report of catalytic chemistry with a VPI-5 molecular sieve. The data show that catalysis can be performed in the extra-large pores of VPI-5. Since (i) rhodium is an extremely active hydrogenation catalyst, (ii) the temperature is low, and (iii) the reaction is occurring in the liquid phase, we expect that the results shown in Figure 9 are strongly influenced by the effects of diffusion for both Rh-VPI-5 and Rh-$AlPO_4$-5. If such is the case, then the data in Figure 9 could be illustrating the relative effective diffusivities of 1-octene and *cis*-cyclooctene in Rh-$AlPO_4$-5 and Rh-VPI-5. The data are consistent with this thought in that 1-octene is converted faster (i) with Rh-VPI-5 than Rh-$AlPO_4$-5 and (ii) than *cis*-cyclooctene in Rh-VPI-5.

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Use of a Recombinant Bacterial Fructose-1,6-diphosphate Aldolase in Aldol Reactions: Preparative Syntheses of 1-Deoxynojirimycin, 1-Deoxymannojirimycin, 1,4-Dideoxy-1,4-imino-D-arabinitol, and Fagomine

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Abstract: A combined enzymatic aldol condensation and catalytic intramolecular reductive amination has been used in the high-yield asymmetric synthesis of polyhydroxylated alkaloids including 1-deoxynojirimycin, 1-deoxymannojirimycin, 1,4-dideoxy-1,4-imino-D-arabinitol, and fagomine. The *Escherichia coli* Zn^{2+}-containing fructose-1,6-diphosphate aldolase overexpressed in *E. coli* was used for the syntheses. The enzyme in aqueous solution containing 0.3 mM $ZnCl_2$ has excellent stability with a half-life of 60 days, compared to 2 days for the enzyme from rabbit muscle. The reactions were carried out under mild conditions without protection of functional groups. Either dihydroxyacetone phosphate or a mixture of dihydroxyacetone and inorganic arsenate can be used as donor in the aldol reactions. The aldol acceptors (*R*)- and (*S*)-3-azido-2-hydroxypropanal were prepared via lipase-catalyzed resolution of the racemic acetal precursor.

Recent studies have demonstrated the synthetic utility of aldolase-catalyzed reactions.[1-10] Of more than 15 aldolases found in nature, the metal-free, Schiff base forming fructose-1,6-diphosphate (FDP) aldolase from rabbit muscle is the most often used.[1-6] The enzyme is highly specific for dihydroxyacetone phosphate (**1a**), but accepts a variety of aldehydes as a second substrate. The donor **1a** can be generated from FDP in situ[1-4] or replaced with a mixture of dihydroxyacetone (**1b**) and inorganic arsenate (As).[2] The stereochemistry of the C-C bond formation is completely controlled by the enzyme and is the same with all substrates tested so far. Although the enzyme is commercially available, the requirement for its isolation from the mammalian source and the instability of this enzyme may circumvent its use in large-scale processes. The Zn^{2+}-containing FDP aldolase was found in many microorganisms such as *Escherichia coli*,[11] but the use of this enzyme in organic synthesis was not reported. We describe here our study of *E. coli* FDP aldolase[12] as a synthetic catalyst.

An interesting finding in this research is that the Zn^{2+}-aldolase seems to have the same substrate specificity as that of rabbit muscle FDP aldolase, accepting all the aldehyde substrates used before[2,3] for the rabbit enzyme. In aqueous solution, the microbial enzyme, however, showed excellent stability in the presence of 0.3 mM $ZnCl_2$. The estimated half-life of the free enzyme at room temperature is 60 days while that of the rabbit enzyme is 2 days (Figure 1).

