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Parenteral formulation and thermal degradation pathways of a potent rebeccamycin based indolocarbazole topoisomerase I inhibitor

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ABSTRACT

The development of a practical and pharmaceutically acceptable parenteral dosage form of **1** is described. A cosolvent formulation strategy was selected to achieve the necessary human dose of **1** for administration via intravenous infusion. The final market formulation of **1** chosen for commercial development and Phase II clinical supplies was the topoisomerase inhibitor dissolved in a 50% aqueous propylene glycol solution vehicle with 50 mM citrate buffered to pH 4. The thermal degradation pathways of **1** in this aqueous propylene glycol vehicle in the pH range of 3–5 were determined by relative kinetics and degradation product identification using LC/MS, LC/MS/MS, and NMR analysis. The primary mode of degradation of **1** in this aqueous cosolvent formulation is hydrolysis affording the anhydride **2** (in equilibrium with the dicarboxylic acid **3**) and release of the hydrazine diol side chain **11**. Subsequent oxidative degradation of **11** occurs in several chemical steps which yield a complicated mixture of secondary reaction products that have been structurally identified.

1. Introduction

The indolocarbazole derivative **1** (12,13-dihydro-2,10-dihydroxy-6-*N*-(1-hydroxymethyl-2-hydroxyethylamino)-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*)-dione, shown in Fig. 1, has been found to be a potent inhibitor of topoisomerase I enzyme. Preclinical pharmacological studies with **1** demonstrated selective activity against solid cancer tumors, and **1** more effectively induces single-strand DNA cleavage as compared to Camptothecin (Yoshinari et al., 1999; Cavazos et al., 2001).

Based on its promising activity and reduced toxicity profile, **1** is currently being developed as an anticancer agent that recently entered Phase III and II clinical trials in the USA and Japan, respectively. In order to facilitate the rapid entry of **1** into early clinical trials the topoisomerase inhibitor was initially formulated in aqueous glucose solution. However, due to poor aqueous of **1** a large volume of intravenous fluids has to be administered. An additional drawback of this simple aqueous based formulation of **1** is insufficient thermal stability requiring refrigerated conditions (at 2–8 °C) for storage and transportation. Therefore, an alternative formulation approach was pursued to address the lack of aqueous solubility and poor thermal stability of the initial formulation. Ideally, a new formulation would be concentrated in **1** to allow for dosing versatility in the clinic as well as ease of handling and transporting of reduced volumes of the drug product. The concentrated formulation would be diluted with an appropriate diluent before intravenous infusion.

There are several types of scientific approaches to achieve solubility enhancement of poorly water-soluble pharmaceutical drugs in parenteral formulations such as introduction of organic cosolvents, complexation with cyclodextrin, micellization, and pH control that are commonly used in parenteral formulations (Yalkowsky and Rubino, 1985; Sweetana and Akers, 1996; Nema et al., 1997; Powell et al., 1998; Zhao et al., 1999; Strickley, 1999, 2000a,b; Jain et al., 2001; Ni et al., 2002). Of these possible formulation approaches a propylene glycol (PG) cosolvent vehicle was found to be feasible for a concentrate formulation of the topoisomerase inhibitor **1** based on the high solubilities that can be achieved in PG–water solvent mixtures relative to the clinical doses.

The thermal stability of **1** in buffered and unbuffered aqueous PG formulations was subsequently examined. The primary chemical reaction of **1** in aqueous propylene glycol is hydrolysis of the imide ring which also releases the hydrazine diol side chain **11**. Buffering

