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Bioconversion of ascomycin and its 6-alkoxyderivatives

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Abstract

Ascomycin is known to be *O*-demethylated to 32-*O*-desmethyl-ascomycin by bioconversion using the strain *Actinoplanes* sp. ATCC 53771. Using modified screening media, the conversion rate could be improved from previously published 15% to up to 35%. Together with 32-*O*-desmethyl-ascomycin, two further metabolites, the already known 32-*O*-desmethyl-19-hydroxymethylascomycin and the new 32-*O*-desmethyl-32-epi-ascomycin were isolated and characterized. Prompted by the observed demethylation capability of ATCC 53771, we studied whether this strain was also able to dealkylate 6-alkoxy derivatives of ascomycin to 6-hydroxy-ascomycin. Although the 6-alkoxy derivatives were demethylated at various positions of the molecule, production of the hypothetical metabolite 6-hydroxy-ascomycin by biotransformation could not be shown. However, work up of the fermentation broth allowed the isolation and characterization of a new metabolite, the 6-ethoxy-15-desmethyl-ascomycin.

Keywords: Bioconversion; *Actinoplanes* sp. ATCC 53771; Ascomycin; 6-alkoxy-ascomycins; 32-*O*-desmethyl-ascomycin; 32-*O*-desmethyl-32-epi-ascomycin; 6-ethoxy-15-desmethyl-ascomycin

1. Introduction

Ascomycin **1** is a macrolactam produced by *Streptomyces hygroscopicus* subsp. *yakushimaensis* No. 7238 [1]. It was originally isolated on the basis of its antifungal activity [2] and later rediscovered in a directed screening for immunosuppressive compounds [3]. It inhibits T-cell proliferation at nanomolar concentrations comparable to FK 506 [4]. Previous studies on microbial conversion aiming at modifications of chemically inaccessible sites of ascomycin revealed that ascomycin can be *O*-demethylated in positions C-13, C-15 and C-32 using the strain *Actinoplanes* sp. ATCC 53771 [5]. Moreover, this strain is also capable to both *N*-demethylate and hydroxylate cyclosporin A [6].

In order to produce sufficient amounts of 32-*O*-desmethyl-ascomycin **1a** for a chemical derivatisation program, the fermentation conditions for microbial conversion using ATCC 53771 were optimized. Based on the already known demethylation and hydroxylation capability of ATCC 53771 [5] we aimed at the dealkylation of 6-alkoxy-ascomycins. A combination of dealkylations at positions *O*-13, *O*-15,

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O-32 and probably *O*-6 may result in the production of various new metabolites.

2. Experimental

2.1. Culture conditions

A seed culture medium was inoculated with ATCC 53771 and incubated at 27°C on a rotary shaker (200 rpm) for 4 days (Table 1). The main culture medium was inoculated with seed culture and incubated at 24°C. Transformation was started by addition of the parent compound. Cultures were harvested after different periods of time (2 h, 4 h, 6 h, 8 h, 24 h).

2.2. HPLC analysis

EtOAc extracts of the main cultures were chromatographed on reversed phase columns. Metabolites were identified by a combination of UV (DAD) detection and LC-MS (EI-MS) measurements.

2.3. Isolation

The EtOAc extract of a 900-l fermentation broth (substrate concentration: 90 g) yielded 18 g of 32-*O*-desmethyl-ascomycin **1a**, 0.64 g of 32-*O*-desmethyl-19-hydroxymethyl-ascomycin **1b** and 0.08 g of 32-*O*-desmethyl-32-epi-ascomycin **1c** after being successively chro-

