An Activated *O* → *N* Acyl Transfer Auxiliary: Efficient Amide-Backbone Substitution of Hindered "Difficult" Peptides

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Overcoming the phenomenon known as "difficult" synthetic sequences has been a major goal in solid-phase peptide synthesis for over 30 years. In this work the advantages of amide backbone-substitution in the solid-phase synthesis of "difficult" peptides are augmented by developing an activated N^{α}-acyl transfer auxiliary. Apart from disrupting troublesome intermolecular hydrogen-bonding networks, the primary function of the activated N^{α}-auxiliary was to facilitate clean and efficient acyl capture of large or β-branched amino acids and improve acyl transfer yields to the secondary N^{α}-amine. We found *o*-hydroxyl-substituted nitrobenzyl (Hnb) groups were suitable N^{α}-auxiliaries for this purpose. The relative acyl transfer efficiency of the Hnb auxiliary was superior to the 2-hydroxy-4-methoxybenzyl (Hmb) auxiliary with protected amino acids of varying size. Significantly, this difference in efficiency was more pronounced between more sterically demanding amino acids. The Hnb auxiliary is readily incorporated at the N^{α}-amine during SPPS by reductive alkylation of its corresponding benzaldehyde derivative and conveniently removed by mild photolysis at 366 nm. The usefulness of the Hnb auxiliary for the improvement of coupling efficiencies in the chain-assembly of difficult peptides was demonstrated by the efficient Hnb-assisted Fmoc solid-phase synthesis of a known hindered difficult peptide sequence, STAT-91. This work suggests the Hnb auxiliary will significantly enhance our ability to synthesize difficult polypeptides and increases the applicability of amide-backbone substitution.

Introduction

The phenomenon of "difficult" sequences has plagued the history of solid-phase peptide synthesis (SPPS)[1−6,67] and continues to be one of the most troublesome aspects of contemporary SPPS. Such sequences are generally identified by irregular or a series of unacceptable N^{α}-acylation or N^{α}-deprotection yields.[3] Although the occurrence and severity of difficult sequences cannot be predicted with confidence, it is generally thought the difficulty arises from the intermolecular association of resin-bound peptide chains into extended β-sheet type structures during sequential assembly.[4,7−9] The formation of such structures is thought to result in a significant proportion of N^{α}-amine groups becoming inaccessible to acylation or deprotection. Even though considerable advances in coupling methods,[10−12] resin properties,[13] and the choice of coupling solvents[8,14] have been made, the problem of difficult synthetic sequences has not been eliminated. Currently the challenge for peptide synthesis clearly lies in the synthesis of homogeneous tailored peptide segments (~40 residues) for the chemical synthesis of small proteins via chemoselective ligation strategies[15−17] and the construction of high quality combinatorial peptide libraries. Consequently, the development of new or improved synthetic strategies to overcome this long standing problem in SPPS remains a continuing goal.

One of the most powerful strategies used to combat difficult sequences is reversible amide-backbone substitution. This strategy involves *N*-substitution of one or more amide bonds several residues before the identified or expected difficult sequence in a peptide is encountered. Amide-backbone substitution appears to prevent or disrupt troublesome intermolecular hydrogen bonding net-

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works by the removal of the native amide hydrogen bond after *N*-substitution and also by alteration of the backbone conformation caused by the introduction of tertiary amide bonds. For this purpose, Sheppard and co-workers introduced the 2-hydroxy-4-methoxybenzyl (Hmb, **1**) N^{α}-auxiliary.[18−21] The usefulness of backbone substitution with the Hmb auxiliary has been demonstrated by the improved Fmoc syntheses of several difficult peptides[22−25] and suppression of solid-phase side reactions.[26−28] However, the full potential of amide-backbone substitution for difficult peptide synthesis has not been realized as only unhindered dipeptide sites are suited to incorporation of the Hmb auxiliary.[20]

Following its incorporation the Hmb auxiliary[29] has a second but equally important function, that is, facilitating *N*-acylation of the secondary N^{α}-amine. This is achieved by acyl capture of activated amino acids through the 2-hydroxyl moiety of the Hmb auxiliary via an internal base-catalyzed mechanism[18,20] followed by $O \rightarrow N$ intramolecular acyl transfer. This template assisted acyl capture and transfer concept was pioneered in the 1950s by Brenner[30,31] and Wieland,[32] who both realized that amide bond formation would be more efficient if the *N*- and *C*-termini were somehow brought and held close together (i.e., resulting in a pseudo increase in local concentration), so that reaction could take place. They independently investigated the facilitation of bimolecular peptide coupling reactions via intramolecular rearrangements through the use of similar "templates". Although superseded by other improved amino acid coupling methods, such as DCC-mediated couplings,[33] this concept reemerged in the early 1980s in the form of Kemp's thiol-capture ligation strategy, which employs a 4-hydroxy-6-mercaptodibenzofuran template.[34−36] Later this concept was further refined into native chemical ligation (NCL)[15,37] and more recently to peptide cyclization via ring contraction.[38,39] Significantly, even though these amide bond forming methods differ in their respective applications, they all have the same fundamental requirements for success: *quantitative acyl capture and efficient acyl transfer*.

With the Hmb auxiliary, acyl capture and acyl transfer reaction between adjacent small residues such as glycine or alanine is acceptable, but when more sterically demanding residues such as phenylalanine, leucine, and valine are encountered, the efficiency of N^{α}-acylation decreases to unacceptable levels.[20] With these more sterically demanding amide bond forming reactions the literature[20] and in-house results[68] indicate this problem is not due to incomplete *O*-acylation of Hmb auxiliary (acyl capture) but rather poor $O \rightarrow N$ acyl transfer reaction kinetics. Furthermore, the use of more reactive acylation agents such as symmetric anhydrides[20] or acid fluorides in toluene at elevated temperatures (60−80 °C)[40] does not provide a satisfactory or general solution to this problem. In practice, the poor acyl transfer characteristics of the Hmb auxiliary limits its use to peptides which contain relatively unhindered dipeptide residues before difficult sections. Moreover, where the preceding residues to the difficult region contain predominantly large or β-branched residues, such as Ile, Leu, Phe, and Val, the introduction and subsequent incompatibility of the Hmb auxiliary ironically adds to the difficulty of the synthesis even before the identified or expected difficult section is reached. Thus, to realize the full potential of amide-backbone substitution there is clearly a need for a new N^{α}-auxiliary with superior acyl transfer efficiency.

