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# Synthesis, spectroscopic characterization, stability assessment and DNA-binding of new 2,6-piperidinedione derivatives

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**Abstract**

This work reports on structural characterization of new antineoplaston (ANP) representatives, namely 3-(benzoylamino)-2,6-piperidinedione (BPD), 3-(4-methoxybenzoylamino)-2,6-piperidinedione (MPD) and 3-(*p*-nitrobenzoylamino)-2,6-piperidinedione (NPD). These compounds were prepared by reacting *N*-(4-substituted benzoyl)-glutamines with *N*-hydroxysuccinimide to afford the corresponding esters, which were heated to produce the corresponding 2,6-piperidinedione (PD) compounds. Non-destructive analytical procedures such as $^1$H NMR and NIR analyses confirmed the postulated chemical structures of these PD compounds. HPLC chromatograms at an ambient temperature or from solutions preheated at 30, 40 or 60 °C displayed only a single peak for each compound. Combination of heat with pH modification had virtually no effect on the obtained peaks, thus attesting to the stability and purity of these compounds. MS analysis displayed molecular mass ions indicative of BPD, MPD and NPD at *m*/*z* 233.4, 263.2 and 278.3, respectively. The fragmentation patterns using MS/MS analyses conformed to the structural and molecular formulae of the prepared compounds. Furthermore, preliminary biological assessments showed the capacity of these compounds to bind to the DNA. NPD, but not BMP or MPD, had a superior affinity to the DNA than the prototype ANP-A10.


*Keywords:* 2,6-Piperidinediones; NIR; HPLC; MS/MS; DNA-binding

## 1. Introduction

Antineoplaston, 3-(phenylacetylamino)-2,6-piperidinedione (A10) is a human endogenous cytodifferentiating agent with a broad range of biological activities [1–4]. A10 was proved to be an efficacious cancer chemopreventive agent when tested on cancer cell lines [5–7] or given to animals with various tumor types [8]. Moreover, A10 and its metabolites showed promising cytostatic responses when challenged on tumor tissue cultures [6,7,9] or administered to human patients with hepatocellular carcinoma [6,7]. Likewise, studies conducted on human cancer cells and cancer patients showed antineoplastons as remarkable cytostatic, immunemodulatory and cytodifferentiating agents [10–14]. In all such studies, antineoplastons acted mostly via nuclear mechanisms that included interaction with the DNA or modulation of apoptic rates in tumor- or host defensive-cells [11]. Despite the numerous biological investigations on antineoplastons, little information, if any, is available on their structure characterization or stability in biological fluids. Accordingly, the present study was undertaken to characterize the structural profiles of antineoplastons and gain insights into their stability at more drastic pH or thermal conditions. Recent analytical procedures [15,16] have been currently utilized to investigate such possibilities. Furthermore, we report on the capacity of these newly

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prepared derivatives to bind to the DNA. This assay is reportedly predictive of the antineoplastic activity of agents that target the genomic structure [14,17].

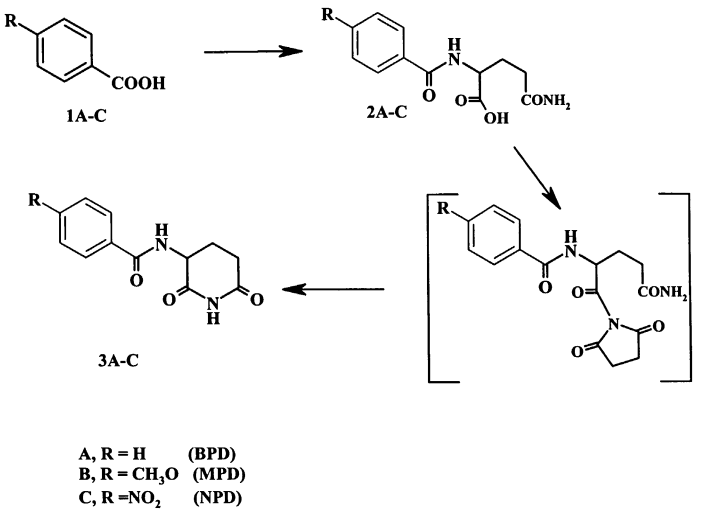
## 2. Experimental

### 2.1. Materials

All chemicals, reagents and solvents used for the chemical synthesis of 2,6-piperidinedione compounds were of analytical grade. HPLC grade solvents were used for HPLC and LC/MS analyses.

