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Short communication

Synthesis of pyrrolo[3,4-*c*]pyridine derivatives possessing an acid group and their *in vitro* and *in vivo* evaluation as aldose reductase inhibitors

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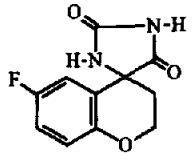
Summary — Derivatives of [pyrrolo[3,4-*c*]pyridin-1,3(2*H*)-dion-2-yl] alkanoic acids were prepared and their *in vitro* aldose reductase inhibitory activity was tested on rat lens enzyme. The acetic derivatives **2**, **5** and **15a–d** proved to be much more potent inhibitors than the propionic derivatives, **7** and **16a–d**, and the iso-propionic derivatives, **3** and **6**. The presence of a second planar aromatic area in the benzoyl derivatives **15a–d** did not result in any increase in activity. Two of the most active compounds *in vitro* (**2** and **5**) were also evaluated *in vivo* as inhibitors of glutathione lens depletion in galactosemic rats. None of the compounds was found to be active in maintaining the rat lens glutathione level, suggesting possible problems of ocular bioavailability and metabolism. The aldose reductase inhibitory activity of compounds **2** and **15d** was also discussed by taking into account their conformational and electronic characteristics evaluated by means of theoretical calculations.

pyrrolo[3,4-*c*]pyridine / aldose reductase inhibitor / glutathione lens depletion inhibitor / structure–activity relationship

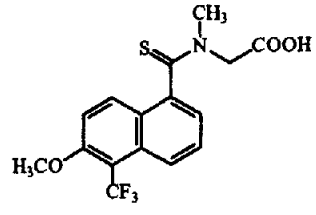
Introduction

The first enzyme in the polyol pathway is aldose reductase (AR), which catalyzes the reduction of glucose to sorbitol, which can be converted in turn by polyol dehydrogenase to fructose. Under physiological conditions, flux through this pathway is probably low in most tissues, but it has not been measured *in vivo*. Certainly, the levels of sorbitol measured in most tissues are negligible, and the affinity of AR for glucose is much lower than that of hexokinase. It is therefore likely that most intracellular glucose is phosphorylated, and that the rate of sorbitol formation is low and matched by its clearance. When this situation is altered, as in diabetes mellitus, sorbitol is produced in excess and accumulated in various human tissues, including peripheral nerves, lens, kidney, and retina, osmotically inducing morphological and functional alterations, such as cataracts. These changes can be ameliorated through inhibition of AR, since aldose reductase inhibitors (ARIs) can provide a direct therapeutic treatment for diabetic complications (and they do not pose the risk of hypoglycemia), which is independent of the control of blood sugar levels [1–3].

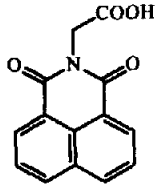
These observations have spurred great interest in the development of ARIs, which can be divided into two general groups, those containing rigid spirohydantoins or related ring systems, such as Sorbinil [4], and those containing a carboxylic acid moiety, like Alrestatin [5], Tolrestat [6] and Zopolrestat [7]. In these molecules, the presence of an acidic proton, in a suitable spatial relationship with a planar aromatic



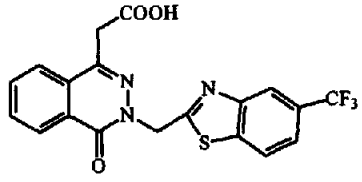
Sorbinil



Tolrestat



Alrestatin

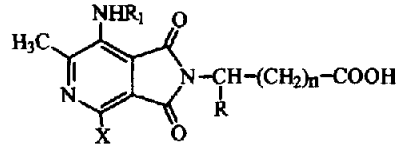


Zopolrestat

group, appears to be essential for the inhibition of the enzyme [8]. Kador *et al* [9–11] have shown that the minimum pharmacophore structural requirements for activity consist of a planar structure with hydrophobic (aromatic) regions and a common region which is susceptible to charge-transfer interactions.

Several spirohydantoins, including the most important member of this class, Sorbinil, show an excellent activity in both *in vitro* and *in vivo* models of diabetic complications. However, due to a certain incidence of hypersensitivity side effects with Sorbinil in clinical trials, the present search for new ARIs is mainly directed towards the family of carboxylic acids. Nevertheless, relatively few carboxylic acids show any *in vivo* activity, which underlines the difficulty of obtaining a good conversion of *in vitro* activity to *in vivo* activity, particularly among compounds of this class. All this seems to be due to the higher polarity of carboxylic acids, which are almost completely ionized at physiological pH, and could thus encounter greater difficulty than the hydantoin derivatives in crossing a biological membrane.

Taking into account the structural features of Alrestatin [5] and new potentially active compounds in which the acetic group is positioned at an imide nitrogen [12], we decided to synthesize, and test for their AR inhibitory activity, several substituted pyridine derivatives with a general formula A, which contain a planar aromatic region and a carboxy group.



A

The pyridine derivatives **2** and **5** (scheme 1) were synthesized to verify whether the presence of a halogen on the aromatic portion could influence the activity through a variation of the lipophilicity of the molecule. With the aim of examining the importance of the acetic group on the AR inibitory activity, the isopropionic derivatives **3** and **6** were also prepared. Moreover, the *n*-propionic derivative **7** was synthesized, since it is reported in the literature that products in which the acetic chain has been lengthened to *n*-propionic or *n*-butyric may maintain the AR inibitory activity [13–16]. Finally, the benzoyl derivatives **15a–d** and **16a–d** of **5** and **7**, respectively, were prepared in order to introduce into the molecule a second aromatic lipophilic region, which might improve interaction with the receptor site, in accordance with the model proposed by Kador [10], and with the results obtained with several recently described inhibitors [17, 18].

The biological data concerning the AR inhibitory activity of these compounds were also evaluated by taking into account their conformational and electronic characteristics determined by means of theoretical calculations.

