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Nitrosopurines en route to Potently Cytotoxic Asmarines**

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Abstract

A nitrosopurine-ene reaction easily assembles the asmarine pharmacophore and transmits remote stereochemistry to the diazepine-purine hetereocycle. This reaction generates potent cytotoxins that exceed the potency of asmarine A (1.2 μM IC_{50}) and supersede the metabolites as useful leads for biological discovery.

Keywords

alkaloids; terpenoids; nitroso; purine; ene reaction

Asmarines A and B (**1**&**2**, Figure 1) were identified in 1998 by Kashman and co-workers as the bioactive constituents of a Red Sea sponge (*Raspailia sp.*) extract, exhibiting cytotoxicity against several cancer cell lines with a minimum EC_{50} of 1.2 μM and 120 nM, respectively.[1] The asmarines are unique among alkaloids by virtue of the embedded N-hydroxypurine diazepine (primary pharmacophore)[2] connected by an ethyl bridge to a

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clerodane decalin (putative secondary pharmacophore). Biosynthetically, the asmarines derive from agelasines (e.g. **3**), which are thought to exert cytotoxicity by Na+/K+ ATPase inhibition[3] or by membrane disruption.[4] However, the uncharged purines **1** and **2** are likely to possess different mechanisms than **3** (*vide infra*). Thus, the driving force for investigation of the asmarines is to determine their mechanism of action and chemical reactivity within the cell. However, no material remains from the original isolation work[5] and the exact identity of the sponge is unknown.[1b] Chemical synthesis is therefore the most feasible way to access material for biological study.\

Procurement of these cytotoxic molecules represents a challenge for synthesis despite efforts by Kashman, Schauss, Tashiro and Gundersen.[6] Their difficulty arises partly from the remoteness of the stereogenic *tert*-alkyl *N*-hydroxyamine moiety (see Figure 1), which frustrates stereocontrol relative to the clerodane subunit. Furthermore, synthesis of the *tert*-alkyl *N*-hydroxy-diazepinepurine pharmacophore is a challenge in itself[6a,b,d] and only one route exists. This successful strategy by Kashman[2] utilizes a carbocationic mode of ring closure (30% HBr/AcOH at 100 °C), which limits functional group compatibility and stereocontrol, and delivers analogs (e.g. **4–7**) that, while bioactive, reach a maximum potency of 4 μM[7] (GI_{50}, HT-29, see Figure 2a). We aimed to secure efficient access to the asmarines in order to understand their SAR and to provide simplified, high potency analogs for further biological study.

A biomimetic strategy to close the seven-membered ring from an agelasine-type intermediate **8** (Figure 2b) appeared to offer high efficiency. However, control of the diazepine stereochemistry during C-N bond formation presented a serious obstacle to this strategy. We wondered if a nitrosoene reaction[8] of linear precursor **8** might relay stereochemistry[9] of the decalin core to the *tert*-alkyl amine stereocenter in **9**, and achieve the necessary Markovnikov selectivity.[10] Here we report 1) the successful implementation of this strategy to relay clerodane stereochemistry to the remote stereocenter, 2) the ability of this strategy to probe the role of stereochemistry in SAR, and 3) the use of this nitrosopurine-ene reaction to synthesize simplified high potency asmarine analogs that exhibit cytotoxicity at nanomolar levels against multiple cancer cell lines.

A short route to the targeted nitrosopurine (**8**) began with 6-chloro-4,5-pyrimidinediamine (**11**) and 4-iodo-1-butyne (**12**). Diamine **11** was monoformylated to amide **13**, and **12** was subjected to the Wipf-modified[11] Negishi carboalumination,[12] followed by alkylation of the intermediate organoaluminum with gaseous formaldehyde[13] to yield iodoalcohol **14**. Formamide **13** was alkylated with iodide **14** and heating effected ring closure to chloropurine **15**.[14] This alcohol was converted to allylbromide **16** (see Supporting Information), which was then trapped with the enolate generated from dissolving metal reduction of (methyl)-Wieland-Miescher ketone[15] ketal **17** (99% ee) in presence of bis(2-methoxyethyl)amine[16] to yield ketone **18** as a single stereoisomer. Selective displacement of the arylchloride of **18** with hydroxylamine proved challenging since condensation with the decalin ketone to form an oxime occurred competitively. We reasoned that the oxime might be cleaved later, and thus decided to push the reaction towards the bis-hydroxyaminated product **19** – an unforeseen but providential choice (see Table 1). Having

obtained the agelasine scaffold **19**, oxidation of the 6-N-hydroxyaminopurine (HAP) moiety to a nitrosopurine was explored in a model system (see Figure 3).

