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Synthesis of imidazo[1,5,4-*de*]quinoxalin-9-ones, benzimidazole analogues of pyrroloiminoquinone marine natural products

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Abstract—The imidazoquinoxalinones **1** and **2** are benzimidazole analogues of indole-based marine natural products called makaluvamins. The stabilized cation **1** and the zwitterion **2** were prepared in ~9 steps from readily available starting materials. Compound **1** is more cytostatic and cytotoxic than **2** and also shows activity in the hollow fiber assay. Unlike the indole-based natural products, **1** and **2** are not potent topoisomerase II inhibitors. Their pattern of cytotoxic and cyostatic activity could be related to inhibition of protein tyrosine kinases.

1. Introduction

A series of compounds known as makaluvamines were isolated from the Fijian sponge *Zyzzya cf. marsailis* in the early 1990's.[1–3] These compounds display in vitro cytotoxicity towards human colon tumor cell-line HCT 116 as well as the topoisomerase II sensitive CHO cell-line xrs-6 and also inhibit human topoisomerase II in vitro.[3] Makaluvamine A and C show in vivo antitumor activity against human ovarian carcinoma Ovcar 3 implanted in athymic mice.[3] Around the same time of the discovery of the makaluvamines, our laboratory reported the 6-acetamidopyrrolo[1,2-α]benzimidazole (APBIs) antitumor agents (Fig. 1).[4–9] The APBIs are structurally and mechanistically similar to the pyrroloiminoquinones of the makaluvamines. Both inhibit topoisomerase II mediated relaxation of supercoiled DNA by intercalation.

The imidazoquinoxalinones **1** and **2** were designed based on the structures of the 6-acetamidopyrrolo[1,2-*a*]benzimidazoles (APBI) antitumor agents and the pyrroloiminoquinone natural products (Chart 1). Thus, structural features of the APBIs (benzimidazole-based and zwitterionic structure) were combined with those of the natural products (presence of an ethylene tether and extended amidine structure). We anticipitated that the hybrid structures **1** and **2** would be excellent topoisomerase II inhibitors.

This article describes the synthesis, chemistry, and biological properties of imidazoquinoxalinones **1** and **2**. The preparation of these compounds was possible in ~9 steps starting with readily available starting materials. The anticipated excellent topoisomerase II inhibitory activity was not realized with inhibition of supercoiled DNA occurring only at high concentrations of **1** and **2**. Compound **1** is more cytotoxic and cytostatic than **2** presumably due to its mimicry of the purine ring.

2. Results and discussion

2.1. Synthesis

The preparation of compound **1** started with the functionalized benzene compound **3** that permitted annulation of the fused tetrahydropyrazine and imidazo rings (Scheme 1). The reported dinitration *ortho* to the methoxy group of an *N*,*N*-diacylanisidine[10] suggested that *p*-methoxydiacetanilide **3**[11] would do likewise. The dinitration product of *p*-methoxydiacetanilide (**4**) was treated with ethanolamine resulting in both nucleophilic aromatic substitution and deacetylation to afford **5**, the precursor of **1**.

Keywords: Imidazoquinoxalinones; Benzimidazole; Pyrroloiminoquinone.
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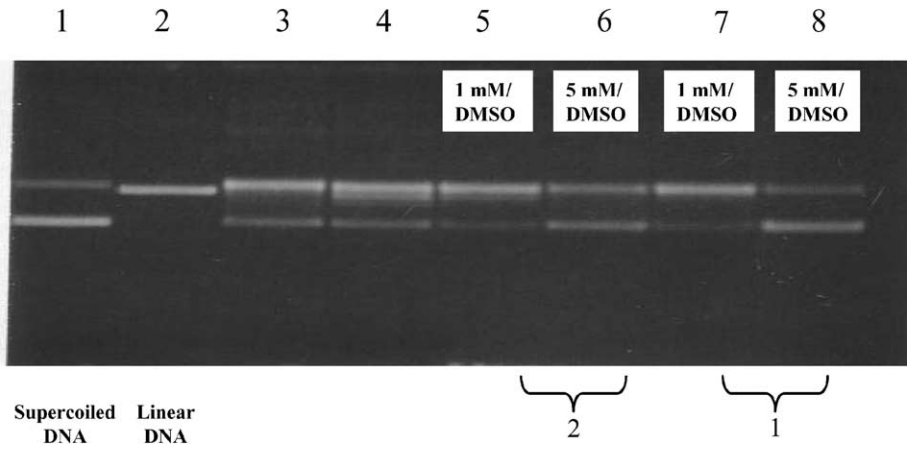


Figure 1. Agarose gel of topoisomerase IIα catalyzed relaxations of pRYG supercoiled DNA (Form I) run in the absence of ethidium bromide. The control lanes include unrelaxed pRYG supercoiled DNA in Lane 1, the linear form of pRYG supercoiled DNA in Lane 2, Lanes 3, and 4 are relaxation reactions carried out with and without DMSO. Lanes 5 and 6 are relaxation reactions carried out in the presence of 1 and 5 mM of **2** and Lanes 7 and 8 likewise carried out in the presence of 1 and 5 mM of **1**.

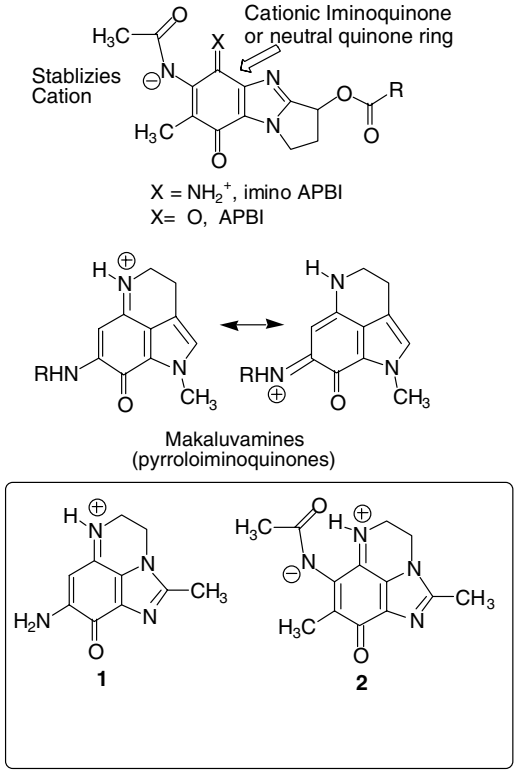


Chart 1.

