DEMETHYLATION AND AUTO-OXIDATION OF DIFFERENT COBALT-BLEOMYCIN COMPLEXES

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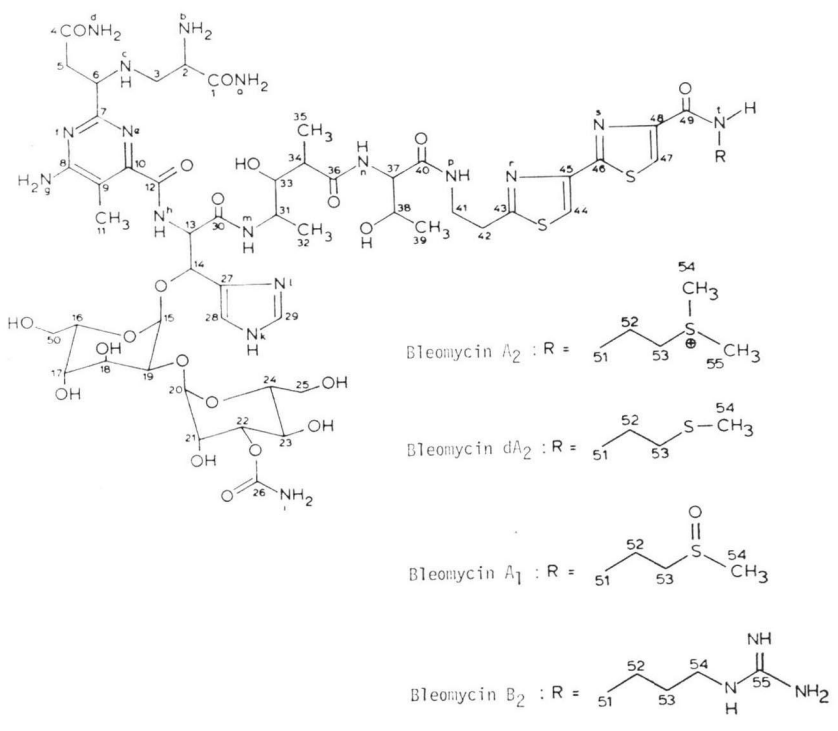
Demethylation of Co-bleomycin A_2 by heating yields three different complexes: form I and form II and "orange" Co-bleomycin-demethyl A_2. These complexes can be separated by HPLC and show different ^1H NMR spectra. Preparation of Co-bleomycin-demethyl A_2 by chelation of bleomycin-demethyl A_2 with cobalt yields a Co-bleomycin-demethyl A_2, which is auto-oxidized into Co-bleomycin A_1.

Cobalt-bleomycin-demethyl A_2 (Co-blm dA_2) is a suitable intermediate in the preparation of tumour-localizing Co-blm complexes[1,2]. When Co-blm dA_2 (Fig. 1) is prepared by heating Co-blm A_2[3], an HPLC-chromatogram of the reaction mixture reveals that another product is formed besides the expected forms I and II of Co-blm dA_2[4]. The underlying study was conducted in order to identify the different products by both HPLC (high performance liquid chromatography) and ^1H NMR (proton nu-

Fig. 1. Structure of bleomycins.



clear magnetic resonance).

Experimental

Bleomycin A_2 (lot U 4300 A_2S) 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_2O. ^1H NMR spectra were recorded on samples containing 0.2~1 mg Co-blm in 0.25 ml D_2O 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 methyliodide 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 ^1H NMR analysis: the ^1H 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 ^1H NMR spectra of Co-blm A_2, whereas in free blm dA_2 this singlet is found at 1.90 ppm. The ^1H NMR assignments are summarized in Table 1.

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

		Observations[b]											References		
		Free (d) A_2	Co-A_2-I	Co-A_2-II	"Orange" Co-A_2	Co-dA_2-I	Co-dA_2-II	"Orange" Co-dA_2	Co-A_1-I	Co-A_1-II	Co-B_2-I	Co-B_2-II	Free A_2[18]	Co-tetrapept. A[17]	"Orange" Co-blm[1]
Assignments[a]															
$C_{(-11)}H_3$		1.86	2.32	2.37	?	2.31	2.36	2.38?	2.33	2.38	2.31	2.37	2.06	2.48	
Terminal amines															
A_2	$C_{(-52)}H_2$	2.05	2.01	2.02	2.09								2.20		2.20
	$C_{(-54,55)}H_3$	2.80	2.81	2.78	2.81								2.94		2.92
dA_2	$C_{(-52)}H_2$	1.81				—	1.82	1.84							1.94
	$C_{(-54)}H_3$	1.90				1.95	1.98	1.98							2.10
A_1	$C_{(-54)}H_3$								2.57	2.58					
B_2	$C_{(-52,53)}H_2$										1.50	1.54			
pH_m		5.7											5.0	6.0	5.2

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.*

Fig. 2. HPLC-chromatogram of Co-bleomycin-demethyl A_2 complexes.

