Affinity Purification of Crosslinked Peptides

Michael B. Strader,[1] Gregory B. Hurst,[1]
Trish K. Lankford,[2] Robert L. Hettich,[1]
Stephen J. Kennel[2]

[1]Chemical Sciences Division
And
[2]Life Sciences Division
Oak Ridge National Laboratory

Overview

- Products obtained using a biotinylated crosslinking reagent are identified.
- A batch-mode affinity purification provides good selectivity for isolating biotinylated crosslinked peptides from tryptic digest mixtures.

Introduction

- Chemical crosslinking is a valuable tool for studying protein structure and protein-protein interactions [1,2]
- Determining the locations of the bonds introduced by crosslinking is a difficult analytical task:
  - Crosslinking reaction yields are generally low.
  - Side reactions to the crosslinking introduce non-informative peptides.
  - The crosslinked peptides thus are minor components of a complex mixture.
- A selective method for isolating crosslinked peptides from such mixtures is desirable. [1]
- We describe here a model system for separating biotinylated, crosslinked peptides from tryptic digests of crosslinked proteins.

Methods

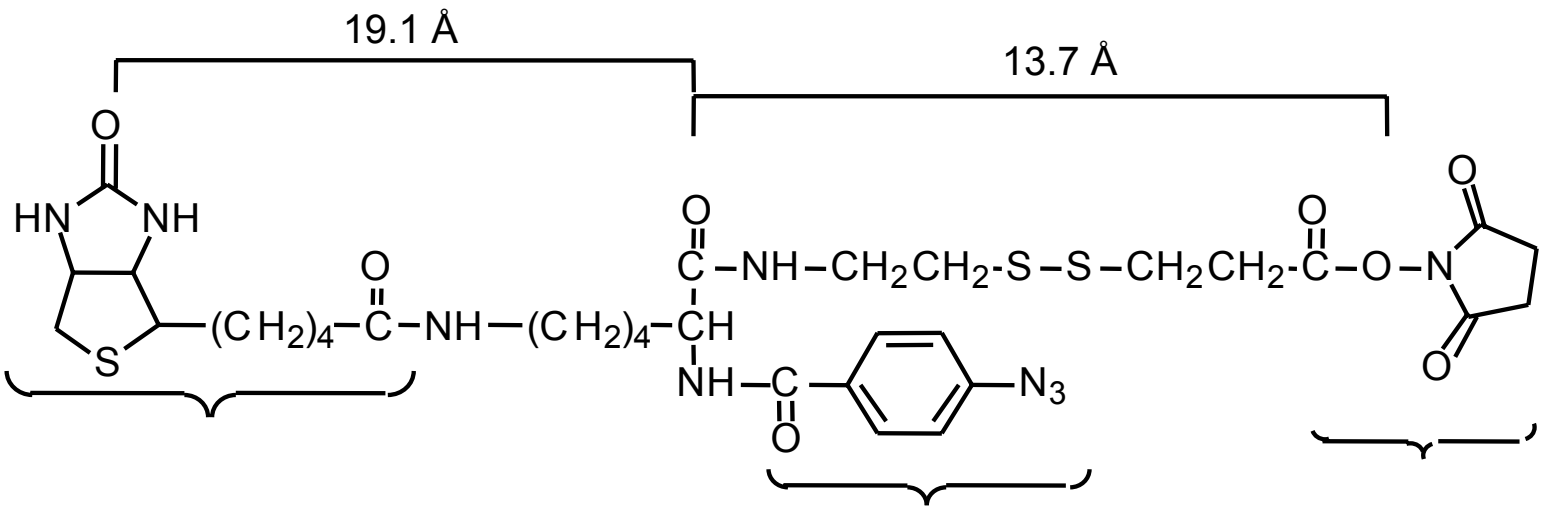
- Crosslink neurotensin intramolecularly using sulfo-SBED, a heterobifunctional crosslinker with a biotin moiety.
  - Characterize reaction products using MALDI-TOF.
- Avidin affinity capture of crosslinked/biotinylated neurotensin from non-crosslinked peptide mixture (tryptic digest of hemoglobin)
  - Characterize separation using MALDI-TOF.

