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Optically Active Amino Acid Synthesis by Artificial Transaminase Enzymes

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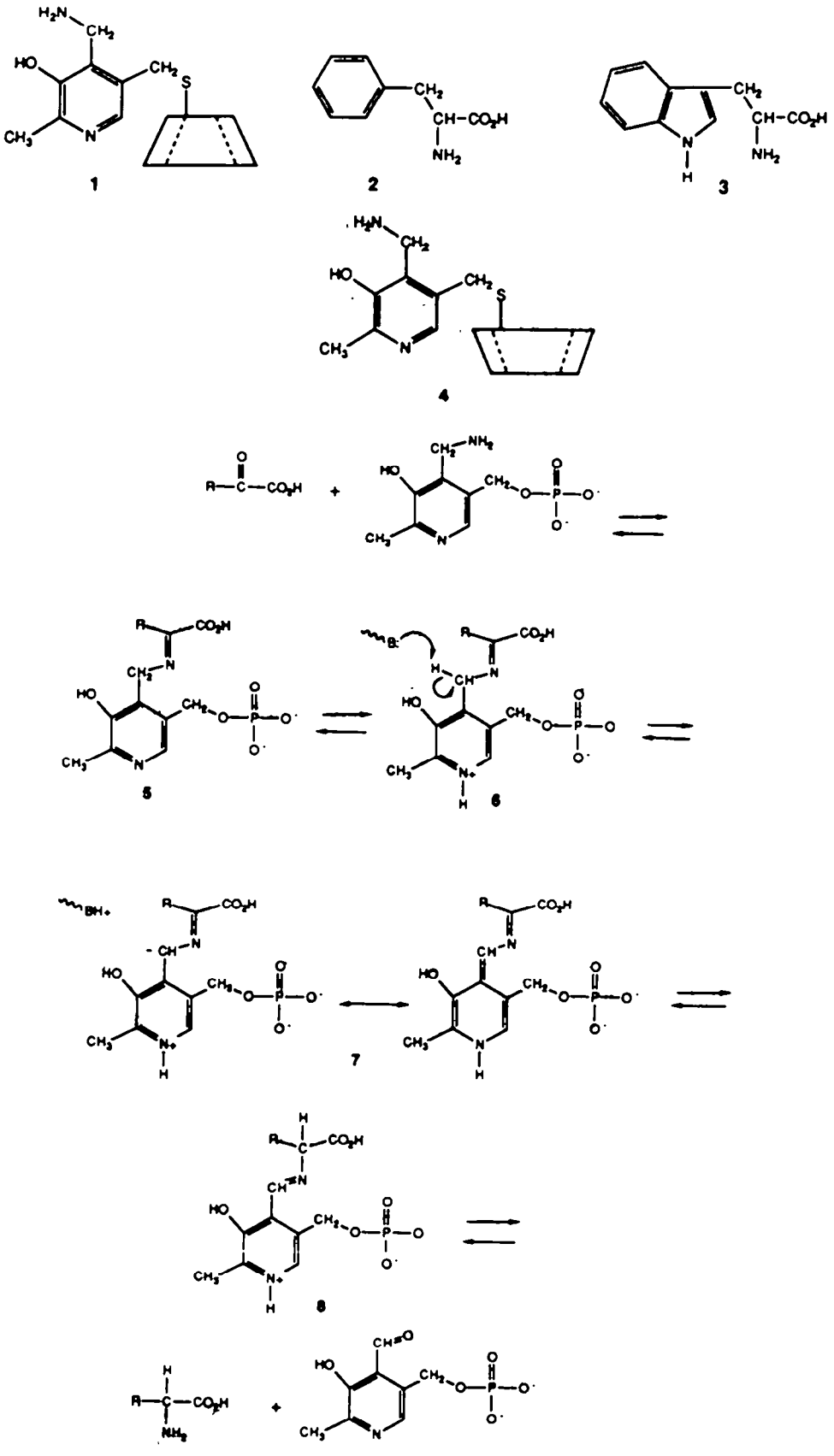
Abstract: The conversion of α-ketoacids to α-aminoacids is performed in some enzyme models by transamination, using pyridoxamine derivatives. When the pyridoxamine is attached to β-cyclodextrin the simple enzyme mimic shows substrate selectivity and also the induction of optical activity in the products. When base catalytic groups are attached to pyridoxamine the proton transfers are catalyzed by the basic groups; if they are mounted chirally on a rigid framework they also induce optical activity in the products. When these lines are combined, artificial enzymes are produced that bind a substrate selectively and then perform a chiral proton transfer to produce optically active amino acids with e.e.'s ranging from 74% to 96%.

