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Synthesis of Peptide Derivatives of 5-Fluorouracil

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Abstract: The preparation of di-, tetra- and pentapeptides carrying 5-fluorouracil as an α-substituent of a terminal glycine moiety is described. The dipeptide compounds were synthetized by addition of ethyl glyoxylate to N-(benzyloxycarbonyl)-amino acid amides, subsequent acetylation of resulting hydroxyl groups,displacement of acetate groups by 5-fluorouracil,and removal of protective group. Tetra- and pentapeptides were prepared from these dipeptides by coupling methods commonly used in peptide chemistry.

INTRODUCTION

Peptide derivatives of many drugs have been prepared as an approach to develop chemotherapeutic agents with improved physicochemical and biological characteristics. Peptide prodrugs are often water soluble and readly cleaved by peptidase enzymes in all parts of the body.[1] The amino acids resulting from hydrolysis are not toxic.[1] The peptide fragment can be designed to be a selective substrate for tumor-associated enzymes, for instance plasmin produced in high levels near many solid tumors.[2,3] A wide variety of microorganisms such as E.coli or Candida albicans possess distinct permease systems for peptides, dependent on the nature and the length of the peptides[1,4,5]. This led to the possibility that normally impermeant inhibitory agents could be transported across bacterial membranes when covalently bound to a peptide.[4,5] Although less well studied, it is known that similar peptide transport systems exist in other bacterial, fungal and animal cells.[1] For example, some L-amino acids participate in transport through mammalian cell membranes.[6]

The design of peptide derivatives as therapeutic active agents has been performed by different approaches. Thus, growth inhibitory amino acids, not recognized by the specific transport systems, have been incorporated into the backbone of a peptide, allowing them to enter the microbial cells by a peptide carrier system.[4,5] Anticancer agents as doxorubicin, daunorubicin,[3] or phenylenediamine mustard,[2,7] have been bound

via their amino group to the carboxyl end of an amino acid or peptide. Some of such derivatives have showed increased solubility, higher cytotoxic activity and improved selectivity compared with the parent drugs.[2,3,7]

Due to the small side chain specificity demonstrated by the peptide transport systems, several attempts have been made to synthetize antimicrobial derivatives carrying the active agent as a side chain of a peptide backbone. 4-(N-2-mercaptoethyl)aminopyridine-2,6-dicarboxylic acid has been attached through a disulfide bond to a cysteine residue of a peptide.[4,5] Nucleophilic compounds have been bound as α-substituents in glycine residues within a peptide chain, through an oxygen (phenol), sulfur (thiophenol) or nitrogen atom (aniline or heterocycles). Such α-substituted glycine containing peptides have been proposed as transport systems for toxophoric agent through microorganism cell membranes,[4,5] or as new analogs for the chemotactic peptide CHO-Met-Leu-Ph-OH.[8]

5-Fluorouracil (**1**) is an antimetabolite with good antimicrobial and antitumor activity, but its administration is accompanied by significant toxic side effects and delivery problems. Its peptide derivatives could emphasize reduced toxicity, improved pharmacokinetic and pharmacodynamic properties. The synthesis of a dipeptide derivative of 5-fluorouracil, namely alanyl-2-(5-fluorouracil-1-yl)glycine, has been already reported by Kingsbury et al.[4,5] as a transport system of drug across E.coli cell membranes.

The present paper describes details of the synthesis of some peptide derivatives of 5-fluorouracil. These peptides contain **1** as an α-substituent in a C-terminal glycine moiety and are designed, by their different constituent amino acid number, sequence, hydrophobicity and configuration, for acting selectively in different biological conditions.

RESULTS AND DISCUSSION

Synthesis of dipeptide derivatives of 5-fluorouracil (7)

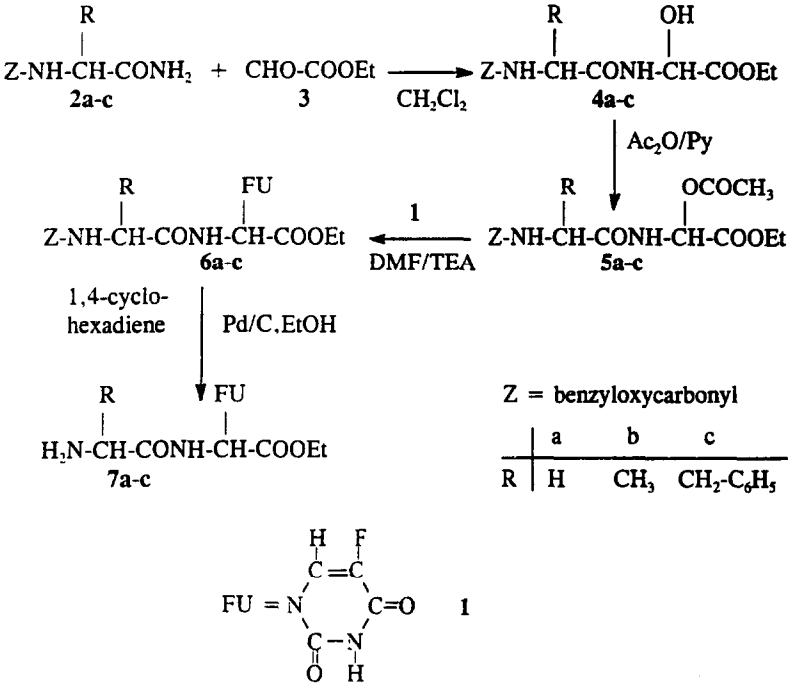
The synthesis of dipeptide derivatives **7** is outlined in Scheme 1. The procedure proposed by Kingsbury et al,[4] for preparing alanyl-2-(5-fluorouracil-1-yl)glycine, was followed with some modifications.

As starting products amino acid amides with benzyloxycarbonyl (Z)-protected α-amino groups were used. In order to obtain dipeptides **7** with a C-terminal ester group, ethyl glyoxylate (**3**) was selected as carbonyl reagent to provide the hydroxy dipeptides **4**. The ethyl ester group is stable to the catalytic hydrogenation used for the final removal of Z-protective group.

