DEMETHYLATION AND AUTO-OXIDATION OF DIFFERENT COBALT-BLEOMYCIN COMPLEXES

CORNELIS M. VOS, DICK SCHIPPER*, JACOBUS D. M. HERSCHEID and GERRIT WESTERA**

RadioNuclide Centre, Free University, P.O. Box 7161, 1007 MC Amsterdam, The Netherlands
*Gist-Brocades N.V., Delft
**Department of Internal Medicine, Free University Hospital, Amsterdam, The Netherlands
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Demethylation of Co-bleomycin A2 by heating yields three different complexes: form I and form II and "orange" Co-bleomycin-demethyl A2. These complexes can be separated by HPLC and show different $^1$H NMR spectra. Preparation of Co-bleomycin-demethyl A2 by chelation of bleomycin-demethyl A2 with cobalt yields a Co-bleomycin-demethyl A2, which is auto-oxidized into Co-bleomycin A1.

Cobalt-bleomycin-demethyl A2 (Co-blm dA2) is a suitable intermediate in the preparation of tumour-localizing Co-blm complexes1,2. When Co-blm dA2 (Fig. 1) is prepared by heating Co-blm A23), an HPLC-chromatogram of the reaction mixture reveals that another product is formed besides the expected forms I and II of Co-blm dA24). The underlying study was conducted in order to identify the different products by both HPLC (high performance liquid chromatography) and $^1$H NMR (proton nu-

Fig. 1. Structure of bleomycins.
clear magnetic resonance).

**Experimental**

Bleomycin A$_2$ (lot U 4300 A$_2$S) was chelated with an equivalent of CoCl$_2$ in 0.05 M phosphate, pH 7.0. Demethylation was performed by heating Co-blm A$_2$ for 18 hours at 100~120°C under reduced pressure (about 10$^{-1}$ mm Hg). The demethylated products were purified by preparative HPLC (column: Nucleosil 10 C$_{18}$ (from Chrompack); eluent: 1% ammoniumacetate - methanol, 6: 4; flow 2 ml/minute; 1 mg per injection$^{5,6}$, after which the methanol was evaporated and the residue lyophilized twice, the second time from D$_2$O. $^1$H NMR spectra were recorded on samples containing 0.2~1 mg Co-blm in 0.25 ml D$_2$O pH$_m$ 5.7 at 250 MHz on a Bruker WM 250 spectrometer.

**Results and Discussion**

Demethylation

In Fig. 2a the HPLC-chromatogram of the reaction mixture of Co-blm A$_2$ after heating is given. It is obvious that at least three products have been formed under the reaction conditions chosen. The peaks 1 and 2 were also obtained when free blm A$_2$ was demethylated by heat, purified and chelated with cobalt to give the known form I and II of Co-blm dA$_2$. These peaks (1 and 2) but also peak 3 (Fig. 2a) disappear upon addition of excess methylidide in methanol reforming Co-blm dA$_2$.$^7$ Therefore it seems that the latter peak also represents a Co-blm dA$_2$ complex. This assignment is supported by $^1$H NMR analysis: the $^1$H NMR spectra (Fig. 3a,b,c) of the isolated peaks 1, 2 and 3 (Fig. 2) all show a singlet at 1.95~1.98 ppm for the S-CH$_3$-group.$^8,9$ This resonance is not present in $^1$H NMR spectra of Co-blm A$_2$, whereas in free blm dA$_2$ this singlet is found at 1.90 ppm. The $^1$H NMR assignments are summarized in Table 1.

Table 1. Chemical shifts (relative to external TMS) in the high field part of $^1$H NMR spectra of several Co-bleomycin complexes.

<table>
<thead>
<tr>
<th>Assignments$^a$</th>
<th>Observations$^b$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{(-11)}$H$_5$</td>
<td>Free (d) A$_2$</td>
<td>Co-A$_2$-I</td>
</tr>
<tr>
<td>C$_{(-52)}$H$_2$</td>
<td>1.86 2.32 2.37</td>
<td>2.31 2.36 2.38</td>
</tr>
<tr>
<td>C$_{(-54,55)}$H$_3$</td>
<td>&quot;Orange&quot; Co-A$_2$</td>
<td>Co-dA$_2$-II</td>
</tr>
<tr>
<td>C$_{(-53)}$H$_3$</td>
<td>&quot;Orange&quot; Co-dA$_2$</td>
<td>Co-A$_2$-I</td>
</tr>
<tr>
<td>C$_{(-53)}$H$_3$</td>
<td>&quot;Orange&quot; Co-dA$_2$</td>
<td>Co-A$_2$-I</td>
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<tr>
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<tr>
<td>C$_{(-52)}$H$_2$</td>
<td>Co-B$_2$-I</td>
<td>Co-B$_2$-II</td>
</tr>
<tr>
<td>C$_{(-52,53)}$H$_2$</td>
<td>Co-B$_2$-II</td>
<td>Co-B$_2$-II</td>
</tr>
<tr>
<td>C$_{(-52)}$H$_2$</td>
<td>Co-ketoppept. A$_{12}$</td>
<td>&quot;Orange&quot; Co-blm$^{12}$</td>
</tr>
</tbody>
</table>

$a)$ Annotations according to Fig. 1.