In this work we have sought to improve acyl transfer rates and yields in hindered difficult peptide synthesis by examining the effect of activated N^{α}-benzyl auxiliaries. Here we demonstrate that the 2-hydroxy-6-nitrobenzyl group (Hnb, **3**)[41] is an effective N^{α}-auxiliary for the assembly of hindered difficult peptides with significantly enhanced $O \rightarrow N$ acyl transfer efficiency when compared to the Hmb group. In a parallel study reported elsewhere,[41] we have found that the superior acyl transfer characteristics of the Hnb auxiliary can also be used to improve the cyclization of small constrained peptides. The Hnb auxiliary is readily introduced by reductive alkylation during routine SPPS and efficiently removed by mild photolysis in a manner orthogonal with respect to Fmoc chemistry. The Hnb auxiliary greatly increases the applicability of amide-backbone substitution and our ability to overcome the long standing problem of difficult peptide sequences.

Results and Discussion

Design of Activated N^{α}-Acyl Transfer Auxiliaries. N^{α}-Auxiliary-assisted acyl transfer reactions can be divided into two steps: acyl capture and acyl transfer. For quantitative N^{α}-acylation after the introduction of the Hmb auxiliary (**1**), complete *O*-acylation of the *o*-hydroxyl group is first required then followed by $O \rightarrow N$ acyl transfer. Previously, Hmb-assisted N^{α}-acylation

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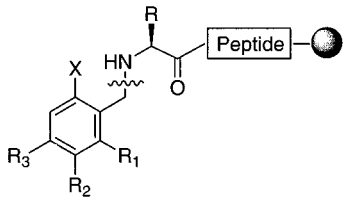
Scheme 1. Chemical Structures of N^{α}-Benzyl-Substituted Auxiliaries (1−6) When Attached to the *N*-Terminal Residue of a Peptide, Where R Indicates Any Amino Acid Side Chain Functionality



1. X = OH, R_1 = H, R_2 = H, R_3 = OCH_3
2. X = OH, R_1 = H, R_2 = NO_2, R_3 = H
3. X = OH, R_1 = NO_2, R_2 = H, R_3 = H
4. X = SH, R_1 = H, R_2 = H, R_3 = H
5. X = SH, R_1 = H, R_2 = NO_2, R_3 = H
6. X = Cl, R_1 = H, R_2 = NO_2, R_3 = H

proceeds well between small residues such as glycine or alanine but not for large or β-branched residues.[20] It appears likely that this inefficiency arises primarily from slow $O \rightarrow N$ acyl transfer with the underlying cause being the relative low reactivity of the methoxyphenyl ester. With this in mind, we considered whether superior acyl transfer rates could be achieved with the use of an activated auxiliary. More specifically, we sought to improve N^{α}-acylation yields by incorporating an electron-withdrawing group. A nitro substituent (1) decreases the pK_a of the *ortho* ionisable group[42] and thereby increases its availability for *O*- or *S*-acylation and (2) enhances the reactivity of the subsequent aryl ester for aminolysis,[43,44] as was observed with aminolysis of *p*-nitrophenol esters of amino acids and with intramolecular $O \rightarrow N$ acyl transfer reactions studied by Kemp and Offer.[34,42,45] Moreover, if the dissociation constants of methoxyphenol and nitrophenols are used as a relative measure of phenolic leaving group efficiency during the acyl transfer reaction, we find *p*-methoxyphenol (Hmb-like alcoholic ester component) has a pK_a value of 10.24, whereas *m*-nitrophenol and *p*-nitrophenol have significantly lower pK_a values of 8.28 and 7.15, respectively.[46−48] On this basis it appeared to us that nitrobenzyl auxiliaries would act as better leaving groups than methoxybenzyl-based auxiliaries (i.e., Hmb) during the acyl transfer reaction. This hypothesis was examined experimentally by evaluating the following activated derivatives in terms of acyl capture and acyl transfer efficiency: N^{α}-2-hydroxy-5-nitrobenzyl (2,5-Hnb, **2**), N^{α}-2-hydroxy-6-nitrobenzyl (Hnb, **3**), N^{α}-2-mercaptobenzyl (Mb, **4**), and N^{α}-2-mercapto-5-nitrobenzyl (Mnb, **5**) (Scheme 1).

Synthesis and Incorporation of N^{α}-Auxiliaries. Two approaches were investigated for the incorporation of these benzyl-based auxiliaries, namely, reductive alkylation[29] and the Fukuyama reaction.[49] However, as a result of competing side reactions of the Fukuyama approach with unprotected *o*-mercaptobenzyl alcohols we chose to use reductive alkylation chemistry for the introduction of the auxiliaries. This synthetic strategy is compatible with Boc- and Fmoc-amino acids but requires use of the benzaldehyde derivatives of the auxiliaries instead of their benzyl alcohols. The various substituted benzaldehydes used in this study were prepared as follows. 2-Mercaptobenzaldehyde (**4**) was synthesized from thiosalicylic acid in good yield.[50] 2-Mercapto-5-nitrobenzaldehyde (**5**) auxiliary was prepared by reaction of 2-chloro-5-nitrobenzaldehyde and sodium disulfide.[51] 2-Hydroxyl-6-nitrobenzaldehyde (**3**) was synthesized from *m*-nitrophenol via the Duff reaction,[52] which involves reaction with hexamethylenetetramine in acidic conditions to give the auxiliary **3** in 34% yield. 2-Chloro-5-nitrobenzaldehyde (**6**), 2-hydroxy-4-methoxybenzaldehyde (**1**), and 2-hydroxyl-5-nitrobenzaldehyde (**2**) were obtained commercially.

Evaluation of Auxiliary Acyl Transfer Efficiency. To establish the relative $O \rightarrow N$ or $S \rightarrow N$ acyl transfer efficiencies of the auxiliaries, a series of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)-mediated[10] amino acid acylation experiments were carried out on solid support.[69] The general reaction pathway for acylation in these experiments is similar to the proposed mechanism for Hmb-assisted N^{α}-acylation (Scheme 2).[20] Initially, the *o*-oxygen or *o*-sulfur atom of the benzyl auxiliary is acylated by the HBTU-activated amino acid to yield **8**. Following this, the acyl group then migrates to the N^{α}-nitrogen atom to form an amide bond **9**. The *o*-oxygen or *o*-sulfur atom, in the presence of excess acylating agent, then becomes available for acylation for a second time with diacylation leading to **10** (Scheme 2).

The resin-bound tripeptide Ala-Gly-Phe was assembled using standard Fmoc-SPPS protocols[53] on chlorotrityl resin,[54,55] and each benzaldehyde auxiliary (**1**−**3**, **6**) incorporated by reductive alkylation. The peptide-resin of each N^{α}-auxiliary substituted peptide was divided into three separate portions and subjected to acylation with 3 equiv of 0.5 M HBTU-activated Fmoc-Ala, Fmoc-Phe, or Fmoc-Val in DMF. Aliquots were removed for analysis after 1, 10, and 60 min. Following reaction, the peptide-resins were washed with DMF, base-treated with piperidine/DMF/water, washed with DMF and then DCM, dried, and cleaved with 0.5% TFA in DCM for 30 min. The base treatments were employed to remove *O*-aryl esters present (**8** and **10**), prior to TFA cleavage,[70] to distinguish between auxiliary (*O*) and N^{α}-amino acylation (Scheme 2). The products were then identified by ES-MS or LC/MS analysis and quantified by reversed phase-HPLC peak integration (Table 1).