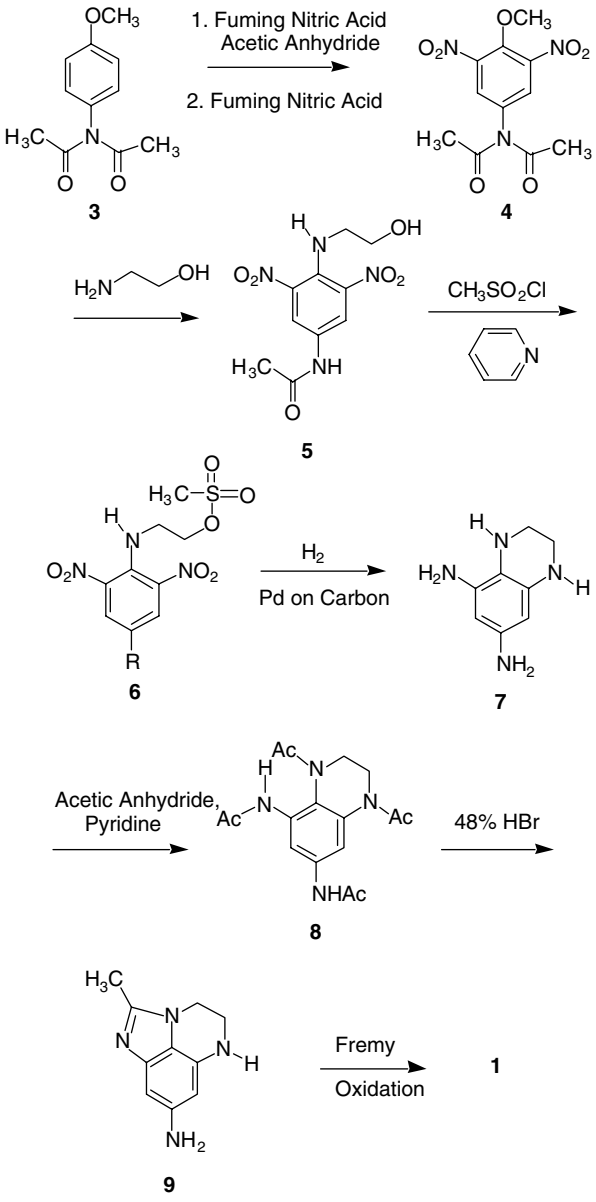
The conversion of **5** to **1** involved methanesulfonation of **5** to afford **6** followed by catalytic reduction of the nitro groups resulting in internal nucleophilic substitution to afford the tetrahydroquinoxoline **7**. Compound **7** is highly susceptible to oxidation and the crude HBr salt is a dark-colored impure solid. Peracetylation of **7** afforded the stable amide **8** that was readily purified by column chromatography. Purified **8** afforded the cyclization product **9** as an analytically pure hydrobromide salt directly from the reaction mixture. Fremy oxidation[4,12] of **9** afforded the iminoquinone **1** as a deep red solid after purification by reverse phase chromatography.

The preparation of **2** started with the nucleophilic aromatic substitution of 4-bromo-3-nitrotoluene **10** with ethanolamine (Scheme 2). Catalytic reduction of **11** followed by peracetylation of the resulting amine afforded **12** that was converted to benzimidazole **13** by treatment with refluxing 4 N HCl. Nitration of **13** with fuming nitric acid afforded both the 4- and the 6-nitro isomers. However, the desired 6-nitro isomer could not be separated from the mixture by column chromatography. The mixture of nitro isomers was catalytically reduced and acetylated with acetic anhydride yielding both acetylated isomers. The desired isomer **14** was purified by column chromatography (5% methanol in chloroform) in a 35% yield from **13**.

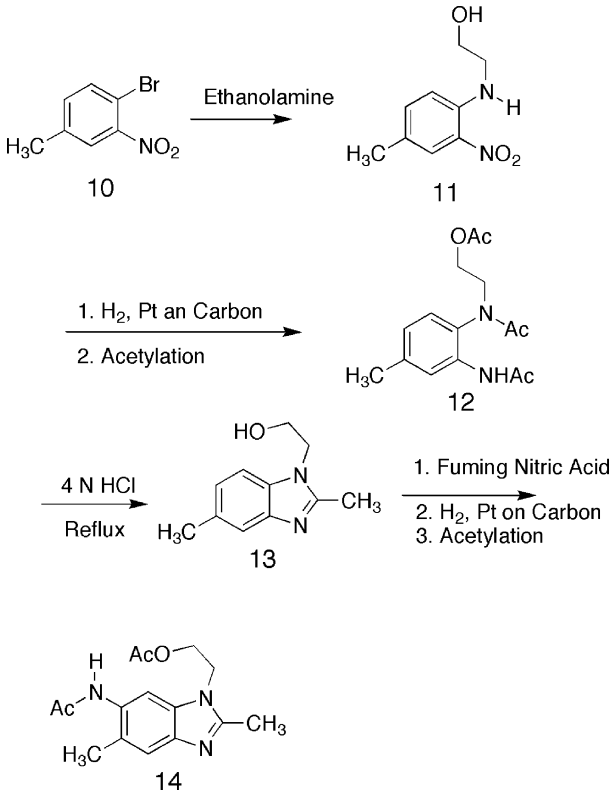
Nitration of **14** with fuming nitric acid at room temperature for 1.5 h yielded the 7-nitro isomer **15** as the sole product (Scheme 3). The unambiguous characterization of **15** as the 7-nitro isomer was obtained by GOESY NMR experiment (NOE interaction between the 5-methyl and 4-H in the desired 7-nitro isomer) and HMBC (three-bond coupling between 4-H and the 5-methyl carbon). Base-catalyzed transesterification of **15** yielded the alcohol that was sulfonated to provide **16**. Ring closure to afford the imidazoquinoxaline **17** was carried out by catalytic reduction of the nitro substituent to the amine followed by heating the reaction at 60 °C for 1 h. Fremy oxidation of **17** afforded the iminoquinone **2** in good yield.

