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JMS Letters

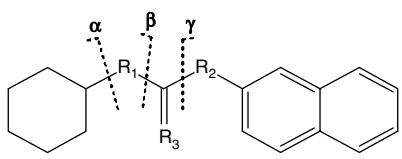
Dear Sir,

The collisional behavior of ESI-generated protonated molecules of some carbamate FAAH inhibitors isosteres and its relationships with biological activity

Recently, we reported some studies meant to rationalize the mode of action of a class of compounds acting as fatty acid amide hydrolase (FAAH) inhibitors[1–3] and characterized by an *N*-alkylcarbamic acid *O*-aryl ester structure.[4,5] The enzyme inactivation is considered to take place through two distinct and consecutive processes, reported in Scheme 1, i.e. formation of a noncovalent complex (recognition step) and a nucleophilic attack by Ser241[6,7] on the carbamate, leading to an irreversible inactivation of the enzyme by carbamoylation (inactivation step). The recognition step, related to the stereoelectronic complementarity between the inhibitors and the enzyme active site, was studied by molecular modeling,[5,8–10] which, however, could not completely account for the inactivation step. As a part of a wider program of application of mass spectrometry (MS) to structure–activity relationships (SARs) studies we hypothesized that the inactivation reaction could be related to the propensity of the C(O)–O bond to cleavage. We started therefore an investigation on collisional experiments on the ESI-generated protonated molecules. Interestingly, the energetics of this process, obtained by breakdown curves,[11] showed a linear correlation between the propensity of the C(O)–O bond to be cleaved under collisional condition and the IC_{50} (half maximal inhibitory concentration of FAAH hydrolysis of [^{3}H]AEA in rat cortical membranes) values for the examined compounds.[12] In a further study the same approach was applied to a series of biphenyl-3-ylcarbamates with electron-withdrawing or electron-donating substituents on the distal or proximal phenyl ring.[13] The results we obtained warned us that caution must be taken when trying to extend previous results to a more complex series of structures, but in general supported the usefulness of MS in SARs, at least when reactivity factors contribute to the biological activity.

During the exploration of the series of the *N*-alkylcarbamic acid *O*-aryl esters a small number of putative bioisosteres (**1–6**) (Fig. 1) were synthesized to establish the role of the carbamate function on the observed FAAH inhibitory behavior.[5] The fact that the compounds **1–6** did not inhibit FAAH[5] led us to investigate their MS behavior to verify whether substantial differences in C(O)–O bond cleavage existed in comparison with the previously studied active *O*-arylcarbamate. Compounds **1–6** were analyzed in ESI conditions and the related $[M + H]^+$ were employed for collisional experiments.

ESI experiments were performed using an LCQ Deca instrument (Thermo, San José, CA, USA) operating in positive ion mode. Compounds **1–6** were dissolved in CH_3OH and their 10^{-6} M solutions were directly infused into the ESI source. Spray voltage, capillary voltage, and entrance capillary temperature were 4 kV, 8 V, and 220 °C, respectively. MS/MS and MS^3 experiments were obtained by resonance excitation[14] of the preselected ion. He pressure inside the trap was kept constant (2.8×10^{-5} Torr directly read by ion gauge, in absence of the N_2 stream). The isolation width was set at 2 mass units and the scan rate was 0.5 s^{-1}.



1: R_1=NH, R_2=CH_2, R_3=O
2: R_1= R_2= NH, R_3=O
3: R_1=NH, R_2=S, R_3=O
4: R_1= R_2=NH, R_3=S
5: R_1=NH, R_2=O, R_3=S
6: R_1=NH, R_2= R_3=S

Figure 1. Structures of compounds **1–6**.

Table 1. Relative abundances of $[M+H]^+$, $[M+Na]^+$, and $[2M+Na]^+$ ions in the ESI spectra of **1–6**

	$[M + H]^+$	$[M + Na]^+$	$[2M + Na]^+$
1	79	95	100
2	100	30	95
3	72	100	65
4	100	22	18
5	55	71	100
6	85	42	100

All the examined compounds gave rise in ESI conditions to abundant protonated molecules, together with $[M + Na]^+$ and $[2M + Na]^+$ species (Table 1).