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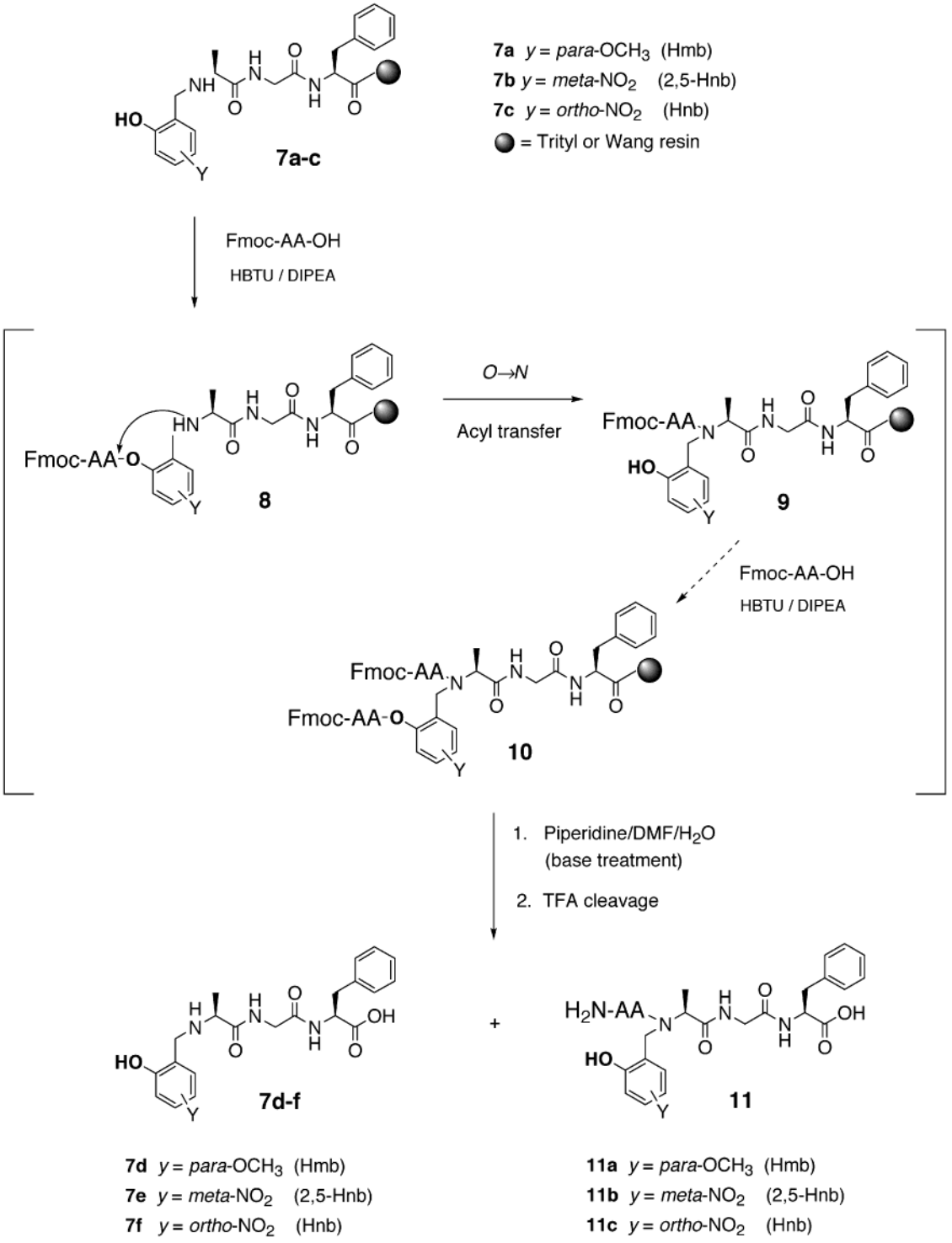
Scheme 2 Proposed Reaction Scheme for the Acylation Reaction of N^{α}-(auxiliary)-Ala-Gly-Phe-resin (7a, 7b, or 7c) with HBTU-Activated Amino Acids[a]



[a] To exclusively determine N^{α}-amino acylation (**11**) a piperidine/DMF/H_2O treatment is carried out before TFA cleavage to hydrolyze any *O*-aryl or *S*-aryl esters.

Initially to determine the extent of direct N^{α}-acylation using HBTU-activated amino acids acylation experiments were carried out with a control auxiliary, N^{α}-2-chloro-5-nitrobenzyl (**6**). As expected no appreciable amount (<1%) of secondary N^{α}-acylation had occurred after 30 min with HBTU-activated alanine, phenylalanine, or valine. Next, the presence of an "inactivated" *o*-hydroxy group as with the Hmb auxiliary (**1**) was examined. We found the formation N^{α}-acylated products did occur by acyl transfer albeit at relatively slow rates. Hmb-mediated acyl transfer proceeds at a reasonable rate for alanine after 1 h (92%), but the transfer efficiency diminishes sharply with increasing steric bulk on amino acid side-chains, as with phenylalanine (48%) or valine (12%). These observations agree well with acylation studies carried out by others[20] and also re-emphasizes the need for *N*-terminal auxiliaries for SPPS with improved acyl transfer efficiency.

In contrast to the Hmb auxiliary, the presence of an

Table 1. Distribution of N^{α}-Acylation Products on the Ala-Gly-Phe Sequence by N^{α}-Auxiliary-Directed $O \rightarrow N$ Acyl Migration

		N^{α}-(auxiliary)Ala-Gly-Phe N^{α}-acylation yield (%)		
auxiliary	*t* (min)	alanine	phenylalanine	valine
6 (control)	30	<1	<1	<1
1 (Hmb)	1	52	39	9
	10	53	42	10
	60	92	48	12
2 (2,5-Hnb)	1	74	42	27
	10	92	79	60
	60	97	85	95
3 (Hnb)	1	80	88	35
	10	93	93	65
	60	97	93	88

o-hydroxyl group in combination with the electron-withdrawing nitro substituent on the benzyl ring of an auxiliary significantly enhances acyl capture and transfer efficiency. *O*-Acylation of both the 2,5-Hnb (**2**) and Hnb (**3**) auxiliaries with HBTU-activated alanine and phenylalanine is essentially complete in less than 1 min, whereas valine requires only 10 min for greater than 95% *O*-acylation.[71] Subsequent acyl transfer (N^{α}-acylation) mediated by the 2,5-Hnb auxiliary (**2**) was rapid for all three amino acids. For alanine and phenylalanine, N^{α}-acylation products formed rapidly, and the reaction was near completion after 10 min. The superior $O \rightarrow N$ acyl transfer efficiency of the 2,5-Hnb auxiliary over the Hmb auxiliary can be readily observed by comparing the formation rate of the desired N^{α}-acylated peptide (**11a** and **11b**) in the crude cleavage RP-HPLC profiles during the reaction time course (Figure 1).

