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Review Article

Autocatalysis and the Generation of Self-Replicating Systems

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From an initial naphthoyl based molecule to a high-affinity biphenyl carbazole replicator, and from a minimalist self-complementary structure to a formal bimolecular replication cycle, the development of a series of self-replicating systems is charted. Successes and failures along the way are noted, with insight into the molecular properties required to generate molecular replication. Characterization of the observed autocatalysis is detailed, confirming the catalytic role of molecular recognition in the various systems.

This review intends to describe the work of our research group in the design of self-replicating systems, with emphasis on the progress made since our earlier review was published[1] in this Journal in 1992. Self-replicating systems are relatively new; work on molecules which produce copies of themselves began in the late 1980's and this undertaking has become a fashionable feature on the landscape of molecular recognition. The superb contributions of our colleagues in the field, mentioned only briefly here, are best read in their original publications.[2–10]

Modern research into self-replication seeks to discover – if only in a synthetic, abiotic environment – the principles which must have governed the molecular transition from chemistry to biology. In this vein, one may propose that very simple molecules or groups of molecules able to reproduce themselves gradually emerged – simple chemical cycles which could scarcely be termed 'life.' Our work did not begin as an investigation into the origins of life on prebiotic earth, however. Instead, our self-replicating molecules, or replicators, grew out of an extensive study of molecular recognition of nucleic acids. Early on, we had discovered molecules that effected the convergence of multiple functionalities at a single location.[11] These were endowed with the ability to recognize certain heterocycles.[12] In 1987, we brought both hydrogen bonding and aryl stacking interactions to bear on the recognition of the purine of adenosine.[13] From these studies of complementarity, replicating molecules were just a simple step away.

The key step was the introduction of self-complementarity into our structures: designing a molecule whose size, shape, and chemical surfaces impart an affinity for itself. While any molecule that enjoys a liquid or solid state has some such affinity, and may therefore be regarded as self-complementary, our more stringent definition invokes selectivity in the self-recognition event. Given such a molecule, one has – in theory – only to break the structure into two parts to design a self-replicating system; alternatively stated, covalent linking of two complementary molecules can give rise to a minimalist, self-complementary, replicating structure. Consider the schematic replicating system depicted in Fig. 1. Two complementary components **A** and **B** react in an intermolecular fashion to form a template (**T**). Because the template is self-complementary, another unit of **A** and **B** can gather on its surface to form a complex (**T**:**A**:**B**). An intracomplex reaction takes place in which the intermediates and transition states along the pathway

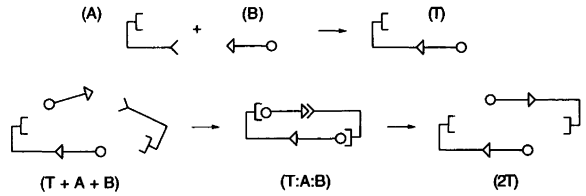

Fig. 1. Schematic representation of template based autocatalysis with self-complementary structures.

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are stabilized by weak intermolecular forces (e.g., hydrogen bonding and aryl stacking). These keep the appropriate ends of **A** and **B** anchored on the template surface while the dimeric product (2**T**) is created. The dimer dissociates and the process – replication – can be repeated.

Autocatalysis in self-complementary systems is generally due to an efficient reaction within the complex (**T**:**A**:**B**). If the reaction has no intermediate, as is the case with the Diels–Alder reaction,[14] or if the formation of an intermediate is rate-limiting, the catalysis arises from the reduction in entropy caused by bringing together the reagents on a template. If the breakdown of a covalent intermediate is rate limiting (as is the case with our amide-forming replicators), the catalysis arises from template stabilization of the tetrahedral intermediate [**A**–**B**]$^{+/-}$ such that product formation is favored over dissociation to **A** and **B**.

The enhancement of reactions by complementary surfaces is a commonly encountered process in the laboratory and in Nature. Template effects, leading to reduced activation entropies or stabilized intermediates, are responsible for the autocatalysis observed not only in our replicators, but also in von Kiedrowski's nucleic acid replicators,[1–5,9] in Kelly's bisubstrate reaction systems,[8] and in a number of other processes,[10] as well as numerous biochemical reactions. The most celebrated biological template is obvious in the structure of double-stranded DNA. It was clear to Watson and Crick that one strand of DNA acts as a template for the other during replication. This feature inspired the experiments of Leslie Orgel and his coworkers at the Salk Institute,[5] where, in 1986, Gunther von Kiedrowski showed that – even without the aid of enzymes – a short, self-complementary segment of DNA could act as a template for its own formation.[2] Complementary trideoxynucleotides were coupled to form a self-complementary hexamer in a process shown to be autocatalytic: the first synthetic self-replicating system. Subsequent improvements led to greater efficiency of autocatalysis and parabolic growth of the hexadeoxynucleotide product could be observed. The exponential nature of an autocatalytic process was revealed.[4]

It is important to distinguish here between self-replication and other forms of autocatalysis; while there are many examples of the latter – the bromination of acetone being among the oldest examples – self-replication is a special subset of autocatalytic reactions in which molecular recognition plays a role. The recognition need not be absolute; indeed, a certain promiscuity is desirable – and even required – if evolution of new and better replicators is to occur. The yields, the solvents, the concentrations or other such variables are unimportant; autocatalysis is a *kinetic* phenomenon. Even the extent of autocatalysis need not be great; any reasonably measurable, reproducible enhancement qualifies. Molecular replication is simply an autocatalytic reaction, as described above, where the product of a chemical transformation acts to catalyze that transformation through the directed production of copies of the molecule.

Even though self-complementarity is the reigning paradigm for our replicators this feature is not *necessary* for self-replication; other types of autocatalytic system exist in which more general physical entities are reproduced. These systems have been extensively explored by Luisi,[6,7] who observed autocatalytic generation of micelles or reversed-micelles in both aqueous and organic media. Binding of a substrate to the micelle enhances its exposure to reagents in these systems and the reaction generates more molecular components of the micelle. The autocatalytic product observed in these systems is the micelle, an aggregate with a more general self-complementarity among its components and a loosely defined size and structure. This system differs from template based replication in that the latter is more strictly defined in its structural fit and the stoichiometry of the recognition event. The difference is sufficiently great that another name for it is warranted; 'self-reproduction' has been proposed as the term for the behavior of the micelle-like systems.[6]

The first generation

Our first self-replicating system was an offshoot of our work on the molecular recognition of adenine. By forging a covalent attachment between an adenosine moiety and its synthetic receptor, a self-complementary molecule such as **1** was created. In the replicating system, the imide outfitted with a naphthoyl-pentafluorophenyl ester **2** reacted with 5′-amino-5′-deoxy-2′,3′-isopropylideneadenosine **3** to form the self-complementary autocatalytic template **1** (Fig. 2).[15] The autocatalytic nature of the reaction was evident from the rate acceleration observed when the reaction product was added to the coupling mixture (Fig. 3).[16] At 8.2 mM initial concentrations of reactants, addition of 20% of compound **1** produced a 43% increase in initial rate of product formation, while addition of 50% of compound **1** produced a 73% increase.

In general, the rate limiting step for ester aminolysis in aprotic solvents is the breakdown of the zwitterionic tetrahedral intermediate.[17–20] We expect that the autocatalysis observed in our system is the result of the product's ability to gather on its framework the two components of which it is formed and stabilize the tetrahedral intermediate thus created (Fig. 2, complex **4**). Non-covalent binding of the two ends of the substrate favors ejection of pentafluorophenol from the tetrahedral intermediate, (and disfavors dissociation) and leads to product. Several geometric possibilities are possible for a productive complex **4** although only the Watson–Crick mode of base-pairing is illustrated. They all result in the covalent coupling of ester and amine to give a replica of the template catalyst in dimeric form. A termolecular complex may or may not be the immediate precursor to **4**, but it need not be invoked; either substrate may be bound first, followed by formation of the intermediate

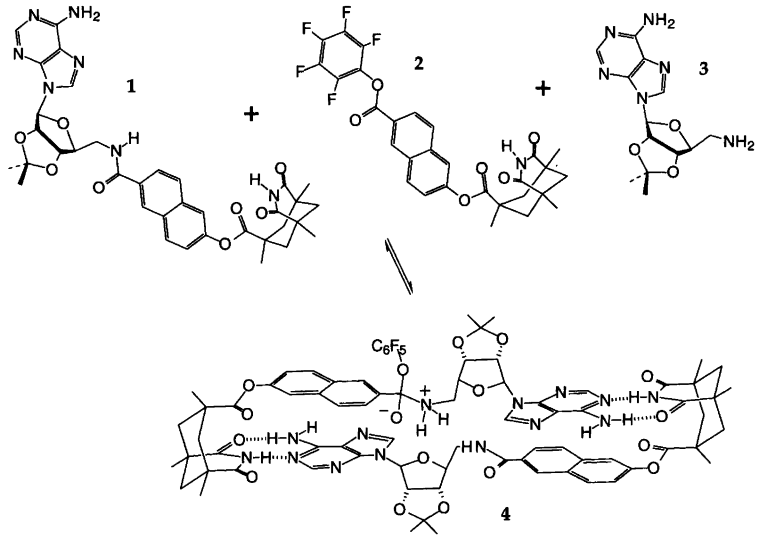

Fig. 2. An abiotic self-replicating system.

