Use of Molecular Recognition To Drive Chemical Evolution: Mechanisms of an Automated Genetic Algorithm Implementation**

Alexey V. Eliseev* and Marina I. Nelen

Dedicated to Professor Fredric M. Menger on the occasion of his 60th birthday

Abstract: A detailed description is provided for a new general approach that allows production of amplified amounts of effective noncovalent binders from pools of compounds that can exist in a dynamic equilibrium. This approach involves a) selective binding of the effective pool components by the immobilized target compound; b) equilibration of the pool remaining after selection, and thereby regeneration of the effective components; c) repetition of the selection–equilibration cycles. It represents a new, automated, implementation of a genetic algorithm/mechanism in a chemical system. Application of the approach to the generation of arginine binders involved the synthesis and screening of eight compounds, each of which was capable of forming a pool of three isomers (*trans,trans*, *trans,cis*, and *cis,cis*) that could be interconverted by photoisomerization. These compounds were screened for their affinity for, or selectivity of binding to, guanidinium derivatives. The most promising compound was then used in our method to generate an amplified amount of the *cis,cis* isomer, the strongest binder from the equilibrating pool. The receptors were selected using an arginine derivative immobilized on the modified silica gel support, which had been found to possess binding affinity and selectivity similar to those of guanidinium salts in solution. Mathematical models of the approach were developed that allowed us to evaluate the importance of various experimental parameters and to assess the applicability of the method to larger combinatorial pools.

Keywords: combinatorial chemistry · genetic algorithms · molecular recognition · photochemistry · template synthesis

Introduction

Among the numerous lessons chemists have learned from biology, that of evolutionary development is probably still the most difficult to follow. Applications of the principles of Darwinian evolution to a wide variety of scientific problems were formalized long ago in terms of so-called genetic algorithms (GAs) which, until recently, have been mostly applied to solving problems in the field of computer science.[1] During the last decade, however, new techniques emerging in chemistry and biochemistry, as well as a vastly increased diversity of molecular structures dealt with on a routine basis, have drawn chemists' attention to the use of GAs as a method that helps to generate or find novel compounds with the required properties.[2]

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[**] Use of Molecular Recognition to Drive Chemical Evolution, Part 2. For Part 1, see ref. 9.

The general sequence of events used by GAs is shown in Figure 1. The initial population, which is simply represented in a chemical system by an array of compounds, undergoes

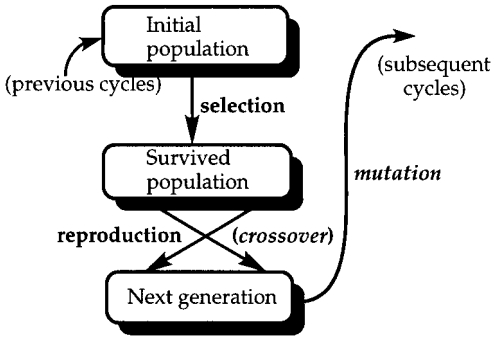

Figure 1. General scheme of the genetic algorithms.

multiple cycles of selection–reproduction to generate a new array (or subarray), possessing new (or improved) properties, such as binding affinity for a particular target compound or the ability to catalyze a chemical reaction.

There are several major approaches aimed at the implementation of various attributes of the GA in chemical and

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biochemical systems. The most remarkable among them include recent work on the optimization of combinatorial libraries,[3] evolutionary enrichment of large nucleic acid pools,[4] mutagenesis/in vivo selection of proteins,[5] and the chemistry of self-replicating systems.[6]

Application of the GA to combinatorial chemistry has been based on manual implementation, that is, stepwise identification and optimization of the effective leads. The other approaches involve chemical or biological selection and/or amplification processes. Thus, the selection of structures can be driven by the thermodynamics of binding (as in nucleic acid evolution[4]), the kinetics of chemical transformations (as in self-replication of individual compounds[6], molecular assemblies,[7] and amplification of chirality in chemical reactions[8]), or the metabolic processes in cell cultures (protein evolution[5]). We believe, therefore, that it may be more appropriate to refer to these approaches as employing genetic mechanisms, rather than genetic algorithms.

We have recently introduced a novel approach that makes it possible to amplify a subset of compounds possessing higher affinity for a given target, within an equilibrating pool of components.[9] This approach involves a more automated implementation of the features of GAs, such as selection and mutation, than the previously known techniques. In this paper we present an example of a general strategy that can be used to design an evolving equilibrating pool, describe mechanisms and mathematical models of this new method, and assess the applicability of the approach to more complex systems.

Results and Discussion

Principle of the approach: The thermodynamic basis of the chemical evolution approach is outlined in Figure 2. The most important requirement for the method is the availability of a

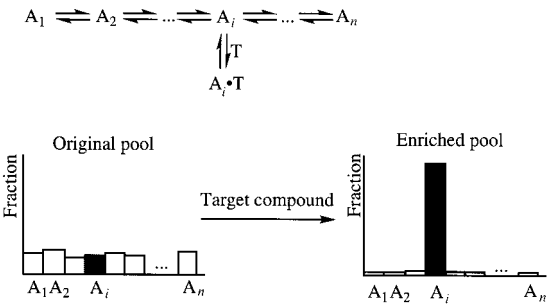


Figure 2. Shift of equilibrium in the dynamic pool by the target compound.

mixture (pool) of components ($A_1 - A_n$) (Figure 2) that can be brought to a dynamic chemical equilibrium by applying specific conditions. The equilibration can be based on any kind of reversible chemical reaction, such as photochemical isomerization (as presented here) or exchange reactions, some of which are mentioned below. The features of the experimental design of such pools are akin to the general principles of combinatorial library design;[10] that is, a single component (A_i, Figure 2) or a small subset of components is expected to show higher affinity for a particular target compound (T, Figure 2) than the majority of the pool components. If the target compound is then introduced into the equilibrating pool, the equilibrium should be shifted towards the formation of complexes $A_i \cdot T$ corresponding to the components possessing higher binding constants with the target.[11] In the ideal situation shown in Figure 2, the strong complexation of one of the components (A_i) will drive the system to the production of an amplified amount of A_i, in the form of the complex $A_i \cdot T$, at the expense of the other components in the pool.

