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NEW ENZYMATIC APPROACH TO THE SYNTHESIS OF CONVENIENT ASPARTIC ACID INTERMEDIATES IN PEPTIDE CHEMISTRY. SYNTHESIS OF N-BENZYLOXYCARBONYL-L-ASPARTIC ACID β-ALLYL ESTER.

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Abstract. Aspartic acid side chain protection by allyl ester functions has been facilitated by the direct synthesis of Z-Asp(OAll)-OH which can be performed by selective hydrolysis of Z-Asp(OAll)-OAll by means of papain catalysis. Both an optimization study and a batch synthesis are reported. Other synthetic routes are discussed.

The β-carboxyl group of aspartic acid, when in protected form, is a source of side reactions in peptide synthesis. Among them, the cyclization to aminosuccinimide (ASU) derivatives has been well characterized. This reaction can take place, for instance, during deprotection of aspartic acid β-benzyl esters under basic and acid treatment and even under hydrogenolysis conditions. This and related problems and their eventual solutions are well documented in the literature (1).

Recently, Kunz et al. introduced the allyl ester function as a temporary protecting group suitable for the synthesis of peptides and glycopeptides (2,3). Advantages such as easy synthesis, mild removal conditions and compatibility with most N-protecting groups could account for the convenience and wide number of possible applications. Mainly due to the mild deprotection conditions required including acid or basic treatment and Rd(I) or Pd(0) catalysis, this group may be of use for temporary side chain protection of aspartic acid. The main goal of examining this application is to avoid the rearrangement reactions of aspartyl residues leading to cyclic imides (4).

The development of methodologies making use of this group should consider the synthesis of intermediates bearing an allyl ester function on the side chain of aspartic acid. To test a synthetic route leading to such an intermediate we have chosen the synthesis of N-benzyloxycarbonyl-L-aspartic acid β-allyl ester (Z-Asp(OAll)-OH) which could be a suitable intermediate for peptide synthesis. For these purposes, synthetic routes such as the ones shown in schemes 1 and 2 can be devised. Regrettably, problems arising from limited compatibility of protecting groups due to the base labile characteristics of the allyl group and from the tendency of dipeptide formation, make compulsory the use of special reagents such as dibenzyl dicarbonate. After testing such routes neither scheme 1 nor 2 satisfactorily yielded the key derivative.

Z-Asp-OBzl ⟶ Z-Asp(OAll)-OBzl ⟶ Z-Asp(OAll)-OH

Scheme 1

Boc-Asp-OtBu ⟶ Boc-Asp(OAll)-OtBu ⟶ Asp(OAll)-OH ⟶ Z-Asp(OAll)-OH

Scheme 2

Although some of these problems can be circumvented by developing reaction pathways such as the one we have also examined (Scheme 3), the derivative Z-Asp(OAll)-OH is always obtained in low overall yields usually requiring purification steps by chromatographic procedures. Being aware of these problems, the present paper deals with the use of protease catalyzed reactions for the direct synthesis of aspartic acid allyl ester derivatives by following Scheme 4. As can be seen, starting from Z-Asp, the cesium salt of this derivative can be easily formed. By further reaction of this salt with allyl bromide, the diallyl ester can be obtained. A final treatment with papain in a buffer (McIlvine buffer, pH:5)-organic solvent (DMF) mixture in the presence of a reducing agent such as dithiothreitol (DDT) can led to Z-Asp(OAll)-OH with good yields. Both the feasibility of the synthesis and the possibility of relatively large scale production of this product have been examined. Moreover, considering reaction factors such as pH, buffer and organic solvent to be critical, an' optimization study has been carried out.

Boc-Asp-OtBu ⟶ Asp-OtBu ⟶ Z-Asp-OtBu ⟶ Z-Asp(OAll)-OtBu ⟶ Z-Asp(OAll)-OH

Scheme 3

Z-Asp-OH $\xrightarrow[2)\text{Allyl bromide}]{1)Cs_2CO_3}$ Z-Asp(OAll)-OAll $\xrightarrow[\text{DTT, DMF, McIlvine, pH:5}]{\text{papain}}$ Z-Asp(OAll)-OH

Scheme 4

In addition, to assist HPLC identification of the products obtanined by the enzymatic reaction, the synthesis of the α-allyl ester of Z-Asp has also been performed following the alternative route shown in scheme 5.

