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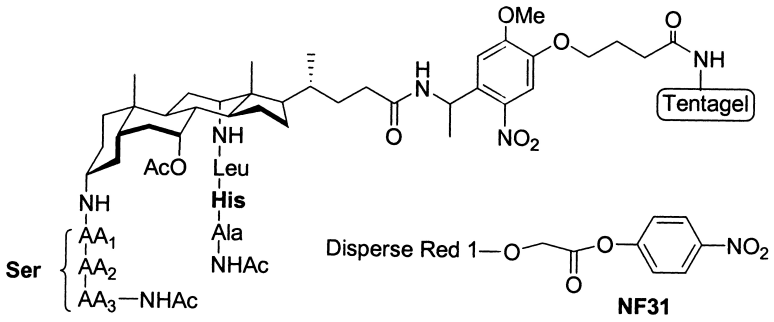

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Articles

Evaluation of a Two-Stage Screening Procedure in the Combinatorial Search for Serine Protease-Like Activity

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Received March 8, 2002

A series of peptidosteroid derivatives containing two independent peptide chains in which Ser and His are incorporated were synthesized by solid-phase peptide synthesis. The activity of the different compounds in the hydrolysis of the activated substrate NF31 was assessed in a stepwise fashion. First, the different resin-bound derivatives **6a**−**l** and **6x**−**z** were individually assayed for serine esterification in the absence of water. The use of a colored substrate allowed for a visual identification of the most active compounds. Through the inclusion of control substances, the involvement of histidine in the mechanism for serine acylation was shown. Second, the hydrolysis and methanolysis of the different acylated derivatives **8a**−**l** and **8x** were evaluated using UV spectroscopy, again indicating the involvement of histidine. The feasibility of applying the above procedures in a combinatorial context was proven via the screening of artificial libraries, created by mixing the different resin-bound peptidosteroid compounds. In this respect, the use of a photocleavable linker allowed for the unambiguous structural characterization of the selected members via application of single-bead electrospray tandem mass spectrometry.

Introduction

The development of artificial catalysts that parallel enzymatic efficiency in terms of rate and turnover constitutes a lasting challenge in organic chemistry.[1] In the context of natural processes, the hydrolysis of unactivated amide bonds as present in peptides and proteins by proteases represents one of the most illustrative cases.[2] Among the known classes of proteases, the serine proteases have been the subject of a thorough investigation.[3] Although new proteases have been discovered in recent years that utilize a serine-lysine dyad mechanism,[4] the action of the classic serine proteases such as chymotrypsin is known to rest with the cooperative involvement of the catalytic triad serine, histidine, and aspartate.[5] This first process involves the transfer of the acyl part of the amide to the Ser residue; subsequently, the acylated enzyme is hydrolyzed by water. At the first stage of the acylation, attack of serine, assisted by the proximate histidine as a general base, leads to a tetrahedral intermediate, the oxyanion in which is stabilized by hydrogen bonding with the backbone peptide bonds. Of the three residues, aspartate is the least important.[6] Its role within the catalytic triad is still under discussion but probably consists of stabilizing the protonated imidazole nucleus.[7] The latter moiety is then involved in the general acid catalyzed breakdown of the tetrahedral intermediate affording the Ser esterified enzyme.

On the basis of this mechanism, the so-called protozymes with peptidase activity have been recently created via engineering of catalytic residues into a protein scaffold that is itself devoid of any catalytic activity.[8] Also, the transition-state analogue-selection approach has led to the development of artificial antibody catalysts for ester hydrolysis.[9] Next to these protein-like structures, less elaborated synthetic protease mimics have also been developed. Among those, one may distinguish two types of model derivatives. One type is characterized by an essentially non-peptide molecular construction consisting of a substrate binding site on which are grafted one or more relevant functional groups.[10] The second group comprises carefully designed peptide assemblies in which the three triad residues are incorporated. As prominent examples of the latter group, the following were reported: (i) a so-called helizyme consisting of a bundle of four short parallel amphipatic helical peptides bearing at their termini the serine protease catalytic residues, reported to show catalytic activity toward *p*-nitrophenyl ester substrates;[11] (ii) a 29-residue cyclic peptide, the trypsin-like activity of which was subsequently questioned;[12] (iii) a computationally de-

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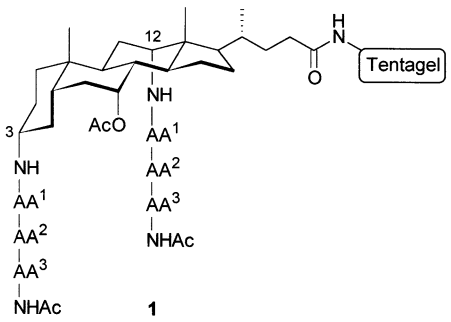
signed cyclic eight-residue peptide that hydrolyzes *p*-nitrophenyl acetate,[13] (iv) a cyclic decapeptide, the low reactivity of which in the hydrolysis of esters was explained by its flexibility and by an inappropriate orientation of the catalytic residues;[14] (v) poly(Asp-Leu-His-Leu-Ser-Leu) with a β-sheet structure, the activity of which in the hydrolysis of chiral phenylalanine-derived *p*-nitrophenyl esters was studied;[15] (vi) a cyclic branched hexapeptide carrying the catalytic triad residues at terminal Lys-amino groups, that showed low activity toward a peptide *p*-nitrophenyl anilide.[16] Despite these numerous efforts, the observed catalytic activity in the above cases is rather modest. Moreover, more often than not, the hydrolyzed substrate is an activated ester. So the challenge of devising a small molecule capable of catalyzing the hydrolysis of an unactivated amide bond with enzymatic efficiency is still intact.

In all previous cases the design of the model peptide has been the result of extensive molecular modeling studies. In contrast, we have become recently intrigued by the possibility of discovering serine-protease-like activity via the *random* generation of large libraries wherein the members possess three independent peptide chains, each containing one of the three residues of the classic triad. The discovery of a genuine catalytic system in a mix−split combinatorial context is inherently elusive because of the difficulty associated with the identification of an eventual active bead-bound member within such a library. However, when the process involves two separate stages as in the serine protease mechanism, one may in principle tackle the problem in a stepwise fashion: (i) the library of potential catalysts is assayed for serine esterification using an adequate acylating agent but in the absence of water; (ii) the reactive members, selected by visual inspection, are pooled to form a new reduced-in-size library that is now assayed for ease of hydrolysis. The reactive members that are revealed in the latter assay could be potential catalysts for the hydrolysis of the acylating agent including turnover. As a preliminary study toward this goal, we will focus here on the development of a construct containing only two independent peptide chains in which serine and histidine are incorporated.

