Structural Investigations of Gossypol Schiff's Bases

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Received: August 27, 1990.

Cálculos teóricos prevêm que o tautômero enamina-cetona da base de Schiff do Gossipol é o mais estável, em concordância com a espectroscopia de RMN ^{13}C e Efeito Nuclear Overhouser em RMN ^{1}H. Derivações com aminas fluorescentes foram investigadas como uma maneira de melhorar a sensibilidade de análise de traços de gossipol, as novas bases de Schiff sintetisadas foram estudadas por HPLC, espectroscopia de massa FAB e fluorimetria.

Theoretical calculations predict the amino-enone tautomer of gossypol Schiff's bases to be the most stable, in agreement with the structure assigned to dianilinogossypol by high field NMR, nOe difference spectroscopy and ^{13}C NMR spectroscopy. Derivatization with fluorescent amines has been investigated as a means of improving the sensitivity of trace analysis of gossypol and the newly synthesised Schiff's bases were studied by HPLC, Fast Atom Bombardment mass spectrometry and fluorimetry.

Key words: *gossypol, Schiff's bases, tautomerism.*

Introduction

Gossypol **1** is a symmetrical binaphthyl dialdehyde and readily forms Schiff's bases with primary aromatic and aliphatic amines. Historically, these Schiff's bases have played a number of important roles: for example, the Schiff's base formed with aniline, known as dianilinogossypol **2: X = NPh**, has been used in the purification of crude gossypol isolated from the cotton plant and, together with other aromatic analogues, has been used in colourimetric assays of gossypol content in products such as cotton seed oil and cotton meal animal feeds[1]. More recently, it has been shown that Schiff's bases of gossypol, unlike gossypol itself, can be resolved on chiral HPLC columns[2] and, furthermore, that Schiff's bases with optically pure, chiral amines can be resolved on non-chiral chromatographic stationary phases[3]. These resolutions of the Schiff's bases have been carried out on a large scale and, after hydrolysis, have liberated large quantities of the individual gossypol isomers[4], biological investigations of which have shown that actvities such as antifertility[5,6], antitumor[7-9], antiamoebic[10] and anti-HIV1[11] properties reside mainly or entirely in the (−) − gossypol enantiomer.

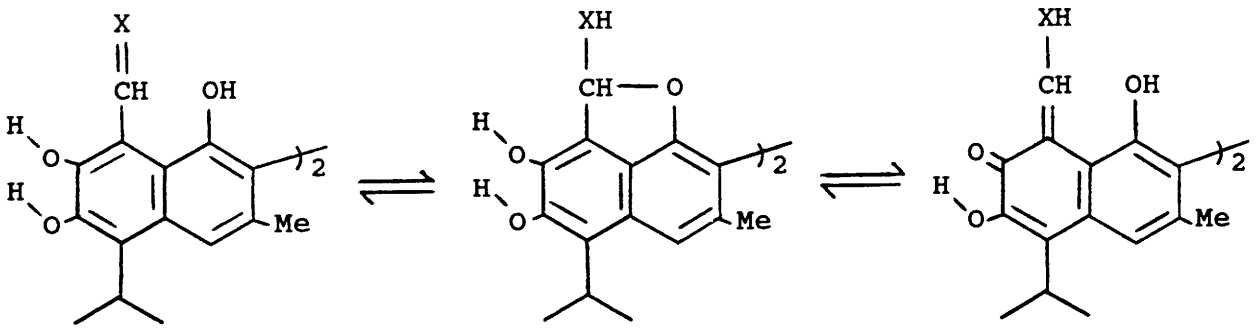
It is notable that, despite these important contributions which gossypol Schiff's bases have made to studies and applications of gossypol, there remains a degree of uncertainty about their exact structure, as a result of the complicating factor of tautomerism.

In gossypol itself, there are three principal tautomeric forms (Scheme 1), although the picture is further complicated by the fact that the hydroxy-enone tautomer **1c** can have two geometric isomers about the double bond and the lactol tautomer **1b** can have R or S configuration at the ketal chiral centre.

