tyn-3-ol [bp 110 °C (45 mm); $[\alpha]^{20}_D$ -4.85 (c 4, $CHCl_3$)] was obtained. Examination of the NMR spectrum in the presence of tris[3-((heptafluoropropyl)hydroxymethylene)-d-camphorato]europium(III) ($Eu(hfc)_3$) indicated an enantiomeric mixture of 97.2% S and 2.8% R (94.4% ee).

In conclusion, NB-Enantrane is an attractive substitute for Alpine-borane prepared from (-)-α-pinene. Owing to its easy preparation from cheap and commercially available nopol, it can be used for the prepartion of (S)-propargyl alcohols in large quantities and high yield. Nopol benzyl ether liberated in the reduction may be easily isolated during purification of the product and recycled.

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Registry No. (-)-1, 35836-73-8; (-)-1 phthalate, 81555-87-5; (-)-1 phthalate α-methylbenzylamine salt, 81601-67-4; (-)-2, 74851-17-5; 3, 81971-15-5; 1-octyn-3-one, 27593-19-7; 2-nonyn-4-one, 81971-16-6; 1-(trimethylsilyl)-1-octyn-3-one, 53210-14-3; 4-heptyn-3-one, 32398-68-8; 1-cyclohexyl-2-octyn-1-one, 81971-17-7; 4-phenyl-3-butyn-2-one, 1817-57-8; ethyl 4-oxo-2-nonynoate, 72036-38-5; (s)-1-octyn-3-ol, 32556-71-1; (s)-2-nonyn-4-ol, 81077-11-4; (s)-4-heptyn-3-ol, 81971-18-8; (s)-1-cyclohexyl-2-octyn-1-ol, 81971-19-9; (s)-1-phenyl-1-octyn-3-ol, 81555-86-4; (s)-ethyl 4-hydroxy-2-nonynoate, 81971-20-2.

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Enzyme-Catalyzed Organic Synthesis: NAD(P)H Cofactor Regeneration Using Ethanol/Alcohol Dehydrogenase/Aldehyde Dehydrogenase and Methanol/Alcohol Dehydrogenase/Aldehyde Dehydrogenase/Formate Dehydrogenase[1]

Summary: An enzyme-catalyzed system potentially applicable to large-scale synthesis is described.

Sir: We have recently described a number of methods for regeneration of NAD(P)H from $NAD(P)^+$ for use in enzyme-catalyzed organic syntheses requiring nicotinamide cofactors.[2-8] In this paper we compare two additional useful schemes, and apply these schemes to syntheses producing 0.1–0.5-mol quantities of products (Figure 1). The first method is based on catalysis by two enzymes—alcohol dehydrogenase (ADH, EC 1.1.1.1) and aldehyde dehydrogenase (AldDH, EC 1.2.1.5)—and converts ethanol to acetate. This scheme has been demonstrated previously

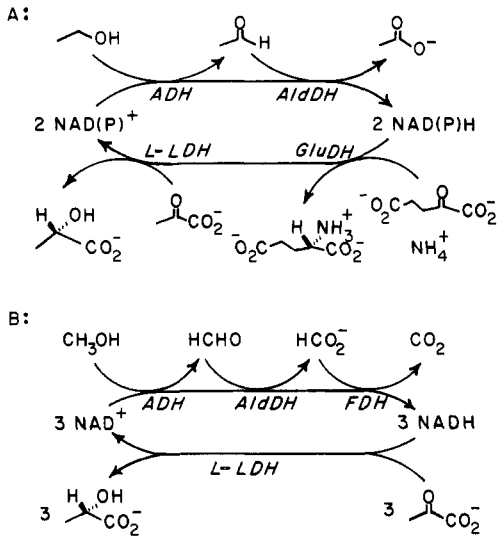

Figure 1. A: Regeneration of NAD(P)H using ethanol and alcohol dehydrogenase/aldehyde dehydrogenases. B: Regeneration of NADH using methanol and alcohol dehydrogenase/aldehyde dehydrogenase/formate dehydrogenase. Abbreviations: ADH, alcohol dehydrogenase from yeast (for NAD) or from *L. mesenteroides* (for NAD or NADP); AldDH, aldehyde dehydrogenase from yeast (for NAD or NADP); L-LDH, L-lactic dehydrogenase; GluDH, glutamic dehydrogenase; FDH, formate dehydrogenase.

