Supporting Information for

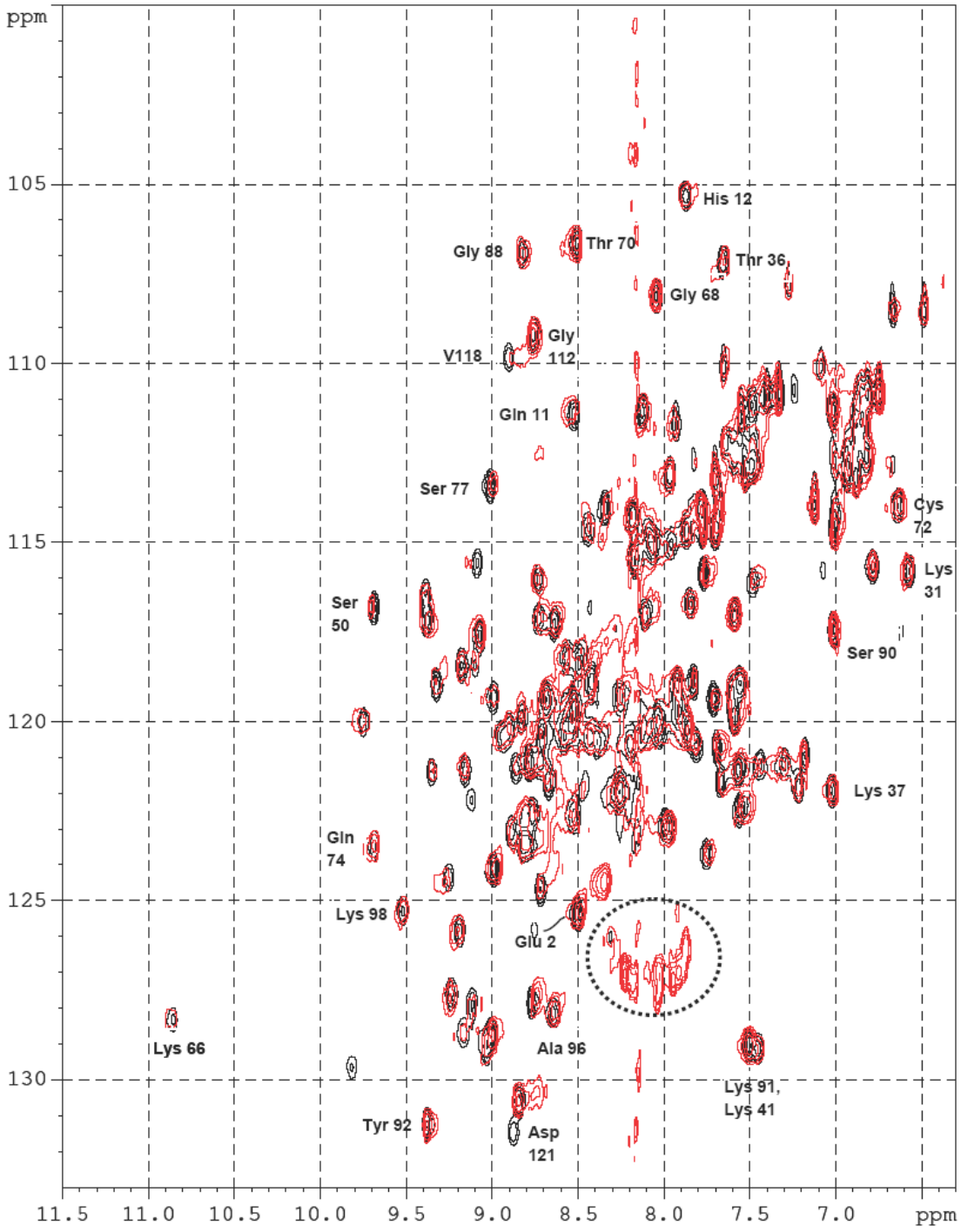
"The Carbodiimide EDC Induces Crosslinks that Stabilize the RNase A C-Dimer Against Dissociation: EDC Adducts Can Affect Protein Net Charge, Conformation and Activity.

