Click Chemistry In Situ: Acetylcholinesterase as a Reaction Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks**

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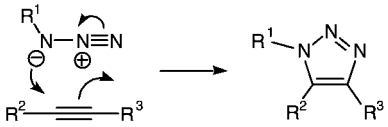
The generation and/or optimization of lead compounds by combinatorial methods has become widely accepted in medicinal chemistry, and is the subject of continued improvement.[1–3] However, most combinatorial strategies remain dependent upon iterative cycles of synthesis and screening. The direct involvement of the target, usually a receptor or enzyme, in the selection, evolution, and screening of drug candidates can accelerate the discovery process by short-circuiting its traditionally stepwise nature.[4–11]

The use of an enzyme target to select building blocks and synthesize its own inhibitor is a relatively unexplored option. This approach depends on the simultaneous binding of two ligands, decorated with complementary reactive groups, to adjacent sites on the protein; their co-localization is then likely to accelerate the reaction that connects them.[12] When the catalysis of such bond formation is blocked by product inhibition, the higher affinity products[12–14] then serve as lead compounds. This and similar approaches that have been adopted by a number of investigators employ one of five types of connecting reactions: formation of hydrazone or Schiff base adducts, disulfide bond formation, alkylation of free thiols or amines, epoxide ring-opening, or olefin metathesis.[5, 6, 8, 11, 15–19] Most closely related to the work described herein is the generation of carbonic anhydrase inhibitors by using the S_N2 reaction of a thiol with an α-chloroketone in the presence of the enzyme target.[16]

Most of the above strategies share the limitation that the reactive groups on the ligand probes (building blocks), being either electrophiles or nucleophiles, are likely to react in undesired ways within biochemical systems. An alternative is offered by the "cream of the crop" among "click reactions"[20]—the Huisgen 1,3-dipolar cycloaddition of azides and acetylenes to give 1,2,3-triazoles [Eq. (1)].[21–23] This water-



(1)

tolerant reaction employs functional groups that are generally compatible with enzymes under physiological conditions[24, 25] and are readily incorporated into diverse organic building blocks. Its dependence on the enforced propinquity and proper alignment of the reactants, which gives rise to large negative values of $\Delta S^{\neq}$, makes it ideal for the purpose at hand. Mock and co-workers established that the rate and regioselectivity of the azide–alkyne cycloaddition can be dramatically enhanced by sequestering the two components inside a host structure.[26–29] Their results with cucurbituril (M_W = 997 Da) as the catalyst in water bear an uncanny resemblance to those reported here for reaction inside a protein host.

We selected the enzyme acetylcholinesterase (AChE), which plays a key role in neurotransmitter hydrolysis in the central and peripheral nervous systems,[30, 31] as the target. AChE contains a narrow gorge approximately 20 Å in depth, lined with aromatic side chains.[32, 33] The active center, comprised of the acylation and choline-binding sites, is located at the gorge base; a "peripheral" site is found at its rim. Small-molecule ligands for each of these sites are known, and inhibitors that span the active center and the peripheral site have also been shown to exhibit tighter binding than the individual components.[34–39]

As a proof of principle AChE was used to select and synthesize a triazole-linked bivalent inhibitor by using known site-specific ligands as building blocks. A selection of site-specific inhibitors based on tacrine[38, 40] and phenanthridinium[38, 41] motifs decorated with alkyl azides and alkyl acetylenes of varying chain lengths (Scheme 1) was prepared by variations of known methods.[40, 42, 43] Although reversible AChE inhibitors are used clinically to treat Alzheimer's dementia,[44] *these compounds should be handled with care, since high-affinity inhibitors are potentially neurotoxic.* The building blocks shown in Scheme 1 allow for the presentation of 98 potential bivalent inhibitors to AChE: 34 regioisomeric pairs (*syn* and *anti* triazoles) of mixed tacrine/phenanthridinium adducts (**TZ2–6/PA2–6** and **TA1–3/PZ6–8**) and 15

