The selection of optimal serum is an important part of the routine in a cell-cultivating laboratory. Quality control of sera, unfortunately, depends mostly on empirical criteria, since neither active component(s), nor the mode of its (their) action are at present known.

Placenta may be a source of substances regulating fetal haemopoiesis. Furthermore, cord blood contains a substantially higher number of CFU-c when compared to adult peripheral blood[7]. Placental blood (20–40 ml) was therefore collected, under sterile conditions, immediately after scission of the umbilical cord during delivery. Serum was obtained following spontaneous coagulation at room temperature and centrifugation at $1,000 \times g$ for 15 min. Small aliquots were frozen at $-25\,^{\circ}C$ and thawed immediately before use. The control fetal calf serum (GIBCO, batch No. 245 501) was selected from 6 commercially available sera. Its quality was independently rated as satisfactory by two other laboratories[8,9].

CFU-c were cultivated essentially according to the method described by ROBINSON and PIKE[10]. Progenitor cells were suspended in 0.3% agar and McCoy 5A modified medium (upper layer). Under layers contained leukocytes isolated from peripheral blood and immobilized in 0.5% agar. To culture medium, 20% of either fetal calf serum (control plates) or human umbilical cord serum (denoted A, B, C or D, i.e. from 4 different placentas) was added. Results are summarized in the Table.

The results suggest that human umbilical cord serum significantly enhanced granulocyte colony formation. The maximum increase of the colony number was obtained when human cord serum was added to both layers of the incubating system. We observed, furthermore, that the number of cells per colony was invariably higher in cultures with human cord serum.

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## A Method for Sequencing Peptides: a Co-operation of Diphenyl Phosphorazidate and 2-Mercapto- or 2-Hydroxypyridine for N-Acyldiketopiperazine Formation

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*Summary.* A new method for sequencing peptides is proposed. As a model experiment for this, Bz-Gly-Pro-OH and Bz-Gly-Sar-OH were conveniently converted to their diketopiperazine derivatives by a co-operation of diphenyl phosphorazidate and 2-mercapto- or 2-hydroxypyridine.

In our previous communications we drew attention to the use of diphenyl phosphorazidate (DPPA)[1] as a new reagent for the racemization-free peptide synthesis[2,3], a modified Curtius reaction[2,4,5], the ester formation reaction of some α-functionalized acetic acids[5], and the direct preparation of thiol esters from carboxylic acids and thiols[6]. During the application of DPPA to peptide sequencing, we found a remarkable effect of combination of DPPA and 2-mercapto- or 2-hydroxypyridine, known as bifunctional catalysts, for the formation of N-acyldiketopiperazines.

Exploitation of the method of peptide sequencing has been attempted[7,8] by thermal degradation of peptides to diketopiperazines. The method is attractive in that both degradation and identification could be carried out in a minute amount of peptides by g.c./m.s. methods. However, the degradative conditions are too severe to obtain diketopiperazines in good yield, and partially degradated peptides could not be used again for sequence determination.

If a diketopiperazine moiety, however, could be formed at the C-terminal residue of peptide, the mild hydrolysis of the resultant N-acyldiketopiperazine would afford diketopiperazine and the peptide, the latter would undergo the stepwise cleavage as shown in Scheme I.

Combination of this method and the selective tritium-labelling method at the C-terminal residue[9] might enable us to know which amino acid residue in the diketopiperazine is present at the C-terminal of the original peptide, and might provide a good prospect for stepwise determination of amino acid sequences.

In preparation for the work, we investigated the diketopiperazine formation from Bz-Gly-Pro-OH and Bz-Gly-Sar-OH[10]. Bz-Gly-L-Pro-OH, m.p. 106–111°, $[\alpha]_D^{20}$ −77° (c 1.2, DMF), was prepared in 60% yield by allowing an equimolecular mixture of Bz-Gly-OH and TEA in tetrahydrofuran to react with DPPA at room temperature for 2 h, followed by the addition of H-L-Pro-OH in aqueous sodium hydroxide. It is interesting that DPPA could be used for the coupling of N-protected peptides with free amino acids or peptides as well as amino acid or peptide esters. This resembles the mixed carbonic anhydride method, but the repetition of the above experiment using ethyl chlorocarbonate afforded Bz-Gly-L-Pro-OH in only 8% yield, proving the superiority of the DPPA method, at least in this case. Bz-Gly-Sar-OH, m.p. 164–166°, was also prepared from Bz-Gly-OH and free H-Sar-OH in a manner similar to above.

