CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200401226

Mass Spectrometric and Quantum-Chemical Study on the Structure, Stability, and Chirality of Protonated Serine Dimers

Ferenc Pollreisz,[a] Ágnes Gömöry,[a] Gitta Schlosser,[a] Károly Vékey,*[a] Iván Solt,[b] and Attila G. Császár*[b]

Abstract: Stability and structure of homo- and heterochiral protonated serine (Ser) dimers were investigated by a combination of mass spectrometry and ab initio quantum chemical calculations. This established that the energy difference between the most stable homo- and heterochiral forms is very small: tandem mass spectrometry with Cooks' kinetic method yielded a negligible difference in Gibbs free energy ($0.2 \pm 0.2\,\mathrm{kJ\,mol^{-1}}$). The various isomeric forms of $(\mathrm{Ser})_2\mathrm{H}^+$ and their energetics were determined by extensive electronic-structure calculations, which yielded homo- and heterochiral forms of the isomers with distinctly different relative energies. The most stable homochiral isomer is stabilized by two hydrogen bonds and is far more stable than any other homochiral isomer. The most stable heterochiral isomer has completely different features, and it is characterized by a salt-bridge structure. This clearly shows that salt-bridge structures do exist in the gas phase even in comparatively small molecules and in the absence of particularly basic or acidic functional groups.

Keywords: amino acids · chirality · focal-point analysis · hydrogen bonds · mass spectrometry

Introduction

Formation and stability of supramolecular assemblies has been investigated extensively, partly to understand the role these assemblies play in chemical and biochemical processes. The processes of interest, which include drug–target interactions, DNA base pairing, and antigen–antibody binding, play key roles in pharmaceutical research, biochemistry, and biology. Supramolecular assemblies are held together by electrostatic forces, apolar interactions, and hydrogen bonds, while chiral recognition determines how the individual elements can fit together. Mass spectrometry, especially after the advent of electrospray ionization (ESI),[1] has become an excellent tool for observing and investigating molecular assemblies. Electronic-structure theory can complement such studies, as it has become a valuable tool for detailed studies of structural features of smaller complexes. Both techniques relate to the gas phase, that is, to a solvent-free environment, so, with some caution, their results are directly comparable.

Amino acids are among the most important building blocks of living systems. Their clustering, and especially the role of chiral discrimination in the formation of such clusters, has been studied by mass spectrometry.[2–11] One of the most intriguing discoveries was that serine forms unusually stable protonated octamers with a marked tendency for homochirality.[2] The preferred formation of homochiral octamers has been implicated in transmission of homochirality to other organic compounds and also to the evolution of living organisms (chirogenesis).[10]

Protonated (and cationized) serine octamers have been observed by ESI[2–8] and by the closely related sonic spray ionization techniques.[9–11] Their molecular structure has been studied by tandem mass spectrometry,[2,5,9] ion-mobility mass spectrometry,[3,5] gas-phase H/D exchange,[11] isotopic labeling,[2,5] and elementary electronic structure theory.[2,3,5,6,8] Ion mobility clearly indicates a very compact, most likely salt-bridge, structure for the octamer. Proton/deuteron exchange shows that the octamer has at least two

---

[a] F. Pollreisz, Á. Gömöry, G. Schlosser, Dr. K. Vékey
Chemical Research Center of the Hungarian Academy of Sciences
1025 Pusztaszeri út 59–67, Budapest (Hungary)
Fax: (+36) 1-325-9105
E-mail: vekey@chemres.hu

[b] I. Solt, Prof. Dr. A. G. Császár
Department of Theoretical Chemistry, Eötvös University
1518 Budapest 112, P. O. Box 32 (Hungary)
Fax: (+36) 1-372-2592
E-mail: csaszar@chem.elte.hu

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the authors.

and possibly more isomeric structures.[11] Besides the exceptional stability of the homochiral octamer, oligomers consisting of an even number of serine units, especially protonated dimers, characteristically show increased stability. Fragmentation of the serine octamer initially occurs by loss of a serine dimer, and subsequent fragmentation also occurs by preferential loss of serine dimers. A common feature of most structures suggested for the protonated octamer $(Ser)_8H^+$ is that they consist of dimeric building blocks. There is practically no contradiction between experimental observations made by various groups on serine clusters, but up to now there is no consensus on the structure of $(Ser)_8H^+$ either. This may be related to the existence of various isomeric forms that are relevant under different experimental conditions. While octamers are out of the reach of both high-quality quantum-chemical calculations and certain experimental studies, the structure of the protonated dimer can be studied in detail. Gas-phase H/D exchange[12] on the protonated homochiral serine dimer suggested that it has a single, rather open structure resembling an ion–molecule complex. While the protonated octamer shows obvious preference for homochirality, no chiral preference was observed for the dimer in ESI spectra.[2,5] This intriguing experimental result prompted us to study the structure and the difference in stability between homo- and heterochiral protonated serine dimers in a joint experimental and computational study.

The kinetic method is well suited to detect small energy differences experimentally. It was originally developed to determine thermochemical values of individual molecules, but has been extended to derive data on molecular complexes.[13,14] The first such application was the determination of gas-phase basicities of molecular pairs, and it was later used for distinguishing amino acid enantiomers, on the basis of the fragmentation of trimeric clusters.[15] The studied protonated amino acid pairs, Pro-Trp, Phe-Ala, Phe-Pro, and Phe-Val, all showed preference for homochirality, and the energy difference between homo- and heterochiral pairs was in the range of 0.4–4 $kJ\,mol^{-1}$. This technique was adopted here to study the protonated serine dimer by using isotopically labeled compounds: D-serine (denoted D) and deuterium-labeled L-serine (with the three CH hydrogen atoms replaced with deuterium; denoted L).

To the best of our knowledge no detailed computational study has been performed to explore the ground electronic state potential energy surface (PES)[16] of $(Ser)_2H^+$. Therefore, to complement the experimental results of this study and to provide basis for their better understanding, elaborate geometry optimizations were performed to identify all low-energy conformers of homo- and heterochiral $(Ser)_2H^+$. Furthermore, definitive ab initio electronic-structure calculations were performed to determine the energy order of the conformers by employing the focal-point approach.[17,18] The converged results of the focal-point approach make it feasible to compare the stability of neutral and zwitterionic forms of $(Ser)_2H^+$. Due to the large size of the system studied the focal-point approach could not be pursued to its usual extent,[19,20] but it is believed that dependable structural and energy data were obtained in this study for all the important conformers of $(Ser)_2H^+$.

Experimental Section

Mass spectrometry: Two types of mass spectrometers were used in the present study. A reverse-geometry double-focusing VG ZAB-SEQ instrument was used in positive-ion mode. D-Serine and deuterium-labeled L-serine were mixed in 1:1 ratio in a 0.1 % aqueous solution of acetic acid, and further mixed with glycerol matrix. Protonated trimer ions were generated by liquid secondary ion mass spectrometry (LSIMS) ion source by using Cs^+ ions accelerated to 30 kV. The metastable fragmentation and collision-induced dissociation (CID) of the mass-selected protonated heterochiral trimer ions were examined by mass-analyzed ion kinetic energy spectrometry (MIKES). Parent ions were accelerated to a potential of 8 kV, the pressure of the collision gas (Ar) was adjusted to give 60 % main-ion beam transmission. To separate CID and metastable components, a small voltage (500 V) was applied to the collision cell.