2.2. In vitro activity

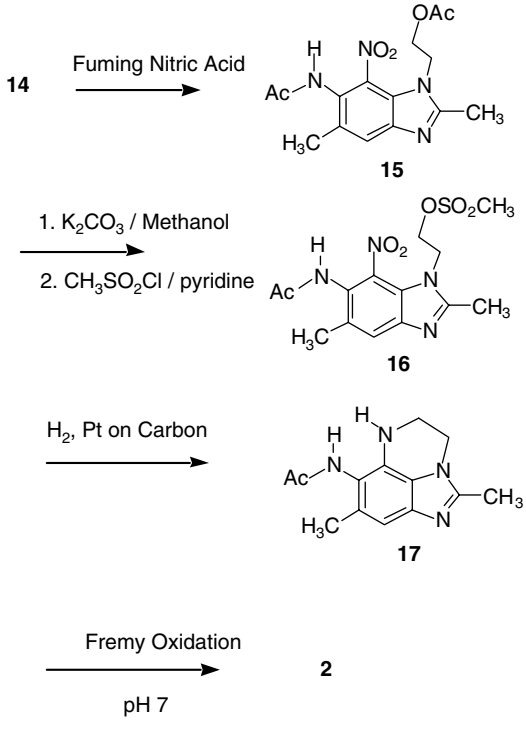
The cytostatic and cytotoxic properties of **1** were compared with those of **2** to assess which structure possesses biological activity. The cytostatic parameters include GI_{50} and TGI, which are the molar concentrations of drug required for 50% growth inhibition and total growth inhibition, respectively. The cytotoxic parameter is the LC_{50}, which is the concentration required for 50% cell kill. These in vitro data were obtained under the In Vitro Cell Line Screening Project at the National Cancer Institute.[13,14] The log mean values for GI_{50},



Scheme 1.



Scheme 2.



Scheme 3.

TGI, and LC_{50} in 60 cell lines are provided in Table 1 along with the log delta value (the maximum sensitivity in excess of the mean) and the log range (the maximum difference between the least sensitive and the most sensitive cell lines). These parameters provided insights into selectivity and potency of antitumor agents.[15] Large values of the delta and range indicate high selectivity for some histological cancers over others.

Inspection of Table 1 reveals that the cytotstatic and cytotoxic parameters for **1** are ($\log GI_{50}$ and TGI) much lower than those of **2**. The large delta and range values of **1** are due to inactivity against leukemia cell lines and activity largely against melanoma and breast cancer cell lines. On the other hand, compound **2** has large delta and range values for $\log GI_{50}$ and TGI because of inactivity against all cell lines except for four breast cell lines (BT-549, $\log GI_{50} < -8$; MDA-MB-435, $\log GI_{50} = -4.72$; MDA-N, $\log GI_{50} = -4.73$; MDA-MB-231/ATCC, $\log GI_{50} = -4.89$). High activity against only one or two cell lines of the 60-cell-line panel is usually considered anomalous, but **1** also shows activity in the

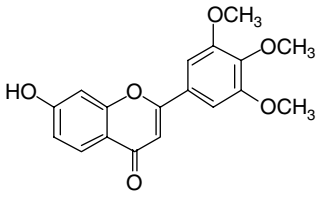
Table 1. Cytostatic and cytotoxic parameters for **1** and **2**

Compd	GI_{50}			TGI			LC_{50}		
	Median	Delta	Range	Median	Delta	Range	Median	Delta	Range
1	−6.11	0.75	2.87	−5.44	1.06	2.50	−4.79	1.43	2.23
2	−4.28	3.72	4.00	−4.09	1.39	1.48	−4.01	0.19	0.21

same panel. Therefore **2** is considered to be much less cytostatic than **1** except against breast cancers. The high median log LC_{50} value of **2**, along with the low delta and range values, indicates the complete absence of cytotoxicity against all cell lines. In contrast, **1** is more cytotoxic with large delta and range values due to the high specificity for melanoma and breast cancers.

COMPARE analysis provided insights into what might be the molecular target of **1**.[13,16] The pattern of cytostatic and cytotoxic parameters (GI_{50}, TGI, and LC_{50} mean graphs) of **1** was compared with those of over 38,000 compounds in the National Cancer Institute's archives. The goals were to find a compound of known mechanism of action and perhaps a molecular target that correlated well with the mean graphs of **1**. Thus, compound **1** was used as a seed compound in the following NCI databases: Standard Agent database, Synthetic Agent database, and the Molecular Targets Database. The Standard Agent Database consists of 175 drugs with cancer treatment applications as well as new compounds with a high level of interest.[14] Molecular target levels (e.g., topoisomerase II, DT-diaphorase, etc.) have been determined in the 60-cell-line panel from RNA measurements and enzyme activity levels.[17]

The results of COMPARE analysis revealed no significant correlations between the GI_{50} and TGI parameters of **1** and those of compounds and molecular targets in the above databases. However, the LC_{50} parameters of **1** correlated well (correlation coefficients > 0.7) with those of the compounds shown in Chart 2 (NCI numbers 649086 and 67616) from the Synthetic Agent database. Compound 649046 is one of a large number of flavonoids that are protein tyrosine kinase inhibitors.[18] Compound 67616 is cytotoxic due to its influence on a number of enzymes including RNA polymerase, PRPP amidotransferase, IMP dehydrogenase, dihydrofolate reductase, thymidine, TMP, and TDP kinases.[19] Both of the compounds in Chart 2 may be purine mimics capable of binding to purine de novo synthetic enzymes and to the ATP binding site of kinases. Proof of the hypothesis requires further study. The high correlation between **1** and the compounds in Chart 2 could be related to their common effect on kinase activity. It is noteworthy that the mean graphs of **1** and **2** did not correlate with topoisomerase II, indicating that this target was not involved in in vitro activity. Indeed, the topoisomerase II assays described below indicate both **1** and **2** are poor inhibitors of this enzyme.



