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The Impact of Chemistry on Biotechnology

Multidisciplinary Discussions

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Chapter 19

Enzymatic Processes for Pheromone Synthesis

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Some general features of enzyme technology applied to synthetic organic chemistry are discussed and recent uses of enzymes to obtain critical configurations in pheromone structures are described. Commercially available lipases were screened to assess their potential to resolve methyl n-alkyl carbinols. A lipase from the fungus Mucor miehei was employed to obtain configurational isomers of the carbinol carbon of 8-methyl-2-decanol, the parent alcohol of a sex pheromone of several economically important Diabrotica (rootworm) species.

The desire to develop alternative methods for controlling insect pests led to the development of research programs in fields such as insect pheromones. Of special concern has been the synthesis of stereoisomers of those pheromones having chirality. Although usually only one stereoisomer is active while the enantiomer has no biological activity, there are exceptions. Therefore, in addition to requiring pure stereoisomers to facilitate identification and to promote fundamental studies in insect behavior, there have been instances where correct stereochemistry was critical to insect attraction (gypsy moth, japanese beetle, several important rootworm species).

A potential resource for those interested in stereochemical syntheses are enzymes, proteins that have biological activity and are responsible for catalyzing large numbers of transformations of biologically important molecules. Judging from the literature about enzymes, one might expect to see them in more general use. A glance at chemical and biochemical catalogs reveals that indeed some enzymes can be purchased, although the majority must be searched out from lists of manufacturers or "home grown". Many have been characterized, but remain yet to be isolated and formulated in a usable fashion. Additionally, the costs may seem high

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for all but specialty products. Stability is another concern; many enzymes will suffer irreversible change in solution during use although others will not. The problems of cost and catalyst longevity are, however, not independent parameters, and would be discussed in concert with considerations of catalytic rate, degree of difficulty of the particular synthetic step, and techniques that stabilize the enzyme and allow for its recovery. Substrate selectivity may be perceived as a technical problem also, though evidence has been rapidly accumulating to indicate that many familiar enzymes accept other than their natural substrates and that catalytic rates, though usually less, may still be useful.

The problems of longevity and reusability have been addressed by an array of immobilization procedures (1) in which enzymes have been alternatively deposited, adsorbed, chemically bound, and included. Deposition can be produced by salting (ammonium sulfate) or adding acetone to a crude solution containing relatively impure enzyme and a powdered solid such as diatomaceous earth. The recovered powder then has enzymatic activity, though exposure to aqueous reaction mixtures will quickly remove the enzyme. Similarly, adsorption to ion exchange resins, though interactions are stronger, serves the purpose of carrying out reactions in non-aqueous media better. Inclusion into gels or capture by controlled pore glass have also been explored. Desorption, or leaching, of enzyme activity is reduced compared to celite deposits. The most permanent technique involves chemical bonding, frequently accomplished by employing the free amino groups from lysine residues that are expected to be present at the surface of globular proteins. Such methodology produces long lasting activity in aqueous reaction mixtures, but the yield of activity is often low. One can imagine that the random binding to a solid support could occasionally produce a structure that could no longer undergo the manifold of conformational changes necessary to catalyze the desired chemical reaction. Given a particular transformation that one wishes to perform, the scale of the reaction, and degree of concern about enzyme recovery, one can screen several common immobilization techniques using the desired catalyst. The following is a brief review of recent reports of asymmetric syntheses that were targeted for selected chiral insect pheromone structures. These can be viewed as exemplifying two broad categories of reaction. In one a prochiral center is reduced asymmetrically; in the other a racemic material is resolved by selective reaction of one enantiomer.

Asymmetric Induction

The reducing power of *Saccharomyces cerevisiae* (Baker's yeast) has been exploited frequently for the preparation of chiral alcohols. Ethyl acetoacetate was reduced by Mori (2) achieving 70-74% ee (enantiomeric excess) of ethyl (*S*)-3-hydroxybutyrate that was then converted to sulcatol, the aggregation pheromone of the Ambrosia beetle, *Gnathotricus sulcatus* (Figure 1). Optical yields in this reduction reported from different laboratories vary, and this may reflect differences in yeast strain and/or conditions of reaction.

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In a subsequent study to produce (R)-4-hexanolide, a pheromone of the dermestid, *Trogoderma glabrum* (Figure 1), an enzymatic reduction of ethyl 3-oxopentanoate was sought. For this substrate Baker's yeast produces the (R)-enantiomer although the stereobias is much less. By enlarging the hydrophobic substituent (ethyl \rightarrow phenylthioethyl) and screening several reducing yeasts, a suitable reductant, *Pichia terricola*, was found that produced the desired configuration in 94% ee (3). Subsequent chemical elaboration of the carbomethoxy group to permit generation of the lactone ring was followed by reductive removal of the phenylthio unit.

