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Characterisation of Anthracyclines from a Cosmomycin D-Producing Species of *Streptomyces* by Collisionally-Activated Dissociation and Ion Mobility Mass Spectrometry

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Running Title: Multiple stages of mass analysis and ion mobility studies of anthracyclines

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Abstract

Cultures of cosmomycin D-producing *Streptomyces olindensis* ICB20 that were propagated for many generations underwent mutations that resulted in production of a range of related anthracyclines by the bacteria. The anthracyclines that retained the two trisaccharide chains of the parent compound were separated by HPLC. Exact mass determination of these compounds revealed that they differed from cosmomycin D (CosD) in that they contained one to three fewer oxygen atoms (loss of hydroxyl groups). Some of the anthracyclines that were separated by HPLC had the same mass. The location from which the hydroxyl groups had been lost relative to CosD (on the aglycone and/or on the sugar residues) was probed by collisionally-activated dissociation using an electrospray ionisation linear quadrupole ion trap mass spectrometer. The presence of anthracyclines with the same mass, but different structure was confirmed using an electrospray ionisation travelling wave ion mobility mass spectrometer.

Keywords: Anthracycline, linear quadrupole ion trap, MS^n, travelling wave, ion mobility, electrospray ionisation mass spectrometry, collisionally-activated dissociation

Introduction

Anthracyclines are natural products produced by *Streptomyces sp*. bacteria of the actinomycetes order. They consist of an anthraquinone ring system (the aglycone) with various levels of glycosylation at C7 and C10 (see Figure 1 for numbering of the ring system). The first anthracyclines, daunorubicin (daunomycin, Dn) and doxorubicin (adriamycin, Dx), were isolated from *Streptomyces peucetius* in the 1960s.[1] These anthracyclines contain one daunosamine sugar moiety (Figure 1) and have been used in the clinic for around thirty years in chemotherapy regimens against breast cancer, childhood solid tumours, soft tissue carcinomas, aggressive lymphomas (Dx) and acute lymphoblastic or myeloblastic leukaemias (Dn).[1] The anticancer activities of these compounds are thought to be the result of their ability to bind DNA. The aglycone moiety intercalates between base pairs and the sugar(s) are involved in non-covalent interactions along the minor groove.[2,3] This interaction results in the stabilization of a normally transient topoisomerase II-DNA adduct, and is proposed to interfere with DNA in the cancer cells.[4] Both the substituents on the anthracycline ring system and the nature of the sugars affect the DNA binding affinity, the DNA sequence selectivity and the efficacy against different tumour cells.[1,4,5] Removal of the sugars results in loss of activity.

Several strategies have been pursued to develop anthracyclines with a spectrum of activity against different tumours. These include screening of natural products produced by different species of *Streptomyces*,[6] synthesis or partial synthesis of new anthracyclines,[7] and genetic manipulation of *Streptomyces sp*.[8] The anthracyclines most commonly used in the clinic, daunorubicin and doxorubicin, contain only one sugar. Cosmomycin D (CosD) was first isolated from *Streptomyces cosmosus* over twenty years

ago.[6] This anthracycline has two trisaccharide chains, each consisting of a rhodinose (Rho) residue linked directly to the aglycone, 2-deoxy-L-fucose (deFuc) as the middle sugar and rhodosamine (RhN) at the terminus of each chain. This structure is less common among anthracyclines, although aclarubicin, which is also used in the clinic[1] has one trisaccharide chain (Figure 1).

In early work, CosD was shown to induce differentiation in Friend (mouse) leukemia cells under conditions where Dn and Dx had no activity,[9] suggesting that CosD was involved in a different set of intermolecular interactions and might be a compound that would have different activity in the clinic. Recently, we isolated and characterised CosD from *Streptomyces olindensis* ICB20[10] and reported a detailed study of its DNA-binding properties.[11] CosD was separated by HPLC as the major component of methanol extracts of the bacterial cultures. When the *S. olindensis* ICB20 cultures had been propagated for many generations, difficulties arose in separation of the CosD by HPLC. There were many peaks in the HPLC chromatogram. Genetic instability in *Streptomyces* is well-documented,[12] and it is these observations that encourage strategies of screening these bacterial cultures for anthracyclines with different clinical properties and genetic engineering to produce novel anthracyclines.

In the current work, a method is presented for separation of anthracyclines containing two trisaccharide chains from the mutants arising from *S. olindensis* ICB20. These were related to CosD, but had "lost" one to three hydroxyl groups from the molecule as judged by exact mass measurements using an electrospray ionisation quadrupole time-of-flight mass spectrometer. The position from which the hydroxyl group had been lost (either from the aglycone or a sugar residue) was determined by

collisionally-activated dissociation (up to MS^5) using an electrospray ionisation linear quadrupole ion trap instrument. These experiments showed that some of the anthracyclines had the same mass, but a hydroxyl group was present on the aglycone rather than on the sugar (or vice versa), or was present on different sugar residues. These results were confirmed by ion mobility mass spectrometry. The ability of the "travelling wave" (Triwave™) ion mobility instrument to separate these structural isomers is discussed.