A PE Sciex API 2000 (Perkin-Elmer Sciex, Toronto, Canada) triple-quadrupole mass spectrometer was also used, equipped with a Turbo Ion Spray source. For CID experiments N_2 was used as collision gas, and the collision energy was varied in the 10–22 eV range.

L-serine, labeled by replacing protons in the three C−H bonds with deuterium, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA), and unlabeled D-serine was obtained from Sigma-Aldrich Kft. (Budapest, Hungary).

Computational methods: A detailed description of the computational methods is given in the Supporting Information, and only an abbreviated version with references to key articles is given here.

The geometry optimizations of this study, that is, mapping minima on the PES of $(Ser)_2H^+$, were carried out in two steps.[19] First, 82 homo- and heterochiral starting structures, constructed by consideration of the intermolecular H-bonding network of $(Ser)_2H^+$, were optimized at the restricted Hartree–Fock (RHF)[21–24] level with the 3-21G basis set.[25–27] The H-bond networks of the 24 lowest-energy minima were considered for further analysis. These structures were employed to generate all homo- and heterochiral starting structures for density functional theory [DFT-(B3LYP)][28–32] geometry optimizations[33] with the 6-311++G** basis set[27] and for MP2_FC/6-31+G* geometry optimizations, where MP2 and FC stand for second-order Møller–Plesset perturbation theory and the usual frozen-core approximation, respectively.[24] The most stable structures of $(Ser)_2H^+$ are shown in Figures 1 and 2. Table 1 lists relative energies of the most stable conformers of $(Ser)_2H^+$ at the three levels of theory for which geometry optimizations were performed. Note that a previous computational study[2] suggested that a favorable interaction can be found between the carboxyl groups of the monomeric units. In this more sophisticated study this structure was found to have a relative energy of at least 40 $kJ\,mol^{-1}$ relative to **I_LL**.

To obtain accurate relative energies for the conformers of $(Ser)_2H^+$ the so-called focal-point approach (FPA)[17,18] was followed, which has been employed successfully for determining the conformational preferences of the neutral amino acids glycine,[19,34,35] alanine,[19,36] and proline.[20] For details of the FPA approach see the Supporting Information and refs. [17–20,34–36] Detailed results and the final FPA relative energies are listed in Tables 2 and 3. The final FPA energies of this study, as judged on the basis of earlier studies, should be accurate to within about ±4 $kJ\,mol^{-1}$.

The correlation-consistent (cc) family of basis sets[37,38] (aug-)cc-p(C)V*X*Z, with cardinal number $X=2$ (D), 3 (T), and 4 (Q), was used for the single-point energy calculations at the RHF and MP2 levels. Extrapolation of the large finite-basis RHF energies $E(\mathrm{RHF})$ and the corresponding MP2 energy increments $\delta E(\mathrm{MP2})$ to the complete basis set (CBS) limits were performed with the three-point[39] $E_X = E_{\mathrm{CBS}} + a\exp(-bX)$ and the two-point[40] $\delta E_X = \delta E_{\mathrm{CBS}} + cX^{-3}$ formulas, respectively, where X is the cardinal number of the correlation-consistent basis set.

The following aug-cc-pVXZ basis sets were employed during extrapolation: $X=2, 3, 4$ for E(RHF) and $X=2, 3$ for δE(MP2). The accuracy of relative energy predictions was increased in this study by augmenting the results of large basis set, essentially CBS, RHF, and MP2 computations, by 6-31G* and (p)VDZ[41] CCSD(T)−-MP2 relative energy increments. In the (p)VDZ CCSD(T) single-point energy computations polarization functions were removed from hydrogen atoms which do not participate in any secondary bonding.

Geometry optimizations, MP2 calculations with large basis sets, and computations of zero-point energy (ZPE) were all carried out with the program suites Gaussian 03[42] and PQS,[43] while the electronic-structure package ACESII[44] was employed for the single-point CCSD(T) computations.

Results and Discussion

The structures of the homo- and heterochiral $(Ser)_2H^+$ conformers can be discussed in terms of inter- and intramolecular hydrogen bonds that stabilize the cluster. Figure 1 shows the two-dimensional network of H-bonds in $(Ser)_2H^+$. Spatial representations of the same structures are shown in Figure 2. The stability order of homo- and heterochiral $(Ser)_2H^+$ isomers is shown in graphical form in Figure 3. Relative energies are listed in Tables 1–3. Selected geometry parameters characterizing the hydrogen-bonded networks are provided in Table 4.

Structurally similar protonated dimers are designated by the same Roman numeral, irrespective of the relative energies of the corresponding homo- and heterochiral isomers. **D**/**L** in the names of the conformers relate to the chirality of the monomeric units within the dimers. The letters A and B are added to the Roman numerals in those cases where the different local minima exhibit very similar hydrogen-bonding networks and the structural differences are more subtle. Mirror-image homochiral (**DD** and **LL**) and heterochiral (**DL** and **LD**) pairs are, of course, energetically identical. Therefore, to conserve space, the energies of mirror images of the isomers discussed are not reported in the tables and figures, although in several cases geometry optimizations resulted in such pairs.

Computed structures: All $(Ser)_2H^+$ isomers are stabilized by two or three intermolecular hydrogen bonds, of which those between OH of the hydroxymethylene group (β-OH) and CO of the carboxyl group are typical of the most favorable structures. Interatomic distances between the anchor atoms of the hydrogen bonds in the range of 2.5–3.0 Å (Table 4) indicate relatively strong interactions.

The homochiral structure **I_LL** is clearly the global minimum on the PES of $(Ser)_2H^+$. It is stabilized by two hydrogen bonds with β-OH and C(O)OH donors and CO and β-OH acceptors, respectively. As Figure 3 clearly reveals, the heterochiral form of this structure, **I_DL**, is far less stable.

