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Synthesis and Mode(s) of Action of a New Series of Imide Derivatives of 3-nitro-1,8 Naphthalic Acid

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Summary. *Four new imide derivatives of 3-nitro-1,8-naphthalic acid have been synthesised. The compounds show strong cytostatic activity against both HeLa and KB cells and are moderately toxic towards both mice and rats (LD_{50} above 4 mg/kg IP). Two of the most active compounds, M-4212 and M-12210, prevented the development of mouse Ehrlich ascites and rat Yoshida carcinoma. All these drugs block cell growth by inhibiting the synthesis of both DNA and RNA. In particular, both M-4212 and M-12210 raise the melting point of double-stranded DNA.*

Introduction

Aristolochic acid, designated as Compound I in Fig. 1, is a natural product possessing a β-nitronaphthalene moiety that is necessary for the reported antitumour activity of the compound [7]. Furthermore, some structural analogues of aristolochic acid, including a number of β-nitro derivatives, have even stronger cytotoxic activities than the natural product in cellular cultures [3]. The antibiotic cycloheximide (Compound II in Fig. 1), a well-known inhibitor of protein synthesis [10], is also a cytotoxic agent, as is 1-(morpholinomethyl)-4-phtalimido piperidin-2,6-dione (CG-603) (Compound III in Fig. 1), which acts by an endocrine mechanism [8]. These two compounds have a glutarimide ring in common, which in the case of Compound III is linked to a side chain. Finally tilorone, an antiviral agent that interacts with DNA [2], has two side chains, each with a tertiary amine moiety (Compound IV in Fig. 1).

We have considered the chemical structures of compounds I–IV and have synthesized four new imide derivatives of 3-nitro-1,8-naphthalic acid. The rationale un-

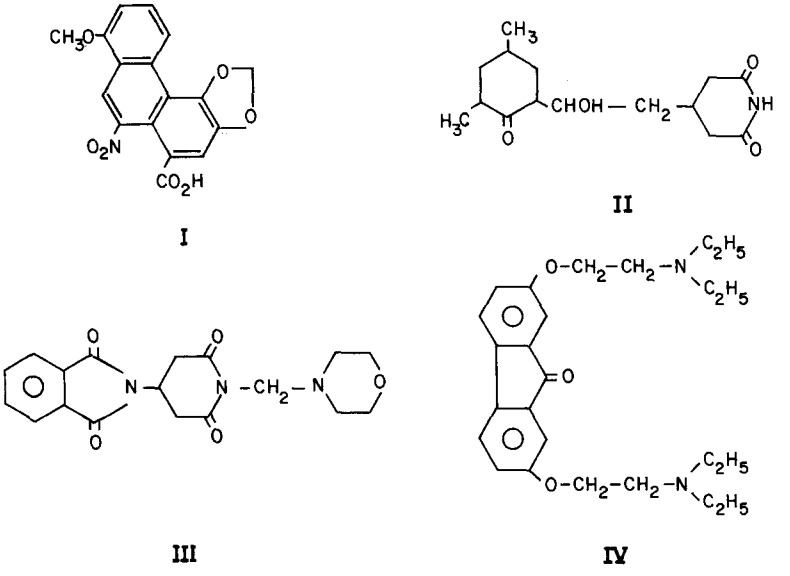

Fig. 1. Chemical structures of aristolochic acid (I), cycloheximide (II), 1-(morpholinomethyl)-4-phtalimido piperidin-2,6-diona (CG-603) (III), and tilorone (IV)

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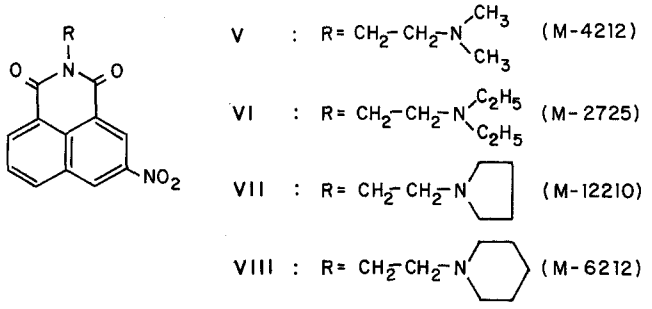


