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Synthesis and Binding Studies of Alzheimer Ligands on Solid Support

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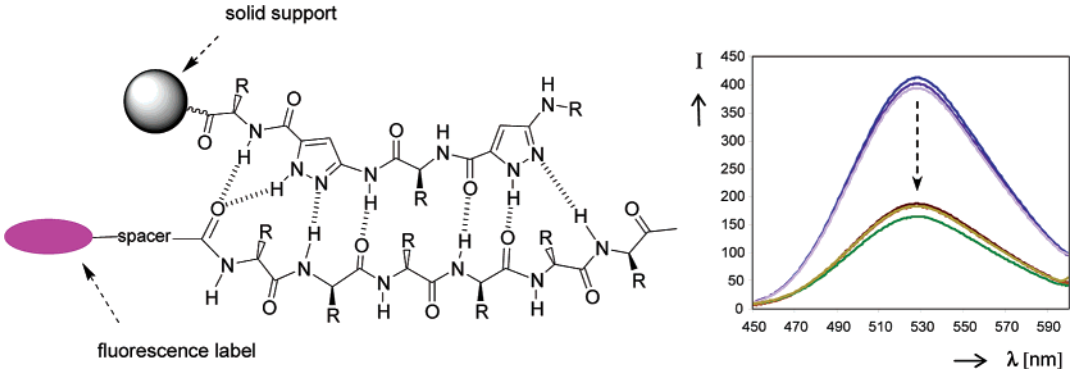

Aminopyrazole derivatives constitute the first class of nonpeptidic rationally designed β-sheet ligands. Here we describe a double solid-phase protocol for both synthesis and affinity testing. The presented solid-phase synthesis of four types of hybrid compounds relies on the Fmoc strategy and circumvents subsequent HPLC purification by precipitating the final product from organic solution in pure form. Hexa- and octapeptide pendants with internal di- and tetrapeptide bridges are now amenable in high yields to combinatorial synthesis of compound libraries for high-throughput screening purposes. Solid-phase peptide synthesis (SPPS) on an acid-resistant PAM allows us, after PMB deprotection, to subject the free aminopyrazole binding sites in an immobilized state to on-bead assays with fluorescence-labeled peptides. From the fluorescence emission intensity decrease, individual binding constants can be calculated via reference curves by simple application of the law of mass action. Gratifyingly, host/guest complexation can be monitored quantitatively even for those ligands, which are almost insoluble in water.

Introduction

Neurodegenerative diseases such as Alzheimer's, Parkinson's, and Creutzfeldt−Jacob challenge modern medicine—because a remedy has not yet been found. Patients suffering from Alzheimer's disease alone constitute an ever growing percentage of the western population and amount to >2 million in the U.S. The underlying cause is well characterized by the term *protein-misfolding diseases*: for widely unknown reasons, native proteins adopt a β-sheet rich abnormal protein conformation which acts as a seed to nucleate protein misfolding and subsequent complex formation, until large insoluble aggregates are formed in the human brain.[1] Medical treatment until today mainly cures symptoms, e.g., through administration of acetylcholine esterase inhibitors,[2] often combined with NMDA receptor antagonists, phytotherapeutics,[3] and antiinflammatory drugs.[4] In the 90s of the past century, the major thrust of pharmaceutical research involved the development of β- and γ-secretase inhibitors; however, new problems arose in the form of low BBB permeability (β) and interference with Notch signaling (γ).[5] Probably the most promising approach has

(1) (a) Lansbury, P. T., Jr. *Acc. Chem. Res.* **1996**, *29*, 317−321. (b) Cohen, F. E.; Kelly, J. W. *Nature* **2003**, *426*, 905−909. (c) Sacchettini, J. C.; Kelly, J. W. *Nat. Rev. Drug Discovry* **2002**, *1*, 267−275.

(2) Acetylcholinesterase inhibitors against AD: Kratzsch, T. *Neurotransmitter* **2002**, *3*, 52−55.

(3) Phytotherapeutics against AD: (a) Perry, G.; Cash, A. D.; Smith, M. A. *J. Biomed. Biotechnol.* **2002**, *2*, 120−123. (b) Aliev, G.; Obrenovich, M. E.; Smith, M. A.; Perry, G. *J. Biomed. Biotechnol.* **2003**, *3*, 162−163.

(4) Ibuprofen against AD: (a) Lim, G. P.; Yang, F.; Chu, T.; Chen, P.; Beech, W.; Teter, B.; Tran, T.; Ubeda, O.; Hsiao Ashe, K.; Frautschy, S. A.; Cole, G. M. *J. Neurosci.* **2000**, *20*, 5709−5714. (b) Mackenzie, I. R. A. *Neurology* **2000**, *54*, 732.

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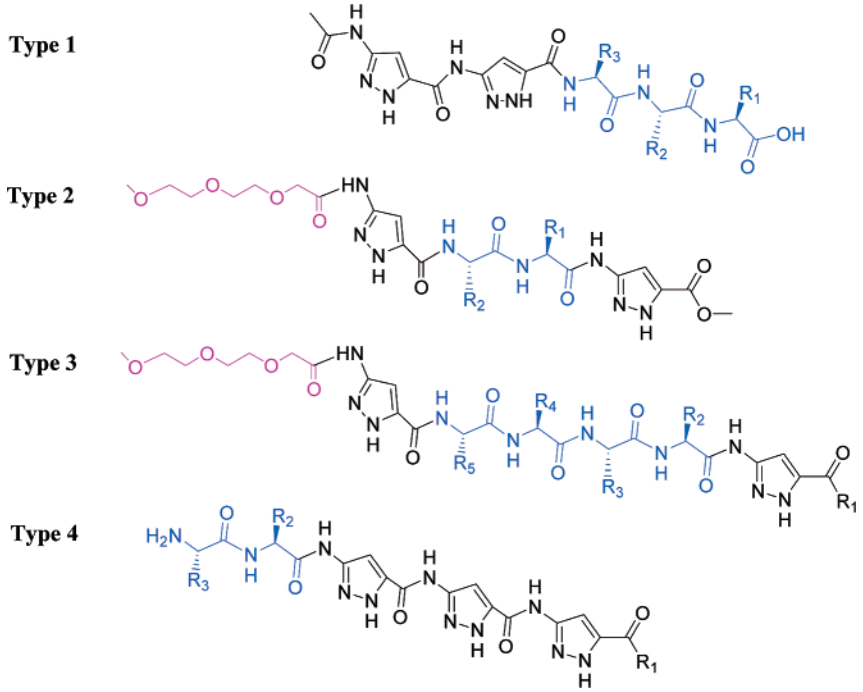
TABLE 1. New Aminopyrazole and Peptide Derivatives with Their Corresponding Yields Obtained from Solution or Solid-Phase Synthesis[a]

entry	molecule	type	synthesis	composition	overall yield (%)
1	**8a**	2	solution (sequ.)	glycole−Pz−K−A−Pz−OMe	11
2	**8b**	2	solution (sequ.)	glycole−Pz−A−K−Pz−OMe	4
3	**8c**	2	solution (sequ.)	glycole−Pz−F−K−Pz−OMe	18
4	**8d**	2	solution (sequ.)	glycole−Pz−A−D−Pz−OMe	6
5	**10a**	1	SPSS (block)	Ac−Pz−Pz−K−F−F−OH	3
6	**13a**	3	SPSS (sequ.)	glycole−Pz−K−L−V−F−Pz−OH	75
7	**13b**	3	SPSS (sequ.)	glycole−Pz-F−F−K−K−Pz−G−OH	80
8	**13c**	3	SPSS (sequ.)	glycole−Pz−K−F−F−K−Pz−G−OH	90
9	**13d**	3	solution (sequ.)	glycole−Pz−F−F−K−V−Pz−OMe	2
10	**13e**	3	solution (sequ.)	glycole−Pz−F−F−K(Dans)−V−Pz−OMe	1
11	**16**	4	solution (block)	Fmoc−K_8−Pz−Pz−Pz−OMe	27
12	**17a**	peptide	SPSS (sequ.)	KKLVFF	95
13	**17b**	peptide	SPSS (sequ.)	KKLVFFAK	80
14	**17c**	peptide	SPSS (sequ.)	KKLVFF−dansyl	75
15	**17d**	peptide	solution (sequ.)	dansyl−glycole−AKLVFF−OMe	25

[a] n.a. = not available; n.d. = not determined; block = fragment coupling of preformed oligomeric building blocks; sequ. = strictly linear nonconvergent elongation protocol.

SCHEME 1. Type 1−4 Aminopyrazole−Peptide Hybrid Compounds Which Were Synthesized and Affinity Tested on Solid Support[a]



[a] Purple: hydrophilic glycole tail. Blue: peptidic part.

reached clinical trials now; it centers around passive or active antibody immunization.[6]

Some years ago, we embarked on a program to design, from modeling studies, a heterocyclic rigid template which is capable of recognizing the β-strand conformation of amyloid structures. The key fragment, a 3-aminopyrazole, binds to every available hydrogen bond donor and acceptor at the top and bottom face of a peptide, as soon as it resides in an extended conformation.[7] In extension of the concept, this central building block was converted into a heterocyclic amino acid and combined with proteinogenic amino acids to hybrid ligands.[8] These retain perfect β-sheet complementarity but introduce, at the same time, an element of sequence selectivity. The in vitro interaction of

(5) β- and γ-secretase inhibitors against AD: (a) Yamada, K.; Ren, X.; Nabeshima, T. *Jpn. J. Pharmacol.* **1999**, *80*, 9−14. (b) Soto, C. *Mol. Med. Today* **1999**, *5*, 343−350.