Two types of combination have been examined. In one of them the optical induction is only fairly good, but in the other the selectivity is excellent. The key seems to be the use of a particular bifunctional basic catalytic group, an ethylenediamine. This group is the best yet examined at catalyzing proton transfers in transamination reactions. Its use in a chiral artificial enzyme system leads to the observed high enantioselectivity.

Introduction. In Nature optically active amino acids are produced by transamination (Scheme 1).[1] An α-ketoacid reacts with pyridoxamine phosphate under the influence of a transaminase enzyme to form the α-amino acid and pyridoxal phosphate (or an enzymatic derivative). Then in a second step a second amino acid is sacrificed, reconverting the pyridoxal phosphate to pyridoxamine phosphate while forming a new α-ketoacid. The optical activity in the product is generally understood to reflect the asymmetric environment of the enzyme.

Such stereoselectivity in an enzymatic reaction is only one of four types of selectivity commonly exhibited by enzymatic processes.[2] They generally show substrate selectivity, reaction selectivity, regioselectivity, and stereoselectivity. All of these are of interest in transamination reactions, including reactions catalyzed by enzyme mimics. We have examined all of them, but here we will focus on stereoselectivity. Specifically, we will describe the current state of our efforts to produce optically active amino acids by using artificial transaminase enzymes.

When we attached β-cyclodextrin to pyridoxamine we produced a compound 1 that showed substrate selectivity.[3,4] That is, it selectively converted α-ketoacids such as phenylpyruvic acid or indolepyruvic acid to phenylalanine 2 or tryptophan 3 because the aromatic ring binds into the cyclodextrin cavity. The reaction for these α-ketoacids is faster with catalyst 1 than it is with simple pyridoxamine, because the binding accelerates the reaction. By contrast, the rate of transamination of pyruvic acid to alanine is essentially unaffected by the cyclodextrin ring. Most interestingly, the phenylalanine or tryptophan produced by 1 was optically active. Transamination in the chiral cyclodextrin cavity leads to optical induction in the product, although the optical ratio was only 5:1 for phenylalanine(2) and 2:1 for tryptophan (3), both preferring the L isomer.



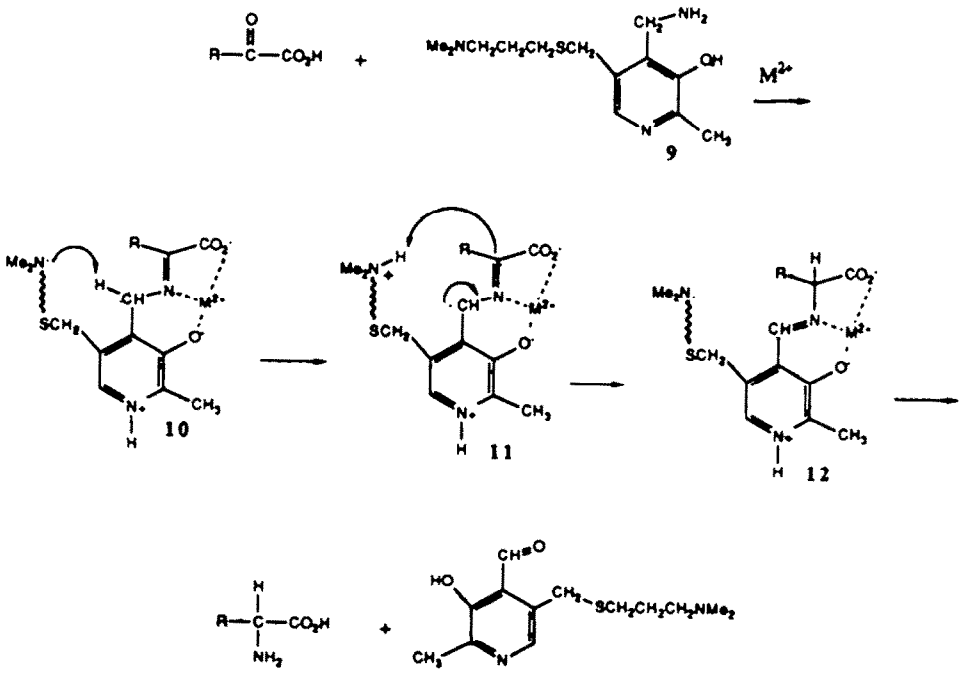
Scheme 1: Enzymatic Transamination

Such accidental production of optical activity, because of the chirality of the cyclodextrin cavity, is not very easily controlled. For instance, with a related compound 4 in which the pyridoxamine is attached on the secondary face of β-cyclodextrin the optical results were quite different.[4,5] This compound showed no optical preference in the formation of phenylalanine, and a 1.8:1 preference for the formation of D-tryptophan. Thus we set out to produce the optical activity by a process related to that used enzymatically: put the proton on the new optical center in the amino acid by transfer from a base group held on the correct face of the reaction intermediate.