Chemistry

Compounds **2** and **3** were obtained by fusion of 3-amino-2-methylpyridin-4,5-dicarboxylic acid **1** [19] with glycine and α-alanine, respectively, at 250°C. Compounds **5–7** were prepared in a similar manner, starting from 3-amino-6-chloro-2-methylpyridin-4,5-dicarboxylic acid **4** [20, 21] with glycine, α-alanine and β-alanine, respectively. Compounds **5–7** were also prepared, in better yields, by hydrolysis of esters **8–10**, obtained by fusion of diacid **4** with the ethyl ester hydrochloride of glycine, α-alanine and β-alanine, respectively, at 160–180°C (scheme 1, tables I–VI). To correspond better to the pharmacophore model of Kador *et al* [10], a second appropriately functionalized lipophilic group was introduced by acylating the 7-amino group of compounds **8** and **10** in a toluene solution in the presence of triethylamine, to give the amide esters **11a–d** and **12a–d** (scheme 1, tables V and VI). As this acylation reaction was carried out using an excess of acyl chloride, the diacylated derivatives **13** and **14** were sometimes isolated by flash chromatography and characterized. The selective hydrolysis of the ester group of compounds **11a–d** and **12a–d** with boron tribromide in a dichloromethane solution [22] or with concentrated hydrochloric acid, respectively, yielded the target acids **15a–d** and **16a–d** (scheme 1, tables I–IV).

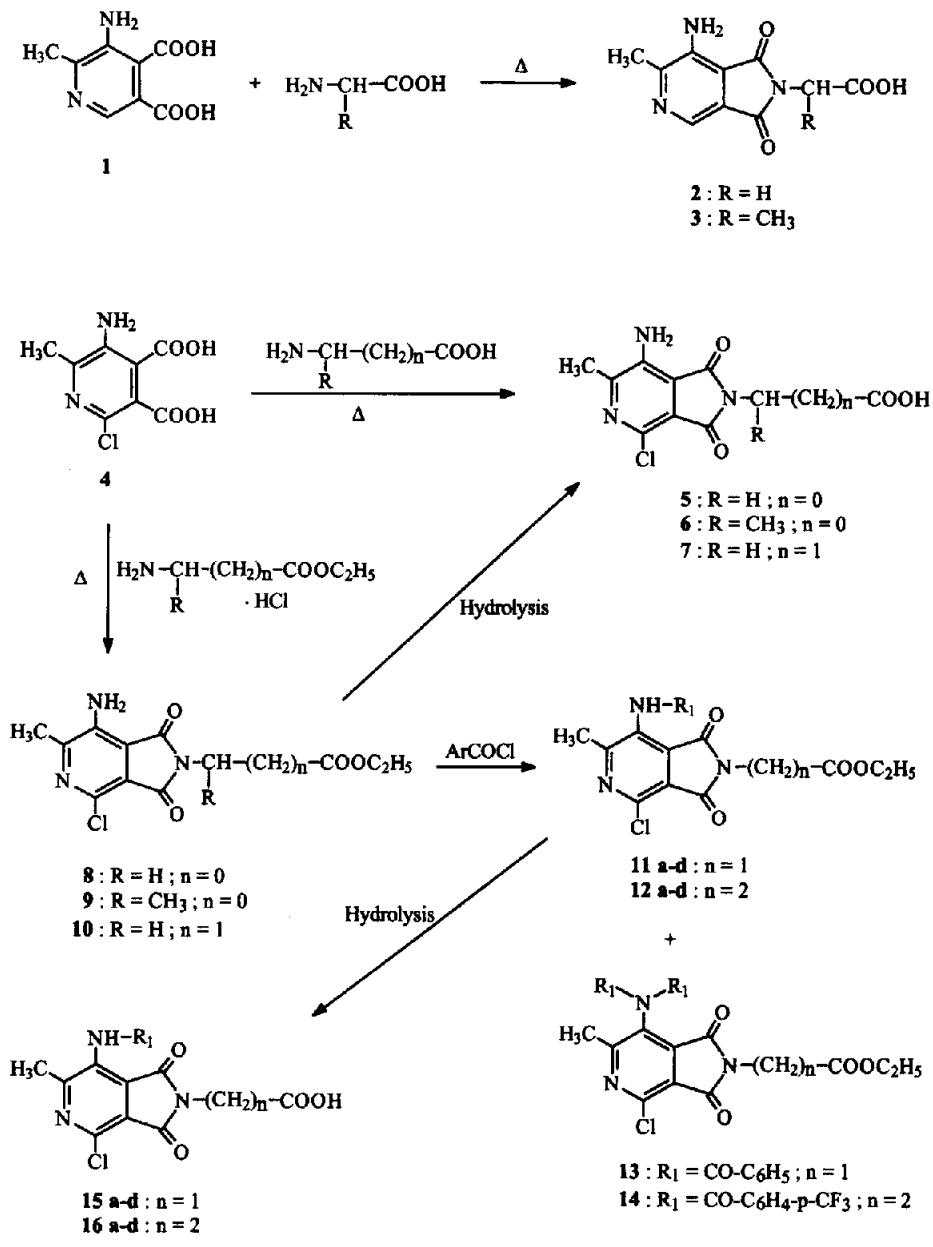
Biochemistry and pharmacology

All the acid derivatives **2**, **3**, **5–7**, **15a–d** and **16a–d** and the ester derivatives **8** and **10** were evaluated *in vitro* for their ability to inhibit the AR enzyme in the water-soluble crude extract of rat lenses [23–26]. IC_{50} values were determined by using linear regression analysis of the log concentration–response curves (tables I and III).

Two of the most active compounds, **2** and **5**, were evaluated *in vivo* as inhibitors of glutathione (GSH) lens depletion in galactosemic rats [27–29], after ocular instillation. The biochemical and pharmacological properties of the new compounds were compared with data obtained with Sorbinil, a potent *in vitro* and *in vivo* ARI [30].

Theoretical calculations

The conformational and electronic properties of substituted pyridine derivatives with a general formula

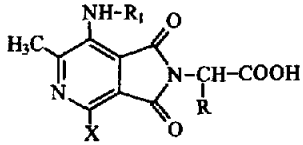


Scheme 1.