To illustrate the use of the microbial enzyme in preparative-scale synthesis, the polyhydroxylated alkaloids 1,5-dideoxy-1,5-imino-D-glucitol (1-deoxynojirimycin, **5**), 1,5-dideoxy-1,5-imino-D-mannitol (1-deoxymannojirimycin, **6**), 1,4-dideoxy-1,4-imino-D-arabinitol (**7**), and 1,2,5-trideoxy-1,5-imino-D-arabinitol (fagomine,

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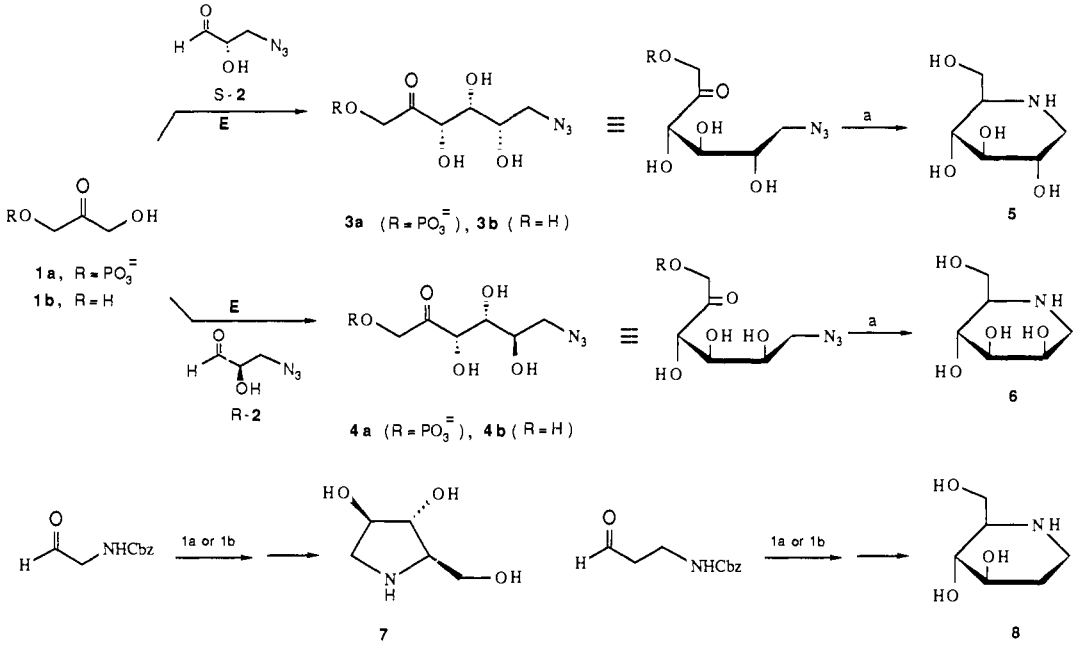
Scheme I. Syntheses of Deoxynojirimycin (**5**), Deoxymannojirimycin (**6**), 1,4-Dideoxy-1,4-imino-D-arabinitol (**7**), and Fagomine (**8**)[a]



[a] E, Zn^{2+}–FDP aldolase or rabbit muscle FDP aldolase. When **1b** was used as substrate, the reaction was in 0.5 M sodium arsenate, pH 6.5. (a) Acid phosphatase (for phosphate product only) then H_2/Pd.

