In Situ Enzymatic Screening (ISES): A Tool for Catalyst Discovery and Reaction Development[**]

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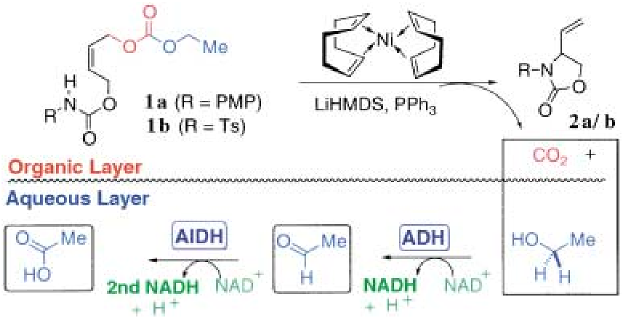
Dedicated to Professor Samuel J. Danishefsky
on the occasion of his 65th birthday

The move from the more deliberate, traditional approach to catalyst discovery to combinatorial approaches, has spurred great interest in the development of parallel-screening methods. As Crabtree and Loch recently put it, ideally one seeks "an appropriate chemical sensor in a rapid parallel assay to detect rate and perhaps selectivity".[1] Herein, we describe the use of enzymes to report rapidly on reaction rate, for a set of parallel reactions. Seto and Abato recently described the use of enzymes to report on (enantio)selectivity.[2] From a broader perspective, these developments may be regarded as adding to the versatility of enzymes as tools for the organic chemist, an area that has seen remarkable expansion from asymmetric processing of unnatural substrates[3–5] and protecting-group cleavage under mild conditions,[6] to the creation of artificial enzymatic pathways.[7, 8]

There is currently great interest in "combinatorial catalysis",[9] especially in transition-metal (TM) catalyzed reactions, for which reaction discovery and optimization often involve varying 1) the metal, 2) the ligand (type, structure, and stoichiometry), and 3) the substrate structure. By choosing such a model reaction, we sought both to establish proof of principle and to assess the ability of the screen to evaluate such variables one at a time.

In our approach, the organic reaction under study is coupled, in situ, with an enzymatic reaction that permits continuous UV spectroscopic monitoring of the reaction. We term this approach 'in situ enzymatic screening' (ISES). This method is complementary to the previously communicated screening techniques,[10–19] in that it provides 1) evidence of product formation (not directly available using the elegant IR-thermography method of the Morken and Reetz groups)[11] and 2) relative rate profiles (not easily available with time-point detection systems employing gas[12] or liquid chromatography[10, 13] or mass spectrometry),[14] 3) without the need to alter the substrate, by installing a chromophore,[15] a fluorophore,[16], or an azo-dye precursor.[17]

Consonant with our interest in developing synthetic methodology toward densely functionalized β,γ-unsaturated amino acids[20] as inhibitors of PLP-dependent (PLP = pyridoxal phosphate) enzymes,[21] we chose a TM-mediated allylic-amination reaction as our model reaction. We were influenced, in this regard, by an important precedent from Trost et al.;[22a] scalemic vinylglycinol had been synthesized through Pd^0-mediated allylic amination.[22] We set out to use ISES to identify other TMs, including less expensive ones, which were capable of catalyzing the intramolecular allylic-amination reaction illustrated in Scheme 1.[23] Success here would, in principle, validate the use of ISES in screening other variants of the allylic-displacement reaction.[24]



Scheme 1. ISES of catalysts for allylic displacement. PMP = *para*-methoxyphenyl.

A UV spectrophotometer fitted with an automated cell changer was chosen for parallel screening, as this equipment is readily available commercially. Six non-palladium TM catalysts were simultaneously monitored for intramolecular allylic amination with model substrates **1a** and **1b**. Because the TM complexes themselves are highly colored, these reactions are run in a biphasic fashion. The organic chemistry takes place in an upper organic layer, above the spectrophotometer beam, and the linked enzymatic chemistry takes place in a lower aqueous layer, in the path of the beam (Scheme 1). Turnover of the ethyl carbonate substrate in the organic layer leads to release of ethanol, which diffuses into the aqueous layer, where it undergoes enzymatic oxidation with concomitant formation of nicotinamide adenine dinucleotide, reduced form (NADH; $\lambda_{max} = 340$ nm). A coupled alcohol-dehydrogenase (ADH)/aldehyde-dehydrogenase (AlDH) enzyme assay was found to be optimal for screening (Figure 1).[25]