In transamination (Scheme 1) the α–ketoacid forms a Schiff base 5 with pyridoxamine phosphate, and the pyridinium form of this 6 is then deprotonated on its alpha carbon by an enzymatic base group to form an intermediate 7 that has both a zwitterionic and a neutral resonance form. This intermediate is then protonated on its gamma carbon to form the new Schiff base 8 that hydrolyzes to form the product amino acid and pyridoxal phosphate. [In the enzymatic reaction, this "hydrolysis" is normally an aminolysis by the lysine ε-NH_2 group of the enzyme. Thus the product is not pyridoxal phosphate, but its Schiff base with the enzyme.] Pyridoxamine phosphate is regenerated by running this process backwards with a second amino acid.

We needed to learn whether we could catalyze the proton transfer from the alpha carbon to the gamma carbon that is part of this sequence, and that generates the new asymmetric center in the amino acid. If so, then a chirally mounted basic catalyst for such proton transfers could produce an optically active product.

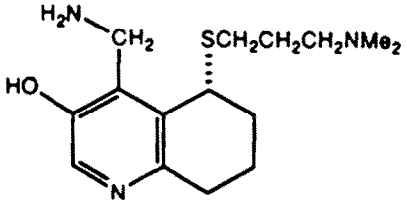
Our first work[4,6] simply established that a basic sidechain attached to pyridoxamine, as in 9, would catalyze the amination of α–ketoacids non-enzymatically (Scheme 2). From the dependence of activity on structure, it was clear that the basic group was catalyzing both parts of the process.[7] That is, the base not only removes a proton from the alpha carbon but it also puts the proton onto the gamma carbon. This is not required by any general principles; it would have been possible that the base only removed the proton in 10 to generate intermediate 11, and the protonation at the gamma carbon to produce intermediate 12 with a new asymmetric center could have been performed by some species in the solvent. However, our studies showed that this was not the case.



Scheme 2

With this mechanistic evidence it was possible to turn our attention to the design of catalysts that could use the geometry of a basic sidechain to induce optical activity in the amino acids, resulting from transamination by a pyridoxamine analog. A simple asymmetric center in a flexible sidechain was not very effective,[4,8] so we decided to fix the sidechain with rigid links.

As we have described,[4,9] a cyclohexanopyridoxamine 13 was synthesized and optically resolved. This molecule gave quite good optical induction in the amino acids produced from α-ketoacids, ca. 20/1 preference. When a non-catalytic chain was fixed in the same spot, in 14, it induced only a 1.5/1 ratio of optically active amino acids, and with the reverse preference. That is, in the same optically resolved series a chain that only blocks one face of the complex in 14 induces a weak tendency for the solvent to deliver proton from the unblocked face, but in the same position a catalytic chain in 13 has a strong preference to deliver a proton selectively. The good selectivity was seen even though the catalytic group used was not our most effective one, as we will discuss below.



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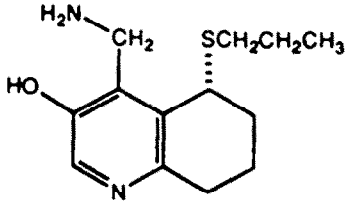

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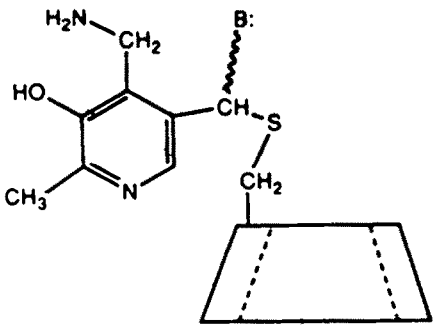
Artificial transaminases carrying binding and catalytic groups. Some years ago we set out to prepare some transaminase mimics with a cyclodextrin unit, a pyridoxamine unit, and a chirally mounted proton transfer group.[10] Our approach was to combine in a single molecule the same types of structures that had been successful in our previous work. We wanted to attach the β-cyclodextrin by a thioether link to the methylene substituent at C-5 in pyridoxamine as in compound 1, and we also wanted to attach a basic sidechain to this same methylene group as in compound 9. Thus the general structure desired was 15.

We thought that such a system would show both substrate specificity and stereospecificity. Furthermore, the binding of a substrate phenyl or indole ring into the cyclodextrin cavity would define the geometry of the system. Such binding produces a large ring (e.g. 16), so that with a single enantiomer of the catalyst the sidechain basic group can reach only one face of the transamination intermediate. Thus it should not be necessary to use a metal ion to complex with the intermediate and define its geometry, as had been needed with our previous catalysts that lack a cyclodextrin binding site. This is of special interest, since transaminase enzymes themselves do not require metal ions as cofactors.