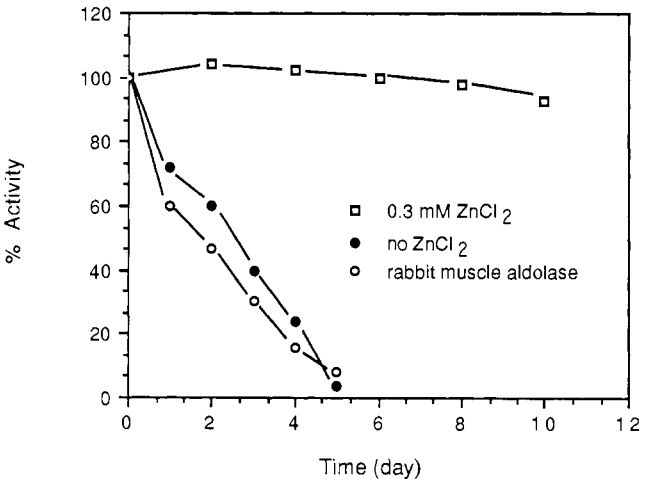


Figure 1. Stabilities of *E. coli* Zn^{2+}–FDP aldolase and rabbit muscle FDP aldolase under air. The enzymes are dissolved in a 0.1 M Tris buffer (pH 7.4, 1 unit/mL). The rabbit enzyme is a metal-free enzyme; no metal ion was added to the solution. The Zn^{2+}–aldolase was studied in the absence and in the presence of 0.3 mM Zn^{2+} to prevent the leakage of metal from the active site. Both enzymes were assayed on the basis of their cleavage of FDP coupled with glycerophosphate dehydrogenase catalyzed reduction of **1a** with NADH.

Table I. Product Distribution of FDP Aldolase Catalyzed Reactions[a]

enzyme source (units used)	substrates (ratio)	reaction time, h	products[b] **5**:**6**	% overall yield[c]
rabbit	**1a** + (*RS*)-**2** (1:2)	10	1:4	52
(340)		20	1:1.2	64
E. coli	**1a** + (*RS*)-**2** (1:2)	10	1:4.1	48
(8)		20	1:1.5	62
rabbit	**1b** + (*RS*)-**2** (1:2)	10	1:4	55
(340)		20	1:4	60
	1b + (*RS*)-**2** (1:3)	10	1:9	58
E. coli	**1b** + (*RS*)-**2** (1:2)	5	1:50	35
(8)		20	1:4	61

[a] Reaction conditions: For rabbit aldolase reactions, 24-mL solutions containing 0.12 M of **1a** and 2 equiv of (*RS*)-**2** at pH 6.5 were used. For reactions with **1b** (0.12 M), conditions were the same except that sodium arsenate (0.5 M) was present; K_m = 0.25 M, V_{max} = 0.67 units/mg for (*R*)-**2** and 0.05 M, 0.083 units/mg for (*S*)-**2**. For *E. coli* enzyme reactions, the volume was 4 mL. All other conditions were unchanged. 1 unit = 1 μmol product formed per min. [b] The ratio was determined by HPLC with a Waters carbohydrate column. For reactions with **1a**, the product phosphate moiety was first removed with phosphatase before HPLC analysis. Mobile phase: CH_3CN/H_2O = 85:15 v/v. Retention time: **3b** (6.4 min), **4b** (7.2 min), **5** (14.8 min), **6** (22.8 min). [c] Determined on the basis of **1a** or **1b** and the isolated **5** plus 6. No significant change of the activity for the microbial enzyme was observed after reactions, whereas 60% activity of the rabbit enzyme was lost in 2 days.

8) were chosen as targets (Scheme I). Compounds **5**–**7** are useful for treatment of carbohydrate-dependent metabolic disorders because of their selective inhibition of specific glycosidases.[13] Compound **8** is a component of the natural product 4-*O*-(β-D-glucopyranosyl)fagomine.[14] Of the methods reported for the synthesis of these compounds,[15] the combined microbial oxidation and reductive amination[15d] for **5** and the intramolecular aminomercuration[15e] for both **5** and **6** are considered to be more efficient.

(12) The FDP aldolase gene was subcloned from plasmid pLC 33-5 isolated from the Clarke and Carbon gene library (Clarke, L.; Carbon, J. *Cell* **1976**, *9*, 91).

