Organic & Biomolecular Chemistry

Dynamic Article Links

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 7071

www.rsc.org/obc

PAPER

Peptide dendrimer enzyme models for ester hydrolysis and aldolization prepared by convergent thioether ligation†

Nicolas A. Uhlich, Tamis Darbre and Jean-Louis Reymond*

Received 2nd June 2011, Accepted 20th July 2011
DOI: 10.1039/c1ob05877k

Peptide dendrimers with multiple histidines or N-terminal prolines efficiently catalyze ester hydrolysis or aldol reactions in aqueous medium. Part of the catalytic proficiency of these dendritic enzyme models stems from multivalency effects observed in G2, G3 and G4 dendrimers displaying multiple catalytic groups in their branches. To study multivalency in higher generation systems, G4, G5 and G6 peptide dendrimers were prepared by a convergent assembly. Thus, peptide dendrimers bearing four or eight chloroacetyl groups at their N-termini underwent multiple thioether ligation with G2 and G3 peptide dendrimers with a cysteine residue at their focal point, to give G4, G5 and G6 dendrimers containing up to 341 amino acids, including multiple histidines or N-terminal prolines. While the efficiency of the esterase catalysts was comparable to that of their lower generation analogs, a remarkable reactivity increase was observed in G5 and G6 aldolase dendrimers.

Introduction

Dendrimers are regularly branched synthetic macromolecules assembled from a variety of dendron building blocks, which display useful properties for technical and biomedical applications.[1] Dendrimers have been compared to proteins because of their roughly globular shape enforced by the dendritic topology. Peptide dendrimers[2] assembled from proteinogenic amino acids and branching diamino acids are particularly well suited to study dendritic analogs of proteins since they are composed of the same building blocks as natural proteins and only differ through their dendritic rather than linear topology. Peptide dendrimers can be obtained by solid phase peptide synthesis (SPPS) alternating standard α-amino acids with branching diamino acids.[3] This method is efficient up to G3 dendrimers with eight end-groups, and lends itself to combinatorial synthesis,[4] enabling the screening of relatively large libraries for function using either on-bead or off-bead assays for binding[5] or catalysis.[6] Upon resynthesis of library hits, the resulting dendrimers exhibit the expected functions, and are also surprisingly resistant towards proteolysis.[7]

Screening peptide dendrimer libraries for various catalytic reactions allowed us to identify peptide dendrimer enzyme models[8] for ester hydrolysis[9,10] and aldolization.[11] In both reaction types significant rate enhancements were observed by multivalency effects, in particular by cooperativity between multiple histidine residues for ester hydrolysis reactions, and between multiple N-terminal proline residues for aldol reactions. However, the limits of the divergent synthesis by SPPS precluded the systematic investigation of multivalency effects in higher generation systems.

Herein, we report on the properties of G4, G5 and G6 catalytic peptide dendrimers obtained by a one-step "dendrimers-on-dendrimer" ligation of peptide dendrimer building blocks themselves prepared by standard SPPS (Fig. 1). Thus, G2 or G3 peptide dendrimer "cores" functionalized with chloroacetyl groups on their four or eight N-termini underwent 4-fold or 8-fold thioether ligation with peptide dendrimer "arms" with a cysteine residue at their focal point, to form G4, G5 and G6 peptide dendrimers such as **G5P5** (Fig. 2). This approach is inspired by convergent approaches to dendrimers.[12] The resulting dendrimers display remarkable catalytic properties in aqueous media for their respective reactions, namely the hydrolysis of acetoxypyrene trisulfonate **1** and isobutyryl fluorescein **3** to give the fluorescent hydroxypyrene trisulfonate **2** and fluorescein **4**, and the aldol addition of acetone to 4-nitrobenzaldehyde **5** to give aldol **6** (Scheme 1).

Results and discussion

Ligation strategy

SPPS of peptide dendrimers gives access to peptide dendrimers up to G3 in excellent yields and purities, and is compatible with an extremely large functional diversity as given by the available amino acid building blocks, including various hydrophilic, hydrophobic, aromatic, basic and acidic functional groups. However SPPS is reliable only up to G3 dendrimers with eight end-groups and up to 40–50 amino acids. To access higher generation peptide dendrimers, we set out to investigate a "dendrimers-on-dendrimer"

Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, 3012 Berne, Switzerland. E-mail: jean-louis.reymond@ioc.unibe.ch

† Electronic supplementary information (ESI) available: HPLC/MS spectra and amino acid analysis data on all dendrimer building blocks and ligation products and additional experimental details. See DOI: 10.1039/c1ob05877k

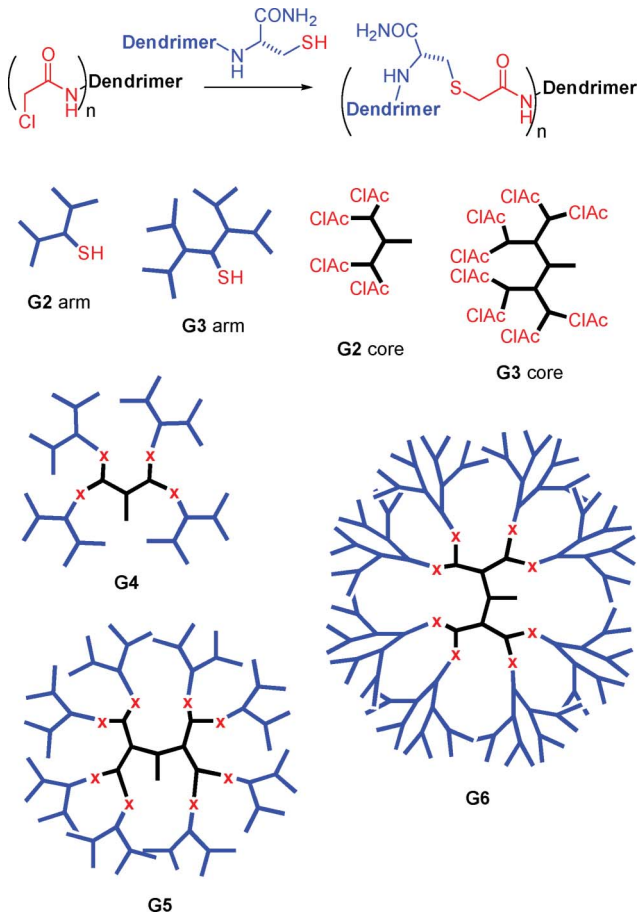
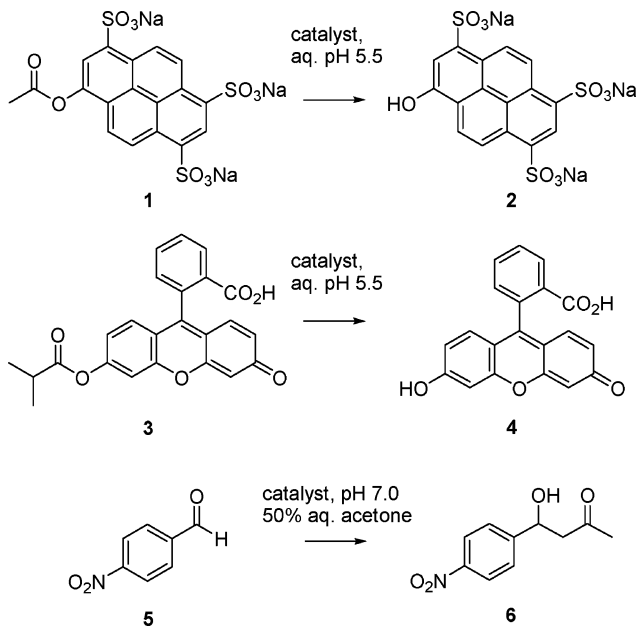


Fig. 1 Multivalent thioether ligation of G4, G5 and G6 enzyme models.



Scheme 1 Reactions catalyzed by multivalent esterase and aldolase enzyme models.

strategy by which a purified peptide dendrimer "core" would undergo multiple ligation at its suitably functionalized N-termini with a purified peptide dendrimer "arm" bearing the corresponding reactive group at its focal point.

Attempts to implement this strategy to prepare higher generation multivalent enzyme models using either click chemistry[13] or native chemical ligation[14] did not yield the desired products, probably due to steric hindrance of the reactive focal points or interference of the multiple catalytic groups, in particular histidine residues. We then turned our attention to the thioether ligation between a cysteine thiol and a haloacetyl group, which has been used previously to couple unprotected linear peptide fragments to form polymers, synthetic proteins and cyclic peptides.[15] The reaction is compatible with unprotected side chains and is reported to proceed in good yields in either buffered water (pH 5.5–8.5) with optional potassium iodide catalyst,[15f] or in DMF with diisopropyl ethylamine (DIEA) as base.[15j] The resulting thioether bond is stable towards hydrolysis and presents a minimal departure from a pure peptide-type structure. The thioether ligation has been used to conjugate the amino termini of PAMAM dendrimers with linear peptides.[16] As discussed below, we found this reaction to be well suited for a one-step "dendrimers-on-dendrimer" convergent assembly to access higher generation aldolase and esterase peptide dendrimer enzyme models.

Synthesis of esterase peptide dendrimers by thioether ligation

The thioether ligation was first used to prepare higher generation analogs of previously reported esterase peptide dendrimers consisting of the repeating (His-Ser)$_2$*Dap* dendron.[9] The corresponding dendrimer cores **G2C1** and **G3C1** with four and eight chloroacetyl groups, respectively, at the N-termini as well as the two G2 dendrimer arms **G2M1** and **G2M2** with a cysteine residue at their focal point were prepared by SPPS and obtained in good yields as pure products after purification by preparative HPLC (Table 1).

The thioether ligation required 1.5 equivalents of cysteine per chloroacetyl group under inert atmosphere and slightly basic conditions, ensuring that the starting materials and products were all soluble. In each case the reaction proceeded to an endpoint where the product composition did not evolve even upon addition of excess dendrimer arm. The ligation products were purified by preparative RP-HPLC, which separated minor amounts of incompletely ligated core and unreacted arm present as disulfide bridged dimers.