Experimental

All reagents used were of the highest grade commercially available and MilliQ™ water was used in all experiments.

Separation of anthracycline fractions

Liquid cultures of (mutant) *Streptomyces olindensis* ICB20 were grown as previously described.[10] Methanol extracts of the culture supernatants contained a range of anthracyclines. The enhanced water solubility of those containing two trisaccharide chains over those containing fewer sugar residues was exploited by dissolving the crude extract in water, sonicating for 5 min and centrifuging in an Eppendorf microfuge (10,000 rpm, 4 °C, 15 min). The supernatant was frozen for 60 hours, thawed slowly to room temperature, and centrifuged as before. The freeze-dried supernatant was dissolved in water (50 mg/mL) and filtered using a 0.45 μm nylon syringe filter. The filtrate was extracted two times with ethyl acetate, and the aqueous phase was freeze-dried using a Savant SpeedVac. The sample was dissolved in an aqueous solution of 100 mM ammonium acetate and separated by HPLC using a Jupiter C4 column (Phenomenex, 10 x 250 mm, 5 μm, 300 Å) equilibrated at 3 mL/min with 100 mM ammonium acetate, pH

7.5, and eluted using a 0-65% acetonitrile gradient over 65 min. Fractions eluting from the column were analysed by measuring the absorbance at 254 nm. Cosmomycin D eluted at 35.2 min and was obtained as a red-purple powder after freeze-drying. Ten other fractions ranging in colour from brown-yellow to red-purple with elution times from 35 to 43 min were collected for further analysis.

Electrospray ionization mass spectrometry and exact mass of anthracyclines

The contents of fractions from the HPLC column (above) were analysed by positive ion electrospray ionization mass spectrometry (ESI-MS) using a Waters Quattro Micro ESI mass spectrometer equipped with a Z-spray ionization source and a quadrupole mass analyzer with a *m/z* range of 4,000. Samples were injected to the mass spectrometer using the instrument syringe pump at a flow rate of 20 μL/min. All spectra were acquired using an RF lens energy of 1.6 V and the cone and capillary voltages were 20 V and 3.0 kV, respectively. The source temperature was 80 °C and the desolvation gas flow rate was 250 L/hr at a temperature of 80 °C. Spectra were acquired in positive ion mode over the range *m/z* 300-2000 and typically 30 scans were summed to obtained a representative spectrum.

The exact masses of anthracyclines in the fractions were determined using a Waters Synapt™ HDMS ESI mass spectrometer equipped with a lockspray attachment using CsI as the standard, a Z-spray ionization source and a quadrupole mass analyzer with a *m/z* range of 4,000. Samples were prepared by dissolving the compounds in a 100 mM solution of ammonium acetate, pH 7.5, with 10 % methanol (to assist desolvation) at a concentration of ~10 μM, then injected to the mass spectrometer using a Harvard Model 22 syringe pump (Natick, MA, USA) at a flow rate of 10 μL/min. All spectra were

acquired using a MCP potential of 1800 V, capillary voltage of 2.5 kV and the sampling and extraction cone voltages were 80 V and 4 V, respectively. The source temperature was 80 °C and the desolvation gas flow rate was 400 L/hr at a temperature of 200 °C. Spectra were acquired in positive ion mode over the range *m/z* 400-2000 and typically 40 acquisitions were summed and the corrected mass was obtained by adjusting the values to the CsI reference ion at *m/z* 1172.1450 Da. The elemental composition was determined by using the MassLynx elemental composition calculator. The instrument was calibrated using a CsI solution (1 mg/mL) over the same *m/z* range.

Collisionally-activated dissociation of anthracyclines

Fragmentation of anthracyclines in each HPLC fraction was performed using a ThermoFisher LTQ linear quadrupole ion trap mass spectrometer equipped with an ESI source. Anthracyclines (20 μM) were injected into the instrument at a flow rate of 5 μL/min. Positive ion ESI mass psectra were acquired using a source voltage, capillary voltage and temperature of 3.71 kV, 60 V and 49 °C, respectively. For all MS^n (n = 1-5) experiments, the isolation width was 2-3 Th, CAD fragmentations were induced using a collision energy of 20-40 V, and the typical activation time was 30 ms. Spectra were acquired over the range *m/z* 350-1300 and 100 scans were summed to obtain a representative spectrum.

Ion mobility ESI mass spectrometry of anthracyclines

The ion mobilities of anthracyclines from HPLC that had the same exact mass were analysed using the Waters Synapt™ HDMS ESI mass spectrometer (see above). All samples were prepared in 100 mM ammonium acetate, pH 7.5, at a concentration of ~5 μM, and injected to the mass spectrometer using the instrument syringe pump at a flow

rate of 5 μL/min. All spectra were acquired using a MCP potential of 1800 V, capillary voltage of 2.0 kV and the sampling and extraction cone voltages were 20 V and 4 V, respectively. The source temperature was 80 °C and the desolvation gas flow rate was 400 L/hr at a temperature of 200 °C. The Triwave™ settings used were as follows: the trap and transfer collision energies were 6.0 V and 4.0 V, respectively, the IMS gas flow was 25 mL/min, the trap DC entrance, bias and exit were 5.0 V, 27.0 V and 5.0 V, respectively; IMS DC entrance and exit voltages were 5.0 V and 2.0 V, the transfer DC entrance and exit were both 2.0 V, and the IMS wave velocity and height were 350 m/s and 14 V, respectively. Spectra were acquired in positive ion mode over the range *m/z* 1000-1300 and 35 acquisitions were summed to obtain a representative spectrum; all drift time results were expressed in ms. The instrument was calibrated using a CsI solution (1 mg/mL) over the range *m/z* 500-2000.