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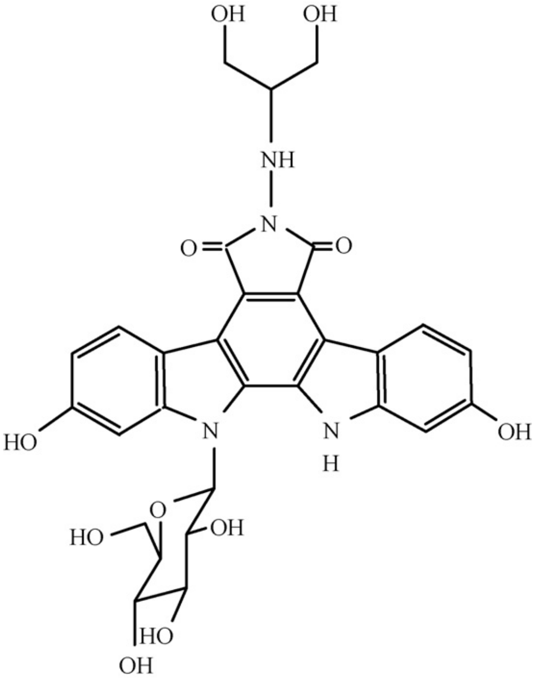


Fig. 1. Chemical structure of **1**.

the aqueous PG formulation at pH 4 vastly slows down the hydrolysis rate of **1**. The hydrazine diol side chain **11** is oxidatively unstable to molecular oxygen and degrades to several products. The degradation products of **11** react with **1** to subsequently give a complex mixture of secondary reaction products that have been structurally identified.

2. Materials and methods

2.1. Chemical materials

The topoisomerase inhibitor **1** was synthesized and characterized at Banyu Pharmaceutical Co., Ltd. All solvents for HPLC and LC/MS analyses were obtained from Fisher Scientific (Pittsburgh, PA) or Wako Pure Chemical Industries (Osaka, Japan) and were HPLC grade or equivalent. Other solvents and reagents were obtained from Fisher Scientific and certified ACS grade or Wako Pure Chemical Industries. Buffer solutions were prepared using a Model 720A pH meter (ORION) which was calibrated using 3 point standards.

2.2. Nuclear magnetic resonance (NMR)

The NMR spectra for some of the isolated degradation products were acquired on a JNMA 500 NMR (JASCO) or a Unity Inova 600 (VARIAN) spectrometer. The NMR samples were approximately 0.1 mg of solid degradate in 50 μL DMSO-d_6 (600 MHz) or 1.5 mg of solid in 200 μL DMSO-d_6 (500 MHz). All spectra were acquired at 25 °C. Proton assignments to their respective chemical shifts were made with the combined use of 1D proton, Nuclear Overhauser Effect (NOE), COSY, and HMBC experiments which unambiguously established the structures of the reaction products.

2.3. Ultraviolet/visible light (UV/vis) spectrophotometry

UV/vis solution spectra were recorded using a Varian Cary 300 spectrophotometer quipped with a thermostated multicell holder temperature controller.

2.4. High performance liquid chromatography (HPLC)

The HPLC instruments used for analytical separation were either Hewlett Packard (1090 or 1100 series) or Waters Alliance (2487 or 2690 series). An Agilent Zorbax Bonus-RP C18 column was employed to achieve quantitative separation of **1** and the degradation products. The mobile phase consisted of 0.1% aqueous phosphoric acid and acetonitrile. The flow rate was 1.0 mL/min and the column was operated at 40 °C. The injection volume was 10 μL and the detection wavelength was 228 nm. Employing this analytical method **1** produces a peak with a retention time of approximately 17 min, and the overall run time was 41 min.

2.5. LC/MS and LC/MS/MS

A Hewlett Packard 1100 series or a Waters Alliance HT 2790 was used for the LC separation in the MS experiments. The gradient conditions were used as follows: column: Zorbax Rx-C8 column (250 mm × 5 mm) 5 μm particle size; column temperature: 40 °C; mobile phase: $H_2O/CH_3CN/CH_3OH/HCOOH$ (850/90/60/1) (A) and $H_2O/CH_3CN/CH_3OH/HCOOH$ (400/360/240/1) (B) (for LC/MS) or H_2O (A) and CH_3CN (B) (for LC/MS/MS); binary gradient program: $t = 0$ min, 100% A; $t = 30$ min, 100% B (for LC/MS) or t = 0 min, 85% A; $t = 30$ min, 80% A; $t = 60$ min, 25% A (for LC/MS/MS); flow rate: 1.0 min/mL; injection volume: 10 μL (for LC/MS) or 15 μL (for LC/MS/MS); detection wave length: 228 nm.