Results and Discussion

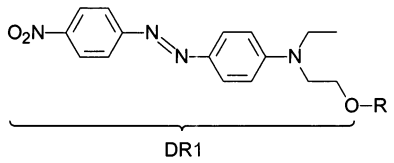
In a previous study, we focused on the acylation stage and identified three TentaGel-bound (TG-NH_2) peptidosteroid derivatives **1a**−**c** (Figure 1), which were selected among the 729 (3^6) members of the mix−split dipodal library **1** on the basis of their reactivity toward acylating agent **2d** (NF31, Figure 2).[17] The use of a solid-phase-bound steroidal scaffold for generating two independent peptide sequences in a combinatorial context was first reported by Still in a seminal paper in which were described the binding properties of a peptidosteroidal receptor library for an enkephalin-like pentapeptide substrate that was tethered to an intensely colored dye (Disperse Red 1).[18] On the basis of the same chromophoric substance as Disperse Red 1, we devised NF31 as an acylating agent for the *covalent* attachment of the acyl chromophore to a reactive solid-phase-bound library member.[19] The choice of NF31 was dictated by the observation that neither TG-Ser-NHAc nor TG-His-NHAc alone led to



C-3 [AA^1, AA^2, AA^3] = [Ser, Phe, Gly]
C-12 [AA^1, AA^2, AA^3] = [His, Leu, Ala]

	C-3			C-12		
	AA^1	AA^2	AA^3	AA^1	AA^2	AA^3
1a	Gly	Phe	Ser	Leu	His	Ala
1b	Phe	Ser	Phe	Leu	His	Ala
1c	Ser	Ser	Phe	Leu	His	Ala

Figure 1. Structures of the mix−split dipodal library **1** and selected members **1a**−**c**.



Disperse Red 1 R = H

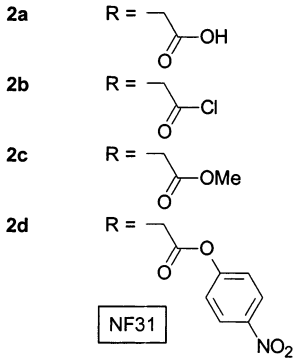
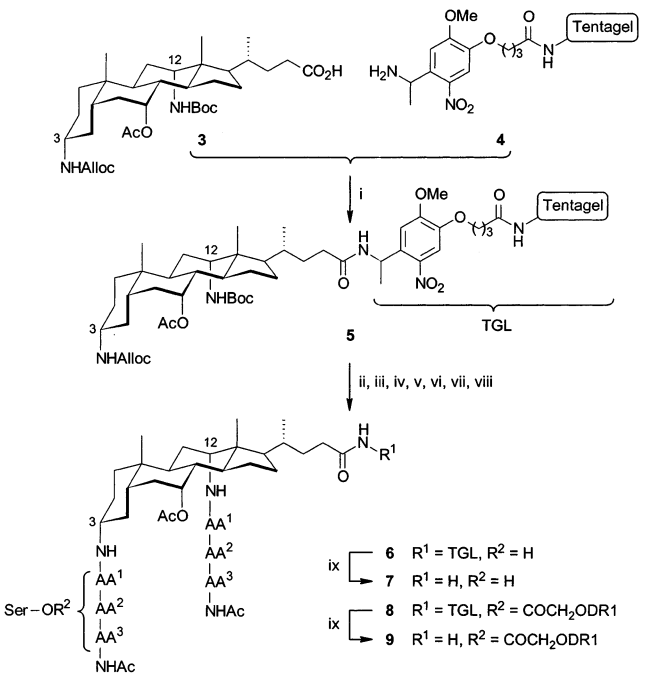


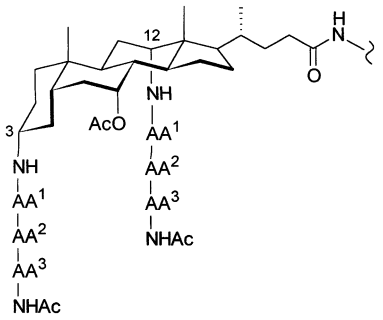
Figure 2. Structures of Disperse Red derivatives **2a**−**d** including the screening substrate NF31.

a significant color reaction under the following dilute screening conditions: exposure of 10 mg beads to 50 μL of a 0.002 M solution of NF31 in 750 μL of CH_3CN, 20 °C, overnight, followed by thorough rinsing with DMF and CH_2Cl_2. Hence, we reasoned that a visual hit would betray a member in which both residues are involved in a cooperative way. The identification of the three selected sequences **1a**−**c** was performed using recursive deconvolution.[20] In this procedure, the amino acids that have been introduced last in the library construction are deciphered first. Inherent in the deconvolution procedure is the increasing difficulty in making choices on the basis of visual inspection while proceeding toward the full identification of an active member. So, while in practice the unraveling of the sequence Leu-His-Ala at C-12 proceeded in a straightforward way, the

Scheme 1. Preparation of the Different Peptidosteroid Derivatives[a]



[a] (i) EDC, DMAP, CH_2Cl_2/DMF (4:1, v/v); (ii) (1) 20% TFA/CH_2Cl_2, (2) 10% DIPEA/DMF; (iii) (1) Fmoc-AA-OH (Leu, His, Ala), HOBt, DIC, CH_2Cl_2/DMF (4:1, v/v), (2) 20% piperidine/DMF, repeated three times; (iv) AcIm, CH_2Cl_2; (v) $Pd(PPh_3)_4$, morpholine, CH_2Cl_2; (vi) (1) Fmoc-AA-OH (Gly, Ser, Phe), HOBt, DIC, CH_2Cl_2/DMF (4:1, v/v), (2) 20% piperidine/DMF, repeated three times; (vii) AcIm, CH_2Cl_2; (viii) (1) 95% TFA/2.5% H_2O/2.5% TIS, (2) 10% DIPEA/DMF; (ix) *hν* (365 nm), 1,4-dioxane, 1% DMSO.



	C-3			C-12		
	AA^1	AA^2	AA^3	AA^1	AA^2	AA^3
a	Gly	Phe	Ser	Leu	His	Ala
b	Phe	Phe	Ser	Leu	His	Ala
c	Gly	Gly	Ser	Leu	His	Ala
d	Phe	Gly	Ser	Leu	His	Ala
e	Gly	Ser	Phe	Leu	His	Ala
f	Phe	Ser	Phe	Leu	His	Ala
g	Gly	Ser	Gly	Leu	His	Ala
h	Phe	Ser	Gly	Leu	His	Ala
i	Ser	Gly	Phe	Leu	His	Ala
j	Ser	Phe	Phe	Leu	His	Ala
k	Ser	Gly	Gly	Leu	His	Ala
l	Ser	Phe	Gly	Leu	His	Ala
x	Gly	Phe	Ser	Leu	Gly	Ala
y	Gly	Phe	Gly	Leu	His	Ala
z	Gly	Phe	Gly	Leu	Gly	Ala

Figure 3. Structures of the different peptidosteroid derivatives containing a fixed amino acid chain at C-12 and a variable amino acid chain at C-3. For simplicity, throughout the text the different compounds are indicated via a three letter code representing the various possible combinations at C-3 (using the one letter code to indicate amino acid residues; e.g., **6a** will be represented by GFS).

identification of reactive tripeptide sequences at C-3, which were introduced first in the construction of library **1**, was difficult. We therefore decided to further focus on those 12 library members that are characterized by the sequence Leu-His-Ala at C-12 and that contain a single Ser residue in the tripeptide sequence at C-3.