(6) Active or passive immunization against AD: (a) Bard, F.; Cannon, C.; Barbour, R.; Burke, R. L.; Games, D.; Grajeda, H.; Guido, T.; Hu, K.; Huang, J.; Johnson-Wood, K.; Khan, K.; Kholodenko, D.; Lee, M.; Lieberburg, I.; Motter, R.; Nguyen, M.; Soriano, F.; Vasquez, N.; Weiss, K.; Welch, B.; Seubert, P.; Schenk, D.; Yednock, T. *Nat. Med.* **2000**, *6*, 916−919. (b) Pepys, M. B.; Herbert, J.; Hutchinson, W. L.; Tennent, G. A.; Lachmann, H. J.; Gallimore, J. R.; Lovat, L. B.; Bartfai, T.; Alanine, A.; Hertel, C.; Hoffmann, T.; Jakob-Roetne, R.; Norcross, R. D.; Kemp, J. A.; Yamamura, K.; Suzuki, M.; Taylor, G. W.; Murray, S.; Thompson, D.; Purvis, A.; Kolstoe, S.; Wood, S. P.; Hawkins, P. N. *Nature* **2002**, *417*, 254−259.

(7) (a) Schrader, T.; Kirsten, C. *J. Chem. Soc., Chem. Commun.* **1996**, 2089. (b) Schrader, T.; Kirsten, C. N. *J. Am. Chem. Soc.* **1997**, *119*, 12061−12068. (c) Rzepecki, P.; Wehner, M.; Molt, O.; Zadmard, R.; Schrader, T. *Synthesis* **2003**, 1815−1826.

(8) (a) Černovská, K.; Kemter, M.; Gallmeier, H.; Rzepecki, P.; Schrader, T.; König, B. *Org. Biomol. Chem.* **2004**, *2*, 1603−1611. (b) Rzepecki, P.; Gallmeier, H.; Geib, N.; Černovská, K.; König, B.; Schrader, T. *J. Org. Chem.* **2004**, *69*, 5168−5178.

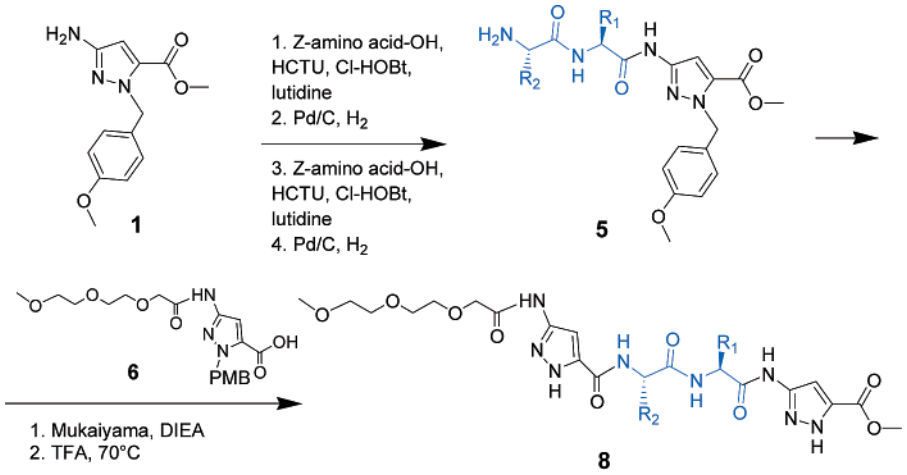


FIGURE 1. Conventional solution synthesis of type 2 hybrid compounds **8** starting from PMB-protected aminopyrazole carboxylate **1**.

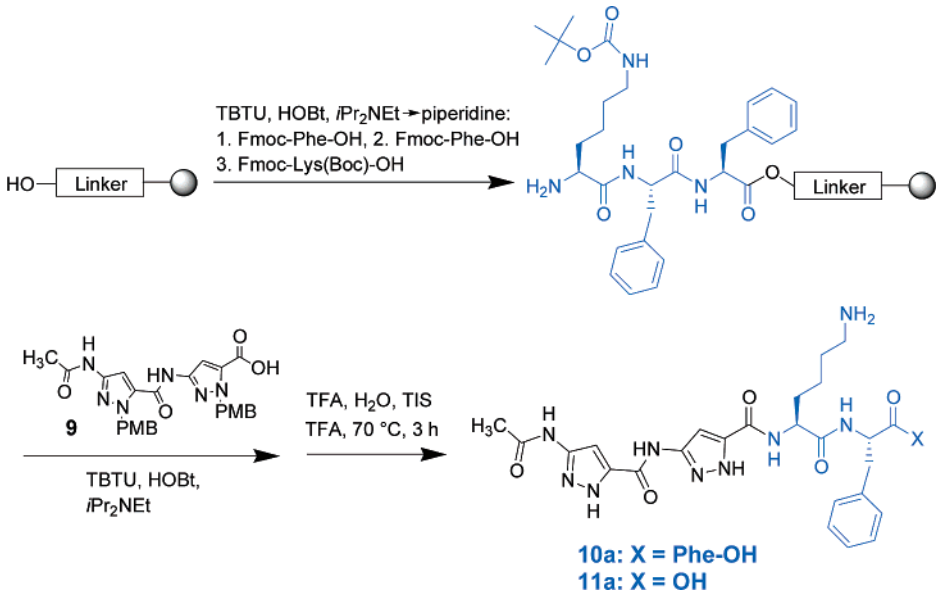


FIGURE 2. Automated synthesis of type 1 hybrid compounds starting from a C-terminal peptide.

such hybrid ligands with peptides taken from Aβ or the whole amyloid peptide(1−42) (aggregation assays with isolated Aβ),[9] however, furnished results conflicting with in vivo experiments (Aβ lesion protocols with living nerve cells), which spurred further investigations.

To understand the diverging experimental results, it hence seemed necessary to develop efficient synthetic and screening protocols for a larger number of aminopyrazole hybrid molecules, in other words, to create an avenue for lead optimization in the frame of this model system. Specifically, we envisioned to facilitate combinatorial screening by a double solid-phase protocol for both synthesis and affinity testing, which is described here.

Results and Discussion

Solid-Phase Synthesis. Four types of hybrid compounds were envisaged (Scheme 1), carrying their peptidic fragment on the N-terminal or the C-terminal end or in between two flanking pyrazole units. Modeling studies restricted the central peptide unit to contain an even number of amino acids to maintain perfect β-sheet complementarity, allowing for a di- or tetrapeptide bridge.[10] In principle, all these ligands can be synthesized from preformed building blocks in solution. However, the multiple synthesis of similar sequences is extremely labor

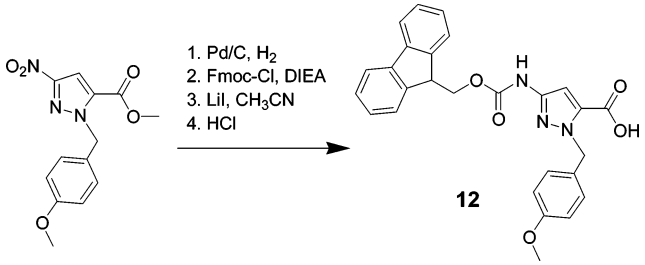


FIGURE 3. Large-scale synthesis of the central PMB- and Fmoc-protected aminopyrazole carboxylic acid **12** from 3-nitropyrazole-5-carboxylate for incorporation into the automated SSPS protocol.

(9) (a) Schrader, T.; Riesner, D.; Nagel-Steger, L.; Aschermann, K.; Kirsten, C.; Rzepecki, P.; Molt, O.; Zadmard, R.; Wehner, M. Patent DE 102 21 052.7 of 10/05/2002. (b) Rzepecki, P.; Nagel-Steger, L.; Feuerstein, S.; Linne, U.; Molt, O.; Zadmard, R.; Aschermann, K.; Wehner, M.; Schrader, T.; Riesner, D. *J. Biol. Chem.* **2004**, *279*, 47479−47505.

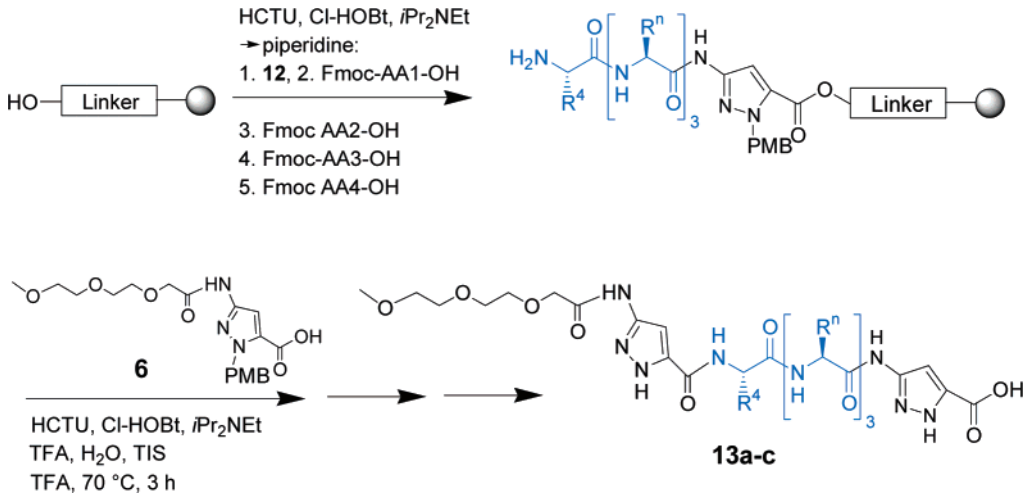


FIGURE 4. Automated synthesis of type 3 hybrid compounds starting from the new PMB- and Fmoc-protected aminopyrazole carboxylate **12**. Note the final double acid cleavage steps for consecutive peptide release as well as Boc and PMB removal.

intensive. We therefore developed an efficient solid-phase synthesis protocol relying on the Fmoc strategy and circumventing subsequent HPLC purification by precipitating the final product from organic solution in pure form (Table 1).

