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Investigating Amine Derivatives of Ambruticin VS-5 and VS-4

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A structure–activity relationship around the amine group of the ambruticin VS series has been developed for antifungal activity. It was shown that the amine can be alkylated through reductive amination without loss of potency. However, if it is converted into either an amide, carbamate, or urea, a significant loss of potency is observed. Of the alkyl amines, small nonpolar groups are optimal for both potency and oral bioavailability. As a result of this study, one compound (KOS-2079) was taken into an animal efficacy model with success.

Introduction

The number of systemic fungal infections has increased rapidly over the past decade. This increase is principally due to the rise in immune-compromised patients, not only those with HIV/AIDS, but also patients undergoing treatment for cancer and organ transplant therapy.[1] However, there are only a few drugs available to treat these infections, of which two classes are most widely used. The azoles, including fluconazole and itraconazole, have limited success against some fungal strains, especially *Aspergillus* spp. These compounds inhibit the enzyme 14α-demethylase, preventing formation of ergesterol, an essential component of the fungal cytoplasmic membrane.[2] Alternatively, amphotericin B, a polyene with a broad spectrum of activity but severe side-effects, is employed. Amphotericin is thought to associate with ergosterol-forming pores, which leads to leakage of K^+ ions and cell death.[3] There remains a need for new and safe antifungal agents.

The ambruticins are a set of polyketide antifungal agents produced by the myxobacterium *Sorangium cellulosum* which vary in the substituent at the 5-position. The S series is characterized by a 5-hydroxy group, and was isolated in the 1970s by researchers at Warner-Lambert.[4] In the 1990s a second series was isolated by researchers at GBF: the VS series, which posses an amine at the 5-position in various methylated states (Figure 1).[5] The ambruticins have in vitro antifungal activities that are similar to amphotericin (with minimum inhibitory concentrations (MICs) in the $\mu g\,mL^{-1}$ range).[5,6] The mechanism of action of the ambruticins was first proposed by Knauth and Reichenbach to be similar to that of the phenylpyrroles,[7] that is, through induction of the high osmolarity glycerol (HOG) signaling pathway.[8] More recently, Vetcher et al. suggested that Hik1, a histidine kinase in the HOG pathway, is the likely target of ambruticin in *S. cerevisiae*.[9]

There has been renewed interest in these molecules, resulting in several total syntheses of ambruticin S (**1**) being reported.[10–13] Our group has investigated the structure–activity relationship (SAR) about the carboxylic acid in the VS series,[14] building on the work of Warner-Lambert researchers on the S series.[15,16] These derivatives showed that the carboxylic acid is not essential for antifungal activity and that there was considerable tolerance for sterically small substituents. There has not been any discussion of the SAR in the amine region of the molecule; however, an ambruticin analogue, KOS-2079, was reported to be effective in two animal models. In the first, a mouse model of invasive aspergillosis,[17] KOS-2079 significantly decreased pulmonary fungal burdens and improved survival over the vehicle control at 200 $mg\,kg^{-1}\,day^{-1}$. The second demonstrated that KOS-2079 prolonged the survival of mice infected with lethal doses of *Coccidioides* over vehicle-treated mice at both 20 and 50 $mg\,kg^{-1}\,day^{-1}$.[18] Herein we describe a series of amine derivatives that led to this compound being taken into these in vivo efficacy animal models.

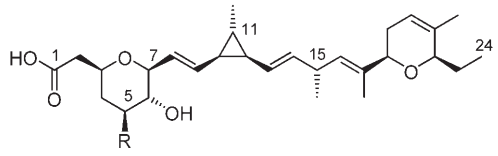

Figure 1. Ambruticin S (**1**; R = OH) and ambruticins VS-5 (**2**; R = NH_2), VS-4 (**3**; R = NHMe), and VS-3 (**4**; R = NMe_2).

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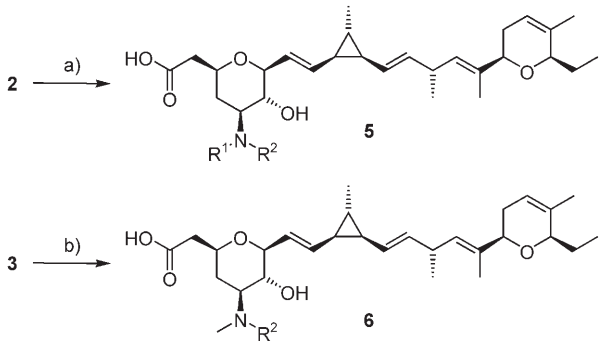
Results and Discussion

The amine functionality of the ambruticin VS series is an attractive site to effect transformations due to its orthogonal reactivity with the remainder of the molecule. It is also an ideal site to modulate the physical and pharmacokinetic properties of the ambruticins. In the natural series the amine appears in all three states of methylation (zero to two in **2**–**4**), with little variation in antifungal activity (see Table 1), suggesting that there is no requirement for a hydrogen bond donor at this position. Thus, we set about derivatizing this site by commencing with both ambruticin VS-4 (**3**; monomethyl) and VS-5 (**2**; free amine), which were both readily accessible through fermentation of the producing organism.

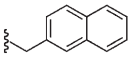
The investigation began with reductive aminations using a range of aldehydes and ketones. The reaction proceeded well in the presence of the unprotected carboxylic acid at C1. In the VS-5 series it was difficult to obtain clean secondary amine product. Instead, reactions were allowed to proceed to the tertiary amine **5**. As expected, the VS-4 series resulted in an *N*-methyl-*N*-alkyl-substituted tertiary amine **6** (Scheme 1).



