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SYNTHESIS OF NEW PHOSPHONATE INHIBITORS OF SERINE PROTEASES

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Abstract: Analogues of phenylalanine and lysine esters were synthesized, and their inhibitory power tested in vitro respectively on chymotrypsin, trypsin and urokinase.

Electrophilic phosphonate analogues of N-acylated amino acids (e. g. structures **4** and **12**) are potent inhibitors of serine proteases[1]. These enzymes are involved in a number of important physiological processes. Their detuning creates a pathological state implying local degradation of the extracellular matrix and breaking of intercellular bridges. Especially, the urokinase type plasminogen activator (uPA) seems to play a key role in inflammatory reactions, ovulation, fertilization, and embryogenesis . Increased uPA activities have been reported in different diseases including pulmonary fibrosis, arthritis, dermatitis and neoplasia[2]. According to its substrate specificity, uPA should be selectively inhibited by the phosphonate mimics of N-acylated lysine or arginine active esters.

This paper describes the preparation of these original compounds. To get the phosphonate analogues of lysine **12b-c**, we first optimized a relevant strategy by synthesizing the phenylalanine mimics **4c-d**. In vitro, **4c-d** inhibit chymotrypsin, and **12b-c** trypsin and (less efficiently) urokinase.

The strategy

The ultimate retrosynthetic terms leading to **12b-c** are N-5 protected derivatives, and the penultimate ones are a phosphonic acid or his monoester. The N-5 protecting group has to withstand the conditions used to transform a phosphonic acid into an active ester, and vice versa, the active ester has to withstand the N-5 deprotection. A suitable protection for the 5-amino function is t-Boc, which can readily be removed under anhydrous acidic conditions, without altering the phosphonate ester. The generated amine is protected from autocondensation by protonation. We had thus to discover a way of transforming phosphonic acids into active esters (a rather tricky step for polyfunctional molecules[3]) that does not cleave t-Boc.

We found that bisarylsulfites (e.g. $(m\text{-}ClC_6H_4O)_2SO$) in pyridine are excellent reagents for this purpose[4]. As an example, step iv in Scheme 1 is described : thionyl chloride (4.8 mmol) was added to a solution of distilled m-chlorophenol (10.2 mmol) in dried pyridine (10 mL) at -40°C. A precipitate of pyridinium chloride rapidly appeared. The mixture was magnetically stirred for one hour at -40°C. Compound **3a** (1.2 mmol), dissolved in pyridine (5 mL), was then added. The mixture was stirred overnight in the cold room (-20°C), evaporated, and the residue was dissolved in a slurry of methylene chloride/phosphate buffer (pH 7). The decanted organic phase was dried over magnesium sulfate, filtrated, and evaporated. The residue was

chromatographed on silica. The excess of m-chlorophenol was first washed out by benzene (TOXIC: use the fume hood), and compound **4d** was then eluted by ethyl acetate. Analytically pure **4d** (0.68 mmol, 57%, oil) was obtained by a subsequent preparative TLC (eluent, benzene/ethyl acetate, 80:20). Analytically pure **4c** (52%, oil) was obtained from **3b** along the same line[5].

The compounds **4c-h** allowed us to optimize the nature of the aryl leaving group, in order to get a rapid inhibition of serine proteases (in this instance, of chymotrypsin) and a relatively slow spontaneous hydrolysis at pH 7 [6]. Nitrophenyl esters **4e-f** were too labile[7]. Phenyl esters **4g-h** reacted too slowly with the enzyme[8]. Phosphonate esters derived from m-chlorophenol **4c-d** constituted a good compromise[9].

Having in hand the required method of activation of phosphonates, we undertook the synthesis of the target lysine analogues.

The synthesis

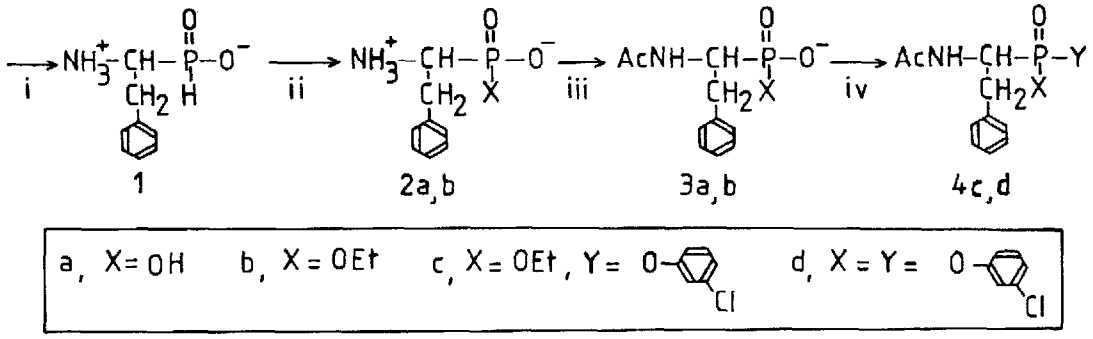
Our synthetic approach is outlined in Scheme 2. We started with the phtalimido protection on N-5, because it appeared to us to be one of the few protecting groups able to resist to the very harsh conditions of the assembly of the N-C-P skeleton according to Baylis et al.[10], and to the selective deprotection of the 1-amino (step ii). A drawback was the excessive sensitivity of the phtalimido function towards ring opening in basic conditions[11]. Accordingly, the acetylation (step iv) was optimized : a suspension of partially dissolved **7b** (3.7 mmol) was stirred in water at 0°C. Triethylamine (6.3 mmol) was added for complete dissolution, and, immediately after, acetic anhydride (5.2 mmol). The reaction was quenched after ca. 1 min by lowering the pH to 5 with 1M acetic acid. The mixture was evaporated, then coevaporated several times with 0.01M HCl to decompose the residual triethylammonium acetate. The residue was taken up in a small amount of pyridine, and the insoluble triethylammonium chloride was removed by filtration. The yield was quantitative.

