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A Convenient Degradation of Polyoxin D to Uracil Polyoxin C: Access to Key Intermediates and Synthesis of Antifungal α-Aminoacyl Derivatives of UPOC

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Abstract: A convenient degradation of readily available polyoxin D under Edman conditions gave carboxyuracil polyoxin C **3** in high yield. Decarboxylation to uracil polyoxin C **5** (UPOC) and ring contraction to imidazolone compound **8**, gave important nikkomycin Z and X intermediates respectively. Syntheses of new polyoxin/nikkomycin analogs **12-27**, some with excellent chitin synthetase inhibition and *Candida albicans* whole cell activity are described. The importance of β-methyl substituted amino acid side chains for whole cell activity is highlighted.

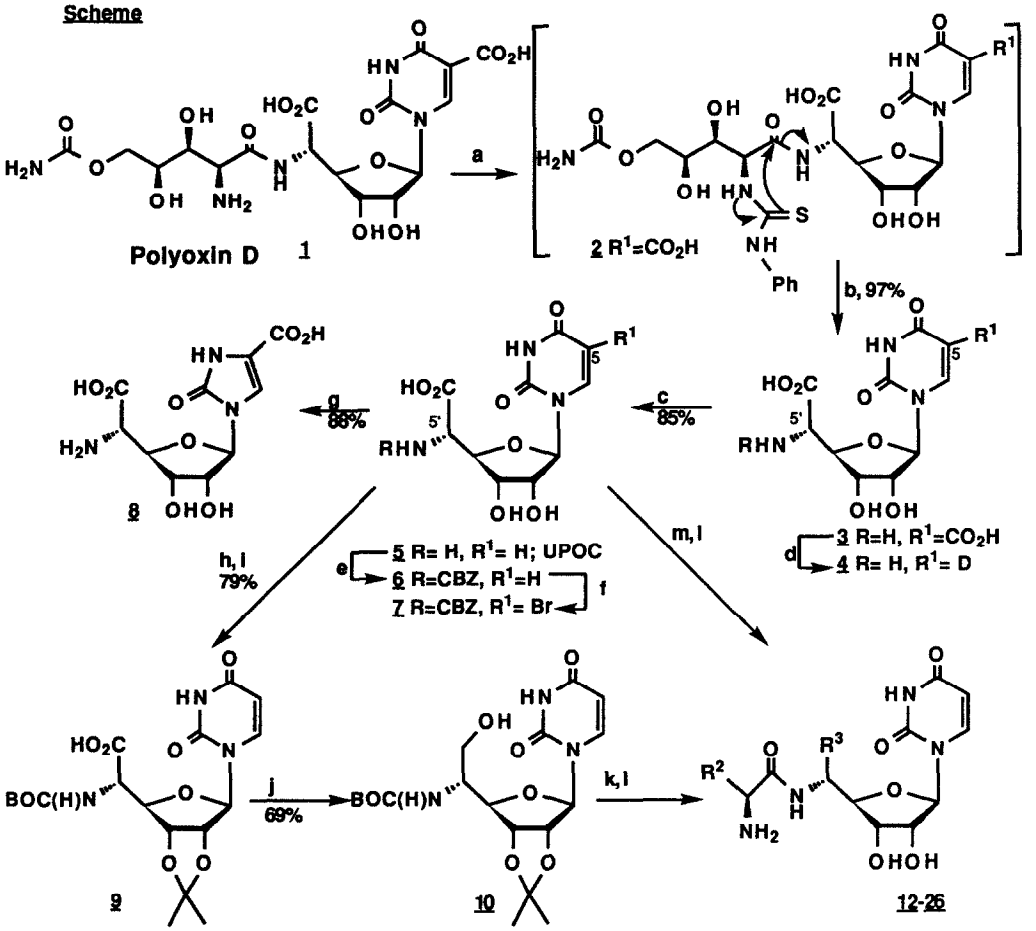
Opportunistic fungal infections can be debilitating and in some cases fatal to the immunocompromised host. Therapy currently available suffers from significant limitations and there still exists a pressing need for more effective and safer antifungal drugs. One main limitation is toxicity, and to minimize this adverse effect a drug should have a mechanism of action that will not interfere with mammalian biochemical processes. One such mechanism of action which recently attracted attention is selective inhibition of the fungal cell wall enzyme chitin synthetase. Two known inhibitors of the chitin synthetase complex are the peptidyl nucleoside antibiotics polyoxin and nikkomycin.[1] These natural products are non toxic to plants or animals and are effective at inhibiting chitin synthetase enzymes from both plant and mammalian pathogenic fungi. They are, however, only weakly active against mammalian pathogenic fungal cells, e.g. *Candida albicans*,[2] presumably due to poor penetration through the cell wall. Nikkomycin X and Z have shown efficacy in murine models.[3]

