Solid-phase peptide synthesis and solid-phase fragment coupling mediated by isonitriles

Ting Wang[a] and Samuel J. Danishefsky[a,b,1]

[a]Laboratory of Bioorganic Chemistry, Memorial Sloan-Kettering Cancer Center, New York, NY 10065; and [b]Chemistry Department, Columbia University, New York, NY 10027

Contributed by Samuel J. Danishefsky, June 4, 2013 (sent for review May 20, 2013)

The synthesis of polypeptides on solid phase via mediation by isonitriles is described. The acyl donor is a thioacid, which presumably reacts with the isonitrile to generate a thio-formimidate carboxylate mixed anhydride intermediate. Applications of this chemistry to reiterative solid-phase peptide synthesis as well as solid-phase fragment coupling are described.

Amide bond formations are arguably among the most important constructions in organic chemistry (1, 2). The centrality of the amide linkage, as found in polypeptides and proteins, in the maintenance of life hardly needs restatement. Numerous strategies, resulting in a vast array of protocols to synthesize biologically active polypeptides and proteins, have been demonstrated (3, 4). Central to reiterative polypeptide bond formations was the discovery and remarkable development of solid-phase peptide synthesis (SPPS) (5, 6). The extraordinary impact of SPPS in fostering enhanced access to homogeneous polypeptides is clear to everyone in the field.

As we have described elsewhere, by classical, mechanistic reasoning, we were led to conjecture about some hitherto-unexplored possibilities relevant to the chemistry of isonitriles (7–14). It was anticipated that isonitriles might be able to mediate the acylation of amines, thus giving rise to amides (15). Early experiments focused on free carboxylic acids as the acylating agents. As our studies progressed, it was found that the combination of thioacids, amines, and isonitriles leads to the efficient formation of amide bonds under stoichiometric or near-stoichiometric conditions (7–13, 16, 17). Although there remain unresolved issues of detail and nuance, the governing mechanism for amide formation under these conditions involves reaction of the thioacid, **1**, with an isonitrile, **2**, to generate a thio-formimidate carboxylate mixed anhydride (thio-FCMA), **3**, which is intercepted by the "acyl-accepting" amine to generate amide, **5**, and thioformamide, **6** (Fig. 1). The efficiency of the amidation was further improved through the use of hydroxybenzotriazole (HOBt) (18), which could well give rise to HOBt ester **7**, although this pathway has not been mechanistically proven.

The potentialities of the isonitrile-mediated amidation method were foreshadowed via its application to the synthesis of cyclosporine (19). The power of the method was particularly well demonstrated in the context of our recent total synthesis of oxytocin (OT) (20), wherein isonitrile mediation was used in each of the peptide bond constructions, leading to the synthesis of the hormone in high yield and excellent purity. This nonapeptide is involved in a range of biological functions including parturition and lactation (21, 22). Signaling of OT to its receptor (OTR) is apparently an important factor in quality maintenance of various CNS functions (23). The ability to synthesize such modestly sized, but bio-impactful peptides in both native (wild-type) form, and as strategically modified variants, is one of the current missions of our laboratory, with the objective of possible applications to the very serious problem of autism (24–26).

Results and Discussion

We wondered whether isonitrile-mediated amide bond formation could be used in the context of SPPS. The enormous impact of SPPS, so ably pioneered by Merrifield (5), clearly heralded a paradigm shift in the synthesis of peptides and even proteins by chemical means. Our program was launched by mixing solid-support bound glutamine **8**, and asparagine-derived thioacid **9**, with *tert*-butyl isocyanide (*t*-BuNC) in dimethylformamide. After cleavage from resin, dipeptide **10** was obtained in 95% yield (Table 1, entry 1). The experiments summarized in Table 1 serve to clarify and integrate the findings herein, conducted under SPPS conditions, with the previously described (20, 27) solution-based amidation of thioacids. Previously, we had demonstrated a highly exploitable oxidatively activable pathway for enhancing the acyl-donating properties of thioacids (18). In solution phase, oxidative amidation is operative even in the absence of isonitrile mediation. In the solution phase experiments, particularly with simple, unhindered thioacids, it proved to be very difficult to avoid significant levels of amide formation, even following attempted avoidance of oxidation and in the absence of isonitrile mediation. This "negative" result could be interpreted as reflecting our inability to fully prevent low levels of oxidation. Alternatively, some amidation may have been triggered through the presence of traces of chain-carrying oxidation impurities (such as diacyldisulfides) with high acyl-donating potential in the substrate samples of thio acid (27). Although these questions cannot yet be definitively answered in the solution phase experiments, the situation under SPPS conditions were more revealing (Table 1). Thus, entry 1 demonstrates the viability of the isonitrile (thio-FCMA) pathway. Although slightly improved yields are obtained with the inclusion of HOBt (entry 2), no meaningful rate increase is observed. Accordingly, it seems that the small improvement in yield between entries 1 and 2 does not suggest a significant change of mechanism. Indeed, entry 3 establishes the dominance of the thio-FCMA pathway, indicating a possible low-yielding acyl activation pathway by HOBt itself, independent of isonitrile mediation or adventitious oxidative activation. Entry 4 demonstrates that, at least under the more discriminating SPPS conditions, we are able to effectively avoid oxidative acylation and there are no discernable acyl-donating properties by thioacids, themselves, with respect to amine nucleophile.