Whereas a targeted equilibrium shift of this kind is essentially predicated on the well-known Le Chatelier principle, its chemical realization may open new routes to generation of combinatorial libraries, leading to discoveries of novel synthetic ligands and receptors. Thus, Venton et al.[12a] and Swann et al.[12b] have introduced a method for using macromolecules as molecular traps for components selected from reversibly synthesized peptide libraries, and have detected amplification of the higher affinity ligands. The utility of such an approach has recently been demonstrated by Huc and Lehn[13] in the formation of virtual libraries of functional imines that have been enriched with carbonic anhydrase inhibitors upon introduction of the target enzyme. Sanders et al. have reported examples of the thermodynamically controlled formation of select macrocycles containing complementary building blocks, as opposed to a multitude of theoretically possible compounds.[14]

Some of the problems that may arise in practical applications of the targeted equilibrium shift in equilibrating pools are related to the presence of the target compound in the mixture. Indeed, one can envision a variety of side-interactions, of covalent or noncovalent types, that may interfere with the binding and equilibration processes. In addition, the target compounds, particularly biopolymers, may be sensitive to the equilibration conditions. Finally, the free and bound pool components always have to be separated from the target.

The experimental method introduced here to overcome the problems mentioned above involves a physical separation of the equilibration and binding sites. In the reaction setup shown in Figure 3, the pool is circulated through two

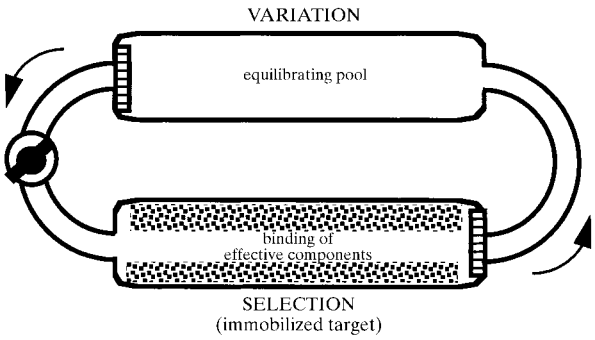


Figure 3. Experimental implementation of the dynamic (evolutionary) molecular diversity approach.

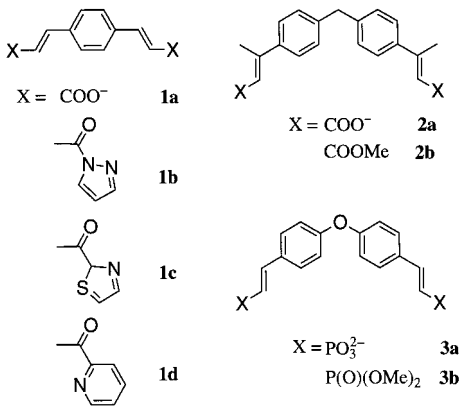
chambers. The selection site (lower chamber) contains the target compound in its immobilized form, which preferentially binds the effective components of the pool, like an affinity chromatography column.[15] As a result of passing

0947-6539/98/0405-0826 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 5

through this column the pool is depleted of the effective components (strong binders). It then enters the upper chamber, in which the pool components are brought to a dynamic equilibrium by physical initiation (e.g., by irradiation with light; see below) or catalysis of the equilibration reaction. Such an equilibration regenerates the fraction of the effective component(s) in the pool, which then moves into the selection chamber again. Repetition of these cycles will gradually lead to amplification of the effective binder(s) in its target-cmplexed form and a decreased concentration of the original mixture in solution. Thus, this method is designed to reorganize the original pool into a subset of compounds enriched with the high-affinity binders. As will be shown below, such stepwise evolutionary-type enrichment leads to results similar to those that might be expected from one-phase equilibration of the pool with the target compound, but makes it possible to avoid undesirable side-reactions between the target and its binders and to simplify isolation of the high-affinity subset.

Application of the approach to the generation of arginine receptors: In developing the approach described, our first goal was to design a relatively simple system containing a limited number of components. In such a system, all the important parameters, such as the affinities of the individual components for the target, the distribution of the components in the mixture, their rates of interconversion, could be separately evaluated and related to the mechanisms of the evolutionary process. Quantitative assessment of the system parameters should then make it possible to evaluate the applicability of the approach to more complex systems.

In order to meet the first requirement of the method, reversible interconversion of the components, we used molecular scaffolds **1**, **2**, and **3**, each containing two double bonds that might undergo *cis*–*trans* isomerization on irradiation with UV light. As defined by the symmetrical structure of the scaffolds, three isomers, *trans,trans*, *cis,trans*, and *cis,cis*, would coexist in the irradiated solution.



Besides the apparent biological importance of the guanidinium fragment, the choice of its derivatives as the target ligands was based on its ability to engage in molecular recognition in various modes, primarily through the formation of salt bridges and of hydrogen bonds.[16] In order to explore both of these possibilities in our receptors, we synthesized a series of compounds, **1a**–**1d**, **2a, 2b**, **3a**, and **3b**, containing either anionic groups or hydrogen-bond acceptors capable of polytopic interactions with the ligand. According to the molecular design, one of the isomers of each receptor, namely *cis,cis*, would have the most favorable geometry for binding to the ligand because of the closeness of its two arms as defined by its isomerization motif (Figure 4).

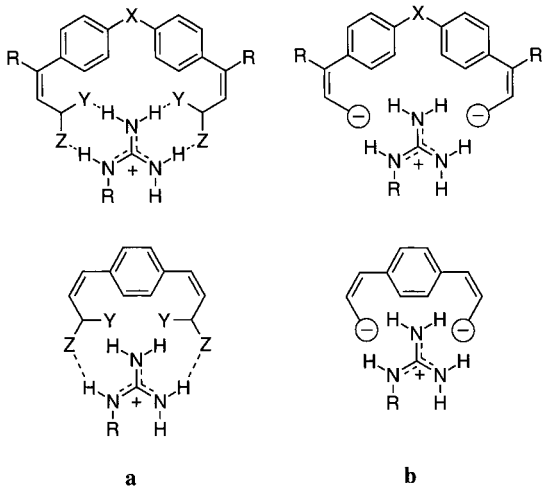


Figure 4. Proposed binding modes of guanidinium salts to the *cis,cis* isomers of compounds **1**–**3**: a) through multiple hydrogen bonds (**1b**–**1d**, **2b**, **3b**); b) through the formation of two salt bridges (**1a**, **2a**, **3a**).