by

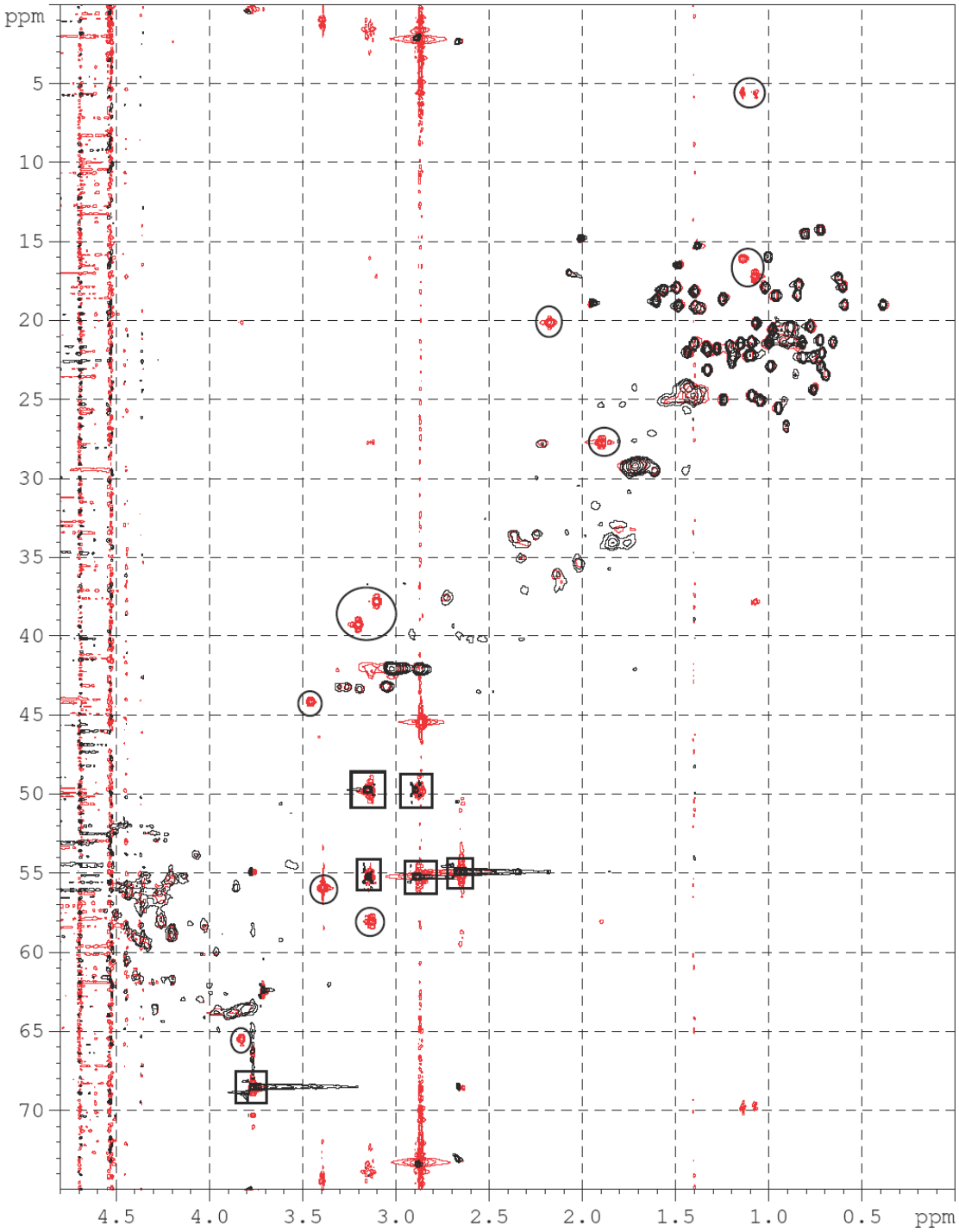
Jorge P. López-Alonso, Fernando Díez, Josep Font, Marc Ribó, María Vilanova, J. Martin Scholtz, Carlos González, Francesca Vottariello, Giovanni Gotte, Massimo Libonati & Douglas V. Laurents

List of Contents:

Supporting Figure 1: A: ^{1}H-^{15}N HSQC Spectra of RNase A (black) and EDC·modified ^{13}C,^{15}N RNase A (red). Some representative resonances in RNase A (based on the assignments from Shimotakahara *et al.* (1997) *Biochemistry 36:* 6915-6929 are labeled. A dotted circle is drawn around new signals appear after EDC treatment which could be due to amide groups near EDC modified carboxylate groups.



Supporting Figure 1B: ^{1}H-^{13}C HSQC Spectra of RNase A (black) and EDC·modified ^{13}C,^{15}N labeled RNase A (red). Ovals are drawn around signals arising from EDC or hydrolyzed EDC and squares surround resonances coming from MES buffer.



Supporting Figure 2:

NMR assignments of EDC, Ac-Asp-Ala-NH_2 and MES

EDC: (200mM, pH 4.7, 25ºC)

CH_3-CH_2-N=C=N-CH_2-CH_2-CH_2-NH^+-(-CH_3)$_2$

δ(ppm) ^{1}H	1.13	3.14			3.46	2.18	3.84		3.40
^{13}C	**16.1**	**42.4**		**150.0**	**44.2**	**20.1**	**65.5**		**55.9**

Hydrolyzed EDC:

CH_3-CH_2-NH-C(=O)-NH-CH_2-CH_2-CH_2-NH^+-(-CH_3)$_2$

δ(ppm) ^{1}H	1.07	3.11	5.99	6.15	3.19	1.89	3.14		2.88
^{13}C	**17.2**	**37.8**		**163.5**	**39.2**	**27.8**	**58.1**		**45.5**
^{15}N			122.4	129.6					

