

Combinatorial Synthesis

A Combinatorial Approach to Catalytic Peptide Dendrimers**

*Anthony Clouet, Tamis Darbre, and
Jean-Louis Reymond**

In de novo protein design one attempts to create artificial proteins with defined structure and function from first principles, usually with the help of trial-and-error procedures

[*] A. Clouet, Priv.-Doz. Dr. T. Darbre, Prof. Dr. J.-L. Reymond
Department of Chemistry and Biochemistry
University of Bern
Freiestrasse 3, 3012 Bern (Switzerland)
Fax: (+41) 31-631-8057
E-mail: jean-louis.reymond@ioc.unibe.ch

[**] This work was financially supported by the University of Bern and the Swiss National Science Foundation. We thank Mr. Urs Kämpfer of the Protein Analytical Service of the Department of Chemistry and Biochemistry, University of Bern, for performing the amino acid analyses.



Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

that scan a large number of possible amino acid sequences.^[1] Our approach to de novo protein design is based on peptide dendrimers. Dendrimers are treelike structures that adopt a globular or disk-shaped structure as a consequence of topology rather than folding.^[2] We have shown that peptide dendrimers that bear histidine residues at the surface catalyze ester hydrolysis in water.^[3] Herein we report a combinatorial approach to peptide dendrimers based on split-and-mix synthesis and on-bead screening. The method is exemplified by the discovery of catalytic and binding peptide dendrimers in a 65 536-membered library.

As peptide dendrimers are synthesized on solid supports, it should be possible to apply the principles of combinatorial peptide synthesis,^[4] which have been exploited successfully for peptide-based and small-molecule catalysts.^[5] To achieve this goal, we selected a dendrimeric architecture containing eight variable positions connected by three successive branching diamino acid units (Figure 1). Preliminary experi-

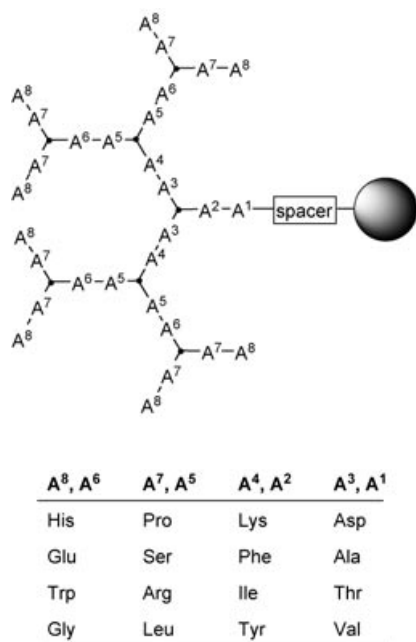


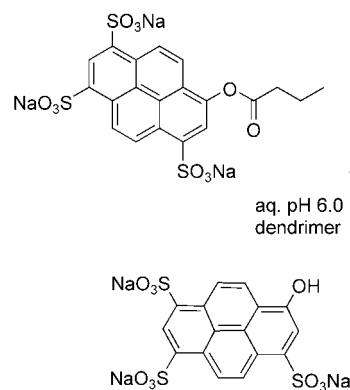
Figure 1. Combinatorial library of 65 536 ($=4^8$) peptide dendrimers obtained by solid-phase split-and-mix synthesis. ● (branching unit) = (S)-2,3-diaminopropanoic acid. Spacer = $\text{NH}(\text{CH}_2)_5\text{CONH}-\text{CH}_2\text{CONH}-$, and $\text{NH}(\text{CH}_2)_5\text{CONHCH}_2\text{CONH}_2$ after cleavage from the support for resynthesized dendrimers. The N terminus at position A⁸ is either acetylated (esterolysis studies) or free (vitamin B₁₂ binding studies). The solid support is tentagel.

ments showed that the sequence of eleven coupling steps to form such dendrimers gave good chemical yields for single sequences when using 2,3-diaminopropanoic acid as the branching unit.^[3b] A split-and-mix library of peptide dendrimers was designed by distributing sixteen proteinogenic amino acids into four groups of four amino acids each. The resulting 65 536-member library required splitting in four portions at each variable sequence step, whereby each group of four amino acids would be used at two positions in different branches of the dendrimer. The single-bead sequencing

problem was solved by taking advantage of the dendritic structure in which amino acids are present in one, two, four, or eight copies, depending on their placement in the different branches. As each amino acid was present at most at two positions in different branches, the sequence could be unambiguously assigned by quantitative amino acid analysis of the dendrimer.^[6]