Receptors **1**–**3** were synthesized from commercially available compounds in two to three steps (see Experimental Section). The double-bond connections between the aromatic moieties and the functional units were built from the corresponding carbonyl compounds through the aldol condensation–dehydration reactions (compounds **1c**, **1d**) and through the Wadsworth–Emmons reactions with the phosphonate derivatives (compounds **2**, **3**).

Each potential receptor, initially obtained in its *trans,trans* form,[17] was then tested for its applicability to our system. The following properties were essential: i) the ability to form all three isomers by reversible light-induced isomerizaton; ii) the affinity of the *cis,cis* isomer for the ligand; iii) the selectivity of binding among the isomers. The isomerization and binding studies were performed in various media, as dictated by the solubility of the receptors and the polarity or hydrogen-bonding properties of the solvents. After irradiation of the receptor solution with broad-band UV–visible light, the content of each of the isomers was determined by NMR spectroscopy and/or HPLC. The receptor–ligand interactions were studied and, wherever possible, binding constants were determined by NMR spectroscopic titrations of the mixture of isomers with the hydrochloride of the corresponding guanidinium derivative. The results of the screening of eight potential receptor systems are summarized in Table 1.

The electrostatic binders (**1a**, **2a**, **3a**), studied in protic solvents with methylguanidinium hydrochloride, showed the highest promise for our approach. Their irradiation led to comparable amounts of all three isomers, which had appreci-

Table 1. Results of molecular scaffold screening for the light-isomerizable arginine receptors.

Receptor	Guest	Solvent	Binding constant (K) [M^{-1}] *cis,cis*	*cis,trans*	*trans,trans*	Selectivity among receptor isomers ($K_{cis,cis}/K_{cis,trans}/K_{trans,trans}$)	Method
1a	methylguanidinium	10% D_2O in [D_4]methanol	86	<20	<20	n/a	^{1}H NMR
1a	methylguanidinium	[D_4]methanol	213 ± 9	120 ± 15	44 ± 17	4.9/2.7/1	^{1}H NMR
1b	dodecylguanidinium	$CDCl_3$	[a]	[a]	[a]		
2a	methylguanidinium	[D_4]methanol	170 ± 12	72 ± 5	<3	ca. 60/25/1	^{1}H NMR
		[D_6]ethanol	980 ± 90	150 ± 50	4 ± 3	250/38/1	^{1}H NMR
		10% D_2O in [D_{10}]*tert*-butyl alcohol	>2500	790 ± 110	40 ± 25	>63/20/1	^{1}H NMR
2b	phenylguanidinium	$CDCl_3$	22 ± 13	7 ± 1	7 ± 2	3/1/1	^{1}H NMR
3a	methylguanidinium	[D_4]methanol	29 ± 3	40 ± 5	160 ± 15	0.2/0.25/1	^{31}P NMR
3b	phenylguanidinium	$CDCl_3$	22 ± 13	21 ± 10	35 ± 17	0.6/0.6/1	^{31}P NMR
3b	phenylguanidinium	$CDCl_3$	73 ± 26	29 ± 9	21 ± 7	3.5/1.4/1	^{1}H NMR

[a] No binding was detected within the guest concentration range 0–0.1M.

able binding affinities for the ligand. The affinities of the isomers of **1a** and **2a** were in line with the predictions obtained from molecular modeling, as described elsewhere.[18] Surprisingly, the *cis,cis* and *cis,trans* isomers of compound **3a** showed comparable affinities for methylguanidinium in methanol, whereas the *trans,trans* isomer was a better binder than the other two. As a possible explanation, we suggest that the strong electrostatic repulsion between the phosphate dianions of **3a** in the complexation mode shown in Figure 4 makes a highly unfavorable contribution to the binding free energy and is not fully compensated by the formation of one salt bridge between the guanidinium and each of the dianions. Therefore, the binding is more likely to occur by the formation of the ion pairs between guanidinium and only one of the phosphates, as opposed to the formation of a chelate complex as shown in Figure 4. The geometry of the *trans,trans* isomer may be more favorable for the formation of such single ion pairs.

Compounds **1c** and **1d** failed to undergo any isomerizations, either upon direct irradiation or in the presence of various sensitizers.[19] We assume that a highly conjugated chalcone structure may prevent the localization of the absorbed light energy to the double bond which is necessary for the formation of a perpendicular intermediate of the isomerization process.[20] Direct irradiation of **1b** led only to the formation of the *cis,trans* isomer. Sensitized irradiation in the presence of 2,3-pentanedione resulted in the formation of a detectable amount of the *cis,cis* isomer. However, no complexation of any of the isomers with dodecylguanidinium hydrochloride in $CDCl_3$ was observed by NMR spectroscopy.

The *cis,cis* isomer of compound **2b** showed marginal selectivity in binding to guanidinium over the other two isomers (Table 1). However, both the absolute affinity and the selectivity of hydrogen bonding in chloroform were quite low. Similar behavior and comparable affinities were observed for the isomers of compound **3b**. We believe that the modest affinities of these receptors are due to the steric hindrance created by methyl substituents at the oxygen atoms that may serve as the hydrogen-bond acceptors. This effect is particularly pronounced in compound **3b**, in which two methyl groups at each phosphate apparently reduce their potentially very strong ability to form hydrogen bonds.[21] There are probably entropic and stereochemical reasons for the low binding selectivity among the isomers of **2a** and **3a**. The formation of chelate complexes (Figure 4a) with multiple hydrogen bonds requires a more precise receptor preorganization than that for electrostatic complexation. Such preorganization in the *cis,cis* isomers of **2a** and **3a** may be disfavored because of both the higher entropy loss upon complexation and the steric hindrance created by the methyl groups.