The reaction of Z-protected amino acid amides **2** with **3** was carried out in dichloromethane at room temperature. At higher temperatures the formation of several side products was noticed. This addition reaction proceeded with a very slow rate, most probably due to the oligomeric acetal form in which **3** resulted after its synthesis from diethyl (-)tartrate, according to the method proposed by Kelly et al.[9] The presence of such oligomeric form has been reported for methyl glyoxylate[9] and was supported by ^{1}H-NMR analysis ($CDCl_3$). In the spectra of both raw and freshly distilled **3**, a very weak signal for aldehyde group

proton was recorded. In turn the occurrence of consistent signals at δ = 5.2-5.4 ppm, characteristic for acetal type protons, was noticed. The acetal form is reversible (it reacted fast with hydroxylamine) but it has to be less reactive than the free aldehyde form.

Scheme 1. Synthesis of dipeptide derivatives of **1**



For establishing an optimum reaction time, some kinetic measurements for the addition of **3** to **2b** were performed. The results presented in Fig.1 indicated a strong influence of the reagent concentration and the molar ratio **3**/**2b** on the progress of the reaction. The presence of small amounts of 4-(N,N- dimethyl)amino-pyridine (DMAP) as catalyst seemed to result in some increase in the reaction rate, but its possible influence on the racemization degree or side reactions would require a more detailed study. Consequently, the preparation of dipeptides **4** was accomplished at a molar ratio **3**/**2** of 1.5-2.0 for 7-10 days, without any catalyst. The pure products **4** were obtained in 40-70% yields after separation by column chromatography (SiO_2 , dichloromethane/2-propanol,volum ratio 9/1). The lowest yield was observed for derivative **4a**. It is due to the formation of a side product resulting from the addition of **3** to both the amide and the urethane groups in **2a**. This was demonstrated by ^{1}H-NMR analysis in $(CD_3)_2SO$. In the spectra of the side product the disappearance of the triplet at δ=7.5 ppm assigned to urethane NH proton was recorded, together with the occurrence of two signals for CH-OH protons (δ=5.5 and 5.7), the intensities of which indicated a double

addition of aldehyde. The addition of formaldehyde to urethane NH in Z-protected glycine amide has already been reported by Bundgaard et al.[10] who also pointed out the difficulty in performing hydroxyalkylation of secondary acyclic amide[11] (an alternative side reaction in our case). The formation of similar side products in the reaction of **2b** or **2c** with **3** was not noticed, even at very high molar ratios **3/2**, probably due to the steric hindrance encountered at the urethane NH bound to a secondary carbon.

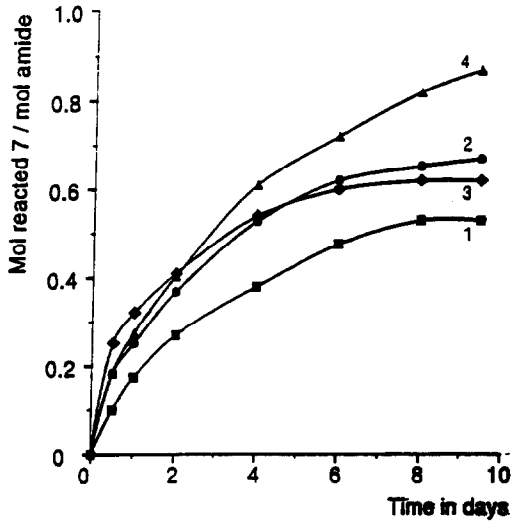


Fig.1. Time course of reaction between N-(benzyloxycarbonyl)alanine amide (**2b**) and ethyl glyoxylate (**3**) in dichloromethane, at 20°C. Concentration of **2b** : 0.11 mmol/ml (curve 1) and 0.22 mmol/ml (curves 2-4); molar ratio **3/2b** : 1.0 (curves 1-3) and 1.5 (curve 4); content in 4-(N,N-dimethyl)aminopyridine : 5mol/100 mol **3** (curve 3).

As the addition reaction resulted in creation of a new asymmetric center, racemic mixtures of S and R enantiomers of dipeptide **4a**, and of S,S and S,R diastereomers of dipeptides **4b-c** were obtained. These isomers could not be separated. Thus, in the subsequent reactions the racemates or diastereomeric mixtures were employed.

The treatment of **4** with acetic anhydride (Ac_2O) in the presence of pyridine (Py) afforded acetoxy group containing dipeptides **5** in 60-70% yields. The subsequent displacement of acetoxy groups in **5** by **1** in N,N-dimethylformamide (DMF)/triethylamine (TEA) had as result derivatives **6** with **1** substituted mainly at N^1 atom of pyrimidine ring, though the formation of N^3-isomers in similar reactions has been reported.[12] The side products separated by column chromatography (SiO_2, dichloromethane/methanol, volum ratio 95/5), representing 5-10% in the crude reaction product, did not exhibit the shift of UV maxima from 265

to 300 nm in the presence of alkali, as is characteristic for N^3-substituted derivatives of **1**.[13] The pure dipeptides **6** were obtained in 70-85% yields.

The attempts to remove the Z-protective group by catalytic hydrogenation in the presence of gaseous hydrogen failed due to the chemical modification of 5-fluorouracil moiety. The ^{1}H-NMR spectrum (in CD_3SOCD_3) of **1** hydrogenated in methanol, in the presence of palladium on charcoal, 4 h at room temperature, indicated a significant modification of the product, including the partial hydrogenation of the double bond in the pyrimidine ring.