The peptide-dendrimer library was prepared from a 400-mg resin batch, which ensured that each sequence would be present in approximately 15 beads. Aromatic, hydrophobic, positively charged, negatively charged, and small amino acids were distributed evenly among the inner positions A¹–A⁴ and the outer positions A⁵–A⁸. Histidine was placed in the outermost layer (A⁶ and A⁸) as surface placement of this residue was known to favor catalysis.^[3] The sequence started with an amino-hexanoyl-glycine spacer to minimize interactions with the solid support. All coupling steps were quantitative, as indicated by the negative TNBS (2,4,6-trinitrobenzenesulfonic acid) staining test. Side-chain protecting groups were removed with trifluoroacetic acid after cleavage of the Fmoc protecting groups and acetylation of the last amino acid, resulting in a functional dendrimer library on beads.

The peptide dendrimer library was screened to find catalytic peptide dendrimers for an ester-hydrolysis reaction. Activity screening was realized by soaking the beads in an aqueous buffered solution of a fluorogenic ester substrate, followed by decantation and spreading the slurry of beads onto a Petri dish. Whereas the solvent quickly evaporated at the glass surface, evaporation inside the beads was slow, so that each bead functioned as a separate microreactor. The procedure was successful for screening the hydrolysis of 8-butyryloxypropylene-1,3,6-trisulfonate, a known fluorogenic lipase substrate (Scheme 1). Approximately 60% of all



Scheme 1. Fluorogenic esterolysis reaction of 8-butyryloxypropylene-1,3,6-trisulfonate catalyzed by peptide dendrimers.

beads showed low-level fluorescence, among which very few beads (10 beads/40 mg resin) showed an intense green fluorescence that indicated product formation (see Supporting Information). These active beads were picked, washed thoroughly with buffer and water, and subjected to amino acid analysis (Table 1). Two consensus sequences and two original sequences were synthesized as single sequences and purified

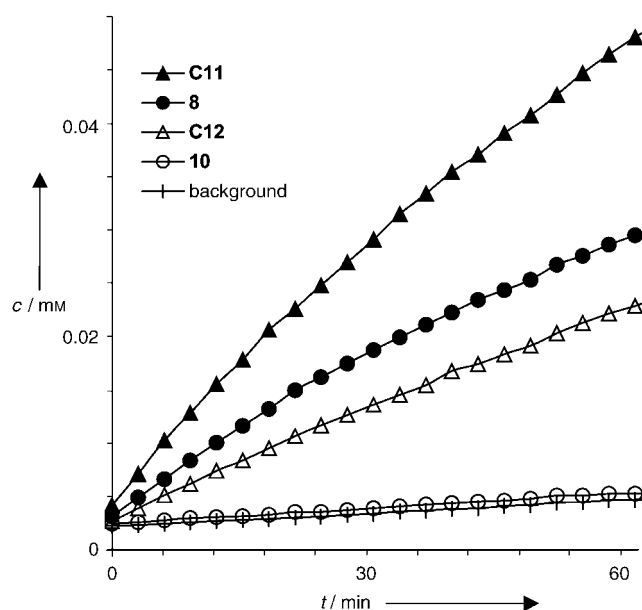
Table 1: Peptide dendrimer sequences identified by amino acid analysis of active beads from the combinatorial library^[6] and the corresponding consensus sequences.^[a]

