Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 1757–1766

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Biological Activities of the Marine Bryozoan Alkaloids Convolutamines A, C and F, and Lutamides A and C

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Received 17 December 1999; accepted 21 March 2000

Abstract—Synthesis of convolutamines and lutamides, new 2,4,6-tribromo-3-methoxyphenethylamine alkaloids isolated from Floridian marine bryozoan *Amathia convoluta*, was accomplished by a sequence of reactions starting from 3-hydroxyphenethylamines. Cytotoxities of the synthetic lutamides, convolutamines and their de-*O*-methyl derivatives were examined using drug-sensitive and -resistant P388 as well as KB cell lines. The bioassay suggests that the 2,4,6-tribromo-3-methoxyphenethylamine is an indispensable unit for detection of the activities. Additionally, a reversal of drug resistance by those alkaloids is recognized.
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Introduction

In recent years, chemical research on marine bryozoans has stimulated new findings of interesting bioactivity in secondary metabolites, the most exciting effort of which is antineoplastic macrolide bryostains isolated from *Bugula neritina*[1] and *Amathia convoluta*.[2] These bryostatins are now in Phase III clinical trials. The other notable alkaloids are amathamides isolated from *Amathia wilsoni*[3,4] and flastramines from *Flustra foliacea*,[5,6] which bear a pyrrolidine ring along with bromo-methoxyphenethylamino group and indoline fused with pyrrolidine, respectively. Our group also obtained several classes of alkaloids, e.g., convolutamides A–F[7] of γ-lactam, convolutamines A–G[8,9] of β-phenethylamines and convolutamydines A–F[10,11] of bromohydroxyindoles, from the Floridian bryozoan *Amathia convoluta* living in the Gulf of Mexico. Recently, we isolated a new β-phenethylamine alkaloid of lutamides A–C from the Floridian bryozoan.[12] A tentative bioassay indicated that the lutamide C has a cell growth inhibitory activity against the human monocyte-like lymphocytic leukemia U937 cells. In particular, the amide shows a strong inhibitory potency against the vincristine-resistant cells of lymphocytic leukemia P388.[12] Although biological evaluation of lutamides and convolutamines is of great interest, a more decisive assay has not been carried out because of the very small quantity isolated. In this paper, we report the synthesis of these novel alkaloids, lutamides A (**1**) and C (**2**), and convolutamines F (**3**), A (**4**) and C (**5**), to investigate their biological activity and to confirm the proposed structures.

Synthesis

As a preliminary effort, *N*-methyl phenethylamine was found to be prepared in 70% yield by heating 1-bromo-2-phenylethane with aqueous methylamine in refluxing ethanol. Therefore, our first approach to synthesis of convolutamine F (**3**) consisted of *N*-methylamination of full substituted phenethyl bromide **11** as shown in Scheme 1. Reduction of phenylacetic acid **6** was accomplished by lithium aluminum hydride (LAH) forming phenethyl alcohol **7**. Treatment of **7** with hydrobromic acid in the presence of phase transfer catalyst afforded 3-hydroxyphenethyl bromide **8** together with 3-methoxyphenethyl bromide **9**, suggesting that the acidic reagent promoted the bromination of alcohol other than the cleavage of methyl ether. Bromination of phenol **8** was examined by several known procedures using pyridinium tribromide, benzyltrimethylammonium tribromide or bromodimethylsulfonium tribromide, but the best yield

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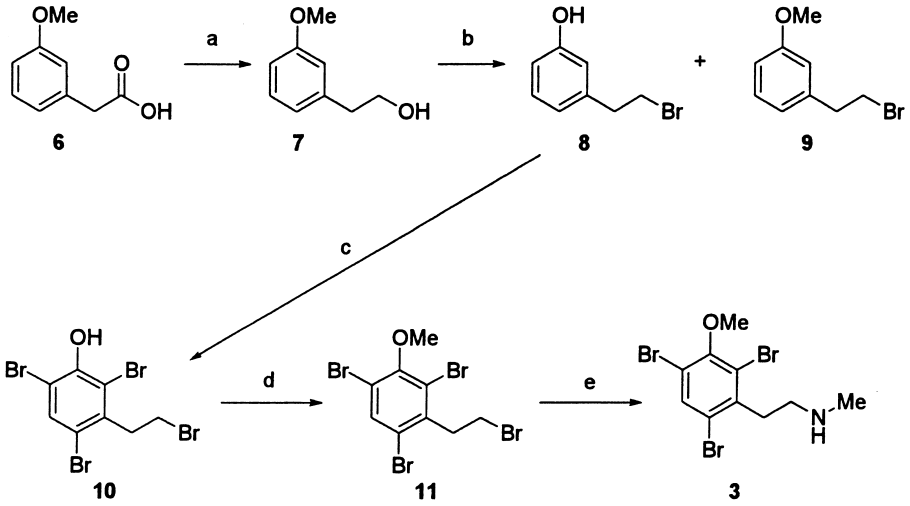
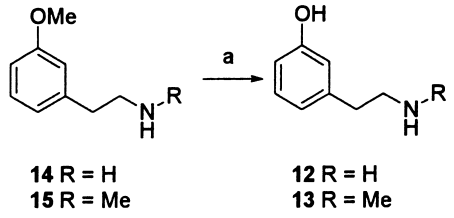
PII: S0968-0896(00)00110-3



Scheme 1. Reagents and conditions: (a) LAH, THF, 87%; (b) 47% HBr, hexadecylPBu$_3$Br, **8** 76%, **9** 5%; (c) Br_2, AcOH, 82%; (d) CH_2N_2, MeOH, −5 °C, 82%; (e) 40% $MeNH_2$, EtOH, 19%.

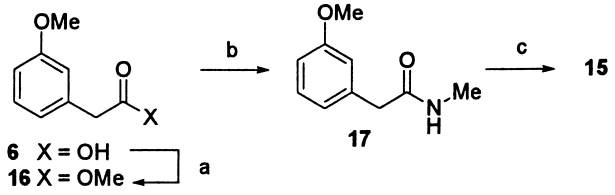
(82%) of **10** was obtained by the classical method treating with bromine in acetic acid. Methylation of phenol **10** was carried out successfully by utilizing diazomethane in methanol to afford anisole **11** in 82% yield. Treatment of **11** with methylamine, however, unexpectedly provided only a 19% yield of the desired convolutamine **3**, and besides this transformation was poorly reproducible.

We next focused on the development of a synthetic route via 3-hydroxyphenethylamines **12** and **13**, which have been prepared by demethylation[13] of the corresponding methoxyphenethylamines **14** and **15** (Scheme 2). The synthesis of precursor **15** has been performed by acylation of 3-methoxyphenethylamine (**14**) with ethyl chlorocarbonate followed by reduction of the resulting carbamate with LAH.[13] Compared to the precedent synthesis of **15**, our current procedure shown in Scheme 3 should be encouraged owing to the accessibility of the starting material. It is noteworthy that conversion of amide **17** into amine **15** was achieved by refluxing with borane in THF, but the amide was not reduced at all by LAH, diisobutylaluminum hydride (DIBAL) or sodium bis(2-methoxyethoxy)aluminum hydride (SMEAH). The methylamine **15** was also synthesized from phenethyl bromide **9** in 78% yield by the procedure utilized in *N*-methylamination of phenethyl bromide **11**. Demethylation of **14** and **15** was completed by refluxing with hydrobromic acid in acetic acid to give phenols **12** and **13**, respectively. In those reactions, phase-transfer catalyst effective in the ether-cleavage of anisole **7** into **8** was not employed because of the difficult separation of the desired product from the catalyst. The more convenient and straightforward synthesis of **13** was accomplished by a three-step sequence of reactions starting from 3-hydroxyphenylacetic acid (**18**) as shown in Scheme 4, in which the reduction of **20** was effected only with borane as for that of the methyl ether derivative **17**.