Catalytic hydrogenation of the protective group in the presence of hydrogen transfer agents[14,15] was tested as an alternative. The cleavage of benzyloxycarbonyl group in the presence of cyclohexene has been already reported for a similar dipeptide derivative of **1**.[4] Our experiments carried out with cyclohexene (in refluxing methanol) and 1,4-cyclohexadiene (in ethanol, at room temperature) revealed the occurrence of some changes in the structure of deprotected products. The ^{1}H-NMR spectra in CD_3OD proved the presence of two products in hydrogenation mixtures. The difference between the side product and the desired dipeptides **7** was indicated by three supplementary signals, as Fig.2A shows for product **7a**. The intensities of the three signals ascribed to the side product were in the ratio 1/1/1. The doublet at δ=5.65 ppm could not be assigned to any proton of compounds **7**. The two hydrogenation products could not be separated by any method. In order to ascertain the side product structure, some additional hydrogenation experiments were performed. **1** alone or physical mixtures of **1** and Z-protected amino acids were reacted under the same conditions above specified for removal of Z-group of dipeptides **6**, with cyclohexene or 1,4-cyclohexadiene as hydrogen donors. The resulting hydrogenation mixtures were separated by reverse phase column chromatography and the separated products were analysed by TLC (eluent n-butanol/water/acetic acid 4/2/2), analytical reverse phase HPLC and ^{1}H-NMR (D_2O and CD_3SOCD_3). The results of these analyses clearly showed that the modification occurred in the structure of **1**, only when it is in the presence of a Z-group containing compound. The modified derivative was eluted before **1** itself, and only in its ^{1}H-NMR spectra the unknown doublet was recorded. These data would indicate the presence of a compound that is more hydrophylic than **1**, and has a supplementary proton. Based on these results, we suggest that the side product formed during the cleavage of Z-group of dipeptides **6** contained a modified **1** moiety, probably due to the hydrogenation of a keto group to an aminal, according to the Scheme 2.

The occurence of the modification of **1** only in the presence of a Z-group containing compound might indicate that the transfer hydrogenation involves a specific donor-acceptor relationship, though such a hypothesis is not unanimously accepted.[14] According to this hypothesis, **1** is not a hydrogen specific acceptor as Z-group is, but it can be hydrogenated by the excess hydrogen produced during the reductive cleavage of a Z-group. The modification seemed to proceed in a larger extent when the removal of Z-group was close to completion.

The extent of modification of the **1** moiety in dipeptide **7** occured in the presence of cyclohexene and 1,4-cyclohexadiene as well. Its extent depended on the reaction conditions, especially on the possibility to

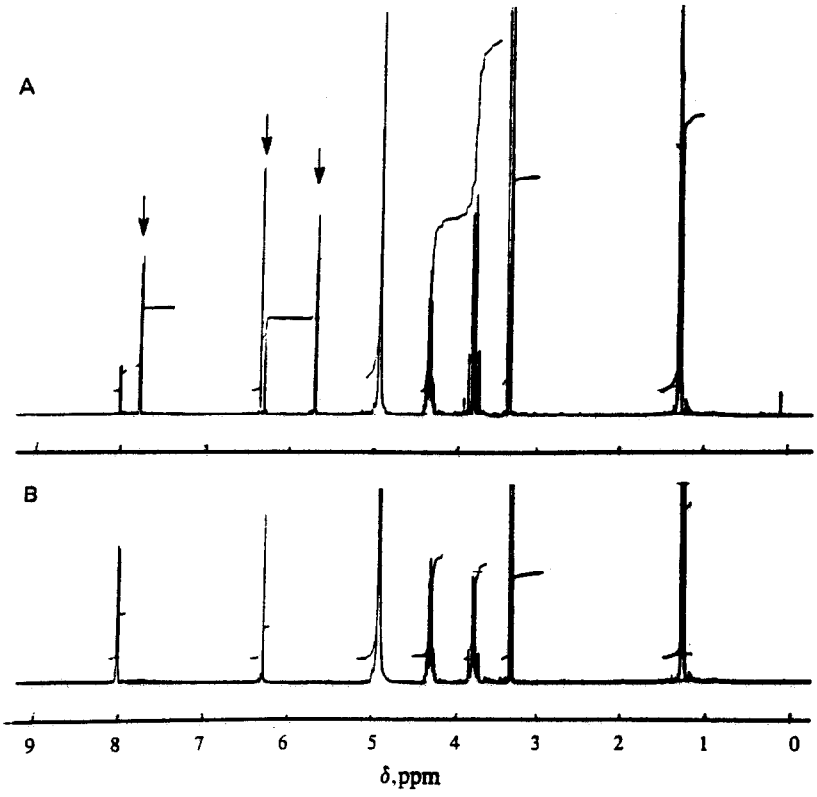
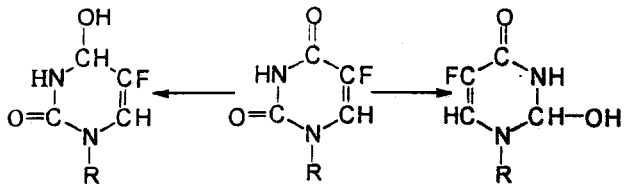


Fig.2 The ^{1}H-NMR spectra (CD_3OD) of products **7a** obtained after hydrogenation of dipeptide **6a**. Reaction condition : cyclohexene, Pd/C, 20 min. in refluxed methanol (A); 1,4-cyclohexadiene, Pd/C, 60 min. in ethanol at 23°C. The arrows indicate the peaks assigned to the side product.

Scheme 2.



R = peptide 7 backbone

control the progress of reductive cleavage. It could not be completely avoided in the presence of cyclohexene, even in very mild reaction conditions: 50-55°C (under 50°C cyclohexene is not active), very low amounts of hydrogenation agent (2-3 mol/mol Z-group) and very short reaction time (20-30 minutes).

This modification could be reduced or even avoided by using 1,4-cyclohexadiene as hydrogen-transfer agent, palladium/charcoal as a catalyst, dry ethanol as a solvent, and 20-25 °C. It could be accomplished by an appropriate choice of the reaction mixture concentration (0.025-0.030 mmol Z-group/ml) and of the molar ratio 1,4-cyclohexadiene/Z-group (5-10/1). Under these conditions, a control of the progress of the Z-group removal (TLC analysis) was possible, and the free amino group containing dipeptides **7** were obtained in 80-90 % yields.

Another side product could be formed during the cleavage of the protective group, by internal cyclization of the dipeptides **7** to the corresponding diketopiperazines. This has been reported for other dipeptides containing amino and ester terminal groups.[8,16,17] The extent of cyclization was found to be dependent on the concentration of the reaction medium, the length of the reaction and the nature of the N-terminal amino acid (Phe < Gly << Ala). Under the reaction conditions we used, the amount of cyclic derivatives formed was lower than 15%, and its increase during the final removal of the solvent was avoided by adding, before evaporation, of small amounts of methanol containing 0.01% acetic acid.

