m/z calcd for $C_{21}H_{21}O_1N_2F_3$ 374.1607, found 374.1610.

Regeneration of Rhodopsin Analogue. Rod outer segments (ROS) were prepared according to Papermaster and Dryer[23] from frozen bovine retinas. Rhodopsin (20 mg) in ROS was bleached in the presence of 50 mM NH_2OH in 10 mM tris-acetate, pH 7.4, with a 300-W lamp using a cutoff filter of 475 nm. Bleached ROS was washed with 200 mL of 10 mM sodium phosphate, pH 6.5, five times, and incubated with a 3-fold molar excess of [^{3}H]-11-*cis*-retinal analogue **3** in 10 mL of the same buffer in the dark at room temperature overnight. The ROS was washed with 7 mL of 10 mM sodium phosphate, pH 6.5, containing 2% bovine serum albumin, seven times to remove excess unbound analogue (see Figure 4). Approximately 70% of unbound retinal was removed. Regenerated rhodopsin was bleached on illumination.

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Registry No. **3**, 127685-64-7; **4**, 5757-66-4; **4** aldehyde, 5769-33-5; **5**, 127666-66-4; **5** alcohol, 17100-59-3; **6**, 127666-67-5; **7**, 127666-68-6; **7** aldehyde, 127666-74-4; **7** (*tert*-butyldimethylsilyl ether), 127666-75-5; **8**, 127666-69-7; **9**, 127666-70-0; 4-bromo-2,6-dimethylaniline, 24596-19-8; trifluoroacetylpiperidine, 340-07-8; 3,5-dimethyl-4-[(*tert*-butyldimethylsilyloxy)methyl]-α,α,α-trifluoroacetophenone, 127666-71-1; 3,5-dimethyl-4-[(*tert*-butyldimethylsilyloxy)methyl]-α,α,α-trifluoroacetophenone oxime, 127666-72-2; 3,5-dimethyl-4-[(*tert*-butyldimethylsilyloxy)-methyl]-α,α,α-trifluoroacetophenone tosyloxime, 127666-73-3; (*E,E,E*)-2,6-dimethyl-8-ethoxy-8-oxo-2,4,6-octatrienyltriphenylphosphonium bromide, 75002-35-6.

Supplementary Material Available: Experimental details and ^{1}H NMR spectral data of the intermediates for the synthesis of **3** (3 pages). Ordering information is given on any current masthead page.

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Application of Two-Dimensional NMR Spectroscopy in the Structural Determination of Marine Natural Products. Total Structural Assignment of the Cembranoid Diterpene Eupalmerin Acetate through the Use of Two-Dimensional ^{1}H-^{1}H, ^{1}H-^{13}C, and ^{13}C-^{13}C Chemical Shift Correlation Spectroscopy[1]

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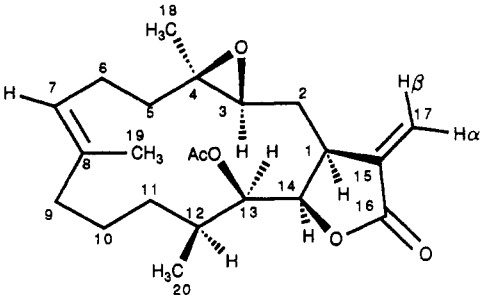
Introduction

Numerous cembranoid diterpenes have been isolated from terrestrial organisms (plants and insects) and especially from marine organisms of the coelenterate phylum.[4] Their carbon skeleton, which is characterized by a 14-membered carbocyclic ring, is the most frequently elaborated by coelenterates. The often dense array of functional groups and stereocenters have made these diterpenoid natural products an attractive target for the natural products chemist.

For the vast majority of cembranoid diterpenes, their structures have been elucidated through the use of either single-crystal X-ray diffraction studies or chemical degradation techniques. However, throughout the last decade, a series of reports have appeared which have described partial or full ^{1}H and ^{13}C NMR spectral assignments in the cembrane series.[5] These studies have helped to establish a data base of sufficient size to be useful in efforts directed at the ^{1}H and ^{13}C NMR based structure elucidation of new cembranoids of unknown structure. However, to the best of our knowledge, only one cembranoid structure has been successfully established using only NMR spectroscopic techniques.[5e] We report here the complete structural assignment of the cembranoid diterpene eupalmerin acetate (**1**), which has been accomplished exclusively on the basis of two-dimensional proton-proton, proton-carbon, and carbon-carbon chemical shift correlation NMR spectroscopy. In the present work, the selection of known cembranolide eupalmerin acetate (**1**) as an appropriate template to probe the effectiveness of modern two-dimensional NMR techniques is based in part on the observation that neither its ^{1}H or ^{13}C NMR spectral data were ever reported following its discovery almost two decades ago.[6] This observation prompted us to initiate a careful and detailed spectral investigation of its molecular structure.