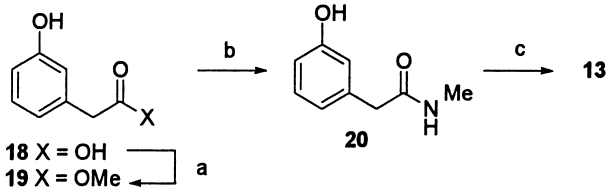


Scheme 2. Reagents and conditions: (a) 47% HBr, AcOH, **12** 73%, **13** 85%.

Treatment of 3-hydroxyphenethylamines **12** and **13** with bromine in acetic acid in the presence of hydrochloric acid to dissolve the substrate gave colorless materials slightly insoluble in organic solvents. These compounds were confirmed to be zwitterions of the desired 2,4,6-tribromo-3-hydroxyphenethylamines due to their high solubility in hot water, and successful conversion to their hydrochloride salts **21** and **22**. Hence, the amino moiety must be required to protect prior to bromination. Treatment of phenethylamines **12** and **13** in refluxing ethyl formate furnished the *N*-formyl compounds without inducing formylation of phenolic hydroxy group. These

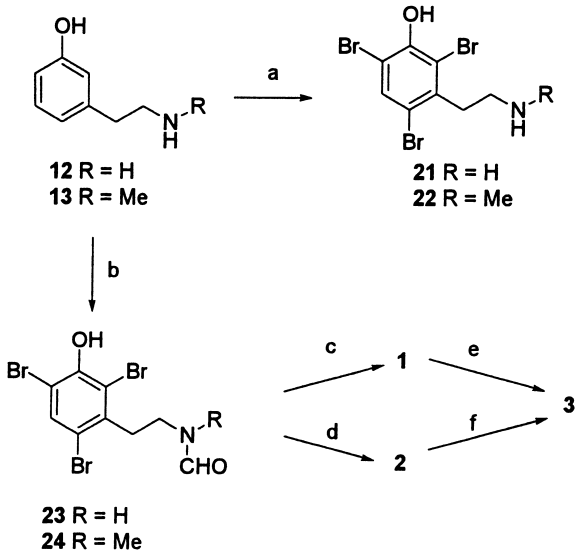


Scheme 3. Reagents and conditions: (a) HCl, MeOH, 91%; (b) 40% $MeNH_2$, EtOH, 99%; (c) BH_3, THF, 84%.



Scheme 4. Reagents and conditions: (a) HCl, MeOH, 83%; (b) 40% $MeNH_2$, EtOH, 91%; (c) BH_3, THF, 83%.

formyl compounds, however, decomposed gradually even at room temperature, so that they were immediately brominated after removal of excess ethyl formate to afford tribromo amides **23** and **24** almost quantitatively. The target lutamides A (**1**) and C (**2**) were obtained by methylation of the phenolic precursors with diazomethane in excellent yields. Hydrolysis of lutamide C (**2**) with methanolic hydrochloric acid provided a 74% yield of convoluamine F (**3**), which was also obtained by borane reduction of lutamide A (**1**). The materials obtained were identical in all respects with the natural samples of convolutamines and lutamides.

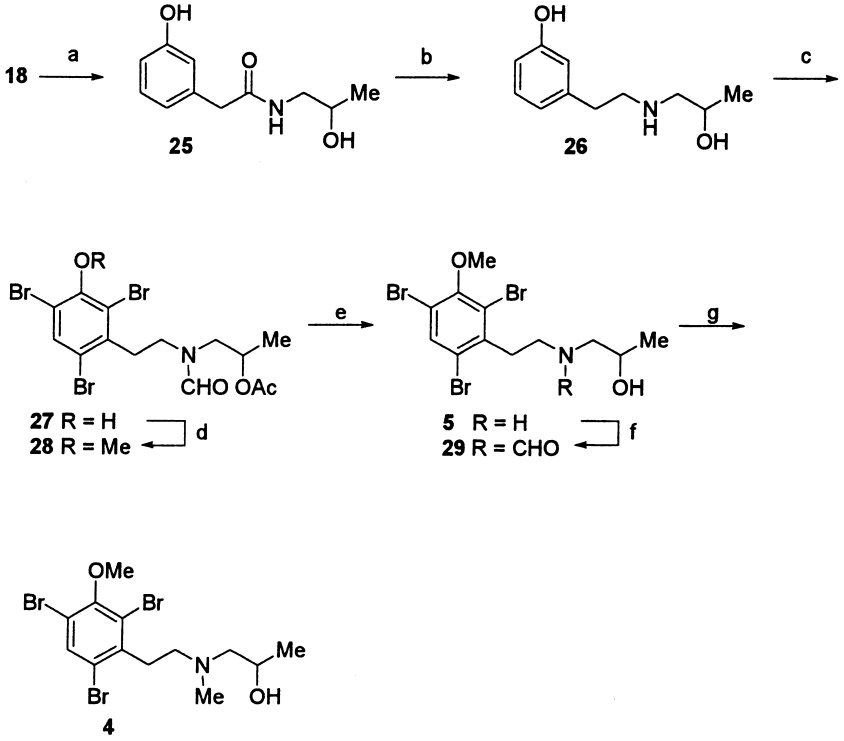


Scheme 5. Reagents and conditions: (a) Br_2, HCl, AcOH, **21** 71%, **22** 54%; (b) i. HCO_2Et; ii. Br_2, AcOH, **23** 97% (2 steps), **24** 100% (2 steps); (c) CH_2N_2, MeOH, −5 °C, 87%; (d) CH_2N_2, MeOH, −5 °C, 95%; (e) BH_3, THF, −5 °C, 67%; (f) 12 M HCl, MeOH, 74%.

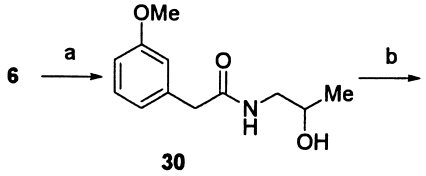
The methodology established above was extended to construction of convolutamines A (**4**) and C (**5**). Condensation of carboxylic acid **18** with 1-amino-2-propanol proceeded using dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), carbonyldiimidazole or diphenylphosphinic chloride, but the yields of amide **25** were 30–40%. Consequently, the amidation was best effected with diethyl cyanophosphonate to afford a 61% yield of a pure product **25**. The following derivatization is illustrated in Scheme 6. *O*-Acetyl compound **28** was successfully hydrolyzed to convolutamine C (**5**), the conversion of which into another alkaloid **4** was achieved by formylation and subsequent borane reduction. Both natural products were identified by the comparison of the ^1H and ^{13}C NMR spectra of these synthetic specimens. Debromoconvolutamine C (**31**) was synthesized from anisole **6** for comparison of biological activities with convolutamines as shown in Scheme 7.

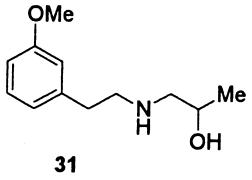
Biological Activities

The synthetic products were examined for their biological activities in vitro, and IC_{50} values for those compounds are summarized in Table 1. Lutamides A (**1**) and C (**2**) did not inhibit the growth of drug-sensitive tumor cells, i.e., murine leukemia P388/S, human oral



Scheme 6. Reagents and conditions: (a) 1-amino-2-propanol, $(EtO)_2PO(CN)$, Et_3N, THF, −7 to −10 °C, 90%; (b) BH_3, THF, 84%; (c) i. HCO_2Et; ii. Br_2, AcOH, 55% (2 steps); (d) CH_2N_2, MeOH, 96%; (e) 12 M HCl, MeOH, 79%; (f) HCO_2Et, 96%; (g) BH_3, thf, −5 °C, 71%.





