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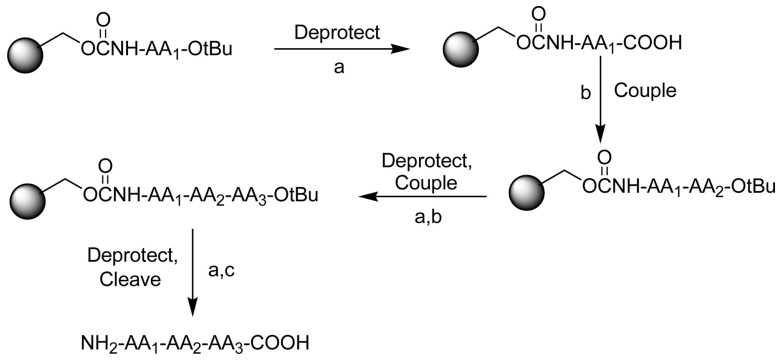

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General Inverse Solid-Phase Synthesis Method for C-Terminally Modified Peptide Mimetics

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Peptide mimetics are of considerable interest as bioactive agents and drugs. C-terminally modified peptide mimetics are of particular interest given the synthetic versatility of the carboxyl group and its derivatives. A general approach to C-terminally modified peptide mimetics, based on a urethane attachment strategy and amino acid *t*-butyl ester-based N-to-C peptide synthesis, is described. This approach is compatible with the reaction conditions generally employed for solution-phase peptide mimetic synthesis. To develop and demonstrate this approach, it was employed for the solid-phase synthesis of peptide trifluoromethyl ketones, peptide boronic acids, and peptide hydroxamic acids. The development of a versatile general approach to C-terminally modified peptides using readily available starting materials provides a basis for the combinatorial and parallel solid-phase synthesis of these peptide mimetic classes for bioactive agent screening and also provides a basis for the further development of solid-phase C-terminal functional group elaboration strategies.

Introduction

Many biological processes are regulated at the level of peptides and proteins interacting with their biological targets. The development of solid-phase approaches to peptide synthesis provided the means to systematically explore peptide and protein biochemistry, for which Merrifield was awarded the Nobel Prize in Chemistry in 1984.[1] Solid-phase peptide chemistry subsequently provided a foundation for the development of combinatorial methods for finding and optimizing peptide-based agents and is now widely used for both peptide- and non-peptide-based agents (reviewed in refs 2, 3). Peptide mimetics are agents closely related to peptides but with key functional group modifications tailored for specific properties and applications. Peptide mimetics are of high interest as bioactive agents and drugs, and a number of drugs in current use are peptide mimetics, including ACE inhibitors,[4] HIV protease inhibitors,[5] and the anti-myeloma agent Velcade.[6] Many biological processes can conceivably be targeted through suitably designed peptide mimetics, and the development of general solid-phase approaches to such agents is expected to greatly facilitate efforts to develop and refine peptide mimetics for such applications.

Many peptide mimetic classes of interest as bioactive agents are modified on the C terminus or are derived from carboxyl group modifications and reactions. Simple C-terminal peptide mimetics include peptide trifluoromethyl ketones,[7−9] peptide boronic acids,[10−13] peptide hydroxamic acids,[14] peptide alcohols,[15,16] and peptide aldehydes.[17−21] Peptide mimetic classes which can be accessed through carboxyl group chemistry also include statine homologues[22−27] and hydroxyethylene isosteres.[27−30] Given the interest in these peptide mimetic classes, a number of approaches to C-terminally modified peptide mimetics have been described (reviewed in ref 31). These approaches can be divided into several subcategories, including attachment through the C-terminal functional group or precursor followed by standard C-to-N peptide synthesis, attachment through the backbone followed by C-to-N peptide synthesis, and attachment through the amino terminus followed by N-to-C (inverse) peptide synthesis (inverse solid-phase peptide synthesis; ISPPS). The first of these general approaches, based on C-terminal functional group specific attachment strategies, is limited to a specific functional group and does not allow further elaboration of the final functional group to be made on the resin, for example for preparing additional derivatives of a C-terminal aldehyde. The second general approach does allow further reaction of the final functional group, but suffers, as does the first general approach, from the limitation that the peptide chain is synthesized in the C-to-N direction, away from the C-terminal functional group. For split-pool combinatorial peptide mimetic synthesis followed by iterative deconvolution to obtain optimized agents, which is arguably one of the better approaches to combinatorial optimization,[32] it is the last residues added that are optimized first. In both the first and second of the above cited general approaches, these are the residues furthest away from the C-terminal functional group. It seems most reasonable when optimizing a C-terminal peptide mimetic for a specific application to optimize the residues closest to the C terminus first.