The diastereoisomers of dipeptides **7b** and **7c** could be separated by reverse phase column chromatography. A good resolution could be obtained by an appropriate choice of the pH of the aqueous eluent and, when necessary, by addition of 5-15% organic solvent. The purity of isomers was checked by ^{1}H-NMR and analytical HPLC. Optical rotation measureaments revealed that for both dipeptides the first eluted diastereomer was levorotatory, the second one dextrorotatory. The absolute configuration of the separated diastereomers could not be determined yet, but the ^{1}H-NMR data could provide some information about their conformation. This aspect will be the subject of a separate study.

At this stage of our research we prefered to continue the work with racemic mixtures. Studies are under way to compare the hydrolytic stability and biological behaviour of the isomers. The results of this study will show to what extent it is necessary to use enantiomeric pure peptides for achieving the most suitable biological activity of synthetized 5-fluorouracil derivatives.

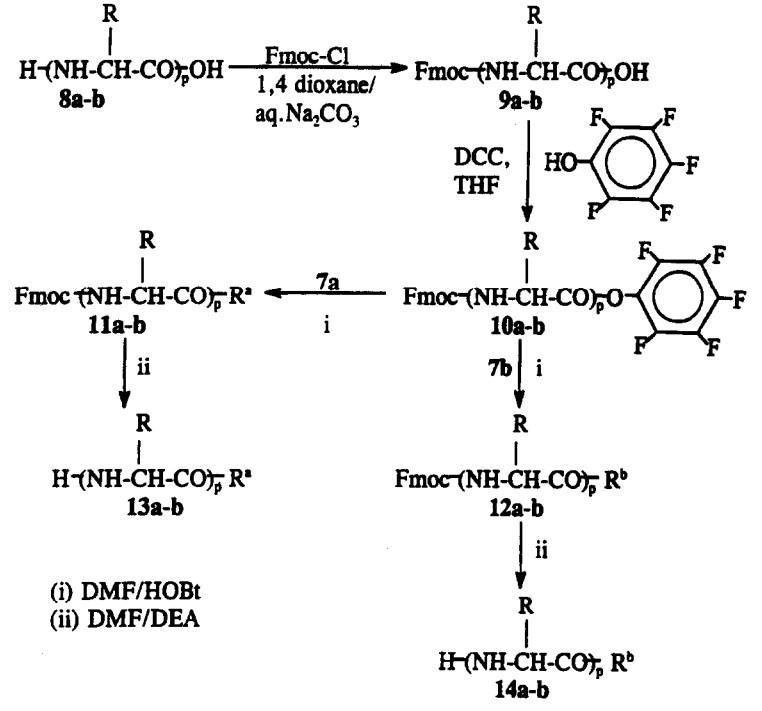
Synthesis of tetra- and peptapeptide of 5-fluorouracil (13,14)

By synthesis of longer peptides of **1** , with different amino acid sequences, we attempted to obtain prodrugs, the biological properties of which may be controlled by an appropriate design of oligopeptide backbone.

The tetra- and pentapeptide derivatives of **1** were synthetized by methods frequently used in peptide chemistry[18] (according to Scheme 3). The amino groups in di- and tripeptides **8** were protected by reaction with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-Cl). The Fmoc-protected peptides **9** were converted to pentafluorophenyl ester derivatives **10** by using N,N'-dicyclohexylcarbodiimide (DCC) as coupling agent.

These reactions proceeded with good yields (70-80%). The coupling of active esters **10** with dipeptides **7a-b** was successfully accomplished in DMF, in the presence of hydroxybenzotriazole (HOBt). The removal of Fmoc-protective group with diethylamine in DMF left the ethyl ester group and **1** moiety unmodified and afforded the oligopeptides **13** and **14** in 80-90% yields

Scheme 3. Synthesis of tetra- and pentapeptide derivatives of **1**



	a	b
$(NH\text{-}CHR\text{-}CO)_p$	Gly-Phe	Gly-Phe-Leu

p =2-3

R^a = NH-CH_2-CO-NH-CH(FU)-COOEt

R^b = NH-CH(CH_3)-CO-NH-CH(FU)-COOEt

The method proposed for the synthesis of these peptides can be successfully used for the design of a large variety of oligopeptide derivatives.

CONCLUSION

A procedure for obtaining several peptide derivatives of 5-fluorouracil was proposed. Every step involved was examined in detail for providing the optimized conditions of synthesis.The different peptide derivatives prepared could represent new and interesting antimicrobial or anticancer agents. Their biological properties can be controlled by an appropriate design of the oligopeptide backbone. These peptide derivatives can be used as such or can be bound, through their primary amino group, to a suitable functionalized polymer. The latter will lead to new macromolecular prodrugs of 5-fluorouracil.

EXPERIMENTAL

Melting points were determined on a hot bench system Kofler (Reichart-Jung) and are uncorrected. ^{1}H-NMR spectra were recorded on a Brucker 360 MHz instrument with tetramethylsilan as internal standard. Chemical shifts are reported in ppm (δ). IR-spectra were measured on a Perkin Elmer 1600 Series FT-IR. UV spectra were obtained on a Uvikon 810 spectrometer (Kontron Instruments). The values of extinction coefficient ϵ are given in l/mol.cm. Microanalyses were performed on a Perkin Elmer elemental analyzer (model 240 C). Mass spectra (MS) were recorded on a Hewlett Packard mass spectrometer (model 5988 A). The ($M^+ + 1$) values are reported for the molecular ion. For column chromatography Merck Silica gel (0.04-0.2 mm) or Merck LiChroprep RP-18 (0.025-0.04 mm) were used. Analytical HPLC was performed on a Zorbax ODS packed column 4.6 mm i.d.x25 cm, and the effluents were monitored by UV. Thin layer chromatography (TLC) was performed with Merck Silica gel 60F-254 glass backed plates (0.25 mm thickness). Optical rotations were taken on a Perkin Elmer 241 polarimeter. The kinetic measurements presented in Fig.1 were performed by withdrawing aliquots from the reaction mixture and determining the aldehyde content by the hydroxylamine hydrochloride method.[19] The reagents were used as such or purified, when necessary, by standard methods.

