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Synthetic studies of carzinophilin. Part 4: Chemical and biological properties of carzinophilin analogues☆

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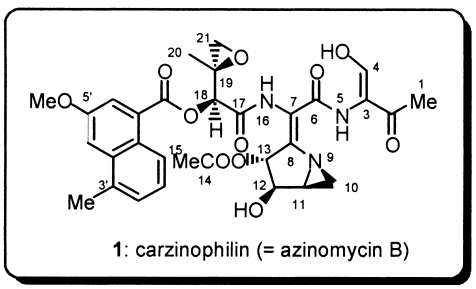
Abstract—Chemical and biological properties of carzinophilin congeners obtained in the course of our synthetic studies were investigated. These studies revealed feasibility for the use of some analogues as a double alkylating agent. Further, analogues carrying the naphthalene and the epoxide parts were found to show remarkable in vitro cytotoxicity and in vivo antitumor activity.

1. Introduction

Antitumor antibiotic carzinophilin (=azinomycin B[2,3], **1**) isolated in 1954[4] has been known as one of the oldest DNA intercalative bis-alkylating agents, before elucidation of its structure.[5] Interaction between **1** and DNA have been reported by Armstrong,[6,7] Saito,[8] Coleman,[9–11] Gates,[12] Shipman[13] and Imanishi.[14] They mainly explored the selectivity in DNA cleavage about the DNA sequence and its denaturing patterns. Interstrand cross-linkage is believed to occur by both aziridine and epoxide ring-openings induced by attacks of bases in DNA. However, the reaction mode in which **1** reacts with nucleophiles has been little discussed.[7,8] On the other hand, the unique structure and strong antitumor activity of **1** attracted organic chemists toward its total synthesis.[15] Recently, Coleman achieved an elegant total synthesis of azinomycin A,[16] the natural analogue of **1** lacking the C4 enol system. In the last decade, we have also made synthetic studies on **1**,[1] and reported preparation of the protected form of **1**[1e] as well as its various congeners.[1a–d] Our studies revealed that the 1-azabicyclo-[3.1.0]hexane system, the central framework of **1**, is susceptible to nucleophilic attacks resulting in aziridine ring-opening. We have further disclosed that some carzinophilin-related compounds exhibit potent in vitro cytotoxicity against P388 murine leukemia in communication forms.[1a–c,e] Now, we would like to describe full details of our studies on chemical and biological properties of the compounds prepared in this series of synthetic studies.



1: carzinophilin (= azinomycin B)

2. Results and discussion

2.1. Chemical stability and reaction mode of the 1-azabicyclo[3.1.0]hexane ring system against nucleophiles

It was found that diethyl (1-azabicyclo[3.1.0]hex-2-ylidene)-malonate (*ent*-**2**) and ethyl (*N*-pivaloyl-1-azabicyclo[3.1.0]-hex-2-ylidene)glycinate (*ent*-**3**) were quite susceptible to nucleophilic attacks. Silica gel column chromatography employed for the purification process decomposed *ent*-**2** completely. Florisil® column chromatography performed quickly only provided a pure sample of *ent*-**2** in low yield (34%) although the crude yield of *ent*-**2** was estimated to be around 80% in its preparation.[1a] The glycine derivative *ent*-**3** seemed to be slightly more stable than *ent*-**2**, because Florisil® column chromatography afforded a pure sample in a little higher yield (54%). The aziridine rings of both *ent*-**2** and *ent*-**3** were found to be cleaved easily at the C10 position (secondary carbon, *carzinophilin numbering*) by treating with thiophenol in the presence of one equivalent of triethylamine, giving phenylthiomethyl, derivatives, *ent*-**4a** and *ent*-**5a**, respectively. Similar aziridine ring-opening took place with an acetate anion under weakly acidic

☆ See Ref. 1a–e.

Keywords: antitumor activity; carzinophilin; anticancer activity; DNA alkylation.

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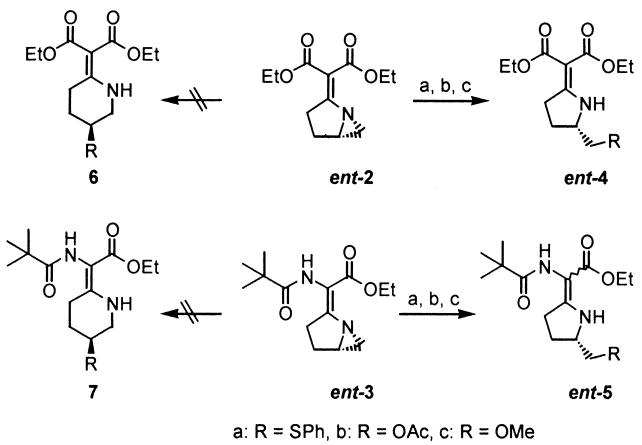


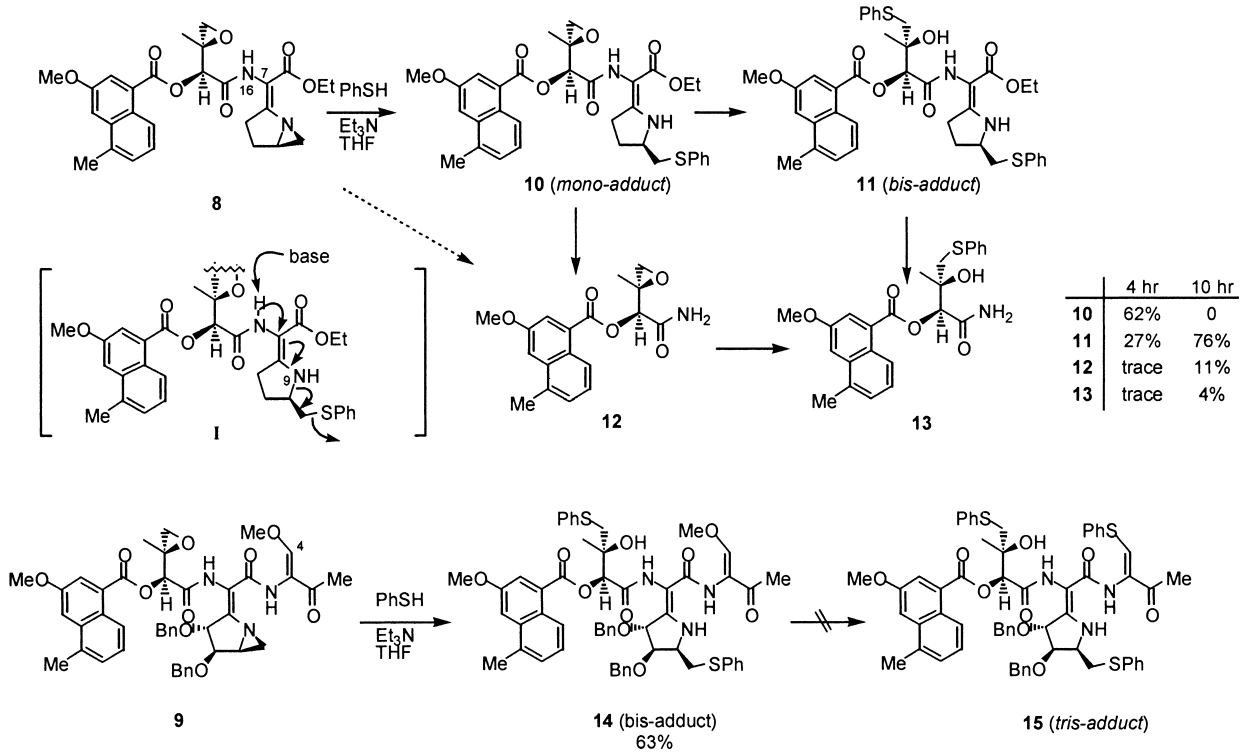
Scheme 1. Reactions of the 1-azabicyclo[3.1.0]hexane derivatives **2** and **3** with nucleophiles. *Reagents and conditions*: (a) PhSH, Et_3N, THF, rt, 62% (**4a**), 67% (**5a**). (b) AcOH, THF, rt, 74% (**4b**), 86% (**5b**), MeOH, rt, 80% (**4c**), 68% (**5c**).

conditions. In those reactions, nucleophilic attack to the C11 position (tertiary carbon, azinomycin numbering), giving the piperidine derivatives **6** or **7** was not observed at all. These bicyclic compounds **2** and **3** were not stable enough even under neutral conditions. Pure samples of both **2** and **3** gradually decomposed in MeOH giving 5-methoxymethyl-pyrrolidine derivatives, *ent*-**4c** and *ent*-**5c** in good yields after standing for several days. The structures of *ent*-**4a**–**c** were confirmed by observing the fragment ion peaks assigned as $[M-RCH_2]^+$ (R=PhS, AcO, or MeO) at *m*/*z*=226 with relatively strong intensity (*ent*-**4a**:11%, *ent*-**4b**:4.1%, *ent*-**4c**:66%). The ^1H NMR spectra of **4a**–**c** also supported the existence of the pyrrolidine rings. Similar observations on *ent*-**5a**–**c** proved their structures as depicted (Scheme 1).

