Table I. Comparison of Activities of *d*-, *l*-, and *dl*-α-Di-*n*-heptylaminomethyl-6-bromo-9-phenanthrenemethanol against *P. berghei*

	ΔMST[a-c]			
	160 mg/kg	80 mg/kg	40 mg/kg	20 mg/kg
d	23.2, 25.5[d]	12.3, 12.1	9.1, 9.5	5.9, 6.1
l	4.7, 4.5	3.5	1.9, 1.7	0.7
dl	9.9, 9.9	9.5, 8.4	7.9, 8.1	5.9, 6.1

[a] ΔMST is the mean survival time (days) above that for controls for an average of five mice at the concentration (mg/kg) given. When two sets of figures are given, such as 23.2, 25.5, the tests were repeated at a different time. [b] All compounds were tested as free bases. We have established that the free base and its hydrochloride essentially have the same activity. [c] All *P. berghei* tests were conducted by the Walter Reed Army Institute of Research. [d] Three cures for five mice.

cally by Amaya.[5] The resolution from a chiral solvent as shown in this paper may be more practical and applicable to other bulky racemates or diastereoisomeric salts which tend to dissociate easily. The most interesting feature of the resolution is that the optically active salt did not crystallize in a 1:1 stoichiometry of amine to acid but rather one amine to several molecules of acid. This fact made the resolution feasible.

The *Plasmodium berghei* test in mice[6] gave the results shown in Table I. Interpretation must be qualified because all forms are active, and differences are small. The *d* form appears more active than *dl* which in turn appears slightly more active than *l*. This sequence suggests that, if the intercalation theory of activity applies,[7,8] the side-chain fixation wrought by amine–phosphate binding together with adjacent hydroxyl group hydrogen bonding to the 2-carbonyl group of a thymine residue does not wholly control the manner or protrusion of the aromatic group into the loop of the helical DNA structure, at least as far as optical antipodes are concerned.

Experimental Section

Stock Solution. The stock solution for resolution was a mixture of 200 g of *d*-tartaric acid, 200 ml of MeOH, and 90 ml of H_2O, the total volume being 396 ml. The solution did not deposit crystals at room temperature but did become cloudy occasionally with the organic base. In this case a few milliliters of a second stock solution (30 g of *d*-tartaric acid in 80 ml of MeOH) was used to clear the cloudiness.

Recovery of Free Base from Salt. Since the rotation values of the base–tartaric acid salt were variable, the salt was reconverted to the free base in the following manner whenever rotations were desired. The tartrate salt was dissolved in hot MeOH. The solution was partially cooled and strongly rotated while concentrated NH_4OH was added dropwise until strongly basic. Excess H_2O was added and the heterogeneous mixture was heated on the steam bath to remove MeOH. The free base was extracted from the cooled aqueous mixture with C_6H_6. This solution was washed with H_2O, dried, and evaporated to give the crystalline free base.

Resolution of α-Diheptylaminomethyl-6-bromo-9-phenanthrenemethanol. The *dl* base (27 g) was dissolved in 225 ml of the stock solution and held at room temperature until crystallization occurred, a time interval of several days to weeks. The crop was redissolved in more stock solution (in the proportion above) and recrystallized, and this process repeated to give ever-diminishing amounts of crop 1. Crops from the mother liquor were taken also. Rotations (αD) of crops varied from +38.2 to +10.2°. The fifth crop, 3.82 g, was recrystallized from the stock solution and monitored as the free base, the weights and αD of which are shown.

Fraction 1	0.65	+43.57°
Fraction 2	0.95	+43.8°
Fraction 3	0.45	+43.6°
Fraction 4	1.5	+38.8°

Fractions 1, 2, and 3 were combined and recrystallized from MeOH with just enough *i*-C_3H_7OH added to dissolve the base at the boiling point to give transparent plates: mp 51.5–56°; αD +43.6°; NMR spectrum identical with the *dl* base. The resolution perhaps could have been accomplished by the systematic triangular crystallization technique.[9]

The crude *l* base from the mother liquors of the above recrystallization was resolved in a manner similar to that of the *d* base except that *l*-tartaric acid was used as the resolving agent. The presumably pure *l* base was obtained as a white powder: mp 55–58°; NMR identical with that of the *dl* base; αD 41° (1 g in 10 ml of *i*-C_3H_7OH). The *dl* base was a white powder of mp 59–62°.

