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The Search for Inosine 5'-Phosphate Dehydrogenase (IMPDH) Inhibitors from Marine Sponges. Evaluation of the Bastadin Alkaloids

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Abstract: Six known bastadins, compounds **1** - **6**, and one new one, bastadin 18 (**8**), have been isolated from the marine sponge *Ianthella Basta* collected in Papua New Guinea. A mixture of compounds **2** and **3** was also isolated from *I. flabelliformis*. Two of these compounds, bastadins 8 (**5**) and 10 (**6**), were found to be moderate inhibitors of IMPDH. A discussion of the enzyme assay is included.

INTRODUCTION

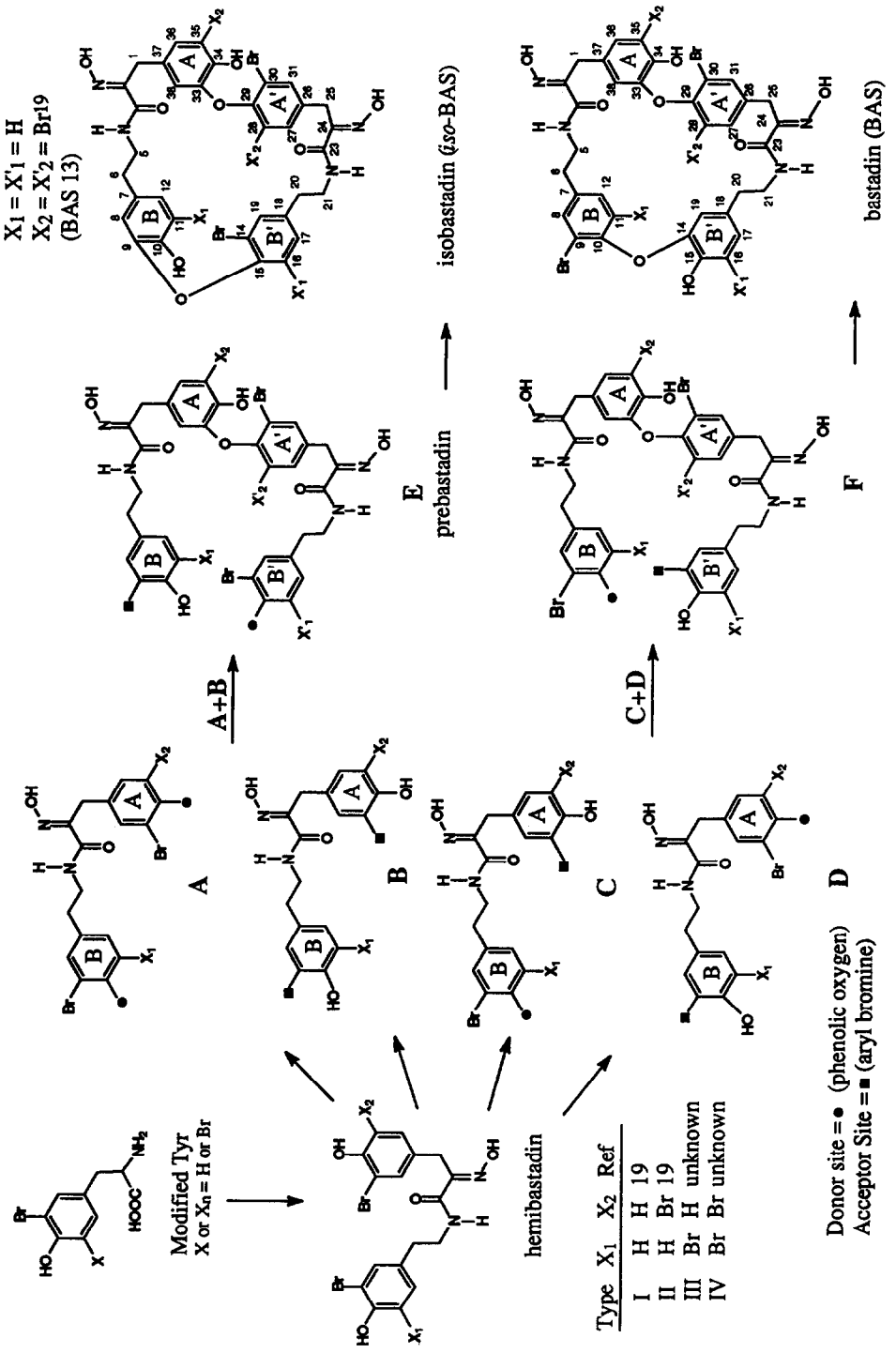
The use of subcellular enzyme targets to direct the isolation of natural products as leads for anticancer chemotherapeutics represents an emerging nontraditional strategy.[1,2,3,4] We have been using inosine 5'-phosphate dehydrogenase in a primary screen to evaluate a wide spectrum of marine invertebrate extracts. An important rationale is that the amount of Type-II IMPDH, which catalyzes the rate-limiting reaction in the *de novo* pathway of guanine nucleotides, is elevated in ovarian tumor and leukemic cells.[5,6] IMPDH enzyme activity is also known to be regulated by the p53 antioncogene.[7]