In contrast to the functional group specific and backbone attachment strategies, the third approach, based on ISPPS, provides the C terminus of the nascent peptide mimetic for elaboration into any desired functional group and for further elaboration into additional derivatives and also allows the residues closest to the C terminus to be optimized first when

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using a split−pool/iterative deconvolution optimization strategy. There have been a number of efforts to develop effective ISPPS strategies. Such an approach was first suggested by Letsinger and Kornet[33] using amino acid ethyl esters. Another early report on ISPPS described the use of amino acid hydrazides.[34] More recently, amino acid 9-fluorenylmethyl (Fm) esters,[35] amino acid tri-*t*-butyloxysilyl esters,[36] and amino acid allyl esters[37] have been used for ISPPS. However, few if any of these amino acid derivatives are currently commercially available. Amino acid silyl esters are difficult to prepare, unstable to store, and unstable under peptide coupling conditions. The Fm ester approach looks attractive considering its similarity to standard Fmoc-based C-to-N SPPS, but Fm esters are not as stable as Fmoc amino acids, and Fm ester-based inverse peptide synthesis apparently suffers from this limitation. The Fm ester approach also suffers from significant racemization during coupling reactions.[35] The allyl ester-based approach is practicable and appears currently to be the method most competitive with the *t*-butyl ester-based ISPPS method described below. However, allyl esters are also not generally available commercially, and deprotection requires the use of 20 mol % of $Pd(PPh_3)_4$, a heavy metal-based reagent. These strategies for ISPPS, therefore, appear less than ideal, especially since suitable amino acid derivatives are not generally available commercially.

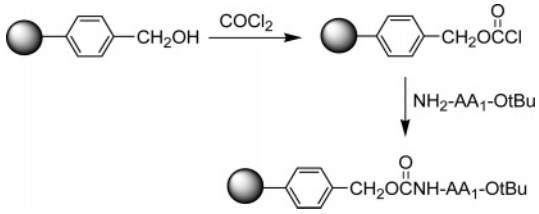
The strategy we are pursuing for ISPPS development is based on amino acid *t*-butyl esters.[38] Favorable features of this approach are that amino acid *t*-butyl esters are stable, a large selection are commercially available, and the synthesis of commercially unavailable monomers is relatively straightforward.[39] The *t*-butyl ester strategy also has the benefit that this approach is exactly the inverse of the well-developed Boc strategy for normal C-to-N peptide synthesis, and the extensive knowledge of side chain protection strategies and other chemical details can therefore be transferred from Boc chemistry to *t*-butyl ester chemistry.

Our initial report on this strategy was based on the use of standard Boc chemistry resins (hydroxymethyl, Pam, and MBHA) combined with dicarboxylic acid linkers, such as succinate, Glu, and Gln.[38] The disadvantage of this approach is that the dicarboxylic acid linker remains in the peptide products, and a "linkerless" strategy which provides the desired peptide/mimetic products without the presence of a linker was desired. Other approaches to ISPPS have solved the attachment problem in several ways, including through tritylamine attachment,[35,37] urethane attachment,[33,34,40] or with photocleavable linkers.[36] In this report, we describe a simple urethane attachment strategy compatible with amino acid *t*-butyl ester-based ISPPS which provides product peptides without linkers, in high purity and yield, and with low racemization of amino acid residues. This strategy is also demonstrated for solid-phase peptide mimetic synthesis by its application to the solid-phase synthesis of peptide trifluoromethyl ketones, peptide boronic acids, and peptide hydroxamic acids.