Column: Nucleosil 10 C_{18}. Eluent: 1% Ammoniumacetate-methanol, 6:4 (v/v), Flow: 2 ml/minute.

a) Co-blm A_2, demethylated by heating at 120°C overnight.

b) 0.7 mM blm dA_2, chelated with cobalt, chromatographed immediately after preparation.

c) preparation b) after 24 hours standing at room temperature.

Assignments: Peak 1: Co-blm dA_2 form I. Peak 2: Co-blm dA_2 form II. Peak 3: "orange" Co-blm dA_2. Peak 4: Co-blm A_1 form I. Peak 5: Co-blm A_1 form II. Peak 6: "orange" Co-blm A_2.

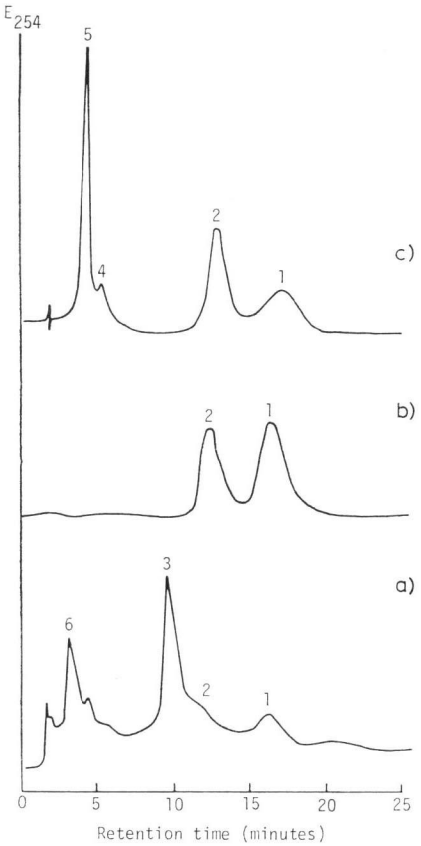
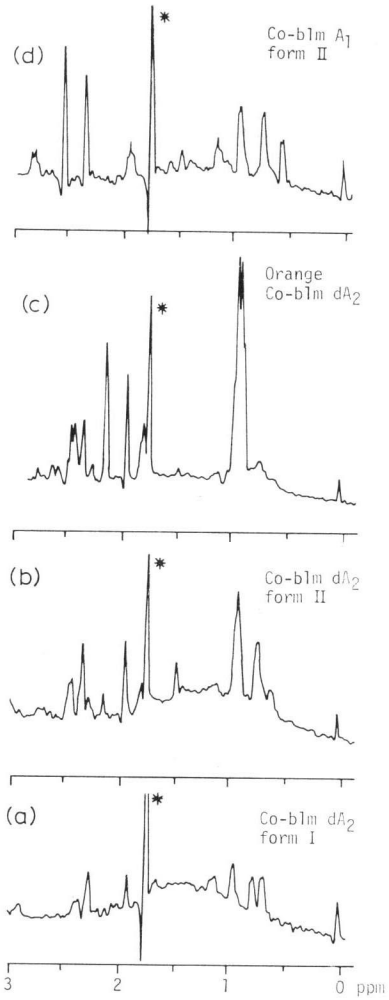


Fig. 3. High field part of ^1H NMR spectra in D_2O (pH_m 5.7).

a) Co-blm dA_2 form I. b) Co-blm dA_2 form II. c) "orange" Co-blm dA_2. d) Co-blm A_1 form II.

Assignments are given in Table 1. The resonances with an asterisk are assigned to acetate, which is present as a residue from the HPLC-eluent.



