Biochimica et Biophysica Acta, 341 (1974) 284–290

BBA 67175

INHIBITION OF α-CHYMOTRYPSIN BY HYDROXYMETHYL ANALOGUES OF D- AND L-*N*-ACETYLPHENYLALANINE AND *N*-ACETYLTRYPTOPHAN OF POTENTIAL AFFINITY LABELING VALUE

J. BRYAN JONES, DAVID W. SNEDDON and ARTHUR J. LEWIS
Department of Chemistry, University of Toronto, Toronto, Ontario, M5S 1A1 (Canada)
(Received October 22nd, 1973)

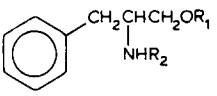
SUMMARY

D- and L-*N*-acetyl- and *N*,*O*-diacetylphenylalaninol and -tryptophanol are found to be competitive inhibitors of α-chymotrypsin with K_i values in the range 4.3–65.7 mM. In each case, the N,O-diacetates are better inhibitors than the corresponding hydroxymethyl compound and although hydrogen bonding of hydroxymethyl groups at the nucleophilic site (*n*) does contribute to binding, interactions of O-acetate functions with this region are much more significant. The tryptophanol derivatives are more effective by a factor of about 5 than those of phenylalaninol and D enantiomers bind approximately twice as strongly as their L counterparts. The results obtained indicate that phenylalaninol, or preferably tryptophanol, structures of the types studied are suitable for use as alkylating group carriers in affinity labelling experiments designed to probe the more remote amino acid residues of the *n*-region of the active site. They are also of potential value for X-ray and NMR studies on the differences between the binding of D and L enantiomers by α-chymotrypsin.

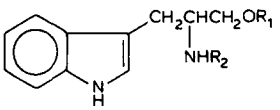
INTRODUCTION

So far, all affinity labelling of α-chymotrypsin by alkylating agent analogues of specific and non-specific substrates has resulted only in modification of amino acid residues at or near to the active site [1, 2]. With a view to achieving specific alkylation of residues *exo* [2] to the active site, such as His-40, we became interested in preparing modified affinity label structures which would combine specificity of interaction with the enzyme with a capacity for elaboration of their functional groups into alkylating agents capable of attacking remote amino acid residues of the amide $(am)^{[3]}$- and nucleophilic $(n)^{[3]}$-active site regions.

The basic structures considered were those of phenylalaninol (Compound 1a) and tryptophanol (Compound 2a). It was hoped that in the binding of such compounds at the active site [4], interaction of the phenyl or indole ring(s) (see next page). with the aromatic locus (*ar*-locus) [3] and hydrogen bonding of the hydroxymethyl groups with Ser-195 would ensure the desired orientation of the amino group (carrying an appropriate [1, 2, 5–7] alkylating function) towards the *am*-binding region.



1



2

a, $R_1 = R_2 = H$
b, $R_1 = R_2 = Ac$
c, $R_1 = H, R_2 = Ac$

Alternatively, with an N-acyl group present, as in compound 1c and compound 2c, selected alkylating functions could be attached to the hydroxymethyl group, thereby enabling the more distant residues of the *n*-locus to be probed. Before proceeding with the alkylation aspect of this approach, the abilities of compounds such as compound 1 and compound 2 to interact with the active site were evaluated. The results obtained established that both D- and L-enantiomers of compounds 1b and 1c and Compounds 2b and 2c act as competitive inhibitors of α-chymotrypsin and that such structures would be suitable as affinity label carriers of the types desired.

MATERIALS AND METHODS

In the preparative work, the criteria of purity applied, the methods and analytical instrumentation used, specifications of chromatographic supplies, solvents etc., were as described previously [5–7]. *N*-Acetyl-L-tyrosine ethyl ester and D- and L-phenylalanine and -tryptophan were purchased from Mann Research Laboratories and the latter were converted into their methyl ester hydrochlorides by the standard thionylchloride–methanol procedure of Brenner and Huber [8]. α-Chymotrypsin (3 times recrystallized) was obtained from the Worthington Biochemical Corp. R_F values refer to thin-layer chromatography analyses on silica gel G. All compounds prepared were purified to constant optical rotation.

