Eur J Med Chem (1990) 25, 309–319

Original article

Pyridinium azolate betaines and their derivatives: a new class of antiprotozoal agents

E Alcalde[1]*, I Dinarés[1], J Elguero[2], J Frigola[3], A Osuna[4], S Castanys[4]

[1]*Lab Química Orgánica, Facultad de Farmacia, Universidad de Barcelona, 08028-Barcelona;*
[2]*Instituto de Química Médica, CSIC, 28006-Madrid;*
[3]*Lab Dr Esteve SA, 08026-Barcelona;*
[4]*Depto Parasitología, Facultad de Farmacia, Universidad de Granada, 18001-Granada, Spain*

(Received 10 April 1989; accepted 14 September 1989)

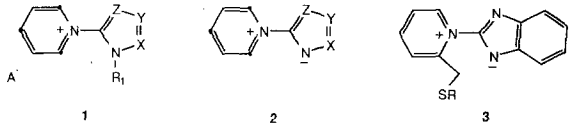
Summary — *N*-azolylpyridinium salts **1** and several betaines of pyridinium azolate **2** have been synthetized in order to study their *in vitro* antiprotozoal activity. A careful ^{1}H and ^{13}C-NMR study has been carried out in order to characterize the different compounds. A significant growth inhibition of *Trypanosoma cruzi* and especially *Leishmania donovani* was observed with the *N*-benzimidazolylpyridinium derivatives. When tested *in vivo* against *Leishmania donovani*, compounds **9** and **40** have had a much greater reducing effect in the parasite than that of the reference drug (glucantime).

Résumé — Bétaïnes d'azolates de pyridinium et leurs dérivés: une nouvelle classe d'antiprotozoaires. *Un nombre élevé de sels de* N*-azolylpyridinium* ***1*** *et plusieurs bétaïnes d'azolate de pyridinium* ***2*** *ont été synthétisés et leur activité antiprotozoaire évaluée. Une étude RMN (^{1}H et ^{13}C) a été effectuée afin de caractériser les différents composés. Les dérivés de* N*-benzimidazolylpyridinium montrent un effet inhibiteur de la croissance des* Trypanosoma cruzi *et, surtout, des* Leishmania donovani. *Dans les essais* in vivo *vis-à-vis de* Leishmania donovani, *les composés* ***9*** *et* ***40*** *se montrent très supérieurs au produit de référence (glucantime).*

***N*-azolylpyridinium salts / pyridinium azolate mesomeric betaines / anti-leishmanial activity**

Introduction

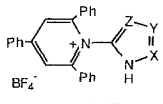
Both the potential pharmaceutical value of pyridinium azolate inner salts and the considerable interest from a theoretical point of view have prompted us to investigate them [2]. As part of an ongoing research project for the discovery of new antiparasitic agents, we describe here the synthesis and antiprotozoal activity of several new *N*-azolylpyridinium salts **1** and the mesomeric heterocyclic betaines of pyridinium azolate class **2**. Antiparasitic agents have very different structures but none are related to that of **1** and **2**, whose structures are, indeed, without precedent in the literature concerning antiparasitic drugs [3, 4]. Only a few mesomeric betaines of pyridinium azolate class **2** and their salts **1** are known [5]. Recently, some substituted pyridinium benzimidazolate betaines **3** and their salts have been described, in relation to the mechanism of action of the gastric acid inhibitor *Omeprazole* and several close analogues [6–9].



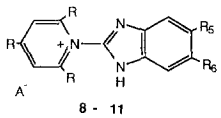
In a previous paper [5] we have reported the synthesis and structural studies of the *N*-azolylpyridinium tetrafluoroborates **4–7**, *N*-benzimidazolylpyridinium salts **8–11** and the pyridinium azolate inner salts **12–17**. Preliminary antiparasitic screening has shown that some of the above-mentioned *N*-azolylpyridinium derivatives demonstrate activity against *Trypanosoma cruzi* and *Leishmania donovani*, especially compounds **4** and **9** (and its betaine **15**).

*Correspondence and reprints

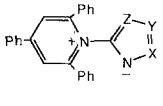
Compounds **7–14** were evaluated against 5 parasites, either *in vitro* (*Naegleria fowleri, Acanthamoeba* sp) or *in vivo* (*Hymenolepis nana, Syphacia* sp, *Giardia muris*) using different compounds as standards. None of these compounds have shown significant activity deserving further studies.



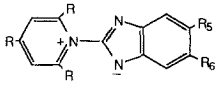
4 - 7



8 - 11



12 - 13



14 - 17

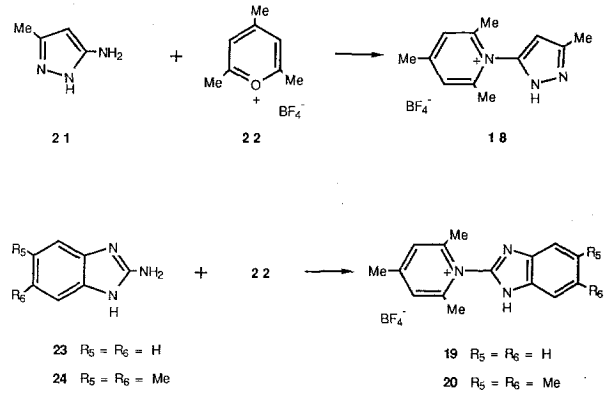
4	X = N; Y = CMe; Z = CH
5, 12	X = Z = N; Y = CH
6, 13	X = Y = Z = N
7	X = Z = N; Y = CNH_2
8, 14	R = Ph; R_5 = R_6 = H
9, 15	R = Ph; R_5 = R_6 = Me
10, 16	R = R_5 = R_6 = H
11, 17	R = H; R_5 = R_6 = Me

Due to the early finding that *N*-azolylpyridinium derivatives **4, 9** and **15** possessed activity, we decided to investigate this new class of potential antiprotozoal agents. Several structural analogues, more or less distant from the most active compounds have been synthetized and evaluated for anti-trypanosomal and antileishmanial activity.

Molecular modifications have been carried out both in the pyridinium and benzimidazole moieties in order to establish a possible structure–activity relationship. Thus, the 2,4,6-triphenylpyrimidium group has been replaced by 2,4,6-trimethylpyridinium, *N-p*-phenylentriphenylpyridinium and *N-p*-phenylentrimethylpyridinium groups. On the other hand, the benzimidazole nucleus has been conveniently substituted in 1-, 5- or/and 6-position.

