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Hydrolytic enzymatic transformation of advanced synthetic intermediates: on the choice of the organic cosolvent

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Abstract

For enzymatic hydrolyses of advanced synthetic intermediates, the use of an organic cosolvent is often warranted. As a model study, the cosolvent dependence (at 10 volume%) of the key enzymatic step in our asymmetric synthesis of (−)-podophyllotoxin was examined. At high protein concentrations and rt, DMSO and the polyethereal organic cosolvents dioxane, diglyme and triglyme emerged as the best organic cosolvents, in terms of yield, % ee and catalytic efficiency (V_{max}/K_m). However, only in 10% triglyme was optimal efficiency maintained at both lower protein concentration and higher temperature.

1. Introduction

As part of a program directed at structure/function studies of the important etoposide family of chemotherapeutic drugs, we recently developed a chemoenzymatic synthesis of (−)-podophyllotoxin, the aglycon from which these drugs are derived (Scheme 1).[1] The synthesis represents the first catalytic, asymmetric synthesis of the natural product, and has the important feature of being modular in ring E, as that sector of the molecule deliberately enters late. The absolute stereochemistry derives from a PPL-mediated desymmetrization of advanced *meso* diacetate **4**, bearing all of the carbon atoms for rings A–D.

In scale-up efforts, we needed to push significant quantities of diacetate **4** through this enzymatic ester cleavage step in order to bring forward multigram quantities of **2**, the pivotal intermediate for SAR studies. We had been using relatively large amounts of enzyme under the standard conditions[1] that we had established for this desymmetrization (13 equiv. by total weight, 3.25 equiv. of protein; 10% DMSO as cosolvent), and had noticed that decreasing the protein concentration produced inferior results, especially in terms of ee. So, it seemed prudent to examine the cosolvent dependence of this transformation, with an eye toward optimizing enzyme performance in this mixed organic–aqueous medium.

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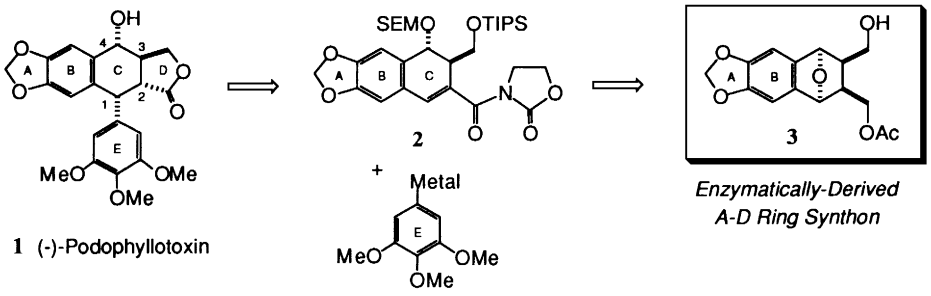
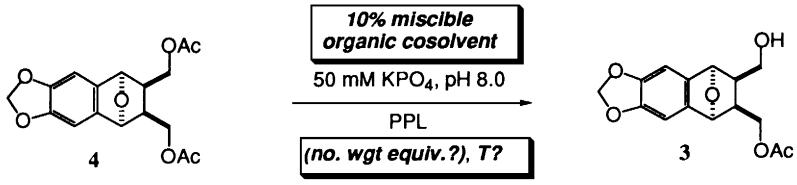


Scheme 1.

Indeed, the issue being examined here is becoming quite a general one in enzyme-assisted organic synthesis today.[2] Particularly when advanced synthetic intermediates (implying a relatively high carbon count) are employed as enzymatic substrates, aqueous solubility is often low. If one is dealing with an esterase or a lipase, it may be possible to run the reaction in the reverse direction (acylation of an alcohol or alcoholysis of an ester).[3–5] For enzymes such as nitrile hydrolases and epoxide hydrolases, the reverse reactions are presumably much more difficult to achieve.[6] Moreover, with lipases, it is quite common to obtain better results (yield, % ee) in the hydrolytic direction than in the acylation direction.[7] In such cases, one may elect to circumvent the problem of aqueous solubility by simply running the reaction as a well-agitated suspension,[7a] or as a biphasic hydrolysis in which the substrate is dissolved in an immiscible organic solvent.[8,9] While such approaches minimize contact of the enzyme with solvent, rates are sometimes unacceptably slow, being of the order of days,[8] if not weeks.[7a]