Ac-Asp-Ala-NH_2: 36 mM, pH 4.78, 25 ºC

180.1 COO^-

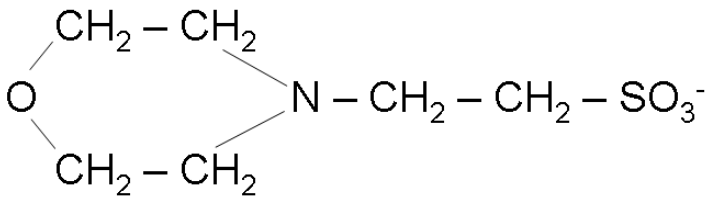
41.5 CH_2 2.68, 2.58 **19.4** CH_3 1.40

CH_3-CO-NH-CH-CO-NH-CH-CO-NH_2

δ(ppm) ^{1}H	2.03		8.26	4.54		8.37	4.28		7.59, 7.02
^{13}C	**24.7**	**180.8**		**54.6**	**176.5**		**52.4**	**177.2**	
^{15}N			125.5			126.4			106.15

Supporting Figure 2 Cont.

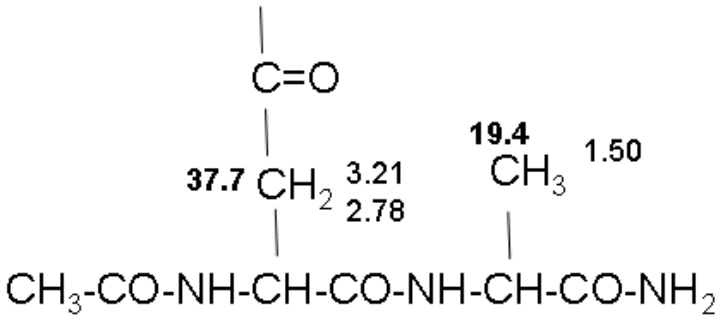
MES 100 mM 25 ºC



δ (ppm) ^{1}H	3.80	2.81	3.19	3.01	pH 6.4
^{13}C	**68.9**	**55.5**	**50.0**	**55.9**	
δ(ppm) ^{1}H	3.96	3.35	3.36	3.51	pH 5.1
^{13}C	**66.7**	**54.8**	**47.6**	**55.2**	

The assignments of these two methylene groups could be swapped. Literature values of the $^{13}CH_2$ α and β to an $-SO_3$ group {F. Freeman & C. N. Angeletakis, Org. Mag. Res. (1983) 31(2) 86-93} and of methyl morpholine (Aldrich Spectral Viewer) suggest that the assignments shown are the most probable.