Neurotensin

pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu

blocked N-terminal amine

Sulfo-SBED

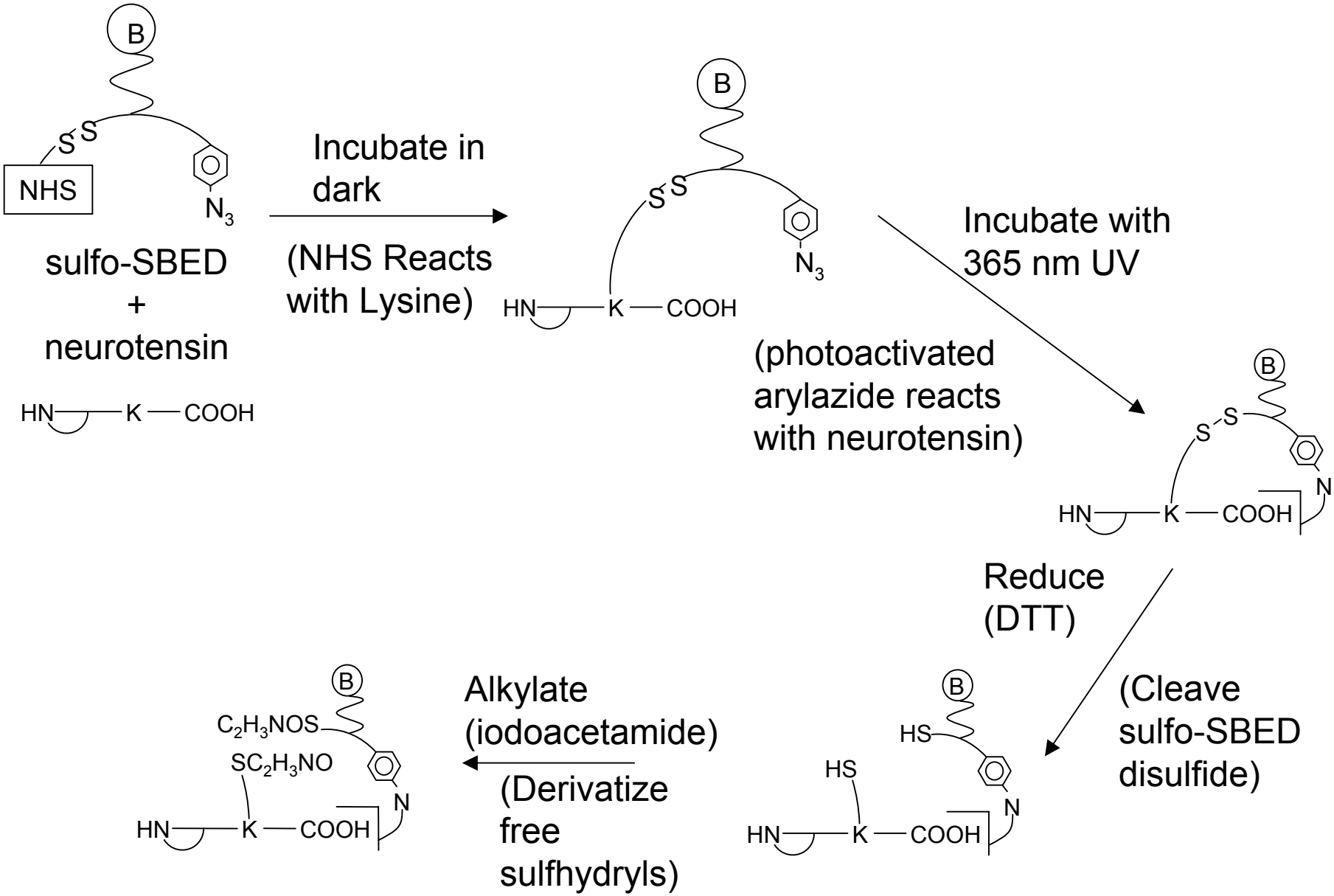


Biotin

Photoactive Group

1º Amine-reactive Group (NHS)