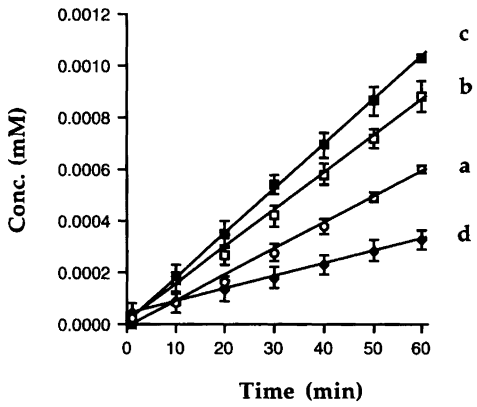

Fig. 3. Formation of **1** in $CHCl_3$ as followed by HPLC, 21 ± 1 °C; 8.2 mM initial concentrations of **2** and **3**, 1% TEA base added; (a) baseline reaction **2** + **3**; (b) baseline reaction plus 0.2 equiv. product **1**; (c) baseline reaction plus 0.5 equiv. product **1**; (d) baseline reaction plus 1.0 equiv. adenine binder **7**.

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 **1**.

But why should this system be regarded as self-replicating – how can it be shown that directed, recognition-based catalysis rather than simple chemical autocatalysis is being observed? After all, **1** bristles with functional groups known for their reactivities: the imide, amide, ribose, and purine functionalities could all contribute to the autocatalysis observed.

Evidence from a wide range of experiments confirmed that template effects were the origins of catalysis in these reactions, and we dwell here at some length on these control experiments. To begin, the system shows autocatalysis at 16.0 mM, 8.2 mM, and 2.2 mM concentrations of starting materials **2** and **3** in $CHCl_3$ at ambient temperature. With varied amounts of added product, the rate enhancements are not directly proportional to product concentration, but rather to its square root.[16] This 'square root law' was described by von Kiedrowski[2,3] to characterize nucleic acid replicators in which the autocatalytic entity exists largely in dimeric form.

While autocatalysis was clearly occurring, we had to make sure of the pathway. Imidazole is a well-known catalyst for acylation reactions, and the purine contains such a subunit. Could not this functionality be causing simple chemical catalysis? To guarantee that autocatalysis did in fact stem from template/substrate recognition, all of the potentially catalytic functions of the product molecule had to be individually tested, *in the structural context in which they appear in* **1**, and under the conditions where autocatalysis was observed.

We proceeded, through a series of overlapping control experiments,[21,22] to exclude each individual function of the product molecule as a source of simple chemical catalysis. The results are summarized in Table 1. In $CHCl_3$ at 2.2 mM concentrations of **2** and **3**, a 50% increase in initial rate was observed when the reaction was seeded with 0.5 equiv. product **1** (Table 1, entry 2).

(1) A simple control experiment with the *N*-methylated imide **5** (Fig. 4) gave strong indications that recognition and template effects – rather than conventional functional group catalysis – was involved in the autocatalytic reaction. In the presence of **5**, no rate enhancement in product formation was seen (within the 5% experimental error) at either high (16 mM) or low (2.2 mM) concentrations of reactants (Table 1, entry 3). Merely *N*-methylating the imide shut down autocatalysis.

(2) Using molecule **6**, the imide was next excluded as a chemical catalyst (Table 1, entry 4). The inability of **6** to catalyze the reaction still left open the possibility that some combination of functions, left untested by **5** or **6**, was responsible. Even so, these studies pointed to base-pairing between the imide and adenine as necessary for autocatalysis by molecule **1**. Support for

Table 1. Effect of various additives on the formation of **1** in $CHCl_3$ as followed by HPLC (see Ref. 21): 2.2 mM initial concentrations of **2** and **3**, 22±1 °C, 1% Et_3N base added.

Entry	Additive (0.5 equiv.)	Avg. initial rate of product formation ±5%/μM min^{-1}	Percentage of baseline rate
1	—	0.54	—
2	**1**	0.81	150
3	**5**	0.55	102
4	**6**	0.56	104
5	**8**	0.52	96
6	**9**	0.55	102
7	**10**	0.50	93
8	**11**	0.56	104
9	**21**	0.63	117
10	**12**	0.57	106

such base-pairing was obtained with the use of **7**. Diacylaminopyridines such as **7** are prized as hydrogen-bonding complements to imides,[23] and addition of **7** *inhibited* the replication reaction (Fig. 3).[16]

(3) Control experiments with added *trans* secondary amides such as **8** showed that an external, secondary amide function (presented in the steric environment of **1**) was unable to catalyze the reaction (Table 1, entry 5). This excluded the amide of **1** as the sole seat of catalysis.

(4) The possibility of purine catalysis (already made suspect by experiments with **5** was further discredited. The addition of 9-ethyladenine **9** and the naphthoylated ribosyl derivative **10** (Table 1, entries 6 and 7) failed to result in enhanced coupling rates between **2** and **3**. These experiments also confirmed that the ribose of **1** had only a spectator's role.

(5) Using control molecules **10** and **11**, we excluded catalytic mechanisms **13** and **14** (Fig. 5) involving combinations of the amide and one of the two base-pairing sites of **1**. Both complexes **13** and **14** propose that the

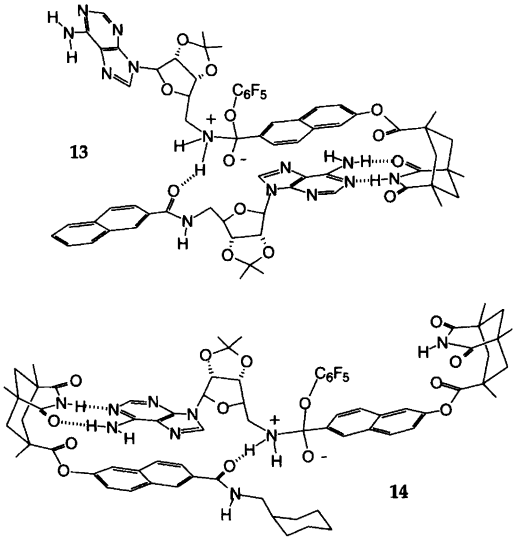

Fig. 5. Two possible mechanisms of catalysis excluded by experiments with molecules **10** and **11**.

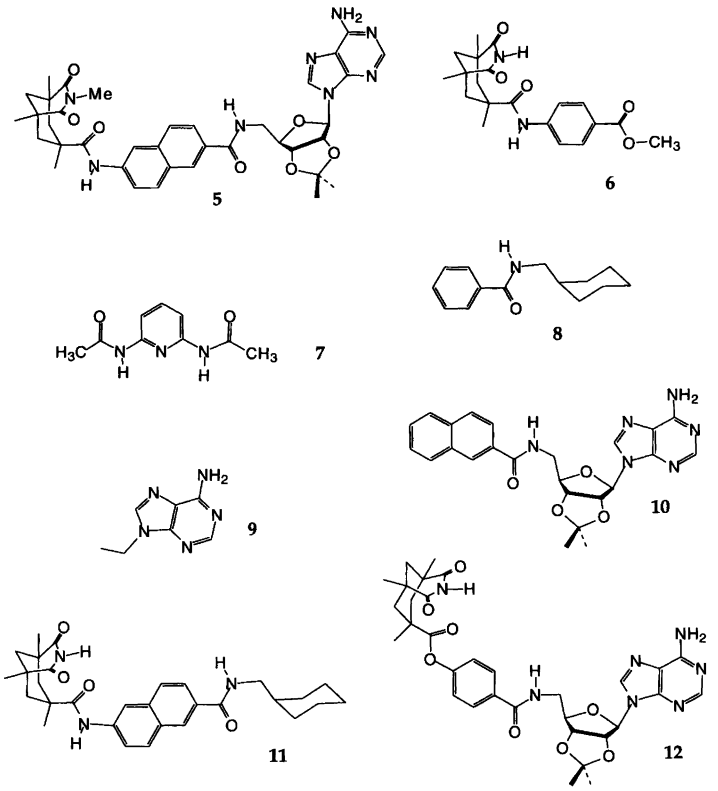

Fig. 4. Control additives bearing diverse functions for the reaction of **2**+**3**.

amide chemically assists the breakdown of the tetrahedral intermediate, but as neither **10** nor **11** was a catalyst under these conditions,[22] it can be concluded that the full template **1** is necessary for autocatalysis (Table 1, entries 7 and 8). Merely positioning one of the two substrates or one end of the tetrahedral intermediate on the template backbone is insufficient.

The isolated, individual features and functionalities of **1** are, therefore, unable to account for the autocatalysis observed. Rather, the whole product molecule is more effective than the sum of its parts. The most economical explanation consistent with these results is Fig. 2, complex **4**. Template catalyzed replication – in which **1** binds **2** and **3**, stabilizes the tetrahedral intermediate that forms, and favors its breakdown to an amide – is the source of autocatalysis.

The naphthoyl-based self-replicating system shown in Fig. 2 was our first success in the field of replication and it has been examined more thoroughly than any other system we created.[15,16,21,22] Even in the light of these experiments, another laboratory contended that the system did not replicate through directed template catalysis;[24] instead, these authors asserted a mechanism of simple (external) amide catalysis by the product **1**. Control experiments with molecules **5**, **6** and **8** – all amides – had already excluded this pathway, and controls with **10** and **11** had further excluded a more subtle pathway of internal amide catalysis[25] (Fig. 5). Nevertheless, additional evidence against amide catalysis by product **1** was desired, and thus experiments were conducted coupling **3** with molecule **15** (Fig. 6). Molecule **15** has neither stacking nor hydrogen bonding capability, and any catalysis by template **1** would support a mechanism of amide catalysis. However, it was shown that **1** did not act as a catalyst to form **16** (Table 2). Accordingly, the full template mechanism of complex **4** was upheld: catalysis is dependent not on the amide functionality of **1**, but rather on the template's ability to bind both substrates through stacking and hydrogen bonding.[26]

Was amide catalysis even a remote possibility under these conditions? This question was answered with control experiments involving even simpler amide-forming reactions with active esters in $CHCl_3$. As shown in Fig. 7 and Table 3, even at 20 mM concentrations of pentafluorophenyl ester **17** and benzylamine **18**, amide **20** was not a significant catalyst of amide formation.[26]

To be sure, catalysts other than the template **1** can be

Table 2. Generation of product **16** as a function of time, as followed by HPLC. All reactions were performed at 2.0 mM initial concentrations of reactants **3** and **15** in $CHCl_3$ with 1.0% TEA base added, 22±1 °C.