Chemistry

N-Azolyl-2,4,6-trimethylpyridinium salts **18–20** were obtained by reaction of 3(5)-amino-5(3)-methylpyrazole **21** and 2-aminobenzimidazoles **23, 24** with 2,4,6-trimethylpyrylium tetrafluoroborate **22** (scheme 1). Isolation of the benzimidazole derivatives **19** and **20** was quite difficult because the solubilities of the starting materials and the final pyridinium salts were quite similar. Failure of the reaction between other *C*-aminoazoles (1,2,4-triazole, tetrazole) and the pyrylium salt **22** was likely due to the weakly nucleophilic character of the amino group of the latter compounds toward the less reactive trimethylpyrylium salt **22**. Transformation of the *N*-azolyl-2,4,6-trimethylpyridinium salts **18–20** into their corresponding betaines of 2,4,6-trimethylpyridinium azolate was unsuccessful. Several attempts have been made using different procedures but coloured oily mixtures were formed which prevented isolation of solid material [10].



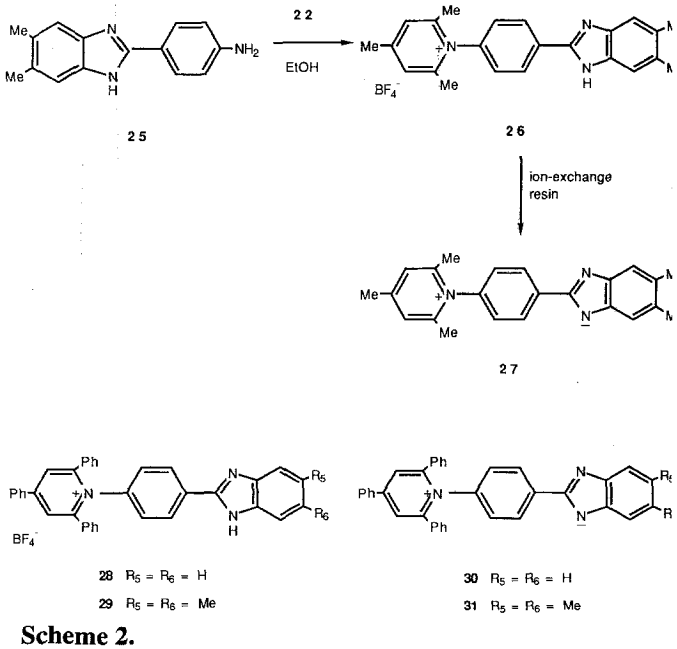
21 **22** **18**

23 R_5 = R_6 = H
24 R_5 = R_6 = Me

19 R_5 = R_6 = H
20 R_5 = R_6 = Me

Scheme 1.

Reaction of 2-(4-aminophenyl)-5,6-dimethylbenzimidazole **25** with the pyrylium salt **22** gave the desired compound **26**, and the inner salt of 2,4,6-trimethylpyridinium benzimidazolate **27** was also obtained as a quite unstable coloured product, which decomposed in solution (see experimental section). Compounds **28–31** have previously been reported [5] (scheme 2).



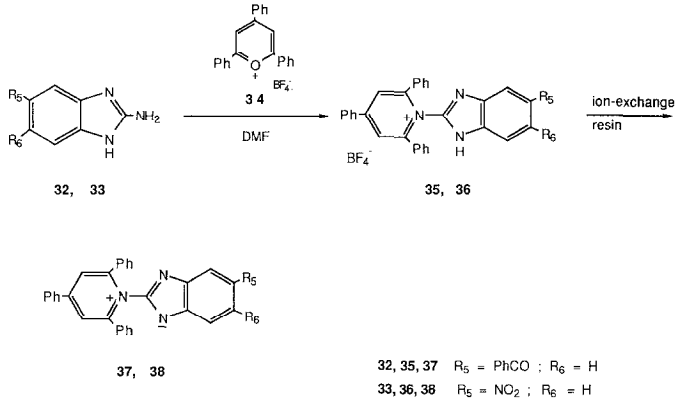
25 **26** **27**

28 R_5 = R_6 = H
29 R_5 = R_6 = Me

30 R_5 = R_6 = H
31 R_5 = R_6 = Me

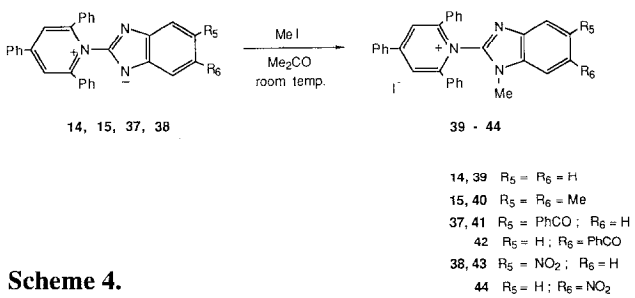
Scheme 2.

As to the benzimidazole moiety, 5-benzoylbenzimidazole and 5-nitrobenzimidazole derivatives have been selected. Thus, 2-aminobenzimidazoles **32** and **33** reacted with 2,4,6-triphenylpyrylium tetrafluoroborate **34** to give the desired compounds **35** and **36**. Then, the pyridinium benzimidazolate inner salts **37** and **38** were obtained by deprotonation of the corresponding *N*-benzimidazolylpyridinium salts **35** and **36** (scheme 3).



Scheme 3.

Due the highly dipolar structure of the mesomeric betaines of the pyridinium azolate class **2** [2, 5], it would be expected that electrophilic attack at a nitrogen atom of the azolate ring should take place under neutral and mild conditions. Indeed, the betaines of pyridinium benzimidazolate **14, 15, 37** and **38** reacted with methyl iodide/acetone at room temperature to afford the *N*-(1-methylbenzimidazol-1-2-yl)-2,4,6-triphenylpyridinium iodides **39–44** (yield > 79%). In the alkylation of benzimidazoles by alkylhalides under neutral conditions, the yields are restricted to around 50 % [11]. When the 5-substituted benzimidazolepyridinium salts **37** and **38** were methylated, a mixture of the two isomeric *N*-methyl derivatives **41** + **42** and **43** + **44** was obtained (scheme 4). In spite of the difficulty of separation of these isomers even by column chromatography and fractional crystallizations, it has been possible to isolate compounds **41** and **43** in a pure state. The



Scheme 4.

physical data of the new compounds described in this work are listed in table I and details of synthesis are given in the Experimental section.

