

Supporting Online Material for

Insights into heterocyclization from two highly similar enzymes

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Figures S1-S8

Table S1

Experimental methods

Figure S1. SDS-PAGE of purified TruD and PatD proteins. From the left, lanes are: (1) TruD (2) Ladder (3) PatD.

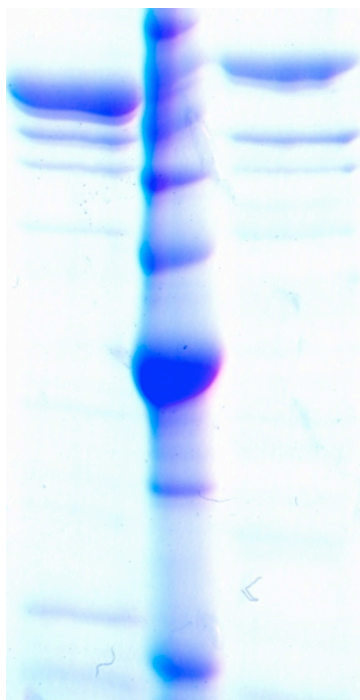


Figure S2. Intact analysis of TruD- and PatD-treated TruE2. (a) shows ESI(-) intact analysis of PatD-treated TruE2. Result is consistent with the formation of two thiazoline rings and a single oxazoline ring; (b) shows ESI(-) intact analysis of TruD-treated TruE2. Result is consistent with the formation of two thiazoline rings; (c) SDS-PAGE showing typical band shifts resulting from TruE2 modification reactions. From right to left, lanes are as follows: (1) TruE2 standard (2) TruD-treated TruE2 (3) TruD-treated TruE2 + TruE2 standard (4) PatD-treated TruE2 (5) PatD-treated TruE2 + TruE2 standard (6) TruE2 standard (7) ladder.

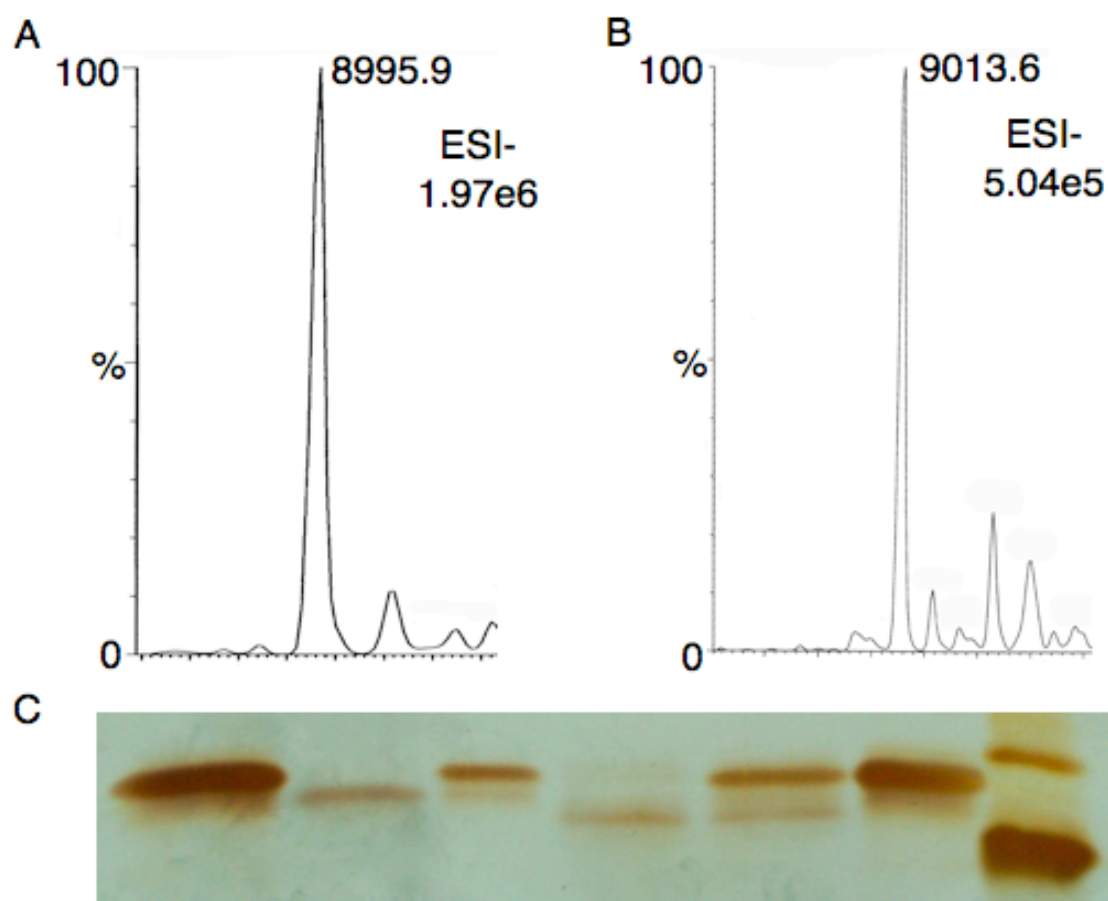
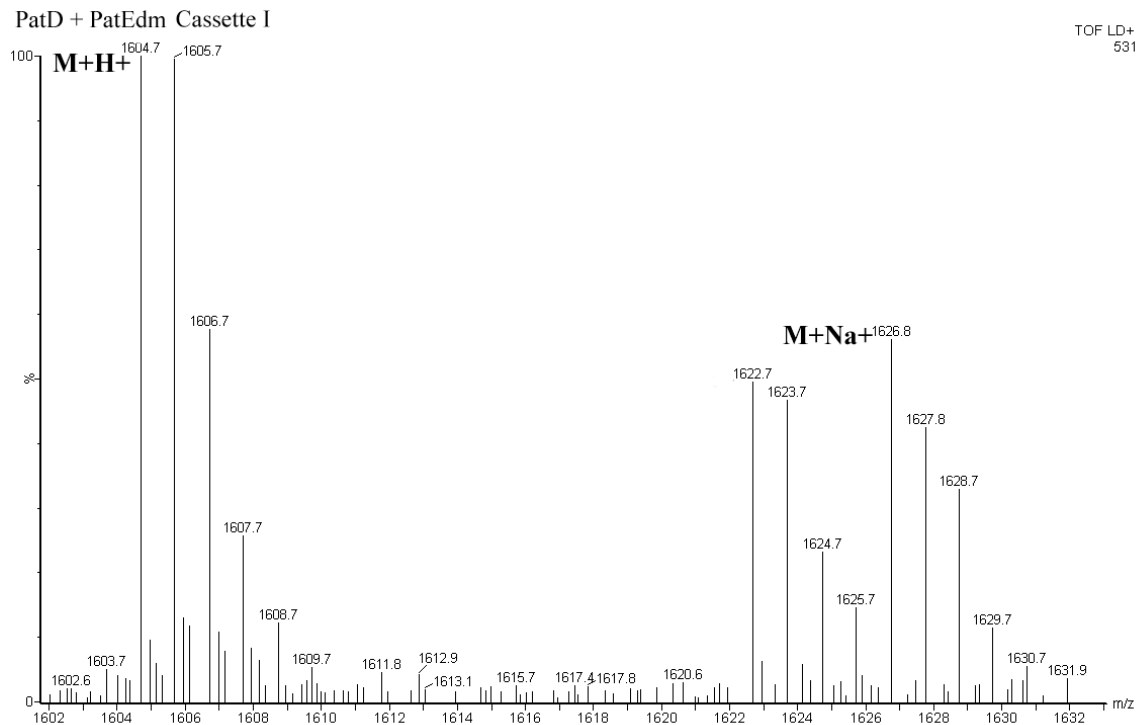
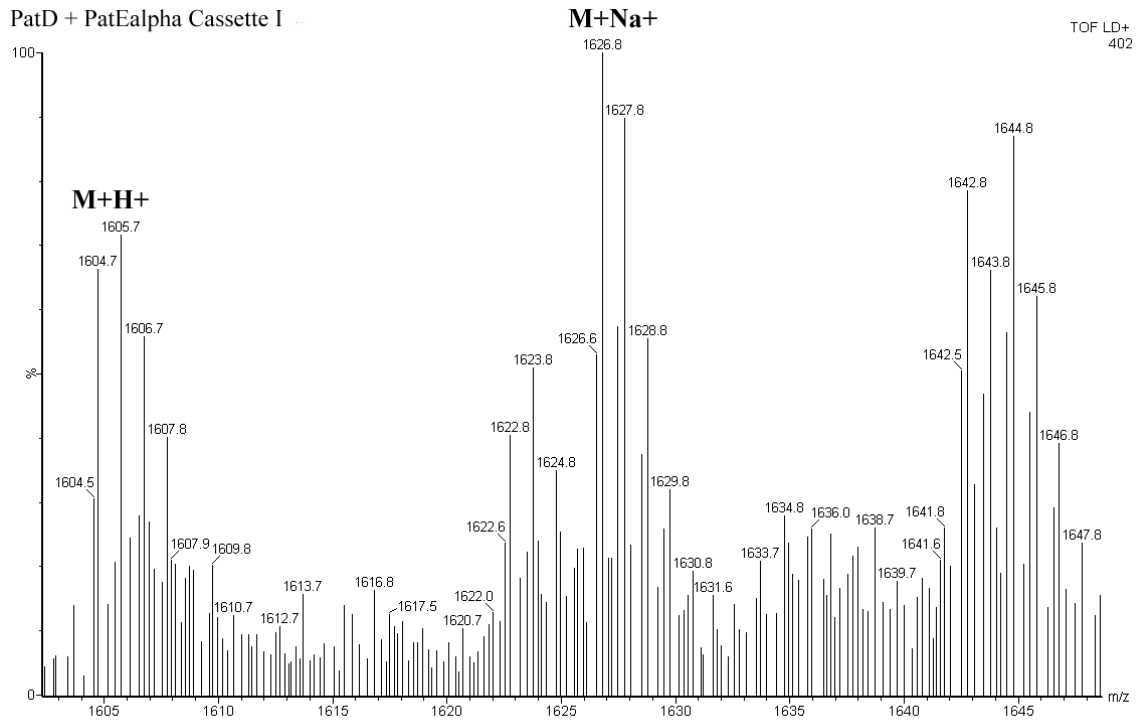
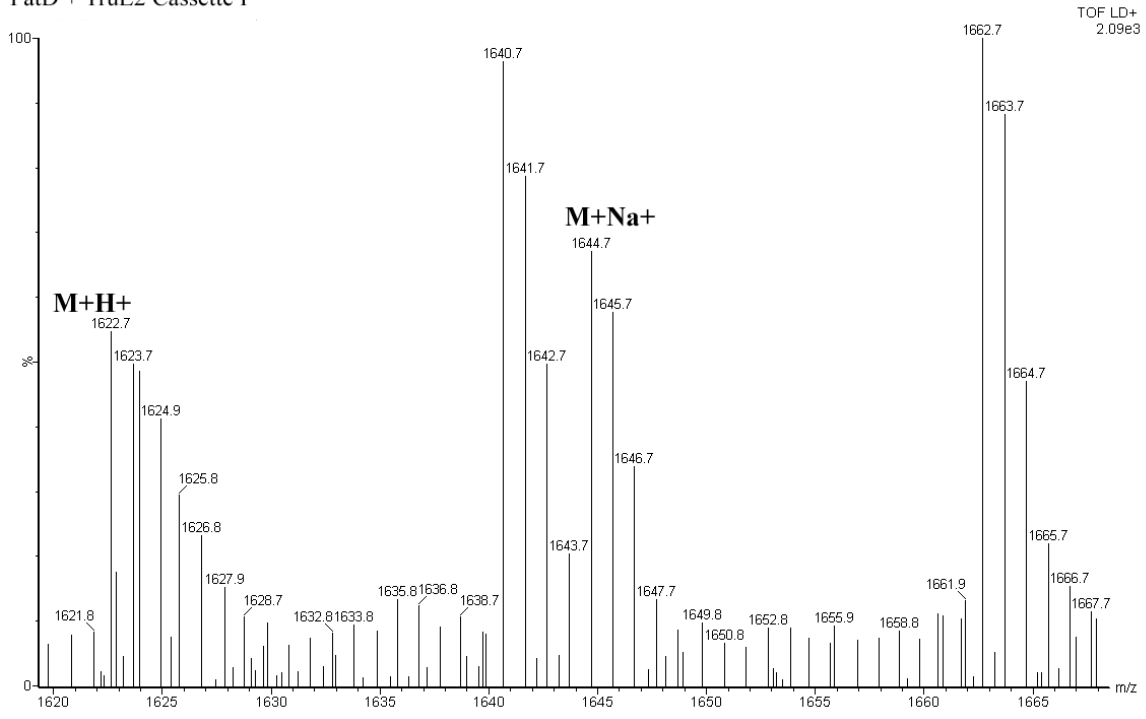