We preferred the alternative here, namely, use of a water-miscible organic solvent (Scheme 2)[10,11] to solubilize the organic substrate so as not to be limited by mass transport. With this approach, choice of cosolvent and its volume proportion are the central issues. In contrast to enzymatic esterifications or biphasic hydrolyses, wherein organic solvents with high log P values[5h,12] are generally preferable,[5c,i,k,l,13] it has been argued that with water-miscible solvents, low log P values are desirable to stabilize protein tertiary structure.[10d] In the case of a transesterification with a suspended enzyme powder in an organic solvent, it is reasoned that high log P solvents have the lowest propensity to strip essential water molecules from the enzymatic surface.[14] For biphasic hydrolyses, the prevailing notion is that by minimizing solubility of the cosolvent in water (and hence choosing solvents with high log P values), one limits enzymatic contact with solvent and maximizes enzyme performance.[4a]



Scheme 2.

With water-miscible solvents, on the other hand, Mozhaev and coworkers have shown that there is generally a threshold concentration, C_{50}, above which rapid denaturation occurs.[10c,d] For most polar organics, the C_{50} values obtained are in the 20–50 volume% range, so we chose to limit our experiments to 10 volume% cosolvent and to assay a broad spectrum of solvents for their compatibility with the key enzymatic deacylation under study here. We chose several traditional organic cosolvents (DMSO, DMF, acetonitrile and acetone) along with a number of non-traditional solvents that we felt might maintain tertiary structure. The use of TFE was motivated by its popularity as a helix-forming solvent in the peptidomimetic community. The glymes were chosen as being intermediate in structure between

glycerol (a widely used stabilizing agent for enzyme storage) and polyethylene glycols (used both as biocompatible linkers and to covalently modify enzymes[15]).

2. Results and discussion

Our results are summarized in Table 1. The values for % ee and isolated yield are then plotted as a function of solvent dielectric[14] in Fig. 1, and as a function of log P in Fig. 2. All of the entries in Table 1 represent identical conditions in terms of buffer, % cosolvent, enzyme concentration, pH and temperature (see Experimental for details).[1,7d] The only variable is the nature of the organic cosolvent present at 10 volume%. Note that, in general, we found it most practical to terminate the fastest reactions at 2/3 completion to completely avoid formation of diol. Unreacted diacetate is then recovered and recycled and this is reflected in the corrected yields presented in Table 1. From the results, it is clear that the choice of cosolvent has a very significant effect on the desymmetrization efficiency in this case. Interestingly, there is a qualitative correlation between yield and % ee. Those solvents in which the enzyme turns over unnatural substrate most rapidly (DMSO and the polyethereal solvents dioxane, diglyme and triglyme) are also those in which the highest level of selectivity is maintained.

Table 1
Solvent effects on PPL-mediated hydrolysis of diacetate **4***

Cosolvent	%ee	Yield	Corrected Yield	Time (h)	log P	ε
Dioxane	**96**	**60**	**97**	**2.5**	**-0.27**	**2.2**
Diglyme	**96**	**60**	**94**	**3**	**-1.22**	**7.2**
Triglyme	**96**	**60**	**84**	**3**	**-1.74**	**7.6**
Pyridine	n.d.	6	57	24	0.71	13
Acetone	81	30	91	24	-0.24	21
TFE	52	16	41	6	0.41	28
Acetonitrile	58	9	20	6	-0.34	37
DMF	83	26	35	7	-1.01	38
DMSO	**95**	**66**	**83**	**3**	**-1.35**	**47**
DMPU	--	no rxn.	--	32		

* Note: All reactions were run at 25 °C as described in the Experimental Section.

From Fig. 1 it is clear that there is no correlation between either % ee or % isolated yield and solvent dielectric. On the other hand, Fig. 2 suggests a weak qualitative correlation between both measures of enzymatic desymmetrization efficiency and the cosolvent log P value, along the lines implied by Mozhaev et al.[10c,d] However, there are a number of notable outliers. Dioxane is an especially good solvent here, providing higher yields and ees than would be predicted from such a graph. Acetone falls 'above the line' in terms of ee, but not yield. Also, both acetonitrile and DMF produce especially low isolated yields, falling well 'below the line' by this measure. So, we would argue against a statistically significant correlation of desymmetrization efficiency and log P for the reaction under study. However, a pattern does emerge from the data. It would appear that polyethereal solvents (bearing 2, 3 and 4 oxygens) are especially compatible with this lipase and provide a viable alternative to DMSO for hydrolyses of advanced synthetic intermediates.

