Evidence against an Alternative Mechanism for a Self-Replicating System

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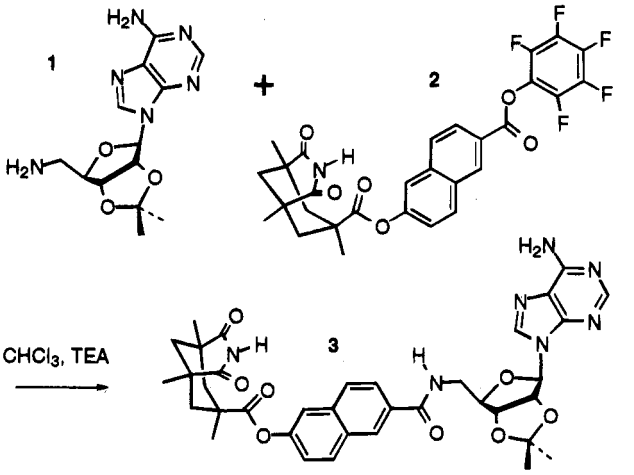
A recent paper by Menger *et al.* described experimental and computational work related to our self-replicating system and concluded that amide catalysis—either external or internal—is the cause of observed rate enhancements. Herein we show that the proposal of Menger *et al.* is inconsistent with published data, that their conclusions overreach the data afforded by their experiments, and that the step which they modeled is likely irrelevant to the autocatalytic nature of the system. We present new results and a refined mechanism in which the transition state is stabilized not by amides but by template-based recognition, the hallmark of self-replicating systems.

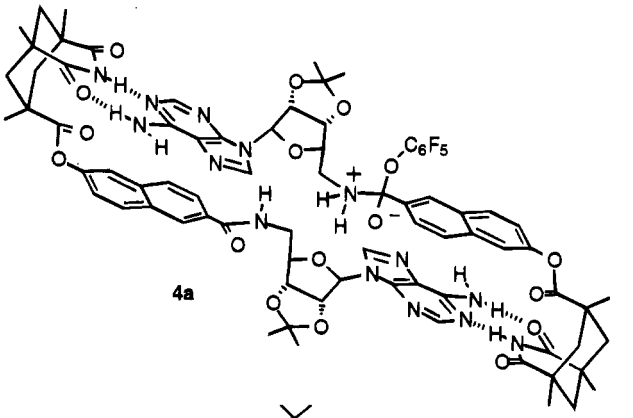
In a recent paper[1] in this journal, F. M. Menger, A. V. Eliseev, N. A. Khanjin, and M. J. Sherrod described experimental work and modeling related to a self-replicating system developed in our group.[2-5] They concluded that amide catalysis—either in a simple, nonspecific manner or within a complex—is the cause of the rate enhancements. The reaction, as shown in Scheme 1, involves the coupling in chloroform of amine **1** with ester **2** in the presence of triethylamine to produce amide **3**. Numerous experiments have shown that the reaction is autocatalytic; adding product **3** to the reaction increases the rate of amide formation. While the exact nature of this autocatalytic effect remains unknown, we show here that the proposal of Menger *et al.* is inconsistent with published data and new results, and we assert a refined mechanism **4a** in which the rate-limiting step is catalyzed by recognition on a template surface in the manner expected of self-replicating systems.

Menger *et al.* suggest a complex such as **4b** (Figure 1), in which an internal amide assists the breakdown of the tetrahedral intermediate. In general, the rate-determining step for ester aminolysis in aprotic solvents has been shown to be the breakdown of the zwitterionic tetrahedral intermediate,[6-9] so **4b** makes sense in that catalysis arises from assisting that breakdown. However, only one end of **3** is involved in recognition in the mechanism of complex **4b**, a stipulation inconsistent with our published data and the experiments which are described below.

