Mass spectrometric analysis of epimeric 3α/3β functionalized steroids

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Abstract

Tandem mass spectrometry (MS/MS) using an atmospheric pressure chemical ionization (APCI) source in positive mode (+) reveals different intensity ratios of two fragment peaks of the epimeric cholestane derivatives synthesized from 3α- or 3β-cholestanol. The investigation of the fragmentation pattern obtained from their full scan and/or collision induced dissociation (CID) spectra in the case of the cholestane skeleton together with the respective electronic ionization (EI) spectra supports a distinction of the epimers.

Keywords: APCI, EI, tandem MS, epimer discrimination

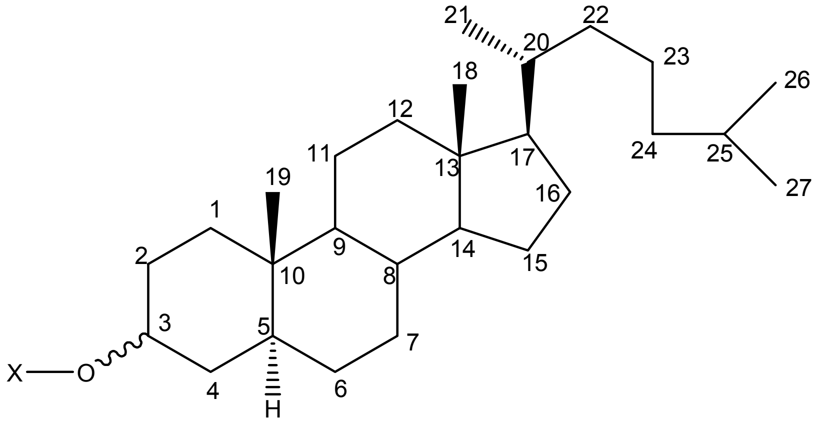
Introduction

Hemisynthetic derivatives of cholestane **1-10** (Figure 1) have been our subject of interest as model precursors in an ongoing study seeking novel strategies for the functionalization of unreactive carbons of terpenes in the presence of ferric catalysts, by means of Patin chemistry.[1] For this purpose, groups containing *O*-, *N*- and *S*-heteroatoms were introduced at the C-3 position in cholestanol **1** and **2**.[2a] These hemisynthetic cholestane derivatives could potentially be useful since the insertion of these functional groups, which are not introduced by biosynthetic processes, result in new biological activities.[2b] In this context, a fragmentation study for structure elucidation and determination of the influence of α- and β- configurations of the substituents at C-3 was performed using APCI and EI. The ^{13}C NMR spectral assignments of the functionalized cholestane 3α-/3β-epimers of **1-10** based on the common cholestane skeleton were previously discussed.[3]

The distinction of epimers on the exclusive basis of their mass spectra is usually difficult, especially in the case of steroids, even when an informative technique such as electron ionization mass spectrometry is used. The analysis of cholestane derivatives by APCI has received some attention in the literature in the recent years.[4-6] However, reliable commercial MS instruments possessing the ion trap technique that has multi-stage MS (MS^n) capability, provide a new and very useful analytical capacity by MS.

Usually, this kind of study is performed by NMR, however, ^1H NMR, with a detection threshold of about 5×10^{-9} mol, is several orders of magnitude less sensitive than MS, which has a detection threshold of 1×10^{-12} mol.[7]

This communication relates to the potential use of tandem mass spectrometry (MS^n) with an APCI source in the positive ion mode as a complementary tool for the distinction between various epimers of several cholestane derivatives. The resulting discrimination will provide distinguishable mass spectra for each epimer that can be further used to perform *on-line* identifications of each epimer in mixtures not completely resolved chromatographically, without time-consuming isolation.



1 α (X = H)

2 β (X = H)

3 α (X = $C(O)C_6H_5$)

4 β (X = $C(O)C_6H_5$)

5 α (X = $C(S)SCH_3$)

6 β (X = $C(S)SCH_3$)

7 α (X = $C(S)C_6H_5$)

8 β (X = $C(S)C_6H_5$)

9 α (X = $C(S)N(C_2H_5)_2$)

10 β (X = $C(S)N(C_2H_5)_2$)

Figure 1. Structures of $3\alpha/3\beta$ functionalized cholestane derivatives **1-10**.