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References and Notes

(1) L. C. Washburn and D. E. Pearson, *J. Med. Chem.*, 17, 676 (1974) (paper 8).
(2) Contribution No. 1306 to the Army Research Program in Malaria. Senior thesis.
(3) P. H. Boyle, *Q. Rev., Chem. Soc.*, 25, 323 (1971), and references cited therein.
(4) C. Buchanan and S. H. Graham, *J. Chem. Soc.*, 500 (1950).
(5) K. Amaya, *Bull. Chem. Soc. Jpn*, 34, 1803 (1961).
(6) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, 10, 431 (1967).
(7) F. E. Hahn, R. L. O'Brien, J. Ciak, J. L. Allison, and J. G. Olenick, *Mil. Med. Suppl.*, 131, 1071 (1966).
(8) D. W. Henry, 13th National Medicinal Chemistry Symposium, Abstracts, Iowa City, Iowa, 1972, p 41.
(9) A. Weissberger, "Technique of Organic Chemistry", Vol. 3, Interscience, New York, N.Y., 1950, p 424.

Substituted 1-[(5-Nitrofurfurylidene)amino]-4-imidazolin-2-ones

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A series of 1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-ones has been prepared. A new synthesis of 4-alkyl-1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-ones involving the oxidative ring closure of 5-nitro-2-furaldehyde 2-(2-hydroxyethylalkyl)semicarbazones is described. The in vitro testing of the compounds against a variety of bacteria is reported.

The discovery that 1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-one (1)[1] is a degradative product of nifuradene[2] and that it possesses antibacterial properties prompted the synthesis of a series of substituted derivatives of 1.

Chemistry. The *N*-hydroxymethyl compound 2 was pre-

Table I. Substituted 1-[(5-Nitrofurfurylidene)amino]-4-imidazolin-2-ones

Compd no.	R	R_1	Yield, %	Mp, °C	Formula[a]	Minimal inhibitory concentration, μg/ml[b]								
						Mi-12[c]	Co-11	SH-378	Es-2	Es-L	StD-25	SaD-13	Ps-44	Pr-91	Ae-6
1[d]	H	H				0.75	12.5	1.5	0.19	0.75	3.1	0.19	>50	12.5	3.1
2	CH_2OH	H	70	205 dec	$C_9H_8N_4O_5$	1.5	3.1	0.38	0.19	0.38	3.1	0.38	>50	25	3.1
3	CH_3	H	30	238–240	$C_9H_8N_4O_4$	6.25	1.5	0.75	0.38	3.1	50	0.75	>100	>50	25
4	H	C_6H_4-*p*-NO_2	85	285–287 dec	$C_{14}H_9N_5O_6$	6.25	50	>50	>50	>50	>50	>50	>50	>50	>50
5	H	C_6H_4-*p*-Cl	60	265–267 dec	$C_{14}H_9ClN_4O_4$	25	>50	>50	>50	>50	>50	>50	>50	>50	>50
7	H	CH_3	77	245 dec	$C_9H_8N_4O_4$	1.5	12.5	1.5	0.19	1.5	3.1	0.38	>50	>50	12.5
9	H	C_2H_5	76	221–223	$C_{10}H_{10}N_4O_4$	1.5	6.25	3.1	1.5	6.25	3.1	1.5	>50	>50	50
11	H	*n*-C_4H_9	79	178–179	$C_{12}H_{14}N_4O_4$	3.1	12.5	25	3.1	>50	12.5	25	>50	>50	>50
Nifuradene[e]						3.1	12.5	1.5	0.38	0.75	6.25	3.5	>50	>50	12.5

[a] The analytical results of these compounds were within ±0.4% of the theoretical values and were obtained for C, H, and N. [b] Minimal inhibitory concentration is the lowest concentration of a compound that prevents visible growth after 24 hr of incubation at 37°. [c] The Norwich Pharmacal Co. strain number: Mi-12 = *Staphylococcus aureus*, Co-11 = *Corynebacterium liquefaciens*, SH-378 = *Shigella flexneri*, Es-2 and Es-L = *Escherichia coli*, StD-25 = *Streptococcus faecalis*, SaD-13 = *Salmonella typhosa*, Ps-44 = *Pseudomonas aeruginosa*. Pr-91 = *Proteus mirabilis*, Ae-6 = *Aerobacter aerogenes*. [d] See ref 1. [e] Nifuradene for comparison.

pared by treating **1** with aqueous HCHO. The *N*-methyl derivative **3** was prepared by a sequence of reactions previously reported for the preparation of **1**[1] but substituting the use of *N*-methylaminoacetaldehyde diethylacetal for aminoacetaldehyde diethylacetal. The 4-aryl compounds **4** and **5** were prepared by the acid hydrolysis of 1-acetamido-4-(4-nitrophenyl)-4-imidazolin-2-one[3] and 1-acetamido-4-(4-chlorophenyl)-4-imidazolin-2-one,[3] respectively, in the presence of 5-nitro-2-furaldehyde.