In the previous investigations on the collisional behavior of $[M + H]^+$ of carbamates,[12,13] the most favored decomposition route was that related to the C(O)–O bond cleavage (as in Fig. 1, cleavage γ), whereas this was not always true for **1–6**, possibly because of the different bond strength of the groups replacing the carbamic one. Thus, the $[M + H]^+$ species of compound **1** leads to the product ion spectra reported in Fig. 2. The primary fragmentation processes might be rationalized by admitting an initial protonation of the nitrogen atom, but it must be taken into account that the protonation can more reasonably take place on the R_3 group, which, because of the electron delocalization, represents the most basic site of the amide (or thioamide) moiety. The protonation on R_3 is confirmed by the high abundance of $[M + H]^+$ ion for **2** and **4**; in these cases the negative charge on the R_3 group is reinforced by both the nitrogen-donating groups. The most abundant species, at *m/z* 186, is due to the cleavage of the cyclohexyl–NH bond with H-rearrangement (Scheme 2, cleavage α). The ion at *m/z* 141 originates from the C(OH)–CH_2 bond cleavage, whereas that of the NH–C(OH) bond leads both to ions at *m/z* 169 and *m/z* 100. MS^3 experiments show that the ion at *m/z* 186 decomposes, through NH_3 and CH_3NO losses, leading to ions at *m/z* 169 and 141, respectively. The latter species is also produced by the acylium ion at *m/z* 169 through a decarbonylation process.

The observed fragmentation pathways can also be explained by an isomerization of $[M + H]^+$ ions into a proton-bound dimer associating cyclohexylamine and naphtylketene, giving account for the formation of protonated cyclohexylamine (*m/z* 100) and the acylium cation at *m/z* 169. Analogous isomerizations can be invoked for the protonated molecules of the other compounds under investigation.

It is to be noted that all the observed fragmentation pathways lead to even electron product ions, in agreement with the even electron rule.[15] Thus, for compound **1** the most favored decomposition route is no longer that observed in the carbamate derivatives (as in Fig. 1, cleavage γ), but rather that due to the cleavage of cyclohexyl–NH bond (as in Fig. 1, cleavage α). This behavior might be explained by the weaker CH–NH bond strength relatively to that of the C(OH)–CH_2. Of course this hypothesis would have to be confirmed by theoretical calculations, but they are not available in our lab.

In the case of $[M + H]^+$ of compound **2** the most abundant collisionally induced fragmentation product was that due to the C(OH)–NH bond cleavage with 2H rearrangement (Fig. 1, cleavage γ; Fig. 3; Scheme 3,