### 2.2. Instruments

Melting point determinations were recorded using Thomas Hoover Capillary Apparatus. Elemental analyses ( $\pm$ 0.4%) were performed by Atlantic Microlab Inc., Nocross, GA. $^1$H NMR spectra were measured in DMSO-$d_6$ using 300 MHz Bruker NMR spectrometer. FT-NIR spectra were collected using Nexus FT-NIR equipment (Nicolet, USA) with NIR source (10 000–4000 cm$^{-1}$). NIR spectra were run in the transmission mode using Nicolet NIR updraft unit. All data processing was controlled by the instrument build-in OMNIC E.S.P 5.1 software (Nicolet). HPLC analyses were performed using an isocratic high-performance liquid chro-




Scheme 1. Chemical reactions for the synthesis of 3-(4-substituted-benzoylamino) 2,6-piperidinedione analogs.

Table 1
Melting points, elemental analysis, and $^1$H NMR analysis of BPD, MPD and NPD

| Comp. | m.p. (°C) | Formula | Analysis (Calc./Found) | $^1$H NMR |
|---|---|---|---|---|
| BPD | 222–224 | $C_{12}H_{12}N_2O_3$ | C 62.06/62.42 | 2.01 (m, 2H, HC–$CH_2$–$CH_2$) |
| | | | | 2.55 (m, 2R, $CH_2$–CO) |
| | | | H 5.17/5.21 | 4.70 (q, 1H, NH–CH–$CH_2$) |
| | | | | 7.85 (m, 5H, Ar–H) |
| | | | N 12.06/12.23 | 8.61 (d, 1H, CONH–CH) |
| | | | | 10.89 (s, 1H, CO–NH–CO) |
| MPD | 213–215 | $C_{13}H_{14}N_2O_4$ | C 59.54/59.35 | 1.98 (m, 2H, HC–$CH_2$–$CH_2$) |
| | | | | 2.45 (m, 2H, $CH_2$–CO) |
| | | | H 5.34/5.39 | 3.91 (s, 3H, $CH_3O$) |
| | | | | 4.65 (q, 1H, NH–CH–$CH_2$) |
| | | | N 10.68/10.65 | 7.75 (m, 4H, Ar–H) |
| | | | | 8.75 (d, 1H, CONH–CH) |
| | | | | 10.50 (s, 1H, CO–NH–CO) |
| NPD | 240–241 | $C_{12}H_{11}N_3O_5$ | C 51.98/52.07 | 2.02 (m, 2H, HC–$CH_2$–$CH_2$) |
| | | | | 2.45 (m, 2H, $CH_2$–CO) |
| | | | H 3.97/4.01 | 4.78 (q, 1H, NH–CH–$CH_2$) |
| | | | | 7.55 (d, 2H, Ar–H) |
| | | | N 15.16/15.09 | 8.25 (d, 2H, Ar–H) |
| | | | | 8.68 (d, 1H, CONH–CH) |
| | | | | 10.60 (s, 1H, CO–NH–CO) |