The unambiguous structural identification of bead-bound library members implies the possibility of releasing them from the solid phase. Therefore, an orthogonal cleavable linker was incorporated in the construct. After some experimentation, the photocleavable amine-based α-methyl-*o*-nitroveratryl linker bound on TentaGel (**4**) was selected (Scheme 1).[21] Subsequent coupling of the cholic acid derivative **3**[22] to resin **4** (EDC, DMAP) led to the solid-phase-bound steroidal scaffold **5**. The synthesis of different hexapeptide sequences analogous to **1** involved the successive generation of two tripeptide strands. In contrast to the construction of library **1**, the synthesis proceeded now by the prior introduction of the more sterically congested strand at C-12, followed by the second strand at C-3. Not only is this particular order the more logical one, it also allows in the present context a more efficient synthesis of a series of compounds that possess the same sequence at C-12. All peptide couplings were effected in a classical way using Fmoc-protected amino acids and DIC/HOBt methodology. After deprotection of the Boc group in **5** (20% TFA, CH_2Cl_2), the tripeptide strand was generated on the free amino group at C-12, using L-Leu, L-His(Trt), and L-Ala and then capped with acetylimidazole (AcIm). After removal of the Alloc group ($Pd(PPh_3)_4$, morpholine, CH_2Cl_2), the second tripeptide strand was introduced at C-3 using L-Phe, L-Ser(O*t*Bu), or Gly and again capped with AcIm. After acidic removal of the amino acid protective groups (95% TFA, 2.5% H_2O, 2.5% TIS), followed by thorough washing, the desired resin-bound peptidosteroid derivatives **6** were obtained. In this way, 15 different resin-bound sequences were prepared separately, corresponding to **6a**−**l** and **6x**−**z** (Figure 3). Derivatives **6a**−**l** are characterized by the presence of the same tripeptide strand Leu-His-Ala at C-12 and a variable second tripeptide strand at C-3 using the three amino acids L-Ser, L-Phe, and Gly chosen in such a way that each derivative contains one (and only one) Ser residue. Derivatives **6x**−**z** will serve as control substrates. The identity of each sequence was confirmed by direct ESI tandem mass spectrometric analysis of **7**, obtained in MeOH solution after photolytic cleavage. The latter process consisted of irradiation of 1 mg beads in MeOH at 365 nm for 3 h. In the context of our search for genuine catalytic activity, we also need to consider the deacylation process. This requires the separate synthesis of the 12 ester derivatives **8a**−**l**, which was effected using acid chloride **2b** (DIPEA, DMAP, CH_2Cl_2). Also, control sequence **6x** was esterified in the same way. For each member, the confirmation of the structure was gained by direct ES-MS analysis of the corresponding substrate obtained by photolytic cleavage (irradiation at 365 nm of 1 mg of resin, CH_3CN, 3 h).

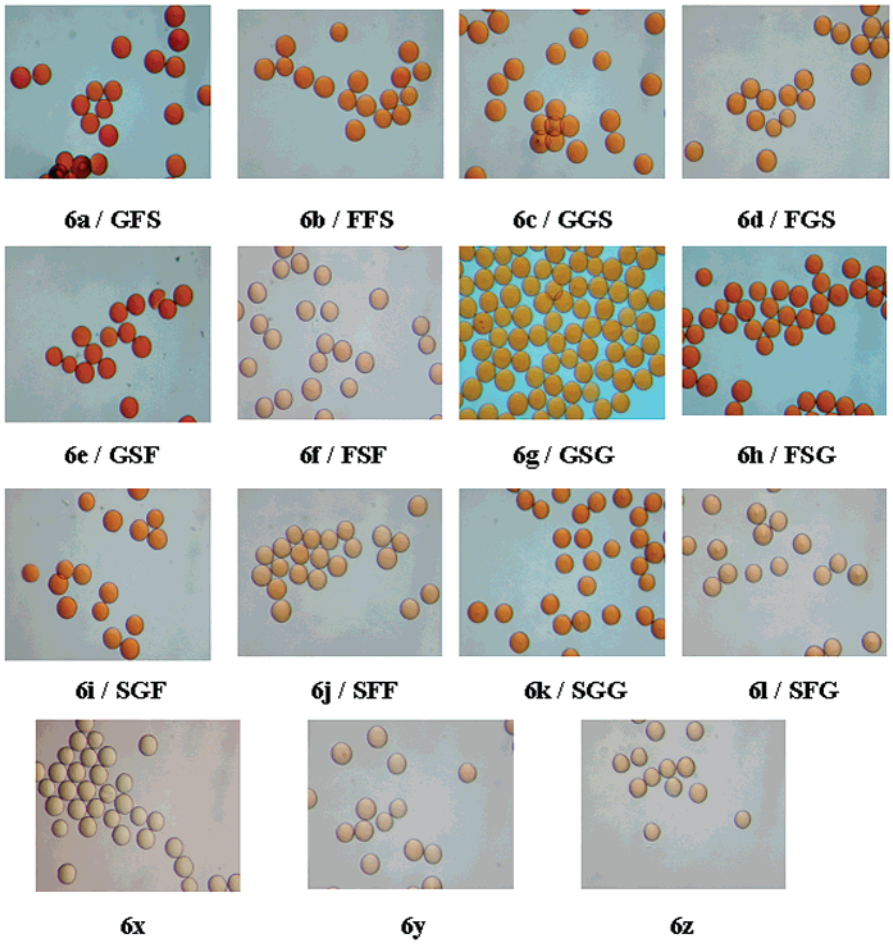


Figure 4. Screening of individual members **6a**−**l** and **6x**−**z** for acylation with NF31. The different members are indicated by a three letter code representing the amino acid chain at C-3.

First, we wished to establish a reactivity order for the acylation of the 12 synthesized sequences of **6** by simple visual inspection. Since acylation of resin-bound peptide **6** may involve in principle Ser esterification or His acylation, we decided to isolate and spectroscopically identify the acylated derivative, at least in one series. Therefore, **6a** was treated with an excess of NF31 (4 days, room temperature, CH_3CN), followed by photolytic cleavage (irradiation at 365 nm of a suspension of 61 mg of resin in 1,4-dioxane containing 1% DMSO for three consecutive periods of 2 h). Chromatographic purification afforded **9a** as a red solid (8 mg, 43% yield based on loading of TentaGel resin). The ester structure of **9a** was established via ^{1}H NMR spectroscopy. In particular, comparison of the spectral data obtained for **7a** and **9a** by using 2D homonuclear correlation spectroscopy (COSY) and nuclear Overhauser effect spectrometry (NOESY) techniques allowed for an unambiguous assignment (relevant information from the 2D COSY and NOESY spectra is included in the Supporting Information).

The result of the screening assays with NF31 (in deficiency) under standard conditions (exposure of 1 mg of resin to 200 μL of a 0.2 mM solution of NF31 in CH_3CN, room temperature, 6 h, followed by thorough rinsing with DMF and brief exposure to MeOH) is shown in Figure 4. For comparison, the result of applying the screening conditions to the control substances **6x**−**z** is also shown. The inclusion of a brief (2 × 1 min) exposure to MeOH in the assay procedure is important. Indeed, when this washing is omitted, one notes that whereas the **6x** beads, which contain Ser but lack His, remain colorless, the **6y** beads, which contain His and lack Ser, become pale-orange, indicating some background reaction of the His residue. However, when the latter beads are briefly exposed to MeOH, they turn colorless.[23] Albeit brief, this exposure to MeOH could also lead to the decoloring of Ser-esterified members through a transesterification reaction. Therefore, we investigated separately the reactivity of the series **8a**−**l** in this process by measuring the release of the methyl ester dye **2c** from the bead into the solution (UV spectroscopy at λ_{obs} = 480 nm). The kinetic results are shown in Figure 5. In comparison with the control substrate **8x** (which reacts slowly), all sequences **8a**−**l** react faster, which implies an active role of histidine in the process. Among the most reactive sequences in this process (GGS (**8c**), GFS (**8a**), and FGS (**8d**)), the GGS and FGS sequences showed moderate reactivity in the acylation process with NF31 (see Figure 4). The latter observation, however, may be due to a partial (and fast) deacylation caused by the methanol washing in the screening experiment. From inspection of Figure 4, it is clear that although it is not possible to establish a complete reactivity sequence by mere visual comparison, one may divide the 12 members into 3 different reactivity groups, with **6a** (GFS), **6e** (GSF), and **6h** (FSG) being the most reactive and with **6f** (FSF), **6j** (SFF), and **6l** (SFG) being the least reactive sequences. In view of the