Conc. of ester **15** and amine **3**/mM	Equiv. template **1**	Avg. initial rate of formation of **16**/μM min^{-1}	Relative rate
2.0	0	15.0	1
2.0	0.5	15.0	1.00
2.0	0.7	15.2	1.01

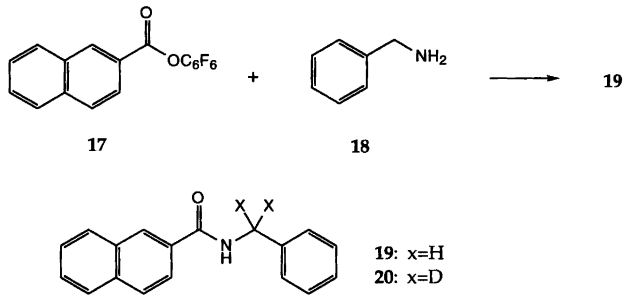

Fig. 7. Molecules for amide formation control experiments.

found for this system. The action of bifunctional catalysts in acylation reactions and glucose mutarotation is well known,[17] and indeed, it was found that the *cis* amide valerolactam (**21**) increases the initial rate of formation of **1** by 17% when added to the reaction of **2** and **3** (Table 1, entry 9). Fig. 8 depicts a possible catalytic role for valerolactam in the breakdown of the tetrahedral intermediate.[17] The acidic and basic sites on valerolactam can stabilize the zwitterionic tetrahedral intermediate and facilitate the required proton transfers for product release, ultimately regenerating the catalyst. Probably by the same mechanism, primary amides such as acetamide catalyzed the reaction of **2** plus **3**.[24] At 2.2 mM concentrations of starting materials, we found that 50% added acetamide increased the initial rate of formation of **1** by 35%.

Valerolactam and primary amides have a common capability: their acidic and basic sites, being on the same edge of the molecule, can act in concert. A *trans* amide, however, such as that in molecule **1**, has no such feature. Su and Watson[19] showed that under certain conditions, small *trans* amides (even tertiary amides such as *N,N*-

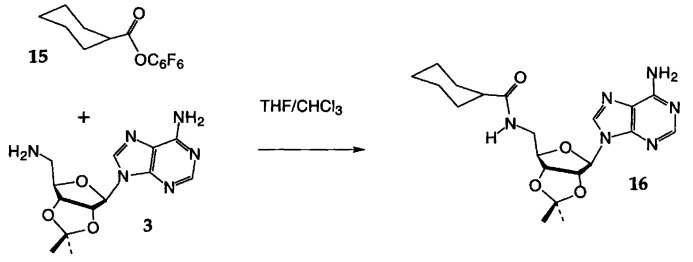

Fig. 6. Control molecules used to test the proposed mechanism of complex **14**.

Table 3. Amide formation control experiments at 25°C as followed by NMR spectroscopy. Coupling of **17** and **18** in $CDCl_3$ with or without addition of amide **20**. Initial velocities of reaction were determined through integration of the methylene peak of the product amide **31** at 4.72 ppm relative to the methylene peak of **18** at 3.88 ppm (see Ref. 26).

Conc. of ester **17** and amine **18**/mM	Equiv. amide **20**	Avg. initial rate of formation of **19**/μM min^{-1}	Relative rate
4	0	42	1
	0.5	42	1.00
8	0	84	1
	0.5	84	1.00
16	0	168	1
	0.5	174	1.04
20	0	258	1
	0.5	282	1.09

dimethylacetamide) can hydrogen bond to the tetrahedral intermediates in related reactions and catalyze their breakdown to products. However, in light of the failure of the many secondary amides in Table 1 to catalyze the reaction of **2** plus **3**, the *trans* amide of **1** cannot be a significant contributor to the autocatalysis observed under these conditions.

The most plausible explanation, then, for the autocatalytic nature of **1** remains its ability to act as a template for its parts. The forces of hydrogen bonding and aryl stacking position the amine and active ester on the template's surface, stabilizing the tetrahedral intermediate formed (complex **4**, Fig. 2). An alternative, but equivalent description involves the trapping of the equilibrium amount of the tetrahedral intermediate by the template. Subsequent collapse to the amide bond is favored over reversion to a termolecular complex, and this results in an exact replica of the catalyst in the form of a template dimer. The weak intermolecular forces which stabilize the dimer also permit its dissociation (a dimerization constant of 630 M^{-1} was measured for the product **3** in $CDCl_3$), and monomeric template is generated. Thus, the template, through specific noncovalent contacts, has produced a copy of itself – the molecule replicates.

In our earliest studies of replicator **1**,[16] a minimalist kinetic model for the formation of product was presented. This took into account all measurable bimolecular complexes and estimated the termolecular complexes of **2**, **3** and **1**. This model gave simulations that followed experimental data at low concentrations (8 mM or less), but did not predict the rate of product formation quantitatively at higher concentrations. Rather it predicted rates which were higher than that observed. We have not pursued such simulations further due to lack of the necessary experimental binding data. In particular, a number of distinct termolecular complexes of **2**, **3** and **1** are likely, only a few of which can be productive. The problems of simulation are compounded by the multiple equilibria that arise from the self-complementarity of **1**; with a dimerization constant, K_d, for **1** in the hundreds, it is likely that trimers, tetramers, etc. appear at higher concentrations. These decrease the amount of available monomeric template accordingly. In addition the oligomeric species (and even one form of the dimer) all feature 'frayed ends'; i.e., unpaired imides and adenines which provide sites for binding their respective complements. Such species would isolate the reaction components from each other rather than gather them, and would inhibit the reaction. An example of this isolation effect is presented later (Fig. 27). Accordingly, our recent modeling efforts have been limited to molecular mechanics minimizations[27] which are useful to estimate distances between functionalities in hydrogen-bonded complexes (e.g., Fig. 9).

The second generation

After many years of testing the system in Fig. 2, we are confident that it is a real, if modest, example of self-replication. It has been our recent goal to enhance the template-directed process relative to the desultory background reactions of amine and ester. Chemical catalysis – positioning acids and bases on the template surface – is one means to this goal; for example, hydrogen-bond-donating and -accepting functions could be trained on the tetrahedral intermediate to assist breakdown of the zwitterion to an amide. To begin, however, we have explored an alternative tactic: reducing the rate of background reactions.

The second generation systems emerged from the question: what are the geometrical requirements for autocatalysis to occur with these structures? The spacing requirements for an effective template were examined by changing the naphthoyl spacer for a longer biphenyl and a shorter phenyl spacer.[28,29] While almost no catalysis was observed with the shorter **12** (Table 1, entry 10), lengthening the spacer to create **22** had beneficial effects.

The reason for heightened replication with **22** was that in the original naphthoyl-drived system, coupling

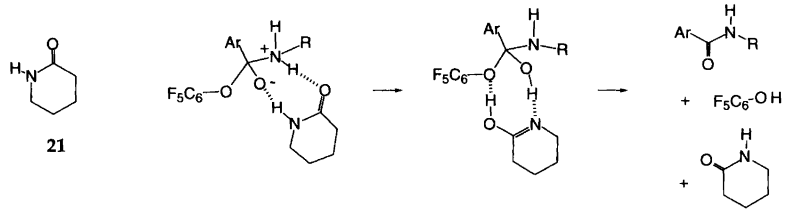

Fig. 8. A possible role for valerolactam in the catalysis of ester aminolysis.[17]

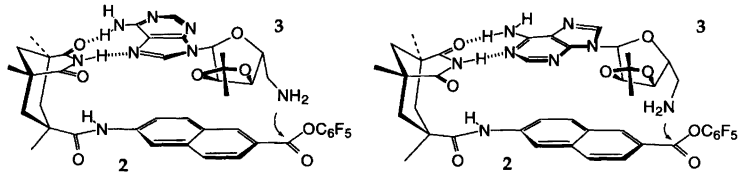

Fig. 9. Hoogsteen- and Watson–Crick-type intracomplex reactions in the naphthoyl self-replicating system.

between the amine and ester could occur within a complex of the *two precursors* (Fig. 9). In fact, this appears to be the major background pathway for product formation.[16] By lengthening the spacer element from a 2,6-naphthoyl to a 4,4′-biphenyl, the ester and amine are moved away from each other.[21] Intracomplex reaction now occurs less frequently; only if the adenosine is bound in the more-extended Watson–Crick mode (as shown in Fig. 10) can it reach the active ester. By reducing the amount of this pre-associative process, the effect of the autocatalytic pathway became apparent in the initial rate of growth of product. Specifically, at 50 mM, 20% of added biphenyl template increased the rate of coupling of **3** and **22** by 60%. Following the time course of the reaction, we observed a gentle sigmoidal product growth curve expected of a more efficient self-replicating system,[30] a feature which had already been observed for nucleic acid replicators.[3] This result was further exploited for a study of molecular competition and 'mutation' using N^6-CBz derivatives of adenosine,[31] (discussed later).

The next available step was to remove the remaining pre-associative bimolecular pathway with a still longer spacer, hence the terphenyl derivative **23** (Fig. 11). Computer modeling showed that this spacer left only two reaction paths to product: random bimolecular collision and template directed stabilization of the tetrahedral intermediate. As shown in Fig. 11, no pre-associative bimolecular path is available to the terphenyl molecule. Unfortunately, this system did not replicate; the only reaction seen was the slow bimolecular background reaction of ester and amine. An NMR titration

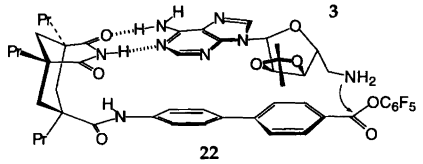

Fig. 10. Intracomplex reaction in the biphenyl self-replicating system.