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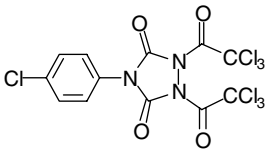

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Chart 2.

2.3. In vivo activity

The activity and novelty of **1** resulted in selection for screening in the hollow fiber assay. Hollow fiber assays were carried out at the National Cancer Institute in the following way. Human tumor cells are cultivated in polyvinylidene flouride hollow fibers, and a sample of each cell line is implanted into each of two physiological compartments (intraperitoneally and subcutaneously). Mice are treated with either a high or a low dose (at 2.4 and 1.6 mg/kg/inj) using a QD × 4 schedule (four daily treatments) administered intraperitoneally. Altogether, 12 cell lines are studied resulting in 48 possible test combinations (12 cell lines × 2 sites × 2 doses). A score of two is given to each test in which there is a % T/C of 50 or less (tumor mass 50% or less than the control). Thus the highest possible score is a 96, but the typical score is only 5 and the highest score achieved so far is a 64. The score is broken down into a intraperitoneal (IP) and a subcutaneaous (SC) score. A good SC score ($\geqslant 8$) indicates that the drug is able to get to the tumor site (subcutaneous) from a distant site (intraperitoneal) of injection. Compound **1** has an IP score of 8 and an SC score 2, for a total of 10, indicating activity according to the National Cancer Institutes criteria.

Compound **1** was evaluated with respect to percent survival and inhibition of tumor growth in the B16 melanoma syngraft assay.[20] Evaluation was carried out at three doses: 1, 2, or 4 mg/kg/day, on days 1, 5, and 9 after subcutaneous tumor implantation of 10^5 cells in the front flank on day 0. Both percent survival and inhibition of tumor growth were monitored until day 29. There was early lethality, or $\geqslant 50\%$ lethality prior to any deaths in the control group, at the 2 and 4 mg/kg/day doses. Mice given the 1 mg/kg/day doses exhibited 20% lethality prior to deaths in the control group. Tumor mass in mice given the 2 mg/kg/day doses was less than that in the control group after day 15, but this

effect was accompanied by 80% lethality. In conclusion, compound **1** did not increase survival in the B16 melanoma syngraft assay due to toxicity.

2.4. Topoisomerase II inhibition

Relaxation assays were carried out with recombinant human topoisomerase II and the completed reactions were assayed on agarose gels that were stained with ethidium bromide. These gels will readily resolve the relaxed (Form II) DNA arising from the supercoiled (Form I) DNA and will show the presence of linear DNA. Catalytic inhibition could then be documented by noting the decrease in relaxation with increasing inhibitor concentration. Stabilization of the cleavable complex (poisoning) will be indicated by the presence of linear DNA in these assays upon treatment with proteinase K.

Shown in Figure 1 are the assays for the relaxation of pRYG supercoiled DNA by p170 human topoisomerase II in the presence of **1** and **2**, as well as control assays. The first four lanes are controls showing supercoiled DNA (Lane 1), linear DNA (Lane 2), and relaxation with and without DMSO (Lanes 3 and 4, respectively). Lanes 5 and 6 show relaxation reactions in the presence of **2** and Lanes 7 and 8 show relaxation reactions in the presence of **1**. Although the assay shows some inhibition of the relaxation of supercoiled DNA by topoisomerase II, this alone is not conclusive evidence that **1** and **2** are topoisomerase II inhibitors. This may simply be due to intercalation of DNA by **1** and **2** at the high concentrations employed.

2.5. Conclusions

Compound **1** is more cytotoxic than **2** and displays minimal activity in the hollow fiber assay and toxicity in vivo. The relative inactivity of **2** is in sharp contrast to the structurally similar APBI zwitterions, and ongoing analogue studies of **2** confirmed this assessment. It is concluded that analogues of **1** should be candidates for further development. Further analogue development of **1** is now ongoing and active analogues closer in structure to the makaluvamines have been prepared. It remains to be seen if analogue development will remove the in vivo toxicity of **1**.

COMPARE analysis suggests that the activity of **1** is related to its purine-like structure, perhaps capable of binding to purine de novo synthetic enzymes and to the ATP binding site of kinases. Inhibition assays revealed that **1** is not a potent inhibitor of topoisomerase II mediated relaxation of supercoiled DNA.

3. Experimental

All analytically pure compounds were dried under high vacuum in a drying pistol over refluxing toluene. Elemental analyses were run at Atlantic Microlab, Inc., Norcross, GA, see Table 2. All TLCs were performed on silica gel plates using a variety of solvents and a fluorescent indicator for visualization. IR spectra were taken as thin films and the strongest absorbances reported. ^{1}H NMR spectra were obtained from a 300 MHz spectrometer. All chemical shifts are reported relative to TMS. The GOESY pulse sequence used to identify 15 was supplied with Varian Software.

3.1. 3,5-Dinitro-*N*,*N*-diacetyl-4-methoxyaniline (4)

This was prepared from **3** by sequential nitrations as described below.