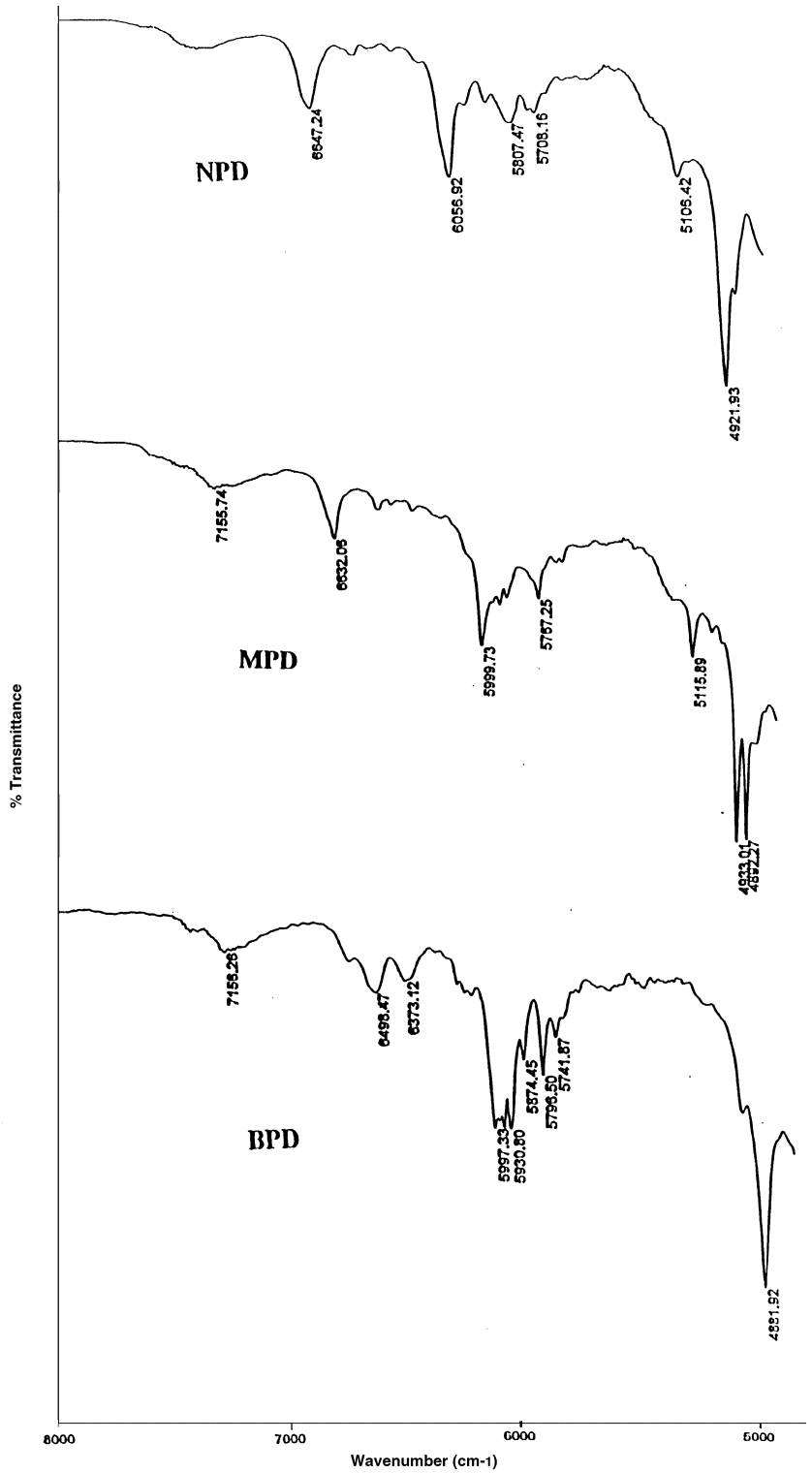



Fig. 1. NIR transmission spectra of BPD, MPD and NPD.

matograph (Waters 2690 Separation Module) equipped with photodiode array detector (Waters 996) and Shim Pack CLC-SIL column (150 × 6 mm id, 5 μm). The mobile phase (methanol/1% acetic acid solution 1:1) was pumped at flow rate 1 ml/min and the eluents were monitored at 262 nm. Analytical data were processed by the instrument build-in Millennium software. MS and MS/MS analyses were performed on the LCQ mass spectrometer (Finnigan) linked to HPLC pump P 2000 Spectra system (TSP). The HPLC column was Shim Pack CLC-CN, 5 μm (15 × 6 mm id) and the mobile phase was a mixture of methanol/acetic acid solution

1% (4:1) flowing at 1 ml/min. APCI was selected as an ionization process. APCI conditions were: corona discharge 4.5 kV, vaporization temperature 430 °C and capillary temperature 147 °C. The scanning modes were full MS and full MS/MS. All data processing was controlled by the instrument LCQ software.

### 2.3. Procedures

#### 2.3.1. Preparation of 2,6-piperidinedione derivatives

*2.3.1.1. Preparation of N-(4-substituted benzoyl)-L-glutamines (**2A–C**).* The acid chloride solutions of **1A–C** (34.25 mmol) in *N*,*N*-dimethylformamide (DMF) (100 ml) were gradually added to a cooled aqueous solution of L-glutamine (34.25 mmol) and $NaHCO_3$ (68.50 mmol) in water (100 ml). The reaction mixture was then stirred at room temperature for 24 h. The solvent was removed in vacuo and the residue was dissolved in water (50 ml) and neutralized with 1N HCl solution. The crude product was purified by flash column chromatography [solvent: ethyl acetate/cyclohexane (1:1)]. The solvent was evaporated in vacuo and the white crystalline product was collected. The yield percentages of compounds (**2A–C**) were 75.1, 69.7 and 64.9, respectively, of the purified products (Scheme 1).