We have also succeeded in synthesizing **8**, the model compound further carrying an epoxide function in the molecule as well as 13-*O*-desacetyl-12,13-di-*O*-benzyl-4-*O*-methylcarzinophilin **9**. These were found to be more stable than the simple models **2** and **3**. For example, **8** and **9** were readily purified by silica gel column or thin-layer chromatography, which conditions had decomposed almost all of **2** and **3**. However, treatment of **8** with thiophenol took place the aziridine ring opening to give the mono-adduct **10** smoothly. Interestingly, the epoxide moiety of **10** was also cleaved gradually by thiophenol, providing the bis-adduct **11** in 76% yield after 11 hours. Under those conditions, amides **12** and **13** were obtained as minor products. Formation of **12** and **13** is tentatively considered to be induced by removal of N16H and following hydrolysis as shown for **I**. However, direct hydrolysis of the C7N16 enamine in **8** might be also possible. Similarly, the *O*-protected carzinophilin derivative **9** also underwent the same reaction to give the bis-adduct **14** in 63% yield. Michael addition of thiophenol to the C4 position of **14** was also conceivable, and subsequent elimination of the methoxy group may give rise to the tris-adduct **15**. However, **15** was not detected so far as we examined. These observations may mimic a putative double-linking property of DNA with **1** although thiophenol was employed as the nucleophile in place of nucleotides (Scheme 2).

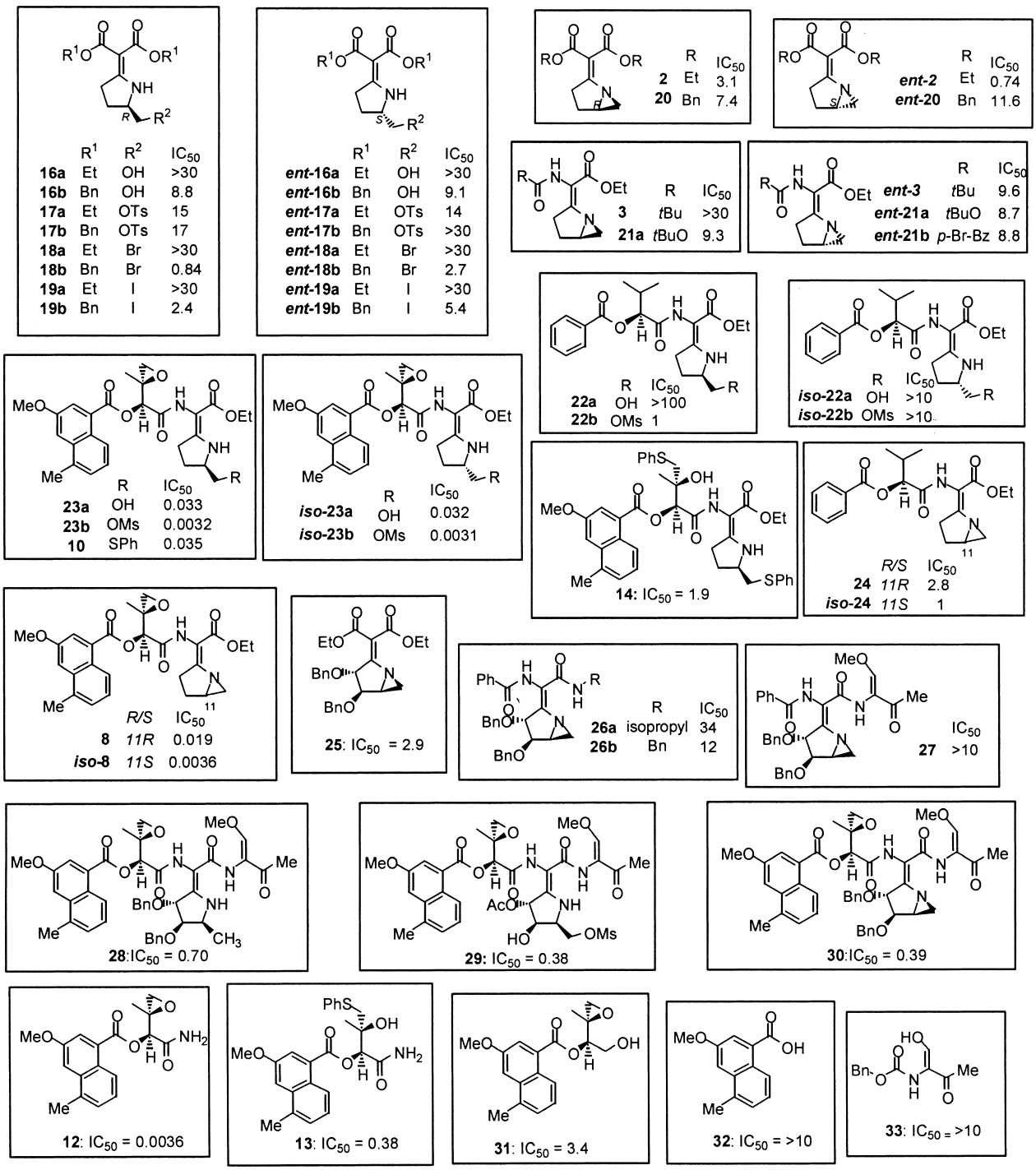
2.2. In vitro cytotoxicity of carzinophilin analogues synthesized in our synthetic studies

We also aimed to develop a novel anticancer drug from the compounds obtained in the series of synthetic studies on **1**. Thus, 51 related compounds were subjected to cytotoxicity assay employing P388 murine leukemia in vitro. The values of IC_{50} are summarized in Table 1. Adriamycin was



Scheme 2. Reactions of thiophenol with highly functional analogues **8** and **9**.

Table 1. IC_{50} Values of carzinophilin related analogues against P388 murine leukemia in vitro (μg/mL)



employed as a reference compound showing the IC_{50} to be $1-3\times10^{-3}$ μg/mL. Most of the monocyclic malonylidenepyrrolidines **16**–**19** and their enantiomers *ent*-**16**–*ent*-**19** showed no cytotoxicity, however, bromide or iodide with dibenzyl ester **18b**, *ent*-**18b**, **19b**, and *ent*-**19b** exhibited weak activity (**18b**:0.84. *ent*-**18b**:2.7, **19b**:2.4, *ent*-**19b**:5.4). Enantiomers carrying 11*R* configuration showed a tendency to exhibit a little stronger activity than those of the 11*S*-isomers. Both enantiomers of bicyclic malonylidene diethyl esters **2** and *ent*-**2** showed weak cytotoxicity, but neither enantiomer of dibenzyl ester **20** nor *ent*-**20** had cytotoxicity. Although the bicyclic models with simple glycinate ester groups (**3**, *ent*-**3**, **21a**, and *ent*-**21a**,**b**) exhibited no cytotoxicity, replacement of the *N*-acyl group with the C17–C21 unit significantly intensified the activity. Especially, mesylates **23b** and *iso*-**23b** inhibited increasing of cell numbers as strongly as adriamycin[17] which is now in clinical usage. Bicyclic molecule *iso*-**8**, which carries the

Table 2. Antitumor activity against P388 leukemia in vivo

Compound	Dose (mg/kg)	*T/C* (%)
12	40	109
	20	109
	10	146
23b	60	141
	30	139
	15	146
ent-**23b**	40	127
	20	127
	10	114

Table 3. Anticellular activity against human uterine cervix carcinoma Hela S_3 cells in vitro

Compounds	IC_{50} (μM)	
	1 h	72 h
12	0.12	0.035
23b	1.8	0.049
ADM	0.53	0.074
MMC	2.0	0.13

