.45 (4 overlapping doublet, $J = 9$ Hz, and multiplet), 8.20, 8.30 (3, overlapping doublets, $J = 15, 9$ Hz); CIMS $M + 1 = 356$.

The Bisacrylate Ester (9). 4-*tert*-Butylphenol (1 equiv) was converted to 2,6-dibromo-4-*tert*-butylphenol by stirring for 1 h with 2.3 equiv of bromine and 2.75 equiv of sodium carbonate in carbon tetrachloride. The crude product was *O*-methylated in the standard manner with dimethyl sulfate and the product purified by distillation under reduced pressure. The dibromide was converted to the dicarboxaldehyde by treatment with 3 equiv of *n*-butyllithium at 0 °C for 15 min to form the dilithio derivative, which was quenched in situ with 5 equiv of *N,N*-dimethylformamide. After chromatography on silica gel, eluting with methylene chloride/hexane (1/1), the dialdehyde was isolated as a crystalline solid (45% based on the dibromo-*tert*-butylanisole). The dialdehyde was elaborated to the diethyl diacrylate using the Wittig reaction described above. This was saponified with 1 equiv of NaOH to give the monoacid, which was separated by extraction and converted to the acid chloride with phosphorus trichloride. With *p*-nitrophenol and pyridine this gave the ethyl *p*-nitrophenyl diester (**9**). This was purified by silica gel chromatography, eluting with methylene chloride, to give a white, crystalline compound: mp 160–161 °C; IR (CHCl₃) 1705, 1740 cm⁻¹; ¹H NMR δ 1.36 (12, overlapping singlet and triplet, $J = 8$ Hz), 3.28 (3, s), 4.30 (2, q, $J = 8$ Hz), 6.60–6.84 (2, t), 7.30–7.50 (2, broad doublet), 7.66 (2, broad singlet) 8.05–8.40 (4, m); CIMS $M + 1 = 454$.

***p*-Nitrophenyl 1-Adamantylpropiolate (10)**. This ester, mp 142–143 °C, was prepared in the standard way from the known¹⁴ acid and had IR (neat) 2220, 1730, 1540 cm⁻¹; ¹H NMR (CDCl₃) δ 8.21 (2, d), 7.28 (2, d), 1.97 (9, m), 1.71 (6, m).

***p*-Nitrophenyl 3-*tert*-Butyl-1-Adamantylpropiolate (11)**. The known 1-bromo-3-*tert*-butyladamantane¹⁵ was converted to the 3-*tert*-butyl-1-Adamantylpropionic acid by a similar procedure to that used¹⁴ for the compound without the *tert*-butyl group. The *p*-nitrophenyl ester, prepared in our standard way, had mp 132–133 °C; IR (neat) 2220, 1735, 1530 cm⁻¹; ¹H NMR (CDCl₃) δ 8.26 (2, d), 7.28 (2, d), 2.0 (2, m), 18.5 (6, m), 1.57 (6, m), 0.80 (9, s).

Cyclohepta(6-*N*-methylformamido)amylose (3). In a modification

of the known procedure,^{12,26} β -cyclodextrin hepta(primary)tosylate was prepared from dried β -cyclodextrin (17 g) and purified *p*-toluenesulfonyl chloride (21.7 g) in pyridine (70 mL). After 24 h at room temperature and 18 h at 5 °C, the solution was dripped into ice water with stirring, and the precipitate collected and washed with H₂O (1 L) and Et₂O (1 L). The crude product was stirred with MeOH (600 mL) at 62–65 °C for 30 min, then collected and dried to afford rather pure heptatosylate (11 g, 33% yield). Anal. Calcd for C₉₁H₁₁₂O₄₂S₇: C, 49.36; H, 5.09; S, 10.13. Found: C, 49.44; H, 5.29; S, 10.12. An additional 18% of the heptatosylate could be obtained by silica gel chromatography of the mother liquors. The pure compound on TLC had R_f 0.30 (MeOH) and 0.80 (pyridine/H₂O/1-butanol, 1:1:1).

