Thienocinnolinone Alkanoic Acid Derivatives as Aldose Reductase Inhibitors

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Abstract: A new series of 8-halogen-4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolinone-*N*2-alkanoic acids was prepared and tested for aldose reductase (ALR2) inhibitory activities. These compounds showed significant inhibitory activity against bovine lens ALR2, with the best compound **2e** showing an IC_{50} value of 31.4 μM. The presence of the C8-substituents here studied (Cl, Br) on the thienocinnolinone scaffold caused a decrease of the inhibitory potency by a factor of about 4 with respect to the unsubstituted parent compound, while the presence of a C8-methyl group, considered in a previous paper [1] decreased the activity by a factor of about 2. Moreover, the length of the *N*2 alkanoic chain influences strongly the enzyme inhibitory activity. While most of the carboxylic acids ALR2 inhibitors are acetic acid derivatives, in the case of thienocinnolinone compounds, homologues higher than acetic acids showed to be more active.

Key Words*:* Diabetic complications, aldose reductase inhibitors, thieno[*h*]cinnolinone carboxylic acids.

INTRODUCTION

Diabetes mellitus, defined as a debilitating disease affecting millions of people worldwide [2], may be classified into two subclasses: (a) type 1, namely insulin-dependent diabetes mellitus (IDDM or juvenile diabetes), characterized by ß-cells destruction caused by an autoimmune process, usually leading to absolute insulin deficiency; and (b) type 2, namely non-insulin-dependent diabetes mellitus (NIDDM or adult onset), characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the ß-cells. Type 2 diabetes is the most common form of disease and it is characterized by excessive hepatic glucose production, impaired glucose disposal in the periphery, impaired lipid metabolism, and an inability of ß-cells to secrete sufficient insulin in a timely manner in order to achieve normalization of glucose and lipid homeostasis. Therefore, diabetes mellitus is the cause of significant morbidity/mortality due to progressive impairment associated with the disease, namely macrovascular (coronary artery disease, abnormal vascular smooth muscle cell proliferation, cancers, and mood disorders) [3] and microvascular (retinopathy, neuropathy, nephropathy, other microangiopathies) complications [4,5]. Damage to these tissues results from biochemical and metabolic alterations occurring in response to chronic hyperglycemia [6].

Considerable evidences suggest that the activation of the polyol pathway, which happens in hyperglycaemic conditions, results in a reduced ratio of NADPH to $NADP^+$, and in an increased ratio of NADH to NAD^+. The first is due to the activity of aldose reductase, ALR2, which converts glucose to sorbitol, and the second is related to the activity of sorbitol dehydrogenase, which biosynthesizes fructose from sorbitol [7], Fig. (**1**). These changes, which alter the reduction potential of the cell, affect the cellular functions causing the appearance of diabetic complications [8].

On the other hand, sorbitol does not readily diffuse across cell membranes, and also its intracellular accumulation has been implicated in the appearance of diabetic complications such as cataract, retinopathy, nephropathy and neuropathy [9]. Thus, ALR2 has long been recognized as an important target for preventing the onset or progression of these complications [10,11]. Aldose reductase inhibitors therefore provide a therapeutically rational means to safely prevent or slow the progression of long-term diabetic complications, despite imperfect control of blood glucose and with no risk of hypoglycemia.

ALR2 inhibitors (ARIs) belong to different chemical classes [12] including: (*i*) hydantoins and related cyclic imide derivatives, typified by Sorbinil and Minalrestat [13]; (*ii*) carboxylic acid derivatives, epitomized by Epalrestat [14] (the only ARI on the market, currently available only in Japan), Alrestatin, Tolrestat and Zopolrestat; (*iii*) heterocyclic-substituted sulfonyl-2*H*-pyridazin-3-one analogues, typified by 6-(5-chloro-3-methylbenzofuran-2-sulfonyl)-2*H*-pyridazin-3-one [15], Fig. (**2**).

At present, however, unacceptable side effects related to toxicity or inadequate pharmacokinetic profiles and therapeutic index [16], have rendered most of the drug candidates undesirable. Thus, owing to the limited number of currently available drugs for the treatment of diabetic complications, the discovery of new drugs appears highly desirable and urgent.

Recently, our group has been greatly interested in the synthesis of new ARIs endowed of aldose reductase

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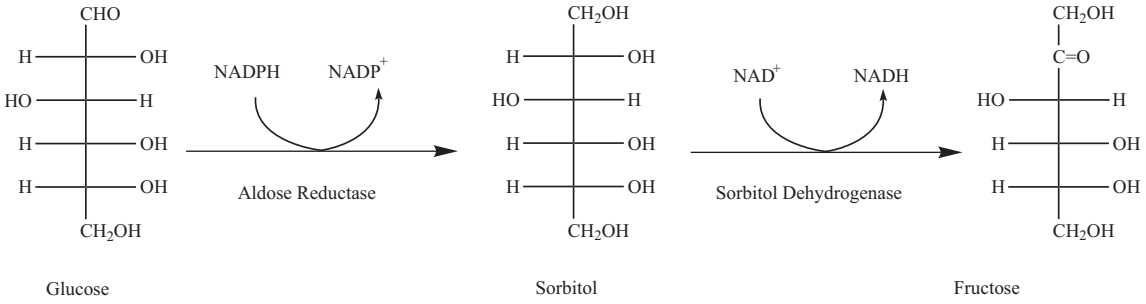


Fig. (1). Polyol pathway.

inhibitory activity based on a thienocinnolinone scaffold of general formula reported in **1** Fig. (**3**). (where R = H, CH_3, Z = $(CH_2)_n$, n = 1-5) [1].