Scheme 7. Reagents and conditions: (a) 1-amino-2-propanol, $(EtO)_2$-PO(CN), Et_3N, THF, 61%; (b) BH_3, THF, 82%.

epidemoid KB/S and human monocyte-like lymphocytic leukemia U937 cells. However, these amides displayed the growth inhibition against adriamycin (ADM)-resistant P388/ADM, vincristine (VCR)-resistant P388/VCR and KB/VJ300 cells in the presence of ADM or VCR whose concentration affected no growth of the cells examined. The best result was obtained at the IC_{50} value (4.8 μg/mL) of lutamide **2** against P388/VCR. In other words, lutamides reverse resistance to ADM and VCR in their resistant tumor cells. On the other hand, convolutamines **3**–**5** inhibited the cell growth of both drug-sensitive and -resistant P388 cell lines and U937, but did not show any growth inhibition against KB cell lines. As can be seen in Table 1, the IC_{50} values for the convolutamines against the drug-resistant P388 cell lines can be ascribed to combined cytocidal effect of convolutamine and the antitumor agents, ADM or VCR, through overcoming drug resistance by convolutamine.

Interleukin-6-induced growth of IL-6-independent mouse myeloid MH-60 cell was weakly inhibited by lutamide **1**, convolutamines **3** and **5**, whose IC_{50} values were 13, 12 and 19 μg/mL, respectively. Conversely, the convolutamine **5** only showed weak growth inhibition against IL-6-dependent MH-60 cells with IC_{50} value of 12 μg/mL. A weak inhibitory activity toward cell division of fertilized sea urchin (*Psedocentrous depressus*) eggs was observed on lutamide **2**, convolutamine **3** and debromoconvolutamine C (**30**) with IC_{50} values of 48, 81 and 20 μg/mL, respectively.

Table 1. Cytotoxic activity of convolutamines, lutamides, and some analogues[a]

Compound	IC_{50} (μg/mL)					
	P388/S	P388/ ADR[b]	P388/ VCR[c]	KB/S	KB/ VJ300[d]	U937
L. A 1	>25	18	13.8	>25	7.5	86.8
L. C 2	>25	>25	4.8	>25	6.5	55.9
C. F 3	13.8	9.5	8.0	>25	>25	12.0
C. A 4	17.5	7.0	3.0	>25	>25	33.6
C. C 5	12.5	3.0	1.4	>25	>25	11.9
22	>25	>25	>25	>25	>25	>100
23	>25	>25	>25	>25	>25	70.7
24	>25	19	>25	>25	>25	>100
27	>25	>25	>25	>25	>25	>100
29	>25	>25	8.0	>25	10.0	36.3
31	>25	>25	>25	>25	>25	68.8

[a]P388/S, P388/ADR, and P388/VCR are drug-sensitive, ADM- and VCR-resistant murine leukemia cells, respectively. KB/S and KB/VJ300 are VCR-sensitive and -resistant human oral epidemoid carcinoma cell lines, respectively.
[b]With added ADM 0.1 μg/mL, which did not affect any growth of the P388/ADR cells.
[c]With added VCR 0.04 μg/mL, which did not affect any growth of the P388/VCR cells.
[d]With added VCR 0.25 μg/mL, which did not affect any growth of the KB/VJ300 cells.

Discussion

In comparison with biological activities of the synthetic products **1**–**5**, their precursors and the related

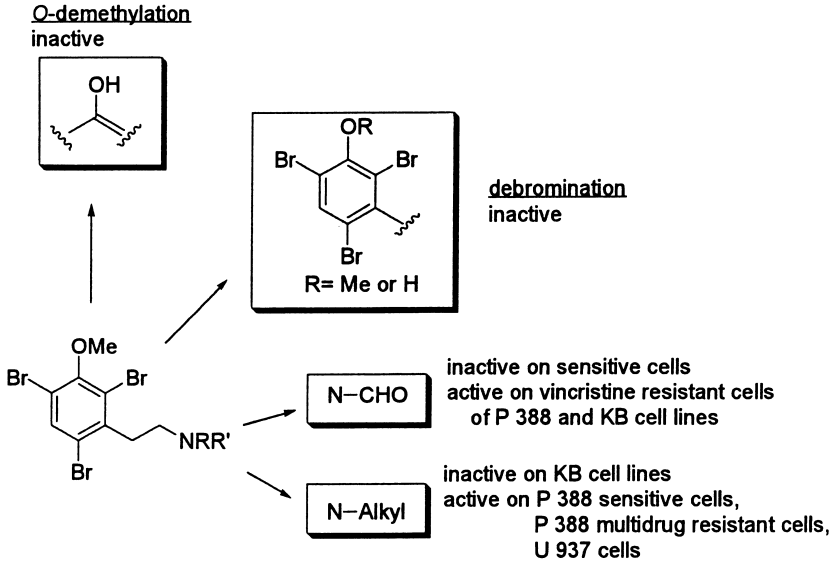
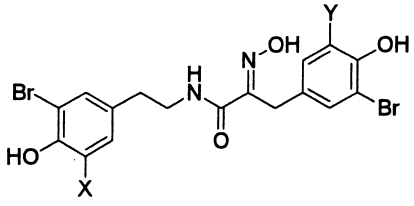


Figure 1. Variations of activities for U937, P388, and KB cells.

compounds, 3-methoxy-2,4,6-tribromophenyl moiety can be rationalized in association with the cytotoxicity. Additional formyl group on phenethylamine rather suppresses inhibitory activities on cell growth of U937 and P388 cell lines. This deduction was further verified by a bioassay of *N*-formylconvolutamine C (**28**), which was synthesized by treatment of convolutamine C (**5**) with ethyl formate, as shown in Table 1. The relationship of structure–activity is illustrated in Figure 1.

In addition to lutamides and convolutamines, amathamides,[3,4] amathaspiramides,[14] volutamides[15] and alteranatamides[16] have been isolated from the marine *Amathia* genus, all of which possess a bromophenyl unit. Regardless of such structural similarity with lutamides and convolutamines, they were believed to have antibacterial activities but not cytotoxicity against tumor cells. In contrast, hemibastadins **32**, **33** and **34**, isolated from marine sponge *Ianthella basta* of Papua New Guinea,[17] displayed the biological activities against P388 cell lines, which are similar to those of the above lutamides and convolutamines, in spite of bromotyrosine derivatives.



hemibastadin 1 (32) X = H, Y = H
hemibastadin 2 (33) X = H, Y = Br
hemibastadin 3 (34) X = Br, Y = H

Conclusion

Analysis of structure–activity relationships may explain why 2,4,6-tribromo-3-methoxyphenethylamine is an indispensable unit for producing biological activities. Another finding is reversal of ADM or VCR resistance by convolutamines and lutamides, and especially the latter alkaloids have a better promise than the former in terms of lack of cytotoxicity against drug-sensitive P388 cells.

Syntheses of the other convolutamines and lutamides are now in progress and will be presented in our subsequent paper.

Experimental

Melting points were determined using a Büchi 535 apparatus and uncorrected. Boiling points were uncorrected. Ot refers to oven temperature for Kugelrohr-distillation. NMR spectra were obtained with a JEOL EX270 spectrometer with solutions in deuteriochloroform unless otherwise noted, containing tetramethylsilane as internal standard. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-AX505H spectrometer.

