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# Impurity analysis of retinoic acid samples

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**Abstract**—The structure of an impurity contained in samples of *all trans*-retinoic acid was established by means of NMR and MS spectra, and confirmed by X-ray diffraction analysis. The chemical structure of the impurity **2** was found to be strictly correlated to the synthetic procedure employed for the preparation of the retinoic acid samples. Single crystal analysis allowed us to characterise the molecular conformation and the crystal structure of **2**.


Frauds in the drug industry could be unearthed by studying the analytical 'fingerprints' of pharmaceutical substances. Efficient analytical methods are necessary to monitor the chemical composition of pharmaceutical products, to detect the effects of process changes on their quality, and to establish whether a product is now on the market that is essentially the same as that originally approved. The identification of trace impurities represents an important step in establishing drug fingerprints, because the structures of by-products are strictly correlated to the synthetic sequence. Our recent work on the impurity analysis of commercial samples of tamoxifen[1] and toremifene[2] has shown that the structural characterisation of impurities can be used to trace the synthetic history of the sample. We report herein on the identification of an impurity contained in commercial samples of *all trans*-retinoic acid (tretinoin (**1**)).

Tretinoin (**1**) is a vitamin A derivative. The biological significance of liposoluble vitamin A was first recognised nearly one hundred years ago.[3] Since then, there has been continuous research in investigating the functions of retinoids in terms of proliferation, differentiation, and immunomodulation, to understand the mechanism of their therapeutic action.[4] In 1968, a group of researchers began work on modifying the structure of vitamin A to achieve new effective derivatives with lesser side effects.[5] The search led first to *all trans*-retinoic acid. Tretinoin is currently used to treat mild to moderate acne, fine wrinkles, dark spots, or rough skin on the face caused by damaging rays of the sun. It is also employed for acute promyelocytic leukemia that is accompanied by specific gene changes.

In the past few years, several methods for the synthesis of **1** have been developed.[6] The traditional industrial method employs vinyl β-ionol as the starting material as per the BASF patent.[7]

Our samples of *all trans*-retinoic acid (**1**) were prepared by treating vitamin A propionate with $Ag_2O$ in methanolic potassium hydroxide at fixed pH. The resulting retinoic acid was found to be contaminated by an impurity (0.3%, HPLC), which stickily adhered to the main compound. Purification of **1** by fractional crystallisation was a difficult task.

The presence of impurities in commercial drugs is strictly regulated by law.[8] All impurities that are present at a level greater than a certain threshold established according to maximum daily dose of the drug have to be identified.

The samples of contaminated tretinoin were submitted to the LC/MS analysis.[9] The APCI/MS spectrum of

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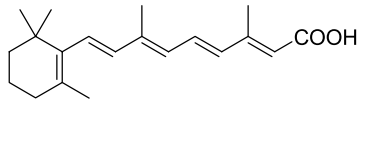


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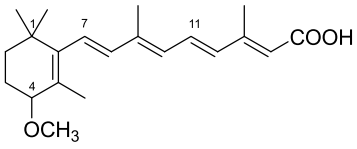



2

the impurity, obtained by the LC/MS analysis, showed the loss of a $CH_3OH$ molecule by fragmentation of the molecular peak. The sample was accurately chromatographed on a silica gel column to isolate the impurity. To the latter structure, compound **2** was assigned on the basis of NMR and MS spectra.[10] The signals at 3.52 ppm ($^1$H NMR spectrum, triplet, 1H) and at 79.34 ppm ($^{13}$C NMR, CH) are characteristic of a methine group linked to an oxygen atom. The signals at 3.39 ppm ($^1$H NMR spectrum, singlet, 3H) and 56.88 ($^{13}$C NMR, $CH_3$) are characteristic of a methoxy group. The presence of two methylene groups was shown by the $^{13}$C DEPT spectrum. The NOE observed between H–C(4) and $CH_3$–C(5) showed that the methine group was spatially nearer to the methyl $CH_3$–C(5). The EI mass spectrum showed the highest peak at *m*/*z* 330, corresponding to the molecular weight of compound **2**. The NMR data and the value of the melting point were in accordance with those found in the literature.[11]

The structure was finally confirmed by X-ray diffraction[12] (Fig. 1).

Several crystallisation procedures of the compound were performed and many crystals were selected for a complete data collection to find a good single crystal matching the structure solution and refinement. However, twinned or very thin platelet-like crystals were always obtained. The poor quality of crystals and some extent of disorder found in the molecular conformation caused the refinement to be rather cumbersome. Nevertheless, this did not significantly influence the results on the conformation and geometry of the molecule.

