Eur J Med Chem (1992) 27, 419–423

Short communication

Synthesis and immunostimulating activity of new 1,4-benzothiazine derivatives

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(Received 1 April 1991; accepted 10 December 1991)

Summary — The synthesis and immunomodulating properties of 1,4-benzothiazine derivatives are reported. Several compounds revealed interesting activity in the test of superoxide anion production by peritoneal macrophages in the prednisolone-immunodepressed mouse.

1,4-benzothiazine derivatives / immunomodulating activity

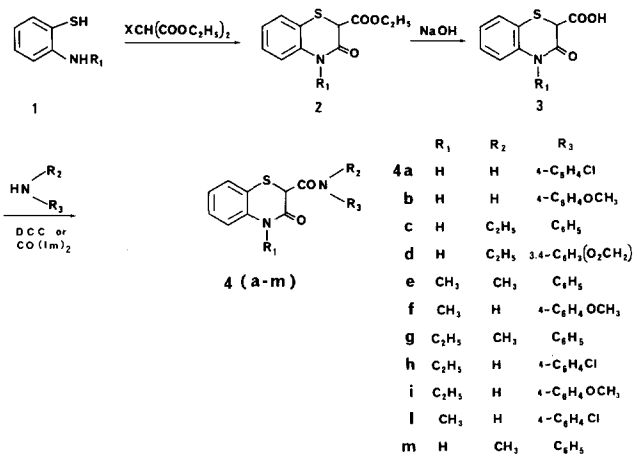
Introduction

In the field of agents capable of playing a regulator role in the disorders of the cellular and humoral immune system, many classes of compounds have been developed. Among these, *N*-phenyl-*N*-methyl-1,2-dihydro-4-hydroxy-1-methyl-quinoline-3-carboxamide (Roquinimex) was recently [1, 2] shown to enhance delayed hypersensitivity reactions in mice and rats [3], to increase the response of spleen cells to concanavalin A, to have immunostimulating action on the macrophages, to stimulate NK cells against metastases of melanoma [4] and to reduce the number and size of dimethylbenzanthracene-induced mammary tumors in rats [1]. On the other hand, the immunomodulating properties of some sulfur-containing compounds such as levamisole, thiabendazole, lotifazole, fenetizole, are known.

In the course of our studies we planned to verify the possibility to retain and enhance the activity in structures that could join, in broad outline, the characteristics of the above-mentioned compounds, developing some 1,4-benzothiazine derivatives such as 2H-1,4-benzothiazine-3,4-dihydro-3-oxo-2-carboxamides and 4H-1,4-benzothiazine-2-carboxamides. Several compounds, tested in comparison with *N*-phenyl-*N*-methyl-1,2-dihydro-4-hydroxy-1-methylquinoline-3-carboxamide, confirmed the expectations and exhibited interesting properties.

Chemistry

The synthesis of new 2H-1,4-benzothiazine-3,4-dihydro-3-oxo-2-carboxamides **4** was carried out as outlined in scheme 1 starting from acids **3** (prepared according to known procedures) [5] by amidation in the presence of *N,N'*-dicyclohexylcarbodiimide or *via* 1,1'-carbonyldiimidazole. The substituted 2-mercaptoanilines **1** were prepared according to Kiprianov and Pazenko [6].



Scheme 1.

Abbreviations: TMS, tetramethylsilane; DMF, *N,N*-dimethyl formamide; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; THF, tetrahydrofuran

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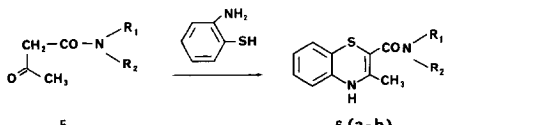
The 4H-1,4-benzothiazine-3-methyl-2-carboxamides **6** were prepared according to scheme 2, by reacting o-aminothiophenol with the substituted acetoacetamides **5** in the presence of triethylamine in ethyl alcohol at room temperature [7]. The acetoacetamides **5** are known and were prepared by the procedure of G Erhardt and I Hemig [8] from diketene and the corresponding *N*-alkylanilines .



5 6 (a-b)

	R_1	R_2
6a	CH_3	C_6H_5
b	C_2H_5	C_6H_5

Scheme 2.

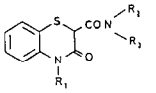
All prepared compounds are listed in tables I and II.

Pharmacological results and discussion

The activity of the compounds on superoxide anion production by macrophages in prednisolone-immunodepressed mice is shown in table III. All the synthesized compounds showed immunostimulating activity in superoxide anion production by macrophages in prednisolone-immunodepressed mice.

