JOURNAL OF NATURAL PRODUCTS

Volume 59, Number 10 October 1996

Rapid Communications

Polyalthidin: New Prenylated Benzopyran Inhibitor of the Mammalian Mitochondrial Respiratory Chain

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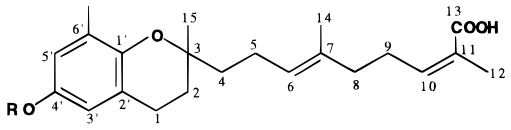
Received May 28, 1996⊗

Abstract: Polyalthidin (**3**), a new benzopyran derivative, was isolated from the stem bark of *Polyalthia cerasoides*. Its structure was established on the basis of chemical and spectral evidence. Polyalthidin has showed potent biological activity as an inhibitor of the mammalian mitochondrial respiratory chain.

From the stem bark of *Polyalthia cerasoides* (Roxb.) Bedd. (Annonaceae), polycerasoidin (**1**) and polycerasoidol (**2**) have been isolated and identified on the basis of their spectral characteristics.[1] The present paper describes further work on this species, which has led to the identification of a new prenylated benzopyran derivative, polyalthidin (**3**). The structural resemblance of these compounds with ubichromanol (a derivative of ubiquinol or reduced coenzyme Q) and some of the well-known inhibitors of the mitochondrial electron-transfer chain, such as rotenone and stigmatellin, prompted us to assay these natural products as inhibitors of the mammalian respiratory chain.

Polyalthidin (**3**)[2,3] was isolated as an oil, and its UV absorption bands at 208 and 292 nm were consistent with the presence of a chromane skeleton. The fragmentation pattern observed in the MS was very similar to that observed for **1**, with a $[M]^+$ in the HREIMS at *m/z* 372.2303 corresponding to the same molecular formula, $C_{23}H_{32}O_4$. The fragment ions at *m/z* 151 and 191 (Figure 1) suggested an identical substitution pattern at the chromane nucleus. ^{1}H-NMR signals for **3** at δ 6.55 (H-5′), 6.44 (H-3′), 3.72 (OMe-4′), 2.14 (Me-6′), 2.72 (H-1), 1.72−1.79 (H-2), and 1.26 (Me-15) specifically indicated the presence of a 2,8-dimethyl-6-methoxychromane fragment. The ^{13}C-NMR chemical shifts also supported these assignments (Table 1). Fragment ions at *m/z* 205, 218, and 259 and inspection of the ^{1}H- and ^{13}C-NMR spectra of **3** led to the identification of the subunit [$-CH_2CH_2CH{=}C(CH_3)-$] and also allowed assignment of the *E* geometry to the $\Delta^{6,7}$ double bond based on the values of the chemical shifts of Me-14 at δ 1.59/δ 15.86 (^{1}H-/^{13}C-NMR).[1]



1: R= CH_3
2: R= H

The remaining portion of the structure of **3** was elucidated by examination of the 1D ^{1}H- and ^{13}C-NMR and DEPT and 2D homonuclear (COSY 45) and 2D ^{1}H−^{13}C-direct heteronuclear correlation (HMQC) NMR data. When the ^{1}H-NMR spectrum of **3** was compared with that of **1**, the only differences displayed were confined to the last isoprene unit of the side chain: two olefinic protons (δ 5.53, H-9, H-10), a methine proton (δ 3.14, H-11), and a methyl (δ 1.27, Me-12) were observed for **3** (Table 1). By analogy, in the ^{13}C-NMR spectrum of **3**, C-9 through C-13 were the only carbon atoms whose chemical shifts differed significantly from the corresponding resonances in **1** and represented a disubstituted double bond (δ 130.68 and 129.53), a methine carbon (δ 42.63), and a methyl carbon (δ 17.22). A carbonyl resonance was observed that shifted downfield from δ 173.63 in **1** to δ 180.51 in **3**, suggesting a

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⊗ Abstract published in *Advance ACS Abstracts*, September 1, 1996.