Experimental Section

Synthesis of cholestane derivatives. Cholestan-3β-ol **2** was synthesized following a described procedure.[8] The epimerization to cholestan-3α-ol **1** (hydroxyl group in the axial position)[9] was accomplished in two steps starting from tosylation of **2**,[10] followed by reaction with KNO_2 in dry DMF. All the derivatives **3-10** were prepared following typical procedures described in the literature or by adaptation of methods already reported.[11-15] The benzoylated derivatives **3** and **4** were prepared from cholestan-3-ol **1** and **2** by the use of benzoyl chloride in dry pyridine.[11] Thiobenzoates **8** and **9** were prepared from benzoates **3** and **4** using Lawesson's reagent.[12] Xanthates **5** and **6** were prepared from cholestan-3-ol **1** and **2** using sodium hydride in dry THF in the presence of a catalytic amount of imidazole, followed by treatment with carbon disulfide and methyl iodide.[13,14] Thiocarbamates **9** and **10** were prepared from xanthates **5** and **6** by reaction with diethylamine in petroleum ether.[15]

Mass spectrometry. APCI (+) mass spectra were acquired using a Bruker Esquire 3000 (Bruker Daltonics, Bremen, Germany) ion trap instrument with an APCI ionization source in the positive ion mode. The vaporizer temperature was 300 ºC, the corona discharge needle current was 4.0 mA and the heated capillary was maintained at 300 ºC with a voltage of 4000 V. The nitrogen sheath gas pressure and auxiliary flow rate were 30 psi and 7 L/min, respectively. The following cone and trap voltages were applied depending on the target mass for each pair of epimers: **1** and **2** (31 Volt for both); **3** and **4** (35.3 and 34.9 Volt); **5** and **6** (34.8 and 34.4 Volt); **7** and **8** (36 and 35.5 Volt); and **9** and **10** (35.8 and 35.3 Volt). Product ion spectra of the $[M+H]^+$ precursor ion were obtained by CID using helium as the collision gas, with a range of fragmentation energies between 0.60 and 1.00 V and an ion isolation width of 1 a.m.u. The APCI spectra were obtained over the range of *m/z* 50-800.

EI(+) mass spectra were acquired with a Kratos MS25RF magnetic sector instrument with a 70 eV electron ionization ion source kept at 200 ºC. The compounds were introduced with a probe which was ballistically heated to 250 ºC. The EI spectra were obtained over the range of *m/z* 35-550.

Results and Discussion

The electron ionization mass spectra of the cholestanes **1** and **2** show significant molecular and $(M\text{-}CH_3)^+$ ions. On the other hand, in the mass spectra of the cholestane derivatives **3-10** those ions were not detected (or detected but with very low intensity for derivative **4**), being the more abundant peaks observed resulted from the fragmentation of molecular ions involving the C-3 substituent. The ions with *m/z* 370 result from the loss of the XOH molecule (Scheme 1).

Data on abundance of this type of fragmentation for cholestane derivatives **1-10** are shown in Table 1. The ion at *m/z* 371 is due to the loss of radical $XO^{\cdot}$. For compounds **1** and **2**, however, this ion might also be derived from thermal dehydrogenation followed by the loss of 15 mass units (-CH_3).

The ion *m/z* 370 may originate from two different sources, in the case of compounds **1** and **2**. It is either the result of the thermal degradation occurring before the electronic ionization or, alternatively, it is produced after the elimination of water, by the molecular ion. Egger[16] reported that thermal dehydration precedes and overlaps with the water elimination after electron ionisation.

Table 1. Relative abundance (% base peak) in EI spectra for cholestane derivatives **1**-**10**

	Relative abundances (% base peak)[a] *m/z*										
Compnd	$M^{+\cdot}$	$(M-CH_3)^+$	371[b]	370	355	316	257	230	216[b]	215	201
1	24	12	8	7	11	4	5	8	10	47	8
2	42	17	11	5	9	3	5	10	15	58	9
3	2	-	-	100	36	30	19	21	24	65	14
4	5	1	-	93	33	32	22	23	29	72	17
5	-	-	1	100	36	50	25	15	26	63	15
6	-	-	6	100	36	57	27	17	26	66	16
7	-	-	1	97	35	50	25	16	24	65	15
8	-	-	3	100	35	52	25	15	23	61	17
9	-	-	-	100	37	56	26	17	27	67	15
10	-	-	-	100	37	59	27	16	27	66	16

[a] Four averaged mass spectra were used to obtain these data
[b] Abundances have been corrected for isotopic contribution
- Abundance less than 1%

From the ion at *m/z* 370 we obtained other ions (Scheme 1) whose fragmentation patterns are very similar to those of the corresponding 2-cholestene.[17, 18]