Synthesis of amino acid derivatives

Since both the D- and L enantiomers of each compound were prepared and purified by identical procedures, experimental details are given for the L series only.

D-*and* L-*phenylalaninol (Compound 1a)*

L-Phenylalanine methyl ester hydrochloride (15 g, 70 mmoles) was added a little at a time during 1 h to a vigorously stirred slurry of lithium aluminum hydride (7 g, 0.18 moles) in dry tetrahydrofuran (250 ml). After stirring the mixture for a further 24 h, wet tetrahydrofuran (200 ml) was added and the mixture filtered. Evaporation of the filtrate gave a colourless oil which was first triturated with diethyl ether and then recrystallized from benzene to give L-phenylalaninol (Compound 1a, 5.3 g), m.p. 91–92 °C; R_F 0.4 butanol–acetic acid–water, 3:1:1; by vol.); $[\alpha]_D^{25}$ (*c* 1.2, ethanol) −21.7° (ref. 9, m.p. 95 °C, $[\alpha]_D$ (*c* 2, methanol) −23.3°); infrared (Nujol) 3500–3000 cm^{-1} (broad), 3290 and 3350 cm^{-1}, and 1580 cm^{-1}; NMR (^3H-labelled chloroform) δ 2.19 (3H, broad s, exchangeable with ^2H_2O, NH_2 and OH), 2.65 (2H, two q, *J* (gem.) = 13 Hz, *J* (vic.) = 5 and 8 Hz, C-3 H), 3.10 (1H, m, C-2 H), 3.50 (2H, two q, *J* (gem.) = 10 Hz, *J* (vic.) = 4 and 7 Hz, C-1 H) and 7.25 ppm (5H, broad s, C_6H_5); mass spectrum, *m/e* 151 (M^+).

The D-enantiomer of Compound 1a had $[\alpha]_D^{25}$ (*c* 1.3, ethanol) +24.6°, and m.p., R_F and spectral properties identical with those of the L enantiomer of Compound 1a.

Analysis. Calcd for $C_9H_{13}NO$: C, 71.48%; H, 8.66% and N, 9.26%. Found: C, 71.26%; H, 8.54% and N, 9.33%.

D- *and* L-*N,O-diacetylphenylalaninol (Compound 1b)*

Acetyl chloride (5.5 g, 70 mmoles) was added dropwise with stirring to a cooled (0 °C) solution of L-phenylalaninol (Compound 1a, 5.2 g, 34 mmoles) in dry benzene (150 ml) containing dry pyridine (5.5 g, 70 mmoles). The mixture was then kept at 23 °C for 4 h and ice (200 g) added. The organic layer was separated and the aqueous solution extracted with ethyl acetate (4 × 50 ml). The combined organic solutions were washed with 1 M HCl until free of pyridine, then with water, dried ($MgSO_4$) and evaporated. The product was then recrystallized from benzene to give *N,O*-diacetyl-L-phenylalaninol (Compound 1b, 5 g) m.p. 127–129°C; R_F 0.7 (chloroform–methanol, 9:1; by vol.); $[\alpha]_D^{25}$ (*c* 2.3, ethanol) −7.4°; infrared (Nujol) 3300, 1730, 1650 and 1580 cm^{-1}; NMR (^{3}H-labelled chloroform) δ 1.91 (3H, s, CO_2CH_3), 2.05 (3H, s, $NHCOCH_3$), 2.85 (2H, d of d, J = 3 Hz, C-3H), 4.15 (2H, d, J = 8 Hz, C-1 H), 4.45 (1H, m, C-2H), 5.80 (1H, broad s, NH) and 7.27 ppm (5H, broad s, C_6H_5); mass spectrum, *m/e* 235 (M^+).

Analysis. Calcd for $C_{13}H_{17}NO_3$: C, 66.33%; H, 7.28 and N, 5.95%. Found: C, 66.54%; H, 7.24% and N, 5.98%.

