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Diketopinic acid-a novel reagent for the modification of arginine[§]

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Abstract. Diketopinic acid has been synthesized and shown to be a reagent of choice for specific, reversible modification of the guanidine groups of arginine residues. Diketopinic acid is a yellow crystalline substance and the carboxyl group of the reagent is a convenient handle for attachment to other molecules. The adducts of diketopinoyl derivatives with the guanidine group are cleaved by 0·2 M *o*-phenylenediamine at pH 8–9. The modification and regeneration of arginine and of arginyl residues in soyabean trypsin inhibitor and insulin are presented as demonstrations of the use of the reagent. The use of diketopinoyl resin in the separation of oxidized A and B chains of insulin has been discussed.

Keywords. Diketone; reversible modification of arginine residues; aminopolymer; diketopolymer.

1. Introduction

Tremendous progress has been made in the development of protecting groups for various functionalities in proteins and amino acids. Protection of functional groups is not only important in the synthesis of polypeptides, but in the form of modification of amino acid residues in proteins it offers a useful technique of selective chemical and enzymatic cleavage of large protein molecules. Modification of specific residues in biologically active proteins may render them inactive or alter their activity and are useful probes for the active sites. Efforts have been directed to develop new amine or carboxyl-protecting groups, which can be introduced and cleaved under natural conditions *e.g.* neutral or near neutral pH, lower temperatures and physiological conditions. The choice is very limited in the selection of protecting groups for the protection of the guanidine function of arginine. Pande *et al* (1980) successfully introduced a new, bifunctional reagent, camphorquinonesulphonic acid for the protection of the guanidino group of arginine in peptides and proteins. Camphorquinonesulphonylnorleucine was found to be a reagent of choice when the work had to be carried out with larger peptides and proteins. The sulphonic acid gave it a high solubility in water, but there were limited ways to activate the group and the isolation and purification of the reagent was cumbersome.

Continuing our work on the development of 1,2-diketones with a suitable handle,

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Abbreviations used: DKPA, Diketopinic acid (2,3-diketo-10-bornanoic acid); STI, soyabean trypsin inhibitor; DMF, dimethylformamide; DCHA, dicyclohexylamine.

we have now developed a bifunctional reagent, the diketopinic acid (DKPA) which possesses a carboxylic group, for the protection of the guanidino group of arginine. The adduct of DKPA is quite stable and resistant to hydrolysis by acid or base. The carboxylic group of the reagent serves as a handle for attachment on to a polymeric support and the 1,2-diketo group specifically protects the guanidino side chain of arginine. The modification is done at pH 7·5–7·8 in borate buffer and is removed by 0·2 M *o*-phenylenediamine.

2. Materials and methods

d-Camphor-10-sulphonic acid was purchased from Aldrich. Soyabean trypsin inhibitor (STI) and insulin were purchased from the Sigma Chemical Co., St. Louis, Mo, USA. Solvents were purified by standard methods before use. Thin-layer chromatography (TLC) was carried out on silica gel-G plates. Quantitative estimation of amino acids was carried out on a Beckman 121 automatic amino acid analyser. Hydrolysis of proteins was carried out with 6N HC1 at 110° for 22 h in sealed evacuated tubes.

2.1 *Preparation of d-camphor-10-sulphonyl chloride*

Chloride of d-10-camphorsulphonic acid was prepared using phosphorous pentachloride according to the method reported by Sutherland and Shriner (1936). The white material was washed several times with cold water. The crude product melted at 81–83°C (yield 80%) and was used as such in the next step.

2.2 *Preparation of ketopinic acid*

Ketopinic acid was prepared by the action of potassium permanganate on d-10-camphorsulphonyl chloride following the method of Bartlett and Knox (1939). Crude ketopinic acid has a melting point (mp) 233–235°C, yield 20%. The product was recrystallized from hot water.

