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Synthetic analogues of the montanine-type alkaloids with activity against apoptosis-resistant cancer cells

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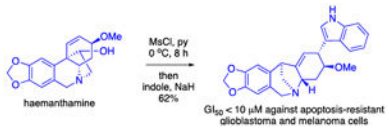
Abstract

In a search of small molecules active against apoptosis-resistant cancer cells, a skeletal rearrangement of alkaloid haemanthamine was utilized to generate a series of compounds possessing the alkaloid montanine ring system. The synthesized compounds were found to inhibit proliferation of cancer cells resistant to apoptosis at micromolar concentrations. Selected compounds were also active against patient-derived glioblastoma cells expressing stem-cell markers. This is the first report describing the preparation of synthetic analogues of the montanine-type alkaloids with antiproliferative activity. The compounds prepared in the current investigation appear to be a useful starting point for the development of agents to fight cancers with apoptosis resistance, and thus, associated with poor prognoses.

Graphical abstract



Keywords

Antiproliferative activity; Apoptosis resistance; Montanine; Haemanthamine; Manthine

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[1]These authors contributed equally,

Cancers with intrinsic resistance to apoptosis are characterized by the lack of responsiveness to current chemotherapeutic agents that generally work by the induction of apoptosis in cancer cells. These cancers include tumors of the lung, melanoma and glioblastoma and they represent a major challenge in the clinic.[1] For example, patients afflicted by glioblastoma multiforme,[2,3] have a median survival expectancy of less than 14 months when treated with the best available protocol that involves surgery, radiation and chemotherapy with temozolomide.[4] Further, the main cause of death of cancer patients are tumor metastases. Metastatic cells are resistant to anoikis, a type of apoptotic cell death triggered by the loss of contact with extracellular matrix or neighboring cells.[5] Resistance to anoikis, and thus apoptosis, renders metastatic cells unresponsive to the large majority of proapoptotic agents as well.[3,6] In addition, it is believed that a population of cancer cells, referred to as cancer stem cells, is particularly unresponsive to pro-apoptotic chemotherapy. Glioblastoma cells exhibiting stem cell markers (GSCs) can be grown in culture as neurospheres. They possess the ability to self-renew, differentiate into multiple neural lineages and faithfully recapitulate human glioblastoma on both histological and genetic levels when injected into the brains of mice.[7-9] Therefore, a search for novel anticancer agents that can overcome cancer cell resistance, including resistance to apoptosis, is an important pursuit.

In this connection, natural products represent a valuable source of not only cytotoxic compounds, but also those capable of overcoming the intrinsic resistance of cancer cells to apoptosis.[10-13] Our recent studies have focused on isocarbostyrils and alkaloids belonging to the *Amaryllidaceae* plant family. The medicinal value of the *Amaryllidaceae* plants was already recognized in the fourth century BC, when Hippocrates of Cos used oil from the daffodil *Narciclasus poeticus L.* for the treatment of cancer.[14] In more recent times, over 100 structurally diverse alkaloids, possessing a wide spectrum of biological activities have been isolated from the *Amaryllidaceae* species.[15] Narciclasine and lycorine (Figure 1) have been actively investigated for their potent anti-tumor effects, both *in vitro* and *in vivo*, in various pre-clinical models of human cancers by us[16-21] and others.[22-25] Our interest in these natural products primarily stems from their ability to overcome apoptosis resistance and display promising activity in orthotopic mouse models of glioblastoma and metastatic melanoma.[16,19,21] Although, not evaluated *in vivo*, crinine-type alkaloids, such as haemanthamine (Figure 1)[17] and bulbispermine,[26] also show promising activity against apoptosis-resistant cancer cells. To investigate whether promising antiproliferative properties are also associated with other representative *Amaryllidaceae* alkaloid groups, we noted that montanine (Figure 1) was shown to be cytotoxic to human cancer cells.[27] The montanine group of alkaloids is characterized by a 5,11-methanomorphanthridine ring system and some examples of the montanine-type alkaloids are shown in Figure 1. The work reported in the current manuscript describes the first systematic attempt to assess the anticancer potential of compounds based on the montanine-type alkaloid structural system.