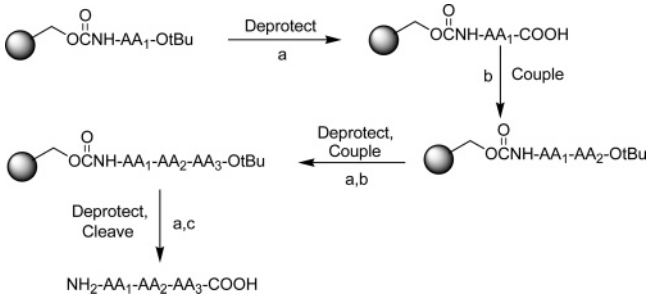
Experimental Section

Resin Activation and First Residue Attachment. The attachment and ISPPS strategy are outlined in Schemes 1 and 2. Hydroxymethyl polystyrene resin (100 mg, 0.1 mmol) was converted to the chloroformate by treating with 10 equiv of phosgene in dichloromethane (DCM) for 30 min and then drying under vacuum.[33] The first amino acid was loaded onto the resin by adding a solution of 10 equiv of amino acid *t*-butyl ester and 10 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) to the dried resin and stirring for 4 h. To assess loading efficiency, Phe was used as the first residue. After loading and washing, Phe was cleaved from the resin by treatment with 10% trifluoromethanesulfonic acid (TFMSA)/trifluoroacetic acid (TFA) for 1 h and quantitated by HPLC. After loading the first residue, possible unreacted hydroxyl groups were capped with acetic anhydride (Ac_2O)/DIPEA as a precaution before performing inverse peptide synthesis reactions.

Scheme 1. Loading of the First Amino Acid *tert*-Butyl Ester



Scheme 2. Amino Acid *tert*-Butyl Ester-Based ISPPS



[a] 50% TFA/DCM; [b] HATU/TMP, 5 equiv of AA-OtBu·HCl; [c] 10% TFMSA/TFA.

Table 1. Amino Acid *t*-Butyl Ester-Based ISPPS Protocol

description	reagent	repetition and duration
OtBu deprotection	25% TFA/DCM	1 × 5 s
	50% TFA/DCM	1 × 30 min
washes	DCM	3 × 5 s
	NMP[a]	2 × 5 s
	DCM	3 × 5 s
activation/coupling	5 × HATU	12 h
	5 × AA-OtBu·HCl	
	10 × TMP in DMF	
washes	DCM	3 × 5 s
	DMF	3 × 5 s

[a] NMP: 1-methyl-2-pyrrolidinone.

Amino Acid *t*-Butyl Ester-Based ISPPS. After loading, the *t*-butyl ester of the first residue was deprotected with 50% TFA/DCM, and synthesis cycles were performed as outlined in Table 1. Cleavage from the resin was accomplished with 10% TFMSA/TFA for 2 h. To demonstrate this approach, seven tripeptides were synthesized (Table 2). These peptides were analyzed by HPLC for purity and yield and for amino acid racemization using Marfey's reagent.[41,42]

Table 2. Molecular Weight Confirmation and Purities of the Synthesized Peptides and Peptide Mimetics

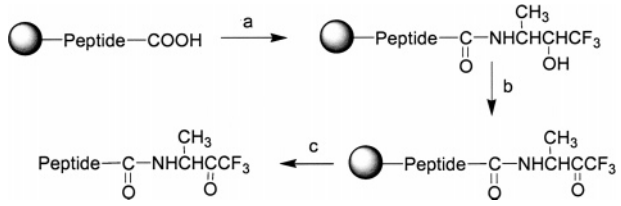
	sample	mol wt from $[M+H]^+$ calcd	mol wt from $[M+H]^+$ found[a]	purity,[b] %
1	Tyr-Ala-Phe	400.2	399.8	88
2	Tyr-Gly-Orn	353.2	352.7	92
3	Tyr-Ala-Val	352.2	351.8	89
4	Asn-D-Val-Leu	345.2	344.8	87
5	Asn-Leu-Glu	375.2	374.8	81
6	Gly-Ile-Thr	290.1	289.7	82
7	Phe-Ala-Gly	294.1	293.6	81
8	Asn-Leu-Glu-boroAla	428.2	427.8	74
9	Phe-Ala-Gly-boroAla	347.0	346.7	75
10	Tyr-Ala-Phe-$NHCH(CH_3)COCF_3$	523.2	523.4	86
11	Tyr-Gly-Orn-$NHCH(CH_3)COCF_3 \cdot H_2O$	494.2	494.5	87
12	Phe-Ala-Gly-NHOH	309.1	308.7	81
13	Phe-Leu-Val-NHOH	393.4	392.9	79