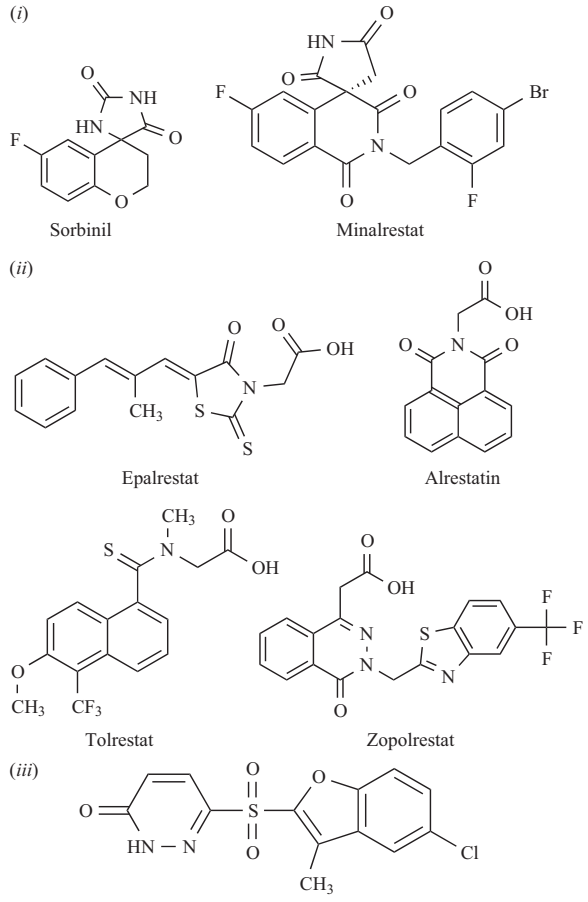


Fig. (2). Structures of different chemical classes of ALR2 inhibitors (ARIs).

This class of compounds is very interesting because the closely related benzocinnolinone derivatives were shown [17] to exert a little inhibitory activity against ALR1 (alcohol: $NADP^+$ oxidoreductase, EC 1.1.1.2, ALR1), the enzyme of the aldo-keto reductase superfamily that possess the highest homology in structure and activity with ALR2. ALR1 is an enzyme that does not appear to participate in polyol formation *in vivo*; even if the physiological function of this enzyme is not completely understood; inhibitors should not bind to this enzyme in order to avoid potential side effects [18,19].

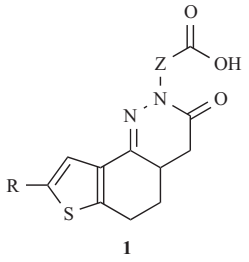
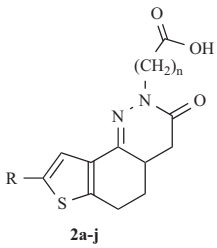


Fig. (3). Thienocinnolinone core.

The thienocinnolinone series incorporate a valid pharmacophore for ARIs activity represented by the thienocinnolinone template linked through a pentamethylene spacer to a carboxylic function, namely 4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolin-3(2*H*)-one-2-hexanoic acid (**1e**). This lead compound is provided with a potency comparable to sorbinil (compare **1e** IC_{50} 7.6 μM versus sorbinil IC_{50} 0.97 μM) [1]. Continuing our interest in this field, we decided to modify the compounds of general formula **1** by the introduction of a halogen atom (*i.e.* chlorine and bromine) at C-8 of the thienocinnolinone core in order to extend the SAR studies in this class of compounds. Thus, we synthesized and tested on ALR2 the new compounds **2a-j**, Fig. (**4**).



2	a	b	c	d	e
n	1	2	3	4	5
R	Cl	Cl	Cl	Cl	Cl

2	f	g	h	i	j
n	1	2	3	4	5
R	Br	Br	Br	Br	Br

Fig. (4). New thienocinnolinonic series.

CHEMISTRY

The target compounds **2a-j** were synthesized *via* the general route depicted in Scheme (**1**).

Compounds **3a-j** were obtained by alkylation of 8-halogen-thienocinnolinones **5**, **6** (1.0 mmol) with ethyl-bromoalkanoate (1.5 mmol) in anh. DMF at 70-85 °C for 1.5-2 h in presence of sodium hydride (1.5 mmol). Subsequent alkaline hydrolysis of the ester **3a-j** with 1 N NaOH in EtOH at reflux gave the title compounds **2a-j**. The structures of the new compounds **2a-j** and **3a-j** were characterized and confirmed by their IR, ^{1}H NMR spectra, mass spectra and elemental analyses. The still unknown 8-halogen-thienocinnolinones (*i.e.* **5, 6)** were prepared by a standard procedure starting from the appropriate 4,4a,5,6-terahydrothieno[2,3-*h*]cinnolin-3-(2*H*)-one **4**.