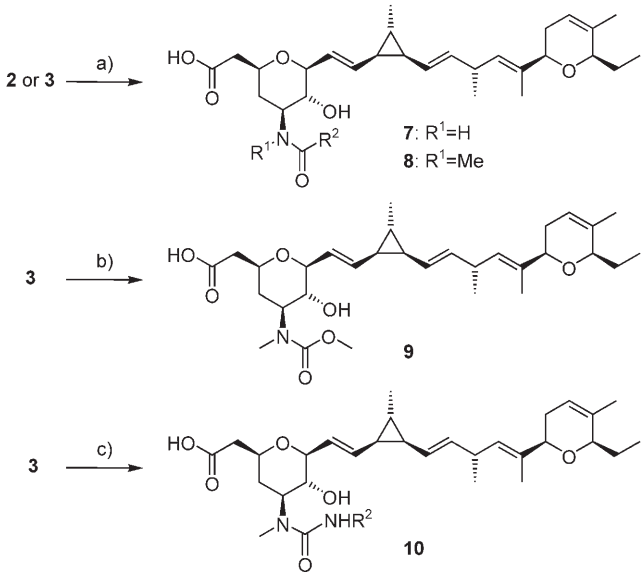
Scheme 1. Reductive aminations: a) R^1CHO, $Na(CN)BH_3$, CH_3OH, room temperature, 16 h; b) R^2CHO or CH_3COCH_3, $Na(CN)BH_3$, CH_3OH, room temperature, 16 h.

The same methodology was used to synthesize a range of tertiary amines from both **2** and **3** to probe both the steric restrictions and the effects of introducing nonpolar, polar, and aromatic groups on the MICs required for antifungal activity against two *Aspergillus* strains (Table 1).[19] The table shows that a range of *N*-alkyl amines can be tolerated. Steric bulk appears to be the determining factor in many cases. For example, comparison of VS-3 with the *N*,*N*-diethyl compound (**4** versus **5a**), and in the VS-4 series comparing *N*-ethyl, *N*-propyl, and *N*-butyl (respectively **6a**, **6b**, **6c**) shows a steady decline in potency. Branched or cyclic substituents are better tolerated than the equivalent straight-chain moieties (compare **6b** with **6d**, and **6c** with **6e** and **6f**). The *N*-(3-hydroxypropyl) compound **6g** suggests that the introduction of a polar group does not have a significant effect on potency. However, the introduction of an imidazole moiety (in **5b** and **6h**) results in a considerable decrease in potency. This may be due in part to the increase in steric bulk.

Table 1. Substituted amine derivatives of ambruticins VS-4 and VS-5 and their MIC values.

Compound	R^1	R^2	MIC [μg mL^{-1}] *A. flavus* 204303	MIC [μg mL^{-1}] *A. fumigatus* 204305
amphotericin			1.71	1.55
ketoconazole			1.50	6.00
1 (ambruticin S)			28	29
2 (VS-5)	H	H	1.15	1.69
3 (VS-4)	Me	H	0.47	0.60
4 (VS-3)	Me	Me	0.71	0.60
5a	Et	Et	2.20	2.10
5b			24	23
6a	Me	Et	2.00	2.00
6b	Me	*n*Pr	2.25	2.25
6c	Me	*n*Bu	4.50	4.25
6d (KOS-2079)	Me	*i*Pr	1.10	1.32
6e	Me	CH_2-*c*Pr	1.50	1.50
6f	Me	cyclopentyl	2.00	2.67
6g	Me	$(CH_2)_3OH$	2.00	1.40
6h	Me		9.00	8.50
6i	Me		4.00	4.25
6j	Me		4.67	8.00

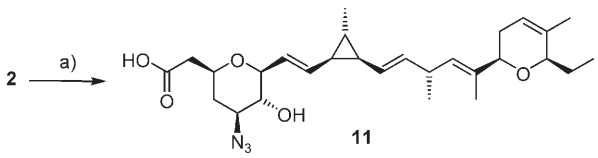
The reactivity of the amine allowed selective reaction with anhydrides, chloroformates, and isocyanates under standard reactions to form the corresponding amides (**7** and **8**), carbamates **9**, and ureas **10** in either the VS-4 or VS-5 series without the need for protection (Scheme 2). In this way the effect of re-



Scheme 2. Amide, carbamate, and urea formation: a) $(R^2CO)_2O$, CH_3OH, room temperature, 20 h; b) CH_3OCOCl, iPr_2NEt, THF, room temperature, 20 h; c) R^2NCO, THF, room temperature, 20 h. THF = tetrahydrofuran.

moving the free amine, which would be protonated under physiological conditions, could be assessed.

In the case of ambruticin VS-5 (**2**) the amine was converted into the azide **11** by reaction with trifluoromethanesulfonyl azide (Scheme 3).[20,21] These compounds were assessed for antifungal activity against our standard strains (Table 2). The results show that removing the amine leads to a significant decrease in potency in all cases. There is little to differentiate between the amides, carbamates, urea or azide. These compounds resemble the potency of ambruticin S (**1**), suggesting that the presence of an amine at C5 is required for the optimal potency.



Scheme 3. Azide formation: a) TfN_3, K_2CO_3, $CuSO_4$, H_2O/CH_2Cl_2, room temperature, 16 h. Tf = trifluoromethanesulfonyl.

Table 2. Amides, carbamates, and ureas of ambruticins VS-5 and VS-4 and the azide and their MIC values.