Our original hypothesis[2,3] concerning the source of autocatalysis in this self-replicating system was a termolecular complex in which the template **3** brought amine **1** and ester **2** into close proximity to enhance the rate of coupling. Recently, we have focused on the

Scheme 1. Autocatalytic Ester Aminolysis System





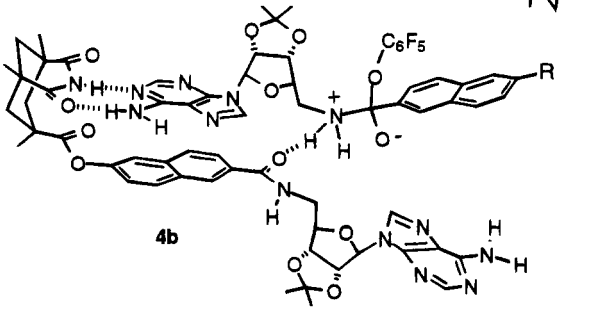


Figure 1. Two proposed mechanisms of catalysis by product **3**.

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(1) Menger, F. M.; Eliseev, A. V.; Khanjin, N. A.; Sherrod, M. J. *J. Org. Chem.* **1995**, *60*, 2870–2878.
(2) Tjivikua, T.; Ballester, P.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 1249–1250.
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(5) Conn, M. M.; Wintner, E. A.; Rebek, J. Jr. *J. Am. Chem. Soc.* **1994**, *116*, 8823–8824.
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(8) Su, C.; Watson, J. W. *J. Am. Chem. Soc.* **1974**, *96*, 1854–1857.
(9) Hogan, J. C.; Gandour, R. D. *J. Org. Chem.* **1992**, *57*, 55–61.

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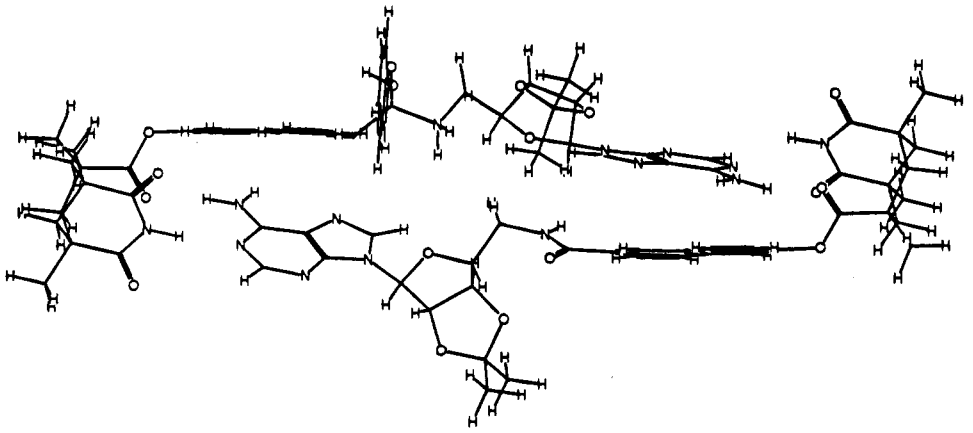


Figure 2. Computer-generated model[10] of catalytic complex **4a**. The distance between the amide carbonyl of **3** and the nitrogen proton of the tetrahedral intermediate is 4.9 Å.

breakdown of the tetrahedral intermediate as the probable rate-limiting step, and we propose refined complex **4a** as a low-concentration, steady-state intermediate. In **4a**, both ends of the template hold their complementary substrates as the tetrahedral intermediate, favoring release of pentafluorophenol over reversion to substrates. This models catalysis of the rate-limiting step just as **4b**, but—as our data requires—stabilization of the transition state relies on molecular recognition *at both ends* in the form of hydrogen bonding and π-stacking interactions, without participation of the amide. Menger *et al.* modeled termolecular complexes in detail,[1] but since these are ground states, they were most likely modeling the wrong step. A more likely complex for the case at hand is **4a**; modeling[10] of this complex shows no undue strain and shows little likelihood of amide catalysis (Figure 2). Given the inexact nature of such modeling, however, we prefer to base our conclusions on experiment.

While the physical differences between complexes **4a** and **4b** may appear small, they are mechanistically quite disparate: **4b** could not be called a replicating mechanism since catalysis requires specific molecular recognition at only one end; **4a**, on the other hand, meets the full requirement of template-directed autocatalysis, recognition of both reaction partners.