An appropriate organic solvent for a biphasic ISES screen such as this must: 1) be essentially immiscible in the aqueous buffer, 2) solubilize all the organic substrates and catalysts (here TM complexes) chosen, 3) be compatible with the reaction under study, 4) permit the diffusion of the (by)-product (enzymatic substrate) into the aqueous-buffer layer. Several organic solvents were examined here, and a mixed organic solvent (THF:toluene:hexane 2:1:1) was chosen to fulfil these criteria best (see Supporting Information).[26, 27]

With this system, one can easily screen six reactions simultaneously, and obtain meaningful relative kinetic data within ten minutes (Figure 2 and Table 1). All screening methods that rely upon the monitoring of a leaving group are susceptible to the false positive that would be observed on

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Supporting information for this article is available on the WWW under http://www.angewandte.com or from the author.

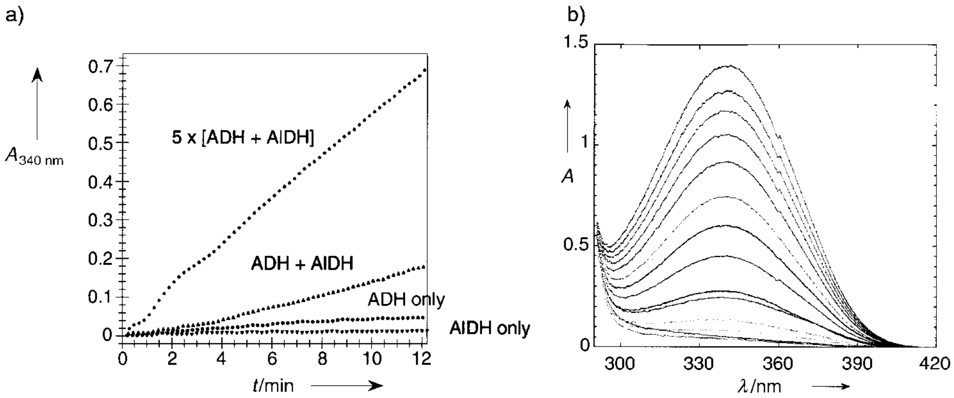
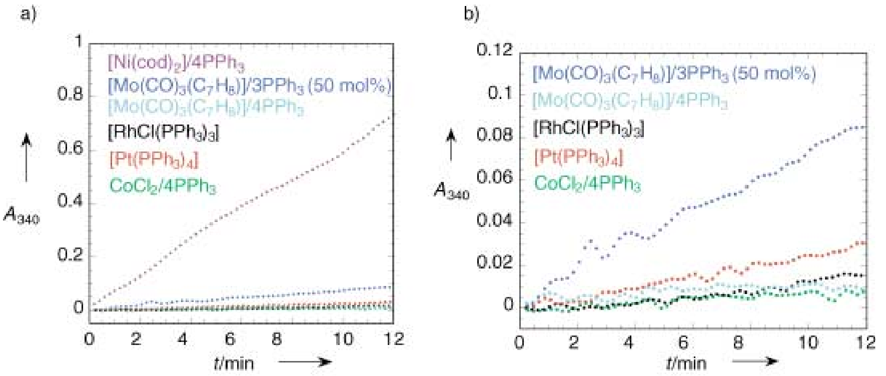


Figure 1. UV spectra showing a) the dependence of NADH formation rate observed upon enzymatic couple used, and right) a clear spectroscopic signature, which indicates that NADH is the species giving rise to the observed 340 nm absorbance increase. All traces are for the reaction in Scheme 1. Spectral scans shown (b) are at two min intervals. The AlDH-only experiment (a) is a negative control and shows no rate, as expected.