An additional important use for reducing-enzymes (Figure 2) is for the hydrogenation of double bonds that are conjugated to a carbonyl. For example, citronellol can be oxidized to an α,β -unsaturated aldehyde that undergoes Baker's yeast reduction at both carbonyl and carbon-carbon double bond generating an (S)-configuration on the new saturated methyl branched carbon atom (4). Using this synthetic sequence (R)-and-(S)-citronellols have been converted to (2S,6R) and (2S,6S)-2,6-dimethyl-1,8-octanediols. These diols can be viewed as useful chiral synthons for the many natural products that are 1,5-dimethylated acyclics such as pheromone structures of pine sawflies, red flour beetle, and tsetse fly species. The authors point out that one could use the (2S,6R)-isomer to build the (R,R)-isomer of 17,21-dimethylheptatriacontane.

Kinetic Resolution

Kinetic resolution makes use of a preferential reaction of one of two enantiomers of a racemate. In contrast to the reductions just described that require cofactors (the cells of the cultures were being used rather than an enzyme preparation), hydrolyzing enzymes (proteases, esterases, lipases) generally do not require cofactors and powders derived from cultures can be employed. Whereas carbonyl reduction can in principle provide a complete conversion to one enantiomer, resolutions are usually performed with the goal of obtaining quantities of both enantiomers pure. Then, if one seeks just one enantiomer, the unwanted isomer must be transformed by stereospecific chemical processes to the desired one. In any case, the selectivity of the reaction must be quite high or much material will be lost before the remaining (slower reacting) enantiomer is pure enough for the purposes of the effort.

A strain of an *Aspergillus* species (Amano Co.) has been cultured for N-deacylase activity. The selectivity for (S)-acylated amino acids is nearly absolute. Racemic threo-2-amino-3-methylhexanoic acid was synthesized and converted to an acetamide (5) (Figure 3). Treatment with the deacylase produced the (2S,3R)-amino acid. Further treatment of recovered deacylated material provided a sample of the enantiomer as its amide that was then hydrolyzed to the (2R,3S)-amino acid. These amino acids were employed initially to obtain the remaining diastereomers by inverting the 2-position, then to prepare the enantiomers of threo-4-methyl-3-heptanol, a pheromone component of the smaller European elm bark beetle, *Scolytus multistriatus*.

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In similar fashion, racemic 2-aminodecanoic acid was resolved as its N-chloroacetyl derivative (6) (Figure 3). The amino-bearing carbon retained its configuration during deamination that replaces NH_2 with OH, and the carboxyl terminus was extended by conventional means that preserved the compound's stereochemistry. The ultimate products were the enantiomeric 4-dodecanolides that are produced in the pygidial glands of rove beetles. This lactone was also synthesized by microbial reduction by another group (7).

A study of hydrolysis of acetates of alkynyl alcohols using *Bacillus subtilis* var Niger was studied (8) (Figure 4). The optical purities obtained were highly dependent on the alkyl group present and, for the 4-methyl-3-pentenyl unit (Figure 4) were only fair. Nevertheless such a structure is convertible to the pheromone of the Japanese beetle and an improved enzymatic process would probably be a worthwhile objective, since the current industrial preparation begins with the unnatural isomer of glutamic acid.

Mathematical equations that describe enzymatically driven resolutions have been developed (9). These allow calculation of the ratio of specificity constants (V_{max}/K_M) for a pair of enantiomers and give a constant value for this ratio with stipulated provisions. A convenient form of this expression that may be more useful generally for synthetically oriented chemists has also appeared (10) (Figure 5). Although the rate ratios are actually ratios of specificity constants with attendant limitations to their validity (9), the use of K_R and K_S was employed for simplicity and the sake of comparison (10). Since lipase catalyzed resolutions to be described always resulted in faster reaction of R-enantiomers, the rate ratios are depicted as k_R/k_S . Operationally, one need only determine the fraction of starting material converted (C) and the enantiomeric excess (ee) of the starting material. The product ee could, of course, be determined instead and transformed to starting material ee. A way to view this is as follows: if you had set the goal of 95% ee in your unreacted starting material, a rate ratio of 2.6 requires that the reaction proceed to 95% conversion. The product ee would only be 5. For an infinitely large rate ratio, one only needs to go to 50% conversion. A "useful" rate ratio is subjective, and can be maximized by screening sources of enzymes, strain selection processes, optimizing reaction conditions and altering substrate choice.