Hela S3 cells (8×10^2/well) were cultured on day 0, and treated with the compounds for 1 and 72 h from day 1. On day 4, the anticellular activity was determined by the neutral red dye uptake method.

left-hand functionalities, also inhibited the cell number growth. The naphthalene and the epoxide moieties seem to be required for the enhanced cytotoxicity, because the activity of benzoates **22a**,**b**, *iso*-**22a**,**b** **24**, and *iso*-**24** was found to be very weak. Functionalization at the C12 and C13 positions did not enhance the cytotoxicity (see **25**–**27**). The analogues with C1–C21 carrying the naphthalene moiety exhibited acceptable cytotoxicity although these compounds did not have the aziridine ring in their molecules (see **28**–**30**). Based on our experiments, these congeners may react with nucleophiles only at the C21 epoxide carbon. Deleting the epoxide function decreased the cytotoxicity remarkably as shown for **13** and **14**. Our studies also revealed that amide **12** exhibits cytotoxicity in a similar level to that of adriamycin though it has been isolated as an inactive constituent from the same culture broth as **1**.[2] The amide **12** possesses the C20C21 epoxide but not the aziridine moiety. Thus, **12** may act as a single alkylating agent. Interestingly, sulfide **13**, the analogue of **12** without epoxide, also showed relatively strong cytotoxicity. The naphthoate part seemed to be the most important for the activity. But simple naphthoic acid **32** was inactive. Quite recently, Coleman et al. also described the role of the left-hand moiety of **1** in cross-link formation with DNA.[11] Finally, the C1–C6 fragment **33** was found to show no activity. As mentioned above, we have succeeded in finding some congeners such as **12**, **23b**, and *ent*-**23b** to be promising as an antitumor agent in the first screening. However, we found those compounds except for **8**, *iso*-**8** and **30** bearing both the epoxide and aziridine moieties, can not make double linked complex with DNA. Those cytotoxicities might be caused by a different mechanism from that of the double alkylation of DNA. Thus, it is difficult at this stage to summarize the structure activity relationship from those results.

Congeners **12**, **23b**, and *ent*-**23b**, showing promising cytotoxic activity were further subjected to in vivo antitumor assay against P388 murine leukemia (Table 2). These experiments revealed that **12** and **23b** showed weak antitumor activity. As for **12** and **23b**, anticellular activity was further determined employing Hela S_3 cells in vitro. It was found that that both of them showed strong activity which is almost the same level as that of adriamycin (ADM) and mitomycin C (MMC)[18] as shown in Table 3. These congeners **12** and **23b** were further subjected to in vivo antitumor activity against murine Sarcoma 180 employing MMC as a reference (Table 4). Although amide **12** did not show remarkable activity in this assay, carzinophilin analogue **23b** was found to shrink Sarcoma 180 more significantly by administrating 60 mg/kg of **23b** intravenously on days 1 and 4 (run 11). This antitumor activity is stronger than that of MMC (see run 12). However, these experiments suggested that a large amount of **23b** was required to be administered for effective tumor degeneration. The amount is excessive from the viewpoint of use as a medicinal drug. In other words, more than two grams of **23b** must be taken once in four days based on the simple calculations. On the other hand, body weight of the mice

Table 4. Antitumor activity against murine Sarcoma 180 in vitro

Run	Compounds	Dose (mg/kg/day)	Schedule	Tumor size (mm^3)	*T/C*	Body weight[a] (g)	Mortality
1	Control	0		1958±270	1.00	+7.6	0/5
2	**12**	1.3	Days 1, 4	2074±275	1.06	+7.3	0/5
3		2.5	Days 1, 4	1666±308	0.85	+5.1	0/5
4		5.0	Days 1, 4	1751±460	0.89	+7.2	0/5
5		10	Days 1, 4	1682±309	0.86	+6.1	0/5
6		20	Days 1, 4	1524±197	0.78	+4.7	0/5
7	**23b**	3.8	Days 1, 4	1788±645	0.91	+7.4	0/5
8		7.5	Days 1, 4	1800±667	0.92	+5.2	0/5
9		15	Days 1, 4	1818±409	0.93	+6.6	0/5
10		30	Days 1, 4	1465±432	0.75	+3.9	0/5
11		60	Days 1, 4	372±208	0.19	+0.3	0/5
12	MMC	6.0	Day 1	648±179	0.33	+2.8	0/5

Sarcoma 180 cells (5×10^6/mouse) were inoculated into male mice (body weight=18–20 g) on day 0. Compounds are administrated intravenously following the indicated schedule.

[a] Body weight change between day 1 and day 7 in Sarcoma 180 bearing mice.

must increase during the experiments. Actually, mice without treatment gained 7.6 g after a week (run 1 as control). However, mice treated with 60 mg/kg of **23b** gained only 0.3 g of their weight. These observations suggest that administering **23b** resulted in health declines of mice for some reasons including toxicity. Although we could not succeed in developing an antitumor drug from carzinophilin-related compounds, we found some molecules which show strong cytotoxicity in vitro and antitumor activity in vivo. We believe these studies provide foundational knowledge for developing a novel antitumor drug from carzinophilin congeners.

3. Conclusion

We succeeded in finding several potent cytotoxic carzinophilin congeners, which inhibit P388 cell increasing in vitro among compounds obtained in our synthetic studies on **1**. Especially, **12** and **23b** also exhibited promising antitumor activity against P388 murine leukemia in vivo. Both **12** and **23b** were also found to induce decline of uterine cervix carcinoma Hela S_3 in vitro. Further investigation revealed **23b** induces decline of Sarcoma 180 remarkably although it brings about some health deterioration in mice.

4. Experimental

4.1. General

See General in the experimental part for Part 1 of this series of papers. Compounds with large molecular weight (MW>750) could not be subjected to high-resolution mass spectroscopy.

4.2. Reactions of diethyl [(*S*)-azabicyclo[3.1.0]hex-2-ylidene]malonate (*ent*-**2**)

4.2.1. Reaction with PhSH giving (*S*)-2-bis(ethoxycarbonyl)methylidene-5-phenylthiomethylpyrrolidine (*ent*-4a). A solution of *ent*-**2** (2.1 mg, 8.8 μmol), PhSH (10 μL), and Et_3N (30 μL) in THF (200 μL) was stirred at room temperature for 1 h, and the mixture was concentrated in vacuo. Purification of the residue by preparative silica gel TLC (benzene/AcOEt=80:20) gave thiophenol adduct *ent*-**4a** (1.9 mg, 62%) as a colourless caramel. IR (film): 3300, 2970, 1645, 1565, 1435, 1280, 1250, 1080, 895, 840, 790 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.29, 1.30 (each 3H, t, *J*=7.1 Hz, C*H*$_3$CH$_2$O×2), 1.78 (1H, dddd, *J*=6.0, 6.8, 9.4, 12.9 Hz, C4H*H*), 2.21 (1H, dddd, *J*=5.7, 7.7, 9.1, 12.9 Hz, C4H*H*), 2.96 (1H, dd, *J*=7.3, 13.4 Hz, C5CH*H*O), 3.05 (1H, ddd, *J*=6.8, 9.5, 18.5 Hz, C3H*H*), 3.07 (1H, dd, *J*=5.8, 13.4 Hz, C5CH*H*O), 3.23 (1H, ddd, *J*=5.7, 9.4, 18.5 Hz, C3H*H*), 4.98 (1H, m, C5*H*), 4.18, 4.20 (each 2H, q, *J*=7.1 Hz, CH$_3$C*H*$_2$O×2), 7.2–7.4 (5H, m, aromatic protons), 9.67 (1H, br s, N*H*). EI-MS (rel. int. %) *m*/*z*=349 (2.1, M^+), 304 (2.1, $[M-EtO]^+$), 226 (11, $[M-PhSCH_2]^+$), 180 (100, $[M-EtOH-PhSOCH_2]^+$). EI-HRMS calcd for $C_{18}H_{23}NO_4S$ (M^+): *m*/*z*=349.1349; found *m*/*z*=349.1343. The ^1H NMR spectrum of this sample was identical to that of the authentic sample independently prepared described below.

