

(+)-5, 139892-76-5; (-)-6, 81445-44-5; (+)-6, 81445-45-6; (+)-7, 94233-72-4; (-)-7, 89104-01-8; (+)-8, 94233-71-3; (-)-8, 89104-02-9; (-)-9, 102735-38-6; (+)-9, 129170-66-7; (+)-10, 102735-37-5; (-)-10, 129170-70-3; (-)-11, 139892-68-5; (+)-11, 129170-67-8; (-)-12,

139892-71-0; (+)-12, 139892-72-1; (-)-13, 501-39-3; (+)-13, 35119-42-7; (-)-14, 139892-69-6; (+)-14, 139892-70-9; (-)-15, 139892-73-2; (+)-15, 139892-74-3; (-)-16, 6793-45-9; (+)-16, 139892-75-4.

## Bioreductive Fluorescent Markers for Hypoxic Cells: A Study of 2-Nitroimidazoles with 1-Substituents Containing Fluorescent, Bridgehead-Nitrogen, Bicyclic Systems

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The oxygen-sensitive bioreductive binding of 2-nitroimidazoles labeled with fluorescent side chains has been used to stain hypoxic mammalian cells selectively. Several novel compounds were synthesized with a 1-substituent containing a fluorescent, bicyclic system having a bridgehead-nitrogen atom. Additional amine and secondary alcohol substituents were also included in the link between the fluorophor and the nitroimidazole to improve water solubility. Their ability to discriminate between hypoxic and oxic cells was compared by flow cytometric analysis. A wide range of cellular fluorescence and hypoxic-oxic differentials in fluorescence was observed when compounds with indolizine fluorophors were incubated with cells, and one such compound was considered suitable for further evaluation in vivo. Two compounds with bimane fluorophors gave very little cellular fluorescence when incubated with hypoxic cells.

Measurements of the fraction of poorly oxygenated (and therefore radio-resistant) cells in tumors<sup>1,2</sup> could be of considerable clinical significance as optimal radiotherapy schedules could be devised for individual patients on the basis of the oxygen status of their tumors. Those patients with significant levels of hypoxic cells could be selected for treatment with hyperbaric or normobaric oxygen to reduce the proportion of hypoxic cells, or with oxygen-mimetic radiosensitizers to increase the radiosensitivity of their hypoxic cells. Alternatively, high linear energy transfer (LET) radiation could be administered, taking advantage of the reduced dependence of radiosensitivity on oxygen levels with this sort of radiation.

One approach to the identification of hypoxic cells has been to take advantage of the inhibition by oxygen of the reductive metabolism of fluorescent nitroaromatic compounds in cells.<sup>3-11</sup> The nitro group quenches the fluorescence of the aromatic ring system, but on bioreduction of the nitro group in hypoxic cells the ring system becomes fluorescent.<sup>7</sup> Numerous nitroaromatic structures have been evaluated in model systems in vitro, but many were large planar molecules with intercalating properties.<sup>6,7,10</sup> Although good results have been obtained with some of these compounds in vitro, their high affinity for DNA could lead to bioavailability problems in vivo. A probe for use in vivo would have to diffuse from blood vessels through several layers of well oxygenated tumor cells to reach those cells that are hypoxic. In addition, the equilibrium between fluorescent metabolites, intercalated with DNA and in free solution, could allow the fluorescent label to diffuse from the hypoxic cells to other, better oxygenated, cells and tissues.

The hypoxia-dependent bioreductive metabolism of 2-nitroimidazoles, and resultant binding of ring fragments with the side chains to cellular constituents, has been the basis of several alternative methods proposed for deter-

mining the hypoxic fraction of tumors. Various isotope labels have been evaluated including <sup>3</sup>H, <sup>14</sup>C, <sup>75</sup>Br, <sup>76</sup>Br, <sup>77</sup>Br, and <sup>19</sup>F.<sup>11-16</sup> In a previous paper,<sup>17</sup> we examined the

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<sup>†</sup> Gray Laboratory.

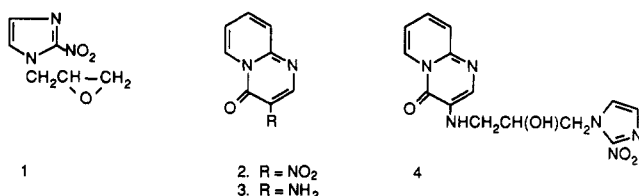
<sup>‡</sup> Brunel University.

use of a fluorescent label covalently bound to a 2-nitroimidazole as a method of identifying hypoxic cells. Such fluorescent probes bind selectively to cellular constituents of hypoxic cells as a result of bioreductive metabolism of the nitroimidazole.

With the current series of compounds, this concept has been improved by choosing bicyclic fluorophors of low molecular weight which were expected to have high fluorescence efficiencies and low intercalation properties. The hydrophilicity of the side chains linking the fluorescent moieties and the nitroimidazoles was also increased. Thus transport in vivo to tumor cells was expected to be better than for the series of compounds described previously,<sup>17</sup> where the fluorophors were tricyclic in several cases. As before, selective binding of these new probes in hypoxic cells was expected to be conferred by reductive metabolism of the 2-nitroimidazole nucleus.

## Chemistry

The pyrido[1,2-*a*]pyrimidone ring system has several qualities as a fluorophor which made it appear, on the limited evidence available<sup>18</sup> when this work was started, to be attractive for our purposes. The nitro compound 2 was conveniently reduced with titanium trichloride to afford the amine 3 and, upon reaction with the epoxide 1, gave the first of this series of potential fluorescent probes 4. Unfortunately, our subsequent investigation<sup>19</sup> with a



number of 3-aminopyrido[1,2-*a*]pyrimidones showed these compounds to have a relatively low quantum yield ( $\phi_f < 0.1$ ), so that, although the excitation and fluorescence emission wavelengths were suitable, the intensity of any fluorescence due to the probe compound in the hypoxic cells was likely to be low.