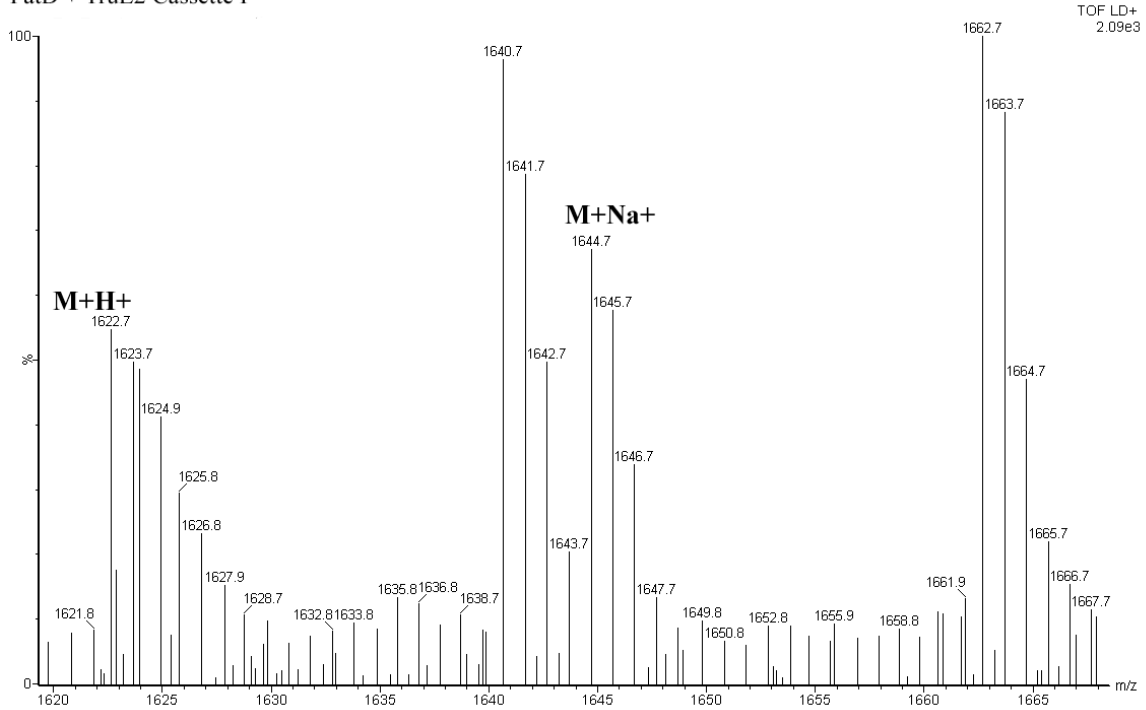
Figure S3. MALDI-MS spectra demonstrating modification of TruE2, PatE α , and PatEdm product cassettes by PatD and TruD; peptides were all digested with PatA prior to analysis.



