1*H*-2-Benzopyran-1-one Derivatives, Microbial Products with Pharmacological Activity. Relationship between Structure and Activity in 6-[[1(*S*)-(3(*S*),4-Dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]-amino]-4(*S*),5(*S*)-dihydroxy-6-oxo-3(*S*)-ammoniohexanoate

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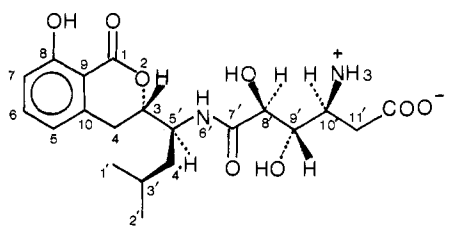
In order to investigate the structural requirements for gastroprotective activity in 6-[[1(*S*)-(3(*S*),4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4(*S*),5(*S*)-dihydroxy-6-oxo-3(*S*)-ammoniohexanoate [AI-77-B, 1], a product of *Bacillus pumilus* AI-77, nine derivatives were prepared and then tested for protective activity against stress-induced ulcers in rats. Neither the chromophore, [1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]ammonium chloride (**2**), nor the side-chain moiety, 4-amino-2,3-dihydroxyhexanedioic acid (**4**), as separate fragments alone showed any significant activity. Hydrolysis of the lactone ring of the 1*H*-2-benzopyran-1-one skeleton, to give 6-[[1-(2-carboxy-3-hydroxyphenyl)-2-hydroxy-5-methylhex-3-yl]amino]-4,5-dihydroxy-6-oxo-3-ammoniohexanoate (**5**), led to a considerable decrease in activity. The formation of a γ-lactone ring in the side chain, 4-[1-hydroxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]-amino]-2-oxoethyl]butan-4-olid-3-yl]ammonium chloride (**6**), resulted in a small decrease in activity. Selective acetylation at the primary amine position of **6**, to give 4-[1-hydroxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (**7**), led to a considerable decrease in activity. Both di- and triacetylated derivatives of **6**, 4-[1-acetoxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (**8**) and 4-[1-acetoxy-2-[[1-(3,4-dihydro-8-acetoxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (**9**), were inactive. Selective methylation of the phenolic hydroxyl group of to give **1**, 6-[[1-(3,4-dihydro-8-methoxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4,5-dihydroxy-6-oxo-3-ammoniohexanoate (**15**), resulted in a small decrease in activity. On the other hand, conversion of the carboxyl group of the side chain to an amide, to give [6-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4,5-dihydroxy-6-oxo-3-hexanamido]ammonium chloride (**10**), caused a considerable increase in both activity and toxicity. The findings showed that modifications at the position of the amino acid moiety of **1** significantly influenced the activity and that the 1*H*-2-benzopyran-1-one skeleton was also required for activity to occur.

One of several novel gastroprotective substances, 6-[1-(*S*)-(3(*S*),4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl)amino]-4(*S*),5(*S*)-dihydroxy-6-oxo-3-(*S*)-ammoniohexanoate [AI-77-B (**1**)], has been isolated



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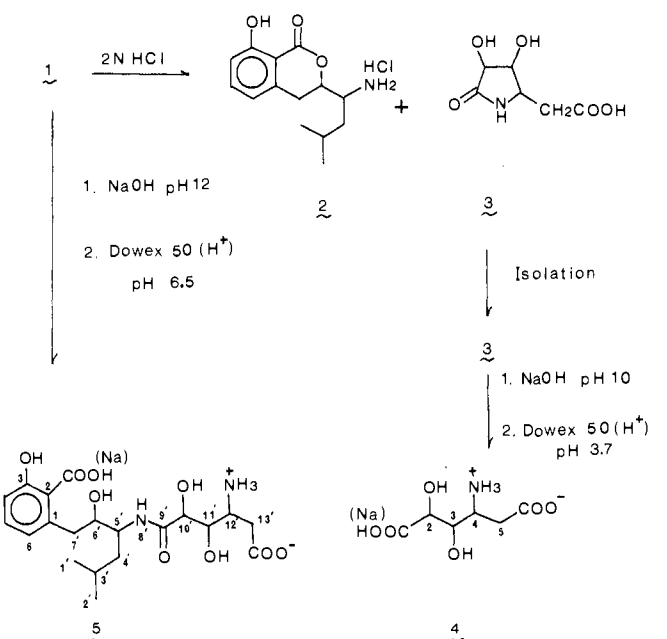
from a culture broth of *Bacillus pumilus* AI-77.[1] Structural and chemical studies of **1** have been reported in our previous paper.[2] A number of naturally occurring 1*H*-2-benzopyran-1-one (isocoumarin) derivatives that display certain biological activities have been reported. These include oosponol[3] (a constrictive activator on the tracheal muscles of guinea pigs and a dopamine β-hydroxylase inhibitor), reticulol[4] (a cyclic adenosine 3′,5′-monophosphodiesterase inhibitor), ochratoxines,[5] cladosporin[6] (an antifungal), and bacipheracin[7] (an antibacterial and antiviral). Interestingly, **1** appears to be part of a unique drug class because it has noncentral suppressive nonanticholinergic and nonantihistaminergic properties in spite of its potent antiulcerogenicity activity against