The parent compound 6-nitrosopurine **20**[17] proved unstable and in our hands could not be isolated. However, we found that treatment of 6-*N*-hydroxyaminopurine **21** with MnO_2 in DMSO and in the presence of excess tetramethylethylene gave the expected *tert*-alkyl methallyl hydroxylamine **22**. A variety of oxidants (I_2, $Mn(OAc)_3$, $PhI(OAc)_2$) gave similar results and on large scale $PhI(OAc)_2$ proved more amenable to purification. When these oxidation conditions were applied to *N*-hydroxyaminopurine **23** in a mixture of methanol and dichloromethane, the targeted 7-membered diazepine purine **24** was obtained as the sole product with no trace of the 8-membered diazocine. Notably, this reaction could be preformed on gram-scale, demonstrating that the chemistry of nitrosopurines (e.g. **25**) is a practical means of procuring material.

When applied to agelasine-type compound **19**, we also obtained the diazepine **26** exclusively, but initial experiments generated this as a 1:1 mixture of diastereomers (entry 1, Table 1). Fortunately, we discovered two influences on diastereoselectivity in the ene reaction. There was a marked solvent effect associated with diastereoselectivity, whereby more polar protic solvents increased selectivity, and water proved the most selective (ethanol was added for solubility). This effect was not due to protonation or hydrogen bonding, since acetic acid had no pronounced effect (entry 6), nor did the strongly hydrogen bond-donating solvent trifluoroethanol[18] (entry 7) compared to ethanol (entry 3). There was also a stark and serendipitous substituent effect: although the incidental oxime caused high stereoselectivity, the ketone **27** and exocyclic methylene **28** showed almost no selectivity in formation of **29** and **30**, respectively (entries 10 and 11). Application of the polarized diradical (PD, **31**) model for the nitroso-ene reaction[19] of **19** suggests that developing positive charge at carbon in the transition state might be stabilized by the proximal oxime (Figure 4). Conformer **32** should be lower in energy than **33**, which suffers a steric clash between the methyl and the aromatic ring. These polarized transition states would be more populated in solvents of high polarity (e,g, water), as predicted by Houk.[19]

This strategy allowed us to directly probe the contribution of diazepine stereochemistry to cytotoxicity. The major diastereomer **26** (Figure 5) exhibited higher potency against HT-29 cells (EC_{50} = 8 μM) than the minor isomer **34** (17 μM), even though **34** corresponds to the stereochemistry of the natural asmarines (determined by x-ray analysis,[20] see Supporting Information). Since the difference in potency is not profound, the stereochemistry in the diazepine seems to play only a minor role in the mechanism of action.

Synthesis also allowed us to probe the role of absolute stereochemistry. We were surprised to find that *ent*-**26** and *ent*-**34** possessed similar biological activity as their enantiomers (EC_{50} = 10 μM for both). Similarly, diketones **35** and *ent*-**35** possess activity that differs only two-fold. Whereas this similarity might suggest a non-specific mechanism of action, it seems more likely that the differences in potencies reflect cell-permeability, affecting accessibility to the target. As such, replacement of the oxime with a more lipophilic methylene delivers compounds with greater potency (**36** and **37**). In this case, the ‘natural’ diazepine **37** is five-fold more potent than the ‘unnatural’ diastereomer **36**, and in fact

matches the potency against HT-29 cells reported for asmarine A (EC_{50} = 1 μM). As control compounds, we also tested the N-H purine **38** and the uncyclized N-hydroxyaminopurine **19**. Both compounds were unable to effectively induce cell death after 72 h. The inactivity of **19** and **37** suggests a different mechanism than the inhibition of ATPases ascribed to the agelasines and their simplified analogs,[21] although further study will be necessary to rule out this target.

Since the ultimate goal of our work was procurement of material for biological study and it was clear that stereochemistry had little influence on the activity of the asmarines, we adapted the route shown in Scheme 1 to the late-stage, divergent appendage of unnatural hydrophobic cores. As shown in Figure 6, a short convergent sequence from **13** and **39** was devised to arrive at iodide **40** on gram scale. From **39**, a variety of substituents were added by a very effective Negishi coupling[22] - a noteworthy step given the acidic proton on the hydroxyamino group and our observation that the N-hydroxyamino purine will chelate metals. Each adduct (**41–48**) was then cyclized via the nitrosopurine-ene reaction to its N-hydroxydiazepine purine **50–57** to generate a small library of cytotoxic compounds.

