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RACEMIZATION AT PROLINE RESIDUES DURING PEPTIDE BOND FORMATION

A STUDY OF DIASTEREOMERIC MIXTURES OF SYNTHETIC ALAMETHICIN FRAGMENTS BY 270 MHz ^{1}H NMR

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Abstract—The stepwise synthesis of amino terminal pentapeptide of alamethicin, Z-Aib-Pro-Aib-Ala-Aib-OMe, by the dicyclohexylcarbodiimide mediated couplings leads to extensive racemization at the Ala and Pro residues. Racemization is largely suppressed by the use of additives like N-hydroxysuccinimide and 1-hydroxybenzotriazole. The presence of diastereomeric peptides may be detected by the observation of additional methyl ester and benzylic methylene signals in the 270 MHz ^{1}H NMR spectra. Unambiguous spectral assignment of the signals to the diastereomers has been carried out by the synthesis and NMR studies of the D-Ala tetra and pentapeptides. The racemization at Pro is of particular relevance in view of the reported lack of inversion at C-terminal Pro on carboxyl activation.

Racemization of amino acids on carboxyl activation constitutes a significant hurdle in the synthesis of configurationally pure peptides.[1,2] The dicyclohexylcarbodiimide (DCC)† condensation leads to appreciable racemization. This may be suppressed by the use of additives like N-hydroxysuccinimide[3] or 1-hydroxybenzotriazole.[4] The loss of optical purity is likely to be marked in peptide bond forming reactions involving a sterically hindered amino component. This problem is particularly pertinent in the synthesis of microbial peptides containing the hindered amino acid, α-aminoisobutyric acid (Aib).[5,6] During the course of studies directed towards the synthesis of alamethicin I, the pentapeptide Z-Aib-Pro-Aib-Ala-Aib-OMe (Fig. 1) was prepared by stepwise elongation from the N-terminal using DCC mediated couplings. The ^{1}H NMR spectrum of this peptide clearly showed the presence of additional resonances attributable to racemization of Ala (4). However, even under conditions where racemization is expected to be suppressed significant amounts of diastereomeric peptides were detected. Further study revealed the possibility of racemization at Pro (2). In this report we provide evidence for racemization at Pro in the Aib-Pro sequence and also emphasise the utility of high field ^{1}H NMR in the analysis of diastereomeric mixtures of these peptides.

RESULTS AND DISCUSSION

The 100 MHz ^{1}H NMR spectrum of an analytically pure sample of Z-Aib-Pro-Aib-Ala-Aib-OMe (2) prepared by the DCC method showed the presence of two ester methyl resonances of unequal intensity at 3.64δ and 3.61δ. The two signals could be clearly resolved in a 4:1 $CDCl_3$–$(CD_3)_2SO$ mixture. The benzylic CH_2 group appeared as an AB quartet centered at 5.15δ and a singlet at 5.11δ. The observation of additional resonances strongly suggests the presence of diastereomeric peptides. The possible centres at which racemization could have occurred are at Pro (2) and Ala (4). However, Pro residues are not expected to readily undergo racemization as reported in the literature. Hence, it is likely that racemization has occurred at Ala (4). Further evidence for this assumption is obtained from the 100 MHz ^{1}H NMR spectrum of the pentapeptide 3 where one equivalent of a racemization suppressing agent like HOSU was added. In this peptide the intensities of the additional resonances in the ester and the benzylic–CH_2 region diminish considerably. In order to assign the extra lines to the appropriate diastereomer, the peptide Z-Aib-Pro-Aib-D-Ala-Aib-OMe 5 was synthesised from D-Ala (4) tetrapeptide acid 4 and Aib-OMe with the addition of one equivalent of HOBT and DCC.

