Proceedings of the 24th American Peptide Symposium
Ved Srivastava, Andrei Yudin, and Michal Lebl (Editors)
American Peptide Society, 2015 *http://dx.doi.org/10.17952/24APS.2015.001*

Orthogonal Solid-Phase Peptide Synthesis

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Introduction

I am deeply honored to receive the Murray Goodman Scientific Excellence & Mentoring Award, and to join the distinguished company of its past recipients. It was a privilege to know Professor Goodman, whose exceptional combination of scientific innovation and mentorship offers a model we can all hope to emulate.

Our theme is *orthogonality*, a concept I introduced to peptide chemistry during my doctoral studies with Bruce Merrifield at The Rockefeller University [1, 2], and which has been a centerpiece of my independent career on the University of Minnesota chemistry faculty.

Orthogonality

In mathematics, "orthogonality" means intersecting at right angles. In law, it refers to multiple issues that are irrelevant to each other, a point that recently came up in arguments at the U.S. Supreme Court, much to the delight of Justices Roberts and Scalia [3]. In crossword puzzles [4], orthogonal clues can hide the theme. For example, if I asked you "Boston airport" (five letters: answer LOGAN), and "Ball girl?" (three letters: answer DEB), you would get an extra "aha" once you realized that the puzzle was about the wedding of my daughter Deb to my new son-in-law Logan that took place the weekend just preceding the award lecture.

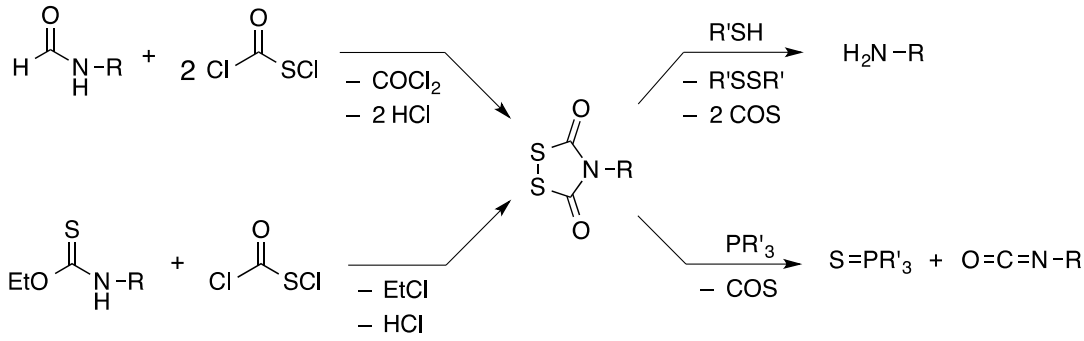
The term "orthogonal" was brought to chemistry in a 1977 *J. Am. Chem. Soc.* communication [1] where we defined an orthogonal system as a set of completely independent protecting groups in which different chemical mechanisms are used to remove each set. Ever since, this word has proven highly useful to succinctly describe a concept of chemoselectivity that chemists and biologists have grasped, at least intuitively, for much longer. Think Emil Fischer's concept of an enzyme lock and a substrate key [5, 6], or E. J. Corey's multi-level protecting group combinations for the total syntheses of complex poly-ols [7].