Results and Discussion

HPLC separation of anthracyclines

Propagation of *Streptomyces olindensis* ICB20 cultures that had originally produced CosD as the major anthracycline mutated over time to produce cultures that synthesised many different anthracycline molecules. Water-soluble material from methanol extracts of these mutant cultures were cleaned by extraction of less soluble anthracyclines into ethyl acetate, and the resulting compounds were separated by HPLC on a C4 column. Figure 2 shows the HPLC elution profile obtained. Fractions ranged in colour from yellow-brown through to red-purple. The elution profile of fractions of interest containing anthracycline molecules with two trisaccharide chains is expanded (inset). A positive ion

ESI mass spectrum of fraction A showed an ion of high abundance at *m/z* 594.8 ($[CosD+2H]^{2+}$) and another at low abundance corresponding to the molecular ion, $[CosD+1H]^{1+}$, at *m/z* 1189.6. The anthracyclines in other fractions had masses consistent with CosD minus one to three hydroxyl groups (-16 for each), with positive ions in the ESI mass spectra at *m/z* 1173.6 (fractions B, C, E, F, H), 1157.6 (fractions D, G, I, K) and 1141.6 (fraction J).

Exact masses of the anthracyclines

The exact masses of the anthracycline in each of the fractions were determined in order to compare these with the calculated masses based on the proposed molecular formulae of the anthracyclines. Table 1 shows the proposed molecular formula for each fraction, its calculated mass, the experimental exact mass and the difference in ppm. In all cases the difference between the calculated and measured masses was within 3 ppm. Pure CosD subjected to elevated temperatures (*e.g*. 40 °C) in the presence of water was subject to degradation, undergoing hydrolysis of the sugar residues. This process was catalysed under acid conditions (not shown). Since the masses of the anthracyclines purified in this work corresponded to molecules that contained two *intact* trisaccharide chains, this supports that the array of anthracyclines separated by HPLC were produced by the bacteria, rather than as a result of sample degradation. Some anthracyclines separated by C4 chromatography had the same mass, suggesting that the hydroxyl group(s) that had been "lost" from CosD (*e.g.* one hydroxyl group in the case of anthracyclines with mass 1173.6) may have been lost in some cases from the sugars, or in other cases from the aglycone, accounting for their significantly different chromatographic behaviour.

Collisionally-activated dissociation of the anthracyclines

There have been many dissociation studies of anthracyclines possessing a variety of sugar chains differing in their length, composition and position of attachment using precursor ions generated by an array of different techniques such as electron ionisation,[13] chemical ionisation,[14] field desorption,[15] and fast atom bombardment.[16] Furthermore, dissociation mechanisms have been proposed for CosD based on negative ion desorption chemical ionization[17] and positive ion field desorption ionization tandem mass spectrometry.[18] Recently, the first positive ion electrospray ESI-MS/MS experiments have been carried out on doxorubicin, daunorubicin and the structurally similar epirubicin and idarubicin using a triple quadrupole mass spectrometer.[19] In the current work, a linear quadrupole ion trap ESI mass spectrometer was used to distinguish among the anthracycline fractions separated by HPLC. In order to determine whether it was the aglycone and/or the sugars which were altered from the structure of CosD, each fraction was subjected to multiple stages of collisionally-activated dissociation (CAD). Figure 3 shows positive ESI-MS/MS (MS^2) spectra obtained for CosD (A) and for the anthracyclines in fractions B (B) and C (C) from the HPLC. The precursor ions ($[M+1H]^{1+}$) for CosD, fractions B and C were 1189.4, 1173.2 and 1173.3, respectively. The product ions from CosD were: 1075.2 (low intensity, -114, consistent with the loss of one of the outermost sugars, 1 or 1′ from Figure 1, Rho); 945.3 (high intensity, -130, consistent with the loss of a middle sugar, 2 or 2′, deFuc); 831.3 (low intensity, -114, consistent with the loss of the outermost sugar on the second trisaccharide chain, 1′ or 1, Rho); 701.3 (high intensity, -130, consistent with the loss of the middle sugar from the second chain, 2′ or 2, deFuc), and 544.3 (low

to moderate intensity, -157, consistent with loss of one of the innermost amino sugars, 3 or 3′, RhN). The relative intensities of the product ions suggest that the glycosidic bond between sugars 2 and 3 (or 2′ and 3′) is more labile under the conditions than the bond between sugars 1 and 2 (or 1′ and 2′). Table 2 shows the product ions obtained in MS^n experiments and gives the relative intensities of each ion in brackets.