A steady state kinetic analysis of the formation of product was then undertaken. The rate of product formation was assessed by ^{1}H NMR and the data worked up as detailed in the Experimental. In terms of V_{max}/K_m, one sees little difference among the top four solvents, at room temperature (Table 2).

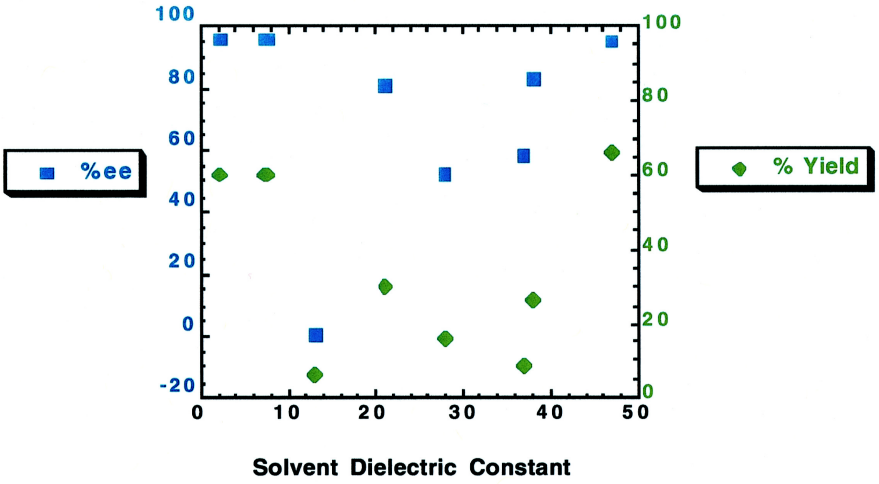


Fig. 1. Desymmetrization efficiency vs cosolvent dielectric

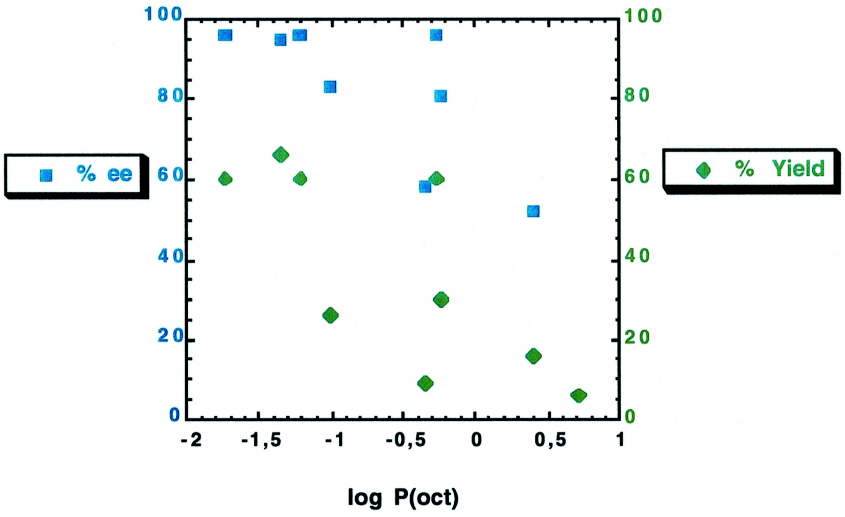


Fig. 2. Desymmetrization efficiency vs log P

Interestingly, this reflects a reduced affinity of the enzyme for **4** in 10% dioxane (increased K_m), that is almost equally compensated for in terms of an increased V_{max}. On the other hand, V_{max}/K_m for DMF, one of the less effective solvents, lies more than an order of magnitude below the values for all of the top solvents. This is largely a reflection of the significantly (4–16-fold) reduced V_{max} in this solvent. It is striking that DMF performs so much more poorly than DMSO as a cosolvent for this enzyme. This observation appears to find parallels (i) in the work of Cabral et al. who saw significantly greater stability of chymotrypsin to DMSO than to DMF (as measured by $AcPheLeuNH_2$ hydrolysis)[10a] and (ii) in the report of Clark and coworkers, who saw a decline in V_{max} and an increase in K_m with trypsin upon surpassing the 10 volume% level with a DMF:DMSO (9:1) mixed cosolvent.[10b]

Table 2
Steady state kinetic parameters by cosolvent

Solvent	K_m (mM)	*V_{max} (mM min^{-1})	V_{max}/K_m (min^{-1})
Dioxane	11	1.1	0.1
Diglyme	2.2	0.25	0.11
Triglyme	2.3	0.3	0.13
DMF	8	0.07	0.0088
DMSO	2.8	0.44	0.16

* Note: In all cases, protein concentration is held fixed at 18 mg protein/mL total volume.