Catalyst	Slope [mAbs/min] [a]	Isolated Yield [%]
[Ni(cod)$_2$]/4PPh$_3$	58 [b]	70
[Ni(cod)$_2$]/4PPh$_3$ [c]		(89)
[Mo(CO)$_3$(C$_7$H$_8$)]/3PPh$_3$ [d]	6.8	18
[Mo(CO)$_3$(C$_7$H$_8$)] [e]		(35)
[Mo(CO)$_3$(C$_7$H$_8$)]/4PPh$_3$	0.6	[f]
[Pt(PPh$_3$)$_4$]	2.5	15 [g]
[RhCl(PPh$_3$)$_3$]	1.5	= 5 [f]
CoCl$_2$/4PPh$_3$	0.7	[e]

Figure 2. a) UV spectra showing the relative rates of turnover of substrate **1**, inferred from enzymatically coupled EtOH oxidation; b) expansion of (a). For each metal, the Table provides a comparison between the NADH-formation rate observed in the cuvette screen[30] and the isolated yield of **2** for the same reaction in a round-bottomed (RB) flask.[31] [a] Slopes of linear least-squares fit lines to the UV spectra shown above. Note: The entries in () represent RB-flask results only, run at higher catalyst loadings; [b] three runs gave values of 55, 58, and 60 mAbs/min, which are consistent with an experimental uncertainty of ±5–15% for these ISES slopes; [c] reaction run at 20 mol% catalyst; [d] in both the cuvette and the RB flask, this reaction was run on **1b** at 138 mM substrate (half the usual concentration) and at 50 mol% catalyst; [e] reaction run on **1b** at 100 mol% catalyst at the usual concentration (275 mM); [f] little to no product detected by crude ^{1}H NMR spectroscopy; [g] for the Pt^0 catalyzed reaction, the rate observed by ISES increases somewhat beyond the initial 12 mins.

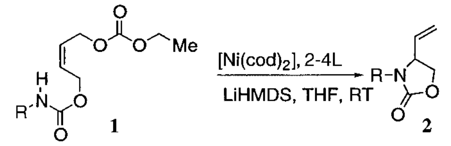
hydrolysis of the substrate. This concern necessitates the independent testing "in the reaction flask" of apparent positive results in the cuvette. Only after a significant body of data of this kind is accumulated will it be possible to establish the extent of this potential limitation. In the case at hand, there is a good correlation between relative NADH-formation rates observed and relative product-formation rates in the upper organic layer, as examined independently by an NMR spectroscopic time-point assay.[28] There is also a correlation, though less direct, as expected, between relative rates observed in the cuvette and yields obtained for the same reactions in neat THF with one h reaction times (Figure 2).

Across a spectrum of non-palladium TM complexes screened, the most favorable results were obtained with Ni^0 catalysts.[29] To our knowledge, only a couple of Ni^0-catalyzed allylic aminations have been reported, and these employ simple secondary amines as nucleophiles. These methods require either stoichiometric amounts of nickel,[23f] or elevated temperatures.[23d, e] The cyclization uncovered here, in which a lithiated carbamate serves as intramolecular nucleophile,[32] is the first example of a room-temperature allylic amination, catalyzed by Ni^0. The screen also uncovered the first Mo^0-mediated allylic amination, of which we are aware, albeit in modest yield.[33]

With Ni^0 centers showing promise, we employed ISES to probe the ligand and nitrogen-PG (PG = protecting group) dependence of this reaction. The results are collected in Table 1 and correspond to data assimilated from three sets of six-cuvette runs. Electron-rich N-protecting groups are beneficial, with the PMP group giving the best rates. This PG was retained for the ligand screens, which turned up three hits (1,4-butanediylbis[(diphenyl)phosphane] (dppb), PPh$_3$, dppf); these ligands include the only two bidentate phosphane ligands examined.

Several other control experiments were run to define more precisely the optimal conditions and/or limitations of this biphasic ISES screen. In one, by varying the amount of one dehydrogenase enzyme with the other held constant,[35] we were able to establish that, under the standard screening conditions chosen here, the ADH-mediated oxidation of ethanol to acetaldehyde is not at all rate-limiting. On the other hand, the AlDH-mediated oxidation of acetaldehyde to acetic acid is partially rate-limiting. So while, in general, relative rates are reproducible at ±5–15% under our standard conditions (Figure 2; this reproducibility points to consistency in AlDH-pipetting), this experimental uncertainty might be reduced even further by expending more AlDH (0.12→0.24 U) per cuvette, to make neither enzymatic step even partially rate limiting).

Table 1. Nitrogen-protecting-group/ligand screens.