The ligation reaction was initially tested by coupling the chloroacetylated cores **G2C1** and **G3C1** with the polyanionic arm **B1G2** (AcGlu-Ala)$_4$(*Lys*-Amb-Tyr)$_2$*Dap*-Cys-AspNH$_2$, whose cysteine thiol was known to be accessible for reaction from its high reactivity with cobalamin (Scheme 2).[17] The reaction proceeded well in aqueous phosphate buffer pH 8.5 and yielded **G4E** and **G5E** in 28% and 30%, respectively, after preparative HPLC purification. The chloroacetylated core **G2C1** reacted under the same conditions with the polyhistidine "arms" **G2M1** and **G2M2** to give the polyhistidine G4 dendrimers **G4H1** and **G4H2**. However, reaction of the G3 core **G3C1** with **G2M1** and **G2M2** led to precipitation and incomplete conversion under a variety of conditions, including aqueous, aqueous–organic, or completely organic conditions. A G5 polyhistidine dendrimer was obtained in the form of dendrimer **G5H** by ligation of core **G3C2**, containing the (Pro-Ser)$_2$*Dap* dendrons (see below), with arm

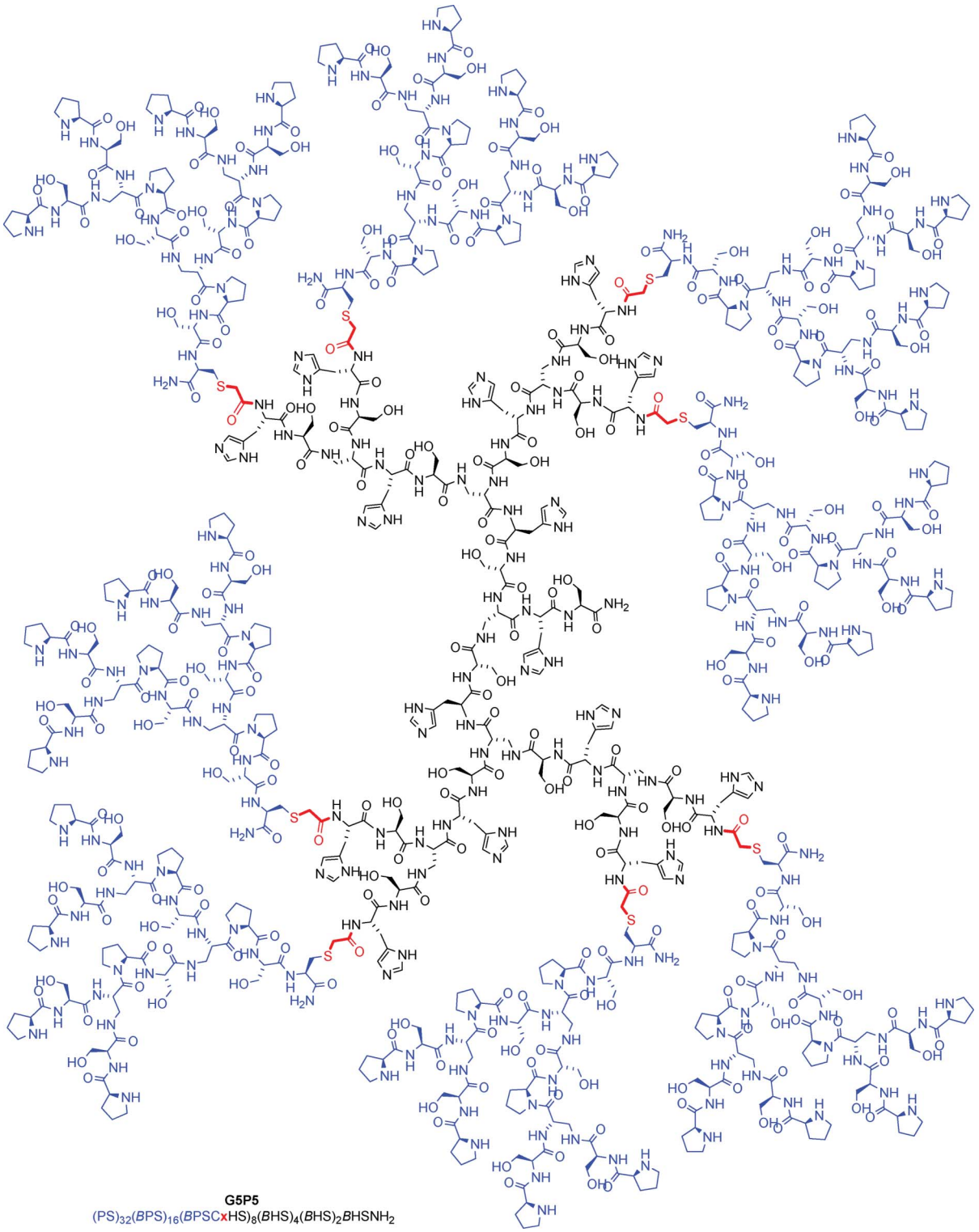


Fig. 2 Structural formula of peptide dendrimer **G5P5**.

G2M2 in 1 : 1 DMF/water with diisopropylethylamine (DIEA) as base in the presence of potassium iodide as catalyst (Table 2).

Synthesis of aldolase peptide dendrimers by thioether ligation

Our previous studies with aldolase peptide dendrimers showed that efficient catalysis could be obtained with G2 and G3 dendrimers with multiple N-terminal prolines as catalytic groups, in particular peptide dendrimers with repetitive (Pro-Thr)$_2$*Dap* or (Pro-Lys)$_2$*Dap* dendrons.[11] We chose to assemble higher generation aldolase dendrimers based on N-chloroacetylated G2 and G3 dendrimer "cores" **G2C2** and **G3C2** composed of the related (Pro-Ser)$_2$*Dap* dendron, and the G3 core **G3C3** assembled from the sterically less congested (Pro-Ser)$_2$*Lys* dendron. Six different

Table 1 Building blocks for the synthesis of esterase dendrimers by thioether ligation

ID[a]	Sequence[b]	Yield[c] %	Yield[c] mg	MW (Da)[d] Found	MW (Da)[d] Calc.
G2C1	$(ClAcHS)_4(BHS)_2BHSNH_2$	44	157	2150.0	2150.8
G3C1	$(ClAcHS)_8(BHS)_4(BHS)_2BHSNH_2$	15	116	4594.0	4594.8
G2M1	$(AcHS)_4(BHS)_2BCNH_2$	40	123	1891.0	1891.9
G2M2	$(AcHS)_4(BHS)_2BHSCNH_2$	42	148	2116.0	2116.1

[a] Synthesized on Tenta Gel S RAM® resin, loading 0.24 mmol g^{-1}. [b] *B* = *Dap* = (*S*)-2,3-diaminopropionic acid branching. [c] Yields were calculated for the TFA salts of the products. [d] ESI+.

Table 2 Esterase peptide dendrimers and analogs prepared by thioether ligation

ID	Sequence[a]	C.[b]	Yield %	Yield mg	MW (Da)[c] Found	MW (Da)[c] Calc.	N_{AA}	+[d]	R_h (nm)[e]
G4E	$(AcEA)_{16}(KXY)_8(BC(D)xHS)_4(BHS)_2BHSNH_2$	I	28	8.23	10562.0	10561.9	85	7	1.98 ± 0.08
G5E	$(AcEA)_{32}(KXY)_{16}(BC(D)xHS)_8(BHS)_4(BHS)_2BHSNH_2$	I	30	13.3	21417.0	21416.1	173	15	2.35 ± 0.02
G4H1	$(AcHS)_{16}(BHS)_8(BCxHS)_4(BHS)_2BHSNH_2$	I	12	4.02	9572.0	9572.5	81	31	2.63 ± 0.06
G4H2	$(AcHS)_{16}(BHS)_8(BHSCxHS)_4(BHS)_2BHSNH_2$	I	8	2.24	10471.0	10470.4	89	35	2.92 ± 0.09
G5H	$(AcHS)_{32}(BHS)_{16}(BHSCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	II	50	13.7	20634.0	20633.6	181	56	2.51 ± 0.07

[a] *B* = *Dap* = (*S*)-2,3-diaminopropionic acid branching; *K* = lysine branching; X = Amb = 4-aminomethylbenzoic acid; x = thioether linkage. [b] Conditions: I: 0.2 M phosphate pH 8.5; II: DMF/H_2O, 55 equiv. DIEA, 20 equiv. KI. [c] ESI+ for all compounds except **G4E**/**G5E** (ESI–). [d] + charges calculated for pH 2. [e] From PGSE diffusion NMR in D_2O, 303 K, pH 2 (as formic acid salts).

Table 3 Peptide dendrimer building blocks for the synthesis of G4–G6 aldolase dendrimers

ID[a]	Sequence[b]	Yield[c] %	Yield[c] mg	MW (Da)[d] Found	MW (Da)[d] Calc.
G2C2	$(ClAcPS)_4(BPS)_2BPSNH_2$	46	104	1869.6	1870.6
G3C2	$(ClAcPS)_8(BPS)_4(BPS)_2BPSNH_2$	25	120	3993.9	3994.4
G3C3	$(ClAcPS)_8(KPS)_4(KPS)_2KPSNH_2$	18	150	4287.9	4289.0
G2M3	$(PS)_4(BPS)_2BPSCNH_2$	47	119	1668.0	1667.8
G2M4	$(PD)_4(BPD)_2BPDCNH_2$	49	111	1863.0	1863.9
G2M5	$(PD)_4(BPD)_2BCNH_2$	55	109	1651.0	1651.7
G2M6	$(PK)_4(BPK)_2BPKCNH_2$	59	196	1955.0	1955.5
G2M7	$(pPE)_4(BPD)_2BPDCNH_2$	45	150	2307.5	2308.4
G3M1	$(PS)_8(BPS)_4(BPS)_2BPSCNH_2$	36	149	3485.0	3485.7

[a] Synthesized on Tenta Gel S RAM® resin, loading 0.24 mmol g^{-1}. [b] *B* = *Dap* = (*S*)-2,3-diaminopropionic acid branching; *K* = lysine branching. [c] Yields were calculated for the TFA salts of the products. [d] ESI+.

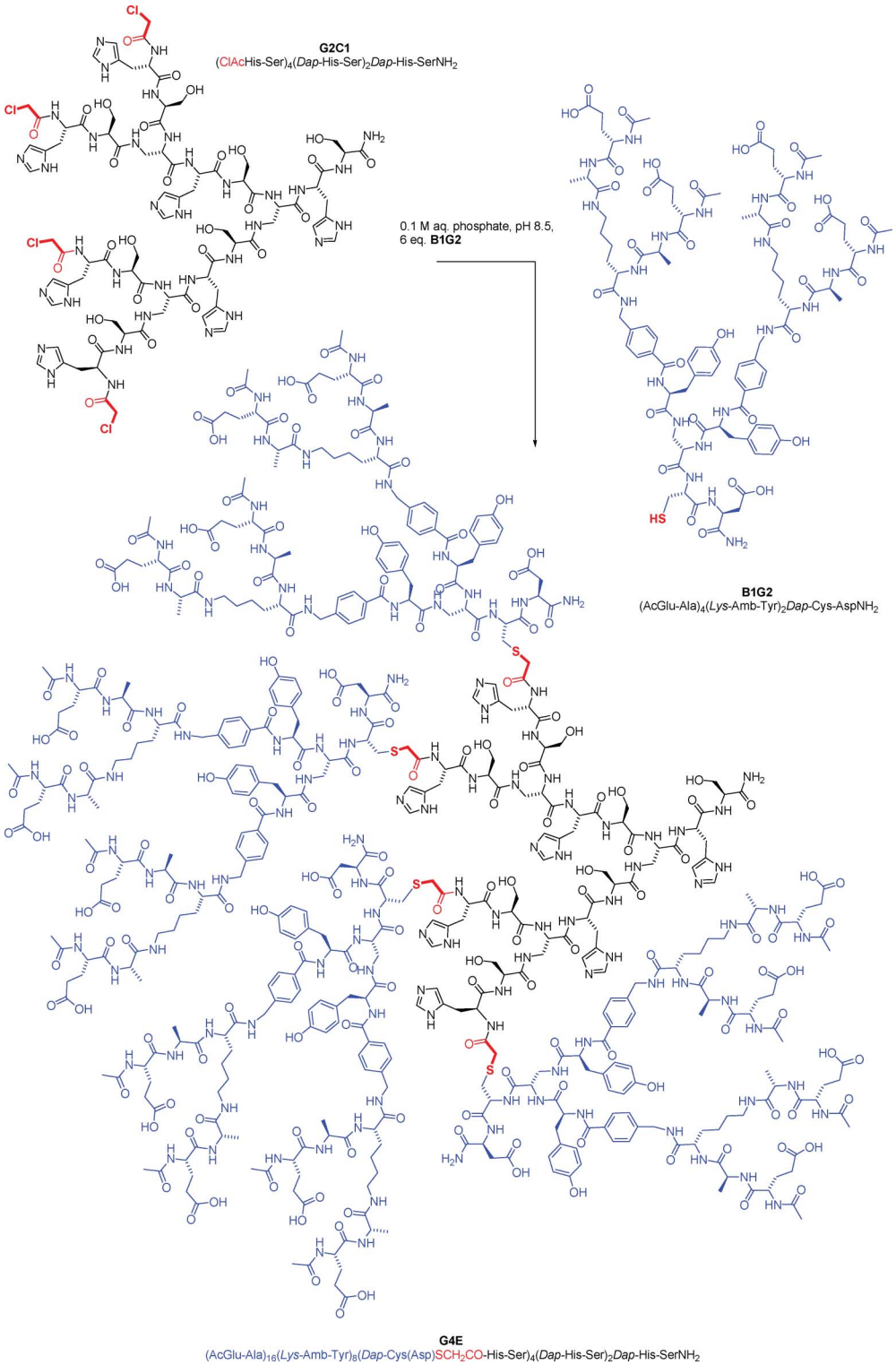
dendrimer "arms" with a cysteine as the focal point were prepared featuring various types of potentially catalytic di- or tri-peptides at their N-termini, including Pro-Ser (**G2M3**, **G3M1**), Pro-Asp (**G2M4**), **G2M5**), Pro-Lys (**G2M6**), and D-Pro-Pro-Glu (**G2M7**) which has been shown to be a particularly active organocatalyst for various aldol-type reactions (Table 3).[18]