Scheme I



(1) Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibukawa, M. *Agric. Biol. Chem.* **1982**, *46*, 1823.
(2) Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibukawa, M.; Iitaka, Y. *Tetrahedron Lett.* **1982**, *23*, 5435.
(3) (a) Yamamoto, I.; Nitta, K.; Yamamoto, Y. *Agric. Biol. Chem.* **1961**, *25*, 405. (b) Yamamoto, I.; Nitta, K.; Yamamoto, Y. *Ibid.* **1962**, *26*, 486. (c) Nitta, K.; Yamamoto, Y.; Yamamoto, I.; Yamatodani, S. *Ibid.* **1963**, *27*, 822.
(4) (a) Mitscher, L. A.; Andres. W. W.; McCrae, W. *Experientia* **1964**, *20*, 258. (b) Lin, J.; Yoshida, S.; Takahashi, N. *Agric. Biol. Chem.* **1971**, *35*, 363. (c) Eaton, M. A. W.; Hutchinson, D. W. *Tetrahedron Lett.* **1971**, 1337. (d) Furutani, Y.; Shimada, M.; Hamada, M,; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1975**, *28*, 558.
(5) van der Merwe, K. J.; Steyn, P. S.; Fourie, L. *J. Chem. Soc.* **1965**, 7083.
(6) Scott, P. M.; van Walbeek, W. *J. Antibiot.* **1971**, *24*, 747.
(7) Okazaki, H.; Kishi, T.; Beppu, T.; Arima, K. *J. Antibiot.* **1975**, *28*, 719.

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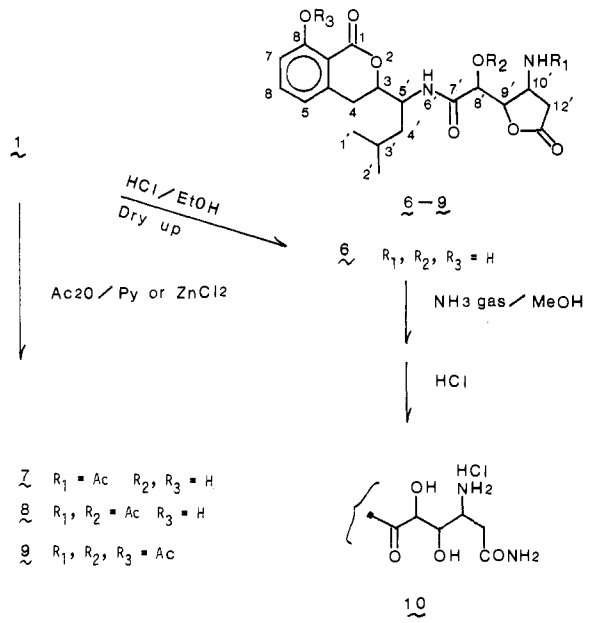
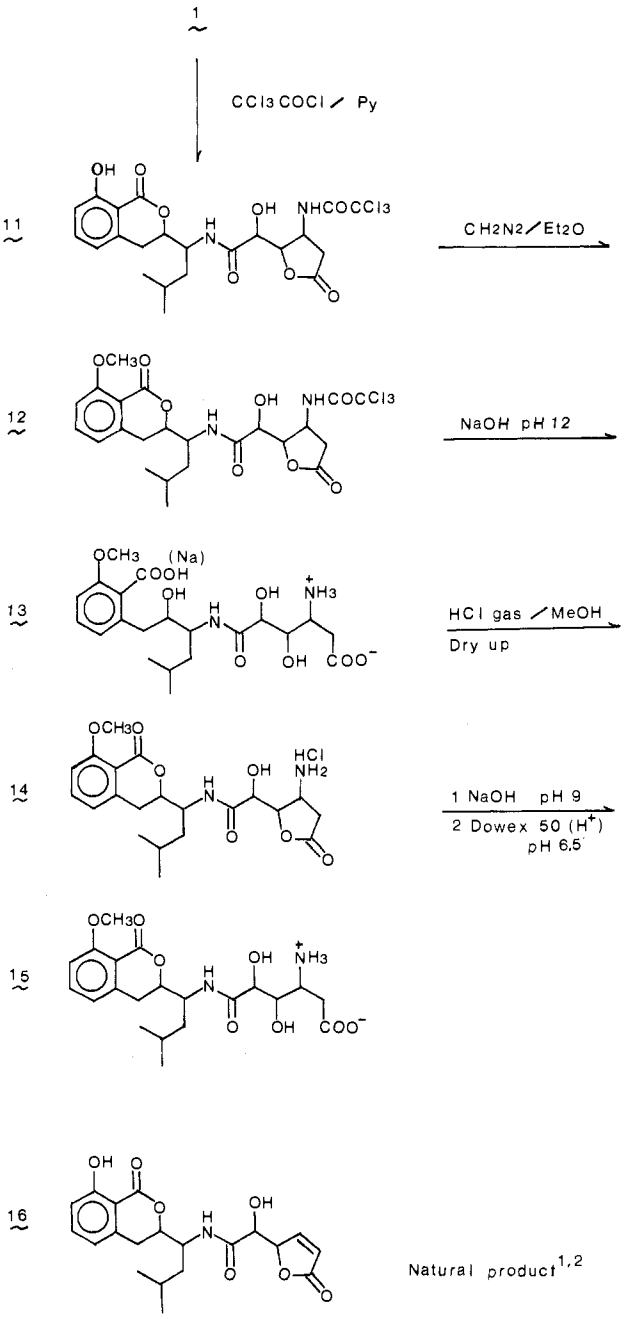
Scheme II