1

Eupalmerin acetate (**1**) was found originally by Ciereszko and co-workers in the Caribbean gorgonian *Eunicea succinea* collected from Florida.[7] Rehm also found it to occur in *Eunicea palmeri* collected near Miami, but not in specimens collected in the Florida Keys.[6] Eupalmerin acetate has also been reported to occur in *Eunicea mammosa* from Puerto Rico.[7] The gross structure of **1** was deduced by Rehm in 1971 primarily on the grounds of

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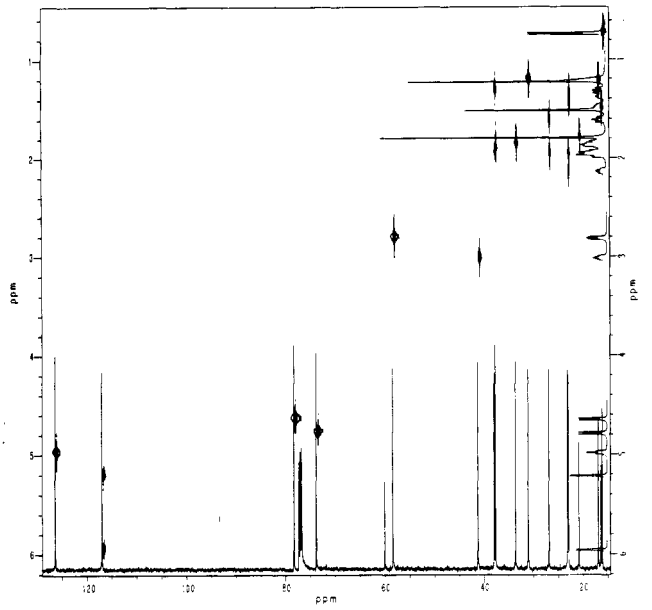

Figure 1. Heteronuclear ($^1H/^{13}C$) 2D chemical shift correlation spectrum of a solution of ca. 60 mg of eupalmerin acetate (1) in 0.6 mL of deuteriochloroform, measured in a 5-mm sample tube at 25 °C with a carbon observation frequency of 125.76 MHz and a proton observation frequency of 500.11 MHz.

chemical methods and without the knowledge of stereochemical details.[6] The molecular structure of eupalmerin acetate (1) was later confirmed by Van der Helm and co-workers who also established its absolute configurations through an X-ray diffraction study.[8]

Results and Discussion

Of the many features of the ^1H and ^{13}C NMR spectra of cembranoid diterpenes that make spectral interpretation difficult, the one that poses the biggest challenge is the highly congested nature of the high-field region (Figure 1). Partial assignment of the ^{13}C NMR spectra can be made with a reasonable degree of confidence on the basis of conventional chemical shift arguments when supplemented by a knowledge of either APT-, DEPT-, or INEPT-based spin multiplicities. Similarly, many resonances in the ^1H NMR spectrum of cembranes may be assigned solely from chemical shift, splitting pattern, coupling constant, and spin decoupling considerations. Thus, for some ^1H NMR resonances (particularly for protons absorbing downfield from 3.0 ppm), the assignment may be straightforward. In contrast, total assignment of the spectrum cannot be made without resorting to additional and more sophisticated experiments. The interpretation of the proton and carbon NMR spectra of cembranes is therefore significantly aided by two-dimensional NMR techniques such as the COSY experiment[9] and the proton–carbon chemical shift correlation experiment (CSCM).[10]