Figures 2 and 3 compare the methyl ester and benzylic–CH_2 regions of the 270 MHz ^{1}H NMR spectra of the pentapeptides 2, 3 and 5. From Fig. 2, the high field methyl ester resonance at 3.61δ may be assigned to the D-Ala (4) diastereomer, while the low field resonance at 3.64δ corresponds to the L-Ala (4) isomer. The benzylic–CH_2 appears as an AB quartet in the L-Ala (4) pentapeptides whereas in D-Ala (4) pentapeptide the corresponding protons appear as a singlet at 5.11δ. The detection of diasteroemeric peptides would suggest that even in the presence of the additives HOBT and HOSU, considerable inversion of configuration occurs. This could possibly be due to the low reactivity of the hindered nucleophile, Aib-OMe. Alternately the possibility that racemization has occurred at Pro (2) needs to be considered. Examination of the 100 MHz ^{1}H NMR spectrum of Z-Aib-Pro-Aib-Ala-OMe in $CDCl_3$ or $(CD_3)_2SO$ or mixtures of these solvents did not show any evidence for the existence of diastereomeric peptides. However, it was considered desirable to also examine the spectra of the tetrapeptide acids.

The benzylic–CH_2 region of the tetrapeptide acids 1

†Abbreviations used: DCC, N,N-dicyclohexylcarbodiimide; Z, benzyloxycarbonyl; Aib, α-aminoisobutyric acid; DCU, dicyclohexylurea; HOSU, N-hydroxysuccinimide; HOBT, 1-hydroxybenzotriazole.

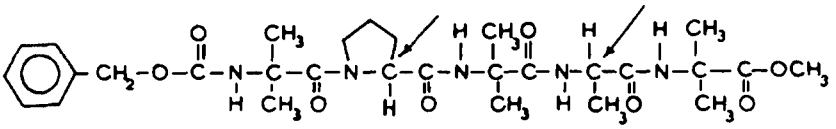

Fig. 1. Z-Aib-Pro-Aib-Ala-Aib-OMe. Arrows indicate sites of configurational inversion.

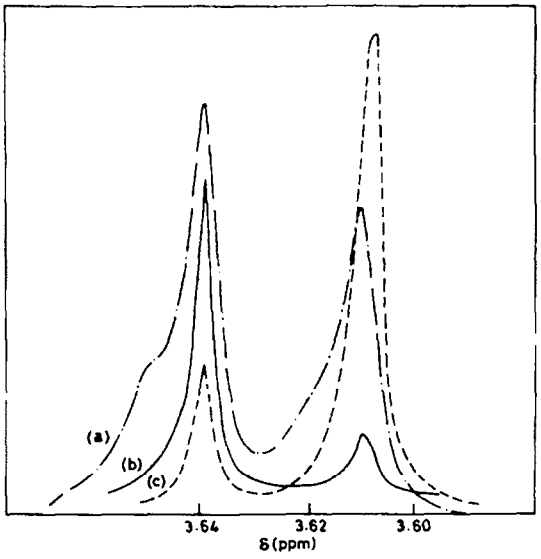

Fig. 2. Ester methyl resonances of the pentapeptides (a) Z-Aib-Pro-Aib-Ala-Aib-OMe 2 (b) Z-Aib-Pro-Aib-Ala-Aib-OMe 3 (c) Z-Aib-Pro-Aib-D-Ala-Aib-OMe 5: in $CDCl_3/(CD_3)_2SO$ (4:1) at 270 MHz.

and 4 is shown in Fig. 4. The presence of two sets of quartets of unequal intensities centred at 5.09δ and 5.08δ clearly indicate the presence of diastereomers due to racemization at Pro (2) in these peptides. Here, the stepwise procedure employed allows only the inversion at optically active Pro (2) on carboxyl activation. The possibility of Ala (4) inversion on base hydrolysis of the ester was discounted by examination of the NMR spectrum of the tetrapeptide acid obtained by acid hydrolysis of the ester. A spectrum identical to that of 4 was obtained, suggesting that racemization must have taken place only at Pro (2) during the formation of Z-Aib-Pro-Aib-OMe. Further evidence for this is obtained from an examination of the benzylic-CH_2 region in the tetrapeptide acid 6, where one equivalent of HOSU was added in the coupling of Z-Aib-Pro-OH to Aib-OMe (Fig. 4c). Only one quartet centred at 5.08δ is observed for this peptide. Hence the quartet of lower intensities centred at 5.09δ in the tetrapeptides 1 and 4 can be assigned to the D-Pro (2)-L-Ala (4) and D-Pro (2)-D-Ala (4) isomers respectively.