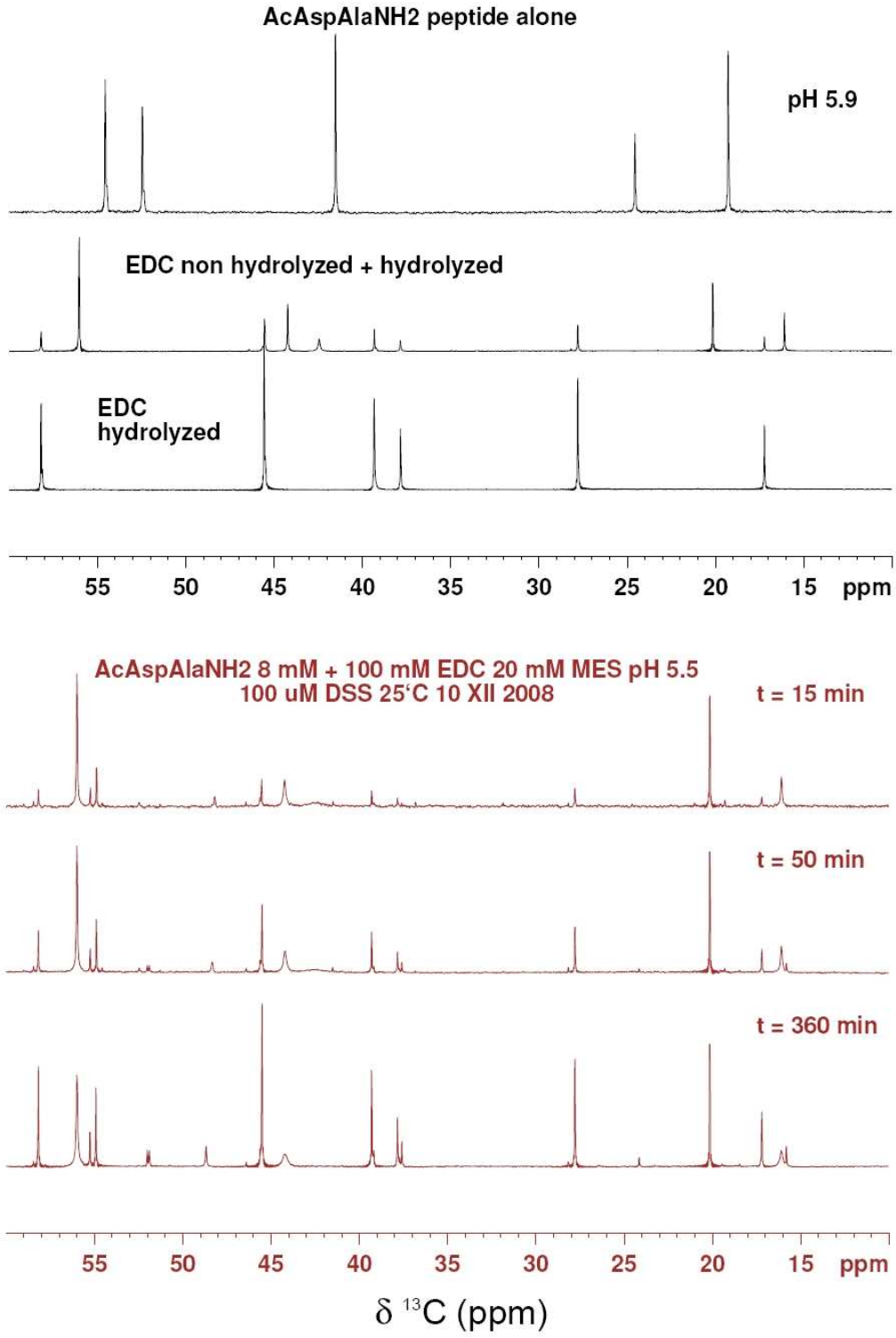
EDC or EDC fragment



δ(ppm) ^{1}H	**2.03**	**8.70 4.52**	**8.33 4.90**	**7.48, 7.19**	
^{13}C	**24.2**	**52.0**	**52.5**		
^{15}N		120.7	125.2	106.7	

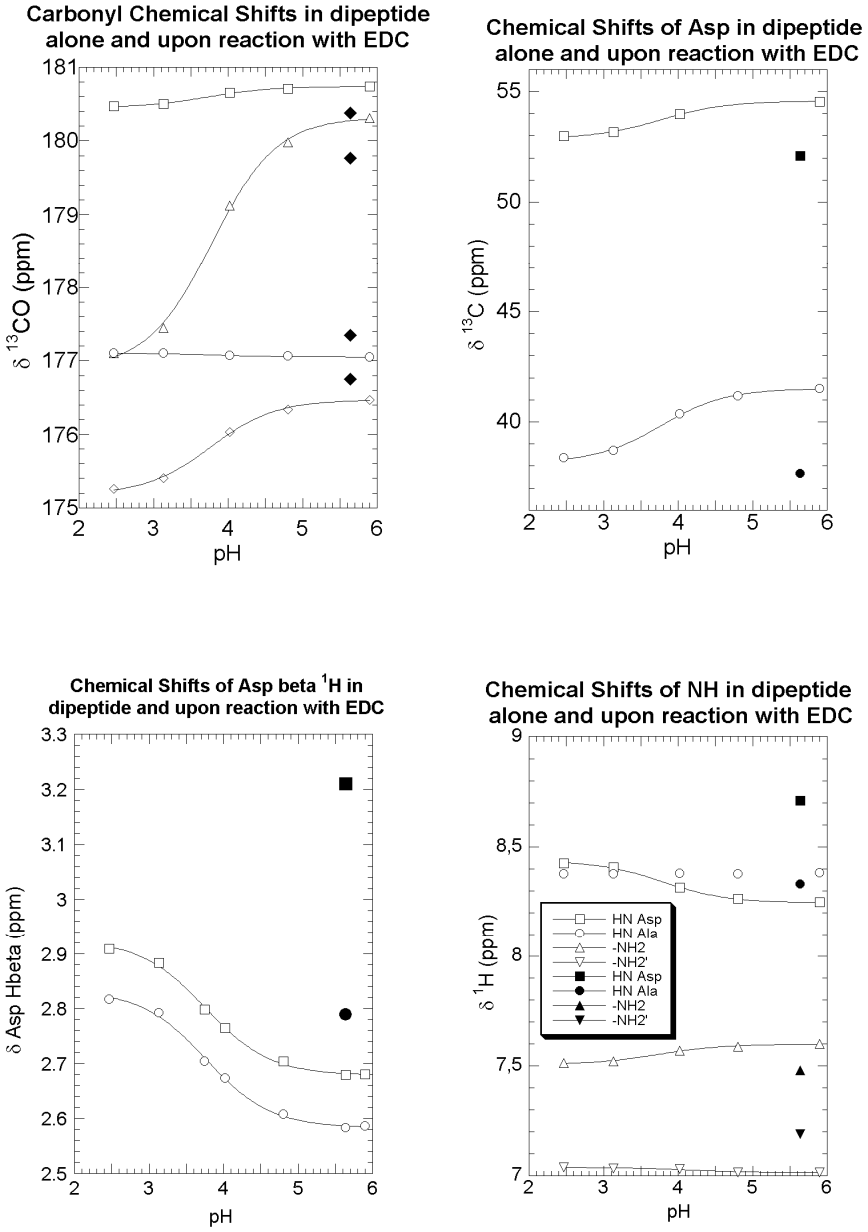
(^{13}CO at 180.4, 179.8, 177.4, 176.8, could not be assigned to individual nuclei)

Supporting Figure 3. 1D ^{13}C Spectra of the Dipeptide Ac-Asp-Ala-NH_2, EDC alone and during their reaction.



