The Microscale Laboratory

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Complete Analysis of a Biologically Active Tetrapeptide: A Project Utilizing Thin-Layer Chromatography and Mass Spectrometry†

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Most experiments in undergraduate organic or biochemistry laboratories that involve peptides focus on either sequence determination (*1*) or synthesis (*2*) of a simple dipeptide. The identification of the amino acid content of a tripeptide has been reported in this *Journal*, but the sequence was not determined (*3*). In no case has the stereochemistry of the individual amino acids been reported. At SUNY Oswego, in addition to our second-year organic and biochemistry laboratories, we offer an advanced chemistry laboratory. In this course we implement projects that have a research flavor and that take several weeks to complete. We describe here a project involving a tetrapeptide, which expands significantly upon the usual dipeptide analysis. Students analyze the biologically active tetrapeptide D-Ala-Gly-L-Phe-D-Leu ([des-Tyr^1-D-Ala^2-D-Leu^5] enkephalin), for amino acid content, stereochemistry, and sequence. The experiment gives students valuable experience in microscale synthesis, both normal and reversed-phase thin-layer chromatography (TLC), stereochemical analysis, and mass spectrometry. The peptide was carefully chosen to include amino acids with both D- and L- stereochemistry. The analyses were successfully done on *less than 125 μg of the tetrapeptide* per pair of students.

Experimental Procedure

The experiment was performed over a four-week period and was divided into four major sections as listed below:

A. N-terminal amino acid analysis

B. Identification of the remaining three amino acids

C. Determination of the stereochemistry of the amino acids

D. Determination of the sequence of the peptide

The first three sections involved synthesis and TLC; the last section was concerned only with the interpretation of the tandem quadrupole mass spectrum (MS/MS) of the peptide that was given to the students. One student of each pair (student 1) performed Section A, and the other (student 2) worked on Section B. Both students worked together on portions of Section B and on Sections C and D.

The day before the first lab period, student 2 began the overnight hydrolysis of a portion of the tetrapeptide to provide the free amino acids needed for Section B. In the first lab period, after an introductory briefing, student 2 converted the resulting four free amino acids to their respective *N*-5-dimethylamino-1-naphthalene sulfonyl (dansyl, DNS) derivatives using dansyl chloride (*4*). Student 1 converted a second portion of the peptide to the *N*-DNS-tetrapeptide. The day before the second lab period, student 1 began the overnight hydrolysis of the DNS-tetrapeptide, freeing the N-terminal amino acid. During the second lab period, student 1 identified the N-terminal amino acid by TLC and student 2 purified the four DNS-amino acids by preparative TLC. During lab periods two and three, both students identified the remaining three DNS-amino acids by TLC. During the fourth lab period, both students determined the stereochemistry of the individual amino acids in Section C by reversed-phase TLC. Finally, the MS/MS spectrum was given to the students so that they could determine the amino acid sequence of the tetrapeptide. The reactions and methods used in Sections A, B, and C are summarized in Figure 1.

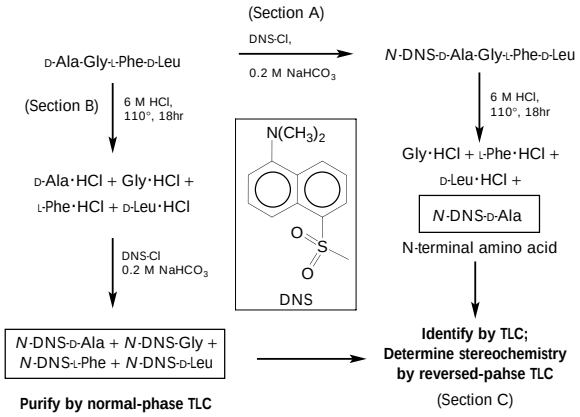

Figure 1. Reactions and methods used to identify the amino acids in the tetrapeptide.

Results and Discussion

The experiment has been successfully performed for four years on either the tetrapeptide or a related pentapeptide. Because DNS-amino acids fluoresce so strongly, the analyses could be done on very small amounts. In Section A only 40 μg of tetrapeptide were used, and in Section B, 80 μg. The students were given access to 15 commercial DNS-amino acids

†Presented in part at the 13th Biennial Conference on Chemical Education, Bucknell University, July 31–August 4, 1994.