The list of known IMPDH inhibitors is extremely short. At the top stands a polyketide fungal natural product, mycophenolic acid,[8,9,10,11] whose Ki = 10 nM makes it a potent standard for studies of IMPDH inhibitors. A different structural class of IMPDH inhibitor is represented by related synthetic and natural product carbohydrate derivatives such as tiazofurin[12] (IMPDH Ki = 115 μM), benzamide riboside (55% inhibition of IMPDH at 5.7 mM),[13] and bredinin-5'-monophosphate[14] (IMPDH Ki = 0.02 μM). Significantly, tiazofurin is currently being evaluated in human clinical trials as an antileukemic agent.[15]

Reported below are the first examples of marine natural products which are active against IMPDH. Seven known bastadins (BAS), compounds **1** - **6**, and one new one, compound **8**, were purified during IMPDH assay guided isolation work. These bastadins, plus **7**, were retested in secondary screens and two compounds, BAS 8 (**5**) and BAS 10 (**6**), are inhibitors of IMPDH, but less potent than those reported to date.

RESULTS AND DISCUSSION

The semipure extracts of two Verongida sponges, collected from Papua New Guinea, exhibited activity in a first generation IMPDH primary screen. These included *Ianthella basta* (coll. no. 91628) whose CH_2Cl_2 solvent partition fractions after Sephadex™ chromatography yielded material exhibiting EC_{50} values of 4 - 8 μg/mL, while the crude extract of *Ianthella flabelliformis* (coll. no. 93128) exhibited an EC_{50} = 4 μg/mL. Parallel work was begun to isolate the active constituents of both organisms which were assumed to contain



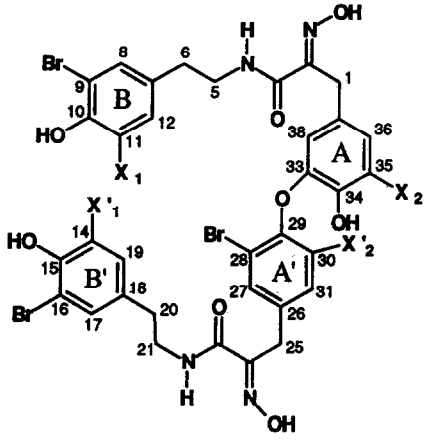
Scheme 1. Biosynthetic Pathway to the Bastadins.

condensed modified tyrosine units. Several publications on *Ianthella basta* have appeared and collectively they contain data on a family of brominated tyrosine tetramers, BAS 1 - 13, 15 - 17,[16,17,18,19,20,21] while BAS 14 has been isolated recently from the Verongid sponge *Psammaplysilla purpurea*.[22] Recently, the 34 sulfato derivative of BAS 13 has been isolated from *Ianthella* sp.[23] There have been no previous reports of bastadins isolated from *I. flabelliformis*.