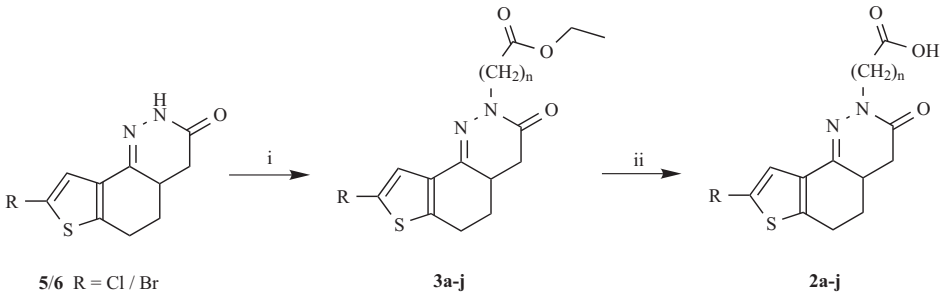
Tricyclic scaffold **4** [20,21] was synthesized in four steps by Mannich reaction between **7**, formaldehyde and dimethyl-amine hydrochloride in acetic anhydride; this allowed the isolation of **8** which was then subjected to cyanation (NaCN) (**9**) followed by the conversion into the corresponding acid **10**; the subsequent condensation with hydrazine hydrate at reflux gave the tricyclic derivative **4**. The intermediate **7** was in turn prepared in three steps by Friedel-Crafts reaction between thiophene **11** with succinic anhydride and $AlCl_3$ in CH_2Cl_2 solution to give **12** followed by Wolff-Kishner reduction of the resulting ketoacid **12** to furnish **13**. Finally, intramolecular cyclization of the thienobutanoic acid carried out under acidic conditions led to the corresponding 4,5,6,7-tetrahydrobenzo[*b*]thiophen-4-one **7** in good yield [22], Scheme (**2**).

ENZYMATIC SECTION

NADPH, D,L-glyceraldehyde, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman. Sorbinil was a gift from Pfizer and was used as standard. All other chemicals were commercial samples of good grade. Calf lenses for the isolation of ALR2 were obtained locally from freshly slaughtered animals. The enzyme was purified by a chromatographic procedure as previously described [17]. Briefly, ALR2 was released by carving the capsule and the frozen lenses were suspended in potassium phosphate buffer pH 7 containing 5 mM DTT and stirred in an ice-cold bath for two hours. The suspension was centrifuged at 4000 rpm at 4 °C for 30 minutes and the supernatant was subjected to ion exchange chromatography on DE-52. Enzyme activity was assayed spectrophotometrically on a Cecil Super Aurius CE 3041 spectrophotometer by measuring the decrease in absorption of NADPH at 340 nm which accompanies the oxidation of NADPH catalysed by ALR2. The assay was performed at 37 °C in a reaction mixture containing 0.25 M potassium phosphate buffer pH 6.8, 0.38 M ammonium sulfate, 0.11 mM NADPH, and 4.7 mM D,L-glyceraldehyde as substrate in a final volume of 1.5 ml. All inhibitors were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was 1%. To correct for the nonenzymatic oxidation of NADPH, the rate of NADPH oxidation in the presence of all the components except the substrate was subtracted from each experimental rate. Each dose-effect curve was generated using at least three concentrations of inhibitor causing an inhibition between 20 and 80 %. Each concentration was tested in duplicate and IC_{50} values as well as the 95 % confidence limits (95 % C.L.) were obtained by using CalcuSyn software for dose effect analysis [23].

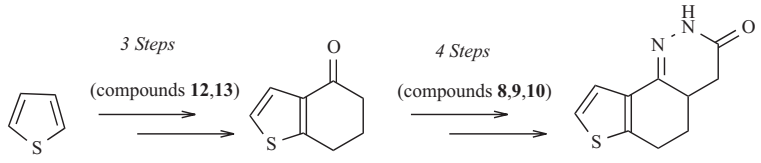
RESULTS AND DISCUSSION

The tetrahydrothienocinnolinones **2a-j** were evaluated for their ability to inhibit bovine lens aldose reductase by an *in vitro* spectrophotometric assay, following the consumption of NADPH at 340 nm with D,L-glyceraldehyde as substrate, Table (**1**). Enzyme inhibition data are expressed as IC_{50}



5/**6** R = Cl / Br **3a-j** **2a-j**

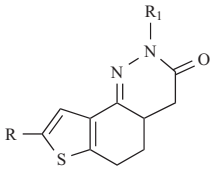
Scheme (1). Synthesis of target compounds **2a-j**: (i) NaH, dry DMF, $Br(CH_2)_nCOOC_2H_5$; (ii) 1 N NaOH, EtOH.



11 **7** **4**

Scheme (2). Synthesis of thieno cinnolinone **4**.