Table 1
Comparison of fermentation conditions for *Actinoplanes* sp. ATCC 53771

	MSD	Novartis
Seed culture medium	Dextrose 0.1%	Glucose 0.7%
	Dextrin 1%	Malt extract 0.5%
	Beef extract 0.3%	Soluble starch 1.0%
	Ardamine pH 0.5%	Yeast extract 0.45%
	N-*Z*-amine type E 0.5%	*N*-*Z*-amine type A 0.25%
	$MgSO_4 \times 7\ H_2O$ 0.005%	$CaCO_3$ 0.005%
	K_2HPO_4 0.037%	Trace elements (Fe, Co, I, Cu, Mn, Zn)
	$CaCO_3$ 0.05%	
	pH 7.1	pH 7.0
Transformation medium	Glucose 1.0%	Glucose 0.5%
	Hycase SF 0.2%	Soluble starch 1.5%
	Beef extract 0.1%	*N*-*Z*-amine type A 1.0%
	Corn steep liquor 0.3%	Brewer's yeast 0.2%
		K_2HPO_4/KH_2PO_4 0.06%/0.03%
		$MgSO_4 \times 7\ H_2O$ 0.01%
		$CaCl_2 \times 2\ H_2O$ 0.01%
		NaCl 0.005%
		Trace elements (Fe, Co, I, Cu, Mn, Zn)
	pH 7.0	pH 6.2–6.5
Inoculation of transformation medium with seed culture medium	5%	10%
Incubation period		
seed culture medium	24 h	96 h
transformation medium	18 h	6–8 h
Temperature		
seed culture medium	27°C	27°C
transformation medium	27°C	24°C
Shaker frequency	220 rpm	200 rpm
Substrate concentration	100 mg/l medium dissolved in DMSO	100 mg/l medium dissolved in ethanol
Turnover of ascomycin into 32-*O*-desmethyl-ascomycin	15%	45.8% small scale, 35.0% large-scale fermentor

matographed on Sephadex UA20 with MeOH and silicagel with hexane/acetone 8:2 (increasing acetone concentration). 6-Ethoxy-15-desmethyl-ascomycin **3b** was isolated out of a 5-l fermentation broth (substrate concentration: 500 mg) using an CH_3CN/H_2O gradient (40% to 70%) on a RP-8 column.

2.4. Structure elucidation

Structures of the metabolites were determined on the basis of UV, IR, ^{1}H- and ^{13}C-NMR data.

2.5. Biological evaluation

Metabolites of ascomycin and its 6-alkoxy derivatives were tested for FKBP12 binding [7], effects on the murine mixed-lymphocyte reaction [8] and in an IL-2 reporter gene assay [9].

3. Results and discussion

3.1. 32-O-Desmethyl-ascomycin, *1a*

In order to obtain sufficient amounts of 32-*O*-desmethyl-ascomycin **1a**, the actinomycete *Actinoplanes* sp. ATCC 53771, which had been described by MSD to demethylate ascomycin **1** to 32-*O*-desmethyl-ascomycin **1a** [10], served for a large scale fermentation. Applying own screening conditions (Table 1), the conversion rate could be improved from 15% (MSD) to up to 45% on a small scale and 35% in a large-scale