For example, type 2 hybrid compounds with dipeptide bridges were prepared in the following conventional way: the PMB-protected aminopyrazole ester **1** was elongated with a *Z*-protected amino acid by HCTU/Cl-HOBt coupling, followed by hydrogenolytic *Z*-removal. These two steps were repeated, and the resulting dipeptide aminopyrazole **5** was coupled with an acylaminopyrazole carboxylic acid **6**, activated with the Mukaiyama reagent (1-methyl-2-chloropyridinium iodide)[11] to yield, after double PMB deprotection with hot TFA, the final product **8** (Figure 1). To increase water solubility, the acyl protecting group at the N-terminus of **8** was chosen to carry an oligoethyleneglycole unit. Overall yields for this six-step procedure varied between 6% and 18%.

By contrast, most type 1, 3, and 4 hybrids were synthesized on solid support. Wang resin was used as such or preloaded with a C-terminal glycine. Fmoc amino acids were coupled with TBTU and HOBt in DMF[12] and deprotected with piperidine. Aminopyrazole carboxylic acids were coupled with HCTU and Cl-HOBt or with Mukaiyama's reagent (vide supra).[13] Release from the resin with concomitant ϵ-amino Boc removal was affected with a cleavage cocktail containing TFA (95%), water (2.5%), and TIPS (2.5%). Only the final PMB deprotection required harsher conditions, i.e., prolonged heating with TFA at 70 °C. The hybrid compounds could be precipitated from cold diethyl ether and were thus obtained, after washing, in spectroscopically pure form.

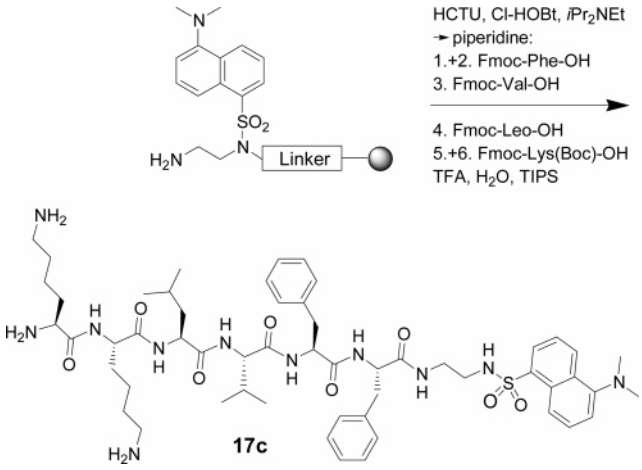


FIGURE 5. Automated synthesis of Dansyl-labeled Aβ fragment **17c** starting from Dansylethylenediamine-preloaded Wang resin.

Compounds **10** and **11** (type 1) were produced in almost quantitative yield, albeit in a 97:3 ratio; obviously, the first coupling step proceeded with incomplete conversion. Here, the three C-terminal amino acids were coupled sequentially, followed by covalent attachment of a preformed dimeric aminopyrazole building block (Figure 2).

The most economic synthesis yielded three prototypes **13a**−**c** of an octapeptide hybrid ligand. To this end, 3-nitropyrazole-5-carboxylic acid was converted into the Fmoc building block unit in a five-step sequence: esterification and PMB protection were followed by hydrogenolytic release of the amine functionality and subsequent Fmoc protection. Mild ester dealkylation with LiI finally furnished the free acid **12** (Figure 3).[14] It was coupled to the resin with HCTU and Cl-HOBt and elongated in a 4-fold repetition with a tetrapeptide bridge, followed by the N-terminal oligoethyleneglycole-protected aminopyrazole carboxylic acid. Double acid treatment effected cleavage from the resin, and complete deprotection of the hybrid compounds, which were again purified by precipitation from

(10) MacroModel 7.2, Force-field: Amber*, OPLS-AA, GBA water solvation model, 2000 steps molecular mechanics, followed by 2000 steps Monte-Carlo simulations, and subsequent MD calculation, 300 K, 10 ps.

(11) (a) Bald, E.; Saigo, K.; Mukayima, T. *Chem. Lett.* **1975**, 1163. (b) Mukaiyama, T. *Angew. Chem.* **1979**, *91*, 798. (c) Li, P.; Xu, J. C. *Tetrahedron* **2000**, *56*, 8119.

(12) Carpino, L. A.; Imazumi, H.; El-Faham, A.; Ferrer, F. J.; Zhang, C.; Lee, Y.; Foxman, B. M.; Henklein, P.; Hanay, C.; Mügge, C.; Wenschuh, H.; Klose, J.; Beyermann, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 441−445.

(13) HCTU: (a) Davis, A. M.; Teague, S. J. *Angew. Chem., Int. Ed* **1999**, *38*, 737. (b) Sabatino, G.; Mulinacci, B.; Alcaro, M. C.; Chelli, M.; Rovero, P.; Papini, A. M. *Lett. Pept. Sci.* **2002**, *9*, 119. (c) Marder, O.; Shvo, Y.; Albericio, F. *Chim. Oggi-Chem. Today* **2002**, *20*, 37.

(14) (a) Karaman, R.; Goldblum, A.; Breuer, E. *J. Chem. Soc., Perkin Trans.* **1989**, 765. (b) Krawczyk, H. *Synth. Commun.* **1997**, *27*, 3151−3161.

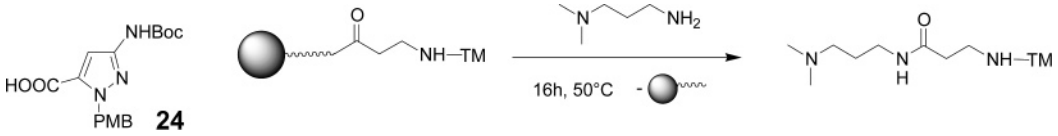
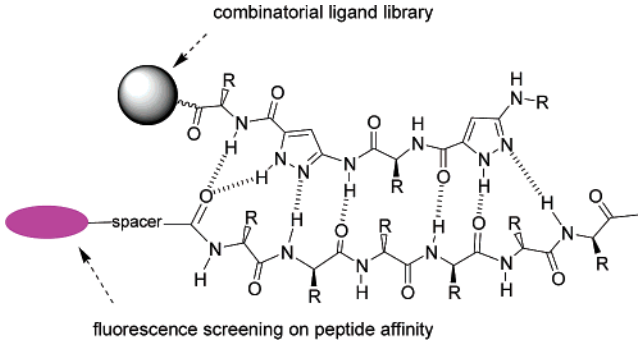


FIGURE 6. Left: Boc-protected 3-aminopyrazole-5-carboxylic acid for the automated synthesis of hybrid ligands on PAM resin. Right: aminolytic release of the deprotected ligand (TM = target molecule) from the PAM resin for analytical purposes.

SCHEME 2. Schematic Illustration of Solid-Phase Screening Assay on Peptide Affinity



diethyl ether. Spectroscopically pure products were obtained in 75−90% total yields over 13 steps on solid support, without the need for HPLC purification (Figure 4).[15]

Type 4 compounds with more than two aminopyrazole nuclei are normally notoriously insoluble in water but turn smoothly water soluble on introduction of one or two lysines. The most critical part is the repetitive coupling of aminopyrazole units to each other because the aromatic amine is hardly nucleophilic. Thus, in spite of multiple coupling steps, total yields remained moderate in this case. For optimal aminopyrazole condensations, a combination of HCTU/Cl-HOBt with Hünig's base was used for all coupling steps.

Finally, the C-terminally Dansyl-labeled KKLVFF peptide **17c** was also prepared on a Dansyl-preloaded resin (Dansyl NovaTag resin), with a short connecting ethylenediamine spacer (Figure 5).[16] For the largest hybrid ligands with a tetrapeptide bridge, a representative octapeptide fragment was truncated from Aβ and synthesized in a nonfluorescent form (H−KKLVF-FAK−OH).

We conclude that, although shorter versions of aminopyrazole hybrid ligands may be synthesized by time-consuming multistep protocols in solution, yields are up to 10-fold higher (up to 90%) with automated solid-phase peptide synthesis (SPPS) procedures, and precipitation from ethereal solution even circumvents HPLC purification. Hence, especially type 2 and 3 hybrid compounds are now amenable to combinatorial synthesis of compound libraries for high-throughput screening purposes. It should certainly be taken into account that the amino acid reactants are used in a 4−5-fold excess for SPPS, so that yields are only high compared to the immobilized growing molecule.

Unfortunately, a direct determination of binding constants between the new hybrid ligands and small Aβ peptide fragments proved futile by fluorescence techniques. The emission intensity change of the weak pyrazole chromophore on hydrogen bonding to the peptides remains minute; evaluation of the resulting almost linear binding isotherms did not proceed to convergence in most cases. Only an NMR titration in water furnished distinct chemical shift changes with some of the larger type 2 and 3 hybrid compounds. However, in the presence of 10 mM phosphate buffer, these changes disappeared completely. This may well originate from the buffer competition overriding the weak pyrazole peptide interaction. In the absence of buffer, it was noted that the pH of the solution shifted toward lower values (e.g., from 7.0 to 6.7), most likely due to the addition of the slightly acidic lysine-containing hybrid ligand. Unfortunately, the diagnostic C-terminal NH proton is also sensitive toward such a pH change, as demonstrated by a titration with small amounts of TFA.[17] We therefore decided to use our new automated synthesis protocol on an acid-resistant PAM resin, so that after PMB deprotection the free aminopyrazole binding sites can be subjected in an immobilized state to on-bead assays with labeled peptides.[18]

Solid-Phase Binding Studies. The underlying idea of this approach focuses on the automated synthesis of aminopyrazole hybrid molecules on the solid phase, their subsequent equilibration with fluorescence-labeled Aβ fragments, and the calculation of individual binding constants from reference curves by simple application of the law of mass action.[19] If this concept proves valid, it can be extended to β-sheet ligand libraries and used for high-throughput screening (Scheme 2).[20] The inverse approach, i.e., immobilization of peptide libraries and treatment with a particular fluorescence-labeled aminopyrazole oligomer, allows us to determine putative sequence selectivities.