Table 1. Structures and Inhibitory Activities Towards ARL2 of the Compounds 2

	R	R_1	ALR2[a]	C.L.[b]
 **2a-j**	**a**/**f** Cl/Br	CH_2COOH	35% at 50 μM / 23% at 50 μM	-
	b/**g** Cl/Br	$(CH_2)_2COOH$	39% at 50 μM / 31% at 50 μM	-
	c/**h** Cl/Br	$(CH_2)_3COOH$	32.0 μM / 59.4 μM	29.2-34.9 / 44.3-79.6
	d/**i** Cl/Br	$(CH_2)_4COOH$	50.5 μM / 55.2 μM	45.9-55.5 / 48.0-63.4
	e/**j** Cl/Br	$(CH_2)_5COOH$	31.4 μM / 34.8 μM	26.5-37.1 / 31.4-38.6
Sorbinil			1.49 μM	1.08 - 2.07
Tolrestat[c]			0.031 μM	0.026 - 0.036
1e[c]	H	$(CH_2)_5COOH$	7.60 μM	6.60 - 8.70

[a]IC_{50} values; ([b]C.L., 95 % confidence limits) or % inhibition at a given concentration; [c][1].

values (the concentration of the inhibitor required to produce 50 % inhibition of the enzyme catalysed reaction). Sorbinil and **1e** were used as reference compounds.

In the present paper, the synthesis and biological evaluation of the new compounds **2a-j** are presented. These novel molecules were obtained from the parent compounds with the general structure **1**, substituting the hydrogen atom at C8-position of the thienocinnolinone core with a chlorine or bromine atom. The compounds here described exert a moderate ALR2 inhibitory activity, Table (**1**). In particular the compounds **2e** and **2j**, namely the 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolin-3-(*2H*)-one-2-hexanoic acid and the corresponding 8-bromo derivative, together with the 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolin-3-(*2H*)-one-2-butanoic acid **2c** were the most potent ALR2 inhibitors within the compounds here studied, with IC_{50} (μM) values of 31.4, 34.8 and 32.0 respectively, Table (**1**). Thus the presence of the C8-substituents here studied (Cl, Br) on the thienocinnolinone scaffold caused a decrease of the inhibitory potency by a factor of about 4 with respect to the unsubstituted parent compound, while the presence of a C8-methyl group, previously considered [1] decreased the activity by a factor of about 2 (compare **2e**, IC_{50} 31.4 μM, *versus* **1e,** IC_{50} 7.6 μM; **2j**, IC_{50} 34.8 μM *versus* the corresponding C8-methyl derivative [1], IC_{50} 18.0 μM).

Considering the effect of the length of the alkanoic chain, it can be seen that the acetic acid derivatives of the two series of compounds are only marginally active (a situation very different to what observed in the benzocinnolinone derivatives [17,24]) lengthening the carboxylic chain led to compounds possessing moderate activity. In particular the 8-Cl butanoic acid (**2c**) and the hexanoic acid derivatives (**2e** and **2j**) exert the same activity. This trend in activity is in agreement with the results of our previous researches, in which the unsubstituted and the 8-methyl derivatives of the thienocinnolinone structure were studied [1]. In that case, the hexanoic acid derivatives were the most active substances.

Thus, the comparison between the results here obtained (compounds **2a-j**) with our previous results relative to the corresponding unsubstituted or C8-methyl derivatives [1] allow to conclude that the length of the *N*2-hexanoic acid side chain can modulate strongly the activity of these compounds depending on the nature of the cinnolinone scaffold. These results will be useful for the obtainment of valuable SAR aimed to find new and more potent ALR2 inhibitors in this class of compounds.

EXPERIMENTAL SECTION

Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Paragon 500 FT IR spectrophotometer (KBr pellets for compounds **2a-j**, **5** and **6**, as film for **3a-j**). ^{1}H NMR spectra were recorded on a VARIAN XL 200 FT NMR spectrometer using $CDCl_3$ as solvent unless otherwise specified. Chemical shifts were reported in δ or ppm downfield from tetramethylsilane (TMS). Multiplicities are recorded as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Elemental analyses were performed by Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari, Italy and are within ± 0.4 % of the theoretical values.

Reactions were monitored by thin-layer chromatography (TLC) using SiO_2 Polygram SIL and ALOX N/UV_{254} precoated plastic sheets and iodine vapor and/or UV light for detection. Flash chromatography was performed using Merck silica gel type 60 (230-400 mesh ASTM). Electron ionization mass spectra (70 eV) were recorded on a Hewlett-Packard 5790-5970 MSD gas chromatograph/mass spectrometer. Atmospheric Pressure Ionization Electrospray (API-ES) mass spectra, when reported, were obtained on a Agilent 1100 series LC/MSD spectrometer. All moisture sensitive reactions were performed under nitrogen atmosphere, using oven-dried glassware. Anhydrous THF and DMF was obtained from Aldrich or Merck. All starting materials and reagents were commercially available from Aldrich, Lancaster and Avocado.