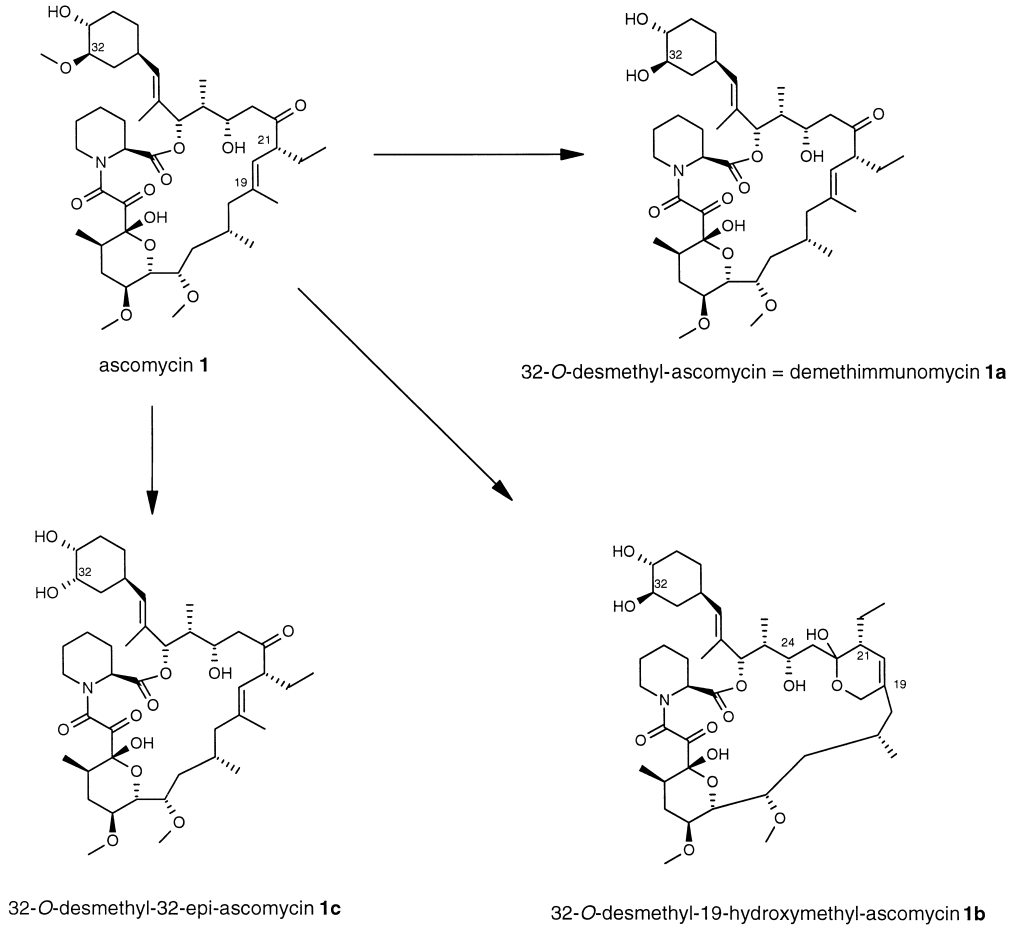

Fig. 1. Bioconversion products of ascomycin with *Actinoplanes* sp. ATCC 53771.

fermenter. Since the main difference between the fermentation conditions can be found in the medium composition (Table 1), we suppose that this parameter influenced the enzyme expression and activity of the strain *Actinoplanes* sp. ATCC 53771 and thus the metabolite profile most.

In addition to 32-*O*-desmethyl-ascomycin **1a**, two further metabolites from the incubation of ascomycin with ATCC 53771 (Fig. 1) were characterized by NMR. One of these metabolites, 32-*O*-desmethyl-19-hydroxymethyl-ascomycin **1b**, has been previously described to be formed by biotransformation of ascomycin with *S. lavendulae* ATCC 55209 [11]. The other bioconversion derivative (Fig. 1) was totally characterized by ^{1}H-NMR spectroscopy and decoupling experiments (H-32/H-31a (3 Hz) and H-32/H-31e (4.5 Hz)). The low values of the coupling constants indicated the equatorial position of the H-32, because an axial position of the H-32 would result in a coupling constant of about 8-13 Hz. On the basis of these data, the structure of 32-*O*-desmethyl-32-epi-ascomycin **1c** was assigned to the new derivative. Both metabolites were up to now not detected by