In Schiff's bases such as dianilinogossypol, similar tautomeric possibilities arise (Scheme 1). Prior to the introduction of modern spectroscopic techniques, Adams, Geissman and Edwards[1] suggested that dianilinogossypol, formed either via the condensation of gossypol with aniline or of apogossypol with diphenyl formamidine, has the hemiaminal structure **2b; X = NPh**. However, NMR data now available can be interpreted only in terms of the forms **a** or **c** (Scheme 1). The ^{13}C NMR spectrum of gossypol has been analysed in detail[12] and indicates the aldehyde tautomer **1a** to be present in deuterated acetone solution. More recently, a Chinese group[13] has discussed the ^{13}C and 90 MHz ^{1}H NMR spectra of the Schiff's base **2; X = NCH(Me)CH$_2$Ph** in $CDC\ell_3$, in terms of the possible tautomersf **2a** and **2c**, drawing attention to the observation of a low-field carbon signal (172.87 ppm) attributed to a carbonyl group and of a coupled CH-NH group (δ 9.5 and 13.5, the latter exchangeable in D_2O, wich supports the assignment of the form **2c**. These spectra were complicated by the presence of the configurationally unstable and rapidly equilibrating diastereoisomers resulting from the use of the chiral secondary alkyl amine.

We now report the results of a theoretical examination of tautomeric stability of some model compounds and an investigation of the tautomeric structure of dianilinogossypol, using modern NMR techniques to make unambiguous assignments of relevant signals.

As a result of the growing interest in the pharmacological properties of gossypol[5-11], sensitive and selective methods are needed for its trace detection in biological fluids



1a X = O
2a X = NR

1b X = O
2b X = NR

1c X = O
2c X = NR

Scheme 1

and tissues. Currently used HPLC methods for the detection of gossypol itself or of HPLC-resolved, diastereomeric Schiff's bases for separate determination of the enantiomers rely on UV or electrochemical methods[14-16]. These methods have sensitivities in the ng/mℓ range for gossypol in serum. Since fluorescence offers the potential of improving sensitivity by several orders of magnitude over UV detection[17], a series of Schiff's bases of gossypol with fluorescent amines was also prepared and preliminary studies made of their properties.

Experimental

Theoretical calculations on model structures **4** and **5** were performed on a VAX 11-750 computer by the AM1 method[18] in the modified self-consistent Reaction Field (SCRF) approach to take account of solvent effects[19].

All mps are uncorrected (Koffler apparatus). Infrared spectra were recorded on a Perkin Elmer 3600 spectrophotometer, all as KBr discs. Fluorescence spectra were obtained on MeOH or $CH_2Cℓ_2$ solutions with an Aminco-Bowman 34-8960a spectrofluorimeter. 100 MHz ^1H NM spectra were recorded on a Jeol MH100 spectrometer, 270 MHz ^1H and 67.8 MHz ^{13}C NMR spectra were recorded on a Jeol G270 machine and 360 MHz ^1H NMR spectra on a Bruker WH-360 machine, all in $CDCℓ_3$ and are reported in parts per million (δ) downfield from tetramethylsilane internal standard. FAB-MS were obtained on a VG Micromass ZAB-E mass spectrometer.

HPLC analyses were performed with a Waters 6000A pump operating at 2.0 mℓ/min, Rheodyne 7125 injector fitted with a 20 μℓ loop and a Waters 441 UV detector set at 254 nm x 0.5 AUfsd. Columns were 25 x 0.45 cm i.d., with stationary and mobile phase conditions as given in Table 4.

(±) - Gossypol-acetic acid was supplied by the World Health Organization. Amines used for Schiff's base formation were obtained from Aldrich. Dianilinogossypol **6** was prepared by the literature method[1,20].

General procedure for the preparation of Schiff's bases: (±) - Gossypol-AcOH (110mg, 0.19 mmoℓ) and the amine (0.58 mmoℓ) were combined in a suitable solvent (25mℓ) and stirred at room temperature for 24h. The product was collected as a precipitate or by evaporation and was washed and recrystallized.

Gossypol-2-amino-9-fluorenone Schiff's base 7: After reaction in $CH_2Cℓ_2$, the product was collected by evaporation and recrystallized from MeOH, 65% yield; mp >320°C; ν_{max} 3488-2955, 1716, 1615 cm^{-1}; δ(WH-360) 1.46 (2x6H, d) and 3.76 (2x1H, m)(Me_2CH), 15.05 (2x1H, bd NH) ppm.

Gossypol-1-naphthylamine Schiff's base 8: After reaction in i-PrOH, the product was collected by evaporation and recrystallized from $CH_2Cℓ_2$-MeOH, 82% yield; mp = 282°C; ν_{max} 3465, 3317-3117, 1626 cm^{-1}; δ(WH-360) 1.46 (2x6H, d) and 3.76 (2x1H, m) (Me_2CH), 10.50 (2x1H, m) and 15.10 (2x1H, m) (CH-NH) ppm.