The ^{13}C NMR spectrum of eupalmerin acetate (1) shows the expected 22 carbon resonances (Table I). With only the ^{13}C DEPT,[11] CSCM, and conventional COSY spectra of eupalmerin acetate, we assigned 14 carbon resonances unambiguously. For instance, some of the most readily assigned group of signals in the CSCM spectrum (Figure 1) are those due to the methyl resonances. Beginning with the H20 methyl protons, which appear as a doublet (J = 7.02 Hz) at about δ 0.84 ppm, we observe a point of intensity in the contour plot that correlates with a carbon resonance at 16.06 ppm. The remaining methyl signals, the H18 methyl singlet, the H19 vinyl methyl singlet, and the H22 acetate methyl singlet at about δ 1.32, 1.61, and 1.90 ppm, respectively, are observed to correlate with the carbon resonances at 16.85, 16.33, and 20.74 ppm, respectively. Similarly, the ^{13}C NMR resonances of carbon atoms C1 through C4, C6 and C7, C12 through C14 and C17 are also assigned with absolute confidence (Table I). However, the unequivocal assignment of the remaining eight carbon resonances of eupalmerin acetate is accomplished only through its proton-detected long-range ^1H–^{13}C NMR spectrum, viz. HMBC (heteronuclear multiple-bond connectivity).[12] With the expanded range of two- and three-bond ^1H–^{13}C correlations detected with the HMBC experiment, we secured the remaining eight resonances, namely C5, C8, C9, C10, C11, C15, C16, and C21. Table

Table I. ^{13}C (125 MHz)[a] and ^1H (500 MHz)[b] NMR Spectral Data of Eupalmerin Acetate (1) in $CDCl_3$ and Protons to Which Long-Range Correlations Were Observed in the HMBC Experiments

atom	^{13}C (mult)[c]	^1H (mult, J (Hz), intgrtn)	HMBC (^1H)
1	41.17 (d)	3.08 (m, 1 H)	H2α, H13, H14, H17α, H17β
2α	26.82 (t)	2.11 (m, 1 H)	H3, H14
2β		1.69 (ddd, 4.0, 8.9, 13.4, 1 H)	
3	58.35 (d)	2.91 (dd, 4.0, 8.9, 1 H)	H2α, H18
4	59.97 (s)		H2α, H6α, H6β, H18
5α	37.86 (t)	1.46 (m, 1 H)	H6α, H6β, H18
5β		1.41 (m, 1 H)	
6α	23.04 (t)	2.24 (m, 1 H)	H7
6β		2.09 (m, 1 H)	
7	126.32 (d)	5.06 (t, 6.4, 1 H)	H6α, H6β, H9β, H19
8	134.99 (s)		H6α, H9β, H19
9α	37.59 (t)	2.04 (m, 1 H)	H7, H19
9β		1.97 (m, 1 H)	
10	22.87 (t)	1.58 (m, 2 H)	H9α, H9β
11	31.01 (t)	1.31 (m, 2 H)	H9β, H12, H13, H20
12	33.56 (d)	1.97 (m, 1 H)	H13, H14, H20
13	73.75 (d)	4.89 (d, 9.6, 1 H)	H14, H20
14	78.23 (d)	4.74 (d, 7.9, 1 H)	
15	138.38 (s)		H1, H14, H17α
16	169.74 (s)		H14, H17α
17α	116.93 (t)	6.08 (d, 3.2, 1 H)	
17β		5.30 (d, 3.2, 1 H)	
18	16.85 (q)	1.32 (s, 3 H)	
19	16.33 (q)	1.61 (s, 3 H)	H7, H9α
20	16.06 (q)	0.84 (d, 7.0, 3 H)	H12
21	169.92 (s)		H13, H22
22	20.74 (q)	1.90 (s, 3 H)	

[a] The chemical shifts are given in δ units (parts per million downfield from TMS). Assignments were aided by ^1H–^1H COSY, ^1H–^{13}C COSY, and ^{13}C–^{13}C COSY experiments. [b] The chemical shifts are given in δ units (parts per million downfield from TMS). Assignments were aided by ^1H–^1H COSY, ^1H–^{13}C COSY, and homonuclear spin-decoupling experiments. [c] Multiplicities of the carbon atoms were revealed by the ^{13}C DEPT experiment [see ref 11].

