Potent Inhibition of Oxidative Phosphorylation by Marine Natural Products

DANA D. JAMIESON ˣ, PAUL J. de ROME, and KENNETH M. TAYLOR

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Abstract □ Many lipid-soluble extracts from various marine organisms have a nonspecific depressant effect on smooth muscle contractions. Novel compounds isolated from such lipid-soluble extracts were tested for their effects on the respiration of rat liver mitochondria and produced potent stimulation or inhibition of oxygen uptake by the mitochondria.

Keyphrases □ Oxidative phosphorylation—effect of marine natural products on mitochondrial respiration □ Adenosine diphosphate—stimulating and inhibitory effects on mitochondrial respiration by compounds from marine organisms, oxidative phosphorylation □ Depressants, smooth muscle—novel marine compounds □ Respiration, mitochondrial—effect of marine natural products

Der Marderosian (1) observed that marine organisms offered enormous potential for the development of new and different pharmaceuticals, and he cited examples of the pharmacological activities from marine sources known at that time. In addition, Der Marderosian stressed the difficulties in handling the crude extracts for biological screening and the problems inherent in the extraction and characterization of the compounds responsible for the biological activity. While research of marine natural products in pharmacology and microbiology has continued since that time, there has been little biochemical experimentation, and marine natural products have not been tested yet in the field of energy metabolism.

During routine screening of compounds isolated from marine organisms in these laboratories, it was observed that many compounds obtained from organic solvent extraction depressed the contractile activity of mammalian smooth muscle preparations. The most sensitive tissue to the inhibitory action was the isolated guinea pig ileum. The inhibition was nonspecific in that the contractions produced by acetylcholine, histamine, nicotine, and 5-hydroxytryptamine were affected equally. One possible mode of action of the compounds was inhibition of energy metabolism. Therefore, in the present experiments, the compounds were tested for their effects on oxygen uptake by isolated rat liver mitochondria.

EXPERIMENTAL

Mitochondria were prepared by homogenizing rat liver in 20 volumes of solution (pH 7.0) containing 225 m*M* mannitol, 75 m*M* sucrose, and 0.5 m*M* ethylenediaminetetraacetic acid using a polytef pestle and a glass tube. From the supernate obtained after centrifugation at 500×*g* for 5 min, the mitochondria were precipitated by centrifugation at 9000×*g* for 5 min. The mitochondrial pellet was washed twice by resuspension in the described solution, followed by centrifugation at 9000×*g* for 5 min. The washed mitochondrial pellet was resuspended in 2 ml of the preparation medium.

Mitochondrial respiratory activity was measured in a medium containing 225 m*M* mannitol, 75 m*M* sucrose, 20 m*M* tromethamine hydrochloride, and 10 m*M* monobasic potassium phosphate adjusted to pH 7.4. The reaction vessel was a 4-ml glass cell, and oxygen uptake was measured with a polytef-covered oxygen electrode connected to a pO_2 module and a pen recorder. The temperature was 23.5°. Additions to the mitochondrial suspension (2.5–3 mg of protein) were made with microsyringes through a rubber seal in the sidearm of the reaction vessel in a manner essentially the same as that described by Estabrook (2).

Because the compounds tested had low water solubility, they were dissolved in dimethyl sulfoxide to suitable dilutions such that 1–10 μl of solution was added to the reaction vessel. Control experiments showed that <50 μl of dimethyl sulfoxide had no effect on the oxygen uptake of the rat liver mitochondria. Protein determinations were carried out according to Lowry *et al.* (3). Results are expressed as micromoles of oxygen consumed per minute per milligram of protein.

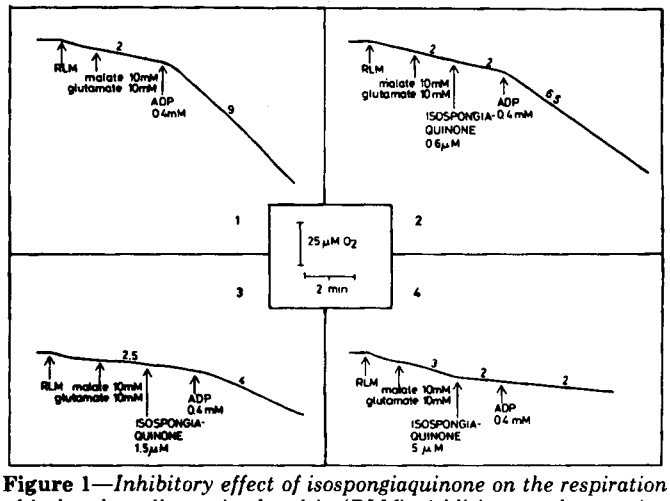

Figure 1—*Inhibitory effect of isospongiaquinone on the respiration of isolated rat liver mitochondria (RLM). Additions to the reaction vessel are as indicated, and numerals on the trace indicate micromoles of oxygen consumed per liter per minute per milligram of mitochondrial protein.*