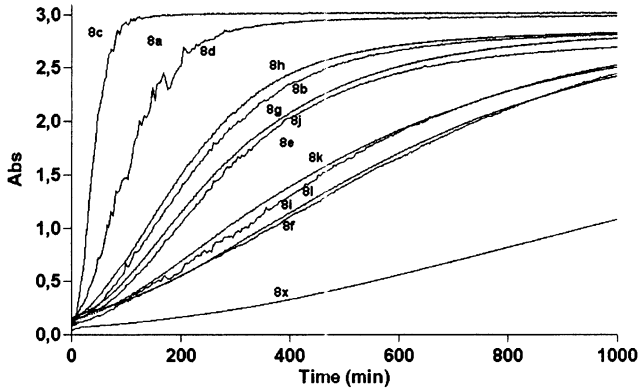


Figure 5. Liberation of methyl 5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)-azo]phenyl}amino-3-oxapentanoate (**2c**) from acylated members **8a**−**l** and **8x** (room temperature, $\lambda_{obs} = 480$ nm). Approximately 1 mg of each resin (ca. 0.2×10^{-6} mol of acylated peptide) was incubated in 1 mL of MeOH.

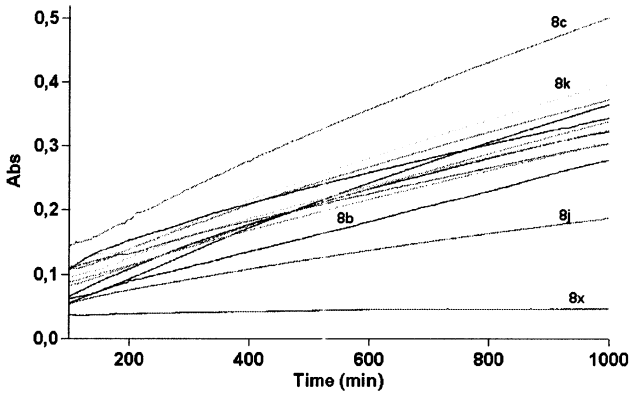


Figure 6. Liberation of 5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}-amino-3-oxapentanoic acid (**2a**) from acylated members **8a**−**l** and **8x** (room temperature, $\lambda_{obs} = 480$ nm). Approximately 1 mg of each resin (ca. 0.2×10^{-6} mol of acylated peptide) was incubated in 1 mL of DMF/pH 7.0 buffer (1:1, v/v).

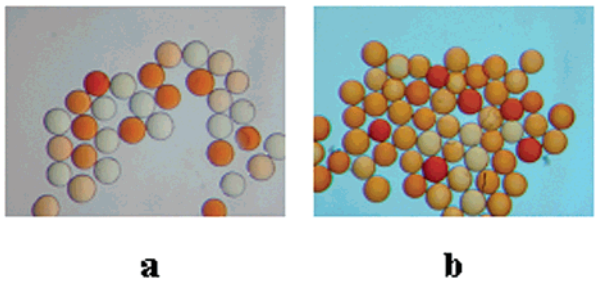


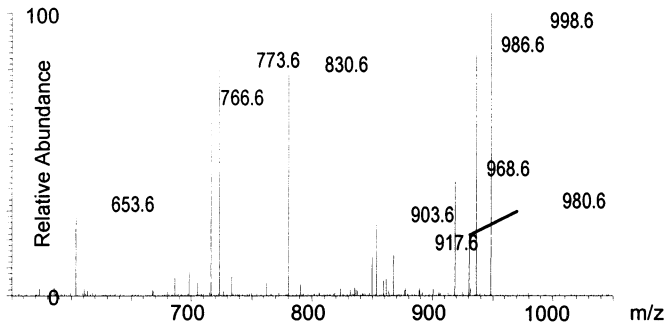
Figure 7. Screening of artificially created libraries. (a) For acylation with NF31, members **6a**−**l** (12×1 mg) and **6x**−**z** (3×5 mg) were pooled, and 6 mg of this library was suspended in 50 μL of 2×10^{-3} M NF31 in 400 μL of acetonitrile for 6 h and subsequently washed with DMF (2×) and MeOH (2×45 min). (b) For deacylation, acylated members **8a**−**l** were pooled and the resulting library was exposed to DMF/pH 7.0 buffer (1:1, v/v) for 11 h at 65 °C.

above-mentioned pitfalls of the recursive deconvolution procedure, we were not so surprised, when comparing the above reactive sequences with the ones (**1a**, **1b**) that were originally identified, to discover other reactive sequences such as **6e** and **6h**, next to **6a** (compare **1a**), but the nonreactivity of **6f** was totally unexpected (compare **1b**). Presumably, the latter observation must result from an incorrect interpretation during the deconvolution of library **1**.

It is interesting to note that all sequences except the three unreactive members **6f**, **6j**, and **6l**, turn out to be more reactive than **6x** and **6y**, which serve here as control substrates, indicating a cooperative effect between the serine and histidine residues. Ester formation may, however, still imply either the direct involvement of the serine hydroxyl group in the attack on the reactive acyl substrate (the GBC pathway) or an acyl transfer following the nucleophilic attack of the histidine imidazole moiety (the Nuc pathway). Obviously a GBC mechanism implies that proton transfer from serine to histidine is geometrically possible. In this particular context, we have established via molecular modeling that irrespective of the position of the serine residue within the tripeptide strand several low-energy conformations involving a short intramolecular distance (<300 pm) between the histidine basic nitrogen and serine hydroxyl oxygen atoms are available (see Supporting Information).

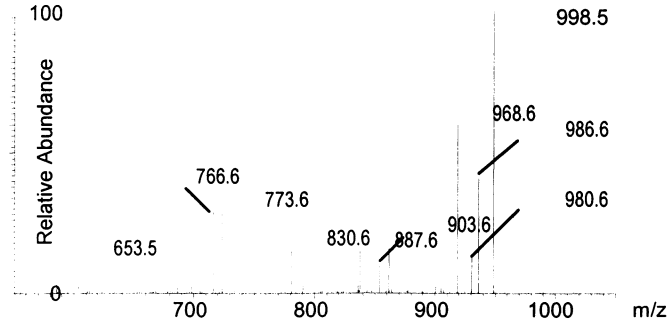
The hydrolysis of **8a**−**l** and **8x** was first examined in buffered aqueous solution at pH 7 in DMF at 25 °C by measuring the release of the acid dye **2a** from the bead into the solution via UV spectroscopy ($\lambda_{obs} = 480$ nm). The kinetic results are shown in Figure 6. Under these conditions, control substrate **8x** is essentially unreactive. The slow reaction that is observed for all derivatives **8a**−**l** thus indicates a participation of the His residue in the deacylation process. We also note that whereas all derivatives react very slowly, the most and least reactive sequences, i.e., GGS (**8c**)and SFF (**8j**), respectively, correpond to those in which the ester functions are presumably the most and least sterically accessible. The same hydrolytic conditions were applied at 65 °C. Pseudo-first-order rate constants were calculated for the individual reactions and give the following reactivity order: k_{obs} (in order of decreasing reactivity) = 10.7 (**8c**), 9.1 (**8g**), 7.9 (**8a**), 7.8 (**8d**), 7.6 (**8h**), 7.5 (**8k**), 7.1 (**8e**), 7.0 (**8f**), 6.2 (**8b**), 5.3 (**8i**), 5.0 (**8l**), 3.4 (**8j**), 1.2 (**8x**) min^{-1}.[24] Whereas at this elevated temperature sequence **8x** is slowly deacylating, we note that the rate at which the other sequences undergo deacylation is still substantially higher, leading to complete loss of the dye after incubation for about 14 h, confirming the importance of His participation also in the deacylation.

