Brief Articles

Novel Inhibitors of Mitochondrial Respiratory Chain: Endoperoxides from the Marine Tunicate *Stolonica socialis*

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Received December 20, 2000

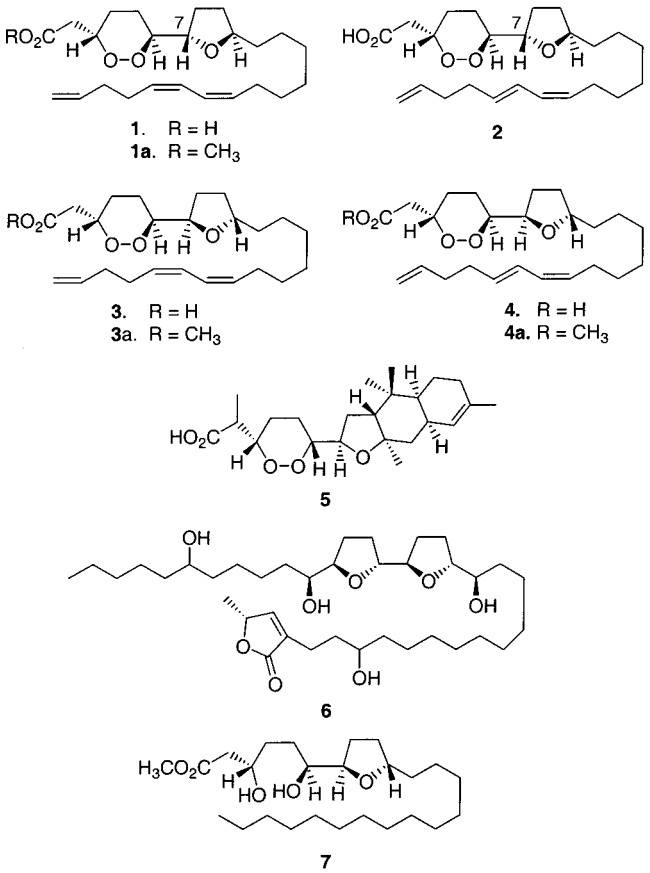
The Mediterranean tunicate *Stolonica socialis* contains a new class of powerful cytotoxic acetogenins, generically named stolonoxides. In this paper, which also details the isolation and chemical characterization of a minor component (**3a**) of the tunicate extract, we report the potent inhibitory activity (IC_{50} < 1 μM) of stolonoxides (**1a** and **3a**) on mitochondrial electron transfer. The compounds affect specifically the functionality of complex II (succinate:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome C oxidoreductase) in mammalian cells, thereby causing a rapid collapse of the whole energetic metabolism. This result, which differs from the properties of similar known products (e.g., **6**), reflects the molecular features of stolonoxides.

Introduction

Cytotoxic peroxides have been isolated from many marine organisms. Recently, we gave an account for the isolation of a new member of this class of natural products, named stolonoxide A (**1/1a**) (Chart 1), from the Mediterranean tunicate *Stolonica socialis*.[1] Soon after the publication of our paper, a series of stolonoxide A-related compounds (**2**−**4**) was independently isolated by bioassay guided screening from researchers of the University of Cadiz (Spain).[2] In this paper, the authors claim a strong cytotoxicity (IC_{50} ≤ 0.1 μg/mL) against several mammalian tumor cell lines for purified and for a crude mixture of **1**−**4.**[2] Differently from other known peroxides, stolonoxides (**1**−**4**) display an unusual structure with a 1,2-dioxane ring directly linked to a tetrahydrofuranyl moiety.[1,2] Such an arrangement, which vaguely resembles the structure of the polycyclic metabolites trunculins (e.g., trunculin B, **5**),[3] seems to be closely related to the adjacent bis-THF group of Annonaceous (custrad-apple family) acetogenins[4] (e.g., **6**), a class of antitumor and pesticidal compounds whose main mode of action is the inhibition of the mitochondrial respiratory chain,[5−7] the fundamental process that drives the ATP synthesis under aerobic metabolism in cells.

The suggestion that the activity of Annonaceous acetogenins may be related to the presence of the bis-adjacent THF moieties[4] prompted us to continue our investigation of the *S. socialis* components. In this paper, we report the full characterization of stolonoxide C (**3**/**3a**),[2] which has been previously isolated in mixture with stolonoxide D (**4**/**4a**), and, moreover, the activity and the mechanism of action of **1a** and **3a** on mitochondrial electron transport system of mammalian cells.