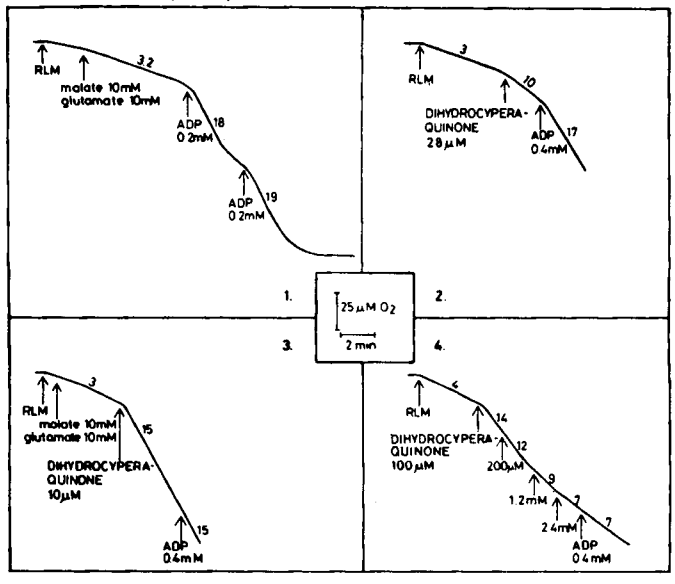

Figure 2—*Effect of dihydrocyperaquinone in stimulating the respiration of rat liver mitochondria (RLM). In Panel 4, increasing concentrations of dihydrocyperaquinone above 100 μM gradually decreased the initially stimulated respiration.*

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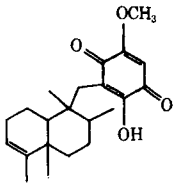
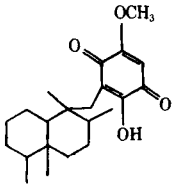
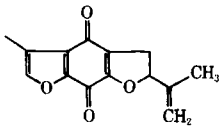
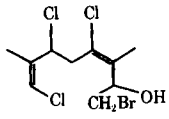
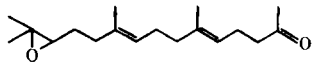
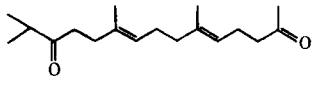
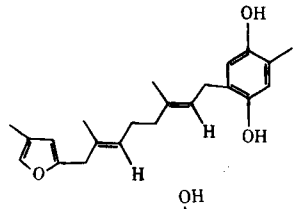
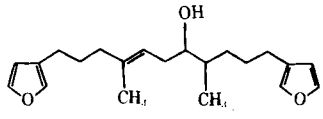
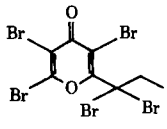
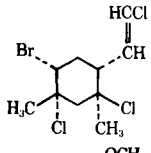
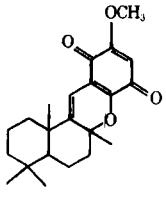
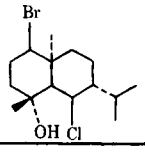
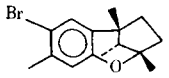
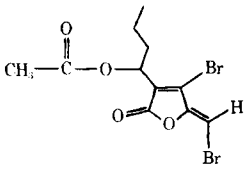
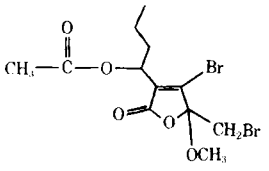
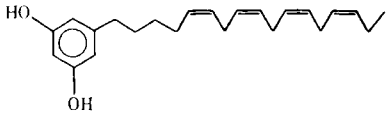
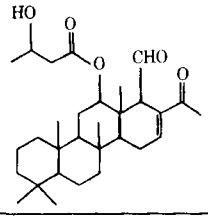
Table I—Effects on Respiration of Isolated Rat Liver Mitochondria by Novel Lipid-Soluble Compounds from Marine Organisms

