Intermolecular β-Sheet Stabilization with Aminopyrazoles

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Abstract: 3-Aminopyrazole derivatives are the first artificial templates that stabilize the β-sheet conformation in *N*/*C*-protected dipeptides by purely *inter*molecular interactions. In the complex two aminopyrazole molecules lie exactly above and below the peptide backbone. Binding to the top face of the peptide is strongly favored because it forms three cooperative hydrogen bonds simultaneously to the receptor molecule, whereas the bottom face has only two. Polymerizable 3-amino- and 3-amidopyrazoles have been made accessible in excellent yields by a general route starting from *p*-toluic acid. With ^{1}H NMR titrations binding constants for the 1:1 complex of up to 880 M^{-1} have been determined in chloroform. The association constants are strongly influenced by the electronic character of the aminopyrazole derivative as well as by the steric demand of the peptide residues. Variable temperature studies prove that the complex is formed by dynamic hydrogen bonds and confirmed the preferential binding of the receptor molecules at the top face. By detailed Karplus-analysis of the *NH*-α-*CH* coupling constants in the complex a remarkable correlation between the dihedral angle θ and the degree of complexation was found, which shows that several amidopyrazoles are capable of forcing the dipeptide into an almost ideal β-sheet conformation. In glycine-containing dipeptides the third hydrogen bond slows down the free rotation around the C−C/C−N bond to almost zero. Intramolecular nuclear Overhauser enhancements (NOE) provide additional evidence for the peptide's extended conformation, while strong reciprocal *inter*molecular NOEs give the final proof of the existence of the critical third hydrogen bond and the postulated mutual orientation of the complexation partners. First ^{1}H NMR titrations with tripeptides show very promising results concerning the application of this concept to oligopeptides.

Introduction

Many biological processes are connected with the creation of a β-sheet structure in peptides and proteins. Lack of control is often fatal: thus β-amyloid deposition leads to Alzheimer's disease,[1] and the conversion of α-helices to larger β-sheet aggregates causes BSE/Creutzfeldt-Jakob and other prion deseases.[2] For reasons like these a better understanding of this important secondary structure and the synthesis of small, soluble model compounds are of high interest and represent an area of intense research.[3−12] The gain to knowledge about protein structure and folding may serve as a basis for future drug design. Several years ago Kemp for the first time achieved the stabilization of the β-sheet conformation in a short peptide, bound to 2,8-diaminoepindolidione.[13] The first two amino acids which were connected with the ring system served as a β-turn element, while the heterocycle itself worked as a rigid template for the construction of the β-sheet. By measuring the relative stability of these artificial β-sheets the propensities of different amino acids for β-sheet formation could be determined. In another concept Kelly used a dibenzofuran derivative as β-turn model and intensively examined the formation of a hydrophobic cluster as a possible cause for the spontaneous development of β-sheets;[14] recently he also turned to biphenyl derivatives to mimic the β-turn.[15] Since 1992 Nowick has been reporting about the development of low molecular weight β-sheet models.[16] He created a "molecular scaffold" on the basis of an oligourea structure, which is able to keep covalently attached peptide strands in close proximity and thus facilitates formation of a β-sheet structure. In his latest works he combined this scaffold with artificial β-sheet templates based on 5-amino-2-methoxybenzamides.[17]

We recently introduced 3-aminopyrazole derivatives for the first *inter*molecular stabilization of the β-sheet conformation in dipeptides.[18] Our efforts serve a dual purpose: we want to

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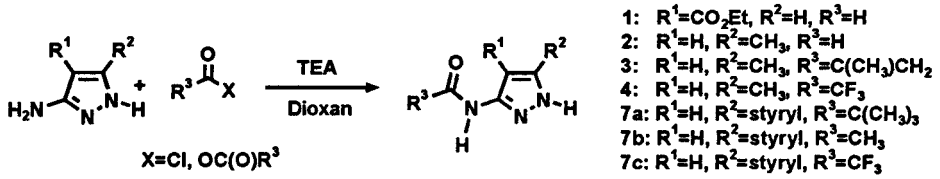
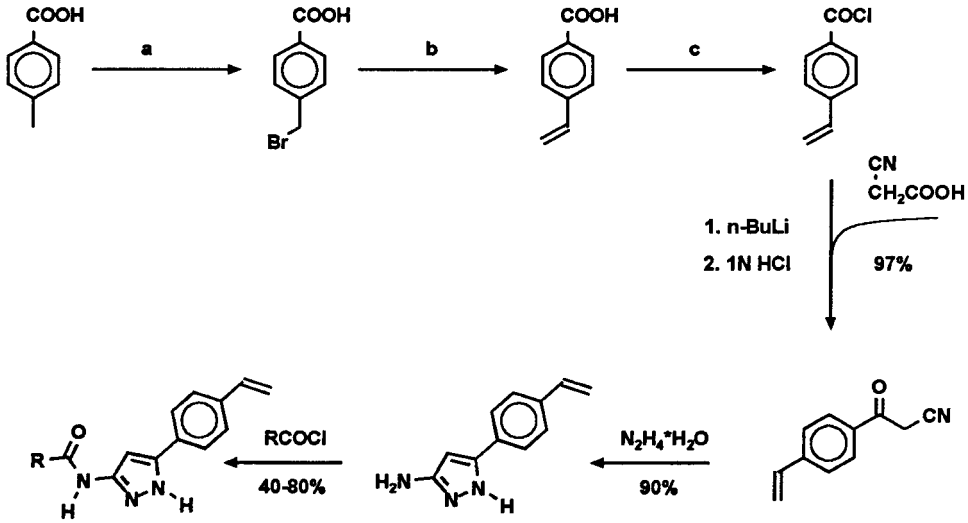
Scheme 1. Acylation of 3-Aminopyrazole Derivatives



Scheme 2. Synthesis of Styryl-Substituted 3-Amidopyrazoles **7**[a]



[a] (a) $Br_2/h^*\nu/CCl_4$ (ref 22); (b) 1. PPh_3/acetone; 2. $H_2CO/NaOH/H_2O$ (ref 23); (c) $SOCl_2$ (ref 24).