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Table II. ^1H–^1H COSY, Long-Range ^1H–^1H COSY, and NOE Connectivities of Eupalmerin Acetate (1)

proton	COSY[a]	RCT–COSY[a]	RCT2–COSY[a]	NOE[b]
1	H2α, H14, H17α/β	H2β, H3		H14, H17α/β, H18
2α	H1, H2β, H3	H14, H17α/β		H2β, H3
2β	H2α, H3	H14	H1, H17α/β	H2α, H3, H17β, H18
3	H2α/β	H1, H18	H14, H17α/β	H1, H2α/β, H13, H18
5α	H5β, H6β	H7, H18	H6α	H6β
5β	H5α, H6β	H7, H18	H6α	H6β
6α	H6β, H7	H19	H5α/β, H7	
6β	H5α/β, H6α, H7	H19	H3	H5α/β, H6α
7	H6α/β, H19	H5α/β		
9α		H7	H6α, H19	
9β	H10	H19		
10	H9β, H11	H12	H13	
11	H10, H12	H9β, H13, H20		
12	H11, H13, H20	H10		H20
13	H12, H14	H20	H10	H3, H14, H17α/β, H18
14	H1, H13	H2α/β, H17α/β	H3, H20	H1, H3, H12, H13, H17α/β, H18
17α	H1, H14	H2α	H2β, H3	H1, H13, H14, H17β
17β	H1	H2α, H14	H2β, H3	H1, H13, H14, H17α
18-Me		H3, H5α/β		H1, H2β, H3, H13, H14
19-Me	H7	H6α/β, H9β	H9α	
20-Me	H12	H10, H13		H12
22-Me				

[a] COSY spectra measured in $CDCl_3$ at 500-MHz. [b] Obtained from the 2D nuclear Overhauser effect spectrum for a sample of ca. 60 mg of 1 in 0.4 mL of $CDCl_3$; a 5-mm probe was used.

I shows all the $^{2-3}J_{C-H}$ correlations from the HMBC experiments for eupalmerin acetate which also allow us to confirm the rest of the assignments obtained previously on the basis of alternative techniques. Alternatively, we can arrive at the carbon–carbon connectivity directly from ^{13}C–^{13}C couplings. The INADEQUATE spectrum[13] of 1 shows the cross peaks of 18 pairs of carbons as illustrated in Figure 2. Correlated long-range ^1H–^{13}C spectra further elucidate the connections between C7 and C8, C15 and C16, and C15 and C17, respectively (Table I). Additional experimental evidence supporting the C15–C16 and C15–C17 connectivities is obtained from the infrared data which is consistent with the presence of the α-methylene γ-lactone ring shown in structure 1. Connection of each carbon pair clarifies the sequence of carbon atoms and confirms the assignment of all carbon resonances in 1 unambiguously.

In order to achieve the complete assignment of the proton spectrum of eupalmerin acetate, we employed a combination of two-dimensional proton experiments which included conventional COSY, RCT, and RCT2–COSY.[14] Primarily due to the fact that we used a proton observation frequency of 500-MHz, these techniques alone allowed us to unravel all the connectivity pathways in the highly congested high-field spectral region of 1 (Table II). Beginning with substructure A (Figure 3), proton–proton connectivities progressing outward from the pivotal H1 resonance can be easily tracked. There are extensive couplings to the neighboring protons H2αβ, H3, H14 and to the more remote exomethylene protons H17αβ. Since the H2–H3 coupling response leads to a termination point, the two fragments, A and B, could not, however, be linked together through either the normal COSY or RCT–COSY experiments alone. On the other hand, the double relayed COSY (RCT2) spectrum of 1 shows key $^{4-5}J_{H-H}$ correlations, which facilitate the assembly of the two structural fragments. Thus, detection of long-range spin coupling

