CEPHALOSPORINS. VI

SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS OF NEW 7β-[(*Z*)-2-ALKOXYIMINO-2-(2-AMINOTHIAZOL-4-YL)ACETAMIDO]-CEPHALOSPORINS WITH A TETRAZOLOPYRIDAZINE AT THE 3-POSITION

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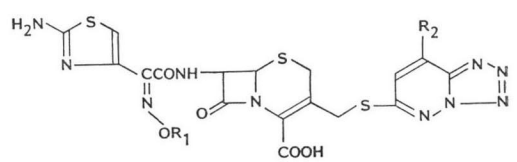
The synthesis and *in vitro* activity of 7β-[(*Z*)-2-alkoxyimino-2-(2-aminothiazol-4-yl) acetamido]cephalosporins with a tetrazolo[1,5-b]pyridazine at the 3-position are described. These cephalosporins showed excellent activity against Gram-negative bacteria, including β-lactamase producing strains. The most interesting compound of the series was 7β-[(*Z*)-2-(2-aminothiazol-4-yl)-2-methoxyimino acetamido]-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)-thiomethyl-3-cephem-4-carboxylic acid (**9**, FCE 20485) because of its extraordinarily long half-life and marked *in vivo* activity.

A turning-point in the history of cephalosporins was made with the discovery of cefotaxime[1~8], a compound which for the first time combined outstanding potency against Gram-negative bacteria with good resistance to most β-lactamases.

Since then several groups, including the authors, have attempted to improve on this basic molecule by modifying the oxyimino group and/or replacing the acetoxy group at the 3-position with different heterocyclic thiols. In particular, because cefotaxime suffered from poor activity against some Gram-positive bacteria, and from a relatively short half-life and metabolic inactivation, our goal was to find derivatives provided with a better balanced spectrum of antibacterial activity, and a more favorable pharmacokinetic profile. After a few orientative experiments, we focused our attention on congeners bearing a tetrazolo[1,5-b]pyridazin-6-ylthiomethyl group at the 3-position, a type of substitution we had previously studied in depth and employed with good results, and which had appropriately balanced the overall antimicrobial spectrum of compounds with strongly hydrophilic 7-side chains[4].

General formula of the products synthesized are shown at the right. Of these, compound **9** (R_1=Me, R_2=COOH), coded FCE 20485, showed prolonged serum half-life and promising therapeutic effectiveness in preliminary *in vivo* experiments in mice. This paper describes the synthesis of new cephalosporins and the results of our structure-activity studies.



R_1=H, Me, Et, CH_2COOH, CMe_2COOH
R_2=H, NH_2, COOH, $CONH_2$

Chemistry

The heterobicyclic thiols and 3-heterocyclylthiomethyl derivatives of 7-ACA have been reported in a previous paper[4]. Cephalosporins **1**~**12** (Table 1) were prepared by conversion of the alkoxyimino acids to the corresponding acyl chlorides followed by coupling with 7-ACA derivatives. The amino group present in the thiazole ring was protected with tritylchloride; the hydroxyl and carboxy group, when present on the imino moiety, were masked respectively as trityl ethers (**4**, **8**) and *tert*-butyl esters (**2**, **3**, **6**, **7** and **11**). Warm aqueous formic acid was usually expedient for the simultaneous removal of all the protecting groups. The oxime *syn* configuration was preserved throughout the synthesis, as shown by high-field resonance of the thiazole proton in the ^1H NMR spectrum[2].

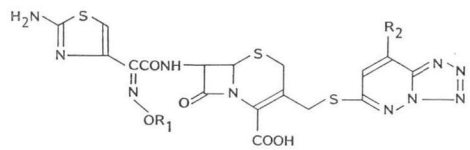
The cephalosporins synthesized are listed in Table 1. Their purity, established by NMR, TLC and analysis was greater than 90%.

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of the new compounds against 2 strains of Gram-positive and 12 strains of Gram-negative bacteria, including β-lactamase producing strains, was determined by the standard two fold serial dilution method using diagnostic sensitivity test agar (Oxoid). The plates were inoculated with about 2×10^5 colony forming units using an automatic inoculator (Denley Tech. Ltd). The results reported in Table 2 are the geometric average of two determinations and are compared with cefazolin, cefuroxime and cefotaxime.