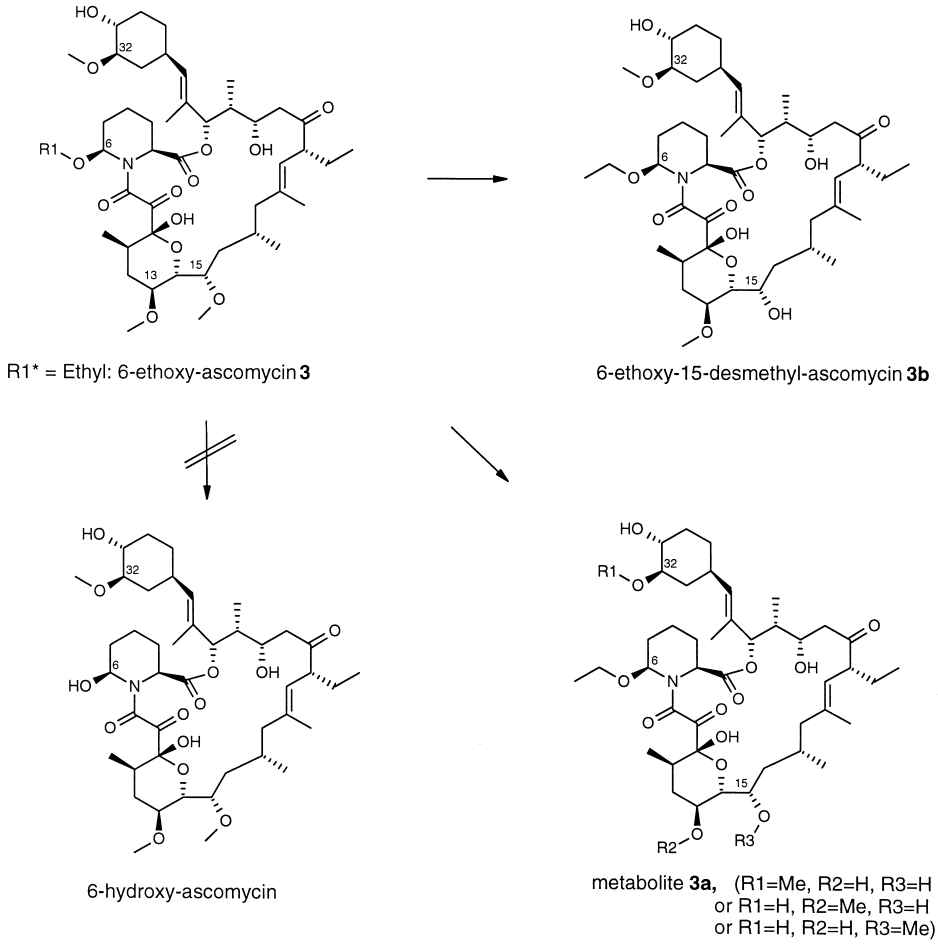

Fig. 2. Bioconversion of 6-ethoxy-ascomycin with *Actinoplanes* sp. ATCC 53771.

Table 2
Selected ^{1}H-NMR data of 6-ethoxy-ascomycin **3** and its metabolite **3b**

	6-Ethoxyascomycin **3**	6-Ethoxy-15-desmethyl-ascomycin **3b**
H-6	5.77 (br, t)	5.74 (br, t)
H-13	3.28 (m)	3.25 (m)
H-15	**3.58 (m)**	**3.97 (m)**
H-32	3.0 (m)	3.02 (m)

$CDCl_3$, (500 MHz), chemical shifts (δ_H) in ppm relative to TMS.

biotransformation of ascomycin with *Actinoplanes* sp. ATCC 53771.

3.2. 6-Alkoxy-ascomycins **2**, **3**, **4**

Introduction of an alkoxy group in the 6-position of ascomycin reduces its FKBP12 binding, activity in MLR and the activity in the IL-2 reporter gene assay [12]; the larger the alkoxy group, the lower the activity (Table 3). We hypothesized that dealkylation of 6-alkoxy-ascomycins to 6-OH-ascomycin might restore the biological activity. Prompted by the known demethylation capability of strain ATCC 53771 [5] in positions *O*-13, *O*-15 and *O*-32, we studied whether this strain was also able to dealkylate 6-alkoxy-derivatives of ascomycin.

Bioconversion of 6-methoxy-ascomycin **2**, 6-ethoxy-ascomycin **3** and 6-propoxy-ascomycin **4**, respectively, which was monitored by LC-MS generated for each substrate three different mono-desmethylated metabolites. Moreover, microbial conversion of 6-ethoxy-ascomycin **3** and 6-propoxy-ascomycin **4** yielded one further metabolite (**3a**, **4a**, respectively). Since LC-MS analysis of metabolites **3a** and **4a** indicated deethylation (-28 mass units) and depropylation (-42 mass units) from **3** and **4**, respectively, while their retention times are identical, **3a** and **4a** could be putatively assigned to 6-OH-ascomycin. This derivative was not formed with 6-methoxy-ascomycin **2**.

In order to isolate and characterize this metabolite, a 5-l fermentation with 6-ethoxy-ascomycin **3**, was performed. Metabolite **3a** was isolated as a single bioconversion product. The evaluation of its ^{1}H-NMR spectra was not totally possible, because of the presence of carbon–hydrogen side-products, probably enriched out of the solvents of the preparative HPLC separation. Nevertheless, the presence of the proton signals at δ 5.73 (t, $J = 2.7$) clearly indicated the existence of the 6-ethoxy-group and thus that **3a** and the putative 6-OH-ascomycin do not correspond (Fig. 2). On the basis of the ^{1}H-NMR and LC-MS data we assume that metabolite **3a** is a di-desmethylated 6-ethoxy-ascomycin.