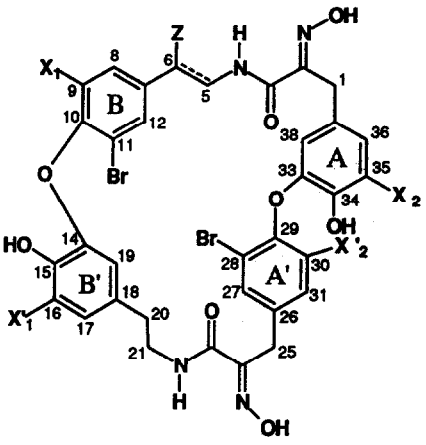
The collective structures of the known bastadins provide a biosynthetic pattern (Scheme 1) which augments the use of spectral data in assembling the entire structure of a BAS. The first biogenetic step proposed in the formation of the macrocyclic ring involves the dimerization of two brominated tyrosines to give **I** - **IV** of which two, the hemiBAS, have been reported.[19] Next, these four hemiBAS precursors represented by the hypothetical structures **A** - **D**, each of which has four possible bromination patterns, can undergo the first of two biaryl ether formations. This condensation occurs by the displacement of an aryl Br (acceptor site ■) by a phenolic oxygen (donor site •) (*cf* synthetic results of Yamamura *et al*[24]). There are 16 possibilities for each of **E** and **F** but just two of these 32 possible preBAS have been isolated,[19] and one analog has been prepared by synthesis.[25] Interestingly, switching the ether bridge between the A/A' rings in **F** from C33/C29 to C34/C30 yields structure **E**. The macro-cyclization of **E** or **F** can occur in only one way to give 16 *iso*BAS and 16 BAS frameworks respectively and seven such skeletons are represented among the 18 known tetramers.[16-23] The above analysis allows a concise approach, illustrated below, to differentiate between a BAS and *iso*BAS skeleton. It begins by establishing that rings A and A' are connected by an ether bridge and then a determination of which A/A' ring is linked by the acyclic residue to which bisoxygenated B/B' ring.

Our strategy for characterizing a purified BAS begins with inspection of the phenolic OH region of a ^{1}H NMR spectrum. The count of phenolic protons differs between the preBAS (3 H's), or the BAS = *iso*BAS (2 H's) types. Dereplication among the seven known cyclic tetramer types is not straight-forward but can be accomplished by tallying the number of symmetrical rings, counting the total number of aromatic protons (Scheme 2) and matching the lowest and highest field aromatic ^{1}H NMR peaks to those of known compounds. In some situations it may be advantageous to obtain HMBC data to identify hemibastadin substructures (I-IV of Scheme 1), which is especially important in the unequivocal differentiation between the two types of cyclophanes (BAS or *iso*BAS). Rigorous distinction between a BAS or *iso*BAS has been accomplished by either X-ray crystallography,[16] total synthesis,[24] or extensive use of two-dimensional NMR data plus mass spectral fragmentation patterns.[22] One or more of these methods have been applied to secure the macrocyclic structures for BAS 4-6,[16] 8,[17] 9,[17] 13,[19] 14,[22] and 16-17.[21] Using one dimensional NMR data, as outlined above, along with the biosynthetic analysis of Scheme 1 allowed us to identify the six known compounds obtained from the CH_2Cl_2 partition fraction of *I. basta* as BAS 1 (**1**),[16] 2 (**2**),[16] 5 (**3**),[16] 6 (**4**),[16] 8 (**5**),[17] 10 (**6**).[17] The purification of these compounds from *I. basta* is described in the Experimental section. In addition, a mixture of BAS 2 (**2**) and 5 (**3**) was also isolated from *I. flabelliformis*.