The percentage of diastereomers in the tetrapeptide acids 1 and 4 and the pentapeptide esters prepared under different conditions are summarized in Table 1. Comparing the values obtained for the tetrapeptides 1 and 4 with the corresponding pentapeptides 2 and 5 and also 2, where no additive was used, it is evident that HOSU and HOBT, indeed suppress racemization effectively during Ala (4) activation. It may be noted that in the case of the pentapeptides NMR cannot distinguish the L-Pro (2)-L-Ala (4) and D-Pro (2)-D-Ala (4) isomers. Similarly the D-Pro (2)-L-Ala (4) and L-Pro (2)-D-Ala (4) isomers are also indistinguishable.

In peptide synthesis racemization of carboxyl terminal Pro has not merited serious attention. This may be due to the fact that base catalyzed oxazolone formation is not

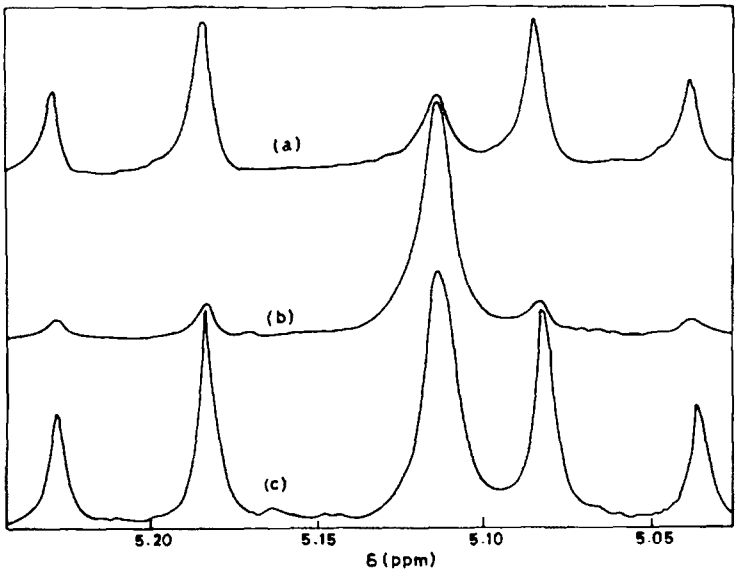

Fig. 3. Benzylic-CH_2 resonances of the pentapetides (a) Z-Aib-Pro-Aib-Ala-Aib-OMe 3 (b) Z-Aib-Pro-Aib-D-Ala-Aib-OMe 5 (c) Z-Aib-Pro-Aib-Ala-Aib-OMe 2: in $CDCl_3/(CD_3)_2SO$ (4:1) at 270 MHz.

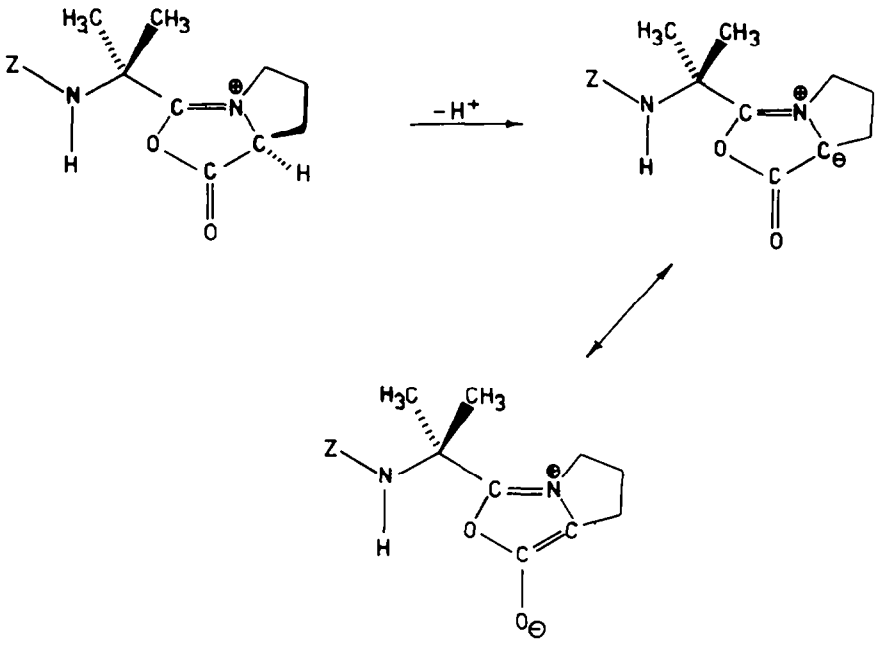

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Table 1. Yield of diastereomeric peptides under different conditions