The thioether ligation was then used to couple various combinations of cores and arms to prepare G4, G5 and G6 aldolase peptide dendrimers (Table 4). The reactions proceeded well under a variety of conditions, including water/DMF as used for **G5H**, or water with $NaHCO_3$ as base and potassium iodide catalyst with or without acetonitrile as cosolvent, to yield the ligation products after preparative RP-HPLC.

Characterization of the peptide dendrimers

The HPLC-purified ligation products gave sharp peaks by analytical UPLC under conditions which clearly separated the starting materials, the disulfide bridged dimer of the arm, and the intermediates of partial ligation. The complete (4-fold or 8-fold) ligation product was in each case the major product detected by MS, either as the only peak, or together with minor peaks corresponding to the incomplete (3-fold, 7-fold or 6-fold) ligation products (Fig. 3A and the ESI†). These peaks could be assigned to an unreacted chloroacetyl (**G5E** and **G4H2**) or iodoacetyl group (**G5P3**, **G5P4**, **G5P6**, **G5P7**), or a hydroxyacetyl group (**G5H** and **G5P5**). The two G6 dendrimers also gave sharp UPLC peaks; however, their MS spectra could not be deconvoluted.

Characterization by SDS-PAGE showed relatively sharp bands for the dendrimers. In several cases such as **G5P3** a second weaker band was separated, which probably corresponded to the incomplete ligation product observed by MS. Surprisingly, the highly cationic **G5P4** did not stain at all with Coomassie blue or silver staining, and **G4P** and **G4E** only stained very weakly (Fig. 3B). The migration behavior of the dendrimers was very different from that of linear peptides and was strongly sequence dependent.



Scheme 2 Thioether ligation between the dendrimer core **G2C1** and the dendrimer arm **B1G2**.

Such anomalous behavior has also been reported with peptide dendrimers obtained by native chemical ligation of linear peptides and proteins to PPI dendrimers.[14b]

Standard PGSE diffusion NMR experiments were used to determine the hydrodynamic radii of the dendrimers.[10,17,19] The analysis indicated that the dendrimers were homogeneous, with hydrodynamic radii (R_h) ranging from 1.98 to 5.51 nm depending on molecular weight and amino acid sequence (Tables 2 and 4). A linear relationship between hydrodynamic radius and molecular weight was observed for the homologous series **G4P→G5P2→G6P1** (Fig. 4A). A comparable increase in hydrodynamic radius as a function of MW occurred in the three peptide

Table 4 Synthesis of aldolase peptide dendrimers and analogs by thioether ligation

ID	Sequence[a] [b]	C.	Yield %	Yield mg	MW (Da)[c] Found	MW (Da)[c] Calc.	N_{AA}	+[d]	R_h (nm)[e]
G4P	$(PS)_{16}(BPS)_8(BPSCxPS)_4(BPS)_2BPSNH_2$	II	55	2.05	8393.0	8395.9	89	16	2.09 ± 0.07
G5P1	$(PD)_{32}(BPD)_{16}(BCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	III	57	10.3	16916.0	16916.1	165	32	2.76 ± 0.05
G5P2	$(PS)_{32}(BPS)_{16}(BPSCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	II	40	1.48	17043.0	17045.1	181	32	3.42 ± 0.04
G5P3	$(PD)_{32}(BPD)_{16}(BPDCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	IV	62	14.7	18620.0	18619.7	181	32	3.12 ± 0.04
G5P4	$(PK)_{32}(BPK)_{16}(BPKCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	IV	77	16.4	19353.0	19352.4	181	88	4.74 ± 0.15
G5P5	$(PS)_{32}(BPS)_{16}(BPSCxHS)_8(BHS)_4(BHS)_2BHSNH_2$	V	11	2.77	17645.0	17645.5	181	47	3.64 ± 0.10
G5P6	$(PD)_{32}(BPD)_{16}(BPDCxPS)_8(KPS)_4(KPS)_2KPSNH_2$	II	66	11.8	18907.0	18908.2	181	32	3.07 ± 0.05
G5P7	$(pPE)_{32}(BPD)_{16}(BPDCxPS)_8(KPS)_4(KPS)_2KPSNH_2$	II	56	15.6	22459.0	22464.8	213	32	3.21 ± 0.08
G6P1	$(PS)_{64}(BPS)_{32}(BPS)_{16}(BPSCxPS)_8(BPS)_4(BPS)_2BPSNH_2$	II	45	17.7	n.d.[f]	31588.4	341	64	5.51 ± 0.07
G6P2	$(PS)_{64}(BPS)_{32}(BPS)_{16}(BPSCxPS)_8(KPS)_4(KPS)_2KPSNH_2$	II	51	18.2	n.d.[f]	31883.0	341	64	4.88 ± 0.18

[a] *B* = *Dap* = (*S*)-2,3-diaminopropionic acid branching; *K* = lysine branching; x = thioether linkage. [b] Conditions: II: DMF/H_2O, 55 equiv. DIEA, 20 equiv. KI; III: 0.1 M aq. phosphate pH 8.5, 20 equiv. KI; IV: 0.5 M $NaHCO_3$ pH 8.0, 20 equiv. KI; V: 0.5 M $NaHCO_3$ pH 8.0/CH_3CN, 20 equiv. KI. [c] ESI+ for all compounds. [d] + charges calculated for pH 2. [e] From PGSE diffusion NMR in D_2O, 303 K, pH 2 (as formic acid salts). [f] n.d. = not detected.

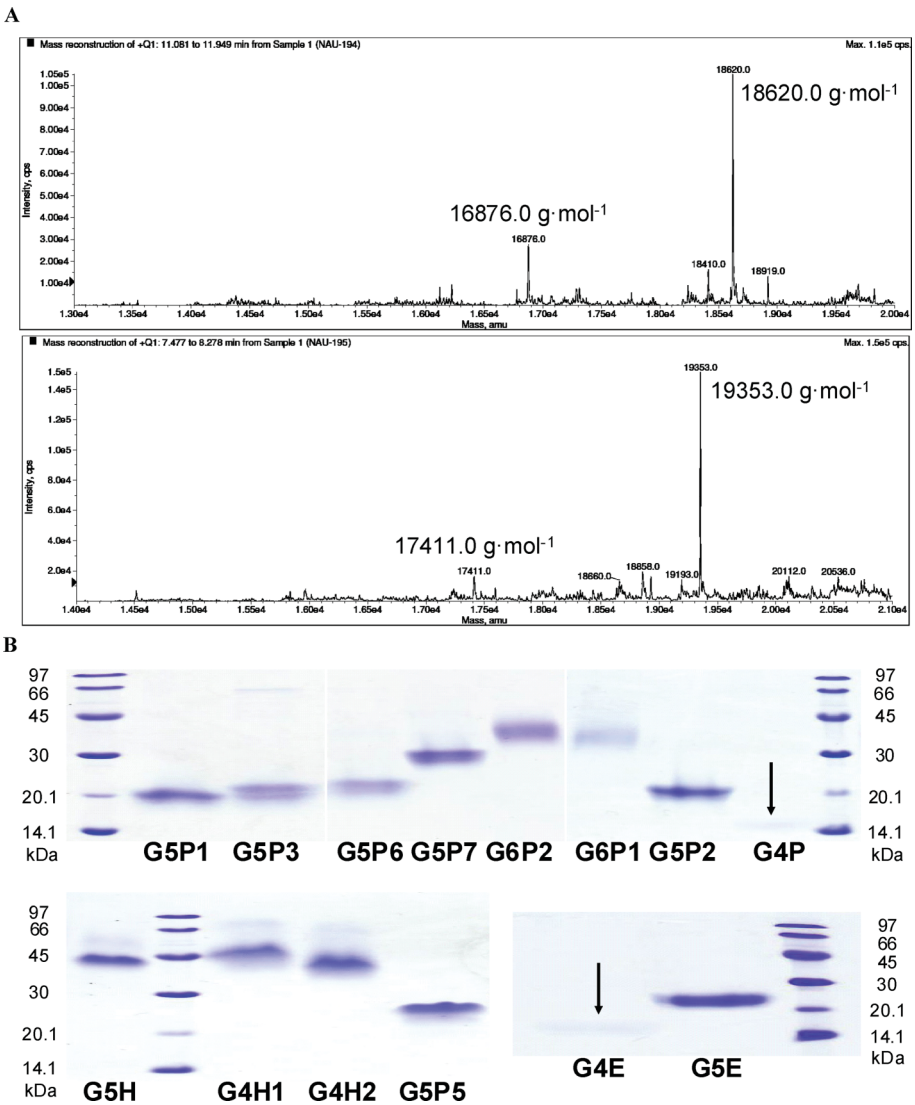


Fig. 3 Analysis of ligation products. **A**. Direct MS (ESI+) of a sample of the HPLC-purified ligated dendrimer **G5P3** $C_{757}H_{1105}N_{221}O_{316}S_8$ (8-fold ligation product, main peak) found/calc. 18620.0/18619.7 $[M + 6H]^+$. 7-Fold ligation product with unreacted iodoacetyl group $C_{682}H_{996}IN_{199}O_{284}S_7$ (peak height 26% of main peak) found/calc. 16876.0/16877.7 $[M]^+$; **G5P4** $C_{869}H_{1497}N_{277}O_{204}S_8$ (8-fold ligation product, main peak) found/calc. 19353.0/19352.4 $[M + 6H]^+$. 7-Fold ligation product with one hydroxyacetyl group $C_{780}H_{1340}N_{248}O_{187}S_7$ (peak height 10% of main peak) found/calc. 17411.0/17411.0 $[M + 2H]^+$. **B**. SDS-PAGE of thioether ligation products.