All the compounds synthesized are in general markedly more active than cefazolin and cefuroxime against Gram-negative bacteria, showing in addition interesting *in vitro* activity against *Pseudomonas aeruginosa*; substituents other than methoxy on the imino group performed noticeably worse. Although *in vitro* no general improvement over cefotaxime was discernible at first sight, some compounds

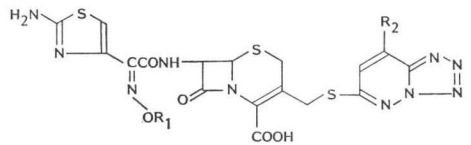
Table 1.



Compound	R_1	R_2	IR (β-lactam) (cm^{-1})	Thiazole 5-H (ppm)	Formula[g]
1	Me	H	1760[c]	7.15[d]	$C_{18}H_{16}N_{10}O_5S_3$
2	CH_2COOH	H	1775[c]	6.80[d]	$C_{19}H_{16}N_{10}O_7S_3$
3	CMe_2COOH	H	1780[b]	7.05[f]	$C_{21}H_{20}N_{10}O_7S_3\cdot HCl$
4	H	NH_2	1760[a]	6.76[d]	$C_{17}H_{15}N_{11}O_5S_3$
5	Me	NH_2	1760[a]	6.82[d]	$C_{18}H_{17}N_{11}O_5S_3$
6	CH_2COOH	NH_2	1770[c]	6.82[d]	$C_{19}H_{17}N_{11}O_7S_3$
7	CMe_2COOH	NH_2	1770[a]	6.75[d]	$C_{21}H_{21}N_{11}O_7S_3$
8	H	COOH	1760[a]	7.59[e]	$C_{18}H_{14}N_{10}O_7S_3$
9	Me	COOH	1765[a]	6.83[d]	$C_{19}H_{16}N_{10}O_7S_3\cdot 2H_2O$
10	Et	COOH	1775[a]	6.82[d]	$C_{20}H_{18}N_{10}O_7S_3$
11	CMe_2COOH	COOH	1780[a]	7.56[e]	$C_{22}H_{20}N_{10}O_9S_3\cdot HCl$
12	Me	$CONH_2$	1780[c]	7.18[d]	$C_{19}H_{17}N_{11}O_6S_3$

[a] KBr; [b] DMSO; [c] Nujol; [d] DMSO-d_6; [e] CF_3COOH-d; [f] DMSO-d_6 - $CDCl_3$; [g] All compounds were analysed for C, H, N, S.

Table 2. *In vitro* antibacterial activity of cephalosporins.



Compound	R_1	R_2	MIC (μg/ml)[a]												
			S. a. (R)	*S. p.*	*E. c.*	*K. p.*	*E. ae.*	*S. t.*	*S. s.*	*P. m.*	*P. v.*	*E. c.t.*	*K. ae.*	*E. cl.* P 99	*P. ae.*	*B. fr.*[b]
1	Me	H	0.7	0.003	0.17	0.003	0.4	0.17	0.35	0.015	≦0.001	0.17	22.6	22.6	8	16
2	CH_2COOH	H	4	0.03	0.25	0.03	0.5	0.25	1	0.002	0.003	0.35	2.8	90.5	11.3	64
3	CMe_2COOH	H	2.8	0.04	0.5	0.12	1	0.35	0.5	0.008	≦0.001	0.71	1	16	8	16
4	H	NH_2	0.5	≦0.002	0.25	0.004	0.5	0.12	0.5	0.015	0.008	1	32	32	8	n.t.
5	Me	NH_2	0.5	0.002	0.25	0.004	0.5	0.12	0.25	0.015	0.001	0.25	4	16	4	n.t.
6	CH_2COOH	NH_2	4	0.03	0.35	0.04	0.5	0.25	0.7	0.004	0.0005	0.35	5.7	90.5	4	32
7	CMe_2COOH	NH_2	1.4	0.06	0.7	0.25	1.4	0.5	0.5	0.004	0.015	0.5	1	32	2.8	16
8	H	COOH	1	0.01	0.17	0.12	0.25	0.17	0.25	0.004	0.006	4	90.5	>128	16	n.t.
9	Me	COOH	2	0.015	0.12	0.06	0.12	0.08	0.12	0.006	≦0.0002	0.17	32	128	2.8	8
10	Et	COOH	2	0.015	0.71	0.17	0.5	0.35	0.5	0.015	0.002	0.35	>128	>128	4	8
11	CMe_2COOH	COOH	8	0.12	2	1	2	1	1.4	0.008	≦0.001	2	4	64	8	16
12	Me	$CONH_2$	0.5	0.004	0.25	0.01	0.25	0.12	0.35	0.03	0.001	0.17	4	16	8	4
Cefazolin			0.57	0.06	1.4	0.7	2	1.4	1.4	5.7	>128	12.5	>128	>128	>128	>128
Cefuroxime			1	0.004	8	0.06	8	2.4	8	0.25	16	8	>128	>128	>128	>128
Cefotaxime			2	0.006	0.12	0.004	0.12	0.03	0.12	0.008	0.003	0.06	5.7	128	4	4