Each molecule in Figure 6 was screened for cytotoxicity against HT-29, Jurkat, and HeLa cells. While compounds with truncated sidechains (**24, 49–53**) show cytotoxicity, the potency is weak, with or without an unsaturated linker (**24** vs. **49**). Small rings like a methylenecyclobutane can be generated in the ene reaction, highlighting the mildness of the reaction conditions, and in stark contrast to the previously reported high temperature/strong acid method of ring closure. Therefore, esters (**52**) and amino acid motifs can be incorporated (**53**); the β,γ-unsaturated amino ester carbamate does not migrate into conjugation under the reaction conditions. This latter example shows some small stereoselectivity (2:1) associated with the presence of the carbamate (compare to methyl, **52**), supporting the idea that a Lewis basic group is necessary for stereoinduction. However, none of these short, polar side chains show significant potency. In contrast, large hydrophobic groups similar to the clerodane cores of asmarines A and B induce high potency. For example, compounds with 1- and 2-naphthyl substituents (**54** and **55**) possess single digit μM activity; **54** is equipotent to asmarine A (1.1 μM against HT-29). 1-Adamantyl (**56**) and 4-biphenyl (**57**) asmarines are more potent still with sub-micromolar activity against all three cell lines (471 nM–714 nM; **56** also inhibits HL60 cells at 199 nM, see Figure 8), approaching the 120 nM IC_{50} value reported for asmarine B (**2**) against HT29 cells. In fact, this study represents a rare example of the deprioritization of isolated metabolites in light of near-equipotent but simpler analogs. Readily-accessible asmarines **56** and **57** supersede the scarce metabolites **1** and **2** as useful tools for biochemical inquiry.

Whereas there is some latitude in the choice of hydrophobic lobe, the N-hydroxy diazepine purine is more conservative. Acyclic *tert*-alkyl N-hydroxyaminopurine **19** was completely inactive (Figure 7), indicating that a ring is necessary. However, the ring-expanded *N*-hydroxy diazocine purine **51** was similarly inactive. This 8-membered ring was the unexpected anti-Markovnikov product of nitrosopurine-ene reaction of cyclopropane **42**, which reacts with ‘twix’ selectivity and avoids the alternative, highly strained methylenecyclopropane (Figure 7).

The potency of **56** and **57** is general, showing sub-micromolar cytotoxicity against HL60 (leukemia), HEK 293, MCF7 (breast cancer), and MDA-MB 231 (breast cancer) cell lines, in addition to the HT29, Jurkat, and Hela cell lines (Figure 8). The activity of **56** is surprising, since the saturated analog was reported by Kashman to posses very weak activity against two cancer cell lines (NSCL A549 and PANC1, EC_{50} > 27 μM). The activity of **57** is noteworthy since installation of functional groups on the aromatic rings should allow a variety of pull down experiments.

This work builds a platform for the discovery of the mechanism of action of the asmarines. We are entertaining three hypotheses: non-covalent, covalent, and radical. The latter two possibilities are supported by some experimental data. Kashman[1b] and Tashiro[6c] have reported the instability of the N-hydroxypurine upon acylation, wherein methanol adds to the purine ring and cleaves the N-O bond (**2**→**58**, Figure 9). This mode of reactivity may also be effected in vivo by acetylation or phosphorylation. Alternatively, we observed that the *tert*-alkyl *N*-hydroxypurine is readily oxidized with mild reagents. The acyclic purine **22** generates stable nitroxide radical **59**, a vibrant red-orange crystalline solid whose structure was confirmed by x-ray analysis.[23] Interestingly, the nitroxides of the diazepines (e.g. **24**) are very unstable, cannot be isolated, and instead appear to disproportionate to the corresponding *N*-H diazepines, suggesting that, if generated, they may react with a target in vivo.