This heptatosylate (2 g) was placed in an 80-mL sealed tube with 35 mL of 50% (v/v) methylamine in methanol. After 3 days at 70 °C, the crude product was collected and chromatographed on carboxymethylcellulose with NH₄HCO₃ solution. The product hepta(*N*-methylamino)cyclodextrin was isolated in 90% yield and characterized by NMR. The ratio of CH₃-N at δ 1.11 to anomeric protons was 21.2/7 (expected 21/7).

To a solution of the hepta-*N*-methylaminocyclodextrin (580 mg) in pyridine (100 mL) at 0 °C was slowly added formic-acetic anhydride prepared from 10 mL of acetic anhydride and 5 mL of formic acid with good stirring. After 2 h at 0 °C and 2 h at room temperature the solvent was stripped, yielding the performylated compound. The NCH₃/formyl proton ratio was 1.02/1 (expected 1:1). The formate ester groups were then removed by stirring the crude product at pH 12, adding NaOH as needed, until base consumption ceased. The product was then isolated by chromatography on Sephadex G-25 or Sephadex G-10 followed by Sephadex SP-25. The ¹H NMR showed a ratio of anomeric:formamide:methyl:others of 7:6.6:22.6:43.6 (expected 7:7:21:42). The compound showed a single spot on TLC.

Cyclohepta(6-*N*-ethylformamido)amylose (4). This was prepared as above, but using ethylamine. The hepta-*N*-ethylaminocyclodextrin had a ratio, in the ¹H NMR, of anomeric:CH₃:others of 7:20.1:56.9 (expected 7:21:56). The hepta-*N*-ethylformamido compound **4** had, in the NMR, a ratio of formamides:anomerics:all others:methyls of 6.1:7:58.5:19.3 (expected 7:7:56:21).²⁷

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(27) Support of this work by the National Institutes of Health, and an NSF Postdoctoral Fellowship to M. Czarniecki, are gratefully acknowledged.

1,*N*⁶-Etheno-Bridged Adenines and Adenosines. Alkyl Substitution, Fluorescence Properties, and Synthetic Applications

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Abstract: It has been shown that the reaction of chloroacetaldehyde with adenosine at pH 4.5 and 37 °C that produces the fluorescent ϵ -adenosine species will not develop interfering fluorescence with *N*⁶-alkyladenosines. The preferred site of methylation and benzylation of ϵ -adenosine and ϵ -adenine was established as N(9) (a) by acidic ring opening of the products to substituted aminobimidazoles in which the two etheno protons were nonequivalent; (b) by reaction of *N*⁶-substituted adenines with chloroacetaldehyde followed by polyphosphoric acid to dehydrate the intermediate to an N(9)-substituted ϵ -adenine for an unequivocal synthesis. The fluorescence of the ϵ -adenosine and ϵ -adenine species at pH 7.0 has again been confirmed, and the fluorescence properties of their N(9)-alkylated derivatives under neutral and acidic conditions have been determined. It has been shown possible, earlier reports to the contrary, to prepare *N*⁶-substituted adenosines through Schiff-base formation on the 6-NH₂. The general method involves the use of sodium cyanohydridoborate to bring about reductive amination of aldehydes and ketones at acidic pH and is exemplified by the synthesis of *N*⁶-ethyladenosine, *N*⁶-benzyladenosine, and *N*⁶-furfuryl-adenosine (kinetin riboside), using large excesses of aldehyde and reducing agent.

Current interest in the tertiary structure^{1–7} and solution properties of tRNA has included the development of reagents capable of causing specific modification at the exposed sites.² Chloroacetaldehyde, an important reagent of this class, has been used to modify adenine and cytosine residues^{8,9} at the nucleoside and nucleotide levels^{10–12} and in tRNA.^{13,14} More recently, it has been shown that at pH ~6.4 there is a slow reaction of chloroacetaldehyde with guanosine.¹⁵ In all these reactions an etheno bridge is introduced between the exocyclic amino group of the base and a ring nitrogen to form the corresponding etheno (or ϵ) compounds.¹⁶ Since methylated and isopentenylated derivatives of adenosine are found in various tRNAs,^{17,18} it is desirable to learn which of these react with chloroacetaldehyde and which furnish fluorescent products as in the case of adenosine.