S0163-3864(96)00492-2 CCC: $12.00

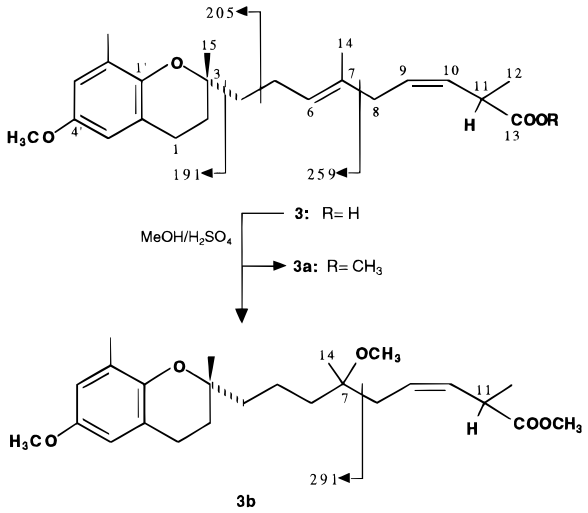

Figure 1. EIMS fragment ions of **3** and methylated derivatives (**3a** and **3b**).

Table 1. ^{1}H-, ^{13}C-, DEPT, COSY 45, and HMQC NMR Spectral Data of **3** ($CDCl_3$, 400 MHz)

position	^{1}H-NMR δ^a	coupling in COSY 45	^{13}C-NMR (DEPT)^b
1	2.72 t	CH_2-2	22.61 (CH_2)
2	1.72−1.79 m	CH_2-1	31.34 (CH_2)
3			75.23 (C)
4	1.60−1.66 m	CH_2-5	39.54 (CH_2)
5	2.01−2.11 m	CH_2-4	22.20 (CH_2)
6	5.16 dt	CH_3-14	125.33 (CH)
7			133.57 (C)
8	2.66 d	CH-9	42.59 (CH_2)
9	5.53 dddd	CH_2-8	129.53 (CH)
10	5.53 dddd	CH-11	130.68 (CH)
11	3.14 m	CH-10, CH_3-12	42.63 (CH)
12	1.27 d	CH-11	17.22 (CH_3)
13			180.51 (C)
14	1.59brs	CH-6	15.86 (CH_3)
15	1.26 s		23.99 (CH_3)
1′			145.97 (C)
2′			120.83 (C)
3′	6.44 d	CH-5′	110.94 (CH)
4′			152.05 (C)
5′	6.55 d	CH-3′	114.70 (CH)
6′			127.18 (C)
CH_3-6′	2.14 s		16.21 (CH_3)
OCH_3-4′	3.72 s		55.59 (CH_3)

^{a} J_{1-2} = 7.4 Hz; J_{5-6} = 7.2 Hz, J_{6-14} = 1.2 Hz; J_{8-9} = 2.8 Hz; J_{9-10} = 6.0 Hz, J_{9-11} < 1 Hz; J_{10-11} = 1.2 Hz; J_{11-12} = 7.0 Hz; $J_{3'-5'}$ = 2.9 Hz. ^{b} Carbon assignments were made by HMQC (quaternary carbons by comparison with **1**).[1]

nonconjugated carboxyl group in agreement with an IR absorption band at 1707 cm^{-1} (**3**) instead of at 1686 cm^{-1} (**1**).

Methylation of **3**[4] furnished a mixture of two compounds (**3a** and **3b**), which were purified by column chromatography to yield the methyl ester derivative **3a**[5] as the main product, thus confirming a carboxyl function in **3**. The ^{1}H- and ^{13}C-NMR spectra (Table 2) and selective ^{1}H-NMR irradiations of **3a** supported the assigned terminal isoprene unit for **3**. Thus, irradiating the multiplet at δ 3.15 (H-11) collapsed the doublet at δ 1.25 (Me-12) to a singlet and simplified the signal at δ 5.52 (H-9, H-10); irradiation of the resonance at δ 5.52 changed the multiplet at δ 3.15 (H-11) to a quartet (J_{11-12} = 7 Hz) and the doublet at δ 2.65 (H-8) to a singlet; irradiation of the doublet at δ 1.25 (Me-12) changed the resonance at δ 3.15 (H-11) to a doublet. The second compound obtained by methylation of **3**, was