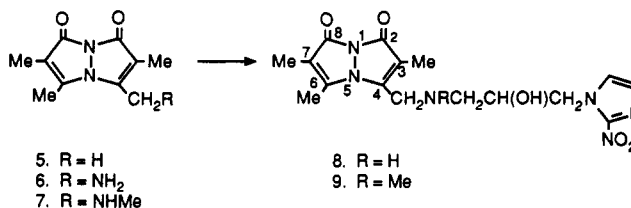
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Table I. Ultraviolet Absorption, Fluorescence Maxima, and Quantum Yields<sup>a</sup>

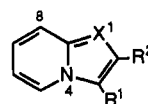
compd	UV absorption $\lambda_{max}$ , nm (log $\epsilon$ )	fluorescence <sup>b</sup>		$\phi_f^b$
		excitation $\lambda_{max}$ , nm	emission $\lambda_{max}$ , nm	
11	295 (3.57)	300	371	0.37
13	342 (3.70)	340	426	0.14
14	329 (4.10)	330	451	0.26
20	300 (3.88)			
	343 (3.89)	343	400	0.4
21	300 (3.81)			
	345 (3.79)	351	399	0.41
	364 (3.57)			
23	304 (3.87)			
	331 (3.95)	320	396	0.78
	363 (3.82)			
24	305 (3.84)			
	331 (3.96)	320	395	0.66
	362 (3.81)			
26	304 (3.57)			
	343 (3.77)	320	400	0.45

<sup>a</sup> The experimental procedure is given in the Experimental Section. <sup>b</sup> Determined for methanolic solution.

A small molecule with a high quantum efficiency is biman (5).<sup>20</sup> Certain of its derivatives have found widespread use in chemical and biochemical investigations since the relatively recent preparation of the ring system. The aliphatic and nucleophilic primary and secondary amines 6 and 7 are known,<sup>21</sup> and the reaction of each with the epoxide 1 gave the candidate hypoxia probes 8 and 9 in good yield.



A relatively small ring system meeting our requirements which has not been utilized as a fluorophor but seemed to us to have some potential applications in this area is the indolizine nucleus (10). The parent compound is



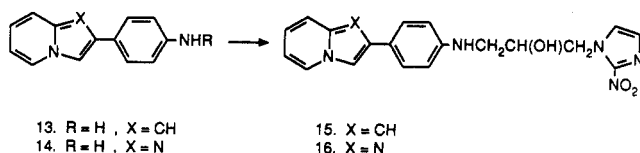
10. R<sup>1</sup> = R<sup>2</sup> = H, X = CH
11. R<sup>1</sup> = R<sup>2</sup> = H, X = N
12. R<sup>1</sup> = CO<sub>2</sub>Et, R<sup>2</sup> = Me, X = CH

known<sup>22</sup> to show a high fluorescence quantum efficiency ( $\phi_f = 0.72$ ). One foreseen potential difficulty associated with attempts to utilize indolizine derivatives is that like other electron-excessive aromatic heterocycles such as pyrrole, indolizine and simple alkyl derivatives are readily susceptible to oxidation.<sup>23</sup> One approach to combating

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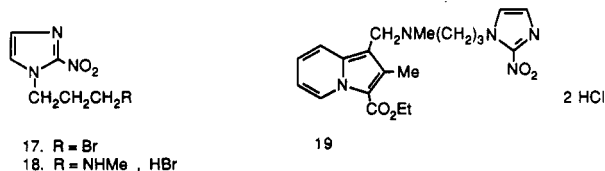
this potential problem is to introduce a phenyl substituent on the five-membered ring so that the negative charge can be delocalized outside the indolizine nucleus. Alternatively, the introduction of a pyridine-like nitrogen atom into the five-membered ring is also expected to produce a decrease in the ease of oxidation of the heterocycles by localization of the negative charge on the nitrogen atom which, of course, is not susceptible to substitution reactions. A suitable example of such a ring system is the imidazo[1,2-*a*]pyridine nucleus (11), and this and the 2-phenylindolizine and 2-phenylimidazo[1,2-*a*]pyrimidine are known<sup>22</sup> to have high fluorescence quantum yields.

To test these ideas, and so that comparable data could be obtained, the two known 2-(*p*-aminophenyl) derivatives 13 and 14 of indolizine and imidazo[1,2-*a*]pyridine, respectively, were prepared<sup>24</sup> and were shown to have fairly high quantum yields (Table 1). The reactions of these amines with the epoxide 1 gave the required probe molecules 15 and 16 in moderate yields, reflecting the lower nucleophilicity of the aromatic amines compared with the aliphatic amines in the biman series. However, determined attempts to optimize the yields were not undertaken.



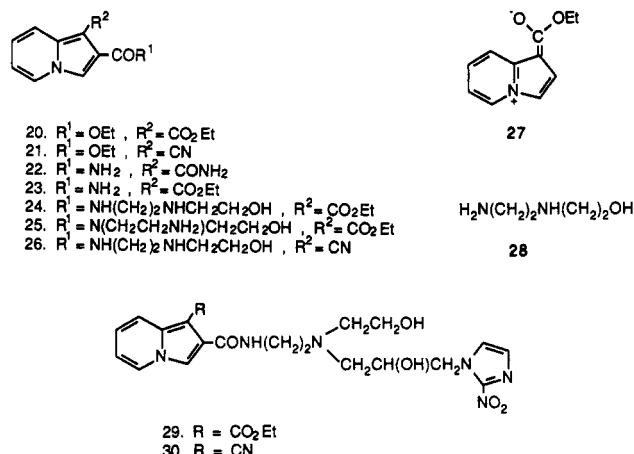
A different and more useful approach, for our purposes, to decreasing the susceptibility of the indolizine nucleus to oxidation was to introduce one or more electron-withdrawing groups into the five-membered ring. This stabilized the nucleus to unwanted oxidation reactions while maintaining or increasing the fluorescence quantum yield. For example, the presence of a 3-carbomethoxy group in 12 produced a stable compound with  $\phi_f = 0.53$ . Fluorescence quantum yields for other compounds prepared in this work are given in Table 1.