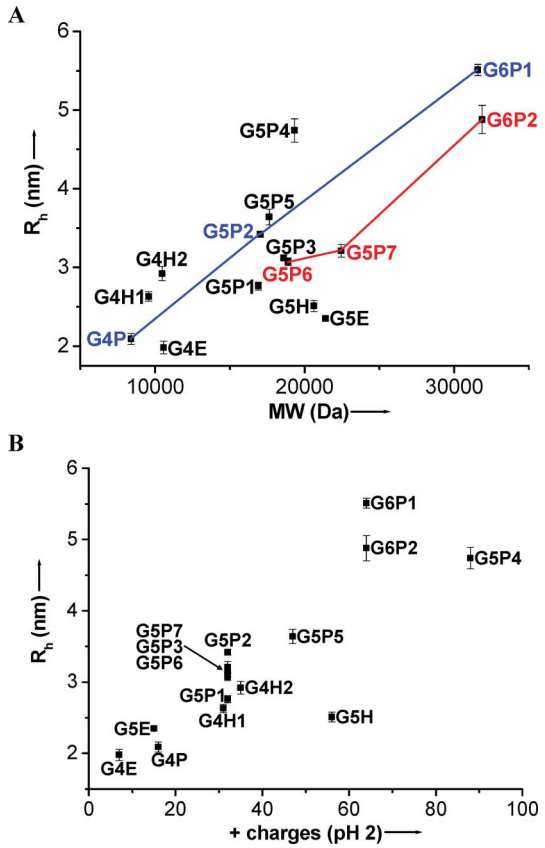


Fig. 4 Hydrodynamic radii R_h of the ligation products in water pH 2 as a function of **A**. molecular weight, and **B**. the number of positive charges calculated at pH 2.

dendrimers with a *Lys*-branched core **G5P6→G5P7→G6P2**. These dendrimers were more compact than their analogs with a *Dap*-branched core probably due to a tighter packing enabled by the longer but more flexible *Lys* branching unit. The hydrodynamic radius also correlated with the number of positive charges (at pH 2 corresponding to the pH of dissolved formic acid salts), suggesting that electrostatic repulsion between dendrimer branches enforced an extended conformation as previously observed with polyanionic peptide dendrimers (Fig. 4B).[17]

Overall, the MS, SDS-PAGE and NMR data on the isolated products were consistent with the products resulting from a 4-fold or 8-fold ligation of the cysteine-containing dendrimer "arms" onto the chloroacetylated dendrimer "cores". The ligation products were also analyzed by quantitative amino acid analysis, in which a weighted sample is hydrolyzed by complete acidic hydrolysis, and the released amino acids are quantified by analytical HPLC as phenylthiocarbamoyl derivatives. The observed amino acid composition was in all cases consistent with the calculated composition. The quality of the samples obtained was judged sufficient to study the effect of multivalency in catalysis in these higher generation peptide dendrimer enzyme models.

Catalytic properties of the esterase enzyme models

Dendrimers **G4H1**, **G4H2** and **G5H**, **G5P5** with multiple His-Ser branches catalyzed the ester hydrolysis of 8-acetoxypyrene-1,3,6-trisulfonate **1** in aqueous buffer with enzyme-like kinetics ($k_{cat}/k_{uncat} \sim 10^4$, $K_M \sim 50$ μM, Table 5). The catalytic proficiency per histidine residue was however independent of dendrimer size, and was in the range of that previously reported for dendrimer **A2** (dendrimer **A2** structure: $(AcHis\text{-}Ser)_4(Dap\text{-}His\text{-}Ser)_2Dap\text{-}His\text{-}SerNH_2$) under the same conditions ($((k_{cat}/K_M)/k_2)/N_{His} = 280$ with $N_{His} = 7$),[9] with no significant additional positive effect from the increased multivalency. Considering that the histidine-containing dendrimer arms **G2M1** and **G2M2** used to prepare the ligated dendrimers correspond to dendrimer **A2** appended with an additional cysteine at the focal point, the data suggest that the extended tripeptide or pentapeptide branch at the level of the thioether bond results in reduced steric congestion preventing additive multivalency.

Hydrolysis of the hydrophobic fluorescein isobutyryl ester **3** was also catalyzed by the G4 and G5 esterase dendrimers. We recently reported the use of substrate **3** to study peptide dendrimer enzyme models, and showed that the substrate reacts faster with more hydrophobic dendrimers than substrate **1**.[20] Ester **3** was converted with efficiencies per histidine residue in the range of $2 < ((k_{cat}/K_M)/k_2)/N_{His} < 20$ for various histidine containing catalysts including linear polyhistidine peptides,[21,22] and G3 peptide dendrimers. The highest values were observed with G3 peptide dendrimers **H7** $(His)_8(Lys\text{-}Phe)_4(Lys\text{-}Pro)_2Lys\text{-}aProNH_2$) and **AcH3** $(AcHis)_8(Lys\text{-}Leu)_4(Lys\text{-}Val)_2Lys\text{-}LysOH$, an effect attributed to the presence of hydrophobic residues in the sequence.[20] The present G4 and G5 peptide dendrimers gave a comparable value of $((k_{cat}/K_M)/k_2)/N_{His} \sim 10$; however, with the important difference that pre-equilibrium substrate binding in the range of $K_M \sim 50$ μM was observed. By contrast, the lower generation peptide dendrimers such as **H7** and **AcH3** gave no detectable substrate binding. This apparent substrate binding in the G4 and G5 esterase dendrimers might indicate more potent hydrophobic interactions with the substrate created by a microenvironment effect possible only in these larger dendrimers.

Catalytic properties of the aldolase enzyme models

The G4, G5 and G6 dendrimers with multiple N-terminal prolines were investigated for catalysis of the aldol addition of acetone to 4-nitrobenzaldehyde **5** to form aldol **6** (Scheme 1). The reaction was conducted in 50% aqueous acetone at neutral pH using 100 mM 4-nitrobenzaldehyde and 0.64 mM (0.64 mol%) N-terminal proline added either as free L-proline or as G4, G5 or G6 dendrimer (Fig. 5A). The reaction was efficiently catalyzed by several of the dendrimers, in particular **G6P1**, **G6P2** and **G5P5**. Catalysis with the best catalyst **G6P1** corresponded to a 150-fold reactivity enhancement per N-terminal proline (over proline itself), which compared favorably to the only 4.3-fold increase in MW per N-terminal proline (Fig. 5B). This relatively strong dendritic effect with the G5 and G6 dendrimers was surprising since previously studied peptide dendrimer aldolases showed only a 4-fold activity increase per N-terminal proline with G2 dendrimers with no further increase at G3.[11] Catalysis with **G6P1** was highest at neutral pH, which might indicate bifunctional catalysis involving a free-base and a protonated proline with depressed pK_a due to multivalency (Fig. S1, ESI†).

The enantioselectivity of the aldol reaction was determined with 1 mol% N-terminal proline under aqueous conditions, as well as under organic conditions (DMSO/acetone) under which the

Table 5 Catalytic parameters for ester hydrolysis of substrates **1** and **3**

		G4H1	G4H2	G5H	G5P5
	N_{His}	31	35	56	15
Substrate 1	K_M (μM)	42 ± 3	50 ± 1	72 ± 3	48 ± 1
	k_{cat} (min^{-1})	0.32 ± 0.0067	0.32 ± 0.0034	0.43 ± 0.0081	0.33 ± 0.0079
	k_{cat}/K_M (min^{-1} M^{-1})	7600 ± 582	6330 ± 170	6050 ± 355	6790 ± 155
	$k_{cat}/K_M/N_{His}$ (min^{-1} M^{-1})	250	180	110	450
	k_{cat}/k_{uncat}	8740	8590	11800	8900
	$(k_{cat}/K_M)/k_2$	7580	6310	6040	6770
	$(k_{cat}/K_M)/k_2/N_{His}$	250	180	110	450
Substrate 3	K_M (μM)	65 ± 10	39 ± 7	29	50
	k_{cat} (min^{-1})	0.047 ± 0.004	0.034 ± 0.004	0.037 ± 0.002	0.017 ± 0.0007
	k_{cat}/K_M (min^{-1} M^{-1})	730 ± 85	880 ± 88	1290 ± 70	340 ± 19
	$k_{cat}/K_M/N_{His}$ (min^{-1} M^{-1})	24	25	23	23
	k_{cat}/k_{uncat}	1940	1390	1540	690
	$(k_{cat}/K_M)/k_2$	320	390	570	150
	$(k_{cat}/K_M)/k_2/N_{His}$	10.3	11.1	10.1	10.0

Conditions: 5 mM aq. citric acid-sodium citrate pH 5.5, with 11% acetonitrile for substrate **3**; 1.39–1.86 μM peptide dendrimers; 120 μL assays in microtiterplate wells were followed by fluorescence (λ_{exc} = 450 ± 25 nm, λ_{em} = 530 ± 12 nm) at 34 °C; all kinetics result from three independent measurements. K_M = reciprocal substrate binding; k_{uncat} (**1**) = 3.9×10^{-5} min^{-1}; k_{uncat}(**3**) = 2.5×10^{-5} min^{-1}; k_2(**1**) = 1.0 $M^{-1}min^{-1}$; k_2 (**3**) = 2.26 $M^{-1}min^{-1}$ (2nd order rate constant with 4-methyl imidazole); N_{His} = number of histidine residues in the dendrimer.

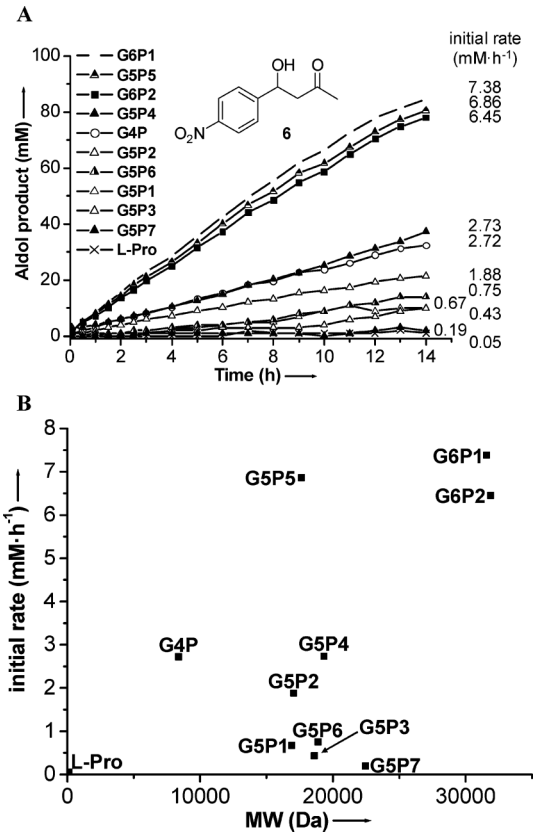


Fig. 5 Dendrimer-catalyzed aldol reaction. **A**. Time-course of product formation with 100 mM 4-nitrobenzaldehyde and 0.64 mol% N-terminal proline in acetone/aq. HEPES pH 7.0 (1/1) at 25 °C. Followed by RP-HPLC. **B**. Initial rates of the dendrimer-catalyzed aldol reaction with 100 mM 4-nitrobenzaldehyde acetone/aq. HEPES pH 7.0 (1/1) as a function of the dendrimers' MW.

reference catalyst L-proline is active at 30 mol% catalyst and gives significant enantioselectivity (76% *ee*).[23] The dendrimers showed only low levels of enantioselectivity in both cases (9–41% ee, Table S1, ESI†).

