Tetrahedron Vol. 44, No. 8, pp. 2137 to 2148, 1988
Printed in Great Britain.

0040-4020/88 $3.00+.00

A Model for the Enzyme-Catalyzed, Coenzyme B12-Dependent Interconversion of β-Methylaspartate with Glutamate

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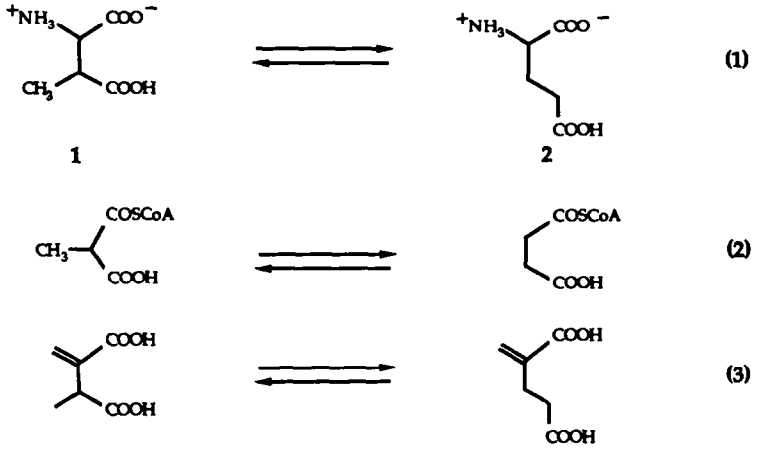
(*Received in USA 9 November* 1987)

Abstract. *A novel rearrangement promoted by vitamin B_{12s} of diethyl 1-keto-2-bromomethyl-2-methylsuccinate* **10** *to diethyl 4-methyl-2-ketoglutarate* **11** *has been discovered. This can serve as a partial model for the coenzyme B_{12} dependent enzyme catalyzed interconversion of β-methylaspartate and glutamate. The Schiff base corresponding to* **10** *does not react with vitamin B_{12s}, but it does rearrange readily under free radical conditions when treated with tri-n-butyltin hydride.*

Introduction. H. A. Barker observed that the bacterium *Clostridium tetanomorphum* uses glutamic acid as a source of energy.[1] The energy-yielding sequence of events begins with the conversion of glutamic acid to β-methylaspartic acid (eq. 1) - an extraordinary rearrangement.[2] A signal discovery; that coenzyme B_{12}, the biologically active form of vitamin B_{12} with 5'-deoxyadenosine attached to cobalt, is a critical cofactor in the rearrangement;[3] was made in the course of this research. As a consequence of this work, coenzyme B_{12} is now recognized to be an essential cofactor for at least twelve enzyme-catalyzed rearrangement reactions.[4]

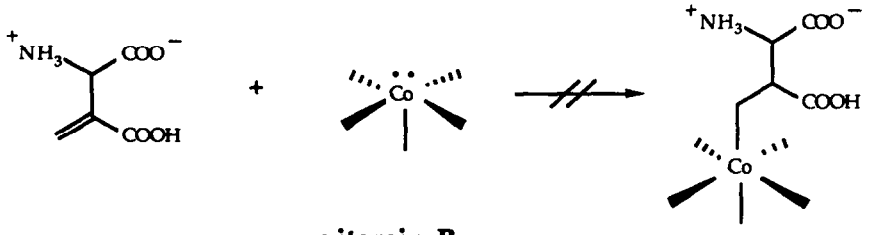
Of these, there are three (eq. 1-3), possibly four,[5] reactions which involve a carbon-skeleton rearrangement. The β-methylaspartic acid (1) ⟷ glutamic acid (2) rearrangement (eq. 1) stands out, even



in the latter class, because it is the only one of the three in which the migrating group, the glycine part of the molecule, is a saturated carbon. The other two carbon-skeleton rearrangements are the methyl-

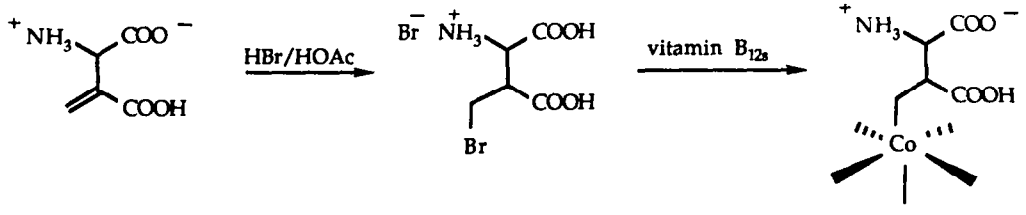
malonyl-CoA to succinyl-CoA[6] (eq. 2) and the methylitaconate to α-methyleneglutarate[7] (eq. 3) transformations. In both instances, the migrating group is unsaturated and one can formulate a reactive intermediate of the cyclopropylcarbinyl or cyclopropyloxy type for these reactions. It has been possible to construct partial models for the latter two rearrangements by attaching the branched substrate, methylitaconate[8] or methylmalonate[9], to the cobalt atom of vitamin B_{12}. The model experiments opened the door to an interesting series of reactions and yielded insight into the rearrangement process. For this reason, we were anxious to complete the series by constructing a model for the β-methylaspartate rearrangement (eq 1) in order to learn how this rearrangement takes place and whether it can be treated in the same terms.

Results. To construct the model with methylaspartic acid (1) attached to cobalt through its methyl group, we attempted to add the nucleophilic vitamin B_{12s} to β-methyleneaspartic acid (3). This series of



3 5

experiments was not successful, because, in contrast to the parent acrylate, the α-substituted acrylate 3 is not a good Michael acceptor. We then made use of a device used earlier[8] to surmount a similar problem in which the Michael addition approach was replaced by an S_N2 reaction. Accordingly, β-methyleneaspartic acid (3) was treated with HBr in acetic acid forming β-bromomethylaspartic

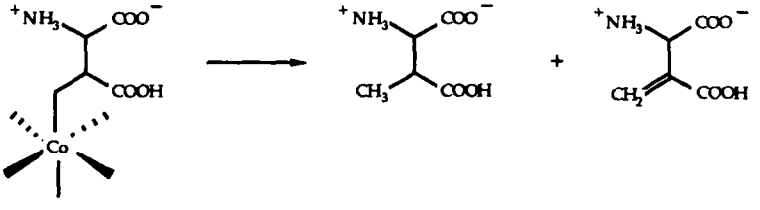


3 4 5

acid hydrobromide (4). Although 4 is rather unstable with respect to the loss of hydrogen bromide, if 4 is used immediately, it reacts readily with vitamin B_{12s} to yield the desired adduct 5.[10]

The new B_{12} adduct 5 exhibits an ultraviolet-visible spectrum in excellent accord with other carbon-cobalt bonded adducts[8,9], and 5 is converted to hydroxocobalamin, following cleavage of the carbon cobalt bond, upon exposure to light. The adduct 5 has been further characterized by its FAB mass spectrum which includes an exact mass determination in good agreement with expectation (see the Experimental Part).