Collisional activation (MS^3, 22 V) of the ion at 544.3 corresponding to the CosD aglycone with 1 RhN sugar remaining gave product ions at 369.0, 351.0 and 323.2. The former is consistent with an aglycone moiety that has lost the OH, most likely from C7, concomitant with dissociation of the last amino sugar from the aglycone. This could result in formation of a double bond between C7 and C8 of ring A, or may be the result of loss of the amino sugar followed by a rapid spontaneous loss of water. The ions at 351.0 and 323.2 are consistent with loss of a second water molecule from the side chain of ring A followed by ethylene. Similar neutral losses (*e.g.* H_2O, CO) from the side chain of ring A of doxorubicin, daunorubicin, epirubicin and idarubicin have previously been observed using a triple quadrupole ESI mass spectrometer[19] as well as in the current work when daunorubicin and doxorubicin were subjected to CAD for comparison with CosD (not shown).

Figures 3(B) and (C) show the MS^2 spectra of the anthracyclines from fractions B and C which possess an identical molecular mass giving molecular ions at 1173.2 and 1173.3, respectively. The fragmentation patterns/sequences of these compounds were markedly different confirming that they differ in structure. The differences could arise from a difference in either the sequence of the sugars in the trisaccharide chains or the number and positions of the oxygen group on the molecule (sugar or aglycone). Since

these anthracyclines have similar retention times when separated by HPLC (Figure 2), it is likely that they possess a general structure resembling CosD. Further, given that the main fragmentation pathway for CosD involves the cleavage of the glycosidic bonds where the 2-3 and 2′-3′ bonds are the most labile, and that charge is retained on the aglycone, the sequence of sugars in compounds B and C can be proposed. Collisionally-activated dissociation of the precursor ion of compound B at 1173.2 gave 1059.1 (-114) → 929.3 (-130) → 815.4 (-114) → 685.3 (-130) → 528.3 (-157) → 334.9 (MS^3; -157 and -H_2O). These dissociation products are consistent with compound B bearing the same sugar residues as CosD, but having a different aglycone. The aglycone of compound B gave a product on MS^3 at 334.9 compared with 351.0 for CosD. This is consistent with the aglycone of compound B containing one fewer hydroxyl groups than CosD. Compound C dissociated via a similar fragmentation pathway suggesting loss of sugar residues, but in this case, the products are consistent with one of the middle sugars (2 or 2′, see Figure 1) having one less hydroxyl group. The product ions from 1173.3 for compound C were 1059.1 (-114) → 945.1 (-114) → 831.2 (-114) → 701.2 (-130)→ 544.3 (-157) → 351.1 (MS^3; -157 and -H_2O). The final product at 351.1 suggests that the aglycone is the same as that for CosD. This however may not necessarily be true, since the aglycone may have the same mass, but may differ in the position of hydroxyl groups. In some cases (*e.g.* fractions C, E, F and H) molecules possessing the same dissociation sequence and the same product ions were well-separated by HPLC indicating that even if the sugar has the same molecular mass they might be somehow different (different position or stereochemistry of functional groups on the sugar, different conformation).

Table 2 summarises the results obtained for the CAD (MS^2 and MS^3) of all the anthracyclines present in the HPLC fractions (A to K). The table indicates the masses of all product ions observed in MS^2 and their intensities, and the mass of the aglycone after loss of water and ethylene in some cases. It also shows the proposed number and position (sugar or aglycone) of hydroxyl groups that were "lost" compared to the structure of CosD. For all the fractions analysed this way, each of the major product ions (corresponding to the loss of one or more sugars) were selected for further CAD experiments (up to MS^5) confirming the sequence of sugars observed in the MS^2 and MS^3 experiments. These experiments were also carried out with the goal of determining whether the aglycone could be dissociated to gather supplementary information on the position its hydroxyl groups. No further fragmentation of the aglycone was observed beyond that described for CosD. The current MS^n experiments have enabled some conclusions to be made about the sugars and aglycones present on these natural products using only small amount of material. Full structural determination will require NMR experiments, but at present these are not possible since these natural products were obtained in less than milligram quantities.

Ion mobility ESI mass spectrometry of the anthracyclines

Since structural isomers of the anthracyclines were separable by HPLC, experiments were carried out to determine whether these were also separable by ion mobility mass spectrometry (IMMS). The instrument used was a Waters Synapt™ HDMS which employs Triwave™ technology. In this mobility cell, a voltage pulse along rings of stacked electrodes generates a travelling voltage wave on which ions can "surf".[20,21] Ions