With an eye toward streamlining the desymmetrization for scale-up, we next examined the effects of fewer wgt. equivalents of protein (down from 3.25 to 1.0, Table 3), as well as increased temperature (from 25 to 35°C, Table 4) on enzymatic hydrolyses with the best cosolvents. Enzymatic yield and pro-chiral arm selectivity dropped off in DMSO at both the lower protein concentration and the higher temperature. The enzyme operated well in 10% dioxane at the lower protein concentration, but exhibited much lower efficiency (56% ee, 12% yield) at 35°C. This is reminiscent of the work of Jones with chymotrypsin, in which he showed a dramatic drop-off in catalytic efficiency of that enzyme upon going from 5 to 25% dioxane.[10e]

Table 3
Effect of reduced protein concentration*

	Time (h)	%ee	Yield	Corrected Yield
DMSO	4	**85**	**22**	70
Triglyme	4	**96**	**58**	73
Dioxane	4	**96**	**59**	88

* Note: In all cases here, protein concentration has been reduced 3.25-fold compared to the entries in Table 1. There 3.25 wgt. equivalents of protein (PPL, Sigma) were employed. Here a single wgt. equivalent is used.

Table 4
Effect of temperature*

Solvent	Temp (°C)	Time (h)	%ee	Yield	Corrected Yield
Triglyme	10	10	96	46	46
Dioxane	10	9	96	45	78
DMSO	10	9	96	34	85
Triglyme	35	6	**96**	**58**	73
Dioxane	35	6	**56**	**12**	88
DMSO	35	6	**64**	**17**	78

* Note: In all cases here, protein concentration has been fixed at the usual 3.25 wgt. equivalents of protein (PPL, Sigma) vis-à-vis diacetate **4**.

Strikingly, only in triglyme does the enzyme perform the title desymmetrization with optimal efficiency under all such conditions. These results suggest that this organic cosolvent may be especially effective, in general, as a stabilizing cosolvent for enzymatic hydrolyses of organic substrates. A thorough

search of the literature revealed no previous examples of such applications for triglyme. However, we did find one report from a French peptide group in which significant concentrations of triglyme are used to displace the usual equilibrium position for amide bond hydrolysis, and thereby permit peptide bond formation in water under peptidase catalysis.[16] Given that report and the results detailed herein, we would suggest that others investigate the use of triglyme as organic cosolvent for enzymatic hydrolyses of advanced synthetic intermediates.

3. Experimental

3.1. General

Monoacetate **3** was synthesized as previously described.[1] Buffer was prepared freshly from KH_2PO_4 and K_2HPO_4 and carefully adjusted to pH 8.0 as needed. All cosolvents were obtained commercially and were of high purity grades. PPL was obtained from Sigma (type II, cat. no. 3126; ≈25% protein; 66 U/mg protein with triacetin; 220 U/mg protein with olive oil). (*R*)-(−)-α-Methoxy-α-trifluoromethylphenylacetyl chloride was prepared from the corresponding (*S*)-acid (Aldrich, 99% ee) with oxalyl chloride (6 equiv.) in refluxing hexane (3 h), and purified by vacuum distillation. ^{1}H NMR experiments were run on a GE-Omega 300 MHz instrument. Residual $CHCl_3$ is used as the internal reference peak and set at 7.25 ppm.

3.2. Spectral characteristics

For diacetate **4**: ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 6H), 2.82–2.85 (m, 2H), 3.24 (dd, *J*=10, 11 Hz, 2H), 3.75 (dd, *J*=6, 11 Hz, 2H), 5.26 (d, *J*=4 Hz, 2H), 5.94 (d, *J*=1 Hz, 1H), 5.99 (d, *J*=1 Hz, 1H), 6.75 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.5, 40.6, 63.1, 82.2, 102.1, 103.8, 136.7, 147.6, 171.2. Anal. calcd for $C_{17}H_{18}O_7$: C, 61.07; H, 5.43. Found: C, 61.20; H, 5.61.