A were evaluated by means of theoretical conformational studies and molecular electrostatic potential (MEP) analysis.

The most active compounds, **2** and **15d** (table I), were considered together with Alrestatin and Zopolrestat, respectively. The preferred conformations of **2** were obtained by means of full geometry optimization, using the molecular mechanics program Discover [31]. The conformation of Alrestatin was optimized in the same way. In the case of **15d** and Zopolrestat, a conformational search was also carried out by varying the torsion angles between the two aromatic systems (pyrrolopyridine and benzoyl for **15d**, the naphthalazine and benzothiazole groups for Zopolrestat). The preferred conformation of Zopolrestat was found to correspond closely to that described by Wilson *et al* [32], who solved the structure of the ternary complex AR-NADPH-Zopolrestat by

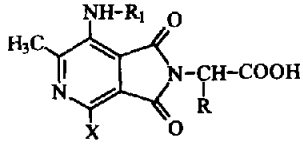
Table I. Physical properties and inhibition of RLAR by acetic acid derivatives **2**, **3**, **5**, **6** and **15a–d**.



N	*X*	*R*	*R_1*	*Reaction time (h)*	*Yield (%)*	*Recrystallization solvent*	*Mp (°C)*	*Formula*[a]	*Inhibition*[b] *% (M)*			*IC_{50}*[c] *(μM)*
									10^{-4}	*10^{-5}*	*10^{-6}*	
2	H	H	H		50	Methanol	>300	$C_{10}H_9N_3O_4$	100	83	43	1.4
5	Cl	H	H		68 (73)[d]	Ethanol	>300	$C_{10}H_8ClN_3O_4$	100	70	33	2.5
3	H	CH_3	H		35	Ethanol	269–272 (dec)	$C_{11}H_{11}N_3O_4$	17			
6	Cl	CH_3	H		34 (63)[d]	Benzene	274–276	$C_{11}H_{10}ClN_3O_4$	43	15		
15a	Cl	H	COC_6H_5	48	46	Methanol	214–216	$C_{17}H_{12}ClN_3O_5$	90	80	19	2.7
15b	Cl	H	COC_6H_4-*p*-Cl	48	30	Methanol	218–220 (dec)	$C_{17}H_{11}Cl_2N_3O_5$	90	80	10	3.7
15c	Cl	H	COC_6H_4-*p*-F	16	75	Methanol	224–228 (dec)	$C_{17}H_{11}ClFN_3O_5$	90	80	23	3.2
15d	Cl	H	COC_6H_4-*p*-CF_3	72	38	Dichloro-methane	180–182	$C_{18}H_{11}ClF_3N_3O_5$	100	90	36	1.7
Sorbinil									100	92	68	0.5

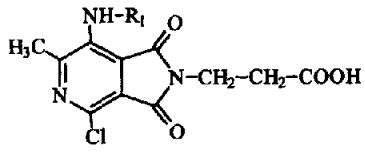
[a]Elemental analyses for C, H, N were within ±0.4% of the calculated values. [b]Percentage inhibition at the indicated inhibitor concentration. [c]IC_{50} values represent the concentration required to produce 50% enzyme inhibition. [d]Yield obtained from the ester hydrolysis reaction.

Table II. Spectral data of acetic acid derivatives **2**, **3**, **5**, **6** and **15a–d**.



N	*X*	*R*	*R_1*	*IR (nujol, cm^{-1})*	*^{1}H-NMR (DMSO-d_6, ppm)*
2	H	H	H	3450, 3350, 1740, 1690, 1610, 750	2.50 (s, 3H, 6-CH_3); 4.27 (s, 2H, CH_2CO); 6.50 (s, 2H, 7-NH_2); 8.20 (s, 1H, ArH)
5	Cl	H	H	3450, 3350, 1750, 1690, 1610, 740	2.47 (s, 3H, 6-CH_3); 4.24 (s, 2H, CH_2CO); 6.54 (s, 2H, 7-NH_2)
3	H	CH_3	H	3450, 3350, 1760, 1700, 1640, 880	1.54 (d, 3H, $CHCH_3$); 2.47 (s, 3H, 6-CH_3); 4.77 (q, 1H, $CHCH_3$); 6.37 (s, 2H, 7-NH_2); 8.06 (s, 1H, ArH)
6	Cl	CH_3	H	3450, 3350, 1740, 1700, 1640, 740	1.59 (d, 3H, $CHCH_3$); 2.50 (s, 3H, 6-CH_3); 4.83 (q, 1H, $CHCH_3$); 6.50 (s, 2H, 7-NH_2)
15a	Cl	H	COC_6H_5	3250, 1770, 1690, 1620, 700	2.61 (s, 3H, 6-CH_3); 4.26 (s, 2H, CH_2CO); 7.57–8.02 (m, 5H, ArH); 10.41 (s, 1H, 7-NH)
15b	Cl	H	COC_6H_4-*p*-Cl	3300, 1760, 1700, 1620, 740	2.60 (s, 3H, 6-CH_3); 4.26 (s, 2H, CH_2CO); 7.60 (d, 2H, ArH); 8.00 (d, 2H, ArH); 10.50 (s, 1H, 7-NH)
15c	Cl	H	COC_6H_4-*p*-F	3300, 1770, 1700, 1660, 750	2.60 (s, 3H, 6-CH_3); 4.26 (s, 2H, CH_2CO); 7.24–8,14 (m, 4H, ArH); 10.48 (s, 1H, 7-NH)
15d	Cl	H	COC_6H_4-*p*-CF_3	3300, 1770, 1720, 1660, 740	2.62 (s, 3H, 6-CH_3); 4.27 (s, 2H, CH_2CO); 7.91 (d, 2H, ArH); 8.17 (d, 2H, ArH); 10.68 (s, 1H, 7-NH)

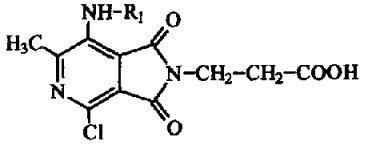
Table III. Physical properties and inhibition of RLAR by propionic acid derivatives **7** and **16a–d**.