We continued our exploration in this method of amide formation using varying amines and thioacids (19, 28, 29). Coupling of solid-support glutamine **8** and leucine-derived thioacid **11** (19) under mediation by *t*-BuNC and HOBt, followed by cleavage from resin, provided dipeptide **12** in 94% yield (Fig. 2, Eq. **1**). The solid-phase isonitrile-mediated peptide bond formation was also shown to be highly efficient by coupling solid-support serine derivative **13** with arginine derivative **14**, to afford a 92% yield of **15** after cleavage from supporting polymer (Fig. 2, Eq. **2**). In a similar fashion, a glycine-leucine dipeptide **17** was obtained in

Author contributions: T.W. and S.J.D. designed research; T.W. performed research; T.W. and S.J.D. analyzed data; and T.W. and S.J.D. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: s-danishefsky@ski.mskcc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1310431110/-/DCSupplemental.

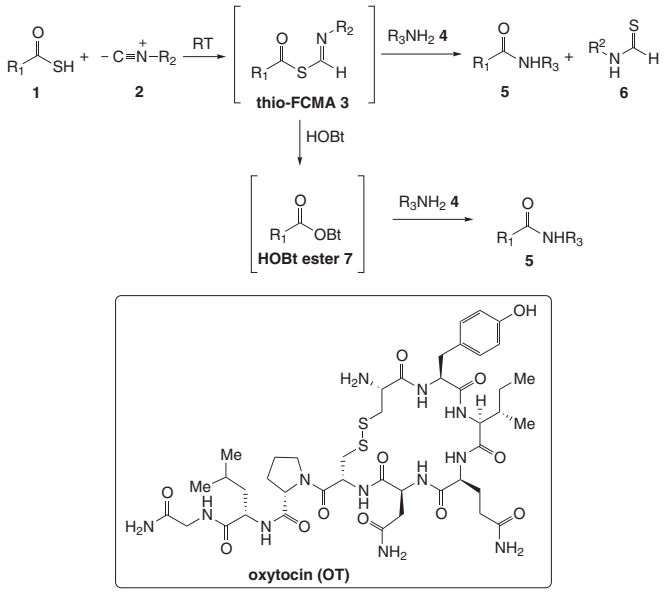
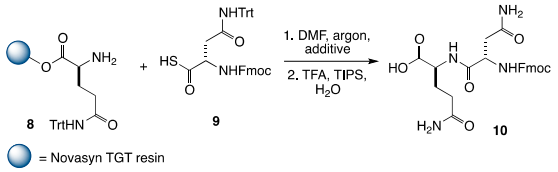


Fig. 1. Isonitrile-mediated amidation; structure of OT.

95% (Fig. 2, Eq. **3**). Also encouraging was the success of the method in producing tetrapeptide **20** through a miniligation. Solid-support–bound **18** underwent amidation with cysteine-proline–derived thioacid **19,** providing ligation product **20** in 87% yield (Fig. 2, Eq. **4**).

Having demonstrated the efficiency of isonitrile-mediated thioacid amidation on solid support, we next investigated the applicability of this chemistry to enable an iterative "peptide homologation" process. The isonitrile-mediated reiterative SPPS sequence started with cleavage of the fluorenylmethyloxycarbonyl (Fmoc) group. The resulting N terminus served to amidate amino acid-derived thioacid to afford solid-support dipeptide. The sequence was reiterated through several cycles (vide infra) through the same two-step sequence of Fmoc deprotection followed by isonitrile (and HOBt)-mediated acylation. Following cleavage from the resin, the polypeptide is obtained. Tripeptide **21** was elaborated after two deprotection/isonitrile-mediated coupling cycles in excellent yield (85%; Fig. 3). Happily, isonitrile-mediated SPPS proved to be extendable to a C-terminal histidine initiated tetrapeptide, thereby providing pentapeptide **22** in 72% yield. The program was further extended to a C-terminal glutamate-bound pentapeptide to afford hexapeptide **23** in 68% yield. It is worth noting that only 1.2–1.5 eq of both thioacids and reagents were used in all of the cases shown in Fig. 3, thus demonstrating

Table 1. Application of thio-FCMA chemistry to SPPS



Entry	Additive	Yield, %
1	*t*-BuNC	95
2	*t*-BuNC, HOBt	99
3	HOBt	20
4	None	ND

ND, not detected; TGT, tentagel 4-carboxytrityl.