The compound crystallises in the triclinic system, a centrosymmetric space group, with cell data: $a = 6.000(1)$, $b = 8.008(1)$, $c = 21.674(4)$ Å, $\alpha = 83.48(2)$, $\beta = 89.26(2)$, $\gamma = 71.77(1)°$, $V = 982.4(3)$ Å$^3$, $Z = 2$, $D_c = 1.114$ g cm$^{-3}$, $F(000) = 358$. A total of 4087 reflections were collected in the range 3.5°–136° of $2\theta$.[13] The six-membered ring and its methyl and methoxy-group substituents were found

**Table 1.** Selected geometrical parameters

| | | | | |
|---|---|---|---|---|
| *Bond length (Å)* | | | | |
| O1–C15 | 1.210(5) | | | |
| O2–C15 | 1.320(4) | | | |
| O3–C4 | 1.403(6) | | | |
| C1–C2 | 1.480(12) | | | |
| C1–C6 | 1.534(5) | | | |
| C2–C3 | 1.340(13) | | | |
| C3–C4 | 1.464(6) | | | |
| C4–C5 | 1.521(6) | | | |
| C5–C6 | 1.331(5) | | | |
| *Bond angles (°)* | | | | |
| C1–C6–C5 | 123.2(3) | | | |
| C1–C6–C7 | 114.7(3) | | | |
| C5–C6–C7 | 122.1(3) | | | |
| O1–C15–O2 | 121.4(4) | | | |
| O1–C15–C14 | 126.5(4) | | | |
| O2–C15–C14 | 112.1(4) | | | |
| *Torsion angles (°)* | | | | |
| C1–C2–C3–C4 | 46.0(17) | | | |
| C2–C1–C6–C5 | 2.7(7) | | | |
| C1–C6–C5-C4 | 2.5(6) | | | |
| C1–C6–C7–C8 | −137.6(4) | | | |
| C13–C14–C15–O2 | 171.8(5) | | | |
| *Hydrogen-bonding (Å, °)* | | | | |
| D–H···A | *d*(D–H) | *d*(H···A) | *d*(D···A) | ∠(DHA) |
| O2–$H_2$O···O1#1 | 1.06(6) | 1.60(6) | 2.658(4) | 173(5) |

Symmetry transformations used to generate equivalent atoms: #1 $1 - x$, $1 - y$, $1 - z$.

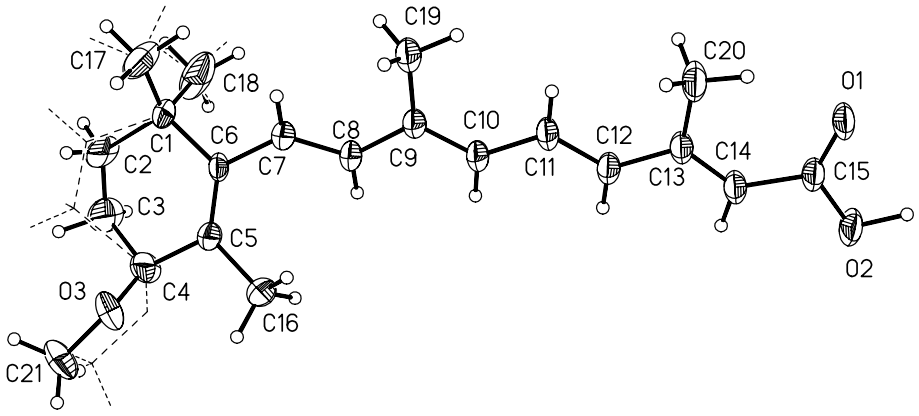
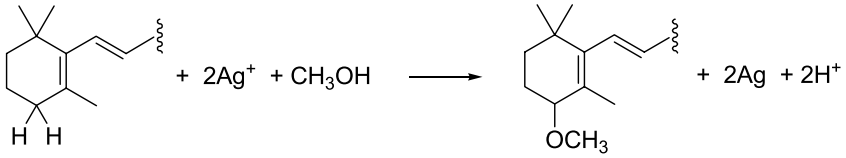



**Figure 1.** View of the molecular structure of **2**, showing the numbering scheme of the atoms. Dashed lines link disordered atoms. Displacement ellipsoids correspond to 25% probability.