Compound	R^1	R^2	MIC [$\mu g\,mL^{-1}$]	
			A. flavus 204303	*A. fumigatus* 204305
7a	H	Me	>32	32
8a	Me	Me	>32	32
7b	H	Et	16	8
8b	Me	Et	>32	>32
9			32	16
10a	Me	allyl	32	16
10b	Me	benzyl	32	32
11	azide		>32	>32

Based on the MIC results, five of the alkyl amine compounds were further investigated for their pharmacokinetic (PK) properties in mice. The molecules were chosen based on potency and also on range of substituent about the amine. Thus, there were two nonpolar compounds **5a** and **6d** displaying *N*-alkyl groups, a more polar compound **6g** having a 3-hydroxypropyl group, and two aromatic compounds **6h** and **6i**, the imidazolylmethyl and pyridylmethyl, respectively. By selecting compounds with a range of substituents we hoped to understand the effect that this portion of the molecule has on the PK properties.

All the molecules tested had high plasma exposures when administered intravenously and low volumes of distribution (V_{ss}) (Table 3). Many of the compounds have good oral bioavailability (%*F*), with the nonpolar side chains being optimal (**5a**, **6d** versus **6g**, **6h**). All the molecules were well tolerated by mice. Based on the excellent potency and high oral bioavailability, compound **6d** (given the number KOS-2079) was chosen for efficacy studies in two mouse models.[17,18] Both studies showed that the molecule is able to prolong the life of the animals relative to vehicle.

Table 3. Pharmacokinetic parameters for selected ambruticin analogues in mice.

Compd	Route	Dose [$mg\,kg^{-1}$]	C_{max} [$\mu g\,mL^{-1}$]	AUC [$\mu g\,mL^{-1}\,h^{-1}$][a]	$t_{1/2}$ [h]	V_{ss} [$L\,kg^{-1}$][b]	%F[c]
5a	p.o.	2.5	1.91	22.22	5.6	1.0	82
	i.v.	1.0	1.84	10.86	5.8	0.8	
6d	p.o.	2.5	2.13	23.47	3.7	0.9	91
	i.v.	1.0	2.05	10.28	4.7	0.7	
6g	p.o.	2.5	0.22	1.21	1.8	12.9	54
	i.v.	1.0	2.10	0.90	2.4	3.2	
6h	p.o.	2.5	0.13	0.44	–	–	27
	i.v.	1.0	3.39	0.64	6.4	4.0	
6i	p.o.	2.5	0.95	3.48	3.4	4.0	80
	i.v.	1.0	1.61	1.74	3.2	1.8	

[a] AUC = area under curve. [b] V_{ss} = volume of distribution. [c] %F = bioavailability.

Conclusions

In summary we have shown that the amine functionality of the VS series is vital for the increased potency of the VS series over the S series of ambruticin. If the amine is acylated the potency decreases to an MIC similar to that of ambruticin S. However, provided that the amine is retained it will tolerate a wide range of substitution. There is no requirement for a proton donor at this site and, in general, it appears that small nonpolar alkyl groups result in optimal potency and oral bioavailability. An example of this type, KOS-2079 (**6d**) has been shown to prolong survival when given orally to mice in two lethal palmary infection models.

The ambruticins do appear to be an interesting scaffold on which to investigate a new range of antifungal agents. They possess an alternative mechanism of action to the widely used clinical agents, and they have high in vitro potency and excellent oral bioavailability. This study shows that there is room for tuning the properties in the VS series, and one of the molecules in this study has been shown to be efficacious in two animal models.[17,18]

Experimental Section

Unless otherwise noted, ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 K using a Bruker DRX 400 spectrometer. HRMS data were obtained by flow injection with manual peak-matching using an Applied Biosystems Mariner TOF spectrometer with a turbo-ion spray source. HPLC purification was performed using a Varian Metasil Basic reversed-phase column eluting with a gradient of acetonitrile in water with 0.1% acetic acid.

The care and husbandry of animals were in conformity with the institutional guidelines in compliance with national and international

laws and policies. All animal work was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC, File #1205) accredited facility at Kosan.

General procedure for reductive aminations on ambruticins VS-5 and VS-4

The appropriate aldehyde or ketone (0.20 mmol, 2.0 equiv) was added to a solution of ambruticin VS-5 (**2**) or VS-4 (**3**) (0.10 mmol, 1 equiv) in CH_3OH (1.0 mL), followed by acetic acid (0.40 mmol, 4.0 equiv) and sodium cyanoborohydride (0.20 mmol, 2.0 equiv). The resulting solution was stirred at room temperature or 50 °C until the starting ambruticin was consumed (typically 12 h). The reaction mixture was concentrated under reduced pressure and filtered through a C_{18} plug. The crude material was purified by HPLC.

5a; ^{1}H NMR (CD_3OD): $\delta = 3.31$ (m, 2H, 2×1H of NCH_2), 3.03 (m, 2H, 2×1H of NCH_2), 1.34 ppm (t, 6H, $^3J_{H,H} = 7.0$ Hz, 2×CH_3); ^{13}C NMR (CD_3OD): $\delta = 137.6$, 134.9, 134.8, 134.7, 129.4, 125.4, 123.9, 120.6, 81.2, 78.0, 73.5, 68.4, 63.4, 45.5, 43.7, 34.8, 30.3, 29.7, 29.3, 28.4, 25.1, 21.0, 20.3, 17.6, 11.9, 11.2, 9.6, 7.2 ppm; HRMS *m/z* 530 $[M+H]^+$ found 530.3842, $C_{32}H_{52}NO_5$ requires 530.3840 (Table 4).

5b; ^{1}H NMR (CD_3OD): $\delta = 7.92$ (s, 2H, 2×ArH), 7.10 (s, 2H, 2×ArH), 3.87 (d AB system, $^3J_{H,H} = 14.0$ Hz, 2H, 2×1H of NCH_2), 3.66 ppm (d AB system, $^3J_{H,H} = 14.0$ Hz, 2H, 2×1H of NCH_2); HRMS *m/z* 634 $[M+H]^+$ found 634.3966, $C_{36}H_{52}N_5O_5$ requires 634.3963 (Table 4).