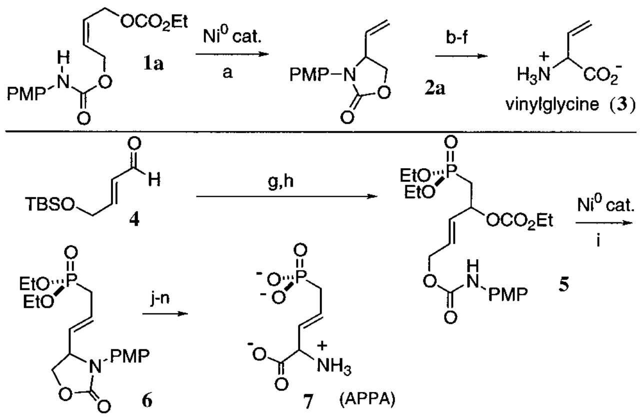
Entry	Ligand (L)	N-Protecting Group (R)	Slope[a] [mAbs/min]
1	PPh_3	H (no protection)	7
2	PPh_3	$CHPh_2$ (benzhydryl)	9
3	PPh_3	**C_6H_2-3,4,5-$(OMe)_3$**	**18**
4	PPh_3	C_6H_4-*p*-NO_2 (PNP)	0.2
5	PPh_3	CO_2*t*Bu (Boc)	1
6	**PPh_3**	**C_6H_4-*p*-OMe (PMP)**	**35**
7	$P(OMe)_3$	C_6H_4-*p*-OMe (PMP)	1
8	P(2,6-di-OMe-$C_6H_3)_3$	C_6H_4-*p*-OMe (PMP)	7
9	$AsPh_3$	C_6H_4-*p*-OMe (PMP)	0.3
10	2,2'-CH_2-(4S)-Ph-box[b]	C_6H_4-*p*-OMe (PMP)	0.6
11	**dppf**[c]	C_6H_4-*p*-OMe (PMP)	**26**
12	P(C_6H_4-*p*-$NMe_2)_3$	C_6H_4-*p*-OMe (PMP)	1
13	$P(C_6F_5)_3$	C_6H_4-*p*-OMe (PMP)	0.3
14	**$Ph_2P(CH_2)_4PPh_2$**	C_6H_4-*p*-OMe (PMP)	**118**
15	P(2-fur)$_3$[d]	C_6H_4-*p*-OMe (PMP)	0.4
16	P(*t*Bu)$_3$	C_6H_4-*p*-OMe (PMP)	0.4

[a] Slopes of least-squares fitted lines to the Abs (absorbance) versus time data over about 10 min.[34] [b] Box = 2,2′-methylenebis[(4S)-4-phenyl-2-oxazoline]. [c] Dppf = 1,1′-bis(diphenylphosphino)ferrocene. [d] fur = furyl.

Perhaps the central issue that arises when one considers the ISES concept is the cocompatibility of enzymatic chemistry and TM-mediated organic chemistry in a single vessel. While the biphasic nature of this assay is expected to offer a considerable degree of compartmentalization for any screened reaction, it will be important to establish whether components of the aqueous phase (i.e. water and acetaldehyde) diffuse into and inhibit the chemistry in the organic layer. Conversely, one must consider the possibility that components of the organic layer (e.g. metal complexes) inhibit the enzymatic reporter groups in the aqueous layer.

A Karl Fischer titration established that up to 1 weight% diffuses into the organic layer under the screening conditions.[35] It is noteworthy that not only is the reaction apparently compatible with stoichiometric amounts of water, but, indeed, a considerable variety of organic transformations display aqueous compatibility (see the Supporting Information for references to such reactions). Two experiments were carried out to assess the affect of enzymatically generated acetaldehyde on the TM-mediated reaction in the organic phase. Neither introduction of acetaldehyde into the aqueous layer, nor into the organic layer directly (at 45 mol% levels) produced a decrease in the ISES-monitored reaction rate.[35] As for the converse direction, control experiments revealed no inhibition of ethanol oxidation in the aqueous layer when standard levels of phosphane ligand and either $[Ni(cod)_2]$ or $[(C_7H_8)Mo(CO)_3]$ were introduced into the organic layer.[35] These results are consistent with the appearance of a characteristic, unperturbed NADH spectrum in the aqueous layer with time, as the metal-complex-mediated chemistry takes place in the upper organic layer (Figure 1), which suggests that indeed little to no diffusion of these organometallic complexes into the aqueous layer is occurring.

Having identified good conditions for this Ni^0-mediated allylic amination at room temperature, these conditions were applied to the syntheses of two important *β,γ*-unsaturated amino acids. Vinylglycine (**3**), a known inactivator of PLP-dependent transaminases for L-aspartate, L-alanine, L-serine, and D-alanine[36] is easily constructed (Scheme 2). It is noteworthy that the key intramolecular allylic-amination step proceeds in excellent yield and that the PPh_3-ligated Ni^0 catalyst may be generated either by ligand exchange from $[Ni(cod)_2]$ or by Zn-mediated reduction of the corresponding dichloro-Ni^{II} complex.