The single new compound isolated from *I. basta* was designated as BAS 18 (**8**). The molecular formula of **8**, $C_{34}H_{28}Br_4N_4O_8$, makes it isomeric with BAS 9[17] (Scheme 2) and BAS 13[19] (Scheme 1). The pattern of the ten aryl proton resonances indicated that each of the four aryl rings was non-symmetrical so a cyclophane skeleton analogous to that of either BAS 7[16] and 10[17] or of an unreported *iso*BAS was indicated. Eventually **8** was concluded to be the 30-debromo analog of BAS 5 (**3**). The ^{13}C NMR spectrum (125 MHz) showed two close characteristic resonances for the oxime carbons at δ 153.3 and 152.6 ppm and the carbonyl groups were found at δ 166.0 and 166.6 ppm. The remaining ^{13}C and ^{1}H signals were assigned from HMBC and HMQC data (Table 1). The HMBC spectrum showed a correlation from C3(δ 166.0) and C2(δ 153.3) to H1(δ 3.67) and also from C23(δ 166.6) and C24(δ 152.6) to H25(δ 3.72). Other COSY and HMBC correlations (Table 1) established two substructures which encompassed the atoms from C23 through C31 and from C3 through to all the carbons on ring A. The LRFABMS data showed a fragment at 461 *m/z* (5.3% $C_{16}H_{16}Br_2N_2O_4$) indicating these two substructures (ring A and A') were connected, and the biosynthetic argument above requires the ether bridge as C29-O-C33. The ^{1}H-^{1}H COSY correlations in Table 1 allowed connections to be established from C5 through C12



prebastadins (preBAS)



bastadins (BAS)

PreBAS No.	BAS No.	Cmpd. No.	Z	X_1	X'_1	X_2	X'_2	Aryl H count	symmetry (ring)
1		1		H	H	Br	H		none
2		2		H	H	Br	Br		A'
	5	3		H	Br	Br	Br	9	A'
	6	4		Br	Br	Br	Br	8	A' + B
	8	5	OH	H	Br	Br	Br	9	A'
	10	6	OH	H	Br	Br	H	10	none
	11	7	5,6Δ	H	H	Br	Br	10	A'
	18	**8a**		H	Br	Br	H	10	none
	4		5,6 Δ	H	Br	Br	Br	9	A'
	7		5,6 Δ	H	Br	Br	H	10	none
	9			H	H	Br	Br	10	A'
	12		OH	Br	Br	Br	H	9	B
	14		5,6 Δ	Br	Br	Br	H	9	B
	15			Br	Br	Br	H	9	B
	16			Br	H	Br	Br	9	A' + B
	17		OH	H	H	Br	Br	10	A'

Scheme 2. Structure Overview of BAS.

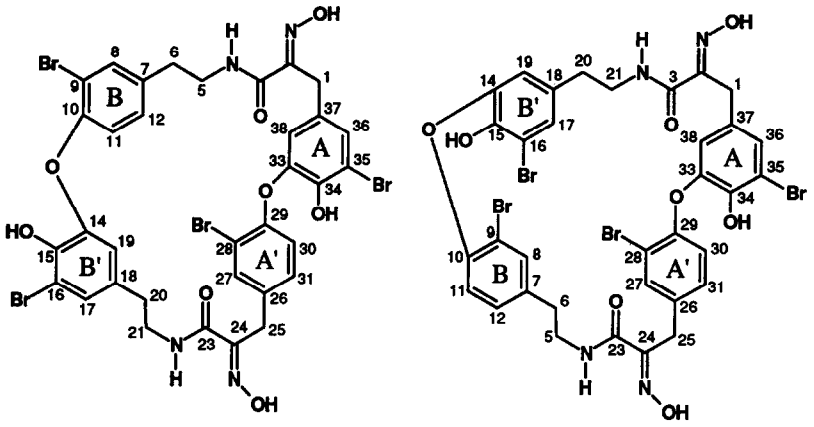
and from C14 through C21. At this point, employing the biosynthetic principle of Scheme 1, two structures were possible, **8a** or **8b** (Scheme 3). An nOe difference experiment was attempted on the tetramethyl derivative of BAS 18 but the sample size was too small, so no interpretable data was obtained. A choice in favor of **8a** was made based on the key HMBC correlation from H5(δ 3.30) to C3(δ 166.0) which established a linkage between the A and B rings. An HMBC correlation from H21(δ 3.34) to C23(δ 166.6) allowed us to use a similar argument to connect the A' and B' rings.