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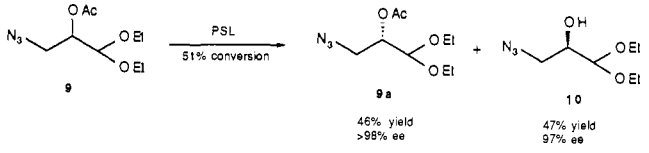
Compounds **5** and **6** can be further converted to castanospermine and the 6-epimer,[16] respectively, both useful as anticancer and antiviral agents. A recent study also indicated that the *N*-butyl derivative of **5** is very effective against the AIDS virus.[17]

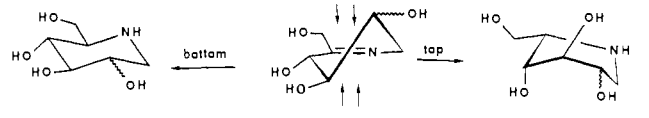
A key component used in the syntheses was 3-azido-2-hydroxypropanal. Both *R* and *S* enantiomers were prepared via lipase-catalyzed resolution of the racemic mixture of the acetal precursor (eq 1). Of different enzymes tested for the resolution,



the lipase from *Pseudomonas* is the most enantioselective. The enzyme can be used as a free form or immobilized on XAD-8. No decrease of activity was observed in 8 days in either case. The initial activity of the immobilized enzyme, however, was 3 times more active than the free enzyme.

Table I indicates the product distribution of aldol condensation with (*RS*)-**2**. Interestingly, the ratio of **5** to **6** increased when the aldol reaction proceeded, indicating a kinetic preference of (*R*)-**2** over (*S*)-**2**. The same situation was observed in the previous aldol reaction catalyzed by the enzyme from rabbit muscle.[3] The aldol products **3** and **4** could be acyclic or form a cyclic furanose. The cyclic form could have different conformations and anomeric configurations with relatively same stability. This was indeed indicated in the 1:1 ratio for **3b** to **4b** after the reaction reached equilibrium. When **1a** was replaced with a mixture of **1b** and 0.5 M sodium arsenate in each of the above aldol reactions, the rate slowed by a factor of 5. In the reaction of **1b**/As with (*RS*)-**2**, the kinetic product **6** was obtained predominantly at different periods of time. This result was expected as the reverse reaction (i.e. aldol cleavage) in this case should be much slower than the forward reaction.[19] Similar results were obtained in the rabbit muscle aldolase catalyzed reactions. Compounds **7** and **8** were prepared similarly as shown in Scheme I.

It is worth noting that, under the reductive amination condition, only one diastereomer is produced. The stereocontrol seems to originate from the diastereoselective hydrogenation of the imine intermediate (eq 2). Attack of hydrogen from the bottom face



seems to be more favorable as it would avoid the torsional strain developed via attack from the top face. This results in a product with C4–C5 in a trans relation. A similar argument could be applied to the synthesis of **7** and **8**.

In summary, this paper demonstrates the use of a recombinant aldolase in the synthesis of derivatives of piperidine and pyrrolidine with important biological activities. The combined enzymatic and catalytic chemical procedure was carried out under mild conditions with minimum protection of functional groups. It requires less reaction steps with higher overall yields compared to the reported procedures. The broad substrate specificity, excellent stability, and the easy accessibility of the Zn^{2+}–aldolase should open a practical route to a number of other polyhydroxylated compounds. Research is in progress to use other aldolases to prepare piperidine derivatives structurally related to L-fucose, L-rhamnose, D-galactose, and sialic acids.

Experimental Section

Construction of an Overproduction *E. coli* Strain. An overproducer of *E. coli* FDP aldolase was constructed in *E. coli*. A 14.4-kb plasmid known to contain the *E. coli* FDP aldolase gene (*fda*),[18] pLC33-5, was obtained from the Clarke and Carbon gene library.[19] An *Eco*RI digest of pLC33-5 was ligated into the unique *Eco*RI site of the *E. coli* multicopy vector pUC8[20] and used to transform the *E. coli* mutant CO100[21]. Recombinants expressing the *fda* gene were isolated by selecting for the ability to metabolize glucose.[22] A recombinant clone containing a 16.4-kb plasmid, pE3, was isolated. This plasmid contained two *Eco*RI fragments (9.6 and 4.1 kb) cloned into the pUC8 vector. The strain was grown in LB containing ampicillin (50 mg/L). Cell-free extracts from the recombinant strain contained 4.0 units/mg specific FDP aldolase activity[23] equivalent to 50-fold overproduction compared to wild type *E. coli*. This recombinant *E. coli* CO100/pE3 was used as a source of bacterial FDP aldolase. A 2-L fermentation typically would produce 10 g of crude extracts, which contain approximately 7000 units FDP aldolase.