As a consequence of the electron availability from the nitrogen atom of the indolizine nucleus, electrophilic substitution at the 1-position or, if it is blocked, at the 3-position, is known to occur readily. It seemed likely that 12 would readily undergo electrophilic substitution at position 1. The availability of the secondary amine 18 by amination of the bromo compound 17<sup>25</sup> allowed the application of the Mannich reaction to give the candidate probe compound 19 in high yield.



The final two probe compounds 29 and 30 were obtained in two steps from the known diester 20 and the cyano ester 21, respectively. The diester was reported<sup>26</sup> to react with

alkylamines to give diamides (22). We were unable to reproduce this result, and even when 20 was heated with alcoholic ammonia in a sealed tube at 85 °C for 36 h only a monoamide was obtained. The two ester functions in 22 were expected to exhibit different reactivities with nucleophiles since electron release from the nitrogen atom was expected to preferentially deactivate the 1-ester function to nucleophilic attack as indicated by the canonical structure 27. The monoamide obtained from 20 was therefore assigned the 2-carboxamido structure 23. The reaction of the diester 20 with [*N*-(2-aminoethyl)amino]ethanol (28) gave a product containing one compound. This compound contained one ethyl group (NMR) so that attack was thought to have occurred at the 2-carbomethoxy group. The attacking species 28 contained three nucleo-



philic centers, though, under the reaction conditions, the amino groups were most likely to react. Inspection of the IR spectrum showed the presence of a hydroxyl group, what appeared to be two separate signals attributable to the amide NH and amine NH, and an amide carbonyl group stretching absorption. The twin peaks expected, indicating the presence of an NH<sub>2</sub> group, were not seen. The proton NMR spectrum showed the presence of two exchangeable signals: one was part of a broad multiplet at  $\delta = 2.68$ –3.43 ppm and included two exchangeable protons; the second exchangeable signal, equivalent to one proton, occurred as a triplet at  $\delta = 9.54$  ppm. Thus, the reaction product was thought to be the secondary amide 24 rather than the isomeric tertiary amide 25. An exhaustive search for the presence of 25 in the reaction mixture was not made, and it may be that the reaction workup procedure selectively isolated one isomer. The reaction of the secondary amine 24 with the epoxide 1 gave a product which showed the low-field peak (equivalent to one proton) ascribed to the amide NH, thus supporting the structural assignment of 24 and showing the structure of the product to be 29. Two corresponding reactions from the cyano ester 21 afforded 26 and subsequently the potential hypoxia probe 30.

## Biological Results and Discussion

The potential hypoxia probes 4, 8, 9, 15, 16, 19, 29, and 30 each consisted of a bicyclic fluorophore containing a bridgehead-nitrogen atom, attached by a side chain to a 2-nitroimidazole. High fluorescence efficiencies and low intercalation properties were expected from most of the fluorophores employed, although chemical investigation showed that the pyrimidone ring system used in 4 had a low quantum yield. The compounds contain at least one

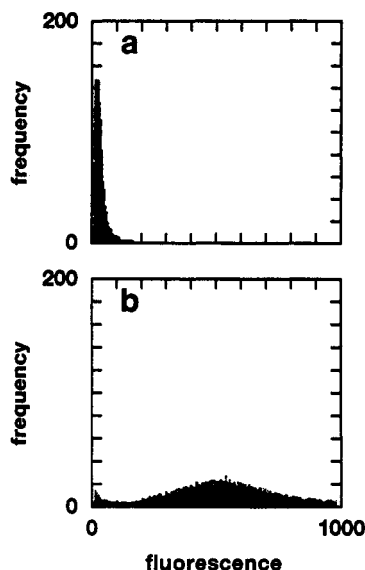
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**Table II.** Flow Cytometric Analysis of Cells Incubated under Oxic and Hypoxic Conditions with the Compounds from Scheme I<sup>a</sup>

compd	intensity hypoxic fluorescence	intensity oxic fluorescence	t max, h
4	1746	603	6
8	89	33	15
9	55	43	15
15	169	107	6
16	1571	417	6
19	765	258	6
29	1144	66	6
30	81	27	6

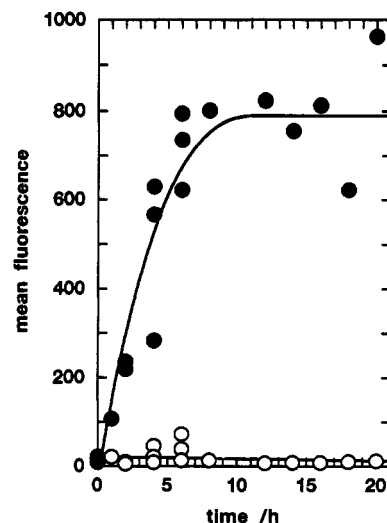
<sup>a</sup> All drug concentrations were  $10^{-4}$  M. Fluorescence was excited at 366 nm and all wavelengths  $>390$  nm were collected, except for compounds 4, 8, 9, and 16 where all wavelengths  $>410$  nm were collected. Mean fluorescence intensities (in arbitrary units) measured by flow cytometry have been normalized for constant PMT gain and laser excitation power.



**Figure 1.** Cytofluorimeter analysis of V79 Chinese hamster cells incubated at 37 °C under (a) oxic and (b) hypoxic conditions with 29 ( $10^{-4}$  M). Data was gated on forward scatter and fluorescence to omit debris.

amine function in the chain which links the fluorophor with the azomycin nucleus and, with one exception (19) a secondary alcohol is also present. These features of the probe molecules were introduced to improve aqueous solubility.