PatD + TruE2 Cassette I

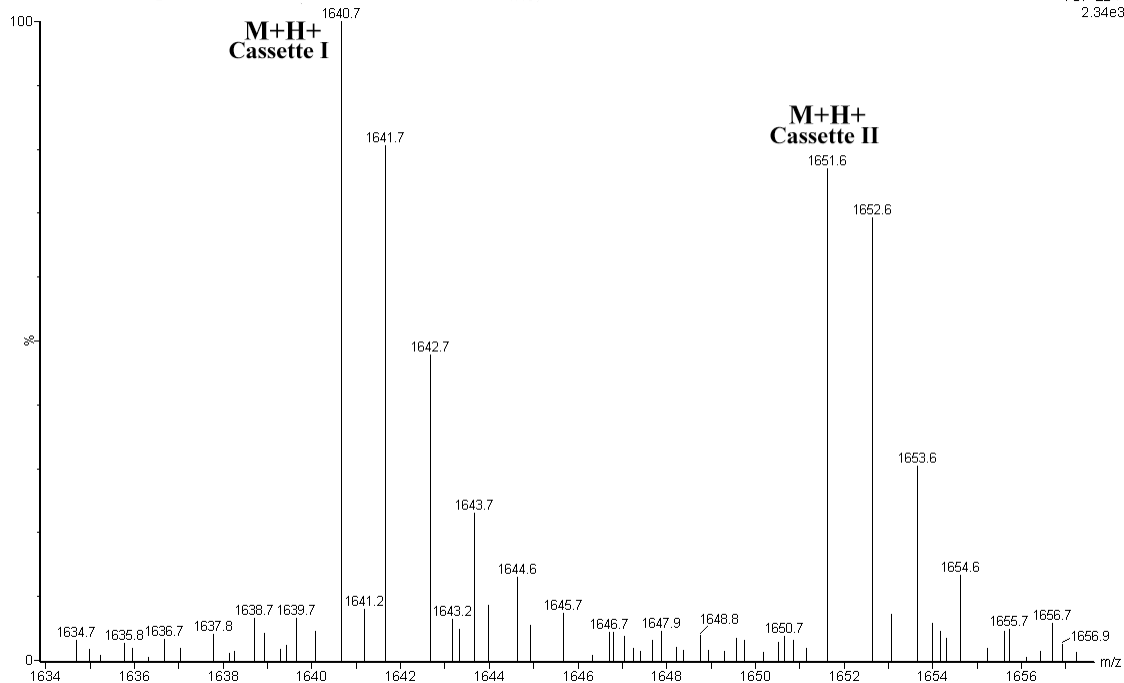


PatD + TruE2 Cassette I



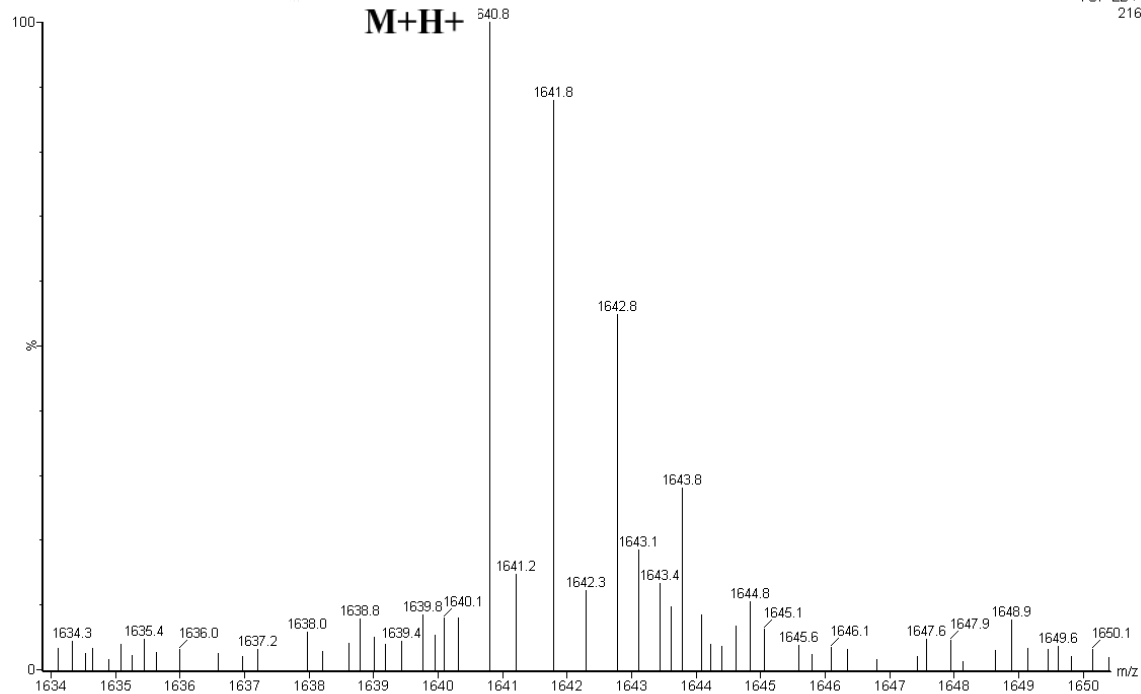
TruD + PatEalalpha cassettes I and II

TOF LD+
2.34e3

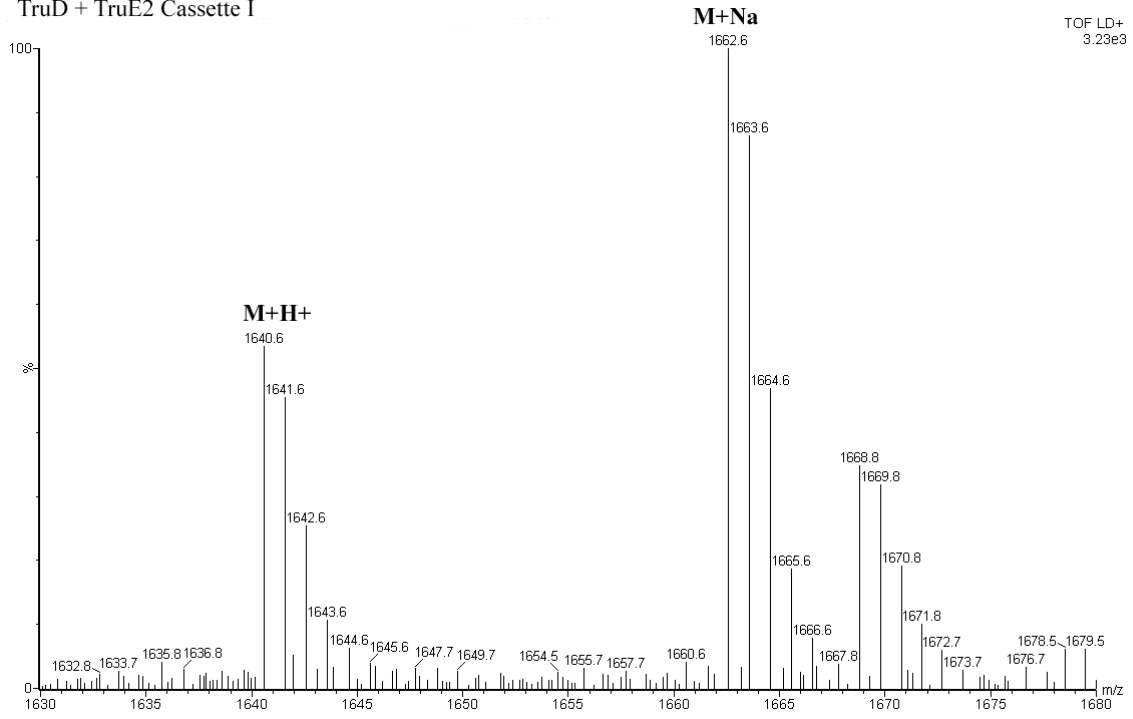


TruD + PatEdm Cassette I

TOF LD+
216



TruD + TruE2 Cassette I



TruD + TruE2 Cassette II

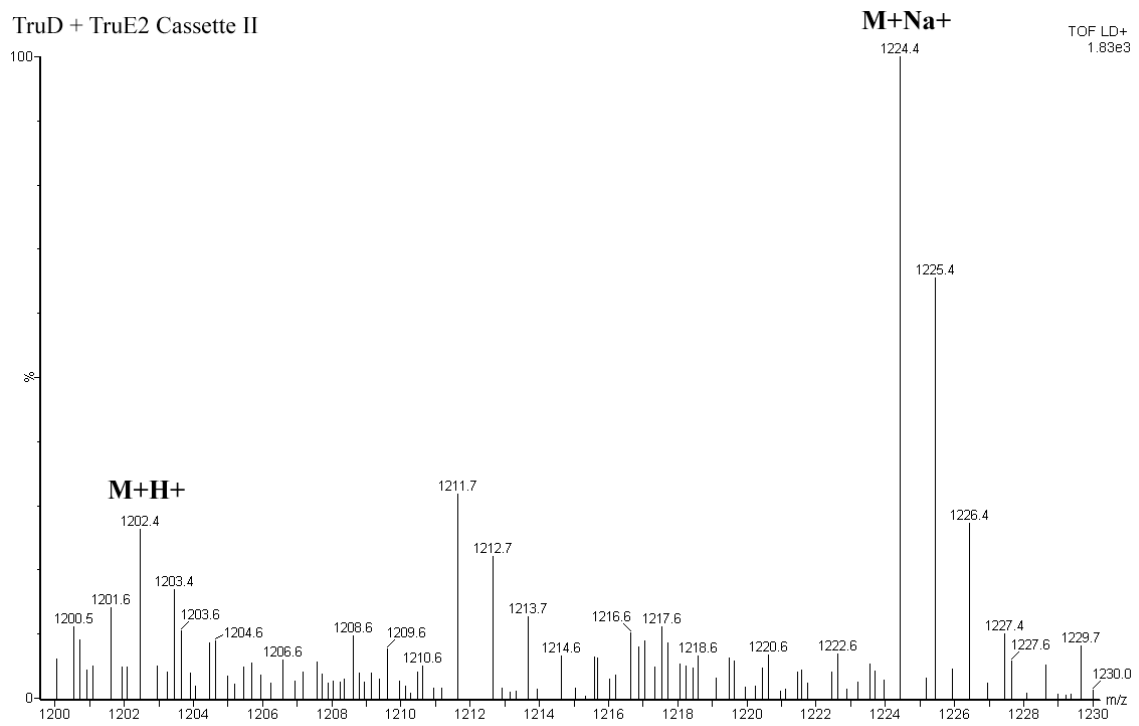
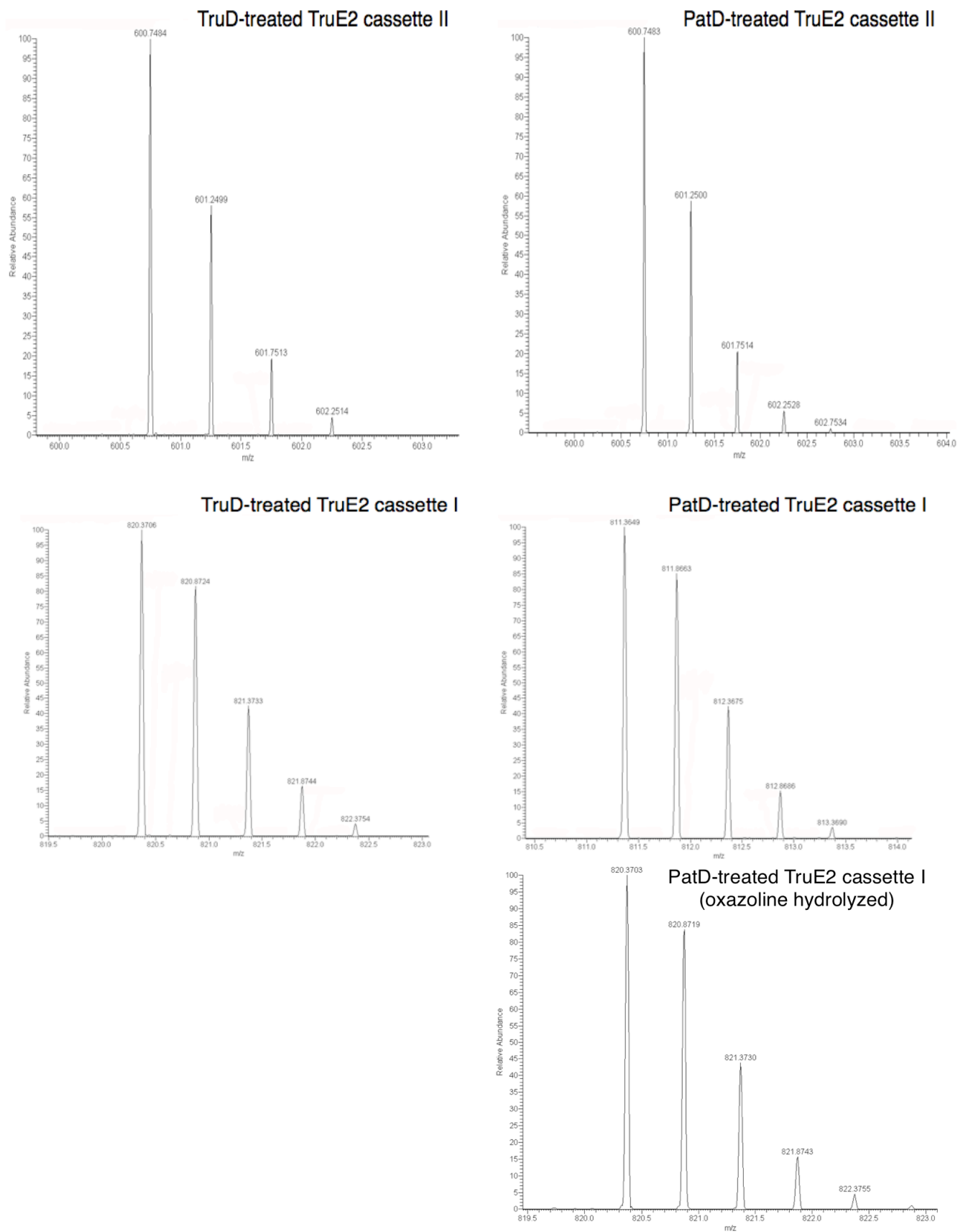


Figure S4. (a) FT-ICR characterization of TruD- and PatD-treated TruE2, digested with PatA. (b) FT-ICR characterization of TruD- and PatD treated PatEdm, digested with PatA.