The polyoxins and nikkomycins enter the fungal cell via a dipeptide transport pathway.[4] Considerable research has been conducted to replace the amino terminal peptide moiety of polyoxins[1] and nikkomycins[5] to produce an analog having the ability to penetrate the cell wall with only partial success.[6] Uracil polyoxin C (UPOC)[1] is the carboxy terminal nucleoside amino acid common to most members of the polyoxin and nikkomycin family of dipeptide compounds, and is a valuable intermediate for the synthesis of analogs. Previous attempts at degradation of polyoxin resulted in low yields of UPOC.[7,8] Low yielding multistep syntheses of UPOC have been employed for the total synthesis of polyoxin analogs.[9]

We sought a convenient procedure which would provide large quantities of UPOC in high yield for rapid analog preparation. The Edman degradation was of interest since it cleaves the amino terminal amino acid from a peptide by acid treatment of its phenylthiocarbamoyl derivative.[10] There were however two potential problems associated with the use of the Edman degradation in the present context. First, it is known that cleavage of serine containing peptides by this method results in very low yields of peptide due to dehydration of the phenylthiocarbamylserine amides in the presence of acid to form the dehydro derivative which can then polymerize;[11] polyoxin D has a β-hydroxyl group which could behave similarly. Second, electron withdrawing groups at the 5-position of uracil can appreciably accelerate the cleavage of the glycosidic bond under acidic conditions used in the Edman degradation; polyoxin D has a carboxyl group at the 5-position of uracil.[12]

We first required an ample source of polyoxin for degradation. Commercial quality polyoxin D zinc salt[13] was purified by sulfonic acid ion exchange resin chromatography, followed by crystallization to provide pure polyoxin D (**1**).[14] Reaction of polyoxin D with phenylisothiocyanate under Edman conditions gave the phenylthiourea **2**, which when treated

Scheme



Reagents/Conditions: a) PhNCS/pyridine, b) TFA c) **5**, $NaHSO_3$, pH=4.5 24 hrs. followed by SO_3H-resin workup, d) $NaDSO_3/D_2O$ followed by NaOH to pH=10.5 followed by SO_3H-resin workup e) benzylchloroformate/NaOH/water, 98%, f) Br_2/H_2O, 60%, g) **5**, Br_2 followed by $NaHCO_3/\Delta$followed by SO_3H-resin workup, h) $(BOC)_2O$/NaOH/water, i)PTSA/dimethoxypropane, j) DCC/HOSu followed by $NaBH_4$, k)TFA followed by N-BOC or CBz blocked aminoacid-succinimide ester/NMM/DMSO followed by RP-18 chromatography, l) TFA/MeOH with BOC protection, H_2/Pd over carbon with CBZ protection, m) **5** , N-BOC or CBz blocked aminoacid-succinimide ester/NMM/DMSO followed by RP-18 chromatography.

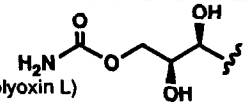
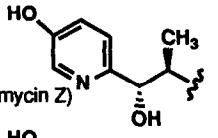
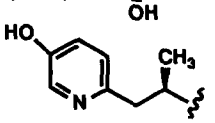
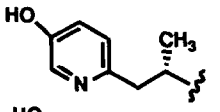
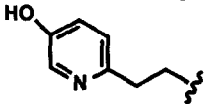
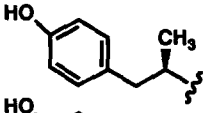
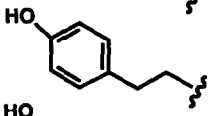
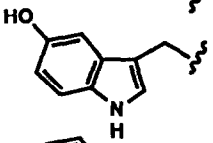
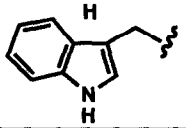
with trifluoroacetic acid (TFA), gave 5-carboxy-UPOC (**3**) in 97 % isolated yield as it's TFA salt (Scheme).[15] Carboxy UPOC was then decarboxylated with sodium bisulfite at pH 4 to give a 85% yield of UPOC (**5**).[16]