stress-induced (by restraint and water immersion) ulcers in rats. The relationship between structure and activity in 1 is an important consideration in designing compounds more effective for gastroprotective action. With this in mind, functional group modifications of 1 were made (γ-lactone, acetamide, acetoxy, methoxy, and amide derivatives), as well as products of hydrolysis (including only a side chain or the chromophoric moiety), and their gastroprotective activities were compared in order to examine the effects of minor structural modifications. In this paper, the preparation of nine derivatives and biological activities of ten derivatives, including a natural analogue,[1,2] are described, and the structural requirements for (1) are discussed.

Chemistry. The hydrolysis products **2–5** were prepared by the procedures outlined in Scheme I. Although the chromophoric moiety **2** was obtained from 1 in an almost quantitative yield by conventional hydrolysis of the amide bond with 6 N HCl, none of the corresponding side chain was obtained from the hydrolysate due to a variety of side reactions. The hydrolysis of 1 by 2 N HCl at 110 °C for 2 days gave predominantly **3**[8] as a corresponding side chain. After purification by column chromatography (Amberlite XAD-2), **3** was subjected to hydrolysis with aqueous NaOH at pH 10 for the purpose of opening the pyrrolidone ring, followed by desalting with Dowex 50 (H^+) to give **4**. The completely salt-free sample of **4** was not obtained due to the ease with which the pyrrolidone ring formed. The δ-lactone open form **5** was obtained by alkaline hydrolysis of 1 at pHs of 10–12, followed by purification with column chromatography. The UV spectrum of **5** revealed blue shifts. The γ-lactone form **6**, the acetylated derivatives **7–9**, and the amide derivative **10** were prepared by the reactions outlined in Scheme II. Treatment of 1 with HCl gave **6** in a high yield. Acetylation of 1 with acetic anhydride under different conditions afforded **7–9**. Reaction of 1 with acetic anhydride in pyridine at 0 °C produced the acetamide **7** exclusively. Treatment of 1 with $ZnCl_2$ in acetic anhydride at 50 °C produced **8**. Exhaustive acetylation of 1 with acetic anhydride in pyridine at 50 °C produced **9**. The UV spectrum of **9** revealed blue shifts with accompanying disappearance of the characteristic fluorescence of the 3,4-dihydro-8-hydroxy-1*H*-2-benzopyran-1-one skeleton. Introduction of ammonia gas into a methanol solution of **6** produced the amide **10**. The 8-methoxy derivative **15** was synthesized from 1 via a reaction sequence composed of five stages (Scheme III), which gave four intermediates, **11–14**. The protection of the primary amine of 1 with trichloroacetyl chloride afforded **11**. Alkylation of **11** with diazomethane gave **12**. The following deprotection process of the trichloroacetyl group with aqueous NaOH was accompanied by the hydrolysis of both the γ- and δ-lactone rings to give **13**. Treatment of **13** with methanol saturated with HCl gas gave **14**. In order for the γ-lactone ring to be selectively opened, **14** was hydrolyzed carefully with aqueous NaOH at pH 7–9 to give **15**. Hydrolysis of the δ-lactone proceeded at pHs greater than 10. The processes from the second to the final stage were carried out without isolation of the intermediates **13** and **14**.

Scheme III



Butenolide derivative **16** (Table I) has been isolated from a culture broth of *Bacillus pumilus* AI-77 as a minor component accompanying 1.[1,2]

(8) Virtanen, A. I.; Ettala, T. *Acta Chem. Scand.* **1957**, *11*, 182.

Table I. Protective Effects on Stress-Induced Ulcers and LD_{50} Values

no.	protection, %,[a,b] at the following ip doses: 50 mg/kg	25 mg/kg	12.5 mg/kg	6.25 mg/kg	LD_{50} in mice,[d] mg/kg ip
1	100	100	76	56	179 (131.6-243.4)[f]
2	33	33	16	NT[e]	
4	43	23	0	NT	
5	50	50	16	NT	
6	83	76	76	17	
7	66	33	16	NT	
8	16	16	0	NT	
9	16	0	0	NT	
10	100	100	100	100[c]	7 (5.0-9.8)
15	83	83	66	50	
16	33	16	0	NT	

[a] Five rats were treated as a group for each dosage. [b] Treatments in which protective values were more than 60% were evaluated as significantly effective ($p < 0.05$). [c] The minimum effective dose = 125 μg/kg (protection 66%). [d] Ten mice were treated as a group for each dosage. [e] NT = not tested. [f] 95% confidence limits.

Results and Discussion

Table I shows the biological evaluation of ten compounds. In the evaluation, those treatments where protective values were greater than 60% were considered significantly effective. Neither the chromophore (**2**) nor the side-chain moiety (**4**) alone showed any significant activity. Even though treatment was conducted at doses up to 100 mg/kg, the protective value of compound **5** did not rise above 50% (data not shown). The formation of a γ-lactone ring in the side chain (compound **6**) resulted in a small decrease in protective values. However, from the standpoint of the minimum effective dose, there was no significant difference between **1** and **5**.