The activation of the phosphonate function (Scheme 2, step vii) and the isolation of **11b-c** were performed exactly as described for the phenylalanine analogues **4c-d**, and similar yields were obtained (**11b**, 61%, oil[12]; **11c**, 60%, oil). 200Mhz ^{1}H NMR ($CDCl_3$) delta$_{TMS}$, ppm : **11b**, 7.2 (4H, m, aryl), 6.7 (1H, d, J=10 Hz, AcNH), 4.65 (2H, m, NCH and t-BocNH), 4.2 (2H, m, $-CH_2-CH_3$), 3.1 (2H, m, NCH_2), 2.0 (3H, s, CH_3CO-), 1.2-2.0 (6H, m, $-(CH_2)_3$-), 1.4 (9H, s, $(CH_3)_3C$-), 1.25 (3H, t, J=7.5 Hz, $-CH_2-CH_3$). **11c**, 7.15 (8H, m, aryls), 6.5 (1H, d, J=10 Hz, AcNH), 4.8 (1H, m, NCH), 4.6 (1H, m, t-BocNH), 3.1 (2H, m, NCH_2), 1.95 (3H, s, CH_3CO-), 1.2-2.0 (6H, m, $-(CH_2)_3$-), 1.4 (9H, s, $(CH_3)_3C$-).

Aliquots of compounds **11b-c** were deprotected by trifluoroacetic acid immediately prior to use in biological tests. The deprotection was complete after half an hour, as demonstrated by NMR monitoring of the $-C(CH_3)_3$ signal in neat CF_3COOD.

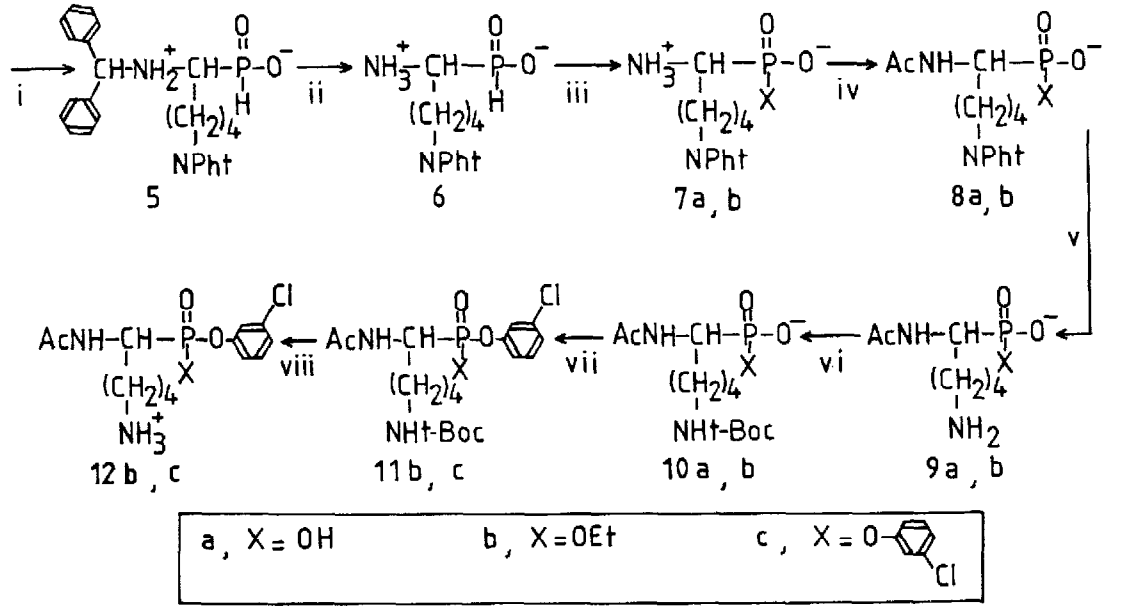
Inhibition of trypsin and urokinase[6,12]

The synthesized lysine analogues were potent inhibitors of trypsin (e.g. **12b**, $k_{(hydrolysis)} = 3\ 10^{-6}$ sec^{-1}, $k_i/K_I = 2.6\ 10^3\ M^{-1}sec^{-1}$). In vitro, compounds **12b** and **12c** inhibited 50 percent of the activity of urokinase in about 30 min at micromolar concentrations. The inhibition of urokinase did not follow a first order kinetics. There was evidence of spontaneous reactivation of the inhibited enzyme.