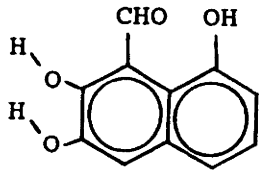
Gossypol-2-aminoquinoline Schiff's base 9: After reaction in MeOH, the precipitate was recrystallized from MeOH, yield 92%; mp >320°C; ν_{max} 3600-3000, 1600 cm^{-1}; δ(WH-360) 1.49 (2x6H, d) and 3.78 (2x1H, m)(Me_2CH), 2.03 (2x3H, s, Me-Ar), 7.57 (2x1H, s, H-Ar), 8.35 (2x1H, bs, HO-Ar), 8.79 (2x1H, bs, HO-Ar), 10.60 (2x1H, d, 10.6Hz) and 16.12 (2x1H, d, 10.6Hz) (CH-NH), and quinoline protons at 7.56-8.26 (2x7H) ppm; m/z (+ve FAB, 3-nitrobenzyl alcohol matrix): see Results and Discussion.

Gossypol-fluoreseceinamine (I) Schiff's base 10: After reaction in i-PrOH, the product was collected by evaporation and recrystallized from MeOH, 84% yield; mp >320°C; ν_{max} 3329-2950, 1725 cm^{-1}; δ(WH-360) 1.46 (2x6H, d) and 3.77 (2x1H, m)(Me_2CH), 10.07 (2x1H, m), and 15.00 (2x1H, m) (CH-NH) ppm; m/z (+ve FAB, thioglycerol matrix): see Results and Discussion.

Results and Discussion

For theoretical calculations, the structure of gossypol was simplified to that of 2,3,8-trihydroxy-1-naphthaldehyde **3** by deletion of the methyl and isopropyl groups and breakage of the internaphthyl bond. These modifications are justified by the non-polar nature of the alkyl groups and by the lack of coplanarity of the naphthyl rings in gossypol which prevents resonance interactions, so that the influences of these factors on the position of the tautomeric equilibria should be minimal. In view of the lack of spectroscopic evidence for the occurence of lactol

or hemiaminal forms in gossypol or its derivatives only the two conjugation-stabilised forms of the Schiff's bases **4** and **5** were examined.



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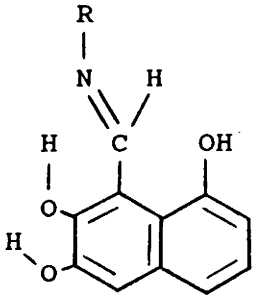

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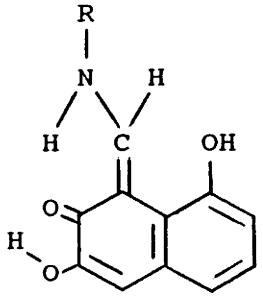

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Results of calculations by the AM1 method of the energy difference between the imine **4** and amino-enone **5** tautomers are shown in Table 1. Three different solvent environments, corresponding to a wide range of dielectric constants, were studied and in all cases it was found that the amino-enone tautomer **5** was the most stable, the relative energy difference between the two forms decreasing with decreasing solvent polarity and also decreasing in the series H>Me>Ph for the substituent on nitrogen. The variation in relative tautomer stability with solvent polarity reflects the change in total dipole moments of the tautomers with the dielectric constant of the medium: in all cases the total dipole moment is larger for the amino-enone and increases more rapidly with increasing solvent dielectric (Table 2).

Table 1. *Difference in stabilization energies of imine 4 and amino-enone 5 tautomers in solvents of different dieletric constants.*

Compound	Energy difference (eV)[a]		
4/5	Dielectric constant of solvent		
R	2.0	30.0	80.0
H	−0.121	−0.414	−0.437
Me	−0.096	−0.344	−0.363
Ph	−0.083	−0.304	−0.323

[a] *Energy difference (eV) between amino-enone tautomer 5 in soluction ($E_{SCRF} - E_{Gaseous}$) and aromatic imine tautomer 4 in solution ($E_{SCRF} - E_{Gaseous}$).*

Table 2. *Total dipole moments of imine 4 and amino-enone 5 tautomers in solvents of different dieletric constants.*