Crosslinking Protocol



Affinity Purification Protocol

Block monomeric avidin beads:
1. Biotin
2. BSA

double blocking reduces non-specific binding

Incubate beads (10 μL) with sample (100 μL)

small volume prevents sample loss

MALDI of supernatant: Figure 3b

Wash beads with phosphate buffer (1-3x)

MALDI Figure 3c

Wash with 20% MeOH, 50 mM NH_4HCO_3

MALDI: Figure 3d

Elute with 50% CH_3CN, 0.4% TFA

MALDI Figure 3e

Results
Identifying Crosslinking Products

- **Figure 1a** and **Scheme 1** show products of the neurotensin/sulfo-SBED reaction
  - Predicted intramolecular crosslink (I)
  - Incomplete ("one-sided") crosslinks (II, III)
  - Oxidized analogs (I_{ox}, I_{ox}, III_{ox})
  - Unreacted neurotensin (NT)
- **Figure 1b, c** show products of reduction (DTT) alkylation (iodoacetamide)
- Major products are:
  - Oxidized intramolecular crosslink
  - "single-sided" product of NHS attachment to lysine

Scheme 1

B=biotin
NHS=N-hydroxysuccinimide
DTT=dithiothreitol
IAM=iodoacetamide
"ox" = addition of an O-atom to a species
Bold numbers: observed peaks in Fig. 1
Monoisotopic, neutral masses