The results of screening show that compound **2a** in its dicationic form possesses the necessary properties to be applied in our method. The approach was further implemented with the isomers of **2a** in ethanol as a solvent, where the optimal ratio of the solubility/binding properties was achieved.[22]

One of the important issues we intended to address in this model study was the necessity for physical separation of the selection and variation sites. Indeed, one might be able to achieve the desired self-organization of the isomeric mixture merely by adding the ligand to the equilibrating system of receptors, thereby shifting the isomerization equilibrium (see Figure 3). In order to check this possibility in our system, we performed long-term irradiation of the receptor solution in the absence and in the presence of an excess of methylguanidinium hydrochloride. Although the addition of the ligand to the solution did cause a modest shift of the photostationary distribution of the isomers (Figure 5), a much stronger effect

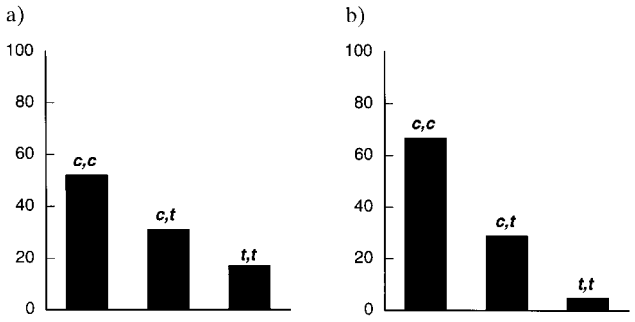


Figure 5. Photostationary distribution [%] of isomers of **2a** (2.6×10^{-4}M in ethanol): a) with no additive; b) in the presence of 0.1M methylguanidinium hydrochloride.

can be predicted (see below) on the basis of the binding-constant values. It is likely that direct addition of methylguanidinium affects the equilibrium by mechanisms other

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than formation of electrostatic complexes. Possible effects may include changes in the extinction coefficients and alteration of the excited-state energies of the receptor isomers due to the complexation.[23] Any of these effects would cause intrinsic changes in the photostationary equilibrium and result in a relatively inefficient enrichment of the system with the effective binder. This fact justifies separation of the selection and isomerization sites and may have important implications for the use of this method in combinatorial chemistry (see below).

Since selection of the effective receptor forms by the immobilized ligand seemed to have advantages over one-phase selection, we then explored various sorbents as possible supports for the guanidinium derivatives.[24] After the sorbents had been modified with different loadings of protected arginine (see Experimental Section), they were equilibrated with an irradiated 3×10^{-4} M solution of the disodium salt of **2a** in ethanol, and the residual distribution of isomers in solution was analyzed by HPLC.[25] As shown in Table 2, the modified silica gel was found to be optimal among the tested supports because of its low nonspecific adsorption, low flow resistance, and high specific surface. This sorbent and agarose, which showed an appreciable nonspecific adsorption, were later used for the circulation experiments. More hydrophilic supports, such as oxirane acrylic beads, Celite, and the Merrifield resin, absorbed a major amount of all the receptor forms and were abandoned in further studies.

Table 2. Adsorption of the isomers of **2a** from ethanol solution on arginine-modified sorbents (5 mM Arg on all supports).

Support	Isomer distribution in solution (*cis,cis*/*cis,trans*/*trans,trans*, relative peak areas)		% of bound isomer (*cis,cis*/*cis,trans*/*trans,trans*)
	before column	after column	
aminopropyl silica	16.5/20.9/8.6	0.8/8.0/5.3	95.32/61.7/37.8
oxirane acrylic beads	16.8/21.6/8.7	0/0/0	100/100/100
Celite	16.3/20.8/8.2	0/0/5.3	100/100/35
agarose	1/6.1/69.5	0/0.59/11.9	> 95/90.4/82.8
Merrifield resin	8.5/21.9/14.0	0/0/0.03	100/100/ > 99

The concentration of the ligand to be attached to the solid support for the preferential selection of the receptor isomers was chosen according to the solution binding constants. Thus, with 5×10^{-3} M immobilized arginine the *trans,trans* isomer would remain mostly in solution, the *cis,trans* isomer would be approximately half-bound to the ligand, and the majority of the *cis,cis* isomer would be bound to the solid support. However, since the solution association constants might not necessarily be the same for binding to supported arginine, we tested the distribution of the isomeric mixture between the solution and the immobilized ligand. Figure 6 shows the distribution of isomers in the mixture before and after passing the column with 5×10^{-3} M ligand immobilized on the silica support. As predicted, equilibration with the support indeed led to preferential binding of the effective isomer. In order to maximize the yield of *cis,cis*-**2a** in our system, a somewhat higher ligand concentration was used for the circulation experiment.

For performing the selection–variation cycles, we used an experimental setup similar to the one shown in Figure 3 in which the variation compartment was represented by a photochemical flow cell immersed in a Rayonet reactor (Figure 7). First, the isomer distribution in the mixture, reached after the solution had been in the irradiation cell at the given flow rate for one radiation cycle, was analyzed by HPLC (Figure 8a). The time (approx. 5 min) was sufficient to achieve only partial isomerization; this allowed us to use higher flow rates, shorten the total experimental time, and minimize the side-reactions. The circulation experiments were then performed, and the compositions of the ligand-bound and the solution-phase receptors were analyzed as described in the Experimental Section.

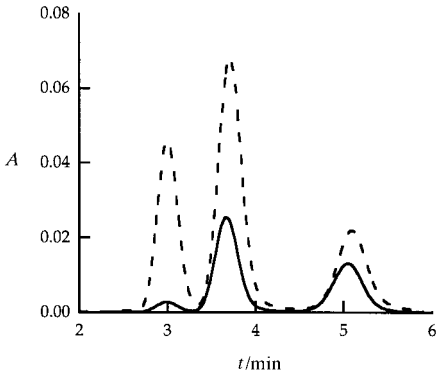

Figure 6. HPLC trace of the isomeric mixture of **2a** before (broken line) and after (solid line) it passed through the silica column with 5×10^{-3} M immobilized arginine (detection at 270 nm); peaks shown correspond to *cis,cis*, *cis,trans*, and *trans,trans* isomers, in increasing order of retention time. A = absorbance.

Using 1×10^{-2} M arginine immobilized on the silica support resulted in the distribution of isomers on the column shown in Figure 8b, which indicated a significant enrichment of the

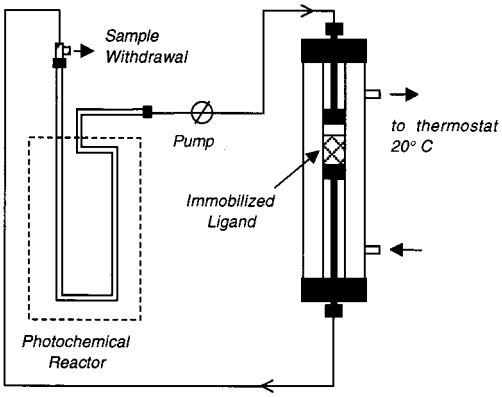

Figure 7. The experimental setup.

system compared with the photostationary distribution in solution (*cis,cis/cis,trans* ratios 6.5:1 and 1.7:1, respectively). Application of the agarose-supported ligand led to a lower enrichment (Figure 8c) (*cis,cis/cis,trans* ratio 2.0:1) due to a strong nonspecific adsorption (see Table 2).