The synthetic challenge was to protect pyridoxamine so that the needed operations could be conducted on the C-5 methylene carbon. After some experimentation we found that iminoether formation did the job. Reaction of pyridoxamine 17 with trimethyl orthobenzoate formed the iminoether 18, and this was oxidized to the aldehyde 19. Then reaction with the Grignard reagent derived from N,N-dimethyl-3-chloropropylamine afforded the alcohol 20, with a basic sidechain attached. This was then converted to the corresponding thiol 21 by Mitsunobu reaction with thiolacetic acid and hydrolysis of the resulting thioester.

At this point we did some studies with the racemic material, although we also resolved the thiol into its enantiomers. Reaction of the thiol 21 with β-cyclodextrin-6-iodide 22 and deprotection afforded the cyclodextrin thioether 23. In comparison with our simple cyclodextrin/pyridoxamine compound 1, the new compound 23 transaminated pyruvic acid 15-17 times faster in our u.v. kinetic assay. However, this acceleration was not as great as we had hoped for from our studies with pyridoxamines carrying basic catalytic groups. The modest base catalysis, suggesting that catalyzed proton transfer does not completely dominate undirected proton transfers from solution, may be responsible for the limited optical specificity eventually seen in this series.



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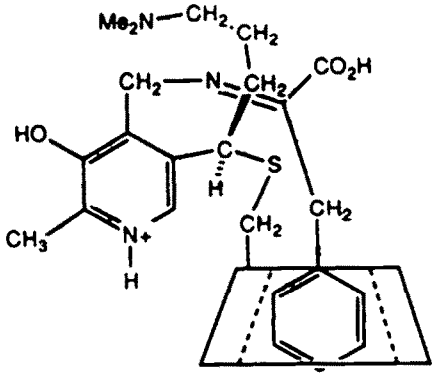

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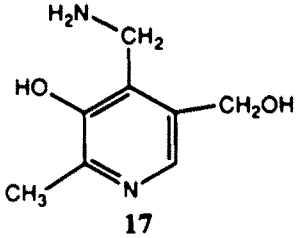

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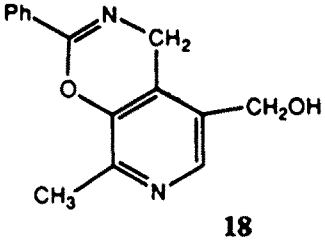

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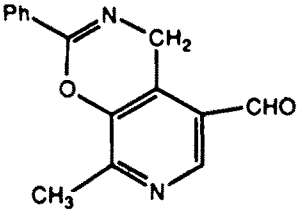

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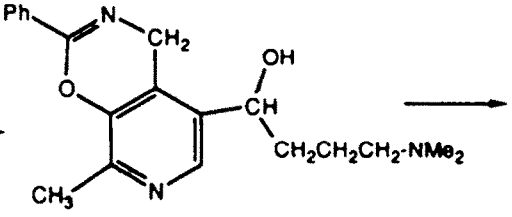

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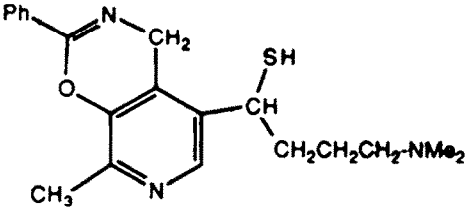

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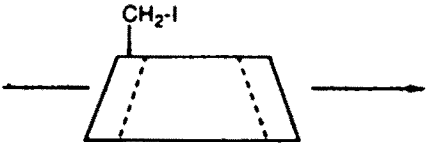

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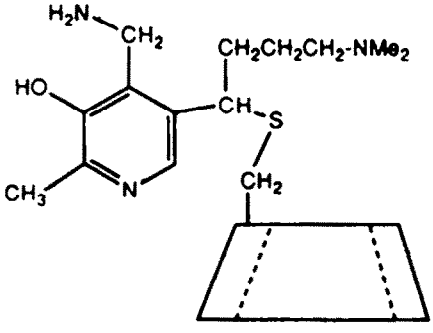

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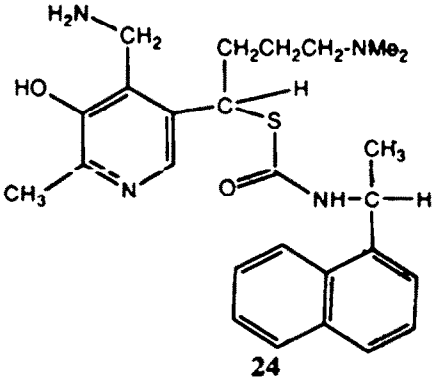