For LC/MS and LC/MS/MS analyses, a PerkinElmer SCIEX-API 365 triple stage quadrupole mass spectrometer or Finnigan LCQ quadrupole ion-trap mass spectrometer operated in ESI negative ion mode was employed. The typical parameters are as follows: capillary temperature, 245 °C; spray voltage, −4.5 kV; sheath gas flow, 60 L/h; cone voltage, 47 V.

2.6. Preformulation studies

2.6.1. pH-rate profile

The pH-rate profile of **1** in buffered aqueous solution was described previously (Breslin et al., in press).

2.6.2. Solubility study

Crystalline solid **1** was placed in screw cap vials containing small aliquots of pure solvents or solvent mixtures at a level beyond saturation to give suspensions of drug. The resulting suspensions of **1** were agitated using a magnetic stirrer at 5 °C or ambient temperature (actual: 21–23 °C) for up to 1 week. The suspensions of **1** were covered with aluminum foil to prevent photoinduced degradation. Subsequently, the suspensions of **1** were centrifuged for 10 minutes and an aliquot of the supernatant fluid was removed. A volumetrically dispensed aliquot of the supernatant was diluted with 40% aqueous ethanol to adjust the concentration of **1** within the linear HPLC analysis range prior to injection.

2.6.3. Thermal stability study

The thermal stability of **1** dissolved at concentration of 5 mg/mL in 50% aqueous PG citrate buffer, was examined as a function of pH. In glass ampoules was placed 1-mL aliquot of the aqueous PG formulation. The ampoules were then flamed sealed and subsequently placed in laboratory ovens that were thermostated at 40, 60, and 80 °C. The ampoules were removed from the ovens at various time points and the formulation was diluted and analyzed by HPLC.

2.6.4. Degradate spiking experiments

Aqueous PG formulations of **1** were spiked with 1 mole equivalent of the proposed degradation products of **11**. The formulations tested for thermal stability consisted of **1** at concentration of 5 mg/mL in 50 mM pH 4 citrate buffered 50% PG/water solution.

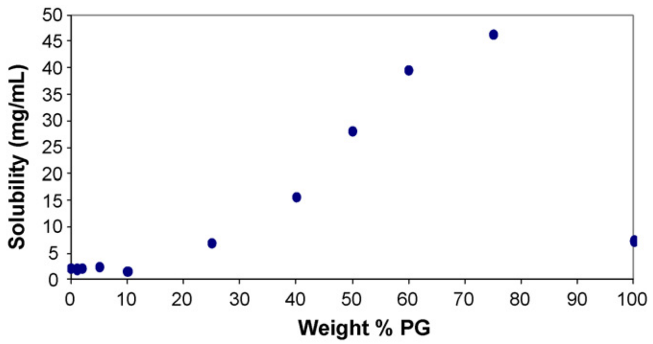


Fig. 2. Solubility of **1** in aqueous PG solvent mixtures at 25 °C.

A 1-mL aliquot of the spiked aqueous PG formulations were placed in a flame sealed glass ampoules that were subsequently placed in thermostated laboratory ovens at 80 °C. The ampoules were then removed from the oven at various time points and the formulation was analyzed by HPLC. Degassed formulations of **1** were prepared by 3 successive freeze-pump–thaw cycles under high vacuum (~5 mTorr). The formulations were analyzed by HPLC after being stored at 80 °C for 3 days in sealed ampoules.

3. Results and discussion

3.1. Preformulation studies

*3.1.1. Aqueous solubility enhancement of **1***

Adjustment of formulation pH to ionize **1** was the first approach considered to raise the aqueous solubility limit. As previously described **1** has maximum stability at pH 4 in a pure aqueous solution (Breslin et al., in press), but **1** is unionized and poorly water soluble at this pH (approximately 0.6 mg/mL at 20 °C). The accessible pK_a values of **1** are the protonation/deprotonation of the phenolic protons at 8.9 and 10.4. The thermal stability of the anionic and dianionic forms of **1** in water is very poor and alkaline solutions of **1** are not amenable for a drug product formulation.