To summarize, we have established a chemical platform for the study of asmarine cytotoxins, enabled by an unusual but highly practical nitrosopurine-ene reaction. This reaction exhibits both high regioselectivity for the Markovnikov product and exhibits high chemoselectivity. Identification of a Lewis base (oxime) as a stereoelectronic control element in the nitroso-ene reaction may be generally useful for linear stereocontrol. Use of the nitrosopurine-ene reaction as a simplifying element in the synthesis of cytotoxic asmarine analogs enables very short syntheses with few redox manipulations and no protecting groups. Notably, this route is diversifiable at a late stage, and has generated potent analogs with cytotoxicity in the nanomolar range. We have also found that 1) increased potency may result from cell permeability, but the N-hydroxypurine diazepine is required; 2) the similar potency of **54** and **55** suggests the hydrophobic moiety does not play a major role in target binding; 3) stereochemistry at the diazepine plays a minor, but not insignificant role in potency; 4) the N-O bond is required for activity, since an N-H analog is inactive; and 5) the seven-membered ring is required for activity, since acyclic variants and a diazocine analog show no cytotoxicity. These findings, coupled with the development of a short, scalable and divergent route to asmarine analogs, and especially the identification of potent biphenyl asmarine **57** lay the groundwork for identification of the mechanism of action of these enigmatic metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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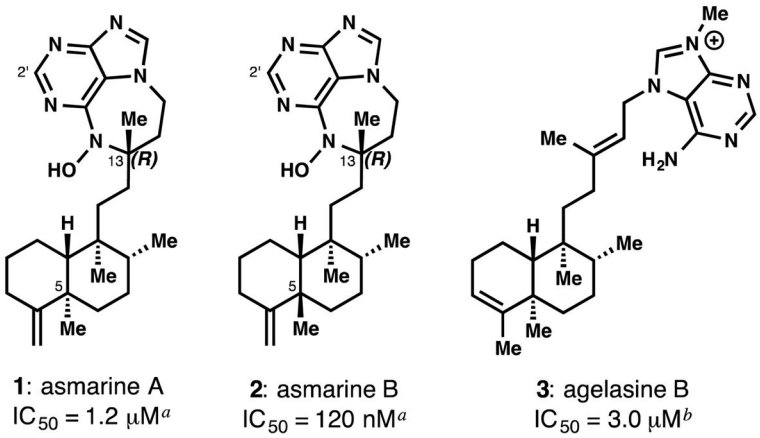


Figure 1.

Asmarines may be derived from agelasines. [a]against HT-29 cells; [b]against MCF-7 cells [3b].

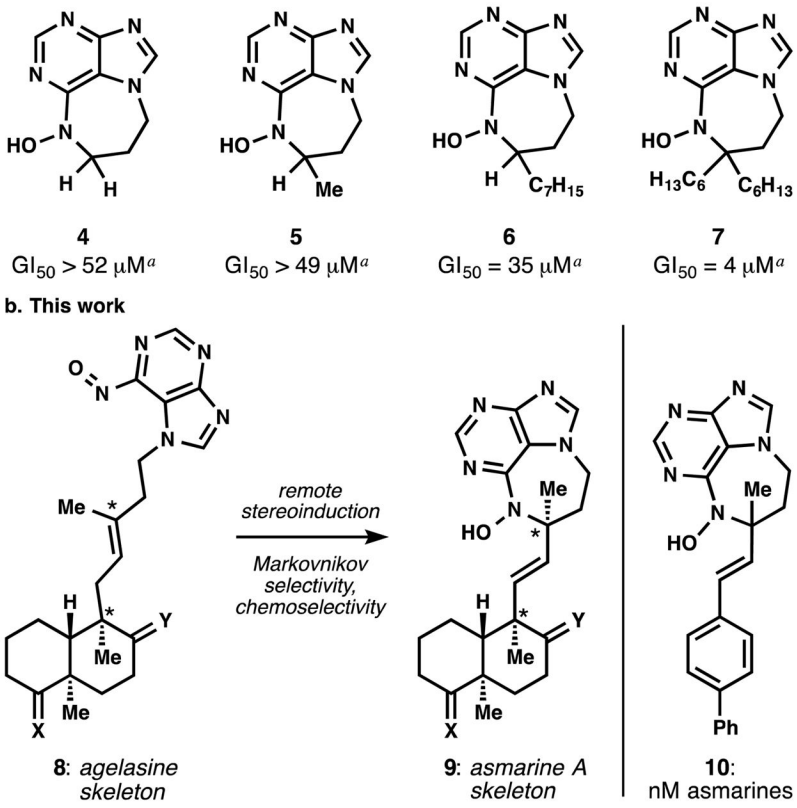


Figure 2.

a. Prior analog work. **b.** Stereochemical relay and high potency analogs via nitrosopurine-ene reaction. [a]cytotoxicity against HT-29 cells (EC50 more appropriate).

