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Structure elucidation and characterization of daunorubicin degradation products

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Summary

The thermal degradation of the cytostatic drug, daunorubicin, in aqueous solution was studied. Degradation products were extracted with chloroform from the degradation mixtures and separated by thin-layer chromatography. The predominant degradation products were identified and characterized with several spectroscopic techniques. All identified compounds were aglycones.

Introduction

The instability of anthracycline cytostatics in aqueous solution has been recognized by several investigators (Wassermann and Bundgaard, 1983; Janssen et al., 1985; Bouma et al., 1986). The degradation kinetics of doxorubicin (Dx) and daunorubicin (Dr) (Fig. 1) in infusion fluids (Benvenuto et al., 1981; Poochikian et al., 1981; Beijnen et al., 1985a) and in aqueous buffers as a function of pH have been reported (Wassermann and Bundgaard, 1983; Beijnen et al., 1985b, 1986a and b). However, information about the isolation, identification and analysis of degradation products is scanty. Degradation products have been noticed but were not identified (Cummings et al., 1984; Lankelma et al., 1982; Janssen et al., 1985; Kaniewska, 1977), except in the study of Abdeen et al. (1985) dealing with the degradation of Dx in aqueous sodium hydroxide solutions. Apart from knowledge about the drug degradation kinetics, it is also essential to elucidate the structures of the degradation products in order to establish de-

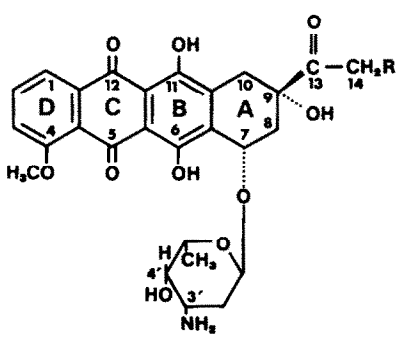

Fig. 1. Structures of daunorubicin (R = H) and doxorubicin (R = OH).

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gradation mechanisms and to develop selective, stability indicating determination methods.

In this paper, the isolation, separation and structure elucidation of the principal degradation products of Dr, arising in acidic and weakly alkaline solution, are described. The reported facile preparation and isolation procedures of the Dr degradation products may serve as a source to obtain reference compounds. This project is a sequel to earlier studies concerning the degradation kinetics of Dr and Dx in aqueous solution (Beijnen et al., 1985a and b, 1986a and b).

Experimental

Materials

Daunorubicin hydrochloride (Dr) was obtained from Rhône-Poulenc Nederland B.V. (Amstelveen, The Netherlands). 7-Deoxydaunorubicinone (IV/VII) and 7,8-9,10-bisanhydrodaunorubicinone (III/VI) reference compounds were generous gifts from Dr. S. Penco, Farmitalia (Milan, Italy). All other chemicals used were of reagent grade and deionized water was used throughout.

Degradation conditions

Dr, 25 mg (0.044 mmol), was dissolved in 500 ml 1 M HCl. This mixture was boiled and refluxed in the dark for 28 h. For the degradation in weakly alkaline solution 25 mg Dr was dissolved in 500 ml 0.01 M phosphate buffer pH 8 and refluxed for 4 h in the dark.

Isolation of the degradation products

The degradation products arising in 1 M HCl and at pH 8 were isolated and separated in the same manner.

After cooling to room temperature the reaction solutions were extracted with chloroform until the aqueous layer was colourless. The chloroform extracts were combined and the organic solvent was partly evaporated under reduced pressure. The resulting red coloured solution was subjected to thin-layer chromatography (TLC) in a zone 15 cm long $\times 0.5$ cm wide. After development of the chromatograms the zones were scraped off and eluted with methanol except for the compounds III/VI which were eluted with chloroform.

Chromatography

TLC was performed on precoated silica gel plates (20×20 cm) (DC-Fertigplatten Kieselgel 60, thickness 0.25 mm, Merck, Darmstadt, F.R.G.). The eluent consisted of chloroform–methanol–6 M ammonia (90 : 10 : 1, v/v/v) and the migrating distance was 15 cm. The coloured products are detected by viewing under daylight.

The equipment and conditions used for high-performance liquid chromatography (HPLC) with fluorescence detection have been described previously (Beijnen et al., 1985b, 1986a). For the present study, the HPLC system was extended with a model 440 dual wavelength UV absorbance detector (Waters Assoc. Milford, MA, U.S.A.) with fixed wavelength filters for detection at 254 nm and 280 nm. The sensitivity of both absorbance detector signals were set equal so that the ratio of the peak heights at these wavelengths yields the molar absorptivity ratio A254/A280.

Spectroscopy

Ultraviolet and visible (UV-VIS) spectra of the degradation products were recorded by rapid scanning with a PU 4021 photo-diode array detector (Pye Unicam, Cambridge, U.K.) coupled with the HPLC system.