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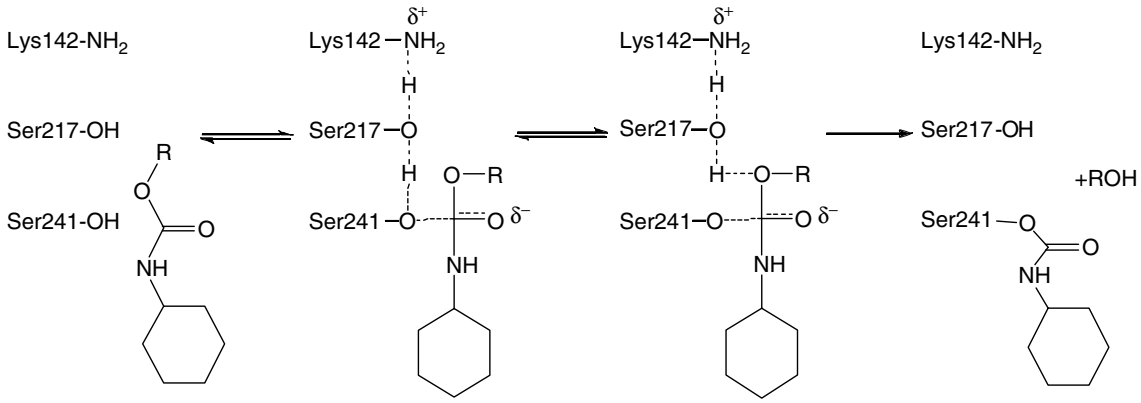
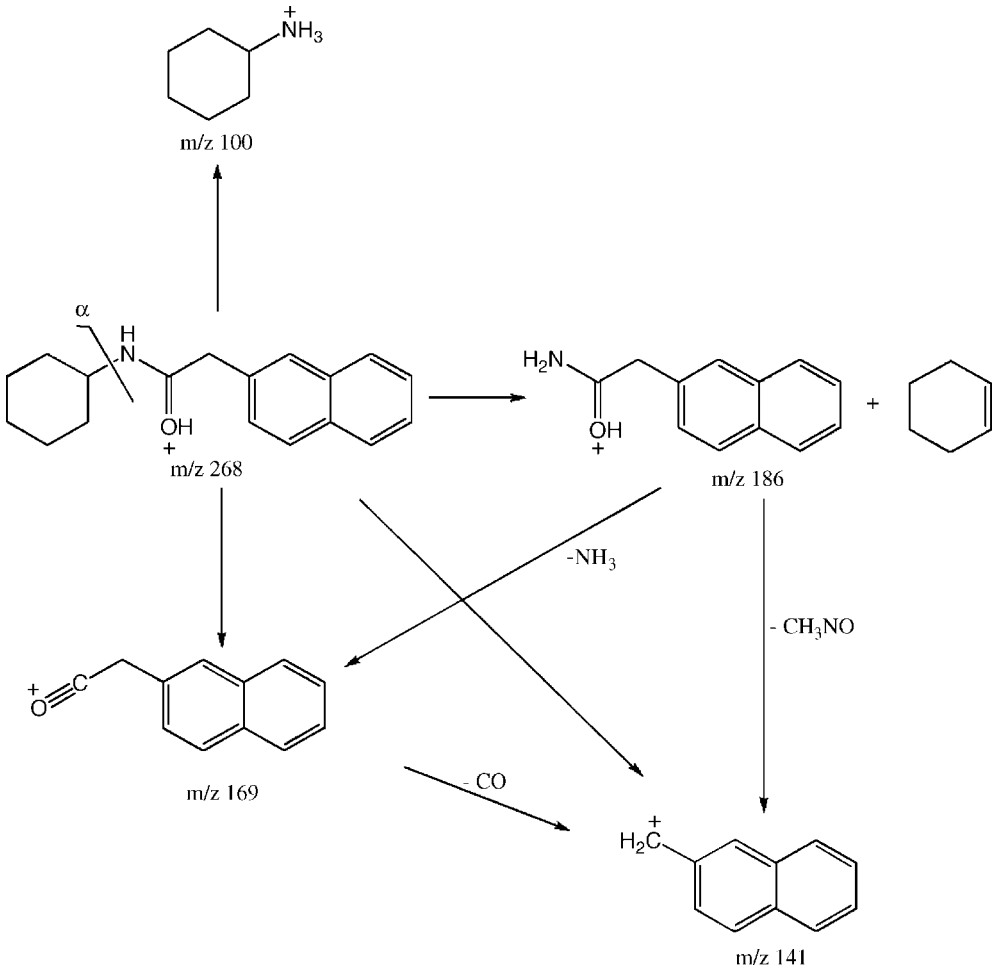


Scheme 1. The two distinct and consecutive processes leading to the enzyme inactivation.



Scheme 2. Collisionally induced decomposition pattern of protonated compound **1**.

cleavage γ), though also cleavage α takes place. Also for compound **2** the formation of a proton-bound dimer allows to rationalize some of the observed decomposition pathways. Thus the proton-bound dimer constituted by cyclohexylamine/naphthyl–N=C=O justifies the formation of the cyclohexyl–NH_3^+ ion (*m/z* 100), while the dimer cyclohexyl–N=C=O/naphthyl–NH_2 gives account for the formation of the naphthyl–NH_3^+ species (*m/z* 144).

The $[M + H]^+$ of **3** showed the most abundant fragment at *m/z* 161, corresponding to naphthyl–SH_2^+. The fragment *m/z* 204 as a result of cyclohexene loss was also present, even if in low abundance (Table 2). This seems to suggest that protonation took place on the sulfur atom. It should be considered, however, that different intramolecular proton-bridged forms can be present, as those shown in Fig. 4, which could explain the observed behavior.

Compound **4** showed the formation of naphthyl–NH_3^+ at *m/z* 144 (Table 2) and a further decomposition product at *m/z* 188, because of the cleavage of the NH–C(SH) bond (Fig. 1, cleavage β) formally corresponding to naphthyl–NH–CH=SH^+ ion.