In their latest paper and its predecessor,[1,11] Menger *et al.* begin by raising the question of "simple amide catalysis":

[At substrate concentrations of 30 mM], "simple amides (e.g. 2-naphthamide, acetamide, and N-methyl-propionamide) also catalyze the aminolysis of ester **2** by amine **1**. Since Rebek's template **3** is itself an amide, concern arose as to whether his catalysis might arise not from a template effect but instead from a more mundane amide acceleration."[1]

In two previous papers,[4,5] we detailed evidence against simple amide catalysis in this system under our conditions (2.2 mM concentrations of **1** and **2**). We saw no catalysis of **1** + **2** by *secondary amides* such as **5**, although we did note catalysis by *primary amides*. As further evidence against simple amide catalysis, we have now performed the additional experiments outlined in

Scheme 2. Amide Formation Control Experiment

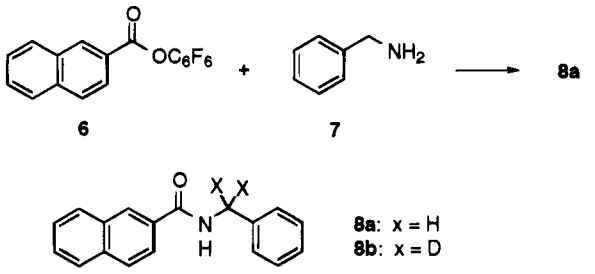


Table 1. Amide Formation Control Experiments at 25 ± 0.3 °C as Followed by NMR[a]

concn of ester **6** and amine **7** (mM)	equiv of amide **8b**	av initial rate of formation of **8a** (μM/min)	relative rate
4	0	42 ± 1	1
	0.5	42 ± 1	1.00 ± 0.03
8	0	84 ± 1	1
	0.5	84 ± 1	1.00 ± 0.02
16	0	168 ± 3	1
	0.5	174 ± 3	1.04 ± 0.03
20	0	258 ± 4	1
	0.5	282 ± 5	1.09 ± 0.03

[a] Coupling of **6** and **7** in $CDCl_3$ with or without addition of amide **8b**. Initial velocities of reaction were determined through integration of the methylene peak of the product amide **8a** at 4.72 ppm relative to the methylene peak of **7** at 3.88 ppm. Error values reflect reproducibility

Scheme 2. In these, ester **6** and amine **7** were coupled in chloroform at various concentrations in the presence or absence of deuterated product **8b** (see Table 1). Formation of amide **8a** could be followed cleanly by NMR without background signal from **8b**. While slight amide catalysis was seen at 20 mM (9%), no catalysis was seen by secondary amides at substrate concentrations of 8 mM or below. Accordingly, at the high concentrations (30 mM) described by Menger,[11] simple amide catalysis can be expected, but at the low concentrations (1.6–16 mM) of all of our published work, the effects are negligible.

Given that there is much evidence against simple amide catalysis in the rate enhancement of **1** + **2** by **3**, the mechanism of catalysis would seem to involve a complex of some kind. As discussed above, the rate-limiting step requires that any such complex involve binding to the tetrahedral intermediate such that breakdown to products is favored in some way over reversion to reactants. The question then remains, to what extent is molecular recognition featured in such a complex: can

(10) MacroModel 3.5X. Mohamadi, F.; Richards, N. G.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440–467.

(11) Menger, F. M.; Eliseev, A. V.; Khanjin, N. A. *J. Am. Chem. Soc.* **1994**, *116*, 3613–3614.