For *N,O*-diacetyl-D-phenylalaninol, $[\alpha]_D^{25}$ (*c* 2.1, ethanol) +7.6°; all other properties as for the L enantiomer.

Analysis. Found: C, 66.59%; H, 7.42% and N, 6.10%.

D- *and* L-*N-Acetylphenylalaninol (Compound 1c)*

Aqueous NaOH (18 ml of 1 M, 18 mmoles) was added with stirring to a cold (0 °C) solution of *N,O*-diacetyl-L-phenylalaninol (Compound 1b, 3.7 g, 16 mmoles) in ethanol (20 ml). After 30 min, thin-layer chromatography analysis showed complete hydrolysis of the O-acetate and the mixture was brought to pH 7 with 0.5 M aqueous H_2SO_4 and extracted with chloroform (3 × 20 ml). The combined chloroform extracts were dried ($MgSO_4$), evaporated, and the residual oil recrystallized from benzene–isopropyl ether to give *N*-acetyl-L-phenylalaninol (Compound 1c, 2.9 g) m.p. 100–102 °C; R_F 0.5 (chloroform: methanol, 9:1; by vol.) 0.5; $[\alpha]_D^{25}$ (*c* 2.7, ethanol) 16.5°; infrared (Nujol) 3300–3000 cm^{-1} (broad), 1640 and 1575 cm^{-1}; NMR (^{3}H-labelled chloroform) δ 1.90 (3H, s, $COCH_3$), 2.86 (2H, d, J = 8 Hz, C-3 H), 3.60 (3H, m, partly exchangeable with ^2H_2O, C-1 H and OH), 4.15 (1H, m, C-2 H), 6.00 (1H, broad s, NH) and 7.30 ppm (5H, s, C_6H_5); mass spectrum, *m/e* 193 (M^+).

Analysis. Calcd for $C_{11}H_{15}NO_2$: C, 68.36%; H, 7.82%; N, 7.24%. Found: C, 68.69%; H, 7.78% and N, 7.10%.

For D-*N*-acetylphenylalaninol, $[\alpha]_D^{25}$ (*c* 2.5, ethanol) +15.7°; all other properties were identical with those recorded above for the L-enantiomer.

Analysis. Found: C, 68.34%; H, 7.87% and N, 7.20%.

D- *and* L-*tryptophanol (Compound 2a)*

L-Tryptophan methyl ester hydrochloride (16.0 g, 63 mmoles) and lithium alu-

minum hydride (7.2 g, 0.19 mmoles) in dry tetrahydrofuran was refluxed for 2 h and then worked up as for Compound 1a. Recrystallization from ethyl acetate–diethyl ether yielded L-tryptophanol (Compound 2a, 6 g) m.p. 75–77°C; R_F (butanol–acetic acid–water, 3:1:1; by vol.) 0.4; $[\alpha]_D^{25}$ (*c* 1.9, ethanol) −20.6°; infrared (Nujol) 3400, 3350 and 1625 cm^{-1}; NMR ([U-^{2}H]acetone) δ 2.0 (2H, m, C-3 H), 3.0 (5H, m, partly exchangeable with ^2H_2O, C-1 H, OH, and NH_2), 3.80 (1H, m, C-2 H), 7.1 (4H, m, C_6H_4) and 7.40 ppm (1H, m, indole C-2 H); mass spectrum, *m/e* 190 (M^+).

Analysis. Calcd for $C_{11}H_{14}N_2O$: C, 69.42%; H, 7.42% and N, 14.73%. Found: C, 69.42%; H, 7.27% and N, 14.62%.

The enantiomeric alcohol, D-tryptophanol had $[\alpha]_D^{25}$ (*c* 2.1, ethanol) +22.8° and all other properties were as reported for the L enantiomer above.

Analysis. Found: C, 69.24%; H, 7.51% and N, 14.63%.