Synthetic derivatization of the montanine-type alkaloids to generate a library of analogues is challenging because their stable and practical supply from the plant sources has not been established to our knowledge. In contrast, we and others have developed practical multigram isolation procedures for narciclasine, lycorine and haemanthamine.[28] We thus drew our attention to an old report of a semisynthetic transformation of haemanthamine-type skeleton to the montanine-type scaffold (Figure 2).[29] This elegant work, which was reproduced in a

later study,[30] described the intramolecular nucleophilic attack of the electron rich aromatic moiety on the activated C11-hydroxyl (**A** in Figure 2) to form the C10a-C11 bond presumably leading to the arenium ion **B**. The C10a-C10b bond then breaks upon the S_N2' attack of a nucleophile at C2 with the translocation of the C1-C2 alkene to the C1-C10b position resulting in the montanine-type skeleton **C** (Figure 2).

The reaction can be conducted without the addition of an external nucleophile for 48 h at rt, in which case it will result in chlorine-containing derivative **1**, where the chloride liberated upon mesylation serves as the nucleophile (Figure 3).[29] Alternatively, the reaction can be run at 0 °C for 8 hours with the subsequent addition of a nucleophilic species. In this case a wide variety of C2-substituted montanine-type synthetic derivatives can be prepared. Thus, the addition of water results in **2**.[29] The addition of methoxide, prepared by the reaction of MeOH with NaH leads to another montanine-type alkaloid manthine, isolated by Wildman from South African plant genus *Haemanthus*.[29,31] Depending on the species the yield varied from 1 to 44 mg per kg of wet bulbs, clearly insufficient for its supply for biological studies.[31] This alkaloid was also obtained by a total synthesis by Sha and coworkers, which involved a 24-step procedure from commercially available quinic acid.[32] The one step synthesis of manthine from haemanthamine, readily available in multigram quantities, is thus significant.

We found that other nucleophiles that can be utilized in this transformation are ethanol, allylic and propargylic alcohols, leading to compounds **3, 4** and **5**. In addition, a primary amine, indole and pyrrole can be added to the C2-position quite readily following their deprotonation with NaH, resulting in **6, 7** and **8** (Figure 3). In the case of indole, only C3-alkylation was observed, although both N1- and C3-alkylations are possible.

Because chloride **1** could be readily prepared on the preparative scale, it was utilized in further transformations, involving *N*-oxidation, *N*-propargylation, and single or double iodination of the aromatic ring, resulting in derivatives **9, 10, 11** and **12**, respectively (Figure 4A). In addition, **2** can be converted to bromide **13** (Figure 3B). Finally, we found that the transformation of the crinine to montanine skeleton can be also achieved using a related alkaloid, haemanthidine (Figure 4C). Here, the C6-mesylated product **14** was isolated in a reasonable yield. The stability of the mesylate is accounted for by the bridgehead nature of the tertiary nitrogen and thus its inability to stabilize the adjacent positive charge that would develop in the transition state of the hydrolysis reaction.[33,34]

The synthesized compounds were evaluated for *in vitro* antiproliferative properties using the MTT colorimetric assay against a panel of six cancer cell lines.[35] This included cells resistant to a number pro-apoptotic stimuli, such as human A549 non-small cell lung cancer (NSCLC), human glioblastoma U373, and human SKMEL-28 melanoma, as well as tumor models, which are largely susceptible to apoptosis-inducing stimuli, such as human Hs683 anaplastic oligodendroglioma, human MCF-7 breast adenocarcinoma and mouse B16F10 melanoma (Table 1). Analysis of these data reveals that the active derivatives displayed comparable potencies in both cell types, irrespective to cell sensitivities to apoptosis induction and suggesting that they are capable of overcoming apoptosis resistance (Table 1).[19]