Solubility screening of **1** in aqueous mixtures of commonly used organic solvents, buffers, and surfactants for parenteral use was tested. It was discovered that aqueous PG mixtures had the highest solubility limit on **1**. An aqueous solubility curve of **1** as a function of PG concentration at room temperature is shown in Fig. 2.

A 50% PG level in the formulation of **1** was chosen after considering chemical and physical attributes of **1** and taking into account pharmaceutical administration requirements (e.g. vehicle viscosity, minimum amount of excipient usage). However, it was found that 50% aqueous PG vehicle was not suitable as a finished drug product due to poor chemical stability. Consequently, the effect of pH on the stability of **1** in 50% aqueous PG was investigated. The solubility of **1** was found to be unaffected by the presence of a buffer to hold the formulation pH constant.

3.1.2. Stability study

The initial stability studies of the unbuffered 50% aqueous PG formulation of **1** indicated that a desirable shelf life, room temperature storage up to 2 years, could not be attained without reaching unacceptable levels of degradation. Thermal stressing of the unbuffered formulation of **1** led to a large number of degradation products. The effect of formulation pH was investigated to stabilize **1** in an aqueous PG solution. As discussed in an earlier manuscript (Breslin et al., in press) **1** was found to have degradation kinetics that were strongly dependent on pH in aqueous solution. In aqueous solution **1** is most stable at pH 4. Therefore, the thermal stability of the aqueous PG formulation was examined around this pH range specifically looking at values of 3, 4, and 5 with a 50 mM citrate buffer. As shown in Fig. 3, the aqueous PG buffered formulations of **1** afforded significant stability improvement drug over an unbuffered formulation when heated to 80 °C for 3 days. The PG cosolvent greatly attenuates the pH dependency on rate of degradation rate compared to a purely aqueous formulation. Upon completion of longer term stability studies a 5 mg/mL 50% aqueous PG vehicle at pH 4 was found to provide the best thermal stability of **1**. A combination of buffer catalysis experiments and solubility limits led to selecting a citrate buffer at a 50 mM concentration for purposes of thermal stabilization of the formulation.

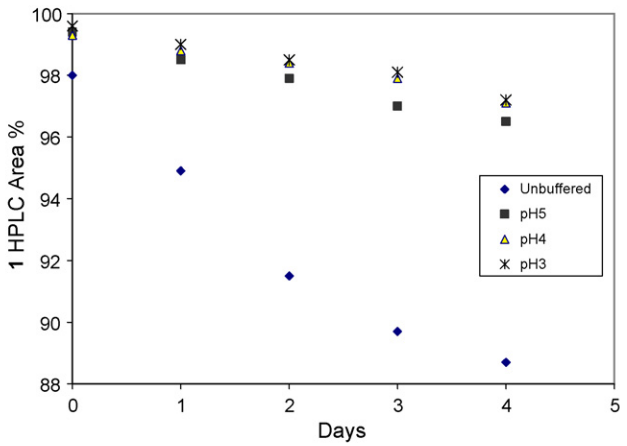


Fig. 3. HPLC chromatograms of **1** at 5 mg/mL in 50% aqueous PG solution after storage at 80 °C.

3.1.3. Identification of degradation products of PG/water formulation

The typical HPLC chromatogram of a 5 mg/mL 50% aqueous PG formulation of **1** after brief storage under accelerated thermal stress at 80 °C is shown in Fig. 4. LC/MS and LC/MS/MS techniques were used for identification of molecular weight and preliminary struc-