	Dend.	A ⁸	A ⁷	A ⁶	A ⁵	A ⁴	A ³	A ²	A ¹
Hits for hydrolysis of 8-butyryloxypropyne-1,3,6-trisulfonate ^[b]	1	W	S	G	R	K	V	I	A
	2	H	L	H	S	Y	A	I	D
	3	H	L	G	L	Y	T	I	V
	4	H	P	G	P	K	T	I	A
	5	E	R	G	S	I	V	I	V
	6	G	R	W	R	I	V	I	A
	7	H	S	H	L	F	A	F	D
	8	H	S	G	R	I	A	I	V
	9	H	S	H	P	K	V	F	V
	10	G	R	G	P	I	V	I	V
consensus sequences	C11	H	S	H	L	K	V	I	V
	C12	H	S	G	S	I	V	I	V
Hits for binding to vitamin B ₁₂ ^[c]	13	W	L	H	S	I	A	K	A
	14	E	P	G	R	Y	T	Y	D
	15	W	P	E	S	Y	A	Y	D
	16	G	P	W	P	Y	V	K	V
	17	G	R	E	R	I	T	I	D
	18	H	L	G	R	K	V	K	D
	19	W	R	E	S	I	V	I	V
consensus sequences	C20	W	P	G	R	Y	V	Y	D
	C21	G	R	E	S	I	T	K	V

[a] Dendrimer structure according to Figure 1. Dendrimers marked bold were resynthesized by Fmoc-SPPS and purified. [b] All N termini N-acetylated. Screening conditions: Beads soaked in 20 mM aqueous Bis-Tris buffer containing 80 μ M 8-butyryloxypropyne-1,3,6-trisulfonate, 25 °C, 30 min. Hits are green-fluorescent under UV radiation (356 nm). [c] N termini are free amine. Screening conditions: 30 min equilibration in aq. PBS (10 mM phosphate, 160 mM NaCl, pH 7.4) containing 400 μ M cyanocobalamin (vitamin B₁₂), followed by washing with PBS and water. Hits are bright orange.

by preparative HPLC. Dendrimer **10**, which did not contain a histidine residue, was not active and represented a false positive.^[7] The three other dendrimers (**8**, **C11**, and **C12**) were catalytically active, and contained histidine at the outermost position A⁸ (Figure 2). These dendrimers displayed enzyme-like catalysis for the ester hydrolysis (Table 2) with multiple turnover and rate enhancement $k_{\text{cat}}/k_{\text{uncat}}$ up to 3000-fold over background and K_{M} values in the 0.05 mM range. In terms of apparent catalytic effect $V_{\text{net}}/V_{\text{uncat}}$, these dendrimers are 2–10 times better than those prepared previously by rational design.^[3]

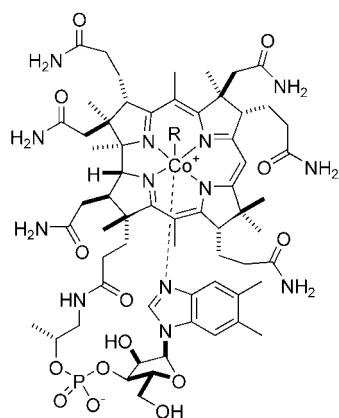
Activity screening of solid-supported combinatorial libraries for binding affinity can be readily carried out by bead staining with a colored ligand.^[4a] The dendrimer library was screened for binding to vitamin B₁₂, a corrinoid-type chromophore for which no artificial binding proteins have been described to date (Figure 3). Vitamin B₁₂-containing enzymes hold the vitamin by multiple hydrogen bonds to the carboxamide groups of the corrin ring.^[8] Equilibration with vitamin B₁₂ in aqueous neutral buffer resulted in staining of two to five beads per experiment (0.01 %). The experiment was repeated twice to give a total of seven colored beads, which were subjected to amino acid analysis (Table 1). Two original sequences and two consensus sequence were resynthesized, cleaved from the resin, and purified by preparative HPLC. In each case a small portion of noncleavable synthesis beads

**Figure 2.** Hydrolysis of 8-butyryloxypropyne-1,3,6-trisulfonate (Scheme 1) catalyzed by peptide dendrimers from the combinatorial library. Conditions: substrate (200 μ M), dendrimer (5 μ M), aqueous 20 mM Bis-Tris pH 6.0, 25 °C. The reactions were run in 96-well, polystyrene, half-area microtiter plates with subsequent detection on a SpectraMAX fluorescence detector with $\lambda_{\text{exc}} = 460$ nm, $\lambda_{\text{em}} = 530$ nm. Fluorescence was converted to product concentration by using a calibration curve, which was linear in the concentration range used.**Table 2:** Kinetic parameters for esterolytic peptide dendrimers from the combinatorial library.^[a]