[a] Organisms included in this Table are: *S. a.* (R), *Staphylococcus aureus* 39/2 (benzylpenicillin resistant); *S. p.*, *Streptococcus pyogenes* C 203; *E. c.*, *Escherichia coli* G; *K. p.*, *Klebsiella pneumoniae* ATCC 10031; *E. ae.*, *Enterobacter aerogenes* ATCC 8308; *S. t.*, *Salmonella typhi* Watson; *S. s.*, *Shigella sonnei* ATCC 11060; *P. m.*, *Proteus mirabilis* ATCC 9921; *P. v.*, *Proteus vulgaris* X 20; *E. c.t.*, *Escherichia coli* TEM; *K. ae.*, *Klebsiella aerogenes* 1082 E; *E. cl.* P 99, *Enterobacter cloacae* P 99; *P. ae.*, *Pseudomonas aeruginosa* ATCC 9027; *B. fr.*, *Bacteroides fragilis* VPI 9032.

[b] n.t.=not tested.

were considered worth further study, either because of their more balanced spectrum (in particular, an up to four fold increase of potency against Gram-positive bacteria was observed for **1**, **4**, **5** and **12**), or for the promise[5~7] of favorable pharmacokinetic properties connected with the presence of a free carboxy group on the heterocyclic moiety (entries **8**~**11**). Furthermore, two derivatives (**5**, **12**) showed useful levels of activity against *Enterobacter cloacae* P 99, producer of type Ia β-lactamase. Compounds **1**, **5**, **9** and **12** were accordingly selected for plasma half-life evaluation in mice.

A single intravenous injection of 50 mg/kg of each compound was given to Crl: CD-1 (ICR) BR mice. Groups of 8 mice (20±1 g) were used for each sampling time. The antibiotic concentration in serum was measured by an agar well technique on Folic TE medium (Difco), with 5% added horse serum, using *Bacillus pumilus* NCTC 8241 as indicator organism. The half-life in serum was calculated by the least squares method in the terminal linear phase of the curve obtained by plotting the logarithms of concentrations against time.

As shown in Table 3 the introduction of a carboxy group in the heterobicyclic ring (compound **9**) markedly increased the plasma half-life, while other modifications left it unchanged (**5**) or even reduced it (**12**). Compound **9** is without any doubt one of the cephalosporins with more prolonged half-life in mice. Compound **12** was discarded because of its too short half-life and compounds **1**, **5** and **9** were compared *in vivo* with cefazolin, cefuroxime and cefotaxime (Table 4). The compounds were tested *in vivo* in mice against experimental infections with *Streptococcus pyogenes* C 203, *Escherichia coli* G and *Proteus mirabilis* ATCC 9921; the most interesting compound **9** was also tested on *Haemophilus influenzae* 10479 and *Salmonella typhi* Watson infections.