6a; ^{1}H NMR (CD_3OD): $\delta = 3.33$ (m, 2H, NCH_2), 2.77 (s, 3H, NCH_3), 1.34 ppm (t, $^3J_{H,H} = 7.0$ Hz, 3H, CH_2CH_3); ^{13}C NMR (CD_3OD): $\delta =$ 177.0, 137.8, 134.9, 134.8, 134.6, 129.4, 125.4, 123.8, 120.6, 81.1, 78.0 (2C), 73.1, 68.3, 65.4, 48.6, 43.3, 35.3, 34.8, 30.3, 29.7, 28.4, 25.1, 21.0, 20.2, 17.6, 11.9, 11.2, 9.1, 7.2 ppm; HRMS *m/z* 516 $[M+H]^+$ found 516.3701, $C_{31}H_{50}NO_5$ requires 516.3684 (Table 4).

6b; ^{1}H NMR $\delta = 3.33$ (m, 1H, 1H of NCH_2), 2.89 (m, 1H, 1H of NCH_2), 2.63 (s, 3H, NCH_3), 1.72 (m, 2H, $NCH_2CH_2CH_3$), 0.92 ppm (t, $^3J_{H,H} = 7.0$ Hz, 3H, CH_3); ^{13}C NMR $\delta = 176.0$, 138.9, 135.5, 135.0 (2C), 129.5, 125.1, 123.8, 120.9, 81.4, 78.0, 77.8, 73.3, 69.2, 65.7, 55.6, 42.9, 36.7, 34.9, 30.5, 30.1, 28.9, 25.5, 21.4, 21.1, 18.9, 18.5, 12.9, 12.2, 11.2, 8.1 ppm; HRMS *m/z* 530 $[M+H]^+$ found 530.3838, $C_{32}H_{52}NO_5$ requires 530.3840 (Table 4).

6c; ^{1}H NMR $\delta = 2.94$ (m, 2H, NCH_2), 2.61 (s, 3H, NCH_3), 1.65 (m, 2H, $CH_2CH_2CH_2$), 1.32 (m, 2H, CH_2CH_3), 0.91 ppm (t, $^3J_{H,H} = 7.5$ Hz, 3H, CH_3); ^{13}C NMR $\delta = 176.0$, 139.0, 135.5, 135.0 (2C), 129.5, 125.1, 123.7, 120.9, 81.5, 78.0, 77.8, 73.4, 69.2, 65.7, 54.0, 43.0, 36.7, 34.9, 30.5, 30.1, 29.0, 27.2, 25.5, 21.4, 21.1, 20.2, 18.9, 13.6, 12.9, 12.2, 8.1 ppm; HRMS *m/z* 544 $[M+H]^+$ found 544.3969, $C_{33}H_{54}NO_5$ requires 544.3997 (Table 4).

6d (KOS-2079); ^{1}H NMR $\delta = 3.34$ (m, 1H, NCH), 2.48 (s, 3H, NCH_3), 1.31 (d, $^3J_{H,H} = 6.5$ Hz, 3H, 1×CH_3), 1.24 ppm (d, $^3J_{H,H} = 6.5$ Hz, 3H, 1×CH_3); ^{13}C NMR $\delta = 176.7$, 137.1, 135.1, 135.0, 134.9, 129.5, 125.3, 124.6, 120.8, 81.2, 77.9, 77.7, 73.3, 68.6, 63.8, 55.8, 43.1, 34.7, 32.3, 31.3, 30.5, 30.0, 28.7, 25.5, 21.1, 21.0, 18.8 (2C), 18.1, 12.9, 12.0, 8.1 ppm; HRMS *m/z* 530 $[M+H]^+$ found 530.3841, $C_{32}H_{52}NO_5$ requires 530.3840 (Table 4).

6e; ^{1}H NMR $\delta = 3.04$ (m, 2H, NCH_2), 2.74 (s, 3H, NCH_3), 1.11 (m, 1H, 1H of cyclopropyl), 0.66 (m, 2H, 2H of cyclopropyl), 0.27 ppm (m, 2H, 2H of cyclopropyl); ^{13}C NMR $\delta = 176.5$, 139.8, 135.6, 135.0 (2C), 129.5, 125.0, 123.5, 120.9, 81.6, 78.0, 77.8, 73.6, 69.4, 65.0, 43.3, 34.9, 31.1, 30.5, 30.1, 29.1, 25.6, 21.5, 21.1, 18.9, 13.0, 12.2, 8.1, 7.0, 5.0, 4.2 ppm; HRMS *m/z* 542 $[M+H]^+$ found 542.3828, $C_{33}H_{52}NO_5$ requires 542.3840 (Table 5).

Table 4. ^{1}H NMR resonances for the ambruticin backbone of compounds **5a–6d**.