Since the reaction sequence used to prepare **1** and **3** gave relatively poor yields, a new synthesis for the preparation of 4-alkyl derivatives of **1** was desired. To this end, a method for the oxidation of 5-nitro-2-furaldehyde 2-(2-hydroxyethylalkyl)semicarbazones (**6**, **8**, and **10**) to the corresponding ketones was sought in the expectation that subsequent ring closure to the imidazolones would occur.

6, R = CH_3
8, R = C_2H_5
10, R = *n*-C_4H_9

7, R = CH_3
9, R = C_2H_5
11, R = *n*-C_4H_9

After several unsuccessful attempts, the desired oxidation was accomplished by the method of Albright and Goldman[4] using a DMSO–Ac_2O mixture. Indeed, when **6**, **8**, and **10** were treated with this oxidation mixture, the corresponding imidazolones **7**, **9**, and **11** were isolated in good yields. The physical constants for the imidazolones are given in Table I.

Screening Results. The 1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-ones were screened in vitro against a variety of bacteria according to procedures previously described.[5] It can be seen from Table I that as a class the alkyl compounds possess activity against gram-positive and gram-negative organisms with but one exception. All of the compounds are inactive against a strain of *Pseudomonas aeruginosa* at the drug levels tested. The 4-aryl compounds appear to have minimal activity at the levels tested.

A new synthetic route has been devised to prepare a series of 4-alkyl-1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-ones. These compounds, which are homologs of a degradative product of nifuradene,[2] possess antimicrobial activity.

Experimental Section

The melting points were taken in an open capillary tube on a Mel-Temp melting point apparatus and are corrected. The physical constants of all the final products are listed in Table I.

1-Hydroxymethyl-3-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-one (2). Compound 1 (89.0 g, 0.4 mol) was added to boiling 5% aqueous HCHO (3500 ml). The mixture was boiled for 15 min, cooled, and filtered. An analytical sample was prepared by recrystallization from $MeNO_2$.

1-Methyl-3-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-one (3). This compound was prepared by the method previously described[1] without isolating the intermediate as follows. To a solution of acetone semicarbazone (115 g, 1.0 mol) in 2-ethoxyethanol (600 ml) was added *N*-methylaminoacetaldehyde diethylacetal (147 g, 1.0 mol). The reaction mixture was heated at reflux for 6 hr. The solution was concentrated under reduced pressure and diluted

with Et_2O until a precipitate formed. The solid (unreacted acetone semicarbazone) was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was added to 10% H_2SO_4 (500 ml). A solution of 5-nitro-2-furaldehyde (113 g, 0.8 mol) in absolute EtOH (500 ml) was added and the mixture was heated on the steam bath for 1 hr. The solution was chilled and filtered to yield 51 g. The product was recrystallized from $MeNO_2$ (charcoal).

1-[(5-Nitrofurfurylidene)amino]-4-(4-nitrophenyl)-4-imidazolin-2-one (4). To an aqueous MeOH solution of 5-nitro-2-furaldehyde (28.2 g, 0.20 mol) was added 1-acetamido-4-(4-nitrophenyl)-4-imidazolin-2-one[3] (52.4 g, 0.20 mol). The mixture was acidified with concentrated HCl and heated on the steam bath for 2 hr. After chilling, the product was collected by filtration and dried to yield 58.0 g. The material was recrystallized from aqueous DMF (charcoal).

In a similar manner, **5** was prepared from 1-acetamido-4-(4-chlorophenyl)-4-imidazolin-2-one[3] and recrystallized from aqueous DMF.

4-Methyl-1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-one (7). To a solution of 5-nitro-2-furaldehyde 2-(hydroxypropyl)semicarbazone (**6**,[6] 37.0 g, 0.14 mol) in anhydrous DMSO (435 ml) was added Ac_2O (290 ml). The mixture was stirred at 50° for 18 hr and poured into a large volume of ice and water. The brick-red precipitate was collected, washed with H_2O, and dried to yield 26 g. The product was purified by recrystallization from $MeNO_2$ (charcoal).

In a similar manner, **9** was prepared from **8** and recrystallized from $MeNO_2$. Material recrystallized from $MeNO_2$ formed a complex which was decomposed by drying at 70° for 2 hr. Compound **8** was prepared by the method of Gever[7] and recrystallized from aqueous EtOH (charcoal): mp 184.5–186°. Anal. ($C_{10}H_{14}N_4O_5$) C, H, N. Compound **11** was synthesized in the above manner from **10**[7] and recrystallized from $MeNO_2$.