N-Benzyloxycarbonyl-L-alanyl-2(R,S)-hydroxyglycine ethyl ester (4b). To a suspension of N-benzyloxycarbonylalanine amide (**2b**) (2 g; 9 mmol) in dichloromethane (30ml), ethyl glyoxalate (**3**) (1.37 g; 13.5 mmol) was added.The mixture was stirred for 7 days at room temperature, whereafter it was filtered. The filtrate was concentrated by evaporation of the solvent in vacuo and the crude product was purified on silica gel using dichloromethane/2-propanol as eluent (9/1 v/v). The title compound was eluted at R_f=0.55 and recovered as a solid (m.p.=121-124°C). Yield 70%. IR (KBr): 3552, 3407, 3309, 2976, 1746, 1693, 1662,1536, 1452,1351, 1250, 1106, 1061, 973, 735 cm^{-1}. ^{1}H-NMR (CD_3OD):δ=7.3(m,5H,phenyl), 5.56(s,1H,C<u>H</u>-OH), 5.11 (s,2H,C<u>H</u>$_2$-C_6H_5), 4.2(two overlapped q,3H,C<u>H</u>-CH_3 and C<u>H</u>$_2$-CH_3), 1.35(d,3H,CH-C<u>H</u>$_3$) and 1,25(t,3H,CH_2-C<u>H</u>$_3$). Anal.calcd. for $C_{15}H_{20}N_2O_6$: C 55.55; H 6.17; N 8.64; found: C 55.31; H 6.23; N 8.62.

N-Benzyloxycarbonyl-L-alanyl-2(R,S)-acetoxyglycine ethyl ester (5b). To a cooled (0°C) suspension of **4b** (1.7 g; 5.24 mmol) in acetic anhydride (10 ml) dry pyridine (8 ml) was added. The resulting solution was stirred for

24 h at O°C. The reaction mixture was diluted with 60 ml ethyl acetate and the organic solution was extracted with cold aqueous 3N HCl (4x20 ml), cold aqueous 5% $NaHCO_3$ (4x 20ml) and cold water (2x20 ml). Then it was dried ($MgSO_4$) and concentrated in vacuo. The residue was recrystallized from diethyl ether/petroleum ether affording product **5b** as a white-yellow solid with m.p.99-101°C (70% yield). IR (KBr): 3302, 3063, 2975, 1758, 1692, 1667, 1537, 1453, 1373, 1335, 1261, 1227, 1142, 1053, 1026, 925, 744, 698 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ=7.54 and 7.45(two d,1H,amide N<u>H</u>), 7.3(m,5H,phenyl), 6.36(m,1H,C<u>H</u>-OCOCH_3), 5.32(bm,1H,urethane N<u>H</u>), 5.12(s,2H,C<u>H_2</u>-Ph), 4.33(q,1H,C<u>H</u>-CH_3), 4.25(q,2H,C<u>H_2</u>-CH_3), 2.1(s,3H,OCOC<u>H_3</u>), 1.39(two overlapped d,3H,CH-C<u>H_3</u>) and 1.27(t,3H,CH_2-C<u>H_3</u>). Anal. calcd. for $C_{17}H_{22}N_2O_7$: C 55.73; H 6.01; N 7.65; found: C 55.41; H 6.20; N 7.60.

N-Benzyloxycarbonyl-L-alanyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (6b). A mixture of **5b** (0.82 g; 1.88 mmol), **1** (0.24 g; 1.84 mmol) and TEA (0.185 g; 1.84 mmol) in dry DMF (4.8 ml) was stirred for 24 h at room temperature. The solvent was then removed by distillation in vacuo. The residue was dissolved in 60 ml ethyl acetate and the solution so obtained was extracted with water (2x20 ml), dried ($MgSO_4$), concentrated in vacuo and purified by silica gel column chromatography (eluent dichloromethane/methanol 95/5 v/v). The product **6b** was eluted at R_f=0.24. Evaporation of collected solution and drying in vacuo afforded 0.71 g pure **6b** (89% yield) as a white solid with m.p.110-115°C. IR (KBr): 3425, 3319, 3069, 1755, 1705, 1662, 1524, 1467, 1366, 1260, 1142, 1024, 750 cm^{-1}. UV (MeOH): $\lambda_{max}(\epsilon)$=264 (8224) nm. ^{1}H-NMR ($CDCl_3$): δ=10.00 and 9.70(two bs,1H,pyrimidine N^3<u>H</u>), 8.61 and 8.42(two d,1H,amide N<u>H</u>), 7.56(two overlapped d,1H,pyrimidine C^6<u>H</u>), 7.3(s,5H,phenyl), 5.88 and 5.8(two d,1H,C<u>H</u>-FU), 5.65 and 5.54(two d,1H,urethane N<u>H</u>), 5.1(m,2H,C<u>H_2</u>-Ph), 4.55 and 4.45(two q,1H,C<u>H</u>-CH_3), 4.25(q,2H,C<u>H_2</u>CH_3), 1.40 and 1.32(two d,3H,CH-C<u>H_3</u>), 1.25(t,3H,CH_2-C<u>H_3</u>). Anal. calcd.for $C_{19}H_{21}FN_4O_7$: C 52.29; H 4.85; N 12.83; found: C 52.01; H 4.79; N 12.5.

L-Alanyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (7b). 6b (0.1 g; 0.23 mmol) was dissolved in dry ethanol (10 ml), whereafter 10% Pd/C (0.1 g) was added and the mixture was purged with nitrogen while stirred. Freshly distilled 1,4-cyclohexadiene (0.25 ml,2.5 mmol) was added with a syringe through a rubber septum. The mixture was stirred at 23-25°C till TLC analysis (dichloromethane/methanol 95/5 v/v) indicated the dissapearance of Z-protected derivative. The catalyst was removed by filtration and the residue on the filter was rinsed with methanol (2x20 ml) containing 0.01% acetic acid. The filtrate was evaporated in vacuo to dryness and 0.062 g (89% yield) **7b** were obtained. M.p.=108-112 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.61. IR (KBr): 3354, 3213, 2986, 1753, 1698, 1625, 1528, 1372, 1217, 1138, 1020, 780, 653 cm^{-1}. UV (MeOH): $\lambda_{max}(\epsilon)$=263 (6930) nm. ^{1}H-NMR (CD_3OD):δ=7.95(d,1H,pyrimidine C^6<u>H</u>), 6.25 and 6.21(two s,1H,C<u>H</u>-FU), 4.27(q,2H,C<u>H_2</u>-CH_3), 3.65(m,1H,C<u>H</u>-CH_3), 1.35 and 1.27(two d,3H,CH-C<u>H_3</u>), 1.25(t,3H,CH_2-C<u>H_3</u>). Anal. calcd.for $C_{11}H_{15}FN_4O_5$: C 43.72; H 5.00; N 18.53; found: C 43.1; H 5.10; N 18.2. MS: m/z 303.16 (M^++1).

Glycyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (7a) and **L-phenylalanyl-2(R,S)-(5-fluorouracil-1-yl)-glycine ethyl ester (7c)** were prepared according to the same procedure described above for the synthesis of

dipeptide **7b**.

7a: M.p.=118-123 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.54. IR(KBr): 3416, 3224, 3071, 2362, 1748, 1701, 1662, 1529, 1464, 1360, 1259, 1124, 1019, 810 cm^{-1}. UV (H_2O): $\lambda_{max}(\epsilon)$=265 (7350) nm. ^{1}H-NMR (D_2O): δ=8.01(d,1H,pyrimidine $C^6\underline{H}$), 6.35(s,1H,C$\underline{H}$-FU), 4.32(q,2H,C$\underline{H_2}$-CH_3), 3.92(m,2H,Gly C$\underline{H_2}$), 1.25(t,3H,CH_2-C$\underline{H_3}$). Anal.calcd.for $C_{10}H_{13}FN_4O_5$: C 41.67, H 4.54, N 19.43; found: C 41.2, H 4.62, N 19.6. MS: m/z 289.23 (M^++1).

7c: M.p.=112-122 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.64 and 0.70 (two spots corresponding to the two diastereoisomers were recorded). IR (KBr): 3422, 2966, 2363, 1750, 1702, 1663, 1555, 1457, 1371, 1260, 1139, 803, 749 cm^{-1}. UV (MeOH): λ_{max} (ϵ)=265 (7222) nm. ^{1}H-NMR (CD_3OD): δ=7.91 and 7.75(2d,1H,pyrimidine $C^6\underline{H}$), 7.31-7.08(m,5H,phenyl), 6.21 and 6.19(2s,1H,C$\underline{H}$-FU), 4.23(q,2H,C$\underline{H_2}$-CH_3), 3.78(m,1H,C$\underline{H}$-CH_2-Ph), 3.08-2.87(m,2H,CH-C$\underline{H_2}$-Ph), 1.26(t,3H,CH_2-C$\underline{H_3}$). Anal.calcd. for $C_{17}H_{19}FN_4O_5$: C 53.96, H 5.06, N 14.8; found: C 53.2, H 5.2, N 14.3. MS: m/z 379.35 (M^++1).

Separation of diastereomers from diastereomeric mixtures 7b and 7c. The separation of the diastereomeric mixtures into pure diastereomers was accomplished by column chromatography on LiChroprep RP 18. The best resolution was obtained using as eluents aqueous solution of acetic acid with pH=3.24 for isomers **7b**, and aqueous solution of acetic acid with pH=2.93, containing 12% acetonitril, for isomers **7c**.

First eluted diastereomer 7b (-) : $[\alpha]_D^{20}$=-65 (c=1; water). ^{1}H-NMR (D_2O): δ=7.93(d,1H,$C^6\underline{H}$ of pyrimidine ring), 6.35(s,1H,C$\underline{H}$-FU), 4.3(q,2H,C$\underline{H_2}$-CH_3), 4.2(q,1H,C$\underline{H}$-CH_3), 1.48(d,3H,CH-C$\underline{H_3}$), 1.25(t,3H,CH_2-C$\underline{H_3}$). The IR and UV spectroscopic data were identical to those of **7b**. Anal. calcd. for $C_{11}H_{15}FN_4O_5{\cdot}CH_3COOH$: C 43.09, H 5.24, N 15.46;found C 43.01, H 5.32, N 15.05.

Second eluted diastereomer 7b (+) : $[\alpha]_D^{20}$=+60 (c=1; water). ^{1}H-NMR (D_2O): δ=7.96(d,1H,$C^6\underline{H}$ of pyrimidine ring), 6.32(s,1H,C$\underline{H}$-FU), 4.3(q,2H,C$\underline{H_2}$-CH_3), 4.12(q,1H,C$\underline{H}$-CH_3), 1.58(d,3H,CH-C$\underline{H_3}$), 1.25(t,3H,CH_2-C$\underline{H_3}$). The other spectroscopic data were identical to those of **7b**. Anal.calcd for $C_{11}H_{15}FN_4O_5{\cdot}CH_3COOH$: C 43.09, H 5.24, N 15.46; found: C 43.25, H 5.35, N 15.14.

First eluted diastereomer 7c (-) : $[\alpha]_D^{20}$= -103 (c=1; water). ^{1}H-NMR (D_2O): δ=7.67(d,1H,$C^6\underline{H}$ of pyrimidine ring), 7.23(m,3H,phenyl) 7.12(m,2H,phenyl), 6.17(s,1H,C$\underline{H}$-FU), 4.26(two overlapped q,3H,C$\underline{H_2}$-CH_3 and C$\underline{H}$-CH_2-Ph), 3.27 and 2.93(two m,2H,CH-C$\underline{H_2}$-Ph), 1.21(t,3H,CH_2-C$\underline{H_3}$). IR and UV spectra were identical to spectra obtained for **7c**. Anal. calcd for $C_{17}H_{19}FN_4O_5 \cdot CH_3COOH$: C 52.05, H 5.25, N 12.78; found: C 51.97, H 5.18, N 12.52.

Second eluted diastereomer 7c (+) : $[\alpha]_D^{20}$= +109 (c=1; water). ^{1}H-NMR (D_2O): δ=7.81(d,1H,$C^6\underline{H}$ of pyrimidine ring), 7.32(m,3H,phenyl), 7.22(m,2H,phenyl), 6.15(s,1H,C$\underline{H}$-FU), 4.26(q,2H,C$\underline{H_2}$-CH_3), 4.08(q,1H,C$\underline{H}$-CH_2-Ph), 3.22 and 3.08(two m,2H,CH-C$\underline{H_2}$-Ph), 1.21(t,3H,CH_2-C$\underline{H_3}$). The IR and UV spectroscopic data were identical to those recorded for **7c**. Anal. calcd for $C_{17}H_{19}FN_4O_5{\cdot}CH_3COOH$: C 52.05, H 5.25, N 12.78;found: C 52.05, H 5.23, N 12.29.