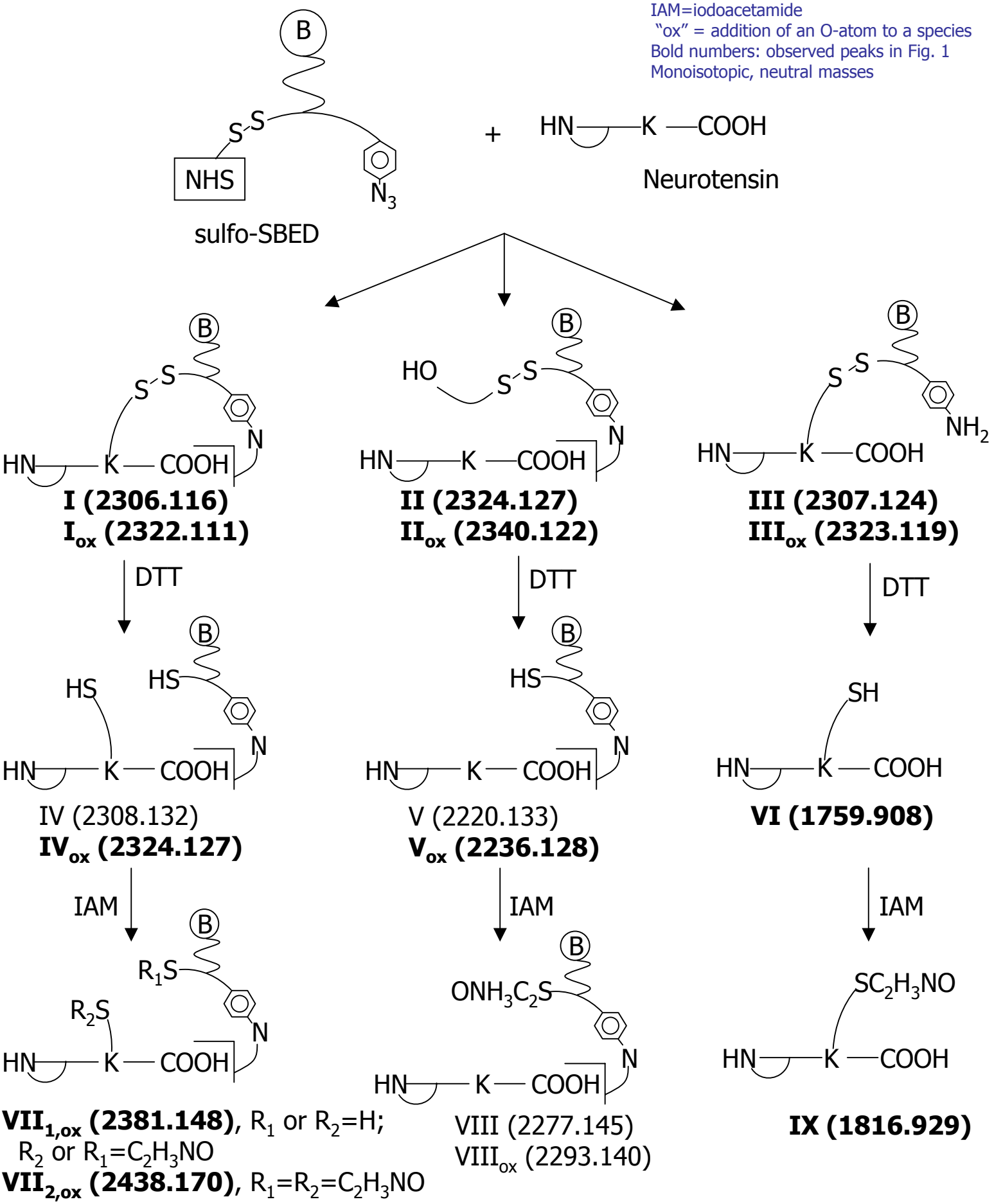