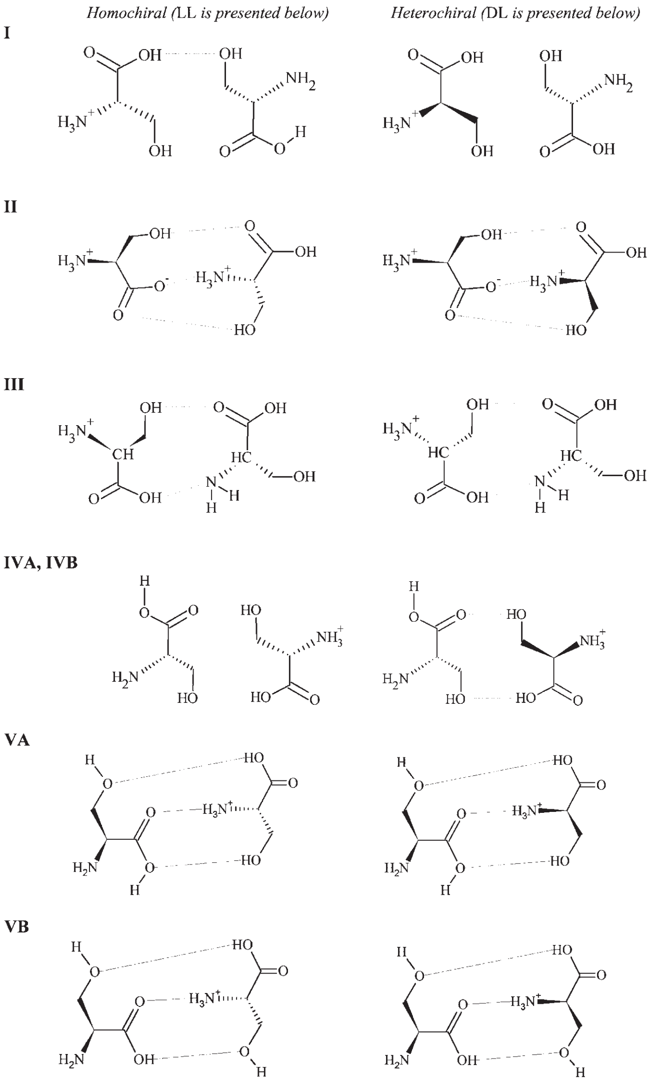

Figure 1. Conformational families of $(Ser)_2H^+$. Only LL and DL forms are presented for the homo- and heterochiral conformers, respectively. Note that the homochiral **VA** structure could not be found during the geometry optimizations, and thus its structure is only indicated here for the sake of completeness.

The most stable heterochiral isomers are **II_DL** and **VA_DL**. The small energy difference between **II_DL** and **VA_DL** is at least partly due to the considerable similarity of their H-bond networks. Intramolecular proton transfer in **VA_DL** from the neutral COOH to the NH_2 group would result in a salt-bridge structure, very similar to **II_DL**. Based on the computed data alone it is impossible to determine whether **II_DL** or **VA_DL** has the lower relative energy. It is the substantial difference in the ZPE increments that makes the final relative energy of **VA_DL** very similar to that of **II_DL**. Given the drastically different δMP2 increments of the two conformers, a small difference (Table 2) may not have too much meaning. It is important to note that **II_DL** (Figures 1 and 2) has a salt bridge between $C(O)O^-$ and NH_3^+ and two OH···CO hydrogen bonds. There is a clear difference between these two hydrogen bonds: that involving β-OH of the monomer with the COO^- residue is substantially weaker, as shown by the respective bond lengths (2.80 versus 3.03 Å, Table 4). Figure 3 also shows that both the most stable homo- (**I_LL**) and the

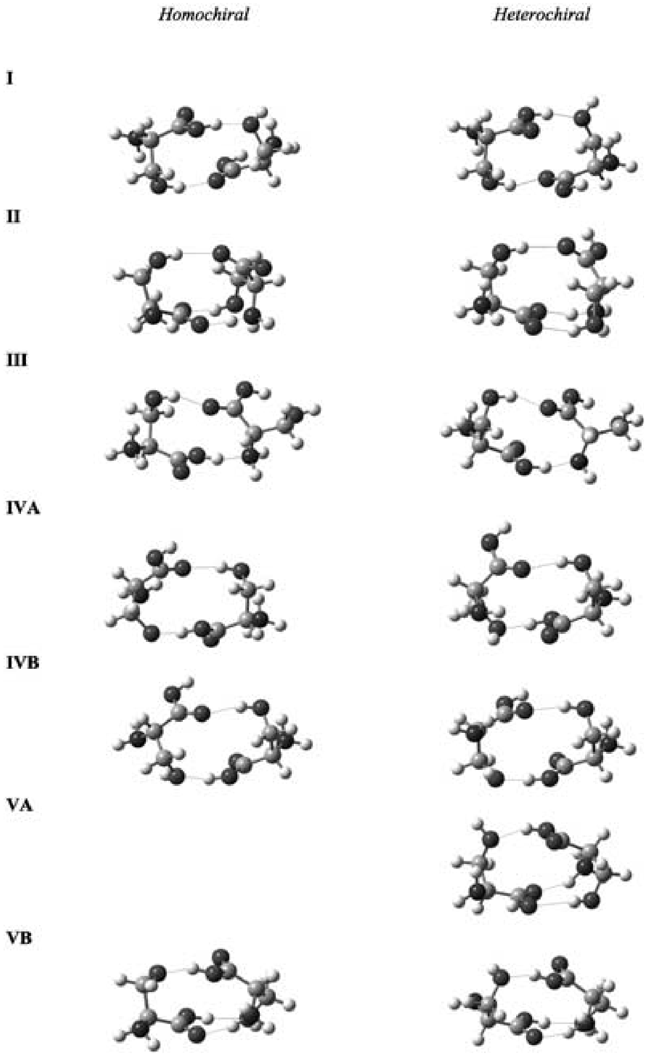

Figure 2. Representation of the spatial structures of the lowest energy isomers of $(Ser)_2H^+$, optimized at the B3LYP/6-311++G** level of theory. **VA** could not be optimized at the levels of theory employed in this study and thus its detailed structure is not presented here.

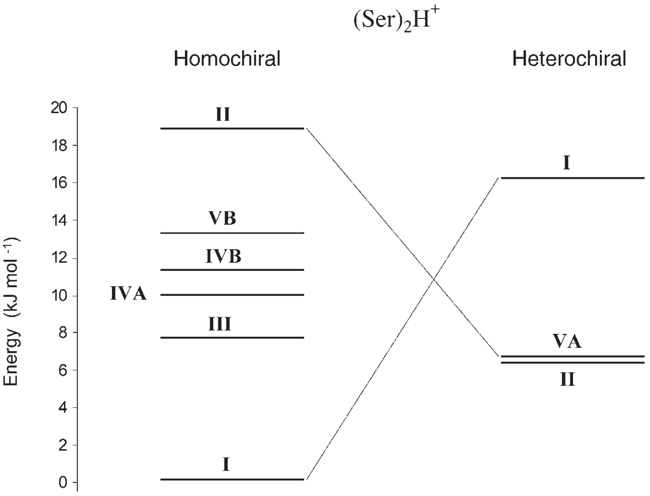

Figure 3. Comparative energy diagram of the most stable $(Ser)_2H^+$ isomers based on data from Table 2.

Table 1. Relative energies [kJ mol^{-1}] of the most stable conformers of $(Ser)_2H^+$ (see Figure 1 for a pictorial depiction of the conformers, obtained from complete geometry optimizations).

Conformer	RHF/3-21G	B3LYP/6-311++G**	MP2/6-31+G*
I_LL	0.00	0.00	0.00
II_DL	−6.18	6.43	-8.05
III_LL	6.28	7.04	9.67
IVA_LL	1.87	9.83	11.48
IVB_LL	−0.44	10.39	13.44
VA_DL	−0.71	10.84	2.10
IVB_DL	−0.02	13.76	
I_DL	4.32	15.59	12.24
II_LL	7.07	16.81	2.84
VB_LL	−6.52	18.18	2.10
VB_DL	−6.91	19.02	
IVA_DL	9.13	24.67	

most stable heterochiral structures (**II_DL** and **VA_DL**) have considerably higher stability than any of the other isomers.