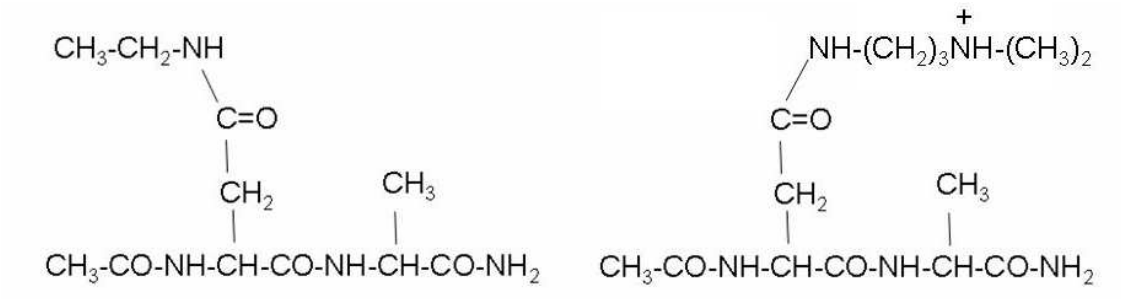
Supporting Figure 4: Comparison of the Chemical Shifts in Ac-Asp-Ala-NH_2 alone and upon incubation with EDC.

In each graph, the chemical shifts of the unmodified dipeptide is shown in open symbols and the chemical shifts of the dipeptide in the EDC reaction mixture is shown in closed symbols. The curve shows the fit of the Henderson-Hasselbach equation to the data. This comparison shows that whereas the chemical shifts of the dipeptide do depend on pH, this pH variation can not account for the new resonances appearing upon incubation with EDC. We therefore conclude that the dipeptide does indeed react with EDC.



Supporting Figure 5

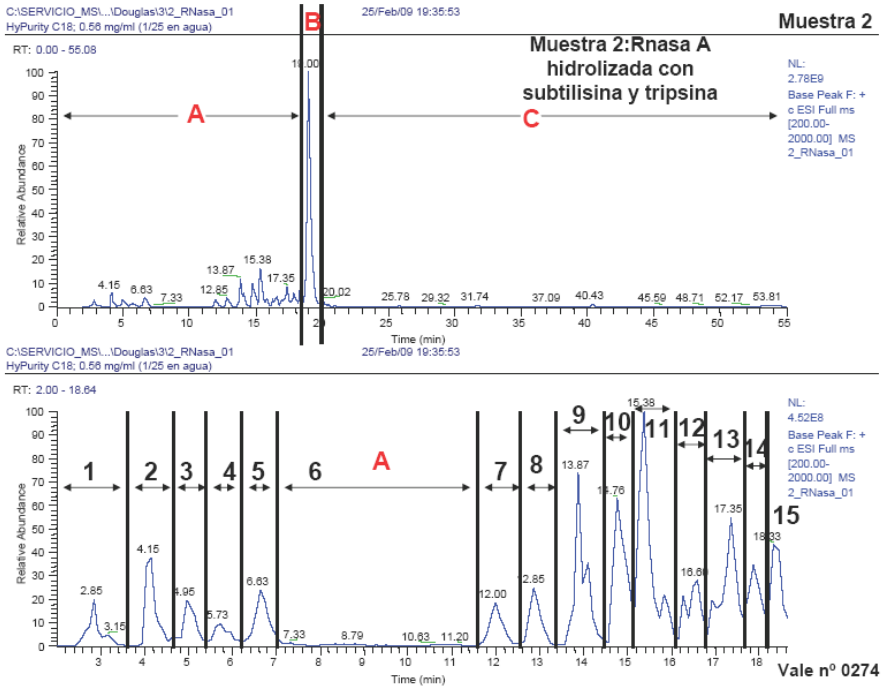
Putative Structure of the Dipeptide – EDC Fragment Adducts Detected by Electrospray Mass Spectrometry