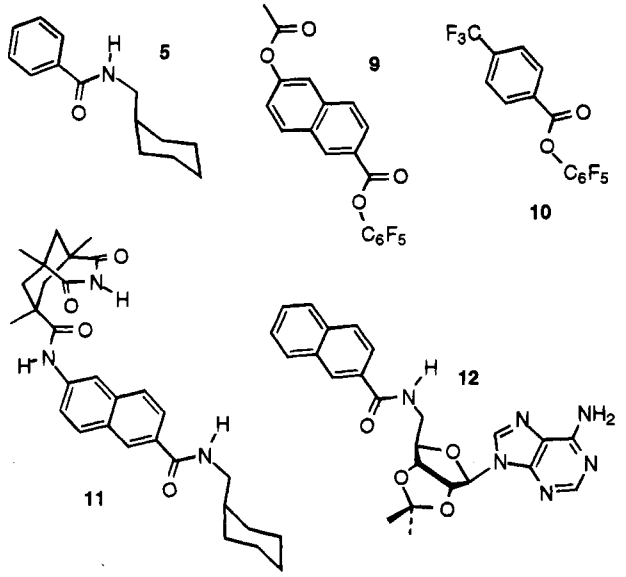


Figure 3. Control molecules used to test the autocatalytic nature of the reaction **1** + **2**.

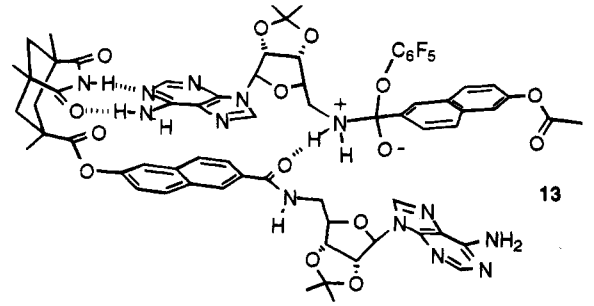


Figure 4. A previously proposed complex of **3** + **1** + **9**.[1]

the system be termed self-replicating, or is it merely a trivial case of autocatalysis?

The bulk of the argument put forward by Menger *et al.* rests on their experiments with **3** as a catalyst for **1** plus "non-hydrogen-bonding" analogues of ester **2**. They show that **3** enhances coupling of **1** with **9** or **10** (Figure 3) and propose complexes such as **13** (Figure 4) to explain this.[1] They claim that since the product molecule **3** also catalyzes other reactions, autocatalysis of **1** + **2** is not a case of self-replication. This reasoning is unsound; the ability of **3** to catalyze any other reaction in no way detracts from it status as a self-replicating molecule.

In their abstract,[1] Menger *et al.* state that their alternate mechanism (complexes **4b** or **13**) is "consistent with all available data." This is a curious statement, since published control experiments[4,5] tested precisely this proposal. These experiments showed that **11** and **12** (Figure 3) are not catalysts for **1** + **2**, thus ruling out complex **14** and its cousin **15** (Figure 5) as pathways for catalysis. In both **14** and **15**, the tetrahedral intermediate is recognized only at one end and presented with an amide in the middle of the structure; the results with **11** and **12** make clear that *both ends of molecule 3 are involved in the catalysis of the coupling of 1 + 2.* Accordingly, to maintain that complexes **4b** or **13** are "consistent with all available data" is to operate outside the normal guidelines of scientific discourse. Whether both ends of molecule **3** are necessary to catalyze coupling of **1** and anything else is another subject, which we now address.

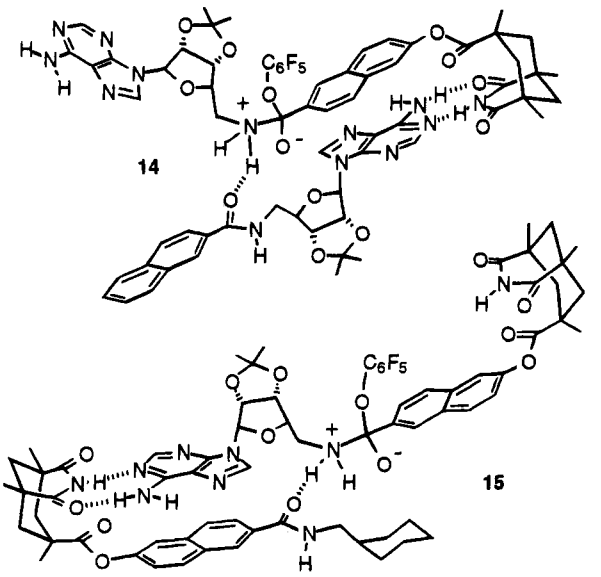
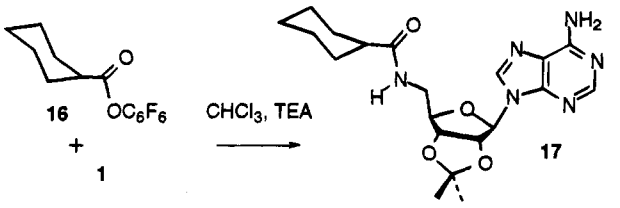


Figure 5. Two possible mechanisms of catalysis excluded by experiments with molecules **11** and **12**.