N	*R_1*	*Hydrolysis reaction*		*Yield (%)*	*Recrystallization solvent*	*Mp (°C)*	*Formula*[a]	*Inhibition*[b] *(%) (M)*	
		Temperature (°C)	*Reaction time (min)*					*10^{-4}*	*10^{-5}*
7	H	Reflux	180	10 (89)[c]	Ethanol	266–270 (dec)	$C_{11}H_{10}ClN_3O_4$	45	15
16a	COC_6H_5	Reflux	30	83	DMF/H_2O	268–270 (dec)	$C_{18}H_{14}ClN_3O_5$	30	13
16b	COC_6H_4-*p*-Cl	Reflux	90	65	Ethanol	243–245	$C_{18}H_{13}Cl_2N_3O_5$	8	0
16c	COC_6H_4-*p*-F	Reflux	30	74	DMF/H_2O	259–261	$C_{18}H_{13}ClFN_3O_5$		0
16d	COC_6H_4-*p*-CF_3	70	5	64	$CHCl_3$/petroleum ether 40–60°C	95–96	$C_{19}H_{13}ClF_3N_3O_5$		ND[d]

[a]Elemental analyses for C, H, N were within ±0.4% of the calculated values. [b]Percentage inhibition at the indicated inhibitor concentration. [c]Yield obtained from the ester hydrolysis reaction. [d]Not determined due to its insolubility in the assay buffer.

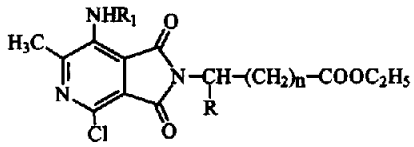
Table IV. Spectral data of propionic acid derivatives **7** and **16a–d**.



N	*R_1*	*IR (nujol, cm^{-1})*	*^{1}H-NMR (ppm)*
7	H	3450, 3350, 1760, 1700, 1640, 760	2.33 (s, 3H, 6-CH_3); 2.50 (t, 2H, CH_2CO); 3.76 (t, 2H, NCH_2); 6.40 (s, 2H, 7-NH_2)[a]
16a	COC_6H_5	3300, 1760, 1700, 1640, 740	2.58 (s, 3H, 6-CH_3); 2.58 (t, 2H, CH_2CO); 3.77 (t, 2H, NCH_2); 7.54–8.08 (m, 5H, ArH); 10.43 (s, 1H, 7-NH)[a]
16b	COC_6H_4-*p*-Cl	3300, 1760, 1700, 1640, 740	2.57 (s, 3H, 6-CH_3); 2.57 (t, 2H, CH_2CO); 3.76 (t, 2H, NCH_2); 7.63 (d, 2H, ArH); 8.04 (d, 2H, ArH); 10.52 (s, 1H, 7-NH)[a]
16c	COC_6H_4-*p*-F	3300, 1760, 1700, 1620, 740	2.57 (s, 3H, 6-CH_3); 2.57 (t, 2H, CH_2CO); 3.76 (t, 2H, NCH_2); 7.29–8.18 (m, 4H, ArH); 10.48 (s, 1H, 7-NH)[a]
16d	COC_6H_4-*p*-CF_3	3300, 1760, 1700, 1660, 740	2.68 (s, 3H, 6-CH_3); 2.75 (t, 2H, CH_2CO); 3.95 (t, 2H, NCH_2); 7.76 (d, 2H, ArH); 8.08 (d, 2H, ArH); 8.85 (s, 1H, 7-NH)[b]

[a]Recorded in DMSO-d_6. [b]Recorded in $CDCl_3$.

Table V. Physical properties of ester derivatives **8–10**, **11a–d** and **12a–d**.



N	*R*	*R1*	*n*	*Reaction time (h)*	*Yield (%)*	*Recrystallization solvent*	*Mp (°C)*	*Formula*[a]
8	H	H	0		64	Ethanol	235–237	$C_{12}H_{12}ClN_3O_4$
11a	H	COC_6H_5	0	48	32	Ethyl acetate/ petroleum ether 60–80°C	171–173	$C_{19}H_{16}ClN_3O_5$
11b	H	COC_6H_4-*p*-Cl	0	48	49	Petroleum ether 100–140°C	177–178	$C_{19}H_{15}Cl_2N_3O_5$
11c	H	COC_6H_4-*p*-F	0	72	31	Ethyl acetate/ petroleum ether 60–80°C	160–161	$C_{19}H_{15}ClFN_3O_5$
11d	H	COC_6H_4-p-CF_3	0	72	54	Ethyl acetate/ petroleum ether 60–80°C	172–173	$C_{20}H_{15}ClF_3N_3O_5$
10	H	H	1		35	Benzene	151–153	$C_{13}H_{14}ClN_3O_4$
12a	H	COC_6H_5	1	30	82	Ethyl acetate/ petroleum ether 60–80°C	111–112	$C_{20}H_{18}ClN_3O_5$
12b	H	COC_6H_4-*p*-Cl	1	24	77	Petroleum ether 60–80°C	150–152	$C_{20}H_{17}Cl_2N_3O_5$
12c	H	COC_6H_4-*p*-F	1	48	86	Ethyl acetate/ petroleum ether 60–80°C	77–78	$C_{20}H_{17}ClFN_3O_5$
12d	H	COC_6H_4-*p*-CF_3	1	8	45	Ethyl acetate/ petroleum ether 60–80°C	103–105	$C_{21}H_{17}ClF_3N_3O_5$
9	CH_3	H	0		54	Ethanol	190–192	$C_{13}H_{14}ClN_3O_4$

[a]Elemental analyses for C, H, N were within ±0.4% of the calculated values.

means of X-ray diffraction studies. In this conformation, the two aromatic planar systems of Zopolrestat are perpendicular.

The MEPs of **2** and Alrestatin were calculated at the *ab initio* SCF STO-3G level, considering the molecules in their preferred conformations, in a three-dimensional grid around the molecules.