4.2.2. Authentic *ent*-4a. A solution of NaSPh in DMF [prepared with NaH (3.0 mg, 125 μmol), and PhSH (20 mg, 181 μmol) in DMF (500 μL)] was added to a solution of (*S*)-2-bis(ethoxycarbonyl)-methylidene-5-methanesulfoxymethylpyrrolidine[1a] (35.0 mg, 85.0 mmol) in DMF (1.0 mL) at room temperature. After stirring at 130°C for 3 h, the mixture was poured into water and extracted with Et_2O. The combined ethereal extracts were washed with brine, dried over $MgSO_4$, then concentrated in vacuo. Purification of the residue by silica gel column chromatography (benzene/AcOEt=90:10) gave *ent*-**4a** (25.9 mg, 87%) as a colourless caramel.

4.2.3. Reaction with AcOH giving (*S*)-2-bis(ethoxycarbonyl)methylidene-5-acetoxymethylpyrrolidine (*ent*-4b). A mixture of *ent*-**2** (14.3 mg, 59.8 μmol) and AcOH (100 μL) in CH_2Cl_2 (5.0 mL) was stirred at room temperature. After stirring for 3 h, the mixture was concentrated in vacuo. Purification of the residue by silica gel column chromatography (CH_2Cl_2/acetone=93:7) gave **4b** (13.3 mg, 74%) as a colourless oil. $[\alpha]_D^{20}$=+43.8° (*c* 1.06, $CHCl_3$). IR (film): 3300, 2970, 1740, 1690, 1645, 1570, 1440, 1370, 1250, 1080, 1040, 800 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.22, 1.24 (each 3H, t, *J*=7.1 Hz, C*H*$_3$CH$_2$O×2), 1.67 (1H, dddd, *J*=6.2, 6.8, 9.3, 13.1 Hz, C4H*H*), 2.03 (3H, s, C*H*$_3$CO), 2.10 (1H, dddd, *J*=5.8, 8.0, 9.4, 13.1 Hz, C4H*H*), 3.02 (1H, ddd, *J*=6.9, 9.4, 18.5 Hz, C3H*H*), 3.13 (1H, ddd, *J*=5.8, 9.5, 18.5 Hz, C3H*H*), 3.93 (1H, dd, *J*=7.6, 11.2 Hz, C5CH*H*O), 4.04 (1H, m, C5*H*), 4.11, 4.13 (each 2H, q, *J*=7.1 Hz, CH$_3$C*H*$_2$O×2), 4.16 (1H, dd, *J*=3.9, 11.2 Hz, C5CH*H*O), 9.53 (1H, br s, N*H*). EI-MS (rel. int. %) *m*/*z*=299 (2.7, M^+), 254 (4.4, $[M-EtO]^+$), 239 (0.94, $[M-AcOH]^+$), 226 (4.1, $[M-AcOCH_2]^+$), 180 (100, $[M-EtOH-AcOCH_2]^+$). EI-HRMS calcd for $C_{14}H_{21}NO_6$ (M^+): *m*/*z*=299.1369; found *m*/*z*=299.1338. The ^1H NMR spectrum of this sample was identical to that of the authentic sample independently prepared described below.

4.2.4. Authentic *ent*-4b. A mixture of (*S*)-2-bis(ethoxycarbonyl)methylidene-5-hydroxymethylpyrrolidine[1a] (28 mg, 109 μmol), and Ac_2O (100 μL, excess) in a mixture of CH_2Cl_2 (1.0 mL) and pyridine (200 μL) was stirred at room temperature for 12 h. After concentration in vacuo, purification of the residue by silica gel column chromatography (acetone/CH_2Cl_2=7:93) gave *ent*-**4b** (30.5 mg, 93%) as a colourless oil. $[\alpha]_D^{20}$=+44.7° (*c* 1.07, $CHCl_3$).

4.2.5. Reaction with MeOH giving (*S*)-2-bis(ethoxycarbonyl)methylidene-5-methoxymethylpyrrolidine (*ent*-4c). A solution of *ent*-**2** (16.0 mg, 66.9 mmol) in MeOH (1.0 mL) was stirred at room temperature for 7 days. After concentration in vacuo, purification of the residue by silica gel column chromatography (benzene/AcOEt=90:10) gave **4c** (14.5 mg, 80%) as an oil. IR (film): 3300, 2970, 1690, 1640, 1570, 1280, 1245, 1080 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 1.28, 1.29 (each 3H, t, *J*=7.1 Hz, C*H*$_3$CH$_2$O×2), 1.68 (1H, dddd, *J*=6.2, 7.0, 9.3, 12.8 Hz, C4H*H*), 2.10 (1H, dddd, *J*=5.7, 8.2, 8.7, 12.8 Hz, C4H*H*), 3.03 (1H, ddd, *J*=7.3, 9.1, 18.4 Hz, C3H*H*), 3.20 (1H, ddd, *J*=5.7, 9.5, 18.4 Hz, C3H*H*), 3.25 (1H, dd, *J*=7.6, 9.4 Hz,

C5CH*H*O), 3.35 (3H, s, CH_3O), 3.42 (1H, dd, *J*=4.2, 9.4 Hz, C5CH*H*O), 4.03 (1H, m, C5*H*), 4.15, 4.17 (each 2H, q, *J*=7.1 Hz, CH_3CH_2O×2), 9.60 (1H, br s, N*H*). EI-MS (rel. int. %) *m*/*z*=271 (15, M^+), 226 (66, $[M-MeOCH_2]^+$), 180 (100, $M-EtOH-MeOCH_2)^+$). EI-HIMS calcd for $C_{13}H_{21}NO_5$: *m*/*z*=271.1420; found *m*/*z*=271.1426.

4.3. Reaction of ethyl *N*-(pivaloyl-[(*S*)-1-azabicyclo-[3.1.0]hex-2-ylidene]glycinate (*ent*-3)

4.3.1. Reaction with PhSH giving ethyl *N*-(pivaloyl-[(*S*)-5-phenylthiomethylpyrrolidin-2-ylidene]glycinate (*ent*-5a). The same treatments of *ent*-**3** (8.7 mg, 32.8 μmol) as described in Section 4.2.1 gave *ent*-**5a** (8.3 mg, 67%) as an oil after silica gel column chromatography. The ^{1}H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=90:10). Assignments of signals for the main isomer and some for the minor isomer are described. ^{1}H NMR (400 MHz, $CDCl_3$, *a*=0.90, *b*=0.10, *carzinophilin numbering*): δ 1.25 [3H×a, t, *J*=7.1 Hz, CH_3CH$_2$O (*E*-isomer)], 1.25 [9H×a, s, (CH_3)$_3$CSi (*E*-isomer)], 1.28 [9H×b, s, (CH_3)$_3$CSi (*Z*-isomer)], 1.72 [1H×a, dddd, *J*=5.8, 7.2, 9.2, 13.0 Hz, C12H*H* (major)], 1.80 [1H×b, m, C12H*H* (minor)], 2.14 [1H×a, dddd, *J*=5.3, 7.3, 9.2, 13.0 Hz, C12H*H* (major)], 2.20 [1H×b, m, C12H*H* (minor)], 2.62 [1H×a, ddd, *J*=7.3, 9.2, 17.2 Hz, C13H*H* (*E*-isomer)], 2.70 [1H×a, ddd, *J*=5.8, 9.3, 17.2 Hz, C13H*H* (*E*-isomer)], 3.00 [2H+1H×b, m, C11H_2, C13H*H* (*Z*-isomer)], 3.19 [1H×b, ddd, *J*=5.3, 9.0, 18.0 Hz, C13H*H* (*Z*-isomer)], 3.88 [1H×b, m, C11*H* (*Z*-isomer)], 3.95 [1H×b, m, C11*H* (*E*-isomer)], 4.11 [2H×a, br q, *J*=7.1 Hz, CH_3CH_2O (*E*-isomer)], 4.13 [2H×b, q, *J*=7.1 Hz, CH_3CH_2O (*Z*-isomer)], 5.95 [1H×b, br, N*H* (*Z*-isomer)], 6.46 [1H×a, br, N*H* (*E*-isomer)], 6.98 [1H×b, br, N*H* (*Z*-isomer)], 7.2–7.4 (5H, m, aromatic protons), 8.16 [1H×a, br, N*H* (*E*-isomer)]. EI-MS (rel. int. %) *m*/*z*=376 (4.7, M^+), 291 (61, $[M-t$BuCO$]^+$), 253 (45, $[M-PhSCH_2]^+$), 207 (27, $[M-PhSCH_2-EtOH]^+$), 57 (100, tBu$^+$).