1 2

**Scheme 1.** Hypothesis for the formation of impurity **2**.

to be affected by the disorder, which created considerable problems during refinement. In the final model accepted to interpret this disorder, the six-membered ring appeared to adopt an envelope conformation, with the apex C atom ($C_3$) disordered over two positions located at the opposite sides of the plane of the other ring atoms.[14]

Conformational disorder on the six-membered ring was also found in 13-*cis*-retinoic acid[15] and in 13-*cis*-5,6-dihydro-5,6-epoxy retinoic acid,[16] and seems to be a common feature of the crystal structures of a number of retinal analogues.[16] The polyene chain is, as expected, in a fully extended *all trans* conformation with the bond lengths of the π system within the expected range (mean value of the double C=C bond length of 1.347(5) and of the single C–C bond of 1.453(5)). The torsion angles of the conjugated chain show a slight deviation from the ideal value of 180°, so that the zigzag backbone appeared slightly arched, a feature usually observed in long-chain aliphatic compounds.[16–18] The carboxyl hydrogen bond is in trans conformation with respect to the C14–C15 double bond. Selected geometrical parameters are given in Table 1. In the crystal, the molecules form centrosymmetric dimers via hydrogen bonds between carboxylic groups.

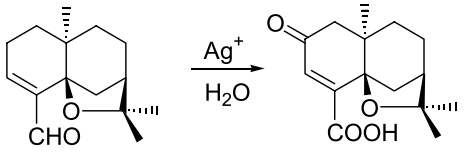
Compound **2** was also prepared by an independent chemical synthesis according to the procedure described in Ref. 11a.

The formation of impurity **2** was tentatively attributed to the allylic oxidation depicted in Scheme 1. The following considerations support our hypothesis.

It is well known that the carbon atom in position 4 of these molecules can be easily oxidised: (i) the metabolism of acid **1** is based on an oxygenation process of the $C_4$ position;[19] (ii) $MnO_2$ allylic oxidation in methylene chloride has been employed for the preparation of 4-oxo-retinoic acid derivatives.[20]

Interestingly enough, it has also been reported[21] that when an *all trans*-retinoic acid methyl ester was oxidised with trifluoromethanesulphonic acid in the presence of lithium chloride and methanol, formation of methyl ester of **2** was observed.

A previous example of allylic oxidation of a $CH_2$ promoted by Ag(I) has been reported in 1997.[22] The treatment of the α,β-unsaturated aldehyde **3** with silver oxide in aqueous ethanol gave enonic acid **4** (Scheme 2).




3 4

**Scheme 2.** A known example of allylic oxidation promoted by silver oxide.

The structure of impurity **2** bears clues of the synthetic steps leading to the preparation of this tretinoin sample. Its presence in any commercial sample is the 'chemical proof' of the involvement of a silver(I)-promoted oxidation. The work highlights how helpful is the structural characterisation of the impurities of a common drug to the definition of the fingerprint of the drug itself.

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9. *HPLC-MS-DAD analysis*. One hundred milligram **1** were dissolved in 100 ml of methanol (1000 ppm solution); samples were directly analyzed by LC-MS-DAD carried out with a Surveyor system equipped with a quaternary pump, a Surveyor UV–vis PhotoDiode Array detector, a Surveyor AS autosampler, a vacuum degasser and a Xalibur software and connected to a Thermo Finnigan LCQ Deca XP plus Ion-Trap Mass Spectrometer. Separations were effected by reverse-phase elution with a Phenomenex Prodigy ODS column (150 × 4.6 mm i.d.; particle size 5 μm) under the following conditions: gradient elution from 15% solvent A (water), 5% solvent B (formic acid 1% in water) and 80% solvent C (methanol) to 7% solvent A, 5% solvent B and 88% solvent C in 25 min; flow rate 1 ml/min; injection volume 20 μl; column temperature 25 °C and UV/DAD detection 350 nm. The autosampler was set at 4 °C during analysis to minimize isomerization and oxidation of **1**. The APCI/MS source was set as follows: vaporizer temperature at 350 °C, source

current of $4.8 \times 10^{-3}$ mA and heated capillary at 200 °C. The mass spectrometer operated in positive-ion mode, with a scan range from *m/z* 100 to 500 (scan rate of 0.5 scan/s). APCI-MS/MS experiments in positive-ion mode were performed by setting the collision energy at 32% (optimized by using the LCQ–Xalibur software) on *m/z* 299 $(M+H-32)^+$, using helium as collision gas. Retention time of the impurity, the APCI/MS [*m/z* 299 $(M+H-32)^+$], and the APCI-MS/MS spectra were in agreement with those of the structure assigned herein.

