RACEMIZATION AT PROLINE RESIDUES DURING PEPTIDE BOND FORMATION

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

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Abstract—The stepwise synthesis of amino terminal pentapeptide of alamethicin, Z-Aib-Pro-Aib-Ala-Aib-OMe, by the dicarboxylate-carbodiimide 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 1H 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 carboxylic activation.

Racemization of amino acids on carboxyl activation constitutes a significant hurdle in the synthesis of configurationally pure peptides.** The dicarboxylate-carbodiimide (DCC)† condensation leads to appreciable racemization. This may be suppressed by the use of additives like N-hydroxysuccinimide or 1-hydroxybenzotriazole. 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). During the course of studies directed towards the synthesis of alanethicin 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 1H 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 1H NMR in the analysis of diastereomeric mixtures of these peptides.

RESULTS AND DISCUSSION

The 100 MHz 1H 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 3.666 and 3.616. The two signals could be clearly resolved in a 4:1 CDCl3-(CD3)2SO mixture. The benzylic CH2 group appeared as an AB quartet centered at 5.156 and a singlet at 5.116. 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 1H 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-CH2 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 and Aib-OOMe with the addition of one equivalent of HOBt and DCC.

Figures 2 and 3 compare the methyl ester and benzylic-CH2 regions of the 270 MHz 1H NMR spectra of the pentapeptides 2, 3 and 5. From Fig. 2, the high field methyl ester resonance at 3.616 may be assigned to the d-Ala (4) diastereomer, while the low field resonance at 3.646 corresponds to the l-Ala (4) isomer. The benzylic-CH2 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 5.116. The detection of diastereomeric 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-OOMe. Alternately the possibility that racemization has occurred at Pro (2) needs to be considered. Examination of the 100 MHz 1H NMR spectrum of Z-Aib-Pro-Aib-Ala-OMe in CDCl3 or (CD3)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-CH2 region of the tetrapeptide acids 1

†Abbreviations used: DCC, N,N-dicyclohexylcarbodiimide; Z, benzoyloxycarbonyl; Aib, α-aminoisobutyric acid; DCC, dicarboxylate; HOBt, N-hydroxysuccinimide; HOSU, 1-hydroxybenzotriazole.

‡Stepwise: The stepwise synthesis of amino terminal pentapeptide of alamethicin, Z-Aib-Pro-Aib-Ala-Aib-OMe, by the dicarboxylate-carbodiimide mediated couplings leads to extensive racemization at the Ala and Pro residues.

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

Fig. 2. Ester methyl resonances of the pentapeptides (a) Z-Alb-Pro-Alb-Ala-Alb-OMe 2 (b) Z-Alb-Pro-Alb-Ala-Alb-OMe 3 (c) Z-Alb-Pro-Alb-o-Ala-Alb-OMe 5: in CDCl$_3$(CD$_3$)$_2$SO (4:1) at 270 MHz.

and 4 is shown in Fig. 4. The presence of two sets of quartets of unequal intensities centred at 5.096 and 5.086 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-Alb-Pro-Alb-OMe. Further evidence for this is obtained from an examination of the benzyl-CH$_3$ region in the tetrapeptide acid 6, where one equivalent of HOSU was added in the coupling of Z-Alb-Pro-OH to Alb-OMe (Fig. 4c). Only one quartet centred at 5.086 is observed for this peptide. Hence the quartet of lower intensities centred at 5.096 in the tetrapeptides 1 and 4 can be assigned to the d-Pro (2)-l-Ala (4) and d-Pro (2)-o-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)-o-Ala (4) homers. Similarly the d-Pro (2)-l-Ala (4) and d-Pro (2)-o-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
Table 1. Yield of diastereomeric peptides under different conditions

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Procedure</th>
<th>Isomer</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Alb-Pro-Ala-Ala-Cie&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DCC</td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-D-Ala(4)</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>23</td>
</tr>
<tr>
<td>Z-Alb-Pro-Ala-Ala-OMe&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DCC + HOSU</td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)- D-Ala(4)</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-Pro(2)-D-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>16</td>
</tr>
<tr>
<td>Z-Alb-Pro-Ala-D-Ala-Ala-Cie&lt;sup&gt;a&lt;/sup&gt;</td>
<td>XC + HOSU</td>
<td>L-Pro(2)-D-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>22</td>
</tr>
<tr>
<td>4-Alb-Pro-Ala-Ala-CH&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Saponification of ester</td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-Pro(2)-L-Ala(4)</td>
<td>21</td>
</tr>
<tr>
<td>Z-Alb-Pro-Ala-D-Ala-Ala&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Saponification of ester</td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-D-Ala(4)</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-Pro(2)-D-Ala(4)</td>
<td>18</td>
</tr>
<tr>
<td>Z-Alb-Pro-Ala-D-Ala-CH&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Acid hydrolysis of ester</td>
<td>L-Pro(2)-D-Ala(4) + D-Pro(2)-D-Ala(4)</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-Pro(2)-D-Ala(4)</td>
<td>16</td>
</tr>
<tr>
<td>2-Alb-Pro-fib-Ala-OH&lt;sub&gt;b&lt;/sub&gt;</td>
<td>HOSU in Pro(2)-Alb(3) + coupling</td>
<td>L-Pro(2)-L-Ala(4) + D-Pro(2)-L-Ala(4)</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isomer amounts evaluated from ester CH<sub>3</sub> resonances.