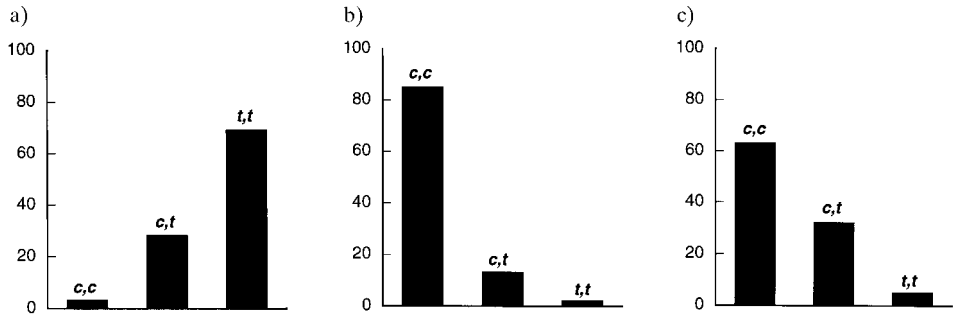

Figure 8. Distribution [%] of the isomers of **2a** in the circulation experiments: a) in 2.6×10^{-4} M ethanol solution, after the first irradiation cycle; b) on the arginine-modified silica column, after 30 irradiation–selection cycles; c) on the arginine-modified agarose column, after 100 irradiation–selection cycles.

As a positive side-effect of the variation–selection cycles, the column-accumulated subset of receptors was free from any detectable by-products of photochemical isomerization. Thus, after an 8 h irradiation of **2a** in solution, leading to a photostationary distribution of the components (see Figure 5a), a total of 66% of all receptor forms remained in solution. HPLC showed the formation of two additional peaks (Figure 9a), probably attributable to the condensation products.[26] In contrast, the mixture removed from the silica support after the evolution experiment (54% of the total original amount) contained no components other than the isomers of **2a** (Figure 9b), all of the by-products having remained in the circulating solution (Figure 9c).

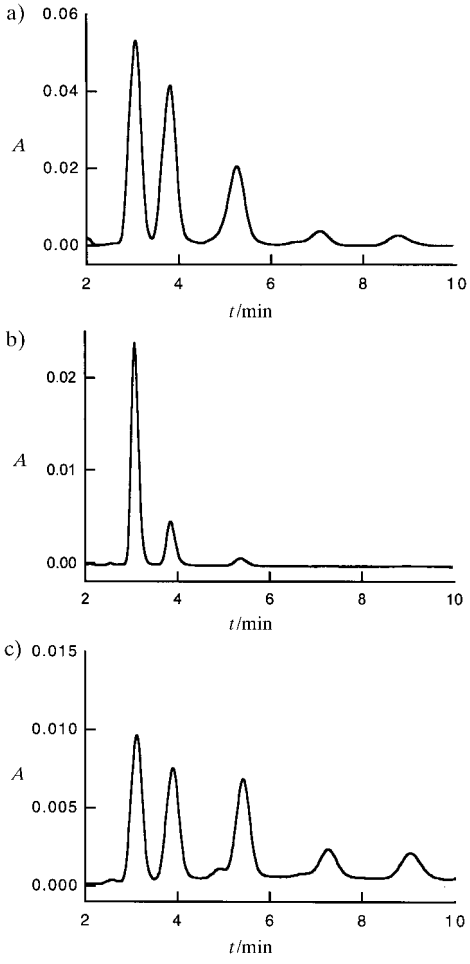

Figure 9. HPLC traces of the isomeric mixtures of **2a**: a) in solution, after 8 h of irradiation; b) on the arginine-modified silica column, after 30 isomerization–selection cycles; c) in solution, after 30 cycles. A = absorbance (278 nm).

In order to check whether the result of evolution was actually caused by differential binding to the immobilized arginine, a control experiment was performed in which the amino groups on the silica support were acetylated instead of being modified with arginine. After 30 cycles with this blank sorbent, only 12% of the isomers had accumulated on the column, their ratio (*cis,cis*/*cis,trans*/*trans,trans* = 55:31:14) being close to the photostationary distribution in solution. After 30 cycles with the same sorbent but without irradiation, the column distribution was *cis,cis*/*cis,trans*/*trans,trans* = 5:20:75 (12% total), probably because of a minor thermal isomerization.

The results obtained prove that application of the selection–isomerization process to the pool of potential receptors allows amplification of the total amount of the best receptor in the reaction setup, and that such an amplification is driven by the differential affinity of the pool components for the immobilized target. Notably, the use of the appropriate immobilization technique makes it possible to maintain the binding affinity and selectivity observed in solution.[27]

Mathematical modeling of the approach: As shown above, the approach described here is designed to shift the equilibrium of the variation process (see Figure 3) to the formation of the most effective binders by means of their complexation with the ligand. However, in the implementation of the method, every variation cycle results in only a limited degree of transformation of the mixture components and does not bring the system to equilibrium. This raises important questions, such as whether the kinetically controlled isomerization would lead to the optimal evolution of the equilibrating pool, and whether and how the degree of variation affects the yield of the effective receptors. In order to address these issues, we have performed mathematical modeling of the real-time circulation process and compared the results with both the experimental data and the calculated equilibrium distribution of the receptor among the isomers in the free and bound forms.

Real-time simulation: The first step in the modeling process involved calculation of the degree of isomerization for the time the mixture spent in the variation chamber during the first cycle. The assumption was made that the rate constant for the transformation of the *cis* form to the *trans* form ($k_{c\rightarrow t}$) and the reverse constant ($k_{t\rightarrow c}$) are independent of the state of the receptor (i.e., $k(trans,trans \rightarrow cis,trans) = k(cis,trans \rightarrow cis,cis)$ and $k(cis,cis \rightarrow cis,trans) = k(cis,trans \rightarrow trans,trans)$. The val-

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ues of the constants were determined from the initial rate of isomerization of *trans,trans*-**2a** ($k_{t\rightarrow c} = 0.0182\ \mathrm{min}^{-1}$) and the equilibrium constant (K_{ps}) of photostationary distribution of the isomeric forms ($k_{c\rightarrow t} = k_{t\rightarrow c}/K_{ps} = k_{t\rightarrow c}[trans]_{ps}/[cis]_{ps} = 0.00874\ \mathrm{min}^{-1}$, where $[trans]_{ps}$ and $[cis]_{ps}$ are the photostationary concentrations of the respective forms). By solving the differential equation for the reversible *cis*–*trans* transformation, one can find the concentrations of the *trans* and *cis* forms at a particular time point (t) [Eqs. (1) and (2); c_0 and t_0 are the initial concentrations of the *cis* and *trans* forms, respectively].