Synthesis

3-(2-Bromoethyl)phenol (8). A suspension of LAH (1.921 g, 0.051 mol) in dry THF (30 mL) was refluxed with stirring for 1 h, and a solution of **6** (6.654 g, 0.040 mol) in dry THF (10 mL) added at a rate to keep the mixture at gentle reflux without external heating. The mixture was refluxed for 3 h and then cooled to rt. EtOAc (30 mL) was added dropwise to decompose the excess of LAH. After adding 6 M HCl (30 mL) followed by saturation with NaCl, the organic layer was separated, and the aqueous layer was extracted with THF (3×20 mL). The combined organic layers were washed with brine (5 mL), dried ($MgSO_4$) and concentrated. The residual oil was chromatographed on silica gel, and an unidentified material was eluted (hexane:EtOAc, 4:1). 2-(3-Methoxyphenyl)ethanol (**7**) was obtained from further elution (hexane:EtOAc, 1:3), which was distilled to give a colorless oil (5.318 g, 87%), bp 113–114 °C/5 mmHg (lit.[18] 141–143 °C/12 mmHg); ^{1}H NMR: δ 2.84 (t, 2H, J = 6.6 Hz), 3.80 (s, 3H), 3.86 (t, 2H, J = 6.6 Hz), 6.77–6.83 (m, 3H), 7.20–7.25 (m, 1H); ^{13}C NMR: δ 39.2, 55.1, 63.5, 116.7, 114.7, 121.3, 129.5, 140.1, 159.7.

A mixture of **7** (3.040 g, 0.020 mol), tributylhexadecylphosphonium bromide (0.508 g, 1.0 mmol) and 47% HBr (25 mL, 0.22 mol) was stirred and refluxed for 1.5 h. Water (100 mL) was added, and the mixture was extracted with $CHCl_3$ (3×20 mL). The organic layer was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel, and the first elution (hexane:EtOAc, 19:1) afforded **9** (0.222 g, 5%), at 100 °C/3 mmHg; ^{1}H NMR: δ 3.14 (t, 2H, J = 7.6 Hz,), 3.56 (t, 2H, J = 7.6 Hz), 3.80 (s, 3H), 6.75–6.81 (m, 3H), 7.21–7.27 (m, 1H); ^{13}C NMR: δ 32.7, 39.5, 55.2, 112.2, 114.5, 121.0, 129.6, 140.4, 159.8. Anal. calcd for $C_9H_{11}OBr$: C, 50.26; H, 5.15. Found: C, 49.94, H, 5.14.

The second elution (hexane:EtOAc, 4:1) gave a colorless oil (3.527 g), which was purified by Kugelrohr-distillation to provide **8** (3.083 g, 76%) as a colorless oil. The analytical sample was obtained by distillation, bp 117 °C/4 mmHg; ^{1}H NMR: δ 3.12 (t, 2H, J = 7.6 Hz), 3.56 (t, 2H, J = 7.6 Hz), 6.70–6.80 (m, 3H), 7.16–7.22 (m, 1H); ^{13}C NMR: δ 32.7, 39.0, 113.9, 115.6, 121.0, 129.7, 140.6, 155.4. Anal. calcd for C_8H_9OBr: C, 47.79; H, 4.51. Found: C, 47.67; H, 4.58.

2,4,6-Tribromo-3-(2-bromoethyl)phenol (10). A solution of bromine (5.660 g, 35 mmol) in AcOH (6 mL) was added dropwise to a stirred solution of **8** (2.016 g, 10 mmol) in AcOH (4 mL), and the mixture was heated at 70–80 °C for 1 h and then concentrated. The residue was dissolved in $CHCl_3$ (20 mL), and the organic layer was decolorized with dilute aqueous $NaHSO_3$ (10 mL). The solution was washed with brine (5 mL), dried ($MgSO_4$) and concentrated. The residue was recrystallized from hexane to afford **10** (3.618 g, 82%) as colorless needles, mp 100 °C; ^{1}H NMR: δ 3.42–3.57 (m, 4H), 5.99 (s, 1H), 7.71 (s, 1H); ^{13}C NMR: δ 27.5, 40.3, 108.8, 113.1, 115.0, 134.8, 138.0, 149.4. Anal. calcd for $C_8H_6OBr_4$: C, 21.95; H, 1.38. Found: C, 22.38; H, 1.29.

2,4,6-Tribromo-3-(2-bromoethyl)anisole (11). An ethereal solution of diazomethane, which was prepared from *p*-tolylsulfomethylnitrosamide (0.175 mg, 8 mmol), was added dropwise to a suspension of **10** (2.177 g, 5.0 mmol) in MeOH (25 mL) at −5 °C until a pale yellow color was developed, and the mixture was stirred for 30 min at the ambient temperature and concentrated. The residue was chromatographed on silica gel (hexane:EtOAc, 4:1), and the oily product was recrystallized from hexane to provide colorless needles (1.785 g, 82%), mp 59 °C; ^{1}H NMR: δ 3.46–3.55 (m, 4H), 3.87 (s, 3H), 7.77 (s, 1H); ^{13}C NMR: δ 27.5, 40.4, 60.5, 117.1, 119.8, 121.8, 135.6, 138.7, 154.2. Anal. calcd for $C_9H_8OBr_4$: C, 23.93; H, 1.78. Found: C, 23.91; H, 1.69.

Methyl (3-methoxyphenyl)acetate (16). A mixture of **6** (8.310 g, 50 mmol) in 10% HCl in MeOH solution (50 mL) was stirred and refluxed with stirring for 5 h and then concentrated. After addition of ice-water (30 mL), the mixture was made basic with $NaHCO_3$ and extracted with EtOAc (3×20 mL). The organic layers were washed with water, dried ($MgSO_4$) and concentrated. Purification by Kugelrohr-distillation provided a colorless oil (8.221 g, 91%). The analytical sample was obtained by short-path distillation, bp 106–107 °C/3 mmHg (lit.[19] 87–90 °C/0.25 mmHg); ^{1}H NMR: δ 3.60 (s, 2H), 3.69 (s, 3H), 3.80 (s, 3H), 6.80–6.88 (series of m, 3H), 7.24 (t, 1H, J = 7.6 Hz); ^{13}C NMR: δ 41.2, 52.1, 55.2, 112.6, 114.9, 121.6, 129.6, 135.4, 159.7, 171.9. Anal. calcd for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71. Found: C, 66.62; H, 6.78.

Methyl (3-hydoxyphenyl)acetate (19). The ester was prepared using **18** in 83% yield by the above procedure for the synthesis of **16** as colorless oil, bp 135–136 °C/2 mmHg; ^{1}H NMR: δ 3.58 (s, 2H), 3.70 (s, 3H), 5.42 (br s, 1H), 6.72–6.84 (series of m, 3H), 7.17 (t, 1H, J = 7.6 Hz); ^{13}C NMR: δ 41.0, 52.3, 114.3, 116.2, 121.3, 129.7, 135.0, 156.0, 172.9. Anal. calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 64.78; H, 6.34.