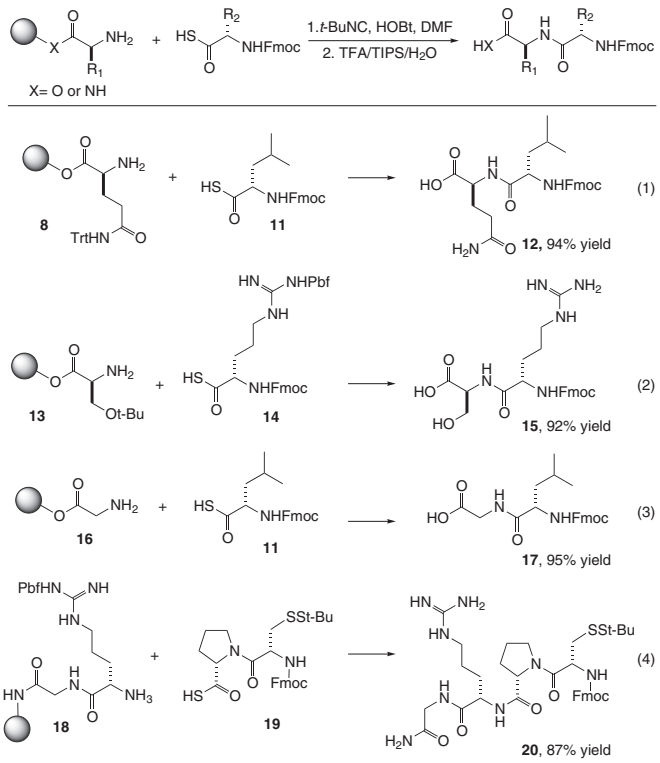


Fig. 2. Isonitrile-mediated SPPS: substrate scope. Entry 1, 2, 3: Novasyn tentagel 4-carboxytrityl (TGT) resin; entry 4: rink amide polyethylene glycol-dimethylacrylamide copolymer (PEGA) resin.

a high level of efficiency in the isonitrile/HOBt-mediated SPPS method.

Having established the feasibility of the core isonitrile-mediated SPPS strategy, we turned to the synthesis of the historically important mammalian hormone vasopressin, **25** (30, 31). Vasopressin is a well-known neurohypophysial hormone found in most mammals. Much of the hormone is maintained in vesicles at the posterior pituitary, awaiting release into the bloodstream to control the reabsorption of molecules in the tubules of the

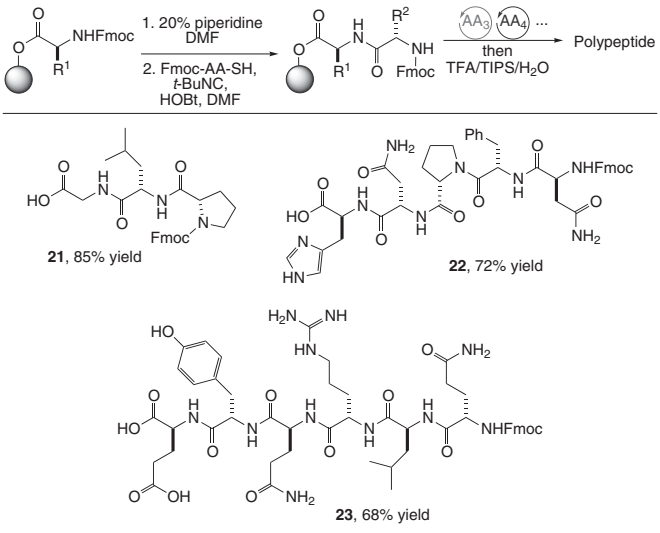


Fig. 3. Reiterative isonitrile-mediated SPPS.

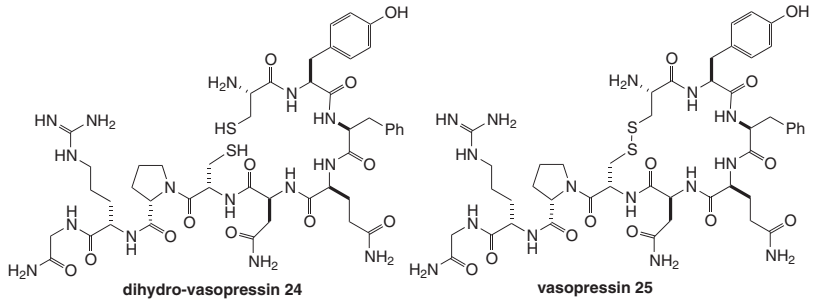


Fig. 4. Structure of vasopressin.

kidneys, by affecting the tissue permeability. However, vasopressin is also released directly into the brain, where it also plays an important role in social behavior and bonding. As seen, vasopressin is structurally similar to the nonapeptide oxytocin (Fig. 4, **25** vs. **8**) (32, 33). Accordingly, the interactivity of these two hormones has been studied extensively. We undertook to revisit the synthesis of vasopressin in the context of isonitrile-mediated SPPS.