The recombinant plasmid expressing the *fda* gene was subcloned in order to reduce the size of the 13.7-kb insert. The plasmid pE3 was partially digested with *Sau*3AI to generate fragments 2–4 kb in size. These fragments were ligated into the *Bam*HI site of pUC8 and transformed into *E. coli* Jm83. A recombinant clone containing a 5.8-kb plasmid, pS4, was isolated. This plasmid contained a 3.1-kb insert. Cell-free extracts from this strain contained 25 units/mg specific FDP aldolase activity, an approximately 300-fold overproduction.

Combined Enzymatic Aldol Reaction and Reductive Amination. A solution (50 mL) containing (*R*)-3-azido-2-hydroxypropanal (12 mmol), FDP–Na_3 (3.0 mmol),[24] Zn^{2+}–FDP aldolase (16 units), triosephosphate isomerase (500 units from Sigma), and $ZnCl_2$ (0.3 mM) at pH 6.5 was stirred slowly for 48 h. The product was recovered, hydrolyzed to remove the phosphate moiety as described previously,[2] and then hydrogenated in 50% MeOH with 300 mg of 10% Pd/C under 40 psi of hydrogen for 10 h. The solution was filtered, concentrated to 4 mL, and chromatographed on a Dowex 50 (Ba^{2+}) column (3.0 × 97 cm) with water as the mobile phase. The fractions (284–310 mL) were collected and lyophilized to yield 0.85 g of **6** (80% yield, $[\alpha]^{25}_D = -29°$ (*c* 0.2, MeOH). Compound **5** was prepared similarly from (*S*)-**2** (0.64 g, 64% yield, $[\alpha]^{25}_D = +47.5°$ (*c* 0.2, H_2O)). The ^1H NMR spectra, optical rotations, and microanalyses of both compounds are in agreement with the reported values.[15a,i]

In a similar manner, compounds **7** and **8** were prepared from FDP and the appropriate acceptor catalyzed by either aldolase (Scheme I). The overall yield for **7** was 30% and that for **8** was 41%. ^1H and ^{13}C NMR and optical rotations of both compounds were consistent with the reported values.[15k,l]

Improved Method for the Preparation of (*RS*)-3-Azido-2-*O*-acetoxypropanal Diethyl Acetal, 9. The procedure is a modification of that reported.[3a] To a stirred suspension of $KHCO_3$ (4.52 g, 45 mmol) in methanol (150 mL) was added acrolein diethyl acetal (45 mL, 38.4 g, 295 mmol), benzonitrile (30 mL), and H_2O_2 (30%, 32 mL, 313 mmol). The solution was warmed to 40 °C in a water bath. After 8 h, 10 mL

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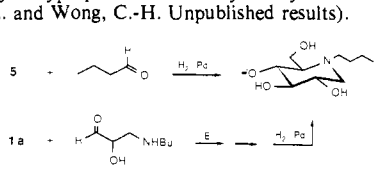
(17) Karpas, A.; Fleet, G. W. J.; Dwek, R. A.; Petursson, S.; Namgoong, S. K.; Ramsden, N. G.; Jacob, G. S.; Rademacher, T. W. *Proc. Natl. Acad. Sci.* U.S.A **1988**, *85*, 9229. *N*-Butyl-1-deoxynojirimycin can be prepared via catalytic reduction (H_2/Pd (50 psi)) of a mixture of **5** and *n*-butanal. Alternatively, it can be prepared via FDP aldolase catalyzed condensation with 3-(*N*-butyl)-2-hydroxypropanal followed by catalytic reductive amination (Pederson, R. L. and Wong, C.-H. Unpublished results).