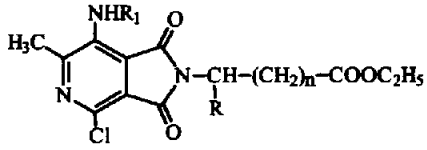
Results and discussion

The biological data obtained indicated that an acidic function is required for *in vitro* activity since the esters **8** and **10** were inactive even at 10^{-4} M (data not reported), while the corresponding acids **5** and **7** inhibited 100 or 45% of the enzyme activity at this concentration, respectively (tables I and III). These results were in agreement with previous observations [17].

The inhibition percentages and IC_{50} data listed in table I show that the inhibitory activity of the acetic derivatives **2**, **5** and **15a–d** was generally slightly lower than that of the reference compound Sorbinil, similar to that of Alrestatin (IC_{50} = 1.5 x 10^{-6} M) [9, 33, 34], and clearly lower than that of Tolrestat (IC_{50} = 3.5 x 10^{-8} M) [6] and Zopolrestat (IC_{50} = 3.1 x 10^{-9} M) [7]. Moreover, they proved to be much more potent inhibitors of AR than the propionic derivatives **7** and **16a–d** and the isopropionic derivatives **3** and **6** (tables I and III).

The lipophilicity of the molecules was varied by introducing a chlorine atom at position 4 of the pyrrolo-

Table VI. Spectral data of ester derivatives **8–10**, **11a–d** and **12a–d**.



N	R	R_1	n	IR (nujol, cm^{-1})	^{1}H-NMR (ppm)
8	H	H	0	3450, 3350, 1740, 1700, 1620, 750	1.22 (t, 3H, $CH_2\underline{CH_3}$); 2.47 (s, 3H, 6-CH_3); 4.17 (q, 2H, $\underline{CH_2}CH_3$); 4.36 (s, 2H, CH_2CO); 6.60 (s, 2H, 7-NH_2)[a]
11a	H	COC_6H_5	0	3300, 1760, 1730, 1700, 1660, 720	1.28 (t, 3H, $CH_2\underline{CH_3}$); 2.70 (s, 3H, 6-CH_3); 4.23 (q, 2H, $\underline{CH_2}CH_3$); 4.39 (s, 2H, CH_2CO); 7.51-8.06 (s, 1H, 7-NH)[b]
11b	H	COC_6H_4-p-Cl	0	3300, 1770, 1740, 1700, 1640, 750	1.28 (t, 3H, $CH_2\underline{CH_3}$); 2.68 (s, 3H, 6-CH_3); 4.24 (q, 2H, $\underline{CH_2}CH_3$); 4.39 (s, 2H, CH_2CO); 7.51 (d, 2H, ArH); 7.94 (d, 2H, ArH); 8.81 (s, 1H, 7-NH)[b]
11c	H	COC_6H_4-p-F	0	3320, 1770, 1740, 1700, 1660, 740	1.28 (t, 3H, $CH_2\underline{CH_3}$); 2.68 (s, 3H, 6-CH_3); 4.22 (q, 2H, $\underline{CH_2}CH_3$); 4.37 (s, 2H, CH_2CO); 7.07-8.07 (m, 4H, ArH); 8.76 (s, 1H, 7-NH)[b]
11d	H	COC_6H_4-p-CF_3	0	3200, 1770, 1740, 1660, 740	1.28 (t, 3H, $CH_2\underline{CH_3}$); 2.69 (s, 3H, 6-CH_3); 4.21 (q, 2H, $\underline{CH_2}CH_3$); 4.37 (s, 2H, CH_2CO); 7.76 (d, 2H, ArH); 8.08 (d, 2H, ArH); 8.82 (s, 1H, 7-NH)[b]
10	H	H	1	3450, 3350, 1750, 1700, 1620, 740	1,23 (t, 3H, $CH_2\underline{CH_3}$); 2,52 (s, 3H, 6-CH_3); 2,70 (t, 2H, CH_2CO); 3,95 (t, 2H, NCH_2); 4,14 (q, 2H, $\underline{CH_2}CH_3$); 5,23 (s, 2H, 7-NH_2)[b]
12a	H	COC_6H_5	1	3300, 1760, 1700, 1660, 720	1,21 (t, 3H, $CH_2\underline{CH_3}$); 2,69 (s, 3H, 6-CH_3); 2,70 (t, 2H, CH_2CO); 3,96 (t, 2H, NCH_2); 4,12 (q, 2H, $\underline{CH_2}CH_3$); 7,51-8,06 (m, 5H, ArH); 8,99 (s, 1H, 7-NH)[b]
12b	H	COC_6H_4-p-Cl	1	3200, 1760, 1700, 1640, 740	1,22 (t, 3H, $CH_2\underline{CH_3}$); 2,67 (s, 3H, 6-CH_3); 2,71 (t, 2H, CH_2CO); 3,96 (t, 2H, NCH_2); 4,14 (q, 2H, $\underline{CH_2}CH_3$); 7,52 (d, 2H, ArH); 7,95 (d, 2H, ArH); 8,87 (s, 1H, 7-NH).[b]
12c	H	COC_6H_4-p-F	1	3300, 1760, 1700, 1660, 740	1,21 (t, 3H, $CH_2\underline{CH_3}$); 2,67 (s, 3H, 6-CH_3); 2,71 (t, 2H, CH_2CO); 3,96 (t, 2H, NCH_2); 4,12 (q, 2H, $\underline{CH_2}CH_3$); 7,10-8,08 (m, 4H, ArH); 8,86 (s, 1H, 7-NH)[b]
12d	H	COC_6H_4-p-CF_3	1	3300, 1760, 1700, 1660, 740	1,22 (t, 3H, $CH_2\underline{CH_3}$); 2,68 (s, 3H, 6-CH_3); 2,71 (t, 2H, CH_2CO); 3,97 (t, 2H, NCH_2); 4,12 (q, 2H, $\underline{CH_2}CH_3$); 7,82 (d, 2H, ArH); 8,13 (d, 2H, ArH); 8,94 (s, 1H, 7-NH)[b]
9	CH_3	H	0	3450, 3350, 1760, 1700, 1670, 1240, 760	1.24 (t, 3H, $CH_2\underline{CH_3}$); 1.60 (d, 3H, $CH\underline{CH_3}$); 2.51 (s, 3H, 6-CH_3); 4.20 (q, 2H, $\underline{CH_2}CH_3$); 4.89 (q, 1H, $\underline{CH}CH_3$); 5.20 (s, 2H, 7-NH_2)[b]