Table 2. ^{1}H-NMR ($CDCl_3$, 250 MHz) and ^{13}C-NMR ($CDCl_3$, 62.5 MHz) Data of **3a** and **3b**

	3a		**3b**^{a}	
	δ_H	δ_C	δ_H	δ_C
1	2.72 t (6.7)	22.64	2.71 t (6.2)	22.65
2	1.76 dd (6.7; 13.0)	31.40	1.76 dd (6.2; 13.1)	31.35
3		75.26		75.42^b
4	1.59−1.62 m	39.61	1.50−1.56 m	37.75
5	2.02−2.11 m	22.23	1.39−1.41 m	17.34
6	5.16 dt (7.5; < 1)	125.29	1.50−1.56 m	40.20
7		133.67		*c*
8	2.65 d (4.2)	42.58	2.19 d (4.1)	40.71
9	5.52 m	127.95	5.52 m	127.63
10	5.52 m	130.14	5.52 m	131.66
11	3.15 m	42.69	3.14 m	42.87
12	1.25 d (7.0)	17.40	1.23 d (7.5)	17.42
13		175.41		175.40
14	1.59 brs	15.86	1.07 s	22.62
15	1.26 s	24.02	1.25 s	24.04
1′		145.98		146.05
2′		120.87		120.89
3′	6.44 d (2.9)	111.00	6.44 d (2.9)	110.99
4′		152.14		152.10
5′	6.55 d (2.9)	114.75	6.55 d (2.9)	114.74
6′		127.20		127.18
CH_3-6′	2.15 s	16.19	2.14 s	16.21
OCH_3-4′	3.72 s	55.62	3.72 s	55.61
OCH_3-7			3.16 s	48.88
$COOCH_3$	3.67 s	51.76	3.67 s	51.75

^{a} Proton assignments were made by COSY 45. ^{b} Recorded in CD_3CN: δ 76.29. ^{c} Shift not observed in $CDCl_3$, recorded in CD_3CN: δ 76.94.

identified as the methyl ester, the 7-methoxy derivative **3b**.[6] Methoxylation at the C-7 position was suggested by the presence of a new EIMS fragment ion at *m/z* 291 (Figure 1), indicating cleavage of the C-7, C-8 bond, and confirmed by examination of spectral data (Table 2). The C-7 carbon resonance was not observed due to an overlapping with the $CDCl_3$ signal. The presence of a new quaternary oxygen-bearing carbon in **3b** was finally confirmed in CD_3CN solution, where two quaternary carbon resonances at δ 76.29 (C-3) and δ 76.94 (C-7) were observed.

The coupling constant of the olefinic H-9, H-10 (J = 6 Hz) bond of **3** proved the geometry to be *Z*, which was also confirmed by the C-8 methylene chemical shift at δ 2.66 (in geometrical isomers this value is shifted upfield to δ 2.0−2.1).[7] The configuration at C-3 was tentatively assigned as *R* by comparison with ^{1}H- and ^{13}C-NMR spectral data of tocopherols and related compounds at the chromane nucleus.[8,9]

The three prenylated benzopyrans (**1**−**3**) were found to be inhibitors of the mitochondrial electron transfer chain with different IC_{50} values (Figure 2).[10] Polycerasoidol (**2**) was found to be the weakest inhibitor, whereas polyalthidin (**3**) was the most potent inhibitor of the integrated respiratory chain with an IC_{50} at <5 μM. Full inhibition of the respiratory chain by **3** was exhibited at 13−14 μM. The residual activity beyond this concentration was the same as that of the nonsensitive NADH oxidation by rolliniastatin-1 (a bis-tetrahydrofuran acetogenin)[11] (25 nM), a new specific and powerful inhibitor of the NADH ubiquinone oxidoreductase (complex I) isolated from Annonaceae species,[11,12] and by rotenone, the classical inhibitor of this respiratory complex.[12]