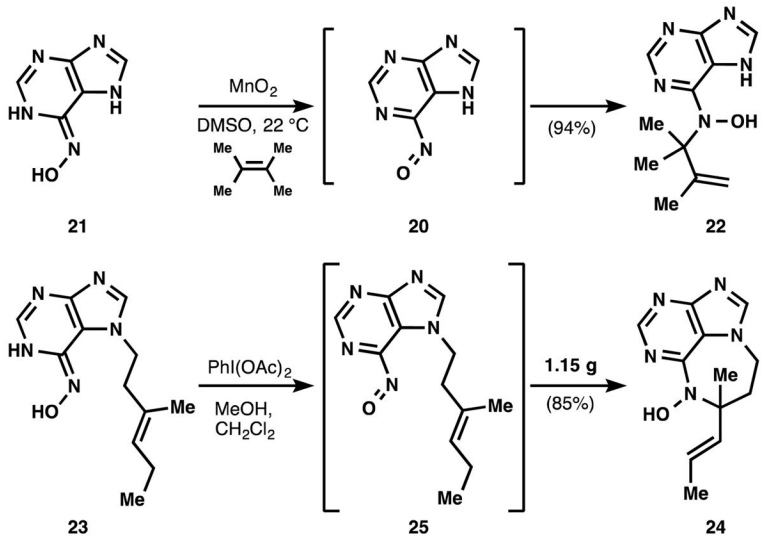


Figure 3.
Proof-of-principle for nitrosopurine-ene reactions.

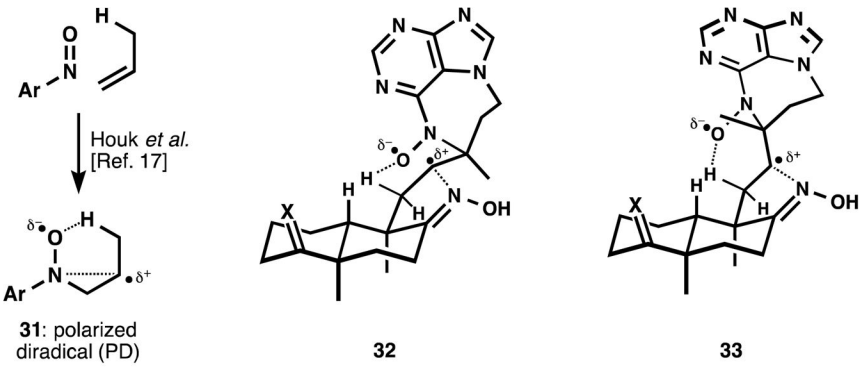


Figure 4.

Water might increase the polar character in a nitrosoene polarized diradical (PD) and the transition state leading to it. Conformers of different energy are stabilized by the proximal oxime; **X** = (-OC_2H_4O-).

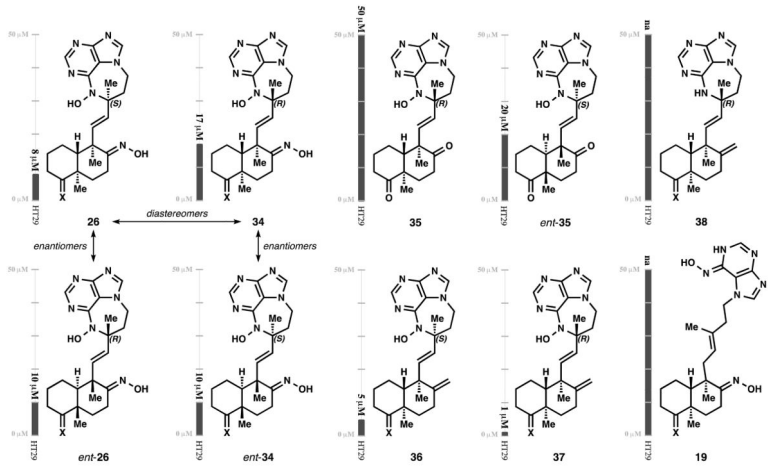


Figure 5.