Flow-cytometric analysis of cells incubated under oxic or hypoxic conditions with compounds from Scheme I is shown in Table II and Figure 1. All of the compounds tested exhibited more fluorescence in hypoxic cells than the background fluorescence from untreated cells. However, the compounds containing the bimeane fluorophors 8 and 9 gave rather low levels of fluorescence in hypoxic cells, even after longer incubation times (15 h) than that used with the other compounds. A wide range of intensities of cellular fluorescence was obtained when hypoxic cells were incubated with the compounds containing the indolizines 15, 19, 29, and 30, the last giving very little cellular fluorescence. The compounds containing a pyrimidone (4) and an imidazopyridine (16) fluorophor gave the most fluorescence in hypoxic cells. It is surprising that 4 gave the greatest hypoxic fluorescence of all the compounds tested, in view of the low quantum yield measured for the pyrimidone fluorophor. However, in general, where fluorescence quantum efficiencies were measured in model fluorophors, they were poorly correlated with the amount



**Figure 2.** The time-dependence of accumulation of fluorescent metabolites for hypoxic (●) and oxic (○) V79 Chinese hamster cells incubated with 29 ( $10^{-4}$  M) at 37 °C.

of cellular fluorescence observed following incubation of the target compounds with hypoxic cells. This may reflect additional cellular metabolism of the fluorophors to more (or less) fluorescent products.

Although the levels of fluorescence that can be observed in hypoxic cells are important in determining the sensitivity with which hypoxia-specific metabolites may be detected, the ratio of hypoxic-oxic fluorescence is also important and, ideally, should be as high as possible. This ratio determines the maximum possible separation between the profiles of fluorescence measured for hypoxic and well-oxygenated cell populations (e.g. Figure 1), and would therefore affect the ability of the probe to identify a small proportion of hypoxic cells among a population of better-oxygenated tumor cells. With these compounds the hypoxic-oxic ratio was generally low (ca. 2–4) after 6-h incubation of cells with the drug, with the exception of 29, for which a much higher hypoxic-oxic ratio of cellular fluorescence was observed.

The time-dependence of accumulation of fluorescent products in cells incubated with 29 is shown in Figure 2. A large differential (17-fold after 6 h) was seen between the fluorescence of cells incubated under hypoxic and oxic conditions. As with other probes for hypoxic cells,<sup>8,10,17</sup> the rate of increase in fluorescence fell with time and a plateau level of cellular fluorescence was observed after incubation of cells with 29 for several hours. By contrast with 29, the poor performance of 30 (Table II) was unexpected, in view of its structural similarity to 29 and the similar quantum efficiencies of the model fluorophors 24 and 26 (Table I).

The hypoxic-oxic differential in cellular fluorescence following incubation of cells with the target compounds must reflect the combined effects of several processes. These will include the different rates of metabolic reduction of the compounds in hypoxic and well-oxygenated cells, the degree with which the fluorescence of the parent compound is quenched, and trapping of parent compound and/or metabolites within the cells. Although the chemical basis for bioreductive metabolism and binding of these compounds and for the development of an hypoxic-oxic differential appears to be well established, we clearly do not understand all the factors contributing to these endpoints. All the compounds have identical one-electron reduction potentials, but other factors such as partition coefficient, pK, and charge will probably influence diffusion into cells, enzymic reduction, and diffusion from the

site of metabolism to that of binding. Differentials in bioreductive metabolism of the identical nitroimidazole moieties would be expected to be relatively small, and the saturated linking chains will have prevented the conjugated fluorophors from influencing significantly the nitroimidazole's electron-affinity and therefore the rate of nitro-group reduction.<sup>27</sup> It also seems unlikely that the fluorophors would have sterically hindered nitro-group metabolism, as hypoxia-specific metabolism of nitroimidazoles with much bulkier fluorophor side chains<sup>17</sup> and metabolism of a variety of heterocyclic compounds<sup>10</sup> have been observed to occur readily in a range of mammalian cells. However, cellular metabolism of nitroaromatic compounds such as nitronaphthalimides has been found previously to produce a range of hypoxia-specific metabolites, with different excitation and fluorescence properties.<sup>6,8</sup> It therefore seems likely that many of the differences observed here in the behavior of compounds of similar structure (e.g. 29 and 30) reflect differential metabolism of the fluorophor group.

Failure of drugs to bind firmly to cellular macromolecules does not necessarily prevent the accumulation of sufficient fluorescent metabolites for analysis by flow cytometry. For example, HPLC analysis shows that 29 is readily removed from cells by washing with physiological buffered saline, although its fluorescent metabolites remain. However, the majority of these fluorescent metabolites are not covalently bound to macromolecules, because permeabilization of cells with Triton-X-100 detergent or by fixing cells in 70% ethanol greatly reduced the amount of fluorescence observed by flow cytometry. Much of the bioreductively bound metabolites of isotopically labeled misonidazole are to acid-soluble components of cells such as glutathione.<sup>28,29</sup> Similarly the easily-removable fluorescent metabolites of 29 may be bound to the low molecular weight fraction of cells, thereby becoming trapped within the cells as long as their membrane is intact, but easily lost if cells are fixed and permeabilized. The small amount of residual fluorescence following ethanol extraction of cells presumably represents the remaining adducts to high molecular weight components of the cell.

Preliminary evaluation of 29 indicates that its toxicity and short half-life (12 min) in mouse plasma may not allow sufficient exposure of hypoxic tumor cells to the drug for sufficient fluorescence to develop to be evaluated by flow cytometry. However, HPLC analysis of extracts of tumors exposed to 29 in vivo has indicated the presence of hypoxia-specific metabolites which are increased in clamped tumors (M.R.L. Stratford, personal communication).