The structures of the new products have been unambiguously characterized on the basis of their spectroscopic data and all of them gave satisfactory elemental analyses.

Both ^{1}H NMR and ^{13}C NMR chemical shifts have been assigned by comparison with data from benzimidazoles [12, 13] and the useful comparative data from *N*-azolylpyridinium salts **4–11** and betaines of pyridinium azolate **12–17** previously reported [5]. If necessary, individual assignments have been made using the appropriate NMR techniques [10]. The ^{1}H NMR data and selected ^{13}C NMR chemical shifts are set out in tables II and III.

Results and Discussion

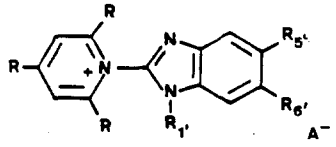
The *in vitro* activity against *Trypanosoma cruzi* has been studied by measuring the growth inhibition *Trypanosoma cruzi* epimastigote forms, nifurtimox being the reference drug [3, 4, 15] (for nifurtimox: *po* LD_{50} in mice, > 1600 mg/kg [16]). The *N*-azolylpyridinium salts and pyridinium azolate betaines **4–15, 18–20, 26, 28–30, 35–41** and **43** have been evaluated, and at 100 μg/ml compounds **4, 9, 15, 35** and **40** showed themselves to be much more active than nifurtimox. Nevertheless, at 1 and 10 μg/ml none of these compounds have shown an activity comparable to that of nifurtimox, with the exception of the *N*-pyrazolyl-2,4,6-triphenylpyridinium salt **4** after 72 h (see table IV).

The fact that all the compounds at the concentrations tested were well tolerated by HeLa cells over a 72 h exposure period is noteworthy, as this precludes the possibility of their being unselective cytotoxins. Moreover, the chemical stability of the compounds in aqueous solution at 50°C has been checked out, given the long incubation times needed (48–72 h) to show effects on parasites by several of the compounds, and all of them were stable in solution.

Almost all the compounds tested have exhibited an interesting profile of anti-leishmanial activity, as they significantly reduce the growth inhibition of *Leishmania donovani* promastigote forms using glucantime, the most widely used drug against leishmaniasis, as reference drug [15]. Results in table V show that the anti-leishmanial activity is maximum for compounds **9, 15, 29** and **40** which have common structural features. Thus, the azole moiety is a 5,6-dimethylbenzimidazole and the pyridinium ring is 2,4,6-trisubstituted by phenyl groups.

The anti-leishmanial activity of compounds **9** and **40** with a LD_{50} in mice > 6400 mg/kg was further

Table I. Physical data of *N*-benzimidazolylpyridinium salts and pyridinium benzimidazolate inner salts.



Compd	*R*	$R_{1'}$	$R_{5'}$	$R_{6'}$	X^-	*mp, °C* [a]	*Recryst solvent* [b]	*Yield* [e] *(%)*	*Method* [d]	*Reaction time (h)*	*Mol formula* [e]
19	Me	H	H	H	BF_4^-	134–135[f]	A	37	A	1	—
20	Me	H	Me	Me	BF_4^-	219–221	B	31	A	2	$C_{17}H_{20}N_3BF_4/2H_2O$
35	Ph	H	PhCO	H	BF_4^-	229–230	C	62	B	1.5	$C_{37}H_{26}N_3OBF_4/1.5H_2O$
36	Ph	H	NO_2	H	BF_4^-	178–179	C	32	B	1.5	$C_{30}H_{21}N_4O_2BF_4$
37	Ph	–	PhCO	H	–	262–263	D	90	C	[g]	$C_{37}H_{25}N_3O/0.5H_2O$
38	Ph	–	NO_2	H	–	180–182	E	96	C	[g]	$C_{30}H_{20}N_4O_2/H_2O$
39	Ph	Me	H	H	I^-	279–280	F	90	D	13	$C_{31}H_{24}N_3I/0.25H_2O$
40	Ph	Me	Me	Me	I^-	292	G	90	D	14	$C_{33}H_{28}N_3I/0.33H_2O$
41+42	Ph	Me	{PhCO; H}		I^-	–	G	96	D	14	—
41	Ph	Me	PhCO	H	I^-	243–245	H	42	D	14	$C_{38}H_{28}N_3OI$
43+44	Ph	Me	{NO_2; H}		I^-	–	I	99	E	14	—
43	Ph	Me	NO_2	H	I^-	279–280	I	20	E	14	$C_{31}H_{23}N_4O_2I$

[a]Melting points are uncorrected. [b]A = methylene chloride-benzene-tetrafluoroboric acid, B = methylene chloride–hexane–tetrafluoroboric acid, C = isopropanol–tetrafluoroboric acid, D = 70% ethanol, E = 50% ethanol, F = ethyl acetate, G = water, H = benzene, I = tetrahydrofuran. [c]Yields not optimized. [d]See Chemistry section. [e]Elemental analysis for C, H and N were within 0.4% of theoretical values. [f]Literature [14] mp 134°C. [g]See Experimental section.

studied by means of an *in vivo* assay. The percentages of leishmanial amastigote reduction were for compound **9** 54.7% and 77.2% for 1,5,6-trimethylbenzimidazolyltriphenylpyridinium salt **40**. In a similar experiment condition, the reduction rate for glucantime was only 47% (see table VI and Experimental section).

The results show that both the azole and the pyridinium moiety are important for the antiprotozoal activity. Concerning the pyridinium residue, the presence of phenyl groups at positions 2, 4 and 6 exerts moderate to high activity, when R =CH_3, slight activity, and when R = H, a total lack of the antiprotozoal activity. Concerning the azole, the most interesting compounds are the benzimidazole derivatives, and among them, the 1,5,6-trimethyl substituted one, **40**, is the most potent. Compounds **35, 37** and the *N*-methyl derivatives **41** and **42** are analogues of mebendazole and they exhibited a significant activity. Thus, the results seem to indicate that the presence of a methyl group at the 1 position of the benzimidazole ring is associated with the anti-leishmanial activity. The pyridinium azolate betaines and their corresponding *N*-azolyl-pyridinium salts have shown almost the same degree of activity, due to the existence of an acid-base equilibrium for these compounds [17].

