GLYCOTHIOHEXIDE α, A NOVEL ANTIBIOTIC PRODUCED BY "*Sebekia*" sp., LL-14E605

III. STRUCTURAL ELUCIDATION

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The chemical structure of a novel thiopeptide antibiotic, glycothiohexide α (**1**), isolated from the fermentation broth of a "*Sebekia*" species was determined based on extensive 2D NMR studies, as well as, IR, UV, and mass spectral data. The chemical structure of glycothiohexide α is closely related to nosiheptide (**3**) and antibiotic S-54832A.

Glycothiohexide α, produced by a "*Sebekia*" species (NRRL 21083), was discovered in the course of screening microbial fermentations for antibacterial activity against antibiotic-resistant bacteria derived from clinical specimens. Preliminary characterization data suggested that glycothiohexide α was related to nosiheptide but could be distinguished from it. The isolation and the preliminary characterization of glycothiohexide α are reported in the preceding paper;[1] its antimicrobial activity will be reported separately.[2] The chemical structure of glycothiohexide α was elucidated based largely on spectroscopic analysis as described in this report.

Results and Discussion

The molecular formula of glycothiohexide α (**1**) (Fig. 1) was established as $C_{58}H_{57}N_{13}O_{15}S_6$ from an analysis of mass spectral, NMR, ESCA and IR data. An initial survey of the ^1H and ^{13}C NMR spectra revealed the presence of at least 43 hydrogens and 50 carbons. An ESCA spectrum revealed the presence of substantial amounts of nitrogen and sulfur in an approximate ratio of 2 : 1. A very strong amide carbonyl stretching band at 1668 cm^{-1} in the IR spectrum confirmed the presence of nitrogen and oxygen. An approximate elemental composition of $C_{50\sim60}H_{40\sim60}N_{7\sim16}O_{4\sim20}S_{4\sim8}$ was established based on the above analysis. A molecular ion $(M+H)^+$ of 1,367±1 amu for glycothiohexide α was observed in electrospray mass spectroscopy while high resolution FAB-MS showed an $(M+Na)^+$ ion at 1,390.2307. Based on the constrains above, the molecular formula of glycothiohexide α was determined to be $C_{58}H_{57}N_{13}O_{15}S_6$ (Δ=0.8 mmu). More detailed analysis of the complete set of NMR data revealed resonances for all 58 carbons and 53 of the protons (4 of the 10 exchangeable protons could not be assigned). Good evidence for the presence of all of the nitrogen, oxygen, and sulfur atoms was also found in the NMR spectra.

Sharp singlets in the ^1H NMR spectrum between 9.0 and 7.7 ppm integrating for one proton each suggested the presence of disubstituted thiazole rings. This possibility was confirmed by inverse detected carbon hydrogen correlation experiments (HMQC and HMBC). All six singlets correlated to carbons

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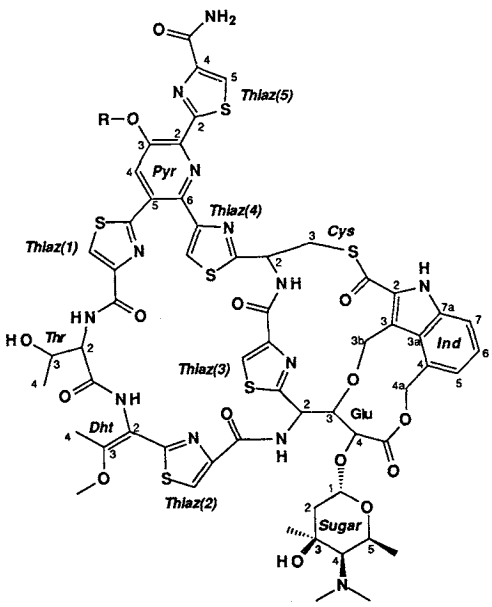
Table 1. NMR data of glycothiohexide α (1) in d_6-DMSO (50°C).