## Conclusions

It was anticipated that bioreductive metabolism of the nitroimidazole moieties of the novel compounds in this work, as of those previously reported,<sup>17</sup> would lead to hypoxia-specific cellular fluorescence which might depend

mainly on the fluorescent properties of the fluorophors. However, the amounts of cellular fluorescence obtained in hypoxic cells, and the hypoxic-oxic differentials, varied in ways that were not easily predicted from the structures of the fluorophor side chains. In this series of compounds, the most promising candidate for in vivo evaluation is 29 with an indolizine side chain, but 30, with a very similar structure, performed badly with very low fluorescence (although an oxic-hypoxic differential of 3 was obtained). Similarly, despite the high quantum efficiency of bimanies, probes incorporating these fluorophors showed little cellular fluorescence. Bioreductive metabolism of the nitroimidazole function is clearly not the only factor contributing to the behavior of these compounds when used as metabolic stains for hypoxic cells. Chemical studies alone cannot be used to predict the potential usefulness of compounds as probes for hypoxic cells, and evaluation in a biological test system is essential.

Most of the target compounds evaluated here are probably unsuitable for use as probes for hypoxic cells because their metabolism gave poor levels of cellular fluorescence and low hypoxic-oxic differential fluorescence. However, 29 showed a very high differential fluorescence between hypoxic and oxic cells and is suitable for further evaluation as a probe for hypoxic cells in tumors in vivo.

## Experimental Section

**Preparative Chemistry.** Melting points, and IR, UV fluorescence, NMR, and mass spectra were obtained as described previously.<sup>17</sup> Light petroleum refers to petroleum ether (bp 60–80 °C) and ether refers to diethyl ether. The starting materials, 1-(2,3-epoxypropyl)-2-nitroimidazole (1),<sup>31</sup> 3-amino-4-oxo-4H-pyrido[1,2-a]pyrimidine (3),<sup>19</sup> aminobimane (6), [N-methylamino]bimane (7),<sup>21</sup> 2-(4-aminophenyl)indolizine (13), 2-(4-aminophenyl)imidazo[1,2-a]pyridine (14),<sup>24</sup> ethyl 2-methylindolizine-3-carboxylate (12),<sup>32</sup> 1-(3-bromopropyl)-2-nitroimidazole (17),<sup>25</sup> diethyl indolizine-1,2-dicarboxylate (20),<sup>33</sup> and ethyl 1-cyanoindolizine-2-carboxylate (21)<sup>34</sup> were prepared by literature methods.

**3-[[2-Hydroxy-3-(2-nitroimidazol-1-yl)propyl]amino]-4-oxo-4H-pyrido[1,2-a]pyrimidine (4).** A solution of 3-amino-4-oxo-4H-pyrido[1,2-a]pyrimidine (3) (0.1 g, 0.679 mmol) and 1-(2,3-epoxypropyl)-2-nitroimidazole (1) (0.12 g, 0.679 mmol) in dry EtOH (5 mL) was refluxed for 15 h. The precipitated solid was filtered off and washed with EtOH to afford 4 as an orange solid (42 mg, 19%): mp 230–232 °C dec; IR (KBr) 3300 (NH), 3100 (OH), 1575 and 1350 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 4.35 (m, 5 H, aliphatic CH), 5.40 (br s, 2 H, exchanged with D<sub>2</sub>O, NH and OH), 7.00 (d, 1 H, *J* = 8 Hz, 9-H), 7.40 (m, 2 H, 7- and 8-H), 7.10 (s, 1 H, 4-H of imidazole), 7.57 (s, 1 H, 5-H of imidazole), 7.76 (s, 1 H, 2-H), 8.64 (d, 1 H, *J* = 9 Hz, 6-H). Anal. (C<sub>14</sub>H<sub>14</sub>N<sub>6</sub>O<sub>4</sub>) C, H, N.

**4-[[N-[2-Hydroxy-3-(2-nitroimidazol-1-yl)propyl]amino]methyl]-3,6,7-trimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (8).** Aminobimane (6) (367 mg, 1.77 mmol), 1-(2,3-epoxypropyl)-2-nitroimidazole (150 mg, 0.89 mmol), dilute NaOH solution (2 drops), and MeOH (2 mL) were heated together at reflux for 14 h. The mixture was cooled to 0 °C, and the yellow solid was filtered off. Recrystallization from aqueous MeOH gave

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8 (230 mg, 70%): mp 185–187 °C; IR (KBr) 3325 (NH), 1570 and 1360 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.72 (s, 3 H, CH<sub>3</sub>), 1.78 (s, 3 H, CH<sub>3</sub>), 2.45 (s, 3 H, 6-CH<sub>3</sub>), 3.78 (s, 2 H, CH<sub>2</sub>), 3.85 (br s, 1 H, NH), 4.25 (m, 3 H, NHCH<sub>2</sub>CHOH and CHOH), 4.57 (m, 2 H, imidazole-CH<sub>2</sub>), 5.22 (d, 1 H, *J* = 6 Hz, OH), 7.14 (s, 1 H, 4-H of imidazole), 7.54 (s, 1 H, 5-H of imidazole); MS *m/z* (%) 376 (2, M<sup>+</sup>), 207 (52), 206 (52), 190 (27), 113 (60), 112 (100), 111 (94). Anal. (C<sub>16</sub>H<sub>20</sub>N<sub>6</sub>O<sub>6</sub>) C, H, N.