In a manner similar to that with the 2,5-Hnb auxiliary, high alanine, phenylalanine, and valine acyl transfer efficiencies were also observed with the Hnb auxiliary (**3**) after 1 h (Figure 2). Importantly, for the more sterically demanding acylation of valine onto N^{α}-(Aux)-Ala-Gly-Phe the yield with both the 2,5-Hnb (>95%) and Hnb (88%) auxiliaries are nearly 8 times higher than with the Hmb auxiliary (**1**, 12%) under identical experimental conditions. The position of the nitro-substituent on the aromatic ring of the auxiliary does not appear to play a crucial role (Table 1). Racemization during the acyl transfer reaction was not evident by chiral amino acid analysis.

With the aim of further enhancing acyl transfer rates preliminary studies were undertaken using *o*-mercapto-substituted benzyl groups. In a manner similar to the studies with hydroxyl-based auxiliaries (**2** and **3**) we have found that the nitro-activated mercapto auxiliary, 2-mercapto-4-nitrobenzyl (Mnb, **5**), is also clearly superior to its "inactivated" 2-mercaptobenzyl analogue (**4**).[56] A more detailed study of mercapto-based auxiliaries is in progress.

To determine if the activating effect of the nitro substituent could also improve acyl transfer rates and yields with more sterically demanding residues a second set of acylation experiments were carried out. In these experiments, auxiliaries **1**−**3** were each introduced onto the resin-bound tetrapeptide, Val-Ala-Gly-Phe, by reductive alkylation and subjected to acylation by HBTU-activated Fmoc-Gly, Fmoc-Phe and Fmoc-Val. As a result of the inherent difficulty associated with the acylation

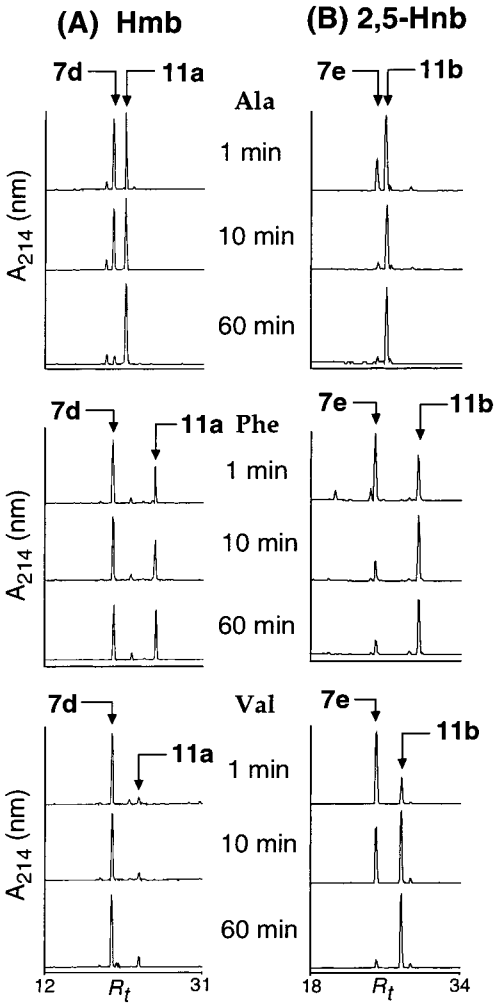


Figure 1. Comparison of $O \rightarrow N$ acyl transfer rates for the Hmb (**1**) and 2,5-Hnb (**2**) auxiliaries by RP-HPLC analysis. (A) RP-HPLC profiles of the crude cleavage products after coupling of Fmoc-Ala, Fmoc-Phe, and Fmoc-Val to N^{α}-(Hmb)-Ala-Gly-Phe-trityl resin (**7a**), piperidine base treatment at $t = 1$, 10, and 60 min, and TFA cleavage. (B) RP-HPLC profiles of the crude cleavage products after coupling of Fmoc-Ala, Fmoc-Phe, and Fmoc-Val to N^{α}-(2,5-Hnb)-Ala-Gly-Phe-trityl resin (**7b**), piperidine base treatment at $t = 1$, 10, and 60 min, and TFA cleavage. The non-N^{α}-acylated peptides (starting material) for the Hmb- and 2,5-Hnb-substituted Ala-Gly-Phe peptides are labeled **7d** and **7e**, respectively. The desired N^{α}-acylation products for the Hmb- and 2,5-Hnb-substituted Ala-Gly-Phe peptides are labeled **11a** and **11b**, respectively. The products were separated on a Vydac reversed-phase C-18 (5 μm, 300 Å, 0.46 cm × 15 cm) column using a linear 0−80% buffer B gradient over 40 min at a flow rate of 1 mL/min.

of sterically hindered secondary amines the acyl transfer reaction time course in these experiments was increased to 1, 6, and 24 h. Following acylation, the peptide-resins were subjected to piperidine base treatment to exclusively observe the N^{α}-amino acylation products (Scheme 2) and then cleaved with 0.5% TFA in DCM for 30 min. The products were identified by ES-MS or LC/MS analysis and quantified by RP-HPLC peak integration (Table 2).

From the acylation results listed in Table 2, it is clear that the nitro-activated auxiliaries strongly enhance $O \rightarrow N$ acyl transfer rates and yields with respect to the Hmb auxiliary (**1**). In all three cases the 2,5-Hnb auxiliary (**2**) and Hnb (**3**) were superior acyl transfer auxiliaries. The 2,5-Hnb-assisted acyl transfer of glycine onto

(56) In a recent publication, Offer and Dawson (*Organic Lett.* **2000**, *2*, in press) demonstrate the use of the 2-mercaptobenzyl group in a chemical ligation approach.