with a greater collisional cross section will tend to fall behind the waves. Anthracyclines from the different HPLC fractions were analysed by IMMS separately, and after mixing aliquots from fractions containing anthracyclines with the same mass as judged by the exact mass measurements (Table 1). The drift times for the different anthracyclines are also shown in Table 1. Some of the fractions of the same mass had different drift times. For example, compounds B and H both have molecular ions at *m/z* 1173.6, but their drift times are 3.98 and 4.95 ms, respectively. Figures 4A and B show the drift and mobility plots that were obtained when fractions B and H were mixed together. The two anthracyclines are clearly resolvable. Figure 4C shows the ESI mass spectrum of the compound that gives rise to each peak in the mobility plot (B), confirming that they have the same elemental composition. Anthracyclines in fractions E and F were separable by HPLC (Figure 2) and had different drift times (4.43 and 3.92 ms, respectively), but had similar CAD mass spectra (Table 2). This is consistent with these two compounds bearing the same sequence of sugars, with differences arising possibly from different conformations of the sugars. In contrast, the compounds in fractions B and C were proposed to have different aglycones and a middle sugar differing by one hydroxyl group as judged by CAD mass spectra. These compounds were separable by HPLC, but not by ion mobility under the conditions of the current experiments. It is likely that the conformation of the sugars has a greater effect on mobility than the presence or absence of a hydroxyl group. It will be interesting to correlate the structures of these anthracyclines (once determined) with their drift times. This will be the subject of a more extensive investigation.

Conclusions

Streptomyces sp. bacteria produce a vast array of natural products. Some of the anthracyclines that they produce are potent anticancer chemotherapeutics. In an effort to discover new anthracyclines with activities against different types of cancer, fewer side-effects, and to counter the development of resistance of tumours to chemotherapy regimens, a range of strategies has been investigated. The most promising methods involve genetic manipulation of the bacteria.[22] These methods have produced novel hydroxylated and glycosylated anthracyclines as a result of relaxed substrate specificity of the enzymes involved in anthracycline biosynthesis (*e.g*. oxygenases and glycosyltransferases).[22] Previously we showed that *Streptomyces olindensis* ICB20 produced CosD. Spontaneous mutations of this bacterium resulted in production of a range of anthracyclines with similar glycosylation patterns to CosD in that they contained two trisaccharide chains linked to the aglycone, but which varied in the numbers of hydroxyl groups on the aglycone and/or the sugars. Application of several mass spectrometric methods (using electrospray ionization) enabled preliminary characterisation of these anthracyclines. Exact mass determination confirmed the elemental composition of the compounds, and MS^n experiments enabled insight into the positions from which hydroxyl groups had been 'lost' from the original CosD anthracycline. Since the two trisaccharide chains of CosD (and most of the related anthracyclines investigated here) are the same (or very similar), it was not possible to determine whether the first sugar residues that were dissociated in the CAD experiments were lost from the trisaccharide attached to C7 or C10 (Figure 1). Since travelling wave ion mobility mass spectrometry is relatively new, it was of interest to determine whether

some of the anthracyclines of the same mass could be separated in this mobility cell. The results were consistent with the proposal that the conformation of the sugars has a greater effect on mobility than the presence or absence of a hydroxyl group. A full understanding of the relationship among the structure of the anthracyclines and drift time in the travelling wave ion mobility mass spectrometer awaits full structural determination of these anthracyclines by NMR spectroscopy. These experiments will be challenging since the large number of anthracyclines produced meant that each was purified in only small quantities. The combination of mass spectrometric techniques used here enabled preliminary characterisation of the compounds using only very small amounts of material.

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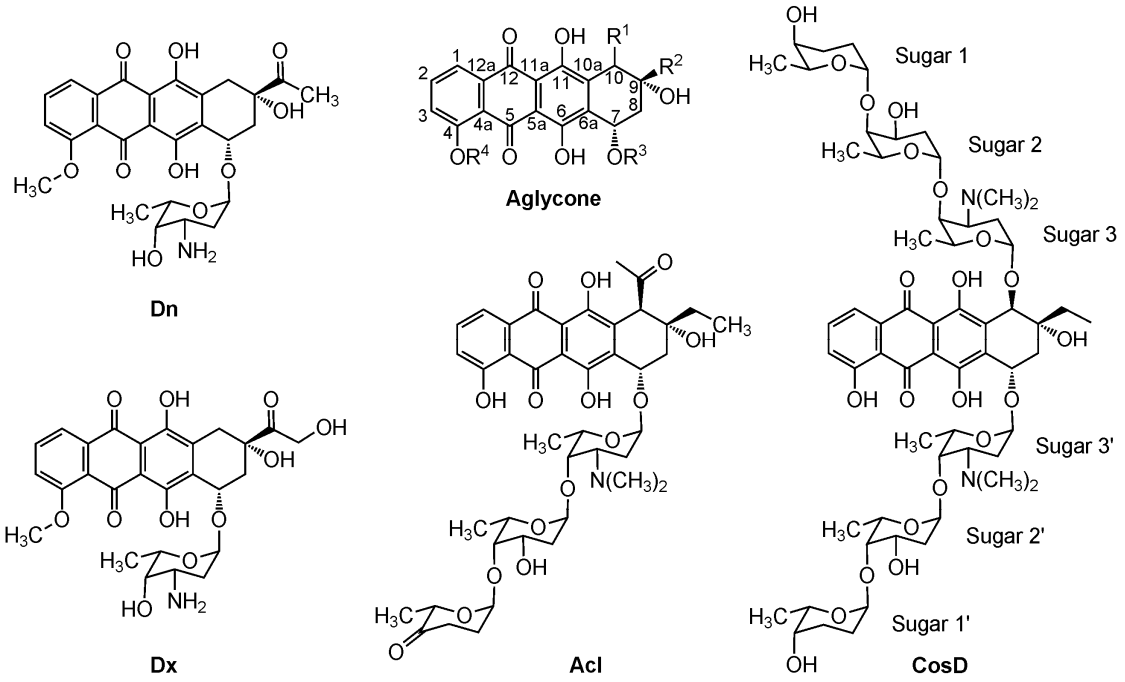