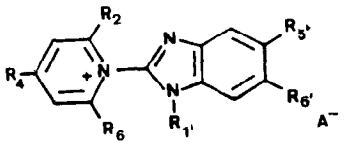
Given the available data, it can be pointed out that lipophilicity, electronic and steric effects may account for the observed activity of these *N*-benzimidazolylpyridinium salts and derivatives. In addition, we think that the present results would be very useful in studies of experimental infections with *Leishmania donovani*, as some of these compounds are highly toxic for this parasite. They produce the total destruction of the cultures after short treatment times. This effect is in contrast with the activity of the glucantime, which only induces growth inhibition and where in some cases glucantime-resistant flagellates appear.

Experimental protocols

Chemistry

Melting points were determined on a CTP-MP 300 hot-plate apparatus and are uncorrected. IR spectra were recorded as KBr disks on a Perkin–Elmer 1430 spectrophotometer. ^1H

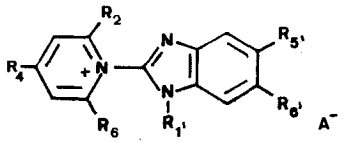
Table II. ^{1}H NMR chemical shifts (δ, ppm) of *N*-benzimidazolylpyridinium salts and pyridinium benzimidazolate inner salts[a].



Compd	*R-2,4,6*	*R-1'*	*R-5'*	*R-6'*	*A*$^-$	*R-2,6*		*R-4*	*R-3,5*	*R-1'*	*H-4'*	*H-7'*	*R-5'*	*R-6'*
19	Me	H	H	H	BF_4^-	2.38		2.63	7.87	(b)	7.66	7.66	7.25	7.25
20	Me	H	Me	Me	BF_4^-	2.45		2.61	7.85	9.14	7.57	7.57	2.38	2.38
						Ph-2,6		Ph-4						
							Ho	Hm,p						
35	Ph	H	PhCO	H	BF_4^-	7.33–7.77	8.45	7.33–7.77	8.81	(b)	7.90	7.33–7.77	7.33–7.77	7.33–7.77
36	Ph	H	NO_2	H	BF_4^-	7.20–7.94	8.28–8.58	7.20–7.94	8.79	(b)	8.28–8.58	7.52–7.94		8.0
37	Ph	–	PhCO	H		7.25–7.60	8.30–8.35		8.55		8.30–8.35		7.70	7.25–7.60
38	Ph	–	NO_2	H		7.15–7.76	8.30	7.15–7.76	8.55		8.12	7.15–7.76		7.75
39	Ph	Me	H	H	I$^-$	7.36–7.74	8.46	7.36–7.74	8.89	3.46	7.60	7.34	7.27*	7.23*
40	Ph	Me	Me	Me	I$^-$	7.36–7.77	8.45	7.36–7.77	8.86	3.38	7.33	7.18	2.23*	2.25*
41	Ph	Me	PhCO	H	I$^-$	7.37–7.75	8.48	7.37–7.75	8.92	3.55	7.92	7.37–7.75	7.37–7.75	
42	Ph	Me	H	PhCO	I$^-$	7.37–7.75	8.48	7.37–7.75	8.92	3.54	7.92	7.37–7.75		7.37–7.75
43	Ph	Me	NO_2	H	I$^-$	7.24–7.62	8.48	7.24–7.62	8.95	3.60	8.54	7.42		8.22
44	Ph	Me	H	NO_2	I$^-$	7.28–7.82	8.47–8.53	7.28–7.82	8.94	3.63	7.86	8.47–8.53	8.15	

*Values can be interchanged. [a]DMSO–d_6 as solvent. [b]Not observed.

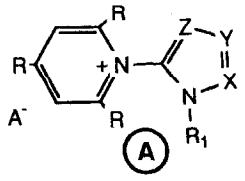
Table III. ^{13}C NMR chemical shifts (δ, ppm) of *N*-benzimidazolylpyridinium salts and pyridinium benzimidazolate inner salts [a,b].

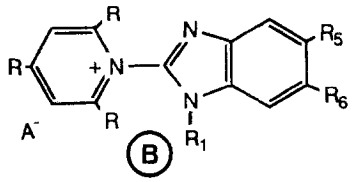


Compd	*R-2,4,6*	*R-1'*	*R-5'*	*R-6'*	*A⁻*	*C-2,6*	*C-3,5*	*C-4*	*R-1'*	*C-2'*	*C-3'a*	*C-4'*	*C-5'*	*C-6'*	*C-7'*	*C-7'a*	*R-5'*	*R-6'*
19	Me	H	H	H	BF_4^-	156.2[c]	127.9	163.7[d]		141.7	137.5	117.3	125.1	125.1	117.3	137.5		
20	Me	H	Me	Me	BF_4^-	155.8[e]	127.3	162.8[f]		140.4	135.7	116.5	133.3	133.3	116.5	135.7	19.9	19.9
35	Ph	H	PhCO	H	BF_4^-	156.9	125.3	158.4		143.6	136.1	119.1	131.4	125.4	116.1	139.1	194.8[g]	
36	Ph	H	NO_2	H	BF_4^-	156.8	125.2	158.4		146.4	137.0	113.7	143.5	118.8	116.6	140.9		
37	Ph	–	PhCO	H	–	156.2	125.1	155.8		155.3	144.2	120.8	127.4	121.1	116.7	149.2	195.9[g]	
38	Ph	–	NO_2	H	–	156.1	125.1	156.0		150.4	140.0	113.6	143.1	114.4	116.7	143.6		
39	Ph	Me	H	H	I^-	156.5	125.7	158.6	30.5	141.3	138.9	119.9	123.6	124.7	111.2	133.5		
40	Ph	Me	Me	Me	I^-	156.6	125.7	158.5	30.3	140.4	137.6	119.7	132.5	134.0	110.0	131.5	20.0*	19.7*
41	Ph	Me	PhCO	H	I^-	156.4	125.8	158.4	31.0	143.2	138.4	122.2	132.9	126.2	111.7	136.3	194.9[g]	
42	Ph	Me	H	PhCO	I^-	156.4	125.8	158.8	30.9	143.8	141.7	119.9	125.3	133.1	113.6	136.6		192.5[g]
43	Ph	Me	NO_2	H	I^-	156.4	125.8	159.0	31.4	144.6	137.9	116.3	144.2	120.0	112.6	137.4		
44	Ph	Me	H	NO_2	I^-	156.4	125.8	159.0	31.4	145.5	142.8	120.9	118.9	144.3	108.7	133.2		

[a]DMSO–d_6 as solvent. [b]δ C of the phenyl rings carbons in 2,4,6-triphenylpyridinium derivatives were found in the range of 128.2 to 134.4 ppm. [c]δ Me-2,6 = 20.4 ppm. [d]δ Me-4 = 22.5 ppm. [e]δ Me-2,6 = 20.0 ppm. [f]δ Me-4 = 22.0 ppm. [g]δ C of the phenyl ring carbons were found in the range of 128.6 to 137.5 ppm.