A Boc approach was selected because in most resins used with the Fmoc strategy the bond between the linker and the substrate is too acid labile. To be able to cleave off small amounts of product for analytical purposes, a β-alanine functionalized PAM resin was chosen.[21] Aminolysis with primary amines at elevated temperatures releases the product from the solid support, so that the conventional HF cleavage can be avoided, which is normally applied during Boc strategy. Boc protection of the PMB-protected pyrazole amino acid was impossible with Boc_2O but achieved in high yield with Boc−HOBt.[22] Subsequent LiOH saponification furnished the key building block **24**, which was used for SPPS (Figure 6).

(15) (a) Similar problems arise during the synthesis of β-peptide oligomers: Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2005**, *127*, 11516−11529. (b) Murray, J. K.; Gellman, S. H. *Org. Lett.* **2005**, *7*, 1517−1520.

(16) Dansyl NovaTag resin (Novabiochem): average loading = 0.59 mmol/g.

(17) We cannot rule out that K_a values determined via NMR titrations in pure water reflect true complex stabilities, but these are always connected with the uncertainty of a potential influence of a minor pH change. The diagnostic NH proton is located next to the free C-terminal carboxylate group; this in turn may be partly protonated by the approaching alkylammonium side chain of the ligand's lysine residue.

(18) A solution-based library for β-peptides: Murray, J. K.; Farooqi, B.; Sadowsky, J. D.; Scalf, M.; Freund, W. A.; Smith, L. M.; Chen, J.; Gellman, S. H. *J. Am. Chem. Soc.* **2005**, *127*, 13271−13280.

(19) (a) Schmuck, C.; Heil, M. *ChemBioChem* **2003**, *4*, 1232−1238. (b) Wennemers, H.; Conza, M.; Nold, M.; Krattiger, P. *Chem.−Eur. J.* **2001**, *7*, 3342−3347.

(20) Yingyongnarongkul, B.; How, S.-E.; Diaz-Mochon, J. J.; Muzerelle, M.; Bradley, M. *Comb. Chem. High Throughput Screening* **2003**, *6*, 577−587.

(21) PAM resin: Mitchel, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845.

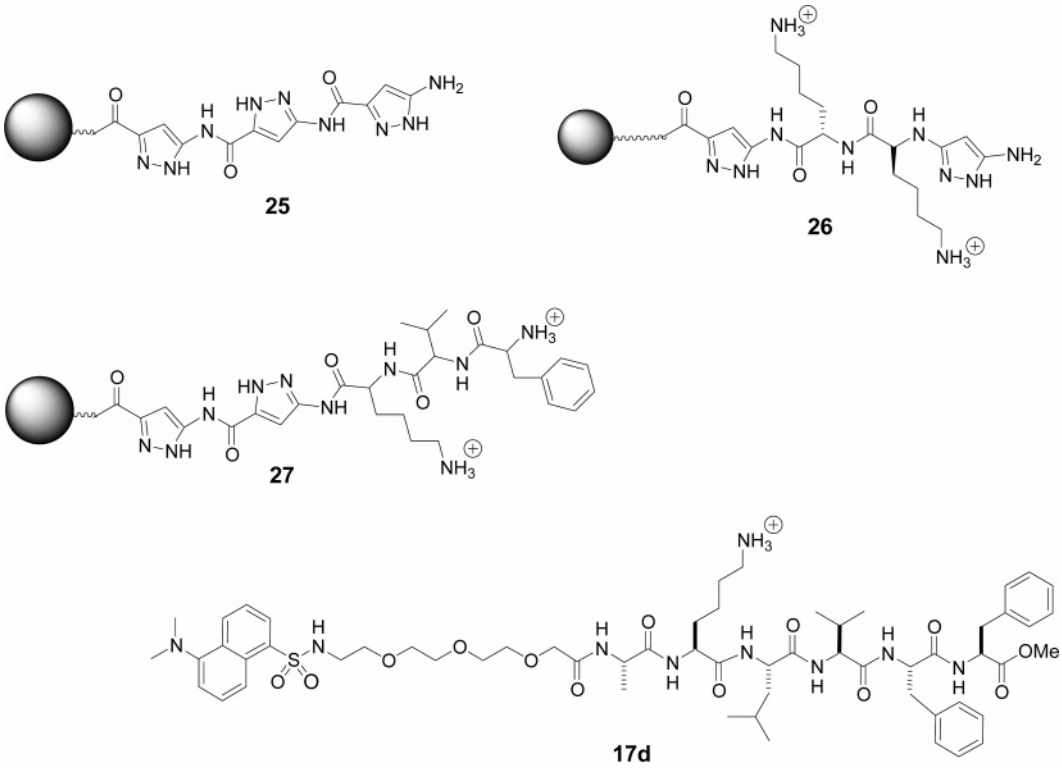


FIGURE 7. Top: Structures of immobilized aminopyrazole ligands **25**−**27**, synthesized on the PAM resin. Bottom: Dansyl-labeled AKLVFF peptide **17d**, synthesized for the on-bead assays.

Three representative oligomeric aminopyrazole compounds were synthesized: trimeric **25**, type 2 hybrid **26**, and type 4 hybrid **27** (Figure 7). The general procedure involved Boc removal from the β-alanine linker, followed by repetitive double coupling with Boc-protected pyrazole amino acid **24** or proteinogenic amino acids; lysine side chains were orthogonally ϵ-Fmoc protected. To ensure complete conversion, **24** was preactivated with HATU/HOAt under sonication, and each coupling step was carried out under argon in the dark.[23] An Nf31 color test proved problematic, most likely due to the lowered nucleophilicity of the aromatic amine.[24] Final deblocking was affected with hot TFA. Gravimetric analyses documented negligible weight losses due to unwanted linker cleavage during the whole solid support synthesis. A small amount of the immobilized ligand was subsequently cleaved off the resin with *N*,*N*-dimethylpropylamine and examined by HPLC-MS (Scheme 1 in the Supporting Information).[25] It revealed that in the case of type 2 hybrid **26** a byproduct lacking the N-terminal aminopyrazole was also formed. However, the same status was found before PMB removal; so, the last coupling step must have been incomplete (single coupling only), and no TFA cleavage of the internal amide bond had occurred.

The fluorescent peptide substrate was synthesized in a convergent approach from a dansyl unit with a hydrophilic oligoethyleneglycole spacer,[26] and the aggregation initiator sequence AKLVFF. Purification via semipreparative HPLC (RP-18) afforded **17d** as a water-, ethanol-, and chloroform-soluble material (Figure 7).

Binding studies were carried out in four different media: chloroform, ethanol, water/acetonitrile 1:1, and phosphate buffer/acetonitrile 1:1. In these solvents, beads with immobilized aminopyrazole ligands were incubated for 20 h, at ambient temperature and in the dark, with the fluorescence-labeled AKLVFF fragment from Aβ (40 μM labeled peptide solution over 1 mg of resin and $\sim$ peptide/ligand ratio 1:100). The starting value was obtained from a sample without beads, whereas a linear reference curve for absolute peptide concentrations was generated from a series of peptide samples (Scheme 3). After filtration of the beads from the peptide solution, a marked decrease in fluorescence intensity was detected (Figure 8). This was not the case if unmodified pure resin was incubated at identical conditions with labeled peptide; hence, nonspecific aggregation can be excluded. From the law of mass action, $K_{ass} = ([R - S])/([R][S])$, the association constants K_{ass} can be directly calculated.[27] They are summarized in Table 2. Because of the spatial separation of aminopyrazole ligands on the resin, exclusive 1:1 complex formation is assumed.

For all aminopyrazole ligands, binding remains relatively weak in unpolar solution, where hydrogen bonds play a crucial role (K_a < 200 M^{-1}). On transition to ethanol, affinity to the Dansyl-labeled hexapeptide is completely lost. However, water

(22) (a) Boc-HOBt: Singh, J.; Fox, R.; Wong, M.; Kissick, T. P.; Moniot, J. L.; Gougoutas, J. Z.; Malley, M. F.; Kocy, O. *J. Org. Chem.* **1988**, *53*, 205. (b) Paquette, L. A. *Encyclopedia of Reagents for Organic Synthesis*; John Wiley & Sons: New York, 1995.

(23) (a) HATU: Carpino, L. A.; Imazumi, H.; El-Faham, A.; Ferrer, F. J.; Zhang, C.; Lee, Y.; Foxman, B. M.; Henklein, P.; Hanay, C.; Mügge, C.; Wenschuh, H.; Klose, J.; Beyermann, M.; Bienert, M. *Angew. Chem., Int. Ed.* **2002**, *114*, 458. (b) Speicher, A.; Klaus, T.; Eicher, T. *J. Prakt. Chem./Chem.-Ztg.* **1998**, *340*, 581.