2.3 *Preparation of DKPA*

Ketopinic acid (1·82 g, 0·01 mol) was dissolved in 20 ml of dioxane. SeO_2 (1·42 g, 0·011 mol) was added and the mixture was refluxed gently for 72 h. Precipitated selenium was filtered and the dioxane solution was concentrated on a rotary evaporator. The oily residue was dissolved in aqueous methanol and SO_2 was passed for 24 h. Colloidal red selenium was removed with the help of celite filter aid. Most of the methanol was removed on a rotavapor. The aqueous solution was extracted with ether several times. The combined extracts were dried over anhydrous sodium sulphate and the solvent was removed to give a yellow solid. This solid was dissolved in sodium bicarbonate solution, the solution washed with ether and the product reprecipitated by acidifying the solution. The solid was recrystallized from ethanol-water. An oil which separated, crystallized on cooling. The crystalline solid was purified by column chromatography on a column of silica

gel. The narrow yellow-coloured band was eluted with chloroform and the product was recrystallized from ethanol-water (yield 51·0%, mp 233–235°C). The homogeneity of the compound was checked on TLC in EtOAc: C_6 H_6: AcOH in the ratio of 10:10:0·2 (R_f, 0·57) and $CHC1_3$: CH_3OH : AcOH, 20:1:0·5 (R_f, 0·83)

IR /cm $^{-1}$: 2640–2720 (COOH, b), 1710 (COOH, s), 1760 (CO, s), 1385 and 1370 (gem-dimethyl) .

^{1}H NMR ($CDC1_3$) δ/ppm: 0·95 (s, 3H, CH_3); 1·15 (s, 3H, CH_3); 2·25 (d, 2H, CH_2), 2·75 (t, 2H, CH_2); 3·3 (d, 1H, -CH); 9·1 (s, 1H, COOH)

Anal: Calculated for C_{10} $H_{12}O_4$: C, 61·22; H, 6·12.
Found: C, 61·17; H, 6·21.

2.4 *Preparation of dicyclohexylamine salt of DKPA*

DKPA (10 mg) was dissolved in solvent ether. To this was added an ethereal solution of dicyclohexylamine (DCHA). Crystals started forming soon afterwards. These were collected by filtration and washed with ether.

Anal: Calculated for C_{22} $H_{35}NO_4$:C, 70·03; H, 9·28; N, 3·71
Found: C, 70·14; H, 9·38; N, 3·75.

2.5 *Arginine binding with DKPA*

Borate buffer was prepared by dissolving a 12·367 g of boric acid in 100 ml of 1 N NaOH solution in one litre of water (sodium tetraborate 0·05 M). The pH of this solution was adjusted to 7·8 by the addition of the required amount of 0·1 N HCl.

To 2 ml of borate buffer in a 50 ml round-bottomed flask, arginine hydrochloride (2·1 mg, 0·01 mmol) and DKPA (2·94 mg, 0·015 mmol) were added. The contents were stirred for 20 h on a magnetic stirrer and the reaction was monitored by TLC in BuOH : AcOH : H_2O (4: 1: 1) and spraying with ninhydrin. After two hours the adduct started showing up. In 20 h all the arginine had disappeared from the reaction mixture. The adduct of arginine and DKPA had an R_f 0·08.

2.6 *Specificity of DKPA for arginine*

A solution containing 2·5 μm each of the 18 amino acids in dilute HCl (calibration mixture for amino acid analysis) was evaporated to dryness. The residue was dissolved in 2 ml of water containing 0·2 ml of triethylamine and lyophilized. The lyophilized residue was taken in 2 ml of 0·2 M sodium borate buffer, pH 9 and the pH was readjusted to 9. Samples (0·7 ml each) of this stock solution were withdrawn. The first sample was treated with 0·3 ml of a solution of DKPA (10 mg/ml) in sodium borate buffer at pH 9. The second sample was added to 0·3 ml of borate buffer. After 12 h samples of each of these mixtures were diluted with sodium citrate buffer, pH 2·2 and subjected to quantitative amino acid analysis. The two solutions had the same amino acid composition except that the arginine

content of the DKPA-treated sample was decreased to 7% of that in the control solution (table 1).

2.7 *Reversal of DKPA-modification of arginine with o-phenylenediamine*

The solution of DKPA-arginine adduct from the experiment described earlier was subjected to preparative TLC. The band containing the DKPA-arginine adduct was scrapped and the material was eluted with warm water. The solution was lyophilized and the residue was taken in 2 ml of 0·2 M solution of *o*-phenylenediamine at pH 8·5. The solution was held at 37°C and analysed at time intervals by TLC after spraying with ninhydrin. The regeneration of arginine from starting material in *o*-phenylenediamine was 75% complete after 4h. After overnight incubation, the starting material had completely vanished and the only ninhydrin-positive spot corresponded to arginine.