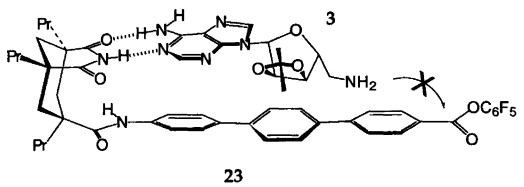

Fig. 11. Intracomplex reaction is prohibited in the terphenyl system.

of the terphenyl molecule **23** with 9-ethyladenine **9** revealed that the structure no longer provided adequate binding of adenosine, a feature that had been overlooked in the design of the molecule and doomed it to sterility.

What was needed was a new adenosine binding structure that could hold its substrate fixed, even in an elongated molecule. The spacer element in the receptor had to be of sufficient length to keep the amine and ester groups from reacting within a bimolecular complex, yet the affinity of the components for each other had to be high. These criteria were fulfilled by using a diaminocarbazole based diimide module developed in our efforts at molecular recognition of polynucleotides;[32] structure **24** (Fig. 12) had proved to be a nearly ideal complement to the purine nucleus of adenine.[33,34] The imides in molecule **24** chelate the purine through simultaneous Watson–Crick and Hoogsteen base-pairing, and the extended heterocyclic surface of the carbazole nestles against the purine. The binding affinity for adenosine derivatives was extremely high ($K_a \sim 10^5$ M^{-1} in $CDCl_3$), and with triplex-like chelation of the adenine moiety,[25] conformational ambiguities (Watson–Crick vs. Hoogsteen) binding modes were eliminated.

Attaching a biphenyl substituent on N^9 of the carbazole-diimide gave a new molecular skeleton for self-replication,[35,36] (shown as the methyl ester in structure **25**). Fig. 13 shows the computer-simulated geometry of a bimolecular complex between **25** and aminoadenosine **3**.[27] From the figure it is clear that the amine and ester are separated by a significant distance, >5.5 Å. This distance is fixed, as only limited motion within the complex is possible for the diimide-bound purine. Since the two reactive centers cannot approach each other within the complex, a bimolecular pre-associative pathway is eliminated. Thus the separation of ester and bound amine was achieved just as in the case of the terphenyl molecule (Fig. 11), yet the binding affinity was uncompromised; The diimide function of the new carbazole structure retained the ability to bind adenosine tenaciously.

The new self-replicating system (**3**+**26** to form the self-complementary template molecule **27**) is pictured in Fig. 14. The reaction was expected to occur either in an unassisted intermolecular fashion or through the template-stabilized complex **27**·**26**·**3**. Kinetic studies of the coupling reaction (Fig. 15) were performed in $CHCl_3$–THF mixtures and product appearance of the amide product **27** was, as before, followed by HPLC. The reaction was found to be autocatalytic; at 6.2 mM

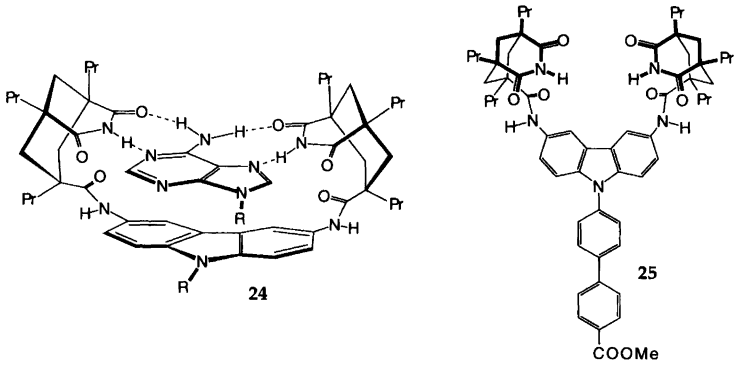

Fig. 12. The carbazolediimide backbone.

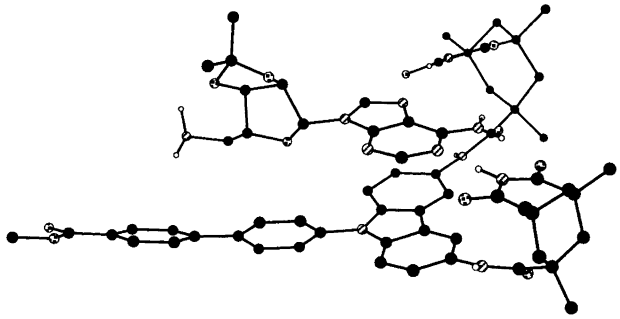

Fig. 13. Computer-generated complex[27] between the biphenylylcarbazole **25** and aminoadenosine **3**.

in 13% THF–$CHCl_3$, 50% added **27** increased the coupling of **3** plus **26** by an average of 53%. While this rate of autocatalysis was only comparable to that which we had previously achieved, it was evidence of termolecular autocatalysis in a system where all other likely pathways had been excluded. The tetrahedral intermediate for the complex **27·26·3** is modeled[27] in Fig. 16.

As with the naphthoyl based replicator, the carbazolediimide system was extensively tested against various control molecules to assure that autocatalysis was due to molecular recognition and not to trivial chemical catalysis by some functionality of the template. The results are summarized in Fig. 15 and Table 4.

Initial experiments revealed that the diimide function alone is not the source of the autocatalysis. Experiments using the diimide methyl ester **25** (Fig. 12) showed no catalysis of the reaction of **26** with **3**; instead, inhibition was observed, probably as a consequence of its sequestering the aminoadenosine in an unproductive complex. Additional experiments with **6** (Fig. 3), which competes, albeit poorly for adenosines, further supported the conclusion that the imide moiety was an ineffective catalyst.

Additional experiments showed that the phenyl amide **26** does not catalyze the reaction. The coupling rate of **26** with **3** was not increased by the addition of the benzoyl derivative **8** (Fig. 4), which presents a secondary amide function in a steric environment similar to the one in **27**, but lacks recognition elements. Finally, earlier experiments with adenosines (9-ethyladenine **9** and naphthoyl derivative **10**) had shown that neither the purine nor the ribose were effective as catalysts for an acylation reaction,[21] and this result was upheld in 13% THF–$CHCl_3$ by the data in Table 5 (*q.v.*).

Separated, the individual features and functionalities of **27** are unable to account for the autocatalysis observed; like the naphthoyl replicator, the effect of the whole molecule is greater than the sum of its parts. The autocatalytic nature of **27** is best explained by postulating that the molecule serves as a template for its own replication: the initial reaction to form **27** is relatively slow, but once present, it can form a productive complex **27·26·3** stabilized by complementary recognition surfaces. Within the complex, hydrogen bonding and aryl stacking hold the tetrahedral intermediate in place (as modeled in Fig. 16) and favor breakdown to amide **27** over reversion to **26** and **3**.

It is important to stress that under these conditions, autocatalysis is not a general feature of ester aminolysis. For example, experiments with the naphthoyl ester **28** and amine **3** (Fig. 17) showed that the amide product **10** did not significantly catalyze its own formation (Table 5). Again, complexation of **3** with the diimide methyl ester **25** inhibited the reaction of **3** with **28** (as it did the reaction of **3** with **26**). In either case, the inhibition is presumably due to the ability of the diimide **25** to sequester **3** in a complex sterically encumbered toward external electrophiles.

What are the consequences of replication through high-affinity complexes? The rate of formation of **27** compared with that of **10** under identical conditions revealed that recognition event *slows* the rate of coupling twofold; in the presence of **3**, active ester species which are unable to complex **3** (e.g., **28**) are twice as reactive toward amines than is ester **26**. For the same reasons, it seems likely that non-complexed esters are also more prone to side reactions. Structures which recognize each other and form complexes become stabilized; the surfaces in contact are protected from external, often destructive reagents, and the protected structures react primarily with molecules which are specifically complexed with them.[37] If this can be generalized, molecular recognition offers advantages for evolution at the molecular level; survival as well as replication is enhanced.

After much experimentation,[36] the shortcomings of the diimide replicator became clear; it suffered from

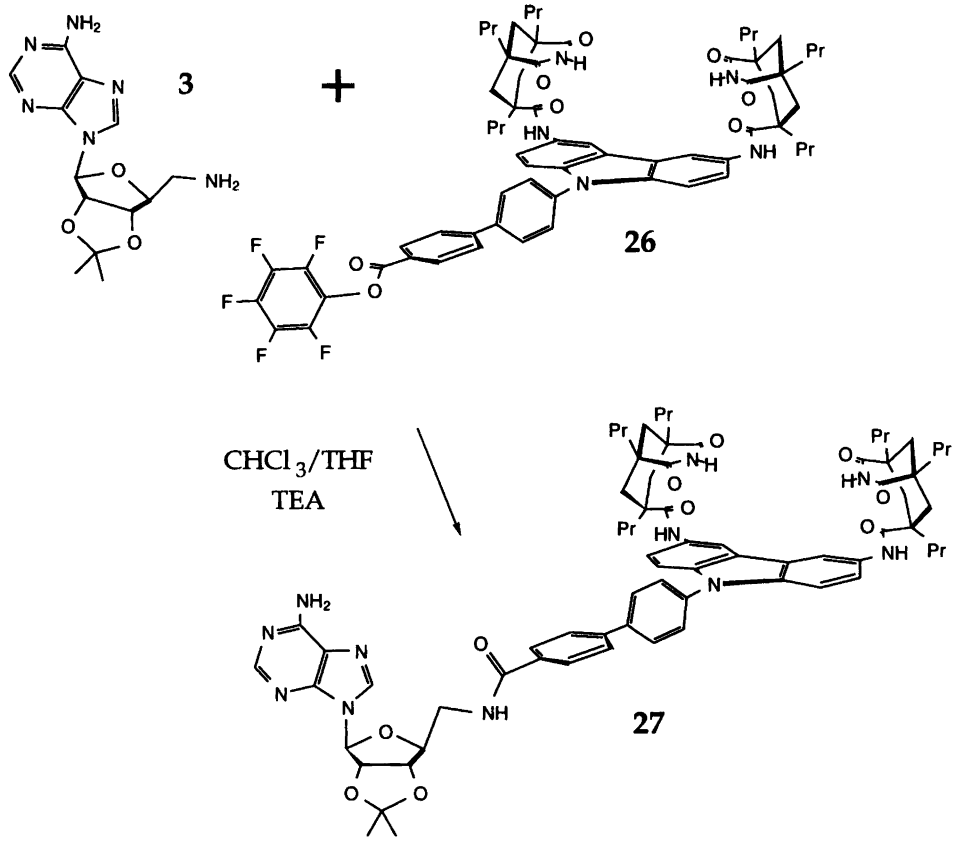

Fig. 14. A diimide-based replicator.