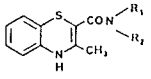
The most interesting compounds (**4g**, **4h**, **4i**, **6a**, **6b**) were studied in comparison with standard (Roquinimex) in the same test at lower dosages. The results reported in table III showed that compounds **4g**, **6a**, **6b** were more active than Roquinimex. Taking into

Table I. 2H-1,4-Benzothiazine-3,4-dihydro-3-oxo-2-carboxamides.



Compound	R_1	R_2	R_3	*Method*	*Yield (%)*	*Mp (°C)*	*Formula*	*MW*	*Analysis*
4a	H	H	4-C_6H_4Cl	A	57	224–226	$C_{15}H_{11}ClN_2O_2S$	318.78	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 4.43 (s, 1H, S-CH); 6.95–7.57 (m, 8H, arom); 10.53 (s, 1H, NH exch); 10.99 (s, 1H, NH-4 exch)
4b	H	H	4-$C_6H_4OCH_3$	A	63	220–222	$C_{16}H_{14}N_2O_3S$	314.36	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 3.72 (s, 3H; OCH_3); 4.39 (s, 1H, S-CH); 6.84–7.44 (m, 8H, arom); 10.23 (s, 1H, NH exch); 10.95 (s, 1H, NH-4 exch)
4c	H	C_2H_5	C_6H_5	B	94	166–168	$C_{17}H_{16}N_2O_2S$	312.39	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 1.00 (t, 3H, CH_3); 3.48–3.60 (m, 2H, CH_2); 3.97 (s, 1H, S-CH); 6.93–7.57 (m, 9H, arom); 10.96 (s, 1H, NH exch)
4d	H	C_2H_5	3,4-$C_6H_3(O_2CH_2)$	B	80	203–205	$C_{18}H_{16}N_2O_4S$	356.40	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 0.99 (t, 3H, CH_3); 3.40–3.80 (m, 2H, CH_2-CH_3); 4.09 (s, 1H, S-CH); 6.11 (s, 2H, CH_2); 6.81–7.31 (m, 7H, arom); 10.85 (s, 1H, NH exch)
4e	CH_3	CH_3	C_6H_5	B	75	113–114	$C_{17}H_{16}N_2O_2S$	312.39	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 3.18 (s, 3H, N-CH_3); 3.36 (s, 3H, N-4-CH_3); 4.15 (s, 1H, S-CH); 7.03–7.49 (m, 9H, arom)
4f	CH_3	H	4-$C_6H_4OCH_3$	A	53	204–206	$C_{17}H_{16}N_2O_3S$	328.39	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 3.43 (s, 3H, N-CH_3); 3.72 (s, 3H, OCH_3); 4.51 (s, 1H, S-CH); 6.85–7.46 (m, 8H, arom); 10.20 (s, 1H, NH exch)
4g	C_2H_5	CH_3	C_6H_5	A	57	110–111	$C_{18}H_{18}N_2O_2S$	326.41	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 1.07–1.22 (m, 3H, CH_2); 3.36 (s, 3H, N-CH_3); 3.92–4.07 (m, 2H, CH_2); 7.02–7.50 (m, 9H, arom)
4h	C_2H_5	H	4-C_6H_4Cl	A	67	175–177	$C_{17}H_{15}ClN_2O_2S$	346.83	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 1.20 (t, 3H, CH_3); 3.90–4.22 (m, 2H, CH_2); 4.52 (s, 1H, S-CH); 7.15–7.56 (m, 8H, arom) 10.48 (s, 1H, NH exch)
4i	C_2H_5	H	4-$C_6H_4OCH_3$	A	50	190–192	$C_{18}H_{18}N_2O_3S$	342.41	(C, H, N); ^{1}H-NMR (DMSO-d_6, δ = ppm from TMS): 1.20 (t, 3H, CH_2CH_3); 3.72 (s, 3H, OCH_3); 3.94–4.17 (m, 2H, CH_2); 4.47 (s, 1H, S-CH) 6.85–7.46 (m, 8H, arom); 10.18 (s, 1H, NH exch)
4l	CH_3	H	4-C_6H_4Cl	A	72	152–154	$C_{16}H_{13}ClN_2O_2S$	332.80	See *Experimental protocols*
4m	H	CH_3	C_6H_5	B	89	171–173	$C_{16}H_{14}N_2O_2S$	298.36	See *Experimental protocols*

Table II. 4H-1,4-Benzothiazine-3-methyl-2-carboxamides.