Scheme 2. a) $[Ni(cod)_2]$, PPh_3, LiHMDS (89%) or Zn^0, $[NiCl_2(PPh_3)_2]$, LiHMDS (84%); b) CAN, MeCN:H_2O 2:1 (78%); c) Boc_2O, NEt_3, CH_2Cl_2 (88%); d) Cs_2CO_3, MeOH (88%); e) CrO_3, H_2SO_4(aq), acetone (82%); f) TFA, CH_2Cl_2 (84%, isolated as the TFA salt); g) MeP(O)$(OEt)_2$, *n*-BuLi, THF, −40 °C, 30 min; EtO_2CCl, 30 min; then 0.3 N HCl, warm to RT (68%); h) *p*-MeO-C_6H_4NCO, pyr, (96%); i) 20 mol% $[Ni(cod)_2]$, dppb, LiHMDS (79%); or 20 mol% $[Ni(cod)_2]$, PPh_3, LiHMDS (77%); Zn^0, or 20 mol% $[NiCl_2(PPh_3)_2]$, LiHMDS (70%); j) CAN, MeCN:H_2O 2:1 (89%); k) Boc_2O, LiHMDS, THF (85%); l) Cs_2CO_3, EtOH (95%); m) CrO_3, H_2SO_4(aq), acetone (76%); n) TMSI, CH_2Cl_2 (80%, isolated as the ammonium salt). CAN = ceric ammonium nitrate, TFA = trifluoroacetic acid, Boc = *tert*-butoxycarbonyl, pyr = pyridine, TMS = trimethylsilyl, cod = cyclooctadiene, HMDS = 1,1,1,3,3,3-hexamethyldisilazane, TBS = *tert*-butyldimethylsilyl.

The same strategy led to an expeditious synthesis of *E*-2-amino-5-phosphono-3-pentenoic acid (**7**; APPA).[37] This *β,γ*-unsaturated phosphonate mimic of homoserine phosphate has been shown recently to inhibit two PLP-dependent enzymes: cystathionine *γ*-synthase (tight-binding reversible inhibitor; $K_i = 27\ \mu M$)[38a] and threonine synthase (irreversible inhibitor; $K_i = 400\ \mu M$; $k_{inact} = 0.25\ min^{-1}$).[38b] The requisite *α*-phosponomethyl allylic carbonate **5** could be assembled easily from aldehyde **4** (Scheme 2). Three operations were carried out in one pot; aldehyde **4** was condensed with diethyl lithiomethylphosphonate at low temperature, followed sequentially by alkoxide trapping with ethyl chloroformate, and in situ desilylation. Pleasingly, with either dppb or triphenylphosphane (tpp) as ligand, the key Ni^0-mediated allylic amination proceeded both efficiently and stereoselectively, and yielded solely the *E* isomer of **6**.

The ISES approach to catalyst screening demonstrated herein is expected to be applicable to a range of synthetically useful transformations, which includes others that involve release of carbonates or alcohols, and their detection with the

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enzyme couple detailed herein, and still others that involve monitoring entirely different products or leaving groups with other enzyme couples.

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[25] The second enzymatic reaction not only forms a second molecule of NADH for each EtOH equivalent oxidized, but it also converts an unfavorable equilibrium into a highly favorable one. Thus, even at pH 7.7, $\Delta G' = +4.6\,\text{kcal}\,\text{mol}^{-1}$ for the (ADH-mediated) oxidation of ethanol with NAD^{+}, whereas $\Delta G' = -15.2\,\text{kcal}\,\text{mol}^{-1}$ for the subsequent (AlDH-mediated) oxidation of acetaldehyde to acetate (for a discussion of the equilibrium constants for these oxidations, see: R. A. Alberty, *Arch. Biochem. Biophys.* **1993**, *307*, 8–14. The $\Delta G'$ values given here assume an ionic strength of 0.1 and have been corrected for the fixed assay pH). Note: the thermodynamics of the ADH-step can be improved by substitution of $APAD^{+}$ (a better oxidant, but more expensive cofactor; redox potential $= -258$ mV, $\lambda_{max} = 365$ nm) for NAD^{+} (redox potential $= -320$ mV; $\lambda_{max} = 340$ nm). For experimental details of this alternative single-dehydrogenase ISES assay, see the Supporting Information under the heading "Effect of Acetaldehyde on TM-Catalyzed Allylic-Amination Rate".