Confident of the structure of the adduct 5, we began to explore its chemical reactivity as a potential model for the β-methylaspartic acid (1) ⟵ glutamic acid (2) rearrangement. The adduct 5 was heated for

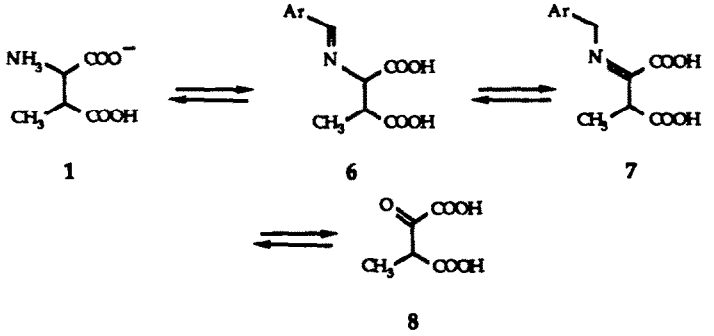


5 1 3

extended periods until the carbon-cobalt bond was no longer evident in the ultraviolet-visible spectrum, or it was photolyzed with and without sodium cyanide under the same boundary condition. β-Methylaspartic acid (1) and β-methyleneaspartic acid (3) were isolated, but glutamic acid (2) could not be detected. However, it has recently been observed[11] that our model B_{12} adduct 5, slightly modified by esterfication of the B_{12} carboxamide groups, upon photolysis in the presence of detergent rearranges to methylaspartate and glutamate. This demonstrates again[8,9] the utility of the carbon-cobalt bond approach to modeling the carbon skeleton rearrangements dependent on coenzyme B_{12}.

In the meantime, the question persists regarding ways to make this a viable reaction under thermal conditions in the dark. Since, it is possible that the enzyme activates the substrate to make it susceptible to rearrangement. We have been exploring means of activating the substrate to rearrangement.

Schiff base activation of amino acids is an attractive device, widely distributed in living systems, for this purpose . In this instance (Scheme 1) Schiff base formation at the amino group of β-methylaspartic acid



1 6 7 8

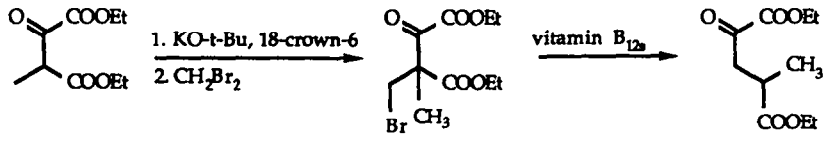
Scheme 1

(1) would yield the aldimine 6. Proton-induced shift of the double bond would then provide the ketimine form 7. This process could be carried to the next step, hydrolysis of the ketimine 7 to the corresponding carbonyl compound 8. The α-keto acid 8 or the α-ketimine acid 7 would then provide the unsaturated carbon to assist the rearrangement.

Several counts mitigate against this scheme. Barker and Suzuki[12] searched for a pyridoxal cofactor and found no evidence for the involvement of vitamin B_6 in the β-methylaspartate (1) ⟷ glutamate (2) rearrangement (eq. 1). However, we have not been able to learn whether the possible occurrence of an enzyme bound transaminating agent such as pyruvate was investigated.[13] Moreover, in order to convert the aldimine 6 to the ketimine 7, the α-proton must be removed from 6, then returned to the benzylic carbon of the Schiff base on 7. Barker[12] found that when the glutamate mutase reaction (eq 1) was carried out in D_2O, no deuterium was incorporated into the substrate. If equilibration between the aldimine 6 and the ketimine 7 does occur in the enzyme system, it must happen in such a way that there is no exchange of hydrogen with the medium. Barker[12] also showed that α-ketoglutarate was not a free intermediate in the enzymic transformation. However, α-ketoglutarate could be an enzyme bound intermediate.

It is attractive to explore the reaction from the standpoint of a Schiff base model, in spite of these limitations, especially since none of them is final. Indeed, model studies are valuable precisely because they encourage one to experiment in areas otherwise limited by enzyme specficity.

Accordingly, we decided to explore the α-keto ester system to learn whether rearrangement was possible. The ideal starting point for this purpose would be oxalylbromopropionate (2-keto-3-bromomethylsuccinic ester). However, because of the acidity of the proton flanked by ketone and ester groups, it was necessary to attach a blocking methyl group to the 3-position to avoid facile elimination of hydrogen bromide. Therefore, the model employed was 10 (Scheme 2), prepared by alkylation of diethyl

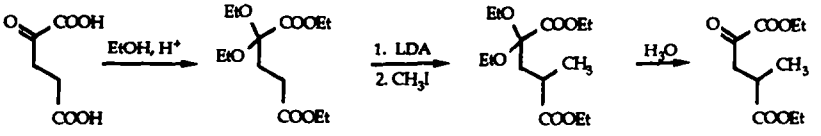


9 10 11

Scheme 2

oxalylpropionate 9 with methylene dibromide. When 10 was treated with vitamin B_{12s}, generated by reduction of hydroxocobalamin with zinc dust, smooth rearrangement to diethyl 4-methyl-2-ketoglutarate (11) occurred in 71% yield (Scheme 2).[14]

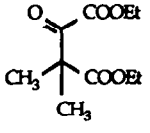
The structure of the rearrangement product 11 was established by comparison with an authentic sample prepared according to the straightforward synthesis outlined below (Scheme 3). Thus, α-



12 13 14 11

Scheme 3

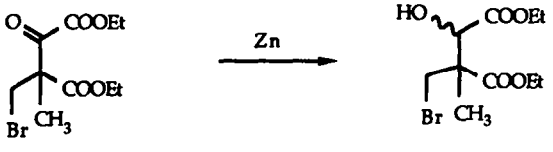
ketoglutaric acid 12 was converted to its diester ketal 13 under standard conditions. Alkylation leading to 14 was effected with lithium diisopropylamide followed by methyl iodide. The alkylated ketal 14 was then hydrolyzed to the alkylated keto ester 11, which was identical to the product from the vitamin B_{12} promoted rearrangement. An authentic sample of unrearranged 3,3-dimethyl-2-ketosuccinate 15 was also



15

prepared as described in the experimental section. None of the latter was found in the rearrangement reaction mixture.