Scheme 3. System Used To Test the Proposed Mechanism of Complex 18



In light of the claims of Menger *et al.* concerning catalysis of **1** + **9** (or **10**) by molecule **3**, the experiment shown in Scheme 3 was undertaken, investigating the effect of **3** on the coupling of **1** plus **16** to form **17**. Cyclohexyl ester **16** has no ability to bind to the adenosine of molecule **3**, as it lacks any hydrogen bonding or π-stacking surfaces (apart from the pentafluorophenyl function shared by all of the esters under consideration). Following formation of **17** by HPLC at 2.0 mM, we found that added **3** was unable to catalyze the ester aminolysis of **1** + **16** (Table 2). The experiment was repeated by NMR at 8 mM with 1 equiv of added **3**. Again, no catalysis by **3** was observed (Figure 6). The fact that **3** does not catalyze the coupling of **1** + **16** is strong evidence against a mechanism such as complex **18** (Figure 7) and hints that the "nonbinding" esters of Menger *et al.* (**9** and **10**) may not be devoid of recognition capabilities. Both **9** and **10** feature electron deficient aromatic surfaces, and π-stacking of adenines with such surfaces can afford several kcal/mol in binding affinity under these conditions.[12] Structure **9** might further hydrogen bond through its acetyl carbonyl. In short, experiments with ester **16** indicate that **9** and **10** are ill-conceived control molecules for understanding the reaction in question: the coupling of **1** + **2** in the presence of **3**.

Given the results that show the necessity of both the imide and the adenosine functions of **3** in catalyzing formation of **1** + **2**, we assert a mechanism involving complex **4a**. We expect that the autocatalysis observed in our system is the result of the product's ability to

(12) Williams, K.; Askew, B.; Ballester, P.; Buhr, C.; Jeong, K. S.; Jones, S.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1989**, *111*, 1090–1094.

Table 2. Generation of Product 17 as a Function of Time, as Followed by HPLC[a]

concn of ester **16** and amine **1** (mM)	equiv of template **3**	av initial rate of formation of **17** (μM/min)	relative rate
2.0	0	15.0 ± 0.4	1
2.0	0.5	15.0 ± 0.5	1.00 ± 0.04
2.0	0.7	15.2 ± 0.3	1.01 ± 0.03

[a] All reactions were performed at 2.0 mM initial concentrations of reactants **3** and **16** in $CHCl_3$ with 1.0% TEA base added, 22 ± 0.5 °C. Error values reflect reproducibility.

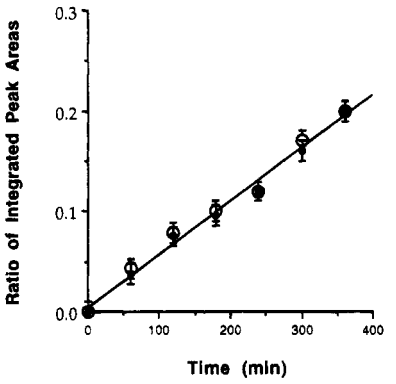


Figure 6. Graph of the ratio of product **17** to reactant amine **1** as a function of time, with (dark circles) or without (open circles) 1.0 equiv of added **3**. Reactions were performed at 8.0 mM initial concentrations of reactants **1** and **16** in $CHCl_3$ with 1.0% TEA base added and followed by NMR at 25 ± 0.3 °C. The rate of both reactions was 3.8 μM/min. Error bars reflect reproducibility.