Assignment[a]	^{13}C shift[b] (ppm)	DEPT	$^{1}J_{CH}$[c] (Hz)	^{1}H shift[d] (ppm)	^{1}H multiplicity (Hz)	HMBC ($^{n}J_{CH}$) correlation	COSY[e] correlation	ROESY[f] (correlation)
Thiaz(1)2	163.54	C				8.85, 7.86		
Thiaz(1)4	150.17	C				8.85		
Thiaz(1)5	126.23	CH	197	8.85	s, 1H			
Thiaz(1)CO	158.14	C				8.85, 7.25		
Thiaz(2)2	162.39	C				8.20		
Thiaz(2)4	145.56	C				8.20		
Thiaz(2)5	124.63	CH	195	8.20	s, 1H			1.42, 0.59
Thiaz(2)CO	160.2	C				8.59, 8.20		
Thiaz(3)2	167.25	C				8.50, 5.73		
Thiaz(3)4	148.89	C				8.50		
Thiaz(3)5	125.28	CH	197	8.50	s, 1H		5.73*	
Thiaz(3)CO	160.2	C				8.50, 7.64		
Thiaz(4)2	168.67	C				7.91		
Thiaz(4)4	154.05	C				7.91		
Thiaz(4)5	119.94	CH	195	7.91	s, 1H		5.93*	
Thiaz(5)2	167.6	C				8.41		
Thiaz(5)4	150.75	C				8.41		
Thiaz(5)5	126.23	CH	193	8.41	s, 1H			
Thiaz(5)CO	161.27	C				8.41		
Pyr2	134.24	C				7.86		
Pyr3	150.49	C				7.86		
Pyr4	127.18	CH	166	7.86	s, 1H			
Pyr5	130.19	C						
Pyr6	142.9	C				7.91, 7.86		
ThrCO	167.64	C				8.77, 7.25, 4.22		
ThrN				7.25	d (7.7), 1H		4.22	4.22, 2.50w, 1.25
Thr2	55.25	CH	147	4.22	dd (7.8, 4.4), 1H	7.25, 1.25	7.25, 2.50	8.77, 7.25, 2.50
Thr3	65.38	CH	151	2.5	m, 1H	4.22, 1.25	4.32, 4.22, 1.25	7.25w, 4.32, 4.22
Thr3OH				4.32	d (6.5)		2.5	8.77, 8.59, 2.50, 1.25
Thr4	17.51	CH_3	125	1.25	d (6.5), 3H	4.22	2.5	7.25, 4.32
DhtN				8.77	br s, 1H		1.97*	4.32, 4.22, 1.97
Dht2	109.94	C				1.97		
Dht3	160.16	C				8.77, 3.88, 1.97		
DhtOMe	55.69	CH_3	149	3.88	s, 3H			1.97
Dht4	12.71	CH_3	130	1.97	s, 3H		8.77*	8.77, 3.88