*2.3.1.2. Preparation of 3-(4-substituted-benzoylamino)-2,6 piperidinediones (**3A–C**).* A solution of 1,3-dicyclohexylcarbodiimide (20 mmol) in dry DMF (50 ml) was gradually added to a mixture of *N*-hydroxysuccinimide (20 mmol) and **2A–C** (20 mmol) in dry DMF (50 ml). The reaction mixture was stirred overnight at room temperature. A white precipitate of 1,3-dicyclohexy-

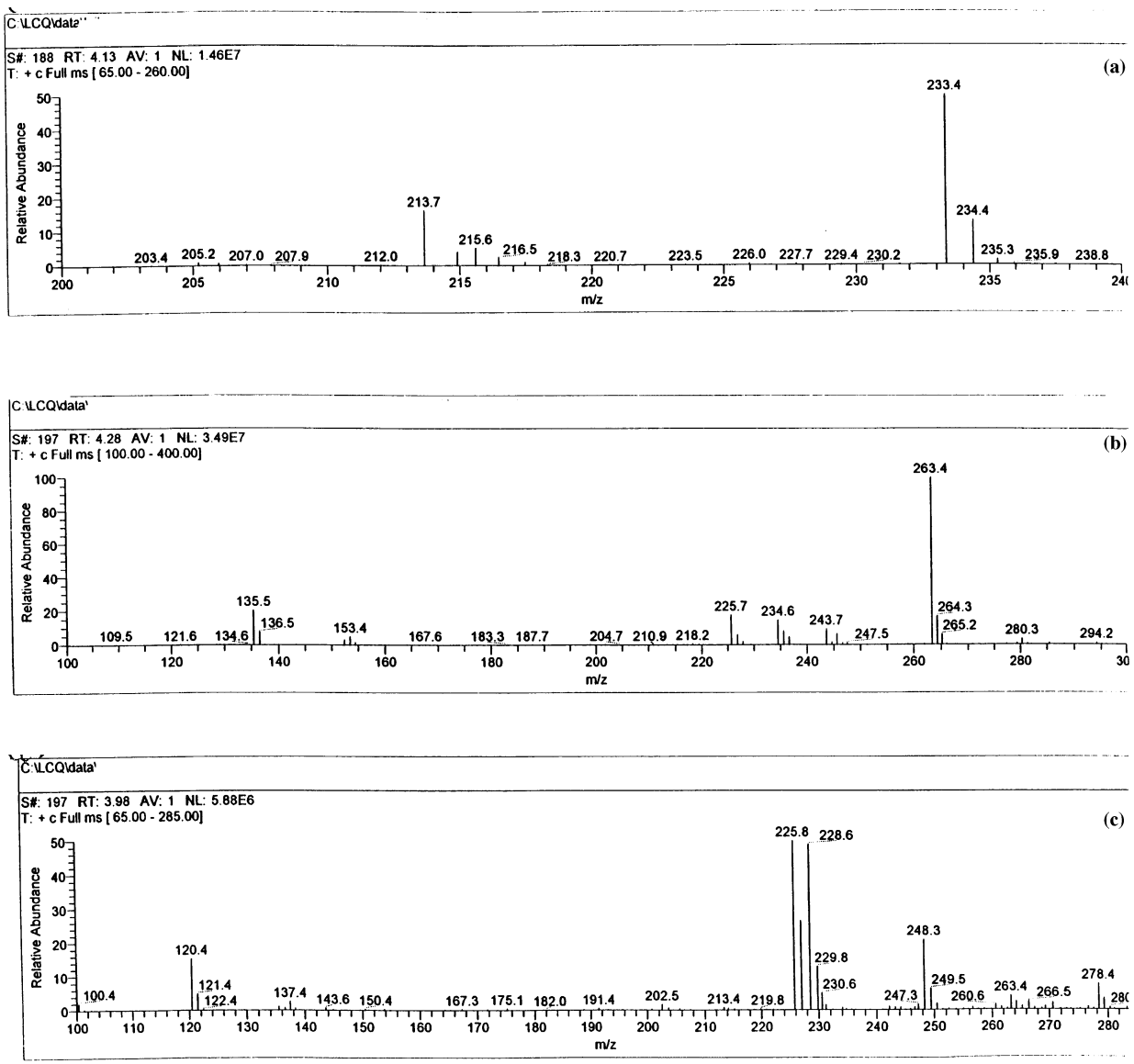



Fig. 2. Full MS spectra of BPD (a); MPD (b) and NPD (c).