4.3.2. Reaction with AcOH giving ethyl (*N*-pivaloyl-[(*S*)-5-acetoxymethylpyrrolidin-2-ylidene]glycinate (*ent*-5b). Similar treatments of *ent*-**3** (11.5 mg, 43.2 μmol) to those described in Section 4.2.3 gave *ent*-**5b** (12.1 mg, 86%) as an oil after silica gel column chromatography. The ^{1}H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=90:10). Assignments of signals for the main isomer and some for the minor isomer are described. ^{1}H NMR (400 MHz, $CDCl_3$, *a*=0.90, *b*=0.10, *carzinophilin numbering*): δ 1.24 [3H×a, t, *J*=7.1 Hz, CH_3CH$_2$O (*E*-isomer)], 1.25 [9H×a, s, (CH_3)$_3$CSi (*E*-isomer)], 1.29 [9H×b, s, (CH_3)$_3$CSi (*Z*-isomer)], 1.67 [1H×a, dddd, *J*=6.3, 7.6, 9.0, 12.9 Hz, C12H*H* (major)], 2.09 [3H×a, CH_3CO (*E*-isomer)], 2.12 [1H×a, dddd, *J*=6.5, 7.5, 8.2, 12.9 Hz, C12H*H* (major)], 2.17 [3H×b, s, CH_3CO (*Z*-isomer)], 2.67 [1H×a, dt, *J*=17.3, 7.7 Hz, C13H*H* (*E*-isomer)], 2.70 [1H×a, ddd, *J*=6.2, 9.0, 17.3 Hz, C13H*H* (*E*-isomer)], 3.14 [2H×b, m, C13H_2 (*Z*-isomer)], 3.88 [1H×a, dd, *J*=7.8, 11.0 Hz, C11CH*H*O (*E*-isomer)], 3.90 (1H×b, dd, *J*=7.1, 11.0 Hz, C11CH*H*O (*Z*-isomer)), 4.09 [1H, m, C11*H*, 4.11 [2H×a, br q, *J*=7.1 Hz, CH_3CH_2O (*E*-isomer)], 4.15 [2H×b, q, *J*=7.1 Hz, CH_3CH_2O (*Z*-isomer)], 4.15 [1H×b, dd, *J*=3.3, 11.0 Hz, C11CH*H*O (*Z*-isomer)], 4.22 [1H×a, dd, *J*=4.0, 11.0 Hz, C11CH*H*O (*E*-isomer)], 5.95 [1H×b, br, N*H* (*Z*-isomer)], 6.45 [1H×a, br, N*H* (*E*-isomer)], 7.13 [1H×b, br, N*H* (*Z*-isomer)], 8.03 [1H×a, br, N*H* (*E*-isomer)]. EI-MS (rel. int. %) *m*/*z*=326 (2.9, M^+), 269 (11, $[M-t$-Bu$]^+$), 253 (12, $[M-AcOCH_2]^+$), 241 (61, $[M-t$-BuCO$]^+$), 57 (100, tBu$^+$). The ^{1}H NMR spectrum of this sample was identical to that of the authentic sample independently prepared described below.

4.3.3. Authentic *ent*-5b. Treatment of ethyl *N*-pivaloyl [(*S*)-5-hydroxymethylpyrrolidine-2-ylidene]glycinate[1a] (8.0 mg, 28.1 μmol) in the same manner as described in Section 4.2.4. *ent*-**5b** (8.0 mg, 87%) as an oil after silica gel column chromatography (CH_2Cl_2/acetone=88:12).

4.3.4. Reaction with MeOH giving ethyl *N*-(pivaloyl-[(*S*)-5-methoxymethylpyrrolidin-2-ylidene]glycinate (*ent*-5c). Treatments of *ent*-**3** (10.0 mg, 37 mmol) in the same manner to that disclosed in Section 4.2.4 gave *ent*-**5c** (7.1 mg, 68%) as an oil after silica gel column chromatography. The ^{1}H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=90:10). Assignments of signals for the main isomer and some for the minor isomer are described. ^{1}H NMR (400 MHz, $CDCl_3$, *a*=0.90, *b*=0.10, *carzinophilin numbering*): δ 1.23 [3H×a, t, *J*=7.2 Hz, CH_3CH$_2$O (*E*-isomer)], 1.25 [9H×a, s, (CH_3)$_3$CSi (*E*-isomer)], 1.26 [3H×b, t, *J*=7.1 Hz, CH_3CH$_2$O (*E*-isomer)], 1.28 [9H×b, s, (CH_3)$_3$CSi (*Z*-isomer)], 1.64 [1H×a, dddd, *J*=7.7, 7.6, 9.2, 13.3 Hz, C12H*H* (*E*-isomer)], 1.76 [1H×b, m, C12H*H* (*Z*-isomer)], 2.05 [1H×a, dddd, *J*=5.9, 7.7, 8.9, 13.3 Hz, C12H*H* (*E*-isomer)], 2.13 [1H×b, m, C12H*H* (*Z*-isomer)], 2.63 [1H×a, ddd, *J*=7.6, 8.9, 17.2 Hz, C13H*H* (*E*-isomer)], 2.69 [1H×a, ddd, *J*=5.9, 9.2, 17.2 Hz, C13H*H* (*E*-isomer)], 3.05, 3.13 [each 1H×b, m, C13H_2 (*Z*-isomer)], 3.28 [1H×a, dd, *J*=7.6, 9.3 Hz, C11CH*H*O (*E*-isomer)], 3.35 (3H, s, CH_3O), 3.40 [1H×a, dd, *J*=4.5, 9.3 Hz, C11CH*H*O (*E*-isomer)], 3.90 [1H×b, m, C11*H* (*Z*-isomer)], 4.02 [1H×a, m, C11*H* (*E*-isomer)], 4.10 [2H×a, br q, *J*=7.2 Hz, CH_3CH_2O (*E*-isomer)], 4.13 [2H×b, q, *J*=7.2 Hz, CH_3CH_2O (*Z*-isomer)], 5.75 [1H×b, br, N*H* (*Z*-isomer)], 6.47 [1H×a, br, N*H* (*E*-isomer)], 7.08 [1H×b, br, N*H*(*Z*-isomer)], 8.07 [1H×a, br, N*H*(*E*-isomer)]. EI-MS (rel. int. %) *m*/*z*=298 (25, M^+), 253 (1.0, $[M-MeOCH_2]^+$), 241 (8.2, $[M-t$Bu$]^+$), 213 (5.4, $[M-t$BuCO$]^+$), 57 (100, t-Bu$^+$).

4.4. Reaction of ethyl 2-[(*R*)-1-azabicyclo[3.1.0]hex-2-ylidene]-2-[(2*S*,3*S*)-3,4-epoxy-2-(3-methoxy-5-methyl-1-naphthoyl)bytyryl]aminoacetate (8) with PhSH

4.4.1. 4 h's reaction. A solution of **8**[15] (5.0 mg, 10.1 μmol), PhSH (10 μL, 91 μmol), and Et_3N (10 mg, 99 μmol) in THF (1.0 mL) was stirred at room temperature. After 4 h, the mixture was directly adsorbed on silica gel column. After elution of excess PhSH with benzene, further elution with AcOEt gave the crude products. Purification by silica gel preparative TLC (benzene/acetone=85:15) gave **10** (3.8 mg, 62%) and **11** (2.0 mg, 27%), amide **12** (trace), and **13** (trace).