Fig. 2. Chemical structures of compounds M-4212 (V), M-2725 (VI), M-12210 (VII), and M-6212 (VIII)

derlying these preparations was to produce a single molecule containing chemical moieties known to be required for activity in compounds I–IV. The compounds synthesised were M-4212 (V), M-2725 (VI), M-12210 (VII), and M-6212 (VIII) (Fig. 2). All are imide derivatives of 1,8-dicarboxylic acid, each with a different basic side chain linked to the *N*-imide. The cytostatic activity and mode of action of these compounds were also studied; the results obtained are reported in the present communication.

Materials and Methods

Cells and Growth Conditions

HeLa 229 and Ehrlich ascites cells were used throughout this work. HeLa cells were grown in monolayer culture in Difco TC-Minimal Medium Eagle and in suspension culture in Difco TC-Minimal Medium Eagle Spinner Modified (Medium S). Both media were supplemented with 10% calf foetal serum. Ascites cells were maintained and grown in Albino Swiss mice. Subculturing was performed every 6–8 days.

Measurement of Cytostatic Activity

HeLa cells in monolayer culture were always used in our tests for cytostatic activity. The method employed was as previously described [4]. Drugs were always in sterile 0.5% carboxymethyl cellulose.

Determination of DNA, RNA, and Protein Synthesis. All experiments were performed with Ehrlich ascites cells, as described elsewhere [6].

Melting Curves

Melting curves for DNA were obtained with the aid of a double-beam spectrophotometer (Beckman Acta CIII) connected to a temperature programmer (Beckman Tm). The cells had a light path of 1 cm and a volume of 1 ml. The temperature was increased at a rate of 1° C/min. Calf thymus DNA (Worthington), bacteriophage φ29 DNA (a gift from Dr. Margarita Salas), and selected drugs were dissolved in SHE buffer (2 m*M* HEPES, 10 μ*M* EDTA, 9.4 m*M* NaCl adjusted to pH 7.0 with NaOH). For determinations of thermal denaturation profiles 125-μl aliquots of the stock DNA solutions were each added to 2.5 ml SHE buffer. Melting curves of DNA were determined by adding 125 μl stock DNA solution to 2.5 ml appropriate drug solution in SHE buffer. The final concentrations of DNA were therefore identical in the presence or absence of drugs. Absorbance at 260 nm was registered with the reference cell containing either SHE buffer or SHE buffer containing the relevant drug in solution.

Results and Discussion

Synthesis of Compounds V–VIII

Synthesis of Compounds V–VIII was carried out by nucleophilic reaction of the amine, corresponding to the required side chain (see Fig. 3) [9]. The selected amine (0.01 ml dissolved in 10 ml absolute ethanol) was added dropwise to a suspension of 0.01 mol 3-nitro-1,8 naphthalic anhydride in 50 ml absolute ethanol with constant agitation. Agitation was maintained for a further 2 h at room temperature after addition of the amine was complete. The solid formed was filtered, dissolved, and crystallized with ethanol.

Both the chemical and the spectroscopic characteristics of Compounds V–VIII are shown in Tables 1 and 2. IR spectra (only the most characteristic bands are given) were obtained by using KBr tablets in a Perkin-Elmer Model 257. The NMR spectra were registered in Cl_3CD in a Perkin-Elmer, Model R-12, TMS being used as a reference. Quantitative analyses were performed at the Laboratorio de Microanálisis del Centro Nacional de Química Orgánica (Madrid), the results obtained having errors of only $\pm$ 0.4%. From a combination of all these data the chemical structures of Compounds V–VIII were deduced (see Fig. 2).