Acetylation of the primary amine (compound **7**) resulted in a decrease in activity compared to **6**. Diacetylated derivative **8** and triacetylated derivative **9** were inactive. The weak activity of **7** may be due to the low levels of **1** generated by in vivo deacetylation. On the other hand, the conversion of the carboxyl group to an amide group (compound **10**) caused a considerable increase in both activity and toxicity. The minimum effective dose of **10** was about 100 times that of **1** (12.5 mg/125 μg). While the LD_{50} of **10** in mice was approximately 25 times that of **1** (179 mg/7 mg). The carboxyl group may not be very important for activity to occur. However, the situation is not so simple because both the γ-lactone derivative **6** and the amide derivative **10** are easily hydrolyzed to **1** under conditions of pH greater than 7. According to our unpublished data in rats, measurements of the concentration of total 1*H*-2-benzopyran-1-one derivatives in blood showed that **1** represented 45% of the total (**1** + **6**) 1 h after administration of **6**, whereas **1** was 20% of the total (**1** + **10**) after a similar administration of the amide **10**. The activity of **6** may be attributed to the formation of **1** in vivo, but the increased activity of **10** cannot be explained on this basis. Judging from the minimum effective dose, the amide derivative **10** itself seems to be one of the active forms. When **10** is administered, high levels of the active forms are very likely retained in the blood for longer periods as compared to the levels after administration of **1**, because **10** is probably metabolized via **1**. Consequently, other modifications of the carboxyl to secondary or tertiary amide may have interesting results. Further studies are required to clarify the contribution of the carboxyl group here. Selective alkylation of the phenolic hydroxyl group resulted in only a small decrease in activity (compounds **15** and **6**). The butenolide derivative **16** showed no significant activity.

The findings showed that modifications at the position of the amino acid moiety of **1** significantly influenced the activity and that the 1*H*-2-benzopyran-1-one skeleton was also required for activity to occur.

Experimental Section

Melting points were determined in capillary tubes by using a silicone oil bath (Yamato) MP-21 and are uncorrected. UV spectra were measured with a Shimadzu UV-210A spectrophotometer, IR spectra were measured with a Hitachi Model 285 spectrophotometer, and mass spectra were measured with a JEOL Model OISG-2 spectrometer. NMR spectra were recored on a JEOL JNM-MH 100 or FX 200 spectrometer with Me_4Si as the internal standard. Atomic absorption analysis was carried out with a Shimadzu AA-630 flame spectrophotometer. Each analytical sample had spectral data compatible with its assigned structure and was found to be a single spot on TLC or paper electrophoresis or to be a single peak in high-performance liquid chromatography (HPLC). TLC was carried out on plates coated with a 0.25-mm layer of silica gel 60 F_{254} (Merck). For preparative separations, plates with a 2-mm layer thickness were used. The location of spots was detected by illumination with a UV lamp or by spraying with ninhydrin reagent. In order for 1-oxopyrrolidinecarboxylic acid spots to be detected, the paper was first treated with chlorine and then with a solution of 1% starch-KI.[8] HPLC was performed on a Waters 200 series chromatography system incorporating a Model 6000A pump operating at a flow rate of 1 mL/min, a Model 440 absorbance detector at 254 nm, a Model U6K injector, and a Waters μBondapak C_{18} column (particle size 10 μm). Wacogel C-100 (40–100 mesh) was used for silica column chromatography.

Biological Assay. The gastroprotective activity of each compound was measured according to the method of Takagi.[9] Male Wistar rats weighing 160–180 g were deprived of food for 24 h and then intraperitoneally administered the test compound in physiological saline solution in order to avoid any effect due to variable gastrointestinal absorption. Rats administered only physiological saline solution were used as controls. One hour after administration of the test compound, each rat was restrained in a net cage and immersed from tail to xiphisternum in a water bath (21 °C) for 6 h. The animals were then sacrified by dislocating the cervical vertabrae, and the stomachs were removed and incised along the greater curvature. An ulcer index corresponding to the severity of hemorrhaging was given to each. Those stomachs without any ulceration were defined as 0, while the others were rated 1, 2, or 3 with increasing ulceration. The percent of ulcer inhibition was calculated as follows:

% of ulcer inhibn (protection) = 100 −
[(total rating of subjects treated with the test drug/total rating of subjects treated with physiological saline)100]

Five rats served as a group for each dosage.