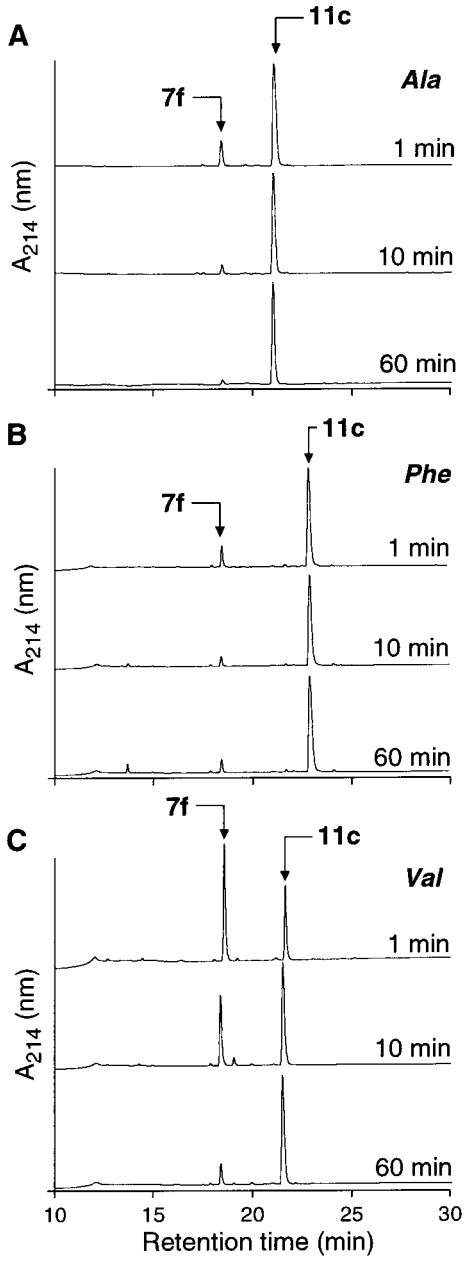


Figure 2. Reversed-phase HPLC analysis of Hnb-assisted *O* → *N* acyl transfer rates for HBTU-activated Fmoc-Ala, Fmoc-Phe, and Fmoc-Val to N^{α}-(Hnb)-Ala-Gly-Phe-trityl resin (**7c**) following piperidine base treatment at $t = 1$, 10, and 60 min, and TFA cleavage. The non-N^{α}-acylated peptide (starting material) is labeled **7f**, whereas the desired N^{α}-acylation products for the Hnb-substituted Ala-Gly-Phe peptide are labeled **11c**. The products were separated on a Vydac reversed phase C-18 (5 μm, 300 Å, 0.46 cm × 15 cm) column using a linear 0−80% buffer B gradient over 40 min at a flow rate of 1 mL/min.

valine proceeded rapidly and was essentially complete (>98%) within 1 h, whereas phenylalanine and valine required 24 h for greater than 95% completion. The Hnb auxiliary (**3**) also induced rapid acyl transfer but nevertheless still required 24 h for approximately 95% N^{α}-acylation for all three amino acids. On the other hand, Hmb-assisted acyl transfer resulted in unacceptably low N^{α}-acylation yields, especially with phenylalanine and valine, even though *O*-acylation proceeds in approximately 50% and 90% yield after 1 h, respectively. More specifically, the Hmb-assisted N^{α}-acylation of valine only proceeds in 23% yield after 24 h and is approximately 4 times lower than with either the 2,5-Hnb (95%) or Hnb (93%) auxiliary. These results strongly indicated that the 2,5-Hnb and Hnb auxiliaries could be of considerable value for the chain assembly of difficult peptide sequences, especially when β-branched or bulky residues are predominant. As a result, we decided to investigate the feasibility of using the 2,5-Hnb (**2**) or Hnb (**3**) auxiliaries in the solid-phase synthesis of a difficult peptide. However, as removal of the auxiliary is essential for reversible *N*-substitution, efficient removal methods of the auxiliaries were sought.

Table 2. Distribution of N^{α}-Acylation Products on the Val-Ala-Gly-Phe Sequence by N^{α}-Auxiliary-Directed *O* → *N* Acyl Migration

		N^{α}-(auxiliary)Val-Ala-Gly-Phe N^{α}-acylation yield (%)		
auxiliary	*t* (h)	glycine	phenylalanine	valine
1 (Hmb)	1	65	4	ND[a]
	6	72	17	<1
	24	77	54	23
2 (2,5-Hnb)	1	>98	37	11
	6	>99	91	42
	24	>99	97	95
3 (Hnb)	1	66	38	31
	6	87	86	75
	24	94	94	93

[a] ND indicates N^{α}-acylation product not detected.

Removal of the Hnb Auxiliary. The next phase of this study focused on the identification of methods for the facile and efficient removal of the 2,5-Hnb (**2**) and Hnb (**3**) auxiliaries when present as amide substituents. We found that mild photolysis was the most convenient and effective method for removal of the Hnb auxiliary. Photolysis of model *N*-Hnb substituted peptides from the above acylation experiments at 366 nm in methanol resulted in the rapid elimination of the auxiliary to generate native amide bonds (data not shown). Other solvent systems such as acetonitrile/water, dioxane, DMF, DMF/water, MeOH/water, and toluene can also be used.[41] The photolytic cleavage mechanism presumably proceeds via an internal oxidation−reduction process, where the auxiliary leaves as the nitrosobenzaldehyde.[57] The expelled nitrosobenzaldehyde is then prone to further transformation in solution to the diazoxybenzene derivative. It has been suggested that this derivative may act as an internal light filter and slow the rate of cleavage,[58,59] however, in our hands, this problem was not encountered during the removal of the Hnb auxiliary. To minimize the possibility of cleavage side reactions during photolysis of the Hnb auxiliary the use of amine-based scavengers (3−5%), such as lysine, is recommended. No general and effective cleavage method for the removal of the 2,5-Hnb auxiliary has been found. This drawback with the 2,5-Hnb auxiliary thus makes the Hnb auxiliary the most appropriate choice for routine SPPS at the present time.

Hnb-Assisted Difficult Peptide Synthesis: Synthesis of STAT-91(699−709). In principle, the Hnb (**3**) and Hmb (**1**) groups should have a similar effect on

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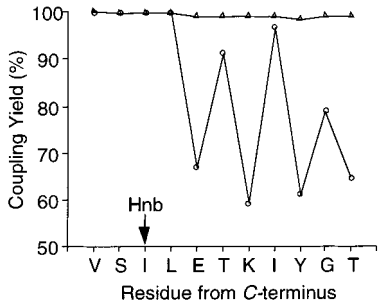

Figure 3. Comparison of the coupling yields for the Fmoc chain-assembly of STAT-91(699−709), **12** and **12a**, using (A) standard 10 min HBTU coupling protocols[53] (−○−), which resulted in an average coupling yield of 83%, and (B) Hnb-assisted 10 min HBTU coupling protocols, with the incorporation of the Hnb auxiliary at Ile^{707} (−Δ−). Average coupling yield, 99.6%.