[a] Recorded in DMSO-d_6. [b] Recorded in $CDCl_3$.

pyridine system, with the aim of verifying the influence on the inhibitory properties of the newly synthesized compounds. This structural variation did not result in any substantial change in the activity, as confirmed by the IC_{50} values of the same order of magnitude for compounds **2** and **5**. The small improvement in the inhibitory properties of compound **6** with respect to **3** was not significant, due to their low activities.

The benzoyl derivatives **15a–d** and **16a–d** were taken into consideration to obtain further insight into structure–activity relationships. They showed an inhibitory potency analogous to or lower than that of the corresponding non-benzoylated products **5** and **7**, respectively. Also in this case, an increase in the overall lipophilicity of the molecule by the introduction of a second planar aromatic area did not seem to play any role in the improvement of the activity.

A comparison of the structures of **2** and Alrestatin may help to explain their similar activity. Figure 1 shows that the structure of **2** is fully superimposable on that of Alrestatin, because it possesses a lipophilic region (the pyrrolopyridine system) at a suitable distance from a group bearing the necessary acidic proton, namely the carboxylic function of the acetic chain. Therefore, it can be hypothesized that **2** and Alrestatin occupy the inhibitory site of AR in a very similar manner. The MEP, which gives information about the chemical reactivity of a molecule, also supports the above hypothesis; as shown in figure 2, the MEP isopotential surfaces corresponding to negative values of –20 kcal/mol and –10 kcal/mol are very similar for **2** and Alrestatin. The only significant difference is due to the nitrogen of the pyridine ring of **2**, which generates an additional area of negative MEP.

Compounds **15a–d** are no more active than **2** (table I), in spite of the introduction of a secondary

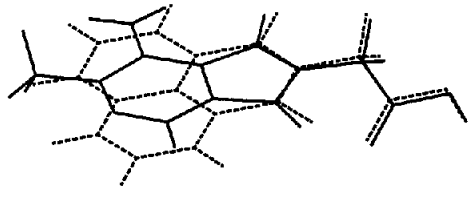


Fig 1. Superimposition of **2** (solid line) and Alrestatin (dashed line) in their preferred conformations.

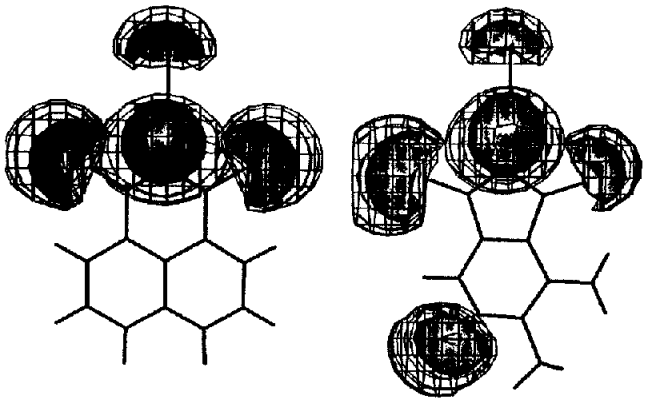


Fig 2. MEP of Alrestatin (left) and **2** (right); the isopotential surfaces corresponding to values of –20 kcal/mol (solid surface) and –10 kcal/mol (lines surface) are shown.

aromatic lipophilic region, in accordance with the model proposed by Kador [9].

Zopolrestat is one of the most active AR inhibitors possessing this kind of aromatic region; its activity (IC_{50} = 3.1 nM) [7] is several times higher than that of Alrestatin and Sorbinil. For these reasons, a comparison of the conformational properties of **15d** and Zopolrestat was carried out. Figure 3 shows the preferred conformation of Zopolrestat, together with one of the four quite similar low-energy conformations of **15d**. The importance of the benzothiazole moiety of Zopolrestat has been stressed, as this group can determine a high increase in activity when inserted into poorly active structures. Mylari *et al* [7] have proposed the hypothesis that there is a still unrecognized binding site on the AR enzyme with a strong affinity for benzothiazoles at some distance from a site which binds to acidic groups. It may be pointed out that because of the rigidity of the benzamide portion the phenyl ring of **15d** cannot occupy the same region as the benzothiazole system of Zopolrestat and therefore it probably cannot interact efficiently with the AR site. Anyway, it is possible to suppose that this group does not occupy a site of steric repulsion on the enzyme, because it does not result in a decrease in activity. At present, our synthetic efforts are aimed at obtaining new molecules in which the spatial disposition of the second lipophilic area may better match the benzothiazole portion of Zopolrestat.

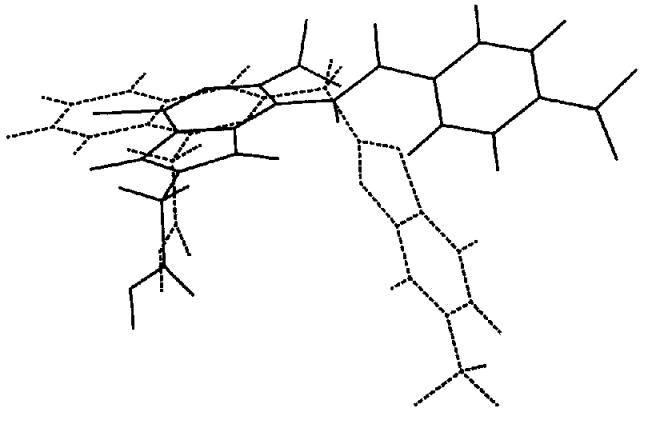


Fig 3. Superimposition of **15d** (solid line) and Zopolrestat (dashed line) in their preferred conformations.