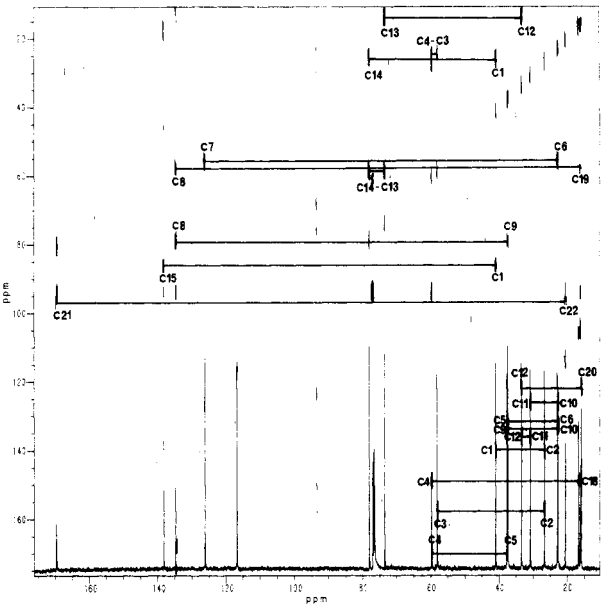

Figure 2. INADEQUATE–2D ^{13}C spectrum of a solution of ca. 100 mg of eupalmerin acetate (1) in 1 mL of deuteriochloroform, measured in a 5-mm sample tube. The adquisition time in t_1 was about 196 ms (256 increments with a spectral width of 20.8 KHz; 64 scans per increment). Since τ was selected for the average aliphatic C–C coupling, the two correlations across double bonds were of low intensity and are not visible in the plot. The final data matrix was of 4096 × 256 complex points; chemical shifts were referenced to internal tetramethylsilane.

between H3 and the H18 methyl protons is consistent with the linking of partial substructures A and B through C3 and C4.

Structural fragment C is assembled on the basis of the connectivity network elucidated through the COSY/RCT–COSY experiments. The observed lack of a spin coupling pathway between either H5α or H5β and H3 is consistent with the separation of fragments B and C by the C4 quaternary carbon. Experimental evidence also supporting the C4–C5 connectivity is provided by long-range coupling between either H5α or β with the H18 methyl protons. Additional support stems from HMBC data which show long-range J_{C-H} correlations between C4 and protons H6α and β, H18 and H2α, respectively.

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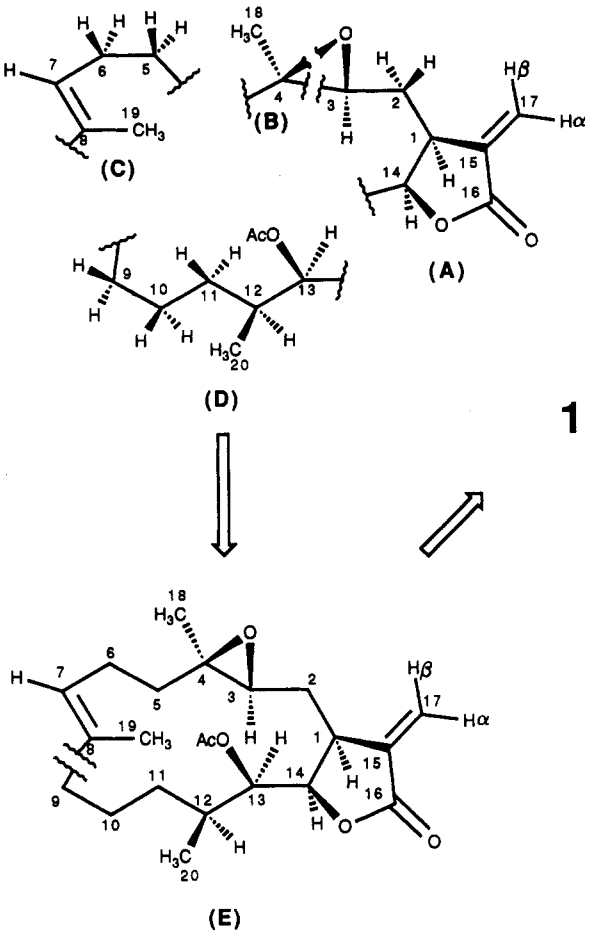

Figure 3. Structural fragments of eupalmerin acetate (1) assembled from the various 2D ^1H–^1H and ^1H–^{13}C experiments performed in the course of the total structure assignment.

The assembly of structural fragments A and D through C13 and C14, however, is not entirely straightforward by COSY due to the very small spin coupling observed between H13 and H14. Very weak points of intensity connecting H13 and H14 are observed in the contour plot of the ^1H–^1H COSY spectrum of 1. The extraction of this proton connectivity is complicated further by the absence of long-range coupling between H13 and H1 and H12 and H14. Therefore, the C13–C14 connectivity is established unequivocally on the basis of HMBC and 2D-INADEQUATE data. Additional experimental evidence stems from simple homonuclear spin decoupling experiments[15] and NOESY[16] data (Table II).

Direct proton–proton connectivity information linking C9 through C13 in substructure D is also obtained from the COSY, RCT, and RCT2–COSY spectra of eupalmerin acetate. The proton resonances for H9α and H9β are located from the proton–carbon heteronuclear chemical shift correlation spectrum. A correlation is observed between H9β resonating at δ 1.97 and the H10 protons absorbing at δ 1.58 ppm. The H10 resonances are, in turn, coupled to the H11 resonances located at δ 1.31 ppm. In addition to strong vicinal coupling with H10 and H12 (the latter accidentally isochronous with H9β), the H11 protons show long-range spin coupling to H13 and H20 methyl protons. Finally, H13 also shows responses for couplings to the H12 and H20 methyl protons, respectively.