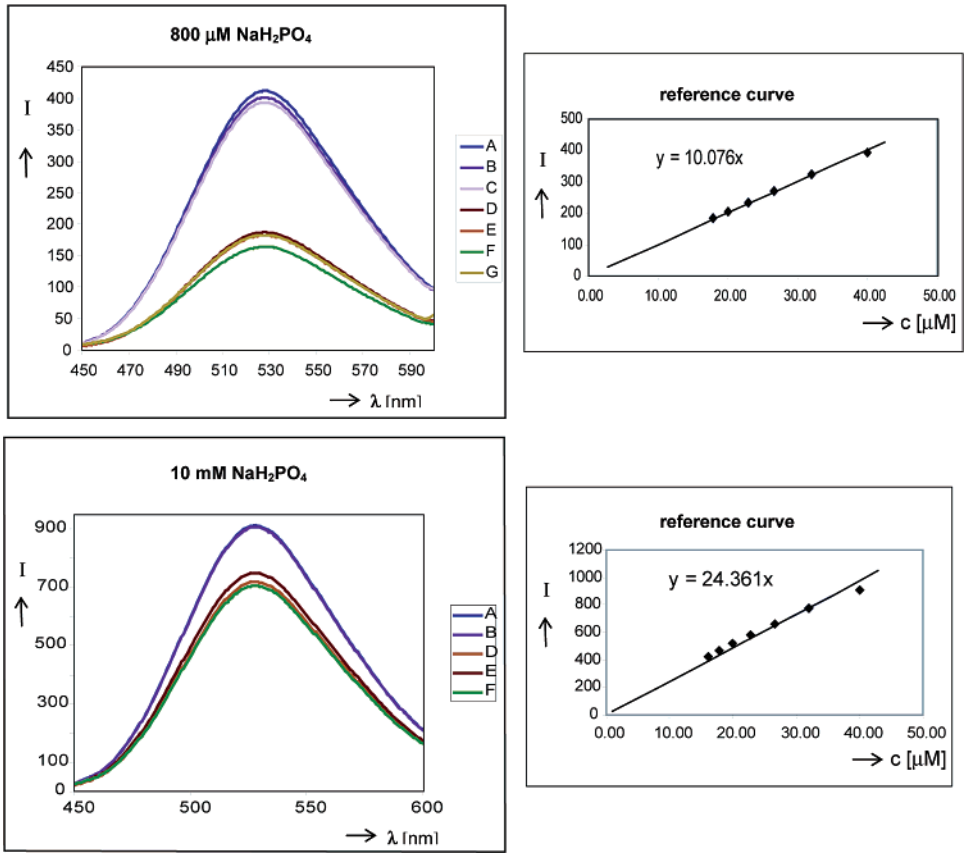
(24) Nf31 color test: Madder, A.; Farcy, N.; Hosten, N. G. C.; De Muynck, H.; De Clercq, P. J.; Barry, J.; Davis, A. P. *Eur. J. Org. Chem.* **1999**, 2787.

(25) Nucleodur C-18 column, 40 °C; gradient elution with acetonitrile/water and 0.1% TFA.

(26) Boumrah, D.; Campbell, M. M.; Fenner, S.; Kinsman, R. G. *Tetrahedron* **1997**, *53*, 6977.

(27) Definition of R and S terms: [S] unbound substrate; [R−S] complex; R [free receptor]. For details see Experimental Section and Supporting Information.

SCHEME 3. Fluorescence Spectra of Peptide 17d Solution after Equilibration with Beads Carrying Immobilized Ligands in Acetonitrile/Buffer = 1:1 (Top, 800 μM Sodium Phosphate; Bottom, 10 mM Sodium Phosphate)[a]



[a] **A** = reference; **B**,**C** = solutions incubated with unmodified PAM resin; **D**,**E** = solutions incubated with PAM resin carrying trimeric **25**; **F**,**G** = solutions incubated with PAM resin carrying type 2 hybrid **26**; right, corresponding reference curves.

TABLE 2. Binding Constants for Hybrid Molecules 25−27, All Calculated from On-Bead Assays with Fluorescent Peptide 17d in Buffered Acetonitrile[a]

aminopyrazole	composition	K_a [M^{-1}] on-bead	1 mM buffer	10 mM buffer	K_a [M^{-1}] free solution
25	trimer (20% impure)	200	180	90	n.a.
25	trimer (pure)	500	430	120	n.a.
26	PzKKPz	660	620	130	1700
27	PzPzKVF	260	n.d.	n.d.	130

[a] n.a. = not available; n.d. = not determined. The experimental error (i.e., the K_a difference between two independent measurements) was calculated at 3−11%.

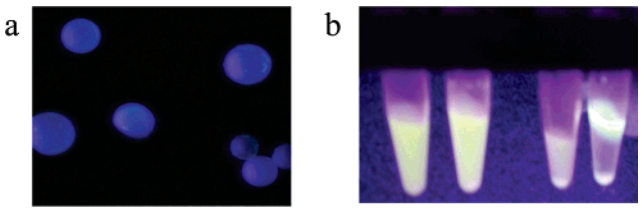


FIGURE 8. (a) Fluorescent PAM resin beads after equilibration with the Dansyl-labeled KLVFF peptide. (b) Fluorescence intensity change of the peptide solution before (left) and after (right) binding to the immobilized aminopyrazole hybrid ligand.

proves to be superior even to less polar chloroform, documenting the obvious importance of additional hydrophobic interactions, most likely exerted by the LVFF side chains (K_a > 500 M^{-1}). A resin carrying trimeric **25** with ∼20% impurities furnished a markedly lower intensity shift than its counterpart with pure **25**. Intriguingly, this eventually leads to a distinctly lowered calculated association constant (∼200 M^{-1}), highlighting the importance of examining pure materials in this assay. In this respect, the value for hybrid **26** must probably be doubled and then compares favorably with the binding constant determined in free solution by Watergate NMR titrations (∼1700 M^{-1}).[28] In 800 mL of phosphate buffer at pH = 7.0, the peptide affinity only slightly decreases by ∼5%; however, raising the buffer to 10 mM produces a competitive medium with K_a values around 100 M^{-1}.

We conclude that the above-described on-bead assay constitutes a valuable new tool to estimate Alzheimer ligand/Aβ peptide affinities in various solvents, by performing only one single new measurement. Gratifyingly, host/guest complexation can be monitored quantitatively even for those ligands, which

(28) (a) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661−665. (b) Sklenar, V.; Piotto, M.; Leppik, R.; Saudek, V. *J. Magn. Res. (A)* **1993**, *102*, 241−245.

are almost insoluble in water (e.g., **25**). This paves the way toward a combinatorial ligand screening as well as toward a deeper understanding of energetic contributions from different structural ligand or guest features. In the case of **25**−**27**, e.g., the hybrid ligand seems to be superior to the other two structures, perhaps because the dipeptide bridge allows for an induced fit toward perfect β-sheet complementarity.

In the future, we intend to attach the Dansyl label via an extended oligoethyleneglycol to our best aminopyrazole hybrid ligands and equilibrate these fluorescent ligands with small libraries of immobilized peptides, preferably truncated overlapping versions of Aβ_1-42. This will allow us to identify the hot spot(s) on Aβ for aminopyrazole binding. Alternatively, a series of ESI-MS measurements of free ligand libraries will help to identify the best binders among them, thus a 1:1 complex could very recently be detected between the above-discussed hybrid peptide **10b** (∼inverted **27**) and Aβ_1-40 (data not shown).

Experimental Section

I. **Solution-Phase Synthesis Protocols for Type 2 Hybrid Compounds.** For each general procedure, one representative example is given in detail. For all other related derivatives, please refer to the Supporting Information material.

General Procedure A for Coupling of *Z*-Protected Amino Acids with PMB-Protected Aminopyrazole Methyl Carboxylate 1. *N*-Benzyloxycarbonyl-(*S*)-amino acid (1.00 mmol, 1.00 equiv) was dissolved under argon in a 3:1 mixture of dry dichloromethane and DMF (5.0 mL) and treated with HCTU (414 mg, 1.00 mmol, 1.00 equiv), Cl-HOBt (424 mg, 2.50 mmol, 2.50 equiv), and 2,6-lutidine (349 μL, 3.00 mmol, 3.00 equiv). For preactivation, this mixture was stirred in an ice bath for 30 min. In a separate flask, 5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester **1 (**261 mg, 1.00 mmol, 1.00 equiv) was dissolved under argon in the same solvent mixture (2.0 mL) and treated with 2,6-lutidine (116 μL, 1.00 mmol, 1.00 equiv). Both solutions were combined, and the resulting mixture was stirred for 3.5 h. The organic phase was washed three times with 1 M HCl, then three times with saturated aq $NaHCO_3$, and finally three times with saturated aq NaCl. After drying over $MgSO_4$ and filtration, the organic solvent was removed in vacuo. The crude product was subsequently purified by chromatography over silica gel eluting with dichloromethane/methanol (35:1). After evaporation of the solvent, the hybrid product was isolated as a colorless solid.

***N*-Benzyloxycarbonyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic Acid Methyl Ester 2a.** A portion of 223 mg (1.00 mmol, 1.00 equiv) of *N*-benzyloxycarbonyl-(*S*)-alanine gave 308 mg (660 μmol, 66%) of product **2a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.43 (d, ^3J = 7.1 Hz, 3H), 3.75 (s, 3H), 3.85 (s, 3H), 4.45 (brs, 1H), 5.11 (d, ^2J = 12.2 Hz, 1H), 5.16 (d, ^2J = 12.2 Hz, 1H), 5.47 (d, ^3J = 6.8 Hz), 5.57 (s, 2H), 6.79 (d, ^3J = 8.7 Hz, 2H), 7.17 (d, ^3J = 8.7 Hz, 2H), 7.25−7.41 (m, 6H), 8.87 (brs, 1H). MS (ESI): m/z = 489 $(M + Na)^+$. HRMS: calcd for $C_{24}H_{26}O_6N_4Na$, 489.1745; found, 489.1743; R_f 0.48 in ethyl acetate.

General Procedure B for *N*-Terminal *Z*-Deprotection from Hybrid Peptides with One Amino Acid. The N-terminally *Z*-protected hybrid peptide (∼500 μmol, 1.00 equiv) was dissolved in methanol (25−50 mL), treated with 5 mol % of Pd/C (70 mg, 0.03 mmol, 0.07 equiv, Degussa Type E101 NE/W), and stirred under a hydrogen atmosphere for 16 h. The reaction mixture was filtered over celite or silica gel and washed three times with methanol, and the organic solvent was evaporated to dryness. The crude product was purified by chromatography over silica gel eluting with dichloromethane/methanol (10:1).