Finally, in the context of our final goal, i.e., the discovery of a true catalyst via a stepwise procedure involving single-bead sequence identification after detachment of the relevant peptidosteroid derivatives from the solid phase, we investigated the acylation and deacylation processes in a combinatorial context. Therefore, a library was artificially created by mixing 1 mg beads of each of the 12 members **6a**−**l** together with 5 mg beads of each of the control sequences **6x**−**z**. This pool was subsequently exposed to NF31 under the standard conditions. After selection of several (nine) among the most colored beads and of a few (three) of the colorless beads (Figure 7a), each bead was individually subjected to photolytic cleavage (irradiation at 365 nm of a suspension of the selected bead in 30 μL CH_3CN for 3 h, followed by treatment with MeOH for the colored beads).[25] The structural identification was performed by direct ESI tandem MS spectroscopic analysis. Among the red beads,



m/z	Fragment	Amino acid (residue mass)
653.6	Yb_0	
766.6	Yb_1	L (113)
773.6	Ya_0	
830.6	**Ya_1**	**G (57)**
903.6	Yb_2	H (137)
917.6	**Ya_2**	**S (87)**
968.6	$[M\text{-}HOAc\text{-}HCOH\text{-}H_2O+Na]^+$	
980.6	$[M\text{-}HOAc\text{-}2H_2O+Na]^+$	
986.6	$[M\text{-}HOAc\text{-}HCOH+Na]^+$	
998.6	$[M\text{-}HOAc\text{-}H_2O+Na]^+$	

a



m/z	Fragment	Amino acid (residue mass)
653.5	Yb_0	
766.6	Yb_1	L (113)
773.6	Ya_0	
830.6	**Ya_1**	**G (57)**
887.6	**Ya_2**	**G (57)**
903.6	Yb_2	H (137)
968.6	$[M\text{-}HOAc\text{-}HCOH\text{-}H_2O+Na]^+$	
980.6	$[M\text{-}HOAc\text{-}2H_2O+Na]^+$	
986.6	$[M\text{-}HOAc\text{-}HCOH+Na]^+$	
998.5	$[M\text{-}HOAc\text{-}H_2O+Na]^+$	

b

Figure 8. ESI-MS/MS/MS spectra for (a) **7g** (GSG) and (b) **7c** (GGS) obtained after screening for deacylation and photocleavage. The spectra represent fragments obtained from fragmentation of the daughter ion $[M - HOAc + Na]^+$ at $m/z = 1016$. Fragment ions Ya have an intact chain on C-12, and ions Yb have an intact chain on C-3. Subscripts indicate the number of amino acids still present on the other (fragmented) chain. In this way, $[Yb_n - Yb_{n-1}]$ reveals the identity of the successive amino acids.

five were identified as **8a** (GFS), two as **8d** (FGS), and one as **8g** (GSG). On the other hand, the three colorless beads corresponded to the sequences **6f** (FSF, twice) and **6j** (SFF). These assignments are in line with the results of the individual sreening assay for acylation (see Figure 4). We were of course also interested in investigating the second stage of the overall process, the deacylation reaction, in a library context. For this purpose, a model library was artificially created by mixing 1 mg beads of each of the acylated members **8a**−**l**. This pool was then exposed to DMF/pH 7 buffer (1:1, v/v). After equilibration for 11 h at 65 °C, two among the most colored beads and two colorless beads were selected (Figure 7b) and separately subjected to photolytic cleavage. The sequence of the two intensely colored beads was found to be SFF (**8j**), corresponding to the least reactive member in the buffer-catalyzed hydrolysis; the colorless beads on the other hand turned out to carry the sequences GSG (**6g**) and GGS (**6c**), two among the most active sequences in the deacylation (see Figure 6 and the above-mentioned k_{obs} values). The ESI tandem MS spectra obtained from the single-bead cleavage, which led to the identification of the last two sequences, are shown in Figure 8.[26]

The above experiments show the feasibility of the proposed method for catalyst discovery. Does this mean that **6c** (GGS) and/or **6g** (GSG) behave as serine-protease-like catalysts in the hydrolysis of NF31? At first glance, the answer to this question seems to be yes. When using resin-bound peptidosteroid **6c** (GGS) as the catalyst in the hydrolysis of NF31 (65 °C, CD_3CN/D_2O, 2:1, v/v), an 88% conversion was observed after approximately 20 h, compared to only 18% conversion for the background hydrolysis of NF31 in the absence of catalyst. However, when Ser-lacking control sequence **6y** (GPG) was used, a similar conversion of 83% was observed after the same period of time. Furthermore, resin-bound N-acylated His hydrolyzes NF31 with essentially the same efficiency (90% conversion after approximately 20 h). It thus seems unlikely that the process involving **6c** implicates Ser participation. Rather, the pathway by which NF31 is hydrolyzed through the involvement of His alone, involving either Nuc or GBC in an *inter*molecular way, is more probable (and the only possibility in the cases of **6y** and N-acylated His). In this context, the recent report[27] about the hydrolytic potential of a series of oligopeptides *including the simple dipeptide serylhistidine* deserves critical attention. Our observation is also reminiscent of a recent report by Berkessel[28] that in the context of discovering new catalysts via combinatorial approaches, (sub-)structure−activity analyses are essential.

Conclusions

The potential of a stepwise approach toward the discovery of catalytic systems that operate in two distinct stages, e.g., serine proteases, was investigated via the parallel synthesis of possible candidates. Since the relative reactivities of the different members were assessed separately, it became possible to evaluate the selection procedure, in particular the single-bead methodology, in a combinatorial context. Whereas

the obtained results were satisfactory, we were not successful in discovering a truly catalytic hydrolase system because of (i) the intrinsic low reactivity of the system that only contains Ser and His among the three amino acid residues of the natural catalytic triad and (ii) the compulsory use of a reactive acyl substrate, i.e., *p*-nitrophenyl ester NF31, in which a hydrolytic process presumably proceeds via nucleophilic catalysis involving histidine so that serine participation is not required.

Future work will focus on the development of tripodal constructs, including each of the essential amino acids, together with the use of less or unactivated acyl substrates.