Acute toxicities in mice were measured as follows. Test compounds were administerd intraperitoneally to groups of 10 male ddy mice (20–25 g); the period of observation was 7 days. The LD_{50} was calculated by the method of Litchfield and Wilcoxon.[10]

[1-(3,4-Dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]ammonium Chloride (2). A solution of **1** (3 g, 7.1 mmol) in 6 N HCl (30 mL) was treated at 110 °C for 18 h with refluxing. After evaporation in vacuo, the hydrolysate was redissolved in 5 mL of boiling water and then cooled to give 1.48 g (73%) of **2**. Recrystallization from boiling water gave the analytical sample: mp >210 °C dec; UV max (MeOH) 314 nm (ε 4100), 246 (6280); IR (KBr) 1680 (hydrogen-bonded lactone C=O) cm^{-1}; ^1H NMR (CD_3OD) δ 10.5 (s, phenolic OH), 7.59 (dd, 1, each J = 8 Hz, aromatic H), 6.95 and 6.85 (2 d, each 1, each J = 8 Hz, aromatic H), 4.97 (m, 1, C_3 H), 3.5 (m, 1 $C_{5'}$ H), 3.35–3.04 (m, 2, C_4 H), 2.16–1.35 (m, 3, $C_{3'}$ and $C_{4'}$ H), 0.99 and 0.92 (2 d,

(9) Takagi, K.; Okabe, S. *Jpn. J. Pharmacol.* **1968**, *18*, 9.
(10) Litchfield, J. T.; Wilcoxon, F. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99.

each 3, each J = 7 Hz, 2 CH_3); mass spectrum, m/z 249.1375 ($C_{14}H_{19}NO_3$ requires 249.1360). Anal. ($C_{14}H_{19}NO_3$·HCl) C, H, N.

4-Amino-2,3-dihydroxyhexanedioic Acid (4). A solution of 1 (2 g, 4.7 mmol) in 2 N HCl (200 mL) was treated at 110 °C for 2 days with refluxing and then diluted with water (200 mL) and passed through a column (Amberlite XAD-2, 300 mL) to remove 2. The column was washed with water (200 mL). The fraction that eluted from the column with water was again passed through another column packed with Dowex 2 (OH^- type, 320 mL) to remove Cl^- and then washed with water (320 mL). The fraction and washings containing 3 were combined and evaporated to a volume of about 100 mL, which was adjusted to pH 10.0 with 1 N NaOH and stirred overnight at room temperature, followed by the addition of Dowex 50 (H^+ type) to a pH of 3.7. The Dowex 50 was filtered away, and the filtrate was lyophilized to obtain 886 mg (87%) of 4 as a mixture of the free acid form and the monosodium salt form: IR (KBr) 1710, 1600, 1570 (sh) cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 4.25 (d, 1, $J_{2,3}$ = 6 Hz, C_2 H), 4.15 (dd, 1, $J_{2,3}$ = 6 Hz and $J_{3,4}$ = 4 Hz, C_3 H), 3.92 (m, 1, C_4 H), 2.96 and 2.79 (2 dd, 2, $J_{4,5a}$ = 4 Hz and $J_{5a,b}$ = 17 Hz, $J_{4,5b}$ = 8 Hz and $J_{5a,b}$ = 17 Hz, C_5 H_a and C_5 H_b); FD mass spectrum, m/z 194 (M + 1), 216 (M + Na); Na, 0.72 equivalent weight (atomic absorption analysis). This sample 4 was found to be a single spot in paper electrophoresis. During electrophoresis on paper [1 mA/cm, 2.5 h, pH 6.5 (pyridine/AcOH/H_2O, 10:0.4:90)] there was a 5-cm migration of 3 to the anode (ninhydrin yellow, Cl_2 and KI–starch bluish black), an 8.0-cm migration of 4 to the anode (ninhydrin purple), and an 8-cm migration of glutamic acid (standard sample) to the anode under the same conditions.

6-[[1-(2-Carboxy-3-hydroxyphenyl)-2-hydroxy-5-methylhex-3-yl]amino]-4,5-dihydroxy-6-oxo-3-ammoniohexanoate (5). A mixture of 1 (2 g, 4.71 mmol) and 0.5 N aqueous NaOH (40 mL) was stirred at room temperature until the UV absorption maximum shifted completely from 314 to 300 nm. Dowex 50 (H^+) was added slowly to the solution until the pH reached 6.5. After the resin was removed, the resulting solution was passed through a column packed with Amberlite XAD-2 (50 mL), and the column was washed with water (50 mL). The washings were concentrated and lyophilized to give 1.92 g (88%) of 5 in the form of a white powder: HPLC (55% MeOH in water containing 5% AcOH) t_R 5.1 min; UV max (MeOH) 300 nm, 245 (sh); IR (KBr) 1670 (sh), 1620 (sh), 1590 cm^{-1}; ^1H NMR (D_2O) δ 7.33 (dd, 1, J = 7 and 8 Hz, aromatic H), 6.87 (2 d, 2, J = 7 and 8 Hz, aromatic H), 4.35 (d, 1, $J_{10',11'}$ = 6 Hz, $C_{10'}$ H), 4.2–3.64 (m, 4, $C_{5'}$, $C_{6'}$, $C_{11'}$, and $C_{12'}$ H), 3.3–2.9 (m, 2, $C_{7'}$ H_a and H_b), 2.8–2.4 (m, 2, $C_{13'}$ H_a and H_b), 1.8–1.25 (m, 3, $C_{3'}$ and $C_{4'}$ H), 0.90 (2 d, 6, each J = 8 Hz, 2 CH_3); Na, 0.82 equivalent weight (atomic absorption analysis). The retention time of 1 was 8.6 min with the same conditions of HPLC analysis.