Despite the large variations in cancer cell line sensitivities, the data indicate that a small C2-substituent is preferred as observed in chloride **1**, alcohol **2** and bromide **13**. Activity of chloride **1** and bromide **13**, in which the halogen atom has the inverted configuration, appears to indicate that the α-C2 configuration is not critical. The variations in cell line sensitivities to both chloride **1** and bromide **13** suggest that they are not indiscriminate alkylating agents, but have distinct intracellular targets. Of the C2-ethers, alkaloid manthine, containing the small C2-methoxy group, stands out by affording single digit GI_{50} values across the entire panel, including the glioblastoma U373 cells for which **1** and **2** are less effective. The replacement of the C2-OMe by the C2-OEt leads to the loss of activity as observed in **3**. The activity is regained by the introduction of a multiple bond as in **4** and **5**. Of the compounds possessing larger C2-substuents, the indole-containing compound **7** exhibited single-digit GI_{50} values against several cell lines. Further, the quaternization of the nitrogen (**9** and **10**) gives inactive compounds as well. It is likely that in this case the charged derivatives cannot cross the cell membrane. Finally, the substitution pattern on the aromatic ring appears to be critical as well, since the derivatization of the C7 and C10 positions (**11** and **12**) leads to inactive compounds.

Selected compounds, showing the highest potency in Table 1 were evaluated against a patient-derived GSC22 cell line that was authenticated for stem-like properties by validating self-renewal (sphere formation), expression of stem-cell markers (CD133, nestin), multi-lineage differentiation, and high efficiency for orthotopic *in vivo* tumor initiation in immunodeficient NOD SCID mice, as reported previously.[36,37] Figure 5 shows that manthine, compound **7** and haemanthamine significantly reduced proliferation at concentrations as low as 1 μM. All tested compounds inhibited proliferation by 80-95% at concentrations of 10 and 30 μM.

In conclusion, utilizing a previously described transformation of the crinine to montanine-type skeletons, we prepared the first series of synthetic analogues of the montanine alkaloids differing by the substituents at C2, N5, C6, C7 and C10 positions. It was found that the reaction readily proceeded not only with oxygen or halogen-based nucleophiles, but also with primary amines and nucleophilic heterocycles. The synthesized compounds were found to inhibit proliferation of cancer cells resistant to apoptosis at micromolar concentrations, with alkaloid manthine, C2-OH and C2-indole-substituted compounds appearing to be most potent. Selected compounds also were active against patient-derived glioblastoma cells expressing stem-cell markers. These preliminary results indicate that compounds based on the montanine skeleton should be investigated as potential anticancer agents active against drug-resistant cancer cells, including cancer stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and notes