severe product inhibition. Based on several NMR studies, the dimerization constant of diimide template **27** was calculated to be $\sim 10^9$ in 13% THF–$CHCl_3$, and thus very little template (of the order of 1 μM)* is present as a monomer in solution. Thus, the system shows promise, but is insufficiently catalytic due to the small amount of free template. That so little free template gave rise to a 54% increase in rate indicates the success of **27** in positioning its substrates for reaction. If chemically useful functional groups could be focused specifically on the transition state of the reaction, a new chapter in self replication might be written. We are now exploring molecules in which functional groups are incorporated into the template at the point of amide formation. These include functionalities which can enhance proton transfers within the tetrahedral intermediate.

To date, two pyridyl templates **29** and **30** (Fig. 18) have been tested as catalysts for the reaction of **3** plus **26**. The pyridine nitrogens were intended to act as general bases to catalyze the breakdown of the tetrahedral intermediate, but neither of these molecules showed enhanced catalytic activity. The bipyridyl molecule **29** was difficult to characterize in solution, showing aggregation (beyond dimerization) by NMR analysis. The mono-pyridyl template **30** acted much as its biphenyl predecessor **27**: 50% of added template catalyzed the reaction by about 54% at 6.2 mM concentrations.

To summarize our progress, we have shown the viability of creating abiotic self-replicating systems. Detailed evidence of self-replication has been obtained. We have modified our molecules to enhance the autocatalytic pathways of the systems, but substantial autocatalysis (e.g., ten-fold rate enhancement) is still an unrealized goal. In the meantime, we have used our existing systems to delve into some of the interesting questions that naturally arise when replicators are at hand, and these explorations are detailed in the next section.

Further experiments with replicating molecules

Once we had functioning replicators, it was not difficult to modify the systems to simulate various aspects of evolution. Anyone may debate the relevance of these model systems to pre-biotic chemistry or biology – we are, after all, dealing only with carefully crafted molecules

* The concentration of template monomer at 3.1 mM total template concentration was calculated by estimating a single binding event at 86 000 M^{-1} in 13% THF–$CHCl_3$ (between 10^5 and 576 M^{-1}) and taking the dimerization of the template to be the square of that value. Values were inserted into a model of autocatalysis developed by James Nowick (see details in Ref. 16). The concentration of template monomer may also be approximated using $K_{dim} = [\text{template dimer}]/[\text{template monomer}]^2$.

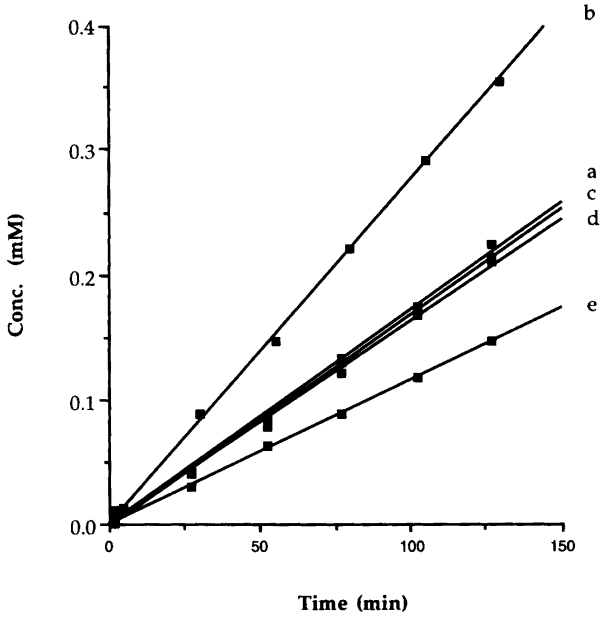

Fig. 15. Representative kinetic plots of the generation of product **27** as a function of time (initial 5% of reaction). All reactions were performed at 6.2 mM initial concentrations of reactants **3** and **26** in 13% THF–$CHCl_3$ with 1.0% TEA base added, 22±1 °C. All individual slopes (reaction rates) are given in Table 1: (a) baseline reaction (**3**+**26**); (b) baseline reaction plus 0.5 equiv. product **27**; (c) baseline reaction plus 0.5 equiv. imide methyl ester **6**; (d) baseline reaction plus 1.0 equiv. amide **8**; (e) baseline reaction plus 0.5 equiv. diimide methyl ester **25**.

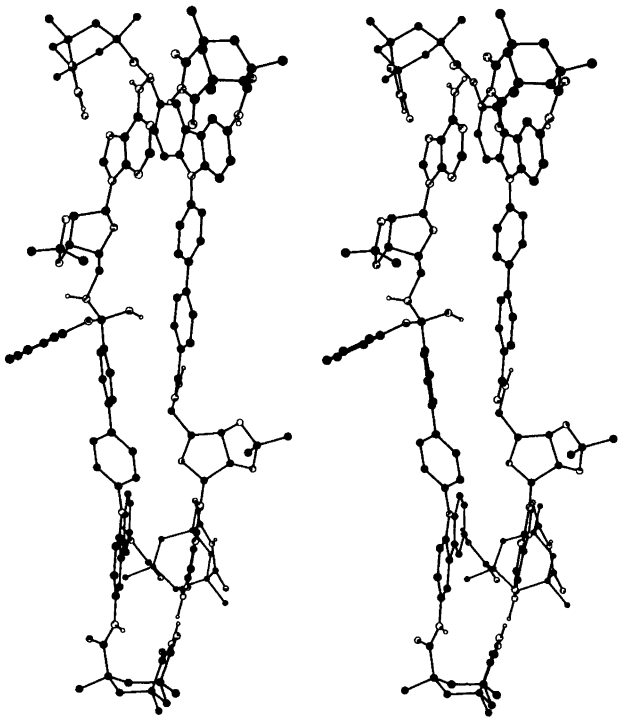

Fig. 16. Computer-generated structure[27] of the tetrahedral intermediate for the complex **27**·**26**·**3**, modeled as the neutral tautomer (stereoview). Hydrogens attached to carbon have been omitted for clarity.

and small rate enhancements –but our fascination with some engaging aspects of self-replication proved irresistible.

Starting with the biphenyl replicating system (**3**+**22**), we decided that the adenine component should be modified (Fig. 19).[31] The exocyclic amine of the purine was acylated with urethane-type blocking groups: a benzyloxycarbonyl was attached to give **31**, and an *o*-nitrobenzyloxycarbonyl was attached to give **32**. Such changes were known to reduce base-pairing possibilities: the blocking groups protrude on the Watson–Crick hydrogen bonding edge of the purine and bias the molecules that bear them toward base-pairing on the Hoogsteen edge.[38] Both **31** and **32** coupled to the biphenyl **8** uneventfully, and the respective self-complementary products (**34** and **35**) are formed. Both were replicators, they did catalyze their own formation. But hobbled as they were in their base-pairing capacities, they could not be efficient in this regard. They also showed promiscuity; as shown in the generic complex **36**, there is no means by which either template can distinguish between those molecules bearing a benzyloxycarbonyl group from those bearing an *o*-nitrobenzyloxycarbonyl group. The mistake-making behavior of these molecules was reciprocal; one catalyzed the formation of the other and *vice versa*.

These experiments were, of course, *contrived* to make a further point. We intended to show that environmental effects could alter the system such that a third replicator was produced which was more efficient than either **34** or **35**. The environmental stress occurred with the introduction of light into the system; when the molecules bearing

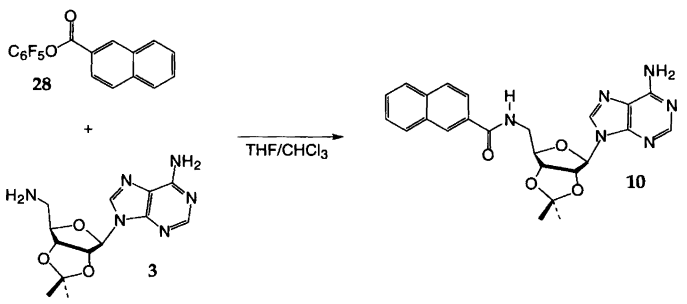

Fig. 17. A non-replicating system.