For monoacetate **3**: ^{1}H NMR ($CDCl_3$) δ 1.43–1.58 (br. s, 1H), 2.06 (s, 3H), 2.77–2.84 (ddd, *J*=6, 9, 13 Hz, 2H), 2.87 (dd, *J*=9, 10 Hz, 1H), 3.23 (dd, *J*=10, 11 Hz, 1H), 3.28 (dd, *J*=6, 10 Hz, 1H), 3.76 (dd, *J*=6, 11 Hz, 1H), 5.25 (d, *J*=4 Hz, 1H), 5.32 (d, *J*=4 Hz, 1H), 5.94 (d, *J*=1 Hz, 1H), 5.98 (d, *J*=2 Hz, 1H), 6.73 (s, 1H), 6.82 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.5, 40.3, 43.8, 61.0, 63.4, 82.3, 82.4, 102.0, 103.7, 103.8, 136.7, 137.2, 147.3, 147.4, 171.5; $[\alpha]_D^{21}$=+52.6 (*c* 0.6, $CHCl_3$); HRMS (FAB, 3-NOBA) calcd for $C_{15}H_{16}O_6$ 292.0947 [M^+], obsd 292.0952. Anal. calcd for $C_{15}H_{16}O_6$: C, 61.64; H, 5.52. Found: C, 61.72; H, 5.65.

3.3. Typical procedure for the enzymatic desymmetrization

To a 25–50 mL RB flask containing PPL (4–13 wgt. equivalents w.r.t. substrate=1–3.25 wgt. equivalents of protein) and buffer solution (50 mM KPO_4, pH 8.0, 9 mL) is added a solution of diacetate **4** (50 mg, 150 μmol) in the organic cosolvent (1 mL). The reaction is run under magnetic stirring with temperature regulation (oil bath for 35°C; controlled cold room for 10°C) and is monitored by TLC. At appropriate times, the reaction is quenched by pouring into EtOAc (25–50 mL). Following separation, the aqueous layer is extracted twice more with EtOAc. After drying ($MgSO_4$), filtration and concentration, flash SiO_2 chromatography (50% EtOAc–hexanes) yields recovered diacetate **4** (R_f≈0.7) in a first fraction, and monoacetate **3** (R_f≈0.5) in a second fraction. The corrected yield is calculated by

comparing the isolated yield of monoacetate with the theoretical 100% monoacetate yield, based upon consumed diacetate (i.e. starting diacetate mass-recovered diacetate mass).

The optical purity of each sample of enzymatically derived monoacetate is determined by conversion to the corresponding Mosher ester(s) {CH_2Cl_2, 10 equiv. NEt_3, cat. DMAP, 2 equiv. (*R*)-Mosher chloride, rt}. Enantiomeric excesses are calculated by comparing the integrals of the aromatic C_5-H protons (podophyllotoxin numbering) for each diastereomeric Mosher ester. Observed chemical shifts in the ^{1}H NMR ($CDCl_3$) for these protons are: δ 6.43 (major diastereomer=(*S*)-Mosher ester/(*R*)-substrate arm); δ 6.58 (minor diastereomer=(*S*)-Mosher ester/(*S*)-substrate arm).

3.4. Steady state kinetics

To determine v_0 as a function of [*S*] for each cosolvent, the rate of monoacetate formation was monitored by NMR. All experiments were run with 50 mg of substrate (as above) and diluted with the appropriate total volumes to achieve the desired fixed substrate concentrations for each run. Dilutions were performed with 90% buffer/10% organic solvent and the enzyme concentration was held fixed at 72 mg solid (18 mg protein) per mL. Time point aliquots (typically 1/5 of the total volume; 4–5 time points for each substrate concentration) were taken periodically, quenched by extraction (EtOAc), and worked up as described above. The ratio of product to starting material was calculated from the ^{1}H NMR ($CDCl_3$) signals of the aromatic CH protons for diacetate **4**: (s, 2H at 6.745 ppm); for monoacetate **3**: (s, 1H at δ 6.82 ppm; resolved) and (s, 1H at 6.73 ppm; overlaps with the diacetate peak). The **3**:**4** ratio=[δ 6.82 integral]÷[{(δ 6.73–6.75 integral)−(δ 6.82 integral)}/2]. Product concentration is then plotted vs time for each fixed [*S*]. Linear regression then gives v_0 for that [*S*]. For each solvent, the v_0 vs [*S*] data are least-squares fit to a hyperbola (Michaelis–Menten equation) using SigmaPlot (version 4.14, Jandel Scientific) to estimate the values for K_m and V_{max}.

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