To ensure that the geometry of the salt-bridge structure is well described and hence that relative energies computed for reference structures optimized at the B3LYP/6-311++G** level are accurate, further geometry optimizations were performed at the MP2 level with 6-31G* and 6-31+G* basis sets. These optimizations confirmed the existence of salt-bridge structure **II**. Subsequent single-point energy calculations (compare relevant results in Tables 2 and 3) confirmed that no substantial error is introduced in the energy calculations by using a single set of well-defined reference geometries in the focal-point approach.

It is instructive to compare the accuracy of the relative energies obtained at different levels of theory. As Tables 1–3 show, the influence of electron correlation, especially the MP2 energy increment, can be more than 20 kJ mol^{-1} for some structures, most importantly for salt-bridge structure **II**. Therefore, the RHF relative energies, even at the CBS limit, are completely misleading for the energy order of the conformers of $(Ser)_2H^+$.

For much larger molecules and those containing strongly acidic and basic functional groups, the occurrence of salt-bridge structures in the gas phase has long been recognized.[45–48] Beauchamp et al. found $(Ser)_6H^+$ as the smallest salt-bridge-containing serine cluster. The results presented here were obtained at a considerably higher level of theory, and clearly indicate that one of the two most stable heterochiral $(Ser)_2H^+$ isomers does have a salt-bridge structure. Note that for a meaningful comparison of the energies of salt-bridge and other structures a relatively high level of theory is required. It is especially striking how different the RHF and MP2 energy predictions are for the relative energy of **II_DL** (Tables 2 and 3). While at the RHF/CBS level **II_DL** is among the least stable conformers of $(Ser)_2H^+$ considered, at the MP2/CBS level it is the most stable heterochiral isomer, only 2.2 kJ mol^{-1} less stable than **I_LL** and more than 5 kJ mol^{-1} more stable than the next conformer. It is also clear that sufficiently large basis sets are required when comparing energetics of such disparate structures; for

Table 2. Relative RHF energies, incremental MP2, CCSD, CCSD(T), and core energy contributions, and zero-point energy corrections of the most stable conformers of $(Ser)_2H^+$, all in kJ mol^{-1}.[a]

	Level	Basis	**I_LL**	**II_DL**	**VA_DL**	**III_LL**	**IVA_LL**	**IVB_LL**	**VB_LL**	**I_DL**	**II_LL**
relative RHF energies	RHF	6-31G*	0.00	14.11	2.44	13.01	7.31	5.29	13.31	16.89	26.57
		(p)VDZ	0.00	20.95	1.42	12.75	9.23	5.07	1.42	17.19	35.41
		6-311++G**	0.00	19.70	2.30		6.48	2.80	13.97	16.86	32.16
		cc-pVDZ	0.00	20.23	2.28	13.37	7.95	4.89	13.15	18.00	33.73
		aug-cc-pVDZ	0.00	20.35	4.95	12.80	7.20	3.92	18.13	18.95	33.35
		cc-pVTZ	0.00	22.36	5.94	14.33	6.66	3.92	19.27	18.95	35.83
		aug-cc-pVTZ	0.00	22.34	6.19	13.75	6.43	3.09	19.24	18.96	35.49
		cc-pVQZ	0.00	22.92	6.36	14.02	6.53	3.31	19.71		36.13
		aug-cc-pVQZ	0.00	22.83	6.39	13.93	6.47	3.07	19.67	19.09	36.03
		CBS	0.00	23.00	6.40	13.95	6.61	3.17	19.89	19.21	36.21
MP2 increments	MP2	6-31G*	0.00	-21.54	1.58	−7.95	3.51	8.37	−4.52	−3.05	−22.98
		(p)VDZ	0.00	−18.17	2.02	−8.92	1.72	6.28	2.02	−3.08	−19.15
		6-311++G**	0.00	−13.02	−1.20		4.69	10.24	−7.12	−3.91	−14.13
		cc-pVDZ	0.00	−14.65	2.74	−8.41	4.28	9.43	−3.04	−7.96	−15.50
		aug-cc-pVDZ	0.00	−21.08	−0.97	−7.20	4.22	9.54	−8.60	−4.57	−22.48
		cc-pVTZ	0.00	−17.83	2.39	−7.00	4.45	9.80	−4.71	−3.12	−18.80
		aug-cc-pVTZ	0.00	−20.90	1.03	−6.75	3.98	9.42	−6.76	−3.84	−22.44
		CBS	0.00	−20.83	1.87	−6.57	3.87	9.37	−5.98	−3.54	−22.43
CCSD increments	CCSD	6-31G*	0.00	6.69	−2.16	2.05	−0.15	−1.76	0.68	0.35	6.65
	CCSD	(p)VDZ	0.00	5.74	−1.92	1.85	0.11	−1.25		0.33	5.66
CCSD(T) increments	CCSD(T)	6-31G*	0.00	−3.65	0.46	−1.46	0.61	1.32	−0.41	−0.34	−3.86
	CCSD(T)	(p)VDZ	0.00	−3.28	0.72	−1.53	0.48	1.24		−0.30	−3.49
zero-point corrections	B3LYP	6-311++G**	0.00	1.68	−0.72	0.14	−1.06	−1.03	−0.64	0.67	2.27
core-correlation energy	MP2	cc-pCVDZ	0.00	−0.03	0.00	−0.02	−0.01	0.03	0.00	0.01	0.02
ZPE-corrected	Final	CBS	**0.00**	**6.27**	**6.34**	**7.83**	**10.02**	**11.53**		**16.38**	**18.23**

[a] The final relative energies were obtained by an exponential extrapolation (see text) of aug-cc-pV*XZ* RHF energies [$X=2$ (D), 3 (T), and 4 (Q)], augmented by CBS MP2 energy increments, obtained by an inverse polynomial extrapolation (see text) of aug-cc-pV*XZ* energies [$X=2$ (D) and 3 (T)], further augmented by 6-31G* and (p)VDZ CCSD and CCSD(T) (only the (p)VDZ results were employed when the final energies of this table are computed) and core correlation energy increments. Zero-point energy (ZPE) corrections, calculated at 298.15 K, were finally added.

Table 3. Relative RHF energies, incremental MP2 and CCSD(T) energy contributions, and zero-point energy (ZPE) corrections for the three most stable conformers of $(Ser)_2H^+$, all given in kJ mol^{-1}.[a]

	Level	Basis	**I_LL**	**II_DL**	**VA_DL**
relative energies	RHF	CBS	0.00	17.87	6.87
	δMP2	CBS	0.00	−15.69	0.55
	δCCSD(T)	(p)VDZ	0.00	1.62	−1.47
ZPE corrections[a]	B3LYP	6-311++G**	0.00	1.68	−0.72
core-correlation energy	MP2	cc-pCVDZ	0.00	−0.03	0.00
ZPE-corrected	final	CBS	**0.00**	**5.45**	**5.22**

[a] The reference geometries employed in the computation of the relative energies reported in this table were optimized at the MP2_FC/6-31+G* level. ZPE corrections are the same as in Table 2, calculated at the B3LYP/6-311++G** level.

example, **II_DL** is more stable than **I_LL** at the MP2/aug-cc-pVDZ level.