4.4.2. 10 h's reaction. A solution of **8** (5.4 mg, 11.0 μmol),

PhSH (10 μL, 91 μmol), and Et_3N (10 mg, 99 μmol) in THF (1.0 mL) was stirred at room temperature. After 10 h, TLC analysis of the mixture indicated disappearance of starting **8**. Work-up similar to that described in Section 4.4.1 gave **11** (6.0 mg, 76%), **12** (0.3 mg, 11%), and **13** (0.2 mg, 4%). The ^{1}H NMR and IR spectra of **12** were identical to those of the natural product.[2]

4.4.3. Physical data of mono-adduct 10. R_f=0.40 (silica gel, acetone/CH_2Cl_2=15:85). IR (film): 3350, 2930, 1720, 1700, 1670, 1600, 1280, 1240, 1080 cm^{-1}. The ^{1}H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=85:15). Assignments of signals for the main isomer and some for the minor isomer are described. ^{1}H NMR (400 MHz, $CDCl_3$, *a*=0.85, *b*=0.15 *carzinophilin numbering*): δ 1.17 [3H×b, t, *J*=7.1 Hz, C*H*$_3$CH$_2$O (*Z*-isomer)], 1.21 [3H×a, t, *J*=7.1 Hz, C*H*$_3$CH$_2$O (*E*-isomer)], 1.59 [3H×a, s, C20*H*$_3$ (*E*-isomer)], 1.60 [3H×b, s, C20*H*$_3$ (*Z*-isomer)], 1.71 [1H×a, m, C12H*H* (*E*-isomer)], 2.15 [1H×a, m, C12H*H* (*E*-isomer)], 2.69 (3H, s, C5′CH3), 2.70 [2H×a, m, C13*H*$_2$ (*E*-isomer)], 2.79 [1H×a, d, *J*=4.6 Hz, C21H*H* (*E*-isomer)], 2.92 [1H×a, dd, *J*=7.2, 13.3 Hz, C11CH*H* (*E*-isomer)], 3.04 [1H×a, dd, *J*=5.7, 13.3 Hz, C11CH*H* (*E*-isomer)], 3.05 [1H×a, d, *J*=4.6 Hz, C21H*H* (*E*-isomer)], 3.95[1H×a, m, C11*H* (*E*-isomer)], 3.98 [3H×a, s, C3′OC*H*$_3$ (*E*-isomer)], 3.99 [3H×b, s, C3'OC*H*$_3$ (*Z*-isomer)], 4.12 (2H, m, CH$_3$C*H*$_2$O), 5.27 [1H×a, s, C18*H* (*E*-isomer)], 5.33 [1H×b, s, C18*H* (*Z*-isomer)], 5.75 [1H×b, br s, N*H* (*Z*-isomer)], 6.93 [1H×a, br s, N*H* (*E*-isomer)], 7.2–7.4 (7H, aromatic protons), 7.49 [1H×a, d, *J*=2.6 Hz, C4′*H* (*E*-isomer)], 7.93 [1H×b, d, *J*=2.6 Hz, C2′*H* (*E*-isomer)], 7.95 [1H×a, d, *J*=2.6 Hz, C2′*H* (*Z*-isomer)], 8.25 [1H×a, br, N*H* (*E*-isomer)], 8.66 [1H×a, m, C8'*H*]. EI-MS (rel. int. %) *m*/*z*=604 (18, M$^+$), 481 (8.9, [M−PhSCH$_2$]$^+$), 199 (100, ArCO$^+$). EI-HRMS calcd for $C_{33}H_{36}N_2O_7S$: *m*/*z*=604.2245; found *m*/*z*=604.2239.

4.4.4. Physical data of bis-adduct 11. R_f=0.46 (silica gel, acetone/CH_2Cl_2=15:85). IR (film): 3350, 2970, 2920, 1720, 1690, 1670, 1595, 1275, 1230, 1080, 730 cm^{-1}. ^{1}H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=85:15). The ^{1}H NMR (400 MHz, $CDCl_3$, *a*=0.85, *b*=0.15, carzinophilin numbering): δ 1.21 [3H, br t, *J*=7.2 Hz, C*H*$_3$CH$_2$O (*E*-isomer)], 1.52 [3H×a, s, C20*H*$_3$ (*E*-isomer)], 1.68 [1H×a, m, C12H*H* (*E*-isomer)], 1.81 [1H×b, m, C12H*H* (*Z*-isomer)], 2.13 [1H×a, m, C12H*H* (*E*-isomer)], 2.20 [1H×b, m, C12H*H* (*Z*-isomer)], 2.68 (3H, s, C5′CH3), 2.70 [2H×a, m, C13*H*$_2$ (*E*-isomer)], 2.90, 3.04 (each 1H, br, C11C*H*$_2$), 3.12, 3.35 (each 1H, d, *J*=13.6 Hz, C21*H*$_2$), 3.89[1H×a, m, C11*H* (*E*-isomer)], 3.97 [3H×b, s, C3′OC*H*$_3$ (*Z*-isomer)], 3.98 [3H×a, s, C3′OC*H*$_3$ (*E*-isomer)], 4.12 (2H, m, CH$_3$C*H*$_2$O), 5.52 [1H×a, br s, C18*H* (*E*-isomer)], 5.81 [1H×b, br s, N*H* (*Z*-isomer)], 7.03 [1H×a, br s, N*H* (*E*-isomer)], 7.05–7.40 (12H, aromatic protons), 7.49 [1H×a, d, *J*=2.6 Hz, C4′*H* (*E*-isomer)], 7.79 [1H×b, C2′*H* (*E*-isomer)], 8.26 [1H×0.9, br, N*H* (*E*-isomer)], 8.61 (1H×0.9, m, C8′*H*). EI-MS (rel. int. %) *m*/*z*=714 (7.3, M$^+$), 704 (8.4, [M−PhSH]$^+$), 548 (3.4, [M−PhSCH$_2$ CMe+H]$^+$), 498 (7.6, [M−ArCO$_2$H]$^+$). EI-HRMS calcd for $C_{39}H_{42}N_2O_7S_2$ (M$^+$): *m*/*z*=714.2436; found *m*/*z*=714.2420.

4.4.5. Physical data of 13. IR (film): 3400, 2920, 1720, 1680, 1610, 1600, 1410, 1270, 1230, 1200, 1180, 1080 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 (3H, s, C4H3), 2.69 (3H, s, C5′CH3), 3.26, 3.38 (each 1H, d, *J*=13.8 Hz, C3CH$_3$SPh), 3.76 (1H, br, OH), 3.98 (3H, s, C3′OC*H*$_3$), 5.54 (1H, s, C2*H*), 5.55, 6.36 (each 1H, br, N*H*$_2$), 7.13–7.40 (7H, aromatic protons), 7.49 (1H×a, d, *J*=2.6 Hz, C4′*H*), 7.83 (1H×b, d, *J*=2.6 Hz, C2′*H*), 8.62 (1H×0.9, m, C8′*H*). EI-MS (rel. int. %) *m*/*z*=439 (0.8, M$^+$), 421 (1.3, [M−H$_2$O]$^+$), 404 (1.0, [M−H$_2$O−NH$_3$]$^+$), 315 (2.0, M−PhSCH$_2$)$^+$), 216 (36, ArCO$_2$H$^+$), 199 (100. ArCO$^+$). EI-HRMS calcd for $C_{24}H_{25}NO_5S$ (M$^+$): *m*/*z*=439.1455; found *m*/*z*=439.1433. The ^{1}H NMR spectrum of this sample was identical to that of the authentic sample independently prepared described below.

4.4.6. Authentic 13. A solution of **12** (4.0 mg, 11.2 μmol) PhSH (10 mg, 91 μmol) and Et_3N (10 mg, 99 mmol) in THF (1.0 mL) was stirred at room temperature for 12 h. After the mixture was concentrated in vacuo, the residue was purified by silica gel column chromatography (acetone/CH_2Cl_2=10:90) gave **13** (4.0 mg, 9.1 mmol, 81%) as an oil.

4.5. Reaction of 13-*O*-dibenzyl-4-*O*-methyl carzinophilin (9) with thiophenol giving (3*R*,4*R*,5*S*)-3,4-dibenzyloxy-2-[(*E*)-1-((2*S*,3*S*)-3-hydroxy-3-methyl-2-(3-methoxy-5-methyl-1-naphthoxy)-4-phenyl-thiobutyrylamino)-1-(*N*-((*Z*)-1-methoxymethylidene-2-oxopropyl)carbamoyl)]-methylidene-5-phenylthiomethyl-pyrrolidine (14)