(i): ref. 10; (ii): **2a**, satd. Br_2/H_2O, 1.5 eqv., 75°C, 40 min, yield 80%; **2b**, I_2/EtOH, 1 eqv., reflux, 2h, yield 75% ; (iii): AcOAc 3 eqv., NEt_3 7 eqv., H_2O, 0°C, 2h, yield 100%; (iv): see text.

SCHEME 1



(i): ref. 10; (ii): neat CF_3COOH under argon, reflux, 1h, yield 81%; (iii): **7a**, satd. Br_2/H_2O, 2 eqv., 70°C, 40 min, yield 60%; **7b**, I_2/EtOH, 1 eqv., reflux, 30 min, yield 87%; (iv): see text; (v): 1°) MeOH, NH_2NH_2 1 eqv, 80°C, 1h, 2°) HCl to pH 2, remove the precipitated phthalhydrazide, 3°) precipitate the product in MeOH by addition of propylene oxyde, yields, **9a**, 60%, **9b**, 73%; (vi): EtOH, NEt_3 5 eqv., $(t\text{-ButOCO})_2O$ 1.4 eqv, R.T., 3h, yield 100%; (vii): see text; (viii): neat CF_3COOH, R.T., 30 min.

SCHEME 2

Interestingly, murine brain acetylcholinesterase was not significantly inhibited by these compounds, even at a 100 micromolar concentration. After 30 min of incubation (37°C, pH 7.4), the activity was reduced by less than 5 percent.

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References and notes

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4 These reagents were used to obtain active esters from N-acylated amino acids : Yamazaki, N., Higashi, F., and Niwano, M., *Tetrahedron* **30**, 1319 (1974).

5 Anal. , **4d,** Calc. for $C_{22}H_{20}Cl_2NO_4P$, C, 56.91; H, 4.34; N, 3.02. Found, C, 56.74; H, 4.56; N, 2.83. **4c**, Calc. for $C_{18}H_{21}ClNO_4P$, C, 56.63; H, 5.54; N, 3.67. Found, C, 57.03; H, 5.80; N, 3.65.

6 The hydrolysis rate was measured in a 0.05M phosphate buffer, pH 7.0. To determine k_i/K_I ($M^{-1}sec^{-1}$), chymotrypsin (or trypsin) was incubated in the same buffer with various concentrations of the inhibitors, and the residual activity was periodically checked using AcPhe(p-nitrophenyl)ester (or Z-Lys(p-nitrophenyl)ester) as substrate. The measurements were performed on mixtures of enantiomers or diastereoisomers.

7 e.g. **4e**, X=methoxy, Y=p-nitrophenoxy, $k_{(hydrolysis)}=4.5\ 10^{-2}\ sec^{-1}$, $k_i/K_I=5.0\ 10^4\ M^{-1}sec^{-1}$; **4f**, X=methoxy, Y=m-nitrophenoxy, $k_{(hydrolysis)}=2.5\ 10^{-4}\ sec^{-1}$, $k_i/K_I=2.1\ 10^4\ M^{-1}sec^{-1}$.

8 e.g. **4g**, X=methoxy, Y=phenoxy, $k_i/K_I=12.0\ M^{-1}sec^{-1}$; **4h**, X=Y= phenoxy, $k_i/K_I=36.0\ M^{-1}sec^{-1}$.

9 **4c**, $k_{(hydrolysis)}=2.1\ 10^{-5}\ sec^{-1}$, $k_i/K_I=1.56\ 10^3\ M^{-1}sec^{-1}$. **4d**, $k_i/K_I=2.55\ 10^4\ M^{-1}sec^{-1}$.

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11 Wolfe, S., and Hasan, S.K., *Can. J. Chem.* **48**, 3572 (1970).

12 Anal., **11b,** Calc. for $C_{20}H_{32}N_2O_6PCl$, C, 51.89; H, 6.97; N, 6.05. Found, C, 51.70; H, 7.03; N, 5.79.

13 The inhibitory activity of **12b** and **12c** was evaluated by incubating urokinase (from Sigma) during 30 min (37°C, pH 7.4) with different concentrations of the compounds. Inhibition was stopped by diluting the preparation in 50 mM Tris-HCl buffer (pH 7.4) containing 0.7 mM S-2251 (a chromogenic substrate of plasmin from Kabi Vitrum) and 0.165 CU/mL of plasminogen. The remaining activity was assessed spectrophotometrically (405 nm) by following the acceleration of p-nitroaniline production as described by Drapier, J.C., Tenu, J.P., Lemaire, G., and Petit, J.F., *Biochimie* **61**, 463 (1978).

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