$$[cis] = \frac{1}{k_{t\rightarrow c} + k_{c\rightarrow t}}[k_{t\rightarrow c}(t_0 + c_0) + (k_{c\rightarrow t}c_0 - k_{t\rightarrow c}t_0)\exp(-(k_{t\rightarrow c} + k_{c\rightarrow t})t)] \quad (1)$$

$$[trans] = \frac{1}{k_{t\rightarrow c} + k_{c\rightarrow t}}[k_{c\rightarrow t}(t_0 + c_0) + (k_{t\rightarrow c}t_0 - k_{c\rightarrow t}c_0)\exp(-(k_{t\rightarrow c} + k_{c\rightarrow t})t)] \quad (2)$$

The concentrations of the *cis* and *trans* forms at the end of one variation cycle obtained in this way were then used to express the concentrations of particular isomers by numerical solution of Equations (3)–(5).

$$2[cis,cis] + [cis,trans] = [cis] \quad (3)$$

$$2[trans,trans] + [cis,trans] = [trans] \quad (4)$$

$$\frac{2[trans,trans]}{[cis,trans]} = \frac{[cis,trans]}{2[cis,cis]} \quad (5)$$

These calculations yield the concentrations of isomers in the solution that leaves the variation chamber and is transferred to the selection column, which were then used to calculate the distribution of isomers between the bound and free forms in the selection column. This was achieved by finding a numerical solution of three complexation equilibria [Eqs. (6)–(8)] and four mass-balance equations, for each of

$$K_{tt} = \frac{[trans,trans]_{col}}{[L][trans,trans]_{sol}} \quad (6)$$

$$K_{ct} = \frac{[cis,trans]_{col}}{[L][cis,trans]_{sol}} \quad (7)$$

$$K_{cc} = \frac{[cis,cis]_{col}}{[L][cis,cis]_{sol}} \quad (8)$$

the isomers [Eqs. (9)–(11)] and for the immobilized ligand [Eq. (12)] ([L] and $[L]_t$ are the receptor-unbound and total concentrations of immobilized arginine, respectively; subscripts col and sol refer to the concentrations of column-bound and free receptor forms, respectively). The resulting array of concentrations is the result of the first cycle, as shown in the first set of points (cycle 1) in Figure 10. The second and following cycles of isomerization–selection were simulated similarly: the concentrations in solution of the isomers from the preceding selection were used as the initial concentrations in the next calculation set of isomerization kinetics.

$$[trans,trans] = [trans,trans]_{col} + [trans,trans]_{sol} \quad (9)$$

$$[cis,trans] = [cis,trans]_{col} + [cis,trans]_{sol} \quad (10)$$

$$[cis,cis] = [cis,cis]_{col} + [cis,cis]_{sol} \quad (11)$$

$$[L]_t = [L] + [trans,trans]_{col} + [cis,trans]_{col} + [cis,cis]_{col} \quad (12)$$

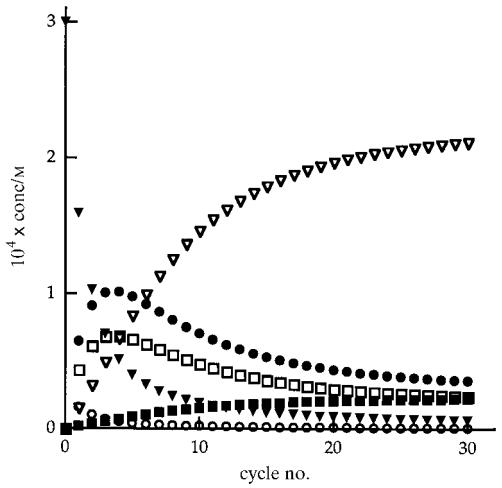

Figure 10. Calculated distribution of the isomers of **2a** in the circulation setup as a function of cycle number: *trans,trans* (▼) in solution and (○) on column; *cis,trans* (□) in solution and (●) on column; *cis,cis* (■) in solution and (▽) on column.

The results of modeling 30 variation–selection cycles are shown in Figure 10. The final distribution of the isomers on the column is in very good agreement with the experimental results. Notably, the system reaches *macro*equilibrium between the solution and the solid state through a steady increase in the *cis,cis* isomer content and a decrease in the *trans,trans* isomer content, whereas the concentration of the *cis,trans* isomer passes its maximum, as is typical for the kinetics of regular consecutive reactions.

Equilibrium simulations: The main purpose of these simulations was to compare the results of the circulation experiment with a hypothetical situation, depicted in Figure 2, in which the ligand introduced into the solution would just shift the isomerization equilibrium by binding the effective receptor isomers. This system was modeled by taking into account the equilibrium distribution of the isomers in the photostationary state [Eqs. (5), (13)] and association of each of them

$$\frac{2[cis,cis]_{sol} + [cis,trans]_{sol}}{2[trans,trans]_{sol} + [cis,trans]_{sol}} = K_{ps} \quad (13)$$

with the ligand [Eqs. (6)–(8)]. Solution of this system together with the corresponding mass-balance equations for our experimental conditions yielded the column distribution shown in Figure 11a, in which the component concentrations exactly matched the level-off values for the components in Figure 10. Similar calculations were also performed taking into account the (80%) nonspecific adsorption of the agarose-supported ligand (Figure 11b). The distribution obtained also corresponds to the one observed experimentally (cf. Figure 8c).

These results indicate that the enrichment of the pool obtained after a sufficient number of cycles in our experimental setup is essentially driven by the shift of the variation

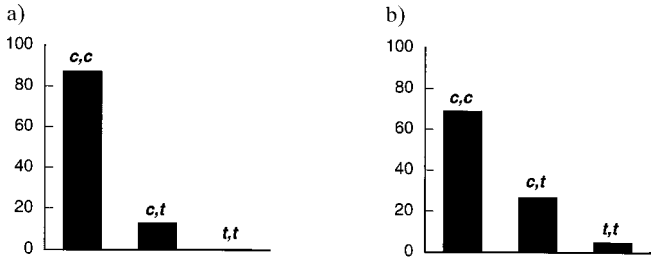

Figure 11. Calculated distribution [%] of isomers of **2a**: a) on the arginine-modified silica column; b) on the arginine column (cf. Figures 8b, 8c, respectively).