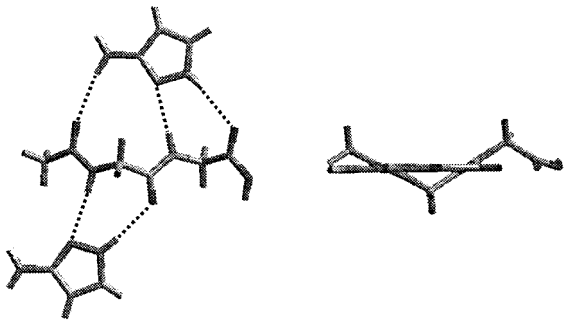


Figure 1. A 2:1 complex between 3-aminopyrazole and Ac-Gly-Gly-OMe. Left: side view; Right: top view. The geometry was energy minimized using CERIUS[2] with the DREIDING 2.21 force field.

develop a new generation of low molecular weight peptide receptors and at the same time introduce a new, efficient binding site for the molecular imprinting technology.[19] Invention of new functional monomers seems to be a key prerequisite to extend the scope of this powerful method for the development of enzyme mimicks, new chiral stationary phases for racemic resolution, and many other purposes.[20] Binding sites which undergo stoichiometric noncovalent interactions with the template combine the advantage of strong binding with a rapid equilibrium between bonded and nonbonded state.

In this article we report about several new, rigid 3-aminopyrazole derivatives and their capability of stabilizing the β-sheet conformation of *N*/*C*-protected dipeptides in solution. Influences of the template as well as the peptide structure will be discussed extensively. We also present our first investigations about the *inter*molecular complexation and β-sheet stabilization of tripeptides.

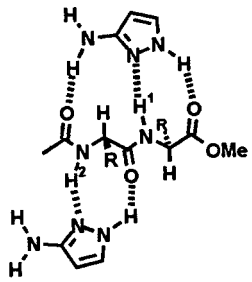


Figure 2. A 2:1 complex between 3-aminopyrazole and a *N*/*C*-protected dipeptide. The top face of the peptide is involved in three hydrogen bonds (ADA) and the bottom face only in two (DA).

Results and Discussion

Molecular modeling of a 3-aminopyrazole/dipeptide 2:1 complex suggests that both amino acids may be clamped together by three simultaneous hydrogen bonds to a rigid 3-aminopyrazole.[21] However, this is only possible while the peptide exists in the β-sheet conformation (Figure 1). Viewed from above (right structure) the aminopyrazoles appear as linear bars lying exactly perpendicular above and below the peptide backbone. This arrangement guarantees an almost optimal linear geometry for all hydrogen bonds.

Top and bottom face of an *N*/*C*-protected dipeptide differ enormously with respect to their hydrogen bond donor and acceptor pattern (Figure 2). While the top face offers three binding sites (ADA), the bottom face has only two (DA).

By formation of three cooperative hydrogen bonds with a 3-aminopyrazole molecular recognition of the peptide's top face should be strongly favored above the bottom face. This should provide a means for experimental proof of the three-point binding mode.

Synthesis of 3-Aminopyrazole Derivatives. Because of our aim of developing new functional monomers for molecular

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(21) For computer modeling the program CERIUS[2] from Molecular Simulations Incorporated was used, utilising a DREIDING 2.21 force field.

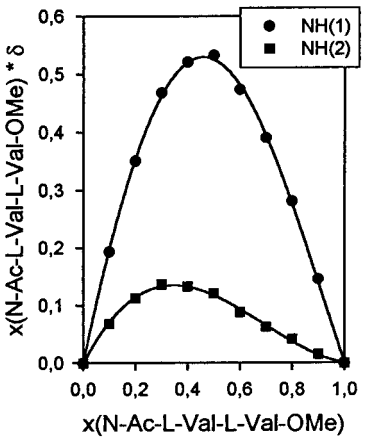

Figure 3. ^{1}H NMR job-plot: 3-amino-5-methylpyrazole **2**/Ac-L-Val-L-Val-OMe **8**.

imprinting we were especially searching for a general entry to polymerizable 3-aminopyrazole derivatives. Simple derivatives such as 3-amino-4-carbethoxypyrazole **1** or 3-amino-5-methylpyrazole **2** can be purchased from chemical suppliers. By reaction of the latter with methacrylic acid chloride or trifluoroacetic acid anhydride one obtains 3-methacryloylamino-5-methylpyrazole (MAMP) **3** and the nonpolymerizable 3-trifluoroacetylamino-5-methylpyrazole (TriFlAMP) **4** in good to excellent chemical yields (40%/99%). Acylation converts the amino functionality into a better hydrogen bond donor, which should lead to markedly stronger complexation of the dipeptide.

For the preparation of styryl-substituted amidopyrazoles the heterocycle must be constructed from open chain precursors. We developed the following optimized procedure (Scheme 2): deprotonation of cyanoacetic acid with *n*-butyllithium at −78 °C and subsequent reaction with *p*-vinylbenzoyl chloride leads polymerization-free and in excellent yields (97%) to β-ketonitrile **5**. This is cyclized almost quantitatively with hydrazine hydrate to afford 3-amino-5-(4-vinylphenyl)pyrazole **6**. Final acylation as described above for 3-amino-5-methylpyrazole **3** gives amidopyrazoles **7**.

^{1}H NMR Chemical Shift Studies and Titrations. A first hint for strong interaction in chloroform was obtained by the drastic solubility increase for the aminopyrazoles on addition of a dipeptide. In the ^{1}H NMR spectra the amide protons undergo large but markedly different downfield shifts on the top and bottom face of the peptide (3.0 ppm vs 0.5 ppm). The stoichiometry of the formed complexes was determined according to Job's method of continuous variations (Figure 3).[25] We found a 1:1 stoichiometry for the top face (NH(1), see Figure 2) but a 1:2 ratio for the bottom face (NH(2)). These results suggest that the top face of the peptide is first saturated by way of the stronger three-point interaction, before weaker two-point binding occurs on the bottom face with a second aminopyrazole molecule.