The synthesis of vasopressin **25** began with coupling solid-supported glycine **26** and arginine-derived thioacid **27** (Fig. 5). Cleavage of the Fmoc protecting group in the resulting dipeptide **28**, followed by ligation with cysteine-proline–derived thioacid **29**, efficiently provided solid-support tetrapeptide **30**. This adventure then progressed through the same two-step deprotection-coupling homologation process using thioacids **31–35**, derived from asparagine, glutamine, phenylalanine, tyrosine, and cysteine, respectively, to afford the vasopressin backbone, **36**, on solid support. Happily, cleavage of the nonapeptide from the resin, using TFA/triisopropylsilane (TIPS)/H_2O, provided dihydro-vasopressin in 43% overall yield, and enabled us to characterize **24**

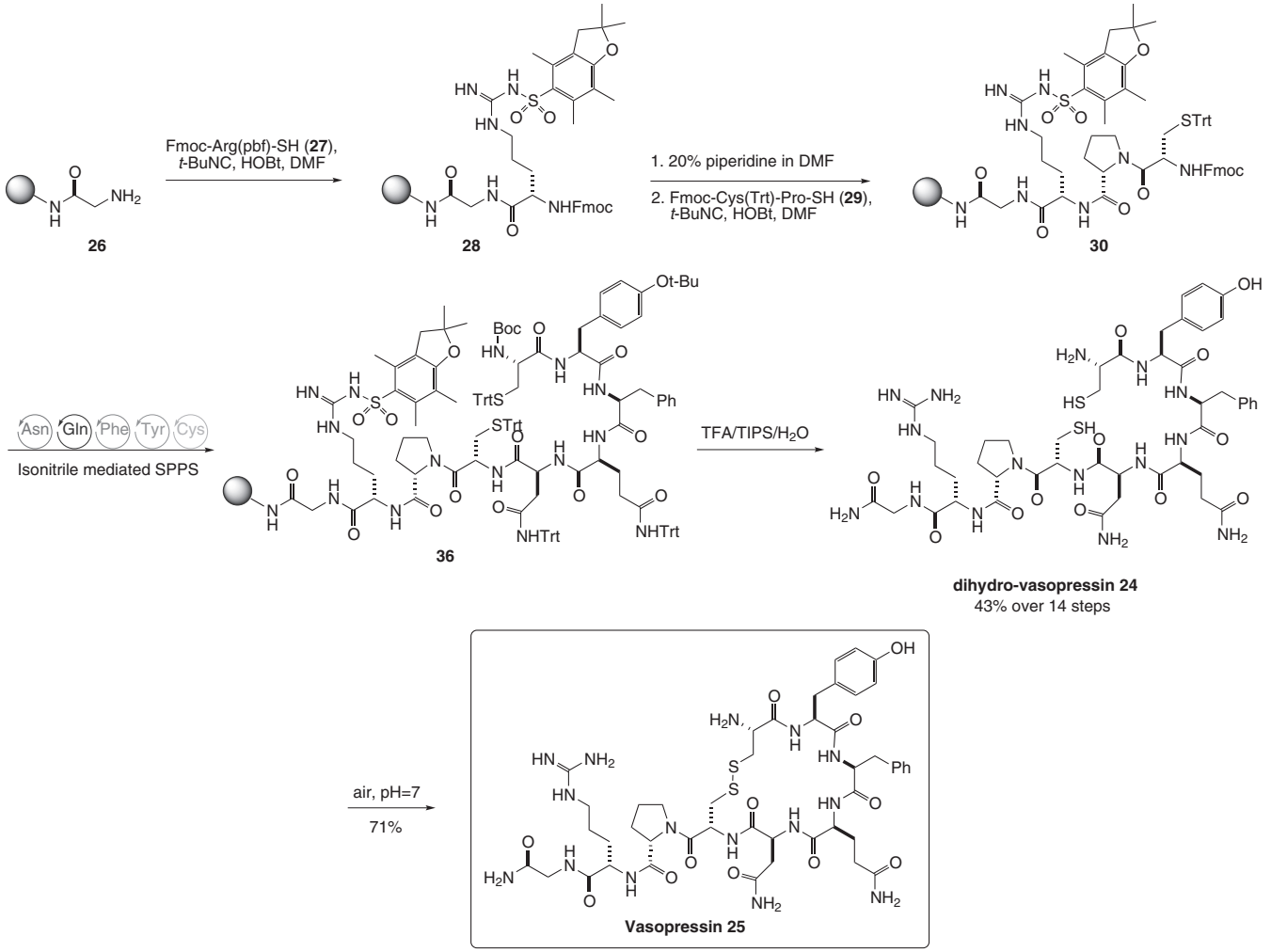


Fig. 5. Synthesis of vasopressin. Reagents in the isonitrile-mediated SPPS: Asn = Fmoc-Asn(Trt)-SH (**31**); Gln = Fmoc-Gln(Trt)-SH (**32**); Phe = Fmoc-Phe-SH (**33**); Tyr = Fmoc-Tyr(*t*-Bu)-SH (**34**); Cys = Boc-Cys(Trt)-SH (**35**).