	5a	5b	6a	6b	6c	6d
H-2a	2.53	2.49	2.54	2.68	2.69	2.66
H-2b	2.31	2.37	2.33	2.31	2.30	2.37
H-3	3.86	3.85	3.88	3.86	3.84	3.90
H-4a	2.14	2.01	2.15	2.20	2.21	2.17–2.07
H-4b	1.50	1.44–1.37	1.57–1.46	1.46	1.36	1.47
H-5	3.40	2.77	3.42–3.38	2.90	3.27	3.21
H-6	3.40	3.45	3.42–3.38	3.36	3.35	3.34
H-7	3.63	3.72	3.62	3.64	3.63	3.65
H-8	5.50–5.43	5.53–5.32	5.53–5.39	5.50–5.40	5.47–5.40	5.54
H-9	5.50–5.43	5.53–5.32	5.53–5.39	5.50–5.40	5.47–5.40	5.46–5.36
H-10	1.13	1.10	1.14	1.09	1.10	1.13
H-11	1.04	1.03	1.08	1.02	1.02	1.04
H-11′	1.04	1.03	1.04	1.02	1.03	1.04
H-12	1.49	1.44–1.37	1.45	1.39	1.47	1.46
H-13	5.16	5.14	5.15	5.04	5.05	5.07
H-14	5.50–5.43	5.53–5.32	5.53–5.39	5.50–5.40	5.47–5.40	5.46–5.36
H-15	3.11	3.09	3.09	3.04	3.04	3.05
H-15′	1.05	1.03	1.06	1.02	1.03	1.04
H-16	5.25	5.24	5.25	5.22	5.22	5.24
H-17′	1.64	1.63	1.64	1.62	1.62	1.64
H-18	3.83	3.83	3.83	3.82	3.82	3.85
H-19a	2.11	2.10	2.09	2.10	2.11	2.17–2.07
H-19b	1.92	1.85	1.86	1.85	1.82	1.86
H-20	5.58	5.57	5.58	5.55	5.55	5.57
H-21′	1.59	1.59	1.59	1.57	1.60	1.59
H-22	4.08	4.08	4.08	4.07	4.08	4.10
H-23a	1.76	1.75	1.75	1.82	1.75	1.77
H-23b	1.52	1.53	1.57–1.46	1.51	1.52	1.53
H-24	0.89	0.88	0.89	0.87	0.87	0.89

6f; ^{1}H NMR $\delta = 2.67$ (s, 3H, NCH_3), 2.29 (m, 1H, 1H of cyclopentyl), 2.09–2.02 (m, 2H, 2H of cyclopentyl), 1.78 (m, 2H, 2H of cyclopentyl), 1.55–1.48 (m, 2H, 2H of cyclopentyl), 1.29–1.24 ppm (m, 2H, 2H of cyclopentyl); ^{13}C NMR $\delta = 176.0$, 135.5, 135.1, 135.0, 129.5, 125.1, 123.6, 120.9, 81.5, 78.0, 77.8, 73.2, 68.9, 42.9, 34.9, 34.5, 30.5, 30.1, 29.1, 29.0, 28.9, 25.5, 23.5, 21.5, 21.1, 18.9, 13.0, 12.2, 8.1 ppm; HRMS *m/z* 556 $[M+H]^+$ found 556.3981, $C_{34}H_{54}NO_5$ requires 556.3996 (Table 5).

6g; ^{1}H NMR (CD_3OD): $\delta = 3.86$ (m, 2H, CH_2OH), 3.23 (m, 1H, 1H of NCH_2), 3.12 (m, 1H, 1H of NCH_2), 2.77 ppm (s, 3H, NCH_3); ^{13}C NMR (CD_3OD): $\delta = 176.7$, 137.5, 134.9, 134.8, 134.6, 129.4, 125.4, 124.0, 120.6, 81.1, 78.0 (2C), 73.3, 68.8, 66.4, 56.0, 54.9, 43.3, 36.6, 34.8, 30.3, 29.7, 29.2, 28.7, 28.4, 25.1, 20.9, 20.2, 17.6, 11.9, 11.2, 7.2 ppm; HRMS *m/z* 532 $[M+H]^+$ found 532.3638, $C_{31}H_{50}NO_6$ requires 532.3633 (Table 5).

6h; ^{1}H NMR (CD_3OD): $\delta = 7.82$ (s, 1H, 1H of ArH), 7.30 (s, 1H, 1H of ArH), 4.22 (d AB system, $^3J_{H,H} = 13.5$ Hz, 1H, 1H of NCH_2), 4.10 (d AB system, $^3J_{H,H} = 13.5$ Hz, 1H, 1H of NCH_2), 2.66 ppm (s, 3H, NCH_3); ^{13}C NMR (CD_3OD): $\delta = 176.4$, 137.4, 136.2, 134.9, 134.8, 134.6, 130.1, 129.4, 125.4, 124.1, 120.6, 119.6, 81.2, 78.0 (2C), 73.3, 68.9, 65.2, 49.9, 43.0, 35.3, 34.8, 30.3, 29.7, 29.2, 28.4, 27.7, 25.1, 20.9, 20.3, 17.7, 12.0, 11.2, 7.3 ppm; HRMS *m/z* 568 $[M+H]^+$ found 568.3719, $C_{33}H_{50}N_3O_5$ requires 568.3745 (Table 5).

6i; ^{1}H NMR $\delta = 8.56$ (s, 1H, ArH-2), 8.53 (d, $^3J_{H,H} = 4.0$ Hz, 1H, ArH-6), 7.78 (d, $^3J_{H,H} = 6.5$ Hz, 1H, ArH-4), 7.36 (t, $^3J_{H,H} = 5.5$ Hz, ArH-5), 3.87 (m, 1H, 1H of NCH_2), 3.68 (d AB system, $^3J_{H,H} = 13.0$ Hz, 1H, 1H of NCH_2), 2.31 ppm (s, 3H, NCH_3); ^{13}C NMR $\delta = 175.3$, 174.2, 149.0, 147.8, 138.1, 137.8, 135.4, 135.0, 133.8, 129.6, 125.3, 124.3, 124.0, 120.9, 81.4, 78.0, 77.8, 73.0, 69.7, 65.4, 55.4, 41.1, 36.4, 34.9, 30.6,

30.1, 28.9, 28.8, 25.5, 21.4, 21.1, 18.9, 13.0, 12.1, 8.1 ppm; HRMS *m/z* 579 $[M+H]^+$ found 579.3827, $C_{35}H_{51}N_2O_5$ requires 579.3893 (Table 5).