Fluorescence spectra of the compounds in 2% (v/v) acetic acid containing methanolic solutions were obtained with a Perkin-Elmer Fluorescence Spectrophotometer 204 (Hitachi, Tokyo, Japan).

Infra-red (IR) spectra are measured on a Perkin-Elmer 580B IR Spectrophotometer. Two methods are used. *Method A (overall spectra).* Solvent, chloroform; pathway, 0.2 mm. *Method B (OH-stretching frequencies).* Solvent, carbon tetrachloride; pathway, 10 mm. Circular dichroism (CD) spectra of most compounds were obtained in methanol (III/VI in chloroform) with a Dichrograph III (Jobin Yvon, Longjumeau, France).

^{1}H-NMR spectra of the compounds I, II and IV/VII were measured on a Bruker WH-90 spectrometer with tetramethylsilane as an internal standard. $CDCl_3$ was used as the solvent.

The conditions and the equipment for field desorption (FD) and chemical ionization (CI) mass spectral analysis of the degradation products were described previously (Beijnen et al., 1985b).

Electrochemistry

Direct current (DC) polarography and controlled potential electrolysis (CPE) were performed using a Bruker E310 modular electrochemical system, equipped with a drop-timer and a Houston model 2000 X–Y recorder (DC) or a Kipp BD-41 y-t recorder (CPE). A water-jacketed 10 ml polarographic cell (Metrohm EA 880-T-5) with a dropping mercury pool electrode (CPE, approx. 7 cm^2), a Metrohm EA 436 Ag/AgCl/3M KCl reference electrode and a platinum wire auxiliary electrode was employed. CPE was carried out in 10 ml of a 1×10^{-4} M solution of the compound in 0.1 M phosphate buffer pH 7. The desired potential of electrolysis was chosen from a DC polarographic curve which was recorded first. The course of the electrolysis was monitored at appropriate time intervals by DC polarography as well as by HPLC.

Results and Discussion

Degradation of Dr in acidic solution

The degradation of Dr in acidic medium (pH < 4) generates initially two products, the tetrahydronaphthacenequinone aglycone daunorubicinone (I) (Fig. 2) and the amino sugar, daunosamine (Arcamone et al., 1964a and b; Beijnen et al., 1985b). Under protracted and more vigorous acid conditions the red aglycone I is subject to progressive degradation. After 28 h at 100°C in 1 M HCl the degradation mixture contained a red precipitate.

TABLE 1

CHROMATOGRAPHIC PROPERTIES, RATIOS OF MOLAR ABSORPTIVITIES AT 254 nm AND 280 nm AND COLOURS IN DAYLIGHT OF REFERENCE COMPOUNDS AND DAUNORUBICIN DEGRADATION PRODUCTS

Compound	R_f [a]	k [b]	A254/A280	Colour
daunorubicinone	0.69	1.1	3.2	orange
7-deoxydaunorubicinone	0.77	1.7	4.0	orange
7,8,9,10-bisanhydrodaunorubicinone	0.89	6.5	0.7	pink
compound I	0.69	1.1	3.2	orange
compound II	0.54	0.8	3.4	orange
compound III/VI	0.88	6.5	0.7	pink
compound IV/VII	0.77	1.7	4.0	orange
compound V	0.86	2.3	3.6	orange
compound VIII	0.49	3.5	0.6	pink
zone IX	0.69	1.1; 1.3	3.2; 0.6	orange/pink
zone X	0.35	0.8	3.2	orange
zone XI	0.40	2.1	n.d [c]	pink

[a] TLC. Stationary phase, silica gel; mobile phase, chloroform–methanol–6 M ammonia (90:10:1, v/v/v).
[b] HPLC. Column, Lichrosorb 10RP8 (30 cm × 3.9 mm i.d.); mobile phase, acetonitrile:0.02 M sodium chloride pH 2.0 (60:40, v/v); flow rate 1.5 ml/min.
[c] n.d. = not determined.

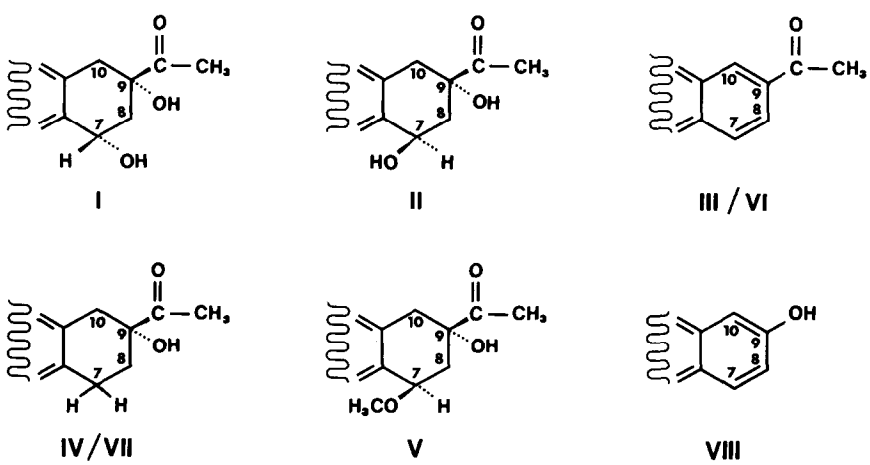

Fig. 2. Structures of daunorubicin degradation products.