2.8 *Modification of STI with DKPA*

STI (20 mg) was added to a solution of DKPA (10 mg) in 4 ml of 0·2 M sodium borate buffer, pH 9. The solution was incubated at 37°C for 24 h in dark. It was then dialysed (3500 molecular weight cut-off membrane) against water (4 × 21) at 4°C and lyophilized. A few mg of modified STI were treated with *o*-phenylenediamine (0·2 M, pH 8·5) for 20 h in dark. The solution was then dialysed, filtered and lyophilized. Native STI, DKPA-STI and *o*-phenylenediamine treated DKPA-STI were subjected to gel electrophoresis on 15% polyacrylamide slabs at pH 8·6 in Tris/borate buffer, pH 9, and a constant current of 35 mA. The protein band was stained with Commassie blue G-250 in 6% (wt/vol) $HC1O_4$. The DKPA-

Table 1. Effect of DKPA incubation of standard amino acid mixture.

Amino acid	Control	DKPA treated
Asp	1·00	1·00
Thr	1·00	0·95
Ser	1·00	0·95
Glu	1·00	1·00
Pro	1·00	1·00
Gly	1·00	0·93
Ala	1·00	1·00
Cys	0·50	0·50
Val	1·00	1·00
Met	1·00	0·96
Ile	1·00	1·00
Leu	1·00	1·00
Tyr	1·00	0·90
Phe	1·00	1·00
Lys	1·00	0·98
His	1·00	0·98
$(NH_4)_2SO_4$	1·00	0·93
Arg	1·00	0·07

STI moved ahead of the native STI in this electrophoretic system. Regenerated STI had the same electrophoretic mobility as the native protein.

2.9 *Modification of insulin by DKPA*

Bovin insulin (10 mg) was added to a solution of DKPA (10 mg) in 4 ml of 0·2 M sodium borate buffer, pH 9. The solution was incubated at 37°C for 24 h in the dark, then dialysed (3500 molecular weight cut-off membrane) against water (four times, 2 litres each) at 4°C and lyophilized.

Modified insulin (10 mg) was dissolved in 5 ml of 0·2 M phosphate buffer, pH 7 and was treated with 0·5 mg of trypsin in 0·5 ml of the same buffer. A control experiment was run with native insulin under the same conditions. After 2 h at 25°C, the solutions were dialysed against aqueous acetic acid (2 ml/1) and lyophilized.

Samples of lyophilized powder were hydrolyzed and subjected to amino acid analysis. The trypsin-treated native insulin showed a significant loss of Phe, Thr and Lys, whereas the trypsin-treated DKPA-modified insulin had almost the same amino acid composition as the native insulin itself (table 2).

2.10 *Preparation of diketopinoyl chloride*

DKPA (570 mg, 5 mmol) was treated with thionyl chloride (2 ml) in dimethyl-formamide (DMF) (2 ml) in a 50 ml round-bottomed flask in an ice bath. The mixture was stirred under exclusion of moisture for 2 h at ice bath temperature and for 1 h at room temperature. The product was freed of thionyl chloride by adding dry benzene and removing the azeotrope on a rotavapor repeatedly and was used without further purification.

Table 2. Amino acid composition of modified insulins.

Amino acid	Native bovine insulin	Trypsin treated insulin	Trypsin treated DKPA-insulin
Asp	2·97	2·89	2·90
Thr	1·00	0·10	0·92
Ser	2·95	2·87	2·88
Glu	6·81	6·90	6·83
Pro	0·88	0·05	0·85
Gly	3·90	2·88	3·88
Ala	2·95	1·85	2·12
Val	5·00	5·00	4·88
Ile	0·98	0·88	0·98
Leu	6·00	6·00	6·00
Tyr	3·82	2·68	3·85
Phe	2·96	0·91	2·87
Lys	0·86	0·00	0·83
His	1·91	1·85	1·83
Arg	0·88	0·87	0·87

2.11 *Preparation of cross-linked polyacrylamine from polyacrylamide, P-NH_2*

Cross-linked polyacrylamide (Biogel P4, 200-400 mesh) 10 g, was treated with 50 ml of sodium hypochlorite "Chlorox" in a cylindrical glass vessel having a sintered disc at the bottom further connected to a stopcock. The reaction was allowed to run with a stream of nitrogen bubbling through the bottom which kept the polymer well-stirred under inert atmosphere. The reaction was allowed to run for 6 h at 4°C after which the polymer was filtered and thoroughly washed under nitrogen. It gave a positive test with Kaiser reagent. This resin was stored under nitrogen.

2.12 *Preparation of diketopinoyl cross-linked polyacrylamine*

Cross-linked polyacrylamine (5 g) was suspended in an alkaline solution of sodium bicarbonate (pH 8·5). Diketopinoyl chloride (589 mg, 3 mmol) was dissolved in dioxane and added to the polymer. The reaction mixture was stirred vigorously for 5 h on a magnetic stirrer after which it was thoroughly washed with aqueous dioxane and water. The resulting polymer had a yellow colour.