Figure 1: Structures of some anthracyclines. Daunorubicin (Dn, daunomycin), doxorubicin (Dx, adriamycin), aclarubicin (Acl) and cosmomycin D (CosD). The sugars on CosD are numbered: Sugar 1 and 1′ is rhodinose (Rho); sugar 2 and 2′ is 2-deoxy-L-fucose (deFuc); sugar 3 and 3′ is rhodosamine (RhN). The numbering system for aglycone ring system is shown in the top centre of the Figure.

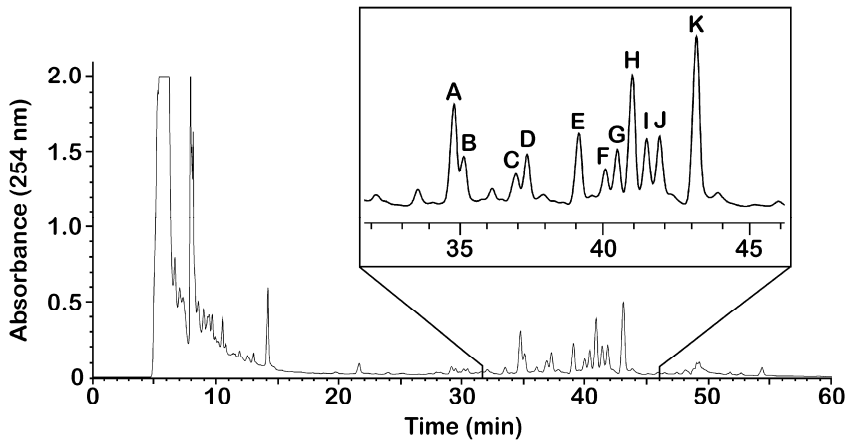

Figure 2: HPLC elution profile of water-soluble fractions from *Streptomyces* culture supernatants. The fractions eluting between 32 and 46 min are shown in detail (inset). The fractions are labeled A-K and fraction A is CosD.

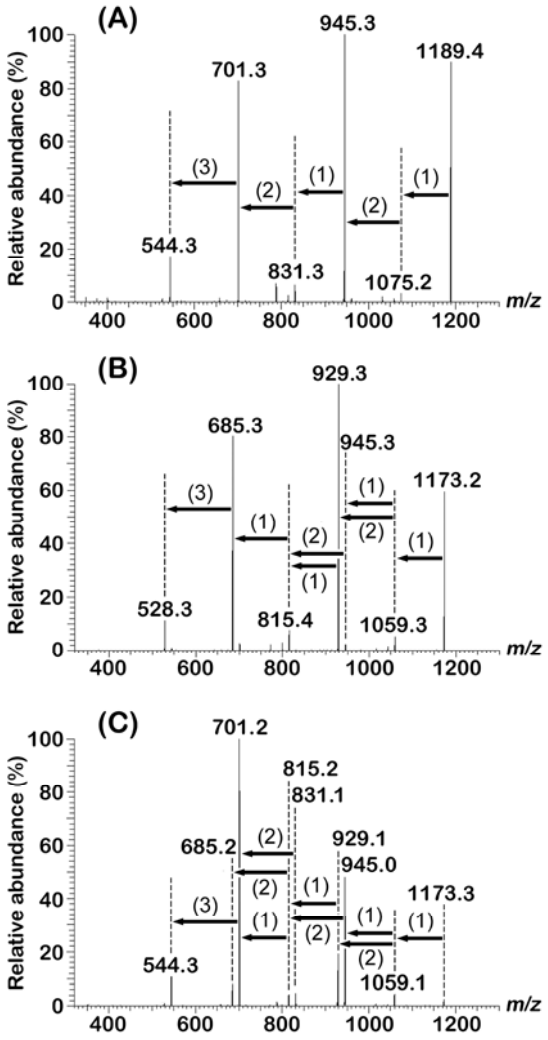

Figure 3: ESI-MS/MS (MS^2) of anthracycline fractions from HPLC. (A) CosD; (B) Fraction B; (C) Fraction C. The activation energies applied to give the spectra in A, B and C were 35, 36 and 38 V, respectively. The dotted lines were drawn to extend the intensities of the observed peaks so that the arrows indicating loss of fragments could be drawn. The numbers (1, 2, 3) on top of the arrows refer to the sugars (Figure 1) that are lost from the structures. Note that it is not possible to distinguish among sugar 1 and1′, or 2 and 2′ or 3 and 3′.