Single-point calculations at the 3-21G, 6-31G*, 6-31+G*, 6-311G*, and 6-311++G** B3LYP level on the optimized 6-311++G** B3LYP reference structures (not detailed in Table 2), resulted in mean absolute deviations of 11.6 (12.6), 3.6 (3.8), 3.2 (3.9), 3.8 (4.6), and 1.7 (1.8) kJ mol^{-1} as compared to the final energy results of Table 2 excluding (including) ZPE. Based on these systematic studies, the lowest recommended level of theory which gives a reasonable estimate for the presence and relative energy of salt-bridge structures is DFT(B3LYP)/6-311++G**.

To discuss the presence of various structures in the gas phase, and to make direct comparison with experiment, entropy contributions to all structures were also obtained, by using the rigid rotor/harmonic oscillator model. The required second-derivative calculations were carried out at the DFT(B3LYP)/6-311++G** level. This simplified treatment suggests that the entropy difference at 298.15 K between isomers **I_LL** and **II_LL** is only 0.005 kJ mol^{-1} K^{-1} in favor of the salt-bridge structure. For the heterochiral isomers the analogous difference between **VA** and **II** is also negligible. The entropy difference between the two most stable conformers of $(Ser)_2H^+$, **I_LL** and **II_DL**, is again very small, only −0.002 kJ mol^{-1} K^{-1} in favor of the global minimum **I_LL**. These relative values suggest that the final relative enthalpy and Gibbs free energies are very close to each other. In other words, the entropy contribution does not change the stability order of the studied conformers.

In summary, the computational results discussed above suggest that the energy difference between the most stable homochiral and heterochiral protonated serine dimers is small, comparable to the intrinsic accuracy of the calculations. Nevertheless, the calculations seem to indicate a homochiral preference for $(Ser)_2H^+$.

Experimental relative energies: The kinetic method is based on studying the kinetics of competing reactions[14,15,49] and is used widely in mass spectrometry to determine thermochemical properties. The kinetic method is simple to use and

Table 4. Heavy-atom distances [Å] in the hydrogen-bond networks of the conformers of $(Ser)_2H^+$.

Conformer	COH···OH	OH···CO	COO^-···HNH_2^+	OH···COH	NH_3^+···CO
I_LL	2.687	2.750			
I_DL	2.679	2.740			
II_LL		2.770/3.020	2.646		
II_DL		2.800/3.030	2.605		
III_LL	2.573	2.840			
III_DL	2.571	2.810			
IVA_LL	2.573	2.840			
IVA_DL	2.571	2.810			
IVB_LL	2.554	2.850			
IVB_DL	2.550	2.862			
VA_LD	2.746			3.020	2.760
VB_LL	2.832/2.600				2.737
VB_DL	2.840/2.610				2.705

[a] The headings refer to bond types; CO and COH stand for the corresponding parts of the carboxyl group, and OH for the hydroxymethylene OH group.

sensitive to very small energy differences. Nevertheless, the method has been criticized as being not always reliable, especially when the two competing reaction channels differ in entropy. The various sources of error of the kinetic method have been discussed in detail,[50–52] and a consensus is starting to emerge. In general, enthalpy errors on the order of 5 kJ mol^{-1} can be expected when there are no complications due to entropy differences between the two product channels. When isomers or conformers are compared, most of the errors of the kinetic method cancel, and a considerably lower error results.

Serine clusters were prepared by LSIMS ionization. Besides protonated monomers, dimers, and matrix peaks the LSIMS spectra showed protonated peaks for homochiral (DDD and LLL) and heterochiral (DDL and DLL) serine trimers. Owing to isotopic labeling these ions are separated by 3 Da and are easily distinguished. The heterochiral DDL cluster was selected by the magnet, and its metastable fragmentation was studied by MIKES. The metastable MIKE spectrum shows only the loss of a serine molecule to form a protonated serine dimer either in the homochiral (DD) or heterochiral (DL) configuration (Figure 4a). Without chiral discrimination between DD and DL and in the absence of an isotope effect, the abundance ratio of the two fragments should be 1:2. Statistically a DDL cluster loses D twice as likely as L. The measured ratio of peak areas is very close to statistical (1:1.91 with standard deviation of 0.06), and this suggests that formation of the homochiral protonated dimer is very slightly favored. An analogous result was also obtained in the case of the DLL trimer (Figure 4b), for which the ratio of LL to DL is 1:1.89 ± 0.06.

The results, which are very close to statistical, can be interpreted by the kinetic method. The kinetic method[14,15,49] is used to evaluate competing reactions on the basis of the Gibbs energy difference between the two competing reaction channels ($\Delta\Delta G$), $\ln(I_1/I_2) = \ln(k_1/k_2) = \Delta\Delta G/RT_{\text{eff}}$, where I_1 and I_2 are the ion abundances (peak areas), k_1 and k_2 are the respective rate constants, R is the universal gas constant, and T_{eff} is the effective temperature, an empirical parameter.[14,15,49]

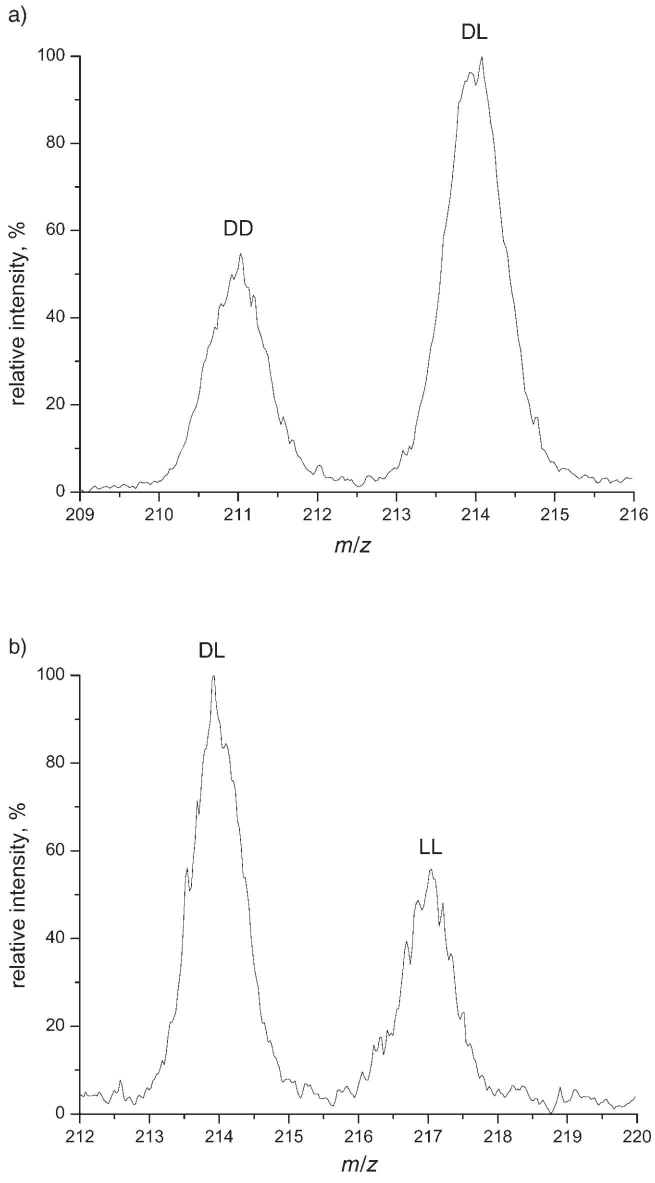

Figure 4. Metastable fragmentation of heterochiral DDL (a) and DLL (b) trimers. The main fragment ions are protonated DD, DL, and LL serine dimers.