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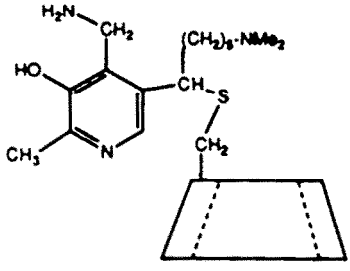
The best optical resolution of the thiol 21 was achieved by reaction with enantiomerically pure (+) 1-(α–naphthyl)ethyl isocyanate, to form the thiourethane 24. Chromatography was used to separate the two diastereomers. The isomer with the higher Rf was converted back to the thiol and allowed to react with β–cyclodextrin-6-iodide to afford a single diastereomer of 23 after deprotection. This was then examined in transamination under a variety of conditions. The highest optical ratio (L/D) in the product amino acid was 6.8, for the synthesis of phenylalanine by transamination of phenylpyruvic acid. Although this is respectable, it is not an exciting selectivity.

Molecular models suggested that the chain in 23 might be a little short, so directed proton transfer does not compete well with undirected protonation from solution. To overcome this we prepared compound 25, with two more methylene groups in the sidechain. The synthesis followed the same path as was used for the shorter 23, except that the Grignard reagent was prepared by lithium reduction of N,N-dimethylaminopentyl phenyl thioether, then addition of $MgBr_2$. With this more flexible compound the optical selectivity in transamination was even poorer. With phenylpyruvic acid under various conditions the maximum optical ratio (L/D) in the product phenylalanine was 2.8. As will be discussed below, it seems likely that the problem here is poor proton transfer catalysis by a simple dimethylamino group on a flexible chain.



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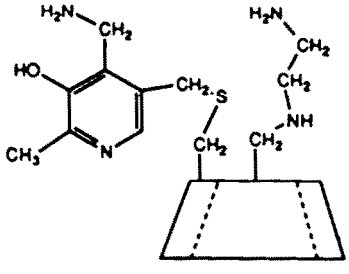

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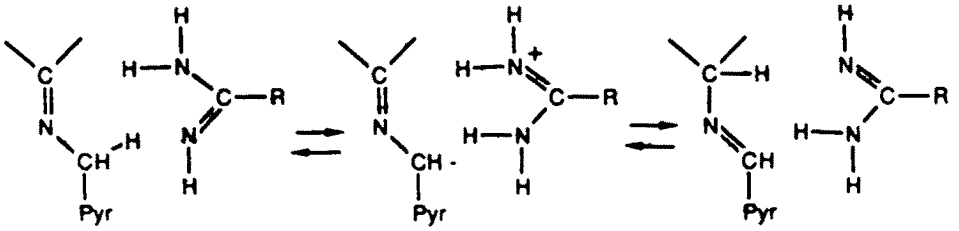
Our early work and proposal of this series of catalysts had stimulated Tabushi to think about another approach to the problem. He had been interested in cyclodextrins carrying two substituents, and had developed methods for the selective attachment of groups to two different glucose residues of β-cyclodextrin.[11] Thus when we proposed the synthesis of our series of compounds, with a basic catalyst and a cyclodextrin attached to pyridoxamine through the C-5 methylene group,[10] he decided to try another approach.[12] Tabushi reacted the 6A,6B-cyclodextrin diiodide with one equivalent of our pyridoxaminethiol, and then with ethylenediamine. The resulting compound 26 had a cyclodextrin ring carrying both a pyridoxamine and a basic chain, as a mixture of A,B and B,A isomers.

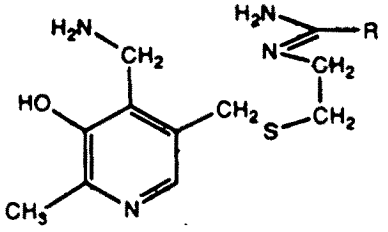
These isomers are actually diastereomers, combining the chirality of the glucose residues with the chirality of a clockwise or anticlockwise arrangement of groups around the cyclodextrin rim. Thus they could be separated by careful chromatography. Remarkably, the separated isomers were outstanding at the production of optically active amino acids. Tabushi reports optical ratios of 98/2 for the production of phenylalanine by one of these isomers, of 95/5 for tryptophan, and of 98/2 for phenylglycine.[12] Furthermore, the rate of amino acid production was observed to be ca. 2000 fold faster than with simple pyridoxamine under the same conditions, an acceleration ascribable to both the cyclodextrin binding and the base catalysis.

This catalyst was used in the absence of metal ions, and in the presence of high concentrations of phosphate buffer. The metal ions are normally needed to accelerate both the original Schiff base formation and the subsequent steps, and to fix the geometry of the intermediates so that proton transfer on a "single face" translates into enantioselectivity. However, Schiff base formation with such a catalyst as 23, 25, or 26 is promoted by cyclodextrin binding, and the net effect is larger without a competing metal ion binding. Also the geometry is fixed by cyclodextrin binding, as we have pointed out above, so the metal ion is not needed for geometric control. Thus the acceleration is quite large when compared with pyridoxamine that is also used without metal ions.