4-[[*N*-(2-Hydroxy-3-(2-nitroimidazol-1-yl)propyl)-*N*-methylamino]methyl]-3,6,7-trimethyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione (9). (*N*-Methylamino)bimane (7) (500 mg, 2.27 mmol), 1-(2,3-epoxypropyl)-2-nitroimidazole (1) (281 mg, 1.66 mmol), dilute aqueous NaOH (3 drops), and MeOH (2.2 mL) were heated together at reflux for 14 h. After cooling of the mixture, the yellow solid was filtered off as 9 (489 mg, 75%): mp 192–193 °C; IR (KBr) 1740 (CO), 1530 and 1350 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.72 (s, 3 H, CH<sub>3</sub>), 1.80 (s, 3 H, CH<sub>3</sub>), 2.31 (s, 3 H, 6-CH<sub>3</sub>), 2.51 (s, 3 H, NCH<sub>3</sub>), 3.59 (s, 2 H, CH<sub>2</sub>), 4.01 (m, 3 H, CH<sub>3</sub>NCH<sub>2</sub>CHOH and CHOH), 4.59 (d, 2 H, *J* = 11 Hz, imidazole-CH<sub>2</sub>), 5.14 (br s, 1 H, OH), 7.14 (s, 1 H, 4-H of imidazole), 7.54 (s, 1 H, 5-H of imidazole); MS *m/z* (%) 390 (2, M<sup>+</sup>), 310 (29), 278 (41), 251 (33), 221 (33), 211 (28), 206 (33), 190 (100). Anal. (C<sub>17</sub>H<sub>22</sub>N<sub>6</sub>O<sub>5</sub>) C, H, N.

2-[4-[[2-Hydroxy-(3-nitroimidazol-1-yl)propyl]amino]phenyl]indolizine (15). A solution of 2-(4-aminophenyl)-indolizine (13) (0.25 g, 1.2 mmol) and 1-(2,3-epoxypropyl)-2-nitroimidazole (0.2 g, 1.2 mmol) in dry EtOH (20 mL) was refluxed for 36 h. The solid was filtered off and washed with hot EtOH to leave the secondary amine 15 (0.14 g, 31%): mp 194–195 °C dec; IR (KBr) 3350 (NH), 3100 (OH) and 1360 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 3.89 to 4.66 (m, 5 H, aliphatic CH), 5.19 (br s, 1 H, exchanged with D<sub>2</sub>O, OH), 5.55 (br s, 1 H, exchanged with D<sub>2</sub>O, NH), 6.83 (m, 3 H, 1-, 6-, and 7-H), 7.37 (m, 5 H, 8-H and C<sub>6</sub>H<sub>4</sub>), 7.07 (s, 1 H, 4-H of imidazole), 7.50 (s, 1 H, 5-H of imidazole), 7.62 (s, 1 H, 3-H), 8.05 (d, 1 H, *J* = 8 Hz, 5-H). Anal. (C<sub>20</sub>H<sub>19</sub>N<sub>5</sub>O<sub>3</sub>) C, H, N.

2-[4-[[2-Hydroxy-3-(2-nitroimidazol-1-yl)propyl]amino]phenyl]imidazo[1,2-*a*]pyridine (16). A mixture of 2-(4-aminophenyl)imidazo[1,2-*a*]pyridine (14) (0.15 g, 0.72 mmol) and 1-(2,3-epoxypropyl)-2-nitroimidazole (0.12 g, 0.72 mmol) in dry EtOH (6 mL) was refluxed for 15 h. The product was filtered off as bright yellow 16 (85 mg, 31%): mp 219–220 °C dec; IR (KBr) 3325 (NH), 3075 (OH) and 1540 and 1350 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 3.90 to 4.69 (m, 5 H, aliphatic CH), 5.25 (br s, 1 H, exchanged with D<sub>2</sub>O, OH), 5.73 (br s, 1 H, exchanged with D<sub>2</sub>O, NH), 6.78 (dd, 1 H, *J* = 8 Hz and *J* = 8 Hz, 6-H), 7.00 (dd, 1 H, *J* = 8, 8 Hz, 7-H), 7.15 (q, 4 H, *J* = 10 Hz, C<sub>6</sub>H<sub>4</sub>), 7.45 (d, 1 H, *J* = 8 Hz, 8-H), 7.13 (s, 1 H, 4-H of imidazole), 7.55 (s, 1 H, 5-H of imidazole), 8.10 (s, 1 H, 3-H), 8.39 (d, 1 H, *J* = 8 Hz, 5-H). Anal. (C<sub>19</sub>H<sub>18</sub>N<sub>6</sub>O<sub>3</sub>) C, H, N.

1-[3-(Methylamino)propyl]-2-nitroimidazole Hydrobromide (18). 1-(3-Bromopropyl)-2-nitroimidazole (4 g, 35.4 mmol) and methylamine (220 mL, 33% solution in EtOH) were stirred together at room temperature for 2 h. The solid was filtered off, and the solution was evaporated under reduced pressure to yield the crude product as an oil which later solidified. Crystallization from concentrated HBr–EtOH mixture (1:19) afforded the secondary amine hydrobromide 18 (3.1 g, 64%): mp 131–132 °C; IR (KBr) 1540 and 1350 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 2.13 (q, 2 H, *J* = 7 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.55 (s, 3 H, CH<sub>3</sub>), 2.92 (t, 2 H, *J* = 7 Hz, CH<sub>3</sub>NHCH<sub>2</sub>), 4.47 (t, 1 H, *J* = 7 Hz, imidazole-CH<sub>2</sub>), 7.20 (d, 1 H, *J* = 2 Hz, 4-H), 7.70 (d, 1 H, *J* = 2 Hz, 5-H), 8.35 (br s, 2 H, NH<sup>+</sup>); MS *m/z* (%) 184 (2, M<sup>+</sup>–HBr), 183 (20), 137 (100). Anal. (C<sub>7</sub>H<sub>13</sub>BrN<sub>2</sub>O<sub>2</sub>) C, H, N.