Contrary to the hitherto known intramolecular β-sheet models the *inter*molecular complexation of peptide by free templates offers the possibility of a precise determination of binding strengths. To this end we performed ^{1}H NMR titrations of the peptides with increasing amounts of aminopyrazole derivatives. We measured the chemically induced shifts (CIS) of the peptide

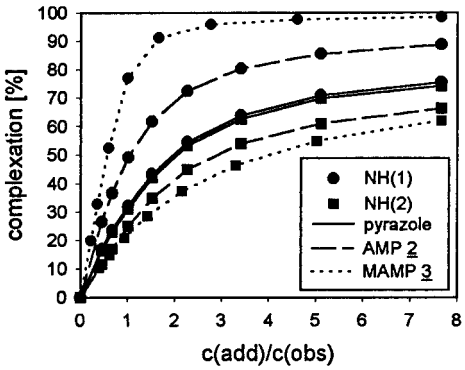

Figure 4. ^{1}H NMR titration curves for the divaline complex with pyrazole, aminopyrazole **2**, and amidopyrazole **3**.

amide protons and calculated the association constants by a nonlinear regression method.[26] Intramolecular NOE experiments allow distinction between NH(1) and NH(2), i.e., between the top and bottom face of the peptide. All obtained data confirm the stronger complexation of the peptide's top face and thus provide additional experimental evidence for the three-point binding mode. We examined the binding strength as it depends on the aminopyrazole as well as on the peptide structure. We began with Ac-L-Val-L-Val-OMe **8**, which is preoriented in a conformation close to that in a β-sheet due to its sterically demanding isopropyl groups.[27]

Even the relatively weakly binding 3-amino-5-methylpyrazole **2** discriminates between top and bottom face of the peptide by a factor of 4 ($K_1 = 10$; $K_2 = 2.5$, Figure 4). Conversion of its amino function into an amide drastically enhances this effect (MAMP **3**: $K_1 = 80$; $K_2 = 2.0$). This is in sharp contrast to pyrazole, which can form only two hydrogen bonds on each side of the peptide. It binds not only weakly but also completely unselectively ($K_1 = K_2 = 4$). The binding constant for the peptide's top face is strongly influenced by the electronic character of the heterocycle as well as the acyl substituent. We obtained the strongest complexation for 3-trifluoroacetylamino-5-methylpyrazole (TriF1AMP) **4**. The association constant in chloroform is 880 M^{-1}. Here the introduction of the strongly electron-withdrawing trifluoromethyl group turns the amide into an optimal hydrogen bond donor, while the methyl substituent increases the electron density within the ring and thus improves its hydrogen bond acceptor capacity. The strength of complexation also depends strongly on the peptide structure. Exchange of *N*-terminal valine for glycine (Ac-Gly-L-Val-OMe **9**) allows almost free rotation around the C−C and the C−N bond in the glycine residue.[28] Therefore in the ^{1}H NMR spectrum the methylene protons appear chemically equivalent and give a simple doublet (Figure 5). On complexation with an amidopyrazole the glycine's rotation can only be hindered when the third binding point is formed. This is indeed the case: on addition of aminopyrazole the methylene protons can immediately be distinguished NMR-spectroscopically and at the end of the titration give two separated doublets of doublets with a shift difference of 0.25 ppm.

The association constants of the glycine-containing dipeptides must be smaller than those of Ac-L-Val-L-Val-OMe **8**, because the amount of energy necessary to stop the bond rotation has to be subtracted from the complex stabilization energy (Table

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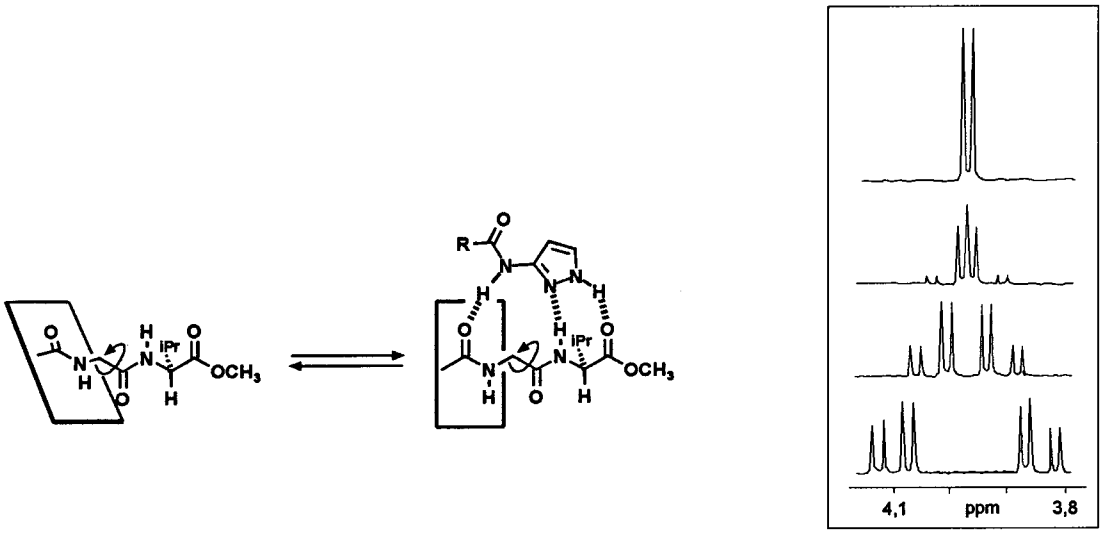

Figure 5. Left: Conformational lock of a rotationally free glycine-containing dipeptide by complexation with 1 equiv of amidopyrazole. Right: ^{1}H NMR signal of the methylene protons in Ac-Gly-L-Val-OMe **9**. From top to bottom: without, with 0.4, 2.3, and 6 equiv of MAMP **3**.

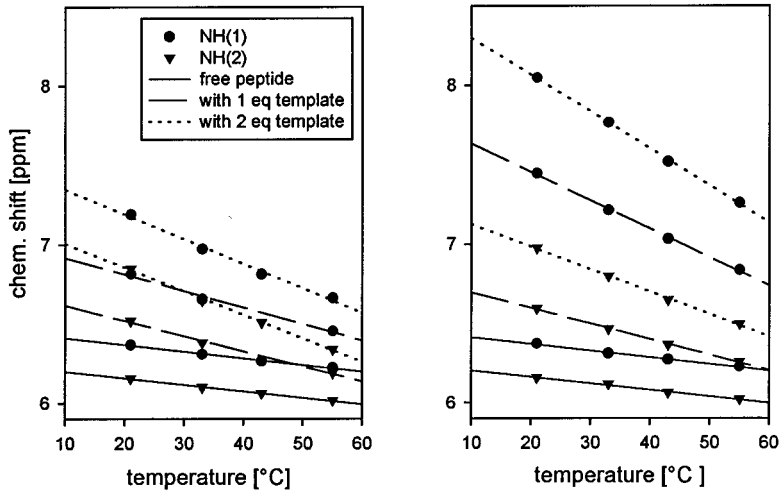

Figure 6. Temperature dependence of the chemical shift for the amide protons of Ac-L-Val-L-Val-OMe **8** on complexation with pyrazole (*left*) and 3-amino-5-methylpyrazole **2** (*right*).