By addition of chloroform, the precipitate dissolved and was effectively extracted into the organic phase. The remaining colourless aqueous phase was subjected to HPLC with UV detection, but no compounds were detected. TLC analysis of the chloroform extracts revealed the presence of 5 zones. The chromatographic behaviour of the compounds, the colour in daylight as well as the A254/A280 ratio are tabulated (Table 1). The colours of the compounds are most obvious when the plates are still moisty after development. After drying, the colours fade.

The structures of the identified substances are illustrated in Fig. 2.

Compound I

Compound I is daunorubicinone and is still present after the 28-h incubation period indicating that the consecutive degradation step of Dr has not been completed yet at this stage. However, characteristics of daunorubicinone have been presented elsewhere (Arcamone et al., 1964a; Arcamone, 1978; Vigevani et al., 1985) some of its physicochemical properties are overviewed here as a reference for the identification of the other degradation products.

The UV-VIS spectrum of I (Fig. 3) is identical to that of Dr with absorption maxima at 234, 255, 292, 476, 496 and 530 nm, characteristic for the 6,11-dihydroxy-4-methoxy-anthraquinone chromophoric part of the molecule.

Fluorescence excitation and emission spectra of I are represented in Fig. 4 showing the same maxima as those found for Dr recorded under identical conditions. The major group vibrations in the IR spectrum of I (Method A) are found at 1530, 1575 and 1615 cm^{-1} (dihydroxy-anthraquinone system) and 1710 cm^{-1} (13-C=O stretching vibration). It must be noted that the value of 1615 cm^{-1} for $\nu_{C=O}$ of the quinone system is extremely low, because of strong intramolecular hydrogen bonds between OH and C=O groups in the dihydroxy anthraquinone system. Consequently, no OH stretching absorptions are found in the spectra of these quinone systems. In the OH region (Method B) maxima are found at 3510 and 3585 cm^{-1}. The ν_{OH} value of 3585 cm^{-1} is characteristic for hydrogen-bonded OH groups, whereas $\nu_{OH} = 3510$ cm^{-1} is somewhat higher than ν_{OH} of the hydrogen-bonded OH group in 1-acetyl cyclohexanol (Lutz, 1986). This is in accordance with a conformation in which the axial

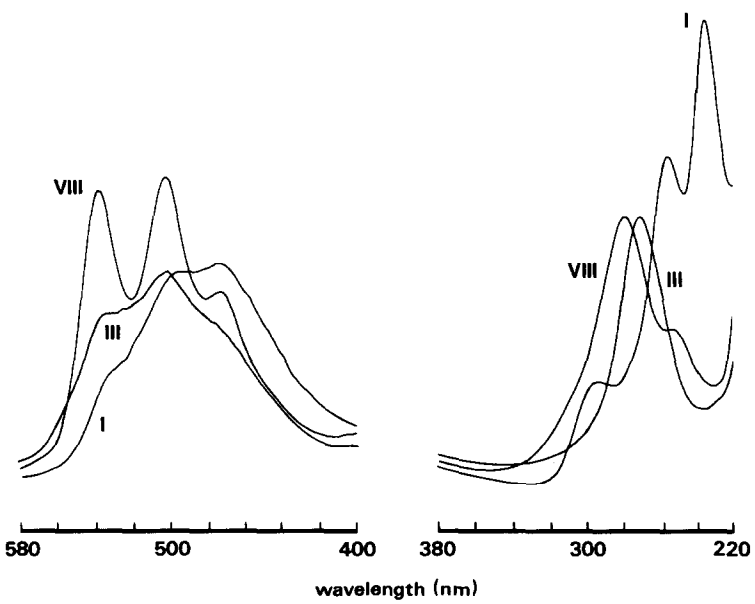

Fig. 3. UV-VIS spectra of daunorubicin degradation products I, III and VIII.