We then prepared a number of UPOC derivatives. Synthesis of the 5-deuterium substituted compound **4** was accomplished by treatment of **3** with 4 equivalents of $NaDSO_3$ in D_2O, as described for **5**.[17] A useful intermediate in uracil chemistry is the introduction of a halogen at the C-5 position. The 5-bromo UPOC derivative could not be obtained by direct bromination of **3**, however treatment of the N-CBZ-blocked UPOC derivative **6** with an aqueous solution of bromine followed by evaporation of the excess bromine gave **7** in 60 % yield.[18] These procedures could be used to prepare tritiated polyoxin or nikkomycin analogs. Nikkomycin X has a 2-oxo-imidazoline ring instead of the uracil ring

common to most of the polyoxins and nikkomycins. The 2-oxo-imidazoline-4-carboxylic acid compound **8** was conveniently prepared utilizing a procedure to convert uridine to the corresponding 2-oxo-imidazoline-4-carboxylic acid compound.[19] Further modifications on the 5-deuterium **4**, 5-bromo **7**, and 2-oxo-imidazoline-4-carboxylic acid **8** compounds will be published elsewhere.

Having ample supplies of UPOC, a variety of new polyoxin and nikkomycin derivatives containing different α-amino acyl groups were synthesized. Initially we prepared several nikkomycin type α-amino acids, by varying the substitution on the butyryl chain linking 5-hydroxypyridine, which were then coupled to UPOC (Table 1).[20] The compounds were tested for their ability to inhibit chitin synthetase (*Saccharomyces cerevisiae*) both before[21] and after subjecting the compound to cellular enzymes from a fungal cell homogenate.[22] The difference was a measure of the stability of the inhibitors to intracellular enzymes. Whole cell activity was determined by taking the geometric mean minimum inhibitory concentration (GMMIC) using various strains of *C. albicans*.[23] It is important to note that nikkomycins and polyoxins were only active against approximately 20% of the *Candida* strains, termed "sensitive strains", and the rest were considered "resistant". The Tables 1 and 2 depict activity only against the sensitive strains.

Table 1

Cpd #	R^2	CS(IC_{50})	CHCS(IC_{50})	GMMIC(μg/ml)*
1 (polyoxin L)		18	21	153
4 (UPOC)		>1000	>1000	>2048
11 (nikkomycin Z)		1.2	2.0	6
12		27	40	9.3
13		61	100	51
14		15.5	59	118
15		4.6	6.5	35
16		20	360	128
17		2.5	10	27
18		30	100	2048

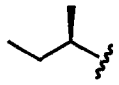
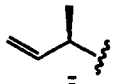
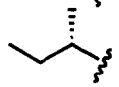
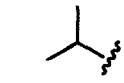
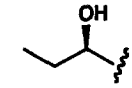
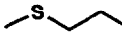
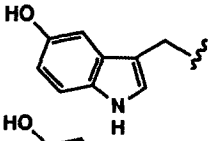
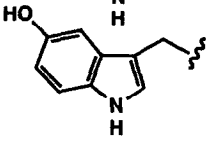
CS= Chitin Synthetase inhibition; CHCS= Chitin synthetase inhibition after treated with cell homogenate.
* GMMIC= Geometric mean minimum inhibitory concentration

Compound **12**, lacking the γ-hydroxyl group of nikkomycin Z, had an average GMMIC comparable to nikkomycin Z but with lower chitin synthetase activity. Epimerization of the β-methyl group as in **13** led to a significant loss in GMMICs and chitin synthetase inhibition. Compound **14** a stripped analog of nikkomycin Z, lacking all substitution on the butyryl side chain. resulted in even lower

activity (higher GMMICs). Interestingly this compound retained significant chitin synthetase activity which was lost when subjected to cell homogenate. Compounds **11** through **13** exhibited reasonable stability to cell homogenate which may be due to a stabilizing effect of the β-methyl group. To further test this hypothesis we prepared the corresponding p-hydroxyphenyl compounds **15** and **16**. Compound **15** the β-methyl compound, compared to compound **16**, exhibited even greater relative stability towards cell homogenate than compounds **12** and **14**. Although compound **15** had a lower CS-IC_{50} relative to compound **12**, the GMMIC of the latter compound was lower (more active). This may be, in part, due to the ability of the pyridine substrates to penetrate the cell wall with greater facility than the phenyl substrates. Compound **16** as expected, lost significant chitin synthetase activity after being subjected to cell homogenate and had a correspondingly higher GMMIC.