For a rate ratio of 10 and a 34% conversion, the product ee still exceeds that of starting material (78:40). But even with this relatively low rate ratio, 90% ee in the starting material is obtained after 63% conversion.

Screening Lipases to Kinetically Resolve Secondary Alcohols

Apart from the evident utility of configurationally pure aliphatic secondary alcohols as chiral building blocks, a number of pheromone structures are esters of methyl alkylcarbinols. In particular, 8-methyl-2-decanol is the parent alcohol for esters that have been identified for several closely related species of

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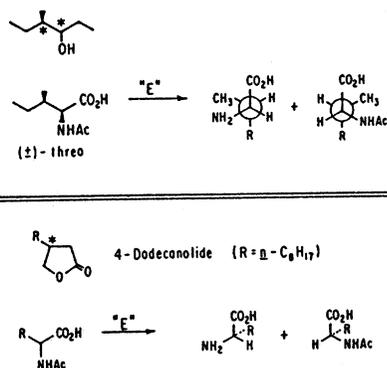


Figure 3. Smaller European Elm Bark Beetle.

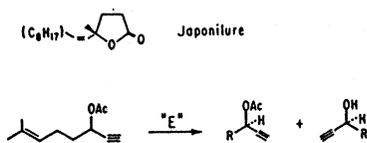


Figure 4. Kinetic resolution of alkynyl alcohols using *Bacillus subtilis*.

RATE RATIOS (K_R/K_S) WERE CALCULATED FROM THE EXPRESSION:

$$\frac{K_R}{K_S} = \frac{\ln(1-C) (1-ee)}{\ln(1-C) (1+ee)}$$

C = FRACTION OF RACEMIC STARTING MATERIAL CONVERTED.

ee = ENANTIOMERIC EXCESS OF RESIDUAL STARTING MATERIAL.

FOR HYDROLYSIS, THE "ee" WAS DETERMINED IN THE PRODUCT AND THEN RELATED TO THE STARTING MATERIAL:

$$ee = \frac{C (ee_B)}{1-C}$$

A REFERS TO STARTING MATERIAL; B TO PRODUCT.

Figure 5. Equations that characterize enzymatic resolution (9). Since R-alcohols and their esters react faster with the lipases studied than S-alcohols, rate ratios are expressed as K_R/K_S .

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rootworm (genus *Diabrotica*). These compounds contain two asymmetric centers, one involving the secondary alcohol (carbon 2) and the other is designated as the hydrocarbon center (carbon 8). The responses of seven species to the stereoisomers in which the hydrocarbon center is *R* are tabulated in Figure 6. The insects apparently do not respond to isomers with an (*S*) hydrocarbon center - synthetics can be racemic at that site and, excepting for effects of dilution, elicit the same response as the corresponding (*R*)-8 stereoisomer. However, responses to the carbinol site are varied, and evidence of inhibition has been obtained for tests of mixtures that contain blends of (*R*)-2 and (*S*)-2 materials. Since the racemic alcohol can be readily synthesized, such as by a procedure developed for the USDA by Zoecon Corporation (12), an efficient enzymatic resolution of the racemic carbinol center could be a valuable synthetic process. The ability to monitor these species selectively by using synthetics as baits in traps may depend critically on the stereochemical constitution of those chemicals.

We initially examined reactions involving 2-octanol and its esters as models using commercially available lipase preparations. Lipases have indeed been employed to perform resolutions (13), but the alcohols (esters) involved were usually alicyclics. Enzymatic resolution of methyl *n*-alkylcarbinols is much more difficult and has only been occasionally reported (13). In addition, general screening of lipases for this purpose does not seem to have been done. The enzymes were calibrated for activity on olive oil (ca. 85% triolein) using an initial rate assay (Figure 7). Considerable differences exist in these lipase preparations, and this can be attributed to the degree of purification as well as to intrinsic differences between the enzymatically active proteins. At this point little is known of the structure or mechanism of action of triglyceride lipases. We opted therefore to treat these materials simply as undefined, but potentially useful catalysts for organic synthesis.

An examination of the esterification of 2-octanol using octanoic acid in hexane at 30°C with several lipases (Figure 8) gave a strong indication that the *M-miehei* lipase sold by NOVO Co. would be useful for our purposes (14). This listing of lipase is far from exhaustive, and merely represents a few readily available materials some of which are in commercial use now.