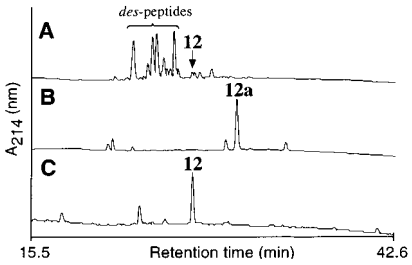

Figure 4. Comparison of the RP-HPLC traces of the crude STAT-91(699−709) cleavage products using (A) standard 10 min HBTU/Fmoc coupling protocols, where the major products correspond to the expected deletion peptides, and (B) Hnb-assisted 10 min HBTU/Fmoc coupling protocols with the incorporation of the Hnb auxiliary at Ile^{707} (−○−). (C) Photolysis of Ile^{707}(N^{α}-Hnb)-STAT-91(699−709) at 366 nm in 1%AcOH/MeOH for 3 h in shown section.

disrupting hydrogen-bonding networks, since they both alter the backbone structure of growing peptide chains and remove a backbone hydrogen-bond donor. To demonstrate this beneficial effect the STAT-91(699−709) sequence, TGYIKTELISV (**12**), which we have previously reported as difficult in standard Fmoc- and Boc-SPPS,[60] was assembled using standard chain assembly protocols[53] and also with the assistance of *N*-Hnb backbone substitution under identical experimental conditions. The STAT-91 peptide was selected because it does not contain a relatively unhindered site before the difficult section is encountered and thus precludes the use of the Hmb auxiliary.

Using standard Fmoc/*tert*-butyl 0.5 M HBTU/DIEA 10 min coupling protocols in DMF,[53] the chain assembly of STAT-91(699−709) proceeds well until residues Glu^{705} and Thr^{704}, which only couple in 67% and 91% yield, respectively (Figure 3). This is then followed by a 59% coupling yield at Lys^{703}, which only increases to 62% upon recoupling after 1 h.[60] Furthermore, substitution of HBTU with HATU did not significantly improve the coupling yield for this residue. Tyrosine^{700} also gave a poor coupling yield (61%) that also does not improve significantly even after recoupling for 1 h. In short, the chain-assembly of STAT-91(699−709) under standard coupling conditions proceeded with an unacceptable average acylation yield of 83%. However, when the Hnb (**3**) auxiliary is incorporated onto the backbone of the growing peptide at Ile^{707} (the third residue from the resin-linker) by reductive alkylation, subsequent chain-assembly proceeded in high efficiency using the same coupling conditions. With the assistance of the Hnb auxiliary,[72] the average coupling yield for the assembly of Ile^{707}(N^{α}-Hnb)-STAT-91(699−709) (**12a**) increased to 99.6% as determined by the quantitative ninhydrin assay (Figure 3). The HBTU-mediated N^{α}-acylation of Leu^{706} onto the N^{α}-Hnb-substituted Ile^{707} was monitored by ES-MS and RP-HPLC analysis of a cleaved resin sample before continuation. The Fmoc-Leu to N^{α}-(Hnb)Ile-Ser-Val-resin acyl transfer reaction was approximately 50% complete after 1 h but apparently *quantitative* after 24 h as determined by RP-HPLC analysis (data not shown). By comparison, for the analogous Fmoc-Leu to N^{α}-(Hmb)-Ile-Ser-Val-resin acyl transfer reaction, N^{α}-acylation only proceeds in 21% yield after 24 h (under identical reaction conditions). This poor Hmb-assisted N^{α}-acylation yield further highlights the limitation of the Hmb auxiliary in the synthesis of hindered or β-branched containing difficult peptides.

Following chain-assemblies, the crude TFA cleavage material of the standard and Hnb-assisted STAT-91(699−709) syntheses were analyzed by ES-MS and RP-HPLC. When employing standard Fmoc/HBTU synthesis protocols no appreciable amount (<1%) of the target STAT-91 peptide was found in the crude product by either ES-MS or RP-HPLC analysis (Figure 4A). With the Hnb-assisted synthesis, the Hnb-substituted STAT-91(699−709) peptide (**12a**) was the major component in the crude cleavage material as determined by ES-MS and RP-HPLC (Figure 4B). The Ile^{707}(N^{α}-Hnb)-STAT-91(699−709) peptide, was then readily photolyzed at 366 nm for 3 h to give the target peptide (**12**) in good yield (Figure 4C). From this comparison, it appears that the Hnb auxiliary can be used to significantly improve the chain-assembly efficiencies of difficult peptides in a manner similar to that of Hmb-substitution but with the key advantage of greatly improved acyl transfer efficiency.[20]

Conclusions

Until now the applicability of backbone substitution for the improved synthesis of hindered difficult peptides has been severely restricted by the relatively poor $O \rightarrow N$ acyl transfer efficiency of the Hmb auxiliary. To address this problem here we have reported that the nitro-activated N^{α}-2,5-Hnb and N^{α}-Hnb auxiliaries facilitate efficient $O \rightarrow N$ acyl transfer, especially with β-branched amino acids, in a manner clearly superior to that of the Hmb auxiliary. On the basis of our results we rank the relative abilities of the examined auxiliary to facilitate acyl transfer in the following order: 2,5-Hnb $\approx$ Hnb $\gg$ Hmb. The superior acyl transfer efficiency of the Hnb auxiliary (**3**) now increases the applicability of amide-backbone substitution to peptides which predominantly contain relatively large side-chain functionality and β-branched amino acids before the difficult sequence. Incorporation of the N^{α}-Hnb auxiliary is readily achieved by reductive alkylation during SPPS in a manner compatible with common side chain protection (*tert*-butyl, benzyl, and Boc) and subsequently removed by mild photolysis.[73] It is expected the Hnb auxiliary can also be introduced during SPPS in a preformed manner similar to commercially available *N*,*O*-bis(Fmoc)-*N*-(Hmb)amino

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acids derivatives. Since its removal is orthogonal to Fmoc chemistry, the Hnb auxiliary also increases the flexibility and versatility of backbone protection for various applications. For example, the Hnb auxiliary may also prove to be useful for amino- or amide-protection in solid-phase organic synthesis, peptide cyclization,[41] and peptide ligation. Furthermore, the Hnb auxiliary could also be used in solubilizing protected peptides or poorly soluble unprotected peptides, as well as improving the quality of combinatorial peptide libraries. In practical terms, use of the Hnb auxiliary should (1) significantly increase the number of dipeptide sites that are compatible with backbone substitution and (2) reduce the reaction time required for acceptable amino acid acylation immediately following and thus minimize the risk of side reactions such as racemization becoming kinetically competitive. In summary, it appears the Hnb auxiliary will significantly enhance our ability to synthesize difficult polypeptides more easily and increase the applicability of amide-backbone protection.