The experimental section records our experiments with the zinc reduction omitting vitamin B_{12} and using activated zinc in protic and aprotic solvents. This was essential because vitamin B_{12s} was generated by reduction with zinc in this series of experiments. We also tried sodium naphthalide as a reductant. No rearrangement product 11 was found in these reactions. Instead, the unrearranged reduction product 16

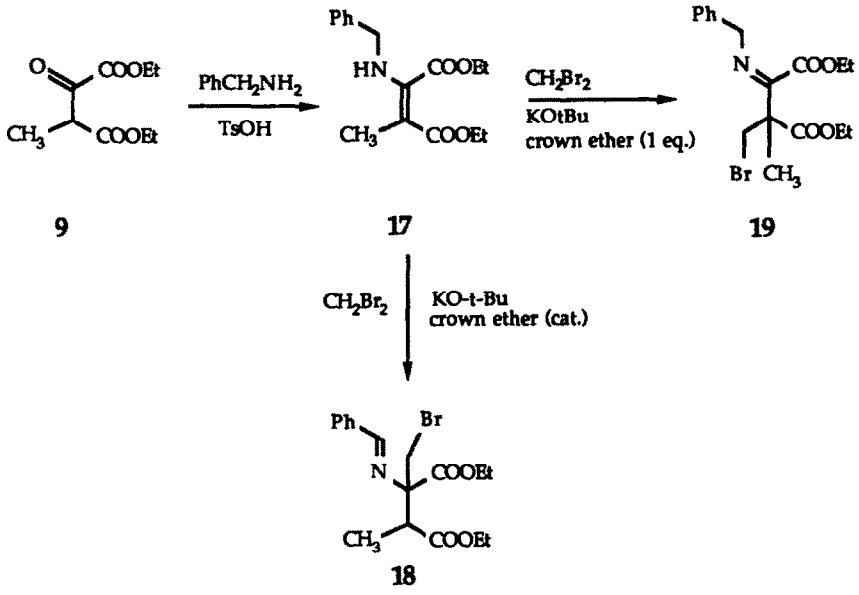


10 16

was isolated as a diasteromeric mixture of alcohols.

Our efforts to extend the model investigation to the corresponding Schiff base have been less successful and have led to a curious paradox. We can prepare the Schiff base of the model as outlined in

Scheme 4 below. Treatment of oxalylpropionate 9 with benzylamine and p-toluenesulfonic acid yielded the



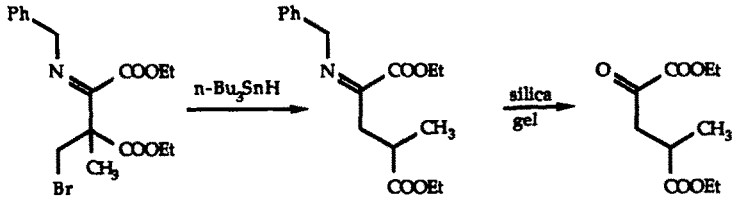
9 17 19

18

Scheme 4

enamine 17. When 17 was alkylated with methylene dibromide in the presence of potassium t-butoxide and a catalytic amount of 18-crown-6, the α-alkylated product 18 was produced in 17% yield. The outcome of this reaction is a consequence of thermodynamic control. When the reaction was carried out under kinetically controlled conditions, using a full equivalent of 18-crown-6, the desired β-alkylated product 19 was produced in 22% yield. These are isolated yields; the two reactions are nearly exclusive of one another.

Many attempts were made to induce 19 to rearrange with vitamin B_{12s}. Only recovered starting material was produced. Curiously, when the bromide 19 was treated with tri-n-butyltin hydride - under



19 20 11

what are presumably free radical conditions - smooth rearrangement to 20 occurred. The latter was isolated as 11 in 80% yield following chromatography.

Attempts to observe stable carbon-cobalt bond formation have not been successful in this series because the carbon carrying the bromide is a neopentyl carbon. When carbon-cobalt bond formation is not consummated, then rearrangement may be circumvented by competitive reactions such as electron transfer to halogen followed by fragmentation and reduction.

Summary. We have observed a rearrangement of a carbonyl analogue of methylaspartate which may point the way to a thermal model for the β-methylaspartate ⟷ glutamate interconversion. Together with the advent of photochemical conversion of the methylaspartate - B_{12} adduct to glutamate[11] substantial progress has been made toward modelling this unusual rearrangement reaction.

Experimental Part

β-Bromomethyl-DL-aspartic Acid Hydrobromide (4). Thirty mL of 32% hydrogen bromide in acetic acid was added, at 0°, to a flask containing 2.5 g (10.3 mmoles) of β-methyleneaspartic acid (3).[15] The reaction was allowed to warm to room temperature and stirred for 20 hr. The solvents were evaporated to yield 3.1 g (96%) of the desired bromide 4 as a light tan hygroscopic foam. The 300 MHz proton nmr spectrum (D_2O/DCl) showed a three-proton multiplet at δ 4.55 (CH, CH_2Br), a four-proton singlet at δ 4.5 (NH^+_3, COOH), and a one-proton multiplet at δ 3.8. The IR spectrum (KBr pellet) showed bands at 3400 (s, OH) and 1700 cm^{-1} (s, CO).

Since the bromide 4 is rather unstable, it is used immediately in the next step.

Reaction of β-Bromomethyl-DL-aspartic Acid Hydrobromide (4) with Vitamin B_{12}. The bromomethylaspartic acid 4 (3.1 g, 9.8 mmoles) was taken up in 30 mL of ice-cold, deionized, deoxygenated water and immediately added in the dark to a fresh solution of vitamin B_{12s}. The vitamin B_{12s} solution had been prepared as follows. In a nitrogen atmosphere, a solution of 4.2 g (110 mmoles) of sodium borohydride in 15.0 mL of water was added to a solution of 7 g (5.2 mmoles) of hydroxocobalamin in 75 mL of water. The reaction was stirred for 30 min at room temperature. The resulting grey-green vitamin B_{12s} solution was then placed in the dark, immersed in an ice-bath, and the solution of the bromide 4 was added. After the addition was complete, the ice bath was removed. When the reaction mixture had stirred for 10 min, a sample was removed and a UV spectrum was obtained. The UV spectrum (H_2O) indicated alkylcobalamin formation. The reaction was poured into a separatory funnel and washed with ten 10-mL portions of 50:50 phenol-methylene chloride. The combined organic layers were then washed with two 20-mL portions of water. The two water washings were combined and reextracted with two 5-mL portions of 50:50 phenol-methylene chloride. The combined organic layers were diluted with 1000 mL of methylene chloride and washed with ten 25-mL portions of water. The combined aqueous layers were concentrated under vacuum yielding 6.6 g of the alkylcobalamin 5, a red solid. The UV-visible spectrum (H_2O) showed maxima at 525, 500, 435, 376, and 335 nm, characteristic of an alkylcobalamin. Photolysis of the sample gave the spectrum of hydroxocobalamin. FAB exact mass; calc'd for $C_{67}H_{97}CoN_{14}O_{18}P$ ($M^+ + H$): 1475.6174. Found: 1475.6146.