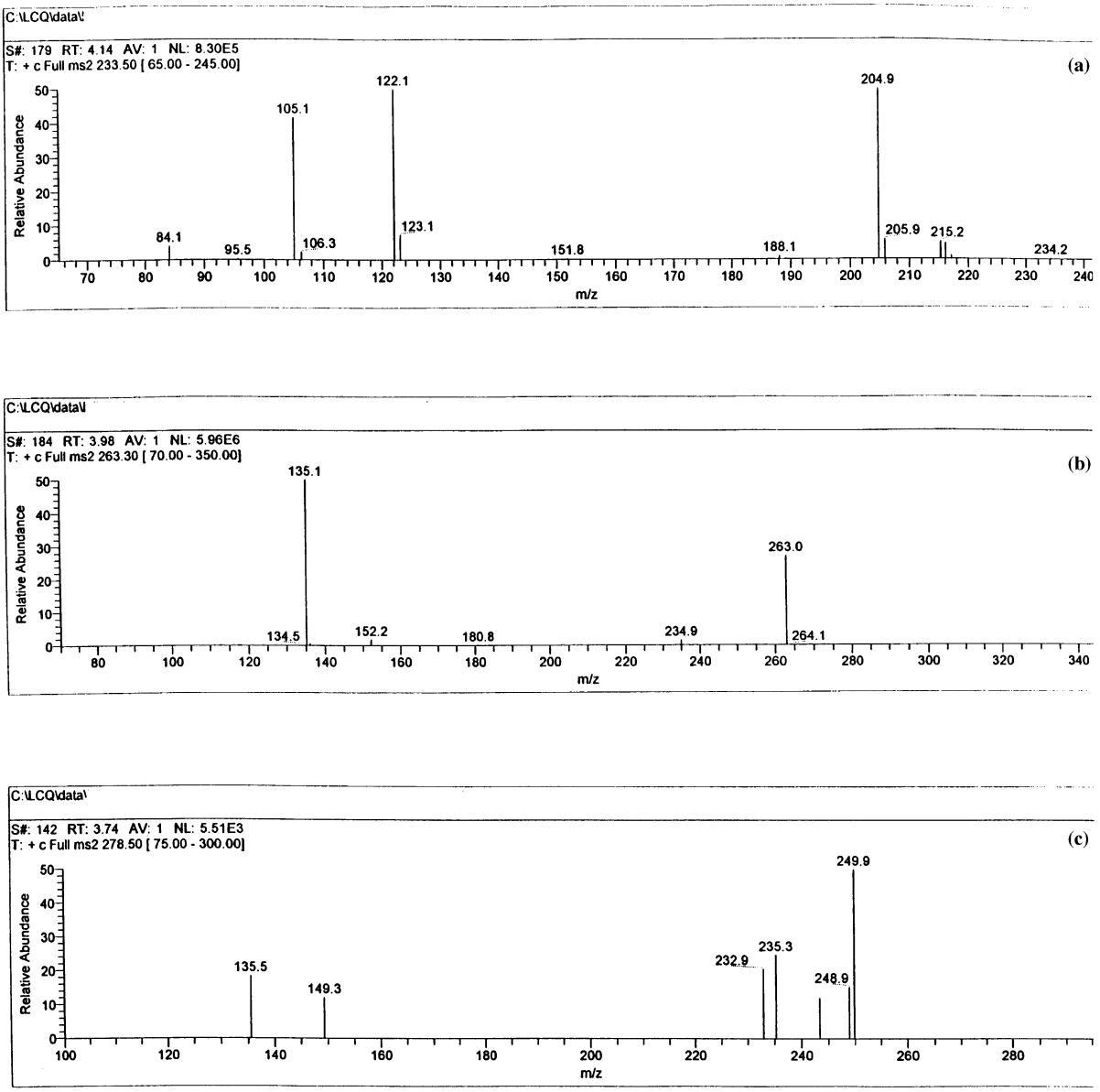



Fig. 3. MS/MS spectra of BPD (a); MPD (b) and NPD (c).

lurea was filtered off and the filtrate was heated under reflux for 4–6 h at 100 °C. The reaction mixture was evaporated in vacuo and the corresponding target compound was purified by flash column chromatography [solvent: chloroform/methanol (10:1)]. The yield% of the purified products was 49.7 of BPD, 55.2 of MPD and 50.0 of NPD.

### 2.3.2. Purity and stability studies

Aliquots (50 μl) of freshly prepared solutions of BPD, MPD or NPD in methanol (1 μg/μl) were transferred into 1 ml glass vials and diluted to volumes with either methanol/water (1:1), 0.1 M HCl solution, or phosphate buffer solution (pH 7.5). The solutions were heated in thermostatically controlled water bath at 30, 40 or 60 °C for 6 h. The vials were cooled and solutions were neutralized (when necessary) and 10 μl aliquots were injected into HPLC.