[26] A referee pointed out that, with ISES, one might encounter a "leveling effect", in which turnover rates that exceed the "instantaneous" release rate of EtOH into the aqueous layer from the solvent of choice cannot be differentiated. Indeed, we do see this "topping-out effect" with dppb as the ligand (Table 1, entry 14). However, we find that we are able to observe faster catalysts such as $[Pd_2(dba)_3]$ (dba = *trans,trans*-dibenzylideneacetone) by reducing the mole percentage of catalyst (to ≤ 2 % in this case). So, while we can circumvent "leveling-effect" issues in the biphasic assay, the need for a viable biphase and for an acceptable leaving-group release rate into the aqueous layer limits the organic solvents that can be employed in biphasic ISES.

[27] Restrictions on solvent composition also arise in the IR-thermography (ref. [11a]) and gel-embedded fluorescent-chemosensor (ref. [18b]) screens, in which other practical issues ("bead floatability" and gel swelling, respectively) arise. While a $THF{:}PhCH_3{:}$hexane mixed solvent proved to be best for ISES with this chemistry, for other reactions, in an inverted configuration, it should be possible to run ISES assays with solvents more dense than water.

[28] As a control experiment, for the cuvette screens of both the Ni^{0}- and Mo^{0}-catalyzed reactions (Figure 2), aliquots were drawn early from the upper organic layers of the cuvettes. After quenching and extractive workup, NMR spectroscopic analysis using an internal standard (2′-acetonaphthone) showed a ratio of initial rates ([P]/time) of ≈8.7–13:1, which is consistent with the ratio of slopes observed for NADH formation (≈8.5:1) in the aqueous layer of the same cuvettes. Full details of these experiments are provided in the Supporting Information. We simply note here that these experiments are both tedious (one time point is obtained for each aliquot taken, worked up and analyzed by NMR spectroscopy, in contrast to the continuous relative [product] versus time data provided by the ISES screen) and difficult (to obtain a valid time point, one needs an instantaneous quench of the reaction, which may be difficult to obtain, and leads to

considerable experimental uncertainty, especially for rapid reactions at short time points).

[29] In light of Evans' work (refs. [23a,b]), one might have expected better results for the Rh^{I} catalyst here. In fact, whereas the unmodified Wilkinson catalyst proved to be much less efficient than the Ni^{0} catalysts found for this reaction involving a lithiated carbamate as an internal nitrogen nucleophile, it proved to be a good catalyst for the reaction of a simple substrate (allyl ethyl carbonate) with a neutral, external nitrogen nucleophile (dibenzylamine; see the Supporting Information).

[30] With one exception, all cuvettes contain an organic layer: **1a** (110 µmol), LiHMDS (100 µmol), catalyst (10 mol%), with added PPh_3, where added, in 400 µL total organic solvent (THF:PhMe:hexane 2:1:1), over an aqueous layer containing: NAD^{+} (7.4 mM), ADH (1.3 U) and AlDH (0.12 U; U = the amount of enzyme that leads to the oxidation of 1 µmol of ethanol under the standard assay conditions given in the Supporting Information) in sodium pyrophosphate buffer (15 mM, pH 8.8); final pH 7.7 in 900 µL total aqueous volume. The exception was the $[Mo(CO)_3(C_7H_8)]/3PPh_3$-catalyzed reaction (Figure 2), which was run at higher catalyst loading (50 mol% catalyst) on substrate **1b**, at half the usual concentration (i. e. 55 µmol in the same volume) for solubility reasons.

[31] RB-flask reactions contained the same concentrations of **1a** or **1b** and catalyst as the cuvette reactions, with one equivalent LiHMDS in THF:hexane (73:27). All reactions were stopped after 60 min and, where appropriate, the product (**2a** or **2b**) was isolated by SiO_2 chromatography.

[32] Of the several bases examined for carbamate deprotonation (*n*-BuLi, NaHMDS, KHMDS, LDA, NaOTMS), LiHMDS gave the best amination rates/yields. Interestingly, Evans and co-workers also settled upon this base for his Rh^{I}-mediated aminations (refs. [23a,b]).