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28. For example, we were able to identify Sterngberia lutea (L.) Ker-Gawl (Autumn daffodil) collected in Apula region, Italy as a rich source of lycorine (9.8 g per kg of dry bulbs) as well as *Narcissus pseudonarcissus* var. King Alfred, readily available from ornamental flower growers in England, as a stable source of narciclasine (0.1 g per kg of dried bulbs) and haemanthamine (0.4 g per kg of dried bulbs).
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33. Selected procedure for the preparation of alcohol 2: A solution of haemanthamine (100 mg, 0.332 mmol) in dry pyridine (3 mL) was treated with methanesulfonyl chloride (0.1 mL, 1.33 mmol) and allowed to stand at 0 °C for 8 h. The mixture was poured into of water (10 mL) containing sodium bicarbonate (100 mg). The solution was again allowed to stand overnight and then extracted with chloroform. The solvent was removed under reduced pressure. The resulting residue was purified by preparative TLC using CH_2Cl_2/MeOH (9:1) as an eluent to yield 74 mg of 2 (74%) as an amorphous white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.54 (s, 1H), 6.49 – 6.42 (m, 1H), 5.87 (dd, *J* = 10.6, 1.4 Hz, 2H), 5.53 (d, *J* = 14.9 Hz, 1H), 4.35 (dd, *J* = 16.6, 7.6 Hz, 1H), 4.06 (s, 1H), 3.86 (dd, *J* = 18.8, 9.8 Hz, 1H), 3.53 (d, *J* = 11.4 Hz, 1H), 3.41 – 3.34 (m, 4H), 3.31 (t, *J* =), 3.19 – 3.02 (m, 2H), 2.85 (s, 1H), 2.32 (ddd, *J* = 12.9, 5.1, 3.5 Hz, 1H), 1.56 (td, *J* = 12.5, 3.1 Hz, 1H); 13C NMR (100 MHz, $CDCl_3$) δ 152.8, 146.9, 146.1, 131.9, 123.8, 115.5, 107.4, 106.78, 100.8, 81.1, 67.1, 60.6, 58.9, 56.9, 55.4, 45.4, 28.3; HRMS (ESI) calcd for $C_{17}H_{20}NO_4^+$, 302.1392 (M +H)$^+$; found, 302.1388.
34. Selected procedure for the preparation of indole 7: A solution of haemanthamine (30 mg, 0.1 mmol) in dry pyridine (2 mL) was treated with methanesulfonyl chloride (33 μL, 0.4 mmol) and allowed to stand at 0 °C for 8 hrs. After another hour at 25 °C the reaction mixture was added via a cannula to an ice-cold THF (3 mL) solution of indole (23 mg, 0.15 mmol), pretreated with NaH (100 mg, 0.45 mmol). After stirring for 30 min, the reaction mixture was poured into water (10 mL) and extracted with chloroform (3 × 5 mL). The solvent was removed under reduced pressure and the residue was purified by preparative TLC using CH_2Cl_2:MeOH (20:1) mixture as the eluent to yield 7 (25 mg, 62%) as a semi-solid substance. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.66 (d, *J* = 7.8 Hz, 1H), 7.46 – 7.34 (m, 1H), 7.27 – 7.22 (m, 1H), 7.21 – 7.14 (m, 1H), 6.90 (d, *J* = 1.8 Hz, 1H), 6.65 (s, 1H), 6.56 (s, 1H), 5.95 (dt, *J* = 8.9, 4.5 Hz, 2H), 5.77 (s, 1H), 4.58 (d, *J* = 16.4 Hz, 1H), 4.16 – 4.01 (m, 1H), 3.90 – 3.85 (s, 1H), 3.80 – 3.71 (m, 2H), 3.64 – 3.56 (m, 1H), 3.49 (s, 3H), 3.43 – 3.27 (m, 2H), 2.53 (s, 1H), 1.75 – 1.65 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.8, 126.2, 122.5, 121.9, 119.7, 118.3, 117.1, 111.5, 107.7, 106.9, 101.0, 79.1, 77.2, 56.9, 56.1, 44.0, 36.9, 30.9; HRMS (ESI) calcd for $C_{25}H_{25}N_2O_3$ (M + H)$^+$: 401.1865, found, 401.1870.
35. The human cell lines: breast carcinoma MCF-7 (DSMZ ACC107), oligodendroglioma Hs683 (ATCC HTB138), non-small cell lung cancer A549 (DSMZ ACC107), glioblastoma U373 (ECACC 08061901), melanoma SKMEL-28 (ATCC HTB72) and the murine melanoma B16F10 (ATCC CRL-6475) were cultured in RPMI supplemented with 10% heat-inactivated FBS (GIBCO code 10270106), 4mM glutamine (Lonza code BE17-605E), 100 μg/mL gentamicin (Lonza code 17-5182), and penicillin-streptomycin (200 units/ml and 200 μg/ml) (Lonza code 17-602E). Cell lines were cultured in flasks, maintained and grown at 37 °C, 95% humidity, 5% CO_2. Antiproliferative effects of the compounds on these cell lines were evaluated through the colorimetric assay MTT. Briefly, cells were trypsinized and seeded in 96 well plates. Prior to treatment compounds were dissolved in DMSO at a concentration of 10 mM. After 24h, cells were treated with the compounds at different concentrations ranging from 10 nM to 100 μM or left untreated for 72 h. Cell viability was estimated by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Bornem, Belgium) mitochondrial reduction into formazan in living cells. The optical density of the untreated control was normalized as 100% of viable cells allowing determination of the concentration that reduced their global growth by 50%.