D- *and* L-*diacetyltryptophanol (Compound 2b)*

Acetyl chloride (3.6 g, 46 mmoles) was added dropwise with stirring at −5 °C to a suspension of L-tryptophanol (Compound 2a, 3.5 g, 18 mmoles) in dry benzene (150 ml) containing dry pyridine (3.6 g, 46 mmoles). The mixture was then heated to 50 °C for 1 h and subsequently stirred for 6 h at 23 °C. Work-up of the reaction mixture as described for Compound 1b followed by recrystallization from benzene yielded *N,O*-diacetyl-L-tryptophanol (Compound 2b, 2.9 g) m.p. 126–127 °C; R_F 0.6 (chloroform–methanol, 9:1; by vol.); $[\alpha]_D^{25}$ (*c* 3.1, ethanol) −1.3°; infrared (Nujol) 3400, 3300, 1725 and 1635 cm^{-1}; NMR (^{3}H-labelled chloroform) δ 1.90 (3H, s, CO_2CH_3), 2.04 (3H, s, $NHCOCH_3$), 2.97 (2H, d, J = 7 Hz, C-3 H); 4.10 (2H, J = 6 Hz, C-1 H), 4.55 (1H, m, C-2 H), 5.87 (1H, broad s, amide NH), 7.20 (4H, m, C_6H_4), and 7.70 ppm (1H, m, indole C-2 H); mass spectrum, *m/e* 274 (M^+).

Analysis. Calcd for $C_{15}H_{18}N_2O_3$: C, 65.65%; H, 6.61% and N, 10.21%. Found: C, 65.62%; H, 6.59% and N, 10.36%.

For the D enantiomer of Compound 2b, $[\alpha]_D^{25}$ (*c* 2.9, ethanol) was +1.3° and all other physical data as for the L enantiomer above.

Analysis. Found: C, 65.72%; H, 6.80% and N, 10.10%.

D- *and* L-*N-acetyltryptophanol (Compound 2c)*

Hydrolyses of the acetate group of the D- and L-enantiomers of the N,O-diacetate (Compound 2b; 2.74 g, 14 mmoles) were effected in 87–88% yields exactly as described in the analogous phenylalaninol experiments (Compound 1b → Compound 1c). The D- and L-*N*-acetyltryptophanols (Compound 2c) could not be crystallized and decomposed during attempted distillation. Their purification was achieved by column chromatography on Florisil with light petroleum (b.p. 30–60 °C)–ethyl-acetate elution. The pure, but relatively unstable, colourless oils obtained were used immediately in the kinetic experiments.

The L enantiomer of Compound 2c had R_F 0.3 (chloroform–methanol, 9:1; by vol.); $[\alpha]_D^{25}$ (*c* 3, ethanol) −12.2°; infrared (film) 3400–3300 cm^{-1} (broad) and 1630 cm^{-1}; NMR ([U-^{2}H]aceton) δ 1.90 (3H, s, $COCH_3$), 3.0 (2H, d, J = 7 Hz, C-3 H), 3.70 (2H, m, C-1 H), 4.0 (1H, m, C-2 H), 7.20 (4H, m, C_6H_4), and 7.75 ppm (1H, m, indole C-2 H); mass spectrum, *m/e* 232 (M^+).

The D-enantiomer of Compound 2c was identical in all properties except that $[\alpha]_D^{25}$ (*c* 3.2, ethanol) was +11.6°.

Evaluation of the D *and* L *enantiomers of Compounds 1b, 1c and Compounds 2b and 2c as competitive inhibitors of α-chymotrypsin*