***N*-Methyl-(3-methoxyphenyl)acetamide (17).** A solution of **16** (5.412 g, 0.030 mol), and 40% aqueous methylamine (40 mL, 0.47 mol) in EtOH (100 mL) was stirred and heated at 70 °C (bath temperature) for 2 h, and then the methylamine (10 mL) was added. After being heated for an additional 1 h, the mixture was concentrated, and Kuagelrohr-distillation provided a colorless oil (5.342 g, 99%), which was redistilled; bp 163–164 °C/4 mmHg, which crystallized to off-white solid at room temperature; ^{1}H NMR: δ 2.84 (d, 3H, J = 5.0 Hz), 3.55 (s, 2H), 3.81 (s, 3H), 5.49 (br s, 1H), 6.80–6.85 (series of m, 3H), 7.27 (t, 1H, J = 7.8 Hz); ^{13}C NMR: δ 26.5, 43.8, 55.3, 112.8, 115.2, 121.8, 130.1, 136.4, 160.1, 171.6. Anal. calcd for $C_{10}H_{13}NO$: C, 67.02; H, 7.31; N, 7.82. Found: C, 67.48; H, 7.55; N, 7.11.

***N*-Methyl-(3-hydoxyphenyl)acetamide (20).** A solution of **19** (4.166 g, 0.025 mol) and 40% aqueous methylamine (32 + 7 mL, 0.46 mol) in EtOH (75 mL) was worked up as described for the synthesis of **17**. Concentration of the reaction mixture and successive refrigeration overnight gave a colorless solid. Recrystallization from EtOH afforded colorless prisms (3.755 g, 91%), mp 150–151 °C; ^{1}H NMR (DMSO-d_6): δ 2.56 (d, 3H, J = 5.0 Hz), 3.28 (s, 2H), 6.59–6.67 (series of m, 3H), 7.06 (t, 1H, J = 7.8 Hz), 9.29 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 25.7, 42.5, 113.4, 115.9, 119.7, 129.1, 137.7, 157.2, 170.6. Anal. calcd for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.75, H, 6.77; N, 8.53.

***N*-Methyl-3-methoxyphenethylamine (15).** From **17**. Borane–THF complex (1.0 M in THF, 100 mL, 0.10 mol) was added to a stirred solution of **17** (3.590 g, 0.020 mmol) in dry THF (100 mL), and the mixture was gently refluxed for 18 h. After being cooled to rt and 6 M HCl added (20 mL), the mixture was concentrated, diluted with water (30 mL) and basified with $NaHCO_3$. The solution was saturated with NaCl and extracted with BuOH (3×20 mL). The combined organic layers were washed with brine (5 mL), dried (K_2CO_3) and concentrated. Kugelrohr-distillation of the residue gave **15** (2.796 g, 84%) as a colorless oil, which was redistilled, bp 89–91 °C/4 mmHg (lit.[20] 118 °C/7 mmHg); ^{1}H NMR: δ 2.43 (s, 3H), 2.75–2.88 (m, 4H), 3.80 (s, 3H), 6.75–6.82 (series of m, 3H), 7.21 (t, 1H, J = 7.8 Hz); ^{13}C NMR: δ 36.2, 36.3, 53.0, 55.1, 111.4, 114.5, 121.1, 129.4, 141.6, 159.7.

From **9**. A solution of **9** (0.206 g, 1.2 mmol) and 40% aqueous methylamine (5.2 mL, 60 mmol) in EtOH (100 mL) was refluxed with stirring for 2 h, and then the methylamine (2.6 mL) was added again. The mixture was further heated for 1 h and concentrated. The residue was dissolved in water (10 mL), and the mixture was made basic with $NaHCO_3$. Extraction with BuOH and successive work up in the above manner gave a colorless oil (0.156 g, 78%).

3-Hydroxyphenethylamine (12). A mixture of 97% 3-methoxyphenetylamine (**14**) (Aldrich, 4.680 g, 0.030 mol), 47% HBr (20 mL) and AcOH (20 mL) was refluxed with stirring for 3 h and then concentrated. The residue was basified with 2 M aqueous NaOH and the solution was washed with ether (2×30 mL). The aqueous layer was acidified with 6 M HCl and then made basic with 28% aqueous ammonium hydroxide. After saturating with NaCl, the solution was extracted with BuOH (3×20 mL), dried (K_2CO_3) and concentrated. Kugelrohr-distillation and recrystallization from EtOH gave colorless prisms (3.011 g, 73%), mp 102.5–103 °C; ^{1}H NMR: δ 2.72 (t, 2H, J = 6.6 Hz), 3.02 (t, 2H, J = 6.6 Hz), 4.45 (br s, 3H), 6.63–6.69 (series of m, 3H), 7.16 (t, 1H, J = 7.8 Hz); ^{13}C NMR: δ 38.6, 42.7, 113.9, 116.0, 120.1, 130.0, 138.4, 140.8. Anal. calcd for $C_8H_{11}NO$: C, 70.04; H, 8.08; N, 10.21. Found: C, 70.00; H, 8.14; N, 10.04.

***N*-Methyl-3-hydroxyphenethylamine (13).** From **15**. This compound was obtained from **15** in 85% yield by the above procedure for the demethylation of **14** to **12**, at 138–139 °C/4 mmHg; ^{1}H NMR: δ 2.42 (s, 3H), 2.80 (t, 2H, J = 6.5 Hz), 2.93 (t, 2H, J = 6.0 Hz), 6.64–6.73 (series of m, 3H), 7.21 (t, 1H, J = 7.9 Hz); ^{13}C NMR: δ 35.3, 35.7, 52.2, 114.1, 116.4, 118.9, 130.3, 140.4, 158.0. Anal. calcd for $C_9H_{13}NO$: C, 71.49; H, 8.67; N, 9.26. Found: C, 71.22; H, 9.03; N, 9.17.

From **20**. The amine was also prepared by borane reduction using **20** in 83% yield by the above procedure for the synthesis of **15** from **17**, as a colorless oil.

From **8**. Amination of **8** with methylamine was accomplished as described for the synthesis of **3** from **11** (72% yield).

2,4,6-Tribromo-3-hydroxyphenethylamine hydrochloride (21). A solution of bromine (1.12 g, 7.0 mmol) in AcOH (0.8 mL) was added dropwise to a stirred solution of **12** (0.275 g, 2.0 mmol) in AcOH (0.8 mL) and 12 M HCl (1.7 mL). The mixture was stirred for 15 min and then heated at 60–70 °C (bath temperature) for 30 min. After concentrating and adding water (10 mL), the mixture was treated with $NaHCO_3$ until the evolution of carbon dioxide ceased and refrigerated overnight. The precipitated material was collected and dissolved in warmed 6 M HCl (20 mL). The solution was concentrated, and the residue was recrystallized from water to afford colorless tiny needles (0.581 g, 71%), mp 274–275 °C (decomp); ^{1}H NMR (DMSO-d_6): δ 2.86 (br s, 2H), 3.25–3.32 (m, 2H), 7.87 (s, 1H), 8.50 (br s, 1H); ^{13}C NMR (DMSO-d_6): δ 34.8, 36.4, 111.2, 113.8, 115.8, 134.4, 135.8, 151.1. Anal. calcd for $C_8H_8NOBr_3$·HCl: C, 23.42; H, 2.41; N, 3.41. Found: C, 23.40; H, 2.32; N, 3.23.

***N*-Methyl-2,4,6-tribromo-3-hydroxyphenethylamine hydrochloride (22).** This compound was prepared by the above procedure using *N*-methylphenethylamine **13**, and the crude product was recrystallized from EtOH–ether giving colorless tiny needles (54% yield); mp 218–219 °C (decomp); ^{1}H NMR (DMSO-d_6): δ 2.62 (s, 3H), 2.91–2.97 (m, 2H), 3.29–3.36 (m, 2H), 7.88 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 32.2, 33.2, 45.2, 111.2, 113.8, 115.8, 134.4, 135.8, 151.1. Anal. calcd for $C_9H_{10}NOBr_3$·HCl: C, 25.47; H, 2.61; N, 3.3. Found: C, 25.86; H, 3.19; N, 2.54.