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(22) For general techniques of cloning, see Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Lab: Cold Spring Harbor, NY, 1982.

(23) To prepare the crude enzyme, the cells were harvested by centrifugation and sonicated for 4 minutes on ice. After centrifugation the supernatant was dialyzed, lyophilized, and used directly without further purification. The aldolase was assayed on the basis of the cleavage of FDP coupled with glycerophosphate dehydrogenase catalyzed reduction of **1a** in the presence of NADH at 37 °C. Protein was determined by the Bio-Rad procedure (Bradford, M. *Anal. Biochem.* **1976**, *72*, 248–54) using bovine serium albumin as standard.

(24) FDP was obtained from Sigma. FDP was converted to **1a** in the presence of aldolase and triosephosphate isomerase. One can also use **1a** as a substrate.

of H_2O_2 was added and, after an additional 8 h, 10 mL more of H_2O_2 was added. The solution was allowed to react for an additional 20 h and then NaN_3 (39 g, 0.6 mol) was added. The pH was adjusted to 7.5 with 1 M H_2SO_4 and the mixture was maintained at that pH by adding 1 M H_2SO_4 with a peristaltic pump. The solution was warmed to 30 °C for 14 h. GC analysis indicated a complete reaction. The methanol was removed under reduced pressure. After 100 mL of water was added, the solution was extracted with dichloromethane (150 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and then evaporated to remove solvent. Hexane was added to precipitate benzamide. This mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in pyridine (36.4 mL) and reacted with acetic anhydride (34 mL, 360 mmol). After 3 h at room temperature, methanol (8 mL) was added to quench the excess acetic anhydride. Ethyl acetate (150 mL) was added, and then the resulting solution was washed with water (100 mL × 2), 1 N HCl (100 mL × 2), saturated $NaHCO_3$, and brine and then dried over Na_2SO_4. The solvent was removed under reduced pressure. Distillation of the residue yielded 52 g of **9** (76% based on acrolein diethyl acetal, bp 88-89 °C (0.4 mmHg)). ^1H NMR data are in agreement with that reported.[3a]

Resolution of Compound 9. Method A. A solution of **9** (2.31 g, 10 mmol) in 100 mL of phosphate buffer solution (0.05 M, pH 7) was mixed with 100 mg of lipoprotein lipase 80 from *Pseudomonas* (from Amano Co.) at room temperature with stirring. The pH was maintained at 7.0 with a peristaltic pump by adding 0.25 N NaOH and the degree of conversion was monitored by the consumption of base. After 50% conversion (12 h), the reaction mixture was extracted with ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and the solvent was removed in vacuo. The products were separated by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:12 to 1:8) to give 1.06 g (46%) of **9a** ($[\alpha]^{25}_D$ -5.1° (*c* = 1.5, $CHCl_3$)) and 0.89 g (47%) of alcohol **10** ($[\alpha]^{25}_D$ +45.5° (*c* = 1.5, $CHCl_3$)). The R_f values of **9a** and **10** were 0.42 and 0.17 (ethyl acetate/*n*-hexane = 1:5), respectively. Compound **10**: ^1H NMR ($CDCl_3$/TMS) δ 1.23 (3 H, t, J = 7.1 Hz), 1.26 (3 H, t, J = 7.1 Hz), 2.47 (1 H, d, J = 6.0 Hz), 3.35 (1 H, dd, J = 6.0 Hz and 12.8 Hz), 3.51 (1 H, dd, J = 3.6 Hz and 12.8 Hz), 3.51 (1 H, dd, J = 3.6 Hz and 12.8 Hz), 3.60 (1 H, m), 3.77 (4 H, m), 4.47 (1 H, d, J = 6.2 Hz). The enantiomeric excess of **9a** was determined to be greater than 98% by ^1H NMR in the presence of Eu(hfc)$_3$ (24 mg). The relative intensities of the acetoxy group at 3.52 (major) and 3.57 (minor) were used for ee determination. On the other hand, compound **10** was treated with (+)-2-methoxy-2-(trifluoromethyl)phenylacetyl chloride and the resulting ester was analyzed by ^1H NMR to establish an ee of 97%. The relative intensities of the methine proton in the acetal group at 4.66 (d, major) and 4.54 (d, minor) were measured for ee determination.