(a)



(b)

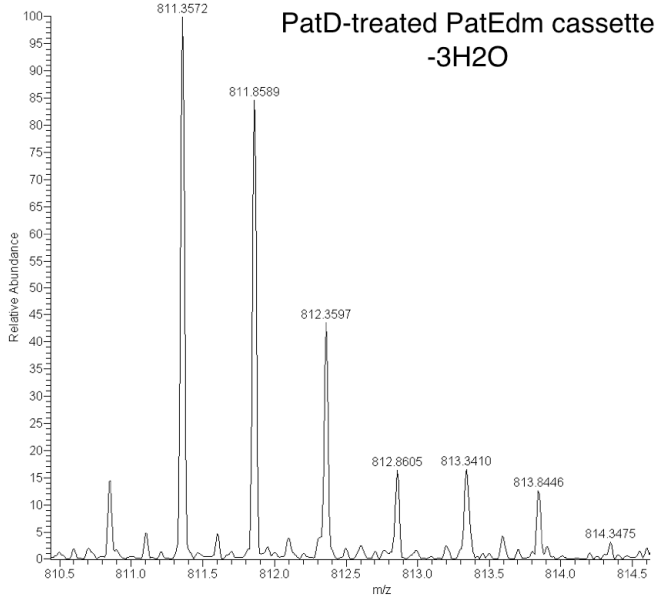
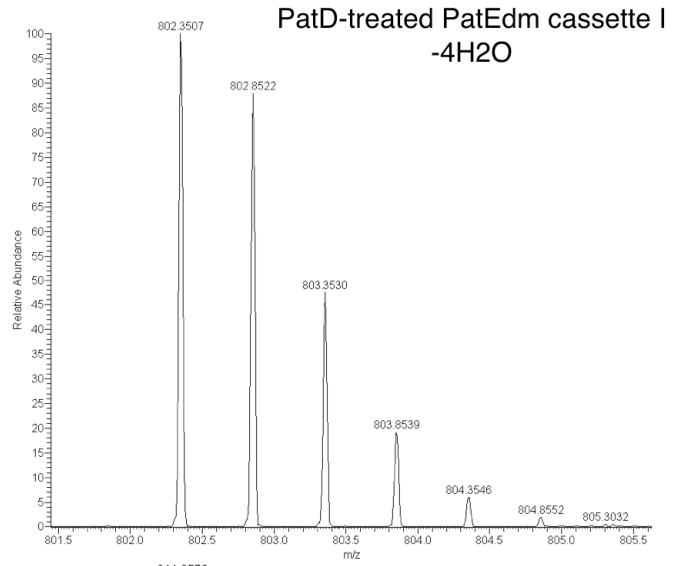
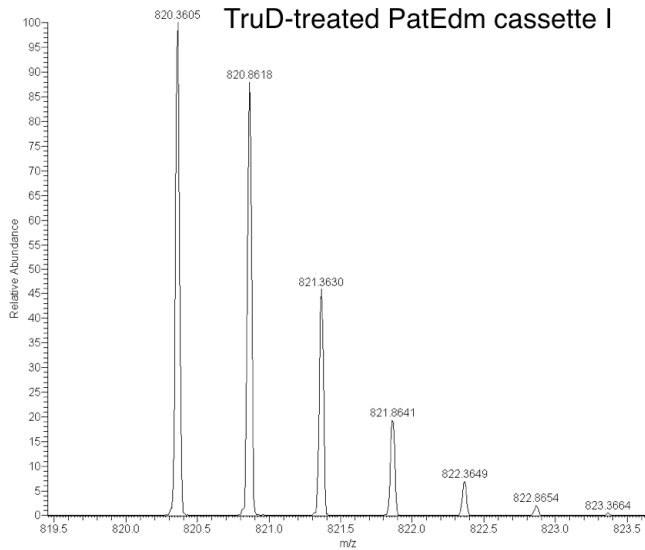
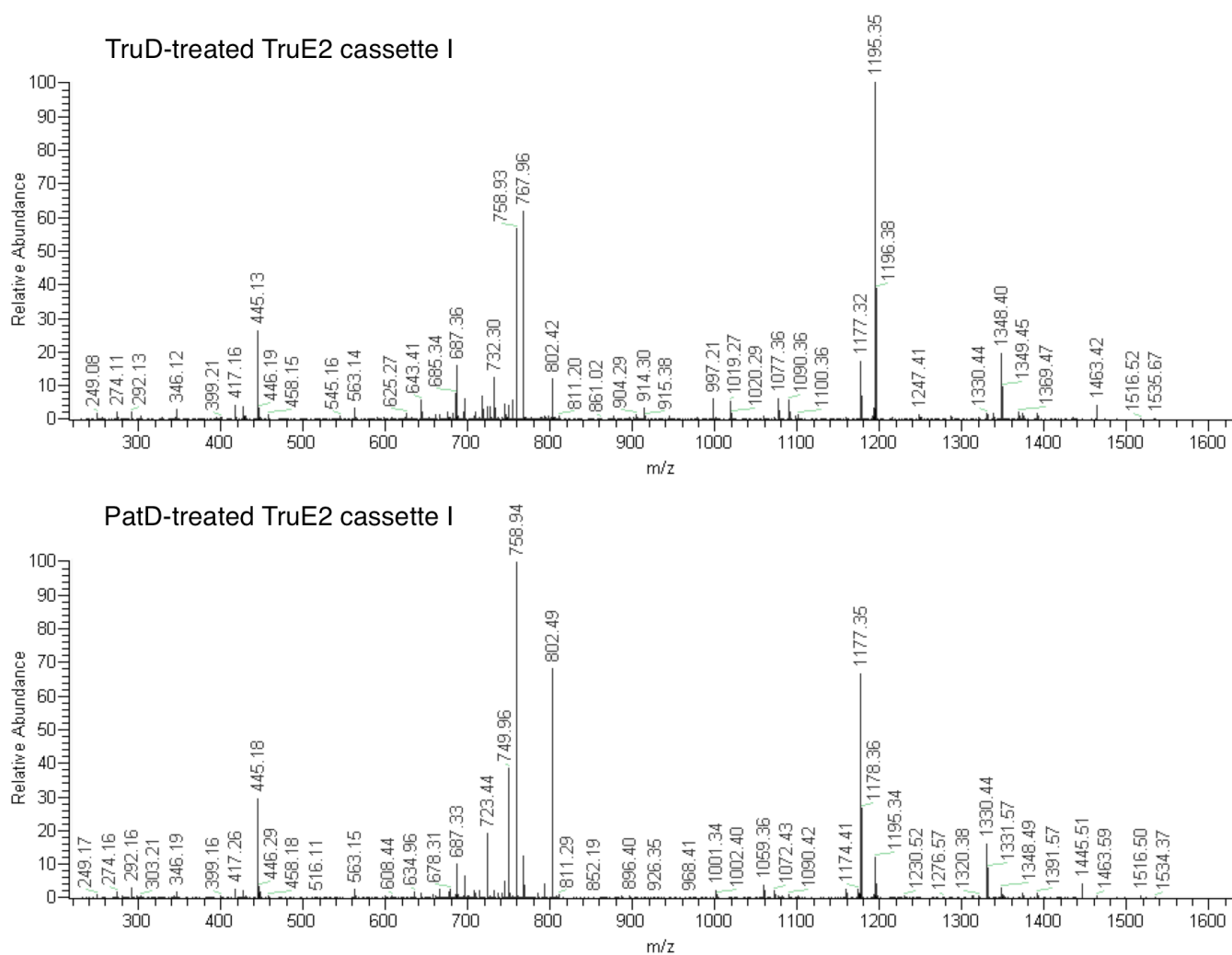
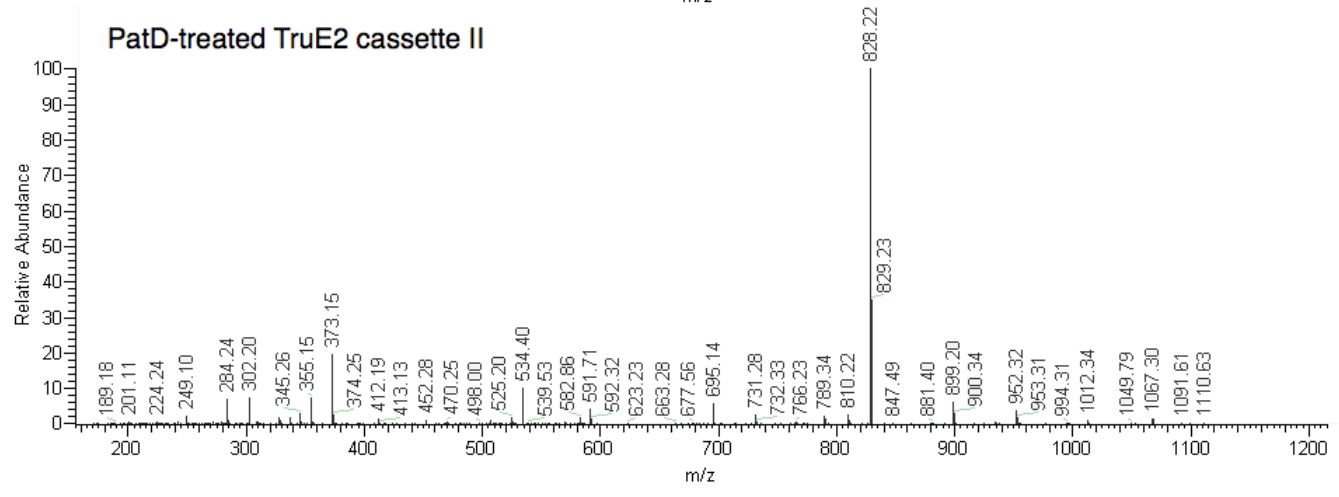
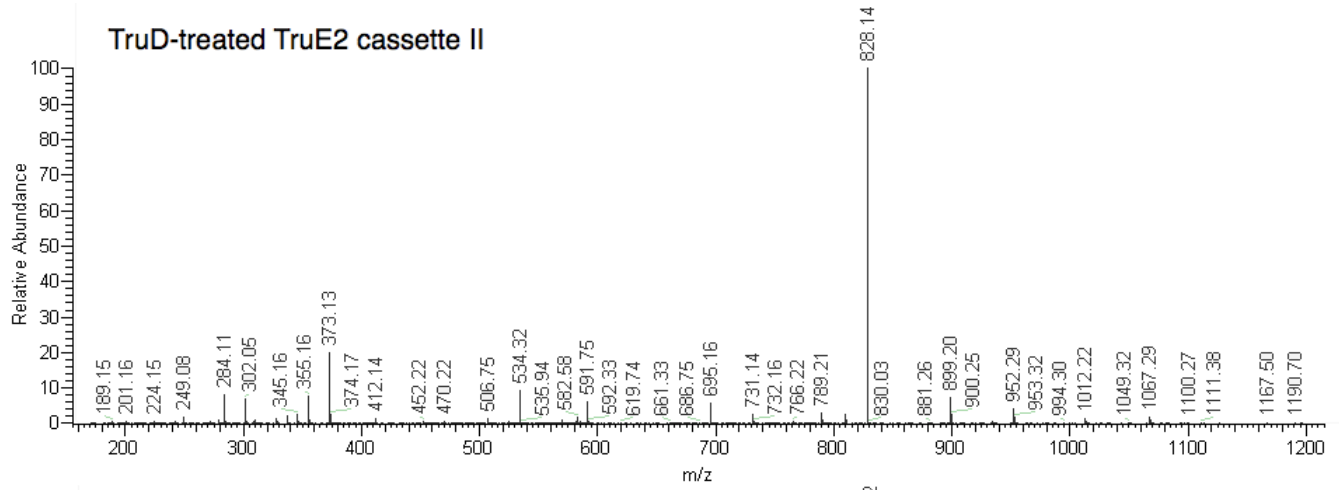


Figure S5. MS-MS of modified peptides and summary tables (a) MS-MS of TruD- and PatD-modified TruE2 cassette I (b) MS-MS of and TruD- and PatD-modified TruE2 cassette II (c) Tables summarizing MS-MS of TruE2 cassettes I and II (d) MS-MS of TruD- and PatD-modified PatEdm; one PatD-modified PatEdm cassette I is $-3\text{H}_2\text{O}$ an due to oxazoline ring-opening; fully modified is $-4\text{H}_2\text{O}$ (e) Tables summarizing MS-MS of TruD- and PatD-modified (both $-3\text{H}_2\text{O}$ and $-4\text{H}_2\text{O}$) PatEdm.