Compound	Dipole moments (Debye)							
4/5	Dielectric constant of solvent (Debye)							
	1.0		2.0		30.0		80.0	
R	4	5	4	5	4	5	4	5
H	2.97	4.85	3.47	5.61	4.55	7.12	4.63	7.28
Me	3.06	5.12	3.51	5.71	4.40	7.14	4.46	7.25
Ph	2.89	4.88	3.28	5.73	4.06	7.65	4.11	7.81

Whatever the accuracy of the absolute energy values for the tautomers **4** and **5** generated by these theoretical studies, it can be concluded from the consistent trends observed that the amino-enone tautomer **5** is more stable. Correspondingly, it is evident that the amino-enone tautomer of gossypol Schiff's bases **2c** should be the preferred form and the correctness of this prediction was examined in detail for the case of dianilinogossypol.

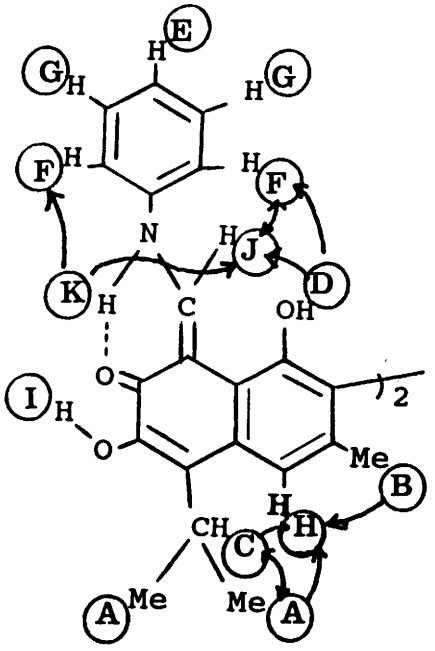
The 100 MHz ^{1}H NMR spectrum of dianilinogossypol in $CDC\ell_3$ confirms it to have a symmetrical structure and shows signals for an isoporpyl group, aryl-methil group, a multiplet for the phenyl hydrogens, one non-coupled aromatic hydrogen, and four further low-field signals, two being broad singlets, one a broad doublet and one a sharp doublet. The latter can be assigned to the CH attached to nitrogen and is coupled to the broad doublet which, like the two broad singlets, is exchangeable (CF_3COOH) and must be located on oxygen or nitrogen. The presence of this vicinal coupling evidently favours tautomers **2b** and **2c** over the simple imine structure **2a**. At 270 MHz, (Table 3) the phenyl protons resolve into separate triplet, doublet and triplet patterns and the isopropyl group is revealed as a 1H septet and a 6H pattern of two overlapping doublets, arising from the diastereotopicity of the methyls in this chiral molecule.

Further information was obtained by use of nuclear Overhauser enhancement difference spectroscopy (nOe'DS), a sensitive technique for the observation of nOe's, which reveals teh presence of close spatial relationships between protons[21]. The results of nOeDS experiments are summarised in Table 3 and lead to the following conclusions: the aromatic hydrogen **H** is spatially located between the methyl and isopropyl groups by the nOe interactions; the CH group **J** attached to nitrogen is close in space to the OH group **D**, the ortho protons **F** of the phenyl group and the NH group **K**. The spatial proximities of this set of protons could be consistent with either of the tautomeric arrangements **2b** or **2c**. However, the CH group **J** has an extremely low-field chemical shift (10.16 ppm), which is difficult to explain on the sole basis of substituent shifts of the aryl, ether and amine functions in hemi-aminal **2b** (calculated: 6.03 ppm) or from the substituent effects on a vinyl hydrogen in enamine **2c** (calculated: 7.35 ppm) and seems best accounted for by the additional, powerful anisotropic deshielding effects of the N-phenyl ring and the delocalised electrons in a rigid H-bonded structure of the enamine form **6**. The observed nOe's are illustrated on this structure, with arrows indicating the direction in wich signal enhancements were produced on double irradiation.

Further support for this structure comes from the ^{13}C NMR spectrum of dianilinogossypol. The lowest field signal was at 174.4 ppm, in the normal range for a carbonyl group. A gated coupled spectrum showed a

Table 3. *^1H NMR and nOe difference spectra of dianilinogossypol 6.*

Proton	δ(ppm)	multiplicity	J (Hz)	No. of H	Protons enhanced in nOeDs
A	1.55	2d	6	6	C H
B	2.15	s		3	H
C	3.72	septet	6	1	A H
D	5.78	s		1	F J
E	7.17	t	6	1	
F	7.29	d	6	2	
G	7.35	t	6	2	
H	7.62	s		1	
I	7.87	bs		1	
J	10.16	d	12	1	F D
K	14.90	bd	12	1	J F



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$^3J_{CCCH}$ of 7.8 Hz to this signal, confirming that it must be C-7, consistent with the formulation of dianilinogossypol as the amino-enone tautomer **6**.