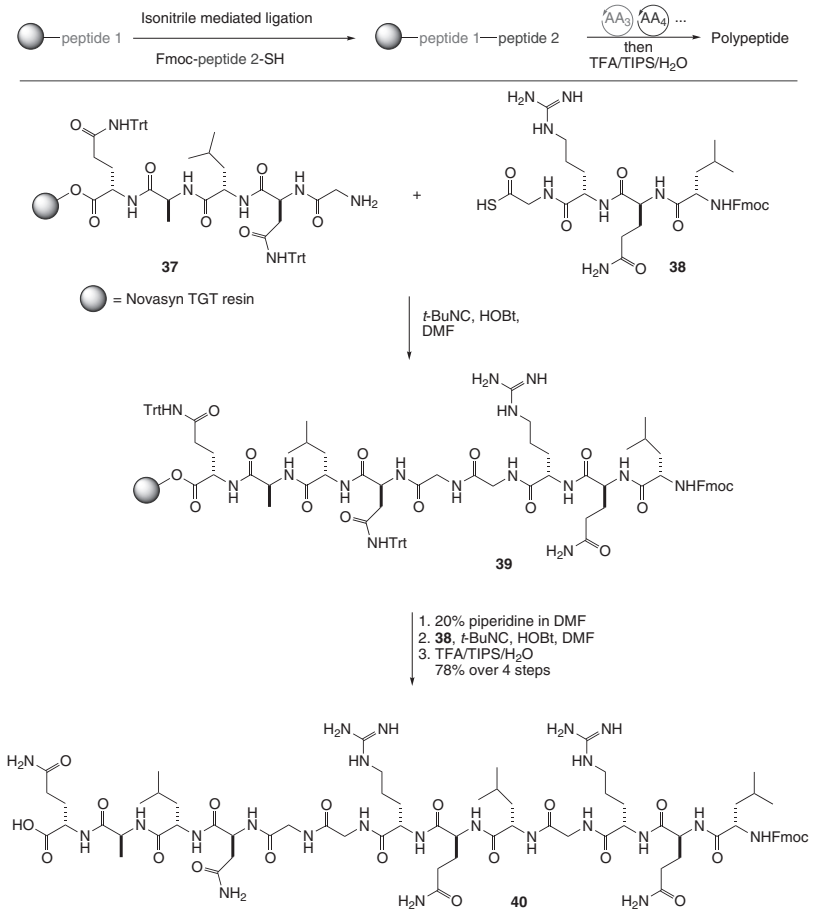


Fig. 6. Isonitrile-mediated SPFC.

as a homogeneous entity. Finally, aeration of **24** in pH 7 aqueous solution provided vasopressin **25** in 71% isolated yield. As in the recently reported case of oxytocin (20), the high field proton spectra of **24** and **25** were quite similar, thus suggesting a high order of preorganization, even in the absence of disulfide bond formation.

We next evaluated the feasibility of a more challenging proposition, namely, the possibility of an iterative chemoselective solid-phase fragment coupling (SPFC) via isonitrile-mediated amidation. Due to the high levels of convergence, SPFC is, in principle, an attractive strategy for the synthesis of large peptides and/or peptides with "difficult" sequences (34–40). Moreover, SPFC may offer considerable advantages in allowing for interim purification, which should simplify obtaining homogeneous end product. However, ligations in the SPPS mode have often been plagued by limitations. Protected peptide fragments, which are used in traditional SPFC, are difficult to purify by HPLC due to their poor solubility in aqueous solvents (34, 35). To solve this problem, it would be ideal if minimally protected peptide fragments could be used in the ligation step. It was in the context of this problem that we could envision a major advantage of the chemistry developed above. Thus, we have shown that a suitably rendered isonitrile could selectively activate a thioacid functional group at room temperature in the presence of carboxylic acid, amide, guanidine, or phenol (18). The resulting thio-FCMA (or its corresponding HOBt ester) acylates primary amine more readily than the alcohol, imidazole, indole, or phenol-based putative acyl acceptors. Given this unique aspect of the method, it seemed possible that one could execute SPFC by using unprotected fragments (except for lysine), thus simplifying purification by HPLC. Ideally, a two-step deprotection–fragment condensation process would allow for assembly of a large peptide or even a protein in an efficient way without the need for postligation purification (Fig. 6).