Conclusion

Esterase and aldolase peptide dendrimers were prepared in a single step by a multivalent dendrimers-on-dendrimer ligation strategy from G2 and G3 peptide dendrimer building blocks, which were themselves prepared readily by SPPS from standard building blocks. The G4 and G5 esterase peptide dendrimers thus obtained showed catalytic proficiencies per histidine residue which were comparable to previously reported G3 dendrimers. On the other hand, G5 and G6 aldolase peptide dendrimers exhibited remarkable aldolase reactivity not present in lower generation analogs, although without significant enantioselectivity. The aldolase reactivity was also largely sequence dependent.

The simple one-step ligation approach presented to assemble higher generation peptide dendrimers gives access to products with MW above 20 kDa, a size range corresponding to proteins and which is not accessible by direct SPPS. This convergent synthesis gave products with satisfactory yields and purities, although minor amounts of incomplete ligation products were also observed. The thioether ligation has the advantage of being compatible with unprotected amino acid side chains. In addition, the reaction does not require any unusual building blocks apart from the chloroacetyl group, which is simply added by acylation of the N-termini at the last step of the dendrimer "core" SPPS. This multivalent dendrimers-on-dendrimer strategy should be generally useful to explore the structure and function of other types of peptide dendrimers.

Experimental section

SPPS

Dendrimers were synthesized by adding 500–800 mg of Tenta Gel S RAM® resin (loading: 0.24 or 0.26 mmol g^{-1}) in a 10 mL polypropylene syringe fitted with a polyethylene frit, a Teflon stopcock and stopper. The resin was swollen in DCM for 15 min. After removal of the DCM the first amino acid was coupled. Stirring of the reaction mixture at any given step described below

was performed by attaching the closed syringe to a rotating axis. The following coupling conditions were used:

Coupling of the Fmoc-protected amino acids

3.0 equivalents of Fmoc-protected amino acid and 3.0 equivalents of PyBOP in 6 mL of NMP were added to the resin. 5.0 equivalents of DIEA were added and the reaction was stirred for 30 min (reaction times were prolonged for the amino acids after 1 branching unit (60 min), after 2 branching units (90 min) and 3 branching units (120 min); for branching units twice the reaction time was used). The resin was then washed (3× each) with NMP, MeOH and DCM. The effectiveness of the coupling was monitored by the trinitrobenzensulfonic acid (TNBS)[24] or chloranil test.

Cleavage of the Fmoc protecting group

The Fmoc protecting group was removed with 6 mL of a solution of DMF/piperidine (1/4, v/v) for 10 min. After filtration the procedure was repeated and finally the resin washed (3× each) with NMP, MeOH and DCM.

N-Acetylation/*N*-chloroacetylation

When necessary the resin was acetylated/chloroacetylated with a solution of acetic acid anhydride/DCM (1/1, v/v) or a solution of chloroacetic acid anhydride (10.0 equivalents)/DCM for 30 min. After filtration the resin was washed (3× each) with NMP, MeOH and DCM.

TFA cleavage

The cleavage was carried out using a TFA/H_2O/TIS (95/2.5/2.5 v/v/v) solution (if the dendrimer contained Cys a TFA/H_2O/TIS/EDT (94/1/2.5/2.5 v/v/v) solution was used instead) for 4.5 h.

Purification

After TFA cleavage the dendrimers were separated from the resin by filtration and precipitated with ice-cold MTBE. The MTBE was removed by centrifugation and the crude product dried under vacuum. It was then dissolved in a water/acetonitrile mixture, purified by preparative RP-HPLC and lyophilized. Yields were calculated for the TFA salts of the products. For characterization by analytical RP-HPLC the following elution solutions were used: A = mQ-deionized H_2O with 0.1% TFA; D = mQ-deionized H_2O/HPLC-grade CH_3CN (40 : 60) with 0.1% TFA.

(ClAc-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G2C1)

G2C1 was obtained as a foamy colourless solid after preparative RP-HPLC (156.7 mg, 53.1 μmol, 44%). Analytical RP-HPLC: t_R = 9.071 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{80}H_{109}Cl_4N_{35}O_{28}$ found/calc. 2150.0/2150.8 $[M]^+$; 2189.0/2189.9 $[M + K]^+$.

(ClAc-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$(G2C2)

G2C2 was obtained as a foamy colourless solid after preparative RP-HPLC (104.0 mg, 55.6 μmol, 46%). Analytical RP-UPLC: t_R = 1.405 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{73}H_{109}Cl_4N_{21}O_{28}$ found/calc. 1869.6/1870.6 $[M]^+$.

(ClAc-His-Ser)$_8$(*Dap*-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$(G3C1)

G3C1 was obtained as a foamy yellowish solid after preparative RP-HPLC (116.2 mg, 18.4 μmol, 15%). Analytical RP-HPLC: t_R = 9.766 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{172}H_{233}Cl_8N_{75}O_{60}$ found/calc. 4594.0/4594.8 $[M]^+$.

(ClAc-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G3C2)

G3C2 was obtained as a foamy yellowish solid after preparative RP-HPLC (119.9 mg, 30.0 μmol, 25%). Analytical RP-UPLC: t_R = 1.515 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{157}H_{233}Cl_8N_{45}O_{60}$ found/calc. 3993.9/3994.4 $[M]^+$; 4015.4/4017.4 $[M + Na]^+$; 4034.3/4033.5 $[M + K]^+$.

(ClAc-Pro-Ser)$_8$(*Lys*-Pro-Ser)$_4$(*Lys*-Pro-Ser)$_2$*Lys*-Pro-Ser-NH$_2$ (G3C3)

G3C3 was obtained as a foamy colourless solid after preparative RP-HPLC (149.7 mg, 34.9 μmol, 18%). Analytical RP-UPLC: t_R = 1.510 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{178}H_{275}Cl_8N_{45}O_{60}$ found/calc. 4287.9/4289.0 $[M]^+$; 4310.0/4312.0 $[M + Na]^+$; 4326.7/4328.1 $[M + K]^+$; 4348.8/4351.1 $[M + Na + K]^+$; 4364.1/4367.2 $[M + 2K]^+$.

(Ac-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-Cys-NH$_2$ (G2M1)

G2M1 was obtained as a foamy colourless solid after preparative RP-HPLC (123.0 mg, 47.8 μmol, 40%). Analytical RP-HPLC: t_R = 7.682 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{74}H_{106}N_{32}O_{26}S$ found/calc. 1891.0/1891.9 $[M]^+$.

(Ac-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-Cys-NH$_2$ (G2M2)

G2M2 was obtained as a foamy colourless solid after preparative RP-HPLC (147.9 mg, 50.7 μmol, 42%). Analytical RP-HPLC: t_R = 7.569 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{83}H_{118}N_{36}O_{29}S$ found/calc. 2116.0/2116.1 $[M]^+$.

(Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-Cys-NH$_2$(G2M3)

G2M3 was obtained as a foamy colourless solid after preparative RP-HPLC (118.8 mg, 56.0 μmol, 47%). Analytical RP-HPLC: t_R = 9.142 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{68}H_{110}N_{22}O_{25}S$ found/calc. 1668.0/1667.8 $[M]^+$; 1709.0/1708.8 $[M + 2H + K]^+$.

(Pro-Asp)$_4$(*Dap*-Pro-Asp)$_2$*Dap*-Pro-Asp-Cys-NH$_2$(G2M4)

G2M4 was obtained as a foamy colourless solid after preparative RP-HPLC (110.5 mg, 59.3 μmol, 49%). Analytical RP-HPLC: t_R = 9.258 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{75}H_{110}N_{22}O_{32}S$ found/calc. 1863.0/1863.9 $[M]^+$; 1902.0/1903.0 $[M + K]^+$.

(Pro-Asp)$_4$(*Dap*-Pro-Asp)$_2$*Dap*-Cys-NH$_2$(G2M5)

G2M5 was obtained as a foamy colourless solid after preparative RP-HPLC (109.0 mg, 66.0 μmol, 55%). Analytical RP-HPLC: t_R = 8.330 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{66}H_{98}N_{20}O_{28}S$ found/calc. 1651.0/1651.7 $[M]^+$; 1690.0/1690.8 $[M + K]^+$.

(Pro-Lys)$_4$(*Dap*-Pro-Lys)$_2$*Dap*-Pro-Lys-Cys-NH$_2$ (G2M6)

G2M6 was obtained as a foamy colourless solid after preparative RP-HPLC (195.9 mg, 71.2 μmol, 59%). Analytical RP-HPLC: t_R = 9.523 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{89}H_{159}N_{29}O_{18}S$ found/calc. 1955.0/1955.5 $[M]^+$; 1996.0/1994.6 $[M + K]^+$; 2069.0/2069.5 $[M + TFA]^+$; 2183.0/2183.5 $[M + 2TFA]^+$; 2297.0/2297.5 $[M + 3TFA]^+$; 2411.0/2411.5 $[M + 4TFA]^+$; 2525.0/2525.5 $[M + 5TFA]^+$.

(DPro-Pro-Glu)$_4$(*Dap*-Pro-Asp)$_2$*Dap*-Pro-Asp-Cys-NH$_2$ (G2M7)

G2M7 was obtained as a foamy colourless solid after preparative RP-HPLC (149.9 mg, 54.2 μmol, 45%). Analytical RP-UPLC: t_R = 1.267 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{99}H_{146}N_{26}O_{36}S$ found/calc. 2307.5/2308.4 $[M]^+$.

(Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-Cys-NH$_2$ (G3M1)

G3M1 was obtained as a foamy colourless solid after preparative RP-HPLC (149.0 mg, 42.8 μmol, 36%). Analytical RP-HPLC: t_R = 8.995 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{144}H_{230}N_{46}O_{53}S$ found/calc. 3485.0/3485.7 $[M]^+$; 3527.0/3526.8 $[M + 2H + K]^+$; 3568.0/3567.9 $[M + 4H + 2K]^+$; 3610.0/3610.0 $[M + 7H + 3K]^+$; 3650.0/3650.2 $[M + 8H + 4K]^+$.