6j; ^{1}H NMR $\delta=7.80$ (m, 3H, 3H of ArH), 7.74 (s, 1H, ArH-1), 7.56 (m, 3H, 3H of ArH), 4.10 (m, 1H, 1H of NCH_2), 3.84 (m, 1H, 1H of NCH_2), 2.33 ppm (s, 3H, NCH_3); ^{13}C NMR $\delta=175.6$, 138.2, 135.4, 135.0 (2C), 133.2, 132.9, 129.6, 128.4, 128.2, 127.8, 127.6 (2C), 126.9, 126.2, 126.0, 125.3, 124.3, 120.981.5, 78.0, 77.8, 73.3, 69.6, 65.5, 58.2, 41.8, 36.4, 34.9, 30.6, 30.1, 28.9, 25.5, 21.4, 21.1, 18.9, 13.0, 12.2, 8.1 ppm; HRMS *m/z* 628 $[M+H]^+$ found 628.3967, $C_{40}H_{54}NO_5$ requires 628.3996 (Table 5).

Table 5. ^{1}H NMR resonances for the ambruticin backbone of compounds **6e–6j**.

	6e	6f	6g	6h	6i	6j
H-2a	2.74	2.70	2.53	2.52	2.74	2.69
H-2b	2.30	2.29	2.32	2.35	2.48	2.47
H-3	3.92	3.89	3.88	3.84	3.89	3.84
H-4a	2.30	2.10	2.13	2.13	2.08	2.15
H-4b	1.51	1.55–1.48	1.55–1.52	1.56–1.49	1.48	1.47
H-5	3.48	3.50	3.36	3.22	2.80	2.97
H-6	3.78	3.40	3.38	3.42	3.33	3.38
H-7	3.67	3.66	3.61	3.56	3.61	3.63
H-8	5.44–5.40	5.46–5.35	5.53–5.37	5.53–5.38	5.52	5.52
H-9	5.44–5.40	5.46–5.35	5.53–5.37	5.53–5.38	5.45–5.41	5.46–5.38
H-10	1.11	1.12	1.14	1.13	1.12	1.12
H-11	1.02	1.03	1.05	1.05	1.03	1.03
H-11′	1.02	1.03	1.05	1.04	1.03	1.03
H-12	1.49	1.48	1.49	1.46	1.42	1.47
H-13	5.04	5.05	5.15	5.15	5.06	5.05
H-14	5.44–5.40	5.46–5.35	5.53–5.37	5.53–5.38	5.45–5.41	5.46–5.38
H-15	3.04	3.05	3.07	3.09	3.05	3.04
H-15′	1.02	1.03	1.05	1.04	1.03	1.03
H-16	5.23	5.23	5.25	5.25	5.23	5.23
H-17′	1.63	1.63	1.64	1.64	1.63	1.64
H-18	3.83	3.84	3.83	3.83	3.83	3.84
H-19a	2.12	2.13	2.10	2.09	2.10	2.12
H-19b	1.85	1.87	1.88	1.86	1.84	1.84
H-20	5.56	5.56	5.58	5.58	5.55	5.53
H-21′	1.58	1.58	1.59	1.59	1.57	1.58
H-22	4.09	4.09	4.09	4.09	4.08	4.10
H-23a	1.76	1.82	1.78	1.75	1.75	1.76
H-23b	1.53	1.55–1.48	1.55–1.52	1.56–1.49	1.50	1.54
H-24	0.88	0.88	0.89	0.87	0.88	0.88

General procedure for amide formation with ambruticin VS-5 or VS-4

Acetic anhydride (1.00 mmol, 10 equiv) was added to a solution of ambruticin VS-5 (**2**) or VS-4 (**3**) (0.10 mmol, 1 equiv) in CH_3OH (1.0 mL). The solution was stirred at room temperature for 20 h before concentrating under reduced pressure. The residue was dissolved in EtOAc (20 mL) and washed with H_2O (20 mL) and brine (20 mL) before drying (Na_2SO_4) and concentrating under reduced pressure. The crude product was purified by HPLC.

7a; ^{1}H NMR $\delta=6.03$ (brs, 1H, NH), 2.00 ppm (s, 3H, $COCH_3$); HRMS *m/z* 516 $[M+H]^+$ found 516.3339, $C_{30}H_{42}NO_6$ requires 516.3320 (Table 6).

8a; ^{1}H NMR $\delta=2.91$ (s, 3H, NCH_3), 2.12 ppm (s, 3H, $COCH_3$); ^{13}C NMR $\delta=174.2$, 173.1, 138.3, 135.4, 135.0, 129.6, 125.2, 124.0, 120.9, 81.7, 78.0, 77.7, 72.4, 70.3, 55.4, 40.7, 34.9, 34.0, 30.6, 30.0, 29.0, 28.9, 25.5, 22.4, 21.4, 21.1, 18.9, 13.0, 12.2, 8.1 ppm; HRMS *m/z* 530 $[M+H]^+$ found 530.3500, $C_{31}H_{48}NO_6$ requires 530.3476 (Table 6).