To evaluate this concept, solid-support–based pentapeptide **37** and unprotected tetrapeptide-derived thioacid **38*** were assembled (Fig. 6). Isonitrile-mediated chemoselective SPFC served to smoothly amidate the C-terminus thioacid, affording solid-support nonapeptide **39**. A deprotection–ligation homologation process followed by cleavage from resin using TFA/TIPS/H_2O furnished polypeptide **40** in 78% yield.

We next addressed the question of C-terminal epimerization during the course of this isonitrile-mediated SPFC. In the simplest case, solid-supported glycine **41** underwent SPFC with peptide-derived thioacid **42*** to provide **43** in 83% yield with no detectable loss of stereointegrity (Fig. 7, Eq. **1**). Similarly, the more complex resin-bound peptide, **37**, readily coupled with **42** to afford decapeptide **44** in 82% yield with no epimerization (Fig. 7, Eq. **2**). However, when the C-terminal phenylalanine-derived thioacid **45*** served as the ligation partner, a significant level of epimerization product (21%) was observed, presumably because Phe is particularly prone to C-terminal epimerization (Fig. 7, Eq. **3**) (41). More detailed studies examining a wide range of C-terminal thioacids and their application in peptide synthesis are underway.

*Peptide-derived thioacids were prepared following the procedure documented in refs. 18 and 20.

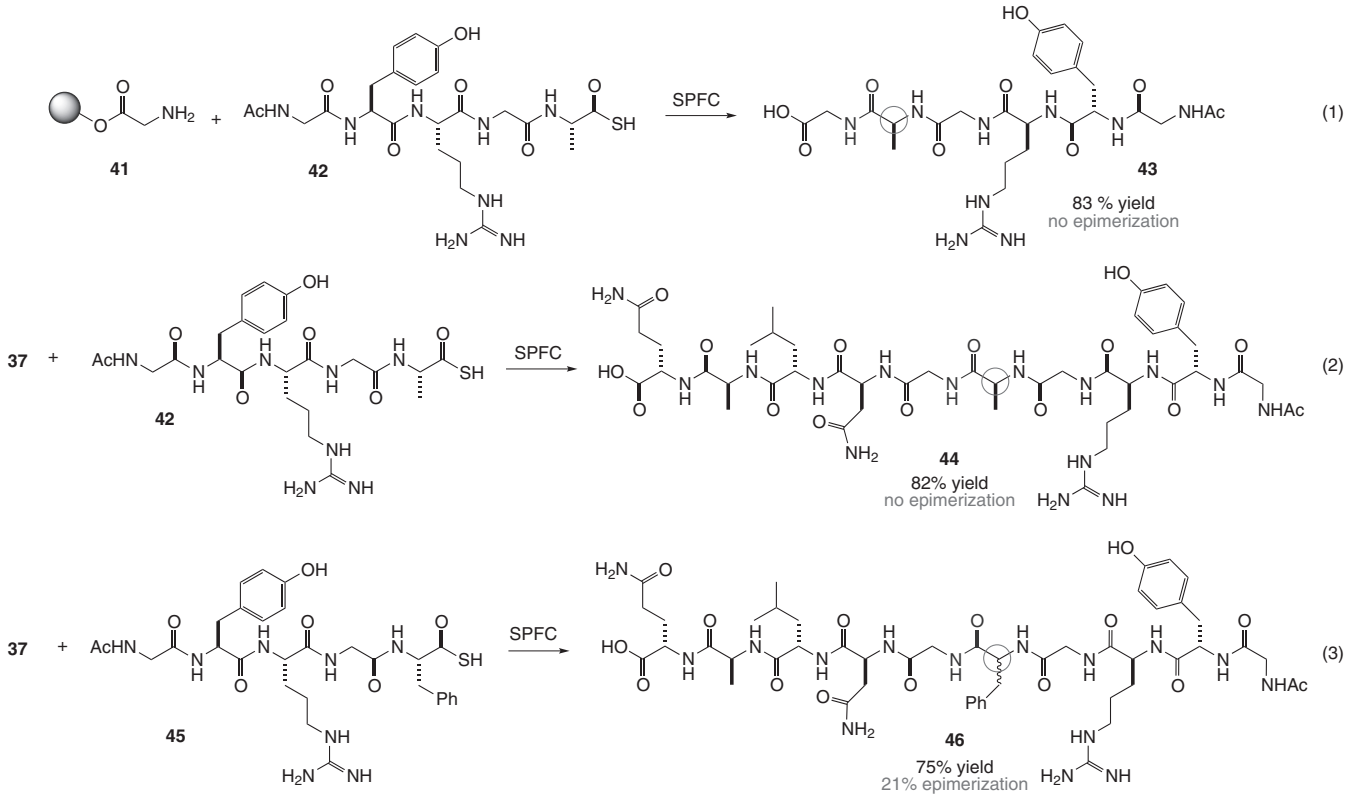


Fig. 7. Isonitrile-mediated SPFC: substrate scope.