Thioether ligation

In a typical experiment a solution of core dendrimer (sequence containing chloroacetyl end groups, ~3 mg, 1.0 equiv.) and KI (20 equiv.) in DMF/H_2O (1/1 v/v) (300 μL) was prepared in a 5 mL pointed glass flask. The mixture was degassed (Ar) during 10 min. In a 5 mL round bottomed glass flask arm dendrimer (Cys containing sequence, 1.5 equiv. per chloroacetyl end group in core sequence) was prepared (without solvent) and the flask degassed (Ar) during 10 min. The core dendrimer solution was transferred to the round bottomed glass flask containing the arm dendrimer with a gas tight syringe. DIEA (55 equiv.) was added and the solution stirred at rt. The reaction was followed by RP-UPLC (1.0 μL reaction mixture taken with a gas tight 10 μL glass syringe + 200 μL of A). After completion (usually overnight for 4th generation and 18 to 22 h for 5th and 6th generation sequences), the reaction was quenched by adding 3.5 mL of A, filtered and directly injected to preparative RP-HPLC. The purified product was lyophilized (3×) with formic acid 1%. Yields were calculated based on amino acid analysis.

Several variants of the reaction were performed in the following solvents instead of DMF/H_2O (1/1 v/v): 0.1 M or 0.2 M phosphate buffer pH 8.5, 0.5 M $NaHCO_3$ buffer pH 8.0, 0.5 M $NaHCO_3$ buffer pH 8.0/CH_3CN (2/1 v/v). The reactions were performed with or without addition of KI.

(Ac-Glu-Ala)$_{16}$(*Lys*-Amb-Tyr)$_8$(*Dap*-Cys(Asp)-x-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G4E)

From starting materials **G2C1** and **B1G2** using the general procedure described above (solvent: 0.2 M phosphate buffer pH 8.5), **G4E** was obtained as a colourless solid after preparative RP-HPLC (8.23 mg). Amino acid analysis: protein content 52.5% (4.32 mg, 0.40 μmol, yield 28%); found/calc. (%) Glu 17.6/18.8, Ala 17.3/18.8, Dap 11.8/8.2, Tyr 10.0/9.4, Amb 9.5/9.4, Lys 9.2/9.4, His 7.8/8.2, Ser 7.1/8.2, Asp 4.8/4.7, CM-Cys 4.8/4.7. Analytical RP-UPLC: t_R = 1.467 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI–): $C_{464}H_{629}N_{119}O_{160}S_4$ found/calc. 10562.0/10561.9 $[M]^-$; 10624.0/10624.0 $[M + Na + K]^-$.

(Ac-Glu-Ala)$_{32}$(*Lys*-Amb-Tyr)$_{16}$(*Dap*-Cys(Asp)-x-His-Ser)$_8$(*Dap*-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G5E)

From starting materials **G3C1** and **B1G2** using the general procedure described above (solvent: 0.2 M phosphate buffer pH 8.5), **G5E** was obtained as a foamy colourless solid after preparative RP-HPLC (13.3 mg). Amino acid analysis: protein content 32.3% (4.29 mg, 0.19 μmol, yield 30%); found/calc. (%) Ala 19.2/18.5, Glu 15.2/18.5, Dap 13.1/8.7, Tyr 9.8/9.2, His 9.7/8.7, Lys 9.2/9.2, Amb 9.0/9.2, Ser 8.7/8.7, CM-Cys 4.2/4.6, Asp 1.9/4.6. Analytical RP-UPLC: t_R = 1.480 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI–): $C_{940}H_{1273}N_{243}O_{324}S_8$ (8-fold ligation product, main peak) found/calc. 21417.0/21416.1 $[M - H]^-$. $C_{844}H_{1143}ClN_{222}O_{291}S_7$ (7-fold ligation product with one unreacted chloroacetyl group, peak height 33% of main peak) found/calc. 19311.0/19313.3 $[M - H]^-$.

(Ac-His-Ser)$_{16}$(*Dap*-His-Ser)$_8$(*Dap*-Cys-x-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G4H1)

From starting materials **G2C1** and **G2M1** using the general procedure described above (solvent: 0.2 M phosphate buffer pH 8.5), **G4H1** was obtained as a foamy colourless solid after preparative RP-HPLC (4.02 mg). Amino acid analysis: protein content 46.6% (1.87 mg, 0.17 μmol, yield 12%); found/calc. (%) His 47.8/38.3, Ser 31.5/38.3, Dap 16.3/18.5, CM-Cys 4.4/4.9. Analytical RP-UPLC: t_R = 1.228 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{376}H_{529}N_{163}O_{132}S_4$ found/calc. 9572.0/9572.5 $[M]^+$.

(Ac-His-Ser)$_{16}$(*Dap*-His-Ser)$_8$(*Dap*-His-Ser-Cys-x-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G4H2)

From starting materials **G2C1** and **G2M2** using the general procedure described above (solvent: 0.2 M phosphate buffer pH 8.5), **G4H2** was obtained as foamy colourless solid after preparative RP-HPLC (2.24 mg). Amino acid analysis: protein content 59.7% (1.34 mg, 0.11 μmol, yield 8%); found/calc. (%) His 48.8/39.3, Ser 32.1/39.3, Dap 14.7/16.9, CM-Cys 4.3/4.5. Analytical RP-UPLC: t_R = 1.230 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{412}H_{577}N_{179}O_{144}S_4$ (4-fold ligation product, main peak) found/calc. 10471.0/10470.4 $[M + H]^+$; 10511.0/10510.5 $[M + 2H + K]^+$. $C_{329}H_{460}ClN_{143}O_{115}S_3$ (3-fold ligation product with one unreacted chloroacetyl group, peak height 9% of main peak) found/calc. 8392.0/8390.7 $[M + H]^+$.

(Ac-His-Ser)$_{32}$(*Dap*-His-Ser)$_{16}$(*Dap*-His-Ser-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G5H)

From starting materials **G3C2** and **G2M2** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G5H** was obtained as foamy colourless solid after preparative RP-HPLC (13.7 mg). Amino acid analysis: protein content 64.1% (8.75 mg, 0.38 µmol, yield 50%); found/calc. (%) His 37.2/30.9, Ser 31.8/39.2, Dap 15.6/17.1, Pro 10.9/8.3, CM-Cys 4.5/4.4. Analytical RP-UPLC: t_R = 1.243 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (LC-MS+): $C_{821}H_{1169}N_{333}O_{292}S_8$ (8-fold ligation product, main peak) found/calc. 20634.0/20633.6 $[M + 2H]^+$. $C_{738}H_{1053}N_{297}O_{264}S_7$ (7-fold ligation product with one hydroxyacetyl group, peak height 23% of main peak) found/calc. 18535.0/18534.5 $[M]^+$.

(Pro-Ser)$_{16}$(*Dap*-Pro-Ser)$_8$(*Dap*-Pro-Ser-Cys-x-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G4P)

From starting materials **G2C2** and **G2M3** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G4P** was obtained as a foamy colourless solid after preparative RP-HPLC (2.05 mg). Amino acid analysis: protein content 65.8% (1.35 mg, 0.15 µmol, yield 55%); found/calc. (%) Pro 44.0/39.3, Ser 33.8/39.3, Dap 16.9/16.9, CM-Cys 5.4/4.5. Analytical RP-HPLC: t_R = 9.156 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (LC-MS+): $C_{345}H_{545}N_{109}O_{128}S_4$ found/calc. 8393.0/8395.9 $[M]^+$.

(Pro-Asp)$_{32}$(*Dap*-Pro-Asp)$_{16}$(*Dap*-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G5P1)

From starting materials **G3C2** and **G2M5** using the general procedure described above (solvent: 0.1 M phosphate buffer pH 8.5, 20 equiv. KI), **G5P1** was obtained as a foamy colourless solid after preparative RP-HPLC (10.3 mg). Amino acid analysis: protein content 76.5% (7.86 mg, 0.43 µmol, yield 57%); found/calc. (%) Pro 39.4/38.2, Asp 30.7/29.1, Dap 19.0/18.8, Ser 6.3/9.1, CM-Cys 4.7/4.8. Analytical RP-UPLC: t_R = 1.245 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{685}H_{1009}N_{205}O_{284}S_8$ found/calc. 16916.0/16916.1 $[M]^+$.

(Pro-Ser)$_{32}$(*Dap*-Pro-Ser)$_{16}$(*Dap*-Pro-Ser-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G5P2)

From starting materials **G3C2** and **G2M3** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G5P2** was obtained as a foamy colourless solid after preparative RP-HPLC (1.48 mg). Amino acid analysis: protein content 62.4% (0.92 mg, 0.05 µmol, yield 40%); found/calc. (%) Pro 44.1/39.2, Ser 33.3/39.2, Dap 17.2/17.1, CM-Cys 5.4/4.4. Analytical RP-HPLC: t_R = 9.132 min (A/D 100/0 to 50/50 in 10 min, λ = 214 nm). MS (ESI+): $C_{701}H_{1105}N_{221}O_{260}S_8$ found/calc. 17043.0/17045.1 $[M]^+$.

(Pro-Asp)$_{32}$(*Dap*-Pro-Asp)$_{16}$(*Dap*-Pro-Asp-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G5P3)

From starting materials **G3C2** and **G2M4** using the general procedure described above (solvent: 0.5 M $NaHCO_3$ buffer pH 8.0, 20 equiv. KI), **G5P3** was obtained as a foamy colourless solid after preparative RP-HPLC (14.7 mg). Amino acid analysis: protein content 63.6% (9.33 mg, 0.46 µmol, yield 62%); found/calc. (%) Pro 40.4/39.2, Asp 31.9/30.9, Dap 17.1/17.1, Ser 6.6/8.3, CM-Cys 4.1/4.4. Analytical RP-UPLC: t_R = 1.245 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (LC-MS+): $C_{757}H_{1105}N_{221}O_{316}S_8$ (8-fold ligation product, main peak) found/calc. 18620.0/18619.7 $[M + 6H]^+$. $C_{682}H_{996}IN_{199}O_{284}S_7$ (7-fold ligation product with one unreacted iodoactyl group, peak height 26% of main peak) found/calc. 16876.0/16877.7 $[M]^+$.

(Pro-Lys)$_{32}$(*Dap*-Pro-Lys)$_{16}$(*Dap*-Pro-Lys-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G5P4)

From starting materials **G3C2** and **G2M6** using the general procedure described above (solvent: 0.5 M $NaHCO_3$ buffer pH 8.0, 20 equiv. KI), **G5P4** was obtained as crystalline colourless solid after preparative RP-HPLC (16.4 mg). Amino acid analysis: protein content 82.3% (13.5 mg, 0.58 µmol, yield 77%); found/calc. (%) Pro 40.8/39.2, Lys 31.5/30.9, Dap 17.8/17.1, Ser 6.4/8.3, CM-Cys 3.5/4.4. Analytical RP-UPLC: t_R = 1.255 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (LC-MS+): $C_{869}H_{1497}N_{277}O_{204}S_8$ (8-fold ligation product, main peak) found/calc. 19353.0/19352.4 $[M + 6H]^+$. $C_{780}H_{1340}N_{248}O_{187}S_7$ (7-fold ligation product with one hydroxyacetyl group, peak height 10% of main peak) found/calc. 17411.0/17411.0 $[M + 2H]^+$.