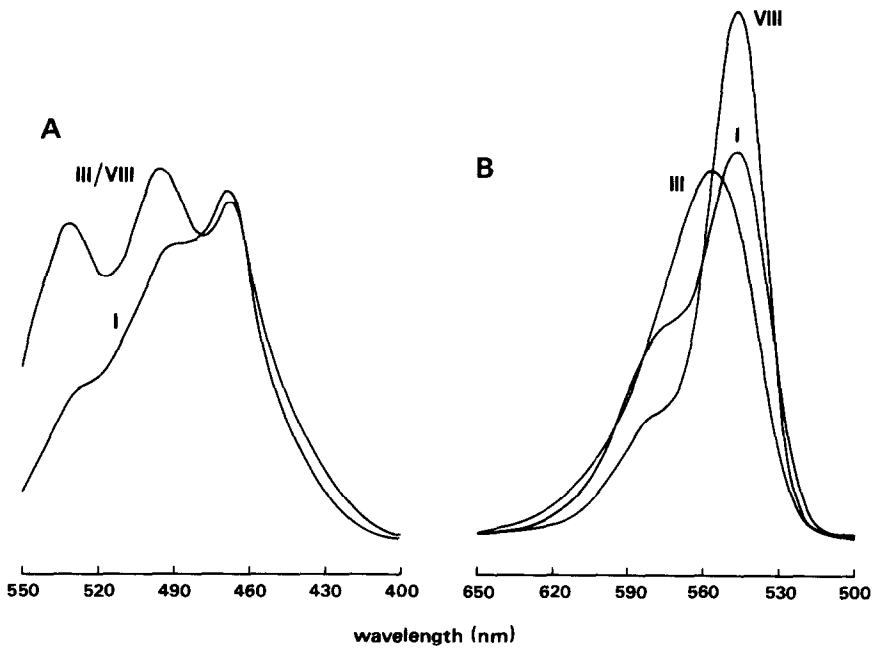

Fig. 4. Fluorescence excitation (A; $\lambda_{emission} = 550$ nm) and emission (B; $\lambda_{excitation} = 470$ nm) spectra of degradation products I, III and VIII in 2% (v/v) acetic acid methanol solution.

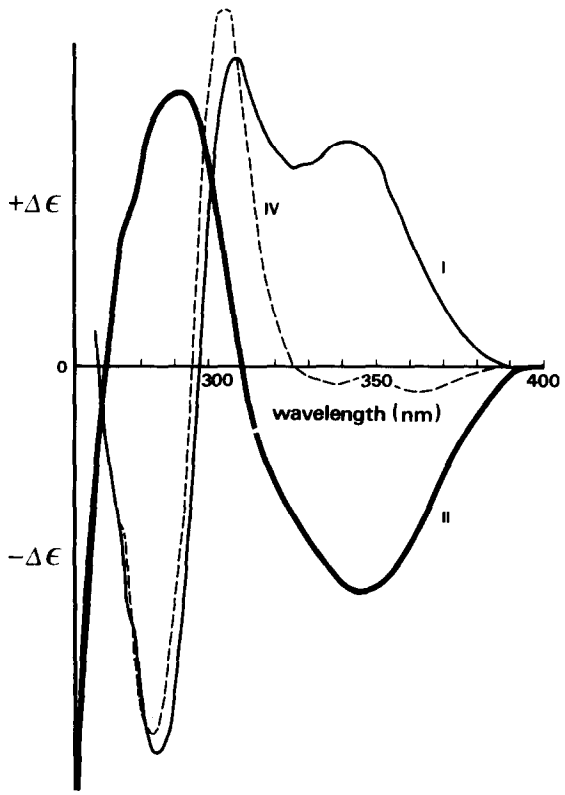

Fig. 5. Circular dichroism spectra of degradation products I, II and IV in methanol. Ellipticity is plotted in arbitrary units.

9-OH group is H-bonded with the 13-C=O, while the 9-OH group is also H-bonded with the axial 7-OH group. The $\nu_{C=O}$ value (1710 cm^{-1} in chloroform) is in accordance with this conformation (Renema, 1972). On acid hydrolysis of the glycosidic linkage of Dr the absolute configurations at C7 (S) and C9 (S) in the aglycone I are retained. This was established on the basis of the similarity of the CD Cotton effects of I and Dr. The CD spectrum of I in the 260–400 nm wavelength region is included in Fig. 5. The maintenance of chirality at C7 in I rules out an SN^2 type nucleophilic water attack at C7 with the elimination of the amino sugar. This reaction would result in an aglycone with a C7 hydroxyl function reversed in position. Probably, the conversion of Dr into I is initiated by protonation of the oxygen atom of the glycosidic bond. Subsequent nucleophilic water attack at daunosamine C1′ and cleavage of the glycosidic O–C1′ bond affords I and the aminosugar.

The ^{1}H-NMR data of I are: δ 13.99 (1H, s, ArOH), 13.31 (1H, s, ArOH), 8.05 (1H, dd, J = 1.5, J = 7.5 Hz, H(3)), 7.74 (1H, t, J = 7.5 Hz, H(2)),

7.37 (1H, dd, J = 7.5, J = 1.5 Hz, H(1)), 5.42–5.25 (1H, m, H(7)), 4.53 (1H, s, OH(9)), 4.07 (3H, s, OCH_3), 3.70 (1H, d, J = 4.5 Hz, OH(7)), 3.23 (1H, A of AB, J = 18.5 Hz, with extra long-range coupling, H(10e)), 2.92 (1H, A of AB, J = 18.5 Hz, H(10a)), 2.42 (3H, s, CH_3(14)), 2.36–2.15 (2H, m, H(8)).