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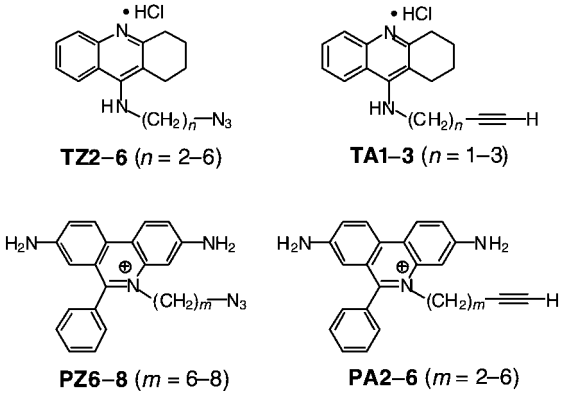

Scheme 1. Azide and acetylene building blocks. Key: T = tacrine, P = phenanthridinium, A = alkyne terminus, Z = azide terminus, n, m = number of CH_2 units in the chain connecting the binding and reactive moieties.

regioisomeric pairs of tacrine/tacrine triazoles (**TA1–3**/**TZ2–6**).[45] Each of the possible binary mixtures was incubated in the presence of *Electrophorus* AChE at room temperature.[46] The rate of reaction under these conditions in the absence of enzyme is negligible,[47] so detectable amounts of triazole products should form only when the azide and alkyne are brought together by the enzyme. Therefore, product formation is a direct indication of a potential "hit".

Examination of the 49 reactions by DIOS mass spectrometry[48, 49] showed only one combination, **TZ2** + **PA6**, in which a detectable amount of the corresponding triazole (compound **1**) was produced, an observation confirmed for a subset of reactions by more cumbersome HPLC-MS methods.[39] Control experiments established that blocking of the enzyme active center in either covalent or noncovalent fashion inhibits the formation of triazole **1**[39] which demonstrates that the binding cleft of AChE serves as a template for the 1,3-dipolar cycloaddition reaction. Furthermore, it was found that 2 ± 1 equivalents of triazole were made per equivalent of active enzyme[39] which suggests that the adduct was bound tightly by AChE.

Authentic samples of triazoles from seventeen of the possible azide–alkyne combinations were prepared by heating the components together at 80 °C in the absence of solvent for six days. The products were obtained in high yield, typically as equimolar mixtures of the *syn* (1,5-triazole) and *anti* (1,4-triazole) regioisomers. When desired, the regioisomers were separated by HPLC and independently characterized by MS and ^{1}H-NMR (nOe). Comparison of the HPLC traces of the enzyme-templated product and the authentic mixture of *syn*- and *anti*-**1** (from thermal cycloaddition between **TZ2** and **PA6** in the absence of enzyme) revealed that the in situ reaction generates predominantly the *syn* isomer (Figure 1).

Detailed kinetic analyses of the binding and inhibitory properties of *syn*- and *anti*-**1** against *Electrophorus*, *Torpedo*, and mouse AChE were performed by using both stopped-flow[50] and conventional (Ellman assay[51]) techniques (Table 1).[39, 52] Dissociation constants (K_d) of *syn*-**1** of 77 to 410 femtomolar (fM) were found, depending on the species, which makes it the most potent noncovalent AChE inhibitor known to date by approximately two orders of magnitude.[53, 54] The *anti*-**1** isomer exhibited K_d between 720 fM and 14 pM, a value as much as 140 times larger than that of the *syn* compound. Thus, the more active *syn*-triazole regioisomer is the same structure that is preferentially assembled by the enzyme.

The dissociation constants for both *syn*- and *anti*-**1** are substantially lower (i.e. higher affinity) than their components (10–100 nM for tacrine and low μM for propidium). We find that both isomers access the enzyme at rates similar to each other and to tacrine, but differ in their rates of dissociation (off-rates), with that for *syn*-**1** being extremely slow. In addition to the entropic benefits expected from tethering two binding elements to each other, the linker assembly, which

