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The First Synthesis of a Member of the Iturin Family, the Antifungal Cyclic Lipopeptide, Iturin-A2

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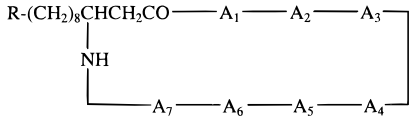
The iturins are a group of fungicidal, cyclic lipopeptides produced by *Bacillus subtilis*.[1−8] The group contains iturins A−E, bacillomycins D, F, and L, and mycosubtilin (Table 1). All members are cyclic octapeptides with seven α-amino acids and one unique *β*-amino acid. In all cases the α-amino acids are arranged in an LDDLLDL configurational sequence. The amino acids D-Tyr[2] and D-Asn[3] are constant, while substitutions for the other amino acids are frequent in nature. Iturin-A, as are all members of the group, is naturally produced as a mixture of up to eight isomers (Table 1). These isomers arise from a mixture of methylene homologs of iturinic acid (Itu), the unusual *β*-amino acid component. Iturinic acid has a side chain of 10−14 carbons (C_{13}−C_{17} total) in the *n*-, *iso*-, or *anteiso*-configuration, giving rise to iturin-A1−A8 (numbered as to C18 HPLC elution pattern).[9] The iturinic acid in the iturins has been determined to have the *R*-configuration at the *β*-carbon.[10] The predominant iturin-A isomer, A2, contains the *n*-C_{14} isomer of iturinic acid.

Previous studies from this laboratory have shown that the side chain of iturinic acid is critically associated with iturin's antifungal activity. When iturinic acid was replaced with *β*-alanine all activity was lost.[11] Several analogs of ituirn-A had the iturinic acid residue replaced by *β*-aspartic acid, functionalized at the α-carboxylate as an ester or amide with various chain lengths.[12] All were found to be inactive against fungal growth. From the natural isomers it was found that activity is proportional to side chain length and is related to the type of branching (*iso* > *normal* ≫ *anteiso*).[13] The synthesis of a member of the iturin family had been hampered by the inability to synthesize the *β*-amino acid component, iturinic acid. Therefore, a new procedure was developed to produce the enantiomerically pure *n*-C_{14} isomer of iturinic acid by lithium diorganocuprate addition to the tosylate derivative of the α-carboxyl-reduced analog of aspartic acid.[14]

The iturins make ideal candidates for biorational fungicides or models for the synthesis of such fungicides. Clinical trials on man and animals have shown iturin to be a valuable drug for its large antifungal spectrum, its low toxicity, and low allergic effect.[15] The synthesis of an iturin was needed both to verify the structure of reported compounds isolated from natural sources and as a route to the synthesis of iturin analogs used for structure−activity relationship studies. We report here the first synthesis of an iturin, iturin-A2.

Table 1. Amino Acid Composition of the Iturin Family and Compositional Differences of the *β*-Amino Acid



Antibiotic	A_1	A_2	A_3	A_4	A_5	A_6	A_7
Iturin A	Asn	D-Tyr	D-Asn	Gln	Pro	D-Asn	Ser
Iturin C	Asp	D-Tyr	D-Asn	Gln	Pro	D-Asn	Ser
Bacillomycin L	Asp	D-Tyr	D-Asn	Ser	Gln	D-Ser	Thr
Bacillomycin F	Asn	D-Tyr	D-Asn	Gln	Pro	D-Asn	Thr
Bacillomycin D	Asn	D-Tyr	D-Asn	Pro	Glu	D-Ser	Thr
Mycosubtilin	Asn	D-Tyr	D-Asn	Gln	Pro	D-Ser	Asn

#	R	type
1	CH_3CH_2-	n-C_{13}
2	$CH_3CH_2CH_2$-	n-C_{14}
3	$CH_3CH_2CH(CH_3)$-	anteiso-C_{15}
4	$(CH_3)_2CHCH_2$-	iso-C_{15}
5	$CH_3CH_2CH_2CH_2$-	n-C_{15}
6	$(CH_3)_2CHCH_2CH_2$-	iso-C_{16}
7	$CH_3CH_2CH_2CH_2CH_2$-	n-C_{16}
8	$CH_3CH_2CH(CH_3)CH_2CH_2$-	anteiso-C_{17}