3-(Ethoxycarbonyl)-2-methyl-1-[[*N*-(3-(2-nitroimidazol-1-yl)propyl)-*N*-methylamino]methyl]indolizine Dihydrochloride (19). Ethyl 2-methylindolizine-3-carboxylate (12) (1.27 g, 6.26 mmol), 1-[3-(methylamino)propyl]-2-nitroimidazole hydrobromide (18) (1.66 g, 6.26 mmol), triethylamine (11 mL, 0.57% w/v in EtOH), formaldehyde (510 mg, 37% w/v in water), glacial acetic acid (1.77 g), and EtOH (54 mL) were stirred together at room temperature for 24 h. The crude product (2.26 g, 93%) was filtered off and crystallized from EtOH–DMF–concentrated HCl mixture to yield the salt 19: mp 173–175 °C; IR (KBr) 1660 (CO), 1530 and 1350 (NO<sub>2</sub>) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.36 (t, 2 H, *J* = 7 Hz, CH<sub>2</sub>CH<sub>3</sub>), 2.30 (q, 2 H, *J* = 5 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.54 (s,

3 H, CH<sub>3</sub>), 2.71 (s, 3 H, NCH<sub>3</sub>), 3.23 (t, 2 H, *J* = 5 Hz, CH<sub>3</sub>NCH<sub>2</sub>), 4.20–4.56 (m, 6 H, OCH<sub>2</sub>, indolizine-CH<sub>2</sub> and imidazole-CH<sub>2</sub>), 7.00 (t, 1 H, *J* = 8 Hz, 6-H), 7.16 (s, 1 H, 4-H of imidazole), 7.27 (t, 1 H, *J* = 8 Hz, 7-H), 7.66 (s, 1 H, 5-H of imidazole), 7.91 (d, 1 H, *J* = 8 Hz, 8-H), 9.38 (d, 1 H, *J* = 8 Hz, 5-H). Anal. (C<sub>19</sub>H<sub>27</sub>Cl<sub>2</sub>N<sub>5</sub>O<sub>4</sub>) C, H, N.

Ethyl 2-Carbamoylindolizine-1-carboxylate (23). A solution of diethyl indolizine-1,2-dicarboxylate (20) (1.0 g, 3.85 mmol) in ethanolic ammonia (30 mL) was heated at 85 °C in a sealed tube for 36 h. The solvent was removed, and the crude product was crystallized from MeOH to give 23 (0.14 g, 52%): mp 212–213 °C; IR (KBr) 3250 and 3140 (amide NH<sub>2</sub>), 1670 (ester CO), 1635 (amide CO) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.34 (t, 3 H, *J* = 8 Hz, CH<sub>3</sub>), 4.27 (q, 2 H, *J* = 8 Hz, CH<sub>2</sub>), 6.86 (dd, 1 H, *J* = 8 Hz, *J* = 8 Hz, 6-H), 7.21 (dd, 1 H, *J* = 8 Hz, 7-H), 8.04 (d, 1 H, *J* = 8 Hz, 8-H), 8.06 (s, 1 H, 3-H), 8.43 (d, 1 H, *J* = 8 Hz, 5-H), 9.01 (br s, 2 H, exchanged with D<sub>2</sub>O, NH<sub>2</sub>). Anal. (C<sub>12</sub>H<sub>12</sub>N<sub>2</sub>O<sub>5</sub>) C, H, N.

Ethyl 2-[[2-(2-Hydroxyethyl)amino]ethyl]carbamoyl]indolizine-1-carboxylate (24). A mixture of diethyl indolizine-1,2-dicarboxylate (20) (0.5 g, 1.91 mmol), [*N*-(2-aminoethyl)amino]ethanol (28) (0.57 g, 5.45 mmol) and dry EtOH (8 mL) were refluxed for 19 h. The solvent was distilled off under vacuum and the residual oil dissolved in hot water (20 mL). The cold solution yielded a solid which was filtered off and shown (TLC) to be the starting diester. The aqueous phase was extracted with ether and then continuously with EtOAc. The latter extract yielded a solid which was crystallized from an EtOAc–light petroleum mixture to afford the amide 24 (260 mg, 40%): mp 130–131 °C; IR (KBr) 3260 (amide NH), 3100 (NH), 2850 (OH), 1650 (CO) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.38 (t, 3 H, *J* = 8 Hz, CH<sub>3</sub>), 2.68–3.43 (m, 10 H, decreased to 8 H on exchange with D<sub>2</sub>O, 2 × CH<sub>2</sub>CH<sub>2</sub>, OH, and NH), 4.33 (q, 2 H, *J* = 8 Hz, CH<sub>2</sub>CH<sub>3</sub>), 6.94 (dd, 1 H, *J* = 8 Hz, 6-H), 7.25 (dd, 1 H, *J* = 8 Hz, *J* = 8 Hz, 7-H), 8.04 (d, 1 H, *J* = 8 Hz, 8-H), 8.10 (s, 1 H, 3-H), 8.49 (d, 1 H, *J* = 8 Hz, 5-H), 9.54 (t, 1 H, exchanged with D<sub>2</sub>O, CONH). Anal. (C<sub>18</sub>H<sub>21</sub>N<sub>3</sub>O<sub>4</sub>) C, H, N.

1-Cyano-2-[[2-(2-Hydroxyethyl)amino]ethyl]carbamoyl]indolizine (26). Ethyl 1-cyanoindolizine-2-carboxylate (21) (0.25 g, 1.17 mmol) and [*N*-(2-aminoethyl)amino]ethanol (28) (2.31 g, 2.95 mmol) in EtOH (8 mL) were refluxed for 12 h and then allowed to stand at 0 °C for 12 h. The deposited crystals were filtered off and washed with ether to afford the amide 26 (156 mg, 49%): mp 140–141 °C; IR (KBr) 3050 (amide NH), 3100 (NH), 2900 (OH), 2200 (CN), 1640 (CN), 1640 (CO) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 2.65–3.41 (m, 10 H, changes to 8 H after addition of D<sub>2</sub>O, 2 × CH<sub>2</sub>CH<sub>2</sub>, OH, and NH), 6.91 (dd, 1 H, *J* = 8 Hz, *J* = 9 Hz, 6-H), 7.19 (dd, 1 H, *J* = 8, 8 Hz, 7-H), 7.62 (d, 1 H, *J* = 8 Hz, 8-H), 8.09 (s, 1 H, 3-H), 8.35 (t, 1 H, exchanged with D<sub>2</sub>O, CONH) and 8.51 (d, 1 H, *J* = 8 Hz, 5-H). Anal. (C<sub>14</sub>H<sub>16</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