Experimental Section

Materials and Methods. Wang resin, N^{α}-*tert*-butoxycarbonyl (Boc)-L-amino acids and N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc)-L-amino acids were purchased from Auspep (Melbourne, Australia), Novabiochem (San Diego, CA), and the Peptide Institute (Osaka, Japan). Chlorotrityl resin[54,55] was purchased from PepChem (Tubingen, Germany). Peptide synthesis grade dichloromethane, *N*,*N*-diisopropylethylamine, *N*,*N*-dimethylformamide, piperidine, and trifluoroacetic acid were obtained from Auspep (Melbourne, Australia). 5-Chloro-2-nitrobenzaldehyde, 2-hydroxy-5-nitrobenzaldehyde, *p*-cresol, *p*-thiocresol, hydrogen peroxide, thiosalicylic acid, *o*-nitrobenzenesulfonyl chloride, diethyl azodicarboxylate (DEAD), tetrahydrofuran, triphenylphosphine, sodium cyanoborohydride, and sodium borohydride were purchased from Aldrich or Fluka (Sydney, Australia). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Richelieu Biotechnologies (Quebec, Canada). Deionized water was used throughout and was prepared by a Milli-Q water purification system (Millipore-Waters). Screw-cap glass peptide synthesis reaction vessels (10 mL) with sintered glass filter frit were obtained from Embell Scientific Glassware (NSW, Australia). Argon, helium, and nitrogen (all ultrapure grade) were from BOC Gases (Queensland, Australia). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz Gemini in CD_3OD or d_6-DMSO, and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$. Reversed-phase high performance liquid chromatography was performed on a Waters 600E solvent delivery system equipped with a 484 UV-absorbance detector and recorded on an Apple Macintosh computer using model 600 software (Applied Biosystems Inc.). RP-HPLC was performed using a detection wavelength of 214 on Vydac analytical C-18 (5 μm, 0.46 cm $\times$ 15 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% CH_3CN, 10% H_2O, 0.09% TFA) over 40 min at a flow rate of 1 mL/min.

Mass Spectrometry. Mass spectra were acquired on a PE-Sciex API−III triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 μL) were injected into a moving solvent (30 μL/min; 50/50 CH_3CN/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d. $\times$ 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100−120 μm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range of 250−2000 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed *m*/*z* values using the Biomultiview 1.2 software package (PE-Sciex Toronto, Canada). Calculated theoretical monoisotopic and average masses were determined using the MacBioSpec program (PE-Sciex Toronto, Canada). LC/MS runs were carried out using a linear gradient on a 140B ABI dual syringe pump solvent delivery system and a Zorbax reversed-phase C-18 (5 μm, 300 Å, 0.21 cm $\times$ 5 cm) column at a flow rate of 200 μL/min. Samples (typically 5 μL of 1 mg/mL solution) were loaded directly on the column, and the eluent was directly connected to the mass spectrometer via a 30 cm, 75 μm i.d. fused silica capillary. The application of Turbo Ionspray (5 L/min N_2 at 500 °C) allowed the introduction of the total eluent without splitting and loss in sensitivity. Acquisition parameters were as described above.

Peptide Synthesis. Peptides were chemically synthesized stepwise using 0.5 M HBTU-DMF activation protocols for Fmoc and Boc chemistry as previously described.[53,61] The syntheses were performed on *p*-benzyloxybenzyl alcohol (Wang) resin or chlorotrityl resins. The following amino acid side chain protection was used: Fmoc-Glu(*O*-*tert*-butyl)-OH, Fmoc-Lys-(Boc)-OH, Fmoc-Ser(*O*-benzyl)-OH, Fmoc-Thr(*O*-*tert*-butyl)-OH, and Fmoc-Tyr(*O*-*tert*-butyl)-OH. Each residue was coupled for 10 min, and coupling efficiencies were determined by the quantitative ninhydrin reaction[62] and the qualitative isatin test.[63]

Reductive Alkylation. Typical Procedure for the Incorporation of the Hnb Auxiliary (3) onto a Growing Peptide Chain during SPPS. A 0.1 mmol portion of the N^{α}-deprotected peptide-resin was washed with 10 mL of DMF/MeOH/AcOH (9:9:2), drained, and then mixed with 3 equiv of the substituted benzaldehyde in 1 mL of DMF/MeOH (1:1) for 30 min. The solution was then drained, and imine formation reaction was repeated. Following drainage, the resin was washed briefly with DMF, and then 5 equiv of sodium cyanoborohydride (for mercaptobenzaldehydes) or sodium borohydride (for hydroxybenzaldehydes) in DMF/MeOH/AcOH (9:9:2) was added and mixed at room temperature for 15 min. The resin was then drained and washed successively with DMF, DMF/H2O, H_2O, MeOH/DCM, DCM, and DMF. A few milligrams (~3 mg) was then taken, dried, cleaved with 97% aqueous TFA, dissolved in 30% buffer B, filtered, and then immediately analyzed by ES-MS and RP-HPLC to determine reaction completion. If necessary, the procedure is simply repeated.

Acylation Experiments. The Ala-Gly-Phe and Val-Ala-Gly-Phe sequences were assembled on chlorotrityl resin (0.96 mmol/g, PepChem) using standard Fmoc/HBTU protocols.[53,55] The resin was then divided into three portions in separate reaction vessels and swollen in DMF for 10 min. Three equivalents of Fmoc-protected glycine, alanine, phenylalanine, and valine were coupled to Ala-Gly-Phe-resin or Val-Ala-Gly-Phe-resin using 2.95 equiv of 0.5 M HBTU in DMF with 4 equiv of DIEA for 1, 10, and 60 min or 1, 6, and 24 h, respectively. To examine N^{α}-acylation exclusively and also remove Fmoc groups, resin samples before cleavage were subjected to 2 cycles of 5 min piperidine/DMF (1:1) and 5 min DMF/piperidine/H_2O (4:4:2) treatments (to hydrolyze *S*- or *O*-esters) and then dried with DCM/MeOH (1:1). Trityl resin samples were cleaved with 0.5% TFA in DCM for 30 min. The TFA cleavage solutions were evaporated with a stream of nitrogen, and the product was dissolved in 100 μL of 50% buffer B. Samples were centrifuged, and supernatant was collected and then immediately analyzed by RP-HPLC and ES-MS or LC/MS.

Photolysis. Photolysis (1−50 mg scale) was carried out in a CAMAG UV-cabinet II, with a lamp wavelength of 366 nm and power of 0.25 A for 2−3 h. Typically 1 mM peptide solutions in 1% AcOH/MeOH were placed in an uncovered wide-mouth vial on a white mat.