A solution of **9** (2.5 mg, 3.2 μmol), PhSH (35 mg, 31.8 μmol), and Et_3N (2.5 μL, 1.8 mg, 18 μmol) was stirred in THF (1.0 mL) at room temperature for 3 h. After concentration, purification of the residue by preparative silica gel TLC developed with AcOEt/hexane (50:50) gave **14** (2.0 mg, 63%) as a caramel. R_f=0.40 silica gel, AcOEt/hexane=50:50). IR (film): 3370, 2940, 1720, 1700, 1650, 1510, 1500, 1480, 1280, 1240, 1210, 1190, 1090, 740, 690 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.51, 2.18, 2.67 (each 3H, s, C20*H*$_3$, C1*H*$_3$, C*H*$_3$Ar, respectively), 3.08 (1H, dd, *J*=7.4, 13.6 Hz, C11C*H*HSPh), 3.24 (1H, dd, *J*=6.8, 13.6 Hz, C11C*H*HSPh), 3.33, 3.37 (each 1H, d, *J*=13.9 Hz, C21*H*$_2$SPh), 3.43 (3H, s, C*H*$_3$O), 3.87 (1H, d, *J*=3.1 Hz, C12*H*), 3.96 (3H, s, C*H*$_3$O), 4.22, 4.47 (each 1H, d, *J*=11.6 Hz, PhC*H*$_2$O), 4.68 (1H, d, *J*=11.1 Hz, PhC*H*HO), 4.78 (1H, br s, alcoholic proton), 4.86 (1H, d, *J*=11.1 Hz, PhC*H*HO), 5.00 (1H, s, C18*H*), 5.13 (1H, d, *J*=3.1 Hz, C13*H*), 6.40 (1H, br s, N*H*), 7.04 (1H, d, *J*=0.8 Hz, C4*H*), 7.05–7.50 (25H, N*H*, aromatic protons), 7.81 (1H, d, *J*=2.5 Hz, C2′*H*), 8.66 (1H, br d, *J*=7.8 Hz, C8′*H*). SIMS (3-nitrobenzylalcohol) *m*/*z*=996 (MH$^+$). Neither EI- nor CI-MS of this sample gave informative signals.

4.6. Physical data of novel compounds in Table 1

The compounds **22a**,**b**, **24**, and their diastereomers *iso*-**22a**,**b**, and *iso*-**24** were prepared only for cytotoxicity assay by employing almost the same procedure as described for the preparation of **2** and **3a**–**c** in Part 1 of this series of papers. So, their physical data are only reported here.

4.6.1. Ethyl 2-[(*R*)-5-hydroxymethylpyrrolidin-2-yl-idene]-2-[(*S*)-2-benzoxy-3-methylbutyrylamino]acetate (22a). IR (film): 3360, 2960, 2920, 1720, 1665, 1590, 1260,

1090, 710 cm^{-1}. The ^1H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=80:20). Assignments of signals for the main isomer and some for the minor isomer are described. ^1H NMR (400 MHz, $CDCl_3$, *a*=0.80, *b*=0.20, *carzinophilin numbering*): δ 1.10 [3H×a, d, *J*=6.9 Hz, C20CH_3 (major)], 1.15 [3H×a, d, *J*=6.8 Hz, C19CH_3 (major)], 1.16 [3H×a, t, *J*=7.2 Hz, C19CH_3 (major)], 1.90 [1H×b, dq, *J*=4.3, 9.0 Hz, C12*H*H (minor)], 2.05 [1H×b, C12*H*H (minor)] 2.48 [1H+1H×a, m, C19*H*, C12*H* (minor)], 2.52 (1H×a, br, OH), 2.65 [1H×a, ddd, *J*=6.5, 8.9, 17.6 Hz, C13*H* (major)], 2.68 [1H×a, ddd, *J*=7.1, 9.2, 17.6 Hz, C13*H* (major)], 3.06 [1H×b, ddd, *J*=5.1, 10.3, 18.4 Hz, C13*H*H (minor)], 3.15 [1H×b, ddd, *J*=7.2, 9.4, 18.4 Hz, C13HH (minor)], 3.30 [1H×b, ddd, *J*=br, OH (minor)], 3.48 [1H×a, dd, *J*=5.9, 11.8 Hz, C11C*H*HO (major)], 3.51 [1H×b, C11*H*H (minor)], 3.68 [1H×a, dd, *J*=3.6, 11.3 Hz, C11C*H*H (major)], 3.73 [1H×b, br d, *J*=11.8 Hz, C11C*H*H (minor)], 3.87 [1H×b, m, C11*H* (minor)], 3.99 [1H×a, C11*H* (major)], 5.06 [1H×b, d, *J*=4.9 Hz, C18*H* (minor)], 5.37 [1H×a, d, *J*=4.4 Hz, C18*H* (major)], 5.50 [1H×b, br, N*H* (minor)], 6.83 [1H×a, br, N*H* (major)], 7.19 [1H×b, br, N*H* (minor)], 7.50 (2H, br t, *J*=7.5 Hz, aromatic protons), 7.63 (1H, m, aromatic proton), 8.11 [2H+1H×a, N*H* aromatic protons, (major)]. EI-MS (rel. int. %) *m*/*z*=404 (10, M^+), 373 (7.2, [M−CH_2OH]$^+$), 227 (5.8, [M−PhCOOCHCH(Me)$_2$]$^+$), 199 (18, [M−PhCOOCHCH(Me)$_2$CO]$^+$), 105 (100, PhCO$^+$). EI-HIMS calcd for $C_{21}H_{28}N_2O_6$ (M^+): *m*/*z*=404.1945; found *m*/*z*=404.1961.

4.6.2. Ethyl 2-[(*R*)-5-methanesulfoxymethylpyrrolidin-2-ylidene]-2-[(*S*)-2-benzoxy-3-methylbutyrylamino]acetate (22b). IR (film): 3350, 2950, 1710, 1670, 1595, 1510, 1350, 1250, 1170, 1100, 950, 710, 520 cm^{-1}. The ^1H NMR spectrum of this sample showed that it consists of the two tautomers derived from its enamine moiety (*E*/*Z*=85:15). Assignments of signals for the main isomer are only described. ^1H NMR (400 MHz, $CDCl_3$, *carzinophilin numbering*): δ 1.11, 1.16 (each 3H, d, *J*=6.5 Hz, C19 (CH_3)$_2$), 1.18 (3H, t, *J*=7.2 Hz, CH_3CH_2O), 1.74 (1H, dddd, *J*=6.0, 6.6, 9.7, 19.9 Hz, C12*H*H), 2.18 (1H, ddd, *J*=6.5, 7.9, 19.9 Hz, C12*H*H), 2.47 (1H, m, C19*H*), 2.70 (1H, ddd, *J*=6.7, 9.5, 17.6 Hz, C13*H*H), 2.71 (1H, ddd, *J*=6.7, 9.5, 17.6 Hz, C13*H*H), 3.04 (3H, s, CH_3SO_3), 4.05 (1H, dd, *J*=6.7, 10.1 Hz, C11C*H*HOMs), 4.07 (2H, q, *J*=7.2 Hz, CH_3CH_2O), 4.17 (1H, m, C11*H*), 4.26 (1H, dd, *J*=3.8, 10.1 Hz, C11C*H*HOMs), 5.26 (1H, d, *J*=4.4 Hz, C18H), 6.80 (1H, br, NH), 7.51 (2H, br t, *J*=7.9 Hz, aromatic protons), 7.64 (1H, br tt, *J*=1.4, 7.9 Hz, aromatic protons), 8.12 (2H, br dd, *J*=1.4, 7.9 Hz, aromatic protons). EI-MS (rel. int. %) *m*/*z*=482 (12, M^+), 305 (6.2), 105 (100, PhCO). EI-HIMS calcd for $C_{22}H_{30}N_2O_8S$ (M^+): *m*/*z*=482.1724; found *m*/*z*=482.1740.

4.6.3. Ethyl 2-[(*S*)-5-hydroxymethylpyrrolidin-2-ylidene]-2-[(*S*)-2-benzoxy-3-methylbutyrylamino]acetate (*iso*-22a). IR (film): 3360, 2960, 1720, 1670, 1590, 1380, 1250, 1100, 710 cm^{-1}. The ^1H NMR spectrum of this sample showed that it consists of the two tautomers arising from the enamine moiety (*E*/*Z*=85:15). Assignments of signals for the main isomer are only described. ^1H NMR (400 MHz, $CDCl_3$, *carzinophilin numbering*): δ 1.11, 1.15 (each 3H, d, *J*=6.5 Hz, C19 (CH_3)$_2$), 1.25 (3H, t, *J*=7.2 Hz, CH_3CH_2O), 1.74 (1H, m, C12*H*H), 2.00 (1H, m, C12*H*H), 2.48 (1H, m, C19*H*), 2.57 (1H, ddd, *J*=6.1, 9.8, 17.4 Hz, C12*H*H), 2.74 (1H, ddd, *J*=6.4, 9.8, 17.4 Hz, C12*H*H), 3.40 (1H, br, OH), 3.52 (1H, dd, *J*=6.0, 11.2 Hz, C11C*H*HOH), 3.57 (1H, br dd, *J*=2.6, 11.7 Hz, C13C*H*H), 3.69 (1H, dd, *J*=3.8, 11.2 Hz, C13C*H*HO), 3.90 (1H, m, C11*H*), 4.05 (2H, m, CH_3CH_2O), 5.29 (1H, d, *J*=4.4 Hz, C18*H*), 6.84 (1H, br, N*H*), 7.50 (2H, m, aromatic protons), 7.60 (1H, m, aromatic proton), 8.10 (3H, NH, aromatic protons). EI-MS (rel. int. %) *m*/*z*=404 (13, M^+), 373 (1.7, [M−CH_2OH]$^+$), 227 (7.2, [M−PhCOOCHCH(Me)$_2$]$^+$), 199 (18, [M−PhCOOCHCH(Me)$_2$CO]$^+$), 105 (100, PhCO$^+$). EI-HIMS calcd for $C_{21}H_{22}N_2O_6$ (M^+): *m*/*z*=404.1945; found *m*/*z*=404.1960.