[a] Determined on an aQa ThermoQuest (Finnigan) LC/MS instrument equipped with atmospheric-pressure ionization (API) electrospray source. [b] Determined by HPLC analysis of the crude product on a Hewlett-Packard series 1050 system equipped with a C18 column (Solvent miser, 2.1 × 250 mm, 5.0 μm particles). Compounds were separated by gradient elution; 0% of solvent B (0.1% TFA in 70% aqueous acetonitrile) in solvent A (0.1% TFA in water) for 1 min, then 0−100% of solvent B in solvent A in 10 min, then 0−100% of solvent C (0.095% TFA in acetonitrile) in solvent B in 5 min.

Table 3. Percentage Racemization Determined with Marfey's Reagent[41,42] for the Indicated Peptides

peptide	AA_1	AA_2	AA_3
Tyr-Ala-Phe	D-Tyr (1.2%)	D-Ala (1.4%)	D-Phe (1.0%)
Tyr-Gly-Orn	D-Tyr (1.3%)	NA	D-Orn (1.2%)
Tyr-Ala-Val	D-Tyr (1.2%)	D-Ala (1.2%)	D-Val (1.4%)
Asn-D-Val-Leu	D-Asn (1.4%)	L-Val (1.5%)	D-Leu (1.1%)

Scheme 3. On-Resin Synthesis of Peptide Trifluoromethyl Ketones

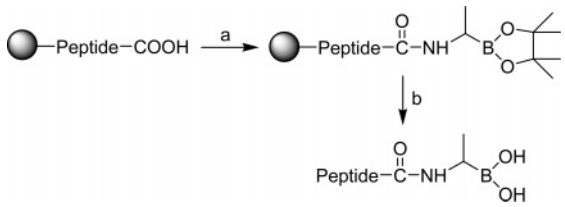


[a] HATU/TMP, 5 equiv of $NH_2CH(CH_3)CH(OH)CF_3$, in DMF for 4 h; [b] 10 equiv of 1,3-dicyclohexylcarbodiimide (DCC)/1 equiv $CHCl_2COOH$/100 μL dimethyl sulfoxide (DMSO)/100 μL of toluene, 18 h, repeat once; [c] 10% TFMSA/TFA, 2 h.

The observed racemization of individual amino acids was <2% (Table 3).

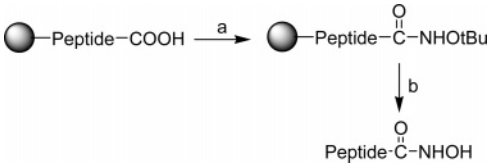
ISPPS of Peptide Trifluoromethyl Ketones. Two peptide trifluoromethyl ketones were synthesized using the approach outlined in Scheme 3. The precursor aminotrifluoromethyl alcohol was synthesized as described previously.[9,43] Resin loaded with Tyr-Ala-Phe or Tyr-Gly-Orn was coupled with this aminotrifluoromethyl alcohol using the HATU/TMP coupling procedure to give the corresponding peptide trifluoromethyl alcohols. Oxidation of the on-resin trifluoromethyl alcohols was performed by Pfitzner−Moffat oxidation[38,44,45] to give the corresponding peptide trifluoromethyl ketones, which were then cleaved from the resin using 10% TFMSA/TFA. Peptide trifluoromethyl ketones are often detected in LC/MS as their hydrates. The lack of detectable trifluoromethyl alcohols indicated quantitative oxidation.