(*S*)-Alaninyl-5-amino-2-(4-methoxybenzyl)-2H-pyrazole-3-carboxylic Acid Methyl Ester 3a. A portion of 296 mg (635 μmol, 1.00 equiv) of *N*-benzyloxycarbony-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester **(2a)** gave 178 mg (536 μmol, 85%) of **2b** as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.42 (d, ^3J = 7.1 Hz, 3H), 3.74 (s, 3H), 3.83 (m, 4H), 5.58 (s, 2H), 6.80 (d, ^3J = 8.7 Hz, 2H), 7.17 (d, ^3J = 8.7 Hz, 2H), 7.26 (s, 1H), 9.97 (brs, 1H). MS (ESI): m/z = 333 $(M + H)^+$. HRMS: calcd for $C_{16}H_{21}O_4N_4$, 333.1557; found, 333.1560. R_f 0.12 in ethyl acetate.

General Procedure C for Coupling of *Z*-Protected Amino Acids with Hybrid Peptides (Amino Acid−Aminopyrazole Methyl Carboxylate) at Their Free N-Termini. *N*-Benzyloxycarbonyl-(*S*)-amino acid (1.00 mmol, 1.00 equiv) was dissolved under argon in a 3:1 mixture of dry dichloromethane and DMF (5.0 mL) and treated with HCTU (414 mg, 1.00 mmol, 1.00 equiv), Cl-HOBt (424 mg, 2.50 mmol, 2.50 equiv), and 2,6-lutidine (349 μL, 3.00 mmol, 3.00 equiv). For preactivation, this mixture was stirred in an ice bath for 30 min. In a separate flask, the respective hybrid peptide (1.00 mmol, 1.00 equiv) was dissolved under argon in the same solvent mixture (2.0 mL) and treated with 2,6-lutidine (116 μL, 1.00 mmol, 1.00 equiv). Both solutions were combined, and the resulting mixture was stirred for 3.5 h. The organic phase was washed three times with 1 M HCl, then three times with saturated aq $NaHCO_3$, and finally three times with saturated aq NaCl. After drying over $MgSO_4$ and filtration, the organic solvent was removed in vacuo. The crude product was subsequently purified by chromatography over silica gel eluting with dichloromethane/methanol (35:1). After evaporation of the solvent, the hybrid product was isolated as a colorless solid.

***N*-Benzyloxycarbonyl-*N*-*tert*-butyloxycarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic Acid Methyl Ester 4a.** A portion of 188 mg (493 μmol, 1.10 equiv) of *N*-benzyloxycarbonyl-*N*-*tert*-butyloxycarbonyl-(*S*)-lysine and 149 mg (448 μmol, 1.00 equiv) of (*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester (**3a**) gave 313 mg (487 mmol, 99%) of **4a** as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.23−1.57 (m, 16H), 1.59−1.68 (m, 1H), 1.86−1.93 (m, 1H), 2.97−3.04 (m, 2H), 3.72 (s, 3H), 3.84 (s, 3H), 4.23 (brs, 1H), 4.72−4.79 (m, 1H), 5.11 (s, 2H), 5.49 (d, ^2J = 8.1 Hz, 1H), 5.59 (d, ^2J = 8.1 Hz, 1H), 6.20 (d, ^3J= 14.7 Hz, 2H), 6.74 (d, ^3J = 14.6 Hz, 2H), 6.92 (d, ^3J = 8.1 Hz, 1H), 7.11 (d, ^3J = 8.6 Hz, 2H), 7.25−7.42 (m, 6H), 9.59 (s, 1H). MS (ESI): m/z = 595 $(M + H)^+$, 717 $(M + Na)^+$. HRMS: calcd for $C_{35}H_{46}N_6O_9Na$, 717.3218; found 717.3205. R_f 0.49 in ethyl acetate.

General Procedure D for N-Terminal *Z*-Deprotection from Hybrid Peptides with Two Consecutive Amino Acids. The *N*-terminally *Z*-protected hybrid peptide (∼500 μmol, 1.00 equiv) was dissolved in methanol (25 mL), treated with 5 mol % of Pd/C (70 mg, 0.033 mmol, 0.07 equiv, Degussa Type E101 NE/W), and stirred under a hydrogen atmosphere for 16 h. The reaction mixture was filtered over celite or silica gel, and the organic solvent was evaporated to dryness. The crude product was purified by chromatography over silica gel eluting with dichloromethane/methanol (10:1). In some cases, a greenish impurity precipitated on dissolving the purified compound in methanol for an NMR sample. This procedure was subsequently used for further purification and explains the suboptimal yields in two cases.

***N*-*tert*-Butyloxycarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic Acid Methyl Ester (5a).** A portion of 293 mg (422 μmol, 1.00 equiv) of *N*-benzyloxycarbonyl-*N*-*tert*-butyloxy-carbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester (**4a**) gave 176 mg (314 mmol, 74%) of **5a** as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.25−1.51 (m, 16H), 1.52−1.76 (m, 1H), 1.76−1.93 (m, 1H), 3.02−3.12 (m, 2H), 3.61−3.71 (m, 1H), 3.72 (s, 3H), 3.81 (s, 3H), 4.63−4.71 (m, 1H, α-H), 4.86 (brs, 1H, α-H), 5.56 (s, 2H), 6.76 (d, ^3J = 8.5 Hz, 2H), 7.13 (d, ^3J = 8.5 Hz, 2H), 7.22 (s, 1H), 8.12 (s, 1H), 9.50 (s, 1H). MS

(ESI): $m/z = 561$ $(M + H)^+$, 583 $(M + Na)^+$. HRMS: calcd for $C_{27}H_{41}N_6O_7$, 561.3031; found, 561.3027. R_f 0.05 in ethyl acetate.

General Procedure E for Coupling of PMB- and Glycole-Protected Aminopyrazole Methyl Carboxylates with Hybrid Peptides at Their Free N-Termini. The respective side chain and nucleus-protected hybrid peptide (~0.50 mmol, 1.00 equiv) was dissolved in dry dichloromethane (10 mL) under argon and treated with 2-(4-methoxybenzyl)-5-{2-[2-(2-methoxy-ethoxy)-ethoxy]-acetylamino}-2*H*-pyrazole-3-carboxylic acid (**6**, 1.05 equiv), 3-chloro-1-methylpyridinium iodide (Mukaiyama's reagent, 1.20 equiv), and diisopropylethylamine (DIEA, 3.00 equiv). The reaction mixture was stirred at ambient temperature for 16 h. Subsequently, the organic layer was washed three times with 1 M HCl, three times with saturated aq $NaHCO_3$, and finally three times with saturated aq NaCl. After drying over $MgSO_4$ and filtration, the solvent was evaporated to dryness. The resulting crude product was purified by chromatography over silica gel eluting with dichloromethane/methanol 50:1 → 10:1 (gradient elution).

2-[2-(2-Methoxy-ethoxy)-ethoxy]-acetyl-*N*-2-(4-methoxybenzyl)-2*H*-pyrazolcarboxyl-*N*-*tert*-butyloxycarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazol-3-carbonsäuremethyl Ester 7a. A portion of 176 mg (313 μmol, 1.00 equiv) of *N*-*tert*-butyloxycarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester (**5a**) and 134 mg (330 μmol, 1.05 equiv) of 2-(4-methoxybenzyl)-5-{2-[2-(2-methoxy-ethoxy)-ethoxy]-acetylamino}-2*H*-pyrazole-3-carboxylic acid (**6**) gave 195 mg (205 μmol, 62%) of **7a** as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.23 (d, $^3J = 6.6$ Hz, 3H), 1.24−1.48 (m, 13H), 1.62−1.77 (m, 1H), 1.77−1.93 (m, 1H), 2.88−3.06 (m, 2H), 3.30 (s, 3H), 3.52−3.71 (m, 8H), 3.71, 3.72 (2s, 6H), 3.83 (s, 3H), 4.11 (d, $^2J = 16.4$ Hz, 1H), 4.19 (d, $^2J = 16.2$ Hz, 1H), 4.48−4.60 (m, 1H), 4.73−4.98 (m, 2H), 5.46−5.69 (m, 4H), 6.75, 6.76 (2d, $^3J = 8.8$ Hz, $^3J' = 8.6$ Hz, 4H), 7.06, 7.18 (2d, $^3J = 8.8$ Hz, $^3J' = 8.6$ Hz, 4H), 7.27, 7.35 (2s, 2H), 8.09 (d, $^3J = 8.1$ Hz, 1H), 9.28, 10.13 (2s, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ [ppm] = 17.6, 22.06, 27.4, 28.5, 29.7, 48.0, 50.9, 52.2, 52.5, 52.8, 54.1, 57.9, 69.1, 69.3, 69.6, 70.3, 70.8, 77.9, 97,9, 101.7, 112.7, 112.8, 127.5, 127.7, 128.1, 128.1, 128.5, 131.0, 133.6, 143.6, 144.8, 155.0, 158.0, 158.1, 159.0, 167.4, 169.3, 170.5. MS (ESI): $m/z = 950$ $(M + H)^+$, 972 $(M + Na)^+$, 988 $(M + K)^+$. HRMS: calcd for $C_{46}H_{63}N_9O_{13}$, 972.4438; found, 972.4424. R_f 0.12 in ethyl acetate.

General Procedure F for Total Deprotection of All Hybrid Peptides in One Step with Hot TFA. A portion of ~200 μmol (1.00 equiv) of the side chain and nucleus-protected complete hybrid peptide was dissolved in dry trifluoroacetic acid (2.0 mL, HPLC grade) and stirred under argon for 3 h at 70 °C. Subsequently, the solution was cooled to 0 °C and treated with ice-cold diethyl ether (~25 mL) in a Falcon tube. The precipitating colorless solid was centrifuged off, washed three times with cold diethyl ether, and dried.