Reference	Compound	Structure	Marine Organism	Concentration for 50% Inhibition of State 3 Respiration, μM	Concentration for 100% Increase of State 4 Respiration, μM
5	Isospongiaquinone		Sponge (Spongiidae)	1	—
5	Dihydroisospongiaquinone		Sponge (Spongiidae)	1	—
6	Dihydrocyperaquinone		Cyperaceae (*Remirea martima*)	1000	4
7	Costatol		Red alga (*Plocamium costatum*)	20–30	6
8	13(*R*),14-Epoxy-6,10,14-trimethyl-5,9-pentadecadiene-2-one		Brown alga (*Cystophora moniliformis*)	5	—
8	6,10,14-Trimethyl-5,9-pentadecadiene-2,13-dione		Brown alga (*Cystophora moniliformis*)	3	—
9	Furanoquinol		Soft coral	10	—
10	Furospongenol		Sponge (Spongia)	20	—
11	—		Red alga (*Ptilonia australasica*)	20	—
12	Plocamene E		Red alga (*Plocamium cartilagineum*)	150–300	30
5	Dehydrocyclospongiaquinone		Sponge (Spongiidae)	30	—
13	Heterocladol		Red alga (*Laurencia filiformis*)	60	60

(*continued*)

Table I—*Continued*

Reference	Compound	Structure	Marine Organism	Concentration for 50% Inhibition of State 3 Respiration, μM	Concentration for 100% Increase of State 4 Respiration, μM
14	Filiformin		Red alga (*Laurencia filiformis*)	150	—
15	Acetoxyfimbrolide		Red alga (*Delisea fimbriata*)	260	130
15	—		Red alga (*Delisea fimbriata*)	260	130
16	—		Brown alga (*Cystophora torulosa*)	300	—
17	—		Sponge (*Phyllospongia dendyi*)	—	200

After a steady State 4 respiration (4) was established with substrates of 10 mM malate and 10 mM glutamate, the test compound was added to the reaction vessel; adenosine diphosphate was added ~5 min later. Thus, the effects on both State 4 (resting, adenosine diphosphate-limited) and State 3 (active, adenosine diphosphate-stimulated) respiration were assessed in each trial. Each compound was tested at three or more concentrations on three batches of mitochondria to estimate an accurate concentration for doubling of State 4 respiration (*i.e.*, uncoupling action) or inhibition of the adenosine diphosphate-stimulated respiration to one-half of its control value.

RESULTS AND DISCUSSION

The chemical isolation and structural determinations of the compounds isolated from marine organisms in these laboratories have been reported, and the references are given in Table I. The structures of the tested compounds, the organisms from which the compounds were extracted, and the mean compound concentration required to produce doubling of oxygen uptake in mitochondria in State 4 (uncoupling effect) or 50% inhibition of oxygen uptake in State 3 respiration are summarized in Table I. The estimated effective potencies for each compound were very similar in the different mitochondrial batches.

Several compounds were very potent in either stimulating or inhibiting respiration. The most potent compounds were two closely related substances, isospongiaquinone and dihydroisospongiaquinone, extracted from a sponge of the family Spongiidae (5). Both compounds produced ~50% inhibition of State 3 respiration at 1 μM; at 5 μM, considerable depression of State 4 respiration also was seen. The effect of isospongiaquinone is illustrated in Fig. 1.

The most potent stimulant of State 4 respiration was dihydrocyperaquinone (6), which approximately doubled State 4 respiration at 4 μM. As the dihydrocyperaquinone concentration was increased, State 3 respiration gradually decreased, as is usual with compounds that uncouple oxidative phosphorylation. However, concentrations of 1 mM were needed to produce 50% inhibition of the State 3 respiration (Fig. 2).

Several other compounds showed mixed effects. The halogenated compounds costatol (7), heterocladol (13), and acetoxyfimbrolide (15) all showed mixed stimulation of State 4 and inhibition of State 3 respiration such that, while State 4 respiration could be approximately doubled at the concentrations shown in Table I, there was little or no further stimulation of respiration by adenosine diphosphate. As the concentration of these compounds was increased further, decreased stimulation of State 4 respiration was observed. However, such mixed effects on mitochondrial respiration were not always apparent with halogenated compounds. Filiformin and the pentabromopyrone from *Ptilonia australasica* (11) produced only inhibition with no uncoupling action.

While the precise site of action of these novel marine compounds in the respiratory chain is unknown, their nonselective inhibitory effects on smooth muscle preparations may be explained largely by the inhibition of electron transport or the uncoupling of oxidative phosphorylation (18, 19). In addition, compounds that uncouple oxidative phosphorylation affect the permeability of many excitable cells (20). Other enzyme systems also may be inhibited by these compounds because it was shown recently that several halogenated compounds isolated from red algae and from the sea hare *Aplysia dactylomela* prolonged pentobarbital-induced sleeping times and blood pentobarbital levels in mice, probably by inhibiting pentobarbital metabolism (21, 22).