(a)

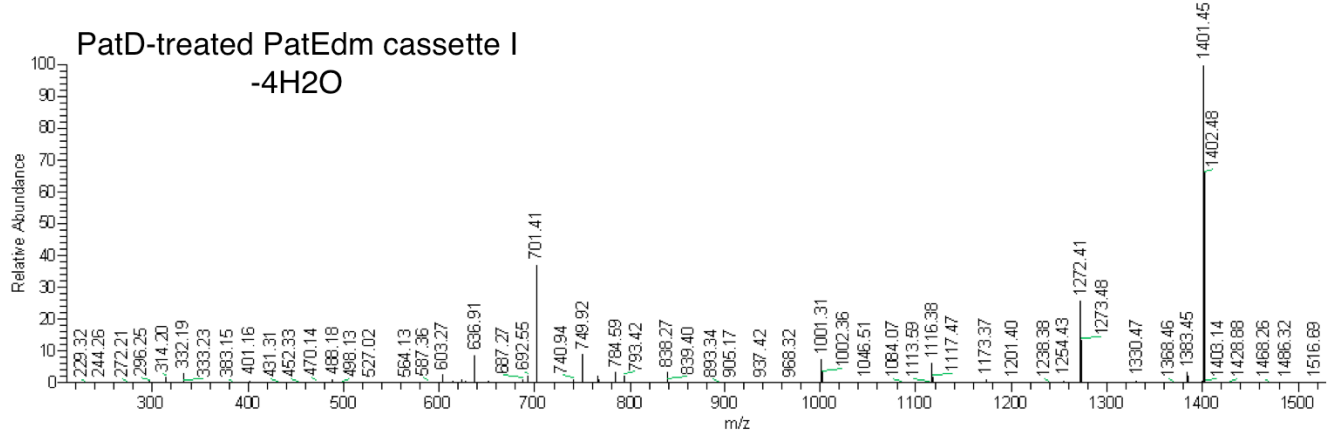
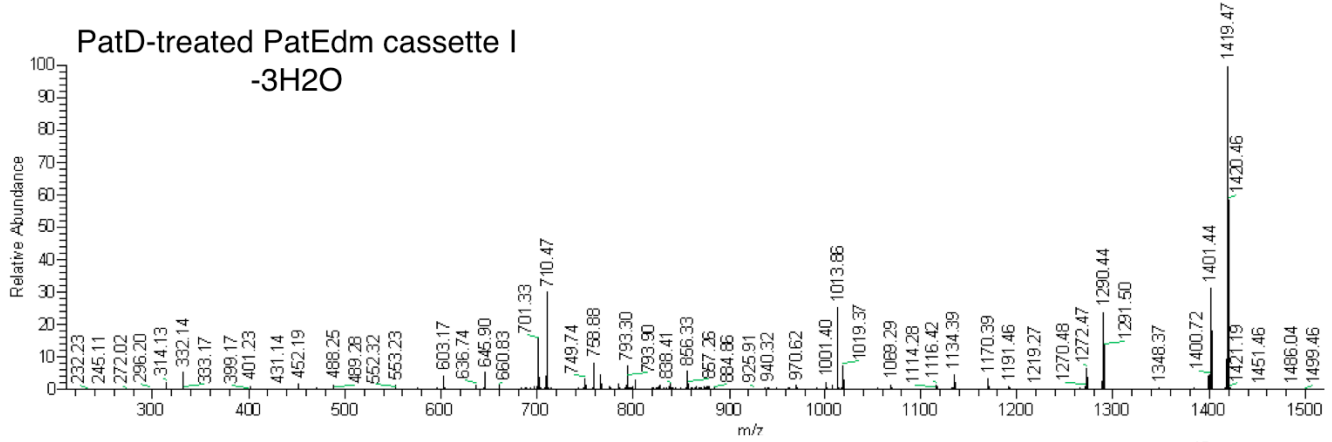
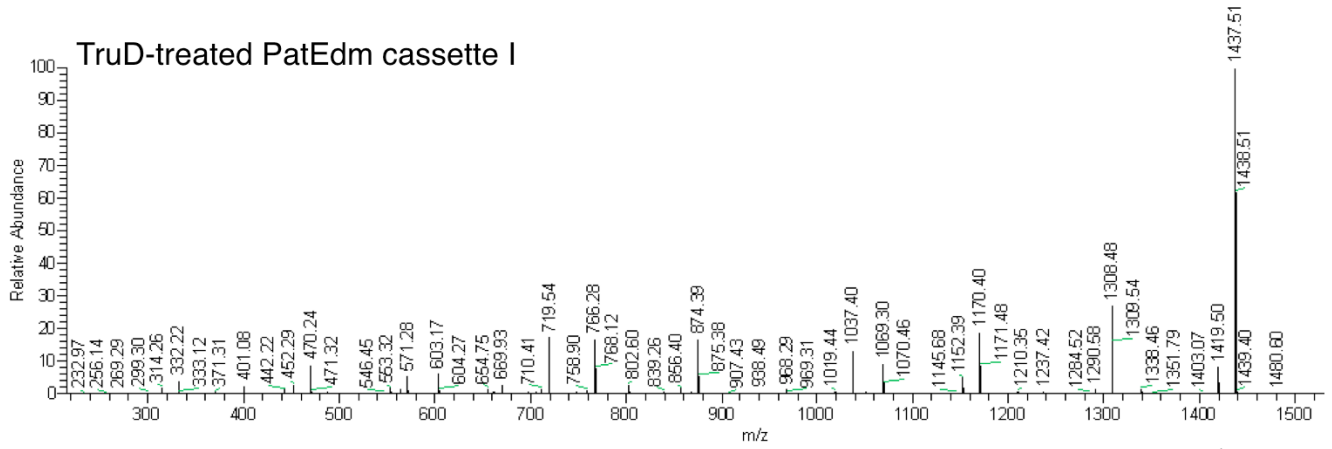


(b)



TruD-treated TrueE2 cassette I	B ⁻ -ions	B ⁻ -ions (+2)	B ⁰ -ions	B ⁰ -ions (+2)	PaD-treated TrueE2 cassette I	B ⁻ -ions	B ⁻ -ions (+2)	B ⁰ -ions	B ⁰ -ions (+2)
TF	249.08	-	-	-	TF	249.08	-	-	-
TFP	346.12	-	-	-	TFP	346.15	-	-	-
TFPV	445.13	-	-	-	TFPV	445.18	-	-	-
TFPVP	-	-	-	-	TFPVP	-	-	-	-
TFPVPPT	643.41	-	-	-	TFPVP(OxZln)	-	-	-	-
TFPVPPTV	-	-	-	-	TFPVP(OxZln)V	-	-	-	-
TFPVPPTV(TZln)	-	-	-	-	TFPVP(OxZln)V(TZln)	-	-	-	-
TFPVPPTV(TZln)S	914.30	-	-	-	TFPVP(OxZln)V(TZln)S	896.28	-	-	-
TFPVPPTV(TZln)SY	1077.36	-	-	-	TFPVP(OxZln)V(TZln)SY	1059.47	-	-	-
TFPVPPTV(TZln)SYD	-	-	-	-	TFPVP(OxZln)V(TZln)SYD	-	-	-	-
TFPVPPTV(TZln)SYDG	-	-	-	-	TFPVP(OxZln)V(TZln)SYDG	-	-	-	-
TFPVPPTV(TZln)SYDGV	1348.40	-	-	-	TFPVP(OxZln)V(TZln)SYDGV	1330.49	-	-	-
TFPVPPTV(TZln)SYDGVDA	1463.43	-	-	-	TFPVP(OxZln)V(TZln)SYDGVDA	1445.48	-	-	-
TFPVPPTV(TZln)SYDGVDA	-	767.96	-	-	TFPVP(OxZln)V(TZln)SYDGVDA	-	758.98	-	749.97
TFPVPPTV(TZln)SYDGVDA	-	-	-	-	TFPVP(OxZln)V(TZln)SYDGVDA	-	-	-	-
Y ⁻ -ions		Y ⁻ -ions (+2)	Y ⁰ -ions	Y ⁰ -ions (+2)	Y ⁻ -ions	Y ⁻ -ions (+2)	Y ⁰ -ions	Y ⁰ -ions (+2)	
AS	-	-	-	-	AS	-	-	-	
DAS	292.12	-	-	-	DAS	292.24	-	-	
VDAS	-	-	-	-	VDAS	-	-	-	
GVDAS	-	-	-	-	GVDAS	-	-	-	
DGVDAS	563.13	-	-	-	DGVDAS	563.13	-	-	
YDGVDA	-	-	-	-	YDGVDA	-	-	-	
SYDGVDA	-	-	-	-	SYDGVDA	-	-	-	
(TZln)SYDGVDA	-	-	-	-	(TZln)SYDGVDA	-	-	-	
V(TZln)SYDGVDA	997.20	-	-	-	V(TZln)SYDGVDA	-	-	-	
TV(TZln)SYDGVDA	-	-	-	-	(OxZln)V(TZln)SYDGVDA	-	-	-	
P(TZln)SYDGVDA	1195.35	1177.32	-	-	P(OxZln)V(TZln)SYDGVDA	1177.33	1159.36	-	
VP(TZln)SYDGVDA	-	-	-	-	VP(OxZln)V(TZln)SYDGVDA	-	-	-	
PVP(TZln)SYDGVDA	-	-	-	-	PVP(OxZln)V(TZln)SYDGVDA	-	-	687.55	
FPVP(TZln)SYDGVDA	-	-	-	-	FPVP(OxZln)V(TZln)SYDGVDA	-	-	-	
TFPVP(TZln)SYDGVDA	-	-	-	-	TFPVP(OxZln)V(TZln)SYDGVDA	-	-	-	
Double cleaved TruD-treated True2 cassette I	Mixed B, Y	Mixed B, Y (+2)	Mixed B ⁰ , Y ⁰	Mixed B ⁰ , Y ⁰ (+2)	Double cleaved TruD-treated TrueE2 cassette I	Mixed B, Y	Mixed B, Y (+2)	Mixed B ⁰ , Y ⁰	Mixed B ⁰ , Y ⁰ (+2)
P(TZln)SYDGVDA	1019.26	-	-	-	P(OxZln)V(TZln)SYDGVDA	1001.41	-	-	-
P(TZln)SYDGVDA	1091.37	-	-	-	P(OxZln)V(TZln)SYDGVDA	1072.40	-	-	-
PVP(TZln)SYDGV	1100.35	-	-	-	PVP(OxZln)V(TZln)SYDGV	1082.53	-	-	-