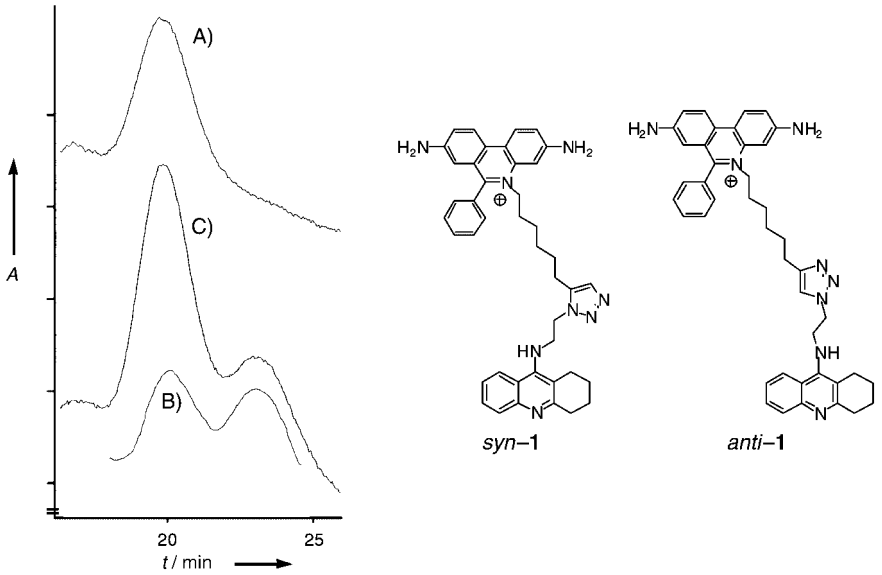

Figure 1. Left: HPLC analysis of thermal and AChE-templated assembly of **TZ2** and **PA6**. A) Product from in situ assembly in the presence of AChE. B) Triazole **1** prepared by thermal reaction; equal amounts of *syn* and *anti* isomers were isolated. C) Solution (A) plus a small amount of solution (B). Right: *syn*- and *anti*-isomers of **1**.

Table 1. Kinetic parameters derived for binding of **1**, and literature data for related noncovalent inhibitors of AChE from various species.

Inhibitor	k_{on} [10^{10} M^{-1} min^{-1}]	k_{off} [min^{-1}]	K_d	AChE source
syn-**1**	1.5	0.0015	99 fM	*E. electricus*
	1.3	0.0011	77 fM	*T. californica*
	1.3	0.0079	410 fM	mouse
anti-**1**	1.8	0.25	14000 fM	*E. electricus*
	3.2	0.026	720 fM	*T. californica*
	2.4	0.30	8900 fM	mouse
tacrine[38]	0.78	138	18 nM	mouse
propidium[38]	1.4	15000	1100 nM	mouse
huprine X[55]	0.044	0.009	26 pM	human
ambenonium[35]	0.31	0.78	250 pM	human

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consists of the two methylene chains and the triazole, may also interact favorably with the enzyme.

Docking of *anti* and *syn*-**1** in AChE from *Torpedo californica* (PDC code 1ACJ with Trp279 adopting the conformation found in 1ACL) with the program AutoDock v.3.05[56] shows that the tacrine portion of the inhibitor can be accommodated at the bottom of the active center gorge (practically superimposed on tacrine in the crystal structure), while the phenanthridinium piece is likely to be located in the peripheral site at the rim of the gorge (Figure 2). Interestingly, the triazole moiety is predicted to lie below (deeper than) the narrowest point of the gorge (defined by Phe330, Tyr334, Phe331, Phe288, Trp233, Phe290, and Tyr121).[57]

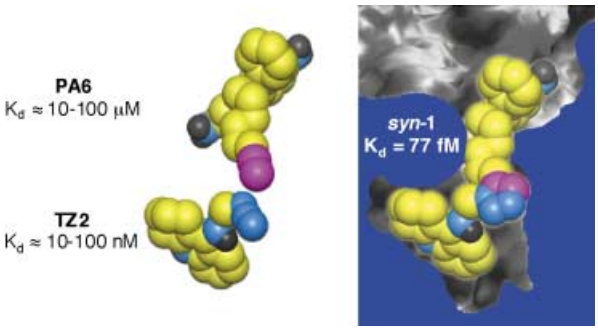

Figure 2. Left: **TZ2** and **PA6** components used for in situ assembly of **1**. The estimated binding constants to AChE shown are those of the tacrine and propidium, respectively, which lack azide or alkyne functional groups. Right: Clipping plane (blue) revealing the *T. californica* AChE active center gorge. The lowest-energy docked conformer of *syn*-**1** is shown. Alkyne and triazole carbon atoms appear in pink, nitrogen atoms in blue, and nitrogen-bound H atoms in black; all other H atoms are omitted for clarity.