One of the most common types of fragmentation in C-17 substituted steroids is the loss of this substituent plus 42 mass units.[17] In all compounds studied, this leads to an abundant ion at *m/z* 215 (a_1, Scheme 1) in the corresponding spectra (Table 1). According to Djerassi *et al.*[17] this ion is formed as a result of the cleavage of two bonds, 13-17 and 14-15, simultaneously with the migration of an H atom to the neutral fragment. For Biemann,[18] the ion formed from the homolytic cleavage of the 13-17 and 14-15 bonds, together with the migration of an H atom (mainly from position 14) to the neutral fragment, contributes to 76-79% of this ion intensity. The remaining 21-24% of the a_1 ion results from the loss of the 19-CH_3 group from the ion with *m/z* 230 (d_1). This fragmentation pattern, without the H migration, leads to the ion at *m/z* 216 (b_1, Scheme 1), through a Retro-Diels-Alder (RDA) mechanism.

Further decomposition of the ion b_1 is accompanied by the loss of the C-19 methyl group and results in an ion at *m/z* 201 (c_1).[18]

The formation of fragment ions at *m/z* 355 results from the loss of a methyl group. Djerassi *et al.*[17] indicated that the corresponding peak in the cholestane skeleton at *m/z* 355 is due to the loss of the angular 18- and 19-CH_3 group in a 1:4 ratio and have suggested the structure shown in Scheme 1.

Ions at *m/z* 316 result from the fragmentation of ions at *m/z* 370, through elimination of one XOH molecule from the molecular ion, followed by a RDA mechanism. This mechanism justifies the double bond in position 2,3 instead of 3,4 in the ion with *m/z* 370. This fragmentation pathway is a common feature of Δ^2-unsaturated steroids and was described for the first time by Reed.[16] It is worth noting the cleavage pattern which supports the structures in Scheme 1, since it indicates fragmentations resulting from an ion originated from an 1,2-elimination.

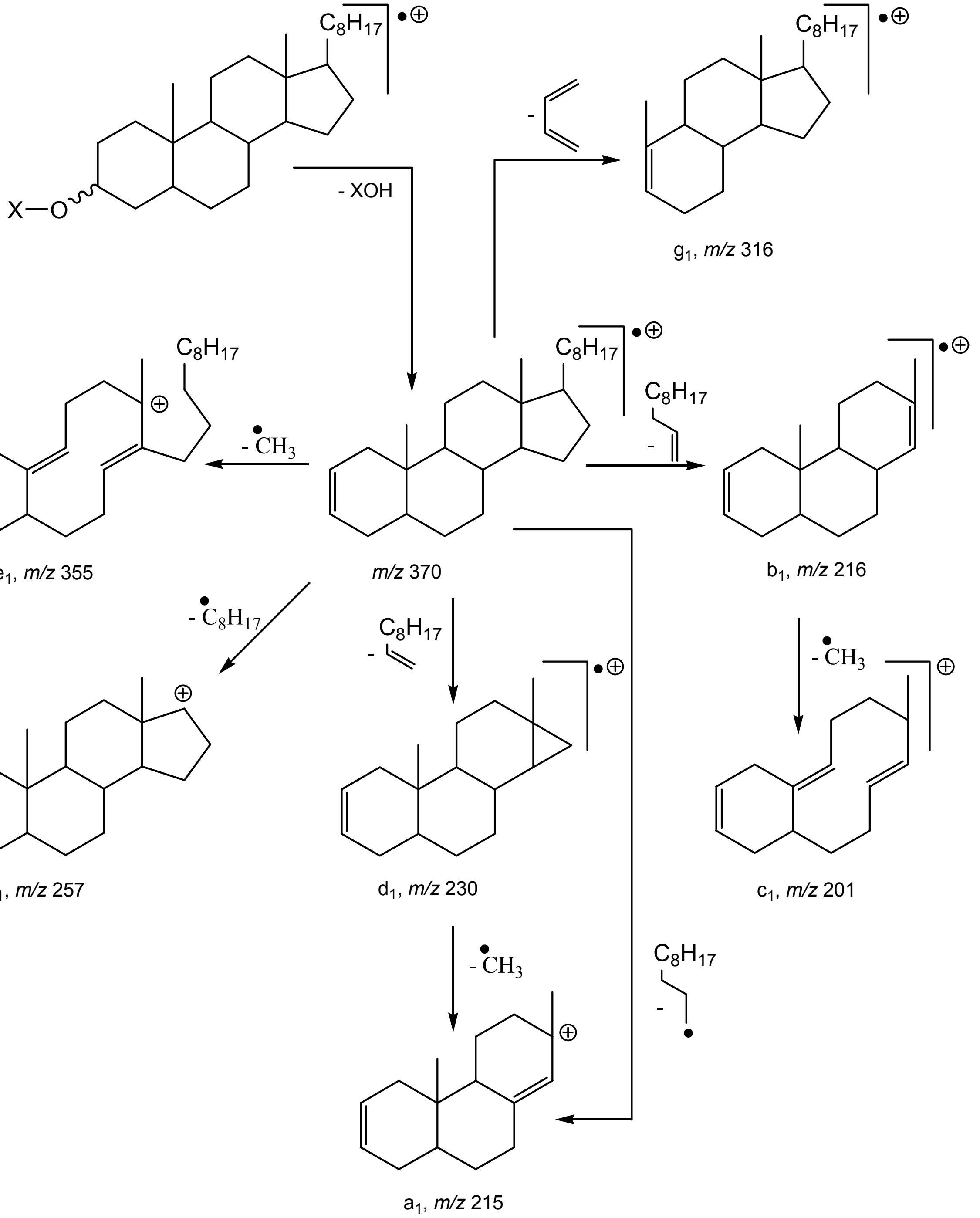
The loss of the substituent at C-17 only results in the ion f_1 at *m/z* 257 with a relative abundance lower that the relative abundance observed for ion a_1.