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Inhibition of *In Vitro* Cytochrome P-450-Catalyzed Reactions by Substituted Pyridines

JERRY L. BORN ˣ and WILLIAM M. HADLEY

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Abstract □ A series of substituted pyridines was investigated as inhibitors of cytochrome P-450-catalyzed reactions. The relative potencies for the *in vitro* inhibition of aminopyrine demethylation and aniline hydroxylation are reported for a series of 2-, 3-, and 4-substituted pyridines.

Keyphrases □ Oxidation—cytochrome P-450, inhibition of *in vitro* metabolism of aniline and aminopyrine, effect of substituted pyridines on inhibition □ Aniline—hydroxylase activity, effect of substituted pyridines on *in vitro* aniline metabolism, cytochrome P-450 oxidation reactions □ Aminopyrine—demethylation activity, effect of substituted pyridines on *in vitro* aminopyrine metabolism, cytochrome P-450 oxidation reactions

Cytochrome P-450 is the terminal oxygenase responsible for the metabolism of many drugs and xenobiotics. Numerous pyridine-containing compounds such as metyrapone and ellipticine are known to inhibit cytochrome P-450-catalyzed reactions (1,2). It has been suggested that potent inhibitors of cytochrome P-450-catalyzed oxidations may be useful therapeutically in the prevention of chemical-induced cancer (2). Another potential use of cytochrome P-450 inhibitors is to increase the half-lives for drugs, thus increasing their therapeutic effectiveness.

The nonselective nature of the known inhibitors of cytochrome P-450-mediated reactions limits their therapeutic application. For example, metyrapone is used to inhibits steroid 11-β-hydroxylase, but it also is a potent inhibitor of many other cytochrome P-450 transformations (1, 3).

To develop leads in designing potent substrate-selective inhibitors of hepatic cytochrome P-450-catalyzed reactions, the ability of some simple pyridine compounds to inhibit the metabolism of aniline and aminopyrine was investigated.

EXPERIMENTAL

Male Sprague–Dawley rats were sacrificed by cervical dislocation, and their livers were excised and placed in ice-cold isotonic 1.15% KCl–tromethamine [tris(hydroxymethyl)aminomethane] buffer (0.1 *M*, pH 7.4). The following steps were performed at 0–4°. The liver was pressed through a tissue press, and the mince was weighed and homogenized[1] in three volumes of ice-cold isotonic potassium chloride–tromethamine buffer.

The homogenate was centrifuged for 20 min at 9000×*g* in an ultracentrifuge[2] using a No. 30 rotor. The supernate was withdrawn and centrifuged at 78,000×*g* for 60 min. Then the supernate was decanted, and the pellet was resuspended in tromethamine buffer so that 1 ml of buffer contained microsomes from 250 mg of liver. The protein content was determined using the method of Lowry *et al.* (4).

Aniline hydroxylase activity was determined by the analysis for *p*-hydroxyaniline. Metabolic reactions were run in 25-ml erlenmeyer flasks. The 5-ml reaction volume contained microsomal protein (10 mg), glucose-6-phosphate (30 μM), nicotinamide adenine dinucleotide phosphate (4 μM), magnesium chloride (50 μM), glucose-6-phosphate dehydrogenase (2 units), inhibitor in tromethamine buffer (pH 7.4), and substrate (1.09 μM) in buffer (pH 7.4). The reaction components, except the substrate, were preincubated for 5 min at 37° in a metabolic incubator under oxygen (flow rate >1000 ml/min) at 100 oscillations/min. Substrate then was added, and the reaction was incubated for 30 min. The production of *p*-hydroxyaniline was analyzed as described by Netter and Seidel (5).

Aminopyrine demethylation was determined by the analysis of formaldehyde. The 5-ml reaction volume contained the same cofactors used for aniline hydroxylation. The aminopyrine concentration was 8 m*M*. Formaldehyde was analyzed by the method of McMahon and Easton (6).

All samples were run in duplicate, and each experiment was repeated. The I_{50} values were calculated from three inhibitor concentrations.

All compounds were prepared by literature methods, and their physical constants corresponded to the literature values (7–9).

RESULTS AND DISCUSSION

The inhibition of aniline and aminopyrine metabolism by the substituted pyridines is shown in Table I. Inhibition of aniline metabolism was highly dependent on the position of substitution on pyridine. The 4-substituted pyridines were the most potent inhibitors of aniline metabolism, and the 3-substituted pyridines were more potent inhibitors than the 2-substituted pyridines. The presence of a hydroxyl group in the inhibitors lowered their ability to inhibit aniline metabolism. The decreased lipid solubility of the hydroxyl-substituted pyridines may be involved in their lower inhibitory activity.

[1] Potter-Elvejhem-type homogenizer.
[2] Beckman L5-40.

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