Compd	R_1	R_2	*Yield (%)*	*Mp (°C)*	*Formula*	*MW*	*Analysis*
6a	CH_3	C_6H_5	55.7	142–143	$C_{17}H_{16}N_2OS$	296.39	See *Experimental protocols*
6b	C_2H_5	C_6H5_5	49	116–118	$C_{18}H_{18}N_2OS$	310.41	(C, H, N); ^{1}H-NMR (DMSO–d_6, δ = ppm from TMS): 1.03 (t, 3H, CH_2-CH_3); 1.78 (s, 3H, =C-CH_3); 3.72 (q, 2H, CH_2); 6.55–7.60 (m, 9H, arom); 8.01 (s, 1H, NH exch)

Table III. Activity on superoxide anion production by macrophages in prednisolone-immunodepressed mice (mean ± SE of six experiments).

Treatment	*Dosages mg/kg ip*	*nmol of reduced cytochrome C/ 10^6 macrophages*	*Dosages mg/kg ip*	*nmol of reduced cytochrome C/ 10^6 macrophages*
5% arabic gum (10 ml/kg per os)	–	11.36 ± 0.30*	–	11.94 ± 0.43*
5% arabic gum + prednisolone	–	1.99 ± 0.23	–	2.04 ± 0.25
Roquinimex + "	200	8.45 ± 0.28*	100	6.19 ± 0.25*
4a + "	200	6.14 ± 0.29*	–	
4b + "	200	5.94 ± 0.30*	–	
4c + "	200	6.47 ± 0.22*	–	
4d + "	200	6.50 ± 0.16*	–	
4e + "	200	6.35 ± 0.29*	–	
4f + "	200	6.50 ± 0.30*	–	
4g + "	200	9.30 ± 0.21*	100	7.78 ± 0.18*
4h + "	200	9.38 ± 0.24*	100	6.57 ± 0.16*
4i + "	200	9.42 ± 0.17*	100	6.76 ± 0.14*
4l + "	200	6.41 ± 0.20*	–	
4m + "	200	6.36 ± 0.30*	–	
6a + "	200	9.40 ± 0.20*	100	8.12 ± 0.19*
6b + "	200	9.26 ± 0.23*	100	7.79 ± 0.18*

*$P \leq 0.01$ Dunnett *t*-test *vs* 5% arabic gum + prednisolone group.

consideration the number of new compounds and the immunostimulating activity in only one test, SAR is not discussed.

The compounds showed no antiulcer, analgesic, antihypertensive, bronchodilator and spasmolytic activities. The oral acute toxicity in mice of the new compounds is > 1600 mg/kg whereas that of Roquinimex is about 1200 mg/kg.

Experimental protocols

Chemistry

Melting points were taken on a Büchi 535 apparatus and are uncorrected. ^{1}H-NMR were recorded on a Varian Geminy (200 MHz) spectrometer using tetramethylsilane (TMS) as an internal standard. Microanalysis values are within ± 0.4% of the theoretical values.

*2H-1,4-Benzothiazine-3,4-dihydro-3-oxo-4-methyl-2-N-(4-chlorophenyl)carboxamide (**4l**). Method A*

To a solution of 3.34 g (0.015 mol) of 2H-1,4-benzothiazine-3,4-dihydro-3-oxo-4-methyl-2-carboxylic acid and 2.10 g (0.0165 mol) of p-chloroaniline in 45 ml of THF stirred at 0°C, 3.24 g (0.0157 mol) of DCC in 15 ml of THF were added. After 30 min at 0°C, the precipitated DCU was filtered off, the solvent evaporated, and the residue shaken with a mixture of 50 ml water and 50 ml ethylacetate. The organic layer was washed with diluted hydrochloric acid and sodium bicarbonate solution, dried over sodium sulphate and the solvent distilled. By adding petrol ether the product crystallized. It was filtered and dried at 40°C, yielding 3.6 g (72%), of product with mp = 152–154°C. Anal for $C_{16}H_{13}ClN_2O_2S$ (C, H, Cl, N, S). ^{1}H-NMR (DMSO–d_6, δ = ppm from TMS): 3.44 (s, 3H, N-CH_3); 4.55 (s, 1H, S-CH); 7.10–7.55 (m, 8H, arom); 10.50 (s, 1H, NH exch).