These results promted us to look at another series of amino acids with a different heterocyclic ring. Tryptophan was chosen from which compounds **17** and **18** were prepared. The 5-hydroxytryptophan derivative **17** had excellent activity against chitin synthetase and a low GMMIC. Interestingly the tryptophan compound **18**, lacking the 5-hydroxyl group, exhibited no GMMIC, although it was reported to cause morphological changes in *C. albicans*.[22] The 5-fluorotryptophan, 5-methoxytryptophan, azatryptophan, and 5-hydroxythiophene derivatives were prepared and these also had no activity (GMMICs greater than 2048 μg/mL).

Table 2

Cpd #	R^2	R^3	GMMIC(μg/ml)*
19		CO_2H	44
20		CO_2H	406
21		CO_2H	>2048
22		CO_2H	87
23		CO_2H	>2048
24		CO_2H	49
25	H	CO_2H	>2048
26		CH_2OH	>2048
27		H	>2048

* GMMIC= Geometric Minimum inhibitory concentration

Having modified the butyryl side chain of 5'-hydroxynikkomycin E, our attention was turned to eliminating the pyridine or heterocyclic ring by preparing compounds **19** through **25**. This involved coupling of simpler amino acids to UPOC. Interestingly, several of these compounds showed good antifungal activity. The alloisoleucine compound **19** had a GMMIC comparable to compounds **13**, **15**, and **17**. Addition of a double bond to give **20** resulted in a ten fold reduction in activity. Epimerization of the methyl group (isoleucine) **21** resulted in total loss of activity while the valine compound **22** had activity in the range of compound **19**. Replacing the methyl group of compound **19** with a hydroxyl group to give **23** resulted in the total loss of whole cell activity. The methionine compound **24** had GMMICs in the range of **19**, and the glycine compound **25** was inactive. Synthesis of the previously unreported α-amino acids used in compounds **12** through **27** will be published in subsequent papers.

In an effort to determine if the 5'-carboxyl group was necessary for activity the 5'-hydroxymethyl derivative of compound **17** was prepared. As shown in the scheme, it was necessary to block the amino and vicinal diol groups of **5**

with the BOC and isopropylidene groups respectively to obtain **9**. The carboxyl group was then converted to the succinimide ester, followed by $NaBH_4$ reduction to obtain **10** in 69 % yield. The 5-hydroxy tryptophan derivative **26** was prepared and it was found that this 5'-hydroxymethyl analog was inactive, thus demonstrating the requirement of a 5'-carboxyl group. Furthermore the decarboxylated compound **27**, prepared from 5'-amino-5'-deoxy-uridine, was also inactive .[24]

A more detailed account of this work along with other polyoxin/nikkomycin derivatives will be reported in future publications.

Acknowledgement: We would like to thank Mr. Allan Morenberg of Schering-Plough Process Developement for purifying the large quantities of polyoxin D required for our work, and Mr. Marvin Cohen and Mr. Robert Runkle for their help in scaling up the UPOC preparation.

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13 Substantial quantities of commercial quality polyoxin D (10-14% polyoxin D) could be obtained as the zinc salt. This product is used in Japan against rice blast disease and sold by Kaken Pharmaceutical Co. LTD. Tokyo, Japan.

14 11.43 gm of 10-14% pure polyoxin D was dissolved in 60 ml of water and added to a 381 ml column of XFS-43279.00 cation exchange resin (Dow Corporation). The column was washed with 1L of water and the compound eluted with 1.5N ammonium hydroxide. The product containing eluent was collected and the water evaporated to obtain 3.93 gm of solid. The solid was dissolve in 13 ml of water and the pH adjusted to 2.5 with TFA. This solution was then dripped into 130 ml of 2-propanol and the solids filtered to give 2.57 grams of semi-purified polyoxin D. The solid was dissolved in water to give a 30% solution and let sit at 5 °C whereupon we obtained 1.06 gm of pure crystalline polyoxin D.