To illustrate, note that for the classic Merrifield scheme, the "temporary" N^{α}-amino Boc protecting group is removed at each cycle by treatment with trifluoroacetic acid (TFA), while all of the "permanent" side-chain protecting groups and the anchor to the support are required to be stable. This makes it necessary to use a much stronger acid, namely anhydrous HF, to achieve the final cleavage, a direct consequence of the fact that the same chemical mechanism is used to cleave both classes [2, 8]. In contrast, a two-dimensional orthogonal scheme can be developed, using the dithiasuccinoyl (Dts) protecting group on the N^{α}-amino group. Dts is very acid stable, but removable under mild conditions by thiolysis–hence fitting our definition of orthogonality. This now allows the Boc (and related) groups previously used for "temporary" protection to be applied to the side-chains, and taken with Wang's *p*-alkoxybenzyl ester support [an electron-donating oxygen substituent on the ring makes the ester more acid-labile; see ref. 9], the entire protection scheme is "frame-shifted" to allow for the relatively milder cleavage by TFA. A third dimension of orthogonality can be added by adopting an acid-stable, non-thiolysable but photolabile *ortho*-nitrobenzyl ester, as first introduced into solid-phase peptide synthesis by Dan Rich [10], to provide anchoring to the support–this all was experimentally implemented with Fernando Albericio, as described in our 1985 *J. Am. Chem. Soc.* full paper [11].

Many others, including James Tam [12], Carolyn Bertozzi [13], Barry Sharpless [14], and Steve Zimmerman [15], have developed the idea in different directions, including to describe specific bond-making chemistries, like ligations, "clicks," "stapling," chemical modifications of proteins, PEGylation's, and so forth [citations to this sentence are highly selective, and many more can be found with minimal effort]. In collaborative work with my former student Bob Hammer and my brother Francis, we exploited orthogonality to create universal "zip-code" arrays for highly specific "DNA-on-a-chip" detection of mutations in genetic diseases and cancer [16].

Dithiasuccinoyl (Dts) Chemistry

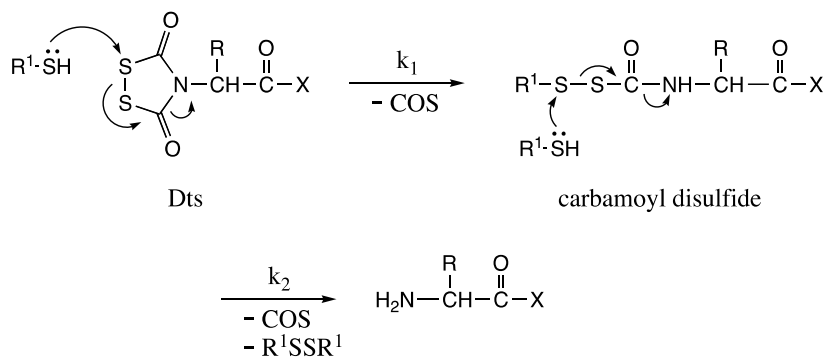
I found the 1,2,4-dithiazolidine-3,5-dione heterocycle in the German patent literature – credit goes to Zumach, Weiss, and Kühle) [17] – and saw how it could be adapted for amino group protection. Groups that cover both free valences of nitrogen are relatively rare. Viewed as a nitrogen atom linked to two molecules of carbonyl sulfide (COS), the analogy to a succinoyl group, but with two sulfurs, led me to coin the dithiasuccinoyl (Dts) moniker. Several multi-step routes to elaborate the Dts heterocycle had been proposed previously, and over the years we adapted and improved the original methods (Scheme 1).



Scheme 1. Preparation and transformations of dithiasuccinoyl (Dts)-amines

In terms of removal, I reasoned that any reductive procedure that cleaved the disulfide would eventually result in the reducing hydrogens ending on the nitrogen. In the process, two COS molecules would be lost, via thiocarbamate intermediates. Later, we realized that reagents like trivalent phosphines, that can "pluck out" a sulfur, make it possible to consider Dts-amines as "masked" isocyanates.

Early on, we established the kinetic and mechanistic details of the thiolytic removal of the Dts group [18, 19], which involved identification of so-called carbamoyl disulfide intermediates (Scheme 2). Although the two steps with rates k_1 and k_2 both involve disulfide cleavage and would nominally appear to be similar, they have rather different features and driving forces … knowledge we would later be able to use in productive ways.