In concert with the isolation work described above were bioassay evaluations on substances isolated above and also obtained from the University of Oklahoma (UO) compound bank. Eight compounds (**1** - **8**) were evaluated. Almost all of these appeared to be quite active in the first generation IMPDH screen as summarized in Table 2 under the column labeled "EZ first, without DTT". Redesign of the assay caused the potency of all compounds to diminish as shown by data "EZ last, with DTT".

Table 1. 125/500 MHz NMR+ Data for Bastadin 18 (8).

Position	^{13}C	^{1}H (*J*, Hz)	HMBC (C - H)	COSY (H-H)
1	28.7	3.67, 2H, s	38	36, 38
2	153.3		1	
3	166.0		1, 5	
4				
5	41.5	3.30, 2H, m (7)	6	6
6	35.4	2.55, 2H, t (7)		5, 8
7	138.6		6	
8	135.1	7.39, 1H, d (2.0)	6	6, 12
9	115.6		11	
10	152.4		11	
11	122.2	6.82, 1H, d (8.3)		8, 12
12	130.9	7.06, 1H, dd (8.3, 2.1)	6, 8	11
13				
14	145.3			
15	146.6		17, 19	
16	111.6			
17	128.5	7.04, 1H, d (2.0)	19, 20	19, 20
18	132.7		20	
19	118.0	6.35, 1H, d (2.0)	17, 20	17
20	35.5	2.66, 2H, t (6.5)	19	17, 21
21	41.7	3.34, 2H, m (7)	20	20
22				
23	166.6		21, 25	
24	152.6		25	
25	29.3	3.72, 2H, s	27	27, 31
26	135.8		25, 30	
27	135.1	7.42, 1H, d (2.0)	31	25, 31
28	115.2		27, 30	
29	153.0		27	
30	121.2	6.67, 1H, d (8.4)		31
31	130.3	6.94, 1H, dd (8.5, 2.1)	25, 27	25, 27, 30
32				
33	145.1			
34	147.0		36, 38	
35	111.7			
36	129.5	7.13, 1H, d (1.9)	1, 38	1, 38
37	129.7		1, 38	
38	118.6	6.46, 1H, d (1.9)	1, 36	1, 36

+ Solvent was CD_3OD, HMBC *J* optimized for 9Hz, 140 Hz decoupled.



8a **8b**

Scheme 3. Possible Cyclic Structures of BAS-18.

The progression in design of the IMPDH assay is represented in Table 2 and it emerged to deal with results that seemed to be quite variable. A major problem was that activity in the primary automated assay was diminished to above the cut-off level when BAS compounds or their semipure extracts were investigated in the manual assay protocol. A major difference between these two assays was in the timing of the addition of enzyme. The activity of the positive control, mycophenolic acid was independent of when the enzyme was added. By contrast, all of the BAS compounds show a time dependency for the enzyme addition but at this point the source of the effect can not be explained. The second problem was that elemental sulfur and organic compounds containing sulfur showed micro molar EC_{50}'s. This latter problem, to be discussed more fully elsewhere, was dealt with by the addition of DTT as illustrated by the data for sublimed sulfur in Table 2. Also seen in Table 2 is that the DTT reagent has a mild to minimum effect on the activity of some BAS compounds. Finally, each of the BAS compounds tested in the initial screen was observed to be a potent IMPDH inhibitor. Alternatively, BAS 8 (**5**) and 10 (**6**) were found to be the most active IMPDH inhibitors in the modified assay.

Table 2. IMPDH Activity Data.

Compound	EC_{50} (μM)			
	Enzyme First		Enzyme Last	
	without DTT	with DTT	without DTT	with DTT[a]
1	–	–	–	> 16
2	3	3	> 32	> 32
3	8	16	> 32	> 32
4	6	12	> 32	> 32
5	6	7	12	17
6	11	12	25	20
7	5	8	19	32
8	–	–	–	> 16
mycophenolic acid	0.010	0.009	0.006	0.007
sublimed sulfur	0.8	not active	2.6	not active

[a] DTT = dithiothreitol

EXPERIMENTAL

General Experimental Procedures. The NMR spectra were recorded at 250, 300 or 500 MHz for ^1H and 62.5, 75 or 125 MHz for ^{13}C respectively. Low and high resolution FAB mass spectra (using Magic Bullet) were obtained either at UCSC, or at the University of Illinois mass spectrometry facility. High performance liquid chromatography (HPLC) was performed on 10μ silica or ODS columns.