An interesting decomposition was observed in the case of **5**, whose collisional spectra of $[M + H]^+$ ions showed the formation of two ions

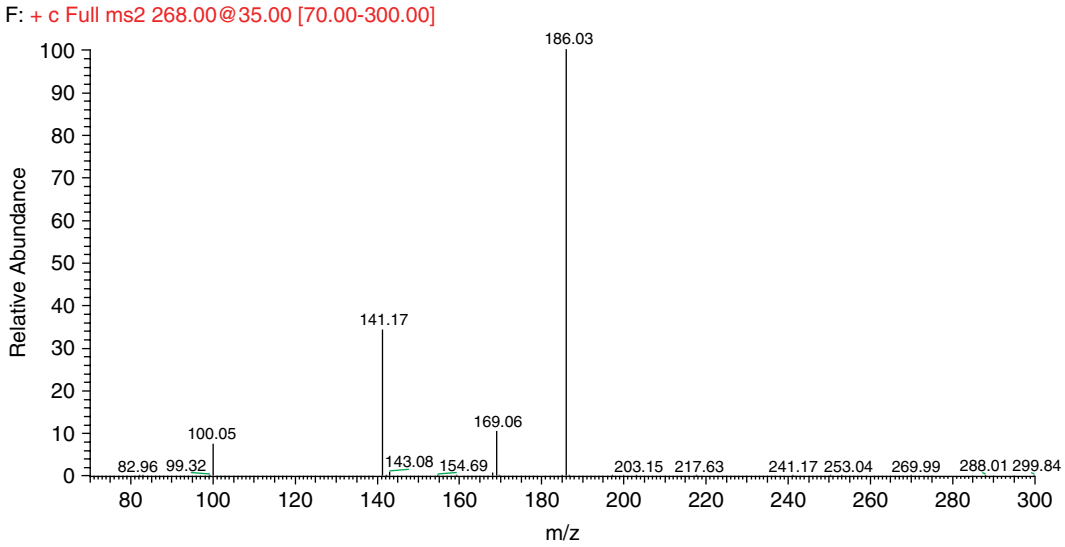


Figure 2. MS^2 spectrum of $[M + H]^+$ ions of compound **1** (*m/z* 268).

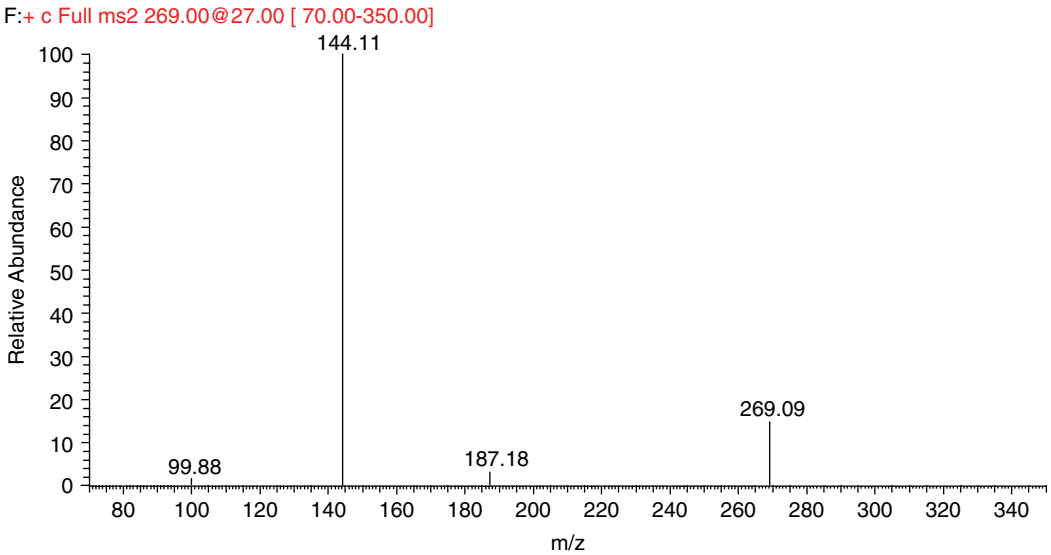


Figure 3. MS^2 spectrum of ion at *m/z* 269 related to $[M + H]^+$ ions of compound **2**.