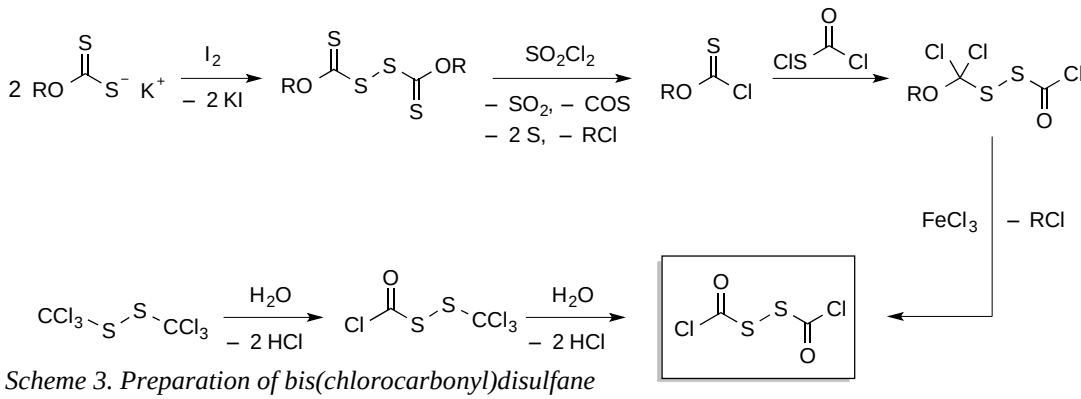


Scheme 2. Thiolytic removal of the dithiasuccinoyl (Dts)-amino protecting group

If we think of Dts as a cyclic carbamoyl disulfide, a myriad of interesting applications have emerged for this family of compounds:

- Primary amino groups that can be protected by Dts include not only the N^{α}-amino group of α-amino acids [1, 20, 21], but also the building blocks for peptide nucleic acids (PNA) [22] and the side-chains of amino sugar building blocks for certain glycopeptides [23].
- Obviously, Dts cannot be used to protect the N^{α}-imino group of proline, but fortunately we were able to optimize open-chain carbamoyl disulfide protection for this residue [24].
- We can "invert" the fundamental mechanism of thiolytic deprotection to develop protecting groups for the sulfhydryl side-chain of cysteine, and for "directed" syntheses of inter- and intramolecular disulfides [25, 26].
- The fact that Dts derivatives are "masked" isocyanates [21] can be very important, given all the difficulties and complications of working directly with isocyanates. Moreover, Dts-amino acids are desulfurized to *N*-carboxyanhydrides (NCA's), and Dts-dipeptides give rise to hydantoins, all under unusually mild conditions [20, 21].
- Focusing on the trivalent phosphorus that can act to desulfurize Dts (and a related compound we call "EDITH"), this chemistry serves as an excellent entry to phosphorothioate DNA and RNA for "anti-sense" applications [27, 28].
- Lastly, Dts chemistry makes it possible to come up with a vastly milder variation to the classic Gabriel synthesis that converts alkyl halides to the corresponding amines – I particularly want to call attention to Mark Wood and collaborators for independent results in this direction [29].

Turning to the preparation of Dts-amines, it was obvious almost from the start that there had to be some way that was more straightforward than what was in the literature. I found a 1973 paper by Kobayashi *et al.* [30] that described a rather roundabout route to bis(chlorocarbonyl)disulfane, the reagent in the box. But look at the structure: it has all of the non-nitrogen atoms that make up Dts, plus two leaving groups – surely this would be a winner. But first, Alayne Schroll, Andy Mott, and David Halsrud in my Minnesota lab had to put in an enormous amount of meticulous work [31] to develop a robust and reproducible route to the desired reagent (Scheme 3).