Figure 3 shows replotted traces of the effect of a fixed concentration of **3** (15 μM) on the time-course reduction of ferricytochrome c by both NADH and succinate.[13−16]

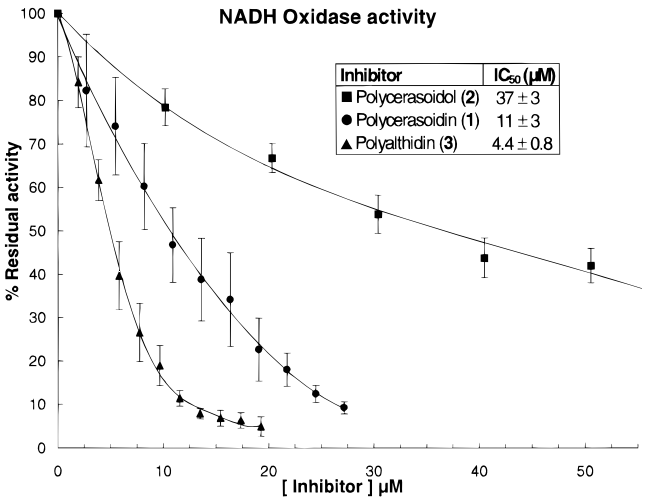

Figure 2. Titration of prenylated benzopyrans against NADH oxidase activity in bovine heart submitochondrial particles. Mitochondrial protein concentration was 6 μg·mL^{-1}. Control activity was approximately 0.45 μmol·min^{-1}·mg^{-1}. Data were means from five determinations for each product.

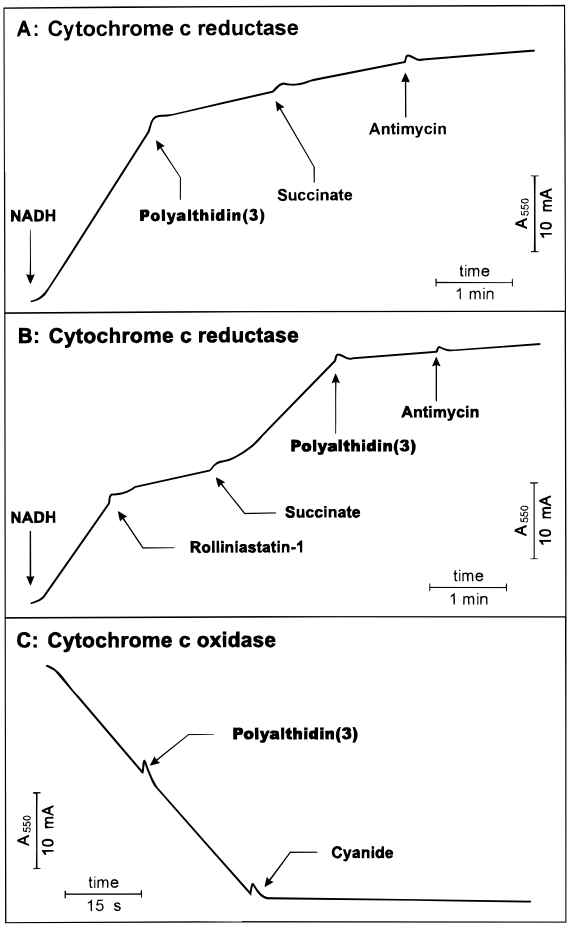

Figure 3. Effect of polyalthidin (**3**) on the time-course reduction and oxidation of cytochrome c in bovine heart submitochondrial particles. Mitochondrial protein concentration was 6 μg·mL^{-1}. Cytochrome c reductase activity was 0.15 and 0.12 μmol·min^{-1}·mg^{-1} for NADH and succinate, respectively. Cytochrome c oxidase activity was 0.42 μmol·min^{-1}·mg^{-1}. Polyalthidin was added at 15 μM, antimycin A at 5 μM, rolliniastatin-1 at 25 nM, and cyanide at 2 mM.