Table 1. Association Constants K_{ass} [L/mol] for the Peptide's Top Face of Different Dipeptide Aminopyrazole Complexes (Errors < 5%)

	Ac-L-Val-L-Val-OMe **8**	Ac-Gly-L-Val-OMe **9**
pyrazole	4	
AMP **2**	10	
MAMP **3**	80	24
TriFlAMP **4**	890	
PivAStyP **7a**	81	21
AcAStyP **7b**	370	106
TriFlAStyP **7c**	570	200

1). This energy is calculated with the Arrhenius equation to be ca. 3 kJ/mol at 23 °C. All binding constants for the complexation of the peptide's top face are summarized in Table 1.

The influence of the acyl group discussed above is beautifully demonstrated by the styryl-substituted aminopyrazole complexes with both examined peptides. Exchange of the pivaloyl group with its strong positive inductive effect for the simple acetyl group increases the binding constant for **8** as well as for **9** by a factor of 5. If the strongly electron withdrawing trifluoroacetyl group is used instead, the binding constant reaches values that are even 7−10 times higher than with the pivaloyl rest.

Variable Temperature (VT) ^{1}H NMR Studies. To which extent are the amide hydrogens involved in hydrogen bonds? This question can be answered by observing the temperature dependence of their chemical shifts in a ^{1}H NMR experiment. In general non-hydrogen bonded amide protons appear in $CDCl_3$ at ca. 6 ppm, whereas hydrogen bonded amide protons are observed at ca. 8 ppm or beyond.[16b,17a] For peptidic amide protons which are either not hydrogen bonded at all or fixed in a very strong locked hydrogen bond, the temperature dependence of their chemical shifts is small (−2 to −4 ppb/K), if the concentration is below the critical value for self-association. Peptidic amide protons in a dynamic hydrogen bond produce much larger effects.[29] We performed VT-NMR experiments with Ac-L-Val-L-Val-OMe **8** and pyrazole as well as 3-amino-5-methylpyrazole **2**. In both experiments the temperature dependence for the free peptide was determined to be −4 ppb/K, which at the chosen concentration rules out self-association. As a consequence, any change in the temperature dependence of the chemical shift must be correlated with the complexation by the pyrazole derivatives. Addition of 1 or 2 equiv of pyrazole leads to identical values for the top and bottom face of the peptide (−10 or −15 ppb/K). This is another proof for the fact that pyrazole cannot distinguish between both peptide faces.

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Table 2. *NH-a-CH*-Coupling Constants in Dipeptide Complexes with Pyrazole Derivatives at a Concentration of 0.5 mol/L[a]. [a]n.d. = not detectable, because of signal overlap; (a) ABX-pattern; $^3J_{AX} \pm {}^3J_{BX}$ is reported.

	Ac-L-Val-L-Val-OMe **8**		Ac-L-Phe-L-Phe-OMe **10**		Ac-Gly-L-Val-OMe **9**	
	NH(1)	NH(2)	NH(1)	NH(2)	NH(1)	NH(2) (a)
free	8.2	8.5	7.6	7.9	8.3	10.4
pyrazole	8.2	9.0	8.0	8.5		
APCE **1**	8.6	9.6	8.5	8.7		
MAMP **3**	9.8	10.0			9.5	11.1
PivAStyP **7a**	9.0	9.7			n.d.	11.3
AcAStyP **7b**	9.6	9.8			n.d.	11.5
TriFlAStyP **7c**	10.0	9.8			10.0	11.8

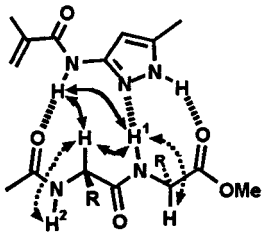

Figure 7. Strong (solid) and weak (dotted) intra- and intermolecular nuclear Overhauser effects in the Ac-L-Val-L-Val-OMe **8**/MAMP **3** complex.

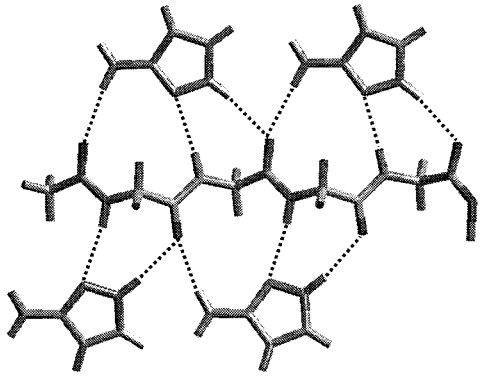

Figure 8. Structure of a tetrapeptide/aminopyrazole complex. The geometry was energy minimized using CERIUS[2] with the DREIDING 2.21 force field.

By contrast 3-amino-5-methylpyrazole **2** recognizes the peptide's top face much stronger than the bottom face. Addition of only 1 equiv leads to a much stronger downfield shift for NH(1) compared to pyrazole. The temperature dependence for the top face is −18 ppb/K, increasing to −23 ppb/K on addition of a second equiv of aminopyrazole. The values for the bottom face are much lower and correspond exactly to those determined for pyrazole (−10 and −15 ppb/K). These results constitute another experimental proof for the fact that the bottom face of the dipeptide is always bound by the same two-point interaction, while the top face by virtue of its third hydrogen bond acceptor is involved in much stronger three-point binding.

^{1}H-NMR Coupling Studies. Direct information about the peptide conformation can be drawn from Karplus analyses of the *NH*-α-*CH* coupling constants which correlate with the characteristic torsion angle θ.[30] In general, coupling constants greater than 7 Hz indicate formation of a β-sheet-conformation, while coupling constants below 6 Hz belong to an α-helix.[31] Peptides with a random coil structure show coupling constants between 7 and 8 Hz. We examined complexes of pyrazole, 3-amino-4-carbethoxypyrazole (APCE) **1**, and several acylated aminopyrazole derivatives with the preoriented peptides Ac-L-Val-L-Val-OMe **8** and Ac-L-Phe-L-Phe-OMe **10** as well as the rotationally free Ac-Gly-L-Val-OMe **9**. Addition of pyrazoles to the peptide solution invariably led to sharper signals and increased ^3J-values. The different ^3J-coupling constants are summarized in Table 2.