A particular concern related to the use of the kinetic method is that the two products, DD and DL, have significantly different structural characteristics, as one is an ion–neutral complex, while the other has a salt bridge. Consequently, one may question whether the two respective transition states are similar or not, and this has consequences for the accuracy of the results obtained from the kinetic method.

Although the structures of DD and DL are different, the transition states leading to the products are in both cases loose ion–neutral complexes between a dimer and a monomer, (DD)$\cdots$H$^+\cdots$L and DL$\cdots$H$^+\cdots$D. Such loose complexes, in most cases, are considered to be similar. In a more quantitative manner, "similarity" between two reaction channels is typically measured by entropy differences. When the entropy difference is negligible, the "simple" kinetic method can be applied in a straightforward manner. When the entropy difference is large, it must be explicitly considered, and results obtained by the kinetic method may be compromised. Partly for this reason, we calculated the entropy difference between the two products DD and DL, as discussed above. These calculations indicate that, in spite of the difference in structural properties, the entropy of the two product states are very close. This indicates that the kinetic method results in dependable estimates of energies in the present case.[51] We also compared the entropy difference between the two transition states experimentally. This was possible only in a semiquantitative manner, by comparing product ratios at different degrees of excitation (high- and low-energy CID and metastable ion spectra; see below). These experimental results show the absence of a considerable entropy difference between the two respective transition states. We believe that these results clearly suggest that the kinetic method can be used to compare energy differences between the protonated DD and DL isomers.

In the present case the energy difference between the two competing reaction channels may derive from two different effects. One is the stability difference between the homo- and heterochiral dimers ($\Delta G_{\mathrm{chiral}}$), which is of principal interest in this study. The other is the kinetic isotope effect, derived from the energy difference between losing labeled versus unlabelled serine ($\Delta G_{\mathrm{isotope}}$). Note that the isotopic labels are not directly involved in dissociation (secondary isotope effect), so the isotope effect is unlikely to be significant. In the present case fragmentation from both DDL and DLL was studied. Fixing the arbitrary zero energy level for the reaction involving the loss of an unlabeled serine and forming a homochiral protonated dimer, reactions and evaluation of the energetics can be expressed by Equations (1)–(4).

$$\begin{aligned}\mathrm{DLL} &\rightarrow \mathrm{LL} + \mathrm{D} \quad 0\\ &\rightarrow \mathrm{DL} + \mathrm{L} \quad \Delta G_{\mathrm{chiral}} + \Delta G_{\mathrm{isotope}}\end{aligned} \tag{1}$$

$$\ln(2\,\mathrm{LL/DL}) = \ln P_{\mathrm{DLL}=}(\Delta G_{\mathrm{chiral}} + \Delta G_{\mathrm{isotope}})/RT_{\mathrm{eff}} \tag{2}$$

$$\begin{aligned}\mathrm{DDL} &\rightarrow \mathrm{DD} + \mathrm{L} \quad \Delta G_{\mathrm{isotope}}\\ &\rightarrow \mathrm{DL} + \mathrm{D} \quad \Delta G_{\mathrm{chiral}}\end{aligned} \tag{3}$$

$$\ln(2\,\mathrm{DD/DL}) = \ln P_{\mathrm{DDL}=}(\Delta G_{\mathrm{chiral}} - \Delta G_{\mathrm{isotope}})/RT_{\mathrm{eff}} \tag{4}$$

In Equations (1)–(4) DLL and DDL represent the protonated trimers, DD, DL, and LL the protonated dimers (and their peak areas), D and L the monomers, the value "2" is used to correct for the symmetry factor, and P_{DLL} and P_{DDL} are the symmetry-corrected fragment-ion abundance ratios measured for the two parent ions. From these equations the following energy differences can be calculated:

$$\Delta G_{\mathrm{chiral}} = (\ln P_{\mathrm{DLL}} + \ln P_{\mathrm{DDL}})RT_{\mathrm{eff}}/2$$

and

$$\Delta G_{\mathrm{isotope}} = (\ln P_{\mathrm{DLL}} - \ln P_{\mathrm{DDL}})RT_{\mathrm{eff}}/2.$$

The experimental results indicate that $\Delta G_{\mathrm{isotope}}$ is negligible, about 10 times smaller than the chiral energy difference $\Delta G_{\mathrm{chiral}}$. The empirical parameter T_{eff} usually lies in the 400–600 K range.[14,15] Taking 500 K, $\Delta G_{\mathrm{chiral}}$ is calculated to be 0.2 kJ mol^{-1}, that is, a very slight preference for homochirality. Even assuming 50 % uncertainty in T_{eff}, the uncertainty in relative stability is small (<0.1 kJ mol^{-1}). Uncertainty in determination of peak ratios causes about ±0.1 kJ mol^{-1} uncertainty in relative stability. The overall uncertainty in the experimental determination of $\Delta G_{\mathrm{chiral}}$ is thus estimated to be less than ±0.2 kJ mol^{-1}.

Analogous results were obtained by CID. To separate CID and metastable components, a small voltage (500 V) was applied to the collision cell.[13,15] Ion ratios observed in CID were close to statistical (2.03 ± 0.10). CID experiments were also performed on a triple-quadrupole instrument (API-2000, N_2 collision gas). Note that in ESI the abundance of the serine trimer depends significantly on experimental conditions, and although it is usually a minor peak, it was sufficient to generate product ion spectra with sufficiently good signal-to-noise ratio to apply the kinetic method. Within experimental error, the intensity ratios were the same as those obtained for metastable ions, and were independent of the collision energy.

The results discussed above show that chiral discrimination in protonated serine dimers is very minor indeed. Relative abundances of homo- and heterochiral fragments differ only by a few percent, corresponding to an energy difference of 0.2 ± 0.2 kJ mol^{-1} in favor of the homochiral form.

Comparison of experimental and computed relative energies: The difference between experimental results and calculations, which only concerns the relative stability of the most stable homo- and heterochiral forms of $(\mathrm{Ser})_2\mathrm{H}^+$, **I_LL** and **II_DL**, is somewhat larger at about 5 kJ mol^{-1} (Table 3) than expected. For this reason potential error sources both in experiment and theory are considered in detail.

In the experiments, the most typical source of error of the kinetic method, that due to entropy effects, can be discounted here on two counts. First, experiments performed under different excitation conditions, for example, metastable and CID, result in ion ratios close to statistical. Second, the theoretical calculations clearly show no difference in entropy contributions among the isomeric structures. The magnitude of errors due to reproducibility and isotope effects were considered (see above). A further potential error source arises if the homochiral dimer is formed not in the most stable, but in a higher lying isomeric form (like **III_LL**).