***N*-(2,4,6-Tribromo-3-hydroxyphenethyl)formamide (23).** A mixture of **12** (0.687 g, 5.0 mmol) in ethyl formate (10 mL) was refluxed with stirring for 6 h, and the clear solution was concentrated. The residue was dissolved in AcOH (2.0 mL), and a solution of bromine (2.81 g, 18 mmol) in AcOH (3.0 mL) was added, when exothermic reaction was observed. After being stirred at ambient temperature for 15 min, the mixture was heated at 60–70 °C (bath temperature) for 30 min and then concentrated. The residue was triturated with water (30 mL), and the solid was collected by filtration and dried in air to give **23** (1.990 g, 97%), which was recrystallized from EtOH as colorless prisms, mp 189–190 °C; ^{1}H NMR (DMSO-d_6): δ 3.04–3.10 (series of m, 2H), 3.23–3.31 (m, 2H), 7.83 and 7.84 (each s, due to geometrical isomer, 1H), 7.89 and 8.00 (each s, due to geometrical isomer, 1H), 8.20 (br s, 1H); ^{13}C NMR (DMSO-d_6): δ 35.2, 36.8, 110.6, 114.1, 115.9, 134.3, 137.8, 150.9, 161.1 and 164.1 (due to geometrical isomer). Anal. calcd for $C_9H_8NO_2Br_3$: C, 26.90; H, 2.01; N, 3.49. Found: C, 26.86; H, 1.96; N, 3.37.

***N*-Methyl-(2,4,6-tribromo-3-hydroxyphenethyl)formamide (24).** This compound was prepared by the above procedure using **13** (0.456 g, 3.0 mmol), ethyl formate (6.0 mL) and bromine (1.68 g, 10.5 mmol). After concentration of the reaction mixture, to the residue was added water (20 mL), and the mixture was extracted with $CHCl_3$ (20 mL + 2×10 mL). The organic layer was washed with water (10 mL) containing $NaHSO_3$ and then water, dried ($MgSO_4$) and concentrated. The residue was crystallized with a small amount of EtOH to give **24** (0.908 g, 100%), which was recrystallized from EtOH to afford colorless tiny needles, mp 145.5–146 °C; ^{1}H NMR: δ 2.99 (s, 3H), 3.20–3.26 (series of m, 2H), 3.40–3.56 (m, 2H), 6.37 (br s, 1H), 7.70 and 7.71 (each s, due to geometrical isomer, 1H, J = 5.0 Hz), 8.04 and 8.05 (each s, due to geometrical isomer, 1H, J = 4.0 Hz); ^{13}C NMR: δ 30.3, 34.7, 35.1, 36.7, 42.5, 47.4, 108.5, 113.3 and 113.5 (due to geometrical isomer), 114.9 and 115.1 (due to geometrical isomer), 134.8 and 134.9 (due to geometrical isomer), 136.9 and 137.9 (due to geometrical isomer), 149.8, 162.7 and 162.8 (due to geometrical isomer). Anal. calcd for $C_{10}H_{10}NO_2Br_3$: C, 28.88; H, 2.42; N, 3.37. Found: C, 29.32; H, 2.36; N, 3.25.

Lutamide A (1). A slurry of **23** (1.208 g, 3.0 mmol) in MeOH (15 mL) was treated with diazomethane, prepared from *p*-tolylsulfomethylnitrosamide (0.105 g, 4.8 mmol), as described for the synthesis of **11**. After concentration, the residue was recrystallized from EtOH to afford colorless needles (1.084 g, 87%), mp 105 °C; ^{1}H NMR: δ 3.22–3.29 (m, 2H), 3.43–3.62 (m, 2H), 3.87 (s, 3H), 5.71 (br s, 1H), 7.77 and 7.78 (each s, due to geometrical isomer, 1H), 8.0 and 8.17 (each s, due to geometrical isomer, 1H); ^{13}C NMR: δ 36.2 and 36.7 (due to geometrical isomer), 38.7 and 39.6 (due to geometrical isomer), 60.5 and 60.6 (due to geometrical isomer), 116.6, 119.9 and 120.0 (due to geometrical isomer), 121.9 and 122.0 (due to geometrical isomer), 135.5 and 135.6 (due to geometrical isomer), 137.3 and 138.4 (due to geometrical isomer), 154.1 and 154.2 (due to geometrical isomer), 161.2 and 164.2 (due to geometrical isomer). Anal. calcd for $C_{10}H_{10}NO_2Br_3$: C, 28.87; H, 2.42; N, 3.36. Found: C, 28.89; H, 2.42; N, 3.24.

Lutamide C (2). This compound was prepared by the above procedure using **24** (0.832 g, 2.0 mmol) in MeOH (10 mL). After concentration of the reaction mixture, the residue was chromatographed on silica gel (hexane: EtOAc, 1:1) providing **2** (0.820 g, 95%), which was recrystallized from EtOH to give colorless needles, mp 69–70 °C; ^{1}H NMR: δ 3.00 (s, 3H), 3.24 (m, 2H), 3.40–3.53 (m, 2H), 3.88 (s, 3H), 7.76 and 7.78 (each s, 1H, due to geometrical isomer), 8.05 (s, 1H); ^{13}C NMR: δ 30.2, 34.7, 35.0, 42.4 and 47.3 (due to geometrical isomer), 60.5 and 60.6 (due to geometrical isomer), 116.6 and 117.0 (due to geometrical isomer), 119.8 and 120.0 (due to geometrical isomer), 121.9 and 122.0 (due to geometrical isomer), 135.5 and 135.7 (due to geometrical isomer), 137.5 and 138.6 (due to geometrical isomer), 154.3, 162.5 and 162.6 (due to geometrical isomer). Anal. calcd for $C_{11}H_{12}NO_2Br_3$: C, 30.73; H, 2.81; N, 3.26. Found: C, 30.77: H, 2.75; N, 3.16.

Convolutamine F (3). From **11**. A solution of **11** (0.455 g, 1.0 mmol) and 40% aqueous methylamine (4.3 mL, 51 mmol) in EtOH (100 mL) was stirred and refluxed for 7 h, and then concentrated. The residue was chromatographed on silica gel, and the starting material (0.328 g, 72%) was recovered from the first elution (hexane:EtOAc, 4:1). The further elution (EtOAc:EtOH, 1:1) gave **3** (0.079 g, 19%) as colorless amorphous; ^{1}H NMR: δ 2.51

(s, 3H), 2.76–2.82 (m, 2H), 3.16–3.21 (m, 2H), 3.86 (s, 3H), 7.74 (s, 1H); ^{13}C NMR: δ 36.2, 37.7, 49.6, 60.6, 116.0, 120.0, 121.8, 135.4, 139.9, 154.0. Anal. calcd for $C_{11}H_{12}NO_2Br_3$: C, 29.88; H, 3.01; N, 3.48. Found: C, 30.02; H, 2.99; N, 3.31.

From lutamide A (**1**). Reduction of lutamide **1** with borane–THF complex was achieved as described for the synthesis of **15** from **17** (67% yield).

From lutamide C (**2**). A mixture of lutamide **2** (0.353 g, 0.82 mmol) in 12 M HCl in MeOH (1:10 v/v, 5.0 mL) was stirred and refluxed for 2 h and then concentrated. The residue was dissolved in water, and $NaHCO_3$ was added until the evolution of carbon dioxide ceased. The mixture was extracted with $CHCl_3$ (3×10 mL), and the organic layers were washed with water, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography on silica gel (EtOAc:EtOH, 1:1) to afford colorless amorphous **3** (0.243 g, 74%). An attempt at purification by Kugelrohr distillation failed, in which unidentified materials were formed.