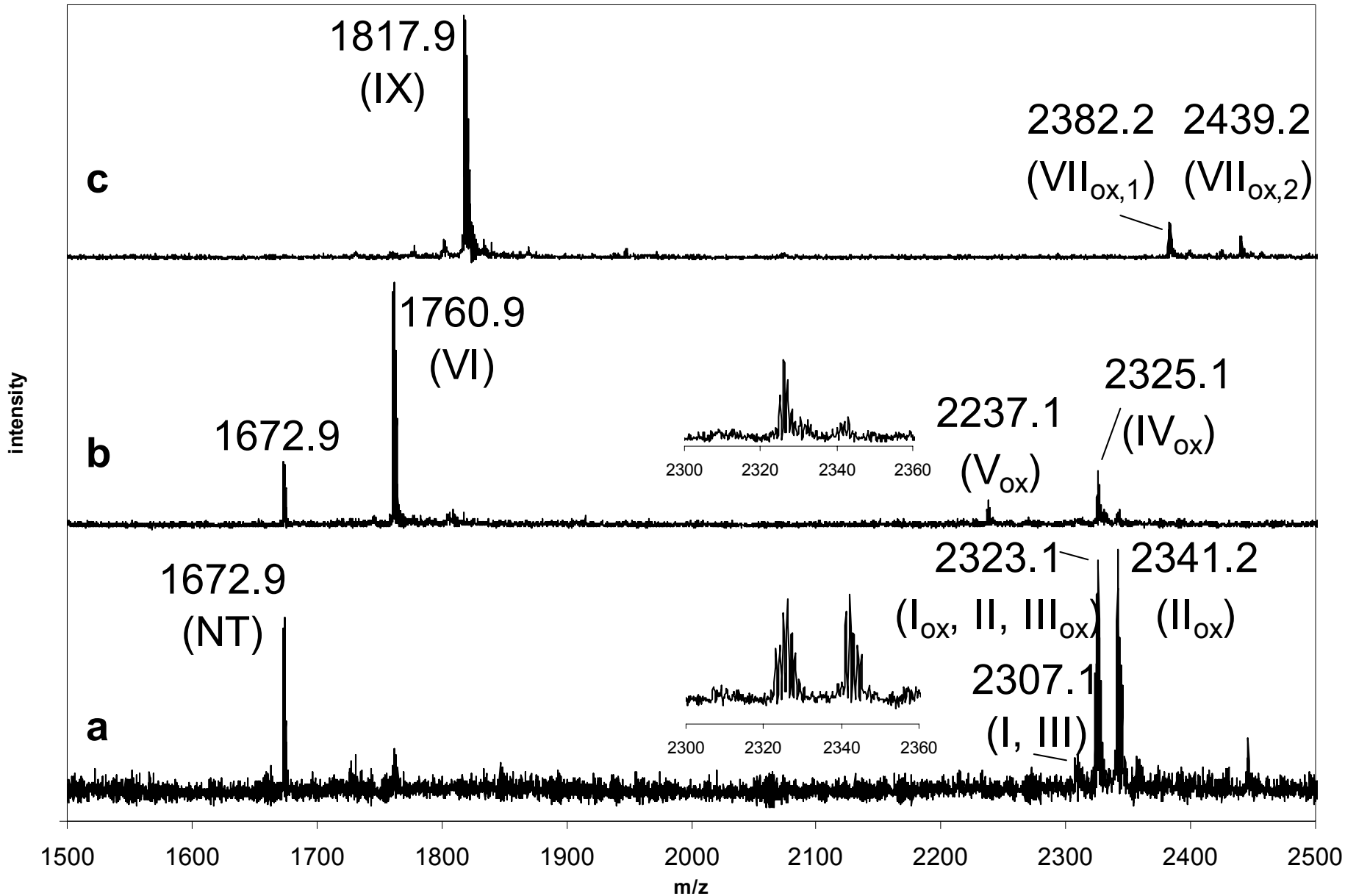