2.13 *Oxidation of insulin*

Performic acid was prepared by adding 0·1 ml of 30% H_2O_2 to 1·9 ml of 99% formic acid and allowing the solution to stand in a stoppered flask at room temperature for 2 h. In another flask 10 mg of insulin was dissolved in 1 ml of 99% formic acid. The insulin and performic acid solutions were cooled to 0°C and mixed slowly. Reaction was allowed to proceed for 3 h at the same temperature. The formic acid was then partly removed on a rotavapor at 20°C. Cold water (5 ml) was added to the reaction mixture, the solution was shell frozen at once and lyophilized.

Table 3. Amino acid composition of bovine insulin chains.

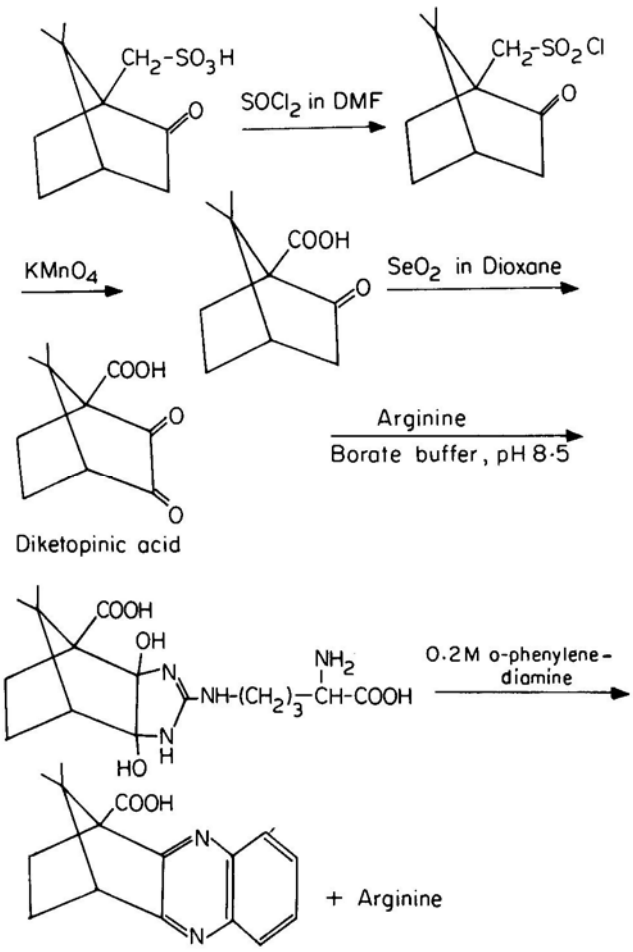
Amino acid	Oxidized A chain (calculated)	Oxidized B chain (calculated)	Fraction A	Fraction B
Cysteic acid	4·00	2·00	3·95	1·84
Asp	2·00	1·00	1·88	0·98
Thr		1·00		0·90
Ser	2·00	1·00	1·98	0·89
Glu	4·00	3·00	3·85	2·88
Pro		1·00		0·97
Gly	1·00	3·00	0·95	3·01
Ala	1·00	2·00	0·93	2·00
Val	2·00	3·00	1·8	2·98
Ile	1·00		1·01	
Leu	2·00	4·00	2·00	4·00
Tyr	2·00	2·00	1·89	1·88
Phe		3·00		2·99
Lys		1·00		0·93
His		2·00		1·85
Arg		1·00		0·88

2.14 *Separation of oxidized insulin A and B chains*

The diketopinoyl-polyacrylamine beads (5 g) were packed in a 20 cm column in 0·2 M sodium borate buffer, pH 8·5. Oxidized insulin dissolved in 5 ml of the same buffer was allowed to pass slowly through the column, which was then eluted with the same borate buffer. The eluate (50 ml) was dialysed in a dialysis bag (2000 molecular weight cut-off) against 4 × 11 distilled water and lyophilized (fraction A).

The column was then eluted with 0·2 M *o*-phenylenediamine (100 ml) in borate buffer pH 9. The eluate was dialysed in a dialysis bag (2000 molecular weight cut-off) against 5 × 11 distilled water, filtered from a little particulate matter and lyophilized (fraction B).