Experimental Section

DMF, MeOH, DMSO, and MeCN were HPLC grade and obtained from Riedel-De-Haën. For coupling reactions, DMF extra-dry from Biosolve was used. CH_2Cl_2 and DIPEA were dried by distillation from CaH_2. 1,4-Dioxane was distilled from $NaBH_4$. KH_2PO_4/Na_2HPO_4 buffer concentrate (FIXANAL, for 500 mL of buffer solution), from Fluka was used to prepare aqueous pH 7 buffer. TentaGel-NH_2 resin (0.29 mmol/g, 90 μm), Fmoc-amino acids, and HOBt were purchased from Novabiochem. Trifluoroacetic acid and piperidine were obtained from Avocado. DIC and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (1% in DMF) were obtained from Fluka. All reagents and solvents were used as received without further purification unless otherwise stated. NF31 was synthesized as previously described.[19] ^{1}H NMR spectra were recorded at 500 MHz on a Brucker AN-500. Mass spectra were recorded using an LCQ ion trap mass spectrometer equipped with an ESI source (ThermoFinnigan, San Jose, CA). All experiments were carried out in the positive ionization mode. Library beads were examined under a Nikon stereoscopic zoom micrsocope SMZ800 equipped with a Nikon Coolpix 950 camera. Deprotection of N^{α}-Fmoc was done using 20% piperidine in DMF (2 × 20 min). Washing volumes were always 1−2 times the volume necessary to swell the resin. Each coupling−deprotection step was monitored using the NF31 test[19] and the TNBS test.[29] UV spectroscopy was performed using a Varian Cary 3E spectrophotometer equipped with a Varian temperature controller. Photolysis was carried out at a distance of 1 cm using a 450 W ACE GLASS incorporated 7225-34 UV lamp set at 365 nm. For small quantities of resin (1 mg) and single-bead cleavage, photolysis was carried out at a distance of 1 cm using a 4 W Bioblock Scientific compact UV lamp set at 365 nm (relative intensity at 15 cm of 340 μW/cm^2).

Synthesis of Resin-Bound Peptidosteroid 6a and Cleavage To Obtain Compound 7a. To a solution of 4-{4-[1-(9-fluorenylmethoxycarbonylamino)ethyl)]-2-methoxy-5-nitrophenoxy}-butanoic acid (Fmoc photolinker, prepared according to the published procedure,[21b] 0.896 g, 1.72 mmol), and HOBt (0.276 g, 2.0 mmol) in 12 mL of DMF was added DIC (0.4 mL, 2.5 mmol). The reaction mixture was stirred under nitrogen at room temperature for 1 h. Subsequently 3 g of preswollen (in DMF) TentaGel-NH_2 resin (0.29 mmol/g, 0.87 mmol) was added to the above solution of OBt-activated Fmoc photolinker (2 equiv, 1.72 mmol). The suspension was shaken at room temperature for 12 h. The resin was then filtered and washed with DMF (3×), MeOH (3×), and CH_2Cl_2 (3×). The Fmoc protecting group was then removed using a solution of 20% piperidine in DMF. The obtained resin **4** was washed with DMF, MeOH, and CH_2Cl_2.

To a solution of steroid **3** (0.951 g, 1.5 mmol) and EDC (0.531 g, 2.77 mmol) in 10 mL of DCM/DMF (4:1, v/v) was added DMAP (15 mg, 0.12 mmol). After stirring the reaction mixture under nitrogen at room temperature for 1 h, resin **4** (2.77 g, 0.638 mmol, theoretical loading of 0.268 mmol/g,) was added to the solution of activated scaffold (1.71 equiv, 1.5 mmol). The suspension was shaken for 12 h, and the beads were subsequently washed with DMF, MeOH, and CH_2Cl_2 to provide resin **5**. The TNBS and NF31 tests showed a complete coupling reaction.

The Boc protecting group was removed by adding 20% TFA in CH_2Cl_2 to resin **5** (2.57 g, 0.591 mmol, theoretical loading of 0.23 mmol/g). The suspension was shaken at room temperature (1 × 20 min, 1 × 1 h). The resin was then washed with DMF and MeOH, neutralized using 10% DIPEA in DMF (2 × 5 min), and washed with DMF, MeOH, and CH_2Cl_2. At this stage, the TNBS test did not work for the sterically hindered secondary amine group. However, reactions at this position could be efficiently followed using the NF31 test. After complete deprotection, Fmoc-Leu-OH (0.626 g, 1.77 mmol, 3 equiv) was activated by HOBt (0.245 g, 1.77 mmol) and DIC (0.28 mL, 1.77 mmol) in 10 mL of CH_2Cl_2/DMF (4:1, v/v) under nitrogen for 1 h. The resulting solution was added to the resin. The suspension was shaken for 12 h. The coupling reaction was repeated twice. In the last coupling, HOAt was used instead of HOBt to ensure complete coupling, which was indicated by the colorless beads obtained in the NF31 test. Following Fmoc deprotection, Fmoc-His(Trt) and Fmoc-Ala were coupled in succession under standard conditions by adding a 3-fold excess of preactived Fmoc-amino acid/OBt ester (HOBt and DIC under nitrogen for 1 h). Coupling reactions were repeated twice, and HOAt was used in the last coupling reaction. After Fmoc deprotection of the last amino acid, Ala, the free amino group was capped by AcIm (12 equiv, 0.788 g, 7.1 mmol) in CH_2Cl_2 (2 × 12 h).

The Alloc protecting group was removed by adding Pd(PPh$_3$)$_4$ (90 mg, 0.077 mmol) under nitrogen to a suspension of resin (2.56 g, 0.53 mmol, theoretical loading of 0.206 mmol/g) in 2.7 mL of morpholine and 14 mL of CH_2Cl_2. The reaction vessel was shaken for 12 h. The deprotection was repeated once. The resin was washed with CH_2Cl_2, DMF, MeOH, and CH_2Cl_2 again and was dried under high vacuum. Coupling of Fmoc-Gly, Fmoc-Phe, and Fmoc-Ser(O*t*Bu) was carried out using standard Fmoc chemistry. Preactivated amino acid/OBt ester (3.5 equiv) in CH_2Cl_2/DMF (3.5 equiv of HOBt, DIC under N_2, 1 h) was added to the resin (250 mg, 51.5 mmol, theoretical loading of 0.206 mmol/g). The suspension was shaken for 17 h. The coupling reaction was repeated twice, and HOAt was used in the last coupling reaction. The resin was washed with DMF (3×), MeOH (3×), and CH_2Cl_2 (3×). Fmoc deprotection was achieved with 20% piperidine in DMF, and the beads were washed with DMF, MeOH, and CH_2Cl_2. The free amino

group was capped with 12 equiv of AcIm (68 mg, 0.624 mmol) in CH_2Cl_2 (2 × 10 h). The resin was washed with CH_2Cl_2, DMF, MeOH, and CH_2Cl_2. The side chain protecting groups (His-Trt and Ser-O*t*Bu) were removed simultaneously by treatment with a solution of 95% TFA, 2.5% H_2O, 2.5% TIS (4 × 1 h). The resin was washed with CH_2Cl_2 and MeOH, neutralized with 10% DIPEA in DMF (3 × 2 min), and finally washed with DMF, MeOH, and CH_2Cl_2.

A 1 mg sample of resin **6a** was irradiated in 200 μL of MeOH at 365 nm for 3 h. The resulting solution was analyzed without further purification by ESI-MS to give the correct molecular weight. Application of ESI-MS/MS/MS confirmed the identity of the sequence (see Supporting Information for corresponding spectra).