The FD-MS and methane CI-MS analysis of I was reported earlier (Beijnen et al., 1985b).

Compound II

Compound II was identified as 7-epidaunorubicinone (Fig. 2) possessing the opposite configuration at C7 (R) compared with I (C7 (S)). For isolation purposes the diastereomeric compounds could be effectively separated by TLC.

I and II have essentially identical UV-VIS and fluorescence excitation and emission spectra.

FD-MS analysis of II revealed the parent mass m/z 398 $(M)^{+\cdot}$. The CI-MS spectrum of II is characterized by m/z values 399 $(M + H)^+$ (5%), 381 $[(M + H)\text{-}H_2O]^+$ (5%), 363 $[(M + H)\text{-}2H_2O]^+$ (80%) and 321 $\{[(M + H)\text{-}2H_2O]\text{-}COCH_2\}^+$ (100%). Consequently, II shows a mass spectrum similar to I (Beijnen et al., 1985b). Our attention was driven towards the difference in chirality between I and II on account of their CD Cotton effects which are markedly different (Fig. 5). However, I and II possess two asymmetric carbon atoms (C7 and C9) and the CD spectra do not reveal the conclusive configurational differences between I and II. The CD spectrum of 7-deoxydaunorubicinone reference (IV) was also recorded (Fig. 5). This compound has only one chiral center (at C9 (S)). Both I and II could be converted into 7-deoxydaunorubicinone by constant potential electrolysis at −400 mV, as evidenced by chromatography, MS and CD, after the compounds were acetylated with acetic anhydride–pyridine (5 : 1, v/v; 1 h, 25°C). This experiment proved that the C9 chiralities in I and II are identical and, therefore, I and II differ stereochemically only at C7. The rate of the elimination reaction of the C7 substituent of anthracyclines by electrolysis depends on the nature of the C7 leaving group. While the daunosamine moiety in Dr is rapidly cleaved, a hydroxyl group at C7 is much more stable (Malatesta et al., 1984; Berg et al., 1982). Acetylation of this 7-hydroxy function strongly promotes the conversion rate into its 7-deoxy derivative.

The major group vibrations in the IR spectrum of II (Method A) are found at 1520, 1585, 1600 and 1620 cm^{-1} (dihydroxy-anthraquinone system) and 1720 cm^{-1} (asymmetric shape, 13-C=O stretching vibration). In the OH region (Method B) maxima are present at 3483, 3570 and 3613 cm^{-1}. This is in accordance with the existence of two conformations:

(1) 9-OH function hydrogen bonded with 13-C=O; ν_{OH} = 3483 cm^{-1}
7-OH function interacted with the π electron system of the anthraquinone system; ν_{OH} = 3616 cm^{-1} (Visser et al., 1986).

(2) 9-OH function free; ν_{OH} = 3615 cm^{-1}
7-OH function hydrogen bonded with 13-C=O, ν_{OH} 3570 cm^{-1}

The shape of the 13-C=O peak at 1720 cm^{-1} is in accordance with the expected values of $\nu_{C=O}$ for conformations 1 and 2: 1710 and 1720 cm^{-1}, respectively (Renema, 1972). As the peaks at 3570 cm^{-1} (ν_{OH}) and 1720 cm^{-1} ($\nu_{C=O}$) are the most intense ones in their region, it can be concluded that conformation 2 is the predominant one. This could be expected for compound II, due to the *cis* position of the 7- and 9-substituents.

The ^{1}H-NMR spectrum of II showed signals at δ 14.40 (1H, s, ArOH), 13.30 (1H, s, ArOH), 8.05 (1H, dd, J = 1.5, J = 7.5 Hz, H(3)), 7.74 (1H, t, J = 7.5 Hz, H(2)), 7.37 (1H, dd, J = 7.5 Hz, J = 1.5 Hz, H(1)), 5.54-5.25 (1H, m, H(7)), 4.33 (1H, d, J = 1.5 Hz, OH(7)), 4.07 (3H, s, OCH_3), 3.84 (1H, s, OH(9)), 3.2–2.8 (2H, m, H(10)), 2.42 (1H, s, CH_3(14)), 2.35-2.15 (2H, m, H(8)). In comparison with the spectrum of I the H(7) has been broadened due to an increased coupling of H(7) with H(8a) indicating the axial position of H(7).