It is apparent that the narrow confines of the AChE gorge impart high selectivity to the assembly reaction. For example, a preliminary survey of the relative potency of a selection of adducts made by thermal 1,3-dipolar cycloaddition as described above shows that the connectivity of the triazole does not seem to be as important as its position (both **TZ2**/**PA6** (**1**) and **TA2**/**PZ6** are highly potent, but **TZ6**/**PA2** is not). While we suspect that the unique adduct preferentially assembled by the enzyme (i.e., *syn*-**1**) is also likely to be the strongest inhibitor among the 98 triazoles which could have been synthesized, further measurements are in progress to confirm or refute this hypothesis.

We have shown that an enzyme can select and synthesize an extremely potent inhibitor from a parallel array of building blocks by using 1,3-dipolar cycloaddition reactions. This process, which is distinguished by its slow background rate and biocompatibility, provides an excellent probe of the AChE binding landscape. Function can be developed in situ as the individual blocks explore the biomolecular target for recognition elements. A permanent nexus in the form of the robust triazole linkage is made only when two cross-reactive blocks find themselves temporarily moored at adjacent sites, locking in topological and/or dynamic information about the biostructure which recruited them.

We anticipate that "false positives" will be relatively rare in the "in situ" approach. Assuming that the enzyme active site or an important allosteric site is the template, and that the background rate of the reaction that connects the blocks is low, the formation of a bond between two blocks in situ virtually guarantees that the resulting adduct will be a valuable hit or lead compound for enzyme inhibition. A potential disadvantage of the application of "in situ" click chemistry to inhibitor discovery is the possibility of "false negatives" (effective inhibitors that are not assembled in the enzyme). Improvements in analytical methods and adjustments in the background rate of reaction of the components will help alleviate this problem.

In principle, target-directed assembly of inhibitors could be monitored by assays of enzyme activity instead of detection of the linked inhibitor molecule. In our view, such screening for function, when feasible, is almost always preferred. However, when function is difficult to measure in high-throughput fashion, the detection of potential inhibitors formed by the target is an attractive alternative, as demonstrated here. This latter approach should also facilitate true combinatorial experiments, in which multiple candidate blocks are incubated with the target.

In general, the in situ and traditional (screening of prefabricated candidates) methods of discovery are complementary, and tend to merge with the use of increasingly reliable synthetic transformations. That such a potent inhibitor as *syn*-**1** was found directly by using the azide–alkyne cycloaddition to unite the probe molecules is interesting, but its broad utility as a search tool remains to be established. Nevertheless, the special qualities of this reaction bode well for its use in creating or amplifying function.

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[23] In the description of "click chemistry," (ref. [20.]) we inadvertently omitted mention of the powerful block cycloaddition-connection strategies pioneered by Warrener and co-workers (see, for example, R. N. Warrener, D. N. Butler, *Aldrichimica Acta* **1997**, *30*, 119 – 129; R. N. Warrener, D. N. Butler, D. Margetic, F. M. Pfeffer, R. A. Russel, *Tetrahedron Lett.* **2000**, *41*, 4671 – 4675). This body of work offers many beautiful examples of modular synthetic sequences for the construction of polycyclic skeletons and other interesting structures. Its success relies, both in design and execution, on a handful of concerted cycloaddition reactions—pure "fusion" events that are premier examples of click chemistry.

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[43] The proposed structures were first examined computationally against AChE from electric ray *Torpedo californica* (PDB codes 1ACJ and 1ACL) with AutoDock 3.05[56] to ensure that tethers likely to be optimal would not be omitted. These calculations predicted that a chain length of 2 – 3 methylenes on the tacrine moiety and 5 – 6 on the phenanthridinium moiety would present favorable conformations to allow the cycloaddition reaction to occur in the enzyme.

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[45] Phenanthridinium/phenanthridinium combinations were not explored as they would not be expected to provide a dual-site inhibitor, the phenanthridinium moiety being too large to function as an active center ligand.

[46] The tacrine components in water were each added to solutions of *Electrophorus* AChE (Sigma) in 2 mM ammonium citrate buffer (pH 7.3 – 7.5) and allowed to stand for 90 minutes at room temperature. The corresponding coupling partner was then added and the solutions were thoroughly mixed. The final concentrations were: AChE, 1.0 mg mL^{-1} of the commercial material (titrated concentration of functioning active centers = 1 μM); tacrine component, 30 μM; phenanthridinium component, 66 μM.