The need for high concentrations of phosphate buffer is curious. In our previous work on catalytic sidechains[4,6] such concentrated buffers were not used, since they would have competed with proton

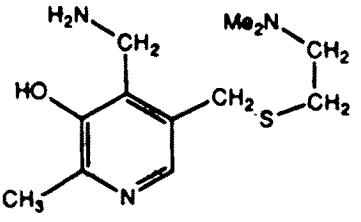


Scheme 3



27 R = CH_3

29 R = CF3



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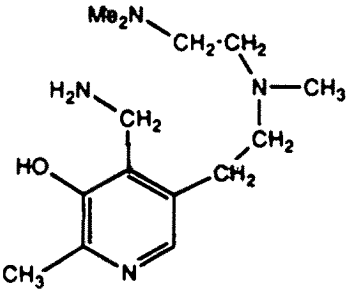

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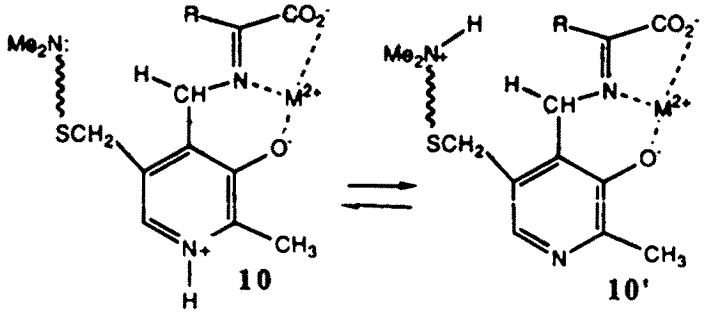


10 **10'**

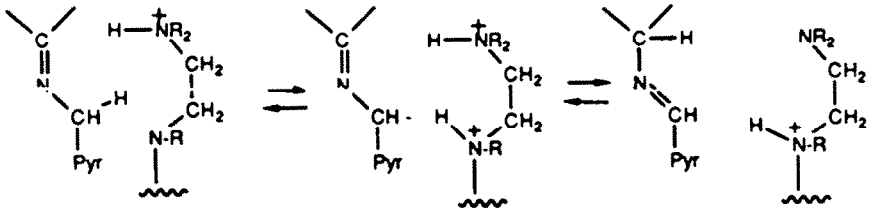
transfer by the catalytic sidearm itself. Of course we had studied only the proton transfer step, by spectroscopic observation of the intermediate Schiff bases. In the overall reaction being studied here there is a condensation step, to form the Schiff base, as well as a proton transfer step to isomerize it and then a hydrolysis of the isomeric Schiff base to liberate the amino acid. Perhaps the phosphate buffer promotes these other steps. We had used this buffer in our studies[3] of simple pyridoxamine-cyclodextrin 1, which is compound 26 without the ethylenediamine group. In 1 the buffer probably catalyzed all the steps, including proton transfers to isomerize the Schiff base intermediates. Our studies on ethylenediamine catalysis, described below, suggest that with this catalytic group the proton transfers are best performed by the amino groups, not by a phosphate ion.

The most striking finding is the very large effectiveness of the Tabushi catalyst 26, in contrast to the catalysts 23 and 25 that we described above. Our recent studies indicate that most of the difference reflects the different way in which the catalytic sidechain is mounted. In principal it could also have involved the special properties of ethylenediamine units as proton transfer catalysts.

Catalytic sidechains; the special effectiveness of ethylenediamine units. In the isomerization of a ketimine intermediate 10 to an aldimine intermediate 12 a base must remove the alpha proton and deliver it to the gamma carbon, producing the new asymmetric center in the product amino acid. We were interested in developing a base catalyst that could perform this 1,3 proton transfer particularly well. At first sight an amidine base might seem optimal, since it could remove a proton with one nitrogen and then deliver another proton from the other nitrogen without moving (Scheme 3). Thus an amidine could be rigidly fixed in space and still perform a 1,3 proton transfer, with such rigid fixation leading to fast rates. However, we found that amidines were not effective catalysts.

When we prepared compound 27, with a simple amidine on a sidearm, it was considerably slower in the isomerization of one pyruvic acid Schiff base (10) to the other (12) than was the corresponding system 28 with a simple dimethylamino group. In this case the problem is probably that amidines are too basic. For transamination the intermediate must be 10, with a proton on the pyridine nitrogen while the basic catalyst group is unprotonated. As is usual in such a competition between equilibrium and reactivity, the equilibrium problem wins out. A strong base group in the sidearm switches the equilibrium away from 10 and toward 10'. The presumed greater rate for the amidine base group in isomer 10 does not make up for the fact that the equilibrium between 10 and 10' is so unfavorable (i.e., the Brønsted coefficient for reaction of 10 is less than 1.0). This relationship, that weaker bases should be better catalysts in our system, is probably part of the reason that we had found imidazole groups to be even more effective than dimethylamino groups in 1,3 proton transfers.[4,6] When we prepared amidines that were more weakly basic, such as 29, the amidine system was hydrolytically unstable under our conditions.