This is certainly a possibility, but it seems fairly unlikely, especially as hydrogen-bonded systems usually isomerize fairly easily and such complications have rarely been reported. Probably the best argument supporting that the results reported here are very close to the "true" energy difference between the homo- and heterochiral dimers comes from ion abundances observed in single-stage mass spectra. It has been reported in electrospray spectra that the relative abundance of homo- and heterochiral dimers is close to statistical,[5] that is, there is no large stability difference between them. These results were reproduced in the present work as well, both with ES and LSIMS ionization. Although it is difficult to translate ion-abundance ratios in ES spectra into energy differences, these observations suggest energy difference between homo- and heterochiral dimers of less than 1–2 kJ mol^{-1}. This corroborates the evidence obtained with the kinetic method that the energy difference between homo- and heterochiral protonated serine dimers is very small.

The ab initio relative energies of the present study, obtained with the focal-point approach and presented in the last row of Table 2, clearly establish that only three isomers need be considered to understand the conformational preferences of chiral (Ser)$_2$H$^+$ relevant to the MS experiment. Among the homochiral conformers, **I** is by far the most stable, and the relative energy of the second most stable homochiral conformer **III_LL** is +8 kJ mol^{-1}; thus, **III_LL** and all homochiral isomers of higher energy can be omitted from further discussion. Among the heterochiral conformers, **II** and **VA** have very similar energies of about 5–7 kJ mol^{-1}. The next heterochiral conformer **I_DL** has a relative energy of more than 16 kJ mol^{-1}. Therefore, it is unnecessary to further consider **I_DL** and all heterochiral conformers of higher energy. These are the arguments for the choice of conformers for the more detailed computations, results of which are presented in Table 3. These relative energies are the final results of this study. The relative energy predictions should be viewed with an overall conservative error estimate of 4 kJ mol^{-1}, that is, 2σ for the present calculations. In summary, these ab initio results seem to suggest a small preference for homochirality for (Ser)$_2$H$^+$.

By far the greatest difficulty in predicting relative energy is for the heterochiral isomer **II_DL**. For conformer **II** not only the δMP2 increment is sizable (−21–22 kJ mol^{-1}), while the next largest increment is +9 kJ mol^{-1} for **IVB_LL**, but also the CCSD(T)−MP2 energy increment is relatively large at 3 kJ mol^{-1} (Table 2), while for most other conformers the CCSD(T)-MP2 energy increment is less than 1 kJ mol^{-1}. The main reason for the unusually large energy increments is the dissimilar, salt-bridge structure of conformer **II** compared to all the other conformers of (Ser)$_2$H$^+$, most importantly to **I**. It seems unlikely that when more extensive and expensive calculations, for example, at the CCSD(T) level, become feasible, the relative energy of the conformers other than **II_DL** will change noticeably. Nevertheless, the relative energy of **II_DL**, the most favorable heterochiral structure, might change somewhat, bridging the gap between experiment and theory.

Note that while the focal-point approach, with its limited CCSD(T) calculations, underestimates the stability of the salt-bridge structures, DFT(B3LYP) may overestimate it. To wit, when the 3–21G, 6-31+G*, and 6–311G* basis sets are employed, the single-point DFT(B3LYP) energy calculations suggest that **II_DL** is the global minimum on the PES of (Ser)$_2$H$^+$.

Conclusion

A combined theoretical and experimental study was presented on the structure, energetics, and chiral properties of the protonated serine dimer. The energy difference between the most stable homo- and heterochiral forms of (Ser)$_2$H$^+$ was accurately measured by tandem mass spectrometry and the kinetic method of Cooks to be 0.2 ± 0.2 kJ mol^{-1} in favor of the homochiral form. This result implies that the pronounced homochiral preference observed in the protonated octamer (Ser)$_8$H$^+$ does not seem to be derived from the structure of the presumed dimeric unit(s), as the homochiral dimer is certainly not more stable than the heterochiral form. The accompanying theoretical study gave detailed structural information on the various isomeric forms of (Ser)$_2$H$^+$. The most stable homochiral isomer **I_LL** is stabilized by two H-bonds (see Figures 1 and 2) and is 8 ± 4 kJ mol^{-1} more stable than any other homochiral isomer. The most stable heterochiral isomer **II_DL** has completely different structural features, and is characterized by a salt-bridge structure (see Figures 1 and 2). The schematic energy diagram presented in Figure 3 also illustrates that the various hydrogen-bonded structures have significantly different energies in their homo- and heterochiral forms.

Existence of salt-bridge structures in the gas phase has long been considered.[45–48] In most cases they are thought to occur when both a very basic (usually arginine) and a strongly acidic functional group is present in a molecule. As the present study illustrates, salt-bridge structures may be formed even in the absence of exceptionally basic or acidic groups, and in quite small species, such as a protonated amino acid dimer. We believe the present systematic theoretical study to be the most accurate published up to now on a salt-bridge structure of a small biomolecule, and has important methodological consequences for studies on larger species.

Obtaining qualitative structural results, that is, information of the preferred bonding arrangements in (Ser)$_2$H$^+$, does not require high levels of electronic-structure theory. Most importantly, the geometry optimizations performed in this study at both the DFT(B3LYP) and MP2 levels indicate that even the dimer of protonated serine can have either a homo- or a heterochiral salt-bridge conformer. The focal-point (FPA) results of this study indicate, however, that fairly elaborate computations are needed to determine the energy order of the conformers of (Ser)$_2$H$^+$. Most of the relative energies obtained at the RHF level are inadequate, not only when small basis sets such as 3-21G and 6-31G* are employed in the calculations, but also at the complete basis-set limit. RHF theory fails especially badly for the relative energy of the salt-bridge structure **II**. Elaborate coupled-cluster calculations result in comparatively small changes in relative energies of the conformers, and the largest effect, as expected, is for the salt-bridge form, the relative energy of which is increased. Inclusion of core correlation has a negligible effect on the final energies. Zero-point energy corrections change the energy order by 1–2 $\mathrm{kJ\,mol^{-1}}$, and thus this effect cannot be neglected when accurate relative energies are to be obtained from electronic structure calculations. DFT(B3LYP) calculations, with moderate triple-zeta-quality basis sets including diffuse and polarization functions, yield quite accurate relative energies. This indicates that the B3LYP/6-311++G** level of theory can be used efficiently for investigation of larger protonated serine clusters, especially $(\mathrm{Ser})_8\mathrm{H}^+$.

Acknowledgements

The work described received minor support from the Hungarian Scientific Research Fund (OTKA), through grants T043538 and T047185, and was also supported by the QLK2-CT-2002-90436 project of the European Union for Center of Excellence in Biomolecular Chemistry.