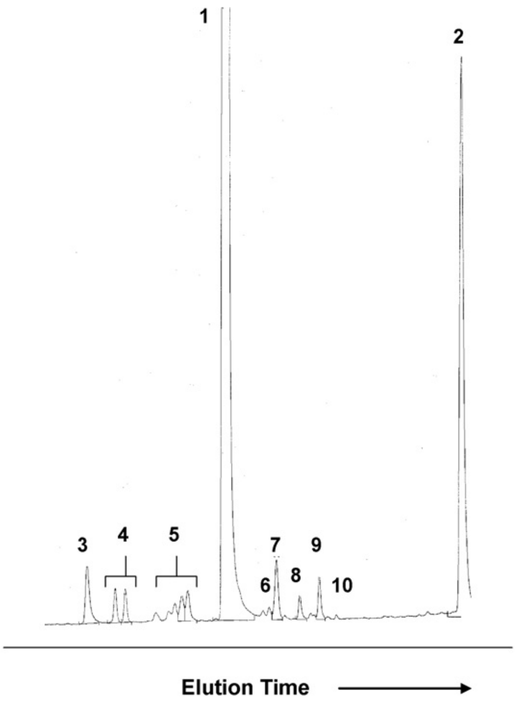
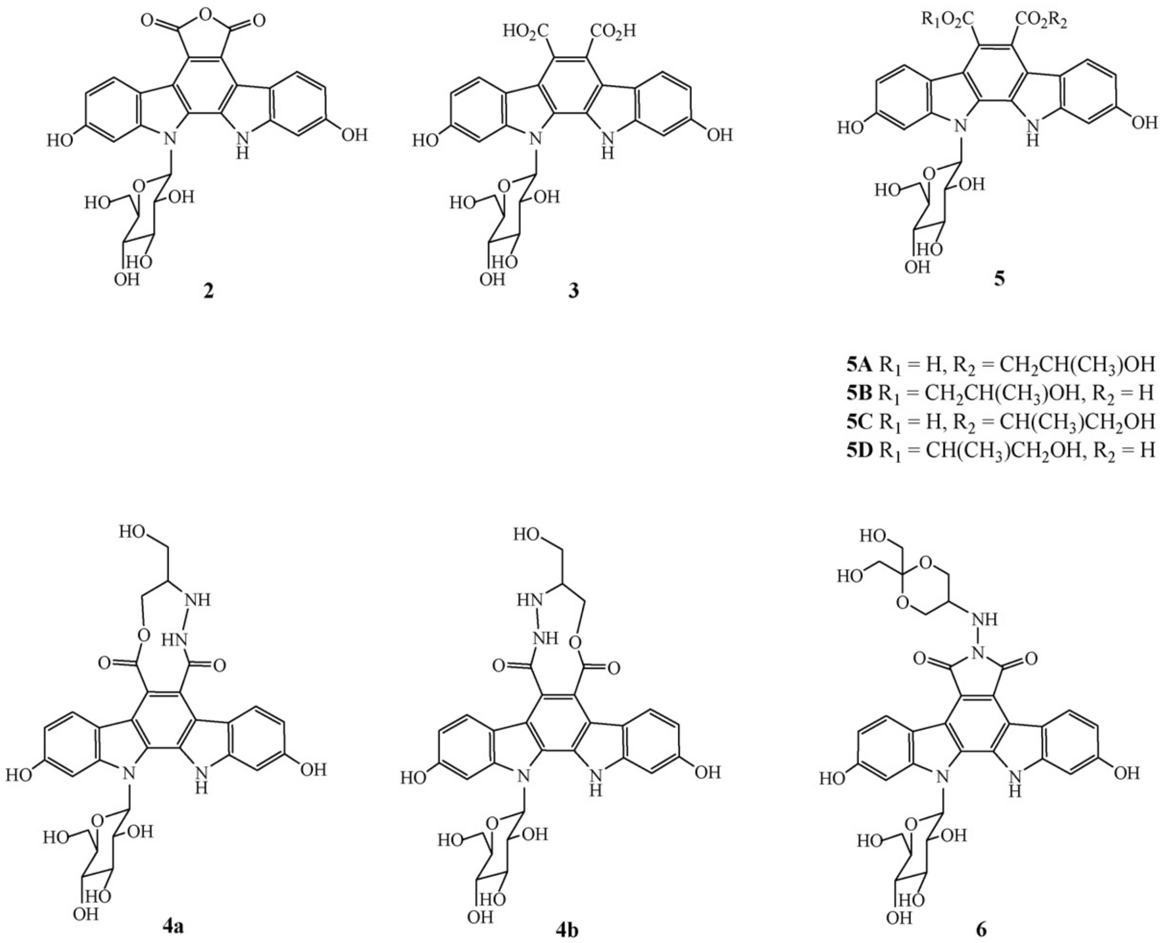


Fig. 4. HPLC assay data of **1** in 50% aqueous PG solution solutions at 80 °C.