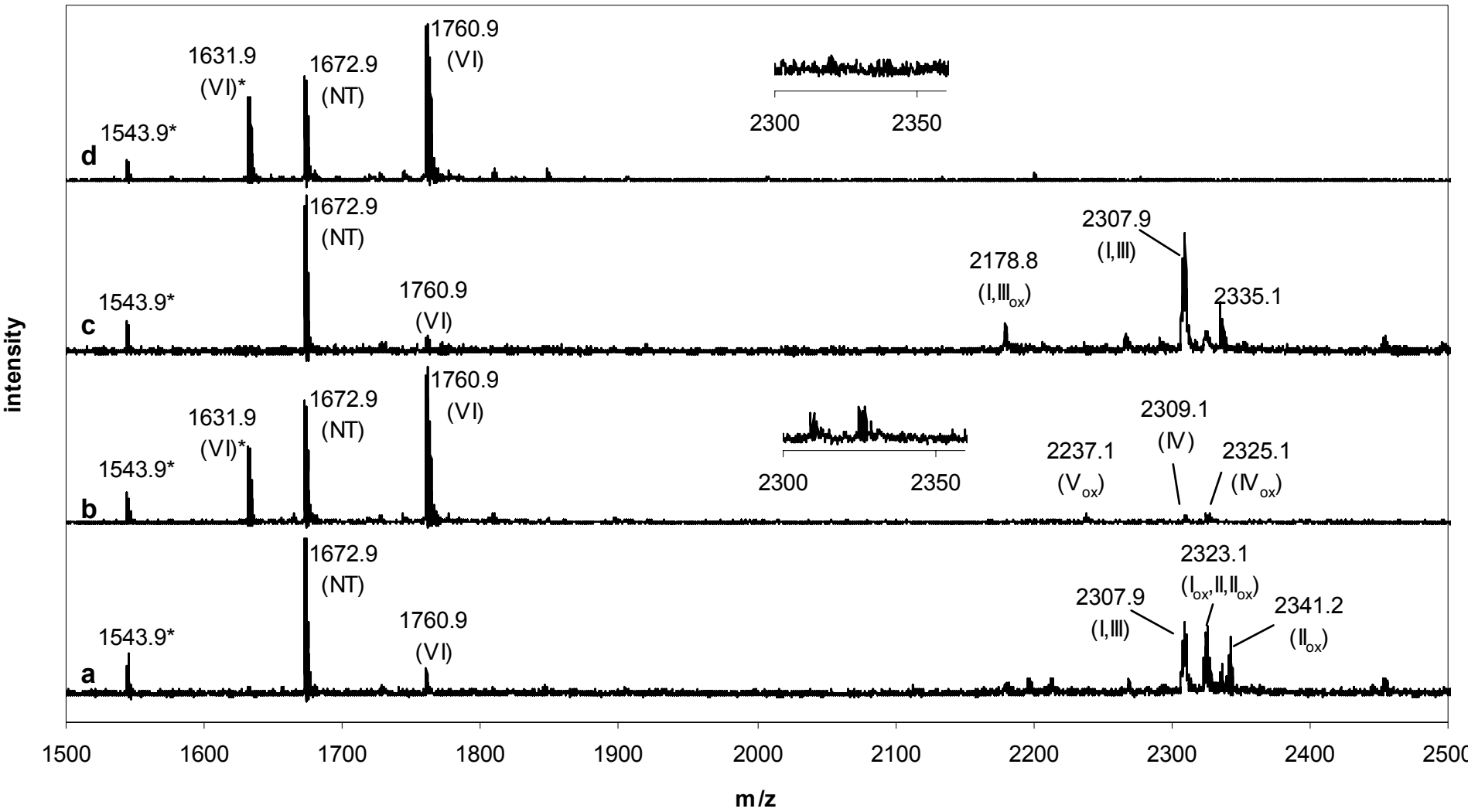
Figure 1


c
1817.9
(IX)
2382.2
(VII$_{ox,1}$)
2439.2
(VII$_{ox,2}$)
b
1672.9
1760.9
(VI)
2237.1
(V$_{ox}$)
2325.1
/(IV$_{ox}$)
2300
2320
2340
2360
a
1672.9
(NT)
2323.1
(I$_{ox}$, II, III$_{ox}$)
2341.2
(II$_{ox}$)
2307.1
(I, III)
2300
2320
2340
2360
intensity
1500
1600
1700
1800
1900
2000
2100
2200
2300
2400
2500
m/z

Results
Minimizing Oxidation

- Figure 2 shows results of shorter UV illumination times in the crosslinking reaction:
  - a: 15 minute UV (b: DTT reduction)
  - c: 0 minute UV (d: DTT reduction)
- Observe more I and IV (Fig. 2a,b) than for 90 minute UV exposure (Fig. 1a,b)
- Oxidation appears to be on biotin sulfur
  - Not on neurotensin
  - Not at disulfide