However, a new metabolite **3b** (Fig. 2) was isolated and characterized on the basis of MS, ^{1}H- and ^{13}C-NMR spectra and ^{1}H-decoupling experiments and C–H correlation spectra. The loss of a methyl group was indicated either by a mass difference of minus 14 with respect to 6-ethoxy-ascomycin and the lack of a OMe-sig-

Table 3
Biological data of ascomycin and bioconversion metabolites

Metabolite	FKBP12 binding IC_{50} (nM)	MLR IC_{50} (nM)	IL-2 reporter gene assay IC_{50} (nM)
FK 506	1.1	0.096	0.1
ascomycin **1**	0.6	2	1.8
32-*O*-desmethyl-ascomycin **1a**	3.72	0.56	0.41
32-*O*-desmethyl-32-epi-ascomoycin **1b**	n.d.	n.d.	0.25
6-methoxy-ascomycin **2**	77	45	24
6-ethoxy-ascomycin **3**	>1000	>1000	260
6-ethoxy-15-desmethyl-ascomycin **3b**	n.d.	n.d.	570
6-propoxy-ascomycin **4**	n.d.	n.d.	>1000

n.d.: no data.

nal in the ^{1}H- and ^{13}C-NMR spectra. Since a CH carrying a hydroxy group is generally 0.1–0.3 ppm downfield shifted relative to a CH–OCH_3, it was reasonable that demethylation had taken place at *O*-15, because the H-15 appeared to be the only proton shifted for 0.39 ppm compared to that of the parent compound (Table 2). Decoupling experiments additionally proved that desmethylation had occurred at O-15. Irradiation at the H-15 (δ 3.97 br, t) resulted in simplification of H-16a (δ 1.44) and H-16b (δ 1.18). The 6-ethoxy-group remained intact which was shown by the presence of the H-6 signal (δ 5.74). All important signals could be assigned through the correlation spectra. On the basis of these data **3b** was identified as the new 6-ethoxy-15-desmethyl-ascomycin. As a consequence of these data we conclude, that the demethylation capability of the enzyme present in *Actinoplanes* sp. ATCC 53771 seemed to be restricted to *O*-13, *O*-15 and *O*-32. Alkoxy groups in position 6 are not modified by bioconversion with ATCC 53771.

3.3. Biological evaluation

The microbial conversion metabolites were evaluated with respect to FKBP12 binding, MLR activity and effects in an IL-2 reporter gene assay. Both 32-*O*-demethylation and an additional C-32-epimerization of ascomycin did not affect the biological profile as compared to ascomycin. The 15-desmethylation of 6-ethoxyascomycin also did not result in a significant loss of the biological activity with respect to the parent compound (Table 3).

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References

[1] H. Hatanaka, T. Kino, S. Miyata, N. Inamura, A. Kuroda, T. Goto, H. Tanaka, M. Okuhara, J. Antibiot. 41 (1988) 1592–1601.
[2] F.J. Dumont, K.M. Byrne, N.H. Sigal, L. Kaplan, R.L. Monaghan, G. Garrity, European Patent Application EP 0323 865 (1989).
[3] M. Morisaki, T. Arai, J. Antibiot. 45 (1992) 126–128.
[4] T. Goto, T. Kino, H. Hatanaka, M. Nishiyama, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, Transplant. Proc. 19 (6) (1987) 4–8, Suppl.
[5] T.S. Chen, B.H. Arison, L.S. Wicker, E.S. Inamine, R.L. Monaghan, J. Antibiot. 45 (1992) 118–123.
[6] M. Kuhnt, F. Bitsch, J. France, H. Hofmann, J.J. Sanglier, R. Traber, J. Antibiot. 49 (1996) 781–787.
[7] T. Fehr, J.J. Sanglier, W. Schuler, L. Gschwind, M. Ponelle, W. Schilling, C. Wioland, J. Antibiot. 49 (1996) 230–233.
[8] T. Meo, in: I. Levkovits, B. Pernis (Eds.), Immunological Methods, Academic Press, New York, 1979, pp. 227–239.
[9] P. Wenner, Internal Report, PKF, Sandoz Pharma, 1993.
[10] E.S. Inamine, B.H. Arison, S.T. Chen, J.S. Wicker, European Patent Application EP-0 349 049, 1989.
[11] S.T. Chen, R.F. White, G. Dezeny, B.H. Arison, T.R. Beattie, A.M. Hale, F. Dumont, European Patent Application EP 526934 A1 930210, 1992.
[12] A. Bulusu, European Patent Application EP 0626385 A, 1994.