### 2.3.3. LC–MS and LC/MS/MS

Aliquots (10 μl) of BPD, MPD or NPD in methanol were injected into the mass spectrometer. The samples were measured in MS and MS/MS scanning modes.

### 2.3.4. Assessment of DNA binding affinity

DNA binding was determined according to the reported procedures [17]. The principle of the assay is that DNA/methyl green forms a stable green-colored complex in neutral solutions ($\lambda$ max, 642–645 nm). When displaced by agents that bind DNA, the liberated

methyl green adds a molecule of water to its structure, thereby forming a colorless 'carbinol'. The reduction in the absorbance of DNA/methyl green complex reflects the DNA binding capacity of the added drug.

DNA/methyl green (20 μg/ml) was suspended in 0.05 M Tris–HCl buffer (pH 7.5) containing 7.5 mM $MgSO_4$, stirred overnight at 37 °C. The newly synthesized piperidinediones were dissolved in dimethyl sulfoxide (DMSO). Solvent was removed in vacuo, and 200 μl of the DNA/methyl green solution was added to each well. Initial absorbance for each well was determined in a microplate reader and samples were then incubated in the dark at ambient temperature. After 24 h, the final absorbance of the samples was determined. Readings were corrected for initial absorbance and normalized as a percentage of the untreated DNA/methyl green absorbance value. $IC_{50}$ values were determined by linear regression of data plotted on a semi-log scale (Table 2).

## 3. Results and discussion

### 3.1. Chemistry of 3-(4-substituted-benzoylamino)-2,6-piperidinedione

The presented 2,6-piperidinedione derivatives were synthesized according to Scheme 1. The intermediate compounds, *N*-(4-substituted benzoyl)-L-glutamines (**2A**–**C**), were firstly prepared by reacting the appropriate 4-substituted benzoyl chlorides (**1A**–**C**) with L-glutamine in aqueous sodium bicarbonate [2]. These intermediates (**2A**–**C**) were then reacted with *N*-hydroxysuccinimide in the presence of 1,3-dicyclohexylcarbodiimide to afford the corresponding *N*-hydroxysuccinimide esters. The obtained esters were heated at 100 °C for 4–6 h to afford the 3-(4-substituted-benzoylamino)-2,6 piperidinedione compounds **3A**–**C**. The yield percentages of the purified BPD, MPD and NPD were 49.7, 55.2 and 50.0, respectively. The melting points, elemental analysis and $^1H$ NMR analysis supported the assigned chemical structures (Table 1).

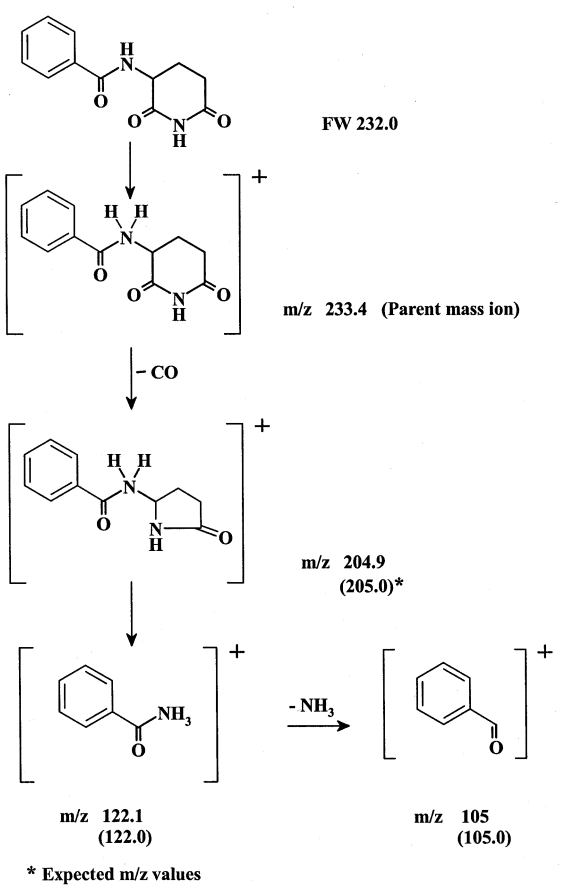
### 3.2. NIR analysis

FT-NIR has now become an increasingly valuable tool in identifying compounds with cognate chemical structures [15]. Currently, the NIR spectra of BPD, MPD and NPD were recorded in the transmission scanning mode using NIR updraft unit in the range of 10 000–4000 $cm^{-1}$. As shown in Fig. 1, despite the structural similarity of investigated compounds, their spectra can be distinguished by fine measurement of the wave number of NIR-bands of interest. Therefore, the 4-substituted analogs, MPD and NPD, can be distinguished from the non-substituted compound, BPD, by having characteristic NIR-bands at 6647.24 and 5115.89 $cm^{-1}$ (MPD) and 6632.06 and 5106.42 $cm^{-1}$ (NPD). BPD was also characterized by the appearance of a 'split band' at 6498.47/6373.12 $cm^{-1}$. MPD also showed a 'split band' at 4933.01/4892.27 $cm^{-1}$.