[4-[1-Hydroxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]butan-4-olid-3-yl]ammonium Chloride (6). An ethanol solution (260 mL) of 1 (10 g, 23.6 mmol) was evaporated with HCl (4.5 mL) in vacuo at 25 °C to dry it and then dissolved in ethanol (60 mL) and reevaporated. The dried residue was dissolved in 0.5 M aqueous $NaHCO_3$ (200 mL) and extracted with ethyl acetate (160 mL). The ethyl acetate layer was dried with Na_2SO_4 and evaporated. The residue was then dissolved in ethanol (260 mL) containing HCl (4.5 mL) and evaporated to give 9.7 g (93%) of 6: TLC ($CHCl_3$/EtOH, 9:1) R_f 0.3; UV max (MeOH) 314 nm, 246; IR (KBr) 1790 (γ-lactone C=O), 1680 (sh), 1660, 1625 cm^{-1}; ^1H NMR (CD_3OD) δ 7.46 (dd, 1, each J = 8 Hz, aromatic H), 6.84 and 6.81 (2 d, 2, each J = 8 Hz, aromatic H), 4.7 (m, 1, $C_{9'}$ H), 4.56 (m, 1, C_3 H), 4.4–4.2 (m, 2, $C_{5'}$ and $C_{8'}$ H), 3.72 (m, 1, $C_{10'}$ H), 3.2–2.95 (m, 3, C_4 H_a and H_b, $C_{11'}$ H_a), 2.70 (dd, 1, J = 4 and 18 Hz, $C_{11'}$ H_b), 1.95–1.20 (m, 3, $C_{3'}$ and $C_{4'}$ H), 0.96 and 0.92 (2 d, 6, each J = 8 Hz, 2 CH_3); mass spectrum, m/z 406.1736 ($C_{20}H_{26}N_2O_7$ requires 406.1733). Anal. ($C_{20}H_{26}N_2O_7$·HCl) C, H, N.

4-[1-Hydroxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (7). To a stirred, ice-cooled solution of thoroughly dried 1 (8.48 g, 20 mmol) in pyridine (50 mL) was added dropwise acetic anhydride (4.08 g, 40 mL). The reaction mixture was cooled and stirred for 2 h following addition. After evaporation of the pyridine in vacuo, the residue was washed twice with water (each 50 mL). The residue was crystallized from ethanol/water to give 5.0 g (56%) of 7 in the form of white needles. A second crop was 1.2 g (14%): mp 210–211 °C dec; TLC (AcOEt) R_f 0.28; UV max 314 nm, 246; IR (KBr) 1775 (γ-lactone C=O), 1690, 1675, 1660, 1620, 1550 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 10.85 (s, phenolic OH), 8.37 (d, J = 7 Hz, CONH), 7.88 (d, J = 9 Hz, $NHCOCH_3$), 4.90–4.04 (m, 5, C_3, C_5, $C_{8'}$, $C_{9'}$, and $C_{10'}$ H), 1.69 (s, 3, $NHCOCH_3$); mass spectrum, m/z 448.1830 ($C_{22}H_{28}N_2O_8$ requires 448.1838). Anal. ($C_{22}H_{28}N_2O_8$) C, H, N.

4-[1-Acetoxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (8). To a solution of thoroughly dried 1 (3.12 g, 7.4 mmol) in acetic anhydride (7.66 g, 75 mmol) was added zinc chloride (50 mg), and the solution was stirred at 50 °C for 2 h. The reaction mixture was poured into ice–water (300 mL), and the resulting precipitate was filtered and dried. The residue was crystallized with ethanol to give 780 mg (22%) of 8 in the form of white needles. A second crop (520 mg) contained trace amounts of 7. The analytical data were obtained from the first crop: mp 216–217 °C dec; TLC ($CHCl_3$/EtOH, 9:1) R_f 0.58; UV max (MeOH) 314 nm, 246; IR (KBr) 1802 and 1790 (sh) (γ-lactone C=O), 1755 (ester C=O), 1685 (sh), 1675, 1660, 1615, 1580 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 10.8 (s, phenolic OH), 8.38 (2 d, J = 7 and 8 Hz, 2 CONH), 5.18 (d, 1, J = 4 Hz, $C_{8'}$ H), 4.17 (m, 1, $C_{10'}$ H), 2.08 (s, 3, $OOCCH_3$), 1.58 (s, 3, $NHCOCH_3$); mass spectrum, m/z 490.1939 ($C_{24}H_{30}N_2O_9$ requires 490.1943). Anal. ($C_{24}H_{30}N_2O_9$) C, H, N.