The method used was as follows: male albino mice IVA: NMRI (SPF), weighing 18~20 g, were infected intraperitoneally with the bacterial suspension (stored at −80°C and diluted at the moment of use in Brain-Heart infusion broth, with or without gastric mucin depending on the virulence of the strain) in quantities corresponding to the LD_{99}. Treatment was given subcutaneously immediately after infection and three hours later, according to a scheme of complete balanced blocks, administering the drugs randomly (2 groups of 6 or 7 mice per dose). The animals were kept under observation for seven days. The medium effective dose (ED_{50} and fiducial limits for P=0.95) were calculated by probit analysis[8].

Of our compounds, entry **9** proved most active against Gram-negative organisms. It was 7 to 23 times more active than **1**, from 5 to 35 times more active than **5**, from 22 to 180 times more active than cefazolin and from 50 to 180 times more active than cefuroxime. It was as active as cefotaxime against *E. coli* G, *P. mirabilis* ATCC 9921 and *S. typhi* Watson; its activity was 6 times that of cefotaxime against *H. influenzae* 10479. Against *Streptococcus pyogenes* C 203 it was as active as compound **1** and cefazolin, but less active than **5**, cefuroxime and cefotaxime. Further work is in progress to fully evaluate the potentialities of this new long-lasting third generation cephalosporin.

Experimental

Infrared spectra were recorded on a Perkin-Elmer spectrometer (model 125). The NMR spectra were determined on a Bruker HX-90 (90 MHz) spectrometer; chemical shifts are reported in parts per million (δ) relative to Me_4Si. Melting points are not corrected and often not accurately reproducible because of extensive decomposition.

7β-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic Acid Dihydrate (**9**)

Table 3. Plasma half-life in mice after i.v. injection of 50 mg/kg.

Compound	$t^1/_2$ (minutes)
1	10
5	10
9	90
12	7
Cefazolin	16
Cefuroxime	14
Cefotaxime	14

Table 4. *In vivo* activity of **1**, **5**, **9**, cefazolin, cefuroxime and cefotaxime in acute systemic infection in mice.

Challenge organism	ED_{50} in mg/kg (Confidence limits for P=0.95)					
	1	**5**	**9**	Cefazolin	Cefuroxime	Cefotaxime
S. pyogenes C 203	0.77 (0.57~1.02)	0.15 (0.07~0.33)	0.38 (0.32~0.45)	0.58 (0.48~ 0.70)	0.04 (0.02~ 0.07)	0.09 (0.056~0.14)
E. coli G	2.28 (1.92~2.71)	1.82 (1.41~2.36)	0.34 (0.29~0.41)	7.53 (6.22~ 9.12)	23.6 (18.5 ~30.8)	0.2 (0.12 ~0.34)
P. mirabilis ATCC 9921	3.53 (2.4 ~5.18)	5.23 (3.69~7.40)	0.15 (0.05~0.46)	14.82 (12.47~17.81)	8.95 (7.02~11.4)	0.14 (0.04 ~0.46)
H. influenzae 10479	n.t.	n.t.	0.06 (0.03~0.09)	11.29 (7.6 ~15.11)	10.88 (7.78~14.11)	0.36 (0.19 ~0.68)
S. typhi Watson	n.t.	n.t.	0.17 (0.13~0.22)	11.83 (8.93~15.67)	9.0 (5.62~14.4)	0.27 (0.14 ~0.53)

n.t.=not tested.