Especially among carboxylic acids, it is very difficult to translate the *in vitro* activity into an *in vivo* form in models of diabetic complications. In addition, even those compounds which can inhibit sorbitol accumulation in the sciatic nerve generally show a low activity in the lens. Nevertheless, since the effective treatment of an ocular disease with topical application is of clinical interest, ophthalmic solutions of compounds **2** and **5** were also evaluated *in vivo* as inhibitors of glutathione lens depletion in galactosemic rats. As shown in figure 4, depletion of lens GSH occurs quickly and drastically, following induction of galactosemia in rats. We found that under hypergalactosemic conditions, glutathione depletion could be prevented by topical Sorbinil, while none of the new compounds tested were active in maintaining the rat lens GSH level, suggesting possible problems of ocular bioavailability and/or metabolism.

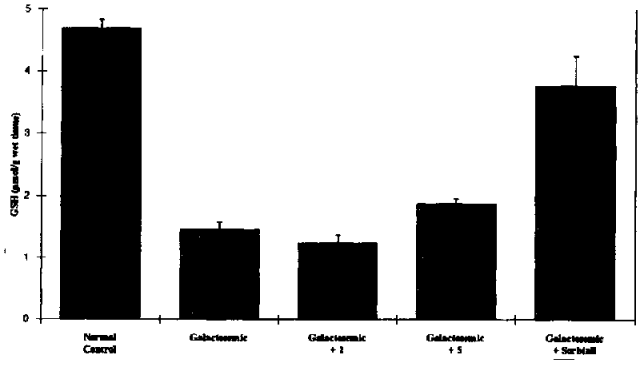


Fig 4. The effects of high galactose diet and treatment with ARI ophthalmic solutions (Sorbinil: 1%; **2** and **5**: 2%) on rat lens GSH. The treated groups were pre-dosed 1 d before switching their diet to galactose-containing chow. Rats were killed after 2 d of galactose diet. Concentrations of GSH (μmol/g wet tissue) are means ± SE (n = 16).

In conclusion, the [pyrrolo[3,4-*c*]pyridin-1,3(2*H*)-dion-2-yl]acetic acids **2**, **5** and **15a–d** represent a new class of derivatives with a promising AR inhibitory activity, even if lower than that of the reference drug, Sorbinil. For these kinds of compounds, it has been demonstrated that the presence of the carboxylic group at an adequate distance from the aromatic pyrrolopyridine system is important for *in vitro* activity.

Understanding of the pharmacophoric requirements necessary for a high activity may lead to the rational modification of molecular portions or substituents, so that they may have the appropriate spatial disposition and, at the same time, can contribute to an optimal hydrophilic-lipophilic balance of the molecules, so as to improve corneal penetration and, therefore, the *in vivo* activity.

Experimental protocols

Melting points were determined on a Köfler hot-stage apparatus and are uncorrected. IR spectra were recorded with a Pye Unicam Infracord Model PU 9516 in Nujol mulls. Routine ^{1}H-NMR spectra were determined on a Varian CFT 20 spectrometer operating at 80 MHz, using tetramethylsilane (TMS) as the internal standard. Magnesium sulfate was always used as the drying agent. Evaporations were made *in vacuo* (rotating evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica-gel aluminium sheets (60 F-254). Silica gel 60 (230-400 mesh) or aluminium oxide (Grade I, 70-230 mesh) were used for column chromatography. Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

Chemistry

*General procedure for the synthesis of 2-[7-amino-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]alkanoic acids **2** and **3**, and 2- or 3-[7-amino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]alkanoic acids **5–7** from amino acids*

A mixture of acid **1** or **4** (5 mmol) and glycine, α-alanine or β-alanine (10 mmol) was heated to fusion at 250°C for 1.5 h (TLC analysis). After cooling, the solid mass was extracted with the appropriate solvent, from which the pure product was obtained by concentration (tables I–IV).

*General procedure for the synthesis of 2- or 3-[7-amino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]alkanoic acid ethyl esters **8–10***

Equimolar amounts of diacid **4** and the ethyl ester hydrochloride of glycine, α-alanine or β-alanine were heated to fusion at 160–180°C for 1–1.5 h (TLC analysis). From the reaction mass, product **8** was obtained pure by recrystallization from ethanol, and products **9** and **10** by recrystallization from the appropriate solvent after filtration on an alumina column using as the eluting system benzene/chloroform 7:3 (tables V and VI).

*General procedure for the synthesis of 2- or 3-[7-amino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]alkanoic acids 5–7 by hydrolysis of esters **8–10***

A suspension of 1 mmol of the appropriate ester in 1–1.5 ml of concentrated hydrochloric acid was heated at reflux (1–3 h) until the disappearance (TLC analysis) of the starting material. After cooling, the crude acid precipitated from the solution obtained was collected and recrystallized (tables I–IV).

*General procedure for the synthesis of 2- or 3-[7-aroylamino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]alkanoic acid ethyl esters **11a–d** and **12a–d***

A solution of the amino ester **8** or **10** (1 mmol), triethylamine (1 mmol) and the appropriate benzoyl chloride (1.5 mmol) was heated at reflux until the disappearance of the starting material (TLC analysis) (8–72 h, table V). After cooling, the triethylamine hydrochloride was filtered off. The solution obtained was concentrated to dryness to give a residue from which pure **11a–d** and **12a–d** were obtained by flash chromatography (eluting system: ethyl acetate/petroleum ether 60–80°C 3.5:6.5) and recrystallization from the appropriate solvent (tables V and VI). In the reaction of **8** with benzoyl chloride and in the reaction of **10** with *p*-trifluoromethylbenzoyl chloride, products **13** and **14**, respectively, were also isolated by flash chromatography and characterized.