A) General Procedure for the Preparation of Compounds 2a-j

To a solution of esters **3a-j** (1.38 g, 0.27 mmol) in ethanol (7 ml) under stirring was added an aqueous solution

1 N NaOH (6 ml). The mixture was refluxed for 1-1.5 h. After evaporation of the solvent *in vacuo*, the mixture was poured into water (20 ml) and acidified with 6 N HCl. The precipitate was filtered, washed with water, and air dried to yield the corresponding acid as solid purified by trituration with isopropyl ether.

8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-acetic Acid (2a)

Yield 51%, mp 180-181 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.29). IR (cm^{-1}): 1731 (COOH); 1650 (CO). ^1H NMR δ: 1.63-1.85 (m, 2H, CH_2-5); 2.26-2.42 (m, 1H, CH-4a); 2.65-3.20 (m, 4H, CH_2-6, CH_2-4); 4.25 (sbr, 1H, COOH, D_2O exchangeable); 4.69 (s, 2H, CH_2-2'); 7.10 (d, 1H, CH-9).LC-MS (*m/z*): 321 $[M+Na]^+$. *Anal*. Calcd. For $C_{12}H_{11}Cl\ N_2O_3S$: C 48.24, H, 3.71, N 9.38. Found C 47.98, H 3.65, N 9.16.

8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-propanoic Acid (2b)

Yield 15%, mp 152-153 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.5). IR (cm^{-1}): 1715 (COOH); 1633 (CO). ^1H NMR ($CDCl_3$): δ: 1.20-1.40 (m, 2H, CH_2-5); 2.17-2.35 (m, 2H, CH_2-6); 2.60-3.00 (m, 6H, CH-4a, CH_2-4, CH_2-2', COOH D_2O exchangeable); 4.10-4.20 (t, 2H, CH_2-3'); 7.28 (d, 1H, CH-9). LC-MS (*m/z*): 335 $[M+Na]^+$. *Anal*. Calcd. for $C_{13}H_{13}ClN_2O_3S$: C 48.92, H 4.19, N 8.96. Found C 48.78, H 4.12, N 8.85.

8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-butanoic Acid (2c)

Yield 81%, mp 155-157 °C. TLC: $CHCl_3$-MeOH 9: 1 (R_f = 0.38). IR (cm^{-1}): 1728 (COOH); 1637(CO). ^1H NMR ($CDCl_3$) δ: 1.67-1.84 (m, 4H, CH_2-3', CH_2-5,); 1.97-2.47 (m, 5H, CH_2-6, CH_2-4a, CH_2-4); 2.62-3.00 (m, 3H, CH_2-2', COOH D_2O exchangeable); 3.80-4.00 (m, 2H, CH_2-4'); 7.27 (d, 1H, CH-9). LC-MS (*m/z*): 349 $[M+Na]^+$. *Anal*. Calcd. for $C_{14}H_{15}ClN_2O_3S$: C 51.45, H 4.63, N 8.57. Found C 51.28, H 4.56, N 8.40.

8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-pentanoic Acid (2d)

Yield 65%, mp 93-94 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.52). IR (cm^{-1}): 1719 (COOH); 1635 (CO). ^1H NMR ($CDCl_3$) δ: 1.58-1.83 (m, 6H, CH_2-3', CH_2-4', CH_2-5); 1.17-2.54 (m, 5H, CH_2-6, CH-4a, CH_2-4,); 2.65-3.00 (m, 5H, CH_2-2', COOH D_2O exchangeable, CH_2-5'); 7.27 (d,1H, CH-9). LC-MS (*m/z*): 364 $[M+Na]^+$. *Anal*. Calcd. for $C_{15}H_{17}ClN_2O_3S$: C 52.86, H 5.03, N 8.22. Found C 52.67, H 4.96, N 7.98.

8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-hexanoic Acid (2e)

Yield 39%, mp 101-102 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.56). IR (cm^{-1}): 1726 (COOH); 1617 (CO). ^1H NMR ($CDCl_3$) δ: 1.34-1.45 (m, 2H, CH_2-5); 1.58-1.78 (m, 6H, CH_2-5', CH_2-4', CH_2-3'); 2.20-2.40 (m, 2H, CH_2-6, CH-4a); 2.62-2.97 (m, 5H, CH_2-4, CH_2-2', COOH D_2O exchangeable); 3.62-3.93 (m, 2H, CH_2-6'); 7.27 (d, 1H, CH-9). LC-MS (*m/z*): 377 $[M+Na]^+$. *Anal*. Calcd. for $C_{16}H_{19}ClN_2O_3S$: C 54.16, H 5.40, N 7.89. Found C 53.98, H 5.36, N 7.67

8-Bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-acetic Acid (2f)

Yield 84%, mp 188-190 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.23). IR (cm^{-1}): 1732 (COOH); 1647 (CO). ^1H NMR ($CDCl_3$) δ: 1.65-1.85 (m, 2H, CH_2-5); 2.19-2.41 (m, 2H, CH_2-6); 2.65-3.06 (m, 3H, , CH-4a, CH_2-4); 4.20-4.58 (brs, 1H, COOH D_2O exchangeable); 4.59 (s, 2H, CH_2-2'); 7.35 (d, 1H, CH-9). LC-MS (*m/z*): 365 $[M+Na]^+$. *Anal*. Calcd. for $C_{12}H_{11}BrN_2O_3S$: C 42.00, H 3.23, N 8.16. Found C 41.89, H 3.18, N 8.25.