2-[2-(2-Methoxy-ethoxy)-ethoxy]-acetylaminopyrazolecarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-aminopyrazolecarboxylic Acid Methyl Ester Trifluoroacetate 8a. A portion of 185 mg (195 μmol, 1.00 equiv) of 2-[2-(2-methoxy-ethoxy)-ethoxy]-acetyl-*N*-2-(4-methoxybenzyl)-2*H*-pyrazolecarbonyl-*N*-*tert*-butyloxycarbonyl-(*S*)-lysinyl-(*S*)-alaninyl-5-amino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid methyl ester (**7a**) gave 79 mg (83 μmol, 43%) of **8a** as a colorless solid. ^{1}H NMR (300 MHz, CD_3OD): δ [ppm] = 1.45 (d, $^3J = 7.1$ Hz, 3H), 1.47−1.58 (m, 2H), 1.60−1.78 (m, 2H), 1.80−1.89 (m, 1H), 1.77−1.93 (m, 1H), 2.89−3.02 (m, 2H), 3.34 (s, 3H), 3.52−3.79 (m, 8H), 3.89 (s, 3H), 4.18 (s, 2H), 4.43−4.66 (m, 2H), 6.90, 6.95 (2s, 2H). ^{13}C NMR (500 MHz, $CDCl_3$): δ [ppm] = 17.9, 23.3, 23.6, 28.1, 29.1, 32.6, 32.8, 40.5, 50.8, 52.5, 52.7, 54.2, 59.0, 71.3, 71.3, 72.2, 72.8, 96.8, 100.3, 161.6, 163.6, 170.8, 172.9, 173.7. MS (ESI): $m/z = 610$ $(M + H)^+$, 632 $(M + Na)^+$, 648 $(M + K)^+$. HRMS: calcd for $C_{25}H_{40}N_9O_9$, 610.29; found, 610.29.

For the related solution-phase synthesis of the type 2 hybrid peptides Glycole−Pz−K−V−Pz−OMe **8e** and Glycole−Pz−K−K−Pz−OMe **8f**, see ref 29.

II. Automated Solid-Phase Synthesis Protocols of Type 1−4 Hybrid Compounds: Type 1 Hybrid Compounds Acyl−Pz−Pz−AA1−AA2−AA3, General Procedure H. The complete synthesis was performed with the Advanced ChemTech model Apex396. Nonpreloaded Wang-resin with an average loading of 1.2 mmol/g was used as the polymeric carrier. Prior to the first coupling step, the resin was swollen in DMF (2 mL) for 2 h. The critical coupling steps with Fmoc amino acids employed coupling reagents TBTU and HOBt and DIEA as a base. Each coupling required 4.0 equiv of Fmoc amino acid, 4.0 equiv of TBTU, 4.0 equiv HOBt, and 8.0 equiv of DIEA. The first amino acid as well as the last building block 3-{[5-acetylamino-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carbonyl]-amino}-2-(4-methoxybenzyl)-2*H*-pyrazole-3-carboxylic acid **9**[7] were doubly coupled. Removal of the Fmoc protecting group was effected with 20% piperidine in DMF (2 × 10 min). Each coupling and Fmoc deprotection was followed by washing the resin with dry DMF.

The peptide was cleaved off the resin concomitant with deprotection of lysine's ϵ-amino-Boc groups via 3-fold treatment with an acidic cleavage cocktail (95% TFA, 2.5% water, and 2.5% TIPS). In the first step, 400 μL of this cocktail was added to the reaction vessel, shaken for 45 min, and filtered off. This procedure was repeated twice (300 μL, 45 min; 200 μL, 15 min).

By bubbling N_2 through the solution, the TFA volume was reduced to ~0.3 mL. It was then cooled to 0 °C, and the PMB-protected hybrid peptide was precipitated with cold diethyl ether. For complete PMB removal, the crude intermediate was redissolved in TFA (2.0 mL) and heated for 2 h with stirring to 70 °C. The solution was cooled in an ice bath and pipetted into cold diethyl ether for precipitation of the final product, which was centrifuged off. The colorless solid was taken up three times in diethyl ether, vortexted, and again centrifuged, to separate impurities.

5-Acetylamino-2*H*-pyrazole-3-carbonyl-amino-2*H*-pyrazole-3-carbonyl-(*S*)-lysinyl-(*S*)-phenylalaninyl-(*S*)-phenylalanine Trifluoroacetate 10a. The NMR spectrum revealed incomplete coupling of the first amino acid Phe and formation of major byproduct **11a**, which could be sparated from **10a** by HPLC chromatography (125/4 Nucleodur 100−5 C-18, Macherey and Nagel). Thus, 48 μg (68 nmol) of **10a** and 979 μg (1.77 mmol) of **11a** were obtained in pure form (MS detection). MALDI-TOF: $m/z = 701$ (**10a** + H)$^+$, 554 (**11a** + H)$^+$.

For the solution synthesis of Ac−Pz−Pz−K−V−F−OMe **10b**, see ref 29.

Type 3 Hybrid Compounds Glycole−Pz−AA1−AA2−AA3−AA4−Pz−OH, General Procedure G. The complete synthesis was performed with the Advanced ChemTech model Apex396. Nonpreloaded Wang-resin with an average loading of 1.2 mmol/g was used as the polymeric carrier. The critical coupling steps with Fmoc amino acids employed coupling reagents HCTU and Cl-HOBt and DIEA as a base. Each coupling required 4.0 equiv of Fmoc amino acid, 4.0 equiv of HCTU, 4.0 equiv of Cl-HOBt, and 8.0 equiv of DIEA. Removal of the Fmoc protecting group was effected with 20% piperidine in DMF (2 × 10 min). Each coupling and Fmoc deprotection was followed by washing the resin with dry DMF.

Prior to the first coupling, the resin was swollen for 2 h, to optimize the accessability of all styrene groups and circumvent sterical problems. The first building block *N*-4-methoxybenzyl-3-(9-fluorenylmethoxycarbonyl)-aminopyrazole-5-carboxylic acid **(12)** was attached to the resin via double coupling (coupling time 2.5 h). Due to the low nucleophilicity of the free aromatic aminopyrazole amino group, the first amino acid was also introduced via a double coupling protocol with extended coupling times of ~2.5 h. The other three amino acids were coupled under standard conditions

(29) Rzepecki, P.; Schrader, T. *J. Am. Chem. Soc.* **2005**, *127*, 3016−3025.

within 1 h. Finally, another double coupling (2.5 h) elongated the growing hybrid molecule by the 2-(4-methoxybenzyl)-5-{2-[2-(2-methoxy-ethoxy)-ethoxy]-acetylamino}-2*H*-pyrazole-3-carboxylic acid fragment **(6)**. The peptide was cleaved off the resin concomitant with deprotection of lysine's ϵ-amino-Boc groups via 3-fold treatment with an acidic cleavage cocktail (95% TFA, 2.5% water, and 2.5% TIPS). In the first step, 400 μL of this cocktail was added to the reaction vessel, shaken for 45 min, and filtered off. This procedure was repeated twice (300 μL, 45 min; 200 μL, 15 min).

By bubbling N_2 through the solution, the TFA volume was reduced to ~0.3 mL. It was then cooled to 0 °C, and the PMB-protected hybrid peptide was precipitated with cold diethyl ether. For complete PMB removal, the crude intermediate was redissolved in TFA (2.0 mL) and heated for 2 h with stirring to 70 °C. The solution was cooled in an ice bath and pipetted into cold diethyl ether for precipitation of the final product, which was centrifuged off. The colorless solid was taken up three times in diethyl ether, vortexted, and again centrifuged, to separate impurities.

2-[2-(2-Methoxy-ethoxy)-ethoxy]-acetylaminopyrazolecarbonyl-(*S*)-lysinyl-(*S*)-leucinyl-(*S*)-valinyl-(*S*)-phenylalaninyl-aminopyrazolecarboxylic Acid Trifluoroacetate 13a. Overall yield 75%. ^{1}H NMR (500 MHz, CD_3OD): δ [ppm] = 0.78 (d, ^3J = 6.6 Hz, 3H), 0.82 (d, ^3J = 6.6 Hz, 3H), 0.88 (d, ^3J = 6.4 Hz, 3H), 0.93 (d, ^3J = 6.4 Hz, 3H), 1.41−1.73 (m, 10H), 2.81−2.96 (m, 2H), 2.83−3.23 (m, 2H), 3.35 (s, 3H), 3−54-3.81 (m, 8H), 4.18 (s, 2H), 4.38−4.51 (m, 1H), 4.52−4.59 (m, 1H, α-H), 4.59−4.68 (m, 2H, α-H's), 6.88 (brs, 2H), 7.06−7.28 (m, 5H). ^{13}C NMR (500 MHz, $CDCl_3$): δ [ppm] = 18.7, 19.8, 21.9, 23.5, 25.9, 28.1, 32.2, 32.6, 39.1, 40.6, 41.6, 59.1, 71.3, 71.4, 72.2, 72.8, 127.8, 129.5, 129.5, 130.3, 130.4, 138.2, 170.8, 173.0. MS (ESI): *m*/*z* = 922 $(M + K)^+$. HRMS: calcd for $C_{41}H_{61}N_{11}O_{11}$, 922.4752; found, 922.4757.

Type 4 Hybrid Compounds AA1−AA2−Pz−Pz−Pz−OMe, General Procedure I. The synthesis followed a very similar protocol to the above-described type 3 hybrid peptides. In this case glycine-preloaded Wang-resin was used, with an average loading of 0.8 mmol/g. All pyrazole building blocks and the first lysine were doubly coupled for 2.5 h.

Automated Solid-Phase Synthesis Protocols for KKLVFF, Dansyl−KKLVFF, and KKLVFFAK. (a) (*S*)-Lysinyl-(*S*)-lysinyl-(*S*)-leucinyl-(*S*)-valinyl-(*S*)-phenylalaninyl-(*S*)-phenylalanine Trifluoroacetate 17a. The complete synthesis was performed with the Advanced ChemTech model Apex396. Nonpreloaded Wang-resin with an average loading of 1.2 mmol/g was used as the polymeric carrier. The first Phe was attached to the resin by a double coupling step. Coupling reagents were HOBt/HBTU or HCTU/Cl-HOBt. Release of the peptide from the resin was affected with the above-decribed cleavage cocktail (95% TFA, 2.5% water, and 2.5% TIS).