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STAT-91(699−709) Syntheses (12 and 12a). The standard and Hnb-assisted synthesis of STAT-91(699−709) was carried out using Fmoc chemistry on Fmoc-Val-Wang resin (Applied Biosystems Inc., 0.70 mmol/g, 0.1 mmol synthesis scale). The standard assembly using 10 min couplings times resulted in an average coupling yield of 83%. The Hnb-assisted Fmoc chain-assembly of STAT-91(699−709)[72] was also performed using 10 min coupling times with an average coupling yield of 99.6%. The Hnb auxiliary was introduced at Ile^{707} by reductive amination (2 × 3 h imine formation reaction using 5 equiv Hnb) and was then followed by the 0.5 M HBTU coupling of Leu^{706} in DMF. N^{α}-Acylation of Leu^{706} onto Ile^{707}(N^{α}-Hnb)-STAT-91(708−709)-resin was complete after 24 h as determined by ES-MS and RP-HPLC analysis following TFA cleavage of a small resin sample. To maintain experimental consistency, Ile^{707} in the standard synthesis was also coupled with HBTU for 24 h. In both cases, the final peptide-resin product was cleaved with 97% aqueous TFA at room temperature for 1 h. After evaporation of the TFA in vacuo, the crude product was washed with cold diethyl ether (2 × 5 mL), dissolved in 30% B (5 mL), and lyophilized. Ile^{707}(N^{α}-Hnb)-STAT-91(699−709) (**12a**) ES-MS: M_r 1374.1 Da, calcd for $C_{63}H_{99}N_{13}O_{21}$ 1373.7 Da (monoisotopic); The Hnb-substituted STAT peptide (**12a**) was then photolyzed as described above to give the target peptide in 76% yield after RP-HPLC purification. STAT-91(699−709) (**12**) ES-MS: M_r 1222.7 Da, calcd for $C_{56}H_{94}N_{12}O_{18}$ 1222.68 Da (monoisotopic).

Synthesis of Auxiliaries. 2-Hydroxy-6-nitrobenzaldehyde (Hnb) (3). Hnb was synthesized as described by Harayama et al.[52] Briefly, hexamethylenetetramine (7.10 g, 50.7 mmol, AJAX chemicals) was added to a stirred solution of *m*-nitrophenol (7.0 g 50.3 mmol, Sigma) in 85% polyphosphoric acid (400 mL, Fluka) at 100 °C, and the mixture was stirred for 45 min. After cooling, the reaction mixture was diluted with cold water, extracted with ethyl acetate, and dried in vacuo. The residue in chloroform was then chromatographed on silica gel (Merck flash silica) and eluted with *n*-hexane, *n*-hexanes−ethyl acetate (9:1) to give 2.3 g of 2-hydroxy-6-nitrobenzaldehyde (34% yield, yellow solid). ^{1}H NMR (300 MHz, d_6-DMSO, ppm) 7.64 (t, J = 8.5 Hz, 1H$_{ar}$), 7.33 (d, J = 8.2 Hz, 1H$_{ar}$), 7.27 (d, J = 8.2 Hz, 1H$_{ar}$), 10.27 (s, 1H, CHO), 11.48 (broad, 1H, OH); ^{13}C 188.67 (CHO), 160.28 (C$_{ar}$), 135.39(C$_{ar}$), 133.49(C$_{ar}$), 121.43(C$_{ar}$), 114.11(C$_{ar}$). **2,2′-Dithiobenzylaldehyde (4)** was prepared as described by Kasmai and Mischke in 38% yield:[50,64] ^{1}H NMR (300 MHz, d_6-DMSO, ppm) 7.29−8.09 (m, 8H$_{ar}$), 10.17 (s, 2H, CHO); ^{13}C 125.40 (C$_{ar}$), 126.61(C$_{ar}$), 133.49(C$_{ar}$), 134.60(C$_{ar}$), 136.01(C$_{ar}$), 137.86(C$_{ar}$), 193.32 (CHO). **Disulfide, bis(2-nitro-5-benzaldehyde) (5)** was synthesized as described by Fries and Brothuhn in 37% yield (yellow solid).[51] **5-Chloro-2-nitrobenzaldehyde (7)**, **2-hydroxy-5-nitrobenzaldehyde (2)**, and **2-hydroxy-4-methoxybenzaldehyde (1)** were obtained from Aldrich and were used without further purification.

Chiral Amino Acid Analysis. Determination of the peptide chirality was performed according to the method of Goodlett[66] as previously described.[41]

Acknowledgment. L.P.M. was supported by an Australian postgraduate research award (APRA) scholarship from the Australian Government. We thank Glaxo Wellcome UK and Glaxo Wellcome Australia for their financial support.

Supporting Information Available: Comparison of the RP-HPLC traces of HBTU-activated Boc-alanine acylation products without base treatment after 1, 10, and 60 min starting with N^{α}-(Aux)-Ala-Gly-Phe-Wang resin peptide, where Aux is Mnb (**5**), 2,5-Hnb (**2**), or Hnb (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

JO991340+

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(67) Abbreviations used: Aux, auxiliary; Boc, *tert*-butoxycarbonyl; CH_3CN, acetonitrile; DA, di-acylation product (*N*- and *O*-); Da: dalton; DCM, dichloromethane; DEAD, diethyl azodicarboxylate; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hmb, 2-hydroxy-4-methoxybenzyl; 2,5-Hnb, 2-hydroxy-5-nitrobenzyl; Hnb, 2-hydroxy-6-nitrobenzyl; LC/MS, liquid chromatography mass spectrometry; MA, mono-acylation product; Mb, 2-mercaptobenzyl; Mnb, 2-mercapto-5-nitrobenzyl; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high performance liquid chromatography; SM, starting material; SPPS, solid-phase peptide synthesis; STAT, signal transducers and activators of transcription; TFA, trifluoroacetic acid; X_{aa}, any amino acid. Standard IUPAC single and triple letter codes for amino acids are used throughout.

(68) The following experiment confirms this. A 30 min HBTU mediated coupling of Fmoc-Val onto (Hmb)Val-Ala-Gly-Phe-WANG resin, followed by TFA cleavage showed near complete acylation by MS. However, when the same resin was first treated with piperidine (to remove all *O*-acylation) prior to TFA cleavage, only nonacylated product was observed.

(69) To be able to make the appropriate on-resin comparisons between Hnb and Hmb we have carried out all *N*-acylation experiments in parallel using identical experimental conditions. HBTU/DMF coupling chemistry at room temperature was employed in this study as it is effective and commonly used in contemporary Fmoc- and Boc-SPPS. It must also be noted that in the very hindered acylation reactions reported here, higher yields can be achieved by the optimization of coupling variables such as the activation method, solvent, temperature, and time.

(70) It should be noted that tertiary amide bond cleavage may occur during prolonged TFA treatment.[65] However, in this study less than 1% of this side reaction was observed during cleavage from trityl resin. In practice, this type of side reaction can be avoided by first carrying out on-resin auxiliary photolysis prior to TFA treatment.

(71) Diacylation (*N* and *O*) yields were used as a gauge for *O*-acylation. Determined by TFA resin cleavage after acylation with Boc-amino acids without prior base treatment and quantification of the di-acylation product by RP-HPLC peak integration after identification by ES-MS.

(72) Throughout the synthesis and handling of the Hnb-substituted peptide care was taken to avoid premature photolysis of the Hnb auxiliary by minimizing exposure to sun and room light. We recommend that the reaction vessel and other glassware used in manipulations be covered with aluminum foil or another type of nontranslucent material.

(73) It is expected the Hnb auxiliary can be introduced during SPPS in a preformed manner similar to commercially available *N*,*O*-bisFmoc-*N*-(Hmb)amino acids derivatives.