[33] While Mo^{0}-mediated allylic alkylation is well-developed chemistry (For leading references, see; a) O. Belda, N. F. Kaiser, U. Bremberg, M. Larhed, A. Hallberg, C. Moberg, *J. Org. Chem.* **2000**, *65*, 5868–5870; b) B. M. Trost, S. Hildbrand, K. Dogra, *J. Am. Chem. Soc.* **1999**, *121*, 10416–10417; c) F. Glorius, A. Pfaltz, *Org. Lett.* **1999**, *1*, 141–144), we have been unable to find a report of Mo^{0}-mediated allylic amination. Kocovsky et al. have developed a mechanistically distinct Mo^{IV}-mediated allylic-amination reaction upon allylic alcohols (ref. [23i]).

[34] The PG screens were run at half the usual substrate concentration (138 mM), but at the usual concentrations of $Ni(cod)_2$ (27.5 mM) and PPh_3 (110 mM). On the other hand, the ligand screens (entries 7–16) were run at the usual concentrations of substrate (275 mM) and metal (27.5 mM), but at half of the typical ligand concentration (55 mM for monodentate ligands or 27.5 mM for bidentate ligands). These changes are apparently approximately compensatory. For example, for the PPh_3 ligand/PMP PG case, the PG-screening conditions gave a rate of 35 mAbs/min, whereas the ligand-screening conditions gave slopes of 36 and 41 mAbs/min, in two independent runs.

[35] A complete description of these experiments is included in the Supporting Information.

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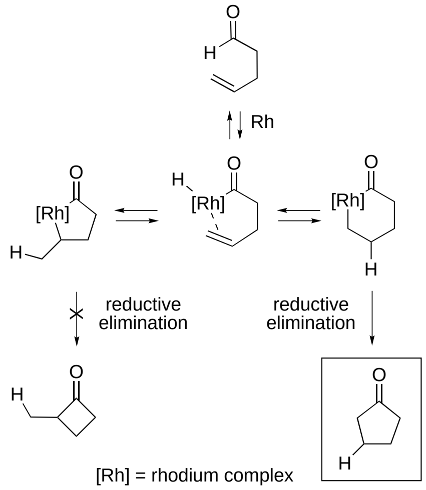
[37] For other syntheses of APPA, see: a) M. Fukuari, I. Ichimoto, K. Mitsunori, *Biosci. Biotech. Biochem.* **1996**, *60*, 680–682; b) H. Allgeier, C. Angst, G. Bold, R. Duthaler, R. Heckendorn, A. Togni, Eur. Pat. Appl. EP 302826 A2 19890208, **1989**.

[38] a) T. Clausen, M. C. Wahl, A. Messerschmidt, R. Huber, J. C. Fuhrmann, B. Laber, W. Streber, C. Steegborn, *Biol. Chem.* **1999**, *380*, 1237–1242; b) B. Laber, K. P. Gerbling, C. Harde, K. H. Neff, E. Nordhoff, H. D. Pohlenz, *Biochemistry* **1994**, *33*, 3413–3423.

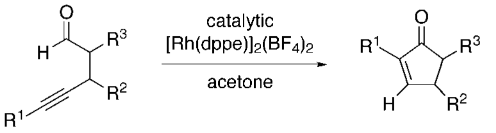
A Novel Rhodium-Catalyzed Reduction–Oxidation Process: Reaction of 4-Alkynals with Phenol to Provide *cis*-4-Alkenoates[**]

Ken Tanaka and Gregory C. Fu[*]

For the rhodium-catalyzed intramolecular hydroacylation of a 4-alkenal, five- and six-membered metallacycles have both been shown to be generated during the course of the reaction, although it is the six-membered metallacycle that selectively reductively eliminates to furnish the cyclopentanone product (Scheme 1).[1–3] We have recently reported that rhodium complexes also catalyze the corresponding cyclization of a 4-alkynal to afford a cyclopentenone [Eq. (1); dppe = 1,1′-bis(diphenylphosphanyl)ethane], presumably through an analogous six-membered metallacycle (see pathway to **A** in Scheme 2).[4]



Scheme 1. Rhodium-catalyzed intramolecular hydroacylation of 4-alkenals to form cyclopentanones.



(1)

This new catalytic process is mechanistically interesting from several standpoints, including the question of whether a five-membered metallacycle (**M**; Scheme 2) is formed tran-

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