8-Bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-propanoic Acid (2g)

Yield 58%, mp 115-117 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.27). IR (cm^{-1}): 1717 (COOH); 1633 (CO). ^1H NMR ($CDCl_3$) δ: 1.60-1.87 (m, 2H, CH_2-5); 2.17-2.38 (m, 2H, CH_2-6); 2.60-3.00 (m, 6H, CH-4a, CH_2-4, CH_2-2', COOH D_2O exchangeable,); 4.06-4.13 (t, 2H, CH_2-3'); 7.45 (d, 1H, CH-9). LC-MS (*m/z*): 379 $[M+Na]^+$. *Anal*. Calcd. for $C_{13}H_{13}BrN_2O_3S$: C 43.71, H 3.67, N 7.84. Found C 43.58, H 3.61, N 7.70.

8-Bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-butanoic Acid (2h)

Yield 37%, mp 137-138 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.5). IR (cm^{-1}): 1735 (COOH); 1635 (CO). ^1H NMR ($CDCl_3$) δ: 1.65-1.87 (m, 2H, CH_2-5); 1.95-2.10 (m, 2H, CH_2-3'); 2.20-2.45 (m, 5H, CH_2-6, CH-4a, CH_2-4); 2.62-3.18 (m, 3H, CH_2-2', COOH D_2O exchangeable); 3.73-3.94 (m, 2H, CH_2-4'); 7.37 (d, 1H, CH-9). LC-MS (*m/z*): 393 $[M+Na]^+$. *Anal*. Calcd. for $C_{14}H_{15}BrN_2O_3S$: C 45.29, H 4.07, N 7.55. Found C 45.08, H 4.02, N 7.35.

8-Methyl-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-pentanoic Acid (2i)

Yield 70%, mp 121-123 °C. TLC: $CHCl_3$–MeOH 9:1 (R_f = 0.26). IR (cm^{-1}): 1717 (COOH); 1633 (CO). ^1H NMR ($CDCl_3$) δ: 1.58-1.87 (m, 6H, CH_2-3', CH_2-4', CH_2-5); 2.17-2.55 (m, 3H, CH_2-6, CH-4a,); 2.62-3.18 (m, 5H, CH_2-4, CH_2-2', COOH D_2O exchangeable); 3.70-3.95 (m, 2H, CH_2-5'); 7.38 (d, 1H, CH-9). LC-MS (*m/z*): 407 $[M+Na]^+$. *Anal*. Calcd. for $C_{15}H_{17}BrN_2O_3S$: C 46.74, H 4.45, N 7.27. Found C 46.63, H 4.36, N 7.08.

8-Methyl-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3(2H)-one-2-hexanoic Acid (2j)

Yield 56%, mp 53-54 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.54). IR (cm^{-1}): 1707 (COOH); 1625 (CO). ^1H NMR ($CDCl_3$) δ: 1.35-1.43 (m, 2H, CH_2-5); 1.58-1.82 (m, 6H, CH_2-5', CH_2-4', CH_2-3'); 2.20-2.40 (m, 3H, CH_2-6, CH-4a); 2.62-3.15 (m, 5H, CH_2-4, CH_2-2', COOH D_2O exchangeable); 3.63-3.95 (m, 2H, CH_2-6'); 7.38 (d, 1H, CH-9). LC-MS (*m/z*): 421 $[M+Na]^+$. *Anal*. Calcd. for $C_{16}H_{19}BrN_2O_3S$: C 48.13, H 4.80, N 7.02. Found C 47.89, H 4.75, N 6.88.

B) General Procedure for the Synthesis of Esters 3a-j

To a stirred solution of sodium hydride (60% dispersion in oil, 1.5 mmol) in anh. DMF (5 ml) was added a solution of the cinnolinone **5** or **6** (1.0 mmol) portionwise at 0 °C. The

reaction mixture was stirred for 0.5 h and a solution of appropriate ethyl bromoalkanoate (1.5 mmol) in dry DMF (2 ml) was added dropwise. The mixture was heated at 80-85 °C for 1.0-1.5 h. After cooling to rt, the mixture was evaporated under reduced pressure, taken up with water and extracted with $CHCl_3$ (6x7 ml). The organic phases were combined, dried (Na_2SO_4), filtered and evaporated under reduced pressure to give the desired product as an oil.

Ethyl 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-acetate (3a)

Yield 83 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.94). IR (cm^{-1}): 1738 ($COOC_2H_5$); 1671 (CO). ^1H NMR ($CDCl_3$) δ: 1.26-1.32 (t, 3H, CH_2CH_3); 1.73-1.83 (m, 2H, CH_2-5); 2.28-2.40 (m, 3H, CH-4a, CH_2-4); 2.73-3.04 (m, 2H, CH_2-6); 4.18-4.24 (q, 2H, CH_2CH_3); 4.53 (s, 2H, CH_2-2'); 7.09-8.03 (s, 1H, CH-9). GC-MS (*m/z*): 326 (M^+). *Anal*. Calcd. for $C_{14}H_{15}ClN_2O_3S$: C 51.45, H 4.63, N 8.57. Found C 51.36, H 4.58, N 8.46.