Table 3. Simulation of the evolutionary enrichment of multicomponent mixtures by the selection–variation method.

No. of components	K_{strong}/K_{weak}	Column yield of the strong binder [%]	Total yield of strong binder [%]
5	5	55.6	34.9
5	10	71.4	51.6
5	50	92.6	84.0
10	10	52.6	33.7
10	50	84.7	71.5
10	100	91.7	83.3
15	15	51.7	33.3
15	50	78.1	62.3
15	100	87.7	76.7

equilibrium by the immobilized ligand. One of the important conclusions resulting from these simulations is that the final result of circulation is independent of the degree of isomerization in each cycle. Thus, just a minor mutation performed in the isomerization chamber would eventually lead to the same distribution of the components that would be achieved by bringing the system to the isomerization equilibrium every time.[28] This conclusion is essential for the application of the method to more complex pools and slow variation reactions. The final total ratio of the isomers in the experimental setup corresponds to their thermodynamic distribution in the situation when the ligand is initially dissolved in the irradiated receptor solution, but there are no side-reactions involved. Therefore, the final evolution result, even in the system with kinetically controlled variation, is determined by the thermodynamics of the interconversion equilibrium and the ligand–receptor interactions. Since this observation sets an upper limit on the enrichment that can be achieved by our method, it was interesting to use the simulations described above to evaluate the applicability of the approach to larger pools.

We considered three sample pools, containing 5, 10, and 15 components respectively, and estimated how the affinity difference between the components would influence the effectiveness of selection by the ligand. For simplicity, all of the pool components were assumed to have equal binding constants to the ligand (K_{weak}), except for one that was assigned a higher constant (K_{strong}). The ligand concentration was chosen to equal $1/K_{weak}$, in order to leave half of each weak binder in solution and, at the same time, to bind the majority of the effective component. The equilibrium calculations, similar to those described above for the three-component mixture, were then performed for different K_{strong} values. The results of these simulations were expressed in terms of yield of the strong binder on the column, defined as the ratio (amount of strong binder on column)/(total amount of receptor on column), and the total yield of the strong binder, defined as (amount of strong binder on column)/(total amount receptor in the experimental setup) (Table 3). As expected, the efficiency of the method is highly dependent on the selectivity of ligand recognition by different receptor forms. The increasing complexity of the calculations did not allow us to evaluate pools larger than 15 components. The general trend shows, however, that the mixture of n components in which $K_{strong}/K_{weak} = n$ would be evolved by repeating the selection–equilibration to afford approximately 50% of the effective component on the column, its total yield being approximately 30%. Such a situation seems to be realistic for combinatorial libraries where the hits may possess a target affinity several orders of magnitude higher than the majority of the components.[29]

Conclusions and Outlook

In this paper we have assessed the mechanism, applicability, and possible extensions of the method of targeted evolution of equilibrating pools driven by the differential affinity of the pool components for a chosen target compound. In a way, this method represents a thermodynamic analogue of template synthesis[30] in which assembly of the reactants on the template leads to a faster coupling of the preassembled units and thereby eliminates the formation of multiple side-products that could have been formed in the absence of a template. It is important, however, to emphasize the differences between evolutionary selection–variation and the traditional template-directed synthesis. Thermodynamic control of the selection process ensures that the compounds accumulated on the support are indeed the best *binders* for the given target. Also, the template (or target) in our method does not interfere with the variation reaction, however severe the conditions that are used.

The latter point is particularly important for the applicability of the technique in combinatorial chemistry, where the targets, such as proteins or nucleic acids, may be particularly fragile. The method may be especially promising for combinatorial chemistry in that it allows one to combine the generation of diversity and screening in one system that, in addition, can be easily automated. Generation of larger equilibrating pools, which is a focus of our current work, can be based on many known types of reversible reactions, such as imine exchange, transesterification, thiol–disulfide exchange, and aldol condensations, to name just a few.

Experimental Section

General: Reagents for synthesis and compound **1a** (the diacid form) were obtained from commercial sources and used without further purification. NMR spectra were obtained and processed on Varian Unity 300 and 400 MHz spectrometers; HPLC analysis was performed on the Beckman Gold system equipped with a Model 168 photodiode array detector.

0947-6539/98/0405-0832 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 5

Mathcad Pro software (version 6.0) running on a Power Macintosh 6100 was used for the numerical simulations.

Synthesis of phenylenediacrylic acid pyrazolylamide (1b): Phenylenediacrylic acid (**1a**) (2 g, 9.17 mmol) was transformed into its dichloroanhydride by refluxing overnight in SO_2Cl_2/CH_2Cl_2 (40 mL, 2 M) with a catalytic amount of DMF, followed by evaporation of the solvent. The dichloroanhydride (1.17 g, 4.6 mmol) was dispersed in anhydrous Et_2O (30 mL) containing pyrazole (0.750 g, 11 mmol), Et_3N (1.53 mL, 11 mmol), and 4-dimethylaminopyridine (DMAP)(20 mg), and heated to reflux for 18 h. The off-white precipitate was then filtered, washed twice with Et_2O, dissolved in $CHCl_3$ (150 mL), extracted three times with water, and dried over $MgSO_4$. Crystallization from $CHCl_3$/hexanes yielded **1b** (833 mg, 57 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.41$,8.40 (d, 2H), 8.07, 8.01, 7.99, 7.94 (dd, 4H), 7.81 (s, 2H), 7.76 (s, 4H), 6.52 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 163.9$, 146.9, 144.6, 137.3, 129.9, 129.3, 117.7, 110.6.

Synthesis of 3,3′-(1,4-phenylene)-bis[1-(2-thiazolyl)-2-propen-1-one] (1c): To a solution of terephthalaldehyde (0.134 g, 1.0 mmol) and 2-acetylthiazole (0.254 g, 2.0 mmol) in MeOH (5 mL) was added saturated NaOH in MeOH (0.1 mL). The mixture was stirred for 3 h at room temperature, then the precipitate formed was filtered off, washed with MeOH, dried, and crystallized from EtOAc to afford **1c** (110 mg, 31 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.10$,8.09 (d, 2H), 8.02 (s, 4H), 7.78 (s, 4H), 7.74,7.73 (d, 2H). Calcd for $C_{18}H_{12}O_2N_2S_2$: C, 61.3, H, 3.43, N, 7.95; found: C, 60.8, H, 3.55, N, 7.79.