To a solution of 10 g (48.25 mmol) of **3** in 10 mL of acetic anhydride, chilled in an ice bath, was added a solution prepared by adding 5 mL of fuming nitric acid to 5 mL of acetic anhydride chilled in an ice bath. The mononitro product crystallized from the reaction mixture toward the end of the addition. The reaction mixture was

Table 2. Calculated and found elemental analyses

Compound #	Formula	Calculated			Found		
		C%	H%	N%	C%	H%	N%
Mononitro precursor to **4**	$C_{11}H_{12}N_2O_5$	52.38	4.80	11.11	52.22	4.80	11.12
4	$C_{11}H_{11}N_3O_7$	44.45	3.73	14.14	44.54	3.78	14.13
5	$C_{11}H_{11}N_3O_7$	42.46	4.26	19.71	43.27	4.21	19.61
6	$C_{11}H_{14}N_4O_8S$	36.47	3.89	15.46	36.56	4.02	14.94
8	$C_{16}H_{20}N_4O_4{\cdot}0.25H_2O$	57.05	6.13	16.63	57.41	6.12	16.45
9	$C_{10}H_{12}N_4{\cdot}H_2O{\cdot}3HBr$	26.75	3.82	12.28	26.52	3.88	12.18
1		66.83	4.60	16.95	65.71	4.53	16.59
11	$C_9H_{12}N_2O_3$	55.09	6.16	14.28	55.02	6.13	14.41
12	$C_{15}H_{20}N_2O_4$	61.63	6.90	9.58	61.73	6.86	9.57
13	$C_{11}H_{14}N_2O{\cdot}0.2NH_3{\cdot}0.6H_2O$	64.62	7.79	15.07	64.39	7.36	15.27
14	$C_{15}H_{19}N_3O_3{\cdot}0.25H_2O$	61.31	6.69	14.30	61.53	6.50	14.41
15	$C_{15}H_{18}N_4O_5$	53.17	5.50	16.54	52.79	5.26	16.21
Deacetylated **15**	$C_{13}H_{16}N_4O_4{\cdot}0.5H_2O$	51.82	5.69	18.59	51.48	5.39	18.27
16	$C_{14}H_{18}N_4O_6S$	45.40	4.90	15.13 S, 8.66	45.25	4.87	14.95 S, 8.47
17	$C_{13}H_{16}N_4O$	56.50	5.84	20.27			
2	$C_{13}H_{14}N_4O_2{\cdot}0.75H_2O$	57.45	5.75	20.61	57.63	5.52	20.45

poured over ice resulting in further crystallization. The pure mononitro product was filtered off and washed with water. The solid was dissolved in a minimum volume of chloroform and the solution dried over sodium sulfate. The dried chloroform was diluted with hexane until cloudy, crystallization occurred with chilling: yield 11.2 g (92% yield); mp 139–140 °C; TLC (ethyl acetate) $R_f = 0.5$; FTIR 3424, 3076, 2948, 1724, 1690, 1522, 1352, 1258, 1012, 858, 626 cm^{-1}; ^1H NMR δ 7.70 (1H, d, $J = 2.7$ Hz), 7.34 (1H, dd, $J = 2.7$ Hz, $J = 8.8$ Hz), 7.18 (1H, d, $J = 8.8$ Hz), 4.017 (s, 3H), 2.318 (s, 6H). Anal. ($C_{11}H_{12}N_2O_5$) C, H, N.

To a mixture of 5 mL acetic anhydride and 5 mL fuming nitric acid, chilled in an ice bath, was added 5 g (0.0198 mol) of the mononitro derivative. The resulting mixture was allowed to come to room temperature and stir 3 h. The reaction was poured over ice resulting in precipitation of **4** that was filtered off and washed extensively with water. The solid was dissolved in a minimum volume of chloroform and the solution dried over sodium sulfate. The dried chloroform was diluted with hexane until cloudy, crystallization of pure **4** occurred with chilling: yield 3 g (51%). Analytically pure **4** was obtained by recrystallization from ethyl acetate: mp 132–133 °C; TLC (ethyl acetate) $R_f = 0.66$; FTIR 3091, 1707, 1548, 1417, 1358, 1214, 1121, 1045, 984, 723, 656, 637, 574 cm^{-1}; ^1H NMR δ 7.80 (s, 2H), 4.14 (s, 3H), 2.36 (s, 6H). Anal. ($C_{11}H_{11}N_3O_7$) C, H, N.

3.2. 4-(2-Hydroxyethylamino)-3,5-dinitroacetanilide (5)

A mixture 5.12 g (0. 017 mol) of **4** and 5 mL of ethanolamine in 50 mL of ethanol was stirred for 12 h at room temperature and then chilled a refrigerator for 8 h. The product was filtered off as red needles: yield 3.66 g (76%); mp 185–186 °C; TLC (ethyl acetate) $R_f = 0.43$; FTIR 3357, 3097, 2937, 1680, 1586, 1526, 1500, 1287, 1245, 1082, 901, 697 cm^{-1}; ^1H NMR δ 10.27 (broad s, 1H), 8.50 (s, 1H), 8.18 (broad t, 1H), 5.00 (t, $J = 6$ Hz), 3.61 and 2.90 (2q, $J = 5$ Hz), 2.01 (s, 3H). Anal. ($C_{11}H_{11}N_3O_7$) C, H, N.

3.3. 4-(2-Methanesulfonoxyethylamino)-3,5-dinitroacetanilide (6)

To a solution of 3 mmol of **5** or **7** in 5 mL of pyridine was added 0.5 mL of methanesulfonyl chloride and the resulting mixture stirred for 1 h. The reaction mixture was combined with 100 mL of methylene chloride and extracted 2× with 100 mL of water followed by 1 × 100 mL of 1 N HCl. The last extraction removed the last traces of pyridine resulting in crystallization of the product in both the organic and aqueous phases: yield 1.02 g (94%); mp 143–144 °C; TLC (ethyl acetate) $R_f = 0.4$; FTIR 2905, 3302, 1666, 1535, 1350, 1278, 1168, 939, 526 cm^{-1}; ^1H NMR δ 10.26 (broad s, 1H, amide NH), 8.50 (s, 2H, aromatic), 8.18 (t, $J = 5$ Hz, 1H, amine NH), 5.00 (t, $J = 5$ Hz, 2H, OCH_2), 3.52 (q, $J = 5$ Hz, $NHCH_2$), 3.06 (s, 3H, sulfonylmethyl), 2.21 (s, 3H, acetamido methyl). Anal. ($C_{11}H_{14}N_4O_8S$) C, H, N.