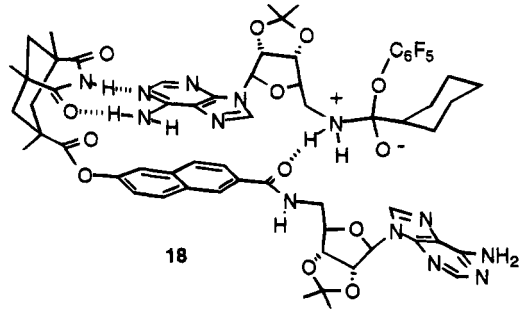


Figure 7. A complex of **3** + **1** + **16** ruled out by the data in Table 2 and Figure 6.

gather on its framework the two components of which it is formed and stabilize the tetrahedral intermediate thus created. Noncovalent binding of the two ends of the substrate favors ejection of pentafluorophenol from the tetrahedral intermediate and disfavors substrate dissociation, thus leading to product. A termolecular complex may be the immediate precursor to **4a**, but it need not be invoked; either substrate may be bound first, followed by formation of the intermediate and then binding of the other end, or the tetrahedral intermediate may form by itself and then be bound by first one and then the other end of **3**.

In conclusion, structure **3** is a molecule which assists in making a copy of itself, and without the recognition afforded by both ends of the structure, the autocatalysis fails. Autocatalytic reactions in which molecular recognition plays a key role are defined as self-replicating; therefore this is a self-replicating system. The fact that similar systems do not require such molecular recognition does not affect the evidence for template replication of **3**, nor does it provide testimony for amide participation. Published evidence against amide participation abounds, particularly in the data generated from our second generation of self-replicating molecules. In this generation (**1** + **19**),[13-15] the amide in question was unambiguously removed from the site of ester aminolysis (Figure 8). To date, we have devised some half-dozen self-replicating systems, each with different spacings between recognition sites and reaction sites. It is possible that some of them are properly positioned to provide intramolecular general base catalysis, but our control experiments with structure **3** make clear that in this case the autocatalysis observed is the result of self-replication.

Experimental Section

Synthesis. Imide–naphthyl–cyclohexylamide **11**. The corresponding imide–napthyl–carboxylic acid[3] (58 mg, 0.14 mmol), 1-ethyl-3-(3,3-dimethyl-1-aminopropyl)carbodiimide (EDC, 40 mg, 0.21mmol), and (dimethylamino)pyridine (DMAP, 5mg, 0.04 mmol) were stirred in 8 mL of anhydrous THF under Ar. Cyclohexylmethylamine (55 μL, 0.42 mmol) was added by syringe, and the solution was stirred for 24 h. The solution was evaporated and the crude solid purified by flash chromatography (40% EtOAC/Hex) to yield a clear oil. Product was precipitated from $CHCl_3$ with hexanes to yield **11** (45mg, 0.09 mmol, 64%) as a white powder: mp 118–123 °C dec; IR (KBr) 3180, 2925, 2851, 1750, 1702, 1645, 1541, 1457, 1314, 1202, 1151 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 10.861 (s, 1H,), 8.591 (t, 1H, J = 4.8 Hz), 8.455 (s, 1H), 8.055 (d, 1H, J = 9.0 Hz), 7.946 (s, 2H), 7.670 (d, 1H, J = 2.1 Hz), 7.316 (dd, 1H, J = 9.0, 2.1 Hz), 3.150 (t, 2H, J = 6.3 Hz), 2.520 (d, 2H, DMSO obsc), 2.018 (d, 1H, J = 13.0 Hz), 1.55–1.80 (m, 6H), 1.502 (d, 1H, J = 12.9 Hz), 1.434 (d, 2H, J = 14.1 Hz), 1.384 (s, 3H), 1.175–1.225 (m, 3H), 1.151 (s, 6H), 0.5–1.0 (m, 2H); HRMS (FAB+) calcd for $C_{30}H_{36}N_2O_5$ (M + H) 505.2702, found 505.2706.