Polyalthidin (**3**) inhibited NADH cytochrome c reductase (integrated activity of complex I and III). When succinate was added, which is oxidized at site II of the electron transport chain, cytochrome c reductase remained blocked (Figure 3A). However, a slight further inhibition was detected after addition of antimycin A, a specific inhibitor of the ubiquinol cytochrome c oxidoreductase (complex III). When NADH ubiquinone oxidoreductase (complex I) was blocked by rolliniastatin-1 or rotenone the cytochrome c reduction by succinate was completely inhibited by **3** (Figure 3B), and no further inhibition was found after antimycin A addition. Cytochrome c oxidase activity (complex IV) was not affected by **3** (Figure 3C), and it was completely blocked by addition of cyanide, a specific inhibitor of this respiratory complex.

The methoxybenzopyran moiety is present in ubichromanol, a derivative of the ubiquinol or reduced coenzyme Q, in rotenone, a well-known inhibitor of the respiratory chain at the complex I site, and in stigmatellin (as a chromone group), an inhibitor of both complexes I and III. Therefore, the most probable site of inhibition of this new prenylated benzopyran is the coenzyme Q junction of the respiratory chain. As expected, cytochrome c oxidase activity was not affected by polyalthidin (**3**), neither at a concentration that fully inhibited the respiratory chain activity nor up to 60 μM (results not shown). Our results seem to evidence that **3** acts primarily as an inhibitor of the ubiquinol cytochrome c oxidoreductase (complex III) by blocking electron transfer between the ubiquinone reduced at NADH and succinate dehydrogenases (complex I and II) and cytochrome c. This respiratory complex has two active sites, namely centers *P* and *N*, where ubiquinol is oxidized and reduced, respectively, to form the Q-cycle.[17] The additive effect of polyalthidin (**3**) with antimycin A (center *N* inhibitor) in NADH oxidation suggest that this and other prenylated benzopyrans could interact with the *P* center like other methoxylchromone inhibitors as stigmatellin.[17] Moreover, we cannot discard a competitive effect with endogenous coenzyme Q in the mitochondrial membrane that could affect the three enzymes involved in the Q junction of the respiratory chain to different extents, being the most sensitive complex III. To resolve these problems, additional experiments are currently being undertaken in our laboratory.

Therefore we have described, for the first time, that these prenylated benzopyrans act as inhibitors of the mammalian respiratory chain, with the most potent representative being polyalthidin (**3**), a new isoprenylated benzopyran derivative with a double bond located at C-9, C-10. The inhibition of the mitochondrial electron-transport chain by these natural products could explain their cytotoxic and antitumor activity described previously.[18,19]

Acknowledgment. This research was supported by the Spanish DGICYT under Grant No. PB 93-0682. We wish to thank Conselleria de Cultura, Educació i Ciència of the Generalitat Valenciana, and Spanish Ministerio de Educación y Ciencia for the scholarship grant to M.C.G. and J.R.T., respectively. We are grateful to K. Sundar Rao, P. Wanganigi, and Max Kuduk for providing and authenticating the plant material, *P. cerasoides* stem bark, collected in December 1992 in the National Park of Varirata, located in the Central Province of Papua, New Guinea.

References and Notes

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(2) Stem bark of *P. cerasoides* (550 g) was macerated with MeOH at room temperature and then further partitioned[1] to give 4.05 g of organic extract. Flash column chromatography on Si gel was applied to this extract and developed using hexane containing gradually increasing amounts of CH_2Cl_2. Fractions 9−11 (hexane−CH_2Cl_2 5:5) were subjected to column chromatography over silica gel 60H (hexane−AcOEt−MeOH, 7:2.4:0.6) yielding 495 mg of **3**.