3.4. 5,7-Diacetamido-1,4-diacetyl-1,2,3,4-tetrahydroquinoxaline (8)

A solution of 3.7 mmol of **6** in 300 mL of methanol was reduced under 50 psi H_2 in the presence of 300 mg of 5% Pd on carbon for 15 h. The catalyst was filtered off using Celite filter aide and the filtrate acidified with a few drops of concd HCl. Concentration in vacuo afforded the dihydrochloride salt of **7** as a dark oily residue (500 mg). To this residue was added 5 mL of dried pyridine and 5 mL of acetic anhydride and the resulting mixture stirred for 2 h. The solvents were completely removed in vacuo and the residue chromatographed on a silica gel column using ethyl acetate–ethanol (80:20). Compound **8** was eluted from a ~50 g silica gel column as a broad band over a period over several hours.

Recrystallization was carried out by dissolution in chloroform followed by addition of hexane: yield 300 mg (50%); mp 206–208 °C; TLC (ethyl acetate–ethanol, [80:20]) $R_f = 0.06$; FTIR 3441, 3304, 3026, 2924, 1668, 1604, 1535, 1492, 1373, 1340, 1259, 698 cm^{-1}; ^1H NMR δ 8.4 (broad s, 1H, amide NH), 8.02 and 7.42 (2s, 2H, aromatic), 4.2 and 3.5 (2 very broad s, 4H, ethylene), 2.36, 2.29, 2.13, and 1.61 (4s, 12H, acetyls). Multiple amide resonance structures cause the ethylene group resonances to become very broad bands. Mass Spectrum (EI mode) m/z 332 (M^+), 290, 273, 247, 230, 205. Anal. ($C_{16}H_{20}N_4O_4 \cdot 0.25H_2O$) C, H, N.

3.5. 8-Amino-5,6-dihydro-2-methyl-4*H*-imidazo[1,5,4-*de*]quinoxaline (9)

A solution of 100 mg (0.3 mmol) of **8** in 2 mL of 48% HBr was heated at reflux for 5 min. The reaction was cooled to room temperature and diluted with 5 mL of ethyl acetate resulting in crystallization of the product as the trihydrobromide salt: 138 mg yield (~99%); mp dec >335 °C; TLC (butanol, acetic acid, water [5:2:3]) $R_f = 0.25$; FTIR 3284, 3029, 2922, 2850, 2604, 1666, 1631, 1574, 1498, 1450, 1386, 1348, 698 cm^{-1}; ^1H NMR δ 6.49 and 6.30 (2s, 2H, aromatic), 4.32 and 3.54 (2t, $J = 5$ Hz, 4H, ethylene), 2.67 (s, 3H, methyl). Mass Spectrum (EI mode) m/z 188 (M^+). Anal. ($C_{10}H_{12}N_4 \cdot H_2O \cdot 3HBr$) C, H, N.

3.6. 8-Amino-2-methyl-4,5-dihydroimidazo[1,5,4-*de*]quinoxalin-9-one (1)

To a solution of 550 mg (1.22 mmol) of the trihydrobromide monohydrate of **9** in 20 mL of 0.2 M pH 7.0-phosphate buffer was added 840 mg of Fremy's salt. The reaction turned to a dark blue color almost immediately. The reaction mixture was placed on a 100 mL Baker Phenyl reverse phase column and eluded with 500 mL of double distilled water to remove salts. The product was eluded as a red band with 0.1 M hydrochloric acid.

Concentration of the product fractions and then reprecipitation of the residue from ethanol/ethyl acetate

afforded the dihydrochloride salt of **1**: 335 mg yield (~99%); mp dec > 320 °C; TLC (butanol, acetic acid, water [5:2:3]) $R_f = 0.29$; FTIR 3410, 2964, 2511, 1718, 1606, 1543, 1346, 1255, 1049, 846 cm^{-1}; ^1H NMR δ 11.0, 9.6, and 8.9 (3s, 3H, NH protons), 5.70 (s, 1H, 7-H), 4.3 and 3.4 (2t, $J = 7$ Hz, 4H, ethylene bridge), 2.41 (s, 3H, 2-methyl). Mass Spectrum (EI mode) *m/z* 202 (M^+). Anal. ($C_{10}H_{10}N_4O \cdot 2HCl$) C, H, N.

3.7. 4-(2-Hydroxyethylamino)-3-nitrotoluene (11)

A solution of 4 mL (5.2 g, 0.03 mol) of 4-chloro-3-nitrotoluene in 4 mL (4.08 g, 0.07 mol) of ethanolamine was refluxed for 2 h. The reaction was then cooled to room temperature and diluted with dichloromethane and water. The organic phase was dried with sodium sulfate, filtered, and concentrated giving red oil. Triturating the oil with hexane yielded a red solid. The solid was filtered off and then washed with hexane and dried. The crude product was recrystallized from dichloromethane: 4.44 g (75% yield); mp 80–81 °C; TLC (chloroform/methanol [90:10]) $R_f = 0.44$; FTIR 3445, 3346, 2957, 2926, 2872, 1637, 1568, 1523, 1433, 1406, 1356, 1311, 1219, 1178, 1151, 1039, 922, 812 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.06 (1H, s, N-(4-amino), 7.98 (1H, s, C(2) aromatic proton), 7.29 and 6.83 (2H, 2d, $J = 8.7$ Hz, C(5) and C(6) aromatic protons), 3.95 and 3.53 (4H, 2t, $J = 4.8$ Hz, methylenes), 2.27 (3H, s, C(1) methyl); MS (EI mode) *m/z* 196 (M^+). Anal. ($C_9H_{12}N_2O_3$) C, H, N.