[47] A second-order rate constant of $1.9 \pm 0.7 \times 10^{-5}$ M^{-1} min^{-1} was determined for the uncatalyzed reaction of **TZ2** and **PA6** over 9 days at 18 °C in 1-butanol. This is comparable to 7.0×10^{-5} M^{-1} min^{-1} obtained by Mock et al. at 40 °C.[26,27] Under these conditions, the reaction would take approximately 40 years to reach 80% completion.

[48] DIOS = Desorption/Ionization on Silicon.

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[52] The association rate constants (k_{on}) were determined by both direct measurements of inhibitor binding to AChE and by measurements of time-dependent loss of AChE activity in reaction with inhibitor. The stopped-flow technique was used to measure rates of quenching of intrinsic AChE tryptophan fluorescence upon binding of inhibitor at micromolar concentrations as previously described.[38] The time-dependent loss of AChE activity was measured upon mixing AChE with picomolar concentrations of inhibitor in ten-fold excess. The AChE activity in aliquots of the reaction mixture was determined by Ellman assay[51] at intervals of several minutes to an hour. The second-order rate constants of inhibitor association were obtained by linear fit of first-order decay rates of either AChE fluorescence or its activity, at several inhibitor concentrations. The first-order dissociation constants (k_{off}) were determined by measurements of the return of AChE activity by Ellman assay upon 5000-fold dilution of 50 – 100 nM concentrations of AChE·**1** complex into 250 μg mL^{-1} solution of herring sperm DNA (Boehringer) in buffer (thereby the reassociation of the inhibitor to AChE by virtue of the affinity of the phenanthridinium part of the molecule for duplex DNA is competitively suppressed). The dissociation constant was determined by nonlinear fit of first-order increase in enzyme activity up to 70 – 80% of the AChE control activity in a mixture containing no inhibitor. All experiments were performed in at least triplicate with the standard error of determination equal to or smaller than 20% of the mean value. The measurements were performed in 0.1M phosphate buffer pH 7.0 at 22 °C on a SX.18 MV stopped-flow instrument (Applied Photophysics) or Cary 1E UV/Vis spectrophotometer (Varian).

[53] The trifluoroketone of Quinn and co-workers forms a hemiketal with the serine residue of the active center and exhibits a K_i value based on the total concentration of inhibitor of 80 pM. Most of this species exists in the inactive hydrated form. When one factors in the equilibrium concentration of the active carbonyl species, the inhibitor constant reaches low femtomolar levels [H. K. Nair, K. Lee, D. M. Quinn, *J. Am. Chem. Soc.* **1993**, *115*, 9939 – 9941; M. Harel, D. M. Quinn, H. K. Nair, *J. Am. Chem. Soc.* **1996**, *118*, 2340 – 2346]. Triazole *syn*-**1** suffers no such equilibrium deactivation.

[54] The inhibitory power of **1** exceeds even that of the fasciculin family of snake venom toxins, which occlude the mouth of the AChE gorge with binding constants of 0.44 – 40 pM, depending on the fasciculin and the source of the enzyme: P. Marchot, A. Khelif, Y. H. Ji, P. Mansuelle, P. E. Bougis, *J. Biol. Chem.* **1993**, *268*, 12458 – 12467; R. Duran, C. Cerveñansky, F. Dajas, K. F. Tipton, *Biochim. Biophys. Acta* **1994**, *1201*, 381 – 388; Z. Radić, R. Duran, D. C. Vellom, Y. Li, C. Cervenansky, P. Taylor, *J. Biol. Chem.* **1994**, *269*, 11233 – 11239; J. Eastman, E. J. Wilson, C. Cerveñansky, T. L. Rosenberry, *J. Biol. Chem.* **1995**, *270*, 19696 – 19701; G. Puu, M. Koch, *Biochem. Pharmacol.* **1990**, *40*, 2209 – 2214.

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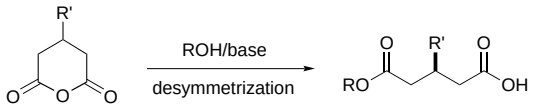
[56] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639 – 1662.
[57] Although the lead inhibitor **1** was assembled by *Electrophorus electricus* AChE, it has a similarly high affinity for the *Torpedo californica* enzyme. This observation suggests a high degree of functional similarity with respect to binding in the active center gorge for these two enzymes (see: S. Simon, J. Massoulié, *J. Biol. Chem.* **1997**, *272*, 33045 – 33055), and justifies our use of the crystallographically well-characterized (2.8 Å resolution) *Torpedo* enzyme for modeling studies. At the present time *Electrophorus* AChE has only been characterized to 4.2 Å resolution (Y. Bourne, J. Grassi, P. E. Bourgis, P. Marchot, *J. Biol Chem.* **1999**, *274*, 30370 – 30376).