Ethyl 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-propanoate (3b)

Yield 45 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.88). IR (cm^{-1}): 1729 ($COOC_2H_5$); 1655 (CO). ^1H NMR ($CDCl_3$) δ: 1.21-1.28 (t, 3H, CH_3); 1.67-1.76 (m, 2H, CH_2-5); 2.16-2.32 (m, 3H, CH-4a, CH_2-4); 2.65-3.10 (m, 6H, CH_2-6, CH_2-2', CH_2CH_3); 4.08-4.18 (m, 2H, CH_2-3'); 8. (s, 1H, CH-9). GC-MS (*m/z*): 340 (M^+). *Anal.* Calcd. for $C_{15}H_{17}ClN_2O_3S$: C 52.86, H 5.03, N 8.22. Found C 52.74, H 4.97, N 8.16.

Ethyl 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-butanoate (3c)

Yield 78 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.78). IR (cm^{-1}): 1731 ($COOC_2H_5$); 1681 (CO). ^1H NMR ($CDCl_3$) δ: 1.18-1.28 (t, 3H, CH_2CH_3); 1.46-1.60 (m, 2H, CH_2-5); 1.86-2.40 (m, 2H, CH_2-3'); 2.45-3.02 (m, 7H, CH-4a, CH_2-6, CH_2-4, CH_2-2'); 3.66-3.88 (m, 2H, CH_2-4'); 4.06-4.20 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 354 (M^+). *Anal*. Calcd. for $C_{16}H_{19}ClN_2O_3S$: C 54.16, H 5.40, N 7.89. Found C 54.28, H 5.31, N 7.95.

Ethyl 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-pentanoate (3d)

Yield 76 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.87). IR (cm^{-1}): 1732 ($COOC_2H_5$); 1667 (CO). ^1H NMR ($CDCl_3$) δ: 1.21-1.28 (t, 3H, CH_2CH_3); 1.46-1.60 (m, 4H, CH_2-3', CH_2-4'); 2.26-2.41 (m, 3H, CH_2-5, CH-4a); 2.65-3.23 (m, 4H, CH_2-6, CH_2-4); 3.78-3.92 (m, 4H, CH_2-2', CH_2-5'); 4.00-4.20 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 368 (M^+). *Anal*. Calcd. for $C_{17}H_{21}ClN_2O_3S$: C 55.35, H 5.74, N 7.59. Found C 55.19, H 5.68, N 7.43.

Ethyl 8-chloro-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-hexanoate (3e)

Yield 85 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.94). IR (cm^{-1}): 1738 ($COOC_2H_5$); 1670 (CO). ^1H NMR ($CDCl_3$) δ: 1.22-1.27 (t, 3H, CH_2CH_3); 1.28-1.69 (m, 6H, CH_2-5', CH_2-4', CH_2-3'); 2.20-2.40 (m, 5H, CH_2-5, CH_2-6, CH-4a); 2.60-3.18 (m, 4H, CH_2-4, CH_2-2'); 3.64-3.88 (m, 2H, CH_2-6'); 4.00-4.23 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 382 (M^+). *Anal*. Calcd. for $C_{18}H_{23}BrN_2O_3S$: C 56.46, H 6.02, N 7.32. Found C 56.35, H 6.12, N 7.16.

Ethyl 8-bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-acetate (3f)

Yield 81 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.88). IR (cm^{-1}): 1748 ($COOC_2H_5$); 1668 (CO). ^1H NMR ($CDCl_3$) δ: 1.25-1.35 (t, 3H, CH_2CH_3); 2.05-2.15 (m, 2H, CH_2-5); 2.67-3.12 (m, 5H, CH_2-6, CH-4a, CH_2-4,); 4.18-4.30 (q, 2H, CH_2CH_3); 4.55 (s, 2H, CH_2-2'); 8.03 (s, 1H, CH-9). GC-MS (*m/z*): 370 (M^+). *Anal*. Calcd. for $C_{14}H_{15}BrN_2O_3S$: C 45.29, H 4.07, N 7.55. Found C 45.37, H 4.11, N 7.43.

Ethyl 8-bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-propanoate (3g)

Yield 52 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.88). IR (cm^{-1}): 1741 ($COOC_2H_5$); 1668 (CO). ^1H NMR ($CDCl_3$) δ: 1.25-1.28 (t, 3H, CH_2CH_3); 1.43-1.61 (m, 2H, CH_2-5); 1.68-2.40 (m, 5H, CH_2-6, CH-4a, CH_2-4); 2.61-2.98 (m, 2H, CH_2-2'); 2.61-3.89 (m, 2H, CH_2-3'); 4.05-4.22 (q, 2H, CH_2-CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 384 (M^+). *Anal*. Calcd. for $C_{15}H_{17}BrN_2O_3S$: C 46.76, H 4.45, N 7.27. Found C 46.63, H 4.38, N 7.15.