Table IV. Selected growth inhibition percentages of *Trypanosoma cruzi* epimastigote forms [a,b].





μg/ml							24 h			48 h			72 h		
Compound	*Structure*	*A⁻*	*R*	*R-1'*	*X*	*Y*	*100*	*10*	*1*	*100*	*10*	*1*	*100*	*10*	*1*
4	A	BF_4^-	Ph	H	CMe	CH	16.1	4.8	1.4	92.7	20.1	1.9	100	85.1	3.1
					R-5'	R-6'									
9[c]	B	BF_4^-	Ph	H	Me	Me	32.6	4.5	1.4	91.5	9.1	2.0	100	9.2	2.8
15	B	–	Ph	–	Me	Me	34.5	4.8	1.4	94.6	9.2	2.0	100	9.2	2.8
35	B	I^-	Ph	Me	PhCO	H	25.8	4.5	1.3	100	8.7	1.5	100	20.1	2.7
40[c]	B	I^-	Ph	Me	Me	Me	22.1	4.2	1.4	100	8.5	1.5	100	19.1	2.7
nifurtimox[d]							33.8	20.0	11.0	45.4	34.4	29.6	85	80.4	50.9

[a]Average number from 5 different experiments. The SEM > 10%. [b]See Experimental section. [c]*po* LD_{50} in mice, > 6400 mg/kg. [d]*po* LD_{50} in mice, > 1600 mg/kg.

NMR spectra were obtained either with a Bruker AM-100 or Perkin–Elmer R-24B spectrometer operating at 100 and 60 MHz respectively, ^{13}C NMR spectra were run on a Bruker AM-100 Fourier transform spectrometer operating at 25.1 MHz. NMR spectra were determined in dimethylsulfoxide-d_6, and chemical shifts are expressed in parts per million (δ) relative to TMS as internal standard or the central peak of dimethylsulfoxide-d_6. MS spectra was recorded on a Hewlett–Packard 5988A spectrometer. TLC was performed on SiO_2 (silica gel 60 F_{250}, Merck), in the following solvent systems: A, methanol-diethyl ether (8:2); B, diethyl ether-methanol (9.5:0.5); C, chloroform–methanol (8.5:1.5) as developing solvent, and the spots were located with UV light. Ion-exchange chromatography was carried out on an anionic (OH-form) ion-exchange resin (Amberlite IRA-401) [5]. If necessary the compounds were dried by heating overnight at 110°C in a vacuum oven. Where microanalyses are indicated by symbols of the elements, the analytical results were within ± 0.4 % of the theoretical values; they were performed on a Carlo Erba 1106 analyzer by the Instituto de Química Bio-orgánica, Barcelona.

The 2-aminobenzimidazoles **23, 24** and 4-nitro-1,2-phenylenediamine are commercially available. 3(5)-Amino-5(3)-methylpyrazole **21** [18], 2,4,6-trimethylpyrylium tetrafluoroborate **22** [19], 2-(4-aminophenyl)-5,6-dimethylbenzimidazole **25** [5], 2-amino-5-benzoylbenzimidazole **32** [20], 2,4,6-triphenylpyrylium tetrafluoroborate **34** [21], *N*-azolyl-pyridinium salts **4–11, 28,** and **29** [5], and pyridinium azolate inner salts **12–17** and **30, 31** [5] were prepared as in the literature.

2-Amino-5-nitrobenzimidazole 33

A solution of cyanogen bromide (10 g, 94.3 mmol) in 80 % aqueous dioxane (50 ml) was added to a suspension of 4-nitro-1,2-phenylenediamine (14.26 g, 93.3 mmol) in 80% aqueous dioxane (285 ml) and the mixture was stirred at room temperature for 28 h, and then evaporated to dryness. The residue was treated with concentrated NH_4OH (50 ml) and the crude product was filtered, washed with water, and dried, to provide 16.45 g (99% yield) of 33: mp 211–212°C (lit [22] mp 222–223°C, 72 % yield).

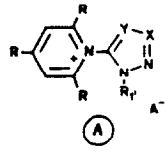
Procedures for N-*azolylpyridinium salts and pyridinium benzimidazolate inner salts (table I)*

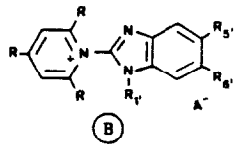
Method A. A solution of the aminoazole **21, 23** or **24** (6 mmol) in 25 ml of ethanol and the pyrylium salt **22** (0.63 g, 3 mmol) were refluxed under stirring for the time specified in table I. The reaction mixture was decolourized (charcoal), the solvent was evaporated to dryness, and the residue of compound **18** was recrystallized twice (see table I). To the crude product **19** or **20** water was added, filtered to remove the insoluble materials, and concentrated to dryness. Subsequent treatment of the residue with dichloromethane (3 x 15 ml), was followed by filtration to remove insoluble material, and the filtrate was evaporated. The residue was recrystallized twice with dichloromethane-hexane and a few drops of tetrafluoroboric acid to give the *N*-benzimidazolyl-2,4,6-trimethylpyridinium tetrafluoroborates **19** and **20**.

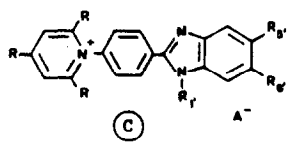
Compound **18**: ^1H NMR (DMSO-d_6) 2.40 (s, 3H), 2.42 (s, 6H), 2.62 (s, 3H), 6.42 (s, 1H) and 7.90 (s, 2H); ^{13}C NMR (DMSO-d_6) 160.5 (C-4), 155.9 (C-2, C-6), 145.5 (C-3'), 142.5 (C-5'), 127.2 (C-3), 100.8 (C-4'), 20.7 [Me-(C-2', C-6')], 21.5 [Me-(C-4')].