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38. Cell proliferation of GC22 glioblastoma cells was measured by quantifying BrdU (Bromodeoxyuridine) incorporation using an immunoassay Brdu Kit (EMD Millipore, code 2750) as described in Zhu W, Carney KE, Pigott VT, Falgoust LM, Clark PA, Kuo JS, Sun D. Carcinogenesis. 2016; 37:839. [PubMed: 27287871] Briefly, GC22 cells (1×10^3 cells/well) were seeded in 96-well plates in 180 μL media. After 24 h in culture cells were incubated for 72 h with compounds of different concentrations ranging from 1 μM to 30 μM and DMSO as vehicle control. BrdU was added in the last 4 h period of 72 h incubation. The incorporation of BrdU into newly synthesized DNA of proliferating cells was detected by using a peroxidase-conjugated antibody which reacts with the thymidine analogue BrdU. Bound anti-BrdU-peroxidase conjugated antibody was measured by a substrate reaction, and then quantified calorimetrically by an ELISA plate reader (Spectra MAX 190, Molecular Devices, Sunnyvale, CA) at dual wavelength of 450/550 nm.

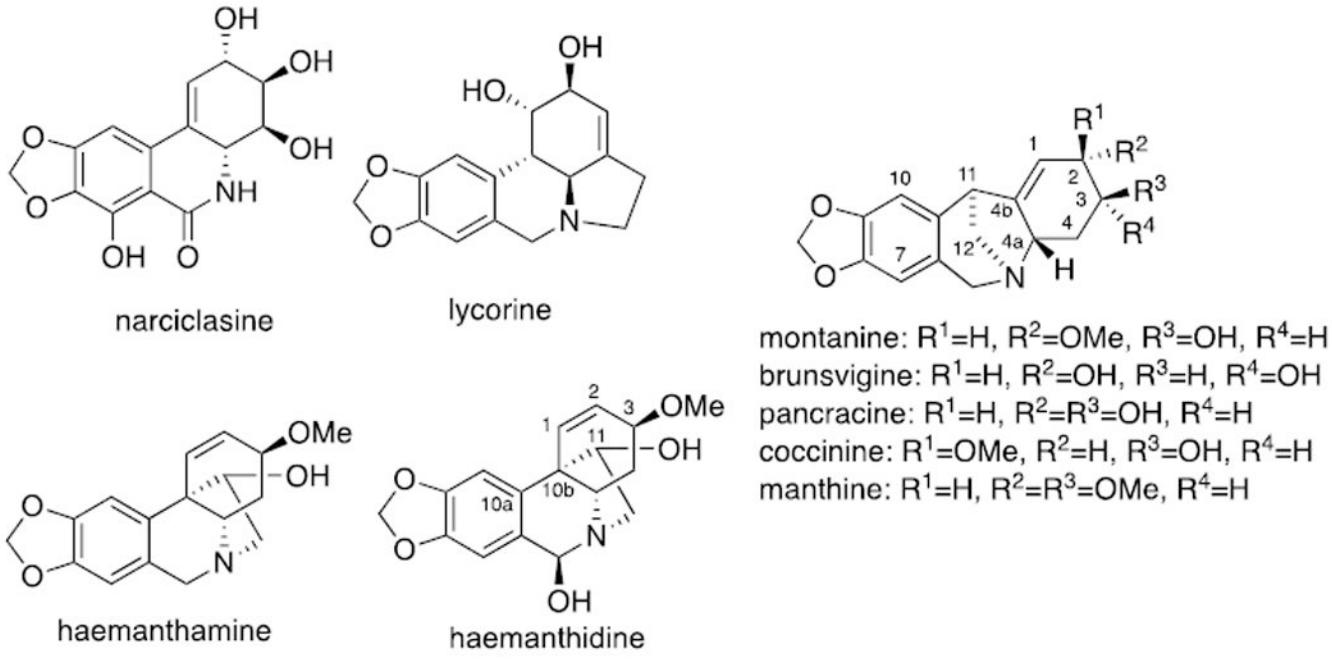

Figure 1.
Structures of Amaryllidaceae constituents with promising anticancer activities