Peptide	Procedure	Isomer	%
Z-Aib-Pro-Aib-Ala-Aib-OMe[a] 2	DCC	L-Pro(2)-L-Ala(4) + D-Pro(2)-D-Ala(4)	57
		L-Pro(2)-D-Ala(4) + D-Pro(2)-L-Ala(4)	43
Z-Aib-Pro-Aib-Ala-Aib-OMe[a] 3	DCC + HOSU	L-Pro(2)-L-Ala(4) + D-Pro(2)-D-Ala(4)	84
		L-Pro(2)-D-Ala(4) + D-Pro(2)-L-Ala(4)	16
Z-Aib-Pro-Aib-D-Ala-Aib-OMe[a] 5	DCC + HOSU	L-Pro(2)-D-Ala(4) + D-Pro(2)-L-Ala(4)	78
		L-Pro(2)-L-Ala(4) + L-Pro(2)-D-Ala(4)	22
Z-Aib-Pro-Aib-Ala-OH 1[b]	Saponification of ester	L-Pro(2)-L-Ala(4)	79
		D-Pro(2)-L-Ala(4)	21
Z-Aib-Pro-Aib-D-Ala-OH 4[b]	Saponification of ester	L-Pro(2)-D-Ala(4)	82
		D-Pro(2)-D-Ala(4)	18
Z-Aib-Pro-Aib-D-Ala-OH[b]	Acid hydrolysis of ester	L-Pro(2)-D-Ala(4)	82
		D-Pro(2)-D-Ala(4)	18
Z-Aib-Pro-Aib-Ala-OH[b]	HOSU in Pro(2)-Aib(3) coupling	L-Pro(2)-L-Ala(4)	100
		D-Pro(2)-L-Ala(4)	-

a. Isomer amounts evaluated from ester CH_3 resonances.

b. Isomer amounts evaluated from benzylic $-CH_2-$ resonances.

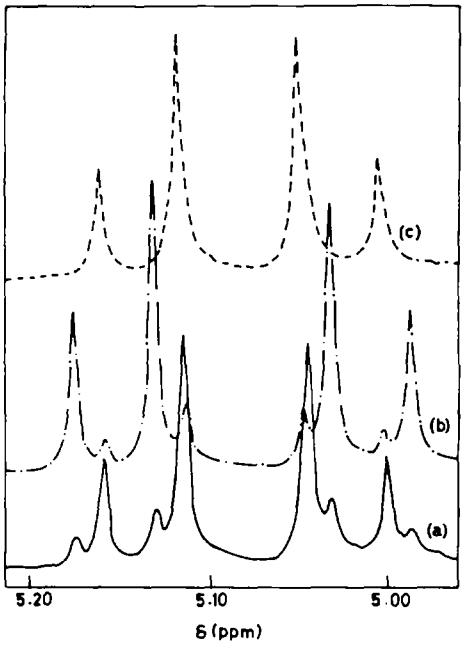

Fig. 4. Benzylic-CH_2 resonances of the tetrapeptide acids (a) Z-Aib-Pro-Aib-Ala-OH 1 (b) Z-Aib-Pro-Aib-D-Ala-OH 4 (c) Z-Aib-Pro-Aib-Ala-OH 6: in $CDCl_3/(CD_3)_2SO$, (4:1) at 270 MHz.

possible due to the lack of an enolisable NH proton.[1] The present study clearly indicates that extensive racemization can occur at C-terminal Pro in X-Pro sequences, where X is a sterically bulky group. In the Aib-Pro sequence the Me groups of the Aib residue may play a part in stabilizing the charged oxazolone 7 and hence favour cyclization. Proton abstraction from a positively charged intermediate should be facile, leading to inversion of configuration at Pro. Such racemization has also been detected in the case of couplings involving pivaloyl-L-Pro (Venkatachalapathi and Balaram, unpublished results). Evidence for the intermediacy of a 5 (4 H)-oxazolone in the activation of C-terminal N-methylamino acids has been provided by trapping reactions using a 1,3-dipolarophile.[8]