Collection and Identification. The sponges, collected from open reef habitats in Papua New Guinea (coll. no. 91628 and 93128), were identified as *Ianthella basta* (order *Verongidae*, family *Iantellidae*) and *Ianthella flabelliformis* (order *Verongidae*, family *Ianthellidae*) by M. C. Diaz. The specimen, 91628, was obtained at a depth of 12 - 15m, and 93128 was collected at a depth of 6 - 18m. The specimens were fan shaped with a thickness < 5 mm. Live specimens ranged in color from purple to green in both cases and specimens with yellow tinges were observed as well. The surfaces of both species are conulated, but very differently. In *Ianthella basta* the fibers form rectangular skeletal meshes oriented in a single plane, producing a very regular pattern of conules distributed in rows parallel to each other. In *Ianthella flabelliformis* the simple single-plane reticulation is extended at right angles from the main two dimensional fan. As a consequence the body of *I. flabelliformis* is thicker and the conules are more pronounced than the ones of *I. basta*. The sponges' consistency is compressible. Fibers (300 - 700 μm in diameter) are of typical verongid construction, with both bark and pith elements, and the characteristic dark-brown color. These species have been described and displayed by Bergquist.[26]

Extraction and Isolation. Sponges were preserved by being immersed in a 50:50 alcohol:H_2O solution. After approximately 24 hr. this solution was decanted and discarded. The damp organisms were placed in nalgene bottles and shipped back to the home lab at ambient temperature. Next 100% MeOH was added and the organisms were soaked for 24 h. This procedure was repeated two more times. The crude oil was then successively partitioned between equal volumes of aqueous MeOH, percent adjusted to produce a biphasic solution, and hexanes followed by CH_2Cl_2. The remaining water solubles were extracted with *sec*-BuOH. The work-up of coll. no. 91628 was as follows. Its CH_2Cl_2 fraction was subjected to Sephadex LH-20 gel filtration chromatography in pure MeOH giving six main fractions. The last fraction was subjected to flash chromatography using a solvent gradient from EtOAc:Hexanes 1:1 to pure EtOAc to pure MeOH. Two fractions were further purified by HPLC chromatography, first normal phase in 1:1 hexanes:EtOAc followed by reverse phase in 35% water in MeOH, giving the known bastadins as colorless amorphous solids.

Assay. The IMPDH inhibition assay followed the protocol reported by Carr *et al.*[27]

Bastadin 1 (**1**) (2mg),-colorless amorphous solid, ^1H, ^{13}C NMR and COSY spectra are in accord with the literature data.[16]

Bastadin 2 (**2**) (17mg),-colorless amorphous solid, ^1H, ^{13}C NMR and COSY spectra are in accord with the literature data.[16]

Bastadin 5 (**3**) (3mg),-colorless amorphous solid, ^1H NMR and COSY spectra are in accord with the literature data.[16]

Bastadin 6 (**4**) (5mg),-colorless amorphous solid, ^1H spectrum was in accord with the literature data.[16]

Bastadin 8 (**5**) (3mg),-colorless amorphous solid, ^1H, ^{13}C NMR and COSY spectra are in accord with the literature data.[17]

Bastadin 10 (**6**) (3mg),-colorless amorphous solid, ^1H NMR spectrum is in accord with the literature data.[17]

Bastadin 18 (**8**) (3mg).-colorless amorphous solid, HRFABMS *m/z* 940.8705 ($C_{34}H_{28}{}^{79}Br_2{}^{81}Br_2N_4O_8$, Δ 2.7 mmu of calcd.); LRFABMS (mb) *m/z* (rel. int.) M^++1, 613 (4.9), 581 (1.8), 461 (5.3), 427 (1.5); ^1H NMR (CD_3OD, 500 MHz) see Table 1.

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