Oxidation

The demethylation product of blm A_2 is described to be sensitive to oxidation leading to blm A_1[10]. On the basis of analogy the preparation of Co-blm A_1 by heating Co-blm A_2 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 dA_2 (prepared by chelation of blm dA_2 with cobalt) is chromatographed several hours after preparation, two new peaks appear at the expense of Co-blm dA_2 (Fig. 2c).

Realizing that, upon chelation of blm with cobalt, a reactive oxygen species is formed[11] and assum-

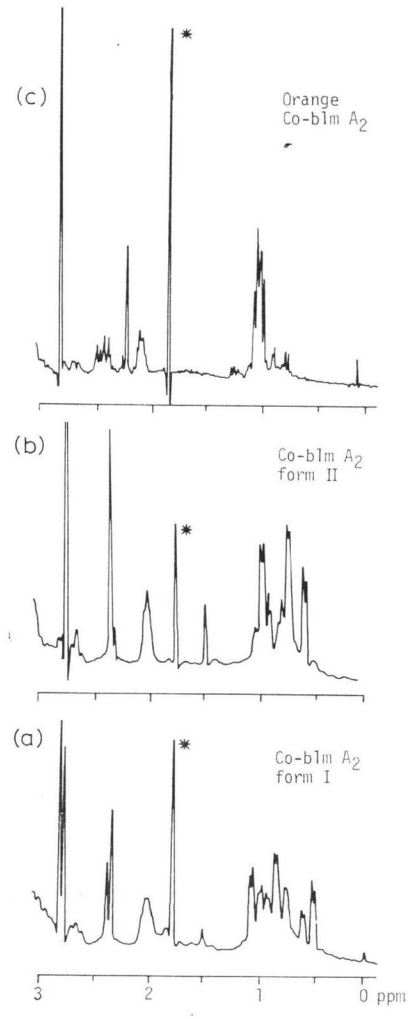
ing that this oxygen species is responsible for the observed phenomenon, this phenomenon should also occur when blm dA_2 is chelated with iron (II). And indeed, it was found that new peaks in the HPLC-chromatogram were formed at the expense of Fe-blm dA_2 immediately after iron (II) was added to blm dA_2. These peaks increase if a reducing agent like mercaptoethanol[12] is added. Upon chelation with iron (III) only a Fe (III)-blm dA_2 complex is formed, because no reactive oxygen species is generated upon chelation with a trivalent iron[12]. By analogy, Cu-blm dA_2 also does not yield Cu-blm A_1. These data suggests an auto-oxidation by the reactive oxygen species to Co-blm A_1 respectively Fe-blm A_1. The hypothesis of oxidation is also supported by ^{1}H NMR analysis. The ^{1}H 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 ^{1}H NMR spectra of Co-blm A_2 and Co-blm dA_2[8,9].

The slow generation of the reactive oxygen species by cobalt (up to about 50% Co-blm dA_2 is oxidized in 24 hours) may be due to a rather stable cobalt-oxygen bond, which supports the results obtained by SUGIURA[13].

Fig. 4. High field part of ^{1}H NMR spectra in D_2O (pH_m 5.7).

a) Co-blm A_2 form I (contaminated with form II). b) Co-blm A_2 form II. c) "orange" Co-blm A_2.

Assignments are given in Table 1. The resonances with an asterisk are assigned to acetate, which is present as residue from the HPLC-eluent.



Different Forms

Although the existence of different forms of Co-blm complexes is firmly established[4,14,15], new evidence can be found from the ^{1}H 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 dA_2 and Co-blm A_1, but also for Co-blm A_2 and Co-blm B_2. Even the resonances of the protons of the sulfonium group are somewhat different for both forms as can be seen in Fig. 4a, in which the ^{1}H NMR spectrum of Co-blm A_2 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[1]. In a previous study it was already proved that the chromatographic behaviour of "orange" Co-blm A_2 differed from those of Co-blm A_2 form I and II[16]. Now it is obvious also from the ^{1}H 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 dA_2 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[1]) as well as on basis of the ^{1}H NMR spectrum, which is quite similar to that of "orange" Co-blm A_2, except of course for the resonances of the different functional groups. DERIEMER *et al.*[1] 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_2, which is not formed when Co-blm dA_2 is prepared by chelation of blm dA_2 with cobalt. The differences between the ^{1}H NMR spectra of the Co-blm complexes strongly suggest conformational differences between these complexes. DABROWIAK *et al.*[17] 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_2 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 ^{1}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[4] 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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