Cytotoxic and Antitumor Activity

Cytotoxic activities of Compounds V–VIII were tested on both HeLa and KB cells following standard techniques previously described [6]. Maximum activity was observed with M-4212 and M-12210, the ID_{50} being below 0.5 μg/ml in both cases (Table 3). Toxicity was studied by IP

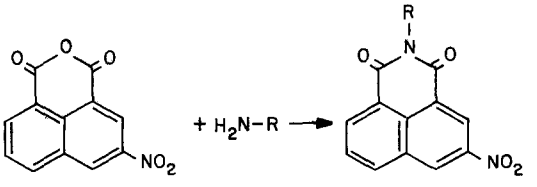
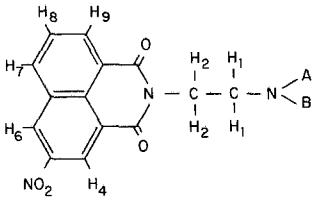


Fig. 3. Chemical reaction required for the synthesis of compounds V–VIII

Table 1. Chemical characterization of Compounds V–VIII

Compound	% Yield	Melting point (°C)	Analysis	IR spectra max. cm^{-1}
V (M-4212)	64	139–140	C, H, N	1710, 1660, 1600, 1540, 1345, 1330, 790, 760
VI (M-2725)	64	120–121	C, H, N	1710, 1655, 1600, 1550, 1350, 1330, 800, 760
VII (M-12210)	58	145–146	C, H, N	1700, 1665, 1600, 1535, 1340, 1325, 785, 760
VIII (M-6212)	57	136–137	C, H, N	1700, 1665, 1600, 1540, 1350, 1340, 895, 760

Table 2. NMR analysis of Compounds V–VIII



Compound	A	B	H_1	H_2	H_4	H_6	H_7	H_8	H_9
V (M-4212)	2.4 (s)	2.4 (s)	2.7 (t)	4.4 (c)	9.3 (d)	9.2 (d)	8.0 (c)	8.5 (c)	8.8 (c)
VI (M-2725)	1.0 (t)	2.7 (m)	2.7 (m)	4.3 (c)	9.3 (d)	9.1 (d)	8.0 (c)	8.5 (c)	8.8 (c)
VII (M-12210)	1.8 (m)	2.7 (m)	2.7 (m)	4.4 (c)	9.3 (d)	9.2 (d)	8.0 (c)	8.5 (c)	8.9 (c)
VIII (M-6212)	1.5 (m)	2.6 (m)	2.6 (m)	4.4 (c)	9.4 (d)	9.2 (d)	8.0 (c)	8.5 (c)	8.9 (c)

Table 3. Cytotoxic activity and acute toxicity of Compounds V–VIII

Compound	ID_{50} μg/ml HeLa	ID_{50} μg/ml KB	LD_{50} mg/kg IP mice	LD_{50} mg/kg IP rats
V (M-4212)	0.15	0.20	10	6.5
VI (M-2725)	2.50	3.50	13	32.5
VII (M-12210)	0.30	0.35	12.6	4.5
VIII (M-6212)	2.0	2.0	40	9.1

injection in mice and rats, and the LD_{50} of the compounds was always above 4 mg/kg (Table 3).

The antitumour activity of the compounds has been assayed previously in a series of 13 animals against both fast-growing tumours (Ehrlich ascites in mice and Yoshida carcinoma in rats) and a slow-growing tumour (SC Ehrlich ascites). The best survival effects were obtained with M-4212 (the international nonpropietary name of OMS is mitonafide) and M-12210 injected IP [1]. These two products are being studied more extensively in vivo by the National Cancer Institute (USA) and the Department of Pathological Anatomy (Faculty of Medicine, Valencia, Spain).