(3) Polyalthidin (**3**): oil; $[\alpha]_D$ +3.75° (*c* 0.8, MeOH); IR (dry film) ν_{max} 3450, 2922, 2849, 1707 (C═O, carboxyl), 1604, 1509, 1479, 1464, 1375, 1276, 1218, 1149, 1099, 1059, 966, 856 cm^{-1}; UV (EtOH) λ_{max} (log ϵ) 208 (4.37), 227 (3.92), 292 (3.67) nm; HREIMS *m/z* $[M]^+$ 372.2303 (96), calcd for $C_{23}H_{32}O_4$ 372.2300, 259.1692 (10), 218.1315 (9), 206.1287 (21), 205.0564 (18), 203.1113 (11), 191.1073 (49), 189.0930 (43), 177.0541 (12), 152.0832 (34), 151.0771 (100), 150.0689 (48), 135.0853 (8), 121.0861 (12), 93.0449 (18); ^{1}H-NMR ($CDCl_3$, 400 MHz), ^{13}C-NMR ($CDCl_3$, 100 MHz), COSY 45, and HMQC NMR data, see Table 1.

(4) Concentrated H_2SO_4 (0.5 mL) was added to a solution of **3** (33 mg) in MeOH (10 mL) and the mixture refluxed with stirring for 2.5 h. Only **3a** was obtained (yield 90%) when **3** was refluxed for 1 h. The reaction mixture was neutralized with $NaHCO_3$ and extracted with CH_2Cl_2, which was subjected to column chromatography on silica gel 60H (hexane−AcOEt 9:1) to afford **3a** (8.5 mg, yield 25%) and **3b** (5.4 mg, yield 15%).

(5) Compound **3a**: oil; $[\alpha]_D$ −10.5° (*c* 0.57, MeOH); IR (dry film) ν_{max} 1735 (C═O, ester), 1602, 1479 cm^{-1}; UV (EtOH) λ_{max} (log ϵ) 208 (4.42), 239 (3.86), 293 (3.65) nm; EIMS *m/z* $[M]^+$ 386 (91), 371 (4), 354 (6), 327 (12), 259 (30), 235 (18), 219 (51), 206 (59), 205 (40), 191 (78), 190 (32), 189 (75), 175 (45), 163 (30), 151 (100), 150 (7), 133 (26); ^{1}H-NMR ($CDCl_3$, 250 MHz) and ^{13}C-NMR ($CDCl_3$, 62.5 MHz) data, see Table 2.

(6) Compound **3b**: oil; $[\alpha]_D$ +13° (*c* 0.53, MeOH); IR (dry film) ν_{max} 1735 (C═O, ester), 1603, 1479 cm^{-1}; UV (EtOH) λ_{max} (log ϵ) 208 (4.40), 235 (4.75), 293 (3.76) nm; EIMS *m/z* $[M]^+$ 418 (9), 386 (73), 291 (8), 259 (8), 235 (6), 218 (8), 206 (18), 205 (12), 191 (31), 189 (35), 175 (13), 163 (7), 151 (100), 150 (32), 133 (7); ^{1}H-NMR ($CDCl_3$, 250 MHz) and ^{13}C-NMR ($CDCl_3$, 62.5 MHz) data, see Table 2.

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(10) Polycerasoidin (**1**), polycerasoidol (**2**), and polyalthidin (**3**) were diluted in ethanol at 5 mM. The solutions were kept in the dark at 4 °C. Accurate titration was performed spectrophotometrically. The biological activity of these compounds was first assayed as inhibition of the integrated electron transfer chain (NADH oxidase activity) in beef heart submitochondrial particles (SMP) by addition of increasing concentrations of each one. IC_{50} values were the final compound concentrations in the assay cuvette that yielded averaged 50% inhibition of NADH oxidase activity.

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(13) For this experiment, the respiratory chain was divided in two segments: that of the coenzyme Q junction, which involves the reducing coenzyme Q complexes I and II and the oxidizing complex III, and that of the cytochrome oxidase complex. The prenylated benzopyrans were assayed as inhibitors of both segments by following the ferricytochrome c reduction by NADH and succinate for the first segment and the ferrocytochrome c oxidation for the last segment as described previously.[16−18]

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