***N*-(2-Hydroxypropyl)-3-hydroxyphenethylacetamide (25).** Hydroxyphenylacetic acid **18** (1.523 g, 10.0 mmol) was added in small portions to a stirred and cooled (−7 to −8 °C) solution of 98% DL-1-amino-2-propanol (0.768 g, 10.0 mmol) and triethylamine (1.022 g, 10.0 mmol) in dry THF (50 mL). At the same temperature, a solution of 93% diethyl cyanophosphonate (1.764 g, 10.1 mmol) in dry THF (10 mL) was added dropwise, and the resulting mixture was stirred below −10 °C for 1 h and then at rt for 1 h. During this period, the slurry became solution. After concentration, the residue was chromatographed on silica gel (EtOAc:EtOH, 9:1), and oily product was ultrasonically dispersed in hexane and concentrated. Drying in vacuo and then addition of $CHCl_3$ gave the crystallized amide **25** (1.918 g, 90%). Recrystallization from EtOAc provided colorless needles (1.288 g, 61%), mp 126–127 °C; ^{1}H NMR (DMSO-d_6): δ 0.99 (d, 3H, J = 6.3 Hz), 2.96–3.00 (m, 2H), 3.32 (s, 2H), 3.60–3.64 (m, 1H), 4.66 (d, 1H, J = 4.6 Hz), 6.58–6.67 (series of m, 3H), 7.06 (t, 1H, J = 7.9 Hz), 7.93 (br s, 1H), 9.27 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 21.1, 42.4, 46.4, 65.2, 113.2, 115.9, 119.6, 129.0, 137.8, 157.1, 170.2. Anal. calcd for $C_{11}H_{15}NO_3$: C, 63.14; H, 7.23; N, 6.69. Found: C, 63.13; H, 7.31; N, 6.55.

***N*-(2-Acetoxypropyl)-*N*-(2,4,6-tribromo-3-hydroxyphenethyl)formamide (27).** Borane–THF complex (1.0 M in THF, 77 mL, 0.077 mol) was added dropwise to a stirred solution of **25** (2.694 g, 12.9 mmol) in dry THF (130 mL), and the mixture was gently refluxed for 20 h. After being cooled to rt and then 6 M HCl added (15 mL), the mixture was worked up as described in the above borane reduction to amine compounds. The crude product was purified by chromatography on silica gel (EtOAc:EtOH, 1:1) to afford **26** as a colorless syrupy material including EtOAc (2.807 g); ^{1}H NMR: δ 1.14 (d, 3H, J = 6.0 Hz), 2.48–2.52 (m, 1H), 2.65–2.94 (series of m, 5H), 3.85 (m, 1H), 6.67–6.72 (series of m, 3H), 7.16 (t, 1H, J = 7.9 Hz); ^{13}C NMR: δ 21.0, 35.5, 50.1, 56.5, 65.8, 114.0, 116.1, 120.0, 130.0, 140.0, 157.0.

A mixture of syrup in ethyl formate (60 mL) was refluxed with stirring for 6 h and then concentrated. The residue was dissolved in AcOH (5.0 mL), and a solution of bromine (6.2 g, 39 mmol) in AcOH (8 mL) was added dropwise. The resulting mixture was heated at 75–80 °C (bath temperature) for 1 h and worked up as described above. After concentration of $CHCl_3$ extract, the residue was chromatographed on silica gel (hexane:EtOAc, 1:1 to EtOAc). A further purification by recrystallization from MeOH afforded **27** as colorless amorphous (3.558 g, 55%); ^{1}H NMR: δ 1.22–1.27 (m, due to geometrical isomer, 3H), 2.03 and 2.04 (each s, due to geometrical isomer, 3H), 3.19–3.76 (series of m, 6H), 5.06–5.21 (m, 1H), 6.15 (br s, 1H), 7.70 and 7.72 (each s, due to geometrical isomer, 1H), 8.05 and 8.06 (each s, due to geometrical isomer, 1H); ^{13}C NMR: δ 17.5 and 17.8 (due to geometrical isomer), 21.0 and 21.3 (due to geometrical isomer), 35.0, 37.0, 41.1, 45.7, 46.7, 52.0, 67.4 and 68.8 (due to geometrical isomer), 109.0, 113.2, 113.4, 114.8 and 114.9 (due to geometrical isomer), 134.6 and 134.7 (due to geometrical isomer), 136.5 and 137.4 (due to geometrical isomer), 149.5, 149.7, 163.1 and 163.3 (due to geometrical isomer), 170.2 and 170.4 (due to geometrical isomer). Anal. calcd for $C_{14}H_{16}NO_4Br_3$: C, 33.50; H, 3.21; N, 2.79. Found: C, 33.71; H, 3.18; N, 2.81.

***N*-(2-Acetoxypropyl)-*N*-(2,4,6-tribromo-3-methoxyphenethyl)formamide (28).** A solution of **27** (1.882 g, 3.75 mmol) in MeOH (20 mL) was treated with diazomethane as described in the above methylation of phenolic hydroxy group to give colorless amorphous (1.864 g, 96%). The analytical sample was obtained by chromatography on silica gel (hexane:EtOAc 1:1), ^{1}H NMR: δ 1.23–1.27 (each d, due to geometrical isomer, 3H), 2.03 and 2.04 (each s, due to geometrical isomer, 3H), 3.19–3.75 (series of m, 6H), 5.09–5.19 (m, 1H), 7.76 and 7.78 (each s, due to geometrical isomer, 1H), 8.05 and 8.07 (each s, due to geometrical isomer, 1H); ^{13}C NMR: δ 17.5 and 17.7 (due to geometrical isomer), 21.0 and 21.3 (due to geometrical isomer), 35.0, 37.0, 40.9, 45.6, 46.7, 51.9, 60.4 and 60.5 (due to geometrical isomer), 67.4 and 68.8 (due to geometrical isomer), 116.5 and 116.9 (due to geometrical isomer), 119.7 and 119.9 (due to geometrical isomer), 121.8 and 121.9 (due to geometrical isomer), 135.4 and 135.6 (due to geometrical isomer), 137.2 and 138.2 (due to geometrical isomer), 154.2, 162.9 and 163.1 (due to geometrical isomer), 170.1 and 170.3 (due to geometrical isomer). Anal. calcd for $C_{15}H_{18}NO_4Br_3$: C, 34.91; H, 3.52; N, 2.71. Found: C, 35.23; H, 3.41; N, 2.50.

Convolutamine C (5). A solution of **28** (0.344 g, 0.67 mmol) and 12 M HCl in MeOH (1:10 v/v, 4.5 mL) was refluxed with stirring for 2 h and then concentrated. The residue was made basic with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$ (3×10 mL). The combined extracts were washed with brine, dried ($MgSO_4$) and concentrated to afford **5** (0.234 g, 79%). Recrystallization from EtOH gave colorless tiny crystals, mp 78–79 °C; ^{1}H NMR: δ 1.18 (d, 3H, J = 6.3 Hz), 2.42–2.50 (m, 1H), 2.78–2.87 (series of m, 3H), 3.17 (t, 2H, J = 7.9 Hz), 3.74–3.81 (m, 1H), 3.86 (s, 3H), 7.74 (s,

1H); ^{13}C NMR: δ 20.5, 38.0, 47.2, 56.4, 60.4, 65.6, 115.9, 119.7, 121.7, 135.3, 139.5, 153.9. Anal. calcd for $C_{12}H_{16}NO_2Br_3$: C, 32.32; H, 3.62; N, 3.14. Found: C, 32.59; H, 3.61; N, 3.06.