Effects on DNA, RNA, and Protein Synthesis in Animal Cell

To obtain information concerning the mode(s) of action of the most active compounds (M-4212 and M-12210), we studied their effects on macromolecular synthesis in Ehrlich ascites tumour cells. Various concentrations of both drugs were tested for effects on DNA, RNA, and protein

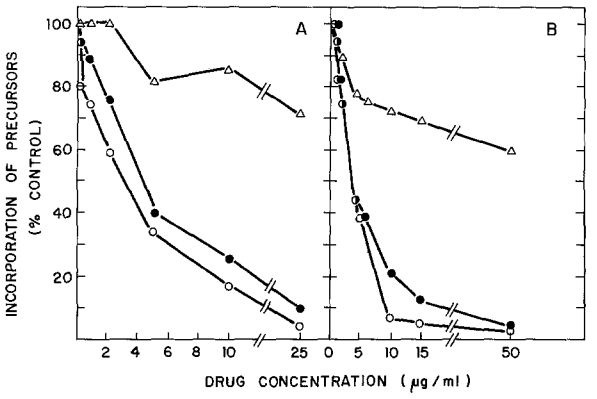


Fig. 4 A and B. Effects of different concentrations of M-4212 (**A**) and M-12210 (**B**) on the synthesis of DNA, RNA, and protein in Ehrlich ascites tumour cells. The drugs were added at the final concentrations indicated to 1.5 ml ascites cells suspended in medium S (5×10^5 cells/ml) containing the appropriate precursor: ^{14}C-thymidine (5 nCi/ml), ^{14}C-uridine (10 nCi/ml) or ^{14}C-proline (30 nCi/ml) to measure DNA (○), RNA (●), or protein (△) synthesis, respectively. The mixtures were incubated for 90 min and then 1-ml samples were diluted with 3 ml cold saline, centrifuged at 800 *g* for 2 min, and the supernatant discarded. Cell pellets were lysed in 1 ml cold water, mixed with 1 ml 10% trichloroacetic acid, and the precipitates were filtered through Whatmann GF/C glassfibre filters, washed with 5% trichloroacetic acid, dried, and counted in a liquid scintillation fluid. The results are presented as a percentage of the incorporation in the appropriate control which contained no inhibitor

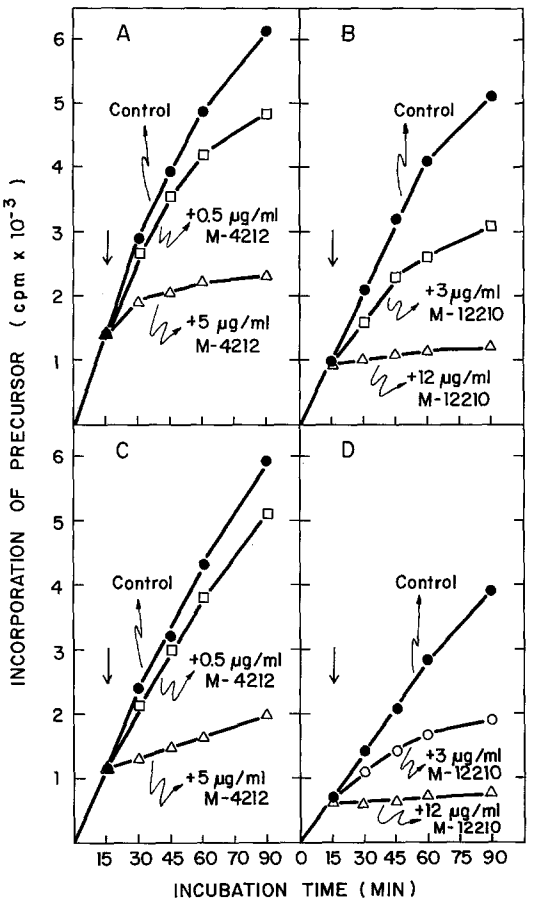


Fig. 5A–D. Time courses showing the effects of M-4212 and M-12210 on the synthesis of DNA (**A** and **B**) and RNA (**C** and **D**) by Ehrlich ascites tumor cells. Concentrations of cells and precursors were as described in the legend to Fig. 4. At the indicated times 1 ml cells was removed and processed for radioactivity as described for Fig. 4. The *arrows* indicate the time of drug addition