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References and Notes

(1) D. L. Pugh, J. Olivard, H. R. Snyder, Jr., and J. P. Heotis, *J. Med. Chem.*, **15**, 270 (1972).
(2) U.S. Adopted Name (USAN) of 1-[(5-nitrofurfurylidene)-amino]-2-imidazolidinone.
(3) H. Beyer and A. Hetzheim, *Chem. Ber.*, **97**, 1031 (1964).
(4) J. D. Albright and L. Goldman, *J. Am. Chem. Soc.*, **87**, 4214 (1965).
(5) R. Freedman and R. E. Chamberlain, *Antimicrob. Agents Chemother.*, 502 (1967).
(6) G. Gever, C. O'Keefe, G. Drake, F. Ebetino, J. Michels, and K. Hayes, *J. Am. Chem. Soc.*, **77**, 2277 (1955).
(7) G. Gever, *J. Am. Chem. Soc.*, **76**, 1283 (1954).

Synthesis of a Fluorescent Derivative of Amethopterin[1a]

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Fluorescein isothiocyanate was treated with excess diaminopentane and the remaining unsubstituted amino group of the product was condensed, via a carbodiimide-promoted reaction, with a carboxyl group of amethopterin. The final product, a fluorescent derivative of amethopterin, was isolated by chromatography on AE-cellulose and preparative electrophoresis on polyacrylamide. It was shown to be homogeneous by analytical polyacrylamide electrophoresis and thin-layer chromatography. Proof of structure was provided by elemental analysis, absorbance spectra (at pH 7.0, λ_{max} at 495 nm; fluorescence emission at 520 nm), and ^1H NMR measurements. The fluorescent derivative of amethopterin inhibited transport of amethopterin into *Lactobacillus casei* and L1210 cells. It was also a good inhibitor of the *L. casei* and L1210 dihydrofolate reductases and could be used to provide a fluorescent label for the enzymes during polyacrylamide electrophoresis.

Amethopterin (Methotrexate, MTX) is a potent inhibitor of dihydrofolate reductases from a variety of sources.[2] Although the affinity of the enzymes for the inhibitor is quite high, the interaction is noncovalent and pH-dependent.[3] These properties have been exploited in numerous studies in which radioactive MTX has been used to detect and quantitate the enzyme. We now wish to report the synthesis of a fluorescein derivative of MTX (MTX-F) which can be used as a *visual* label for dihydrofolate reductases and for MTX-transport systems.

Derivatization of MTX was accomplished via one of the terminal carboxyl groups, since this region of the molecule is minimally involved in binding to dihydrofolate reductases. This is shown by the fact that various esters and amides of MTX are only slightly less inhibitory than the parent compound[4,5] and by the successful use of MTX linked through its carboxyl groups to soluble starch[6] or Sepharose[7–9] in the purification of these enzymes by affinity chromatography. Preparation of MTX-F was carried out in two steps. Initially, fluorescein isothiocyanate was treated with excess of 1,5-diaminopentane in dimethyl sulfoxide and the resulting thiourea was purified by chromatography on DEAE-cellulose. Homogeneity was confirmed by thin-layer chromatography (TLC) on cellulose in two solvent systems, and the presence of the remaining amino group was demonstrated by reactivity with ninhydrin. The infrared spectrum indicated a loss of the characteristic isothiocyanate peak at 2100 cm^{-1}. In the second step, MTX was allowed to react with 1 equiv of the preceding product in the presence of carbodiimide. The reaction mixture was chromatographed on AE-cellulose and elution was achieved with a linear gradient of ammonium bicarbonate. The major component was purified further by preparative polyacrylamide electrophoresis and rechromatography on AE-cellulose. Acid precipitation afforded the desired product (MTX-F) as a yellow, amorphous solid. The purity and authenticity of MTX-F was confirmed by the absorbance spectrum, TLC, elemental analysis, and the ^1H NMR spectrum (which indicated a 1:1 superimposition of the aminoalkylfluorescein aromatic proton resonances on the aromatic side-chain resonances of MTX in the region 7–8 ppm).

From the method of preparation and analytical data, it is concluded that the diaminopentane spacer group is linked covalently to either the α- or γ-carboxyl group of MTX. The natural occurrence of γ-linked folate polyglutamates (reviewed by Wagner and Folkers[10]) and the large size of