Intriguingly, the esterification's stereochemical consequences are dependent on the length of the fatty acid chain (Figure 9). Acetic acid did not become esterified in the presence of the *M-miehei* lipase, and stereoselection for (*R*)-2-octanol increased proceeding to hexanoic acid, diminished beyond nonanoic, and increased again to hexadecanoic. Substituting 2-hexanol as the alcohol to be esterified produced a similar profile that showed overall lowered stereoselection. For example, the rate ratio calculated for 2-octanol and hexanoic acid was >50:1. The corresponding rate ratio for 2-hexanol was 9.5:1. A similar profile was also obtained for 2-decanol; rate ratios were >50:1 for a wider range of fatty acids. The usual conceptualization of the mechanism of hydrolase activity invokes the intermediacy of an

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INSECT	RESPONSE TO CARBINOL CENTER		
	2 (R)	2(S)	ESTER
<u>D. VIRGIFERA VIRGIFERA</u>			
WESTERN CORN ROOTWORM	++	+	PROPIONATE
<u>D. VIRGIFERA ZEA</u>	++	+	PROPIONATE
MEXICAN CORN ROOTWORM			
<u>D. BARBERI</u>	++	I	PROPIONATE
NORTHERN CORN ROOTWORM			
<u>D. PORRACEA</u>	-	++	PROPIONATE
<u>D. LEMNISCATA</u>	+	++	PROPIONATE
<u>D. LONGICORNIS</u>	I	++	PROPIONATE
<u>D. CRISTATA</u>	-	++	ACETATE

D = DIABROTICA; I = INHIBITORY

THOSE INSECTS WITH TRIVAL NAMES ARE ECONOMIC PESTS

Figure 6. Responses of Diabrotica (rootworm) species to esters of 8-(R)- methyl-2(R or S)-decanol.

LIPASE	MANUFACTURER-CODE	UMOLMIN ⁻¹ MG ⁻¹	P. SELECTIVITY ^B	F.A. SELECTIVITY ^C
ASPERGILLUS NIGER,1	AMANO-AP	0.154	1,3	18(Δ9)
ASPERGILLUS NIGER,2	AMANO-K	11.2	1,2,3	10,12
CANDIDA RUGOSA	ENZECO	9.82	1,2,3	18(Δ9)
	SIGMA	9.70	1,2,3	18(Δ9)
MUCOR MIEHEI	AMANO-MAP	2.67	1,3	<12
	GIST BROCADES-S	40.0	1,3	<12
	NOVO (POWDER)	5.33	1,3	<12
	NOVO 3A (RESIN)	0.095	1,3	<12
PORCINE PANCREATIC ^E	SIGMA	15.6	1,3	4
RHIZOPUS ARRHIZUS ^F	GIST BROCADES	33.4	1,3	8,10

Figure 7. INITIAL RATE ASSAYS OF COMMERCIAL LIPASES ON OLIVE OIL.^A

^APH MAINTAINED AT 7.3 UNLESS OTHERWISE INDICATED. ^BPOSITION SELECTIVITY WITH RESPECT TO TRIGLYCERIDE HYDROLYSIS. ^CFATTY ACID SELECTIVITY PREVIOUSLY ESTABLISHED; THE PREFERENCES ARE SLIGHT IN MOST CASES. ^DTHE POWDER FORM IS NOT CURRENTLY AVAILABLE, THOUGH THE LIPASE IS NOW FORMULATED ON BOTH AN ION EXCHANGE RESIN SOLD AS "3A" AND AS A SOLUTION AS "225". ^EpH 8.0 WITH 0.2 ML OF 0.3 M CaCl₂ TO 5 ML EMULSION. ^F0.2 ML OF 0.3 CaCl₂ TO 5 ML EMULSION.

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ESTERIFICATION OF (±)-2-OCTANOL WITH OCTANOIC ACID (HEXANE, 40 C)

LIPASE	k_R/k_S
ASPERGILLUS NIGER (AMANO "AP")	TOO SLOW
ASPERGILLUS NIGER (AMANO "K")	6.1
CANDIDA RUGOSA (ENZECO)	1.1
MUCOR MIEHEI (AMANO "MAP")	TOO SLOW
MUCOR MIEHEI (G.B "S")	2.8
MUCOR MIEHEI (NOVO)	100
RHIZOPUS ARRHIZUS (SIGMA)	5.5
PANCREATIC (SIGMA)	5.4

Figure 8. Rate ratios for esterification of racemic 2-octanol with octanoic acid using several commercial lipase preparations.

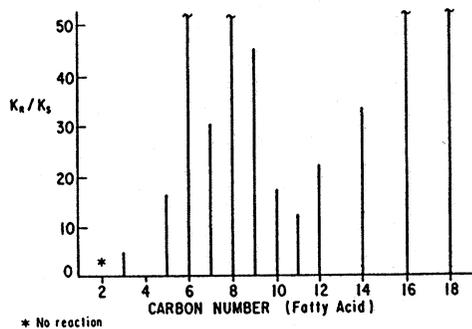


Figure 9. Enantiomeric ratio for esterification of racemic 2-octanol versus fatty acid chain length using Mucor miehei lipase.