2 **3** **5**

5A $R_1 = H, R_2 = CH_2CH(CH_3)OH$
5B $R_1 = CH_2CH(CH_3)OH, R_2 = H$
5C $R_1 = H, R_2 = CH(CH_3)CH_2OH$
5D $R_1 = CH(CH_3)CH_2OH, R_2 = H$

4a **4b** **6**

Fig. 5. Proposed chemical structures of **1** solvolytic degradation products.

tural assignment for each degradate peak in the HPLC trace. Based solely on the LC/MS and LC/MS/MS data the chemical structures of some degradates can be proposed, without further data, as shown in Fig. 5. In contrast, **7**, **8**, **9**, and **10**, cannot be assigned chemical structures without additional information since regioisomers are possible. The molecular weight data from the MS experiment indicated that products **7** and **8** are dimers of **1** connected by methylene unit while **9** and **10** are formaldehyde condensation products of **1**. In order to further elucidate the chemical structures of reaction products **7**, **8**, **9**, and **10**, the degradates were then isolated by preparative HPLC and analyzed using 1 and 2 D NMR spectroscopy. The resulting chemical structures of the degradation products **7**, **8**, **9**, and **10** are proposed from the NMR data as shown in Fig. 6.

3.1.4. Investigation on degradation pathways to formaldehyde adducts

Many of the degradation products could be assigned to solvolysis reactions of **1** with water or propylene glycol. However, the formaldehyde derived adducts **7**, **8**, **9**, and **10** were unexpected, and furthermore the source of formaldehyde was not unclear. Formaldehyde related degradates of **1** were readily generated by stressing a purely aqueous solution (data is not shown). Direct measurement of formaldehyde levels present in the water could not account for the high level of formaldehyde derived degradates. The only remaining possibility was that a secondary decomposition step was generating formaldehyde. The most likely source of formaldehyde was thought to originate from decomposition of the hydrazine diol side chain **11** that is released upon hydrolysis of the imide ring.

A possible mechanism for the degradation of the hydrazine diol to formaldehyde is proposed as shown in Fig. 7. The hydrazine moiety of **11** should be readily oxidized by molecular oxygen to yield the hydrazone diol and hydrogen peroxide. The resulting hydrazone diol **12** can give dihydroxyacetone and hydrazine by hydrolysis. In the final reaction step hydrogen peroxide reacts with **13** to produce formaldehyde and glycolic acid.

Degradate spiking experiments were performed on a 50% aqueous PG formulation of **1** to test the proposed mechanism (Table 1). The proposed degradates were intentionally added to a formulation of **1** at 1 molar equivalent ratio to drug and then thermally stressed at 80 °C. Overall the results of the spiking experiment are consistent with the proposed mechanism where the initial oxidative degradation of **11** yields **13** and hydrogen peroxide. The participation of molecular oxygen to effect the conversion of **11** to **13** is clearly demonstrated by the degassing samples. Removal of oxygen from the **11** spiked for-

Table 1
Degradate products yields observed after thermal stressing of spiking samples.

	7	8	9	10
H_2O_2	+	–	–	+
11 (degassed)	–	–	–	+
11	+++	++	+	+
11 + H_2O_2	+++	++	+	+
13	–	–	–	+
13 + H_2O_2	++	+	+	+

"–" = ND or less than 0.1 area%, "+" = 0.1–0.4 area%, "++" = 0.4–0.8 area%, and "+++" = 0.8–1.2 area%.