Having demonstrated spectroscopically how substructure E can be assembled, all that remains to be assigned is the bridge linking C8 and C9. Long-range ^1H–^1H spin couplings detected between proton H9α with resonances already assigned unambiguously (H7, H6α and the H19 methyl protons) are consistent with the closure of substructure E through the C8–C9 bridge.

The relative stereochemical orientation of all six chiral centers of eupalmerin acetate is established through a two-dimensional nuclear Overhauser effect spectrum (NOESY).[16] The H3/H18, H1/H3, H1/H14, H14/H13, and H14/H12 pairs are oriented as shown on the basis of strong responses visible in the contour plot of the NOESY spectrum of 1 (Table II). The upfield exomethylene proton H17β, resonating at δ 5.30 (doublet, J = 3.2 Hz) is assigned on the basis of strong observed NOE's to the H1 proton (δ 3.08) and the H2β proton (δ 1.69). Experimental evidence supporting this assignment is also provided by strong three-bond ^1H–^{13}C correlation between C1 and H17β. The absolute orientation shown is taken from the X-ray diffraction study reported previously by Van der Helm.[8] Finally, the Me-19 signal in the ^{13}C NMR spectrum (δ 16.33) is at high field which indicates that the trisubstituted double bond in 1 is trans with respect to the continuous chain of carbons.[17] Also, since no enhancement of the H7 proton is observed when the associated methyl resonance is irradiated (Table II), the double bond is assigned as *E*.

Conclusions

The complete structural assignment of the cembranoid diterpene eupalmerin acetate (1) was accomplished by using only two-dimensional (2D) NMR techniques, including 2D homo- and heteronuclear chemical shift correlation spectroscopy. Analysis of the spectral data presented provides a direct method for determining both the structure and the complete and unambiguous ^1H and ^{13}C assignments for molecules in the cembrane series. As a model system, eupalmerin acetate, which contains 16 nonequivalent protons within the region from 2.24 to 0.84 ppm and 12 nonequivalent carbons within the region from 16.06 to 41.17 ppm, was chosen. In addition to providing a sufficiently complex model on which to probe the effectiveness of modern two-dimensional NMR techniques, the present work records the spectral and physical data of eupalmerin acetate which have not been previously reported.

Experimental Section

General Procedures. Column chromatography was performed on Analtech silica gel (35–75 mesh), and TLC analyses were carried out using Analtech glass packed precoated silica gel plates. All solvents used were either spectral grade or were distilled from glass prior to use.

Extraction and Isolation Procedures. The Caribbean gorgonian *E. mammosa* (phylum Cnidaria; class Anthozoa; subclass Alcyonaria; order Gorgonacea) was collected off Desecheo Island, Puerto Rico, on March 18, 1989. The collection was stored at 0 °C for a few hours prior to freezing. The combined MeOH extracts (3 × 150 mL) of the wet specimen (384.5 g) upon filtration followed by concentration gave a residue (17.46 g) which was triturated with hexane followed by chloroform. The hexane extract, after evaporation (13.42 g), was chromatographed over Bio-Beads S-X2 (toluene) to give a group of fractions which were combined on the basis of TLC analyses and evaporated to give an oily residue (8.64 g). A portion of the light-green residue (4.18

(15) Irradiation of H14 (δ 4.74, doublet, J = 7.87 Hz) caused the doublet absorbing at δ 4.89 (H13, J = 9.57 Hz) to sharpen and vice versa.

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g) was further purified by successive chromatography over Sephadex LH-20 (500 g; methanol/chloroform, 1:1) and silica gel (200 g; hexane/EtOAc, 9:1) to give pure **1** (1.41 g; 17%) as a white crystalline solid.