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acyl-enzyme structure. Evidently information involving fatty acid chain length can be transmitted back to the vicinity of the acyl-bearing portion of the complex resulting in altered selection for enantiomeric alcohols. This phenomenon may be general for ester hydrolases and does not appear to have been investigated.

We subsequently evaluated resolution via ester hydrolysis and transesterification using these lipases. Hydrolysis of several esters of racemic 2-octanol are shown in Figure 10. Again, the octanoate ester appeared to be the best substrate for resolution. Transesterification with selected triglycerides gave interesting results, but it was felt that workup procedures would be more complicated. The reason for evaluating triglycerides as sources of acid residues for the resolution was that naturally occurring triglycerides such as vegetable oils or animal fats might prove to be cheaper reagents than organic fatty acids.

The information that we had obtained in evaluating esterification and hydrolysis of racemic 2-octanol was then employed to resolve 8-methyl-2-decanol, the pheromone precursor. Throughout these studies resolutions were monitored by derivatizing the recovered alcohols from reactions with (S)- α -methylbenzylisocyanate. The resulting diastereomeric carbamates are easily separated by capillary GLC. Figure 11 shows the chromatogram of the carbamates formed from the racemic alcohol; the (S,S)-diastereomer eluted first. The accompanying chromatogram is of the carbamate derivative of the hydrolysis product (26% yield after distillation of 98.8% R). Typically, esterifications produced 93% (S)-alcohol, and gave ester that was saponified to 95% (R)-alcohol each in greater than 80% theoretical yield.

Prospects

It seems likely that the current interest in enzyme technology will spur studies resulting in more complete information on the enzymes that in many cases are currently being generated by recipes designed empirically to satisfy an industrial customer. Better reference material that will tabulate reactions conducted with homogeneous enzymatically active substances would be useful. New sources of enzymatic activity will lead to a greater range of choices of substrate structure. Enzymes that have been altered chemically may become available, or will be prepared by the user. For example, we are currently working with *C. rugosa* lipase that has been derivatized to add polyethylene glycol chains thereby rendering the protein soluble in organic solvents. Such a procedure allows reactions to occur homogeneously in benzene, methylene chloride, etc, and offers some interesting alternatives to conducting reactions with the native material (15). The potential for changing enzyme selectivity by such conditions has yet to be investigated. Finally, the methods of recombinant DNA would allow the evaluation of site-selective amino acid replacements that would lead to enzymes whose catalytic activity was better tailored for the desired substrate.

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HYDROLYSIS OF (+)-2-OCTANOL ESTERS
(M. MIEHEI LIPASE = NOVO, 25°C, PH STAT 7.0)

FATTY ACID	k_R/k_S
C ₃	31
C ₈	110
C ₁₂	24

TRANSESTERIFICATION TECHNIQUES (30°C)

ESTER (CONDITIONS)	k_R/k_S
TRIBUTYRIN (NEAT)	13
TRIBUTYRIN (HEXANE)	5.8
TRIBUTYRIN (~H ₂ O)	100
TRIBUTYRIN (HEXANE, ~H ₂ O)	100
TRIPROPIONIN (HEXANE, ~H ₂ O)	28
TALLOW (HEXANE, ~H ₂ O)	6

Figure 10. Rate ratios for the transfer of the indicated fatty acid residue to racemic 2-octanol using Mucor miehei lipase.

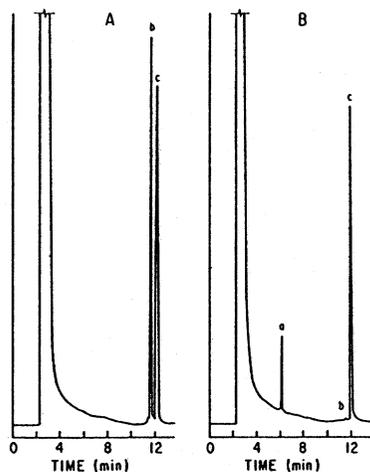


Figure 11. (A)-Chromatogram of diastereomeric carbamates formed from (S)- α -methylbenzyl isocyanate and racemic 8-methyl-2-decanol; (B)- product of lipolysis with Mucor miehei lipase: peak (a)=octanoate ester, (b) = carbamate of 2(S)-alcohol, and (c) = carbamate of 2(R)-alcohol.

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