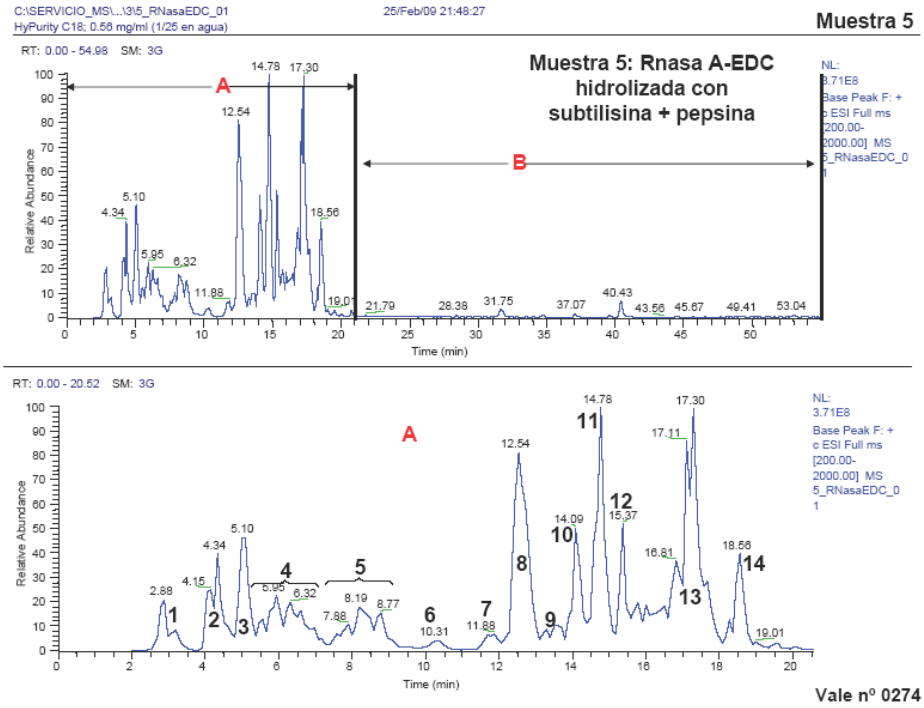
Adduction to a small EDC fragment Adduction to a large EDC fragment

Supporting Figure 6. Representative Peptide Mapping Results

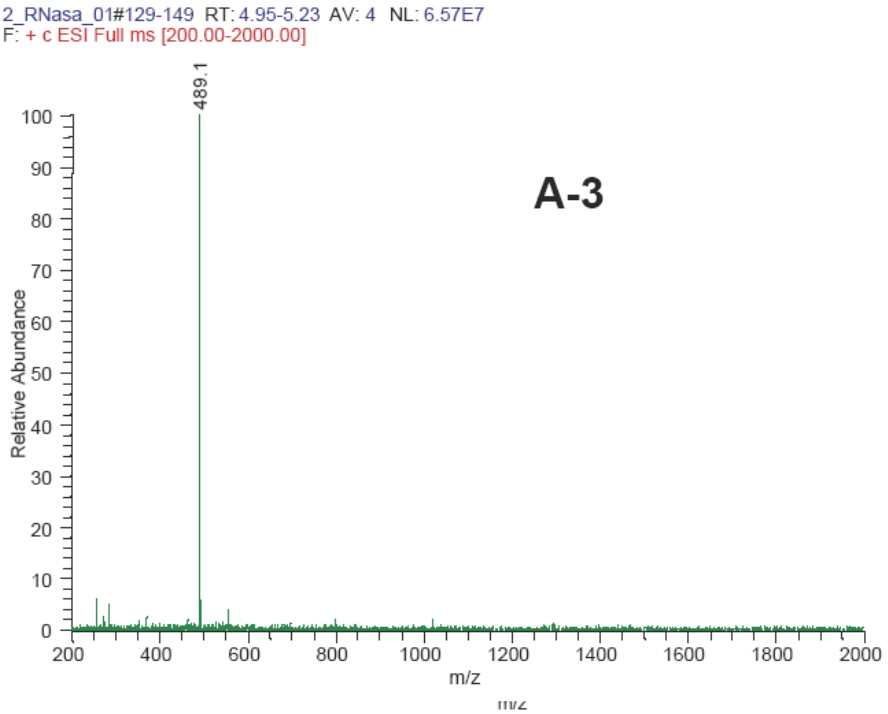
A. HPLC chromatogram of unmodified RNase A digested by subtilisin and trypsin :