10. The sample of the impurity **2** was dissolved in deuterated chloroform, and the proton and carbon spectra were acquired on a Bruker ARX 400 instrument at a temperature of 305 K. The hydrogen and carbon chemical shifts are referred to the signals of the solvent taken, respectively, at 7.26 and 77.23 ppm from tetramethylsilane (TMS). The coupling constants are expressed in Hz. The assignment of the $^1$H and $^{13}$C NMR spectra was performed by a comparison with the known spectra of the parent compound tretinoin. $^1$H NMR of impurity **2**: $\delta$ (ppm) 11.0 (1H, br s, COOH), 7.03 (1H, dd, $J$ = 11.3 and 15.3 Hz, H–C(11)), 6.32 (1H, d, $J$ = 15.3 Hz, H–C(12)), 6.25 (1H, d, $J$ = 16.2 Hz, H–C(7) or H–C(8)), 6.17 (1H, d, $J$ = 16.2 Hz, H–C(8) or H–C(7)), 6.16 (1H, d, $J$ = 11.3 Hz, H–C(10)), 5.81 (1H, s, H–C(14)), 3.52 (1H, t, $J$ = 4.3 Hz, H–C(4)), 3.39 (3H, s, $OCH_3$), 2.36 (3H, s, $CH_3$C(13)), 2.00 (3H, s, $CH_3$C(9)), 1.79 (3H, s, $CH_3$–C(5)), 1.87–1.62 (3H, m), 1.36 (1H, m), 1.03 (3H, s, $CH_3$–C(1)), 1.02 (3H, s, $CH_3$C(1)). $^{13}$C NMR: $\delta$ (ppm) 171.97 (COOH), 155.19 ($C_{13}$), 142.37 ($C_6$), 139.94 ($C_9$), 129.41 ($C_5$), 138.40 ($C_8$), 135.63 ($C_{12}$), 131.77 ($C_{11}$), 130.30 ($C_{10}$), 128.40 ($C_7$), 118.19 ($C_{14}$), 79.34 ($C_4$), 56.88 ($OCH_3$), 34.98 ($C_1$), 34.92 ($C_2$), 29.23 ($C_1$), 27.67 ($C_1$), 23.32 ($C_3$), 19.08 ($C_5$), 14.30 ($C_{13}$), 13.11 ($C_9$).
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12. *X-ray single crystal diffraction* for molecular and crystal structure determination. The crystal used in X-ray analysis, with approximate dimensions of $0.4 \times 0.5 \times 0.01$ mm, was obtained upon slow crystallisation from hexane. Intensity data were collected, at room temperature, on a Siemens P4 diffractometer with graphite monochromated Cu-K$\alpha$ radiation ($\lambda$ = 1.54179 Å), using $\theta/2\theta$ scan technique, voltage 40 kV and current 40 mA. Unit cell parameters were determined using 45 reflections in the range $12.00° \leqslant 2\theta \leqslant 45.6°$. A total of 4087 reflections (3321 unique, $R_{int}$ = 0.1095) were collected up to 136° in $2\theta$ and the index range: $-1 \leqslant h \geqslant 7$, $-8 \leqslant k \geqslant 9$ and $-25 \leqslant l \geqslant 25$. Three standard reflections, monitoring every 100 reflections, showed no intensity decay. No empirical adsorption correction was deemed necessary.
13. The structure was solved by a direct method using the SIR97 program (Altomare, A.; Burla, M. C.; Camalli, M.; Cascarano, G. L.; Giacovazzo, C.; Guagliardi, A.; Moliteni, A. G. G.; Polidori, G. P.; Spagna, R. *J. Appl. Crystallogr.* **1999**, *32*, 115), which revealed the position of all non H-atoms. The refinement was carried out on $F^2$ by a full-matrix least-squares procedure with SHELXL97 (Sheldrick, G. M. SHELXL97. Release 97-2. Program for the Refinement of Crystal Structures. (1997). University of Göttingen, Germany) for 273 parameters, with anisotropic temperature factors for non-H atoms.
14. The site occupation factors converged to about 80% and 20% for the two positions, respectively. The final stage converged to $R$ = 0.0712 ($R_w$ = 0.159) for 1509 observed reflections, with $I \geqslant 2\sigma(I)$, and $R$ = 0.163 ($R_w$ = 0.208) for 3321 unique reflections after merging the equivalents. H atoms, except for the carboxyl H atom, which was freely refined with individual isotropic temperature factors, were placed in geometrically calculated positions and refined in a riding model.
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