The EI mass spectra obtained provide structural information, however, the data can not be used to discriminate the α or β position of any of the C-3 substituents in the cholestane backbone. In order to resolve this limitation, all the cholestane derivatives were subsequently ionized by APCI in the positive mode yielding $[M+H]^+$ ions in their mass spectra with minimal fragmentation. Aiming at distinction of the pairs of epimers based in the relative intensities of the corresponding peaks, we should report fragmentation spectra of the pseudomolecular ions (MS^2) and not to the full scan spectra (MS) because this latter fragmentation is not intentionally induced (“in source” CID), and thus it is not possible to select the precursor ion to fragment.

Several measurements performed on the same samples showed results sufficiently reproducible to allow distinction of epimers.

The epimeric pairs of compounds **1** and **2** are readily distinguished through the APCI(+)-MS^2 spectral behaviour since the relative intensities of the ions $[M+H-XH]^+$ and $[M+H-XOH]^+$ at *m/z* 387 and 371, respectively, are of 100:65 for the alcohol **1** and of 56:100 for the alcohol **2** (Table 2), with the $[M+H-XH]^+$ ion being the base peak for alcohol **1** and the $[M+H-XOH]^+$ ion for alcohol **2**, which corresponds, for the latter case, to the loss of water from the $[M+H]^+$ ion.

With the benzoates **3** and **4**, the base peak is the same for both compounds corresponding to the $[M+H-XOH]^+$ ion at *m/z* 371. In the case of the $[M+H]^+$ ion at *m/z* 493, it has a relative intensity lower than 1% for both compounds. Just for the case of the $[M+H-XH]^+$ and $[M+H-XH-H_2O]^+$ ions at *m/z* 387 and 369, respectively, it is possible to observe a clear distinction between the pair of epimers, with a relative intensity of approximately 1:2 between **3** and **4**. The most significant difference is observed in the fragmentation spectrum of the ion at *m/z* 493 (Table 3) that corresponds to the fragment at *m/z* 369, having relative intensities (relative to the base peak at *m/z* 371) of 1:17 between compounds **3** and **4**.



Scheme 1. Fragmentation by EI of cholestane derivatives **1-10.**

Table 2. Relative abundance (% base peak) with the APCI source in the positive ion mode for cholestane derivatives **1-10**

Compound	Relative Abundances (% base peak)			
	$[M+H]^+$	$[M+H-XH]^+$	$[M+H-XOH]^+$	$[M+H-XH-H_2O]^+$
1	6	100	65	-
2	3	56	100	-
3	< 1	10	100	15
4	< 1	22	100	23
5	4	25	100	41
6	16	13	100	32
7	12	37	100	44
8	28	19	100	35
9	8	49	100	67
10	25	40	100	54

Table 3. Relative abundance (% base peak) of most important ions in MS^2 of the $[M+H]^+$ ion for cholestane derivatives **3-10**

	Relative abundances (% base peak)	
Compound	$[M+H-XOH]^+$	$[M+H-XH-H_2O]^+$
3	100	1
4	100	17
5	100	2
6	100	2
7	100	3
8	100	6
9	100	18
10	100	75

For the xanthates **5** and **6**, the relative intensities of the $[M+H]^+$ ion at *m/z* 479 has a ratio of 1:4 relative to the same base peak (Table 2). In addition, the relative intensity of the $[M+H-XH]^+$ ion at *m/z* 387 is approximately 2:1 relative to the same base peak.