Chart 1



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10.1021/jm0011373 CCC: $20.00
Published on Web 05/25/2001

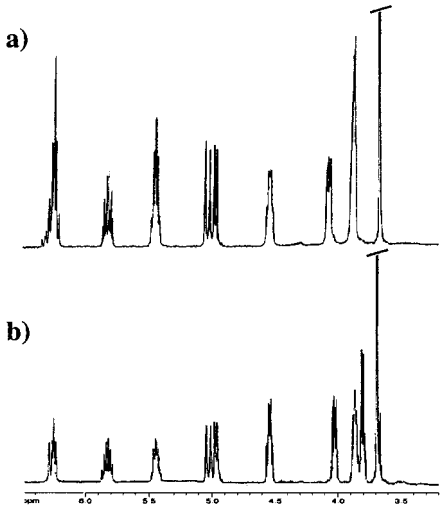

Figure 1. ^{1}H NMR (500 MHz, $CDCl_3$) spectra (between δ 3.2 and 6.6) of (a) stolonoxide A methyl ester (**1a**) and (b) stolonoxide C methyl ester (**3a**).

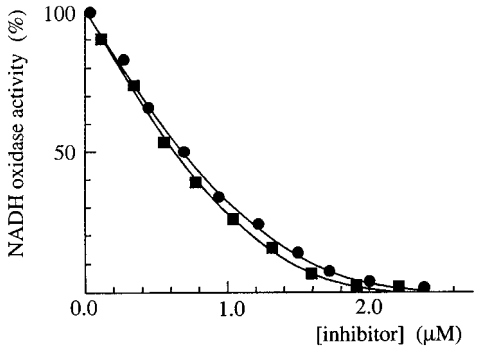

Figure 2. Titration of compounds **1a** and **3a** against NADH oxidase activity in bovine heart submitochondrial particles (SMP). Mitochondrial protein concentration was 6 μg/mL. Control activity was approximately 0.95 μmol min^{-1} mg^{-1}. Data are means from three determinations for each product. (●) Compound **1a**, $IC_{50} = 0.682 \pm 0.021$ μM. (■) Compound **3a**, $IC_{50} = 0.635 \pm 0.017$ μM.

Results and Discussion

The tunicate was collected in Tarifa, Straits of Gibraltar (Spain), in June 1996.[1] The oily residue from the acetone extract was partitioned between water and Et_2O to give 456 mg of ether soluble material that was fractionated by Sephadex LH-20 ($CHCl_3$/MeOH 1:1). To assist the purification of the unstable stolonoxides, the fractions containing the peroxides were combined and methylated by CH_2N_2 in Et_2O. The methyl derivatives **1a** and **3a** were finally purified by two sequential SiO_2 columns with Et_2O in petroleum ether.

The methyl ester **3a** showed spectroscopic properties close to those of stolonoxide A (**1a**).[1] Both compounds had the parent ion at *m*/*z* 420 in EIMS, and their NMR data suggested a similar skeletal structure featured by the molecular formula $C_{25}H_{40}O_5$.[1] On the other hand, **1a** and **3a** differed significantly for the polarimetric values, −33° (*c* 0.1, $CHCl_3$) and +28° (*c* 0.1, $CHCl_3$), respectively. Minor differences were also observable in the ^{1}H NMR signals of the adjacent bicyclic system where H-7 appeared at δ 3.87 in **1a** and at δ 3.80 in **3a** (Figure 1). The suggestion that these effects may be dependent on a different stereochemistry of the substituents at C6 and C7 appeared reasonable since no other changes were observable in the remaining parts of the molecules. Moreover, NOESY spectra of **1a** and **3a** indicated an identical relative stereochemistry of the cyclic parts, with the equatorial orientation of the main substituents of the 1,2-dioxane ring and the pseudo-*trans* geometry of the THF side chains.[1] Reduction of stolonoxide C methyl ester (**3a**) with H_2 on Pd gave quantitatively the diol **7**, for which the observed chemical shift of H-6 (δ 3.77) is significantly close to that of *erythro* THF flanked-hydroxyl moiety in Annonaceous acetogenins.[4] A final confirmation of the *erythro* stereochemistry at C6/C7 was obtained by comparing the NMR data of model compounds[4,8−13] that demonstrated the *trans*/*erythro*/*trans* configuration of **3a** respect to the *trans*/*threo*/*trans* stereochemistry previously reported for stolonoxide A (**1a**).[1] It was, however, impossible to determine the absolute stereochemistry of **3a**, since the Mosher method was unsuccessful because the small amount of available product.