in millimole-scale syntheses.[9,10] The second uses ADH, AldDH, and formate dehydrogenase (FDH, EC 1.2.1.2) and converts methanol to CO_2. The first generates 2 equiv of reduced nicotinamide cofactor/equiv of ethanol, and accepts either NAD^+ or $NADP^+$; the second generates 3 equiv of reduced nicotinamide cofactors/equiv of methanol but accepts only NAD^+. The relevant kinetic parameters for the enzymes in these schemes are summarized in Table I. The only feature of these parameters which requires specific comment concerns ADH: the enzyme from *Saccharomyces cerevisiae* has high specific activity with ethanol but is specific for NAD^+; that from *Leuconostoc mesenteroides* reduces both NAD^+ and $NADP^+$ but has lower specific activity.

In a representative procedure for NADH regeneration using ethanol as ultimate reducing agent (A, Figure 1), a 500-mL solution containing potassium pyruvate (15.1 g, 120 mmol), NAD^+ (50 μmol), ethanol (3.3 g, 70 mmol), and β-mercaptoethanol (39 mg, 0.5 mmol) was mixed with PAN-immobilized yeast ADH (90 units, 0.5 mL of gel),[11] AldDH (110 units, 10 mL of gel), and L-lactic dehydrogenase (L-LDH, 200 units, 0.5 mL of gel). The reaction mixture was stirred at 25 °C under argon, and the pH of the solution was controlled automatically at 8.0–8.2 by adding 2 N KOH through a peristaltic pump. More ethanol (3.3 g, 60 mmol) was added after 1 day. The reaction was complete in 2 days. The solution, after removal of the enzyme-containing gel, was concentrated to 20 mL and acidified with concentrated H_2SO_4 to pH 2.8, followed by addition of ethanol (200 mL). The precipitates were separated by filtration and discarded, and the filtrate was concentrated at room temperature to an oily residue. The residue was diluted with water (150 mL) and neu-

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Table I. Kinetic Parameters for Alcohol Dehydrogenase, Aldehyde Dehydrogenase, and Formate Dehydrogenase

enzyme	substrate K_m, mM	sp act., units mg^{-1}
alcohol dehydrogenase[a]	EtOH (13), NAD (0.074)	400
S. cerevisiae	MeOH (80), NAD	10
alcohol dehydrogenase[b]	EtOH, (50), NAD (0.5)	70
L. Mesenteroides	EtOH (50), NADP (0.085)	90
	MeOH (70), NAD	1.5
	MeOH (70), NADP	2
aldehyde dehydrogenase[c]	CH_3CHO (0.009), NAD (0.03)	80
S. cerevisiae	CH_3CHO (0.009), NADP (0.03)	8
	HCHO (0.7), NAD	36
	HCHO (0.7), NADP	4
formate dehydrogenase[d]	HCO_2^- (13), NAD (0.09)	3
Candida boidinii		

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Table II. Advantages and Disadvantages of NAD(P)H Regeneration Systems

system	advantages	disadvantages
EtOH/ADH/AldDH	1. high equilibrium constant for formation of NAD(P)H	1. requires two enzymes
	2. applicable to NAD^+ and $NADP^+$	2. ADH and AldDH are sensitive to oxidation
	3. generates a relatively innocous product (Ac^-)	3. HOAc may complicate some product isolations
	4. good specific activities for NAD^+ and EtOH	4. specific activities are only moderate for $NADP^+$ (AldDH)
	5. enzymes commercially available	
	6. good TN for NAD(P)(H)	
CH_3OH/ADH/AldDH/FDH	1. inexpensive reducing agent	1. requires three enzymes
	2. ultimate product (CO_2) is volatile	2. ADH, AldDH sensitive to oxidation
	3. restricted to NAD^+ (at least while using this FDH)	3. specific activities are only moderate for MeOH, H_2CO, and HCO_2H
	4. enzymes commercially available	
	5. good TN for NAD(H)	

tralized by adding $ZnCO_3$ (8.2 g, 65 mmol) to obtain crystalline zinc L-lactate (32.6 g of solid containing 96% zinc bis(L-lactate) trihydrate (106 mmol); 88% yield and 94% ee).[3] The turnover number (TN) and residual activities were as follows: NAD^+, 2400 (92%); ADH, 6×10^7 (88%); AldDH, 5×10^6 (87%); L-LDH, 1×10^7 (96%). The recovered enzymes were used for repeated preparations of L-lactate on the same scale; five preparations in a period of 11 days generated 0.5 mol of L-lactate. The recovered enzyme activities after these five cycles of synthesis were as follows: ADH, 70%; AldDH, 71%; L-LDH, 78%.