The base peak for the thiobenzoates **7** and **8** is the ion at *m/z* 371, which corresponds to the $[M+H-XOH]^+$ ion. The $[M+H]^+$ ion at *m/z* 509 shows an relative intensity of approximately 1:3 for compounds **7** and **8**, respectively. Besides this difference, the fragmentation spectrum of the $[M+H]^+$ ion shows a relative intensity ratio of the $[M+H-XH-H_2O]^+$ ion at *m/z* 369 of 1:2 for **7** and **8**, respectively.

The base peak for the carbamates **9** and **10** corresponds to the ion at *m/z* 371 ($[M+H-XOH]^+$ ion). The relative intensities of the $[M+H]^+$ ions at *m/z* 504 are of approximately 1:3 between compounds **9** and **10**, respectively. Substantially different fragmentation is observed for $[M+H]^+$

ion in this pair of epimers where the $[M+H-XH-H_2O]^+$ ion at *m/z* 369 shows a relative intensity ratio of 1:4 for the α and β derivatives.

Conclusions

The investigated five 3α/3β functionalized cholestane epimer pairs were tentatively distinguished using the APCI(+)-IT-MS^2. The epimeric configuration assignment could only be performed by interpretation of spectra using the APCI technique since it exhibits the diagnostic $[M+H]^+$ ions for assignment of the molecular mass of compounds. In conclusion, different fragmentation patterns with respect to both the quality and the intensity of the fragment ions $[M+H-XH-H_2O]^+$ ions formed from the pairs of epimers, may be considered very sensitive to produce a difference that discriminates the α or β position of epimeric 3α/3β functionalized cholestanes. This occurs probably through a preferred loss of water as the limiting step for the fragmentation. The exception was encountered for compounds **5** and **6**, where it was observed that a relative intensity ratio of 1:X, where $X \geq 2$ for the α conformation relative to the β suggests that the bulky S-CH_3 substituent induces a stereochemical constraint for the elimination of water at the fragmentation step.

Acknowledgements

S.S. Ramos thanks the *Fundação para a Ciência e Tecnologia*, Portugal (SFRH/BD/1237/2000) for granting her a Ph.D. scholarship.

References

1. Byrdwell, W. C. *Lipids* **2001**, *36*, 327.
2. (a) Razzazi-Fazeli, E.; Kleineissen, S.; Luf, W. *J. Chromatogr. A* **2000**, *896*, 321. (b) Iida, T., Ogawa,S., Shiraishi,K., Kakiyama, G., Goto,T., Mano,N.,and Goto, J., *ARKIVOC* **2003,** (viii), 171.
3. Raith, K.; Brenner, C.; Farwanah, H.; Müller, G.; Eder, K.; Neubert, R. H. H. *J. Chromatogr. A* **2005**, *1067*, 207.
4. Sumner, L. W.; Mendes, P.; Dixon, R. A. *Phytochemistry* **2003**, *62*, 817.
5. Patin, H.; Mignani, G. J. *J. Chem. Soc., Chem. Commun.* **1979**, *16*, 685.
6. Ramos, S. S. Ph.D. Thesis, Universidade da Beira Interior, 2006.
7. Ramos, S. S.; Santos, L.; Almeida, P.; Motherwell, W. B.; Costa, M. C. *Magn. Reson. Chem.* **2005**, *43*, 861.
8. Augustine, R. L. *Org. React. Steroid Chem.* **1972**, *18*, 111.
9. Radüchel, B. *Synthesis* **1980**, *4*, 292.

10. King, L. C.; Bigelow, M. J. *J. Am. Chem. Soc.* **1952**, *74*, 3338.
11. Gawronski, J. K.; Reddy, S. M.; Walborsky, H. M. *J. Am. Chem. Soc.* **1987**, *109*, 6726.
12. Jang, D. O.; Song, S. H.; Cho, D. H. *Tetrahedron* **1999**, *55*, 3479.
13. Barton, D. H. R.; Choi, S.-Y. *Tetrahedron Lett.* **1996**, *37*, 2695.
14. Dembele, A.; Deshayes, H.; Pete, J.-P. *Bull. Soc. Chim. Fr.* **1988**, *4*, 671.
15. Barrett, A. G. M.; Prokopiou, P. A.; Barton, D. H. R. *J. Chem. Soc., Perkin Trans. 1* **1981**, 1510.
16. Zaretskii, Z. V. *Mass Spectrometry of* Steroids, John Wiley & Sons., Inc.: New York, 1976, p 1.
17. Brooks C. J. W. *Phil. Trans. R. Soc. Lond. A* **1979**, *293*, 53.
18. Biemann K. *Mass Spectrometry: Applications to Organic Chemistry*; McGraw-Hill: New York, 1962, p 345.