Schiff's bases of gossypol were prepared using a series of powerfully fluorescent aromatic amines. Conditions were established for the reverse phase HPLC analysis of each product. A variety of different, commercial ODS packings were equally suitable (Table 4) and for all the compounds mobile phases of low pH were found necessary to suppress peak tailing due to ionization of the phenolic groups.

Initial scans at fixed emission wavelength and variable excitation wavelength, followed by scans at the best excitation wavelength and variable emission wavelength were carried out to establish optimum conditions for fluorescence measurements of fluorescence intensity versus concentration were then made for each compound. The derivatives of 6-aminoquinoline and fluoresceinamine gave the best results in terms of linearity and wide dynamic range of linear response (Figure 1).

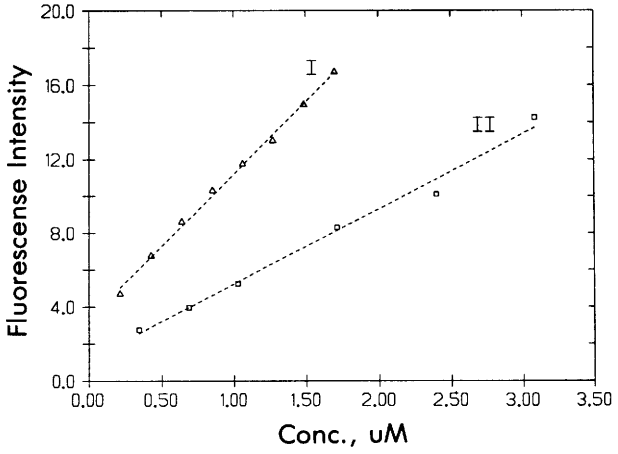


Figure 1. *Least squares fit of fluorescence itensity versus concentration for gossypol Schiff's bases with fluorescamine (I) and 6-aminoquinoline (II).*

The Schiff's bases **9** and **10** were selected for further characterization by mas spectrometry. In previous work[14] we have shown that gossypol Schiff's bases do not show molecular ions in EI-MS, owing to rapid fragmentation. Molecular ions could be observed[14] under FD-MS conditions, accompanied by stepwise amine elimination reactions leading to the ion for anhydrogossypol (m/z 482). To complement this work, we have carried out an investigation of the behaviour of Schiff's bases **9** and **10** by Fast Atom Bombardment (FAB) mass spectrometry, which would be expected to give a richer set of fragment ions than FD-MS.

The positive-ion FAB-MS of the 6-aminoquinoline Schiff's base **9** did not show peaks in the molecular ion region (MW 770), but had significant ions at m/z 645 (M+3H−A), 631 (M+3H−B), 629 (M+1H−B), 627 (M-1H−B) and 607 (M−8H−V), as well as an ion at 145 (B+3H), clearly indicating the loss of the first side chain from the bis (amino) derivative.