In the 1:1 complex of defined and constant concentration a remarkable correlation of the ^3J-coupling with the degree of complexation is observed: at the given concentration (0.5 mol/L) only 50% of the dipeptide is bound to pyrazole, which leads to a very small increase of the ^3J-coupling constant. From this it is clear that pyrazole is not capable of stabilizing the β-sheet conformation in a dipeptide. Addition of 1 equiv of 3-amino-4-carbethoxypyrazole (APCE), **1**, however, effects a complexation degree of 65%. The increase in the ^3J-value for NH (2) of 1.1 Hz compared to the free peptide correlates with an increase in the dihedral angle θ_2 of 11° to 168°. Complexation of the dipeptide with 1 equiv of the acylated aminopyrazoles leads to a much higher amount of fixed peptide molecules, e.g., for MAMP **3** 85%, for PivAStyP **7a** 86%, for AcAStyP **7b** 93%, and for TriFlAstyP **7c** even 94%. The increase in ^3J always follows the binding strength, i.e., the degree of complexation. For two of the amidopyrazoles one coupling constant even reaches 10 Hz—which corresponds to the ideal β-sheet conformation with a dihedral angle θ of 180°. In the case of Ac-Gly-L-Val-OMe **9** with free rotation the Karplus analysis becomes especially impressive.[30b] Not only does the complexation rise θ_1 but also it slows down the free rotation in the glycine residue to almost zero, producing an average dihedrale angle θ_2 of up to 172°. Even with just one molecule of amidopyrazole the conformational lock for the glycine is so strong that the rotation is almost completely "frozen" by means of three dynamic hydrogen bonds (Table 2).

Intra- and Intermolecular ^{1}H NMR NOE Studies. A detailed investigation of intra- and especially intermolecular nuclear Overhauser effects (NOE) provides valuable information about the conformation of the dipeptide and the mutual orientation of the complexation partners.[32] We examined the 1:1 complex between Ac-L-Val-L-Val-OMe **8** and 3-methacryloylamino-5-methylpyrazole (MAMP) **3**. Irradiation into the amide signal of the peptidic top face NH(1) gave rise to a strong *inter*residue NOE with the α-proton of *N*-terminal valine, which was almost four times stronger than the *intra*residue NOE to the α-proton of *C*-terminal valine (18.0% vs 5.0%). Irradiation into the α-proton of *N*-terminal valine produces an analogous effect: the NOE with the amide proton of the neighbor amino acid reaches 10.3%, whereas that to the own amide gives only 1.8%. This strongly indicates an extended conformation of the dipeptide.[31c] No NOEs could be detected between the two peptidic amide protons, as one would expect from their large

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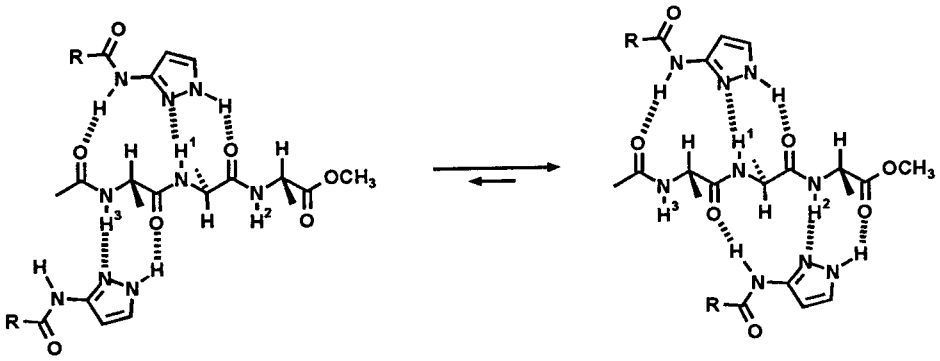

Figure 9. Equilibrium during the ^{1}H NMR titration of a tripeptide with an amidopyrazole: two competitive binding sites at the peptide's bottom face.

distance in a β-sheet, a third proof for the peptide's extended conformation. *Inter*molecular NOEs would be especially conclusive, because they prove close proximity and steric orientation of the peptide and its template. Above all, an NOE between the amide proton of the acylated aminopyrazole and the N-terminal α-proton would provide striking evidence for the existence of the critical third hydrogen bond to the *N*-terminus. We were indeed able to detect large *inter*molecular NOEs on irradiation into NH(1) (1.5%) as well as into the N-terminal α-proton (0.8%). Both reciprocal NOEs appeared on irradiation into the amide proton of the acylated aminopyrazole (1.1% and 1.2%). All observed NOEs agree with our postulated complex structure and at the same time confirm the stabilization of the peptide's β-sheet conformation.

Complexation of the tripeptide Ac-L-Ala-L-Ala-L-Ala-OMe. Can the aminopyrazole concept also be applied to larger peptides? Molecular modeling studies suggest that in oligopeptides the aminopyrazole molecules may be lined up on both sides of the peptide strand, each one being involved in three-point binding.[21] Each amino acid is attached to two receptor molecules by a total of three hydrogen bonds (Figure 8).

We titrated the tripeptide Ac-L-Ala-L-Ala-L-Ala-OMe **11** with 3-trifluoroacetylamino-5-(4-vinylphenyl)pyrazole (TriFlAStyP) **7c** and measured the CIS of the three peptide amide protons. In this case the top face consists of just one three-point binding site, while the bottom face offers two competitive binding sites, i.e., one for two-point and one for three-point binding (Figure 9).

Addition of substoichiometric amounts of the amidopyrazole causes the largest downfield shift for the top face amide proton NH(1). The amide protons at the peptide's bottom face experience weaker (NH(2)) and very small (NH(3)) downfield shifts. As the amount of amidopyrazole added surpasses 1 equiv, the downfield shifts of the top face proton NH(1) and the initially weaker bottom face proton NH(2) become almost identical. NH(3) with its very small downfield shift, however, shifts back to higher field and at great amidopyrazole excess it reaches again the parent chemical shift corresponding to the uncomplexed peptide (Figure 10). From this we conclude that because of the competition between two- and three-point binding at the bottom face first the top face binds the receptor, for here only a single three-point binding mode is possible. When the top face is saturated, those pyrazoles that have been bound at the bottom face by initial two-point binding will be more and more replaced by pyrazoles occupying the three-point binding site. Finally both the peptide's top and bottom face are complexed with one amidopyrazole molecule, each in the three-point binding mode. At this time, the *N*-terminal amide proton is no longer hydrogen bonded, so that its chemical shift must correspond to that in the free peptide.