Photolysis of the β-Methylaspartic Acid Cobalamin Adduct 5. A solution of 2.6 g (1.76 mmoles) of the β-methylaspartic acid cobalamin adduct 5 in 500 mL of water (pH 9) was placed in a 500-mL, two-neck, round-bottom flask equipped with a serum cap and an adapter carrying a argon-filled balloon. The solution was evacuated and flushed with argon five times, then the balloon was firmly inflated with argon. The solution was irradiated with a Westinghouse 250 watt sunlamp placed 35 cm distant from the reaction vessel. The progress of the reaction was monitored by observing the development of the intense visible absorption band at 352 nm corresponding to the formation of hydroxocobalamin. The spectral change was complete after 86 hr of irradiation.

The red solution was concentrated to 25 mL. The pH of the resulting, concentrated solution was lowered to 7.02 by the addition of 10% hydrochloric acid and the sample was placed on 18 mL of Dowex 50W (H^+ form, 400 mesh). The solution was allowed to drop to the top of the resin over 1.5 hr, then the resin was washed with 100 mL of water until the eluent was colorless. The amino acids were selectively eluted by treating the column with 200 mL of 3N NH_4OH until the eluent was negative to a ninhydrin spot test. All the amino acids were passed through before 200 mL of eluent was consumed. To remove traces of cobalamin, the reddish eluent was treated with 2 g of Norit. The charcoal was removed by gravity filtration and washed with three 20-mL portions of hot water. The combined filtrates were concentrated under vacuum affording 61.7 mg of a yellow semi-solid, shown by examination of the ^1H NMR spectrum to contain β-methyleneaspartic acid (3) and β-methylaspartic acid (1). Rearranged product, glutamic acid (2), was not detected within the limits (>0.1%) of the nmr method. The same result was obtained when the photolysis was conducted in the presence of sodium cyanide.

Dark, Thermal Cleavage of the β-Methylaspartic Acid Cobalamin Adduct 5. A solution of 1.53 g (1.04 mmoles) of the β-methylaspartic acid cobalamin adduct 5 in 325 mL of water was prepared. The pH of the solution was 7.02. The reaction was allowed to stand at 37 °C under argon, in the dark for 242 hr, and was

monitored by daily withdrawal of a small sample for spectral analysis. A steady increase in intensity of the band at 352 nm signaled the appearance of hydroxocobalamin. After 242 hr when the increase in intensity of the 352 nm band had ceased, the reaction was concentrated to dryness. The resulting red residue was dissolved in 20 mL of water and then applied to a column of 20 mL of Dowex 50W (H^+ form, 400 mesh). The solution was then allowed to drop to the top of the resin over 12 hr. The resin was then washed with 500 mL of water until the eluent was colorless. The amino acids were selectively eluted by treating the column with 250 mL of 3N NH_4OH until the eluent was negative to a ninhydrin spot test. All the amino acids were eluted before 200 mL of eluent was consumed. The reddish eluent was treated with 3 g of Darco-G60. The charcoal was removed by gravity filtration and washed with three 20-mL portions of hot water. The combined filtrates were concentrated affording 37.2 mg of a white semi-solid, shown by examination of the ^1H NMR spectrum revealed β-methyleneaspartic acid (**3**) and β-methylaspartic acid (**1**). Rearranged product, glutamic acid (**2**), was not detected within the limits (>0.1%) of nmr method.

Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate (10). Under an atmosphere of nitrogen, 4.088 g (0.036 moles) of potassium *t*-butoxide was placed in a three-necked, 250-mL, round-bottom flask. The flask was fitted with a reflux condenser and 250 mL of dry benzene was added. To this solution 6.624 g (0.032 moles) of diethyl oxalpropionate (**9**) was added at a rate of 0.13 mL/min. Methylene dibromide (37.0 g, 0.21 moles) and 0.79 g (0.003 moles) of 18-crown-6 were added. The reaction mixture was allowed to reflux for 6 hr and, upon cooling to 5°, was poured into a separatory funnel containing 150 mL of ether and 50 mL of ice cold 7% hydrochloric acid. The ether layer was washed with 50 mL of ice cold 7% hydrochloric acid, and with two 25-mL portions of water, then it was dried over sodium sulfate, filtered, and concentrated in vacuo to 8.59 g of a dark brown oil. The crude product was placed on 300 g of silica gel and was eluted with 75:25 hexane-ethyl acetate affording 2.5 g (26%) of the desired bromide **10**. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two two-proton methylene quartets (J = 7.2, 7.1 Hz) at δ 4.34 and at δ 4.23, a bromomethyl AB quartet (J = 10.7 Hz) at δ 4.0 and δ 3.7, a three-proton methyl singlet at δ 1.5, and two three-proton triplets (J = 7.1 Hz) at δ 1.2 and δ 1.1. The IR spectrum (neat) showed bands at 2970 (w, CH), 1750 (m, CO), and 1725 cm^{-1} (s, CO). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 251, 249 (1, M^+-OEt), 223,221 (75, M^+-COOEt), 195, 193 (25, M^+-COCOOEt), 115 (100, $C_6H_{11}O_2^+$). Exact mass calc'd for $C_8H_{10}O_4{}^{79}Br$; 248.9762. Found: 248.9762.