5′-((2-Naphthylcarbonyl)amino)-5′-deoxy-2′,3′-isopropylideneadenosine (12). Aminoadenosine **1** (51 mg, 0.17 mmol) and 2-naphthoyl chloride (37 mg, 0.19 mmol) were dissolved in anhydrous THF (10 mL) with an excess of TEA (9 equiv) under Ar, accompanied by the immediate formation of a white precipitate. The reaction was stirred at room temperature for 15 h and filtered to remove TEA·HCl. After concentration, the residue was purified by flash chromatography (5% MeOH/$CHCl_3$) to yield **12** (76 mg, 0.165 mmol, 97%) as a white powder: mp 145–150 °C dec; IR (KBr) 3322, 3172, 2928, 1644, 1598, 1533, 1474 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ 8.836 (t, 1 H, J = 5.5 Hz), 8.433 (s, 1 H), 8.346 (s, 1 H), 8.073 (s, 1 H), 8.03–7.88 (m, 4 H), 7.65–7.55 (m, 2 H), 7.355 (br s, 2 H, amine), 6.166 (d, 1 H, J = 2.8 Hz), 5.500 (dd, 1 H, J = 6.3, 2.8 Hz), 5.091 (dd, 1 H, J = 6.3, 3.3 Hz), 4.351 (m, 1 H), 3.588 (m, 2 H), 1.530 (s, 3 H), 1.314 (s, 3 H); HRMS (EI) calcd for $C_{24}H_{24}N_6O_4$ 460.1859, found 460.1862.

Deuterated Benzylamine 7. To an ice-cooled solution of $LiAlD_4$ (1 g, 0.024 mol) in 50 mL of anhydrous THF was added a solution of benzyl nitrile (2.2 mL, 0.022 mol) in 50 mL of anhydrous THF dropwise under argon atmosphere. After addition was completed, the mixture was allowed to warm to room temperature and was stirred overnight. The mixture was then cooled with an ice water bath, and excess $LiAlD_4$ was quenched with 10% NaOH. After fizzing had stopped, the white slurry was extracted three times with ethyl acetate. The organics were combined and dried over anhydrous $MgSO_4$. Solvent was removed by rotory evaporation and dried under vacuum: yield 52%; ^1H NMR (300 MHz, $CDCl_3$) δ 7.3 (m, 5H), 1.5 (s, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 143.81, 128.93, 127.49, 127.17, 46.30 (p, J = 20.4 Hz).

Deuterated 2-((Benzylamino)carbonyl)naphthalene 8b. To a mixture of deuterated benzylamine (0.567 g, 5.3 mmol)

(13) Wintner, E. A.; Conn, M. M.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1994**, *116*, 8877–8884.
(14) Conn, M. M.; Wintner, E. A.; Rebek, J., Jr. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1577–1579.
(15) Lipkin, R. *Sci. News* **1994**, *146*, 362–363.