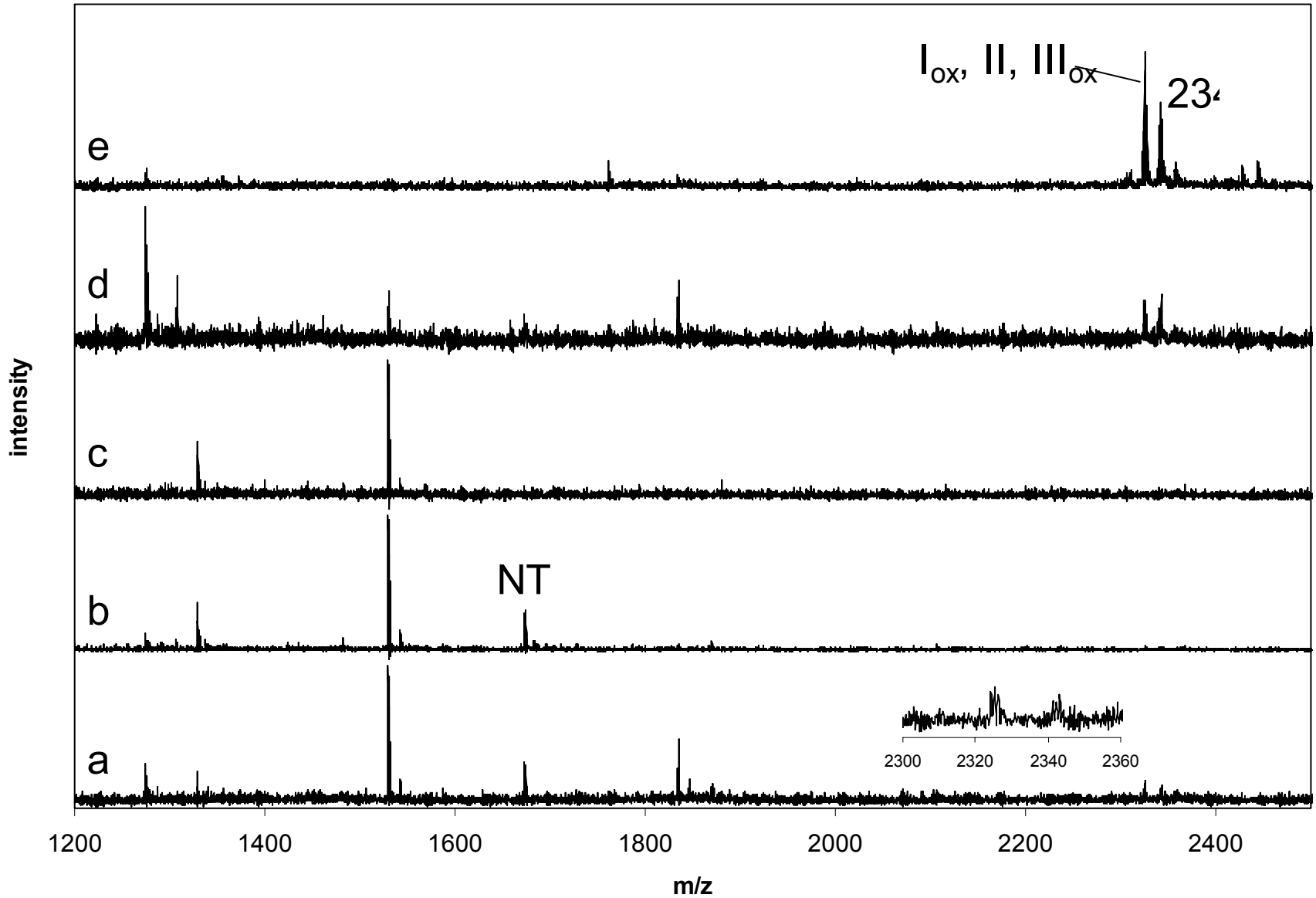
Figure 2


d
1543.9*
1631.9
(VI)*
1672.9
(NT)
1760.9
(VI)
2300
2350
c
1543.9*
1672.9
(NT)
1760.9
(VI)
2178.8
(I,III$_{ox}$)
2307.9
(I,III)
2335.1
b
1543.9*
1631.9
(VI)*
1672.9
(NT)
1760.9
(VI)
2300
2350
2237.1
(V$_{ox}$)
2309.1
(IV)
2325.1
(IV$_{ox}$)
a
1543.9*
1672.9
(NT)
1760.9
(VI)
2307.9
(I,III)
2323.1
(I$_{ox}$,II,II$_{ox}$)
2341.2
(II$_{ox}$)
intensity
1500
1600
1700
1800
1900
2000
2100
2200
2300
2400
2500
m/z

Results
Isolation of Crosslinked Peptides

- Figure 3 shows MALDI spectra from avidin affinity purification of SBED-crosslinked neurotensin from a tryptic digest of hemoglobin
  - 3a: before purification: crosslinked products barely detected (inset)
  - 3b: supernatant after avidin capture
  - 3c: 0.1 M phosphate wash
  - 3d: 20% methanol, 50 mM NH_4HCO_3 wash
  - 3e: eluent shows
    - enrichment of crosslinked peptides
    - minimal non-specific binding

Figure 3


I_{ox}, II, III_{ox}
23
e
d
c
b
NT
a
2300
2320
2340
2360
intensity
1200
1400
1600
1800
2000
2200
2400
m/z

Conclusions

- The neurotensin/sulfo-SBED conjugate is a useful model system for
  - Identifying the range of reaction products obtained (side reactions, oxidation products)
  - optimizing an affinity purification strategy
- These results form a basis for understanding crosslinking/MS data on the more interesting problem of crosslinking and digesting proteins and protein complexes.

References

1. Trester-Zedlitz, M.; Kamada, K.; Burley, S.K.; Fenyo, D.; Chait, B.T.; Muir, T.W. A modular cross-linking approach for exploring protein interactions. *J. Am. Chem. Soc.* **2003**, *125,* 2416-2425.

2. Young, M.M.; Tang, N.; Hempel, J.C.; Oshiro, C.M.; Taylor, E.W.; Kuntz, I.D.; Gibson, B.W.; Dollinger, G. High throughput protein fold identification by using experimental constraints derived from intramolecular cross-links and mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **2000,** *97,* 5802-5806.

Acknowledgements

- This research sponsored by the Office of Biological and Environmental Research, U.S. Department of Energy, and the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory (ORNL). ORNL is managed by UT-Battelle, LLC, for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.