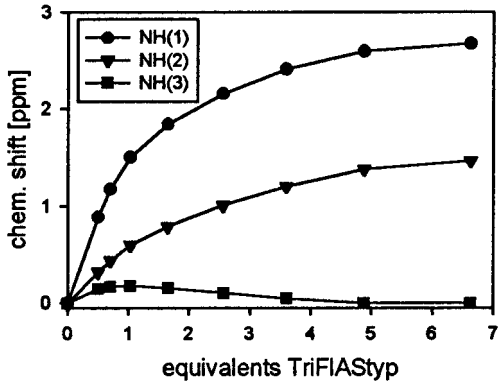

Figure 10. Dependence of the chemical shift of the three peptidic amide protons of Ac-L-Ala-L-Ala-L-Ala-OMe **11** during ^{1}H NMR titration with TriFlAStyP **7c**.

Conclusions and Outlook

Our investigations demonstrate that 3-aminopyrazoles are able to efficiently stabilize the β-sheet conformation in protected dipeptides by three cooperative intermolecular hydrogen bonds. First experiments with tripeptides show very promising results concerning the application of this concept to oligopeptides. The association constant of up to almost 10^3 M^{-1} for dipeptides should rise markedly when aminopyrazole molecules are lined up on both sides of a larger peptide. We have thus taken the first step toward the development of new peptide receptors. In the future we want to create a building-block system for sequence selective peptide recognition by synthesizing a library of convergently functionalized aminopyrazoles. In addition to that, with our route to polymerizable aminopyrazole derivatives, we introduce for molecular imprinting a new class of functional monomers, which fulfills all requirements for an efficient binding site. At present we examine the racemic resolution of dipeptides with imprinted polymers using the new functional monomers. In batch experiments separation factors α of up to 2.7 have been achieved with some of these polymers; a full account of this work will be published in due course. Experiments to use aminopyrazoles for catalysis with imprinted polymers are underway in this laboratory.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded on a Varian VXR 300 NMR spectrometer at 23 °C. Chemical shifts (δ) are reported in ppm downfield from internal tetramethylsilane (TMS). $CDCl_3$ was

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dried over powdered molecular sieve 4Å and freshly distilled for each measurement. Optical rotations were measured on a Perkin Elmer 241 MC digital polarimeter equipped with a 1 dm cell operating at 589 nm (sodium D line). Thin layer chromatographic (TLC) analyses were performed on silica gel 60 F-254 with a 0.2 mm layer thickness. Preparative chromatography columns were packed with Kieselgel 60 (70−230 mesh). L-Amino acids, *N*-acetylamino acids, amino acid methyl ester hydrochlorides, 3-amino-4-carbethoxypyrazole **1**, and 3-amino-5-methylpyrazole **2** were purchased from Aldrich Chemical Co. Pyrazole was purchased from Fluka Chemical Co. All solvents were dried and freshly distilled before use.

^{1}H NMR Titrations. A solution of the binding site molecule was added in portions via microsyringe to a solution of a peptide (concentration equal to the estimated dissociation constant) in a septum-capped NMR tube. Volume and concentration changes were taken into account during analysis.[26]

Job Plots. Equimolar solutions (0.2 M) of binding site molecule and peptide were prepared and mixed in various amounts. ^{1}H NMR spectra of the mixtures were recorded, and the chemical shifts were analyzed by Job's method modified for NMR results.[25]

Temperature-Dependent NMR Spectra. Variable-temperature (VT) NMR studies were carried out at 0.02 M (concentration of peptide) in the range of 21−55 °C. After temperature change the sample was equilibrated for 10 min before shimming, and then measurements were made.

^{1}H{^{1}H}-Nuclear Overhauser Effect Measurements. All samples were prepared under a dry argon atmosphere, exhaustively degassed by the freeze−thaw method, and sealed under vacuum. Nuclear Overhauser enhancements were obtained by saturation of the desired resonance during a preacquisition time (set to 5 times the longest T_1 of the sample). Percent NOEs were calculated by setting the integral for the saturated resonance equal to −100 (inverted signal). The percent NOEs are reported as percentages of this inverted signal.

***N*-Acetyl-L-valyl-L-valine Methyl Ester Ac-L-Val-L-Val-OMe 8.**[33] A suspension of Ac-L-Val (10 mmol), L-Val-OMe·HCl (10 mmol), *N*-ethylmorpholine (10 mmol), and 3-hydroxybenzotriazole (HOBT) (20 mmol) in tetrahydrofuran (30 mL) was cooled to 0 °C, and DCC (10 mmol) was added. The reaction mixture was stirred vigorously for 1 h and afterwards for another 1 h at room temperature. The mixture was filtered, and the solvent was removed under reduced pressure. The residue was resolved in ethyl acetate and washed with saturated aqueous sodium bicarbonate, 2 N citric acid, sodium bicarbonate and water. After drying over magnesium sulfate the solvent was evaporated and the residue was purified by silica gel chromatography (ethyl acetate; $R_f = 0.35$). ^{1}H NMR ($CDCl_3$) δ 0.90−0.98 (4 d, 12H, $J = 6.7$, CH-(CH_3)$_2$), 2.02 (s, 3H, C(O)CH_3), 2.04−2.20 (m, 2H, CH(CH_3)$_2$, 3.74 (s, 3H, OCH_3), 4.43−4.51 (2dd, 2H, C$^\alpha H$), 6.75 (d, 1H, $J = 8.5$, NH-(2)), 7.14 (d, 1H, $J = 8.2$, NH(1)); ^{13}C NMR ($CDCl_3$) δ 17.91, 18.45, 18.96, 19.06, 23.03, 30.77, 31.40, 52.01, 57.50, 58.36, 170.33, 172.05, 172.16; $[\alpha]_{32}^{589}$ (*MeOH*) = −59.6°. Anal. Calcd for $C_{13}H_{24}N_2O_4$ (272.3): C, 57.33; H, 8.88; N, 10.29. Found: C, 57.45; H, 8.84; N, 10.37.

***N*-Acetyl-L-phenylalanyl-L-phenylalanine Methyl Ester (Ac-L-Phe-L-Phe-OMe) 10. 10** was synthesized from L-Phe-OMe·HCl (10 mmol) and *N*-Ac-L-Phe (10 mmol) as in the foregoing experiment. ^{1}H NMR ($CDCl_3$) δ 1.91 (s, 3H, C(O)CH_3), 2.94−3.06 (m, 4H, CH_2), 3.66 (s, 3H, OCH_3), 4.68−4.76 (m, 2H, C$^\alpha H$), 6.37 (d, 1H, $J = 7.9$, NH(2)), 6.54 (d, 1H, $J = 7.6$, NH(1)); ^{13}C NMR ($CDCl_3$) δ 22.99, 37.89, 38.23, 52.26, 53.50, 54.27, 126.90, 127.07, 128.52, 129.19, 129.31, 135.71, 136.47, 170.09, 170.86, 171.34; $[\alpha]_{20}^{589}$ (*CHCl*$_3$) = +24.3°; Anal. Calcd for $C_{21}H_{24}N_2O_4$ (368.4): C, 68.46; H, 6.57; N, 7.60. Found: C, 68.30; H, 6.43; N, 7.52.