7b; ^{1}H NMR $\delta=5.82$ (brs, 1H, NH), 2.25 (q, $^3J_{H,H}=7.5$ Hz, 2H, NCH_2), 1.15 ppm (t, $^3J_{H,H}=7.5$ Hz, 3H, CH_3); ^{13}C NMR $\delta=176.1$, 173.4, 138.7, 135.4, 135.0, 129.6, 125.2, 123.7, 120.9, 81.4, 78.0, 77.8, 74.8, 71.8, 52.8, 40.0, 36.4, 34.9, 30.6, 30.0, 29.5, 29.0, 25.5, 21.4, 21.1, 18.9, 13.0, 12.2, 9.7, 8.1 ppm; HRMS *m/z* 530 $[M+H]^+$ found 530.3457, $C_{31}H_{48}NO_6$ requires 530.3476 (Table 6).

8b; ^{1}H NMR $\delta=2.90$ (s, 3H, NCH_3), 2.36 (m, 2H, OCH_2), 1.11 ppm (m, 3H, CH_3); ^{13}C NMR $\delta=176.1$, 174.0, 138.2, 135.3, 134.9, 126.7, 125.3, 124.0, 120.9, 81.7, 78.0, 77.7, 72.2, 70.4, 55.5, 40.4, 34.9, 34.0, 30.6, 30.0, 29.7, 28.9, 27.4, 25.5, 21.4, 21.1, 18.9, 13.0, 12.1, 9.1, 8.1 ppm; HRMS *m/z* 566 $[M+Na]^+$, 544 $[M+H]^+$ found 544.3644, $C_{32}H_{50}NO_6$ requires 544.3633 (Table 6).

Table 6. ^{1}H NMR resonances for the ambruticin backbone of compounds **7a–8b**.

	7a	8a	7b	8b
H-2a	2.67	2.67	2.67	2.66
H-2b	2.44	2.43	2.48	2.44
H-3	3.96	3.87–3.83	3.93	3.95
H-4a	1.84	2.15–2.09	2.16	1.82
H-4b	1.50	1.32	1.38	1.54–1.42
H-5	4.67	3.87–3.83	3.93	4.72
H-6	3.26	3.06	3.14	3.27
H-7	3.69	3.64	3.66	3.71
H-8	5.51–5.42	5.47–5.42	5.49–5.42	5.5
H-9	5.51–5.42	5.47–5.42	5.49–5.42	5.47–5.38
H-10	1.11	1.12	1.15	1.11
H-11	1.02	1.05–1.03	1.03	1.02
H-11′	1.04	1.03	1.04	1.03
H-12	1.50	1.56–1.59	1.49	1.49
H-13	5.06	5.06	5.06	5.05
H-14	5.51–5.42	5.47–5.42	5.49–5.42	5.47–5.38
H-15	3.06	3.08	3.06	3.05
H-15′	1.04	1.04	1.04	1.03
H-16	5.24	5.23	5.24	5.23
H-17′	1.63	1.64	1.64	1.68
H-18	3.78	3.87–3.83	3.84	3.83
H-19a	2.18–2.12	2.15–2.09	2.13	2.12
H-19b	1.87	1.84	1.85	1.86
H-20	5.56	5.57	5.56	5.55
H-21′	1.58	1.58	1.58	1.57
H-22	4.10	4.10	4.10	4.09
H-23a	1.76	1.77	1.79–1.74	1.79–1.73
H-23b	1.51	1.56–1.59	1.54	1.54–1.42
H-24	0.88	0.88	0.88	0.88

Procedure for carbamate formation with ambruticin VS-5 or VS-4

Diisopropylethylamine (0.30 mmol, 3.0 equiv) was added to a suspension of ambruticin VS-4 (**3**) (0.10 mmol, 1.0 equiv) in THF (1 mL) followed by methyl chloroformate (0.20 mmol, 2.0 equiv). The mixture was stirred at room temperature for 20 h before concentrating under reduced pressure. The residue was dissolved in EtOAc (20 mL), washed with HCl (0.1 M, 20 mL) and brine (20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was purified by HPLC to yield **9** (KOS-1972); ^{1}H NMR $\delta=$

3.70 (s, 3H, OCH_3), 2.81 ppm (s, 3H, NCH_3); HRMS *m/z* 568 $[M+Na]^+$ found 568.3227, $C_{31}H_{47}NO_7Na$ requires 568.3248.

Procedure for the synthesis of the ureas

Alkyl isocyanate (0.50 mmol, 5.0 equiv) was added to a suspension of ambruticin VS-4 (**3**) (0.10 mmol, 1.0 equiv) in THF (1 mL). The mixture was stirred at 50 °C for 20 h before adding PS-$TsNHNH_2$ resin (Argonaut, CA, USA) and stirring at room temperature for 16 h. The mixture was diluted with CH_3OH (10 mL) and filtered to remove the resin. The filtrate was concentrated under reduced pressure, and the residue was dissolved in EtOAc (20 mL). The solution was washed with HCl (0.1 M, 20 mL) and brine (20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was purified by HPLC.

10a; ^{1}H NMR $\delta = 5.85$ (m, 1H, C*H*CH_2), 5.15 (d, $^3J_{H,H} = 17.0$ Hz, 1H, *trans*-CH_2), 5.08 (d, $^3J_{H,H} = 10.0$ Hz, 1H, *cis*-CH_2); 3.82 (s, 2H, NC*H*$_2$CH) 2.78 ppm (s, 3H, NCH_3); ^{13}C NMR $\delta = 173.8$, 159.9, 138.3, 135.4, 135.1, 134.9 (2C), 129.6, 125.2, 124.0, 120.9, 115.8, 81.7, 78.0, 77.8, 72.4, 70.7, 57.1, 43.5, 40.5, 34.9, 34.7, 30.5, 30.0, 28.9, 28.5, 25.5, 21.4, 21.1, 20.8, 18.9, 13.0, 12.2, 8.1 ppm; HRMS *m/z* 593 $[M+Na]^+$ found 593.3582, $C_{33}H_{50}N_3O_6Na$ requires 593.3561 (Table 7).