As already stated, the molecular arrangement of stolonoxides resembles a group of natural products, generically named Annonaceous acetogenins (e.g., **6**[10]), that show a wide variety of biological activities including antitumor, antimicrobial, and pesticidal properties.[4] The Annonaceous acetogenins, in particular, are the most powerful of the known inhibitors[4] of mitochondrial complex I, one of the enzymes involved in the respiratory chain. The structural analogies of stolonoxides (**1**−**4**) with these natural products prompted us to investigate whether the '*Stolonica*' endoperoxides could be new inhibitors of the respiratory chain and thus, products with relevance for biomedical and insecticide usage. Stolonoxide methyl esters (**1a** and **3a**) were found to be inhibitors of the integrated electron transfer chain (NADH oxidase activity) in beef heart submitochondrial particles (SMP)[14] with very close potency (Figure 2). Inhibitory concentration 50% (IC_{50}) was below 1 μM for both compounds, whereas full inhibition of rotenone-sensitive NADH oxidase activity was achieved at approximately 2 μM.

In the integrated electron transfer chain, electrons are first carried from complex I (NADH:ubiquinone oxidoreductase) or from complex II (succinate:ubiquinone oxidoreductase) to complex III (ubiquinol:cytochrome *c* oxidoreductase) by ubiquinone, and then from complex III to complex IV (cytochrome C oxidase) by the peripheral membrane protein cytochrome *c*. After addition of compound **1a**, cytochrome *c* reductase activity was fully inhibited with either NADH or succinate as electron donor.[15] The reductase activity did not recover even after addition of the second donor, succinate or NADH (Figure 3). Instead, compound **1a** did not affect the cytochrome *c* oxidase activity (complex IV) (data not shown). Identical results were obtained with the methyl ester of stolonoxide C (**3a**).

Finally, the effect of stolonoxides on the activity of complex I or complex II was assayed by using decylubiquinone (DB) in agreement with ref 16. Both compounds slightly affected the ubiquinone-sustained NADH oxidation, specific activity of complex I (Figure 4a), but they strongly reduced the specific activity of complex II, succinate-sustained ubiquinone reduction, at low

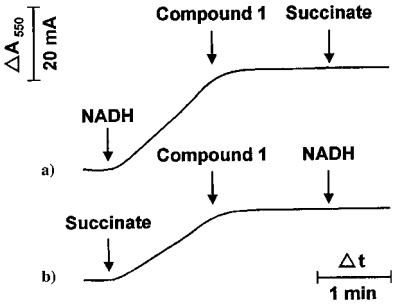

Figure 3. Effect of fixed concentration of **1a** on the time-course reduction of cytochrome *c* in bovine heart submitochondrial particles. Mitochondrial protein concentration was 6 μg/mL. Cytochrome *c* reductase activity was 0.15 and 0.12 μmol min^{-1} mg^{-1} for NADH (a) and succinate (b), respectively. Compound **1a** was added at 2 μM.

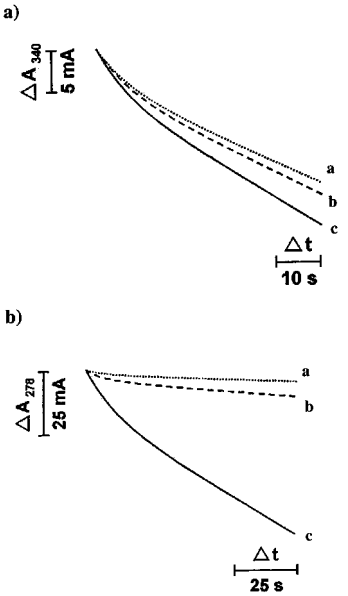

Figure 4. Effect of stolonoxides (**1a** and **3a**) on the specific activities of complex I (a) and II (b) in bovine heart submitochondrial particles (SMP). Mitochondrial protein concentration was 6 μg/mL. Activities were 0.57 and 0.49 μmol min^{-1} mg^{-1} for complex I and complex II, respectively. Compounds **1a** and **3a** were added at the reported concentration. (···, a) Compound **3a** (10 μM). (--, b) Compound **1a** (10 μM). (—, c) Control.