Ethyl 2-[[2-(2-Hydroxyethyl)-*N*-(2-hydroxy-3-(2-nitroimidazol-1-yl)propyl]amino]ethyl]carbamoyl]indolizine-1-carboxylate (29). The amino alcohol 24 (0.5 g, 1.56 mmol) and the epoxide 1 (0.27 g, 1.56 mmol) in MeOH (10 mL) were refluxed together for 56 h. The solvent was removed under reduced pressure, and the oil was triturated with EtOAc (10 mL) and kept at 0 °C for 12 h. The solid was filtered off and washed with cold EtOAc to afford the imidazole derivative 29 (0.28 g, 37%): mp 77–78 °C; IR (KBr) 3450 (amide NH) 3100 (NH), 2900 (OH), 1670 (CO), 1640 (CO) cm<sup>-1</sup>; NMR  $\delta$  (Me<sub>2</sub>SO-*d*<sub>6</sub>) 1.31 (t, 3 H, *J* = 8 Hz, CH<sub>3</sub>), 2.43 to 2.81 (m, 8 H, (CH<sub>2</sub>CH<sub>2</sub>)<sub>2</sub>N), 3.77 to 4.72 (m, 9 H, integrates to 7 H after D<sub>2</sub>O exchange, CH<sub>2</sub>CH(OH)CH<sub>2</sub>, CH<sub>2</sub>CH<sub>3</sub>, and CH<sub>2</sub>OH), 6.87 (dd, 1 H, *J* = 8 Hz, 6-H), 7.20 (dd, 1 H, *J* = 8, 8 Hz, 7-H), 7.07 (s, 1 H, imidazole 5-H), 7.46 (s, 1 H, imidazole 4-H), 7.97 (d, 1 H, *J* = 8 Hz, 8-H), 8.07 (s, 1 H, 3-H), 8.45 (d, 1 H, *J* = 8 Hz, 5-H) and 9.61 (br s, 1 H, exchanged with D<sub>2</sub>O, CONH). Anal. (C<sub>22</sub>H<sub>28</sub>N<sub>6</sub>O<sub>7</sub>) C, H, N.

1-Cyano-2-[[2-(2-Hydroxyethyl)-*N*-(2-hydroxy-3-(2-nitroimidazol-1-yl)propyl]amino]ethyl]carbamoyl]indolizine (30). The amino alcohol 26 (0.2 g, 0.735 mmol), the epoxide 1 (0.12 g, 0.735 mmol), and MeOH (5 mL) were refluxed together for 36 h. The solvent was removed in vacuo, and the residual oil was triturated with EtOAc (ca. 5 mL) and kept at 0 °C for 12 h. The crystals were filtered off and washed with cold EtOAc to afford the pale yellow 30 (140 mg, 43%): mp 173–174 °C; IR (KBr) 3290 (amide NH), 3080 (NH), 2900 (OH), 2290 (CH), 1630 (CO),

1560 and 1360 ( $\text{NO}_2$ )  $\text{cm}^{-1}$ ; NMR  $\delta$  ( $\text{Me}_2\text{SO}-d_6$ ) 2.49 to 3.39 (m, 8 H,  $(\text{CH}_2\text{CH}_2)_2\text{N}$ ), 4.33 to 4.96 (m, 7 H changes to 5 H on addition of  $\text{D}_2\text{O}$ ,  $\text{CH}_2\text{CH}(\text{OH})\text{CH}_2$  and OH), 6.90 (dd, 1 H,  $J = 8$ , 8 Hz, 6-H), 7.19 (dd, 1 H,  $J = 8$ , 8 Hz, 7-H), 7.07 (s, 1 H, 5-H), 7.49 (s, 1 H, 4-H), 7.57 (d, 1 H,  $J = 8$  Hz, 8-H), 8.00 (s, 1 H, 3-H), 8.24 (br s, 1 H, exchanged with  $\text{D}_2\text{O}$ , CONH) and 8.43 (d, 1 H,  $J = 8$  Hz, 5-H). Anal. ( $\text{C}_{20}\text{H}_{23}\text{N}_7\text{O}_5$ ) C, H, N.

### Biology

Drugs were initially dissolved at  $(1-2) \times 10^{-2}$  M in dimethyl sulfoxide (DMSO), and small volumes were added to cell suspensions to give the appropriate drug concentration. The final concentration of DMSO was 1% or less. DMSO and other reagents were BDH AnalaR grade. Special gases (air + 5%  $\text{CO}_2$ , nitrogen + 5%  $\text{CO}_2$ ) were obtained from British Oxygen Co.

V79 379A Chinese hamster cells were maintained as exponentially growing suspension cultures in Eagle's minimal essential medium with Earle's salts, modified for suspension culture, with 7.5% foetal calf serum. The method of incubating cells with drugs for measuring the production of fluorescent products has been described.<sup>8,35</sup> Flow-cytometry analysis of cells for fluorescent products

was carried out in an Ortho Systems 50 cytofluorograph using a Coherent 5W laser operated at 365 nm with an output of 100–200 mW. The total fluorescent emission from the drug metabolites was collected at  $90^\circ$  to the incident beam and separated from the scattered excitation light by a cut-off filter that passed all wavelengths above 390 nm or 410 nm (Table II). Forward-scattered light at the excitation wavelength was also collected and used to discriminate noncellular debris (low scatter).

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