<sup>b</sup> Isomer amounts evaluated from benzylic -CH<sub>2</sub> resonances.
possible due to the lack of an ionizable NH proton. 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 Alb-Pro sequence the Me groups of the Alb residue may play a part in stabilizing the charged oxazolone and hence favours 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 (4H)-oxazolone in the activation of C-terminal N-methyleneamino acids has been provided by trapping reactions using a 1,3-dipolarophile.

270 MHz 1H 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 Alb containing peptides. It appears that in cases where dynamic averaging is limited, NMR analysis may prove routinely useful in detection of racemization.

**EXPERIMENTAL**

Amino acid methyl esters were obtained from the corresponding hydrochlorides. By dissolving in sat NaHCO₃ and extracting with CH₂Cl₂ followed by evaporation of the solvent. All compounds were chromatographically homogenous by TLC on silica gel (10% MeOH/CHCl₃). 100 MHz 1H NMR spectra were obtained on a Varian HA-100 instrument. 270 MHz 1H NMR were recorded on a Bruker WH-270 instrument at the Bangalore NMR Facility.

Z-Alb-Pro-Ala-Ala-OMe

0.5 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 Na₂SO₄ and evaporated to give the tetrapeptide acid, yield 1.3 g, 80%, m.p. 185°.

**Preparation of Z-Alb-Pro-Alb-Ala-Alb-OMe from 1 and Alb-OMe**

(a) DCC (2). Z-Alb-Pro-Alb-Ala-OMe (1.2 g, 2.5 mmol) was dissolved in 15 ml CH₂Cl₂ and cooled to 0°. Alb-OMe (0.287 g, 2.5 mmol) in CH₂Cl₂ (3 ml) was added following 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, H₂O and 1 M NaHCO₃ and dried over Na₂SO₄. Evaporation of CH₂Cl₂ followed by purification with petroleum ether (40-60°), yielded the peptide as a solid, yield 1.3 g, 89%, m.p. 129-132°. Recrystallized from MeOH/ether. [α]D = 24° (c = 0.5, MeOH). (Found: C, 52.61; H, 6.87; N, 12.09. Calc. for C₁₁H₁₉O₅N: C, 52.56; H, 6.75; N, 12.00.)

(b) DCC-N-hydroxysuccinimide (3). Z-Alb-Pro-Alb-Ala-OMe (0.300 g, 0.7 mmol) was dissolved in CH₂Cl₂ (10 ml) and cooled to 0°. Alb-OMe (0.100 g, 0.87 mmol). N-hydroxysuccinimide (0.070 g, 0.7 mmol) was 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, 88%, m.p. 175-176°. [α]D = 42° (c = 0.5 in MeOH). NMR (270 MHz, 4:1 CDCCl₃/CD₃SO₃), 7.276, s, 1 H (AlbNH); 7.356, s, 2 H (Alb+ AlbNNH); 7.386, b, 6 H (AlbNH₂+benzyl ring protons); 5.156, q, 2 H (benzyl CH₂), 4.256, m, 1 H, 4.366, m, 1 H (Pro+C₄H₉N); 3.866, d, 2 H; (Pro+C₄H₉N); 3.646, s, 3 H (OCH₃); 1.756, m, 4 H (Pro+C₄H₉N+C₄H₉); 1.33-1.506, m, 21 H (Alb+Alb C₄H₉). Peaks of diminished intensity were observed around 7.7, 7.55 and 3.66 corresponding to 4 protons.

(c) DCC-N-hydroxysuccinimide (3). Z-Alb-Pro-Alb-Ala-OH (1) was also saponified in the same way as for 1 to give the tetrapeptide acid, yield 0.80%, m.p. 185°.

Z-Alb-Pro-Alb-Ala-Alb-OMe (0.583 g, 1.2 mmol) was dissolved in DMF (50 ml) and cooled to 0°. Alb-OMe (0.138 g, 1.2 mmol) was added following 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 H₂O, 1 N HCl, 1 M NaHCO₃ and dried over Na₂SO₄. 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°. [α]D = 15° (c = 0.5, MeOH). NMR (270 MHz, 4:1 CDCCl₃/CD₃SO₃): 8.706, s, 1 H (AlbNH); 7.486, s, 2 H (Alb+ AlbNNH). 7.376, broad s, 6 H (benzyl ring protons + AlbNH); 5.116, s, 1 H (benzyl-CH₂); 4.266, m, 2 H (Alb+Pro+C₄H₉N); 3.786, m, 1 H, 3.306, m, 1 H (Pro+C₄H₉N); 3.616, s, 3 H (OCH₃); 1.756, m, 4 H (Pro+C₄H₉N+C₄H₉); 1.33-1.506, m, 21 H (Alb+Alb C₄H₉). Peaks of diminished intensity were observed at 67.5, 5.1 and 3.64 corresponding to 2 protons.
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REFERENCES