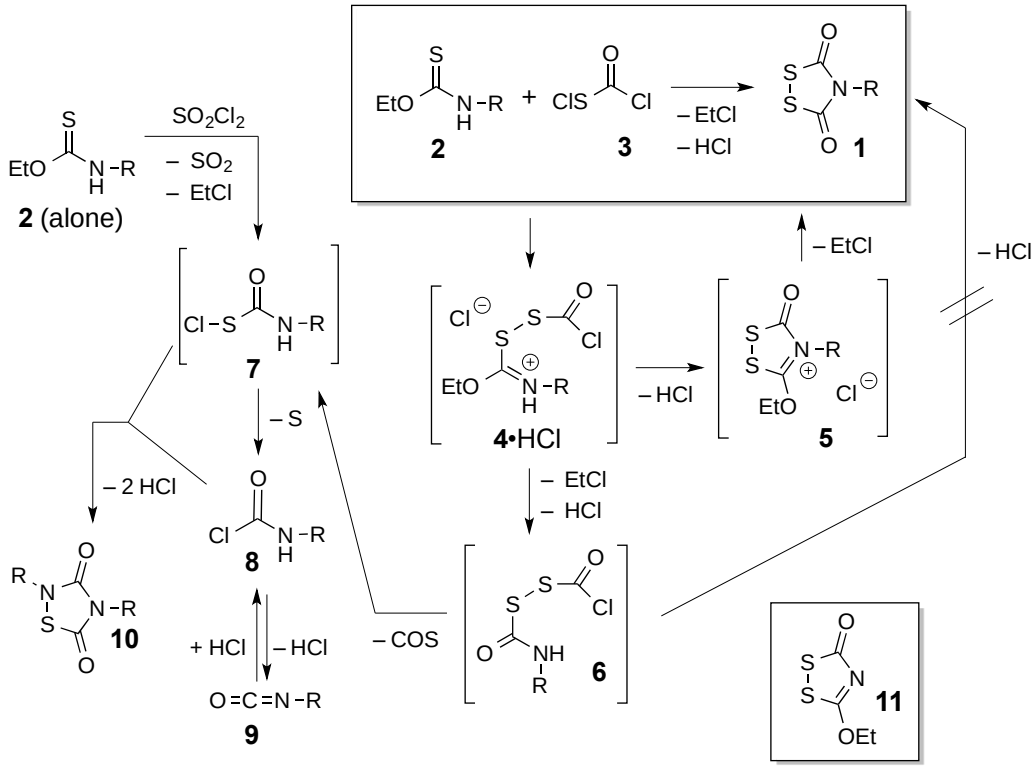
Scheme 3. Preparation of bis(chlorocarbonyl)disulfane

After successfully producing the hard-won reagent, we tried our best to react it with primary amines or amino acids, but none of these experiments gave even the slightest trace of the desired Dts heterocycle. Instead, the products were either isocyanates directly, or derivatives thereof. Therefore, we next investigated additional ways to access the required (chlorocarbonyl)(carbamoyl)disulfane intermediate – which had been postulated by Zumach, Weiss, and Kühle [17] to be an obligatory intermediate in the mechanism for Dts formation – but again, none of these approaches gave Dts.

We had more or less reconciled ourselves to the fact that using bis(chlorocarbonyl)disulfane as a reagent for a single-step synthesis of the Dts heterocycle was not meant to be, when my son Michael joined my lab for a summer while he was still in high school. I remembered a discussion years earlier with Bob Hammer, where he suggested that some of our problems might be due to the fact that our

"leaving group" was HCl, and that maybe they could be circumvented by changing the leaving group to TMS-Cl. In other words, the idea was to use trimethylsilyl (TMS) groups as "big protons" … and it worked, as communicated to the *J. Am. Chem. Soc*. in 2005 [32]. Since the seeds of this work had been planted while I was still in graduate school, I asked my mentor Bruce Merrifield to be a coauthor, and in fact, this is the last publication of Bruce's amazing career.