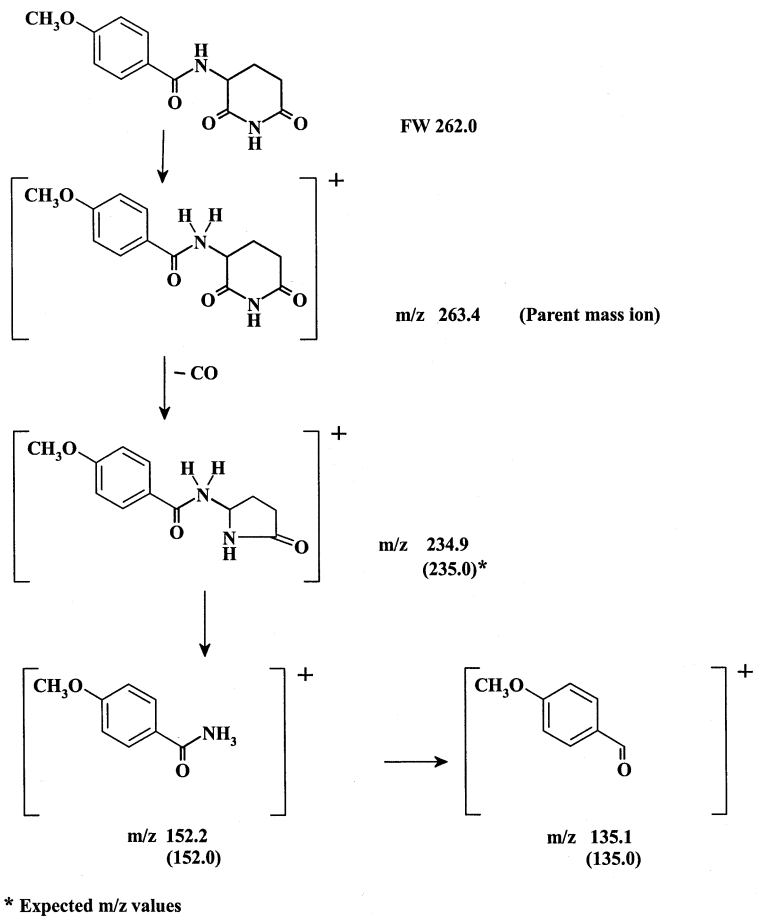
### 3.3. Purity and stability assessment

The purity of BPD, MPD and NPD was assessed by HPLC analysis. The chromatograms showed only a single peak for each compound, thus indicating the lack of degradation products or reaction intermediates. Further evidence for purity of these compounds comes from LC/MS analyses wherein the TIC of compounds exhibited single peaks that were indicative of the mass ions of BPD, MPD and NPD.

Stability of the prepared compounds was also verified by running HPLC analyses following application of more drastic levels of heat or pH. The peak areas of BPD, MPD and NPD were insignificantly changed after acidic or alkaline incubations for 6 h at 30, 40 and 60 °C. Furthermore, no extra peaks due to degradation products were observed in the chromatograms after these treatments. These observations indicate the resistance of the investigated compounds to hydrolysis under conditions similar to those encountered in gastric or intestinal fluids.




Scheme 2. A proposed MS/MS fragmentation pattern of BPD.




Scheme 3. A proposed MS/MS fragmentation pattern of MPD.

### 3.4. LC–MS and LC–MS/MS analyses

Fig. 2a–c shows the MS spectra of BPD, MPD and NPD, respectively. Molecular mass ions $[M-H]^+$ at $m/z$ were 233.4, 263.2 and 278.3, respectively. MS/MS analysis was further used to define the fragmentation pattern and confirm the molecular formulae of these compounds. Fig. 3a shows the MS/MS spectra of BPD at $m/z$ 233.4 and 10% of collision energy. The major fragments of BPD, at $m/z$ 204.9, 122.1 and 105.1, correspond to structures in Scheme 2. Fig. 3b displays the MS/MS of MPD at $m/z$ 263.2 and 7% of collision. The fragment ions, at $m/z$ 235.0, 152.0 and 135.0, refer to structures in Scheme 3. Fig. 3c shows the MS/MS fragmentation of NPD at $m/z$ 278.3 and 20% of collision. The major fragments, observed at $m/z$ 249.9, 235.3, 149.3 and 135.5, are congruent to the structures in Scheme 4. In general, the predicted and reported $m/z$ values were in good agreement with all proposed structures (Schemes 2–4). A mass range difference of $\sim 0.1$ amu between the expected and experimental values of the base fragment peaks was observed.