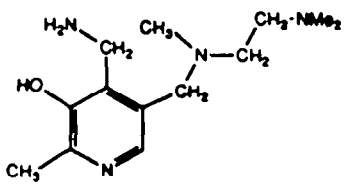
We have found that ethylenediamine systems are remarkably good catalysts. In compound 30, under our standard spectroscopic assay for catalyzed Schiff base isomerization of 10 to 12 with pyruvic acid, the ethylenediamine group led to an acceleration of 110 fold compared to a simple alkyl sidechain, the largest acceleration we had seen. This is because the diamine is acting as a bifunctional catalyst, with one amino group removing the proton from the alpha carbon while the other puts a proton on the gamma carbon (Scheme 4). Another possible explanation has to do with basicities, but we were able to exclude it.



Scheme 4

In ethylenediamine the two amino groups have pK_a's of 7.0 and 10.0, because of electrostatics. After the first proton is added the resulting positive charge decreases the basicity of the second amino

group by ca. 3 units. As we have pointed out above, such decreased basicity should be catalytically useful because it increases the fraction of intermediate in the correct protonation state, but this is not the reason for the greater catalytic effectiveness of the ethyenediamine unit in 30. In compound 31 the ethylenediamine unit is attached too close to the pyridoxamine ring, so both nitrogens cannot perform proton transfers. This compound shows the same rate of isomerization of the intermediate as does compound 28, with the innermost nitrogen replaced by sulfur. Thus in 31, in which only an electrostatic effect of the second nitrogen is possible, no remarkable acceleration occurs. The acceleration in 30, where both nitrogens can actively play a role in proton transfers to the Schiff base system, must be due to this bifunctional catalysis shown in Scheme 4.



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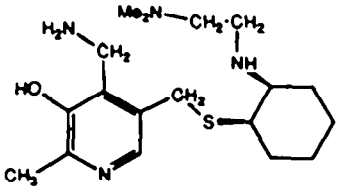

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An even larger effect is seen in compound 32. Here the rate acceleration by the basic sidearm is 200 fold compared with a simple pyridoxamine. In both of these ethylenediamine cases 30 and 32 it thus seems likely that bifunctional catalysis occurs (Scheme 4). The ethylenediamine system starts off singly protonated, and in the Schiff base intermediate the pyridine nitrogen has a proton as well. Then the nearer amino group of the catalytic sidearm removes the proton from the alpha carbon of the ketimine, and the remote ammonium group puts a proton onto the gamma carbon of the intermediate. Molecular models show that this mechanism is possible for both 30 and 32, but not for 31 in which only a slow rate was seen.

Future prospects. Obviously the incorporation of such a strongly catalytic group into an asymmetric system, or improved versions of it under study, should lead to catalysts with great optical selectivity. The goal is to develop biomimetic catalysts for the production of amino acids with high rates and turnovers, and with high optical selectivities. With the progress that has been made this goal seems in sight.

Experimental Section

1. Synthesis of Compounds:

Iminoether 18 was prepared by heating 1.33 g of pyridoxamine with 1.63 ml. of trimethyl orthobenzoate in 15 mL dry DMF at reflux under argon for 2 hours. The product precipitated on cooling as yellow needles in 32% yield. It had the expected NMR spectrum, and a CI MS peak at 255 (M + 1).

Aldehyde 19 was prepared by reaction of 640 mg. of 18 with 2.84 g. of barium manganate in CH_2Cl_2 suspension with stirring for 6 hours at room temperature. Workup and chromatography afforded 19 in 68% yield as a white solid.

Dimethylamino carbinol 20 was prepared by first reacting 1.2 g. of 3-dimethylaminopropyl chloride in 1 ml. dry THF with 0.26 g. of Mg in 1 ml. of dry THF after activating the Mg with 10 microliters of ethylene dibromide. Then the Grignard solution was transferred by cannula into a solution of 0.28 g. of aldehyde 19 in 5 ml. THF. After 2 hours, workup and chromatography afforded 20 as a yellow oil in 85% yield. It showed the expected NMR signals, and a CI MS peak at 340 (M + 1).