The fractions were hydrolysed and subjected to amino acid analysis. Amino acid composition of fraction A matched with that of oxidized A chain. Fraction B had the same amino acid composition as that of oxidized B chain (table 3).

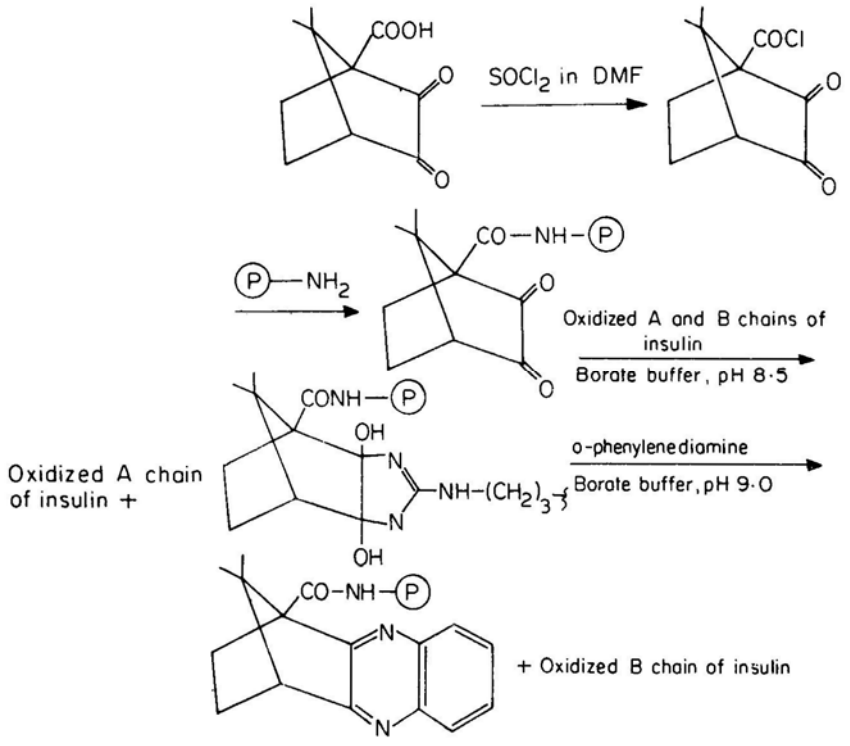


Scheme 1.

3. Results and discussion

DKPA synthesized from camphor-10-sulphonic acid in three steps has been found to be a promising reagent for the reversible protection of the guanidino group of arginine or arginine containing peptides (scheme 1). DKPA like the camphor-quinonesulphonic acid is a bifunctional reagent, the diketo moiety of which offers reversible protection to arginine. The carboxyl function of the reagent serves as a handle for covalent attachment to an insoluble support. DKPA reacted only with arginine from a standard mixture of amino acids in borate buffer. Insulin was modified at its arginine residue and the product resisted proteolysis with trypsin. STI was modified with DKPA as evidenced from the alteration of its electrophoretic mobility. Treatment with *o*-phenylenediamine removed the protection. The regenerated STI had the same electrophoretic mobility as the native STI.

Cross-linked polyacrylamine was prepared by the reaction of sodium hypochlorite on cross-linked polyacrylamide under nitrogen. During this reaction, the carboxamide groups of the polymer were converted into amino groups on a matrix which is compatible with aqueous media. Amino functions of this polymer were acylated with DKPA through its chloride. The derivatized polymer which



Scheme 2.

possessed 1,2-diketo ligands, when packed in a column in borate buffer, picked up only arginine from a mixture of amino acids passing through it. Furthermore, when a mixture of oxidized A and B chains of insulin in borate buffer, pH 8·5 was passed through the column, it retained only the B chain by reacting with its arginine residue. The A chain which did not contain arginine passed through the column unretarded. The column was then eluted with *o*-phenylenediamine at pH 9. Oxidized B chain was released in 98% yield (scheme 2). A column of this kind is, therefore, useful in batch type seperation of peptides with arginine residues from those which do not possess arginine.

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References

Pande C S, Pelzig M and Glass J D 1980 Camphorquinone-10-sulfonic acid and derivatives, convenient reagents for reversible modification of arginine residues; *Proc, Natl. Acad. Sci. USA* **77** 895–899

Sutherland H and Shriner R L 1936 Anamalous mutarolation of salts of Reychler acid IV; *J. Am. Chem. Soc.* **58** 62–63

Bartlett P D and Knox L H 1939 Bicyclic structures prohibiting the Walden inversion; *J. Am. Chem. Soc.* **61** 3184–3192