Rearrangement of Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate 10 under the Influence of Vitamin B_{12}. A solution of 37.4 mg (0.127 mmoles) of diethyl 3-bromomethyl-3-methyl-2-ketosuccinate (**10**) in 8 mL of absolute ethanol was placed in the right side (Flask B) of a special double flask.[8b] Zinc dust (6.4 g, 97.9 mmoles) was placed in the left side (Flask A). A three way stopcock with attached balloon was joined to the top of the curved tube connecting the two flasks. The apparatus was thoroughly purged of oxygen by evacuating (oil pump) and flushing with argon ten times. The balloon was then firmly inflated with argon. A solution of 1.0 g (0.75 mmoles) of hydroxocobalamin in 90 mL of a deoxygenated solution of 10% (w/v) NH_4I in absolute ethanol was added to the zinc through a syringe. The mixture was stirred at room temperature. After 25 min, the grey-green color characteristic of vitamin B_{12s} was observed. The apparatus was removed to a dark room and the grey-green solution of vitamin B_{12s} was filtered through a fritted disk into the solution of the bromide **10**. A uv spectrum, taken after 5 min reaction time, showed hydroxocobalamin. The reaction mixture was centrifuged to remove zinc. The supernatant was evaporated to dryness. The resulting residue was treated with 25 mL of water and extracted with ten 13-mL portions of ethyl acetate. The combined organic phases were dried over K_2CO_3, filtered, and concentrated yielding 30.6 mg of a reddish oil.

The oil was placed on 2.0 g of silica gel and eluted with 3:1 hexane-ethyl acetate affording 19.5 mg (71%) of the rearranged product **11**. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two two-proton methylene quartets.(J = 7.1 Hz) at δ 4.32 and at δ 4.12, a two-proton doubled AB quartet at δ 3.31 (J_{AB} = 18.3, 8.3 Hz) and δ 2.85 (J_{AB} = 18.3, 5.1 Hz, -CH_2CH(Me)COOMe), a one-proton methine multiplet at δ 2.99, two three-proton methyl triplets (J = 7.1 Hz) at δ 1.36 and at δ 1.25, and a three-proton methyl doublet (J = 7.0 Hz) at δ 1.24. The IR spectrum (neat) showed bands at 2920 (s, CH), 2849 (s, CH), and 1728 cm^{-1} (vs, CO). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 216 (6, M^+), 171 (10, M^+-OEt), 143 (100, M^+-COOEt), 115 (58, M^+-COCOOEt). Exact mass calc'd for $C_{10}H_{16}O_5$: 216.0998. Found: 216.0994.

Control Reaction of Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate (10) with Zinc under Rearrangement Conditions. In this experiment, an attempt was made to follow the procedures of the rearrangement experiment exposing the bromosuccinate **10** to the reducing conditions of the rearrangement reaction.

Thus, a solution of 5.0 mg (0.017 mmoles) of the bromomethylsuccinate **10** in 10 mL of a deoxygenated solution of 10% (w/v) NH_4I in absolute ethanol was added to the zinc dust through a syringe and stirred at room temperature under an argon atmosphere for 10 min. The reaction mixture was centrifuged to remove zinc. The supernatant was concentrated to dryness. The resulting residue was treated with 1 mL of water and extracted with eight 2-mL portions of ethyl acetate. The combined organic phases were dried over K_2CO_3, filtered, and concentrated to afford 3.9 mg (73.2%) of a brown oil. The ^1H NMR spectrum of this substance revealed the presence of the reduced product diethyl 3-bromomethyl-3-methyl-2-hydroxysuccinate (**16**). No absorption at δ 2.85 characteristic of the rearranged product **11** was observed. Column chromatography on 2 g of silica gel (elution with 2:1 hexane-ethyl acetate) gave 2.7 mg (54%) of the diastereomeric mixture of the hydroxylsuccinates **16** as a colorless oil. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two one-proton methine doublets at δ 4.5 (J = 5.8 Hz) and 4.34 (J = 7.1 Hz) from each diastereomer, an eight-proton methylene multiplet at δ 4.18-4.31 from the two diastereomers, two bromomethyl AB quartets at δ 3.81 and 3.60 (J = 10.1 Hz) and at 3.73 and 3.69 (J = 10.1 Hz) from each diastereomer, two one-proton hydroxyl doublets at δ 3.66 (J = 7.1 Hz) and 3.36 (J = 5.8 Hz) from each diastereomer, a twelve-proton methyl multiplet at δ 1.37-1.23 from the two diastereomers. The IR spectrum (neat) showed bands at 3599-3476 (br, OH), 2982 (m), 2922 (m), 2851 (m), 1732 cm^{-1} (vs, CO ester). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 223 and 225 (16, M^+-COOEt). Exact mass calc'd for $C_7H_{22}O_3{}^{79}Br$: 222.9970. Found: 222.9970.

Control Reaction of Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate (10) with Activated Zinc under Rearrangement Conditions. A 50-mL, round-bottomed flask, fitted with a magnetic stirring bar and a rubber septum, was charged with zinc dust (802 mg, 12.3 mmoles) and then purged of oxygen by evacuating and flushing twice with argon. To the zinc was added 10.0 mL of 10% hydrochloric acid solution. After stirring for 10 min the hydrochloric acid solution was removed with a syringe. The resulting activated zinc was then washed with two 10-mL portions of deoxygenated water and two 10-mL portions of a deoxygenated solution of 10% (w/v) NH_4I in absolute ethanol. The zinc was then treated with 5 mL of a deoxygenated solution of 10% (w/v) NH_4I in absolute ethanol. A solution of 5.0 mg of diethyl 3-bromomethyl-3-methyl-2-ketosuccinate (**10**) in a deoxygenated solution of 10% (w/v) NH_4I in absolute ethanol was added through a syringe. The mixture was allowed to stir for 10 min at room temperature. The reaction mixture was then centrifuged to remove zinc. The supernatant was concentrated to dryness. The resulting residue was treated with 1 mL of water and extracted with eight 2-mL portions of ethyl acetate. The combined organic phases were dried over K_2CO_3, filtered, and concentrated to afford 3.4 mg (68%) of diethyl 3-bromomethyl-3-methyl-2-hydroxysuccinate (**16**). Neither unreacted starting material **10** nor rearranged product **11** were observed.

Control Reaction of Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate (10) with Zinc under Aprotic Conditions. Diethyl 3-bromomethyl-3-methyl-2-ketosuccinate (**10**) (3.9 mg, 0.0132 mmoles) was treated with zinc dust (120 mg, 1.84 mmoles) in 4.5 mL of anhydrous dimethoxyethane (DME). The mixture was vigorously stirred at room temperature for 11 hr. Removal of zinc by centrifugation and concentration of the supernatant in vacuo afforded 3.6 mg (92.3%) of the starting material **10** as a light yellow oil. Rearranged product **11** was not observed.