3.8. 3-Acetamido-4-(*N*-acetyl-2-acetoxyethylamino)toluene (12)

A suspension consisting of 11.8 g (0.0602 mol) of **11** dissolved in methanol and 2.36 g of 5% Pd on carbon was shaken under 50 psi H_2 for 3 h. The reaction mixture was then filtered through Celite. The methanol was removed from the filtrate by evaporation and the residue dissolved in an excess solution of acetic acid/acetic anhydride (1:1) and refluxed for 1 h. The reaction was cooled to room temperature, made basic with saturated sodium bicarbonate solution (pH = 8) and extracted with chloroform. The chloroform extracts were dried with sodium sulfate, filtered, and concentrated to yield a crude product 15.3 g (77% yield). An analytically pure sample was obtained by recrystallization from chloroform and hexane: mp 145–147 °C; TLC (chloroform/methanol [95:5]) $R_f = 0.53$; FTIR 3235, 1711, 1663, 1607, 1514, 1468, 1395, 1337, 1231, 1038, 826 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.24 (1H, s, amide proton), 7.61 (1H, s, aromatic proton), 7.14 and 7.01 (2H, 2d, $J = 8.1$ Hz, aromatic protons), 4.11 and 3.99 (4H, 2t, $J = 3.0$ Hz, methylenes), 2.28, 2.04, 1.88, and 1.66 (12H, 4s, methyls); MS (EI mode) *m/z* 292 (M^+). Anal. ($C_{15}H_{20}N_2O_4$) C, H, N.

3.9. 1-(2-Hydroxyethyl)-2,5-dimethylbenzimidazole (13)

To 100 mL of 4 N HCl was added 3.78 g (0.0129 mol) of crude **12**. This mixture was then heated under reflux for 3 h. The reaction was cooled to room temperature followed by cooling in an ice bath. Concentrated ammonium hydroxide was then added drop wise until the pH was equal to 8 resulting in crystallization of a white solid. These crystals were then filtered and dried under high vacuum: 2.4 g (96%) yield; mp 131–133 °C, TLC (chloroform/methanol [95:5]), $R_f = 0.13$; FTIR 3420, 3146, 2920, 2843, 1670, 1512, 1404, 1357, 1074, 856 cm^{-1}; ^1H NMR ($CDCl_3$), δ 7.26 (1H, s, aromatic proton), 7.06 and 6.93 (2H, 2d, $J = 8.7$ Hz, aromatic protons), 4.19 and 4.05 (4H, 2t, $J = 4.2$ Hz, methylenes), 2.51 (3H, s, methyl), 2.32 (3H, s, methyl); MS (EI mode) *m/z* 190 (M^+). Anal. ($C_{11}H_{14}N_2O \cdot 0.2NH_3 \cdot 0.6H_2O$) C, H, N.

3.10. 6-Acetamido-1-(2-acetoxyethyl)-2,5-dimethylbenzimidazole (14)

To 3 mL of 90% nitric acid, cooled in an ice-salt bath (0 °C), was added 100 mg (0.53 mmol) of **13** in small portions while keeping the temperature below 10 °C. After the addition, the reaction was allowed to stir at 0 °C for 10 min, poured over ice, and neutralized with sodium bicarbonate solution (pH = 8). The mixture is then extracted with chloroform and dried with sodium sulfate. The product is concentrated by evaporation and crystallized with chloroform/hexane: 111 mg (89%) yield. These crystals are a mixture of isomers that cannot be separated. As a result the mixture was taken to the next step where it was reduced and acetylated. A suspension of 500 mg (2.23 mmol) of the mixture in 100 mL of methanol and 100 mg of 5% Pd on carbon and shaken under 50 psi of H_2 for 3 h at room temperature. The catalyst was removed by filtering through Celite and the filtrate was concentrated to a residue. The residue was dissolved in an excess solution of acetic acid/acetic anhydride (1:1) and stirred at room temperature for 15 h. The acetic acid/acetic anhydride solution was evaporated by high vacuum and the resulting solid was taken up in saturated sodium bicarbonate solution and extracted with chloroform. The organic phase was dried with sodium sulfate, filtered, and concentrated to yield a mixture of isomers that were separated by column chromatography (5% methanol in chloroform) to give 200 mg (33% yield) of the desired isomer: mp 223–225 °C; TLC (chloroform/methanol [90:10]) $R_f = 0.58$; FTIR 3281, 2924, 1745, 1655, 1535, 1473, 1402, 1234, 1053, 868 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.99 (1H, s, aromatic proton), 7.46 (1H, s, aromatic proton), 7.04 (1H, s, amide proton), 4.35 (4H, 2t, $J = 2.7$ Hz, methylenes), 2.59 (3H, s, methyl), 2.36 (3H, s, methyl), 2.24 (3H, s, methyl), 2.09 (3H, s, methyl); MS (EI mode) *m/z* 289 (M^+). Anal. ($C_{15}H_{19}N_3O_3$).

3.11. 6-Acetamido-1-(2-acetoxyethyl)-2,5-dimethyl-7-nitrobenzimidazole (15)

To 10 mL of 90% nitric acid at room temperature was added 900 mg (3.11 mmol) in small portions. After the addition, the reaction was stirred at room temperature for 1.5 h. The reaction was then made basic with sodium bicarbonate solution (pH = 8) and extracted with chloroform, dried with sodium sulfate and concentrated. The resulting pale yellow solid was recrystallized from chloroform and hexane: 930 mg (89%) yield; mp

157–158 °C; TLC (chloroform/methanol [90:10]) $R_f = 0.33$; FTIR 3421, 3294, 3011, 1734, 1662, 1529, 1371, 1234, 1035, 790 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.74 (1H, s, amide proton), 7.34 (1H, s, aromatic proton), 4.39 and 4.20 (4H, 2t, $J = 3.0$ Hz, methylenes), 2.66 (3H, s, methyl), 2.39 (3H, s, methyl), 2.22 (3H, s, methyl), 1.95 (3H, s, methyl); A double pulsed field gradient spin-echo transient NOE experiment (GOESY) and HMBC in $CDCl_3$ was done to determine which position was nitrated from the above reaction. The GOESY revealed a correlation between the aromatic proton and the methyl bonded to the benzene with only a 2% peak enhancement. Further confirmation of the assigned structure was the three bond coupling between the C-4 proton at δ 7.73 and the 5-methyl carbon at δ 18.5 in the HMBC spectrum. MS (EI mode) *m/z* 334 (M^+). Anal. ($C_{15}H_{18}$- N_4O_5) C, H, N.