Activity (EC_{50}, 48 h) against HT-29 cells. Although there is clear SAR, stereochemistry does not play a major role. **X** = (-OC_2H_4O-)

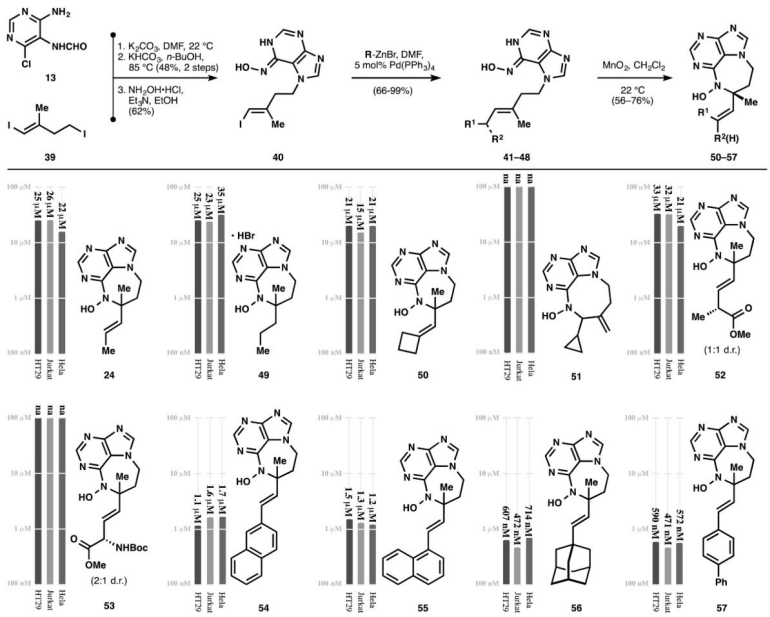


Figure 6.

A late-stage divergent route to unnatural asmarines and their activities (EC_{50}, 48 h) against HT-29 and HeLa cells. Potencies are on a logarithmic scale.

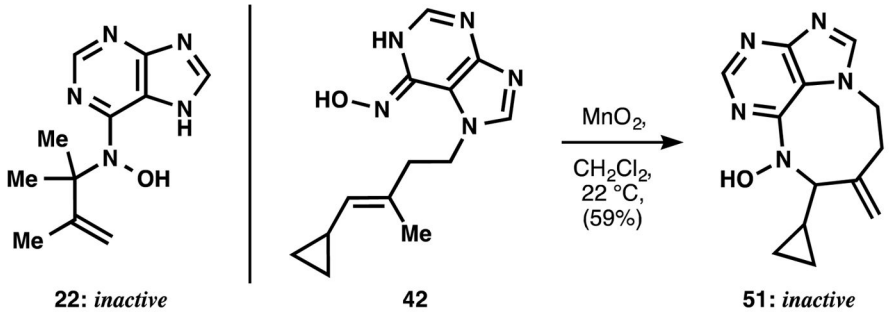


Figure 7.
Structural specificity for activity.

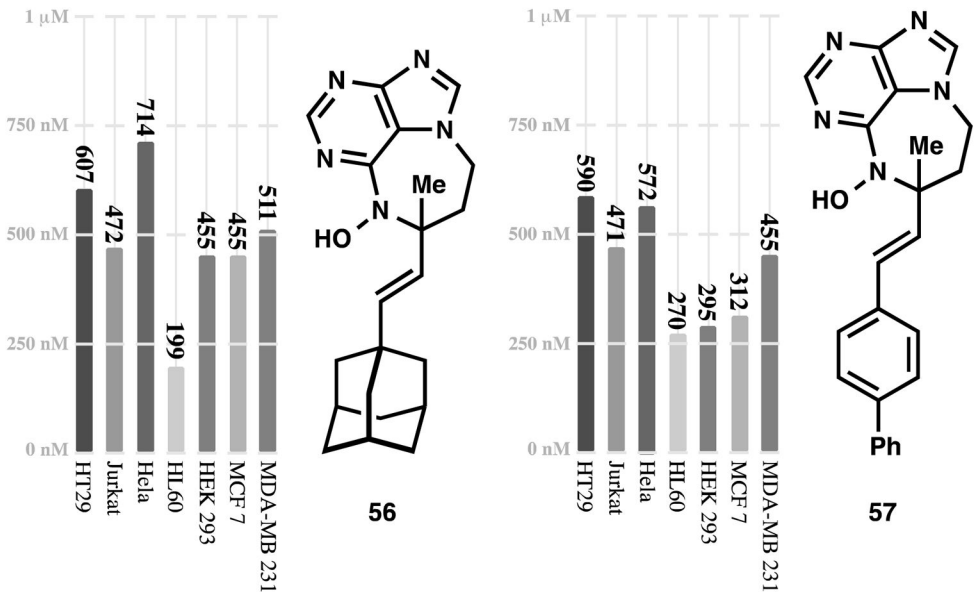


Figure 8.