Conclusion

In summary, isonitrile/HOBt-mediated solid-phase amidation has been demonstrated. In an iterative fashion, this highly efficient methodology was used in SPPS, requiring consumption of only small excesses of reagents. Adoption of this central strategy enabled the total synthesis of homogeneous dihydrovasopressin **24**, which was then oxidatively cyclized to furnish vasopressin, **25**. In addition to single amino acid homologation, an iterative chemoselective SPFC concept, using unprotected peptide-derived thioacids, has been reduced to practice. This ligation method, although not free of C-terminal epimerization issues, already offers enough ligation sites so as to provide the basis for a widely general method for ligating in the SPPS mode. We call this method SPFC. As practiced herein, SPFC offers the advantage of minimal protection of side chains. For the moment, only lysine residues appear to require protection. Moreover, although standard approaches generally require multiple equivalents of costly amino acid precursors, our isonitrile-based protocols permit the use of near-stoichiometric levels of amino acid coupling partners, thereby providing a valuable economic advantage over prevailing SPPS methods. Presumably, this method is also applicable to substrates bearing unnatural side chains and extended backbones. Furthermore, SPFC could conceivably accommodate a recombinant coupling partner (albeit one lacking a lysine residue). Given these attributes, the methods described herein are likely to be quite useful in polypeptide synthesis. More detailed studies and further application to the synthesis of large peptides and proteins will be disclosed in due course. Full experimental details and spectra are included in the *SI Appendix*.

ACKNOWLEDGMENTS. We thank Dr. George Sukenick, Hui Fang, and Sylvi Rusli of Sloan-Kettering Institute's NMR core facility for assistance. We thank Prof. Phil Dawson for sharing valuable insights in the field of SPFC. Support for this research was provided by National Institutes of Health Grant HL25848 (to S.J.D.).

1. Arthur G (2000) *The Amide Linkage: Selected Structural Aspects in Chemistry, Biochemistry, and Material Science* (Wiley-Interscience, New York).
2. Pattabiraman VR, Bode JW (2011) Rethinking amide bond synthesis. *Nature* 480(7378):471–479.
3. Humphrey JM, Chamberlin AR (1997) Chemical synthesis of natural product peptides: Coupling methods for the incorporation of noncoded amino acids into peptides. *Chem Rev* 97(6):2243–2266.
4. Kimmerlin T, Seebach D (2005) "100 years of peptide synthesis": Ligation methods for peptide and protein synthesis with applications to beta-peptide assemblies. *J Pept Res* 65(2):229–260.
5. Merrifield RB (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J Am Chem Soc* 85(14):2149–2154.
6. Fields GB, Noble RL (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res* 35(3):161–214.
7. Li X, Danishefsky SJ (2008) New chemistry with old functional groups: On the reaction of isonitriles with carboxylic acids—a route to various amide types. *J Am Chem Soc* 130(16):5446–5448.
8. Li X, Yuan Y, Berkowitz WF, Todaro LJ, Danishefsky SJ (2008) On the two-component microwave-mediated reaction of isonitriles with carboxylic acids: Regarding alleged formimidate carboxylate mixed anhydrides. *J Am Chem Soc* 130(40):13222–13224.
9. Li X, Yuan Y, Kan C, Danishefsky SJ (2008) Addressing mechanistic issues in the coupling of isonitriles and carboxylic acids: Potential routes to peptidic constructs. *J Am Chem Soc* 130(40):13225–13227.
10. Yuan Y, Zhu J, Li X, Wu X, Danishefsky SJ (2009) Preparation and reactions of *N*-thioformyl peptides from amino thioacids and isonitriles. *Tetrahedron Lett* 50(20):2329–2333.
11. Wu X, Yuan Y, Li X, Danishefsky SJ (2009) Thio-mediated synthesis of derivatized N-linked glycopeptides using isonitrile chemistry. *Tetrahedron Lett* 50(32):4666–4669.
12. Wu X, Li X, Danishefsky SJ (2009) Thio-mediated two-component coupling reaction of carboxylic acids and isonitriles under mild conditions. *Tetrahedron Lett* 50(14):1523–1525.
13. Stockdill JL, Wu X, Danishefsky SJ (2009) Coupling reactions of hindered isonitriles and hindered alkyl thioacids: Mechanistic studies. *Tetrahedron Lett* 50(36):5152–5155.
14. Wilson RM, et al. (2012) A fascinating journey into history: Exploration of the world of ionitriles en route to complex amides. *Angew Chem Int Ed* 51(12):2834–2848.
15. Rao Y, Li X, Danishefsky SJ (2009) Thio FCMA intermediates as strong acyl donors: A general solution to the formation of complex amide bonds. *J Am Chem Soc* 131(36):12924–12926.