The kinetic studies were carried out in CO_2-free aqueous solution at 25 °C under nitrogen using pH stat (Radiometer) controlled addition of $0.5 \cdot 10^{-2}$–$2 \cdot 10^{-2}$ M aqueous NaOH to maintain the pH at 7.8. Stock solutions of α-chymotrypsin (in 10^{-3} M HCl) and of the substrate, *N*-acetyl-L-tyrosine ethyl ester (in water), were made up and appropriate aliquots taken to enable 7–10 runs per experiment to be made under steady conditions within the substrate concentrations range 0.1–4.0 mM and with enzyme concentrations $1 \cdot 10^{-8}$–$1.7 \cdot 10^{-8}$ M. The enzyme concentrations were determined spectrophotometrically [10]. A reaction volume of 10 ml of constant ionic strength (0.1 M in KCl) was used throughout. Aqueous stock solutions of each inhibitor were freshly prepared before each evaluation and final inhibitor concentrations in the range 6–10 mM were studied. Use of higher concentrations for the poorer inhibitors was precluded by their low water solubilities. Determinations of the initial rates of enzyme-catalyzed hydrolysis of substrate, with and without inhibitor present, were performed at least in duplicate and were analyzed by the Lineweaver–Burk method. All data were subjected to least squares non-linear regression analysis. The results are recorded in Table I.

Enzyme concentrations of up to $1.75 \cdot 10^{-7}$ M were used in evaluating the substrate activities of the D- and L-diacetates, Compound 1c and Compound 2c.

RESULTS

The syntheses of the desired inhibitors were accomplished in good yields and without major problems. All compounds were readily purified except for D- and L-*N*-acetyltryptophanol (Compound 2c) which could not be crystallized and, being somewhat unstable, required careful chromatographic purification immediately prior to

TABLE I

INHIBITION CONSTANTS OF THE D AND L ENANTIOMERS OF COMPOUNDS 1b, 1c AND OF COMPOUNDS 2b AND 2C

The inhibition constants were determined at 25 °C at pH 7.8 in aqueous 0.1 M KCl solutions using *N*-acetyl-L-tyrosine ethyl ester (0.1–4.0 mM) as the substrate and with α-chymotrypsin concentrations of $1.2 \cdot 10^{-8}$–$1.75 \cdot 10^{-8}$ M. For the uninhibited runs, k_{cat} and K_m values of $2.13 \pm 0.03 \cdot 10^2$ sec^{-1} and 0.66 ± 0.05 mM, respectively, were recorded (ref. 10 quotes $2.1 \cdot 10^2$ sec^{-1} and 0.7 mM). All determinations were carried out at least in duplicate and data were subjected to least squares regression analyses. The error limits given are standard deviations.

Inhibitor	Concn of inhibitor (mM)	K_I (mM)
L Enantiomer of Compound 1b	10.0	24.5 ± 10.3
L Enantiomer of Compound 1c	10.0	65.7 ± 53.9
D Enantiomer of Compound 1b	10.0	16.4 ± 1.5
D Enantiomer of Compound 1c	10.0	30.5 ± 4.7
L Enantiomer of Compound 2b	6.0	6.9 ± 0.9
L Enantiomer of Compound 2c	7.0	12.3 ± 3.1
D Enantiomer of Compound 2b	7.5	4.3 ± 0.3
D Enantiomer of Compound 2c	7.5	6.4 ± 0.7

use. Each of the N,O-diacetates, Compounds 1b and 2b and the N-acetates, Compounds 1c and 2c was found to be a competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester and the K_i values observed are recorded in Table I. None of the N,O-diacetates, the D and L enantiomers of Compound 1b and Compound 2b showed any substrate activity even when subjected to relatively high enzyme concentrations. The data obtained show clearly that, in each case, inhibition by a tryptophanol derivative is four to five times more effective than that of the corresponding phenylalaninol compound. Furthermore, for each pair of N,O-diacetates and N-acetates, the inhibitor with the free hydroxymethyl function invariably has the higher K_i value and the D enantiomers form stronger EI complexes than their L counterparts.