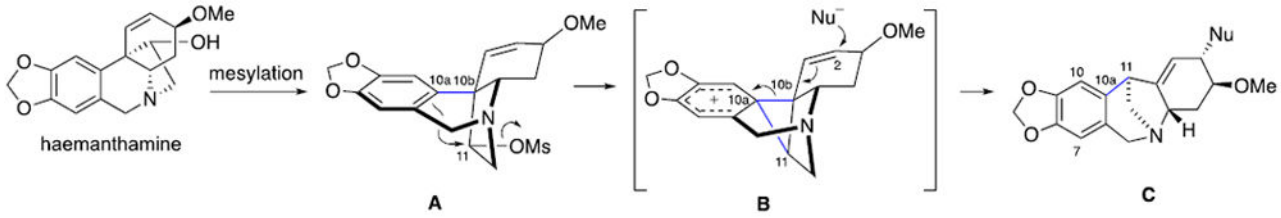

Figure 2.
Hypothetical mechanism for haemanthamine-montanine skeletal rearrangement

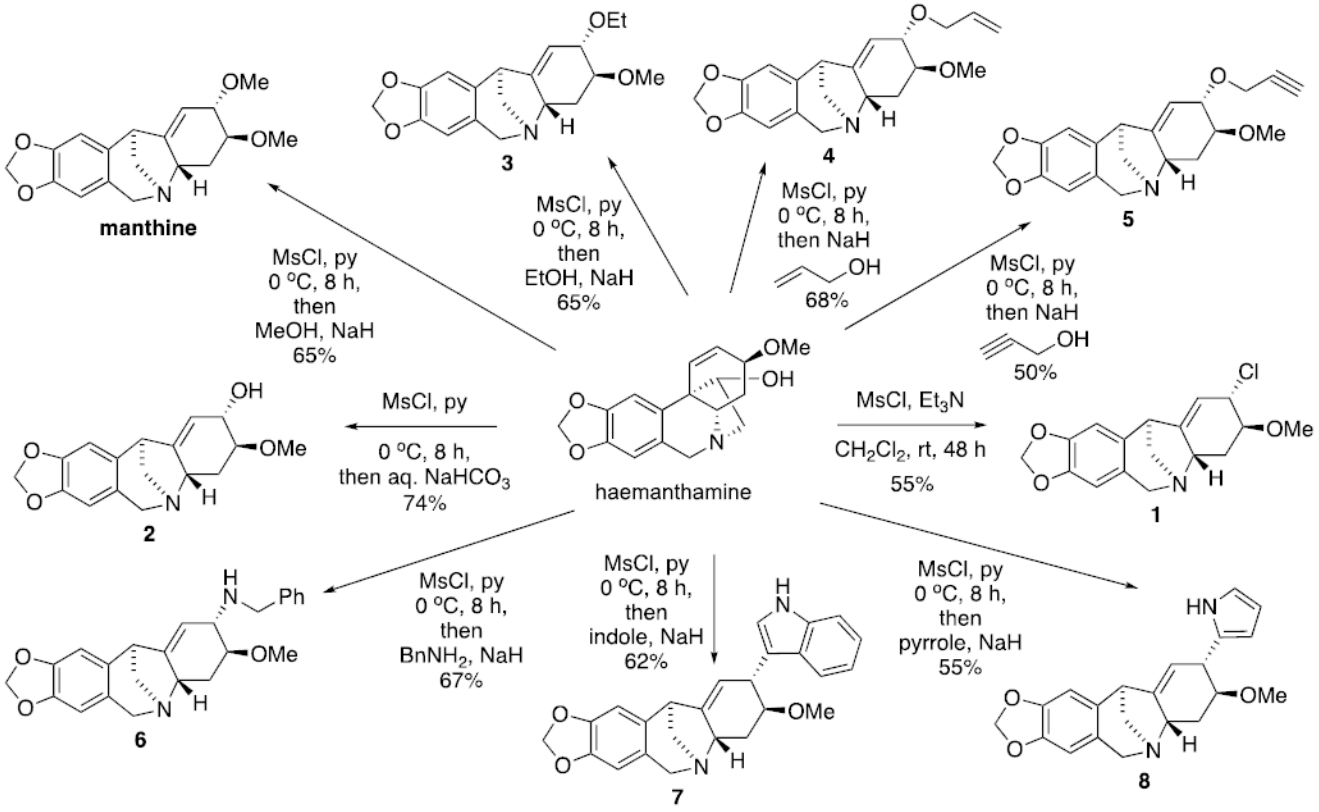

Figure 3.