Method B. A solution of 2-aminobenzimidazole **32** or **33** (16.1 mmol) in 7 ml of anhydrous DMF and the pyrylium salt **34** (5.31 g, 14.41 mmol) were refluxed under stirring for the time specified in table I. After cooling, diethyl ether

Table V. Growth inhibition percentages of *Leishmania donovani* promastigote form[a,b].







							24 h			48 h			72 h		
μg/ml							*100*	*10*	*1*	*100*	*10*	*1*	*100*	*10*	*1*
Compound	*Structure*	*A⁻*	*R*	*R-1'*	*X*	*Y*									
4	A	BF_4^-	Ph	H	CMe	CH	10.7	5.9	1.5	22.6	9.9	4.9	83.6	32.5	20.2
5	A	BF_4^-	Ph	H	CH	N	10.7	5.9	0.7	11.5	9.9	4.8	38.8	21.0	8.1
6	A	BF_4^-	Ph	H	N	N	14.9	6.9	1.1	15.1	7.8	4.9	23.4	12.7	10.9
7	A	BF_4^-	Ph	H	CNH_2	N	10.7	5.8	1.3	10.8	8.6	4.8	25.5	12.7	8.1
12	A	–	Ph	–	CH	N	14.9	5.9	0.9	10.8	8.1	5.1	39.8	21.7	10.6
13	A	–	Ph	–	N	N	14.0	5.8	1.4	15.1	8.6	4.9	23.4	12.7	7.1
18	A	–	Me	H	CMe	CH	9.0	5.9	0.7	21.9	6.0	2.3	100	35.1	5.5
					R-5'	R-6'									
8	B	BF_4^-	Ph	H	H	H	16.0	6.0	1.0	15.0	12.0	5.0	82.9	30.0	17.0
9[c]	B	BF_4^-	Ph	H	Me	Me	100	11.0	1.0	100	37.0	5.0	100	53.0	8.0
10	B	Cl^-	H	H	H	H	12.6	4.1	0.5	20.7	11.7	5.1	50.0	22.6	11.0
11	B	Cl^-	H	H	Me	Me	10.1	4.1	0.5	20.7	11.9	5.1	39.3	21.6	10.8
14	B	–	Ph	–	H	H	16.0	6.0	1.0	15.0	12.0	5.0	82.0	30.0	17.0
15	B	–	Ph	–	Me	Me	90.0	6.0	1.0	100	18.0	3.0	100	28.0	5.0
19	B	BF_4^-	Me	H	H	H	18.1	5.0	0.7	18.6	6.0	2.3	38.6	15.1	8.0
20	B	BF_4^-	Me	H	Me	Me	35.6	6.1	0.6	56.1	20.1	3.6	82.1	35.6	11.5
26	C	BF_4^-	Me	H	Me	Me	53.4	20.8	0	64.3	26.8	0	68.6	41.8	0
28	C	BF_4^-	Ph	H	H	H	87.5	55.1	35.6	93.2	57.3	37.3	96.3	69.2	59.1
29	C	BF_4^-	Ph	H	Me	Me	73.2	32.9	28.6	96.6	39.3	30.2	97.5	68.6	42.5
30	C	–	Ph	–	H	H	68.6	38.2	26.5	42.4	16.3	25.3	95.6	40.3	23.5
35	B	BF_4^-	Ph	H	PhCO	H	45.0	7.0	1.0	70.0	10.0	3.0	100	14.0	5.0
36	B	BF_4^-	Ph	H	NO_2	H	40.0	20.0	0	89.5	32.0	15.0	90.4	40.0	20.0
37	B	–	Ph	–	PhCO	H	20.0	7.0	1.0	47.0	22.0	3.0	83.0	31.0	5.0
38	B	–	Ph	–	NO_2	H	45.5	8.9	2.0	49.5	15.5	6.0	100	65.0	33.0
39	B	I^-	Ph	Me	H	H	40.0	11.0	1.0	69.0	20.0	2.0	100	53.0	13.0
40[c]	B	I^-	Ph	Me	Me	Me	100	10.0	1.0	100	10.0	5.0	100	100	16.0
41	B	I^-	Ph	Me	PhCO	H	91.0	10.0	2.0	100	13.0	5.0	100	35.0	10.0
41+42	B	I^-	Ph	Me	PhCO·H		90.0	10.0	2.0	100	13.0	5.0	100	50.0	12.0
43	B	I^-	Ph	Me	NO_2	H	81.5	20.8	10.0	100	53.5	28.0	100	68.0	36.0
43+44	B	I^-	Ph	Me	NO_2·H		83.5	23.0	8.0	100	35.0	20.0	100	60.0	28.0
Glucantime							25.0	7.0	2.0	20.0	13.0	7.0	39.5	20.4	10.5

[a]Average number from 5 different experiments, SME > 10%. [b]See Experimental section. [c]*po* LD_{50} in mice, > 6400 mg/kg.

Table VI. Mean number of leishmanial amastigotes per spleen weight (g)[a].

Expt	*Amastigotes/spleen* (10^6)	*Spleen weight* (g)	*Amastigotes/g spleen* (10^6)	*Reduction* [a] (%)
Control	12.81	0.6114	21.15	—
9	4.50	0.4701	9.57	54.73
40	2.75	0.5842	4.82	77.23
Glucantime	6.46	0.5765	11.20	47.04

[a]See Experimental section.

(3 x 15 ml) was added to give an oily product **35** and the solution was decanted from the oily residue which was then triturated with water. The yellow to orange triturate was filtered, washed with water, and recrystallized (see table I).

In a similar manner, the oily residue of **36** was treated with methanol (20 ml) and the insoluble materials removed by filtration. The filtrate was evaporated to dryness, and the residue triturated with water, filtered, and washed with water. The crude product was treated with chloroform and filtered to remove insoluble material (2-aminobenzimidazole **33**). The chloroform solution was evaporated to dryness and then recrystallized from 2-propanol/tetrafluoroboric acid (table I).

An analytical sample of compound **36** was obtained by passing a solution of the above mentioned recrystallized salt in 70% ethanol through a column with anion-exchange resin (IRA-401. OH^- form; see later). The neutral eluates were concentrated to dryness and the solid residues recrystallized in 2-propanol/tetrafluoroboric acid (table I).

Method C. Preparation of pyridinium benzimidazolate inner salts 27, 37 and 38. A column packed with anion-exchange Amberlite resin IRA-401 was used and the chloride-form was converted to the hydroxide-form [4]. A solution of *N*-benzimidazolylpyridinium salt **26, 35** or **36** (1 mmol) in 70 % ethanol (50 ml) was passed through the column. The neutral eluates were concentrated in a rotary evaporator at 40°C to give coloured solids, which were recrystallized (table I).