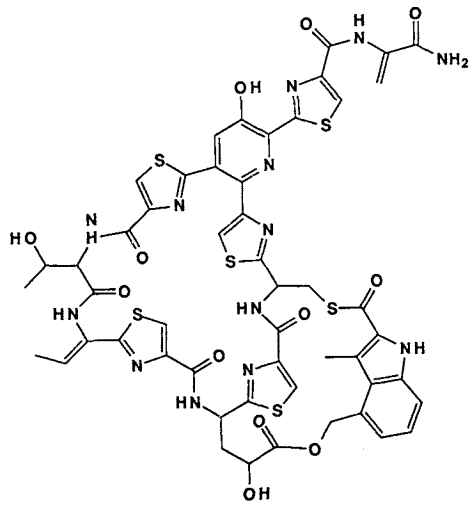
GluN				8.59	d (8.7), 1H		5.73	5.73, 4.32, 4.27, 3.97, 1.25
Glu2	50.3	CH	140	5.73	br d (8.7), 1H	4.27	8.59, 8.50*, 3.97	8.59, 4.27, 3.97, 1.42w
Glu3	79.82	CH	150	3.97	br d (9.7), 1H	4.27, 4.94	5.73, 4.27	8.59, 5.73, 4.94
Glu4	70.44	CH	152	4.27	d (9.8)		3.97	5.73, 4.96
Glu5	171.35	C				6.00, 4.27		
Sug1	94.91	CH	174	4.96	br d (4.5), 1H	4.27, 1.80	1.91, 1.80	4.27, 2.51w, 1.91w, 1.80
Sug2	40.21	CH_2	124	1.91	dd (14.3, 4.5), 1H	1.42	4.96, 1.80, 1.42*	4.96w, 1.80, 2.51
			129	1.80	br d (14.3), 1H		4.96, 2.06*, 1.91	4.96, 1.91, 1.42
Sug3	67.26	C				4.96, 1.91, 1.80, 1.42		
Sug3Me	30.15	CH_3	126	1.42	s, 3H	2.06, 1.91, 1.80	1.91*	8.20w, 5.73w, 3.79, 2.06, 1.80
Sug4	68.24	CH	133	2.06	br s, 1H	2.51, 1.81, 0.59	3.79, 1.80*	3.79, 2.51, 1.42, 0.59
Sug4NMe_2	44.04	CH_3	133	2.51	s, 6H	2.51, 2.06		4.96w, 2.06, 1.91, 0.59
Sug5	66.09	CH	140	3.79	m, 1H	4.96, 0.59	2.06, 0.59	2.06, 1.42, 0.59
Sug6	17.61	CH_3	126	0.59	d (6.5), 3H		3.79	8.20w, 3.79, 2.51, 2.06
IndN				11.37	br s, 1H			
Ind2	132.5	C				4.94, 4.09		
Ind3	112.93	C				4.94, 4.09		
Ind3a	124.18	C				5.03, 4.94, 4.09		
Ind3b	64.11	CH_2	151	4.94	d (10.4), 1H	3.97	4.09	4.09, 3.97
			140	4.09	d (10.4), 1H		4.94	6.00, 4.94
Ind4	128.33	C				7.34, 6.00, 5.03		
Ind4a	67.22	CH_2	146	6.00	d (12.4), 1H	7.17	7.78*, 7.34*, 7.17*, 5.03	5.03, 4.09
			152	5.03	d (12.5), 1H		7.17*, 6.00	7.17, 6.00
Ind5	122.73	CH	162	7.17	d (7.0), 1H	7.78, 6.00, 5.03	7.78*, 7.34, 6.00*, 5.03*	7.34, 5.03
Ind6	123.96	CH	161	7.34	dd (8.1, 7.2), 1H	7.78, 7.17	7.78, 7.17, 6.00*	7.17
Ind7	115.93	CH	168	7.78	d (8.4), 1H	7.17	7.34, 7.17*, 6.00*	
Ind7a	137.36	C						
IndCO	183.73	C				3.48		
CysN				7.64	d (10, 7), 1H		5.93	5.93
Cys2	50.17	CH	142	5.93	br d (10.6), 1H	7.64	7.91*, 7.64, 3.74, 3.48	7.64
Cys3	30.05	CH_2	147	3.74	dd (18.2), 1H		5.93, 3.48	
Cys3			143	3.48	m, 1H		5.93, 3.74	

[a] The numbering system used for nosiheptide in ref 4 was adopted.
[b] 75 MHz, d_6-DMSO 50°C.
[c] Determined from the splitting pattern in a coupled HMQC experiment.
[d] 300 MHz, d_6-DMSO 50°C.
[e] *, Observed in a COSY experiment optimized for the detection of small couplings.
[f] w, Weak correlation.

Fig. 1. The chemical structures of glycothiohexide α (**1**), *O*-methyl-glycothiohexide α (**2**), and nosiheptide (**3**).



Glycothiohexide α (**1**) R=H
O-Methyl-glycothiohexide α (**2**) R=CH_3



Nosiheptide (**3**)

between 119 and 128 ppm in the proton decoupled $^1J_{CH}$ HMQC experiment (see Table 1). Five of these six proton resonances showed $^1J_{CH}$ coupling constants between 193 and 197 Hz in a coupled HMQC experiment (Table 1). The combination of proton and carbon chemical shifts with the larger than normal $^1J_{CH}$ coupling constant is diagnostic for 2,4-disubstituted thiazoles where the proton is attached to a carbon adjacent to the sulfur.[3] An HMBC experiment optimized for $^nJ_{CH}$ coupling constants of 7 Hz revealed the expected C-2 and C-4 carbons of each thiazole ring.

An examination of the structures of published thiazole containing antibiotics revealed that glycothiohexide α shared many structural features with nosiheptide (**3**) as was suggested by their nearly identical UV spectra.[1,4] A comparison of chemical shifts, homo and heteronuclear scalar (COSY, HMQC, HMBC) and homonuclear NOEs (ROESY) correlations (Table 1) showed that glycothiohexide α and nosiheptide have the same overall structure, but differ in the five areas discussed below.