270 MHz ^{1}H NMR has proved successful in the analysis of the diastereomeric mixtures described above, since each component yields distinct NMR resonances for one or two groups of protons. This is presumably a consequence of the limited range of conformations accessible to Aib containing peptides.[9,10] It appears that in cases where dynamic averaging is limited, NMR analysis may prove routinely useful in detection of racemization.[11,12]

EXPERIMENTAL

Amino acid methyl esters were obtained from the corresponding hydrochlorides, by dissolving in sat $NaHCO_3$ and extracting with CH_2Cl_2 followed by evaporation of the solvent. All compounds reported were chromatographically homogenous by tlc on silica gel (10% MeOH/$CHCl_3$). 100 MHz ^{1}H NMR spectra were obtained on Varian HA100 instrument. 270 MHz ^{1}H NMR were recorded on a Bruker WH-270 instrument at the Bangalore NMR Facility.

Z-Aib-Pro-Aib-Ala-OH (1). Z-Aib-Pro-Aib-Ala-OMe[9] (1.6 g, 3.2 mmol) was dissolved in MeOH (5 ml) and 2 N NaOH (4 ml) added to it. After 12 hr, 15 ml of water was added and the aqueous phase was extracted with EtOAc (10 ml), to remove unsaponified tripeptide ester. The aqueous was acidified with 2 N HCl and extracted with EtOAc (3 × 25 ml). The organic layer was dried over Na_2SO_4 and evaporated to give the tetrapeptide acid, yield 1.3 g, 80%, m.p. 190°.

*Preparation of Z-Aib-Pro-Aib-Ala-Aib-*OMe *from* 1 *and Aib*-OMe.

(a) *DCC* (2). Z-Aib-Pro-Aib-Ala-OH (1.2 g, 2.5 mmol) was dissolved in 15 ml CH_2Cl_2 and cooled to 0°. Aib-OMe (0.287 g, 2.5 mmol) in CH_2Cl_2 (3 ml) was added followed by DCC (0.515 g, 2.5 mmol). The mixture was then stirred at room temp for 24 hr. The dicyclohexylurea (DCU) was then filtered off and the organic layer washed with 1 N HCl, H_2O and 1 M $NaHCO_3$ and dried over Na_2SO_4. Evaporation of CH_2Cl_2 followed by trituration with petroleum ether (40–60°) yielded the peptide as a solid, yield 1.3 g 89%, m.p. = 129–132°. Recrystallized from MeOH/ether. $[\alpha]_D^{25}$ = 24° (c = 0.5, MeOH). (Found: C, 58.61; H, 6.87; N, 12.09. Calc. for $C_{29}H_{43}O_8N_5$: C, 59.08; H, 7.3; N, 11.88%.) NMR (270 MHz) showed the presence of additional peaks ~7–8δ, ~5δ and 3.6δ, corresponding to 4 protons.

(b) *DCC + N-hydroxysuccinimide*(3). Z-Aib-Pro-Aib-Ala-OH (0.300 g, 0.7 mmol) was dissolved in CH_2Cl_2 (10 ml) and cooled to 0°. Aib-OMe (0.100 g, 0.87 mmol), N-hydroxysuccinimide (0.070 g, 0.7 mmol), DCC (0.135 g, 0.7 mmol) were added successively and the mixture stirred at room temp for 24 hr. The work up was done as in procedure (a), yield 0.310 g 86%, m.p. = 175–176°, $[\alpha]_D^{25}$ = 42° (c = 0.5 in MeOH) NMR (270 MHz, 4:1 $CDCl_3/(CD_3)_2SO$). 7.772δ, s, 1 H (AibNH), 7.55δ, s + d, 2 H (Aib + AlaNH), 7.38δ, b, 6 H (AibNH + benzylic ring protons); 5.15δ, q, 2 H (benzylic CH_2), 4.25δ, m, 1 H, 4.3δ, m, 1 H (Pro + Ala, C^αH); 3.8δ, m, 2 H, (Pro $C^\delta H_2$), 3.64δ, s, 3 H (OCH_3), 1.75δ, m, 4 H (Pro $C^\beta H_2 + C^\gamma H_2$), 1.33–1.50$\delta$, m, 21 H (Aib + Ala $C^\beta H_3$). Peaks of diminished intensity were observed around 7.7, 5.5 and 3.6δ corresponding to 5 protons.