Synthesis of C2-substituted montanine-type compounds

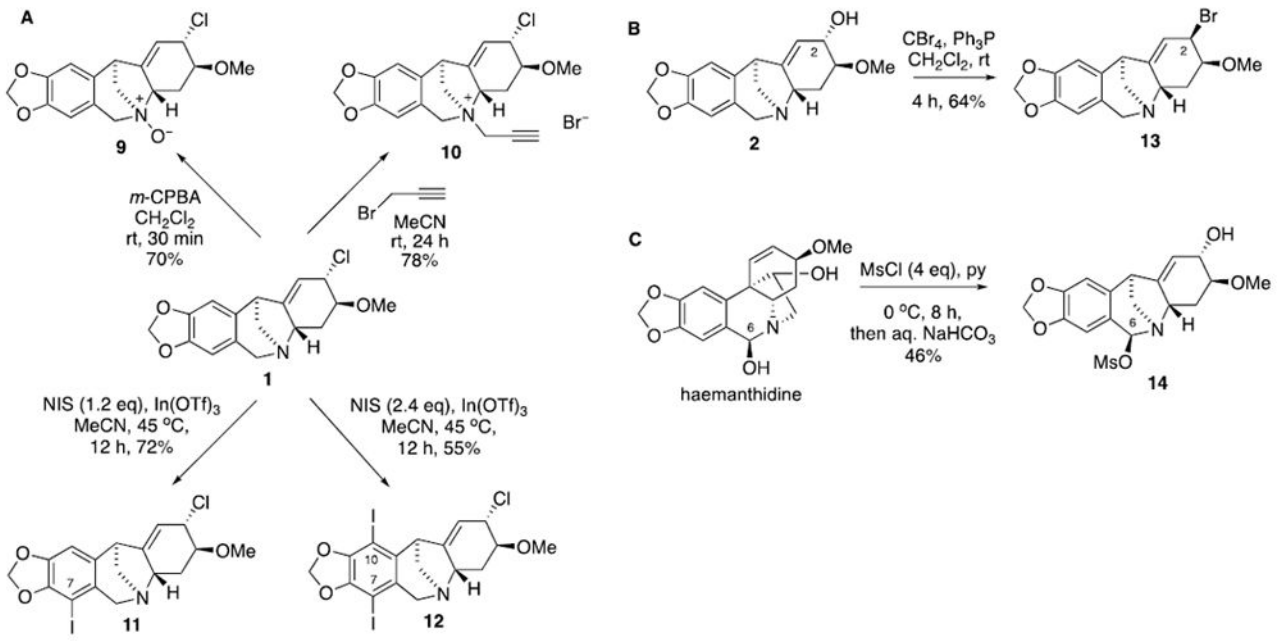

Figure 4.

Derivatization of **1** (A), **2** (B) and a similar rearrangement of alkaloid haemanthidine (C).