The conversion of I into II may proceed by SN^1 type of reaction initiated by protonation of the C7 hydroxyl group, leaving as water. The carbonium ion at C7 is stabilized by resonance with the adjacent anthraquinone chromophore. Successive trapping of a water molecule may result in the return of I but also in the formation of II when the nucleophilic water molecule enters from the *cis* side, with regard to the C9 acetyl

group. II can be easily converted back into I by treatment with trifluoroacetic acid 1 H; 25°C).

II is earlier reported as a side product in the total synthesis of anthracyclines (Smith et al., 1976; Kende et al., 1976; Arcamone, 1981).

Compound III

The pink coloured compound III was identified as 7,8-9,10-bisanhydrodaunorubicinone (Fig. 2). The chromatographic behaviour as well as the spectroscopic properties of III and the bisanhydro reference were identical. The compound shows a bad peak symmetry in TLC and HPLC, which is more pronounced when lower quantities are subjected to chromatography. A strong adsorptive tendency towards the stationary phase may account for this chromatographic behaviour. A HPLC chromatogram of a mixture of the degradation products, III included, is shown in Fig. 6.

Compound III is almost insoluble in methanol but dissolves in solvents such as chloroform, dimethylsulfoxide and acetone. The fully aromatized tetracyclic ring system in III leads to distinct UV-VIS spectral and fluorescence differences with regard to I (Figs. 3 and 4) and loss of chirality. Consequently, III exhibits no CD activity.

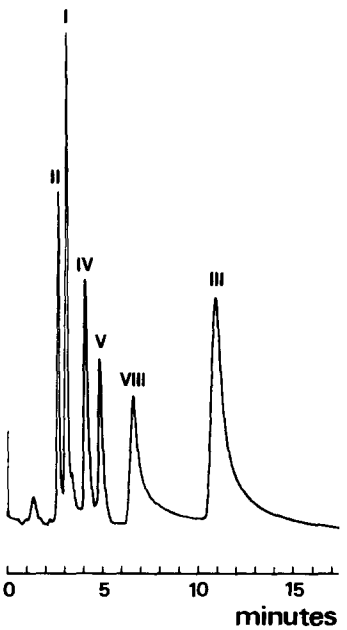

Fig. 6. HPLC chromatogram of a mixture of daunorubicin degradation products. Column: Lichrosorb 10RP8; mobile phase: acetonitrile–0.02 M sodium chloride pH 2.0 (60:40, v/v); temperature 25°C; flow rate 1.5 ml/min; fluorescence detection: $\lambda_{excitation}$ 465 nm; $\lambda_{emission}$ 550 nm.

The FD-MS spectrum showed only one peak at m/z 362 $(M)^{+\cdot}$ and the methane CI-MS spectrum 3 peaks at m/z 391 (12%), m/z 363 $(H + H)^+$ (100%) and m/z 321 $[(M + H)\text{-}COCH_2]^+$ (80%). The m/z 391 signal may be a $C_2H_5^+$ adduct with III.

In the IR spectrum of III (Method A), the quinone peaks are found at 1580 and 1615 cm^{-1}. The $\nu_{C=O}$ at 1685 cm^{-1} is characteristic for an aryl-conjugated acetyl group (Renema, 1972).

In acidic medium the conversion reaction of I into III is likely to start with protonation of the C7-OH group and subsequent cleavage as water yielding a carbonium ion at C7, just as with the formation of II. Subsequent loss of a C8 hydrogen and C9-C10 dehydration yields III. The driving force of this reaction is the high stability of the bisdehydrated end product following the full aromatization of the anthracyclinone system.

Compound IV

On degradation of Dr in acidic medium traces of IV were recovered. The chromatographic behaviour (HPLC, TLC) as well as the UV-VIS and fluorescence characteristics of this substance appeared to be identical to 7-deoxydaunorubicinone reference. By means of FD-MS the parent mass m/z 382 $(M)^{+\cdot}$ could be deduced. The yield of compound IV was too low to permit further spectral analysis. The data obtained so far indicate compound IV to be 7-deoxydaunorubicinone.

Compound V

V is also found in trace amounts. The compound elutes near III on TLC. Yet, effective separation is possible when the V containing zone is accurately scraped off and extracted with methanol. III is practically insoluble in this solvent and contaminations of III remain on the silica gel. Similarly, for the isolation of III the layer material scraped off should be washed with methanol to remove traces of V, after which elution of III with chloroform follows. Until now the structure of compound V is not completely certain.

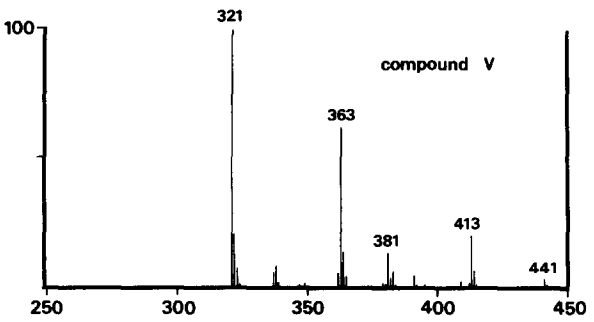

Fig. 7. Methane CI mass spectrum of compound V.