To a stirred solution of (*Z*)-2-methoxyimino-2-(2-tritylaminothiazol-4-yl)acetic acid (11.35 g, 0.0256 mol) and triethylamine (3.61 ml) in dry methylene chloride (200 ml), cooled to 0°C, phosphorus pentachloride (5.6 g, 0.027 mol) was added portionwise. After stirring for 15 minutes at 0°C and 1 hour at room temperature, the mixture was evaporated under reduced pressure, taken up with dry benzene and evaporated again to remove any trace of phosphorus oxychloride. This treatment was repeated twice (2×50 ml). The residue was taken up in 50 ml of dry acetone and the separated triethylamine hydrochloride removed by filtration. The acetone solution of the acyl chloride was then dropped into a cold (0°C), vigorously stirred solution of 7β-amino-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid (10 g, 0.0232 mol) and $NaHCO_3$ (15 g) in a mixture of water (500 ml) and acetone (250 ml). After stirring for 30 minutes at 0~5°C and 90 minutes at room temperature, any undissolved matter was filtered off and most of the acetone was removed *in vacuo*. The resulting aqueous solution was adjusted to pH 2.0 with 8% HCl and extracted with ethyl acetate (3× 400 ml). Washing the organic extracts with aqueous NaCl solution, drying over Na_2SO_4 and removal of the solvent yielded a residue, which was triturated with ethyl ether, filtered, washed with fresh ethyl ether and dried to give 7β-[(*Z*)-2-methoxyimino-2-(2-tritylaminothiazol-4-yl)acetamido]-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid as a white solid. This compound was added portionwise to a stirred warm (55°C) solution of 50% aqueous formic acid (140 ml).

After 30 minutes at 55°C the solid (triphenylmethanol) was filtered off, the filtrate was evaporated *in vacuo* and the residue triturated with water to give the crude product. This was dissolved in aqueous CH_3COONa solution (at pH 5.2), filtered from any undissolved material and chromatographed on Al_2O_3 previously treated with pH 5.1 phosphate buffer. Elution with pH 5.4 phosphate buffer followed by 2% aqueous CH_3COONa solution and subsequent acidification of the appropriate fractions with 8% HCl gave a solid which was filtered and dried to give 9.1 g (60%) of **9**: mp 255°C (dec.); TLC on silica gel gave a single spot with chloroform - methanol - formic acid - water (140: 75: 20: 20), Rf 0.25. IR (KBr): 3500~2300, 1765, 1710, 1650, 1620~1580, 1540 cm^{-1}; NMR (DMSO-d_6): δ 3.68 (1H, d, 2-CH_2), 3.86 (1H, d, 2-CH_2), 3.92 (3H, s, OCH_3), 4.36 (1H, d, 3-CH_2), 4.69 (1H, d, 3-CH_2), 5.21 (1H, d, 6-H), 5.85 (1H, d-d, 7-H), 6.83 (1H, s, 5-H on thiazole ring), 7.30 (2H, br-s, NH_2 on thiazole ring), 8.02 (1H, s, 7-H on pyridazine ring), 9.38 (1H, d, CONH).

Anal. Calcd. for $C_{19}H_{16}N_{10}O_7S_3 \cdot 2H_2O$: C 36.30, H 3.20, N 22.28, S 15.30.
Found: C 35.88, H 3.25, N 21.75, S 15.05.

7β-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-hydroxyiminoacetamido]-3-(8-aminotetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic Acid (**4**)

An acetone solution of (*Z*)-2-(2-tritylaminothiazol-4-yl)-2-trityloxyiminoacetyl chloride was prepared from 2.25 g (3.5 mmol) of the parent acid[2], triethylamine (0.47 ml) and phosphorus pentachloride (0.697 g) following an experimental procedure analogous to that described for preparation of compound **9**. The above solution was dropped into a cold (0°C), vigorously stirred solution of 7β-amino-3-(8-aminotetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid (0.76 g, 2 mmol), triethylamine (0.562 ml, 4 mmol) and $NaHCO_3$ (0.281 g) in water (35 ml) and acetone (25 ml). After 30 minutes at 0°C and 90 minutes at room temperature, the reaction mixture was poured into ethyl acetate (350 ml), diluted with water (50 ml) and then adjusted to pH 2.0 with 8% HCl. The organic phase was separated, washed with aqueous NaCl solution, dried (Na_2SO_4) and evaporated to dryness. The resulting foam was triturated with ethyl ether to give 1.98 g of crude 7β-[(*Z*)-2-(2-tritylaminothiazol-4-yl)-2-trityloxyiminoacetamido]-3-(8-aminotetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid, contaminated by a considerable amount of the starting acid, which could in part be removed by dissolving the mixture in dioxane (10 ml) and dropping the resulting solution into ethyl ether (70 ml). The purified intermediate (1 g) was added under stirring to a 50% aqueous formic acid solution (40 ml) kept at 55°C (oil bath). After 35 minutes the mixture was cooled to 25°C and filtered; the solid was washed with fresh 50% formic acid (20 ml) and then with distilled water and discarded. The acidic solutions were combined and evaporated under reduced pressure. The residue was taken up in 99% ethanol, evaporated to a small volume (5 ml) and filtered.