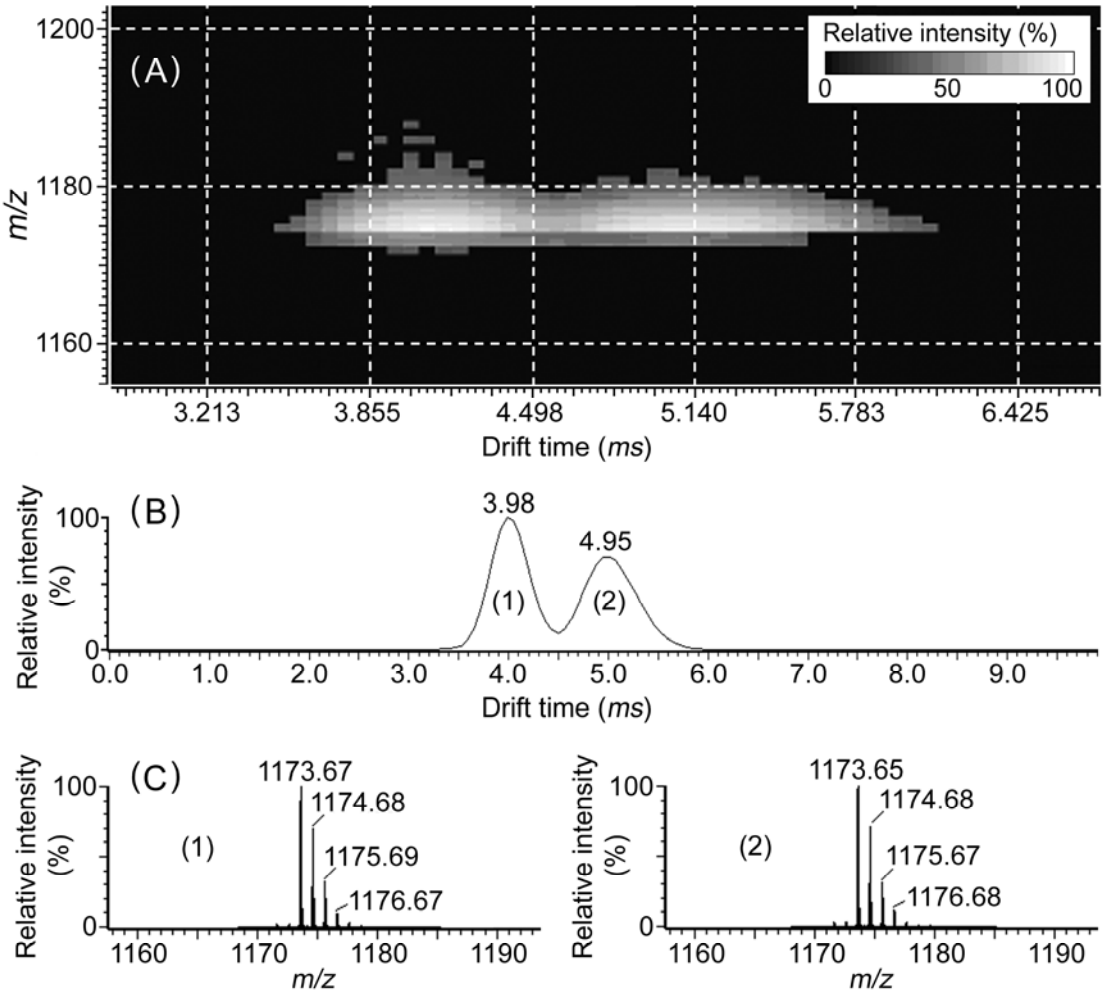

Figure 4: Waters Synapt™ HDMS ion mobility mass spectra of a mixture of fractions B and H. A. drift plot of a mixture of the two fractions. B. Mobility of the two anthracyclines. The ion (1) with a drift time of 3.98 ms was confirmed as fraction B in experiments where each fraction was analysed separately, and ion (2) at 4.95 ms was from fraction H. C. ESI mass spectra of peaks at drift time 3.98 ms (1) and 4.95 ms (2).

Figure Legends

Figure 1: Structures of some anthracyclines. Daunorubicin (Dn, daunomycin), doxorubicin (Dx, adriamycin), aclarubicin (Acl) and cosmomycin D (CosD). The sugars on CosD are numbered: Sugar 1 and 1′ is rhodinose (Rho); sugar 2 and 2′ is 2-deoxy-L-fucose (deFuc); sugar 3 and 3′ is rhodosamine (RhN). The numbering system for aglycone ring systems is shown in the top centre of the Figure.

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Table 1. Exact mass determination and drift times of the anthracyclines contained in the different fractions separated by HPLC.