Z-Asp(OtBu)-OH ⟶ Z-Asp(OtBu)-OAll ⟶ Z-Asp-OAll

Scheme 5

For the selective hydrolysis of one of the two ester bonds on the Z-Asp(OAll)-OAll intermediate, the thiol protease papain was chosen due to its broad substrate specificity. Since trial experiments showed that the selective cleavage of the α-allyl ester was possible, an optimization study of reaction conditions was undertaken. Thus, the effect of pH, organic solvent and buffer nature on the yield of the hydrolysis product was studied. Two sets of experiments either containing dimethylformamide (DMF) or acetonitrile (ACN) as cosolvents have been performed. Moreover, two pH values chosen following the controversy about the optimum pH for hydrolytic or synthetic activities of the enzyme (5-9) have been examined by means of four different buffer compositions. Results are summarized in Table I.

Nearly quantitative yields were always achieved except for carbonate-ACN containing reactions. In this instance, no major differences could be observed among the conditions tested. However, reactions performed in ACN were much slower. This could be due to enzyme

denaturation caused by the solvent (10).

Minor differences in reaction performance could be noticed when DMF was used. Thus, pH:5 gave better yields than pH:9. Moreover, despite the fact that, in basic media carbonate led to slightly better yields than borate, the nature of the buffer solution did not dramatically influenced reaction yields.

Table I. Reaction yields of the papain catalyzed hydrolysis of Z-Asp(OAll)-OAll at different reaction conditions. Organic solvent proportion in the reaction media was always 58%. The mean values of three experiments are reported.

			Reaction yield (%)		
pH	Buffer	Cosolvent	1h	2.30h	24h
5	McIlvine	DMF	88.9	99.6	
5	McIlvine	ACN	31.2		97.0
5	Acetate	DMF	88.1	99.8	
5	Acetate	ACN	32.8		94.8
9	Carbonate	DMF	87.7	97.0	
9	Carbonate	ACN	7.4		18.1
9	Borate	DMF	84.7	93.0	
9	Borate	ACN	31.3		96.4

Buffer molarities were the following: McIlvine buffer (0.07M) (citric acid (0.1M) / dibasic sodium phosphate (0.2M), pH:5); Acetate buffer (0.1M) (acetic acid (0.2M) / sodium acetate (0.2M), pH:5); Carbonate buffer (0.05M) (sodium carbonate (0.2M) / sodium bicarbonate (0.2M), pH:9); Borate buffer (0.06M) (boric acid (0.2M) / borax (0.05M), pH:9).

On the other hand, although carbonate buffer in the presence of ACN was the worst reaction media, the remaining ACN containing reactions were not significantly influenced by pH. In such cases, a residual chemical hydrolysis has always been detected when carbonate (0.5% after 1h) and borate (1.2% after 1h) buffers were used.

To test the performance of this method at preparative level, a gram scale hydrolysis of Z-Asp(OAll)-OAll has also been successfully conducted. In summary, this and the above results make the enzymatic process a method of choice since higher chemical yields can be obtained, purification procedures can be avoided and bulk synthesis is possible.

Experimental.

The following aspartic acid derivatives Z-Asp, Z-Asp(OtBu)-OH and Z-Asp-OBzl were supplied by Bachem (Switzerland) while Boc-Asp(OtBu)-OH was obtained from Novabiochem (Switzerland). Papain (EC 3.4.22.2) from Papaya latex, type N, 2 x crystallized (13 U/mg of solid) was purchased from Sigma. Both 1,4 dithio-DL-threitol (DTT) and dibenzyl dicarbonate, commercially available from Fluka A.G. (Switzerland) were used as received. All the amino acid derivatives used, unless stated, were of the L-configuration.

Analytical thin layer chromatography (TLC) was routinely performed on precoated silica gel 60 plates of 0.2 mm thickness supplied by Merck, using two solvent systems: (1) petroleum ether/ethyl acetate/acetic acid (6:4:0.5)(v:v:v) (2) petroleum ether/ethyl acetate (8.1:1.9)(v:v). Spots were visualised by spraying the plates either with 40% perchloric acid

and charring or toluidine solutions. Column flash chromatography was performed using silica gel (40-63 μm) Merck on 15 x 5 cm column beds at a 5 cm/min of flow rate (11).