Reaction of Diethyl 3-Bromomethyl-3-methyl-2-ketosuccinate (10) with Sodium-naphthalide. Naphthalene (640 mg, 5 mmoles) and 50 mL of dry THF was placed in a 100-mL flask. Sodium metal (115 mg, 5 mmoles) was added and the reaction was stirred at room temperature under an argon atmosphere for 3 hr to yield a deep dark green sodium-naphthalide solution. The sodium-naphthalide solution (1.7 mL, 0.169 mmoles) was placed in a 5-mL flask and cooled to -78° and 4.98 mg (0.0169 moles) of the bromide **10** in 0.8 mL of dry THF was injected dropwise. After stirring for 15 min at -78°, the reaction was quenched with 22 μL of acetic acid. On addition of acetic acid, the dark green color of the reaction mixture disappeared. The reaction was concentrated to a white solid. The ^1H NMR of this substance was too complex to describe. Accordingly, the crude product was passed through a short column of silica gel (1 g) with 4:1 hexane-ethyl acetate to remove the naphthalene. Evaporation of the solvent gave 4.9 mg of an oil whose ^1H NMR and

TLC were complex and gave no evidence of the presence of rearranged product **11** nor of the starting material **10**.

Preparation of an Authentic Sample of Rearranged Product 11. Diethyl 2,2-diethoxyglutarate (13). Acetyl chloride (1.3 g, 16.8 mmoles) was slowly added to 25 mL of ethanol in a 100-mL round-bottomed flask fitted with a Soxhlet extractor containing 3Å molecular sieves. 2-Ketoglutaric acid (**12**) (5 g, 27 mmoles) was added, followed by triethyl orthoformate (13.2 g, 88 mmoles). The reaction was refluxed for 100 hr then concentrated to 10.2 g of a yellow oil. Column chromatography on 300 g of silica gel (elution with 75:25:1 hexane-ethyl acetate-Hunig's base) provided 2.46 g of the desired ketal **13** as a colorless oil, R_f 0.46 (3:1 hexane-ethyl acetate). The 300 MHz proton nmr spectrum ($CDCl_3$) showed two two-proton methylene quartets (J = 7.1 Hz) at δ 4.24 and δ 4.10, a four-proton ketal methylene multiplet at δ 3.51, a four-proton multiplet at δ 2.25, and a twelve-proton multiplet at δ 1.31. The IR spectrum (neat) showed bands at 2980 (m, CH), 2936 (w, CH), 1740 (vs, CO), 1182 (m), and 1080.0 cm^{-1} (m). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 231 (16, M^+-OEt) and 203 (100, M^+-COOEt). Exact mass calc'd for $C_{11}H_{19}O_5$: 231.1233. Found: 231.1231.

Diethyl 2,2-Diethoxy-4-methylglutarate (14). At 0° under an atmosphere of nitrogen 0.271 mL (0.43 mmoles) of 1.6 M n-butyllithium was added to a stirring solution of diisopropylamine (43 mg, 0.43 mmoles) in 2 mL of dry tetrahydrofuran (THF). After 30 min the reaction mixture was cooled to -78° and the ketal **13** (100 mg, 0.362 mmoles) in dry THF (4 mL) was added dropwise for 10 min. After stirring at -78° for 1 hr, 0.68 mL (1.1 mmoles) of methyl iodide was added. The reaction mixture was allowed to stir for 1 hr at -78° then 30 min at -30°. Upon warming to 0° the reaction mixture was poured into a separatory funnel containing 2 mL of water and 10 mL of ether. The aqueous layer was washed with 4 mL of ether. The combined ether layers were dried over sodium sulfate, filtered, and concentrated to 113 mg (100%) of the desired product **14**, a light yellow oil. The 300 MHz proton nmr spectrum ($CDCl_3$) showed a two-proton double quartet (J = 7.1 Hz) at δ 4.11 and 4.10, a four-proton ketal methylene multiplet at δ 3.5, a one-proton methine multiplet at δ 2.5, a doubled AB quartet at δ 2.49 (J_{AB} = 13.3, 7.7 Hz) and δ 1.87 (J_{AB} = 13.3, 4.0 Hz), and a fifteen-proton multiplet at δ 1.2.

Diethyl 4-Methyl-2-ketoglutarate (11). Diethyl 4-methyl-2,2-diethoxy-glutarate (**14**) (115 mg, 0.362 mmoles) was placed in 5 mL of ice cold 3:1 H_2SO_4-H_2O solution. After stirring for 1.5 hr at 0°, the reaction was diluted to 10 mL with cold water and washed with five 7-mL portions of ether. The combined ether layers were dried over sodium sulfate, filtered, and concentrated to 69 mg of a light yellow oil. The crude product was placed on 1 g of silica gel and eluted with a 75:25 hexane-ethyl acetate mixture affording 57 mg (73%) of diethyl 4-methyl-2-ketoglutarate (**11**) as a colorless oil. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two two-proton methylene quartets (J = 7.1 Hz) at δ 4.3 and δ 4.1, a two-proton doubled AB quartets at δ 3.3 (J_{AB} = 18.3, 8.3 Hz) and δ 2.9 (J_{AB} = 18.3, 5.1 Hz), a one-proton multiplet at δ 3.0, a three-proton methyl triplet at δ 1.4 (J = 7.1 Hz), a three-proton methyl triplet at δ 1.26 (J = 7.1 Hz), and a three-proton methyl doublet at δ 1.25 (J = 7.0 Hz). The IR spectrum (neat) showed bands at 2920 (s, CH), 2849 (s, CH), and 1728 cm^{-1} (vs, CO). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 216 (6, M^+), 171 (19, M^+-OEt), 143 (100, M^+-COOEt), 115 (58, M^+-COCOOEt). Exact mass calc'd for $C_{10}H_{16}O_5$: 216.0998. Found: 216.0994.

Diethyl 3,3-Dimethyl-2-ketosuccinate (15). Under an atmosphere of nitrogen, 1.27 g (0.011 mmoles) of potassium *t*-butoxide was placed in a 250-mL, round-bottomed flask. The flask was fitted with a three way stopcock with attached balloon and 125 mL of dry benzene was added. To this solution 2.058 g (0.01 moles) of diethyl oxalpropionate **9** was added dropwise. 18-Crown-6 (0.25 g, 0.95 mmoles) was added followed by dropwise addition of 9.9 g (0.0576 moles) of iodomethane. The reaction mixture was allowed to stir for 20 hr at room temperature and was poured into a separatory funnel which contained 300 mL of ether. The ether was washed with two 30-mL portions of water, dried over sodium sulfate, filtered, and concentrated in vacuo to 1.6 g of a yellow oil. The oil was distilled under reduced pressure (0.04 mmHg, bp 63-65°) yielding 1.21 g of the desired ketoester, a colorless oil. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two two-proton methylene quartets (J = 7.1 Hz) at δ 4.31 and δ 4.17, a six-proton methyl singlet at δ 1.44, and two three-proton methyl triplets (J = 7.1 Hz) at δ 1.35 and δ 1.22. The IR spectrum (neat) showed bands at 2986 (m, CH), 2941 (w, CH), 1732 (vs, CO), 1472 (m), 1258 (s), and 1150 cm^{-1} (s). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 216 (2, M^+), 171 (9, M^+-OEt), 143 (70, M^+-COOEt), and 115 (70, M^+-COCOOEt). Exact mass calc'd for $C_{10}H_{16}O_5$: 216.0998. Found: 216.0998.