DISCUSSION

From studies using models it appeared that with the N-acetyl and aromatic groups in the *am*- and *ar*-sites, respectively, of the enzyme, the hydroxymethyl groups of the L enantiomers of Compound 1c and of Compound 2c were oriented such that strong hydrogen bonding with Ser-195 could take place. However, although each of the D and L enantiomers of Compound 1c and of Compound 2c are competitive inhibitors of α-chymotrypsin, their K_i values (Table I) are 1–2 orders of magnitude greater than the K_m or K_i values of the corresponding methyl ester substrates and inhibitors [11]. This shows that *n*-site hydrogen bonding of Compound 1c and Compound 2c does not occur to the extent hoped for. Nevertheless, the fact that the D and L enantiomers of Compound 2c are as good, or better, inhibitors than D- or L-*N*-acetyltryptophan (K_i 4.8 and 17.5 mM, respectively [11]) indicates that some hydroxymethyl–*n*-site interaction is occurring. The inhibition constants of Table I show that acetylation of Compound 1c and Compound 2c improves their inhibitory properties in each case, with the most marked changes being observed in the phenylalaninol series. That the N,O-diacetates, Compounds 1b and 2b, have lower K_i values than the corresponding hydroxymethyl inhibitors is ascribable to better binding of the less polar (than CH_2OH) O-acetate group with the hydrophobic [12, 13] *n*-region and/or to improved hydrogen bonding or nucleophilic interaction of Ser-195 with the acetate carbonyl. The better inhibiting characteristics of D enantiomers over L enantiomers and of tryptophanol over phenylalaninol derivatives, and the relative magnitudes of the respective K_i differences, are in accord with the literature data [11] on the corresponding amino acid derivatives.

From the affinity label viewpoint, the fact that the O-acetate functions of the D and L enantiomers of Compounds 1b and 2b are not hydrolyzed by α-chymotrypsin means that probing the *n*-binding region may be feasible via appropriate esterification of Compound 1c or Compound 2c. Furthermore, uncharged O- and/or N-acylated specific substrate analogues of Compound 1a and Compound 2a would seem to be attractive candidates for X-ray and NMR comparisons [4, 14–16] of the binding of D and L enantiomers with α-chymotrypsin.

ACKNOWLEDGEMENT

This work was supported by the National Research Council of Canada.

REFERENCES

1 Shaw, E. (1970) The Enzymes (Boyer, P. D., ed.), Vol. 1, 3rd edn, pp. 94–102, Academic Press, New York
2 Baker, B. R. (1967) Active-Site Directed Irreversible Alkylating Agents, J. Wiley and Sons, Inc., New York
3 Cohen, S. G., Milovanovic, A., Schultz, R. M. and Weinstein, S. Y. (1969) J. Biol. Chem. 244, 2664–2674
4 Blow, D. (1971) The Enzymes (Boyer, P. D., ed.), Vol. 3, 3rd edn, pp. 185–212, Academic Press, New York
5 Jones, J. B. and Hysert, D. W. (1971) Can. J. Chem. 49, 325–332
6 Jones, J. B. and Hysert, D. W. (1971) Can. J. Chem. 49, 3012–3019
7 Jones, J. B. and Hysert, D. W. (1972) Biochemistry 11, 2726–2732
8 Brenner, M. and Huber, W. (1953) Helv. Chim. Acta 36, 1109–1115
9 Kapil, R. S., Gautam, B. C., Vohra, M. M. and Nitya, N. (1966) J. Ind. Chem. Soc. 4, 177–187
10 Cunningham, L. W. (1954) J. Biol. Chem. 207, 443–458
11 Cunningham, L. (1965) Comprehensive Biochemistry (Florkin, M. and Stotz, E. H., eds), Vol. 16, pp. 85–188, Elsevier, New York
12 Inward, P. W. and Jencks, W. P. (1965) J. Biol. Chem. 240, 1986–1996
13 Lin, Y. Y., Palmer, D. N. and Jones, J. B. (1974) Can. J. Chem. in the press
14 Smallcombe, S. H., Ault, B. and Richards, J. H. (1972) J. Am. Chem. Soc. 94, 4585–4590
15 Gerig, J. T. and Rinerman, R. A. (1972) J. Am. Chem. Soc. 94, 7565–7569
16 Nicholson, B. C. and Spotswood, T. M. (1973) Austr. J. Chem. 26, 135–145