Scheme 4. On-Resin Synthesis of Peptide Boronic Acids



[a] HATU/TMP, 5 equiv of DL-boroAla-pinacol (HCl salt) in DMF for 4h; [b] 10% TFMSA/TFA, 2h.

Scheme 5. On-Resin Synthesis of Peptide Hydroxamic Acids



[a] HATU/TMP, 5 equiv of *O*-(*t*-butyl)hydroxylamine hydrochloride in DMF for 4 h; [b] 10% TFMSA/TFA, 2h.

ISPPS of Peptide Boronic Acids. Peptide boronic acids were synthesized using the approach outlined in Scheme 4. DL-BoroAla-pinacol was synthesized as described previously.[11,43] Resin loaded with Phe-Ala-Gly or Asn-Leu-Glu was coupled with boroAla-pinacol (HCl salt) using the HATU/TMP coupling protocol, followed by cleavage with 10% TFMSA/TFA to give the corresponding peptide boronic acids.

ISPPS of Peptide Hydroxamic Acids. Peptide hydroxamic acids were synthesized using the approach outlined in Scheme 5. Resin loaded with Phe-Ala-Gly or Phe-Leu-Val was coupled with *O*-(*t*-butyl)hydroxylamine (HCl salt) using HATU/TMP, followed by cleavage with 10% TFMSA/TFA to give the corresponding hydroxamic acids.

Results and Discussion

Resin Activation and First Amino Acid Loading. Following the procedure outlined above with Phe as the loaded residue, quantitation of Phe by HPLC with detection at 260 demonstrated >95% loading efficiency with this method.

ISPPS on Urethane-Attached Nascent Peptides. After loading and capping, ISPPS was performed using the synthesis cycles outlined in Table 1. Seven tripeptides were synthesized in good yield and purity, as determined by HPLC and LC/MS (Table 2), using this approach. Analysis of four of these tripeptides for racemization using Marfey's method demonstrated low levels of racemization using this ISPPS approach (Table 3). In a previous study based on the use of dicarboxylic acid linkers,[38] succinyl linkers had previously been observed to give high levels of racemization in ISPPS, a problem which could be solved by the use of N^{α}-Cbz protected Glu as the dicarboxylic acid linker. The presence of an α-urethane presumably suppressed racemization through oxazalone formation. The presence of Glu-based linkers in peptide products from this original attachment strategy was undesirable for most applications of ISPPS, and a urethane

attachment strategy seemed likely to be compatible with *t*-butyl ester-based ISPPS, to suppress first residue racemization and to provide peptide/mimetics without an unwanted linker. Results with *t*-butyl ester-based ISPPS using such a urethane attachment strategy demonstrate that this approach can provide short peptides in high yields and purity (Table 2) and with low racemization in all residues (Table 3).

C-Terminal Peptide Mimetic Synthesis. The principle motivation for developing an effective method for ISPPS based on readily available amino acid *t*-butyl ester monomers is our interest in developing novel peptide mimetics as antibacterial agents.[43] To demonstrate the potential of the approach described here for peptide mimetic synthesis, two peptide boronic acids, two peptide trifluoromethyl ketones, and two peptide hydroxamic acids were synthesized following the procedures outlined above. These products were also obtained in good yield and purity (Table 2, entries 8−13), demonstrating for the first time a general, convenient, and effective ISPPS-based approach to C-terminally modified peptide mimetics.

Summary

t-Butyl ester-based ISPPS was demonstrated with a urethane attachment strategy. This approach provides peptides in high yield and purity, with low racemization in all residues and without a linker residue appearing in the final products. To demonstrate the potential of this approach for solid-phase peptide mimetic synthesis, it was also demonstrated for the synthesis of three C-terminally modified peptide mimetic classes—peptide boronic acids, peptide trifluoromethyl ketones, and peptide hydroxamic acids—also in high yield and purity. Its seems reasonable to expect that many peptide mimetic classes will be accessible with this methodology, providing for the first time an effective general solid-phase approach to accessible peptide mimetic classes using readily available starting materials. This approach is well-suited for use with combinatorial chemistry-based strategies for drug and bioactive agent discovery and optimization.

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