Ethyl 2-Benzylamino-3-carboethoxybut-2-enoate (17). A solution of diethyl oxalpropionate (9) (20 g, 99 mmoles), benzylamine (10.8 mL, 99 mmoles), and p-toluenesulfonic acid (0.8 g, 4.2 mmoles) in 250 mL of dry benzene was heated at reflux for 26 hr. Water was removed using a Soxhlet extractor containing calcium hydride. The reaction mixture, upon cooling to room temperature, was concentrated under reduced pressure yielding 36 g of a light yellow oil. The oil was placed on 250 g of silica gel and eluted with 7:1 hexane-ethyl acetate containing Hunig's base (0.5%) while taking 35 mL fractions. Fractions 8-34 provided 8.9 g of the desired enamine **17** as a colorless oil. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two three-proton methyl triplets at δ 1.25 (J = 7.0 Hz) and at δ 1.27 (J = 7.2 Hz), a three-proton methyl singlet at δ 1.7, two two-proton methylene quartets at δ 4.24 (J = 7.2) and at δ 4.15 (J = 7.0 Hz), a two-proton benzylic doublet (J = 5.8 Hz) at δ 4.27, and a five-proton aromatic multiplet at δ 7.29. The IR spectrum (neat) showed bands at 3275 (w, NH), 2975 (m, CH), 1730 (s, CO), 1630 (s, C=C), and 1595 cm^{-1} (s). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 291 (100, M^+), 262 (17, M^+-Et), 246 (40, M^+-EtO), and 218 (53, M^+-COOEt). Exact mass calc'd for $C_{16}H_{21}NO_4$: 291.1471. Found: 291.1467.

Diethyl Benzylidene-α-bromomethyl-β-methylaspartate (18). Under an atmosphere of nitrogen, 0.31 g (2.77 mmoles) of potassium t-butoxide was placed in a 10-mL, round-bottomed flask. The flask was fitted with a three way stopcock with attached balloon and 2 mL of dry benzene was added. A solution of 0.73 g (2.5 mmoles) of ethyl 2-benzylamino-3-carboethoxybut-2-enoate (**17**) and 70 mg (0.26 mmoles) of 18-crown-6 in 2 mL of dry benzene was added. After stirring at room temperature for 2 min, dibromomethane (4.8 mL, 68.5 mmoles) was added rapidly. The reaction mixture was stirred for 31 hr at room temperature then poured into a separatory funnel with 50 mL of ethyl acetate. The organic layer was washed with four 5-mL portions of saturated potassium chloride solution, dried over sodium sulfate, filtered, and concentrated in vacuo to 2.08 g of a yellow oil. This oil was placed on 90 g of silica gel and eluted with 7:1 hexane-ethyl acetate containing Hunig's base (0.5%), while taking 15 mL fractions. Fractions 17-34 provided 167 mg (17%) of the bromide **18** as a 2:1 ratio of diastereomeric mixture. The 300 MHz proton nmr spectrum ($CDCl_3$) showed a nine-proton multiplet at δ 1.1-1.37, a pair of methine quartets (J = 7.0 Hz) at δ 3.25 and δ 3.36 from each diastereomer, a bromomethyl AB quartet (J_{AB} = 10.7 Hz) at δ 3.81 and at δ 3.96 from the major diastereomer, a bromomethyl AB quartet (J_{AB} = 11.7 Hz) at δ 3.94 from the minor diastereomer, a four proton methylene multiplet at δ 4.08-4.34, aromatic multiplets at δ 7.41 and at δ 7.81, and a pair of aldimine proton singlets at δ 8.42 and at δ 8.58 from each diastereomer. The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 356, 354 (18, M^+-Et) and 312, 310 (100, M^+-COOEt). Exact mass calc'd for $C_{15}H_{17}NO_4{}^{79}Br$: 354.0341. Found: 354.0340.

Diethyl 3-Bromomethyl-3-methyl-2-benzyliminosuccinate (19). Under an atmosphere of nitrogen, 1.3 g (11.6 mmoles) of potassium t-butoxide was placed in a 25-mL, round-bottomed flask. The flask was fitted with a three-way stopcock with attached balloon, and 8 mL of dry benzene was added. A solution of 2.59 g (8.93 mmoles) of ethyl 2-benzylamino-3-carboethoxybut-2-enoate **17** and 3.1 g (11.6 mmoles) of 18-crown-6 in 7 mL of dry benzene was rapidly added to the slurry above. The reaction mixture became dark red in color in ca. 30 sec and then dibromomethane (8.73 mL, 124.6 mmoles) was added rapidly. The reaction mixture was stirred for 20 hr at room temperature then poured into a separatory funnel with 180 mL of ethyl acetate. The organic layer was washed with four 10-mL portions of saturated potassium chloride solution, dried over sodium sulfate, filtered, and concentrated in vacuo to 3.548 g of a yellow oil. This oil was placed on 280 g of silica gel and eluted with 7:1 hexane-ethyl acetate containing Hunig's base (0.5%), taking 30 mL fractions. Fractions 24-32 provided 754 mg (22%) of the desired Schiff base **19**. The 300 MHz proton nmr spectrum ($CDCl_3$) showed two three-proton methyl triplets at δ 1.27 (J = 7.2 Hz) and δ 1.33 (J = 7.3 Hz), a three-proton methyl singlet at δ 1.58, a bromomethyl AB quartet (J_{AB} = 10.3 Hz) at δ 4.03 and δ 3.71, a four proton methylene multiplet at δ 4.32-4.13, a two-proton benzylic singlet at δ 4.73, a five-proton aromatic multiplet at δ 7.4-7.32. The ^{13}C nmr spectrum ($CDCl_3$, proton noise decoupled) showed 15 lines at: δ 170.3 (s), 161.9 (s), 160.7 (s), 138.2 (s), 128.0 (d, J = 159.3 Hz), 127.2 (d, J = 158.5 Hz), 126.6 (d, J = 160.7 Hz), 61.42 (t, J = 148.6 Hz), 61.3 (t, J = 148.6 Hz), 57.3 (t, J = 135.7 Hz), 54.3 (s), 37.5 (t, J = 158.2 Hz), 20.1 (q, J = 131.0 Hz), 13.75 (q, J = 127.0 Hz), and 13.69 (q, J = 127.0 Hz). The IR spectrum ($CHCl_3$) showed bands at 2955 (m, CH), 1715 (s, CO), and 1640 cm-1 (m, C=N). The mass spectrum (15 eV) showed peaks at m/z (rel. Int.): 385, 383 (19, M^+), 356, 354 (5, M^+-Et), 312, 310 (59, M^+-COOEt), and 290 (65, M^+-$BrCH_2$). Exact mass calc'd for $C_{17}H_{22}NO_4{}^{81}Br$: 385.0712. Found: 385.0712.