Ethyl 8-bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-butanoate (3h)

Yield 41 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.79). IR (cm^{-1}): 1731 ($COOC_2H_5$); 1668 (CO). ^1H NMR ($CDCl_3$) δ: 1.22-1.35 (t, 3H, CH_2CH_3); 1.46-1.60 (m, 2H, CH_2-5); 1.86-2.40 (m, 2H, CH_2-3'); 2.41-2.68 (m, 5H, CH-4a, CH_2-4, CH_2-6); 2.60-2.98 (m, 2H, CH_2-2'); 3.66-3.88 (m, 2H, CH_2-4'); 4.10-4.20 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 398 (M^+). *Anal*. Calcd. for $C_{16}H_{19}BrN_2O_3S$: C 48.13, H 4.80, N 7.02. Found C 48.25, H 4.73, N 7.11.

Ethyl 8-bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-pentanoate (3i)

Yield 80 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.87). IR (cm^{-1}): 1732 ($COOC_2H_5$); 1668 (CO). ^1H NMR ($CDCl_3$) δ: 1.21-1.32 (t, 3H, CH_2CH_3); 1.46-1.60 (m, 4H, CH_2-3', CH_2-4'); 2.26-2.41 (m, 3H, CH_2-5, CH-4a); 2.65-3.23 (m, 6H, CH_2-6, CH_2-4, CH_2-2'); 3.78-3.92 (m, 2H, CH_2-5'); 4.06-4.17 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 412 (M^+). *Anal*. Calcd. for $C_{17}H_{21}BrN_2O_3S$: C 49.40, H 5.12, N 6.78. Found C 49.26, H 5.07, N 6.73.

Ethyl 8-bromo-4,4a,5,6-tetrahydrothieno[2,3-h]cinnolin-3 (2H)-one-2-hexanoate (3j)

Yield 85 %, TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.86). IR (cm^{-1}): 1731 ($COOC_2H_5$); 1668 (CO). ^1H NMR ($CDCl_3$) δ: 1.22-1.27 (t, 3H, CH_2CH_3); 1.28-1.69 (m, 6H, CH_2-3', CH_2-4', CH_2-5); 2.2-2.4 (m, 5H, CH-4a, CH_2-5', CH_2-6); 2.60-3.18 (m, 4H, CH_2-4, CH_2-2'); 3.64-3.88 (m, 2H, CH_2-6'); 4.0-4.23 (q, 2H, CH_2CH_3); 8.02 (s, 1H, CH-9). GC-MS (*m/z*): 426 (M^+). *Anal*. Calcd. for $C_{18}H_{23}BrN_2O_3S$: C 50.59, H 5.42, N 6.56. Found C 50.46, H 5.37, N 6.38.

C) 8-Chloro-4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolin-3 (2*H*)-one (5)

To a stirred solution of cinnolinone **4** (2.4 mmol) in acetic acid (3 ml) was added *N*-chlorosuccinimide (2.7

mmol), and the solution was gradually carried out at reflux for 1 h and then at rt. The precipitate was filtered, washed (aqueous 5% $NaHCO_3$ solution and ice-water), and dried *in vacuo*. Yield 0.54 g (93 %) (yellowish solid), mp 222-223 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.75). IR (cm^{-1}): 3190 (NH); 1700 (CO). ^1H NMR ($CDCl_3$) δ: 1.67-1.82 (m, 2H, CH_2-5); 2.16-2.32 (m, 2H, CH-4a); 2.57-3.32 (m, 4H, CH_2-6, CH_2-4); 7.30 (s, 1H, CH-9); 9.57 (s, 1H, NH D_2O exchangeable). GC-MS (*m/z*): 240 (M^+). *Anal*. Calcd. for $C_{10}H_9ClN_2OS$: C 49.90, H 3.71, N 11.64. Found C 49.72, H 3.63, N 11.46.

D) 8-Bromo-4,4a,5,6-tetrahydrothieno[2,3-*h*]cinnolin-3(2*H*)-one (6)

To a stirred solution of cinnolinone **4** (2.9 mmol) in acetic acid (11 mmol) was added dropwise a solution of bromine (4.1 mmol) in acetic acid (4 ml) at 0 °C. The reaction mixture was kept at rt for 1 h, then a saturated aqueous sodium acetate solution (2 ml) was added under stirring. The precipitate formed was filtered, washed with water and dried. Yield 0.81 g (92%) (beige solid), mp 237-238 °C. TLC: $CHCl_3$-MeOH 9:1 (R_f = 0.69). IR (cm^{-1}): 3200 (NH); 1703 (CO). ^1H NMR ($CDCl_3$) δ: 1.69-1.79 (m, 2H, CH_2-5); 2.16-2.35 (m, 2H, CH-4a); 2.57-3.30 (m, 4H, CH_2-6, CH_2-4); 7.39 (s, 1H, CH-9); 9.60 (s, 1H, NH D_2O exchangeable). GC-MS (*m/z*): 284 (M^+). *Anal*. Calcd. for $C_{10}H_9BrN_2OS$: C 42.12, H 3.18, N 9.82. Found C 41.98, H 3.25, N 9.73.

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