(Pro-Ser)$_{32}$(*Dap*-Pro-Ser)$_{16}$(*Dap*-Pro-Ser-Cys-x-His-Ser)$_8$(*Dap*-His-Ser)$_4$(*Dap*-His-Ser)$_2$*Dap*-His-Ser-NH$_2$ (G5P5)

From starting materials **G3C1** and **G2M3** using the general procedure described above (solvent: 0.5 M $NaHCO_3$ buffer pH 8.0/CH_3CN (2/1 v/v), 20 equiv. KI), **G5P5** was obtained as a foamy colourless solid after preparative RP-HPLC (2.77 mg). Amino acid analysis: protein content 52.2% (1.45 mg, 0.07 µmol, yield 11%); found/calc. (%) Pro 37.5/30.9, Ser 31.2/39.2, His 10.7/8.3, Dap 16.0/17.1, CM-Cys 4.6/4.4. Analytical RP-UPLC: t_R = 1.144 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (ESI+): $C_{716}H_{1105}N_{251}O_{260}S_8$ (8-fold ligation product, main peak) found/calc. 17645.0/17645.5 $[M]^+$. $C_{648}H_{997}N_{229}O_{236}S_7$ (7-fold ligation product with one hydroxyacetyl group, peak height 19% of main peak) found/calc. 15995.0/15995.7 $[M]^+$.

(Pro-Asp)$_{32}$(*Dap*-Pro-Asp)$_{16}$(*Dap*-Pro-Asp-Cys-x-Pro-Ser)$_8$(*Lys*-Pro-Ser)$_4$(*Lys*-Pro-Ser)$_2$*Lys*-Pro-Ser-NH$_2$ (G5P6)

From starting materials **G3C3** and **G2M4** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G5P6** was obtained as a foamy colourless solid after preparative RP-HPLC (11.8 mg). Amino acid analysis: protein content 80.0% (9.41 mg, 0.46 µmol, yield 66%); found/calc. (%) Pro 39.6/39.2, Asp 31.7/30.9, Dap 12.3/13.3, Ser 7.5/8.3, CM-Cys 4.6/4.4, Lys 4.3/3.9. Analytical RP-UPLC: t_R = 1.214 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (LC-MS+): $C_{778}H_{1147}N_{221}O_{316}S_8$ (8-fold ligation product, main peak) found/calc. 18907.0/18908.2 $[M]^+$. $C_{703}H_{1038}IN_{199}O_{284}S_7$ (7-fold ligation product with one unreacted iodoacetyl group, peak height 28% of main peak) found/calc. 17165.0/17172.3 $[M]^+$. $C_{628}H_{929}I_2N_{177}O_{252}S_6$ (6-fold ligation product with two unreacted iodoacetyl groups, peak height 11% of main peak) found/calc. 15467.0/15436.3 $[M]^+$.

(DPro-Pro-Glu)$_{32}$(*Dap*-Pro-Asp)$_{16}$(*Dap*-Pro-Asp-Cys-x-Pro-Ser)$_8$(*Lys*-Pro-Ser)$_4$(*Lys*-Pro-Ser)$_2$*Lys*-Pro-Ser-NH$_2$ (G5P7)

From starting materials **G3C3** and **G2M7** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G5P7** was obtained as foamy colourless solid after preparative RP-HPLC (15.6 mg). Amino acid analysis: protein content 60.7% (9.46 mg, 0.40 μmol, yield 56%); found/calc. (%) Pro 51.7/48.4, Glu 15.3/15.0, Asp 11.6/11.3, Dap 10.6/11.3, Ser 6.6/7.0, CM-Cys 4.2/3.8, Lys 3.7/3.3. Analytical RP-UPLC: t_R = 1.304 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm). MS (LC-MS+): $C_{970}H_{1435}N_{253}O_{348}S_8$ (8-fold ligation product, main peak) found/calc. 22459.0/22464.8 $[M]^+$. $C_{871}H_{1290}IN_{227}O_{312}S_7$ (7-fold ligation product with one unreacted iodoacetyl group, peak height 12% of main peak) found/calc. 20270.0/20271.9 $[M]^+$.

(Pro-Ser)$_{64}$(*Dap*-Pro-Ser)$_{32}$(*Dap*-Pro-Ser)$_{16}$(*Dap*-Pro-Ser-Cys-x-Pro-Ser)$_8$(*Dap*-Pro-Ser)$_4$(*Dap*-Pro-Ser)$_2$*Dap*-Pro-Ser-NH$_2$ (G6P1)

From starting materials **G3C2** and **G3M1** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G6P1** was obtained as a foamy colourless solid after preparative RP-HPLC (17.7 mg). Amino acid analysis: protein content 65.8% (11.6 mg, 0.34 μmol, yield 45%); found/calc. (%) Pro 44.2/39.6, Ser 30.3/39.6, Dap 22.9/18.5, CM-Cys 2.6/2.3. Analytical RP-UPLC: t_R = 1.189 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm).

(Pro-Ser)$_{64}$(*Dap*-Pro-Ser)$_{32}$(*Dap*-Pro-Ser)$_{16}$(*Dap*-Pro-Ser-Cys-x-Pro-Ser)$_8$(*Lys*-Pro-Ser)$_4$(*Lys*-Pro-Ser)$_2$*Lys*-Pro-Ser-NH$_2$ (G6P2)

From starting materials **G3C3** and **G3M1** using the general procedure described above (solvent: DMF/H_2O (1/1 v/v), 20 equiv. KI), **G6P2** was obtained as a foamy colourless solid after preparative RP-HPLC (18.2 mg). Amino acid analysis: protein content 68.6% (12.5 mg, 0.36 μmol, yield 51%); found/calc. (%) Pro 44.2/39.6, Ser 32.9/39.6, Dap 17.4/16.4, CM-Cys 3.0/2.3, Lys 2.6/2.1. Analytical RP-UPLC: t_R = 1.202 min (A/D 100/0 to 0/100 in 5 min, λ = 214 nm).

PGSE diffusion NMR

Standard PGSE diffusion NMR experiments were performed using a Bruker DRX500 with dilute solutions of dendrimer (1–5 mM) in D_2O (pH 2–3, at 303 K). The gradient with a maximum strength of 50 × 10^{-4} T cm^{-1} was calibrated using the HOD proton signal in D_2O (99.997%). The diffusion time Δ was 110 ms and the gradient duration δ was 4 ms. Data analysis was performed by using the Bruker Simfit software and the diffusion coefficient D [m^2 s^{-1}] was derived from peak integrals or intensities. The hydrodynamic radii were calculated from the diffusion coefficient D using the Stokes–Einstein equation $R_h = kT/6\pi\eta D$ with Boltzmann constant k = 1.380 × 10^{-23} J K^{-1}, temperature T in K and viscosity η = 1.089 mPa s for D_2O at 303 K.

Kinetics of ester hydrolysis

All necessary solutions were prepared in a 96-deep well plate (2000 μL). Peptide dendrimer solutions were freshly prepared in mQ-deionized H_2O. The actual peptide content of the solutions was determined by amino acid analysis which lead to the following concentrations in the deep well plate: 5.37 μM (**G4H2**), 4.17 μM (**G4H1**), 4.53 (**G5P5**), 5.58 μM (**G5H**). 8-Acetoxypyrene-1,3,6-trisulfonic acid trisodium salt (**1**) or isobutyryl fluorescein (**3**) were used as substrates. Dilutions by 2/3 with mQ-deionized H_2O (for **1**) or mQ-deionized H_2O/CH_3CN (2/1, v/v) (for **3**) gave eight substrate concentrations ranging from 0.18 to 3 mM (for **1**) or 0.018 to 0.3 mM (for **3**). 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) and fluorescein (fluo) were used as fluorescent standards. Dilutions of stock solutions of HPTS or fluorescein in mQ-deionized H_2O or mQ-deionized H_2O/CH_3CN (2/1, v/v), respectively, gave eight fluorescent standard concentrations ranging from 0 to 0.3 mM (HPTS) or 0 to 0.03 mM (fluo). Citric acid–sodium citrate 15 mM at pH 5.5 was used as buffer and the pH was adjusted to the desired value with HCl 1.0 M or NaOH 1.0 M using a Metrohm 692 pH/ion meter.

40 μL of buffer were mixed with 40 μL of dendrimer solution and 40 μL of substrate solution in a 96-well half area flat bottom plate (190 μL). Thus all initial concentrations of the prepared solutions were divided by 3 to give eight final substrate concentrations ranging from 58.5 to 1000 μM in buffer (for **1**) or 5.85 to 100 μM in CH_3CN/buffer (11 : 89) (for **3**) for each Michaelis–Menten plot. The eight final fluorescent standard concentrations ranged from 0 to 100 μM in buffer (HPTS) or 0 to 10 μM in CH_3CN/buffer (11 : 89) (fluo) and were used for the calibration curve.

The formation of HPTS or fluorescein was followed by fluorescence emission using an absorbance filter 450/50 and an emission filter 530/25. The calibration curve and the background (40 μL buffer, 40 μL mQ-deionized H_2O and 40 μL substrate solution) were recorded for every experiment in parallel. Prior to every experiment the gain was adjusted to a signal of ~50 000 for the well with the maximum concentration of HPTS (for **1**) or fluorescein (for **3**). The temperature inside the instrument was set to 34 °C. Kinetic experiments were followed for 3 h and the data points were measured every 90 s. Fluorescence data were converted into product concentration by means of the calibration curve. Initial reaction rates were calculated from the steepest linear part observed in the curve "fluorescence *vs.* time". All values result from three independent measurements.

Kinetic parameters k_{cat} and K_M

V_{cat} is the apparent rate in the presence of dendrimer catalyst; V_{uncat} is the rate in buffer alone. The observed rate enhancement is defined as V_{net}/V_{uncat} with $V_{net} = V_{cat} - V_{uncat}$. Michaelis–Menten parameters k_{cat} (rate constant) and K_M (Michaelis–Menten constant) were determined by fitting the data to the Michaelis–Menten model. The rate constant k_{uncat} without dendrimer catalyst was calculated from the slope of the linear curve that gives V_{uncat} (as product concentration per time) *vs.* substrate concentration [S]. The following values were obtained: k_{uncat} (for **1**, pH 5.5) = 3.891 × 10^{-5} min^{-1}, k_{uncat} (for **3**, pH 5.5) = 2.512 × 10^{-5} min^{-1}.

Second order rate constant k_2 of reference catalyst 4-methylimidazole

The solutions of 4-methylimidazole (4-MeIm) were prepared by serial dilution (2/3) from stock solutions (3 mM for the use with

8-acetoxypyrene-1,3,6-trisulfonic acid trisodium salt (**1**) or 0.3 mM for the use with isobutyryl fluorescein (**3**)) adjusted to the desired pH value using HCl 1 M. The reaction rate with 4-MeIm was obtained under the same conditions as described above. The final concentrations in the 96-well half area flat bottom plate were 0, 88, 132, 198, 296, 444, 667 and 1000 μM of 4-MeIm (for **1**) or 0, 8.78, 13.2, 19.8, 29.6, 44.4, 66.7 and 100 μM of 4-MeIm (for **3**); 200 μM (for **1**) or 20 μM (for **3**) of substrate and 5 mM (citrate buffer pH 5.5) of buffer. The second order rate constants k_2 were calculated from linear regression of the experimentally measured pseudo first order rate constants k_2' as a function of 4-MeIm concentrations. The second order rate constants k_2 is given by $k_2 = k_2'/[S]$. The following values were obtained: k_2 (for **1**, pH 5.5) = 1.00 min^{-1} M^{-1}, k_2 (for **3**, pH 5.5) = 2.26 min^{-1} M^{-1}.