For a further example of how an initially disappointing result could bear fruit, we were long aware of the classic Nefkens Reagent [33] that allows a one-pot method, in aqueous basic solution, to create phthaloyl amino acids, but were repeatedly unsuccessful in creating its Dts analogue, let alone using such a reagent to create Dts-amino acids. In trouble-shooting the chemistry, we found that the Zumach-Weiss-Kühle-type reaction that for other substrates is essentially instantaneous under normal conditions, now slowed down enough to allow us to isolate, characterize, and/or trap relevant intermediates. As revealed at this meeting [34], we found four structures, all of which could be solved at the atomic level by x-ray crystallography, and all of which model stages in the classic Zumach-Weiss-Kühle mechanism towards Dts-amines. In particular, we believe that delving into the molecular geometries will explain why bis(chlorocarbonyl)disulfane fails to give Dts when reacted with primary amines, but successfully gives Dts when reacted with bis(TMS)-amines.



Scheme 4. A full picture of the Zumach-Weiss-Kühle reaction mechanism

Our best current understanding (Scheme 4) is as follows: thiocarbamates **2** react with (chlorocarbonyl)sulfenyl chloride (**3**) to generate an initial adduct **4**, which can cyclize to **5** first, and then lose EtCl, to give Dts (**1**). If however EtCl is lost first, the (chlorocarbonyl)(carbamoyl)disulfane intermediate **6** is surprisingly stable, but does not go to Dts. We also understand the formation of other by-products, such as 1,2,4-thiadiazolidine-3,5-dione (Tda) (**10**), and the outcomes under specialized conditions [like 3-ethoxy-1,2,4-dithiazolin-5-one (EDITH)

(**11**), formed when R = H]. Thus, we are finally on the verge of a complete understanding of the Zumach-Weiss-Kühle reaction … and it took less than 40 years.

Orthogonal Solid-Phase Peptide Synthesis

All along, these investigations into fundamental organosulfur chemistry have informed significant developments in orthogonal solid-phase peptide synthesis. In particular:

- Tracking down a low-level but nonetheless troublesome side reaction in the preparation of Dts-amino acid building blocks, Samuel Zalipsky was compelled to prepare any number of novel polyethylene glycol (PEG) derivatives [20]. A mechanistic control experiment led to the isolation of a PEG-amino acid, and that was used, in turn, to create the first PEG-PS resin [35]. Later work with Fernando Albericio, Jane Chang, Derek Hudson, and Nuria Solé led to more practical formulations, suitable for commercial production [36]. Once peptide chemists realized that their more challenging synthetic targets required support materials that were compatible with both organic solvents and with aqueous media, PEG-PS became the resin support of choice for many experiments, including combinatorial chemistry designs that culminate in biological testing.
- Later, lightning hit a second time, when Maria Kempe parlayed her experience and insights in the field of molecular imprinting to develop the highly counter-intuitive, but very effective, CLEAR family of supports for SPPS [37].
- The signature step of solid-phase synthesis is the anchor to the support, and many of my co-workers, including Fernando Albericio, Jordi Alsina, Yongxin Han, Knud Jensen, Nancy Kneib-Cordonier, Michael Songster, Josef Vágner, and Scott Yokum, made significant contributions to a veritable alphabet soup (or rhyme scheme) of handles (or linkers), like PAL, HAL, XAL, and BAL [38–41].
- I have already alluded to the underlying motivation for orthogonal peptide synthesis – development of milder reaction conditions for the key steps, so that the overall scheme would be conducive to the preparation of the sort of labile constructs needed to solve important biological problems. While our initial focus was to apply Dts chemistry, it later turned out that many of our ideas could be implemented with Carpino's Fmoc group instead [42]. In this regard, I want to call attention to the important work of Meienhofer's group at Hoffmann-LaRoche [43] and the Cambridge group of Bob Sheppard and Eric Atherton [44] in expediting the transition from Boc to Fmoc in a sizeable portion of the peptide synthesis laboratories in academia and in the private sector worldwide. In my lab, many students contributed, especially Liz Ottinger who was the first to create phosphopeptides by Fmoc chemistry in the mid '90's [45].
- A central interest of ours has been the management of cysteine residues, including the development of new protecting groups and the regioselective creation of disulfide bridges both in solution and on-resin [the latter exploiting Steve Mazur's concept, as per ref. 46, of *pseudo-dilution*. Starting with Ioana Annis and Lin Chen, and continuing in collaboration with Arno Spatola, Deanna Long, and Krys Darlak then all at Peptide International, we developed a polymer-supported Ellman's reagent for oxidation of cysteine-containing peptides under extraordinarily mild conditions, in essence an artificial "chaperone."
- Lin Chen, Bob Hammer, and others helped to homologate our chemistry to create peptide trisulfides [47], a class of compounds that were speculative novelties when we started, but have since appeared in Nature in surprising ways.
- A productive collaboration with Clare Woodward provided numerous insights into fundamental questions in protein folding research, particularly on the role of disulfide bridges. Using the 58-residue three-disulfide small protein bovine pancreatic trypsin inhibitor (BPTI) as a model, Marc Ferrer, Elisar Barbar, Chris Gross, Hong Pan, Judit Tulla-Puche, Irina Getun, and Natàlia Carulla synthesized and characterized the parent structure along with numerous analogues that were made more flexible by replacing cysteine moieties by pairwise α-amino-*n*-butyric acid (Abu) residues [48–53]. Most dramatically, we designed, synthesized, and characterized BetaCore, the first four-stranded antiparallel β-sheet that folds in water [54]. The synthetic chemistry required orthogonal oxime ligation chemistry that we developed and optimized [55].