Reaction of Diethyl 3-Bromomethyl-3-methyl-2-benzyliminosuccinate (19) with Vitamin B_{12}. A solution of 32.5 mg (0.085 mmoles) of diethyl 3-bromomethyl-3-methyl-2-benzyliminosuccinate (19) in 1 mL of absolute methanol was placed in the right side of the double flask.[8b] Zinc dust (353 mg, 5.41 mmoles) was placed in the left side. A three way stopcock with attached balloon was joined to the top of the curved tube connecting the two flasks. The apparatus was thoroughly purged of oxygen by evacuating (oil pump) and flushing with argon ten times. The balloon was then firmly inflated with argon. To the zinc was added 3.0 mL of 10% hydrochloric acid. After stirring for 10 min, the hydrochloric acid was removed. The resulting activated zinc was then washed with two 3-mL portions of deoxygenated water and two 3-mL portions of a deoxygenated solution of 10% (w/v) NH_4I in absolute methanol. The zinc was then treated with 3-mL of a deoxygenated solution of 10% (w/v) NH_4I in absolute methanol. A solution of 133 mg (0.085 mmoles) of hydroxocobalamin in 2 mL of a deoxygenated solution of 10% (w/v) NH_4I in absolute methanol was added through a syringe. The mixture was allowed to stir for 20 min at room temperature. After 25 min, the grey-green color characteristic of vitamin B_{12s} was observed. The apparatus was removed to the dark room and the grey-green solution of vitamin B_{12s} was added to the bromide 19 by tilting the double flask. A UV spectrum, taken after 5 min reaction time, showed hydroxocobalamin. The reaction mixture was evaporated to dryness. The resulting residue was treated with 10 mL of brine and extracted with ten 10-mL portions of ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtered, and concentrated yielding 20.4 mg (63%) of a light yellow oil. A 300 MHz proton nmr showed that the starting Schiff base 19 was the major component of the crude reaction mixture. Rearrangement product was not observed. Further purification via column chromatography on 2 g of silica gel (elution with 87.5:12:0.5 hexane-ethyl acetate-Hunig's base) provided 7.4 mg (23%) of the pure starting material along with 3.6 mg (11%) of unidentified material. ^1H NMR analysis of every fraction from the column showed neither the presence of the rearranged Schiff base nor the rearranged and hydrolyzed product, diethyl 4-methyl-2-ketoglutarate (11). The rearranged Schiff base was found to hydrolyze during column chromatography (see the next experiment).

Rearrangement of Diethyl 3-Bromomethyl-3-methyl-2-benzylimino-succinate (19) with Tri-n-butyltin Hydride. To a 25-mL, round-bottomed flask fitted with a magnetic stirring bar and a reflux condenser were added diethyl 3-bromomethyl-3-methyl-2-benzyliminosuccinate (19) (10 mg, 0.026 mmoles), dry benzene (5.5 mL), and tri-n-butyltin hydride (9.0 mg, 0.026 mmoles). AIBN (3 mg, 0.018 mmoles) was added, and the flask was heated for 19 hr at reflux in a 105° oil bath. The reaction mixture was cooled to room temperature and evaporated on the rotary evaporator yielding an oil, which was dissolved in 25 mL of dichloromethane and washed with ten 1-mL portions of 10% potassium fluoride solution, dried over K_2CO_3, filtered, and concentrated to an oil. The resulting oil was taken up in 25 mL of acetonitrile, washed with four 5-mL portions of hexane, and concentrated to 5.6 mg of the rearranged Schiff base 20 as a colorless oil. The ^1H NMR of this substance was quite complex, probably as consequence of coexistence of the four possible diasteromers. However, it revealed the presence of neither the starting material 19 nor the rearranged and hydrolyzed product, diethyl 4-methyl-2-ketoglutarate (11), which exhibits a characteristic one-proton AB quartet at δ 3.31. The IR spectrum (neat) showed bands at 2980 (w, CH), 1730 (m, CO), 1650 (w), and 1454 cm^{-1} (w). The mass spectrum (15 eV) showed peaks at m/z (rel. int.): 305 (3.6, M^+), 260 (4.5 M^+-OEt), 232 (17, M^+-COOEt), 204 (25), and 91 (100, $PhCH_2^+$). Exact mass calc'd for $C_{17}H_{23}O_4N$: 305.1627. Found: 305.1627.

Column chromatography on 2 g of silica gel (elution with 80:20:0.1 hexane-ethyl acetate-Hunig's base) provided 4.5 mg (80%) of the rearranged and hydrolyzed diethyl 4-methyl-2-ketoglutarate (11) as a colorless oil whose nmr and IR spectra were identical to those of an authentic sample.

Acknowledgement. This research was generously supported by the Institute for General Medical Sciences of the National Institutes of Health under grant GM 19906. We thank Katherine Dowd for her assistance. We are grateful to the University of Illinois Mass Spectrometry Laboratory, supported by NIH grant GM 27029 for the FAB mass spectrum of the cobalamin 5.

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(13) We have observed that glutamate mutase is irreversibly inhibited by sodium borohydride and by sodium cyanoborohydride. This may indicate that a reducible Schiff base or carbonyl group might be involved in the rearrangement, but clearly much remains to be done to establish the specific group or groups involved in the reduction and their connection to the glutamate mutase rearrangement, if any.

(14) The ketone **10** also rearranges readily when treated under free radical conditions. See Dowd, P.; Choi, S.-C. *J. Am. Chem. Soc.* **1987**, *109*, 3493.

(15) Dowd, P.; Kaufman, C. *J. Org. Chem.* **1979**, *44*, 3956.