To fully characterize compound **7a**, a 66 mg sample of resin **6a** (theoretical loading of 0.206 mmol/g, corresponding to ca. 13.6 mmol of peptidosteroid) in 4 mL of 1,4-dioxane (containing 1% DMSO) was irradiated at 365 nm under nitrogen for 3 h. This process was repeated once. Both filtrates were collected and concentrated. The peptidosteroid was precipitated in cold diethyl ether and further purified by column chromatography (CH_2Cl_2/MeOH/33% ammonia, 70:20:2, v/v/v) to provide 8.8 mg of **7a** (7.7 mmol, 56%) as a white solid. IR(KBr) 3419, 2925, 1654, 1542, 1458 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 0.59 (3H, d, J = 5.4 Hz), 0.67 (3H, d, J = 5.5 Hz), 0.71 (3H, s), 0.76 (3H, d, J = 5.7 Hz), 0.87 (3H, s), 1.28 (3H, m), 1.51 (2H, m), 1.82 (3H, s), 2.02 (6H, s), 2.81 (1H, dd, J = 13.8, 9.4 Hz), 3.01 (1H, m), 3.07 (1H, dd, J = 13.9, 4.1 Hz), 3.26 (1H, m), 3.49 (2H, m), 3.53 (1H, m), 3.70 (1H, dd, J = 15.9, 5.2 Hz), 3.99 (1H, m), 4.05 (1H, m), 4.25 (1H, q, J = 6.4 Hz), 4.48 (2H, m), 4.72 (1H, br s), 5.02 (1H, br s), 6.64 (2H, s), 6.86 (1H, s), 7.18 (1H, m), 7.22 (5H, m), 7.30 (1H, d, J = 7.5 Hz), 7.37 (1H, m), 7.54 (1H, m), 7.64 (1H, br s), 7.92 (1H, d, J = 7.6 Hz), 8.00 (1H, d, J = 7.6 Hz), 8.18 (1H, m), 8.80 (1H, m), 9.09 (1H, d, J = 7.4 Hz) (see Supporting Information for more detailed 2D COSY and 2D NOESY assignment); MS-ES$^+$ *m/z* 1145 [[MH$^+$, 4%]], 1167 [[MNa$^+$, 100%]], 1183 [[MK$^+$, 7%]].

Synthesis of Resin-Bound Peptidosteroids 6b−l and 6x−z and Cleavage To Obtain Compounds 7b−l and 7x−z. Analoguous procedures as described above for **6a** were used for the synthesis and characterization of peptidosteroid derivatives **6b−l** and **6x−z**. In all cases, at the end of the synthesis a 1 mg sample was irradiated in MeOH at 365 nm to photolytically cleave the compound from the resin and the resulting solution was analyzed by electrospray mass spectrometry. MS-ES$^+$ *m/z* **7b** 1234 [[MH$^+$, 10%]], 1256 [[MNa$^+$, 100%]]; **7c** 1054 [[MH$^+$, 75%]], 1076 [[MNa$^+$, 100%]]; **7d** 1144 [[MH$^+$, 38%]], 1166 [[MNa$^+$, 100%]]; **7e** 1166 [[MNa$^+$, 100%]]; **7f** 1234 [[MH$^+$, 13%]], 1256 [[MNa$^+$, 100%]]; **7g** 1054 [[MH$^+$, 9%]], 1076 [[MNa$^+$, 100%]]; **7h** 1144 [[MH$^+$, 28%]], 1166 [[MNa$^+$, 100%]]; **7i** 1144 [[MH$^+$, 100%]], 1166 [[MNa$^+$, 68%]]; **7j** 1234 [[MH$^+$, 14%]], 1256 [[MNa$^+$, 100%]]; **7k** 1054 [[MH$^+$, 41%]], 1076 [[MNa$^+$, 100%]]; **7l** 1144 [[MH$^+$, 13%]], 1166 [[MNa$^+$, 100%]]; **7x** 1086 [[MNa$^+$, 100%]]; **7y** 1114 [[MH$^+$, 59%]], 1136 [[MNa$^+$, 100%]]; **7z** 1056 [[MNa$^+$, 100%]]. See Supporting Information for the corresponding ESI-MS/MS/MS spectra.

Quantitative Acylation of Resin-Bound Compounds via the Acid Chloride Method and Cleavage To Obtain Compound 9a. To a solution of 5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentanoic acid (51 mg, 0.137 mmol) in 3.5 mL of CH_2Cl_2 was added thionyl chloride (45 μL, 0.616 mmol, 4 equiv) at 0 °C. The solution was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the acid chloride was dried under high vacuum to provide 53.9 mg of acid chloride as a purple solid which was immediately used without further purification. A solution of the acid chloride (0.37 mL, 0.027 M in CH_2Cl_2, 5 equiv) was added to a mixture containing 10 mg of resin **6a**, 8 mg of DMAP, and 10 μL of DIPEA in 50 μL of CH_2Cl_2. The reaction mixture was shaken at room temperature for 4 h. The acylation was repeated using 3 equiv of the acid chloride. The beads were washed with DMF (5 × 30 min), MeOH (2 × 10 min), and CH_2Cl_2 (5 × 30 min) and were dried under high vacuum overnight to provide the red resin **8a**. Subsequently 1 mg of red resin **8a** was photocleaved in acetonitrile at 365 nm for 3 h to provide a red solution of **9a**. The solution was analyzed by electrospray mass spectrometry without any purification. MS-ES$^+$ *m/z* 1498 [[MH$^+$, 6%]], 1520 [[MNa$^+$, 100%]].

Quantitative Acylation of Resin-Bound Compounds 8b−l, 8x via the Acid Chloride Method and Cleavage To Obtain Acylated Compounds 9b−l, 9x. Analoguous procedures as described above for **8a** were used for the synthesis and characterization of acylated peptidosteroid derivatives **8b−l** and **8x**. In all cases, at the end of the synthesis a 1 mg sample was irradiated at 365 nm in acetonitrile to photolytically cleave the compound from the resin and the resulting solution was analyzed by electrospray mass spectrometry without any purification. MS-ES$^+$ *m/z* **9b** 1588 [[MH$^+$, 37%]], 1610 [[MNa$^+$, 100%]]; **9c** 1408 [[MH$^+$, 13%]], 1430 [[MNa$^+$, 100%]]; **9d** 1520 [[MNa$^+$, 100%]]; **9e** 1498 [[MH$^+$, 51%]], 1520 [[MNa$^+$, 100%]]; **9f** 1588 [[MH$^+$, 40%]], 1610 [[MNa$^+$, 100%]]; **9g** 1430 [[MNa$^+$, 100%]]; **9h** 1498 [[MH$^+$, 18%]], 1520 [[MNa$^+$, 100%]]; **9i** 1498 [[MH$^+$, 28%]], 1520 [[MNa$^+$, 100%]]; **9j** 1610 [[MNa$^+$, 100%]]; **9k** 1408 [[MH$^+$, 38%]], 1430 [[MNa$^+$, 100%]]; **9l** 1498 [[MH$^+$, 39%]], 1520 [[MNa$^+$, 100%]]; **9x** 1440 [[MNa$^+$, 100%]].