The resulting powder was dissolved in 2% aqueous $NaHCO_3$ solution (20 ml), charcoal was added, and the filtered solution was adjusted to pH 2.0 with 2 N HCl. After 5 minutes stirring, the precipitate

was collected by filtration, washed in sequence with water and a small amount of ethanol, and dried at 65°C for 16 hours, to give 0.25 g of **4**; mp 205°C (dec.). TLC on silica gel gave a single spot with chloroform - methanol - formic acid (160: 70: 30), Rf 0.36. IR (KBr): 3400, 3000, 1760 cm^{-1}; NMR (DMSO-d_6): δ 3.61 (1H, d, 2-CH_2), 3.89 (1H, d, 2-CH_2), 4.16 (1H, d, 3-CH_2), 4.60 (1H, d, 3-CH_2), 5.21 (1H, d, 6-H), 5.86 (1H, d-d, 7-H), 6.42 (1H, s, 7-H on pyridazine ring), 6.76 (1H, s, 5-H on thiazole ring), 7.20 (2H, br-s, NH_2 on thiazole ring), 8.02 (2H, br-s, NH_2 on pyridazine ring), 9.56 (1H, d, CONH), 11.66 (1H, br-s, OH).

Anal. Calcd. for $C_{17}H_{15}N_{11}O_5S_3$: C 37.15, H 2.75, N 28.03, S 17.20.
Found: C 36.81, H 2.83, N 27.73, S 16.92.

7β-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-(2-carboxy-2-propoxyimino)acetamido]-3-(8-carboxytetrazolo-[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic Acid, Hydrochloride (**11**)

Starting from (*Z*)-2-(2-*tert*-butoxycarbonyl-2-propoxyimino)-2-(2-tritylaminothiazol-4-yl)acetic acid[9] and 7β-amino-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid, and following the procedure described for the preparation of **4**, 7β-[(*Z*)-2-(2-*tert*-butoxycarbonyl-2-propoxyimino)-2-(2-tritylaminothiazol-4-yl)acetamido]-3-(8-carboxytetrazolo[1,5-b]pyridazin-6-yl)thiomethyl-3-cephem-4-carboxylic acid was obtained as a white powder (65%).

An ice-cold solution of this compound (1.5 g) in 99% formic acid (7 ml) and 37% hydrochloric acid (0.4 ml) was stirred for 1 hour at 0°C and 20 minutes at room temperature. The precipitate was removed by filtration and isopropyl ether was added to the solution. The separated amorphous solid was collected and taken up in dry ethanol (20 ml). After stirring for 20 minutes at room temperature, ethyl ether (20 ml) was added, causing the precipitation of a brownish portion which was discarded. The filtrate was diluted with isopropyl ether (70 ml); the white precipitate was filtered, washed with isopropyl ether and dried *in vacuo* to give 0.62 g (57%) of **11**, mp 200°C (dec.). TLC on silica gel gave a single spot with chloroform - methanol - formic acid - water (140: 75: 20: 20), Rf 0.20. IR (KBr): 3600~3200, 3000~2400, 1780, 1725, 1675, 1630, 1540, 1445~1360, 1260~1170, 790 cm^{-1}; NMR (CF_3COOH-*d*): δ 1.87 (6H, s, Me_2), 4.01 (2H, ABq, 2-CH_2), 4.78 (2H, ABq, 3-CH_2), 5.40 (1H, d, 6-H), 6.13 (1H, d, 7-H), 7.56 (1H, s, 5-H on thiazole ring), 8.39 (1H, s, 7-H on pyridazine ring).

Anal. Calcd. for $C_{22}H_{20}N_{10}O_9S_3 \cdot HCl$: C 37.69, H 3.02, N 19.98, Cl 5.06, S 13.72.
Found: C 37.23, H 3.15, N 19.78, Cl 4.87, S 13.41.

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