Methoxy-dehydrothreonine Residue

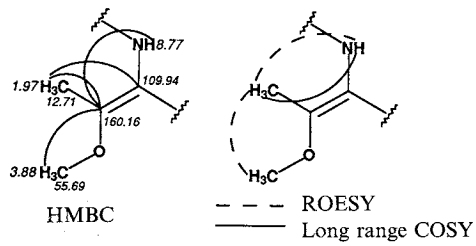
Three proton singlets (δ_H 8.77 1H, 3.88 3H, 1.97 3H) and four carbon resonances (δ_C 160.16, 109.94, 55.69, 12.71) were assigned to a methoxy-dehydrothreonine residue on the basis of chemical shifts, HMBC and ROESY correlations as illustrated in Fig. 2. This fragment replaces the 2,3-dehydro-2-aminobutyric acid residue in nosiheptide (**3**). None of the proton resonances in this structural fragment showed coupling in the normal COSY experiment performed on glycothiohexide α (**1**) or its methyl ether (**2**). The ^1H singlet (δ_H 8.77) showed no attachment to a carbon in the HMQC experiment, and was assigned on this basis as an amide NH. The 3H singlet at δ_H 3.88 was correlated to a methoxy carbon at δ_C 55.69 while the 3H singlet at δ_H 1.97 to a methyl carbon at δ_C 12.71 in the HMQC. In the HMBC experiment, the protons

of the methoxy group showed correlation to a non-protonated carbon (δ_C 160.16). Since a methoxy ether group can only long-range J_{CH} couple into its point of attachment, this carbon was assigned to the α-carbon of an enol ether. The NH proton resonance also coupled to the carbon at δ_C 160.16, while the remaining proton singlet (δ_H 1.97, 3H), showed coupling to this and another non-protonated carbon (δ_C 109.94). A coupling between the NH and CH_3 protons was observed in a COSY experiment optimized for long-range couplings indicating that these two groups are on opposite ends of the tetra-substituted double bond. The observed ROESY correlations from the olefinic methyl to both the NH and OCH_3 are consistent only with the *E* substitution pattern illustrated in Fig. 2.

Modified Glutamate Residue

In glycothiohexide α, the modified glutamate residue of nosiheptide is further hydroxylated at the 3 position. A four proton spin system, that showed an obvious linear vicinal coupling pattern in the COSY and ^1H experiments, was assigned to this modified glutamate residue. An amide NH, (δ_H 8.59, d, 8.7 Hz; no correlation in the HMQC) was coupled to a methine proton (δ_H 5.73, br d 8.7 Hz) that showed attachment to a methine carbon (δ_C 50.30) in the HMQC. This methine is adjacent to a carbinol methine, (δ_H 3.97, br d 9.7 Hz; δ_C 79.82) which in turn is adjacent to a third carbinol methine (δ_H 4.27, d, 9.8 Hz; δ_C 70.44) as revealed by COSY and HMQC correlations. The attachment of an ester carbonyl (δ_C 171.35) was indicated by an HMBC correlation to this carbon from the δ_H 4.27 methine resonance. Further evidence for the presence of a lactone in glycothiohexide α was also found in the IR spectrum where a small C=O stretching band at 1743 cm^{-1} (1741 cm^{-1} in the methyl ether) was observed.[1)]

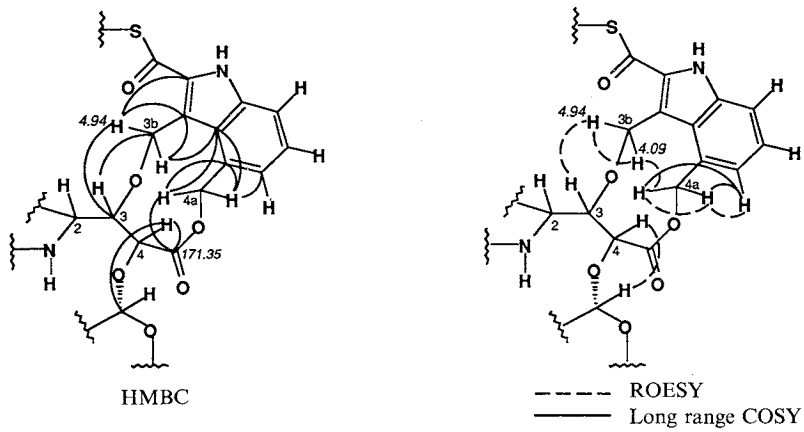
Fig. 2. Selected NMR data and correlations of the dehydrothreonine residue.