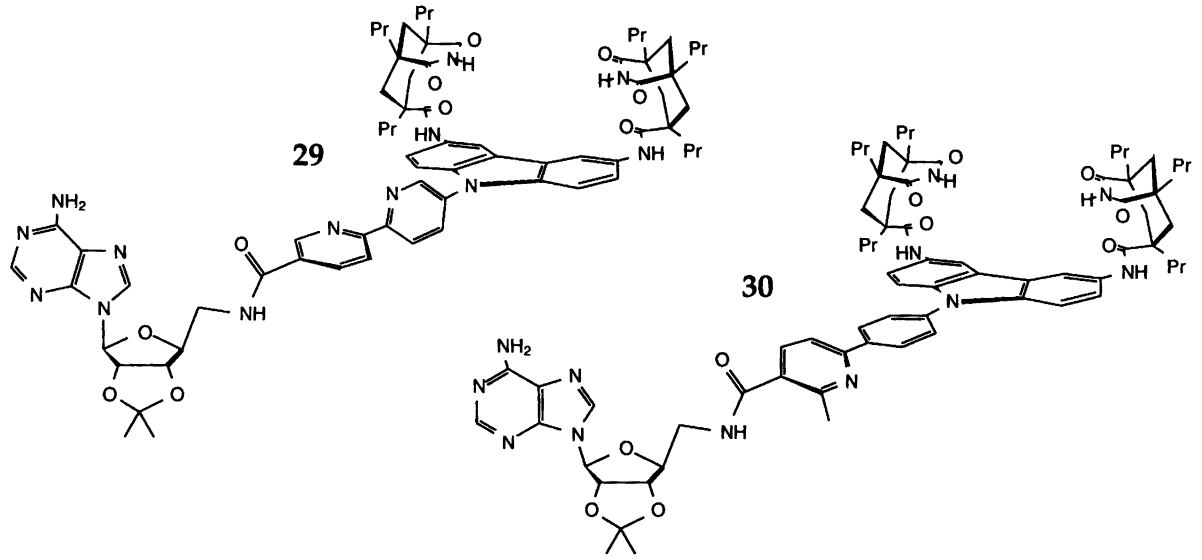

Fig. 18. Pyridyl templates intended to confer chemical catalysis at the point of amide formation.

Table 4. Generation of product **27** as a function of time. All reactions were performed at 6.2 mM initial concentrations of reactants **3** and **26** in 13% $CHCl_3$–THF with 1.0% Et_3N base added, 22 ± 1 °C.

Reaction	Additive	Avg. initial rate of product formation/μM min^{-1}	Relative rate
a	Nothing	1.71 (6)	1.00
b	Product **27** (0.5 equiv.)	2.63 (11)	1.54 (8)
c	Imide **6** (0.5 equiv.)	1.72 (2)	1.01 (4)
d	Amide **8** (1.0 equiv.)	1.56 (8)	0.91 (6)
e	Diimide **25** (0.5 equiv.)	1.18 (10)	0.69 (6)

Table 5. Generation of product **10** as a function of time. All reactions were performed at 6.2 mM initial concentrations of reactants **3** and **28** in 13% $CHCl_3$–THF with 1.0% TEA base added, 22 ± 1 °C.

Additive	Individual initial rates of product formation (μM/min)	Avg. initial rate of product formation (μM/min)	Relative rate
Nothing	3.76 3.84 3.73	3.78 (6)	1.00
Amide **10** (0.5 equiv.)	4.11 4.05 4.07	4.08 (3)	1.08 (2)
Diimide **25** (1 equiv.)	2.32 2.64 2.85	2.60 (27)	0.69 (7)

the *o*-nitrobenzyloxycarbonyl group were irradiated, the photolabile blocking group was cleaved.[39]

In one experiment,[31] amines **31** and **32** were allowed to compete for a limited amount of active ester **8**.

The result (Fig. 20) was that the nitro derivative was a slightly more effective replicator; more **35** was formed than **34**. The reaction solution was then irradiated, causing the photolabile nitro groups to be removed (the photocleavage product of **32** is **3** and that of **35** is **33**). Because the new replicator **33** generated in this manner permitted both Watson–Crick and Hoogsteen base-pairing, it had a statistical advantage over either **34** or **35**. Accordingly, when additional amounts of active ester were added to the solution, the new molecule was more effective at replication. The resources of the system were quickly consumed by the product of the irradiation. The permanent change in chemical structure caused by light created a self-replicating molecule that proved to be better fitted to the environment of the experiment.

We were next intrigued with the question, could synthetic replicators shuffle their components to generate new hybrid replicators? This notion was inspired by recent computer-generated model systems,[40] which have allowed many evolutionary problems to be explored. In our answer, we made use of a self-replicating system developed in parallel with the adenine biphenyl replicator, but which had never been fully explored. The replicator, pictured at the top of Fig. 21, was based on molecular recognition of thymine ester **38** and xanthene amine **37** on template **39**. Coupling of the components

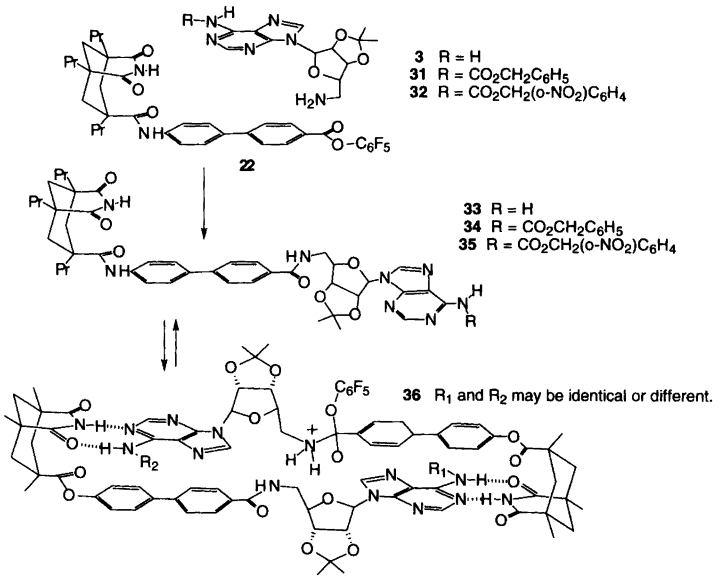

Fig. 19. Molecules of a mutation experiment.

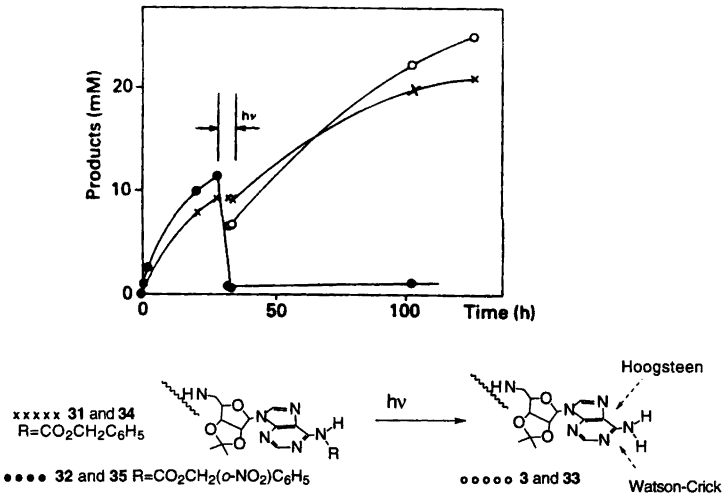

Fig. 20. Kinetics of a 'mutation' experiment.

through an amide bond gave self-complementary structure **39**, which showed autocatalytic behavior when added to its starting materials[41] as expected.

In the recombination experiment, the components of the new replicator were shuffled with the components of our biphenyl replicator.[42] We expected to generate four self-complementary systems, and assumed that self-complementarity of the structure was sufficient for replication. After all, our replicators (and those of others)[43] all shared this feature. The possible combinations of **3**, **22**, **37** and **38** were duly synthesized (Fig. 21), and their behavior gave us a new insight concerning molecular shape. One of the recombinants, the adenine–thymine product **40**, resembles DNA, but with an amide backbone. It turned out to be the most effective synthetic replicator we have encountered to date (perhaps this is not mere coincidence). The other recombinant **41** was unable to catalyze its own formation.

The reasons for the differing activities of **40** and **41** are a consequence of their molecular shapes. The adenine–thymine hybrid can present its recognition surfaces (arrows on structure **40**) in such a way that a productive complex can be assembled with its precursors. No such conformation is available to the unfortunate **41**. The overall conformation of **41** is either a C-shape (shown) or an S-shape (not shown). In the C-shaped conformation, recognition surfaces converge (arrows on structure **41**), and the parts required for replication cannot fit within the cleft. In the S-shaped conformation, the termolecular complex can form, but it cannot be productive; the recognition surfaces diverge and the reactive centers are too far apart to form a covalent bond. Self-complementarity is thus shown to be insufficient for replication; it is necessary for the replication product to be able to achieve a reactive conformation.