***N*-Acetylglycyl-L-Valine Methyl Ester (Ac-Gly-L-Val-OMe) 9.**[34] A solution of acetylglycine (10 mmol) and triethylamine (10 mmol) in acetonitrile (40 mL) was cooled to −10 °C and treated with isobutyl chloroformate (10 mmol). After 10 min a suspension of L-valine methyl ester hydrocyloride (10 mmol) and triethylamine (10 mmol) in acetonitrile (10 mL) was added. The mixture was stirred for 1 h at −10 °C to 0 °C and then for 1 h at room temperature. The precipitated triethylamine hydrochloride was removed by filtration, and the solvent was replaced by 50 mL of hot tetrahydrofuran. The tetrahydrofuran solution was allowed to stand in a refrigerator overnight. The precipitate was removed again, the filtrate was evaporated in vacuo, and the residue was purified by silica gel chromatography (ethyl acetate/ethanol 3:1; $R_f = 0.53$). ^{1}H NMR ($CDCl_3$) δ 0.92 + 0.95 (2 d, 6H, J = 6.9, CH(CH_3)$_2$), 2.05 (s, 3H, C(O)CH_3), 2.11−2.22 (m, 1H, CH(CH_3)$_2$), 3.74 (s, 3H, OCH_3), 4.02 (d, 2H, $J = 5.22$, C$^\alpha H_2$), 4.50 + 4.55 (dd, 1H, $J = 5.0, 8.7$, C$^\alpha H$), 6.55 (br, 1H, NH(2)), 6.88 (d, 1H, J = 8.3, NH(1)); ^{13}C NMR ($CDCl_3$) δ 17.72, 18.98, 22.91, 31.04, 43.32, 52.23, 57.36, 169.10, 170.75, 172.16; $[\alpha]_{32}^{589}$ (MeOH) = −27.4°. Anal. Calcd for $C_{10}H_{18}N_2O_4$ (230.3): C, 52.16; H, 7.88; N, 12.17. Found: C, 52.13; H, 8.01; N, 12.17.

3-(Methacryloylamino)-5-methylpyrazole (MAMP) 3. A solution of 3-amino-5-methylpyrazole **2** (10 mmol) and triethylamine (10 mmol) in dioxane (25 mL) was cooled to 10 °C. Under vigorous stirring methacryloylchloride (10 mmol) was added very slowly. The reaction mixture was stirred for 1 h at 10 °C and for another 1 h at room temperature. The precipitated triethylamine hydrochloride was removed by filtration, and the solvent was evaporated to dryness. The residue was resolved in water and washed three times with dichloromethane. The organic layer was dried over magnesium sulfate, and the solvent was evaporated in vacuo. The mixture of regioisomers was purified by recrystallization from ethyl acetate. Yield: 40%; mp 162 °C; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H, ring CH_3), 2.29 (s, 3H, CH_3), 5.47 + 5.83 (2s, 2H, CH_2), 6.50 (s, 1H, H_{arom}), 8.81 (s, 1H, C(O)NH); ^{13}C NMR (DMSO) δ 10.77, 18.69, 96.06, 120.13, 138.43, 139.54, 146.88, 165.605; MS *m*/*z* 165 (M^+, 18%), 164 (19), 150 (3), 137 (9), 120 (10), 110 (9), 97 (12), 96 (12), 69 (21), 41 (100). Anal. Calcd for $C_8H_{11}N_3O$ (165.2) C, 58.17; H, 6.71; N, 25.44. Found: C, 58.05; H, 6.79; N, 25.50.

3-Trifluoroacetylamino-5-methylpyrazole (TriFlAMP) 4. Preparation follows the procedure described above. When the oily residue is treated with water, pure product is obtained by precipitation. Yield: 99% mp 203 °C; ^{1}H NMR (DMSO) δ 2.25 (s, 3H, CH_3), 6.32 (s, 1H, CH_{arom}), 11.87 (s, 1H, C(O)NH), 12.42 (s, 1H, NH_{arom}); ^{13}C NMR (DMSO) δ 10.60, 96.60, 115.91, 139.14, 144.88, 153.98; MS *m*/*z* 193 (M^+, 72%), 124 (61), 96 (40), 81 (5), 69 (39), 41 (100). Anal. Calcd for $C_6H_6F_3N_3O$ (193.13): C, 37.31; H, 3.13; N, 21.76. Found: C, 37.48; H, 3.11; N, 21.69.

3-(*p*-Vinylphenyl)-3-oxopropanenitrile 5. A solution of cyanoacetic acid (80 mmol) in tetrahydrofuran (400 mL) is cooled to −78 °C with stirring under an argon atmosphere. The mixture is titrated with *n*-butyllithium (1.6 M in hexane, 160 mmol), while the reaction temperature slowly rises to 0 °C. When the red color persists at 0 °C, the slurry is again cooled to −78 °C, and a solution of *p*-vinylbenzoyl chloride[24] (40 mmol) in tetrahydrofuran is added dropwise. The slurry is stirred at −78 °C for 1 h and then allowed to gradually warm to room temperature over a period of 1 h. Hydrochloride acid (1 M, 200 mL) is added to the mixture. The resulting solution is extracted twice with chloroform (300 mL and 100 mL), and the combined organic layers are washed with saturated aqueous sodium bicarbonate (200 mL) and then with saturated aqueous sodium chloride (200 mL). The organic layer is dried over magnesium sulfate, filtered, and evaporated in vacuo (bath temperature 30 °C). The residual crude product is used without further purification. Yield 6.6 g (97%); mp 99 °C; ^{1}H NMR ($CDCl_3$) δ 4.12 (s, 2H, CH_2), 5.46 (d, 1H, $J = 10.7$, C$H_{2,cis}$), 5.91 (d, 1H, J = 17.6, C$H_{2,trans}$), 7.51 ± 7.86 (AB pattern, 4H, $J_{AB} = 8.5$, H_{arom}); ^{13}C NMR ($CDCl_3$) δ 29.45, 114.12, 126.73, 128.89, 133.28, 135.46, 143.62, 186.76; MS *m*/*z* 171 (M^+, 59%), 131 (100), 103 (62), 77 (65), 51 (80). Anal. Calcd for $C_{11}H_9NO$ (171.2): C, 77.17; H, 5.30; N, 8.18. Found: C, 77.23; H, 5.21; N, 7.92.