Intramolecular cyclization of Bz-Gly-L-Pro-OH by DPPA in the presence of a large excess of TEA in DMF was attempted to yield a considerable mixture of products detected on a thin-layer plate. Diethyl phosphorocyanidate (DEPC), a new reagent for the racemization-free peptide synthesis[11], was also proved to be fruitless. Replacement of DPPA with ethyl chlorocarbonate under the same reaction conditions afforded Bz-Gly-Pro[12] in 45% yield together with Bz-Gly-Pro-OEt in 7% yield. In an attempt to improve the yield of Bz-Gly-Pro, several cyclization reagents were investigated: phenyl chlorocarbonate (16%)[13], diphenyl phosphorochloridate (50%), triphenylphosphine and carbon tetrabromide[14] (38%). The most satisfactory results were, however, obtained by the use of an equimolecular mixture of DPPA and 2-mercaptopyridine: To a stirred mixture of Bz-Gly-L-Pro-OH (0.56 g, 2 m*M*), DPPA (0.56 g, 2 m*M*), and 2-mercaptopyridine (0.22 g, 2 m*M*) in DMF (3 ml) was added TEA (1 ml) in DMF (1 ml), and the mixture was stirred at room temperature overnight. After dilution with benzene followed by aqueous acid and alkali work-

ups, the crude product was purified by silica-gel column chromatography with chloroform and diethyl ether (9:1) to give Bz-Gly-Pro (0.39 g, 75%) as colorless prisms, m.p. 133–135° (chloroform-diethyl ether); $[\alpha]_D^{20} \approx 0$ (c 2, $CHCl_3$). Co-operative effect of DPPA and 2-hydroxypyridine was also observed, but less dominant (64% yield). Interestingly, DEPC had no such combined action with the bifunctional catalysts.

The structure of Bz-Gly-Pro was confirmed by comparisons with the sample prepared from Z-L-Pro-OH and H-Gly-OEt by condensation with DPPA and TEA, hydrogenolysis over 5% palladium-carbon, diketopiperazine formation with ethanolic ammonia (3 steps, 40% yield), followed by treatment with benzoyl chloride in pyridine (racemization) as shown in Scheme 2.

Bz-Gly-Sar-OH was cyclized with diphenyl phospohrochloridate to give Bz-Gly-Sar, m.p. 153–154°, in 8% yield. Combination of DPPA and 2-mercaptopyridine under the same reaction conditions as above raised the yield of the diketopiperazine to 38%. In this case, however, the action of 2-hydroxypyridine was much more effective and afforded Bz-Gly-Sar in 50% yield under coexistence of DPPA.

As a model experiment for the removal of diketopiperazines from the C-terminal of peptides, Bz-Gly-Pro was treated with *n*-butylamine in ethanol to give Gly-Pro in 85% yield. This, combined with diketopiperazine formation at the C-terminal by DPPA and 2-mercapto or 2-hydroxypyridine, might promise the stepwise cleavage of the peptides, outlined in Scheme 1.

Extension of the method to various peptides and clarification of the cyclization mechanism are now under investigation.


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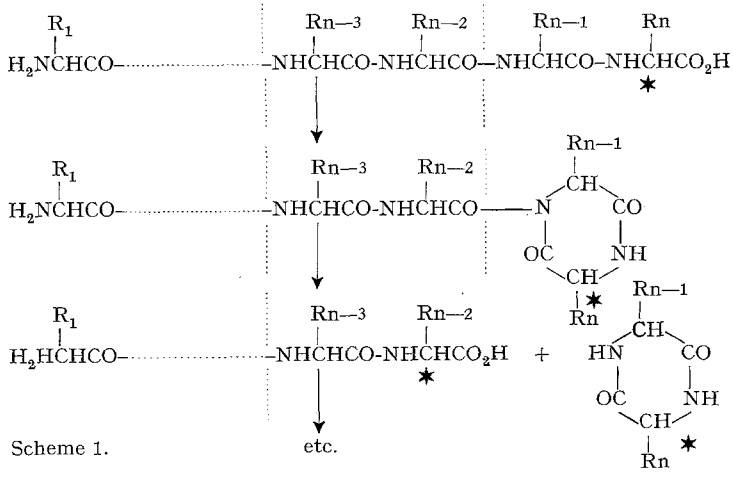
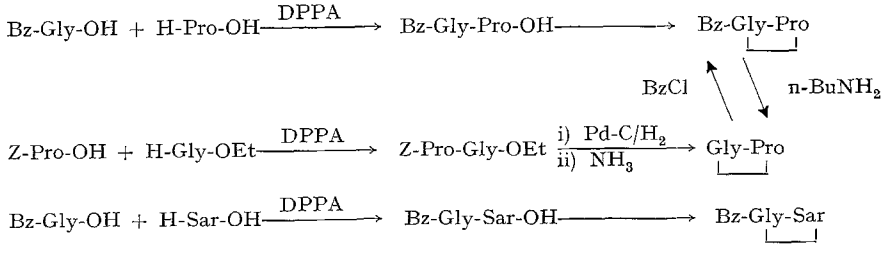



Scheme 1.




Scheme 2.