Indole Residue

The methyl substituent at C-3 of the indole fragment of nosiheptide is replaced by a carbinol methylene (δ_H 4.94 and 4.09 d, 10.4 Hz; δ_C 64.11) in glycothiohexide α. This assignment was established based on HMBC correlations from the methylene protons to three carbons of the indole

Fig. 3. Selected NMR correlations of the indole and glutamate residues.

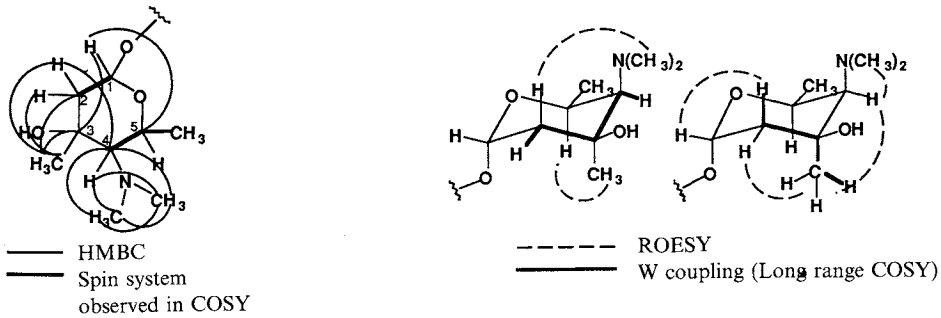


ring, and the observation of a ROESY correlation between the upfield methylene proton (δ_H 4.09) and a proton (δ_H 6.00) of the Ind4a carbinol methylene as illustrated in Fig. 3. The ether linkage between the Ind3b carbinol methylene and the carbinol methine at C-3 of the glutamate residue was indicated by an HMBC correlation from the proton at Glu3 to the Ind3b carbinol methylene carbon, and a similar correlation from the downfield carbinol methylene proton (δ_H 4.94) to the Glu3 carbon. Further evidence was found in the ROESY experiment where a cross peak was observed between the downfield proton of Ind3b and the Glu3 proton.

Glycosidic Residue

A number of non-olefinic carbon and hydrogen resonances with no analogy in nosiheptide were attributed to an amino-dideoxy-pyranose moiety. A carbon hydrogen pair (δ_C 94.91; δ_H 4.96) observed in the HMQC experiment was assigned as the anomeric center of a glycoside on the basis of their chemical shifts. In the normal COSY experiment, the anomeric proton showed coupling to a pair of methylene protons (δ_H 1.91, dd, δ_H 1.80, br d; δ_C 40.21; C-2) which were not correlated to other protons. A methine proton (δ_H 2.06; δ_C 68.24; C-4) coupled to a second methine (δ_H 3.79; δ_C 66.09; C-5) which was coupled to a methyl doublet (δ_H 0.59, d, 6.5 Hz, 3H; δ_C 17.61; C-6) constituted a second spin system. These two spin systems were assembled together with a quaternary carbinol carbon (δ_C 67.26; C-3), a methyl singlet (δ_H 1.42, 3H; δ_C 30.15), and an *N,N*-dimethyl moiety (δ_H 2.51, 6H; δ_C 44.04, 2C) to form the pyranose as discussed below. In addition to the chemical shift and the number of equivalent protons, evidence for the presence of two equivalent methyls attached to a single nitrogen was provided by an unusual HMBC three bond correlation from the protons of one methyl to the carbon of the other. The aliphatic methyl singlet at δ_H 1.42 showed HMBC correlations to the carbinol carbon at δ_C 67.26 and the methylene carbon (C-2) of the first proton spin system, indicating that the methyl was attached to the carbon at δ_C 67.26 (C-3) which is in turn attached to the methylene (C-2). This assignment was confirmed by three bond HMBC correlations from the anomeric proton to the carbinol carbon (C-3), and from both methylene protons to the methyl carbon (Fig. 4). The attachment of the *N,N*-dimethyl group to one end of the second spin system was established by three bond HMBC correlations from the C-4 methine proton (δ_H 2.06; δ_C 68.24) to the *N*-methyl carbons, and from the *N*-methyl protons to the C-4 methine carbon. A three bond HMBC correlation from one of the C-2 methylene protons (δ_H 1.80) to the C-4 methine carbon established the connection between C-3 and C-4 of the glycosidic ring. A three bond HMBC correlation from H-1 to C-5 through oxygen completed the pyranose ring. The attachment of the glycosidic unit to the aglycon through a glycosidic linkage to the C-4 carbinol methine of the modified glutamate residue was revealed by

Fig. 4. Selected NMR correlations of the glycosidic residue.



correlations from the Glu4 proton to the anomeric carbon in the HMBC experiment, and to the anomeric proton in the ROESY experiment (Fig. 3).