$b)$ Abbreviations used: dA$_2$: bleomycin-demethyl A$_2$; Co-A$_2$-I: cobalt-bleomycin A$_2$ form I; etc.

References
Oxidation

The demethylation product of blm A₂ is described to be sensitive to oxidation leading to blm A₁₁. On the basis of analogy the preparation of Co-blm A₁ by heating Co-blm A₂ in the presence of oxygen was attempted. The reaction mixture however, gives the same HPLC-chromatogram as does the reaction mixture, prepared by heating in vacuo.

On the other hand when Co-blm dA₂ (prepared by chelation of blm dA₂ with cobalt) is chromatographed several hours after preparation, two new peaks appear at the expense of Co-blm dA₂ (Fig. 2c).

Realizing that, upon chelation of blm with cobalt, a reactive oxygen species is formed and assum-
ing that this oxygen species is responsible for the observed phenomenon, this phenomenon should also occur when blm dA₂ is chelated with iron (II). And indeed, it was found that new peaks in the HPLC-chromatogram were formed at the expense of Fe-blm dA₂ immediately after iron (II) was added to blm dA₂. These peaks increase if a reducing agent like mercaptoethanol is added. Upon chelation with iron (III) only a Fe (III)-blm dA₂ complex is formed, because no reactive oxygen species is generated upon chelation with a trivalent iron. By analogy, Cu-blm dA₂ also does not yield Cu-blm A₁. These data suggest an auto-oxidation by the reactive oxygen species to Co-blm A₁, respectively Fe-blm A₁. The hypothesis of oxidation is also supported by ¹H NMR analysis. The ¹H NMR spectra (Table 1, Fig. 3d) of the isolated peaks 4 and 5 (Fig. 2c) both show a singlet at 2.57–2.58 ppm, which singlet is not present in the ¹H NMR spectra of Co-blm A₂ and Co-blm dA₂.

The slow generation of the reactive oxygen species by cobalt (up to about 50% Co-blm dA₂ is oxidized in 24 hours) may be due to a rather stable cobalt-oxygen bond, which supports the results obtained by Sugiyama.

Different Forms

Although the existence of different forms of Co-blm complexes is firmly established, new evidence can be found from the ¹H NMR spectra (Fig. 3 and 4). Analysis of the "methyl-region" of these spectra reveals a difference in chemical shift of the protons of the pyrimidine-methyl group of about 0.05 ppm between the so called forms I and II (see Table 1). This difference is not only observed for Co-blm dA₂ and Co-blm A₂, but also for Co-blm A₁ and Co-blm B₂. Even the resonances of the protons of the sulphonium group are somewhat different for both forms as can be seen in Fig. 4a, in which the ¹H NMR spectrum of Co-blm A₂ form I (contaminated with form II) is given.

Besides the forms I and II of each Co-blm, a third complex exists, the so called "orange" Co-blm. In a previous study it was already proved that the chromatographic behaviour of "orange" Co-blm A₁ differed from those of Co-blm A₂ form I and II. Now it is obvious also from the ¹H NMR spectra, that "orange" Co-blm differs from Co-blm form I and II (see the region around 1 ppm and the singlet at 2.18 ppm which occurs only in "orange" Co-blm). The third Co-blm dA₂ complex (peak 3 in Fig. 2a)
has also been assigned as "orange" Co-blm on basis of chromatographic behavior (similar retention times for the complex prepared in this study to that prepared by DeRiemer) as well as on basis of the H NMR spectrum, which is quite similar to that of "orange" Co-blm A, except of course for the resonances of the different functional groups. DeRiemer et al. prepared "orange" Co-blm by heating Co-blm overnight at 50°C followed by 2 hours at 110°C. Besides demethylation another process probably occurs upon heating of Co-blm, because demethylation by heating yields "orange" Co-blm dA, which is not formed when Co-blm dA is prepared by chelation of blm dA with cobalt. The differences between the H NMR spectra of the Co-blm complexes strongly suggest conformational differences between these complexes. Dabrowski et al. recently described the analysis of the cobalt complex of pseudotetrapeptide A of bleomycin, obtained by hydrolysis of "orange" and "green" (form I and II?) Co-blm A and found both hydrolysis products to be identical. This result may be explained by a distortion of the conformation of Co-blm by the rigorous hydrolysis method used.

The H NMR spectra may also be used to confirm the assignment form I and II of Co-blm complexes. The assignment form I and II has been made on the basis of the sequence of elution from a CM Sephadex C25 column and it was assumed that this sequence was similar for the different Co-bleomycins. Furthermore the chemical shifts of the methyl groups of the so called forms I appear to be identical (see Table 1) as are the chemical shifts in the case of the forms II.

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References

8) Sadtlter Standard Spectra: No. 533 M (DMO; δ 2.52 ppm in CCl₄) and No. 6344 M (DMS; δ 2.06 ppm in CCl₄) (According to Ref. 9 these values are 3.20, 2.50 ppm respectively). Sadtlter Research Laboratories, Philadelphia