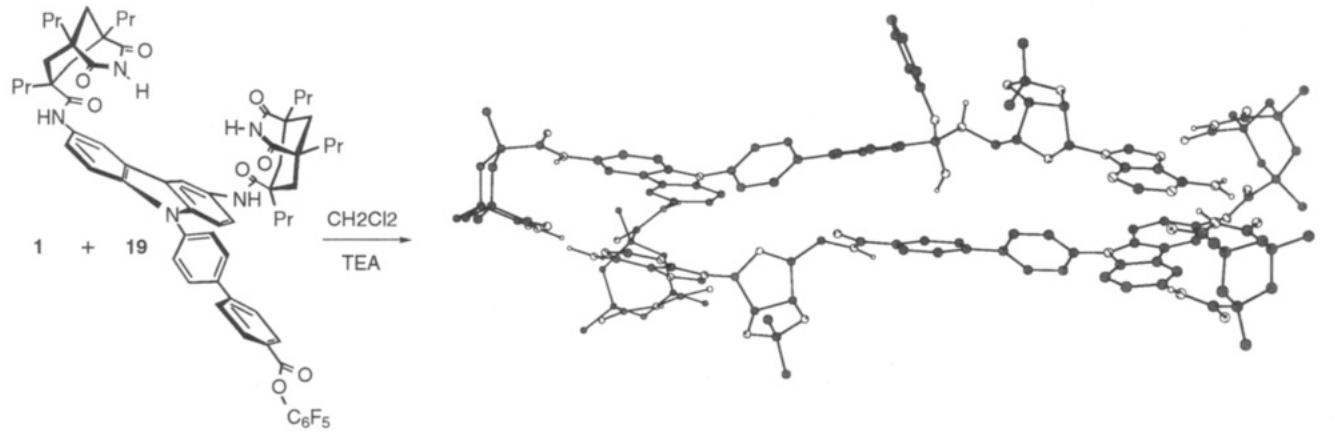


Figure 8. A diimide-based replicator: computer-generated complex[10] of the tetrahedral intermediate formed by the biphenylylcarbazole **19** and aminoadenosine **1** on its own template. Hydrogens not participating in the reaction have been omitted for clarity.

and Et_3N (0.72 mL, 5.2 mmol) in anhydrous THF (50 mL) was slowly added a solution of 2-naphthoyl chloride (1 g, 5.2 mmol) in 50 mL of anhydrous THF at room temperature and under argon atmosphere. This was stirred overnight and Et_3N·HCl salt was removed by filtration through Celite. The solvent was removed by rotory evaporation. Chromatography on silica gel with 1:1 mixture of ethyl acetate/hexanes as eluant gave the deuterated amide: yield 31%; mp 133−135 °C dec; IR (KBr) 3289, 3054, 1636, 1624, 1535, 1504, 1400, 1316 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.3 (s, 1H), 7.9 (m, 4H), 7.5 (m, 2H), 7.4 (m, 5H), 6.5 (s, 1H); HRMS (EI) calcd for $C_{18}H_{13}D_2NO$ 463.1277, found 463.1272.

NMR Kinetics. All ^1H NMR spectra were taken in $CDCl_3$ on a Varian Unity 300 MHz or Varian VXR 500 MHz spectrometer with temperature control. Chemical shifts in parts per million are reported relative to residual solvent peak.

Coupling reactions of **6** + **7** were carried out at 25 ± 0.3 °C by adding benzylamine **7** in $CDCl_3$ to a solution of naphthoyl pentafluorophenyl ester **6** in $CDCl_3$ and 0.01 equiv of Et_3N with or without the deuterated amide **8b**. Spectra were taken every 2 h until at least 10% of the product was formed. Initial velocities of the reactions were determined through integration of the methylene peak of the product amide **8a** at 4.72 ppm relative to the methylene of benzylamine **7** at 3.88 ppm.

Coupling reactions of **1** + **16** were carried out at 25 ± 0.3 °C by adding adenosineamine **1** in $CDCl_3$ to a solution of cyclohexyl pentafluorophenyl ester **16** in $CDCl_3$ and 0.01 equiv of Et_3N with or without 1.0 equiv of molecule **3**. Spectra were taken every hour until at least 10% of the product was formed. Initial velocities of the reactions were determined through integration of the C2 aromatic adenosine proton of the product **17** at 8.29 ppm relative to the C2 aromatic adenosine proton of the amine **1** at 8.35 ppm.

HPLC Kinetics. All reactions were performed in 3 mL Teflon-capped autoinjector vials at 2.0 mM initial concentrations of reactants in $CHCl_3$ with 1% TEA base. Solvent loss, other than the 3 μL per injection volume, was not observed during the reaction. Formation of product **17** was followed by HPLC at 270 nm on a Waters 600E instrument with a Waters 717 autosampler (with heater/cooler option) and a Waters 490E UV detector. Temperature inside the autosampler was constant at 22 ± 0.5 °C. Separation was achieved using a Microsorb MV C-18 column, 4.6 mm i.d. × 25 cm length, with gradient elution from 1% to 5% MeOH/$CHCl_3$.

Acknowledgment. We thank co-worker Dr. Morgan Conn for his help, especially the synthesis of the biphenyl carbazole replicator. E.W. thanks the American Chemical Society, Organic Division, for a Predoctoral Fellowship, and B.T. thanks the NSF. We are grateful to the National Science Foundation for financial support.

Supporting Information Available: NMR spectra (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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