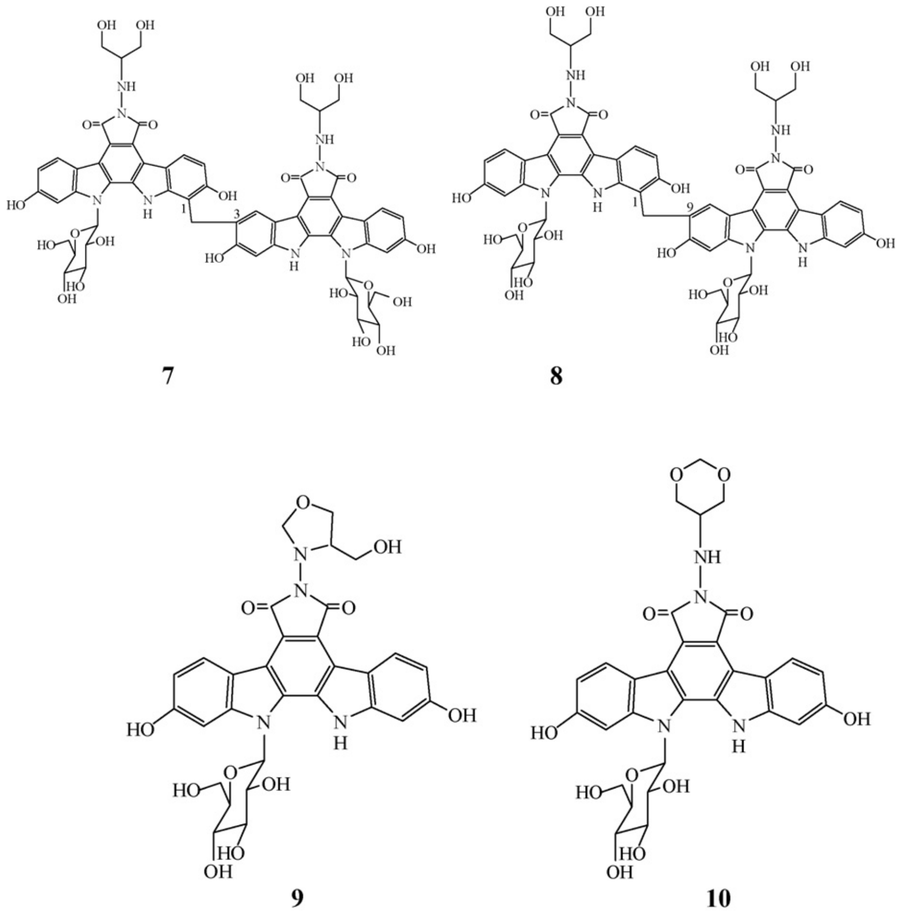


Fig. 6. Proposed chemical structures of **1** formaldehyde related degradation products.

mulation did not exhibit any growth of formaldehyde derived degradates. However in air saturated solution additional **11** was found to accelerate the formation of formaldehyde degradates. The proposal that hydrogen peroxide is needed to react with **13** to give formaldehyde has also been shown. Without hydrogen peroxide the formulation spiked with **13** did not exhibit any additional growth of formaldehyde degradates of **1**.

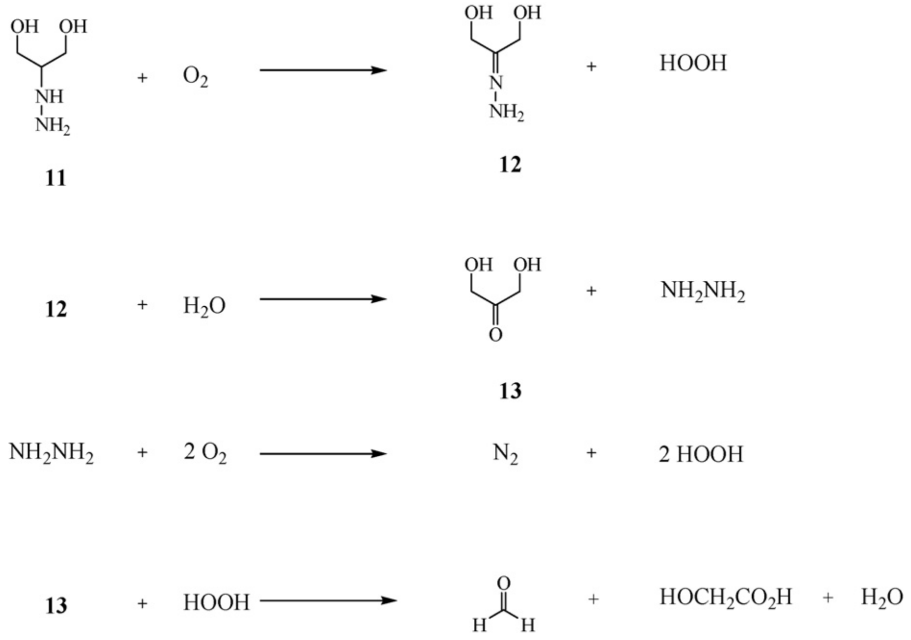


Fig. 7. Proposed mechanisms for the production of formaldehyde from degradation of **1**.