concentration (Figure 4b). Furthermore, combined with the result of the cytochrome *c* reductase activity, these data suggested that both stolonoxides behave as specific inhibitors of complex II and complex III.[15]

Conclusion

The Mediterranean tunicate *S. socialis* contains a mixture of novel long chain acids, generically named stolonoxides (**1**−**4**).[1,2] These compounds are featured by an adjacent dioxane−THF system, the stereochemistry of which has been determined as 3*S*,6*S*,7*S*,10*R* in the major compound (**1a**)[1,2] and 3*S**,6*S**,7*R**,10*S** in the minor product (**3a**). Stolonoxides are structurally related to Annonaceous acetogenins, powerful inhibitors of the electron transport systems in mammalian and insect cells. Compounds **1a** and **3a** indeed inhibit the mitochondrial electron transfer chain acting in the ubiquinone junction by affecting both complex II (succinate: ubiquinone oxidoreductase) and complex III (ubiquinol: cytochrome *c* oxidoreductase). Such properties, however, differ from those of Annonaceous acetogenins (e.g., **6**) that, generally, inhibit mitochondrial complex I.[4−7] The inhibitory mechanisms of stolonoxides and Annonaceous acetogenins against their protein targets may reflect the differences of the molecular features. Our study may suggest that a bis-adjacent ring arrangement seems to be critical for the activity of both classes of compounds.[4] Furthermore, it seems very likely that the length of the alkyl chain and the absence of the γ-lactone at the end of the chain may be essential for the selectivity of action.[4] The stereochemistry of the oxygenated rings does not seem to be crucial for the potency of stolonoxides, since **1a** and **3a** showed similar activities in both cytotoxicity[2] and electron transport inhibitory assays. This reflects a characteristic of Annonaceous acetogenins for which changes of the stereochemistry surrounding the THF rings seem to have very little effect on their biological properties.[4]

In view of a possible biological usage, stolonoxides are placed in a middle range with respect to the most interesting respiratory inhibitors. However, the specificity of their action on complex II and complex III may make these compounds very promising for future applications in the biomedical and pest-control fields.

Experimental Section

General Methods. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. 1D and 2D-NMR spectra were recorded on a 500 MHz Bruker DRX 500 spectrometer. The $CHCl_3$ resonances at δ 7.26 and 77.0 were used as the internal standard. IR spectra were recorded in liquid film by a Bio-rad FTS7 spectrophotometer. AEI MS30 (EIMS) instruments were used for obtaining mass spectra. UV and CD data were obtained on a JASCO J700 spectrophotometer.

Biological Material. The tunicate *Stolonica socialis* was collected in Tarifa (Spain) during the summer of 1996. Submitochondrial particles for biological assays were obtained from beef heart prepared according to Fato et al.[14] by extensive ultrasonic disruption of frozen-thawed mitochondria to produce open membrane fragments.

Extraction and Isolation of Stolonoxides. The frozen tunicate (456 g wet wt) was extracted and fractionated as previously described[1] to give 8.2 mg of **1a** and 1.6 mg of **3a**.

Stolonoxide C methyl ester (3a): colorless oil; $[\alpha]_D$ + 28.0° (*c* 0.1, $CHCl_3$); IR (liquid film) 2935, 2865, 1753, 1150 cm^{-1}; UV λ_{max} (ETOH) 244, 237 nm; ^{1}H NMR ($CDCl_3$) δ 6.25 (bt, 8.0 Hz, 2H), 5.82 (m, 1H), 5.45 (m, 2H), 5.05 (bd, 17.0 Hz, 1H), 4.95 (bd, 10.8 Hz, 1H), 4.55 (ddd, 8.8, 7.6 and 5.6 Hz, 1H), 4.06 (ddd, 11.0, 5.9 and 5.6 Hz, 1H), 3.90 (m, 1H), 3.80 (bdd, 13.1 and 5.9 Hz, 1H), 3.69 (s, 3H), 2.48 (dd, 15.6 and 7.6 Hz, 1H), 2.37 (dd, 15.6 and 5.4 Hz, 1H), 2.30 (m, 2H), 2.15 (m, 4H), 1.99 (m, 2H), 1.98 (m, 2H), 1.92 (m, 2H), 1.80 (m, 1H), 1.68 (m, 1H), 1.55 (m, 3H), 1.45 (m, 1H), 1.35 (m, 3H), 1.30 (m, 4H), 1.27 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 170.0, (s) 138.4 (d), 132.4 (d), 124.0 (d), 114.7 (t), 83.7 (d), 79.9 (d), 78.5 (d), 77.8 (d), 51.9 (q), 38.4 (t), 35.6 (t), 31.6 (t), 29.6 (t), 29.6 (t), 29.2 (t), 28.9 (t), 28.0 (t), 27.0 (t), 25.7 (t); EIMS *m*/*z* 420 (5), 229 (30), 159 (60), 121 (100); HREIMS *m*/*z* 420.2880 (required 420.2876 for $C_{25}H_{40}O_5$).