Further confirmation of the connectivity, and establishment of the relative stereochemistry of the glycosidic unit was provided by data from the long range COSY and ROESY experiments. The anomeric proton was assigned equatorial as its coupling to both of the C-2 methylene protons was less than 6 Hz. The upfield C-2 methylene proton (δ_H 1.80) is coupled through four bonds to the methine proton at C-4, which is possible only if both substituents are equatorial. The downfield C-2 methylene proton (δ_H 1.91) showed a four-bond W coupling to the protons of the methyl at C-3. A coplanar arrangement of the four bonds involved is possible only if both substituents are axial (Fig. 4). The observed ROESY correlation between the C-3 methyl and the C-5 methine resonances is consistent with a $1\sim3$ diaxial relation between these two substituents on a six membered ring in a chair conformation. Similarly, a $1\sim3$ diaxial NOE is observed between the methyls of the dimethylamine substituent and the axial proton (downfield) at C-2. Thus, the glycosidic unit was assigned to be 2,4-dideoxy-4-dimethylamino-3-methyl-α-fucopyranoside.

Dehydro-alanine Residues

All of the resonances for the dehydro-alanine residue found in nosiheptide are missing in glycothiohexide α. The chemical shift of the carbonyl attached to the terminal thiazole ring is similar to those in the rest of the molecule leading to its assignment as a primary amide terminus, consistent with the molecular formula.

Conclusion

The chemical structure of glycothiohexide α (**1**) is closely related to that of antibiotic S-54832A disclosed in a US patent.[5] A partial listing of carbon chemical shifts and a proton spectrum were disclosed in the patent with no details of the structural assignment. The proposed structure of antibiotic S-54832A has a different sugar, a hydroxyl substitution on the indole nitrogen and an ester, instead of the thioester linkage. It also has the dehydro-2-aminobutyric acid and the terminal dehydro-alanine residues found in nosiheptide.

Experimental

General

NMR spectra of glycothiohexide α were obtained on a Bruker AMX 300 instrument, while a GE Omega 500 MHz NMR was used to obtain the spectra of *O*-methyl-glycothiohexide α. Chemical shifts were determined in parts per million relative to the solvent signals of d_6-DMSO at 2.49 (^{1}H) and 39.5 (^{13}C) ppm. HMBC experiments were optimized for $^nJ_{CH}=7$ Hz or 4 Hz. FAB mass spectra were recorded using a VG-ZAB SE high performance mass spectrometer and a VG 11-250 data system.

Preparation of *O*-Methyl-glycothiohexide α (2)

To a solution of partially purified glycothiohexide α (109 mg in 2 ml each of MeOH and CH_2Cl_2) was added an excess of freshly prepared diazomethane etherate. The reaction mixture was stirred for 1 hour and was concentrated *in vacuo* to give 104 mg of 80% pure **2** which was further purified by countercurrent chromatography. The purification was conducted with a Sanki Centrifugal Partition Chromatograph using the following solvent system: toluene-dichloromethane-methanol-buffer (0.1 M trifluoroacetic acid, adjusted to pH 2.0 with concentrated NH_4OH), 108: 792: 700: 400 in the ascending mode to give 46 mg of analytically pure *O*-methyl-glycothiohexide α (**2**): CD λ_{max}^{MeOH} nm (θ) 221 ($-127{,}500$), 255 (85,000), 326 (10,000), 354 (22,500); UV λ_{max}^{MeOH} nm (ε) 296 (33,200), 349 (23,800); IR ν_{max} (KBr) cm^{-1} 3391, 2938, 1741, 1667, 1534, 1481; HRFAB-MS: 1,404.2474 $(M+Na)^+$. 2D NMR data, in addition to confirming the assignment for **1** in Table 1, showed correlation of Cys3H to Thiaz(4)2C, IndNH to Ind2C,

and Ind3C to Ind7aC.

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