3-Amino-5-(*p*-vinylphenyl)pyrazole 6. Oxonitrile **5** (30 mmol) and hydrazine hydrate (90 mmol) dissolved in ethanol (150 mL) are heated under reflux for 2.5 h. The reaction mixture is filtered, and the solvent is removed under reduced pressure. The crude product is recrystallized from toluene. Yield 90%. mp 159 °C; ^{1}H NMR ($CDCl_3$) δ 5.25 (d, 1H, $J = 11.0$, C$H_{2,cis}$), 5.76 (d, 1H, $J = 17.7$, C$H_{2,trans}$), 5.88 (s, 1H, CH_{arom}), 6.70 (dd, 1H, $J = 11.0, 17.7$, CH), 7.41 + 7.60 (AB pattern, 4H, $J_{AB} = 8.3$, H_{arom}); ^{13}C NMR ($CDCl_3$) δ 89.14, 113.91, 125.33, 126.50, 130.36, 136.28, 136.90, 145.32, 153.60; MS *m*/*z* 185 (M^+, 100%), 156 (30), 143 (11), 128 (29), 115 (11), 103 (18), 93 (25), 77 (28). Anal. Calcd for $C_{11}H_{11}N_3$ (185.2): C, 71.33; H, 5.99; N, 22.69. Found: C, 71.16; H, 5.85; N, 22.59.

3-Trimethylacetylamino-5-(*p*-vinylphenyl)pyrazole (PivAStyP) 7a. A solution of 3-amino-5-(*p*-vinylphenyl)pyrazole **6** (20 mmol) and triethyl amine (20 mmol) in dioxane (100 mL) is cooled to 12 °C. Trimethylacetic acid chloride (20 mmol) is added dropwise and the mixture is stirred for 3 h. After the first hour the reaction mixture is allowed to gradually warm to room temperature over a period of 1 h. The solvent is removed under reduced pressure (bath temperature 30 °C), and the oily residue is dissolved in water. After extraction with ethyl acetate the organic layer is dried over sodium sulfate, filtered, and evaporated in vacuo (bath temperature 30 °C). The crude product is purified by silica gel chromtography (ethyl acetate/*n*-hexane 1:1; R_f = 0.29). Yield: 40%; dp. 193 °C; ^{1}H NMR ($CDCl_3$) δ 1.31 (s, 9H, C*H*$_3$), 5.27 (d, 1H, J = 10.8, C$H_{2,cis}$), 5.76 (d, 1H, J = 17.6, C$H_{2,trans}$), 6.69 (dd, 1H, J = 10.8, 17.6, C*H*), 7.263 (s, 1H, CH_{arom}), 7.41 ± 7.58 (AB pattern, 4H, J_{AB} = 8.3, H_{arom}), 8.28 (s, 1H, C(O)N*H*), 11.2 (br, 1H, NH_{arom}); ^{13}C NMR ($CDCl_3$) δ 27.50, 39.38, 93.75, 114.48, 125.51, 126.76, 129.30, 136.11, 137.68, 144.74, 146.91, 176.59; MS *m*/*z* 269 (M^+, 34%), 185 (81), 156 (6), 128 (30), 102 (8), 77(8), 57 (100), 41 (30). Anal. Calcd for $C_{16}H_{19}N_3O$ (269.35): C, 71.35; H, 7.11; N, 15.60. Found: C, 71.25; H, 7.05; N, 15.82.

3-Acetylamino-5-(*p*-vinylphenyl)pyrazole (AcAStyP) 7b. This compound is prepared in a similar procedure as described above. The crude product is recrystallized from methanol. Yield 65%; dp 203 °C; ^{1}H NMR (DMSO) δ 2.07 (s, 3H, C*H*$_3$), 5.30 (d, 1H, J = 11.0, C$H_{2,cis}$), 5.90 (d, 1H, J = 17.9, C$H_{2,trans}$), 6.76 (dd, 1H, J = 11.0, 17.9, C*H*), 6.95 (s, 1H, CH_{arom}), 7.55 + 7.73 (AB pattern, 4H, J_{AB} = 8.3, H_{arom}), 10.49 (s, 1H, C(O)N*H*), 12.87 (s, 1H, NH_{arom}); ^{13}C NMR (DMSO) δ 23.08, 93.67, 114.61, 125.08, 126.69, 128.73, 136.03, 136.71, 141.43, 148.35, 167.55; MS *m*/*z* 227 (M^+, 77%), 185 (100), 156 (19), 128 (34), 102 (11), 77 (16), 43 (67). Anal. Calcd for $C_{13}H_{13}N_3O$ (227.27) C, 68.71; H, 5.77; N, 18.49. Found: C, 68.62; H, 5.83; N, 18.70.

3-Trifluoroacetylamino-5-(*p*-vinylphenyl)pyrazole (TriFlAStyP) 7c. Preparation follows the procedure described above. When the oily residue is treated with water, the crude product precipitates and is subsequently purified by silica gel chromatography (ethyl acetate/*n*-hexane 1:1; R_f = 0.34). Yield: 80%. dp 205 °C; ^{1}H NMR (DMSO) δ 5.31 (d, 1H, J = 11.0, C$H_{2,cis}$), 5.92 (d, 1H, J = 17.2, C$H_{2,trans}$), 6.77 (dd, 1H, J = 11.0, 17.2, C*H*), 6.96 (s, 1H, CH_{arom}), 7.58 + 7.75 (AB pattern, 4H, J_{AB} = 8.3, H_{arom}), 12.08 (s, 1H, C(O)N*H*), 13.27 (s, 1H, NH_{arom}); ^{13}C NMR (DMSO) δ 94.92, 114.91, 115.85, 125.31, 126.79, 128.22, 135.99, 137.13, 142.24, 145.77, 154.19; MS *m*/*z* 281 (M^+, 100%), 212 (84), 184 (11), 169 (15), 156 (21), 142 (21), 128 (81), 102 (28), 77 (56), 69 (40), 51 (40). Anal. Calcd for $C_{13}H_{10}F_3N_3O$ (281.24): C, 55.52; H, 3.58; N, 14.94. Found: C, 55.47, H, 3.50, N, 14.97.

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