TruD-treated TrueE2 cassette II		B-ions	B-ions (+2)	B°-ions	B°-ions (+2)	PatD-treated TrueE2 cassette II		B-ions	B-ions (+2)	B°-ions	B°-ions (+2)
TS		189.15	-	-	-	TS		189.18	-	-	-
TSI		302.05	-	284.11	-	TSI		302.20	-	284.24	-
TSIA		373.13	-	355.16	-	TSIA		373.15	-	355.15	-
TSIAP		470.22	-	452.22	-	TSIAP		469.95	-	-	-
TSIAPF		-	-	-	-	TSIAPF		-	-	-	-
TSIAPF(TzIn)		702.06	-	-	-	TSIAPF(TzIn)		702.41	-	-	-
TSIAPF(TzIn)S		789.21	-	-	-	TSIAPF(TzIn)S		789.32	-	-	-
TSIAPF(TzIn)SY		952.29	-	-	-	TSIAPF(TzIn)SY		952.32	-	-	-
TSIAPF(TzIn)SYD		1067.29	534.31	-	-	TSIAPF(TzIn)SYD		1067.30	534.40	-	525.20
TSIAPF(TzIn)SYDD		-	-	-	591.75	TSIAPF(TzIn)SYDD		-	-	-	591.71
		Y-ions	Y-ions (+2)	Y°-ions	Y°-ions (+2)			Y-ions	Y-ions (+2)	Y°-ions	Y°-ions (+2)
DD		249.08	-	-	-	DD		249.10	-	-	-
YDD		412.14	-	-	-	YDD		412.19	-	-	-
SYDD		-	-	-	-	SYDD		-	-	-	-
(TzIn)SYDD		-	-	-	-	(TzIn)SYDD		-	-	-	-
F(TzIn)SYDD		731.15	-	-	-	F(TzIn)SYDD		731.28	-	-	-
PF(TzIn)SYDD		828.14	-	810.16	-	PF(TzIn)SYDD		828.22	-	810.22	-
APF(TzIn)SYDD		899.20	-	-	-	APF(TzIn)SYDD		899.20	-	-	-
IAPF(TzIn)SYDD		1012.22	-	-	-	IAPF(TzIn)SYDD		1012.34	-	-	-
SIAPF(TzIn)SYDD		-	-	-	-	SIAPF(TzIn)SYDD		-	-	-	-
TSIAPF(TzIn)SYDD		-	-	-	591.75	TSIAPF(TzIn)SYDD		-	-	-	591.71
		Mixed B, Y	Mixed B, Y (+2)	Mixed B°, Y°	Mixed B°, Y° (+2)			Mixed B, Y	Mixed B, Y (+2)	Mixed B°, Y°	Mixed B°, Y° (+2)
Double cleaved TruD-treated TrueE2 cassette II		695.16	-	-	-	Double cleaved PatD-treated TrueE2 cassette II		695.14	-	-	-
PF(TzIn)SYD		-	-	-	-	PF(TzIn)SYD		-	-	-	-



(e)

TruD-treated PatEdm cassette I					PaD-treated PatEdm cassette I (-3H2O)				
	B-ions	B-ions (+2)	B ⁻ -ions	B ⁻ -ions (+2)		B-ions	B-ions (+2)	B ⁻ -ions	B ⁻ -ions (+2)
VT	-	-	-	-	V(Oxzn)	-	-	-	-
VTA	-	-	-	-	V(Oxzn)/A	-	-	-	-
VTA(Tzn)	470.25	-	452.28	-	V(Oxzn)/A(Tzn)	452.19	-	-	-
VTA(Tzn)I	571.29	-	553.31	-	V(Oxzn)/A(Tzn)I	553.22	-	-	-
VTA(Tzn)IT	-	-	-	-	V(Oxzn)/A(Tzn)IT	-	-	-	-
VTA(Tzn)ITF	-	-	-	-	V(Oxzn)/A(Tzn)ITF	-	-	-	-
VTA(Tzn)ITFF(Tzn)	-	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)	-	-	-	-
VTA(Tzn)ITFF(Tzn)/A	874.38	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/A	856.34	-	-	-
VTA(Tzn)ITFF(Tzn)/AY	1037.40	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AY	1019.37	-	-	-
VTA(Tzn)ITFF(Tzn)/AYD	1152.38	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYD	-	-	1098.55	-
VTA(Tzn)ITFF(Tzn)/AYDG	1210.33	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDG	-	-	-	-
VTA(Tzn)ITFF(Tzn)/AYDGV	1308.47	654.78	1290.58	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDGV	1290.44	645.92	1272.49	-
VTA(Tzn)ITFF(Tzn)/AYDGVPE	1437.52	719.53	1419.50	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDGVPE	1419.47	710.48	1401.47	701.25
VTA(Tzn)ITFF(Tzn)/AYDGVPEP	-	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDGVPEP	-	758.88	-	749.80
VTA(Tzn)ITFF(Tzn)/AYDGVPEPS	-	-	-	802.61	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDGVPEPS	-	-	-	793.29
EP	Y-ions	Y-ions (+2)	Y ⁻ -ions	Y ⁻ -ions (+2)	EP	Y-ions	Y-ions (+2)	Y ⁻ -ions	Y ⁻ -ions (+2)
EPS	332.22	-	314.25	-	EPS	332.15	-	314.16	-
VEPS	-	-	-	-	VEPS	-	-	-	-
GVEPS	-	-	-	-	GVEPS	488.23	-	-	-
DGVEPS	603.17	-	-	-	DGVEPS	603.17	-	-	-
YDVEPS	766.27	-	-	-	YDVEPS	766.25	-	-	-
AYDVEPS	-	-	-	-	AYDVEPS	-	-	-	-
(Tzn)/AYDVEPS	-	-	-	-	(Tzn)/AYDVEPS	-	-	-	-
FT(zn)/AYDVEPS	1069.31	-	-	-	FT(zn)/AYDVEPS	1069.29	-	-	-
TF(Tzn)/AYDVEPS	1170.40	-	-	-	TF(Tzn)/AYDVEPS	1170.38	-	-	-
ITFF(Tzn)/AYDVEPS	-	-	-	-	ITFF(Tzn)/AYDVEPS	-	-	-	-
(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-	(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-
AT(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-	AT(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-
TA(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-	O(xzn)/A(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-
VTA(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-	V(Oxzn)/A(Tzn)ITFF(Tzn)/AYDVEPS	-	-	-	-
Double cleaved TruD-treated PatEdm cassette I	Mixed B, Y	Mixed B, Y (+2)	Mixed B ^o , Y ^o	Mixed B ^o , Y ^o (+2)	Double cleaved TruD-treated PatEdm cassette I -3H2O	Mixed B, Y	Mixed B, Y (+2)	Mixed B ^o , Y ^o	Mixed B ^o , Y ^o (+2)
DGVE	401.09	-	-	-	DGVE	401.24	-	-	-
TA(Tzn)ITFF(Tzn)/AY	938.48	-	-	-	TA(Tzn)ITFF(Tzn)/AY	-	-	-	-
TF(Tzn)/AYDGVPE	968.29	-	-	-	TF(Tzn)/AYDGVPE	-	-	-	-
TA(Tzn)ITFF(Tzn)/AYDGVPE	1338.47	669.93	-	-	TA(Tzn)ITFF(Tzn)/AYDGVPE	-	-	-	-

TruD-treated PatEdm cassette I	B-ions	B-ions (+2)	B°-ions	B°-ions (+2)	PatD-treated PatEdm cassette I (-4H2O)	B-ions	B-ions (+2)	B°-ions	B°-ions (+2)
VT	-	-	-	-	V(Oxzn)	-	-	-	-
VT	-	-	-	-	V(Oxzn)A	-	-	-	-
VT	-	-	-	-	V(Oxzn)A(Tzn)	-	-	-	-
VT	470.25	-	452.28	-	V(Oxzn)A(Tzn)I	-	-	-	-
VT	571.29	-	553.31	-	V(Oxzn)A(Tzn)(Oxzn)	-	-	-	-
VT	-	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F	-	-	-	-
VT	-	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)	-	-	-	-
VT	874.38	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)A	838.26	-	-	-
VT	1037.40	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AY	1001.32	-	-	-
VT	1152.38	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYD	1116.36	-	-	-
VT	1210.33	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYDDG	1173.42	587.22	-	-
VT	1308.47	654.78	1290.58	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYDDG	1272.37	636.90	1254.58	-
VT	1437.52	719.53	1419.50	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYDDGVE	1401.47	701.43	1383.47	692.55
VT	-	-	-	-	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYDDGVEP	-	749.91	-	740.94
VT	-	-	-	802.61	V(Oxzn)A(Tzn)(Oxzn)F(Tzn)AYDDGVEPS	-	-	-	793.42
Y-ions	Y-ions	Y-ions (+2)	Y°-ions	Y°-ions (+2)	Y-ions	Y-ions (+2)	Y°-ions	Y°-ions (+2)	
EP	-	-	-	-	EP	-	-	-	-
EPS	332.22	-	314.25	-	EPS	332.10	-	314.15	-
VEPS	-	-	-	-	VEPS	-	-	-	-
GVEPS	-	-	-	-	GVEPS	488.17	-	470.15	-
DGVEPS	603.17	-	-	-	DGVEPS	603.27	-	-	-
YDGEPS	766.27	-	-	-	YDGEPS	766.25	-	-	-
AYDGEPS	-	-	-	-	AYDGEPS	-	-	-	-
(Tzn)AYDGEPS	-	-	-	-	(Tzn)AYDGEPS	-	-	-	-
F(Tzn)AYDGEPS	1069.31	-	-	-	F(Tzn)AYDGEPS	-	-	-	-
TF(Tzn)AYDGEPS	1170.40	-	-	-	(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
ITF(Tzn)AYDGEPS	-	-	-	-	I(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
(Tzn)ITF(Tzn)AYDGEPS	-	-	-	-	(Tzn)I(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
A(Tzn)ITF(Tzn)AYDGEPS	-	-	-	-	A(Tzn)I(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
TA(Tzn)ITF(Tzn)AYDGEPS	-	-	-	-	(Oxzn)A(Tzn)I(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
VT	-	-	-	-	V(Oxzn)A(Tzn)I(Oxzn)F(Tzn)AYDGEPS	-	-	-	-
Double cleaved TruD-treated PatEdm cassette I	Mixed B, Y	Mixed B, Y (+2)	Mixed B°, Y°	Mixed B°, Y° (+2)	Double cleaved TruD-treated PatEdm cassette I -4H2O	Mixed B, Y	Mixed B, Y (+2)	Mixed B°, Y°	Mixed B°, Y° (+2)
DGVE	401.09	-	-	-	DGVE	401.20	-	-	-
TA(Tzn)ITF(Tzn)AY	938.48	-	-	-	TA(Tzn)ITF(Tzn)AY	-	-	-	-
TF(Tzn)AYDDGVE	968.29	-	-	-	TF(Tzn)AYDDGVE	-	-	-	-
TA(Tzn)ITF(Tzn)AYDDGVE	1338.47	669.93	-	-	TA(Tzn)ITF(Tzn)AYDDGVE	-	-	-	-