Among the methyladenosines found in tRNA, 1-methyladenosine (m¹Ado)¹⁹ would not be expected to and did not produce an etheno-bridged product,¹⁰ and *N*⁶,*N*⁶-dimethyladenosine (m₂⁶Ado),²⁰ a questionable tRNA component, also would not be able to form a bridged compound. The reactions of chloroacetaldehyde with the remaining methyl components *N*⁶-methyladenosine (m⁶Ado) (**1a**)^{21,22} and 2-methyladenosine (m²Ado) (**2a**)^{20,21,23} (as 2-methyladenine (**2c**)) were examined. We had observed earlier that *N*⁶-(Δ^2 -isopentenyl)adenosine (**1b**) reacted quantitatively with chloroacetaldehyde to yield 7,8-dihydro-8-hydroxy-9-(Δ^2 -isopentenyl)3- β -D-ribofuranosylimidazo[2,1-*i*]purinium chloride (**3b**).⁹ Although the position of the hydroxyl group was initially not fully confirmed, the X-ray determination of the structure of the reaction product of α -chloro-*n*-butyraldehyde and adenosine²⁴ left no doubt that the direction of attachment of the aldehydic carbon was to the exocyclic nitrogen. *N*⁶-Methyladenosine (**1a**) reacted with chloroacetaldehyde to give the compound analogous to **3b**, namely, 7,8-dihydro-8-hydroxy-9-methyl-3- β -D-ribofuranosylimidazo[2,1-*i*]purinium chloride (**3a**). Similarly, *N*⁶-methyladenine (**1c**) gave the corresponding compound **3c**. None of the "hydrated" forms

(**3a–c**) obtained from chloroacetaldehyde and members of the *N*⁶-substituted series was appreciably fluorescent. Accordingly, *N*⁶-substituted adenosines will not interfere with the reaction of chloroacetaldehyde with adenosine at pH 4.5 and 37 °C that develops fluorescence from the neutral ϵ -adenosine species (**4a**).²⁵

We hoped to relate the site of alkylation of ϵ -adenosine (**4a**) to the *N*⁶-substituted compounds (**3**) in a manner parallel to our assignment of structure of the alkylated ϵ -cytidines.²⁶ Alkylation of **4a** could conceivably take place at several of the nitrogens in ϵ -adenosine, although N(9) (see **3** for numbering system) as the nucleophilic center for attack on an alkyl halide would permit the most favorable delocalization of positive charge in the transition state. ϵ -Adenosine (**4a**) was converted efficiently to its monomethyl or monobenzyl derivative by alkylation with methyl iodide or benzyl bromide, respectively, in dimethylacetamide and metathesis to the corresponding chloride. The elemental analyses and mass spectra of the major product in each case were indicative of the introduction of one such group, as were the NMR spectra, i.e., a singlet at δ 4.38 ppm for the methyl compound or a singlet at δ 5.97 and a multiplet at δ 7.3–7.5 for the benzyl compound. Compounds of the imidazo[2,1-*i*]purine type (as in postulated **5**) are known to undergo ring opening to aminobimidazoles readily on treatment with 2 M HCl at 100 °C.²⁷ Only an N(9)-substituted tricyclic compound (**5**) would generate a biimidazole (**6**) in which the two etheno protons would be nonequivalent and thus should exhibit a pair of AB doublets in the NMR spectrum. When the major product of methylation of ϵ -adenosine (**4a**) was hydrolyzed with HCl, the initial NMR finding was that the methyl-substituted aminobimidazole in DCl or D₂O showed only a two-proton singlet (100 MHz) at δ 7.62 or 7.45, respectively. However, when the solvent was changed to CDCl₃ for the free base, a pair of etheno doublets was observed, δ 6.86 and 7.08, $J = 1.8$ Hz, demanding the N-substitution shown in **6a**. Benzylation of ϵ -adenosine followed by HCl hydrolysis produced a benzyl-substituted aminobimidazole for which