Highly Enantioselective Desymmetrization of Anhydrides by Carbon Nucleophiles: Reactions of Grignard Reagents in the Presence of (−)-Sparteine**

Ryo Shintani and Gregory C. Fu*

The desymmetrization of *meso* and other prochiral compounds represents a powerful approach to asymmetric synthesis,[1] and a number of enantioselective total syntheses have been based on this strategy.[2] The desymmetrization of anhydrides has been a particular focus of interest. Most investigations of this family of substrates have employed an alcohol as the nucleophile[3] [for example, a chiral alcohol[4] or an achiral alcohol in combination with a chiral catalyst;[5] Eq. (1)]. In addition, success has been reported for reactions with a stoichiometric quantity of an enantiopure reducing agent[6] or amine.[7]

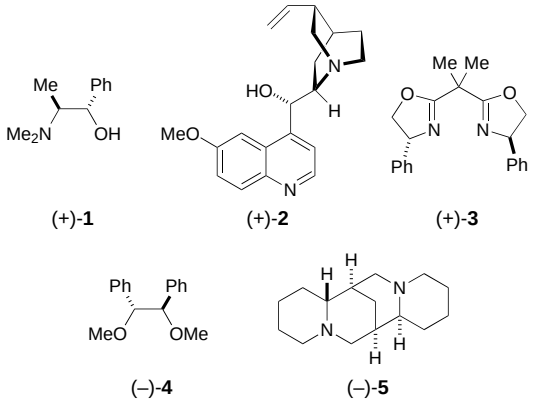


(1)

ROH and/or the base is chiral

On the other hand, very little progress has been described for the desymmetrization of anhydrides with carbon-based nucleophiles. In fact, to the best of our knowledge, only one report has begun to successfully address this challenge, a study by Real and co-workers that focused on the reaction of a single substrate, a bicyclic anhydride, with Grignard reagents bearing chiral oxazolidine auxiliaries.[8] In an attempt to remedy this methodological deficiency, we have recently initiated an investigation of the desymmetrization of anhydrides by carbon nucleophiles. Rather than covalently attaching a chiral auxiliary to the nucleophile and then releasing it, we chose to concentrate our efforts on the use of chiral ligands as the source of asymmetry. Here we report that (−)-sparteine-bound Grignard reagents effectively desymmetrize an array of cyclic anhydrides to furnish ketoacids in very good enantiomeric excess.

In our initial work, we decided to explore the ring-opening of 3-phenylglutaric anhydride by phenylmagnesium chloride. We examined a structurally diverse set of chiral ligands (Scheme 1) that have proved useful in a number of other

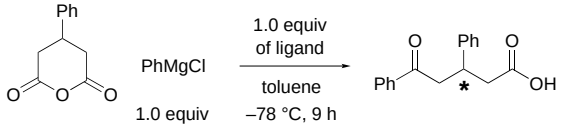


Scheme 1. Ligands used in preliminary experiments.

enantioselective processes, including a simple aminoalcohol (Table 1, entry 1), a cinchona alkaloid (entry 2), a bisoxazoline (entry 3), and a dimethyl ether (entry 4). Disappointingly, all were rather ineffective at desymmetrizing the anhydride (<40% *ee*). Fortunately, however, we discovered that readily available (−)-sparteine accomplishes the ring opening with high enantioselectivity (88% *ee*; entry 5).

Of course, we are not the first to document the remarkable capacity of (−)-sparteine to control enantioselection. Pioneering observations by Nozaki et al. in the 1960's[9] have been followed by fascinating studies by a number of groups, including those of Hoppe and Beak.[10, 11] The large majority

Table 1. Desymmetrization of 3-phenylglutaric anhydride by PhMgCl: a survey of chiral ligands.[a]



Entry	Ligand	*ee* [%]	Yield [%]
1[b]	(+)-**1**	1	76
2[b]	(+)-**2**	12	76
3	(+)-**3**	32	66
4	(−)-**4**	39	77
5	(−)-**5**	88	63

[a] All data are the average of two runs. [b] 2.0 equiv of PhMgCl was used.

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