Synthesis of Resin-Bound Acylated Peptidosteroid 8a Using NF31 and Cleavage To Obtain Acylated Compound 9a. To 61 mg of resin **6a** was added a solution of NF31 (21 mg, 3.5 equiv) in 6.6 mL of acetonitrile. This slurry was shaken at room temperature (2 × 2 days). The beads were then washed with DMF (5 × 30 min), MeOH (2 × 30 min), and CH_2Cl_2 (5 × 30 min) and were dried under high vacuum to provide a red resin **8a**. The resin (theoretical loading of 0.192 mmol/g) was subsequently irradiated at 365 nm in 1,4-dioxane (containing 1% DMSO) under nitrogen (3 × 2 h). The filtrate was collected and concentrated, and the residue was purified by flash column chromatography (CH_2Cl_2/MeOH, 7:2, v/v) to provide 8 mg (43%) of **9a** as a red solid. IR (KBr) 3420, 2924, 2370, 1654, 1661, 1521 cm^{-1}; UV (λ_{max}, acetonitrile) 282, 484 nm; ^1H NMR (500 MHz, DMSO-d_6) δ 0.57 (3H, m), 0.62 (3H, d, J = 6.3 Hz),

0.64 (3H, s), 0.72 (3H, d, $J = 6.3$ Hz), 0.79 (3H, s), 1.11 (3H, t, $J = 6.9$ Hz), 1.21 (3H, m), 1.76 (3H, s), 1.95 (3H, s), 1.97 (3H, s), 2.45 (2H, t, $J = 1.8$ Hz), 2.76 (2H, m), 2.98 (2H, m), 3.20 (1H, br s), 3.50 (4H, m), 3.60 (5H, m), 3.90−3.95 (2H, m), 4.02 (1H, dd, $J = 10.9$, 7.2 Hz), 4.06 (2H, s), 4.16 (1H, dd, $J = 10.9$, 5.1 Hz), 4.42 (1H, m), 4.47 (1H, m), 4.51 (1H, m), 4.67 (1H, br s), 6.59 (2H, s), 6.78 (1H, s), 6.83 (2H, d, $J = 9.3$ Hz), 7.16 (6H, m), 7.29 (1H, br s), 7.32 (1H, br s), 7.54 (1H, s), 7.59 (1H, s), 7.78 (2H, d, $J = 9.2$ Hz), 7.88 (2H, d, $J = 9.0$ Hz), 8.08 (3H, m), 8.31 (2H, d, $J = 9.0$ Hz), 8.69 (1H, s), 8.96 (1H, br s); MS-ES$^+$ *m*/*z* 1520 [[MNa$^+$, 100%]].

Screening Conditions for Acylation of Members in Parallel Using NF31. Approximately 1 mg of resins **6a**−**l** and **6x**−**z** (corresponding to ca. 0.2 μmol of peptidosteroid) was incubated with a 2×10^{-3} M solution of NF31 in acetonitrile (200 μL, 0.04 μmol, 20% molar ratio to peptidosteroid). The resulting suspensions were shaken at room temperature for 6 h. The beads were washed with DMF (3×) and with MeOH (2 × 1 min). The beads were transferred to a small glass plate and photographed using a stereomicroscope (see Figure 4).

Transesterification of Acylated Compounds 8a−**l and 8x.** The transesterification rate of resins **8a**−**l** and **8x** was measured by following the increase in UV absorbance due to liberation of methyl 5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]-phenyl}amino-3-oxapentanoate at 480 nm. Therefore, to 1 mg resin was added 1 mL of MeOH, and the reaction was monitored at 25 °C for 20 h (see Figure 5).

Hydrolysis of Acylated Compounds 8a−**l and 8x.** The rate of hydrolysis of resins **8a**−**l** and **8x** was measured by following the increase in the UV absorbance due to liberation of 5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentanoic acid at 480 nm. Therefore, 1 mg resin was incubated in 1 mL of DMF/buffer pH 7 (1:1, v/v) and the reaction was monitored at 25 °C for 40 h (see Figure 6). The experiment was also performed at 65 °C. In this case pseudo-first-order rate constants were obtained by calculation using the Cary WinUV-Kinetics software program.

Screening Conditions for Acylation of the Artificially Created Model Library Using NF31. The 12 members **6a**−**l** (12 × 1 mg) together with 5 mg of **6x**, 5 mg of **6y**, and 5 mg of **6z** were mixed in a vessel. This mixture was shaken in DMF (1 × 5 min) and CH_2Cl_2 (1 × 5 min), washed with CH_2Cl_2, and dried under high vacuum. Approximately 6 mg of the pooled resin (corresponding to 0.53 μmol of Ser or His) was suspended in 50 μL of 2×10^{-3} M NF31 (0.1 μmol of NF31) in 400 μL of acetonitrile. This mixture was shaken at room temperature for 6 h, and subsequently the beads were washed with DMF (2×) and MeOH (2 × 45 min). A total of nine red beads and three colorless beads were collected and analyzed by ESI tandem MS after single-bead cleavage.

Screening Conditions for Deacylation of the Artificially Created Model Library. The 12 acylated red members **8a**−**l** were pooled (12 × 1 mg). This mixture was shaken in CH_2Cl_2 (2 × 5 min), filtered, and dried under high vacuum for 3 h. The pooled resin (1.5 mg) was incubated in 2 mL of DMF/buffer pH 7 (1:1, v/v) at 65 °C for 11 h. Two red beads and two white beads were collected, photolytically cleaved, and analyzed by ESI tandem MS.

Single-Bead Resin Cleavage and ESI Tandem MS Analysis. The beads were placed on a glass dish, incubated in DMF and observed under a microscope. A single bead (containing approximately 80 pmol of peptidosteroid) was picked up using the tip of a 10 μL Eppendorf Transferpette, transferred to a 35 mm × 6 mm i.d. heavy-walled glass tube and irradiated in MeCN at 365 nm for 3 h. MeCN was removed under vacuum, and the dried bead was mixed with 30 μL of MeCN/H_2O (1:1, v/v) (acylated samples are incubated for 1 h in MeOH at 55 °C prior to analysis). The resulting solution (approximately 2.7 μM) was infused in the ESI source at 2 μL/min without a make-up flow using a syringe pump. The MS settings were as follows: nitrogen sheath gas flow-rate, 30 (arbituary units); nitrogen auxiliary gas flow-rate, 5 (arbituary units); spray voltage, 4.25 kV; capillary temperature, 200 °C; capillary voltage, 39 V; tube lens offset, 35 V. The identity of the sequences was determined by the mass difference between fragmentation peaks (see Figure 8 for an illustrative example; ESI-MS/MS/MS spectra of compounds **7a**−**l** and **7x**−**z** have also been included in the Supporting Information).

Hydrolysis of NF31 Using Resin-Bound Peptidosteroids as Catalysts. To a solution of 500 μL of 3×10^{-3} M NF31 in CD_3CN and 250 μL of D_2O was added **6c** (GGS, 1.96 mg, 0.412×10^{-3} mmol). The hydrolysis of NF31 at 65 °C was then followed by NMR spectroscopy, and the conversion was calculated from integration of the characteristic ^{1}H NMR signals. This experiment was performed in the same way using **6y** (GPG, 1.98 mg, 0.412×10^{-3} mmol) and resin-bound N-acylated His (1.5 mg, 0.412×10^{-3} mmol).

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Supporting Information Available. Fifteen ESI-MS/MS/MS spectra for compounds **7a**−**l** and **7x**−**z**, illustrative NMR data obtained from 2D COSY and 2D NOESY spectra for compounds **7a** and **9a**, pictures illustrating the decay of the color as a function of time when His-containing beads were washed with MeOH, figures representing low-energy H-bonded conformations for compounds **6c** (GGS), **6g** (GSG), and **6k** (SGG) obtained via molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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