Enzymatic reactions were monitored by analytical HPLC on a Kontron Series 200 instrument using a 250 x 4 mm Spherisorb ODS-2 (10 μ) column, eluting with water (milli-Q grade) containing 0.05% trifluoroacetic acid (TFA) (HPLC grade) and acetonitrile of the same TFA content with a programmed gradient from 65/35 to 35/65 in 5 min and isocratic conditions for 8 min at a flow rate of 0.8 ml/min. Elution was spectrophotometrically monitored at 215 nm. The amount of eluted substances was calculated from peak areas by the external standard method on a D-2000 Hitachi-Merck chromatointegrator. HPLC solvents were filtered through 0.2 m filters and degassed with Helium prior use.

^{1}H-n.m.r. spectra were recorded on a Brucker (80 MHz) instrument from $CDCl_3$ or CD_3OH solutions. Chemical shifts are reported downfield from tetramethylsilane in ppm of the applied field. Optical rotations were measured on a Perkin Elmer 141 polarimeter using a 1 dm path cell.

Abbreviations currently used troughout the text are: *DMF (dimethylformamide)*, *ACN (acetonitrile)*, *DTT (1,4 dithio-DL-threitol)*, *THF (tetrahydrofurane)*, *MeOH (methanol)* and TFA (trifluoroacetic acid). Petroleum ether refers to the fraction boiling between 40-60°C.

1.- N-Benzyloxycarbonyl-L-aspartic acid diallyl ester. Z-Asp(OAll)-OAll. The method of Wang et al. (12) was used with minor modifications. Z-Asp (3.5 g, 13.1 mmol) was dissolved in 15 ml of peroxide-free THF and water was added dropwise until the solution became turbid. Neutralization was accomplished by addition of Cs_2CO_3 (4.3 g, 26.3 mmol) in 5 ml of water at room temperature. After a few minutes the solution was evaporated to dryness and the salt obtained was suspended in 15 ml of DMF and treated with 30 mmols of allyl bromide. The mixture was stirred 4h at room temperature. Evaporation of the solvent and excess reagent afforded an oil which was redissolved in ethyl acetate and washed with 5% $NaHCO_3$ solution amd water. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated. The pale yellow oil yielded after crystallization from a MeOH/water mixture a white solid (3.2 g, 9.4 mmol). M.P. 43-44°C. Yield 72%. Rf (1): 0.56. $[\alpha]_D^{25}$= -17.1 (c=1, MeOH). ^{1}H-n.m.r. ($CDCl_3$) s, 7.3, arom Z; m, 5.9, -CH= (All); m, 5.5, $=CH_2$ (All); s, 5.1, $-CH_2$ (Z); m, 4.6, C_α H (Asp), $-CH_2$ (All); t, 3.0, C_βH (Asp).

2.- N-Benzyloxycarbonyl-L-aspartic acid α-allyl ester. Z-Asp-OAll. The α-allyl ester of Z-Asp was synthesized following route depicted in Scheme 1 using the cesium salt method as described above. Thus, 2.0 g (6.2 mmol) of Z-Asp(OtBu)-OH were dissolved in 7 ml of THF and water was added. Neutralization was carried out with a 2.5 ml water solution containing 1 g (6.2 mmol) of Cs_2CO_3. After solvent evaporation the residue was suspended in 7 ml of DMF and 6.5 mmols of allyl bromide were added. The reaction mixture was kept at room temperature under stirring for 30 min. The mixture was worked up as already described and 2.1 g (5.9 mmol) of Z-Asp(OtBu)-OAll were obtained. Rf (1): 0.60. Yield 95%. Treatment of 1.6 g (4.5 mmol) of this intermediate with TFA/CH_2Cl_2 (5:5) during 60 min resulted in a complete hydrolysis of the tertbutyl ester group, affording an oily product (1.3 g, 4.2 mmol). Yield 94%. Rf (1): 0.28. $[\alpha]_D^{25}$= -20.6 (c=1, MeOH) ^{1}H-n.m.r. (CD_3OH) same chemical shifts as the diallyl intemediate except: d, 2.85, C_βH Asp.