Method B. The same substrate solution as described in method A was mixed with an immobilized lipase (prepared by stirring 100 mg of the enzyme and 5 g of XAD-8 (from Sigma) in 5 mL of 0.05 M phosphate, pH 7, overnight at 8 °C) at room temperature with stirring. The resulting solution was then treated in the same way as in method A. When the conversion reached 50% (5 h), the immobilized enzyme was removed by filtration and washed with dichloromethane (the immobilized enzyme recovered had 80% activity retained). The combined filtrate was separated and then the water layer was extracted with ethyl acetate to recover the product.

Hydrolysis of 9a and Determination of Stereochemistry. To a solution of **9a** (1.19 g) in 10 mL of methanol cooled with an ice water bath was added NaH (12 mg, 0.5 mmol). The solution was stirred for 30 min at the same temperature and then for 2 h at room temperature. After 10 mL of brine solution was added, the resulting mixture was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1:5) to give 0.916 g of a product which is the enantiomer of **10**. To generate the aldehyde, the acetal was treated with 0.1 N HCl according to the procedure described previously.[3a] The stereochemistry of **2** was determined on the basis of the product obtained after aldol reaction and reductive amination.

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2,4-Dimethylene-1,3-cyclobutanediyl, the Non-Kekulé Isomer of Benzene. Synthesis, EPR, and Electronic Spectroscopy

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Abstract: The preparation and direct observation of triplet 2,4-dimethylene-1,3-cyclobutanediyl (**1**), the non-Kekulé isomer of benzene, is described. The biradical was generated by photolysis of 5,6-dimethylene-2,3-diazabicyclo[2.1.1]hex-2-ene (**2**) (which was synthesized in several steps from benzvalene) under cryogenic, matrix isolation conditions. Biradical **1** was characterized by EPR spectroscopy ($|D/hc|$ = 0.0204 cm^{-1}, $|E/hc|$ = 0.0028 cm^{-1}) and found to have a triplet ground state. The Δm_s = 2 transition displays hyperfine splitting attributed to a 7.3-G coupling to the ring methine and a 5.9-G coupling to the exocyclic methylene protons. Several experiments, including application of the magnetophotoselection (mps) technique in the generation of biradical **1**, have allowed a determination of the zero-field triplet sublevels as x = -0.0040, y = +0.0136, and z = -0.0096 cm^{-1}, where x and y are, respectively, the long and short in-plane axes and z the out-of-plane axis of **1**. Triplet **1** is yellow-orange and displays highly structured absorption (λ_{max} = 506 nm) and fluorescence (λ_{max} = 510 nm) spectra, with vibronic spacings of 1520 and 620 cm^{-1} for absorption and 1570 and 620 cm^{-1} for emission. The spectra were unequivocally assigned to triplet **1** by the use of a novel technique that takes advantage of the biradical's photolability. The absorption has ϵ = 7200 M^{-1} cm^{-1} and f = 0.022, establishing that the transition is spin-allowed. Further use of the mps technique has demonstrated that the transition is x-polarized, and the excited state is therefore of B_{1g} symmetry, in accord with theoretical predictions.

A large variety of thermal and photochemical reactions are generally believed to involve biradicals as short-lived intermediates, proceed via biradical-like electronic states, or traverse transition structures envisioned as possessing biradical character.[2] While this has historically provided much of the motivation for the study of biradicals, the attention of investigators in the field has recently

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