Synthesis of 3,3′-(1,4-phenylene)-bis(1-(2-pyridyl)-2-propen-1-one) (1d): The same procedure as that for **1c** was used with 2-acetylpyridine instead of 2-acetylthiazole. Yield 46 %. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.78$–8.77, 8.77–8.76 (dq, 2H), 8.39, 8.34 (d, 2H), 8.22, 8.20 (dt, 2H), 7.97, 7.92 (d, 4H), 7.92, 7.90, 7.87 (td, 2H), 7.79 (s, 4H), 7.54–7.49 (qd, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 189.8$, 154.7, 149.4, 144.1, 137.8, 137.6, 129.8, 127.5, 123.5, 122.5.

The dicarboxylic acid **2a** and its dimethyl ester **2b** were prepared and characterized as described in ref. [18].

4-(4-Formylphenoxy)benzaldehyde: This intermediate, used in the synthesis of **3b**, was prepared by the reaction of 4-hydroxybenzaldehyde with 4-fluorobenzaldehyde as described previously.[31] Yield 89 %. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.98$ (s, 2H), 7.95, 7.20 (dd, $J = 9.0$ Hz, 8H).

Tetramethyldiphosphonate 3b: A solution of tetramethylmethylenediphosphonate (1 g, 4.3 mmol) in anhydrous THF (2 mL) was added dropwise to a stirred suspension of NaH (0.099 g, 4.1 mmol) in THF (2 mL) at 0 °C in an argon atmosphere. The mixture was adjusted to room temperature and kept for 40 min until the solution became transparent and no further gas formation was observed. A solution of 4-(4-formylphenoxy)benzaldehyde (0.406 g, 1.8 mmol) in THF (2 mL) was added dropwise within 30 min at 10–15 °C, and the resulting mixture was stirred for 2 h at ambient temperature. Then the solvent was removed in vacuo, and the residue was redissolved in Et_2O, extracted twice with water and then with a saturated solution of NaCl, and dried over $MgSO_4$. The crude product was purified by chromatography on silica gel in 15 % (v/v) MeOH in EtOAc, yielding the analytically pure product (0.380 g, 48 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 7.57, 7.52, 7.50, 7.44 (q, 2H), 7.52, 7.50, 7.05, 7.02 (dd, 8H), 6.20, 6.14, 6.09 (t, 2H), 3.80, 3.77 (d, 12H); ^{31}P NMR (160 MHz, $CDCl_3$): $\delta =$ 22.8.

Diphosphonate 3a: **3b** (0.056 g, 0.128 mmol) was dissolved in dry CH_2Cl_2 (7 mL), and TMS bromide (0.1 mL, 0.76 mmol) was added at 0 °C. After this mixture had been stirred for 10 min, the temperature was adjusted to ambient, and stirring was continued for 5 h. Then the solvent was removed; the dry residue was redissolved in MeOH (7 mL), kept overnight, and then heated to reflux for another 2.5 h. Removal of the solvent in vacuo resulted in analytically pure **3a** (48 mg, 98 %). ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta = 7.66$, 7.64 (d, 4H), 7.25, 7.19, 7.18, 7.12 (q, 2H), 7.06, 7.04 (d, 4H), 6.47, 6.42, 6.36 (t, 2H); ^{31}P NMR (160 MHz): $\delta = 13.8$.

NMR titrations: Solutions of the *trans,trans* forms of the receptors (**1a** and **2a** as their disodium salts, and **3a** as the di(tetrabutylammonium) salt) in appropriate deuterated solvents were irradiated in NMR tubes with a mercury lamp in a Rayonet photochemical reactor for 20–30 min to generate mixtures of all the isomers at a total concentration of 0.5–4 mM; these were titrated with the chloride of the corresponding guest cation G, with an increasing concentration of the latter from [G] = 0 to 130 mM. The individual binding constants K of all the isomers were then determined from the changes in chemical shifts ($\Delta\delta$) of the host signals by nonlinear curve fitting to Equation (14) for the 1:1 complexation mode.

$$\Delta\delta = \Delta\delta_\infty K[G]/(1 + K[G]) \quad (14)$$

With a few exceptions, the variations of the binding constants determined from the shifts of different protons did not exceed the experimental error (approx. 10–15 %).

Ligand immobilization: *N*-(*α*-*tert*-Butoxycarbonyl)arginine was attached to the solid support by carbodiimide coupling with chromatographic sorbents modified with amino groups (aminopropyl silica was prepared by a procedure described in ref. [32]; other supports were commercially available). In a typical procedure (for silica), the amino sorbent (4 g, 6.7 mL dry volume) was dispersed in dry DMF (15 mL); the solutions of the arginine derivative (10.5 mg, 0.0335 mmol) in DMF (2 mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (89 mg, 0.3 mmol) in DMF (2 mL) were added. The resulting slurry was shaken for 18 h on a device with a wrist-shaking action. Then Ac_2O (2 mL, 21.2 mmol) and Et_3N (3 mL, 22 mmol) were added and the mixture was shaken for another 24 h, then filtered, and washed successively with DMF, water, and ethanol.

Circulation experiments: The circulation experiments were performed at a typical flow rate of 0.5–1 mL min^{-1} for 8–12 h, resulting in 30–100 cycles. The transparent flow cell made from borosilicate glass was irradiated with broad-band UV–visible light in a Rayonet reactor; the same cell was used to determine the photostationary distribution in solution. The experiments were terminated when the HPLC analysis of the circulating solutions showed no further changes in the concentrations of the components in solution. When cycling had been terminated, the sorbent removed from the column was treated with several portions of 1 M NaCl in water and aqueous ethanol. Combined extracts were concentrated in vacuo and analyzed by HPLC as described in ref. [9].

Acknowledgments: This work was supported by a start-up grant to A.V.E. from SUNY/Buffalo. We also acknowledge the Donors of the Petroleum Research Fund administered by the American Chemical Society for partial support of this research.

Received: July 10, 1997 [F762]

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[28] Apparently, the smaller the degree of isomerization, the more cycles are necessary for achieving the macroequilibrium. The total time for macroequilibration is, however, comparable with the time necessary to reach a similar solution equilibrium and can be estimated from straightforward kinetic studies in solution.
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