Z-Aib-Pro-Aib-D-Ala-OH (4). Z-Aib-Pro-Aib-OH (1.0 g, 2.4 mmol) was suspended in CH_2Cl_2 (10 ml) and cooled to 0°. D-Ala-OMe (0.300 g, 2.4 mmol) in CH_2Cl_2 (3 ml) was added and a clear soln was obtained. DCC (0.500 g, 2.4 mmol) in CH_2Cl_2 (5 ml) was added and the mixture stirred at room temp for 24 hr. The DCU was then filtered off and the organic layer washed with 1 N HCl, H_2O, 1 M $NaHCO_3$ and dried over Na_2SO_4. On evaporating CH_2Cl_2 and trituration with petroleum ether (40–60°), the tetrapeptide was obtained was a solid, yield 1.04 g, 86%, m.p. = 133° $[\alpha]_D^{25}$ = 62.5° (c = 0.6 methanol).

NMR ($CDCl_3$) 7.35δ, d, 1 H (AlaNH), 7.23δ, s, 5 + 1 (benzylic ring protons), 7.20δ, s, 1 H (AibNH), 5.63δ, s, 1 H (AibNH), 5.16δ, q, 2 H (benzylic-CH_2); 4.36δ, m, 2 H (Ala + Pro C^αH); 3.66δ, s + m, 3 + 2 H (OCH_3, Pro $C^\delta H_2$); 1.73δ, m, 2 H (Pro $C^\beta H_2$), 1.40–1.56δ, m, 17 H (Pro $C^\gamma H_2$, Aib + Ala $C^\beta H_3$).

The tetrapeptide ester was saponified in the same way as for 1 to give the tetrapeptide acid, yield 80%, m.p. 185°.

Z-Aib-Pro-Aib-D-Ala-Aib-OMe (5). Z-Aib-Pro-Aib-D-Ala-OH (0.583 g, 1.2 mmol) was dissolved in DMF (50 ml) and cooled to 0°. Aib-OMe (0.138 g, 1.2 mmol) in DMF (1 ml) was added followed by HOBT (0.164 g, 1.2 mmol) and DCC (0.247 g, 1.2 mmol). The mixture was stirred at room temp for 24 hr. DCU was filtered off and the DMF layer was diluted with EtOAc (20 ml). The EtOAc layer was washed with H_2O, 1 N HCl, 1 M $NaHCO_3$ and dried over Na_2SO_4. The EtOAc was evaporated and the residue was triturated with petroleum ether (40–60°) to give the pentapeptide as a solid, yield 0.570 g, 86%, m.p. = 123–124°. $[\alpha]_D^{25}$ = −15° (c = 0.5, MeOH). NMR (270 MHz, 4:1 $CDCl_3/(CD_3)_2SO$) 7.80δ, s, 1 H (AibNH), 7.48δ, d + s, 2 H (Aib + AlaNH), 7.37δ, broad S, 6 H (benzylic ring protons + AibNH), 5.11δ, s, 1 H (benzylic-CH_2), 4.2δ, m, 2 H (Ala + Pro C^αH); 3.78δ, m, 1 H, 3.30δ, m, 1 H (Pro $C^\delta H_2$); 3.61δ, s, 3 H (OCH_3); 1.75δ, m, 4 H (Pro $C^\beta H_2 + C^\gamma H_2$); 1.33–1.50$\delta$, m, 21 H (Ala + Aib $C^\beta H_3$). Peaks of diminished intensity were observed at δ7.7, 5.1 and 3.64 corresponding to 2.

Z-Aib-Pro-Aib-Ala-OH (6) was also prepared using one equivalent of HOSU in the coupling between Z-Aib-Pro-OH to Aib-OMe. Subsequent steps were same as in the case of 1, m.p. = 200° $[\alpha]_D^{25}$ = 12.5° (c = 0.16, MeOH).

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