Results and Discussion

The synthesis of the linear octapeptide precursor of iturin-A2, Itu-Asn-D-Tyr-D-Asn-Gln-Pro-D-Asn-Ser, **1**, was accomplished by solid phase methods using Fmoc chemistry. Coupling was performed by in-line preformation of the hydroxybenzotriazole (HOBt) ester with diisopro-

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pylcarbodiimide (DIPCDI). The N-terminal iturinic acid was coupled as the Boc-protected amino acid.[14] On cleavage of the peptide from the resin with TFA, the Boc-group and all side chain protection was removed, giving **1** in 74% yield after purification by HPLC. Characterization was determined by ^{1}H and ^{13}C NMR and HPLC/electrospray mass spectrometry (ESMS). The crude linear octapeptide (76% purity) was cyclized with diphenylphosphoryl azide (DPPA) to give cyclo(Itu$_{nC14}$-Asn-D-Tyr-D-Asn-Gln-Pro-D-Asn-Ser-), iturin-A2, **2**. Crude cyclic product was obtained in 91% yield. Analysis by HPLC showed product to be 73% of the total. Since the starting material was only 76% pure, this calculates to an amazing 87% yield for the cyclization reaction. Most cyclization reactions are performed on the protected peptide; however, the natural folding inherent in this possible native precursor peptide may have enhanced the yield of cyclization. Purification by HPLC gave the product in 46% yield. The major impurity was characterized as iturin-A2 with a Tmob-protecting group still attached. Unfortunately, the Tmob could not be removed from this impurity by treatment with TFA. The results indicate that proper scavengers were not used for the deprotection of the linear peptide, resulting in reattachment of the cleaved Tmob-group. Because the cyclization was performed on crude linear peptide, the side product was also cyclized. The pure product was characterized by comigration with authentic iturin-A2 on RP-HPLC and by ESMS.

The lipopeptide's action on *Penicillium expansum* was tested, showing the synthetic analog to be comparable to natural iturin-A2, isolated from *B. subtilis*,[13] in inhibiting fungal growth. Iturin-A2, isolated from natural sources by HPLC, was found to have a minimum inhibitory concentration (MIC) of 12 μg/disk, while the synthetic compound was measured to have an MIC of 8 μg/disk. The Tmob-iturin impurity was inactive.

In conclusion, the first total synthesis of a member of the iturin family, iturin-A2, has been accomplished. Its execution was based on the development of a new synthesis of enantiomerically pure iturinic acid. Accounting for nonoptimized procedures, yields were remarkably high. Biological results show the activity of the synthetic compound to match the natural compound. On the basis of these results, further research is in progress for the full determination of the antifungal, antibacterial, and other biological properties of the iturin family.

Experimental Section

Fmoc-protected amino acids and solid support were purchased from Millipore (currently Perseptive Biosystems).

TFA·Itu-Asn-D-Tyr-D-Asn-Gln-Pro-D-Asn-Ser (1). To 0.82 g (0.39 mmol/g; 0.32 mmol) of Fmoc-Ser(tBu)-PAC resin (Millipore) was coupled the necessary Fmoc-amino acid; L-Asn and L-Gln were protected with Tmob; D-Tyr was protected with tBu; D-Asn was unprotected; Itu was coupled as the Boc-Itu derivative. Amino acids were activated in-line with DIPCDI + HOBt. Coupling times were 1 h except for Gln^5 and Asn^2, which were coupled for 2 h; Boc-Itu was coupled for 4 h. Amino acid concentrations were 0.4 M with 6.67 equiv added. The Fmoc group was deblocked with 30% piperidine in DMF/toluene (1:1) for 10 min. The deblocking of the dipeptide Fmoc-D-Asn-Ser-(tBu)-PAC was modified to be a 50% piperidine solution for 5 min to prevent DKP formation. The fully protected octapeptide resin was washed with methylene chloride and methanol and dried *in vacuo* over KOH. The dried resin was mixed with 25 mL of TFA/H_2O (95:5) and mixed by nitrogen bubbling for 3.5 h. The resin was removed by filtration (rinsed with 95% TFA twice) and the filtrate was concentrated *in vacuo* to a dark red oil. Ether (35 mL) was added to the oil, which solidified, with scraping, to a yellow solid. The solid was filtered (washed with ether twice) and dried *in vacuo* over KOH to give 0.362 g (96%) of the linear peptide, TFA salt, as a yellow solid. TLC of the solid [BAW(4:1:5)] showed product at R_f 0.28 with two minor impurities. HPLC [POROS II R/M, C18, 4.6 × 100 mm column; 10 mM NH_4OAc/acetonitrile, gradient of 20 to 100% B over 5 min, 4 mL/min; detection at 215 and 278 nm] showed the product to be 76% pure. A UV scan of product peak at 2.0 min had 99.9% correlation with standard iturin. On-line ESMS established the identity of the product peak with MH$^+$ of 1061. With increased repeller voltage the peptide was fragmented between Gln^5 and Pro^6, giving MH$^+$ of 317 and 745. A 50 mg sample of product was purified by displacement HPLC [same column as above; 20 min at 0% B, 5 min gradient to 20% B, 10 min at 20% B, 5 min gradient to 60% B, 10 min at 60% B; 4 mL/min; 2 mL injection]. Peaks eluted at 2, 27, 35, and 40 min. Pure product was obtained from the first and third peaks, as determined by HPLC and ESMS. The first peak at the solvent front must have been due to overloading. From the first and third peaks there were obtained 19 and 18 mg (74% total) of white solid, respectively. ^{1}H and ^{13}C NMR of this linear peptide closely matched that of the natural cyclic peptide, iturin-A.