Thiol 21 was prepared as its S-acetate ester, and liberated when needed. An adduct was formed between triphenylphosphine (90 mg., 0.36 mmol.) and diisopropyl azodicarboxylate (70.8 microliters, 0.36 mmol.) formed in 2 ml. of dry THF at O °C over 2 hours. Then thiolacetic acid (25.7 microliters,

0.36 mmol.) was added and a solution of 60 mg. of 20 in 2 ml. THF. After 2 hours workup and chromatography afforded S-acetyl21 as a yellow oil in 81% yield. It had the expected NMR spectrum, and a CI MS peak at 398 (M + 1).

The racemic catalyst 23 was prepared by deacetylation of 68 mg. of the above thioacetate in 5 ml. dry DMF with 85 mg. K_2CO_3 and 60 microliters of methanol for 15 min. at room temperature. Then 311 mg. of β-cyclodextrin-6-iodide was added, and the solution was heated at 60° for 2 hours under argon. Solvent was evaporated, and the product was isolated by Sephadex G-15 chromatography, then ion exchange CM-25 chromatography with 0.5 M NH_4CO_3. The resulting iminoether derivative of 23 was obtained in 19% yield as a white fluffy powder. The MS (FAB/ glycerol) peak came at 1471 (Calc. 1472). This was then deprotected to afford 23 itself by heating 62 mg. of the iminoether with 187 mg. of o-phenylenediamine dihydrochloride in 12 ml. ethylene glycol at 60° for 3 hours. Chromatography as above afforded 23 in 47% yield as a white solid, with the expected NMR spectrum and MS (FAB/ glycerol) peak at 1387 (Calc. 1386).

The thiol 21 was optically resolved by deacetylation of S-acetyl 21 with K_2CO_3 in methanol, quenching with acetic acid, and reaction of the thiol 21 with (+)-α-(1-naphthyl)ethyl isocyanate and DABCO in benzene. The resulting mixture of thiocarbamates 24 was separated by repeated preparative TLC on silica with 5% MeOH, 4% MeOH saturated with NH_3, and 91% CH_2Cl_2 into a high R_f and a low R_f isomer of 24. Optical purity was checked by reaction of each thiol with (-)-camphanic acid chloride after liberating the thiol from the thiourethane 24 with 1 ml. concentrated NH_4OH and 1 ml. MeOH for 10 minutes at room temperature. The camphanate ester from the high R_f isomer of 24 had a three proton NMR peak at δ 0.98, while that from the low R_f isomer had a corresponding signal at δ 0.82. No contamination could be seen, and the addition of 2.3% by weight of the low R_f product to the high R_f product gave a clear signal at δ 0.82. Thus the optical contamination in the separated isomers of 24 is less than 2%. These resolved thiols were converted to the two separate diastereomers of 23 by the procedure described above.

REFERENCES

1. For a review, cf. A.E. Martell and Y. Matsushima, "Pyridoxal Catalysis: Enzymes and Model systems"; Interscience: New York, 1963.

2. R. Breslow, "Artificial Enzymes and Enzyme Models," Advances in Enzymology and Related Areas of Molecular Biology, Alton Meister, Ed., John Wiley & Sons, Inc., 1986, 58, 1-60.

3. R. Breslow, M. Hammond, and M. Lauer, *J. Am. Chem. Soc.*, 1980, **102**, 421.

4. R. Breslow, A.W. Czarnik, M. Lauer, R. Leppkes, J. Winkler, and S. Zimmerman, *J. Am. Chem. Soc.*, 1986, **108**, 1969.

5. R. Breslow and A.W. Czarnik, *J. Am. Chem. Soc.*, 1983, **105**, 1390.

6. S.C. Zimmerman, A.W. Czarnik, and R. Breslow, *J. Am. Chem. Soc.*, 1983, **105**, 1694.

7. Cf. also W. Weiner, J. Winkler, S. C. Zimmerman, A. W. Czarnik, and R. Breslow, *J. Am. Chem. Soc.*, **107**, 4093 (1985).

8. S. Zimmerman, Ph.D. thesis, Columbia University,1983.

9. S.C. Zimmerman and R. Breslow, *J. Am. Chem. Soc.*, 1984, **106**, 1490.

10. R. Breslow, "Approaches to Artificial Enzymes," in Biomimetic Chemistry, Z.-I. Yoshida and N. Ise, eds., Kodansha Ltd. Publishing Co., Tokyo, Japan, 1983, pp. 1-20; see also D. Foley, Ph.D. thesis, Columbia University, 1982.

11. Cf. I. Tabushi, *Acc. Chem. Res.*, 1982, **15**, 66; see also R. Breslow, P. Bovy, and C. Lipsey Hersh, *J. Am. Chem. Soc.*, 1980, **102**, 2115.

12. I. Tabushi, Y. Kuroda, M. Yamada, H. Higashimura, and R. Breslow, *J. Am. Chem. Soc.*, 1985, **107**, 5545.