*[7-Dibenzoylamino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]acetic acid ethyl ester **13**.* Mp 128–130°C (petroleum ether 100–140°C); yield 26%. Anal for $C_{26}H_{20}ClN_3O_6$ (C, H, N). IR ν cm^{-1}: 1760, 1720, 1680, 1640, 1220, 680. ^{1}H-NMR ($CDCl_3$): δ 1.26 (t, 3H, CH_2CH_3); 2.66 (s, 3H, 6-CH_3); 4.23 (q, 2H, CH_2CH_3); 4.38 (s, 2H, CH_2CO); 7.34–7.79 (m, 10H, ArH).

*3-{7-[Di(4-trifluoromethylbenzoyl)amino]-4-chloro-6-methylpyrrole[3,4-c]pyridin-1,3(2H)-dion-2-yl}propionic acid ethyl ester **14**.* Mp 163–165°C (petroleum ether 100–140°C); yield 19%. Anal for $C_{29}H_{20}ClF_6N_3O_6$ (C, H, N). IR ν cm^{-1}: 1760, 1720, 1660, 1300, 1180, 840. ^{1}H-NMR ($CDCl_3$): δ 1.22 (t, 3H, CH_2CH_3); 2.63 (s, 3H, 6-CH_3); 2.71 (t, 2H, CH_2CO); 3.96 (t, 2H, NCH_2); 4.11 (q, 2H, CH_2CH_3); 7.50–8.22 (m, 8H, ArH).

*General procedure for the synthesis of [7-aroylamino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]acetic acids **15a–d***

A dichloromethane solution of BBr_3 1 M (5 ml) was added, under stirring and cooling at –10°C, to a solution of 1 mmol of ester **11a–d** in 10 ml dichloromethane. Once the addition was complete, the reaction mixture was maintained under stirring at 0°C for 1 h, and then at room temperature until the disappearance of the starting material (TLC analysis) (16–72 h, table I). The solution was then slowly poured onto ice and the solid formed was collected, washed with water and recrystallized (tables I and II).

*General procedure for the synthesis of 3-[7-aroylamino-4-chloro-6-methylpyrrolo[3,4-c]pyridin-1,3(2H)-dion-2-yl]propionic acids **16a–d***

A suspension of ester **12a–d** (1 mmol) in 1–1.5 ml of concentrated hydrochloric acid was heated at the temperature and for the times indicated in table III. After cooling and dilution with water, the product precipitated from the solution was collected and purified by recrystallization (tables III and IV).

Biology

Enzyme preparation

Lenses were quickly removed from normal killed rats (Sprague Dawley, 3 months old). The lenses were homogenized in cold distilled water (Glas-Potter) and then the homogenate was centrifuged at 12 000 rpm at 4°C for 60 min. The supernatant was used as a crude extract for the assay.

Enzymatic bioassay

The AR activity of the supernatant was assayed at 30°C by evaluating spectrophotometrically the oxidation of NADPH to NADP at 340 nm, using D,L-glyceraldehyde as the substrate.

The reaction mixture contained 0.5 ml of 0.1 M phosphate buffer (pH 6.2); 0.1 ml distilled water; 0.5 ml of 0.104 mM NADPH; 0.2 ml enzyme supernatant; 0.2 ml AR enzyme inhibitor solution; 0.5 ml of 10 mM D,L-glyceraldehyde, in a total volume of 2 ml. All the above reagents (except glyceraldehyde) were incubated at 30°C for 10 min; glyceraldehyde was then added to start the reaction, which was monitored for 5 min.

The decrease in NADPH concentration/min was determined using a Beckman DU-64 kinetics software program (SolfPac™ Module). Enzyme activity was calibrated by diluting the supernatant to obtain an average reaction rate of 0.011 ± 0.0010 absorbance units/min for each control sample.

To correct for the non-enzymatic oxidation of NADPH and absorption by the compounds tested, a reference blank containing all the above assay components except glyceraldehyde, substituted with water, was prepared.

The uninhibited AR activity of the crude enzyme supernatant from rat lenses was fixed as 100%. The percentage inhibition for each component was calculated by comparing the reaction rate of the solution containing the inhibitor with that of the control. All the inhibitors were dissolved in water; the solubility was facilitated by adjustment to a favorable pH. After complete dissolution, the pH was readjusted to 7. The inhibitory effect of the new derivatives was routinely estimated at a concentration of 10^{-4} M. Those compounds found to be active were tested at additional concentrations between 10^{-5} and 10^{-7} M. Each inhibitor concentration was tested in duplicate and the determination of the IC_{50} values was performed by using linear regression analysis of the log concentration–response curves.

Pharmacology

Determination of in vivo biological activity: levels of glutathione (GSH) in lenses of galactosemic rats

Groups of eight male Sprague–Dawley rats weighing 45–55 g were used. The galactose diet consisted of a pulverized mixture of 50% D-galactose and 50% MIL laboratory chow, and the control diet consisted of normal MIL. Control and experimental animals had access to both diet and water *ad libitum*.

Groups treated with ARIs were pre-dosed 1 d before switching their diet to galactose-containing chow. Test compounds were administered four times daily for 3 d as 2% ocular drops and animals were fed with the galactose diet for 2 d.

An untreated group was given access to the galactose diet for 2 d. For proper comparison, both the normal control group (on a normal diet) and the untreated group were dosed with the same vehicle in which ARIs were contained. A 1% ophthalmic solution of Sorbinil was the reference standard in this assay. On day 4, the animals were killed and lenses were removed.

Glutathione determination

Lens GSH was assayed in accordance with Ellman's method, modified by Lou *et al* [26]. Pairs of lenses from individual animals were homogenized in 10% trichloroacetic acid (5% w/v) and centrifuged at 6000 rpm for 20 min at 0–5°C.

The supernatant (10 µl) was mixed with 0.89 ml of 1.0 M Tris, pH 8.2, 0.02 M EDTA; 70 µl of DTNB solution (99 mg in 25 ml of methanol) was added to this mixture and the colored product was monitored at 412 nm.

GSH standard curves (10–80 µg/ml) were produced for each assay. The lens GSH value was expressed as micromoles per gram wet weight of tissue.

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