	8	C11	C12
K_{M} (mM)	0.065	0.066	0.051
k_{cat} (min ⁻¹)	0.094	0.14	0.054
$k_{\text{cat}}/k_{\text{uncat}}$	2140	3100	1200
$k_{\text{cat}}/K_{\text{M}}/k_2$	1800	2540	1300
$V_{\text{net}}/V_{\text{uncat}}$	39	51	22

[a] Conditions: 50–800 μ M 8-butyryloxypropyne-1,3,6-trisulfonate, 5 μ M dendrimer, 20 mM aqueous Bis-Tris pH 6.0, 25 °C. The kinetic constants given are derived from the linear double-reciprocal plots of $1/V_{\text{net}}$ versus $1/[S]$. $V_{\text{net}}/V_{\text{uncat}}$ is the apparent rate enhancement observed with $S = 200$ μ M and 2.5 mol% catalytic peptide dendrimer (5 μ M). $V_{\text{net}} = V_{\text{app}} - V_{\text{uncat}}$ with V_{app} being the apparent hydrolysis rate in the presence of dendrimer and V_{uncat} the hydrolysis rate in buffer alone ($k_{\text{uncat}} = 4.4 \times 10^{-5}$ min⁻¹). $k_2 = 8.1 \times 10^{-4}$ mM⁻¹ min⁻¹ is the catalytic rate constant for hydrolysis by 4-methyl-imidazole under the same conditions.

were used in the synthesis, and these beads bound vitamin B₁₂ as observed during screening. Beads carrying a catalytic sequence were not stained under these conditions. Bead staining by vitamin B₁₂ was inhibited in the presence of > 0.5 mM of soluble purified dendrimer **C21**, but not with the same amount of the catalytic dendrimer **C10**, indicating a relatively weak, yet highly selective, binding interaction. While aquocobalamin also bound the dendrimers, there was no binding with cobinamide, which lacks the nucleotide loop, therefore suggesting that this structural element is involved in the dendrimer–vitamin B₁₂ interaction (Table 3).



vitamin B₁₂-cyano (R = CN)
vitamin B₁₂-aquo (R = OH)

Figure 3. Structure of vitamin B₁₂. In cobinamide the 3'-phosphonucleoside is absent and the cobalt center has two cyanide ligands.

Table 3: Interaction between dendrimers and vitamin B₁₂ derivatives.^[a]

Dendrimer	Vitamin B ₁₂ -cyano		Vitamin B ₁₂ -aquo		Cobinamide	
	400 μ M	100 μ M	400 μ M	100 μ M	400 μ M	100 μ M
19	+++	+	+++	++	—	—
14	+++	—	+++	++	—	—
C20	+++	—	+++	++	—	—
C21	+++	—	+++	++	—	—
C11	—	—	—	—	—	—

[a] Intensity of bead staining (orange/red coloration) after equilibration of solid-supported dendrimer with the given concentration of vitamin B₁₂ in aqueous phosphate buffer pH 7.4 for 30 min, followed by washing.

The experiments above demonstrate a general approach to functional peptide dendrimers through split-and-mix combinatorial libraries. Library design and the dendritic architecture allow single-bead sequencing by quantitative amino acid analysis, such that bead encoding^[9] is not necessary. Selection for catalysis was realized by soaking the beads with fluorogenic substrates and spreading on a dry surface, which allows direct selection for catalytic turnover. The procedure is, to our knowledge, new and is suitable for screening reactions with soluble fluorogenic or chromogenic substrates or sensor systems.^[10] The method provides a useful complement to previously reported on-bead screening methods based on the covalent attachment of substrate^[11] or sensor^[5c] to the beads, the use of precipitating dyes,^[5a] or IR thermography.^[12] The exploration of focused libraries should enable the refinement and improvement of the properties of the isolated dendrimers, and the exploration of further problems in binding and catalysis.