By bubbling N_2 through the solution, the TFA volume was reduced to ~0.3 mL. It was then cooled to 0 °C, and the PMB-protected hybrid peptide was precipitated with cold diethyl ether. The colorless solid was taken up three times in diethyl ether, vortexted, and again centrifuged, to separate impurities. ^{1}H NMR (500 MHz, CD_3OD): δ [ppm] = 0.78 (d, ^3J = 6.6 Hz, 3H), 0.83 (d, ^3J = 6.6 Hz, 3H), 0.88 (d, ^3J = 6.4 Hz, 3H), 0.93 (d, ^3J = 6.4 Hz, 3H), 1.27−2.03 (m, 16H), 2.80−2.98 (m, 4H), 2.98−3.21 (m, 4H), 3.96 (brs, 1H), 4.16 (d, 1H, ^3J = 7.6 Hz), 4.36−4.50 (m, 2H), 4.55−4.71 (m, 2H), 7.12−7.30 (m, 10H), 7.88 (d, 1H, ^3J = 6.4 Hz, amide-H), 8.16, 8.42, 8.66 (3brs, 3H, amide-H). MS (ESI): *m*/*z* = 782 $(M + H)^+$, 804 $(M + Na)^+$, 820 $(M + K)^+$. HRMS: calcd for $C_{41}H_{65}N_8O_7$, 781.4971; found, 781.4965.

(b) (*S*)-Lysinyl-(*S*)-lysinyl-(*S*)-leucinyl-(*S*)-valinyl-(*S*)-phenylalaninyl-(*S*)-phenylalaninyl-(S)-alaninyl-(S)-lysine Trifluoroacetate 17b. The synthesis was performed on the Advanced ChemTech model Apex396. Nonpreloaded chlorotrityl-chloride resin with an average loading of 1.4 mmol/g was used as the polymeric carrier. Couplings were carried out with 3.0 equiv of Fmoc amino acid, 3.0 equiv of HBTU, 6.0 equiv of HOBt, and 6.0 equiv of DIEA. Coupling times were ~1 h; prior to each coupling step and after each Fmoc deprotection, the resin was washed with DMF. The Fmoc group was mildly removed with 20% piperidine in DMF (2 × 10 min). Peptide release from the resin was affected with the cleavage cocktail (95% TFA, 2.5% water, and 2.5% TIS). The mixture was cooled to 0 °C, and the PMB-protected hybrid peptide was precipitated with cold diethyl ether. ^{1}H NMR (500 MHz, CD_3OD): δ [ppm] = 0.77 (d, ^3J = 6.6 Hz, 3H), 0.83 (d, ^3J = 6.6 Hz, 3H), 0.88 (d, ^3J = 6.4 Hz, 3H), 0.93 (d, ^3J = 6.4 Hz, 3H), 1.38 (d, 3H, ^3J = 7.0 Hz), 1.43−2.07 (m, 22H), 2.78−3.19 (m, 10H), 3.89−3.99 (m, 1H), 4.06−4.17 (m, 1H), 4.28−4.46 (m, 4H), 4.52−4.68 (m, 2H), 7.13−7.33 (m, 10H). MS (ESI): *m*/*z* = 980 $(M + H)^+$. HRMS: calcd for $C_{50}H_{82}N_{11}O_9$, 980.6291; found, 980.6292.

(c) [5-Dimethylamino-naphthyl-1-sulfonic acid]-(*S*)-lysinyl-(*S*)-lysinyl-(*S*)-leucinyl-(*S*)-valinyl-(*S*)-phenylalaninyl-(*S*)-phenylalaninyl-(2-amino-ethyl)-amide Trifluoroacetate 17c. The synthesis was performed on the Advanced ChemTech model Apex396. Dansyl NovaTag resin with an average loading of 0.59 mmol/g was used as the polymeric carrier. The synthesis followed a very similar protocol as that for **17a**. All amino acids were coupled twice. Coupling reagents were HCTU/Cl-HOBt. Peptide release from the resin was affected with the cleavage cocktail (95% TFA, 2.5% water, and 2.5% TIS). The mixture was cooled to 0 °C, and the PMB-protected hybrid peptide was precipitated with cold diethyl ether. ^{1}H NMR (500 MHz, CD_3OD): δ [ppm] = 0.79 (d, ^3J = 6.6 Hz, 3H), 0.83 (d, ^3J = 6.7 Hz, 3H), 0.87 (d, ^3J = 6.4 Hz, 3H), 0.92 (d, ^3J = 6.4 Hz, 3H), 1.35−2.07 (m, 16H), 2.60−3.22 (m, 18H), 3.95 (t, 1H, ^3J = 6.2 Hz), 4.13 (d, 1H, ^3J = 7.4 Hz), 4.34−4.49 (m, 3H), 4.57 (d, ^3J = 5.9 Hz, 1H, Phe α-H_α), 4.60 (d, ^3J = 5.9 Hz, 1H, Phe α-H_β), 7.03−7.23 (m, 10H), 7.27 (d, ^3J = 7.4 Hz, 1H, Naphthyl-H), 7.57 (d, ^3J = 7.6 Hz, 1H, Naphthyl-H), 7.60 (d, ^3J = 7.6 Hz, 1H, Naphtalin-H), 8.18 (d, ^3J = 7.4 Hz, 1H, Naphtalin-H), 8.32 (d, ^3J = 8.5 Hz, 1H, Naphthyl-H), 8.58 (d, ^3J = 8.5 Hz, 1H, Naphthyl-H). MS (ESI): *m*/*z* = 1057 $(M + H)^+$, 1079 $(M + Na)^+$. HRMS: calcd for $C_{55}H_{82}N_{11}O_8S$, 1056.6063; found, 1056.6043.

Synthesis of PAM-Immobilized Aminopyrazole Ligands 25−27 Employing the Boc Strategy. Immobilized 3-{[5-({5-[(5-Amino-1*H*-pyrazole-3-carbonyl)-amino]-1*H*-pyrazole-3-carbonyl}-amino)-1*H*-pyrazole-3-carbonyl]-amino}-propionyl-PAM (PAM-β-Ala−Pz−Pz−Pz) 25. For these ligands, a manual solid-phase synthesis protocol was employed. PAM resin was used as a polymer support, with an average loading of 0.8 mmol/g. Coupling of Boc-protected amino acids was accomplished with HATU/HOAt and lutidine according to the following method: For each coupling step [5-*tert*-butoxycarbonylamino-2-(4-methoxybenzyl)-2*H*-pyrazol-3-yl]-carboxylic acid **24** (5.0 equiv),[22] HATU (4.5 equiv), HOAt (12.5 equiv), and lutidine (15 equiv) were used in DMF solution. To this end, the aminopyrazole carboxylic acid (**24**) was preactivated ~10 min prior to the peptide coupling in an ultrasound bath with HATU/HOAt and lutidine in DMF in a brown glass flask. Removal of the Boc-protecting group occurs by treatment with TFA (1.6 mL), dichloromethane (400 μL), and triisopropylsilane (100 μL, 1 × 5 min, 1 × 20 min). The resin was washed thoroughly with dichloromethane before and after each deprotection step. To cleave the PMB protecting groups on the pyrazole nucleus, the resin was heated under argon in dry TFA for 2.5 h to 70 °C. The solution was subsequently filtered, and the resin was washed with dichloromethane and dried in vacuo. The complete protocol is listed in a summarizing table, found in the Supporting Information.

On-Bead Assay: Calculation of Binding Constants. The on-bead assays were conducted under rigorous light exclusion. Solvents were dry dichloromethane, acetonitrile (HPLC-grade), bidistilled water, and sodium phosphate buffer (pH 7.0). A defined amount of resin was weighed into an Eppendorf tube (870 μg−5.50 mg) and treated with a defined amount of a 40 μM solution of fluorescence-labeled peptide **17d** (ratio ligand/peptide = 100:1). Usually, [the amount of resin with a loading of 0.8 mmol/g in

milligrams] × 200 = the amount of the solution in microliters. In all cases, two to three samples of immobilized **25**−**27**, two samples of nonfunctionalized Boc-β-alanine PAM resin, and one sample without a polymer were preincubated overnight under light exclusion at 20 °C. The last mentioned sample served as a reference; it revealed potential unspecific fluorescence intensity changes because it was treated under the same conditions as all resin-containing samples. To achieve an optimal distribution of polymer beads, each solution was treated with an ultrasound for 30 s under light exclusion prior to incubation. After 16−18 h the solutions were pipetted off the resin, and the remaining fluorescence intensity was measured. The labeled peptide was measured at various concentrations in the appropriate solvent to obtain a reference curve.

Instrumental Values. Excitation wavelength: 310 nm. Measurement range: 450−600 nm. Data pitch: 1 nm. Scanning speed: 1000 nm/min. Response: medium. Sensitivity: medium. Bandwidth(Ex): 5 nm. Bandwidth(Em): 10 nm.

From the fluorescence intensities of peptide reference and solutions after incubation, the association constant can be calculated, if a 1:1 complex is formed.

$$K_{ass} = \frac{[R - S]}{[R][S]}$$

[S]: Concentration of unbound substrate which can be determined from the remaining fluorescence intensity of the solution with a reference curve.

[R − S]: Complex concentration which is obtained from the difference between original fluorescence intensity and fluorescence intensity after incubation, $[R - S] = [S_0] - [S]$.

[R]: Concentration of the free receptor which can be calculated from the employed amount of receptor (amount of resin, loading, and correction factor for the molar mass of the bound receptor) and the concentration of the receptor−substrate complex.

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Supporting Information Available: Full characterization of compounds **2b**−**20**. Detailed information about the solid-phase synthesis protocol for **25**−**27** on the PAM resin. Scheme 1: HPLC traces for **25-PMB** and free **25**. ^{1}H NMR spectra of reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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