Many replicating systems are based on cycles. Nucleic

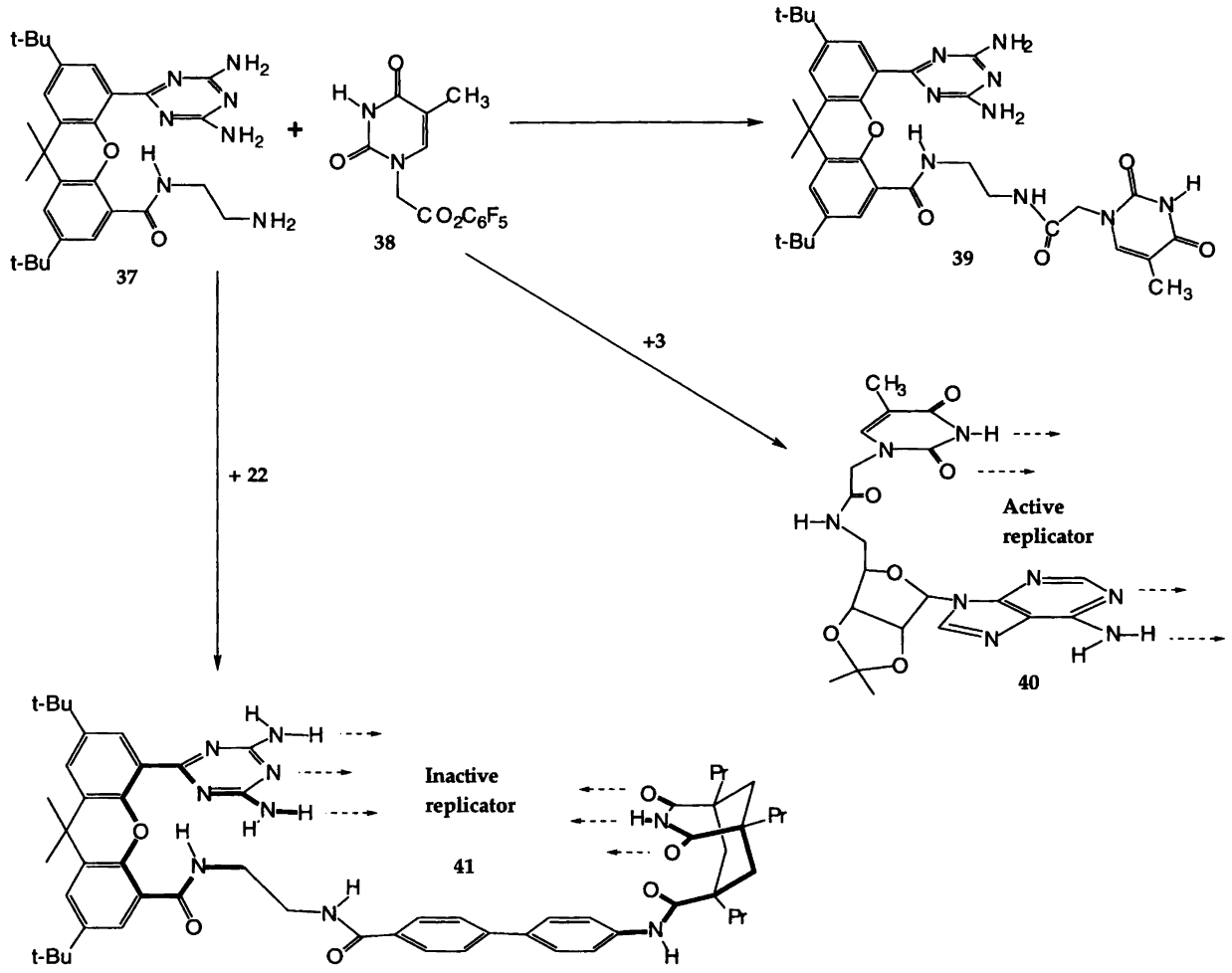

Fig. 21. A recombinant experiment with two different replicators.

acid replication is the paradigm in biology: one strand acts as a template for the other, and the new strand in turn acts as a reciprocal template. This general pattern of template directed catalysis has been used before in abiotic systems,[44-49] and we were able to modify our carbazolediimide replicator to establish the bicyclic system shown schematically in Fig. 22.

Both the carbazole portion of the receptor and the ribose portion of adenosine are well-suited to synthetic elaboration into a reciprocal system. Each component was outfitted with amine nucleophiles and active ester electrophiles for covalent coupling reactions,[50] that is, two amines and two *p*-nitrophenyl esters were prepared (**3**, **42**, **43** and **44**, Fig. 23). Next, the two templates **45** and **46** (Fig. 24) were synthesized. Two templates **47** and **48** were also prepared; these control systems contained many of the structural features of template **46** but lacked various recognition sites.

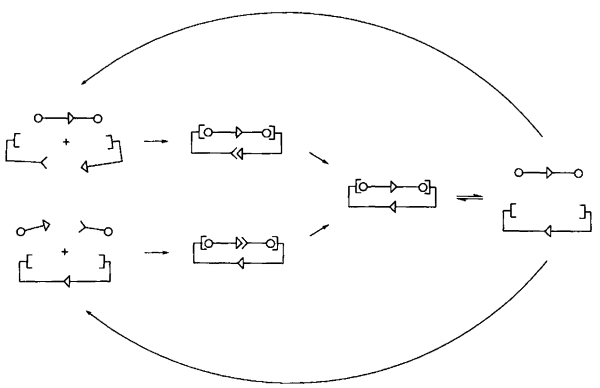

Fig. 22. Schematic representation of a replication cycle of reciprocal templates.

Template effects on the coupling of **3**+**42** and **43**+**44** were then individually measured.[50] The results are summarized in Table 6. The coupling between **3** and **42** proceeded with an initial rate of 1.5×10^{-8} M min^{-1} (Fig. 25) to give the product amide **45**. The reaction was then run in the presence of 1 equiv. of **46** (the product of **43** and **44**). Template **46** accelerated the coupling reaction between **3** and **42** by a factor of ten. This acceleration was reduced when competitive binders were added. For example, the product **45** (1 equiv.) reduced the acceleration to threefold, while excess 9-ethyladenine (**3**, 10 equiv.) lowered the acceleration to twofold. The rate of the uncatalyzed reaction was unaffected by the presence of these compounds. Compound **48**, having one

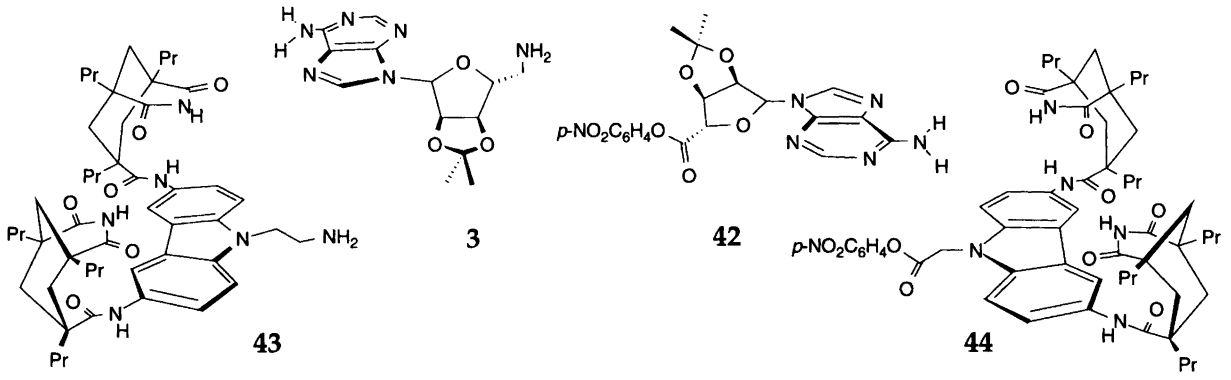

Fig. 23. Components of a replication bicycle.

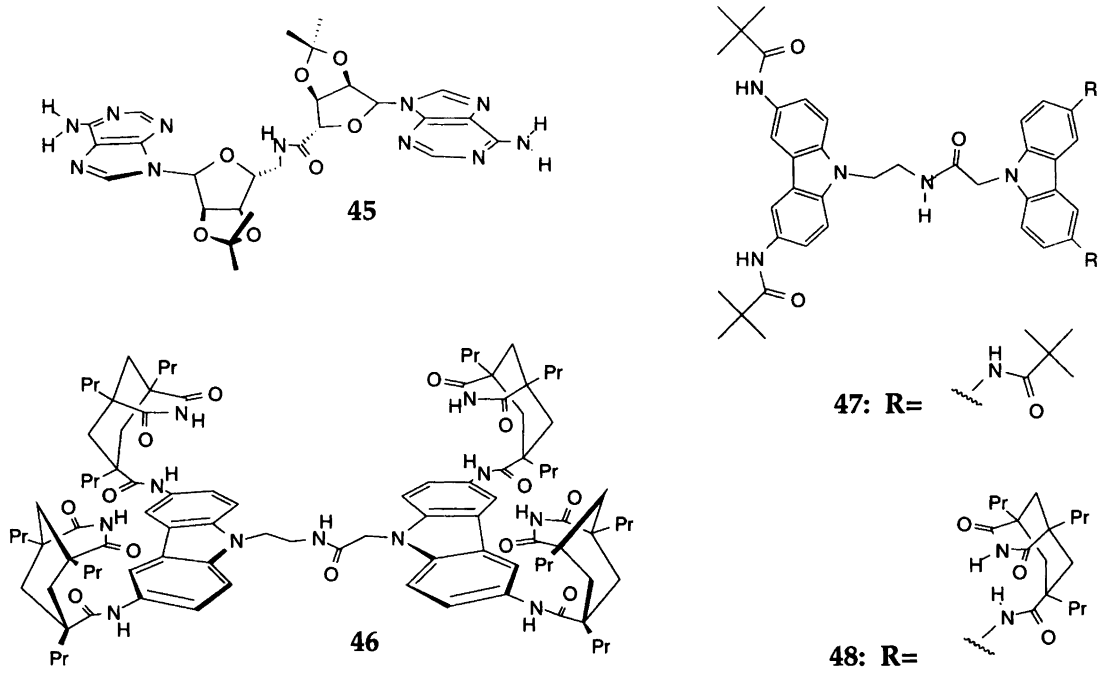

Fig. 24. Templates for the evaluation of coupling reactions between **3**, **45**, **46** and **47**

receptor site, slightly *reduced* the initial coupling rate Thus, molecules bound by **48** are less accessible for reaction with other molecules in solution. The control **47**, which lacks both receptor sites and contains only amide functionalities, had no effect on the rate of the reaction. This is in keeping with earlier studies of amide catalysis of nitrophenyl ester aminolysis[19] in which amide catalysis appeared only at concentrations over a hundred times greater than in our studies.

Reciprocity was established when it was observed that **45** catalyzed the formation of **46**. An initial rate of 4.3×10^{-9} M min^{-1} was measured for the coupling of **43** and **44** (Table 6), but addition of one equivalent of **45** increased the initial coupling rate five fold. Again either product (**46**) or 9-ethyladenine acted as competitive inhibitors; both reduced the acceleration while having no significant effect on the background reaction rate.