[1] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **1989**, *246*, 64–71.
[2] R. G. Cooks, D. X. Zhang, K. J. Koch, F. C. Gozzo, M. N. Eberlin, *Anal. Chem.* **2001**, *73*, 3646–3655.
[3] A. E. Counterman, D. E. Clemmer, *J. Phys. Chem. B* **2001**, *105*, 8092–8096.
[4] R. Hodyss, R. R. Julian, J. L. Beauchamp, *Chirality* **2001**, *13*, 703–706.
[5] R. R. Julian, R. Hodyss, B. Kinnear, M. F. Jarrold, J. L. Beauchamp, *J. Phys. Chem. B* **2002**, *106*, 1219–1228.
[6] K. J. Koch, F. C. Gozzo, D. X. Zhang, M. N. Eberlin, R. G. Cooks, *Chem. Commun.* **2001**, 1854–1855.
[7] K. J. Koch, F. C. Gozzo, S. C. Nanita, Z. Takats, M. N. Eberlin, R. G. Cooks, *Angew. Chem.* **2002**, *114*, 1797–1800; *Angew. Chem. Int. Ed.* **2002**, *41*, 1721–1724.
[8] C. A. Schalley, P. Weis, *Int. J. Mass Spectrom.* **2002**, *221*, 9–19.
[9] Z. Takats, S. C. Nanita, R. G. Cooks, G. Schlosser, K. Vékey, *Anal. Chem.* **2003**, *75*, 1514–1523.
[10] Z. Takats, S. C. Nanita, R. G. Cooks, *Angew. Chem.* **2003**, *115*, 3645–3647; *Angew. Chem. Int. Ed.* **2003**, *42*, 3521–3523.
[11] Z. Takats, S. C. Nanita, G. Schlosser, K. Vékey, R. G. Cooks, *Anal. Chem.* **2003**, *75*, 6147–6154.
[12] O. Geller, C. Lifshitz, *Int. J. Mass Spectrom.* **2003**, *227*, 77–85.
[13] K. Vékey, G. Czira, *Rapid Commun. Mass Spectrom.* **1995**, *9*, 783–787.
[14] R. G. Cooks, J. T. Koskinen, P. D. Thomas, *J. Mass Spectrom.* **1999**, *34*, 85–92.
[15] K. Vékey, G. Czira, *Anal. Chem.* **1997**, *69*, 1700–1705.
[16] A. G. Császár, W. D. Allen, Y. Yamaguchi, H. F. Schaefer III in *Computational Molecular Spectroscopy, Vol. III* (Eds.: P. Jensen, P. R. Bunker), Wiley, Chichester, **2000**, pp. 15–68.
[17] A. L. L. East, W. D. Allen, A. G. Császár in *Structures and Conformations of Non-Rigid Molecules* (Eds.: J. Laane, M. Dakkouri, B. van der Veken, H. Oberhammer), Kluwer, Dordrecht, **1993**, pp. 343–373.
[18] A. G. Császár, W. D. Allen, H. F. Schaefer III, *J. Chem. Phys.* **1998**, *108*, 9751–9764.
[19] A. G. Császár, A. Perczel, *Prog. Biophys. Mol. Biol.* **1999**, *71*, 243–309.
[20] E. Czinki, A. G. Császár, *Chem. Eur. J.* **2003**, *9*, 1008–1019.
[21] C. C. J. Roothaan, *Rev. Mod. Phys.* **1951**, *23*, 69–89.
[22] C. C. J. Roothaan, *Rev. Mod. Phys.* **1960**, *32*, 179–185.
[23] P. Pulay, *Mol. Phys.* **1969**, *17*, 197-.
[24] W. J. Hehre, L. Radom, P. von R. Schleyer, J. A. Pople, *Ab Initio Molecular Orbital Theory*, Wiley, New York, **1986**.
[25] M. J. Frisch, J. A. Pople, J. S. Binkley, *J. Chem. Phys.* **1984**, *80*, 3265–3269.
[26] T. Clark, J. Chandrasekhar, G. W. Spitznagel, P. von R. Schleyer, *J. Comput. Chem.* **1983**, *4*, 294–301.
[27] R. Krishnan, J. S. Binkley, R. Seeger, J. A. Pople, *J. Chem. Phys.* **1980**, *72*, 650–654.
[28] P. A. M. Dirac, *Proc. Cambridge Phil. Soc.* **1930**, *26*, 376.
[29] A. D. Becke, *Phys. Rev. A* **1988**, *38*, 3098–3100.
[30] A. D. Becke, *J. Chem. Phys.* **1993**, *98*, 5648–5652.
[31] C. T. Lee, W. T. Yang, R. G. Parr, *Phys. Rev. B* **1988**, *37*, 785–789.
[32] S. H. Vosko, L. Wilk, M. Nusair, *Can. J. Phys.* **1980**, *58*, 1200–1211.
[33] M. J. Frisch, J. A. Pople, J. E. Delbene, *J. Phys. Chem.* **1985**, *89*, 3664–3669.
[34] A. G. Császár, *J. Am. Chem. Soc.* **1992**, *114*, 9568–9575.
[35] A. G. Császár, *J. Mol. Struct.* **1995**, *346*, 141–152.
[36] A. G. Császár, *J. Phys. Chem.* **1996**, *100*, 3541–3551.
[37] T. H. Dunning, *J. Chem. Phys.* **1989**, *90*, 1007–1023.
[38] R. A. Kendall, T. H. Dunning, R. J. Harrison, *J. Chem. Phys.* **1992**, *96*, 6796–6806.
[39] D. Feller, *J. Chem. Phys.* **1992**, *96*, 6104–6114.
[40] T. Helgaker, W. Klopper, H. Koch, J. Noga, *J. Chem. Phys.* **1997**, *106*, 9639–9646.
[41] A. Schafer, H. Horn, R. Ahlrichs, *J. Chem. Phys.* **1992**, *97*, 2571–2577.
[42] Gaussian 03, Revision C.02, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople, Gaussian, Inc., Wallingford, CT, **2004**.
[43] P.Q.S., PQS version 3.1, 2013 Green Acres Road, Fayetteville, Arkansas 72703.
[44] J. F. Stanton, J. Gauss, J. D. Watts, W. J. Lauderdale, R. J. Bartlett, *Int. J. Quantum Chem.* **1992**, 879–894.
[45] R. A. Jockusch, W. D. Price, E. R. Williams, *J. Phys. Chem. A* **1999**, *103*, 9266–9274.
[46] S. W. Lee, H. S. Kim, J. L. Beauchamp, *J. Am. Chem. Soc.* **1998**, *120*, 3188–3195.
[47] J. Gonzalez, V. Besada, H. Garay, O. Reyes, G. Padron, Y. Tambara, T. Takao, Y. Shimonishi, *J. Mass Spectrom.* **1996**, *31*, 150–158.
[48] P. D. Schnier, W. D. Price, R. A. Jockusch, E. R. Williams, *J. Am. Chem. Soc.* **1996**, *118*, 7178–7189.
[49] R. G. Cooks, P. S. H. Wong, *Acc. Chem. Res.* **1998**, *31*, 379–386.
[50] K. M. Ervin, P. B. Armentrout, *J. Mass Spectrom.* **2004**, *39*, 1004–1015.
[51] L. Drahos, C. Peltz, K. Vékey, *J. Mass Spectrom.* **2004**, *39*, 1016–1024.
[52] G. Bouchoux, M. Sablier, F. Berruyer-Penaud, *J. Mass Spectrom.* **2004**, *39*, 986–997.

Received: November 30, 2004
Revised: April 16, 2005