Method D. A solution of methyl iodide (1.83 g, 12.88 mmol) in 10 ml of dry acetone was added dropwise at 0–5°C to a suspension of the pyridinium benzimidazolate inner salts **14, 15** and **37** (3.22 mmol) in 50 ml of dry acetone under an atmosphere of nitrogen, and stirring was continued at room temperature for the time specified in table I. The progress of the reaction was monitored by TLC (chloroform-methanol, 8.5, 1.5) and by ^1H NMR of aliquots.

The resulting solution was evaporated to dryness, and the residue washed successively with ethyl acetate and water to give compounds **39** and **40**. A similar procedure, but washing the residue with benzene, gave a mixture of the *N*-methyl isomers **41** and **42**. 0.58 g of this mixture (**41** + **42**) was treated with 50 ml of boiling benzene, the solid was collected by filtration, washed with benzene, and dried to afford compound **41** in a pure state. The percentage of isomers obtained was determined by running the ^1H NMR of the reaction mixture in DMSO–d_6: **41** + **42** (54 %–46 %).

Method E. A solution of methyl iodide (2.37 g, 16.69 mmol) in dry acetone (10 ml) was added dropwise at 0–5°C to a stirred solution of the mesomeric betaine **38** (2.22 g, 4.74 mmol) in 100 ml of dry acetone under an atmosphere of nitrogen. When the addition was complete the mixture was heated under reflux for 14 h and allowed to cool to room temperature. The solvent was removed gently in the rotary evaporator and the residue triturated with diethyl ether to give a mixture of **43** + **44** (60 % –40 %), TLC (THF-diethyl ether 7:3). The mixture was isolated and purified by repeated recrystallized from THF giving 0.55 g of pure **43** (table I). The work-up procedure required extreme care because the *N*-methyl derivatives **43** and especially **44** were unstable in solution at temperatures above 60°C; therefore, attempted isolation of compound **44** from the mother liquours in a sufficiently pure form was precluded. Moreover, compound **44** was transformed under the purification conditions into 1-methyl-6-nitro-benzimidazol-2-one, mp 267–270°C; IR (KBr): 1660 cm^{-1}; MS: *m/z* 193 (M^+, 8), 192 (M-1, 100).

1-[4-(5,6-Dimethyl-1H-benzimidazol-2-yl)phenyl]-2,4,6-trimethylpyridinium tetrafluoroborate 26

A solution of **25** (2.68 g, 11.3 mmol) and the pyrylium salt **19** (1.98 g, 9.43 mmol) in absolute ethanol (150 ml) was stirred under reflux for 24 h. The ethanol was evaporated to dryness, the residue triturated with diethyl ether, and the solid residue washed with hot 2-propanol, giving compound **26**, mp 205–207°C (2.03 g, 50%); ^1H NMR 8.54 (m, 2H), 7.85 (s, 2H), 7.74 (m, 2H), 7.42 (s, 2H), 2.59 (s, 3H), 2.32 (s, 12H); ^{13}C NMR 159.2 (C-4), 154.6 (C-2), 126.6* (C-3), 127.2* and 128.3* (C-2', C-3'), 148.7 (C-2"), 138.6 (C-3a", C-7a"), 115.4 (C-4", C-7"), 131.3 (C-5", C-6"), 20.1 [Me-(C-5", C-6")], 21.4 [Me-(C-4)] and 21.6 [Me-(C-2, C-6)].

5,6-Dimethyl-2-[4-(2,4,6-trimethyl-1-pyridinio)phenyl]-benzimidazolate 27

A solution of the *N*-benzimidazolylpyridinium salt **26** (0.19 g, 0.44 mmol) in ethanol (30 ml) was passed through a column packed with anion-exchange resin IRA-401 (OH^- form). The neutral eluates were concentrated in a rotary evaporator at room temperature affording the betaine **27** as an unstable brown solid: mp > 252°C (0.145 g, 96%); ^1H NMR (DMSO–d_6) 8.47 (m, 2H), 7.91 (s, 2H), 7.56 (m, 2H), 7.26 (s, 2H), 2.59 (s, 3H), 2.28 (s, 12H).

Biological evaluation

Parasites

The strain of *Trypanosoma cruzi* used in this study was obtained from the Institute of Malariology in Maracay (Venezuela) and had been isolated from human in a clinical case.

The leishmania strains used were *Leishmania donovani* (LRC-L133).

Epymastigote forms of *Trypanosoma cruzi* were cultured in liquid *Trypanosoma medium* (LTM) composed of Hank's solution (Gibco, Grand Island, NY, USA) 900 ml, lacto-albumin hydrolysate (Difco, Detroit, MI, USA) 5 g yeast extract (Difco) 0.1 g and bovine haemoglobin 0.2 mg. Medium prepared this way was supplemented with 10% of fetal calf serum (FCS) [23] inactivated at 56°C for 30 min. The pH was adjusted to 7.2.

Promastigote forms of *Leishmania donovani* were cultured in TC 199 Medium (Gibco) supplemented with 10% of Fetal bovine serum. The parasites were cultured at 28°C.

Compounds

Substances were initially dissolved in dimethylsulfoxide and further diluted with cell culture medium before use. The concentration of the solvent (DMSO) was about 0.2%, which is non toxic for the parasites.

Compounds **9** and **40** were entrapped in liposomes, as interesting results have been reported with liposome-encapsulated drugs in experimental leishmaniasis [24–26]. Multillamellar liposomes were prepared as described by Heath *et al* [24] from the method of Bangham *et al* [26].

Glucantime (100 mg/ml) and compounds **9, 40** (100 mg/ml) were entrapped in liposomes, and the amount of product entrapped was 46% for compound **9** and 55% for compound **40**. Liposomes were shown to be multilamellar by negative staining techniques [26] and liposome preparations were stored at 4°C. The amount of unentrapped material was removed by gel filtration on a Sephadex G-50 column and determined spectrophotometrically at λ = 243 nm. All doses of liposomes were freshly prepared and used within 4 h of preparation.