synthesis, the results obtained being illustrated in Fig. 4. Although protein synthesis is only weakly affected, nucleic acid synthesis is strongly inhibited, with DNA synthesis being blocked preferentially (Fig. 4). The slight effect on protein synthesis might, therefore, result indirectly from inhibition of RNA synthesis. The effects on nucleic acid synthesis become apparent within 5 min after addition of the drugs (Fig. 5), whereas no inhibition of ^{14}C-proline incorporation into the protein fraction was detected during 60 min of incubation in the presence of the drugs (results not shown). The inhibitory effects of M-4212 and M-12210 are effectively reversed after centrifugation of the cells followed by resuspension in fresh medium (Fig. 6). This result suggest that the drugs do not bind covalently to their target site(s).

The inhibitors may block DNA and RNA synthesis either by acting as structural analogues of nucleosides or deoxynucleosides or by inhibiting the synthesis of these precursors. We eliminated these two possibilities by examining the effects of M-4212 and M-12210 on the incorporation of both ^{14}C-thymidine into DNA and ^{14}C-uridine into RNA in the presence of any of the following precursors alone or a mixture of all of them: cytidine, 2′-deoxyuridine, thymidine, deoxyadenosine, deoxycytidine and deoxyguanosine, each present at 6.6×10^{-2} *M* concentration. In all cases the precursors failed to reverse the inhibitory action of the drugs on either DNA or RNA synthesis (results not shown).

Effects of M-4212 and M-12210 on the Melting Curves of DNA

The interaction of certain drugs with double-stranded DNA frequently affects the unfolding of the two chains that normally takes place on heating and results in an increase in the 'denaturing temperature'. Since both M-4212 and M-12210 induce such an effect (Fig. 7), it is clear that they bind to DNA. Thus, in the presence of 300 μg M-4212/ml, the usual melting temperature of calf thymus DNA (76.2° C) is increased to 87° C (A = 10.8° C) (Fig. 7A). Similarly, 350 μg/ml of M-12210 increases the melting temperature of bacteriophage φ29 from 73° C–83.5° C (A = 10.5° C) (Fig. 7B). The decreased absorption of DNA in the presence of drugs compared with the controls is frequently observed as a consequence of the binding of the inhibitors to DNA [2, 5].

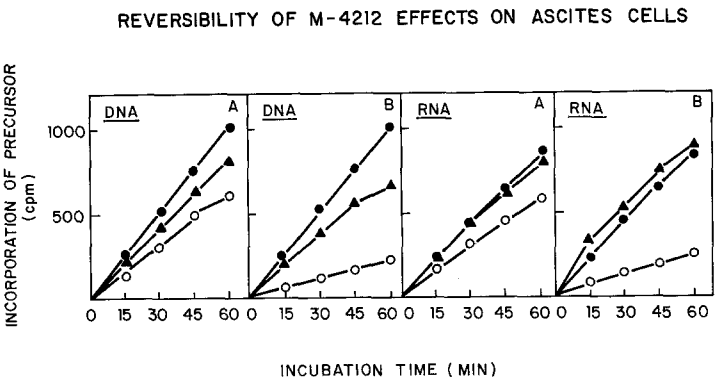


Fig. 6. Reversal of the inhibitory effect of M-4212 on Ehrlich ascites tumor cells. Control cells (●); 5×10^5 cells/ml) were incubated without drug for 1 h and then washed and incubated with either ^{14}C-thymidine or ^{14}C-uridine. ▲, Cells were preincubated with 2 μg/ml (**A**) or 10 μg/ml (**B**) of M-4212 for 1 h and then washed, resuspended in fresh medium and incubated with ^{14}C-thymidine or ^{14}C-uridine. ○, Cells were preincubated with 2 μg/ml (**A**) or 10 μg/ml (**B**) M-4212 for 1 h, then washed, resuspended in fresh medium containing either 2 μg/ml (**A**) or 10 μg/ml (**B**) of M-4212 and incubated with ^{14}C-thymidine or ^{14}C-uridine. At the indicated times 1-ml samples were removed and processed for radioactivity as indicated under Fig. 4