Fraction name	Predicted formula	Calculated mass (Da)	Experimental mass (Da)	Error (mDa)	Error (ppm)	Drift time (ms)
A	$C_{60}H_{89}N_2O_{22}^+$	1189.5907	1189.5922	1.5	1.3	4.56
B	$C_{60}H_{89}N_2O_{21}^+$	1173.5958	1173.5933	-2.5	-2.1	3.98
C	$C_{60}H_{89}N_2O_{21}^+$	1173.5958	1173.5940	-1.8	-1.5	3.98
D	$C_{60}H_{89}N_2O_{20}^+$	1157.6009	1157.5990	-1.9	-1.6	3.98
E	$C_{60}H_{89}N_2O_{21}^+$	1173.5958	1173.9620	0.4	0.3	4.43
F	$C_{60}H_{89}N_2O_{21}^+$	1173.5958	1173.5966	0.8	0.7	3.92
G	$C_{60}H_{89}N_2O_{20}^+$	1157.6009	1157.5978	-3.1	-2.7	3.86
H	$C_{60}H_{89}N_2O_{21}^+$	1173.5958	1173.5979	2.1	1.8	4.95
I	$C_{60}H_{89}N_2O_{20}^+$	1157.6009	1157.6002	-0.7	-0.6	4.30
J	$C_{60}H_{89}N_2O_{19}^+$	1141.6060	1141.6079	1.9	1.7	4.30
K	$C_{60}H_{89}N_2O_{20}^+$	1157.6009	1157.5984	-2.5	-2.2	5.20

Table 2. Main product ions observed in the MS^2 and MS^3 spectra of the anthracyclines in fractions A to K with their relative abundances (RA %), and proposed assignment of the position(s) from which –OH group(s) were 'lost' from CosD.

	Compound										
	A	B	C	D	E	F	G	H	I	J	K
Collision activation energy applied (MS^2)	35 V	36 V	38 V	39 V	37 V	36 V	36 V	37 V	36 V	37 V	38 V
$[M+H]^+$	1189.4 (90)	1173.2 (59)	1173.1 (2)	1157.3 (39)	1173.3 (26)	1173.3 (36)	1157.2 (9)	1173.3 (38)	1157.2 (11)	1141.3 (62)	1157.1 (13)
$[M–114+H]^+$	1075.2 (5)	1059.3 (5)	1059.1 (5)	1043.1 (6)	1059.2 (3)	1059.2 (5)	1043.3 (6)	1059.5 (2)	1043.2 (17)	1027.3 (3)	1043.5 (2)
$[M–2\times114+H]^+$	-	945.3 (2)	945.1 (48)	929.1 (61)	945.2 (78)	945.1 (98)	929.2 (100)	945.2 (100)	929.1 (7)	913.2 (94)	929.2 (86)
$[M–114–130+H]^+$	945.3 (100)	929.3 (100)	929.1 (19)	913.1 (22)	929.2 (26)	929.1 (11)	913.2 (9)	929.2 (14)	-	-	-
$[M–3\times114+H]^+$	-	-	831.2 (5)	815.2 (10)	831.3 (3)	831.3 (4)	815.3 (4)	831.3 (3)	815.3 (6)	799.2 (6)	815.4 (4)
$[M–2\times114–130+H]^+$	831.3 (7)	815.4 (8)	815.2 (4)	799.2 (7)	815.3 (3)	815.3 (4)	799.2 (3)	815.3 (3)	-	-	-
$[M–4\times114+H]^+$	-	-	-	-	-	-	-	-	701.3 (100)	685.3 (100)	701.2 (100)
$[M–3\times114–130+H]^+$	-	-	701.2 (100)	685.2 (100)	701.3 (100)	701.3 (100)	685.3 (93)	701.3 (83)	685.4 (4)	-	-
$[M–2\times114–2\times130+H]^+$	701.3 (83)	685.3 (80)	685.3 (8)	-	-	-	-	-	-	-	-
$[M–4\times114–157+H]^+$ [a]	-	-	-	-	-	-	-	-	544.3 (8)	528.2 (12)	544.2 (13)
$[M–3\times114–130–157+H]^+$ [a]	-	-	544.2 (11)	528.2 (14)	544.3 (19)	544.3 (10)	528.3 (10)	544.3 (13)	-	-	-
$[M–2\times114–2\times130–157+H]^+$ [a]	544.3 (17)	528.3 (11)	-	-	-	-	-	-	-	-	-
Collision activation energy applied (MS^3)	22 V	24 V	22 V	24 V	22 V	23 V	23 V	24 V	24 V	23 V	21 V
Aglycone – H_2O [b]	369.0 (64)	-	369.2 (7)	-	369.1 (80)	369.1 (16)	-	369.0 (100)	369.0 (6)	-	369.1(45)
Aglycone – 2 H_2O [b]	351.0 (100)	334.9 (100)	351.1 (100)	335.0 (100)	351.1(100)	351.1 (100)	335.0 (100)	351.6 (38)	351.1 (100)	335.0 (100)	351.1 (33)
Aglycone – 2 H_2O – CH_2=CH_2 [b]	323.2 (33)	-	323.1 (15)	307.2 (12)	323.2 (10)	323.2 (22)	-	323.1 [c]	323.2 (19)	307.4 [c]	323.2 (9)
Position of the 'missing' oxygen	Number of 'missing' oxygens										
on sugar 2 or 2' [d]	-	-	1	1	1	1	1	1	2	2	2
on the aglycone	-	1	-	1	-	-	1	-	-	1	-

[a] m/z selected to obtain the aglycone fragment by MS^3
[b] obtained by MS^3 of the fragment selected in [a]
[c] observed by MS^4 experiments
[d] see Figure 1