Conclusions

In this contribution, I have shared with you a number of scientific stories with the common denominator of "orthogonality." What started as a simple word – to express and clarify an idea that chemists have implicitly known for much longer – has blossomed significantly in the 21st century as a critical design consideration when carving out new directions in chemical biology. It is particularly appropriate to note that in the last lecture that I heard Murray Goodman give, at a 2001 symposium honoring Bruce Merrifield's 80th birthday, he spoke about ways that orthogonality had formed his own research.

Acknowledgments

Throughout my scientific career, I have been fortunate to have mentors and role models who cared about me and influenced my development. I made my first professional scientific presentations at age 17, at a Federation meeting in Atlantic City and, a few months later, at a Cold Spring Harbor Symposium [56]. I want to pay tribute to my parents, Michael and Kate Bárány, who guided me into the family business, and Bruce Merrifield, who patiently accepted me into his laboratory when I was still very green. In 1999, it was my privilege to co-Chair the 16th American Peptide Symposium, and welcome a "who's who in peptide science" (Figure 1) to offer their perspectives for the new millennium [57]. Jed Fisher has been a good friend from the time we met in 1980 through the present day.

Fig. 1. From left to right: Victor Hruby, Charles Deber, Tom Muir, Robert Hodges, Bruce Merrifield, Robin Offord, Murray Goodman, Arno Spatola, Daniel Veber, and George Barany

Over the years, I've mentored about two dozen Ph.D. students, over 30 postdoctoral fellows, and over 100 undergraduates. Beyond what they achieved in my laboratory, almost all of them have gone on to productive independent careers in academia, industry, biotechnology, and government. A full listing appears on my

webpage [58], but I want to single out Alayne Schroll and Bob Hammer, wonderful friends … and we're still writing papers together. Several of my mentees have achieved recognition of their own, for example, my long-time friend and collaborator, Fernando Albericio, was recognized two years ago with the Vincent du Vigneaud award of the American Peptide Society, and Simon Shannon, the first member of his family to attend college, let alone earn a Ph.D., received a major national achievement award from TRIO in November 2014. Matt Henley, some of whose organosulfur research discoveries were reported at this, his first American Peptide Symposium, won two prizes for undergraduate research this past spring at a local symposium [I also thank Matt for his help in preparing this manuscript].

I view mentoring as a life-long commitment, and my students are my extended family. I thank all of them for what they contributed that has led to my Goodman Award.

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