1-adamantyl-asmarine (**55**) and 4-biphenyl-asmarine (**56**) possess nM activity against seven cell lines.

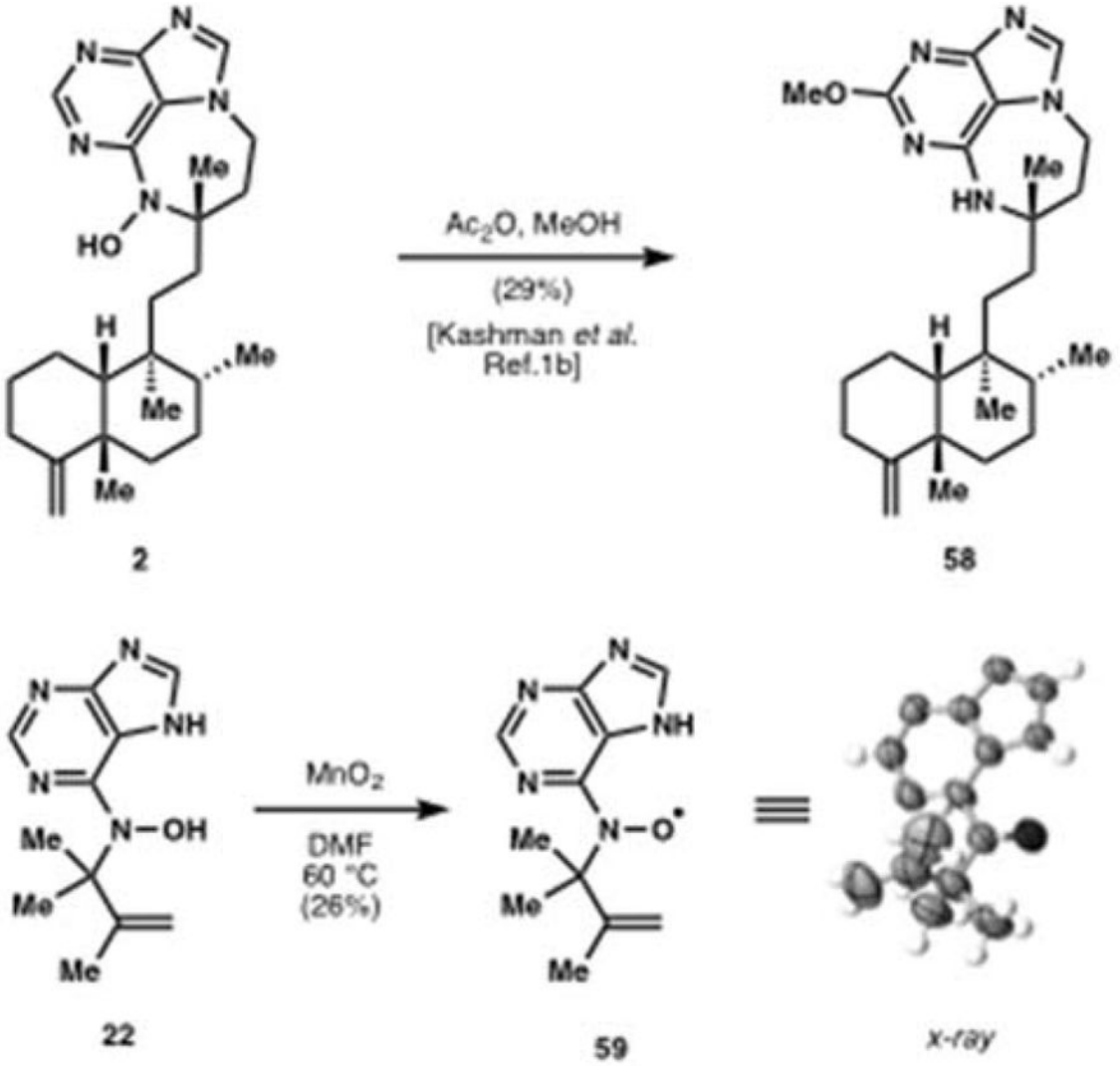
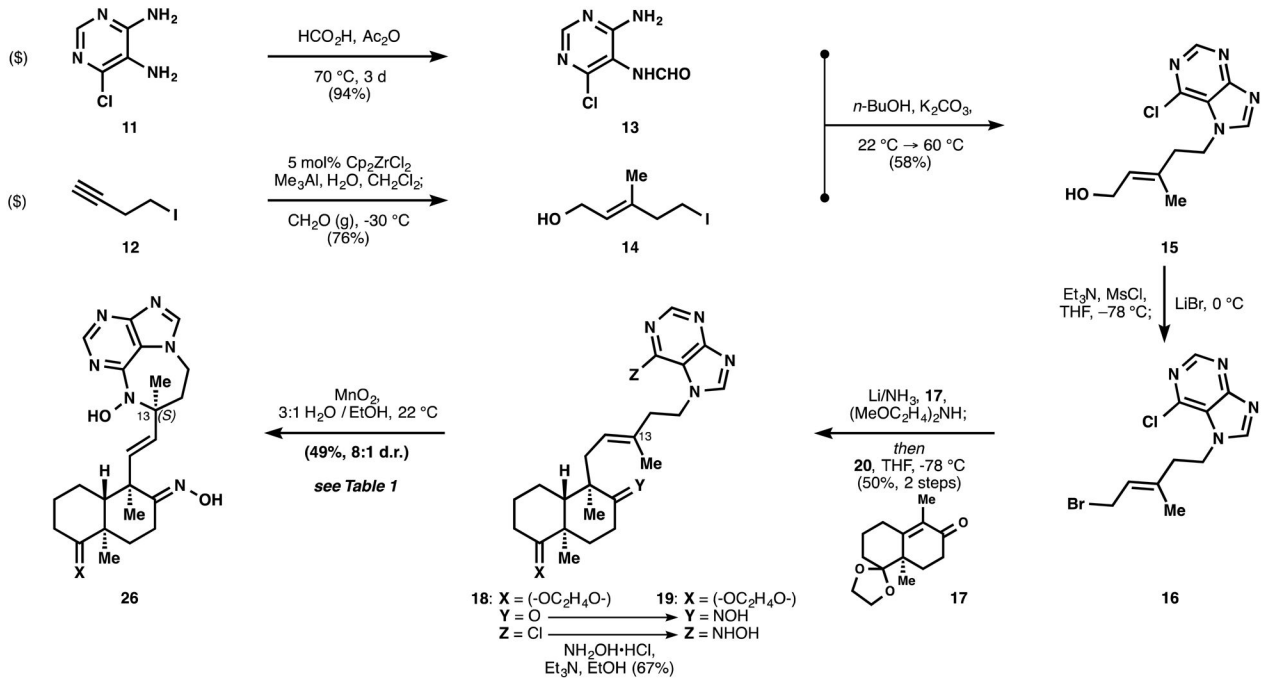