Figure S6. Shown below are intact ESI analyses of TruE2, demonstrating that one dehydration catalyzed by PatD is an oxazoline ring. After treatment with mild base, PatD-modified TruE2 (a) is $-3\text{H}_2\text{O}$, while TruE2 modified by TruD (b) is $-2\text{H}_2\text{O}$. However, after treatment with mild acid, PatD-modified TruE2 (c) is $-2\text{H}_2\text{O}$, while the mass of TruE2 modified by TruD (d) is unchanged. Thus, two acid- and base-stable dehydrations are catalyzed by both PatD and TruD, based on this and MS-MS analysis these are thiazoline rings. Additionally, PatD catalyzes formation of a further base-stable, acid-labile dehydration, which by MS-MS analysis localized to Thr. Based on the chemical properties of this third dehydration, it can only be oxazoline.

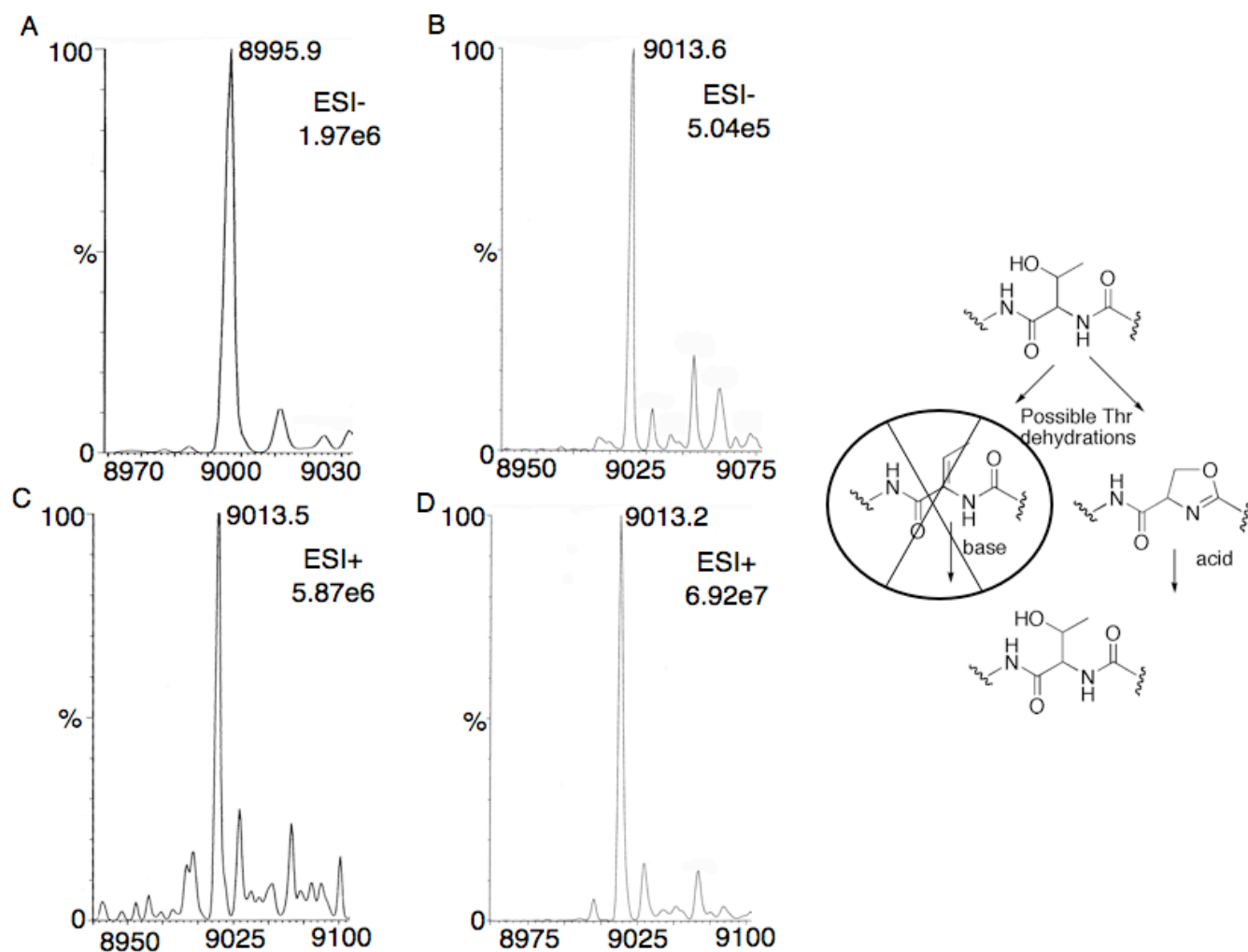


Figure S7. ESI-MS(-) and SDS-PAGE analyses demonstrating modification of TruE5 by TruD and PatD. (a) From left-to-right unmodified TruE5, TruD-modified TruE5, and PatD-modified TruE5 are shown. The predominant product of both TruD and PatD by MS is a singly dehydrated species that most likely represents a peptide with the Cys in the first cassette cyclized. However, in both TruD and PatD reactions, some amount of fully modified TruE5 is present, corresponding to products that contain, respectively, one and two oxazoline rings in the first cassette. (b) SDS-PAGE gel showing modification of TruE5. From left to right, lanes are: (1) ladder (2) TruE5 standard (3) TruE2 + PatD (4) TruE2 + TruD (5) TruE2 + PatD (6) TruE2 + TruD (7) TruE5 + PatD (8) TruE5 + TruD.

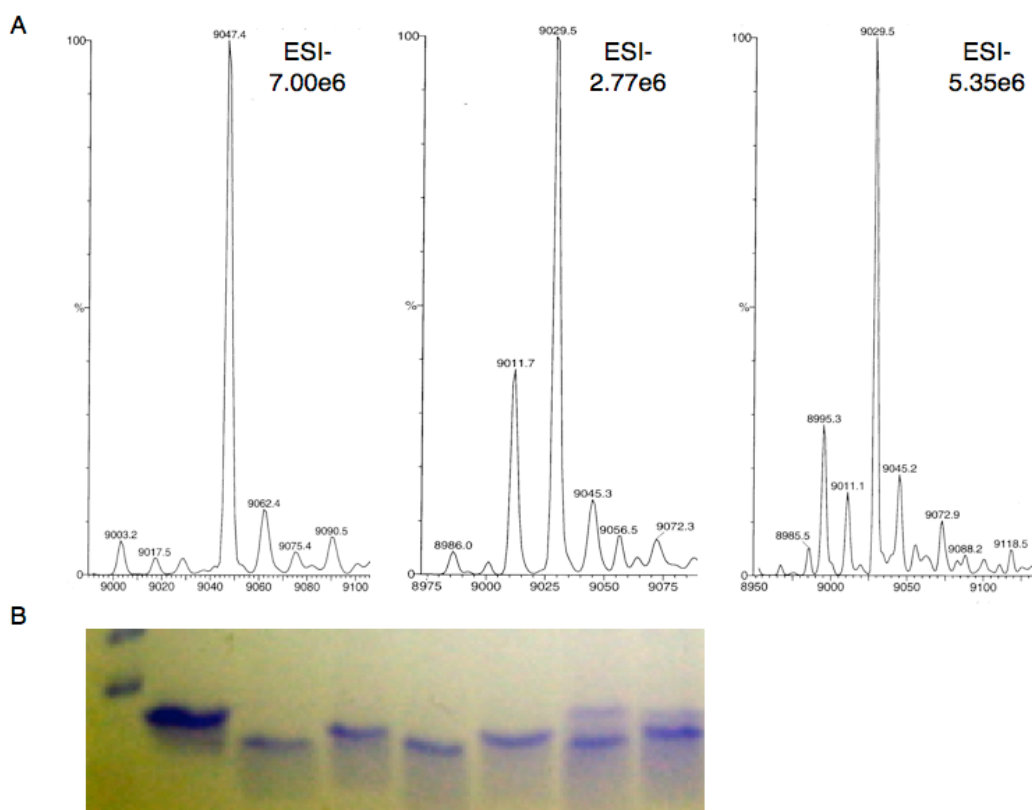


Figure S8. ESI-MS(-) data demonstrating modification of TruE4 by a single dehydration event localized to the single product cassette that it contains. At left is intact analysis of unmodified TruE4; middle is intact analysis of TruD-modified TruE4, and at right is ESI-MS(-) of PatD-treated TruE4, digested with PatA, showing the cassette is -H₂O (1164.6 Da).