In a typical procedure illustrating the operation of ADH/AldDH for NADPH regeneration, a 500-mL solution containing monopotassium α-ketoglutarate (22 g, 120 mmol), $NADP^+$ (50 μmol), and ethanol (3.3 g, 70 mmol) was neutralized with NH_4OH to pH 8.0–8.2. β-Mercaptoethanol and PAN-immobilized ADH from *L. mesenteroides* (90 units based on $NADP^+$, 1 mL of gel), AldDH (92 units based on $NADP^+$, 25 mL of gel) and glutamic dehydrogenase (GluDH, 180 units, 1 mL of gel) were added. The reaction was conducted at pH 8.2 and was complete in 2 days. The decanted solution was concentrated to ~100 mL, adjusted to pH 6.5, and treated with ethanol (80 mL) until the solution became turbid. A crystalline solid (20 g) containing 97% of monopotassium L-glutamate (104 mmol, 86% yield) was obtained after cooling. The TN and residual activities were as follows: $NADP^+$, 2400 (82%); GluDH, 1×10^7 (88%); ADH, 2×10^7 (86%); AldDH, 1×10^6 (86%).

The operation of the redox system using NAD^+ as cofactor and methanol as ultimate reducing agent is illustrated by a preparation of L-lactate. A 600-mL solution containing potassium pyruvate (15 g, 120 mmol), NAD^+ (10 μmol), yeast ADH (30 units, 15 mL of gel, activity based on methanol as substrate), AldDH (34 units, 6 mL of gel, activity based on formaldehyde as substrate), FDH (30 units, 12 mL of gel), and L-LDH (50 units, 0.5 mL of gel) was deoxygenated with Ar, followed by addition of methanol (1.5% v:v, 0.3 M) and mercaptoethanol (final concentration, 4 mM). The pH of the reaction mixture was controlled at 8.0–8.2. After 4 days, L-lactate was isolated as its zinc salt (16.2 g, 94% purity, 86% yield, 92% ee). The TN and residual activities were as follows: NAD^+, 1200 (90%); FDH, 3.2×10^5 (88%); ADH, 3×10^5 (82%); AldDH, 9×10^5 (86%); L-LDH, 1.8×10^7 (96%).

The most important feature of these preparations is the use of ratios of AldDH/ADH/(FDH) such that the first step (formation of aldehyde) is the slow step. This protocol insures that the aldehyde concentration in the reactor remains as low as possible and protects the enzymes from deactivation.

The advantages and disadvantages of these systems are listed in Table II. The balance of these characteristics is that these systems are potentially more economical for nicotinamide cofactor regeneration in connection with large-scale synthesis than most others but are somewhat less convenient for laboratory-scale (~0.1–10 mol) syntheses than those based on glucose 6-phosphate[3] or formate.[2] We note that it should be relatively straightforward by recombinant DNA techniques to produce microorganisms having high contents of the two or three enzymes required in these schemes in the correct ratios

for optimum performance. The use of immobilized whole organisms of this type, or of crude extracts from them, would be the most efficient method for preparation of the required enzymatic activities.

Registry No. NADH, 58-68-4; NAD^+, 53-84-9; NADPH, 53-57-6; $NADP^+$, 53-59-8; ADH, 9031-72-5; AldDH, 9028-88-0; FDH, 9028-85-7; ethanol, 64-17-5; methanol, 67-56-1.