10b; ^{1}H NMR $\delta = 7.32$ (m 5H, ArH), 5.14 (br s, 1H, NH), 4.36 (m, 2H, CH_2Ar), 2.73 ppm (s, 3H, NCH_3); ^{13}C NMR $\delta = 175.0$, 159.9, 139.1, 137.9, 135.3, 135.0, 134.9, 129.6, 128.6 (2C), 127.6 (2C), 127.2, 125.3, 124.3, 120.9, 81.5, 78.0, 77.7, 72.7, 70.6, 57.0, 45.0, 41.2, 34.9, 34.7, 30.5, 30.0, 28.9, 28.5, 25.5, 21.4, 21.1, 18.9, 13.0, 12.2, 8.1 ppm; HRMS *m/z* 643 $[M+Na]^+$ found 643.3746, $C_{37}H_{52}N_2O_6Na$ requires 643.3718 (Table 7).

Procedure for the synthesis 5-azidoambruticin 11

Potassium carbonate (0.56 mmol, 1.0 equiv) was added to a solution of ambruticin VS-5 (**2**) (0.56 mmol, 1.0 equiv) in H_2O (2.0 mL) and CH_3OH (4.0 mL), followed by TfN_3 (3.0 mL of a 0.37 M solution in CH_2Cl_2, 1.11 mmol, 2.0 equiv). The mixture was made homogeneous by the addition of CH_3OH (2 mL), and copper sulfate (0.006 mmol, 0.01 equiv) was added. The solution was stirred at room temperature for 16 h before concentrating under reduced pressure. The residue was loaded onto silica. Column chromatography (silica, 5→10% CH_3OH/CH_2Cl_2) yielded **11**; ^{13}C NMR $\delta = 174.1$, 140.3, 138.8, 135.1, 135.0, 129.6, 125.0, 123.0, 120.9, 81.4, 78.0, 77.9, 73.9, 71.6, 62.6, 39.9, 35.8, 35.0, 30.4, 30.1, 29.2, 25.6, 21.7, 21.1, 18.9, 13.0, 12.3, 8.2 ppm; HRMS *m/z* 501 $[M+H]^+$ (Table 7).

General procedure for the antifungal activity assay

The NCCLS protocol was used.[19] Compounds were dissolved in DMSO and prepared as a stock solution in RPMI media at 2× concentration. A stock solution of the spores for the filamentous fungi ($1–10\times10^4$ cells mL^{-1}) was made in RPMI media at 2× concentration. Aliquots of 100 μL of each solution were added to each well to a final volume of 200 mL. Drug-free and fungi-free controls were included. Plates were incubated at 37 °C for 48 h. To determine the MIC for filamentous fungi, the optical density (OD) of each well was measured at 405 nm with a spectrophotometer. After subtraction of the OD of the control not containing fungal spores, the percentage growth relative to the drug-free well was calculated. Relative OD values were grouped into five levels: 0 = 0–5%; 1 = 6–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%. The MICs reported refer to 50% inhibition.

Table 7. ^{1}H NMR resonances for the ambruticin backbone of compounds **9–11**.

	9	10a	10b	11
H-2a	2.63	2.67	2.63	2.67
H-2b	2.46	2.42	2.39	2.50
H-3	3.95	3.92	3.90	3.91
H-4a	1.87	1.90	1.89	2.14–2.10
H-4b	1.52–1.48	1.47	1.50–1.44	1.52–1.44
H-5	4.23	4.27	4.36	3.51
H-6	3.31	3.23	3.19	3.23
H-7	3.65	3.68	3.65	3.92
H-8	5.48–5.43	5.50–5.42	5.46–5.34	5.50–5.41
H-9	5.48–5.43	5.50–5.42	5.46–5.34	5.50–5.41
H-10	1.12	1.10	1.04	1.14
H-11	1.02	1.02	1.01	1.05
H-11′	1.04	1.03	1.02	1.05
H-12	1.52–1.48	1.51–1.47	1.50–1.44	1.52–1.44
H-13	5.06	5.04	5.04	5.08
H-14	5.48–5.43	5.46–5.38	5.46–5.34	5.50–5.41
H-15	3.07	3.05	3.05	3.07
H-15′	1.04	1.03	1.02	1.05
H-16	5.24	5.23	5.23	5.25
H-17′	1.64	1.63	1.63	1.64
H-18	3.85	3.82	3.84	3.84
H-19a	2.18–2.09	2.16	2.12	2.14–2.10
H-19b	1.84	1.82	1.86–1.80	1.87
H-20	5.57	5.55	5.56	5.57
H-21′	1.59	1.57	1.58	1.59
H-22	4.10	4.09	4.10	4.11
H-23a	1.79–1.74	1.76–1.73	1.77	2.14–2.10
H-23b	1.54	1.52	1.52	1.52–1.44
H-24	0.89	0.87	0.88	0.89

General procedure for PK studies

Compounds were dissolved in ethanol at 20× above the targeted concentration. One part of the concentrate was diluted with 19 parts of a diluent consisting of 12% hydroxypropyl-β-cyclodextrin in phosphate-buffered saline. Mice were dosed as indicated in Table 3 through either oral gavage or i.v. bolus injection through the tail vein. Plasma specimens were taken at various time points (up to 24 h post-dose). The plasma concentration of each compound was determined by LC–MS–MS using a Waters Alliance 2795 HPLC instrument coupled to a Waters QuattroMicro mass spectrometer. Pharmacokinetic parameters were calculated using Kinetica 4.1 (Thermo Electron).

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Keywords: ambruticin · antifungal agents · polyketides · structure–activity relationships

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