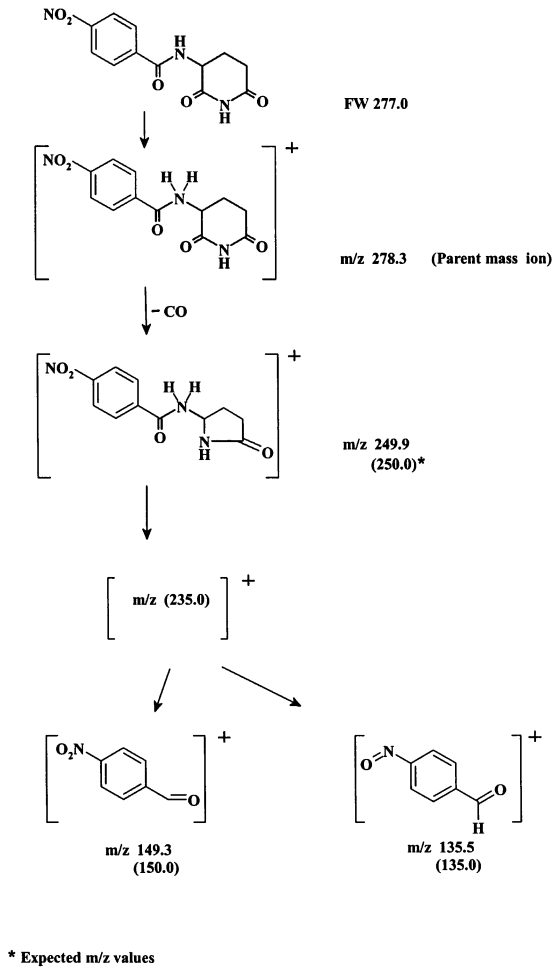
### 3.5. DNA binding of A10 analogs

This assay was carried out to gain insights into the potential of the newly prepared A10 analogs to interact with DNA. It has been shown that the prototype ANP-A10 may interact reversibly with DNA, thereby competing with carcinogens that form covalent linkage with DNA [6]. Present data, supported by statistical analyses, revealed that compound NPD bound to the DNA and displaced methyl green with a significantly higher potency than the prototype A10 ($P < 0.05$, Table 2). Compounds BPD and MPD showed lower affinity to the DNA. Taken together, these data suggest that interaction with DNA is a possible nuclear mechanism for the action of A10-analogs. This dogma is in agreement with A10 studies on DNA fragmentation, cell cycle and apoptosis in various malignant cell lines [7,11].

## 4. Conclusions

The present study is the first to fully characterize the spectroscopic profiles of 2,6-piperidinedione, a now well-recognized class of antineoplastic agents. Accord-

ingly, three new such derivatives were synthesized for that purpose. Their structures were confirmed by conventional spectroscopic methods as well as by advanced analyses such as LC/MS, LC/MS/MS and NIR. These procedures permitted identification, differentiation and confirmation of the molecular/structural formulae of 2,6-piperidinediones. Also, they provided unequivocal evidence for the stability of these compounds under challenging pH or thermal conditions, similar to those encountered in biological fluids or during pharmaceutical preparations. Because the antineoplastic activity known for the lead is largely dictated by its DNA interaction, we verified the potential of the prepared compounds to bind to the DNA in vitro. A reasonable to appreciable affinity to bind the DNA was obtained.




Scheme 4. A proposed MS/MS fragmentation pattern of NPD.

Table 2
DNA binding capacity of A10 analogs in the 'DNA/methyl green' binding assay

| Comp. | $IC_{50}$ (μM) |
|---|---|
| A10 | $99 \pm 7.3$ |
| BPD | $108 \pm 4.2$ |
| MPD | $136 \pm 8.7$ |
| NPD | $43 \pm 2.1$ [a] |

Data are presented as means ± standard error means, $n = 4$–5.
[a] Significantly lower than A10 value; $P < 0.05$.

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