The UV-VIS and fluorescence spectra of V and I are nearly identical, indicative of their chromophoric similarities.

The CD spectrum of V shows the same characteristics as found for II. The molecular mass $(M)^{+\cdot}$ of V was found at m/z 412, with the aid of FD-MS, so 14 mass units higher than I. A high resolution mass spectrum exhibited the molecular ion at m/z 412.1165 with composition $C_{22}H_{20}O_8$.

The CI-MS spectrum of the compound is shown in Fig. 7, with fragments m/z 381 and m/z 363 corresponding to analogous fragments in the MS spectra of I/II and a molecular ion $(M + H)^+$ m/z = 413. Loss of the C9 acyl function on the m/z 363 fragment gives the ion of m/z 321. The ion at m/z = 441 may represent an adduct of V and $C_2H_5^+$, originating from the reactant gas.

The data obtained indicate compound V to be a methylated degradation product. Although the location of the methyl substituent is not yet clear, the 7-OH is favorable since during the course of degradation a reversal of the chirality at C7 has occurred demonstrating the reactivity of this function. In that case the structure V (Fig. 2) must be assigned to compound V. Further investigations are in progress to provide conclusive evidence as well as to get more insight into the mechanism of the process.

Degradation of Dr in weakly alkaline solution

Alkaline decomposition conditions were chosen at pH 8. At higher pH values a rapid, full discolouration of the solution occurs indicating a rigorous destruction of the chromophore. The degradation pattern at pH 8 is more controlled and also reproducible.

After the 4-h period at 100°C precipitates were present but dissolved readily in the extraction solvent. TLC analysis of the extracts showed 7 zones. The zone with R_f 0.05 is residual Dr.

The major degradation products were identified.

Compound VI

Compound VI is the major degradation product of Dr at pH 8. It was isolated and demonstrated identical chromatographic and spectroscopic properties as III and the reference compound 7,8,9,10-bisanhydrodaunorubicinone. The properties of the compound are summarized in the section of the paper applying to III.

Compound VII

The chromatographic (TLC, HPLC), spectroscopic (UV-VIS, fluorescence, IR, CD, MS, ^{1}H-NMR) properties of VII are identical to 7-deoxydaunorubicinone reference.

The UV spectrum of VII and I show similar maxima and minima but differ slightly which is clearly expressed in the absorptivity ratios at the maxima 234 and 255 nm. The ratio A234/A255 is for I 1.5 and for VII 1.0. A slight intensity difference was also found in the VIS spectrum. For I the ratio A476/A496 was 1.01 and for VII 0.96. These findings may indicate the existence of an interaction of the C7 hydroxyl group with the adjacent anthraquinone chromophore.

The fluorescence emission and excitation spectra of I and VII almost coincide. However, in the emission spectrum of VII the shoulder at 575 nm is less pronounced in comparison to I.

In the IR spectrum of VII (Method A) the quinone carbonyl stretching vibrations exhibit absorptions at 1585 and 1615 cm^{-1}. In the OH region (Method B) only one peak is found at 3485 cm^{-1}, in accordance with a 13-C=O bonded 9-OH group (Lutz, 1986). The $\nu_{C=O}$ of 1710 cm^{-1} corresponds with this conformation (Renema, 1972).

FD-MS showed one peak at m/z 382 related to the molecular $(M)^{+\cdot}$ ion. With CI-MS m/z values at 383 (100%), 365 (25%) and 323 (60%) were detected. The most abundant peak at m/z 383

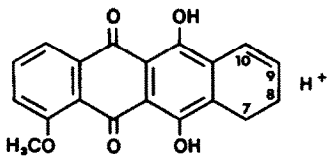

Fig. 8. Proposed structure of m/z = 323 fragment in the mass spectrum (CI) of 7-deoxydaunorubicinone.

corresponds with the protonated molecular ion. Successive loss of H_2O from C9 and C10 and the C9 acyl group yields the fragments m/z = 365 and m/z = 323, respectively. The structure of the last-mentioned fragment is illustrated in Fig. 8. In the CI mass spectrum of daunorubicinone (I) a related fragment at m/z 321, possessing double bonds between C7-C8 and between C9-C10, is observed. The presence of the C7 hydroxyl function in I allows full aromatization of the A ring by dehydration (Beijnen et al., 1985b) which is not possible for VII, due to the absence of the 7-OH function. The ^1H NMR spectrum of VII showed the following signals: δ 13.87 (1H, s, ArOH), 13.48 (1H, s, ArOH), 8.05 (1H, dd, J = 1.5, J = 7.5 Hz, H(3)), 7.78 (1H, t, J = 7.5 Hz, H(2)), 7.37 (1H, dd, J = 7.5 Hz, J = 1.5 Hz, H(1)), 4.09 (3H, s, OCH_3), 3.75 (1H, s, OH(9)), 3.22–2.83 (4H, m, H(7) + H(10)), 2.39 (3H, s, CH_3(14)), 2.14–1.82 (2H, m, H(8)).