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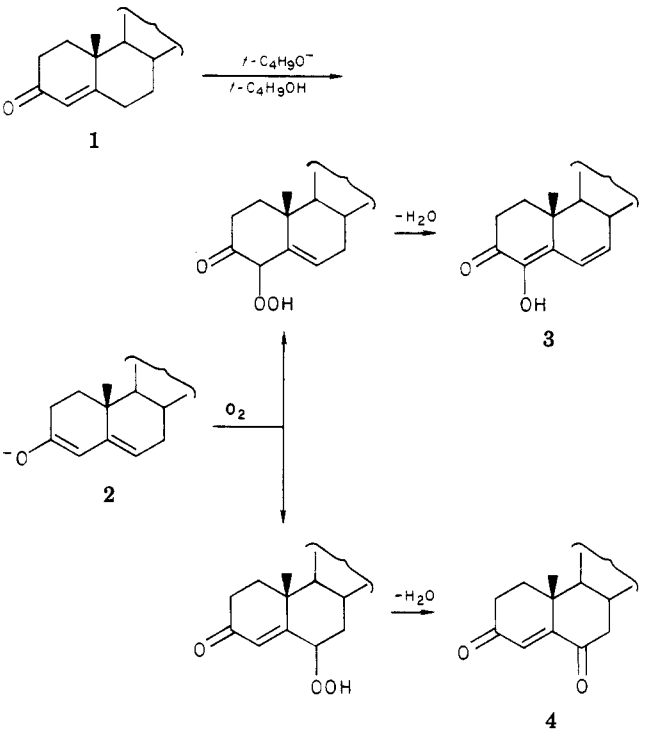
Superoxide-, *tert*-Butoxide-, and Hydroxide-Mediated Autoxidation of 3-Oxo-Δ^4 Steroids in Aprotic Media

Summary: The course of the base-catalyzed autoxidation of 3-oxo-Δ^4 steroids in aprotic media differs sharply both in yield and product distribution from that reported for protic solvents and involves the oxygenation of the kinetic dienolate.

Sir: The base catalyzed autoxidation[1] of various 3-oxo-Δ^4 steroids (1) in protic media was studied by Camerino and co-workers[2] in the early sixties. They reported that enones 1 react with oxygen in the presence of potassium *tert*-butoxide in *tert*-butyl alcohol, generating in low yield the corresponding 4-hydroxy-Δ^6-dehydro and 6-keto derivatives (ketones **3** and **4**, respectively). For example, when 4-cholesten-3-one is the substrate (1), enones **3** and **4** are isolated in 15% and 5% yield, respectively; no information is supplied by the authors as to the identity or fate of the remaining 80% of the product. In any case, it would seem clear that **3** and **4** result from oxygenation of the α(C-4) and γ(C-6) carbons of the thermodynamic[3] dienolate **2**.

Our interest in the base-catalyzed autoxidation of enones and enols in aprotic solvents,[4] particularly those mediated by the biologically important superoxide anion radical,[5] led us to examine the reaction of the steroidal analogues. As the results outlined in Scheme I indicate, the course of these oxidations differs substantially both in product distribution and yields from those reported by Camerino et al.[2] In a typical reaction, 4-cholesten-3-one,18-crown-6, and potassium superoxide (KO_2) in a 1:2:4 molar ratio were dissolved in dry benzene (100 mL/mmol of steroid) and



1 2 3 4

stirred under dry air at ambient temperature until all the steroid had been essentially consumed (~20 h). The reaction mixture was then acidified with 10% HCl and extracted 3 times with $NaHCO_3$ solution during which time an orange precipitate formed which floated in between the aqueous and organic layers. The organic phase was dried, concentrated, and chromatographed (preparative TLC on silica gel, using 25% acetone in hexane as eluent), yielding lactol **9** (30% yield) and a mixture of lactols **10** (4%) and **11** (6%). The latter were readily separable once they were converted by Ag_2O/CH_3I[6] to the corresponding keto esters **16** and **17**. The aforementioned precipitate was dissolved in 10% HCl (overnight) and the resulting solution was extracted with chloroform. Evaporation of the extracts gave a white precipitate (60% yield), which was treated with diazomethane. Separation of the resulting mixture of esters by preparative TLC gave **18**, **19**, and **20** in a molar ratio of 4:1:1. Esters **15–20** could be obtained directly from the reaction mixture by treating the latter with excess methyl iodide prior to aqueous workup. Similar results were obtained with testosterone.

Several important observations should be made regarding this reaction. Firstly, the course of this reaction remains essentially unchanged when KO_2 is replaced by KOH or potassium *tert*-butoxide except that the rate of reaction is fastest with the latter and slowest with KOH. Hence it is probable that the mechanism of the superoxide anion radical mediated process (like *tert*-butoxide and hydroxide) entails initial proton (not hydrogen-atom) abstraction,[4,7] which is followed by the various steps typical of base-catalyzed autoxidation.[1]

Secondly, when the reaction is quenched at shorter reaction times, in addition to starting material substantial amounts of enol **6** are obtained. The conversion of the latter to lactol **9** under the reaction conditions is quantitative and is likely to proceed by the mechanism suggested for 2-hydroxy-2,5-cyclohexadien-1-ones.[4b] Similarly lactol

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