***N*-(2-Hydroxypropyl)-*N*-(2,4,6-tribromo-3-methoxyphenethyl)formamide (29).** A mixture of **5** (0.364 g, 0.82 mmol) and ethyl formate (5 mL) was stirred and refluxed for 6 h and then concentrated. The residue was purified by chromatography on silica gel (hexane: EtOAc, 1:3) to give colorless amorphous (0.370 g, 96%); ^{1}H NMR: δ 1.21–1.25 (each d, due to geometrical isomer, 3H), 2.91 (br d, 1H), 3.21–3.58 (series of m, 6H), 3.87 (s, 3H), 4.10 (m, 1H), 7.76 and 7.78 (each s, due to geometrical isomer, 1H), 8.10 and 8.13 (each s, due to geometrical isomer, 1H); ^{13}C NMR: δ 20.6 and 21.4 (due to geometrical isomer), 35.1 and 37.3 (due to geometrical isomer), 41.1, 46.7, 51.7, 55.1, 60.5 and 60.6 (due to geometrical isomer), 64.9 and 67.0, 116.5, 117.0, 119.8, 119.9, 121.8, 121.9, 135.4 and 135.6 (due to geometrical isomer), 137.1 and 138.3 (due to geometrical isomer), 154.0 and 154.2 (due to geometrical isomer), 163.8 and 164.1 (due to geometrical isomer). Anal. calcd for $C_{13}H_{16}NO_3Br_3$: C, 32.94; H, 3.40; N, 2.96. Found: C, 33.24; H, 3.42; N, 2.64.

Convolutamine A (4). A solution of **29** (0.238 g, 0.50 mmol) in dry THF (10 mL) was reduced by borane–THF complex (1 M in THF, 3.0 mL, 3 mmol), and the mixture was worked up as described in the above borane reduction. The crude product was purified by chromatography on silica gel (hexane:EtOAc, 1:3) to give colorless amorphous (0.164 g, 71%); ^{1}H NMR: δ 1.15 (d, 3H, J = 6.3 Hz), 2.30–2.47 (m, 2H), 2.44 (s, 3H), 2.55–2.79 (m, 2H), 3.08–3.25 (m, 2H), 3.78–3.84 (m, 1H), 3.87 (s, 3H), 7.74 (s, 1H); ^{13}C NMR: δ 19.9, 34.7, 41.9, 55.1, 60.5, 63.1, 64.8, 116.0, 119.8, 121.7, 135.4, 139.7, 154.1. HRFABMS $(M+H)^+$ obsd 457.8889, calcd 457.8966 for $C_{13}H_{19}NO_2{}^{79}Br_3$. Anal. calcd for $C_{13}H_{18}NO_2Br_3$: C, 33.94; H, 3.94; N, 3.04. Found: C, 34.31; H, 3.89, N, 2.72.

***N*-2-Hydroxypropyl-(3-methoxyphenyl)acetamide (30).** This compound was prepared by the above procedure for the synthesis of **25**, using **6** instead of **18** (61% yield). The analytical sample was obtained by chromatography on silica gel (EtOAc) as a colorless oil; ^{1}H NMR: δ 1.12 (d, 3H, J = 6.3 Hz), 3.04–3.13 (m, 1H), 3.33–3.42 (m, 1H), 3.57 (s, 2H), 3.80 (s, 3H), 3.83–3.89 (m, 1H), 5.97 (br s, 1H), 6.82–6.86 (m, 3H), 7.27 (t, 1H, J = 7.6 Hz); ^{13}C NMR: δ 20.5, 43.3, 47.0, 55.0, 66.8, 112.5, 114.9, 121.4, 129.7, 136.2, 159.7, 172.0. Anal. calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 64.66; H, 8.00, N, 5.83.

1-(3-Methoxyphenethylamino)-2-propanol (31). This compound was prepared by reduction of **30** with borane by the above procedure to give colorless oil (82% yield), at 110 °C/4 mmHg; ^{1}H NMR: δ 1.14 (d, 3H, J = 6.0 Hz), 2.35–2.94 (series of m, 6H), 3.80 (s, 3H), 6.75–6.81 (series of m, 3H), 7.22 (t, 1H, J = 9.2 Hz); ^{13}C NMR: δ 20.5, 35.8, 50.4, 55.1, 56.4, 65.0, 111.6, 114.4, 121.0, 129.5, 140.9, 159.7. Anal. calcd for $C_{12}H_{19}NO_2$: C, 66.87; H, 9.15; N, 6.69. Found: C, 66.97; H, 9.43; N, 6.31.

Biological Procedure

Cytotoxic activity tests

Murine leukemia P388/S, P388/ADR, and P388/VCR cells as well as human oral epidermoid carcinoma KB/S and KB/VJ300 were maintained in culture flasks in MEM medium supplemented with 10% fetal bovine serium and kanamaycin (100 μg/mL). For the in vitro drug treatment experiments, tumor cells (2×10^4 cells) were seeded in 0.2 mL of culture medium/well in 96-well plates (Corning Glass Works). The cells were treated in triplicate with graded concentrations of antitumor agents in the presence or absence of VCR or ADM and were then incubated in a carbon dioxide incubator at 37 °C for 72 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was used to measure the cytotoxic effect.

The human monocyte-like lymphocytic leukemia U937 cells were maintained in tissue culture flasks and grown in 96-well microtiter plates for assay. The cells were treated in triplicate with graded concentrations of antitumor agents. After 72 h incubation at 37 °C in 5% carbon dioxide atmosphere, the survival rates of cells in the cultures were evaluated by the MTT method. The effect was shown as IC_{50} values.

Cytotoxicity in mouse myeloid MH-60 cells was measured using stock cultures of MH-60 cells washed twice with RPMI 1640 medium in order to remove completely recombinant human IL-6 (rhIL-6; Wako). Washed cells (5×10^3 cells) were suspended in 100 μL of RPMI medium containing 10% FCS and plated in a 96-well culture plate. Inoculated plates were incubated with 5 μL of test samples for 72 h at 37 °C in the presence of 0.002 U rhIL-6 (100 μL) in 5% carbon dioxide atmosphere. The cell growth was evaluated by the MTT method.

IL-6-independent MH-60 cell line was established by gradually decreasing IL-6 concentration in the medium. The growth rate of IL-6-independent cells in the medium without IL-6 was almost the same as that of IL-6-dependent cells, and the cytotoxicity on this cell line was examined by the method described above.

The impediment in sea urchin egg division was performed using dry sperms of sea urchins obtained by intracoelomic injection of 0.5 M KCl, which were stored in cold until use. Eggs were spawned into natural sea water (NSW) by intracoelomic injection of 0.5 M KCl, sedimented by a hand centrifuge and washed three times by resuspension in NSW and allowed to settle until use. The eggs divided around 90 min after insemination at 18 °C. At 5 min after insemination when fertilization membranes were elevated, the eggs were treated with sample solution in MeOH (100, 50, 25, 12, 6, 3 μg/mL). The rate of egg division of fertilized sea urchin eggs was evaluated by observing through a microscope after 90 min.

Acknowledgements

We are very pleased to acknowledge the very helpful support of Dr. A. Yamashita of Kanagawa University.

We also thank Ms. H. Hiraide of Kanagawa University for sea urchin egg assay.

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