All of the observations are consistent with a mechanism in which the template stabilizes the tetrahedral intermediate. A structure of the tetrahedral intermediate in the coupling of **3** and **42** is shown in Fig. 26. The model suggests that both adenosine derivatives can be accommodated simultaneously by template **46**, and that the binding permits close proximity of the two reactive functions. In the case of the reaction of **43** with **44**, acceleration is likely due to a similar complex, but here the 'outside' amide is formed instead of the 'inside' one. The two sets of reactions **3** plus **42** and **43** plus **44** are related in a special way, since the product of one reaction is a template for the other: these reactions comprise a formal *replication cycle*.[44]

Additional support for this mechanism and an unusual result emerged from experiments in which the concentration of added template was varied. Increasing the amount of **46** increased the initial coupling rate of **3** plus **42** with a maximum at 2 equiv. template (Fig. 27). Further addition resulted in *lower* coupling rates. Apparently, the two reactive components became increasingly isolated as

Table 6. Initial rates of amide formation.[50c]

Reaction[a]	Concentration/mM [45]	[46]	[9-Et-Ad.] [9]	[47]	[48]	Init. rate/nM min^{-1}[b]	Relative rate
3+**42**	—	—	—	—	—	15	1
3+**42**	0.05	—	—	—	—	16	1.1
3+**42**	—	0.05	—	—	—	150	10
3+**42**	0.05	0.05	—	—	—	42	2.8
3+**42**	—	0.05	0.5	—	—	30	2
3+**42**	—	—	0.5	—	—	15	1
3+**42**	—	—	—	0.05	—	15	1
3+**42**	—	—	—	—	0.05	11	0.7
43+**44**	—	—	—	—	—	4.3	1
43+**44**	0.05	—	—	—	—	23	5.3
43+**44**	—	0.05	—	—	—	4.3	1
43+**44**	0.05	0.05	—	—	—	13	3
43+**44**	0.05	—	0.5	—	—	15	3.5
43+**44**	—	—	0.5	—	—	4.8	1.1
42+**43**	—	—	—	—	—	53000	1
42+**43**	—	—	0.5	—	—	14000	0.3
3+**44**	—	—	—	—	—	2200	1
3+**44**	—	—	0.5	—	—	600	0.3

[a] Both components were present at 0.05 mM in $CHCl_3$ at 25 °C with 4 mM triethylamine. [b] Values are averaged from multiple independent runs. Standard deviations are ±15%.

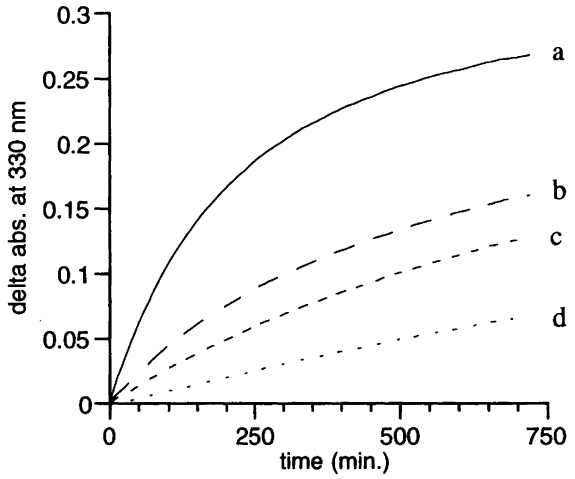

Fig. 25. Reaction between **3** and **42** (both at 0.05 mM) in $CHCl_3$ at 25 °C with 4 mM Et_3N and with (a) 1 equiv. of **46**; (b) 1 equiv. of **46**+1 equiv. of **45**; (c) 1 equiv. of **46**+10 equiv. of 9-ethyladenine **9**; (d) no additives (background).

they exist as bimolecular complexes on different template molecules. As shown by the solid line in Fig. 27, this data fits a curve[50b,c] calculated for such a system in which the template binds each substrate with a K_a of 14000 M^{-1}. This value is in good agreement with the experimentally observed association constants for similar carbazole diimide receptors for adenosines.[34]

The final coupling combinations of the starting materials (Fig. 23) are the reactions between **3** and **44** and between **42** and **43**. These reactions involve complementary bimolecular components and are very rapid. Both are about three orders of magnitude faster than those previously discussed (Table 6). The high rates are due to the association of the two reaction partners in complexes where the reactive groups are forced into close proximity[51] (Fig. 28 depicts complex **42**·**43**). These combinations 'suffer' from a preassociative bimolecular effect. In experiments involving all four reactants (**3**, **42**, **43** and **44**), preassociative bimolecular pathways dominate, template effects of **45** and **46** become negligible and a meaningful test of replicator efficiency cannot be arranged. Nevertheless, reciprocal templates **45** and **46** undergo a formal replication cycle in which covalent coupling reactions are individually accelerated by up to 13-fold. These rate enhancements are comparable to those observed by Kelly in reaction templates for bimolecular S_N2 reactions,[8] and are considerably larger than those observed in template effects involving the *self*-complementary structures (above). It is perhaps no accident that the strands of DNA are complementary, and not *self*-complementary. Whether reciprocal cycles are generally more efficient than the minimalist self-complementary replicators is the subject of on-going research.

We have now explored replication, mutation and recombination with admittedly simplistic molecular analogues, and perhaps how evolution might function at the molecular level. Our compounds are clearly abiotic, soluble in chloroform and a far cry from the structure of DNA. But these chemicals exhibit traits which one generally associates with biology. Perhaps our results are trying to tell us that if structures capable of selective molecular recognition are at hand, life is no coincidence but rather a inevitable phenomenon of covalent chemistry just waiting to occur.

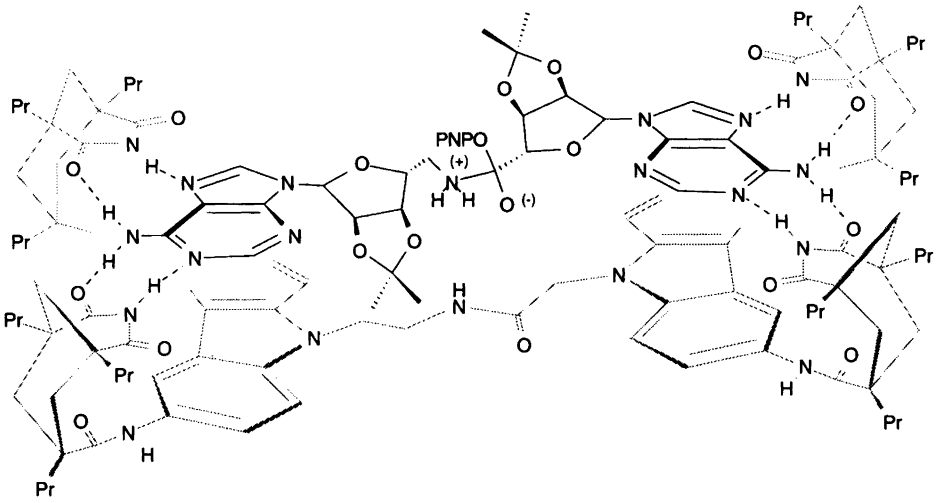

Fig. 26. Structure of the tetrahedral intermediate from **3**+**42**+**46**.

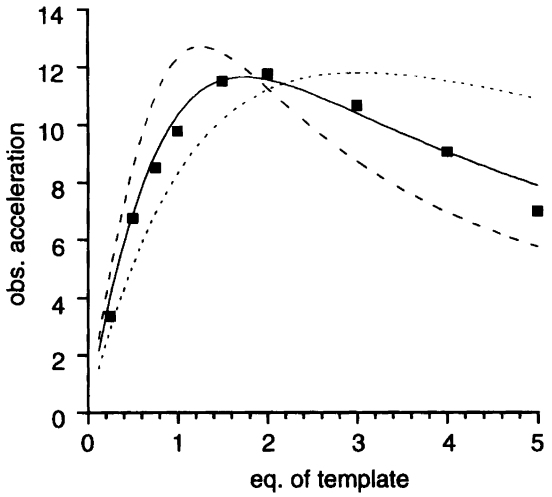

Fig. 27. Plotted points: observed acceleration of **3**+**42** vs. the amount of template **46**, (0.05 μM of each reagent, 4 μM NEt_3 in $CHCl_3$ at 25 °C). Solid line: calculated concentration (under the same conditions) of productive complex (**3**·**42**·**46**) for a diimide–adenine affinity of 14 000 M^{-1}. Dotted line: calculated concentration of productive complex (**3**·**42**·**46**) for K=5000 M^{-1}. Dashed line: calculated concentration of productive complex (**3**·**42**·**46**) for K=45 000 M^{-1}. Note than no vertical scale is intended for the calculated data, as the scale is different for each line. Calculations were based on the assumption that the template binds each substrate independently and with the same intrinsic affinity K.[50b,c]

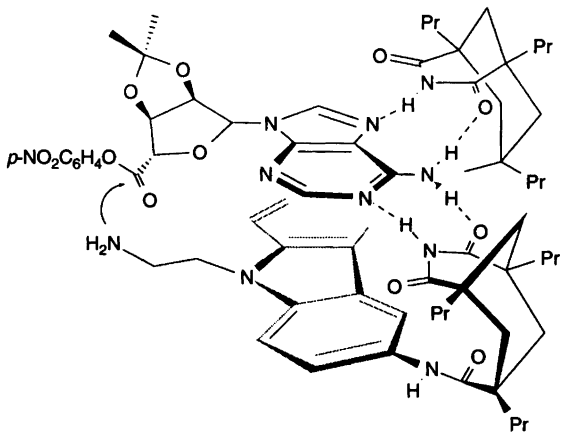

Fig. 28. Bimolecular complex proposed for the fast reaction between **42** and **43**.

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