*2H-1,4-Benzothiazine-3,4-dihydro-3-oxo-2-N-phenyl-N-methylcarboxamide (**4m**). Method B*

To 4.18 g (0.02 mol) of 2H 1,4-benzothiazine-3,4-dihydro-3-oxo-2-carboxylic acid in 40 ml of THF 3.55 g (0.022 mol) of 1,1'-carbonyldiimidazole were added and the mixture was stirred until gas evolution ceased. To the suspension a solution of 2.67 g (0.025 mol) of *N*-methylaniline in 7.5 ml of THF was added, and the temperature was kept at 40°C for 5 h. The solvent was evaporated and the residue was partitioned with 50 ml diethylether and 50 ml saturated aqueous sodium bicarbonate solution, the organic layer was separated and washed with diluted hydrochloric acid. By usual work-up 5.3 g (89%) of product, mp = 171–173°C was obtained. Anal for $C_{16}H_{14}N_2O_2S$ (C, H, N, S). ^{1}H-NMR (DMSO–d_6), δ = ppm from TMS); 2.95 (s, 3H, N-CH_3); 4.0 (s, 1H, S-CH); 6.90–7.60 (m, 9H, arom); 10.80 (s, 1H, NH exch).

*4H-1,4-Benzothiazine-3-methyl-2-N-phenyl-N-methyl-carboxamide (**6a**)*

To a solution of 9.43 g (0.03 mol) of *N*-methyl-*N*-phenyl-acetoacetamide and 4.5 ml (0.032 mol) of triethylamine in 30 ml of ethanol, 3.75 g (0.03 mol) of *o*-aminothiophenol in 7.5 ml of ethanol were added at 20°C. The solution was kept at room temperature for 6 h, then poured into water, extracted with ethyl acetate and the organic layer separated and washed with diluted hydrochloric acid and water. The solution was concentrated to 1/3 of its vol and the crystallized product was filtered, washed with cold ethanol and dried to give 3.3 g (55.7%) of product, mp = 142-143°C. Anal for $C_{17}H_{16}N_2OS$ (C, H, N, S). ^{1}H-NMR (DMSO–d_6, δ = ppm from TMS): 1.78 (s, 3H, =C-CH_3); 3.25 (s, 3H, N-CH_3); 6.59–7.35 (m, 9H, arom); 8.05 (s, 1H, NH exch).

*2H-1,4-Benzothiazine-3,4-dihydro-3-oxo-4-ethyl-2-carboxylic acid (**3**, $R_1 = C_2H_5$)*

This compound was prepared in a 73% yield according to the mentioned procedure [5] as oil. ^{1}H-NMR (DMSO–d_6, δ = ppm from TMS): 1.2 (t, 3H, CH_2CH_3); 3.71–3.98 (m, 2H, CH_2CH_3); 4.5 (s, 1H, S-CH); 6.9–7.5 (m, 4H, arom).

Pharmacology

Activity on superoxide anion production by macrophages in prednisolone-immunodepressed mice

Male mice CD-1 (C River), 20–22 g, were treated with prednisolone (0.5 mg/kg die sc for 11 days) and the compounds (100–200 mg/kg die os for 11 days twice daily). The peritoneal macrophages were isolated and the superoxide anion production was determined by measuring the nmol of reduced cytochrome *C* by 10^6 macrophages [9].

The peritoneal macrophages were isolated from the mouse following ip administration of 5 ml of RPMI-1640 (Sigma). The isolated cells were centrifuged, washed twice in RPMI-1640 and diluted in RPMI with 10% bovine foetal serum at a concentration of 10^6 macrophages/ml. Cell suspension 1 ml aliquot was incubated with 0.25 ml horse heart cytochrome C (20 mg/ml) (Sigma), 0.05 ml phorbol myristate acetate (1 mg/ml) (Sigma) and 0.7 Krebs-Ringer solution with 5 mmol/l glucose. The assay was performed at pH 7.4. After incubation for 15 min at 37°C, the reaction was stopped by adding 1.0 ml ice-cold Krebs-Ringer solution with superoxide dismutase (Sigma) to each tube.

The contents of the tubes were promptly centrifuged at 4°C, 600 *g* for 10 min. Absorbance of the supernatant at 550 nm was determined in a spectrophotometer using a blank without phorbol myristate acetate. Results were converted to nanomoles cytochrome *C* reduced by using the extinction coefficient *E* (550) = 2.1×10^4 M^{-1} cm^{-1}.

Acute toxicity

The acute oral toxicity of the compounds was evaluated in male CD-1 mice (C River). Each compound, suspended in 5% arabic gum, was administered at various doses (4 animals per dose) and the animals were observed for 7 days [10].

Other pharmacological activities

The compounds were assayed for antiulcer activity in the rat gastric ulcers by the indomethacin test [11], as analgesic activity in the mouse phenylquinone test [12], as antihypertensive activity in the SH rats test [13], as bronchodilator activity in the guinea-pig histamine bronchospasm test [14] and as spasmolytic activity in the *in vitro* guinea-pig ileum stimulated by histamine, acetylcholine and serotonine test [15].

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