(9-Fluorenylmethyloxycarbonyl)glycyl-L-phenylalanine (9a). Glycyl-L-phenylalanine **(8a)** (0.5 g; 2.25 mmol)

was dissolved in aqueous 10% Na_2CO_3 (20 ml), the mixture was cooled at O°C and a solution of 9-fluorenylmethyloxycarbonyl chloride (0.58 g; 2.25 mmol) in 1,4-dioxane (6 ml) was added dropwise for 1/2 h. The mixture was stirred for 4 h at room temperature, whereafter the resulting solution was diluted with 150 ml water and acidified to pH 2 with concentrated HCl. The mixture was extracted first with diethyl ether (2x50 ml), then with ethyl acetate (2x200 ml). The ethyl acetate solution was dried on $MgSO_4$, filtered and the filtrate was evaporated to dryness. The resulting product (0.85 g; 85% yield) had m.p.215-217°C. IR (KBr): 3322, 3026, 2964, 2602, 1729, 1596, 1536, 1449, 1411, 1356, 1326, 1256, 1233, 1159, 1088, 1045, 994, 916, 820, 784, 760, 741, 701, 640, 613 cm^{-1}. ^{1}H-NMR (CD_3SOCD_3):δ=8.11 (d,1H,amide NH), 7.9 and 7.7(two d,4H,fluorenyl group), 7.4 and 7.3(two m,4H,fluorenyl group), 7.28 and 7.15(m,5H,phenyl), 7.5(t,1H,urethane NH), 4.43(m,1H,CH-CH_2-Ph), 4.22 (m,3H,CH_2-CH of protective group), 3.64 and 3.57(two dd,2H,Gly CH_2), 3.04 and 2.87(two dd,2H,CH-CH_2-Ph). Anal.calcd.for $C_{26}H_{24}N_2O_5$: C 70.25, H 5.44, N 6.30; found: C 71.03, H 5.36, N 6.05.

(9-Fluorenylmethyloxycarbonyl)glycyl-L-phenylalanine pentafluorophenyl ester (10a). **9a** (0.2 g; 0.45 mmol) and pentafluorophenol (0.1 g; 0.53 mmol) were dissolved in dry THF (6 ml), the solution was cooled at O°C in an ice bath, and DCC (0.108 g; 0.52 mmol) was added. The mixture was stirred for 3 h at O°C and for other 20 h at room temperature. The precipitate formed during the reaction was filtered and the filtrate evaporated in vacuo. The residue was dissolved in ethyl acetate (20 ml). The mixture was filtered, the filtrate precipitated in dry hexane and the precipitate recovered by filtration and dried in vacuo. The resulting white solid (0.236 g; 86%) showed a single spot at TLC analysis (R_f=0.69, chloroform/methanol 9/1 v/v). M.p.131-134°C. IR (KBr): 3305, 3066, 2934, 1740, 1697, 1657, 1521, 1449, 1346, 1262, 1131, 996, 758, 741, 698 cm^{-1}. Anal.calcd. for $C_{32}H_{23}F_5N_2O_5$: C 62.95, H 3.79, N 4.58; found: C 62.10, H 3.85, N 4.13.

(9-Fluorenylmethyloxycarbonyl)glycyl-L-phenylalanylglycyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (11a). To a solution of **10a** (0.104 g; 0.17 mmol) in dry DMF, HOBt (0.032 g; 0.22 mmol) and **7a** (0.052 g; 0.18 mmol) were added. The mixture was stirred for 24 h at room temperature, till the TLC analysis (chloroform/methanol 9/1 v/v) indicated the dissapearance of the spot for free amino group containing reagent. The solvent was removed by distillation in vacuo and the residue redissolved in 30 ml ethyl acetate. The resulting solution was extracted with aqueous 5% $NaHCO_3$ (3x20 ml) and water (2x20 ml), dried ($MgSO_4$) and evaporated to dryness, giving 0.108 g (78% yield) of **11a**. M.p.105-110°C. IR (KBr): 3550, 3410, 3067, 2963, 1758, 1706, 1654, 1516, 1450, 1363, 1261, 1156, 1101, 1016, 802, 760, 741, 700, 621, 539 cm^{-1}. UV (MeOH): λ_{max} (ϵ)= 262 (6978) nm. ^{1}H-NMR (CD_3SOCD_3):δ=11.5 (s,1H,pyrimidine N^3H), 9.2(m,1H,NH-CH-FU), 8.4(m,1H,Gly NH), 8.1 (m,1H,Phe NH), 7.9(d,2H,fluorenyl group), 7.7(m,3H,fluorenyl group and pyrimidine C^6H), 7.5(t,1H,urethane NH), 7.4 and 7.3(two m,4H,fluorenyl group), 7.2(m,5H,phenyl), 6.38(m,1H,CH-FU), 4.53(m,1H,CH-CH_2-Ph), 4.28-4.1(bm,3H,CH_2-CH of protective group and 2H,CH_2CH_3), 3.85(m,2H,Gly CH_2), 3.65 and 3.50(two dd,2H,Gly CH_2), 3.02 and 2.76(two dd,2H,CH-CH_2-Ph), 1.18(t,3H,CH_2-CH_3). Anal.calcd.for $C_{36}H_{35}FN_6O_9$: C 60.49, H 4.93, N 11.76; found: C 60.09, H 4.56, N 11.25.