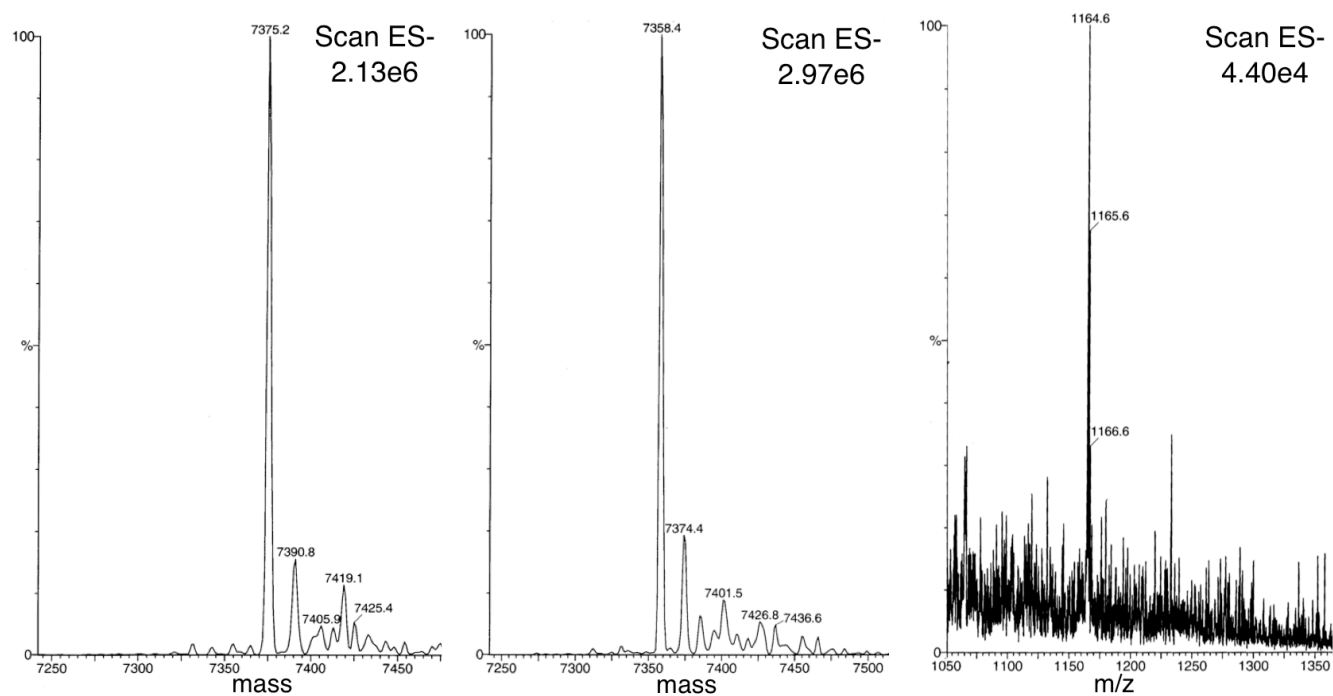


Table S1. Table of primers used in this study.

	Primer Name	
1	PatE-F	AACATATGGACTTAAATTGACAGGCTTC
2	PatE α -R	GCATCACTTTTTGCGCTTATGATGGTGTGGAGCCATCTCATCACCACCACCATCACCATCACGCTTACGATGGTGAATAA
3	TruE-R	AATTCGGTACCTTAGTCGTCGTAAGAGCAGAG
4	TruD-F	TTCATGCAACCAACCGCCCTCCAAATTAAG
5	TruD-R	AACATATGGACTTAAATTGACAGGCTTC

Experimental

General methods. Isopropyl β -D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT), leupeptin, pepstatin, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), and phenylmethanesulfonyl fluoride (PMSF) were purchased from ISC Bioexpress. Metal-free nitric acid (Optima, Fisher Scientific) was purchased from Fisher Scientific. Ultra-pure MgCl_2 , β,γ -methylene-adenosine triphosphate (β,γ -methylene-ATP) were purchased from Sigma-Aldrich. γ - ^{32}P -labeled ATP was purchased from Perkin-Elmer. Ni-NTA resin was purchased from Qiagen. ZipTip C18 pipette tips were purchased from Millipore. All expression vectors were purchased from Novagen. *Escherichia coli* strain DH5 α was used for all cloning steps, while *E. coli* strain BL21(DE3)Star was used for all protein expressions. Protein concentrations were determined by absorbance at 280 nm; extinction coefficients were predicted as described elsewhere.¹⁶

Gene cloning. Genes were obtained from ascidian symbiont metagenomes as previously described.² *patD* was cloned into pET15b with an N-terminal His-tag. *TruD* was cloned into pCDF Duet-1. Both *patD* and *truD* have an internal PstI site located 700 bp into the coding region and are 99% identical in this N-terminal region. Thus, to construct *truD*, the His-tagged N-terminus of *patD* was obtained by PstI / NdeI digest and ligated into homologous restriction sites in the *truD* construct. *TruE2* and *truE4* were cloned untagged into pRSF-Duet vector between the NdeI and KpnI restriction sites. The N-terminal His tag was then moved from pET28b into the pRSF vectors through the restriction sites MluI and NdeI. Clones were verified by restriction digests and DNA sequencing. *patE α* was modified from a previously described *patE* variant, *patE2*. *TruE5* was modified from *truE2* using the Quikchange site directed mutagenesis kit (Stratagene). The *patE2* gene was amplified using the primers PatEf and

PatE α r, subcloned into pCR2.1-TOPO (Invitrogen), and then cloned into pRSFDuet-1. PatA and PatEdm were cloned as previously described.¹⁷

Expression of PatD and TruD. Seed cultures were inoculated into 1 L of LB and grown in a Fernbach at 30°C with shaking at 225 rpm until the cells reached 0.4 OD₆₀₀. Expression was induced with 0.1 mM IPTG at 15°C and left overnight. Cells were pelleted by centrifugation and resuspended in 40 mL of lysis buffer (1 M NaCl, 10 mM imidazole, 50 mM HEPES pH 7.5, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 mM AEBSF). Cells were lysed using a Vibracell sonicator and centrifuged. Filtered lysates were applied to a gravity column containing 10mL of wet Ni-NTA resin. The column was then washed with 10 column volumes of lysis buffer, 10 column volumes of wash buffer (500 mM NaCl, 25 mM Imidazole pH 7.8), and the protein was eluted with elution buffer (750 mM NaCl, 250 mM Imidazole pH 7.8). The purified protein was dialyzed twice against dialysis buffer (200 mM NaCl, 25 mM HEPES pH 7.5) at 4°C, and then against dialysis buffer w/ 10% glycerol. Dialyzed protein was aliquoted, flash frozen with liquid nitrogen, and stored at -80°C.

Expression of precursor peptides. TruE2-expressing cells were grown to density in a manner identical to that for PatD and TruD. Expression of TruE2 was induced by addition of IPTG up to a final concentration of 1 mM followed by overnight growth at 37°C. Cell pellets were resuspended in lysis buffer at a concentration of 4 mL per gram of cell paste. After centrifugation, supernatants were discarded, while the pellets were resuspended in 50 mL of buffer B (8 M urea, 0.1 M NaH₂PO₄, 10 mM tris pH 8.0) and then sonicated to affect further resolubilization. The resuspended material was again centrifuged, and filtered lysates were applied to a Ni-NTA column, which was then washed with 2 x 25 mL of buffer C (8 M urea, 0.1 M NaH₂PO₄, 10 mM tris pH 6.3), 4 x 12.5 mL of buffer D (8 M urea, 0.1 M NaH₂PO₄, 10 mM tris pH 5.9), and eluted with 4 x 12.5 mL of buffer E (8 M urea, 0.1 M NaH₂PO₄, 10 mM tris pH 4.5). The eluents were dialyzed twice against dialysis buffer (200 mM NaCl, 100 mM proline, 1 mM DTT, 10% glycerol, 20 mM HEPES pH 8.0). The eluents were then combined, aliquoted, flash frozen, and stored at -80°C.

Expression of TruE4 was performed in a manner identical to that of TruE2, except that owing to the larger amount of insoluble material obtained, pellets were resuspended in 100 mL of buffer B, and the Ni-NTA column was washed with 100 mL of buffer C.

Expression of PatE α was performed in a manner identical with that of TruE2, with the following exceptions: the lysis buffer used consisted of 500 mM NaCl, 20 mM NaH₂PO₄ pH 7.8. Pellets were resuspended in 8 M urea rather than buffer B, and the Ni-NTA column was washed using 500 mM NaCl, 25 mM imidazole, and protein was eluted using 3 x 10 mL of elution buffer (750 mM NaCl, 250 mM imidazole pH 7.8). Eluents were combined and dialyzed twice against dialysis buffer (500 mM NaCl, 2 mM DTT, 25 mM HEPES pH 7.8).

Expression of TruE5 was performed in a manner identical with that of TruE2.

PatA and PatEdm were expressed as previously described.¹⁷

Enzyme reactions. Reaction mixtures were incubated at 34°C in an MJ research minicycler for varying amounts of time. Enzyme, precursor peptide, and ATP concentrations varied, and are described below. The following additives were present in standard reactions but were varied in early optimization experiments: 40 mM tris pH 8.0, 8 mM DTT, and 4 mM MgCl₂.

Enzyme reactions generally contained the optimized additive mixture and 0.6 μ M PatD or TruD, 8 μ M TruE2, or 12 μ M TruE4, or 42 μ M PatE α , or 22 μ M PatEdm, and 0.8 mM ATP. Reactions were run using varying times, from 15 min to 27 h. To confirm modification, 10 μ L of the reaction mixture was removed and analyzed by SDS-PAGE. At minimum, at least three separate experiments were performed for each enzyme-substrate concentration.

For reactions requiring cleavage by PatA, mixtures as described above were incubated for 27 h, with and without ATP and with and without PatD / TruD. PatA was then added to 1.7 μ M final concentration followed by incubation for a further 17 h. After completion of PatA-containing reactions, reaction mixtures were frozen at -80°C until analyzed by MALDI-TOF, FT-MS, and / or ESI-MS.

SDS-PAGE assays. 18% acrylamide gels were used for all assays. Prior to electrophoresis, samples were brought up in 1X SDS sample buffer diluted from 6X SDS sample buffer and then boiled for 3 min.¹⁸ After electrophoresis, gels were placed in boiled fixing solution (53% H₂O, 40% ethanol, 7% acetic acid), incubated 10-20 min with gentle rocking, and then placed in boiled stain (0.02% w/v Coomassie R250 in 85% H₂O, 10% acetic acid, 5% ethanol), and then destained in (85% H₂O, 10% acetic acid, 5% ethanol) for several h, and then photographed. For TruE4 inhibition experiments, gels were imaged using a Li-Cor Odyssey infrared scanner.

Mass spectrometry analysis. Samples for MALDI-TOF were prepared by desalting with ZipTip C18 pipette tips according to the manufacturer's instructions. Desalted peptide samples were mixed 1:1 with α -cyano-4-hydroxycinnamic acid resin (10 mg/mL CHCA in 50:50 H₂O:methanol w/ 0.1% trifluoroacetic acid), and spotted. MALDI was performed using a Micromass MALDI micro MX (Waters) instrument using an automated targeting protocol. LC-FTMS, ESI-MS(+), and ESI-MS(-) were run at the University of Utah Mass Spectrometry and Proteomics Core Facility. FT was performed using an LTQ FT Ultra Hybrid Mass Spectrometer (Thermo Scientific). ESI used a Micromass Quattro-II (Waters).