15 a) Polyoxin D trifluoroacetic acid salt (23 gm, 36 mmol) was dissolved in 400 ml of a 50% mixture of pyridine:water (v/v). The pH was adjusted to 8.5 with 4N NaOH and phenylisothiocyanate (6.4 ml, 53 mmol) was added and the reaction mixture stirred at room temperature while maintaining the pH at 8.5 with NaOH. After 3 hours, 3 ml more of phenylisothiocyanate was added. After a total of 20 hours, water and pyridine wereevaporated at 55 °C under vacuum to obtain an amber solid. The solid was dissolved in 400 ml of water and washed twice with equal volumes of ethylacetate. The aqueous layer was filtered and evaporated to 70 ml and added to 600 ml of stirring methanol. 300 ml of diethyl ether was added and the solids filtered to obtain 32.3 gm of crude 2"-n-phenylthiocarbonyl-polyoxin D as the sodium salt after drying in a vacuum oven at 40 °C over P_2O_5 for 18 hours. This was used without purification for the next reaction. ^1H NMR of the compound as the free acid (200MHz, D_2O) δ_H: 8.27 (1H,s), 7.24-7.46 (5H, m), 5.86 (1H, d, J=6 Hz), 5.27 (1H, d, J= 6Hz), 4.80 (1H, d,J=4 Hz), 4.47 (1H, t, J=4 Hz), 4.36 (1H, t, J= 6Hz), 4.13-4.23 (4H, m), 3.89 (1H, m); FABMS m/e (relative intensity) 657 (M^+ +1, 20), 327 (19), 326 (100), 307 (14). b) 2"-n-phenylthiocarbonyl-polyoxin D (32 gm) was dissolved in trifluoroacetic acid (200 ml)and stirred under a nitrogen atmosphere at 20 °C for 25 minutes. The reaction mixture was dripped into vigorously stirring diethyl ether (1300ml). The resulting solids were filtered and washed with diethyl ether, and dried in a vacuum oven at 35 °C for 18 hours. The solids were dissolved in 800 ml of water and filtered. The aqueous layer was washed with 500 ml of ethylacetate three times. The aqueous layer was then added to a column of1180 ml of XFS-43279.00 sulfonic acid resin and the resin washed with 2L of dist. water. The product was eluted with 1.5 % ammonium hydroxide. The product containing fractions from the column were combined and evaporated to 100 ml whereupon some of the product crystallizes out of solution. The crystals were filtered and the filtrate added to 500 ml of stirring methanol to precipitate the rest of the product to obtain a total of 11.85 gm, 35 mmol of 5-carboxy UPOC. The overall yield of 5-carboxy UPOC was 97 % of theory.

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17 The decarboxylation of carboxy-UPOC gave UPOC with 87% deuterium incorporation at the C-5 position of uracil as evidenced by the disappearance of the H-5 signal at δ5.93 in the PMR. See ref. 16

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19 Addition of excess bromine to an aqueous solution of UPOC followed by immediate evaporation of the excess bromine and refluxing the solution with 10 equivalents of $NaHCO_3$ for 15 hours gave, after sulfonic acid cation exchange resin workup and chromatogrphy on RP-18 silica gel, an 88% yield of **8**. See Otter, B. A.; Falco, E. A.; Fox, J. J. *Tetrahedron Lett.* **1968**, 2967-2970

20 A typical coupling reaction involved the addition of 1.5 mmol of the N-BOC or N-CBZ derivative of the L-α-amino acid hydroxysuccinimide ester to a mixture of 1 mmol of UPOC in 40 ml DMSO and 0.8 ml NMM and stirring the reaction mixture under dry nitrogen for 72 hrs at ambient temperature. Yields of the coupled products ranged from 30 to 50 % after RP-HPLC of the crude reaction mixtures. In cases where D,L-amino acids were used, the final coupled diastereomeric mixtures were separated by RP-HPLC.

21 The *Candida albicans* chitin synthetase assay was performed following : Cabib, E. *Antimicrob. Agents and Chemother.* **1991**, 35, 170-173 and references cited therein.

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23 Microtiter MICs were performed in yeast nitrogen base (Difco) pH 5.4 with 0.2% glutamic acid added. The compounds were screened against a total of 10 "nikkomycin sensitive" yeast strains, each adjusted to give a final inoculum of 3×10^3 cells/mL. Plates were incubated at 37 °C and read after 48 hours incubation. The lowest concentration of compound to inhibit the organism growth was read as the MIC. The geometric mean MIC (GMMIC) was calculated using the 10 "sensitive" strains. Seven of the strains were clinical isolates of *C. albicans* and 3 were obtained from ATCC; *C. albicans* ATCC 38245, ATCC 32247, and *C. stellatoidea* ATCC 11006.

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