Glycyl-L-Phenylalanylglycyl-2(R,S)-(5-fluorouracil-yl)glycine ethyl ester (13a). To a solution of **11a** (0.04 g; 0.0576 mmol) in DMF (1 ml), 0.1 ml DEA were added and the mixture was stirred at room temperature for 2 h. The solvent was removed by distillation in vacuo, then diethyl ether (20 ml) was added. The suspension was stirred for 2 h and filtered. The solid product on the filter was washed with diethyl ether till the TLC analysis of the filtrate showed the disappearance of the spots for benzofulvene and its derivatives formed during the removal of Fmoc group. The solid was dissolved in methanol, the solution was filtred and the filtrate was evaporated to dryness, affording 0.023g (82%) of **13a**. M.p.=132-140 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.62 and 0.65. IR (KBr): 3550, 3413, 3281, 3063, 2363, 1749, 1697, 1669, 1522, 1494, 1453, 1369, 1261, 1149, 1010, 748, 702 cm^{-1}. UV (MeOH): λ_{max} (ϵ)=264 (8204) nm. ^{1}H-NMR (CD_3OD): δ=7.75(dd,1H,pyrimidine $C^6\underline{H}$), 7.35-7.2(m,5H,phenyl), 6.3(s,1H,C$\underline{H}$-FU), 4.62(m,1H,C$\underline{H}$-CH_2-Ph), 4.26(q,2H,C$\underline{H_2}$-CH_3), 3.95 and 3.8(two dd,2H,Gly C$\underline{H_2}$), 3.6 and 3.44(two dd,2H,Gly C$\underline{H_2}$), 3.17 and 2.92(two dd,2H,CH-C$\underline{H_2}$-Ph), 1.27(t,3H,CH_2-C$\underline{H_3}$). Anal.calcd.for $C_{21}H_{25}FN_6O_7$: C 51.21, H 5.11, N 17.06; found: C 50.85,H 5.03, N 16.89. MS: m/z 493.4 (M^++1).

Glycyl-L-phenylalanyl-L-alanyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (14a), glycyl-L-phenylalanyl-L-leucylglycyl-2(R,S)-(5-fluorouracil-1-yl)glycine ethyl ester (13b) and **glycyl-L-phenylalanyl-L-leucyl-L-alanyl-2(R,S)-(5-fluorouracyl-1-yl)glycine ethyl ester (14b)** were prepared by the same procedure described for the synthesis of **13a**.

13b: M.p.=138-158 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.71 and 0.76. IR (KBr): 3548, 3483, 3413, 2924, 2849, 2360, 1752, 1672, 1638, 1616, 1540, 1456, 1382, 1261, 1113, 1027, 802, 621 cm^{-1}. UV (MeOH): λ_{max} (ϵ)=259.5 (7295) nm. ^{1}H-NMR (CD_3OD):δ=7.9(two overlapped d,1H,pyrimidine $C^6\underline{H}$), 7.35-7.1(m,5H,phenyl), 6.3 and 6.28(2s,1H,C$\underline{H}$-FU), 4.65(m,1H,Phe C$\underline{H}$), 4.55(m,1H, Leu C$\underline{H}$), 4.25(q,2H,ethyl C$\underline{H_2}$), 3.9(m,2H,Gly C$\underline{H_2}$), 3.65(m,2H,Gly C$\underline{H_2}$), 3.2 and 3.0(two m,2H,CH-C$\underline{H_2}$-Ph), 1.6(m,3H,Leu C$\underline{H}$-C$\underline{H_2}$), 1.25(t,3H,ethyl C$\underline{H_3}$), 0.9(m,6H,Leu C$\underline{H_3}$). Anal.calcd.for $C_{27}H_{36}FN_7O_8$: C 53.57, H 5.99, N 16.19; found: C 53.13, H 5.87, N 15.94. MS: m/z 606.6 (M^++1).

14a: M.p.=135-150 °C. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.65 and 0.69. IR (KBr): 3414, 3064, 2931, 1751, 1702, 1664, 1528, 1456, 1371, 1261, 1022, 804, 747, 702, 620 cm^{-1}. UV (MeOH): λ_{max} (ϵ)=265 (5488) nm. ^{1}H-NMR (CD_3OD):δ=7.9(two overlapped d,1H,pyrimidine $C^6\underline{H}$), 7.3-7.1(m,5H,phenyl), 6.21 and 6.20(2s,1H,C$\underline{H}$-FU), 4.62(m,1H,Phe C$\underline{H}$), 4.37(m,1H,Ala C$\underline{H}$), 4.27(q,2H,ethyl C$\underline{H_2}$), 3.72(m,2H,Gly C$\underline{H}$), 3.15 and 2.9(m,2H,CH-C$\underline{H_2}$-Ph), 1.35(d,3H,Ala C$\underline{H_3}$), 1.25(t,3H,ethyl C$\underline{H_3}$). Anal.calcd.for $C_{22}H_{27}FN_6O_7$: C 52.17, H 5.37, N 16.59; found: C 52.25, H 5.14, N 16.02. MS: m/z 507.48 (M^++1).

14b: M.p.=145-165. R_f (n-BuOH/CH_3COOH/H_2O 4/2/2) 0.77 and 0.81. IR (KBr): 3540, 3492, 3410, 2924, 2849, 2343, 1762, 1692, 1658, 1609, 1540, 1458, 1382, 1261, 1118, 1028, 802, 632 cm^{-1}. UV (MeOH): λ_{max} (ϵ)=262 (7364) nm. ^{1}H-NMR (CD_3OD):δ=7.9(two overlapped d,1H,pyrimidine $C^6\underline{H}$), 7.35-7.15(m,5H,phenyl), 6.23 and 6.21(2s,1H,C$\underline{H}$-FU), 4.68(m,1H,Phe C$\underline{H}$), 4.50(m,1H,Leu C$\underline{H}$), 4.40(m,1H,Ala C$\underline{H}$), 4.25(q,2H,ethyl C$\underline{H_2}$) 3.75(m,2H,Gly C$\underline{H_2}$), 3.15 and 2.95(two dd,2H,CH-C$\underline{H_2}$-Ph), 1.62(m,3H,Leu CH-C$\underline{H_2}$-C$\underline{H}$), 1.37(two overlapped d,3H,Ala C$\underline{H_3}$), 1.27(t,3H, ethyl C$\underline{H_3}$), 0.9(m,6H,Leu C$\underline{H_3}$). Anal.calcd.for $C_{28}H_{38}FN_7O_8$: C 54.27, H 6.18, N 15.82; found: C 53.95, H 6.29, N 15.28. MS: m/z 620.64 (M^++1).

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