Beforehand, the abundant presence of VII in degradation mixtures was not anticipated because this compound was earlier known to occur only by enzymatic and electrochemical reductive deglycosidation of Dr (Bachur and Gee, 1976; Malatesta et al., 1984; Berg et al., 1982). The hydroquinone form of Dr easily undergoes elimination of the sugar moiety to give the 7-deoxy compound after reoxidation. However, no reducing agents were added to the degradation medium. In Dr degradation experiments at 50°C and 70°C VII was not produced. So, high temperatures seem to be a requisite for the formation of VII at pH 8. Thermal splitting of the glycosidic bond as well as reductive deglycosidation, by which other hydroquinone anthracycline molecules act as reducing electron-donating agents, may account for our observations.

Compound VIII

Just as compound III/VI, compound VIII shows a pink colour, indicating the chromophoric (aromatized) resemblances between the two degradation products. The UV-VIS and fluorescence emission spectra of VIII, therefore, correspond to a great extend with the spectra of VI but are not identical (Figs. 3 and 4). While VI is soluble in chloroform and sparingly soluble in methanol the reverse applies to VIII. The pink colour of the compound is most obvious in methanolic solution. Both FD-MS and methane CI-MS analysis of VIII provided only one peak in each spectrum at m/z 336 $(M)^{+\cdot}$ and at m/z 337 $(M + H)^+$, respectively. In the IR spectrum of VIII (Method A), the quinone peaks are found at 1580 and 1610 cm^{-1} The non-presence of an aryl conjugated acetyl group in VIII appears from the absence of a peak around 1685 cm^{-1} in the IR spectrum.

When Dx is subjected to degradation at pH 8 a pink coloured product could also be isolated, having the same chromatographic, UV-VIS and fluorescence properties and molecular mass as VIII.

On account of the above-mentioned analytical characterization of VIII the compound is identified as 7,8-dehydro-9,10-desacetyldaunorubicinone (Fig. 2).

Full aromatization of the A ring system has occurred in VI and VIII whereby in the first case the 9-OH group is cleaved while for VIII the 9-acetyl group has disappeared. Both reactions can be considered to start with abstraction of a C10 benzylic proton in Dr. VIII has also been reported to be formed when Dr solutions (pH 7.05) are irradiated with UV light (Gray and Phillips, 1981).

When Dr is degraded under the experimental conditions VI, VII and VIII emerge in greatest quantities, on account of colour intensities. Besides, products in trace amounts could be detected. The low yield of these substances prevented full analytical characterization and structure elucidation. The so far collected data are enumerated.

Zone IX

HPLC analysis of this zone revealed the presence of two fluorescent compounds (Table 1). One

of these compounds (k′ 1.1) has identical chromatographic (TLC, HPLC) and UV-VIS properties to I. The other compound (k′ 1.3) exhibits an A254/A280 ratio of 0.6 and its UV-VIS spectrum resembles that of VIII.

Zone X

Zone X contains one compound, verified with HPLC. The k′ value of the compound appeared to be identical to that of II (Table 1). However, the TLC behaviour of these two degradation products revealed unambiguously their different identities. The UV-VIS and fluorescence spectra of I and the compound of zone X have the same shape. The CD spectra of these compounds also show a certain similarity except for the maximum around 310 nm in the spectrum of I which is absent in the case of the compound from zone X.

Zone XI

Zone XI has the same pink colour as compound VIII and the VIS spectra are similar except for the fact that the maxima of the latter show a bathochromic shift of about 5 nm. Moreover, the fluorescence emission and excitation spectra of the compounds do not differ substantially.

Concluding remarks

In this study coloured degradation products of Dr, extractable with chloroform from the degradation mixtures, were analyzed. No attempts have been made to track down the amino sugar residues. It is noteworthy that only aglycone degradation products could be traced and no compounds with an intact glycosidic linkage. The amount of aglycones in a pharmaceutical anthracycline preparation should always be limited because these compounds are devoid of antitumour activity (Driscoll et al., 1974) and suspected in eliciting cardiotoxity (Mhatre et al., 1971; Herman et al., 1974).

With the identification of some degradation products more knowledge on the degradation processes of anthracyclines has been gathered although further research is necessary. In order to form a full picture of the degradation it is essential to involve the degradation kinetics (Beijnen et al., 1985b, 1986a and b) and to complete a mass balance of the disappearance of the drug studied and the appearance of degradation products.

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