4.6.4. Ethyl 2-[(*S*)-5-methanesulfoxymethylpyrrolidin-2-ylidene]-2-[(*S*)-2-benzoxy-3-methylbutyrylamino]acetate (*iso*-22b). IR (film): 3350, 2960, 1720, 1670, 1600, 1350, 1260, 1170, 1100, 950, 710 cm^{-1}. The ^1H NMR spectrum of this sample showed that it consists of the two tautomers arising from its enamine moiety (*E*/*Z*=85:15). Assignments of signals for the main isomer are only described. ^1H NMR (400 MHz, $CDCl_3$, *carzinophilin numbering*): δ 1.10, 1.16 (each 3H, d, *J*=6.5 Hz, C19 (CH_3)$_2$), 1.19 (3H, t, *J*=7.2 Hz, CH_3CH_2O), 1.80 (1H, m, C12*H*H), 2.14 (1H, m, C12*H*H), 2.47 (1H, m, C19*H*), 2.62 (1H, ddd, *J*=5.9, 9.6, 17.5 Hz, C13*H*H), 2.77 (1H, ddd, *J*=6.6, 8.5, 17.5 Hz, C13*H*H), 3.07 (3H, s, CH_3SO_3), 4.02–4.19 (4H, CH_3CH_2O, C11*H*, C19*H*), 4.28 (1H, dd, *J*=3.8, 10.1 Hz, C11C*H*HOMs), 5.26, (1H, d, *J*=4.4 Hz, C18H), 6.81 (1H, br, NH), 7.51 (2H, br t, *J*=7.9 Hz, aromatic protons), 7.64 (1H, br tt, *J*=1.4, 7.9 Hz, aromatic protons), 8.12 (2H, br dd, *J*=1.4, 7.9 Hz, aromatic protons). EI-MS (rel. int. %): *m*/*z*=482 (10, M^+), 386 (2.6, [M−MsOH]$^+$), 305 (5.9), 105 (100, PhCO). EI-HIMS calcd for $C_{22}H_{30}N_2O_8S$ (M^+): *m*/*z*=482.1724; found *m*/*z*=1702.

4.6.5. Ethyl 2-[(*R*)-1-azabicyclo[3.1.0]hexan-2-ylidene]-2-[(*S*)-2-benzoxy-3-methylbutyrylamino]acetate (24). IR (film): 3350, 2960, 1720, 1680, 1490, 1360, 1100, 710 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, *carzinophilin numbering*): δ 1.08 (3H, d, *J*=7.0 Hz, C19CH_3), 1.14 (3H, d, *J*=6.8 Hz, C19CH_3), 1.27 (3H, t, *J*=7.2 Hz, CH_3CH_2O), 1.68 (1H, dd, *J*=0.8, 4.3 Hz, C10*H*), 2.16 (2H, m, C12H_2), 2.31 (1H, d, *J*=5.1 Hz, C10*H*), 2.50 (1H, m, C19*H*), 2.70 (1H, dt, *J*=19.5, 9.3 Hz), 2.76 (1H, m, C11H), 3.15 (1H, ddd, *J*=4.0, 10.1, 19.5 Hz, C13*H*), 4.19, 4.22 (each 1H, dq, *J*=10.0, 7.2 Hz, CH_3CH_2O), 5.44 (1H, d, *J*=4.2 Hz, C18*H*), 7.48 (2H, tt, *J*=1.5, 7.9 Hz, aromatic protons), 7.61 (1H, tt, *J*=1.5, 7.9 Hz, aromatic proton), 7.68 (1H, br, N*H*), 8.11 (2H, m, aromatic protons). EI-MS (rel. int. %) *m*/*z*=386 (1.5, M^+), 105 (100, PhCO$^+$).

4.6.6. Ethyl 2-[(*R*)-1-azabicyclo[3.1.0]hexan-2-ylidene]-2-[(*S*)-2-benzoxy-3-methylbutanoyl-amino]acetate (*iso*-24). IR (film): 3350, 2960, 1720, 1680, 1380, 1100 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, *carzinophilin numbering*): δ 1.10, 1.15 (each 3H, d, *J*=6.9 Hz, C19CH_3), 1.17 (3H, t, *J*=7.1 Hz, CH_3CH_2O), 1.70 (1H, d, *J*=5.1 Hz, C10*H*), 2.20 (2H, m, C12H_2), 2.35 (1H, d, *J*=5.1 Hz, C10*H*), 2.46 (1H, m, C19*H*), 2.71 (1H, ddd, *J*=9.1, 9.1, 19.6 Hz, C13*H*), 2.81 (1H, m, C11*H*), 3.16 (1H, ddd, *J*=4.1, 10.6, 19.6 Hz,

C13*H*), 4.20 (2H, m, CH_3CH_2O), 5.38 (1H, d, J=4.6 Hz, C18*H*), 7.48 (2H, tt, J=1.5, 7.9 Hz, aromatic protons), 7.61 (1H, tt, J=1.5, 7.9 Hz, aromatic proton), 7.68 (1H, br, N*H*), 8.11 (2H, m, aromatic protons). EI-MS (rel. int. %) m/z= 386 (1.8, M^+), 105 (100, PhCO).

4.7. Cytotoxicity assay against P388 murine leukemia in vitro

Growing cells of murine P388 *lymphocytic leukemia* were suspended at 2×10^4 cells/mL in PRMI-1640 medium containing 10% fetal bovine, 10 μM 2-hydroxyethyldisulfate and kanamycin (100 μg/mL), and the samples dissolved in MeOH were added. The mixtures were incubated at 37°C for 4 days in a CO_2 incubator with an atmosphere containing 5% CO_2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (1.0 mg/mL PBS) was added and incubation was continued for 4 h. To the supernatant solution was added DMSO and it was mixed thoroughly to dissolve the formazan. The absorbance was measured at 550 nm (Ref 630 nm), indicating the mass of viable cells. A 50% cell growth inhibitory concentration (IC_{50}) was determined from the calculated cell growth inhibitory rates at several concentrations.

4.7.1. Antitumor assay against P388 murine leukemia in vivo. P388 murine leukemia cells (10^6 cells/mouse) were inoculated i.p. into CDF[1] mice (6 mice). After 1 and 5 days, solutions of the sample were administered. The effectively (*T*/*C* value) was calculated by the following method:

$$T/C = T/C \times 100$$

T: median survival days for group incubated the samples, *C*: median survival days for control, Compounds, of which the *T*/*C* values are >120, were regarded to be effective.

4.8. Cytotoxicity assay against Sarcoma 180

Human uterine cervix Hela S_3 cells (8×10^2/well) were cultured in MEM medium containing 10% bovine serum at 37°C for 12 h in a CO_2 incubator with an atmosphere containing 5% CO_2. Samples dissolved in DMSO were added to the culture medium. After incubation for 72 h, the cytotoxicity was estimated by neutral red dye uptake method.

4.9. Antitumor assay against Sarcoma 180

Sarcoma 180 cells (5×10^6/mouse) were inoculated s.c. into male ddY mice (body weight=18–20 g). After 1 and 4 days, methanol solutions of the sample were administered. The size of the tumor was measured by the following method;

$$\text{tumor volume} = \frac{(\text{major axis}) \times (\text{minor axis})^2}{2}$$

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