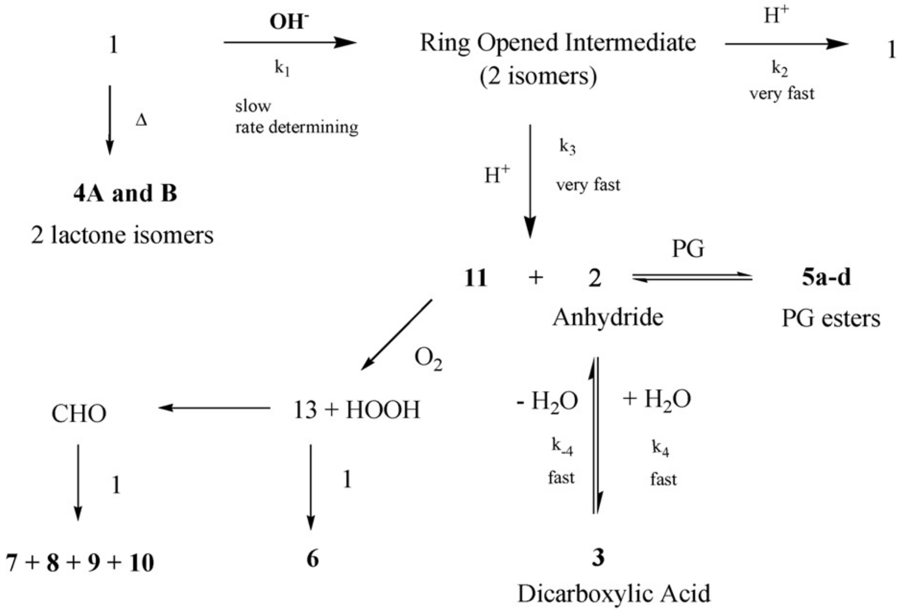


Fig. 8. Summary of thermal degradation pathways of **1** in 50% aqueous PG.

*3.1.5. Degradation pathways of **1** in aqueous propylene glycol*

The overall thermal degradation pathways of **1** observed in 50% aqueous PG solution is summarized in Fig. 8. The initial nucleophilic attack of water on **1** opens the imide ring. The ring opened intermediate, which is observable in water at higher pH (Breslin et al., in press), can either close down to regenerate **1** or undergo a second hydrolysis step to release **11** and generate **2**. The anhydride derivative **2** can react with water to give the open diacid **3** or react with propylene glycol to yield a complicated mixture of regioisomeric esters **5**. It also appears that an intramolecular ring opening of **1** can take place to yield two isomeric products **4**. The hydrazine diol fragment **11**, which is concomitantly produced with **2**, appears to be oxidatively unstable ultimately yielding formaldehyde. Consistent with the proposed degradation scheme of **11**, reaction product **6** observed as which is a dihydroxyacetone adduct formed with **1**. The methylene bridged dimer products **7** and **8** are formed via the Lederer-Manasse reaction that takes place between phenolic compounds and formaldehyde.

4. Conclusion

The parenteral formulation of a poorly water-soluble topoisomerase inhibitor **1** was developed using a PG cosolvent for solubility. Formulation buffering was employed to provide a thermally robust and commercially viable parenteral solution of **1**. It was found that the primary degradation route of **1** is hydrolysis that can be effectively controlled by choice of pH at 4. Secondary degradation of the hydrazine diol **11** released upon hydrolysis accounts for a majority of the other degradate products. A mechanism was proposed to account for the formation of formaldehyde, and spiking experiments are consistent with this mechanism. Formaldehyde can react with electron rich **1** to yield formaldehyde dimer products **7** and **8**.

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