Figure 9.
Reactivity of potential relevance to bioactivity.



Scheme 1.

Union of clerodane and purine cores, and a nitrosopurine-ene reaction with remote stereocontrol.

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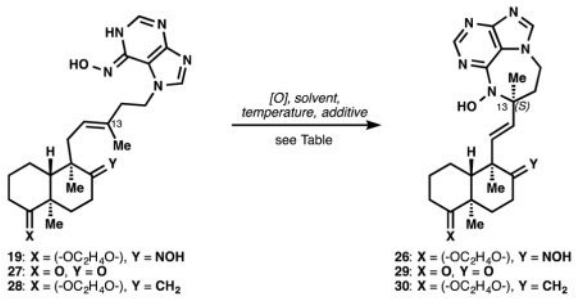
Table 1

Solvent and substituent control stereoinduction.



Entry	Y	Solvent	Temperature	Additive	13-(*S*) : 13-(*R*)
1	**NOH**	DMF	22 °C	–	1.5 : 1.0
2	**NOH**	CH_2Cl_2	22 °C	–	1.5 : 1.0
3	**NOH**	EtOH	22 °C	–	3.8 : 1.0
4	**NOH**	EtOH	−20 °C	–	3.0 : 1.0
5	**NOH**	1:9 MeOH/CH_2Cl_2	22 °C	NH_4OH (1%)	2.1 : 1.0
6	**NOH**	EtOH	22 °C	AcOH (1 equiv.)	2.7 : 1.0
7	**NOH**	TFE	22 °C	–	2.3 : 1.0
8	**NOH**	1:1 EtOH/H_2O	22 °C	–	4.7 : 1.0
9	**NOH**	**1:3 EtOH/H_2O**	**22 °C**	**–**	**7.9 : 1.0**
10	**O**	1:4 EtOH/H_2O	22 °C	–	1.2 : 1.0
11	**CH_2**	1:4 EtOH/H_2O	22 °C	–	1.1 : 1.0