Reduction of 3a. A total of 1.1 mg of ester **3a** in 600 mL EtOH was hydrogenated on 5% Pd/C. The reaction was kept at room temperature overnight and then filtered off. The filtrate was evaporated to give 0.7 mg of **7**: colorless solid, IR (liquid film) 3400, 2950, 2880, 1750 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.05 (1H, m, H-3), 3.95 (1H, m, H-10), 3.87 (1H, m, H-7), 3.77 (1H, m, H-6), 3.70 (3H, s, OCH_3), 2.50 (2H, d, J = 3.1 Hz, H_2-2), 2.05 (1H, m, H-9a), 1.89 (1H, m, H-8), 1.68 (1H, m, H-4), 1.62 (1H, m, H-5a), 1.56 (1H, m, H-9b), 1.50 (1H, m, H-11a),

1.40 (1H, m, H-11b), 1.29 (22H, m), 1.27 (2H, m, H_2-23), 0.87 (3H, t, $J = 5.9$ Hz, H_3-24).

Biological Methods. The inhibitory potency of the compounds was assayed by using submitochondrial particles (SMP) from beef heart. SMP were diluted to 0.5 mg/mL in 250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.4, and treated with 300 μM NADH to activate complex I before starting experiments. The enzymatic activity was assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA with SMP diluted to 6 μg/mL. Stock solutions (15 mM in absolute EtOH) of **1a** and **3a** were prepared and kept in the dark at −20 °C. Stolonoxides were added to diluted SMP preparations with 5 min of incubation in ice after each addition. NADH activity was measured as the aerobic oxidation of 75 μM NADH. Reaction rates were calculated from the linear decrease of NADH concentration (λ 340 nm, ϵ 6.22 mM^{-1} cm^{-1}) in an end-window photomutiplier spectrophotometer ATI-Unicam UV4-500. Given values are means ± SD of three assays for each compound.

Cytochrome *c* reductase activity sustained by both NADH (integrated activity of complex I and III) and succinate (integrated activity of complexes II and III) was measured while monitoring the reduction of 40 μM ferricytochrome *c* at 550 nm (ϵ 19.1 $mM^{-1}cm^{-1}$) in the presence of 2 mM KCN to block its reoxidation, starting the reaction with either 75 μM NADH or 12.5 mM succinate. Cytochrome *c* oxidase (complex IV) was measured as the aerobic oxidation of 30 μM ferrocytochrome *c* in the presence of 2 μM antimycin A for preventing the reduction of cytochrome *c* by complex III.

NADH:ubiquinone oxidoreductase (specific complex I activity) was measured with 75 μM NADH and 30 μM decylubiquinone (DB) as a soluble short-chain analogue of ubiquinone in the presence of 2 μM antimycin and 2 mM potassium cyanide to block any reaction downstream of complex I, following the NADH oxidation rate. Succinate:ubiquinone oxidoreductase (specific complex II activity) was measured similarly by changing NADH for 12.5 mM succinate and following the reduction of decylubiquinone (DB) at 278 nm (ϵ 14.0 mM^{-1} cm^{-1}). SMP were preincubated for 10 min in the presence of 1.25 mM succinate to fully activate complex II.

Supporting Information Available: NMR assignment of stolonoxide methyl esters and spectroscopic data (^1H and ^{13}C NMR spectra) of **1a** and **3a** in $CDCl_3$ (500 MHz). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0011373