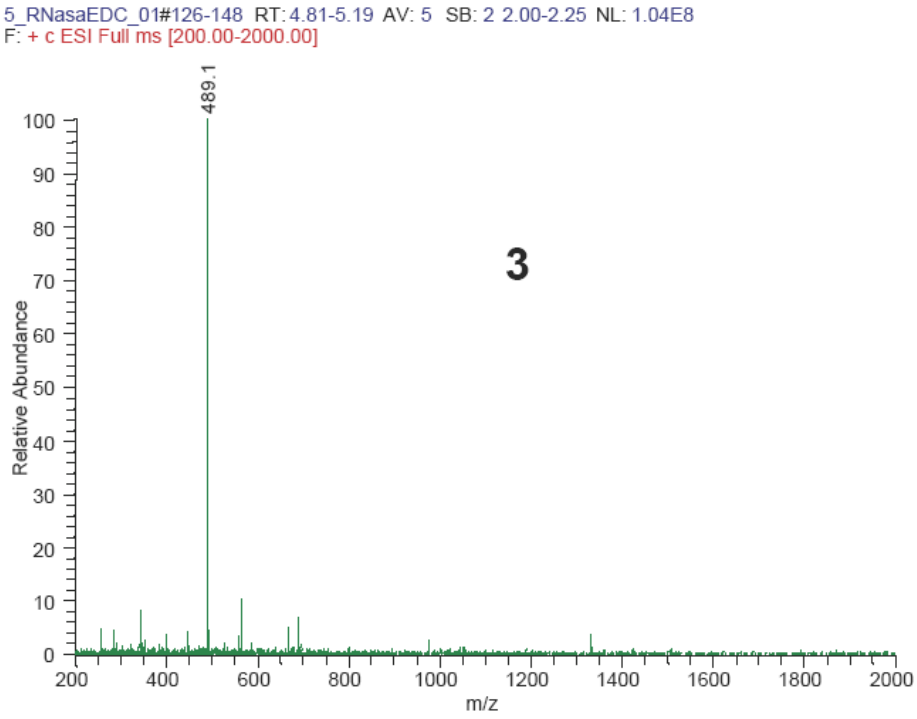


B. HPLC chromatogram of RNase A modified by EDC and digested by subtilisin and trypsin:



C. Observe that peaks A3 (top panel) and 3 (bottom panel) elute in similar positions in both chromatograms. The mass spectra of these peaks:

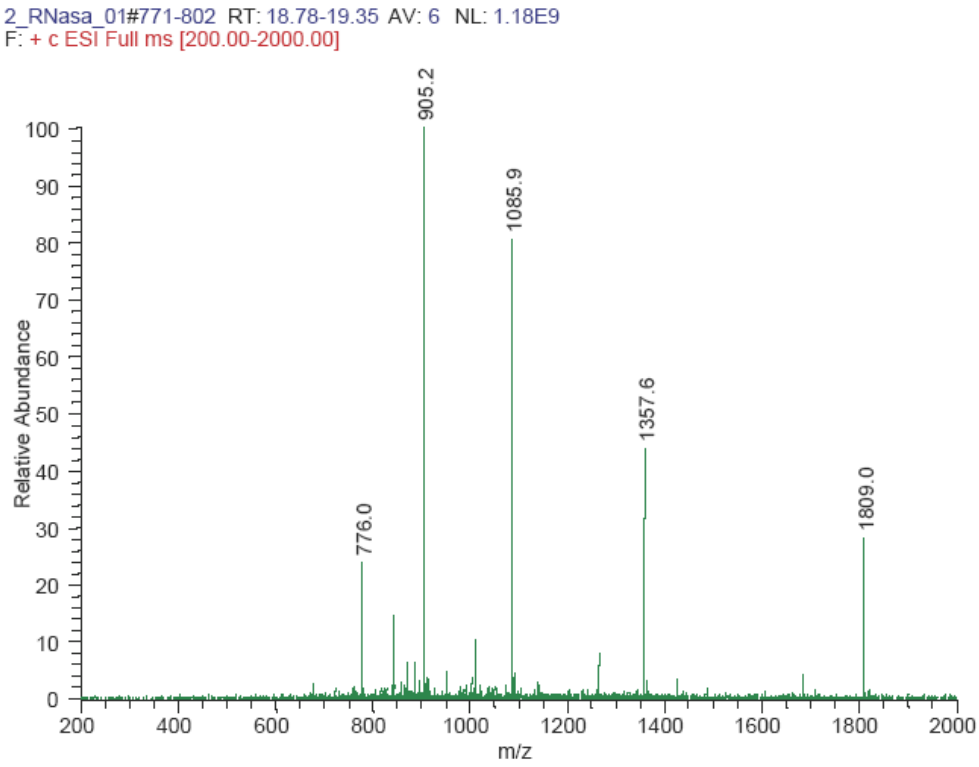




reveal identical peaks corresponding to Fragment II + K^+. Since Fragment II contains Glu 9, these data are good evidence that Glu 9 is not modified or that it is modified in a minority of RNase molecules treated with EDC.

D. Observe that the peak "B" eluting after 18 minutes in the top panel is missing in the bottom panel. The mass spectrum of this peak:

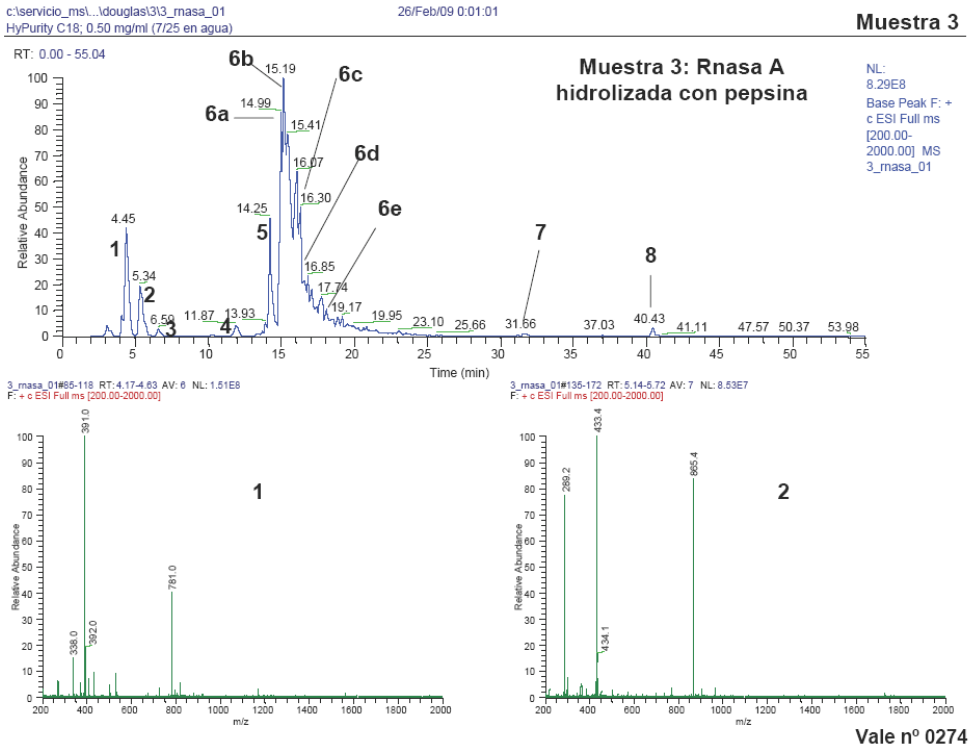
B

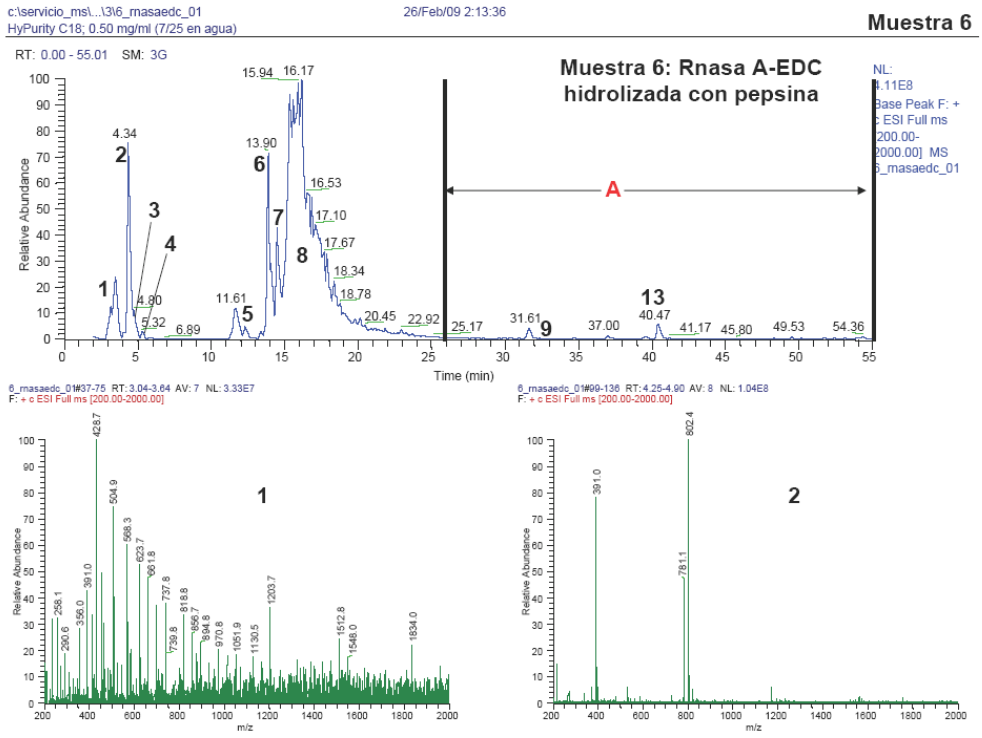


reveals peaks corresponding to fragment XIII + IX (5425.2 Da) with the peaks 1809, 1357.6, 1085.9 and 905.2 carrying 3, 4, 5 and 6 positive charges (H+), respectively. The fact that fragments XIII + IX are not separated is not unexpected as the bond between Lys 41 and Pro 42 is known to be only slowly cleaved by trypsin.

The absence of this peak in the chromatogram of RNaseA · EDC cleaved by trypsin suggests that one or more of the carboxylates in this portion of the protein have been modified by EDC. These carboxylates are Glu 49, Asp 53, Glu 111, Asp 121 and Val 124

E. Regarding the chromatograms of RNase A (top panel) and RNase A · EDC (bottom panel) hydrolyzed by pepsin:





One can see that peak 1 in the chromatogram of RNase A cleaved by pepsin corresponds in elution time to peak 2 in the chromatogram of RNase A · EDC cleaved by pepsin and that both have a peak whose mass corresponds to the C-terminal tetrapeptide Asp121-Ala122-Ser123-Val-124. Therefore, we can conclude that Asp 121 and Val 124 are not modified or that they are modified in a minority of RNase molecules treated with EDC.

On the basis of the other data and a similar way of reasoning, we have reached the following conclusions:

Not modified or modified in a minority of RNase A molecules treated with EDC	Modified in many if not a majority of RNase A molecules treated with EDC
Glu 2	Asp 38
Glu 9	Glu 49
Asp 14	Asp 53
Asp 83	Glu 111
Glu 86	
Asp 121	
Val 124 (C-terminal carboxylate)	