4-[1-Acetoxy-2-[[1-(3,4-dihydro-8-acetoxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-(acetylamino)butan-4-olide (9). To a solution of thoroughly dried 1 (2.13 g, 5 mmol) in pyridine (30 mL) was added acetic anhydride (50 mmol), and then the solution was stirred for 2 h at 50 °C. After evaporation of the pyridine in vacuo, cold water was added to the residue, and the precipitate thus obtained was filtered and washed with water (50 mL). The resulting white solid was crystallized from ethanol/ethyl ether to give 1.98 g (74%) of 9 in the form of white needles. Recrystallization from ethanol gave the analytical sample: mp 170 °C dec; TLC ($CHCl_3$/EtOH, 9:1) R_f 0.55 (fluorescence no longer appeared); UV max (MeOH) 288 nm (ϵ 1805), 236 (7150); IR (KBr) 1790 (γ-lactone C=O), 1775 and 1755 (2 $OOCCH_3$ C=O), 1730 (δ-lactone C=O), 1690 (sh), 1678, 1672, 1660 (sh), 1650 (sh), 1640 (sh), 1613 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 2.27 and 2.09 (2 s, each 3, 2 $OOCCH_3$), 1.59 (s, 3, $NHCOCH_3$); mass spectrum, m/z 532.2028 ($C_{26}H_{32}N_2O_{10}$ requires 532.2048). Anal. ($C_{26}H_{32}N_2O_{10}$) C, H, N.

[6-[[1-(3,4-Dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4,5-dihydroxy-6-oxo-3-hexanamido]ammonium Chloride (10). Dried ammonia gas (50 mL) was bubbled into dried methanol (100 mL) containing 6 (2.5 g, 6.6 mmol) while the methanol was being cooled in water. Then the reaction system was closed and stirred for 2 h. The solvent was removed in vacuo, and the residue was dissolved in methanol (50 mL). Hyflo-supercel (6 g) was added to the solution. After the solution was stirred, the solvent was removed. The residue was placed in a column packed with silica gel (250 g) in chloroform. The column was washed with chloroform (500 mL) and then eluted with chloroform/methanol (3:1, 500 mL). The fractions containing 10 were combined and concentrated to as small a volume as possible. Methanol saturated with HCl gas was added dropwise to the residue with stirring to a pH of 2.0, and the solution was left standing until a white precipitate had formed. The precipitate was collected on filter paper and washed with ethyl acetate, followed by drying in vacuo to give 0.61 g (23%) of 10 in the form of a white powder: TLC ($CHCl_3$/MeOH, 1:1) R_f 0.11, ninhydrin pink; HPLC [28% tetrahydrofuran in water containing PIC B-7 (Waters)] t_R 5.1 min; UV max (MeOH) 314 nm, 246; IR (KBr) 1680 (sh), 1650, 1635 (sh), 1615, 1580, 1520 cm^{-1}; ^1H NMR (200 MHz, CD_3OD) δ 7.46 (dd, 1, each J = 8 Hz, aromatic H), 6.84 and 6.79 (2 d, 2, each J = 8 Hz, aromatic H), 4.68 (m, 1, C_3 H), 4.34 (m, 1, $C_{5'}$ H), 4.21 (d, 1, $J_{8',9'}$ = 6 Hz, $C_{8'}$ H), 4.03 (dd, 1, $J_{8',9'}$ = 6 and $J_{9',10'}$ = 4 Hz, $C_{9'}$ H), 3.74 (m, 1, $C_{10'}$ H), 3.05–2.85 (m, 3, C_4 H and $C_{11'}$ H_a), 2.67 (dd, 1, $J_{10',11'b}$ = 10 and $J_{11'a,b}$ = 16 Hz, $C_{11'}$ H_b), 1.90–1.57 (m, 2, $C_{3'}$ H and $C_{4'}$ H_a), 1.50–1.35 (m, 1, $C_{4'}$ H_b), 0.98 and 0.94 (2 d, 6, each J = 6 Hz, 2 CH_3); FD mass spectrum, m/z 423 (M + 1). Anal. ($C_{20}H_{29}N_2$-

O_7·HCl) C, H, N. The retention time of 1 was 8.0 min under the same conditions as the above HPLC analysis.

4-[1-Hydroxy-2-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-[(trichloroacetyl)amino]butan-4-olide (11). To a solution of thoroughly dried 1 (10 g, 23.6 mmol) in pyridine (40 mL) cooled to 0 °C was added dropwise trichloroacetyl chloride (9.1 g, 50 mmol), and the reaction mixture was stirred for 4 h at 0 °C. After the pyridine had been evaporated in vacuo, the residue was dissolved in ethyl acetate (300 mL) and then extracted three times with 0.1 N aqueous HCl (200 mL). This was followed by washing with water (200 mL). The ethyl acetate layer was dried with Na_2SO_4 and evaporated to give 9.8 g of a white solid. The white solid was crystallized from ethanol/water to give 6.3 g (48%) of 11 in the form of white needles. A second crop was 2.1 g (16%). The samples were used without further purification, although TLC analysis showed a trace of another spot corresponding to the disubstituted deriviative. If the contaminant is the disubstituted derivative, both 11 and the contaminant will form the same compound if the deprotection procedure is followed. On TLC ($CHCl_3$/EtOH, 9:1) the R_f value of 11 was 0.57 and that of the contaminant was 0.62.

4-[1-Hydroxy-2-[[1-(3,4-dihydro-8-methoxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-2-oxoethyl]-3-[(trichloroacetyl)amino]butan-4-olide (12). To a solution of 11 (6 g, 10.8 mmol) in 1,2-dichloroethane (60 mL) was added diazomethane (400 mmol) in ethyl ether, and the solution was stirred overnight. After the excess diazomethane had been destroyed with acetic acid, the solution was filtered, and the filtrate was dried in vacuo to give 5.75 g of crude 12, which was used without further purification: TLC ($CHCl_3$/EtOH, 9:1) R_f 0.60 and ($CHCl_3$/MeOH, 3:1) R_f 0.82; UV max (MeOH) 306 nm, 244.