Table 4. *HPLC and fluorescence parameters for gossypol Schiff's bases 7 - 10.*

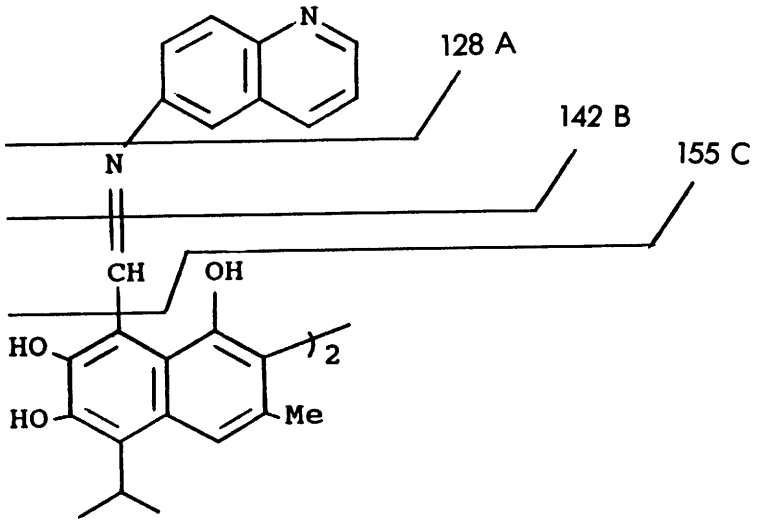
Compound No.	Amine in Schiff's base	Column	Mobile phase MeOH/0.01N KH_2PO_4 v:v	pH	K'	λexcit nm	λemis nm
1	None	Hypersil-ODS	97.5:2.5	2.1	0.36	-	-
7	2-Amino-9-fluoreneone	Hypersil-ODS	97.5:2.5	2.1	1.21	270	270
8	1-Naphthylamine	Novapak-ODS	97.5:2.5	2.1	8.86	260	260
9	6-Aminoquinoline	Novapak-ODS	90:10	3.0	2.42	360	470
10	Fluoresceinamine(I)	Bondapak-ODS	97.5:2.5	4.5	0.60	490	520

The positive-ion FAB-MS of the fluoresceinamine (I) Schiff's base **10** (MW 1176) showed pseudo-molecular ion peaks at m/z 1200 (M + Na + H), 1199 (M + Na), 1180 (M + 4H), 1179 (M + 3H), 1178 (M + 2H) and 1177 (M + 1H) and fragment ions at 848 (M + 3H − A), 846 (M + 1H − A), 832 (M + 1H − B), 831 (M − B), 830 (M − 1H − B), 822 (M + 4H − C), 820 (M + 2H − C), 818 (M − C) and 810 (M − 8H − C), as well as an ion at 348 (B + 3H), a very similar fragmentation pattern to that of **7** and again clearly indicating the loss of the first side chain from the bis (amino) derivative.

Further work on the application of the fluorecent Schiff's base derivatives **9** and **10** for trace determination of gossypol is in progress.

Acknowledgements

We thank the World Health Organization Special Progremme of Research, Development and Research Training in Human Reproduction and FAPESP for support, WHO for a supply of pure gossypol-acetic acid, D. Smith (Beecham Pharmaceuticals) and SERC High Field NMR Service, Edinburgh, for running the high field NMR



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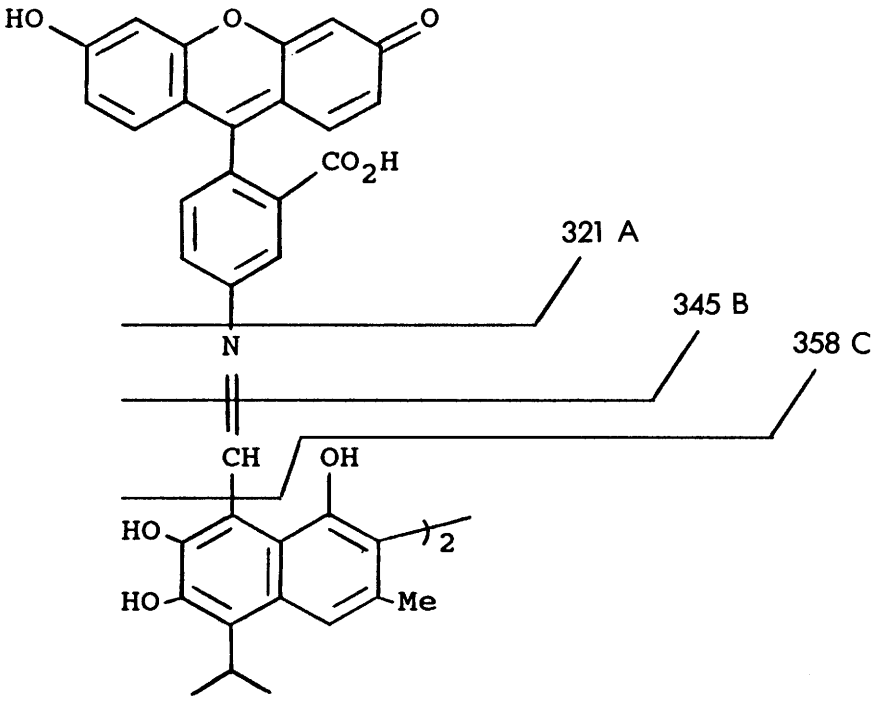

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spectra and the SERC Mass Spectromery Service, Swansea, for providing the FAB-MS spectra.

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