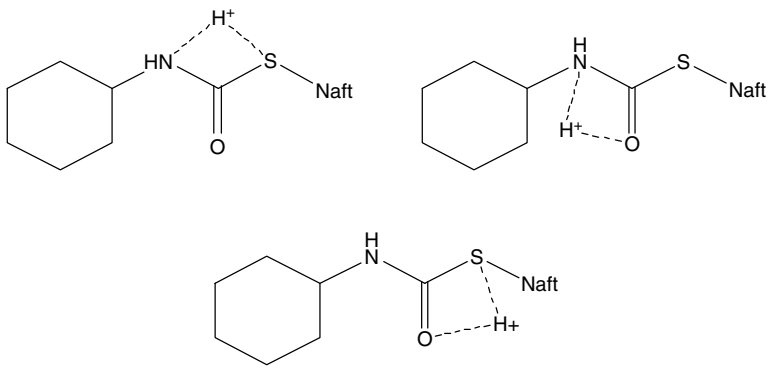
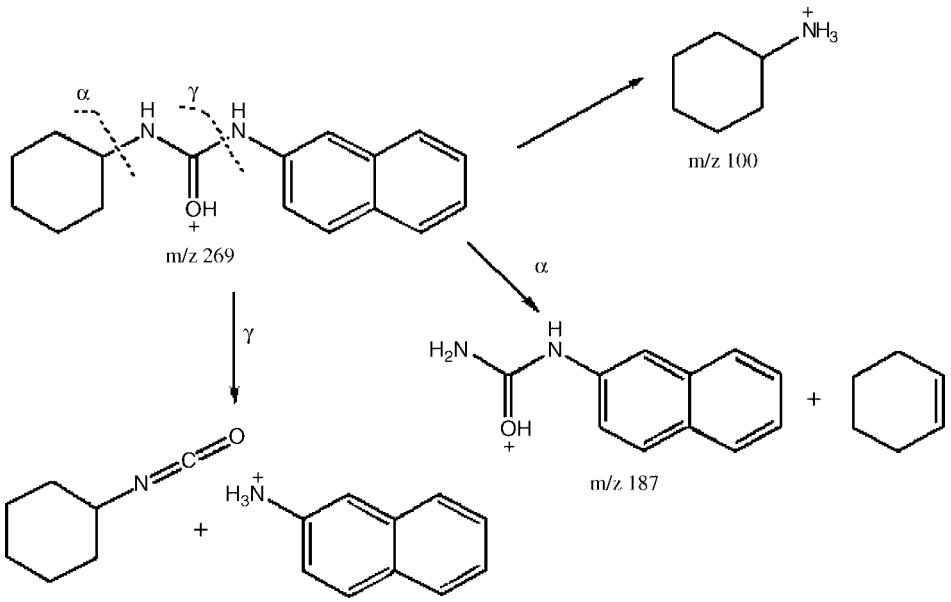


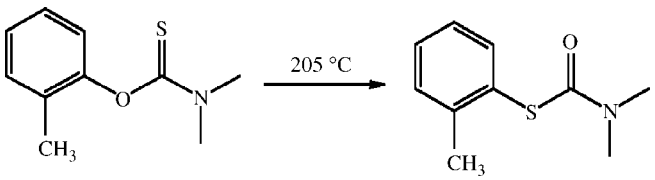
Figure 4. Possible intramolecular H^+ bridged structures of $[MH]^+$ of **3**.



Scheme 3. Collisionally induced decomposition pattern of protonated compound **2**.

Table 2. Collisionally generated fragmentation products of $[MH]^+$ of **1–6**

	$[M+H]^+$	$[MH-C_6H_{10}]^+$	Naphthyl–R_2–CR_3^+	Naphthyl–R_2^+	Others
1	268	186	169	141	–
2	269	187	–	144 (H rearr.)	–
3	286	204	–	161 (2H rearr.)	–
4	285	–	188 (H rearr.)	144 (H rearr.)	–
5	286	–	–	145 (H rearr.)	161
6	302	–	–	161 (2H rearr.)	142



Scheme 4. Example of the Newman-Kwart rearrangement, converting phenols to thiophenols.

at *m/z* 145 and *m/z* 161. In the case of the former ion the structure naphthyl–OH_2^+ could be easily assigned, while for the latter the ion naphthyl–SH_2^+ could be proposed; this ion would originate from a Newman–Kwart rearrangement (converting phenols to thiophenols, as shown by the example reported in Scheme 4)[16] induced by a protonation reaction leading to compound **3**. In fact, the most abundant ion from **3** is the naphthyl–SH_2^+ one, which is also observed in the spectrum of **6**, together with an ion at *m/z* 142, because of the cleavage of the C(S)–S bond, with charge localization on the cyclohexyl-containing species.

The above results indicate that compounds **1–6** behave quite differently from what was previously observed in the case of *N*-alkylcarbamic acid *O*-aryl esters. The naphthyl–R_2H^+ ion is produced by collision of $[M+H]^+$ in the present case as well, but many concurrent decomposition pathways are present, mainly because of protonation sites and bond strengths different from those of the carbamates. The lack of FAAH inhibitory activity of compounds **1–6** may thus be explained also on the basis of the MS data reported here, clearly indicating a different reactivity of the putative bioisosteres in comparison with that of the parent carbamic acid ester FAAH inhibitors.

Yours,

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