In vitro *antiprotozoal activity*

The *in vitro* anti-trypanosomal and anti-leishmanial activities of the test compounds have been evaluated. Once the flagellate forms were obtained in the culture medium, they were pelleted at 450 g/10 min. The flagellate forms were suspended in the culture medium and aliquots adjusted to 10^7 flagellates/ml were taken and placed in culture flasks containing the compounds to be tested at the concentrations of 100, 10 and 1 μg/ml. An equivalent quantity of the solvent (DMSO) was added to the control parasite culture. The antiprotozoal activity was determined 24, 48 and 72 h after addition of the different products. Nifurtimox and glucantime which are at present used in medical treatment [3, 4] were used as reference drugs on *Trypanosoma cruzi* and *Leishmania donovani* respectively.

The total number of flagellates were counted in a Coulter counter (DN, Coulter Electronics LTD, Harpenden, Harts, England). In order to establish parasite viability, flagellate suspensions were observed under a phase contrast microscopet which determine the percentage of forms with normal motility and appearance. The percentage of growth inhibition was determined as previously described [27].

Culture of HeLa cells

HeLa cells (FLOW strains) were cultured in MEM medium supplemented with 10% of inactivated FCS containing different concentrations of compounds (100, 10 and 1 μg/ml). After a 72 h incubation period at 37°C in a 5% CO_2 incubator, cell growth was measured by a colorimetric method [28] using a Kontron STL 210 Spectrophotometer.

In vivo *anti-leishmanial activity*

Groups of 6 Nestle rats (98–100 g) were infected intraperitoneally with 5 x 10^7 infective promastigote metacyclics of *Leishmania donovani* (LRC-L133) taken from the stationary-phase *in vitro* cultures of the parasite (TC 199, 10% FCS). Thirteen days after infection, compounds **9** and **40** were administered intravenously at a single dose of 34 mg/kg given once a week for three consecutive weeks. Five d after the last dose, the animals were killed and the total number of intracellular spleen amastigotes determined by counts of impression smears after staining with Giensa. The percentage reduction was calculated: % R = 100-(Tx100/C), where T is the number of amastigotes in 500 nucleated cells from treated rats and C is the number of amastigotes in 500 ml nucleated cells from control rats. Additionally in the same standard protocol, a group of 6 rats were treated with glucantime as the reference drug.

Acknowledgments

This work was supported by the Fondo de Investigaciones Sanitarias (projects No 86/1082, 87/1570, 88/0832). We gratefully acknowledge the Ministerio de Educacion y Ciencia for the postgraduate scholarship to one of us (ID). Part of this work was awarded by the Sociedad Española de Química Terapéutica.

References

1 Part of this work has been presented at the 10th Int Symp on Medicinal Chemistry (Budapest, August 1988)
2 Alcade E, Dinarés I, Fayet JP, Vertut MC, Elguero J (1986) *J Chem Soc Chem Comm* 734–735
3 Ross WJ (1979) *In: Burger's Medicinal Chemistry* (Wolff ME, ed) John Wiley & Sons, NY, vol 2, 439–479
4 James DM, Guilles HM (1985) *In: Human Antiparasitic Drugs, Pharmacology and Usage*. John Wiley & Sons, Chichester
5 Alcalde E, Dinarés I, Elguero J, Fayet JP, Vertut MC, Miravitlles C, Molins E (1987) *J Org Chem* 52, 5009–5115 and references therein
6 Ankner KF, Brandström AE, Lindberg PL, Nordberg P, Wallmark B (1986) E Patent 181.846
7 Lindberg P, Nordberg P, Alminger T, Brandström A, Wallmark B (1986) *J Med Chem* 29, 1327–1329
8 Sturm E, Krüger V, Senn-Bilfinger J, Figala V, Klemm K, Kohl B, Rainer G, Schaefer H, Blake TJ, Darkin DW, Ife RJ, Leach CA, Mitchell RC, Pepper ES, Salter CJ, Viney NJ, Huttner G, Zsolnai L (1987) *J Org Chem* 52, 4573–4581
9 Sturm E, Krüger V, Senn-Bilfinger J, Figala V, Klemm K, Kohl B, Rainer G, Schaefer H, Blake TJ, Darkin DW, Ife RJ, Leach CA, Mitchell RC, Pepper ES, Salter CJ, Viney NJ, Huttner G, Zsolnai L (1987) *J Org Chem* 52, 4582–4592
10 Dinarés I (1988) PhD Thesis, Barcelona
11 Preston PN (1981) *Chem Heterocycl Comp* 40 (part I) 86
12 Pappalardo L, Elguero J, Fruchier A (1975) *Anal Quim* 71, 598–602

13 Fruchier A, Pappalardo L, Elguero J (1980) *Anal Quim* 76, 79–84 and references therein
14 Dinculescu A, Balaban AT (1980) *Rev Roum Chim* 23, 1505–1528
15 Sharma S (1988) *Drugs of Today* 25, 249–261
16 Bock M, Haberkorn A, Herlinger H, Meyer KH, Petersen S (1972) *Arzneim-Forsch* 22, 1564–1569
17 Gonzalez E, Alcalde E, Dinarés I, Elguero J (1987) *Bull Soc Chim Fr* 604–606
18 Alcalde E, de Mendoza J, Garcia-Marquina JM, Almera C, Elguero J (1974) *J Heterocycl Chem* 11, 423–429
19 Balaban AT, Boulton AJ (1981) *Org Synth* coll V, 1112–1113
20 Ram S, Skinner M, Kalvin D, Wise DS, Townsend LB, McCall JW, Worth D, Ortwine D, Werbel LM (1984) *J Med Chem* 27, 914–917
21 Dimroth K, Reichardt C, Vogel K (1969) *Org Synth* 49, 121–124
22 Perg SS, Parnce EW (1961) *J Chem Soc* 5275–5284
23 Ruiz-Pérez LM, Osuna A, Castanys S, Gamarro F, Craciunescu D (1986) *Arzneim-Forsch* 36, 13–16
24 Heath S, Chance ML, New RRC (1984) *Mol Biochem Parasitol* 12, 49–60
25 Chapman WL, Hanson WL, Alwing CR, Hendricks LD (1984) *Am J Vet Res* 45, 1028–1030 and references therein
26 Bangham AD, Horne RW (1964) *J Mol Biol* 8, 660–668
27 Osuna A, Castanys S, Mascaro MC, Adroher FJ, Braña MF, Roldan CM (1983) *Rev Inst Med Trop Sau Paulo* 25, 254–260
28 Finlay GJ, Baguley BC, Wilson WR (1984) *Anal Biochem* 139, 272–275