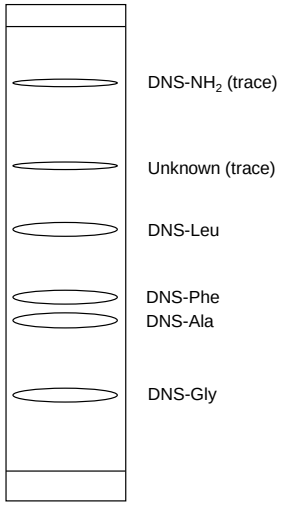

Figure 2. Normal-phase TLC separation. Solvent: toluene–pyridine–AcOH, 30:10:1; 5 × 20-cm plate.

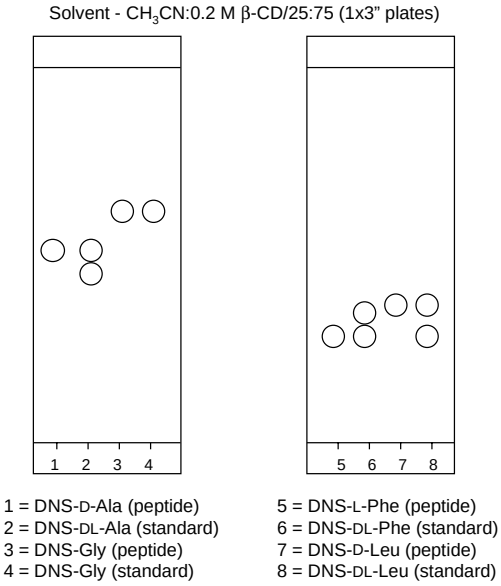

Figure 3. Reversed-phase TLC separations.

that were used as TLC standards for identifying the four amino acids in the tetrapeptide. These four DNS-amino acids separate well on normal-phase TLC using toluene–pyridine–acetic acid (AcOH), 30:10:1 (v/v/v) as the mobile phase (*5*), as shown in Figure 2.

After students had correctly identified the four amino acids, they began the stereochemical analysis of each. The analyses were done with 1 × 3-in. C-18 reversed-phase TLC plates using the chiral mobile phase additive β-cyclodextrin (β-CD) in an aqueous solvent containing acetonitrile (CH_3CN). The β-CD forms diastereomeric inclusion complexes with DNS-D- and L-amino acids, which are separable by reversed-phase TLC. The DNS-D-isomers always elute ahead of the corresponding L-isomers, making the stereochemical assignments of individual DNS-D- or L-amino acids easy by comparison with the corresponding DL-racemates (*6*). Students were sent to the literature (the first two references in *6*) to determine these facts. Achiral glycine, of course, showed no such separation. The DNS–DL-alanine standard must be synthesized because only the L-isomer is commercially available. A simple procedure for its preparation is included in the Instructors' Notes.[W] Typical reversed-phase TLC separations are shown in Figure 3.

After all the wet chemistry was performed the students were given the MS/MS spectrum of the tetrapeptide (*7*). With this information they successfully determined the sequence of the amino acids in the peptide, and proposed a structure that included stereochemistry. In our experience the students have really enjoyed the experiment, and it has been a great success. All the materials for the experiment are easily obtainable, and the results are reproducible. The tetrapeptide is available from Sigma in 5-mg quantities, which is enough for approximately 40 pairs of students. The experiment can be successfully performed without a tandem quadrupole mass spectrometer, since the MS/MS spectrum of the tetrapeptide is provided for students in Student Handout #2.[W] The MS/MS spectrum of a hexapeptide and a step-by-step interpretation of it are also included.

Acknowledgments

We thank the students in Che 334L, Advanced Chemistry Laboratory, for their hard work on this project. We also gratefully acknowledge the State University of New York at Oswego for financial support. Finally, we thank Linda LeFevre for typing the manuscript.

[W]Supplemental Material

Detailed student procedures, questions, and Instructors' Notes are available in this issue of *JCE Online*. The Instructors' Notes include comments on the experiment, tables of TLC retention factors (R_f values), MS/MS fragmentation pattern data, answers to questions, equipment and reagent lists, detailed procedures for preparing reagents, and hazard alerts.

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