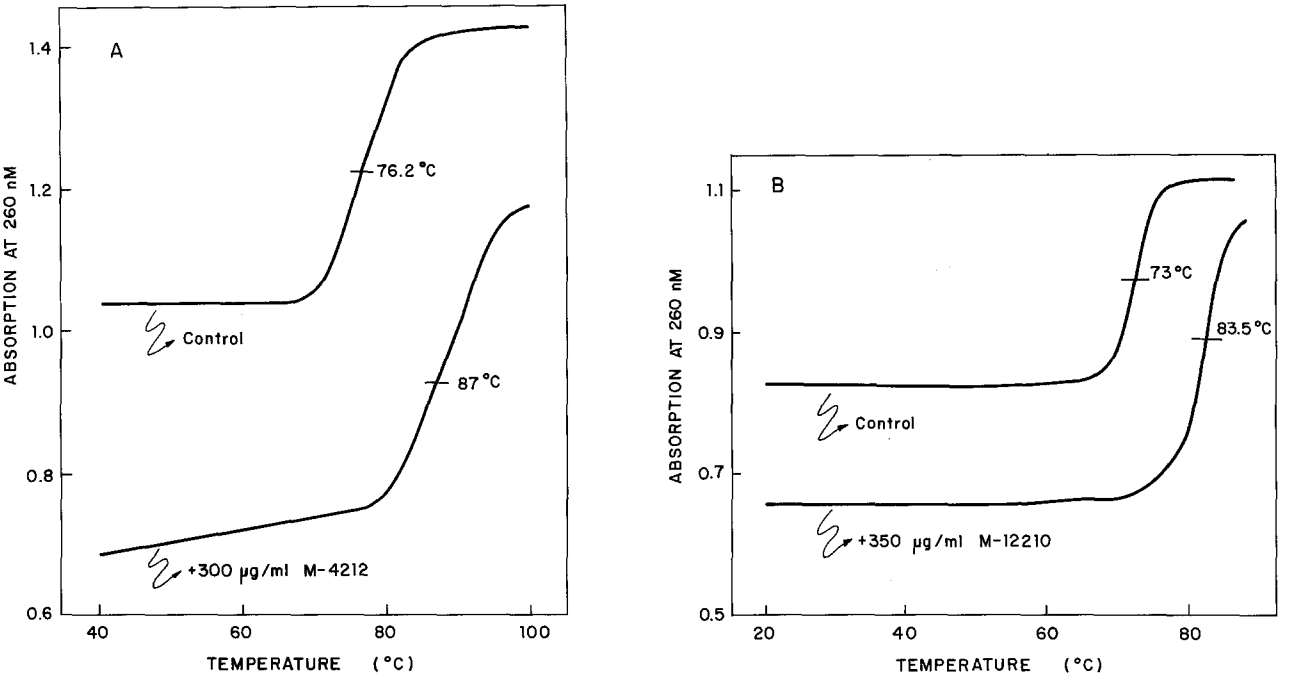


Fig. 7 A and B. The effects of M-4212 and M-12210 on the melting curves of DNA. **A** Calf thymus DNA; **B** Bacteriophage φ29 DNA

Discussion

Our results with the imide derivatives of 3-nitro-1,8 naphthalic acid, synthesised and characterized as described above, have shown these compounds to possess strong cytotoxic activity. Since in addition their inhibitory effects on nucleic acid synthesis are reversed it seems that these compounds might be useful for further studies with experimental tumours.

The most active imide derivatives synthesised (M-4212 and M-12210) certainly have cytotoxic and cytostatic activities. It is unlikely that the rationale underlying these preparations was correct in this case; thus M-4212 and M-12210 do not inhibit primarily protein synthesis as cycloheximide does, even at rather high concentrations. However, both drugs inhibit DNA and RNA synthesis and bind to DNA. Therefore these new imide derivatives (Fig. 2) might possibly act as tilorone, which has two side chains with a tertiary amine moiety (Fig. 1, Compound IV) and intercalates between the bases of the DNA molecule [2].

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