16. Hou JL, Ajami D, Rebek J, Jr. (2008) Reaction of carboxylic acids and isonitriles in small spaces. *J Am Chem Soc* 130(25):7810–7811.
17. Restorp P, Rebek J, Jr. (2008) Reaction of isonitriles with carboxylic acids in a cavitand: Observation of elusive isoimide intermediates. *J Am Chem Soc* 130(36):11850–11851.
18. Wang P, Danishefsky SJ (2010) Promising general solution to the problem of ligating peptides and glycopeptides. *J Am Chem Soc* 132(47):17045–17051.
19. Wu X, Stockdill JL, Wang P, Danishefsky SJ (2010) Total synthesis of cyclosporine: Access to *N*-methylated peptides via isonitrile coupling reactions. *J Am Chem Soc* 132(12):4098–4100.
20. Wang T, Danishefsky SJ (2012) Revisiting oxytocin through the medium of isonitriles. *J Am Chem Soc* 134(32):13244–13247.
21. Dale HH (1906) On some physiological actions of ergot. *J Physiol* 34(3):163–206.
22. Lee H, Macbeth AH, Pagani JH, Young WS, 3rd (2009) Oxytocin: The great facilitator of life. *Prog Neurobiol* 88(2):127–151.
23. Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: Structure, function, and regulation. *Physiol Rev* 81(2):629–683.
24. Witt DM, Winslow JT, Insel TR (1992) Enhanced social interactions in rats following chronic, centrally infused oxytocin. *Pharmacol Biochem Behav* 43(3):855–861.
25. Andari E, et al. (2010) Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. *Proc Natl Acad Sci USA* 107(9):4389–4394.
26. Green L, et al. (2001) Oxytocin and autistic disorder: Alterations in peptide forms. *Biol Psychiatry* 50(8):609–613.
27. Wang P, et al. (2011) Encouraging progress in the ω-aspartylation of complex oligosaccharides as a general route to β-N-linked glycopolypeptides. *J Am Chem Soc* 133(5): 1597–1602.
28. Fu X, et al. (2007) Design and synthesis of novel bis(L-amino acid) ester prodrugs of 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) with improved anti-HBV activity. *Bioorg Med Chem Lett* 17(2):465–470.
29. Wu X, Stockdill JL, Park PK, Danishefsky SJ (2012) Expanding the limits of isonitrile-mediated amidations through a second-generation synthesis of cyclosporine: On the remarkable stereosubtleties of macrolactam formation from *seco*-cyclosporins. *J Am Chem Soc* 134(4):2378–2384.
30. Du Vigneaud V, Lawler HC, Popenoe EA (1953) Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary. *J Am Chem Soc* 75(19):4880–4881.
31. Caldwell HK, Young WS (2006) *Handbook of Neurochemistry and Molecular Neurobiology: Neuroactive Proteins and Peptides* (Springer, Berlin), 3rd Ed, p 573.
32. Donaldson ZR, Young LJ (2008) Oxytocin, vasopressin, and the neurogenetics of sociality. *Science* 322(5903):900–904.
33. Heinrichs M, von Dawans B, Domes G (2009) Oxytocin, vasopressin, and human social behavior. *Front Neuroendocrinol* 30(4):548–557.
34. Lloyd-Williams P, Albericio F, Giralt E (1993) Convergent solid-phase peptide synthesis. *Tetrahedron* 49(48):11065–11133.
35. Borgia JA, Fields GB (2000) Chemical synthesis of proteins. *Trends Biotechnol* 18(6): 243–251.
36. Aucagne V, et al. (2012) Towards the simplification of protein synthesis: Iterative solid-supported ligations with concomitant purifications. *Angew Chem Int Ed Engl* 51(45):11320–11324.
37. Heinlein C, et al. (2011) Fragment condensation of C-terminal pseudoproline peptides without racemization on the solid phase. *Angew Chem Int Ed Engl* 50(28):6406–6410.
38. Canne LE, et al. (1999) Chemical protein synthesis by solid phase ligation of unprotected peptide segments. *J Am Chem Soc* 121(38):8720–8727.
39. Brik A, Keinan E, Dawson PE (2000) Protein synthesis by solid-phase chemical ligation using a safety catch linker. *J Org Chem* 65(12):3829–3835.
40. Krambovitis E, Hatzidakis G, Barlos K (1998) Preparation of MUC-1 oligomers using an improved convergent solid-phase peptide synthesis. *J Biol Chem* 273(18): 10874–10879.
41. Kovacs J (1980) Racemization and coupling rates of N-α-protected amino acid and peptide active esters: Predictive potential. *The Peptides*, eds Gross E, Meienhofer J (Academic, New York), Vol 2, pp 485–539.