Received: March 31, 2004

Keywords: cobalamines · combinatorial chemistry · dendrimers · enzyme models · peptides

- [1] a) D. A. Moffet, M. H. Hecht, *Chem. Rev.* **2001**, *101*, 3191–3203; b) D. N. Bolon, C. A. Voigt, S. L. Mayo, *Curr. Opin. Chem. Biol.* **2002**, *6*, 125–129.
- [2] a) A. W. Bosman, H. M. Janssen, E. W. Meijer, *Chem. Rev.* **1999**, *99*, 1665–1688; b) D. K. Smith, *Tetrahedron* **2003**, *59*, 3797–3798; c) S. Hecht, J. M. J. Fréchet, *Angew. Chem.* **2001**, *113*, 76–94; *Angew. Chem. Int. Ed.* **2001**, *40*, 74–91; d) G. R. Newkome, C. N. Moorefield, F. Vögtle, *Dendritic Molecules: Concepts, Synthesis, Perspectives*, Wiley-VCH, Weinheim, **1996**.
- [3] a) A. Esposito, E. Delort, D. Lagnoux, F. Djojo, J. L. Reymond, *Angew. Chem.* **2003**, *115*, 1419–1421; *Angew. Chem. Int. Ed.* **2003**, *42*, 1381–1383; b) D. Lagnoux, E. Delort, C. Douat-Casassus, A. Esposito, J. L. Reymond, *Chem. Eur. J.* **2004**, *10*, 1215–1226.
- [4] a) K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82–84; b) R. A. Houghten, C. Pinilla, C. S. E. Blondelle, J. R. Appel, C. T. Dooley, J. H. Cuervo, *Nature* **1991**, *354*, 84–86.
- [5] a) A. Berkessel, *Curr. Opin. Chem. Biol.* **2003**, *7*, 409–419; b) C. Gennari, U. Piarulli, *Chem. Rev.* **2003**, *103*, 3071–3100; c) C. A. Evans, S. J. Miller, *Curr. Opin. Chem. Biol.* **2002**, *6*, 333–338; d) B. Linton, A. D. Hamilton, *Curr. Opin. Chem. Biol.* **1999**, *3*, 307–312; e) K. W. Kunz, M. L. Snapper, A. H. Hoveyda, *Curr. Opin. Chem. Biol.* **1999**, *3*, 313–319.
- [6] Single dendrimer-containing resin beads were hydrolyzed with aqueous HCl (6M) at 110 °C for 22 h, and their amino acid composition was determined quantitatively by HPLC after derivatization with phenyl isothiocyanate (PITC). Such amino acid analyses are routine for protein-composition analysis. The analysis detects as little as 5 pmol per amino acid, which is sufficient for single-resin-bead analysis, as these contain 50–200 pmol of dendrimers.
- [7] False positives are most likely due to manipulation errors during bead picking. Sometimes more than one bead is transferred to the pipette, and the fluorescence staining is diluted when the beads are washed down the pipette used for picking, which does not allow staining to be rechecked afterwards.
- [8] *Chemistry and Biochemistry of B₁₂* (Ed.: R. Banerjee), Wiley, New York, **1999**.
- [9] M. H. J. Ohlmeyer, R. N. Swanson, L. W. Dillard, J. C. Reader, G. Asouline, R. Kobayashi, M. H. Wigler, W. C. Still, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 10922–10926.
- [10] a) *Enzyme Assays: A Practical Approach* (Eds.: R. Eisinger, M. Danson) Oxford University Press, Oxford **2002**; b) S. Gul, S. K. Sreedharan, K. Brocklehurst, *Enzyme Assays: Essential Data*, Wiley, New York, **1998**; c) M. T. Reetz, *Angew. Chem.* **2001**, *113*, 292–320; *Angew. Chem. Int. Ed.* **2001**, *40*, 284–310; d) D. Wahler, J.-L. Reymond, *Curr. Opin. Chem. Biol.* **2001**, *5*, 152–158; e) F. Beisson, A. Tiss, C. Rivière, R. Verger, *Eur. J. Lipid Sci. Technol.* **2000**, *133*–153; f) D. Wahler, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2001**, *12*, 535–544.
- [11] a) P. Krattiger, C. McCarthy, A. Pfaltz, H. Wennemers, *Angew. Chem.* **2003**, *115*, 1763–1766; *Angew. Chem. Int. Ed.* **2003**, *42*, 1722–1724; b) I. Lingard, G. Bhalay, M. Bradley, *Chem. Commun.* **2003**, 2310–2311.
- [12] S. J. Taylor, J. P. Morken, *Science* **1998**, *280*, 267–270.