6-[[1-(3,4-Dihydro-8-methoxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4,5-dihydroxy-6-oxo-3-ammoniohexanoate (15). To the above crude 12 (5.5 g) in a solution of ethanol/water (1:1, 250 mL) was added dropwise 1 N aqueous NaOH with stirring until the pH reached 12. The pH was held constant at 12 by the addition of more of the same alkali. Alkaline hydrolysis for the purpose of deprotection was monitored with TLC until it had been completed. This step caused the opening of the γ- and δ-lactone rings. The UV absorption maximum of the solution shifted from 306 to 277 nm due to opening of the δ-lactone. To the above hydrolysate containing 13 was added methanol (20 mL) saturated with HCl gas (about 68 mmol) while cooling with ice. The mixture was stirred for 30 min and then dried in vacuo to give 14. Th residue was redissolved in ethanol/water (1:1, 100 mL). In order for the γ-lactone ring to be opened, 0.1 N aqueous NaOH was dripped into the above solution with stirring to a pH of 9.0. The pH was held at 9.0 by the addition of alkali until the spot of 14 could no longer be detected on TLC, and then the pH was adjusted to 6.5 with 0.1 N HCl. The resulting solution was passed through a column packed with Amberlite XAD-2 (300 mL) in water. The column was washed with methanol/water (1:4, 600 mL) and eluted with methanol/water (3:2, 500 mL). Fractions containing only 15 were combined and dried in vacuo to give 2.25 g of 15. The overall yield was 30.7%. The sample 15 obtained above showed UV max (MeOH) 306 nm (ε 3973), 244 (ε 5573); IR (KBr) 1720 (δ-lactone C=O), 1655, 1590, 1575 cm^{-1}; ^1H NMR (CD_3OD) δ 7.48 (dd, 1, J = 7 and 8 Hz, aromatic H), 6.84 and 6.98 (2 d, each 1, J = 8 and 7 Hz, aromatic H), 4.6–4.3 (m, 2, C_3 and $C_{5'}$H), 4.19 (d, 1, J = 7 Hz, $C_{8'}$ H), 3.98 (m, 1, $C_{9'}$ H), 3.87 (s, 3, OCH_3), 3.66 (m, 1, $C_{10'}$ H), 3.1–2.8 (m, 2, C_4 H_a and H_b), 2.8–2.4 (m, 2, $C_{11'}$ H_a and H_b), 1.10–1.95 (m, 3, $C_{3'}$ and $C_{4'}$ H), 0.96 and 0.92 (2 d, 6, each J = 7 Hz, 2 CH_3). Anal. ($C_{21}H_{30}N_2O_9$) C, H, N. The R_f values of derivatives 13–15 on TLC were as follows: R_f ($CHCl_3$/MeOH, 3:1) for 13, 0.05; 14, 0.78; 15, 0.12; R_f ($CHCl_3$/MeOH, 1:1) for 13, 0.42; 14, 0.75.

Registry No. 1, 77674-99-8; 2, 86527-24-4; 3, 86527-25-5; 4, 86527-26-6; 4·Na, 86527-27-7; 5, 77675-03-7; 6, 86594-32-3; 7, 77675-00-4; 8, 77676-66-5; 9, 77676-74-5; 10, 86561-37-7; 11, 77675-97-9; 12, 77676-98-3; 13, 86527-28-8; 14, 86594-33-4; 15, 86527-29-9; 16, 77675-02-6.

Synthesis and Transport Applications of 3-Aminobicyclo[3.2.1]octane-3-carboxylic Acids

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The isomeric 3-aminobicyclo[3.2.1]octane-3-carboxylic acids were synthesized and compared with the widely used (1*R*,2*S*,4*S*)-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid as to specificity to the Na^+-independent membrane transport system L of the Ehrlich ascites tumor cell and of the rat hepatoma cell line HTC. The presence of an additional methylene group in the ring system leads to an optically symmetrical amino acid, with the advantages that the product is devoid of isomeric contamination. Hence, optical resolution is not necessary to secure a homogeneous test substrate for discrimination of amino acid transport systems. Through its inhibitory action on the cellular uptake of known system-specific amino acids, the bicyclo[3.2.1]octane amino acid proved more reactive than the bicycloheptane analogue with the Na^+-independent amino acid transport system of the test cells and not perceptibly reactive with the accompanying Na^+-dependent systems. Recent evidence of the presence of a second component of Na^+-independent amino acid transport, beyond system L, increases the importance of securing a variety of possibly discriminatory model substrates.

Metabolism-resistant amino acid analogues for a given membrane transport system have greatly assisted in the discrimination of the routes of uptake of each amino acid by various animal cells.[1-4] For example, N-methylation of 2-aminoisobutyric acid or alanine limits their uptake to Na^+-dependent system A. At the same time, this alteration eliminates their inhibition of uptake by other transport systems. A second Na^+-dependent system called ASC does not tolerate the *N*-methyl group but responds favorably to a side-chain hydroxyl or sulfhydryl group. System ASC is usually somewhat narrowed, relative to

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