(7*E*,1*S*,3*S*,4*R*,12*S*,13*R*,14*R*)-13-Acetoxy-3,4-epoxycembra-7,15-dien-16,14-olide [Eupalmerin Acetate (1)]. The solid was recrystallized twice from hexane/benzene mixtures: mp 151-153 °C (lit.[4] mp 157-159 °C); $[\alpha]_D^{25} = +8.26°$ (c = 1.76 g/100 mL, $CHCl_3$) (lit.[4] $[\alpha]_D^{25} = +8°$); λ_{max} ($CHCl_3$) 242 nm (ϵ 300); IR (KBr) 1772 (s), 1736 (s), 1669 (w), 1459 (w), 1384 (m), 1234 (s), 1106 (s), 1048 (s), 1040 (s), 1032 (s), 970 (m), 940 (m), 925 (m), 814 (m), 679 (m), 580 (m) cm^{-1}; HREIMS M^+, 1%, m/z obsd 376.2240, $C_{22}H_{32}O_5$ requires 376.2250, 334 (4), 316 (7), 298 (4), 122 (36), 107 (58), 94 (100), 81 (97), 69 (53), 55 (87); ^1H and ^{13}C NMR (see Table I). In vitro screening data for eupalmerin acetate show potent cytotoxicity against CHO-K1 cells (ED_{50} = 9.3 μg/mL) and antimicrobial activity against *Shigella flexneri* and *Proteus vulgaris* (MIC = 1 μg/mL).

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Supplementary Material Available: 500-MHz ^1H NMR and COSY spectra of compound 1 (2 pages). Ordering information is given on any current masthead page.

Reactions of 2,3-Dihydro-9,10-dihydroxy-1,4-anthracenedione (Leucoquinizarin) with Hydrazine and Substituted Hydrazines

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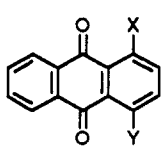
Introduction

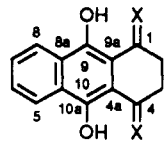
Our previous attempts to prepare 1,4-bis(hydrazino)-anthracene-9,10-dione (**1a**) by ipso substitutions of the fluorides of **1b** by *N,N*-dimethylhydrazine have been unsuccessful.[1] The N-N bond cleavage products **1c** (48%) and **1d** (40%) were isolated from this reaction.



1a, X = Y = $NHN(CH_3)_2$
b, X = Y = F
c, X = F, Y = $N(CH_3)_2$
d, X = Y = $N(CH_3)_2$
e, X = Y = NH_2
f, X = OH, Y = NH_2

(1) Krapcho, A. P.; Avery, K. L., Jr. *J. Org. Chem.* **1988**, *53*, 5927.

Treatment of 2,3-dihydro-9,10-dihydroxy-1,4-anthracenedione (**2a**) (leucoquinizarin) with primary amines leads to the corresponding bis(imines). These



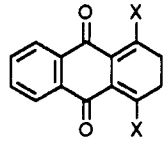
2a, X = O
b, X = NNH_2
c, X = $NNHCH_3$
d, X = $NN{=}C(CH_3)_2$

bis(imines) generally undergo facile air oxidations to yield the 1,4-bis(aminoalkyl)anthracene-9,10-diones. Numerous anthracene-9,10-dione analogues have been prepared by this procedure for antitumor evaluations.[2]

A literature survey uncovered only one related process in which a product formulated as **2b** was obtained by treatment of **2a** with aqueous hydrazine.[3] No evidence was presented for the structure being this particular tautomeric form. We report our results of an investigation of the reactions of **2a** with hydrazine and substituted hydrazines, which has uncovered some interesting N-N bond cleavages.

Results and Discussion

Treatment of **2a** with hydrazine or methylhydrazine at room temperature yielded bis(hydrazones) **2b** and **2c**, respectively. Both are rather unstable and quite difficult to purify. Attempts to oxidize these compounds to the corresponding 1,4-bis(hydrazino) 9,10-diones were unsuccessful. However, **2b** on treatment with acetone formed a crystalline derivative formulated as **2d**. These structures rather than the alternative tautomeric forms **3a-c** are



3a, X = $NHNH_2$
b, X = $NHNHCH_3$
c, X = $NHN{=}C(CH_3)_2$

based on ^1H NMR and ^{13}C NMR data. In particular, since the resonance peak for the carbonyl carbon (C-9,10) of an anthracene-9,10-dione is characterized by an absorption at about 180 ppm,[4-8] the absence of this peak would appear to exclude these structures.

The pertinent ^{13}C data are tabulated in Table I.

When hydrazine was refluxed with **2a**, a complex reaction mixture was obtained that proved difficult to char-

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