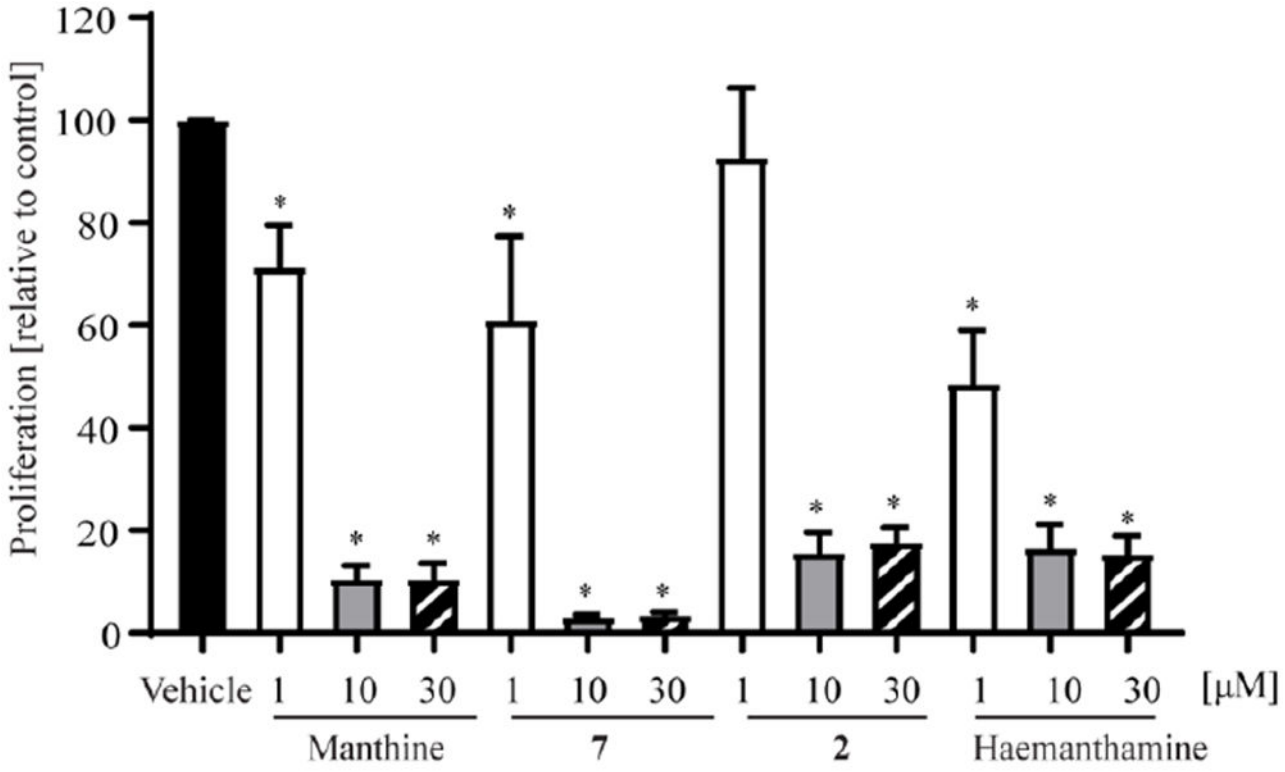

Figure 5.

Brdu incorporation in GSC22 cells following 72 h drug treatment.[38] Data are means ± SEM, n=3; *P < 0.05 versus vehicle control.

Table 1

***In vitro* growth inhibitory effects of compounds 1–4 and alkaloid manthine**

compound	GI_{50} *in vitro* values (μM)[a]					
	resistant to apoptosis			sensitive to apoptosis		
	A549	SKMEL-28	U373	MCF7	Hs683	B16F10
1	6	26	51	17	6	7
2	5	8	31	13	4	8
manthine	3	4	5	4	3	3
3	59	> 100	> 100	82	> 100	40
4	10	14	20	20	7	7
5	23	28	42	28	24	10
6	59	65	72	44	67	10
7	18	9	9	23	24	4
8	86	67	>100	68	95	11
9	> 100	> 100	> 100	> 100	> 100	> 100
10	> 100	> 100	> 100	> 100	> 100	> 100
11	> 100	> 100	> 100	> 100	> 100	> 100
12	78	> 100	> 100	78	71	39
13	9	18	25	19	5	7
14	> 100	> 100	> 100	> 100	> 100	72

[a]Mean concentration required to reduce the viability of cells by 50% after a 72 h treatment relative to a control, each experiment performed in sextuplicates, as determined by MTT assay.