3.12. 6-Acetamido-1-(2-methanesulfonoxyethyl)-2,5-dimethyl-7-nitrobenzimidazole (16)

To 900 mg (2.69 mmol) of **15** in 180 mL of methanol, was added 540 mg of K_2CO_3. This reaction mixture was stirred at room temperature for 3 h. The methanol was removed by evaporation leaving an oily residue that was diluted with 30 mL of water. After adding the water, yellow product crystallized from solution: 777 mg (98%) yield; mp 225–230 °C; TLC (chloroform/methanol [90:10]) $R_f = 0.12$; FTIR 3283, 2928, 1666, 1527, 1408, 1271, 1080, 792 cm^{-1}; ^1H NMR ($CdCl_3$) δ 7.89 (1H, s, amide proton), 7.36 (1H, s, aromatic proton), 4.15 and 3.93 (4H, 2t, $J = 4.2$ Hz, methylenes), 2.63, 2.24, and 2.21 (9H, 3s, methyl protons); MS (EI mode) *m/z* 292 (M^+). Anal. ($C_{13}H_{16}N_4O_4$) C, H, N.

To 710 mg (2.43 mmol) of alcohol in 8 mL of dry pyridine, was added 0.4 mL of methanesulfonyl chloride. This reaction mixture was stirred at room temperature for 3 h, during which the product crystallized from solution. These crystals were collected by filtration washed with ice cold pyridine and dried under high vacuum to give 670 mg of **16** (74% yield); mp 230–230 °C; TLC (chloroform/methanol [85:15]) $R_f = 0.36$; FTIR 3327, 3007, 2930, 1670, 1631, 1167, 920, 810 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.70 (1H, s, amide proton), 7.72 (1H, s, aromatic proton), 4.63 (4H, 2t, $J = 3.0$ Hz, methylenes), 3.02, 2.58, 2.25, and 1.99 (12H, 3s, methyl protons); MS (EI mode) *m/z* 370. Anal. ($C_{14}H_{18}N_4O_6S$) C, H, N, S.

3.13. 7-Acetamido-5,6-dihydro-2,8-dimethyl-4*H*-imidazo[1,5,4-*de*]quinoxaline (17)

A suspension of 200 mg (0.541 mmol) of **16** and 160 mg of 5% Pd on carbon in methanol was shaken under 50 psi H_2 for 30 min. The catalyst was removed by filtering through Celite. The remaining methanol solution was then heated to 60 °C and stirred at that temperature for 1.5 h. After the reaction is complete the methanol was evaporated leaving a crude product. This crude product was then recrystallized using methanol and ethyl acetate: 103 mg (79%) yield; mp 228–229 °C; TLC (chloroform/methanol [85:15]) $R_f = 0.14$; FTIR 3261, 3007, 1637, 1508, 1194, 1058, 785 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.12 (1H, s, amide proton), 6.80 (1H, s, aromatic proton), 4.33 and 3.54 (4H, 2t, $J = 4.2$ Hz, methylenes), 2.69, 2.21, and 2.05 (9H, 3s, methyl protons); MS (EI mode) *m/z* 244. Anal. ($C_{13}H_{16}N_4O$) C, H, N.

3.14. 7-Acetamido-2,8-dimethyl-4,5-dihydroimidazo-[1,5,4-*de*]quinoxalin-9-one (2)

To 25 mg (0.102 mmol) of **17** in 1 mL of phosphate buffer (pH = 7), was added 140 mg of Fremy salt. This reaction mixture is stirred at room temperature for 5 min. The reaction mixture was extracted with chloroform and the extracts dried over (Na_2SO_4), filtered, concentrated to a residue. The residue was recrystallized from chloroform and hexane: 8.5 mg (31%) yield; mp > 160 °C dec; TLC (chloroform/hexane [90:10]) $R_f = 0.17$; IR (KBr pellet) 3421, 3223, 2993, 1656, 1518, 1375, 1280, 1033 cm^{-1}; ^1H NMR ($CdCl_3$) δ 4.33 and 4.06 (4H, 2t, $J = 7.2$ Hz, metheylenes), 2.46, 2.27, and 1.60 (9H, 3s, methyl protons); MS (EI mode) *m/z* 258. Anal. ($C_{13}H_{10}N_4O_2$) C, H, N.

3.15. Topoisomerase II inhibition assays

The topoisomerase II relaxation reactions were carried out with 0.25 μg of pRYG supercoiled DNA (form I), 4 units of recombinant human topoisomerase IIα (TopoGEN Inc.) in a total volume of 20 μL 50 mM Tris Buffer (pH 8.0), containing 120 mM of KCL, 10 mM of $MgCl_2$, 0.5 mM of dithiothreitol, and 0.5 mM of ATP in microcentrifuge tubes. Varying amounts of drugs were added to each reaction. The reactions were run at 37 °C for 45 min. The reactions were then stopped by the addition of 2 μL of 10% SDS to each tube and 1 μL of proteinase K (1 mg/mL) was also added. The resulting mixtures were incubated for 15 more min at 37 °C. Each reaction mixture was extracted once with 20 μL of chloroform/isoamyl alcohol (24:1) and then combined with 2 μL of 10× loading dye (0.25% bromophenol blue and 50% aqueous glycerol). The resulting mixtures were then loaded onto a 1% agarose gel in 1× TAE buffer and run at 2 V/cm for 5–7 h. The gels either contained or not contained 0.5 μg/mL of ethidium bromide. After running, gels without ethidium bromide were soaked in 1× TAE buffer containing 0.5 μg/mL of ethidium bromide for 30 min followed by destaining in 1× TAE buffer for 20 min.

3.16. In vivo evaluation

The B-16 melanoma in C57/bl mice syngraft model was employed to determine in vivo activity.[20] Each agent was evaluated at three doses: 1, 2, or 4 mg/kg/day, on days 1, 5, and 9 after subcutaneous tumor implantation of 10^5 cells in the front flank on day 0. 'Toxic' means that there was early lethality, or $\geqslant$50% lethality prior to any deaths in the control group. The treated over control values (T/C) were measured at day 25 of the study. A T/C value <40% is considered active. The control was obtained with drug-free animals.

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