cyclo(Itu-Asn-D-Tyr-D-Asn-Gln-Pro-D-Asn-Ser-), Iturin-A2 (2). To a solution of **1** (245 mg, 209 μmol) in 150 mL of DMF, under a nitrogen atmosphere and cooled to 0 °C, were added, dropwise, diphenyl phosphorazidate (DPPA) (49 μL, 229 μmol) and $NaHCO_3$ (87 mg, 1.04 mmol). The reaction was monitored by HPLC showing a linear decrease in **1** (to zero on day 8) and a concurrent increase in product with no other peaks observed except that of DPPA. The retention time of natural iturin-A2 was used to identify product peak. After 8 days at 0 °C, H_2O (40 mL) and 5 g of Bio-Rad AG501-X8(D) mixed bed resin were added to the reaction mixture. After the mixture was stirred for 3 h, the resin was removed by filtration and washed with DMF/H_2O (4:1) (2 × 20 mL). The combined filtrate was concentrated *in vacuo* to give 197 mg (91%) of beige solid. HPLC analysis [Alltech, C18-DVB, 4.6 × 250 mm column; 10 mM NH_4-OAc/MeOH, 65−95% B gradient over 30 min, 1.0 mL/min; detection at 215 and 278 nm] showed the product peak, eluting at 14 min, to be 73% of the total (peak area at 215 nm), with the major impurity, eluting at 25 min, accounting for 15%. ESMS of the impurity gave a mass indicative of Tmob-protected iturin-A2. Treatment of the HPLC-isolated peak with 95% TFA for 18 h did not remove the Tmob-group. HPLC purification of 5 mg [Alltech, C18-DVB, 4.6 × 250 mm column; 10 mM NH_4-OAc/MeOH gradient of 40 (15 min)−40 (25 min)−65 (25 min)−65 (25 min)−90% B, 1.0 mL/min; detection at 215 and 278 nm] gave product, with an elution time of 55.0−66.7 min, in 50% yield (2.5 mg, 46% yield overall); ESMS MH$^+$ = 1043. The Tmob-impurity eluted at 89.0−92.9 min and was isolated in 5% yield (0.25 mg); ESMS MH$^+$ = 1222.

Bioassay. Petri plates with PDA (20 mL) were spread with *P. expansum SRRC 1134* (100 μL) culture broth. Sterilized filter paper disks (Whatman #4), cut to 6 mm diameter, were impregnated with 5 μL of methanolic solution of **2**, at concentrations to give 32, 24, 16, 8, and 4 μg/disk. The disks were air dried for 20 min and applied to the PDA plates. After incubating 3 days at 25 °C, plates were visualized and the inhibition zone was measured. For concentrations of 32, 24, 16, 8, and 4 μg/disk, inhibition zone diameters were 12, 10, 8, 6, and 0 mm, respectively. An MIC value of 8 μg/disk corresponds to the lowest concentration at which the paper disks were not overgrown.

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