3.- N-Benzyloxycarbonyl-L-aspartic acid β-allyl ester. Z-Asp(OAll)-OH. The chemical

synthesis of this intermediate was achieved following Scheme 3. Boc-Asp-OtBu (0.81 g, 2.8 mmol) was treated with 10 ml of TFA/CH_2Cl_2 (7:3) solution at room temperature during 30 min. After solvent evaporation 0.92 g (2.8 mmol) of a white solid residue were obtained in quantitative yield. This TFA.Asp-OtBu salt (0.88 g, 2.76 mmol) was dissolved in 10 ml of dioxan and approx. 10 ml of a 0.27N NaOH solution were added until neutral pH, under stirring at room temperature. To this solution, dibenzyl dicarbonate (0.79 g, 2.76 mmol) dissolved in 10 ml of dioxan was added dropwise. The reaction was left to continue for 30 min and acidified with diluted HCl to pH:2. After solvent evaporation the residue was dissolved in ethyl acetate and washed with 5% citric acid solution and water. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated affording 0.8 g (2.6 mmol) of an oil. Yield 94%. Rf (1): 0.64. $[\alpha]_D^{25}$= -15.9 (c=1, MeOH). ^{1}H-n.m.r. ($CDCl_3$) chemical shifts corresponding to Z and Asp are coincident to those of the diallyl intermediate and s, 1.45, tert-butyl.

The allyl ester of Z-Asp-OtBu was synthesized following the cesium salt method. Thus, 0.65 g (2.03 mmol) of Z-Asp-OtBu were treated as described above and a crude, oily residue was obtained. Purification by flash chromatography using as a solvent system a mixture of petroleum ether/ethyl acetate (81:19) yielded 0.24 g (0.66 mmol) of a pure oil. Yield 32%. Rf (1): 0.58, Rf (2): 0.22. $[\alpha]_D^{25}$= -2.16 (c=1, MeOH). ^{1}H-n.m.r. ($CDCl_3$) same chemical shifts as the diallyl intermediate and Z-Asp-OtBu.

Hydrolysis of the tert-butyl ester was achieved as follows. The intermediate Z-Asp(OAll)-OtBu (0.12 g, 0.33 mmol) was treated with 2 ml of TFA/CH_2Cl_2 (7:3) solution during 30 min. The solvent mixture was evaporated and the residue dissolved in ethyl acetate, washed with 5% citric acid solution and water. After drying, evaporation of the organic layer gave 0.1 g (0.31 mmol) of a pure oil. Yield 95%. Rf(1): 0.25. $[\alpha]_D^{25}$= -4.39 (c=1, MeOH). ^{1}H-n.m.r. (CD_3OH) same chemical shifts as the diallyl intermediate except: d, 2.85, C_βH Asp.

4.- Optimization of the papain catalyzed synthesis of N-benzyloxycarbonyl-L-aspartic acid β-allyl ester. As example of the enzymatic methodology followed through the optimization study a typical experiment is reported. To a 4.2 ml solution of Z-Asp(OAll)-OAll (0.10 g, 0.3 mmol) in either DMF or ACN, 5.8 ml of a buffer solution containing 4.5 mg of papain and 30 mg (0.19 mmol) DTT were added. The reactions were kept in a thermostatic bath at 25°C under stirring either 2.5 or 24 h depending on the reaction conversion rate. Monitoring was performed by removal of 100 μl samples which were diluted 1/11 with ACN/water (5:5), frozen and subsequently analyzed by HPLC. Chemically synthesized intermediates Z-Asp(OAll)-OAll, Z-Asp-OAll and Z-Asp(OAll)-OH were used for calibration purposes.

5.- Batch synthesis of N-benzyloxycarbonyl-L-aspartic acid β-allyl ester. To a solution obtained by dissolving 5.3 mg of papain and 40 mg (0.26 mmol) of DTT in 30 ml of McIlvine buffer pH:5, 10 ml of a DMF solution containing 1 g (3 mmol) of Z-Asp(OAll)-OAll were added dropwise during 4 h. The reaction was allowed to continue under stirring at 25°C until a clear solution was obtained. The pH of the solution was lowered to pH:2 with dilute HCl and lyophilized. The residue was suspended in ethyl acetate and washed with water. After drying and evaporation the organic layer rendered a pure oil (0.78 g, 2.56 mmol) with identical physical and spectroscopic properties to the chemically synthesized Z-Asp(OAll)-OH. Yield 86%.

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