Aldol reactions

A freshly prepared solution of 4-nitrobenzaldehyde in acetone (200 mM, 50 μL) was mixed with a solution of dendrimer catalyst in buffer (50 μL) in a 1 mL glass vial and stirred for 14 to 24 h at rt by means of a miniature stirring ball. The initial reaction concentrations of dendrimers were determined using amino acid analysis: 0.04 mM (= 1/25 mol%) for the 4th generation compound, 0.02 mM (= 1/50 mol%) for 5th generation compounds or 0.01 mM (1/100 mol%) for 6th generation compounds corresponding to 0.64 mol% N-terminal Pro. Aq. bicine buffer 0.1 M at pH 8.5, aq. HEPES buffer 0.1 M at pH 7.0 or aq. citric acid–sodium citrate buffer 0.1 M at pH 6.0 or 5.0 were used as buffers and the pH was adjusted to the desired value with HCl 1.0 M or NaOH 1.0 M using a Metrohm 692 pH/ion meter. A variant of the reaction consisted in replacing the solvent system by DMSO/acetone (4/1 v/v), buffered with x equiv. of *N*-methylmorpholine (NMM, where x = number of formic acid–amine salts for the dendrimer tested). An aliquot (1 μL) of the reaction mixture was taken every 30 min or 1 h, quenched with A (mQ-deionized H_2O with 0.1% TFA, 200 μL) and injected to analytical RP-UPLC. A gradient of A/D 100/0 to 0/100 in 5 min was used and the reaction followed at $\lambda = 254$ nm. Conversion was calculated using the area under the peaks. The initial reaction rate was deduced from the initial slope (first 5 h) of the curves "aldol product **6** [mM] *vs.* time [h]". L-Pro (0.64 mM) was used as reference catalyst and the background (4-nitrobenzaldehyde (100 mM) in buffer or DMSO/acetone (4/1 v/v)) was recorded for every experiment in parallel. The enantiomeric excess was determined by means of a chiral Daicel Chirlapak AS® column with a gradient of iPrOH/hexane 20/80 isocratic in 20 min ($\lambda = 254$ nm).

Acknowledgements

This work was supported financially by the Swiss National Science Foundation, the Marie-Curie Training Network IBAAC, and the University of Berne.

References

1 (*a*) G. R. Newkome, C. N. Moorefield, F. Vögtle, *Dendritic Molecules: Concepts, Synthesis, Applications*, VCH: Weinheim, 2001; (*b*) C. C. Lee, J. A. MacKay, J. M. J. Fréchet and F. C. Szoka, *Nat. Biotechnol.*, 2005, **23**, 1517–1526; (*c*) D. Astruc, E. Boisselier and C. Ornelas, *Chem. Rev.*, 2010, **110**, 1857–1959.
2 (*a*) L. Crespo, G. Sanclimens, M. Pons, E. Giralt, M. Royo and F. Albericio, *Chem. Rev.*, 2005, **105**, 1663–1681; (*b*) P. Niederhafner, J. Sebestik and J. Jezek, *J. Pept. Sci.*, 2005, **11**, 757–788; (*c*) J. Kofoed and J.-L. Reymond, *Curr. Opin. Chem. Biol.*, 2005, **9**, 656–664.
3 (*a*) A. Esposito, E. Delort, D. Lagnoux, F. Djojo and J.-L. Reymond, *Angew. Chem., Int. Ed.*, 2003, **42**, 1381–1383; (*b*) C. Douat-Casassus, T. Darbre and J.-L. Reymond, *J. Am. Chem. Soc.*, 2004, **126**, 7817–7826; (*c*) T. Darbre and J.-L. Reymond, *Acc. Chem. Res.*, 2006, **39**, 925–934.
4 (*a*) A. Clouet, T. Darbre and J.-L. Reymond, *Angew. Chem., Int. Ed.*, 2004, **43**, 4612–4615; (*b*) N. Maillard, A. Clouet, T. Darbre and J.-L. Reymond, *Nat. Protoc.*, 2009, **4**, 132–142.
5 (*a*) E. Kolomiets, E. M. V. Johansson, O. Renaudet, T. Darbre and J.-L. Reymond, *Org. Lett.*, 2007, **9**, 1465–1468; (*b*) E. Kolomiets, M. A. Swiderska, R. U. Kadam, E. M. V. Johansson, K.-E. Jaeger, T. Darbre and J.-L. Reymond, *ChemMedChem*, 2009, **4**, 562–569; (*c*) N. A. Uhlich, P. Sommer, C. Bühr, S. Schürch, J.-L. Reymond and T. Darbre, *Chem. Commun.*, 2009, 6237–6239.
6 N. Maillard, T. Darbre and J.-L. Reymond, *J. Comb. Chem.*, 2009, **11**, 667–675.
7 (*a*) P. Sommer, V. S. Fluxà, T. Darbre and J.-L. Reymond, *ChemBioChem*, 2009, **10**, 1527–1536; (*b*) E. M. V. Johansson, R. U. Kadam, G. Rispoli, S. A. Crusz, K. M. Bartels, S. P. Diggle, M. Cámara, P. Williams, K. E. Jaeger, T. Darbre and J.-L. Reymond, *Med. Chem. Commun.*, 2011, **2**, 418–420.
8 (*a*) R. Breslow, *Acc. Chem. Res.*, 1995, **28**, 146–153; (*b*) A. J. Kirby, F. Hollfelder, *From Enzyme Models to Model Enzymes*, Royal Society of Chemistry: Cambridge, 2009; (*c*) L. Baltzer and J. Nilsson, *Curr. Opin. Biotechnol.*, 2001, **12**, 355–360.
9 (*a*) E. Delort, T. Darbre and J.-L. Reymond, *J. Am. Chem. Soc.*, 2004, **126**, 15642–15643; (*b*) E. Delort, N.-Q. Nguyen-Trung, T. Darbre and J.-L. Reymond, *J. Org. Chem.*, 2006, **71**, 4468–4480.
10 (*a*) S. Javor, E. Delort, T. Darbre and J.-L. Reymond, *J. Am. Chem. Soc.*, 2007, **129**, 13238–13246; (*b*) S. Javor and J.-L. Reymond, *J. Org. Chem.*, 2009, **74**, 3665–3674; (*c*) S. Javor and J.-L. Reymond, *Isr. J. Chem.*, 2009, **49**, 129–136.
11 J. Kofoed, T. Darbre and J.-L. Reymond, *Org. Biomol. Chem.*, 2006, **4**, 3268–3281.
12 (*a*) S. M. Grayson and J. M. J. Fréchet, *Chem. Rev.*, 2001, **101**, 3819–3867; (*b*) G. R. Newkome and C. Shreiner, *Chem. Rev.*, 2010, **110**, 6338–6442.
13 (*a*) H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021; (*b*) K. L. Killops, L. M. Campos and C. J. Hawker, *J. Am. Chem. Soc.*, 2008, **130**, 5062–5064; (*c*) G. Franc and A. Kakkar, *Chem. Commun.*, 2008, 5267–5276.
14 (*a*) P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776–779; (*b*) I. van Baal, H. Malda, S. A. Synowsky, J. L. J. van Dongen, T. M. Hackeng, M. Merkx and E. W. Meijer, *Angew. Chem., Int. Ed.*, 2005, **44**, 5052–5057; (*c*) V. Y. Torbeev and S. B. H. Kent, *Angew. Chem., Int. Ed.*, 2007, **46**, 1667–1670; (*d*) A. Dirksen, E. W. Meijer, W. Adrieaens and T. M. Hackeng, *Chem. Commun.*, 2006, 1667–1669.
15 (*a*) W. Lindner and F. A. Robey, *Int. J. Pept. Protein Res.*, 1987, **30**, 794–800; (*b*) F. A. Robey and R. L. Fields, *Anal. Biochem.*, 1989, **177**, 373–377; (*c*) P. E. Dawson and S. B. H. Kent, *J. Am. Chem. Soc.*, 1993, **115**, 7263–7266; (*d*) D. R. Englebretsen, B. G. Garnham, D. A. Bergman and P. F. Alewood, *Tetrahedron Lett.*, 1995, **36**, 8871–8874; (*e*) S. Futaki, M. Aoki, M. Fukuda, F. Kondo, M. Niwa, K. Kitagawa and Y. Nakaya, *Tetrahedron Lett.*, 1997, **38**, 7071–7074; (*f*) D. R. Englebretsen, B. Garnham and P. F. Alewood, *J. Org. Chem.*, 2002, **67**, 5883–5890; (*g*) F. M. Brunel and P. E. Dawson, *Chem. Commun.*, 2005, 2552–2554; (*h*) Y. Sako, J. Morimoto, H. Murakami and H. Suga, *J. Am. Chem. Soc.*, 2008, **130**, 7232–7234; (*i*) Y. Sako, Y. Goto, H. Murakami and H. Suga, *ACS Chem. Biol.*, 2008, **3**, 241–249; (*j*) J. O. Freeman, W. C. Lee, M. E. P. Murphy and J. C. Sherman, *J. Am. Chem. Soc.*, 2009, **131**, 7421–7429.
16 M. Sakamoto, A. Ueno and H. Mihara, *Chem. Commun.*, 2000, 1741–1742.
17 (*a*) P. Sommer, N. A. Uhlich, J.-L. Reymond and T. Darbre, *ChemBioChem*, 2008, **9**, 689–693; (*b*) N. A. Uhlich, A. Natalello, R. U. Kadam, S. M. Doglia, J.-L. Reymond and T. Darbre, *ChemBioChem*, 2010, **11**, 358–365 .
18 M. Wiesner, G. Upert, G. Angelici and H. Wennemers, *J. Am. Chem. Soc.*, 2010, **132**, 6–7.

19 (*a*) Y. Cohen, L. Avram and L. Frish, *Angew. Chem., Int. Ed.*, 2005, **44**, 520–554; (*b*) D. K. Wilkins, S. B. Grimshaw, V. Receveur, C. M. Dobson, J. A. Jones and L. J. Smith, *Biochemistry*, 1999, **38**, 16424–16431; (*c*) B. Fritzinger and U. Scheler, *Macromol. Chem. Phys.*, 2005, **206**, 1288–1291; (*d*) W. Ong, J. Grindstaff, D. Sobransingh, R. Toba, J. M. Quintela, C. Peinador and A. E. Kaifer, *J. Am. Chem. Soc.*, 2005, **127**, 3353–3361.
20 N. Maillard, R. Biswas, T. Darbre and J.-L. Reymond, *ACS Comb. Sci.*, 2011, **13**, 310–320.
21 R. Biswas, N. Maillard, J. Kofoed and J.-L. Reymond, *Chem. Commun.*, 2010, **46**, 8746–8748.
22 (*a*) L. Vial and P. Dumy, *J. Am. Chem. Soc.*, 2007, **129**, 4884–4885; (*b*) L. Vial and P. Dumy, *ChemBioChem*, 2008, **9**, 2950–2953; (*c*) C. Schmuck, U. Michels and J. Dudaczek, *Org. Biomol. Chem.*, 2009, **7**, 4362–4368.
23 B. List, R. A. Lerner and C. F. Barbas, III, *J. Am. Chem. Soc.*, 2000, **122**, 2395–2396.
24 W. S. Hancock and J. E. Battersby, *Anal. Biochem.*, 1976, **71**, 260–264.

